Universidade Estadual de Londrina

CARLA FABIANA SOUZA GUAZELLI

EFEITO TERAPÊUTICO E MECANISMOS DE AÇÃO DA HESPERIDINA METIL CHALCONA, TRANS-CHALCONA E SUAS FORMAS MICROENCAPSULADAS EM MODELO DE COLITE ULCERATIVA EM CAMUNDONGOS

Londrina
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Londrina – UEL.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Jr.

Co-orientadora: Profa. Dra. Marcela Maria Baracat.

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RESUMO

As doenças inflamatórias intestinais (DII) são condições inflamatórias crônicas intermitentes que acometem preferencialmente adultos jovens e representam um sério problema de saúde, pois cursam com recidivas frequentes e apresentam formas clínicas de alta gravidade. Assim, resultam em altos custos a longo prazo tanto para o paciente, quanto para o sistema de saúde e para a sociedade. A inflamação intestinal está associada ao aumento tecidual na produção de citocinas e quimiocinas, recrutamento de neutrófilos e estresse oxidativo. Os tratamentos convencionais utilizados para as DII não são completamente eficazes e a utilização a longo prazo está associada a efeitos adversos que comprometem a adesão ao tratamento. Desta forma, a descoberta e o desenvolvimento de novas terapias que promovam a melhora na qualidade de vida dos portadores destas doenças são necessários. Em geral, flavonoides são consideradas moléculas promissoras no tratamento de DII ao passo que apresentam baixa incidência de efeitos colaterais. Por isso objetivamos desenvolver microcápsulas contendo hesperidina metil chalcona (HMC) e *trans*-chalcona (TC), e avaliar o efeito terapêutico e mecanismos de ação da HMC, TC e suas formas microencapsuladas na colite ulcerativa induzida por ácido acético em camundongos. Neste sentido, buscamos verificar se há aumento da potência dos flavonoides microencapsulados em comparação com os flavonoides não microencapsulados. O tratamento com ambos os fármacos não microencapsulados, HMC e TC, reduziu significativamente o recrutamento de neutrófilos, o edema e as lesões macro e microscópicas induzidas pela administração intracolonica de ácido acético a 7,5%. Estes efeitos foram acompanhados pela preservação da capacidade antioxidante, que contribuiu para a diminuição do estresse oxidativo, e pela redução da produção de citocinas pró-inflamatórias e da ativação do NF-κB no cólon. As microcápsulas de HMC não foram eficazes em reduzir o processo inflamatório induzido por ácido acético no cólon dos animais. A alta solubilidade do fármaco em água dificultou o processo de desenvolvimento das microcápsulas. Por isso, o processo de desenvolvimento desta formulação microecapsulada contendo HMC será aprimorado a fim de obtermos melhores resultados. Por outro lado, a TC quando veiculada na formulação microencapsulada MT1:3 com liberação modificada do fármaco, torna-se suficiente para reduzir significativamente o recrutamento de neutrófilos para o cólon, o edema, as lesões macro e microscópicas no tecido, o estresse oxidativo, os níveis de citocinas (TNF-α, IL-1β, IL-6 e IL-33) e a ativação do fator de transcrição NFκB em dose 10 vezes menor do que a dose necessária do fármaco não microencapsulado, demonstrando o aumento da potência da TC microencapsulada em comparação com a TC não microencapsulada. Finalmente, estes resultados sugerem a aplicabilidade da HMC, TC e microcápsulas de TC (MT1:3) para reduzir o estresse oxidativo e inflamação durante a colite induzida por ácido acético. Além disto, demonstram a importância do desenvolvimento farmacêutico de sistemas de liberação modificada para reduzir a dose necessária de fármaco para se obter

efeitos anti-inflamatórios e antioxidantes.

Palavras-chave: Hesperidina metil chalcona. *Trans*-chalcona. Microcápsulas. Colite ulcerativa.

GUAZELLI, Carla Fabiana Souza. **Therapeutic efficacy and mechanisms of action of hesperidin methyl chalcone, *trans*-chalcone and their microencapsulated forms on ulcerative colitis model in mice**. 2017. 126p. Thesis (Doctorate degree in Health Sciences) - Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

Inflammatory bowel diseases (IBD) are chronic intermittent inflammatory conditions that primarily affect young adults and represent a serious health problem, because they present frequent relapses and high severity clinical forms. Thus, they result in high long-term costs for the patient, the health system and society. Intestinal inflammation is associated with increased cytokines and chemokines production, neutrophils recruitment and oxidative stress. Conventional IBD treatments are not completely effective and the long-term use is associated with adverse effects that compromise the adherence to treatment. In this way, it is necessary to discovery and develop new therapies that improve the quality of patients life. Flavonoids are promising molecules in the IBD treatment, therefore we aimed to develop microcapsules containing hesperidin methyl chalcone (HMC) and *trans*-chalcone (TC), and to evaluate the therapeutic effect and mechanism of action of HMC, TC and their microencapsulated forms on acetic acid-induced ulcerative colitis in mice. In this sense, we verify if microencapsulated formulations have the potential for improving the therapeutic effect of non microencapsulated flavonoids. Treatment with both non-microencapsulated drugs, HMC and TC, significantly reduced neutrophil recruitment, edema, and macroscopic and microscopic lesions induced by intracolonic administration of 7.5% acetic acid solution. These effects were accompanied by the preservation of antioxidant capacity, which contributed to the reduction of oxidative stress, and by the reduction of pro-inflammatory cytokines production by inhibiting the activation of NF-κB in the colon. HMC microcapsules were not effective in reducing the inflammatory process induced by acetic acid in the colon of animals.The high solubility of the HMC in water hindered the microencapsulation process. Therefore the microencapsulation of HMC will be improved in order to obtain better results. On the other hand, when TC is conveyed in microencapsulated MT1:3 formulation, a product with modified drug delivery characteristics, it becomes sufficient to significantly reduce neutrophil recruitment, edema, macro and microscopic tissue damage, oxidative stress, cytokine levels (TNF-α, IL-1β, IL-6 and IL-33) and the activation of the NFκB transcription factor in the colon at a dose of 10 times lower than the dose of non-microencapsulated drug, demonstrating the increased potency of microencapsulated TC compared to non-microencapsulated TC. Finally, these results suggest the applicability of HMC, TC and TC-loaded microcapsules (MT1:3) to reduce oxidative stress and inflammation during acetic acid-induced colitis. In addition, they demonstrate the importance of pharmaceutical development of modified delivery systems to reduce the required dose of drugs to obtain anti-inflammatory and antioxidant effects.

Keywords: Hesperidin methyl chalcone. *Trans*-chalcone. Microcapsules. Ulcerative colitis.

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Desenvolvimento de Microcápsulas de Hesperidina Metil Chalcona

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LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2-azinobis (3-etilbenzotiazolina-6-sulfonato, sal de diamônio)
CU	Colite Ulcerativa
DC	Doença de Crohn
DSC	*Differential Scanning Calorimetry* (Calorimetria Diferencial de Varredura)
DII	Doenças Inflamatórias Intestinais
DSS	Dextran Sulfato de Sódio
EDTA	*Ethylenediaminetetraacetic Acid* (Ácido Etilenodiaminotetracético)
ELISA	*Enzyme-linked Immunosorbent Assay* (Ensaio de Imunoabsorção por Ligação Enzimática)
EROs	Espécies Reativas de Oxigênio
FRAP	*Ferric Reducing Antioxidant Power* (Capacidade Antioxidante Redutora do Íon Ferro)
GSH	Glutationa Reduzida
IL-10	Interleucina-10
IL-1β	Interleucina-1β
IL-33	Interleucina-33
IL-6	Interleucina-6
iNOS	*Inducible Nitric Oxide Synthase* (Óxido Nítrico Sintase Induzível)
MAPK	*Mitogen-Activated Protein Kinase* (Proteínas Quinases Ativadas por Mitógeno)
MPO	Mieloperoxidase
SLF	Sistemas de Liberação de Fármacos
TNBS	*Trinitrobenzene Sulfonic Acid* (Ácido Trinitrobenzeno Sulfônico)
TNF-α	*Tumor Necrosis Factor-α* (Fator de Necrose Tumoral-α)
TPTZ	2,4,6-tripiridil-s-triazina
Trolox	*6-hydroxy-2,5,7,8-tetramethylchroman-2-Carboxylic Acid* (Ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico)

SUMÁRIO

1 INTRODUÇÃO

1.1 Colite Ulcerativa

O sistema imunológico intestinal enfrenta uma delicada tarefa de fornecer uma resposta imune rápida e eficaz contra bactérias patogênicas, mantendo a tolerância em relação a antígenos alimentares e da própria microbiota (1). O desequilíbrio entre o sistema imunológico e a microbiota intestinal pode resultar em respostas inflamatórias anormais que conduzem a uma inflamação intestinal crônica, como no caso das Doenças Inflamatórias Intestinais (DII) (1). As DII são caracterizadas por um processo inflamatório crônico intermitente, no qual os períodos de doença ativa alternam com períodos de remissão (2), e incluem a doença de Crohn (DC) e a Colite Ulcerativa (CU). Embora ambas compartilhem muitas características imunológicas em comum, incluindo aumento da produção de citocinas pró-inflamatórias e acúmulo de células imunológicas nas áreas afetadas, a DC é uma inflamação transmural profunda que acomete principalmente a região terminal do íleo, mas pode ocorrer em qualquer local do trato gastrointestinal, desde a boca até o ânus, enquanto que a CU é caracterizada por inflamação contínua e ulcerosa da camada mucosa, geralmente restrita ao cólon (3).

No desenvolvimento da CU, fatores ambientais estressantes associados a predisposição genética do indivíduo podem conduzir a quebra da homeostasia da mucosa (4). Sendo assim, a inflamação intestinal resulta da estimulação do sistema imunológico da mucosa por produtos de bactérias comensais presentes no lúmen que penetram a barreira mucosa e induzem a produção de citocinas e quimiocinas envolvidas no recrutamento e ativação de leucócitos (5), como neutrófilos. Os neutrófilos são células do sistema imune inato capazes de destruir patógenos intra e extracelulares, pois produzem enzimas proteolíticas e proteínas bactericidas, como a catepsina G, elastase, lisozima e mieloperoxidase (MPO) (6), que é utilizada como um importante marcador de recrutamento de neutrófilos para o tecido. Além disto, os neutrófilos ativados também liberam espécies reativas de oxigênio (ERO) e nitrogênio (ERN) nos tecidos intestinais superando a capacidade antioxidante local, contribuindo para o estresse oxidativo (5, 7). A produção excessiva de ERO provoca danos ao DNA e às proteínas, e induz a peroxidação lipídica das membranas prejudicando a integridade das células epiteliais intestinais e aumentando a permeabilidade da mucosa intestinal (8). As ERO também estimulam o recrutamento de mais neutrófilos (9) e induzem a produção de citocinas, como o fator de necrose tumoral (TNF-)α, a interleucina (IL)-1 e IL-6, através da ativação do fator de transcrição nuclear kappa B (NF-κB) (8).

O NF-κB é um fator de transcrição composto por duas subunidades, a p50 (NF-κB1) e a p65 (RelA). Em condições fisiológicas, o NF-κB está presente no citoplasma associado ao inibidor de Kappa B-α (IκBα), sua subunidade inibitória. Em resposta a estímulos pró-inflamatórios, a IκB quinase (IKK) é ativada e fosforila o IκBα nos resíduos serina / treonina, resultando na liberação do NF-κB. Consequentemente, ocorre a translocação do NF-κB para o núcleo, onde ele se liga a sequências específicas promovendo a transcrição de genes que codificam citocinas, quimiocinas, moléculas de adesão e outros mediadores pró-inflamatórios (10, 11). Assim como as ERO, as citocinas também medeiam sua ação via ativação de fatores de transcrição como o NF-κB (8).

Sabe-se que os níveis de várias citocinas pró-inflamatórias estão aumentados na mucosa intestinal de pacientes com CU (12), dentre elas podemos citar o TNF-α, a IL-1β e a IL-33 que estão envolvidos na ativação de fatores de transcrição e na consequente expressão de mais citocinas pró-inflamatórias, quimiocinas, moléculas de adesão, ciclooxigenase, óxido nítrico sintase induzível e metaloproteinases de matriz (11, 13), que amplificam a resposta inflamatória. Os níveis de IL-6 também estão aumentados no colon de pacientes com CU e esta citocina está associada ao desenvolvimento de tumores associados à inflamação (14).

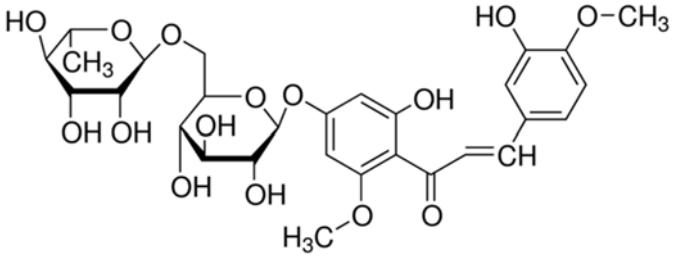
A ativação exacerbada do sistema imunológico durante a CU é caracterizada por vasodilatação, infiltração de leucócitos, liberação de mediadores inflamatórios, tais como TNF-α, IL-6, IL-1β, IL-33, e superprodução sustentada de ERO e ERN levando ao dano tecidual(15). Portanto, o recrutamento de neutrófilos, o estresse oxidativo, a ativação do NF-κB e o aumento da produção de citocinas desempenham papel fundamental na iniciação, amplificação e perpetuação da doença, uma vez que são responsáveis pela lesão da mucosa na CU.

Os tratamentos para a CU têm o objetivo de suprimir a inflamação e induzir a cicatrização da mucosa, reduzindo os sintomas e melhorando a qualidade de vida dos pacientes. Estes tratamentos incluem os salicilatos (sulfasalazina, mesalazina), corticosteroides (cortisona e budesonida), imunomoduladores (tiopurinas e metotrexato) e biológicos (infliximab, adalimumab) (16). No entanto, as reações adversas, a toxicidade e a ineficácia terapêutica são as principais limitações dos tratamentos atualmente aprovados e fazem com que muitos pacientes interrompam o uso destes medicamentos(16). Novas e promissoras abordagens terapêuticas têm sido sugeridas, tais como anticorpos contra citocinas, integrinas e metaloproteinases, estratégias anti-quimiocinas, e terapias com compostos naturais, como os flavonoides (17).

1.2 Hesperidina Metil Chalcona

Os flavonoides são uma classe de compostos polifenólicos bioativos amplamente distribuídos na natureza (principalmente em frutos, legumes e plantas em geral) que apresentam várias atividades biológicas, incluindo propriedades antioxidantes e anti-inflamatórias. A naringenina, quercetina e rutina são alguns exemplos de flavonoides utilizados como tratamento em modelos experimentais de DII em roedores (18). A hesperidina, um flavonoide pertencente à classe das flavanonas, melhorou a colite experimental induzida por sulfato sódico de dextrana, através da redução das lesões no cólon, dos níveis de malondialdeído (MDA), da atividade da mieloperoxidase (MPO), e da produção de IL-6 (19). No entanto, a hesperidina apresenta baixa solubilidade em água e é pouco absorvida no intestino delgado como muitos outros flavonoides (20). O processo de metilação da hesperidina sob condições alcalinas produz a hesperidina metil chalcona (HMC; Figura 1), que apresenta maior solubilidade em água do que a hesperidina (21). O processo de metilação dos flavonoides pode aumentar a absorção intestinal, a biodisponibilidade e a distribuição nos tecidos (22).

Figura 1 – Estrutura química da hesperidina metil chalcona.



A HMC é amplamente utilizada em formulações farmacêuticas associada ao extrato de *Ruscus aculeatus* e ácido ascórbico para o tratamento da doença venosa crônica(23), mostrando-se capaz de reduzir a gravidade dos sintomas e melhorar a qualidade de vida dos pacientes. Recentemente, nosso grupo de pesquisa demonstrou os efeitos anti-inflamatórios e antioxidantes da HMC em diferentes modelos experimentais. O tratamento com HMC reduziu a dor, o edema de pata, o recrutamento de neutrófilos, o estresse oxidativo, a produção de citocinas e a ativação do NF-kB em modelo de inflamação plantar induzida por carragenina em camundongos (20). Além disto, a administração tanto sistêmica quanto tópica de HMC inibiu o estresse oxidativo e a inflamação induzidos pela radiação UVB na pele de camundongos (24, 25). É importante ressaltar que não foram detectados sinais de toxicidade após tratamento com HMC durante 7 dias em camundongos (20), e a

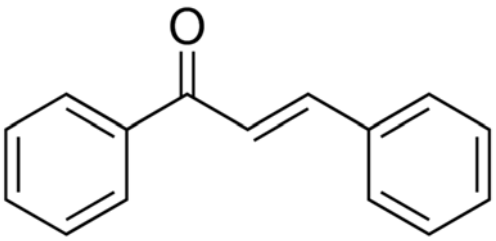
HMC mostrou-se segura mesmo quando administrada em doses massivas de 15 g/dia (26). Estes resultados sugerem o uso potencial da HMC para tratar outras doenças inflamatórias, como CU, uma vez que não há atualmente nenhuma evidência científica quanto a capacidade da HMC em prevenir e / ou tratar a colite.

1.3 *trans*-Chalcona

As chalconas são os principais precursores da biossíntese dos flavonoides e formam o núcleo central de uma variedade de compostos biológicos importantes obtidos a partir de plantas ou preparados sinteticamente (27, 28). Elas consistem basicamente em dois anéis aromáticos ligados por uma enona de três carbonos (29) e podem existir em duas formas isoméricas, *cis* e *trans*, das quais a forma *trans* é termodinamicamente estável (30). Devido à sua abundante disponibilidade, estrutura simples, variedade e diferentes formas de ciclização, esta classe de compostos tem se tornado importante na busca de moléculas com potencial terapêutico (27). Os derivados de chalcona possuem propriedades anti-inflamatórias, antioxidantes, antifúngicas, antibacterianas, antimalárica, anti-tuberculose, anti-tumorais e antivirais (31). Os efeitos anti-inflamatórios e antioxidantes reportados estão associados a inibição da expressão de moléculas de adesão celular (CAM), e de outros mediadores inflamatórios, tais como óxido nítrico (NO), TNF-α, IL-1β, ciclo-oxigenase-2 (COX-2) e óxido nítrico sintase induzível (iNOS), além de indução da expressão da heme oxigenase (HO)-1 e supressão das vias de sinalização do NF-kB e MAPKs (31, 32).

A *trans*-chalcona (TC; Figura 2) é uma chalcona que apresenta a estrutura base deum polifenol. Embora existam poucos dados relativos ao seu efeito farmacológico, alguns estudos já mostraram sua propriedade anti-inflamatória e antioxidante. Sikander e col. (2011) (29) demonstraram que a TC protege células de carcinoma hepatocelular (HepG2) do estresse oxidativo induzido por peróxido de hidrogênio (H_2O_2). O tratamento com TC também reduz a expressão do fator de crescimento endotelial vascular (VEGF) e da molécula de adesão celular ICAM-1 ao inibir as vias de sinalização do NF-κB e STAT3 em modelo de retinopatia isquêmica em camundongos (33). Além disto, o tratamento com TC reduz significativamente o estresse oxidativo e a inflamação em modelo de lesão hepática em ratos (34). Resultados promissores demonstraram que a liberação modificada da TC utilizando o polímero ácido poli-(lático/glicólico) é capaz de maximizar o efeito leishmanicida desta substância (35). Mesmo assim, ainda existe um vasto campo a ser investigado sobre os efeitos farmacológicos da TC, e mais importante, não há dados na literatura sobre o uso desta substância no tratamento da CU.

Figura 2 – Estrutura química da *trans*-chalcona



1.4 Sistema Microencapsulado para Liberação Modificada

Várias abordagens têm sido utilizadas para melhorar a atividade de fármacos, reduzir a toxicidade e as limitações dos tratamentos para as DII, tais como o desenvolvimento de sistemas de liberação direcionados ao cólon, incluindo pró-fármacos, os sistemas de liberação pH-dependentes, tempo-dependentes e enzima-dependentes (36). A concepção de sistemas de liberação adequados também é considerada um passo fundamental para resolver problemas como concentração insuficiente de moléculas ativas, rápido metabolismo e eliminação, baixa solubilidade e alta flutuação dos níveis plasmáticos devido à biodisponibilidade imprevisível (37).

Microcápsulas poliméricas são uma tecnologia desenvolvida para permitir a administração oral de fármacos, uma vez que podem protegê-los das alterações de pH e da degradação enzimática, além de possibilitar o aumento da permeabilidade de fármacos através do epitélio intestinal e proporcionar liberação modificada (38). Diferentemente da mucosa do cólon saudável, verifica-se o aumento da deposição de micropartículas nas regiões de tecido inflamado (39). A utilização de polissacarídeos biodegradáveis, como a pectina, é uma alternativa promissora no desenvolvimento de sistemas de liberação, pois estas substâncias permanecem estáveis no interior do trato gastrointestinal superior e são degradados pela microbiota bacteriana presente no cólon (39). A coacervação complexa da pectina com o polímero natural caseína em meio aquoso ocorre por interação eletrostática e resulta na formação de sistemas microencapsulados capazes de modificar a liberação do fármaco no trato gastrointestinal (40, 41). Sendo assim, temos como hipótese que o fármaco microencapsulado será liberado por difusão após degradação / bioerosão do invólucro polimérico da microcápsula (42) quando atingir o cólon. Nosso grupo já demonstrou que a liberação modificada da quercetina utilizando microcápsulas poliméricas de pectina / caseína potencializou os efeitos anti-inflamatórios e antioxidantes do fármaco em modelo de colite ulcerativa em camundongos (41). Portanto, a microencapsulação de um fármaco

utilizando o complexo polimérico de pectina / caseína pode ser uma estratégia para liberação modificada do fármaco melhorando o tratamento da CU.

As evidências de que a HMC e a TC são capazes de modular negativamente as respostas inflamatórias e o estresse oxidativo nos levaram ao desenvolvimento de sistemas microencapsulados contendo HMC e TC a fim de investigar se os efeitos destas substâncias seriam potencializados com a liberação modificada em modelo de colite ulcerativa induzida por ácido acético em camundongos, um método padronizado de inflamação intestinal que compartilha características histológicas, macroscópicas e os parâmetros bioquímicos relacionados a CU em humanos.

2 OBJETIVOS

2.1 Geral

Avaliar o efeito anti-inflamatório e antioxidante da HMC e TC na colite experimental e desenvolver microcápsulas de liberação modificada contendo HMC e TC com o objetivo de reduzir as doses dos fármacos necessárias para que o efeito anti-inflamatório e antioxidante ocorra no cólon.

2.2 Específicos

- Avaliar o efeito do tratamento com HMC e TC sobre o recrutamento de neutrófilos, edema, lesões macro e microscópicas, níveis das citocinas TNF-α, IL-1β, IL-6, IL-33 e IL-10, e ativação do fator de transcrição NF-κB no tecido colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com HMC e TC sobre a capacidade antioxidante total e os níveis de glutationa reduzida (GSH) após a indução da colite com solução de ácido acético;
- Preparar as microcápsulas de liberação modificada contendo HMC e TC;
- Avaliar a morfologia, a distribuição do tamanho da partícula e o perfil de liberação *in vitro* das microcápsulas contendo HMC e TC;
- Avaliar se as microcápsulas contendo HMC e TC potencializam os efeitos anti-inflamatórios e antioxidantes, em doses menores, porém mais eficazes dos respectivos fármacos não microencapsulados, em modelo de colite induzida por solução de ácido acético.

3 MATERIAIS E MÉTODOS

3.1 Materiais

Pectina GENU® 8003 (ref. 13595) foi obtida da CP Kelco (Limeira, Brasil). Caseína da Kauffman & Co (Kehl, Germany). Ácido cítrico; hidróxido de sódio; ácido clorídrico; fosfato de sódio e formaldeído foram adquiridos da Merck (Darmstadt, Germany). HMC e TC da Santa Cruz Biotechnology (Santa Cruz, USA). Brometo de hexadecil trimetil-amonio (HTAB); dihidrocloreto de O-dianisidina; tween 80; glutationa reduzida (GSH); EDTA; cloreto de ferro hexahidratado; 2,4,6-tripiridil-s-triazina (TPTZ); 2,2-azinobis (3-etilbenzotiazolina-6-sulfonato, sal de diamônio; ABTS); Trolox (ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico); e persulfato de potássio foram adquiridos da Sigma Chemical Co. (St. Louis, USA). Kits para dosagem de TNF-α, IL-1β, IL-6, IL-33 e IL-10, e para NF-κB foram adquiridos da eBioscience (San Diego, USA) e Cell Signaling (Beverly, USA), respectivamente. Todos os reagentes utilizados foram de grau analítico.

3.2 Preparação das Microcápsulas

Foram preparadas duas formulações microencapsuladas para cada um dos fármacos, HMC e TC, pela técnica de coacervação complexa, como descrito anteriormente (40), com diferentes proporções de fármaco:polímeros. A pectina e caseína foram dispersas em água destilada sob agitação mecânica constante e o pH de 8,0 ± 0,1 foi ajustado com hidróxido de sódio (4,0 M). Na primeira formulação de cada fármaco, tanto a HMC quanto a TC foram adicionadas na proporção de 1:5 (fármaco:polímeros) e na segunda formulação, os fármacos foram adicionados na proporção 1:3, sendo assim, obtivemos 4 formulações: microcápsulas de HMC (MH)1:5, MH1:3, microcápsulas de *trans*-chalcona (MT)1:5 e MT1:3 (Tabela I). As microcápsulas foram obtidas por redução lenta e gradual do pH de 8,0 ± 0,1 para 3,0 ± 0,1 com ácido cítrico (1 M). As formulações microencapsuladas foram secas por spray dryer (Lab Plant, modelo SD-05) usando bico atomizador do tipo duplo fluido com mistura externa e orifício de saída de 1 mm. Os parâmetros do processo foram: temperatura do ar de entrada mantida constante a 180 °C e temperatura de saída de 104-110 °C; a taxa de alimentação foi de 377,8 mL / h, e o fluxo de ar de 66 m^3 / h.

Tabela I - Proporção fármaco:polímeros das microcápsulas de HMC (MH) e TC (MT)

Formulação	Descrição	Proporção Fármaco:Polímeros
Microcápsulas de HMC	MH1:5	1:5
	MH1:3	1:3
Microcápsulas de TC	MT1:5	1:5
	MT1:3	1:3

3.3 Eficiência de Encapsulação

Para quantificação da HMC microencapsulada, 100 mg de cada uma das formulações (MH1:5 e MH1:3) foram dispersas em 10 mL de etanol absoluto e centrifugadas a 0,8*g* por 5 min. Este passo foi repetido três vezes. A HMC presente no sobrenadante foi quantificada pela técnica da capacidade sequestradora do radical ABTS ao reagir com a solução do radical cátion ABTS+• por 6 minutos seguida da leitura da absorvância a 730 nm (43).Utilizou-se uma curva padrão de HMC para determinar a quantidade do fármaco nos sobrenadantes. A eficiência de encapsulação (EE) foi calculada de acordo com a equação:

$$\mathrm{EE}(\%) = \left(\frac{\text{HMC na formulação (mg)} - \text{HMC no sobrenadante(mg)}}{\text{HMC na formulação (mg)}}\right) \text{x } 100$$

Para quantificação da TC microencapsulada, 100 mg de cada uma das formulações (MT1:5 e MT1:3) foram dispersas em 10 mL de acetonitrila e centrifugadas a 0,8 g por 5 min. Este passo foi repetido três vezes. O sobrenadante foi analisado por espectrofotometria a 337 nm para determinar o teor de TC (44), utilizando uma curva padrão de TC. A eficiência de encapsulação (EE) foi calculada de acordo com a equação:

$$\mathrm{EE}(\%) = \left(\frac{\text{TC na formulação (mg)} - \text{TC no sobrenadante(mg)}}{\text{TC na formulação (mg)}}\right) \text{x } 100$$

3.4 Microscopia Eletrônica de Varredura

Amostras de HMC, TC e de cada uma das formulações microencapsuladas foram cobertas com ouro sob atmosfera de argônio e analisadas em microscópio eletrônico de

varredura (JOEL JSM-T330A). As fotomicrografias foram obtidas sob aumento de 2400 vezes.

3.5 Análise de Tamanho de Partícula

A distribuição do tamanho de partícula das microcápsulas foi analisada em equipamento Zeta-APS system (Matec Applied Sciences, Northborough, MA, USA) imediatamente após a dispersão das formulações em água ultrapura a 25 °C. O tamanho de partícula foi obtido por análise cumulativa usando o software Zeta-APS.

3.6 Difração de Raios X

Os perfis de difração de raios X dos fármacos não microencapsulados e das formulações microencapsuladas foram obtidos em difratômetro Bruker D8 (Bruker AXS, Madison, WI, EUA), utilizando a geometria de Bragg-Brentano, de modo contínuo com velocidade de 0.05 °/ s. Uma radiação Cu Kα (λ = 1,5405 Å) foi operada a 40 kV e 30 mA, e os ângulos 2θ foram obtidos de 3 a 40 °C.

3.7 Calorimetria Exploratória Diferencial (DSC)

As curvas de DSC dos fármacos não microencapsulados e das formulações microencapsuladas foram obtidas em equipamento Shimadzu DSC-60. As amostras foram aquecidas de 30 a 500 °C com razão de aquecimento de 5 °C/min sob atmosfera de nitrogênio.

3.8 Liberação *In Vitro*

O ensaio de dissolução das formulações microencapsuladas foi realizado durante 14 horas em dissolutor Erweka DT800, utilizando aparato USP tipo 1 e velocidade de 50 rpm. Durante as 2 primeiras horas, o meio de dissolução utilizado foi 500 mL de HCl 0,1 M, contendo 2,5% de Tween® 80, pH 1,2 ± 0,1, a 37 °C, simulando as condições do suco gástrico. Nas próximas 12 horas, o meio de dissolução foi 500 mL de KOH-KH_2PO_4 0,1 M, contendo 2,5% de Tween® 80, pH 7,1 ± 0,1, simulando as condições do suco intestinal. Em intervalos de tempos definidos, as amostras dos meios de dissolução foram coletadas e filtradas em papel de filtro Millipore de 0,45 mícrons. Cada amostra foi analisada para quantificação dos fármacos no meio de dissolução e os resultados foram expressos em % de fármaco liberado em função do tempo.

A cinética de liberação dos fármacos a partir das microcápsulas foi analisada de acordo com as seguintes equações:

$$\text{Ordem zero: } Q = k_0 t \quad (1)$$

$$\text{Primeira ordem: } \ln (100 - Q) = \ln (Q_0) - k_1 t \quad (2)$$

$$\text{Higuchi: } Q = k_H t^{1/2} \quad (3)$$

Onde, Q: concentração do fármaco liberado no meio de dissolução no instante "t"; Q_0: concentração do fármaco não dissolvido no tempo 0; k_0, k_1 e k_H são as constantes de liberação para cada equação.

3.9 Animais Experimentais

Foram utilizados camundongos Swiss machos, pesando aproximadamente 25 g, provenientes do Biotério Central da Universidade Estadual de Londrina. Durante os experimentos, os animais foram mantidos no Departamento de Ciências Patológicas da Universidade Estadual de Londrina, em caixas plásticas, forradas com maravalha, em ciclo de 12 horas claro/escuro e temperatura de 23 °C ± 2. A água e ração foram disponibilizadas *ad libitum*, com exceção das 24 horas que antecediam os experimentos, período no qual os animais foram deixados em jejum sólido. O protocolo de experimentação foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Estadual de Londrina (n° processo: 1494.2015.56).

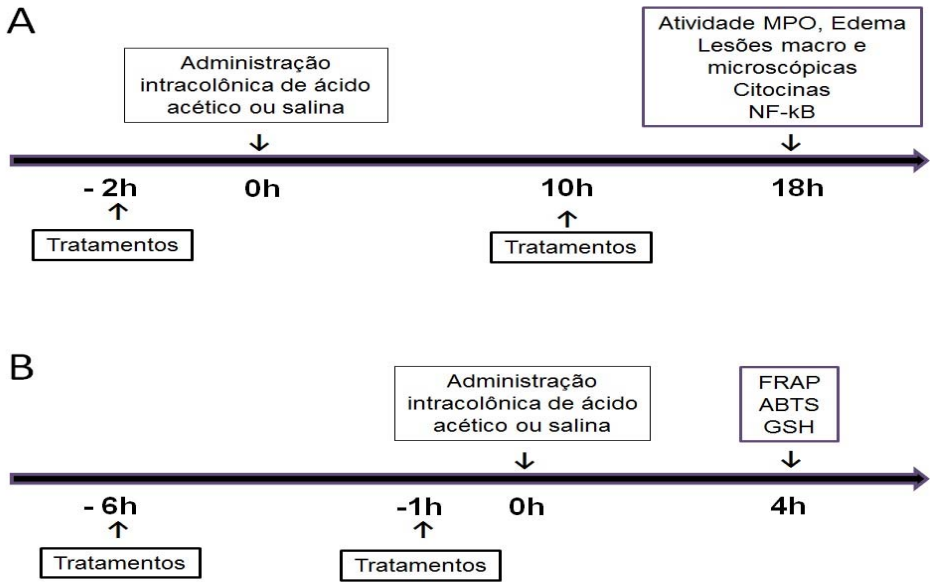
3.10 Indução da Colite Experimental

A colite foi induzida em camundongos de acordo com um método descrito previamente (41). Após 24 h de jejum sólido, os animais foram anestesiados com quetamina (80 mg/kg, im) e xilazina (10 mg/kg, im). Foi utilizada uma cânula de polietileno de 3 cm de comprimento para a administração das soluções via retal, por enema. Primeiramente, os animais foram submetidos à administração via retal de 100 µL de solução salina estéril para lavagem do cólon. Em seguida, a indução da colite foi realizada através da administração via retal de 200 µL de solução de ácido acético 7,5% (v/v) em salina. Os animais foram mantidos de cabeça para baixo por 3 minutos para impedir a saída da solução de ácido acético. Os animais do grupo controle negativo receberam solução salina via retal ao invés da solução de ácido acético.

3.11 Protocolos Experimentais e Tratamentos

Os grupos experimentais foram: controle negativo (sem colite), que recebeu apenas salina intracolônica, sem tratamento; controle colite, que recebeu administração intracolônica de solução de ácido acetico a 7,5%, sem tratamento; e grupos tratados, que além da administração intracolônica de solução de ácido acético a 7,5%, foram tratados por via oral com HMC (10, 30 e 100 mg/kg, solubilizada em salina), TC (0,3, 1 e 3 mg/kg, solubilizada em 20% de tween 80 e 80% de salina), microcápsulas inertes (0,3 mg/kg, suspensas em salina), MH1:5 e MH1:3 (10 mg/kg, suspensas em salina), MT1:5 e MT1:3 (0,3 mg/kg, suspensas em salina). Foram utilizados dois protocolos experimentais, como já descrito por nosso grupo de pesquisa (41): (A) Para avaliação dos parâmetros inflamatórios, os animais foram tratados 2 horas antes e 10 horas após a indução da colite, e foram submetidos a eutanasia seguida de laparotomia com exposição do intestino na 18ª hora após a indução. Após avaliação das lesões macroscópicas e comprimento do cólon, amostras da porção distal foram coletadas para determinação do infiltrado neutrofílico pela atividade da mieloperoxidase (MPO) e para avaliação do edema. Além disso, foram coletadas amostras para análise histológica, quantificação de citocinas (TNF-α, IL-1β, IL-6, IL-33 e IL-10) e avaliação da ativação do NF-κB no cólon (Figura 3A). (B) Para avaliação do estresse oxidativo, os animais foram tratados 6 e 1 hora antes da indução da colite, e foram submetidos a eutanasia na 4ª hora após a indução da colite. Após a laparotomia, as amostras da porção distal do colon foram coletadas para realização dos ensaios de FRAP, ABTS e GSH (Figura 3B). Os experimentos foram realizados em duplicata.

Figura 3 – Representação esquemática dos protocolos experimentais para tratamento e indução da colite.



Para avaliação dos parâmetros inflamatórios, os camundongos foram tratados 2 horas antes e 10 horas após a indução da colite, e amostras do cólon foram coletadas na 18ª hora (A). Para avaliação do estresse oxidativo, os camundongos foram tratados 6 e 1 hora antes da indução da colite, e amostras do cólon foram coletadas na 4ª hora (B) (41).

3.12 Determinação da Atividade da Mieloperoxidase (MPO)

A mieloperoxidase é uma enzima secretada por neutrófilos ativados no local da inflamação (45) e a sua quantificação é diretamente proporcional a quantidade de neutrófilos no tecido. O recrutamento de neutrófilos induzido pela administração intracolônica de solução de ácido acético foi avaliado através de um ensaio colorimétrico (46). Amostras da porção distal do cólon foram coletadas em tampão fosfato de potássio 50 mM (pH 6,0) contendo HTAB (brometo de hexadecil trimetil-amonio) 13,72 mM e armazenadas a -20°C. No dia do ensaio, as amostras foram homogeneizadas, centrifugadas (16100 g, 2 min, 4°C) e o sobrenadante foi utilizado para a reação colorimétrica em placa de 96 poços. A cada alíquota de 50 µL de amostra foi adicionado 200 µL da solução de reação contendo 52,64 mM de dihidrocloreto de O-dianisidina e 0,05% de H_2O_2 30% em tampão fosfato de potássio 50 mM (pH 6.0). As absorvâncias foram determinadas em espectrofotômetro (Multiskan GO ThermoScientific) a 450 nm, e o número de neutrófilos por mg de tecido foi determinado utilizando-se uma curva padrão de neutrófilos.

3.13 Avaliação do Edema

Fragmentos da porção distal dos cólons dos animais, medindo 1 cm de comprimento, foram coletados e pesados para avaliação do edema no tecido. Após determinação do peso, em gramas, de 1 cm de tecido colônico, os resultados foram expressos em % de aumento [do peso (g) / comprimento do tecido colônico (cm)], em relação ao grupo controle sem colite (47).

3.14 Avaliação das Lesões Macroscópicas e Comprimento do Colon

A análise macroscópica dos cólons foi realizada logo após a eutanásia dos camundongos. O cólon foi exposto, aberto longitudinalmente e os escores de lesão foram determinados de acordo com os achados macroscópicos descritos por Morris et al. (1989) (48), com modificações conforme Tabela II. O comprimento do cólon foi determinado com uma régua para avaliação do encurtamento do intestino.

Tabela II - Índices macroscópicos de colite segundo Morris et al. (1989), com modificações.

Escore	Achados macroscópicos
0	Sem danos
1	Hiperemia sem ulcerações
2	Ulcerações lineares sem inflamação significativa
3	Ulcerações lineares com inflamação em um local
4	Dois ou mais locais de inflamação e ulceração
5	Área da lesão > 1 cm ao longo do cólon
6-10	Área de lesão > 2 cm ao longo do comprimento do cólon. A quantificação é aumentanda em 1 para cada centímetro adicional

3.15 Avaliação das Lesões Microscópicas

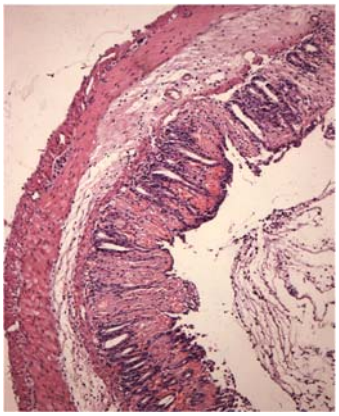
Amostras da porção distal do cólon foram coletadas em solução de formalina a 10%. Após fixação durante 24 horas, as amostras foram embebidas em parafina para técnica

histológica de rotina e cortes de 5 µm foram corados com hematoxilina / eosina. Em seguida, as lâminas contendo as amostras já coradas foram analisadas em microscópio de luz (Olympus OX31, Tokyo, Japan), acoplado à câmera digital (Lumenera Infinity 1, Ottawa, Canada). As alterações histopatológicas no cólon foram avaliadas de acordo com os critérios descritos por Appleyard e Wallace (1995)(49), com modificações conforme Tabela III e Figura 4. O escore de lesão microscópica final de cada amostra foi obtido pela soma dos escores determinados para cada um dos achados.

Tabela III - Índices microscópicos de colite segundo Appleyard e Wallace (1995), com modificações.

Escore	Achados microscópicos
0 – 3	Perda da arquitetura da mucosa
0 – 3	Infiltração celular
0 – 3	Espessamento da muscular
0 – 3	Formação de abscesso em cripta
0 – 3	Redução de células caliciformes

Figura 4 – Achados microscópicos de lesão no cólon.



Infiltração celular (a); Espessamento da muscular (b); Formação de abscesso em cripta (c); Redução de células caliciformes (d).

3.16 Dosagem de Citocinas

Amostras do tecido colônico foram coletadas e homogeneizadas em salina estéril. Após homogeneização, as amostras foram centrifugadas (0,8*g*, 4°C, 10 minutos) e o

sobrenadante foi utilizado para avaliar os níveis de citocinas por ELISA (enzyme-linked immunosorbent assay), utilizando kits ELISA Ready-SET-Go! (eBioscience). Primeiramente, as placas de 96 poços foram incubadas a 4°C por toda a noite com anticorpos contra as proteínas de interesse (10 µg/mL). No dia seguinte, as placas foram lavadas e incubadas durante 1 hora com solução de albumina bovina a 1%, no intuito de evitar ligações inespecíficas. Após esse bloqueio e lavagem das placas, as curvas-padrão de cada uma das citocinas analisadas e alíquotas das amostras foram adicionadas e incubadas a 4°C por 24 h. As placas foram novamente lavadas e os anticorpos policlonais diluídos foram adicionados (100 µl/poço). Após incubação em temperatura ambiente por 1 hora, as placas foram lavadas e 50 µl de enzima avidina-HRP diluída 1:5000 foi adicionada. Em seguida (trinta minutos após), 50 µl do reagente colorido OPD (solução 0,4 mg OPD – 0,4 µl H_2O_2 – 1 mL tampão) foi adicionado e as placas mantidas no escuro, em temperatura ambiente, por 15-20 min. A reação enzimática foi interrompida com H_2SO_4 (1 M, 50 µl/poço) e as absorbâncias determinadas em 450 nm (Multiskan GO ThermoScientific). A quantificação de proteína nos homogenatos do tecido colônico foi feita pelo método de Lowry (50) e os resultados expressos em pg de citocina por mg de proteína.

3.17 Determinação da Capacidade Redutora do Ferro (FRAP)

Este teste avalia a capacidade antioxidante através da redução do complexo Fe^{3+}-TPTZ (ferritripiridiltriazina) a Fe^{2+}-TPTZ (tripiridiltriazina ferroso) por antioxidantes doadores de elétrons. Esta capacidade redutora foi avaliada nas amostras do tecido colônico de acordo com Katalinic et al. (2005) (43), com adaptações do método para microplacas. Amostras da porção distal do cólon dos animais foram coletadas, homogeneizadas em solução de cloreto de potássio e centrifugadas a 0,2*g*, a 4°C, por 10 min. Alíquotas dos sobrenadantes reagiram com o reagente de FRAP (composto por tampão acetato de sódio 0,3 mM pH 3,6, TPTZ 10 mM em ácido hidroclorídrico 40 mM, e cloreto de ferro hexahidratado 20 mM) durante 6 minutos, e as absorvâncias foram determinadas a 595 nm (Multiskan GO ThermoScientific). A capacidade redutora das amostras foi determinada a partir de uma curva padrão de Trolox e os resultados foram expressos em nmol equivalente ao Trolox por mg de tecido, que representa a quantidade de Trolox (em nmol) com um potencial antioxidante equivalente a 1 mg do tecido.

3.18 Determinação da Capacidade Sequestradora do Radical ABTS+•

Este ensaio baseia-se na habilidade dos antioxidantes em sequestrar o radical cátion 2,2-azinobis (3-etilbenzotiazolina-6-sulfonato, sal de diamônio) - ABTS+•. Primeiramente, o

ABTS foi dissolvido em água para uma concentração final de 7 mM. Para gerar o radical cátion, foi adicionado persulfato de potássio 2,45 mM à solução de ABTS, e esta mistura foi mantida no escuro, a temperatura ambiente, por 12-16 horas. Esta solução do radical cátion ABTS+• foi diluída em quantidade suficiente de tampão fosfato de potássio (pH 7,4) até atingir uma absorvância de 0,8 a 730 nm (43). Amostras da porção distal do cólon dos animais foram coletadas, homogeneizadas em solução de cloreto de potássio e centrifugadas a 0,2*g*, a 4°C, por 10 min. Alíquotas dos sobrenadantes reagiram com a solução do radical cátion ABTS+• durante 6 minutos, e as absorvâncias foram determinadas a 730 nm (Multiskan GO ThermoScientific). A capacidade sequestradora do radical nas amostras foi determinada a partir de uma curva padrão de Trolox e os resultados foram expressos em nmol equivalente ao Trolox por mg de tecido.

3.19 Determinação da Glutationa Reduzida (GSH)

A GSH faz parte do sistema antioxidante endógeno e sua quantificação é um parâmetro de estresse oxidativo. Os níveis de GSH nas amostras da porção distal do cólon dos camundongos foram determinados por método espectrofotométrico previamente descrito, com algumas modificações (51). Amostras colônicas foram coletadas e armazenadas a -80°C. No dia do ensaio, as amostras foram homogeneizadas em solução de EDTA 0,02 M. Os homogenatos foram tratados com ácido tricloroacético 30% e centrifugados (1,5 g, 4°C, 15 min). Em seguida, adicionou-se tampão Tris-HCl 0,4 M (pH 8.9) às alíquotas do sobrenadante de cada amostra. Após homogeneização, 10 µl de DTNB (ácido ditionitrobenzóico) 0,01 M em metanol foi adicionado. A leitura da absorvância foi determinada após 5 min de reação, a 412 nm (Multiskan GO ThermoScientific). As quantidades de GSH nas amostras foram determinadas a partir de uma curva padrão de GSH e os resultados foram expressos em µM de GSH por mg de proteína.

3.20 Avaliação da Ativação do NF-κB

Os níveis da subunidade p65 do NF-kB total e fosforilado foram determinados nas amostras de tecido colônico por ELISA utilizando kit PathScan® (Cell Signaling) de acordo com as recomendações do fabricante. Os resultados foram expressos como densidade óptica das amostras (p65 total/ p65 fosforilado) a 450 nm (Multiskan GO ThermoScientific).

3.21 Análise Estatística

Os dados foram expressos como média ± erro padrão da média. As diferenças estatisticamente significativas entre os grupos foram determinadas através do teste paramétrico ANOVA de uma via seguida do pós-teste de Newman-Keuls. Para a análise dos escores micro e macroscópicos de lesão colônica, foi utilizado o teste não paramétrico de Kruskall-Wallis seguido pelo teste de Dunn. As análises estatísticas foram realizadas usando-se o software GraphPad Prism 5 (GraphPad Software Inc., San Diego, EUA). As diferenças foram consideradas estatisticamente significativas para valores correspondentes a $p < 0,05$, ou seja, nível de significância de pelo menos 5%.

4 RESULTADOS

4.1 Antioxidant and anti-inflammatory effects of hesperidin methyl chalcone in experimental ulcerative colitis

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Running title: HMC inhibits acetic-acid induced colitis.

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Abstract

Neutrophil infiltration, oxidative stress and pro-inflammatory cytokines have been implicated in development and progression of ulcerative colitis (UC), an inflammatory bowel disease (IBD) characterized by ulcerating inflammation of the mucosal layer generally restricted to the colon. The side effects, safety and human intolerance are limitations of the currently approved treatments for UC. Hesperidin methyl chalcone (HMC) is a flavonoid used to treat chronic venous disease, which shows anti-inflammatory and antioxidant properties in pre-clinical studies, however its effects on colitis have never been described. Therefore, we aimed to evaluate the protective effects of HMC in a mouse model of acetic acid-induced colitis. Treatment with HMC significantly reduced neutrophil infiltration, edema, colon shortening, macro and microscopic damages induced by intracolon administration of 7,5% acetic acid. The improvement of colitis after HMC treatment is related to the preservation of antioxidant capacity, contributing to the reduction of oxidative stress, and the inhibition of pro-inflammatory cytokines TNF-α, IL-6, IL-1β and IL-33 production associated to the reduction of NF-κB activation in the colon. Finally, these results demonstrate a novel applicability of HMC to reduce oxidative stress and inflammation during acetic acid-induced colitis suggesting it as a promising drug for treatment of ulcerative colitis.

Keywords: Hesperidin methyl chalcone; ulcerative colitis; oxidative stress; inflammation.

1. Introduction

The intestinal immune system faces a delicate task of providing a rapid and effective immune response against pathogenic bacteria, while maintaining tolerance towards food and microbiota antigens [1]. The imbalance between the intestinal immune system and the microbiota may result in aberrant inflammatory responses leading to chronic intestinal inflammation, such as inflammatory bowel disease (IBD) [1]. IBD is considered a chronic intermittent inflammatory process, in which active disease alternates with variable periods of remission [2], and includes Crohn's disease (CD) and Ulcerative colitis (UC), two distinct conditions with some different clinical presentations. Although both share many immunological features in common including increased cytokine secretion and accumulation of inflammatory cells in the affected areas, CD is a deep transmural inflammation that mostly occurs in the terminal ileum but can occur anywhere from the mouth to the anus, while UC is an ulcerating inflammation of the mucosal layer generally restricted to the colon [3]. Besides that, adaptive T-cell responses are thought to differ between the two diseases. T-helper (Th)1/Th17 responses dominate CD, whereas aberrant Th2 responses have been suggested to drive UC [4].

The uncontrolled immune system activation during UC is characterized by vasodilation, leukocyte infiltration, release of inflammatory mediators, such as TNF-α, IL-6, IL-1β, IL-33, and sustained overproduction of reactive oxygen and nitrogen metabolites, leading to tissue damage [5]. However, the severity of colitis can be modified therapeutically by drugs that influence neutrophil infiltration, free radicals generation, and pro-inflammatory agents production [6]. The goal of medical treatment in IBD is to suppress inflammation and induce mucosal healing. The current treatment regimen includes salicylate (e.g., sulfasalazine, mesalamine), corticosteroids (i.g., cortisone and budesonide), immunomodulators (e.g., thiopurines and methotrexate), and biologics (e.g., infliximab, adalimumab) [7], but the side effects, safety and human intolerance are limitations of the currently approved treatments for IBD. For this reason, many novel therapeutic alternatives have been investigated in experimental models of colitis and in humans [8].

Flavonoids are a class of bioactive polyphenolic compounds which are widespread in nature and display a number of biological activities, including antioxidant and anti-inflammatory properties. Citrus fruits, such as grapefruit (*Citrus paradisi*), orange (*Citrus sinensis*), tangerine (*Citrus reticulata*) and lemon (*Citrus limon*), are significant sources of flavonoids, mainly flavanones, which are present in both the juice and pericarp that are ingested when fruit segments are consumed [9]. Naringenin, quercetin, rutin, kaempferol and myricitrin are some examples of flavonoids used as treatments in rodent IBD models [10]. Hesperidin, a flavonoid belonging to the class of flavanone [11], ameliorate DSS-induced

experimental colitis by reducing colon injury, malondialdehyde (MDA) and myeloperoxidase (MPO) activity as well as suppressing IL-6 levels [12]. However, hesperidin presents low water solubility and is poorly absorbed in the small intestine as many other flavonoids [13]. The methylation process of hesperidin under alkaline conditions produces hesperidin methyl chalcone (HMC), which presents higher water solubility than hesperidin [14]. The increase of water solubility of flavonoids by methylation process can enhance the intestinal absorption, bioavailability and tissue distribution [15], thus, an improvement in the anti-inflammatory effect of HMC compared to hesperidin would be expected.

Hesperidin methyl chalcone (HMC) is widely used in pharmaceutical formulations associated to *Ruscus aculeatus* extract and ascorbic acid for the treatment of chronic venous disease [16]. HMC reduces the severity of symptoms and improve the quality of life of patients. Recently, our group has demonstrated the anti-inflammatory and antioxidant effects of HMC in different experimental models. Treatment with HMC reduced pain, paw edema, neutrophil recruitment, oxidative stress, cytokine production, and NF-kB activation during inflammation induced by carrageenan in mice [13]. Besides that, systemic and topical administration of HMC inhibits UVB induced skin oxidative stress and inflammation [17, 18]. Importantly, no signs of toxicity were found by HMC treatment during 7 days in mice [13], and it was reported that HMC is safe even when administrated in massive doses of 15 g daily in patients with abnormal capillary fragility [19]. However, to our knowledge, there is currently no scientific evidence regarding the ability of HMC to prevent and/or treat colitis.

The induction of colitis by acetic acid in mice is one standardized model of intestinal inflammation due to their simplicity and reproducibility, and the fact that it features some characteristics of human ulcerative colitis, such as colonic damage resulting in immune cell activation, prolonged infiltration of neutrophils, oxidative stress, and up-regulation of inflammatory mediators. Therefore, the present study was designed to evaluate the effects of HMC on neutrophil recruitment, edema, colon lesions and shortening, oxidative stress, cytokines production and NF-κB activation in a preclinical model of ulcerative colitis induced by acetic acid in mice.

2. Materials and methods

2.1 Materials

HMC was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Hexadecyltrimethylammonium bromide (HTAB), o-dianisidine dihydrochloride, Tween 80, reduced glutathione (GSH), EDTA, ferric chloride hexahydrate, 2,4,6-tri(2-pyridyl)-s-triazine, diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), Trolox, and potassium persulfate (dipotassium peroxidisulfate) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mouse TNF-α, IL-1β, IL-6, IL-33 and IL-10 ELISA kits were obtained from eBioscience (San Diego, CA, USA). Total and phospho NF-κB kits were obtained from Cell Signaling (Beverly, USA). Other reagents were of commercially available analytical grade.

2.2 Animals

Male Swiss mice (25 g) from the Londrina State University (Londrina, Brazil) were used in this study. Mice were kept in standard clear plastic cages with free access to tap water and food, and temperature-controlled room (21−24 °C) under a 12:12 h light/dark cycle. The Ethics Committee of the Londrina State University approved the animal care and handling procedures of this study (process number 1494.2015.56).

2.3 General experimental procedures

Colon inflammation was induced by a single intracolon administration of 7.5% acetic acid (200 µL) as previously described [20]. The colitis groups received per oral treatment with vehicle or HMC (10, 30 or 100 mg/kg; in saline) by gavage. In the negative control group, animals received intracolon saline (200 µL) instead of acetic acid solution and no treatment. Two experimental protocols were followed [20], as shown in Figure 1: (A) For assessment of inflammatory parameters, mice were treated 2 h before and 10 h after colitis induction, and they were euthanized at the 18th hour after induction. The distal colon was collected for myeloperoxidase activity assay, edema determination, macroscopic damage, colon length and microscopic damage evaluation, cytokine and NF-κB activation measurements. (B) To evaluate the ability of the colon samples to resist oxidative stress, mice were treated 6 h and 1 h before colitis induction and euthanized at the 4th hour after induction. The ferric reducing antioxidant power (FRAP), ABTS radical cation scavenging and reduced glutathione (GSH)

levels were determined in the samples of the distal colon. Experiments were performed twice.

2.4 Myeloperoxidase Activity Assay

The acetic acid-induced neutrophil recruitment to the colon of mice was evaluated by myeloperoxidase colorimetric assay [21]. Tissue samples were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 13.72 mM HTAB and homogenized using a Turrax IKA (T10 basic). The homogenates were centrifuged (16100*g*, 2 min, 4 °C) and the resulting supernatants were mixed with K_2HPO_4 buffer (50 mM, pH 6.0) containing o-dianisidine dihydrochloride (0.0167%) and hydrogen peroxide (0.05%). The absorbance was determined after 5 min at 450 nm (Multiskan GO ThermoScientific). The myeloperoxidase activity of samples was compared to a standard curve of neutrophils and presented as the number of neutrophils per mg of tissue.

2.5 Edema

Distal colon segments measuring 1 cm were weighed in order to determinate the colon weight/length ratio. The results were expressed in percentage of increase in colon weight (g)/ length (cm) ratios compared to a normal control group without colitis [22].

2.6 Macroscopic Damage Score and Colon Length

The colon was excised, opened longitudinally and the damage score was determined using the following criteria outlined by [23], with modifications: no damage (score 0); localized hyperemia but no ulcers (score 1); linear ulcers with no significant inflammation (score 2); linear ulcers with inflammation at one site (score 3); two or more sites of ulceration and inflammation (score 4); one site of inflammation >1 cm along the length of the colon (score 5); site of inflammation >2 cm along the length of the colon, with quantification increased by 1 for each additional centimeter (score 6−10). The length of the colon was measured with a ruler for evaluation of intestinal shortening.

2.7 Microscopic Damage Score

Formalin-fixed distal colon samples were embedded in paraffin and sections (5 µm) were stained with hematoxylin and eosin. The stained tissues were observed with a light microscope (Olympus OX31, Tokyo, Japan), coupled with a digital camera (Lumenera Infinity

1, Ottawa, Canada). The histological assessment of damage was graded semiquantitatively as described previously [24], with modifications: (1) loss of mucosal architecture (score 0−3), (2) cellular infiltration (score 0−3), (3) muscle thickening (score 0−3), (4) crypt abscess formation (score 0−3), and (5) goblet cell depletion (score 0−3). The final score was determined by adding the scores above for each of the samples.

2.8 Total antioxidant capacity assays

The ability of samples to resist oxidative damage was determined by its ferric reducing antioxidant power (FRAP assay) and free radical scavenging (ABTS assay) property. The tests were adapted to a 96-well microplate format from previously described assays [25]. Colon tissue samples were homogenized in KCl solution (1.15%), centrifuged (0.2*g*, 4 °C, 10 min) and the supernatant aliquots were used in both assays. For FRAP assay, the supernatants were mixed with the FRAP reagent and the absorbance was measured at 595 nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland) after 6 min of incubation. For ABTS assay, diluted ABTS solution was mixed with the sample supernatant and after 6 min of incubation, the absorbance was measured at 730 nm (Multiskan GO ThermoScientific). The results of ABTS and FRAP assays were equated against a standard Trolox curve (0.4–400 nmol).

2.9 Reduced glutathione (GSH) Assay

The GSH levels were determined in colon samples using a spectrophotometric method [26], with modification. The samples were homogenized in 0.02 M EDTA solution, treated with 30% trichloroacetic acid and centrifuged at 1.5*g* at 4 °C for 15 min. The supernatant was mixed with 0.4 M Tris-HCl (pH 8.9) and 0.01 M dithiobisnitrobenzoic acid. After 5 min, the absorbance was determined at 412 nm (Multiskan GO ThermoScientific). Protein levels in the colon homogenates was measured using the Lowry method [27]. The results were expressed as μmol of GSH per mg of protein, using a standard GSH curve (2–2000 nmol).

2.10 Cytokine Measurements

Samples of the distal colon were collected and homogenized in sterile saline. The homogenates were centrifuged at 0.8*g* at 4 °C for 10 min, and TNF-α, IL-1β, IL-6, IL-33 and IL-10 levels were evaluated using ELISA Ready-SET-Go! kits (eBioscience) according to the manufacturer’s recommendations. Protein levels in the colon homogenates were measured

using the Lowry method [27]. The results were expressed as pg of cytokine per mg of protein.

2.11 NF-κB Activity

The levels of phosphorylated and total NF-κB p65 subunit in colon samples were determined by ELISA using PathScan® kits (Cell Signaling) according to the manufacturer's recommendations. The results are expressed as OD of samples (total p65/phospho-p65 ratio) at 450 nm (Multiskan GO ThermoScientific).

2.12 Statistical Analysis

All data are expressed as means ± SEM. Statistical significance of differences between the groups was determined by ANOVA followed by the Newman Keuls's test. For categorical variables, the Kruskal−Wallis test followed by Dunn's test was performed. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). The criterion for significance was set at $p < 0.05$.

3. Results

3.1 HMC reduces acetic acid-induced colon inflammatory myeloperoxidase activity

Myeloperoxidase is secreted by activated neutrophils at the site of inflammation [28] and its quantification is directly proportional to tissue neutrophil content. The anti-inflammatory effect of HMC in acetic acid-induced colitis was firstly determined through neutrophil recruitment evaluation in the colon (Figure 2). Acetic acid (7.5%, 200 µl, in saline) administration induced neutrophil recruitment to the colon of mice compared to negative control group (36.382 ± 3.146 neutrophils/mg of tissue and 6.109 ± 1.442 neutrophils/mg of tissue, respectively, $p< 0.05$). It is a common feature of ulcerative colitis and corroborates with previous results [3, 29]. Furthermore, treatment with HMC at 30 and 100 mg/kg, but not at 10 mg/kg (p.o., in saline), significantly ($p< 0.05$) reduced neutrophil recruitment induced by acetic acid (19.775 ± 3.502 neutrophils/mg of tissue and 20.916 ± 5.059 neutrophils/mg of tissue, respectively) (Figure 2), suggesting its capacity of modulating the inflammatory process in terms of neutrophils recruitment during ulcerative colitis. Thus, as there was no difference between the treatments with 30 and 100 mg/kg, we chose the lowest dose of HMC (30 mg/kg) that significantly reduced neutrophil recruitment for the following experiments that evaluate the effect of HMC in acetic acid-induced colitis.

3.2 HMC reduces acetic acid-induced weight/length ratio (edema) in the colon

The wet weight of the inflamed colon tissue can be considered an indicator of the severity and extent of inflammatory response [30]. The administration of acetic acid (7.5%, 200 µl, in saline) induced significant edema in the colon of mice compared to negative control group. On the other hand, the treatment with HMC (30 mg/kg, p.o., in saline) significantly reduced the acetic acid-induced inflammatory edema of the colon compared to colitis control group (32,01 ± 11,09% and 81,60 ± 16,25%, respectively, $p< 0.05$) (Figure 3).

3.3 HMC improves macroscopic lesions and prevents colon shortening induced by acetic acid

Macroscopically, acetic acid (7.5%, 200 µl, in saline) caused severe inflammation in the colon besides causing its shortening. The negative control group presented no macroscopic damage while colitis control group showed sites of inflammation > 1 cm along the length of the colon (score of 0 and mean score of 5.5, respectively, $p< 0.05$) (Figure 4A, 4c1 and 4c2). Besides, there was colon shortening in colitis control group compared to negative control

group (6,03 ± 0,18 cm and 9,41 ± 0,29 cm, respectively, $p< 0.05$) (Figure 4B, 4c1 and 4c2). The treatment with HMC (30 mg/kg, p.o., in saline) attenuated the macroscopic lesions (mean score of 2.5) and prevents colon shortening (7,83 ± 0,42, $p< 0.05$) compared to colitis control group (Figure 4A, 4B and 4c3).

3.4 HMC prevents acetic acid-induced microscopic lesions in the colon

We analyzed tissue morphology by hematoxylin/eosin (H/E) staining of paraffin embedded colon sections. Negative control group showed the expected morphology (Figure 5A), with mucosal architecture preserved and no significant changes related to cellular infiltration, muscle thickening, crypt abscess formation and goblet cell depletion. The intracolon administration of acetic acid (7.5%, 200 µl, in saline) led to the typical signs of colitis, with lost of mucosal architecture, cellular infiltration, muscle thickening and goblet cell depletion (Figure 5B). The treatment with HMC (30 mg/kg, p.o., in saline) clearly diminished the acetic acid-induced morphological changes (Figure 5C), improving the mucosal architecture, reducing cellular infiltration and preserving goblet cells. The histopathological score of colon inflammation was higher in colitis control group compared to negative control group (mean score of 10.5 and score of 0, respectively, $p< 0.05$). However, HMC (30 mg/kg, p.o., in saline) significantly reversed the lesion scores (mean score of 4, $p< 0.05$) (Figure 5D).

3.5 HMC reduces acetic acid-induced oxidative stress

Since oxidative stress is another important feature of colitis, partly as a consequence of excessive neutrophil recruitment [31], we analyzed the antioxidant capacity on colon samples. Acetic acid (7.5%, 200 µl, in saline) administration induce severe oxidative stress in the colon of mice, resulting in decrease of total antioxidant capacity, assessed by FRAP (3.81 ± 0.22 nmol Trolox equiv/mg tissue, $p< 0.05$) and ABTS radical cation scavenging (10.34 ± 0.50 nmol Trolox equiv/mg tissue, $p< 0.05$), and GSH levels (0.44 ± 0.03 µmol/mg protein, $p< 0.05$). HMC (30 mg/kg, p.o., in saline) treatment prevent the reduction of FRAP (5.77 ± 0.44 nmol Trolox equiv/mg tissue, $p< 0.05$), ABTS scavenging (13.46 ± 1.03 nmol Trolox equiv/mg tissue, $p< 0.05$) and GSH levels (0.66 ± 0.06 µmol/mg protein, $p< 0.05$) induced by acetic acid (Figure 6A, 6B and 6C, respectively).

3.6. HMC reduces acetic acid-induced colon inflammation by targeting cytokine production and NF-κB activation

Inflammatory cytokines are cardinal biomarkers released in intestinal mucosa upon hyper-activation of transcription factors such as NF-κB in immune cells in IBD [32]. Therefore, we investigated the effects of HMC on TNF-α, IL-1β, IL-6, IL-33 and IL-10 levels in the colon after acetic acid administration (Figure 7). We also evaluated the activation of NF-κB in colon samples (Figure 8). The levels of TNF-α (45.55 ± 7.16 pg/mg protein), IL-1β (108.45 ± 30.82 pg/mg protein), IL-6 (84.88 ± 4.16 pg/mg protein) and IL-33 (239.48 ± 51.90 pg/mg protein) were elevated in colitis control group compared to negative control group. On the other hand, the treatment with HMC (30 mg/kg, p.o., in saline) significantly ($p< 0.05$) reduced acetic acid-induced TNF-α (21.61 ± 4.34 pg/mg protein; Figure 7A), IL-1β (49.79 ± 14.78 pg/mg protein; Figure 7B), IL-6 (37.99 ± 11.53 pg/mg protein; Figure 7C) and IL-33 (116.83 ± 24.49 pg/mg; Figure 7D) production in the colon compared to acetic acid control group (7.5%, 200 µl, in saline). The acetic acid administration also reduced the colon levels of IL-10 (30.00 ± 2.48 pg/mg protein), and although we can see a trend towards increase of IL-10 production by HMC treatment (30 mg/kg, p.o., in saline), this effect was not statistically significant (37.61 ± 4.26 pg/mg protein). The colon inflammation induced by acetic acid is characterized by increased activation of NF-κB compared to negative control group, and treatment with HMC (30 mg/kg, p.o., in saline) also significantly reduced the activation of this transcription factor (Figure 8).

4. Discussion

Hesperidin is a flavonoid, belonging to flavanones class, widely distributed in citrus fruits and juices, the major sources of flavanones intake by humans. The process of methylation of hesperidin to obtain HMC increases the water solubility of this flavonoid, improving its intestinal absorption [15]. The HMC has been used clinically in pharmaceutical formulations associated to *Ruscus aculeatus* extract and ascorbic acid for the treatment of chronic venous disease [16]. Treatment with HMC significantly reduced neutrophil infiltration, edema, macroscopic and microscopic colon damage, colon shortening, oxidative stress and pro-inflammatory cytokine production by decreasing NF-κB activation in the colon. Therefore, the present data demonstrate, to our knowledge, the first evidence that HMC inhibits acetic acid-induced colitis in mice and its mechanisms.

Firstly, we observed that oral treatment with HMC dose-dependently inhibited acetic acid-induced MPO activity increase in the colon. MPO serves as an indirect marker of neutrophil counts in inflamed gastrointestinal tissue [33]. A dose selected in this dose-response curve was used in the following experiments that demonstrated HMC also inhibited acetic acid-induced colon edema. Corroborating these results, HMC inhibits carrageenan- and capsaicin-induced paw skin neutrophil recruitment and paw edema [13], and ultraviolet B irradiation-induced skin MPO activity and edema [17, 18]. Neutrophils contribute markedly to tissue damage and mucosal dysfunction in UC [32]. Histologic examination of biopsies obtained from patients with active disease reveal the presence of large numbers of leukocytes such as neutrophils, monocytes, and lymphocytes in the colon. Concurrently with this inflammatory infiltrate, there is extensive mucosal and/or transmural injury including edema, loss of goblet cells, decreased mucous production, crypt cell hyperplasia, erosions, and ulcerations [5]. Macroscopically, intracolon administration of acetic acid induced severe and extensive inflammation and significant colon shortening as previously described [20]. Histological evaluation of colon confirmed the development of inflammation in the colitis control group, with significant destruction of mucosal crypt, cellular infiltration, muscle thickening and goblet cell depletion. On the other hand, the treatment with HMC significantly attenuated the severity and extent of macroscopic damage and colon shortening as well as preserved the colon crypts and globet cell, and reduced cellular infiltration into colon tissue. Therefore, HMC consistently inhibited acetic acid-induced colon damage. These findings are corroborated by studies demonstrating that flavonoids such as hesperidin [12], naringenin [34], quercetin [20] and rutin [35] inhibited colon damage in experimental models of colitis.

Activated neutrophils produce reactive oxygen (ROS) and nitrogen (RNS) species within intestinal mucosa. Both ROS and RNS at low and moderate concentrations are signalling molecules involved in mitogenic response as well as in defense against infectious agents [6]. However, excessive production of ROS and RNS or their inefficient scavenging leads to tissue oxidative and nitrosative stress, respectively, which play a significant role in the pathogenesis of ulcerative colitis. Furthermore, ROS are also key regulators of neutrophil chemotaxis [36] and induce pro-inflammatory cytokine production [37], [38]. The inflammatory response initiated by acetic acid increases ROS production which in turn upregulates hydroxyl radicals and peroxide production leading to mucosal erosion and loss of crypts [32]. Our results support this concept showing that acetic acid induces oxidative stress in the colon of animals while increased neutrophils recruitment and tissue damage are also observed. In agreement with other studies demonstrating the antioxidant capacity of HMC during inflammation induced by carrageenan [13], and skin damage induced by ultraviolet B irradiation [17], we observed that HMC reduces oxidative stress by increasing the total antioxidant capacity and GSH levels in the colon. Accordingly, the inhibition of oxidative stress by HMC contributed to reduced neutrophil infiltration and mucosal damage in inflamed tissue, reflecting the antioxidant and anti-inflammatory activity of HMC in acetic acid-induced colitis.

Endogenous antioxidants are part of a compensatory system to limit oxidative stress-induced tissue damage and inflammation consisting of enzymatic (such as catalase, superoxide dismutase and glutathione peroxidase) and non-enzymatic (such as reduced glutathione; GSH) molecules antioxidants [39]. Nevertheless, the endogenous antioxidant mechanism can be overwhelmed in inflammatory diseases. For instance, neutrophils produce superoxide anion via NOX2 during inflammation resulting in endogenous antioxidant mechanisms depletion, including GSH [13]. GSH is a water-soluble tripeptide composed of the amino acids glutamine, cysteine and glycine, containing the cysteine-derived thiol group, which is a potent reducing agent. GSH is highly abundant in the cytoplasm (1–11mM), nucleus (3–15 mM) and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments [6]. In its reduced form, GSH is capable of scavenging ROS/RNS, thereby contributing to the control of redox homeostasis. Like many cysteine-containing molecules, GSH is readily oxidized when ROS donate one electron to its molecule, forming a disulfide bridge between two GSH molecules that yield the oxidized dimer (GSSG) [40]. Sufficient concentration of GSH in the jejunal and colonic epithelial cells prevents tissue degradation by eliminating harmful peroxides, while loss of GSH/GSSG redox balance contributes to tissue hyperplasia, mucosal inflammation, and symptoms of clinical colitis [41].

Oxidative stress besides playing a key role in the development of intestinal damage during IBD, also causes deregulated redox signaling, leading to activation of NF-kB and the

subsequent overexpression of pro-inflammatory cytokines and further boosting ROS production-related enzymes [2]. In this sense, pro-inflammatory cytokines, such as TNF-α, as well as ROS and RNS have a long-standing implication in both the etiology and the progression of UC [6]. TNF-α, released by macrophages, DCs and T cells, may exert various pro-inflammatory functions in colitis by binding to its receptor TNFR1 and initiating a cascade of events including the activation of transcription factors such as mitogen-activated protein kinases (MAPKs) and nuclear factor-kappaB (NF-kB), thus activating the gene transcription of cytokines (such as IL-1β, IL-6 and TNF-α), chemokines and adhesion molecules [42], known to contribute to the pathogenesis of IBD. TNF-α also induces stromal cell differentiation into activated myofibroblasts, which together with lamina propria mononuclear cells (LPMCs), produce large and unbalanced amounts of matrix metalloproteinases (MMPs), digesting the extracellular matrix and the basement membrane, determining the apoptosis of epithelial cells and the development of ulcers [43].

The clinical efficacy of the anti-TNF-α monoclonal antibodies supports the crucial pathogenic role of TNF-α in UC [43]. However, approximately one-third of patients do not respond to anti-TNF-α therapy, and of those who initially benefit, the majority eventually lose their response to treatment or develop intolerance to one or more of the medications within the anti-TNF class [44]. In addition, increased incidence of severe infections and malignancies are associated to patients treated with anti-TNF-α, emphasizing the need for further therapies [44]. We observed significant reduction in TNF-α levels by treatment with HMC during acetic acid-induced colitis, showing the anti-TNF-α activity of this drug associated to its anti-inflammatory property. In agreement, other flavanones such as naringenin, eriodictyol and hesperetin also are capable of inhibiting TNF-α release [42, 45, 46]. Luteolin, luteolin-7-glucoside and quercetin all inhibited LPS-induced TNF-α and IL-6 release in RAW 264.7 cells [42].

The IL-6 levels are elevated in patients with IBD and in experimental colitis [47], including acetic acid-induced colitis model used herein. IL-6 binds to the soluble IL-6R (sIL-6R), and by a trans-signaling mechanism, the IL-6–sIL-6R complex activates intestinal target cells, including antigen presenting cells and T cells, by binding to the gp130 surface molecule (also known as IL-6R subunit-β) [48]. The IL-6–sIL-6R complex prevents apoptosis of mucosal T cells and activates pro-inflammatory cytokine production by these cells [47]. Moreover, the IL-6/STAT3 axis of JAK/STAT signaling is believed to be involved in the transition of inflammatory lesions to tumors leading to colitis-associated cancer [49], a common neoplastic complication of long-term UC. Importantly, the blockade of IL-6 signaling ameliorated colitis in mouse models and also had beneficial effects in a clinical trial of patients with Crohn’s disease [50].

The IL-1 family consists of 11 molecules, including IL-1β and IL-33, that are centrally involved in regulating inflammatory responses [51]. IL-1β is a pleiotropic cytokine produced by cells of the immune system (macrophages, dendritic cells, monocytes) and from non-immune cells such as epithelial cells, involved in the pathology of several chronic conditions including UC. Caspase-1 regulates the cleavage of inactive pro-IL-1β to active mature IL-1β upon nucleotide oligomerization domain (NOD)-like receptors (NLRs)-stimulation [52]. By binding to its receptor IL-1R1, IL-1β recruits MyD88 and phosphorylates several kinases (IRAK), culminating to the translocation of NF-kB to the nucleus, thereby activating pro-inflammatory genes [52], and adhesion molecule expression to promote the adhesion and transmigration of leukocytes [53]. IL-33 exerts its biological effects through the binding to its receptor ST2, also known as IL-1 receptor-like 1 (IL1RL1), to activate NF-kB and mytogen-activated protein kinases, resulting in inflammatory cytokine production and cellular activation [54]. In this sense, mice treated with recombinant (r)IL-33 displayed epithelial hyperplasia and eosinophil/neutrophil infiltration in the colonic mucosa [55]. Therefore, increased levels of IL-1β and IL-33 contribute to perpetuation of inflammatory response in IBD. The levels of IL-1β and IL-33 are increased in experimental models of colitis induced by acetic acid [20], and in patients with Crohn's disease and UC. The established clinical relevance of targeting TNF-α, IL-6 and IL-1β in IBD, further support the importance of the HMC inhibition of acetic acid-induced TNF-α, IL-6, IL-1β and IL-33 in the colon. Furthermore, these four cytokines induce NF-kB activation [42][51][53], which is consistent with the HMC inhibition of acetic acid-induced activation of NF-κB in the colon.

However, progression and flares of IBD are not only due to increased production of pro-inflammatory mediators such as free radicals, TNF-α, IL-6, IL-1β and IL-33. The balanced production of the anti-inflammatory cytokine IL-10 also presents a relevant role. IL-10 is a pleiotropic cytokine that suppresses pro-inflammatory cytokine production by antigen-presenting cells and T cells and induces STAT3 signaling in regulatory T cells [48]. IL-10-deficient mice shows enhanced responses to intestinal bacterial antigens, which induce severe colitis [56]. Similarly, in humans rare genetic deficiencies in IL-10, IL-10R or its downstream signaling cascade lead to loss of tolerance to intestinal microbial antigens, contributing to development of IBD [57]. HMC treatment reduced IL-10 production induced by UVB radiation [17, 18] and carrageenan [13]. The colon administration of acetic acid solution induces a reduction of IL-10 production, and treatment with HMC at a dose of 30 mg/kg showed a tendency ($p = 0.0655$) to increase its levels, but there was no statistical difference compared to the colitis control group.

In conclusion, the present data demonstrates the anti-inflammatory effect of HMC in acetic acid-induced colitis. HMC inhibited colitis-induced tissue oxidative stress, inflammatory cells infiltration and pro-inflammatory cytokines production by inhibiting NF-kB activation. HMC

also significantly reduces colon edema, macroscopic lesions, colon shortening and histological damage, showing a significant improvement in the colon inflammation. Taken together, these results suggest the potential applicability of oral treatment with HMC in inflammatory diseases such as IBD.

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Figures

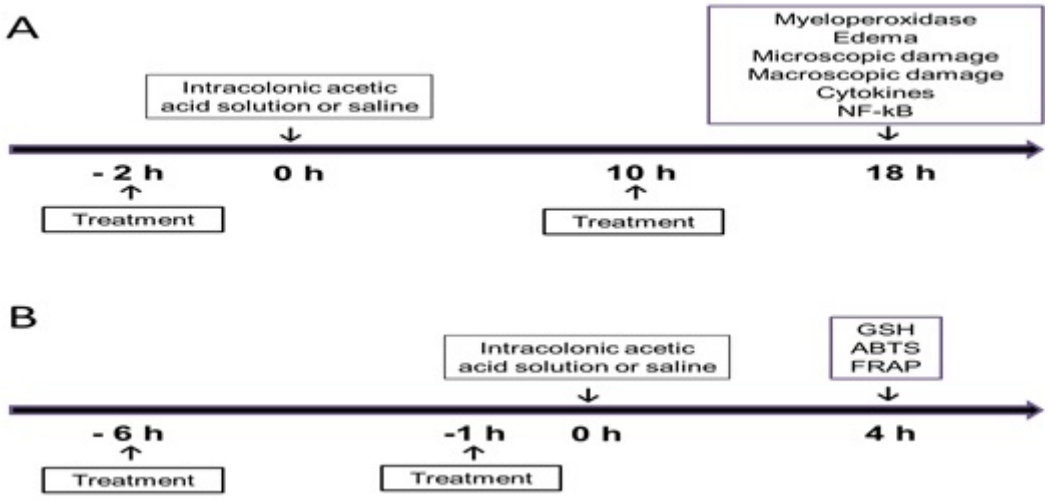


Figure 1. Schematic representation of experimental protocols for treatments and colitis induction. For assessment of inflammatory parameters, the mice were treated 2 hours before and 10 hours after colitis induction, and colonic samples were collected on the 18 hour (A). To evaluate the oxidative stress in the colon, the mice were treated at 6 and 1 hour before colitis induction, and colonic samples were collected on the 4 hour (B) [20].

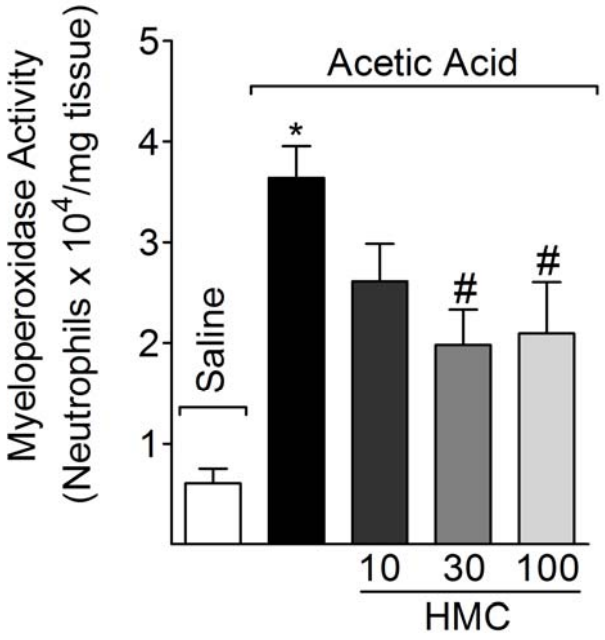


Figure 2. HMC reduces neutrophil recruitment to the colon of mice with acetic acid-induced colitis. Mice were treated with HMC (10, 30 or 100 mg/kg, p.o.), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). Myeloperoxidase activity was evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

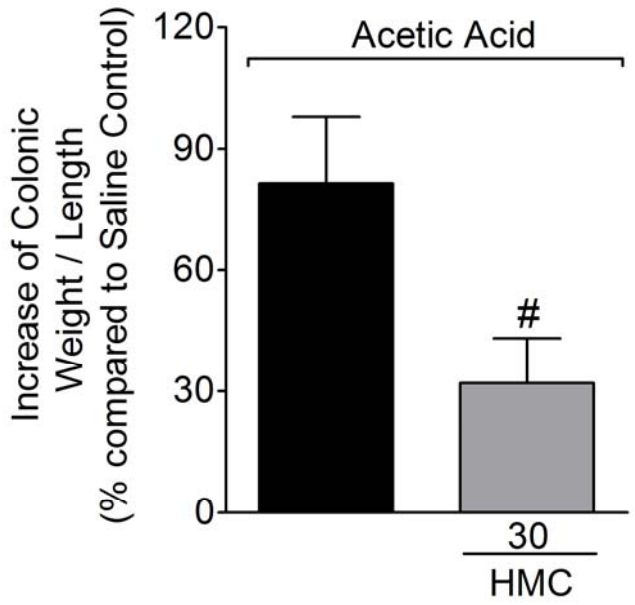


Figure 3. HMC reduces weight / length (edema) in the colon of mice with acetic acid-induced colitis. Mice were treated with HMC (30 mg/kg, p.o.), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). Edema was evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

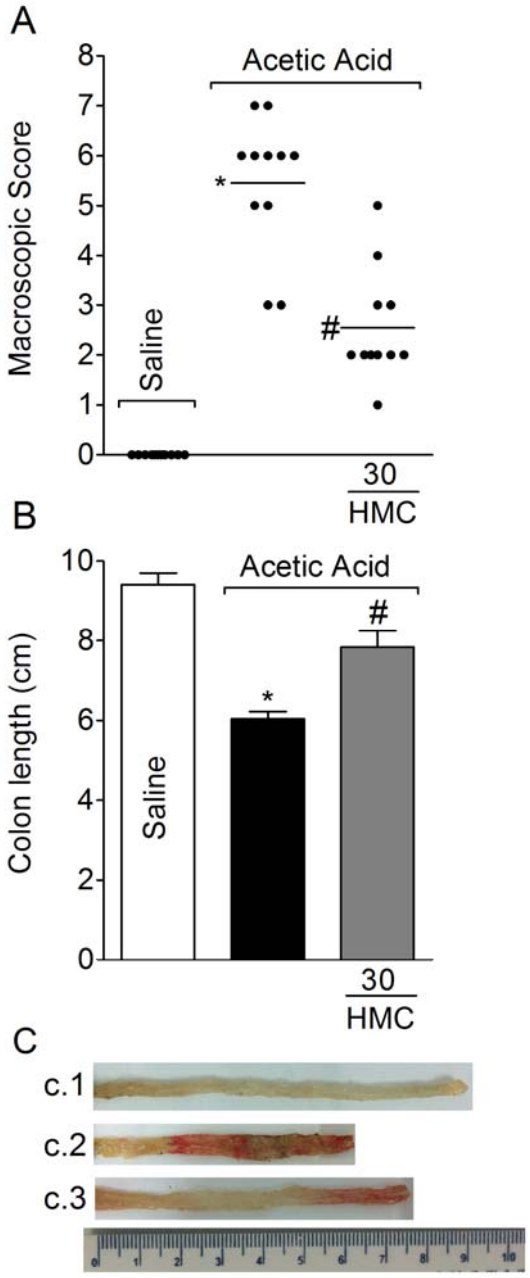


Figure 4. HMC reduces macroscopic damage and prevents the reduction of colon length in mice with acetic acid-induced colitis. Mice were treated with HMC (30 mg/kg, p.o.), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). The macroscopic damage score (A) and colon length (B) were evaluated 18 hours after colitis induction in the negative control group (c.1), colitis control group (c.2) and HMC treated group (c.3). n=11 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test for macroscopic damage score and ANOVA followed by Newman-Keuls's test for colon length.

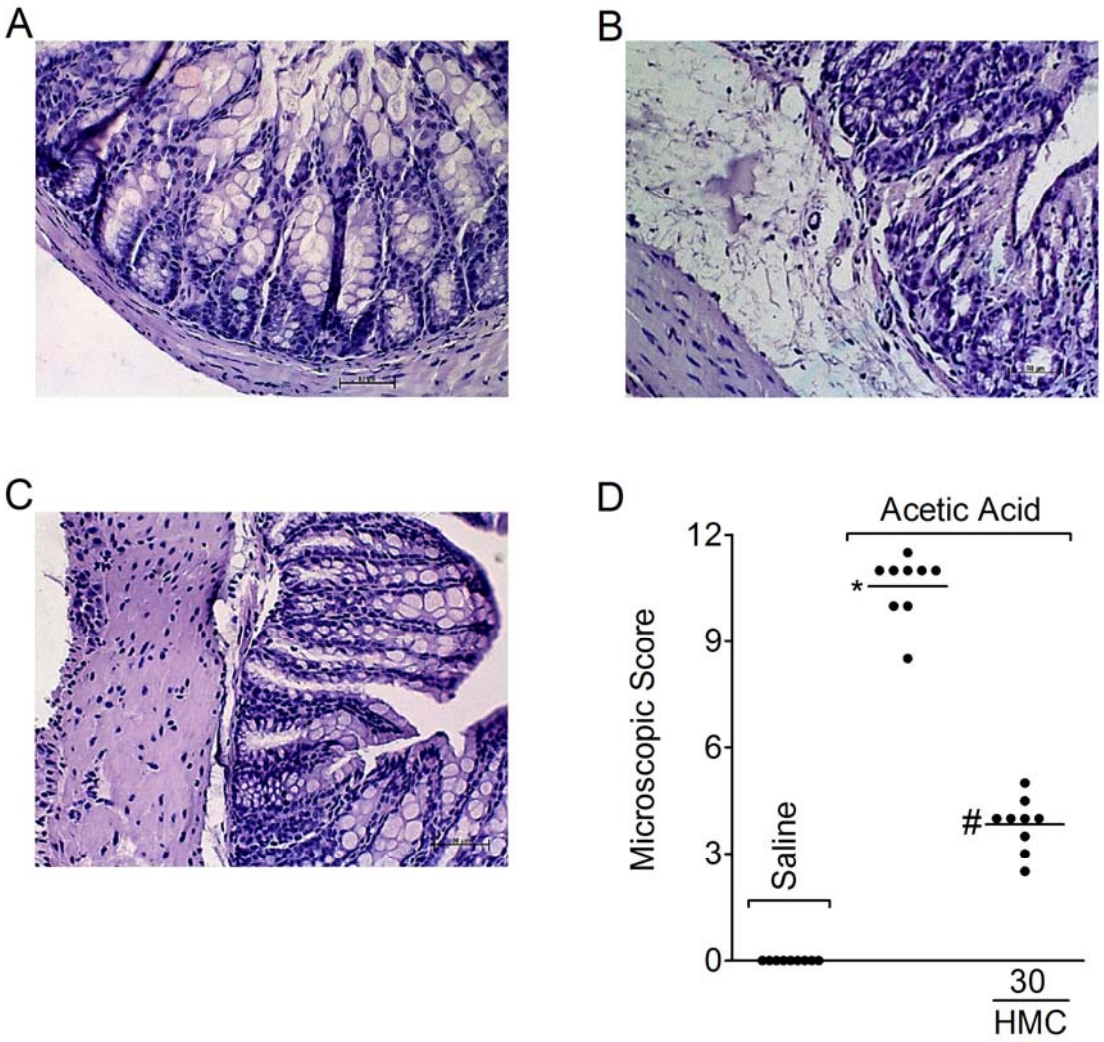


Figure 5. HMC reduces microscopic damage in the colon of mice with acetic acid-induced colitis. Mice were treated with HMC (30 mg/kg, p.o.), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). The microscopic damage score (D) were evaluated 18 hours after colitis induction in the negative control group (A), colitis control group (B) and HMC treated group (C). n=9 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test.

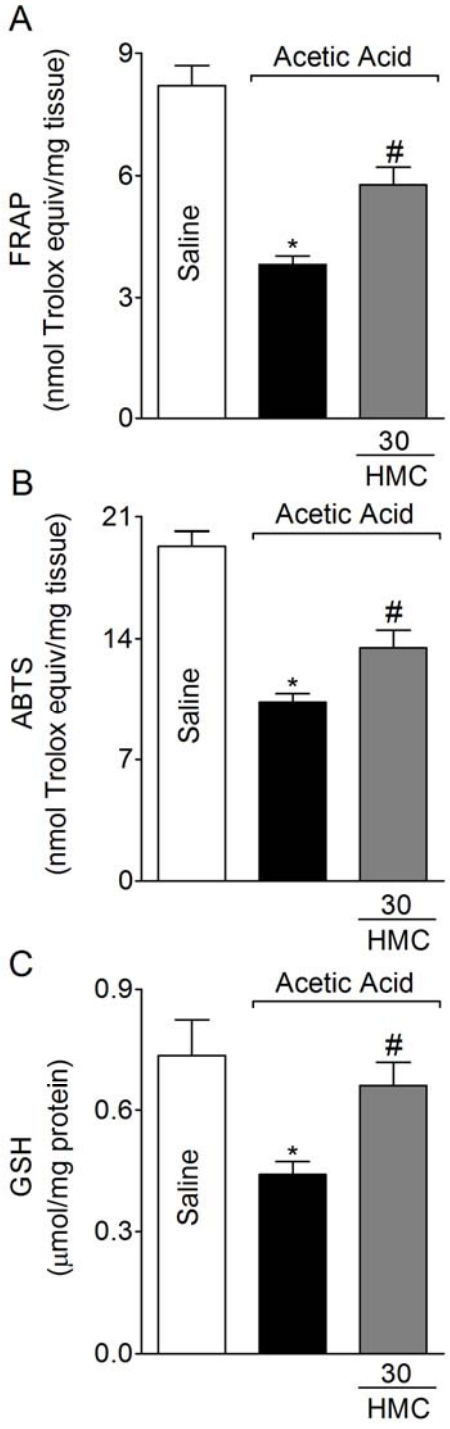


Figure 6. HMC improves antioxidant capacity in the colon of mice with acetic acid-induced colitis. Mice were treated with HMC (30 mg/kg, p.o.), 6 and 1 h before intracolon administration of acetic acid (7,5%, 200µL). The ferric reducing antioxidant power - FRAP (A), ABTS radical scavenging ability (B) and reduced GSH levels (C) were determined 4 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

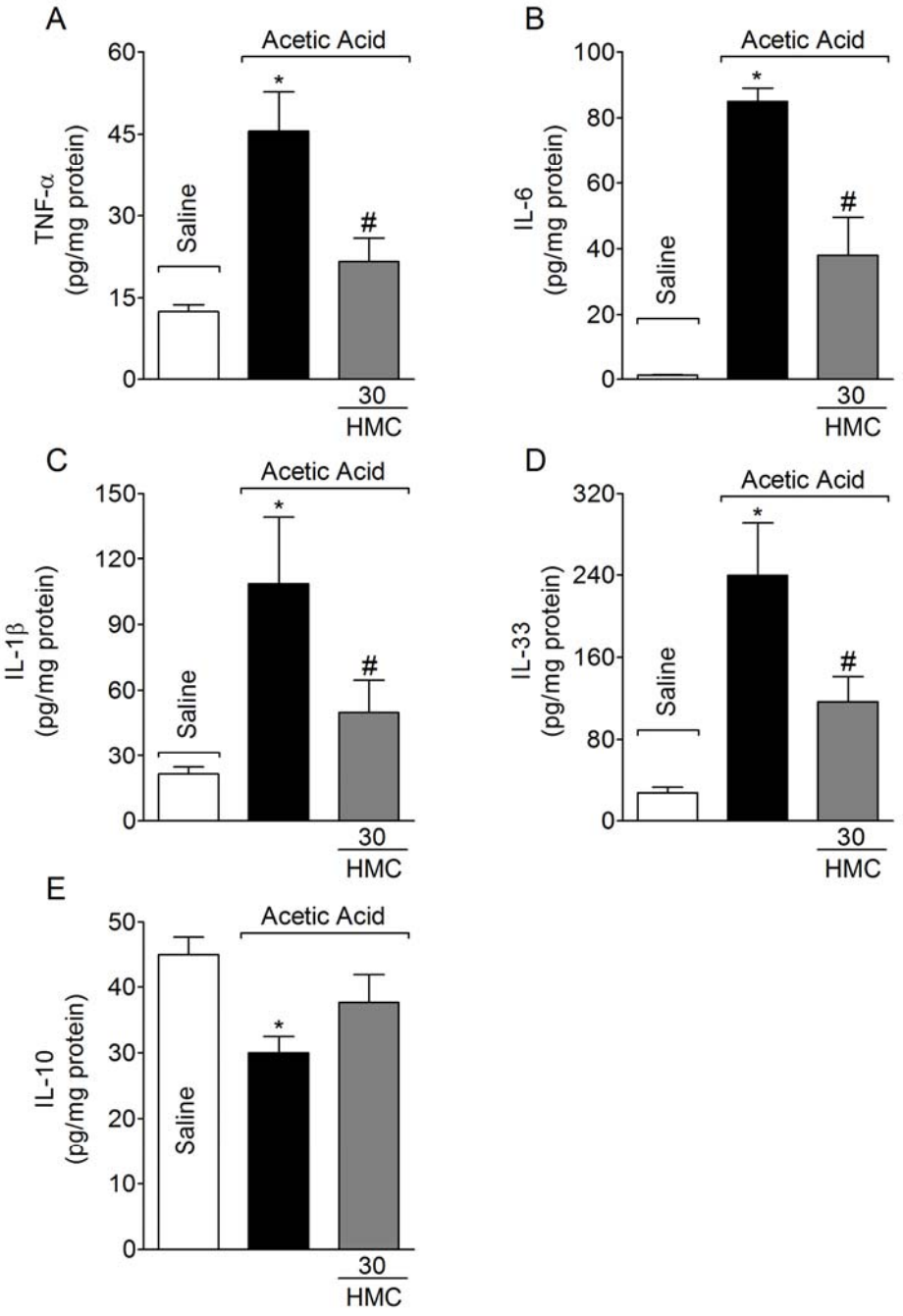


Figure 7. HMC reduces pro-inflammatory cytokines production in the colon of mice with acetic acid-induced colitis. Mice were treated with HMC (30 mg/kg, p.o.), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). TNF-α (A), IL-6 (B), IL-1β (C), IL-33 (D) and IL-10 (E) levels were evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

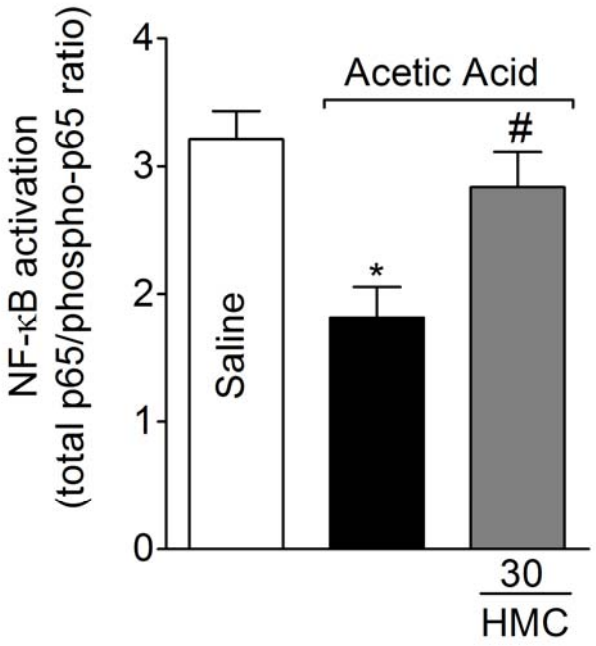


Figure 8. HMC inhibits NF-kB activation in the colon of mice with acetic acid-induced colitis. Mice were treated with HMC (30 mg/kg, p.o.), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200μL). The NF-kB activation was determined 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

4.2 Desenvolvimento de Microcápsulas de Hesperidina Metil Chalcona

Foram desenvolvidas duas formulações microencapsuladas contendo HMC (MH) com diferentes proporções fármaco:polímeros (MH1:5 e MH1:3). O processo de microencapsulação da HMC resultou em microcápsulas com eficiência de encapsulação de 46% e 39%, respectivamente.

A distribuição do tamanho de partículas de ambas as formulações foi semelhante, sendo de 0.60 ± 1.48 μm para MH1:5 e de 0.56 ± 1.69 μm para MH1:3 (Figura 1). A HMC é uma substância com propriedade amorfa (Figura 2A). O complexo polimérico de pectina e caseína utilizado no preparo das microcápsulas também apresenta propriedade amorfa. Por este motivo não detectamos diferença entre o fármaco isolado (Figura 2A) e as formulações microencapsuladas (MH1:5 – Figura 2B e MH1:3 – Figura 2C) no difratograma obtido por difração de raios X.

Já na análise térmica (DSC; Figura 3), observamos que as formulações microencapsuladas (MH1:5 e MH1:3) não apresentam o pico de degradação da HMC próximo a 500 graus, então provavelmente houve a mistura ou revestimento do fármaco (HMC) pelo complexo polimérico.

Em relação a dissolução, ambas as formulações, MH1:5 e MH1:3, apresentaram o mesmo perfil, com liberação de aproximadamente 100% do fármaco dentro de 240 minutos em meio que simula as condições do suco entérico (Figura 4). *In vivo*, o tratamento com as formulações microencapsuladas MH1:5 e MH1:3 não proporcionaram melhora alguma no recrutamento de neutrófilos para o tecido colônico nas doses propostas (10 mg/kg), não evidenciando assim, qualquer efeito adicional ao tratamento com o fármaco não microencapsulado na mesma dose em modelo de colite ulcerativa induzida por ácido acético em camundongos.

Portanto, o desenvolvimento das formulações microencapsuladas contendo HMC deve ser aprimorado utilizando uma técnica para encapsulação de fármacos hidrossolúveis (como coacervação em fase orgânica, extrusão com alginato ou pectinato de cálcio, emulsificação) (52) a fim de se obter microcápsulas com maior eficiência de encapsulação e que permitam liberação modificada do fármaco hidrossolúvel, para então testá-las em modelo experimental de colite e verificar se há ou não melhora no efeito terapêutico do fármaco quando este é veiculado em microcápsulas de liberação modificada.

Figures

Figura 1 - Distribuição do tamanho de partícula da MH1:5 (A) e MH1:3 (B).

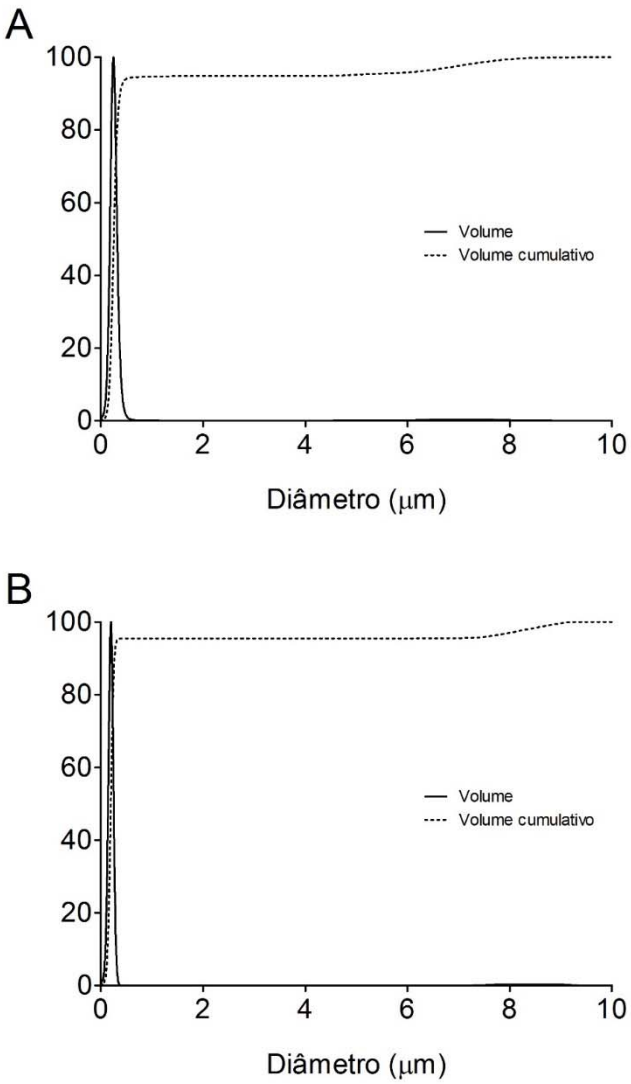


Figura 2 - Difratogramas de raios X da HMC (A), MH1:5 (B) e MH1:3 (C).

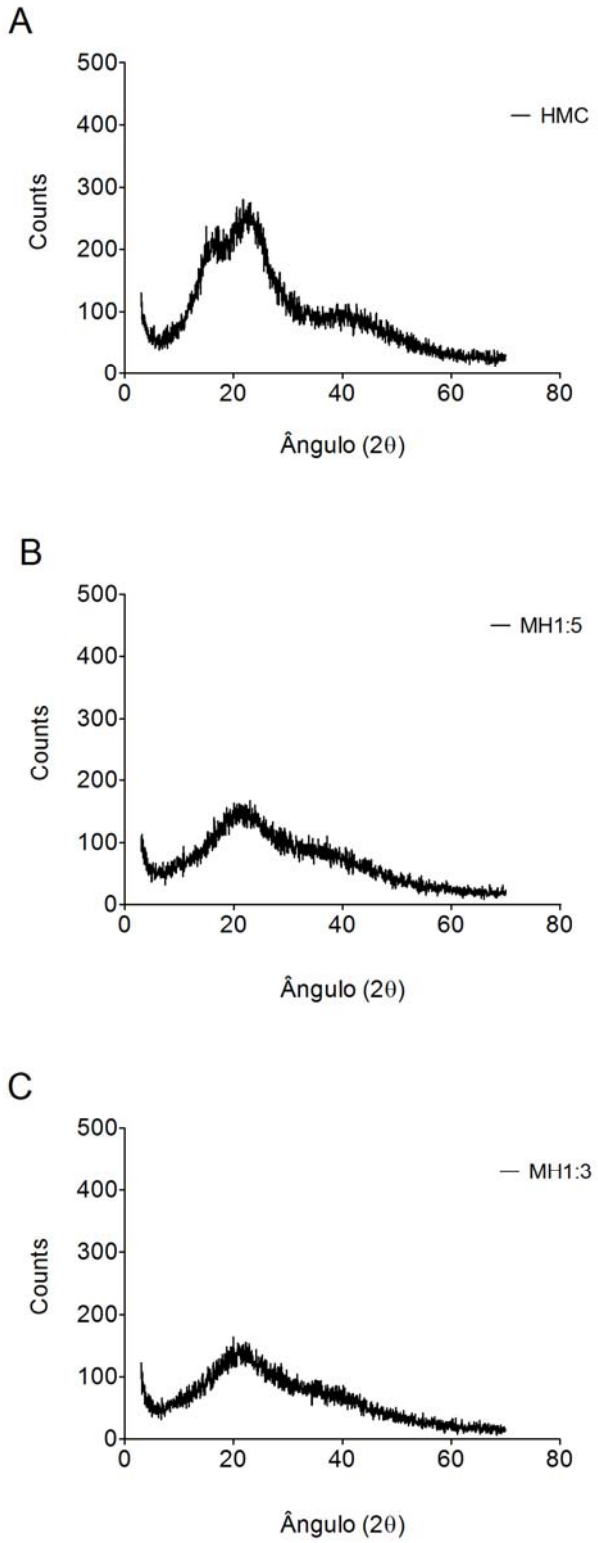


Figura 3 - Termogramas de calorimetria diferencial de varredura (DSC) da HMC, MH1:5 e MH1:3.

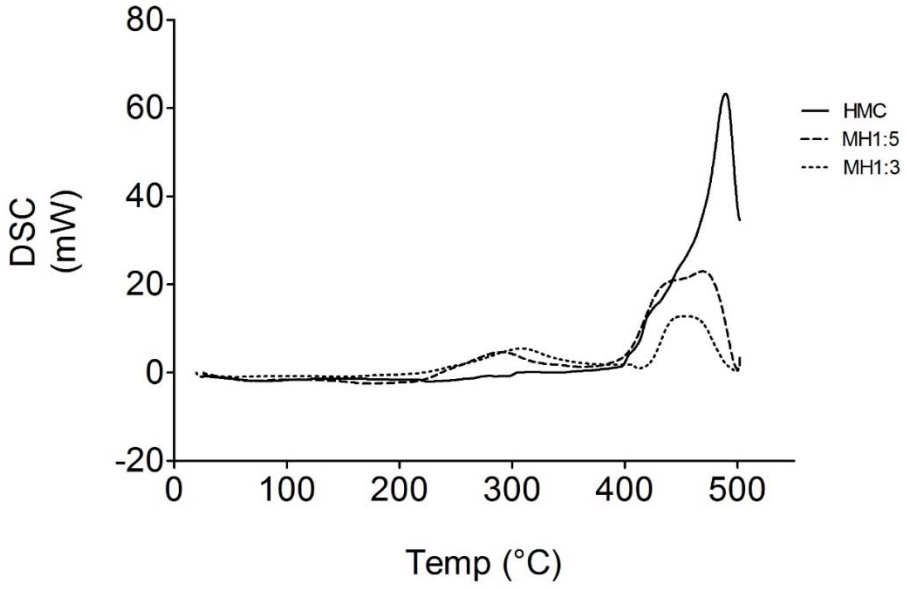


Figura 4 - Perfil de liberação *in vitro* da MH1:5 e MH1:3.

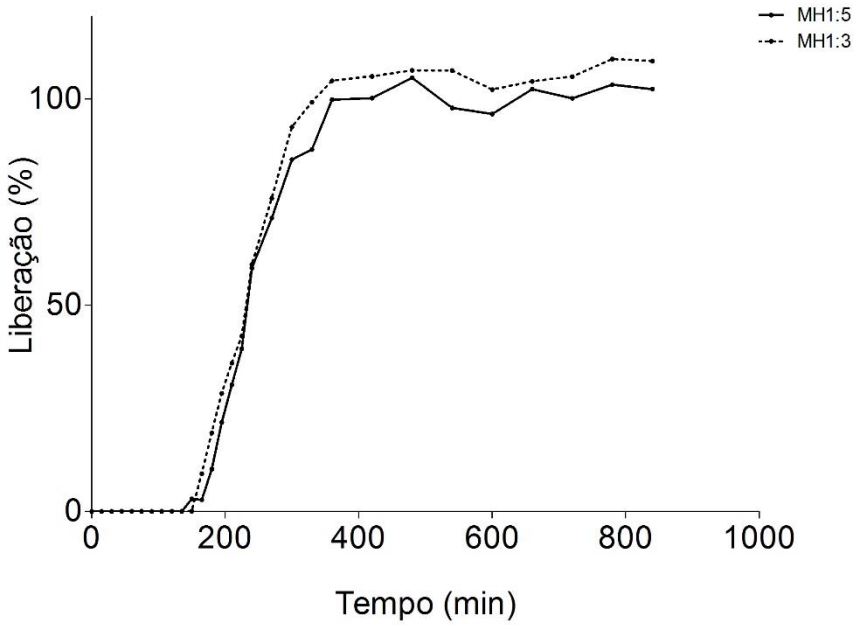
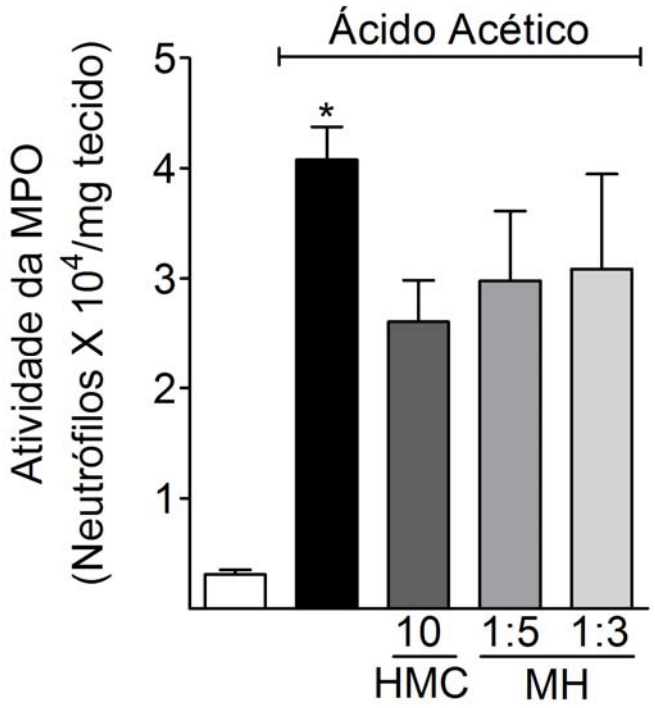


Figura 5 - Efeito do tratamento com HMC, MH1:5 e MH1:3 sobre a atividade da mieloperoxidase (MPO) no cólon de camundongos com colite induzida por ácido acético.



Os animais foram tratados com HMC, MH1:5 ou MH1:3 (10 mg/kg, via oral), 2 horas antes e 10 horas após a administração intracolônica de solução de ácido acético (7,5%, 200 µL). A atividade da MPO foi avaliada 18 horas após a indução da colite. n=8. Resultados representativos de 2 experimentos separados. * $p < 0.05$ comparado ao grupo controle negativo (sem colite).

4.3 *trans*-Chalcone ameliorates ulcerative colitis in mice: reduction of oxidative stress and cytokine production

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ABSTRACT

Ulcerative colitis (UC) is a colon disorder characterized by leucocyte infiltration, oxidative stress and upregulation of cytokines. *trans*-Chalcone (**1**) is one of the precursor of flavonoids and has antioxidant and anti-inflammatory properties, but its effects and mechanisms on experimental ulcerative colitis are still unknown. Hence, the aim of this study was to evaluate the potencial of **1** on reduce neutrophil recruitment, colon lesions, oxidative stress and cytokines production during acetic acid-induced colitis in mice. Male Swiss mice were treated with **1** (0.3 – 3 mg/kg, po) or vehicle (saline) before and after inflammatory stimuli. The treatment with **1** significantly reduced neutrophil infiltration, edema, macro and microscopic damage and colon shortening induced by intracolon administration of 7,5% acetic acid. Furthermore, **1** also increased total antioxidant capacity and GSH levels, and reduced the levels of TNF-α, IL-6, IL-1β and IL-33 by inhibiting NF-κB activation in the colon. In this study, for the first time, we demonstrated the potencial of **1** on ameliorate experimental ulcerative colitis through reduction of neutrophil recruitment, prevention of oxidative stress, and inhibition of pro-inflammatory cytokines production in the colon, suggesting **1** as a promising therapeutic approach for inflammatory bowel diaseases (IBD).

Chalcones (1,3-diaryl-2-propene-1-ones) is an open-chain flavonoids having two aromatic rings joined by a three-carbon enone moiety[1]. *trans*-Chalcone (**1**) is a basic polyphenolic structure precursor in the biosynthesis of flavonoids and isoflavonoids and form the central core of a variety of important biological compounds obtained from plants or prepared synthetically[2, 3]. Due to their easy availability, their simple structure, variety and different ways of cyclization, this class of compounds has emerged as important in the search for molecules with therapeutic potential[2]. Notable pharmacological activities of chalcone derivatives include anti-inflammatory, antioxidant, antifungal, antibacterial, antimalarial, antituberculosis, antitumor, and antiviral effects[4]. The reported anti-inflammatory and antioxidant effects have been associated with the inhibition of cell adhesion molecules (CAM) expression, down-regulation of inflammatory mediators, such as NO, TNF-α, IL-1β, cyclooxygenase-2 (COX-2) and inducible oxide nitric synthase (iNOS), induction of heme oxigenase (HO)-1 and suppression of NF-kB and MAPKs signaling pathways[4, 5].

Although there are few data concerning the pharmacological effect of **1**, some studies have already shown its antioxidant and anti-inflammatory property. Sikander et al. (2011)[1] demonstrated that **1** protects hepatocellular carcinoma (HepG2) cells from oxidative stress induced by hydrogen peroxide (H_2O_2). Furthermore, treatment with **1** prevents VEGF and ICAM-1 expression by down-regulating NF-kB- and STAT3-dependent signaling pathways in the ischemic retina of mice in a model of oxygen-induced retinopathy[6]. Even so, there is still a vast field to be investigated on the pharmacological effects of **1**, and importantly there is no data in the literature about the use of **1** in ulcerative colitis (UC).

Ulcerative colitis is a chronic inflammatory disease that affects the rectum and colon of the body, characterized by continuous inflammation of the intestinal lamina propria[7]. The incidence and prevalence of UC are increasing with time and in different regions around the world, indicating its emergence as a global disease[8]. Its pathogenesis involves many different factors, such as genetic susceptibility, environmental triggers, and immune response, which all are necessary, but none of them is sufficient itself to induce the disease[7]. During the development of UC, genetic and environmental pressures lead to failure of single

or multiple components of mucosal homeostasis[9]. Intestinal inflammation results from stimulation of the mucosal immune system by products of commensal bacteria in the lumen that penetrate the mucosal barrier or stimulate the surface epithelium to produce cytokines and chemokines that recruit and activate immune cells[10], such as neutrophils. The activated neutrophils release reactive oxygen species (ROS) into intestinal tissues overcoming local antioxidant capacity and causing mucosal damage, which further stimulates the infiltration of more leukocytes through induction of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α[10, 11]. Therefore, neutrophil recruitment, oxidative stress, and cytokines play a key role in initiation, augmentation, and perpetuation of the disease, since they are directly responsible for the mucosal injury in UC.

The goal of medical treatment in UC is to suppress inflammation and induce mucosal healing, improving the quality of life of patients[12]. Conventional UC treatment include 5-aminosalicylic acid (5-ASA) compounds, steroids and immunomodulators[12]. However, adverse reactions and toxicity are limitations of these therapies and make patients discontinue use[12]. New and promising therapy approaches has been suggested, such as antibodies directed against cytokines (e.g., anti-TNF and anti-IL-6R therapy), integrins, and matrix-metalloproteinases; anti-chemokine strategies; therapies with natural compounds (e.g., flavonoids), among others[13]. Existing evidence of **1** and its derivatives acting as negative modulators of inflammatory responses[1, 4, 6] led us to evaluated the effects and mechanisms of **1** on neutrophil recruitment, oxidative stress and cytokines production during acetic acid-induced colitis in mice, one standardized method of intestinal inflammation which shares classic course of histological, macroscopical and biochemical parameters related to those of human UC.

RESULTS AND DISCUSSION

***trans*-Chalcone (1) Reduces Neutrophil Recruitment and Edema in the Colon of Mice with Acetic Acid-induced Colitis.** Biopsies obtained from patients with active ulcerative colitis reveal the presence of large numbers of neutrophils in the colon[14]. As an abundant effector cell, neutrophils are recruited from peripheral blood to inflammatory sites and play an important role in development and progression of IBD[15]. Neutrophils are innate immune cells able to destroy extracellular and intracellular invading pathogens, and produce significant quantities of ROS/RNS and cytokines[16]. They have two granule population, azunophil and specific granules, that contain several proteolytic enzymes and a wide range of bactericidal proteins including cathepsin G, elastase, lysozyme and myeloperoxidase (MPO)[17]. This latter is used as an important marker of neutrophil recruitment to different tissues and monitoring the number and the migration activity of neutrophils is an efficient way to evaluate inflammatory responses. We observed increased neutrophil recruitment to the colon after acetic acid (7.5%, 200 µl, in saline) administration, which was significantly inhibited by treatment with **1** at 3 mg/kg, but not with 0.3 and 1 mg/kg (Figure 1A). Corroborating our results, chalcone derivates also reduce neutrophil recruitment. It was demonstrated that a newly synthesized chalcone analoge reduces wound-induced neutrophil recruitment in living zebrafish[18], and 2`-hydroxychalcone inhibits the expression of cell adhesion molecules involved in recruitment and subsequent migration of the leukocytes to the site of inflammation[19]. 1,3-Diphenyl-2-propenone, the chalcone itself, also reduced neutrophil recruitment to the colon of rats during trinitrobenzene sulfonic acid-induced colitis[20], an experimental model of Crohn's Disease. Thus, the treatment with **1** can ameliorate colon inflammation by reducing neutrophil recruitment, since they are important sources of inflammatory mediators such as ROS/RNS and cytokines.

Co-incident with the inflammatory infiltrate observed in patients with UC is the colon edema. Increases in vascular permeability could account for the edema and tissue dysfunction observed during acute flares of UC[14]. Colon weight/length ratio, an index of colon

edema associated with acetic acid-induced inflammatory reaction, was significantly increased in colitis control group compared to negative control group. The treatment with **1** at 3 mg/kg significantly reduced the colon weight/length ratio (Figure 1B). However, the 0.3 and 1 mg/kg doses of **1** did not provide significant effect compared to colitis control group. The oral administration of 3,4,5-trimethoxy-4'-fluorochalcone significantly inhibited paw edema in rat adjuvant-induced arthritis[21], and other chalcone derivates also inhibited formalin-induced paw edema in rats[22]. So, the inhibition of edema seems to be consistent among chalcone class.

***trans*-Chalcone (1) Reduces Macroscopic Damage and Prevents the Reduction of Colon Length in Mice with Acetic Acid-induced Colitis.** During ulcerative colitis, the recruitment of immune cells to colon tissue accompanied by the over-expression of pro-inflammatory mediators, such as ROS and cytokines, leads to development of ulcers and tissue destruction with consequent release of extracellular matrix-degrading enzymes metalloproteinases (MMPs)[15]. Colitis control group showed sites of inflammation > 1 cm along the colon (Figure 2A) and shortening in approximately 25% of total length compared to negative control group (Figure 2B, 2c1 and 2c2). However, the treatment with **1** at 3 mg/kg significantly attenuated the macroscopic lesions and prevents colon shortening induced by acetic acid in colitis control group (Figure 2A, 2B and 2c3). This result lines up well with the reduction of neutrophil recruitment by treatment with **1** observed in this study. Corroborating, previously study demonstrated that Cardamonin (2',4'-dihydroxy-6'-methoxychalcone) markedly prevent colon shortening in a mouse model of dextran sulfate sodium-induced colitis, and this effect was correlated with a decline in MPO activity and nitric oxide, TNF-α and IL-6 production in the colon[5].

***trans*-Chalcone (1) Reduces Microscopic Damage in the Colon of Mice with Acetic Acid-Induced Colitis.** One of the most prominent histological features observed in UC is infiltration of neutrophils into the inflamed mucosa and subsequently into the intestinal

lumen, resulting in the formation of so-called crypt abscesses[17]. Along with the neutrophil infiltrate, there is extensive mucosal and/or transmural injury including edema, loss of goblet cells, decreased mucous production, crypt cell hyperplasia, erosions, and ulcerations[14]. The lesion score of colitis control group was significantly higher compared to negative control group (Figure 3A), that presented the expected morphology (Figure 3B), with mucosal architecture preserved and no significant changes related to cellular infiltration, muscle thickening, crypt abscess formation and goblet cell depletion. Histological changes in the colon of mice with acetic acid-induced colitis showed loss of mucosal architecture with edema in sub-mucosa, cellular infiltration, slight muscle thickening, crypt abscess formation and goblet cell depletion (Figure 3C). Treatment of mice with **1** at 3 mg/kg significantly improved the lesion score compared to colitis control group (Figure 3A), through preservation of mucosal architecture and goblet cell, and decreasing cellular infiltration, muscle thickening and crypt abscess formation in colon sections (Figure 3D). So, **1** significantly attenuated the severity of histological signs of colon damage during acetic acid-induced colitis in mice and it is consistent with the reduction of neutrophil recruitment (Figure 1A), edema (Figure 1B) and macroscopic lesions (Figure 2). Similarly, the chalcone cardamonin also ameliorate the histological injury in dextran sulfate sodium-induced colitis[5].

***trans*-Chalcone (1) Improves Antioxidant Capacity in the Colon of Mice with Acetic Acid-Induced Colitis.** Oxidative stress has been well documented in UC with increased ROS and decreased antioxidant levels in the inflamed mucosa, which contribute to inflammatory process[23]. Corroborating, we observed that colitis control group shows significant reduction in colon total antioxidant capacity (Figure 4A and 4B) and GSH levels (Figure 4C) compared to negative control group. The inability to efficiently regulate the activation of tissue macrophages as well as the recruitment and activation of neutrophils, will result in increased production of ROS within the colon via activation of NADPH oxidase[14]. This enzyme transfer an electron to oxygen inside and outside cells generating superoxide radicals ($O_2^{-\cdot}$), which serves as the starting material for the production of a vast assortment

of reactive oxidants, including oxidized halogens, free radicals, and singlet oxygen[24]. Excessive ROS production, in addition to induce damage to DNA and protein modifications, leads to lipid peroxidation that impairs the integrity of the intestinal epithelial cells and increases the intestinal mucosal permeability[25]. Furthermore, accumulation of ROS in ulcerative colon tissues stimulates inflammation responses and secretion of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, through NFkB activation[25].

Enzymatic (SOD, catalase, GSH peroxidase) and nonenzymatic (GSH, ascorbate, α-tocopherol) antioxidants are responsible to decompose the injurious oxidizing agents thereby limiting tissue damage. However, the uncontrolled overproduction of ROS, as would occur during active episodes of UC, could easily overwhelm these protective mechanisms resulting in oxidative damage to cells and tissue[14]. So, preserve or increase the antioxidant capacity in colon tissue to reduce the damage caused by excessive ROS are extremely important. In this sense, antioxidant molecules have attracted considerable attention as therapeutic candidates for the treatment of UC. We observed that treatment with **1** prevented acetic acid-induced decrease of total antioxidant capacity (Figure 4A, 4B) in the colon of mice, which was evaluated through ferric reducing antioxidant power (FRAP) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging abilities. Importantly, treatment with **1** also preserved GSH levels in the colon after acetic acid administration (Figure 4C). The depletion of total antioxidant capacity and GSH levels occurs because they have been used to neutralize the excessive ROS production[26]. However, **1** reduces the consumption or increases endogenous antioxidants production, offering protection against oxidative stress. Consistent with our results, Sikander et al. (2011)[1] reported the ability of **1** exert protective effect by delaying the consumption of GSH and other cellular antioxidants during oxidative stress induced by hydrogen peroxide (H_2O_2) in hepatocellular carcinoma (HepG2) cells.

***trans*-Chalcone (1) Reduces Pro-Inflammatory Cytokines Production by Inhibiting NF-κB activation in the Colon of Mice with Acetic Acid-Induced Colitis.** Concentrations of pro-inflammatory cytokines are increased in intestinal mucosa of patients with UC and neutrophils are important source of these cytokines[27]. The release of cytokines is induced by ROS[25] and the overproduction of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, leads to increased production of ROS, amplifying the inflammatory response, and resulting in target tissue damage[28]. As well as ROS, cytokines also mediate their action via NF-kB and mitogen-activated protein kinase (MAPK) signalling pathways[25]. Our data show that colon administration of acetic acid, besides increases neutrophil recruitment (Figure 1A) and reduces endogenous antioxidants (Figure 4), induces significant increase in TNF-α, IL-6, IL-1β and IL-33 levels (Figure 5A, 5B, 5C and 5D) by stimulating NF-kB activation in colon tissue (Figure 6). These effects are also described in human ulcerative colitis. Nevertheless, the administration of **1** could inhibit the production of these cytokines (Figure 5A, 5B, 5C and 5D) by inhibiting NF-kB signalling pathway (Figure 6) and contributing to ameliorate acetic acid-induced colitis in mice.

TNF-α is a pro-inflammatory cytokine that plays significant role in the pathogenesis of UC. The clinical efficacy of anti-TNF-α monoclonal antibodies supports its crucial pathogenic role[15]. This cytokine exists in two forms: a transmembrane (tmTNF) and a soluble (sTNF) form, and both mediate their biological function by binding to one of the two TNF-α receptors: TNFR1 or TNFR2[29]. Induction of TNF-α signaling results in activation of the transcription factor NF-κB promoting the expression of pro-inflammatory cytokines (e.g., IL-6 and IL-1β), chemokines, adhesion molecules, and other inflammatory mediators[30]. So, TNF-α acts within a complex network of pro-inflammatory cytokines and mediators involving several positive-feedback loops that together amplify the inflammatory response and facilitate recruitment, activation, differentiation, and proliferation of various immune cells[29]. Similar to TNF-α, IL-6 has been shown to be a central regulator of the immune response in various types of inflammatory diseases[31]. In inflamed bowel, IL-6 is largely derived from macrophages, neutrophils and mast cells, and its expression is increased in both UC and CD, perhaps with

higher levels in UC[32]. IL-6 is a pleiotropic cytokine that plays a crucial role in inflammation, because it is a strategic bridge between the innate and the adaptive system[33]. Furthermore, IL-6 has emerged as main mediator of inflammation-associated tumorigenesis, and its signaling essentially depends on signal-transducer-and-activator-of-transcription (STAT)-3[31]. Therefore, targeting IL-6 might not only reduce intestinal inflammation, but also reduce the increased risk for colorectal cancer (CRC) development in IBD patients[31].

The IL-1 family consists of 11 agonist and antagonist molecules that are centrally involved in regulating inflammatory responses, including IL-1β and IL-33. Together with TNF-α, they are defined as “alarm cytokines” that are secreted by macrophages and initiate inflammatory responses by increasing the expression of integrins on endothelial cells, stromal cells and leukocytes promoting cell infiltration; and inducing a cascade of other pro-inflammatory genes, including of cyclooxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOS), chemokines/cytokines and matrix metalloproteinases (MMPs)[34], that amplify the inflammatory response. Both have been found to be upregulated in human UC. However, the inhibition of IL-1β-converting enzyme (ICE; also known as caspase 1) - an enzyme that cleaves IL-1β into active cytokine - protected mice from DSS-induced colitis[35], which suggests that blockade of IL-1β may be relevant for the therapy of UC[36]. Accordingly, neutralisation of the IL-33/ST2 signalling pathway ameliorates DSS-induced colitis, which suggests that targeting of IL-33 may also represent an efficient therapy in IBD[37].

Considering that NF-kB is a critical transcription factor that regulates the production of ROS and cytokines in activated macrophages during inflammation[2, 25], the inhibition of this transcription factor by **1** seems to be an effective therapeutic approach for treatment of UC. Several chalcones, such as butein, cardamonin and 2’-hydroxychalcone, inhibit NF-κB-mediated inflammation[2]. Park et al. (2012)[20] demonstrated that 1,3-diphenylpropenone (chalcone) possesses anti-inflammatory activity in TNBS-induced rat colitis, by suppressing NF-kB activation and its target pro-inflammatory cytokine expression. Concluding, **1** has already shown its ability on down-regulate NF-kB activation, preventing further pathological neovascularization in a model of ischemic retinopathy in mice[6]. So, it is the first time shown

that **1** can prevent acetic acid-induced colitis in mice and we believe that the anti-inflammatory effects of **1** are related, at least in part, to its antioxidant property and the inhibitory effect of this drug on neutrophil recruitment and NFkB activation, with the consequent reduction of TNF-α, IL-6, IL-1β and IL-33 cytokines expression.

Experimental Section

General Experimental Procedures. *trans*-Chalcone (**1**) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Hexadecyltrimethylammonium bromide (HTAB), o-dianisidine dihydrochloride, Tween 80, reduced glutathione (GSH), EDTA, ferric chloride hexahydrate, 2,4,6-tri(2-pyridyl)-s-triazine, diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), Trolox, and potassium persulfate (dipotassium peroxidisulfate) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mouse TNF-α, IL-1β, IL-6 and IL-33 kits were obtained from eBioscience (San Diego, CA, USA). NF-κB kit from Cell Signaling (Beverly, USA). Other reagents were of commercially available analytical grade.

Animals. Male Swiss mice (25 g) from the Londrina State University (Londrina, Brazil) were used in this study. Mice were housed in standard clear plastic cages with free access to water and food, and temperature of 23 °C ± 2. A 12/12 h light/dark cycle was used with lights on at 6 h and off at 18 h. Animal care and handling procedures were approved by the Ethics Committee of the Londrina State University (process number 1494.2015.56).

Acetic Acid-induced Colitis. Colitis was induced in mice according to a published method [38]. The animals received, into the lumen of the colon, acetic acid solution (7.5%, in saline, 200 μL) stimulation after 24 h fasted, through a 3 cm polyethylene cannulum. The negative control group underwent intracolon instillation with saline instead. Animals were left upside down for 3 min to prevent the leakage of intracolon instillation.

Experimental Design and Treatments. Two experimental protocols were followed [38] to evaluate the effect of **1** on inflammatory (A) and oxidative (B) parameters of acetic acid induced-colitis in mice. (A) Neutrophil recruitment, edema, macro and microscopic damage, colon length and cytokine levels were assessed 18 h after colitis induction. In this protocol, the animals were treated 2 h before and 10 h after colitis induction with **1** (0.3, 1 or 3 mg/kg, Tween 80 20% diluted in saline, po). (B) The ferric reducing antioxidant power (FRAP), ABTS radical cation scavenging and reduced GSH levels were used to evaluate the ability of the colon samples to resist oxidative stress 4 h after colitis induction. In this case, the

animals were treated at 6 h and 1 h before colitis induction with **1** (3 mg/kg, Tween 80 20% diluted in saline, po). The results are representative of two separated experiments.

Myeloperoxidase Activity Assay. The neutrophil recruitment to the colon was evaluated by myeloperoxidase kinetic-colorimetric assay[39]. Colon samples were homogenized using a Turrax IKA (T10 basic) in K_2HPO_4 buffer (50 mM, pH 6.0) containing HTAB (13.72 mM). The homogenates were centrifuged (16100g, 2 min, 4 °C) and the resulting supernatants reacted with K_2HPO_4 buffer (50 mM, pH 6.0) containing o-dianisidine dihydrochloride (0.0167%) and hydrogen peroxide (0.05%). The absorbance was determined at 450 nm (Multiskan GO ThermoScientific). The myeloperoxidase activity of samples was compared to a standard curve of neutrophils and presented as the number of neutrophils per mg of tissue.

Edema. It was weighed 1 cm of the distal colon segments to determinate the colon weight/length ratio. The results were expressed in percentage of increase in colon weight (g)/ length (cm) ratios, compared to a negative control group, without colitis [40].

Macroscopic Damage Score and Colon Length. The macroscopic damage was determined using the following criteria outlined by Morris, et al. [41] with modifications: no damage (score 0); localized hyperemia but no ulcers (score 1); linear ulcers with no significant inflammation (score 2); linear ulcers with inflammation at one site (score 3); two or more sites of ulceration and inflammation (score 4); one site of inflammation >1 cm along the length of the colon (score 5); site of inflammation >2 cm along the length of the colon, with quantification increased by 1 for each additional centimeter (score 6−10). The length of the colon was measured with a ruler for evaluation of intestinal shortening.

Microscopic Damage Score. The distal colon samples were fixed in 10% formalin, dehydrated with increasing concentrations of ethanol, embedded in paraffin and cut into 5 µm sections. The paraffin sections were mounted on slides, deparaffinized and rehydrated, then stained with hematoxylin and eosin to determine the score of microscopic damage. The stained tissues were observed with a light microscope (Olympus OX31, Tokyo, Japan), coupled with a digital camera (Lumenera Infinity 1, Ottawa, Canada). The histological

assessment of damage was graded semiquantitatively as described previously [42], with modifications: (1) loss of mucosal architecture (score 0−3), (2) cellular infiltration (score 0−3), (3) muscle thickening (score 0−3), (4) crypt abscess formation (score 0−3), and (5) goblet cell depletion (score 0−3). The final score was determined by adding the scores above for each of the samples.

FRAP Assay. The ferric reducing antioxidant power in colon samples was determined as previously described [43], adapted to a 96-well microplate format. Colon samples were homogenized in KCl solution (1.15 %) and centrifuged at 0.2*g*, 4 °C for 10 min. The supernatant aliquots reacted with the FRAP reagent, composed by ferric chloride (20 mM) and 2,4,6-tripyridyl-s-triazine (TPTZ, 10 mM) in acetate buffer (0.3 mM, pH 3.6). After 6 min of incubation, the absorbance was read at 595 nm (Multiskan GO ThermoScientific). The reducing ability of the tissue was equated against a Trolox standard curve (0.4−400 nmol). The results were expressed as nmol Trolox equivalents per mg of tissue, that is, the amount of Trolox (nmol) with an equivalent reducing antioxidant power to 1 mg of the tissue under investigation.

ABTS Assay. The free radical scavenging property of colon samples was determined as previously described [43], adapted to a 96-well microplate format. Colon samples were homogenized in KCl solution (1.15 %) and centrifuged at 0.2*g*, 4 °C for 10 min. The supernatant aliquots reacted with the diluted ABTS solution (7mM in potassium persulfate) and the absorbance was measured at 730 nm (Multiskan GO ThermoScientific) after 6 min of incubation. The results were equated against a standard Trolox curve (0.4–400 nmol) and expressed as nmol Trolox equivalents per mg of tissue, that is, the amount of Trolox (nmol) with an equivalent scavenging property to 1 mg of the tissue under investigation.

GSH Assay. The reduced GSH levels in colon were determined using a spectrophotometric method [44], with modification. The colon samples were homogenized in EDTA solution (0.02 M), treated with trichloroacetic acid (30 %) and centrifuged at 1.5*g* at 4 °C for 15 min. The supernatant reacted with Tris-HCl (0.4 M, pH 8.9) and dithiobisnitrobenzoic acid (0.01 M). After 5 min, the absorbance was determined in 412 nm

(Multiskan GO ThermoScientific). Protein levels in the colon homogenates was measured using the Lowry method [45]. The results were expressed as μmol of GSH per mg of protein, using a standard GSH curve (2–2000 nmol).

Cytokine Measurements. Colon samples were homogenized in sterile saline to evaluate the pro-inflammatory cytokine levels. The homogenates were centrifuged at 0.8g at 4 °C for 10 min, and TNF-α, IL-1β, IL-6 and IL-33 levels were evaluated using ELISA Ready-SET-Go! kits (eBioscience) according to the manufacturer's recommendations. Protein levels in the colon homogenates was measured using the Lowry method [45]. The results were expressed as pg of cytokine per mg of protein.

NF-κB Activity. The levels of phosphorylated and total NF-κB p65 subunit in colon samples were determined by ELISA using PathScan® kits (Cell Signaling) according to the manufacturer's recommendations. The results are expressed as OD of samples (total p65/phospho-p65 ratio) at 450 nm (Multiskan GO ThermoScientific).

Statistical Analysis. All data are expressed as means ± SEM. Statistical significance of differences between the groups was determined by ANOVA followed by the Newman Keuls's test. For categorical variables, the Kruskal−Wallis test followed by Dunn's test was performed. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). The criterion for significance was chosen at $p < 0.05$.

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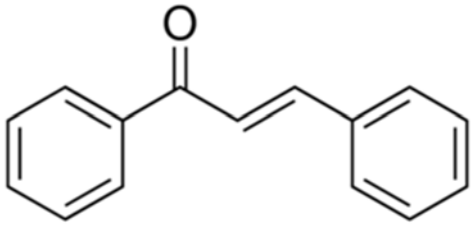
Table of Contents Graphic

***trans*-Chalcone ameliorates ulcerative colitis in mice: reduction of oxidative stress and cytokine production.** Carla F. S. Guazelli, Victor Fattori, Barbara B. Colombo, Rubia Casagrande, Marcela M. Baracat, and Waldiceu A. Verri, Jr.*



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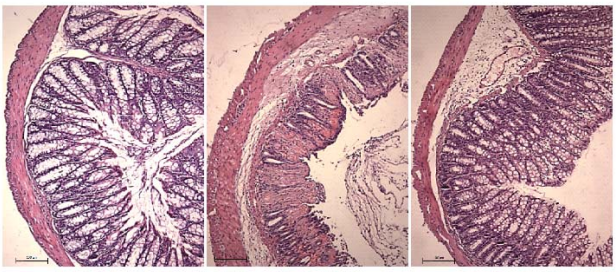

Figures

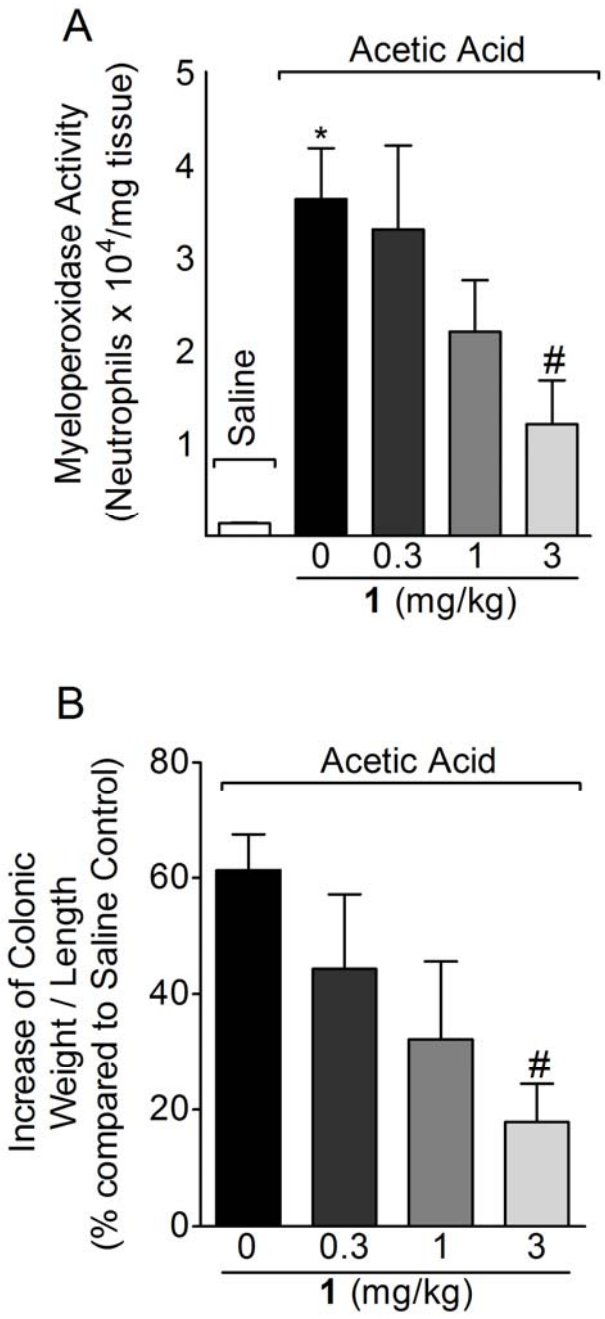

Figure 1. *trans*-Chalcone (**1**) reduces neutrophil recruitment and weight / length (edema) in the colon of mice with acetic acid-induced colitis. Mice were treated with **1** (0.3, 1 or 3 mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7.5%, 200µL). Myeloperoxidase activity (A) and edema (B) were evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

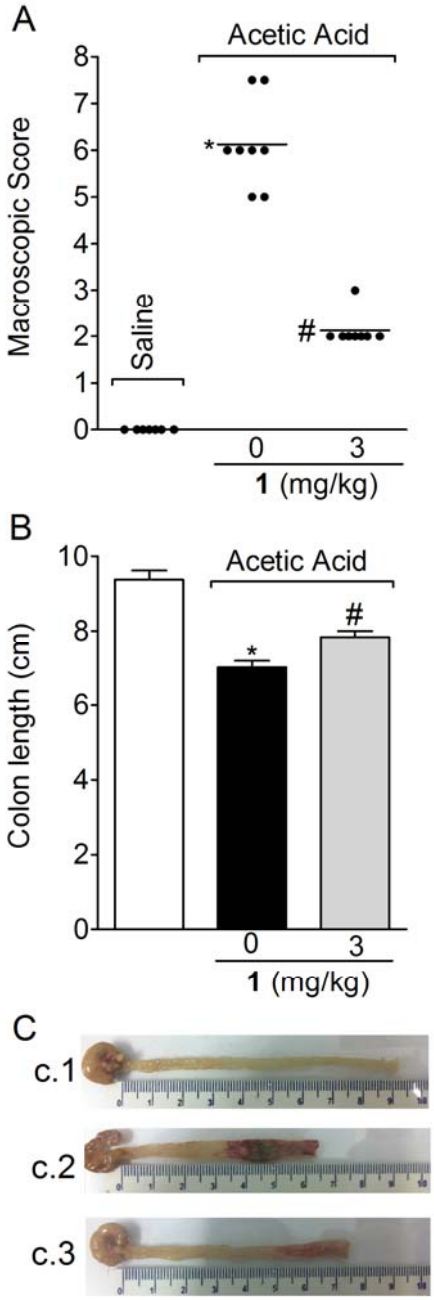

Figure 2. *trans*-Chalcone (**1**) reduces macroscopic damage and prevents the reduction of colon length in mice with acetic acid-induced colitis. Mice were treated with **1** (3 mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7.5%, 200µL). The macroscopic damage score (A) and colon length (B) were evaluated 18 hours after colitis induction in negative control group (c.1), colitis control group (c.2) and **1** treated group (c.3). n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test for macroscopic damage score and ANOVA followed by Newman-Keuls's test for colon length.

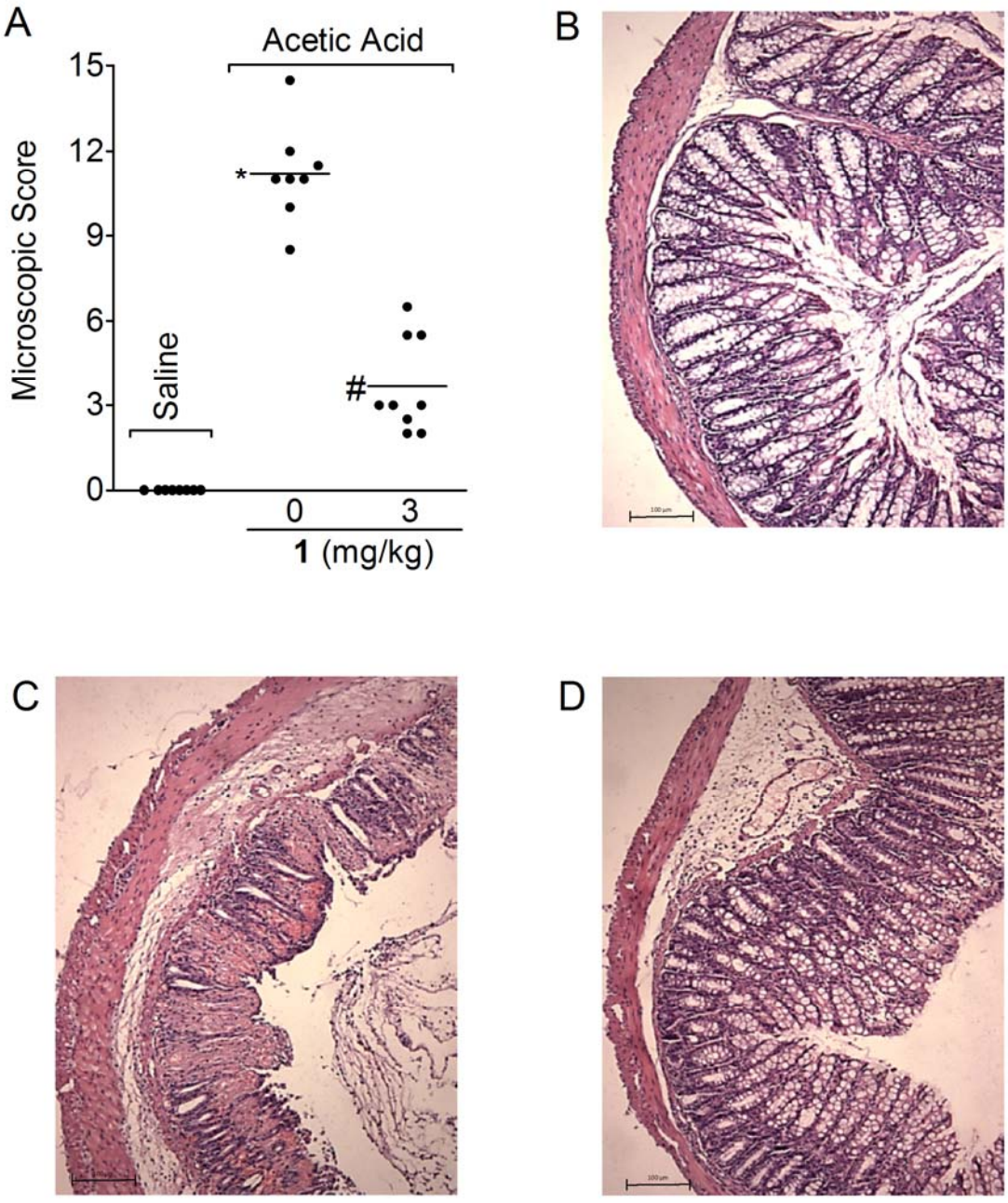

Figure 3. *trans*-Chalcone (**1**) reduces microscopic damage in the colon of mice with acetic acid-induced colitis. Mice were treated with **1** (3 mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7.5%, 200µL). The microscopic damage score (A) were evaluated 18 hours after colitis induction in the negative control group (B), colitis control group (C) and **1** treated group (D). n=9 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test.

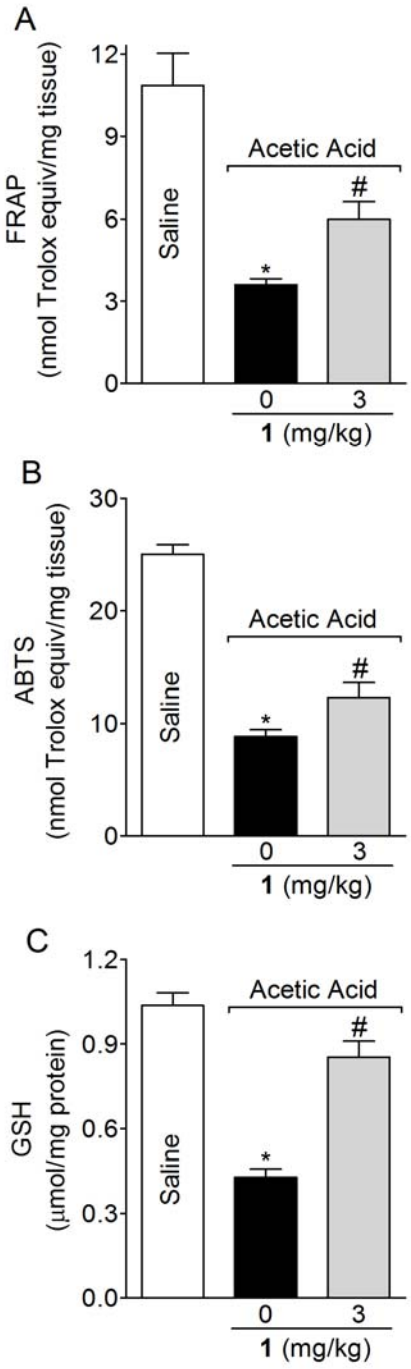

Figure 4. *trans*-Chalcone (**1**) improves antioxidant capacity in the colon of mice with acetic acid-induced colitis. Mice were treated with **1** (3 mg/kg, po), 6 and 1 h before intracolon administration of acetic acid (7.5%, 200µL). The ferric reducing antioxidant power - FRAP (A), ABTS radical scavenging ability (B) and reduced GSH levels (C) were determined 4 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

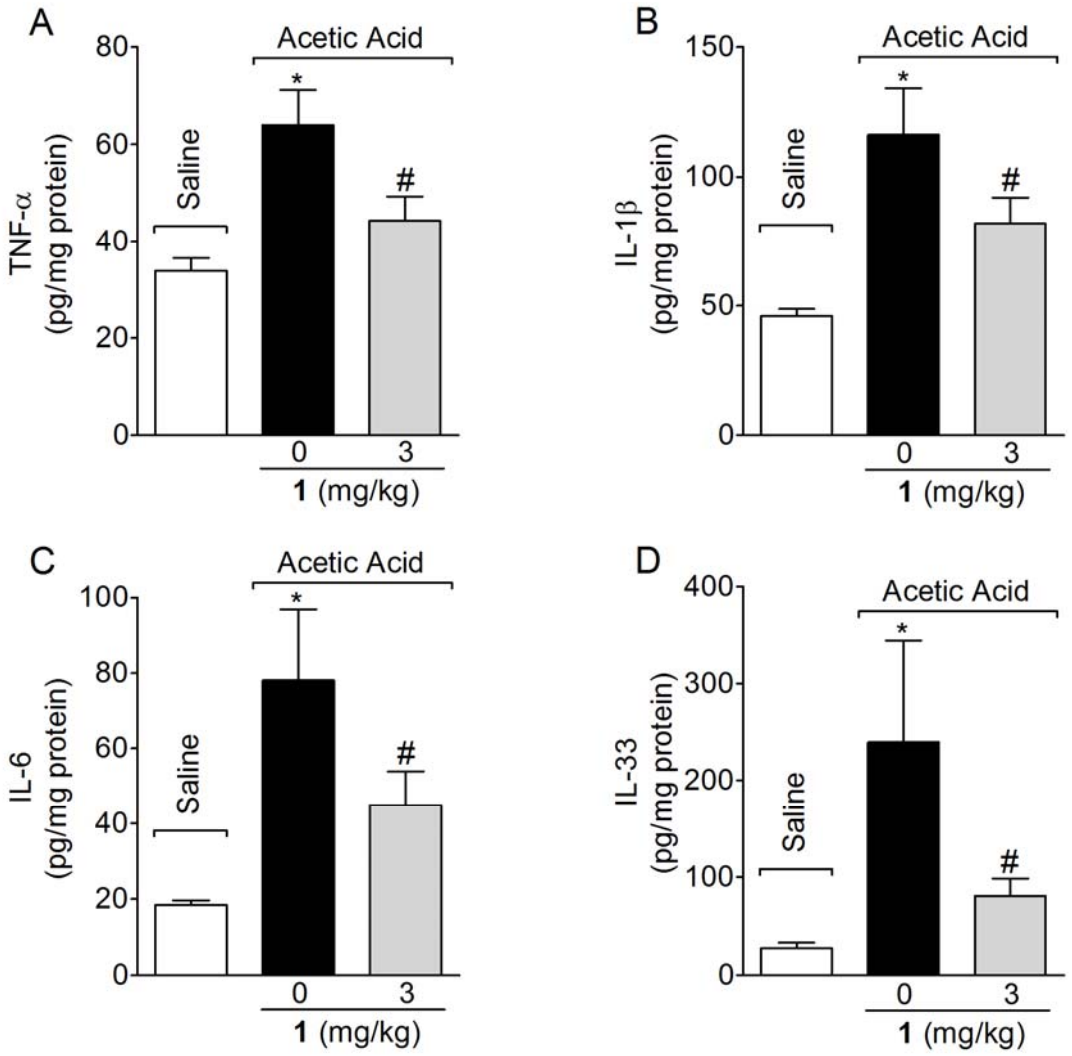

Figure 5. *trans*-Chalcone (**1**) reduces pro-inflammatory cytokines production in the colon of mice with acetic acid-induced colitis. Mice were treated with **1** (3 mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7.5%, 200µL). TNF-α (A), IL-1β (B), IL-6 (C) and IL-33 (D) levels were evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

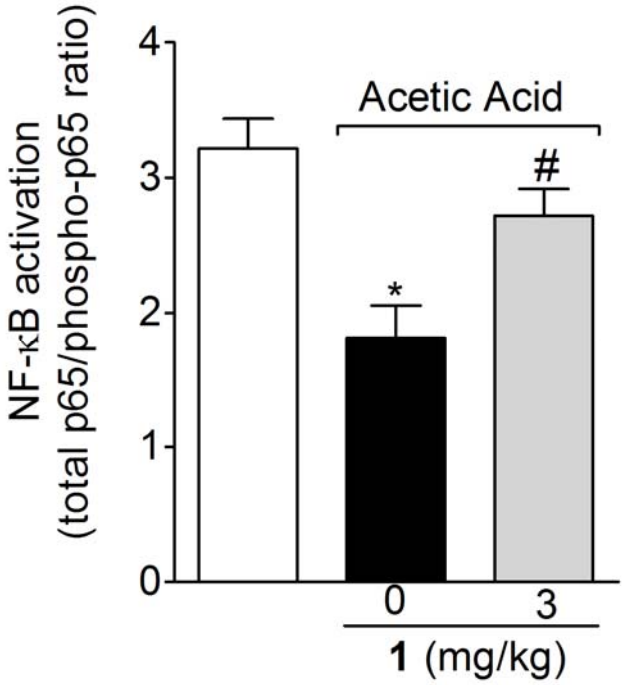

Figure 6. *trans*-Chalcone (**1**) inhibits NF-kB activation in the colon of mice with acetic acid-induced colitis. Mice were treated with **1** (3 mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7.5%, 200μL). The NF-kB activity was determined 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

4.4 Microencapsulation of *trans*-Chalcone for the Treatment of Ulcerative Colitis: Microcapsule Characterization and *In vivo* Antioxidant and Anti-inflammatory Mechanisms

THE AAPS JOURNAL

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Running Head: Microencapsulated *trans*-Chalcone Improves Colitis

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ABSTRACT. Polymeric biocompatible microcapsules obtained by complex coacervation of pectin and casein provide modified release of the drug in gastrointestinal tract, which makes them useful for the design of systems for colon drug delivery. In order to improve the anti-inflammatory and antioxidant effects of *trans*-chalcone (T), we developed and characterized microencapsulated systems of pectin/casein polymer containing T and evaluated the therapeutic effect and mechanisms of *trans*-chalcone-loaded microcapsules (MT) in acetic acid-induced colitis in mice. Complex coacervation process employed yielded microcapsules with surface irregularities, but no cracking. *trans*-Chalcone (T) was released *in vitro* in simulated intestinal fluid, but not in simulated gastric fluid, predominantly by diffusion. In experimental colitis, per oral treatment with MT, but not with non-encapsulated T at the same dose, significantly reduced neutrophil recruitment, attenuated macro and microscopic colon lesions, improved antioxidant capacity and decreased TNF-α, IL-1β, IL-6 and IL-33 levels, by reducing NF-kB activation in the colon tissue. Our results demonstrate that microencapsulated systems of pectin/casein polymer containing T improved anti-inflammatory and antioxidant effects of the drug in experimental model of ulcerative colitis in mice. Therefore, we observed the importance of pharmaceutical development of modified release systems to reduce the required dose of drugs to obtain anti-inflammatory and antioxidant effects.

KEY WORDS: *trans*-chalcone; microencapsulated delivery system; oxidative stress; nuclear factor-κB; ulcerative colitis.

1. INTRODUCTION

Several approaches have been used to improve the activity of drugs, reduce the toxicity and the limitations of treatments for inflammatory bowel diseases (IBD), such as development of colon-targeted delivery, including prodrugs, pH-dependent release systems, time-dependent release systems, and enzyme-dependent release systems (1). The design of suitable carrier systems is also considered a key step to solve problems like insufficient concentration of active molecules due to poor absorption, rapid metabolism and elimination, poor solubility and high fluctuation of plasma levels due to unpredictable bioavailability (2). A simple and inexpensive way to modify drug delivery characteristics of dosage forms is the coating with biocompatible polymers (3).

Polymeric microcapsules are one technology developed to enable clinically feasible oral delivery, because it can protect the drug from a wide range of pH environments and enzymatic degradation, increase drug permeability across the intestinal epithelium and provide controlled release (4). In contrast to healthy colonic mucosa, the accumulation of microparticles in inflamed tissue is characterized by an increased deposition of particles in inflamed tissue areas (3). Biodegradable polysaccharides, like pectin, remain stable within the physiological environment of the upper gastrointestinal tract, and are degraded by colonic bacterial flora in the colon (3), so they are useful for the construction of systems for colon drug delivery. The complex coacervation of pectin with the natural polymer casein in aqueous environment occurs by electrostatic interaction and forms microencapsulated systems able to modify the drug delivery on gastrointestinal tract (5, 6). We supposed that drugs that are entrapped within a microcapsule are released by diffusion upon degradation/bioerosion of the polymeric shell (7). Thus, microencapsulation of a drug using pectin/casein complex can be used for targeted delivery to the colon improving the treatment of IBD.

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major types of IBD, both characterized as a chronic inflammatory disease of the gastrointestinal tract associated with a variety of intestinal complaints, such as bloody diarrhoea, fever, weight loss, abdominal pain, or vomiting (3). Genetically based interactions between the human intestinal microbiome and mucosal immune system and the manner in which environmental factors modify these relationships appear particularly relevant for the development of IBD (8). UC affects the colon, exclusively, with the inflammatory reaction being confined to the mucosa and spreading continuously from the anus and extends proximally (9). On the other hand, CD can affect any part of the gastrointestinal tract from mouth to anus and is characterized by transmural inflammations and the involvement of discontinuous segments of the intestine (9).

The medical treatment for IBD includes salicylate, corticosteroids, immunomodulators, and biologics (10), administrated via parenteral, per oral and rectal dosage forms, whose purpose is to suppress inflammation and induce mucosal healing, but the low efficacy, side effects, intolerance, and the consequent reduced patient compliance are limitations of the currently approved treatments for IBD. As opposed to most oral delivery applications that require the therapeutic to reach the bloodstream, the goal for UC treatment is local delivery of therapeutics to immune cells in the intestines (11). Furthermore, the oral administration is a desirable alternative because of the convenience and increased compliance by patients, especially for chronic diseases that require frequent administration (11).

Lately, there has been increasing attention dedicated to chalcones as they have shown diverse biological activities (12), including anti-inflammatory and antioxidants. Chalcones consist of two aromatic rings joined by a three-carbon enone moiety (12) and may exist in two isomeric forms, *cis* and *trans*, of which the trans form is thermodynamically favorable (13). *trans*-Chalcone (T) down-regulates NF-kB- and STAT3-dependent signaling pathways in a model of oxygen-induced retinopathy in mice (14) and protects hepatocellular carcinoma (HepG2) cells from oxidative stress induced by hydrogen peroxide (H_2O_2) (12). Furthermore, T treatment has significantly attenuated oxidative stress and inflammation on experimentally-induced hepatic injury in rats (15). Promising results, reported by Piñero et al. (2006) (16), demonstrated the potential to maximize the leishmanicidal effect of T using controlled release polylactic/glycolic acid polymer that provide the local delivery of the drug; and we had also demonstrated that pectin/casein polymer microcapsules loaded with quercetin improved the anti-inflammatory and antioxidant effects of the drug in experimental colitis (6).

In this sense, we aimed to develop and characterize two microencapsulated formulations containing T in different proportions of drug:polymer (MT1:5 and MT1:3)because it can influence the release rate and *in vivo* effects, and investigate if the controlled release of T potentiates its anti-inflammatory and antioxidant effects in experimental UC induced by acetic acid in mice. We suggest that microencapsulated formulation of T has potential to improving its therapeutic effect through reducing the necessary dose to achieve the anti-inflammatory and antioxidant effects, and consequently diminishing the possible adverse effect of this drug.

2. MATERIALS AND METHODS

2.1. Materials

Pectin GENU® 8003 (ref. 13595) was obtained from CP Kelco (Limeira, SP, Brazil), and casein from Katuffman & Co. (Kehl, BW, Germany). Citric acid, sodium hydroxide and acetonitrile were purchased from Merck (Darmstadt, HE, Germany). *trans*-Chalcone (T) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Hexadecyltrimethylammonium bromide (HTAB), o-dianisidine dihydrochloride, Tween 80, reduced glutathione (GSH), EDTA, ferric chloride hexahydrate, 2,4,6-tri(2-pyridyl)-s-triazine, diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), Trolox, and potassium persulfate (dipotassium peroxidisulfate) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mouse TNF-α, IL-1β, IL-6 and IL-33 kits were obtained from eBioscience (San Diego, CA, USA). NF-κB kit from Cell Signaling (Beverly, USA). Other reagents were of commercially available analytical grade.

2.2. Preparation of T-loaded Microcapsules (MT)

It was prepared two microencapsulated formulations by complex coacervation technique, as we previously described (5), with different proportions of drug:polymers. Pectin and casein were dispersed in distilled water under constant mechanical shaking and the pH of 8.0 ± 0.1 was adjusted with sodium hydroxide (4.0 M). In the first formulation, T was added in the 1:5 proportion (MT1:5) and in the second formulation, it was used the 1:3 proportion (MT1:3).Microcapsules were obtained by slow and gradual reduction of the pH from 8.0 ± 0.1 to 3.0 ± 0.1 with citric acid (1 M). The microencapsulated formulations were spray dried with a laboratory scale spray (Lab Plant, model SD-05) using a double-fluid type atomizer nozzle with external mixture and an outlet orifice of 1 mm. The process parameters were: the inlet air temperature was kept constant at 180 °C and the outlet temperature was at 104-110 °C; the feed rate was 377,8 mL/h, and the airflow was 66 m^3/h.Inert microcapsules (IM) were prepared without the drug (T) using the same methodology.

2.3. Encapsulation Efficiency (EE)

The content of T encapsulated in MT1:5 and MT1:3 was determined using 100 mg of each microcapsules dispersed in 10 ml of acetonitrile followed by centrifugation at 0.8 g for 5 min. This step was repeated three times. The supernatant was analyzed by spectrophotometer at 337 nm to determine drug content (17), using a standard curve of pure T. Encapsulation efficiency was calculated using the following equation (1):

$$EE(\%) = \left(\frac{inicial\ drug\ added - free\ drug}{inicial\ drug\ added}\right) x\ 100$$

(1)

2.4. Scanning Electron Microscopy (SEM) Study

The pure T and spray-dried MT1:5 and MT1:3 were coated under argon atmosphere with gold and examined under a scanning electron microscope (JOEL JSM-T330A). Pictures were taken at 2400 magnification.

2.5. Particle Size Measurement

The particle size distribution was analyzed by light scattering using a Zeta-APS system (Matec Applied Sciences, Northborough, MA, USA). The pure T, IM, MT1:5 and MT 1:3 were dispersed in double distilled water and measured at 25°C for analysis. The particle size was obtained by cumulate analysis using the Zeta-APS software.

2.6. X-ray Diffraction

The X-ray diffraction patterns of pure T, IM, MT1:5 and MT 1:3were obtained in a Bruker D8 powder diffractometer (Bruker AXS, Madison, WI, USA) using the Bragg–Brentano geometry in a continuous mode with a scan speed of 0.05°/s. A Cu Kα (λ = 1.5405 Å) line focus radiation tube was operated at 40 kV and 30 mA, and 2θ angle were taken from 3 to 40°.

2.7. Differential Scanning Calorimetry (DSC)

DSC analyses of pure T, IM, MT1:5 and MT 1:3were performed using a Shimadzu DSC-60. The samples were heated from 30°C to 500°C at scan rate of 5°C/min of nitrogen gas flow.

2.8. *In Vitro* Release

The *in vitro* release of T from MT1:5 and MT1:3 were performed during 14 h in Erweka DT800 dissolution system using USP Apparatus 1 at a rotational speed of 50 rpm. During the first 2 h 500 mL of HCl 0,1 M, added to 2.5% Tween® 80, at pH 1.2 ± 0.1 at 37°C were used to simulate the gastric fluid (SGF) conditions. In the next 12 h, 500 mL of KOH-KH_2PO_4 0,1 M, added to 2.5% Tween® 80, were used to simulate intestinal fluid (SIF) conditions reaching pH 7,1 ± 0,1. At the indicated time points, samples were collected and filtered through 0.45-µm Millipore filter paper. An equal volume of dissolution medium was replaced at each sampling time to maintain a constant media volume. Each sample was analyzed spectrophotometrically at 337 nm to determine drug content (17), using a standard curve of pure T. The results were expressed as mean (±SEM) of three determinations.

The mechanism of T release from MT1:5 and MT1:3 was performed by fitting the release rate data into the following equations:

$$\text{Zero-order model equation: } Q = k_0 t \quad (1)$$

$$\text{First-order model equation: } \ln (100 - Q) = \ln (Q_0) - k_1 t \quad (2)$$

$$\text{Higuchi's square-root equation: } Q = k_H t^{1/2} \quad (3)$$

In equations, Q is the percentage of drug released at time t; Q_0 is the percent of drug remaining to be released at time 0, and k_0, k_1, and k_H are the release rate constants for equations.

2.9. Animals

Animal care and handling procedures were approved by the Ethics Committee of the Londrina State University (process number 1494.2015.56). Male Swiss mice (25 g) from the Londrina State University (Londrina, Brazil) were used. They were kept in standard plastic cages with free access to water and food, temperature of 23 °C ± 2 and a 12/12 h light/dark cycle was used.

2.10. Acetic Acid-induced Colitis

Colitis was induced in mice according to a published method (6). The animals received, into the lumen of the colon, acetic acid solution (7.5%, in saline, 200 µL) stimulation after 24 h fasted, through a 3 cm polyethylene cannulum. The negative control group underwent intracolon instillation with saline instead. Animals were left upside down for 3 min to prevent the leakage of intracolon instillation.

2.11. Experimental Design and Treatments

The dose of 0.3 mg/kg of pure T, MT1:5 or MT1:3 were used because this dose of pure T did not provide anti-inflammatory and antioxidant effects in acetic acid-colitis (according to previous experiments in our laboratory). Two experimental protocols were followed (6) to evaluate whether MT provides additional effects to the pure T on acetic acid induced-colitis in mice. The first protocol (A) was used to assess inflammatory parameters. The animals were treated with pure T, MT1:5 or MT1:3 (0.3 mg/kg) 2 h before and 10 h after colitis induction. Distal colon were collected for myeloperoxidase activity, edema, macroscopic damage, colon length, microscopic damage and cytokine production evaluation 18 h after colitis induction. The second protocol (B) was used to evaluate the antioxidant

capacity on colon samples. The animals were treated with pure T, MT1:5 or MT1:3 (0.3 mg/kg) 6 h and 1 h before colitis induction. The ferric reducing antioxidant power (FRAP), ABTS radical cation scavenging and reduced GSH levels were determined in the samples of the distal colon 4 h after colitis induction. The results are representative of two separated experiments.

2.12. Myeloperoxidase Activity Assay

Colon samples were homogenized using a Turrax IKA (T10 basic) in K_2HPO_4 buffer (50 mM, pH 6.0) containing HTAB (13.72 mM). The homogenates were centrifuged (16100g, 2 min, 4 °C) and the resulting supernatants reacted with K_2HPO_4 buffer (50 mM, pH 6.0) containing o-dianisidine dihydrochloride (0.0167%) and hydrogen peroxide (0.05%). The absorbance was determined at 450 nm (Multiskan GO ThermoScientific). The myeloperoxidase activity of samples was compared to a standard curve of neutrophils and presented as the number of neutrophils per mg of tissue (18).

2.13. Macroscopic and Histologic Features of Colitis

The macroscopic damage was determined using the criteria outlined by Morris, Beck (19) with modifications (6), considering the presence and extension of hyperemia, ulcers and inflammation. For histological damage evaluation, colon samples were immediately fixed in buffered formalin (10%). After fixation, the specimens were routinely processed and embedded in paraffin. Serial histology sections of 5 μm thickness were obtained, mounted on slides and stained with hematoxylin-eosin. The assessment of damage was graded considering the loss of mucosal architecture, cellular infiltration, muscle thickening, crypt abscess formation and goblet cell depletion (6, 20).

2.14. Total Antioxidant Capacity Assays

The ferric reducing antioxidant power (FRAP) and ABTS radical cation scavenging property in colon samples was determined as previously described (21), adapted to a 96-well microplate format. Colon samples were homogenized in KCl solution (1.15%) and centrifuged at 0.2g, 4 °C for 10 min. The supernatant aliquots reacted with the FRAP reagent, composed by ferric chloride (20mM) and 2,4,6-tripyridyl-s-triazine (TPTZ, 10 mM) in acetate buffer (0.3 mM, pH 3.6), or with ABTS radical cation solution (7mM in potassium persulfate). After 6 min of incubation, the absorbance was read at 595 nm and 730 nm (Multiskan GO ThermoScientific), respectively. The reducing ability and free radical scavenging property of the tissue were equated against a Trolox standard curve (0.4−400 nmol). The results were expressed as nmol Trolox equivalents per mg of tissue.

2.15. Reduced GSH Assay

Colon samples were homogenized in EDTA solution (0.02 M), treated with trichloroacetic acid (30%) and centrifuged at 1.5g at 4 °C for 15 min. The supernatant reacted with Tris-HCl (0.4 M, pH 8.9) and dithiobisnitrobenzoic acid (0.01 M). After 5 min, the absorbance was determined in 412 nm (Multiskan GO ThermoScientific) (22). Protein levels in the colon homogenates was measured using the Lowry method (23). The results were expressed as μmol of GSH per mg of protein, using a standard GSH curve (2–2000 nmol).

2.16. Cytokine Measurements

TNF-α, IL-1β, IL-6 and IL-33 levels were evaluated in colon samples using ELISA Ready-SET-Go! kits (eBioscience) according to the manufacturer's recommendations. Previously, colon samples were homogenized in sterile saline, centrifuged at 0.8g at 4 °C for 10 min and the cytokine levels were determined in supernatant. Protein levels in the colon homogenates was measured using the Lowry method (23). The results were expressed as pg of cytokine per mg of protein.

2.17. NF-κB Activity

The levels of phosphorylated and total NF-κB p65 subunit in colon samples were determined by ELISA using PathScan® kits (Cell Signaling) according to the manufacturer's recommendations. The results are expressed as OD of samples (total p65/phospho-p65 ratio) at 450 nm (Multiskan GO ThermoScientific).

2.18. Statistical Analysis

All data are expressed as means ± SEM. Statistical significance of differences between the groups was determined by ANOVA followed by the Newman Keuls's test. For categorical variables, the Kruskal−Wallis test followed by Dunn's test was performed. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). The criterion for significance was chosen at $p < 0.05$.

3. RESULTS

3.1. Characterization of MT

We prepared two formulations of microcapsules from pectin/casein complex containing different proportion of drug (T)/polymer, called MT1:5 and MT1:3. The first formulation, MT1:5, was prepared with 1 part of T plus 5 parts of pectin/casein complex, while the second, MT1:3, used 1 part of T and 3 parts of polymer complex. The encapsulation efficiency of microcapsules were 82.9% for MT1:5 and 75.7% for MT1:3 (Table I). The photomicrographs of the T, MT1:5 and MT1:3 are shown in Figure 1A, 1B and 1C, respectively, where we can observed that the complex coacervation process employed yielded microcapsules with surface irregularities (depressions), but no cracking (Figure 1B and 1C). The median particle size was 0.76 ± 0.42 μm for T (Figure 2A), 0.94 ± 1.83 μm for IM (Figure 2B), 0.65 ± 1.77 μm for MT1:5 (Figure 2C; Table I), and 0.80 ± 1.94 μm for MT1:3 (Figure 2D; Table I).

Powder X-ray diffraction (XRD) data of T, IM, MT1:5, and MT1:3 are shown in Figure 3. The XRD pattern of free T showed a number of distinct peaks characteristic of high crystalline nature (Figure 3A). However, no diffraction peaks were observed in IM (Figure 3B). There are peaks of crystalline T in XRD pattern of MT1:5 (Figure 3C) and MT1:3 (Figure 3D). The DSC analysis of T (Figure 4A) showed one endothermic peak at 56.5°C corresponding to the melting point of T; nevertheless, this peak was not visible in IM (Figure 4B), MT1:5 (Figure 4C) and MT1:3 (Figure 4D).Furthermore, we observed other exothermic peaks for IM (Figure 3B), MT1:5 (Figure 3C), and MT1:3 (Figure 3D), but not for T (Figure 4A), at approximately 290°C and between 430-470°C, which probably corresponds to polymer degradation peaks.

Table I. Proportion of drug (T)/polymer, encapsulation efficiency of T and particle size of MT1:5 and MT1:3

Formulation	Proportion drug (T):polymer	Encapsulation efficiency (%)	Particle size (μm)
MT1:5	1:5	82.9	0.65 ± 1.77
MT1:3	1:3	75.7	0.80 ± 1.94

3.2. *In Vitro* Release

trans-Chalcone (T) release from MT1:5 and MT1:3 in simulated gastric and intestinal fluid conditions at 37°C for 14 h was shown in Figure 5A. Corroborating with previous results using similar pectin/casein complex to prepare microcapsules (6), T was released *in vitro* in simulated intestinal fluid, but not in simulated gastric fluid and there was no significant difference in drug-release profile between the MT1:5 and MT1:3 in the first 2 h. The release of T from MT1:5 initiated in simulated intestinal fluid conditions and achieved nearly 37.5 ± 0.7% of release within 10 h. This was accompanied by a slower and more sustained release of T from MT1:5 over the period of 14 h. In contrast, the release profile of T from MT1:3 achieved 49.5 ± 3.9% in 14 h.

In addition, the release data of MT1:5 and MT1:3 were fitted into different release mechanism models (Table II). The kinetic evaluation of formulations started at 2 hour and 15 min (Figure 5A) until the release rate reached steady state. For both formulations, MT1:5 and MT1:3, there was a partial linearization between the T release rate and the square root of time, suggesting the release kinetics can be explicated by Higuchi's equation, indicating that the drug release was predominantly controlled by diffusion.

Table II. Correlation coefficient (R^2) of different kinetic models for MT1:5 and MT1:3 formulations

Formulation	Dissolution time (min)	Zero-order $Q = k_0 t$	First-order $\ln (100 - Q) = \ln (Q_0) - k_1 t$	Higuchi $Q = k_H t^{1/2}$
MT1:5	135 - 420	0.9849	0.9903	0.9921
MT1:3	135 - 420	0.9340	0.9542	0.9657

Q: the percentage of drug released at time t; Q_0: the percentage of drug remaining to be released at time 0; k_0, k_1, k_H: the release rate constants for equations.

3.3. MT Reduces Myeloperoxidase (MPO) Activity in the Colon of Mice with Acetic Acid-induced Colitis

The administration of acetic acid (7.5% in saline; 200 µL) increased MPO activity, an indirect measurement of neutrophil recruitment, in the colon of mice compared to negative control group, which received intracolon saline (200 µL) instead of acetic acid solution (Figure 5B). Treatment with IM, T and MT1:5 at a dose of 0.3 mg/kg did not significantly reduce the acetic acid-induced neutrophil recruitment to the colon, while a significant

reduction was observed by treatment with MT1:3 at the same dose (Figure 5B). So, we used this formulation (MT1:3) for the next experiments.

3.4. MT Reduces Macroscopic Damage in the Colon of Mice with Acetic Acid-induced Colitis

Extensive inflammation characterizes the macroscopic lesions induced by intracolon administration of acetic acid (7.5% in saline; 200 µL) in colitis control group (mean score of 6.25; Figure 6A and 6b.2), while no lesion was observed in negative control group (score 0; Figure 6A and 6b.1). Treatment with IM (0.3 mg/kg) and T (0.3 mg/kg) were ineffective in reducing the macroscopic damage in the colon of animals with acetic acid-induced colitis (mean score of 5.5; Figure 6A, 6b.3 and 6b.4). On the other hand, treatment MT1:3 (0.3 mg/kg) showed a progressive reduction of acetic acid-induced macroscopic damage in the colon (mean score of 3.5; Figure 6A and 6b.5).

3.5. MT Reduces Microscopic Damage in the Colon of Mice with Acetic Acid-induced Colitis

The samples of negative control group showed the expected morphology, with typical histological colon structure and no lesions (score 0; Figure 7A and 7B). On the other hand, the intracolon administration of acetic acid (7.5% in saline; 200 µL) led to typical signs of colitis, characterized by pronounced loss of mucosal architecture, leukocyte recruitment and goblet cell depletion, and moderated crypt abscess formation (mean score of 11; Figure 7A and 7C). Treatment with IM (0.3 mg/kg) and T (0.3 mg/kg) were ineffective in reducing histological lesions in the colon of animals with acetic acid-induced colitis (mean score of 9.4 and 8.6, respectively; Figure 7A, 7D and 7E). Interestingly, treatment with MT1:3 (0.3 mg/kg) significantly ameliorated the histopathological scoring of inflammation compared to colitis control group (mean score of 5.9; Figure 7A), through partial preservation of mucosal architecture, reduction of leukocyte recruitment, prevention of goblet cell depletion, and avoiding crypt abscess formation (Figure 7F).

3.6. MT Improves Antioxidant Capacity in the Colon of Mice with Acetic Acid-induced Colitis

The colitis control group showed significant reduction in the ferric reducing antioxidant power (Figure 8A), ABTS radical cation scavenging property (Figure 8B), and GSH levels (Figure 8C) in the colon after administration of acetic acid (7.5% in saline; 200 µL), compared

to negative control group. Treatment with MT1:3 (0.3 mg/kg), but not with IM and T at the same doses, significantly prevented colon antioxidants depletion in acetic acid-induced colitis, improving the ferric reducing antioxidant power (Figure 8A), ABTS radical cation scavenging property (Figure 8B), and GSH levels (Figure 8C), therefore preserving the colon tissue from oxidative damage that characterizes intestinal inflammation.

3.7. MT Reduces Pro-inflammatory Cytokines Production and NF-κB Activation in the Colon of Mice with Acetic Acid-induced Colitis

Intracolon administration of acetic acid (7.5% in saline; 200 µL) significantly increased the levels of the pro-inflammatory cytokines TNF-α (Figure 9A), IL-1β (Figure 9B), IL-6 (Figure 9C) and IL-33 (Figure 9D) in the colon, compared to the negative control group. In agreement, there was increased NF-κB activation in colitis compared to negative control group (figure 10). Treatment with MT1:3 (0.3 mg/kg) significantly reduced TNF-α (Figure 9A), IL-1β (Figure 9B), IL-6 (Figure 9C) and IL-33 (Figure 9D) levels in the colon of mice compared to the colitis control group. Importantly, we demonstrated that MT1:3 (0.3 mg/kg) also reduced NF-κB activation in the colon of mice with acetic acid-induced colitis. On the other hand, IM (0.3 mg/kg) and T (0.3 mg/kg) did not affect significantly the production of TNF-α (Figure 9A), IL-1β (Figure 9B), IL-6 (Figure 9C) and IL-33 (Figure 9D), and the activation of NF-κB induced by acetic acid (Figure 10).

4. DISCUSSION

trans-Chalcone-loaded microcapsules (MT) were prepared by complex coacervation using the pectin/casein complex polymer under mild conditions (e.g., absence of organic solvents), thus, it is of great pharmaceutical interest (5). Previous results showed that microcapsules obtained from pectin/casein polymer provide modified release, allowing a slow and gradual release of a test compound under conditions that simulate the gastrointestinal environment *in vitro*(5, 6), and improve the anti-inflammatory and antioxidant effects of quercetin in acetic acid-induced colitis (6). Therefore, this system is appropriate to meet the aims of this study.

Surface of MT did not exhibit any sign of cracking, as a result of the drying process, which suggest high integrity of the encapsulating material. The surface irregularities observed may improve the release properties of the MT due to the increased surface area of the capsule (24). The proportion of drug:polymer used to prepare MT1:5 and MT1:3 affected the encapsulation efficiency and particle size. The encapsulation efficiency decreased and the mean particle size become slightly higher with the increase of the proportion drug:polymer from 1:5 to 1:3. Corroboranting, increasing quercetin concentrations in quercetin-nanoparticle formulations lead to higher values of average size (25). The XRD pattern of free T and MTs (both 1:5 and 1:3) provide evidence that the encapsulation does not alter the crystallinity of the sample. In DSC analysis, the absence of endothermic peak at 56.5°C, corresponding to the melting point of T, in MT formulations is possibly due to entrapment of drug molecules into and in the surface of microcapsules.

The MT1:5 formulation presented higher drug encapsulation efficiency (82.9%) and lower drug release within 14 h (38.73 ± 1.94%) than MT1:3 (75.7% and 49.55 ± 3.91%, respectively). Thus, increased encapsulation efficiency diminishes the drug release in simulated intestinal fluid conditions (5), because of the greater amount of polymer in the formulation, corroborating to other studies using microcapsules (5, 27). Furthermore, MT1:5 exhibited a biphasic release pattern with an effect during the first 10 hours followed by a sustained release until 14 h; while MT1:3 maintained a constant release of the drug without reaching the plateau at 14 h. Thus, although MT1:3 presented lower encapsulation efficiency, it showed a constant and higher *in vitro* delivery of T predominantly controlled by diffusion within 14 h. Since the T provides beneficial effects in colitis in doses above of 3 mg/kg (unpublished data), we propose to investigate if microencapsulation allows reduction of the required dose of T to exercise the antioxidants and anti-inflammatory effects in acetic acid-induced colitis model. So, we used a dose of 0.3 mg/kg of MT1:5 and MT1:3 formulations, IM and T.

As noted earlier with quercetin-loaded microcapsules (6), we also observed that MT obtained from pectin/casein polymer provide modified release and improve the anti-inflammatory and antioxidant effects of T in experimental colitis. Firstly, we evaluated the effect of both formulations (MT1:5 and MT1:3) on neutrophil recruitment to the colon tissue of mice induced by acetic acid. Neutrophils, as well as monocytes and lymphocytes, is widely present in the colon tissue of patients with active disease and contributes markedly to tissue damage and mucosal dysfunction in UC (28), through the production of significant quantities of ROS/RNS and cytokines (29). We observed that treatment with MT1:3, but not IM, non microencapsulated T and MT1:5, significantly reduced neutrophil recruitment to the colon of mice. Although there was a tendency of reduction with MT1:5 treatment, this effect was not enough to promote significant differences compared to colitis control group. We suggest that the different formulations do not have the same effects *in vivo*, probably due to smaller amount of T released by MT1:5 compared to MT1:3 over the time, which was not enough to significantly reduce neutrophil recruitment. Considering this result, we continue evaluating the therapeutic effects of microcapsules using MT1:3 formulation in order to preserve the number of animals in study, since neutrophils are extremely important in the injury process and significant effect was provided only by MT1:3.

The reduction of neutrophil recruitment by MT1:3 lines up to the preservation of tissue damage, observed in macroscopic and histological analyzes of colon lesions induced by acetic acid. This finding corroborate with our (unpublished data) and other studies demonstrating that T and its derivates also reduce neutrophil recruitment (30-32). However, here we could use the dose of 0.3 mg/kg of MT1:3 to obtain significant effects, while the treatment with the same dose of 0.3 mg/kg of non-encapsulated T does not reduce the neutrophil recruitment and colon lesions. As well as in ulcerative colitis, the inflammatory response promoted by acetic acid induces oxidative stress in colon tissue leading to mucosal erosion, distortion and loss of crypts (28), such as observed in histological analyses.

Activated neutrophils are also important sources of reactive oxygen species (ROS) within intestinal mucosa and ROS are key regulators of neutrophil chemotaxis (33). Normally, the tissues possess sufficient amounts of protective enzymatic (SOD, catalase, GSH peroxidase) and nonenzymatic (thiols, ascorbate, α-tocopherol) antioxidants that will decompose most of the injurious oxidizing agents thereby limiting tissue damage (34). However, an excessive production of ROS occurs in UC, leading to oxidative damage in tissues, so it is necessary to seek antioxidant therapies to reduce the damage generated (35). In this sense, we demonstrated that treatment with MT1:3, besides reduces neutrophil recruitment and the colon lesions, also prevented the depletion of total antioxidant capacity (FRAP and ABTS assays) and GSH levels, contributing to the reduction of oxidative stress induced by acetic acid. Over again, the same dose of non-encapsulated T does not

prevented the depletion of antioxidant capacity in colon tissue. Corroborating with our results, it was reported that T delays the consumption of GSH and other cellular antioxidants during oxidative stress induced by hydrogen peroxide (H_2O_2) in hepatocellular carcinoma (HepG2) cells (12). So, we suggest that treatment with MT1:3 can reduce the consumption of endogenous antioxidants, offering protection against oxidative stress during acetic acid induced colitis in mice.

Pro-inflammatory cytokines are cardinal biomarkers also released in intestinal mucosa by activated neutrophils, and are increased in intestinal mucosa of patients with UC (36). Briefly, TNF-α, IL-1β and IL-33 are involved in the activation of transcription factors and the resulting expression of more pro-inflammatory cytokines, chemokines, adhesion molecules, cyclooxygenase, inducible nitric oxide synthase, and matrix metalloproteinases (37, 38), that amplify the inflammatory response. Furthermore, IL-6 has emerged as main mediator of inflammation-associated tumorigenesis (39). Then, reducing pro-inflammatory cytokine levels results in improvement of inflammation characteristic of UC. Our result demonstrates that treatment with MT1:3, but not non-encapsulated T, significantly reduces the colon levels of TNF-α, IL-1β, IL-6 and IL-33 compared to colitis control group, besides decreases neutrophil recruitment and oxidative stress. As well as ROS, cytokines also mediate their action via activation of NF-κB signaling pathway (40) and it was demonstrated increased activation of NF-κB in patients with UC, accompanied by marked secretion of TNF-α and IL-6, which are directly involved in the mucosal tissue damage occurring in UC (41). Therefore, the effective inhibition of NF-κB activation by MT1:3 probably decreased cytokines and other pro-inflammatory molecules expression and improved the inflammation in acetic acid-induced colitis. In agreement, T and several chalcones, such as butein, cardamonin and 2’-hydroxychalcone, also inhibit NF-κB-mediated inflammation (14, 32, 42).

5. CONCLUSIONS

We prepared and tested two microencapsulated formulations from pectin/casein complex containing T in different proportions (MT1:5 and MT1:3) by complex coacervation process in order to reduce the required dose of drug to obtain anti-inflammatory and antioxidant effects in acetic acid-induced colitis model in mice. The microcapsules presented surface depressions, but no cracking. However, only the treatment with MT1:3 significantly reduced neutrophil recruitment to the colon of mice after acetic acid-induced colitis. At the same time, treatment with MT1:3 also reduces macro and microscopic lesions in the colon, improves antioxidant capacity and decreases pro-inflammatory cytokines levels, by reducing NF-kB activation. It is important to mention that treatment with non-encapsulated T at the same dose of MT1:3 (0.3 mg/kg) was not sufficient to provide the benefic effects obtained with MT1:3 in acetic acid-induced colitis model. But, when T is conveyed in MT1:3 formulation, that provides modified release of the drug due to the properties of the polymer to swell and to release gradually, it becomes sufficient to significantly reduce NF-kB activation and the other parameter of inflammation and oxidative stress induced by acetic acid. It once again demonstrates the importance of pharmaceutical development of modified release systems to reduce the required dose of drugs to obtain anti-inflammatory and antioxidant effects. So, treatment with MT1:3 prevents acetic acid-induced colitis in mice and this protective effect is related to its anti-inflammatory and antioxidant actions. However, further investigations are necessary to evaluate whether a similar efficacy can be achieved in other models of experimental colitis and in clinical application.

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Figures

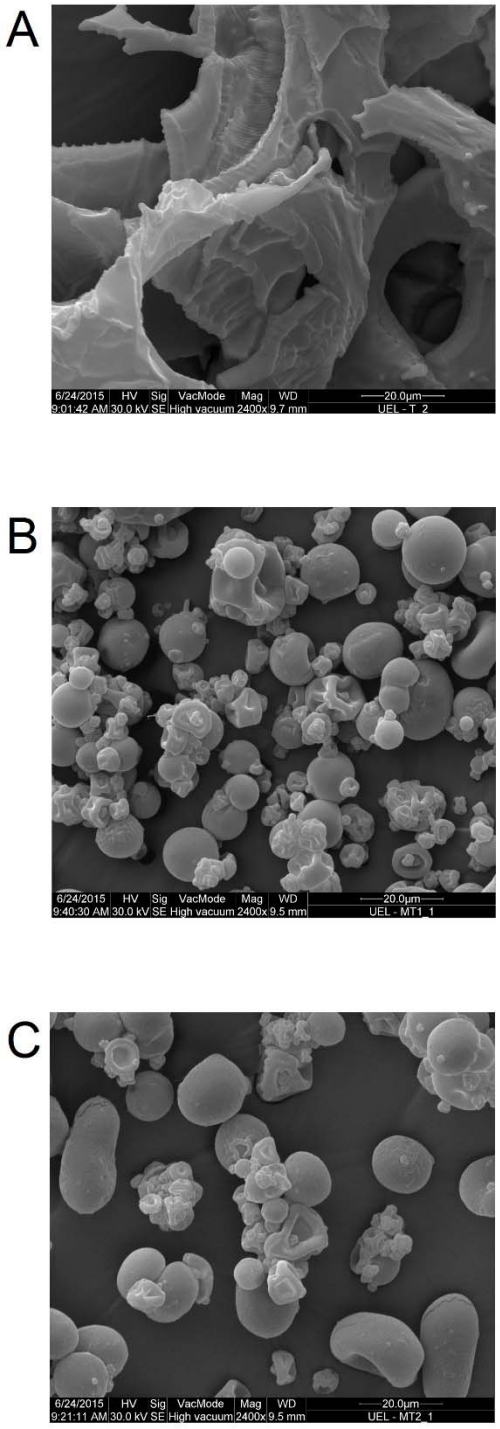

Figure 1. SEM photomicrographs of pure T and MT. Original magnification x 2400 of T (A), MT1:5 (B) and MT1:3 (C).

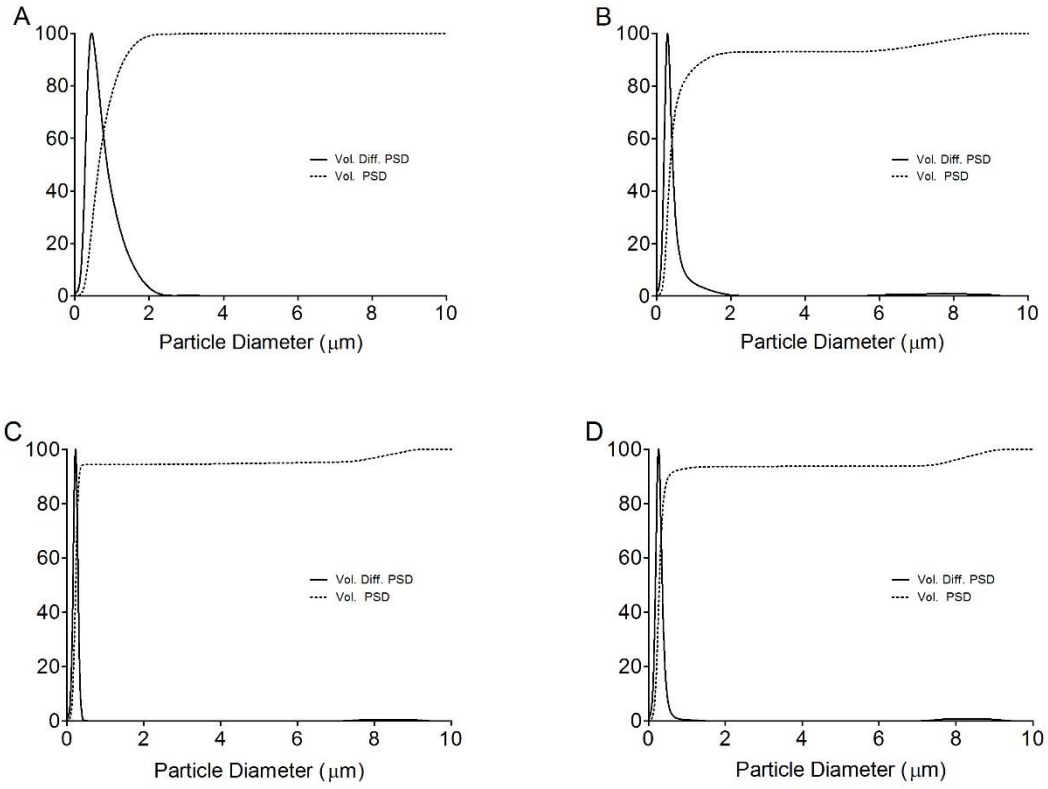

Figure 2. Particle size distribution of pure T (A), IM (B), MT1:5 (C) and MT1:3 (D).

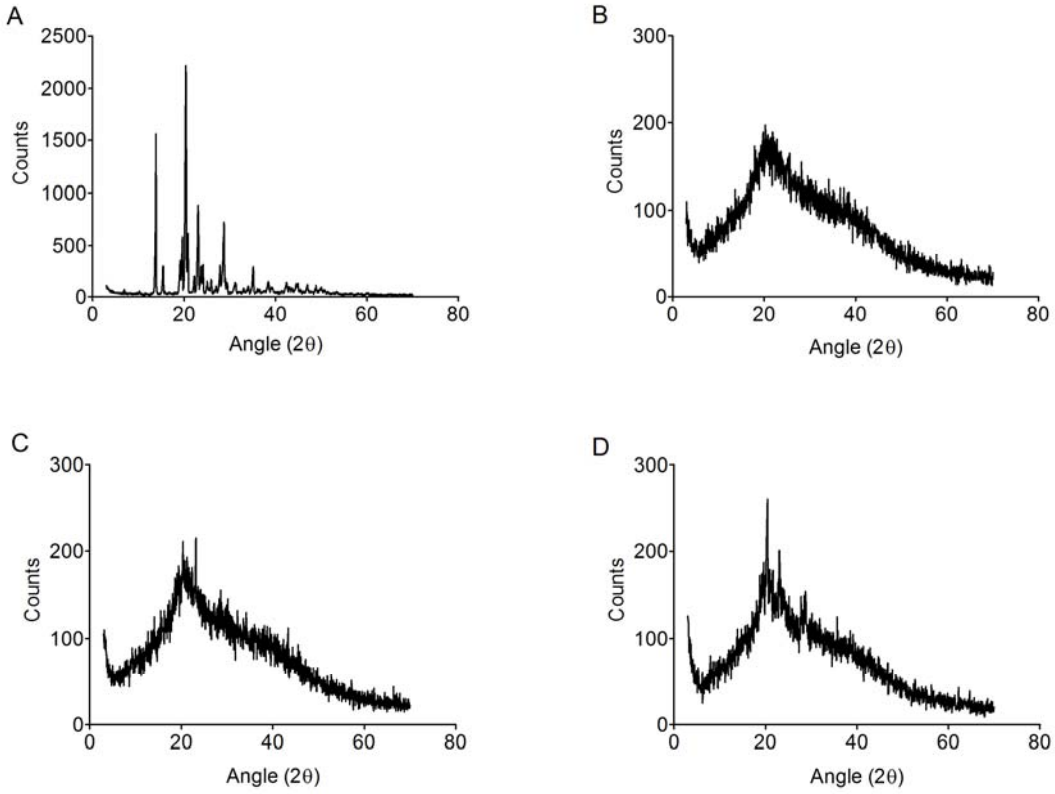

Figure 3. Powder X-ray diffraction pattern of pure T (A), IM (B), MT1:5 (C) and MT1:3 (D).

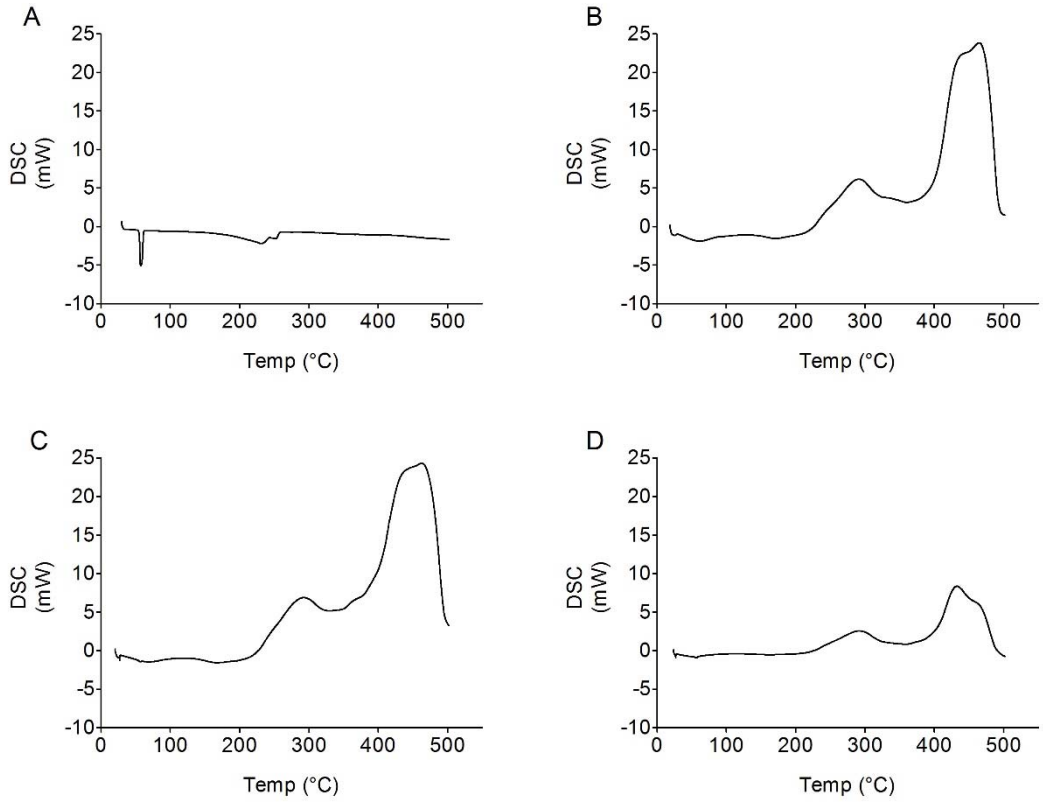

Figure 4. DSC thermograms of pure T (A), IM (B), MT1:5 (C) and MT1:3 (D).

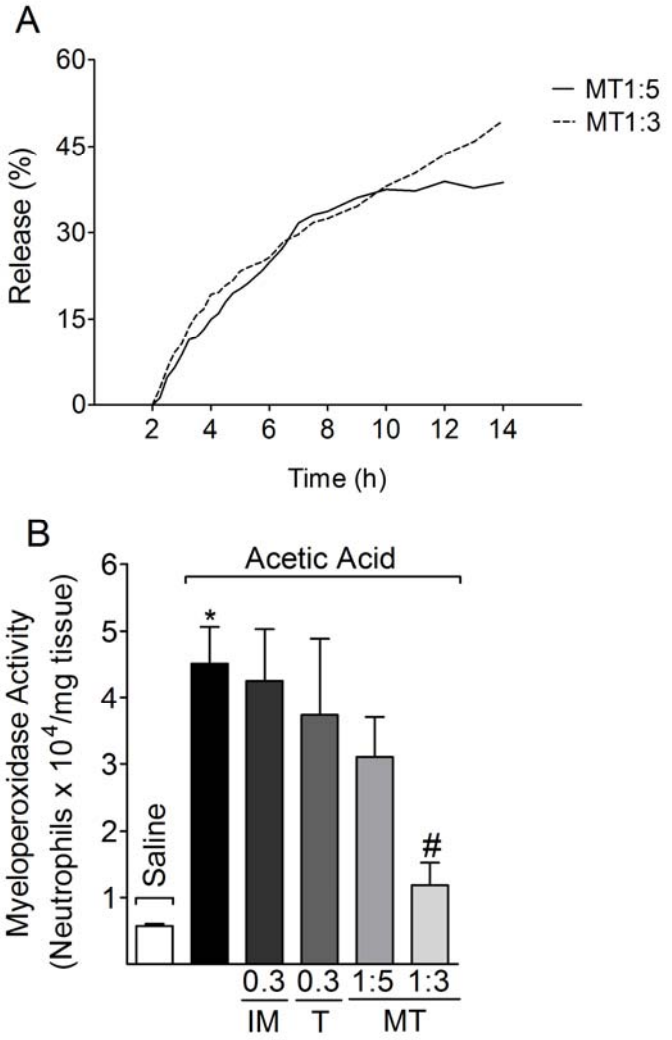

Figure 5. *In vitro* release profile of MT (A) and the reduction of neutrophil recruitment (B) by MT in the colon of mice with acetic acid-induced colitis. Mice were treated with IM (0,3mg/kg, po), T (0,3mg/kg, po), MT1:5 (0,3mg/kg, po) or MT1:3 (0,3mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). Myeloperoxidase activity (B) were evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

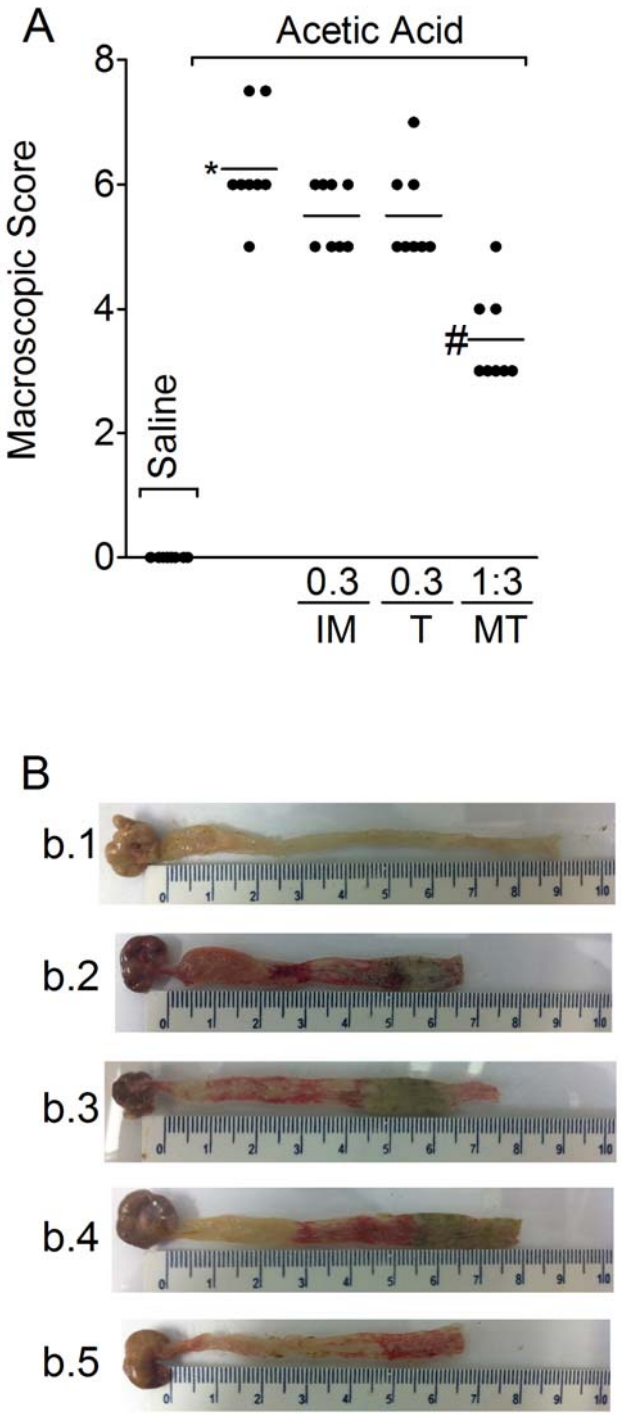

Figure 6. MT reduces macroscopic damage in the colon of mice with acetic acid-induced colitis. Mice were treated with IM (0,3mg/kg, po), T (0,3 mg/kg, po)or MT1:3 (0,3mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). The macroscopic damage score (A) of negative control group (b.1), colitis control group (b.2), IM treated group (b.3), T treated group (b.4) and MT treated group (b.5) were evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test.

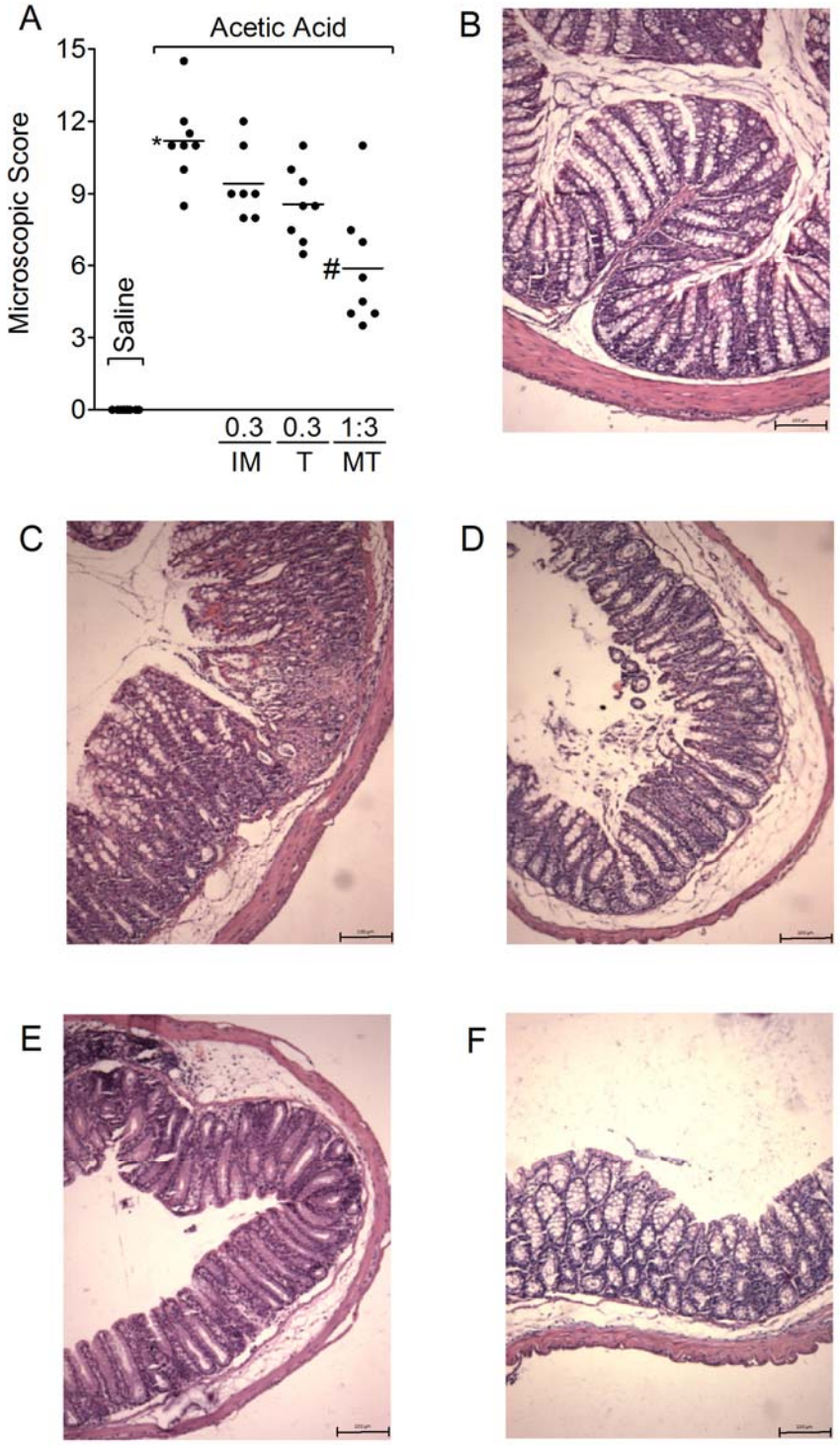

Figure 7. MT reduces microscopic damage in the colon of mice with acetic acid-induced colitis. Mice were treated with IM (0,3mg/kg, po), T (0,3 mg/kg, po)or MT1:3 (0,3mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). The microscopic damage score (A)of negative control group (b.1), colitis control group (b.2), IM treated group (b.3), T treated group (b.4) and MT treated group (b.5) were evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test.

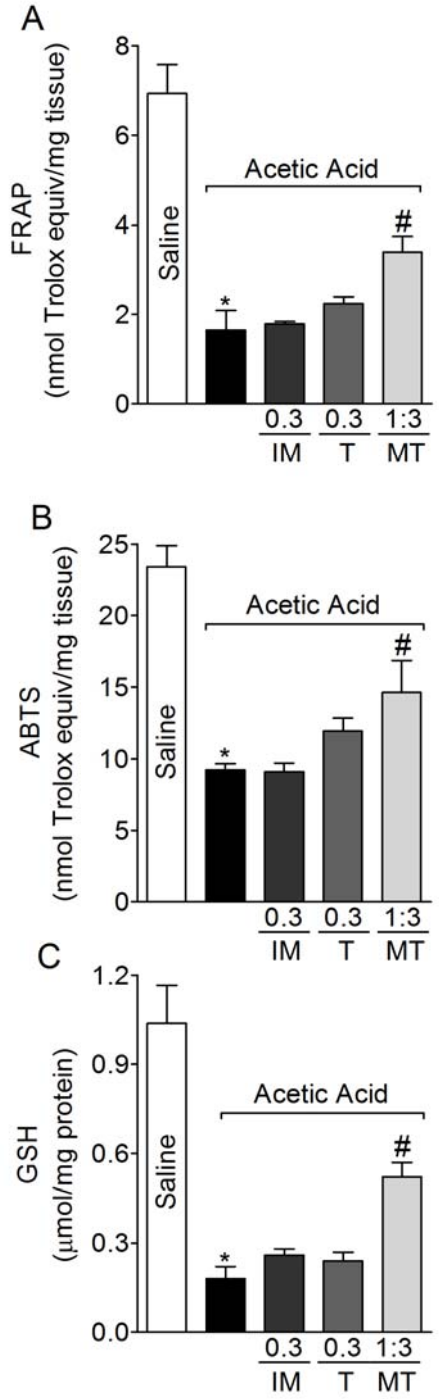

Figure 8. MT improves antioxidant capacity in the colon of mice with acetic acid-colitis. Mice were treated with IM (0,3mg/kg, po), T (0,3 mg/kg, po)or MT1:3 (0,3mg/kg, po), 6 and 1 h before intracolon administration of acetic acid (7,5%, 200µL). The ferric reducing antioxidant power - FRAP (A), ABTS radical scavenging ability (B) and reduced GSH levels (C) were determined 4 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

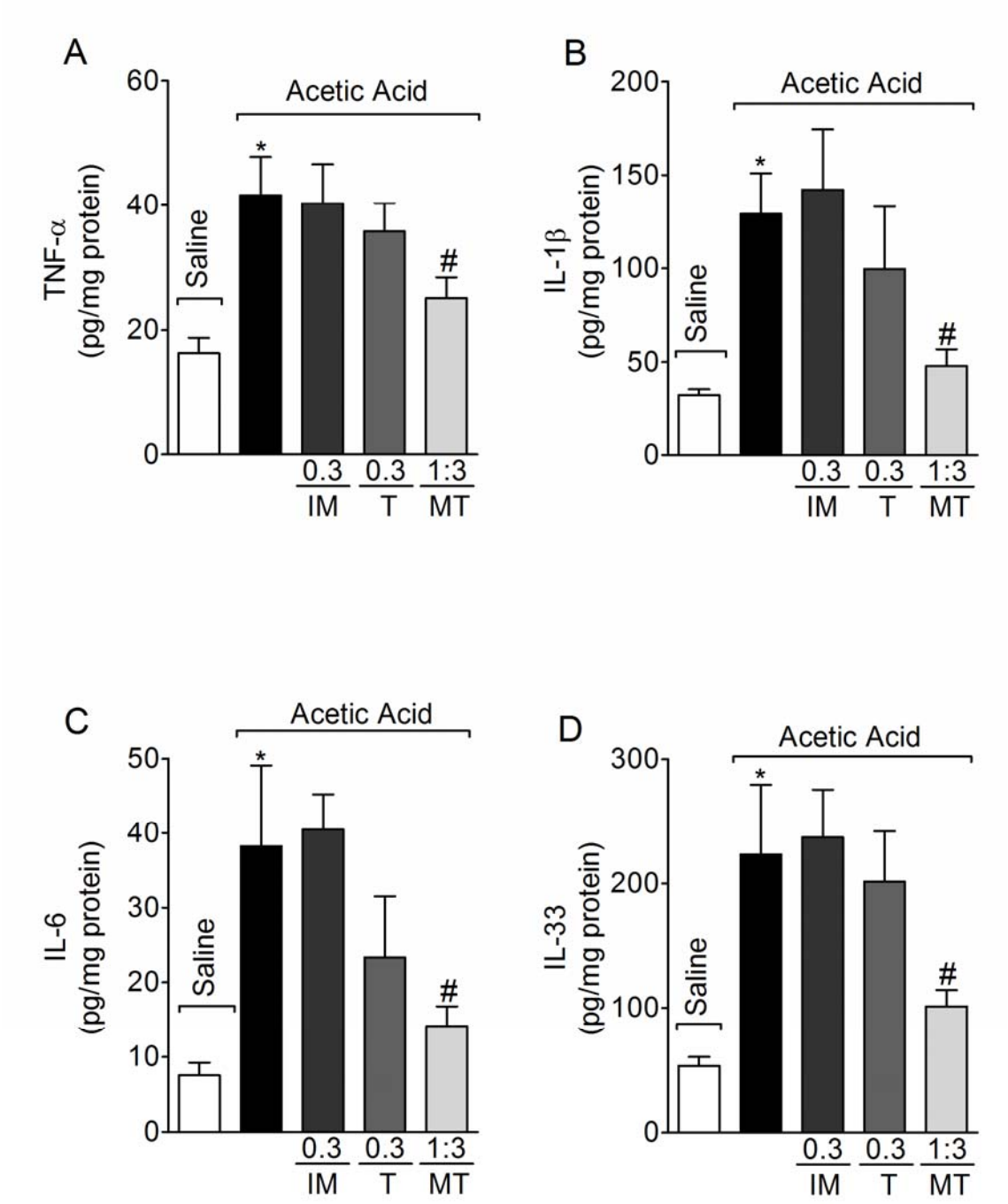

Figure 9. MT reduces pro-inflammatory cytokines production in the colon of mice with acetic acid-induced colitis. Mice were treated with IM (0,3mg/kg, po), T (0,3 mg/kg, po)or MT1:3 (0,3mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). TNF-α (A), IL-1β (B), IL-6 (C) and IL-33 (D) levels were evaluated 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

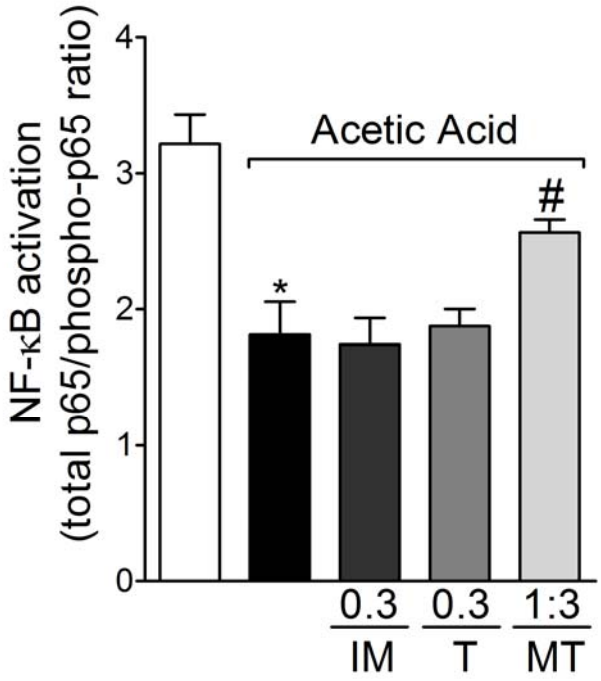

Figure 10. MT reduces NF-kB activation in the colon of mice with acetic acid-induced colitis. Mice were treated with IM (0,3mg/kg, po), T (0,3 mg/kg, po)or MT1:3 (0,3mg/kg, po), 2 hours before and 10 hours after intracolon administration of acetic acid (7,5%, 200µL). The NF-kB activity was determined 18 hours after colitis induction. n=8 and the results are representative of two separated experiments. * $p < 0.05$ compared to negative control group, # $p < 0.05$ compared to colitis control group. ANOVA followed by Newman-Keuls's test.

5 CONCLUSÕES

Os resultados desse estudo demonstraram que o tratamento com HMC e TC por via oral melhora a colite ulcerativa induzida por ácido acético em camundongos. Os efeitos anti-inflamatórios destes fármacos estão relacionados a redução significativa da atividade da mieloperoxidase, do edema, das lesões microscópicas e macroscópicas, dos níveis de citocinas pró-inflamatórias e da ativação do fator de transcrição NF-κB no cólon dos camundongos. Além disto, o tratamento com HMC e TC também reduziu o estresse oxidativo ao prevenir a diminuição das capacidades antioxidantes endógenas no tecido colônico.

O processo de microencapsulação da HMC resultou em microcápsulas com baixa eficiência de encapsulação e com perfis semelhantes de liberação em meio que simula as condições do suco entérico, embora tenhamos utilizado diferentes proporções fármaco:polímeros. O tratamento com as formulações microencapsuladas MH1:5 e MH1:3 não proporcionaram qualquer efeito adicional ao tratamento com o fármaco não microencapsulado, utilizando as mesmas doses, sobre o recrutamento de neutrófilos induzido por ácido acético no cólon dos animais. Portanto, o desenvolvimento das formulações microencapsuladas contendo HMC deve ser aprimorado a fim de se obter microcápsulas com maior eficiência de encapsulação e que permitam liberação modificada do fármaco, para então testá-las quanto à eficácia terapêutica em modelo experimental de colite e verificar se há ou não melhora no efeito terapêutico do fármaco quando este é veiculado em microcápsulas de liberação modificada.

Por outro lado, a microencapsulação da TC com o objetivo de melhorar os efeitos anti-inflamatórios e antioxidantes deste fármaco no modelo de colite induzida por ácido acético em camundongos resultou em microcápsulas sem rachaduras, com boa eficiência de encapsulação. No entanto, apenas o tratamento com uma das formulações, a MT1:3, reduziu significativamente o recrutamento de neutrófilos para o cólon dos camundongos após a colite induzida por ácido acético. Além disto, o tratamento com MT1:3 também reduziu as lesões macro e microscópicos no cólon, melhorou a capacidade antioxidante e diminuiu os níveis de citocinas pró-inflamatórias, ao reduzir a ativação do NF-kB no cólon dos animais. É importante mencionar que o tratamento com TC não microencapsulada com a mesma dose utilizada de MT1:3 (0,3 mg/kg) não foi suficiente para proporcionar os efeitos benéficos obtidos com MT1:3 no modelo de colite induzida por ácido acético. Mas quando a TC é veiculada na formulação microencapsulada MT1:3, que proporciona liberação modificada do fármaco, torna-se suficiente para reduzir significativamente a ativação do NF-kB e outros parâmetros inflamatórios e de estresse oxidativo induzidos pelo ácido acético.

Sendo assim, nossos resultados sugerem a potencial aplicabilidade da HMC e TC no tratamento de doenças inflamatórias intestinais e demonstram a importância do desenvolvimento farmacêutico de sistemas de liberação modificada para reduzir a dose necessária de fármaco para se obter efeitos anti-inflamatórios e antioxidantes na colite experimental. No entanto, são necessárias investigações adicionais para avaliar se eficácia similar pode ser obtida em outros modelos de colite experimental e na aplicação clínica.

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