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

FORMULAÇÕES NANOESTRUTURADAS CONTENDO RUTINA: DESENVOLVIMENTO, ATIVIDADE ANTIOXIDANTE *IN VITRO* E EFEITO SOBRE A CICATRIZAÇÃO CUTÂNEA

DISSERTAÇÃO DE MESTRADO

Juliana Severo de Almeida

Santa Maria, RS, Brasil
2010

FORMULAÇÕES NANOESTRUTURADAS CONTENDO RUTINA: DESENVOLVIMENTO, ATIVIDADE ANTIOXIDANTE *IN VITRO* E EFEITO SOBRE A CICATRIZAÇÃO CUTÂNEA

por

Juliana Severo de Almeida

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**

Orientador: Prof. Dr. Ruy Carlos Ruver Beck

Santa Maria, RS, Brasil
2010

Universidade Federal de Santa Maria
Centro de Ciências da Saúde
Programa de Pós-Graduação em Ciências Farmacêuticas

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

FORMULAÇÕES NANOESTRUTURADAS CONTENDO RUTINA: DESENVOLVIMENTO, ATIVIDADE ANTIOXIDANTE *IN VITRO* E EFEITO SOBRE A CICATRIZAÇÃO CUTÂNEA

elaborada por
Juliana Severo de Almeida

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

Comissão Examinadora:

Prof. Dr. Ruy Carlos Ruver Beck
(Orientador)

Profª Dra. Marta Palma Alves
(UNIFRA)

Profª. Dra. Margareth Linde Athayde
(UFSM)

Santa Maria, 16 de agosto de 2010.

Ao meu pai e irmãos pelo amor incondicional em todos os momentos da minha vida e o constante incentivo para que eu chegasse até aqui.

AGRADECIMENTOS

À Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Ciências Farmacêuticas pelas oportunidades oferecidas.

Ao Departamento de Farmácia Industrial pela estrutura física oferecida.

Ao Prof. Dr. Ruy Carlos Ruver Beck, pela orientação, dedicação, amizade, incentivo e exemplo profissional demonstrados no decorrer deste trabalho.

Aos professores e funcionários do Departamento de Farmácia Industrial pela amizade, apoio e incentivo.

Ao Corpo Docente do PPGCF, pelos conhecimentos transmitidos.

À Profª. Drª. Sílvia S. Guterres e Profª. Drª Adriana R. Pohlmann da UFRGS pelas contribuições científicas.

Ao Prof. Dr. Luiz Otávio Bulhões pelas análises de tamanho de partícula e potencial zeta.

Ao Prof. Dr. Leandro Machado de Carvalho pelo fornecimento de amostras para o estudo e pela oportunidade de executar o experimento de atividade anti-oxidante in vitro em seu laboratório.

À Profª. Dra. Marilise Escobar Bürger pelo auxílio e atenção prestados nos trabalhos desenvolvidos no Laboratório de Farmacologia.

Aos colegas de laboratório de Tecnologia Farmacêutica pelos bons momentos de convivência, companheirismo e auxílio prestados. Em especial a Aline, Karine, Luana, Lucas, Márcia, Marila e Roberta. E também às colegas do Laboratório de Farmacologia Dalila, Raquel e Nardeli e Fernanda do Laboratório de Química.

Ao meu amor, Laziê pelo apoio e incentivo para que eu seguisse me especializando cada vez mais.

À minha amada mãe, Vilma pelo exemplo de força e carinho e por estar sempre em pensamento e oração.

À Rede de Nanocosméticos CNPq/MCT pelo apoio financeiro.

À CAPES, órgão financiador da bolsa de estudos.

E a todos aqueles que, de alguma forma, contribuíram para a realização deste trabalho.

RESUMO

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

FORMULAÇÕES NANOESTRUTURADAS CONTENDO RUTINA: DESENVOLVIMENTO, ATIVIDADE ANTIOXIDANTE *IN VITRO* E EFEITO SOBRE A CICATRIZAÇÃO CUTÂNEA

AUTORA: Juliana Severo de Almeida
ORIENTADOR: Ruy Carlos Ruver Beck
Data e Local da Defesa: Santa Maria, 16 de agosto de 2010.

Este trabalho teve como principal objetivo o desenvolvimento de formulações nanoestruturadas contendo rutina, a partir de nanopartículas contendo óleos vegetais até então não estudados no preparo destas formulações. No primeiro capítulo foi demonstrada a viabilidade de preparar nanocápsulas e nanoemulsões contendo o óleo de semente de uva e de amêndoas doce como fase oleosa. As suspensões de nanocápsulas e nanoemulsões foram preparadas pelo método da deposição interfacial do polímero pré-formado e emulsificação espontânea, respectivamente. Todas as formulações apresentaram tamanho médio nanométrico, índice de polidispersão inferior a 0,30, potencial zeta negativo e valores de pH entre 6,5 e 7,5, permanecendo estáveis após 6 meses de armazenamento. As formulações promoveram a fotoproteção do ativo frente à degradação UV, independente do tipo de fase oleosa e do tipo de vesícula. No segundo capítulo, as formulações contendo o óleo de semente de uva foram selecionadas para o estudo do desenvolvimento de nanopartículas contendo rutina, bem como avaliação de sua atividade antioxidante *in vitro* e fotoestabilidade. Após o preparo, todas as formulações apresentaram tamanho nanométrico de partículas, baixo índice de polidispersão, valores de pH ácido, potencial zeta negativo e eficiência de encapsulação próxima à 100%. As nanopartículas protegeram a rutina frente à fotodegradação UV, quando comparadas à solução etanólica. No estudo da atividade antioxidante *in vitro*, as nanocápsulas e nanoemulsões apresentaram uma menor taxa de decaimento da rutina comparada com a solução etanólica, quando expostas à radiação UV em presença do radical ·OH. No entanto, a presença das nanocápsulas levou a uma atividade antioxidante *in vitro* mais prolongada comparada com as nanoemulsões contendo rutina. Finalmente, no terceiro capítulo estudamos o desenvolvimento de hidrogéis contendo rutina livre ou associada a nanocápsulas poliméricas, avaliando a sua atividade sobre a cicatrização de lesões cutâneas em ratos. As formulações desenvolvidas apresentaram propriedades adequadas para aplicação tópica. A resposta *in vivo* do efeito cicatrizante foi avaliada através da regressão de lesões na pele após 6 dias de tratamento. Marcadores do estresse oxidativo nas lesões dos ratos foram também avaliados, como os níveis da peroxidação lipídica através do método de TBARS, níveis de proteína carbonilada, níveis de proteínas totais, níveis de glutationa (GSH), vitamina C e avaliação da enzima antioxidante catalase (CAT). Este terceiro capítulo demonstrou pela primeira vez a potencialidade do emprego dermatológico de hidrogéis contendo rutina para a promoção de cicatrização de feridas *in vivo*.

Palavras-chave: atividade antioxidante, cicatrização cutânea, nanocápsulas, nanoemulsões, óleo de semente de uva, óleo de amêndoas doce, rutina.

ABSTRACT

Master Dissertation
Programa de Pós-Graduação em Ciências Farmacêuticas

NANOSTRUCTURED FORMULATIONS CONTAINING RUTIN: DEVELOPMENT, *IN VITRO* ANTIOXIDANT ACTIVITY AND EFFECT ON THE CUTANEOUS WOUND HEALING

AUTHOR: Juliana Severo de Almeida
ADVISER: Prof. Dr. Ruy Carlos Ruver Beck
Place and Date of Defense: Santa Maria, August 16, 2010

This work had as main objective the development of nanostructured formulations containing rutin, using nanoparticles prepared with alternative vegetables oils. In the first chapter it was demonstrated the feasibility of preparing nanocapsules and nanoemulsions using grape seed or almond kernel oil. Nanocapsule suspensions and nanoemulsions were prepared by the interfacial deposition of preformed polymer and spontaneous emulsification, respectively. All formulations presented nanometric mean size, polydispersity index below 0.30, negative zeta potential, pH values between 6.5 and 7.5 remaining stable after 6 storage months. These formulations promoted the protection of the active against UV degradation, regardless of the type of the oily phase or vesicle. In the second chapter, formulations prepared with grape seed oil were selected for the development of rutin-loaded nanoparticles, as well as to evaluate its *in vitro* antioxidant activity and photostability. All formulations presented nanometric size, low polydispersity index, acid pH values, negative zeta potential and encapsulation efficiency close to 100 %. Nanoparticles were able to protect rutin against UV photodegradation, if compared to rutin ethanolic solution. In the study of the *in vitro* antioxidant activity, rutin-loaded nanocapsules and nanoemulsions showed a lower rutin decay rate compared to the rutin ethanolic solution when exposed to UV radiation in the presence of OH radical. However, its presence in nanocapsules led to a prolonged *in vitro* antioxidant activity compared to the rutin-loaded nanoemulsions. Finally, in the third chapter we studied the development of hydrogels containing rutin (free or associated to polymeric nanocapsules). Their activity on the cutaneous wound healing in rats was evaluated. The developed formulations showed adequate properties regarding their cutaneous administration. *In vivo* response concerning the healing effect of hydrogels was evaluated by the regression of skin lesions after six days of treatment. Markers of oxidative stress in the lesions of rats were also evaluated, as levels of lipid peroxidation analyzed by the method of thiobarbituric acid reactive substances (TBARS), determination of protein carbonyls levels, total proteins levels, glutathione (GSH) levels, vitamin C and evaluation of the antioxidant enzyme catalase (CAT). This last chapter showed for the first time the feasibility of the dermatological use of such formulations containing rutin to promote the *in vivo* wound healing.

Keywords: antioxidant activity, cutaneous wound healing, nanocapsules, nanoemulsions, grape seed oil, almond kernel oil, rutin.

LISTA DE FIGURAS

REVISÃO DA LITERATURA

CAPÍTULO 1: Nanopartículas contendo óleos vegetais alternativos (óleo de semente de uva ou óleo de amêndoas doce): preparação, caracterização e avaliação da estabilidade

CAPÍTULO 2: Sistema nanoestruturado contendo rutina: estudo de atividade antioxidante *in vitro* e fotoestabilidade

CAPÍTULO 3: Hidrogéis contendo rutina para administração cutânea: desenvolvimento, caracterização reológica e cicatrização de feridas *in vivo*

LISTA DE TABELAS

CAPÍTULO 1: Nanopartículas contendo óleos vegetais alternativos (óleo de semente de uva ou óleo de amêndoas doce): preparação, caracterização e avaliação da estabilidade

SUMÁRIO

INTRODUÇÃO

INTRODUÇÃO

Atualmente existe um crescente interesse no estudo dos antioxidantes devido às recentes descobertas sobre o efeito dos radicais livres no organismo, os quais se encontram envolvidos na produção de energia, fagocitose, regulação do crescimento celular, entre outros (BARREIROS *et al*., 2006). O estresse oxidativo representa uma condição que ocorre em um sistema quando uma geração de espécies reativas de oxigênio (EROs) ultrapassa a capacidade de neutralização e eliminação dos mesmos do sistema. Este desequilíbrio pode resultar de uma falta de capacidade antioxidante causada por perturbações na produção, distribuição ou por uma abundância de espécies de oxigênio reativo a partir de fontes endógenas ou condições ambientais estressantes. O estresse oxidativo tem sido associado a uma lista crescente de doenças como as doenças cardiovasculares e neurodegenerativas, câncer, artrite, choque hemorrágico, catarata assim como nos processos de envelhecimento (BRENNEISEN *et al*., 2005; BARREIROS *et al*., 2006; CORNELLI, 2009).

Antioxidantes são moléculas naturais ou sintéticas que impedem a formação descontrolada do oxigênio reativo ou inibe suas reações com estruturas biológicas. As defesas antioxidantes envolvem várias estratégias enzimáticas e não-enzimáticas. São conhecidos três sistemas enzimáticos antioxidantes: o primeiro é composto por dois tipos de enzimas superóxido dismutase (SOD), que catalisam a destruição do radical ânion superóxido, convertendo-o em oxigênio e peróxido de hidrogênio. O segundo é formado pela enzima catalase (CAT) que atua na dismutação do peróxido de hidrogênio em oxigênio e água. O terceiro sistema é composto pela glutationa peroxidase (GPx) que reduz o peróxido de hidrogênio em água (BARREIROS, *et al*., 2006). Os compostos que atuam como antioxidantes não-enzimáticos compreendem tocoferóis, carotenóides, ascorbato, glutationa, ubiquinona e os flavonóides (BRENNEISEN *et al*., 2005; BARREIROS *et al*., 2006).

Os flavonóides são também conhecidos como polifenóis e geralmente ocorrem em plantas na forma de glicosídeos (DEGÁSPARI e WASZCZYNSKYJ, 2004; DWECK *et al*., 2009). A atividade antioxidante dos flavonóides depende da sua estrutura e pode ser determinada pela sua reatividade como agente doador de prótons e elétrons, estabilidade do radical flavonoil formado, reatividade frente a outros antioxidantes, capacidade de quelar metais de transição e solubilidade

e interação com as membranas (OGA *et al*., 2008). A atividade de seqüestro está diretamente relacionada ao potencial de oxidação dos flavonóides e das espécies a serem seqüestradas. Quanto menor o potencial de oxidação do flavonóide, maior é sua atividade como seqüestrador de radicais livres. A estabilidade do radical livre flavonoil formado depende da habilidade do flavonóide em deslocar o elétron desemparelhado. A remoção de metais de transição livres no meio biológico é fundamental para a proteção antioxidante do organismo, visto que esses catalisam reações de formação de radicais livres. A lipofilia do flavonóide indica a tendência da sua incorporação pelas membranas celulares que são alvo da maioria dos radicais livres (BARREIROS *et al*., 2006). Além da atividade antioxidante, os flavonóides possuem atividade anti-inflamatória, antialergênica, hepato-protetora, antitrombótica (CEMELI *et al*., 2009), e estão envolvidos nos processos de cicatrização (PATTANAYAK *et al*., 2008).

A rutina (quercetina-3-ramnosilglicose) é um flavonóide derivado da flavona com propriedades antioxidantes significativas em espécies como o radical hidroxila e o radical superóxido. É amplamente utilizada no tratamento de diversas doenças por possuir atividade antialérgica, anti-inflamatória, vasoativa, entre outras. Por ser uma molécula não tóxica e não oxidável, a rutina oferece uma vantagem sobre alguns flavonóides, que em algumas ocasiões comportam-se como agentes pró-oxidantes e catalisam a produção do radical oxigênio. Porém a sua baixa solubilidade em água ainda impõe restrições ao seu uso em formas farmacêuticas e cosméticas (CALABRÒ *et al*., 2005; KUNTI´C *et al*., 2007; MAULUDIUN et al., 2009[a]; MAULUDIUN *et al*., 2009[b]). Além destes compostos, os óleos vegetais também podem apresentar ação antioxidante, devido à presença de compostos fenólicos em sua estrutura, destacando-se o óleo de semente de uva e o de amêndoas doce, amplamente empregados nas indústrias de alimentos e cosméticos (MARTÌN-CARRATALÁ *et a*l., 1999; BEVERIDGE *et al*., 2005).

A cicatrização é um processo complexo e multifatorial, resultado da contração e fechamento da ferida e a restauração de uma barreira funcional. A reparação de feridas nos tecidos ocorre como uma seqüência de eventos, que inclui inflamação, proliferação e migração de diferentes tipos celulares. É aceito que as EROs são deletérias ao processo de cicatrização da ferida, devido aos efeitos nocivos sobre as células e tecidos. Aplicações tópicas de compostos com propriedades antioxidantes em pacientes mostraram melhora significativa na cicatrização de

feridas e proteção aos tecidos dos danos oxidativos (PATTANAYAK *et al*., 2008). Diversos fatores afetam o processo de cicatrização de feridas, tais como a idade, o estado nutricional e a neuropatia pós-ferimento. O processo de cicatrização é promovido por diversos produtos naturais e vegetais, os quais são compostos por substâncias ativas como triterpenos, alcalóides, flavonóides e biomoléculas. Estes agentes geralmente influenciam uma ou mais fases do processo de cura (SUMITRA *et al*., 2005; PANCHATCHARAM *et al*., 2006).

Os sistemas nanoestruturados podem ser empregados com a finalidade de modificar a biodisponibilidade e distribuição de fármacos através da pele. Eles apresentam uma enorme área superficial, o que os torna altamente eficazes para a aplicação de substâncias lipofílicas podendo promover uma liberação homogênea do fármaco (GUTERRES *et al*., 2007). Esses sistemas apresentam algumas vantagens para aplicação tópica como a liberação sustentada suprindo a pele com o fármaco por um período prolongado de tempo e efeitos sistêmicos mínimos (ALVES *et al*., 2007; MARCHIORI *et al*., 2010). No entanto, o transporte de fármacos ou ativos cosméticos através do estrato córneo é complexo, representando uma barreira para o direcionamento de muitas moléculas nos tratamentos cutâneos (GUTERRES *et al*., 2007). As nanopartículas poliméricas são sistemas carreadores de fármacos com diâmetro inferior a 1 μm e são denominadas nanocápsulas ou nanoesferas de acordo com a presença ou ausência de óleo em suas formulações, respectivamente. As nanoemulsões apresentam a fase oleosa, mas não apresentam o polímero (SCHAFFAZICK *et al*., 2006; MARTINI *et a*l., 2007; POHLMANN *et al*., 2008).

A partir do exposto, o presente trabalho visa estudar o desenvolvimento de nanocápsulas poliméricas e nanoemulsões empregando óleos vegetais alternativos como o óleo de semente de uva e o óleo de amêndoas doce para a veiculação de compostos destinados a aplicação cutânea. Inicialmente foi avaliada a possibilidade de obtenção dos sistemas nanoestruturados contendo tais óleos e a sua capacidade de veicular e proteger um filtro solar orgânico da radiação UV. Posteriormente, foi estudada a incorporação de um composto antioxidante (rutina) nestes sistemas, considerando a possibilidade de se aumentar a sua fotoestabilidade e atividade antioxidante *in vitro*. Finalmente, foi delineado um estudo baseado na obtenção de formas semissólidas contendo o composto antioxidante associado ou não ao sistema nanoestruturado avaliando-se o seu emprego no tratamento do processo de cicatrização cutânea *in vivo* após a

aplicação tópica e o efeito da nanoencapsulação. Até o momento não há relatos na literatura da incorporação da rutina a formulações semissólidas focados na obtenção de benefícios na cicatrização cutânea.

OBJETIVOS

1.1 Objetivo geral

Desenvolver formulações nanoestruturadas contendo rutina, a partir da otimização de formulação contendo um óleo vegetal alternativo (óleo de semente de uva ou óleo de amêndoas doce), estudando as potencialidades de associação de compostos de interesse farmacêutico e/ou cosmético a esses sistemas, tais como: aumento na fotoestabilidade, melhora na atividade antioxidante *in vitro* e potencialidade no tratamento de processos de cicatrização cutânea.

1.1.2 Objetivos específicos

- Avaliar o emprego do óleo de semente de uva e de amêndoas doce como matérias-primas (fase oleosa) para a preparação de nanocápsulas poliméricas e nanoemulsões, utilizando uma substância modelo, a benzofenona-3;

- Associar a rutina aos sistemas nanoestruturados propostos, avaliando a sua influência sobre as características físico-químicas dos sistemas, o perfil de liberação *in vitro* e o potencial de prevenção da fotodegradação, além de avaliar o efeito da nanoencapsulação sobre a atividade antioxidante *in vitro*;

- Estudar o desenvolvimento de uma forma semissólida (hidrogel) contendo rutina livre ou associada à nanopartículas poliméricas, avaliando a sua aplicabilidade na cicatrização cutânea em modelo animal.

REVISÃO DA LITERATURA

REVISÃO DA LITERATURA

1.1 Estresse oxidativo e antioxidantes

Espécies reativas de oxigênio (EROs) são geradas pelos processos metabólicos normais em todos os organismos que utilizam o oxigênio. Porém, a produção excessiva de EROs pode superar as defesas celulares antioxidantes e levar a uma condição chamada de estresse oxidativo (BARREIROS *et al*., 2006; PEREIRA *et al*., 2009). O estresse oxidativo é um distúrbio no equilíbrio pró-oxidante/antioxidante resultando em uma diminuição dos níveis antioxidantes e/ou aumento da produção de espécies reativas (CEMELI *et al*., 2009). Este distúrbio tem sido implicado no desenvolvimento de uma série de doenças degenerativas, tais como doenças cardiovasculares, câncer, processos de envelhecimento do corpo, dentre outras (CEMELI *et al*., 2009; PEREIRA *et al*., 2009), além de danificar vários tipos de biomoléculas, incluindo proteínas, DNA e lipídios (MARITIM *et a*l., 2003; BRENNEISEN *et al*., 2005; SOUSA *et al*., 2007; FANG *et al*., 2009). As doenças cardiovasculares constituem um grande problema do mundo moderno, sendo a principal causa de morte de homens e mulheres. Níveis altos de colesterol estão associados a essas doenças, porém o consumo de ácidos graxos saturados vem diminuindo e dando lugar a ácidos graxos mono e poliinsaturados. Essa substituição promove aumento das HDLs e diminuição dos níveis séricos de LDLs e de triglicerídeos, além de tornar as LDLs menos suscetíveis à oxidação. Por sua vez, a ingestão de fibras, probióticos e antioxidantes, como vitamina E, vitamina C, β-caroteno, licopeno e flavonóides têm recebido muita atenção nos últimos anos, pelo seu efeito na redução do risco de doenças cardiovasculares e de outras doenças degenerativas (COSTA e BORÉM, 2003).

O mecanismo e a sequência de eventos pelas quais os radicais livres interferem com as funções celulares não está bem definido, mas um dos eventos mais importantes parece ser a peroxidação lipídica, que resulta em dano nas membranas celulares. Este dano celular muda a pressão osmótica da célula levando a inchação e eventualmente morte celular (NIJVELDT *et al*., 2001; SOUSA *et al*., 2007). As EROs também estão envolvidas nos processos de oxidação de substâncias orgânicas reduzindo assim a vida de prateleira de produtos alimentícios

industrializados bem como das matérias-primas em geral (DEGÁSPARI e WASZCZYNSKYJ, 2004).

EROs é um termo utilizado para espécies derivadas de oxigênio e não inclui apenas radicais de oxigênio como o radical ânion superóxido ($O_2^{\cdot -}$), radicais hidroxilas ($^{\cdot}OH$), peroxila ($ROO^{\cdot}$) e alcoxila ($RO^{\cdot}$), mas também alguns derivados de oxigênio não-radicalares como o peróxido de hidrogênio (H_2O_2), oxigênio singlete ($^1O_2$), que são espécies eletronicamente excitadas quando o oxigênio no estado fundamental absorve energia (OGA *et al*., 2008) e ácido hipocloroso (BARREIROS *et al*., 2006). São moléculas geralmente muito pequenas devido à presença de um ou mais elétrons não-pareados ocupando orbitais atômicos ou moleculares. Durante a produção de EROs, o oxigênio molecular normalmente age como aceptor de elétrons para se tornar um radical livre de oxigênio. Adicionalmente, óxidos de nitrogênio ($^{\cdot}NO$, $^{\cdot}NO_2$) são também radicais livres e frequentemente chamados de espécies reativas de nitrogênio (ERNs) e tem importância similar aos EROs (FANG *et al*., 2009).

O radical $^{\cdot}OH$ é o mais deletério ao organismo devido a sua curta meia-vida, dificultando o seu seqüestro *in vivo*. Estes radicais atacam as moléculas por abstração de hidrogênio e adição a insaturações. Ele é formado no organismo principalmente pela reação do H_2O_2 com metais de transição e homólise da água por exposição à radiação ionizante (Equação 1). A incidência da radiação ultravioleta pode produzir a formação do radical $^{\cdot}OH$ na pele. O ataque intensivo e frequente desse radical podem originar mutações ao DNA e, consequentemente desenvolvimento de câncer em seres humanos ao longo dos anos (BARREIROS *et al*., 2006).

$$H_2O \xrightarrow{\text{luz UV}} HO^{\cdot} + H^{\cdot} \qquad (1)$$

O peróxido de hidrogênio pode se difundir facilmente através das membranas celulares. Pelo fato das células possuírem metais de transição, ocorre a formação do radical $^{\cdot}OH$ no seu interior (BARREIROS *et al*., 2006), como mostrado na equação 2:

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + HO^{\cdot} + HO^{-} \qquad (2)$$

Vários outros mecanismos também têm sido sugeridos na formação de radicais livres como a oxidação da glicose (MARITIM *et al*., 2003). A implicação dos EROs em diversas doenças, como mencionado anteriormente, se dá devido a sua alta reatividade e citotoxicidade. Em condições patológicas como infecções crônicas e inflamatórias esses metabólitos altamente reativos induzem dano letal à integridade celular reversível ou irreversível ao tecido. Desse modo, o desenvolvimento de terapias para suprimir EROs faz-se necessário, sendo que a maior parte das pesquisas voltam-se para o estudo de agentes antioxidantes com esse objetivo (FANG *et al*., 2009).

Antioxidantes são substâncias que retardam o processo de oxidação inibindo a polimerização iniciada por radicais livres e outras subseqüentes reações de oxidação (CÈSPEDE *et al*., 2008) podendo estes terem origem endógena, como as enzimas catalase (CAT) e superóxido dismutase (SOD) ou serem provenientes da dieta alimentar ou outras fontes (SOUSA *et al*., 2007).

A catalase está presente em todos os órgãos do corpo, mas é particularmente encontrada no fígado. Localizada nos peroxissomas decompõe o H_2O_2 em H_2O e O_2 (MARITIM *et al*., 2003; CEMELI *et al*., 2009) e exerce sua função de redução em altas concentrações de H_2O_2. Por outro lado, em baixas concentrações de H_2O_2 sua eficiência diminui. A razão para isso se dá pela necessidade da catalase requerer duas moléculas de H_2O_2 para executar sua redução (CEMELI *et al*., 2009).

A terapia com a superóxido dismutase é utilizada em situações em que o aumento da geração de radical superóxido deve ser controlado (OGA *et al*., 2008). Existem diferentes tipos de SOD e são denominadas de acordo com os íons que possui como Cu-Zn-SODs que são enzimas estáveis presentes no citosol, particularmente nos lisossomas e no núcleo, sendo que sua atividade não é afetada pelo estresse oxidativo. As Mn-SOD estão presentes nas mitocôndrias de animais e sua atividade aumenta com o estresse oxidativo (BARREIROS *et al*., 2006) enquanto que Au-SOD não são encontradas nos tecidos animais (CEMELI *et al*., 2009). Outro sistema antioxidante é formado pela glutationa (GSH), um tripeptídeo formado por resíduos de glicina, cisteína e ácido glutâmico. A glutationa é um seqüestrador de radicais hidroxila e de oxigênio singlete (OGA *et al*., 2008) e age em conjunto com as enzimas glutationa peroxidase (GPx) e glutationa redutase (GR). Esse sistema também catalisa a dismutação do peróxido de hidrogênio em água e oxigênio, sendo que a glutationa opera em ciclos entre sua forma oxidada e sua forma

reduzida. Além da glutationa, destacam-se no combate ao estresse oxidativo os compostos não-enzimáticos como os carotenóides, a bilirrubina, a ubiquinona, o ácido úrico, os tocoferóis, o ácido ascórbico e os flavonóides (BARREIROS *et al*., 2006).

1.2 Flavonóides

Os flavonóides pertencem a um grupo de substâncias naturais encontrados em frutas, vegetais, grãos, raízes, flores, chás e vinhos. Esses produtos naturais são conhecidos por seus efeitos benéficos para a saúde e podem ser divididos em várias classes com base na sua estrutura molecular (NIJVELDT *et al*., 2001).

Eles consistem de vários grupos relacionados quanto a sua estrutura, que são frequentemente identificados como polifenóis (DWECK, 2009), porém sua concentração varia de planta para planta ou até em órgãos diferentes da mesma planta (SULTANA e ANWAR, 2008). São encontrados sem a presença da ligação com o açúcar, denominados agliconas ou em sua forma de glicosídeos denominados glicosídeos (YAO *et al*., 2004). Esses são moléculas substituídas em um ou mais grupos hidroxila do composto, como a galactose, glicose, manose ou ramnose (DWECK, 2009).

Os compostos fenólicos oferecem não apenas propriedades sensoriais importantes sendo responsáveis pela cor, aroma e sabor das plantas, mas também porque podem desempenhar um papel importante na prevenção de diversas doenças associadas ao estresse oxidativo (CHIRINOS *et al*., 2009). Os flavonóides na dieta são considerados antioxidantes mais eficientes que as vitaminas C e E (SULTANA e ANWAR, 2008). Estudos *in vitro* e *in vivo* têm mostrado que os flavonóides possuem várias outras atividades como anti-inflamatória, antialérgica, hepato-protetora, antitrombótica, antiviral, anticarcinogência e fotoprotetora (PEKKARINEN *et al*., 1999; MARITIM *et al*., 2003; GONZÁLEZ *et al*., 2008; CEMELI *et al*., 2009; PEREIRA *et al*., 2009). A mais bem descrita propriedade de quase todo o grupo dos flavonóides é a capacidade de atuar como antioxidante (NIJVELDT *et al*., 2001). Os flavonóides inibem as enzimas responsáveis pela produção do $O_2^{\cdot -}$. O seu baixo potencial redox permite termodinamicamente a redução de radicais livres tais como $O_2^{\cdot -}$, $RO^{\cdot}$ e $HO^{\cdot}$ (DEGÀSPARI e WASZCZYNSKYJ, 2004; CEMELI *et al*., 2009).

Desta forma, os flavonóides podem prevenir o dano causado por radicais livres através de diferentes sistemas. Eles são oxidados pelos radicais, resultando em um radical mais estável, e menos reativo. Por causa da alta reatividade dos grupos hidroxilas dos flavonóides os radicais são desativados, como mostra a equação 3:

$$\text{Flavonóide (OH)} + R^{\cdot} \longrightarrow \text{Flavonóide } (O^{\cdot}) + RH \qquad (3)$$

onde $R^{\cdot}$ é um radical livre e $O^{\cdot}$ é um radical livre de oxigênio.

A xantina desidrogenase é uma forma de enzima presente sob condições fisiológicas, mas muda para xantina oxidase durante condições de isquemia. A xantina oxidase é uma fonte de radicais livres de oxigênio que quando reage libera radicais livres superóxido. Assim, alguns flavonóides podem inibir a atividade da xantina oxidase resultando em diminuição do dano oxidativo. Os quatro principais grupos de flavonóides são as flavonas, flavanonas, catequinas e antocianinas (NIJVELDT *et al*., 2001).

A rutina (quercetina-3-rutinosideo, Figura 1), substância escolhida para este estudo, é um composto fenólico natural derivado da flavona encontrada em grandes concentrações em frutos e sementes com significativas propriedades sequestrantes de espécies oxidantes, tais como radical $^{\cdot}OH$, superóxido e peroxila. Consequentemente, a rutina tem sido amplamente utilizada no tratamento de várias doenças. Seu emprego farmacológico inclui atividades antialérgica, anti-inflamatória e vasoativa, além de citoprotetora, antiespasmódica e anticarcinogênica (CALABRÒ *et al*., 2005; HAN *et al*., 2009; MAULUDIN *et al*., 2009[a]; MAULUDIN *et al*., 2009[b]). Por aumentar a força dos capilares e reduzir a sua permeabilidade, a rutina ajuda a prevenir hemorragias e rupturas nos capilares e tecido conectivo, além de ser frequentemente usada no tratamento de insuficiência venosa crônica e hemorragias (KUNTI´C *et al*., 2007).

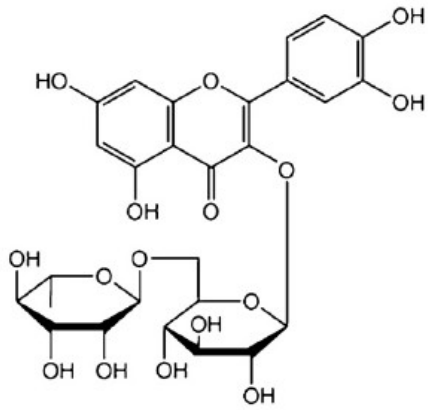

Figura 1. Estrutura química da rutina

Kormaz e Kolankaya (2009) investigaram os efeitos da rutina na lesão por isquemia e reperfusão induzida em rim de ratos. Constataram que a atividade da Mn-SOD foi restabelecida após o tratamento dos ratos com esse flavonóide, assim como os níveis de glutationa (GSH), sugerindo que a rutina mostrou um efeito protetor contra o dano renal, provavelmente por inibição das EROs.

Itagaki e colaboradores (2010) verificaram o efeito protetor da rutina no dano oxidativo intestinal ocasionado por isquemia e reperfusão em ratos. A mucosa intestinal é extremamente sensível às EROs, assim este estudo mostrou uma significativa inibição da peroxidação lipídica no intestino de ratos após tratamento com a rutina através da sua atividade de seqüestro de radicais hidroxila.

Apesar da sua potencial aplicação na terapêutica, algumas características físico-químicas da rutina, como a sua baixa solubilidade aquosa (12,5g/ 100mL) especialmente para administração tópica e oral, impõem algumas restrições ao seu uso em formas farmacêuticas (CALABRÒ *et al*., 2005; MAULUDIN *et al*., 2009[a]). Na administração oral, a rutina é atualmente recomendada por alguns autores pela sua aplicação como suplemento nutricional diário (MAULUDIN *et al*., 2009[a]).

Mauludin e colaboradores (2009[b]) desenvolveram nanocristais de rutina, preparados por liofilização com posterior incorporação em comprimidos, como estratégia para melhorar a solubilidade do ativo em formas farmacêuticas sólidas. As propriedades físico-químicas como redispersibilidade, tamanho de partícula, morfologia e dissolução foram avaliadas. Os resultados mostraram que os nanocristais de rutina melhoraram a sua solubilidade e particularmente a sua

velocidade de dissolução, sugerindo que esses nanocristais podem superar problemas de biodisponibilidade associados com a baixa solubilidade aquosa. Outros autores estudaram a complexação da rutina com ciclodextrinas, oferecendo vantagens físico-químicas, como o aumento da sua solubilidade em água e biodisponibilidade. Estes resultados mostraram que a solubilidade do ativo aumenta proporcionalmente com o aumento da sua interação com ciclodextrinas, além de melhorar a atividade antioxidante *in vitro* da rutina quando complexada, provavelmente pela sua solubilidade aumentada (CALABRÒ *et al*., 2005).

1.3 Óleos vegetais

Óleos e gorduras são substâncias insolúveis em água, podendo ser derivados de fontes vegetais e animais (JOHN *et al*., 2004). São amplamente utilizados em indústrias alimentares (CUNHA e OLIVEIRA, 2006) e não-alimentares pelo seu caráter renovável e biodegradável (BLAYO *et al*., 2001). Os óleos vegetais apresentam papéis funcionais e sensoriais importantes em produtos alimentícios, além de conterem vitaminas A, D, E e K. Eles também fornecem energia e ácidos essenciais responsáveis pelo crescimento e são um dos importantes ingredientes usados na fabricação de sabões, cosméticos e produtos farmacêuticos (FASINA *et al*., 2008).

Os óleos vegetais são constituídos principalmente de triacilgliceróis (95–98%) e misturas complexas de compostos secundários (2–5%) de natureza química variada. Os principais grupos de constituintes secundários presentes nestes óleos são: alcoóis graxos, hidrocarbonetos, ésteres, tocoferóis e tocotrienos, compostos fenólicos e voláteis, pigmentos, triglicerídeos secundários, fosfolipídios e ácidos triterpênicos (CERT *et al*., 2000).

O óleo de semente de uva é um óleo vegetal cada vez mais popular na culinária, em produtos farmacêuticos e cosméticos, principalmente pelo seu alto nível de ácidos graxos insaturados (PASSOS *et al*., 2009). Este óleo é rico em ácido linoléico, tocoferóis e apresenta alto nível de vitamina E, com considerável estabilidade oxidativa, o que contribui para o efeito anticolesterêmico. Este efeito é caracterizado pela redução da lipoproteína de baixa densidade (LDL), aumento dos níveis da proteína de alta densidade (HDL) conferindo um efeito protetor contra problemas cardíacos (BEVERIDGE *et al*., 2005). O óleo de semente de uva apresenta significativas quantidades de compostos fenólicos, porém muitas vezes são destruídos em

diferentes estágios do processo de refinamento (CERT *et al*., 2000). Por outro lado, os ácidos graxos poliinsaturados tais como o ácido linoléico e linolênico são essenciais para o organismo humano, porque não podem ser sintetizados pelo organismo (COSTA e BORÉM, 2003; BAYDAR *et al*., 2007).

As sementes de uvas constituem cerca de 5% do peso da uva, contendo 10 a 20% de óleo com um elevado teor de vitamina E. O α-tocoferol é a forma ativa da vitamina E *in vivo* e é considerado um antioxidante intracelular devido a sua atividade na inibição da peroxidação de ácidos graxos poliinsaturados em membranas biológicas (CHOI e LEE, 2009). A habilidade dos tocoferóis em agir como antioxidantes é resultante da presença do seu grupo fenólico.

O óleo de amêndoas doce também apresenta altas concentrações de ácido linoléico e oléico, como principais constituintes, além das vitaminas A, B1, B2, B6 e vitamina E tornando-o importante na indústria alimentar (MARTÍN-CARRATALÁ *et tal*., 1999; ESPÍN *et al*., 2000, ZHANG *et al*., 2009). Também é utilizado na composição de formulações cosméticas, servindo como excelente emoliente e contribuindo para manter o equilíbrio da umidade da pele. Devido às inúmeras vantagens, o interesse no óleo de amêndoas tem aumentado muito nos últimos anos (ZHANG *et al*., 2009). O seu emprego destaca-se também contra doenças cardiovasculares reduzindo os níveis de LDL e elevando os níveis de HDL, assim como também apresenta ações anti-inflamatórias, anti-hepatotóxicas (AHMAD, 2010), antitumorais, hipocolesterêmicas, antiulcerativas e propriedades antifúngicas e antibacterianas devido a presença de compostos secundários , os fitosteróis (CHERIF *et al*., 2009).

1.4 Nanotecnologia e sistema de liberação de fármacos

A nanotecnologia é uma área interdisciplinar, onde o termo ‘nano’ deriva do grego e significa ‘anão’. É amplamente aplicada com muitos benefícios e facilidades em diversas áreas como a engenharia, eletrônica, física até sistemas mais eficientes para veicular e aumentar a eficácia de fármacos no organismo (SAHOO e LABHASETWAR, 2003; SCHMALTZ *et al*., 2005; SAHOO *et al*., 2007).

As nanopartículas são menores que as células humanas, porém semelhantes em tamanho a moléculas biológicas como enzimas e receptores. Marcadas com anticorpos, colágeno e

micromoléculas, são usadas para diagnóstico precoce de doenças como o câncer. Podem auxiliar no pronunciamento dos efeitos benéficos máximos dos fármacos, com redução dos seus efeitos colaterais, além de poderem ser ativadas por estímulos como substâncias químicas, campo magnético, calor e pH (SANDHIYA *et al*., 2009).

As nanopartículas poliméricas tem sido extensamente estudados na área farmacêutica com a finalidade de promover um sistema de liberação direcionada e controlada de fármacos, aumentando sua eficácia e/ou diminuindo sua toxicidade (BECK *et al*., 2004; BECK *et al*., 2005; BECK *et al*., 2006; CRUZ *et al*., 2006; SANDHIYA *et al*., 2009). Apresentam diâmetro inferior a 1 μm (ZAHR *et a*l., 2005) e são classificadas como nanocápsulas e nanoesferas de acordo com sua composição e estrutura. As nanocápsulas apresentam um invólucro polimérico disposto ao redor de um núcleo oleoso podendo o fármaco estar dissolvido no núcleo ou adsorvido à parede polimérica. Já as nanoesferas não apresentam óleo em sua composição, são formadas por uma matriz polimérica podendo o fármaco estar retido ou adsorvido na matriz. As nanocápsulas e nanoesferas são estabilizadas por tensoativos na interface partícula/água, os quais ajudam a impedir a aglomeração destas partículas (COUVREUR *et al.*, 2002; SCHAFFAZICK *et al.*, 2003).

Com relação a outras nanoestruturas com potencial aplicação na terapêutica, as nanopartículas poliméricas são mais comumente utilizadas, em relação aos lipossomas porque estes possuem menor estabilidade *in vivo*. Os lipossomas consistem de pequenas vesículas esféricas compostas por uma bicamada de fosfolipídios envolvendo um núcleo aquoso. São estudados há mais tempo e são amplamente utilizados em cosméticos (SHARMA *et al*., 1997; GUTERRES *et al*., 2007). Entretanto, altas doses de fosfolipídios aplicados topicamente por longo período podem levar a irritações na pele normal e seca. Os trabalhos de desenvolvimento de lipossomas têm sido limitados por seus problemas estruturais como baixa eficiência de encapsulação e baixa estabilidade durante o armazenamento (SCHMALTZ *et al*., 2005). As nanopartículas de lipídio sólido são sistemas carreadores alternativos às emulsões, lipossomas e nanopartículas poliméricas, sendo dispersões lipídicas em meio aquoso e estabilizadas por um tensoativo, quando necessário. As nanopartículas lipídicas possuem ampla utilização na área dermocosmética (PARDEIKE *et al*., 2009). No entanto, estas formulações apresentam desvantagens como baixa capacidade de associação do fármaco, presença simultânea de outras estruturas coloidais (micelas, lipossomas, nanocristais do fármaco) e instabilidade física durante o armazenamento devido à complexidade do estado físico do lipídio (GUTERRES *et al*., 2007).

Nanoemulsões, preparadas sem o polímero, são emulsões submicrométricas e também tem sido estudadas como nanocarreadores de fármacos (MARTINI *et al*., 2007; OURIQUE *et al*., 2008; FONTANA *et al*., 2009) como na liberação ocular (CALVO *et al*., 1996) e tópica (ALVES *et al.*, 2007).

O eficiente desenvolvimento farmacotécnico de formulações de nanopartículas poliméricas depende da escolha do sistema polimérico mais apropriado para se obter uma máxima encapsulação (alta eficiência de encapsulação) e melhor estabilidade físico-química. Além disso, busca-se nesse desenvolvimento a obtenção de formulações com perfis de liberação diferenciados, um maior direcionamento do fármaco e maior impacto terapêutico, quando comparadas com formulações convencionais, onde o fármaco encontra-se livre. A capacidade de direcionamento é influenciada pelo tamanho da partícula, carga e modificação da superfície e hidrofobicidade. O tamanho e a distribuição do tamanho das nanopartículas são importantes para determinar a interação do fármaco com as membranas celulares e sua penetração através das barreiras fisiológicas (KUMARI *et al*., 2010), além de garantir a sua característica nanotecnológica.

Os nanossistemas podem ser administrados por diferentes vias. Para a administração oral de fármacos eles são projetados de modo que permaneçam estáveis no trato gastrintestinal, interajam com o epitélio e que sejam produzidos com materiais seguros. Na administração nasal, os estudos mostram que as nanopartículas atravessam o epitélio e que a composição da superfície promove uma melhora do transporte nasal de macromoléculas quando associadas aos nanossistemas, o que também pode ser observado nos estudos de liberação ocular do fármaco (ALONSO, 2004). Como vetores de fármacos para liberação cerebral, as nanoestruturas têm sido estudadas devido a sua alta habilidade em promover o acesso do fármaco pela barreira hemato-hencefálica (SCHAFFAZICK *et al*., 2008; BERNARDI *et al*., 2009). Além das vantagens do ponto de vista da ação farmacológica, as nanopartículas podem apresentar outras vantagens, como aumentar a estabilidade de substâncias ativas, proteger as substâncias sensíveis à degradação química induzida pela luz UV e fatores como pH (OURIQUE *et al*., 2008; MORA-HUERTAS *et al*., 2010).

Em relação à aplicação tópica, o tamanho reduzido facilita a sua incorporação em produtos dermatológicos, podendo promover uma liberação sustentada do fármaco na pele, suprindo-a com o fármaco por um maior período de tempo (ALVES *et al*., 2007; GUTERRES *et al*., 2007). Neste caso, o mecanismo de ação das nanopartículas pode ser atribuído à sua associação com a

superfície da pele, sendo que a pequena granulometria assegura um contato estrito com o estrato córneo. É por isso que a quantidade do agente encapsulado facilita o transporte do fármaco através da pele alterando o coeficiente de partição veículo/estrato córneo (ALVAREZ-ROMÀN *et al*., 2004; ALVES *et al*., 2007).

Na preparação de nanopartículas poliméricas, os polímeros biodegradáveis e biocompatívies são os materiais de maior interesse, pois promovem a sua biodegradação *in vivo* e conseqüente remoção do organismo (KALLINTERI *et al*., 2005). Esses sistemas podem ser preparados por diversos métodos como a polimerização de monômeros dispersos, deposição interfacial do polímero pré-formado (nanoprecipitação) ou dispersão de polímeros pré-formados, dentre outros (SCHAFFAZICK *et al*., 2006). A técnica de deposição interfacial do polímero pré-formado foi utilizada neste trabalho, e descrita por Fessi e colaboradores em 1988 (FESSI *et al*., 1988), e consiste no emprego de um solvente miscível com a água (por exemplo, a acetona), que irá solubilizar o polímero, um estabilizador e o fármaco sob agitação magnética e temperatura controlada por um determinado período de tempo. Após, esta solução será injetada a uma solução aquosa contendo um estabilizante hidrofílico. O solvente é então removido da fase aquosa por pressão reduzida à 40 °C até ajuste do volume final da formulação.

A caracterização físico-química destas suspensões é realizada através da sua análise morfológica, distribuição do tamanho de partícula, massa molar e distribuição de massa molar do polímero, potencial zeta, pH, taxa de associação do fármaco, perfil e cinética de liberação e estabilidade das suspensões de nanopartículas ao longo do período de armazenamento (POHLMANN *et al*., 2002; SCHAFFAZICK *et al*., 2003).

Muitos estudos tem sido realizados utilizando formulações de base nanotecnológica com o intuito de vetorizar os fármacos (NAGARWAL *et al*., 2009; ZHU *et al*., 2009), modificar as suas características de liberação (ALONSO, 2004; BECK *et al*., 2004; BECK *et al*., 2005; CRUZ *et al*., 2006; BECK *et al*., 2007; FONTANA *et al*., 2009; LIN TAN *et al*., 2010), melhorar a biodisponibilidade e solubilidade de fármacos (CALABRÒ *et al*., 2005; MAULUDIN *et al*., 2009[a]; MAULUDIN *et al*., 2009[b]), bem como a proteção de fármacos contra a peroxidação lipídica (SCHAFFAZICK *et al*., 2005; SCHAFFAZICK *et al*., 2008). Entretanto, até o momento não existem relatos na literatura da preparação destes sistemas empregando óleos vegetais como o óleo semente de uva e de amêndoas na preparação de nanocápsulas poliméricas e nanomulsões, tampouco a associação do flavonóide rutina nestes sistemas.

CAPÍTULO 1: Nanopartículas contendo óleos vegetais alternativos (óleo de semente de uva ou óleo de amêndoas doce): preparação, caracterização e avaliação da estabilidade

CAPÍTULO 1: Nanopartículas contendo óleos vegetais alternativos (óleo de semente de uva ou óleo de amêndoas doce): preparação, caracterização e avaliação da estabilidade

1.1 Introdução

Os sistemas coloidais tem sido extensamente estudados no campo farmacêutico nos últimos anos para a liberação de fármacos no sítio específico, ou seja, nos tecidos oculares, nasais cerebrais, intestinais e na pele (ALONSO, 2004; ALVAREZ-ROMÁN *et al*., 2004; KIM e MARTIM., 2006; NAGARWAL *et al*., 2009). Além disso, diferentes estudos científicos tem sido realizados para se entender o mecanismos de formação das nanopartículas (SCHAFFAZICK *et al*., 2003; GUTERRES *et al*., 2007; MARTINI *et al*., 2007; KUMARI *et al*., 2010; MORA-HUERTAS *et al*., 2010), sua estrutura supramolecular (POHLMANN *et al.*, 2002; JÄGER et al., 2007), sua estabilização (SCHAFFAZICK *et al*., 2007; FONTANA *et al*., 2009; FONTANA *et al*., 2010) e a influência dos tipos de excipientes utilizados no seu preparo (SCHAFFAZICK *et al.*, 2006; OURIQUE *et al*., 2008; SCHAFFAZICK *et al*., 2008). No entanto, não há estudos relacionados ao emprego dos óleos de semente de uva e óleo de amêndoas na preparação de nanocápsulas poliméricas e nanoemulsões.

Diante disso, este capítulo descreve o desenvolvimento de suspensões de nanocápsulas poliméricas e nanoemulsões utilizando dois óleos vegetais inéditos para esse tipo de formulação, o óleo de semente de uva e de amêndoas doce. Esses óleos foram selecionados por apresentar considerável estabilidade oxidativa e por serem uma alternativa de fase oleosa para sistemas desenhados para incorporação de moléculas com atividade antioxidante. Neste estudo, foi avaliada a influência do tipo de óleo nas características físico-químicas das formulações bem como o tipo de sistema nanoestruturado empregado. As formulações foram avaliadas quanto ao teor do ativo, eficiência de incorporação, diâmetro médio, índice de polidispersão, pH e estabilidade frente ao armazenamento e frente a radiação UV. O ativo escolhido como modelo para esse estudo foi a benzofenona-3.

PUBLICAÇÃO 1: Oil-based nanoparticles containing alternative vegetable oils (grape seed oil and almond kernel oil): preparation and characterization

Artigo publicado no periódico Latin American Journal of Pharmacy

Oil-Based Nanoparticles Containing Alternative Vegetable Oils (Grape Seed Oil or Almond Kernel Oil): Preparation and Characterization

Juliana S. ALMEIDA[1], Luciane JEZUR[2], Márcia C. FONTANA[1], Karina PAESE[3], Cristiane B. SILVA[2], Adriana R. POHLMANN[4], Sílvia S.GUTERRES[3] & Ruy C. R. BECK[1*]

[1] Programa de Pós-Graduação em Ciências Farmacêuticas, Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Santa Maria, RS Brazil;
[2] Curso de Farmácia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil;
[3] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Ipiranga, 2752, 90610-000, Porto Alegre, RS, Brazil
[4] Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul (UFRGS), CP 15003, 91501-970, Porto Alegre, RS, Brazil

* Corresponding author:
Tel +55 55 3220-9373 Fax: +55 55 3220-8248
E-mail address: ruybeck@smail.ufsm.br (R. C. R. Beck)

SUMMARY

The use of two alternative vegetable oils (grape seed oil and almond kernel oil) to prepare nanoparticulated delivery systems (nanocapsules and nanoemulsions) for active substances was evaluated. They were prepared by interfacial deposition of preformed polymer (poly-ε-caprolactone) or spontaneous emulsification, respectively. All formulations presented nanometric size, polydispersity index below 0.30, negative zeta potential and spherical-shaped particles. Benzophenone-3, as a model substance was efficiently entrapped in these systems, independent on the type of oily phase. Its association did not alter significantly the physicochemical properties of the nanoparticle dispersions, which remained adequate until 6 months of storage. Nanocapsules and nanoemulsions prepared with both vegetable oils were suitable to delay benzophenone-3 photodegradation under UV radiation.

Keywords: Almond kernel oil, Grape seed oil, **N**anoparticles.

INTRODUCTION

Nanostructured materials such as polymeric nanoparticles, nanoemulsions, liposomes and dendrimers have been widely studied in the pharmaceutical and cosmetic field over the last years [1-4]. Active substances can have some properties improved by its incorporation in these biomedical nanomaterials, such as aqueous solubility, photostability, distribution after topical or systemic application, efficacy and bioavailability [2,5-9].

Nanocapsules are submicrometric particles (mean size below 1 μm) composed by an oily core surrounded by a thin polymer wall [10-12]. They are formulated in presence of surfactants in order to stabilize the particles [13]. Nanoemulsions are nanometric-sized emulsions having droplets up to 500 nm, being also formulated in presence of surfactants [14]. Because of their small size allowing them to permeate through biological barriers these systems showed potential use following topical (ocular, dermal) or systemic administration [15,16].

The development of suitable nanocapsule suspensions or nanoemulsions for pharmaceutical or cosmetic application requires the adequate selection of their adjuvants, like polymers (for NC only), surfactants, and oils [11,17-19]. Nowadays, special attention has been taking to biodegradable polymers in the preparation of nanocapsules due to its degradation to non-toxic and non-reactive metabolites, which can be excreted by the organism [20]. Surfactants are necessary to obtain small and stable oil droplets. Their type and concentration can affect the physicochemical properties of the nanoparticles, such as size, polydispersity index and drug loading [11,17-19]. In addition, the type of the oily phase used as the core in preparation of polymeric nanocapsules or nanoemulsions can also have an influence mainly on the mean particle size and polydispersity index due to the difference in its viscosity, hydrophobic characteristic and interfacial tension [11,18].

Grape seed oil and almond kernel oil are vegetable oils with known antioxidant activities, based in their chemical constitution. Grape seed oil is rich in phenolic components, linoleic acid and tocopherols [21,22]. Almond kernel oil also presents high concentrations of linoleic acid [23,24]. This way, both oils could be interesting alternatives to prepare nanocapsules or nanoemulsions as delivery systems for cosmetic or pharmaceutical active substances.

To the best of our knowledge, there is no report in the literature on the use of these vegetable oils as functionally adjuvants in the preparation of nanocapsule and nanoemulsions. In this context, the aim of this work was to evaluate the feasibility of their use as alternative

vegetable oil to prepare oil-based nanomaterials, as delivery systems of active substances. Nanocapsules and nanoemulsions were prepared and characterized by means of mean size, polydispersity index, pH, zeta potential, and stability under storage. In order to demonstrate a practical application of these alternative oils in the development of the oil-based nanomaterials, we evaluated the incorporation of a model substance (benzophenone-3) to the systems and their properties to delay benzophenone-3 photodegradation under UV radiation.

MATERIAL AND METHODS

Materials

Grape seed oil, almond kernel oil and benzophenone-3 (B3) were obtained from PharmaSpecial (São Paulo, Brazil). Poly-ε-caprolactone (PCL) and sorbitan monostearate (Span 60®) were acquired from Sigma (São Paulo, Brazil). Polysorbate 80 (Tween 80®) was supplied by Henrifarma (São Paulo, Brazil). All others chemicals and solvents presented pharmaceutical or HPLC grade and were used as received.

Preparation of nanocapsules and nanoemulsions

Nanocapsule suspensions (NC) were prepared (n = 3) by the interfacial deposition of preformed polymer method as described by Fessi *et al.* [25]. Briefly, an organic solution consisted of the oily phase – GSO or AKO (3.3 mL), a low HLB (hydrophilic-lipophilic balance) surfactant – Span 60® (0.776 g), the polymer (PCL) (1.0 g) and acetone (267.0 mL) was added under moderate magnetic stirring to an aqueous solution (533.0 mL) containing a high HLB surfactant - Tween 80® (0.776 g). The magnetic stirring was maintained for 10 min. Thus, the acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure to a final volume of 100 mL (10 $mg.mL^{-1}$ of polymer and 3.30 % v/v of oil). Nanoemulsions (NE) were prepared (n = 3) by the spontaneous emulsification method as described by Martini *et al.* [26]. To prepare the nanoemulsions it was omitted the presence of the polymer in the organic solution. All formulations were storage protected from the light.

Benzophenone-3-loaded nanoparticles were prepared at a final concentration of 1.00 $mg.ml^{-1}$, being dissolved (100 mg) in the organic solution during the nanoparticle preparation. Quali-quantitative composition of each formulation is described in Table 1.

Characterization of nanocapsules and nanoemulsions

Particle size analysis and polydispersity indices

Particle sizes and the polydispersity indices (n = 3) were measured by photon correlation spectroscopy after adequate dilution of an aliquot of the suspension in purified water (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK).

Table 1. Quali-quantitative composition of nanoparticles – 100 ml (NC: nanocapsules; NE: nanoemulsion; B3: benzophenone-3) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase.

Formulation	PCL	Span 60®	GSO	AKO	Tween 80®	B3
NC-AKO	1.00 g	0.77 g	---	3.3 ml	0.77 g	---
NE-AKO	---	0.77 g	---	3.3 ml	0.77 g	---
NC-GSO	1.00 g	0.77 g	3.3 ml	---	0.77 g	---
NE-GSO	---	0.77 g	3.3 ml	---	0.77 g	---
B3-NC-AKO	1.00 g	0.77 g	---	3.3 ml	0.77 g	0.10 g
B3-NE-AKO	---	0.77 g	---	3.3 ml	0.77 g	0.10 g
B3-NC-GSO	1.00 g	0.77 g	3.3 ml	---	0.77 g	0.10 g
B3-NE-GSO	---	0.77 g	3.3 ml	---	0.77 g	0.10 g

Zeta potential

Zeta potentials were determined after dilution of the samples in 1 mmol L^{-1} NaCl aqueous solution using Zetasizer Nano Series (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK).

pH

The pH values of suspensions were determined by immersion of the electrode directly in the suspension using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil), at room temperature.

Benzophenone-3 content

Benzophenone-3 content ($mg.ml^{-1}$) was determined (n = 3) after dissolution of nanocapsules or nanoemulsions in acetonitrile (1 ml of suspension to 25 ml of acetonitrile) and assayed by high performance liquid chromatography – HPLC. The chromatographic system consisted of a Gemini RP-18 column (150 x 4.60 mm, 5 µm, Phenomenex, Torrance, USA) and a Shimadzu instrument (LC-10AVP Pump, UV-VIS SPD-10AVP Module, Class-VP Software, Shimadzu, Tokyo, Japan). The mobile phase at a flow rate of 1.0 $ml.min^{-1}$ consisted of acetonitrile/water (85:15% v/v) containing 1 % of acetic acid. The volume injected was 20 µL and benzophenone-3 was detected at 286 nm. Validation of the HPLC assay demonstrated that this method was linear ($y = 74529x – 1860.3$, $r^2 = 0.9999$, $n = 5$) in the range of 1 – 20 $\mu g.ml^{-1}$ and precise (RSD: 0.65 % for repeatability and 0.68 % for intermediate precision). The specificity was tested in presence of the nanoparticle adjuvants and demonstrated that they did not alter the benzophenone-3 assay [27,28].

Encapsulation efficiency

Free benzophenone-3 was determined in the clear supernatant following separation of nanoparticles (NC and NE) from aqueous medium by a combined ultrafiltration-centrifugation technique (Ultrafree-MC® 10,000 MW, Millipore, Bedford, USA). Encapsulation efficiency (%) was calculated by the difference between the total and free benzophenone-3 concentrations determined in the nanoparticles (drug content) and in the ultrafiltrate, respectively, using the HPLC method described above.

Morphological analyses

Morphological analyses were conducted by transmission electron microscopy (TEM; Jeol, JEM 1200 Exll, *Centro de Microscopia* - UFRGS) operating at 80 kV. The diluted suspensions were deposited in Formvar-Carbon support films on specimen grid (Electron Microscopy Sciences, 400 mesh), negatively stained with uranyl acetate solution (2% m/v) and observed at different magnifications.

Stability studies

All NC and NE formulations were stored at room temperature and protected from light for 6 months. Particle size, polydispersity index, zeta potential and pH were evaluated after 6 months. Drug content and encapsulation efficiency were determined after 3 and 6 months of storage.

Photostability study

After preparation, the formulations (NC and NE) were placed in transparent quartz cells with 5 mm optical path and exposed to UVC radiation (Phillips TUV lamp – UVC long life, 30 W). The cells were irradiated in a box of mirror for 168 hours (7 days). The formulations were placed at a distance of 10 cm from the fluorescent lamps. After 72 and 168 hours (3 and 7 days, respectively), the total concentration of benzophenone-3 was quantified. Benzophenone-3 was assayed by HPLC after the dissolution of 200 µl of samples with acetonitrile according to the method previously described in section 3.3.4. A benzophenone-3 methanolic solution was used as a model of free benzophenone-3, prepared at a concentration of 1 $mg.ml^{-1}$.

Statistical analysis

Formulations were prepared and analyzed in triplicate. Results are expressed as mean ± SD (standard deviation). One-way analysis of variance (ANOVA) or two-way analysis of variance (ANOVA) were employed in the comparison of the experimental data. Post-hoc multiple comparisons were done by Tukey's test for significance at p–values less than 0.05. All analyses were run using the SigmaStat Statistical Program (Version 3.0, Jandel Scientific, USA).

RESULTS AND DISCUSSION

Nanoparticulated systems have been widely studied in the pharmaceutical and cosmetic area in the last years [2,4]. These drug delivery systems presented advantages compared to other systems and classical dosage forms for parenteral, oral and topical administration. Aiming to study the potential to use alternative vegetable oils to prepare nanostructured delivery systems, we prepared nanocapsules and nanoemulsions containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase.

Preparation and characterization

Nanocapsules and nanoemulsions were prepared by interfacial polymer deposition and spontaneous emulsification, respectively. All formulations appeared macroscopically homogeneous and their aspects were similar to a milky bluish opalescent fluid (Tyndall effect), regardless of the type of oily phase (GSO or AKO) or the vesicle structure (nanocapsule or nanoemulsion). The physicochemical characteristics of the formulations are presented in Table 2. All formulations presented mean particle size in the nanometric range (220 – 280 nm), acidic pH and negative zeta potential (between -6.0 and -9.0 mV). By a one-way ANOVA analysis, these parameters were similar for all formulations ($p > 0.05$). On the other hand, NC presented a narrowed particle size distribution, as can be observed by their lower polydispersity indices (0.19 – 0.20) compared to NE (0.27 – 0.30, $p \leq 0.05$). Polydispersity indices below 0.25 indicating an adequate homogeneity of these systems [7].

In order to obtain a better evaluation of the influence of the oily phase and the vesicle structure on the physicochemical characteristics of the formulations, we also carried out a two-way ANOVA analysis. This analysis showed the influence of the oily phase on the mean particle size as well as on the zeta potential. Formulations prepared with AKO showed a higher mean size and lower zeta potential ($p \leq 0.05$). According to Schaffazick *et al.* [11], the kind of the oily phase used as the core in preparation of polymeric nanocapsules could have a great influence on the particle mean size and polydispersity index due to the difference in its viscosity, hydrophobic characteristic and interfacial tension. Polydispersity indices were influenced by the vesicle structure ($p \leq 0.05$). NC presented lower polydispersity indices than NE, independent on the kind of the oily phase, as showed previously by the one-way ANOVA analysis.

Considering the overall results just after the preparation, the formulations prepared with GSO or AKO as oily phase presented physicochemical characteristics similar to those prepared with capric/caprilic triglyceride mixture [9,19], an oily phase widely used to prepare NC and NE [1,3,29,30].

Figure 1 shows the images obtained by transmission electron microscopy, revealing that nanocapsules and droplets of the nanoemulsions were spherical in shape. The results corroborate the particle size analysis, showing that the particles are present in the nanometric range (close to 200 nm). The morphological analysis allows observing the influence of the type of oily phase on

the mean particle size, as demonstrated by the two-way analysis of variance previously commented.

Table 2. Physicochemical characteristics of nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
NC-AKO	243 ± 06[a]	0.20 ± 0.00[a]	-7.34 ± 0.67[a]	6.97 ± 0.24[a]
NE-AKO	275 ± 49[a]	0.27 ± 0.01[b]	- 6.94 ± 0.64[a]	6.51 ± 1.19[a]
NC-GSO	228 ± 07[a]	0.19 ± 0.02[a]	-8.22 ± 1.34[a]	6.82 ± 0.02[a]
NE-GSO	239 ± 03[a]	0.30 ± 0.02[b]	-8.80 ± 1.11[a]	7.16 ± 0.08[a]

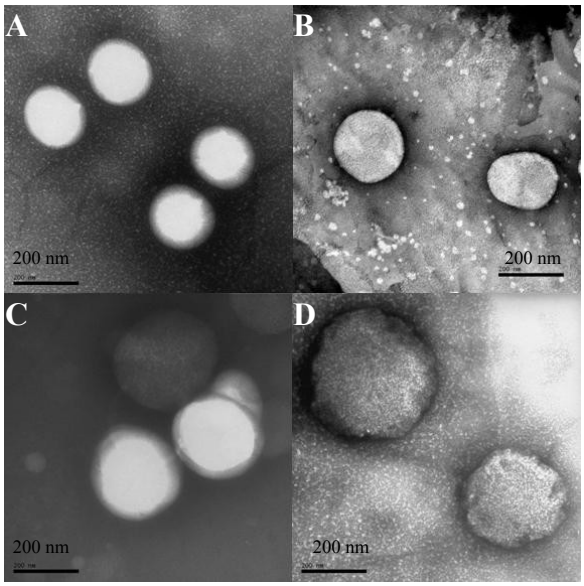

Figure 1. Transmission electron microscopy images of nanocapsules containing grape seed oil (**A**), nanocapsules containing almond kernel oil (**B**), nanoemulsion containing grape seed oil (**C**) and nanoemulsion containing almond kernel oil (**D**). Bar = 200 nm (150,000 x).

After showing the feasibility to obtain colloidal systems (NC and NE) with two alternatives vegetable oils, we choose a substance model to be entrapped in these systems. Benzophenone-3 was chosen because its sun protection properties, which could be efficiently associated to the antioxidant capacities of the oil phases. The physicochemical characteristics of

the benzophenone-3-loaded systems are presented in Table 3. All results are similar to those presented by the unloaded-systems, presenting nanometric mean particle size (230 – 252 nm), polydispersity index below 0.30, acidic pH range and negative zeta potential (- 7.0 to – 9.5 mV). The presence of the sunscreen did not modify the colloidal characteristics of both systems. Regarding the benzophenone-3 content and its encapsulation efficiency, all formulations showed content according to the theoretical concentration and encapsulation efficiency close to 100 % for all formulations (Table 4), independent on the oily phase or the vesicle structured. These results showed that the use of these vegetable oils could be an interesting alternative to be used in the development of dermatological and cosmetic formulations.

Table 3. Physicochemical characteristics of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
B3-NC-AKO	242 ± 11[a]	0.21 ± 0.01[a]	-8.76 ± 0.96[a]	7.03 ± 0.04[a]
B3-NE-AKO	233 ± 06[a]	0.28 ± 0.06[b]	-9.37 ± 1.35[a]	7.08 ± 0.02[a]
B3-NC-GSO	242 ± 11[a]	0.16 ± 0.02[a]	- 7.28 ± 1.00[a]	6.99 ± 0.11[a]
B3-NE-GSO	237 ± 12[a]	0.23 ± 0.04[a,b]	- 7.03 ± 0.34[a]	7.20 ± 0.15[a]

Table 4. Total content and encapsulation efficiency of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase (n = 3, mean ± standard deviation).

Formulation	Benzophenone-3 content ($mg.ml^{-1}$)	Encapsulation efficiency (%)
B3-NC-AKO	0.95 ± 0.04	99.98 ± 0.01
B3-NE-AKO	0.96 ± 0.04	99.98 ± 0.01
B3-NC-GSO	1.00 ± 0.06	99.98 ± 0.01
B3-NE-GSO	0.97 ± 0.04	99.99 ± 0.00

Stability studies

Taking in account the possibility of using these systems in the development of new products, we stored the developed formulations (containing or not benzophenone-3) at room temperature (25 °C) and protected from light. After 6 months, the physicochemical characteristics of the systems were reanalyzed (Table 5 and 6 for unloaded- and benzophenone-3-loaded nanoemulsions and nanocapsules).

As can be observed in Table 5, after 6 months of storage unloaded-NC and NE show similar mean particle size and polydispersity indices compared to the initial characteristics (Table 2), confirmed by a two-way ANOVA. However, all formulations presented a significant decrease in pH values, regardless of the formulation. The pH decline was more pronounced for those formulations containing GSO as oily core. Regarding NC, this decrease is usually explained due to the polymer hydrolysis or the relaxation of the polymer chains [11]. However, in our study this decrease was present in formulations with or without polymer (NC and NE, respectively). Thus, the decrease in pH values observed in our study could be explained by hydrolysis of the triglyceride chains and the respective increase of the free fatty acids content [31-34]. AKO and GSO are oils with high amounts of linoleic acid and other unsaturated fatty acids [21-23], which could undergo oxidative and hydrolytic reactions in contact with aqueous medium and temperature below 100 °C, forming free fatty acids [32,33].

The occurrence of oxidative/hydrolytic reactions during the storage time was confirmed by the development of a characteristic odor in all formulations after 6 months of storage. These results show that it is necessary to add antioxidant substances like butylated hydroxy anisole (BHA) or butylated hydroxy toluene (BHT) in future formulations in which these systems could be incorporated. In addition, as the decrease in pH values was higher for GSO formulations, these formulations presented a significant increase (in module) in their potential zeta values, explained also by the higher concentration of free fatty acids on the particle surface. Similar results were observed for benzophenone-3-loaded nanocapsules and nanoemulsions comparing the results presented in Table 3 and 5. In addition, the results showed no influence of this model substance in the stability of these systems (Table 5 and 6). Benzophenone-3 content and its encapsulation efficiency was not altered during the storage time, presenting values between 90 – 110 % and close to 100 %, respectively, after 3 and 6 months of storage (Table 7).

Based on the results obtained by instrumental analysis, no differences on the physicochemical stability of the different vesicles (NC or NE). However, macroscopic analysis allowed us to observe the occurrence of reversible aggregation (creaming) in the NE, regardless of the type of the oily phase. This finding shows the importance of the polymer layer around the oil droplet to improve the physical stability of the systems. Creaming could probably not be detected by the particle size and polydispersity index measurements due to its reversible characteristic by little agitation.

Table 5. Stability evaluation - Physicochemical characteristics of nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase after 6 months of storage at room temperature and protected from light (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
NC-AKO	222 ± 09^{a}	$0.18 \pm 0.00^{a,b}$	-08.59 ± 0.62^{a}	4.88 ± 1.14^{a}
NE-AKO	238 ± 42^{a}	$0.26 \pm 0.06^{a,b}$	-10.23 ± 1.31^{a}	4.78 ± 0.80^{a}
NC-GSO	220 ± 04^{a}	0.15 ± 0.05^{a}	$-10.30 \pm 1.41^{a,b}$	3.25 ± 0.17^{a}
NE-GSO	239 ± 23^{a}	0.34 ± 0.11^{b}	-13.00 ± 0.47^{b}	3.58 ± 0.35^{a}

Table 6. Stability evaluation – Physicochemical characteristics of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase after 6 months of storage at room temperature and protected from light (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
B3-NC-AKO	237 ± 10^{a}	0.19 ± 0.02^{a}	-09.79 ± 0.88^{a}	$4.77 \pm 0.85^{b,c}$
B3-NE-AKO	216 ± 16^{a}	0.29 ± 0.06^{a}	-12.19 ± 0.58^{a}	5.91 ± 1.95^{c}
B3-NC-GSO	221 ± 12^{a}	0.20 ± 0.04^{a}	-10.35 ± 0.63^{a}	$3.17 \pm 0.21^{b,c}$
B3-NE-GSO	214 ± 10^{a}	0.25 ± 0.03^{a}	-12.82 ± 2.02^{a}	$2.86 \pm 0.15^{a,b}$

Table 7. Stability evaluation - Total content and encapsulation efficiency of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase after 3 and 6 months of storage at room temperature and protected from light (n = 3, mean ± standard deviation).

Formulation	Benzophenone-3 content ($mg.ml^{-1}$)		Encapsulation efficiency (%)	
	3 months	6 months	3 months	6 months
B3-NC-AKO	0.99 ± 0.04	0.98 ± 0.02	99.37 ± 0.12	94.94 ± 3.56
B3-NE-AKO	1.02 ± 0.07	0.99 ± 0.10	97.89 ± 0.67	98.51 ± 0.49
B3-NC-GSO	0.99 ± 0.07	0.96 ± 0.07	99.07 ± 0.41	99.18 ± 0.18
B3-NE-GSO	1.04 ± 0.06	0.96 ± 0.04	99.39 ± 0.13	98.44 ± 0.15

Photodegradation studies

In order to evaluate the potential of these nanoparticulated systems prepared with alternative vegetable oils to prevent the photodegradation of some substances under UV radiation and to highlight their potential application in pharmaceutical and cosmetic field, we carried out a photodegradation study of benzophenone-3-loaded nanoparticles by their exposure to UVC radiation during 7 days. UVC was chosen due to its more energetic characteristic and the relative photostability of benzophenone-3 [35], allowing to reduce the experimental time. Although NE showed a lower physicochemical stability compared to the NC, photodegradation studies were carried out with all formulations, in order to compare the influence of the kind of the oily phase and the vesicle structure. A benzophenone-3 methanolic solution was used as a model of free benzophenone-3. The results of the photodegradation study are shown in Table 8. At the beginning of the experiment, all formulations as well as the methanolic solution presented similar benzophenone-3 content close to the theoretical concentration (1.00 $mg.ml^{-1}$). After 72 hours, benzophenone-3 content in the methanolic solution decreased to 0.74 $mg.ml^{-1}$, which was significantly lower ($p \leq 0.05$) than almost all nanoparticulated formulations (0.84 to 0.86 $mg.ml^{-1}$). However, after 168 hours of UV radiation, the protective effect of the entrapment of benzophenone-3 in NC and NE prepared with GSO and AKO against photodegradation was more pronounced. Benzophenone-3 content in methanolic solution decayed from 0.92 to 0.21 $mg.ml^{-1}$

(close to 80 % of photodegradation), whereas for formulations it decayed only around 10 – 30 % reaching to mean concentrations between 0.71 and 0.81 $mg.ml^{-1}$.

By a two-way analysis of variance, it could be observed that the kind of oily phase and the vesicle structure did not influence the protection of benzophenone-3 against the photodegradation. However, the photostability of benzophenone-3 was improved by its entrapment in the nanoparticle systems. This result suggests that benzophenone-3 is dissolved in the oily phase of the particles in a higher proportion [36] compared to its possible adsorption on the nanoparticle surfaces [11]. Solubilization of a sunscreen in the internal phase of coarse emulsions could led to a higher photostability of formulations [37]. The results from photodegradation obtained in the present study corroborate to previous studies reported in the literature to other substances. Weiss-Angeli *et al.* [38] showed the increase in the photostability of octyl methoxycinnamate against UVA radiation after its incorporation in polymeric nanocapsules. A previous study of our research group demonstrated the lower photodegradation of tretinoin if it is loaded in nanocapsules or nanoemulsion compared to a free tretinoin solution [9]. Formulations developed in these two cited studies were prepared with a capric/caprylic triglyceride mixture as oily phase, pointing out the potential of use the vegetable oils suggested in our study as alternative to compose the oily phase of such kind of nanoparticles structures (nanocapsules and nanoemulsions).

Table 8. Benzophenone-3 content of free benzophenone-3 solution (methanolic solution – MS) and benzophenone-3-loaded nanocapsules or nanoemulsions after 0, 72 and 168 h of UV irradiation (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Benzophenone-3 content ($mg.ml^{-1}$)		
	0 h	72 h	168 h
B3-NC-AKO	0.95 ± 0.00^{a}	$0.84 \pm 0.04^{a,b}$	0.79 ± 0.10^{b}
B3-NE-AKO	0.94 ± 0.00^{a}	0.86 ± 0.05^{b}	0.81 ± 0.10^{b}
B3-NC-GSO	0.99 ± 0.04^{a}	0.86 ± 0.03^{b}	0.78 ± 0.06^{b}
B3-NE-GSO	0.99 ± 0.02^{a}	0.85 ± 0.01^{b}	0.71 ± 0.02^{b}
MS	0.92 ± 0.03^{a}	0.74 ± 0.06^{a}	0.21 ± 0.12^{a}

CONCLUSIONS

This work showed for the first time the feasibility to prepare nanocapsules and nanoemulsions using grape seed oil and almond kernel oil, as alternative oily phases. Nanocapsules and nanoemulsions prepared with these oils presented nanometric size range and negative zeta potential. These parameters remained adequate after 6 months of storage at room temperature although a decline of pH was observed for all formulations. However, nanoemulsions presented higher polydispersity indices and occurrence of creaming after 6 months of storage, regardless of the type of the oily phase. The application of these alternative vegetable oils in the preparation of oil-based nanomaterials like nanocapsules and nanoemulsions with pharmaceutical or cosmetic purposes was demonstrated by an efficient entrapment of a model substance (benzophenone-3) to the nanoparticles as well as by showing the use of them to delay its photodegradation under UV radiation.

Acknowledgement. We thank the financial support received from Rede Nanocosméticos CNPq/ MCT.

REFERENCES

1. Beck, R.C.R., A.R. Pohlmann. & S.S. Guterres (2004) *J. Microencapsul.* **21**: 499–512.
2. Kidane, A. & P.Bhatt (2005) *Curr. Opin. Chem. Biol.* **9**: 347–51.
3. Beck, R.C.R., A. R. Pohlmann, C. Hoffmeister, M.R. Gallas, E. Collnot, U.F. Schaefer, S.S. Guterres & C.M. Lehr (2007) *Eur. J. Pharm. Biopharm.* **67**: 18–30.
4. Guterres, S.S., M.P. Alves & A.R. Pohlmann (2007) *Drug Target Insights* **2**: 147–57.
5. Beck, R.C.R., S.S. Guterres, R.J. Freddo, C.B. Michalowski, I. Barcellos & J.A.B. Funck (2003) *Acta Farm. Bonaerense* **22**: 11–5.
6. Beck, R.C.R., S.E. Hass, S.S. Guterres, M.I. Ré, E.V. Benvenutti & A.R. Pohlmann (2006) *Quim. Nova* **29**: 990–6.
7. Alves, M.P., A.L. Scarrone, M. Santos, A.R. Pohlmann & S.S. Guterres (2007) *Int. J. Pharm.* **341**: 215–20.
8. Fachinetto, J.M., A.F. Ourique, G. Lubini, S.B. Tedesco, A.C.F. Silva & R.C.R. Beck (2008) *Lat. Am. J. Pharm.* **27**: 668–73.
9. Ourique, A.F., A.R. Pohlmann, S.S. Guterres & R.C.R. Beck (2008) *Int. J. Pharm.* **352**: 1–4.
10. Barratt, G. (2003) *Cell. Mol. Life Sci.* **60**: 21–37.
11. Schaffazick, S.R., L.L. Freitas, A.R. Pohlmann & S.S. Guterres (2003) *Quim. Nova* **25**: 726–37.
12. Jäger, A., V. Stefani, S.S. Guterres & A.R. Pohlmann (2007) *Int. J. Pharm.***338**: 297–305.
13. Pohlmann, A.R., V. Weiss, O. Mertins, N. Pesce da Silveira & S.S. Guterres (2002) *Eur. J. Pharm. Sci.* **16** : 305–12.
14. Anton, N., J-P. Benoit & P. Saulnier (2008) *J. Control. Release* **128**: 185–99.
15. Couvreur, P., C. Dubernet & F. Puisieux (1995) *Eur. J. Pharm. Biopharm.* **41**: 2–13.
16. Alonso, M.J. (2004) *Biomed. Pharmacother.* **58**: 168–72.
17. Alvarez-Román, R., G. Barré, R.H. Guy & H. Fessi (2001) *Eur. J. Pharm. Biopharm.* **52**: 191–95.
18. Bouchemal, K., S. Briançon, E. Perrier & H. Fessi (2004) *Int. J. Pharm.* **280**: 241–51.
19. Friedrich, R.B., M.C. Fontana & R.C.R. Beck (2008) *Quim. Nova* **31**: 1131–6.
20. Kallinteri, P., S. Higgins, G.A. Hutcheon, C.B. Pourçain & M.C. Garnett (2005) *Biomacromolecules* **6**: 1885–94.

21. Beveridge, T.H.J., B. Girard, T. Kopp & J.C.G. Drover (2005) *J. Agr. Food Chem.* **53**: 1799–804.
22. Baydar, N.G., G. Ozkan & E.S. Cetin (2007) *Grasas Aceites* **58**: 29–33.
23. Martín-Carratalá, M.L., C. Lorens-Jordá, V. Berenguer-Navarro & N. Grané-Teruel (1999) *J. Agr. Food Chem.* **47**: 3688–92.
24. Espín, J.C., C. Soler-Rivas & H.J. Wichers (2000) *J. Agr. Food Chem.* **48**: 648–46.
25. Fessi, H., F. Puisieux & J.P. Devissaguet (1988) *Eur. Patent* 0274961 Al.
26. Martini, É., E. Carvalho & H. Teixeira (2007) *Quim. Nova* **30**: 930–34.
27. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (2005) *Gudeline on Validation of Analytical Procedure Q2 (R1): Text and Methodology.*
28. The United States Pharmacopeia 31 ed. (2008) United States Pharmacopeial Convention, Rockville, Maryland, USA.
29. Guterres, S. S., H. Fessi, G. Barrat, J.P. Devissaguet & F. Puisieux (1995) *Int. J. Pharm.* **113**: 57–63.
30. Teixeira, M., M.J. Alonso, M.M.M. Pinto & C.M. Barbosa (2005) *Eur. J. Pharm. Biopharm.* **59**: 491–500.
31. Calvo, P., J.L. Vila-Jato & M.J. Alonso (1996) *J. Pharm. Sci.* **85**: 530–36.
32. Fox, N.J. & G.W. Stachowiak (2007) *Tribol. Int.* **40**: 1035–46.
33. Reda, S.Y. & P.I.B. Carneiro (2007) Revista Analytica **27**: 60–7.
34. Schaffazick, S.R., A.R. Pohlmann & S.S. Guterres (2007) *Pharmazie*.**62**: 354–60.
35. Ribeiro, C (2006) "Cosmetologia aplicada a dermoestética", Pharmabooks, São Paulo.
36. Manconi, M., V. Donatella, C. Sinico, G. Loy & A.M. Fadda (2003) *Int. J. Pharm.* **260**: 261–72.
37. Milesi, S.S. & S.S. Guterres (2002) *Caderno de Farmácia* **18**: 81–7.
38. Weiss-Angeli, V., F.S. Poletto, L.R. Zancan, F. Baldasso, A.R. Pohlmann & S.S. Guterres (2008) *J. Biomed. Nanotechnol.* **4**: 80–9.

CAPÍTULO 2: Sistemas nanoestruturados contendo rutina: estudos de atividade antioxidante *in vitro* e fotoestabilidade

CAPÍTULO 2: Sistemas nanoestruturados contendo rutina: estudos de atividade antioxidante *in vitro* e fotoestabilidade

2.1 Introdução

No capítulo anterior, foi demonstrada a viabilidade do desenvolvimento de suspensões de nanocápsulas poliméricas e nanoemulsões empregando o óleo de semente de uva e de amêndoas doce, como fase oleosa, apresentando características compatíveis com o preparo e desenvolvimento de formulações nanoestruturadas.

Na continuidade dos estudos, neste capítulo, o óleo de semente de uva foi selecionado como fase oleosa para o desenvolvimento de formulações contendo rutina. Avaliou-se a atividade antioxidante *in vitro* mediante fotólise direta da rutina nas suspensões de nanocápsulas e nanoemulsões, visando avaliar a sua atividade antioxidante quando associada aos nanossistemas, e também o estudo da sua fotoestabilidade.

Para o desenvolvimento dessas formulações, foi necessária a escolha da melhor e mais estável concentração de rutina, considerando que esta apresenta solubilidade limitada em água e em alguns solventes orgânicos (CALABRÒ *et al*., 2005; MAULUDIN *et al*., 2009[a]; MAULUDIN *et al*., 2009[b]). Assim, foram realizados estudos preliminares, que levaram a utilização de uma mistura acetona e etanol na composição da fase orgânica da preparação.

A partir da metodologia descrita por POHLMANN e colaboradores (2008), foi avaliada a presença de nanocristais de rutina nas suspensões de nanocápsulas poliméricas, obtendo-se assim a maior concentração possível de ser encapsulada, que foi de 0,25 mg/ml. A rutina foi escolhida para esse estudo por ser uma substância com atividade antioxidante bem descrita e pela ausência de relatos na literatura da sua incorporação em nanocápsulas poliméricas e nanoemulsões.

PUBLICAÇÃO 2: Nanostructured systems containing rutin: *In vitro* antioxidant activity and photostability studies

Artigo aceito para publicação no periódico Nanoscale Research Letters

Nanostructured systems containing rutin: *in vitro* antioxidant activity and photostability studies

"Nanostructured systems containing rutin: antioxidant activity and photostability"

Juliana S. Almeida[a], Fernanda Lima[b], Simoní Da Ros[b], Luis O. S. Bulhões[c], Leandro M. de Carvalho[a,b], Ruy C. R. Beck[d*]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas, Av. Roraima, 1000, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil

[b] Departamento de Química, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil

[c] Departamento de Química, Universidade Federal de São Carlos, São Carlos, SP, 13565-905, Brazil

[d] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, RS, 90610-000, Brazil

* Corresponding author: Ruy C. R. Beck

Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, CEP 90610-000, Porto Alegre, RS, Brazil

Tel +55 51 3308-5951 Fax: +55 55 3308-5090

E-mail address: ruy.beck@ufrgs.br

Abstract

The improvement of the rutin photostability and its prolonged *in vitro* antioxidant activity were studied by means of its association to nanostructured aqueous dispersions. Rutin-loaded nanocapsules and rutin-loaed nanoemulsion showed mean particle size of 124.30 ± 2.06 and 124.17 ± 1.79, respectively, polydispersity index below 0.20, negative zeta potential, and encapsulation efficiency close to 100 %. The *in vitro* antioxidant activity was evaluated by the formation of free radical ·OH after the exposure of hydrogen peroxide to a UV irradiation system. Rutin-loaded nanostructures showed lower rutin decay rates [(6.1 ± 0.6) 10^{-3} and (5.1 ± 0.4) 10^{-3} for nanocapsules and nanoemulsion, respectively] compared to the ethanolic solution [(35.0 ± 3.7) 10^{-3} min^{-1}] and exposed solution [(40.1 ± 1.7) 10^{-3} min^{-1}] as well as compared to exposed nanostructured dispersions [(19.5 ± 0.5) 10^{-3} and (26.6 ± 2.6) 10^{-3}, for nanocapsules and nanoemulsion, respectively]. The presence of the polymeric layer in nanocapsules was fundamental to obtain a prolonged antioxidant activity, even if the mathematical modeling of the *in vitro* release profiles showed high adsorption of rutin to the particle/droplet surface for both formulations. Rutin-loaded nanostructures represent alternatives to the development of innovative nanomedicines.

Keywords: antioxidant activity, nanocapsules, nanoemulsions, nanoparticles, photostability, rutin.

1. Introduction

Currently there is a growing interest in the study of antioxidants [1,2]. Recent discoveries have pointed out the effects of free radicals in the body, which are involved in energy production, phagocytosis, and regulation of cell growth among others [3]. Oxidative stress is a condition that occurs in a system when the generation of reactive oxygen species exceeds the capacity of neutralization and disposal system. The instability may result from a lack of antioxidant capacity caused by disturbances in the production, distribution, or by an abundance of reactive oxygen species from endogenous sources or stressful environmental conditions. Oxidative stress has been implicated in a growing list of diseases such as cardiovascular and neurodegenerative diseases, cancer, arthritis, hemorrhagic shock, cataracts, as well as in aging processes [3-5].

Among the various classes of antioxidants, naturally occurring phenolic compounds have received much attention in recent years due to their inhibition of lipid peroxidation and lipoxygenase [4]. The antioxidant activity of phenolic compounds is mainly related to their reducing properties and chemical structure [6]. These characteristics play an important role in the neutralization or sequestration of free radicals and chelation of transition metals, acting both in the initiation step and in the propagation of oxidation [6]. The intermediate species formed by the action of antioxidant phenolic compounds are relatively stable due to the resonance of the aromatic ring in their structure [7].

Natural flavonoids are phenolic compounds known for their significant scavenging properties on oxygen radicals both *in vivo* and *in vitro* [8,9]. Many studies have showed the importance of their antiradical activity [10-13]. Moreover, their actions in humans have been subject of extensive research and natural flavonoids have been described to possess several biological activities such as antioxidant, anti-inflammatory, antitumor, and antiviral properties [14].

Rutin (quercetin-3-O-rutinoside), the glycoside of quercetin, is abundantly found and distributed in plants such as in buckwheat seed, fruits and fruit rinds, especially citrus fruits (orange, grapefruit, lemon). It presents important properties in human health like its significant scavenging properties on oxidizing species such as hydroxyl radical, superoxide radical, and peroxyl radical [15], as shown by many *in vitro* and *in vivo* experiments [16-18]. Furthermore, rutin has several pharmacological activities including anti-allergic, anti-inflammatory and vasoactive properties [15, 19, 20]. Rutin offers an advantage over other flavonoids, which in some occasions behave as pro-oxidant agents and catalyze oxygen production [15]. Therefore, it is considered a non-toxic

molecule and not oxidized. On the other hand, the main disadvantage of the molecule is its poor solubility in aqueous media, explaining its poor oral or topical bioavailability [19] and being a drawback to its conversion in adequate dosage forms.

In the last decade an alternative drug delivery approach was developed to overcome the poor water solubility of rutin by reducing its particle size [19]. A decrease in the drug particle size leads to an increase in the saturation solubility, to an enlarged surface area and to a higher dissolution velocity [21]. Formulating rutin as drug nanocrystals has significant importance to improve its physicochemical properties, especially its oral bioavailability. Some studies have shown techniques for lyophilization and spray drying nanocrystals of rutin in order to ensure their redispersibility as separate and non-aggregated particles. This characteristic is a critical point to improve the dissolution behavior of drugs, especially from a tablet dosage form [19, 20].

On the other hand, polymeric nanoparticles have been designed to encapsulate lipophilic drugs not only to improve their physicochemical properties, but also to target organs or tissues, to avoid drug degradation, to improve drug efficacy, and to circumvent drug toxicity [22]. Nanocapsules are polymeric nanoparticles composed of an oily core surrounded by a polymeric wall stabilized by surfactants at the particle/water interface [23]. The potential use of nanocapsules include the protection of drugs against inactivation in the gastrointestinal tract [21], delivery of poorly water-soluble compounds [24,25], and protection of sensitive materials to chemical degradation induced by UV light [26-28]. As the polymeric nanoparticles, nanoemulsions have been also investigated as drug delivery systems in the last years [29-32]. These colloidal systems are composed of oily nanodroplets stabilized by surfactants in an aqueous medium.

All these considerations into account, the main objective of our study was to develop aqueous rutin delivery systems and to evaluate the influence of the association of rutin to different nanostructures (nanocapsules and nanoemulsions) on its *in vitro* antioxidant activity and UV photostability. Nanocapsules and nanoemulsions were compared to establish the importance of the polymeric layer around the nanodroplets by means of a systematic physicochemical characterization of both formulations. The development of such formulations aimed to obtain aqueous systems containing rutin, as an intermediate or final pharmaceutical product. To the best of our knowledge, no report on the association of rutin to polymeric nanocapsules is currently available in the scientific literature. The administration of flavonoids by means of nanovectors may be useful for the development of advanced delivery systems for these powerful compounds,

in view of their adoption in primary and secondary disease prevention, either for oral or parenteral administration [33].

2. Material and Methods

2.1. Materials

Rutin, poly-(ε-caprolactone) (PCL) and sorbitan monostearate (Span 60®) were acquired from Sigma-Aldrich (São Paulo, Brazil). Polysorbate 80 (Tween 80®) was supplied by Henrifarma (São Paulo, Brazil). Grape seed oil was obtained from Dellaware (Porto Alegre, Brazil). All other chemicals and solvents presented pharmaceutical or HPLC grade and were used as received.

2.2. Preparation of nanoparticles

Rutin-loaded nanocapsule suspensions (R-NC) and nanoemulsions (R-NE) were prepared in triplicate (n = 3) by the interfacial deposition of preformed polymer method and spontaneous emulsification, respectively [34,35]. For the preparation of nanocapsules, an organic solution containing the grape seed oil (3.3 ml), sorbitan monostearate (0.776 g), the polymer (1.0 g), and acetone (147 ml) was subjected to magnetic stirring, at a temperature of 40 ° C. After 1 hour, an ethanolic solution containing the rutin (120 ml) was added to this organic phase maintaining the agitation for a further 10 min. Then, the organic phase was injected into an aqueous phase (534 ml) containing Tween 80® (0.776 g). Acetone was removed and the aqueous phase concentrated to 100 ml by evaporation at 40° C under reduced pressure reaching a final concentration of 0.25 mg ml^{-1} of rutin. All formulations were stored at room temperature and protected from light (amber glass flasks). Nanoemulsions were prepared using the same experimental conditions, but omitting the presence of the polymer in the organic phase. In addition, in order to evaluate the influence of rutin in the physicochemical characteristics of such nanostructures, blank formulations (placebo) were prepared similarly, but omitting the presence of rutin. These formulations were called B-NC and B-NE, for blank nanocapsules and blank nanoemulsions, respectively. Table 1 summarizes the abbreviation presented in Tables and Figures.

Table 1. List of nomenclatures presented in tables and figures.

Abbreviation	Meaning
B-NC	Blank nanocapsules (nanocapsules prepared without drug)
R-NC	Rutin-loaded nanocapsules
B-NE	Blank nanoemulsion (nanoemulsion prepared without drug)
R-NE	Rutin-loaded nanoemulsion
R-ES	Rutin ethanolic solution
k	Release rate constant according (monoexponential kinetic)
k_1	Release rate constant of the burst phase (biexponential kinetic)
k_2	Release rate constant of the sustained phase (biexponential kinetic)
a	Initial concentration of rutin at the burst phase (biexponential kinetic)
b	Initial concentration of rutin at the sustained phase (biexponential kinetic)
r	Regression coefficient
MSC	Model selection criteria

2.3. Characterization of nanoparticles

2.3.1. Rutin content

Rutin was assayed by liquid chromatography (LC). The chromatographic system consisted of a Gemini RP-18 column (150 x 4.60 mm, 5 µm, Phenomenex, Torrance, USA) and a Shimadzu instrument (LC-10AVP Pump, UV-VIS SPD-10AVP Module, Class-VP Software, Shimadzu, Tokyo, Japan). The mobile phase consisted of methanol/water (50:50% v/v) acidified with phosphoric acid (apparent pH 4.0) pumped at a flow rate of 1.0 $ml.min^{-1}$. The volume injected was 20 µl and rutin was detected at 352 nm. Rutin content ($mg.ml^{-1}$) was determined (n = 3) after its extraction with methanol from nanocapsules or nanoemulsions (1.6 ml of the formulation to 10 ml of methanol) and dilution with the mobile phase to a concentration of 20 $\mu g.ml^{-1}$. Validation of the LC assay demonstrated that this method was linear (y = 30322x – 6233.7, r = 0.9999, n = 5) in the range of 5 – 30 $\mu g.ml^{-1}$, precise (RSD: 1.86 % for repeatability and 0.70 % for intermediate precision) and accurate (RSD: 1.70 %). The specificity was tested in presence of the nanoparticle adjuvants and demonstrated that they did not alter the rutin assay [36].

2.3.2. Encapsulation efficiency

Free rutin was determined in the ultrafiltrate after separation of the nanoparticles by ultrafiltration/centrifugation technique (Microcon 10,000 MW, Millipore). Encapsulation efficiency (%) was calculated by the difference between the total and free rutin concentrations determined in the nanoparticles (drug content) and in the ultrafiltrate, respectively, using the LC method previously described.

2.3.3. pH measurements

The pH values of formulations were determined by immersion of the electrode directly in the dispersions using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil), at room temperature.

2.3.4. Particle size analysis, polydispersity indices and zeta potential

Particle sizes and polydispersity indices (n = 3) were measured by photon correlation spectroscopy after adequate dilution of an aliquot of the suspension in reverse osmosis water (Brookhaven Instruments Corporation, USA). The zeta potentials were determined after dilution of the samples in 10 mM NaCl aqueous solution (Brookhaven Instruments Corporation, Holtsville, USA).

2.3.5. Morphological analyses

Morphological analyses were conducted at Centro de Microscopia (UFRGS, Brazil) by transmission electron microscopy (TEM; Jeol, JEM 1200 Exll, Japan) operating at 80 kV. Diluted suspensions and nanoemulsions were deposited on specimen grid (Formvar-Carbon support films, Electron Microscopy Sciences), negatively stained with uranyl acetate solution (2% w/v) [37] and observed at different magnifications.

2.4 Antioxidant activity and photostability study

The system for the irradiation of the samples was developed by Carvalho et al. [38] and consisted of a 400 W high-pressure mercury lamp (Silvania) as UV radiation source, a cooling system based on air and water circulation, a thermo-regulator for the temperature control, a holder for 12 quartz tubes, and an aluminum-based block. 10 ml of each sample (R-NC, R-NE and a rutin ethanolic solution – R-ES) were placed in quartz tubes and exposed to UV radiation for 30

minutes (n = 3). Every 5 minutes, samples were taken from the tubes and analyzed by LC to determine the content of rutin. In order to determine the antioxidant activity, 40 µl H_2O_2 was added to the sample before starting the irradiation process. The amount of H_2O_2 was added in excess, since the concentration of H_2O_2 is 10 times the concentration of the sample and the lamp has a limiting quantum yield for the photolysis of H_2O_2. The artificial generation of the hydroxyl radical was carried out by the decomposition of H_2O_2 in the presence of UV radiation (Equation 1):

$$H_2O_2 + h\nu \rightarrow 2 \cdot OH \quad (1)$$

The kinetic constant for the reaction of rutin with the ·OH radical was determined by subtracting the contribution of direct photolysis of rutin from the ·OH radical reaction kinetic constant, according to the following equation (Equation 2):

$$k_{OH+h\nu} - k_{h\nu} = k_{OH} \quad (1)$$

where, $k_{OH+h\nu}$ represents the kinetic constant determined by the rutin decay after UV photolysis in the presence of H_2O_2, $k_{h\nu}$ represents the kinetic constant determined by the direct photolysis of rutin in absence of H_2O_2, and k_{OH} represents the liquid free radical reaction kinetic constant of the ·OH radical with the antioxidant.

2.5 In vitro rutin release assay

In vitro drug release profiles from rutin-loaded nanostructures were evaluated (n = 3) by the dialysis bag method, using water/ethanol (65:35 v/v) as medium, at 37°C [39]. The dialysis bag (Spectra Por 7, 10 Kd, Biosystems), containing 1 ml of the sample (0.25 mg ml^{-1}) was put into a glass test-tube containing 200 ml of dissolution medium. This system was maintained under constant moderate stirring during all the time. The withdrawal of 2 ml of the external medium from the system was done at predetermined time interval, replaced by an equal volume of fresh medium, and filtered through a 0.45 µm membrane. Rutin was assayed in the samples by LC according to the method previously described. However, the injection sample volume was increased to 100 µl to allow the assay of rutin at lower concentrations. This LC method was validated according to the following characteristics: linearity (y = 167806x + 1170.3, n = 3, r = 0.9997), concentration range (0.1 – 2.0 mg ml^{-1}), and precision (RSD: 0.90%).

In order to obtain a better understanding of the influence of the type of nanoparticle structure on the rutin release behavior, the mathematical modeling (MicroMath® Scientist® for Windows) was used to analyze the drug release profiles. Monoexponential ($C = C_0e^{-kt}$) and biexponential ($C = ae^{-k_1t} + be^{-k_2t}$) models were used to evaluate the rutin release profiles. The release rate constants are k, k_1 and k_2 and the initial concentration of rutin are C_0, a and b. The selection of the model that best fit the release profiles was based on the best correlation coefficient, the best model selection criteria (MSC), both provided by the software and the best graphic adjustment. The meaning of all these abbreviations is presented in Table 1.

2.6. Statistical analysis

Formulations were prepared and analyzed in triplicate. Results are expressed as mean ± SD (standard deviation). Two-way analysis of variance (ANOVA) was employed in the comparison of the experimental data. Post-hoc multiple comparisons were done by Tukey's test for significance at p–values ≤ 0.05. All analyses were run using the SigmaStat Statistical Program (Version 3.0, Jandel Scientific, USA).

3. Results and discussion

3.1. Preparation and physicochemical characterization of aqueous nanostructured systems

All formulations appeared macroscopically homogeneous and their aspects were similar to a milky bluish opalescent fluid (Tyndall effect). The physicochemical characteristics of the formulations are presented in Tables 2 and 3. As can be seen, the formulations presented drug content close to their theoretical value (0.24 and 0.25 mg ml^{-1}), mean particle size in the nanometric range (122 – 126 nm), acidic pH and negative zeta potential (between -26.0 and -27.0 mV). Polydispersity indices below 0.20 indicated an adequate homogeneity of these systems [26]. In addition, neither the presence of rutin nor the presence of the polymer influenced the physicochemical characteristics of the structures. Moreover, nanocapsules and droplets of the nanoemulsions were spherical in shape as showed by the images obtained by transmission electron microscopy (Figure 1). Although the type of the nanostructured system did not show any influence on the physicochemical characteristics, the morphological analyses by TEM revealed that rutin-loaded nanoemulsions (R-NE) presented rutin nanocrystals around the droplets when compared to its respective blank formulation (B-NE). Rutin nanocrystals were not observed in

nanocapsule formulation (R-NC). These results can be explained by the leakage of rutin from nanoemulsion due to the absence of the polymeric layer around the doplets and its subsequent crystallization in the aqueous dispersed medium as previously demonstrated for dexamethasone under storage [37,40].

Table 2 - Drug content, encapsulation efficiency and pH of rutin-loaded nanocapsules (R-NC) and rutin-loaded nanoemulsion (R-NE) as well as their respective blank formulations (B-NC and B-NE) (n = 3).

Formulation	Drug content (mg mL^{-1})	Encapsulation efficiency (%)	pH
B-NC	-	-	5.79 ± 0.03
R-NC	0.25 ± 0.01	93.33 ± 0.63	5.69 ± 0.08
B-NE	-	-	5.94 ± 0.10
R-NE	0.24 ± 0.01	93.83 ± 0.41	5.59 ± 0.01

- Not determined.

Table 3 - Particle size, polydispersity index and zeta potential of rutin-loaded nanocapsules (R-NC) and rutin-loaded nanoemulsion (R-NE) as well as their respective blank formulations (B-NC and B-NE) (n = 3).

Formulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)
B-NC	120.37 ± 2.44	0.11 ± 0.06	-20.55 ± 3.63
R-NC	124.30 ± 2.06	0.12 ± 0.02	-27.12 ± 9.19
B-NE	128.06 ± 3.38	0.13 ± 0.03	-26.03 ± 2.27
R-NE	124.17 ± 1.79	0.10 ± 0.02	-26.92 ± 1.45

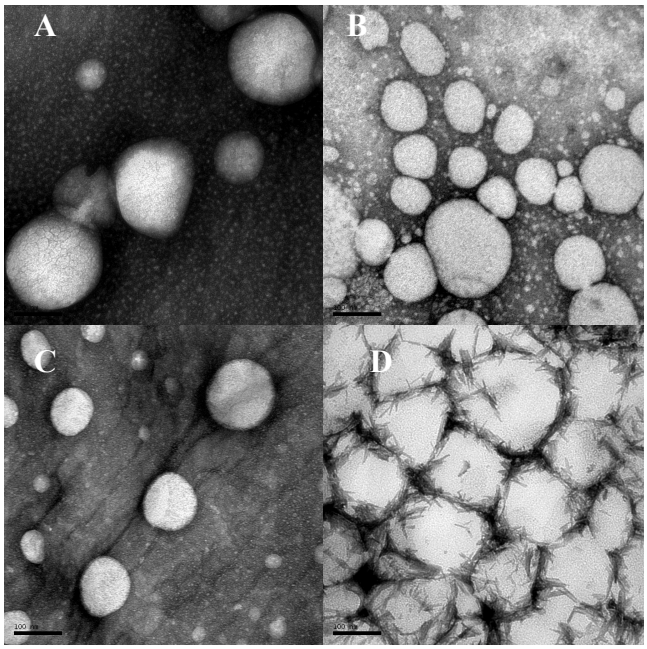

Fig. 1. Transmission electron microscopy images of (A) B-NC, (B) R-NC, (C) B-NE and (D) R-NE. Bar 100 nm (200,000 x)

3.2 Antioxidative activity,UV protodegradation and in vitro release assay

Figure 2 shows the rutin photodegradation obtained for both formulations (R-NC and R-NE), as well as for rutin ethanolic solution (R-ES). The degradation profiles of rutin in the nanostructured formulations (R-NC and R-NE) were according to a first kinetic order, and their respective degradation constants were not significantly different [$k = (6.1 \pm 0.6)\ 10^{-3}\ min^{-1}$, $k = (5.1 \pm 0.4)\ 10^{-3}\ min^{-1}$, respectively] (ANOVA, $p > 0.05$). However, compared to the rutin ethanolic solution [$k = (35.0 \pm 3.7)\ 10^{-3}\ min^{-1}$], both nanostructured formulations showed a significant lower rutin degradation rate (ANOVA, $p \leq 0.05$). These results showed that the association of rutin to the nanocapsules or nanoemulsions led to an increase of 5.3 and 6.9 times in the rutin photostability, respectively, during the 30 minutes of exposure to UV radiation.

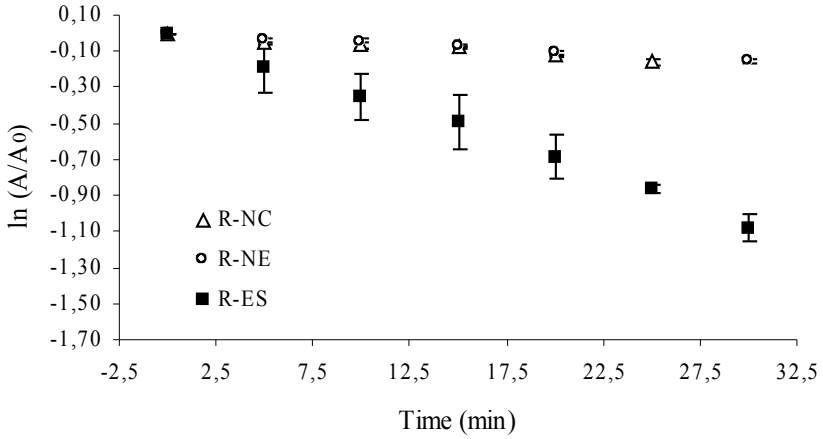

Fig. 2. Photodegradation profile of rutin-loaded nanostructures and rutin ethanolic solution during 30 minutes of UV irradation.

The antioxidant activity (radical scavenging activity) of rutin was evaluated by adding an amount of H_2O_2 in excess to the samples (before the irradiation) leading to the quantitatively controlled formation of ·OH radicals, which are the most harmful radicals formed under physiological conditions. In this case, the decay kinetics were significantly different among all samples (R-NC, R-NE and R-SE) and according to a first order kinetics (ANOVA, $p \leq 0.05$). The nanoparticle formulations (R-NC and R-NE) showed decay kinetics about 2.1 and 1.5 times slower than the solution [$k = (19.5 \pm 0.5)\ 10^{-3}\ min^{-1}$, $k = (26.6 \pm 2.6)\ 10^{-3}\ min^{-1}$ and $k = (40.1 \pm 1.7)\ 10^{-3}\ min^{-1}$, for R-NC, R-NE and R-SE, respectively], indicating a prolonged antioxidant activity of rutin, when associated to the nanostructures (Figure 3). The difference between the decay kinetics of the different nanoparticles (ANOVA, $p \leq 0.05$) could be explained by the presence of rutin nanocrystals adsorbed on the surface of the nanodroplets of nanomulsions (as observed by TEM - Figure 1), making rutin more readily accessible to react with the ·OH free radical.

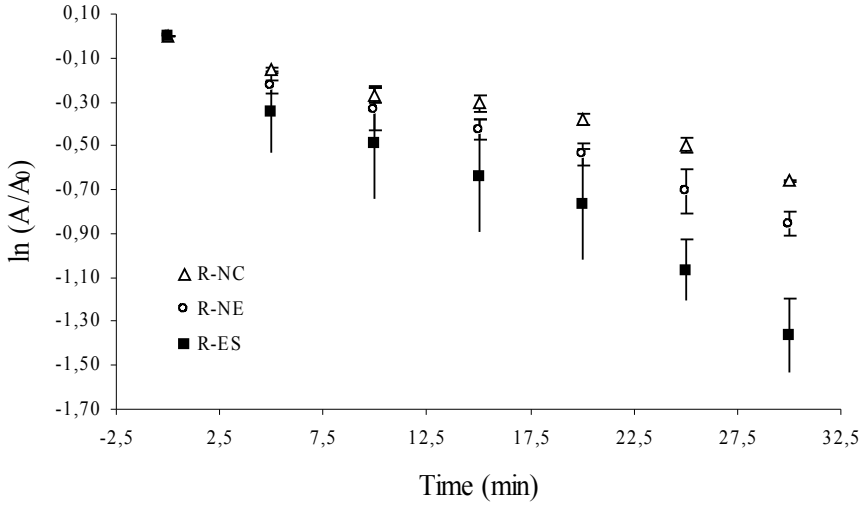

Fig. 3. *In vitro* antioxidant activity of rutin-loaded nanostructures and ethanolic solution after 30 minutes of UV irradiation.

In order to evaluate if the rutin release rate from the different nanoparticles could also play an important role in this different antioxidant behavior we carried out an *in vitro* drug release experiment using the dialysis bag technique. Figure 4 shows the *in vitro* drug release profiles from rutin-loaded nanocapsules (R-NC) and rutin loaded-nanoemulsions (R-NE). Both formulations promoted a rapid and similar release of rutin (release close to 100% in 24 hours). The release profiles were modeled using the monoexponential and biexponential equations. According to the values of the correlation coefficients and the criterion for model selection (MSC) the data fit better to the biexponential equation (Table 4) for both formulations, showing a burst release at an early stage followed by a sustained phase.

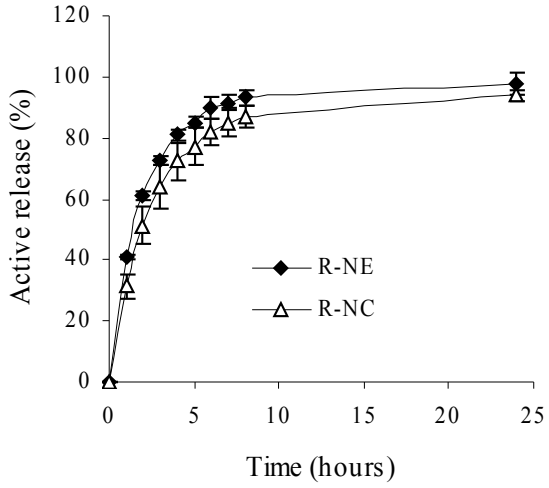

Fig. 4. *In vitro* rutin release profile from nanocarriers (R-NC and R-NE) using the dialysis bag method (n = 3).The lines correspond to the fitting to the biexponential equation.

Table 4 - Rate constants, correlation coefficients and MSC obtained by the mathematical modeling of drug release data from the different nanocarriers (R-NC and R-NE). (n=3)

	R-NC	R-NE
Monoexponential		
k (h^{-1})	0.3215 ± 0.0674	0.4336 ± 0.0242
r (range)	0.9979 ± 0.0010	0.9983 ± 0.0016
MSC (range)	2.9935 ± 0.2326	3.0585 ± 0.9828
Biexponential		
k_1 (h^{-1})	0.4113 ± 0.0765	0.8164 ± 0.6039
k_2 (h^{-1})	0.0310 ± 0.0163	0.1393 ±0.1993
a (mg mL^{-1})	0.8447 ± 0.0597	0.6107 ± 0.3337
b (mg mL^{-1})	0.1285 ± 0.0370	0.3201 ± 0.4125
r (range)	0.9997 ± 0.0002	0.9994 ± 0.0006
MSC (range)	6.5420 ± 0.5612	6.0185 ± 0.8945

The rate constants for the burst phase (k_1) were 0.4113 ± 0.0765 h^{-1} (R-NC) and 0.8164 ± 0.6039 h^{-1} (R-NE) and for the sustained phase the rate constants (k_2) were 0.0310 ± 0.0163 (R-NC) and 0.1393 ± 0.1993 h^{-1} (R-NE). For both phases, R-NC showed lower rate constants compared to R-NE. The percentage of rutin related the burst phase (a) for nanocapsules and nanoemulsions was about 85 % and 65 %, respectively. On the other hand, the percentage related to the sustained

phase was about 13 % for R-NC and 32 % for the R-NE. Such values showed that rutin is about 60-80% adsorbed on the surface of nanostructures [41,42], suggesting that the radical scavenging property of rutin against the ·OH radical occurred mostly at the interface particle/water. However, the influence of the drug release from the inner compartment of the nanocarriers cannot be discarded, considering the slower drug release rate from R-NC at both release phases. This hypothesis can be reinforced by the analysis of the release half-life of rutin, calculated according to the biexponential model. R-NC showed higher values (1.7 h and 22 h for the burst and sustained phase, respectively) compared to R-NE (0.8 h and 5 h for the burst and sustained phase, respectively). This faster drug release from R-NE can be explained by the absence of the polymer around the oily droplets as well as by the presence of nanocrystals on their surface, as observed by TEM.

4. Conclusions

This study showed for the first time the development of rutin-loaded nanocapsules and nanoemulsions, as aqueous intermediate or final systems to the development of nanomedicines containing rutin. Both formulations presented an increase in the rutin photostability and a prolonged *in vitro* antioxidant activity, even if the main mechanism of association of rutin was the adsorption on the particle/droplet surface, as determined by the mathematical modeling of drug release data. Moreover, the presence of the polymer did not show any significant influence in the increase of rutin photostability. However, its presence in nanocapsules led to a slower release rate and to a prolonged antioxidant activity against the strongly reactive ·OH radicals compared to the rutin-loaded nanoemulsions. The results showed that such nanostructured systems are a potential alternative to the preparation of rutin delivery systems to treat different diseases related to oxidative stress, including aging processes caused by the action of free radicals.

Acknowledgements

JSA thanks CAPES/Brazil for the fellowship. We thank Rede Nanocosméticos CNPq/MCT/Brazil, PRONEX/FAPERGS/CNPq, CAPES and CNPq for the financial support received as well as M.B. da Rosa for his contribution in this work.

References

1. C.M.M. Sousa, H. Rocha e Silva, G.M. Vieira-Jr., M.C.C. Ayres, C.L.S. Costa, D.S.Araújo, L.C.D. Cavalcante, E.D.S. Barros, P.B.M. Araújo, M.S. Brandão, M.H. Chaves, Quim. Nova 30, 351 (2007)
2. A.S. Darvesh, R.T. Carroll, A. Bishayee, W.J. Geldenhuys, C.J. Van der Schyf, Expert Rev. Neurother. 10, 729 (2010)
3. A.L.B.S. Barreiros, J.M. David, Quim. Nova 29, 113 (2006)
4. P. Brenneisen, H. Steinbrenner, H. Sies, Mol. Aspects Med. 26, 256 (2005)
5. U. Cornelli, Clin. Dermatol. 27, 175 (2009)
6. A. Michalak, Polish J. Environ. Stud. 15, 523 (2006)
7. S.S. Chun, D.A. Vatem, Y.T. Lin, K. Shetty, Process Biochem. 40, 809 (2005)
8. M.J. Abad, P. Bermejo, A. Villar, Gen. Pharmacol. 26, 815 (1995)
9. C. La Casa, I. Villegas, C. A. de la Lastra, V. Motilva and M. J. Martín Calero, J. Ethnopharmacol. 71, 45 (2000)
10. A. Solomon, S. Golubowicz, Z. Yablowicz, M. Bergman, S. Grossman, A. Altmann, Z. Kerem, M.A. Flaishman, J. Agric. Food Chem. 58, 6660 (2010)
11. K.L. Krishna, K. Mruthunjaya, J.A. Patel, I. J. Pharmacol. 6, 72 (2010)
12. O.A. Fawole, S.O, Amoo, A.R. Ndhlala, M.E. Light, J.F. Finnie, J. Van Staden, Journal of Ethnopharmacol. 127, 235 (2010)
13. H. Jiang, W.Q. Zhan, X. Liu, S.X. Jiang. Nat. Prod. Res. 22, 1650 (2008)
14. J. Yang, J. Guo, J. Yuan, LWT 41, 1066 (2008)
15. M.L. Calabrò, S. Tommasini, P. Donato, R. Stancanelli, D. Raneri, S. Catania, C. Costa, V. Villari, P. Ficarra, R. Ficarra, J. Pharm. Biomed. Anal. 36, 1019 (2005)
16. Korkmaz, D. Kolankaya, J. Surg. Res. DOI: 10.1016/j.jss.2009.03.022 (2009)
17. A.R. Verma, M. Vijayakumar, C.S. Mathela, C.V. Rao, Food Chem. Toxicol. 47, 2196 (2009)
18. S. Itagaki, J. Oikawa, J. Ogura, M. Kobayashi, T. Hirano, K. Iseki, Food Chem. 118, 426 (2010)
19. R. Mauludin, R.H. Müller, C.M. Keck, Int. J. Pharm. 370, 202 (2009a)
20. R. Mauludin, R.H. Müller, C.M. Keck, Eur. J. Pharm. Sci. 36, 502 (2009b)

21. P. Couvreur, G. Barrat, E. Fattal, P. Legrand, C. Vauthier, Ther. Drug Carrier Systems 19, 99 (2002)
22. C.E. Mora-Huertas, H. Fessi, A. Elaissari, Int. J. Pharm. 385, 113 (2010)
23. A.R. Pohlmann, V. Weiss, O. Mertins, N. P. da Silveira, S.S. Guterres, Eur. J. Pharm. Sci. 16, 305 (2002)
24. J.M. Fachinetto, A.F. Ourique, G. Lubini, S.B. Tedesco, A.C.F. Silva, R.C.R. Beck, Lat. Am. J. Pharm. 27, 668 (2008)
25. M. Teixeira, M.J. Alonso, M.M.M. Pinto, C.M. Barbosa, Eur. J. Pharm. Biopharm. 59, 491 (2005)
26. A.F. Ourique, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck, Int. J. Pharm. 352, 1 (2008)
27. J.S. Almeida, L. Jezur, M.C. Fontana, K. Paese, C.B. Silva, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck, Lat. Am. J. Pharm. 28, 168 (2009)
28. M.C. Fontana, K. Coradini, S.S. Guterres, A.R. Pohlmann, R.C.R. Beck, J. Nanosci. Nanotechnol. 10, 3091 (2010)
29. S.R. Schaffazick, L.L. Freitas, A.R. Pohlmann, S.S. Guterres, Quim. Nova 25, 726 (2003)
30. S.S. Guterres, M.P. Alves, A.R. Pohlmann, Drug Target Insights 2, 147 (2007)
31. A.R. Pohlmann, G. Mezzalira, C. De G. Venturini, L. Cruz, A. Bernardi, E. Jäger, A.M.O. Battastini, N. P. da Silveira, S.S. Guterres, Int. J. Pharm. 359, 288 (2008)
32. L. Mei, Y. Zhang, Y. Zheng, G. Tian, C. Song, D. Yang, H. Chen, H. Sun, Y. Tian, K. Liu, Z. Li, L. Huang, Nanoscale Res. Lett. 4, 1530 (2009)
33. G. Leonarduzzi, G. Testa, B. Sottero, P. Gamba, G. Poli, Curr. Med. Chem. 17, 74 (2010)
34. H. Fessi, F. Puisieux, J.-P. Devissaguet, European Patent 0274961 Al (1988)
35. É. Martini, E. Carvalho, H. Teixeira, Quim. Nova 30, 930 (2007)
36. ICH – International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use: Gudeline on Validation of Analytical Procedure Q2 (R1): Text and Methodology (2005)
37. R.C.R. Beck, S.S. Guterres, J.A.B. Funck, R.J. Freddo, C.B. Michalowski, I. Barcellos, Acta Farm. Bonaer. 22, 11 (2003)
38. L.M. Carvalho, C. Spengler, J.C. Garmatz, P.C. Nascimento, D. Bohrer, L. Del-Fabro, G. Radis, A.A. Bolli, A.M. Moro, S.C. Garcia, M.B. Rosa, Quim. Nova 31, 1336 (2008)
39. H. Li, X. Zhao, Y. Ma, G. Zhai, L. Li, H. Lou, J. Control. Release 133, 238 (2009)

40. R. B. Friedrich, M. C. Fontana, A. R. Pohlmann, S. S. Guterres, R. C. R. Beck, Quim. Nova, 31, 1131 (2008)
41. L. Cruz, L.U. Soares, T. Dalla Costa, G. Mezzalira, N.P. Silveira, S.S. Guterres, A.R. Pohlmann, Int. J. Pharm. 313, 198 (2006)
42. M.C. Fontana, K. Coradini, S.S. Guterres, A.R. Pohlmann, R.C.R. Beck, J. Biomedi. Nanotechnol. 5, 254 (2009)

CAPÍTULO 3 Hidrogéis contendo rutina para administração cutânea: desenvolvimento, caracterização reológica e cicatrização de feridas *in vivo*

CAPÍTULO 3: Hidrogéis contendo rutina para administração cutânea: desenvolvimento, caracterização reológica e cicatrização de feridas *in vivo*

3.1 Introdução

Há muitos anos, as plantas tem sido empregadas em diversas partes do mundo, não apenas como fonte de alimentos, mas como fontes de substâncias relacionadas a diversos processos de cura de doenças, desempenhando um papel importante na vida dos humanos. Várias espécies vegetais apresentam atividades biológicas como antimicrobiana, hipoglicêmica, anti-inflamatória e antifúngica, estando envolvidas também no controle de doenças respiratórias e cutâneas (KHALIL *et al*., 2007). Estudos indicam que os flavonóides representam uma das classes de compostos de origem vegetal amplamente responsáveis por esses efeitos, sendo muito investigados principalmente nas doenças relacionadas ao estresse oxidativo e nos processos de cura de lesões cutâneas (SUMITRA *et al*., 2005; PATTANAYAK *et al*., 2008).

Até o momento foi apresentada neste trabalho a possibilidade de emprego de nanocápsulas e nanoemulsões utilizando óleos vegetais com reconhecida atividade antioxidante e a associação a essas nanoestruturas de um flavonóide (rutina). Essa associação resultou na proteção da rutina frente à radiação UV e o prolongamento da sua atividade antioxidante *in vitro*. A atividade antioxidante *in vitro* foi influenciada pelo tipo de nanocarreador, sendo que as nanocápsulas apresentaram uma atividade durante um período de tempo maior em relação às nanoemulsões.

A partir destes resultados, neste capítulo é apresentado o desenvolvimento de hidrogéis contendo rutina livre ou associada às nanocápsulas poliméricas e o seu efeito sobre a cicatrização cutânea e o perfil oxidativo na pele lesada. Até o momento não existem relatos na literatura a respeito da utilização dermatológica de formulações contendo rutina para melhorar a cicatrização cutânea, tampouco do efeito da associação da rutina sobre essa atividade.

PUBLICAÇÃO 3: Hydrogels containing rutin intended to cutaneous administration: development, rheological characterization and *in vivo* wound healing

Artigo em redação para ser submetido ao periódico *Soft Materials*

Hydrogels containing rutin intended to cutaneous administration: development, rheological characterization and *in vivo* wound healing

J.S. Almeida,[1] K. Coradini,[4] D.M. Benvengnú,[2] N. Boufleur,[3] P. Reckziegel,[2] R.C.S. Barcelos, [2] L.M. de Carvalho,[1] M.E. Bürger,[2] R.C.R. Beck,[4*]

[1] Programa de Pós-Graduação em Ciências Farmacêuticas, Av. Roraima, 1000, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil

[2] Programa de Pós-Graduação em Farmacologia, Av. Roraima, 1000, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil

[3] Departamento de Fisiologia e Farmacologia, Av. Roraima, 1000, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil

[4] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, RS, 90610-000, Brazil

* Corresponding author: Ruy C. R. Beck

Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, CEP 90610-000, Porto Alegre, RS, Brazil

Tel +55 51 3308-5951 Fax: +55 55 3308-5090

E-mail address: ruy.beck@ufrgs.br

ABSTRACT

Hydrogels containing rutin at 0.025 % (w/w) were developed and their *in vivo* efficacy on cutaneous wound healing was evaluated in rats. The effect of the association of rutin to polymeric nanocapsules on this activity was also investigated. Hydrogels showed adequate pH values (5.50 - 6.50) and pseudoplastic non-Newtonian behavior. *In vivo* healing effect of hydrogels was evaluated by the regression of skin lesions. After 6 days of treatment, hydrogels containing rutin presented a reduction of wound area compared to the control hydrogels. Analyses of the oxidative stress showed a decrease of levels of lipid peroxidation, protein carbonil and total proteins as well as an increase of the levels of glutathione, vitamin C and catalase after the treatment with hydrogels containing rutin. This study shows for the first time the feasibility of using dermatological formulations containing rutin to improve skin wound healing. However, this activity could not be improved by the association of rutin to polymeric nanocapsules prior to the preparation of hydrogels.

Keywords: rutin, nanocapsules, hydrogels, polyphenols, rheology, wound healing.

INTRODUCTION

Cutaneous wound healing involves a series of processes such as inflammation, migration and proliferation of cells such as fibroblasts and contraction of the formed collagen (1, 2). As a result of inflammation, reactive oxygen species (ROS) are generated continuously (3) and are deleterious to wound healing process due to the harmful effects on cells and tissues (4).

The process of wound healing is promoted by many natural resources which are composed of active substances like flavonoids (5–8). Several pharmacological effects have been reported for flavonoids, such as anti-inflammatory (7, 9), antimicrobial (4), neuroprotective (10) and radical-scavenging activities (11–13).

Rutin (quercetin-3-rutinoside) (Fig. 1) a polyphenolic compound is known as one of the most common naturally occurring flavonoids with a variety of biochemical and pharmacological activities (9). The important properties of rutin are significant free radical scavenging properties such as hydroxyl radical, superoxide radical and peroxyl radical. However, it imposes restraints to pharmaceutical use due to its poor solubility in aqueous media (14). In addition, several studies were successfully carried out to overcome the poor water solubility of rutin by reducing its particle size (15, 16) and also improved its antioxidant activity as a result of the increased solubility when complexed to ciclodextrins (14).

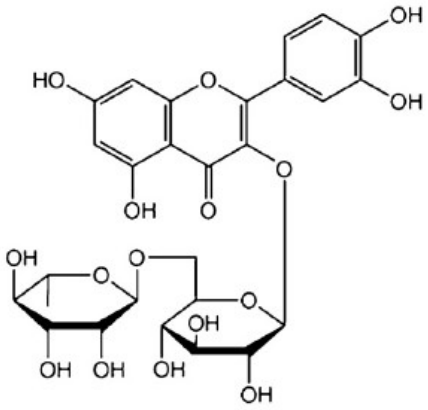

FIGURE 1 Chemical structure of rutin.

Polymeric nanoparticles are submicronic systems and have been extensively studied for oral and parenteral administration (17–20). Recently, their use for skin delivery of substances has been investigated (21–24). Nanocapsules are a particular type of nanoparticles presenting vesicular structure stabilized by surfactants (18, 25). Their small size facilitates its formulation in

dermatological products and confortable application to the skin (26). Besides, other advantages of cutaneous administration of formulations containing polymeric nanocapsules include the improvement of the beneficial activity of the loaded substances (23, 27), protection of sensitive materials to chemical degradation induced by UV light (19, 27–29), retention of substances on the upper layers of skin (21) and the control of the drug release from the dermatological formulation (24).

Previous *in vitro* and *in vivo* studies showed that polymeric nanoparticles are able to improve the antioxidant effect of drugs against lipid peroxidation (18, 30). Recently, we reported a study focused on the evaluation of the *in vitro* antioxidant and photoprotective activities of rutin-loaded nanocapsules and nanoemulsions (31). This study showed a prolonged antioxidant activity against the strongly reactive ·OH radicals for rutin-loaded nanocapsules and an improvement of its photostability.

Taking all these aspects into account, the objective of this study was to develop a hydrogel containing rutin and to study the efficacy of this formulation on the *in vivo* cutaneous wound healing by the analysis of the regression of skin and markers of oxidative stress in the lesions. In addition, we evaluate the potential improvement of such biological activity by loading rutin in polymeric nanocapsules. To the best of our knowledge there is no report in the literature demonstranting the efficacy of hydrogels containing rutin or rutin-loaded nanocapsules in the skin wound healing

MATERIAL AND METHODS

Materials

Rutin, poly(ε-caprolactone) (PCL) and sorbitan monostearate (Span 60®) were acquired from Sigma-Aldrich (São Paulo, Brazil). Polysorbate 80 was supplied by Henrifarma (São Paulo, Brazil). Grape seed oil was obtained from Delaware (Porto Alegre, Brazil). Carbopol Ultrez® 10 NF and triethanolamine were acquired from DEG (São Paulo, Brazil). All other chemicals and solvents presented pharmaceutical or HPLC grade and were used as received.

Preparation and characterization of nanocapsules

Rutin-loaded nanocapsule suspensions (R-NC) were prepared (n = 3) by the interfacial deposition of preformed polymer method (32), at a rutin concentration of 0.25 mg mL^{-1} (31). An

organic solution containing the grape seed oil (3.3%), sorbitan monostearate, the polymer, and acetone was subjected to magnetic stirring, at a temperature of 40 ° C. After 1 hour, an ethanolic solution containing the rutin was added to this organic phase maintaining the agitation for a further 10 min. Then, the organic phase was injected into an aqueous phase containing polysorbate 80 Acetone was removed and the aqueous phase concentrated to 100 ml by evaporation at 40° C under reduced pressure. All formulations were stored at room temperature and protected from light (amber glass flasks). Blank NC formulations (B-NC) were also prepared, without the addition of rutin to the organic phase.

After preparation, the nanocapsules were characterized based on the following parameters: rutin content, encapsulation efficiency, pH, particle size, polydispersity index and zeta potential. Rutin was assayed by liquid chromatography according to a method previously validated by our research group (31). Rutin content (mg mL^{-1}) was determined after extraction of rutin from nanocapsules with methanol. Encapsulation efficiency was determined by the quotient of the rutin entrapped and the total rutin content by an ultrafiltration/centrifugation technique (Microcon 10,000 MW, Millipore). pH values were determined directly in the dispersion using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil). Particle sizes and polydispersity indices were estimated by photon correlation spectroscopy (PCS) after adequate dilution of an aliquot of the suspension in reverse osmosis water (Brookhaven Instruments Corporation, Holtsville, USA). The zeta potentials were determined after dilution of the samples in 10 mM NaCl aqueous solution (Brookhaven Instruments Corporation, Holtsville, USA).

Preparation of hydrogels

Hydrogels were prepared according to the quali-quantitative formula described in Table 1. Briefly, Carbopol Ultrez® 10 NF was dispersed using free rutin aqueous solution in polysorbate 80. This dispersion was neutralized with triethanolamine to obtain an adequate semisolid formulation for skin administration (HG-R). Imidazolidinyl urea was added as preservative. Using the same method, hydrogel containing rutin-loaded nanocapsules suspension (R-NC) was also prepared. Final concentration of rutin in hydrogels was set to 0.025 %. All formulations were prepared in triplicate. In addition, in order to study the influence of the presence of rutin and rutin-loaded nanocapsules on the physicochemical characteristics of hydrogels, formulations containing blank nanocapsules (HG-B-NC) and a hydrogel base (HG-C) were prepared.

TABLE 1 Quali-quantitative composition of hydrogels.

Component	HG-R	HG-R-NC	HG-B-NC	HG-C
Carbopol Ultrez® 10 NF	0.5 g	0.5 g	0.5 g	0.5 g
Imidazolidinyl urea	0.6 g	0.6 g	0.6 g	0.6 g
Triethanolamine	0.2 mL	0.2 mL	0.2 mL	0.2 mL
Rutin	0.025 g	-	-	-
R-NC	-	ad 100 g	-	-
B-NC	-	-	ad 100 g	-
Ultra pure water	ad 100 mg	-	-	ad 100 mg

Physicochemical characterization of hydrogels

Rutin content in hydrogels

Rutin was assayed in hydrogels by liquid chromatography (LC). Approximately 3.0 g of each formulation was placed in a 25 mL volumetric flask. Methanol was added and the flask was maintained under moderate stirring by ultrasound for 30 minutes. This sample was centrifuged, diluted with the mobile phase and then filtered through a filter (Quantitative filter, JP41 – 28 μm) and a 0.45 μm nylon membrane (Millipore®) before LC analysis. The chromatographic system consisted by a Gemini RP-18 column (150 x 4.60 mm, 5 μm particle size, 110 Å pore diameter, Phenomenex, Torrance, USA) and a Shimadzu LC-20A system, (LC-20AT pump, SPD-20AV detector, CBM-20A system controller, SIL-20A auto sampler, Shimadzu, Tokyo, Japan). The mobile phase at a flow rate of 1.0 mL min^{-1} consisted by methanol-water (50:50 v/v) acidified with phosphoric acid (pH 4.0). The volume injected was 20 μL and the rutin was detected at 352 nm. The method was linear ($r^2 = 0.9998$, $y = 36139x - 10939$) in the range of 5.0 – 30.0 μg mL^{-1}, precise (repeatability: 2.37 %; intermediate precision: 2.74 %), accurate (99.97 %) and specific. The specificity was tested in presence of all components of the formulation and demonstrated that these components did not alter the rutin assay.

Determination of pH

pH values of hydrogels (n = 3) were determined in the dispersion of an aliquot of the formulation in ultrapure water (10 %, w/v) using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil).

Evaluation of the rheological properties of hydrogels

The aim of this characterization was to evaluate the influence of rutin as well as of nanocapsules on the rheological properties of hydrogels to attest adequate properties to cutaneous administration. Rheological analysis was carried out at 25 ± 1 °C using a rotational viscosimeter (LVDV II+ Pro model, Brookfield, USA) and a spindle SC4-25 with a small sample adapter. The data obtained were analyzed with the Rheocalc software (V3.1-1 version, Brookfield, USA). The shear stress ramp was applied for 1200 s and 10 different points were recorded, using a shear rate interval of 0.05 s^{-1}. The rheograms were analyzed using different flow models: Power Law ($\tau = \kappa\gamma^{0.5}$), Casson ($\tau = \tau_o^{0.5} + \eta^{0.5}\gamma^{0.5}$), Binghan ($\tau = \tau + \eta\gamma$) and Herschel-Bulkley ($\tau = \tau_o + \kappa\gamma^{0.5}$), where τ is the shear stress, τ_o is the yield stress, η is the viscosity, n is the index of flow, κ is the index of consistency and γ is the shear rate (33).

Determination of the spreadability

The sample was introduced in a central hole (1 cm) of a mold glass plate. The mold plate was carefully removed and the sample was pressed subsequently with glass plates of known weights, with intervals of 1 min between each plate (34). Spreading areas reached by samples between each addition of a glass plate were measured in millimeters in vertical and the horizontal axes. Results were expressed in terms of the spreading area as a function of the applied mass according to the following equation Eq. (1) and represent the mean of three determinations:

$$S_i = \frac{d^2 \cdot \pi}{4} \qquad (1)$$

in which S_i is the spreading area (mm²) after the application of a determined mass i (g), and d is the mean diameter (mm) reached by each sample. The spreading area was plotted against the plate weights to obtain the spreading profiles.

The spreadability factor (S_f) was also calculated and represents the spread a formulation is able to expand on a smooth horizontal surface when a gram of weight is added on it, under the

conditions described in the methodology above. The following equation Eq. (2) is used to calculate the spreadability factor (24, 35):

$$S_f = \frac{A}{W} \qquad (2)$$

in which S_f(mm^2 g^{-1}) is the spreadability factor resulting from the ratio between (A) the maximum spread area (mm^2) after the addition of the sequence of weights used in the experiment and (W) the total weight added (g).

In vivo wound healing effect

Animal treatment

Male Wistar rats (300-350g) purchased from Bioterio Central of the Federal University of Santa Maria (UFSM) were used in this experiment. The animals were kept under temperature (22 ± 2ºC) and controlled illumination (12 h light/dark cycles), 1 week before and during the experiments. They had free access to food and water and were kept in individual cages of clean metal. This experiment followed the model of Pattanayak and co-workers (4) with modifications. The experimental procedure was approved by the Animal Ethics Committee of the Federal University of Santa Maria (Protocol number 23081.006726/2010-89).

Animals were anesthetized with xilasin (20 mg/kg – IM) and ketamine (100 mg/kg – IM). After tricotomization and assepsia with ethanol 70%, two square cutaneous excisions (4 cm^2) were made on the back of each animal until the fascia muscular exhibition. The wounds were then washed and measured. Animals were divided into 5 groups (n = 7) and received the treatment with hydrogels containing free rutin and rutin-loaded nanocapsules (HG-R and HG-R-NC, respectively), hydrogel containing blank nanocapsules and a hydrogel base, like negative controls (HG-B-NC and HG-C, respectively). In order to evaluate the healing activity of hydrogel containing free rutin and rutin-loaded nanocapsules, alantoin hydrogel (1 %) was prepared as positive control (HG-A). The topical administration (0.5 g/animal) was done twice a day during the whole experimental period (6 days). The amount of hydrogel was enough to cover the wounds. After the sixth day, animals were anesthetized and killed by decapitation with guillotine. The wounded skin was removed for the stress oxidative tests.

Morphometry of the wounds

The maximum length and width of each wound were measured on the day the wound was made and at days 3 and 6. The area of the wound was calculated from these measurements. The degree of contraction of the wound was determined from the difference between the initial and final areas (36). The wounds contraction rate was determined through the delimitation of the lateral edges of the lesion area with pen hydrographic help and transparent sheet. The measure of the surface area was made through the program AutoCad 2007® and the results expressed in wound reduction percentage.

Biochemical analyses

The samples of the wounded skin were homogenized in 10 volumes (w/v) of buffer 0.01 M Tris-HCl, pH 7.4. Part of the sample was centrifuged to 3000 rpm for 15 minutes and the supernatant was used to the other biochemical analyses. The extent of lipid peroxidation (LPO) was determined by analysing the levels of thiobarbituric acid reactive substances (TBARS) according to Ohkawa and co-workers (37). Another part of the sample was separated for protein carbonyl determination (38). The proteins levels were estimate by the method of Lowry and co-workers (39). Glutathione (GSH) level was determined according to the method of Boyne and Ellman (40), after modifications. Vitamin C levels like described by Galey and co-workers (41) with modifications for Jacques-Silva and co-workers (42) and endogenous antioxidant status was evaluated by catalase activity (CAT) by the method of Aebi and co-workers (43).

Statistical analysis

All formulations were prepared and analyzed in triplicate. Results are expressed as mean ± SD (standard deviation). One-way analysis of variance (ANOVA) was employed in the comparison of the experimental data. Post-hoc multiple comparisons were done by Tukey's test for significance at p–values ≤ 0.05. All analyses were run using the SigmaStat Statistical Program (Version 3.0, Jandel Scientific, USA). Results of the *in vivo* experiments were subjected to the one-way analysis of variance (ANOVA) followed by the Duncan's test. Values were represented by mean ± S.E.M. (standard error of mean).

RESULTS AND DISCUSSION

Physicochemical characteristics of rutin-loaded nanocapsules

R-NC presented a macroscopic homogeneous appearance, rutin content close to their theoretical value (0.25 mg mL^{-1}), and encapsulation efficiency close to 100 %. All formulations presented acid pH values, nanometric particle mean size between 100 and 150 nm, low polydispersity index (< 0.20) and negative zeta potential, according to our previous study (31).

Physicochemical properties of hydrogels

All hydrogels showed satisfactory organoleptic characteristics in terms of odor, color and appearance. Hydrogels containing rutin-loaded nanocapsules or blank nanocapsules presented a homogeneous white color. On the other hand, hydrogels containing free rutin presented glossy, yellowish color. Although the lower aqueous solubility of rutin, its incorporation in the hydrogels was feasible by using an aqueous dispersion of polysorbate 80. The base hydrogel presented a transparent aspect. Table 2 shows the rutin content and pH obtaining for the different hydrogels. HG-R and HG-R-NC formulations presented rutin content close to their theoretical value (0.25 mg g^{-1}). All hydrogels presented pH values around 5.7, which is adequate for the cutaneous administration. These values were not influenced by the presence of the rutin or nanocapsules (ANOVA, $p \leq 0.05$). These results are in agreement to those related by Marchiori and co-workers (24), which developed hydrogels containing dexamethasone-loaded nanocapsules.

TABLE 2 Physicochemical characteristics of hydrogels (mean ± standard deviation, n = 3).

Formulation	Rutin content (mg mL^{-1})	pH
HG-R	0.255 ± 0.007	5.80 ± 0.08
HG-R-NC	0.243 ± 0.008	5.76 ± 0.06
HG-B-NC	-	5.64 ± 0.10
HG-C	-	5.77 ± 0.06

Figure 2 shows the rheograms of all formulations obtained by plotting the applied shear rate as a function of the shear stress. Formulations showed non-Newtonian behavior, in which the

viscosity decreases with increasing the shear rate, as better observed in Figure 3. Rheological properties of pharmaceutical formulations are of great importance, because they assist in evaluation of their quality and stability (26, 34). Rheograms obtained for all formulations were modeled using different equations in order to establish the better rheological behavior (plastic or pseudoplastic). They fitted better to the Herschel-Bulkley's model, showing regression coefficients higher than 0.97 (Table 3). The yield stress (YS), flow indices (n) and consistency (κ) were determined for all formulations after preparation and the results are shown in Table 4. According to the selected model, almost all flow indices were lower than 1 indicating a pseudoplastic behavior. The yield stress (YS) is the minimum tension demanded so that a material starts to flow. In this parameter, no difference was observed between all formulations (ANOVA, $p>0.05$). Consistency index (k) was not significantly different (ANOVA, $p>0.05$) among hydrogels containing nanocapsules with and without rutin (k = 31868 ± 4752 mPa.s and k = 37002 ± 3391 mPa.s for HG-R-NC and HG-B-NC, respectively) and hydrogels containing free rutin (k = 29276 ± 4070 mPa.s). Also, no difference (ANOVA, $p>0.05$) in consistency index was observed between formulations containing rutin (HG-R-NC and HG-R) and the base hydrogel, showing that its presence did not influence the viscosity of the formulations. However, a significant difference in consistency index (ANOVA, $p\leq0.05$) was observed for hydrogels containing blank nanocapsules (k = 37002 ± 3391 mPa.s) compared to base hydrogel (k = 24589 ± 5059 mPa.s). According to this results, the presence of nanocapsules tend to increase the consistency of those semisolids, explained by the presence of other polymer [poly(ε-caprolactone)] than Carbopol.

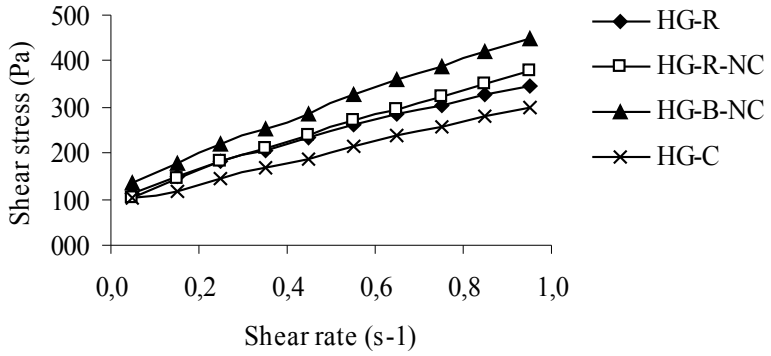

FIGURE 2 Rheograms of hydrogels (n = 3).

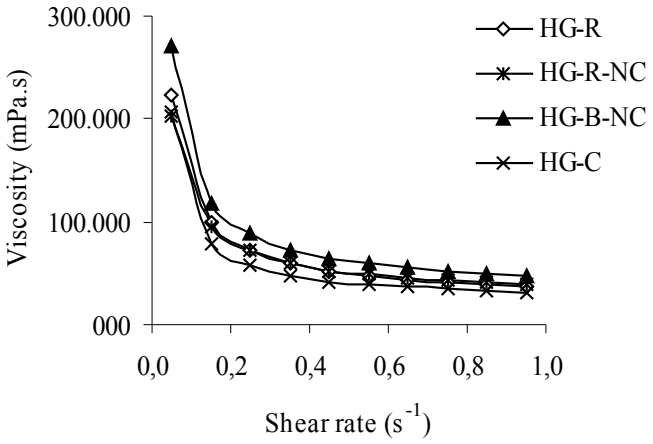

FIGURE 3 Graphic representation of viscosity (mPa.s) of hydrogels in relation to the shear rate (s^{-1}) (n = 3).

TABLE 3 Regression coefficient (r^2) for various flow models in shear rate-shear stress curve.

Formulation	Power Law	Casson	Binghan	Herschel-Bulkley
HG-R	0.919 ± 0.030	0.964 ± 0.013	0.922 ± 0.023	**0.971 ± 0.020**
HG-R-NC	0.905 ± 0.015	0.959 ± 0.007	0.918 ± 0.015	**0.971 ± 0.009**
HG-B-NC	0.911 ± 0.009	0.965 ± 0.003	0.924 ± 0.006	**0.973 ± 0.002**
HG-C	0.926 ± 0.025	0.976 ± 0.010	0.953 ± 0.007	**0.987 ± 0.012**

TABLE 4 Flow index (*n*), consistency index (κ), yield stress (YS) and spreadability factor (S_f) of the hydrogels.

Formulation	*n*	κ (mPa.s)	YS (Pa)	S_f ($mm^2.g^{-1}$)
HG-R	0.79 ± 0.08^{a}	$29276 \pm 4070^{a,b}$	6.41 ± 0.91^{a}	$4.45 \pm 0.47^{a,b}$
HG-R-NC	0.92 ± 0.01^{a}	$31868 \pm 4752^{a,b}$	6.84 ± 1.20^{a}	3.23 ± 0.24^{a}
HG-B-NC	0.92 ± 0.05^{a}	37002 ± 3391^{b}	9.26 ± 0.86^{a}	2.99 ± 0.17^{a}
HG-C	0.94 ± 0.15^{a}	24589 ± 5059^{a}	6.79 ± 2.17^{a}	5.24 ± 0.18^{b}

Means, in column, with the same letter are not statistically different (Anova, TuKey test, $p \leq 0.05$).

Spreadability profiles of hydrogels are shown in Figure 4. Hydrogels containing free rutin (HG-R) and the hydrogel base (HG-C) presented a higher spreadability compared to those formulations containing nanocapsules (HG-R-NC and HG-B-NC) regardless of the presence of the rutin. On the other hand, both formulations containing nanocapsules presented similar spreadability behavior. In order to have a better comparison of the spreadability, spreadability factor (S_f) for the different hydrogels was calculated (Table 4). Spreadability factor was significantly higher for HG-R and HG-C (4.45 ± 0.47 $mm^2.g^{-1}$ and 5.24 ± 018 $mm^2.g^{-1}$, respectively) compared to formulations containing nanocapsules regarding the containing (S_f = 3.23 ± 0.24 $mm^2.g^{-1}$ and 2.99 ± 0.17 $mm^2.g^{-1}$ for HG-R-NC and HG-B-NC, respectively) (ANOVA, $p \leq 0.05$). These results are in accordance to those reported by Marchiori and co-workers (24).

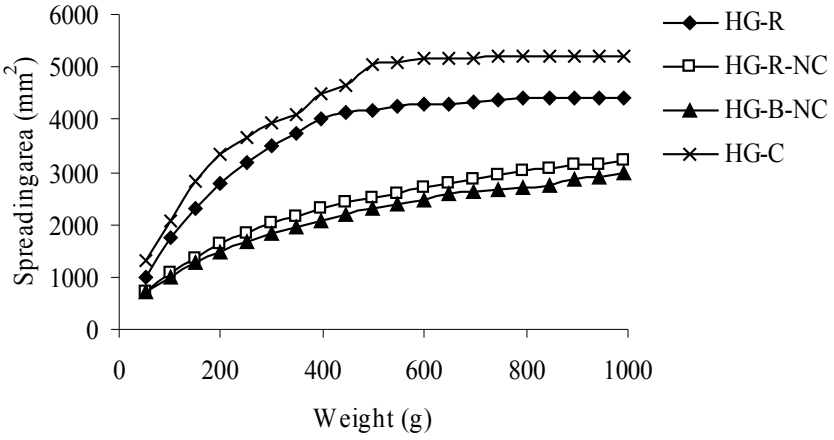

FIGURE 4 Graphic representation of spreadability of hydrogels (n = 3).

Evaluation of the *in vivo* wound healing

Would healing involves different phases, as epithelialization where keratinocytes migrate from the lower skin layers and divide, deposition of connective tissue and contraction that is the process where the wound contracts, narrowing or closing the wound (2, 4). The healing activity of hydrogels containing rutin was investigated by regression of the cutaneous lesion in rats after the cutaneous treatment during 6 days. In addition, markers of oxidative stress in the lesions of rats were evaluated after the treatment. Hydrogel containing alantoin (HG-A) was used as a positive control (44) and presented similar characteristics compared to other hydrogels regarding

pH (pH = 5.5). In addition, consistency index and spreadability factor showed similar values (k = 25237.66 ± 3514.22 mPa.s and S_f = 2.65 ± 0.30 $mm^2.g^{-1}$) regarding the hydrogels developed in this study. As a negative control, hydrogels containing all other components without rutin or nanocapsules was employed (HG-C).

The size of the wound area and percentage of contraction in rats were monitored during the experimental period (6 days) to assess the wound healing potential of the hydrogels. The results are shown in Table 5. At the third day, only a difference in the percentile of the wound area between the group treated with HG-R-NC and HG-B-NC was observed (ANOVA, $p \leq 0.05$). On the other hand, after six days of treatment, both hydrogels containing rutin (free or associated to polymeric nanocapsules) presented a lower percentage of wound area compared to the negative control (HG-C) and similar wound area compared to the positive control (HG-A) (ANOVA, $p \leq 0.05$). Formulation prepared with blank nanocapsules presented a similar wound area compared to the negative control formulation. These results demonstrated that hydrogels containing rutin at the concentration of 0.025 % (HG-R or HG-R-NC) present a similar healing effect compared to the formulation containing alantoin. No significant difference was observed between HG-R and HG-R-NC, which could be explained by the preferencial localization of rutin on the particle surfaces, as reported in our previous work (31). It should be highlighted the differences in the concentration of both substances in the hydrogels. Hydrogels containing rutin at 0.025 % showed a similar effect compared to a hydrogel containing alantoin at 1 %. In addition, nor a healing effect neither a delay in the regression of the wound area was observed after the administration of the hydrogels containing blank nanocapsules, showing the feasibility of using such formulations in those treatments as well as the absence of a significant healing activity of the grape seed oil at 3.3 % in nanocapsules and in the HG-B-NC. This result could be explained by the concentration of oil in the formulation as well as the loss of substances during its refining process (45).

TABLE 5 Percentage of the wound area after 3 and 6 treatment days

Formulation	Day 3	Day 6
HG-C	100.27 ± 2.54	75.21 ± 1.39
HG-A	101.38 ± 6.45	61.89 ± 1.64 [b]
HG-B-NC	112.20 ± 4.29	70.67 ± 3.30
HG-R	96.78 ± 3.30	63.17 ± 2.04 [b]
HG-R-NC	95.26 ± 3.23 [a]	64.26 ± 1.14 [b]

Results are expressed as mean ± S.E.M (n = 7). [a] Statistical evaluation of the experimental results regarding HG-B-NC, [b] Statistical evaluation of the experimental results regarding HG-C ($p \leq 0.05$).

Süntar and co-workers (5) investigated in vivo wound healing activity in extracts of *Sambucus ebulus* L. at 1 % concentration. It was demonstrated wound contraction close to contraction value of the reference drug (Madecassol® 1 %) when treated topically. A flavonoid component, quercetin 3-O-glucoside was isolated from active fraction as one of the active wound healing ingredient. The same research group (6) evaluated the wound healing and anti-inflammatory activities of extracts of *Hyperilum perforatum* L. using in vivo models. In this investigation, the active fractions revealed the presence flavonoids such as rutin and isoquercitrin suggesting that anti-inflammatory activity of the active fractions might have contributed in the wound healing effect of the plant. However, although wound healing activity of flavonoids in extracts have been previously reported, to the best of our knowledge, this activity for isolated rutin incorporated in an alternative dermatological formulation has not been reported yet.

In order to study the mechanism involved in the wound healing activity of rutin present in our innovative formulations, different markers of the oxidative stress were studied in the wound tissues after the different treatments. The use of antioxidants has been an effective strategy for therapeutic approaches to disorders like fibrosis as well as in the acceleration of wound healing (13). At high concentrations, reactive oxygen species (ROS) play a major role in the tissue damage. The antioxidant activity may be due to potent radical-scavenging activity of the phenolics present in the extract that is mainly due to their redox properties (4). Polyphenols such

as flavonoids also inhibit the activity of enzymes including lipoxygenases, cyclooxygenases, collagenases and also increase the activity of antioxidant enzymes, such as SOD yet improved inflammatory response and antioxidant status of cells (46). The results obtained by Lopes and co-workers (2) in the evaluation of extracts of *Stryphnodendron polyphyllum* Mart. and *Stryphnodendron obovatum* Benth. (at 2.5 %) on the wound healing in rats showed that the antioxidant activity observed for these extracts contributed favorably to the wound healing.

Figure 5 shows the effect of the administration of the different hydrogels on the cutaneous lesions in rats regarding different oxidative markers. Measurement of end products of lipid peroxidation is a widely used assay for oxidative damage, including the TBARS method (47). TBARS levels after the treatment of the wounds (Fig. 5A) showed that the groups treated with rutin (HG-R and HG-R-NC) presented lower TBARS levels compared to the groups treated with the negative control formulation (HG-C) and similar TBARS levels compared to the group treated with the alantoin hydrogel (ANOVA, $p > 0.05$). In addition, the group treated with hydrogel containing rutin-loaded nanocapsules (HG-R-NC) showed lower TBARS levels compared to the group treated with hydrogel containing blank nanocapsules (ANOVA, $p \leq 0.05$). No difference was observed between the negative control formulation and the hydrogel containing blank nanocapsules.

Regarding the carbonyl protein levels (Fig. 5B), all groups showed a decrease in this marker compared to the negative control formulation (HG-C) (ANOVA, $p \leq 0.05$). Many diseases and pathological processes have been connected with protein oxidation in damages promoted by the oxidative stress (46). Carbonyl protein can be formed from the reaction of aldehydes (formed by lipid peroxidation and sugar oxidation) with proteins. Increased levels of carbonyl protein are present in many stress conditions and pathologies associated with oxidative stress as cystic fibrosis, Alzheimer's and Parkinson's diseases, diabetes among others (46). Figure 5C shows the level of total proteins after the cutaneous treatment. All groups presented higher levels of total proteins, compared to the control group treated with HG-C as well as to the group treated with HG-B-NC (ANOVA, $p \leq 0.05$). The increase of total proteins content indicates the presence of an active synthesis and deposition of matrix proteins in the granulation tissues (13).

Hydroxyl radicals are involved in the initiation of lipid peroxidation in biological membranes (46). The most important endogenous antioxidant is glutathione (GSH). This

tripeptideo is a scavenging hydroxyl radical and is presenting in high concentration in kidney and liver cells. ROS produced by the cellular metabolism are maintained in low intracellular concentration by the enzymes SOD, GPx and CAT (46). The decrease of the activity of antioxidant enzyme is one responsible for the increased in lipid peroxidation. This can lead to a loss of membrane fluidity, membrane integrity and loss of cell functions leading to circulation of toxic metabolites and free radicals (48). Figure 6 shows the effects of all treatments of the cutaneous lesions in rats on the antioxidant defenses. Groups treated with HG-R-NC presented higher GSH levels regarding the groups treated with HG-C, HG-B-NC and HG-A (ANOVA, $p \leq 0.05$) as showed in figure 6A. There was no statistical difference between the groups treated with hydrogels containing free rutin or rutin-loaded nanocapsules. On the other hand, higher vitamin C levels (Fig. 6B) were observed for the group treated with HG-R-NC compared to all other groups, including that treated with free rutin (ANOVA, $p \leq 0.05$) except the group treated with HG-C and group treated with HG-A with showed a similar effect (ANOVA, $p > 0.05$).

Finally, a significant difference (ANOVA, $p \leq 0.05$) was observed for group treated with HG-R compared to group treated HG-C, HG-B-NC, HG-A regarding catalase enzyme levels (Fig. 6C). However, no difference (ANOVA, $p > 0.05$) was observed between formulations containing rutin-loaded nanocapsules (HG-R-NC) and free rutin (HG-R). The antioxidant enzymes, such as catalase are known to quench the superoxide radical and thus prevent the damage of cells caused by free radicals. Thus the enhanced wound healing may be due to the free radical scavenging action of the flavonoid (49). These results are similar to the study developed by Verma and co-workers (45) that evaluated the antioxidant activity of a series of fractions of plant against the lipid peroxidation. An increase of glutatione (GSH) and catalase (CAT) levels after treatment in rats were observed. The investigation of the antioxidant components in such fractions indicated the presence of rutin.

The results observed for the oxidative markers are well related to the results obtained by the morphometric analysis. The presence of rutin showed a important antioxidant activity in the skin, but its nanoencapsulation did not improved it. As earlier discussed, this result could be explained by the main mechanism of association of rutin, showed in our previous work (31): the adsorption of rutin on the nanocapsule surface.

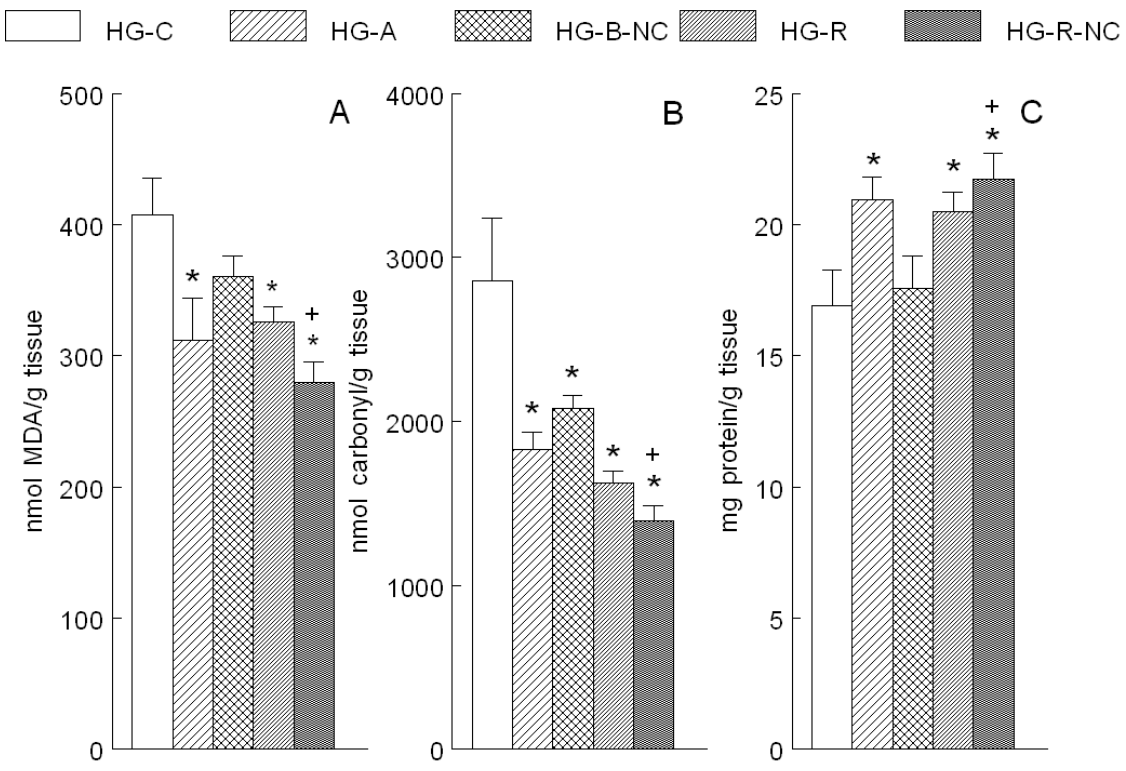

FIGURE 5 Effect of treatment with hydrogels on the cutaneous lesions in rats. A) Levels of lipid peroxidation by the method of thiobarbituric acid reactive substances (TBARS), B) determination protein carbonyl levels and C) total plasma proteins levels. Results are expressed as mean ± S.E.M. (n = 7). Statistical evaluation of the experimental results regarding: $^{+}$HG-B-NC, * HG-C ($p \leq 0.05$)

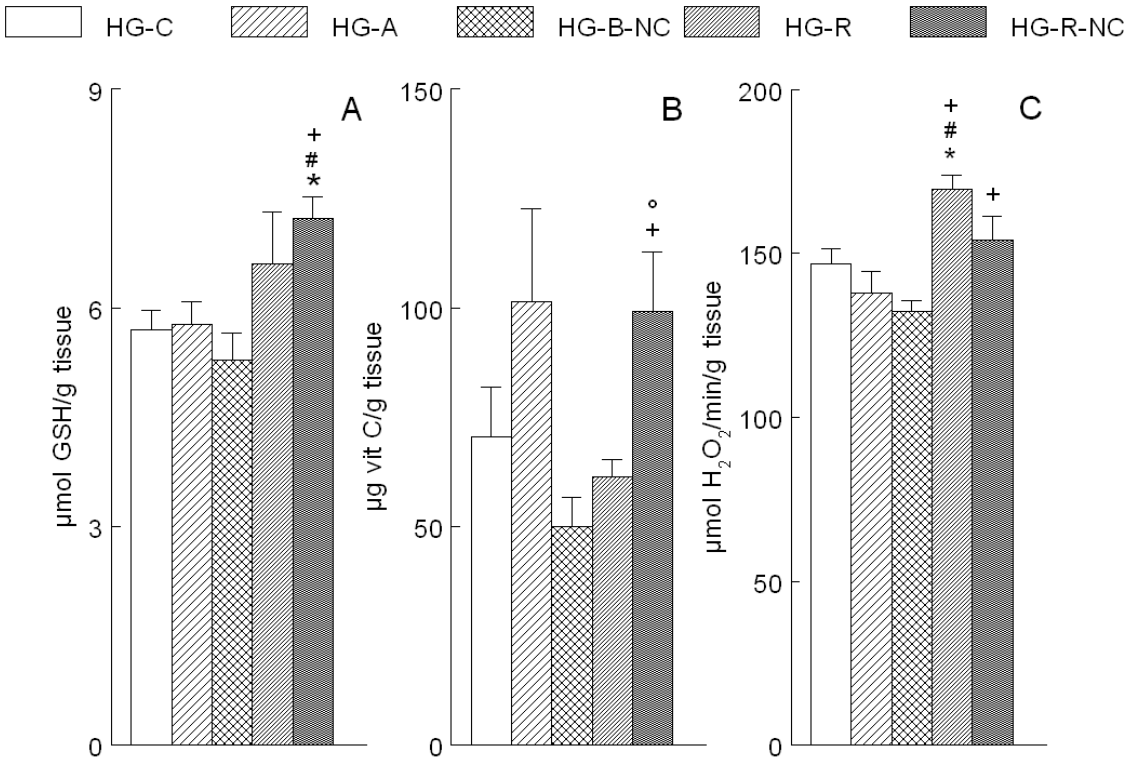

FIGURE 6 Effect of treatment on the antioxidant defenses in the cutaneous lesions in rats. A) glutathione (GSH) levels, B) Vitamin C levels, C) enzyme catalase levels. Results are expressed means ± S.E.M. (n = 7). Statistical evaluation of the experimental results regarding: $^{+}$ HG-B-NC, * HG-C, $^{\#}$ HG-A, ° HG-R ($p \leq 0.05$)

CONCLUSIONS

This work showed the development of two different hydrogels containing rutin (free rutin or rutin associated to polymeric nanocapsules) at 0.025 % (w/w), as potential dermatological formulations. Both formulations showed physicochemical properties compatible to their cutaneous administration, including the pH and rheological behavior, as a pseudoplastic non-Newtonian hydrogel. Regarding the biological meaning of our study, we showed for the first time the feasibility of using formulations containing rutin to improve skin wound healing in an animal model. Grape seed oil at 3.3 % in the formulations did not show a significant wound healing

effect. Analysis of oxidative stress markers suggests that the radical-scavenging activity and the increase in the activity of antioxidant enzymes are intimately related to the efficacy of rutin in such treatment. On the other hand, this activity could not be improved by its association to polymeric nanocapsules prior to the preparation of hydrogels at a rutin concentration of 0.025 % (w/w).

ACKNOWLEDGEMENTS

JSA thanks CAPES/Brazil for the fellowship. We thank Rede Nanocosméticos CNPq/MCT/Brazil for the financial support received.

REFERENCES

[1] Mensah, A.Y., Sampson, J., Houghton, P.J., Hylands, P.J., Westbrook, J., Dunn, M., Hughes, M.A., and Cherry, G.W. (2001) Effects of *Buddleja globosa* leaf and its constituents relevant to wound healing. *J. Ethnopharmacol*. 77: 219–226.

[2] Lopez, G.C., Sanches, A.C.C, Nakamura, C.V., Dias Filho, B.P., Hernandes, L., Mello, J.C.P. (2005) Influence of extracts of *Stryphnodendron polyphyllum* Mart. and *Stryphnodendron obovatum* Benth. on the cicatrisation of cutaneouns wounds in rats. *J. Ethnopharmacol.* 99: 265–272.

[3] Rajasekaran, N. S., Nithya, M., Rose, C., and Chandra, T.S. (2004) The effect of finger millet feeding on the early responses during the process of wound healing in diabetic rats. *Biochim. Biophys. Acta*. 1689: 190–201.

[4] Pattanayak, S.P., and Sunita, P. (2008) Wound healing, anti-microbial and antioxidant potential of *Dendrophthoe falcata* (L.f) Ettingsh. *J. Ethnopharmacol.*120: 241–247.

[5] Süntar, I.P., Akkol, E.K., Yalçin, F.N., Koca, U., Keles, H., and Yesilada, E. (2010[a]) Wound healing potential of Sambucus ebulus L. leaves and isolation of an active component, quercetin 3-O-glucoside. *J. Ethnopharmacol*. 129: 106–114.

[6] Süntar, I.P., Akkol, E.K., Yilmazer, D., Baykal, T., Kirmizibekmez, H., Alper, M., and Yesilada, E. (2010[b]) Investigations on the in vivo wound healing potential of Hypericum perforatum L. *J. Ethnopharmacol*. 127: 468–477.

[7] Koca, U., Süntar, I.P., Keles, H., Yesilada, E., and Akkol, E.K. (2009) *In vivo* anti-inflammatory and wound healing activities of Centaurea iberica Trev. ex Spreng. *J. Ethnopharmacol*. 126: 551–556.

[8] Panchatcharam, M., Miriyala, S., Gayathri, V.S., and Suguna, L. (2006) Curcumin improves wound healing by modulation collagen and decreasing reactive oxygen species. *Mol.Cell. Biochem*. 290: 87–96.

[9] Han, Y. (2009) Rutin has therapeutic effect on septic arthritis caused by *Candida albicans*. *Int. Immunopharmacol*. 9: 207–211.

[10]Khan, M.M., Ahmad, A., Ishrat, T., Khuwaja, G., Srivastawa, P., Khan, M.B., Raza, S.S., Javed, H., Vaibhav, K., Khan, A., and Islam, F. (2009) Rutin protects the neural damage induced by transient focal ischemia in rats. *Brain Res*. 1292: 123–135.

[11]Colomé, L.M., Raffin, R.P., Assumpção, E.R., Pohlmann, A.R. and Guterres, S.S. (2010) Theospheres Based on Theobroma Grandiflorum Seed Butter: Development of Innovative Nanoparticles for Skin Application. *Soft Materials*. 8: 72–88.

[12]Chaudhuri, S., Banerjee, A., Basu, K., Sengupta, B., and Sengupta, P. K. (2007) Interaction of flavonoids with red blood cell membrane lipids and proteins: Antioxidant and antihemolytic effects. *Int. J. Biol. Macromol.* 41: 42–48.

[13]Sumitra, M., Manikandan, P., and Suguna, L. (2005) Efficacy of *Butea monosperma* on dermal wound healing in rats. *Int. J. Biochem. Cell B.* 37: 566–573.

[14]Calabrò, M.L., Tommasini, S., Donato, P., Stancanelli, R., Raneri, D., Catania, S., Costa, C., Villari, V., Ficarra, P, and Ficarra, R. (2005) The rutin/β-cyclodextrin interactions in fully aqueous solution: spectroscopic studies and biological assays. *J. Pharmaceut. Biomed.* 36: 1019–1027.

[15]Mauludin, R., Müller, R.H., and Keck, C.M. (2009[a]) Development of an oral rutin nanocrystal formulation. *Int. J. Pharm*. 370: 202–209.

[16]Mauludin, R., Müller, R.H., and Keck, C.M. (2009[b]) Kinetic solubility and dissolution velocity of rutin nanocrystals. *Eur. J. Pharm. Sci.* 36: 502–510.

[17]Couvreur, P., Barrat, G., Fattal, E., Legrand, P., and Vauthier, C. (2002) Nanocapsuletechnology : A review. *Crit. Rev. Ther. Drug*. 19: 99–134.

[18]Schaffazick, S.R., Siqueira, I.R., Badejo, A.S., Jornada, D.S., Pohlmann, A.R., Netto, C. A., and Guterres, S.S. (2008) Incorporation in polymeric nanocapsules improves the antioxidant effect of melatonin against lipid peroxidation in mice brain and liver. *Eur. J. Pharm. Biopharm.* 69: 64–71.

[19]Ourique, A.F., Pohlmann, A.R., Guterres, S.S., and Beck, R.C.R. (2008) Tretinoin-loaded nanocapsules : Preparation, physicochemical characterization, and photostability study. *Int. J. Pharm*. 352: 1–4.

[20]Mora-Huertas, C.E., Fessi, H., and Elaissari, A. (2010) Polymer-based nanocapsules for drug delivery. *Int. J. Pharm*. 385: 113–142.

[21]Alves, M.P., Pohlmann, A.R., and Guterres, S.S. (2005) Semisolid topical formulations containing nimesulide-loaded nanocapsules, nanospheres or nanoemulsion: development and rheological characterization. *Pharmazie.* 60: 900-904.

[22]Luengo, J., Weiss, B., Schneider, M., Ehlers, A., Stracke, F., König, K.,Kostka, K.H., Lehr, C.M., and Schaefer, U.F. (2006) Influence of nanoencapsulation on human skin transport of flufenamic acid. *Skin Pharmacol. Physiol.* 19: 190–197.

[23]Paese, K., Jäger A., Poletto F.S., Fonseca E.P., Rossi-Bergmann B., Pohlmann, A.R., and Guterres, S.S. (2009) Semisolid Formulation Containing a Nanoencapsulated Sunscreen: Effectiveness, *In Vitro* Photostability and Immune Response. *J. Biomed. Nanotechnol.* 5: 240–246.

[24]Marchiori, M.L., Lubini, G., Dalla Nora, G., Friedrich, R.B., Fontana, M.C., Ourique, A.F., Bastos, M.O., Rigo, L.A., Silva, C.B., Tedesco, S.B., and Beck, R.C.R. (2010) Hydrogel cotaining dexamethasone-loaded nanocapsules for cutaneous administration: preparation, characterization and in vitro drug release study. *Drug Dev. Ind. Pharm. in press*.

[25]Cruz, L., Soares, L.U., Costa, T.D., Mezzalira, G., Silveira, N.P., Guterres, S.S., and Pohlmann, A.R. (2006) Diffusion and mathematical modeling of release profiles from nanocarriers. *Int. J. Pharm*. 313: 198–205.

[26]Guterres, S.S., Alves, M.P., and Pohlmann, A.R. (2007) Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. *Drug Target Insights*. 2: 147–157.

[27]Almeida, J.S., Jezur, L., Fontana, M.C., Pease, K., Silva, C.B., Pohlmann, A.R., Guterres, S.S., and Beck, R.C.R. (2009) Oil-based nanoparticles containing alternative vegetable oils (grape seed oil and almond kernel oil): preparation and characterization. *Lat. Am. J. Pharm.* 28: 165–172.

[28]Fontana, M.C., Coradini, K., Guterres, S.S., Pohlmann, A.R., and Beck, R.C.R. (2009) Nanoencapsulation as a way to control the release and to increase the photostability of clobetasol propionate: influence of the nanostructured system. *J. Biomed. Nanotechnol.* 5: 254–263.

[29]Fontana, M.C., Coradini, K., Guterres, S.S., Pohlmann, A.R., and Beck, R.C.R. (2010) Nanocapsules prepared from amorphus polyesters: Effect on the physicochemical characteristics, drug release and photostability. *J. Nanosci. Nanotechnol.* 10: 3091–3099.

[30]Schaffazick, S.R., Pohlmann, A.R., Cordova, C.A.S., Creczynski-Pasa, T.B., and Guterres, S.S. (2005) Protective properties of melatonin-loaded nanoparticles against lipid peroxidation. *Int. J. Pharm*. 289: 209–213.

[31]Almeida, J.S., Lima, F., Da Ros, S., Bulhões, L.O.S., Carvalho, L.M., and Beck, R.C.R. (2010) Nanostructured systems containing rutin: *in vitro* antioxidant activity and photostability studies. *Nanoscale Res. Lett. in press*.

[32]Fessi, H., Puisieux, F., and Devissaguet, J.P. (1988) Procede de préparation dês systèmes coloïdaux d'une substance sous forme de nanocapsules. *European Patent* 0274961 Al.

[33]Kim, J., Song, J., Lee, E., Park, S. (2003) Rheological properties and microstructures of Carbopol gel network system. *Colloid Polymer Sci*. 281: 614–623.

[34]Borghetti, G.S., and Knorst, M.T. (2006) Desenvolvimento e avaliação da estabilidade física de loções O/A contendo filtros solares. *Rev. Bras. Cienc. Farm.* 42: 531–537.

[35]Milan, A.L.K., Milão, D., Souto, A.A., and Corte, T.W.F. (2007) Estudo da hidratação da pele por emulsões cosméticas para xerose e sua estabilidade por reologia. *Rev. Bras. Cienc. Farm.* 43: 649–657.

[36]Nithya, M., Suguna, L., and Rose, C. (2003) The effect of nerve growth factor on the early responses during the process of wound healing. *Biochim. Biophys. Acta* 1620: 25–31.

[37]Ohkawa, H.. Ohishi, N., and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95: 351–358.

[38]Yan, L, Traber, M.G., and Packer, L. (1995) Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low-density lipoproteins. *Anal. Biochem.* 228: 349–351.

[39]Lowry, O.H., Rosebrouh, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin-Phenol reagents. *J. Biol. Chem.* 193: 265–275.

[40]Boyne A.F, and Ellman G.L. (1972) A methodology for analysis of tissue sulfhydryl components. *Anal. Biochem.* 46: 639–653.

[41]Galley, H., Davies, M.J., and Webster, N.R. (1996) Ascorbil radical formation in patients with sepsis: effects of ascorbate loading. *Free Radical Bio. Med.* 20: 139–143.

[42]Jacques-Silva, M.C., Nogueira, C.W., Broch, L.C., Flores, E.M., and Rocha, J.B. (2001) Diphenyl diselenides and ascorbic acid changes deposition of selenium and ascorbic acid in brain of mice. *Pharmacol. Toxicol***.** 44: 119–125.

[43]Aebi, U., Chiu, W., and Milligan, R. (1995) Role of catalase on antioxidative defenses. *J. Struct. Biol.* 2: 117–118.

[44]Jorge, M.P., Madjarof, C., Ruiz, A.L.T.G., Fernandes, A.T., Rodrigues, R.A.F., Sousa, I.M.O., Foglio, M.A., and Carvalho, J.E. (2008) Evaluation of wound healing properties of *Arrabidaea chica* Verlot extract. *J. Ethnopharmacol.* 118: 361–366.

[45]Cert, A., Moreda, W., and Péres-Camino, M.C. (2000) Chromatographic analysis of minor constituents in vegetable oils. *J. Chromatogr. A*. 881: 131–148.

[46]Hermes-Lima, M. (2004) Oxygen in biology and biochemistry: role of free radicals. In: STOREY, K.B. (ed.) Functional Metabolism: regulation and adaptation. New York: John Wiley & Sons, Inc., 319–368.

[47]Liu, J., Yeo, H. C., Doniger, S.J., and Ames, B.N. (1997) Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. *Anal. Biochem.* 245: 161–166.

[48]Verma A.R., Vijayakumar, M., Mathela, C.S., and RAO, C.V. (2009) *In vitro* and *in vivo* antioxidant properties of different fractions of Moringa oleifera leaves. *Food Chem. Toxicol.* 47: 2196–2201.

[49]Lodhi, S., Pawar, R.S., Jain, A.P., and Singhai, A.K. (2006) Wound healing potential of *Tephrosia purpurea* (Linn.) Pers. in rats. *J. Ethnopharmacol.* 108: 204–210.

DISCUSSÃO GERAL

DISCUSSÃO GERAL

A rutina é um flavonóide de ocorrência natural que apresenta diversas atividades bioquímicas e farmacológicas interessantes do ponto de vista terapêutico (HAN, 2009) incluindo ações antialérgicas, anti-inflamatórias e vasoativas, destacando-se as propriedades de seqüestro de espécies oxidativas, tais como os radicais hidroxil e superóxido e peróxido de hidrogênio (CALABRÒ *et al*., 2005). Apesar do relato em diversos estudos comprovando sua atividade antioxidante no tratamento de diversas doenças, a rutina ainda impõe restrições de uso em formas farmacêuticas pela sua baixa solubilidade aquosa, o que explica a sua baixa biodisponibilidade oral e tópica (MAULUDIN *et al*., 2009[a]).

Assim, com o intuito de minimizar essas limitações, várias alternativas para a liberação de fármacos tem sido desenvolvidas nos últimos anos, como a redução do tamanho de partículas. Através da redução do tamanho de partículas, alguns compostos podem aumentar a sua eficácia *in vivo*, devido a um aumento na solubilidade de saturação, área da superfície e velocidade de dissolução, e subseqüente aumento na biodisponibilidade (MAULUDIN *et al*., 2009[b]). Além da diminuição do tamanho de partículas, algumas outras técnicas têm sido desenvolvidas para aumentar a solubilidade de alguns compostos com solubilidade limitada, incluindo a complexação com ciclodextrinas (CALABRÒ *et al*., 2005).

Nos últimos anos, os sistemas nanoestruturados têm sido extensamente estudados como carreadores de fármacos e têm sido desenvolvidos visando inúmeras aplicações terapêuticas, sendo planejados, principalmente, para administração parenteral, oral, oftálmica e cutânea. Diante destes aspectos, num primeiro momento este projeto foi realizado no sentido de desenvolver e preparar suspensões coloidais poliméricas e nanoemulsões contendo os óleos de semente de uva e óleo de amêndoas doce, por tratar-se de alternativas interessantes no preparo de formulações farmacêuticas e cosméticas. Estes óleos foram selecionados com base na ausência de literatura sugerindo o seu uso como adjuvantes funcionais na preparação destes sistemas e também no seu potencial emprego no desenvolvimento de formulações voltadas ao tratamento de doenças ou processos associados ao estresse oxidativo.

Desse modo, na primeira etapa do trabalho (capítulo 1) foram desenvolvidas suspensões de nanocápsulas poliméricas e nanoemulsões, contendo os óleos de semente de uva e amêndoas doce a 3,3 % (v/v). As formulações foram caracterizadas quanto ao tamanho médio de partículas,

índice de polidispersão, pH, potencial zeta e estabilidade frente ao armazenamento. A fim de demonstrar uma aplicação prática desses óleos alternativos no desenvolvimento das formulações nanoestruturadas, foi avaliada a incorporação de uma substância modelo (benzofenona-3) aos sistemas na concentração de 1 mg/ml e a fotoestabilidade das formulações frente à radiação UVC.

Todas as formulações apresentaram aspecto macroscópico homogêneo, semelhante a um fluido opalescente com um reflexo azulado frente à observação à luz natural, independente do tipo de fase oleosa ou estrutura vesicular empregada. Este reflexo azulado, conhecido como efeito Tyndal é resultado do tamanho nanométrico e ao movimento Browniano das partículas (MAGENHEIM e BENITA, 1991). O tipo de fase oleosa influenciou as características de tamanho médio de partículas e potencial zeta das formulações, de acordo com as medidas de espalhamento de luz e mobilidade eletroforética, respectivamente. Aquelas preparadas com o óleo de amêndoas doce apresentaram um maior diâmetro médio de partículas e menor potencial zeta comparado com as formulações preparadas com óleo de semente de uva. A análise morfológica através de microscopia eletrônica de transmissão corroborou esses resultados. Conforme relatos anteriores, o tipo de fase oleosa empregada nas preparações de nanocápsulas poliméricas pode ter uma grande influência no tamanho médio de partículas e índice de polidispersão devido à diferença na sua viscosidade, características hidrofóbicas e tensão interfacial (SHAFFAZICK *et al*., 2003). Além disso, os índices de polidispersão foram influenciados pela estrutura vesicular. As nanocápsulas apresentaram menor índice de polidispersão comparadas com as nanoemulsões, independente do tipo de fase oleosa. Nas formulações contendo benzofenona-3, os resultados mostraram uma semelhança em suas características físico-químicas com as formulações preparadas sem a substância, demonstrando que a presença do ativo não modificou as características coloidais dos sistemas.

No estudo de estabilidade após 6 meses de armazenamento, as formulações sem o ativo apresentaram tamanho médio de partícula e índice de polidispersão semelhantes àqueles apresentados inicialmente. No entanto, todas as formulações apresentaram um significativo decréscimo nos valores de pH, independente da formulação. Em relação às nanocápsulas esse decréscimo é explicado pela hidrólise do polímero ou pelo relaxamento das cadeias poliméricas. Porém, nesse caso, as formulações com e sem polímero, nanocápsulas e nanoemulsões, respectivamente, apresentaram diminuição dos valores de pH. Isto pode ser explicado pela

hidrólise das cadeias de triglicerídeos e o respectivo aumento do conteúdo de ácidos graxos livres. Os óleos de semente de uva e de amêndoas doce são óleos com altas quantidades de ácido linoléico e outros ácidos graxos insaturados, que podem sofrer reações hidrolíticas e oxidativas em contato com meio aquoso a temperaturas abaixo de 100°C. A ocorrência destas reações foi reforçada pelo desenvolvimento do odor em todas as formulações após 6 meses de armazenamento. Os resultados mostraram também que a substância modelo não influenciou na estabilidade dos sistemas nanoestruturados, permanecendo aproximadamente 100% encapsulada após o período do estudo. Além disso, foi observada a ocorrência de uma agregação reversível (creaming) nas nanoemulsões, independente do tipo de fase oleosa, o que demonstrou a importância da parede polimérica ao redor do núcleo oleoso para melhorar as características de estabilidade física destes sistemas.

O estudo da fotodegradação da benzofenona-3 associada aos sistemas nanoestruturados foi realizado a fim de avaliar o potencial destes sistemas na prevenção de substâncias frente à radiação UV e sua aplicação no campo farmacêutico e cosmético. As nanopartículas contendo benzofenona-3 foram expostas a radiação UVC por 7 dias. Embora as nanoemulsões tenham mostrado uma menor estabilidade físico-química, o estudo de fotodegradação foi conduzido em todas as formulações para se avaliar a influência do tipo de fase oleosa e estrutura supramolecular. Foi empregada uma solução metanólica de benzofenona-3 como modelo do ativo livre. Os resultados mostraram que até 72 horas de exposição à radiação UVC, o conteúdo do ativo livre em solução foi significativamente menor em relação ao ativo associado às nanoestruturas. Após 168 horas, o conteúdo de benzofenona-3 decaiu cerca de 80% na solução metanólica, enquanto que o decaimento do ativo associado às nanoestruturas foi entre 10 e 30%. Assim, foi observado que o tipo de fase oleosa e o tipo de estrutura supramolecular não influenciaram na proteção da benzofenona-3 contra a fotodegradação, sendo que a sua fotoestabilidade foi aumentada pela associação aos sistemas nanoestruturados.

Na etapa seguinte (capítulo 2), foi selecionado o óleo de semente de uva para o preparo de nanocápsulas e nanoemulsões contendo a substância rutina, um flavonóide com ampla atividade antioxidante. Neste estudo, fez-se necessário a seleção da melhor e mais estável concentração do flavonóide, uma vez que a rutina possui limitada solubilidade aquosa. A partir da metodologia proposta por POHLMANN e colaboradores (2008), foi investigada a presença de nanocristais de rutina nas suspensões coloidais poliméricas em duas concentrações diferentes, 0,25 mg/mL e 0,5

mg/ml. Após 5 dias, verificou-se um decréscimo no teor de rutina nas formulações na concentração de 0,5 mg/ml, de cerca de 60%, com a presença de precipitados. Para as formulações contendo rutina na concentração de 0,25 mg/ml não houve diferença em sua concentração em relação ao primeiro dia do estudo (cerca de 100%).

A partir destes resultados, os sistemas nanoestruturados contendo rutina a uma concentração de 0,25 mg/ml (nanocápsulas e nanoemulsões) foram preparados e caracterizados físico-quimicamente. As suspensões apresentaram tamanho nanométrico de partícula (120–128 nm), índice de polidispersão inferior a 0,20, potencial zeta negativo e eficiência de encapsulação próxima a 100%, sendo que nem a presença da rutina nem a parede polimérica influenciaram as características avaliadas. Embora o tipo de sistema nanoestruturado não tenha influenciado as características físico-químicas das nanoestruturas, a análise morfológica revelou a presença de nanocristais de rutina ao redor das nanoemulsões, o que não foi observado nas nanoemulsões sem a rutina e nem nas nanocápsulas.

No estudo de fotodegradação, as constantes de degradação para a rutina associada as diferentes nanopartículas não foram significativamente diferentes. No entanto, quando comparadas à solução etanólica de rutina, ambas formulações mostraram uma menor velocidade de degradação da rutina. Estes resultados mostraram que a associação da rutina às nanocápsulas e nanoemulsões levou a um aumento de 5,3 a 6,9 vezes na fotoproteção da rutina, durante os 30 minutos de exposição à radiação UV.

A atividade antioxidante *in vitro* da rutina foi avaliada pela formação do radical livre OH após a exposição do peróxido de hidrogênio a um sistema de irradiação UV, proposto por CARVALHO e colaboradores (2008). As nanoestruturas mostraram uma cinética de decaimento cerca de 2 e 1,5 vezes menores que a solução etanólica contendo rutina livre, indicando um prolongamento da atividade antioxidante da rutina quando associada aos sistemas nanoestruturados. No entanto, houve diferença significativa no decaimento da rutina para os diferentes tipos de nanopartículas, sendo que a velocidade de decaimento foi menor para a rutina associada a nanocápsulas. Isso pode ter ocorrido provavelmente devido a presença de nanocristais de rutina adsorvidos na superfície das nanoemulsões, conforme observado através da microscopia eletrônica de transmissão, expondo uma quantidade maior de rutina para a reação com o radical OH.

A diferença no prolongamento da atividade antioxidante *in vitro* também poderia estar associada com uma liberação mais lenta do fármaco a partir dos diferentes sistemas. Assim, através do estudo de liberação *in vitro* da rutina a partir dos sistemas nanoestruturados, utilizando a técnica dos sacos de diálise, foi comparada a velocidade de liberação da rutina a partir das diferentes nanopartículas. Ambas as formulações promoveram uma rápida e semelhante liberação (próxima a 100% em 24 horas). Entretanto, observou-se uma liberação mais rápida da rutina nas nanoemulsões, o que pode ser explicado pela ausência do polímero e também pela presença dos nanocristais na superfície dessas estruturas, o que justifica a atividade antioxidante mais prolongada obtida pela sua associação com as nanocápsulas poliméricas.

Finalmente, no capítulo 3 estudamos o desenvolvimento de um hidrogel contendo rutina livre ou associada à nanocápsulas poliméricas, empregando Carbopol Ultrez® 10NF como polímero formador do gel, e avaliamos a eficácia destas formulações na cicatrização de feridas cutâneas *in vivo* através da análise de regressão da área da ferida e marcadores do estresse oxidativo nas lesões. A partir dos resultados anteriores, expostos no capítulo 2, foram selecionadas as formulações de nanocápsulas poliméricas contendo rutina para se avaliar uma potencial melhora na cicatrização em razão da não observação de nanocristais de rutina por microscopia eletrônica de transmissão e também do maior prolongamento da atividade antioxidante. A fim de se verificar a influência da rutina, bem como das nanocápsulas de rutina nas propriedades reológicas das formulações, hidrogéis contendo nanocápsulas brancas e um hidrogel base foram também preparados. Apesar da baixa solubilidade aquosa da rutina, foi possível a obtenção de um hidrogel contendo esse flavonóide livre através da sua redispersão em água com auxílio de polissorbato 80 a 0,77 % (p/v). Os hidrogéis foram caracterizados através dos seguintes parâmetros: teor de rutina, pH, análise reológica e espalhabilidade.

Todos os hidrogéis apresentaram características organolépticas satisfatórias em relação ao odor, cor e aparência. Os hidrogéis contendo nanocápsulas apresentaram coloração branca homogênea ao contrário dos hidrogéis contendo rutina livre que apresentaram uma coloração amarelada brilhosa. O hidrogel base apresentou aparência transparente. As formulações apresentaram teor de rutina próximo ao valor teórico (0.25 mg/g) e todos os hidrogéis apresentaram pH compatível para administração cutânea (entre 5,5 e 6,5). Estes valores não foram influenciados pela presença da rutina ou das nanocápsulas na formulação, estando de acordo com os resultados de MARCHIORI e colaboradores (2010). Em relação à reologia, todos

os hidrogéis apresentaram um comportamento não-Newtoniano e com propriedades pseudoplásticas, indicando que a viscosidade dos géis diminui com o aumento da taxa de cisalhamento seguindo o modelo de Herschel-Bulkley. Estes resultados corroboram o estudo de FONTANA (2009). Os índices de fluxo foram similares para todas as formulações. Por outro lado, embora o índice de consistência tenha sido similar para as formulações contendo nanocápsulas com ou sem rutina e os hidrogéis contendo rutina livre, foi observada diferença neste parâmetro para as formulações contendo nanocápsulas brancas comparada com o hidrogel base mostrando uma tendência no aumento da consistência destas formulações pela presença do polímero poli(ε-caprolactone) nas mesmas. Os hidrogéis contendo rutina e o hidrogel base apresentaram índices de consistência similares demostrando que a rutina não influenciou na viscosidade dos hidrogéis.

Com relação a espalhabilidade, os hidrogéis base e contendo rutina livre apresentaram maior espalhabilidade comparada com as formulações contendo nanocápsulas, independente da presença da rutina. Por outro lado, ambas as formulações contendo nanocápsulas apresentaram comportamento similar, como também foi observado por MARCHIORI e colaboradores (2010).

Na avaliação da atividade cicatrizante dos hidrogéis contendo rutina, o percentual da área da ferida foi monitorado durante todo o tratamento (6 dias). Após 6 dias de tratamento, os hidrogéis contendo rutina livre e rutina nanoencapsulada apresentaram uma menor área percentual da lesão comparada com a formulação controle. Essa redução foi similar àquela observada para as feridas tratadas com formulação controle positivo (hidrogel contendo alantoína a 1%), que apresentava características reológicas próximas aos demais hidrogéis. As formulações contendo as nanocápsulas brancas apresentaram área da ferida similar a formulação controle negativo, demonstrando que apenas a presença do óleo de semente de uvas na formulação, na concentração utilizada (3,3 % v/v), não apresenta efeito benéfico na cicatrização. Esse resultado pode ser explicado pela destruição de algumas substâncias antioxidantes durante o processo de refinamento do óleo ou então pela sua presença abaixo da dose terapêutica. O emprego de concentrações superiores do óleo de semente de uva nos hidrogéis não foi avaliado neste estudo pela característica das formulações de nanocápsulas, que dificultam a encapsulação de concentrações maiores de óleo, sem que haja a formação simultânea de nanoemulsões estabilizadas por tensoativos (JÄGER et al., 2007). Por outro lado, esse resultado demonstra também que essa formulação não apresenta qualquer efeito que possa dificultar os mecanismos

de cicatrização cutânea, pois ao final do tratamento apresenta uma área da lesão semelhante ao grupo controle negativo. Assim, o conjunto dos resultados demonstra que os hidrogéis contendo rutina (0.025%), livre ou associada a nanocápsulas poliméricas, apresentam um efeito cicatrizante similar às formulações contendo alantoína 1%, sugerindo seu potencial emprego em tratamentos dermatológicos. Embora se esperasse um efeito positivo da nanoencapsulação da rutina sobre a cicatrização cutânea, o mesmo não foi evidenciado. Esse resultado pode ser explicado pela localização da rutina na estrutura das nanocápsulas. Nosso estudo anterior demonstrou que a rutina encontra-se majoritamente adsorvida à superfície das nanocápsulas (Capítulo 2) e portanto mais exposta ao seu rápido deslocamento para a fase dispersante da formulação semissólida.

Na avaliação dos diferentes marcadores do estresse oxidativo, a análise da peroxidação lipídica após o tratamento das feridas mostrou que os grupos tratados com rutina apresentaram menores níveis de TBARS comparados com o grupo tratado com a formulação controle negativo (hidrogel base) e níveis similares ao grupo tratado com o hidrogel de alantoína a 1 % (controle positivo). Além disso, o grupo tratado com o hidrogel contendo rutina associada às nanocápsulas mostrou menores níveis de TBARS em relação ao grupo tratado com o hidrogel contendo nanocápsulas brancas. Em relação aos níveis de proteína carbonilada, todos os hidrogéis mostraram um decréscimo neste marcador comparado com a formulação controle negativo. Da mesma forma, na análise dos níveis de proteínas totais, todos os grupos apresentaram maiores níveis de proteínas comparados com o grupo tratado com o hidrogel base (controle negativo), exceto o grupo tratado com o hidrogel contendo nanocápsulas brancas.

Com relação aos níveis de glutationa (GSH), os grupos tratados com os hidrogéis contendo rutina associada a nanocápsulas apresentaram níveis de GSH mais altos em relação aos grupos tratados com os hidrogéis controle negativo e positivo e também com o hidrogel contendo nanocápsulas brancas. No entanto, não foi observado diferença entre os grupos tratados com os hidrogéis contendo rutina livre e nanoencapsulada para este marcador. Por outro lado, níveis mais altos de vitamina C foram observados para os grupos tratados com hidrogéis de nanocápsulas de rutina comparado com os outros hidrogéis, inclusive com o grupo tratado com rutina livre. Porém, um efeito similar foi obtido entre os grupos tratados com os hidrogéis de rutina associada a nanocápsulas, hidrogéis de alantoína e o hidrogel controle negativo (hidrogel base).

Avaliando-se o efeito dos tratamentos sobre os níveis da enzima catalase, foi demonstrando que o tratamento com as formulações contendo rutina livre apresentaram níveis

maiores da enzima comparados aqueles obtidos para os grupos tratados com os hidrogéis controle negativo (hidrogel base) e positivo (alantoína), bem como para os hidrogéis contendo nanocápsulas brancas. No entanto, não foram observadas diferenças entre as formulações de rutina livre e aquelas contendo rutina associada a nanocápsulas.

Assim, os resultados obtidos para os marcadores de estresse oxidativo corroboram aqueles obtidos na avaliação morfométrica das lesões cutâneas (efeito sobre a cicatrização). As formulações contendo rutina livre ou associada a nanocápsulas apresentaram um efeito similar sobre esses marcadores, o que pode novamente ser explicado pela localização superficial da rutina nas nanocápsulas, conforme discutido anteriormente. Essa atividade similar corrobora os resultados obtidos na avaliação da atividade antioxidante in vitro, onde a nanoencapsulação da rutina não promoveu um aumento na atividade antioxidante, mas apenas um efeito antioxidante mais prolongado. Além disso, somente a presença do óleo de semente de uva na concentração de 3,3 % nas nanocápsulas brancas não foi suficiente para alterar os níveis dos marcadores de estresse oxidativo investigados neste trabalho, pois os hidrogéis preparados com as nanocápsulas brancas apresentaram comportamento similar ao hidrogel base (controle negativo), cuja justificativa pode estar baseada na perda de substâncias antioxidantes durante o refinamento do óleo ou da concentração empregada estar abaixo da concentração terapêutica, conforme já discutido anteriormente.

Apesar de não termos observado um efeito superior para os hidrogéis contendo rutina associada a nanocápsulas, foi demonstrada nesse estudo a possibilidade de preparação de hidrogéis contendo rutina, com características compatíveis com a aplicação cutânea e sem a utilização de solventes orgânicos, como o etanol, por exemplo. Em relação à análise biológica, foi mostrada pela primeira vez a viabilidade de uso de formulações semissólidas contendo rutina na cicatrização de feridas cutâneas em modelo animal. A análise dos marcadores do estresse oxidativo sugere que a atividade de seqüestro dos radicais e o aumento da atividade de enzimas antioxidantes estão intimamente relacionados com a eficácia da rutina sobre a cicatrização cutânea.

CONCLUSÕES

CONCLUSÕES

- Foram desenvolvidas nanocápsulas e nanoemulsões empregando os óleos vegetais de semente de uva e de amêndoas doce, como fases oleosas alternativas, que apresentaram características físico-químicas promissoras para o seu emprego no desenvolvimento de futuras formas farmacêuticas e cosméticas;

- A associação da benzofenona-3 a nanocápsulas poliméricas e nanoemulsões preparadas com óleo de semente de uva e óleo de amêndoas doce demonstrou que essas formulações são capazes de proteger esse filtro orgânico do efeito da radiação ultravioleta, independente do tipo de fase oleosa e do tipo de vesícula empregada;

- Foi demonstrada de maneira inédita a possibilidade do desenvolvimento de suspensões de nanocápsulas poliméricas e nanoemulsões como sistemas intermediários ou finais no desenvolvimento de nanomedicamentos contendo rutina.

- As nanocápsulas e nanoemulsões contendo rutina promoveram um aumento na sua fotoestabilidade, bem como o prolongamento da sua atividade antioxidante *in vitro* frente ao radical OH. As nanocápsulas promoveram um maior prolongamento desta atividade em relação às nanoemulsões. Neste caso, a presença da parede polimérica foi fundamental para se obter esses resultados.

- A modelagem matemática dos perfis de liberação de rutina a partir das nanopartículas demonstrou um comportamento biexponencial e uma alta adsorção da rutina à sua superfície, independente da sua estrutura vesicular (nanocápsulas ou nanoemulsões);

- Os hidrogéis de Carbopol Ultrez® 10 NF contendo rutina a 0,025 % (livre ou associada às nanopartículas poliméricas) apresentaram propriedades compatíveis com a sua administração cutânea. Os hidrogéis foram preparados sem a utilização de solventes orgânicos, apesar da baixa solubilidade da rutina em água.

- Através do estudo biológico foi demonstrado que a rutina promoveu uma melhora na cicatrização de feridas cutâneas em modelo animal a uma concentração de 0,025%, sugerindo que as formulações desenvolvidas nesse estudo apresentam-se potenciais alternativas para tratamentos dermatológicos relacionados à atividade de cicatrização cutânea;

- O estudo dos marcadores de estresse oxidativo ao final dos tratamentos, nos tecidos cutâneos envolvidos no processo de cicatrização cutânea, sugere que o seqüestro de radicais livres e o aumento da atividade de enzimas antioxidantes estão envolvidos com a atividade de promoção da cicatrização cutânea observada nesse estudo;

- A nanoencapsulação da rutina e a presença do óleo de semente de uva, nas concentrações avaliadas nesse trabalho, não demonstraram efeito sobre a cicatrização cutânea, considerando o modelo animal empregado. Assim, tanto as nanocápsulas quanto o óleo de semente de uva, apesar de não apresentarem uma melhora neste processo, também não apresentam qualquer efeito que poderia retardá-lo ou dificultá-lo.

REFERÊNCIAS

REFERÊNCIAS

AHMAD, Z. The uses and properties of almond oil. **Complementary Therapies in Clinical Practice**, v. 16, p. 10–12, 2010.

ALONSO, M. J. Nanomedicines for overcoming biological barriers. **Biomedicine & Pharmacotherapy**, v. 58, p. 168-172, 2004.

ALVARES-ROMÁN, R.; NAIK, A.; KALIA, Y. N.; GUY, R. H.; FESSI, H.Skin penetration and distribution of polymeric nanoparticles. **Journal of Controlled Release**, v. 99, p. 53-62, 2004.

ALVES, M. P.; SCARRONE, A. L.; SANTOS, M.; POHLMANN, A. R.; GUTERRES, S. S. Human skin penetration and distribution of nimesulide from hydrophilic gels containing nanocarriers. **International Journal of Pharmaceutics**, v. 341, p. 215-220, 2007.

BARREIROS, A. L. B. S.; DAVID, J. M. Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. **Química Nova**, v.29, p. 113–123, 2006.

BAYDAR, N. G.; OZKAN, G.; CETIN, E. S. Characterization of grape seed and pomace oil extracts. **Grasas y Aceites**, v. 58, p. 29–33, 2007.

BLAYO, A.; GANDINI, A.; LE NEST, J-F. Chemical and rheological characterizations of some vegetable oils derivatives commonly used in printing inks. **Industrial Crops and Products**, v. 14, p. 155–167, 2001.

BECK, R. C. R.; POHLMANN, A. R.; GUTERRES, S. S. Nanoparticle-coated microparticles: preparation and characterization. **Journal of Microencapsulation**, v. 21, n. 5, p. 499-512, 2004.

BECK, R. C. R.; POHLMANN, A. R.; BENVENUTTI, E. V.; COSTA, T. D.; GUTERRES, S. S. Nanostructure-coated diclofenac-loaded microparticles: preparation, morphological, characterization, *in vitro* release and *in vivo* gastrointestinal tolerance. **Journal of the Brazilian Chemical Society**, v. 16, n. 6a, p. 1233-1240, 2005.

BECK, R. C. R.; HAAS, S. E.; GUTERRES, S. S.; RE, M. I.; BENVENUTTI. E. V.; POHLMANN, A. R. Nanoparticle-coated organic-inorganic microparticles: experimental design and gastrointestinal tolerance evaluation. **Química Nova**, v. 29, n. 5, p. 990-996, 2006.

BECK, R. C. R.; POHLMANN, A. R.; HOFFMEISTER, C.; GALLAS, M. R.; COLLNOT, E.; SCHAEFER, U. F.; GUTERRES, S. S.; LEHR, C. M. Dexamethasone-loaded nanoparticle-coated microparticles: correlation between *in vitro* drug release and drug transport across caco-2 cell monolayers. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 67, p. 18-30, 2007.

BERNARDI, A.; BRAGANHOL, E.; JÄGER, E.; FIGUEIRÓ, R.; EDELWEISS, M. I.; POHLMANN, A. R.; GUTERRES, S. S.; BATTASTINI, A. M. O. Indomethacin-loaded nanocapsules treatment reduces *in vivo* glioblastoma growth in a rat glioma model. Cancer Letters, v. 281, n. 1, p. 53–63, 2009.

BEVERIDGE, T. H. J.; GIRARD, B.; KOPP, T.; DROVER, J. C. G. Yield. Composition of grape seed oils extracted by supercritical carbon dioxide and petroleum ether: varietal effects. **Journal of Agricultural and Food Chemistry**, v. 53, p. 1799–1804, 2005.

BRENNEISEN, P.; STEINBRENNER, H.; SIES, H. Selenium, oxidative stress, and health aspects. **Molecular Aspects of Medicine**. v. 26, p. 256–267, 2005.

CALABRÒ, M. L.; TOMMASINI, S.; DONATO, P.; STANCANELLI, R.; RANERI, D.; CATANIA, S.; COSTA, C.; VILLARI, V.; FICARRA, P; FICARRA, R. The rutin/β-cyclodextrin interactions in fully aqueous solution: spectroscopic studies and biological assays. **Journal of Pharmaceutical and Biomedical Analysis**, v. 36, p. 1019–1027, 2005.

CALVO, P.; VILA-JATO, J. L.; ALONSO, M. J. Comparative in vitro evaluation of several colloidal systems, nanoparticles, nanocapsules, and nanoemulsions, as ocular drug carriers. **Journal of Pharmaceutical Sciences**, v. 85, n. 5, 530-536, 1996.

CARVALHO, L.M., SPENGLER, C., GARMATZ, J.C., NASCIMENTO, P.C., BOHRER, D., DEL-FABRO, L., RADIS, G., BOLLI, A.A., MORO, A.M., GARCIA, S.C., ROSA, M.B. Determinação voltamétrica de metais em águas e fluidos biológicos empregando mineralização de amostras com radiação ultravioleta. **Química Nova**. v. 31, p. 1336–1342, 2008.

CEMELI, E.; BAUMGARTNER, A.; ANDERSON, D. Antioxidants and the comet assay. **Mutation Research**, v. 681, p. 51–67, 2009.

CERT, A.; MOREDA, W.; PÉRES-CAMINO, M. C. Chromatographic analysis of minor constituents in vegetable oils. **Journal of Chromatography A**, v. 881, p. 131–148, 2000.

CÉSPEDES, C. L.; EL-HAFIDI, M.; PAVON, N.; ALARCON, J. Antioxidant and cardioprotective activitiesof phenolic extracts from fruits of Chilean blackberry *Aristotelia chilensis* (Elaeocarpaceae), Maqui. **Food Chemistry**, v. 107, p. 820–829, 2008.

CHERIF, A.; Belkacemi, K.; Kallel, H.; Angers, P.; Arul, J.; Boukhchina, S. Phytosterols, unsaturated fatty acid composition and accumulation in the almond kernel during harvesting period: Importance for development regulation. **C. R. Biologies**, v. 332, p. 1069–1077, 2009.

CHIRINOS, R.; BETALLELUZ-PALLARDEL, I.; HUAMÁN, A.; ARBIZU, C. HPLC-DAD characterisation of phenolic compounds from Andean oca (*Oxalis tuberosa* Mol.) tubers and their contribution to the antioxidant capacity. **Food Chemistry**, v. 113, p. 1243–1251, 2009.

CHOI, Y.; LEE, J. Antioxidant and antiproliferative properties of a tocotrienol-rich fraction from grape seeds. **Food Chemistry**, v. 114, p. 1386–1390, 2009.

COUVREUR, P.; BARRAT, G.; FATTAL, E.; LEGRAND, P.; VAUTHIER, C. Nanocapsule Technology: A Review. **Critical Reviews in Therapeutic Drug Carrier Systems**, v. 19, n. 2, p. 99-134, 2002.

COSTA, N. M. B.; BORÉM, A. (Eds.). **Biotecnologia e Nutrição**. 1ª ed. São Paulo: Nobel. 2003.

CORNELLI, U. Antioxidant use in nutraceuticals. **Clinics in Dermatology**, v. 27, p. 175–194, 2009.

CRUZ, L.; SOARES, L. U.; DALLA COSTA, T.; MEZZALIRA, G.; SILVEIRA, N. P.; GUTERRES, S. S.; POHLMANN, A. R. Diffusion and mathematical modeling of release profiles from nanocarriers. **International Journal of Pharmaceutics**, v. 313, p. 198–205, 2006.

CUNHA, S. C.; OLIVEIRA, M. B. P. P. Discrimination of vegetable oils by triacylglycerols evaluation of profile using HPLC/ELSD. **Food Chemistry**, v. 95, p. 518–524, 2006.

DEGÁPARI, C. H.; WASZCZYNSKYJ, N. Propriedade antioxidantes de compostos fenólicos. **Visão Acadêmica**, v. 5, n. 1, p. 33–40, 2004.

DWECK, A. C. The internal and external use of medicinal plants. **Clinics in Dermatology**, v. 27, p. 148–158, 2009.

ESPÍN, J. C.; SOLER-RIVAS, C.; WICHERS, H.J. Characterization of the Total Free Radical Scavenger Capacity of Vegetable Oils and Oil Fractions Using 2,2-Diphenyl-1-picrylhydrazyl Radical. **Journal Agricultural Food and Chemical**, v. 48, p. 648–656, 2000.

FANG, J.; SEKI, T.; MAEDA, H. Therapeutic strategies by modulating oxygen stress in cancer and inflammation. **Advanced Drug Delivery Reviews**, v. 61, p. 290–302, 2009.

FASINA, O. O. ; CRAIG-SCHMIDT, M.; COLLEY, Z.; HALLMAN, H. Predicting melting characteristics of vegetable oils from fatty acid composition. **LWT**, v. 41, p. 1501–1505, 2008.

FESSI, H.; PUISIEUX, F.; DEVISSAGUET, J. P. Procede de préparation dês systèmes coloïdaux d'une substance sous forme de nanocapsules. European Patent, 0274961 A1, 1988.

FONTANA, M.C., CORADINI, K., GUTERRES, S.S., POHLMANN, A.R., BECK, R.C.R. Nanoencapsulation as a way to control the release and to increase the photostability of clobetasol propionate: Influence of the nanostructured system. **Journal of Biomedical Nanotechnology**, n. 5, p. 254–263, 2009.

FONTANA, M. C.; CORADINI, K.; GUTERRES, S. S.; POHLMANN, A. R.; BECK, R. C. R. Nanocapsules prepared from amorphus polyesters: Effect on the physicochemical characteristics, drug release and photostability. **Journal of Nanoscience and Nanotechnology**, v. 10, p. 3091–3099, 2010.

FONTANA, M.C. Nanopartículas contendo propionato de clobetasol: preparação, caracterização e incorporação em hidrogéis. 2009. 148f. Dissertação (Mestrado em Ciências Farmacêuticas) – Universidade Federal de Santa Maria, Santa Maria, 2009.

GONZÁLEZ, S.; FERNÁNDEZ-LORENTE, M.; GILABERTE-CALZADA, Y. The latest on skin photoprotection. **Clinics in Dermatology**, v. 26, p. 614–626, 2008.

GUTERRES, S. S.; ALVES, M. P.; POHLMANN, A. R. Polymeric nanoparticles, nanospheres and nanocápsulas, for cutaneous applications. **Drug Target Insights**, v. 2, p. 147–157, 2007.

HAN, Y. Rutin has therapeutic effect on septic arthritis caused by *Candida albicans*. **International Immunopharmacology**, v. 9, p. 207–211, 2009.

ITAGAKI, S.; OIKAWA, S.; OGURA, J.; KOBAYASHI, M.; HIRANO, T.; ISEKI, K. Protective effects of quercetin-3-rhamnoglucoside (rutin) on ischemia-reperfusion injury in rat small intestine. **Food Chemistry**, n. 118, p. 426–429, 2010.

JÄGER, A.; STEFANI, V.; GUTERRES, S. S.; POHLMANN, A. R. Physicol-chemical characterization of nanocapsule polymeric wall using fluorescent benvavole probes. I**nternational Journal of Pharmaceutics**, n. 338, p. 297–305, 2007.

KHALIL, E. A.; AFIFI, F.; AL-HUSSAINI, M. Evaluation of the wound healing effect of some Jordanian traditional medicinal plants formulated in Pluronic F127 using mice (*Mus musculus*). **Journal of Ethnopharmacology**, n. 109, p. 104–112, 2007.

JOHN, J.; BHATTACHARYA, M.; RAYNOR, P., C. Emulsions containing vegetable oils for cutting fluid application. **Colloids and Surfaces A: Physicochem. Eng. Aspects**, v. 237, p. 141–150, 2004.

KALLINTERI, P.; HIGGINS, S.; HUTCHEON. G. A.; POURÇAIN, C. B. St.; GARNETT, M. C. Novel functionalized biodegradable polymers for nanoparticle drug delivery systems. **Biomacromolecules**, v. 6, p. 1885–1894, 2005.

KIM, D.; MARTIN, D. C. Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery. **Biomaterials**, v. 27, p. 3031–3037, 2006.

KORKMAZ, A.; KOLANKAYA, D. Protective Effect of Rutin on the Ischemia/Reperfusion Induced Damage in Rat Kidney. **Journal of Surgical Research**, 2009 *in press*.

KUMARI, A.; YADAV, S. K.; YADAV, S. C. Biodegradable polymeric nanoparticles based drug delivery systems. **Colloids and Surfaces B: Biointerfaces**, v. 75, p. 1–18, 2010.

KUNTI´C, V.; PEJI´C, N.; IVKOVI´C, B.; VUJI´C, K. I.; MI´CI´C, S.; VUKOJEVI´C, V. Isocratic RP-HPLC method for rutin determination in solid oral dosage forms. **Journal of Pharmaceutical and Biomedical Analysis**, v. 43, p. 718–721, 2007.

LIN TAN, M.; CHOONG, P. F. M.; DASS, C. R. Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. **Peptides**, n. 31, p. 184–193, 2010.

MARCHIORI, M. L.; LUBINI, G.; DALLA NORA, G.; FRIEDRICH, R. B.; FONTANA, M. C.; OURIQUE, A. F.; BASTOS, M. O.; RIGO, L. A.; SILVA, C. B.; TEDESCO, S. B.; BECK, R. C. R. Hydrogel containing dexamethasone-loaded nanocapsules for cutaneous administration: preparation, characterization and in vitro drug release study. **Drug Development and Industrial Pharmacy**, 2010, *in press*.

MARTÍN-CARRATALÁ, M. L.; LORENS-JORDÁ, C.; BERENGUER-NAVARRO, V.; GRANÉ-TERUEL, N. Comparative Study on the Triglyceride Composition of Almond Kernel Oil. A New Basis for Cultivar Chemometric Characterization. **Journal of Agricultural and Food Chemistry**, v. 47, p. 3688–3692, 1999.

MARTIM. A., C.; SANDERS, R. A.; WATKINS, J. B. Diabetes, Oxidative Stress, and Antioxidants: A Review. **Journal Boichemical and Molecular Toxicology**, v. 17, n. 1, p. 24–38, 2003.

MARTINI, E.; CARVALHO, E.; TEIXEIRA, H.; LEÃO, F.; MÔNICA, C. O. Adsorção de oligonucleotídeos em nanoemulsões obtidas por emulsificação espontânea. **Química Nova**, v. 30, n. 4, p. 930–934, 2007.

MAULUDIN, R.; MÜLLER, R. H.; KECK, C., M. Development of an oral rutin nanocrystal formulation. **International Journal of Pharmaceutics**. v. 370, p. 202–209, 2009[a].

MAULUDIN, R.; MÜLLER, R. H.; KECK, C. M. Kinetic solubility and dissolution velocity of rutin nanocrystals. **European Journal of Pharmaceutical Sciences**, v. 36, p. 502–510, 2009[b].

MAGENHEIM, B.; BENITA, S. Nanoparticle characterization: a comprehensive physicochemical approach. **STP Pharma Sciences**, v. 1, p. 221-241, 1991.

MORA-HUERTAS, C. E.; FESSI, H.; ELAISSARI, A. Polymer-based nanocapsules for durg delivery. **International Journal of Pharmaceutics**, v. 385, p. 113–142, 2010.

NAGARWAL, R. C.; KANT, S.; SINGH, P. N.; MAITI, P.; PANDIT, J. K. Polymeric nanoparticulate system: A potential approach for ocular drug delivery. **Journal of Controlled Release**, v. 136, p. 2–13, 2009.

NIJVELDT, R. J.; NOOD, E. V.; HOORN, D. E. C. V.; BOELENS, P. G.; NORREN, K. V.; LEEUWEN, P. A. M. V. Flavonoids: a review of probable mechanisms of action and potential applications. **The American Journal of Clinical Nutrition**, v. 74, p. 418–425, 2001.

OGA, S.; CAMARGO, M. M. A.; BATISTUZZO, J. A. O. **Fundamentos de toxicologia**. 3ª ed. São Paulo: Atheneu. 2008.

OURIQUE, A. F.; POHLMANN, A. R.; GUTERRES, S. S.; BECK, R. C. R. Tretinoin-loaded nanocapsules: Preparation, physicochemical characterization, and photostability study. **International Journal of Pharmaceutics**, v. 352, p.1–4, 2008.

PANCHATCHARAM, M.; MIRIYALA, S.; GAYATHRI, V. S.; SUGUNA, L. Curcumin improves wound healing by modulating collagen and decreasing reactive oxygen species. **Molecular and Cellular Biochemistry**. v. 290, p. 87–96, 2006.

PARDEIKE, J.; HOMMOSS, A.; MÜLLER, R. H. Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. **International Journal of Pharmaceutics**, v. 366, p. 170–184, 2009.

PASSOS, C. P.; SULE YILMAZ, S.; CARLOS M. SILVA, C. M.; COIMBRA, M. A. Enhancement of grape seed oil extraction using a cell wall degrading enzyme cocktail, **Food Chemistry**, v. 115, p. 48-53, 2009.

PATTANAYAK, S. P.; SUNITA, P. Wound healing, anti-microbial and antioxidant potential of *Dendrophthoe falcata* (L.f) Ettingsh. **Journal of Ethnopharmacology**, v. 120, p. 241–247, 2008.

PEKKARINEN, S.; HEINONEN, M.; HOPIA, A. Flavonoids quercetin, myricetin, kaemferol and (+)-catechin as antioxidants in methyl linoleate. **Journal of the Science of Food and Agriculture**, v. 79, p. 499–506, 1999.

PEREIRA, R. P.; FACHINETTO, R.; PRESTES, A. S.; PUNTEL, R. L.; SILVA, G. N. S.; HEINZMANN, B. M.; BOSCHETTI, T. K.; ATHAYDE, M. L.; BÜRGER, M., E.; MOREL, A. F.; MORSCH, V. M.; ROCHA, J. B. T. Antioxidant Effects of Different Extracts *from Melissa officinalis*, *Matricaria recutita* and *Cymbopogon citratus*. **Neurochemical Research**, v. 34, p. 973–983, 2009.

POHLMANN, A. R; WEISS, V.; MERTINS, O.; PESCE DA SILVEIRA, N.; GUTERRES, S. S. Spray-dried Indomethacin-Loaded Polyester Nanocapsules and Nanospheres: Development, Stability Evaluation and Nanostructure Models. **European Journal of Pharmaceutical Sciences**, v. 16, n. 4/5, p. 305-312, 2002.

POHLMANN, A. R.; MEZZALIRA, G.; VENTURINI, C. DE G.; CRUZ, L., BERNARDI, A.; JÄGER, E.; BATTASTINI, A. M. O.; PESCE DA SILVEIRA; N., GUTERRES, S. S. Determining the simultaneous presence of drug nanocrystals in drug-loaded polymeric nanocapsule aqueous suspensions: A relation between light scattering and drug content. **International Journal of Pharmaceutics**, v. 359, p. 288–293, 2008.

SAHOO, S. K.; LABHASETWAR, V. Nanotech approaches to drug delivery and imaging. **Drug Discovery Today**, v. 8, n. 24, p. 1112–1120, 2003.

SAHOO, S. K.; PARVEEN, S.; PANDA, J. J. The present and future of nanotechnology in human health care. **Nanomedicine: Nanotechnology, Biology, and Medicine**, v. 3, p. 20–31, 2007.

SANDHIYA, S.; DKHAR, S. A.; SURENDIRAN, A. Emerging trends of nanomedicine – an overview. **Fundamental & Clinical Pharmacology**, v. 23, p. 263–269, 2009.

SCHAFFAZICK, S. R.; FREITAS, L. L.; POHLMANN, A. R.; GUTERRES, S. S. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. **Química Nova**, v. 25, n. 5, p. 726–737, 2003.

SCHAFFAZICK, S. R.; POHLMANN, A. R.; CORDOVA, C. A. S.; CRECZYNSKI-PASA, T. B.; GUTERRES, S. S. Protective properties of melatonin-loaded nanoparticles against lipid peroxidation. **International Journal of Pharmaceutics**, v. 289, p. 209–213, 2005.

SCHAFFAZICK S. R.; POHLMANN, A. R.; MEZZALIRA, G.; GUTERRES, S. S. Development of nanocapsule suspensions and nanocapsule spray-dried powders containing melatonin. **Journal Brazilian Chemical Society**, v. 17, n.. 3, p. 562–569, 2006.

SCHAFFAZICK, S. R.; POHLMANN, A. R.; GUTERRES, S. S. Nanocapsules, nanoemulsion and nanodispersion containing melatonin: preparation, characterization and stability evaluation. **Pharmazie**, v. 62, p. 354–360, 2007.

SHAFFAZICK, S. R.; SIQUEIRA, I. R.; BADEJO, A. S.; JORNADA, D. S.; POHLMANN, A. R.; NETTO, C. A.; GUTERRES, S. S. Incorporation in polymeric nanocapsules improves the antioxidant effect of melatonin against lipid peroxidation in mice brain and liver. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 69, p. 64–71, 2008.

SHARMA, A.; SHARMA, U. Lipossomes in drug delivery: progress and limitations. **International Journal of Pharmaceutics**, v. 154, p. 123–140, 1997.

SCHMALTZ, C.; SANTOS, J. V; GUTERRES, S. S. Nanocápsulas como uma nova tendência promissora na área cosmética: a imensa potencialidade deste pequeno grande recurso. **Infarma**. v. 16, p. 13–14, 2005.

SOUSA, C. M. M.; SILVA, H. R.; VIEIRA-JR, G. M.; AYRES, M. C. C.; COSTA, C. L. S.; ARAÚJO, D. S.; CAVALCANTE, L. C. D.; BARROS, E. D. S.; ARAÚJO, P. B. M.; BRANDÃO, M. S.; CHAVES, M. H. Fenóis totais e atividade antioxidante de cinco plantas medicinais. **Química Nova**, v. 30, n. 2, p. 351–355, 2007.

SULTANA, B.; ANWAR, F. Flavonols (kaempeferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants. **Food Chemistry**, v. 108, p. 879–884, 2008.

SUMITRA, M.; MANIKANDANA, P.; SUGUNAB, L. Efficacy of *Butea monosperma* on dermal wound healing in rats. **The International Journal of Biochemistry & Cell Biology**, v. 37, p. 566–573, 2005.

ZAHR, A. S.; VILLIERS, M.; PISHKO, M. V. Encapsulation of drug nanoparticles in self-assembled macromolecular nanoshells. **Langmuir**, v. 21, p. 403-410, 2005.

ZHANG, Q-I.; ZHANG, Z-Q.; YUE, X-F.; FAN, X-H.; LI, T.; CHEN, S-F. Response surface optimization of ultrasound-assisted oil extraction from autoclaved almond powder. **Food Chemistry**, 2009 *in press*.

ANEXOS

Artigos publicados

Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense*)
Lat. Am. J. Pharm. **28** (2): 165-72 (2009)

Original Article

Received: October 30, 2008
Accepted: December 8, 2008

Oil-Based Nanoparticles Containing Alternative Vegetable Oils (Grape Seed Oil and Almond Kernel Oil): Preparation and Characterization

Juliana S. ALMEIDA [1], Luciane JEZUR [2], Marcia C. FONTANA [1], Karina PAESE [3], Cristiane B. SILVA [2]; Adriana R. POHLMANN [4], Silvia S.GUTERRES [3] & Ruy C.R. BECK [1]*

[1] *Programa de Pós-Graduação em Ciências Farmacêuticas, Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Santa Maria, RS Brazil;*
[2] *Curso de Farmácia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil;*
[3] *Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Ipiranga, 2752, 90610-000, Porto Alegre, RS, Brazil;*
[4] *Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul (UFRGS), CP 15003, 91501-970, Porto Alegre, RS, Brazil*

SUMMARY. The use of two alternative vegetable oils (grape seed oil and almond kernel oil) to prepare nanoparticulated delivery systems (nanocapsules and nanoemulsions) for active substances was evaluated. They were prepared by interfacial deposition of preformed polymer (poly-ε-caprolactone) or spontaneous emulsification, respectively. All formulations presented nanometric size, polydispersity index below 0.30, negative zeta potential and spherical-shaped particles. Benzophenone-3, as a model substance was efficiently entrapped in these systems, independent on the type of oily phase. Its association did not alter significantly the physicochemical properties of the nanoparticle dispersions, which remained adequate until 6 months of storage. Nanocapsules and nanoemulsions prepared with both vegetable oils were suitable to delay benzophenone-3 photodegradation under UV radiation.

INTRODUCTION

Nanostructured materials such as polymeric nanoparticles, nanoemulsions, liposomes and dendrimers have been widely studied in the pharmaceutical and cosmetic field over the last years [1-4]. Active substances can have some properties improved by their incorporation in these biomedical nanomaterials, such as aqueous solubility, photostability, distribution after topical or systemic application, efficacy and bioavailability [2,5-9].

Nanocapsules are submicrometric particles (mean size below 1 µm) composed of an oily core surrounded by a thin polymeric wall [10-12]. They are formulated in presence of surfactants in order to stabilize the particles [13]. Nanoemulsions are nanometric-sized emulsions having droplets up to 500 nm, being also formulated in presence of surfactants [14]. Because of their small size, which allows them to permeate through biological barriers, these systems showed potential use following topical (ocular, dermal) or systemic administration [15,16].

The development of suitable nanocapsule suspensions or nanoemulsions for pharmaceutical or cosmetic application requires the adequate selection of their adjuvants, like polymers (for NC only), surfactants, and oils [11,17-19]. Nowadays, special attention has been given to the use of biodegradable polymers in the preparation of nanocapsules due to its degradation to non-toxic and non-reactive metabolites, which can be excreted by the organism [20]. Surfactants are necessary to obtain small and stable oil droplets. Their type and concentration can affect the physicochemical properties of the nanoparticles, such as size, polydispersity index and drug loading [11,17-19]. In addition, the type of oily phase used as the core in preparation of polymeric nanocapsules or nanoemulsions can also

KEY WORDS: Almond kernel oil, Grape seed oil, Nanoparticles.

* Author to whom correspondence should be addressed. *E-mail:* ruybeck@smail.ufsm.br

have an influence mainly on the mean particle size and polydispersity index due to the difference in its viscosity, hydrophobic characteristic and interfacial tension [11,18].

Grape seed oil and almond kernel oil are vegetable oils with known antioxidant activities, based on their chemical constitution. Grape seed oil is rich in phenolic components, linoleic acid and tocopherols [21,22]. Almond kernel oil also presents high concentrations of linoleic acid [23,24]. This way, both oils could be interesting alternatives to prepare nanocapsules or nanoemulsions as delivery systems for cosmetic or pharmaceutical active substances.

To the best of our knowledge, there is no report in the literature on the use of these vegetable oils as functionally adjuvants in the preparation of nanocapsule and nanoemulsions. In this context, the aim of this work was to evaluate the feasibility of their use as alternative vegetable oils to prepare oil-based nanomaterials as delivery systems of active substances. Nanocapsules and nanoemulsions were prepared and characterized by means of mean size, polydispersity index, pH, zeta potential, and stability under storage. In order to demonstrate a practical application of these alternative oils in the development of the oil-based nanomaterials, we evaluated the incorporation of a model substance (benzophenone-3) to the systems and their properties to delay benzophenone-3 photodegradation under UV radiation.

MATERIAL AND METHODS

Materials

Grape seed oil (GSO), almond kernel oil (AKO) and benzophenone-3 (B3) were obtained from PharmaSpecial (São Paulo, Brazil). Poly-ε-caprolactone (PCL) and sorbitan monostearate (Span 60®) were acquired from Sigma (São Paulo, Brazil). Polysorbate 80 (Tween 80®) was supplied by Henrifarma (São Paulo, Brazil). All other chemicals and solvents presented pharmaceutical or HPLC grade and were used as received.

Preparation of nanocapsules and nanoemulsions

Nanocapsule suspensions (NC) were prepared (n = 3) by the interfacial deposition of preformed polymer method as described by Fessi *et al.* [25]. Briefly, an organic solution consisted of the oily phase – GSO or AKO (3.3 mL), a low HLB (hydrophilic-lipophilic balance) surfactant – Span 60® (0.776 g), the polymer (PCL) (1.0 g) and acetone (267.0 mL) was added under moderate magnetic stirring to an aqueous solution (533.0 mL) containing a high HLB surfactant - Tween 80® (0.776 g). The magnetic stirring was maintained for 10 min. Then, acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure to a final volume of 100 mL (10 $mg.mL^{-1}$ of polymer and 3.30% v/v of oil). Nanoemulsions (NE) were prepared (n = 3) by the spontaneous emulsification method as described by Martini *et al.* [26]. To prepare the nanoemulsions, the presence of the polymer in the organic solution was omitted. All formulations were storage protected from the light.

Benzophenone-3-loaded nanoparticles were prepared at a final concentration of 1.00 $mg.ml^{-1}$, being dissolved (100 mg) in the organic solution during the nanoparticle preparation. Quali-quantitative composition of each formulation is described in Table 1.

Characterization of nanocapsules and nanoemulsions

Particle size analysis and polydispersity indices

Particle sizes and polydispersity indices (n = 3) were measured by photon correlation spectroscopy after adequate dilution of an aliquot of the suspension in purified water (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK).

Formulation	PCL	Span 60®	GSO	AKO	Tween 80®	B3
NC-AKO	1.00 g	0.77 g	–	3.3 ml	0.77 g	–
NE-AKO	–	0.77 g	–	3.3 ml	0.77 g	–
NC-GSO	1.00 g	0.77 g	3.3 ml	–	0.77 g	–
NE-GSO	–	0.77 g	3.3 ml	–	0.77 g	–
B3-NC-AKO	1.00 g	0.77 g	–	3.3 ml	0.77 g	0.10 g
B3-NE-AKO	–	0.77 g	–	3.3 ml	0.77 g	0.10 g
B3-NC-GSO	1.00 g	0.77 g	3.3 ml	–	0.77 g	0.10 g
B3-NE-GSO	–	0.77 g	3.3 ml	–	0.77 g	0.10 g

Table 1. Quali-quantitative composition of nanoparticles – 100 ml (NC: nanocapsules; NE: nanoemulsion; B3: benzophenone-3) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase.

Zeta potential

Zeta potentials were determined after dilution of the samples in 10 mmol L^{-1} NaCl aqueous solution using Zetasizer Nano Series (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK).

pH

pH values of suspensions were determined by immersion of the electrode directly in the suspension using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil), at room temperature.

Benzophenone-3 content

Benzophenone-3 content ($mg.ml^{-1}$) was determined (n = 3) after dissolution of nanocapsules or nanoemulsions in acetonitrile (1 ml of suspension to 25 ml of acetonitrile) and assayed by high performance liquid chromatography – HPLC. The chromatographic system consisted of a Gemini RP-18 column (150 x 4.60 mm, 5 µm, Phenomenex, Torrance, USA) and a Shimadzu instrument (LC-10AVP Pump, UV-VIS SPD-10AVP Module, Class-VP Software, Shimadzu, Tokyo, Japan). The mobile phase at a flow rate of 1.0 ml.min-1 consisted of acetonitrile/water (85:15% v/v) containing 1% of acetic acid. The volume injected was 20 µL and benzophenone-3 was detected at 286 nm. Validation of the HPLC assay demonstrated that this method was linear ($y = 74529x - 1860.3$, $r^2 = 0.9999$, n = 5) in the range of 1 – 20 $\mu g.ml^{-1}$ and precise (RSD: 0.65% for repeatability and 0.68 % for intermediate precision). The specificity was tested in presence of the nanoparticle adjuvants and demonstrated that they did not alter the benzophenone-3 assay [27,28].

Encapsulation efficiency

Free benzophenone-3 was determined in the clear supernatant following separation of nanoparticles (NC and NE) from aqueous medium by a combined ultrafiltration-centrifugation technique (Ultrafree-MC® 10,000 MW, Millipore, Bedford, USA). Encapsulation efficiency (%) was calculated by the difference between the total and free benzophenone-3 concentrations determined in the nanoparticles (drug content) and in the ultrafiltrate, respectively, using the HPLC method described above.

Morphological analyses

Morphological analyses were conducted by transmission electron microscopy (TEM; Jeol, JEM 1200 Exll, *Centro de Microscopia* - UFRGS) operating at 80 kV. The diluted suspensions were deposited in Formvar-Carbon support films on specimen grid (Electron Microscopy Sciences, 400 mesh), negatively stained with uranyl acetate solution (2% m/v) and observed at different magnifications.

Stability studies

All NC and NE formulations were stored at room temperature and protected from light for 6 months. Particle size, polydispersity index, zeta potential and pH were evaluated after 6 months. Drug content and encapsulation efficiency were determined after 3 and 6 months of storage.

Photostability study

After preparation, the formulations (NC and NE) were placed in transparent quartz cells with 5 mm optical path and exposed to UVC radiation (Phillips TUV lamp – UVC long life, 30 W). The cells were irradiated in a box of mirror for 168 h (7 days). The formulations were placed at a distance of 10 cm from the fluorescent lamps. After 72 and 168 h (3 and 7 days, respectively), the total concentration of benzophenone-3 was quantified. Benzophenone-3 was assayed by HPLC after the dissolution of 200 µl of samples with acetonitrile according to the method previously described. A benzophenone-3 methanolic solution was used as a model of free benzophenone-3, prepared at a concentration of 1 $mg.ml^{-1}$.

Statistical analysis

Formulations were prepared and analyzed in triplicate. Results are expressed as mean ± SD (standard deviation). One-way analysis of variance (ANOVA) or two-way analysis of variance (ANOVA) was employed in the comparison of the experimental data. Post-hoc multiple comparisons were done by Tukey's test for significance at p–values less than 0.05. All analyses were run using the SigmaStat Statistical Program (Version 3.0, Jandel Scientific, USA).

RESULTS AND DISCUSSION

Nanoparticulated systems have been widely studied in the pharmaceutical and cosmetic area in the last years [2,4]. These drug delivery systems presented advantages compared to other systems and classical dosage forms for parenteral, oral and topical administration. Aiming to study the potential to use alternative vegetable oils to prepare nanostructured delivery systems, we

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
NC-AKO	243 ± 06 [a]	0.20 ± 0.00 [a]	-7.34 ± 0.67 [a]	6.97 ± 0.24 [a]
NE-AKO	275 ± 49 [a]	0.27 ± 0.01 [b]	- 6.94 ± 0.64 [a]	6.51 ± 1.19 [a]
NC-GSO	228 ± 07 [a]	0.19 ± 0.02 [a]	-8.22 ± 1.34 [a]	6.82 ± 0.02 [a]
NE-GSO	239 ± 03 [a]	0.30 ± 0.02 [b]	-8.80 ± 1.11 [a]	7.16 ± 0.08 [a]

Table 2. Physicochemical characteristics of nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

prepared nanocapsules and nanoemulsions containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase.

Preparation and characterization

Nanocapsules and nanoemulsions were prepared by interfacial polymer deposition and spontaneous emulsification, respectively. All formulations appeared macroscopically homogeneous and their aspects were similar to a milky bluish opalescent fluid (Tyndall effect), regardless of the type of oily phase (GSO or AKO) or the vesicle structure (nanocapsule or nanoemulsion). Physicochemical characteristics of the formulations are presented in Table 2. All formulations presented mean particle size in the nanometric range (220 - 280 nm), acidic pH and negative zeta potential (between -6.0 and -9.0 mV). By a one-way ANOVA analysis, these parameters were similar for all formulations ($p > 0.05$). On the other hand, NC presented a narrowed particle size distribution, as can be observed by their lower polydispersity indices (0.19 - 0.20) compared to NE (0.27 – 0.30, $p \leq 0.05$). Polydispersity indices below 0.25 indicate an adequate homogeneity of these systems [7].

In order to obtain a better evaluation of the influence of the oily phase and the vesicle structure on the physicochemical characteristics of the formulations, we also carried out a two-way ANOVA analysis. This analysis showed the influence of the oily phase on the mean particle size as well as on the zeta potential. Formulations prepared with AKO showed a higher mean size and lower zeta potential ($p \leq 0.05$). According to Schaffazick *et al.* [11], the kind of oily phase used as the core in the preparation of polymeric nanocapsules could have a great influence on the particle mean size and polydispersity index due to the difference in its viscosity, hydrophobic characteristic and interfacial tension. Polydispersity indices were influenced by the vesicle structure ($p \leq 0.05$). NC presented lower polydispersity indices than NE, regardless of the kind of oily phase, as showed previously by the one-way ANOVA analysis.

Considering the overall results just after the preparation, the formulations prepared with GSO or AKO as oily phase presented physicochemical characteristics similar to those prepared with capric/caprilic triglyceride mixture [9,19], an oily phase widely used to prepare NC and NE [1,3,29,30].

Figure 1 shows the images obtained by transmission electron microscopy, revealing that nanocapsules and droplets of the nanoemulsions were spherical in shape. The results corroborate the particle size analysis, showing that the particles are present in the nanometric range (close to 200 nm). The morphological analysis allows observing the influence of the type of oily phase on the mean particle size, as demonstrated by the two-way analysis of variance previously commented.

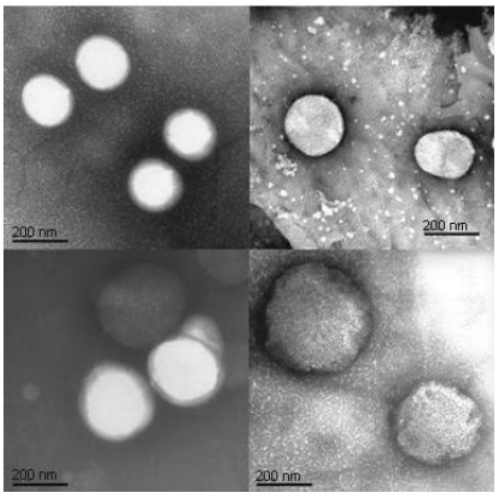

Figure 1. Transmission electron microscopy images of nanocapsules containing grape seed oil (**A**), nanocapsules containing almond kernel oil (**B**), nanoemulsion containing grape seed oil (**C**) and nanoemulsion containing almond kernel oil (**D**). Bar = 200 nm (150,000 x).

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
B3-NC-AKO	242 ± 11 [a]	0.21 ± 0.01 [a]	-8.76 ± 0.96 [a]	7.03 ± 0.04 [a]
B3-NE-AKO	233 ± 06 [a]	0.28 ± 0.06 [b]	-9.37 ± 1.35 [a]	7.08 ± 0.02 [a]
B3-NC-GSO	242 ± 11 [a]	0.16 ± 0.02 [a]	- 7.28 ± 1.00 [a]	6.99 ± 0.11 [a]
B3-NE-GSO	237 ± 12 [a]	0.23 ± 0.04 [a,b]	- 7.03 ± 0.34 [a]	7.20 ± 0.15 [a]

Table 3. Physicochemical characteristics of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Benzophenone-3 content ($mg.ml^{-1}$)	Encapsulation efficiency (%)
B3-NC-AKO	0.95 ± 0.04	99.98 ± 0.01
B3-NE-AKO	0.96 ± 0.04	99.98 ± 0.01
B3-NC-GSO	1.00 ± 0.06	99.98 ± 0.01
B3-NE-GSO	0.97 ± 0.04	99.99 ± 0.00

Table 4. Total content and encapsulation efficiency of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase (n = 3, mean ± standard deviation).

After observing the feasibility to obtain colloidal systems (NC and NE) with two alternative vegetable oils, we chose a substance model to be entrapped in these systems. Benzophenone-3 was chosen because of its sun protection properties, which could be efficiently associated to the antioxidant capacities of the oily phases. Physicochemical characteristics of the benzophenone-3-loaded systems are presented in Table 3.

All results are similar to those presented by the unloaded-systems, presenting nanometric mean particle size (230 – 252 nm), polydispersity index below 0.30, acidic pH range and negative zeta potential (-7.0 to – 9.5 mV). The presence of the sunscreen did not modify the colloidal characteristics of both systems. Regarding benzophenone-3 content and its encapsulation efficiency, all formulations showed content according to the theoretical concentration and encapsulation efficiency close to 100 % (Table 4), regardless of the oily phase or the vesicle structure. These results showed that the use of these vegetable oils could be an interesting alternative to be used in the development of dermatological and cosmetic formulations.

Stability studies

Taking into account the possibility of using these systems in the development of new products, we stored the developed formulations (containing benzophenone-3 or not) at room temperature (25 °C) and protected from light. After 6 months, the physicochemical characteristics of the systems were reanalyzed (Tables 5 and 6 for unloaded- and benzophenone-3-loaded nanoemulsions and nanocapsules, respectively).

As can be observed in Table 5, after 6 months of storage unloaded-NC and NE showed similar mean particle size and polydispersity indices compared to the initial characteristics (Table 1), confirmed by a two-way ANOVA. However, all formulations presented a significant decrease in pH values, regardless of the formulation. The decline of pH values was more pronounced for those formulations containing GSO as oily core. Regarding NC, this decrease is usually explained due to the polymer hydrolysis or the relaxation of the polymer chains [11]. However, in our study this decrease was present in formulations with or without polymer (NC and NE, respectively). Thus, the decrease in pH values observed in our study could be explained by the hydrolysis of the triglyceride chains and the respective increase of the free fatty acids

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
NC-AKO	222 ± 09 [a]	0.18 ± 0.00 [a,b]	-08.59 ± 0.62 [a]	4.88 ± 1.14 [a]
NE-AKO	238 ± 42 [a]	0.26 ± 0.06 [a,b]	-10.23 ± 1.31 [a]	4.78 ± 0.80 [a]
NC-GSO	220 ± 04 [a]	0.15 ± 0.05 [a]	-10.30 ± 1.41 [a,b]	3.25 ± 0.17 [a]
NE-GSO	239 ± 23 [a]	0.34 ± 0.11 [b]	-13.00 ± 0.47 [b]	3.58 ± 0.35 [a]

Table 5. Stability evaluation - Physicochemical characteristics of nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase after 6 months of storage at room temperature and protected from light (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Mean size (nm)	PDI	Zeta potential (mV)	pH
B3-NC-AKO	237 ± 10 [a]	0.19 ± 0.02 [a]	-09.79 ± 0.88 [a]	4.77 ± 0.85 [b,c]
B3-NE-AKO	216 ± 16 [a]	0.29 ± 0.06 [a]	-12.19 ± 0.58 [a]	5.91 ± 1.95 [c]
B3-NC-GSO	221 ± 12 [a]	0.20 ± 0.04 [a]	-10.35 ± 0.63 [a]	3.17 ± 0.21 [b,c]
B3-NE-GSO	214 ± 10 [a]	0.25 ± 0.03 [a]	-12.82 ± 2.02 [a]	2.86 ± 0.15 [a,b]

Table 6. Stability evaluation – Physicochemical characteristics of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase after 6 months of storage at room temperature and protected from light (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

Formulation	Benzophenone-3 content (mg.ml^{-1})		Encapsulation efficiency (%)	
	3 months	6 months	3 months	6 months
B3-NC-AKO	0.99 ± 0.04	0.98 ± 0.02	99.37 ± 0.12	94.94 ± 3.56
B3-NE-AKO	1.02 ± 0.07	0.99 ± 0.10	97.89 ± 0.67	98.51 ± 0.49
B3-NC-GSO	0.99 ± 0.07	0.96 ± 0.07	99.07 ± 0.41	99.18 ± 0.18
B3-NE-GSO	1.04 ± 0.06	0.96 ± 0.04	99.39 ± 0.13	98.44 ± 0.15

Table 7. Stability evaluation - Total content and encapsulation efficiency of benzophenone-3-loaded nanoparticles (nanocapsules - NC and nanoemulsion - NE) containing grape seed oil (GSO) or almond kernel oil (AKO) as oily phase after 3 and 6 months of storage at room temperature and protected from light (n = 3, mean ± standard deviation).

content [31-34]. AKO and GSO are oils with high amounts of linoleic acid and other unsaturated fatty acids [21-23], which could undergo oxidative and hydrolytic reactions in contact with aqueous medium and temperature below 100 °C, forming free fatty acids [32,33].

The occurrence of oxidative/hydrolytic reactions during the storage time was confirmed by the development of a characteristic odor in all formulations after 6 months of storage. These results show that it is necessary to add antioxidant substances like butylated hydroxy anisole (BHA) or butylated hydroxy toluene (BHT) in future formulations in which these systems could be incorporated. In addition, as the decrease in pH values was higher for GSO formulations, these formulations presented a significant increase (in module) in their potential zeta values, explained also by the higher concentration of free fatty acids on the particle surface. Similar results were observed for benzophenone-3-loaded nanocapsules and nanoemulsions comparing the results presented in Table 3 and 5. In addition, the results showed no influence of this model substance in the stability of these systems (Table 5 and 6). Benzophenone-3 content and its encapsulation efficiency was not altered during the storage time, presenting values between 90–110 % and close to 100%, respectively, after 3 as well as 6 months of storage (Table 7).

Based on the results obtained by instrumental analysis, no differences on the physicochemical stability of the different vesicles (NC or NE) could be observed. However, macroscopic analysis allowed us to observe the occurrence of reversible aggregation (creaming) in the NE, regardless of the type of oily phase. This finding shows the importance of the polymer layer around the oil droplet to improve the physical stability of the systems. Creaming could probably not be detected by the particle size and polydispersity index measurements due to its reversible characteristic by little agitation.

Photodegradation studies

In order to evaluate the potential of these nanoparticulated systems prepared with alternative vegetable oils to prevent the photodegradation of some substances under UV radiation and to highlight their potential application in pharmaceutical and cosmetic field, we carried out a photodegradation study on benzophenone-3-loaded nanoparticles by their exposure to UVC radiation during 7 days. UVC was chosen due to its more energetic characteristic and the relative photostability of benzophenone-3 [35], allowing to reduce the experimental time. Although NE showed a lower physicochemical stability compared to NC, photodegradation studies were carried out with all formulations in order to compare the influence of the kind of oily phase

Formulation	Benzophenone-3 content (mg.ml^{-1})		
	0 h	72 h	168 h
B3-NC-AKO	0.95 ± 0.00 [a]	0.84 ± 0.04 [a,b]	0.79 ± 0.10 [b]
B3-NE-AKO	0.94 ± 0.00 [a]	0.86 ± 0.05 [b]	0.81 ± 0.10 [b]
B3-NC-GSO	0.99 ± 0.04 [a]	0.86 ± 0.03 [b]	0.78 ± 0.06 [b]
B3-NE-GSO	0.99 ± 0.02 [a]	0.85 ± 0.01 [b]	0.71 ± 0.02 [b]
MS	0.92 ± 0.03 [a]	0.74 ± 0.06 [a]	0.21 ± 0.12 [a]

Table 8. Benzophenone-3 content of free benzophenone-3 solution (methanolic solution – MS) and benzophenone-3-loaded nanocapsules or nanoemulsions after 0, 72 and 168 h of UV irradiation (n = 3, mean ± standard deviation). Means, in column, with the same letter are not significantly different (ANOVA, $p \leq 0.05$).

and the vesicle structure. A benzophenone-3 methanolic solution was used as a model of free benzophenone-3. The results of the photodegradation study are shown in Table 8. At the beginning of the experiment, all formulations as well as the methanolic solution presented similar benzophenone-3 content close to the theoretical concentration (1.00 mg.ml^{-1}). After 72 h, benzophenone-3 content in the methanolic solution decreased to 0.74 mg.ml-1, which was significantly lower ($p \leq 0.05$) than almost all nanoparticulated formulations (0.84 to 0.86 mg.ml^{-1}). However, after 168 h of UV radiation, the protective effect of the entrapment of benzophenone-3 in NC and NE prepared with GSO and AKO against photodegradation was more pronounced. Benzophenone-3 content in methanolic solution decayed from 0.92 to 0.21 mg.ml^{-1} (close to 80% of photodegradation), whereas for formulations it decayed only around 10-30% reaching mean concentrations between 0.71 and 0.81mg.ml^{-1}.

By a two-way analysis of variance, it could be observed that the kind of oily phase and the vesicle structure did not influence the protection of benzophenone-3 against the photodegradation. However, the photostability of benzophenone-3 was improved by its entrapment in the nanoparticle systems. This result suggests that benzophenone-3 is dissolved in the oily phase of the particles in a higher proportion [36] compared to its possible adsorption on the nanoparticle surfaces [11]. Solubilization of a sunscreen in the internal phase of coarse emulsions could lead to a higher photostability of formulations [37]. The results from photodegradation obtained in the present study corroborate to previous studies reported in the literature to other substances. Weiss-Angeli *et al*. [38] showed the increase in the photostability of octyl methoxycinnamate against UVA radiation after its incorporation in polymeric nanocapsules. A previous study of our research group demonstrated a lower photodegradation of tretinoin when loaded in nanocapsules or nanoemulsion compared to a free tretinoin solution [9]. Formulations developed in these two cited studies were prepared with a capric/caprylic triglyceride mixture as oily phase, pointing out the potential use of the vegetable oils suggested in our study as an alternative to compose the oily phase of such nanoparticles structures (nanocapsules and nanoemulsions).

CONCLUSIONS

This work showed for the first time the feasibility to prepare nanocapsules and nanoemulsions using grape seed oil and almond kernel oil, as alternative oily phases. Nanocapsules and nanoemulsions prepared with these oils presented nanometric size range and negative zeta potential. These parameters remained adequate after 6 months of storage at room temperature, although a decline of pH was observed for all formulations. However, nanoemulsions presented higher polydispersity indices and occurrence of creaming after 6 months of storage, regardless of the type of oily phase. The application of these alternative vegetable oils in the preparation of oil-based nanomaterials like nanocapsules and nanoemulsions with pharmaceutical or cosmetic purposes was demonstrated by an efficient entrapment of a model substance (benzophenone-3) to the nanoparticles as well as by showing their use to delay its photodegradation under UV radiation.

Acknowledgement. We thank the financial support received from Rede Nanocosméticos CNPq/MCT.

REFERENCES

1. Beck, R.C.R., A.R. Pohlmann. & S.S. Guterres (2004) *J. Microencapsul.* **21**: 499-512.
2. Kidane, A. & P. Bhatt (2005) *Curr. Opin. Chem. Biol.* **9**: 347-51.
3. Beck, R.C.R., A.R. Pohlmann., C. Hoffmeister, M.R. Gallas, E. Collnot, U.F. Schaefer, S.S. Guterres & C.M. Lehr (2007) *Eur. J. Pharm. Biopharm.* **67**: 18-30.
4. Guterres, S.S., M.P. Alves & A.R. Pohlmann (2007) *Drug Target Insights* **2**: 147-57.
5. Beck, R.C.R., S.S. Guterres, R.J. Freddo, C.B. Michalowski, I. Barcellos & J.A.B. Funck (2003) *Acta Farm. Bonaerense* **22**: 11-5.
6. Beck, R.C.R., S.E. Hass, S.S. Guterres, M.I. Ré, E.V. Benvenutti & A.R. Pohlmann (2006) *Quim. Nova* **29**: 990-6.
7. Alves, M.P., A.L. Scarrone, M. Santos, A.R. Pohlmann & S.S. Guterres (2007) *Int. J. Pharm.* **341**: 215-20.
8. Fachinetto, J.M, A.F. Ourique, G. Lubini, S.B. Tedesco, A.C.F. Silva & R.C.R. Beck (2008) *Lat. Am. J. Pharm.* **27**: 668-73.
9. Ourique, A.F., A.R. Pohlmann, S.S. Guterres & R.C.R. Beck (2008) *Int. J. Pharm.* **352**: 1-4.
10. Barratt, G. (2003) *Cell. Mol. Life Sci.* **60**: 21-37.
11. Schaffazick, S.R., L.L. Freitas, A.R. Pohlmann & S.S. Guterres (2003) *Quim. Nova* **25**: 726-37.
12. Jäger, A., V. Stefani, S.S. Guterres & A.R. Pohlmann (2007) *Int. J. Pharm.* **338**: 297-305.
13. Pohlmann, A.R., V. Weiss, O. Mertins, N. Pesce da Silveira & S.S. Guterres (2002) *Eur. J. Pharm. Sci.* **16**: 305-12.
14. Anton, N., J.P. Benoit & P. Saulnier (2008) *J. Control. Release* **128**: 185-99.
15. Couvreur, P., C. Dubernet & F. Puisieux (1995) *Eur. J. Pharm. Biopharm.* **41**: 2-13.
16. Alonso M.J. (2004) *Biomed. Pharmacother.* **58**: 168-72.
17. Alvarez-Román, R., G. Barré, R.H. Guy & H. Fessi (2001) *Eur. J. Pharm. Biopharm.* **52**: 191-5.
18. Bouchemal, K., S. Briançon, E. Perrier & H. Fessi (2004) *Int. J. Pharm.* **280**: 241-51.
19. Friedrich, R.B., M.C. Fontana, R.C.R. Beck., A.R. Pohlmann & S.S. Guterres (2008) *Quim. Nova* **31**: 1131-6.
20. Kallinteri, P., S Higgins, G.A. Hutcheon, C.B. Pourçain & M.C. Garnett (2005) *Biomacromolecules* **6**: 1885-94.
21. Beveridge, T.H.J., B. Girard, T. Kopp & J.C.G. Drover (2005) *J. Agr. Food Chem.* **53**: 1799-804.
22. Baydar, N.G., G. Ozkan & E.S. Cetin (2007) *Grasas Aceites* **58**: 29-33.
23. Martín-Carratalá, M.L., C. Lorens-Jordá, V. Berenguer-Navarro & N. Grané-Teruel (1999) *J. Agr. Food Chem.* **47**: 3688-92.
24. Espín, J.C., C. Soler-Rivas & H.J. Wichers (2000) *J. Agr. Food Chem.* **48**: 648-56.
25. Fessi, H., F. Puisieux & J.P. Devissaguet (1988) *Eur. Patent* 0274961 A1
26. Martini, É., E. Carvalho, H. Teixeira (2007) *Quim. Nova* **30**: 930-4.
27. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (2005) *Gudeline on Validation of Analytical Procedure Q2 (R1): Text and Methodology.*
28. The United States Pharmacopeia 31 ed. (2008) United States Pharmacopoeial Convention, Rockville, Maryland, USA.
29. Guterres, S.S., H. Fessi, G. Barrat, J.P. Devissaguet & F. Puisieux (1995) *Int. J. Pharm.* **113**: 57-63.
30. Teixeira, M., M.J. Alonso, M.M.M. Pinto & CM Barbosa (2005) *Eur. J. Pharm. Biopharm.* **59**: 491-500.
31. Calvo, P., J.L. Vila-Jato & M.J. Alonso (1996) *J. Pharm. Sci.* **85**: 530-6.
32. Fox, N.J. & GW Stachowiak (2007) *Tribol. Int.* **40**: 1035-46.
33. Redá, S.Y. & P.I.B. Carneiro (2007) *Revista Analytica* **27**: 60-7.
34. Schaffazick, S.R., A.R. Pohlmann & S.S. Guterres (2007) *Pharmazie* **62**: 354-60.
35. Ribeiro, C (2006) "Cosmetologia aplicada a dermoestética", Pharmabooks, São Paulo.
36. Manconi, M., V. Donatella, C. Sinico, G. Loy & A.M. Fadda (2003) *Int. J. Pharm.* **260**: 261-72.
37. Milesi, S.S. & SS Guterres (2002) *Caderno de Farmácia* **18**: 81-7.
38. Weiss-Angeli, V., F.S. Poletto, L.R. Zancan, F. Baldasso, A.R. Pohlmann & S.S. Guterres (2008) *J. Biomed. Nanotechnol.* **4**: 80-9.

Nanoscale Res Lett
DOI 10.1007/s11671-010-9683-1

NANO EXPRESS

Nanostructured Systems Containing Rutin: In Vitro Antioxidant Activity and Photostability Studies

Juliana S. Almeida · Fernanda Lima · Simoní Da Ros · Luis O. S. Bulhões · Leandro M. de Carvalho · Ruy C. R. Beck

Received: 7 May 2010 / Accepted: 30 June 2010

Abstract The improvement of the rutin photostability and its prolonged in vitro antioxidant activity were studied by means of its association with nanostructured aqueous dispersions. Rutin-loaded nanocapsules and rutin-loaded nanoemulsion showed mean particle size of 124.30 ± 2.06 and 124.17 ± 1.79, respectively, polydispersity index below 0.20, negative zeta potential, and encapsulation efficiency close to 100%. The in vitro antioxidant activity was evaluated by the formation of free radical ·OH after the exposure of hydrogen peroxide to a UV irradiation system. Rutin-loaded nanostructures showed lower rutin decay rates [$(6.1 \pm 0.6)\ 10^{-3}$ and $(5.1 \pm 0.4)\ 10^{-3}$ for nanocapsules and nanoemulsion, respectively] compared to the ethanolic solution [$(35.0 \pm 3.7)\ 10^{-3}\ min^{-1}$] and exposed solution [$(40.1 \pm 1.7)\ 10^{-3}\ min^{-1}$] as well as compared to exposed nanostructured dispersions [$(19.5 \pm 0.5)\ 10^{-3}$ and $(26.6 \pm 2.6)\ 10^{-3}$, for nanocapsules and nanoemulsion, respectively]. The presence of the polymeric layer in nanocapsules was fundamental to obtain a prolonged antioxidant activity, even if the mathematical modeling of the in vitro release profiles showed high adsorption of rutin to the particle/droplet surface for both formulations. Rutin-loaded nanostructures represent alternatives to the development of innovative nanomedicines.

Keywords Antioxidant activity · Nanocapsules · Nanoemulsions · Nanoparticles · Photostability · Rutin

Introduction

Currently, there is a growing interest in the study of antioxidants [1, 2]. Recent discoveries have pointed out the effects of free radicals in the body, which are involved in energy production, phagocytosis, and regulation of cell growth among others [3]. Oxidative stress is a condition that occurs in a system when the generation of reactive oxygen species exceeds the capacity of neutralization and disposal system. The instability may result from a lack of antioxidant capacity caused by disturbances in the production, distribution, or by an abundance of reactive oxygen species from endogenous sources or stressful environmental conditions. Oxidative stress has been implicated in a growing list of diseases such as cardiovascular and neurodegenerative diseases, cancer, arthritis, hemorrhagic shock, cataracts, as well as in aging processes [3–5].

Among the various classes of antioxidants, naturally occurring phenolic compounds have received much attention in recent years due to their inhibition of lipid peroxidation and lipoxygenase [4]. The antioxidant activity of phenolic compounds is mainly related to their reducing properties and chemical structure [6]. These characteristics play an important role in the neutralization or sequestration

J. S. Almeida · L. M. de Carvalho
Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria, Av. Roraima, 1000, Santa Maria, RS 97105-900, Brazil

F. Lima · S. D. Ros · L. M. de Carvalho
Departamento de Química, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

L. O. S. Bulhões
Departamento de Química, Universidade Federal de São Carlos, São Carlos, SP 13565-905, Brazil

R. C. R. Beck (✉)
Faculdade de Farmácia, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, RS 90610-000, Brazil
e-mail: ruy.beck@ufrgs.br

Published online: 15 July 2010

of free radicals and chelation of transition metals, acting both in the initiation step and in the propagation of oxidation [6]. The intermediate species formed by the action of antioxidant phenolic compounds are relatively stable due to the resonance of the aromatic ring in their structure [7].

Natural flavonoids are phenolic compounds known for their significant scavenging properties on oxygen radicals both in vivo and in vitro [8, 9]. Many studies have showed the importance of their antiradical activity [10–13]. Moreover, their actions in humans have been subject of extensive research, and natural flavonoids have been described to possess several biological activities such as antioxidant, anti-inflammatory, antitumor, and antiviral properties [14].

Rutin (quercetin-3-O-rutinoside), the glycoside of quercetin, is abundantly found and distributed in plants such as in buckwheat seed, fruits, and fruit rinds, especially citrus fruits (orange, grapefruit, lemon). It presents important properties in human health like its significant scavenging properties on oxidizing species such as hydroxyl radical, superoxide radical, and peroxyl radical [15], as shown by many in vitro and in vivo experiments [16–18]. Furthermore, rutin has several pharmacological activities including anti-allergic, anti-inflammatory, and vasoactive properties [15, 19, 20]. Rutin offers an advantage over other flavonoids, which in some occasions behave as pro-oxidant agents and catalyze oxygen production [15]. Therefore, it is considered a non-toxic molecule and not oxidized. On the other hand, the main disadvantage of the molecule is its poor solubility in aqueous media, explaining its poor oral or topical bioavailability [19] and being a drawback to its conversion in adequate dosage forms.

In the last decade, an alternative drug delivery approach was developed to overcome the poor water solubility of rutin by reducing its particle size [19]. A decrease in the drug particle size leads to an increase in the saturation solubility, to an enlarged surface area and to a higher dissolution velocity [21]. Formulating rutin as drug nanocrystals has significant importance to improve its physicochemical properties, especially its oral bioavailability. Some studies have shown techniques for lyophilization and spray drying nanocrystals of rutin in order to ensure their redispersibility as separate and non-aggregated particles. This characteristic is a critical point to improve the dissolution behavior of drugs, especially from a tablet dosage form [19, 20].

On the other hand, polymeric nanoparticles have been designed to encapsulate lipophilic drugs not only to improve their physicochemical properties but also to target organs or tissues, to avoid drug degradation, to improve drug efficacy, and to circumvent drug toxicity [22]. Nanocapsules are polymeric nanoparticles composed of an oily core surrounded by a polymeric wall stabilized by surfactants at the particle/water interface [23]. The potential use of nanocapsules includes the protection of drugs against inactivation in the gastrointestinal tract [21], delivery of poorly water-soluble compounds [24, 25], and protection of sensitive materials to chemical degradation induced by UV light [26–28]. As the polymeric nanoparticles, nanoemulsions have been also investigated as drug delivery systems in the last years [29–32]. These colloidal systems are composed of oily nanodroplets stabilized by surfactants in an aqueous medium.

All these considerations into account, the main objective of our study was to develop aqueous rutin delivery systems and to evaluate the influence of the association of rutin to different nanostructures (nanocapsules and nanoemulsions) on its in vitro antioxidant activity and UV photostability. Nanocapsules and nanoemulsions were compared to establish the importance of the polymeric layer around the nanodroplets by means of a systematic physicochemical characterization of both formulations. The development of such formulations aimed to obtain aqueous systems containing rutin, as an intermediate or final pharmaceutical product. To the best of our knowledge, no report on the association of rutin to polymeric nanocapsules is currently available in the scientific literature. The administration of flavonoids by means of nanovectors may be useful for the development of advanced delivery systems for these powerful compounds, in view of their adoption in primary and secondary disease prevention, either for oral or parenteral administration [33].

Materials and Methods

Materials

Rutin, poly-(ε-caprolactone) (PCL), and sorbitan monostearate (Span 60®) were acquired from Sigma–Aldrich (São Paulo, Brazil). Polysorbate 80 (Tween 80®) was supplied by Henrifarma (São Paulo, Brazil). Grape seed oil was obtained from Dellaware (Porto Alegre, Brazil). All other chemicals and solvents presented pharmaceutical or HPLC grade and were used as received.

Preparation of Nanoparticles

Rutin-loaded nanocapsule suspensions (R-NC) and nanoemulsions (R-NE) were prepared in triplicate ($n = 3$) by the interfacial deposition of preformed polymer method and spontaneous emulsification, respectively [34, 35]. For the preparation of nanocapsules, an organic solution containing the grape seed oil (3.3 ml), sorbitan monostearate (0.776 g), the polymer (1.0 g), and acetone (147 ml) was

subjected to magnetic stirring, at a temperature of 40°C. After 1 h, an ethanolic solution containing the rutin (120 ml) was added to this organic phase maintaining the agitation for a further 10 min. Then, the organic phase was injected into an aqueous phase (534 ml) containing Tween 80® (0.776 g). Acetone was removed and the aqueous phase concentrated to 100 ml by evaporation at 40°C under reduced pressure reaching a final concentration of 0.25 mg ml^{-1} of rutin. All formulations were stored at room temperature and protected from light (amber glass flasks). Nanoemulsions were prepared using the same experimental conditions, but omitting the presence of the polymer in the organic phase. In addition, in order to evaluate the influence of rutin in the physicochemical characteristics of such nanostructures, blank formulations (placebo) were prepared similarly, but omitting the presence of rutin. These formulations were called B-NC and B-NE, for blank nanocapsules and blank nanoemulsions, respectively. Table 1 summarizes the abbreviation presented in Tables and Figures.

Characterization of Nanoparticles

Rutin Content

Rutin was assayed by liquid chromatography (LC). The chromatographic system consisted of a Gemini RP-18 column (150 × 4.60 mm, 5 µm, Phenomenex, Torrance, USA) and a Shimadzu instrument (LC-10AVP Pump, UV–VIS SPD-10AVP Module, Class-VP Software, Shimadzu, Tokyo, Japan). The mobile phase consisted of methanol/water (50:50% v/v) acidified with phosphoric acid (apparent pH 4.0) pumped at a flow rate of 1.0 ml min^{-1}. The volume injected was 20 µl, and rutin was detected at 352 nm. Rutin content (mg ml^{-1}) was determined ($n = 3$) after its extraction with methanol from nanocapsules or nanoemulsions (1.6 ml of the formulation to 10 ml of methanol) and dilution with the mobile phase to a concentration of 20 µg ml^{-1}. Validation of the LC assay demonstrated that this method was linear ($y = 30{,}322\times - 6{,}233.7$, $r = 0.9999$, $n = 5$) in the range of 5–30 µg ml^{-1}, precise (RSD: 1.86% for repeatability and 0.70% for intermediate precision) and accurate (RSD: 1.70%). The specificity was tested in presence of the nanoparticle adjuvants and demonstrated that they did not alter the rutin assay [36].

Encapsulation Efficiency

Free rutin was determined in the ultrafiltrate after separation of the nanoparticles by ultrafiltration/centrifugation technique (Microcon 10,000 MW, Millipore). Encapsulation efficiency (%) was calculated by the difference between the total and free rutin concentrations determined in the nanoparticles (drug content) and in the ultrafiltrate, respectively, using the LC method previously described.

pH Measurements

The pH values of formulations were determined by immersion of the electrode directly in the dispersions using a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, São Paulo, Brazil), at room temperature.

Particle Size Analysis, Polydispersity Indices, and Zeta Potential

Particle sizes and polydispersity indices ($n = 3$) were measured by photon correlation spectroscopy after adequate dilution of an aliquot of the suspension in reverse osmosis water (Brookhaven Instruments Corporation, USA). The zeta potentials were determined after dilution of the samples in 10 mM NaCl aqueous solution (Brookhaven Instruments Corporation, Holtsville, USA).

Table 1 List of nomenclatures presented in tables and figures

Abbreviation	Meaning
B-NC	Blank nanocapsules (nanocapsules prepared without drug)
R-NC	Rutin-loaded nanocapsules
B-NE	Blank nanoemulsion (nanoemulsion prepared without drug)
R-NE	Rutin-loaded nanoemulsion
R-SE	Rutin ethanolic solution
k	Release rate constant according (monoexponential kinetic)
k_1	Release rate constant of the burst phase (biexponential kinetic)
k_2	Release rate constant of the sustained phase (biexponential kinetic)
a	Initial concentration of rutin at the burst phase (biexponential kinetic)
b	Initial concentration of rutin at the sustained phase (biexponential kinetic)
r	Regression coefficient
MSC	Model selection criteria

Morphological Analyses

Morphological analyses were conducted at Centro de Microscopia (UFRGS, Brazil) by transmission electron microscopy (TEM; Jeol, JEM 1200 Exll, Japan) operating at 80 kV. Diluted suspensions and nanoemulsions were deposited on specimen grid (Formvar-Carbon support films, Electron Microscopy Sciences), negatively stained with uranyl acetate solution (2% w/v) [37] and observed at different magnifications.

Antioxidant Activity and Photostability Study

The system for the irradiation of the samples was developed by Carvalho et al. [38] and consisted of a 400 W high-pressure mercury lamp (Silvania) as UV radiation source, a cooling system based on air and water circulation, a thermo-regulator for the temperature control, a holder for 12 quartz tubes, and an aluminum-based block. In quartz tubes, 10 ml of each sample (R-NC, R-NE and a rutin ethanolic solution—R-ES) was placed and exposed to UV radiation for 30 min ($n = 3$). Every 5 min, samples were taken from the tubes and analyzed by LC to determine the content of rutin. In order to determine the antioxidant activity, 40 µl H_2O_2 was added to the sample before starting the irradiation process. The amount of H_2O_2 was added in excess, since the concentration of H_2O_2 is 10 times the concentration of the sample and the lamp has a limiting quantum yield for the photolysis of H_2O_2. The artificial generation of the hydroxyl radical was carried out by the decomposition of H_2O_2 in the presence of UV radiation (Eq. 1):

$$H_2O_2 + hv \rightarrow 2 \cdot OH \quad (1)$$

The kinetic constant for the reaction of rutin with the ·OH radical was determined by subtracting the contribution of direct photolysis of rutin from the ·OH radical reaction kinetic constant, according to the following equation (Eq. 2):

$$k_{OH+hv} - k_{hv} = k_{OH} \quad (2)$$

where $k_{OH + hv}$ represents the kinetic constant determined by the rutin decay after UV photolysis in the presence of H_2O_2, k_{hv} represents the kinetic constant determined by the direct photolysis of rutin in absence of H_2O_2, and k_{OH} represents the liquid free radical reaction kinetic constant of the ·OH radical with the antioxidant.

In Vitro Rutin Release Assay

In vitro drug release profiles from rutin-loaded nanostructures were evaluated (n = 3) by the dialysis bag method, using water/ethanol (65:35 v/v) as medium, at 37°C [39]. The dialysis bag (Spectra Por 7, 10 Kd, Biosystems) containing 1 ml of the sample (0.25 mg ml^{-1}) was put into a glass test-tube containing 200 ml of dissolution medium. This system was maintained under constant moderate stirring during all the time. The withdrawal of 2 ml of the external medium from the system was done at predetermined time interval, replaced by an equal volume of fresh medium, and filtered through a 0.45-µm membrane. Rutin was assayed in the samples by LC according to the method previously described. However, the injection sample volume was increased to 100 µl to allow the assay of rutin at lower concentrations. This LC method was validated according to the following characteristics: linearity ($y = 167{,}806\times + 1{,}170.3$, $n = 3$, $r = 0.9997$), concentration range (0.1–2.0 mg ml^{-1}), and precision (RSD: 0.90%).

In order to obtain a better understanding of the influence of the type of nanoparticle structure on the rutin release behavior, the mathematical modeling (MicroMath® Scientist® for Windows) was used to analyze the drug release profiles. Monoexponential ($C = C_0e^{-kt}$) and biexponential ($C = ae^{-k_1t} + be^{-k_2t}$) models were used to evaluate the rutin release profiles. The release rate constants are k, k_1, and k_2 and the initial concentration of rutin are C_0, a, and b. The selection of the model that best fit the release profiles was based on the best correlation coefficient, the best model selection criteria (MSC), both provided by the software and the best graphic adjustment. The meaning of all these abbreviations is presented in Table 1.

Statistical Analysis

Formulations were prepared and analyzed in triplicate. Results are expressed as mean ± SD (standard deviation). Two-way analysis of variance (ANOVA) was employed in the comparison of the experimental data. Post hoc multiple comparisons were done by Tukey's test for significance at p–values ≤ 0.05. All analyses were run using the SigmaStat Statistical Program (Version 3.0, Jandel Scientific, USA).

Results and Discussion

Preparation and Physicochemical Characterization of Aqueous Nanostructured Systems

All formulations appeared macroscopically homogeneous and their aspects were similar to a milky bluish opalescent fluid (Tyndall effect). The physicochemical characteristics of the formulations are presented in Tables 2 and 3. As can be seen, the formulations presented drug content close to their theoretical value (0.24 and 0.25 mg ml^{-1}), mean

Table 2 Drug content, encapsulation efficiency, and pH of rutin-loaded nanocapsules (R-NC) and rutin-loaded nanoemulsion (R-NE) as well as their respective blank formulations (B-NC and B-NE) ($n = 3$)

Formulation	Drug content (mg ml^{-1})	Encapsulation efficiency (%)	pH
B-NC	–	–	5.79 ± 0.03
R-NC	0.25 ± 0.01	93.33 ± 0.63	5.69 ± 0.08
B-NE	–	–	5.94 ± 0.10
R-NE	0.24 ± 0.01	93.83 ± 0.41	5.59 ± 0.01

– Not applicable

Table 3 Particle size, polydispersity index, and zeta potential of rutin-loaded nanocapsules (R-NC) and rutin-loaded nanoemulsion (R-NE) as well as their respective blank formulations (B-NC and B-NE) ($n = 3$)

Formulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)
B-NC	120.37 ± 2.44	0.11 ± 0.06	−20.55 ± 3.63
R-NC	124.30 ± 2.06	0.12 ± 0.02	−27.12 ± 9.19
B-NE	128.06 ± 3.38	0.13 ± 0.03	−26.03 ± 2.27
R-NE	124.17 ± 1.79	0.10 ± 0.02	−26.92 ± 1.45

particle size in the nanometric range (122–126 nm), acidic pH, and negative zeta potential (between −26.0 and −27.0 mV). Polydispersity indices below 0.20 indicated an adequate homogeneity of these systems [26]. In addition, neither the presence of rutin nor the presence of the polymer influenced the physicochemical characteristics of the structures. Moreover, nanocapsules and droplets of the nanoemulsions were spherical in shape as showed by the images obtained by transmission electron microscopy (Fig. 1). Although the type of the nanostructured system did not show any influence on the physicochemical characteristics, the morphological analyses by TEM revealed that rutin-loaded nanoemulsions (R-NE) presented rutin nanocrystals around the droplets when compared to its respective blank formulation (B-NE). Rutin nanocrystals were not observed in nanocapsule formulation (R-NC). These results can be explained by the leakage of rutin from nanoemulsion due to the absence of the polymeric layer around the droplets and its subsequent crystallization in the aqueous dispersed medium as previously demonstrated for dexamethasone under storage [37, 40].

Antioxidative Activity, UV Protodegradation, and In Vitro Release Assay

Figure 2 shows the rutin photodegradation obtained for both formulations (R-NC and R-NE), as well as for rutin ethanolic solution (R-ES). The degradation profiles of rutin

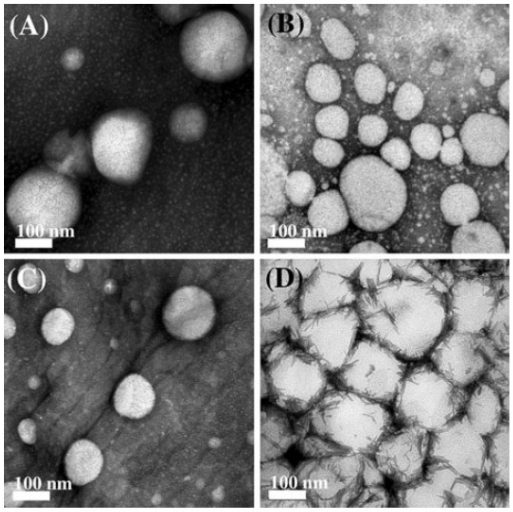

Fig. 1 Transmission electron microscopy images of **a** B-NC, **b** R-NC, **c** B-NE, and **d** R-NE. Bar 100 nm (200,000×)

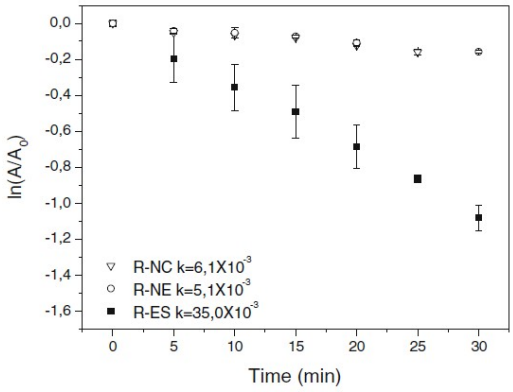

Fig. 2 Photodegradation profile of rutin-loaded nanostructures and rutin ethanolic solution during 30 min of UV irradiation

in the nanostructured formulations (R-NC and R-NE) were according to a first kinetic order, and their respective degradation constants were not significantly different [$k = (6.1 \pm 0.6)\ \ 10^{-3}\ \text{min}^{-1}$, $k = (5.1 \pm 0.4)\ \ 10^{-3}\ \text{min}^{-1}$, respectively] (ANOVA, $p > 0.05$). However, compared to the rutin ethanolic solution [$k = (35.0 \pm 3.7)\ 10^{-3}\ \text{min}^{-1}$], both nanostructured formulations showed a significant lower rutin degradation rate (ANOVA, $p \leq 0.05$). These results showed that the association of rutin to the nanocapsules or nanoemulsions led to an increase of 5.3 and 6.9 times in the rutin photostability, respectively, during the 30 min of exposure to UV radiation.

The antioxidant activity (radical scavenging activity) of rutin was evaluated by adding an amount of H_2O_2 in excess to the samples (before the irradiation) leading to the quantitatively controlled formation of ·OH radicals, which are the most harmful radicals formed under physiological conditions. In this case, the decay kinetics were significantly different among all samples (R-NC, R-NE and R-SE) and according to a first-order kinetics (ANOVA, $p \leq 0.05$). The nanoparticle formulations (R-NC and R-NE) showed decay kinetics about 2.1 and 1.5 times slower than the solution [$k = (19.5 \pm 0.5)\ 10^{-3}\ \text{min}^{-1}$, $k = (26.6 \pm 2.6)\ 10^{-3}\ \text{min}^{-1}$ and $k = (40.1 \pm 1.7)\ 10^{-3}\ \text{min}^{-1}$, for R-NC, R-NE and R-SE, respectively], indicating a prolonged antioxidant activity of rutin, when associated to the nanostructures (Fig. 3). The difference between the decay kinetics of the different nanoparticles (ANOVA, $p \leq 0.05$) could be explained by the presence of rutin nanocrystals adsorbed on the surface of the nanodroplets of nanoemulsions (as observed by TEM—Fig. 1), making rutin more readily accessible to react with the ·OH free radical.

In order to evaluate if the rutin release rate from the different nanoparticles could also play an important role in this different antioxidant behavior we carried out an in vitro drug release experiment using the dialysis bag technique. Figure 4 shows the in vitro drug release profiles from rutin-loaded nanocapsules (R-NC) and rutin-loaded nanoemulsions (R-NE). Both formulations promoted a rapid and similar release of rutin (release close to 100% in 24 h). The release profiles were modeled using the monoexponential and biexponential equations. According to the values of the correlation coefficients and the criterion for model selection (MSC), the data fit better to the biexponential equation (Table 4) for both formulations, showing a burst release at an early stage followed by a sustained phase.

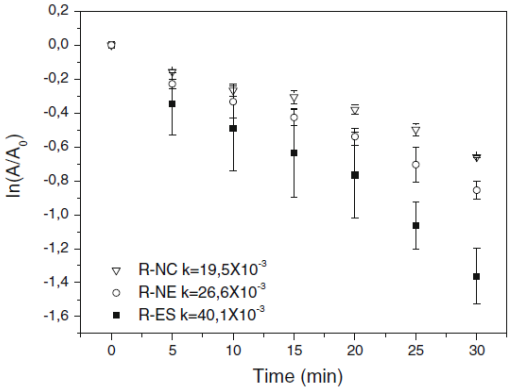

Fig. 3 In vitro antioxidant activity of rutin-loaded nanostructures and ethanolic solution after 30 min of UV irradiation

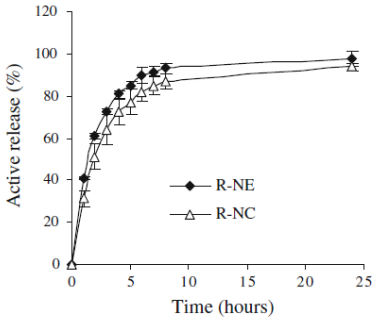

Fig. 4 In vitro rutin release profile from nanocarriers (R-NC and R-NE) using the dialysis bag method ($n = 3$).The lines correspond to the fitting to the biexponential equation

Table 4 Rate constants, correlation coefficients, and MSC obtained by the mathematical modeling of drug release data from the different nanocarriers (R-NC and R-NE)

	R-NC	R-NE
Monoexponential		
k (h^{-1})	0.3215 ± 0.0674	0.4336 ± 0.0242
r (range)	0.9979 ± 0.0010	0.9983 ± 0.0016
MSC (range)	2.9935 ± 0.2326	3.0585 ± 0.9828
Biexponential		
k_1 (h^{-1})	0.4113 ± 0.0765	0.8164 ± 0.6039
k_2 (h^{-1})	0.0310 ± 0.0163	0.1393 ± 0.1993
a (mg ml^{-1})	0.8447 ± 0.0597	0.6107 ± 0.3337
b (mg ml^{-1})	0.1285 ± 0.0370	0.3201 ± 0.4125
r (range)	0.9997 ± 0.0002	0.9994 ± 0.0006
MSC (range)	6.5420 ± 0.5612	6.0185 ± 0.8945

The rate constants for the burst phase (k_1) were $0.4113 \pm 0.0765\ h^{-1}$ (R-NC) and $0.8164 \pm 0.6039\ h^{-1}$ (R-NE) and for the sustained phase the rate constants (k_2) were 0.0310 ± 0.0163 (R-NC) and $0.1393 \pm 0.1993\ h^{-1}$ (R-NE). For both phases, R-NC showed lower rate constants compared to R-NE. The percentage of rutin related the burst phase (a) for nanocapsules and nanoemulsions was about 85 and 65%, respectively. On the other hand, the percentage related to the sustained phase was about 13% for R-NC and 32% for the R-NE. Such values showed that rutin is about 60–80% adsorbed on the surface of nanostructures [41, 42], suggesting that the radical scavenging property of rutin against the ·OH radical occurred mostly at the interface particle/water. However, the influence of the drug release from the inner compartment of the nanocarriers cannot be discarded, considering the slower drug

release rate from R-NC at both release phases. This hypothesis can be reinforced by the analysis of the release half-life of rutin, calculated according to the biexponential model. R-NC showed higher values (1.7 and 22 h for the burst and sustained phase, respectively) compared to R-NE (0.8 and 5 h for the burst and sustained phase, respectively). This faster drug release from R-NE can be explained by the absence of the polymer around the oily droplets as well as by the presence of nanocrystals on their surface, as observed by TEM.

Conclusions

This study showed for the first time the development of rutin-loaded nanocapsules and nanoemulsions, as aqueous intermediate or final systems to the development of nanomedicines containing rutin. Both formulations presented an increase in the rutin photostability and a prolonged in vitro antioxidant activity, even if the main mechanism of association of rutin was the adsorption on the particle/droplet surface, as determined by the mathematical modeling of drug release data. Moreover, the presence of polymer did not show any significant influence in the increase of rutin photostability. However, its presence in nanocapsules led to a slower release rate and to a prolonged antioxidant activity against the strongly reactive ·OH radicals compared to the rutin-loaded nanoemulsions. The results showed that such nanostructured systems are a potential alternative to the preparation of rutin delivery systems to treat different diseases related to oxidative stress, including aging processes caused by the action of free radicals.

Acknowledgments JSA thanks CAPES/Brazil for the fellowship. We thank Rede Nanocosméticos CNPq/MCT/Brazil, PRONEX/FAPERGS/CNPq, CAPES and CNPq for the financial support received as well as M.B. da Rosa for his contribution in this work.

References

1. C.M.M. Sousa, H. Rocha e Silva, G.M. Vieira Jr., M.C.C. Ayres, C.L.S. Costa, D.S. Araújo, L.C.D. Cavalcante, E.D.S. Barros, P.B.M. Araújo, M.S. Brandão, M.H. Chaves, Quim. Nova **30**, 351 (2007)
2. A.S. Darvesh, R.T. Carroll, A. Bishayee, W.J. Geldenhuys, C.J. Van der Schyf, Expert Rev. Neurother. **10**, 729 (2010)
3. A.L.B.S. Barreiros, J.M. David, Quim. Nova **29**, 113 (2006)
4. P. Brenneisen, H. Steinbrenner, H. Sies, Mol. Aspects Med. **26**, 256 (2005)
5. U. Cornelli, Clin. Dermatol. **27**, 175 (2009)
6. A. Michalak, Polish J. Environ. Stud. **15**, 523 (2006)
7. S.S. Chun, D.A. Vatem, Y.T. Lin, K. Shetty, Process Biochem. **40**, 809 (2005)
8. M.J. Abad, P. Bermejo, A. Villar, Gen. Pharmacol. **26**, 815 (1995)
9. C. La Casa, I. Villegas, C.A. de la Lastra, V. Motilva, M.J. Martín Calero, J. Ethnopharmacol. **71**, 45 (2000)
10. A. Solomon, S. Golubowicz, Z. Yablowicz, M. Bergman, S. Grossman, A. Altmann, Z. Kerem, M.A. Flaishman, J. Agric. Food Chem. **58**, 6660 (2010)
11. K.L. Krishna, K. Mruthunjaya, J.A. Patel, Int. J. Pharmacol. **6**, 72 (2010)
12. O.A. Fawole, S.O. Amoo, A.R. Ndhlala, M.E. Light, J.F. Finnie, J. Van Staden, J. Ethnopharmacol. **127**, 235 (2010)
13. H. Jiang, W.Q. Zhan, X. Liu, S.X. Jiang, Nat. Prod. Res. **22**, 1650 (2008)
14. J. Yang, J. Guo, J. Yuan, LWT **41**, 1066 (2008)
15. M.L. Calabrò, S. Tommasini, P. Donato, R. Stancanelli, D. Raneri, S. Catania, C. Costa, V. Villari, P. Ficarra, R. Ficarra, J. Pharm. Biomed. Anal. **36**, 1019 (2005)
16. A. Korkmaz, D. Kolankaya, J. Surg. Res. (2009). doi: 10.1016/j.jss.2009.03.022
17. A.R. Verma, M. Vijayakumar, C.S. Mathela, C.V. Rao, Food Chem. Toxicol. **47**, 2196 (2009)
18. S. Itagaki, J. Oikawa, J. Ogura, M. Kobayashi, T. Hirano, K. Iseki, Food Chem. **118**, 426 (2010)
19. R. Mauludin, R.H. Müller, C.M. Keck, Int. J. Pharm. **370**, 202 (2009)
20. R. Mauludin, R.H. Müller, C.M. Keck, Eur. J. Pharm. Sci. **36**, 502 (2009)
21. P. Couvreur, G. Barrat, E. Fattal, P. Legrand, C. Vauthier, Ther. Drug Carrier Syst. **19**, 99 (2002)
22. C.E. Mora-Huertas, H. Fessi, A. Elaissari, Int. J. Pharm. **385**, 113 (2010)
23. A.R. Pohlmann, V. Weiss, O. Mertins, N.P. da Silveira, S.S. Guterres, Eur. J. Pharm. Sci. **16**, 305 (2002)
24. J.M. Fachinetto, A.F. Ourique, G. Lubini, S.B. Tedesco, A.C.F. Silva, R.C.R. Beck, Lat. Am. J. Pharm. **27**, 668 (2008)
25. M. Teixeira, M.J. Alonso, M.M.M. Pinto, C.M. Barbosa, Eur. J. Pharm. Biopharm. **59**, 491 (2005)
26. A.F. Ourique, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck, Int. J. Pharm. **352**, 1 (2008)
27. J.S. Almeida, L. Jezur, M.C. Fontana, K. Paese, C.B. Silva, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck, Lat. Am. J. Pharm. **28**, 168 (2009)
28. M.C. Fontana, K. Coradini, S.S. Guterres, A.R. Pohlmann, R.C.R. Beck, J. Nanosci. Nanotechnol. **10**, 3091 (2010)
29. S.R. Schaffazick, L.L. Freitas, A.R. Pohlmann, S.S. Guterres, Quim. Nova **25**, 726 (2003)
30. S.S. Guterres, M.P. Alves, A.R. Pohlmann, Drug Target Insights **2**, 147 (2007)
31. A.R. Pohlmann, G. Mezzalira, C. De, G. Venturini, L. Cruz, A. Bernardi, E. Jäger, A.M.O. Battastini, N.P. da Silveira, S.S. Guterres, Int. J. Pharm. **359**, 288 (2008)
32. L. Mei, Y. Zhang, Y. Zheng, G. Tian, C. Song, D. Yang, H. Chen, H. Sun, Y. Tian, K. Liu, Z. Li, L. Huang, Nanoscale Res. Lett. **4**, 1530 (2009)
33. G. Leonarduzzi, G. Testa, B. Sottero, P. Gamba, G. Poli, Curr. Med. Chem. **17**, 74 (2010)
34. H. Fessi, F. Puisieux, J.-P. Devissaguet, European Patent 0274961 Al (1988)
35. É. Martini, E. Carvalho, H. Teixeira, Quim. Nova **30**, 930 (2007)
36. ICH – International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use: Gudeline on Validation of Analytical Procedure Q2 (R1): Text and Methodology (2005)

37. R.C.R. Beck, S.S. Guterres, J.A.B. Funck, R.J. Freddo, C.B. Michalowski, I. Barcellos, Acta Farm. Bonaer. **22**, 11 (2003)
38. L.M. Carvalho, C. Spengler, J.C. Garmatz, P.C. Nascimento, D. Bohrer, L. Del-Fabro, G. Radis, A.A. Bolli, A.M. Moro, S.C. Garcia, M.B. Rosa, Quim. Nova **31**, 1336 (2008)
39. H. Li, X. Zhao, Y. Ma, G. Zhai, L. Li, H. Lou, J. Control. Release **133**, 238 (2009)
40. R.B. Friedrich, M.C. Fontana, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck, Quim. Nova **31**, 1131 (2008)
41. L. Cruz, L.U. Soares, T. Dalla Costa, G. Mezzalira, N.P. Silveira, S.S. Guterres, A.R. Pohlmann, Int. J. Pharm. **313**, 198 (2006)
42. M.C. Fontana, K. Coradini, S.S. Guterres, A.R. Pohlmann, R.C.R. Beck, J. Biomedi. Nanotechnol. **5**, 254 (2009)