CARLA FABIANA SOUZA GUAZELLI

AUMENTO DA EFICÁCIA DA QUERCETINA PELA MICROENCAPSULAÇÃO: EFEITO TERAPÊUTICO EM MODELO DE COLITE EM CAMUNDONGOS

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Londrina, como requisito à obtenção do título de Mestre em Ciências da Saúde.

Orientador: Prof. Dr. Waldiceu Ap. Verri Jr

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“O sucesso parece ser, em grande parte, uma questão de persistir quando os outros desistiram”.
William Feather

GUAZELLI, Carla Fabiana Souza. **Aumento da eficácia da quercetina pela microencapsulação**: efeito terapêutico em modelo de colite em camundongos. 2012. 89f. Dissertação (Mestrado em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2012.

RESUMO

A quercetina é um flavonóide com atividades anti-inflamatória e antioxidante expressivas. Estudos mostram que ela é capaz de inibir enzimas liberadas por neutrófilos e macrófagos, reduzir a produção de citocinas pró-inflamatórias, além de sequestrar radicais livres e quelar íons metálicos. No entanto, o tratamento com quercetina por via oral não reduz a inflamação em modelo experimental de colite. Sendo assim, o objetivo deste estudo foi preparar uma nova formulação microencapsulada contendo quercetina para liberação modificada do fármaco, alcançando a região colônica, e investigar os efeitos terapêuticos desta formulação em um modelo experimental de colite induzida por ácido acético em camundongos. As microcápsulas de quercetina foram preparadas a partir do polímero biodegradável pectina/caseína, e o efeito terapêutico no modelo de colite foi avaliado através dos seguintes parâmetros: atividade da mieloperoxidase, edema, escores de lesões histológicas e macroscópicas, níveis de citocinas e capacidade antioxidante no cólon. O tratamento com microcápsulas de quercetina por via oral reduziu significativamente a atividade da mieloperoxidase, o edema, as lesões histológicas e macroscópicas, os níveis de IL-1β e IL-33 no cólon dos camundongos quando comparados ao grupo controle colite sem tratamento. Além disto, o tratamento com esta nova formulação também preveniu a redução dos níveis de IL-10 e da capacidade antioxidante no tecido colônico. O tratamento com quercetina não encapsulada não mostrou efeitos significativos sobre a colite experimental. Estes resultados mostram que a microencapsulação da quercetina, utilizando o polímero pectina/caseína, proporciona efeitos adicionais à quercetina não encapsulada neste modelo experimental de colite, sendo esta nova formulação uma proposta promissora para o tratamento de doenças inflamatórias intestinais.

Palavras-chave: Quercetina. Microcápsulas. Liberação modificada. Colite.

GUAZELLI, Carla Fabiana Souza Guazelli. **Increasing the efficacy of quercetin by microencapsulation:** therapeutic effect on experimental colitis in mice. 2012. 89p. Dissertation (Master in Health Sciences) – Universidade Estadual de Londrina, Londrina, 2012.

ABSTRACT

Quercetin is a flavonoid with expressive anti-inflammatory and antioxidant activity, such as inhibition of enzyme release by neutrophils, reduction of pro-inflammatory cytokines, in addition to scavenge free radicals and chelate transition metal ions. However, the oral administration of quercetin does not provide beneficial effects in experimental colitis. Therefore, the aim of this study was to prepare a novel microencapsulated formulation for quercetin modified-release, reaching the colonic region, and investigate its therapeutic effects on experimental colitis induced by acetic acid in mice. Quercetin microcapsules were prepared using pectin/casein polymer and their efficacy on experimental colitis model was evaluated by myeloperoxidase activity, edema, histological and macroscopical scoring analysis and cytokines levels, as well as antioxidant capacity in colon samples. The oral administration of quercetin microcapsules significantly decreased myeloperoxidase activity, edema, histological and macroscopical colon damage, and IL-1β and IL-33 levels in the colon compared to colitis control group. Furthermore, this novel formulation also prevented the reduction of IL-10 and antioxidant capacity in the colon tissue. Treatment with non-encapsulated quercetin did not provide significant effect on experimental colitis. These preliminary results indicate that the microencapsulation of quercetin using pectin/casein polymer provided additional effects to quercetin compared to non-encapsulated quercetin in experimental colitis, therefore, suggesting quercetin loaded microcapsules as a promising treatment for inflammatory bowel disease.

Keywords: Quercetin. Microcapsules. Modified drug delivery. Colitis.

LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2-azinobis (3-etilbenzotiazolina-6-sulfonato, sal de diamônio)
CU	Colite Ulcerativa
DC	Doença de Crohn
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-1β	Interleucina-1β
IL-33	Interleucina-33
IL-10	Interleucina-10
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 Doençãs Inflamatórias Intestinais

Doenças Inflamatórias Intestinais (DII) é um termo geral para um grupo de desordens inflamatórias crônicas que inclui a Doença de Crohn (DC) e a Colite Ulcerativa (CU). A etiologia precisa ainda é desconhecida, mas sabe-se que fatores genéticos, o sistema imunológico do indivíduo, fatores ambientais e a própria microbiota intestinal contribuem para a patogênese destas doenças (HILL; ARTIS, 2010). A DC é mais regulada geneticamente do que a CU (HALME et al., 2006). Porém, nenhum fator ou agente sozinho é responsável pelo desenvolvimento destas doenças.

As DII ocorrem em todo o mundo, acometem igualmente ambos os sexos (MOLODECKY et al., 2012) e representam um sério problema de saúde, pois atingem preferencialmente adultos jovens, na faixa etária entre 20 e 40 anos. Além disso, cursam com recidivas frequentes e apresentam formas clínicas de alta gravidade (HANAUER, 1990; KWON et al., 2005), resultando em custos a longo prazo tanto para o paciente, quanto para o sistema de saúde e para a sociedade (YU et al., 2008; COHEN et al., 2010).

A incidência das DII é maior nos países ocidentais, sendo o Canadá o país com as maiores taxas, seguido pelos países do norte da Europa e Austrália (MOLODECKY et al., 2012). No Brasil, os dados epidemiológicos obtidos a partir dos sistemas de informação da saúde podem ser comprometidos pela subnotificação do número de internações, bem como pela falta de padronização dos critérios diagnósticos e de profissionais especializados (OLIVEIRA; EMERICK; SOARES, 2010). Apesar de poucos dados epidemiológicos de países em desenvolvimento, estudos mostram que as taxas de incidência das DII têm aumentado em diferentes regiões do mundo, possivelmente devido a fatores ambientais associados ao desenvolvimento industrial, como hábitos alimentares e estilos de vida, indicando sua emergência como doença global (VICTORIA; SASSAK; NUNES, 2009; MOLODECKY et al., 2012).

A DC pode comprometer qualquer um dos segmentos do trato gastrointestinal, desde a boca até o ânus, mas geralmente acomete o íleo e o cólon de maneira descontínua, ou seja, com presença de regiões inflamadas intercaladas a regiões não inflamadas. O envolvimento intestinal na maior parte das vezes é transmural, comprometendo a mucosa, submucosa, camada muscular e serosa. Esta doença também pode estar associada à presença de granulomas, estreitamento e fístulas no intestino (ABRAHAM; CHO, 2009). Os

sintomas incluem dor abdominal, diarréia, perda de peso, mal-estar e, em alguns casos, pode ocorrer obstrução intestinal (CARTER; LOBO; TRAVIS, 2004).

Já a CU acomete o cólon e o reto de maneira difusa, sendo que a inflamação geralmente é caracterizada pela presença de abscessos nas criptas intestinais e infiltrado de células inflamatórias na lâmina própria, e não está associada à presença de granulomas, estreitamento e fístulas (ABRAHAM; CHO, 2009). É uma doença marcada por períodos de exacerbação e remissão, e os sintomas mais frequentes são: diarréia, enterorragia, dor abdominal em cólicas, urgência em evacuar e tenesmo (ISKANDAR; CIORBA, 2012). Cerca de 5% dos pacientes com DII, que compromete a região do cólon, apresentam características clínicas, radiológicas, endoscópicas e/ou patológicas de ambas condições (DC e CU). Nestes casos, a doença é denominada colite "não classificada" (MOWAT et al., 2011).

As manifestações clínicas ocorrem principalmente devido às desordens inflamatórias resultantes da infiltração transmural de neutrófilos, macrófagos e linfócitos na mucosa e submucosa do cólon (ROBERTS-THOMSON et al., 2011). Os neutrófilos e macrófagos são considerados as células responsáveis pela destruição da integridade epitelial e desenvolvimento da injúria colônica observada em pacientes com CU (GRISHAM; YAMADA, 1992). Inúmeros mediadores, incluindo leucotrienos e várias citocinas inflamatórias, contribuem para a quimiotaxia de neutrófilos nesta doença (VILLEGAS et al., 2003). Os níveis de IL-1β apresentam-se aumentados na mucosa de pacientes com CU (BAMIAS; KALTSA; LADAS, 2011). Esta citocina merece atenção considerável pois contribui para a infiltração celular e destruição da mucosa durante a inflamação intestinal (RADDATZ et al., 2001). A IL-33 também contribui para a migração de neutrófilos através da ativação de macrófagos e consequente produção de quimiocinas e citocinas (como a própria IL-1β), e também agindo diretamente sobre os neutrófilos (VERRI et al., 2010). Estudos recentes mostraram que os níveis de IL-33 estão aumentados no cólon de pacientes com CU ativa (BELTRAN et al., 2010; SEIDELIN et al., 2010; KOBORI et al., 2010), sugerindo o papel desta citocina na patogênese desta doença. Sendo assim, o bloqueio da IL-1β e da IL-33 durante a CU poderia ajudar na redução da severidade da doença.

Por outro lado, a IL-10 é considerada uma citocina importante na manutenção da homeostase intestinal (PAUL; KHARE; GASCHE, 2012). Camundongos geneticamente deficientes para IL-10 desenvolvem colite espontânea, sendo este um modelo experimental genético para doenças inflamatórias intestinais (KÜHN et al., 1993). Além disso, alterações genéticas no locus da IL-10 também contribuem para o desenvolvimento da CU (PAUL; KHARE; GASCHE, 2012). Neste sentido, fica clara a participação da IL-10

como um mediador que previne o desenvolvimento da colite. Desta maneira, tratamentos que evitem a queda nos níveis de IL-10 no cólon durante a CU podem ser uma boa alternativa para a redução do processo inflamatório nestes casos.

Durante a CU, os neutrófilos e macrófagos infiltrados e ativados também representam uma importante fonte de espécies reativas de oxigênio (EROs), como ânion superóxido e radical hidroxil (ROESSNER et al, 2008). O dano oxidativo no cólon também é um fator importante na patogênese da CU, uma vez que a inflamação colônica é acompanhada pelo aumento na produção de EROS, associado à redução na resposta antioxidante local (PRAVDA, 2005; REZAIE; PARKER; ABDOLLAHI, 2007; KIM; KIM; HAHM, 2012). O excesso de EROs, associado à redução das defesas antioxidantes durante a CU, causam danos oxidativos a componentes celulares (como proteínas, lipídios e material genético), levando a progressão da injúria, disfunção e inflamação, o que culmina com a exacerbação do processo patológico da CU (ZHU; LI, 2012).

Vários modelos experimentais de DII têm sido descritos com o objetivo de elucidar os mecanismos envolvidos na patogênese destas doenças e também avaliar a eficácia terapêutica de novos fármacos ou formulações. Embora os modelos não reproduzam exatamente a doença em humanos, eles têm sido úteis para estudar muitos aspectos importantes destas condições. Os modelos mais comumente utilizados são aqueles induzidos pela administração de um agente químico exógeno, como ácido acético, sulfato sódico de dextrana (DSS), ácido trinitrobenzóico (TNBS), entre outros.

Em 1978, MacPherson e Pfeiffer mostraram que a administração de solução de ácido acético por via retal era capaz de induzir de maneira dose-dependente uma inflamação aguda, difusa e reprodutível na porção distal do cólon de ratos. Esta indução da inflamação pelo ácido acético está relacionada à sua capacidade de “destruir” a barreira de células epiteliais e aumentar a exposição das bactérias colônicas aos fagócitos da mucosa. Uma consequência provável desta mudança na função da barreira epitelial é que os fagócitos da mucosa são ativados por componentes da própria microbiota, e isto, por sua vez, resulta na liberação de citocinas pró-inflamatórias e o desenvolvimento da inflamação (STROBER; FUSS; BLUMBERG, 2002).

A injúria colônica induzida pela administração via retal de ácido acético em murinos apresenta características similares à CU em humanos, já que ambas são caracterizadas por inflamação difusa da mucosa, ulcerações superficiais e redução das células caliciformes (RANI et al., 2011). Além disso, também é possível verificar infiltração de polimorfonucleares no tecido, liberação de citocinas pró-inflamatórias, como a IL-1β e o fator

de necrose tumoral (TNF)-α (AMIRSHAHROKHI; BOHLOOLI; CHINIFROUSH, 2011; LUO et al., 2010), além de aumento na produção de EROs e redução das defesas antioxidantes (MILLAR et al., 1996; TAHAN et al., 2011).

1.2 Tratamentos Convencionais para a Colite Ulcerativa

Os tratamentos convencionais para as DII incluem os aminosalicilatos (mesalazina e sulfasalazina), corticosteróides (prednisolona, metilprednisolona e budesonida), imunosupressores não esteroidais (azatioprina, 6-mercaptopurina e metotrexato) e terapia anti-TNF (infliximab, adalimumab e certolizumab pegol) (BURGER; TRAVIS, 2011). Nenhuma estratégia terapêutica já estabelecida é completamente eficaz no tratamento das DII. No entanto, os avanços recentes no entendimento da fisiopatologia das DII têm direcionado o desenvolvimento de ferramentas terapêuticas cada vez mais eficazes (KAWADA; ARIHIRO; MIZOGUCHI, 2007). Os objetivos terapêuticos na CU são: a indução e manutenção da remissão, e o restabelecimento da integridade da mucosa, a fim de evitar intervenção cirúrgica e diminuir a probabilidade do desenvolvimento de câncer (NG; KAMM, 2009), além de melhorar a qualidade de vida do paciente.

A maioria dos pacientes com formas leves ou moderadas de CU respondem ao tratamento com aminosalicilatos. A sulfasalazina é um pró-fármaco que corresponde a uma molécula de mesalazina ligada a sulfapiridina. Quando atinge o cólon, esta molécula é clivada por enzimas bacterianas liberando mesalazina e sulfapiridina. Embora a mesalazina seja um salicilato, seu efeito terapêutico não está relacionado com a inibição da ciclooxigenase, mas sim com a inibição da produção de IL-1β e TNF-α, além da inibição da via da lipoxigenase, sequestro de radicais livres e inibição da ativação do fator nuclear (NF)-kB, um factor de transcrição essencial para a produção de mediadores inflamatórios (PITHADIA; JAIN, 2011).

Já os corticosteróides são indicados para as formas moderadas ou graves da doença e também para pacientes que não respondem ao tratamento com aminosalicilatos (NG; KAMM, 2009). Os corticosteróides atuam sobre as primeiras manifestações da inflamação, reduzindo a permeabilidade vascular, a vasodilatação e a infiltração de neutrófilos, além de inibir a ativação de fibroblastos, a proliferação vascular e a deposição de colágeno. Os corticóides também reduzem a produção de citocinas inflamatórias por inibir a ativação do NF-kB (TRIANTAFILLIDIS; MERIKAS; GEORGOPOULOS, 2011). Porém, tratamentos a longo prazo e/ou com altas doses de corticosteróides estão relacionados a efeitos adversos que limitam seu uso, dentre eles: complicações cutâneas, oculares, endócrinas, músculo-

esqueléticas, gastrointestinais e infecciosas (ARDIZZONE; PORRO, 2002).

Para pacientes cortico-resistentes ou cortico-dependentes, é indicado o uso de azatioprina ou 6-mercaptopurina. A azatioprina é um pró-fármaco convertido no plasma a 6-mercaptopurina, seu princípio ativo. Os efeitos farmacológicos estão relacionados à produção de metabólitos ativos como a 6-tioguanina (PAOLUZI et al., 2002), que atua como análogo das purinas, interferindo na síntese protéica e impedindo o crescimento e a proliferação dos linfócitos T e B (AL HADITHY et al., 2005). Os efeitos adversos, como supressão da medula óssea, elevação progressiva dos níveis das enzimas hepáticas e pancreatite, podem ocorrer (geralmente nas primeiras semanas) e exigem cancelamento imediato do tratamento (MEIER; STURM, 2011).

A administração endovenosa de ciclosporina é eficaz na fase aguda da CU em pacientes refratários ao uso de aminosalicilatos e corticosteróides, mas está associada a efeitos adversos raros e potencialmente fatais, como nefrotoxicidade e infecções oportunistas (REGUEIRO et al., 2006). Os pacientes que respondem à ciclosporina necessitam de azatioprina ou 6-mercaptopurina para manutenção da remissão (REGUEIRO et al., 2006). O tacrolimus é geralmente bem tolerado e também pode ser considerado uma terapia alternativa nesses casos (NG; KAMM, 2009; BURGER; TRAVIS, 2011). A ciclosporina e o tacrolimus são imunosupressores que atuam através da inibição da calcineurina e possuem mecanismo de ação semelhantes. Ao inibirem a calcineurina, estas substâncias impedem a desfosforilação e ativação do fator nuclear de células T ativadas (NF-AT) e a consequente transcrição de genes que codificam citocinas, como IL-2, TNF-α e IFN-γ (OGATA et al., 2006; NAGANUMA; FUJII; WATANABE, 2011), o que resulta na redução do recrutamento e ativação dos linfócitos T. Os efeitos adversos relacionados ao tratamento com tracolimus, tais como nefrotoxicidade, são dose-dependentes, porém reversíveis com a redução da dose ou interrupção do tratamento. Entretanto, são necessários estudos para determinar a eficácia e segurança a longo prazo na CU (TRIANTAFILLIDIS; MERIKAS; GEORGOPOULOS, 2011). Não existem evidências da eficácia do uso de metotrexato em pacientes com CU, por isso seu uso não é recomendado para estes pacientes (CARBONNEL, 2011; TRIANTAFILLIDIS; MERIKAS; GEORGOPOULOS, 2011).

O infliximab (anticorpo monoclonal anti-TNF) é reservado para pacientes com CU ativa moderada ou severa, e pacientes não tolerantes ou não responsivos à terapia com tiopurinas (NG; KAMM, 2009; BURGER; TRAVIS, 2011). Tem sido utilizado para a redução dos sintomas, indução e manutenção da remissão, e para a eliminação do uso de corticosteróides em pacientes refratários (WILHELM et al., 2008). O infliximab se liga ao

TNF-α, impedindo-o de se ligar a seus receptores celulares e, além disso, também é capaz de induzir a apoptose em linfócitos e monócitos (RUTGEERTS; VAN ASSCHE; VERMEIRE, 2004; TRAVASSOS; CHEIFETZ, 2005), o que resulta em redução do processo inflamatório associado à doença. Os efeitos adversos associados ao uso de terapias anti-TNF incluem infecções oportunistas, formação de anticorpos contra o anti-TNF, linfomas e reações à infusão (HOENTJEN; VAN BODEGRAVEN, 2009).

1.3 Estratégia Terapêutica Alternativa

Uma vez que nenhuma estratégia terapêutica é completamente eficaz no tratamento da CU, e que os efeitos adversos associados ao uso dos medicamentos convencionais são significativos e comprometem a adesão ao tratamento, é necessário o estudo e desenvolvimento de novos fármacos e medicamentos para o tratamento desta doença, a fim de aumentar a eficácia terapêutica, reduzir os efeitos adversos e melhorar a qualidade de vida dos pacientes. Ao considerarmos a participação de mediadores pró-inflamatórios, como citocinas e radicais livres na patogênese da colite, associado ao fato de que a mucosa colônica é relativamente pobre em antioxidantes endógenos, a utilização de fármacos com propriendades anti-inflamatórias e antioxidantes, como a quercetina, para tratamento da CU apresenta-se como uma abordagem terapêutica promissora.

1.3.1 Quercetina

Recentemente, várias investigações têm sido descritas sobre a utilização da quercetina, um flavonóide abundante e eficientemente extraído da espécie *Dimorphandra mollis*. A fácil extração juntamente com expressivo efeito antioxidante comparada a outros flavonóides favorece a sua utilização na terapêutica (METODIEWA et al., 1999; SKIBOLA; SMITH, 2000; CASAGRANDE et al., 2006, 2007). A quercetina tem sido estudada como potencial agente terapêutico contra uma série de doenças, tais como, asma, câncer, diabetes, doenças cardiovasculares e inflamatórias, entre outras (FORMICA; REGELSON, 1995; MIDDLETON; KANDASWAMI; THEOHARIDES, 2000; BOOTS; HAENEN; BAST, 2008; ROGERIO et al., 2010; KELLY, 2011).

A quercetina é capaz de inibir a atividade da mieloperoxidase (MPO), uma enzima secretada por neutrófilos e macrófagos ativados durante o processo inflamatório. Casagrande e colaboradores (2006) mostraram que formulações tópicas contendo quercetina

são capazes de impedir o aumento da atividade da MPO na pele de camundongos expostos à radiação UVB. Neste contexto, sabe-se que a quercetina é capaz de reduzir o recrutamento de neutrófilos por inibição da polimerização da actina (SOUTO et al., 2011), além de inibir a expressão de moléculas de adesão (CRESPO et al., 2008) e se ligar a regiões específicas na molécula da MPO, inibindo sua ação (SHIBA et al., 2008). Portanto, o efeito da quercetina sobre a redução da atividade da MPO pode estar associada à diminuição no recrutamento dos neutrófilos e/ou inibição de sítios ativos da própria molécula da MPO.

As propriedades anti-inflamatórias da quercetina também estão associadas à redução da expressão de mediadores pró-inflamatórios, como as citocinas IL-1β e TNF-α (COMALADA et al., 2005; VALÉRIO et al., 2009; CARVALHO et al., 2010), e as enzimas óxido nítrico sintase induzível (iNOS) e ciclooxigenase (COX)-2 (CRESPO et al., 2008); inibição da ativação do NF-kB (COMALADA et al., 2005), modulação da ativação da via JAK-STAT e das proteínas quinases ativadas por mitógenos (MAPK) (MUTHIAN; BRIGHT, 2004; YING et al., 2009) . Além disso, a quercetina foi capaz de aumentar os níveis de IL-10, uma citocina com propriedades anti-inflamatórias, em modelo de pancreatite aguda em camundongos (CARVALHO et al., 2010), e de miocardite em ratos (MILENKOVIĆ et al., 2010).

A quercetina pode inibir o processo de formação de radicais livres em três etapas diferentes: na iniciação (pela interação com íons superóxido), na formação de radicais hidroxil (por quelar íons de ferro) e na peroxidação lipídica (por reagir com radicais peroxi de lipídeos) (AFANAS'EV et al., 1989; TERAO; PISKULA, 1999), além de modular os níveis de antioxidantes endógenos, como a glutationa reduzida (VALÉRIO et al., 2009; PADMA et al., 2012). O mecanismo pelo qual a quercetina promove aumento nos níveis de antioxidantes endógenos parece estar relacionado à ativação do Nrf2, um fator de transcrição responsível ao estresse, que promove aumento na expressão de enzimas antioxidantes (GRANADO-SERRANO et al., 2012).

Entretanto, a administração de quercetina por via oral não reduziu a inflamação em modelo de colite experimental induzida por sulfato sódico de dextrana, enquanto que suas formas glicosiladas, quercetrina e rutina, demonstraram eficácia (CAMUESCO et al., 2004; COMALADA et al., 2005; KWON et al., 2005). Isso ocorre pois a quercetina pode ser metabolizada e absorvida no estômago (CRESPY et al., 2002) e intestino delgado (MANACH et al., 2004), impedindo com que ela atinja o cólon em quantidades suficientes para exercer efeito anti-inflamatório e antioxidante local sobre as lesões colônicas.

1.3.2 Sistema Microencapsulado para Liberação Modificada

Com o desenvolvimento dos sistemas de liberação de fármacos (SLF), permitiu-se prever e controlar a velocidade de liberação do fármaco, prolongando, portanto, a atividade terapêutica e/ou proporcionando liberação sítio específica. Os avanços das pesquisas em SLF devem-se ao reconhecimento das vantagens clínicas e terapêuticas e a fatores econômicos como, por exemplo, a possibilidade de proteção patentária. O planejamento racional do SLF é uma etapa crucial para a modulação da liberação do fármaco, adequada às necessidades clínicas e farmacocinéticas e aos sítios de absorção (SASTRY; NYSHADHAM; FIX, 2000; ZALFEN et al., 2008).

A veiculação de fármacos para o cólon oferece várias vantagens do ponto de vista terapêutico no tratamento da CU. Sistemas de liberação modificada, visando a liberação colônica do fármaco, evitam a rápida e completa liberação do fármaco, permitindo que o mesmo atinja o cólon. A concentração do fármaco que atinge o local de ação é maior, resultando em melhor eficácia terapêutica (KARROUT et al., 2009) e adesão ao tratamento. Para garantir a liberação modificada, visando a liberação colônica de maneira satisfatória, o fármaco precisa ser protegido da degradação, liberação e/ou absorção na porção superior do trato gastrointestinal. Além disso, a forma farmacêutica deve permitir a liberação de quantidades suficientes do fármaco na região colônica (ASGHAR; CHANDRAN, 2006).

Algumas estratégias são empregadas para possibilitar a liberação modificada, visando a liberação colônica de fármacos, dentre elas a veiculação do fármaco em uma matriz polimérica (MONTEIRO et al., 2007; ALIAS; GONI; GURRUCHAGA, 2007), ligação química a resinas de permuta iônica, incorporação em bomba osmótica e utilização de revestimentos monolíticos ou multiparticulados (COLLETT; MORETON, 2005; KARROUT et al., 2009). As microcápsulas são sistemas multiparticulados que têm como vantagem maior reprodutibilidade no tempo de trânsito intestinal em relação ao monolítico, contribuindo para minimizar sítios de alta concentração, reduzindo e até evitando irritações e ulcerações (FELL, 1992), além de reduzir a variação inter- e intra-individuais na resposta ao tratamento (ASGHAR e CHANDRAN, 2006).

O complexo polimérico pectina/caseína é uma alternativa promissora na constituição de sistemas de liberação modificada visando liberação colônica do fármaco (BARACAT, 2004; REDIGUIERI, 2008) já que a pectina é um polissacarídeo capaz de resistir à ação das proteases e amilases presentes no TGI superior, porém pode ser digerida por pectinases presentes no cólon (ITOH, 2007 apud YU et al., 2009).

Com o objetivo de propor uma nova forma de tratamento para a CU utilizando a quercetina, o presente trabalho visa preparar microcápsulas de quercetina utilizando o polímero biodegradável pectina/caseína, que resistam às condições do TGI superior, alcançando a região intestinal e liberando o fármaco por difusão e pela degradação da pectina.

2 OBJETIVOS

2.1 Gerais

Preparar microcápsulas de pectina/caseína contendo quercetina e demonstrar *in vivo* a sua eficácia em modelo de colite induzida por ácido acético em camundongos.

2.2 Específicos

- Preparar a forma farmacêutica microencapsulada contendo quercetina;
- Quantificar a quercetina na formulação;
- Avaliar o efeito do tratamento com as microcápsulas contendo quercetina sobre o recrutamento de neutrófilos para o tecido colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com as microcápsulas contendo quercetina sobre o edema colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com as microcápsulas contendo quercetina sobre as lesões micro e macroscópicas no tecido colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com as microcápsulas contendo quercetina sobre os níveis das citocinas IL-1β, IL-33 e IL-10 no tecido colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com as microcápsulas contendo quercetina sobre a capacidade antioxidante no tecido colônico, através dos níveis de glutatina reduzida (GSH), da capacidade sequestradora do radical ABTS e redutora do íon ferro, após a indução da colite com solução de ácido acético;

3 MATERIAIS E MÉTODOS

3.1 Materiais

Pectina USP foi obtida da CP Kelco (Limeira, Brasil). Caseina da Kauffman & Co (Kehl, Germany). Ácido citrico; hidróxido de sódio; ácido clorídrico; fosfato de sódio; formaldeído foram adquiridos da Merck (Darmstadt, Germany). Quercetina da Acros (New Jersey, USA). Brometo de hexadecil trimetil-amonio (HTAB); dihidrocloreto de O-dianisidina; tween 80; glutationa reduzida (GSH); glutaraldeído; 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 IL-1β, IL-33 e IL-10 foram adquiridos da eBioscience (San Diego, USA). Todos os reagentes utilizados foram de grau analítico.

3.2 Preparação das microcápsulas

O preparo das microcápsulas foi realizado de acordo com Freitas et al. (2007) e Baracat et al. (2012). Foram realizadas dispersões aquosas de caseína e pectina (8,34%, w/v) sob agitação mecânica constante. O pH foi ajustado a 8,0 ± 0,1 pela adição de hidróxido de sódio 4,0 M. Após a dispersão dos polímeros, a quercetina foi adicionada na proporção 1:5 (quercetina / polímero). As microcápsulas foram obtidas pela redução lenta e gradual do pH para 3,5 ± 0,1 com adição de ácido cítrico 1,0 M. As paredes das microcápsulas foram enrijecidas pela adição de glutaraldeído (50 µL/g de polímero) sob agitação constante por mais 30 min. Para a preparação das microcápsulas inertes foi utilizada a mesma metodologia, porém, sem adição da quercetina. As microcápsulas, inertes e carregadas, obtidas a partir de dispersões aquosas de caseína/pectina foram secas por atomização com o uso de secador *spray dryer* (Lab Plant, SD-05), com as seguintes características operacionais: bico atomizador do tipo duplo fluido, com mistura externa e orifício de saída de 0,5 mm; temperatura do ar de entrada de 180º C e de saída de 100º C; pressão de ar de 4 bar e vazão de ar comprimido de 1,1 m^3/min; vazão de alimentação da dispersão de 7,75 mL/min, regulada por bomba peristáltica.

3.3 Quantificação da quercetina na formulação

A quantificação da quercetina na formulação foi realizada por extração do fármaco após o processo de secagem das microcápsulas, de acordo com o método para quantificação de flavonóides totais descrito por Georgetti et al. (2006). Amostras das microcápsulas de quercetina foram dispersas em etanol 80%, agitadas por 15 minutos e centrifugadas por 10 minutos a 3000 rpm. Alíquotas do sobrenadante foram misturadas a cloreto de alumínio ($AlCl_3$) 2%, preparado em etanol 80%. Após 1 h de incubação à temperatura ambiente, a absorvância foi determinada em 420 nm. Este processo foi repetido na mesma amostra até a total extração da quercetina da formulação. A quantidade de quercetina presente na formulação foi calculada utilizando-se a curva de flavonóides totais preparada com quercetina padrão nas concentrações 6, 8, 12, 16, 20, 24 e 30 µg/mL de etanol 80%. O resultado foi expresso em % de quercetina em relação ao total de quercetina adicionada à formulação.

3.4 Animais experimentais

Foram utilizados camundongos Swiss machos, pesando 25 ± 5g, 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 entre 21 e 24°C. A água e ração foram administradas *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 pelo Comitê de Ética em Experimentação Animal (CEEA) da Universidade Estadual de Londrina (n° processo: 26511/2009).

3.5 Indução da colite experimental

A indução da colite foi realizada de acordo com Nosál'ová, Cerná e Bauer (2000) e Luo et al. (2010), com algumas modificações. 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.

3.6 Tratamentos e protocolos experimentais

Os grupos experimentais foram: controle 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 microcápsulas inertes (suspensas em salina), microcápsulas de quercetina (1, 10 e 100 mg/kg, suspensas em salina), quercetina (10 e 100 mg/kg, solubilizada em 20% de tween 80 e 80% de salina) ou tween 80 (20% em salina). Foram desenvolvidos dois protocolos experimentais: (A) Para avaliação dos parâmetros inflamatórios, os animais foram tratados 2 horas antes e 10 horas após a indução da colite, e foram eutanasiados na 18ª hora. Após avaliação das lesões macroscópicas, amostras da porção distal do cólon foram coletadas para determinação do infiltrado neutrofílico pela atividade da mieloperoxidase e para avaliação do edema. Além disso, foram coletadas amostras para análise histológica e quantificação de citocinas (IL-1β, IL-33 and IL-10) no cólon (figura 1A). (B) Para avaliação do estresse oxidativo, os animais foram tratados 6 e 1 hora antes da indução da colite, e foram eutanasiados na 4ª hora após a indução da colite. Amostras da porção distal do colon foram coletadas para realização dos ensaios de FRAP, ABTS e GSH (figura 1B).

Figura 1 – 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).

3.7 Determinação da atividade da mieloperoxidase (MPO)

A mieloperoxidase (MPO) é uma enzima encontrada dentro dos grânulos azurófilos dos neutrófilos. Sua dosagem é muito utilizada para avaliar o processo inflamatório e a injúria tecidual (KO; LAM; CHEUNG, 2005), através da quantificação indireta no número de neutrófilos nos tecidos. A avaliação do infiltrado neutrofílico no cólon dos camundongos foi feita por método colorimétrico, descrito por Bradley et al. (1982). 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 (14000 rpm, 2 min, 4°C) e o sobrenadante foi utilizado para a reação colorimétrica em placa de 96 poços. A cada alíquota de 15 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 leituras foram realizadas em espectrofotômetro (Victor^3 1420 multilabel counter) a 450 nm, e o número de neutrófilos por mg de tecido foi determinado utilizando-se uma curva padrão de neutrófilos.

3.8 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 (LEE et al., 2009; BARBOSA, 2011).

3.9 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 7 μm foram corados com hematoxilina / eosina. Em seguida, as lâminas contendo as amostras já coradas foram analisadas em microscópio de luz (Olympus OX31), acoplado à câmera digital (Lumenera Infinity 1). As alterações histopatológicas no cólon foram avaliadas de acordo com os critérios descritos por Appleyard e Wallace (1995), com modificações, conforme tabela 1. O escore de lesão microscópica final de cada amostra foi obtido pela soma dos escores determinados para cada um dos achados.

Tabela 1 – 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

3.10 Avaliação das lesões macroscópicas

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), conforme tabela 2.

Tabela 2 – 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 aumentando em 1 para cada centímetro adicional

3.11 Dosagem de proteína

O teor de proteínas nas amostras de tecido colônico foi determinado pelo método espectrofotométrico descrito por Lowry et al. (1951), modificado por Miller (1959), seguido de algumas adaptações. Albumina bovina foi utilizada como padrão. Durante a reação, alíquotas das amostras do tecido colônico foram misturadas ao reagente cúprico (composto por carbonato de sódio a 10% em NaOH 5N, tartarato de sódio a 2% e sulfato de cobre a 1%) e folin-ciocalteu, em meio básico, resultando na formação de um complexo colorido, cuja absorvância foi determinada a 660 nm. A intensidade da cor é proporcional à concentração de proteína na amostra. Os resultados foram expressos em mg de proteína por mg de tecido.

3.12 Dosagem de citocinas

Amostras do tecido colônico foram coletadas e homogeneizadas em salina estéril. Após homogeneização, as amostras foram centrifugadas (3000 rpm, 4°C, 10 minutos) e o sobrenadante foi utilizado para avaliar os níveis de citocinas por ELISA (*enzyme-linked immunosorbent assay*), utilizando kits comerciais. 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 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 foram 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 foram determinadas em 450 nm. Os resultados foram obtidos comparando a densidade óptica com as densidades das curvas padrões, e expressos em pg por mg de proteína.

3.13 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 (ELLMAN, 1959; SEDLAK E LINDSAY, 1968). 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 (4000 rpm, 4°C, 15 min). Em seguida, foram adicionados 200 µl de tampão Tris-HCl 0,4 M (pH 8.9) às alíquotas de 150 µl 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 em espectrofotômetro (Spectrum SP-2100) após 5 min de reação, a 412 nm. A curva padrão foi preparada com 0,5 µM de GSH, e os resultados expressos em nM de GSH por mg de proteína.

3.14 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+• (VASCONCELOS et al., 2007). 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 (RE et al., 1999; KATALINIC et al., 2005). Amostras da porção distal do cólon dos animais foram coletadas, homogeneizadas em solução de cloreto de potássio e centrifugadas a 1500 rpm, a 4°C, por 10 min. Alíquotas dos sobrenadantes reagiram com 1 mL da solução do radical cátion ABTS+• durante 6 minutos, e as absorvâncias foram determinadas a 730 nm. A capacidade sequestradora do radical nas amostras foi determinada a partir de uma curva padrão de Trolox (1,5 – 30 µmol/L) e os resultados foram expressos em mmol equivalente ao Trolox por g de tecido, que representa a quantidade de Trolox (em mmol) com um potencial antioxidante equivalente a 1 g do tecido.

3.15 Determinação do FRAP (*Ferric Reducing Antioxidant Power*)

Este teste avalia a capacidade antioxidante através da redução do complexo Fe^{3+}-TPTZ (ferritripiridiltriazina) a Fe^{2+}-TPTZ (ferroso-tripiridiltriazina) por antioxidantes doadores de elétrons, em valor de pH baixo (VASCONCELOS et al., 2007). Esta capacidade redutora foi avaliada nas amostras do tecido colônico de acordo com Benzie e Strain (1996) e Katalinic et al. (2005). O reagente de FRAP foi preparado com 100 mL de tampão acetato de sódio 0,3 mM (pH 3,6), 10 mL de TPTZ 10 mM em ácido hidroclorídrico 40 mM, e 10 mL de cloreto de ferro hexahidratado 20 mM. Todas as soluções foram preparadas no dia do ensaio. Amostras da porção distal do cólon dos animais foram coletadas, homogeneizadas em solução de cloreto de potássio e centrifugadas a 1500 rpm, a 4°C, por 10 min. Alíquotas dos sobrenadantes reagiram com 1,5 mL do reagente de FRAP durante 30 minutos a 37°C, e as absorvâncias foram determinadas a 595 nm. A capacidade redutora das amostras foi determinada a partir de uma curva padrão de Trolox (0,4–2 mmol) e os resultados foram expressos em mmol equivalente ao Trolox por g de tecido.

3.16 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 4 (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 Artigo Científico

O artigo seguinte segue as normas do periódico científico escolhido para submissão, *Journal of Natural Products*.

Quercetin loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms.

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ABSTRACT

Quercetin (**1**) is an anti-inflammatory and antioxidant flavonoid. However, the oral administration of quercetin did not present beneficial effects in experimental colitis models, which involve cytokines and oxidative stress. A possible explanation is that the absorption profile of **1** prevents its activity. Therefore, we reason that the controlled release of **1** would improve its therapeutic effect. Thus, therapeutic effect and mechanisms of **1** loaded microcapsules in acetic acid-induced colitis in mice were evaluated. Microcapsules were prepared using pectin/casein polymer and **1**. The oral administration of **1** loaded microcapsules decreased neutrophil recruitment, attenuated histological alterations and reduced macroscopical damage, edema and IL-1β and IL-33 production in the colon samples. Quercetin (**1**) loaded microcapsules also prevented the reduction of anti-inflammatory cytokine IL-10 and antioxidant capacity of colon. These pre-clinical data indicate that **1** loaded microcapsules of pectin/casein polymer improved the anti-inflammatory and antioxidant effects of **1** compared to non-encapsulated drug. Therefore, **1** seems to be a promising active molecule in inflammatory bowel disease if provided adequate drug controlled release.

The two major clinically defined forms of inflammatory bowel disease, ulcerative colitis and Crohn's disease, are chronic remittent or progressive inflammatory conditions that may affect the colon or the entire gastrointestinal tract.[1] The precise etiology of inflammatory bowel disease remains unclear, but accumulating evidence suggests that host immune system, intestinal microbiota, genetic and environmental factors contribute to the pathogenesis of such diseases.[2] The intestinal injury caused by acetic acid in rodents shares some characteristics with ulcerative colitis in human patients, as both are characterized by diffuse mucosal inflammation, superficial ulceration and goblet cell depletion.[3] Neutrophils and macrophages play an important role in disrupting epithelial integrity and causing colon injury in ulcerative colitis.[4] Moreover, the imbalance of pro-inflammatory (including cytokines, reactive oxygen and nitrogen species) and anti-inflammatory mediators (including IL-10 and antioxidant systems) is a key factor in ulcerative colitis pathogenesis since inflammation protects the host intestine mucosa from pathogenic bacteria, but excessive inflammation causes tissue lesions.

Cytokines are central components of the inflammatory pathways that take place during the active and chronic phases of ulcerative colitis. The levels of IL-1β and IL-33 are increased at the inflamed mucosa and of pathogenic relevance in ulcerative colitis.[5-8] IL-1β receives considerable attention as a potential mediator of inflammatory cell infiltration and mucosal barrier disruption that accompanies gut inflammation.[9] IL-33, which is a member of IL-1 family of cytokines, also induces neutrophil migration by activating macrophages to produce chemokines and cytokines (such IL-1β) and by directly chemoattracting neutrophils.[10] Therefore, the blockade of IL-1β and IL-33 during ulcerative colitis may help to reduce the severity of colitis in these patients. On the other hand, the anti-inflammatory cytokine IL-10 acts as a key mediator for maintaining gut homeostasis.[11] In agreement, IL-10 knockout mice are well established as a genetic model for inflammatory bowel disease,[12] and sequence variants in the IL-10 locus contribute to ulcerative colitis in humans.[11]

Oxidative damage is also an important pathogenic factor in ulcerative colitis because intestinal inflammation is accompanied by increased production of reactive oxygen and nitrogen species (ROS/RNS) in conjunction with an imbalanced antioxidant response.[13-15] In ulcerative colitis, the overproduction of ROS/RNS associated with reduction of antioxidant defenses cause oxidative damage to cellular constituents, which leads to mucosal injury progression, dysfunction and inflammation, exacerbating the pathological process of ulcerative colitis.[15]

Considering the involvement of pro-inflammatory mediators such as cytokines and free radicals in the pathological process of ulcerative colitis, the effect of **1** in experimental model of colitis has been investigated since this flavonoid presents significant anti-inflammatory and antioxidant effects. It has been demonstrated that **1** may inhibit myeloperoxidase activity, an enzyme secreted by activated neutrophils and macrophages at the site of inflammation,[16,17] inhibit the recruitment of neutrophils,[18] reduce the production of pro-inflammatory cytokines such IL-1β,[19,20] and increase the production of anti-inflammatory cytokines such IL-10.[21,22] Moreover, **1** is also able to scavenge free radicals and/or chelate transition metal ions,[23,24] and modulate endogenous antioxidants such reduced glutathione.[20,25,26] These mechanisms that were amenable by treatment with **1** in other models of inflammation are also important in the context of ulcerative colitis.[5,7,11,14,15,27] However, the oral administration of **1** did not show beneficial effects in experimental colitis models.[19,28,29] This occurs because like others aglycones, **1** can be absorbed in the stomach[30] and small intestine,[31] preventing it to reach the colon in pharmacologically effective concentrations to exert its local anti-inflammatory and antioxidant effects.

In these sense, we reason that the controlled release of **1** would improve its efficacy in ulcerative colitis. A possible strategy to obtain such results would be to microencapsulate **1** using a pectin/casein complex to modify its delivery. The pectin/casein mixture in aqueous

dispersion forms microencapsulated systems in pH values lower than the isoelectric point (pI) of the original compounds.[32] Thus, the water penetration could be retarded by the microcapsules coating layer and the physical contact between the drug and the gastrointestinal fluid would be inhibited till the microcapsules reach the colon where pectinolytic enzymes degrade the layer,[33,34] allowing the delivery of **1** to have a local effect on colonic tissue.

In the present study, **1** loaded microcapsules were prepared and the anti-inflammatory and antioxidants effects of this formulation were tested in the acetic acid-induced colitis in mice, which presents common features with ulcerative colitis.

RESULTS AND DISCUSSION

Preparation of Quercetin (1) loaded Microcapsules and Quantification of 1. The pectin/casein mixture in aqueous dispersion forms multiparticle organized systems.[32] The quantification of **1** in the formulation was performed by exhaustive extraction in which 92% of **1** was detected in comparison with the initial amount of this drug added to prepare the formulation. Thus, 8% of **1** added was lost during the process. For *in vivo* experiments, the doses were calculated considering the loss during the process. The pectin/casein polymer provides modified release, allowing a slow and gradual release of a drug under conditions that simulate gastrointestinal environment *in vitro*.[32] Therefore, it should be appropriate for the aims of this study.

Quercetin (1) Loaded Microcapsules Reduce Myeloperoxidase Activity in the Colon of Mice with Acetic Acid-induced Colitis. Neutrophil infiltration into the lamina propria is a common feature of ulcerative colitis and probably accounts for significant nonspecific injury in the disease.[35] The myeloperoxidase is an enzyme mainly found in

azurophilic granules of neutrophils that produce the microbicidal molecule hypochlorite, a strong oxidant, upon reaction with H_2O_2 and Cl^-.[17] It can serve as a good marker of inflammation, tissue injury and neutrophil infiltration in gastrointestinal tissues.[36] Myeloperoxidase activity of the colon tissues was significantly higher in the colitis control group than in the negative control group 12 and 18 hours after colitis induction with acetic acid (Figure 2A). Therefore, the next experiments to evaluate the anti-inflammatory effects of **1** loaded microcapsules were performed at 18 hours following the treatment protocol described in Figure 1A.

The increase in myeloperoxidase activity was dose-dependently diminished by **1** loaded microcapsules (1, 10 and 100 mg/kg; in saline; po; protocol as in Figure 1A), but only the dose of 100 mg/kg significantly reduced myeloperoxidase activity compared to colitis control group. Inert microcapsules (100 mg/kg; in saline; po) and tween 80 (vehicle of non-encapsulated **1;** 20% diluted in saline; po) treated groups had no significant effect on myeloperoxidase activity compared to colitis control group. Although the treatment with non-encapsulated **1** (10 and 100 mg/kg; tween 80 20% diluted in saline; po; protocol as in Figure 1A) had showed a tendency to reduce myeloperoxidase activity in the colon, this effect was not significant compared to colitis control group (Figure 2B).

It was demonstrated that quercetin may reduce myeloperoxidase activity *in vitro*[37] and in a model of UVB-induced skin inflammation in hairless mice.[16] The effect of **1** in reducing the myeloperoxidase activity may be related to the reduction on neutrophil recruitment by **1**[18] and/or the binding of **1** to myeloperoxidase active site, leading to the inhibition of the myeloperoxidase-derived oxidative reactions.[17] In this sense, as neutrophils are the main sources of this enzyme and contribute importantly to the development of the oxidative stress and colonic injury during ulcerative colitis,[4,38] this effect of **1** loaded microcapsules treatment

in reducing the myeloperoxidase activity on colonic tissues may contribute to the reduction of the oxidative stress and colonic injury during the disease.

Quercetin (1) Loaded Microcapsules Prevent the Increase in Colonic Weight / Length (Edema) in the Colon of Mice with Acetic Acid-Induced Colitis.

Colon weight / length ratio, a measurement indicative of colonic tissue edema and / or hyperplasia,[39] is used as an indicator of disease-associated intestinal wall thickening and intensity of inflammation.[40] The intracolonic administration of acetic acid (7,5% in saline; 200 µL) induced significant increase of colon edema (colonic weight / length ratio in comparison to negative control group; Figure 3). Treatment with **1** loaded microcapsules (1, 10 and 100 mg/kg; in saline; po; protocol as in Figure 1A) reduced the acetic acid-induced inflammatory edema of the colon while no significant effect was observed by treatment with inert microcapsules (100 mg/kg; in saline; po), tween 80 (20% diluted in saline; po) and non-encapsulated **1** (10 and 100 mg/kg; tween 20% diluted in saline; po; protocol as in Figure 1A) (Figure 3). The present data confirms that **1** inhibits inflammatory edema as previously demonstrated,[20,41] and that the modified release of **1** is essential to guarantee its efficacy in this experimental model of colitis.

Quercetin (1) Loaded Microcapsules Reduce Microscopic Damage in the Colon of Mice with Acetic Acid-induced Colitis.

Using the same protocols described for Figure 3, microscopy images revealed typically histological colonic mucosal structure in negative control group (Figure 4A). The colitis control group showed loss of mucosal architecture, intense cell infiltration, crypt abscess formation and goblet cell depletion (Figure 4B). The treatment with inert microcapsules (100 mg/kg; in saline; po; Figure 4C), **1** loaded microcapsules 1 mg/kg and 10

mg/kg (in saline; po; Figure 4D and 4E, respectively), non-encapsulated **1** (10 and 100 mg/kg; tween 20% diluted in saline; po; Figure 4G and 4H, respectively) or tween 80 (20% diluted in saline; po; Figure 4I) was ineffective in reducing the microscopic lesions in the colon of mice with acetic acid-induced colitis. On the other hand, the treatment with **1** loaded microcapsules at the dose of 100 mg/kg (in saline; po; Figure 4F) showed significant restoration of the colonic structure and goblet cell, improvement of the mucosal architecture and mild inflammatory cell infiltration compared to colitis control group. The scores of microscopic damage are shown in Figure 4J.

The improvement of microscopic injury observed by **1** loaded microcapsules treatment is probably associated to the reduction of inflammatory cell infiltration into the colonic tissue by **1** and corroborates to the result presented in Figure 2B, in which the same treatment reduced myeloperoxidase activity in the colon. Neutrophils and macrophages were attributed as the responsible cells for disrupting epithelial integrity and causing colon injury in ulcerative colitis,[42] so the reduction of neutrophil recruitment by **1** loaded microcapsules treatment contributes to their protective effect against colonic tissue injury during the colitis.

Quercetin (1) Loaded Microcapsules Reduce Macroscopic Damage in the Colon of Mice with Acetic Acid-Induced Colitis. Using the same protocols described for Figure 3, the negative control group showed no macroscopic damage (score 0; Figure 5A) in the colon, whereas the colitis control group showed expressive inflammation (Figure 5B). The treatment with inert microcapsules (100 mg/kg; in saline; po; Figure 5C) was ineffective in reducing the macroscopic damage in the colon of animals with acetic acid-induced colitis. On the other hand, the treatment with **1** loaded microcapsules (1, 10 and 100 mg/kg; in saline; po) showed a progressive reduction of the acetic acid-induced macroscopic damage in the colon (Figure 5D, 5E and 5F, respectively), but only the dose of 100 mg/kg showed significant effects

compared to colitis control group (Figure 5J). Treatments with non-encapsulated **1** (10 and 100 mg/kg; tween 20% diluted in saline; po; Figure 5G and 5H, respectively) and tween 80 (20% diluted in saline; po; Figure 5I) did also not affect the macroscopic damages in the colon, when compared to colitis control group (Figure 5J). Interestingly, flavonoid-rich fractions or extracts and isolated flavonoids such as rutin (**1** is the aglycone form of rutin) reduce gastric ulcers induced by aspirin and indomethacin, and a cream containing **1** heals aphtous ulcers.[43] Therefore, this inhibition or healing of ulcers by treatment with **1** seems to be constant among a variety of models.

Quercetin (1) Loaded Microcapsules Reduce IL-1β and IL-33 Production, and Increase IL-10 Levels in the Colon of Mice with Acetic Acid-Induced Colitis. Intracolonic administration of acetic acid significantly increased the levels of the pro-inflammatory cytokines IL-1β (Figure 6A) and IL-33 (Figure 6B), and reduced the anti-inflammatory cytokine IL-10 (Figure 6C) in the colon, compared to negative control group (Figure 6). Treatment with **1** loaded microcapsules (100 mg/kg; in saline; po) significantly reduced IL-1β (Figure 6A) and IL-33 (Figure 6B) levels in the colon of mice compared to colitis control group. On the other hand, inert microcapsules (100 mg/kg; in saline; po), non-encapsulated **1** (100 mg/kg; in tween 20% diluted in saline; po) and tween 80 (20% diluted in saline; po) did not affect the production of IL-1β (Figure 6A) and IL-33 (Figure 6B) induced by acetic acid.

Cytokines play an important role in the modulation of the intestinal immune system. Levels of several pro-inflammatory cytokines are elevated in patients with inflammatory bowel diseases. In the present study, two cytokines of the IL-1 family were selected due to previous evidence of their participation in ulcerative colitis functioning as chemoattractants to neutrophils, activators of resident cells and stimulators of the production of additional inflammatory mediators.[7,8,44-46] IL-1β is a pro-inflammatory cytokine produced by

macrophages, monocytes and dendritic cells. By ligation with its receptor (IL-1R1) on endothelial cells, this cytokine stimulates the increase of adhesion molecules expression promoting adhesion and transmigration of leukocytes.[47] Therefore, the reduction of IL-1β levels by **1** loaded microcapsules (100 mg/kg; in saline; po; Figure 6A) lines up well with the reduction of the recruitment of leukocytes as shown in the myeloperoxidase activity (Figure 2B) and histological analysis (Figure 4J). Additionally, it has been demonstrated that **1** reduce the production of IL-1β[20,48] and the mechanisms responsible for **1** anti-inflammatory effects can be likely related to the blockade of NF-κB activation and the resultant down-regulation of the pro-inflammatory genes.[48]

IL-33 is a member of the IL-1 family (also named IL-1F11), produced by fibroblasts, smooth muscle cells, endothelial cells, dendritic cells and activated macrophages. IL-33 binds to the IL-1 family receptor ST2 to activate NF-kB and MAP kinases resulting in inflammatory cytokine production and cellular activation.[49,50] Increased serum levels of IL-33 in patients with inflammatory bowel diseases reflect an active inflammatory state and represent a potential biomarker for disease activity.[51] In agreement, IL-33-deficient mice are protected from dextran sulfate sodium-induced intestinal immunopathology.[44] To our knowledge, this is the first study to demonstrate that **1** inhibits the production of IL-33 and indicates that the inhibition of IL-1β and IL-33 production by **1** loaded microcapsules has beneficial effects in acetic acid-induced colitis.

Furthermore, the treatment with **1** loaded microcapsules (100 mg/kg; in saline; po) prevented the reduction of IL-10 level in the colon of mice with acetic acid induced-colitis (Figure 6C). The treatment with non-encapsulated **1** (100 mg/kg; tween 20% diluted in saline; po), inert microcapsules (100 mg/kg; in saline; po) and tween 80 (20% diluted in saline; po) did not improve the levels of IL-10 (Figure 6C) in the colon, when compared to colitis control group. IL-10 is considered the most important anti-inflammatory cytokine in humans. It is

secreted by a variety of cells including macrophages, dendritic cells, granulocytes and epithelial cells,[11,52] and down-regulates the production of pro-inflammatory cytokines, such IL-1β[53] by dendritic cells, macrophages and monocytes.[54] A growing amount of evidence indicates that IL-10 plays a relevant role in preventing the development of inflammatory bowel disease, since IL-10 deficient mice spontaneously develop enterocolitis. Similar findings were observed in infants with homozygous mutations in the IL-10 receptor genes.[12,55,56] Therefore, the protective mechanisms of **1** loaded microcapsules against colitis development may also depend on prevention of IL-10 levels decrease in the colonic tissue.

Treatment with 1 Loaded Microcapsules Prevents the Decrease of Antioxidant Capacity in the Colon of Mice with Acetic Acid-Induced Colitis. Antioxidants can deactivate radicals by two mechanisms: hydrogen atom transfer and single electron transfer. Reduced gluthatione (GSH) assay is a method that measure the former, and ABTS and FRAP represent the latter.[57]

Concentrations of endogenous antioxidants, such as GSH, were significantly decreased in patients with inflammatory bowel disease and in experimental models of colitis.[14,45,46,58] Therefore, the effect of **1** loaded microcapsules and non-encapsulated **1** on colonic antioxidant capacity in this acetic acid model of experimental colitis was evaluated using the treatment protocol shown in Figure 1B, once at 18th hour there was no statistical difference between the colitis control group and the negative control group (data not show).

The colitis control group showed significant reduction on the endogenous antioxidant GSH levels in the colon at 2, 4 and 6 hours after intracolonic administration of acetic acid (7,5% in saline), compared to the negative control group (Figure 7A). The ABTS radical cation scavenging and ferric reducing abilities (FRAP) were also significantly reduced in colitis control group at 2 and 4 hours after colitis induction, compared to negative control

group (Figure 7B and 7C). The time point of 4 h after acetic acid-induced colitis was selected to evaluate the antioxidant capacity (GSH, ABTS and FRAP assays) because significant differences were observed compared to negative control and it allowed a greater lag time between treatment and sample collection.

The mechanism by which GSH quenches radical is related to its ability of transfer a hydrogen atom. Our study shows that the treatment with **1** loaded microcapsules (100 mg/kg, in saline; po; protocol as shown in Figure 1B) significantly prevented colonic GSH depletion in acetic acid-induced colitis (Figure 7D), preserving the colonic tissue from the oxidative damage that characterizes intestinal inflammation. Although the treatment with non-encapsulated **1** (100 mg/kg; tween 20% diluted in saline; po; protocol as shown in Figure 1B) has a tendency to prevented the GSH depletion, there was no statistical difference compared to colitis control group (Figure 7D). The control groups treated with inert microcapsules (100 mg/kg; in saline; po) and tween 80 (20% diluted in saline; po) also showed no statistical difference in GSH levels in the colon compared to colitis control group (Figure 7D).

To measure the antioxidant capacity in the colon related to electron transfer, we chose ABTS and FRAP assay, both evaluate the total antioxidant capacity in the samples. Although the antioxidant activity of a compound against a free radical does not necessarily match its reducing ability, there seems to be a strong correlation between values obtained with FRAP and ABTS assay.[59] Corroborating this observation, a correlation between FRAP and ABTS assays in colonic samples was detected herein. The **1** loaded microcapsules (100 mg/kg, in saline; po; protocol as shown in Figure 1B) treated-group showed a significant increase in ABTS radical cation scavenging and ferric reducing ability when compared to colitis control group. However, this prevention of antioxidant decrease was not observed in the non-encapsulated **1** (100 mg/kg; tween 20% diluted in saline; po), inert microcapsules (100 mg/kg;

in saline; po) and tween 80 (20% diluted in saline; po)-treated groups (Figure 7E and 7F, respectively).

Oxidative stress has been proposed to play an important role in the pathogenesis of inflammatory bowel disease,[14] and is related to the neutrophil infiltration within the inflamed colonic mucosa. The recruitment and activation of neutrophils during acute inflammation contribute to the overproduction of reactive oxygen and nitrogen species that overwhelms the tissue antioxidant protective mechanisms, resulting in oxidative stress, which perpetuates colonic inflammation.[42,60] Furthermore, it was demonstrated that neutrophils depleted of ROS display reduced chemotaxis efficiency, indicating that ROS are essential players for modulating neutrophil chemotaxis.[61] Thus, a rapid inhibition of free radical generation could have contributed to a lower level of neutrophil infiltration into the inflamed tissue, thus preventing colonic tissue from inflammatory cellular infiltration.

The treatment with non-encapsulated **1** was unable to prevent the inflammatory process in this experimental colitis model. The absorption of **1** in the stomach and small intestine, and distribution through the blood stream, prevent **1** to reach the colon in pharmacological effective concentrations to exert anti-inflammatory and antioxidant effects.[19] However, the group treated with **1** loaded microcapsules showed significant reduction in neutrophils influx (myeloperoxidase activity), edema, histological and macroscopical damage scores in the colon compared to colitis control group. Furthermore, this treatment also down-regulated the levels of pro-inflammatory cytokines IL-1β and IL-33, prevented the anti-inflammatory cytokine IL-10 reduction, and preserved the endogenous antioxidants levels in the colon of mice with acetic acid induced-colitis. These protective effects and mechanisms have not been observed by the treatment with non-encapsulated **1** in experimental colitis models,[19,29] which is corroborated by this study. Thus, the controlled release of **1** by microencapsulation improved the therapeutic effects of **1** in experimental colitis.

These beneficial effects of **1** loaded microcapsules may be explained by the prolonged release of the drug, achieved by the **1** microencapsulation using a pectin/casein complex. This complex enables the delay of drug release by diffusion on upper segments of the gastrointestinal tract, thus reaching the colon, where the drug can be released by diffusion, as shown in *in vitro* dissolution test,[32] and also by the action of the enzymes, such as azoreductase and polysaccharidase, secreted by intestinal bacteria, which may metabolize the pectin[33,62] releasing large amounts of drug to have a local effect on colonic inflammation.

In summary, the present study showed that **1** loaded microcapsules reduced the inflammation in the colon in acetic acid-induced colitis by a mechanism dependent on inhibition of pro-inflammatory cytokine production and preserving antioxidant defenses and anti-inflammatory cytokine IL-10 resulting in reduced colon inflammation. Therefore, we envisage that this novel formulation containing **1** merits further pre-clinical and clinical investigation on its applicability in inflammatory bowel disease. Conceptually, this study also raises the importance of pharmaceutical development of controlled release systems to increase the efficacy of drugs for better use of natural resources.

Experimental Section

General Experimental Procedures. Pectin USP (68% of esterification) was obtained from CP Kelco (Limeira, Brazil). Casein from Katuffman & Co (Kehl, Germany). Citric acid; sodium hydroxide; chloride acid; sodium phosphate; formalin from Merck (Darmstadt, Germany). Quercetin from Acros (New Jersey, USA). Hexadecyltrimethylammonium bromide (HTAB); o-dianisidine dihydrochloride; tween 80; reduced glutathione (GSH); glutaraldehyde; EDTA; ferric chloride hexahydrate; 2,4,6-tri-(2-pyridil)-s-triazine (TPTZ); 2,2’-azinobis(3-etil-benzotiazolin-6-sulfonic acid dominium salt; ABTS); trolox; and potassium persulfate (di-potassium peroxidisulfate) from Sigma Chemical Co. (St.Louis,

USA). Kits of mouse IL-1β, IL-33 and IL-10 were obtained from eBiscience (San Diego, USA).

Microcapsules were prepared by dispersion of the polymers pectin and casein in distilled water under constant mechanical shaking.[32] Sodium hydroxide (4,0 M) was used to adjust pH to 8.0 ± 0.1. After dispersion, quercetin was added at the proportion of 1:5 (drug / polymer). Microcapsules were obtained by slow and gradual reduction of pH from 8.0 ± 0.1 to 3.0 ± 0.1 with 1M citric acid. The microcapsules wall was then hardened by the addition of glutaraldehyde (50 μL/g polymer), with constant shaking for an additional 30 min. The same methodology was used to prepare inert microcapsules. The sample was then 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 0.5 mm. The process parameters were: inlet air temperature was kept constant at 180°C and the outlet temperature was at 100°C; feed rate was 7.75 mL/min and airflow was 1.1 m^3/min.

Colonic inflammation was induced according to the technique described by Nosál'ová, Cerná and Bauer (2000)[35] and Luo, et al. (2010),[63] with modifications. Animals were fasted for 24 h and anesthesized with cetamine (80 mg/kg, im) and xylazine (10 mg/kg, im). After colon lavage with 100 μL of saline, a single intracolonic dose of 7.5% acetic acid (in saline; 200 μL) was inserted into 3 cm to the anus through a polyethylene cannula. The mice were kept in a head-down position for 3 min to prevent leakage of intracolonic acetic acid. In the negative control group, animals received intracolonic saline (200 μL) instead of acetic acid solution.

The experimental groups were: a negative control group that received intracolonic saline and no treatment; a colitis control group that received intracolonic 7.5% acetic acid solution with no treatment; and the treated groups that received intracolonic 7.5% acetic acid solution and orally inert microcapsules (100 mg/kg; in saline), quercetin microcapsules (1, 10

and 100 mg/kg; in saline), quercetin (10 and 100 mg/kg; in tween 80 20% in saline) or tween 80 (20% in saline) by means of an esophageal catheter. Two experimental protocols were followed: (A) For assessment of inflammatory parameters, the animals were treated 2 hours before and 10 hours after colitis induction, and they were euthanasiated on the 18th hour. The macroscopic damage was evaluated and samples of distal colon were collected to myeloperoxidase activity assay, edema determination, histological evaluation and cytokine measurements (Figure 1A). (B) To evaluate the oxidative stress in the colon, the animals were treated 6 and 1 hour before colitis induction, and they were euthanasiated on the 4th hour. Samples of distal colon were collected to GSH, ABTS radical cation scavenging and ferric reducing abilities (FRAP) measurements (Figure 1B).

Quercetin quantification in the formulation. To quantify the quercetin in the formulation by drug extraction, we used the method described by Georgetti, et al. (2006).[64] Samples of microcapsules were dispersed in 80% ethanol and then stirred for 5 min, followed by centrifugation at 3000 rpm for 10 min. The supernatant aliquots react with 2% $AlCl_3$ for 1 hour at room temperature, and the absorbance was determinate spectrophotometrically at 420 nm. This process was repeated on the same sample until complete extraction of quercetin on the formulation. The standard curve was prepared with quercetin (6 - 30 µg/mL) and was used to calculate the concentration of quercetin in the liquid extractor. The results are presented as % of quercetin in the formulation, relative to the total quercetin added on formulation.

Mice. Male Swiss mice (25 ± 5 g) from Universidade Estadual de Londrina (Londrina, Brazil) were used in this study. The animals were kept in standard plastic cages in a temperature controlled room (21 - 24 °C) under a 12:12 h light/dark cycle, with free access to tap water and food. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 26511/2009).

Myeloperoxidase Activity Assay. The acetic acid-induced leukocyte migration to the colon of mice was evaluated using the myeloperoxidase kinetic-colorimetric assay as previously described (Bradley, 1982).[65] Tissue samples were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 13.72 mM HTAB (hexadecyl trimethyl ammonium bromide) and stored at -20°C until assayed. The samples were homogenized using a turrax IKA (T10 basic). After that, homogenates were centrifuged (14000 rpm, 2 min, 4°C) and the resulting supernatant assayed spectrophotometrically for myeloperoxidase activity determination at 450 nm (Victor^3 1420 multilabel counter). The myeloperoxidase activity of samples was compared to a standard curve of neutrophils. Briefly, 15 µL of sample was mixed with 200 µL of 50 mM phosphate buffer (pH 6.0), containing 52.64 mM o-dianisidine dihydrochloride and 0.05% H_2O_2 30%. The results are presented as number of neutrophils per mg of tissue.

Edema. The sectioned 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 normal control group, without colitis.[40,66]

Histological Analysis and Microscopic Damage Score. Samples of distal colon were immediately fixed in 10% formalin solution. Formalin-fixed colonic samples were embedded in paraffin, and sections (7 µm) were stained with hematoxylin and eosin. Then, 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 semi-quantitatively as described by Appleyard and Wallace (1995),[67] 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.

Macroscopic Damage Score. To evaluate the severity of the macroscopic damage, the colon was excised, opened longitudinally and rinsed with saline. Then, a damage score was determined using the criteria outlined by Morris, et al. (1989),[68] with slight 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, whose quantification is increasing by 1 for each additional centimeter (score 6 – 10).

Protein Assay. Total protein content on colon samples was estimated spectrophotometrically by a method described previously.[69,70] During the reaction, the addition of cupric reagent, composed by 10% sodium carbonate in 5N NaOH, 2% sodium tartarate and 1% copper sulfate, and folin phenol reagent in an alkaline medium forms a colored complex, whose absorbance was determined at 660 nm. The color intensity is proportional to protein concentration in the sample. The bovine serum albumin was used as standard. Results are expressed as mg protein per mg of tissue.

Cytokine Measurements. Samples of distal colon were collected and homogenized in sterile saline. The homogenates were centrifuged at 3000 rpm at 4°C for 10 min, and supernatants were stored at -80°C until further analysis. IL-1β, IL-33 and IL-10 levels were evaluated through ELISA kits according to the manufacturer's recommendations. The results were expressed as pg per mg of protein.

GSH Assay. Colon samples were collected and frozen at –80°C until GSH assay. The GSH levels were determined using a spectrophotometric method, with modification.[71,72] The samples were homogenized in 0.02 M EDTA solution. Homogenates were treated with 30% trichloroacetic acid and centrifuged at 4000 rpm at 4°C for 15 minutes. Then, 150 µL of sample was mixed with 200 µl of 0.4 M Tris-HCl (pH 8.9). After vortex-mixing, 10µl of 0.01

M dithiobisnitrobenzoic acid (DTNB) in methanol was added. The samples were vortex-mixed again and the absorbance at 412 nm was read after 5 min. The standard curve was prepared with 0.50 μmol GSH and was used for calculate the concentration of GSH in the colonic tissues. The results are presented as μmol GSH per mg protein.

ABTS Assay. The ability of antioxidants to quench the stable radical cation formed from 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) - ABTS+• was evaluated in the colon samples by a published method.[59,73] Briefly, ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation was generated by reacting ABTS solution with 2.45 mM potassium persulfate and allowing the solution to sit in the dark at room temperature for 12–16 h before use. This solution of deep blue-green ABTS radical cation was diluted in sufficient potassium phosphate (pH 7.4) to give an absorvance of 0.8 at 730 nm. Colonic tissue samples were homogenized in KCl solution, centrifugated (1500 rpm, 4°C, 10 min), and the supernatant aliquots reacted with the solution of ABTS radical cation. The decrease in the absorvance at 730 was read after 6 min. The free radical scavenging capacity of the samples was equated against a Trolox standard curve (1-8 mmol). The results were expressed as mmol Trolox equivalents per g of tissue, that is, the amount of Trolox (mmol) with an equivalent antioxidant potential to 1 g of the tissue under investigation.

FRAP (Ferric Reducing Antioxidant Power) Assay. The reducing capacity in colon samples was determined by FRAP assay, according to Benzie and Strain (1996)[74] and Katalinic, et al. (2005).[59] This method measures the antioxidant capacity due to the formation of a blue colored Fe^{II}-tripyridyltriazine compound from oxidized Fe^{III} form by the action of electron donating antioxidants. The FRAP reagent consist in 0.3 mM acetate buffer (pH 3.6), 10mM TPTZ in 40 mM hydroclorid acid and 20 mM ferric chloride. Colonic tissue samples were homogenized in KCl solution, centrifugated (1500 rpm, 4°C, 10 min), and the supernatant aliquots reacted with the FRAP reagent. The reaction mixture was incubated at

37°C for 30 min, and the absorbance was read at 595 nm. In the FRAP assay, the reducing ability of the tissue homogenate under the test was equated against a Trolox standard curve (0.4–2 mmol). The results are expressed as mmol Trolox equivalents per g of tissue, that is, the amount of Trolox (mmol) with an equivalent antioxidant potential to 1 g of the tissue under investigation.

Statistical Analysis. All data are expressed as means ± SEM. Statistical significance of differences between the groups was determined by one-way ANOVA followed by Student Newman-Keuls's test. For categorical variables, Kruskal-Wallis followed by Dunn's test was performed. Statistical analyses were performed by using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Criterion for significance was chosen to be at $p < 0.05$.

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Legends to 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 18th hour (Panel A). To evaluate the oxidative stress in the colon, the mice were treated 6 and 1 hour before colitis induction, and colonic samples were collected on the 4th hour (Panel B).

Figure 2. Quercetin (**1**) loaded microcapsules reduce neutrophil infiltration within the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. Myeloperoxidase activity was evaluated in samples of distal colon 6, 12, 18 and 24 hours after colitis induction (Panel A). n=8. Mice were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) and tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid, and the samples were collected 18 hours after colitis induction (Panel B). n=20. [* $p < 0.05$ compared to saline (negative control) group, ** $p < 0.05$ compared to untreated (colitis control) group. One-way ANOVA followed by Student Newman-Keuls's test].

Figure 3. Quercetin (**1**) loaded microcapsules prevent the increase in colonic weight / length (edema) in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) or tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The increase in colonic weight / length in relation to

saline group was evaluated in samples of distal colon 18 hours after colitis induction. n=20. [* $p < 0.05$ compared to colitis control group. One-way ANOVA followed by Student Newman-Keuls's test].

Figure 4. Quercetin (**1**) loaded microcapsules reduce microscopic damage in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) or tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The damage score was determined 18 hours after colitis induction and the parameters analyzed were loss of mucosal architecture, cellular infiltration, muscle thickening, crypt abscess formation and goblet cell depletion. Negative control group (Panel A); Colitis control group (Panel B); Colitis + MCIn (Panel C); Colitis + **1**MC 1 mg/kg (Panel D); Colitis + **1**MC 10 mg/kg (Panel E); Colitis + **1**MC 100 mg/kg (Panel F); Colitis + **1** 10 mg/kg (Panel G); Colitis + **1** 100 mg/kg (Panel H); Colitis + Tween 80 (Panel I); Microscopic scores (Panel J). The samples were stained with hematoxylin and eosin. Original magnification 10×. n=7. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test].

Figure 5. Quercetin (**1**) loaded microcapsules reduce macroscopic damage score in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) or tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The macroscopic damage score was determined 18 hours after colitis induction,

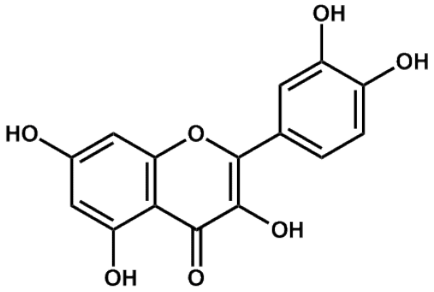
according to the extension as well as the severity of colonic damage. Negative control group (Panel A); Colitis control group (Panel B); Colitis + MCIn (Panel C); Colitis + **1**MC 1 mg/kg (Panel D); Colitis + **1**MC 10 mg/kg (Panel E); Colitis + **1**MC 100 mg/kg (Panel F); Colitis + **1** 10 mg/kg (Panel G); Colitis + **1** 100 mg/kg (Panel H); Colitis + Tween 80 (Panel I); Microscopic scores (Panel J). n=7. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test].

Figure 6. Quercetin (**1**) loaded microcapsules reduce IL-1β and IL-33, and prevent the decrease of IL-10 levels in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 100 mg/kg, po), **1** (100 mg/kg, po) or tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The IL-1β (Panel A), IL-33 (Panel B) and IL-10 (Panel C) levels were evaluated in samples of distal colon 18 hours after colitis induction by commercial kits. n=6. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. One-way ANOVA followed by Student Newman-Keuls's test].

Figure 7. Quercetin (**1**) loaded microcapsules prevent the decrease of antioxidant capacity in the colon of mice with acetic acid colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. Antioxidant capacity was evaluated in samples of distal colon 2, 4 and 6 hours after colitis induction (Panels A, B and C). Mice were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 100 mg/kg, po), **1** (100 mg/kg, po) or tween 80 (20% in saline, po), 6 and 1 hour before intracolonic administration of acetic acid, and the samples were collected 4 hours after colitis induction (Panels D, E and F).

n=6. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. One-way ANOVA followed by Student Newman-Keuls's test].

Structure Sheet



1

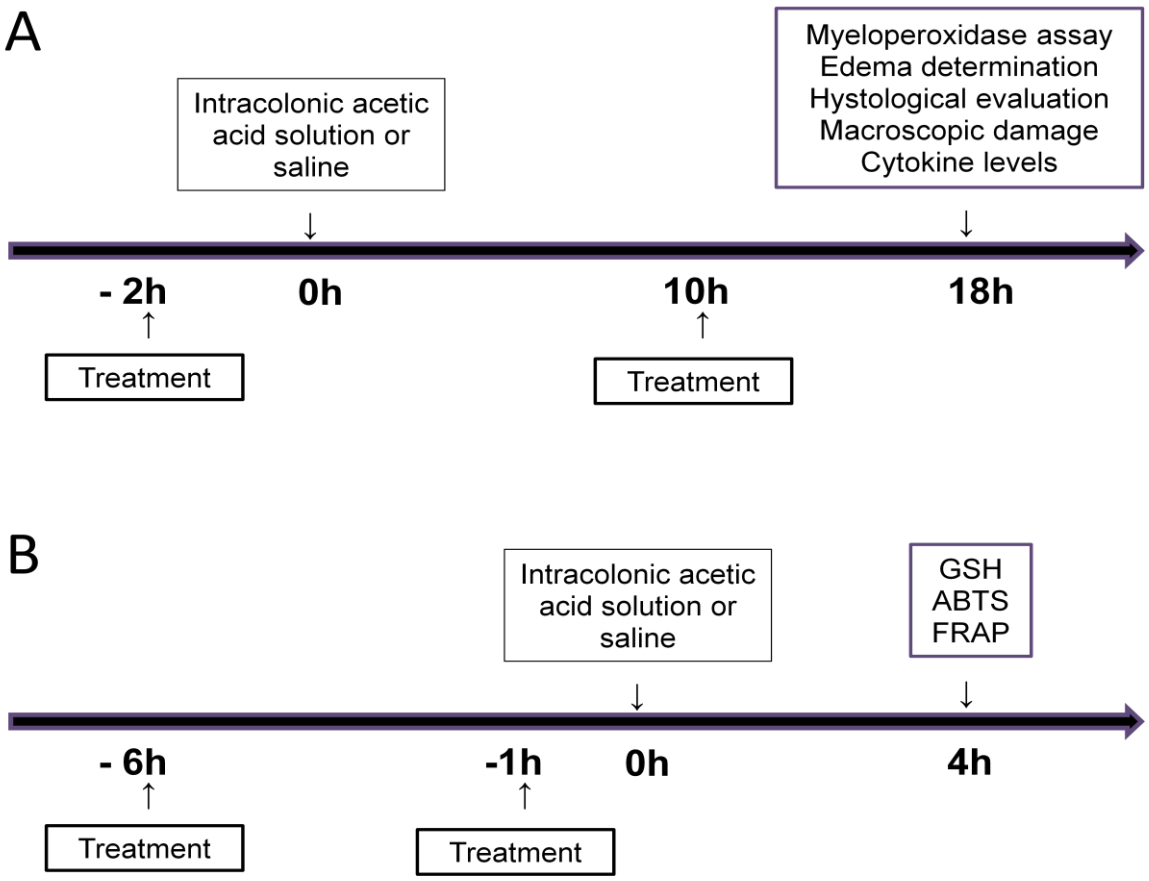


Figure 1. Schematic representation of experimental protocols for treatments and colitis induction. For assessment of inflammatory parameters, the mice were treated 2 hours before and 10 hours after colitis induction, and colonic samples were collected on the 18th hour (Panel A). To evaluate the oxidative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

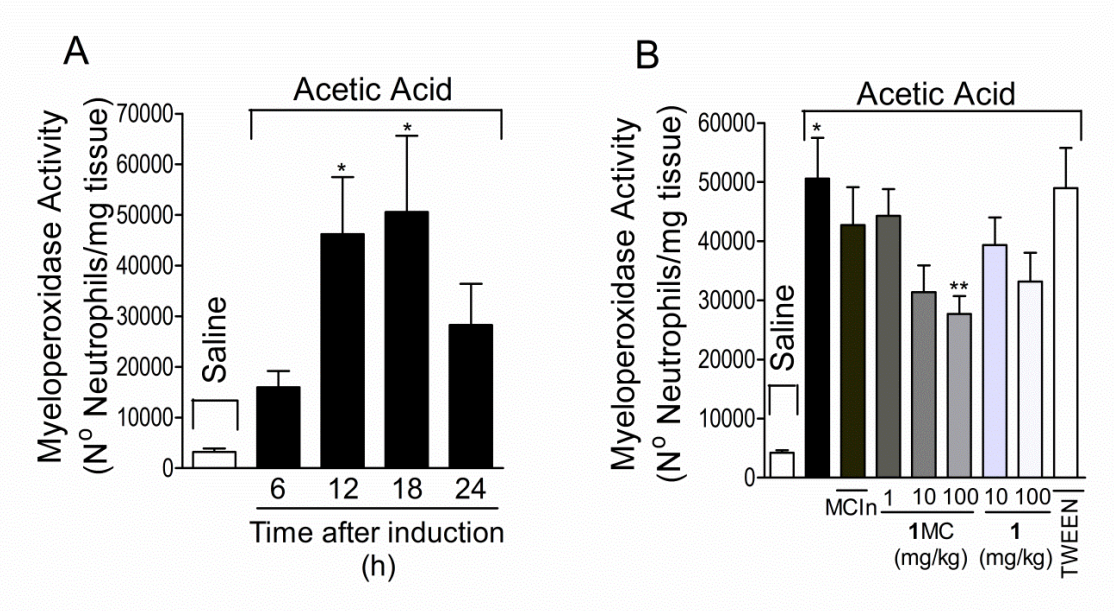


Figure 2. Quercetin (**1**) loaded microcapsules reduce neutrophil infiltration within the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. Myeloperoxidase activity was evaluated in samples of distal colon 6, 12, 18 and 24 hours after colitis induction (Panel A). n=8. Mice were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) and tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid, and the samples were collected 18 hours after colitis induction (Panel B). n=20. [* $p < 0.05$ compared to saline (negative control) group, ** $p < 0.05$ compared to untreated (colitis control) group. One-way ANOVA followed by Student Newman-Keuls's test].

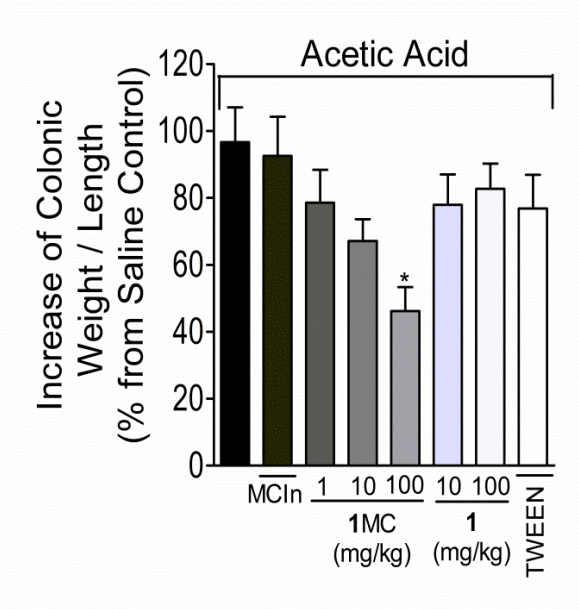
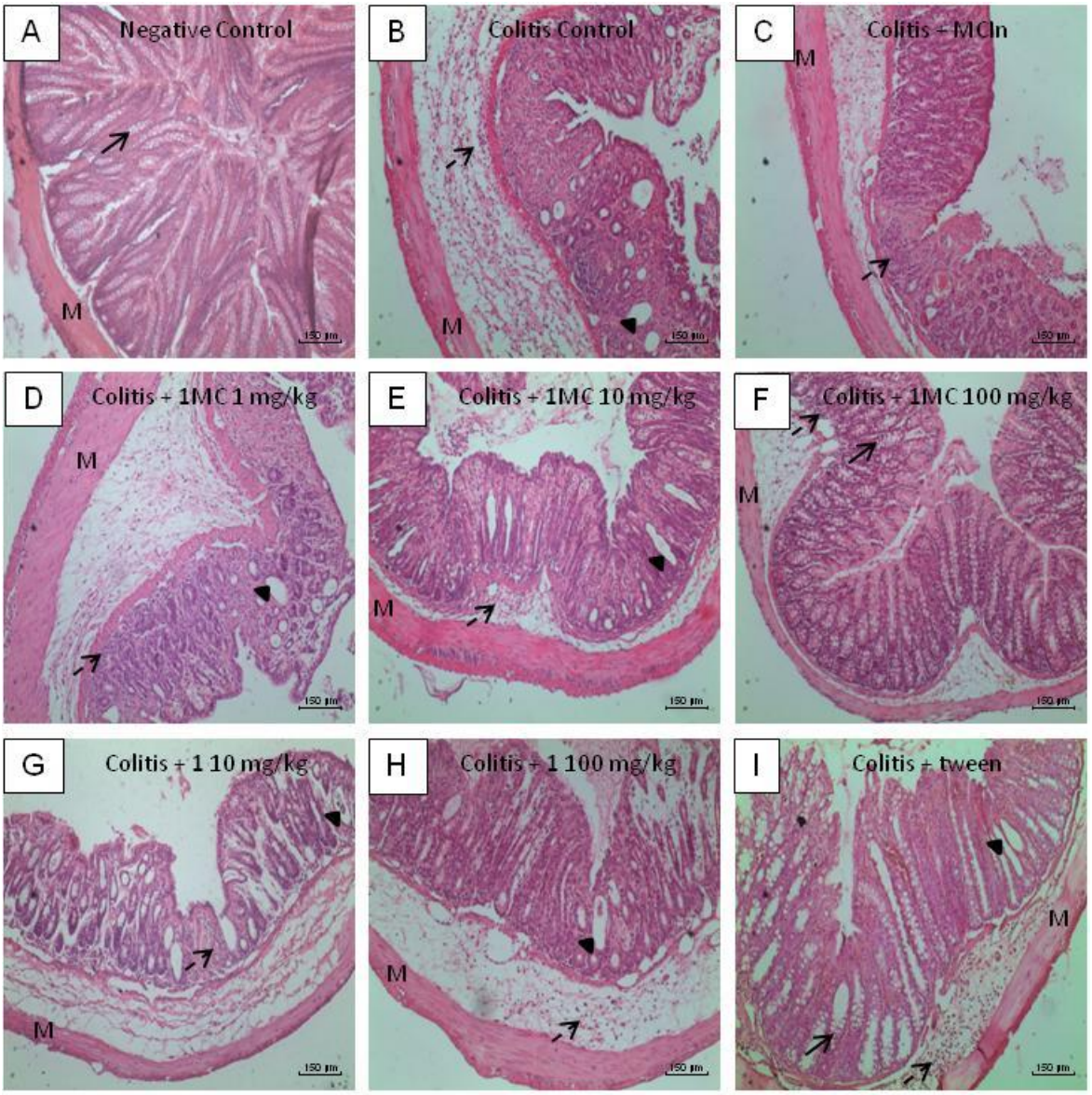


Figure 3. Quercetin (**1**) loaded microcapsules prevent the increase in colonic weight / length (edema) in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) or tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The increase in colonic weight / length in relation to saline group was evaluated in samples of distal colon 18 hours after colitis induction. n=20. [* $p < 0.05$ compared to colitis control group. One-way ANOVA followed by Student Newman-Keuls's test].


A
Negative Control
M
B
Colitis Control
M
C
Colitis + MCln
M
D
Colitis + 1MC 1 mg/kg
M
E
Colitis + 1MC 10 mg/kg
M
F
Colitis + 1MC 100 mg/kg
M
G
Colitis + 1 10 mg/kg
M
H
Colitis + 1 100 mg/kg
M
I
Colitis + tween
M
150 µm

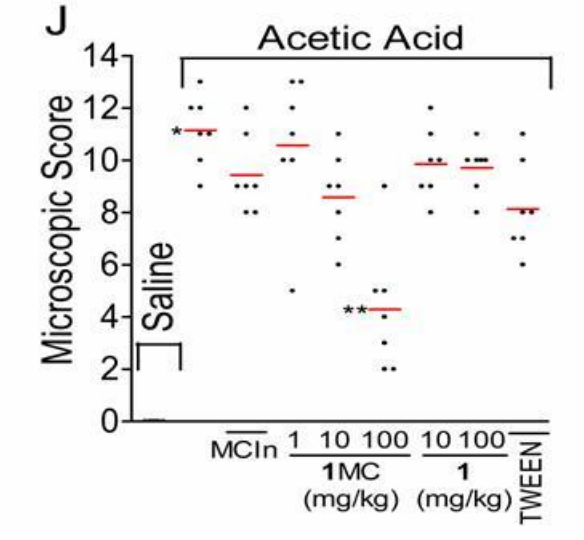

J
Acetic Acid
Microscopic Score
14
12
10
8
6
4
2
0
Saline
*
**
MCln
1
10
100
1MC
(mg/kg)
10
100
1
(mg/kg)
TWEEN

Figure 4. Quercetin (**1**) loaded microcapsules reduce microscopic damage in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) or tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The damage score was determined 18 hours after colitis induction and the parameters analyzed were loss of mucosal architecture, cellular infiltration (dotted arrow), muscle (M) thickening, crypt abscess (arrowhead) formation and goblet cell (arrow) depletion. Negative control group (Panel A); Colitis control group (Panel B); Colitis + MCIn (Panel C); Colitis + **1**MC 1 mg/kg (Panel D); Colitis + **1**MC 10 mg/kg (Panel E); Colitis + **1**MC 100 mg/kg (Panel F); Colitis + **1** 10 mg/kg (Panel G); Colitis + **1** 100 mg/kg (Panel H); Colitis + Tween 80 (Panel I); Microscopic scores (Panel J). The samples were stained with hematoxylin and eosin. Original magnification 10×. n=7. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test].

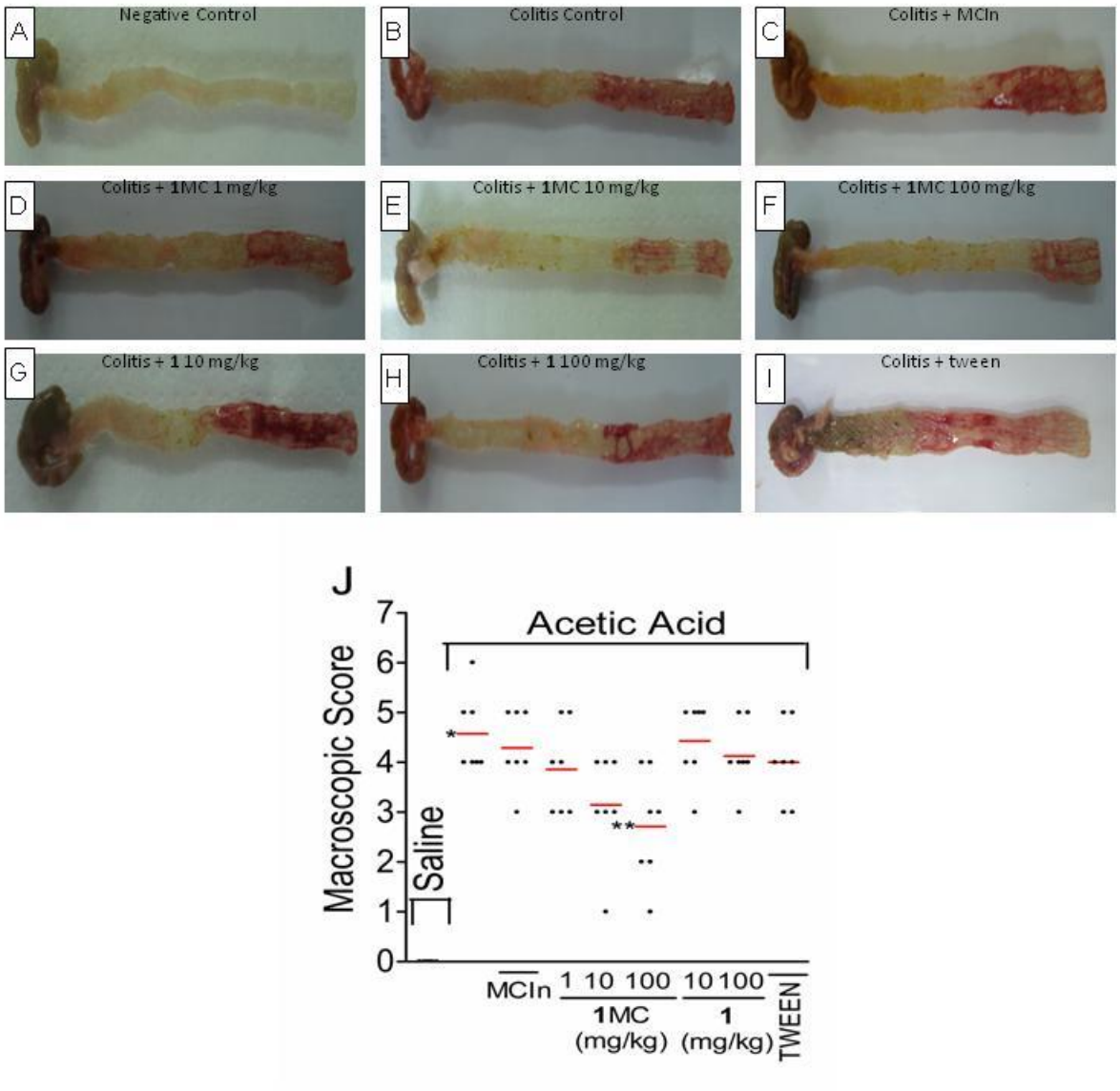


Figure 5. Quercetin (**1**) loaded microcapsules reduce macroscopic damage score in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 1, 10 and 100 mg/kg, po), **1** (10 and 100 mg/kg, po) or tween (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The macroscopic damage score was determined 18 hours after colitis induction, according to the extension as well as the severity of colonic damage. Negative control group (Panel A); Colitis control group (Panel B); Colitis + MCIn (Panel C); Colitis + **1**MC 1 mg/kg (Panel D); Colitis + **1**MC 10 mg/kg (Panel E); Colitis + **1**MC 100 mg/kg (Panel F); Colitis + **1** 10 mg/kg (Panel G); Colitis + **1** 100 mg/kg (Panel H); Colitis + Tween 80 (Panel I); Microscopic scores (Panel J). n=7. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. Kruskal-Wallis followed by Dunn's test].

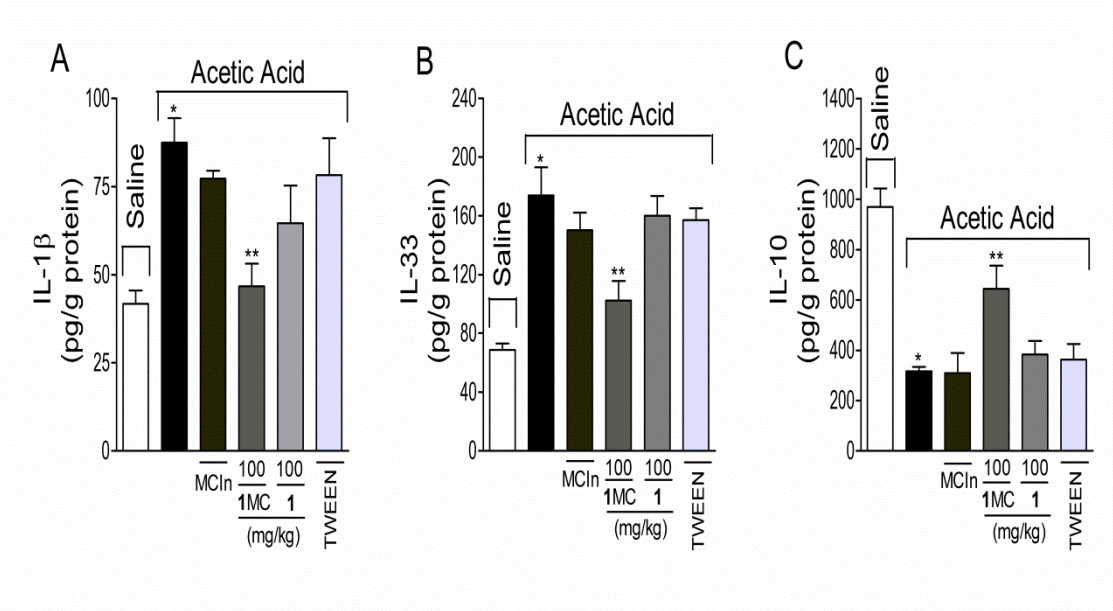


Figure 6. Quercetin (**1**) loaded microcapsules reduce IL-1β and IL-33, and prevent the decrease of IL-10 levels in the colon of mice with acetic acid-induced colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. They were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 100 mg/kg, po), **1** (100 mg/kg, po) or tween 80 (20% in saline, po) 2 hours before and 10 hours after intracolonic administration of acetic acid. The IL-1β (Panel A), IL-33 (Panel B) and IL-10 (Panel C) levels were evaluated in samples of distal colon 18 hours after colitis induction by commercial kits. n=6. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. One-way ANOVA followed by Student Newman-Keuls's test].

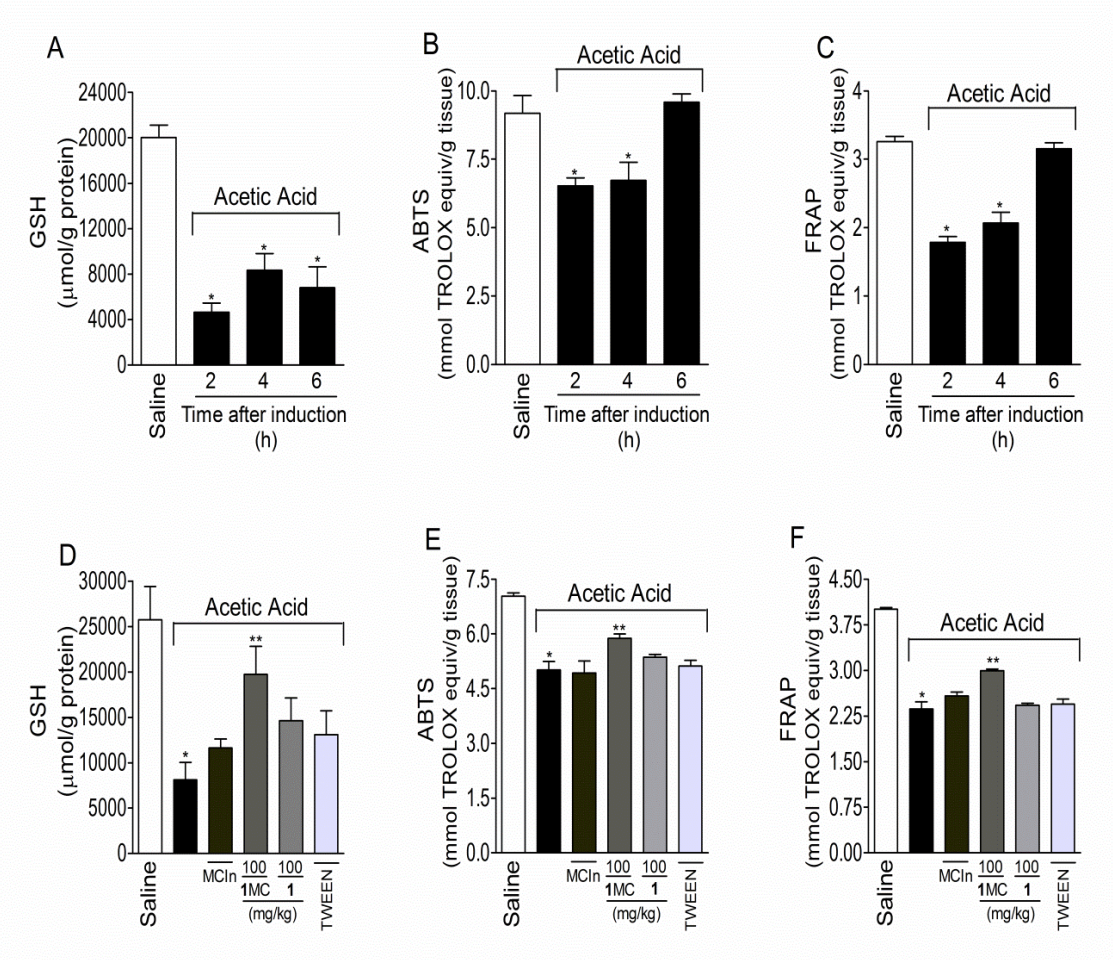


Figure 7. Quercetin (**1**) loaded microcapsules prevent the decrease of antioxidant capacity in the colon of mice with acetic acid colitis. Acetic acid (7,5%, 200 µL) or saline (200 µL) was injected into the colon of Swiss mice. Antioxidant capacity was evaluated in samples of distal colon 2, 4 and 6 hours after colitis induction (Panels A, B and C). Mice were treated with inert microcapsules (MCIn; 100 mg/kg), **1** loaded microcapsules (**1**MC; 100 mg/kg, po), **1** (100 mg/kg, po) or tween 80 (20% in saline, po), 6 and 1 hour before intracolonic administration of acetic acid, and the samples were collected 4 hours after colitis induction (Panels D, E and F). n=6. [* $p < 0.05$ compared to negative control group, ** $p < 0.05$ compared to colitis control group. One-way ANOVA followed by Student Newman-Keuls's test].

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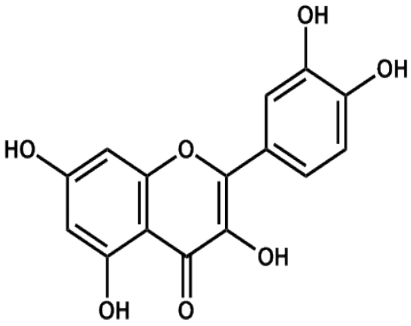
Quercetin loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms.

Carla F.S. Guazelli, Victor Fattori, Barbara B. Colombo, Sandra R. Georgetti, Fabiana T.M.C. Vicentini, Rubia Casagrande, Marcela M. Baracat, Waldiceu A. Verri, Jr.*



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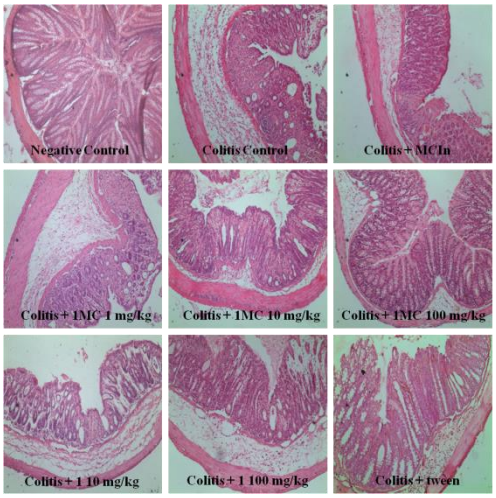

5 CONCLUSÕES

Através dos resultados obtidos, podemos concluir que:

- O processo de microencapsulação da quercetina utilizando o polímero biodegradável pectina/caseína permite a obtenção de uma nova formulação microencapsulada contendo quercetina de maneira eficiente, com perda de apenas 8% do fármaco durante o processo. As microcápsulas obtidas possibilitam a liberação modificada do fármaco e apresentam-se como uma nova proposta terapêutica a ser avaliada em modelo de colite em camundongos, visando ação local da quercetina sobre as lesões colônicas.

- O tratamento com as microcápsulas de quercetina por via oral reduziu significativamente a atividade da mieloperoxidase, o edema, as lesões microscópicas e macroscópicas, e os níveis das citocinas IL-1β e IL-33 no cólon dos camundongos com colite induzida por ácido acético, quando comparados ao grupo controle colite sem tratamento. Além disso, o tratamento também preveniu a queda nos níveis de IL-10 e das capacidades antioxidantes endógenas no tecido colônico, avaliadas através da glutationa reduzida, da capacidade sequestradora do radical $ABTS^{\cdot}$ e redutora do íon Fe^{3+}. Entretanto, o tratamento com quercetina não encapsulada não mostrou efeitos significativos sobre os parâmetros avaliados no cólon dos animais, quando comparados ao grupo controle colite sem tratamento. Portanto, verificamos que a microencapsulação proporciona efeitos adicionais à quercetina não incorporada a microcápsulas sobre a colite induzida por ácido acético em camundongos.

- Sendo assim, o presente estudo mostrou que as microcápsulas contendo quercetina reduzem a inflamação e preservam as capacidades antioxidantes no intestino em modelo de colite induzida por ácido acético em camundongos. Sugerimos que estes efeitos anti-inflamatórios e antioxidantes das microcápsulas de quercetina estejam relacionados à obtenção do sistema de liberação modificada, visando a liberação colônica, através da microencapsulação utilizando o polímero pectina/caseína. Portanto, os efeitos benéficos apresentados pelo tratamento com as microcápsulas de quercetina justificam um pedido de patente deste novo produto, além deste estudo pré-clínico indicar a importância da investigação da eficácia clínica das microcápsulas contendo quercetina.

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ANEXO

Normas técnicas para a publicação na revista científica *Journal of Natural Products.*

Preparation and Submission of Manuscripts
(Revised March 2012)

Contents (click on the topic)

Title Page
The title should appear on a separate page and should be followed by the author names and the institution name and address. The title, author name(s), and affiliations should all appear on their own respective line of text. Place an asterisk after the name of the author to whom enquiries regarding the paper should be directed and include that author's telephone and fax numbers and e-mail address. Author affiliations must be footnoted using the following symbols in order (which should be used as superscripts): †, ‡, §, ⊥, ∥, ∇, O. In article titles, the words "new" or "novel" (with the latter referring specifically to a compound based on an unprecedented carbon skeleton) should not be included, and the number of new substances obtained should not be specified.

Abstract
The abstract, detailing, in one paragraph, the problem, experimental approach, major findings, and conclusions, should appear on the second page. It should be double spaced and should not exceed 200 words for Full Articles and Reviews or 100 words for Notes and Rapid Communications. Compounds mentioned in the abstract, and given as specific Arabic numerals that are bolded in the text, should also be accompanied in the abstract by the same bolded numerals. The abstract should be on a separate page and should be provided with the bolded and capitalized heading "ABSTRACT".

Introduction
The manuscript should include an untitled introduction stating the purpose of the investigation and relating the manuscript to similar research.

Results and Discussion
The "Results and Discussion" should be presented as a coherent whole section, in which the results are presented concisely. The discussion should interpret the results and relate them to existing knowledge in the field in as clear and brief a fashion as possible. Tables and figures should be designed to maximize the presentation and comprehension of the experimental data. Authors submitting a manuscript as a Note should omit the heading "Results and Discussion". For Full Articles of unusual length, subheadings may be included within the "Results and Discussion" section. The major heading "RESULTS AND DISCUSSION" should be bolded and capitalized , with the text starting on the line following. Subheadings are indented, followed by a period, and are a mix of uppercase and lowercase letters. The text follows on the same line as the subheading.

Bolded structural code numbers should only be used for new compounds and for those known compounds for which new biological data or spectroscopic values are being reported. Other known compounds should be referred to in the text by name, wherever necessary. Sugar units in glycosides should not be inferred as D or L based solely on NMR data analysis, but should be determined by supporting experimental work such as measurement of their optical rotations following acid hydrolysis or by the preparation of chiral derivatives and comparison with standards using a chromatographic analytical method. If the aglycone of a glycoside is also a new compound, then it should be isolated and its physical constants and spectroscopic parameters stated. Authors are advised to use correctly the terms "relative and absolute configuration" instead of "relative and absolute stereochemistry". In, for example, a carbocyclic compound, only a stereogenic carbon or a stereogenic element, such as an axis, possesses configuration. Substituents such as methyl groups are either alpha or beta oriented and are not alpha or beta configured. Care should be taken not to make erroneous configurational conclusions via NMR NOE associations from ring to side-chain protons of, for example, sterols and tetracyclic triterpenoids. The term "spectral" should be avoided in a structure elucidation discussion, when "spectroscopic" or "spectrometric" are meant instead.

In manuscripts that present results of biological studies with tumor cell lines or animal-based tumor models, authors should pay special attention to the U.S. National Cancer Institute (NIH) guidelines for cancer drug discovery studies. Compounds that suppress the growth of, or kill, isolated tumor cell lines grown in culture should be referred to as either "cytostatic" or "cytotoxic", as appropriate. Only compounds that inhibit the growth of tumors in animal-based models should be called "antitumor". The term "anticancer" should be reserved for compounds that show specific activity in human-based clinical studies (see Suffness, M.; Douros, J. J. Nat. Prod. 1982, 45, 1– 14). Some flexibility in this system is afforded in the description of compounds that show activity in molecular-targeted antitumor assays. Compounds should be compared against a suitable positive control substance and follow accepted guidelines when represented as "active". For example, a cytotoxic pure substance when tested against a cancer cell line would exhibit an IC50 value of <10 μM (or 4–5 μg/mL).

Experimental Section

The presentation of specific details about instruments used, sources of specialized chemicals, and related experimental details should be incorporated into the text of the Experimental Section as a paragraph headed General Experimental Procedures. The general order for inclusion should be as follows: melting points; optical rotations; UV spectra; CD spectra; IR spectra; NMR spectra; mass spectra; and chromatographic and other techniques.

In a separate paragraph, experimental biological material should be reported as authenticated if cultivated or from a natural habitat, and the herbarium deposit site and voucher number should be recorded. The month and year when the organisms were collected should be stated, and it is recommended that the exact collection location be provided using a GPS navigation tool. All microorganisms used experimentally should bear a strain designation number and the culture collection in which they are deposited. The scientific name (genus, species, authority citation, and family) should be presented when first mentioned in the body of the manuscript. Thereafter, the authority should be eliminated, and the generic name should be reduced (except in tables and figure legends) to the first capital letter of the name (but avoid ambiguity, if two or more generic names have the same first letter).

If the biological material has not been identified as to species, the manuscript will not be considered for publication unless a special protocol has been followed. Thus, a voucher specimen of the organism should be deposited with a recognized taxonomist for the particular group of organisms in question. The taxonomist should then assign to the specimen an identifying number unique to the organism so that any additional collections of the same organism would bear this same number. The number will be retained until the organism is completely identified. The taxonomist should write a brief taxonomic description to be included in the manuscript, which should state how the organism in question relates morphologically to known species. Contributors should use DNA sequence analysis to assist with the taxonomic identification of unknown microorganisms, and to deposit these data in GenBank (http://www.ncbi.nlm.nih.gov/). Photographs of incompletely identified organisms may be included as Supporting Information. Authors should be aware of the fact that the large-scale collection of marine or terrestrial organisms may have negative ecological effects. Authors describing an investigation derived from large-scale collections should thus include a statement in their manuscript (in the "Biological Material" paragraph of the Experimental Section) explaining why the collection had no significant adverse ecological effect or justifying such effect in terms of the benefit from the resulting work.

Authors who purchase dried "herbal remedies" or other materials from companies must make provision for their proper deposit in a herbarium, for access by future workers. When a commercially available extract is obtained, the extraction procedure from the organism of origin must be specified. The identification of the extract should be supported by an HPLC trace of known secondary metabolite constituents of the organism, which should be included with the manuscript as Supporting Information.

When physical and spectroscopic data are presented in the body of the manuscript, the following general style must be used (with the various commonly used techniques presented in this same order):

Romucosine (1): colorless needles (CHC_3); mp 152–153 °C; $[\alpha]_D^{25}$ –110 (c 0.4, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 235 (4.23), 275 (4.18), 292 (sh) (3.52), 325 (3.41) nm; IR (Nujol) ν_{max} 1680, 1040, 920 cm^{-1}; 1H NMR (CDC_3, 400 MHz) δ 8.11 (1H, d, J = 7.6 Hz, H-11), 7.54–7.28 (2H, m, H-9, H-10), 7.27 (1H, m, H-8), 6.59 (1H, s, H-3), 6.10, 5.97 (each 1H, d, J = 1.5 Hz, OCH_2O), 4.86 (1H, dd, J = 13.7, 4.4 Hz, H-6a), 4.44 (1H, m, H-5a), 3.77 (3H, s, $NCOOCH_3$), 3.06 (1H, m, H-7a), 2.99 (1H, m, H-5b), 2.91 (1H, m, H-7b), 2.82 (1H, m, H-4a), 2.61 (1H, m, H-4b); 13C NMR ($CDCl_3$, 100 MHz) δ 155.8 (C, $NCOOCH_3$), 146.8 (C, C-2), 143.0 (C, C-1), 135.8 (C, C-7a), 130.7 (C, C-11a), 128.7 (CH, C-8), 127.79 (C, C-3a), 127.78 (CH, C-9), 127.2 (CH, C-10), 127.0 (CH, C-11), 125.6 (C, C-3b), 117.3 (C, C-1a), 107.6 (CH, C-3), 100.9 (CH_2, OCH_2O), 52.7 (CH_3, $NCOOCH_3$), 51.7 (CH, C-6a), 39.2 (CH_2, C-5), 34.5 (CH_2, C-7), 30.4 (CH_2, C-4); EIMS m/z 323 $[M]^+$ (98), 308 (28), 292 (5), 262 (20), 248 (21), 236 (81), 235 (100), 206 (17), 178 (27), 88 (17); HREIMS m/z 323.1152 (calcd for $C_{19}H_{17}O_4N$, 323.1158).

The correct presentation of NMR spectroscopic data is shown in the table below.

The correct format to present elemental analysis data is: anal. C 72.87, H 11.13%, caled for

C37H68O6, C 73.02, H 11.18%. The structures of compounds are expected to be supported by high-resolution mass spectrometry or elemental analysis. Melting point determinations should not be provided for compounds described as "amorphous solids". The unit of concentration to be used for optical rotation measurements is grams per 100 mL. UV extinction coefficient data should be provided as log ϵ values, to two places of decimals. In reporting
1
H NMR data of diastereotopic methylene protons, the one at lower field should be listed as the "a" proton and that at the higher field as the "b" proton, as in "H-10a" and "H-10b", respectively. If two proton or carbon signals in an NMR spectrum appear at the same chemical shift but are still distinguishable, an additional decimal place (three for 1
H NMR data and two for 13C NMR data)
may be used to designate the resonance in question. Carbon-13 NMR data should be reported to the nearest 0.1 ppm with the number of attached protons designated using the C, CH, CH2, and CH3 notation.

Acknowledgment
The Acknowledgment section should include credits [initial(s) and last name] for technical assistance, financial support, and other appropriate recognition.

References and Notes
References to the literature and all notes, regardless of their nature, should be numbered in order of appearance in the manuscript and cited in the text with superscript numbers. Each reference may have its own citation number, or alternatively, references referring to the same topic may be grouped under a common number using alphabetical subdesignations (e.g., 1a, 1b, 1c, etc.). Each note should be assigned its own number. References and notes should follow the format shown:
(1) Dumdei, E.; Andersen, R. J. J. Nat. Prod. 1993, 56, 792–794.
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(5) Meyer, B. N. Brine Shrimp Toxicity: Certain Components of Stapelia, Coryphantha, Lupinus, and Quinoa. Ph.D. Thesis, Purdue University, West Lafayette, IN, 1983, p 35.
(6) Davis, R. U.S. Patent 5,708,591, 1998.
(7) The biogeographic zone comprising Madiera, the Canary Islands, the Cape Verde Islands, and the Azores.
For additional information on the reference and note format to use, see The ACS Style Guide, 3rd ed. (2006) (http://pubs.acs.org/books), available from Oxford University Press, Order Department, 2001 Evans Road, Cary, NC 27513 (http://www.oup.com). The author is responsible for the accuracy and completeness of all references. In particular, authors must cite all of the references from their own work on a particular topic, such as all papers published or submitted on the constituents of a given organism under consideration.
Because subscribers to the Web edition are now able to click on the "CAS" tag following each reference to retrieve the corresponding CAS abstract, reference accuracy is critical. Journal abbreviations should be those used by Chemical Abstracts [see Chemical Abstracts Service Source Index (CASSI) 1907–2004].
The author should supply the Editor with copies of related manuscripts that are cited as "in

press" or "submitted" for use by the editors and the reviewers in evaluating the manuscript under consideration.

Nomenclature

It is the responsibility of the authors to provide correct nomenclature. All nomenclature must be consistent and unambiguous and should conform with current American usage. Insofar as possible, authors should use systematic names similar to those used by Chemical Abstracts Service, the International Union of Pure and Applied Chemistry, and the International Union of Biochemistry and Molecular Biology.

Chemical Abstracts (CA) nomenclature rules are described in Appendix IV of the Chemical Abstracts Index Guide. A list of ring systems, including names and numbering systems, is found in the Ring Systems Handbook, American Chemical Society, Columbus, OH, 2003, and its latest cumulative supplement. For CA nomenclature advice, consult the Manager of Nomenclature Services, Chemical Abstracts Service, P.O. Box 3012, Columbus, OH 43210-0012. A name generation service is available for a fee through CAS Client Services, 2540 Olentangy River
Road, P.O. Box 3343, Columbus, OH 43210-0334; tel: (614) 447-3870; fax: (614) 447-3747; or
e-mail: answers@cas.org.

For IUPAC rules, see:

- Nomenclature of Inorganic Chemistry, Recommendations, 1990; Blackwell Scientific Publications: Oxford, England, 1990.
- A Guide to IUPAC Nomenclature of Organic Compounds, Recommendations, 1993; Blackwell Scientific Publications: Oxford, England, 1993.
- Nomenclature of Organic Chemistry, Sections A–F and H; Pergamon Press: Elmsford, NY, 1979.
- Compendium of Macromolecular Nomenclature; Blackwell Scientific Publications: Oxford, England, 1991.
- Biochemical Nomenclature and Related Documents, 2nd ed.; Portland Press, Ltd.: London, England, 1992.
- Selected IUPAC recommendations can be found on the Web at http://www.chem.qmw.ac.uk/iupac/iupac.html.
- The ACS Web site has links to nomenclature recommendations: http://chemistry.org.

Abbreviations

Abbreviations are used without periods. Standard abbreviations should be used throughout the manuscript. All nonstandard abbreviations should be kept to a minimum and must be defined in the text following their first use. The preferred forms of some of the more commonly used abbreviations are mp, bp, °C, K, s, min, h, mL, μL, kg, g, mg, μg, cm, mm, nm, mol, mmol, μmol, ppm, TLC, GC, NMR, MS, UV, CD, and IR. For further information, refer to The ACS Style Guide (2006).

Graphics

The quality of the illustrations depends on the quality of the originals provided. Figures cannot be modified or enhanced by the journal production staff. The graphics must be submitted as part of the manuscript file and are used in the production of the Journal (material deposited as Supporting Information will not be published in the print edition). Contrast is important.

Remove all color from graphics, except for those graphics that you would like to have considered for publication in color (see Color section below for details). (1) Layout. In preparing structures for publication, layout is critical. Equations, schemes, and blocks of structures are presented in the Journal either in one-column or two-column format.

For efficient use of journal space, single-column illustrations are preferred.

Authors are advised that structural material labeled as a "Figure" is placed at the top or bottom of a page, as is all two-column material. All structural material that should immediately follow certain text must be designed to fit the one-column format, and its location in the text must be indicated on the manuscript. Structures, arrows, and compound designators should be arranged so as to make maximum use of the width afforded by the one-column or two-column format.
(2) Content. Abbreviations such as Me for CH3, Et for C2H5, and Ph (but not φ) for C6H5 are acceptable. Make liberal use of "R and X groups" in equations, schemes, and structure blocks to avoid the repetition of similar structures. Do not repeat a structure; the number alone of an earlier structure can be used if a compound occurs several times. Schemes are numbered with Arabic numerals. Within schemes, structures should be numbered with boldface Arabic numerals, consecutively from left to right, top to bottom, regardless of the order in which the compounds are discussed in the text. Schemes should be footnoted in the manner described below for Tables.
It is not necessary to give reagents and conditions in complete detail, since this detail is contained in the Experimental Section. Where needed, numbers such as NMR chemical shifts may be included directly on structural formulas.
(3) Dimensions. For best results, illustrations should be submitted in the actual size at which they should appear in the Journal. Original illustrations that do not need to be reduced to fit a single or double column will yield the best quality. Lettering should be no smaller than 4.5 points. (Helvetica or Arial type works well for lettering.) Lines should be no thinner than 0.5 point. Lettering and lines should be of uniform density. If artwork that should be reduced must be submitted, larger lettering and thicker lines should be used so that, when reduced, the artwork meets the above-mentioned parameters.
Complex textures and shading to achieve a three-dimensional effect should be avoided. To show a pattern, a simple cross-hatch design should be used.
Digital graphics should be submitted as TIFF images with the following minimum resolution requirements:
Black and white line art 1200 dpi
Grayscale art 600 dpi
Color art 300 dpi Chemical Structures
Structures should be produced with the use of a drawing program such as ChemDraw. Structure drawing preferences (preset in the ACS Stylesheet in ChemDraw) are as follows:

1. As drawing settings select:
chain angle 120º
bond spacing 18% of width
fixed length 14.4 pt (0.508 cm, 0.2 in.)
bold width 2.0 pt (0.071 cm, 0.0278 in.)
line width 0.6 pt (0.021 cm, 0.0084 in.)
margin width 1.6 pt (0.056 cm, 0.0222 in.)
hash spacing 2.5 pt (0.088 cm, 0.0347 in.)

2. As text settings select:
font Arial/Helvetica
size 10 pt
3. Under the preferences choose:
units points
tolerances 5 pixels
4. Under page setup choose:
Paper US Letter
Scale 100%
5. Using the ChemDraw ruler or appropriate margin settings, create structure blocks, schemes, and equations having maximum widths of 11.3 cm (one-column format) or 23.6 cm (two-column format). Note: if the foregoing preferences are selected as cm values, the ChemDraw ruler is calibrated in cm. ChemDraw graphics will be reduced to 75% during production.
6. Embolden compound numbers, but not atom labels or captions. 7. Authors are urged to use only a single configurational descriptor (heavy line or dashed line, but not both) when defining a stereocenter in a chemical structure. Atoms should be kept outside of rings wherever possible. Rather than rectangular solid and dashed lines, authors should use solid and dashed wedges to indicate configurations, as shown below. Dots at ring junctions intended to represent hydrogen atoms should not be used. Structures should be drawn in a neat manner ready for direct reproduction, and should not be cluttered or overlapping. Any arrows and numbering used for atoms in figures should not come into contact with bonds or ring systems. See an example of a prepared structure using ChemDraw with the specified preferences below. In molecules containing a chiral biphenyl axis, it is recommended that one of the aromatic rings be drawn in the plane of the paper and the second one be rotated out of the plane of the paper, to reflect the P or M conformation about the biphenyl bond.

Authors using other drawing packages should, in as far as possible, modify their program's parameters so that they reflect the above guidelines.

Tables
These should be numbered consecutively with Arabic numerals and should be placed as they should appear in the paper. Footnotes in tables should be given lowercase letter designations and be cited in the table by italic superscript letters. The sequence of letters should proceed by line rather than by column. If a footnote is cited both in the text and in a table, insert a lettered footnote in the table to refer to the numbered footnote in the text. Each table should be provided with a descriptive heading, which, together with the individual column headings, should make the table, as nearly as possible, self-explanatory. In setting up tabulations, authors are requested to keep in mind the type area of the journal page (17.8 × 25.4 cm) and the column width (8.5cm), and to make tables conform to the limitations of these dimensions. Arrangements that leave many columns partially filled or that contain much blank space should be avoided.

Figures
Figures should be constructed in keeping with the column width, line width, and font size specified above (see Structural Drawings). All illustrations should be numbered as "Figures", with Arabic numerals. Blocks of chemical structures should not be designated as "Figures". Each figure must be identified outside the frame of the figure.

(1) Photographs. Digital photographs are accepted. Photographs that are single or double column width so that they will not have to be reduced work best.
(2) Color. Color reproduction, if approved by the Editor, will be provided at no cost to the author. Color illustrations should be submitted only if essential for clarity of communication. The inclusion of a color photograph is particularly recommended for manuscripts based on the constituents of organisms that are not identified beyond the genus level.

Table of Contents/Abstract Graphic
A graphic must be included with each manuscript that will be used for both the abstract and the Table of Contents (TOC) of the Web edition of the Journal issue in which the Communication, Review, Full Article, or Note will appear. This graphic should capture the reader's attention and, in conjunction with the manuscript's title, should give the reader a quick visual impression of the type of chemistry described and/or the biological results obtained. Structures in the TOC graphic should be constructed as specified in the 'Chemical Structures' section above. The TOC graphic may be up to 4.7 in. (12.0 cm) wide and 1.8 in. (4.6 cm) tall. (See detailed instructions at the Paragon Plus Web site.) Text should be limited to labels for compounds, reaction arrows, and figures. The use of color to enhance the scientific value is highly encouraged. The TOC graphic should be inserted on a separate page at the end of the manuscript file. The title and author list will be added during production.

Recommendations for Crystal Structure Papers
Although the results of crystal structure determinations are frequently of interest to readers of the Journal, details of crystal structure experiments are generally not. Results appropriate for the Journal are not, however, sufficient to allow referees to assess the quality of an X-ray structure determination. Thus, it is recommended that manuscripts involving such determinations be accompanied by material provided for the benefit of the reviewers only. Authors should submit the following minimum materials, in tabular form where possible, for each compound for which X-ray crystallographic supplementary data are available.

Published Manuscript:
(1) Crystal data, including chemical formula, formula weight, crystal system and space group, cell dimensions (with uncertainties), number of formulas per unit cell, calculated density, radiation used, and wavelength.
(2) Final fractional atomic coordinates. Hydrogen atom coordinates should be included only if they have been experimentally determined or refined. Calculated coordinates should be provided as reviewer's material.
(3) A brief outline of procedures used for data collection and refinement, including the method used for intensity measurement, 0 limits, portion of the full sphere collected, handling of absorption (if applicable), method of refinement, number of reflections used in the refinement and criteria for their choice, treatment of hydrogen atoms, and final R factor.
(4) A perspective diagram (perhaps prepared by ORTEP, PLUTO, or similar programs) that gives the atom-numbering scheme if it is not unambiguous from the remainder of the paper. If the figure is a stereoview, it should be provided reduced to correct size, about 55–60 mm between images. Besides a description of the structure, other information (i.e., important distances, torsion angles, results of best plane calculations, etc.) may be included if appropriate. A note should be cited at an appropriate place in the manuscript and included in the References and Notes Section:
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