UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

DESENVOLVIMENTO DE NANOCÁPSULAS PARA A LIBERAÇÃO CONTROLADA DE CRISINA: AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE E DA CITOTOXICIDADE *IN VITRO*

DISSERTAÇÃO DE MESTRADO

Alessandra Scherer Lorenzoni

Santa Maria, RS, Brasil
2015

DESENVOLVIMENTO DE NANOCÁPSULAS PARA A LIBERAÇÃO CONTROLADA DE CRISINA: AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE E DA CITOTOXICIDADE *IN VITRO*

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração em Controle e Avaliação de Insumos e Produtos Farmacêuticos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Mestre em Ciências Farmacêuticas

Orientadora: Profa. Dra. Scheila Rezende Schaffazick

Santa Maria, RS, Brasil
2015

Ficha catalográfica elaborada através do Programa de Geração Automática da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a)

Lorenzoni, Alessandra Scherer
DESENVOLVIMENTO DE NANOCÁPSULAS PARA A LIBERAÇÃO CONTROLADA DE CRISINA: AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE E DA CITOTOXICIDADE *IN VITRO* / Alessandra Scherer Lorenzoni. – 2015.
152 f. ; 30 cm

Orientadora: Scheila Rezende Schaffazick
Dissertação (mestrado) – Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós-Graduação em Ciências Farmacêuticas, RS, 2015.

1. Crisina 2. Nanocápsulas 3. Liberação controlada I. Schaffazick, Scheila Rezende II. Titulo.

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Centro de Ciências da Saúde
Programa de Pós-Graduação em Ciências Farmacêuticas

A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

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elaborada por
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como requisito parcial para obtenção do grau de
Mestre em Ciências Farmacêuticas

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Santa Maria, 25 de setembro de 2015.

Dedico esta dissertação à minha família,
em especial aos meus pais, João e Jane,
e à minha irmã Bruna, pelo carinho,
amor e apoio incondicional.

AGRADECIMENTOS

Primeiramente, gostaria de agradecer a Deus, por sempre me guiar e iluminar o ı caminho e minhas escolhas.

Agradeço aos meus pais, João Carlos e Jane e à minha irmã Bruna por serem os meus alicerces, por me darem todo o apoio, carinho, amor e incentivo.

Ao meu namorado Alexandre por ter me dado todo o suporte e apoio durante este trabalho. Além de ter compreensão e paciência durante os momentos de estresse.

Agradeço à minha orientadora Scheila Rezende Schaffazick pela oportunidade, confiança e ensinamentos adquiridos e amizade.

Agradeço também à professora Letícia Cruz pelos conhecimentos adquiridos, pela amizade, carinho e ajuda. Agradeço à professora Cristiane de Bona pela disponibilidade do equipamento Zetasizer.

Aos meus amigos de coração por toda a amizade, apoio, carinho, paciência, por entederem meus momentos de ausência, pelas risadas e conselhos. Em especial à Mariana, Adélia, Natássia, Marcel, Anelise, Carla, Lauren, Vanessa, Lucas e Bruna.

Agradeço à minha IC amada que esteve comigo desde o começo, Gabriele, que além de ser uma menina estudiosa e dedicada, sua ajuda foi essencial durante esses dois anos, tornando-se uma amiga muito especial e que deixou os experimentos mais intermináveis em momentos descontraídos e alegres. Agradeço também à Camila pela ajuda nos experimentos, pela amizade e momentos alegres. Vocês duas foram muito importantes para mim durante esses dois anos.

Aos colegas e amigos do Laboratório de Tecnologia Farmacêutica Luana, Natháli, Mailine, Flábio, Carolina, Marila, Sabrina, Patrícia, Juliane, Taiane, Milena, Tainara, Gabriela, Allanna, Laura, Luiz Eduardo, Verônica, Daniela, Luan, Felipe, Cristina e Douglas agradeço pela amizade, parceria, ajuda e momentos de descontração. Agradeço também aos colegas do laboratório de Farmacotécnica e do Controle de Qualidade, pela ajuda, coleguismo e boas conversas. Agradeço às funcionárias Rose e Carminha pela ajuda, conversas e amizade.

Agradeço a Profª Maria Beatriz e suas alunas Lariane Cargnelutti e Paula Bitencourt pela análise da atividade antioxidante. À Profª Clarice Rolim e Daniele Nogueira pelo estudo da citotoxicidade. Faço um agradecimento em especial à Daniele pela ajuda e os conhecimentos transmitidos.

À Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Ciências Farmacêuticas pela oportunidade e à CAPES pelo suporte financeiro.

Agradeço a todos aqueles que, de alguma forma, contribuíram para a realização deste trabalho, muito obrigada.

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Farmacêuticas
Universidade Federal de Santa Maria

DESENVOLVIMENTO DE NANOCÁPSULAS PARA A LIBERAÇÃO CONTROLADA DE CRISINA: AVALIAÇÃO DA ATIVIDADE ANTIOXIDANTE E DA CITOTOXICIDADE *IN VITRO*

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ORIENTADORA: SCHEILA REZENDE SCHAFFAZICK
Data e Local da Defesa: Santa Maria, 25 de setembro de 2015.

A crisina (5,7-dihidroxiflavona) é um flavonoide pertecente à classe das flavonas, o qual possui atividade antioxidante e antitumoral. No entanto, apresenta uma baixa solubilidade e, consequentemente, sua biodisponibilidade no organismo é baixa, limitando a aplicação no campo farmacêutico. Dessa forma, nanocápsulas são alternativas para veicular esta substância ativa. Este trabalho objetivou o desenvolvimento de nanocápsulas contendo crisina (0,3 e 0,75 mg/mL), utilizando a etilcelulose e os óleos de amedoim, coco ou triglicerídeos de cadeia média (TCM), a fim de avaliar a atividade antioxidante pelo método do DPPH, citotoxicidade *in vitro* em células normais (fibroblastos - 3T3) e tumorais (câncer de mama - MCF-7; melanoma -SK-MEL-28), tendo em vista as suas potencialidades terapêuticas. As suspensões de nanocápsulas (0,75 mg/mL) também foram liofilizadas, empregando trealose a 10 % (p/v) como crioprotetor. Conforme os resultados, foi possível a preparação de nanocápsulas contendo crisina com características físico-química adequadas e alta eficiência de encapsulamento. As formulações contendo 0,3 mg/mL ou 0,75 mg/mL de crisina foram estáveis durante 30 dias ou 50 dias, respectivamente, quanto ao diâmetro médio de partículas e podispersão (PdI). Entretanto, o teor de crisina diminuiu após 30 dias. Todas as formulações mostraram liberação controlada da crisina em comparação ao flavonoide livre, em tampão acetato pH 5,0: etanol (70:30). As suspensões de nanocápsulas aumentaram a atividade antioxidante da crisina na seguinte ordem: óleo de amendoim, óleo de coco e TCM. Na avaliação *in vitro* da citotoxicidade, todas as suspensões de nanocápsulas não apresentaram citotoxicidade em células de fibroblastos, preparadas com ou sem crisina. As nanocápsulas contendo crisina apresentaram atividade antiproliferativa em células de câncer de mama e melanoma, de maneira dose-dependente e com influência do tipo de ensaio (MTT ou NRU), havendo diferença entre os possíveis mecanismos de toxicidade. Nas células de câncer de mama, sugere-se um efeito inicial sobre a atividade metabólica e mitocondrial da célula e, em um segundo momento, na integridade de membrana e atividade lisossomal. Já para as células de melanoma, o contrário foi observado. As nanocápsulas sem crisina também foram citotóxicas para as células tumorais, dependendo da concentração avaliada e do ensaio final. Além disso, foi possível a obtenção de produtos secos redispersíveis a partir da liofilização das suspensões de nanocápsulas. A análise por microscopia eletrônica demonstrou a presença de estruturas coloidais esféricas após a liofilização. Os produtos secos apresentaram teor de crisina estável durante os 50 dias de estudo. Considerando esses resultados, as nanocápsulas de etilcelulose contendo crisina são interessantes sistemas carreadores intermediários, tendo em vista futuros tratamentos, em função de suas atividades antioxidantes e antiproliferativas.

Palavras-chaves: crisina, nanocápsulas, atividade antioxidante, citotoxicidade *in vitro*, efeito antiproliferativo, liberação controlada, liofilização.

ABSTRACT

Master Dissertation
Programa de Pós-Graduação em Ciências Farmacêuticas
Universidade Federal de Santa Maria

DEVELOPMENT OF NANOCAPSULES FOR CONTROLLED RELEASE OF CHRYSIN: EVALUATION OF ANTIOXIDANT ACTIVITY AND *IN VITRO* CYTOTOXICITY

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ADVISOR: SCHEILA REZENDE SCHAFFAZICK
Place and Date of Defense: Santa Maria, September 25th, 2015.

Chrysin (5,7-dihydroxyflavone) is a flavonoid (class of flavones), which presents antioxidant and antitumoral activities. However, it has low solubility and hence its bioavailability in the body is low, limiting its application in the pharmaceutical field. Thus, nanocapsules are alternatives to delivery of this active substance. This study aimed the development of chrysin-loaded nanocapsules (0.3 and 0.75 mg/mL), using ethylcellulose and peanut oil, coconut oil or medium chain triglycerides (MCT), in order to evaluate the antioxidant activity (DPPH assay), *in vitro* cytotoxicity in normal cells (fibroblasts - 3T3) and tumoral cell lines (breast cancer - MCF-7; SK-MEL-28 melanoma), in view of its therapeutic potentials. The nanocapsule suspensions (0.75 mg/mL) were also lyophilized using trehalose (10% w/v) as cryoprotectant. According to the results, it was possible to prepare nanocapsules containing chrysin with suitable physicochemical characteristics and high encapsulation efficiency. The formulations containing 0.3 mg/ml or 0.75 mg/ml of chrysin were stable for 30 days or 50 days, respectively, considering the average particle diameter and polydispersion indexes (PdI). However, the chrysin content decreased after 30 days. All formulations were able to control release of chrysin in relation to the diffusion of this flavonoid in methanolic solution, in buffer acetate pH 5.0: ethanol (70:30). The chrysin-loaded nanocapsule suspensions showed increase in antioxidant activity as following: peanut oil, coconut oil, and MCT. Considering the *in vitro* cytotoxicity, all developed nanocapsule suspensions (with chrysin or without) did not show cytotoxicity in fibroblasts. The chrysin-loaded nanocapsules showed antiproliferative activity in breast cancer cells and melanoma at dose-dependent manner. Also, there was the influence of the type of endpoint (MTT or NRU), suggesting differences between the possible mechanisms of toxicity. In breast cancer cells, it is suggested that an initial effect can occur on the metabolic and mitochondrial activity of the cell and, in a second step, the membrane integrity and lissosomal activity can be affected. For the melanoma cells, the contrary mechanism can be observed. The nanocapsules without chrysin were also cytotoxic to tumoral cells, depending on both the concentration of the assay and the endpoint test. Moreover, it was possible to obtain dispersible dried products from freeze-drying of nanocapsule suspensions. Analysis by electron microscopy revealed the presence of colloidal spherical structures after lyophilization. The dried products showed stable chrysin content during 50 days of the study. Considering these results, the ethylcellulose nanocapsules containing chrysin are interesting intermediate systems that can be used in future treatments, due to its antioxidant and antiproliferative activities.

Keywords: chrysin, nanocapsules, antioxidant activity, *in vitro* cytotoxicity, antiproliferative effect, controlled release, lyophilization.

LISTA DE ABREVIATURAS E SIGLAS

CLAE-UV	cromatografia a líquido de alta eficiência; detecção na região do ultravioleta
CLN	carreador lipídico nanoestruturado
CO	óleo de coco
DMSO	dimetilsulfóxido
DPPH	2,2-difenil-1-picril-hidrazila
EE	eficiência de encapsulamento
EROs	espécies reativas de oxigênio
FBS	soro fetal bovino
IC_{50}	concentração de fármaco que causa 50% de morte da população de células
IPd/PdI	índice de polidispersão
MTT	2,5-difenil-3-(4,5-dimetil-2-tiazolil) tetrazólio
NC	nanocápsulas
NC-PO-C	nanocápsulas preparadas com óleo de amendoim e com crisina
NC-PO	nanocápsulas preparadas com óleo de amendoim e sem crisina
NC-CO-C	nanocápsulas preparadas com óleo de coco e com crisina
NC-CO	nanocápsulas preparadas com óleo de coco e sem crisina
NC-MCT-C	nanocápsulas preparadas com triglicerídeos de cadeia média e com crisina
NC-MCT	nanocápsulas preparadas com triglicerídeos de cadeia média e sem crisina
NE	nanoemulsões
NL ou NLS	nanopartícula lipídica ou nanopartículas lipídicas sólidas
NLC	carreadores lipídicos nanoestruturados
NRU	vermelho neutro
NS	nanoesferas
PBS	tampão salina fosfato
PO	óleo de amendoim
RSD	desvio padrão relativo
SEM	microscopia eletrônica de varredura
S_f/S_i	índice de ressuspensão (S_f: diâmetro médio final; S_i: diâmetro médio inicial)
$t_{1/2}$	tempo necessário para liberar 50% da dose contida na forma farmacêutica
TCM/MCT	triglicerídeos de cadeia média

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REVISÃO DA LITERATURA

CAPÍTULO 1: Desenvolvimento de suspensões de nanocápsulas de etilcelulose contendo crisina: avaliação da atividade antioxidante e citotoxicidade *in vitro*

CAPÍTULO 2: Liberação controlada de crisina associada a nanocápsulas: influência no efeito antiproliferativo *in vitro* e estudo da liofilização sobre a estabilidade do sistema

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SUMÁRIO

INTRODUÇÃO

Espécies reativas de oxigênio (EROs) são fisiologicamente produzidas no organismo através de reação química, onde ocorre a oxidação de substâncias. Quando ocorre excesso de EROs, o organismo encontra-se em estresse oxidativo, o qual é responsável pelo dano às macromoléculas biológicas, como o DNA, o que pode provocar patologias como câncer, diabetes, doenças cardiovasculares e degenerativas, além de danos cutâneos (WU et al., 2011; MANKE et. al., 2013; PLAZA et. al., 2014; LUSHCHACK et. al., 2014).

Os antioxidantes têm a finalidade de neutralizar as EROs e são responsáveis por prevenir danos em componentes celulares, os quais surgem como consequência de reações químicas que envolvem os radicais livres (PROCHÁZKOVÁ et. al., 2011). O organismo possui antioxidantes enzimáticos e não enzimáticos. No entanto, quando há excesso na produção de EROs, os antioxidantes presentes no organismo se esgotam, sendo necessário aporte adicional de substâncias antioxidantes, como flavonoides (MANKE et. al., 2013; SANTHAHUMAR et. al., 2014).

A crisina (5,7-dihidroxiflavona) é um flavonoide pertecente à classe das flavonas (MERCAN et. al., 2006; KIM et. al., 2011). Possui potente atividade antioxidante (PUSHPAVALLI et. al., 2010; REHMAN et. al., 2013; ANITHA; RAJADURAI, 2014; SOUZA et. al., 2015), anti-inflamatória (SHIN, et. al., 2009; BAE; LEE; KIM, 2011) e antitumoral (TOBIN et. al., 2006; SAMARGHANDIAN; AFSHARI; DAVOODI, 2011; REHMAN et. al., 2013; LI et. al., 2015). WU e colaboradores (2011) sugeriram que a crisina tem potencial uso em fotoproteção, pois resultados mostraram benefícios deste flavonoide na proteção de queratinócitos contra danos induzidos por radiação UVA/UVB. Outro estudo sugeriu seu uso potencial como inibidor de hiperpigmentação (KIM et al., 2011). Além disto, pesquisas têm demonstrado seu efeito citotóxico *in vitro* em culturas celulares de melanomas (KASALA et al., 2015).

No entanto, a crisina apresenta uma baixa solubilidade, consequentemente sua biodisponibilidade no organismo é baixa, limitando a aplicação no campo farmacêutico (KIM; KIM; JUNG, 2008; CHAKRABORTY et. al., 2010).

Os sistemas nanoestruturados têm sido desenvolvidos para a veiculação de substâncias pouco solúveis, para aumentar o potencial terapêutico, promover a liberação da substância ativa no local específico de ação e de forma controlada, além de serem alternativas para melhorar a biodisponibilidade de substâncias ativas no organismo e melhorar a estabilidade

das mesmas, podendo, ainda, reduzir efeitos adversos (COUVREUR et. al., 2002; SCHAFFAZICK et. al., 2003; SESSA et. al., 2014).

Neste sentido, com a finalidade de aumentar a biodisponibilidade e/ou a eficácia terapêutica de flavonoides, os sistemas nanoestruturados têm sido pesquisados, para aumentar a atividade antioxidante e antitumoral, como demonstrado na incorporação de quercetina (WU et. al., 2008; PANDEY et. al., 2015), naringenina (KRIHSNAKUMAR et. al., 2011; KUMAR et. al., 2015) e rutina (ALMEIDA et al., 2010). Recentemente, trabalhos relatando o desenvolvimento de nanoesferas (MOHAMMADINEJAD et. al. 2015) e de nanopartículas lipídicas sólidas (AISHWARYA et. al., 2015) contendo crisina foram reportados. A utilização de sistemas coloidais para a veiculação de diferentes substâncias antioxidantes, como a idebenona (LOHAN et. al., 2015), quercetina (BOSE et. al., 2013), coenzima Q10 (MONTENEGRO et. al., 2012) e naringenina (KUMAR et. al., 2015), também têm sido alvo de pesquisas, visando à administração tópica.

As nanocápsulas são sistemas vesiculares, constituídas por um invólucro polimérico disposto ao redor de um núcleo, geralmente oleoso (estrutura *core-shell*), onde o fármaco pode estar dissolvido neste núcleo e/ou adsorvido à parede polimérica (GUTERRES; SCHAFFAZICK; POHLMANN, 2007; MORA-HUERTAS; FESSI; ELAISSARI, 2010). A etilcelulose é um polímero utilizado na indústria farmacêutica, cosmética e alimentícia. Existem estudos utilizando etilcelulose como material polimérico em sistemas nanoestruturados (RAVIKUMARA et. al., 2009; SAHU et. al., 2013; CHASSOT et al., 2014).

O óleo que compõe o núcleo das nanocápsulas é responsável por solubilizar o fármaco e aumentar sua concentração no sistema. Os triglicerídeos de cadeia média (TCM) ou óleos vegetais são utilizados como núcleo das nanocápsulas (MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010; SANTOS et al., 2014).

O óleo de amendoim apresenta elevada estabilidade e propriedades antioxidantes (CARRÍN; CARELLI, 2010). Recentemente, SESSA e colaboradores (2014) desenvolveram nanoemulsões contendo resveratrol, utilizando o óleo de amendoim como componente lipídico. O óleo de coco é muito utilizado na indústria alimentícia e vem atraindo a atenção da indústria farmacêutica e cosmética, por apresentar atividade antioxidante (MARINA et. al., 2009; DeBMANDAL et. al., 2011), antimicrobiana (OGBOLU et. al., 2007; AGARWAL et. al., 2010) e antifúngica (DeBMANDAL et. al., 2011). Na literatura, estudos reportando o uso

do óleo de coco na preparação de nanocápsulas associando um antioxidante (BRENDLE, 2013) e na associação de um antifúngico para a liberação vaginal (SANTOS et. al., 2014) são encontrados.

No entanto, as suspensões de nanocápsulas podem apresentar instabilidade físico-química durante o armazenamento. Para minimizar os problemas de instabilidade das suspensões de nanopartículas, processos de liofilização (ABDELWAHED et. al.. 2006a; SCHAFFAZICK et. al., 2003; KHAYATA et. al., 2012) ou secagem por aspersão (MARCHIORI et.al., 2011; OURIQUE et. al., 2014) são utilizados.

Neste sentido, o objetivo do presente estudo foi desenvolver nanocápsulas poliméricas contendo o antioxidante crisina, em função do potencial destes sistemas vesiculares poliméricos na associação de substâncias pouco solúveis, na capacidade de promover liberação controlada e no incremento da atividade antioxidante de substâncias ativas associadas. O trabalho foi baseado na comparação de nanocápsulas de etilcelulose com diferentes núcleos oleosos (óleo de amendoim, óleo de coco ou triglicerídeos de cadeia média) para a veiculação deste flavonoide, em relação às características físico-químicas, estabilidade, controle de liberação, conversão em liofilizados redispersíveis, atividade antioxidante *in vitro* e citotoxicidade (linhagens tumorais e não tumoral). Cabe mencionar que não foram encontrados estudos relativos à associação da crisina a nanocápsulas poliméricas e ao emprego de óleo de amendoim como núcleo oleoso de nanocápsulas.

OBJETIVOS

1.1 Objetivo Geral

Preparar e caracterizar suspensões e liofilizados de nanocápsulas poliméricas contendo crisina, empregando o óleo de amendoim ou óleo de coco, em comparação aos triglicerídeos de cadeia média (TCM), além de avaliar a atividade antioxidante e a citotoxidade *in vitro* deste flavonoide associado às estruturas coloidais.

1.2 Objetivos Específicos

- Preparar nanocápsulas poliméricas contendo crisina, empregando o óleo de amendoim, óleo de coco ou TCM como núcleo, utilizando a etilcelulose como polímero;
- Caracterizar os sistemas nanovesiculares quanto ao pH, diâmetro médio, índice de polidispersão (IPd), potencial zeta, teor e eficiência de encapsulamento da crisina;
- Efetuar estudo de estabilidade das formulações, em temperatura ambiente e protegidas da luz;
- Determinar a cinética de liberação *in vitro* do flavonoide a partir das nanocápsulas desenvolvidas, através da técnica de difusão em sacos de diálise;
- Realizar estudos relativos à capacidade antioxidante *in vitro* da crisina associada às nanocápsulas, frente ao radical DPPH (2,2 difenil-1-picril-hidrazila);
- Avaliar a citotoxicitade *in vitro* das suspensões de nanocápsulas contendo crisina, em comparação ao flavonoide livre, empregando linhagem celular não tumoral (fibroblastos 3T3);
- Comparar a viabilidade de células tumorais *in vitro*, utilizadas como modelo (SK-MEL-28 – melanoma ou MCF-7 - câncer de mama) frente à crisina livre e nanoencapsulada;
- Preparar e caracterizar liofilizados redispersíveis, a partir das suspensões de nanocápsulas, empregando a trealose como crioprotetor solúvel, além de avaliar a estabilidade destes produtos secos.

REVISÃO BIBLIOGRÁFICA

1.1 Estresse oxidativo, antioxidantes e flavonoides: aspectos gerais

Radicais livres são definidos como qualquer espécie química que apresenta um ou mais elétrons desemparelhados, altamente reativos, apresentando meia-vida curta e podendo danificar qualquer biomolécula do organismo (LUSHCHACK et. al., 2014). Espécies reativas de oxigênio (EROs) são produzidas no organismo através de reação química, onde ocorre a oxidação de substâncias. As EROs incluem radicais livres e outras espécies que, embora não possuam elétrons desemparelhados, são muito reativas em decorrência de sua instabilidade. As principais EROs são o ânion superóxido ($O_2^{\cdot}$), o radical hidroxila ($OH^{\cdot}$) e o peróxido de hidrogênio (H_2O_2) (NIJVELDT et. al., 2001; GENESTRA, 2007; MANKE et. al., 2013; SANTHAHUMAR et. al., 2014).

No entanto, quando o fluxo de elétrons e a produção de energia aumenta, ocorre excesso de EROs no organismo, havendo um desequilíbrio entre a produção destas espécies e a capacidade do sistema biológico em neutralizá-las, sendo caracterizado o estresse oxidativo. As EROs atacam as macromoléculas biológicas como lipídios, proteínas e DNA, induzindo-os à oxidação, resultando em danos nas membranas biológicas e no DNA, o que pode provocar doenças como câncer, diabetes, doenças cardiovasculares e degenerativas (MANKE et. al., 2013; SANTHAHUMAR et. al., 2014; PLAZA et. al., 2014).

Os antioxidantes tem a finalidade de neutralizar as EROs e essas substâncias são responsáveis por retardar, impedir ou eliminar o dano oxidativo, ou seja, são responsáveis em prevenir danos em componentes celulares, os quais surgem como consequência de reações químicas que envolvem as EROs (PROCHÁZKOVÁ et. al., 2011).

O organismo possui antioxidantes enzimáticos (superóxido dismutase, catalase, glutationa peroxidase) e antioxidantes não enzimáticos (glutationa, ácido ascórbico, α-tocoferol, ubiquinonas). Muitos antioxidantes atuam doando elétrons às EROs, dessa forma agem reduzindo ou prevenindo o dano oxidativo. No entanto, quando há excesso na produção de EROs, os antioxidantes presentes no organismo se esgotam, sendo necessária a adição de substâncias antioxidantes, como flavonoides, a fim de neutralizá-las, minimizando o dano oxidativo (NIJVELDT et. al., 2001; GENESTRA, 2007; MANKE et. al., 2013; SANTHAHUMAR et. al., 2014).

Os flavonoides são componentes naturais com grupamentos fenólicos, encontrados principalmente em frutas, legumes, uva, chá e vinho. São considerados compostos antioxidantes importantes na dieta humana, porém, não são considerados nutrientes. Podem

ser divididos em quatro classes, entre elas flavonas, flavononas, catequinas e antocianinas (NIJVELDT et. al., 2001). Os flavonoides são compostos por um anel benzeno (A) condensando com um anel de seis membros pirano (C), juntamente com um anel fenila (B) na posição 2 ou 3. A figura 1 mostra a estrutura química das flavonas (PROCHÁZKOVÁ et. al., 2011; PLAZA et. al., 2014).

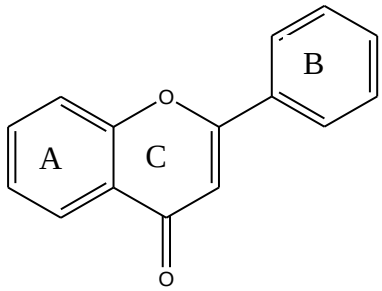

Figura 1 – Estrutura química da flavonona

Os flavonoides são conhecidos por apresentarem potente atividade antioxidante, além de atividade antitumoral, anti-inflamatória, antialérgica e antiviral (PLAZA et al., 2014). Os mecanismos de neutralização dos radicais livres pelos flavonoides são: (a) através da doação de hidrogênio/elétrons para o radical livre, neutralizando-o; (b) quelação de íons metálicos (ferro e cobre), devido à capacidade dos íons metálicos em interferir nas reações de oxidação/redução; (c) inibição das enzimas responsáveis pela produção de radicais livres, como a xantina oxidase, proteína quinase, cicloxigenase e lipoxigenase (NIJVELDT et. al., 2001; SANTHAHUMAR et. al., 2014; PLAZA et. al., 2014).

Para que ocorra a atividade antioxidante dos flavonoides, é necessário que estes sejam absorvidos, metabolizados e estejam biodisponíveis no organismo. A absorção, o metabolismo, a biodisponibilidade e a atividade antioxidante dependem das características estruturais do flavonoide, como tamanho molecular, solubilidade e dose administrada (SANTHAHUMAR et. al., 2014; PLAZA et. al., 2014).

Devido à atividade antioxidante e por serem compostos naturais, os flavonoides tem atraído a atenção em diversas áreas como biológica, química, farmacêutica, médica e alimentícia (PLAZA et. al., 2014).

1.2 Crisina

A crisina (5,7-dihidroxiflavona) é um flavonoide pertecente à classe das flavonas. É encontrada em plantas, pricipalmente do gênero *Passiflora*, no mel e no própolis (RAPTA et. al., 1995; MERCAN et. al., 2006; PICHICHERO et. al., 2010; BARBERIC et. al., 2011). A crisina apresenta massa molar de 254,25 g/mol, sendo caracterizada por um pó fino amarelado, praticamente insolúvel em água e solúvel em solventes orgânicos como metanol, etanol, dimetilsulfóxido e dimetilformamida (CASTRO; FERRETTI; BLANCO, 2005). A estrutura química da crisina é mostrada na figura 2.

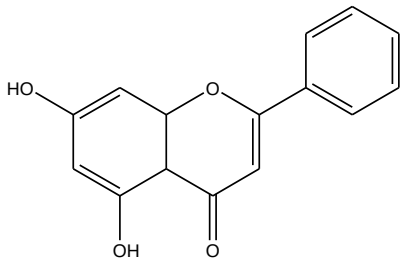

Figura 2 – Estrutura química da crisina

Uma atividade biológica relatada para a crisina é a sua ação inibitória da enzima aromatase, apresentando efeito antiestrógeno, o qual aumenta a produção de testosterona, impedindo a sua conversão em estrogênio. A aromatase é uma enzima do citocromo P-450 que cataliza a conversão de andrógenos em estrógenos. A androstenediona e a testosterona atuam como substratos para a aromatase. Os efeitos inibidores da aromatase promovidos pela crisina tornaram-na popular entre alguns fisioculturistas e atletas que utilizam andrógenos como anabolizantes, pois aumenta os níveis de testosterona (CAMPBELL; KURZER, 1993; KUIPER et. al., 1998; JEONG et. al., 1999).

Além da inativação da aromatase, a crisina possui potente atividade antioxidante (PUSHPAVALLI et. al., 2010; SULTANA; VERMA; KHAN, 2012; REHMAN et. al., 2013; ANITHA; RAJADURAI, 2014; SOUZA et. al., 2015), anti-inflamatória (SHIN et. al., 2009; BAE; LEE; KIM, 2011), antitumoral (TOBIAN et. al., 2006; SAMARGHANDIAN; AFSHARI; DAVOODI, 2011; REHMAN et. al., 2013; LI et. al., 2015), atividade nefroprotetora (CIFTCI et. al., 2012; SULTANA; VERMA; KHAN, 2012), neuroprotetora (HE et. al., 2012), hepatoprotetora (PUSHPAVALLI et. al., 2010; GLORY; THIRUVENGADAM, 2012), anticonvulsivante (MEDINA et. al., 1990) e ansiolítica (WOLFMAN et. al., 1994).

Em relação à atividade antioxidante da crisina, seu mecanismo ainda não está bem relatado. Acredita-se que a crisina reduza a peroxidação lipídica, restaurando os níveis dos antioxidantes enzimáticos presentes no organismo (SULTANA; VERMA; KHAN, 2012; REHMAN et. al., 2013). Na atividade antitumoral, o possível mecanismo também não está bem elucidado, porém, acredita-se que a crisina atue através dos efeitos antiproliferativos e apoptóticos em células de câncer (SAMARGHANDIAN; AFSHARI; DAVOODI, 2011).

PUSHPAVALLI e colaboradores (2010) estudaram o efeito antioxidante e hepatoprotetor da crisina, utilizando a silimarina como referência nesse estudo, e a hepatotoxicidade foi induzida por D-galactosamina. A crisina e a silimarina foram administradas pela via oral na dose de 25 mg/kg/peso. Após o 8° dia de administração, os animais foram sacrificados e o sangue foi coletado e utilizado para realizar a avaliação bioquímica. Nesse estudo, foi observado que o tratamento com crisina diminuiu os níveis das enzimas marcadoras de dano hepático e de produtos da peroxidação lipídica (espécies reativas ao ácido tiobarbitúrico, hidroperóxidos lipídicos e dienos conjugados) e aumentou os níveis de antioxidantes não enzimáticos (vitamina C e E, glutationa) e antioxidantes enzimáticos (superóxido dismutase, catalase e glutationa peroxidase). Dessa forma, a crisina mostrou um promissor efeito hepatoprotetor e antioxidante, podendo ser comparada à silimarina.

SAMARGHANDIAN, AFSHARI e DAVOODI (2011) estudaram o efeito antitumoral da crisina para o tratamento do câncer de próstata (linhagem celular de PC-3), observando a viabilidade celular frente à crisina, utilizando o ensaio MTT. A crisina foi avaliada nas concentrações de 10 a 40 μM, em 24, 48 e 72 horas. Após as 24 horas de tratamento com crisina, foram observados efeitos inibitórios significativos na concentração de 40 μM. Nesse estudo, a crisina induziu a citotoxicidade e a apoptose no modelo celular estudado, sugerindo que essa substância ativa poderia ser utilizada para o tratamento do câncer de próstata.

Em outro estudo, REHMAN e colaboradores (2013) avaliaram a eficácia da crisina como agente antitumoral e a sua atividade antioxidante no câncer renal. Para ocorrer atividade antitumoral e o estresse oxidativo, o câncer renal foi induzido por N-nitrosodietilamina. A crisina foi administrada por via oral a ratos, nas doses de 20 e 40 mg/kg/peso. Após 24 semanas, os ratos foram sacrificados, o sangue foi coletado e os rins foram removidos para a avaliação bioquímica, imunológica e histopatológica. A crisina mostrou-se eficiente na proteção contra a peroxidação lipídica induzida, restaurou os níveis de ureia e de creatinina, apresentando uma proteção na toxicidade renal. Além disso, suprimiu as respostas

inflamatórias do tecido renal e aumentou a atividade antioxidante enzimática. Foi observada também a diminuição do tumor dos animais tratados com a dose de 20 mg/kg/peso em relação aos animais tratados somente com crisina e com a dose de 40 mg/kg/peso. Dessa maneira, a crisina atuou como potente antioxidante e agente antitumoral, podendo ser utilizada no tratamento e na prevenção da carcinogênese renal.

Existem poucos trabalhos relativos ao emprego de crisina por via cutânea. WU e colaboradores (2011) avaliaram o seu potencial uso em fotoproteção e verificaram benefícios deste flavonoide na proteção de queratinócitos contra danos induzidos por radiação UVA/UVB. KIM e colaboradores (2011) avaliaram seu uso potencial como inibidor de hiperpigmentação. Estudos têm demonstrado também seu efeito citotóxico *in vitro* em culturas celulares de melanomas (murino e humano) (KASALA et al., 2015).

A crisina apresenta uma baixa absorção no organismo, o que pode limitar a sua aplicação no campo farmacêutico. Essa baixa absorção ocorre devido ao grupamento fenólico em sua estrutura, havendo uma baixa solubilidade em água (KIM; KIM; JUNG, 2008; CHAKRABORTY et. al., 2010). Acredita-se também que a baixa biodisponibilidade da crisina ocorra por seu metabolismo ser muito rápido, quando ocorre a sua permeação pelas membranas biológicas (WALLE et. al., 2001). Existem estudos associando a crisina a β-ciclodextrinas, com a finalidade de aumentar a solubilidade do flavonoide em água e, consequentemente, melhorar a sua biodisponibilidade no organismo (WALLE et. al., 2001; KIM; KIM; JUNG, 2008; CHAKRABORTY et. al., 2010; HADARUGA et. al., 2012).

WALLE e colaboradores (2001) realizaram um estudo associando crisina à β-ciclodextrinas, onde foi relatado um aumento da solubilidade da mesma e também melhora no seu potencial antioxidante (peroxidação lipídica) devido a esta complexação.

KIM e colaboradores (2008) estudaram a associação de ciclodextrina com diferentes flavonoides (crisina, apigenina e luteolina) em soluções aquosas, com a finalidade de aumentar a solubilidade dos mesmos. Foi observado um aumento da solubilidade desses compostos quando associados com a β-ciclodextrina, incrementando também a estabilidade desses compostos.

Alternativamente, sistemas nanoestruturados poliméricos ou lipídicos foram, recentememente, relatados na literatura para a veiculação de crisina (MOHAMMADINEJAD et al., 2015; AISHWARYA et al., 2015).

MOHAMMADINEJAD e colaboradores (2015) desenvolveram nanoesferas de PLGA-PEG contendo crisina, apresentando diâmetro médio de partículas entre 20 e 75 nm. Foi realizado ensaio de citotoxicidade em linhagem celular de câncer de mama, na faixa de concentração de 5 a 640 µM. Após 72 horas de experimento, houve redução da viabilidade celular, tanto para as nanoesferas contendo crisina, quanto para a crisina livre, sendo que a redução da viabilidade celular ocorreu de maneira dose-dependente. Dessa forma, esse estudo sugere que o sistema nanoestruturado manteve a atividade antitumoral da crisina em células de câncer de mama. Frente a isso, foi possível desenvolver nanoesferas de PLGA-PEG associando crisina, sendo um sistema promissor para a terapia desta patologia.

Outro estudo, AISHWARYA e colaboradores (2015) desenvolveram nanopartículas lipídicas sólidas (NLS) contendo crisina com adequadas características físico-químicas (240 nm; -40,4 mV) e com capacidade de promover liberação deste flavonoide (88,80% em 72 horas), em meio composto de água:etanol (50:50 v/v). Em relação ao estudo de citotoxicidade (1,56 a 200 µg/mL), quando se aumentou a concentração de crisina, houve diminuição da viabilidade célular em linhagem *vero*. Por sua vez, as NLS não apresentam toxicidade a nível celular, sendo interessantes sistemas para veicular este flavonoide.

1.3 Nanopartículas Poliméricas

Os sistemas nanoestruturados têm chamado a atenção de cientistas e da indústria farmacêutica e cosmética por apresentarem um aumento do potencial terapêutico, podendo liberar a substância ativa no local específico de ação, ocorrendo a vetorização desta. Esses apresentam tamanho submicrométrico, portanto, inferior a 1000 nm. Conforme o processo e a composição utilizada em sua preparação, podem ser classificados em lipossomas, nanocápsulas, nanoesferas, nanopartículas lipídicas sólidas, carreadores lipídicos nanoestruturados, nanoemulsões, nanocápsulas de núcleo lipídico, entre outros (COUVREUR et. al., 2002; ALONSO, 2004; MOHANRAJ; CHEN, 2006; GUTERRES; SCHAFFAZICK; POHLMANN, 2007; JAGER et. al., 2009).

Em relação às nanocápsulas, estas são sistemas vesiculares, constituídos por um invólucro polimérico disposto ao redor de um núcleo, geralmente oleoso, onde o fármaco pode estar dissolvido neste núcleo e/ou adsorvido à parede polimérica. O polímero em torno do núcleo é essencial ao sistema, por proteger a integridade do mesmo em condições agressivas e interagir favoravelmente com as barreiras biológicas, melhorando sua

estabilidade. Nestes sistemas, existe a possibilidade do fármaco não apresentar contato direto com o tecido ou a célula, o que reduz o potencial de irritação (COUVREUR et. al., 2002; ALONSO, 2004; MOHANRAJ; CHEN, 2006; GUTERRES; SCHAFFAZICK; POHLMANN, 2007; SAVIAN et al., 2015). Nanocápsulas de núcleo aquoso, para a veiculação de princípios ativos hidrofílicos (MAGLIO et. al., 2011; CUOMO et. al., 2014; COSCO et. al., 2015), ou de núcleo lipídico, contendo fármacos lipofílicos, (JAGER et. al., 2009; RIGO et. al., 2014; SCHULTZE et. al., 2014; ROVERSI et. al., 2015; SAVIAN et. al., 2015) também têm sido desenvolvidas para diversos fins terapêuticos.

Por sua vez, as nanoesferas são caracterizadas por serem sistemas matriciais poliméricos, não apresentando um núcleo oleoso, onde o fármaco pode estar retido ou adsorvido (COUVREUR et. al., 2002; ALONSO, 2004; MOHANRAJ; CHEN, 2006; GUTERRES; SCHAFFAZICK; POHLMANN, 2007). A figura 3 esquematiza os tipos de nanopartículas poliméricas.

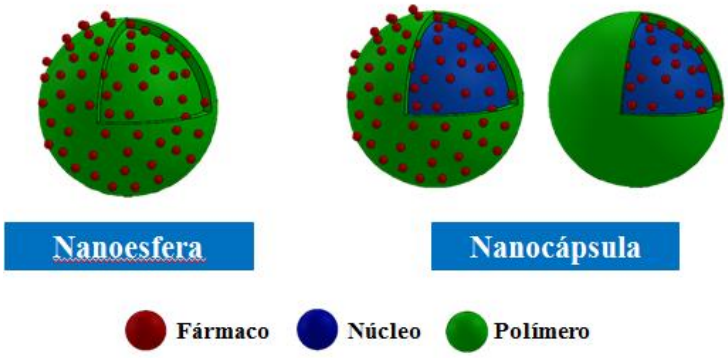

Figura 3 – Desenho esquemático dos tipos de nanopartículas poliméricas.

As nanocápsulas são constituídas por um material polimérico, que deve ser biocompatível e pode ou não ser biodegradável, um ou mais tensoativos, um óleo, além de uma substância ativa associada. Os polímeros são formados por reações de polimerização, sendo empregados os mais diversos tipos na preparação destes sistemas. Eles ajudam a proteger o fármaco e controlam o perfil de liberação. Podem ser naturais (como a gelatina, quitosana, alginato de sódio), semissintéticos, como etilcelulose, ou ainda sintéticos, como a poli(ε-caprolactona), o poli(ácido lático) e os polimetacrilatos [Eudragit®S, Eudragit®RS, Eudragit®EPO]. Os polímeros sintéticos tendem a apresentar maior pureza e melhor reprodutibilidade do que os polímeros naturais (COUVREUR et. al., 2002; SCHAFFAZICK et. al., 2003a; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Quando o núcleo das nanocápsulas poliméricas é oleoso, é interessante que o fármaco tenha a mais alta solubilidade possível no óleo, sendo este importante para a eficácia do carreador, pois pode inclusive aumentar a concentração do fármaco no sistema. Além disto, deve apresentar ausência de toxicidade, elevada estabilidade físico-química, ser incapaz de solubilizar e degradar o polímero e vice-versa, ser biocompatível e apresentar alta afinidade pelo fármaco a ser encapsulado. Frente a isso, é fundamental a sua escolha adequada (COUVREUR et. al., 2002; SCHAFFAZICK et. al., 2003a; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

TCM são os óleos mais utilizados para a composição desses sistemas, devido ao fato de solubilizarem diferentes fármacos (SANTOS et. al., 2013; ABBAS et. al., 2015; RIGO et. al., 2015; SAVIAN et. al., 2015). Atualmente, também vêm sendo desenvolvidas nanocápsulas com diferentes óleos vegetais, como o óleo de semente de uva (ALMEIDA et. al., 2010), óleo de palma, óleo de coco (BRENDLE, 2013; SANTOS et. al., 2014), óleo essencial de melaleuca (FLORES et. al., 2011; FLORES et. al., 2013), óleo de amêndoas (CHASSOT et. al., 2014), óleo de farelo de arroz (RIGO et. al., 2015), entre outros.

As nanopartículas poliméricas podem ser preparadas por diferentes métodos e matérias-primas. A seleção dos materiais a serem utilizados depende do tamanho de partícula requerido, das propriedades do fármaco, como solubilidade e estabilidade em água, características do invólucro polimérico, como a carga de superfície e a permeabilidade e, ainda, do perfil de liberação desejado para o fármaco (MOHANRAJ; CHEN, 2006).

Neste sentido, existem diferentes métodos de preparação de nanopartículas poliméricas relatados, que, de uma forma geral, podem ser classificados em métodos baseados na polimerização *in situ* de monômeros dispersos, na precipitação de polímeros pré-formados (ex: nanoprecipitação, emulsificação-difusão) ou por métodos empregados para polímeros naturais, como, por exemplo, a gelificação iônica. O método de precipitação de polímeros pré-formados é um dos mais utilizados (SCHAFFAZICK et. al., 2003a; MOHANRAJ; CHEN, 2006; GUTERRES; SCHAFFAZICK; POHLMANN, 2007).

No método de polimerização *in situ* de monômeros, são utilizados monômeros como cianoacrilato de alquila. No entanto, nesse método podem ocorrer reações químicas entre o monômero e o fármaco durante o processo, o que é uma desvantagem em relação ao método que emprega polímeros pré-formados, além da possibilidade de apresentar monômeros

residuais no produto final (COUVREUR et. al., 2002; MOHANRAJ; CHEN, 2006; GUTERRES; SCHAFFAZICK; POHLMANN, 2007).

O método de deposição interfacial de polímeros pré-formados foi desenvolvido para a preparação de nanocápsulas, a partir do método de nanoprecipitação ou deslocamento de solvente. De acordo com essa técnica, o preparo das nanocápsulas ocorre através de duas fases, uma fase solvente e outra não solvente, as quais são denomindas fase orgânica e fase aquosa. No entanto, é possível utilizar duas fases orgânicas ou duas fases aquosas, desde que as condições de solubilidade e miscibilidade sejam adequadas (MORA-HUERTAS; FESSI; ELAISSARI, 2010).

A fase orgânica, contendo o polímero, o óleo, um tensoativo lipofílico opcional (baixo EHL) e o fármaco, dissolvidos em um solvente orgânico (geralmente acetona ou etanol), é vertida em uma fase aquosa que contém um tensoativo hidrofílico (alto EHL). Ocorre a formação de uma emulsão espontânea, devido à mútua miscibilidade entre a água e o solvente orgânico. Como o polímero é insolúvel tanto no óleo quanto na fase aquosa, ele se deposita na interface dos dois componentes imiscíveis. Finalmente, o solvente orgânico é evaporado, sob pressão reduzida (Figura 4), formando como produto final uma suspensão coloidal (COUVREUR et. al., 2002; SCHAFFAZICK et. al., 2003a; MOHANRAJ; CHEN, 2006; GUTERRES; SCHAFFAZICK; POHLMANN, 2007).

As principais variáveis do processo são aquelas associadas com as condições de adição da fase orgânica na fase aquosa, tais como a taxa de injeção de fase orgânica, taxa de agitação da fase aquosa e a relação fase orgânica/fase aquosa. Do mesmo modo, as características de nanocápsulas são influenciadas pela natureza e concentração dos seus componentes (MORA-HUERTAS; FESSI; ELAISSARI, 2010). No entanto, esse método apresenta algumas desvantagens como utilização de solvente orgânico, podendo haver risco de toxicidade/inflamabilidade, e somente substâncias ativas que dissolvem em solventes miscíveis com a água podem ser utilizadas (SCHFFAZICK et. al., 2003a; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

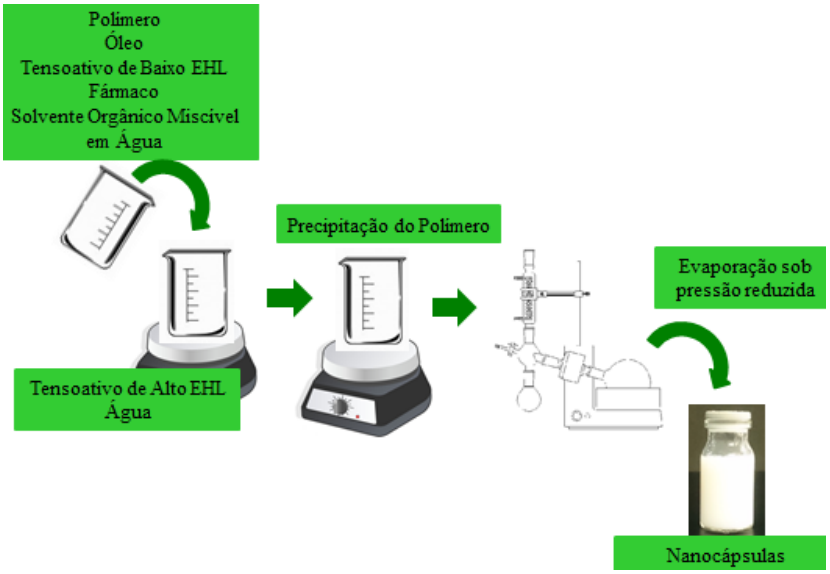

Figura 4 – Esquema do método de preparação das nanocápsulas poliméricas, através de deposição interfacial de polímero pré-formado.

Alguns parâmetros são essenciais no desenvolvimento dos sistemas nanoestruturados, como o diâmetro médio de partículas e a distribuição de tamanho (índice de polidispersão - PdI), características de superfície (potencial zeta) e a eficiência de encapsulamento do fármaco. O tamanho médio de partículas e sua distribuição podem ser determinados através da espectroscopia de correlação de fótons. Estas características são importantes, pois podem influenciar a distribuição *in vivo* das nanoestruturas, toxicidade, perfil de liberação do fármaco, estabilidade do sistema e capacidade de atravessar as barreiras biológicas (COUVREUR et. al., 2002; MOHANRAJ; CHEN, 2006).

Em relação à superfície das nanocápsulas, o potencial zeta é um parâmetro importante a ser avaliado, pois tem relação com a estabilidade do sistema, reduzindo o risco de agregação das partículas, sendo influenciado pela mudança de componentes, especialmente polímero e tensoativos. De uma forma geral, potenciais zeta elevados (em módulo) demonstram alta repulsão entre as partículas, dificultando a agregação das mesmas (COUVREUR et. al., 2002; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010). Entretanto, um potencial zeta elevado nem sempre é necessário para manter a formulação estável, pois a presença de um tensoativo não-iônico, como o polissorbato 80, pode atuar como estabilizador físico na interface partícula/água. Neste sentido, os sistemas coloidais podem ser estabilizados por mecanismo eletrostático ou estérico (RIGO et. al., 2013).

A eficiência de encapsulamento depende da afinidade do fármaco pelos sistemas desenvolvidos. Fármacos hidrofílicos tendem a uma eficiência de encapsulamento mais baixa,

enquanto a eficiência de incorporação para fármacos lipofílicos é maior do que 70%. Por sua vez, a solubilidade, a capacidade de difusão e a biodegradação do polímero governam a liberação do fármaco (MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Frente a isso, as nanocápsulas têm sido desenvolvidas como sistemas de liberação de fármacos por possuírem tamanho reduzido, o que permite a penetração por meio de pequenos capilares, facilitando a sua captura por células e possibilita a permeabilidade pelas barreiras biológicas, proporcionando uma liberação sustentada e controlada do fármaco em sítios específicos de ação (COUVREUR et. al., 2002; ALONSO, 2004; MOHANRAJ; CHEN, 2006; GUTERRES; SCHAFFAZICK; POHLMANN, 2007). Além disso, são sistemas capazes de promover a vetorização do fármaco, aumentando a sua concentração no local de ação e reduzindo os efeitos adversos. Também, estes carreadores podem aumentar a estabilidade físico-química da substância ativa associada frente à degradação enzimática e pela luz, promover a permeabilidade através da pele, reduzir a toxicidade e os efeitos adversos, aumentando a eficácia terapêutica do fármaco (COUVREUR et. al., 2002; SCHAFFAZICK et al., 2003a; MORA-HUERTAS; FESSI; ELAISSARI, 2010). Estudos mostram a eficiência dos sistemas nanoestruturados no encapsulamento de peptídeos e proteínas, protegendo-os contra a degradação enzimática e hidrolítica, aumentando a sua biodisponibilidade e permeação, no tratamento com agentes infecciosos e do câncer, além da liberação cerebral de substâncias ativas, pois conseguem atravessar a barreira hematoencefálica (SCHAFFAZICK et al., 2003a). Neste sentido, as nanocápsulas têm sido utilizadas como sistemas de liberação de fármaco em diferentes vias de administração como oral, ocular, tópica, nasal, subcutânea, intramuscular e, inclusive, intravenosa, sem riscos de embolia (COUVREUR et. al., 2002; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

1.3.1 Nanoestruturas para a veiculação de substâncias antioxidantes

Sistemas nanoestruturados também têm sido empregados para a liberação controlada de substâncias antioxidantes, como a melatonina e o ácido lipoico, aumentando tanto a estabilidade físico-química quanto a atividade antioxidante dos mesmos contra a lipoperoxidação (SCHFFAZICK et al., 2005; SCHAFFAZICK et al., 2008; KULKAMP et al., 2009; KULKAMP et al., 2011). Melhora na atividade antioxidante para substâncias como

idebenona (MONTENEGRO et. al., 2012), quercetina (WU et. al., 2008; BOSE et. al., 2013), coenzima Q10 (LOHAN et. al., 2015), rutina (ALMEIDA et. al., 2010) e naringenina (KUMAR et. al., 2015), objetivando a administração tópica, tem sido estudada.

Os polifenois, como a quercetina, o resveratrol, a rutina, entre outros, apresentam baixa solubilidade em água e, consequentemente, uma baixa biodisponibilidade no organismo. Dessa forma, sistemas nanoestruturados contendo polifenois têm aumentado a absorção dessas substâncias ativas no organismo e a sua atividade terapêutica (LI et. al., 2009; FANG; BHANDARI, 2010; SESSA et. al., 2014). Na tabela 1, pode-se observar trabalhos onde foram desenvolvidos sistemas nanoestruturados contendo flavonoides.

Tabela 1 – Estudos relativos à associação de flavonoides com sistemas nanoestruturados.

Sistema	Flavonoide	Método de Preparação	Características Físico-químicas	Resultados Obtidos	Referências
NS	Quercetina	Nanoprecipitação	82 nm; PdI 0,22; EE 99,9%	Aumento da atividade antioxidante	Wu et. al., 2008
NC	Quercetina	Inversão de Fase	50-102 nm; PdI inferior a 0,05; EE superior 99%	Aumento da estabilidade da quercetina durante 70 dias	Barras et. al., 2009
NC; NE	Rutina	Deposição Interfacial de Polímero Pré-Formado	122-126 nm; PdI inferior a 0,2; -27 mV; EE 93,33%	Aumento da fotoestabilidade da rutina e pronlongamento da atividade antioxidante *in vitro*	Almeida et. al., 2010
NS	Naringenina	Nanoprecipitação	90 nm	Liberação sustentada da naringinina, redução da toxicidade em células de câncer cervical	Krihsnakumar et. al., 2011
CLN	Quercetina	Solidificação-evaporação	215 nm; -20,10 mV	Melhora da permeação de quercetina na pele	Chen-Yu et. al., 2012
NL	Quercetina	Emulsificação-Ultrassonicação	351 nm; PdI 0,245; -35 mV	Melhora na estabilidade, liberação sustentada, melhoria na permeação na pele	Bose et. al., 2013
NLS	Luteolina	Utrassonicação-microemulsão a quente	47 nm; -9,62 mV; EE 74,76%	Aumento da concentração da luteolina no plasma, aumento da	Dang et. al., 2014

				biodisponibilidade	
NS	Quercetina	Nanoprecipitação	242 nm; -22,5 mV; EE 73,3%	Aumento na atividade antioxidante	Sambandam et. al., 2015
NS	Quercetina	Emulsificação-Nanoprecipitação	32 a 153 nm; EE 51 a 65%	Liberação controlada da quercetina, atividade antitumoral	Pandey et. al., 2015
NS	Naringenina	Gelificação Iônica	53-407 nm; 22-58 mV	Melhora na atividade antioxidante, atividade antitumoral, ausência de toxicidade em fibroblastos	Kumar et. al., 2015

NS: nanoesferas; NC: nanocápsulas; NE: nanoemulsão; CLN: carreador lipídico nanoestruturado; NL: nanopartícula lipídica; NLS: nanopartícula lipídica sólida; PdI: índice de polidispersão; EE: eficiência de encapsulamento.

Deve-se ressaltar que as nanocápsulas têm sido pesquisadas para a liberação cutânea de substâncias ativas, apresentando várias vantagens de sua ulitização, como possuir elevada área superficial, o que as torna especialmente interessantes para a aplicação de substâncias lipofílicas, promovendo liberação homogênea. Estas nanovesículas podem atuar como reservatórios de fármacos lipofílicos, controlando a liberação, e aumentar a permanência de substâncias ativas na pele (GUTERRES; ALVES; POHLMANN, 2007). Por apresentarem tamanho de partículas de 10 to 1000 nm, são capazes de aumentar a permeação de substâncias ativas na pele (PROW et. al., 2011). Na literatura, estudos empregando nanocápsulas de núcleo lipídico (OURIQUE et. al., 2011; SILVA et. al., 2013) e realizando comparação entre nanocápsulas e nanoemulsão (FLORES et. al., 2015) para a aplicação cutânea de substâncias têm sido relatados.

1.3.2 Liofilização

Como o produto final no preparo dos sistemas nanoestruturados é uma suspensão, pode ocorrer instabilidade físico-química. As instabilidades mais comuns nos sistemas nanoestruturados são sedimentação, cremação, aglomeração, crescimento de cristais ou mudança do estado cristalino (SCHFFAZICK et al., 2003a). Para minimizar os problemas de instabilidade das suspensões de nanopartículas, processos como a liofilização (ABDELWAHED et. al.. 2006a; KHAYATA et. al., 2012) ou secagem por aspersão (MARCHIORI et.al., 2011; OURIQUE et. al., 2014; THSWEU et. al., 2104) podem ser

utilizados. O produto final de ambos processos são produtos sólidos, os quais podem sofrer redispersão para a administração (WU et. al., 2011; KASPER et. al., 2013).

O processo de liofilização (*freeze-drying*) consiste na remoção de água de um produto congelado por meio de sublimação sob vácuo. A liofilização é o processo de escolha para uma ampla gama de aplicações farmacêuticas como vacinas, antibióticos lábeis, proteínas, peptídeos, terapia genética (ácidos nucleicos), sistemas micro e nanoparticulados a fim de evitar problemas de instabilidade físico-química (ABDELWAHED et. al.. 2006a; KASPER et. al., 2013).

A liofilização apresenta três etapas básicas: (a) congelamento ou solidificação, onde o líquido é congelado; (b) secagem primária (sublimação), em que ocorre a sublimação do gelo a partir da amostra congelada, obtendo no final um produto seco e poroso; (c) secagem secundária (dessorção), ocorrendo a remoção da água adsorvida a partir da formulação (ABDELWAHED et. al.. 2006a). O congelamento é considerado o ponto mais importante no processo de liofilização, sendo reportadas, na literatura, diversas formas de congelamento, como a -20ºC (SCHAFFAZICK et. al., 2003b), -45ºC (KHAYATA et. al., 2012); -70ºC (ZHAO et. al., 2013), -80ºC (ABDELWAHED et. al., 2006b) ou em nitrogênio líquido a -196°C (CHACON et al., 1999).

Quando as nanopartículas são submetidas ao congelamento da amostra, pode ocorrer aglomeração/desestabilização das mesmas. A fim de reduzir o estresse do processo sobre as partículas durante o congelamento/liofilização, são utilizados crioprotetores. Os crioprotetores mais utilizados são trealose, sacarose, manitol, lactose e glicose. A trealose é o crioprotetor mais usado para biomoléculas, por apresentar baixa higroscopicidade e baixa reatividade química (ABDELWAHED et. al., 2006a).

KHAYATA e colaboradores (2012) desenvolveram nanocápsulas de policaprolactona contendo vitamina E, a fim de avaliar a sua estabilidade e os parâmetros ótimos de liofilização, usando diferentes crioprotetores (PVP, sacarose, manitol e glicose todos a 10%; congelamento a -45ºC por 2 horas). Foi relatada uma boa estabilidade das suspensões de nanocápsulas durante seis meses de armazenamento. Em relação à liofilização, os índices de ressuspensão foram de 40,59; 5,65; 1,50 e 23,34 para PVP, manitol, sacarose e glicose, respectivamente, sugerindo que o crioprotetor mais adequado é a sacarose por apresentar índice de ressuspensão mais próximo de 1,0.

Em outro estudo realizado por FONTE e colaboradores (2014), nanopartículas de poli(ácido lático-co-ácido glicólico) contendo insulina foram desenvolvidas e liofilizadas, avaliando-se diferentes crioprotetores como trealose, glicose, sacarose, frutose e sorbitol (10% p/v). Foi observado que o processo de liofilização melhorou a estabilidade da insulina e a adição de crioprotetores melhorou a estabilidade das nanopartículas. Houve aumento significativo de partículas para todos os crioprotetores utilizados, em todas as condições de armazenamento estudadas (4ºC, 25ºC/60% UR e em 40ºC/75% UR). No entanto, entre os crioprotetores utilizados, o sorbitol apresentou melhor estabilidade em todas as condições de armazenamento, em comparação com os outros crioprotetores, ocorrendo um menor aumento de partículas e, consequentemente, uma menor agregação.

1.4 Etilcelulose

A etilcelulose é um de derivado semissintético da celulose, sendo um polímero utilizado na indústria farmacêutica, cosmética e alimentícia (ROWE; SHESKEY; QUINN, 2009). Na indústria farmacêutica, é utilizada em formulações orais ou tópicas, empregada como agente de revestimento, promotor de viscosidade, na modificação da liberação de fármacos e na melhora da estabilidade da formulação (ROWE; SHESKEY; QUINN, 2009). A etilcelulose é estável, não apresenta toxicidade, praticamente insolúvel em glicerina, propilenoglicol e água, sendo solúvel em etanol, metanol, tolueno, clorofórmio, acetato de etila e tetrahidrofurano (WU et. al., 2003; LAI et. al., 2010). Na figura 5, é apresentada a estrutura química da etilcelulose.

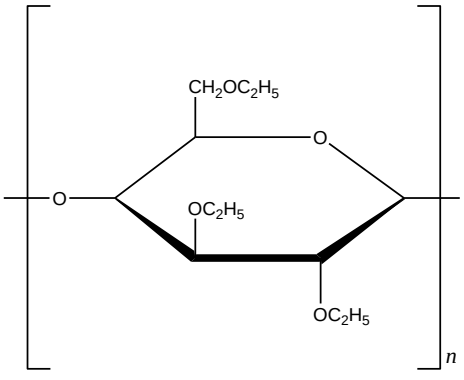

Figura 5 – Estrutura química da etilcelulose

Existem estudos utilizando a etilcelulose como polímero em sistemas nanoestruturados. RAVIKUMARA e colaboradores (2009) desenvolveram nanopartículas de etilcelulose contendo nimesulida (244 a 1710 nm), capazes de promoverem liberação lenta e

sustentada do fármaco, sendo sistemas promissores para o tratamento de doenças, mediante administração pela via oral.

Em outro estudo, realizado por SAHU e colaboradores (2013), nanopartículas de etilcelulose contendo quercetina (229 nm; -17 mV) para a liberação tópica do fármaco, no tratamento do câncer de pele, foram desenvolvidas. Conforme os resultados, a eficiência de encapsulamento da quercetina nestes sistemas foi de 53,93%. As nanopartículas desenvolvidas foram capazes de prolongar a liberação deste flavonoide, permanecendo mais tempo na pele e, assim, podendo reduzir a frequência de administração. Observou-se, também, uma redução da absorção sistêmica.

CHASSOT e colaboradores (2014) desenvolveram nanocápsulas contendo diproprionato de beclometasona, utilizando etilcelulose como polímero e o óleo de amêndoas como núcleo. Foram relatadas características físico-químicas adequadas (158 a 185 nm; -11,7 a -12,0 mV). A citotoxicidade das nanocápsulas desenvolvidas foi avaliada em fibroblastos (1 e 10 µM; 3T3), onde ausência de toxicidade foi encontrada. Além disso, as nanocápsulas desenvolvidas não apresentaram toxicidade pulmonar em ratos. Dessa forma, nanocápsulas de etilcelulose contendo diproprionato de beclometasona podem ser sistemas promissores para a liberação pulmonar.

1.5 Óleo de Amendoim

O óleo de amendoim é extraído da semente do amedoim da árvore *Arachis hypogea*, a qual pertence à família Leguminosae. O óleo de amedoim é nativo da América do Sul, na área conhecida como região do Chaco, que compreende os países Argentina, Bolívia, Paraguai e Brasil. No entanto, também é encontrado no Senegal e Índia. Senegal, Argentina e Índia dominam a exportação do óleo de amendoim. O óleo é extraído por prensagem mecânica ou por solvente, o rendimento da semente em óleo é de 45%. Apresenta uma coloração amarelo-pálida, elevada estabilidade oxidativa e solidifica de 0 a 3°C. Esse óleo é utilizado, principalmente, na indústria farmacêutica, cosmética e alimentícia (ANVISA, 1999; CARRÍN; CARELLI, 2010).

As propriedades físico-químicas do óleo de amendoim são determinadas pela composição de ácidos graxos e suas posições na molécula de triacilglicerol. Os principais ácidos graxos são ácido palmítico (9,3 a 13%), ácido oleico (35,6 a 58,3%) e o ácido linoleico (20,9 a 43,2%); em menor proporção está o ácido esteárico (1,1 a 3,6%) e o ácido araquidônico (0,3 a 2,4%). A variação dos ácidos graxos presentes no óleo depende do

processamento utilizado para a obtenção do mesmo (ANVISA 1999; CARRÍN; CARELLI, 2010).

O óleo de amendoim apresenta propriedades antioxidantes e nutricionais. As propriedades antioxidantes do óleo devem-se à presença de tocoferois, principalmente, do α-tocoferol, o qual é o componente majoritário (18 a 57%). O óleo de amendoim bruto apresenta de 30 a 40% de tocoferois. Além destes compostos, o amendoim é uma fonte de resveratrol, o qual apresenta potente atividade antioxidante, além da atividade antiplaquetária, anti-inflamatória, antitumoral e antimutagênica. Sua atividade antioxidante reduz a peroxidação lipídica, oxidação e nitração de proteínas de plaquetas e de plasma. Como o resveratrol é constituinte do amendoim, também está presente no óleo (CARRÍN; CARELLI, 2010).

SESSA e colaboradores (2014) desenvolveram nanoemulsões contendo resveratrol e, como componente lipídico, o óleo de amendoim (128 a 235 nm; IPd 0,12 a 0,33). Foi observado um aumento da biodisponibilidade oral da substância ativa, além do aumento da estabilidade do resveratrol. Assim, nanoemulsões contendo resveratrol ou outros componentes com baixa solubilidade em água podem ser encapsulados a fim de aumentar a sua estabilidade e biodisponibilidade.

1.6 Óleo de Coco

O óleo de coco é extraído do fruto da árvore *Cocos nucifera L.*, a qual pertence à família *Palmae*. É cultivado principalmente na Índia, Indonésia e Felipinas. Apresenta coloração branca a amarela clara ou incolor, alto valor nutricional, solidifica a 15ºC, a 20°C apresenta-se semissólido e seu ponto de fusão é entre 23 a 26ºC (MARINA et. al., 2009; ROWE; SHESKEY; QUINN, 2009; DeBMANDAL et. al., 2011).

Em sua composição, o óleo de coco apresenta cerca de 90% de ácidos graxos saturados, sendo considerado gordura saturada. O óleo de coco é rico em ácido láurico (40-50%), apresentando também ácido mirístico (15 a 20%), ácido caprílico (5 a 11%), ácido oleico (4-10%), ácido palmítico (7 a 12%), ácido cáprico (4 a 9%), além de fenois como ácido cafeico, e ácido vanílico (MARINA et. al., 2009; ROWE; SHESKEY; QUINN, 2009).

O óleo de coco é muito utilizado na indústria alimentícia, e, atualmente, vem atraindo a atenção da indústria farmacêutica e cosmética. Em relação à indústria farmacêutica/cosmética, por não apresentar toxicidade, ele é utilizado como emoliente e base na fabricação de pomadas, emulsões, soluções intranasais e para a aplicação tópica (MARINA et. al., 2009; ROWE; SHESKEY; QUINN, 2009). Além disso, o óleo de coco tem

apresentado atividade antioxidante (MARINA et. al., 2009; DeBMANDAL et. al., 2011), antimicrobiana (OGBOLU et. al., 2007; AGARWAL et. al., 2010) e antifúngica (DeBMANDAL et. al., 2011).

Na literatura, já existem estudos reportando o uso do óleo de coco na preparação de nanocápsulas. BRENDLE (2013) desenvolveu nanocápsulas de Eudragit®RS100 (166 nm; +11,5 mV), utilizando o óleo de coco como núcleo para a veiculação do antioxidante idebenona. Foi possível a preparação de liofilizados redispersíveis a partir destas suspensões e foi verificada a capacidade do produto seco em aumentar a estabilidade da idebenona, em comparação com as suspensões de nanocápsulas.

SANTOS e colaboradores (2014) desenvolveram nanocápsulas contendo clotrimazol, utilizado o Eudragit®RS100 e o óleo de coco (169 a 173 nm; +14 mV), onde foi observada a capacidade destes sistemas em controlar a liberação do fármaco e aumentar a sua fotoestabilidade (UV), além de apresentar um aumento da atividade antifúngica do clotrimazol contra espécies de *Candida spp*. Estes sistemas foram considerados promissores para o tratamento de candidíase vulvovaginal.

CAPÍTULO 1: Desenvolvimento de suspensões de nanocápsulas de etilcelulose contendo crisina: avaliação da atividade antioxidante e da citotoxicidade *in vitro*.

CAPÍTULO 1: *Desenvolvimento de suspensões de nanocápsulas de etilcelulose contendo crisina: avaliação da atividade antioxidante e da citotoxicidade in vitro.*

Apresentação

No presente capítulo, será apresentado o desenvolvimento de suspensões de nanocápsulas poliméricas contendo crisina (0,3 mg/mL), preparadas com etilcelulose, um polímero biocompatível adequado à aplicação tópica cutânea, e com óleos de diferentes origens, para compor o núcleo destes sistemas vesiculares. Óleos vegetais (coco ou amendoim) foram escolhidos em função de possuírem potencial antioxidante, os quais foram comparados ao TCM, óleo sintético, comumente empregado para a preparação de nanocápsulas, segundo a literatura (SCHAFFAZICK et. al., 2003; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

As formulações foram caracterizadas após a preparação e durante 30 dias. Além disto, o perfil de liberação da crisina, a partir das diferentes nanocápsulas foi determinado em meio aquoso simulando o pH da pele, com adição de etanol (30%) para manter a condição *sink*, através da técnica de difusão em sacos de diálise.

Após o desenvolvimento destes sistemas, a atividade antioxidante *in vitro* mediante o radical DPPH foi avaliada, em comparação à crisina livre, às respectivas nanocápsulas sem fármaco, preparadas com os diferentes óleos, e à solução etanólica dos óleos correspondentes, nas mesmas concentrações em que se encontram nas nanocápsulas. Suspensão de nanoesferas (sem crisina e sem óleo) também foi testada para verificar o efeito dos outros componentes/nanoestruturas no mesmo ensaio, considerando as mesmas alíquotas, em volume, correspondentes às nanocápsulas contendo crisina. Cabe evidenciar que suspensões de nanoesferas contendo crisina não foram viáveis, em função da rápida precipitação deste flavonoide, após a preparação. Buscou-se neste comparativo um possível efeito sinérgico dos óleos vegetais e da crisina, devido à presença de compontentes com atividade antioxidante nestes.

Por fim, avaliou-se a citotoxicidade *in vitro* dos sistemas desenvolvidos em fibroblastos, células muito empregadas para uma avaliação geral de toxicidade, inclusive em sistemas nanoestruturados (KROLL et. al., 2009), importante para preceder futuros testes *in vivo*.

PUBLICAÇÃO 1: Development of chrysin-loaded ethylcellulose nanocapsule suspensions: evaluation of the antioxidant activity and *in vitro* cytotoxicity

Artigo a ser submetido para publicação em periódico classificado como B1 pelo sistema WebQualis (área da Farmácia)

Development of chrysin-loaded ethylcellulose nanocapsule suspensions: evaluation of the antioxidant activity and *in vitro* cytotoxicity

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Abstract

Chrysin is a naturally occurring compound that presents important antioxidant activity. However, this flavonoid has low solubility in water and there is only a few studies intended for cutaneous applications. The present study aimed to develop chryin-loaded ethylcellulose nanocapsule suspensions (0.3 mg/mL), prepared with vegetal oils (peanut oil or coconut oil) in comparison with the medium chain triglycerides (MCT), intended for skin delivery. We evaluated the *in vitro* release profile, antioxidant activity, system stability and cytotoxicity (3T3 cells) of chrysin-lodaded nanocapsules. The results showed suitable physical-chemical characteristics and relative stability of nanocapsules during 30 days. Furthermore, all nanocapsules were able to control release of chrysin compared to the free flavonoid. Chrysin-loaded nanocapsule suspensions prepared with vegetal oils, mainly peanut oil, presented higher DPPH scavenging activity compared to the ones prepared with MCT. Finally, the chryin-loaded ethylcellulose nanocapsule suspensions showed no toxicity in cell line fibroblasts compared to the free chrysin, by MTT assay, suggesting safety of the proposed formulation in this study. Therefore, the developed nanocapsule suspensions are an interesting platform for future delivery of chrysin in skin.

Keywords: Chrysin, nanocapsules, antioxidant activity, flavonoid, *in vitro* cytotoxicity, skin delivery, controlled release.

1 Introduction

Reactive oxygen species (ROS) are produced in the organism through chemical reaction (NIJVELDT et. al., 2001; GENESTRA, 2007; MANKE et. al., 2013; SANTHAHUMAR et. al., 2014). In presence of large amounts of ROS, there is an unbalance between production and the capacity of the biological system to neutralize them, being characterized oxidative stress condition. The oxidative stress can promote several diseases (cancer, cardiovascular, degenerative diseases) and skin disorders (WU et al., 2011; MANKE et. al., 2013; SANTHAHUMAR et. al., 2014; PLAZA et. al., 2014). The antioxidant substances are intended to neutralize ROS, which are responsible for reduction, prevention or elimination the oxidative damage (PROCHÁZKOVÁ et. al., 2011).

Chrysin (5,7-dihydroxyflavone) is a natural compound (flavonoid) that occurs in several plants and foods, mainly in propolis and honey (RAPTA et. al., 1995; MERCAN et. al., 2006; PICHICHERO et. al., 2010; BARBERIC et. al., 2011). Recently, several studies showed biological activities and pharmacological effects of chrysin, including potent antioxidant activity (PUSHPAVALLI et. al., 2010; WU et al., 2011; SULTANA et. al., 2012; REHMAN et. al., 2013; ANITHA; RAJADURAI, 2014; SOUZA et. al., 2015) and antitumor effect (TOBIN et. al., 2006; SAMARGHANDIAN et. al., 2011; REHMAN et. al., 2013; LI et. al., 2015). However, chrysin is practically insoluble in water, therefore it has low bioavailability in the organism, which can limit its application in the pharmaceutical area (KIM et. al., 2008; CHAKRABORTY et. al., 2010). In order to increase the antioxidant activity and bioavailability of chrysin, nanostructured systems are an alternative. Recently, PLGA-nanospheres containing chrysin were developed to treat *in vitro* breast cancer (MOHAMMADINEJAD et. al., 2015). Chrysin-loaded solid lipid nanoparticles were also studied in order to improve the therapeutic efficacy and bioavailability of chrysin (AISHWARYA et. al., 2015).

However, chrysin has rarely been applied in skin care despite their therapeutic potential. WU and co-workers (2011) suggested that chrysin has a potential use in skin photoprotection since the results shown significant benefits of this flavonoid on the protection of keratinocytes against UVA/UVB-induced injuries. In addition, a study indicated that chrysin may be employed as inhibitor of hyperpigmentation (KIM et al., 2011).

In several works based on nanostructured systems advantages of their use have been reported as promising carriers of therapeutic drugs. These systems are characterized by the

submicrometric size. They can increase the therapeutic efficacy and bioavailability of active substances in the organism, besides can promote drug control release, transport to specific sites of action, and reduction the toxicity of drugs (COUVREUR et. al., 2002; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010). Nanocapsules are vesicular systems constituted by a polymeric shell disposed around a core, generally oily, where the drug can be dissolved in the core and/or adsorbed to the polymeric shell. The polymer for preparation of nanocapsules must be biocompatible and not necessarily biodegradable. Ethylcellulose is a stable polymer, without toxicity and biocompatible. Studies have developed nanoparticles (RAVIKUMARA et. al., 2009; SAHU et. al., 2013) and nanocapsules (CHASSOT et. al., 2014) using ethylcellulose as polymer.

It should be noted that the nanocapsules have been researched for skin delivery of active substances, presenting several advantages. Due to the high surface area, these systems are especially interesting for the application of lipophilic substances, promoting homogeneous release in the skin. These nanovesicles may act as reservoirs of lipophilic substances, controlling their release and increasing the time of permanence in the skin (GUTERRES; ALVES; POHLMANN, 2007). By presenting particle size of 10 to 1000 nm, nanocapsules can be useful for increasing the permeation of active substances into the skin (PROW et. al., 2011). In the literature, studies using lipid-core nanocapsules (OURIQUE et al, 2011; SILVA et al, 2013) and polymeric nanocapsules (FLORES et al, 2015) for skin application have been reported (GUTERRES; ALVES; POHLMANN, 2007).

The oil is an essential constituent of the polymeric nanocapsules. Medium chain triglycerides (MCT) are a synthetic oils widely used as the core of these systems for solubilizing a wide range of substances (MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010). Recently, the use of vegetable oils as core of the nanocapsules is increasing, such as grape seed oil (ALMEIDA et. al., 2010), coconut oil (SANTOS et. al., 2014), melaleuca oil (FLORES et. al., 2011) and almond oil (CHASSOT et. al., 2014).

Peanut oil has antioxidant activity due to the presence os natural substances, such as tocopherols (α-form: 18-57%) and resveratrol. Furthermore, this oil shows anti-inflammatory and anti-tumor effects (CARRIN et. al., 2010). Peanut oil was employed for compose a nanoemulsion containing resveratrol (SESSA et. al., 2014).

Another vegetable oil interesting is the coconut oil, which is extracted from the tree Cocos nucifera L. (Palmae). Coconut oil presents as 90% of saturated fatty acids and the

major is lauric acid (40-50%) and myristic acid (15-20%). This oil has antioxidant activity (MARINA et. al., 2009; DeBMANDAL et. al., 2011), antimicrobial effect (OGBOLU et. al., 2007; AGARWAL et. al., 2010) and antifungal activity (DeBMANDAL et. al., 2011). SANTOS and co-workers (2014) developed Eudragit®RS100 nanocapsules containing coconut oil as core for controlled release of clotrimazole (vaginal delivery).

In this context, the aim of this study was to develop chrysin-loaded nanocapsules using ethylcellulose as polymer and the peanut oil (PO), coconut oil (CO) or medium chain triglycerides (MCT) as the core. These systems, intended for potential skin delivery, were evaluated for their stability at room temperature, *in vitro* chrysin release, antioxidant activity (DPPH assay) and *in vitro* cytotoxicity (3T3 cells).

2 Materials and methods

2.1 Materials

Chrysin (99%) was purchased from Fagron (São Paulo, Brazil). Peanut oil was kind gift from Campestre – vegetable oils (São Bernado do Campo, Brazil). Virgin Coconut oil was donated by TheraHerb (Niterói, Brazil). Medium chain triglycerides was supplied by Alpha Química (Porto Alegre, Brazil). Ethycellulose (Ethocel™ Standard 20 Premium) was donated from Colorcon® (Cotia, Brazil). Span 80® (sorbitan monooleate) was obtained from Sigma-Aldrich (São Paulo, Brazil) and Tween 80® (polysorbate 80) was purchased from Delaware (Porto Alegre, Brazil). DPPH (1'1-dipphenyl-2-picrylhydrazyl) was acquired from Sigma-Aldrich (São Paulo, Brazil). MTT (5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl tetrazolium bromide), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and trypsin-EDTA solution (170,000 U l-1 trypsin and 0.2 g l-1 EDTA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM), supplemented with L-glutamine (584 mg/L) and antibiotic/antimicotic (50 mg/mL gentamicin sulphate and 2 mg/L amphotericin B), were obtained from Vitrocell (Campinas, SP, Brazil). Methanol and acetonitrile (HPLC-grade) was supplied by Tedia (Rio de Janeiro, Brazil) and other solventes/chemicals were of analytical grade and employed as received.

2.2 Apparatus and chromatograpic conditions

The experiments were perfomed on a Shimadzu LC-10A HPLC system (Kyoto, Japan) equipped with a model LC-20AT pump, a UV/VIS SPD-M20A detector (Diode array

detectors – PDA), an auto-sampler model SIL-20A HT (injection volume of 20 µL) and a CBM-20A system controller. The separation was obtained using a RP C_{18} Phenomenex column (150 x 4.60 mm; 5 µm; 110 Å) coupled with a C_{18} guard column (4 x 3 mm), at room temperature.

The isocratic mobile phase consisted of acetonitrile:0.1% phosphoric acid (50:50 v/v) at flow rate of 1.0 mL/min and the detection was performed at 255 nm (DMITRIENKO et al., 2012). The peak areas were integrated automatically by a computer (Shimadzu LC solution software program).

2.3 Method validation

The method for the assay of chrysin in ethylcellulose nanocapsules was previously validated according to specific guidelines (ANVISA, 2003; ICH, 2005) and it was based on the method described by DMITRIENKO and co-wokers (2012), with modifications. The parameters considered for the validation of the RP-HPLC-UV method were: specificity, linearity, accuracy and precision.

A stock standard solution of chrysin (0.5 mg/mL) was prepared in methanol. This solution was diluted to prepare five different standard solutions (5.0, 10.0, 15.0, 20.0 and 25.0 µg/mL), in metanol, and were filtered (0.45 µm) prior to being injected into the HPLC system, for linearity evaluation (n=3, in three different days, using linear least-square regression analysis).

The specificity of the chromatographic method was evaluated by comparison of chromatograms obtained for chrysin-loaded nanocapsule suspensions and the corresponding formulations without this flavonoid (empty nanocapsules).

In order to evaluated the accuracy of the method, recovery assay was performed by spiking chrysin-loaded nanocapsule suspensions of known concentrations (NC-CO-C) with three different concentrations (n=3) of standard solution (1.5, 9.0 and 16.5 µg/mL), corresponding to the final concentrations of 7.5, 15.0 and 22.5 µg/mL chrysin (lower, medium and high concentrations, respectively).

The repeatability and intermediate precision were performed by assaying six different samples of suspensions, containing chrysin (NC-CO-C), at the same concentration (15.0 µg/mL; n=12), under the similar experimental conditions, during the same day in two

different days, respectively. In the sequence, the relative standard deviation (RSD %) was calculated for this method. The samples were prepared as described in 2.5.3 (total content).

2.4 Dissolution/swelling experiments of polymer films

In order to evaluate the interaction between the polymer and the oils (vegetable oils or MCT) used in the preparation of polymeric nanocapsules, the dissolution/swelling assay of ethylcellulose films was performed (MORA-HUERTAS et al., 2010; GUTERRES et al., 2000). In this experiment, 2 g ethylcellulose were solubilized in acetone, followed by evaporation of the organic solvent at room temperature, resulting in ethylcellulose polymeric film. In the sequence, about 600 mg of the polymeric film was weighed and immersed completely in PO, CO or MCT (n=3). PO and MCT remained at room temperature. CO remaim stored at 37ºC. At 0, 1, 2, 3, 7, 15, 30, 45, 60 and 90 days, the films were removed from the contact with the oil and dried with the aid of an absorbing paper. Weight variation was determined using an analytical balance.

2.5 Preparation of nanocapsule suspensions

Ethylcellulose nanocapsule suspensions were prepared through the interfacial deposition of a preformed polymer, according to the method describe by FESSI and co-workers, in 1989. An organic phase constitued of ethylcellulose (0.2500 g), Span 80® (0.1925 g), the oil (PO, CO or MCT; 0.3750 g) and acetone (133 mL) was kept for 50 minutes under moderate magnetic sitrring at 40°C. After complete dissolution of the componentes, chrysin (0.0075 g) was added to the organic phase and dissolved under stirring. In the sequence, the organic phase was poured to 133 mL of an aqueous phase of Tween®80 (0.1925 g) and the magnetic stirring was maintained for 10 minutes. At the end of the process, the organic solvente and part of the water were eliminated by evaporation under reduced pressure to achieve a final volume of 25 mL and chrysin concentration of 0.3 mg/mL. For comparation, formulations without the flavonoid (empty nanocapsules) were also prepared. All formulations were made in triplicate. For the best understanding, the following abbreviations are denominated as: NC-PO-C (chrysin-loaded nanocapsules containig peanut oil); NC-PO (nanocapsules containig peanut oil without chrysin), NC-CO-C (chrysin-loaded nanocapsules containig coconut oil), NC-CO (nanocapsules containig coconut oil without chrysin), NC-MCT-C (chrysin-loaded nanocapsules containig medium chain triglycerdies) and NC-MCT (nanocapsules containig medium chain triglycerdies without chrysin).

2.6 Characterization of nanocapsule suspensions

2.6.1 Particle size analysis, polydispersity index (PdI) and zeta potential

Particle sizes and polydispersity indexes (n=3) were measured by photon correlation spectroscopy, using a Zetasizer Nanoseries Malvern Instruments (UK) after diluting the sample in ultrapure water (1:500). Zeta potentials were determined employing the same equipment after the dilution of samples in 10 mM NaCl (1:500).

2.6.2 pH

The pH values of the nanocapsules were verified by directly immersing the electrode of a calibrated potentiometer (Model pH 21, Hanna Instruments, Brazil) in the suspensions. Measurements were performed in triplicate and made at room temperature (25 ± 2ºC).

2.6.3 Chrysin content and encapsulation efficiency

Total chrysin content in coloidal systems (n=3) was determined by diluting an aliquot of the suspensions in methanol (10 mL) and submitting it to sonication for 40 minutes to extract the chrysin (15 µg/mL). The samples were filtered in a 0.45 µm membrane before injecting them into the chromatographic system. To assayed encapsulation efficiency, an aliquot of the suspension was placed in Amicon®Ultra centrifugal filter device (10,000 MW; Milipore) and free chrysin was separated from the nanoparticles employing the ultrafiltration/centrifugation technique (3,615 x g for 10 minutes). The encapsulation efficiency (%) was calculated as the diference between total and free concentration of chrysin determined in the nanocapsule suspensions and ultrafiltrate, respectively, according to the following equation 1:

$$EE = \frac{\text{Total Content - Free Content}}{\text{Total Content}} \text{ x } 100 \text{ (Equation 1)}$$

2.7 Stability studies

All nanocapsule suspensions were monitored after preparation and during 30 days of storage to evaluate flavonoid content, mean particle size, polydispersity index (PdI), zeta potential and pH value. The nanocapsule suspensions were stored at room temperature and protected from the light (amber glass bottle). This study was performed in triplicate of batches.

2.8 *In vitro* chrysin release study

Chrysin release profiles were obtained by dialysis diffusion technique in 200 mL buffer acetate pH 5.0:ethanol (70:30 v/v) to keep the sink condition, at 37ºC and under

continuos magnetic stirring. The samples, either chrysin methanolic solution (MS-C; 0.3 mg/mL) or nanocapsule suspensions (NC-PO-C; NC-CO-C; NC-MCT-C) were placed in the dialysis bag (MWCO = 12,000 to 14,000 Da, Sigma-Aldrich). Aliquots of 1 mL were drawn at predetermined periods and replaced by the same volume of fresh medium. The amount of chrysin released was assessed using HPLC (injection volume of 50 µL). The experiment was conducted in triplicate. For this study, we used the same chromatographic conditions previously described (2.2), using a RP C_{18} Phenomenex Kinetex column (250 x 4.60 mm; 5 µm; 100 Å) and a linear standard curve from 0.125 to 16.0 µg/mL, in release medium (y = 468661x - 26823, r = 0.9998), confirmed by analysis of variance (ANOVA, $p<0.05$).

In order to elucidate the mathematical model and the chrysin release mechanism from the developed nanocapsules, the data were analyzed using dependent models, where the model most adequate was monoexponential (equation 2). The half-life was calculated from the monoexponential model, following the equation 3. To elucidate the release mechanism was used Power Law/Korsmeyer-Peppas model (equation 4). The Scientist 2.0 software (MicroMath®, USA) was used to perform the mathematical modeling.

$$C=C_0.e^{-kt} \text{ (Equation 2)}$$

$$t_{1/2}=\frac{0,693}{k} \text{ (Equation 3)}$$

$$f_t = a.t^n \text{ (Equation 4)}$$

Where *C* is the concentration at time *t*, C_0 is the initial concentration of the chrysin, *k* is the kinetic rate constant, $t_{1/2}$ is the time to release 50% of the chrysin presente, *C* is the fraction of the flavonoid released at time *t* (hours), *a* is a constant which incorporates structural and geometric characteristics of the release system and *n* is the exponent that indicates the drug release mechanism.

2.9 DPPH radical scavenging assay

The antioxidant assays was carried out according to JOSHI and co-workers (2011). To aliquots of each sample (chrysin-loaded nanocapsule suspensions or ethanolic solution of chrysin), 1.5 mL of DPPH ethanolic solution (6 mM) was added at final chrysin concentration of 0.002, 0.010, 0.025, 0.030 or 0.050 mg/mL and incubated for 30 minutes, at room temperature. After this period, the absorbance values were measured at 518 nm and the percentage of DPPH scavenging activity was calculated as equation 5. Nanocapsule

suspensions containing chrysin in ethanol (without DPPH) were used as a blank. As a negative control, the DPPH solution was used. Moreover, for comparison, gallic acid was used as substance with known antioxidant activity (0.002, 0.010, 0.025, 0.030 or 0.050 mg/mL).

$$SC\ (\%) = \frac{Abs_s - Abs_b}{Abs_c} \times 100 \quad \text{(Equation 5)}$$

Where: *SC %*: Percentage of scavenging capacity; Abs_s: sample absorbance; Abs_b: blank absorbance; Abs_c: control absorbance.

For comparison, nanocapsule suspensions without chrysin (NC-PO; NC-CO; NC-MCT), nanospheres without this flavonoid (NS) and oil solutions (PO, CO, MCT) were also evaluated. The corresponding blank samples were prepared without DPPH, in ethanol.

2.10 Cell Culture

To assess the cytotoxic effects of the formulations, the normal fibroblast cells (3T3; murine Swiss albino fibroblasts) were grown in DMEM medium supplemented with FBS (10% v/v), L-glutamine (584 mg/L) and antibiotic/antimicotic (50 mg/mL gentamicin sulfate and 2 mg/L amphotericin B), at 37°C with 5% CO_2. In 75 cm^2 culture flasks, these cells were routinely cultured and were harvest using trypsin-EDTA when the cells reached about 80% confluence.

2.11 Cytotoxicity assays

Into the central 60 wells of a 96-well plate, the normal fibroblast 3T3 cells were seeded at a density of 1 x 10^5 cells/mL. After incubation during 24 h (n=3), under condition of 5% CO_2 at 37ºC, the spent medium was replaced with 100 µL of fresh medium supplemented with 5% FBS containing ethanolic solution of chrysin or chrysin-loaded nanocapsule suspensions at 1.25 – 5.0 µg/mL concentration range of this flavonoid, corresponding also to a range of 62.5-250 µg/mL concentration of each oil in these formulations. For comparison, nanocapsule suspensions without chrysin (1.5 % PO, CO or MCT) or ethanolic solution of these oils (1.5 %) were also evaluated at the same concentration above described (62.5-250 µg/mL). Untreated control cells were incubated with only the medium added with 5% FBS (v/v). The cells were exposed for 24 h to each treatment, and their viability was measured by

MTT assay. The MTT endpoint is a measurement of cell metabolic activity (MOSSMANN, 1983).

After 24 h of each treatment, the medium was withdraw, and 100 µL of MTT in PBS (5 mg/mL) diluted 1:10 in medium without FBS was added to the wells containing the 3T3 cells. In the sequence, the plates containing the cells were incubated for 3 h, the medium was removed. Subsequently, DMSO (100 µL) was inserted to each well to promote the dissolution of the purple formazan products. After shaking at room temperature for 10 min, the resulting absorbance from the samples was measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 550 nm. Lastly, cell viability for MTT assay was calculated as the percentage of tetrazolium salt reduced by viable cells in each sample. The viability values of 3T3 cells were expressed as percentage of untreated control cell (100% viability was set at the mean optical density of cells treated only with the medium). These experiments were performed in triplicate of nanocapsule suspension batches.

2.12 Statistical analysis

The nanocapsule suspensions containing chrysin were prepared and analyzed in triplicate and results are expressed as mean ± SD (standard derivation). Data were analyzed by Student's *t*-test or One-way analysis of variance (ANOVA) followed by post-hoc Tukey's test, using the Prism Program (version 5). For cytotoxicity assay, results are expressed as mean ± standard error (SE) and the statistical analyses were performed using one-way ANOVA followed by Bonferroni's post-hoc test. A p value < 0.05 was considered to be statiscally significant.

3 Results

3.1 Dissolution/swelling experiments of polymer films

The compatibility of the oils and ethylcellulose was studied by monitoring the weight changes in the polymer films, during 90 days (figure 1). Compared to the initial weight, it was noted that there was no swelling/dissolution of the polymer films immersed in PO or CO ($p>0.05$). On the other hand, it can be seen that ethylcellulose films presented a slight increase in weight after 15 days of immersion in TCM, if compared to the initial time ($p<0.05$).

3.2 Method validation

The Figure 2 shows the chromatogram obtained from analysis of chrysin-loaded nanocapsule suspensions, prepared with diferent oily core, in comparison to the respective formulations without chrysin (empty nanocapsules). The specificity test showed that no interference from the constituents of nanostructures in the chrysin peak was verified. Besides, the purity of the chrysin peak was confirmed by analyzing the samples employing a photodiode array (PDA detector; peak purity index > 0.9999). Therefore, the method is specific for the determination of chrysin in the developed nanocapsule suspensions (NC-PO-C, NC-CO-C and NC-MCT-C).

Regarding the linearity evaluation, a linear relationship between the peak area and the concentration of chrysin was verified, considering the concentration range of 5.0 to 25.0 µg/mL, with the equation (n = 3) calculated by the least square method (y = 97.788x – 21.488; correlation coefficient: r = 0.9998), confirmed by analysis of variance (ANOVA, $p < 0.05$).

The precision data of the method for chrysin in nanocapsule suspension (NC-CO-C) at 15 µg/mL is exposed in Table 1. The results showed an adequate intra-day (repeatability) and inter-day precision since the RSD values were lower than 2.0 %. Also, the obtained average recovery (three levels of concentration) was found as 106 ± 2%. Therefore, this method is accurate for determination of chrysin in the ethylcellulose nanocapsules.

3.3 Preparation and characterization of nanocapsule suspensions

Table 2 shows the physicochemical characteristics of chrysin-loaded ethylcellulose nanocapsule suspensions, prepared with the different oily components (NC-PO-C; NC-CO-C; NC-MCT-C). All formulations presented mean particle diameter in a nanometric scale (below 161 nm), polydispersity index (PdI) values were less than 0.2 and electrophoretic mobility analysis gave negative zeta potential. Acid pH values were verified (5.12-6.00), which is suitable for skin delivery. The total content of chrysin was near the theoretical value (97-100 %) and encapsulation efficiency was close to 100 %. The type of oil was not a factor that influenced the mean particle diameter, PdI and zeta potential of the formulations, after preparation ($p>0.05$). On the other hand, the formulations containing MCT had pH more acid compared to the formulations prepared with vegetable oils (PO or CO).

Besides, all nanocapsule suspensions presented aspect of white/bluish opalescent liquids, without visible precipitation.

Empty nanocapsules presented similar characteristics that the respective formulations prepared with chrysin (NC-PO: 125 nm, PdI 0.11, -11.6 mV; NC-OC: 133 nm, PdI 0.14, -11.3 mV; NC-MCT: 157 nm, PdI 0.05, -10.4 mV).

3.4 Stability evaluation

The ethylcellulose nanocapsule suspensions containing chrysin were stored (room temperature/protected from light) and the physicochemical characteristics were evaluated during 30 days, as can be shown in figure 3. After this period, no change of macroscopic characteristics (odor, color, visual aspect and precipitation) was observed for all suspensions. Also, there were no significant differences ($p>0.05$) in relation to mean diameters, PdI, zeta potentials and pH values (figure 3A, 3B, 3C, 3D) regardless the oily component. Chrysin content was stable during 15 days ($p>0.05$), but a slight reduction was verified after 30 days (figure 3E).

3.5 *In vitro* release study

In vitro chrysin release experiment was conducted by diffusion in dialysis bags at 37°C and the figure 4 shows the release profiles of this flavonoid from the nanocapsules prepared with the different oily components (NC-PO-C; NC-CO-C or NC-MCT-C), compared with chrysin methanolic solution (MS-C). The suspensions prepared with PO, CO or MCT showed an average release of 89.32 ± 6.72%, 85.00 ± 4.22% and 85.71 ± 2.94%, respectively, after 30 hour of assay. On the other hand, free chrysin completed its diffusion in 12 hours (99.59 ± 3.41%).

Considering the mathematical modeling (Scientist® 2.0 software, Micromath), the experimental data obtained for chrysin from *in vitro* release study had a good fit to the first order equation ($r>0.99$; monoexponential; table 3) regardless the composition of the formulations. There are significant diferences between the *k* values (kinetic rate constant) of all nanocapsule suspensions and the chrysin solution ($p<0.05$). Besides, the statistical analysis show no difference between the colloidal systems in relation to *k* values ($p<0.05$). Also, there was an increase in the half-lifes of chrysin release from the developed nanocapsules in relation to the free flavonoid ($p<0.05$).

In the sequence, the release mechanism of chrysin from the nanocapsule suspensions was modeled by Korsmeyer-Peppas equation, presenting a good fit ($r>0.99$; table 3). As

related in the literature, for systems with spherical geometry, limits considered for the release exponent (n) include: (a) Fickian diffusion type for n = 0.43; (b) anomalous transport for 0.43 < n < 0.85; (c) case II transport type considering n ≥ 0.85 (RITGER; PEPPAS, 1987). In this work, the values of "n" were in a range of 0.59 to 0.64 (table 3), indicating a release mechanism of chrysin based on anomalous transport from all nanocapsule suspensions.

3.6 Antioxidant activity

In order to investigate the ability of nanostructured systems to increase the antioxidant activity of chrysin, we evaluated the samples of chrysin-loaded nanocapsule suspensions (NC-PO-C; NC-CO-C; NC-MCT-C) in comparison with free chrysin solution, oil solutions (PO, CO or MCT) and nanocapsules without chrysin (NC-PO, NC-CO or NC-MCT). To evaluate the influence of the other components of these systems (except oils and chrysin), nanospheres were also studied. Figures 5, 6 and 7 show the results obtained.

In this work, the DPPH scavenging ability of chrysin was between 19 to 22%, which is lower if compared with gallic acid, employed as standard for radical scavenger (figure 5).

According to figure 5A, DPPH radical scavenging activity of chrysin-loaded nanocapsules prepared with peanut oil (NC-PO-C) was higher than of free chrysin at concentration above 0.025 mg/mL. On the other hand, chrysin nanoencapsulated in NC-CO-C and NC-MTC-C (figure 5B, C) exhibited higher DPPH scavenging activity as compared to free chrysin only at 0.050 mg/mL ($p<0.05$).

It was observed that the vegetal oils (PO and CO) presented antioxidant activity at all concentrations studied (0.10-2.50 mg/mL; figure 6A, B), in contrast with the MTC solution that presented no DPPH scavenging activity (figure 6C). The nanocapsules without chrysin exhibited higher DPPH radical scavenging activity as compared to the respective oil solutions at 1.25-2.50 mg/mL for NC-PO and 2.50 mg/mL for NC-CO ($p<0.05$).

Analyzing only the nanocapsule suspensions containing chrysin (figure **7**), it is possible to note that the NC-PO-C had greater DPPH scavenging activity compared with the NC-CO-C (concentrations of 0.025 and 0.030 mg/mL; $p<0.05$) and NC-MCT-C at concentrations above 0.025 mg/mL ($p<0.05$). Also, NC-CO-C showed higher antioxidant activity than NC-MCT-C at concentration of 0.050 mg/mL ($p<0.05$). Besides, nanosphere suspensions presented lower antioxidant activity ($p>0.05$) and was similar to free chrysin and NC-MCT.

3.7 Cytotoxicity assays

In order to evaluate the *in vitro* cytotoxicity of the nanocapsule suspensions, 3T3 fibroblasts were used as cell model and the endpoint MTT was applied to determine the cell viability rates. As shown by the MTT assay, there was no significant difference ($p>0.05$) between chrysin-loaded nanocapsule suspensions and free chrysin (figure 8A, 8B, 8C). Finally, the unloaded nanocapsules displayed low levels of cytotoxicity in the entire concentration range tested, as shown by MTT assay (figure 8A, 8B, 8C).

4 Discussion

Polymer and oil are essential in the composition of core-shell structure of the nanocapsules (COUVREUR et al., 2002; SCHAFFAZICK et al., 2003; GUTERRES; SCHAFFAZICK; POHLMANN, 2007; MORA-HUERTAS; FESSI, ELAISSARI, 2010). In this sence, the polymer swelling/dissolution experiments have been considered an important pre-formulation test for development of nanocapsules, employing a simple method destined to evaluate the compatibility between a polymer and an oil destined to compose the core of these systems (GUTERRES et al., 2000; SCHAFFAZICK et al., 2002; CHASSOT et al., 2014; SANTOS et al., 2014; SILVA et al., 2013; RIGO et al., 2014). In this work, PO, CO and MCT were considered suitable materials for development of ethylcellulose nanocapsules, since that there was no observed dissolution of polymer films (figure 1), but only slight increase when this material was immersed in MCT (6.7 % of initial weight after 90 days).

Nanocapsule development needs the use of suitable characterization tools for determining parameters as mean particle size, PdI, zeta potential, drug content, encapsulation efficiency and drug release, in order to guarantee the nanotechnological behavior and better understand the performace of these systems (COUVREUR et. al., 2002; SCHAFFAZICK et al., 2003; MORA-HUERTAS; FESSI; ELAISSARI, 2010; RIGO et al., 2015).

In this work, it was possible to prepare chrysin-loaded nanocapsule suspensions, employing ethylcellulose and diferent oils (table 2). Hence, chrysin-loaded ethylcellulose nanocapsules presented size and polydispersion compatible with those of colloidal suspensions, according to the range obtained for others polymeric nanocapsules (OURIQUE et al., 2008; ALMEIDA et al., 2010; SANTOS et al., 2014; RIGO et al., 2014; CHASSOT et al., 2014; SAVIAN et al., 2015). The PdI was lower than 0.2, indicating an adequate homogeneity of particle size distribution for these nanocapsules containing the flavonoid, as described in literature for this parameter (SCHAFFAZICK et. al., 2003; MOHANRAJ et. al.,

2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010; RIGO et al., 2014; FRANK et al., 2014).

In relation to the zeta potential, which reflects the surface potential of the particles (SCHAFFAZICK et al., 2003, WEI et. al., 2011), all nanocapsule suspensions presented negative values (-11 to -12 mV) that are related to the negative surface density of the interfacial components of these colloidal particles. Hence, the influency of chemical nature of the polymer is very important (SCHAFFAZICK et al., 2003). CHASSOT and co-workes (2014) also obtained ethylcellulose nanocapsules (158-185 nm) with negative zeta potential, using almond oil and polysorbate 80/Span® 80 as core and surfactants, respectively (-12 mV to -16 mV).

The oil type (vegetal or MCT) did not have any influence on the mean particle size, PdI and zeta potential ($p>0.05$). RIGO and co-workers (2014) also verified that the vegetal oils (rice bran, sunflower or soya) did no influence the mean particle size of poly(ε-caprolactone) nanocapsules (226-235 nm), prepared by interfacial deposition of preformed polymer technique. In this sence, these same authors (2015) obtained nanocapsules with similar mean diameter, prepared with MCT (195 nm) or rice bran oil (194 nm).

The chrysin content was adequate for all formulations without significant differences between suspensions ($p>0.05$), indicating that the process did no cause degradation or loss of this flavonoid during the production of the nanocapsules at maximum temperature of 40°C. As expected, the encapsulation efficiency was high (about 99%) evincing the most affinity of chrysin for all nanocapsules, avoiding its partitioning in dispersing aqueous phase, which corroborate to the low solubility of this polyphenol in water (KIM et. al., 2008; CHAKRABORTY et. al., 2010).

It should be emphasized that the analytical method was validated to assure the determination of chrysin in these nanocapsules (figure 2; table 1). Hence, according to the data, the method was specific, linear, precise and accurate to quantification of this flavonoid.

Regarding the stability (figure 3), the nanocapsule suspensions were stable considering the parameters evaluated, during 30 days, regardless the oil employed. It should be noted that the zeta potential is influenced by nanoparticle coating with polysorbate 80, a non-ionic surfactant, which acting as a physical stabilizer at the interface between particles and water (SILVA et al., 2013; RIGO et al., 2014). As in the present work, high zeta potential was not necessary to achieve stable colloidal suspensions, due to the interfacial film of polysorbate 80

rather than a great electrical repulsion of the nanocapsules. In addition, chrysin content was relatively stable during 30 days of storage, remaining above 91%.

The results presented in Figure 4 and Table 3 show that the release of chrysin from ethylcellulose nanocapsules occurred more slowly as compared with the release of free flavonoid. In this way, it was observed that the nanocapsule suspensions were able to control release of chrysin with similar kinetics (*k* values between 0.060 and 0.077 h^{-1}), showing no influency of oil used to prepare these systems ($p>0.05$).

According to the model that best described the release data, the release rate depends only on chrysin concentration (first order equation), being described by an equation constituted by a single exponential term (monoexponential). In this way, the release profile of chrysin occurred in a single step, without burst effect. This data suggests that probably chrysin is confined in the core of the nanocapsules, independent of the oil used, corroborating with the high encapsulation efficiency (table 2).

There was no significant difference in the half-lifes (9.0-11.5 h) obtained for nanocapsule suspensions ($p>0.05$). Thus, it was no observed significant influence of type of oils in relation to the release profile of chrysin. The nanocapsules effectively increase the half-lifes of chrysin release about 3.0-3.8 fold, if compared with the free flavonoid, reflecting the influence of polymeric wall on its controlled release. These results corroborate with literature, since several works have been shown that polymeric nanocapsules can promote the controlled release of hydrophobic drugs (FERRANTI et al., 1999; SANTOS et al., 2014; RIGO et al., 2014; CHASSOT et al., 2014; SAVIAN et al., 2015).

In order to evaluated the chrysin release mechanism from the nanocapsules, Power Law (Korsmeyer-Peppas model), a semiempirical model, was used. This equation employs a single exponent to describe the release process, which is usefull for investigate the controlled release of substances from polymeric systems, mainly when the mechanism is not known and/or involving more than one mechanism (RITGER; PEPPAS, 1987), as in the case of nanocapsules (SILVA DE MELO et al., 2012; SANTOS et al., 2014; SAVIAN et al., 2015). In the present work, the release exponent obtained (table 3) indicated that chrysin release is driven by anomalous transport regardless the oily component, where this flavonoid release is controlled by ethylcellulose relaxation followed by Fickian diffusion of chrysin. These results corroborate with the literature, since ethylcellulose nanocapsules also promoted controlled release of beclomethasone dipropionate, presenting a first order kinetic and anomalous transport as release mechanism (CHASSOT et al., 2014).

The rapid and simple DPPH method was selected to measuring the antioxidant activity of chrysin-loaded nanocapsule suspensions prepared with different oil core. The stable DPPH radical is a very employed scavenging model assay for verifying the ability of natural compounds (e.g. polyphenols), oils and plant extractions in free radical scavenging (SANCHEZ-MORENO et al 2002; ZOU et al., 2014). Besides, it has been also used for evaluating the free radical scavenging activity of formulations containing antioxidant substances, including nanostructured systems (WU et al., 2008; KUMAR et al., 2015; SEBAALY et al., 2015). This radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors and the more employed technique is evaluating the decoloration assay, which measures the absorbance decrease at 515-528 nm produced by inclusion of an antioxidant sample to a DPPH solution in ethanol or methanol (SANCHEZ-MORENO et al., 2002).

In this work, the DPPH scavenging ability of chrysin was lower if compared with gallic acid, employed as standard for radical scavenger**.** The free radical-scavenging activity of chrysin is attributed to its ability of hydrogen-donation due to the presence of phenolic hydroxyl group in the structure. In this way, the radical scavenging by flavonoids happens by electron donation from the free hydroxyls on the nucleus with the generation of a less reactive flavonoid radical, which is stabilized through resonance and then presents a moderate role in the propagation of damage in biological systems induced by radicals (JABBARI et al., 2012; PLAZA et. al., 2014). According to JABBARI and co-workers (2012), the radical scavenging activity (DPPH assay) of chrysin was lower than about 11% and 36% in different water-DMSO mixtures in presence or absence of CE (IV) metal ion, respectively, and the experiments confirmed the contribution of both specific and non-specific solute-solvent interactions on the radical scavenging activity of some flavonoids.

At effect dependent of chrysin concentration and type of oil, the antioxidant activity of chrysin-loaded nanocapsules was more effective than free chrysin at concentrations above 0.025 mg/mL. The data obtained indicate that the order of effectiveness for antioxidant activity of the formulations is as follows (figures 5 and 7): peanut oil (NC-PO) > coconut oil (NC-CO) > MCT (NC-MCT).

As expected, the vegetal oils presented higher DPPH scavenging ability in chrysin-loaded nanocapsules, empty nanocapsules and oil solution in comparison with the respective samples based on MCT, at concentrations between 1.25 and 2.50 mg/mL. This fact can be attributed the related presence of natural substances with antioxidant activity in peanut oil and

coconut oil (MARINA et. al., 2009; CARRÍN; CARELLI, 2010), in contrast with the synthetic oil (MCT). Peanut oil is rich in α-tocopherol and resveratrol, which are potent antioxidants (CARRÍN; CARELLI, 2010). Coconut oil has as constituents some phenols such as caffeic acid and vanillic acid. However, it has a lower proportion of antioxidants in their composition compared to peanut oil (MARINA et al., 2009).

The nanoencapsulation of chrysin in NC-MCT-C significantly increased the antioxidant effect of this flavonoid only at the highest concentration evaluated (0.050 mg/mL). The increase of DPPH scavenging activity observed for NC-PO-C and NC-CO-C was corresponding with the better antioxidant effect also for empty nanocapsules (NC-PO and NC-CO), which shown the essential contribution of these vegetal oils for this ability. There is a synergic effect between the chrysin and formulations prepared with peanut oil or coconut oil due to their significanty higher antioxidante activity in comparison with NC-MCT-C (Figure 7). Also, this hypothesis is reforeced by the results obtained from others samples, since that nanosphere suspensions and NC-MCT ($p > 0.05$) presented lower antioxidant activity (17-24%; 16-24%) and was similar to free chrysin (19 to 24 %). The nanosphere suspensions (113 nm) were prepared in the same manner that the nanocapsules, but without any oil and omitting the chrysin, for evaluating the contribution of the nanostructures/others components in the antioxidant activity assay.

These results corroborate with others studies. Hence, the encapsulated form of naringenin, a citrus flavonone, was also evaluated in DPPH assay (KUMAR et al., 2015). In this study, the free radical scavenging activity of naringenin-loaded chitosan nanoparticles (about 50-82 %) was significantly higher that the free flavonoid (about 34-64%). Chitosan nanoparticles without naringenin exhibited about 10-28% inhibition of DPPH. Also, nanostructured lipid carriers prepared with pomegranate seed oil revealed good antioxidant properties using the chemiluminescence method (NICULAE et al 2014). According to the authors, the antioxidant properties of the pomegranate oil were enhanced by bringing this oil at nanometer scale into nanoparticles. In the present work, the peanut oil and coconut oil also presented higher antioxidant activity ($p<0.05$) when incorporated in nanocapsules if compared to the corresponding oil solutions at range of 1.25-2.50 mg/mL and 2.50 mg/mL, respectively.

Nanostructured systems have been developed to improve the efficacy of different drugs in addition to sustained release in the organism. However, before any in vivo application, it is important to assess the potential toxicity of these systems. Currently, *in vitro* cytotoxicity studies have been widely performed for nanostructured systems, as cell-based

tests are considered the initial step in biological screening approaches (KROLL et al., 2009; ARORA et al., 2012; MONTEIRO-RIVIERE et al. 2009).

Here, we studied the cellular viability of chrysin-loaded nanocapsules in 3T3 fibroblasts (Figure 8), in order to evaluate the toxicity of these nanosytems in healthy cells by measuring the likely reduction in cellular viability, using MTT assay as endpoint. MTT assay is based on the reduction of the MTT salt into an insoluble formazan in viable cells and is associated with the cell mitochondrial activity (KROLL et. al., 2009; MONTEIRO-RIVIERE et. al., 2009).

As demonstrated by the MTT assay, there was no significant reduction of cell viability after any treatment with the nanocapsule suspensions or free chrysin in relation to the control cells ($p>0.05$). These results corroborate with studies found in the literature, where chrysin showed no cytotoxicity in fibroblasts when assayed in the concentration of 5.0 µg/mL, after 72 hours of incubation (CÁRDENAS et. al., 2006). Also, according to CHASSOT and co-workers (2014), ethylcellulose nanocapsules with beclomethasone dipropionate or without this drug did not show a significant *in vitro* cytotoxic effect on 3T3 cells (MTT assay) in relation to the control (DMSO) and poly(ε-caprolactone) nanocapsules, after 24 h of incubation.

5 Conclusion

It was possible to prepare chrysin-loaded nanocapsules containing peanut oil, coconut oil or medium chain triglycerides, which had suitable physicochemical characteristics (particle size, PdI, zeta potential, pH and chrysin content) and high encapsulation efficiency. After 30 days of storage, the formulations were stable and remained above 90% of chrysin content. The nanocapsule suspensions showed sustained release of flavonoid compared to the free chrysin, besides showing an increased antioxidant activity, especially nanocapsule suspensions containing peanut oil. Additionally, chrysin-loaded nanocapsule suspensions showed no cytotoxicity in cell line 3T3 fibroblasts by the MTT assay. Therefore, chrysin-loaded ethylcellulose nanocapsule suspensions are a promising system as an intermediary product for topical administration.

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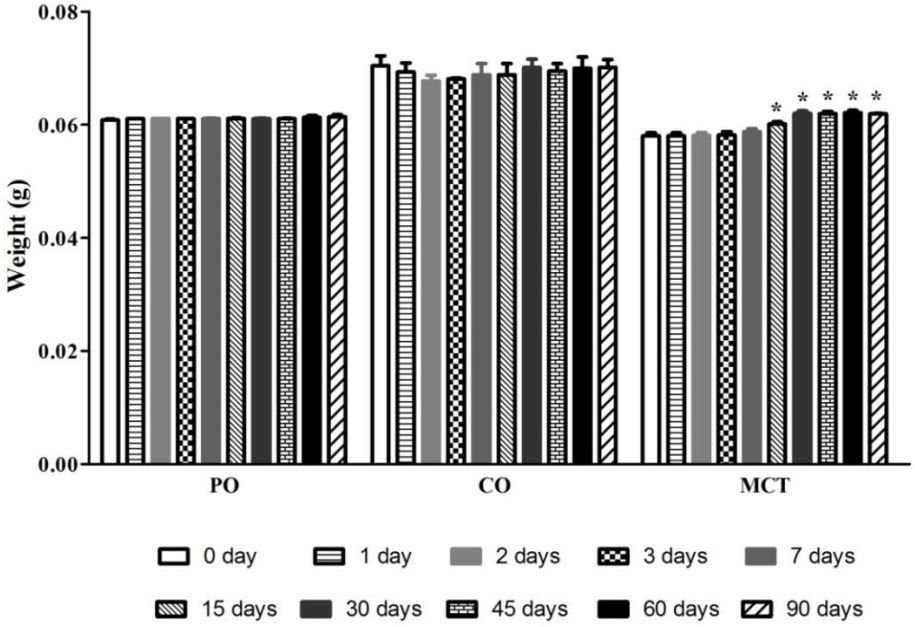

Figure 1 – Weights of ethylcellulose films after immersion in the oils (PO: peanut oil; CO: coconut oil: MCT: medium chain triglycerides).
(*) $p<0.05$. Significant difference compared to the initial weight.

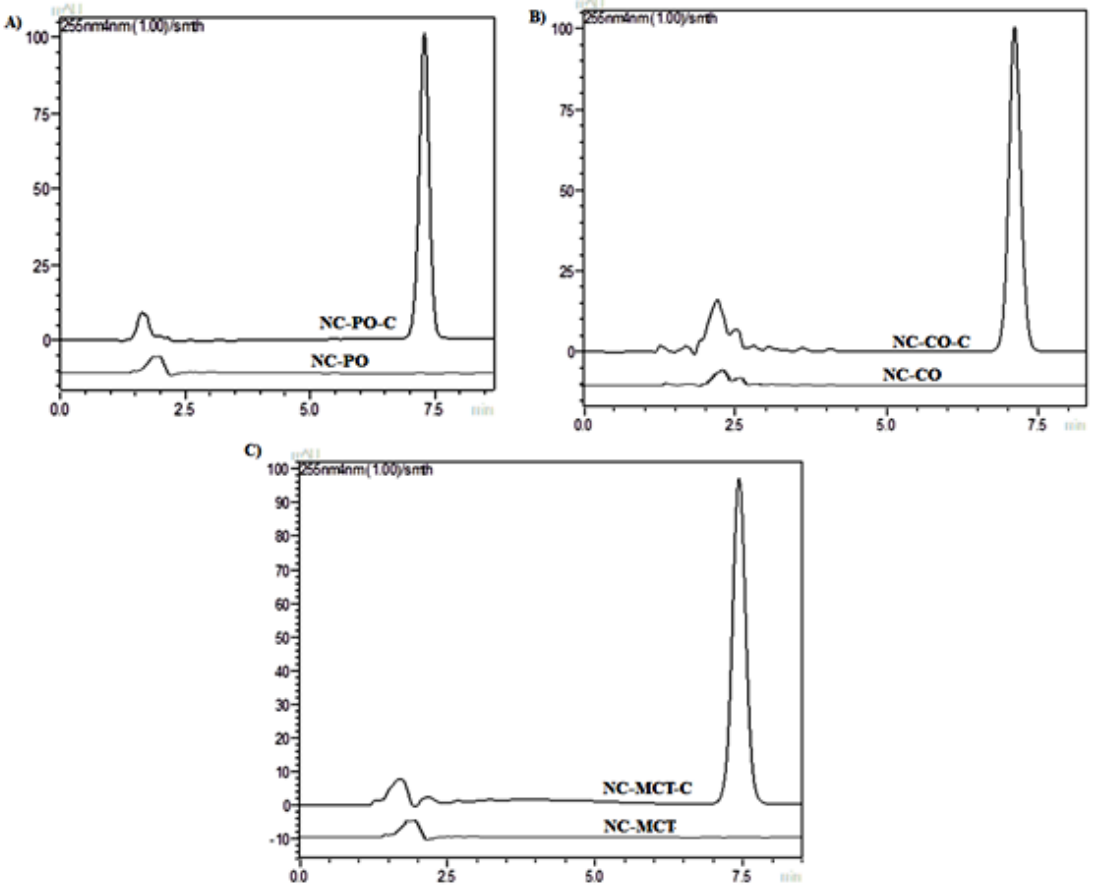

Figure 2 –Chromatograms obtained by HPLC-UV for chrysin determination.
a) NC-PO-C: chrysin-loaded nanocapsules containing peanut oil; NC-PO: nanocapsules containing peanut oil and without chrysin.
b) NC-CO-C: chrysin-loaded nanocapsules containing coconut oil; NC-CO: nanocapsules containing coconut oil and without chrysin.
c) NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides; NC-MCT: nanocapsules containing medium chain triglycerides and without chrysin.

Table 1 – Precision test (repeatability and inter-day precision).

	Theorical concentration (µg/mL)	Experimental concentration (µg/mL)	Recovery (%)	RSD* (%)
Day 1(n=6)	15.00	14.54 ± 0.18	96.93	1.21
Day 2 (n=6)	15.00	14.68 ± 0.10	97.87	0.65
Mean ± SD	15.00	14.76 ± 0.16	98.40	1.06

*RSD: Relative Standard Deviation

Table 2 – Physicochemical characteristics of chrysin-loaded nanocapsule suspensions

Formulation	Mean diameter (nm)	PdI	Zeta Potencial (mV)	pH	Chrysin content (mg/mL)	EE (%)
NC-PO-C	130 ± 06	0.10 ± 0.02	-10.7 ± 1.41	6.00 ± 0.29	0.30 ± 0.00	99.94
NC-CO-C	136 ± 04	0.13 ± 0.01	-11.1 ± 0.67	5.86 ± 0.04	0.30 ± 0.01	99.94
NC-MCT-C	161 ± 46	0.09 ± 0.06	-12.2 ± 1.72	5.12 ± 0.16	0.29 ± 0.01	99.93

NC-PO-C: chrysin-loaded nanocapsules containing peanut oil
NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides.

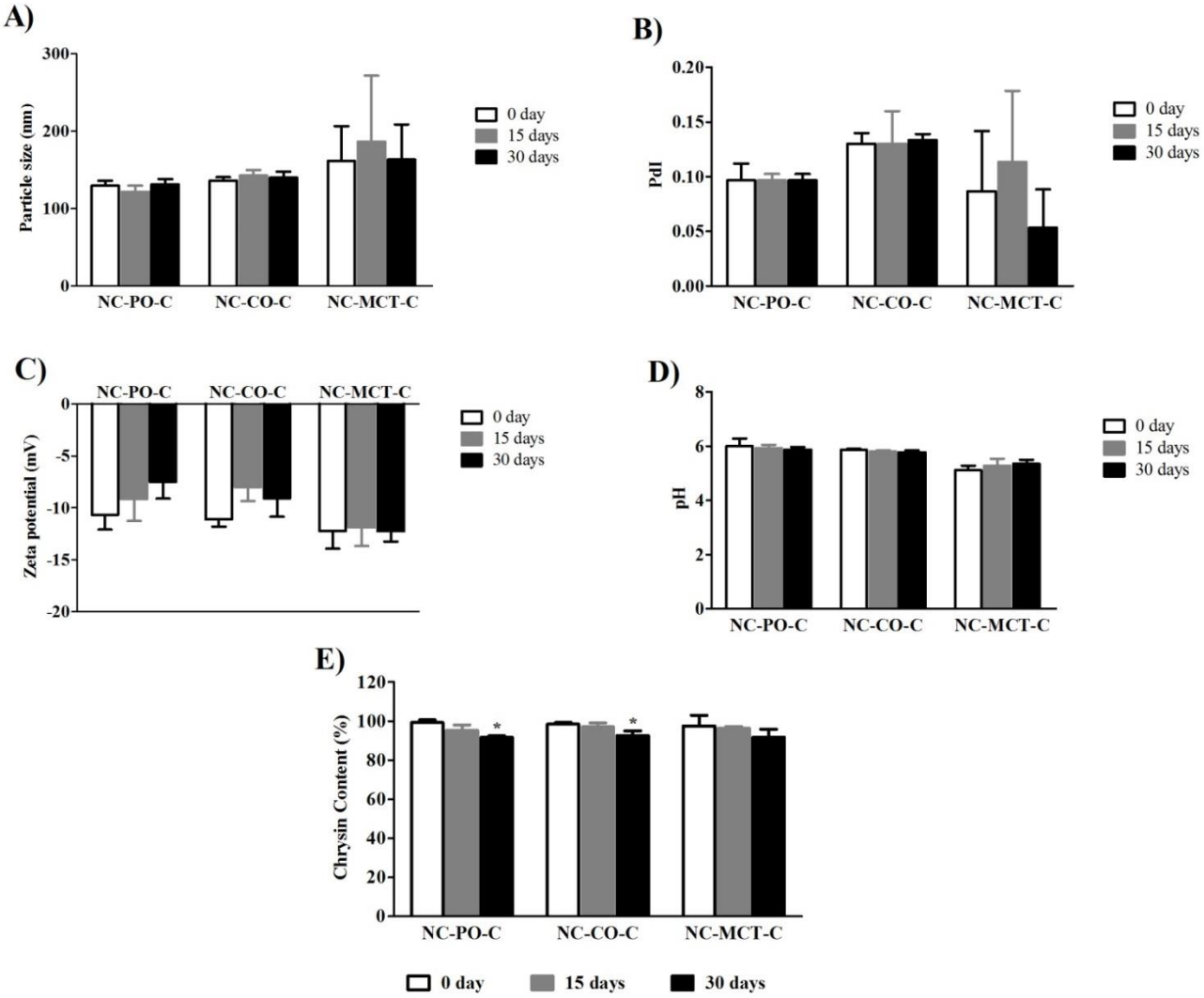

Figure 3 – Stability study of chrysin-loaded nanocapsule suspensions considering as parameters mean particle size (A), PdI values (B), zeta potential (C), pH values (D) and chrysin content (E).
NC-PO-C: chrysin-loaded nanocapsules containing peanut oil
NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides
(*) $p < 0.05$ significant difference compared to the initial time.

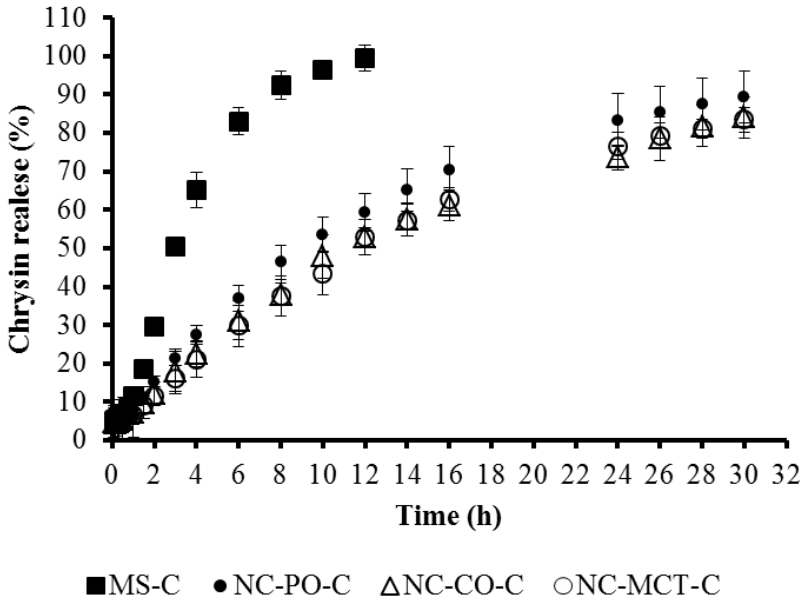

Figure 4 – *In vitro* realese profile of chrysin from ethylcellulose nanocapsules or methanolic solution (MS-C).
NC-PO-C: chrysin-loaded nanocapsules containing peanut oil
NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides

Table 3 - Calculated parameters by first-order and Korsmeyer-Peppas model for chrysin-loaded nanocapsules and free chrysin.

	NC-PO-C	NC-CO-C	NC-MCT-C	MS-C
Monoexponential				
r	0.9994 ± 0.0010	0.9993 ± 0.0004	0.9991 ± 0.0003	0.9912 ± 0.0010
k (h^{-1})	0.0766 ± 0.0132	0.0611 ± 0.0050	0.0604 ± 0.0031	0.2344 ± 0.0158
$t_{1/2}$ (h)	9.0 ± 1.43	11.3 ± 0.98	11.5 ± 0.62	3.0 ± 0.20
Korsmeyer-Peppas model				
r	0.9947 ± 0.0005	0.9964 ± 0.0008	0.9960 ± 0.0008	-
a	0.0128 ± 0.0003	0.0097 ± 0.0008	0.0082 ± 0.0009	-
n	0.5885 ± 0.0028	0.6186 ± 0.0100	0.6419 ± 0.0141	-

NC-PO-C: chrysin-loaded nanocapsules containing peanut oil
NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides
MS-C: methanolic solution of chrysin

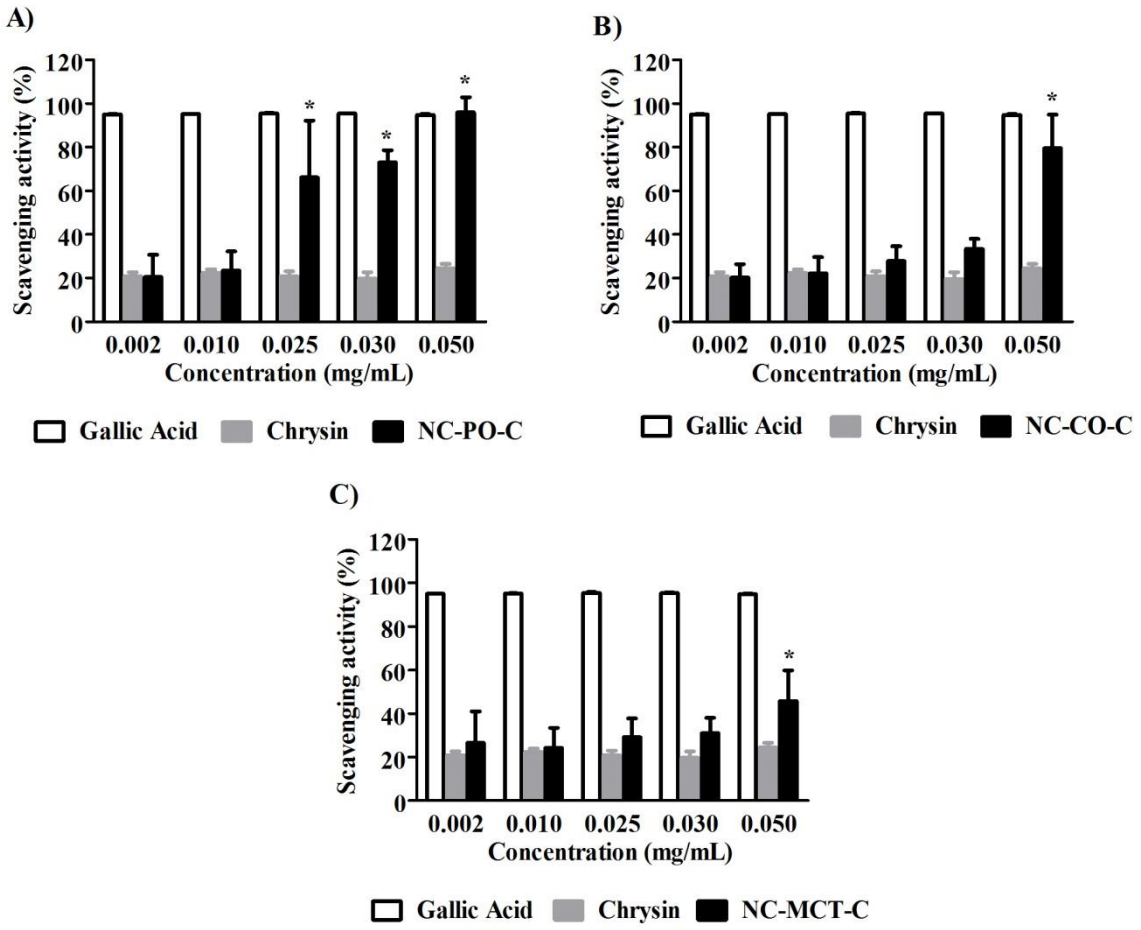

Figure 5 – *In vitro* antioxidant activity of chrysin-loaded nanocapsule suspensions [NC-PO-C (A), NC-CO-C (B), NC-MTC-C (C)] in comparison with free chrysin.
(*) $p<0.05$. Significant differences between free chrysin and nanocapsule suspensions.

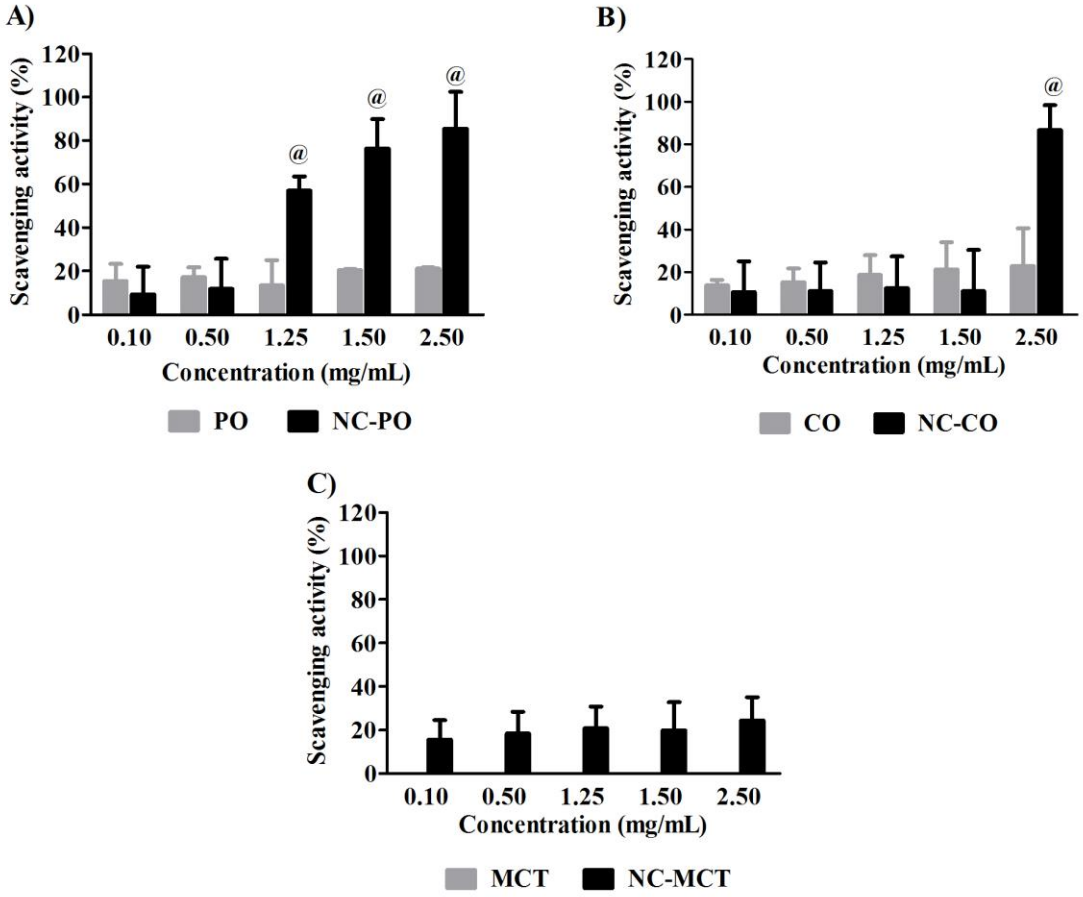

Figure 6 – *In vitro* antioxidant activity of empty nanocapsules [NC-PO (A), NC-CO (B), NC-MCT (C) in comparison with the oil ethanolic solutions.
(@) $p<0.05$. Significant differences between nanocapsule suspensions (without chrysin) and oil solutions.

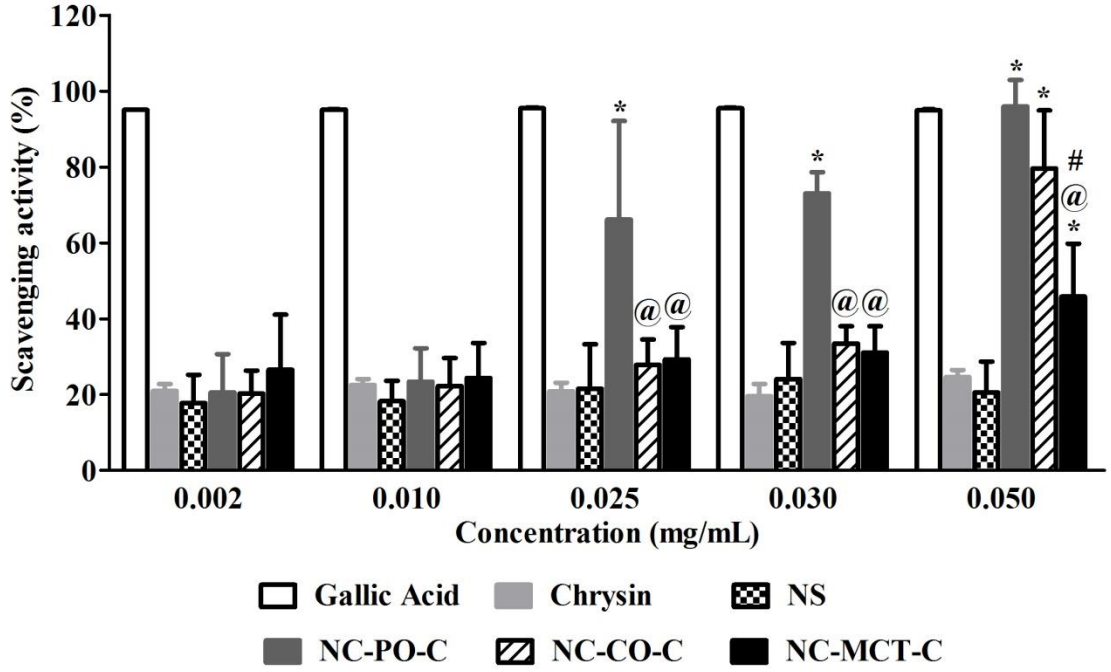

Figure 7 – *In vitro* antioxidant activity of chrysin-loaded nanocapsule suspensions (NC-PO-C; NC-CO-C; NC-MCT-C) in comparison with free chrysin and nanosphere suspension (NS).
(*) Significant difference between nanocapsule suspensions and free chrysin ($p<0.05$).
(@) Significant difference between nanocapsules suspensions (containing CO or MCT) and nanocapsules with PO ($p<0.05$).
(#) Significant difference between nanocapsule suspensions with MCT and ones with coconut oil ($p<0.05$).

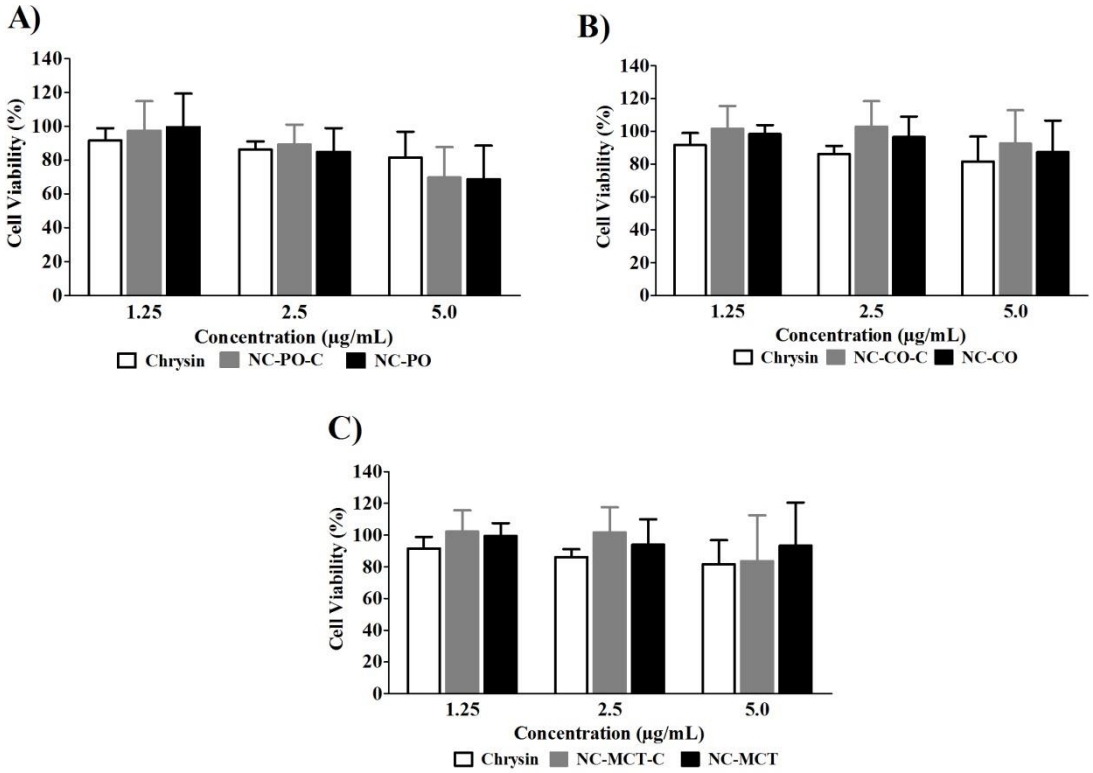

Figure 8 – *In vitro* cytotoxicity assay of free chrysin and nanocapsule suspension with or without chrysin in 3T3 fibroblasts cell line. Chrysin loaded-ethylcellulose nanocapsules prepared with peanut oil (A), coconut oil (B) and medium chain triglycerides (C). * Significant difference from free chrysin ($p < 0.05$).

CAPÍTULO 2: Liberação controlada de crisina associada a nanocápsulas: influência no efeito antiproliferativo *in vitro* e estudo da liofilização sobre a estabilidade do sistema.

CAPÍTULO 2: *Liberação controlada de crisina associada a nanocápsulas: influência no efeito antiproliferativo in vitro e estudo da liofilização sobre a estabilidade do sistema.*

Apresentação

No presente capítulo, será apresentado o desenvolvimento de suspensões de nanocápsulas poliméricas contendo crisina, em maior concentração (0,75 mg/mL) em relação ao primeiro capítulo, preparadas também com etilcelulose e com dois tipos de óleo. O óleo vegetal de coco foi mantido nesta parte do trabalho, em função de suas propriedades antioxidantes, assim como o óleo sintético TCM, para fins comparativos. Não foi possível continuar o trabalho com o óleo de amendoim, pois houve precipitação de crisina, para esta maior concentração de substância ativa.

As formulações foram caracterizadas após a preparação e durante 50 dias, e a capacidade das nanocápsulas em promover a liberação controlada deste flavonoide também foi avalidada. Estudos foram conduzidos para a avaliação da citotoxicidade *in vitro* dos sistemas desenvolvidos em modelos de células não tumorais (fibroblastos) e tumorais (câncer de mama e melanoma), a fim de verificar o potencial uso destes sistemas.

Ao final deste estudo, avaliou-se a possibilidade de liofilizar as suspensões de nanocápsulas com a finalidade de melhorar a estabilidade das mesmas, visando à obtenção de sistemas sólidos redispersíveis, empregando a trealose como adjuvante crioprotetor.

PUBLICAÇÃO 2: Controlled release of chrysin by association with nanocapsule suspensions: influence on *in vitro* antiproliferative effect and study of freeze-drying on system stability

Artigo a ser submetido para publicação em periódico classificado como B1 pelo sistema WebQualis (área da Farmácia)

Controlled release of chrysin by association with nanocapsule suspensions: influence on *in vitro* antiproliferative effect and study of freeze-drying on system stability

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Abstract

Chrysin is a flavonoid with antioxidant and antiproliferative activity in several cell models. However, this natural compound shows poor aqueous solubility, difficulting its biodisponibility in the organism. Hence, the present study aimed to prepare chrysin-loaded ethylcellulose nanocapsule suspensions (0.75 mg/mL), employing coconut oil or medium chain triglycerides as oily core. Studies of the *in vitro* cytotoxicity in normal fibroblast cells (3T3) and antiproliferative effect on human tumoral cells (breast adenocarcinoma or melanoma) was conducted, comparing the colloidal systems and free chrysin. Redispersible lyophilized products, from these liquid formulations, were prepared for improving the stability of chrysin. Our results showed that it was possible to prepare nanocapsules and freeze-dried products containing chrysin with adequate physicochemical characteristics regardless the oily component. Chrysin-loaded nanocapsule suspensions showed controlled release of this flavonoid. In addition, chrysin-loaded nanocapsule suspensions showed no cytotoxic effect on 3T3 cells (MTT and NRU endpoints). Antiproliferative effect of chryin-loaded nanocapsules was observed on tumoral cells (MCF-7 and SK-MEL-28) with diferent sensibility, at dependence of the dose and endpoint assay. Finally, the freeze-drying of nanocapsule suspensions was able to improve the stability of chrysin compared to the liquid formulations. Therefore, these colloidal formulations could be promising systems for delivery of chrysin in future treatments of tumors.

Keywords: chrysin, nanocapsules, flavonoid, controlled release, cytotoxicity, *in vitro* antitumor activity, freeze-drying.

1 Introduction

Chrysin, 5,7-dihidroxiflavona, is a natural compound that belongs to the class of flavones. It is found mainly in honey and propolis (MERCAN et. al., 2006; PICHICHERO et. al., 2010; BARBERIC et. al., 2011). Chrysin has potent antioxidant activity (PUSHPAVALLI et. al., 2010; SULTANA et. al., 2012; REHMAN et. al., 2013; SOUZA et. al., 2015), anti-inflammatory effect (SHIN et. al., 2009; BAE et. al., 2011) and antitumor activity (TOBIAN et. al., 2006; SAMARGHANDIAN et. al., 2011; REHMAN et. al., 2013; LI et. al., 2015). However, chrysin has a low absorption in the organism, which limits its application in the pharmaceutical field (KIM et. al., 2008; CHAKRABORTY et. al., 2010).

In the literature, chrysin-loaded nanospheres for treatment of breast cancer were developed in order to increase the antitumor activity of chrysin for possible local treatment (MOHAMMADINEJAD et. al., 2015). Furthermore, solid lipid nanoparticles containing chrysin were produced with the aim to improve its bioavailability and to promote sustained drug release (AISHWARYA et. al., 2015).

Nanostructured systems have been developed as drug delivery carriers and have sizes lower than 1000 nm, which allows penetration through small capillaries, facilitating their capture by cells and enables the biological barrier permeability, providing sustained or controlled release of the active substances (MORA-HUERTAS; FESSI; ELAISSARI, 2010). Moreover, these systems can promote drug targeting, increasing its concentration in the site-specific of action, improving the active substance therapeutic efficacy. Besides, increasing of the physicochemical stability of the active substance and reduction of the toxicity can be obtained (COUVREUR et. al., 2002; SCHAFFAZICK, 2003a). According to the preparation technique/composition, nanostructured systems are classified as liposomes, nanocapsules, nanospheres and solid lipid nanoparticles (COUVREUR et. al., 2002; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Nanocapsules, core-shell structures, are formed by a shell based on a polymer disposed around an oily core (generally), where the drug can be dissolved in this core and/or adsorbed to the polymeric material (COUVREUR et. al., 2002; ALONSO, 2004; MOHANRAJ; CHEN, 2006). The polymer employed must be biocompatible and its characteristics are important to promote control drug release (SCHAFFAZICK et. al. 2003a; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010). Nanostructured systems have been used in different routes of administration as cutaneous,

oral, ocular, nasal, subcutaneous, intramuscular and even intravenous, without risk of occurs embolism (MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Also, nanocapsules have been studied for delivery of polyphenols such as quercetin, resveratrol, rutin, due to their low solubility/ bioavailability in the organism. Thus, nanostructured systems containing polyphenols have increased the absorption of these active substances, improving their therapeutic activities (LI et. al., 2009; FANG; BHANDARI, 2010; SESSA et. al., 2014).

Ethylcellulose (semisynthetic derivative of cellulose) is a polymer largely used in pharmaceutical industry for compose formulations destined to oral or topical applications, generaly for modify the drug release profile and improve the formulation stability (ROWE; SHESKEY; QUINN, 2009). There are studies using ethylcellulose as polymer for preparation of nanostructured systems intended for the oral (RAVIKUMARA et. al., 2009), topical (SAHU et. al., 2013) or pulmonar administration (CHASSOT et. al., 2015) for delivery of drugs.

The selection of the oily component is very important for the performance of nanocapsules, since it can solubilize the drugs, increasing its concentration into nanosystem. The medium chain triglycerides (MCT) are the most commonly used oil for the composition of these nanovesicles, due to their great ability for solubilizing different substances (SCHAFFAZICK, 2003a; MOHANRAJ; CHEN, 2006; MORA-HUERTAS; FESSI; ELAISSARI, 2010). Nanocapsules produced with vegetable oils such as coconut oil (SANTOS et. al., 2014), grape seed oil (ALMEIDA et. al., 2010), melaleuca (FLORES et. al., 2011) and almonds oil (CHASSOT et. al., 2014) were described in the literature.

Coconut oil, extracted from the fruit of the coconut tree *Coco nucifera L*. (*Palmae*), is rich in lauric acid (40-50%) and is widely used in the food industry, and also can be used in pharmaceutical and cosmetic industries (MARINA et. al., 2009). Moreover, coconut oil shown antioxidant activity (MARINA et. al., 2009; DeBMANDAL et. al., 2011), antimicrobial effect (OGBOLU et. al., 2007; AGARWAL et. al., 2010) and presents antifungal properties (DeBMANDAL et. al., 2011). Recently, the coconut oil was used to prepare Eudragit® RS nanocapsules containing clotrimazole, for the vaginal administration (SANTOS et. al., 2014).

However, nanocapsules are liquid formulations and can presenting physical-chemical instability (e.g. particle agglomeration, sedimentation, cremation, crystal growth or change of

crystalline state) during the storage. In order to minimizing these instability phenomena, processes such as lyophilization or spray-drying have been described in the literature (ABDELWAHED et al., 2006; KHAYATA et al., 2012). The final dried products obtained by both processes can undergo redispersion before administration (WU et. al., 2011; KASPER et. al., 2013), when suitable adjuvants are incorpored into original suspensions, as carbohydrates.

Hence, the aim of this study was to evaluate the performance of ethylcellulose nanocapsules, prepared with coconut oil (CO) or medium chain triglycerides (MCT), as systems for controlled release of chrysin and to generate redispersible lyophilized products for improvement of the flavonoid stability. In addition, the chrysin-loaded nanocapsule suspensions were evaluated on in vitro cytotoxicity assay employing normal cells (3T3 fibroblasts) or tumoral cells (MCF-7 - human breast adenocarcinoma and SK-MEL-28 - human melanoma).

2 Materials and methods

2.1 Materials

Chrysin (99%) was purchased from Fagron (São Paulo, Brazil). Virgin Coconut oil was donated by TheraHerb (Niterói, Brazil) and medium chain triglycerides was purchased from Alpha Química (Porto Alegre, Brazil). Ethycellulose (Ethocel™ Standard 20 Premium) was donated by Colorcon® (Cotia, Brazil). Span 80® (sorbitan monooleate) was acquired from Sigma-Aldrich (São Paulo, Brazil) and Tween 80® (polysorbate 80) was purchased by Delaware (Porto Alegre, Brazil). Trehalose was supplied by Attivos Magistrais (São Paulo, Brazil). Dimethyl sulfoxide (DMSO), 5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye, trypsin-EDTA solution (170,000 U l-1 trypsin and 0.2 g l-1 EDTA) ad phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 Medium, supplemented with L-glutamine (584 mg/L) and antibiotic/antimicotic (50 mg/mL gentamicin sulphate and 2 mg/L amphotericin B) and fetal bovine serum (FBS) were acquired from Vitrocell (Campinas, SP, Brazil). HPLC-grade metanol and acetonitrile were supplied by Tedia (Rio de Janeiro, Brazil). Other chemicals and solvents presented analytical grade.

2.2 Preparation of nanocapsule suspensions

Nanocapsule suspensions (n=3) were produced through the interfacial deposition of a preformed polymer (Fessi et. al., 1989), using ethylcellulose as polymer. An organic phase constitued of ethylcellulose (0.2500 g), Span 80® (0.1925 g), coconut oil (CO) or medium

chain triglycerides (MCT; 0.3750 g) and acetone (133 mL) was kept for 50 minutes under moderate magnetic sitrring (about 40°C). After complete dissolution of the components, chrysin (0.0075 g) was added to the organic phase under stirring. After dissolution of this flavonoid, the organic phase was added into 133 mL of an aqueous phase containing Tween®80 (0.1925 g) under moderate magnetic stirring for 10 minutes. In the sequence, the organic solvent and part of the water were removed at 40°C by evaporation under reduced pressure to achieve a final volume of 10 mL and chrysin concentration of 0.75 mg/mL chrysin. Then, the suspensions were stored in amber glass bottle at room temperature. For comparison, formulations without chrysin were also prepared following the same procedure, but without the flavonoid. To clarify, the nanocapsule suspensions were denominated as: NC-CO-C (chrysin-loaded nanocapsules containig coconut oil), NC-CO (nanocapsules containig coconut oil without chrysin), NC-MCT-C (chrysin-loaded nanocapsules containig medium chain triglycerdies) and NC-MCT (nanocapsules containig medium chain triglycerdies without chrysin).

2.3 Characterization of nanocapsule suspensions

2.3.1 Particle size analysis, polydispersity index (PdI), zeta potential and pH

The nanocapsule sizes and polydispersity indexes (n=3) were determined through dynamic light scattering (Zetasizer Nanoseries Malvern Instruments, UK) by diluting of suspensions in ultrapure water (1:500). For the determination of the zeta potentials, the ethylcellulose nanocapsule suspensions were diluted in 10 mM NaCl (1:500) and electrophoretic mobility was performed using the Zetasizer Nanoseries.

The pH analysis of nanocapsule suspensions (n=3) was performed by direct immersion of an electrode, previously calibrated, in the formulations (Model pH 21, Hanna Instruments, Brazil).

2.3.2 Chrysin content and encapsulation efficiency

Chrysin content in nanocapsule suspensions (n=3) was determined by diluting an aliquot of the formulation in methanol (10 mL) and submitting it to sonication (40 minutes). Samples were filtered through a 0.45 µm membrane and analyzed by high performance liquid chromatography in reverse phase (HPLC-UV), in previously validated method.

The methodology was based on the work of DMITRIENKO and co-wokers (2012) with modifications, using a RP C_{18} Phenomenex column (150 x 4.60 mm; 5 µm; 110 Å),

coupled with a C_{18} guard column, at room temperature. The isocratic mobile phase consisted of acetonitrile:0.1% phosphoric acid (50:50 v/v) at flow of 1.0 mL/min and the separation were performed on a Shimadzu LC-10A HLPC system (Kyoto, Japan; model LC-20AT pump, a CBM-20A system controller, an UV/VIS SPD-M20A detector and auto-sampler model SIL-20A HT). The injection volume was 20 µL and the detection of chrysin was performed at 255 nm. The peak areas of chrysin were integrated automatically by a computer, containing a Shimadzu LC solution software program.

For encapsulation efficiency, an aliquot of the chrysin-loaded nanocapsule suspensions was added in a centrifugal filter device (10,000 MW; Amicon®Ultra Milipore) and free chrysin was separated from the colloidal particles by ultrafiltration/centrifugation technique (3.615 x *g*/10 minutes). The encapsulation efficiency (EE %) was measured as the diference between total and free concentrations of chrysin determined in the ethylcellulose nanocapsule suspensions and in the ultrafiltrate, respectively, according to the following equation 1:

$$EE = \frac{\text{Total Content - Free Content}}{\text{Total Content}} \text{ x } 100 \text{ (Equation 1)}$$

2.4 Stability studies of nanocapsule suspensions

Chrysin-loaded nanocapsule suspensions (n=3) were stored in an amber glass botle during 50 days at room temperature and monitored to flavonoid loading, mean particle size, polydispersity index, zeta potential and pH value.

2.5 *In vitro* chrysin release study

Chrysin release profiles were obtained by dialysis diffusion technique in 250 mL buffer acetate pH 5.0:etanol (70:30 v/v) to keep the sink conditions. The samples (600 µL), either chrysin methanolic solution (MS-C; 0.75 mg/mL) or chrysin-loaded nanocapsule suspensions (NC-CO-C; NC-MCT-C), were placed in the dialysis bag (MWCO = 12,000 to 14,000 Da, Sigma-Aldrich). These bags were kept under continuos agitation at 37°C. Aliquots of 1.0 mL were withdrawn at predetermined periods and replaced by the same volume of fresh medium keep also at 37°C. The amount of chrysin released was determined by HPLC. The experiment was conducted in tripicate. For this experiment, was used a RP C_{18} Phenomenex Kinetex column (250 x 4.60 mm; 5 µm; 100 Å; 50 µL of injection volume). The standard curve (0.125 to 16.0 µg/mL) was linear ($y = 46.8661x - 26.823$, $r = 0.9998$; ANOVA, $p<0.05$).

The Scientist 2.0 software (MicroMath®, USA) was used to perform the mathematical modeling in order to evaluate the mathematical behavior and the chrysin release mechanism from the developed nanocapsules. The data were analyzed using dependent models [zero-order or first-order (monoexponential or biexponential model)]. The experimental data was better fitted to first order equation (monoexponential model; $C=C_0.e^{-kt}$). The half-life was calculated from the monoexponential model, following the equation $t_{1/2}=0.693/k$. To elucidate the release mechanism was used Power Law/Korsmeyer-Peppas model ($f_t=a.t^n$). In these equations, *C* is the time concentration at time *t*, C_0 is the initial concentration of the chrysin, *k* is the kinetic rate constant, $t_{1/2}$ is the time to release 50% of this flavonoid presente, f_t is the fraction of drug released at time *t* (hours), *a* is a constant which incorporates structural and geometric characteristics of the release system and *n* is the exponent that indicates the release mechanism for the active substance.

2.6 Preparation of freeze-dried nanocapsules

Chrysin-loaded nanocapsule suspensions (0.75 mg/mL) were fractionated (5 ml) in wide mouth glass bottles. In the sequence, 10% trehalose was added to suspensions (w/v), in each bottle. After the dissolution of this cryoprotector, under magnetic stirring for 10 minutes, the samples were frozen at -80°C for 2 hours. After, these systems were subjected to dehydration in a freeze-dryer (LIOTOP L 101, Liobras, São Paulo, Brazil) at temperature of about -55°C for 24 hours. For comparative purposes, an aqueous solution of carbohydrate (10% trehalose w/v) was also lyophilized. The dry products (L-NC-CO-C; L-NC-MCT-C) were prepared in triplicate and stored in a desiccator (glass bottles protected from light).

2.7 Physico-chemical characterization of lyophilized nanocapsules

2.7.1 Particle size analysis after freeze-drying

The mean particle diameter was determined by dynamic light scattering (DLS), employing Zetasizer® Nano Series (model ZEN 3600; Malvern Instruments) as equipment. The freeze-dried chrysin-loaded nanocapsules were weighed and redispersed in ultrapure water to obtain the same concentration of components than the original suspensions. Samples (n = 3) were subjected to magnetic stirring for 15 minutes and an aliquot was removed and diluted with purified water (1:500 v/v). In the sequence, the samples were filtered through a hydrophilic membrane (0.45 µm) and the resuspension index was calculated. The resuspension index indicates the recovery of the average particle size in relation to nanocapsule suspensions and was calculated by equation 2.

$$\text{Resuspension Index} = Sf/Si \quad \text{(Equation 2)}$$

Where, *Sf* is the mean particle diameter of the lyophilized product after redispersion in water and *Si* is the mean particle diameter of the original chrysin-loaded nanocapsule suspensions.

2.7.2 Chrysin content in freeze-dried nanocapsules

Samples of the lyophilized products were exactly weighed, corresponding to the theoretical amount of 150 µg of chrysin, transferred to a volumetric flask and dispersed in metanol, under sonication for 40 minutes. In the sequence, the volume of the flask was completed to give a theoretical concentration of 15 µg/mL chrysin. After, the samples were filtrated in regenerated cellulose membrane (0.45 µm) and injected into the HPLC, using the methodology described above for the nanocapsule suspensions.

2.7.3 Scanning Electron Microscopy

The morphological analysis of freeze-dried chrysin-loaded nanocapsules was performed by JSM-6360 Scanning Microscope (Jeol, Japan). Hence, the samples were placed on a metallic support and gold sputtered for 90 seconds (Sputter Desk II, Denton Vacuum, USA). Subsequently, the samples were analyzed using an accelerating voltage of 10 kV. For comparison, the threalose solution (10% w/v) was also observed after freeze-drying in the same microscope.

2.8 Stability study of lyophilized nanocapsules

Lyophilized products containing chrysin (L-NC-CO-C; L-NC-MCT-C) were stored for 50 days at room temperature with protection from light and humidity. At predetermined time, chrysin content of the lyophilizates was determined by HPLC according to above described.

2.9 Cell culture

The cell lines 3T3 (murine Swiss albino fibroblasts) and MCF-7 (human breast adenocarcinoma) were grown in DMEM medium. SK-MEL-28 cells (human melanoma) were grown in RPMI 1640 medium. All cell lines were supplemented with 10% (v/v) FBS, L-glutamine (584 mg/L) and antibiotic/antimicotic (50 mg/mL gentamicin sulfate and 2 mg/L amphotericin B), at 37 °C and with 5% CO_2. The 3T3, MCF-7 and SK-MEL-28 cells were routinely cultured in 75 cm^2 culture flasks and were harvested using trypsin-EDTA when the cells reached about 80% confluence.

2.10 Cytotoxicity assays

3T3 and MCF-7 cells were seeded into the central 60 wells of a 96-well plate at a density of 1 x 10^5 cells/mL. SK-MEL-28 cells were seeded at a density of 5 x 10^4 cells/mL or 2.5 x 10^4 cells/mL for the assays performed during 24 or 48 h, respectively. After incubation for 24 h or 48 h under 5% CO_2 at 37 ºC, the spent medium was replaced with 100 µL of fresh medium supplemented with 5% FBS containing nanocapsules without chrysin (NC-CO; NC-MCT; at 62.5-500 µg/mL concentration range, referred to total oil content in the nanocapsule suspensions) or chrysin-loaded nanocapsules (NC-CO-C; NC-MCT-C; at 1.25 – 10 µg/ml concentration range, referred to total chrysin content in the nanocapsule suspensions). Untreated control cells were treated with medium only (containing 5% (v/v) FBS). The cell lines were exposed for 24 h or 48 h to each treatment, and their viability was assessed by two different assays, MTT and NRU (neutral red uptake).

The MTT endpoint is a measurement of cell metabolic activity and the assay is based on the first protocol described by Mossmann (1983). On the other hand, the NRU assay is based on the protocol described by Borenfreund and Puerner (1985), and reflects the functionality of the lysosomes and cell membranes. After complete the treatment time (24 or 48 h), the NC containing medium was removed, and 100 µL of MTT in PBS (5 mg/mL) diluted 1:10 in medium without FBS was then added to the cells. Similarly, 100 µL of a 50 µg/mL NR solution in DMEM without FBS was added in each well for the NRU assay. The plates were further incubated for 3 h, after which the medium was removed, and the wells of the NRU assay were washed once in PBS. Thereafter, 100 µL of DMSO was then added to each well to dissolve the purple formazan product (MTT assay). Likewise, for the NRU assay 100 µL of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added to extract the dye. After 10 min shaking at room temperature, the absorbance of the resulting solutions was measured at 550 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA) microplate reader. Cell viability for MTT and NRU was calculated as the percentage of tetrazolium salt reduced by viable cells in each sample or as the percentage of uptake of NR dye by lysosomes, respectively. The viability values were normalized by the untreated cell control (cells with medium only).

2.11 Statistical analysis

The liquid and solid formulations were produced and analyzed in triplicate. The results obtained are expressed as mean ± SD (standard deviation) and the Prism program (version 5) was employed for preformed the analysis. One-way analyses of variance (ANOVA) followed

by post-hoc Tukey's tests was conducted. Student's t-test was used for physical and chemical characterization of the formulations and were considered to be statically significant at $p<0.05$. For cytotoxicity assay it was used one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni. The individual IC_{50}-values were performed using Student's t-test or one-way analysis of variance (ANOVA) to determine the differences between the sets of data. A p value < 0.05 was considered to be statically significant and results are expressed as mean ± standard error.

3 Results

3.1 Preparation and characterization of nanocapsule suspensions

After preparation, both chrysin-loaded nanocapsule suspensions prepared with CO or TMC presented homogeneous appearance as a bluish opalescent liquid, without the presence of precipitates. Table 1 shows the physical and chemical characteristics of the nanocapsule suspensions containing chrysin with diferent oils as core. The hydrodynamic diameters and polydispersity index (PdI) of the formulations, measured by DLS, were at nanoscale range and lower than 0.2, respectively. All liquid formulations showed acid pH and the zeta potentials of the nanocapsules were negative.

In relation to the chrysin content, both formulations had adequate levels (97-99%), close to the theoretical value (0.75 mg/mL), and the encapsulation efficiency of this flavonoid was high.

Considering the parameters evaluated, the type of oil only influenced the mean particle size ($p<0.05$) of chrysin-loaded nanocapsules. The other physicochemical characteristics did not were changed by oily composition ($p>0.05$).

Unloaded-nanocapsules presented similar characteristics that the respective suspensions prepared with chrysin (NC-CO: 136 nm, PdI 0.11, -30.0 mV; NC-MCT: 104 nm, PdI 0.14, -30.8 mV).

3.2 Stability study of nanocapsule suspensions

Figure 1 shows the results obtained from the stability study of the nanocapsule suspensions containing chrysin, during 50 days of storage (room temperature/protected from light). Concerning the results, no significant changes ($p>0.05$) in the mean diameter (figure 1A) and PdI (figure 1B) were observed for both nanocapsules, during 50 days of storage.

On the other hand, for all nanocapsule suspensions, the values of zeta potentials (figure 1C) showed a decreasing, in modulus, already after 15 days of storage ($p<0.05$). Also, the pH values (figure 1D) showed a slight increase after 15 days of storage ($p<0.05$) in relation to the initial time, but there was a tendency of stabilization after this period. For all formulations, a reduction of chrysin contents (figure 1E) was verified after 30 days of preparation. The nanocapsule suspensions containing coconut oil (NC-CO-C) presented chrysin content of about 92% ($p<0.05$) after 50 days, while contents of approximately 87% were obtained for the nanocapsule suspensions prepared with MCT (NC-MCT-C) at the same period.

3.3 *In vitro* chrysin release

Chrysin release profiles from nanocapsules are shown in Figure 2. At 8 hours after the beginning, chrysin completed its diffusion (102%). On the other hand, the mean amount of chrysin released from ethylcellulose nanocapsules was 58% (NC-CO-C) and 50% (NC-MCT-C) after 32 hours of the assay.

The profiles were modeled according to different equations and the best model was selected through the parameters evaluated [graphic adjustment, correlation coefficient (r) and model selection criteria (MSC) – MicroMath® Scienctist]. Hence, the first order kinetic was the equation that best described the chrysin release from nanocapsules (monoexponential model; Table 2). The rate constants (k) obtained for the colloidal formulations differed significantly ($p<0.05$) compared to the free chrysin in methanolic solution. However, there was significant difference ($p<0.05$) between the half-lives of NC-CO-C compared to NC-MCT-C.

According to the Korsmeyer-Peppas model (Table 2), chrysin release from ethylcellulose nanocapsules had a high regression coefficient (>0.99) and the release exponent (n) of about 0.66 for both nanocapsules. For delivery systems that present spherical geometry, the ranges considered for the release exponent (n) are: (1) $n = 0.43$, the active substance release is based on Fickian diffusion type; (2) $0.43 < n < 0.85$, the release mechanism is driven by anomalous transport; (3) $n \geq 0.85$; the active substance release is based on case II transport type (RITGER; PEPPAS, 1987).

3.4 Cytotoxicity assays

Figure 3 shows the cytotoxic effects of chrysin-loaded nanocapsules in 3T3 fibroblasts. In this study, no cytotoxicity was observed ($p>0.05$) compared with free chrysin in both assays performed (MTT or NRU).

Figure 4 shows the *in vitro* cytotoxicity of chrysin-loaded nanocapsule suspensions in MCF-7 cells after 24 h incubation. By the MTT assay, it was observed that NC-CO-C reduced the cell viability at all concentrations analyzed ($p<0.05$; figure 4A) in comparison with free chrysin. NC-MCT-C induced significant cytotoxicity only at concentrations of 2.5 and 5.0 µg/mL ($p< 0.05$; figure 4A). In contrast, NRU assay showed no significant reduction in cell viability at any concentration analyzed ($p>0.05$) for all chrysin-loaded nanocapsule suspensions (figure 4A). Concerning the nanocapsule suspensions without chrysin, it was observed lower cytotoxic effects for both formulations, especially by the NRU assay (figure 4B).

Figure 5 shows the results obtained by the MTT and NRU assays in the melanoma cell line SK-MEL-28 after 24 and 48 h incubation. After 24 h, the MTT assay showed reduction in cell viability only at a concentration of 10 µg/mL for all chrysin-loaded nanocapsule suspensions ($p<0.05$; figure 5A) compared with free chrysin. By the NRU assay, it was observed significant cytotoxicity induced by NC-CO-C in comparison with the free chrysin at all concentrations analyzed ($p<0.05$), while NC-MCT-C reduced cell viability ($p<0.05$) only in the concentration 5 and 10 µg/mL (figure 5A). The nanocapsulas without chrysin displayed negligible reduction in cell viability after 24 h incubation ($p>0.05$) by the MTT assay (figure 5C). On the other hand, the NRU assay detected more pronounced cytotoxic effects at concentrations of 250 µg/mL (NC-CO) and 500 µg/mL (NC-CO; NC-MCT; figure **5C**).

After 48 h incubation with SK-MEL-28 cell line, the MTT assay showed that NC-CO-C and NC-MCT-C induced great reduction in cell viability ($p<0.05$) at concentrations of 5 and 10 µg/mL in comparison to the free chrysin (figure 5B). In this assay, there was a significant difference between NC-CO-C and NC-MCT-C only in the concentration of 5 µg/mL. By the NRU assay, all chrysin-loaded nanocapsule suspensions induced greater cytotoxicity than free chrysin, at all concentrations analyzed ($p<0.05$; figure 5B). The unloaded nanocapsule formulations showed reduction of cell viability at 250 and 500 µg/mL by the MTT assay, while significant cytotoxic effects was detected by the NRU assay at all concentrations analyzed (figure 5D). In addition, there was significant difference ($p<0.05$) between NC-CO and NC-MCT only at the lowest concentration tested (125 µg/mL).

Table 3 shows the IC_{50} (concentration causing 50% death of the cell population) values for the cancer cell lines MCF-7 and SK-MEL-28 when treated with free chrysin and chrysin-loaded nanocapsule suspensions. For the MCF-7 cells, no significant differences ($p>0.05$) were observed between the IC_{50} values in both assays. All formulations displayed significantly great activity ($p<0.05$) in comparison to free chrysin in SK-MEL-28 cell line. For this cell line, both formulations showed lower IC_{50} values than the free chrysin ($p<0.05$) at 24 and 48 h by the NRU assay. By the MTT assay, a significant reduction of IC_{50} values for both nanocapsules compared to free chrysin ($p < 0.05$) was observed only after 48 h treatment.

3.5 Preparation and characterization of lyophilized

The lyophilization method (freeze-drying) was chosen to obtain dried products from chrysin-loaded nanocapsule suspensions. The cryoprotectant trehalose at 10% (w/v) was used to obtain redispersible products.

Table 4 shows the physico-chemical characteristics of the freeze-dried chrysin-loaded nanocapsules. Considering the results, there was no significant difference ($p>0.05$) in the mean diameter of resuspended particles in relation to original nanocapsule suspensions. In comparison, the resuspension of freeze-dried trehalose resulted in mean particle sizes of 628 nm, which is higher than the size range obtained for resuspension of freeze-dried nanocapsules containing this cryoprotector.

There was no significant difference between the type of freeze-dried nanocapsules in relation to resuspension index (table 4; $p>0.05$). Besides, the content of chrysin remained close to the theoretical value after lyophilization (97-99%) for both colloidal formulations. Moreover, there was no significant difference in the chrysin content between lyophilized products and the corresponding original ethylcellulose nanocapsule suspensions ($p>0.05$).

Figure 6 showed the micrographs of chrysin-loaded ethylcellulose nanocapsules after freeze-drying by scanning electron microscopy (SEM). The lyophylized nanocapsules (L-NC-CO-C; L-NC-MCT-C) showed a rough surface and the presence of spherical structures at nanoscopic range can be observed. In contrast, lyophylized trealose showed a smooth surface, without spherical particles.

3.6 Stability study of lyophilized

Figure 7 shows the data obtained from stability study of freeze-dried chrysin-loaded nanocapsules during 50 days of storage. There was no significant difference ($p>0.05$) in the chrysin content between the initial time and after 50 days of storage for both nanoformulations.

4 Discussion

According to the manufacturing process (FESSI et al., 1989), the diffusion rate of the acetone (water miscible organic solvent) was rapid and the formed spontaneous nanodroplets immediately resulted in the ethylcellulose nanocapsules by precipitation of this coating polymer due to its reduced solubility at interface of the oily component (CO or TMC). Hence, at the used conditions, the method of interfacial deposition of preformed polymer was able to generate chrysin-loaded ethylcellulose nanocapsule suspensions (0.73-0.74 mg/mL) with good physicochemical characteristics (table 1), presenting particles with narrow distribution (low PdI) and at nanoscale range comparable with other nanocapsules obtained for this same preparation technique (SCHAFFAZICK et al. 2003a; GUTERRES; SCHAFFAZICK; POHLMANN, 2007; OURIQUE et al., 2008; MORA-HUERTAS; FESSI; ELAISSARI, 2010; ALMEIDA et al., 2010; SANTOS et al., 2014; CHASSOT et al., 2014; RIGO et al., 2014; CAMPONOGARA et al., 2014; SAVIAN et al., 2015; CHARÃO et al., 2015).

Also, these results corroborate with studies based on others flavonoid and others ethylcellulose nanocapsules. ALMEIDA and co-workers (2010) developed rutin-loaded nanocapsules (PCL) with size of 120 to 124 nm. CHASSOT and co-workers (2014) developed beclomethasone bipropionate-loaded nanocapsules (ethylcellulose) with size in the range of 158-185 nm (IPd lower than 0.2).

Zeta potential is an important parameter to be evaluated, because it reflects the surface potential of the dispersed particles and it can indicate a good physico-chemical stability of nanocapsule suspensions, due to the high values can reduce the aggregation of the colloidal particles (SCHAFFAZICK et al., 2003a). In the present work, the zeta potential of the nanocapsule suspensions was negative, due to the chemical nature of the ethylcellulose. Other studies also developed nanospheres (SAHU et. al., 2013) and nanocapsules (CHASSOT et. al., 2014) using ethylcellulose as polymer with negative zeta potentials (-12 a -16 mV).

High encapsulation efficiency was obtained, demonstrating the preferential affinity of chrysin, a polyphenolic substance, for the nanocapsules compared with the aqueous phase. In the literature, PLGA-PEG nanospheres (MOHAMMADINEJAD et. al., 2015) and solid lipid

nanoparticles (AISHWARYA et. al., 2015) containing chrysin showed encapsulation efficiency of 98.6% and 86.29%, respectively, corroborating with the high encapsulation efficiency found in this study.

Here, the formation of colloidal suspensions with similar characteristics ($p>0.05$) and with a low SD among the batches (n=3) was obtained with both oily components, showing a good reproducibility of the process. The oil nature influenced only the mean diameter, since the nanocapsules with CO presented higher size than ones with MCT ($p<0.05$). In fact, the nature of oil is a parameter important in the development of these systems and the interfacial tension and viscosity of the oil are factors that can influence the particle sizes (MOSQUEIRA et al., 2000; SCHAFFAZICK et al., 2003a).

Considering the stability study, the chrysin-loaded nanocapsule suspensions (NC-CO-C; NC-MCT-C) exhibit stable mean particle sizes, as well as size distribution (PdI) without significant variation, during 50 days of storage. Although there was a significant reduction of zeta potentials, no important influence of the stabilization in these colloidal systems was verified. Hence, high zeta potential was not necessary to achieve stable nanodispersion under storage, due to the interfacial film of non-ionic surfactant (polysorbate 80) acting as a physical stabilizer at the surface of the nanocapsules, rather than a great mutual electrical repulsion of these structures (SILVA et al., 2013; RIGO et al., 2014).

Regardless the oil employed, all nanocapsule suspensions showed reduction in chrysin content after 30 days compared to the initial time. The nanocapsule suspensions prepared with MCT showed a higher decrease ($p<0.05$) than CO formulations, after 50 days of storage (87.19% and 92.72%, respectively). As hypothesis, it can suggest that the presence of antioxidant components in the CO could act reducing chrysin degradation.

In the release study, it was observed the ability of ethylcellulose nanocapsules in to promote control release of chrysin, since occurred more slowly that the diffusion of the non-encapsulated chrysin (methanolic solution). There was slight influence of the oily component in the chrysin release from the nanocapsule suspensions, because more sustained release of the NC-MCT-C was observed in relation to NC-CO-C ($p<0.05$), after 10 hours of assay.

Furthermore, the best fit to the experimental data was found to be first order kinetics, indicating that the release of chrysin depends on its concentration. Besides, no rapid initial release of this flavonoid was verified, since only one rate constant (k) was associated with the release process (a single step occurred; monoexponential). Hence, the absence of burst effect

in the nanostructure profiles suggests that probably all chrysin is associated into the oily core of the nanovesicles (NC-CO-C or NC-MCT-C), corroborating with the high encapsulation efficiency obtained (<99%).

The *k* values for the suspensions were similar ($p>0.05$). Also, the half-lives associated with chrysin release from NC-CO-C or NC-MCT-C ($p<0.05$) were approximately 9 and 12 times greater than those of non-encapsulated chrysin (2.5 h), respectively, evidencing the controlled release of this flavonoid by these core-shell nanostructures. Several studies have been demonstrated the ability of different polymeric nanocapsules in to promote control release of drugs (FERRANTI et al., 1999; SANTOS et. al., 2014; CHASSOT et. al., 2014; RIGO et al., 2014; SAVIAN et al., 2015).

Power Law (Korsmeyer-Peppas model) was used in order to evaluate the chrysin release mechanism from developed nanocapsule suspensions. Both ethylcellulose nanocapsules showed "n" values of about 0.66, indicating that the chrysin release mechanism is based on anomalous transport. Thus, chrysin release from nanocapsules depends on the relaxation of the ethylcellulose chains followed by chrysin diffusion. CHASSOT and co-workers (2014) developed nanocapsules containing beclomethasone dipropionate, using ethylcellulose as the polymer and almond oil as the oil core. These systems also were able to control release of this drug and the proposed release mechanism was anomalous transport.

Nanostructured systems have attracted the attention of industry and scientists. Therefore, it is important to study the impact of these systems on human healthy. In this context, *in vitro* cytotoxicity assays have been performed to evaluate the potential toxicity of novel drug delivery systems (KROLL et. al., 2009). Here, the toxicity of NC suspensions was firstly assessed in 3T3 fibroblasts cell line and no cytotoxicity was observed in both trials analyzed. CÁRDENAS and co-workers (2006) reported absence of toxicity of chrysin in non-tumor cells, such as fibroblasts, corroborating the results found in our study.

Secondly, the *in vitro* antitumor activity of chrysin-loaded NC suspensions was assessed in two tumor cell lines (breast cancer MCF-7 and melanoma SK-MEL-28) by using two different cytotoxicity assays (MTT and NRU). The results obtained showed different sensitivity of the cell models to the cytotoxic effects of NCs. Moreover, the cytotoxic responses given by the endpoints were not the same, which indicate that there are different mechanisms underlying the potential antitumor activity of the formulations. It is well known that MTT assay measures the metabolic and cellular mitochondrial activity, while the NRU

assay evaluates the integrity of cell membranes and lysosomal activity (KROLL et. al., 2009; MONTEIRO-RIVIERE et. al., 2009).

The breast cancer cell line MCF-7 showed higher sensitivity in detecting the toxic effects of free and encapsulated chrysin by the MTT assay than by NRU assay, suggesting that chrysin-loaded NC suspensions affected, primarily, the mitochondrial compartments and cell metabolism and, in a second time, interfered with lysosomal activity and membrane integrity.

On the other hand, the melanoma cell line SK-MEL-28 showed a different behavior as compared to the MCF-7 cells. SK-MEL-28 cells also showed higher cytotoxicity after treatments with chrysin-loaded NC suspensions than with free chrysin. However, here the NRU assay was more sensitive than the MTT assay, suggesting a different mechanism underlying the cytotoxic effects. It can be inferred that chrysin-loaded NC suspensions have a first effect on the integrity of membranes and lysosomal activity in SK-MEL-28 cells and, subsequently, metabolic and mitochondrial activity of the cell is affected (KROLL et. al., 2009; MONTEIRO-RIVIERE et. al., 2009). These results were corroborated by the IC_{50} values calculated for each treatment. The IC_{50} values obtained for the NRU assay was lower than those obtained for the MTT assay, indicating the greater cytotoxicity detected by the NRU endpoint. NOGUEIRA and co-workers (2013) developed methotrexate-loaded nanoparticles and evaluated this system in different types of tumor and non-tumor cells using MTT and NRU assays. The authors also reported different sensitivities of the cell lines and endpoints used to the toxic effects of each treatment.

Noteworthy that it was observed a reduction in cell viability after treatments with unloaded NCs for both tumor cell lines analyzed (MCF-7 and SK-MEL-28). The toxic effects of the formulations without chrysin may occur due to the excipients and their concentrations used in the preparation of the formulations. Polysorbate 80 (Tween®80) is a component of the NCs that has showed toxic effects in human carcinoma colorectal Caco-2 cell line at the concentration of 0.05% after 24 h of exposure (HODAEI et. al., 2014). Moreover, NOGUEIRA and co-workers (2011) evaluated different lysine-based surfactants in different cell models (tumor and non-tumor) and *in vitro* cytotoxicity assays (MTT and NRU), and reported different sensitivity of the cell lines and assays used in detecting the cytotoxic responses of the surfactants.

In the final part of this study, the lyophilization process (freeze-drying) was used to evaluate the conversion of the nanocapsule suspensions in solid nanoformulations, aiming to obtain redispersible dried products in order to improve the stability of the developed liquid formulations.

Considering the physical-chemical characteristics of dry products, an important parameter to be evaluated is the particle size after lyophilization process. Particle sizes, after resuspension in water, can present significant changes. Therefore, it is important to evaluate this parameter in order to observe the maintenance of the mean particle sizes during the steps of the process (ABDELWAHED et. al., 2006; KUMAR et. al., 2014). In this work, after lyophilization, no changes was observed ($p>0.05$) in the mean particle sizes of resuspended nanoformulations in water (113-145 nm) if compared to the original nanocapsule suspensions (104-137 nm). Hence, the resuspension indexes were close to 1.0, which is considered adequate, indicating that there was no importante change in mean particle size during the lyophilization process, without aggregation of the nanostructures (CHACÓN e. al., 1999).

Scanning electron microscopy (SEM) was performed in order to evaluate both the morphology of the chrysin-loaded nanocapsules and the integrity of these particles after freeze-drying. For lyophilized nanocapsules, it can be observed the presence of colloidal spherical structures, which was not visible in the smooth surface of the sugar employed as cryoprotectant. The formation of an amorphous matrix of the cryoprotectant around the nanostructures is reported in the literature (LOKHANDE et. al., 2013).

Besides, chrysin content in lyophilized nanocapsules remained close to the theoretical value (about 98%) after preparation and during 50 days of storage, indicating no decline in the chrysin during the lyophilization process and the period of storage, regardless the oily component.

Due to the fact that nanocapsule suspensions are liquid systems, during the period of storage can occur physical-chemical instability as chemical degradation of the polymer, drug or other excipients. Thus, the lyophilization process removes the water, which increase the protection of these colloidal formulations against possible reactions of the components. Moreover, the lyophilization is a process conducted at low temperatures, which is an advantage for dehydratation of sensible substances (SCHAFFAZICK et. al., 2003a,b; ABDELWAHED et. al., 2006; KASPER et. al., 2013). In this context, the lyophilization process improved the stability of the chrysin-loaded ethylcellulose nanocapsules (Figure 7) in comparison with these systems in the liquid form (Figure 1E).

5 Conclusion

Chrysin-loaded nanocapsule suspensions with suitable physicochemical characteristics were obtained, employing coconut oil or medium chain triglycerides as oily core. Results showed a prolonged release of chrysin from both ethylcellulose nanocapsules without burst effect. Chrysin-loaded nanocapsule suspensions no showed cytotoxicity on non-tumoral cells (MTT and NRU assays) at the evaluated concentrations. Moreover, chrysin-loaded nanocapsule suspensions reduced *in vitro* cell viability of human breast cancer and melanoma, presenting differences in the effective concentration and sensitivity of the assays. Lyophilization was a good method to improve the stability of the nanocapsule suspensions containing chrysin, presenting also appropriated resuspension ability. Therefore, ethylcellulose nanocapsules can be an alternative platform for delivery of chrysin considering its potential as antiproliferative agent.

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Table 1 – Physicochemical characteristics of chrysin-loaded nanocapsule suspensions after preparation

Formulation	Mean diameter (nm)	PdI	Zeta Potential (mV)	pH	Chrysin content (mg/mL)	EE (%)
NC-CO-C	137 ± 2.08	0.12 ± 0.02	-29.3 ± 0.81	5.33 ± 0.21	0.74 ± 0.00	99.96
NC-MCT-C	104***** ± 1.56	0.14 ± 0.01	-31.0 ± 0.29	5.24 ± 0.19	0.73 ± 0.02	99.96

NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides
* $p < 0.05$. Significant difference in comparison with NC-CO-C

Table 2 - Calculated parameters by first-order and Korsmeyer-Peppas model for chrysin-loaded nanocapsules and free chrysin.

	NC-CO-C	NC-MCT-C	CMS
Monoexponencial			
r	0.9998 ± 0.0004	0.9998 ± 0.0004	0.9850 ± 0.0062
k (h^{-1})	0.0295 ± 0.0021	0.0231 ± 0.0015	0.2811 ± 0.0200
$t_{1/2}$ (h)	23.5 ± 1.67	30.0 ± 2.05	2.5 ± 0.17
Korsmeyer-Peppas Model			
r	0.9987 ± 0.0004	0.9984 ± 0.0007	-
a	0.0069 ± 0.0006	0.0063 ± 0.0007	-
n	0.6643 ± 0.0098	0.6689 ± 0.0138	-

NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides

Table 3 – Cytotoxicity of chrysin-loaded nanocapsule suspensions expressed as IC_{50} values in the cell lines MCF-7 and SK-MEL-28

Cell Line	Assay / Treatment Time	Formulation	IC_{50} (µg/mL)
MCF-7	MTT / 24 h	Chrysin	16.63[a] ± 2.89
		NC-CO-C	12.10[a] ± 0.29
		NC-MCT-C	10.23[a] ± 1.23
MCF-7	NRU / 24 h	Crisina	13.98[a] ± 0.00
		NC-CO-C	16.90[a] ± 0.00
		NC-MCT-C	16.86[a] ± 2.52
SK-MEL-28	MTT / 24 h	Chrysin	>10[b]
		NC-CO-C	25.14[a] ± 5.88
		NC-MCT-C	20.33[a] ± 0.67
SK-MEL-28	NRU / 24 h	Chrysin	24.06[a] ± 0.00
		NC-CO-C	9.83[*] ± 0.55
		NC-MCT-C	8.74[*] ± 0.58
SK-MEL-28	MTT / 48 h	Chrysin	>10[b]
		NC-CO-C	6.42[*] ± 0.10
		NC-MCT-C	5.71[*] ± 0.26
SK-MEL-28	NRU / 48 h	Chrysin	14.74[a] ± 0.91
		NC-CO-C	5.22[*] ± 0.42
		NC-MCT-C	2.96[*] ± 0.64

* $p < 0.05$ indicates significant difference in comparison with free chrysin;
[a] Estimated values; [b] Unable to estimate, because all viability values were near 100%.

Table 4 – Physicochemical characteristics of nanocapsules after freeze-drying process

Formulation	Mean diameter (nm)	Chrysin content (%)	Sf/Si
L-NC-CO-C	145 ± 18.45	98.66 ± 0.86	1.06 ± 0.15
L-NC-MCT-C	113 ± 6.43	97.48 ± 2.70	1.09 ± 0.06
Trehalose	628 ± 3.64	-	-

NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides
Sf/Si: ratio of chryisn-loaded nanocapsules after and before freeze-drying.

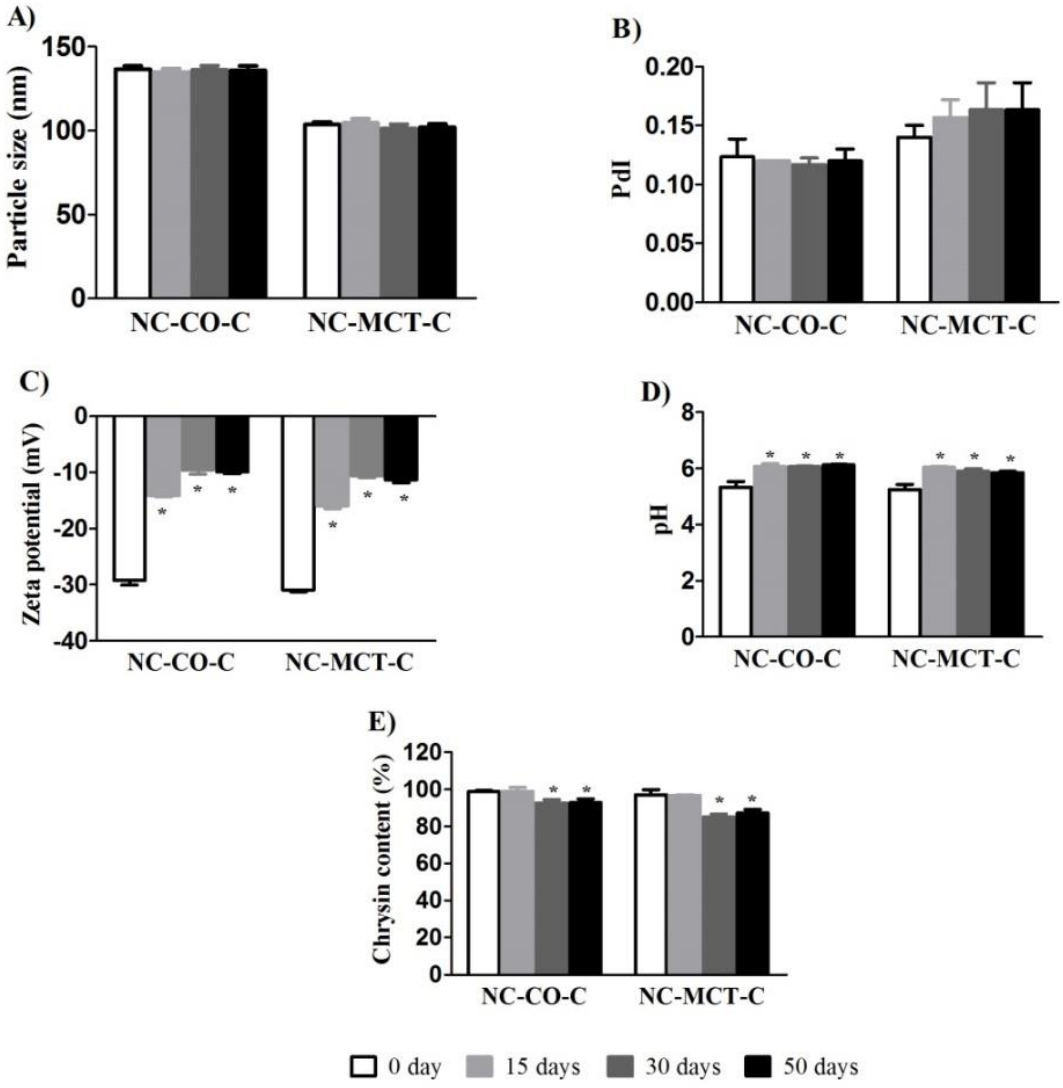

Figure 1 – Stability study of chrysin-loaded nanocapsule suspensions. A) Particle size (nm); B) PdI values; C) Zeta potential (mV); D) pH values; D) Chrysin content (%). (*) Significant difference compared to the initial time ($p < 0.05$).
NC-CO-C: chrysin-loaded nanocapsules containing coconut oil
NC-MCT-C: chrysin-loaded nanocapsules containing medium chain triglycerides

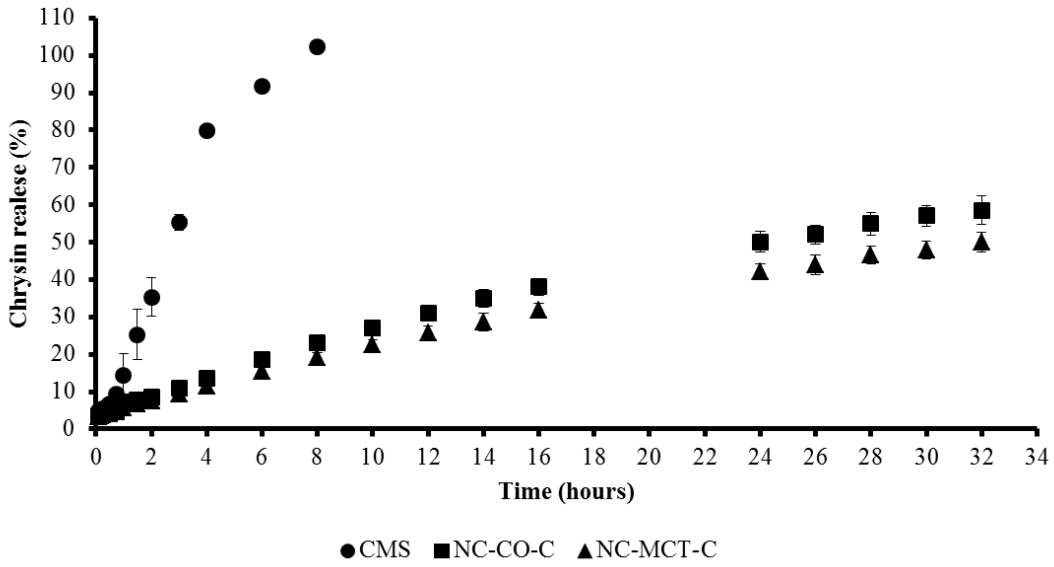

Figure 2 – *In vitro* chrysin release profile from nanocapsules (NCs) and chrysin methanolic solution. CMS: chrysin methanolic solutin; NC-CO-C: chrysin-loaded nanocapsules contataining coconut oil; NC-MCT-C: chrysin-loaded nanocapsules contatining medium chain triglycerides.

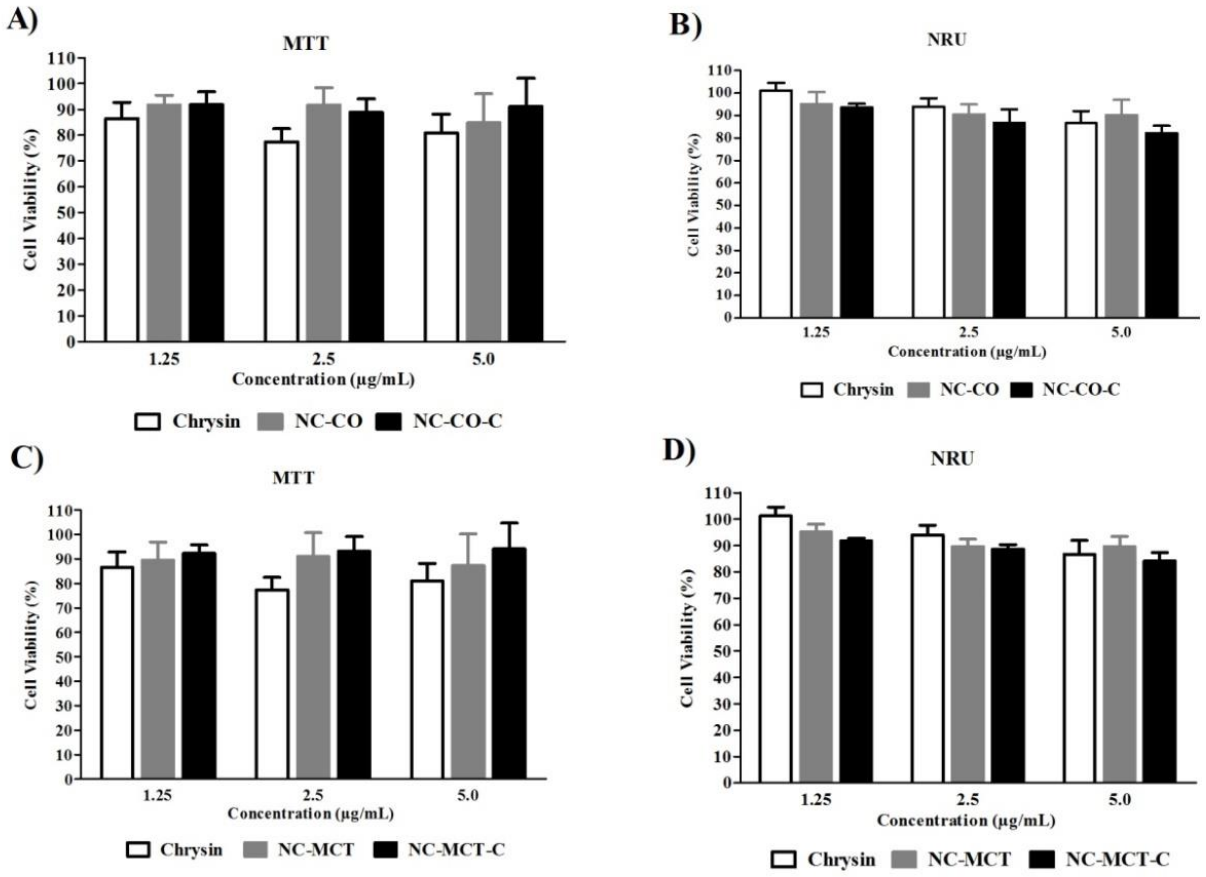

Figure 3 – *In vitro* cytotoxicity assay of nanocapsule suspensions with or without chrysin and free chrysin solution in 3T3 fibroblasts. Chrysin-loaded ethylcellulose nanocapsules prepared with coconut oil (NC-CO) (A and B) and with medium chain triglycerides (NC-MCT) (C and D).

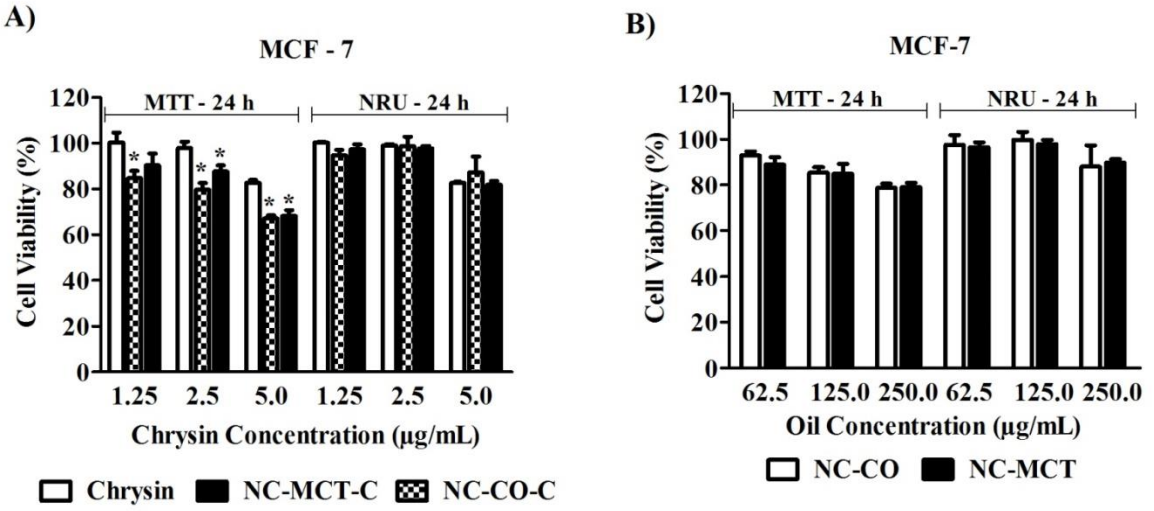

Figure 4 – *In vitro* cytotoxicity of free chrysin and nanocapsule suspensions with or without chrysin in MCF-7 breast cancer cell line after 24 h incubation. Chrysin ethylcellulose-loaded NCs prepared with coconut oil (NC-CO) or medium chain triglycerides (NC-MCT; A) and NCs without chrysin (B). (*) significant difference from free chrysin ($p < 0.05$).

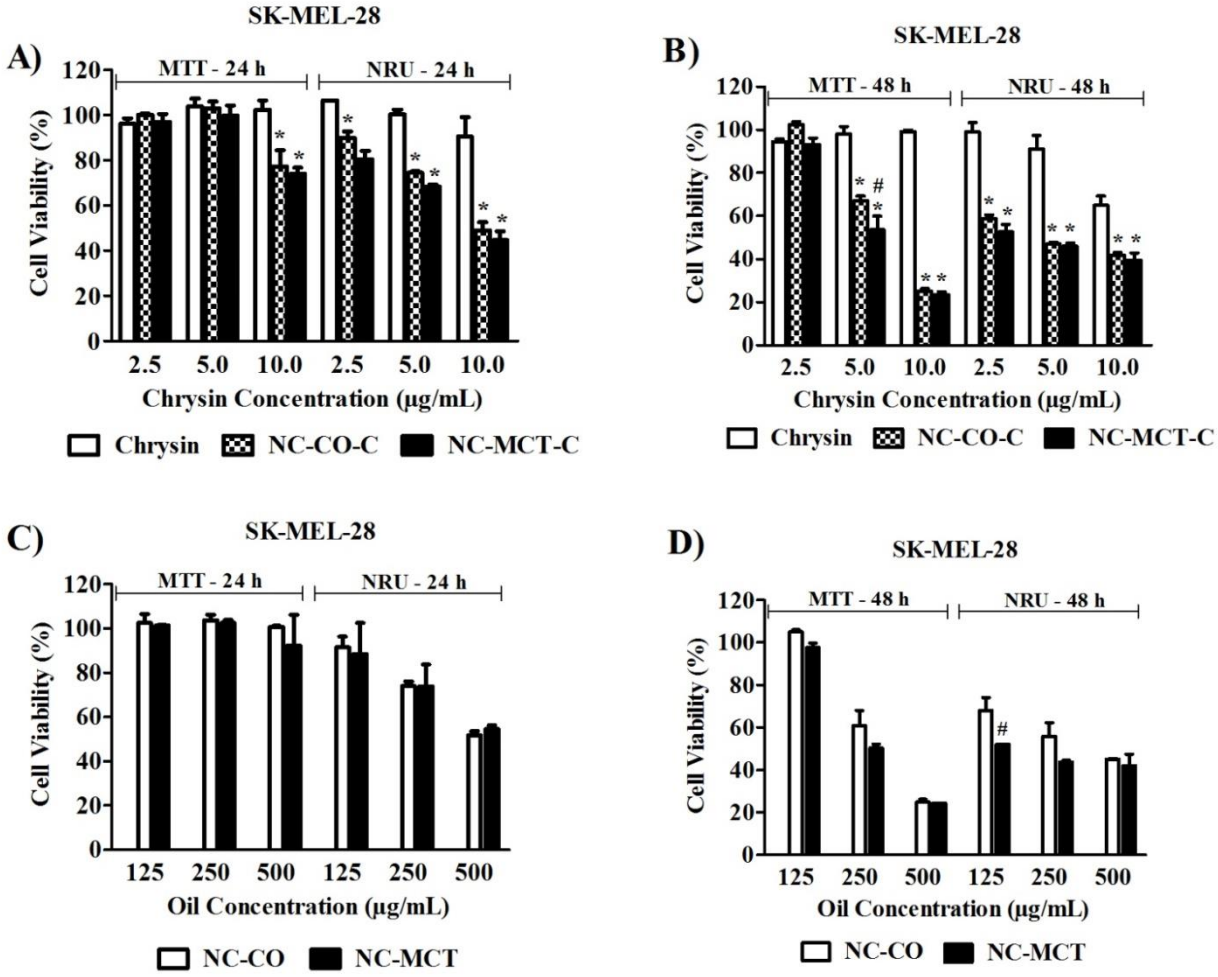

Figure 5 – *In vitro* cytotoxicity of free chrysin and NC suspensions with or without chrysin in SK-MEL-28 melanoma cell line after 24 or 48 h incubation. Chrysin ethylcellulose-loaded NCs prepared with coconut oil (NC-CO) or medium chain triglycerides (NC-MCT) at 24 h (A); NC-CO or NC-MCT at 48 h (B); NCs without chrysin at 24 h (C); NCs without chrysin at 48 h (D). (*) Significant difference from free chrysin ($p < 0.05$) and (#) significant difference from NC-CO formulation ($p < 0.05$).

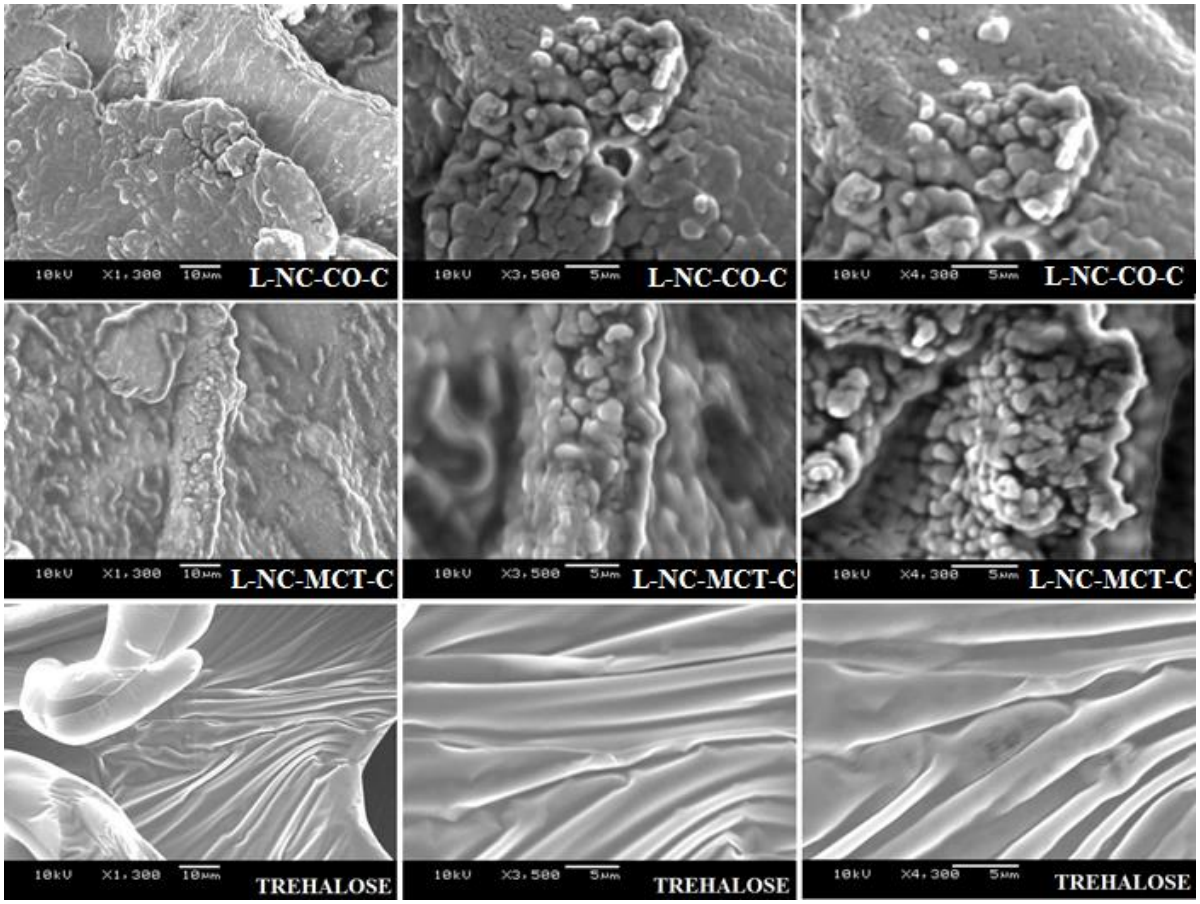

Figure 6 – Morphological analysis of freeze-dried nanocapsules (1,300X – bar 10 μm; 3,500 X – bar 5 μm bar; 4,300X – bar 5μm) compared to the freeze-dried trehalose. NC-CO-C: chrysin-loaded nanocapsule containing coconut oil; NC-MCT-C: chrysin-loaded nanocapsule containing medium chain triglycerides.

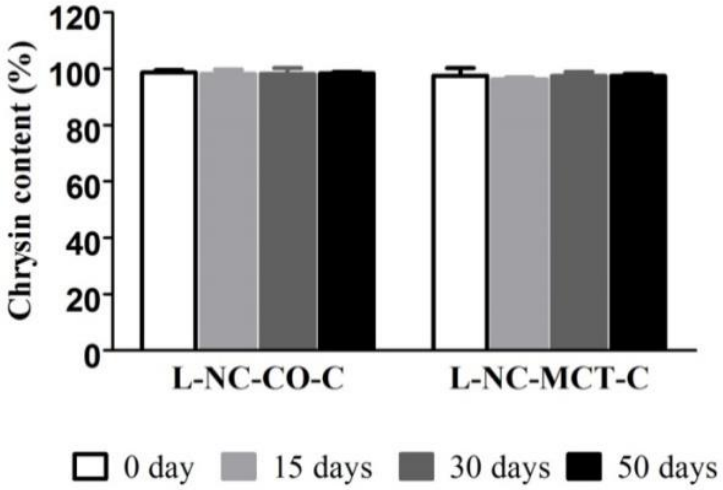

Figure 7 – Chrysin content in lyophilized nanocapsules during 50 days. L-NC-OC-C: lyophilized chrysin-loaded nanocapsule containing coconut oil; L-NC-MCT-C: lyophilized chrysin-loaded nanocapsule containing medium chain triglycerides.

DISCUSSÃO GERAL

A nanotecnologia e a nanociência, nos últimos anos, têm atraído de forma crescente a atenção de pesquisadores e de indústrias farmacêuticas. Os sistemas nanoestruturados têm sido explorados na liberação controlada de substâncias ativas e/ou na vetorização destas a sítios específicos de ação. Além disso, esses carreadores são capazes de aumentar a biodisponibilidade de fármacos no organismo, melhorar a sua eficácia terapêutica, reduzir possíveis efeitos adversos, além de promover uma maior permeação do fármaco pelas barreiras fisiológicas, como a barreira sangue-cérebro e a barreira do trato gastrointestinal (SCHAFFAZICK, 2003; MEI et. al., 2013). Frente ao exposto, os sistemas nanoestruturados têm sido estudados para a prevenção e tratamento de diferentes doenças como tumorais e neurodegenerativas (MATTHEOLABAKIS et. al., 2012; MEI et. al., 2013).

Estas estruturas coloidais podem ser administradas através de diferentes vias como oral, cutânea, ocular, pulmonar, parenteral e, inclusive, intravenosa (sem risco de embolia) (SCHAFFAZICK, 2003; WU et. al., 2011). Várias destas têm sido desenvolvidas, diferindo quanto à composição e método de preparação, incluindo as nanopartículas poliméricas (SCHAFFAZICK, 2003; MARCATO, DÚRAN, 2008). Dentre as últimas, estão inseridas as nanocápsulas, que geralmente apresentam uma estrutura polimérica ao redor de um óleo oleoso (COUVREUR et. al., 2002; MOHANRAJ; CHEN, 2006; MORA-HUERTAS, 2010).

As nanocápsulas são capazer de aumentar a biodisponibilidade do fármaco no organismo, promover sua vetorização e liberação sustentada, reduzindo a frequência de doses administradas, além de proteger o fármaco contra a degradação enzimática, imunológica e química (MORA-HUERTAS et. al., 2010; KAUR et. al., 2014). Estes sistemas têm sido explorados para aplicação cutânea, sendo que, em função da elevada área de superfície, são muito interessantes para promover aplicação de substâncias lipofílicas e homogênea liberação destas, podendo modificar a penetração e a atividade, além de aumentar a adesividade ou o tempo de permanência de substâncias ativas na pele (GUTERRES; ALVES; POHLMANN, 2007).

A crisina foi selecionada como substância ativa neste trabalho em função de ser um composto natural polifenólico, pouco solúvel, e com inúmeras potencialidades terapêuticas, devido às suas propriedades como antioxidante, anti-inflamatória e anticancerígena (KASALA et al., 2015). Além disto, quando este trabalho foi iniciado, não havia sido ainda explorada a nanotecnologia para a veiculação desta molécula, sobretudo em nanocápsulas.

Primeiramente, o objetivo deste estudo foi desenvolver nanocápsulas contendo crisina, utilizando o óleo de amendoim ou triglicerídeos de cadeia média (TCM) como núcleo oleoso das nanocápsulas. Como polímero, foi utilizado poli($\mathcal{E}$-caprolactona) (PCL) e as concentração de 0,5 e 1,0 mg/mL de crisina. No entanto, após a preparação, ocorreu a precipitação das suspensões de nanocápsulas com ambos os óleos utilizados, indicando instabilidade do sistema. Frente a isso, nanocápsulas foram preparadas utilizando a etilcelulose como material polimérico e os óleos de coco, amendoim ou TCM, como componentes do núcleo, contendo crisina nas concentrações de 0,3 a 1,0 mg/mL. Após o preparo, foi observada precipitação de todas as formulações preparadas na concentração de 1,0 mg/mL. Após cerca de 6 dias, as formulações contendo 0,5 mg/mL de crisina apresentaram também precipitados. Para as formulações com 0,3 mg/mL de crisina, não foram observados precipitados e o teor manteve-se adequado, em 30 dias.

A fim de tentar aumentar a concentração de crisina nas nanocápsulas, foi testado concentrar as formulações preparadas na concentração de 0,3 mg/mL (2,5 vezes) até a concentração de 0,75 mg/mL de crisina. Após o preparo, somente a formulação que continha óleo de amendoim apresentou precipitados logo após o preparo. Foi possível concentrar as formulações contendo óleo de coco ou TCM na concentração de 0,75 mg/mL, onde as características físico-químicas foram adequadas.

Portanto, os óleos de coco, amendoim ou TCM foram utilizados como núcleos oleosos e a etilcelulose como polímero, na primeira parte do trabalho (0,3 mg/mL de crisina). Na segunda etapa, formulações de nanocápsulas mais concentradas, constituídas de óleo de coco ou TCM como componente oleoso e a etilcelulose como polímero, foram estudadas (0,75 mg/mL de crisina).

Previamente à determinação do teor de crisina nas nanocápsulas, em triplicata de lote, o método analítico foi validado, o qual foi baseado na metodologia descrita por DMITRIENKO e colaboradores (2012), constituído por cromatografia a líquido de alta eficiência (CLAE-UV). Para a validação do método analítico, os seguintes parâmetros foram avaliados: especificidade, linearidade, precisão e exatidão. A suspensão de nanocápsulas escolhida foi a preparada com óleo de coco, selecionada como matriz mais complexa, por ser um óleo vegetal, rico em vários componentes e devido ao seu estado físico.

Para a especificidade, os cromatogramas de todas as formulações contendo crisina foram comparados com os respectivos cromatogramas das nanocápsulas sem este flavonoide,

não havendo interferência dos excipientes no tempo de retenção da crisina, indicando que o método é específico. A linearidade é outro parâmetro essencial a ser avaliado, pois determina a capacidade do método em gerar resultados proporcionais à concentração da substância ativa em estudo (ANVISA, 2003). O método foi linear, pois apresentou um coeficiente de correlação (r) de 0,9998 e regressão linear significativa ($p<0,05$, ANOVA).

A precisão do método analítico avalia a proximidade dos dados mediante uma série de análises a partir de uma mesma amostra (ANVISA, 2003). Neste parâmetro, foi avaliada a repetibilidade (precisão intra-dia) e a precião intermediária (precisão inter-dia). Com relação à exatidão, esse parâmetro analisa a proximidade dos dados obtidos com o valor verdadeiro/conhecido (ANVISA, 2003). A exatidão do método foi avaliada através do teste de recuperação do analito. O método foi considerado preciso (desvio padrão relativo < 1,22 %), segundo o recomendado, e com adequada exatidão (cerca de 106 %) (ANVISA, 2003).

Todas as formulações preparadas apresentaram características físico-químicas adequadas, onde o tamanho médio de partículas permaneceu na faixa nanométrica (104 a 161 nm) e com baixos índices de polidispersão (0.09-0.14), indicando a homogeneidade do sistema, apresentando um pico de população, através de espalhamento de luz dinâmico.

O potencial zeta foi negativo, devido à natureza química do polímero (CHASSOT et al., 2014). O pH mostrou-se ácido para todas as suspensões (5,12-6,00), que é adequado considerando o uso cutâneo. Após a preparação, o teor de crisina foi adequado (97 a 100%), próximo ao valor teórico (0,3 ou 0,75 mg/mL).

Com relação à estabilidade das suspensões de nanocápsulas, as formulações contendo 0,3 mg/mL de crisina foram estáveis durante os 30 dias de estudo (diâmetro médio, PdI, potencial zeta e pH), havendo uma leve redução no teor em comparação ao tempo inicial ($p<0,05$), porém o teor permaneceu acima de 90%.

Por sua vez, as suspensões contendo 0,75 mg/mL de crisina, durante 50 dias de armazenamento, apresentaram-se estáveis. No entanto, as nanocápsulas contendo óleo de coco apresentaram teor acima de 92% após 50 dias, enquanto que, para as nanocápsulas contendo TCM, o teor reduziu a 87% após o mesmo período. Em hipótese, este fato pode estar relacionado à presença de substâncias antioxidantes no óleo de coco, protegendo a crisina. Além disto, cabe evidenciar que as variações nos valores de potencial zeta não causaram aumento do diâmetro médio e do PdI em função do tempo, para as formulações mais concentradas.

A liberação da crisina *in vitro* foi estudada através da técnica de difusão em sacos de diálise a 37ºC. A fim de padronizar o meio de liberação foram testados os seguintes meios: tampão fosfato pH 5,6; tampão fosfato pH 5,6:etanol (70:30 v/v); tampão acetato pH 5,0; tampão acetato pH 5,0:etanol (70:30 v/v) e água:etanol (70:30 v/v). Em relação aos meios testados, não houve quantificação da crisina nos meios sem etanol (tampão fosfato pH 5,6 e tampão acetato pH 5,0), quando analisados por CLAE. Em relação aos outros meios, não houve influência no pico correspondente ao flavonoide, quando verificado por cromatografia a líquido. Tendo em vista a aplicação tópica das nanocápsulas, considerando o pH ácido da pele (4,5 a 5,5) e os dados preliminares de doseamento da solubilidade de crisina nos meios, importantes para a manutenção da condição *sink*, o meio escolhido para o estudo de liberação *in vitro* foi o tampão acetato pH 5,0:etanol (70:30). Cabe ressaltar que SAPINO e colaboradores (2015) empregaram o meio de liberação baseado em tampão acetato pH 5,0:etanol (80:20) para avaliar o perfil de liberação de quercetina a partir de nanopartículas, visando à aplicação tópica.

Através do presente estudo, foi observada uma liberação controlada de crisina a partir de todas as suspensões de nanocápsulas desenvolvidas, em comparação com a solução metanólica de crisina (0,3 mg/mL e 0,75 mg/mL). As suspensões de nanocápsulas preparadas com menor concentração de flavonoide liberaram aproximadamente 89% de crisina após 30 horas de experimento, em comparação com crisina livre, que completou sua difusão em 12 horas (99%). As formulações com maior concentração de crisina liberaram menor percentual de flavonoide (50-58%, em 32 h) em relação às suspensões de 0,3 mg/mL. Já a crisina livre completou sua difusão (102%) após 8 horas de experimento. Todas as suspensões de nanocápsulas desenvolvidas (0,3 e 0,75 mg/mL) apresentaram cinética de liberação de primeira ordem, através do modelo monoexponencial, o que indica que a liberação da crisina ocorreu em uma única etapa (ausência do efeito *burst*) e dependeu somente da concentração do flavonoide, sugerindo que a crisina ficou confinada no interior do núcleo oleoso. O tipo de óleo não influenciou a liberação de crisina a partir das formulações com menor concentração do flavonoide, apresentando constantes cinéticas próximas (0,060-0,076 h^{-1}; $t_{1/2}$ entre 9 e 11,5 h). Considerando as formulações mais concentradas, a meia-vida foi significativamente mais elevada para as formulações preparadas com TCM ($t_{1/2}$ 30 h ; *k* de 0,023 h^{-1}) em relação àquelas preparadas com óleo de coco ($t_{1/2}$ 23,5 h ; *k* de 0,0295 h^{-1}). Já a difusão da crisina não encapsulada resultou em meias-vidas próximas (2,5-3,0 h^{-1}) para as duas concentrações estudadas.

Verificou-se, através dos valores de *k*, que a liberação da crisina foi significativamente mais lenta a partir das formulações com maior concentração deste flavonoide em relação às suspensões preparadas com 0,30 mg/mL ($p<0,05$), nas mesmas condições experimentais. Apesar de, geralmente, o maior gradiente de concentração favorecer a liberação, as formulações contendo 0,75 mg/mL de crisina apresentam também maior concentração de óleo e de polímero (2,5 vezes mais), o que pode ter levado a esta difusão mais lenta a partir do sistema, apesar da concentração de crisina também estar maior.

Foi inferido o mecanismo de liberação da crisina a partir das nanocápsulas de etilcelulose, através da Lei da Potência (modelo de Korsmeyer-Peppas), onde foi observado que todas as formulações apresentaram mecanismo de liberação do tipo transporte anômolo, sugerindo que ocorre a liberação do flavonoide através do relaxamento das cadeias poliméricas da etilcelulose, seguido pela sua difusão. Desta forma, o mecanismo foi o mesmo independente do tipo de óleo e da concentração de crisina.

Devido ao tamanho submicrométrico das nanocápsulas, ocorre um aumento da área superficial das partículas em relação ao volume, o que pode resultar em um aumento da atividade da substância ativa encapsulada, tanto *in vitro* quanto *in vivo* (WU et. al., 2011). Neste sentido, avaliou-se a atividade antioxidante *in vitro* da crisina nanoencapsulada em comparação com a sua forma livre. Assim, neste estudo, foi utilizado o radical 1’1-difenil-2-picrilhidrazil (DPPH) como ensaio simples e rápido. Esse estudo foi primeiramente desenvolvido por Blois (1958) e modificado por diversos autores, cujo princípio consiste na presença de um agente antioxidante doador de hidrogênio em meio geralmente alcoólico, no qual o DPPH é reduzido à difenil picrilhidrazina (KOLEVA et. al., 2002). O grau de redução é acompanhado pela mudança da coloração de violeta para amarela e medido espectrofotometricamente.

A atividade antioxidante, realizada com as nanocápsulas contendo de 0,3 mg/mL de crisina, foi efetuada também com as nanocápsulas sem crisina (óleo de coco, amendoim e TCM), solução etanólica dos óleos (mesma concentração em que se encontram nas suspensões) e com solução etanólica de crisina (0,3 mg/mL). Conforme os resultados, foi observado aumento da atividade antioxidante tanto da crisina quando dos óleos vegetais quando presentes na formulação nanoestruturada ($p<0,05$). Neste sentido, a atividade antioxidante dependeu da concentração de crisina e do tipo de óleo, sendo que em concentrações acima de 0.025 mg/mL as suspensões de nanocápsulas foram mais efetivas do

que a crisina livre em neutralizar o radical DPPH. A ordem de efetividade das formulações foi óleo de amendoim, seguido do de coco e, por fim, o óleo sintético (TCM), o que pode ser atribuído à presença de substâncias antioxidante nos óleos vegetais (MARINA et. al., 2009; CARRÍN; CARELLI, 2010). Pode-se dizer que houve um efeito sinérgico entre a crisina e as formulações preparadas com os óleos vegetais (coco e amendoim) devido ao maior efeito antioxidante em comparação com as formulações preparadas com TCM. Aumentos semelhantes na capacidade de sequestrar o radical DPPH também foram obtidos para as nanocápsulas preparadas com os óleos vegetais e sem crisina, evidenciando a contribuição dos óleos na atividade antioxidante. As nanoesferas (sem óleo e sem crisina) e as nanocápsulas com TCM (sem crisina) apresentaram baixa atividade antioxidante pelo método avaliado.

É importante destacar que KUMAR e colaboradores (2015) desenvolveram nanoesferas de quitosana contendo ou não ao flavonoide naringenina e avaliaram a atividade antioxidante pelo mesmo ensaio, obtendo aumento da atividade antioxidante da naringenina quando associada às nanoestruturas, corroborando com os resultados encontrados neste estudo. Em outro estudo, óleo de semente de romã apresentou melhores propriedades antioxidantes quando em escala coloidal compondo carreadores lipídicos nanoestruturados (NICULAE et al 2014).

Devido ao grande potencial que os sistemas nanoestruturados podem agregar à terapêutica, é importante avaliar o potencial toxicológico desses sistemas frente aos seres humanos e ao meio ambiente. Neste sentido, testes de citotoxicidade *in vitro* vêm sendo realizados. Esses testes avaliam a viabilidade celular, a qual mensura o estado real das células cultivadas *in vitro* (KROLL et. al., 2009).

Em vista disso, dois ensaios de citotoxicidade *in vitro* foram usados, o ensaio do MTT e do Vermelho Neutro (Neutral Red Uptake – NRU). O ensaio do MTT foi desenvolvido primeiramento por MOSSMANN (1983), o qual avalia o metabolismo e a atividade mitocondrial celular através da redução do MTT em um formazano insolúvel em células viáveis (KROLL et. al., 2009; MONTEIRO-RIVIERE et. al., 2009). Por sua vez, o ensaio do vermelho neutro foi desenvolvido por BORENFREUND e PUERNER (1985), o qual avalia a integridade da membrana e a atividade lisossomal das células, sendo que o corante se acumula nos lisossomas de células viáveis (KROLL et. al., 2009; MONTEIRO-RIVIERE et. al., 2009).

As suspensões de nanocápsulas contendo crisina foram avaliadas em diferentes linhagens celulares, tumorais (câncer de mama MCF-7 e melanoma SK-MEL-28) e não tumorais (fibroblastos 3T3).

Em relação à linhagem celular normal (fibroblastos 3T3), as suspensões de etilcelulose apresentaram baixa citotoxicidade, comparável à crisina livre, tanto empregando o ensio do MTT (0,3 mg/mL e 0,75 mg/mL) quanto o NRU (0,75 mg/mL), independente do óleo empregado. CHASSOT e colaboradores (2014) também avaliaram a citotoxicidade de nanocápsulas de etilcelulose, contendo diproprionato de beclometasona, em fibroblastos, e observaram baixa citotoxicidade pelo ensaio do MTT.

Quanto às linhagens tumorais humanas (MCF-7 e SK-MEL-28), apenas as formulações preparadas com 0,75 mg/mL de crisina foram avaliadas.

Considerando as células MCF-7, após 24 h de incubação, as suspensões de nanocápsulas contendo crisina reduziram a viabilidade celular nas concentrações estudadas (1,25-5,0 µg/mL), segundo o ensaio MTT, em comparação com a crisina livre. Entretanto, com o NRU, não houve redução significativa em todas as concentrações avaliadas para ambas as formulações. As suspensões sem crisina mostraram menor efeito citotóxico, principalmente pelo ensaio NRU. Assim, houve maior sensibilidade do método MTT em detectar os efeitos tóxicos do sistema, em relação ao NRU, sugerindo que as suspensões de nanocápsulas contendo crisina, primariamente interferem nos compartimentos mitocondriais e metabolismo celular, e, em um segundo momento, afetam a atividade lisossomal e integridade de membrana.

No ensaio com as células SK-ME L-28, após 24 h de incubação, o ensaio MTT mostrou redução na viabilidade celular somente na maior concentração (10 µg/mL), para todas as formulações em comparação com a crisina livre. Já pelo NRU, em todas as concentrações observou-se significativa redução na viabilidade celular considerando a formulação com óleo de coco (2,5-10 µg/mL) e a partir de 5,0 µg/mL para aquelas preparadas com TCM. As nanocápsulas sem crisina apresentaram negligenciável redução na viabilidade celular pelo ensaio MTT, entretanto, pelo NRU, pronunciados efeitos tóxicos foram observados nas concentrações de óleo de 250 e 500 µg/mL (correspondente às concentrações de 5,0 e 10 µg/mL de crisina, respectivamente).

Após 48 h de incubação, ambas as nanocápsulas contendo crisina provocaram maior redução na viabilidade em comparação ao flavonoide livre, pelo método do MTT (5,0 and 10

µg/mL) e NRU (2,5-10 µg/mL). As nanocápsulas sem crisina também reduziram a viabilidade celular em ambos os métodos (250 and 500 µg/mL para MTT e 125-500 µg/mL para NRU).

Para as células de melanoma, o ensaio do NRU foi mais sensível do que o MTT, diferentemente das células de câncer de mama, o que pode sugerir que primeiro ocorre dano à integridade das membranas/atividade lisossomal e depois ocorre alteração na atividade metabólica e mitocondrial (KROLL et. al., 2009; MONTEIRO-RIVIERE et. al., 2009).

Os efeitos tóxicos detectados para as formulações sem crisina, para ambas as linhagens tumorais, podem ser atribuídos aos excipientes e suas concentrações. O tensoativo polissorbato 80, empregado neste trabalho, já foi reportado como sendo tóxico em carcinoma colorretal humano (Caco-2; HODAEI et. al., 2014). Na literatura, são encontrados estudos em que comparam diferentes linhagens celulares com diversos ensaios de citotoxidade *in vitro* avaliando exicipientes de formulações ou sistemas nanoestruturados, sendo relatadas variadas respostas toxicológicas em distintos ensaios e linhagens celulares (NOGUEIRA et. al., 2011; NOGUEIRA et. al., 2013).

Devido ao fato das suspensões de nanocápsulas apresentarem-se como líquidos, é mais fácil ocorrer instabilidade físico-química da formulação. Os fatores mais associados com a instabilidade destes sistemas envolvem a agregação e a sedimentação de partículas, além da degradação de componentes. Frente a isso, é necessário avaliar a estabilidade das formulações desenvolvidas (BRASIL, 2005; WU et. al., 2011).

A fim de manter o teor de crisina das nanocápsulas próximo ao teórico, durante um determinado período de armazenamento, minimizando os possíveis problemas de instabilidade, o processo de liofilização foi utilizado, como etapa final do trabalho. A liofilização (*freeze-drying*) consiste em um processo de sublimação, ou seja, remoção de água a partir de uma amostra congelada (ABDELWAHED et. al.. 2006a; KASPER et. al., 2013). As suspensões de nanocápsulas (0,75 mg/mL) foram liofilizadas e apresentaram adequados índices de ressuspensão, próximos a unidade (1,06-1,09), indicando a manutenção do diâmetro das suspensões de origem (CHACÓN et. al., 1999; ABDELWAHED et. al., 2006; KUMAR et. al., 2014). Através de microscopia eletrônica de varredura (MEV) foi possível observar a presença de estruturas coloidais na matriz formada pelo crioprotetor trealose, o qual apresentou, após a liofilização, superfície lisa.

No que diz respeito à estabilidade dos liofilizados obtidos a partir das suspensões de nanocápsulas, durante os 50 dias do estudo, o teor de crisina manteve-se próximo ao valor

teórico (97-99%) tanto para as nanocápsulas contendo óleo de coco quanto aquelas com TCM, não apresentando diferença significativa ($p>0,05$). Portanto, o processo de liofilização provocou melhoria na estabilidade das nanocápsulas de etilcelulose em relação aos sistemas líquidos, podendo ser interessantes intermediários para o uso futuro de crisina encapsulada.

Por fim, as nanocápsulas poliméricas desenvolvidas neste trabalho apresentaram-se como importantes estratégias para a veiculação de crisina, considerando suas inúmeras potencialidades terapêuticas.

CONCLUSÕES

Como conclusões, foi possível a preparação de nanocápsulas de etilcelulose contendo crisina, com diferentes núcleos oleosos (amendoim, coco ou triglicerídeos de cadeia média), em duas concentrações do flavonoide (0,3 e 0,75 mg/mL), através de deposição interfacial de polímero pré-formado. As suspensões de nanocápsulas apresentaram características físico-químicas adequadas, elevadas eficiências de encapsulamento e foram estáveis quanto ao tamanho médio e polidispersão, por 30 ou 50 dias. Entretanto, apresentaram tendência à redução do teor de crisina após 30 dias de armazenamento. Este fato foi resolvido pelo emprego da liofilização, que se apresentou como uma alternativa viável para preparar crisina nanoencapsulada em formulação sólida e estável, apresentando boas características de ressuspensão. Os produtos liofilizados, a partir das suspensões de nanocápsulas, não alteraram significativamente o tamanho médio de partículas durante a liofilização e após a ressuspensão em água, sem influência do tipo de óleo. A presença de partículas esféricas e coloidas na superfície das amostras pôde ser verificada por microscopia eletrônica.

As nanocápsulas desenvolvidas foram capazes de controlar a liberação de crisina, sem liberação rápida inicial da mesma (sem efeito *burst*), seguindo a cinética de primeira ordem. Foi observado um aumento da atividade antioxidante da crisina quando associada às nanocápsulas, em comparação com a crisina livre, sugerindo um possível sinergismo entre este flavonoide e os óleos vegetais (amendoim e coco). A formulação desenvolvida com o óleo de amendoim e crisina apresentou maior efeito antioxidante em relação às demais.

Foi observada ausência de toxicidade para as nanocápsulas contendo ou não crisina frente às células normais estudadas (fibroblastos), em todas as concentrações avaliadas. Além disso, redução da viabilidade celular de linhagens tumorais humanas (mama e melanoma) foi evidenciada quando tratadas com nanocápsulas contendo crisina. Diferentes mecanismos de toxicidade foram apontados, segundo os dois ensaios realizados (MTT e NRU). As nanocápsulas de etilcelulose sem crisina também apresentaram toxicidade dose-dependente e seletiva para as células tumorais, segundo as condições e concentrações do estudo.

Frente ao exposto, as nanocápsulas de etilcelulose contendo crisina, um polifenol pouco solúvel, são sistemas promissores para futuros tratamentos associados a sua atividade antioxidante e antiproliferativa.

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