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DESENVOLVIMENTO E AVALIAÇÃO BIOLÓGICA DE SISTEMAS NANO- E MICROPARTICULADOS CONTENDO 3,3'-DIINDOLMETANO

Santa Maria, RS, Brasil, 2019

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração em Desenvolvimento e Avaliação de Produtos Farmacêuticos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do grau de **Doutor em Ciências Farmacêuticas**.

Orientadora: Prof.ª Dr.ª Letícia Cruz
Coorientador: Dr. Marcel Henrique Marcondes Sari

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Santa Maria, 26 março de 2019

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RESUMO

DESENVOLVIMENTO E AVALIAÇÃO BIOLÓGICA DE SISTEMAS NANO- E MICROPARTICULADOS CONTENDO 3,3'-DIINDOLMETANO

AUTORA: JULIANE MATTIAZZI
ORIENTADORA: LETÍCIA CRUZ
CO-ORIENTADOR: MARCEL HENRIQUE MARCONDES SARI

O 3,3´-diindolmetano (DIM) é um bioativo originado a partir de reações químicas que ocorrem em vegetais crucíferos, após a ingestão destes. Este bioativo tem demonstrado vários benefícios ao organismo, como ação antioxidante, anti-inflamatória e antineoplásica. Entretanto, o DIM é insolúvel em água, apresentando biodisponibilidade menor que 1% por via oral, além de degradar-se quando exposto à luz ou a altas temperaturas. Na tentativa de contornar algumas destas limitações e melhorar o potencial terapêutico do DIM, uma estratégia que pode ser adotada é a incorporação do composto em nanocápsulas (NCs) ou micropartículas (MPs) poliméricas, as quais proporcionam proteção ao ativo e melhora no perfil de dissolução de substâncias hidrofóbicas. Dessa forma, esta tese visou o desenvolvimento de NCs e de MPs contendo DIM e a avaliação da performance destes sistemas frente à fotoestabilidade, ação antioxidante e antitumoral *in vitro* com as NCs, bem como o efeito antinociceptivo do DIM livre ou associado aos sistemas carreadores em modelos animais de dor aguda. Os protocolos descritos foram aprovados pelo Comitê de Ética no Uso de Animais da Universidade Federal de Santa Maria, sob o nº 4428090217/2017. As suspensões de NCs de DIM foram preparadas pelo método de deposição interfacial de polímero pré-formado e apresentaram características físico-químicas adequadas, com tamanho de partícula na faixa de 100-300 nm, índice de polidispersão abaixo de 0,25, potencial zeta negativo ou positivo de acordo com a natureza do polímero empregado, pH levemente ácido, teor de ativo próximo ao teórico (1 mg/mL) e eficiência de encapsulamento próxima a 100%. Quanto ao estudo de fotodegradação frente à radiação UVC, a concentração final de ativo nas NCs foi 3x maior que a de ativo livre após 5h. O perfil de liberação do DIM a partir das NCs foi avaliado através da técnica de difusão em saco de diálise, demonstrando que as NCs prolongaram a liberação do bioativo, apresentando cinética de primeira ordem e mecanismo por transporte anômalo. A avaliação da atividade sequestrante frente aos radicais DPPH e ABTS foi realizada após diluição (2-4-6 µg/mL) das amostras (NCs e ativo livre), seguida da mistura destas com cada radical. O DIM apresentou neutralização dos radicais tanto na forma livre quanto nanoencapsulada, sendo esta mais acentuada para as NCs. O estudo do efeito citotóxico do DIM livre ou nanoencapsulado frente a células de glioma U87 foi realizado nas concentrações de 3-6-12-24 µg/mL e demonstrou que, de maneira geral, a nanoencapsulação aumentou o efeito citotóxico do DIM (**Artigo 1**). Além disso, foi demonstrada a viabilidade de preparação de MPs contendo DIM, as quais apresentaram características físico-químicas adequadas, como diâmetro médio na faixa de 200-400 µm, estreita distribuição granulométrica, teor de DIM de aproximadamente 150 mg/g e eficiência de encapsulamento em torno de 84%, bem como liberação controlada do ativo. Avaliações da forma de associação do DIM às nano- e micropartículas demonstraram a viabilidade dos sistemas em incorporar o DIM, sem apresentar interações com outros constituintes dos sistemas. Ainda, foi realizada a avaliação do efeito antinociceptivo do DIM em diferentes modelos animais (Teste da chapa quente, Modelo de nocicepção induzida pela formalina, Modelo de dor inflamatória aguda induzida pelo Adjuvante Completo de Freund). Para tal, foram utilizados camundongos Swiss machos (25-35 g), os quais foram tratados por via intragástrica (doses de 2,5, 5 ou 10 mg/Kg) nos tempos de 0,5-8h de tratamento. Os resultados demonstraram a ação antinociceptiva/anti-hipernociceptiva do DIM, tanto livre quanto associado aos sistemas, apresentando efeito farmacológico prolongado e mais acentuado devido à nano e a microencapsulação (**Manuscritos 1 e 2**). Portanto, o presente trabalho demonstrou a viabilidade de preparação de NCs e MPs contendo DIM, bem como a capacidade dos sistemas em promover liberação prolongada e fotoproteção, além de melhorar as atividades antinociceptiva e antioxidante deste bioativo, sendo assim uma abordagem inovadora para a liberação oral do DIM no tratamento da dor.

Palavras-chaves: Indol-3-carbinol; nanocápsulas poliméricas; microesferas; fotoestabilidade; atividade antioxidante; nocicepção.

ABSTRACT

DEVELOPMENT AND BIOLOGIC EVALUATION OF NANO- AND MICROPARTICULATE SYSTEMS CONTAINING 3,3'-DIINDOLYLMETHANE

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3,3'-Diindolylmethane (DIM) is a phytochemical compound originated after the ingestion of some vegetables. Despite the several health benefits showed by this bioactive (like antioxidant, anti-inflammatory and antineoplasic effects), it is water-insoluble, presenting oral bioavailability of 1% and it is thermo and photolabile, which restrains its pharmaceutical applications. In attempt to overcome these issues and improve DIM therapeutic potential, an interest approach is the incorporation of the compound in polymeric microparticles (MPs) or nanocapsules (NCs), which provide protection of the drug and improved dissolution profile for hydrophobic substances. Therefore, this thesis aimed to the development of DIM-loaded MPs. The *in vitro* photostability, antioxidant and antitumoral (glioma cells) actions of NCs were assessed as well as the evaluation of DIM antinociceptive effect, in its free or nano/microencapsulated forms, using animal models of acute pain. The Ethical Research Committee of Federal University of Santa Maria approved all experimental procedures carried out in the present study, which are register under the nº 4428090217/2017. The NCs suspensions were prepared by the interfacial deposition of pre-formed polymers and demonstrated suitable physicochemical characteristics: particles in nanometric diameter (100-300 nm), polydispersity indexes below 0.25, positive or negative zeta potentials depending on the polymer feature, acid pH, drug content close to the theoretical (1 mg/mL) and encapsulation efficiency about 100%. In the photostability assay, after 5h under UVC exposure, NCs presented DIM content 3-fold higher than free DIM (methanolic solution). The DIM release from the NCs was evaluated by the dialysis bag technique, demonstrating that the NCs prolonged the DIM release, which fits first order kinetic and anomalous transport as release mechanism. For DIM scavenging capacity test, the DPPH or ABTS reagents were added to NC samples containing 2-4-6 µg/mL of DIM. DIM presented scavenging activity in both free and nanoencapsulated forms, which was more pronounced for the NCs. The cytotoxic effect of free DIM or DIM-loaded NCs against U87 human glioblastoma cells was performed at 3-6-12-24 µg/mL and demonstrated that nanoencapsulation generally enhanced the antitumor effect of DIM (**Article 1**). Besides, it was demonstrated the feasibility of preparation of DIM-loaded MPs, which have shown suitable physicochemical characteristics, as micrometric diameter (200-400 µm), narrow size distribution, DIM content of 150 mg/g and encapsulation efficiency of 84%, as well as controlled release of the active. Other physicochemical evaluations demonstrated that NCs and MPs were able to adequately encapsulate DIM without interactions of the active with the other materials of the formulations. In addition, the antinociceptive effect was evaluated using distinct animal models of acute pain (Thermic nociception model - Hot plate; Chemical nociception model induced by formalin; Model of acute inflammatory pain induced by Complete Freund's Adjuvant). Male Swiss mice (25-35 g) were intragastrically treated (DIM doses of 2,5, 5 ou 10 mg/Kg) and the behavioral tests were performed after 0,5-8h. The results demonstrated DIM antinociceptive/anti-hypernociceptive action, in both free and nano/microencapsulated forms, presenting prolonged and enhanced effect in the NCs or MPs in comparation with the free form (**Manuscripts 1 and 2**). Therefore, the present work showed the feasibility of preparation of DIM-loaded NCs and MPs, in addition to the ability of these systems of promoting DIM prolonged release and photoprotection, as well as improve the antinociceptive and antioxidant activity this bioactive, consisting in an innovative approach to DIM oral delivery for pain management .

Key-words: Indole-3-carbinol; polymeric nanocapsules; microspheres; photostability, antioxidant capacity; nociception.

“A mente que se abre a uma nova ideia jamais volta ao seu tamanho original.”

Albert Einstein (1879 - 1955).

LISTA DE ABREVIATURAS E SIGLAS

ABTS	do inglês *2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)*
CFA	adjuvante completo de Freund (do inglês *Complete Freund's Adjuvant*)
CLN	carreadores lipídicos nanoestruturados
COX-2	ciclooxigenase-2
DIM	3,3'-diindolmetano
DMBA	7,12-dimetilbenzantraceno
DNA	ácido desoxirribonucleico
DPPH	do inglês *1-1-diphenyl-2-picrylhydrazyl*
EC	etilcelulose
EE%	eficiência de encapsulamento
EGFR	receptor do fator de crescimento epidérmico (do inglês *Epidermal Growth Factor Receptor*)
ERL	Eudragit RL
ERS	Eudragit RS
FBS	soro fetal bovino
GABA	ácido gama-aminobutírico
HPLC/CLAE	cromatografia líquida de alta eficiência
HPMC	Hidroxipropilmetilcelulose
I3C	indol-3-carbinol
IFNγ	interferon-gama
IL-1β	interleucina-1β
IL-6	interleucina-6
IL-8	interleucina-8
iNOS	enzima óxido nítrico sintase induzível
MMP-9	do inglês *Matrix metalloproteinase 9*
MPs	micropartículas poliméricas
MTT	do inglês *3(4, 5-dimethyl)-2,5diphenyl tetrazolium bromide*
NC-PEC-D	nanocápsula de etilcelulose e óleo de prímula contendo DIM
PDI/IPD	índice de polidispersão
PGE_2	prostaglandina E_2

PLA_2	Fosfolipase A_2
PLGA	poli(ácido lático-co-glicólico)
QS	Quitosana
TGI	trato gastrointestinal
TNF-α	fator de necrose tumoral α
UVA	ultravioleta A
UVB	ultravioleta B
UVC	ultravioleta C
VF	(filamentos de) Von Frey

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Introdução

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1. INTRODUÇÃO

1 INTRODUÇÃO

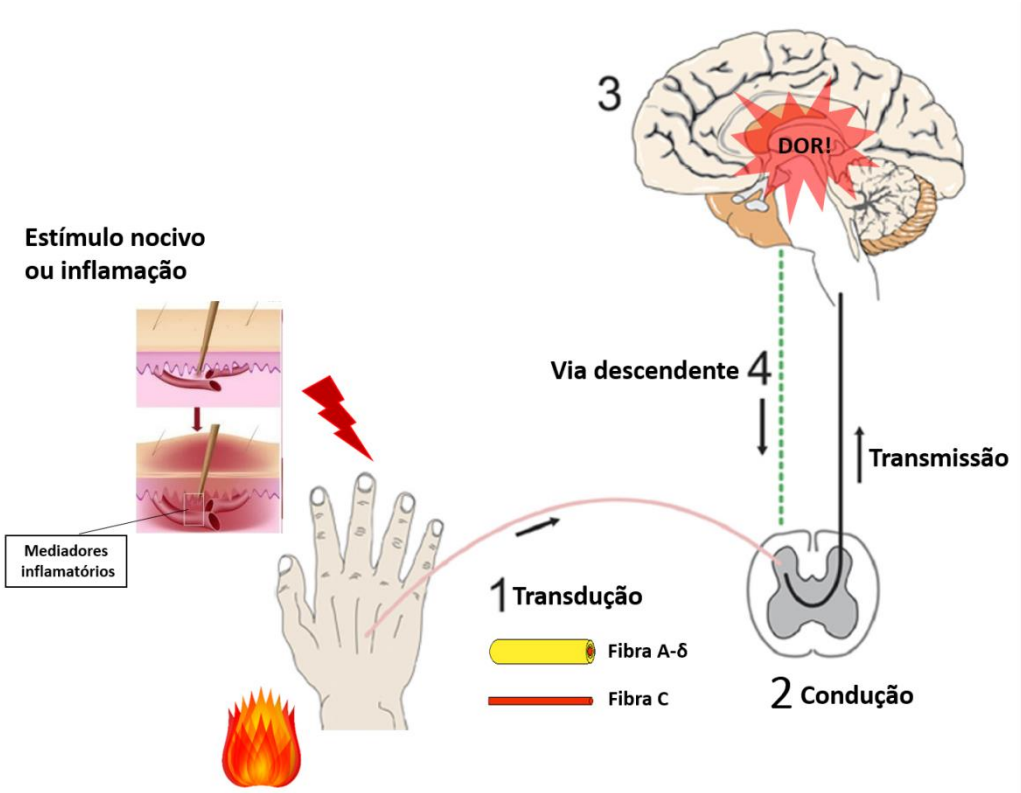
1.1 DOR/NOCICEPÇÃO

Segundo a Associação Internacional para o Estudo da Dor, a dor consiste em uma experiência sensorial e emocional desagradável associada ao dano tecidual real ou potencial, ou descrita em termos deste dano (KUMAR, ELAVARASI, 2016). Em outras palavras, a dor é considerada uma experiência complexa, que envolve múltiplos fatores periféricos e centrais, a cognição e a emoção (BUSHNELL, ČEKO, LOW, 2013). Ainda, constitui um fenômeno essencial à sobrevivência humana, pois transmite informações sobre o estímulo nocivo, como localização e intensidade, atuando assim como alerta para preservar o organismo da lesão tecidual e para manter a homeostasia dos tecidos (SCHOLZ, WOOLF, 2002; LOESER, TREEDE, 2008). Entretanto, quando persistente, a dor pode inviabilizar a execução de tarefas diárias dos indivíduos, podendo acarretar em perda na qualidade de vida, além do desenvolvimento de comorbidades, como depressão e ansiedade, e aumento da susceptibilidade a outros quadros patológicos (JULIUS, BASBAUM, 2001).

O sistema nervoso possui fibras nervosas periféricas, como os nociceptores A-δ e C, os quais detectam os estímulos nocivos externos e os transduzem em impulsos elétricos, sendo estes então conduzidos até regiões específicas do sistema nervoso central (SNC) envolvidas na resposta à dor. Este processo é denominado de nocicepção (**Figura 1**) (SCHOLZ, WOOLF, 2002; OLESEN et al., 2012). Com isso, os estímulos oriundos da periferia são detectados e levados através de neurônios de primeira ordem para a medula espinhal, fazendo conexões com neurônios de segunda ordem, que transmitem a informação sensorial para estruturas supraespinhais (KIDD, URBAN, 2001; SCHOLZ, WOOLF, 2002; BASBAUM et al., 2009). Esta transmissão de informações entre neurônios de primeira e segunda ordem ocorre por mediadores químicos, tais como aminoácidos excitatórios (glutamato) ou inibitórios (GABA e glicina) e neuropeptídeos (substância P) (BASBAUM et al., 2009; OLESEN et al., 2012). Na ausência de um estímulo potencialmente prejudicial ou enquanto não ocorre dano tecidual, inflamação ou injúria ao sistema nervoso, os nociceptores, em condições fisiológicas, ficam silenciosos. Entretanto, quando ocorre estímulo mecânico, térmico (frio ou calor) ou

químico, ou ainda, processo inflamatório ou tumoral, a função dos nociceptores é modificada, havendo sensibilização destes.

Figura 1 – Mecanismos de detecção, condução e processamento do estímulo nocivo envolvidos na sinalização da nocicepção. 1-Transdução: nociceptores periféricos (fibras A-δ e C) detectam estímulos nocivos ou prejudiciais. 2-Condução: a informação codificada é conduzida através de nervos aferentes primários para a medula espinhal. 3-Transmissão: a informação é levada a centros supraespinhais, incluindo o tálamo, o tronco encefálico e o córtex somatosensorial. 4-Via descendente: as vias descendentes inibitórias modulam a atividade da medula espinhal liberando neurotransmissores como serotonina e noradrenalina, controlando a transmissão da dor.



Adaptado de: OLESEN et al., 2012.

Sabe-se que processo inflamatório em si também participa de maneira importante na defesa do organismo frente a lesões e estímulos nocivos, levando à ocorrência de uma série de sinais e sintomas, os quais, classicamente, incluem a dor. Em adição, a dor e a inflamação apresentam mediadores e efetores em comum (por exemplo, a interleucina 1-β), apresentando assim uma inter-relação intrínseca (LIBBY, 2007; REN, TORRES, 2009).

Apesar de a dor inflamatória ser considerada um tipo de dor nociceptiva (dor cujos nociceptores periféricos são ativados por uma lesão), em alguns casos, como quando se torna crônica, a dor inflamatória pode modificar funcionalmente o processo da nocicepção (LOESER, TREEDE, 2008). De maneira geral, a inflamação é acompanhada por um estado de hipersensibilidade à dor, a qual pode ser uma resposta aumentada em decorrência de um estímulo nocivo (hiperalgesia) ou um estímulo normalmente inócuo que acaba gerando dor (alodínia) (WOOLF et al., 1997; KIDD, URBAN, 2001).

Este fenômeno de hipersensibilização dos nociceptores pode ser atribuído à ação de mediadores inflamatórios produzidos e liberados a partir das fibras nervosas e de outras células (mastócitos, plaquetas), os quais são chamados de “sopa inflamatória” (BASBAUM et al., 2009; SCHOLZ, WOOLF, 2002). Esses mediadores podem sensibilizar (ou seja, diminuir o limiar) ou excitar os neurônios nociceptivos de diversas maneiras: i) ao interagirem com receptores ou moléculas alvo, como canais iônicos no caso de prótons extracelulares, da serotonina e da prostaglandina E_2 (PGE2); ii) ao ligarem-se em receptores expressos na superfície neuronal, como o que ocorre com a bradicinina; ou, ainda, iii) estimulando indiretamente a liberação de outros agentes, como o que se observa para as interleucinas na inflamação crônica (WOOLF et al., 1997; KIDD, URBAN, 2001; BINSHTOK et al., 2008; JULIUS, BASBAUM, 2009; GUDES et al., 2015).

Além da ativação do nociceptor transmitir mensagens aferentes para a medula espinhal, e então, para o cérebro, também realiza uma função eferente, liberando peptídeos e neurotransmissores (por exemplo, substância P) a partir de seus terminais periféricos. Esse processo induz vasodilatação e extravasamento de plasma, bem como ativação de muitas células não-neuronais, as quais contribuem na produção da sopa inflamatória, sendo este fenômeno conhecido como inflamação neurogênica (JULIUS, BASBAUM, 2001; BASBAUM, 2009).

Apesar de, fisiologicamente, a dor ser um fenômeno de proteção essencial ao organismo, muitas vezes ela se torna prejudicial, desgastante e até mesmo incapacitante, podendo persistir por muito tempo após a causa inicial ter sido identificada e restaurada. Além disso, fatores psicológicos desempenham um papel fundamental tanto no início como no progresso das patologias relacionadas à dor, comprometendo a qualidade de vida dos pacientes (BUSHNELL, ČEKO, LOW,

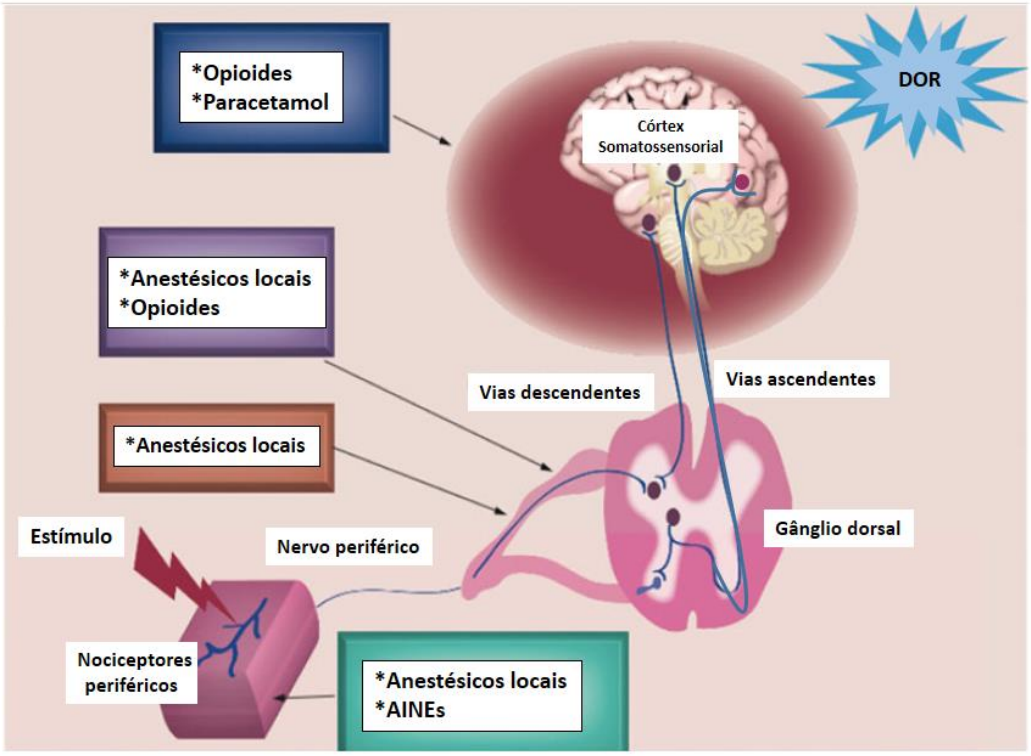
2013; KUMAR, ELAVARASI, 2016). Com as recentes mudanças no estilo de vida e com o envelhecimento da população, a prevenção e o manejo da dor tornam-se aspectos cada vez mais importantes na área médica e de cuidados com a saúde mundialmente (OLESEN et al., 2012; KUMAR, ELAVARASI, 2016). Dessa forma, a busca por ferramentas para o controle e alívio da dor torna-se extremamente necessária (OLESEN et al., 2012).

Embora muitos estudos evidenciem a alta prevalência da dor na população em geral, as pesquisas no âmbito da terapia da dor e a farmacoterapia disponível na atualidade ainda falham em contemplar vários aspectos do diagnóstico e do tratamento da dor (KUMAR, ELAVARASI, 2016; ANDREU, ARRUEBO, 2018). Uma das causas desta dificuldade é o fato de a dor ser uma experiência subjetiva e multidimensional, relacionada com a percepção sensorial, cognitiva, afetiva e até cultural de cada indivíduo, gerando uma grande variabilidade interindividual na resposta clínica (SPRINTZ et al., 2011; BUSHNELL, ČEKO, LOW, 2013; KUMAR, ELAVARASI, 2016; ANDREU, ARRUEBO, 2018). Outro desafio diz respeito à complexidade patofisiológica da dor e ao envolvimento de diferentes vias de sinalização no processo nociceptivo, o que requer, além das terapias não medicamentosas, a utilização de um arsenal de fármacos na tentativa de atenuá-la (CARTER et al., 2014; MORADKHANI, KARIMI, NEGAHDARI, 2017; ANDREU, ARRUEBO, 2018) (**Figura 2**).

Alguns dos fármacos disponíveis na atualidade para o tratamento farmacológico da dor incluem os opioides, os anestésicos e os anti-inflamatórios não-esteroides (AINEs) (CARTER et al., 2014), os quais nem sempre são efetivos no alívio da dor e, por serem utilizados em tratamentos de longa duração, tem a probabilidade aumentada de desencadear efeitos adversos (CARTER et al., 2014; ANDREU, ARRUEBO, 2018). No caso dos AINEs, por exemplo, o uso prolongado pode ocasionar reações tais como desenvolvimento de úlceras no trato gastrointestinal (TGI), complicações cardiovasculares, nefrotoxicidade e até mesmo desorientação, em pacientes idosos (CARTER et al., 2014; WONGRAKPANICH et al., 2018). Com relação aos fármacos opioides, os pacientes seguidamente relatam constipação, a qual é um efeito adverso extremamente desconfortável. Além disso, as evidências clínicas apontam para o risco da terapia a longo prazo devido à atual epidemia de abuso de opioides, a qual é relacionada à tolerância analgésica e

dependência de seu uso, levando até à morte de pacientes (MANCHIKANTI et al., 2010; CARTER et al., 2014).

Figura 2 – Classes de analgésicos comumente utilizados no tratamento da dor.



Adaptado de: MORADKHANI, KARIMI, NEGAHDARI, 2017.

Todos estes efeitos indesejados frequentemente resultam na descontinuidade do tratamento e assim, na ineficácia da terapia. Portanto, é cada vez mais importante a busca por novas abordagens farmacológicas, se possível que atuem em múltiplos alvos e sejam mais eficazes, ao mesmo tempo que gerem menos reações adversas (CHAKRAVARTY et al., 2017). Alguns dos modelos animais que podem ser utilizados na avaliação da atividade antinociceptiva de novos compostos analgésicos serão abordados a seguir.

1.1.1 Modelos animais de avaliação da atividade antinociceptiva de compostos

Com relação aos modelos comportamentais utilizados na triagem de compostos/substâncias/formulações com potencial antinociceptivo, é necessário

utilizar protocolos experimentais que permitam a diferenciação de uma possível ação antinociceptiva envolvendo modulação direta de determinados alvos (como agonistas/antagonistas de receptores) daqueles envolvendo mecanismos periféricos, os quais levam à produção de mediadores mais tardiamente, em especial os mediadores inflamatórios. Além disso, considerando a complexidade do fenômeno da dor, a qual até hoje ainda não se encontra totalmente compreendida, faz-se necessário o uso de diferentes modelos comportamentais para melhor estabelecer o possível efeito terapêutico ou profilático de uma substância neste âmbito. A seguir estão descritas algumas considerações sobre os modelos animais de avaliação de dor utilizados no presente estudo.

1.1.1.1 Modelo de nocicepção química induzida pela formalina

O teste da formalina, descrito pela primeira vez por Dubuisson e Dennis em 1977, consiste na injeção de uma solução de formalina sob a superfície ventral da pata traseira do roedor, a qual gera respostas agudas e tônicas à lesão tecidual desencadeada. Esta resposta é verificada através do comportamento do animal de lamber e/ou morder o local onde o estímulo nocivo ocorreu, sendo contabilizado o tempo cumulativo de lambida da pata como indicativo de nocicepção (DUBUISSON, DENNIS, 1977; LE BARS, GOZARIU, CADDENS, 2001; WU et al., 2016).

Este teste é classificado como um modelo químico de dor persistente, sendo utilizado para avaliar duas fases distintas da dor: a neurogênica e a inflamatória, sendo o período transcorrido entre estas duas fases denominado de intervalo de quiescência. A primeira fase tem sua resposta iniciada imediatamente após a injeção da formalina e dura em torno de 5 min. Esta fase envolve estimulação química dos nociceptores das fibras-C pela formalina, levando à liberação de aminoácidos excitatórios, óxido nítrico e substância P, o que indica que esse período envolve mecanismos centrais. A segunda fase inicia-se em torno de 15 min após a injeção de formalina e leva à sensibilização dos nociceptores por uma série de mediadores inflamatórios liberados localmente (histamina, serotonina, prostaglandinas, interleucinas, fator de necrose tumoral-α), ocasionando edema local e hipernocicepção (SCHOLZ, WOOLF, 2002; BANNON, MALMBERG, 2007; SARI et al., 2014).

Enquanto fármacos opioides apresentam ação antinociceptiva em ambas as fases do teste de nocicepção induzida pela formalina, anti-inflamatórios não-esteroides (como a indometacina e o cetoprofeno) são ineficazes na fase neurogênica. Esse fato demonstra a importância do uso deste modelo na investigação e elucidação das propriedades analgésicas de novos compostos, diferenciando o potencial anti-inflamatório do potencial analgésico, como no caso dos AINEs clássicos. Além disso, permite também distinguir se a ação farmacológica estaria relacionada com a modulação de mecanismos centrais (fase neurogênica), periféricos (fase inflamatória) ou ambos.

De fato, existem inúmeros trabalhos recentes na literatura relatando o teste da formalina como uma ferramenta na avaliação da atividade antinociceptiva, incluindo substâncias de origem vegetal (NASCIMENTO et al., 2016; WU et al., 2016) e/ou ativos incorporados a sistemas nanoestruturados (HATANAKA et al., 2011; MARIANECCI et al., 2014; VILLALBA et al., 2014; GOH et al., 2015, AZIZI et al., 2017, GEHRCKE et al., 2018) e micropartículas (PIGNATELLO et al., 2001; BORODINA et al., 2016).

1.1.1.2 Modelo de nocicepção térmica pelo teste da placa quente (Hot plate)

O modelo da chapa quente utiliza a temperatura como estímulo nociceptivo agudo. Foi descrito em 1944 por Woolfe e MacDonald, mas permanece sendo um dos métodos experimentais mais utilizados na detecção de sustâncias analgésicas de ação no SNC até a atualidade. Os animais (roedores) são tratados com a substância teste ou com o controle e colocados, um a um, em contato com uma chapa metálica aquecida (podendo variar a temperatura conforme o protocolo experimental) até reagirem ao estímulo térmico com o comportamento de levantar/lamber uma das patas ou pular. Assim, mede-se a latência de resposta ao estímulo cronometrando-se o tempo que o animal leva até demonstrar a primeira reação, sendo o aumento significativo na latência após o tratamento com o composto/formulação, interpretado como uma resposta antinociceptiva (BANNON, MALMBERG, 2007; GUNN et al., 2011).

Segundo Le Bars e col. (2001), por estimular receptores cutâneos, o aumento gradual de temperatura da pele até a faixa considerada nociva provoca a ativação

sucessiva de terminais axônicos periféricos específicos, incluindo fibras termossensíveis e em seguida as fibras-C não mielinizadas e as A-δ pouco mielinizadas. Portanto, o teste da chapa quente é específico para avaliar compostos que tenham ação antinociceptiva mediada principalmente por mecanismos do SNC, envolvendo predominantemente estruturas supraespinhais. O estímulo nocivo ativa os receptores vaniloides presentes nas fibras-C, em especial os do tipo TRPV2 (VRL-1), que possuem limiar de ativação em torno de 52 ºC. Esta ativação é conduzida à medula espinhal e depois ao córtex, onde o estímulo é devidamente processado e a sensação de dor é atribuída (LE BARS, GOZARIU, CADDENS, 2001; SCHOLZ, WOOLF, 2002).

Dessa forma, apenas os analgésicos que modulam algum mecanismo do SNC aumentam o tempo de resposta no teste da chapa quente. Além disso, tem sido demonstrada uma adequada correspondência entre os compostos que apresentam ação antinociceptiva neste modelo e os clinicamente utilizados na terapia da dor. No caso de compostos nano- e microencapsulados, assim como para o modelo experimental da formalina, a literatura recente apresenta diversos estudos utilizando o teste da chapa quente (TANG et al., 2012; TARTAU, CAZACU, MELNIG, 2012; SHEN et al., 2013; LALANI et al., 2014; VILLALBA et al., 2014; ARORA et al., 2015; GOH et al., 2015; FORNAGUERA et al., 2015; PATIL, SURANA, 2016; SARI et al., 2017).

1.1.1.3 Modelo de dor inflamatória aguda induzida pelo Adjuvante Completo de Freund

A inflamação plantar induzida por Adjuvante Completo de Freund (CFA) tem sido amplamente utilizada como modelo experimental para determinações quali e quantitativas da atividade anti-inflamatória de compostos em roedores, pois reproduz condições clínicas e histológicas semelhantes à osteoartrite humana (NAGAKURA et al., 2003; HEGEN et al., 2008). A injeção intraplantar do CFA desencadeia uma lesão à cartilagem local, provocada pela liberação de vários mediadores inflamatórios e nociceptivos (como citocinas e prostaglandinas), estimulando um processo inflamatório agudo e assim o surgimento de edema, vasodilatação e hiperalgesia (WOOLF et al., 1997; HEGEN et al., 2008). Essa inflamação local causa

dor patológica inflamatória aguda, a qual provoca aumento da sensibilidade a estímulos mecânicos.

A avaliação desse aumento da sensibilidade dos nociceptores a estímulos mecânicos inócuos (alodínia), frequentemente é realizada através do teste de filamentos de Von Frey (VF). Este método consiste em aplicar na região plantar da pata do animal uma fibra de um certo diâmetro, exercendo-se uma pressão linearmente crescente, até a observação de uma resposta como a elevação ou retirada da pata estimulada. Ao empregar-se fibras com diferentes diâmetros e assim aplicando-se diferentes pressões no centro da pata do animal, é possível determinar o limiar nociceptivo que evoca uma resposta, como um reflexo de flexão, por exemplo (LE BARS, GOZARIU, CADDENS, 2001).

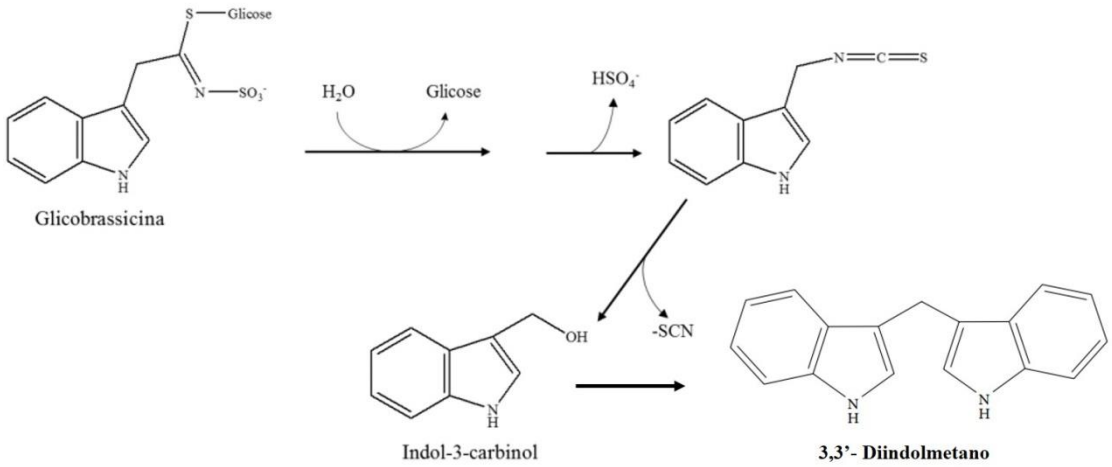
Vários estudos em periódicos de alto impacto científico reportam a utilização de protocolos semelhantes ao utilizado no presente trabalho, utilizando o modelo do CFA e/ou o teste de VF (LIU et al., 2005; TANG et al, 2012; OLIVEIRA et al., 2014; ARORA et al., 2015; BHALEKAR et al., 2015; HAN et al., 2015; ARRIGO et al., 2016; CRISCIONE et al., 2016; NASCIMENTO et al., 2016; WU et al., 2016; DEWANGAN et al., 2017; DAI et al., 2018), incluindo trabalhos de nosso grupo de pesquisa envolvendo a avaliação da ação antinociceptiva de ativos nanoencapsulados (FERREIRA et al., 2016; SARI et al., 2018b).

1.2 3,3’-DIINDOLMETANO

Vegetais crucíferos (gênero *Brassica*) como couve-flor, brócolis, repolho, nabo, entre outros, contêm precursores fitoquímicos chamados de glicosinolatos (dentre os quais o principal é conhecido como glicobrassicina). A mirosinase, uma enzima que catalisa a hidrólise dos glicosinolatos, encontra-se fisicamente separada destes nas células vegetais intactas. Quando estes legumes são esmagados, mastigados ou cortados, a parede celular é quebrada, ocorrendo a interação entre a mirosinase e a glicobrassicina (VIRTANEN, 1965). Com isso, ocorre hidrólise enzimática da glicobrassicina que forma como produto um isotiocianato instável, o qual se degrada a um composto bioativo conhecido como indol-3-carbinol (I3C) (BANERJEE et al., 2011; LI et al., 2013). Este, sob condições ácidas, sofre oligomerização não-enzimática e forma uma mistura de compostos (SHERTZER,

SENFT, 2000), sendo que o maior produto de condensação do I3C *in vivo* é o seu dímero, conhecido como 3,3'-diindolmetano (DIM) (BANERJEE et al., 2011). A **Figura 3** apresenta a sequência de reações químicas que dão origem ao DIM.

Figura 3 - Sequência das reações de síntese do 3,3'-diindolmetano.



Fonte: O próprio autor.

Em um estudo realizado por Reed e colaboradores (2006), o DIM foi o único composto derivado do I3C detectado no plasma de mulheres após ingestão do I3C, sugerindo que o DIM é o ativo responsável pelo efeito biológico proveniente dos glicosinolatos. Portanto, acredita-se que a produção do DIM a partir do I3C seja um pré-requisito para que este exerça seus efeitos benéficos ao organismo (LI et al., 2013). Ainda, o DIM não sofre transformações metabólicas no organismo humano, sendo um composto mais estável. Estas distinções entre o DIM e seu precursor fitoquímico tornam a sua escolha preferível visando a composição de formulações farmacêuticas, em detrimento do I3C.

No que tange suas propriedades biológicas, o DIM é um fitoquímico que apresenta diversos efeitos promissores para aplicação terapêutica, como por exemplo, ação antioxidante, anti-inflamatória e antitumoral, propriedades estas que podem propiciar benefícios no tratamento de inúmeras patologias (ZELIGS, 2004; ZELIGS, 2008; BANERJEE et al., 2011; ZELIGS, 2011; MARUTHANILA,

POORNIMA, MIRUNALINI, 2014; WU et al., 2014; KISELEV, 2016). Existem muitos mecanismos pelos quais o DIM atua no organismo, os quais são bastante complexos e vêm sendo elucidados nos últimos anos. Com relação ao seu efeito antitumoral, tem sido relatado na literatura que o DIM pode reduzir o estresse oxidativo por estimular a expressão de genes que conduzem à resposta antioxidante, protegendo a célula dos danos ao DNA, além de regular o metabolismo do estrogênio (BONNESE, EGGLESTON, HAYES, 2001; CHEN et al., 2001; BENABADJI et al., 2004; DALESSANDRI et al., 2004; FAN et al., 2013). Ainda, o DIM ativa o citocromo P-450, inibe a invasão das células cancerígenas e a angiogênese e causa interrupção no ciclo e divisão celular e estimula apoptose celular (MANSON et al., 1998; LEIBELT et al., 2003; DEL PRIORE et al, 2010; ROY et al, 2013). Outro mecanismo pelo qual o DIM possivelmente exerce sua ação antitumoral envolve a redução das vias de sinalização do fator de crescimento EGFR (Epidermal Growth Factor Receptor) relacionado à ocorrência de metástases e à radiorresistência e quimiorresistência (RAHIMI; HUANG; TANG, 2010).

Estes achados demonstram o potencial do DIM na terapia do câncer em combinação com antineoplásicos convencionais ou isoladamente. Atualmente inúmeros estudos tem sido conduzidos no sentido de testar os efeitos do tratamento com DIM em culturas de células, em animais ou em pacientes com diferentes tipos de cânceres, como próstata (GARIKAPATY et al., 2005; GOLDBERG et al., 2014; LI, SARKAR, 2016), mama (STAUB, ONISKO, BJELDANES, 2006; MARQUES et al., 2014), estômago (YE et al., 2015), nasofaringe (CHEN et al., 2013; WU et al., 2014; LI et al., 2015), pulmão (ICHITE et al., 2010), fígado (LI et al., 2015b; PONDUGULA et al., 2015), entre outros (KISELEVEV, 2016). Um dos tipos de câncer que mais vem sendo estudado atualmente e merece destaque é o glioma, tumor maligno que se desenvolve a partir das células da glia ou neuroglia e corresponde à maior parte dos casos de neoplasias cerebrais malignas em adultos (GOODENBERGER, JENKINS, 2012). Estudos demonstram que 50% dos gliomas apresentam amplificação do gene EGFR, o qual é um dos alvos de ação do DIM, suscitando interesse no estudo deste bioativo no tratamento do glioma. De fato, pelo menos dois autores já avaliaram a influência do tratamento com DIM na viabilidade de células de glioma humano *in vitro* (RAHIMI, HUANG, TANG, 2010; SHERER et al., 2017).

Com relação à sua ação anti-inflamatória, o DIM demonstrou inibir a ativação do fator nuclear kappa-B (NF-κB), o qual está bastante envolvido com os processos inflamatórios, pois regula a expressão de diversos genes associados com a inflamação. Também promove redução nos níveis de quimiocinas, como a interleucina-8 (IL-8) e metaloproteinases de matriz, como a MMP-9, bem como aumento na secreção de interferon-gama (IFNγ) (BANERJEE et al., 2011; CHEN et al., 2013; MARUTHANILA, POORNIMA, MIRUNALINI, 2014). Além disso, em um modelo de neuroinflamação *in vitro* com células microgliais BV-2, o DIM inibiu a atividade das enzimas ciclooxigenase 2 (COX-2) e óxido nítrico sintase induzível (iNOS), bem como reduziu a intensidade do processo inflamatório no hipocampo de ratos (KIM et al., 2014). Estudos também demonstram que o DIM estimula o sistema imune de roedores e atenua a inflamação em modelos animais de edema de orelha e de colite (XUE et al., 2008; KIM et al., 2009; KIM et al., 2010).

Ainda, duas patentes contemplam a proposição do uso do DIM ou outros derivados indólicos no tratamento de desordens inflamatórias e envolvendo dor aguda ou crônica: a endometriose, a mastalgia e a fibromialgia (ARFFMANN, ANDRUS, 1999; ZELIGS, 2004). Arffmann e Andrus (1999) relataram que a administração oral diária (5-1000 mg) do derivado 1H-indol-3-metanol em pacientes atenuou consideravelmente os sintomas mais graves da fibromialgia logo após o primeiro dia ou em uma semana de tratamento, diminuindo a dor e a fadiga, bem como melhorando a qualidade do sono e o bem-estar dos indivíduos. Zeligs (2004), por sua vez, demonstrou que a administração oral diária de DIM (150-500 mg) preveniu ou reduziu os sintomas associados à mastalgia e endometriose em diversas pacientes.

Apesar de vários estudos epidemiológicos relatarem a relação entre o consumo de vegetais crucíferos e a prevenção do câncer e outras doenças (VERHOEVEN et al., 1996; HIGDON et al., 2007; VASANTHI, MUKHERJEE, DAS, 2009), faz-se necessária uma ingesta diária muito grande destes vegetais para atingir-se doses adequadas de DIM (em torno de 1,6 mg/Kg). Esse fato pode ser devido ao DIM ser um composto lipofílico, apresentando pouca solubilidade nos fluidos biológicos, o que acarreta em uma baixa biodisponibilidade (1-3%) após administração oral (ROY et al., 2013; WU et al., 2015). O DIM também possui limitada capacidade de permeação de membranas biológicas e alto grau de ligação

às proteínas plasmáticas, estando também envolvido em várias interações não-específicas no sangue (PALTSEV et al., 2013).

Além disso, estudos farmacocinéticos realizados em roedores quantificaram o DIM em diferentes tecidos, tais como coração, cérebro, rins, pulmões e, em maior concentração, no fígado. Também foi sugerido que a molécula de DIM esteja sujeita a transportadores de efluxo (como a Glicoproteína-P) na barreira sangue-cérebro e que a eliminação sistêmica do DIM ocorra apenas por via renal, na forma não modificada (ANDERTON et al., 2004, PALTSEV et al., 2013). Dessa forma, o DIM é utilizado principalmente como suplemento alimentar, estando disponível em farmácias de manipulação, na forma de cápsulas de uso oral na concentração de 100 mg, sendo indicado o uso de 1-4 cápsulas/dia.

Em adição, alguns estudos avaliaram a degradação de derivados de glicosinolatos quando expostos à radiação UVA e a diferentes temperaturas (entre 37-100º C), sugerindo que a molécula de DIM apresenta relativa instabilidade nas condições testadas (VALLEJO, TOMAS-BARBERAN, GARCIA-VIGUERA, 2002; LUO et al., 2013).

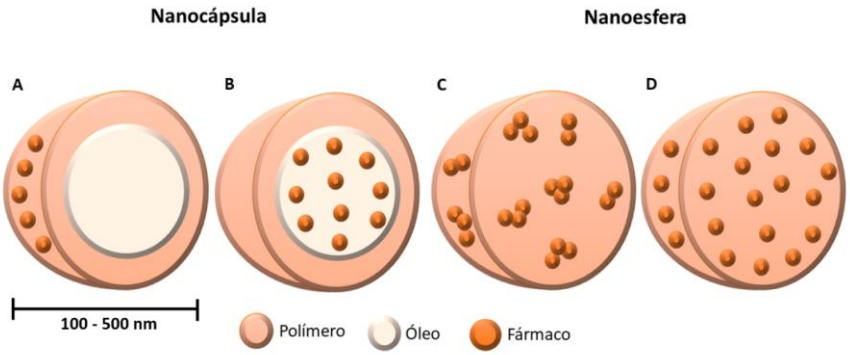
Dessa forma, considerando-se as potencialidades terapêuticas do DIM e as dificuldades de se incorporar este bioativo em fórmulas farmacêuticas para sua aplicação clínica adequada, devido às suas características físico-químicas desfavoráveis, torna-se interessante estudar novas abordagens, como os sistemas de liberação modificada de fármacos, para sua utilização na prática, prerrogativa esta que serviu de base para a construção da hipótese de trabalho da presente tese.

1.3 NANOCÁPSULAS POLIMÉRICAS

Nos últimos anos, é crescente o número de trabalhos científicos descrevendo o uso da nanotecnologia nas mais diversas áreas da ciência. O desenvolvimento de formulações nanotecnológicas destinadas ao tratamento de diversas enfermidades vem destacando-se devido aos diversos benefícios proporcionados pela nanotecnologia na terapêutica, como liberação modificada de fármacos e melhora no efeito farmacológico (DIMER et al., 2013; BHOKARE et al., 2015, FRANK et al., 2015).

Dentre os nanocarreadores mais promissores no tratamento e prevenção de doenças destacam-se os lipossomas, as nanopartículas lipídicas, as nanopartículas poliméricas, entre outros (DIMER et al., 2013). As nanopartículas poliméricas são partículas de tamanho coloidal, apresentando diâmetro médio usualmente na faixa de 100 a 500 nanômetros e incluem as nanoesferas e as nanocápsulas (NCs), as quais diferem quanto à sua composição e organização estrutural (FATTAL; VAUTHIER, 2002; NICOLAS et al., 2013, SCHAFFAZICK et al., 2003). As nanocápsulas são dispersões sólido-líquido nas quais um invólucro polimérico circunda um núcleo geralmente oleoso, sendo por isso consideradas sistemas reservatórios (**Figura 4**). As nanoesferas, por outro lado, apresentam caráter matricial. O fármaco pode encontrar-se fisicamente disperso, dissolvido, ou quimicamente ligado à cadeia polimérica (SCHAFFAZICK et al., 2003; NICOLAS et al., 2013).

Figura 4 - Estrutura e forma de associação do fármaco às nanocápsulas e nanoesferas. A: fármaco adsorvido ao invólucro polimérico das nanocápsulas; B: fármaco dissolvido no núcleo oleoso das nanocápsulas; C: fármaco retido na matriz de polímero das nanoesferas; D: fármaco adsorvido ou molecularmente disperso na rede polimérica das nanoesferas.



Fonte: O próprio autor.

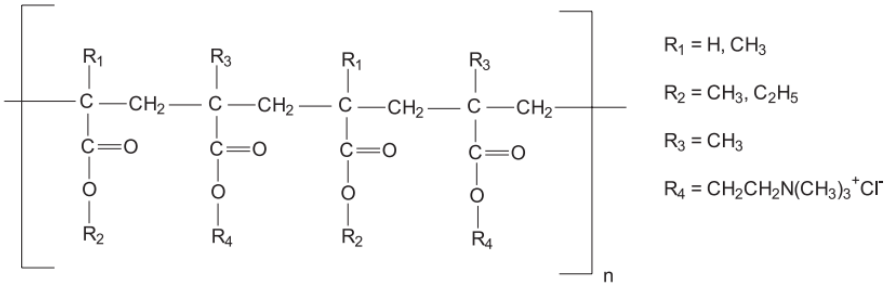
As nanocápsulas oferecem muitas vantagens em comparação aos sistemas terapêuticos convencionais, incluindo a possibilidade de se utilizar matérias-primas biodegradáveis em sua constituição, além do encapsulamento promover proteção ao fármaco (frente à degradação física, química ou enzimática) e melhora no perfil de dissolução de substâncias hidrofóbicas. Isso possibilita redução na dose

administrada de fármaco, o que por consequência reduz a toxicidade e os efeitos adversos, aumentando a eficácia terapêutica (OURIQUE et al, 2008; MORA-HUERTAS; FESSI; ELAISSARI, 2010; FRANK et al., 2015). Ainda, as nanocápsulas permitem a modulação das propriedades de superfície, possibilitando que as partículas escapem da captação pelo sistema retículoendotelial e assim prolongando seu tempo de circulação no organismo (NICOLA et al., 2013; JAIN et al., 2013).

Alguns dos polímeros mais amplamente utilizados para formular nanocápsulas são os poli(metacrilatos), polímeros derivados do ácido metacrílico com metacrilato de metila, acrilato de etila, metacrilato de butila, metacrilato de cloreto de trimetilamônio de etila ou metacrilato de dimetilamino etila. Estes polímeros são conhecidos comercialmente como Eudragit® e exibem funções variadas: estabilizadora, protetora e moduladora da liberação (entérica e sustentada), sendo que algumas das vias de administração mais utilizadas são oral, oftálmica, tópica e vaginal (VILLANOVA; ORÉFICE; CUNHA, 2010; NIKAM et al., 2011; SANTOS et al., 2013; GEHRCKE et al., 2018).

No presente trabalho, o polímero Eudragit® RS100 (**Figura 5**) (nome químico/IUPAC: *Poly (ethyl acrylate-co-methyl methacrylate-corimethylammonioethyl methacrylate chloride*) 1:2:0,1) foi utilizado na preparação das nanocápsulas. O Eudragit® RS100 é sintético, biocompatível, apresenta peso molecular de 32000 g/mol e é insolúvel em água e solúvel na maioria dos solventes orgânicos. Apresenta-se na forma de grânulos e proporciona perfil de liberação independente do pH e baixa permeabilidade (devido à presença de grupamentos amônio quaternário na forma de sais), permitindo que a liberação do fármaco seja controlada em todo o TGI, o que promove melhora no efeito terapêutico e, assim, na adesão do paciente ao tratamento (EVONIK INDUSTRIES, 2015). Além disso, por ser um polímero de caráter catiônico, pode proporcionar aumento no tempo de retenção dos nanocarreadores na mucosa do TGI, conferindo mucoadesão ao produto final (DAMGÉ; MAINCENT; UBRICH, 2007).

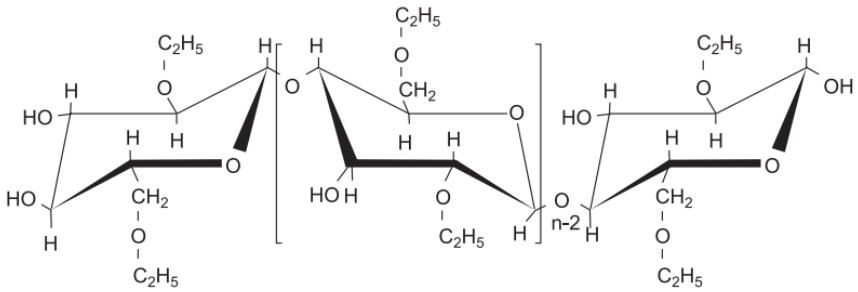
Figura 5 – Estrutura química do Eudragit® RS100.



Adaptado de DOMINGUES et al., 2008.

Outro polímero bastante versátil na preparação de sistemas nanoestruturados e que foi utilizado no presente estudo é a etilcelulose (**Figura 6**). Este polímero é um derivado semissintético da celulose, possuindo repetidas unidades de anidroglicose em sua estrutura, cada uma delas contendo 3 sítios -OH reativos. A etilcelulose é biocompatível, organosolúvel e não-iônica, sendo empregada como agente de revestimento, aromatizante e aglutinante em comprimidos, além de ser utilizada na granulação e na preparação de micro e nanopartículas, principalmente em formulações para uso tópico ou oral, permitindo controle de liberação da substância ativa (MURTAZA, 2012; LOKHANDE et al., 2013; CHASSOT et al., 2014; MARCHIORI et al., 2017).

Figura 6 – Estrutura química da etilcelulose.



Adaptado de MURTAZA, 2012.

Além do polímero, o tipo de óleo utilizado também apresenta influência nas características finais das nanocápsulas, como diâmetro e dispersidade das partículas, devido às diferentes viscosidades, características hidrofóbicas e tensão

interfacial apresentadas pelos óleos (SCHAFFAZICK et al. 2003). Em adição, grande parte dos óleos vegetais apresenta propriedades de interesse na formulação de sistemas de liberação em produtos farmacêuticos, tais como atividades antioxidante, antibacteriana e antifúngica, e por isso estes vem sendo amplamente estudados na concepção de nanocápsulas (ALMEIDA et al., 2009; FLORES et al., 2011; SANTOS et al., 2014; CHASSOT et al., 2014; GEHRCKE et al., 2017; MARCHIORI et al., 2017). No presente trabalho, os óleos de escolha foram os óleos de prímula e o de semente de damasco.

O óleo de prímula é extraído da planta *Primula veris* L*. (Primulaceae),* nativa de países do hemisfério norte, mais especificamente Europa e oeste da Ásia (OKRSLAR et al., 2007; GHEDIRA, GOETZ, 2013). Esta planta (raízes e flores) apresenta composição rica em saponinas, flavonoides e taninos, sendo utilizada na medicina tradicional para tratamento das mais diversas enfermidades, como bronquite e outras doenças respiratórias, gastrite e gota, além de apresentar efeito sedativo, diurético e anti-inflamatório (OKRSLAR et al., 2007; GHEDIRA, GOETZ, 2013). Além disso, extratos aquosos e etanólicos das folhas de prímula demonstraram efeito antiepilético e anticonvulsivante (JAGER et al., 2006).

Da semente da prímula se obtém seu óleo, extraído por prensagem a frio, o qual apresenta interessantes ácidos graxos poli-insaturados em sua composição, como o ɣ-linolênico (18:3) e o estearidônico (18:4) (SAYANOVA, NAPIER, SHEWRY, 1999). A presença de ácido gama-linolênico no óleo é interessante por se tratar de um ácido graxo essencial ao organismo. O crescente interesse em aplicações clínicas e farmacêuticas deste ácido graxo reside também no fato deste lipídeo ser um precursor dos eicosanóides da série 1 (prostaglandina E1) e exercer efeito inibitório na síntese de leucotrienos, desta maneira apresentando atividade anti-inflamatória (MONTSERRAT-De LA PAZ et al., 2014). O ácido estearidônico também apresenta diversos benefícios à saúde humana. Estudos recentes demonstram o papel deste óleo no tratamento de diferentes tipos de tumores, tanto em testes *in vitro* quanto *in vivo* e sugerem seu potencial no tratamento e/ou prevenção de doenças cardiovasculares e artrite reumatoide (RINCÓN, VALENZUELA, VALENZUELA, 2015).

O damasco (*Prunus armeniaca* L.*, Rosaceae*) é originário da China, Coréia e Japão, mas atualmente tem sido cultivado também na Europa e na América (LEE et

al., 2014). Na medicina tradicional oriental, as sementes de damasco têm sido amplamente utilizadas no tratamento de doenças respiratórias (BENSKY et al., 2004) e doenças da pele, como *acne vulgaris* e caspa (LEE et al., 2014). Além disso, já foi relatado na literatura que as sementes apresentam maior atividade antioxidante e quantidade de compostos fenólicos do que a própria fruta (SOONG; BARLOW, 2004). Estudos *in vitro* vêm demonstrando seus efeitos anti-inflamatórios em patologias como colite ulcerativa, efeitos antioxidantes frente a radicais livres e efeitos anticarcinogênicos frente a células de câncer de mama (MCF-7), cólon (HCT-116), e hepatocelular (HepG2) (MINAIYAN et al., 2015; CHANG et al., 2005; KOREKAR et al., 2011; GOMAA, 2013).

O óleo de semente de damasco é extraído por prensagem a frio das sementes do damasco, apresentando cor levemente amarelada e odor discreto (ABD-EL AAL; KHALIL; RAHMA, 1986). A composição quali e quantitativa de ácidos graxos deste óleo é muito semelhante ao do óleo de amêndoas. Compreende principalmente ácidos graxos insaturados, como o oléico (18:1; ω-9) e o linoléico (18:2; ω-3), somando em torno de 93% do total de ácidos graxos constituintes. Isto confere a este óleo grande valor nutricional, já que o ácido linoleico é um dos três ácidos graxos essenciais ao organismo (ABD-EL AAL; KHALIL; RAHMA, 1986; FEMENIA et al., 1995).

Em adição a isso, o óleo de semente de damasco tem atraído atenção por possuir propriedades antibacteriana e antifúngica (IBRAHIM; EL-SALAM, 2015; HAMMER; CARSON; RILEY, 1999; LEE et al., 2014) e sua alta concentração de ácidos graxos insaturados também poderia conferir a este óleo outras propriedades de interesse na área farmacêutica, como a redução dos níveis de colesterol sérico (FEMENIA et al., 1995).

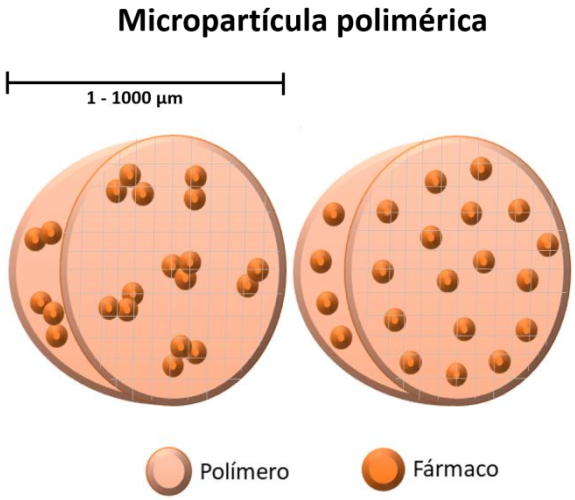
1.4 MICROPARTÍCULAS POLIMÉRICAS

Além das nanopartículas poliméricas, outro tipo de sistema de liberação de fármacos muito estudado são as micropartículas poliméricas (MPs). As micropartículas consistem em sistemas multiparticulados formados por partículas sólidas, geralmente esféricas, que apresentam diâmetros variando entre 1 e 1000 µm. Usualmente, são constituídas por polímeros biodegradáveis ou não e podem

conter o fármaco em sua forma microcristalina ou dissolvida na matriz polimérica. Dividem-se em dois tipos de estruturas: as microcápsulas, as quais apresentam um núcleo sólido, líquido ou gasoso envolto por uma parede polimérica; e as microesferas, constituídas por uma matriz polimérica sólida micrométrica (**Figura 7**) (MURTAZA et al., 2012). Porém, o termo micropartículas poliméricas é geralmente utilizado como sinônimo tanto de microcápsulas quanto microesferas e será adotado para se referir ao sistema desenvolvido no presente trabalho.

Embora sejam baseadas em tecnologias na escala “micro”, a exemplo das nanopartículas, estes sistemas também agregam uma série de características vantajosas em relação aos sistemas convencionais, como a encapsulação de ativos tanto hidro quanto lipofílicos, proteção do fármaco da degradação pela luz, oxigênio, calor ou pelos fluidos gastrointestinais, proteção do TGI de efeitos adversos irritantes de fármacos (como os AINEs), bem como liberação sustentada ou sítio-específica. Além disso, são bastante empregadas em estudos farmacêuticos para melhorar características das substâncias ativas, como estabilidade e perfil de liberação e também para mascarar sabores e odores desagradáveis (FREIBERG, ZHU, 2004; RAFFIN et al., 2008; CRUZ et al., 2009; VELASQUEZ et al., 2012; MURTAZA, 2012; FERREIRA et al., 2015).

Figura 7 – Estrutura esquemática das micropartículas poliméricas.



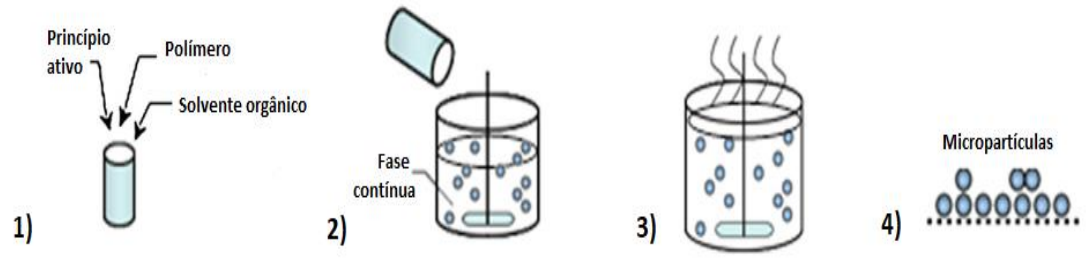
Fonte: O próprio autor.

Por geralmente poderem ser obtidas ou convertidas na forma de um pó seco, as micropartículas podem apresentar uma maior estabilidade em relação às nanopartículas, pois são menos suscetíveis ao desenvolvimento de micro-organismos ou à ocorrência de reações químicas indesejadas. Também podem ser convertidas facilmente à forma farmacêutica final, constituindo, por exemplo, pós-inalatórios ou comprimidos por compressão direta. Além disso, as formas farmacêuticas multiparticuladas demonstram ser superiores aos sistemas unitários de ação prolongada, pois permitem distribuição mais rápida e uniforme do fármaco no TGI, maior reprodutibilidade de doses e menor risco de provocar efeitos tóxicos (WANG, 1997; VILOS, VELASQUEZ, 2012).

As micropartículas podem ser produzidas por vários métodos, como a coacervação, secagem por aspersão, emulsificação-evaporação de solvente, entre outros (FREIBERG, ZHU, 2004; DESHMUKH, WAGH, NAIK, 2016). A técnica de emulsificação-evaporação de solvente é uma das mais comuns na literatura para o preparo de micropartículas utilizando-se polímeros insolúveis em água, por ser um método fácil, reprodutível e compatível com vários polímeros. Isto permite a incorporação de vários compostos hidrofóbicos em micropartículas, utilizando-se um método que não requer agentes químicos ou equipamentos caros (JELVEHGARI; MONTAZAM, 2012).

A versão mais simples deste método baseia-se na formação de uma emulsão simples óleo/água (O/A), a qual é útil na incorporação de ativos que possuam pouca ou nenhuma solubilidade em água. A preparação pode ser dividida em quatro etapas (**Figura 8**).

Figura 8 – Etapas da microencapsulação de fármacos pelo método de emulsificação-evaporação de solvente.



Fonte: Adaptado de LI, ROUAUD, PONCELET, 2008.

A primeira etapa consiste na dissolução do polímero e do composto ativo em um solvente ou mistura de solventes voláteis apropriados (geralmente clorofórmio, diclorometano ou acetato de etila). A segunda etapa compreende a emulsificação dessa fase orgânica em uma fase contendo um não-solvente para o polímero (usualmente água) e um agente estabilizante, assim formando-se a emulsão (O/A). Na etapa seguinte, após estabilização dessa emulsão, o solvente é removido da fase dispersa por evaporação e consequentemente ocorre a formação das micropartículas sólidas. Por fim, ocorre a lavagem das micropartículas para retirada do tensoativo, seguida da coleta e secagem das micropartículas obtidas (O'DONNELL, McGINITY, 1997; LI, ROUAUD, PONCELET, 2008; DESHMUKH, WAGH, NAIK, 2016).

Diversos polímeros podem ser utilizados na preparação de micropartículas, dentre os quais destaca-se a etilcelulose. Como mencionado anteriormente, a etilcelulose é um polímero não-biodegradável de uso versátil, sendo constituinte principalmente de micropartículas visando administração oral de fármacos, pois permite o controle da liberação ao longo do TGI. A **Tabela 1** resume alguns dos trabalhos de relevância utilizando a etilcelulose na constituição de micropartículas poliméricas.

Tabela 1 – Estudos utilizando a etilcelulose na preparação de micropartículas.

Polímero	Ativo	Tipo de microestrutura	Principais resultados	Referência
EC e ERL	Nifedipino	Microesfera	EE%: 11-33%. Diâmetro médio: 10–20 µm. Estáveis por 3 meses a 40ºC.	Huang, Wigent, Schwartz, 2008
EC	Salbutamol	Microcápsula	EE%: 86-97%. Partículas de 40-80 µm, agregadas e de superfície irregular. Liberação controlada (3,5h), seguindo modelo de Higuchi e transporte anômalo.	Murtaza et al., 2009
EC	Tolmetina	Microesfera	EE% aumentada com aumento da concentração de ativo e da miscibilidade no solvente orgânico. Liberação lenta (9h), efeito *burst* e transporte anômalo.	Jelvehgari et al., 2010.
EC	Ácido acetilsalicílico	Microesfera	EE%: 46-58%. Partículas de 328-989 µm, uniformes e esféricas. Liberação controlada (8h).	Patel et al., 2012
EC e ERS	Aceclofenaco	Microesfera	EE%: 70-83%. Partículas de 20-200 µm, ovais, discretas e superfície lisa. Liberação prolongada (12h).	Deshmukh, Naik, 2014
EC, HPMC	Timol	Microesfera	EE%: 61%. Partículas de 1 mm, superfície rugosa e porosas. Liberação prolongada (24h), seguindo difusão Fickiana. Estáveis por 90 dias.	Zamani et al., 2015
EC	Nifedipino	Microesfera	Diâmetro médio das partículas: 50-1000 µm. Diferentes tipos de morfologia de superfície observados.	Parida et al., 2016
EC	Ácido *p*-aminobenzoico	Microesfera	EE%: 37-79%. Partículas esféricas de superfície porosa, diâmetros entre 793-870 µm quando utilizado Tween 80. Liberação prolongada (50h), seguindo modelo de Higuchi e difusão Fickiana.	Mouffok et al., 2016
QS, EC, HPMC	Metronidazol	"Micropartículas efervescentes flutuantes"	EE%: 44-66%. Partículas de 2 µm, uniformes. Liberação controlada (4h), seguindo modelo de Higuchi e transporte anômalo.	Nohemann, Almeida, Ferreira, 2017

EC: etilcelulose; EE%: eficiência de encapsulamento; ERL: Eudragit RL; ERS: Eudragit RS; HPMC: hidroxipropilmetilcelulose; QS: quitosana.

1.5 INCORPORAÇÃO DO DIM EM SISTEMAS DE LIBERAÇÃO MODIFICADA DE ATIVOS

Ao mesmo tempo que a utilização clínica do DIM tem se mostrado cada vez mais relevante, considerando-se suas diversas potencialidades terapêuticas, devido às suas limitações, o preparo de formas farmacêuticas contendo DIM ainda representa um grande desafio. Dessa forma, novas formulações contendo DIM vêm sendo desenvolvidas a fim de se contornar estes inconvenientes e se aprimorar a biodisponibilidade do DIM, bem como melhorar a eficiência com que este bioativo é liberado no foco das doenças. De fato, tem sido relatado na literatura diferentes estratégias a fim de se contornar estas limitações, como o desenvolvimento de nanoesferas poliméricas (LUO et al., 2013; ISABELLA, MIRUNALINI, 2016; BHOWMIK et al., 2017), nanocontêineres (KISELEV et al., 2013), além da complexação do DIM com ciclodextrinas (ROY et al., 2013), e formação de microdispersões (ZELIGS, JACOBS, 2000), entre outros sistemas (KISELEV, 2016).

Zeligs e Jacobs (2000) desenvolveram uma formulação contendo DIM para uso oral, conhecida como BioResponse-DIM (BR-DIM), a qual encontra-se patenteada. Esta formulação compreende microdispersões de DIM formadas por uma matriz solúvel em água, contendo succinato de D-alfa-tocoferil polietilenoglicol 1000 (Vitamina E TGPS) e fosfatidilcolina. Em estudos preliminares em humanos, a administração por via oral do BR-DIM promoveu aumento de 50% na biodisponibilidade do DIM em comparação ao composto cristalino. Atualmente, a formulação está em fase de testes clínicos (Fases I, II e III), sendo estudada para diversas aplicações, como no tratamento do câncer de próstata e da displasia cervical (ZELIGS, JACOBS, 2000; ANDERTON et al., 2004; REED et al., 2008; DEL PRIOE et al., 2010; LI, SARKAR, 2016).

Kiselev e colaboradores (2013) desenvolveram uma nanoformulação contendo DIM baseada em um copolímero não iônico, o Pluronic F127. A formulação proposta aumentou consideravelmente a biodisponibilidade do fitoquímico em relação ao DIM na forma cristalina após administração por via intragástrica em ratos (aumento de aproximadamente 7,2 vezes da Área sob a Curva e 17,1 vezes da C_{max}), além de promover uma maior solubilidade do DIM em água (no mínimo 3 mg/mL). Baseado nestes resultados, um novo produto chamado Cineton® está sendo

desenvolvido pelos autores para aplicação comercial em diferentes áreas, já tendo sido testado para o tratamento de pacientes com hiperplasia endometrial em ensaio clínico (ANDRIANOVA et al., 2015).

Utilizando uma abordagem diferente, Luo e colaboradores (2013), prepararam nanopartículas de zeína e carboximetilquitosana contendo DIM, pelo método de separação de fases líquido-líquido. Devido ao DIM ser um composto hidrofóbico, altas eficiências de encapsulamento foram obtidas (60-80 %), resultando em um perfil de liberação controlada do fármaco a partir das nanopartículas e em uma melhor estabilidade do DIM frente à degradação térmica e luz UVA. Entretanto, o teor de DIM presente nas nanopartículas foi de apenas 50 µg/mL.

Recentemente, Isabella e Mirunalini (2016, 2017a,b) formularam nanopartículas de quitosana contendo DIM e comprovaram sua ação quimioterápica em modelo de câncer de mama induzido por 7,12-dimetilbenzantraceno (DMBA) em ratas, sendo a dose de 0,5 mg/Kg via intragástrica a mais efetiva. Os autores observaram diminuição no número e no volume dos tumores das ratas tratadas em comparação ao grupo controle. Além disso, análises bioquímicas e histopatológicas sugeriram que as nanopartículas promoveram diminuição da lipoperoxidação e melhora no *status* oxidativo das células, o que aliado à liberação sustentada poderia levar a melhora no efeito antitumoral apresentado pelo DIM.

Bhowmik e col. (2017) nanoencapsularam o DIM em nanopartículas de poli (D,L-lactídeo-co-glicolídeo) (PLGA) modificadas com um ligante específico a ser reconhecido por células neoplásicas cerebrais. As nanopartículas apresentaram diâmetros entre 27-87 nm, eficiência de encapsulamento de 70% e liberaram aproximadamente 70% do DIM em 96 h. Além disso, a nanoencapsulação promoveu aumento da captação pelas células de glioma, culminando no acúmulo seletivamente no sítio tumoral, além de proporcionarem aumento de 12 vezes na biodisponibilidade em comparação com o bioativo puro.

Em adição, existem também trabalhos relatando a nanoencapsulação de derivados do DIM. Boakye e col. (2016) desenvolveram lipossomas catiônicos contendo 1,1-bis(3’-indol)-1-(*p*-clorofenil)metano (DIM-D), denominados UltraFLEX-Nano, os quais foram incorporados em géis de hidroxipropilmetilcelulose (HPMC). A formulação apresentou características físico-químicas adequadas e demonstrou melhorar a liberação do composto, resultando na diminuição do aparecimento de

tumores na pele de cobaias expostas à radiação UVB. Godugu e col. (2016) produziram carreadores lipídicos nanoestruturados (CLN) contendo derivados do DIM C-substituídos, os quais apresentaram ação antitumoral frente a células de câncer de mama triplo negativo, em testes *in vitro* e *in vivo*. Os CLN promoveram melhora na biodisponibilidade oral destes compostos e diminuição no volume dos tumores nos animais tratados com os CLN em comparação ao grupo tratado com os compostos não encapsulados.

1.6 ASSOCIAÇÃO DE FÁRMACOS A SISTEMAS NANO- E MICROPARTICULADOS NA TERAPIA DA DOR

Sistemas de liberação de fármacos como as NCs e as MPs tem surgido como tecnologias inovadoras na terapia da dor, pois, como citado acima, são capazes de proporcionar vantagens únicas, como absorção ou distribuição direcionadas a sítios específicos e aumento da biodisponibilidade de fármacos, além de promoverem a liberação prolongada destes, aumentando o potencial farmacológico e reduzindo os efeitos adversos tóxicos dos ativos. Em conjunto, estas melhorias podem resultar em maior adesão do paciente à terapia e, apesar de serem tecnologias inicialmente caras, a longo prazo podem gerar redução no custo do tratamento. De fato, reforçando a importância da utilização destes sistemas no manejo da dor, diversas revisões de literatura recentes relatam a utilização de dispositivos na escala nano e micrométricas em etapas pré-clínicas e ainda outros já utilizados na clínica (MOUNTZIARIS, KRAMER, MIKOS, 2009; SPRINTZ et al., 2011; MORADKHANI, KARIMI, NEGAHDARI, 2017; ANDREU, ARRUEBO, 2018; MAUDENS, JORDAN, ALLÉMAN, 2018).

Alguns dos inúmeros trabalhos da literatura científica que relatam o papel dos nanocarreadores em proporcionar melhora no efeito analgésico/antinociceptivo de diferentes compostos, incluem: opioides, como tapentadol (PATIL, SURANA, 2016); anestésicos, como lidocaína (YIN et al., 2009); AINEs, como indometacina (BERNARDI et al., 2009), nimesulida (LENZ et al., 2012), meloxicam (VILLALBA et al., 2014) e cetoprofeno (FERREIRA et al., 2016); além de fitoquímicos, como curcumina (DEWANGAN et al., 2017); entre outros (LIU et al., 2005; TRONINO et al., 2016; SARI et al., 2017; SARI et al., 2018a,b). Ainda, algumas nano-formulações

demonstraram sucesso na sua utilização em estudos clínicos, como implantes de liberação controlada baseados em nano-canais de silicone e o dispositivo “BioNanoScaffold”, o qual é aplicado em fraturas ósseas; além de nanocarreadores que já se encontram no mercado, como o DepoDur®, uma formulação injetável para a liberação prolongada da morfina, que foi aprovada pela agência americana “Food and Drug Administration” em 2004, visando o tratamento da dor pós-cirúrgica (SPRINTZ et al., 2011; ANDREU, ARRUEBO, 2018).

Com relação às MPs, alguns autores vêm investigando os benefícios da microencapsulação de diferentes compostos para o manejo da dor. A literatura relata MPs contendo opioides como morfina, tramadol, hidromorfona e loperamida (RUSSO et al., 2006; et al., 2009; HAN et al., 2015; BORODINA et al., 2016), bem como diflunisal (PIGNATELLO et al., 2001), bupivacaína (TARABALLI et al., 2014), além de fármacos alternativos aos analgésicos convencionais (RUDNIK-JANSEN et al., 2017; DAI et al., 2018), incluindo um registro de patente (CRISCIONE et al., 2016), no qual foram desenvolvidas MPs de PLGA para a liberação lenta da carbamazepina, administradas na forma de uma injeção de depósito (“depot injection”).

Ainda, é fundamental destacar um recente estudo conduzido em nosso laboratório, o qual demonstrou ineditamente a ação antinociceptiva do indol-3-carbinol (I3C), precursor fitoquímico do 3,3’-diindolmetano (DIM), o qual é o bioativo foco do presente trabalho, em sua forma livre e nanoencapsulada (GEHRCKE et al., 2018). Este fato, aliado aos conhecidos efeitos anti-inflamatórios do DIM e considerando-se as informações supracitadas a respeito da dor e da necessidade de se descobrir alternativas inovadoras para seu manejo, despertou nosso interesse em investigar o efeito antinociceptivo do DIM, bem como o impacto da sua incorporação em NCs e MPs neste efeito.

Diante do exposto, esta tese de doutorado foi delineada visando o desenvolvimento de nanopartículas e micropartículas poliméricas contendo DIM, no sentido de melhorar a performance biológica deste bioativo, bem como estudar, de forma inédita, seus potenciais efeitos farmacológicos em modelos animais de dor. É importante ressaltar que até o momento não existem relatos na literatura sobre a preparação de NCs e MPs de DIM e tampouco relatos da investigação da ação antinociceptiva deste ativo.

2. OBJETIVOS

2 OBJETIVOS

2.1 OBJETIVO GERAL

O presente trabalho tem por objetivo o desenvolvimento de nanocápsulas e micropartículas poliméricas contendo o bioativo 3,3'-diindolmetano, bem como a avaliação do impacto da sua associação nestes sistemas nas suas propriedades biológicas, através de ensaios *in vitro* e *in vivo*.

2.2 OBJETIVOS ESPECÍFICOS

Considerando os aspectos mencionados, os objetivos específicos desta tese compreendem:

- Preparar suspensões de nanocápsulas de etilcelulose ou Eudragit® RS100 contendo DIM, utilizando os óleos de prímula ou damasco como núcleo oleoso;
- Caracterizar as suspensões de nanocápsulas quanto ao teor e eficiência de encapsulamento do DIM, diâmetro de partícula, índice de polidispersão, potencial zeta, pH, morfologia, forma de associação do DIM e interações/incompatibilidades entre os constituintes;
- Avaliar o perfil de liberação *in vitro* do DIM a partir dos sistemas nanoestruturados e determinar o mecanismo de liberação;
- Avaliar a capacidade das suspensões de nanocápsulas em prevenir a fotodegradação do DIM;
- Avaliar a atividade sequestradora de radicais livres do DIM livre e nanoencapsulado;
- Determinar o potencial antitumoral *in vitro* do DIM livre e nanoencapsulado frente às células de glioblastoma maligno humano (U87);
- Preparar micropartículas poliméricas de etilcelulose contendo DIM;
- Caracterizar as micropartículas de DIM quanto ao teor e eficiência de encapsulamento do ativo, diâmetro médio de partícula, distribuição granulométrica, morfologia, forma de associação do DIM e

interações/incompatibilidades entre os constituintes e propriedades de fluxo do pó;

- Avaliar o perfil de dissolução do DIM a partir das micropartículas e propor o mecanismo de liberação;
- Investigar se o DIM, na forma livre ou associado aos sistemas propostos, apresenta efeito antinociceptivo em diferentes modelos de dor em camundongos (Modelo de nocicepção térmica – Teste da chapa quente; Modelo de nocicepção química induzida pela formalina; Modelo de dor inflamatória aguda induzida pelo CFA), comparando-se o potencial antinociceptivo do bioativo nos diferentes sistemas.

3. CAPÍTULO 1

3. CAPÍTULO 1: PREPARAÇÃO, CARACTERIZAÇÃO E AVALIAÇÃO BIOLÓGICA *IN VITRO* E *IN VIVO* DE NANOCÁPSULAS POLIMÉRICAS CONTENDO 3,3'-DIINDOLMETANO

Apresentação

O primeiro capítulo desta tese relata o desenvolvimento de nanocápsulas contendo DIM e a avaliação da fotoestabilidade, da ação sequestrante de radicais livres e do efeito citotóxico frente à linhagem celular de glioblastoma humano *in vitro*, além da investigação do efeito antinociceptivo em diferentes modelos de dor em animais.

Neste capítulo encontra-se o **artigo 1**, publicado na revista "*AAPS PharmSciTech*" (2019 Jan 7;20(2):49. doi: 10.1208/s12249-018-1240-8), no qual são relatadas a preparação e caracterização de nanocápsulas contendo DIM, usando dois diferentes polímeros (EC e ERS) e dois óleos vegetais (prímula e damasco), bem como a avaliação do perfil de liberação do DIM a partir das nanoestruturas, avaliação da sua fotoestabilidade e da capacidade sequestrante frente a dois radicais livres sintéticos (ABTS e DPPH), bem como avaliação da ação antitumoral frente a células de glioma humano (U87). A autorização da reprodução do conteúdo do artigo pela revista encontra-se no **Anexo B** desta tese.

A partir dos resultados do **artigo 1**, a formulação de NCs de etilcelulose e óleo de prímula contendo DIM (NC-PEC-D) foi escolhida para dar seguimento aos estudos contemplados na segunda parte deste capítulo, dando origem ao **Manuscrito 1.** Este manuscrito descreve a caracterização físico-química mais detalhada e completa da formulação NC-PEC-D, fornecendo maiores informações sobre a forma de associação do DIM e interações entre o ativo e o polímero nas nanoestruturas. Em adição, este também aborda a investigação dos efeitos benéficos do nanoencapsulamento do DIM na formulação NC-PEC-D em melhorar o seu efeito antinociceptivo em diferentes modelos animais de nocicepção.

3.1 ARTIGO 1

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Research Article

Incorporation of 3,3′-Diindolylmethane into Nanocapsules Improves Its Photostability, Radical Scavenging Capacity, and Cytotoxicity Against Glioma Cells

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Abstract. 3,3′-Diindolylmethane (DIM) is a phytochemical that presents health benefits (antitumor, antioxidant, and anti-inflammatory effects). However, it is water insoluble and thermo- and photolabile, restraining its pharmaceutical applications. As a strategy to overcome such limitations, this study aimed the development and characterization of DIM-loaded nanocapsules (NCs) prepared with different compositions as well as the *in vitro* assessment of scavenging activity and cytotoxicity. The formulations were obtained using the interfacial deposition of preformed polymer method and were composed by Eudragit® RS100 or ethylcellulose as polymeric wall and primula or apricot oil as the core. All the formulations had adequate physicochemical characteristics: nanometric size (around 190 nm), low polydispersity index (< 0.2), pH value at acid range, high values of zeta potential, drug content, and encapsulation efficiency (~ 100%). Besides, nanoencapsulation protected DIM against UVC-induced degradation and increased the scavenging activity assessed by the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and 1-1-diphenyl-2-picrylhydrazyl methods. The developed DIM-loaded nanocapsules were further evaluated regarding the *in vitro* release profile and cytotoxicity against a human glioblastoma cell line (U87 cells). The results demonstrated that the nanoencapsulation promoted a sustained release of the bioactive compound (in the range of 58–78% after 84 h) in comparison to its free form (86% after 12 h), as well as provided a superior cytotoxic effect against the U87 cells in the highest concentrations. Therefore, our results suggest that nanoencapsulation could be a promising approach to overcome the DIM physicochemical limitations and potentialize its biological properties.

KEY WORDS: indole-3-carbinol; polymeric nanoparticles; photodegradation; antioxidant activity; antitumor.

INTRODUCTION

3,3′-Diindolylmethane (DIM) is a bioactive compound originated from the oligomerization of the indole-3-carbinol, after the ingestion of cruciferous vegetables, such as broccoli and cauliflower. The scientific literature highlights many health benefits provided by DIM, for example, its antioxidant, anti-inflammatory, and antitumor properties, and recent studies investigated its application against several pathologies (1–3). This phytochemical is a free radical scavenger, lipid peroxidation inhibitor, and modulator of different signaling pathways associated with inflammatory and carcinogenic processes (1,4). Despite its beneficial actions, DIM is highly susceptible to photodegradation and also has poor aqueous solubility and low oral bioavailability (5–7). Therefore, developing a pharmaceutical DIM dosage form is a challenging task, reinforcing that technological approaches should be applied to better explore its promising therapeutic application.

In this context, the development of nanoparticles-based drug delivery systems has been recognized as a potential alternative to circumvent physicochemical limitations and maximize the biological properties of drugs (8,9). Several advantages are attributed to nanocarrier systems in comparison to conventional therapy, for instance controlled delivery of active substances, reduction of adverse effects, drug protection against chemical and enzymatic degradation, and improvement of their aqueous apparent solubility (10–12). Among the nanocarriers, polymeric nanocapsules (NCs) are colloidal systems composed by a polymeric wall surrounding an oily core. Of particular importance, the investigation of novel oils from natural sources, such as

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vegetable oils, has been an interesting approach in the formulation of NCs. The oil core is a structural component of the NCs that could provide additional pharmacological properties to the formulation (13–15). Primula (*Primula veris*, L.) and apricot (*Prunus armeniaca*, L.) oils are of special interest for the presence of unsaturated and poly-unsaturated fatty acids in their composition (16–18). Despite their potential, as far as we know, there is no nanocapsule formulation composed by such oils.

Concerning the literature, there are a few investigations about DIM incorporation into polymeric nanocarriers. Kiselev and co-workers (19) developed matrix nanoparticles to improve DIM bioavailability. DIM-loaded nanoparticles of zein/carboxymethyl chitosan were developed to increase drug stability (20). Isabella and Mirunalini (21,22) also formulated DIM-matrix nanoparticles containing chitosan and showed the antitumor effect of the formulation against a rat mama tumor. In addition, a recent report showed that a formulation of PLGA matrix nanoparticles containing DIM demonstrated antitumor effects (23). However, there are neither studies about DIM incorporation into NCs composed by primula and apricot oils nor the investigation of the potential impact of such association in DIM biological effects.

Therefore, the current study aimed the development and characterization of novel NC formulations to DIM encapsulation. Different polymers, ethylcellulose (EC) or Eudragit® RS (ERS), and oils, primula or apricot, were used to prepare the NC suspension. The photoprotective potential and scavenging properties of the formulations were assessed. Besides, these formulations were further studied regarding the *in vitro* DIM release profile from the nanostructures and cytotoxic effect against a malignant glioblastoma cell line (U87 cells).

MATERIALS AND METHODS

Materials

DIM (99.2% purity) was obtained from Active Pharmaceutica (Brazil). Ethylcellulose (EC) was a gift from Colorcon (Brazil). Tween® 80 (polysorbate 80), Span® 80 (sorbitan monooleate), 1-1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 3(4,5-dimethyl)-2,5diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (USA). Primula oil (PO) and apricot kernel oil (AO) were supplied by Mundo dos Óleos (Brazil). Eudragit® RS100 (ERS) was donated by Almapal (Brazil). Dulbecco's modified Eagle's medium (DMEM), fungizone, penicillin/streptomycin, 0.25% trypsin/EDTA solution, and fetal bovine serum (FBS) were obtained from Gibco (USA). All other chemicals and solvents were analytical grade and used as received.

Analytical Procedures

The analytical method for DIM quantification in the NC suspensions was validated according to the ICH guidelines. A LC-10A HPLC system (Shimadzu, Japan), equipped with a LC-20AT pump, an UV–vis SPD-M20A detector, a CBM-20A system controller, and a SIL-20A HT valve sample automatic injector was used to perform DIM quantification. The separation was performed at room temperature using a Kinetex C_{18} Phenomenex column (250 mm × 4.60 mm, 5 μm; 110 Å) coupled to a C18 guard column. The DIM was detected at 288 nm, using an isocratic mobile phase composed by acetonitrile and water (60:40, *v*/*v*) at 1 mL/min flow rate. For the calibration curve, DIM was properly dissolved in methanol (DIM solutions in a concentration range of 0.75–20.0 μg/mL). The method was considered specific, linear (r = 0.9996), accurate, and precise (relative standard deviation ≤ 2.0%) in the above-mentioned concentration range.

DIM Solubility in the Vegetable Oils

The DIM solubility evaluation in the oils was carried out adding an excess amount of the compound in 2 mL of each vegetable oil. The systems were kept under moderate magnetic stirring overnight to assure maximum DIM solubilization. Following, each sample was centrifuged at 3000 rpm for 10 min and supernatant's aliquot was diluted with methanol, filtered, and injected into the HPLC system to DIM detection, using the chromatographic conditions mentioned in the previous section.

Dissolution/Swelling Test of Polymer Films

Polymer films of EC or ERS were prepared by previous dissolution of each polymer in acetone. After solvent evaporation, the resulting films were accurately weighted (approximately 40 mg to EC and 90 mg to ERS) and separately immersed in an aliquot of each oil at room temperature (n = 6). Over a period of 60 days, at predetermined time intervals, the films were removed from the oil and carefully dried with an absorbing paper. Weight variation was measured in an analytical balance in order to determine if the polymers have swollen and/or dissolved during the contact with the oil.

Preparation of Nanocapsule Suspensions

NCs were prepared (n = 6) by the interfacial deposition of preformed polymers method (24). Briefly, an organic phase containing acetone, vegetable oil, Span® 80, polymer, and DIM was kept under magnetic stirring for 60 min at 40°C. This phase was injected into an aqueous dispersion of Tween® 80 and kept under moderate magnetic stirring for 10 min longer. Then, the acetone and water were eliminated under reduced pressure to achieve 10 mL of NC suspension (final DIM concentration at 1 mg/mL). Blank NCs were prepared using the same method, without the active. All formulations were packaged in amber glass flasks, stored at 4°C, and used in the assays within a period of 2 days. The qualitative-quantitative composition of each formulation is available in Table I.

Physicochemical Characterization of Nanocapsule Suspensions

Mean Particle Diameter, Zeta Potential, and pH value

The average particle size, polydispersity index, and zeta potential of the NCs were measured using the ZetaSizer NanoSeries (Malvern Instruments, UK). This equipment employs the photon correlation spectroscopy methodology to determine particle size distribution (samples diluted in

Table I. DIM-Loaded NC Suspensions Composition

Component	Formulation			
	NC-PEC-D	NC-AEC-D	NC-PERS-D	NC-AERS-D
Aqueous phase				
Water (mL)	53	53	53	53
Tween® 80 (g)	0.077	0.077	0.077	0.077
Organic phase				
Acetone (mL)	27	27	27	27
EC (g)	0.1	0.1	-	-
ERS (g)	–	–	0.1	0.1
PO (mL)	0.15	–	0.15	–
AO (mL)	–	0.15	–	0.15
Span® 80 (g)	0.077	0.077	0.077	0.077
DIM (g)	0.01	0.01	0.01	0.01

EC, ethylcellulose; *ERS*, Eudragit RS; *PO*, primula oil; *AO*, apricot oil. Blank nanocapsules were prepared following the table without adding DIM

ultrapure water 1:500), while zeta potential is determined by microelectrophoresis after diluting the samples in 10 mM NaCl (1:500). The autocorrelation treatment function method for size distribution analysis was the general purpose and at least 10 runs were performed to each evaluation. For zeta potential, Smoluchowski's approximation was used for the measurement. The pH value of the formulations was measured by directly immersing the electrode of a calibrated potentiometer in the suspensions (Model pH21, Hanna Instruments, Brazil). All these evaluations were made with 6 (six) independent experiments in triplicate for each batch and at room temperature.

DIM Quantification in the Nanocapsule Suspensions

For total DIM content in the NC suspensions ($n = 6$), an aliquot of 100 μL of each formulation (NC-PEC-D, NC-PERS-D, NC-AEC-D, NC-AERS-D, see Table I) was diluted in 10 mL of methanol, filtered through a 0.45-μm cellulose membrane and injected into the HPLC system. The DIM encapsulation efficiency was assayed as follows: 300 μL of the NC suspension was transferred to a centrifugal device containing an ultrafilter (Amicon® Ultra, 10,000 MW, Millipore), which was subjected to the ultrafiltration/centrifugation technique (2200×*g* for 10 min). Free DIM was determined in the ultrafiltrate, while the entrapped active compound was calculated according to the following equation:

$$\mathrm{EE} = \frac{\text{Total DIM content} - \text{Free DIM content}}{\text{Total DIM content}} \times 100 \quad (1)$$

In Vitro Assessment of Biological Properties of DIM Nanoformulations

Photostability Evaluation of DIM-Loaded Nanocapsule Suspensions

For the photostability evaluation, an aliquot of each formulation containing DIM (700 μL) was placed in plastic cuvettes equidistantly organized inside a mirrored chamber (1 m × 25 cm × 25 cm) containing an UVC source of light (Phillips TUV lamp, 30 W). At predetermined intervals, an aliquot of the formulations was withdrawn and the DIM content was determined by the previously described HPLC method. For comparison purposes, a methanolic DIM solution (free compound) was simultaneously evaluated. In addition, a dark control (cuvettes protected from light) was carried out to discard possible degradation caused by temperature or any other influence of experimental conditions. The evaluation was performed with 6 (six) independent experiments in triplicate for each batch.

Determination of ABTS and DPPH Radical Scavenging Capacity

The ABTS and DPPH radical scavenging capacity of the formulations was assessed as described by Re *et al.* (25) and by Sharma and Bhat (26), respectively. DIM-loaded NCs and blank NCs were diluted in distilled water, pure DIM in ethanol, pure PO, and pure AO in DMSO. The samples were evaluated at concentrations of 2.0, 4.0, and 6.0 μg/mL. The ABTS radical cation solution (0.3763 mM) was prepared by mixing ABTS stock solution (7 mM) with potassium persulfate (140 mM), 12 h before the assay (final ABTS concentration 42.7 μM). The DPPH was dissolved in methanol and used as obtained (50 μM). The samples were incubated with ABTS or DPPH solution during 30 min under light protection. Following, each sample was mixed with sodium lauryl sulfate (10% *w/v*) and the absorbance was measured using a UV/Vis spectrophotometer (Shimadzu, Japan) at 734 nm (ABTS assay) or 517 nm (DPPH assay). Pure DPPH or ABTS and ascorbic acid solutions were used as negative and positive controls, respectively. The radical scavenging activity for both tests was expressed as percentage of scavenging capacity, as follows:

$$\mathrm{SC}\% = 100 - \frac{(\mathrm{Abs} - \mathrm{Ab}b) \times 100}{\mathrm{Ab}c} \quad (2)$$

where SC% is the scavenging capacity in percentage, Abs is the absorbance of the incubated sample with DPPH, Abb is the blank sample absorbance, and Abc is the negative control absorbance. These experiments were carried out with 6 (six) independent experiments in triplicate for each batch.

In Vitro Release Studies

The evaluation of DIM release from the formulations NC-PEC-D, NC-PERS-D, NC-AEC-D, and NC-AERS-D was performed using the dialysis bag diffusion method. An aliquot of 2 mL of the formulation was placed inside the dialysis membrane (molecular weight cut-off 10,000 Da, Sigma-Aldrich) and immersed in 200 mL of the release medium (phosphate buffer pH 7.4/ethanol 70:30). This system was kept at 37 ± 2°C under magnetic stirring, and sink condition was maintained over the experiment. At predetermined periods, 1 mL of the external medium was collected and the same volume of fresh medium was replaced. The quantity of DIM released from the NC suspensions was assessed by the HPLC method previously described. For comparison purposes, a methanolic solution of DIM (free compound) was simultaneously evaluated. The experiment was conducted with 6 (six) independent experiments in triplicate for each batch. The results were expressed as percentage of DIM released over time.

The mathematical modeling was performed to determine the kinetic and mechanism of DIM release from the NCs. The experimental data fitted to first order equation (Eq. 3) and Korsmeyer-Peppas model (27) (Eq. 5), using the Scientist software 2.0 (MicroMath®, USA).

$$C = C_0.e^{-kt} \tag{3}$$

$$t_{1/2} = \frac{0.693}{k} \tag{4}$$

$$ft = a.t^n \tag{5}$$

where C is the concentration of DIM at the time t, C_0 is the initial concentration of the DIM, k is the first order rate constant, ft is the fraction of drug released at the time t, a is the constant incorporating structural and geometric characteristics of the nanostructured system, and n is the exponent related to the release mechanism ($n = 0.43$ for drug diffusion; $n = 0.85$ for polymer degradation; $0.43 < n < 0.85$ for anomalous transport).

General Cell Culture Procedures

The human malignant glioblastoma cell line (U87MG) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown and maintained in low-glucose DMEM containing fungizone (0.1%) and penicillin/streptomycin (100 U/L) and supplemented with FBS (10%). After the cultures reached confluency, glioma cells were seeded in 96-well plates at 5×10^3 cells/well in DMEM/FBS (10%) and were kept at 37°C in a humidified atmosphere with 5% CO_2 for 24 h.

Cell Viability Assay

The plate containing U87 cells was incubated with free DIM, free oils (PO and AO), NC-PEC-D, NC-PERS-D, NC-AEC-D, and NC-AERS as well as with the respective blank formulations, at concentrations of 3.0, 6.0, 12, and 24 μg/mL. Control cells were treated with vehicle, *e.g.*, 1% of DMSO. Following 72 h of treatment, cell viability was determined by 3(4,5-dimethyl)-2,5diphenyltetrazolium bromide (MTT) assay. This method is based on the ability of viable cells to reduce MTT to blue formazan products. The MTT solution was added to the incubation medium in the wells at a final concentration 0.5 mg/mL, under light protection until the formation of violet formazan crystals (around 90 min). Later, the solution was then removed and an amount of DMSO was added to each well. The optical density of each plate was measured at 492 nm and the results were expressed by cell viability (%). Six independent experiments were performed using triplicates.

Statistical Analysis

The results were expressed as mean ± standard deviation. Data normality was evaluated by the Kolmogorov-Smirnov normality test. The statistically significant difference was calculated by means of one-way ANOVA of ordinary or repeated measures followed by Tukey's test. The GraphPad Prism software version 7 (San Diego, CA, USA) was used to perform these analyses. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Preformulation Studies

The DIM solubility in both PO and AO was investigated. Values obtained were 4.3 ± 0.7 mg/mL and 7.1 ± 1.2 mg/mL, respectively. Regarding a possible undesirable interaction between the vegetable oils and polymers used to develop DIM NCs, the film masses modified less than 5% after 60 days of experiment ($p < 0.05$), suggesting no polymer solubilization in the tested oils.

Preparation and Characterization of NC Suspensions

Macroscopically, all formulations presented turbid aspect and opalescent bluish reflection, which are characteristics of the chaotic motion presented by the colloidal particles. Table II depicts the physicochemical characterization of the NC suspensions. The mean diameter was not significantly influenced by the different oils and polymers used in the preparation of the nanostructures ($p > 0.05$). The polydispersity indexes were less than 0.23 to all the formulations ($p > 0.05$). The zeta potential values were negative for NCs composed by EC, while suspensions prepared with ERS presented positive values. The pH values were slightly more acidic for the DIM-loaded NCs containing ERS than those formulated with EC ($p < 0.05$). Concerning DIM content, all formulations presented values close to the theoretical concentration (1 mg/mL), regardless the type of polymer and oil used to prepare the formulations, indicating minimal losses and discarding a possible compound degradation throughout the formulation preparation process. Encapsulation efficiency was

Table II. Physicochemical Characterization of the NC Suspensions ($n = 6$ ± mean)

Nanocapsule	Mean diameter (nm)	PDI	Zeta potential (mV)	pH	Drug content (mg/mL)
NC-PEC-B	165 ± 25	0.23 ± 0.09	− 13.4 ± 1.0	6.2 ± 0.6	–
NC-PEC-D	157 ± 39	0.10 ± 0.01	− 5.9 ± 3.2	5.8 ± 0.2	0.95 ± 0.37
NC-AEC-B	171 ± 42	0.15 ± 0.02	− 13.2 ± 2.0	5.8 ± 0.6	–
NC-AEC-D	156 ± 13	0.11 ± 0.02	− 8.9 ± 2.0	5.9 ± 0.2	0.96 ± 0.33
NC-PERS-B	200 ± 70	0.19 ± 0.08	8.7 ± 2.6	5.2 ± 0.2	–
NC-PERS-D	154 ± 10	0.13 ± 0.04	10.9 ± 4.6	5.9 ± 0.0	0.97 ± 0.60
NC-AERS-B	180 ± 25	0.20 ± 0.08	11.5 ± 0.9	5.5 ± 0.7	–
NC-AERS-D	194 ± 34	0.18 ± 0.07	11.8 ± 6.4	5.0 ± 0.3	0.96 ± 0.26

PDI, polydispersity index

97% for NC-PEC-D and NC-PERS-D, 94% for NC-AEC-D, and about 92% for NC-AERS-D.

Photostability Evaluation

The results of photostability studies are depicted in Fig. 1. After 150 min of experiment, the DIM content reduced to 40.0 ± 1.8% in the methanolic solution while the formulations had around 90% of DIM content. At the end of the experiment, only 28 ± 0.6% of DIM remained in the methanolic solution, while the final DIM content was 81 ± 7.1%, 76 ± 5.0%, 80 ± 2.5%, and 84 ± 5.4% to NC-PEC-D, NC-PERS-D, NC-AEC-D, and NC-AERS-D, respectively, demonstrating a significant decrease in DIM content in comparison to the DIM-loaded NC suspensions ($p < 0.001$). These results showed that the nanoencapsulation increased around 3-folds the photostability of DIM independent of the type of oil and polymer used to prepare the nanostructure. The dark control exhibited 100% of DIM, which indicates that the degradation occurred only because of the radiation exposure (data not shown).

Determination of DPPH Scavenging Capacity

Figure 2 displays the results of DPPH assay. Except the NC-PERS-D formulation, the nanoencapsulation provided an increase in the DIM DPPH scavenging activity in comparison to its free form. At 2 μg/mL, the formulation NC-AERS-D presented DPPH scavenging capacity of 41.0 ± 2.5%, which was significantly higher than that of the pure DIM (25.0 ± 6.0%) ($p < 0.05$). At 6 μg/mL, NC-AERS-D and NC-PEC-D improved the antiradical capacity (65.0 ± 8.2% and 71.0 ± 6.6%, respectively) in comparison to DIM methanolic solution (47.0 ± 1.6%; $p < 0.05$). Besides, at this concentration, the formulation NC-PEC-D demonstrated no significant difference for scavenger property with ascorbic acid solution, a powerful antioxidant used as reference (95.0 ± 0.4%) ($p > 0.05$). Both vegetable oil solutions showed low scavenger activity, about 6.0 ± 0.5% and 3.0 ± 0.8% for PO and AO, respectively. In contrast, the blank NCs containing the oils had superior scavenging capacity in comparison to their pure forms ($p < 0.05$).

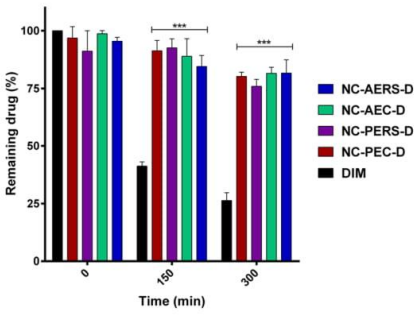

Fig. 1. DIM remaining proportion after the NC suspension and the methanolic solution exposure to UVC radiation. Each bar represents the mean ± SD of six independent experiments. The asterisks denote the significant levels when compared to DIM methanolic solution (DIM) (one-way ANOVA of repeated measures, followed by Tukey's test). ***$p < 0.001$

Determination of ABTS Scavenging Capacity

The results displayed in Fig. 3 show that regardless the NC composition and concentration tested, all the formulations containing DIM had scavenging capacity (63–100%) superior than free DIM (50–63%; $p < 0.05$). In addition, the blank NCs containing the oils increased their scavenger capacity in comparison to the pure oils ($p < 0.05$).

In Vitro Release Studies

The results of DIM release profile are shown in Fig. 4. The nanoencapsulation controlled the DIM release in comparison to the methanolic solution, regardless the nanoformulation, after the first 30 min of experiment ($p < 0.05$). The free DIM (methanolic solution) released around 100% of its content after 12 h of experiment, while NC-PEC-D released 78.0 ± 4.0% and NC-AEC-D, NC-PERS-D, and NC-AERS-D released 68 ± 2.9%, 66 ± 3.5%, and 58 ± 5.3%, respectively, over a period of 84 h. Besides, NC-PERS-D release profile was not significantly different from NC-PEC-D and NC-AEC-D, regardless the time of experiment ($p > 0.05$). NC-AERS-D showed a significant slower release ($p < 0.01$) than NC-PEC-D after 12 h of experiment and differed from NC-PERS-D and NC-AEC-D after 24 h ($p < 0.001$). NC-PEC-D and NC-AEC release profiles only showed significant difference at 72 h and 84 h ($p < 0.01$ and $p < 0.001$, respectively).

The mathematical modeling of the experimental data showed that free DIM and DIM-loaded NCs fitted to the first order equation ($r > 0.989$ for DIM and $r > 0.992$ for DIM-loaded NCs). The dissolution rate constant (k) was approximately

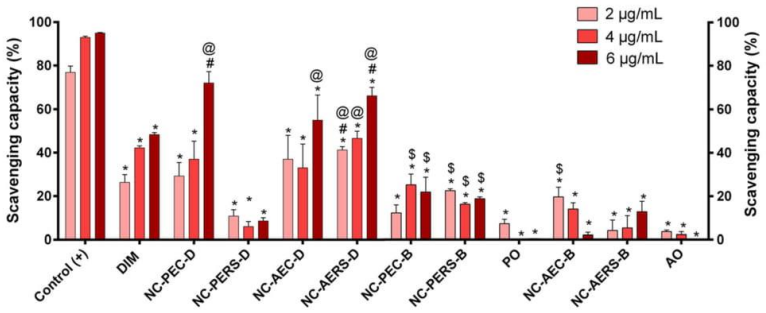

Fig. 2. DPPH radical scavenging capacity of pure DIM, pure oils, NCs with DIM, and the respective blank NCs. Each bar represents the mean ± SEM of six independent experiments (ordinary one-way ANOVA, followed by Tukey's test). *$p < 0.05$. Significant difference between samples and positive control. #$p < 0.05$. Significant difference between pure DIM and DIM-loaded NCs. @$p < 0.05$. Significant difference between NCs with and without DIM. $$p < 0.05$. Significant difference between blank NCs and pure oils

0.01 h^{-1} for NC-AERS-D and 0.02 h^{-1} for other formulations, while for pure DIM it was 0.28 h^{-1}, showing slower DIM dissolution from the NCs than from the pure drug. In addition, the release exponent calculated according to the Korsmeyer-Peppas equation suggested anomalous transport as a release mechanism of the NC suspension (Table III).

Evaluation of Nanocapsule Cytotoxicity Against Glioma Cells

The *in vitro* antiglioma effect of all the nanocapsule suspensions was investigated in U87MG cell line employing MTT test (Fig. 5). Free DIM reduced the U87MG cell viability only at higher concentrations (12 and 24 µg/mL, 78.0 ± 1.5% and 38.0 ± 3.0%, respectively; $p < 0.01$; $p < 0.001$), while the DIM-loaded NCs had antiglioma effect in all concentrations tested. Importantly, NC-PEC-D and NC-AERS-D at the concentration 3 µg/mL presented significantly higher cytotoxic effect (77.0 ± 0.5% and 85.3 ± 0.5%, respectively) in comparison to free DIM (100.0%) ($p < 0.001$). At 6 µg/mL, besides NC-PEC-D and NC-AERS-D, NC-PERS-D also presented higher cytotoxic effect (83.6 ± 1.0%) than free DIM (96.0 ± 3.4%) ($p < 0.01$). At 24 µg/mL, free DIM and all the NCs showed significant lower cell viability than the control and it was possible to observe that NC-PEC-D (38.0 ± 2.0%) showed similar cytotoxic potential to free DIM. Overall, the incubation with blank formulations also reduced the U87MG cell viability, but the pure oils had no significant antitumor effect independent of the concentration tested ($p > 0.05$). Nevertheless, it is important to mention that the highest concentration tested (24 µg/mL) in the formulation NC-PEC-D caused a significant higher cytotoxic effect ($p < 0.05$) in comparison to the respective blank formulation, NC-PEC-B. Regardless the concentration tested, the statistical analysis showed that NC-PEC-D presented the most cytotoxic effect among all the DIM-loaded NCs.

DISCUSSION

Preformulation evaluations are very important for this study, since there are no reports in the literature about the preparation of DIM-loaded polymeric NCs as well as the association of EC or ERS with the proposed oils (PO or AO). To ensure the formation and maintenance of this type of supramolecular structure, the chosen polymer must be insoluble in both oily and aqueous phases. Besides, the absence of polymer swelling by the oil is also required (8,28). Our results indicate that there was no modification in

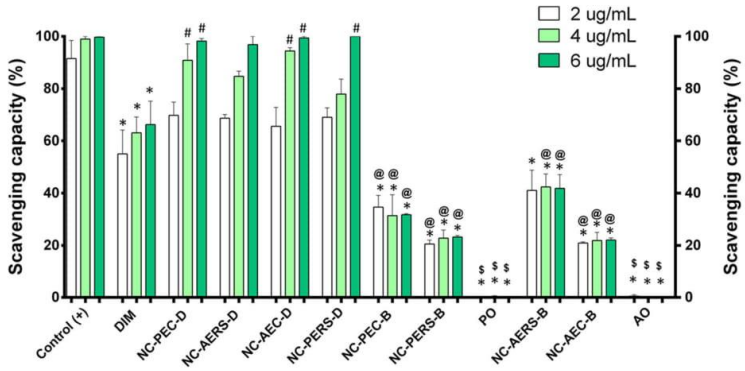

Fig. 3. ABTS radical scavenging capacity of pure DIM, pure oils, NCs with DIM, and the respective blank NCs. Each bar represents the mean ± SEM of six independent experiments (ordinary one-way ANOVA, followed by Tukey's test). *$p < 0.05$. Significant difference between samples and ascorbic acid (positive control). #$p < 0.05$. Significant difference between pure DIM and DIM-loaded NCs. @$p < 0.05$. Significant difference between NCs with and without DIM. $$p < 0.05$. Significant difference between blank NCs and pure oils

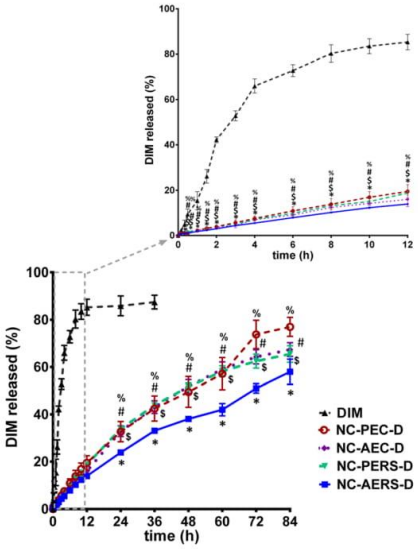

Fig. 4. DIM release profiles from methanolic solution (DIM) and DIM-loaded nanocapsules (NC-PEC-D, NC-AEC-D, NC-PERS-D, NC-AERS-D). Each point represents the mean ± SD of six independent experiments. %$p < 0.05$. Significant difference between NC-PEC-D and control (DIM). #$p < 0.05$. Significant difference between NC-AEC-D and control (DIM). \$$p < 0.05$. Significant difference between NC-PERS-D and control (DIM). *$p < 0.05$. Significant difference between NC-AERS-D and control (DIM). (One-way ANOVA of repeated measures, followed by Tukey's test)

the weight of the polymeric films over time, suggesting that both PO and AO were suitable materials for the NC preparation. Furthermore, due to the high DIM lipophilicity, its solubilization in both oils was already expected, which reinforces the feasibility of preparation of DIM-loaded NCs with primula or apricot oily core. Thus, these data demonstrate that the materials used to prepare the NC formulation are compatible.

Several scientific studies report that the nanoprecipitation methods usually generate particles in the range of 100–500 nm (13–15,29–33), which are in accordance with the present study. NCs are instantaneously obtained by rapid solvent diffusion with slow injection of an organic solution (formed by a semi-polar solvent, an aqueous insoluble polymer, oil, and drug) into an aqueous phase, in the presence of surfactants, which allows the encapsulation of poorly soluble drugs. Besides, this method is suitable to deliver compounds that are intended for pharmaceutical or dietary application, because the organic solvent can be completely removed from the formulation (8,11,34). In our study, colloidal particles were obtained in the nanoscale (154–200 nm), regardless the oil or polymer used in the NC composition and the presence of DIM. All formulations presented low polydispersity indexes (< 0.25), indicating narrowed particle size distribution and appropriate system homogeneity.

The zeta potential of colloidal systems depends on the composition of the particle as well as on its characteristics in the external phase of the suspension. Such parameter is used as an indirect measurement of the nanoparticle's surface charge nature and intensity (8,11,35). In our study, the oil type did not influence this parameter, but the formulations composed of ERS showed positive values probably due to the presence of quaternary ammonium groups in the polymer chains (13,29,30), while EC formulations were negatively charged, reflecting the polymer anionic nature. Besides, in an attempt to minimize the *in vivo* opsonization process and to improve the NC stabilization, the polysorbate 80, a biodegradable copolymer with hydrophilic segments, was used as a non-ionic stabilizing agent. Hence, the values found in this study are in accordance with the properties presented by the raw materials employed in the constitution of the NCs and predicted adequate system stabilization. As observed for zeta potential, pH value results depended on the polymer used in the NC preparation. The formulations containing ERS showed pH values in the range of 5.0–5.9, while EC NCs exhibited higher values (5.9–6.2), which were similar to previous studies that used the same polymers (13,15,36).

The results of drug content indicate minimal active leakage during the NC preparation. Also, all formulations presented high values of entrapment efficiency, which could be explained considering that (i) the interfacial deposition of the preformed polymer method generally ensures adequate active encapsulation; (ii) DIM has high solubility in both vegetable oils tested; and (iii) DIM is a water-insoluble compound, with a high log *P* value (4.05). Considering such characteristics, it is possible to suggest that DIM presented a superior affinity with the oily core and/or with the hydrophobic polymeric wall than to the external aqueous phase of the colloidal suspension, which prevented its partitioning to the aqueous phase.

In addition, the physicochemical properties found in this study are in agreement with previous studies that reported the development of DIM-loaded nanostructures. Luo and collaborators (20) developed zein and zein/carboxymethyl chitosan nanoparticles containing DIM, presenting mean size in the

Table III. Calculated Parameters for First Order and Korsmeyer-Peppas Equations for Free DIM, and DIM-Loaded Nanocapsules (NC-PEC-D, NC-AEC-D, NC-PERS-D, NC-AERS-D)

	DIM	NC-PEC-D	NC-AEC-D	NC-PERS-D	NC-AERS-D
First order					
R	0.989	0.997	0.995	0.992	0.997
k (h^{-1})	0.28	0.02	0.02	0.02	0.01
t½ (h)	2.5	46.2	36.4	43.3	63.9
Korsmeyer-Peppas					
R	–	0.998	0.995	0.995	0.998
N	–	0.73	0.80	0.77	0.74

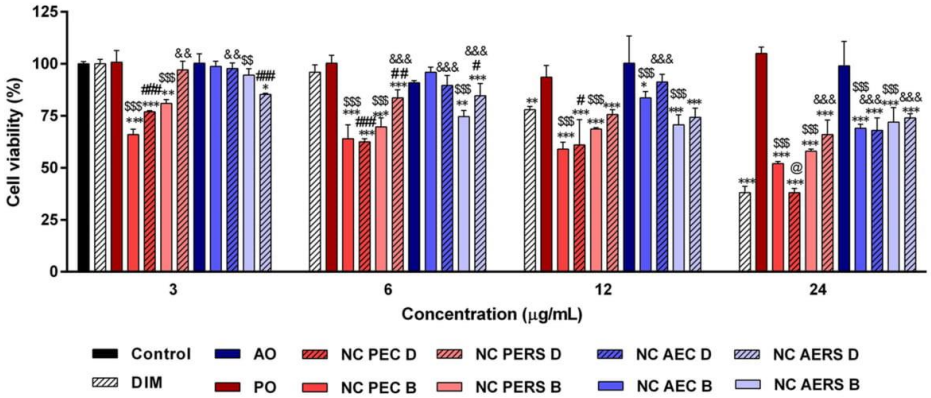

Fig. 5. In vitro cell viability of human glioblastoma cells (U87MG) after 72 h of incubation with crescent concentrations of pure DIM, PO, AO, DIM-loaded nanocapsules (NC-PEC-D, NC-PERS-D, NC-AEC-D, NC-AERS-D), and blank nanocapsules (NCPEC-B, NC-PERS-B, NC-AEC-B, NC-AERS-B) by the MTT reduction assay. Each bar represents the mean ± SEM of six independent experiments. (Ordinary one-way ANOVA, followed by Tukey's test). (*) $p :< 0.05$, (**) $p < 0.01$, (***) $p < 0.001$: significant difference between treatments and control. (#) $p < 0.05$, (##) $p < 0.01$, (###) $p < 0.001$: significant difference between pure DIM and DIM-loaded NCs. (@) $p < 0.05$: significant difference between NCs with and without DIM. (&&) $p < 0.01$, (&&&) $p < 0.001$: significant difference in comparison with NC-PEC-D at the same concentration. ($) $p < 0.05$, ($$) $p < 0.01$, ($$$) $p < 0.001$: significant difference between blank NCs and pure oils

range 89–250 nm, polydispersity indexes lower than 0.20, and negative zeta potentials. However, it is important to mention that the NCs developed in our study showed higher encapsulation efficiency as well as a DIM content efficiency 20-folds greater than that of the nanoparticles obtained by Luo and collaborators (encapsulation efficiency and content efficiency of 69–78% and 50 μg/mL, respectively). These findings could be explained by a better solubilization/dispersion of DIM promoted by the vegetable oil contained in the inner core of the NCs. Indeed, another study supports the hypothesis of improvement of DIM encapsulation in the presence of oily substances. Boakye and col. (37) also reported high entrapment efficiency values for a DIM-derivative compound encapsulated in cationic liposomes. The authors developed a formulation containing a mixture of phospholipids, cholesterol, and polysorbate 80 by the ethanolic injection method and obtained liposomes presenting DIM-derivative encapsulation rate of approximately 91% and drug content of 50 μg/mL.

Considering that it was already defined that DIM photolability was due to the presence of aromatic rings in its structure (20), we evaluated if its nanoencapsulation could prevent its degradation in an accelerated condition (under UVC light). The results demonstrated that independent of the NC composition, all formulations enhanced DIM photostability. This improvement may be due to the structural characteristics of the NCs, which can absorb or scatter the incident radiation, acting as a physical barrier against the UV light (13,30,38,39). Luo and collaborators (20) also observed that DIM nanoencapsulation improved its photostability, which was attributed to the double coating of zein and carboxymethyl chitosan nanoparticles.

It is well known that compounds with high radical scavenging capacity are promising candidates to the management of several diseases. In our study, the scavenger potential of the formulations was determined *in vitro* using the DPPH and ABTS radical assays. Both methods are simple, precise, and useful to evaluate the antioxidant potential of vegetable oils and extracts, pharmaceutical formulations, and pure or nanoencapsulated compounds (25,40,41).

The DPPH assay is based on a reaction that reduces DPPH to diphenyl-picrylhydrazine, which changes the reaction medium from deep-violet to light-yellow color that can be measured on a UV/*v*isible light spectrophotometer at 517 nm (41,42). However, this technique lacks selectivity because of spectral interferences. Hence, the association of DPPH assay with another method is recommended. In this context, ABTS cation solution presents a blue/green color and its reduction by a hydrogen-donating antioxidant is measured by the extent of decolorization and consequent decrease of its characteristic absorption at 734 nm (40). This method is considered more versatile, because the measure is not interfered by the spectra of complex products and it is useful to evaluate the antiradical activity of compounds in several media, including phosphate-buffered solution pH 7.4, which simulates a physiological environment (25,40). In our study, ABTS findings corroborated the results obtained for DPPH assay.

The DIM scavenging capacity could be explained by the presence of two N–H groups in its structure, which can act as H-donating groups and neutralizes free radicals (40,43). Overall, the results of our study showed that the nanoencapsulation increased the radical scavenging activity in comparison to pure DIM and pure oils. Previous studies suggested that the nanometric size of the particles in suspension could provide superior contact surface area between the H donator and DPPH or ABTS molecules, facilitating the access of the hydrogen atom to the radical site (14,15,44,45). Besides, blank NCs also showed scavenging capacity. Therefore, the antioxidant substances present in both PO and AO could contribute for the improved effect verified for DIM-loaded NCs. This finding is in accordance with a previous study of our group that reported superior scavenger capacity of pomegranate oil NCs than the pure oil (15).

The *in vitro* release profiles demonstrated that DIM release from all the DIM-loaded NCs fitted to first order equation, indicating that release rate depends on the concentration and occurs in a single step. Furthermore, the absence of burst release

reinforces the total DIM encapsulation in the oil core, corroborating the values of DIM encapsulation efficiency. It should be noted that even pure DIM showed a slow release, which can be attributed to its high lipophilicity. In the NCs, DIM may be dissolved in the oily core and/or adsorbed onto the hydrophobic polymeric wall. The presence of the oil and the polymer could act as additional barriers to DIM release, slowing down its diffusion from the nanostructures to the medium. Regarding the release mechanism, *n* values of 0.73–0.80 were found for NCs, which suggests that drug release is driven by anomalous transport, where the drug diffuses from the oil to the particle/water interface, followed by the polymer chain relaxation. These results are in accordance with the characteristic of the polymers used in the NC preparation, because both EC and ERS are water-insoluble polymers used to promote sustained-release profiles (13,15,29,46).

Considering the promising results showed by the developed NCs, their cytotoxicity was tested against a human U87MG glioma cell line to evaluate the biological performance of formulations. This study demonstrated interesting results even with lowest concentrations because the DIM-loaded NCs caused a significant increase in the cytotoxicity in comparison with free DIM. Observing the highest concentration, both compound forms as well as the blank formulation reduced the U87MG cell viability. Such results could be explained by the DIM slow and incomplete release from the nanocapsules within the incubation time (72 h), whereas the solubilized DIM could be more available to interact with the cells, as shown in previous studies (47–50). In addition, the cytotoxicity observed for blank nanocapsules can be possibly explained by two reasons: (i) the presence of polysorbate in the constitution of the formulation. This component may disrupt the cell membranes and increase cell permeability; (ii) the nanoparticles can adhere to the cell membrane causing the release of cytotoxic products or uptake triggering cell death (51–54). In comparison with the blank nanocapsules, the improvement of the cytotoxic effect by the DIM nanoencapsulation could be evidenced in the highest concentration (24 μg/mL). At this concentration, the formulation NC-PEC-D was remarkably more cytotoxic than NC-PEC-B, highlighting the role of DIM and reinforcing the positive impact of its nanoencapsulation in biological properties of DIM.

The DIM antitumor property was previously reported, but its physicochemical restrictions could represent a limitation to further studies aiming at its therapeutic application (5–7). It is important to highlight that the DIM nanoencapsulation enhanced the antiglioma effect because in concentrations where the free compound had no action, the NC-PEC-D caused a reduction in U87MG cells viability. These results are in accordance with other authors that reported an improvement of *in vitro* and *in vivo* biological effects by associating active substances in nanocarrier systems (14,30–32,55).

Taking into account the above-mentioned results, together with the other *in vitro* experiments (determination of DPPH and ABTS scavenging capacity), the present study clearly showed the advantages of the DIM-loaded NCs, especially NC-PEC-D. The superior photostability, antioxidant effect, and controlled drug release presented by the developed NCs reinforce the positive impact of DIM nanoencapsulation in enhancing its biological properties.

CONCLUSION

In conclusion, this study showed the feasibility of preparing DIM-loaded NCs based on two different vegetable oils and polymers. All formulations presented adequate physicochemical characteristics and were able to enhance DIM photostability and radical scavenger property. The NC suspension composed by PO and EC seemed to be the most promising system because it provided a prolonged DIM release as well as increased its cytotoxic effect. Overall, the nanoencapsulation promoted a general improvement in the physicochemical characteristics and biological properties of DIM, indicating that such approach is an interesting alternative for future studies regarding the management of different pathological conditions.

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3.2 MANUSCRITO 1

3,3'-DIINDOLYLMETHANE NANOENCAPSULATION IMPROVES ITS ANTINOCICEPTIVE ACTION: PHYSICOCHEMICAL AND BEHAVIORAL STUDIES

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Abstract

This study aimed to characterize the physicochemical properties of 3,3'-diindolylmethane (DIM)-loaded nanocapsules (NCs) as well as the antinociceptive effect using distinct animal models (hot plate test, formalin-induced nociception and complete Freud's adjuvant induced paw inflammation). The DIM-loaded NCs (composed by primula oil and ethylcellulose) were characterized using differential scanning calorimetry, thermogravimetric analysis, Fourier-transformed infrared spectroscopy, X-ray diffractometry and scanning electron microscopy. The physicochemical characterization demonstrated that DIM could be molecularly dispersed into the NCs, whose size was nanometric with a spherical shape. An improvement in DIM thermal stability was achieved by its encapsulation and and there were no interactions among the formula components. For the nociceptive evaluation, male adult *Swiss* mice were pretreated with the NCs or free DIM by the intragastric route at the dose of 10 mg/Kg (time-response curve), 5 or 2.5 mg/Kg (dose-response curve). The behavioral tests were performed over an experimental period of 0.5-8 h. Both free and nanoencapsulated DIM reduced the mechanical hypernociception induced by CFA, mitigated nociceptive behavior of formalin-induced neurogenic and inflammatory pain and increased paw withdrawal latency assessed by the hot-plate test. Importantly, the DIM nanoencapsulation promoted a rapid initiation and prolonged the bioactive antinociceptive action (up to 8 h) as well as reduced the effective dose in comparison to its free form. In summary, this study reported that the NCs had adequate nanometric size, increased DIM stability and its antinociceptive action in different animal models, suggesting that the formulation may be a possible therapeutic alternative to the management of pain and inflammatory-related pathologies.

Key-words Indole-3-carbinol; Phytochemical; Nanoparticles; Inflammation; Pain; Animal model.

1. Introduction

Pain is a complex experience involving several peripheral and central physiological modulators as well as cognition and emotional processes [1,2]. Physiologically, pain exerts an essential role in survival, acting as an alarm to protect the body from any

hazardous stimuli [3,4]. It is well known that pain and inflammation are related processes [5,6]. Exacerbated inflammation presents classical signs including pain and function loss of the affected tissue. Besides, hypersensitivity of the nociceptors at the site of damage and in adjacent tissue occurs due to the presence of inflammatory mediators such as prostaglandin E_2, bradikynin, tumor necrosis factor α (TNF-α), ions and arachidonic acid [7,8]. However, when persistent, pain becomes stressful, triggering the impairment of several economic and social aspects and disrupting the patients' quality of life [1,4]. The class of anti-inflammatory drugs is usually employed as analgesics in the clinical pain management of inflammatory pain. However, they lack efficacy and have several adverse effects and safety issues which compromise treatment efficacy [9,10].

In this sense, the use of nanotechnology aiming at developing nanocarriers as drug delivery platforms can be considered a promising approach to improve the delivery of pharmacologically active drugs. For analgesic drug delivery systems, the nanostructures provide protection of the loaded drug from degradation, prolong drugs release and duration of their pharmacological action, and, consequently, reduce adverse effects and toxicity [4,11]. In fact, previous scientific studies reported the positive impact of the nanoencapsulation in enhancing the antinociceptive effect and safety of different non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin [12], nimesulide [13], meloxicam [14] and ketoprofen [15].

In this context, a recent study conducted by our research group reported for the first time the antinociceptive action of indole-3-carbinol, the phytochemical precursor of 3,3'-diindolylmethane (DIM) [16]. The DIM is the indole-3-carbinol dimerized form, which is produced after the ingestion of cruciferous vegetables and is reported to present anti-inflammatory effects. This bioactive substance inhibits the activity of cyclooxygenase-2, matrix metalloproteinases and inducible nitric oxide synthase; besides, DIM decreases interleukin-8 levels and increases interferon-gamma production [17–20]. Interestingly, Asirvatham and collegues [21] reported that the presence of aromatic ring and lipophilic substituents are important molecular features in the indole structure, suggesting that such groups play an important role in anti-inflammatory action elicited by 3-substituted indole derivatives. It is worthy to mention that those structures are also observed in the DIM molecule, which

reinforce the hypothesys regarding the therapeutic actions. Despite its therapeutic potential, DIM is water-insoluble and photolabile, which compromises its pharmaceutical use [22–25].

In order to circumvent these limitations and improve DIM biological properties, we recently developed a novel DIM-loaded nanocapsule formulation (NC-PEC-D) containing primula oil and ethylcellulose. Nanocapsules (NCs) are composed by an oily core surrounded by a polymeric membrane and are considered a suitable system to incorporate lipophilic drugs. The DIM nanoencapsulation enabled a controlled/sustained release from the nanostructures, increased its photostability and enhanced the DIM scavenging capacity and *in vitro* antitumor effect against a glioma cell line (U87) [26]. Despite these data, no information is known regarding the possible antinociceptive properties of the free compound and the impact of nanoencapsulation on the DIM pharmacological properties.

Taking the aforementioned considerations into account, we aimed to supply further information regarding the physicochemical properties of DIM-loaded NCs. In addition, this study investigated the antinociceptive effect of free DIM or DIM-loaded NCs, in an inflammatory pain model induced by complete Freund's adjuvant (CFA) as well as in the hot plate and formalin-induced nociceptive tests.

2. Materials and methods

2.1 Chemical and reagents

DIM (99.2% purity) was supplied by Active Pharmaceutica (Brazil). Ethylcelullose (EC) was a gift from Colorcon (Brazil). Tween® 80 (polisorbate 80), Span® 80 (sorbitan monooleate), Complete Freund's Adjuvant (CFA) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co (USA). Primula oil was purchased from Mundo dos Óleos (Brazil). All other chemicals and solvents were analytical grade and used as received.

2.2 Animals

The present study was approved by the Institutional Committee for Animal Care and Use of Federal University of Santa Maria (register number:

4428090217/2017), affiliated to the Council for Control of Animal Experiments (CONCEA) and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The studies were performed using male adult *Swiss* mice weighing 25-35 g, which were obtained from the Federal University of Santa Maria breeding colony. The animals were kept in plastic boxes with controlled temperature (22 ± 2 ºC) and humidity (60%), receiving standard food and water *ad libitum* (GUABI, RS, Brazil), under a 12 h light/dark cycle (7 am to 7 pm). The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent bioactive effects. At the end of the experiments, mice were euthanized by cervical displacement.

2.3 DIM loaded-NCs suspension

NC-PEC-D (DIM final concentration at 1 mg/mL) and NC-PEC-B (NCs without DIM) were prepared and characterized as described previously [26]. Briefly, an organic phase (acetone, ethylcellulose, primula oil, Span® 80 and DIM) was injected into an aqueous dispersion of Tween® 80 and kept under magnetic stirring for 10 min. Later, the acetone and excess water were removed under reduced pressure (rotary evaporator) to concentrate the NCs suspension (1 mg/mL of DIM). To further characterize these NCs, the samples of NC-PEC-D and NC-PEC-B were freeze-dried using 10% lactose (w/v – cryoprotectant) and after 24 h of freezing, the NCs were placed in a freeze-dryer (LIOTOP L101, Liobras, Brazil) and dehydrated during 24 h at -55 ºC. The differential scanning calorimetry (DSC), thermogravimetry (TG), Fourier-transformed infrared spectroscopy (FTIR) and X-Ray powder diffraction (XRPD) techniques were performed. For the Scanning electron microscopy with field emission guns (FEG-SEM) analysis, the NCs were used as obtained (suspensions).

2.4 Thermal analyses

2.4.1.1 Differential scanning calorimetry (DSC).

DSC was performed using aluminum crucibles with 2.5 ± 0.1 mg of sample under dynamic N_2 atmosphere (flow rate: 50 mL/min) in a DSC-60 calorimeter (Shimadzu, Japan). The temperature range was 25–500 °C with a heating rate of 10 °C/min. An empty aluminum pan was used as reference. The DSC cell was calibrated with

indium (m.p. = 156.45 °C; ΔH_{fusion} = 28.54 J/g) and zinc (m.p. = 419.45 °C). The samples analyzed were: pure lactose, pure DIM, pure EC, physical mixture (DIM + EC 1:1) and the freeze-dried formulations.

2.4.1.2 Thermogravimetry analyses (TG)

The raw materials, the pure compound and the freeze-dried NCs were evaluated with the aid of a thermobalance (TGA-50, Shimadzu, Japan), which was previously calibrated with copper sulphate pentahydrate. The temperature ranged between 25–650 °C, using platinum crucibles with 5.0 ± 0.1 mg of sample under dynamic N_2 atmosphere (flow rate: 50 mL/min) and heat flow of 10 °C/min.

2.5 Fourier-transformed infrared spectroscopy (FTIR) analyses

The analyses of samples (raw materials, the pure compound and the freeze-dried NCs) were recorded on an Infrared Prestige-21 spectrophotometer (Shimadzu, Japan), from 4000 to 400 cm^{-1}, employing KBr pellets with 32 scans and resolution of 4 cm^{-1}.

2.6 X-Ray powder diffraction (XRD) analyses

For an XRD assay of raw materials, the pure compound and the freeze-dried NCs, a Shimadzu X-ray diffractometer (Shimadzu XRD-6000, Japan) was employed. The 2θ value was increased from 5º to 80º at a scan rate of 2º/min using a Cu-Kα source (λ = 1.5418 Å) at 40 kV and 40 mA.

2.7 Scanning electron microscopy with field emission guns (FEG-SEM)

The FEG-SEM analyses were carried out with the pure DIM and the NCs suspensions. The samples were mounted on aluminum stubs, sputtered with gold (IC-50 Ion Coater, Shimadzu, Japan) and subsequently analyzed using FEG-SEM equipment (TESCAN, model MIRA3, Czech Republic) at 10 kV with different magnifications.

2.8 *Evaluation of the antinociceptive effect of DIM-loaded nanocapsules*

In order to eliminate the effect of stress, before testing, the animals were acclimatized in an appropriate behavioral room with controlled temperature (25 °C)

and humidity, adequate brightness and soundproofing. The behavioral tests were performed only during the light cycle phase (8 am to 4 pm).

2.8.1 Experimental design and general procedures

This study was divided into two distinct protocols. Experimental protocol I was carried out to determine the time- and dose-response curves of DIM pharmacological action using the formalin-induced nociceptive behavior and hot plate test as well as the locomotor and exploratory activities (**Fig 1S.I**). Then, in Experimental protocol II another set of animals was treated to evaluate the effect of DIM treatment in an acute inflammatory pain model induced by CFA (**Fig 1S.II**).

To assess the pharmacological effects before the behavioral tests, the animals received intragastric (i.g.) administration of DIM or DIM-loaded nanocapsules suspension at a constant volume of 10 mL/Kg of body weight. The free DIM was dissolved in an aqueous solution of Tween® 80 10% and DMSO 10% (vehicle). The formulations (NC-PEC-B and NC-PEC-D) were prepared as previously described (Section 2.3) and used as obtained or further diluted in distilled water to achieve lower DIM doses (for Experimental protocol I). Appropriate vehicle-treated groups were simultaneously assessed to discard any vehicle effect.

2.8.2 Experimental protocol I

The time- and dose-response curves were performed with different sets of animals that were randomly assigned to five groups (n = 6-8 animals/group) for each time and dose evaluated, receiving the following treatments:

- Negative control - the animals received the vehicle (10 mL/Kg);
- DIM - the animals received DIM dissolved in the vehicle (10 mg/Kg);
- NC-PEC-D - the animals received DIM incorporated into the NCs suspension (10 mg/Kg);
- NC-PEC-B - the animals received the NCs suspension (10 mg/Kg);

- Positive control (Ketoprofen group) - the animals received ketoprofen at a dose of 10 mg/Kg [27] dissolved in the vehicle (10 mL/kg), 2 h before the behavioral tests (only for the formalin-induced test).

At pre-determined times after the administration of the treatments (30 min, 1, 2, 4, 6 and 8 h), the animals were subjected to a locomotor and exploratory evaluation (section 2.8.2.1), followed by the hot-plate test (section 2.8.2.2) and formalin-induced nociceptive behavior (section 2.8.2.3). The treatment time-curve was conducted with DIM at a dose of 10 mg/Kg. The specific time of administration used to perform the dose-response curve was chosen according to the best effect obtained in the time-response curve and was carried out with DIM-loaded NCs or free DIM at 5 and 2.5 mg/Kg doses.

2.8.2.1. Locomotor and exploratory activities

To discard non-specific effects of treatments that could be misinterpreted as antinociceptive effect the spontaneous locomotor behavior of mice was evaluated before the nociceptive tests. The animals were individually placed inside the locomotor activity monitor (LAM) center, which consists in an open field Plexiglas chamber (45 cm^3) surrounded by a frame of 32 photocells placed on opposite walls (16L x 16W, 2 cm apart from each other), that continuously tracks the animal movements. The animals were allowed to freely explore the arena during 4 min and the following parameters were recorded: number of crossings, average speed (mm/s) and total distance traveled (cm).

2.8.2.2 Hot plate-induced nociceptive behavior

The hot plate test was used to measure latency time for the nociceptive behavior caused by a thermal stimulus and the experiment was performed as described by Woolfe and MacDonald (1944) [28], with minor modifications. The animals were individually placed in a glass cylinder onto a hot plate metallic surface that was maintained at a temperature of 55 ± 0.5 °C (diameter: 24 cm, Ugo Basile, model DS37). The time between the animal placement onto the apparatus and the nociceptive response occurrence (licking or shaking the hind paws or jumping off the surface) was recorded as paw withdrawal latency (s). The animals were tested before

(pretreatment latency) and after (post-treatment latency) the treatment in order to estimate the possible antinociceptive effect triggered by the tested bioactive/formulation. In order to avoid any damage to animal paws, a cut-off time of 25 s (maximum latency) was established. The results were expressed as percentage maximum possible effect (%MPE), as described in the equation below:

$$\%MPE = \frac{(\text{Post treatment latency}) - (\text{Pre treatment latency})}{(\text{Maximum latency}) - (\text{Pre treatment latency})} \times 100$$

2.8.2.3 Nociception and paw edema induced by formalin

The nociception induced by the formalin procedure was carried out according to Hunskaar and Hole (1987) [29]. After treatment 20 µL of a 2.5% percent formalin solution (0.92% of formaldehyde) were injected under the plantar left hind paw of the mice. Immediately after the formalin injection, the animals were individually placed in acrylic boxes and the time spent licking or biting the injected paw was recorded over 0-5 min (neurogenic phase) and 15-30 min (inflammatory phase). The results were expressed as licking time, in seconds (s).

At the end of the behavioral test, the animals were euthanized as described in Section 2.2 and both hind paws were cut at the ankle joint and weighed on an analytical balance. The paw edema was measured by comparing the difference between the weight of the left paw and the right paw. The results were expressed in mg.

2.8.3 Experimental protocol II

For this protocol, the antinociceptive effect of DIM was assessed through the acute inflammatory pain induced by the CFA injection. The behavioral parameter evaluated was the mechanical hypernociception using the Von Frey Hair (VFH) test. The time-response curve was performed with animals randomly allocated in six groups (*n* = 8-10 animals/group), each one receiving the following treatments:

- Control - the animals received a subcutaneous intraplantar saline injection in the hindpaw and were treated with the vehicle (10 mL/Kg);

- Induced (CFA + vehicle) - the animals received a subcutaneous intraplantar CFA injection in the hindpaw and were treated with vehicle (10 mg/Kg);
- Free DIM (CFA + DIM) - the animals received a subcutaneous intraplantar CFA injection in the hindpaw and were treated with DIM dissolved in the vehicle (10 mg/Kg);
- NC-PEC-D (CFA + NC-PEC-D)- the animals received a subcutaneous intraplantar CFA injection in the hindpaw and were treated with DIM incorporated into the NCs suspension (10 mg/Kg);
- NC-PEC-B (CFA + NC-PEC-B) - the animals received a subcutaneous intraplantar CFA injection in the hindpaw and were treated with the NCs suspension (10 mg/Kg);
- Positive control (CFA + Keto) - the animals received a subcutaneous intraplantar CFA injection in the hindpaw and were treated with ketoprofen at a dose of 10 mg/Kg [30] dissolved in the vehicle (10 mL/kg).

2.8.3.1 CFA-induced inflammatory pain model

The paw inflammation was induced by a subcutaneous intraplantar injection of 20 µL of CFA (1 mg/mL of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate) in the left hind paw of mice. Before the induction, mice were submitted to a screening of their basal paw withdrawal response frequency, determined using the VFH test (Section 2.8.3.2). The animals that had a mean withdrawal frequency >15% were not used in the experiment. Then CFA or saline (control) was injected and 24 h later the animals performed the VHF test.

2.8.3.2. Mechanical hypernociception evaluation

The mechanical hypernociception was estimated using the VFH test (Stoelting, Chicago, IL) according to Bortalanza et al. (2002) [31]. First, the animals were acclimatized during 1 h in individual clear plastic chambers (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the plantar surface of the paws. After 24 h of induction, in order to confirm the mechanical hypernociception induced by CFA, the response frequency was measured after ten applications (duration of 1-2 s each) of a calibrated nylon VFH of 1.0 g on the plantar surface of the left hind paw of each animal. The animals that presented a response frequency <70% were

excluded from the study. Then, the animals were treated as described in Section 2.4.3 and the VFH test was carried out at specific times (30 min, 1, 2, 4, 6 and 8 h). The results were expressed as response frequency to VFH stimulation (%).

2.9 Statistical analysis

The results were expressed as mean ± standard error of the *n* observation means (S.E.M.). Data normality was evaluated by the Kolmogorov-Smirnov normality test. GraphPad Prism software (version 6.0, USA) was used for repeated or ordinary one-way analyses of variance (ANOVA), followed by *post-hoc* Newman-Keuls' test. Values of $p<0.05$ were considered statistically significant.

3 Results

3.1 Thermal analysis

Figure 1 summarizes the DSC curves. Ethylcellulose showed an endothermic peak at 378 ºC (Supplementary material; **Fig. 2S.A**), while pure DIM had a sharp endothermic event around 181 ºC (Supplementary material; **Fig. 2S.B**), which corresponds to its melting temperature. Monohydrate lactose presented two sharp endothermic events at 163 ºC and 235 ºC, corresponding to its dehydration and melting endothermic temperatures, respectively (Supplementary material; **Fig. 2S.C**). As expected, the physical mixture revealed the same endothermic events as the pure substances (Supplementary material; **Fig. 2S.D**). On the other hand, the typical melting event of DIM was not observed in the DSC curve of the freeze-dried NC-PEC-D. Both NCs formulation had melting endothermic events similar to those of lactose (Supplementary material; **Fig. 2S.E** and **Fig. 2S.F**).

Regarding the thermogravimetric analyses, monohydrate lactose exhibited a first degradation step at 150 ºC, which is related to the loss of crystallization water (**Fig. 2**). The next weight loss stages that were referred to lactose degradation started around 220 ºC and 280 ºC (Supplementary material; **Fig. 3S.C**). The pure ethylcellulose degradation step started at 315 ºC (Supplementary material; **Fig. 3S.A**) and pure DIM presented weight loss after 250 ºC (Supplementary material; **Fig. 3S.B**). For the physical mixture (Supplementary material; **Fig. 3S.D**) and both

formulations (Supplementary material; **Fig. 3S.E** and Fig. **3S.F**) the endothermic events started at intermediate temperatures compared to the raw materials.

3.2 FTIR analyses

The FTIR spectra for samples are shown in **Figure 3**. The pure lactose presented a broad band at 3450-3150 cm^{-1} related to O-H stretching vibration (Supplementary material; **Fig. 4S.C**). The ethylcellulose had C-O-C stretching vibrations at 1103 cm^{-1}, that is typical of cyclic ether (Supplementary material; **Fig. 4S.A**). Absorption bands at 1380-1485 cm^{-1} corresponding to CH_X and 2871-2973 cm^{-1} assigned to CH were also recorded. The pure DIM spectrum displayed typical sharp peaks of the indolyl group: NH stretching vibration of the secondary amine functional group at 3400 cm^{-1}, characteristic bands related to C=C aromatic stretching vibrations at 1450-1617 cm^{-1} and bands at 1030 and 1090 cm^{-1}, which are referred to plane C-H bending and absorption signal at 740 cm^{-1} of C-H bending vibration out of plane (Supplementary material; **Fig. 4S.B**). No specific absorption band of DIM was observed in NC-PEC-D spectra (Supplementary material; **Fig. 4S.F**). In general, band assignments for physical mixture (Supplementary material; **Fig. 4S.D**) and both NCs formulations correspond to FTIR spectra superposition of raw materials.

3.3 XRD analyses

Figure 4 depicts X-ray diffraction patterns of raw materials and formulations NC-PEC-B and NC-PEC-D. The pure ethylcelullose profile depicted two crystalline peaks, at 7.78º and 20.51º (Supplementary material; **Fig. 5S.A**). For pure DIM, several diffraction peaks were observed, at 6.45º, 13.48º (main peak), 18.60º, 21.85º and 27.87º, attributed to its crystalline state (Supplementary material; **Fig. 5S.B**). Monohydrate lactose showed few crystalline peaks at 12.44º, 16.20º, 19.94º and 21.04º (Supplementary material; **Fig. 5S.C**). The physical mixture presented a similar profile of each raw material (Supplementary material; **Fig. 5S.D**). In addition, the diffractograms of both formulations (Supplementary material; **Fig. 5S.E** and **Fig.**

5S.F) had some diffraction peaks assigned to lactose (cryoprotectant). The diffraction angles pattern of pure DIM were not observed in the NC-PEC-D diffractogram.

3.4 FEG-SEM

Figure 5 shows the representative image of pure DIM and prepared nanostructures. The pure DIM presented a crystal morphology, with several typical crystalline shapes, while the NCs had a spherical shape and smooth surface, without pores. DIM crystals were not observed on the NC-PEC-D surface. The images also confirmed the nanometric size of the nanocapsules, with an average diameter of 163.40 ± 27.50 nm.

3.5 Evaluation of the antinociceptive effect of DIM-loaded nanocapsules

3.5.1 Locomotor and exploratory activities

One-way ANOVA analysis showed that regardless of the treatment, the number of crossings, distance and average speed evaluated in the LAM were not significantly different (p>0.05) among experimental groups (Supplementary material; **Table 1S**).

3.5.2 Hot plate-induced thermal nociceptive behavior

Figure 6 depicts the results obtained in the hot plate-induced nociceptive test. Both free and encapsulated DIM forms caused an antinociceptive action against the thermal stimuli, but different profiles of effect were observed. While the treatment with free DIM (10 mg/Kg) elicited a significant increase in the latency time 1 h after administration (p<0.001), the group of animals that received the NC-PEC-D showed an antinociceptive effect that began at 1 h and lasted up until 2 h after treatment, in comparison to the control group. Interestingly, at a dose of 5 mg/Kg, only the NC-PEC-D maintained the antinociceptive action (p<0.05) 2 h after treatment (Supplementary material; **Fig. 6S**). Independent of the DIM form, doses lower than 5

mg/Kg did not have any effect on the latency response time. The administration of NC-PEC-B had no effect on the hot plate test.

3.5.3 Nociception and paw edema induced by formalin

The time- and dose response curves showed the effect of DIM administration on its free or nanoencapsulated form in both phases of nociception (neurogenic and inflammatory) (**Figure 7**). In the neurogenic phase (**Fig. 7A**), nociception was blocked by DIM after 2 hours and remained significant until 4 h after treatment (p<0.01). The NC-PEC-D triggered an antinociceptive effect that started at 0.5 h of treatment and remained significant until 8 h after formulation administration. Both free DIM and NC-PEC-D reached the highest antinociceptive effect at 2 h after administration (E_{max}: 34.3 ± 3.6% and 39.3 ± 5.1 for DIM and NC-PEC-D, respectively). Similarly, during the inflammatory phase (**Fig. 7B**), free DIM also produced a significant reduction of the licking time after 2 h (E_{max} 47.8 ± 8.1%) and 4 h of treatment (E_{max} 39.0 ± 4.5%) (p<0.01). Interestingly, the DIM nanoencapsulation anticipated (E_{max} 49.5 ± 9.8% at 1h) and prolonged the antinociceptive action compared to its free form. NC-PEC-D presented a significant reduction of the licking time after 0.5 h of treatment, which was maintained until 4 h later (p<0.01).

In addition, the antinociceptive effect observed for DIM, free or nanoencapsulated, was similar (p>0.05) to the effect produced by ketoprofen (positive control) administration (10 mg/Kg), in both nociception phases. Furthermore, NC-PEC-B also had an effect in both nociception phases, but only after 0.5 h of treatment (p<0.05). Regarding the paw edema formation, independent of the treatment received, no group had a reduction in this parameter (p>0.05) (Supplementary material; **Fig. 7S**).

The results of the dose-response curve of free DIM and NC-PEC-D after 2 h of treatment are depicted in supplementary material (**Figure 8S**). Concerning free bioactive action, no antinociceptive effect was observed regardless of the dose administered (5 or 2.5 mg/Kg; p>0.05). Interestingly, NC-PEC-D at 5 mg/Kg decreased the licking time in both neurogenic (**Fig. 8SA**) and inflammatory phases (**Fig. 8SB**) of the formalin test (p<0.05). Moreover, in the inflammatory phase, the nociceptive inhibition was maintained at the lowest dose tested (2.5 mg/Kg).

3.5.4 CFA-induced inflammatory pain model

The time-course effect of free DIM or NC-PEC-D treatment on the CFA-induced inflammatory pain was investigated by the mechanical hypernociception using the VFH test (Fig. 8A). One-way ANOVA of repeated measures demonstrated that intraplantar injection of CFA triggered a mechanical hipernociception, which persisted throughout the experimental period. The treatment with free DIM (10 mg/Kg, i.g.) reduced the mechanical hypernociception starting at 0.5 h and remaining significant up to 4 h after the administration (p<0.05), reaching its action peak at 2 h (E_{max} 61.7 ± 7.5%). The statistical analysis revealed that the group of animals treated with NC-PEC-D had a significant reduction of the mechanical stimulus response that also started 0.5 h after the treatment and reached its maximum effect at 2 h (E_{max} 56.1 ± 10.1 %), lasting up until 6 h after the administration (p<0.05). The group of animals that received NC-PEC-B did not present a reduction in the mechanical hypernociception over the experimental period. Ketoprofen administration (10 mg/Kg, i.g.) also reduced the percentage of response to VFH stimulation, but in a distinct time profile. The anti-hypernociceptive action of ketoprofen started at 0.5 h and remained significant only until 2 h.

Figure 8B shows the area under the curve (AUC) of the time-response in the VFH test. The free DIM, NC-PEC-D and the ketoprofen treatments reduced the AUC values in comparison to the CFA group (treated with vehicle) (p<0.001). Despite the distinct temporal effect shown by free DIM and NC-PEC-D administration, the ordinary One-way ANOVA demonstrated that no statistical significance between the values of AUC (p>0.05) was observed between these groups. Importantly, the *post hoc* analysis revealed a statistically significant reduction in the AUC values of animals treated with NC-PEC-D in comparison to those of animals that received CFA and were treated with the ketoprofen (p<0.05). NC-PEC-B did not reduce AUC values in comparison to the CFA group (p>0.05).

4 Discussion

The current study provided further information about the physicochemical properties of DIM-loaded NCs. Additionally, an investigation concerning the antinociceptive action of DIM in its free and nanoencapsulated forms was carried out

using distinct animal models of pain. It is important to elucidate the physicochemical characteristics of the nanostructured systems because their features directly impact on the product stability, and *in vivo* distribution, toxicity and consequently pharmacological effects. For instance, ionic interactions between the nanoparticle components and the drug could decrease the release rate of drug from the nanoparticles [32]. Taken together, our results suggest that DIM nanoencapsulation promoted an increase in its thermal stability as well as improved DIM antinociceptive action without causing any alteration in locomotor and exploratory activities of mice.

One technique that is used as a tool to investigate chemical interactions among substances and to identify functional groups in molecules is the Fourier-transformed infrared spectroscopy (FTIR) analysis. Considering our results, chemical bond formation between DIM and EC was not detected because their main bands can be seen in the NCs spectra and new peaks or changes in peak shape were not observed. These data indicate the absence of interaction among the components of the formulation. Besides, the typical band assignments of DIM could be overlapped by the lactose and polymer peaks.

Regarding thermal analyses, a pure DIM thermogravimetric profile revealed thermal degradation steps different from the NCs, which presented a lower weight loss ratio, resisting higher temperatures. These results suggest that the DIM nanoencapsulation promoted an improvement in its physicochemical and stability properties in comparison to its free form. The DSC thermograms revealed the suitable DIM encapsulation within the NCs. The endothermic event of the bioactive compound disappeared in the thermogram for the nanoencapsulated form, suggesting that a remarkable reduction of its crystallinity may have occurred in the NCs. This result corroborates the high DIM encapsulation efficiency (close to 100%) that was previously demonstrated for this formulation, which could be attributed to the DIM solubilization in the oily core [26]. Further, this effect may enhance the apparent solubility and the dissolution profile of poorly water-soluble drugs and, consequently, had a positive impact on the biological effect [33,34].

Reinforcing these results, the X-ray diffraction patterns of DIM-loaded NCs did not present crystalline peaks, indicating that DIM could be molecularly dispersed in the NCs oil core. Lastly, the FEG-SEM analysis showed NCs presenting a particle diameter in the nanometric range, confirming previous results assessed using

different techniques (photon correlation spectroscopy) [26] as well as homogeneous and spherical shape. Overall, those data provided a detailed physicochemical characterization of NC-PEC-D formulation and highlighted many advantages attributed to encapsulating molecules.

Taking into account the anti-inflammatory effect already described for indolyl compounds and the antinociceptive action reported for the DIM precursor, indole-3-carbinol; and considering the relevance of investigating new therapeutic approaches to pain management, the present study evaluated the antinociceptive potential of DIM in its free form and incorporated in primula oil-based NCs, using distinct *in vivo* behavioral models of nociception.

In the experimental protocol I, the first model performed was the hot plate test, which is a rapid and precise model of thermal nociception that is useful for the screening of new potential analgesic drugs. The results showed that the treatment with DIM caused an increase in the paw withdrawal latency, independent of the DIM form administered after 1 h. Interestingly, DIM nanoencapsulation prolonged the antinocieptive action compared to the free compound and maintained this effect even for a lower dose (5 mg/Kg). These results suggest that DIM nanoencapsulation could improve the compound biological effect. Several scientific reports demonstrated a similar profile of pharmacological action after encapsulating molecules [34–40]. In addition, the results suggest that DIM antinociceptive action could be at least partly related to the modulation of the central nervous system processes, mainly supraspinal structures, because of the sites activated by the thermal stimulus [9,41]. Indeed, our previous study [16] showed similar results for indole-3-carbinol in the same behavioral test, attributing this effect to centrally mediated components.

The nociception induced by formalin is a model of persistent pain that evaluates two distinct phases of nociception: neurogenic and inflammatory. The acute neurogenic pain evokes the local release of excitatory amino acids (such as glutamate), nitric oxide and substance P as a result of the direct nociceptor stimulation by formalin. Therefore, this phase is mediated by central sensitization. The second phase reflects the peripheral sensitization and leads to an inflammatory response, with the release of histamine, serotonin, interleukins and prostaglandins, triggering local edema formation and hyperalgesic behavior [9,39,42]. Our results demonstrated that both free and nanoencapsulated DIM had a rapid onset of action

in this test. However, it is noteworthy that DIM incorporation into NCs anticipated and prolonged the DIM antinociceptive properties in formalin-induced neurogenic and inflammatory phases of pain. In addition, while indole-3-carbinol showed antinociceptive action only in the inflammatory phase [16], DIM elicited an antinociceptive effect in both phases, demonstrating that this bioactive substance could act by distinct mechanisms of action and its analgesic potential was superior to its phytochemical precursor. In addition, our results demonstrated that the administration of NC-PEC-B (0.5 h) elicited a reduction in the nociceptive behavior in both phases of the formalin test, which could be attributed to the components of the primula oil. It was already reported that flavonoids and different fatty acids were identified in primula oil [43,44]. Particularly important is that the anti-inflammatory action was assigned to ɣ-linolenic and stearidonic acids, the main fatty acids of primula oil [44,45]. In this context, our research group previously showed the potential anti-inflammatory action of blank nanocarriers prepared with different vegetable oils, such as pomegranate oil [15] and rose hip oil [16], reinforcing the possible contribution of the oils to the global pharmacological effect of the formulation.

These findings also indicate that DIM triggered a reduction in licking time in both the neurogenic and inflammatory phases, thereby DIM probably presents peripheral and central antinociceptive actions, corroborating the results obtained in the hot plate test. The potential DIM anti-inflammatory effects were already reported by other authors; the prostaglandin and cytokine degradation and inhibition of cyclooxygenase-2 and nitric oxide synthase enzyme activity seem to be the possible mechanisms related to the aforementioned property [20,46]. Reinforcing the hypothesis regarding the DIM centrally mediated action mechanisms in the inflammatory process, this phytochemical also suppressed LPS-induced neuroinflammation in mice [19], suggesting that besides its anti-inflammatory effect, DIM antinociceptive action could be also related to central mediated actions. It is also interesting to underscore that DIM has in its molecular structure the amine group, which is also present in the chemical structure of some anti-inflammatory drugs, such as dipyrone, diclofenac, mefenamic acid and nimesulide, among others [47,48], suggesting a possible structure-activity relationship. However, further studies regarding the specific pharmacophore group of DIM are necessary to confirm this

hypothesis. In this sense, based on the results obtained, it can be suggested that the DIM incorporation into NCs positively modifies its antinociceptive action by anticipating and prolonging such an effect in distinct animal models of pain.

For the second protocol, a model of acute inflammatory pain induced by CFA injection in mice was used to assess the possible therapeutic potential of DIM-loaded NCs. The intraplantar injection of CFA triggers a local injury, releasing several inflammatory and nociceptive mediators (such as cytokines and prostaglandins), which promote an acute inflammatory process characterized by paw edema, vasodilatation, allodynic and hypernociception behaviors as well as sensitization of central nervous regions associated with pain modulation [49,50]. This model is a well-recognized experimental protocol for screening novel compound for inflammatory pain [51]. The evaluation of the nociceptive sensitization to innocuous mechanical stimuli (hypernociception) was performed using the VFH test. Our results showed that the treatment with free DIM or DIM-loaded NCs reduced the response frequency of VFH stimulation in a distinct time-response profile. The NCs had more prolonged anti-hypernociceptive action on the paw inflammation than the free compound. These findings corroborate our previous results as well as other scientific research studies that tested nanoencapsulated drugs in animal models of nociception [14,15,34,39,40,52–55]. The improvement and prolongation of the DIM-loaded NCs antinociceptive effect compared to the free bioactive substance could be due to DIM sustained release and improved apparent solubility [26], in addition to better absorption/distribution promoted by the nanometric size of the nanoparticles. Furthermore, it should be mentioned that the decrease in the nociceptive behavior presented by DIM-loaded NCs was not significantly different from the antinociceptive effect presented by ketoprofen (positive control), a classic NSAID used to attenuate the peripheral pain and inflammation. Finally, since the treatment with free DIM, NC-PEC-D or NC-PEC-B (*data not shown*) did not modify the evaluated parameters in the locomotor activity chamber, we could discard any non-specific effects of such treatments.

5. Conclusion

In summary, we performed a physicochemical characterization of the DIM-loaded NCs, using DSC, thermal analysis, FTIR and XRD techniques, which

indicated that DIM was not in the free crystalline or precipitated state, but solubilized in the oil core of the NCs. In addition, nanometric sized and thermally stable particles were successfully prepared. These data are useful to predict the possible *in vivo* biological effects of the formulation. Furthermore, the results provided, for the first time, important knowledge about the DIM antinociceptive potential and demonstrated that its nanoencapsulation could be a novel pharmacological approach intended for oral administration to treat inflammatory and painful related pathologies.

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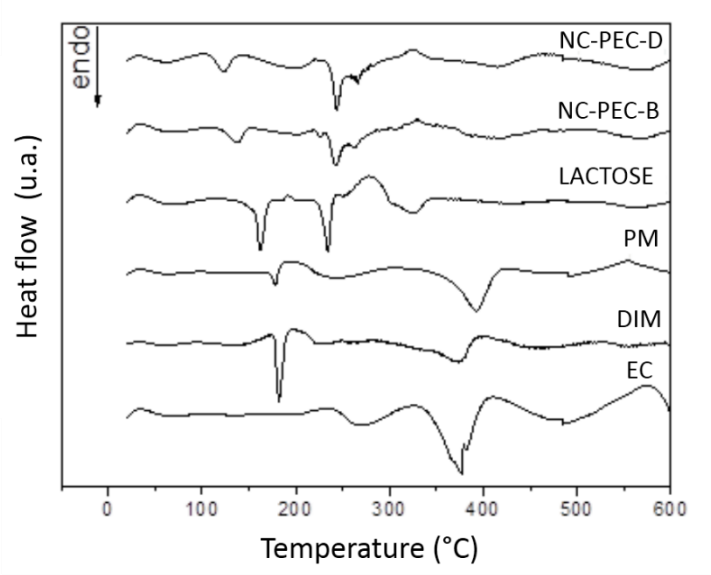


Figure 1 - DSC curves of ethylcellulose (EC), DIM, physical mixture (PM), monohydrate lactose (lactose) and freeze-dried NCs (NC-PEC-B and NC-PEC-D).

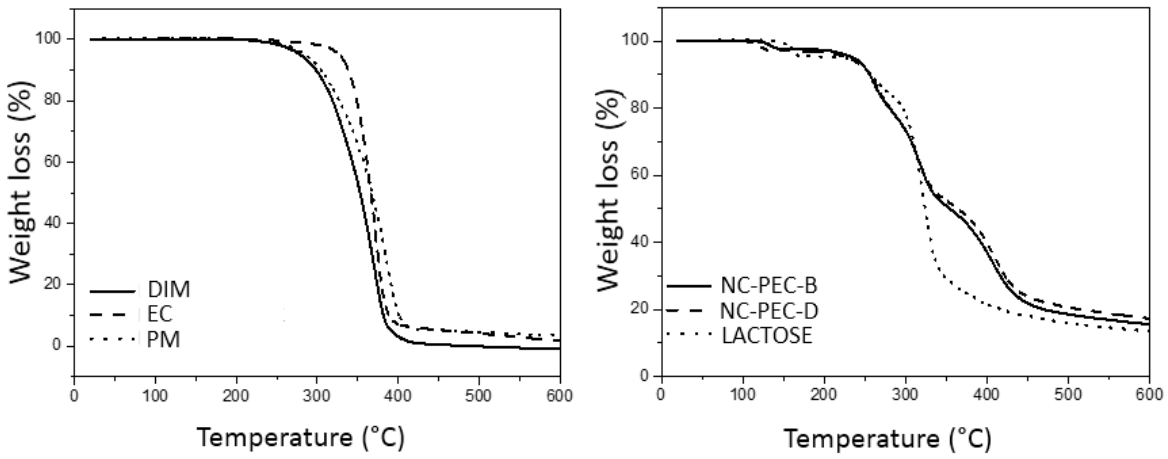


Figure 2 - Thermogravimetric profiles of ethylcellulose (EC), DIM, physical mixture (PM), monohydrate lactose (lactose) and freeze-dried NCs (NC-PEC-B and NC-PEC-D).

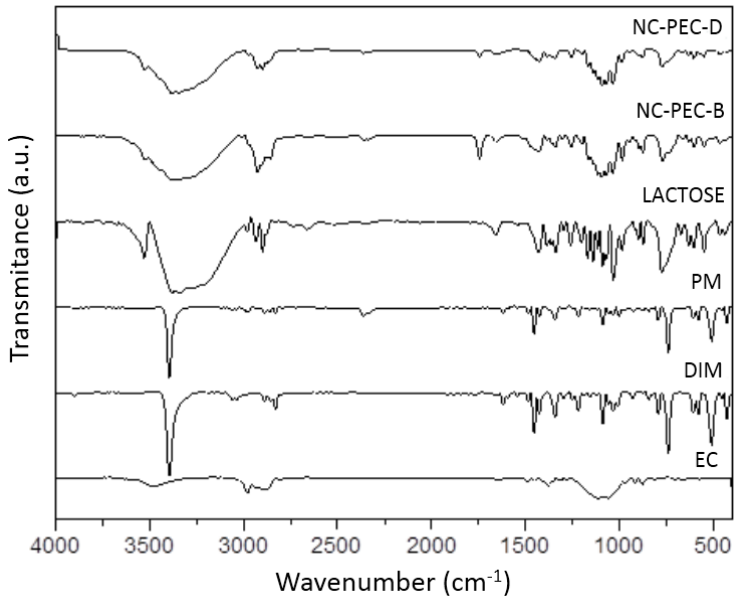


Figure 3 - FTIR spectra for ethylcellulose (EC), DIM, physical mixture (PM), monohydrate lactose (lactose) and freeze-dried NCs (NC-PEC-B and NC-PEC-D).

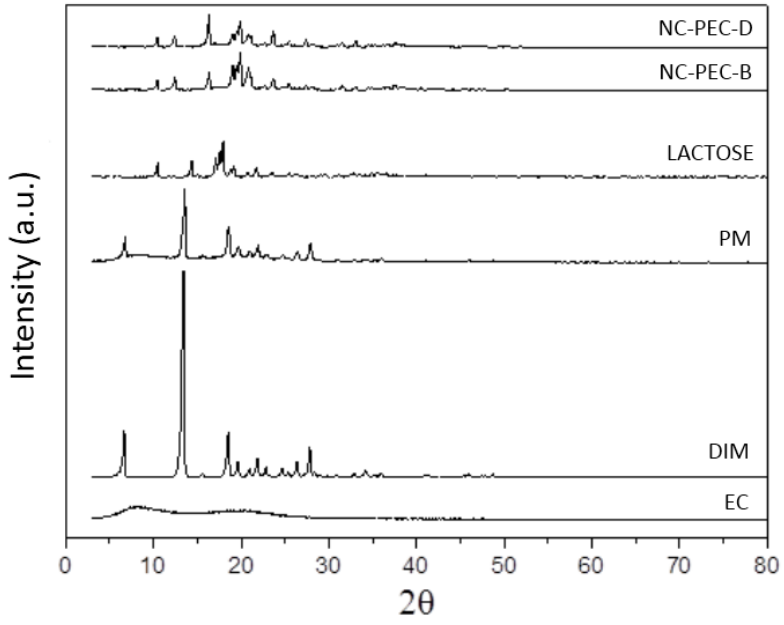


Figure 4 - X-ray diffraction patterns of ethylcellulose (EC), DIM, physical mixture (PM), monohydrate lactose (lactose) and freeze-dried NCs (NC-PEC-B and NC-PEC-D).

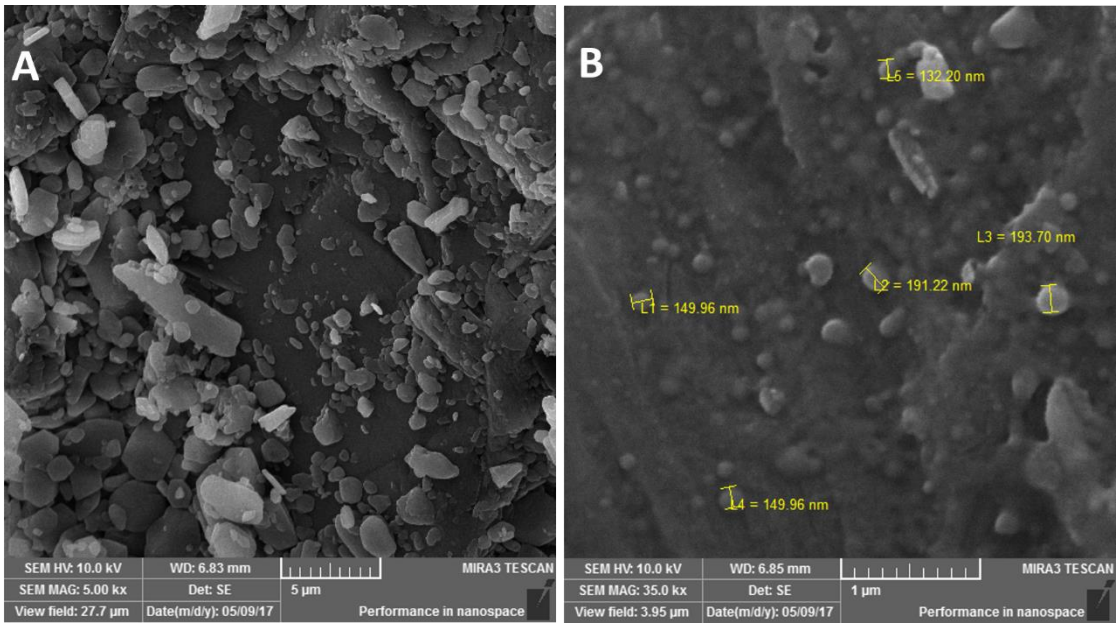


Figure 5 - FEG-SEM micrographs of pure DIM (A) and DIM-loaded NCs (B). Experimental conditions: accelerating voltage: 10.0 kVA; magnification: 5.00 kx (A) and 35.0 kx (B).

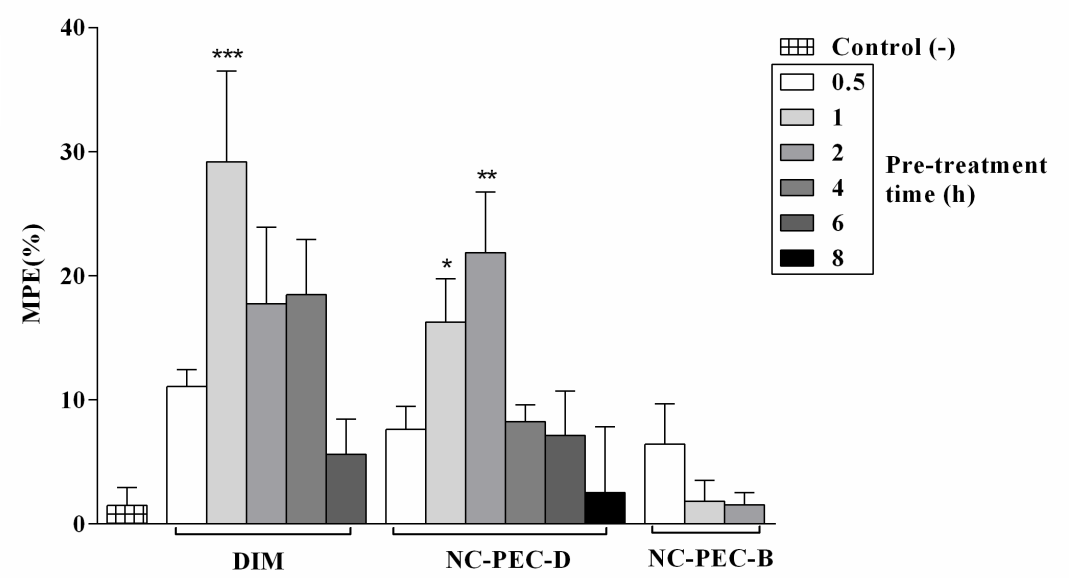


Figure 6 - Time-response curve of the antinociceptive effect of free DIM, NC-PEC-D and NC-PEC-B in hot plate test (10 mg/Kg, i.g.). Each column represents the mean ± S.E.M. of 6–8 mice/group. Asterisks denote the significance levels, when compared to the negative control group (One-way ANOVA followed by Newman-Keuls' test) (*) $p<0.05$ (**)$p<0.01$ (***) $p<0.001$.

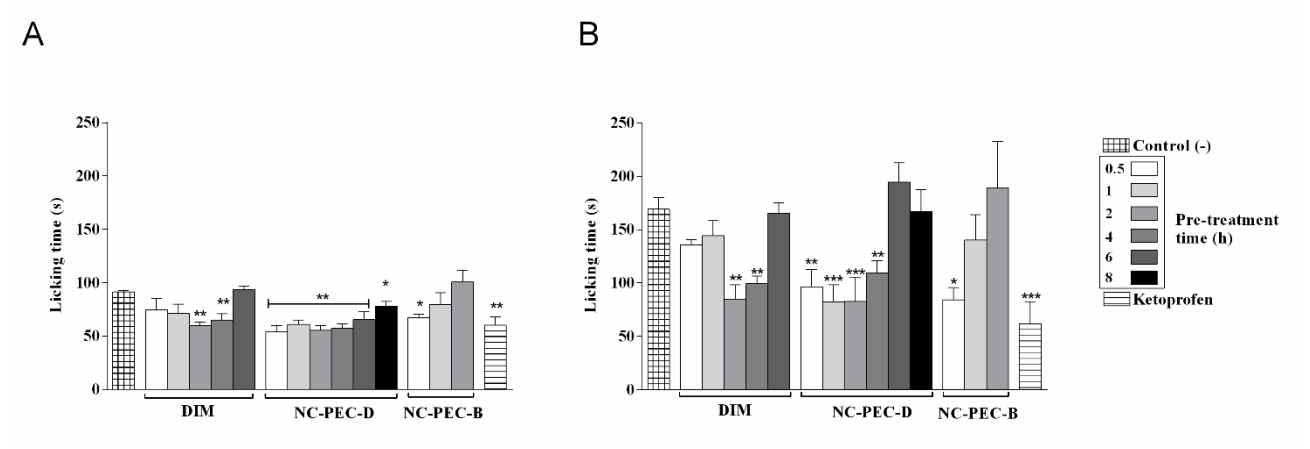


Figure 7 - Time- response curve of free DIM, NC-PEC-D (10 mg/Kg, i.g.), and NC-PEC-B antinociceptive effect induced by formalin in mice, on neurogenic phase (A) and inflammatory phase (B). Each column represents the mean ± S.E.M. of 6-8 mice/group. Asterisks denote the significance levels, when compared to the control group, analyzed by one-way ANOVA followed by Newman-Keuls' test. (*) $p<0.05$ (**)$p<0.01$ (***) $p<0.001$.

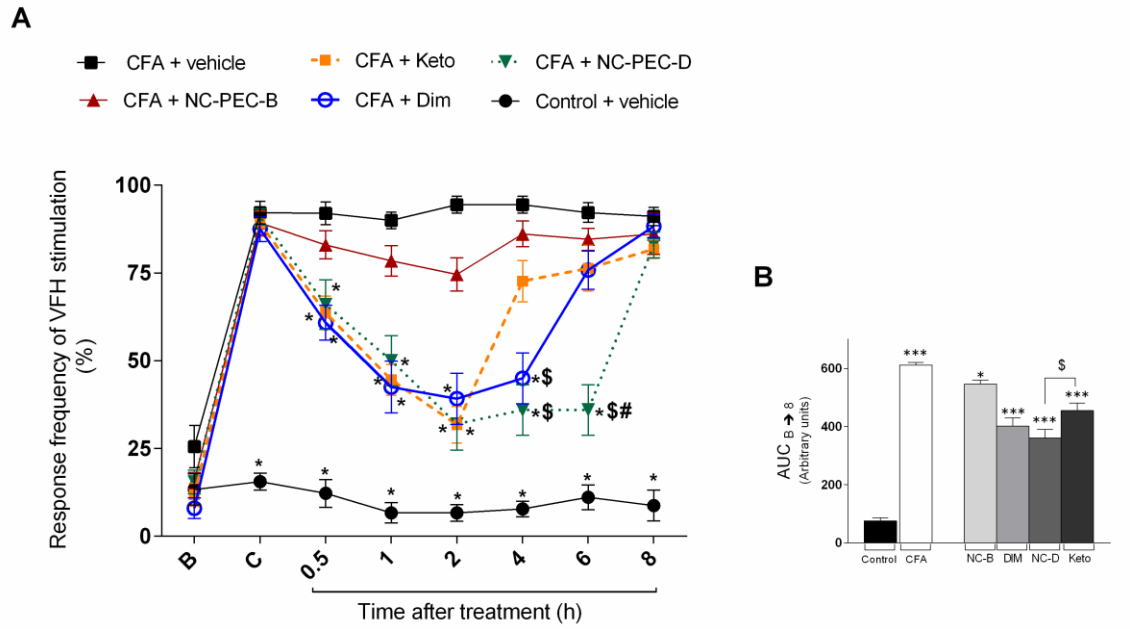


Figure 8. Time-response curve of DIM treatment effect on the mechanical hypernociception induced by an acute inflammatory pain model (A) and the respective area under the curve (basal response [B] to 8 h) (B). The results represent means ± S.E.M. of 8–10 animals/group. CFA: animals induced with CFA and treated with vehicle; B: basal response frequency, before CFA injection; C: confirmatory response frequency to VFH stimulation, 24 h after CFA injection. The asterisks denote significant difference in comparison to the CFA + vehicle group, (*) $p<0.05$ (***) $p<0.001$; sharps denote significant difference in comparison to the CFA + DIM group, (#) $p<0.05$; ciphers represent significant difference in comparison to CFA + Keto group, ($) $p<0.05$. One-way ANOVA of repeated measures followed by Newman Keuls' test was performed to time-response curve.

Fig. 1S. Schematic view of the experimental study design. (I) Time- and dose-response curves were carried out to evaluate the antinociceptive effect using the formalin-induced nociceptive behavior and hot plate test. (II) Another set of animals were treated in order to evaluate the anti-hypernociceptive action of DIM in the von Frey hair (VFH) test. Abbreviations: Pre H.P. – pretreatment latency to the hot plate test; LAM - locomotor activity monitor evaluation; B – baseline response to the VFH test; C – confirmatory of mechanical hyperalgesia induction with CFA in the VFH test.

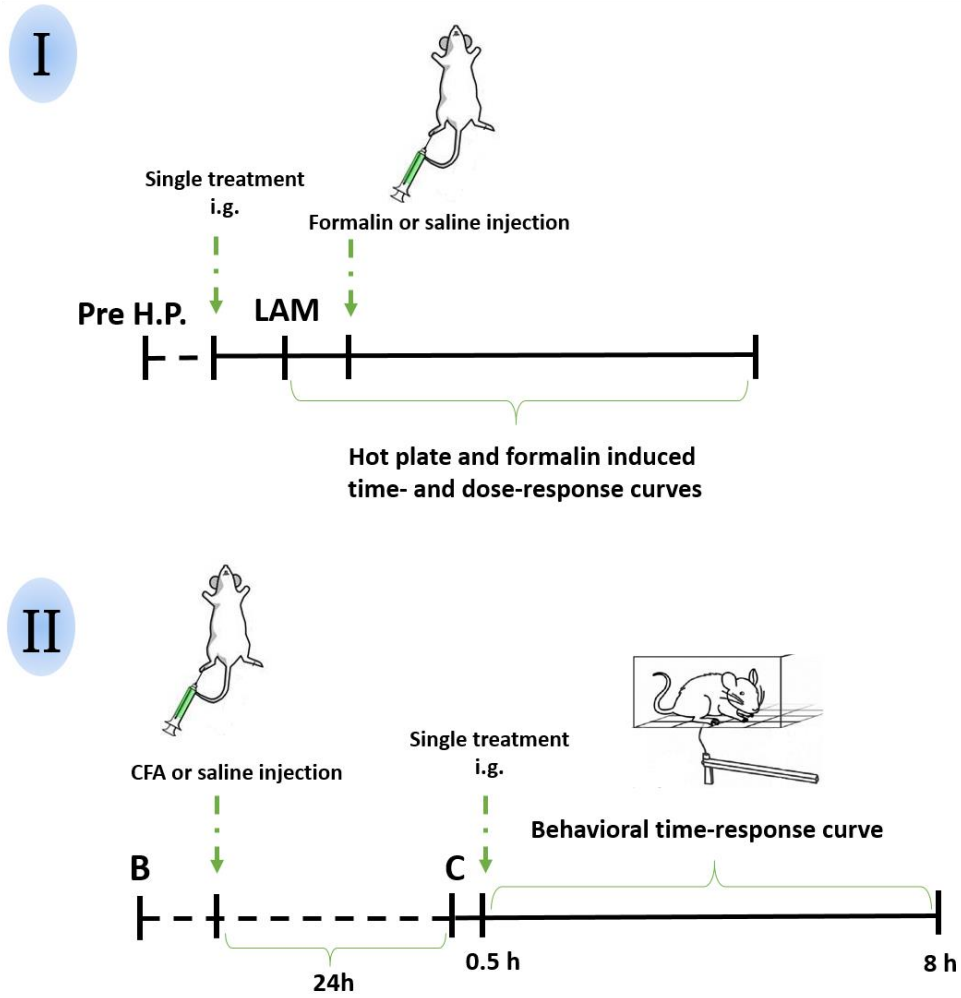


Fig. 2S. DSC curves of ethylcellulose (A), DIM (B), monohydrate lactose (C), physical mixture (D), NC-PEC-B (E) and NC-PEC-D (F).

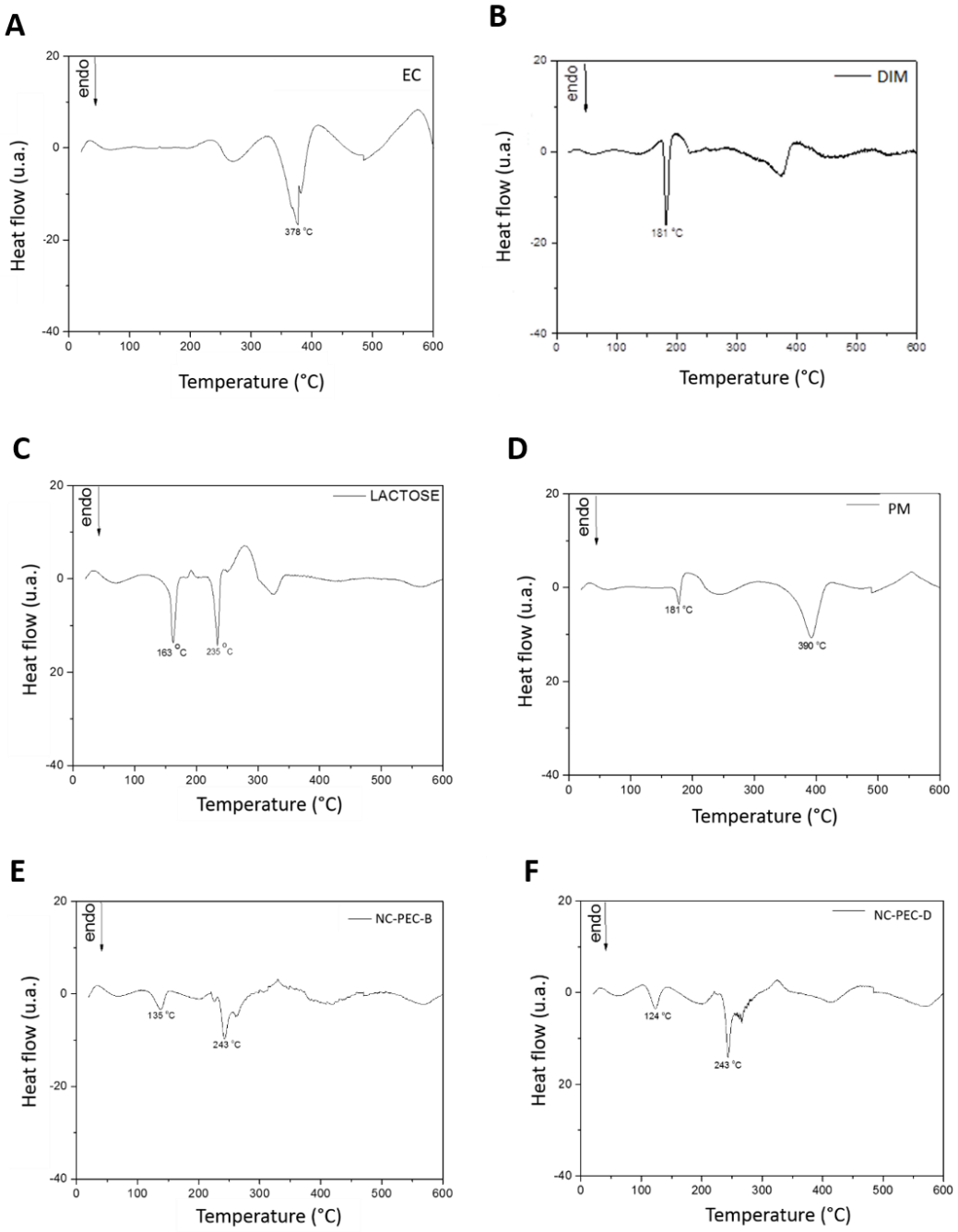


Fig. 3S. Thermogravimetric profiles of ethylcellulose (A), DIM (B), monohydrate lactose (C), physical mixture (D), NC-PEC-B (E) and NC-PEC-D (F).

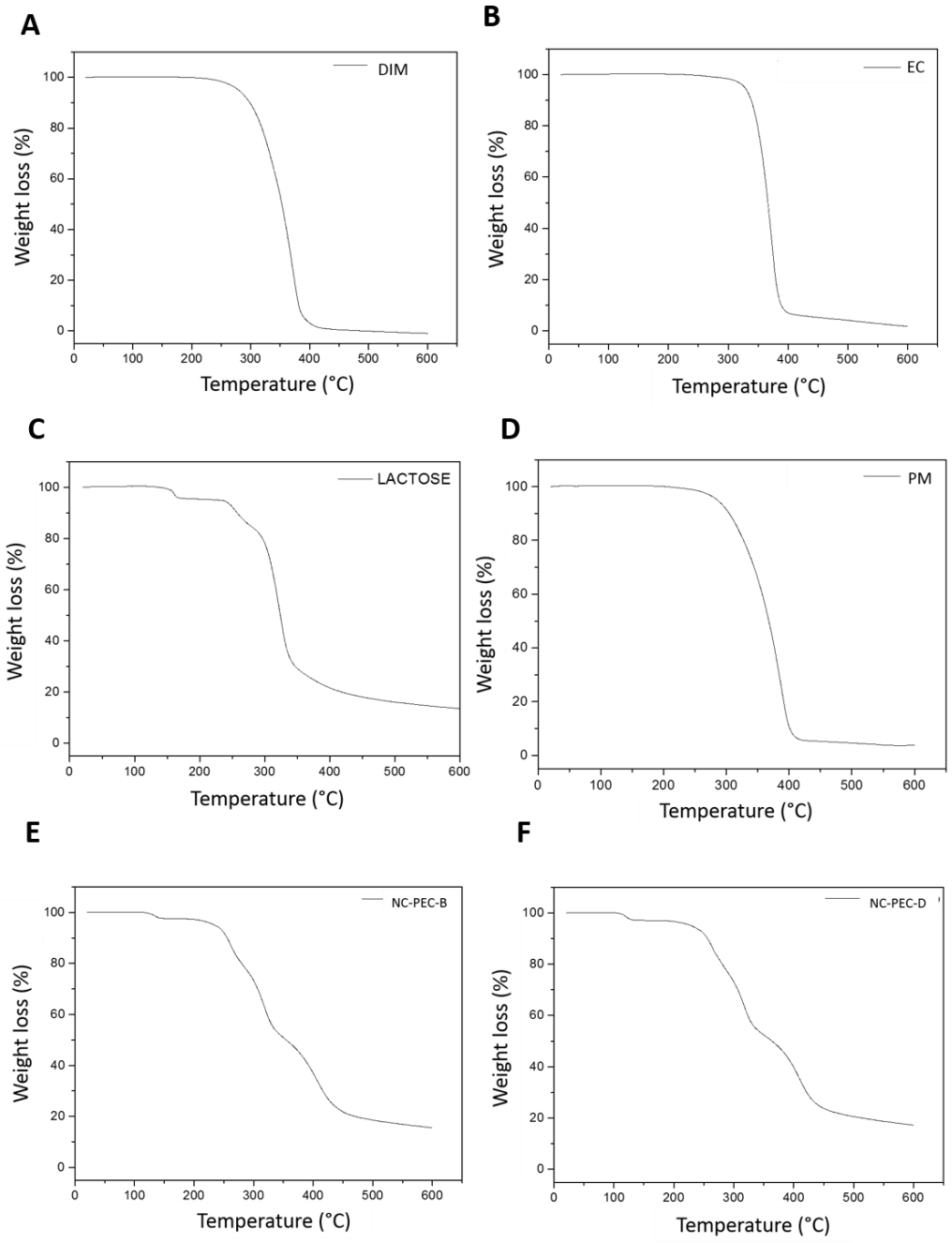


Fig. 4S. FTIR spectra for ethylcellulose (A), DIM (B), monohydrate lactose (C), physical mixture (D), NC-PEC-B (E) and NC-PEC-D (F).

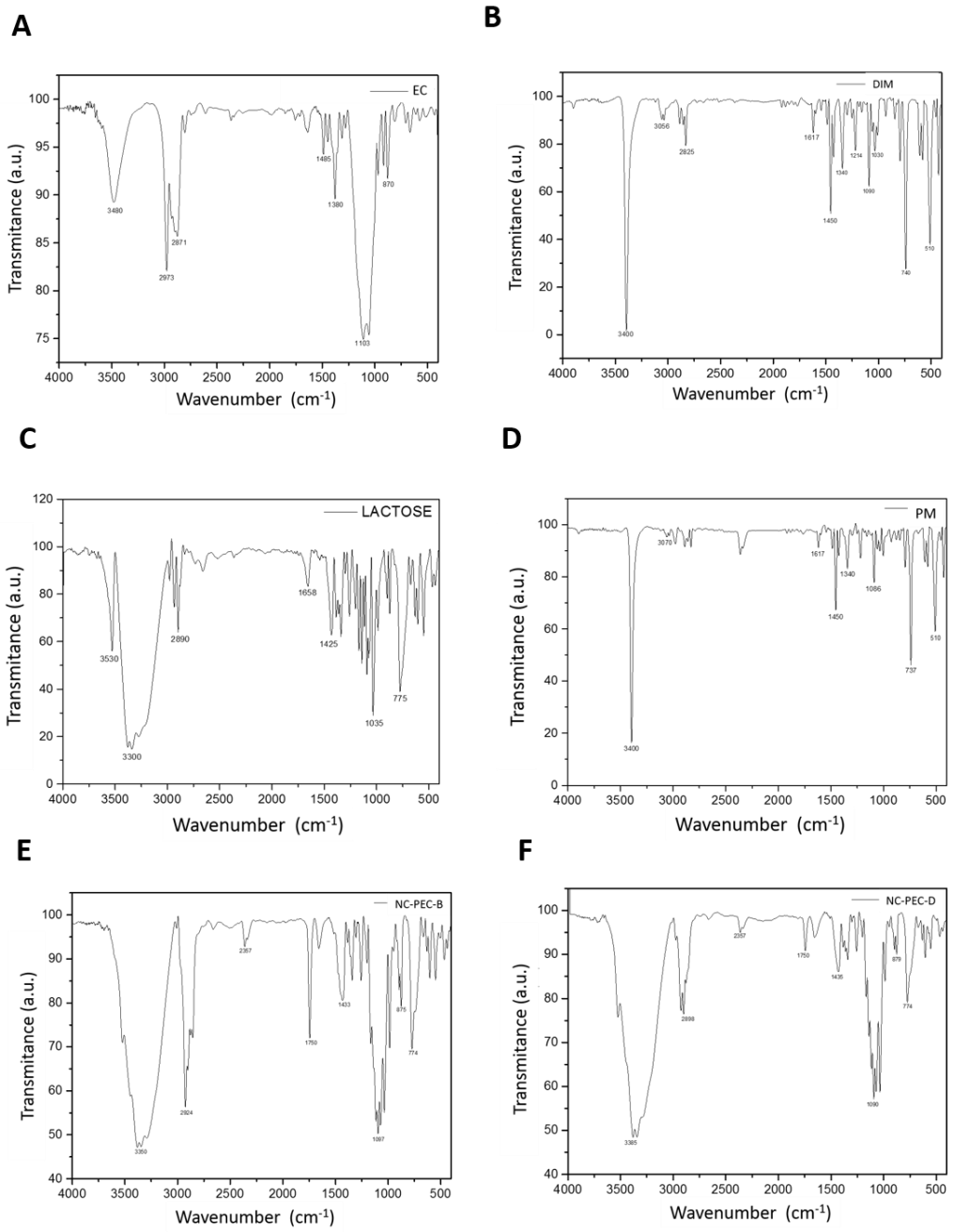


Fig. 5S. X-ray diffraction patterns of ethylcellulose (A), DIM (B), monohydrate lactose (C), physical mixture (D), NC-PEC-B (E) and NC-PEC-D (F).

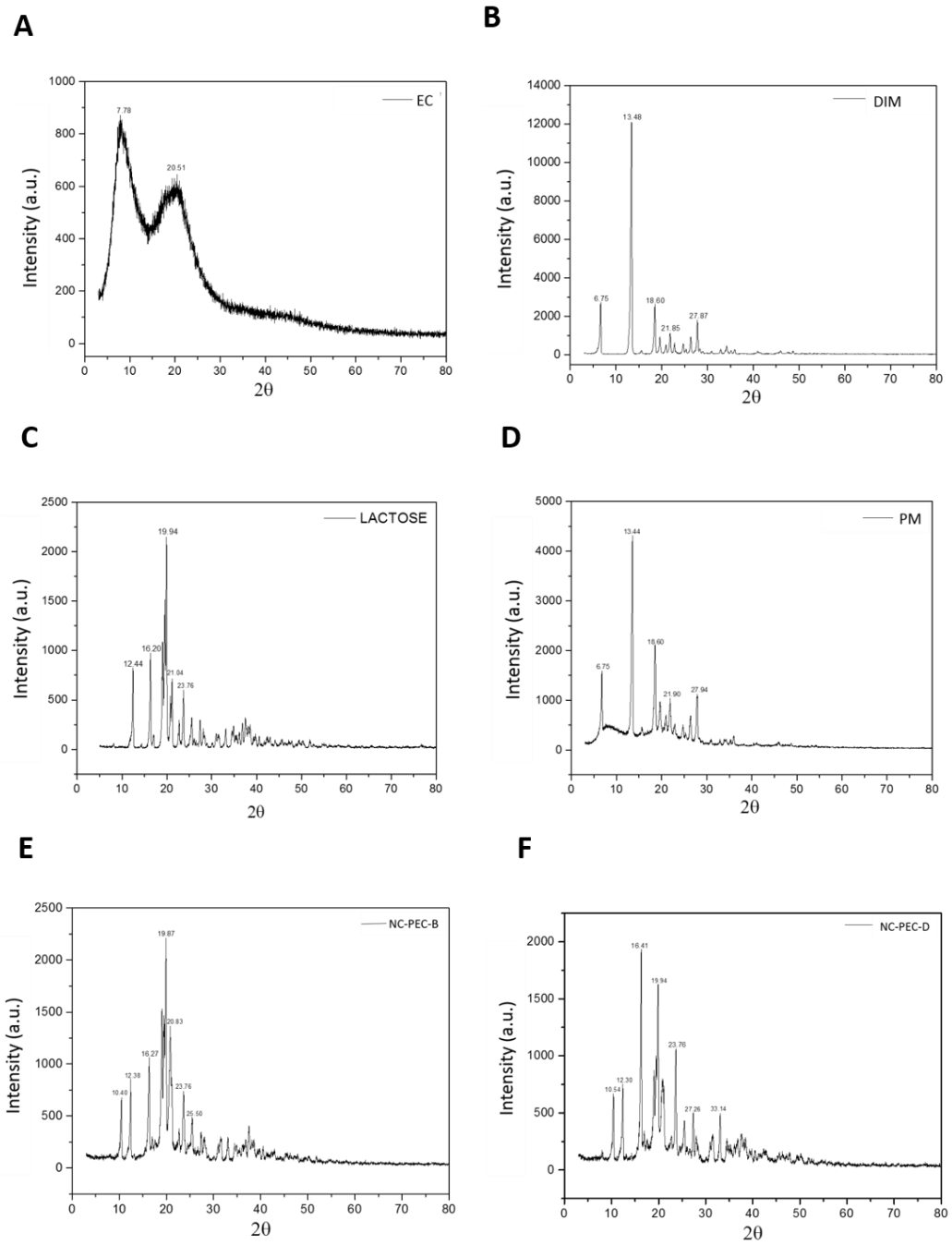


Fig. 6S. Dose-response curve of free DIM and DIM-loaded NCs (i.g.) antinociceptive effect 2 h after treatment on hot-plate test. Each column represents the mean ± S.E.M. of 6–8 mice/group. Asterisks denote the significance levels, when compared to the negative control group (One-way ANOVA followed by Newman-Keuls' test) (*) $p<0.05$.

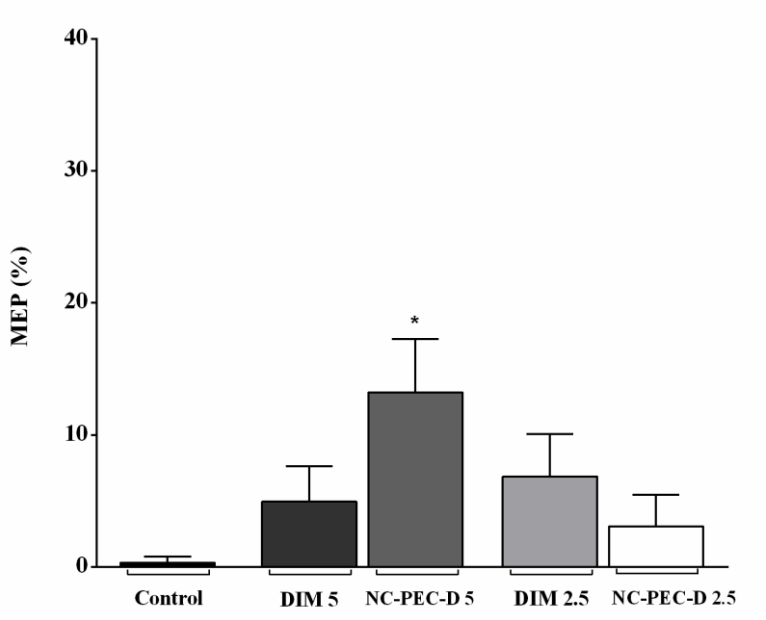


Fig. 7S. Time-response curve of free DIM, DIM-loaded NCs (10 mg/Kg, i.g.) and blank nanocapsules antinociceptive effect on paw edema (mg) induced by formalin in mice. Each column represents the mean ± S.E.M. of 6–8 mice in each group.

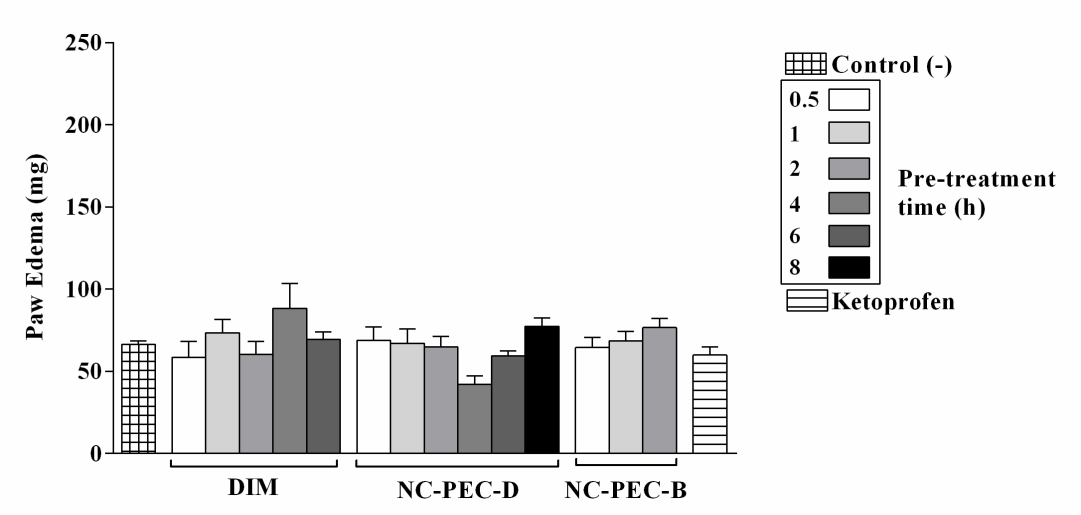
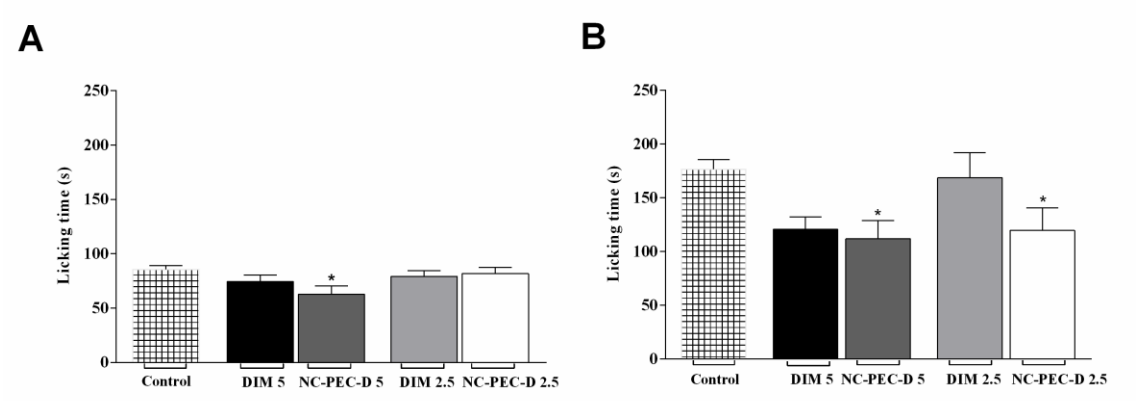


Fig. 8S. Dose-response curve of free DIM and NC-PEC-D (5 mg/Kg or 2.5 mg/Kg, i.g.) antinociceptive effect induced by formalin in mice, on neurogenic phase (A) and inflammatory phase (B), 2 h after treatment. Each column represents the mean ± S.E.M. of 6-8 mice/group. Asterisks denote the significance levels, when compared to the control group, analyzed by one-way ANOVA followed by Newman-Keuls' test. (*) $p<0.05$.



Table

Table 1S - Results of spontaneous locomotor and exploratory activities to free DIM and DIM-loaded NCs time- and dose-response curve (One-way ANOVA followed by Newman-Keuls' test).

Group	DIM			NC-PEC-D		
	Crossings	**Distance (mm)**	**Average Speed (mm/s)**	**Crossings**	**Distance (mm)**	**Average Speed (mm/s)**
Control (-)	502.3 ± 32.5	9468 ± 411	38.8 ± 1.5	502.3 ± 32.5	9468 ± 411	38.8 ± 1.5
Control (+)	567.6 ± 77.5	7781 ± 1253	36.9 ± 2.5	567.6 ± 77.5	7781 ± 1253	36.9 ± 2.5
0.5 h	495.8 ± 65.4	8355 ± 1301	42.0 ± 7.4	425.5 ± 26.3	7680 ± 664.0	36.0 ± 3.2
1 h	478.8 ± 51.1	7842 ± 1952	41.3 ± 3.6	520.8 ± 67.3	8588 ± 1152	37.6 ± 4.0
2 h	478.0 ± 59.9	8073 ± 1293	36.8 ± 3.8	431.3 ± 23.2	8120 ± 550.0	37.6 ± 3.1
4 h	528.0 ± 74.0	9350 ± 1236	41.7 ± 6.6	536.8 ± 54.8	10147 ± 1173	41.6 ± 4.6
6 h	573.0 ± 81.6	8091 ± 1554	40.6 ± 6.3	609.8 ± 64.9	9985 ± 1226	45.1 ± 7.2
8 h	-	-	-	531.4 ± 45.0	9429 ± 847.0	39.1 ± 3.1
5 mg/Kg	666.9 ± 66.6	10928 ± 1260	46.0 ± 5.2	562.5 ± 60.8	9713 ± 903	42.7 ± 5.3
2.5 mg/Kg	630.4 ± 64.7	9936 ± 789	42.82 ± 3.0	613.3 ± 57.2	9707 ± 620	43.3 ± 2.4

4. CAPÍTULO 2

4. CAPÍTULO 2: DESENVOLVIMENTO DE MICROPARTÍCULAS DE 3,3'-DIINDOLMETANO E AVALIAÇÃO DA AÇÃO ANTI-HIPERNOCICEPTIVA EM MODELO ANIMAL DE DOR INFLAMATÓRIA AGUDA

Apresentação

Neste capítulo estão descritos o desenvolvimento das micropartículas contendo DIM e avaliações quanto à atividade anti-hipernociceptiva do DIM na forma pura ou incorporada às micropartículas.

O **Manuscrito 2** relata a preparação e caracterização de micropartículas de etilcelulose contendo DIM, incluindo técnicas que permitem a avaliação do estado físico do DIM nas micropartículas, bem como sua forma de associação e possíveis interações entre o ativo e o polímero nas micropartículas, além da avaliação do perfil de liberação do DIM a partir das micropartículas desenvolvidas. Adicionalmente, foi demonstrada a melhora no efeito anti-hipernociceptivo pela formulação em comparação ao composto livre em modelo animal de dor inflamatória aguda em camundongos.

4.1 MANUSCRITO 2

Microencapsulation enhances 3,3'-diindolylmethane anti-hypernociceptive action in an animal model of acute inflammatory pain

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ABSTRACT

The 3,3′-diindolylmethane (DIM) is a natural molecule that has anti-inflammatory and antinociceptive actions. However, it is water insoluble, besides degrading when exposed to light. Drug carrier systems, such as polymeric microparticles (MPs), have emerged as an interesting approach in order to overcome such issues. The present study aimed to develop DIM MPs as well as evaluate the anti-hypernociceptive potential of the formulation. For this, MPs were prepared by the O/W solvent emulsion-evaporation method and characterized by particle diameter,

DIM content and encapsulation efficiency and DIM release profile. Thermogravimetry, differential exploratory calorimetry and X-ray diffraction techniques were also performed. The anti-hypernociceptive action was evaluated in the animal model of acute inflammatory pain induced by Complete Freund's Adjuvant (CFA) in mice. The results demonstrated that the MPs had mean diameter in the micrometric range (368 ± 31 μm), narrow particle size distribution (*Span* of 2.0 ± 0.2), DIM content of approximately 150 mg/g and encapsulation efficiency around 84%, as well as prolonged the compound release. Evaluations of the association form of DIM to MPs demonstrated the feasibility of the systems to incorporate DIM, without presenting interactions with other constituents. It was also possible to observe an improvement in DIM anti-hypernociceptive action by the formulation compared to the free compound, since this increased and prolonged the pharmacological effect. Therefore, the MPs developed represent a promising formulation for oral DIM administration in the treatment of inflammatory pain.

Key-words: microspheres; indole-3-carbinol; drug delivery; complete Freund's adjuvant; inflammatory pain; Von Frey filament; allodynia.

1. INTRODUCTION

The 3,3′-diindolylmethane (DIM) is a dimer of the indol-3-carbinol, phytochemical originated from chemical reactions that occur in cruciferous vegetables (cauliflower and broccoli) (Banerjee et al., 2011). Several biological properties have already been described for DIM as the anti-inflammatory, antioxidant and antitumoral action (Chen et al., 2013; X. J. Li et al., 2013; Y. Li et al., 2013; Maruthanila et al., 2014; Wu et al., 2014). The scientific literature reports that the anti-inflammatory effect of DIM is related to the inhibition of myeloperoxidase enzyme activity, a decrease in the production of nitric oxide (NO) and prostaglandins (PGE_2), as well as the reduction in the levels of proinflammatory cytokines, such as the tumoral necrosis factor α (TNF-α), interleukins (IL-1β and IL-6), phospholipase A2 (PLA_2), among other mechanisms (E. J. Kim et al., 2009; Kim et al., 2010; Y. H. Kim et al., 2009; Maruthanila et al., 2014). In addition, recently, the DIM antinociceptive action in animal models of acute and inflammatory nociception was demonstrated by our research group (unplublished data – ***Manuscript 1***). These data reinforce DIM

biological potentialities and suggest it as a promising candidate for the treatment of various disorders, including the clinical conditions of pathological pain (Scholz and Woolf, 2002).

Pain is essentially a biological mechanism that provides protection to the organism (Woolf and Salter, 2000). However, depending on the situation, it may become persistent and intense, assuming a pathological character (Jang et al., 2018; Kumar and Elavarasi, 2016). The impact caused by pain, especially chronic pain, leads to numerous psychosocial and functional consequences, negatively affecting the quality of life of patients (Bushnell et al., 2013; Julius and Basbaum, 2001). In addition, the scenario is not positive regarding the pharmacological management of pain, which remains a critical challenge because it often causes several adverse effects, for instance: dependence and tolerance, as is the case of opioids (Manchikanti et al., 2010), and damage to the mucosa of the gastrointestinal tract (Carter et al., 2014; Lanas, 2006) and even nephrotoxicity (Wongrakpanich et al., 2018) (i.e., prolonged use of nonsteroidal anti-inflammatory drugs). Thus, there is an urgent need for seeking new analgesic drugs to supply better options to pain treatment. In this sense, in view of the anti-inflammatory and antinociceptive properties already reported for DIM in preclinical models, more studies that support its application in the therapy of pathological pain are relevant.

Although several epidemiological studies report a link between cruciferous vegetable consumption and disease prevention (Higdon et al., 2007; Vasanthi et al., 2009; Verhoeven et al., 1996), a large daily intake of these vegetables is required to achieve adequate doses of DIM (about 1.6 mg/Kg). This may be due to the high DIM lipophilicity and low bioavailability (1-3%) after oral administration (Roy et al., 2013; Wu et al., 2015). In addition, it may degrade after exposure to light or high temperatures (Luo et al., 2013; Vallejo et al., 2002). In order to meet these clinical needs, drug delivery systems have emerged as an interesting alternative as they are able to circumvent the limitations of conventional systems and optimize the treatment, presenting considerable positive impact on therapy (Bhokare et al., 2015; Li et al., 2008) (dos Santos et al., 2016).

Among these approaches is the development of polymeric microparticles (MPs), which arises as a pharmaceutic technological tool of drug delivery system. MPs are drug delivery systems composed by a polymerized material with a size

ranging from 1 to 1000 µm (Bale et al., 2016; O'Donnell and McGinity, 1997). These systems enable the encapsulation of both hydrophilic and lipophilic drugs, providing protection of the compound against degradation by light, oxygen, heat or gastrointestinal fluids. Besides, microencapsulation could mask unpleasant odors and flavors as well as promote sustained or site-specific release of drugs. Interestingly, MPs formulation may enhance the stability of compounds and may themselves constitute a pharmaceutical form or be included in capsules and tablets (Cruz et al., 2010; Ferreira et al., 2015; Freiberg and Zhu, 2004; Han et al., 2015; Murtaza, 2012; Raffin et al., 2008; Taraballi et al., 2014; Velasquez et al., 2014; Vilos and Velasquez, 2012).

To date, there are no scientific reports concerning preparation of polymeric MPs containing DIM. Only MPs containing other indole derivatives were prepared (Murase et al., 2015). Thus, the objective of the present study was to prepare and characterize novel ethylcellulose DIM-loaded MPs and investigate its potentialities as a DIM controlled oral release system in the treatment of acute inflammatory pain in animal model.

2. MATERIALS AND METHODS

2.1 Materials

DIM (99.2% purity) was purchased from Active Pharmaceutica (Brazil). Ethylcellulose (EC) was donated from Colorcon (Brazil). Tween® 80 (polysorbate 80), Complete Freund's Adjuvant (CFA) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co (USA). Gellan gum was donated by CP Kelco (Brazil). All other chemicals and solvents were analytical grade and used as received.

2.2 Analytical procedure

For DIM quantification, an analytical method was developed by reverse phase high performance liquid chromatography (HPLC), using an LC-10A chromatograph (Shimadzu, Japan), composed of a CBM-20A controller system, an LC-20AT pump, a UV-vis SPD-M20A with diode-array detection and an SIL-20A HT auto-injector. The chromatographic conditions used were: isocratic mobile phase containing acetonitrile/water (60:40, v/v); flow rate 1 mL/min; λ = 288 nm; pre-column and

column C_{18} Phenomenex (250 mm × 4.60 mm, 5 µm; 110 Å), kept at room temperature. The method was previously validated, being considered specific, linear at the concentration range of 5-25 µg/mL (r = 0.9999), precise and accurate (coefficient of variation <3% for all parameters).

2.3 Preparation of the MPs

DIM-loaded MPs (MP-DIM) were prepared in triplicate by the O/W solvent emulsion-evaporation method, using the ratio 1:5 (drug/polymer). Briefly, ethylcellulose (1.0 g) and DIM (0.2 g) were solubilized in a mixture of ethanol and dichloromethane (20 mL - 3:2). The system was then injected into an aqueous phase (80 mL) containing Tween® 80 (0.4 g) under stirring (200 rpm; IKA® mechanical stirrer, Brazil). Thereafter, the organic solvent was evaporated under mechanical stirring (800 rpm) for 2 h. The MPs were collected by vacuum filtration (Quanty® JP42 - 8 µm filter paper), washed with water and dried in an oven (SOLAB SL-100, Brazil) at 30°C for approximately 12h, and then stored in a desiccator. For comparative purposes, formulations without DIM (MP-B) were also prepared. The yield was calculated by the ratio between the experimental mass of powder (g) and the total mass of the solid constituents (g), discounting the mass of Tween® 80.

2.4 Characterization of MPs

2.4.1 Granulometric analysis

The particle diameter and the granulometric distribution (Span) were determined by the laser diffraction technique using Mastersizer® 3000E (Malvern Instruments, United Kingdom), after dispersion of the samples (triplicate) in a water and surfactant mixture. The particle diameter was expressed as the mean diameter based on particle volume (D[4;3]), expressed in µm, while the polydispersity (*Span*) was expressed by the following equation:

$$Span = \frac{D_{90\%} - D_{10\%}}{D_{50\%}} \quad (1)$$

Where the values of $D_{90\%}$, $D_{10\%}$ and $D_{50\%}$ indicate the percentage of particles in the particle size distribution curve having a diameter equal to or less than the determined value.

2.4.2 Determination of powder flow properties

For this determination, 1.0 g of the MP-DIM formulation was weighed and transferred to a 10 mL graduated beaker, and the bulk density (D_b) in g/cm^3 was calculated from the volume of powder obtained. The sample was then placed in a PT-TD Tapped Density Testing Instrument (Pharma Test, Germany) and compacted 1250 times, in order to obtain the tapped density (D_t), also expressed in g/cm^3. The changes occurred in the arrangement of MPs subjected to compaction were expressed as two parameters, Carr index (CI) and Hausner factor (HF), which allow to infer about the flow of powders (Equations 2 and 3). Each parameter was determined in triplicate.

$$CI = \frac{Dt - Db}{Dt} \quad (2)$$

$$HF = \frac{Dt}{Db} \quad (3)$$

2.4.3 DIM content and encapsulation efficiency in MPs

The content and efficiency of encapsulation of DIM were determined by HPLC (section 2.2), in batch triplicate, after weighing 0.1 g of MPs and diluting the samples in 100 mL of methanol using the validated analytical method mentioned above. The DIM content was calculated by dividing the drug mass in the MPs by the total mass of MPs, all then multiplied by 100 (expressed in mg/g). The encapsulation efficiency was expressed as a percentage, being calculated by relating the theoretical and experimental DIM concentrations.

2.4.4 Thermal analyses

For the thermogravimetric analysis, the samples (MP-B, MP-DIM, pure DIM, ethylcellulose and physical mixture of the constituents) were placed in alumina calorimetric cell. The instrument STA 6000 (Perkin Elmer, United States) was calibrated using indium (In; P.F.= 156.6 ºC; ΔH_{fusion} = 28.54 J/g) as the standard. The samples were heated at a constant rate of 10 °C/min, from 20 to 600 °C, under constant nitrogen flow (50 mL/min). The analysis of differential exploratory calorimetry was performed on the same equipment, STA 6000 (Perkin Elmer, USA), using alumina calorimetric cell, where the samples were heated at a constant rate of 10 °C/min, from 20 to 400 °C, under constant nitrogen flow (50 mL/min).

2.4.5 X-ray powder diffraction analysis

The MPs were examined in a Shimadzu XRD-6000 X-ray diffractometer, scan of 2°/min^{-1} and 2θ scan of 5° to 80°, copper Kα radiation (λ = 1.5418 Å), 40 mA current and 40 kV voltage, for the observation of possible peaks indicative of crystallinity. The diffractograms of the pure DIM, the MPs containing DIM, the ethylcellulose, the physical mixture of the constituents and the blank formulation were evaluated.

2.4.6 Morphological evaluation

The particle morphology was analyzed by optical microscopy (Olympus CH30, Brazil) at 50 Hz and 0.15 A, by observation of a blade of MPs dispersed in mineral oil, with a x40 objective lens and ocular lens equipped with a graduated vernier. A scanning electron microscopy with field emission guns (FEG-SEM) (TESCAN, Mira 3 model, Czech Republic) was also employed. The samples were sputtered with gold in a Shimadzu IC-50 Ion Coater (Japan). To obtain the electromicrographs the software *Electron Optical Design* and an acceleration voltage of 15 kV were used.

2.4.7 DIM In vitro *release assay*

The evaluation of DIM release profile from the MPs was performed by the beaker method. The MPs (equivalent to 10 mg DIM) were assayed in 200 mL of release medium (phosphate buffer pH 7.4/2% Tween 80®), which was maintained at

37 °C and under stirring. At predetermined intervals, an aliquot of 2 mL of the dissolution medium was collected, filtered on cellulose membrane (0.45 µm) and analyzed by HPLC (section 2.2) to determine the amount of DIM released. After each collection, the same volume of fresh medium was replaced to maintain a constant medium volume. The experiments were conducted in triplicate and also performed with the bulk compound.

The experimental data were modeled using the software Excel® (Microsoft Office, 2016) according to the first-order (4) and Higuchi (5) kinetics equations.

$$Q = Q_0.e^{-kt} \quad (4)$$

$$Q = K_H.\sqrt{t} \quad (5)$$

Where *Q* is the amount of compound released at time *t*, Q_0 is the initial amount of compound in solution, *k* is the first order release constant and K_H represents the Higuchi release constant (which reflects structural and geometric characteristics of the system).

2.5 *In vivo* evaluation of DIM-MPs anti-hypernociceptive action

2.5.1 Animals

Male Swiss adult mice, weighing between 25 and 35 g, from the Federal University of Santa Maria (UFSM) Central Vivarium were used. The animals were placed in separate boxes (10 animals/box), under temperature conditions of 22 ± 2 ºC and kept in a cycle of 12 h light/12 h dark. They received a diet consisting of commercial ration (GUABI, RS, Brazil) and fresh water ad libitum. All experiments were approved by the Insitutional Committee for Animal Care and Use of the UFSM (Protocol approved under No. **4428090217/2017**). The project was conducted according to the guidelines of the National Council for Control of Animal

Experimentation (Brazil) and other ethical guidelines for the management of laboratory animals.

The tests were carried out during the light phase of the cycle (between 8 h and 16 h), in an appropriate behavioral room (temperature of 25 ± 2 ºC; humidity controlled; suitable brightness and soundproofing). Prior the experimental evaluation, the animals were acclimatized for at least 1 h to adaptation to the room. In addition, all efforts were made to minimize the suffering and the total number of animals used in the experiments. At the end of the procedures, the animals were euthanized by cervical dislocation.

2.7.2 Animal model of acute inflammatory pain induced by CFA

The anti-hypernociceptive action of free DIM or DIM-MPs was evaluated in the inflammatory pain model induced by intraplantar CFA injection, which consists of an oil suspension of inactivated Mycobacterium tuberculosis, diluted in 85% paraffin oil and 15% mannide monooleate (1 mg/ml). The behavioral parameter analyzed for this model was the mechanical hypernociception, through the Von Frey filaments test (VF). Initially, the animals were habituated for 1 h in elevated individual boxes (9 × 7 × 11 cm), whose floor is made of non-malleable wire in the form of a net, in order to allow access to the hind legs of the animal. The right hindpaw was stimulated through 10 applications (duration 1-2 sec each) with a 1.0 g VF filament (Stoelting, USA). Prior to the inflammation induction, the basal response to the stimulation by the filament was evaluated, being considered an adequate frequency of response, around 15%. The intraplantar injection of CFA suspension or saline (20 µL) was then performed on the right hindpaw of mice, according to the experimental group (section 2.7.3).

Twenty-four hours after this procedure, in order to confirm the induction of mechanical hypernociception, all animals were submitted to the VF test, being excluded from the experiment those that presented less than 70% response. Afterwards, the animals were treated by intragastric route and submitted to behavioral analysis to record the frequency of response to VF over time.

2.7.3 Experimental groups and treatments

The animals were randomly divided into six groups (*n* = 8-10), each one receiving the following treatment:

- CFA Group – animals induced with CFA and treated with vehicle (1.5% gellan gum; 10 mL/Kg);
- CFA + DIM Group – animals induced with CFA and treated with DIM in the free form (10 mg/Kg previously dissolved in DMSO (10%) and incorporated into the vehicle);
- CFA + MP-B Group – animals induced with CFA and treated with MPs without DIM (MP-B) incorporated into the vehicle;
- CFA + MPDIM Group – animals induced with CFA and treated with MPs with DIM (MP-D; 10 mg/Kg) incorporated into the vehicle;
- Ketoprofen Group – positive control, animals induced with CFA and treated with ketoprofen (10 mg/kg previously dissolved in DMSO (10%) and incorporated into the vehicle).
- Control Group - without induction of inflammation, only saline was injected into the paw of these animals and treated with vehicle (1.5% gellan gum).

The treatment was performed 24 h after the induction of the experimental model (section 2.7.1), by intragastric gavage (i.g.), at the dose of 10 mg/kg, in an administration volume of 10 mL/kg. The behavioral test was performed at the times of 0.5, 1, 2, 3, 4, 5 and 6 h after the administration of the treatments.

2.8 Statistical analysis

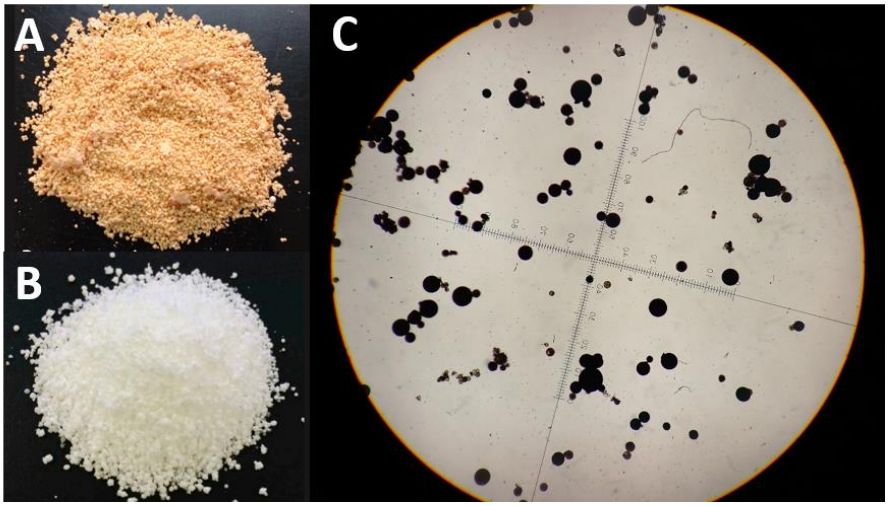
Data obtained are expressed as mean ± standard deviation or mean ± standard error. The normality of the data was evaluated by the Kolmogorov-Smirnov test. The results were evaluated using the *t-test* or the one-way analysis of variance, ANOVA (ordinary or repeated measures), followed by the Tukey test. Values of $p<0.05$ were considered significant. The software GraphPad Prism 6® was used to graphs generation and carry out the statistical tests.

3 RESULTS

3.1 Preparation and characterization of MPs

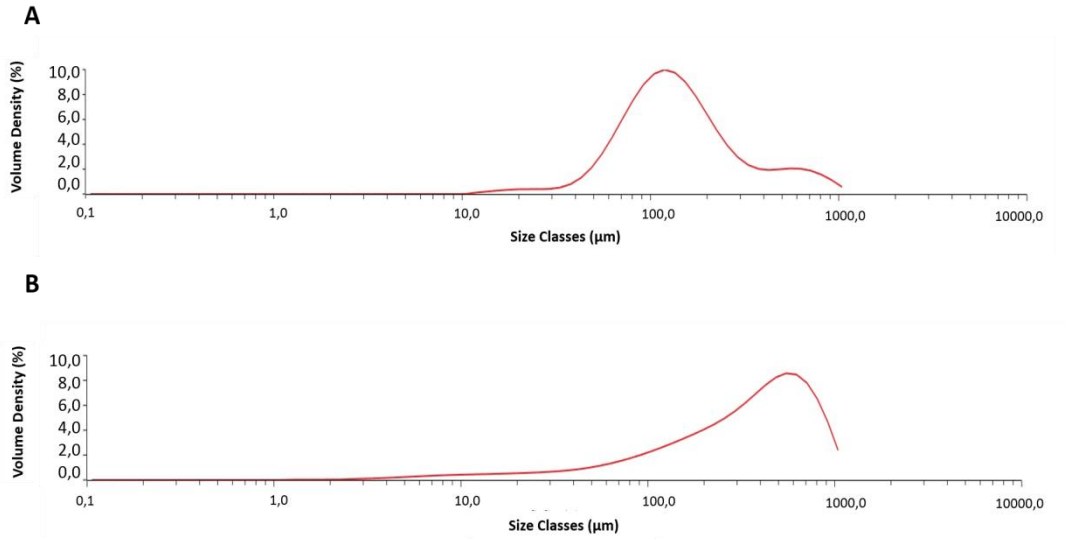
The macroscopic evaluation showed that the MPs constitute a powder of homogeneous appearance (**Figure 1**), white (MP-B) or yellow-orange in the case of the MP-DIM, which can be attributed to the presence of the compound in the formulation. Figure 1C shows the image obtained by optical microscopy (magnification 40x), which demonstrated that the DIM-loaded MPs have regular spherical shape and no presence of crystals of non-encapsulated drug.

Figure 1 – Macroscopic and microscopic aspect of the ethylcellulose MPs. A: DIM-loaded microparticles (MP-DIM); B: blank microparticles (MP-B); C: optical microscopy images of the MP-DIM (magnification 40x).



The yield of the formulation was adequate for both blank and DIM-loaded MPs (93 ± 0% for MP-B and 91 ± 2% for MP-DIM). In relation to the DIM content and encapsulation efficiency, the values obtained were 145.8 ± 13.0 mg/g and 84 ± 3%, respectively. Regarding the granulometric distribution (**Figure 2**), the DIM-loaded MPs presented D[4;3] of 368 ± 31 µm, $D_{10\%}$ of 64 ± 4 µm, $D_{50\%}$ of 324 ± 46 µm and $D_{90\%}$ of 769 ± 17 µm. In addition, the DIM microencapsulation lead to a smaller *Span* values (2.900 ± 0.500 for MP-B and 2.000 ± 0.200 for MP-DIM), which usually indicates adequate homogeneity of the system.

Figure 2 – Graphical representation of the granulometric distribution by volume density. (A): MP-B; and (B): MP-DIM.



From the determination of the powder densities (0.20 ± 0.02 g/cm^3) and compaction (0.23 ± 0.02 g / cm3) of the MP-DIM formulation, it was possible to calculate the CI and HF values, which were 9.6 ± 1.4% and 1.10 ± 0.01, respectively.

The physical state of DIM in the MPs developed was evaluated by differential exploratory calorimetry. The curves obtained for MP-B and MP-DIM formulations as well as their pure constituents are shown in **Figure 3**. Both pure ethylcellulose and MP-B showed an endothermic event at 378 °C, which can be attributed to the melting point of this polymer. In the pure DIM profile, it is observed a more intense peak at 178 °C, temperature near its melting point. The physical mixture of raw materials presented these same events, whereas in the profile of the MP-DIM formulation the only marked endothermic event occurred around 380 ºC, temperature similar to the ethylcellulose melting peak.

Figure 3 – Differential scanning calorimetry curves of MPs and pure constituents. MP: physical mixture between DIM and polymer; MP B: blank microparticles; MP DIM: DIM-loaded microparticles; EC: ethylcellulose.

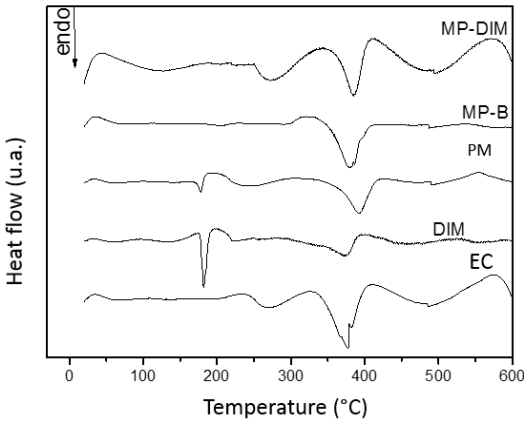
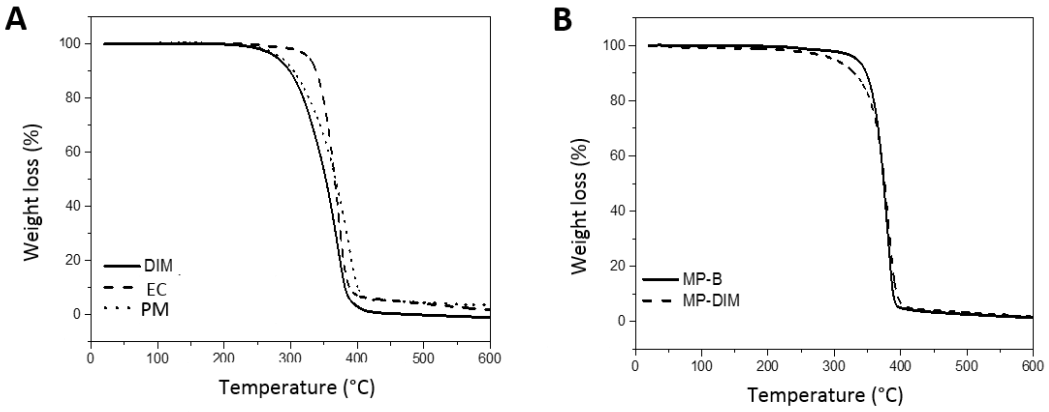


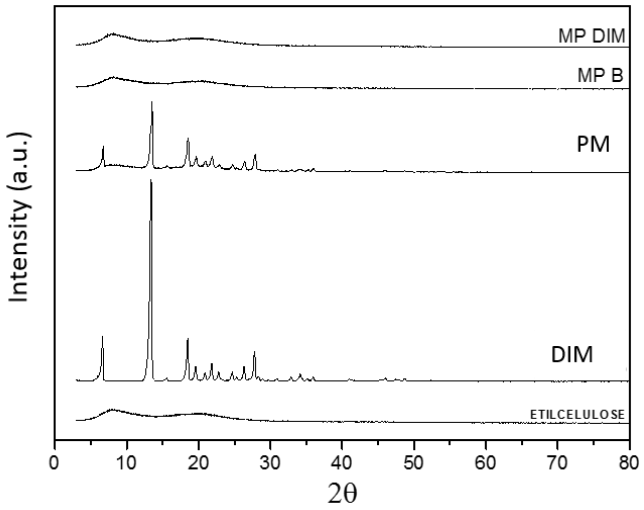
Figure 4 demonstrates the mass loss steps of samples in the thermogravimetry technique. All the samples presented only one mass loss event. The pure ethylcellulose began to degrade at around 315 ºC, which is in accordance with a previous study (LOMAKIN et al., 2011). Regarding the physical mixture, there was an event starting at intermediate temperatures between the physical mixture of ethylcellulose and pure DIM, which showed loss from approximately 250 °C. However, both MP-DIM and MP-B formulations demonstrated mass loss events at higher temperatures than the isolated substances, around 350 °C for the MP-B and 310 °C for MP-DIM.

Figure 4 – Thermograms of MPs and pure constituents. **A**: pure DIM, ethylcellulose, MP (physical mixture between DIM and polymer).



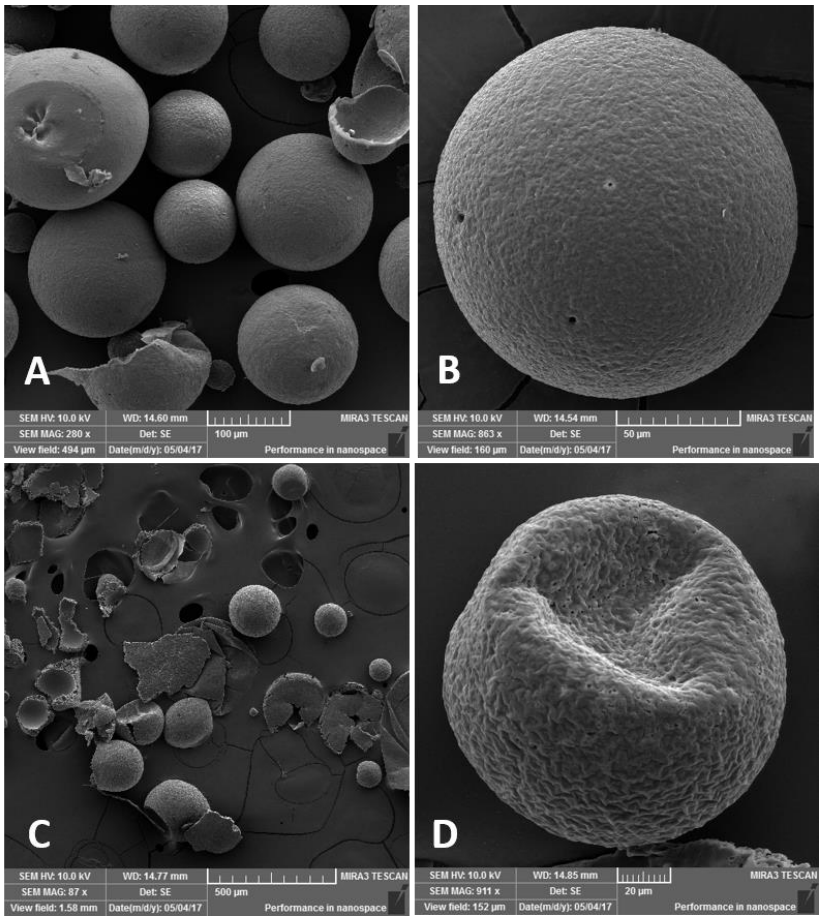
The results of the x-ray analysis are shown in **Figure 5**. The profile of the pure ethylcellulose is characterized by two crystalline peaks at 7.78° and 20.51°. The DIM presented several crystalline peaks, at 6.45º, 13.48º (highest intensity peak), 18.60º, 21.85º, 27.87º. In the case of the MPs, only peaks at angles close to those of ethylcellulose are observed, regardless of DIM presence in the MPs.

Figure 5 – Diffractogram patterns of the MPs and pure constituents. MP: physical mixture between DIM and polymer; MP B: blank microparticles; MP DIM: DIM-loaded microparticles; EC: ethylcellulose.



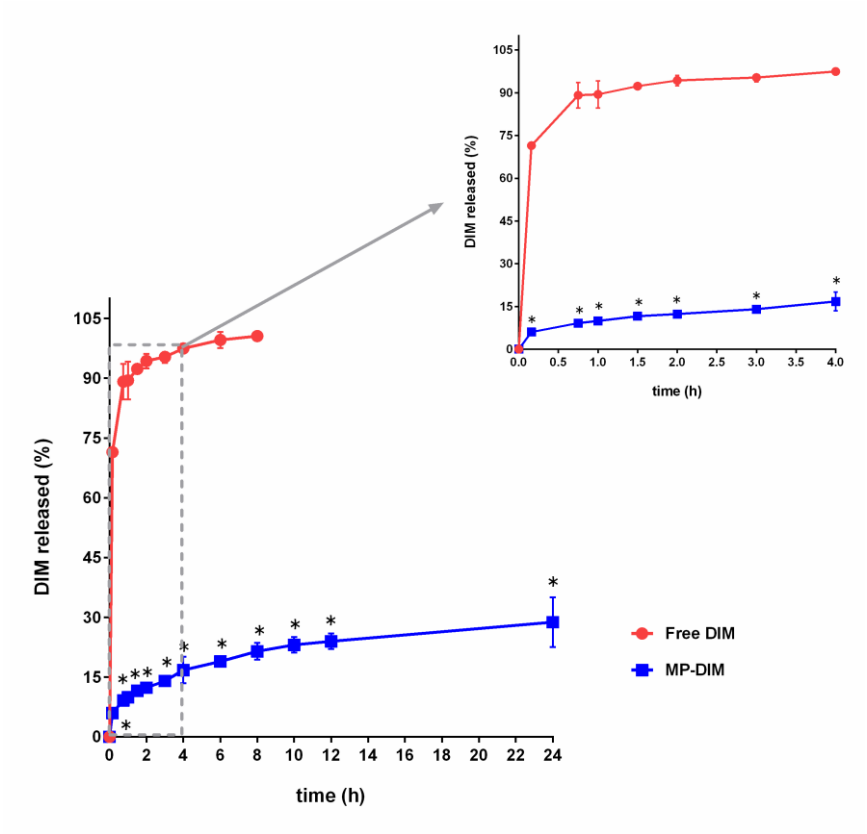
The images obtained by electron microscopy revealed that the MP-DIM formulation presented spherical shape, hollow and rough surface without DIM crystals or clusters (**Figure 6**). The same morphological characteristics were observed to the MP-B (**Figure 6 - C and D**).

Figure 6 – Images obtained by field emission scanning electron microscopy of the Ethylcellulose MPs (A and B: MP-DIM; C and D: MP-B). Experimental conditions: acceleration voltage: 10.0 kVA; magnitude: 280x (A); 863x (B); 87x (C); 911x (D).



The results of DIM release profile from the MPs formulation and the pure DIM are depicted in **Figure 7**. The experiment showed that around 100% of pure DIM (free form) was dissolved after 6 h, while the MPs released only 28 ± 6% of the compound after 24 h of experiment.

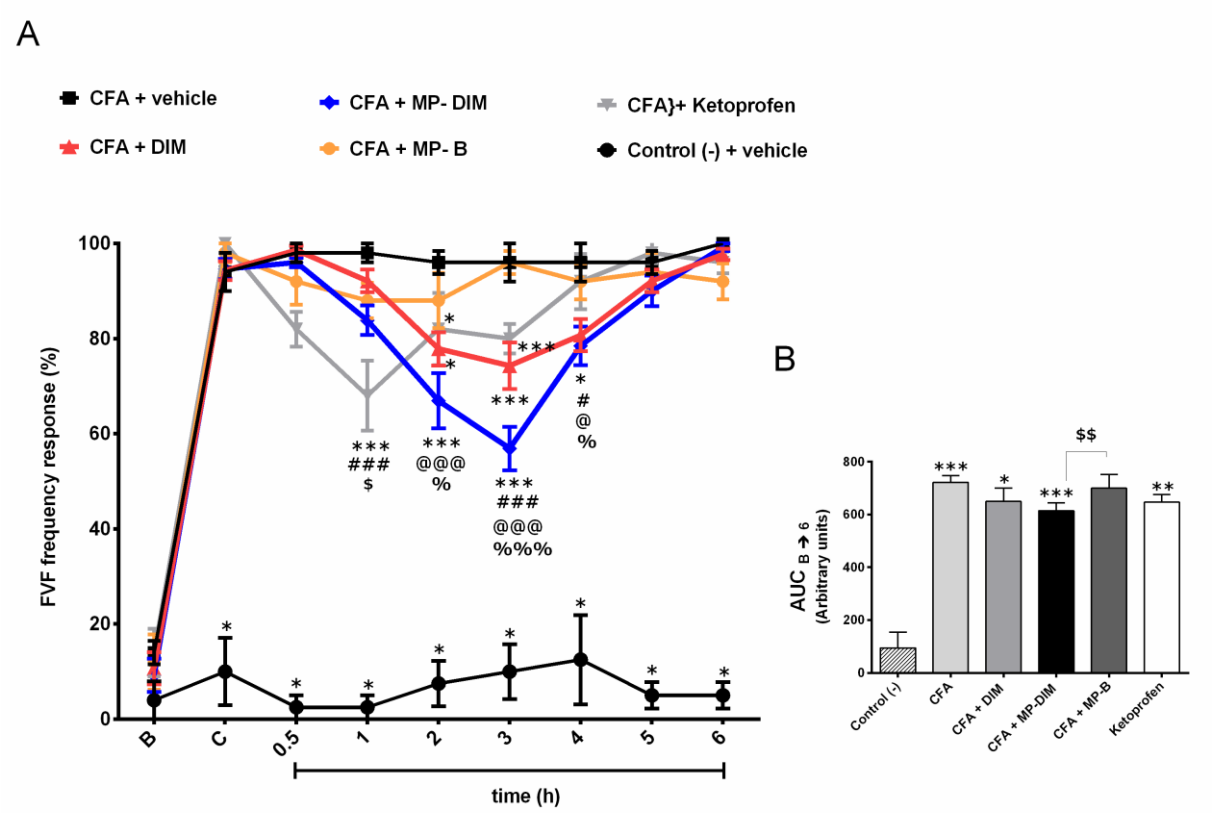
Figure 7 – *In vitro* release profile of DIM in its free (Free DIM) or microencapsulated (MP-DIM) forms. Each point represents the mean ± SD of six independent experiments.* $p<0.05$. Significant difference between MP-DIM and control (Free DIM). (One-way ANOVA of repeated measures, followed by Tukey's test).



3.2 Anti-hypernociceptive action of MP-DIM in the acute inflammatory pain model induced by CFA

The effect of the treatment of the free DIM or MP-DIM in the acute inflammatory pain model induced by intraplantar CFA injection was investigated by the mechanical stimulus test using VF filaments (**Figure 8**). The percentage of response to the filament stimulation was increased at 24 h after the intraplantar CFA injection, characterizing the mechanical hypernociception. Regardless of the time of analysis, the CFA group treated with the vehicle had a high frequency of response to the stimulus compared to the negative control group over all experimental evaluation ($p < 0.05$) (**Figure 8A**).

Figure 8. Time-response curve of DIM treatment effect on the mechanical hypernociception induced by an acute inflammatory pain model (A) and the respective area under the curve (basal response [B] to 8 h) (B). The results represent means ± S.E.M. of 8–10 animals/group. CFA: animals induced with CFA and treated with vehicle; B: basal response frequency, before CFA injection; C: confirmatory response frequency to VFH stimulation, 24 h after CFA injection. The asterisks denote significant difference in comparison to the CFA + vehicle group (CFA), (*) p<0.05 (***) p<0.001; sharps denote significant difference in comparison to the CFA + DIM group, (#) p<0.05 (###) p<0.001; ciphers represent significant difference in comparison to CFA + MP-DIM group, ($) p<0.05 ($$) p<0.01; arrobas represent significant difference in comparison to CFA + MP-B group, (@) p<0.05 (@@@) p<0.001; percents denote significant difference in comparison to the ketoprofen group, (%) p<0.05 (%%%) p<0.001. One-way ANOVA of repeated measures followed by Newman Keuls' test was performed to time-response curve.



Regarding the treatments, the administration of both free DIM and MP-DIM reduced mechanical hypernociception starting at 2 h after treatment (p<0.05 and p<0.001, respectively), with maximum effect occurring after 3 h, being this effect

more pronounced for MP-DIM (p<0,001) (E_{max} 19.0 ± 3.8% for free DIM and 31.9 ± 6.1% for MP-DIM). Notably, the administration of the MP-DIM promoted a reduction in the response to the mechanical stimulus up to 4 h of experiment, in contrast to the compound in the free form and to ketoprofen (positive control), in which the decrease in hypernociception was verified only up to 3 h. The group of animals treated with ketoprofen showed maximum effect after 1 h (E_{max} of 38,9 ± 8,0 %). The MP-B formulation did not show anti-hypernociceptive effect at any treatment time.

Confirming the results of the behavioral analysis, **Figure 8B** shows the area under the curve (AUC) values. The animals treated with free DIM, ketoprofen or MP-DIM showed a significant difference in hypernociception in comparison to the non-treated group (CFA), being this difference significantly higher for MP-DIM, followed by ketoprofen and then by free DIM (p<0.001; p<0.01; and p<0.05, respectively). In addition, the AUC graphic also demonstrates the role of DIM in the anti-hypernociceptive effect presented by MPs, since the MP-DIM showed a significantly greater effect than MP-B (p<0.01).

4 DISCUSSION

The current study demonstrated the preparation of ethylcellulose MPs using a simple and low-cost method. Collectively, the results showed that MP-DIM had adequate physicochemical properties as well as provided a controlled-release profile of the active. In addition, DIM encapsulation increased and prolonged its therapeutic effect in the acute inflammatory pain model after intragastric administration to mice.

Regarding the methodology selected for formulating the MPs, it was observed that the preparation yield by the emulsification-solvent evaporation technique was close to 100%, indicating small loss of mass of the constituents, which is expected for this method. In addition, MPs presented mean diameter in accordance with previous studies that used the same polymer and/or preparation method (Deshmukh and Naik, 2014; Nadendla et al., 2013; Patel et al., 2012; Zamani et al., 2015). It is also important to note that DIM-loaded MPs presented a lower *Span* value (closer to 2.0) than blank MPs, indicating a more adequate size homogeneity for this system. Regarding the evaluation of flow properties, CI and HF values lower than 25% and 1.20, respectively, indicate that the powder has good flow properties, which is related with the spherical homogeneous shape of the particles (as observed in the FEG-SEM

analysis – **Figure 6**) and it is an important characteristic for the preparation of solid pharmaceutical forms.

The encapsulation efficiency and the compound content obtained are also similar to other MPs developed by the same preparation method (Arunkumar et al., 2016; Behera et al., 2008; Nadendla et al., 2013; Shanmugarathinam et al., 2011) which suggests adequate compatibility among the materials. This hypothesis is confirmed by the analyses of differential scanning calorimetry, thermogravimetry, X-ray diffraction, and Infrared spectroscopy. The encapsulation efficiency of a compound in the polymeric matrix MPs depends on its solubility in the solvent and in the continuous phase. Thus, it is important to take the lipophilicity/hydrophilicity of the compound into account in the choice of the type of emulsification intended (O/W, W/O/W, O/O/W, etc.) (Behera et al., 2008; Deshmukh et al., 2016). Therefore, considering the lipophilicity of DIM and the method chosen for the preparation of the MPs (emulsification-evaporation of solvent O/W), a high encapsulation rate of the compound was expected. In addition, the results of differential scanning calorimetry and X-ray diffraction techniques demonstrated that there was a reduction in DIM crystallinity in the MPs, confirming the high encapsulation efficiency observed. In addition, differential scanning calorimetry and X-ray diffraction techniques, which are used to evaluate the crystalline structure of compounds, demonstrated that there was a significant reduction in the crystallinity of the compound in the MPs and its consequent dispersion in the system, confirming the high encapsulation efficiency observed.

On the other hand, approximately 16% of DIM was lost during the preparation. Such loss may be caused by the solubilization of DIM by the surfactant (Tween® 80) in the aqueous external phase, thus being removed together with the surfactant in the filtration/washing step. In addition, some loss of compound may have occurred during the solvent evaporation process, since this step was conducted under atmospheric pressure. This process is slower than the solvent extraction under reduced pressure, which usually generates particles with higher encapsulation efficiency rates (O'Donnell and McGinity, 1997). Furthermore, the fact that the emulsification-solvent evaporation method involves several stages of preparation and exchange of glassware could explain the loss of raw materials during the MPs fabrication.

In order to evaluate the possible loss of mass due to decomposition and/or evaporation processes, the thermogravimetry technique was used (Zayed et al., 2017). The results suggest that MP-DIM formulation has higher thermal stability in comparison to raw materials. The morphological evaluation revealed MPs with uniform surface without DIM crystals on the surface. These results demonstrate the preparation efficacy and suggest that DIM could be adequately incorporated into the polymer matrix. Other authors also developed ethylcellulose microspheres by the O/W emulsification-evaporation method and obtained particles presenting oval morphology, with a uniform surface and little porosity (Deshmukh and Naik, 2014). Furthermore, hollow MPs were also obtained by the same method of preparation (Kohli et al., 2017; Sharma, 2015; Sharma et al., 2017, 2014). According to (Jain et al., 2005), the hollow microspheres are formed by the injection of an organic dispersion containing the polymer and the drug into an aqueous solution containing the surfactant, which leads to the rapid distribution of ethanol in the external aqueous phase and the precipitation of the polymer, covering the droplets of dichloromethane. When the entrapped solvent evaporates at ambient pressure, the formation of the cavities within the MPs occurs.

Regarding the release profile, the extensive control on the release of DIM from the MPs can be attributed to ethylcellulose as the polymer forming the matrix structure of the microparticle, since this polymer can act as a permeation barrier. In fact, the application of ethylcellulose in the development of controlled release systems has been demonstrated a promising profile of drugs release rate (Park et al., 2012; Rogers and Wallick, 2012a). In addition, DIM is a highly lipophilic and water-insoluble compound, resulting in its greater affinity with the hydrophobic polymer matrix than with the aqueous release medium. Thus, such characteristics lead us to suggest that DIM tends to be entrapped in the MPs polymeric matrix and to not easily diffuse to external medium, explaining its slow release rate from the formulation.

Concerning the mathematical modeling of drug release, MPs composed of ethylcellulose often present first-order kinetics as the best model to explain the release profile of compounds from the polymer matrix (Rogers and Wallick, 2012a, 2012b, 2011). By adjusting the data to the first-order reaction kinetic equation, the value found for the rate constant k was 0.0116 h^{-1} with a correlation coefficient of 0.9313. These results indicate that the DIM release from the MPs occurs

proportionally to the remaining compound concentration within the microstructure. The medium diffuses through the polymeric matrix and dissolves DIM, reducing its amount in the MPs, thus, the quantity released will exponentially decrease over time.

Further, in order to establish the possible mechanism of DIM dissolution, the data of cumulative release *versus* time were fitted to the Higuchi equation. The K_H value was 6.03, with a correlation of 0.9974, suggesting that the mechanism of DIM release follows the model proposed by Higuchi. This model can be applied to describe the drug release rate from insoluble/hydrophobic matrices in which the release of the encapsulated drug occurs by Fickian diffusion (Higuchi, 1963; Lopes et al., 2005; Manadas et al., 2002). In the case of the MPs containing DIM, it is suggested that the release of the bioactive occurs mainly by diffusion through the pores or channels formed from the penetration of the medium into the ethylcellulose matrix. This process contributes to slowly dissolve DIM and explains the sustained release observed. Thus, our current data suggest that mechanisms of polymeric swelling or erosion could be negligible.

Among the several properties attributed to DIM, the antinociceptive and anti-inflammatory actions are of particular interest in regard to their application in the treatment of pathological pain conditions. Since the chronic pain is complex and involves several pathophysiological mechanisms, it is interesting that candidates for new drugs have an effect on multiple targets, a characteristic that is presented by DIM (Banerjee et al., 2011; Maruthanila et al., 2014). Thus, after the development of DIM-loaded MPs formulation, the next step was to evaluate the performance of this system concerning the biological effects. The present study showed that the DIM microencapsulation positively impacted in the anti-hypernociceptive action of the compound assessed in the acute inflammatory pain model induced by CFA intraplantar injection. The CFA administration induces inflammation, triggering local pain and edema in the injected site. This inflammation leads to the release of several inflammatory mediators, such as glutamate and C-fiber neuropeptides, which sensitize peripheral nociceptors at the lesion site and also at the spinal cord level, producing local hypersensitivity and thus a lasting increase in the frequency of paw withdrawal in response to stimulation with VF filaments (Hegen et al., 2008; Ren and Dubner, 1999; Samad et al., 2001).

The results of the present study demonstrated that the treatment with DIM in both forms (free or microencapsulated) reduced the response frequency of the animals to the mechanical stimulus, but with different time-response profiles. While the free DIM showed anti-hypernociceptive effect up to 3 h, the MP-DIM demonstrated to prolong the pharmacological action of the compound up to 4 hours. In a previous study, our research group developed ethylcellulose nanocapsules, which also promoted prolonged release of the compound *in vitro*, as well as a longer duration of the anti-hypernociceptive effect in the same model of acute inflammatory pain (unpublished data – ***Manuscript 1***). In addition, a faster onset of action was observed for DIM- loaded nanocapsules than for free DIM. This finding was attributed to the controlled release of the compound from the nanocapsules and to the reduced size of the nanostructures, which favors their absorption in the gastrointestinal tract, due to a larger area of contact with the biological membranes. In comparison with the MPs developed in the current study, the nanocapsules also improved and prolonged the DIM anti-hypernociceptive effect for a longer period. Such differences could be explained by the smaller diameter of nanocapsules as well as because they are obtained in the form of a suspension (liquid form), while the MPs are obtained in the form of dry powders. Thus, in the case of the nanocapsules, the process of drug release from the system and its consequent absorption could start more rapidly, which undergo a faster dissolution process in the gastrointestinal tract than the MPs, providing faster drug availability. On the other hand, because they are a solid powder material, the MPs offer the advantage of having greater physicochemical and microbiological stability than the NCs, besides allowing the incorporation of greater amount of compound in the system (145 mg/g DIM in the MP *x* 1 mg/mL DIM in the NC).

Importantly, in comparison to the free DIM, the prolongation of the pharmacological effect of the MP-DIM could be attributed to the high DIM association with the MPs. It can generate stronger chemical bonds between the particle matrix and the compound than the bonds between molecules of pure DIM. This prolonged release allows the DIM plasma concentration to be maintained at therapeutic levels for longer periods, which may possibly justify the pharmacological action profile observed for the MP-DIM. In addition, less frequent doses would be necessary, which may reduce the risk of toxicity of the compound (Rogers and Wallick, 2012a).

In addition, the MP may promote protection to DIM against enzymatic or chemical degradation in the GIT, releasing the compound only at the site of absorption. Another favorable feature of the MPs is the bioadhesion property, which can remain longer at the site of absorption and also distribute more broadly and evenly in the GIT than the compound in the free form (Bale et al., 2016; Freiberg and Zhu, 2004; Rogers and Wallick, 2012a).

5 CONCLUSION

In conclusion, the current study contemplated the development of novel DIM-loaded MPs, which were successfully prepared and demonstrated to adequately encapsulate the compound. In addition, the MPs presented suitable physicochemical characteristics compatible with oral drug delivery systems, sustained DIM release from the MPs as well as improved anti-hypernociceptive action compared to the free compound. Thus, data obtained herein showed that the proposed MPs could be considered a promising system for the prolonged oral release of DIM aiming at the management of inflammatory pain.

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5. DISCUSSÃO GERAL

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A primeira etapa deste trabalho compreendeu a preparação e caracterização de suspensões de nanocápsulas contendo DIM. Para tal, com fins comparativos, foram utilizados dois diferentes polímeros (EC e ERS) e dois óleos vegetais (prímula e damasco). Considerando-se o ineditismo da preparação de NCs contendo DIM, bem como da utilização destes óleos vegetais, foram realizados alguns estudos de pré-formulação, os quais demonstraram a compatibilidade entre os constituintes das nanoestruturas. Além disso, a partir dos resultados de caracterização foi possível verificar a viabilidade de preparação das formulações, as quais apresentaram características adequadas independentemente do tipo de óleo ou polímero empregado, o que também foi observado com relação à fotoestabilidade do DIM nas diferentes NCs. Ainda, como uma importante etapa de desenvolvimento das formulações, conduziu-se o estudo do perfil de liberação/diálise do DIM a partir das NCs. Os resultados demonstraram que o DIM foi dialisado mais lentamente a partir das NCs, ou seja, estas prolongaram a sua liberação/diálise, o que pode ser explicado pelo fato de o bioativo estar adequadamente associado ao sistema, corroborando os resultados anteriores.

Na sequência, a fim de avaliar-se o desempenho *in vitro* das formulações e possível influência do nanoencapsulamento na ação antioxidante do DIM, procedeu-se a determinação da capacidade sequestrante do DIM frente aos radicais sintéticos ABTS e DPPH. Como resultado, foi verificada uma melhor performance das NCs em comparação ao DIM livre ou aos óleos livres, em sequestrar ambos os radicais. De maneira interessante, esta avaliação nos permitiu observar diferenças entre as formulações, destacando a NC-PEC-D pela sua maior capacidade sequestrante entre as demais formulações desenvolvidas. Além disso, nossos resultados ressaltam a superioridade do potencial antioxidante do DIM em comparação ao I3C. No presente trabalho, concentrações muito menores (2-4-6 µg/mL) de DIM utilizadas em relação às previamente relatadas para o I3C (10-25-50-75 µg/mL) por Gehrcke e col. (2016) no teste do DPPH. Isso pode ser devido ao DIM apresentar dois grupos doadores de hidrogênio para estabilizar a molécula do radical, enquanto que o I3C apresenta apenas um grupo doador.

Como próximo passo, considerando-se o já relatado efeito do DIM em suprimir a resposta inflamatória induzida pelo lipopolissacarídeo (LPS) em macrófagos de ratos (linhagem RAW264.7) (CHO et al., 2008), tentamos realizar a avaliação da ação anti-inflamatória *in vitro* do DIM livre e das NCs. Foram testados diferentes protocolos, em parceria, primeiramente, com a Prof.ª Dr.ª Michele Rorato Sagrillo, da Universidade Franciscana (UFN) e, em um segundo momento, com a Prof.ª Dr.ª Elizandra Braganhol, da Universidade Federal de Ciências da Saúde de Porto Alegre. Os testes incluíram a indução da inflamação pela fitohemaglutinina ou pelo LPS, em macrófagos de ratos (linhagem RAW265) ou em linhagem de glioblastoma maligno humano resistente (U87 e U138), os quais foram tratados tanto com as suspensões de NCs quanto com o DIM livre. Embora tenham sido testadas diversas concentrações dos indutores e das NCs, diferentes tempos e formas de incubação das amostras, além de distintos métodos de avaliação da viabilidade celular (MTT e violeta de genciana), não obtivemos êxito na realização de nenhum dos protocolos. Assim, não foi possível evidenciar o efeito anti-inflamatório *in vitro* do DIM e das formulações desenvolvidas no presente estudo por questões de limitação experimental.

Dessa forma, no intuito de se dar continuidade à avaliação da performance biológica das nanoestruturas e levando-se em conta o efeito antitumoral do DIM e sua possível aplicabilidade no tratamento do glioma (RAHIMI, HUANG, TANG, 2010; SHERER et al., 2017), conduziu-se a avaliação da citotoxicidade *in vitro* do DIM associado às NCs frente à linhagem celular de glioblastoma maligno humano resistente (U87). A faixa de concentração testada foi próxima à utilizada na avaliação da atividade antioxidante (3-6-12-24 µg/mL) e a determinação da viabilidade celular foi realizada pelo ensaio de redução do MTT. Os resultados demonstraram que o DIM reduziu a viabilidade celular das células U87, sendo tal efeito aumentado pelas NCs. Este resultado pode ser atribuído ao fato de que o processo de nanoencapsulação pode promover um aumento na captação do ativo pelas células no meio de cultura, além do tamanho nanométrico propiciar grande área superficial às NCs, intensificando sua interação com as células e aumentando, assim, seu potencial citotóxico contra a linhagem de células U87 (BERNARDI et al., 2008; FONTANA et al., 2014; GEHRCKE et al., 2016; PEGORARO et al., 2017; SARI et al., 2017). Quanto ao mecanismo de citotoxicidade de nanopartículas

contendo DIM no glioma, um estudo prévio relatou que nanopartículas de PLGA contendo DIM com superfície modificada por um peptídeo (“*SSTR2*”) foram adequadamente internalizadas pelas células de glioma após administração intravenosa em ratos, resultando em apoptose das células tumorais através da inibição da via do fator de crescimento epidermal (*EGFR*) (BHOWMIK et al., 2017). Ainda, as NCs foram testadas em células gliais saudáveis (astrócitos), nas mesmas concentrações utilizadas no tratamento das células tumorais, porém não reduziram a viabilidade celular deste tipo celular, demonstrando a seletividade do efeito citotóxico das NCs (resultados não publicados). Adicionalmente, é importante salientar que até esta etapa do trabalho, a formulação NC-PEC-D mostrou-se como a formulação desenvolvida mais promissora, sendo então escolhida para dar continuidade ao estudo.

É sabido que a dor, além do calor, rubor e edema, é um dos sinais/sintomas do processo inflamatório. Na inflamação, ocorre a síntese e liberação de diversos mediadores pelos leucócitos presentes no tecido afetado como uma resposta adaptativa, podendo ocasionar aumento na nocicepção e contribuir para o surgimento da dor (KIDD, URBAN, 2001; SCHOLZ, WOOLF, 2002). Os tratamentos existentes consistem em abordagens múltiplas e uso de vários medicamentos, porém tais terapias ainda apresentam falhas quanto à eficácia e segurança, além de provocarem efeitos adversos desagradáveis nos pacientes. Assim, há uma necessidade urgente de se desenvolver novas abordagens farmacológicas que possam melhor suprir o manejo clínico da dor (CARTER et al., 2014; ANDREU, ARRUEBO et al., 2018). Neste sentido, a investigação do potencial analgésico de ativos que tenham ação anti-inflamatória relatada pode ser uma estratégia relevante. Recentemente, nosso grupo de pesquisa relatou pela primeira vez a ação antinociceptiva do composto I3C, precursor fitoquímico do DIM, livre e nanoencapsulado em modelos animais de dor aguda, sugerindo que este efeito poderia estar relacionado à sua atividade anti-inflamatória (GEHRCKE et al., 2018). Este fato, aliado aos conhecidos efeitos anti-inflamatórios do DIM, despertaram nosso interesse em avaliar a possível ação antinociceptiva da formulação NC-PEC-D e do DIM não-encapsulado. Para tal, foram testados três modelos distintos de nocicepção: o modelo de nocicepção térmica pelo teste da placa quente (*Hot plate*),

o modelo de nocicepção química induzida pela formalina e o modelo de dor inflamatória aguda induzida pelo CFA.

No teste da placa quente, a nanoencapsulação do DIM prolongou a ação antinociceptiva em comparação ao composto livre e manteve este efeito mesmo para uma menor dose (5 mg/Kg). Outros estudos também demonstraram uma melhora na ação farmacológica de ativos através da sua associação às nanoestruturas (BERNARDI et al., 2009; VILLALBA et al., 2014; FERREIRA et al., 2016; SARI et al., 2017). De fato, esta hipótese é reforçada pelo trabalho de GEHRCKE e col. (2018), onde observou-se que as NCs contribuíram para uma modificação no perfil de ação *in vivo* do I3C (5 mg/Kg) neste mesmo modelo, desencadeando um rápido início de ação (após 30 min), o qual se manteve até 6 h. Ainda, este modelo evidenciou o efeito de ambos os compostos na nocicepção não-inflamatória, uma vez que o mecanismo nociceptivo descrito para o teste envolve respostas supraespinhais integradas, sugerindo que o efeito antinociceptivo dos ativos pode estar relacionado com a modulação de componentes do SNC.

Interessantemente, no teste da formalina, os resultados encontrados indicam que o DIM apresenta ação antinociceptiva nas duas fases da dor, tendo esta ação sido antecipada e prolongada pelo nanoencapsulamento. Este resultado pode ser explicado por efeitos anti-inflamatórios já comprovados deste bioativo, como diminuição na produção de prostaglandinas, em especial PGE_2, redução da produção de citocinas pró-inflamatórias (como TNF-α, IL-1β, PLA_2 e IL-6), além da inibição da atividade de enzimas como COX-2 e iNOS (KIM et al., 2010; MARUTHANILA et al., 2014). Reforçando esta hipótese, o DIM também já demonstrou eficácia em um modelo animal de neuroinflamação, pois reduziu o processo inflamatório no hipocampo de ratos e, consequentemente, promoveu a proteção dos neurônios corticais primários, reforçando não somente a ação deste fitoquímico no SNC, como também, que o efeito antinociceptivo do DIM poderia estar relacionado à ação anti-inflamatória do mesmo (KIM et al., 2014). Além disso, é importante mencionar que o I3C não apresentou efeito na primeira fase da dor no modelo da formalina (GEHRCKE et al., 2018), demonstrando que o DIM pode apresentar mecanismos de ação diferentes e potencial analgésico superior ao seu precursor fitoquímico.

Além disso, foi realizado um teste adicional de nocicepção inflamatória em relação ao estudo de GEHRCKE e col. (2018), no qual a avaliação da hipernocicepção causada pela injeção intraplantar de CFA foi realizada pelo teste dos filamentos de VF. Este modelo é amplamente utilizado pela comunidade científica por levar a alterações que reproduzem as condições clínicas e patofisiológicas da dor inflamatória (HEGEN et al., 2008; REN e DUBNER, 1999).

Vale ressaltar que o presente trabalho é o primeiro a investigar a ação farmacológica do DIM em um modelo de inflamação em termos de ação periférica e central, demonstrando resultados interessantes para este composto. Nossos resultados mostraram que os tratamentos tanto com o DIM livre quanto nanoencapsulado reduziram a frequência de resposta após estimulação com o filamento de VF, com distintos perfis de resposta temporal. Novamente, a ação observada para as NCs foi prolongada e melhorada em comparação ao composto livre, corroborando nossos resultados anteriores e também trabalhos de outros autores (BERNARDI et al., 2009; LENZ et al., 2011; HUA, CABOT, 2013; FERREIRA et al., 2016; SARI et al., 2018b).

Em conjunto, estes resultados demonstram a eficácia da administração do DIM, tendo sido investigado o impacto da nanoencapsulação na ação antioxidante, antitumoral e anti-inflamatória já descritas para este bioativo, bem como as diferenças na sua performance *in vivo* com relação ao I3C. Além disso, de forma inédita, esta tese relata o potencial antinociceptivo do DIM em diferentes modelos animais de dor aguda, o que reforça o quão promissor este fitoquímico é e serve de estímulo para futuros estudos visando à aplicação de suas propriedades na terapêutica.

Como uma segunda parte do presente trabalho, considerando-se a relevância do desenvolvimento tecnológico deste tipo de sistema de liberação de fármacos, MPs poliméricas contendo DIM foram preparadas. A microencapsulação de ativos é vista como uma área estratégica no desenvolvimento da indústria farmacêutica, pois é uma ferramenta que permite a liberação controlada de ativos ao longo do tempo e/ou espaço, aumentando a performance terapêutica em comparação às formas farmacêuticas convencionais (VILOS, VELASQUEZ et al, 2012). Além disso, com relação às nanopartículas poliméricas, as MPs trazem a vantagem de serem obtidas como um material pulvéreo seco, apresentando assim maior estabilidade físico-

química e microbiológica e ainda podem constituir por si próprias uma forma farmacêutica ou serem incluídas em cápsulas ou na formulação de comprimidos multiparticulados (VELASQUEZ et al., 2012; VELASQUEZ et al., 2014; FERREIRA et al., 2015 a,b).

Até o presente momento, não há relatos científicos sobre a preparação de MPs poliméricas contendo DIM, apenas com outros derivados indólicos (MURASE et al., 2015). Dessa forma, esta etapa do trabalho esteve focada na preparação e caracterização de MPs inéditas contendo DIM, utilizando-se os mesmos polímeros empregados na formulação das nanocápsulas de DIM previamente desenvolvidas. Apesar das várias tentativas realizadas a fim de se preparar MPs de DIM à base de, primeiramente, Eudragit® RS100, e depois, com as variações EPO e L100, não obteve-se sucesso no preparo de MPs de DIM pelo método de emulsificação-evaporação de solvente com estes polímeros. Esse fato nos indicou uma possível incompatibilidade entre estes materiais. Portanto, com a finalidade de realizar-se futuras comparações da performance do DIM entre as NCs e MPs, prosseguimos com a preparação de MPs de etilcelulose. Como demonstrado no **manuscrito 2**, as formulações de MPs de etilcelulose contendo DIM foram preparadas pelo método de emulsificação-evaporação de solvente O/A e apresentaram características tecnológicas adequadas. Foram realizadas diversas avaliações da forma de associação do DIM às MPs, as quais demonstraram a viabilidade do sistema em incorporar o composto, sem apresentar interações com os outros constituintes.

Além disso, as MPs promoveram liberação prolongada do DIM, seguindo cinética de primeira ordem e modelo de Higuchi. Segundo Manadas, Pina e Veiga (2002), o processo de liberação de uma substância ativa a partir de uma forma farmacêutica de liberação modificada é controlado por diversos fatores inerentes à substância ativa, ao solvente e ao sistema, como a afinidade entre o solvente e a substância ativa, bem como a forma na qual o sistema a libera. Em alguns casos, substâncias capazes de alterar o perfil de dissolução do fármaco no meio podem ser incorporadas na forma farmacêutica, como é o caso das MPs poliméricas. Nesse caso, além das propriedades do fármaco, as características do polímero (como biodegradabilidade e intumescimento) e da própria microestrutura (como tamanho de partícula e capacidade de erosão) também irão influenciar diretamente na velocidade e no mecanismo de liberação (De MELLO, RICCI-JÚNIOR, 2011).

Outras formulações de MPs desenvolvidas previamente também apresentaram este mecanismo de controle de liberação. Amperiadou e Georgarakis (1995) desenvolveram MPs de etilcelulose contendo salbutamol pelo método de emulsificação-evaporação de solvente, as quais exibiram perfil de dissolução *in vitro* características do modelo de Higuchi. Mouffok e col. (2016) também propuseram a difusão Fickiana (modelo de Higuchi) como o melhor mecanismo para explicar a liberação do ácido *p*-aminobenzóico a partir de MPs de etilcelulose preparadas pelo método de emulsificação-evaporação de solvente. Os autores atribuíram esse tipo de liberação ao encapsulamento adequado do ativo no sistema, corroborando os testes de Calorimetria exploratória diferencial e Difração de raio-X. Da mesma forma, no presente trabalho, a liberação prolongada do DIM a partir da formulação MP-DIM pode estar relacionada com a alta eficiência de associação com o polímero e também por possíveis interações em nível molecular entre o ativo e o sistema, o que poderia limitar sua difusão para o meio aceptor.

Após o desenvolvimento das MPs contendo DIM, o passo seguinte foi investigar suas potencialidades como um sistema de liberação oral controlada no tratamento da dor em modelo animal de nocicepção inflamatória aguda, avaliando-se comparativamente o desempenho deste sistema frente aos efeitos biológicos já descritos para o ativo. Para tal, escolheu-se avaliar a ação anti-hipernociceptiva do DIM pelo modelo de dor inflamatória aguda induzida pela injeção intraplantar de CFA em camundongos, através da redução na frequência de resposta ao estímulo mecânico no teste de VF, por este ser um modelo comportamental relativamente simples, rápido e adequado para a avaliação de possíveis efeitos anti-inflamatórios.

Nos últimos anos, alguns trabalhos científicos propuseram o desenvolvimento de MPs com a proposta de possível tratamento para a dor. MPs contendo opioides, como morfina e tramadol, visando a liberação controlada destes ativos, a qual é uma abordagem interessante no tratamento da dor já foram preparadas (RUSSO et al., 2006; AAMIR et al., 2009). Outros autores relataram a preparação de MPs contendo fármacos alternativos aos analgésicos convencionais e demonstraram sua liberação sustentada (desde horas até semanas) em diferentes modelos de dor (PIGNATELLO et al., 2001; TARABALLI et al., 2014; HAN et al., 2015; BORODINA et al., 2016; RUDNIK-JANSEN et al., 2017; DAI et al., 2018), incluindo um registro de patente (CRISCIONE et al., 2016). Pignatello e col. (2001) desenvolveram microesferas de

Eudragit RS100 contendo diflunisal e avaliaram o efeito da administração intraperitoneal destas no modelo de nocicepção induzida pela formalina. Os autores atribuíram a ação antinociceptiva das microesferas ao estado disperso do composto no sistema, o qual favoreceu uma rápida dissolução do ativo e assim um efeito biológico significativamente maior do que a dispersão de fármaco puro. Dai e colaboradores (2018), por sua vez, desenvolveram MPs de PLGA contendo carbamazepina e observaram liberação local (perineural) controlada por longo período (14 dias), proporcionando alívio da dor em modelo de dor neuropática, mas sem provocar efeitos adversos observáveis nas análises comportamentais e histoquímicas.

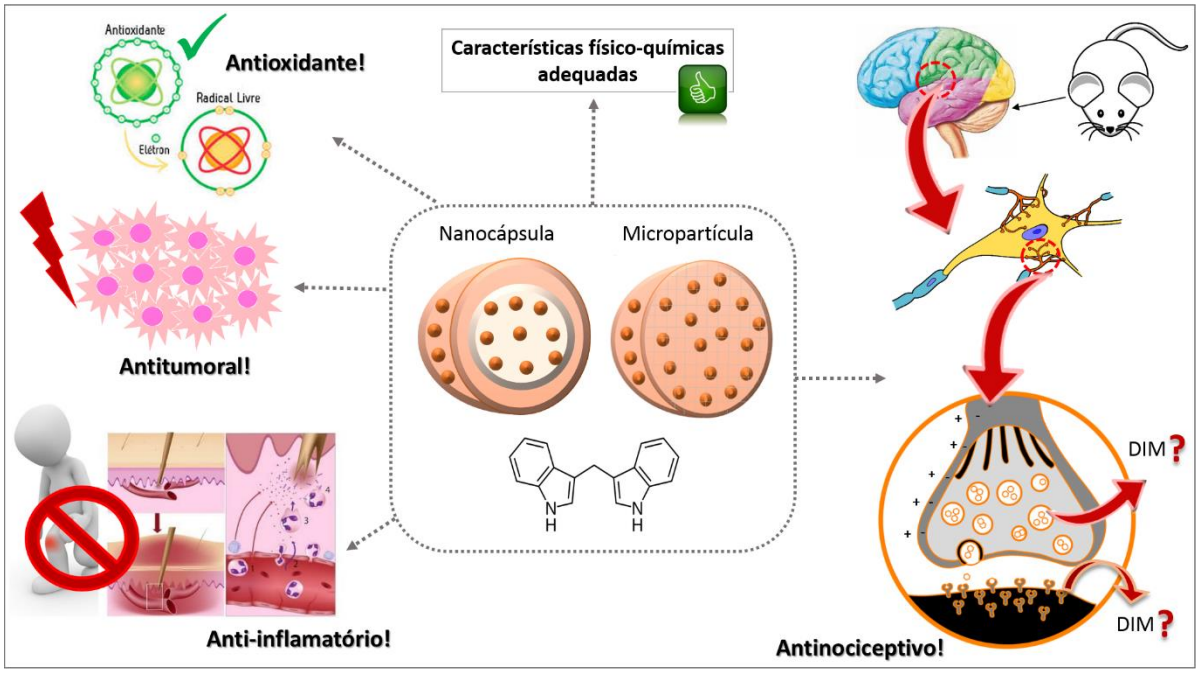
Em nosso estudo, os resultados demonstraram que o tratamento com DIM em ambas as formas (livre ou microencapsulada) reduziu a frequência de resposta dos animais ao estímulo mecânico, porém com diferentes perfis de tempo-resposta. Assim como o observado para as NCs, a MP-DIM demonstrou prolongar a ação anti-hipernociceptiva do bioativo, entretanto, esta ação foi adiantada e mais duradoura para as NCs. Isso pode ser atribuído ao fato de o DIM ser liberado mais lentamente a partir das MPs do que das NCs, e consequentemente sua absorção e seu efeito farmacológico poderiam iniciar mais rapidamente no caso das NCs. Além disso, as NCs poderiam garantir concentração plasmática de ativo em níveis terapêuticos por períodos mais longos em comparação às MPs. Por outro lado, por serem um material sólido, as MPs constituem um sistema mais estável do que as NCs, além de terem proporcionado a incorporação de maior quantidade de ativo no sistema (145 mg/g DIM na MP *x* 1 mg/mL DIM na NC).

Já o prolongamento do efeito do DIM microencapsulado em comparação ao ativo livre pode ser atribuído ao fato do DIM estar eficientemente associado às MPs, ocasionando fortes interações químicas e assim promovendo uma liberação lenta do bioativo a partir do sistema. Em adição, a MP poderia permanecer mais tempo no local de absorção do DIM ou ainda promover liberação sítio-específica do ativo apenas no local de absorção.

Por fim, pode-se concluir a partir do que foi discutido, que esta tese reforça a importância do uso do DIM como uma alternativa para contornar os efeitos adversos das terapias convencionais e que os resultados da sua incorporação em nanocápsulas ou micropartículas podem servir de base para estudos mais

aprofundados contemplando uma nova abordagem farmacológica para a liberação oral do DIM visando o manejo da dor. A **Figura 9** resume os resultados encontrados nesta tese.

Figura 9 - Resumo dos experimentos e resultados apresentados nesta tese.



6. CONCLUSÃO

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Em suma, os resultados obtidos a partir dos estudos conduzidos nesta tese demonstraram a viabilidade de preparação de nanocápsulas e micropartículas poliméricas contendo DIM, apresentando características físico-químicas adequadas e proporcionando sua liberação prolongada. Além disso, as nanocápsulas aumentaram a fotoestabilidade do DIM e sua atividade antioxidante, bem como o seu efeito antitumoral, em comparação ao composto não-encapsulado. Ainda, a associação do DIM a esses sistemas prolongou e aumentou a ação antinociceptiva/anti-hipernociceptiva do composto em distintos modelos animais de dor. Dessa maneira, o presente estudo mostrou que as nanocápsulas e micropartículas propostas constituem um sistema promissor para a liberação oral prolongada do DIM visando o manejo da dor inflamatória.

6.1 PERSPECTIVAS

Com base no conjunto de resultados obtidos, sugere-se os seguintes trabalhos futuros a serem desenvolvidos:

- Investigar o efeito antinociceptivo dos sistemas contendo DIM em protocolos de tratamento mais prolongados (por exemplo, tratamento da dor crônica) e explorar diferentes vias de administração;
- Pesquisar os exatos mecanismos que medeiam a ação antinociceptiva do DIM;
- Aprofundar os estudos com relação às avaliações bioquímicas e toxicológicas do DIM livre e também dos sistemas;
- Conduzir estudos relacionados ao perfil farmacocinético do bioativo na sua forma livre e incorporada aos sistemas;

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ANEXO A

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "DESENVOLVIMENTO DE MICRO E NANOPARTÍCULAS CONTENDO 3,3□-DIINDOLMETANO E AVALIAÇÃO DO POTENCIAL ANTINOCICEPTIVO E ANTI-INFLAMATÓRIO EM MODELOS ANIMAIS", protocolada sob o CEUA nº 4428090217, sob a responsabilidade de **Letícia Cruz** *e equipe; Juliane Mattiazzi; Marcel Henrique Marcondes Sari; Cristina Wayne Nogueira* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 23/03/2017.

We certify that the proposal "Development of micro and nanoparticles containing 3,3´-diindolylmethane and evaluation of antinociceptive and anti-inflammatory potential in animal models", utilizing 514 Heterogenics mice (514 males), protocol number CEUA 4428090217, under the responsibility of **Letícia Cruz** *and team; Juliane Mattiazzi; Marcel Henrique Marcondes Sari; Cristina Wayne Nogueira* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/23/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 03/2017 a 03/2020 Área: Farmácia Industrial

Origem:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Machos	idade:	2 a 2 meses	N:	514
Linhagem:	Swiss			Peso:	25 a 35 g		

Resumo: O 3,3´-diindolmetano (DIM) é um bioativo originado a partir de reações químicas que ocorrem em vegetais crucíferos, como couve-flor e brócolis (BANERJEE et al., 2011). Este composto tem sido foco de muitos estudos devido aos seus efeitos terapêuticos em diversas doenças, atuando na redução do estresse oxidativo, diminuindo processos inflamatórios, além de apresentar atividade antineoplásica (ROY et al., 2013; WU et al., 2014; MARUTHANILA et al., 2014). A capacidade anti-inflamatória do DIM é relacionada a efeitos como redução da atividade da enzima mieloperoxidase, diminuição na produção de óxido nítrico

(NO) e prostaglandinas (PGE2), bem como diminuição nos níveis de citocinas pró-inflamatórias, como TNF-, IL-1, PLA2 e IL-6, entre outros mecanismos (KIM et al., 2010; MARUTHANILA et al., 2014). Embora o DIM possua uma ampla gama de aplicações clínicas, seu uso apresenta limitações, pois é insolúvel em água, apresentando baixa biodisponibilidade após administração oral, além de degradar-se com facilidade após exposição à luz ou a altas temperaturas (ROY et al., 2013; VALLEJO, TOMAS-BARBERAN, GARCIA-VIGUERA, 2002). Novas abordagens podem ser utilizadas no sentido de contornar estas limitações e melhorar a eficácia do composto, como o desenvolvimento de micropartículas e nanopartículas poliméricas. As micropartículas consistem em sistemas de liberação de fármaco nos quais um material polimerizado forma uma rede tridimensional, dando origem à partículas exibindo tamanho entre 1 e 1000 µm, enquanto que as nanopartículas apresentam diâmetro inferior a 1 µm, nas quais o fármaco está confinado em uma cavidade oleosa ou aquosa rodeada por uma membrana de polímero (O□DONNELL, McGINITY, 1997; SCHAFFAZICK et al., 2003; NICOLAS et al., 2013). Neste contexto, este projeto foi delineado visando o desenvolvimento de micro e nanopartículas poliméricas contendo DIM, visando melhora na estabilidade deste bioativo, bem como a avaliação da performance destes sistemas frente a testes de inflamação e nocicepção em modelos animais.

Local do experimento: sala 3209, prédio 19

Santa Maria, 24 de março de 2017

Avenida Roraima, 1000, Reitoria, 2º andar - CEP 97105-900 Santa Maria, RS - tel: 55 (55) 3220-9362 / fax:
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Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

Profa. Dra. Daniela Bitencourt Rosa Leal
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Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

ANEXO B

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Mar 01, 2019

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