Universidade Estadual de Londrina

DANIELA CRISTINA DE MEDEIROS

DESENVOLVIMENTO DE SISTEMAS MICROENCAPSULADOS PARA LIBERAÇÃO MODIFICADA DE FRUTOSE-1,6-BIFOSFATO E RUTINA E AVALIAÇÃO DA SUA EFICÁCIA *IN VIVO* EM MODELOS DE INFLAMAÇÃO

Londrina
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Londrina para obtenção do título de Doutor em Ciências da Saúde.

Orientador: Prof. Dr. Waldiceu A. Verri Jr
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Londrina, 27 de março de 2015.

DEDICATÓRIA

A Deus, que está sempre junto de mim, abençoando e iluminando o meu caminho.

Ao Erik, meu companheiro e amigo, pelo suporte, amor e compreensão nesta jornada.

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Eclesiastes 3: 1.

Medeiros, Daniela C. **Desenvolvimento de sistemas microencapsulados para liberação modificada de frutose-1,6-bifosfato e rutina e avaliação da sua eficácia *in vivo* em modelos de inflamação.** 2015**.** 221 f. Tese de Doutorado (Pós-Graduação em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2015.

RESUMO

O desenvolvimento de novos sistemas de liberação de fármacos é uma estratégia promissora para a melhoria do perfil biofarmacêutico de medicamentos. O objetivo deste trabalho foi desenvolver sistema microencapsulado para modificação da liberação dos fármacos frutose-1,6-bifosfato (FBP) e rutina, com intuito de melhorar a eficácia terapêutica desses compostos. Para atingir este objetivo, microcápsulas contendo rutina ou FBP foram preparadas pelo método de coacervação complexa e analisadas em relação a: a) morfologia das partículas por microscopia eletrônica de varredura; b) tamanho e distribuição das partículas pela técnica de dispersão de luz; c) eficiência de encapsulação pelo método de extração; e d) dissolução *in vitro* das microcápsulas contendo FBP. Foi realizada análise por calorimetria exploratória diferencial (DSC) e difração de raios X para verificar a presença de fase amorfa. A atividade analgésica e anti-inflamatória das formulações foi avaliada *in vivo* em modelo de inflamação induzida pela injeção intraplantar de carragenina em camundongos Swiss. Os camundongos foram pré-tratados por via oral com rutina ou FBP microencapsuladas, rutina ou FBP não microencapsulada, ou microcápsulas sem fármaco, e submetidos ao estímulo com carragenina. A intensidade da hiperalgesia mecânica e a formação do edema na pata foram avaliadas com analgesímetro digital e paquímetro, respectivamente. O efeito anti-inflamatório e antioxidante de formulações tópicas contendo rutina microencapsulada também foi avaliado em modelo de inflamação cutânea induzida por radiação ultravioleta B (UVB) em camundongos sem pêlos e comparado com o fármaco não microencapsulado. Para quantificação da FBP microencapsulada foi desenvolvido e validado um método por cromatografia líquida de alta eficiência. As microcápsulas contendo os fármacos apresentaram forma esférica e tamanho homogêneo. A eficiência de encapsulação das microcápsulas contendo rutina (fármaco lipossolúvel) foi de 76,9%, enquanto que para as microcápsulas contendo FBP (fármaco hidrossolúvel) o valor obtido foi de 25%. No ensaio de dissolução *in vitro* das microcápsulas contendo FBP foi observado um prolongamento da liberação do fármaco quando comparado com o fármaco não microencapsulado. No modelo de inflamação na pata induzida por carragenina em camundongos, as microcápsulas aumentaram a eficácia analgésica de ambos os compostos. Neste mesmo modelo, as microcápsulas de FBP provocaram redução significativa do edema de pata. No modelo de inflamação/estresse oxidativo induzido por radiação UVB, microcápsulas contendo rutina, administradas topicamente, apresentaram efeito anti-inflamatório e antioxidante. Na análise por DSC e difração de raios X foi constatado que a rutina está dispersa em matriz polímerica amorfa enquanto que a FBP apresentou mudança da forma cristalina para amorfa, após o processo de microencapsulação. A presença de fase amorfa, caracterizada pelos polímeros utilizados na preparação das microcápsulas, pode ter influenciado positivamente no aumento da eficácia terapêutica dos fármacos. Sendo assim, a microencapsulação pode ser utilizada para modificação da liberação, contribuindo para o aumento da eficácia terapêutica dos fármacos rutina e FBP.

Palavras-chaves: Microcápsulas. Rutina. Frutose-1, 6-bifosfato. Inflamação. Dor.

Medeiros, Daniela C. **Development of microencapsulated systems for modified release of fructose-1,6-bisphosphate and rutin and evaluation of its efficacy *in vivo* in inflammation models.** 2015**.** 221 p. Doctoral Thesis (Pós-Graduação em Ciências da Saúde) – Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

Development of new drug release systems is a promising strategy for improvement of biopharmaceutical drug profile. The aim of this study was development of microencapsulated system for modify release of fructose-1,6-bisphosphate (FBP) and rutin, with the intent to improve therapeutic efficacy of these drugs. Microcapsules containing either rutin or FBP were prepared by complex coacervation and analyzed for: a) morphology of the particles by scanning electron microscopy; b) size and distribution of the particles by light scattering technique; c) microencapsulation efficiency by extraction method; and d) *In vitro* dissolution of FBP microcapsules. The analyzed by differential scanning calorimetric (DSC) and X ray diffraction was performed to verify the presence of amorphous phase. The analgesic and antiinflamatory activity of the formulations was evaluated *in vivo* in model of inflammation induced by intraplantar carrageenan injection in Swiss mice. Mice were pre-treated per oral with microencapsulated rutin or FBP, non-microencapsulated rutin or FBP, or microcapsules without drug, and were stimulated with carrageenan. The mechanical hyperalgesia intensity and formation of paw edema were evaluated by electronic anesthesiometer and analog caliper, respectively. The anti-inflammatory and anti-oxidant effects of topic formulations containing microencapsulated rutin were also assessed in model of skin inflammation induced by ultraviolet B radiation (UVB) in hairless mice. For quantification of microencapsulated FBP was developed and validated a method of high performance liquid chromatography. Microcapsules containing the drugs showed spherical form and homogeneous size. The microencapsulation efficiency for rutin (a liposoluble drug) was 76.9%, while for FBP (a water soluble drug) was obtained a value of 25%. In the *in vitro* dissolution assay with microcapsules containing FBP, was observed a prolonged drug release comparing with non-microencapsulated FBP. In the model of carrageenan-induced paw inflammation in mice, microcapsules improved the analgesic efficacy of both drugs. In this same model, the FBP microcapsules showed significant reduction of paw edema. For the inflammation/ oxidative stress induced by ultraviolet B irradiation (UVB), topical formulation containing microencapsulated rutin presented anti-inflammatory and anti-oxidant effects. DSC and X Ray diffraction analysis demonstrated rutin dispersed in polymeric amorphous matrix, while FBP changed from crystalline to amorphous form after microencapsulation process. The presence of amorphous phase, characterized by the polymers used in the preparation of microcapsules, may have influenced positively in the increased drugs therapeutic efficacy. Thus, microencapsulation can be used for modification drug release, improving the therapeutic efficacy of the drugs rutin and FBP.

Keywords: Microcapsules. Rutin. Fructose-1,6-bisphosphate. Inflammation. Pain.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2’ azinobis (3-etilbenzotiazolina-6-ácido sulfônico)
ANOVA	Análise de variância
$CaCl_2$	Cloreto de cálcio
CAT	Catalase
CLAE	Cromatografia líquida de alta eficiência
DNA	Ácido desoxirribonucleico
DPPH	2,2-difenil-1-picrilidrazil
DSC	Calorimetria Exploratória Diferencial
DTNB	Ácido 5,5’-ditio-bis-(2-nitrobenzóico)
EDTA	Ácido etilenodiamino tetra-acético
EE	Eficiência de encapsulação
EPM	Erro padrão da media
EROs	Espécies reativas de oxigênio
FBP	Frutose-1,6-bifosfato
$FeCl_3.6H_2O$	Cloreto férrico hexahidratado
FRAP	Poder antioxidante de redução férrica
Gapdh	Gliceraldeído-3-fosfato desidrogenase
Gp91phox	Glutationa peroxidase 91fox
GSH	Glutationa reduzida
HCl	Ácido clorídrico
$HO^{\bullet}$	Radical hidroxil
HTAB	Brometo de hexadecil trietil amônio
H_2O_2	Peróxido de hidrogênio
IL	Interleucina
KCl	Cloreto de potássio
KOH	Hidróxido de potássio
MEV	Microscopia electronica de varredura
MMPs	Metaloproteinases da matriz
NaCl	Cloreto de sódio
NBT	Nitroblue tetrazolium
NF-κB	Fator nuclear-κB
$O_2^{\bullet-}$	Anion superóxido

OD	Densidade optica
PCR	Reação em cadeia de polimerase
PGE2	Prostaglandina E2
RNA	Ácido ribonucléico
SDS	Duodecil sulfato de sódio
SOD	Superóxido dismutase
TCA	Ácido tricloro acético
TNF	Fator de necrose tumoral
TPTZ	2,4,6 tripiridil-S-triazina
Tris	Hidroximetil aminometano
UV	Radiação ultravioleta

SUMÁRIO

1 INTRODUÇÃO

O desenvolvimento de novos sistemas de liberação de fármacos, tanto para substâncias novas quanto para as já utilizadas clinicamente, é considerado uma estratégia promissora para a melhoria do perfil biofarmacêutico de medicamentos (RATTES; OLIVEIRA, 2007). O desenvolvimento de diferentes sistemas de liberação para fármacos já existentes é justificável, principalmente, por apresentarem vantagens clínicas e terapêuticas significativas, tais como melhoria da eficácia terapêutica, redução de efeitos adversos devido à menor flutuação da concentração plasmática do fármaco, redução da dose e maior tempo entre as administrações em relação às formas convencionais (GRUNDY; FOSTER, 1996).

No entanto, o desenvolvimento de novos sistemas de liberação de fármacos ou aprimoramento dos já existentes é um desafio para a área farmacêutica (GALAMBOS; STRUCHIO, 1998). Vários aspectos estão envolvidos no desenvolvimento desses sistemas, como velocidade de agitação no processo de preparação (CHOW *et al*., 1998), relação fármaco:polímero (ARORA; BUDHIRAJA, 2012), processo de secagem (BARACAT *et al*., 2004a) e características do fármaco, como solubilidade, peso molecular, forma e tamanho da partícula (BARACAT, 2004b; DAÍ *et al*., 2005).

A proposta deste trabalho foi desenvolver sistema microencapsulado para modificação da liberação do fármaco, com intuito de melhorar a eficácia terapêutica do mesmo em modelos de inflamação. Com este objetivo, foram utilizados dois fármacos com solubilidade bastante diferentes: a rutina, um flavonoide com baixa solubilidade em água, e a frutose-1,6-bifosfato (FBP), altamente solúvel em água, o que representa um grande desafio tecnológico.

Desta forma, os tópicos a seguir irão abordar: aspectos gerais do desenvolvimento de sistemas microencapsulados, mecanismos fisiopatológicos dos modelos de inflamação utilizados neste trabalho e propriedades físico-químicas e farmacológicas da rutina e FBP.

1.1 Aspectos gerais do desenvolvimento de sistemas microencapsulados

A indústria farmacêutica tem buscado desenvolver novas formulações para liberação modificada de fármacos cujos efeitos terapêuticos já estão estabelecidos, pois o tempo e o custo para a pesquisa e o desenvolvimento de novos fármacos são grandes. A

utilização de sistemas de liberação é uma alternativa viável para o aperfeiçoamento de formulações, objetivando o aumento da eficácia terapêutica de fármacos já utilizados convencionalmente no tratamento de inúmeras patologias (VERRI *et al*., 2013).

O avanço das pesquisas com sistemas de liberação de fármacos deve-se ao reconhecimento das vantagens clínicas e terapêuticas e a fatores econômicos. O planejamento racional de sistemas de liberação é uma etapa crucial para a modulação da liberação do fármaco, adequada às necessidades clínicas e farmacocinéticas e aos locais de absorção (RUBINSTEIN, 1990). A veiculação de fármacos em sistemas de liberação modificada apresenta várias vantagens do ponto de vista terapêutico no tratamento de diversas patologias, podendo aumentar a segurança da terapia e a estabilidade do fármaco no sistema biológico, reduzir os efeitos adversos, além de reduzir a dose do fármaco ou frequência de administração, resultando em melhor eficácia terapêutica e adesão do paciente ao tratamento (ARORA, 2012).

Algumas estratégias são empregadas para possibilitar a liberação modificada de fármacos, dentre elas a veiculação do fármaco em uma matriz polimérica, ligação química a resinas de permuta iônica, incorporação em bomba osmótica e utilização de revestimentos monolíticos ou multiparticulados, como os microencapsulados (AULTON, 2005). Microcápsulas consistem em pequenas partículas ou gotículas de substância(s) ativa(s) envolvidas ou incorporadas em uma matriz homogênea ou heterogênea, geralmente de material polimérico, formando pequenas cápsulas que podem variar de um micrômetro a alguns milímetros de diâmetro. Várias técnicas têm sido utilizadas para microencapsulação, como *spray-drying*, extrusão, coacervação, liofilização, emulsificação, entre outras (VERRI *et al*., 2012).

O método de microencapsulação por coacervação consiste na separação de fases de um ou mais hidrocolóides a partir de uma solução inicial e a subsequente deposição da fase polimérica envolvendo a substância ativa que se encontra suspensa ou emulsificada no mesmo meio. Microcápsulas produzidas por coacervação são insolúveis em água e possuem boa característica de controle da liberação do fármaco. O processo de coacervação pode ser dividido em dois tipos: simples ou complexo, dependendo do número de polímeros utilizados (DONG *et al*., 2008).

O mecanismo de liberação do fármaco encapsulado no interior da partícula envolve processos de natureza difusional e/ou a erosão da rede polimérica previamente formada (LINHARD, 1988; BATYCKY *et al*., 1997). Vários fatores podem afetar a liberação do fármaco a partir das microcápsulas, como as propriedades dos polímeros utilizados, relação

fármaco:polímero, tamanho das partículas e interação entre o fármaco e o polímero (KIM *et al*., 2014).

A versatilidade das técnicas empregadas no uso desta tecnologia permite uma ampla adequabilidade no que se refere às classes de medicamentos, uma vez que fatores passíveis de otimização, tais como as características físico-químicas do polímero e do fármaco empregados e os parâmetros relacionados com a técnica de preparação, determinam as propriedades do produto final (MEDEIROS, 2003).

1.1.1. Microcápsulas formadas pelo complexo polimérico pectina/caseína

As pectinas constituem uma classe de polissacarídeos complexos encontrados nas paredes celulares das plantas superiores. Sua estrutura básica “in natura” é composta por poli(1,4-α-D ácido galacturônico) (MANO; MENDES, 1999; RIDLEY; O´NEILL; MOHNEN, 2001). Em água, fomam dispersões altamente viscosas mesmo em baixas concentrações, apresentando alta velocidade de intumescimento no fluído do TGI (LORENZO-LAMOZA *et al.*, 1998).

A caseína é uma glicofosfoproteína encontrada no leite. A caseína de origem bovina é constituída de quatro cadeias denominadas: α_{s1}, α_{s2}, β e κ, com peso molecular total de aproximadamente 86.240 e ponto isoelétrico em 4,55. Esta substância vem sendo estudada como material para preparação de sistemas de liberação modificada de fármacos por sua capacidade formadora de filme (DE CARVALHO, 1996), obtenção de sistemas matriciais (HERNANDES; FREITAS; PEREIRA, 1998) e como material para preparação de microcápsulas (BARACAT *et al.*, 2012; GUAZELLI *et al*., 2013; VERRI *et al*., 2013).

O complexo polimérico pectina/caseína foi descrito anteriormente no documento PI 0700557-1, que trata do processo de preparação de complexos biodegradáveis utilizando polímeros de caseína e pectina que, após passarem por diferentes etapas, formam micropartículas que podem ser utilizadas em sistemas de liberação de fármacos (FREITAS *et al.*, 2007).

A utilização de sistema de liberação multiparticulado tem como vantagem maior reprodutibilidade no tempo de trânsito intestinal em relação ao monolítico, pois o sistema multiparticulado se distribui de maneira mais uniforme, minimizando locais de alta

concentração do fármaco, além de reduzir a variação inter e intra-individuais na resposta ao tratamento (FELL *et al.*, 1992; VERRI *et al*., 2013).

Além disto, o preparo das microcápsulas utilizando o complexo polimérico pectina/caseína é de baixo custo, realizado em meio aquoso, em condições brandas e isenta de solventes orgânicos, o que significa uma grande vantagem em relação a muitos polímeros disponíveis no mercado que utilizam solvente orgânico em sua preparação e apresentam custo elevado. Aliado ao baixo custo está à possibilidade de produção das microcápsulas em grande escala.

Após administração oral, as microcápsulas devem sofrer a ação dos fluidos biológicos, o que provocaria o intumescimento gradual da parede das micropartículas, permitindo a liberação modificada do fármaco para o meio biológico. Neste sentido, a liberação seria controlada pelo complexo polimérico pectina/caseína, o que torna este sistema atrativo para o tratamento de patologias crônicas que requerem a administração de medicamentos por longos períodos de tempo, nos quais a liberação modificada é mais conveniente por reduzir a frequência de administração.

Um outro fator determinante para fármacos administrados oralmente é a absorção do mesmo através do TGI, que é dependente da solubilidade e permeabilidade do fármaco. Sabe-se que substâncias amorfas são mais solúveis que as cristalinas, porém substâncias cristalinas são mais estáveis fisicamente (HANCOCK e ZOGRAFI, 1997). Desta forma, a presença de fase amorfa pode favorecer a absorção do fármaco a partir das microcápsulas, levando ao aumento da sua eficácia terapêutica. De fato, a forma cristalina da substância pode ser modificada durante o processo de microencapsulação para estado amorfo (KIM *et al*., 2014).

1.2 Mecanismos fisiopatológicos dos modelos de inflamação

Nosso grupo de pesquisa tem estudado vários modelos de inflamação, como carragenina e UVB, averiguando a efetividade de novos fármacos ou modulando a liberação de fármacos já existentes. Dessa forma, serão apresentados os mecanismos fisiopatológicos envolvidos nos modelos de inflamação utilizados neste trabalho.

1.2.1 Modelo de inflamação induzida por carragenina

A carragenina tem sido amplamente utilizada como estímulo inflamatório para investigar o potencial terapêutico de novos compostos. Como consequência, a injeção intraplantar de carragenina induz a resposta inflamatória local, com aumento da permeabilidade vascular, migração de neutrófilos e produção de mediadores inflamatórios (SELLOUM *et al*., 2003).

As reações vasculares da inflamação aguda consistem em mudanças no fluxo sanguíneo e na permeabilidade dos vasos. A vasodilatação é uma das primeiras manifestações da inflamação aguda, resultando em aumento do fluxo sanguíneo, que é a causa do aparecimento dos sinais cardinais calor e rubor (eritema) no local da inflamação. A vasodilatação é induzida pela ação de diversos mediadores, entre eles a histamina e óxido nítrico (NO), que atuam no músculo liso dos vasos sanguíneos. Este fenômeno é seguido pelo aumento da permeabilidade vascular, com o extravasamento de líquido rico em proteínas para o tecido extravascular, causando edema. A contração das células endoteliais, resultando em aumento dos espaços interendoteliais, é o mecanismo mais comum de extravassamento vascular, e é induzida por histamina, bradicinina, leucotrienos, e outros mediadores químicos (KUMAR, 2010).

O aumento do diâmetro dos vasos leva ao aumento do fluxo sanguíneo, causando concentração de células vermelhas em pequenos vasos e aumento da viscosidade do sangue. Estas alterações resultam na dilatação destes vasos, que ficam congestionados devido a movimentação lenta das células sanguíneas, uma condição denominada estase ou congestão vascular. Ao mesmo tempo, as células endoteliais são ativadas por mediadores produzidos no local da inflamação, e expressam aumento dos níveis de moléculas de adesão. Os leucócitos então aderem ao endotélio, e logo depois migram através da parede vascular para o tecido intersticial (KUMAR, 2010).

Como vimos, no modelo de inflamação induzida por carragenina existe uma liberação coordenada de mediadores, que vão atuar também na sensibilização do neurônio nociceptivo aferente primário. Em camundongos, o estímulo com carragenina induz a produção das citocinas TNFα e CXCL1 (quimiocina derivada de queratinócitos, KC), ambas atuam por induzir a produção de IL-1β, que por sua vez induz a produção de prostaglandina E_2 (PGE_2). A PGE_2 é responsável pela ativação e sensibilização neuronal pela fosforilação de canais de sódio resistentes à tetrodotoxina, o que aumenta os níveis intra-neuronais de sódio e facilita a despolarização induzida por estímulos mecânicos e químicos como outros

mediados como a bradicinina. A CXCL1 também induz a liberação de aminas simpáticas que tem papel semelhante à PGE_2 (CUNHA *et al*., 2005).

Ocorre ainda um aumento da sensibilidade à dor (hiperalgesia), um sinal clínico da resposta inflamatória que reduz a qualidade de vida e, por este motivo, é o principal motivo de busca ao atendimento médico. Dessa forma, o edema e hiperalgesia induzidas por carragenina são importantes ferramentas para a avaliação de compostos com potencial terapêutico no tratamento da dor e inflamação (CUNHA *et al*., 2005).

1.2.2 Modelo de inflamação/estresse oxidativo induzido pela radiação UVB

Radicais livres são definidos como qualquer espécie química capaz de existência independente, que possuem um ou mais elétrons desemparelhados estando esses sozinhos no orbital (ARUOMA, 1998). Radicais livres altamente reativos, especialmente radicais derivados de oxigênio, formados por processos metabólicos endógenos ou produtos químicos exógenos ao organismo, são capazes de oxidar biomoléculas, resultando em morte celular e danos aos tecidos (YANG *et al*., 2000). Nas últimas décadas, as espécies reativas de oxigênio (EROs) têm chamado a atenção de pesquisadores devido a sua participação em vários eventos patológicos como em processos inflamatórios, câncer e envelhecimento precoce (JI, 2007). O desequilíbrio entre a formação de EROs e a atividade de defesa antioxidante é denominado estresse oxidativo (CASAGRANDE *et al*., 2006; VICENTINI *et al*., 2008a; MAGDALENA, TAK YEE, 2010).

A pele é uma interface biológica com o meio ambiente continuamente exposta a uma variedade de ataques biológicos, químicos e físicos que ameaçam a integridade das estruturas celulares e desencadeiam o estresse oxidativo. A radiação ultravioleta (UV) é o fator físico que, devido a grande abundância no meio ambiente, representa uma das principais causas de danos na pele, causando lesões pré-cancerosas e cancerosas e aceleração do envelhecimento cutâneo (SAIJA *et al*., 2000; CASAGRANDE *et al*., 2007; TOUITOU; GODIN, 2008; QUAN *et al*., 2009).

A hipótese provável para o aparecimento de patologias de pele devido exposição à radiação solar é a formação de EROs e mobilização de metais de transição. As EROs podem ser geradas pela radiação UV diretamente por meio da interação com os grupos cromóforos ou indiretamente por meio da resposta inflamatória (WEI *et al*., 2002). Com o aumento do fluxo de EROs, a regeneração de antioxidantes endógenos torna-se insuficiente ocasionando a sua depleção (VICENTINI *et al*., 2008a; FONSECA *et al*., 2009). O resultado

do desequilíbrio entre EROs e antioxidantes modula vias de sinais de transdução celular redox-sensível e expressão de genes. Estas mudanças moleculares podem estar envolvidas na patogênese dos danos fotoxidativos (FUCHS, 1998; CASAGRANDE *et al*., 2006; VICENTINI *et al*., 2008b).

A exposição aguda à radiação UV determina uma resposta inflamatória imediata, com aparecimento de eritema, edema e infiltrado leucocitário, além da diminuição dos níveis dos antioxidantes endógenos como a glutationa reduzida (GSH), que é um tripeptídeo sensível ao estresse oxidativo mediado pela radiação UVB (MONTENEGRO *et al*., 1995; CARINI *et al*., 2000; MELONI; NICOLAY, 2003; CASAGRANDE *et al*, 2006; SAWANE *et al*., 2011). A radiação UV pode também levar a danos nas fibras elásticas, no colágeno e nas glicosaminoglicanas da matriz extracelular da derme, perdendo a elasticidade, e contribuindo para o fotoenvelhecimento da pele. A exposição à radiação UV induz fatores de transcrição de genes de metaloproteinases, enzimas proteolíticas produzidas principalmente por células polimorfonucleares, macrófagos, queratinócitos, fibroblastos e células tumorais (FISHER *et al*., 1997). O aumento da produção de metaloproteinases pelos queratinócitos epidermais e fibroblastos da derme resulta na degradação do colágeno e outras proteínas da matriz extracelular. Este processo é seguido pelo fotoenvelhecimento e danos severos à pele (RIEGER, 1999; QUAN *et al*., 2009).

Ainda, a radiação UV aguda pode causar destruição da função imune mediada pelas células T que está associada com a geração de população de linfócitos T supressores (FISHER; KRIPKE, 1982). Sugere-se que a modulação de proteínas quinase redox-sensível e de fatores de transcrição como o fator de transcrição nuclear κB (NF-κB) pelas EROs são eventos precoces e essenciais na indução de reações inflamatórias (FUCHS *et al*., 2001). O NF-κB é um fator de transcrição sensível ao estresse oxidativo, o qual ativa genes envolvidos na produção de substâncias pro-inflamatórias, incluindo o fator de necrose tumoral (TNF-α) e interleucinas (por exemplo: IL-1α, IL-1β e IL-6) que promovem a ativação e recrutamento de células efetoras como leucócitos e células endoteliais e induzem a liberação de proteases (FUCHS, 1998; CARINI, *et al*., 2000; VICENTINI *et al*., 2011). Ademais, existe um ciclo de estimulação recíproca entre EROs e citocinas, por exemplo, a citocina TNFα ativa a NADPH oxidase induzindo a produção de ânion superóxido e consequentemente outras EROs (KILPATRICK *et al*., 2010).

Para enfrentar o elevado grau de exposição, a pele possui uma variedade de mecanismos de defesa interligados para a proteção contra as EROs induzidas pela radiação UV (GEORGETTI *et al*., 2008; VICENTINI *et al*., 2008a, ROBBINS *et al*., 2011). O sistema de defesa antioxidante é composto por dois grupos principais, os antioxidantes enzimáticos

(superóxido dismutase (SOD), catalase, peroxidase e algumas enzimas de apoio como glicose-6-fosfato dehidrogenase e glutationa redutase) e os de baixo peso molecular (ascorbato, glutationa, tocoferol e ubiquinona) (PODDA, *et al*., 1998). Entretanto, após exposição crônica ou excessiva à radiação UV, as EROs podem sobrepor a capacidade de defesa antioxidante da pele (STEENVOORDEN *et al*., 1997; VICENTINI *et al*., 2008a). Antioxidantes de baixo peso molecular, bem como os enzimáticos podem ser diretamente consumidos ou inativados pela radiação solar e pelas EROs (FUCHS, 1998). Neste contexto, uma maneira de controlar a instalação do estresse oxidativo e o desenvolvimento de patologias cutâneas é a utilização de antioxidantes de fontes exógenas, os quais reduzem a formação de EROs e/ou protegem a degradação de antioxidantes endógenos (HECK *et al*., 2004; CASAGRANDE *et al*., 2006).

1.3 Frutose-1,6-bifosfato

A via glicolítica (glicólise) é a via metabólica utilizada pelas células para extrair parte da energia contida na molécula de glicose, produzindo duas moléculas de piruvato, duas moléculas de ATP e dois equivalentes reduzidos de $NADH^{+}$. Esta sequência metabólica é composta por um conjunto de dez reações catalisadas por enzimas livres no citosol. Entre estas enzimas estão a hexoquinase, fosfofrutoquinase e a piruvatoquinase, que são as responsáveis pela regulação da via, controlando a produção de ATP (LEHNINGER, 1984).

A FBP (figura 1) é um intermediário altamente energético da via glicolítica, formada por um monossacarídeo frutose fosforilado nos carbonos C-1 e C-6, com atividade óptica dextrógira. Esta substância apresenta diversos efeitos farmacológicos, incluindo: inibição da aderência leucocitária e disfunção microvascular no músculo esquelético em modelos de isquemia e reperfusão (AKIMITSU *et al*., 1995); redução do edema de pata induzido por carragenina em ratos (PLANAS *et al*., 1993); efeito protetor para células do miocárdio, com redução do dano tecidual associado a isquemia (MARKOV, 1986); proteção dos hepatócitos contra dano celular induzido pela galactosamina (DE OLIVEIRA *et al*., 1992); efeito protetor sobre a função renal e parênquima renal em modelo de nefrotoxicidade induzida por cisplatina em ratos (AZAMBUJA *et al*., 2011); inibição da agregação plaquetária induzida pela adenosina difosfato e melhora nas alterações de coagulação em sepse experimental em ratos (DE OLIVEIRA *et al*., 2010).

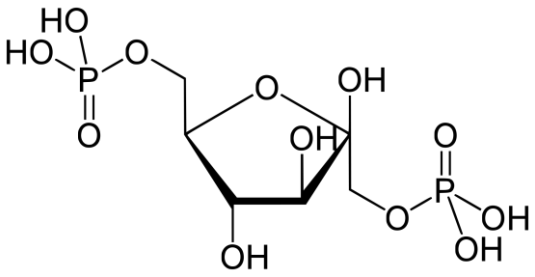

Figura 1. Estrutura molecular da frutose-1,6-bifosfato

Os efeitos farmacológicos da FBP parecem estar relacionados com sua interferência na via glicolítica, atuando como substrato para essa cascata reacional. Como a FBP situa-se após as enzimas hexoquinase e fosfofrutoquinase, pontos de regulação da via, com a entrada da FBP na célula a regulação da via estaria prejudicada (BAILEY *et al*., 1968), logo a produção de ATP seria estimulada. Além disso, a entrada de uma molécula exógena de FBP na glicólise intracelular pouparia a despesa de dois ATPs (um pela hexoquinase e outro pela fosfofrutoquinase), fornecendo duas vezes mais rendimento que o da energia glicolítica, quatro ATPs por molécula de substrato (MARKOV, 1986). Ainda, além de atuar como substrato para a via glicolítica e, consequentemente, elevar a produção de ATP, a FBP também pode elevar os níveis de ATP ao facilitar a ressíntese deste a partir de ADP (LOGUERCIO *et al*., 1996).

Uma atividade importante da FBP é o seu efeito analgésico, o qual foi descrito recentemente em um modelo de inflamação induzida pela carragenina (VALÉRIO *et al*., 2009). Nesse modelo, o efeito analgésico da FBP foi relacionado ao aumento sérico de adenosina (formada pela degradação do ATP e ADP), a qual atua perifericamente via receptores para adenosina A1. Apesar de outras evidências sugerirem que a frutose inibe a migração de leucócitos e a produção de citocinas pró-inflamatórias, não foi detectada inibição nesse modelo, descartando que a FBP tenha efeito analgésico por inibir o recrutamento de neutrófilos e produção de citocinas na inflamação induzida pela carragenina (VALÉRIO *et al*., 2009). É interessante ressaltar que os neutrófilos podem produzir mediadores nociceptivos, e as citocinas podem tanto atuar diretamente nos nociceptores (receptores responsáveis pela detecção de estímulos dolorosos) ou induzir a produção de outros mediadores como a prostaglandina E_2 (VERRI, *et al*., 2006). Um fator limitante para a atividade da FBP pode ter sido a sua farmacocinética. Por exemplo, como a FBP atua por aumentar os níveis de adenosina, e a adenosina é metabolizada pela adenosina deaminase, numa administração em bolus, ao ser alcançada a ocupação máxima

dos receptores A1, a adenosina restante não teria tempo suficiente para atuar nos seus receptores, pois seria metabolizada. Por outro lado, a liberação constante da FBP favoreceria o aumento de adenosina de maneira mais proporcional aos receptores e reduziria a adenosina degradada pela adenosina deaminase que não teve oportunidade de ativar os receptores A1. Assim, um sistema de liberação prolongada para FBP seria útil tanto para aumentar a sua eficácia quanto o seu tempo de ação.

1.3.1 Desenvolvimento e validação de metodologia para quantificação da FBP microencapsulada

Métodos de cromatografia líquida de alta eficiência (CLAE) são bastante eficientes na determinação de fármacos, e apresentam um aumento significativo na sensibilidade quando comparados a outros métodos (MARONA; SCHAPOVAL, 1999). No entanto, antes de um método analítico ser implementado para uso, o mesmo deve ser validado para demonstrar a eficácia para o objetivo pretendido (ROZET *et al*., 2007).

A metodologia para quantificação da FBP nas microcápsulas baseou-se, inicialmente, na metodologia geral para determinação de carboidratos, previamente descrita (TAVARES *et al*., 2003). No entanto, devido a interferência dos polímeros pectina e caseína na determinação da FBP, foi necessário desenvolver e validar um método para análise da FBP microencapsulada.

Os parâmetros de validação analisados foram: especificidade, linearidade, limite de detecção e limite de quantificação, precisão, exatidão e robustez.

- Especificidade e Seletividade: é a capacidade de um método de medir exatamente um composto em presença de outros compostos tais como impurezas, produtos de degradação e os demais componentes da matriz (ANVISA, 2003);

- Linearidade: é a capacidade de uma metodologia analítica de demonstrar que os resultados obtidos são diretamente proporcionais a concentração do analito da amostra, dentro de um intervalo especificado (ANVISA, 2003);
- Limite de Detecção (LD) – Menor quantidade do analito presente em uma amostra que pode ser detectado, porém não necessariamente quantificado, sob as condições experimentais estabelecidas (ANVISA, 2003);

- Limite de Quantificação (LQ) ou determinação – Menor quantidade do analito presente em uma amostra que pode ser determinada com precisão e exatidão aceitáveis sob as condições experimentais estabelecidas (ANVISA, 2003);
- Precisão: a precisão é a avaliação da proximidade dos resultados em uma série de medidas de uma amostragem múltipla de uma mesma amostra (ANVISA, 2003);
- Exatidão: a exatidão de um método analítico é a proximidade dos resultados obtidos pelo método em estudo em relação ao valor verdadeiro (ANVISA, 2003);
- Robustez: a robustez de um método analítico é a medida de sua capacidade em resistir a pequenas e deliberadas variações de parâmetros analíticos. Indica sua confiança durante o uso normal (ANVISA, 2003).

1.4 Rutina

Os flavonoides são polifenóis, metabólitos secundários de plantas e definidos quimicamente como substâncias compostas por um núcleo comum de fenilcromanoma (C6-C3-C6) com substituição em uma ou mais hidroxilas, incluindo derivados ligados a açúcares (BIRT *et al*., 2001).

Uma propriedade importante destes compostos é a inibição da atividade de enzimas relacionadas à produção de eicosanoides, incluindo fosfolipase A2, ciclooxigenase e lipooxigenase, reduzindo assim a produção e liberação de prostanóides e leucotrienos, mediadores inflamatórios (SILVA *et al*, 2002). Devido a essas propriedades e sua significante atividade *in vivo*, os flavonóides tem sido considerados como promissores candidatos a uma nova categoria de drogas anti-inflamatórias.

Adicionalmente, estas substâncias têm sido descritas como potentes antioxidantes, com múltiplos mecanismos, que incluem eliminação de radicais livres, indução de apoptose e inibição da promoção tumoral (CASAGRANDE *et al*., 2006).

Entre os flavonoides, a rutina (figura 2) é conhecida por apresentar efeitos anti-inflamatórios, antioxidantes (BECHO *et al*., 2009) e antinociceptivos, além de múltiplas atividades farmacológicas, com efeitos antibacteriano, antiviral (PANASIAK *et al*., 1989), antitumoral (DESCHNER *et al*., 1991), antialérgico (CHEN *et al*., 2000), vasodilator (CHUNG *et al*., 1993), antidiarreico (DI-CARLO *et al*., 1993), antimutagênico (BEAR; TEEL, 2000), entre outras. Esta substância pode ser encontrada em várias fontes alimentares como cebola, uva, trigo serraceno, feijão vermelho, maçã, tomate e bebidas como vinho e chá preto (HOLLMAN *et al*., 1996).

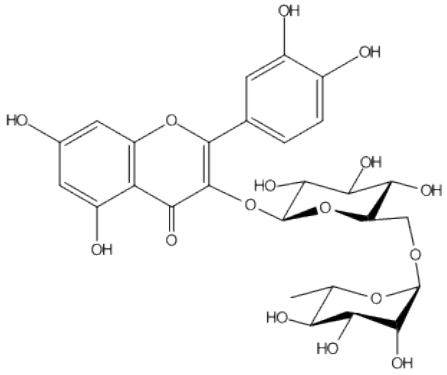

Figura 2. Estrutura molecular da rutina

Assim, o desenvolvimento de um sistema microencapsulado contendo rutina para modular a liberação e/ou absorção do fármaco pode otimizar a sua utilização na terapêutica.

2 OBJETIVOS

2.1 Objetivo geral

Desenvolver e caracterizar microcápsulas contendo rutina ou FBP, avaliar o efeito terapêutico das microcápsulas administradas por via oral em modelo de inflamação na pata induzido por carragenina bem como avaliar formulação tópica contendo rutina microencapsulada em modelo de inflamação/estresse oxidativo cutâneo induzido por radiação UVB.

2.2 Objetivos Específicos

- Desenvolver microcápsulas de liberação modificada contendo rutina, empregando-se o conjugado caseína/pectina para a obtenção da matriz polimérica;
- Desenvolver microcápsulas de liberação modificada contendo FBP, empregando-se o conjugado caseína/pectina para a obtenção da matriz polimérica;
- Caracterizar as microcápsulas a partir dos seguintes ensaios:
  - Avaliação morfológica das partículas;
  - Avaliação do tamanho e distribuição do tamanho das partículas;
  - Determinação da eficiência de encapsulação;
  - Avaliação do comportamento térmico por calorimetria exploratória diferencial;
  - Avaliação da cristalinidade por difração de raios X.
- Quantificar a rutina nas microcápsulas pelo método de DPPH;
- Desenvolver e validar um método para quantificação da FBP microencapsulada;
- Avaliar o perfil de dissolução *in vitro* das microcápsulas contendo FBP;
- Avaliar a eficácia terapêutica *in vivo* das microcápsulas contendo FBP ou rutina administradas via oral em modelo de inflamação na pata induzida por carragenina em camundongos;
- Avaliar a eficácia terapêutica *in vivo* das microcápsulas contendo rutina administradas via tópica em modelo de inflamação/estresse oxidativo induzido por radiação UVB.

3 MATERIAIS E MÉTODOS

3.1 Materiais

Os seguintes materiais foram obtidos a partir das fontes indicadas: Pectina USP (68% de esterificação), CPKelco (Limeira, SP, Brasil); caseína, Katuffman & Co (Kehl, Baden-Württemberg, Alemanha); ácido cítrico, hidróxido de sódio, ácido clorídrico e fosfato de potássio, Merck (grau analítico, Darmstadt, Hessen, Alemanha); tween 20®, Synth (Diadema, SP, Brasil); carragenina, Santa Cruz Biotechnology Inc (Dallas, Texas, USA); rutina, terc-butil hidroperoxido, Acrós Organics (Geel, Antwerp, Bélgica); azul brilhante R, glutationa reduzida (GSH), brometo de hexadeciltrimetil amônio (HTAB), *o*-dianisidina, dihidrocloreto de 1,2 fenilenodiamina, ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB), nitroblue tetrazolium (NBT), bisacrilamida e frutose-1,6-bifosfato, Sigma (St Louis, MO, USA); isoflurano, Abbott (Chicago, IL, USA); xileno cianol e Tris, Amresco (Solon, OH, USA); acrilamida, dodecil sulfato de sódio (SDS), glicerol, Superscript® III, Oligo(dT) primers, Platinum SYBRGreen®, Invitrogen (Carlsbad, CA, USA). Todos os outros reagentes utilizados foram de grau analítico.

3.2 Métodos

3.2.1 Preparação das microcápsulas

As microcápsulas de rutina e de FBP foram preparadas conforme metodologia descrita por Baracat e colaboradores (2004a). Foram realizadas dispersões aquosas de caseína e pectina a 10%, sob agitação mecânica constante. O pH foi ajustado para 8,0 ± 0,1 pela adição de hidróxido de sódio 4,0 M. Após a dispersão dos polímeros, o fármaco foi adicionado na proporção de 1:5 (fármaco/polímero). As microcápsulas foram obtidas pela redução lenta e gradual do pH para 4,0 ± 0,1 com adição de ácido cítrico 1,0 M. Nas microcápsulas contendo FBP, devido a alta solubilidade do fármaco em meio aquoso, as paredes das microcápsulas foram enrijecidas pela adição de glutaraldeído (50 µL/g de polímero) sob agitação constante por 30 minutos. As microcápsulas de rutina e de FBP obtidas foram secas em liofilizador (Edwards – Pirani 501) por 24h.

3.2.2 Caracterização das microcápsulas

3.2.2.1 Avaliação morfológica das partículas

Para avaliação da morfologia externa das microcápsulas, as amostras foram revestidas com ouro sob atmosfera de argônio e examinadas em microscópio eletrônico de varredura (MEV) (FEI – Quanta 200), sob aumento de 2500 vezes (microcápsulas de rutina) e 10000 vezes (microcápsulas de FBP).

3.2.2.2 Avaliação do tamanho e distribuição do tamanho das partículas

A análise do tamanho e distribuição de tamanho das partículas foi realizada pela técnica do espalhamento de luz utilizando-se o equipamento Zetasizer Nano system ZS (Malvern Instruments).

3.2.2.3 Determinação da eficiência de encapsulação

3.2.2.3.1 Microcápsulas contendo rutina

Para determinar a eficiência de encapsulação, 108,8 mg de microcápsulas foram dispersas em 10 ml tampão fosfato pH 6,8 adicionado de 5,0% de tween® 20 e agitada por 5 min, seguida de centrifugação a 3600 rpm por 10 min. O sobrenadante foi filtrado em filtro nylon syringe (0,45 µm), e o filtrado foi analisado pelo método de DPPH e quantificado espectrofotometricamente a 517 nm (item 3.2.3). A eficiência de encapsulação foi calculada usando a seguinte equação (1):

Eficiência de encapsulação (%EE) = (fármaco inicialmente adicionada – fármaco livre /fármaco inicialmente adicionada) x 100

(1)

3.2.2.3.2 Microcápsulas contendo FBP

Para determinar a eficiência de encapsulação, 5,0 mg de microcápsulas foram dispersas em 10 ml água ultrapura e agitada por 5 min, seguida de centrifugação a 9000 rpm por 10 min. O sobrenadante foi tratado com tampão acetato pH 4,7 (1:4) e etanol absoluto (1:2), filtrado em filtro 0,45 µm HV PVDF (Millipore®) e analisado por CLAE utilizando coluna HPX-87C Aminex Biorad com vazão de fase móvel (água ultrapura) de 0,6ml/min. Foram realizadas corridas de 20 min e a FBP foi detectada utilizando detector de índice de refração. O volume injetado foi 20 µL sob temperatura de 80 ºC. A eficiência de encapsulação foi calculada utilizando a equação (1).

3.2.2.4 Avaliação do comportamento térmico por calorimetria exploratória diferencial (DSC)

A análise por DSC foi realizada com o fármaco não microencapsulado, microcápsulas contendo o fármaco e microcápsulas sem fármaco. As amostras foram aquecidas de 30 a 500 ºC com velocidade de 5 ºC/min sob atmosfera de nitrogênio utilizando microcalorimetro Shimadzu DSC-60.

3.2.2.5 Avaliação da cristalinidade por difração de raios X

Os experimentos de difração de raios X foram executados num difratômetro Bruker D8 para difratometria do pó, utilizando-se a geometria Bragg-Brentano em modo contínuo e com velocidade de varredura de 0.05°/s. Um tubo com radiação cobre Kα (lambda = 1.5405 Å) focada em linha foi alimentado com 40 kV de tensão e 30 mA de corrente elétrica. O intervalo de ângulo 2-teta varrido foi de 5 a 70° para frutose e de 3 a 40° para rutina. As fases cristalinas do fármaco puro e do fármaco microencapsulado foram identificadas com programa X'Pert HighScore Plus com banco de dados PDF2DBP.

3.2.3 Quantificação da rutina nas microcápsulas pelo método de DPPH

O DPPH é um radical livre estável que potencialmente reage com compostos capazes de doar um átomo de hidrogênio. A atividade sequestradora do radical pela

rutina foi determinada pela mudança colorimétrica medida espectrofotometricamente em 517 nm. Essa mudança ocorre devido à redução do radical DPPH (BLOIS, 1958; CASAGRANDE *et al.*, 2007).

Para a medida do sequestro do radical livre, 50 µL de amostra foi adicionada à mistura reacional contendo 1 mL de tampão acetato 0,1M (pH 5,5), 1 mL de etanol e 0,5 mL de solução etanólica de DPPH 250 µM. Foi preparada uma curva padrão com concentrações de 0,1 a 10µg/mL de rutina. A mudança na absorvância foi medida espectrofotometricamente após 15 minutos de incubação à temperatura ambiente. As amostras foram analisadas em triplicata. O branco foi constituído de 1 mL de tampão acetato 0,1M (pH 5,5) e 1,5 mL de etanol. O controle positivo não continha amostra; assim, indica o máximo de elétrons livres do DPPH, o qual é considerado 100% de radical livre na solução para calcular-se a capacidade doadora de hidrogênio (%) da rutina por meio da seguinte equação (2):

% Atividade = (1 - absorvância da amostra/absorvância do controle) x 100 (2)

3.2.4 Desenvolvimento e validação de um método de CLAE para quantificação da FBP nas microcápsulas

3.2.4.1 Condições cromatográficas

O sistema cromatográfico utilizado consistiu em um equipamento Shimadzu LC-10A equipado com um loop de 20 µL e detector de índice de refração RID-10A. A integração dos picos foi realizada com software Class-VP. A coluna cromatográfica utilizada foi a Bio-Rad Aminex® HPX-87C (5 µm; 300 x 7.8 mm), utilizando agua ultrapura como fase móvel e fluxo de 0,6 mL/min, sob temperatura de 80 ºC. As amotras foram preparadas em triplicada.

3.2.4.2 Preparação das soluções padrão

O padrão de FBP foi dissolvido em água ultrapura obtendo uma solução de 1000 µg/mL. Esta solução foi diluída para se obter as seguintes concentrações: 50, 100, 200, 300 and 500 µg/mL.

3.2.4.3 Preparação das amostras de microcápsulas

Microcápsulas (0,05g) contendo o equivalente a 8,33 mg de FBP foram transferidas para tubo Falcon e foi adicionado 10 mL de água ultrapura. Esta mistura foi agitada em vortex por 5 min e centrifugada a 9000 rpm por 10 min. Ao sobrenadante (1 mL) foram adicionados 3 mL de tampão acetato (pH 4,7), mantendo a mistura em repouso por 30 minutos para precipitação da caseína. Esta solução foi centrifugada separando-se a caseína. A 1 mL do sobrenadante resultante adicionou-se 1 mL de etanol absoluto para precipitação da pectina. A mistura foi centrifugada e o sobrenadante injetado no sistema cromatográfico, após filtração. O mesmo processo foi realizado para microcapsulas sem fármaco.

3.2.4.4 Validação do método

A validação do método cromatográfico foi realizada de acordo com o guia para validação de processos analíticos ICH, Os parâmetros analisados foram: especificidade, linearidade, limite de detecção e quantificação, precisão, exatidão e robustez.

3.2.4.4.1 Especificidade

A especificidade do método foi avaliada pela análise das soluções de microcápsulas. As amostras foram preparadas como descrito no item 3.2.4.3, e analisadas como descrito nas condições cromatográficas. Todas as análises foram realizadas em triplicata. A presença de interferentes ou sobreposição com o pico da FBP foi verificada através dos cromatogramas obtidos.

3.2.4.4.2 Linearidade

Para avaliação da linearidade do método, soluções de FBP foram preparadas em cinco concentrações entre 50 – 500 µg/mL, como descrito no item 3.2.4.2. As soluções

foram preparadas em triplicata. A linearidade foi avaliada por regressão linear, através do método dos mínimos quadrados.

3.2.4.4.3 Limite de detecção e limite de quantificação

Os parâmetros do limite de detecção (LOD) e do limite de quantificação (LOQ) foram determinados com base na area do pico e na inclinação (“slope”) da equação de regressão. As análises foram realizadas em triplicata.

3.2.4.4.4 Precisão

A precisão do ensaio foi determinada pela repetibilidade (intra-ensaio) e precisão intermediária (inter-ensaio). Para repetibilidade foram analisadas diferentes concentrações de FBP nas amostras de microcápsulas (100, 150 e 200 μg/mL, em triplicata), no mesmo dia e sob as mesmas condicões experimentais. A precisão intermediária foi realizada através da análise de soluções preparadas de microcápsulas contendo FBP, nas mesmas concentrações descritas acima, em três dias diferentes.

3.2.4.4.5 Exatidão

Os experimentos para determinação da exatidão foram realizados utilizando o método para determinação da FBP nas microcápsulas. Soluções padrão (100, 300 and 500 μg/mL) foram adicionadas na proporção de 1:1 nas amostras de microcapsulas (preparadas conforme descrito no item 3.2.4.3). Os resultados representam a média de recuperação de cinco amostras independentes para cada concentração.

3.2.4.4.6 Robustez

A robustez da metodologia foi avaliada utilizando um planejamento fatorial completo (2^3) (tabela 1). As variáveis selecionadas foram: concentração de etanol, pH do tampão

acetato, ambos utilizados para preparação das amostras de microcápsulas, e temperatura do forno de acondicionamento da coluna cromatográfica. O delineamento experimental foi obtido utilizando o programa Statistic 6.0, revelando os efeitos e nível de significância para cada variável.

Tabela 1. Planejamento fatorial completo (2^3) para avaliação da robustez

Ensaios	Concentração de Etanol (%)	pH do Tampão	Temperatura do forno (^oC)	Concentração de Etanol (%)	pH do Tampão	Temperatura do forno (^oC)
1	-1,0	-1,0	-1,0	25,0	4,5	75,0
2	1,0	-1,0	-1,0	75,0	4,5	75,0
3	-1,0	1,0	-1,0	25,0	4,9	75,0
4	1,0	1,0	-1,0	75,0	4,9	75,0
5	-1,0	-1,0	1,0	25,0	4,5	70,0
6	1,0	-1,0	1,0	75,0	4,5	70,0
7	-1,0	1,0	1,0	25,0	4,9	70,0
8	1,0	1,0	1,0	75,0	4,9	70,0
9	0,0	0,0	0,0	50,0	4,7	80,0
10	0,0	0,0	0,0	50,0	4,7	80,0
11	0,0	0,0	0,0	50,0	4,7	80,0

3.2.5 Avaliação do perfil de dissolução *in vitro*

O teste de dissolução *in vitro* para microcápsulas contendo FBP e FBP não microencapsulada, ambas em quantidade correspondente a 133,0 mg de FBP, foi realizado em aparelho Erweka DT-6, de acordo com as especificações da Farmacopéia Americana 35ª ed, com adaptações. Foi utilizado sistema de agitação mecânica a 100rpm e meio de dissolução água ultrapura com temperatura do meio igual a 37°C. As amostras foram coletadas e repostas nos tempos: 5min, 10min, 15min, 30min, 45min, 1h, 1h15, 1h30, 1h45, 2h, 2h30, 3h, 3h30 e 4h. Alíquotas de 1 ml foram retiradas e misturadas com 3ml de tampão acetato pH 4,7. Após 30min esta mistura foi centrifugada por 10min a 9000rpm e 1 ml do sobrenadante foi misturado com 1ml de etanol e centrifugada por 10min a 9000rpm. O

sobrenadante foi retirado e a FBP foi quantificada por CLAE. Os resultados foram expressos como média (±EPM) de três determinações.

3.2.6 Avaliação da eficácia terapêutica *in vivo*

3.2.6.1 Modelo de inflamação na pata induzida por carragenina em camundongos

3.2.6.1.1 Animais Experimentais

Foram utilizados camundongos Swiss macho pesando 25-30g. Os camundongos foram criados no biotério Central da Universidade Estadual de Londrina e mantidos no Biotério do Departamento de Ciências Patológicas da Universidade Estadual de Londrina pelo menos 2 dias antes dos experimentos. Em ambos os biotérios foi utilizado ciclo de claro/escuro (12/12 h) com livre acesso a água e ração e temperatura controlada. Todos os testes comportamentais foram conduzidos durante o ciclo claro e em sala com temperatura controlada. A quantidade de animais e os procedimentos utilizados foram aprovados pelo Comitê de Ética para uso de animais da Universidade Estadual de Londrina, processo número 3321.2013.29 (FBP) e 3324.2013.58 (rutina). Todos os esforços foram realizados para reduzir o número de animais utilizados e o seu sofrimento.

3.2.6.1.2 Indução da inflamação por carragenina

A carragenina tem sido amplamente utilizada como estímulo inflamatório para indução de edema, migração celular e hiperalgesia. Nesse modelo foram administrados 300 µg (POSSEBON *et al*., 2014) ou 100 µg (CUNHA *et al*., 2005) de carragenina em 25 µl de salina via subcutânea na face plantar da pata do camundongo. Os animais foram tratados via oral com o fármaco não microencapsulado, sendo 300mg/kg de FBP (VALÉRIO *et al*., 2009) ou 100mg/kg de rutina (dados não publicados Sandra S. Mizokami e colaboradores); fármaco microencapsulado (equivalente a 300mg/kg de FBP ou 100mg/kg de rutina) ou microcápsulas sem fármaco (equivalente a quantidade de microcápsulas com fármaco). Os tratamentos foram realizados 15min (FBP) (VALÉRIO *et al*., 2009) ou 1h (rutina) (dados não publicados Sandra S. Mizokami e colaboradores) antes da aplicação do estímulo. O grupo controle negativo da inflamação recebeu injeção intraplantar de salina (25 µl). Nos

experimentos com a FBP foram avaliados a hiperalgesia mecânica (“dor”) pelo teste de pressão crescente na pata e o edema por paquímetro, previamente ao estímulo e 1, 3 e 5h após o estímulo. No experimento com a rutina foi avaliada a hiperalgesia mecânica (“dor”) pelo teste de pressão crescente na pata, previamente ao estímulo e 30min, 1, 3, 5 e 7 h após o estímulo.

a) Avaliação da hiperalgesia mecânica (“dor”) pelo teste de pressão crescente na pata

A hiperalgesia mecânica (aumento da sensibilidade ao estímulo mecânico doloroso) foi avaliada por uma versão eletrônica dos filamentos de Von Frey como previamente descrito (CUNHA *et al*., 2004). Brevemente, os animais foram colocados em gaiolas de acrílico (12×10×17 cm) com piso de grade de arame, 15-30min antes do início do teste. O teste consistiu em realizar uma pressão na pata traseira do animal com um transdutor de força (analgesímetro eletrônico; Insight) adaptado com uma ponteira de polipropileno (0.5 mm^2). O investigador foi treinado para aplicar a ponteira perpendicularmente na área central da pata com um aumento gradual da pressão, caracterizando comportamento nociceptivo. O ponto final foi caracterizado pela remoção da pata seguida de movimento de sacudida. Após a retirada da pata, a intensidade da pressão foi registrada automaticamente. O valor foi obtido a partir da média de três medições. Os animais foram testados antes e após o tratamento. Os resultados foram expressos pelo delta (Δ) do início da retirada (in g) calculado pela subtração da média dos valores obtidos em 30min, 1, 3, 5 e 7 h (rutina) ou 1, 3, 5h (FBP) após o estímulo e a média dos valores obtidos no tempo zero. Os dados foram apresentados como media ± EPM de 6 camundongos por grupo por experimento, e os experimentos foram realizados em duplicata.

b) Avaliação do edema de pata

As alterações no volume da pata (formação de edema) foram determinadas utilizando paquímetro. Foram realizadas medições no tempo 0 (antes da injeção intraplantar do estímulo) e 1, 3 e 5 horas após o estímulo nociceptivo com carragenina. A variação do volume da pata foi determinada pelo delta entre os tempos decorridos após a administração do estímulo e o tempo zero ou basal do animal, e o resultado expresso em mm/pata

(ZARPELON *et al*., 2013). Os dados foram apresentados como media ± EPM de 6 camundongos por grupo por experimento, e os experimentos foram realizados em duplicata.

3.2.6.1.3 Análise estatística dos resultados

Os dados obtidos foram analisados utilizando ANOVA de uma via seguida do teste de comparações múltiplas de Tukey. As diferenças foram consideradas significativas quando $P < 0.05$ foi obtido. As análises estatísticas foram realizadas utilizando o software GraphPad Prism® 4.0.

3.2.6.2. Modelo de inflamação/estresse oxidativo induzido por radiação UVB

3.2.6.2.1 Preparo das formulações tópicas

Primeiramente foi preparada uma formulação base de creme não iônico, utilizando a cera auto-emulsionante Polawax®. Nesta preparação, o polawax (10%) e triglicerídeos de ácido cáprico e caprílico (5%) foram misturados e aquecidos a 70^{o}C. Separadamente, foram misturados propilenoglicol (6%) e agua deionizada (qsp 100ml) e aquecido a 70^{o}C. Após aquecimento as fases foram misturadas e agitadas até resfriamento (40^{o}C). Foi então acrescentada uma solução etanólica de metilparabeno (10%) e propilparabeno (2%). A essa formulação foram incorporadas quantidades de rutina em pó ou rutina microencapsulada correspondentes a 1% da formulação preparada. As microcápsulas de rutina foram previamente preparadas conforme descrito no item 3.2.1.

3.2.6.2.2 Animais experimentais

Foram utilizados camundongos sem pelo da linhagem HRS/J, machos ou fêmeas, adultos e com peso de 20 a 30 g. Os animais foram fornecidos e mantidos no biotério do Hospital Universitário de Londrina-PR, em sala com temperatura controlada, ciclo claro/escuro de 12h e com livre acesso a água e ração. Os experimentos foram conduzidos

conforme as normas do Comitê de ética em experimentação animal da Universidade Estadual de Londrina (processo n° 27025.2013.10).

3.2.6.2.3 Sistema e fonte de radiação UVB

A fonte de luz utilizada nos experimentos para indução de estresse oxidativo foi uma lâmpada UVB fluorescente modelo PHILIPS TL/12 40W RS (Medical). A lâmpada emite radiação (4,14J/cm^2) na faixa de λ de 270 a 400 nm com pico máximo de emissão em torno de 313 nm. A lâmpada foi instalada em um compartimento de madeira desenvolvido para a indução do estresse oxidativo. A medida da irradiância foi realizada utilizando-se um radiômetro (IL 1700) com detectores para radiação UV (SED 005) e, especialmente, para UVB (SED 240). Os animais foram colocados em caixas de plástico e cobertos com uma tela plástica para garantir a total exposição da região dorsal. Um rodízio com as caixas contendo os animais foi realizado devido às variações de radiação ao longo da lâmpada. Os animais ficaram a 20 cm da fonte de radiação e movimentavam-se livremente na caixa (CASAGRANDE *et al*., 2006).

3.2.6.2.4 Protocolo experimental para administração das formulações tópicas

Os camundongos foram aleatoriamente distribuídos em diferentes grupos com 5 animais cada:

- grupo 1: controle não irradiado

- grupo 2: controle irradiado

- grupo 3: irradiado e tratado com formulação tópica sem fármaco

- grupo 4: irradiado e tratado com formulação tópica contendo rutina não microencapsulada

- grupo 5: irradiado e tratado com formulação tópica contendo microcápsulas sem fármaco

- grupo 6: irradiado e tratado com formulação tópica contendo rutina microencapsulada.

Os camundongos foram tratados topicamente, na parte dorsal, com 0,5g de formulação contendo 1% de rutina (em pó ou microencapsulada), 12h, 6h, imediatamente antes e 6 h após o início da sessão de irradiação UVB (Figura 3). Os grupos foram

irradiados simultaneamente. Os animais foram terminalmente anestesiados com 1,5% de isoflurano 12 h (edema, FRAP, ABTS, GSH e MMP-9), 2h (NBT e catalase) ou 4h (citocinas e PCR) após exposição a radiação UVB, e a pele da parte dorsal foi removida e lavada com NaCl 145 mM. Nos testes em que a coleta foi realizada 2 h e 4 h após exposição à radiação, os animais foram decapitados imediatamente após anestesia e amostras de pele da região dorsal foram coletadas. As amostras foram armazenadas a -70°C para análises subsequentes, exceto as amostras coletadas para verificação do edema cutâneo, que foram pesadas após serem removidas e não foram congeladas (CASAGRANDE *et al*., 2006; IVAN *et al*., 2014).

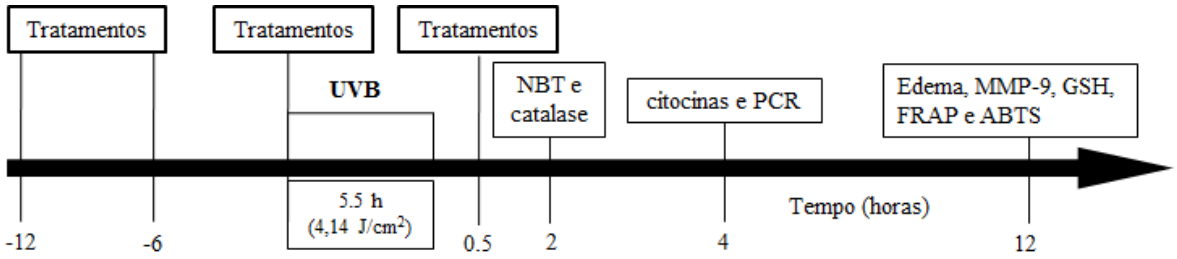

Figura 3. Esquema geral dos protocolos experimentais para avaliação da eficácia de formulação tópica contendo rutina microencapsulada. Os animais foram irradiados com radiação UVB durante 5 horas e 30 min (4,14 J/cm^2). Doze horas, seis horas, imediatamente antes da radiação e trinta minutos após o início da radiação os animais foram tratados com as formulações tópicas. Os animais foram eutanasiados e amostras de pele foram coletadas 2 horas (para os testes de NBT e atividade da CAT), 4 horas (para citocinas e expressão de RNAm por PCR quantitativo) e 12 horas (para os testes de edema, atividade de metaloproteinase-9 (MMP-9), níveis de glutationa reduzida (GSH), avaliação do poder antioxidante redutor de ferro (FRAP) e transferência de elétrons ao radical ABTS), após o fim da radiação UVB.

3.2.6.2.5 Avaliação do edema de pele

Após remoção da pele do dorso de cada animal, uma área padronizada de pele (5 mm de diâmetro) foi coletada com auxílio de um molde de área fixa, e posteriormente pesada (AFAQ; ADHAMI; AHMAD, 2003; BATHIA *et al*., 2011). O efeito das formulações no edema induzido por radiação UVB foi mensurado pelo aumento da espessura da pele na

região dorsal. A análise foi realizada pela comparação do peso da pele entre os diferentes grupos e o resultado foi expresso em mg de pele.

3.2.6.2.6 Avaliação da redução da secreção/atividade da metaloproteinase 9 (MMP-9)

Para determinação de MMP-9 com atividade gelatinase foi utilizada zimografia em gel de poliacrilamida com duodecil sulfato de sódio (SDS-PAGE).

As amostras de pele foram diluídas (1:4) e homogeneizadas em tampão Tris/HCl 50 mM (pH 7,4) contendo cloreto de cálcio ($CaCl_2$) 10 mM e 1% de inibidores de proteinases (fenantrolina, fluoreto de fenilmetilsulfonila e N-etilmaleimida) com auxílio de triturador de tecido (Tissue-Tearor, Biospec). O homogenato foi duplamente centrifugado a 12.000 *g* por 10 min a 4°C. Alíquota de 50 µL do sobrenadante foi coletada e diluída em 10 µL de tampão Tris/HCl (pH 6,8) contendo 20% de glicerol, 4% de duodecil sulfato de sódio (SDS) e 0,005% de xileno cianol. Para aplicação no gel de eletroforese foi utilizado 25 µL dessa mistura. O gel de eletroforese foi preparado utilizando-se 10% de acrilamida e 0,025% de gelatina. Após a eletroforese, o gel foi lavado com solução de triton X-100 2,5% por 1 hora, sob constante agitação e incubado por 12 horas em tampão tris-HCl 50mM (pH 7,4), contendo $CaCl_2$ 5mM e azida sódica 0,02% a 37°C. Após incubação, o gel foi corado com Coomassie Blue 350-R e descorado com ácido acético 20%. Zonas de atividade enzimática foram detectadas como regiões de coloração negativa contra um fundo escuro. A atividade proteolítica foi analisada comparando as bandas dos diferentes grupos pelo programa ImageJ (NIH, Bethesda, MD, USA). A dosagem de proteína das amostras foi realizada utilizando-se o método de Lowry (LOWRY *et al*., 1951).

3.2.6.2.7 Avaliação do poder antioxidante redutor de ferro (ensaio de FRAP)

As amostras de pele (aproximadamente 30 mg) foram homogeneizadas em 500 µL de KCl (1,15%) com auxílio de um triturador de tecido (Tissue-Tearor, Biospec) e centrifugadas a 1.000 *g* por 10 min a 4°C, com retirada do sobrenadante para a análise. Para a reação, foram utilizados 30 µL do sobrenadante e 1 mL de reagente FRAP. O reagente de FRAP foi preparado adicionando 2,5 mL de uma solução 10 mM de 2,4,6 tripiridil-S-triazina (TPTZ) em HCl 40 mM a 2,5 mL de $FeCl_3.6H_2O$ e 25 mL de tampão acetato 0,3 mM (pH 3,6), e esta solução foi incubada a 37°C por 30 min antes do uso. Foi realizada a leitura da reação em espectrofotômetro a 595 nm (EnSpire, Perkin Elmer).

Soluções padrão com diferentes concentrações de trolox (0,01-20 nmol) foram utilizadas para calibração. Os resultados foram expressos como nmol equivalente de Trolox/mg de pele (KATALINIC *et al*., 2005).

3.2.6.2.8 Avaliação do sequestro do radical 2,2',azinobis(3-etilbenzotiazolina-6-ácido sulfônico) ($ABTS^{+}$) (ensaio de ABTS)

O ensaio é baseado na diminuição da absorvância devido ao sequestro do radical ABTS. Amostras de pele (aproximadamente 30 mg) foram homogeneizadas em 500 µL de KCl (1,15%) com auxílio de um triturador de tecido (Tissue-Tearor, Biospec) e centrifugadas a 1.000 *g* por 10 min a 4°C, com retirada do sobrenadante para análise. Para a reação foi adicionado 40 µL do sobrenadante a 1 mL de solução de ABTS diluída. A solução de ABTS foi preparada reagindo 7 mM da solução de ABTS com 2,45 mM de persulfato de potássio. A mistura foi armazenada em frasco âmbar e em temperatura ambiente por no mínimo 16 horas antes do uso. A solução ABTS para uso na reação foi diluída com tampão fosfato (pH 7,4) até uma absorbância de 0,700 a 0,800 em 730 nm. A reação foi incubada por 6 min e posteriormente foi realizada a leitura em 730 nm (EnSpire, Perkin Elmer). Soluções padrão com diferentes concentrações de trolox (0,01-20 nmol) foram utilizadas para calibração. Os resultados foram expressos como nmol equivalente de Trolox/mg de pele (KATALINIC *et al*., 2005; IVAN *et al*., 2014).

3.2.6.2.9 Avaliação dos níveis do antioxidante endógeno glutationa reduzida (GSH)

O GSH é um dos principais antioxidantes endógenos sendo seus níveis diminuídos pela exposição da pele a radiação UV (FUCHS, 1998). O método baseia-se na detecção do ácido 5-mercapto-2-nitrobenzóico, um composto amarelo, liberado pela quebra da ligação dissulfeto do ácido 5',5'-ditio-bis-(2-nitrobenzóico) (DTNB) pelo grupo sulfidrila da glutationa (HUBER; ALMEIDA; FÁTIMA, 2008).

As amostras de pele foram diluídas (1:4) em EDTA 0,02 M e homogeneizadas com auxilio de triturador de tecido (Tissue-Tearor, Biospec). Ao homogenato foi adicionado ácido tricloroacético (TCA) 50% na proporção de 1:0,2 de EDTA e TCA, respectivamente. A mistura foi centrifugada a 2.700 *g* por 10 min a 4°C. O sobrenadante foi novamente centrifugado a 2.700 *g* por 10 min a 4°C, e o sobrenadante final foi retirado para análise. O ensaio para quantificação dos níveis de GSH na pele foi realizado em microplaca por adição

de 50 µL de amostra ao meio reacional contendo 100 µL de tampão Tris 0,4 M (pH 8,9) e 5 µL de uma solução de ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB) em metanol (1,9 mg/mL de metanol). A absorvância foi determinada após 5 min de incubação a 405 nm (Asys Expert Plus, Biochrom). A curva analítica foi preparada com 5 a 150 µM de GSH. Os resultados foram expressos em µM de GSH/ mg de pele (MORON; DEPIERRE; MANNERVIK, 1979; SRINIVASAN; SABITHA; SHYAMALADEV, 2007).

3.2.6.2.10 Avaliação dos níveis do antioxidante endógeno catalase (CAT)

A atividade da CAT foi avaliada através da medida da redução da concentração de peróxido de hidrogénio (H_2O_2) e produção de oxigênio, conforme descrito anteriormente (AEBI, 1984). Amostras de pele (aproximadamente 100 mg) foram homogeneizadas em 500 µL de EDTA 0,02 M com auxílio de um triturador de tecido (Tissue-Tearor, Biospec) e centrifugadas duas vezes a 2700 *g* por 10 min a 4°C. O sobrenadante da amostra (10 µL) foi misturado com 160 µL de tampão Tris-HCl 1 M contendo EDTA 5 mM pH 8,0, 20 µL de água deionizada e 20 µL de H_2O_2 200 mM. A medida da atividade da CAT foi estimada através da diferença entre a leitura inicial e a leitura realizada 30 segundos após a adição de H_2O_2 a 240 nm em espectrofotômetro (EnSpire, Perkin Elmer) a 25ºC. Os resultados foram expressos como unidade de CAT/mg de pele/minuto.

3.2.6.2.11 Avaliação da produção de ânion superóxido ($O_2^{\bullet-}$)

Após exposição a radiação UVB, $O_2^{\bullet-}$ é produzido em grande quantidade (CAMPANINI *et al*., 2013), aumentando os níveis de H_2O_2, o que favorece a geração do radical hidroxil ($HO^{\bullet}$), um derivado citotóxico (GUTTERIDGE, 1986). A quantificação da produção de $O_2^{\bullet-}$ nos homogenatos de tecido (aproximadamente 100 mg em 500 µL de EDTA 0,02 M) foi realizada utilizando o ensaio de NBT (*nitroblue tetrazolium*) (CAMPANINI *et al*., 2013). Resumidamente, 50 µL de cada homogenato foi incubado em placa com 96 poços por 1 h. O sobrenadante foi cuidadosamente retirado e as células aderidas nos poços foram incubadas com 100 µL de NBT (1 mg/ml) por 15 min. O sobrenadante foi cuidadosamente removido e as células foram fixadas com 20 µL de metanol. Partículas de formazan foram dissolvidas pela adição de 120 µL de KOH 2M e 140 µL de dimetilsulfoxido. A redução do NBT para formazan foi medida em 600 nm utilizando espectrofotometro (Asys

Expert Plus, Biochrom) e os resultados foram expressos como densidade optica (OD) por 10 mg de pele.

3.2.6.2.12 Transcrição reversa e reação em cadeia de polimerase quantitativa (RT-qPCR)

RT-qPCR foi realizada conforme previamente descrito (CAMPANINI *et al*., 2013). Amostras de pele foram homogeneizadas em reagente de trizol, e o RNA total foi isolado de acordo com as instruções do fabricante. A pureza do RNA total foi medida com espectrofotômetro e a taxa de absorção em 260/280 nm ficou entre 1,8 e 2,0 para todas as preparações. Transcrição reversa do RNA total para cDNA, e qPCR foi realizada utilizando kit GoTaq® 2-Step RT-qPCR (Promega) e primers específicos. A qPCR foi realizada em sistema StepOnePlusTM PCR em tempo real (Applied Biosystems®). A expressão do gene relativo foi quantificada utilizando o método comparativo $2^{-(\Delta\Delta Cq)}$. A expressão de Gapdh mRNA foi utilizada como controle. Os primers utilizados foram: *Gp91phox*, sense: 5'-AGCTATGAGGTGGTGATGTTAGTGG-3', antisense: 5'-CACAATATTTGTACCAGACAGACTTGAG-3'; IL-1beta sense 5'-GAAATGCCACCTTTTGACAGTG-3', antisense 5'- TGGATGCTCTCATCAGGACAG-3'; TNF-alfa sense 5'- TCTCATCAGTTCTATGGCCC -3', antisense 5'-GGGAGTAGACAAGGTACAAC -3'; IL-10 sense 5'-TCTCATCAGTTCTATGGCCC-3', antisense 5'-GGGAGTAGACAAGGTACAAC-3'; e *Gapdh* sense 5-ATGACATCAAGAAGGTGGTG-3, antisense: 5'-CATACCAGGAAATGAGCTTG-3.

3.2.6.2.13 Análise estatística dos resultados

Os resultados foram expressos como média ± erro padrão da média (EPM) de 5 animais por grupo por experimento e são representativos de dois experimentos separados. Os dados obtidos foram analisados utilizando ANOVA de uma via seguida do teste de comparações múltiplas de Tukey. As diferenças foram consideradas significativas quando $P < 0.05$ foi obtido. As análises estatísticas foram realizadas utilizando o software GraphPad Prism® 4.0.

4 RESULTADOS E DISCUSSÃO – ARTIGOS CIENTÍFICOS

4.1 Development and validation of HPLC method for quantification of fructose-1,6-bisphosphate-loaded microcapsules

Journal of the Brazilian Chemical Society

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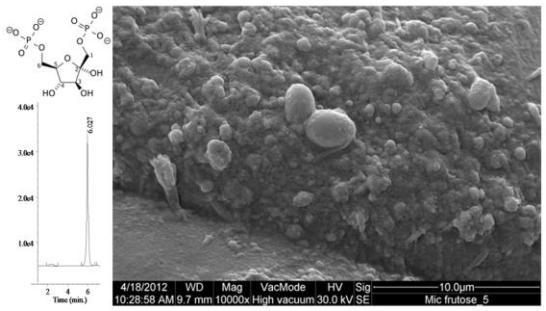
Graphical Abstract



Photomicrographs of fructose-1,6-bisphosphate-loaded microcapsules obtained by scanning electron microscopy, increase of 10000x.

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR QUANTIFICATION OF FRUCTOSE-1,6-BISPHOSPHATE-LOADED MICROCAPSULES

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Abstract

This study validated a high performance liquid chromatography (HPLC) method for the quantitative evaluation of fructose-1,6-bisphosphate (FBP)-loaded microcapsules. The method was linear within 50 – 500 μg/mL range with a correlation coefficient of 0.9999, and without interference in the FBP peak. The detection and quantitation limits were 1.98 and 6.60 μg/mL, respectively. The intra- and inter-assay precisions presented relative standard deviation values between 1.3 and 13.2%. An average of 90.33%, 96.21% and 78.78% of FBP was recovered for 100, 300 and 500 μg/mL concentrations, respectively. The results demonstrated acceptable linearity, sensitivity and accuracy for the purpose of the analysis. The method reported is a reliable HPLC technique useful for FBP-loaded microcapsules evaluation.

Keywords: Fructose-1,6-bisphosphate; microcapsules; pectin/casein.

Introduction

Fructose-1,6-bisphosphate (FBP), is a intermediate of glycolytic route whose pharmacological effects include: reduction of paw edema induced by carrageenan in rats;[1] apoptosis and oxidative stress in galactosamine sensitized hepatocytes;[2] protective effect on renal function and renal parenchyma in model of cisplatin-induced nephrotoxicity in rats;[3] inhibition of platelet aggregation induced by adenosine diphosphate in experimental sepsis in rats[4] and reduction of mechanical hyperalgesia (decrease in the nociceptive threshold)[5] in experimental models.

Microencapsulation technology has been widely used in the pharmaceutical area, mainly in the preparation of drug delivery systems.[6] The placement of drugs in modified release systems prevents rapid and complete drug release, resulting in better therapeutic efficacy and patient adherence to treatment.[7]

The ability of an analytical method to quantify a drug is of great importance to ensure quality, safety and efficacy pharmaceuticals. High performance liquid chromatography (HPLC) methods are very useful in the determination of drugs and present significant improvement of sensitivity compared to previous methods.[8] However, before an analytical method can be implemented for use, it must be validated to demonstrate that it is efficient for the intended purpose.[9] In this sense, this work demonstrate a valuable tool in the pharmaceutical analysis, which a HPLC validated assay for the analysis of FBP-loaded microcapsules. To our knowledge, this is the first study to demonstrate the validation of method for quantification of the microcapsules containing FBP.

Experimental

Materials

Fructose-1,6-bisphosphate was obtained from Sigma **(**St Louis, MO, USA) and used as standard. Pectin USP (68% of esterification) from CPKelco (Limeira, SP, Brazil); casein from Katuffman & Co (Kehl, Baden-Württemberg, Germany); citric acid, sodium hydroxide, chloride acid and potassium phosphate from Merck (analytical grade, Darmstadt, Hessen, Germany); tween 20® from Synth (Diadema, SP, Brazil). All other reagents used were pharmaceutical or chromatographic grade.

Microcapsules

FBP-loaded microcapsules were prepared by dispersion in distilled water (solid content 10%, w/v) of the polymers pectin and casein under constant mechanical shaking. Sodium hydroxide (4.0 M) was used to adjust pH to 8.0 $\pm$ 0.1.[10] After complete dispersion, FBP was added at the proportion of 1:5 (drug:polymer). Microcapsules were obtained by slow and gradual reduction of pH from 8.0 $\pm$ 0.1 to 4.0 $\pm$ 0.1 with 1.0 M citric acid. Then, the wall of microcapsules was hardened by adding glutaraldehyde (50 µl/g polymer) with constant shaking for additional 30 minutes.[11,12] The same methodology was used to prepare microcapsules containing no drug (blank microcapsules). Microcapsules were dried in a lyophilizer (Edwards, model Pirani 501) during 24 h.

Chromatographic conditions

The chromatographic system consisted of a Shimadzu LC-10A equipped with a 20 µL loop and RID-10A refractive index detector. Integration of the chromatographic peaks was achieved with software Class-VP. Chromatography was performed on a Bio-Rad Aminex® HPX-87C column (5 µm; 300 x 7.8 mm i.d.), with a mobile phase of ultrapurified water and flow rate of 0.6 mL/min under a temperature of 80 ºC. Each sample was prepared in triplicate. The volume injected was 20 µL.

Preparation of standard solutions

Standard FBP was dissolved in ultrapure water obtaining a solution of 1000 µg/mL. Then, this FBP aqueous solution was diluted with mobile phase to yield the following concentrations: 50, 100, 200, 300 and 500 µg/mL.

Preparation of samples of microcapsules

Accurately weighed 0.05 g of microcapsules containing the equivalent to 8.33 mg of FBP was transferred to Falcon tube and added 10 mL of ultrapure water. This mixture was stirred in vortex for 5 min and centrifuged at 9000 rpm for 10 min. The supernatant (1 mL) was mixture with 3 mL of acetate buffer pH 4.7 and resting for 30 min. This solution was centrifuged (9000 rpm for 10 min), and the supernatant (1 mL) was mixture with 1mL of absolute ethanol. This solution was centrifuged (9000 rpm for 10 min) and the supernatant was injected, after filtration. The same procedure was performed for blank microcapsules (without drug).

Method validation

The validation was performed based on the ICH Guidance,[13] taking into account the parameters required for assaying dosage forms.

Specificity

The specificity of the method was evaluated by analyzing of microcapsules solutions. The samples were prepared as described in the preparation of samples of microcapsules section, and analyzed as described in the chromatographic conditions. All analyses were performed in triplicate. The system response was examined through the presence of interference or overlaps with the FBP responses.

Linearity

For linearity experiments, solutions of FBP were prepared at five concentrations within the range of 50 – 500 μg/mL as described in the preparation of standard solutions section. The

solutions were prepared in triplicate. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) parameters were determined on the basis of peak area and the slope of the regression equation. Analysis was performed in triplicate.

Precision

The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay). Repeatability was evaluated by analysis of three determinations at different concentrations of FBP in microcapsule samples (100, 150 e 200 μg/mL, in triplicate), in the same day under the same experimental conditions. The intermediate precision was studied by assaying freshly prepared solutions of FBP-loaded microcapsules on three different days.

Accuracy

The accuracy experiments were performed applying the method to quantify FBP-loaded microcapsules. The standard solutions (100, 300 and 500 μg/mL) were added in proportion of 1:1 in microcapsules samples. The results represent the mean of recovery for five independent samples at each concentration.

Robustness

The robustness of the methodology was evaluated using a full factorial design (2^3). The variables selected were: ethanol concentration, pH of buffer solution used for preparation of

samples of microcapsules and temperature of the chromatographic column conditioning. The experimental design was obtained using the Statistic 6.0 Program,[14] revealing the effects and significance level for each variable.

Results and Discussion

Specificity

Firstly, analytical experiments addressed whether the polymers of microspheres would interfere over FBP quantification since microparticles are prepared with pectin and casein components. The specificity was carried out through the comparison of the peak retention time of the FBP-loaded microcapsule samples and blank microcapsules samples, before and after treatment with acetate buffer and ethanol. In the employed chromatographic conditions, FBP presented a retention time of approximately 6.1 minutes for standard solution (Figure 1A). Figure 1B and 1C shows the chromatogram of casein and pectin with retention time of approximately 6.3 and 5.88 minutes, respectively. Chromatogram of blank microcapsules sample and FBP-load microcapsules sample (Figure 2A and 2B, respectively), before treatment with acetate buffer and ethanol, presenting the polymers interference in 6.27 and 6.19 minutes, very close of retention time detected in FBP standard solution. However, no interference was detected after treatment (Figure 3A and 3B), since no peak was detected in the same retention time of FBP (Figure 3A), demonstrating the importance of microcapsule samples treatment for evaluation of FBP-loaded microcapsules for proposed method.

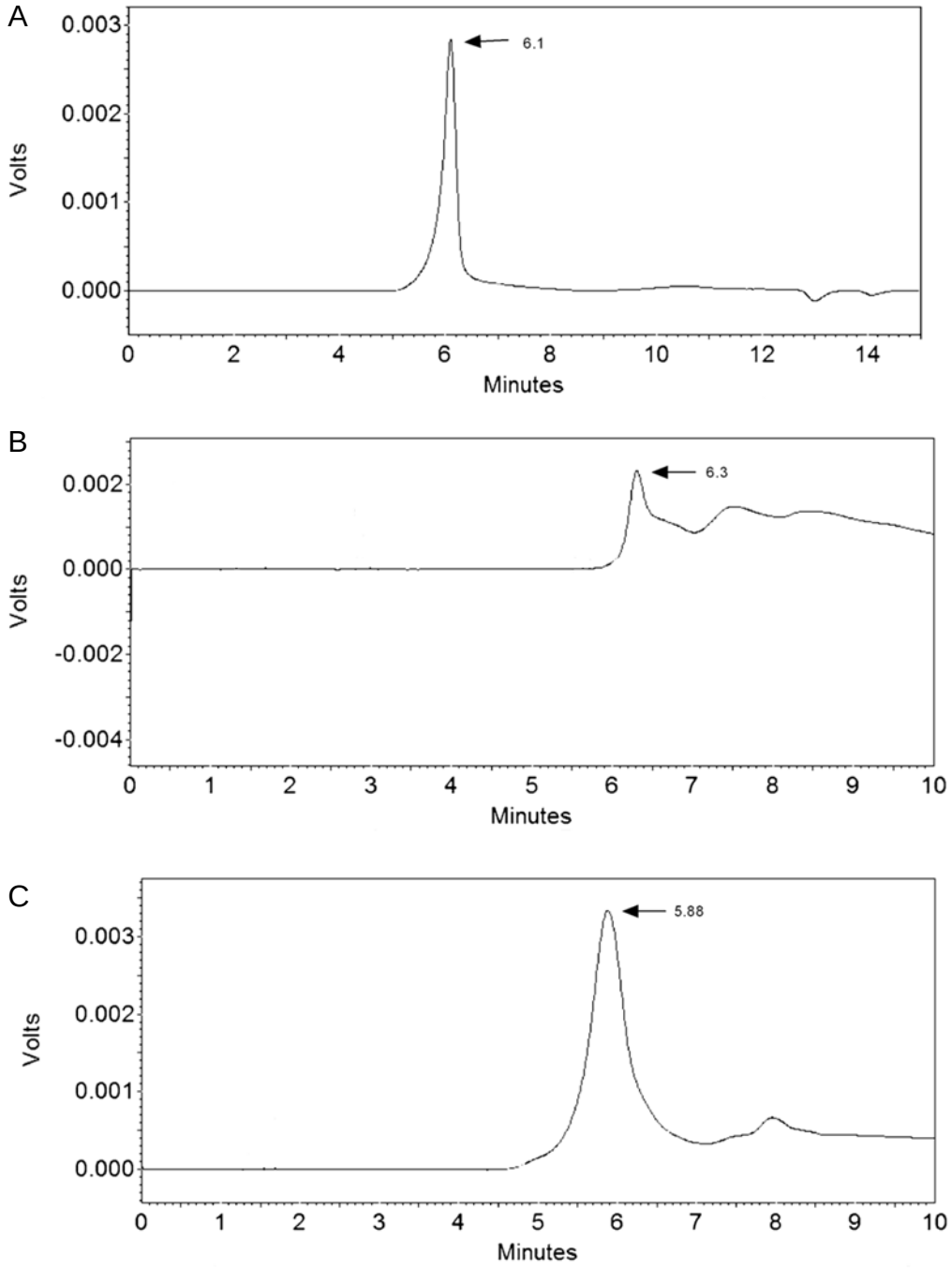

Figure 1. Chromatograms of fructose-1,6-bisphosphate (FBP) standard solution (A); solution of casein (B) and pectin (C). Chromatographic conditions: Bio-Rad Aminex® HPX-87C column, 250 x 4.6 mm, mobile phase: ultrapurified water, 0.6 mL/min.

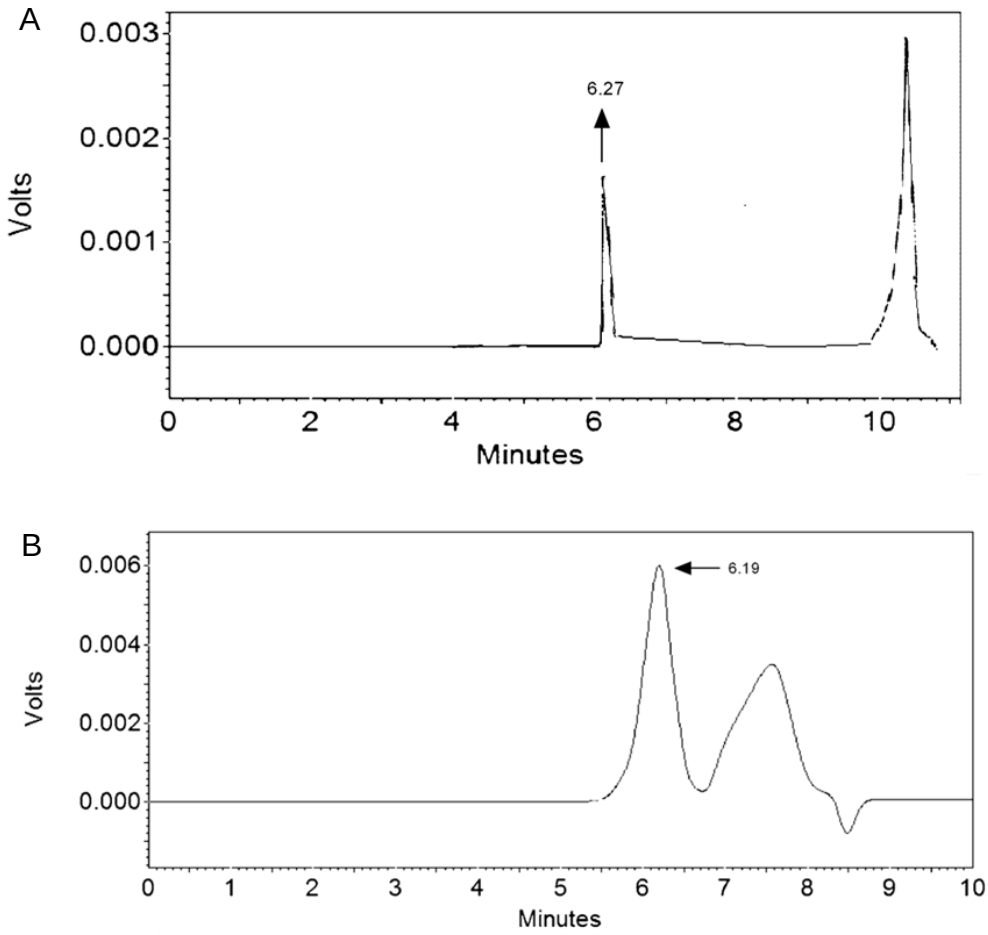

Figure 2. Chromatograms of blank microcapsules sample (A) and FBP-load microcapsules sample (B), before treatment. Chromatographic conditions: Bio-Rad Aminex® HPX-87C column, 250 x 4.6 mm, mobile phase: ultrapurified water, 0.6 mL/min.

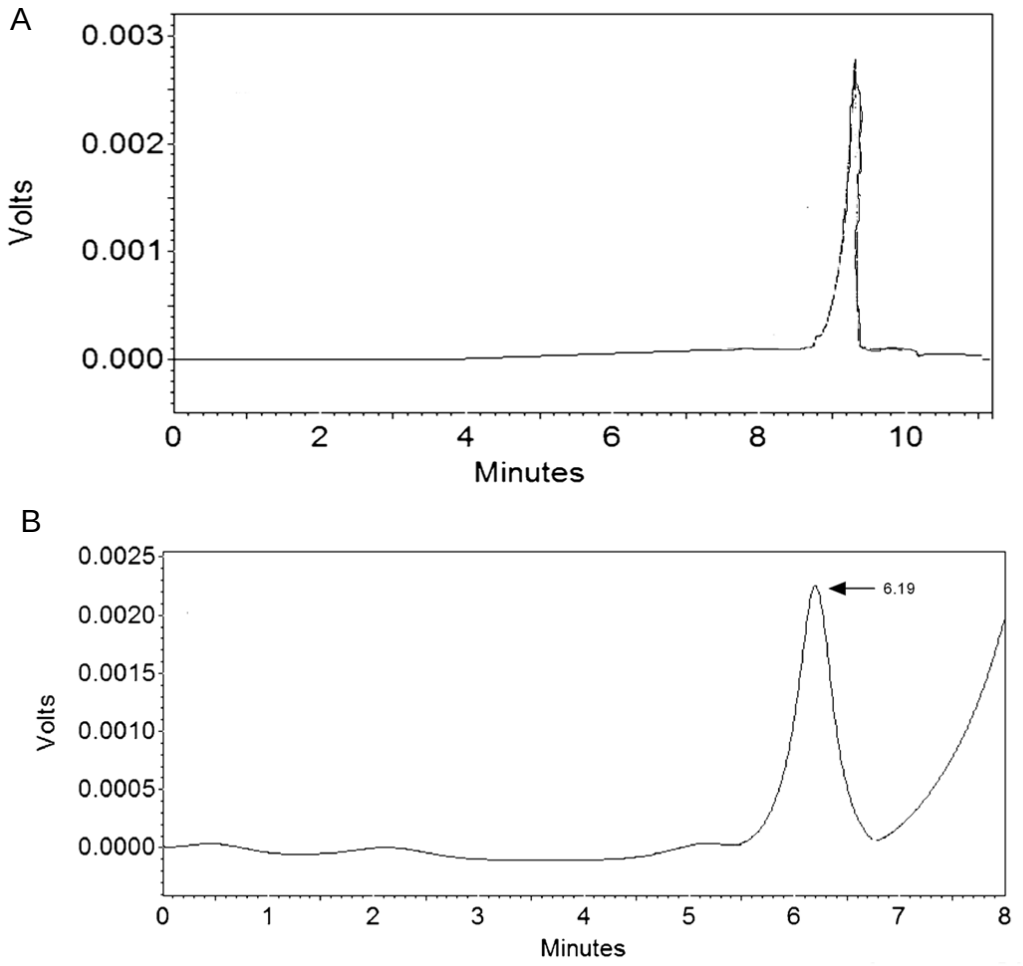

Figure 3. Chromatograms of blank microcapsules sample (A) and FBP-loaded microcapsules sample (B), after treatment. Chromatographic conditions: Bio-Rad Aminex® HPX-87C column, 250 x 4.6 mm, mobile phase: ultrapurified water, 0.6 mL/min.

Linearity

Analytical curves for FBP were fitted by plotting concentration *versus* the corresponding peak area. Satisfactory linearity was detected in the 50 – 500 μg/mL range. The representative linear equation was y = 194.0x - 679.2, with a highly significant correlation coefficient (r = 0.9999) for the method. The detection and quantitation limits were calculated based on the standard deviation of the peak area and the slopes of the calibration curves. The values were found to be 1.98 and 6.60 μg/mL, respectively, indicating the good sensitivity of the method.

Precision and accuracy

The precision of the method was determined by repeatability (intra-assay) and intermediate precision (inter-assay), and shown as the relative standard deviation (R.S.D) in Table 1. The intra- and inter-assay precisions presented R.S.D. values lower than 14%. Considering that regulatory agency[15] recommended that the precisions should be up to 15%, and seeing the complexity of the sample, values obtained by the HPLC method are acceptable for both intra and inter-day evaluations. The accuracy of the method was determined and the mean recoveries for 100, 300 and 500 μg/mL were 90.33%, 96.21% and 78.78%, respectively.

Table 1. Intra-assay and inter-assay precision for HPLC determination of FBP-loaded microcapsule samples

Concentration (μg/ml)	Precision	
	RSD[a] (Intra-assay)	RSD[a] (Inter-assay)
100	1.70 – 8.50	13.21
150	2.60 – 6.88	7.41
200	1.31 – 5.46	5.31

[a]RSD: Relative standard deviation.

Robustness

In robustness tests, the significant variables were related to microcapsule samples preparation, especially the large effect of ethanol concentration (p: 0,00007). The buffer acetate pH used in treatment of samples was also significant (p: 0,022); while the temperature of column oven has no presented significant effect in studied variation range (p: 0,86).

Conclusions

Concluding, the present work proposed a reliable HPLC technique useful for routine quality control for FBP-loaded microcapsules evaluation. The treatment of microcapsule samples ethanol and acetate buffer enabled quantification of FBP.

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References

1. Planas, M. E.; Sánchez, S.; González, P.; Rodrigues de Oliveira J, Bartrons R. *Eur J Pharmacol.* **1993**, *237*, 251.

2. Calafell, R.; Boada, J.; Santidrian, A. F.; Gil, J.; Roig, T.; Perales, J. C.; Bermudez, J. *Eur J Pharmacol.* **2009**, *610*, 128.

3. Azambuja, A. A.; Lunardelli, A.; Nunes, F. B.; Gaspareto, P. B.; Donadio, M. V.; Poli de Figueiredo, C. E.; de Oliveira, J. R. *Inflammation*. **2011**, *34*, 67.

4. de Oliveira, L. M.; Pires, M. G.; Magrisso, A. B.; Munhoz, T. P.; Roesler, R.; de Oliveira, J. R. *J Thromb Thrombolysis*. **2010**, *29*, 387.

5. Valerio, D. A.; Ferreira, F. I.; Cunha, T. M.; Alves-Filho, J. C.; Lima, F. O.; De Oliveira, J. R.; Ferreira, S. H.; Cunha, F. Q.; Queiroz, R. H.; Verri, W. A. Jr. *Br J Pharmacol*. **2009**, *158*, 558.

6. Kim, K. H.; Cho, S. A.; Lim, J. Y.; Lim, D. G.; Moon, C.; Jeong, S. H. *Arch Pharm Res.* **2014**, *37*, 1570.

7. Rattes, A. L. R.; Oliveira, W. P. *Powder Technol.* **2007**, *171*, 7.

8. Marona, H. R.; Schapoval, E. E. *J Pharm Biomed Anal.* **1999**, *20*, 413.

9. Rozet, E.; Ceccato, A.; Hubert, C.; Ziemons, E.; Oprean, R.; Rudaz, S.; Boulanger, B.; Hubert, P. J Chromatogr A. **2007**, *1158*, 111.

10. Freitas, O.; Mestriner-Jr, W.; Conceição, E. C.; Baracat, M. M.; Moreschi, P. E. *Br PI 0700557-1*, **2007**.

11. Baracat, M. M.; Nakagawa, A. M.; Casagrande, R.; Georgetti, S. R.; Verri, W. A. J.; de Freitas, O. *AAPS PharmSciTech*. **2012**, *13*, 364.

12. Guazelli, C. F.; Fattori, V.; Colombo, B. B.; Georgetti, S. R.; Vicentini, F. T.; Casagrande, R.; Baracat, M. M.; Verri, W. A. Jr. *J. Nat. Prod.* **2013**, *76*, 200.

13. ICH steering committee international conference on harmonization of technical requirements for registration of pharmaceuticals for human use, validation of analytical procedures: text and methodology. Geneva, **2005**, 13.

14. Statsoft; STATISTICA – Data analysis software system. Versão 7.1, Statsoft Inc., 2005.

15. Agência Nacional de Vigilância Sanitária (ANVISA), Resolução RE n^{o} 899, 29/05/2003.

4.2 Microencapsulation improves the analgesic and anti-inflammatory efficacy of fructose-1,6-bisphosphate.

Journal of Pharmacy and Pharmacology

Daniela C. Medeiros[1], Sandra S. Mizokami[1], Josiane A. Vignoli[2], Maria A. Celligoi[2], Sandra R. Georgetti[3], Flávia I. S. Ferreira[4], Rubia Casagrande[3], Alexandre Urbano[4], Marcela M. Baracat[2], Waldiceu A. Verri Jr[1].

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Publicação relacionada: Depósito de patente BR 10 2013 032 152 4 – ANEXO A

Microencapsulation improves the analgesic and anti-inflammatory efficacy of fructose-1,6-bisphosphate.

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Running head: Efficacy of fructose-1,6-bisphosphate microcapsule

Abstract

Objectives Improve the analgesic and anti-inflammatory effects of fructose-1,6-bisphosphate (FBP) by modulating its release.

Methods Microcapsules were prepared by coacervation, particle size was evaluated by light scattering, encapsulation efficiency was quantified by HPLC, dissolution study was performed according USP specifications, the analgesic and anti-inflammatory effects were performed in carrageenan-induced paw inflammation in mice, crystallinity was investigated by differential scanning calorimetric (DSC) and X Ray diffraction.

Key findings Microcapsules presented spherical shape and homogeneous size with an average of 2650 nm. Microencapsulation efficiency was 25%. FBP was immediately available when dissolved in water while FBP was gradually released from microcapsules reaching maximal levels after 45 min demonstrating its prolonged release. FBP-loaded microcapsules showed greater analgesic efficacy (62%) than non-microencapsulated FBP (44%) although there was no statistical difference. Importantly, FBP-loaded microcapsules reduced by 59% carrageenan-induced paw edema with significant statistical differences compared to non-microencapsulated FBP (17%). DSC and X Ray diffraction analysis showed that FBP form was changed from crystalline to amorphous state by microencapsulation process.

Conclusions FBP microencapsulation prolonged its release and changed its form from crystalline to amorphous state explaining the increase of analgesia and anti-inflammatory efficacy of FBP. Therefore, microencapsulation can be used to successfully improve FBP therapeutic efficacy.

Keywords: Delivery systems, microcapsules, Fructose-1,6-bisphosphate, inflammation, pain.

Introduction

The development of novel approaches of drug release are widely applied in pharmacotherapy since improve their biopharmaceutical profile. The achievement of more constant and longer plasma profiles has proved to be important characteristics for adherence of the patient to treatment and therapeutic efficacy.[1] Multiparticulate systems such as microcapsules and microspheres have been developed to control drug release applying varied polymeric matrices and methodologies. Microcapsules are formed by covering droplets, dispersions or small solid particles by a layer or a polymer wall[2] while microspheres are particles of spherical shape in which drugs are found dissolved or scattered in a matrix.[3] Microparticles size varies between 10 μm and 2500 μm.[3, 4]

The release mechanism from microcapsules involves processes of diffusional nature and/or enzymatic degradation of the polymer net.[5-9] Microencapsulation is a versatile process allowing the controlled release of varied drugs by optimizing physicochemical character of the polymer and drug as well as using suitable preparation techniques to each drug.[10-12]

Fructose-1,6-bisphosphate (FBP) is a high energy intermediate of glycolytic pathway with many pharmacological activities including reduction of tissue damage associated with ischemia, shock and toxic injury;[13] prevention of inflammation induced by carrageenan in rats;[13] preservative effect of myocardial cells;[14] hepatocyte protection against galactosamine-induced cell damage;[15] protective effect over renal function and renal parenchyma in a rat experimental model of cisplatin-induced nephrotoxicity;[16] inhibition of platelet aggregation induced by adenosine diphosphate and ameliorates coagulation alterations in experimental sepsis in rats.[17] These effects support the therapeutic potential of FBP.[18]

Analgesia is an important activity of FBP, which has been described in a model of carrageenan-induced paw inflammation.[19] In this model, the analgesic effect of FBP was

related to increasing serum levels of adenosine, which acts peripherally at neuronal adenosine A1 receptors to induce analgesia.[19, 20] Furthermore, FBP did not affect carrageenan-induced production of the hyperalgesic cytokines and neutrophil recruitment, disproving these would be mechanisms underlying its analgesic effect.[19] In this previous study, we used 15 min of pretreatment with FBP because longer pretreatment time lessened the analgesic effect of FBP. A probable explanation for the influence of time of pretreatment would be that bolus administration of FBP rapidly increases adenosine levels, however, adenosine deaminase degrades adenosine.[21] Therefore, an increase of adenosine at a single time point would not allow binding of all adenosine molecules to adenosine receptors due to its degradation. In agreement with this possibility, treatment with adenosine deaminase reversed the anti-inflammatory effects of FBP demonstrating its activity is sensible to the activity of this enzyme.[22] Therefore, we reason that prolonging the release of FBP could contribute to improve its analgesic and anti-inflammatory efficacy by allowing more adequate timing of FBP-induced increase of adenosine levels and activation of adenosine A1 receptors.

In view of the information presented above, microcapsules containing FBP were developed and characterized aiming to improve the analgesic and anti-inflammatory efficacy of FBP.

Materials and methods

Materials

Materials were obtained from the indicated sources**:** Pectin USP (68% of esterification) from CPKelco (Limeira, SP, Brazil); casein from Kauffman & Co (Germany); citric acid, sodium hydroxide, chloride acid and potassium phosphate from Merck (Darmstadt, Hessen, Germany); tween 20® from Synth (Diadema, SP, Brazil); carrageenan from Santa Cruz Biotechnology Inc (Dallas, Texas, USA) and fructose-1,6-bisphosphate from Sigma (St Louis, MO, USA).

Animals

Male Swiss mice (25-30 g) from Londrina State University were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h, and controlled temperature. Behavioral testing was conducted during the light cycle. Animal care and handling procedures were approved by the Ethics Committee for Animal Use of Londrina State University, process number 3321.2013.29 of March, 12, 2013. Experimenters were blinded to the treatments.

Preparation of microcapsules

Microcapsules were prepared by dispersion in distilled water (solid content 10%, w/v) of the polymers pectin and casein under constant mechanical shaking. Sodium hydroxide (4.0 M) was used to adjust pH to 8.0 $\pm$ 0.1.[8] After complete dispersion, FBP was added at the proportion of 1:5 (drug:polymer). Microcapsules were obtained by slow and gradual reduction of pH from 8.0 $\pm$ 0.1 to 4.0 $\pm$ 0.1 with 1.0 M citric acid. Then, the wall of microcapsules was hardened by adding glutaraldehyde (50 μl/g polymer) with constant shaking for additional 30

minutes (Brazil Patent, PI 0700557-1).[8, 9] The same methodology was used to prepare microcapsules containing no drug (blank microcapsules). Microcapsules were dried in a lyophilizer (Edwards, model Pirani 501) during 24 h.

Scanning electron microscopic study

The microcapsules were coated under argon atmosphere with gold/palladium and examined under a scanning electron microscope (FEI – Quanta 200). Images were obtained at 10000x magnification. The morphology of the particles was evaluated from scanning electron photomicrographs.

Particle size analysis and distribution

The analysis of size and distribution of the particles was performed by light scattering using a Zetasizer Nano system ZS (Malvern Instruments) followed by polydispersity index determination.

Determination of encapsulation efficiency and *in vitro* dissolution studies

To determine the amount of drug loaded in microcapsules, 5 mg of microcapsules were dispersed in 10 ml of ultrapure water and then stirred for 5 min followed by centrifugation at 9000 rpm for 10 min to extract FBP. Supernatant was treated with phosphate buffer pH 4.7 (1:4) and absolute ethanol (1:2), and then filtered through a 0,45 µm HV (Millipore®) PVDF filter and analyzed by HPLC using Bio-Rad Aminex HPX-87^{o}C column with output of the mobile stage (ultrapurified water) of 0.6 ml/min. Twenty minutes long applications were performed and FBP was detected using refractive index detector. The injection volume was 20 µL under a temperature of 80 ^{o}C.

Encapsulation efficiency was calculated using the following equation (1):

Encapsulation efficiency (%EE) = (initial FBP added – free FBP /initial FBP added) x 100 (1)

Dissolution studies of FBP-loaded microcapsules and non-microencapsulated FBP were performed (Erweka DT-6), according to the specifications of United States Pharmacopeia 35th edition[23] with adaptations. Drug release patterns were studied during 4h using USP Apparatus 1 at a rotational speed of 100 rpm, 37°C and amount corresponding to 133 mg of FBP. The ultrapure water was used as dissolution medium. Samples were collected at the indicated time points. Each sample was treated and analyzed by HPLC method in the same conditions of encapsulation efficiency analyses. The results were expressed as mean (± SEM) of three determinations.

Evaluation of therapeutic effect of FBP-loaded microcapsules in carrageenan-induced paw inflammation in mice

The analgesic and anti-inflammatory effects of FBP, blank microcapsules and FBP-loaded microcapsules were compared in the carrageenan-induced paw inflammation model. Mice were treated with FBP (300 mg/kg, per oral),[19] blank microcapsules (equivalent to the amount of FBP-loaded microcapsules) or FBP-loaded microcapsules (amount equivalent to 300 mg of FBP /kg, per oral) 15 min before intraplantar (subcutaneous injection in the plantar face of hind paw) injection of carrageenan solution (300 µg in 25 µl of saline).[24] The negative inflammation control received i.pl. injection of saline (25 µl).

Mechanical hyperalgesia (increased sensitivity to painful mechanical stimulus) was evaluated by an electronic anesthesiometer (Insight®) adapted with a 0.5 mm^2 polypropylene tip.[25] Briefly, the test consisted of evoking a hindpaw flexion reflex with a hand-held force

transducer and end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. Paw edema was determined using an analog caliper.[26] Mice were tested before and after carrageenan i.pl. injection. The results are expressed by delta (Δ) withdrawal threshold (in g) or Δ mm/paw calculated by subtracting the measurements at 1, 3 and 5 h after stimulus from the zero-time measurements. Data are present as mean ± S.E.M. of 6 mice per group per experiment, and experiments were performed twice.

Differential scanning calorimetric analysis (DSC)

The DSC analysis was performed on the FBP, blank microcapsules and FBP-loaded microcapsules. The samples were heated from 30 to 500 ºC at a heat ingrate of 5 ºC/min under nitrogen atmosphere using Shimadzu DSC-60 microcalorimeter.

X Ray diffraction analysis

The X-ray diffraction experiments were performed in a Bruker D8 powder diffractometer using the Bragg–Brentano geometry in a continuous mode with a scan speed of 0.05°/s. A Cu Kα (λ = 1.5405 Å) line focus radiation tube was operated at 40 kV and 30 mA, and diffractions were taken from 5 to 70° (2θ). The pure FBP and FBP-loaded microcapsules crystalline phases were identified using X'Pert HighScore Plus with PDF2DBP analytical database.

Statistical analysis

Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant when $P < 0.05$ was obtained. Statistical analyzes were performed using GraphPad Prism® 4.0 software.

Results

Scanning electron microscopy

Microcapsules were analyzed by scanning electron microscope and the morphology was evaluated through the photomicrographs obtained in magnification of 10000x (Figure 1A, blank microcapsules and figure 1B, FBP-loaded microcapsules). Both microcapsules presented spherical forms with coalescence of particles. **(Include figure 1 here).**

Particle size analysis and distribution

The analysis of size and distribution of the particles was performed by light scattering and the particle size distribution was evaluated by determining polydispersity, which enables the measurement of homogeneity of particles. FBP-loaded microencapsules presented an average diameter of 2650 nm and polydispersity index of 0.023 (Figure 2) indicating homogeneity of particle size. **(Include figure 2 here).**

Determination of encapsulation efficiency and *in vitro* dissolution studies

The microencapsulation efficiency obtained was 25%.

Dissolution test revealed that microencapsulation prolonged the time lapse to achieve maximal concentration of FBP from microcapsules (Figure 3). The peak of FBP concentration was achieved after 45 min with FBP-loaded microcapsules compared to 5 min (this was the first time point analyzed) when using FBP powder. This result demonstrates that microencapsulation prolonged FBP release to the dissolution medium **(Include figure 3 here).**

Evaluation of therapeutic effect of FBP-loaded microcapsules in carrageenan-induced paw inflammation in mice

Mice were treated with FBP-loaded microcapsules (300 mg/kg, p.o.), FBP (300 mg/kg, p.o.) or blank microcapsules 15 min before intraplantar injection of carrageenan (300 µg/paw). Figure 4A shows carrageenan induced significant mechanical hyperalgesia in mice compared to the negative group that received the vehicle of carrageenan (saline) at all the time points evaluated (Figure 4A). Non-microencapsulated FBP reduced carrageenan-induced mechanical hyperalgesia between 1-5 h. FBP-loaded microcapsules significantly inhibited carrageenan-induced mechanical hyperalgesia at all the time points tested (1–5 h). Blank microcapsules did not affect carrageenan-induced mechanical hyperalgesia, therefore this data were not shown in the graph. Importantly, the anti-hyperalgesic effect of FBP-loaded microcapsules was greater than that of non-microencapsulated FBP with inhibition of 62% and 44% at 3h, and 49% and 30% at 5h, respectively (Figure 4A).

Using the same protocol as for Figure 4A, its was observed that FBP-loaded microcapsules reduced carrageenan-induced paw edema at 3 and 5 h after stimulus injection achieving 59% and 57% of anti-edematogenic activity while non-microencapsulated FBP and blank microcapsules did not affect significantly carrageenan-induced paw edema (Figure 4B). **(Include figure 4 here).**

Differential scanning calorimetric (DSC) analysis

The DSC analysis of FBP (Figure 5, line A) showed endothermic events corresponding to a decomposition process at 116°C and 130°C. In the thermogram of FBP-loaded microcapsules (Figure 5, line B) these events were not observed. The thermogram of blank microcapsules (Figure 5, line C) did not evidenced endothermic events at 116°C and 130°C. **(Include figure 5 here)**

X Ray diffraction analysis

The X-ray diffraction experiments were performed to determine whether there would be difference of non-microencapsulated FBP and FBP-loaded microcapsules regarding crystalline and/or amourphous states. Figure 6 shows the X-ray diffraction pattern of the non-microencapsulated FBP (Figure 6, line A) and FBP-loaded microcapsules (Figure 6, line B). The FBP phase was indentified (reference code 00-036-1944) in pure sample. Pure non-microencapsulated FBP diffractogram showed high crystallinity and FBP-loaded microcapsules diffractogram an amourphous phase. The amourphous samples, blank microcapsules (Figure 6, line C) and FBP-loaded microcapsules, showed a typical Gaussian dispersion around 20º (2θ) that can be related to a 4.2 Å of atomic separation average without symmetry. This result is consistent with DSC results that FBP was shifted from crystalline to an amorphous state after microencapsulation. **(Include figure 6 here).**

Discussion

Achieving steady-state drug levels at tissue and blood to grant and improve therapeutic efficacy is a primary goal of novel therapies and pharmaceutical formulation development.[27] Microencapsulation process has been established as a technique to accomplish drug controlled release[28] with a wide range of polymers of which natural polymers have been attracting considerable attention for several years in modified drug release due to being biodegradable polymers.[29] In the present study, FBP-loaded microcapsules were prepared using biodegradable polymers by complex coacervation method. FBP-loaded microcapsules were homogeneous, and importantly, microencapsulation delayed FBP release to dissolution medium and shifted FBP form from crystalline to amorphous state, which together explain the better analgesic and anti-inflammatory therapeutic profile observed with FBP-loaded microcapsules comparing to non-microencapsulated FBP.

Microencapsulation by complex coacervation consists of processes in which drugs in form of small particles or droplets are surrounded by coating with homogeneous or heterogeneous matrix, generally of polymeric materials, forming capsules at micrometer size range.[30] FBP-loaded microcapsules presented spherical forms with microparticle coalescence forming polymer plate. Drying process can be an explanation for the coalescence of microcapsules.[31] In fact, microcapsules prepared with the same polymer (pectin/casein) and dried by spray drying did not present coalescence.[31] Nevertheless, spray drying was not a suitable drying process since the melting temperature of FBP is 116^{o}C [32] and spray drying uses 180^{o}C.[8]

The average diameter of the FBP-loaded microcapsules was 2650 nm with polydispersity index of 0.023. Many factors can affect the size and distribution of microcapsules, such as drying process and characteristics of the encapsulated drug.[33] The

incorporation of poorly soluble drugs (indomethacin) increased the mean diameter in spray-dried microparticles to a greater degree than did the incorporation of the water soluble drug (acetaminophen).[33] Therefore, it is conceivable that drying process and high solubility of FBP might explain the small particle size.

In general, high encapsulation efficiency with water-soluble molecules is a difficult task to achieve due to the rapid diffusion of water-soluble drugs from the hydrophobic phase into the aqueous phase during the preparation of microparticles.[34] In fact, soluble drug entrapment efficiency normally leads to low amount of the microencapsulated drug.[34-36] Therefore, the encapsulation efficiency of 25% for FBP was reasonable.

The *in vitro* dissolution studies for FBP-loaded microcapsules demonstrated that microencapsulation did not allow an immediate rise of dissolved FBP levels as observed with non-microencapsulated FBP. Maximal FBP concentration was achieved after 45 min while non-microencapsulated FBP was already dissolved in the beginning of testing. Therefore, despite the high water solubility of FBP, microencapsulation was able to effectively control its release. The microencapsulation process used in the present study was previously shown to effectively microencapsulate acetaminophen[8] further corroborating its applicability to control the release of highly soluble drugs. The delayed or prolonged drug release is also attributed to the low solubility of pectin/casein complex in aqueous medium[9, 37] as well as by its drug releasing mechanism that depends on gradual swelling of pectin.[38]

The analgesic effect of FBP in the carrageenan-induced paw inflammation was previously demonstrated[19] justifying the selection of this model. Non-microencapsulated FBP reduced carrageenan-induced mechanical hyperalgesia. The analgesic effect of FBP-loaded microcapsules was slightly higher than of non-microencapsulated FBP at 3 and 5 h after carrageenan stimulus. FBP-loaded microcapsules also reduced carrageenan-induced paw

edema with statistically significant higher anti-edematogenic effect than non-microencapsulated FBP at 3 and 5 h. This result is a clear demonstration that the microencapsulation was effective in enhancing the activity of FBP, at least, on edema. Of note edema is an important clinical sign present in most of inflammatory diseases. In fact, it was noticeable that a higher analgesic and anti-inflammatory effect was achieved with FBP-loaded microcapsules than with bolus treatment with non-microencapsulated FBP. FBP induces increase of adenosine levels[19] and in turn adenosine mediates the analgesic and anti-inflammatory effects of FBP.[19, 22] Adenosine activates its A1 receptors[19] triggering an antinociceptive cascade comprising nitric oxide/ cyclic guanosine monophosphate/ protein kinase G/ and ATP-sensitive K+ channels.[20] Adenosine is metabolized by adenosine deaminase[21] and therefore, bolus administration or massive increases of its levels do not allow the best kinetics of adenosine receptor occupancy. In fact, treatment with adenosine deaminase reversed the anti-inflammatory effects of FBP.[22] In this sense, slower and gradual increase of adenosine levels by FBP-loaded microcapsules administration would improve the kinetics of occupancy of adenosine receptors diminishing the role of adenosine metabolism prior to receptor binding.

However, a better releasing profile seems not to be the solely explanation for the enhanced analgesic and anti-inflammatory effect of FBP-loaded microcapsules compared to non-microencapsulated FBP since both presented activity within 1 h. In this sense, the DSC curves of FBP were consistent with the literature of D-fructose[32,39] showing endothermic events corresponding to a decomposition process at 116°C and 130°C, probably indicating the melting point of 116°C. On the other hand, FBP-loaded microcapsules did not present endothermic events indicating FBP was probably dispersed or covered in the polymer matrix of pectin/casein with amorphous property. The amorphous state of FBP in microcapsules may be related to an interaction with the polymers.[40] These results are consistent with literature

describing that the crystalline form of a drug can be modified during the microencapsulation process to amorphous state.[41] X Ray diffractogram corroborates FBP changed from crystalline to amorphous form by microencapsulation. Crystalline FBP is highly water soluble presenting high polarity and low lipophilicity decreasing its permeability. In fact, FBP is taken up by varied cell types, but FBP administration using lipossomes increases its permeability.[42, 43] Therefore, amorphous state of FBP-loaded microcapsules favored its absorption and enhanced its analgesic and anti-inflammatory effects in the carrageenan paw inflammation model together with FBP prolonged release.

Conclusions

In present study, pectin/casein microcapsules containing FBP were prepared using complex coacervation method. Microcapsules presented, predominantly, spherical shape and uniform distribution of particle size. The *in vitro* dissolution test demonstrated prolonged FBP release from microcapsules compared to non-microencapsulated FBP. DSC and X Ray diffraction analysis revealed FBP changing from crystalline to amorphous state after microencapsulation. As a result of prolonged release and changing from crystalline to amorphous state of FBP-loaded microcapsules, there was an improvement of FBP analgesic and anti-inflammatory effects in the carrageenan model of inflammation. Therefore, microencapsulation with pectin-casein polymer by complex coacervation is a promising strategy to improve the therapeutic effects of FBP.

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References

1. Rattes ALR, Oliveira WP. Spray drying conditions and encapsulating composition effects on formulation and properties of sodium diclofenac microparticles. *Powder Technol.* 2007; 171(1):7-14.

2. Deasy PB. *Drug and The Pharmaceutical Sciences: Microencapsulation and Related Drug Process.* New York: Marcel Dekker, 1984.

3. Pristas LN et al. *Tecnologia Farmacêutica.* 5rd ed. Lisboa: Fundação Calouste Gulbenkian, 1996.

4. Rinaldi APT et al. Preparo e caracterização de micropartículas de acetobutirato de celulose e poli(3-hidroxibutirato) contendo piroxicam. *ActaSciHealthSci.* 2009;31(1):51-56.

5. Linhard R. *Biodegradable polymers for controlled release of drugs. In: Rosoff M. Controlled Release of Drugs: Polymers and aggregate systems.* New York: VCH Publisher Inc, 1988. p.53-85.

6. Batycky RP et al. A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. *J. Pharm. Sci*. 1997;86(12):1464-1477.

7. Morais WA et al. Encapsulation and release characteristics of DCTN/PLGA microspheres. *J. Microencapsulation*. 2009;26(6):529-534.

8. Baracat MM et al. Preparation and characterization of microcapsules based on biodegradable polymers: pectin/casein complex for controlled drug release systems. *AAPS PharmSciTech*. 2012;13(2):364-372.

9. Guazelli CF et al. Quercetin-loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms. *J. Nat. Prod.* 2013;76(2):200-208.

10. Bagheri-Khoulenjani S et al. Particle size modeling and morphology study of chitosan/gelatin/nanohydroxyapatite nanocomposite microspheres for bone tissue engineering. *J. Biomed. Mater. Res., Part A*. 2013;101(6):1758-1767.

11. Arora S, Budhiraja RD. Chitosan-alginate microcapsules of amoxicillin for gastric stability and mucoadhesion. *J. Adv. Pharm. Technol. Res.* 2012;3(1):68-74.

12. Patel AS et al. Effects of spray drying conditions on the physicochemical properties of the Tramadol-Hcl microparticles containing Eudragit((R)) RS and RL. *J Pharm Bioallied Sci*. 2012;4(Suppl 1):S50-53.

13. Planas ME et al. Protective effect of fructose 1,6-bisphosphate against carrageenan-induced inflammation. *Eur J Pharmacol*. 1993;237(2-3):251-255.

14. Markov AK. Hemodynamics and metabolic effects of fructose 1-6 diphosphate in ischemia and shock--experimental and clinical observations. *Ann Emerg Med*. 1986;15(12):1470-1477.

15. De Oliveira JR et al. Effect of galactosamine on hepatic carbohydrate metabolism: protective role of fructose 1,6-bisphosphate. *Hepatology*. 1992;15(6):1147-1153.

16. Azambuja AA et al. Effect of fructose-1,6-bisphosphate on the nephrotoxicity induced by cisplatin in rats. *Inflammation*. 2011;34(1):67-71.

17. de Oliveira LM et al. Fructose-1,6-bisphosphate inhibits in vitro and ex vivo platelet aggregation induced by ADP and ameliorates coagulation alterations in experimental sepsis in rats. *J Thromb Thrombolysis*. 2010;29(4):387-394.

18. Calafell R et al. Fructose 1,6-bisphosphate reduced TNF-alpha-induced apoptosis in galactosamine sensitized rat hepatocytes through activation of nitric oxide and cGMP production. *Eur J Pharmacol*. 2009;610(1-3):128-133.

19. Valerio DA et al. Fructose-1,6-bisphosphate reduces inflammatory pain-like behaviour in mice: role of adenosine acting on A1 receptors. *Br J Pharmacol*. 2009;158(2):558-568.

20. Lima FO et al. Direct blockade of inflammatory hypernociception by peripheral A1 adenosine receptors: involvement of the NO/cGMP/PKG/KATP signaling pathway. *Pain*. 2010;151(2):506-515.

21. Golembiowska K et al. Modulation of adenosine release from rat spinal cord by adenosine deaminase and adenosine kinase inhibitors. *Brain research*. 1995;699(2):315-320.

22. Sola A et al. Fructose-1,6-biphosphate and nucleoside pool modifications prevent neutrophil accumulation in the reperfused intestine. *J Leukoc Biol.* 2003;73(1):74-81.

23. *The United States Pharmacopeia. 35th revision. The National Formulary 30th edition.* Rockville, MD: The United States Pharmacopiel Convention; 2012.

24. Possebon MI et al. Pimaradienoic acid inhibits inflammatory pain: inhibition of NF-κB activation and cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. *J Nat Prod.* 2014;77(11):2488-96.

25. Cunha TM et al. An electronic pressure-meter nociception paw test for mice. *Braz J Med Biol Res*. 2004;37(3):401-407.

26. Zarpelon AC et al. IL-33/ST2 signalling contributes to carrageenin-imduced innate inflammation and inflammatory pain: role of cytokines, endothelin-1 and prostaglandin E2. *Br J Pharmacol*. 2013;169:90-101.

27. Abdelbary A et al. Microencapsulation Approach for Orally Extended Delivery of Glipizide: In vitro and in vivo Evaluation. *Indian J Pharm Sci*. 2012;74(4):319-30. doi: 10.4103/0250-474X.107063.

28. Chowdary KP, Rao YS. Design and in vitro and in vivo evaluation of mucoadhesive microcapsules of glipizide for oral controlled release: a technical note. *AAPS PharmSciTech*. 2003;4(3):E39.

29. Jones OG et al. Effect of Polysaccharide Charge on Formation and Properties of Biopolymer Nanoparticles Created by Heat Treatment of β-Lactoglobulin-Pectin Complexes. *Food Hydrocoll.* 2010;24:374-83.

30. Verri WA Jr et al. *Studies in Natural Products Chemistry*. In: Atta-ur-Rahman, editor. Elsevier Amsterdam. 2012.

31. Baracat MM et al. Microcapsule Processing in a Spouted Bed. *Can J Chem Eng*. 2004;82:134-41.

32. Wungtanagorn R, Schmidt SJ. Phenomenological study of enthalpy relaxation of amorphous glucose, fructose, and their mixture. *Thermochim Acta*. 2001; 369(1-2): 95-116.

33. Marreto RN et al. Impact of cross-linking and drying method on drug delivery performance of casein-pectin microparticles. *AAPS PharmSciTech*. 2013;14(3):1227-1235.

34. Palamoor M, Jablonski MM. Poly(ortho ester) Nanoparticle-Based Targeted Intraocular Therapy for Controled Release of Hidrophilic Molecules. *Mol Pharm*. 2013;10:701-708.

35. Chitkara D, Kumar N. BSA-PLGA-based core-shell nanoparticles as carrier system for water-soluble drugs. *Pharm Res*. 2013;30(9):2396-2409.

36. Shah M et al. Solid lipid nanoparticles of a water soluble drug, ciprofloxacin hydrochloride. *Indian J Pharm Sci*. 2012;74(5):434-442.

37. Dong ZJ et al. Optimization of cross-linking parameters during production of transglutaminase-hardened spherical multinuclear microcapsules by complex coacervation. *Colloids Surf B Biointerfaces*. 2008;63(1):41-47.

38. Kim TH et al. Release of albumin from chitosan-coated pectin beads in vitro. *Int J Pharm*. 2003;250(2):371-383.

39. Hurtta M et al. Melting behaviour of D-sucrose, D-glucose and D-fructose. *Carbohydr Res.* 2004;339(13):2267-2273.

40. Medeiros DC et al. Potential use of Cellulose Acetate Butyrate and Pluronic F68 Blends in the Modulation of the Diclofenac Sodium Release from Microspheres. *Lat. Am. J. Pharm*. 2011; 30(9): 1792-7.

41. Kim KH et al. Preparation of microcapsules with the evaluation of physicochemical properties and molecular interaction. *Arch Pharm Res.* 2014.

42. Wheeler TJ et al. Permeability of fructose-1,6-bisphosphate in liposomes and cardiac myocytes. *Mol Cell Biochem.* 2004;259(1-2):105-114.

43. Martinez MN, Amidon GL. A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals. *J Clin Pharmacol.* 2002;42(6):620-643.

Figure legends

Figure 1. Photomicrographs of microcapsules obtained by scanning electron microscopy, increase of 10000x (A – blank microcapsules and B – FBP-loaded microcapsules).

Figure 2. Particle size distribution of FBP-loaded microcapsules.

Figure 3. In vitro dissolution profile of FBP-loaded microcapsules. The ultrapure water was used as dissolution medium. In all cases, mean values ± SEM (n = 3) are presented.

Figure 4. Microencapsulation of FBP increases its analgesic and anti-inflammatory effects. Mice received per oral treatment with FBP-loaded microcapsules (300 mg of FBP/ kg) or non-microencapsulated FBP (300 mg of FBP/ kg) 15 min before intraplantar stimulus with carrageenan (300 µg/paw). Mechanical hyperalgesia (A) and paw edema (B) were evaluated prior stimulus injection (base line values) and at indicated time points after carrageenan injection. Blank microcapsules did not affect carrageenan-induced mechanical hyperalgesia and edema, therefore this result was not shown in the figure. Results are presented as means ± S.E.M. of 6 mice per group per experiment and are representative of two separated experiments. One-way ANOVA followed by Tukey´s multiple comparison test. * P <0.05 compared to saline group, # P <0.05 compared with untreated carrageenan group; $^{\#\#}$ P <0.05 compared to carrageenan group and FBP group.

Figure 5. Differential scanning calorimetric analysis thermograms of non-microencapsulated FBP (A), FBP-loaded microcapsules (B) and blank microcapsules (C).

Figure 6. X-ray powder diffraction pattern of non-microencapsulated FBP (A), FBP-loaded microcapsules (B) and blank microcapsules (C).

Figure 1

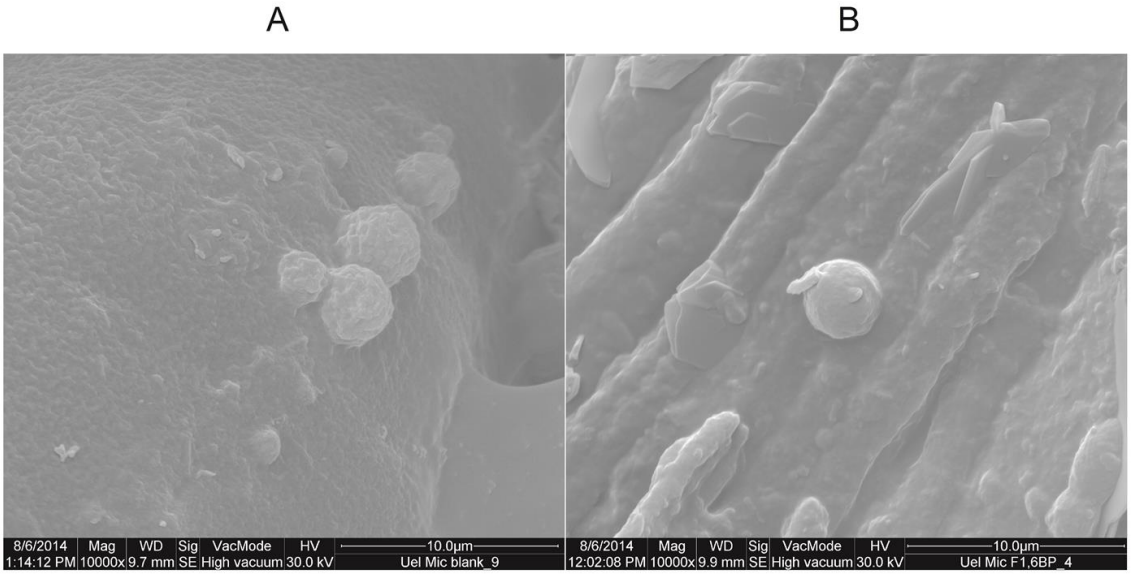


Figure 2

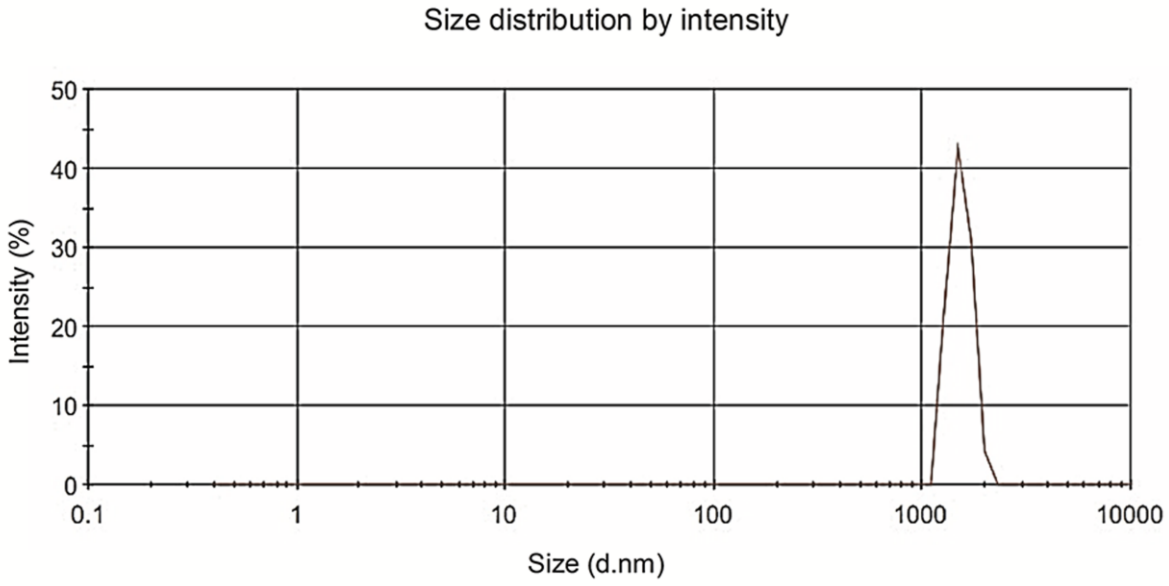


Figure 3

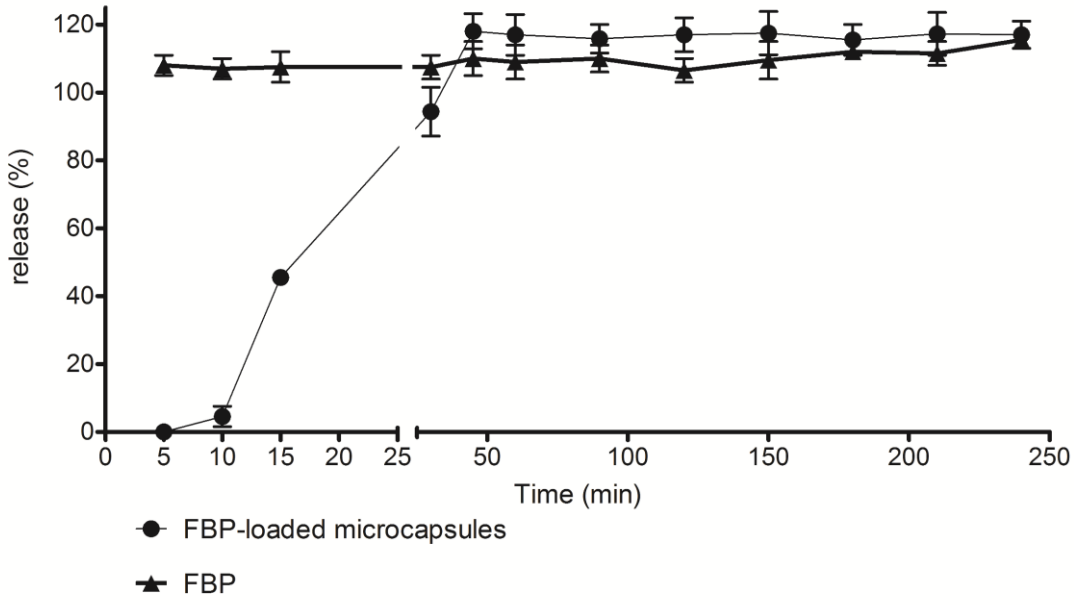


Figure 4

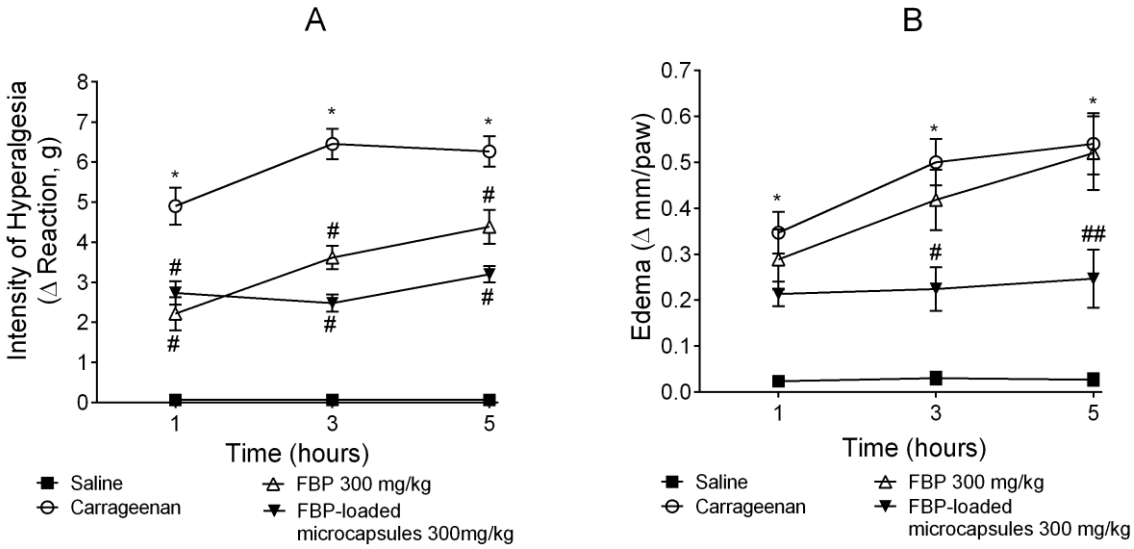


Figure 5

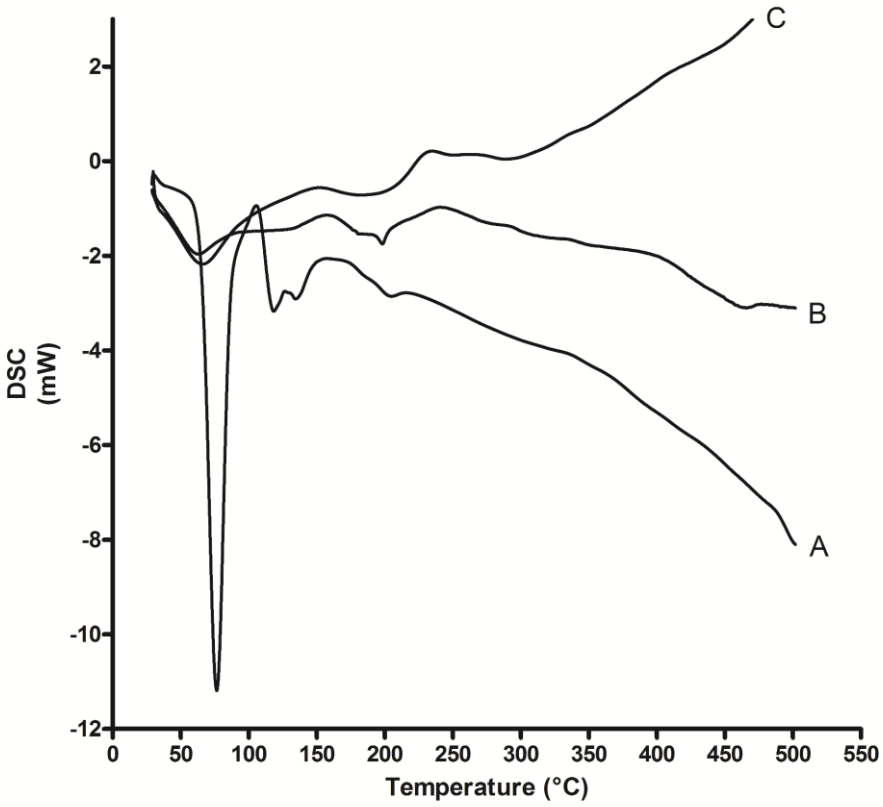
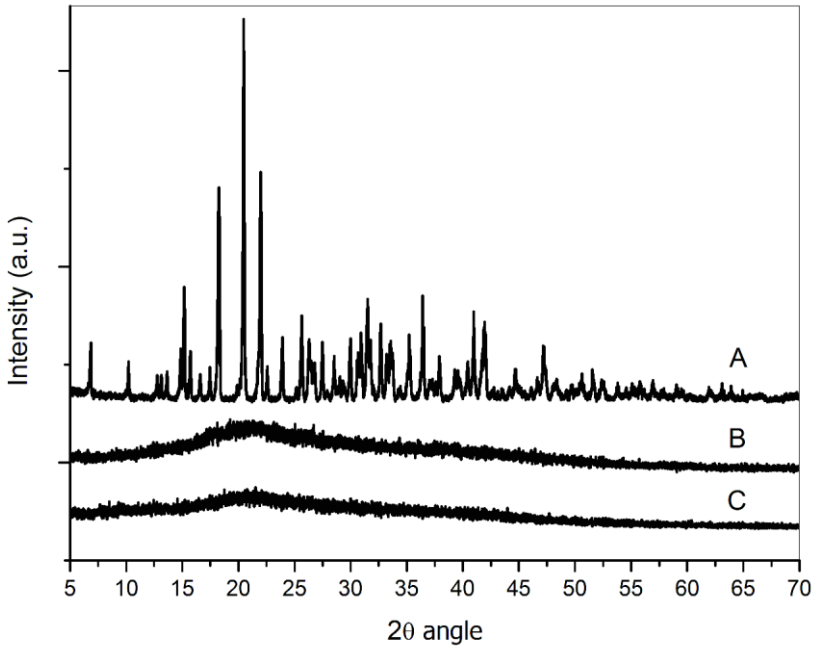


Figure 6



4.3 Preparation and characterization of microcapsules containing rutin: improvement of the analgesic efficacy in mice

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Publicação relacionada: Depósito de patente BR 10 2013 015 911 5 – ANEXO B

Preparation and characterization of microcapsules containing rutin: improvement of the analgesic efficacy in mice

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Running head: Microencapsulation improves efficacy of rutin

ABSTRACT

The aim of this study was to develop and characterize microcapsules containing rutin to improve its analgesic activity. Rutin-loaded microcapsules were obtained by complex coacervation physicochemical method, with an average particle size of 4.903 μm, spherical and of homogeneous size. The encapsulation efficiency was 76.9% quantified by the antioxidant activity. *In vivo*, rutin-loaded microcapsules showed greater efficacy (64%) than non-microencapsulated rutin (28%) to inhibit carrageenan-induced mechanical hyperalgesia. The X Ray diffraction shows that rutin was dispersed in an amorphous matrix and its cristalographic structure and crystal size did not exhibit changes. Differential scanning calorimetric studies confirmed that rutin was dispersed in amorphous matrix in microcapsules. The process was successful to obtain rutin-loaded microcapsules with improved analgesic activity compared to non-microencapsulated rutin.

Keywords: Delivery systems, microcapsules, rutin, pain

INTRODUCTION

Developing novel pharmaceutical forms of drug delivery to improve the therapeutic profile of medication is crucial (1). Research advances in drug delivery systems (DDS) are directly related to recognition of clinical and therapeutic improvements (2). The rational design of DDS is a crucial step to improve drug release, pharmacokinetic, absorption sites, and therefore, obtaining appropriate clinical profile (3). In general, DDS lead to reduced dosage or frequency of administration, reflecting positively on the therapeutic efficacy and adherence to treatment (4, 5), reducing the toxicity and adverse effects, and enabling novel therapeutic regimens (6).

Some strategies to modify the release of drugs include drug delivery in a polymer matrix, chemical bonding to ion exchange resins, incorporation in osmotic pump and use of coating monolithic or multiparticulate systems such as microcapsules (7). The development of modified release systems depends on polymers with appropriate physicochemical characteristic and on effective and economically viable large-scale processes. There is strong tendency of applying biodegradable polymers and preparations in aqueous medium (8, 9). Among these systems, microparticulate systems are useful to control and/or obtain site-specific release (10).

In general, microcapsule comprise layers of polymer ranging from 1 μm to 1 mm that act as protective film for the active molecule (11). This polymer layer crumbles under specific conditions such as enzyme action, pH and contact time with the gastrointestinal environment, allowing the release of the active molecule on the site and/or appropriate moment (12-15).

Microencapsulation by complex coacervation is accomplished by phase separation of hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media (16). Microcapsules produced by coacervation are water insoluble, possessing excellent

controlled release characteristics and heat resistant properties (17). The factors influencing the rate of release include drug particle size, loading, drug solubility and molecular weight and polymer composition (18).

Rutin is a flavonoid found in many dietary sources such as onion, grape, apple, tomato and drinks such as wine and black tea (19). Several investigations have been reporting on the multiple pharmacological activities of rutin including antibacterial, antiviral (20), antiprotozoal (21), antitumor (22), antiallergic (23), anti-inflammatory (24), antiplatelets (25), antidiarrhoeal (26), antiulcer (27), antispasmodic (28), antimutagenesis (29), myocardial protecting (30), vasodilator (31) and immunomodulator (23) activities. Its various pharmacological activities suggests its therapeutic usefulness (19, 32).

The present study reports on the development of rutin-loaded microcapsules to modify drug release aiming to increase rutin therapeutic effect using the physicochemical complex coacervation method. Morphological characteristics of microcapsules were determined. The analgesic efficacy of rutin-loaded microcapsules was compared with the non-microencapsulated rutin in a model of inflammatory pain in mice.

MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated sources**:** Pectin USP (68% of esterification) from CPKelco (Limeira, SP, Brazil); casein from Katuffman & Co (Kehl, Baden-Württemberg, Germany); citric acid, sodium hydroxide, chloride acid and potassium phosphate from Merck (analytical grade, Darmstadt, Hessen, Germany); tween 20® from Synth (Diadema, SP, Brazil); carrageenan from Santa Cruz Biotechnology Inc (Dallas, Texas, USA) and rutin from Acrós Organics (Geel, Antwerp, Belgium).

Animals

Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Londrina, Parana, Brazil were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h, and controlled temperature. All behavioral testings was conducted during the light cycle in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee for Animal Use of the Universidade Estadual de Londrina under the process number 3324.2013.58. All efforts were made to minimize the number of animals used and their suffering. Experiments were double blinded.

Preparation of microcapsules

Microcapsules were prepared by dispersion in distilled water (solid content 10%, w/v) of the polymers pectin and casein under constant mechanical shaking. Sodium hydroxide (4.0 M) was used to adjust pH to 8.0 $\pm$ 0.1 (15). After complete dispersion, rutin was added at the proportion of 1:5 (drug:polymer). Microcapsules were obtained by slow and gradual reduction of

pH from 8.0 ± 0.1 to 4.0 ± 0.1 with 1.0 M citric acid. The same methodology was used to prepare microcapsules containing no drug (blank microcapsules). The sample was dried in a lyophilizer (Edwards, model Pirani 501) during 24h.

Scanning electron microscopic study

The microcapsules were coated under argon atmosphere with gold/palladium and examined under a scanning electron microscope (FEI – Quanta 200). Images were obtained at 2500x magnification. The scanning electron photomicrographs (SEM) were evaluated.

Particle size analysis and distribution

The analysis of size and distribution of the particles was performed by light scattering using a Zetasizer Nano system ZS (Malvern Instruments).

Determination of encapsulation efficiency

To determine the encapsulation efficiency, 101.8 mg of microcapsules were dispersed in 10 ml of phosphate buffer pH 6.8 added 5.0% Tween ® 20 and then stirred for 5 min, followed by centrifugation at 3600 rpm for 10 min. The supernatant was filtered through a 0.45 µm nylon syringe filter, and the filtrate was analyzed by DPPH method and quantified spectrophotometrically at 517 nm to determine drug content (33, 34).

Encapsulation efficiency was calculated using the following equation (1):

Encapsulation efficiency (%EE) = (initial drug added – free drug /initial drug added) x 100 (1)

Evaluation of the analgesic effect of rutin-loaded microcapsules in carrageenan-induced paw inflammation in mice

The analgesic effects of rutin, blank microcapsules and rutin-loaded microcapsules were compared in the carrageenan-induced paw inflammation in mice. Mice were treated with rutin (100 mg/kg, per oral), blank microcapsules (equivalent to the amount of rutin-loaded microcapsules) or rutin-loaded microcapsules (100 mg of rutin/kg, per oral) 1 h before intraplantar (i.pl., subcutaneous injection in the plantar face of hind paw) injection of carrageenan solution (300 µg) in saline (25 µl). The negative inflammation control received i.pl. injection of saline (25 µl).

The mechanical hyperalgesia (increased sensitivity to painful mechanical stimulus) was evaluated by an electronic version of von Frey filaments as previously described in detail (35). Briefly, in a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15-30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements at 0.5, 1, 3, 5 and 7h after stimulus from the zero-time mean measurements. Data are present as mean ± S.E.M. of 6 mice per group per experiment, and experiments were performed twice.

Differential scanning calorimetric analysis (DSC)

The DSC analysis was performed on the rutin, blank microcapsules and rutin-loaded microcapsules. The samples were heated from 30 to 500 ◦C at a heat ingrate of 5 ◦C/min under nitrogen atmosphere using a microcalorimeter (Shimadzu DSC-60).

X Ray diffraction analysis

The X-ray diffraction experiments were performed in a Bruker D8 powder diffractometer using the Bragg–Brentano geometry in a continuous mode with a scan speed of 0.05°/s. A Cu Kα (λ = 1.5405 Å) line focus radiation tube was operated at 40 kV and 30 mA, and 2θ angle were taken from 3 to 40°. The crystalline pure rutin and rutin-loaded microcapsules phases were identified using X'Pert HighScore Plus with PDF2DB Panalytical database. Scherrer equation (2) was used to calculate the crystallite size for both samples, taking $\beta_{2\theta\text{-instrumental}}$ as 0.07º (0.0012 rad), using a Lorentz function to fit the peak, and considering form factor as k = 0.9 (36-39). To Lorentz function the $\beta_{2\theta}$ value for Scherrer equation was obtained by subtracting the FWHM of the sample (β_{sample}) from the instrument (β_i), as equation 3.

$$D = \frac{k\lambda}{\beta_{2\theta}\cos\theta} \quad (2)$$

$$\beta_{2\theta} = \beta_s - \beta_i \quad (3)$$

Statistical analysis

Data were statistically analyzed by one-way ANOVA, followed by Tukey´s multiple comparisons test. Differences were considered significant when $P < 0.05$ was obtained. Statistical analyzes were performed using GraphPad Prism® 4.0 software.

RESULTS

Scanning electron microscopy

Blank microcapsules (Figure 1A) and rutin-loaded microcapsules (Figure 1B) presented spherical shape and irregular surface, with clusters of microparticles. Rutin-loaded microcapsules presented drug adsorbed on the external surface of microcapsules.

Particle size analysis and distribution

Particle size distribution of rutin-loaded microcapsules presented an average diameter of 4.903 µm ± 4.421 (Figure 2). Ten percent (10%) of particles presented a diameter of less or equal to 1.535 µm (d10 = 1.535 µm) and 90% of particles presented a diameter of less or equal to 7.462 µm (d90 = 7.462 µm). Blank microcapsules presented an average diameter of 19.15 µm ± 18.04 (data not shown), d10 of 2.098 µm and d90 of 44.23 µm.

Determination of microencapsulation efficiency

The microencapsulation efficiency was determined through the antioxidant activity of the drug, and calculated according to equation 1. Firstly, microcapsules were submitted to extraction followed by antioxidant activity determination by DPPH method. A microencapsulation efficiency of 76.9% was achieved.

Evaluation of therapeutic effect of rutin-loaded microcapsules in carrageenan-induced mechanical hyperalgesia in mice

The evaluation of in vivo efficacy of rutin-loaded microcapsules was carried out in carrageenan-induced paw inflammation model. Mice were treated with rutin (100 mg/kg, per

oral), blank microcapsules (equivalent to the amount of rutin-loaded microcapsules) or rutin-loaded microcapsules (100 mg/kg, per oral) 1 h before intraplantar administration of carrageenan (100 µg/paw diluted in 25 µl of saline). Mechanical hyperalgesia ("pain") was evaluated using an electronic pressure-meter test at indicated time points after carrageenan injection (Figure 3). Carrageenan induced significant mechanical hyperalgesia in mice at all-time points tested peaking at 3 h compared to saline vehicle control group. Blank microcapsules did not affect carrageenan-induced mechanical hyperalgesia, therefore this data were not shown in the graph. Non-microencapsulated rutin reduced carrageenan-induced mechanical hyperalgesia at 3, 5 and 7 h. The analgesic effect of rutin-loaded microcapsules was significant since 0.5 h until 7 h and importantly, this analgesic effect was significantly higher than of non-microencapsulated rutin at 3, 5 and 7h (Figure 3). At the peak of carrageenan-induced mechanical hyperalgesia, rutin-loaded microcapsules produced 64% analgesia compared to 28% analgesia of non-microencapsulated rutin. Thus, rutin-loaded microcapsules presented earlier and higher analgesia compared to non-microenpcasulated rutin.

Differential scanning calorimetric (DSC) analysis

The DSC thermograms for non-microencapsulated rutin (A) and rutin-loaded microcapsules (B) are presented at figure 4. Non-microencapsulated rutin presented the first peak of phase transition at 176^{o}C confirming changing in sample behavior related to the molecular rearrangement of the rutin polymorphic state in a plastic substance (40). The subsequent peaks of phase transition at 214, 232, 240, 248 ^{o}C and at 214 ^{o}C provide evidence for the boiling process. The data obtained in the DSC curve are consistent with those presented by the literature showing rutin decomposition occurring at 214^{o}C (40, 41). However this peak did not appeared in rutin-

loaded microcapsules, suggesting rutin was uniformly dispersed in an amorphous matrix of pectin/casein.

X Ray diffraction analysis

The X-ray diffraction experiments were performed to determine whether there would be a difference of non-microencapsulated and rutin-loaded microcapsules regarding crystal structure and crystallite size. Figure 5 shows the X-ray diffraction pattern of the non-microencapsulated rutin (Figure 5A) and rutin-loaded microcapsules (Figure 5B). The crystalline rutin phase was identified as reference code 00-005-044 in both samples. Rutin-loaded microcapsules peak intensity was lower than pure rutin (e.g. non-microencapsulated rutin) because it is dispersed in an amorphous polymer phase. Crystallite averaged size D was measured using 5.30º and 26.83º (2θ) peaks resulting in a 59.51 ± 10.00 nm for pure rutin and 79.73 ± 10.00 nm for rutin-loaded microcapsules .

DISCUSSION

The main goal of drug delivery research is to develop formulations that fulfill the therapeutic needs related to particular pathological conditions. Technological advancements have brought many innovative drug delivery systems into commercial circulation. Biodegradable and non biodegradable biocompatible microparticulate systems have shown clear advantages by modified drug release, controlled release and drug targeting for parenteral and oral drug delivery (42).

The process of coacervation to obtaining microparticles has been used by many authors (15, 17, 43, 44). For instance, there are reports on obtaining microcapsules of sulfamerazine by pectin-gelatin complex coacervates (44), and we have applied the present methodology to obtain microcapsules containing acetaminophen (15) and quercetin (12). The complex coacervation of casein (protein) and pectin (polysaccharide) occurs by electrostatic interaction between the polysaccharides and the aminogroups of proteins at pH values above the isoelectric point (pI) of polysaccharides (pI_{po}~3.0) and below the pI of the proteins (pI_{pr}~4.55) (9, 10, 45, 46). In the present study, complex coacervation method was successfully applied to obtain pectin/casein microcapsules containing rutin.

Rutin-loaded microcapsules presented spherical forms and irregular surface with clusters of microparticles and drug crystals attached and/or adsorbed on the external surface of microcapsules. Changing in production parameters leads to formation of microparticles with different morphological characteristics (47). The drying process can be an explanation for the coalescence of particles. In fact, microcapsules prepared with the same polymer (pectin/casein) and dried by spray drying did not lead to formation of agglomerated particles (48).

Factors affecting size distribution of microcapsules include core-to-wall ratio (49), stirring speed of the preparation process (49), drying process (42), drug: polymer ratio (50) and homogenization cycles (51). The average diameter of the rutin-loaded microcapsules was 4.903 μm ± 4.421 in the present study. Additionally, the d10 was 1.535 μm, which means that 10% of particles have the diameter lesser than or equal to 1.535 μm; and the d90 was 7.462 μm, therefore 80% of the particles were between 1.535 μm and 7.462 μm. Blank microcapsules presented an average diameter of 19.15 μm ± 18.04, d10 of 2.098 μm and d90 of 44.23 μm, which means that 80% of particles are between 2.098 μm and 44.23μm. The greater homogeneity of size of rutin-loaded microcapsules than of unloaded microcapsules might be related to the presence of the drug. In fact, increase of homogeneity was observed in acetaminophen-loaded microcapsules compared to formulations without acetaminophen (15).

In addition to microcapsules morphology and diameter, microencapsulation efficiency is also an important parameter. Rutin-loaded microcapsules presented a microencapsulation efficiency of 76.9%, which is consistent with the literature and represents a high efficiency rate. For instance, others have obtained 89% methotrexate encapsulation efficiency in microcapsules prepared through biocompatible hyaluronic acid and sodium alginate (52). Similar result was obtained in a study with amoxicillin-loaded microcapsules, in which microcapsules prepared by ionotropic gelation of sodium alginate with chitosan in presence of calcium chloride presented drug entrapment efficiency of 84% (50).

Importantly, microencapsulation significantly increased the analgesic effect of rutin in the carrageenan-induced paw inflammation model. Microencapsulation of quercetin also improved its anti-inflammatory effect in acetic acid-induced colitis, which was attributed to the local delivery of quercetin by intestinal pectinase degradation of pectin/casein microcapsules (12). In fact, microencapsulation can increase the total time of effect. For instance, there was an increase of efficacy and extension of local anesthetic effect of bupivacaine incorporated in biodegradable

microcapsules after subcutaneous infiltration (53). However, the earlier analgesic effect of rutin-loaded microcapsules compared to non-microencapsulated rutin indicated that microencapsulation modified something else in addition to controlling/delaying rutin release and extending its analgesic effect.

A plausible hypothesis for the increase of rutin-loaded microcapsules analgesia compared to non-microencapsulated rutin is the presence of amorphous phase. Differential scanning calorimetric and X Ray diffraction analysis confirm that rutin was dispersed in amorphous polymer matrix in microcapsules. There has been an increase in the number of newly developed drug molecules that exhibit poor water solubility as well as poor bioavailability, rendering necessary to improve the wettability and oral bioavailability of these novel drugs. An efficient approach to improve the wettability of a drug is the physical modifications that increase the surface area by means of particle size reduction (54) or generation of amorphous state drugs (55). Particle size reduction by micronization of drugs increase drug surface area. The rate of dissolution is proportional to the surface area. In case of practically insoluble pharmaceutical compounds or compounds of very low solubility, micronization is not sufficient to achieve the desired blood concentration of drugs. On the other hand, amorphous state substances show higher solubility than the crystalline ones (51). However, crystalline substances are physically more stable compared with the amorphous forms (51). The fact of rutin be dispersed in amorphous matrix in microcapsules seemed to be capable of providing enhanced absorption resulting in improved analgesic efficacy compared to non-microencapsulated rutin**.**

CONCLUSION

The process described in the present study was successful to obtain rutin-loaded microcapsules. Microcapsules were predominantly of spherical form and with uniform particle size distribution. X Ray diffraction studies confirmed that the drug was dispersed in an amorphous matrix keeping its original crystallographic structure. DSC showed that rutin is in fact dispersed in amorphous matrix in microcapsules. The presence of amorphous phase in microcapsules while maintaining drug crystallinity might be suitable to enhance dissolution velocity, providing improved activity compared to the crystalline phase alone**.** In agreement, microencapsulation increased the analgesic efficacy of rutin.

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REFERENCES

1. Rattes ALR, Oliveira WP. Spray drying conditions and encapsulating composition effects on formulation and properties of sodium diclofenac microparticles. Powder Technology. 2007;171(1):7-14.

2. Annual report on drug delivery - Controlling their destiny. Med Ad News. 1996; 15: 1-32.

3. Rubinstein A. Microbially controlled drug delivery to the colon. Biopharm Drug Dispos. 1990;11(6):465-75.

4. Gandhi R, Lal Kaul C, Panchagnula R. Extrusion and spheronization in the development of oral controlled-release dosage forms. Pharm Sci Technol To. 1999;4(2):160-70.

5. Smith A. Drug delivery system in the 20th century: Merely scratching the surface (CR). Pharm Sci Technol To. 1999;2: 225-227.

6. Colombo P, Bettini R, Santi P, Peppas NA. Swellable matrices for controlled drug delivery: gel-layer behaviour, mechanisms and optimal performance. Pharm Sci Technol To. 2000;3(6):198-204.

7. Aulton ME. Delineamento de formas farmacêuticas, 2nd edn. Porto Alegre: Artmed, 2005.

8. Ortiz SEM, Mauri A, Monterrey-Quintero ES, Trindade MA, Santana AS, Favaro-Trindade CS. Production and properties of casein hydrolysate microencapsulated by spray drying with soybean protein isolate. Food Sci Technol. 2009;42: 919-23.

9. Tunçay M, Franssen O, Hennink WE. A novel preparation method for polymeric microparticles without the use of organic solvents. Int J Pharm. 1998;168:1-7.

10. Sopimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. J Control Release. 2001;70:1-20.

11. Herman FM. Microencapsulation. Encyclopedia of Polymer Science and Technology, 3th edn. 2005;12.

12. Guazelli CF, Fattori V, Colombo BB, Georgetti SR, Vicentini FT, Casagrande R, et al. Quercetin-loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms. J Nat Prod. 2013;76(2):200-8. doi: 10.1021/np300670w.

13. Medeiros DC, Mazon-Cardoso T, Lemos-Senna E, Poli A. Comparative in vitro and in vivo evaluations of oral sustained-release formulations of diclofenac sodium in beagle dogs. Die Pharmazie. 2009;64(10):648-52.

14. Medeiros DC, Soldi V, Poli A, Lemos-Senna E. Potential use of Cellulose Acetate Butyrate and Pluronic F68 Blends in the Modulation of the Diclofenac Sodium Release from Microspheres. Lat Am J Pharm. 2011;30(9): 1792-7.

15. Baracat MM, Nakagawa AM, Casagrande R, Georgetti SR, Verri WA, Jr., de Freitas O. Preparation and characterization of microcapsules based on biodegradable polymers: pectin/casein complex for controlled drug release systems. AAPS PharmSciTech. 2012;13(2):364-72. doi: 10.1208/s12249-012-9752-0.

16. Gouin S. Microencapsulation: industrial appraisal of existing technologies and trends. Trends Food Sci. Technol. 2004;15:330-47.

17. Dong ZJ, Xia SQ, Hua S, Hayat K, Zhang XM, Xu SY. Optimization of cross-linking parameters during production of transglutaminase-hardened spherical multinuclear

microcapsules by complex coacervation. Colloids Surf B Biointerfaces. 2008;63(1):41-7. doi: 10.1016/j.colsurfb.2007.11.007.

18. Dai C, Wang B, Zhao H, Li B. Factors affecting protein release from microcapsule prepared by liposome in alginate. Colloids Surf B Biointerfaces. 2005;42(3-4):253-8. doi: 10.1016/j.colsurfb.2004.12.020.

19. Becho JRM, Machado H, Guerra MO. Rutina – Estrutura, Metabolismo e Potencial Farmacológico. Revista Interdisciplinar de Estudos Experimentais 2009;1:21-25.

20. Panasiak W, Wleklik M, Oraczewska A, Luczak M. Influence of flavonoids on combined experimental infections with EMC virus and Staphylococcus aureus in mice. Acta Microbiol Pol. 1989;38(2):185-8.

21. Iwu MM, Obidoa O, Anazodo M. Biochemical mechanism of the antimalarial activity of Azadirachta indica leaf extract. Pharmacol Res Commun. 1986;18(1):81-91.

22. Deschner EE, Ruperto J, Wong G, Newmark HL. Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. Carcinogenesis. 1991;12(7):1193-6.

23. Chen S, Gong J, Liu F, Mohammed U. Naturally occurring polyphenolic antioxidants modulate IgE-mediated mast cell activation. Immunology. 2000;100(4):471-80.

24. Aleksandrov PN, Speranskaia TV, Bobkov Iu G, Zagorevskii VA, Zykov DA. [Effect of rutin and esculamine on models of aseptic inflammation]. Farmakol Toksikol. 1986;49(1):84-6.

25. Swies J, Robak J, Dabrowski L, Duniec Z, Michalska Z, Gryglewski RJ. Antiaggregatory effects of flavonoids in vivo and their influence on lipoxygenase and cyclooxygenase in vitro. Polish journal of pharmacology and pharmacy. 1984;36(5):455-63.

26. Di Carlo G, Autore G, Izzo AA, Maiolino P, Mascolo N, Viola P, et al. Inhibition of intestinal motility and secretion by flavonoids in mice and rats: structure-activity relationships. J Pharm Pharmacol. 1993;45(12):1054-9.

27. La Casa C, Villegas I, Alarcon de la Lastra C, Motilva V, Martin Calero MJ. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. J Ethnopharmacol. 2000;71(1-2):45-53.

28. Mata R, Rojas A, Acevedo L, Estrada S, Calzada F, Rojas I, et al. Smooth muscle relaxing flavonoids and terpenoids from Conyza filaginoides. Planta Med. 1997;63(1):31-5. doi: 10.1055/s-2006-957598.

29. Bear WL, Teel RW. Effects of citrus flavonoids on the mutagenicity of heterocyclic amines and on cytochrome P450 1A2 activity. Anticancer Res. 2000;20(5B):3609-14.

30. Pozin VM, Skuratovskaia SG, Pocheptsova GA. [Changes in the vascular wall and ischemic damages to the myocardium in reversible episodes of heart muscle ischemia]. Fiziol Zh. 1996;42(1-2):10-6.

31. Chung MI, Gan KH, Lin CN, Ko FN, Teng CM. Antiplatelet effects and vasorelaxing action of some constituents of Formosan plants. J Nat Prod. 1993;56(6):929-34.

32. Verri WA, Jr., Vicentini FTMC, Baracat MM, Georgetti SR, Cardoso RDR, Cunha TM, Ferreira SH, Cunha FQ, Fonseca MJV, Casagrande R. In Studies in Natural Products Chemistry; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam. 2012;36(9):297−322.

33. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958; 181(4617): 1199-1200.

34. Casagrande R, Georgetti SR, Verri WA, Jr., Borin MF, Lopez RFV, Fonseca MJV. In vitro evaluation of quercetin cutaneous absorption from topical formulations and its functional stability by antioxidant activity. International Journal of Pharmaceutics. 2007;328:183-190.

35. Cunha TM, Verri WA, Jr., Vivancos GG, Moreira IF, Reis S, Parada CA, et al. An electronic pressure-meter nociception paw test for mice. Braz J Med Biol Res. 2004;37(3):401-7.

36. Gonçalves NS, Carvalho JA, Lima ZM, Sasaki JM. Size–strain study of NiO nanoparticles by X-ray powder diffraction line broadening. Materials Letters. 2012;72:36–38.

37. Weibel A, Bouchet R, Boulc F, Knauth P. The Big Problem of Small Particles: A Comparison of Methods for Determination of Particle Size in Nanocrystalline Anatase Powders. Mater. 2005;17:2378-2385.

38. Burton AW. On the estimation of average crystallite size of zeolites from the Scherrer equation: A critical evaluation of its application to zeolites with one-dimensional pore systems. Microporous and Mesoporous Materials. 2009;117:75-90.

39. Patterson AL. The Scherrer Formula for I-Ray Particle Size Determination. Physical review, Novem Her. 1939;15:56.

40. Costa EM, Barbosa Filho JM, Nascimento TG, Macêdo RO. Thermal characterization of the quercetin and rutin flavonoids. Thermochimica Acta. 2002; 392-393: 79-84.

41. Merck index: an encyclopedia of chemicals, drugs and biological. 13ed. Whitehouse Station: Merck Research Laboratories, 2001.

42. Patel AS, Soni T, Thakkar V, Gandhi T. Effects of spray drying conditions on the physicochemical properties of the Tramadol-Hcl microparticles containing Eudragit® RS and RL. J Pharm Bioallied Sci. 2012;4(Suppl 1):S50-3. doi: 10.4103/0975-7406.94134.

43. Marreto RN, Ramos MF, Silva EJ, de Freitas O, de Freitas LA. Impact of cross-linking and drying method on drug delivery performance of casein-pectin microparticles. AAPS PharmSciTech. 2013;14(3):1227-35. doi: 10.1208/s12249-013-0012-8.

44. McMullen JN, Newton DW, Becker CH. Pectin-gelatin complex coacervates II: Effect of microencapsulated sulfamerazine on size, morphology, recovery, and extraction of water-dispersible microglobules. J Pharm Sci. 1984;73(12):1799-803.

45. Makino K, Fujita Y, Takao K, Kobayashi S, Ohshima H. Preparation and properties of thermosensitive hydrogel microcapsules. Colloids Surf B Biointerface. 2001;21(4):259-63.

46. Xie HQ, Xiang Q. Surface-graft polymerization of doped polyaniline with hydrophilic monomers. Eur Polym J. 2000;36:509-17.

47. Bagheri-Khoulenjani S, Mirzadeh H, Etrati-Khosroshahi M, Shokrgozar MA. Particle size modeling and morphology study of chitosan/gelatin/nanohydroxyapatite nanocomposite microspheres for bone tissue engineering. J Biomed Mater Res Part A. 2013;101(6):1758-67. doi: 10.1002/jbm.a.34481.

48. Baracat MM, Nakagawa A, Freitas LAP, Freitas O. Microcapsule Processing in a Spouted Bed. Can J Chem Eng. 2004;82:134-41.

49. Chow AHL, Ho SSS, Tong HHY, Ma HHM. Parameters affecting in-liquid drying microencapsulation and release rate of cefaclor. Int J Pharm. 1998;172:113-25.

50. Arora S, Budhiraja RD. Chitosan-alginate microcapsules of amoxicillin for gastric stability and mucoadhesion. J Adv Pharm Tech Res. 2012;3(1):68-74. doi: 10.4103/2231-4040.93555.

51. Sahoo NG, Kakran M, Shaal LA, Li L, Muller RH, Pal M, et al. Preparation and characterization of quercetin nanocrystals. J Pharm Sci. 2011;100(6):2379-90. doi: 10.1002/jps.22446.

52. Genc L, Buyuktiryaki S. Preparation and characterization of methotrexate-loaded microcapsules. Pharm Dev Technol. 2014;19(1):42-7. doi: 10.3109/10837450.2012.751405.

53. Pedersen JL, Lilleso J, Hammer NA, Werner MU, Holte K, Lacouture PG, et al. Bupivacaine in microcapsules prolongs analgesia after subcutaneous infiltration in humans: a dose-finding study. Anesth Analg. 2004;99(3):912-8. doi: 10.1213/01.ANE.0000143791.77608.0B.

54. Subramaniam B, Rajewski RA, Snavely K. Pharmaceutical processing with supercritical carbon dioxide. J Pharm Sci. 1997;86(8):885-90. doi: 10.1021/js9700661.

55. Hancock BC, Zografi G. Characteristics and significance of the amorphous state in pharmaceutical systems. J Pharm Sci. 1997;86(1):1-12. doi: 10.1021/js9601896.

LEGEND TO FIGURES

Figure 1. Photomicrographs of microcapsules obtained by scanning electron microscopy (SEM), increase of 2500x: (A) blank microcapsules and (b) rutin-loaded microcapsules.

Figure 2. Particle size distribution of rutin-loaded microcapsules.

Figure 3. Microencapsulation of rutin increases its analgesic effects. Mice received per oral treatment with rutin-loaded microcapsules (100 mg of rutin/ kg) or non-microencapsulated rutin (100 mg of rutin/ kg) 1 h before intraplantar stimulus with carrageenan (300 µg/paw). Mechanical hyperalgesia was evaluated prior stimulus injection (base line values) and at indicated time points after carrageenan injection. Results are presented as means ± S.E.M. of 6 mice per group per experiment and are representative of two separated experiments. One-way ANOVA followed by Tukey's multiple comparisons test. * P <0.05 compared to saline group, # P <0.05 compared with untreated carrageenan group; ** P <0.05 compared to the group carrageenan and non-microencapsulated rutin group.

Figure 4. Differential scanning calorimetric analysis thermograms of non-microencapsulated rutin (A) and rutin-loaded microcapsules (B).

Figure 5. X-ray diffraction patterns of non-microencapsulated rutin (A) and rutin-loaded microcapsules (B).

Figure 1

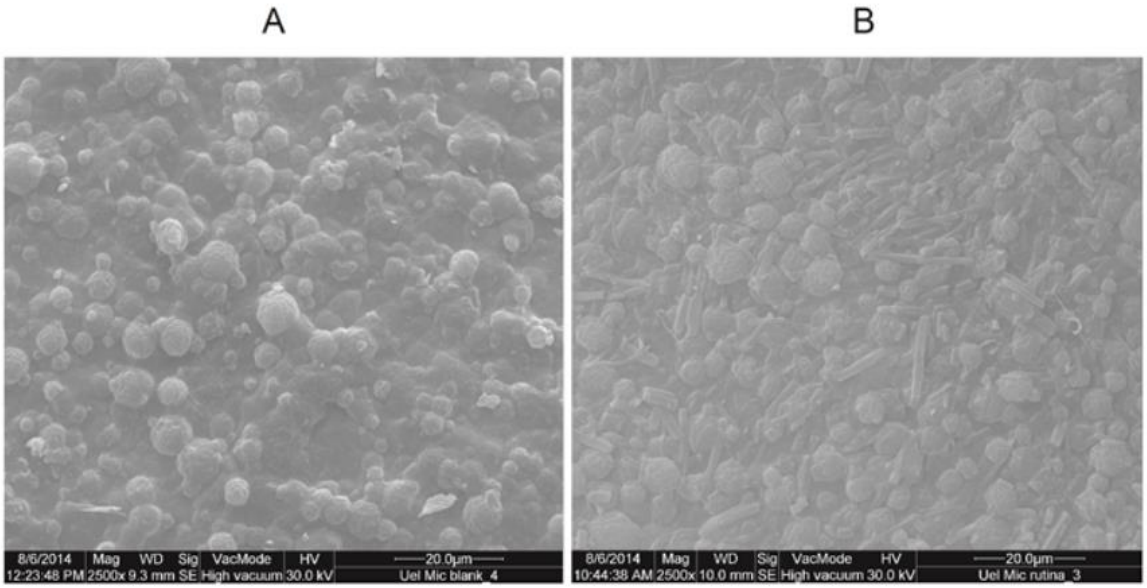


Figure 2

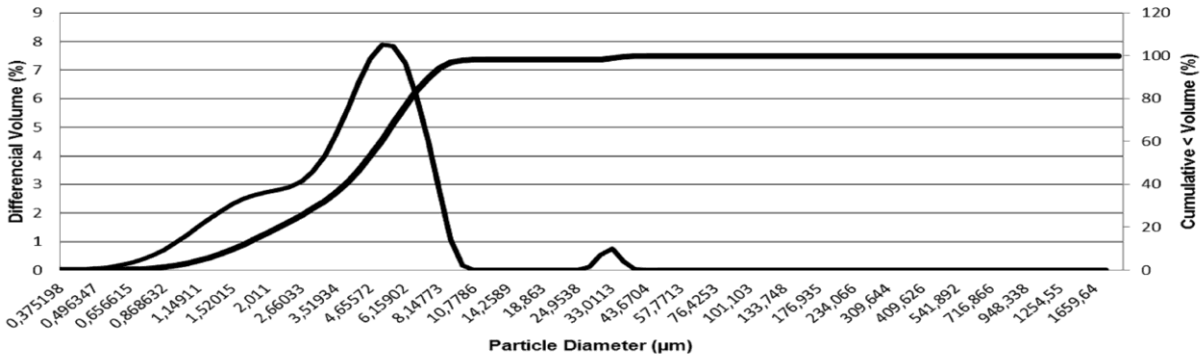


Figure 3

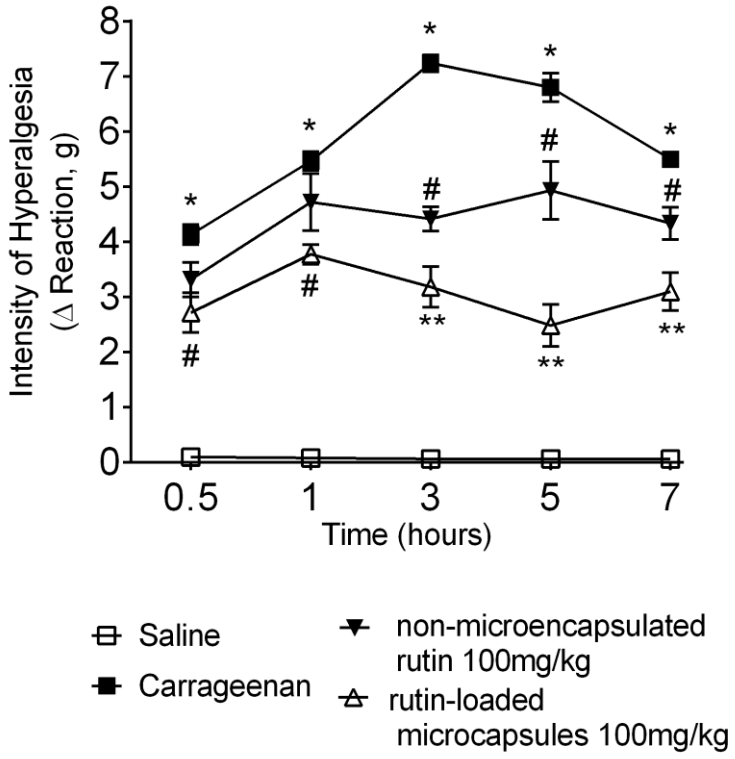


Figure 4

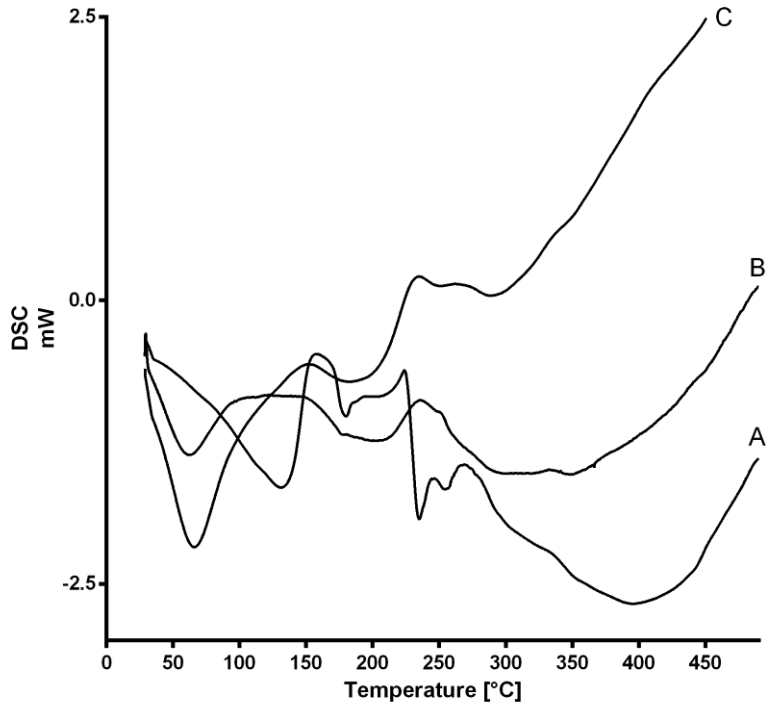
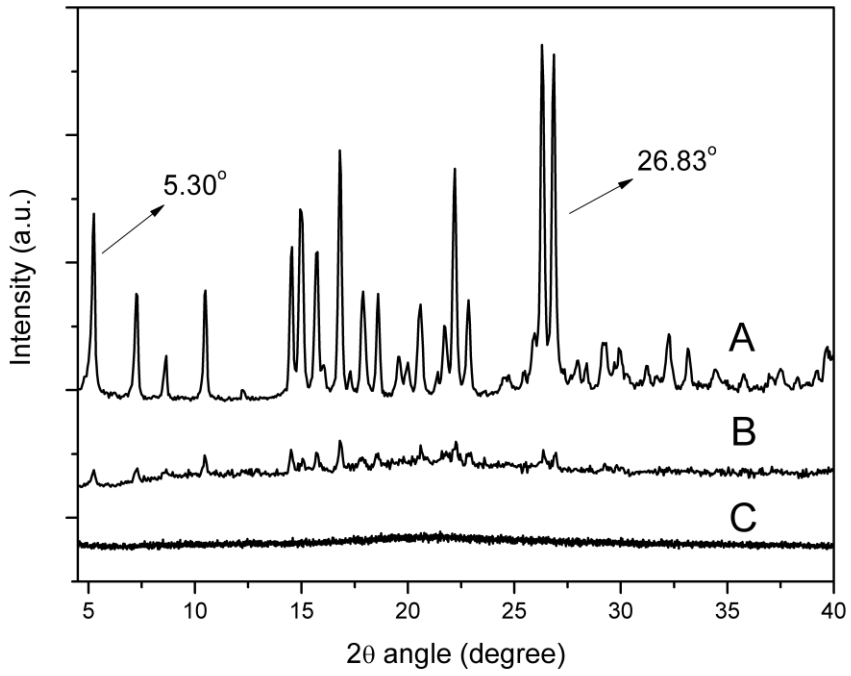


Figure 5



4.4 Topical Formulation Containing Microencapsulated Rutin Flavonoid Reduces Inflammation and Photooxidative Skin Damage Induced by UVB Irradiation.

Journal of Natural Products

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Topical Formulation Containing Microencapsulated Rutin Flavonoid Reduces Inflammation and Photooxidative Skin Damage Induced by UVB Irradiation.

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ABSTRACT

Many novel delivery systems have been designed for topical application of drugs since they can overcome the stratum corneum barrier and increase drug permeability, improving the prevention of ultraviolet B (UVB) irradiation-induced skin damage. The present study addressed the anti-inflammatory and anti-oxidant mechanisms of topical administration with microcapsules containg rutin (**1**), a flavonoid with significant antioxidant and anti-inflammatory activity, on UVB-induced skin inflammation in hairless mice. Edema was evaluated by skin weight, matrix metalloproteinase-9 activity by zymography, oxidative stress was evaluated by measuring of ferric reducing antioxidant power (FRAP), ability to scavenge the radical $ABTS^{\cdot}$**,** reduced glutathione levels, catalase activity, and superoxide anion production. It was also investigated the mRNA expression of NADPH oxidase sub-unit gp91phox and cytokines by qPCR. Topical treatment with microencapsulated **1** prevented skin inflammation by reducing skin edema, and also inhibited matrix metalloproteinase-9 activity. Topical formulation containing microencapsulated **1** administration also inhibited the mRNA expression of gp91phox, cytokines (TNF-α, IL-1β, and IL-10) and oxidative stress. These effects were not observed with the application of topical formulation containing non-microencapsulated **1**. These results suggest that topical application with microencapsulated **1** may represent an important therapeutic approach to reduce skin inflammation and photo-oxidative damage induced by UVB irradiation.

The sun generates various types of irradiation, including ultraviolet irradiation (UVR), which can be divided into UVC (200–280 nm), UVB (280–320 nm) and UVA (320–400 nm) wavelengths.[1] UVA and UVB irradiation cause skin damage, but the ability of UVB to induce sunburn is much higher.[2] Acute exposure to UVB induces sunburn, hyperpigmentation, hyperplasia and inflammatory processes in the skin, including erythema, edema, pain and heat.[1] Exposure to UVB irradiation accounts for 90% of the symptoms of premature skin aging and skin cancer.[3-6]

The damage caused by UVB irradiation is related to its ability of generating reactive oxygen species (ROS), which cause oxidative modification of cellular macromolecules such as lipids, proteins and nucleic acids.[4] The ROS formed by exposure to UVB irradiation leads to oxidative stress, a result of an imbalance between ROS and the skin's endogenous antioxidants, and damages important molecular structures in the cells of the skin.[1] ROS are formed as a consequence of exposure to UVB irradiation *per se*, but its production is further increased by the pro-inflammatory cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α).[7] Furthermore, the exposure to UV irradiation induces the expression of matrix metalloproteinases (MMP), proteolytic enzymes produced mainly by polymorphonuclear cells, macrophages, keratinocytes, fibroblasts and tumor cells.[8]

An alternative to prevent the deleterious effects of the ROS generated from the excessive exposure to UVB irradiation is the exogenous supplementation of antioxidants.[9-12] In this context, antioxidants from natural sources may be an effective approach to the treatment and prevention of damage caused by UVB irradiation.[13] Rutin (**1**) is a flavonoid with significant antioxidant and anti-inflammatory activity, besides antimutagenic and anticancer pharmacological effects.[4,14]

Emulsions are widely used as dermatological bases for incorporation of drugs and have good consumer acceptance because they are easy to apply and spread.[15] However, the

effectiveness of the active substance present in topical formulations depends on factors controlling penetration into the skin.[16]

The use of delivery systems is a viable alternative for the development and improvement of formulations, aiming to increase the therapeutic efficacy of drugs.[17] In this sense, many novel delivery systems have been designed for topical application of drugs since they can overcome the stratum corneum barrier and increase drug permeation,[18-22] penetration[23-25] and retention.[19,21,22,26,27] Multiparticulate systems such as microcapsules have been developed to modified drug release applying varied polymeric matrices and methodologies.[28]

Thus, we investigated whether microencapsulation can improve the photochemical protective properties of **1** against UVB-induced inflammatory and oxidative damage in the skin of hairless mice.

RESULTS AND DISCUSSION

Preparation of Rutin (1)-Loaded Microcapsules and Quantification of 1. The pectin/casein mixture in an aqueous dispersion forms multiparticle organized systems. The quantification of **1** in the system was performed by exhaustive extraction in which 82% of **1** was detected in comparison with the initial amount of this drug added to prepare the formulation. For incorporation in topical formulation, the amount was calculated considering the loss shown during processing.

Topical Formulation Containing Microencapsulated Rutin (1) Reduces UVB Irradiation-Induced Edema in the Skin. Acute exposure to UV irradiation induces an inflammatory response with the development of skin edema.[11] Standard-sized punches of skin were collected 12 h after UVB exposition and weighted in order to evaluate the capacity of topical formulation containing microencapsulated **1** to reduce UVB irradiation-induced edema. As shown in Figure 1, UVB irradiation induced significant skin edema when compared to unexposed mice. The UVB irradiation-induced skin edema was significantly inhibited (100%) by treatment with topical formulation containing microencapsulated **1.** This effect was not observed with the application of topical formulation containing non-microencapsulated drug (Figure 1).

An explanation for the improvement in the efficacy of **1** when microencapsulated is a higher penetration in the skin. In fact, previous studies have demonstrated that **1** exhibits low skin permeability[16,29] similarly to other flavonoids.[22,25,29,30]

Topical Formulation Containing Microencapsulated Rutin (1) Reduces UVB Irradiation-Induced Matrix Metalloproteinase-9 (MMP-9) Activity in the Skin. The exposure to UV irradiation induces the expression of matrix metalloproteinases (MMP), proteolytic enzymes produced mainly by polymorphonuclear cells, macrophages, keratinocytes, fibroblasts and tumor cells.[8] The increased production of MMP by epidermal keratinocytes and

dermal fibroblasts results in degradation of collagen and other extracellular matrix proteins. This process is followed by photoaging and severe skin damage.[31,32] Considering the role of MMP-9 in skin diseases, we investigated the effects of topical formulation containing microencapsulated **1** treatment in MMP-9 activity in the skin after UVB exposition. The MMP-9 activity was measured in skin samples collected 12 h after UVB irradiation by SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) zymography. As observed in Figure 2, UVB irradiation induced significant increases of MMP-9 activity in the skin of hairless mice, and this increase was inhibited by treatment with topical formulation containing microencapsulated **1**. However, no inhibitory effect on MMP-9 activity was observed with topical formulation containing non-microencapsulated **1**. Similarly, microemulsion containing quercetin prevented the UVB irradiation-induced secretion/activity of MMP,[25] which further corroborates that modulating flavonoid delivery is an important approach to increase the efficacy of this class of molecules.

Treatment with Topical Formulation Containing Microencapsulated Rutin (1) Prevents the Decrease of Antioxidant Capacity of Skin Induced by UVB Irradiation. UVB irradiation leads to formation of reactive oxygen species (ROS), which can be generated directly through interaction with chromophores groups or indirectly through inflammatory response.[33] With the increased flow of ROS induced by UVB, regenerating of endogenous antioxidants becomes insufficient leading to their depletion.[25,34] The result of unbalance between ROS and antioxidants modulates signaling transduction cellular pathways of redox-sensitive and gene expression. Such molecular changes might be involved in the pathogenesis of photoxidatives damage.[11,35,36]

Therefore, samples were collected 12 h after UVB exposition to determine whether the topical formulation containing microencapsulated **1** prevents the decrease of skin antioxidant

capacity. As expected, UVB irradiation reduced the antioxidant capacity of skin compared with the non-irradiated control as determined by the FRAP and ABTS assays (Figure 3A and 3B, respectively). The treatment with topical formulation containing microencapsulated **1** inhibited UVB-induced reduction of skin antioxidant (Figures 3), while no effect was observed with the application of topical formulation containing non-microencapsulated **1**.

UVB irradiation also lowered the levels of reduced glutathione (GSH) at 12h (Figure 4A) and the activity of catalase (CAT) at 2h (Figure 4B), compared with non-irradiated group. On the other hand, treatment with topical formulation containing microencapsulated **1** inhibited UVB irradiation-induced GSH depletion (69%), whereas this effect was not observed with non-microencapsulated **1** (Figure 4A). Treatment with topical formulation containing microencapsulated **1** also reversed significantly the UVB irradiation-induced loss of CAT activity (74%), an effect that was not observed with non-microencapsulated **1** (Figure 4B).

Corroborating our results, delivery systems increase penetration and retention of natural antioxidant molecules, preventing the decrease or restoring the antioxidant capacity of skin.[25,26]

Treatment with Topical Formulation Containing Microencapsulated Rutin (1) Prevents UVB-Induced gp91phox mRNA Expression and Cytokine Production in the Skin. In order to evaluate the effects of topical formulation containing microencapsulated **1** over NADPH oxidase 2 (NOX2), skin samples were collected 4 h after UVB exposure, and the mRNA expression of NOX2 sub-unity gp91phox was evaluated by quantitative PCR (qPCR). Gp91phox sub-unity catalyzes electron transfer to O_2, generating large amounts of $O_2^{\bullet -}$ during inflammatory responses. UVB irradiation increased gp91phox mRNA expression, and this increase was inhibited by topical formulation containing microencapsulated **1**. This effect was not observed with the application of topical formulation containing non-microencapsulated **1** (Figure 5).

Treatment with Topical Formulation Containing Microencapsulated Rutin (1) Reduces Superoxide Anion ($O_2^{\bullet-}$) Production in the Skin Induced by UVB Irradiation. UVB induces the production of large amounts of $O_2^{\bullet-}$,[10] increasing the levels of H_2O_2, which favors the generation of hydroxyl radical ($HO^{\bullet}$), a cytotoxic derivative.[37] In the present experimental condition, UVB also induced significant increase of $O_2^{\bullet-}$ levels in the skin of irradiated mice compared to non-irradiated control, and the treatment with topical formulation containing microencapsulated **1** significantly reduced UVB irradiation-induced $O_2^{\bullet-}$ production (Figure 6). This effect was not observed with the application of topical formulation containing non-microencapsulated **1** (Figure 6). It is well known that ROS can inflict damage on all classes of cellular macromolecules including lipids and nucleotides, thus, leading to cell death or causing important cellular alterations.[10,22] In this sense, antioxidant suppression of ROS generation has been proved to be effective in preventing UVB-induced cell injury.[22,25,38]

Despite its role in pathogen killing, $O_2^{\bullet-}$ is very deleterious to the host in the absence of infection and causes even more damage when endogenous antioxidants are lowered by exposition to UVB. Furthermore, $O_2^{\bullet-}$ mediates cytokine production and neutrophil recruitment during inflammatory response and thus contributes to sustaining inflammation.[39,40] Thus, we investigated wether, by reducing gp91phox expression and, consequently, $O_2^{\bullet-}$ production, the treatment with topical formulation containing microencapsulated **1** could modulate the production of cytokines. UVB irradiation induced significant mRNA expression of tumor necrosis factor alpha (TNF-α), pro-interleukin 1 beta (pro-IL-1β), and IL-10 (Figure 7), while the treatment with topical formulation containing microencapsulated **1** reduced the mRNA expression of all these cytokines, an effect not observed with non-microencapsulated **1** (Figure 7). These results suggest that topical formulation containing microencapsulated **1** protected the skin from UVB-induced oxidative stress and inflammation by reducing mRNA expression of

gp91phox, TNF-α and pro-IL-1β. Expression of IL-10, an immunosuppressive cytokine induced by UVB,[41,42] was also inhibited by topical formulation containing microencapsulated **1**, which supports its application in a wide range of skin diseases induced by UVB.

In summary, the present study showed that with the incorporation of microencapsulated **1** allowed to modify the release of the drug, probably resulting in improved skin penetration and retention. This improvement led to increased antioxidant efficacy of rutin, with reduction of skin photoxidative damage induced by UVB irradiation. The possible mechanism was proposed for this improvement in skin absorption. The microcapsules act as carriers to deliver the encapsulated drug into the skin.

Experimental Section

General Experimental Procedures. Brilliant blue R, reduced glutathione (GSH), hexadecyltrimethylammonium bromide (HTAB), N-ethylmaleimide, *o*-dianisidine dihydrochloride, phenylmethanesulfonyl fluoride, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and nitroblue tetrazolium (NBT), bisacrylamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rutin (**1**) from Acrós Organics **(**USA). Pectin USP (68% of esterification) from CPKelco (Limeira, SP, Brazil). Casein from Katuffman & Co (Germany). Tert-butyl hydroperoxide from Acros (Pittsburgh, PA, USA). Xylene cyanol and Tris were obtained from Amresco (Solon, OH, USA). Isoflurane from Abbott (Chicago, IL, USA). ELISA kits for determination of cytokine were obtained from eBioscience (San Diego, CA, USA). Acrylamide, sodium dodecyl sulfate (SDS), glycerol, Superscript® III, Oligo(dT) 12-18 primers, Platinum SYBRGreen® and primers from Invitrogen (Carlsbad, CA, USA). All other reagents used were of pharmaceutical grade.

Preparation of microcapsules. Microcapsules were prepared by dispersion in distilled water (solid content 10%, w/v) of the polymers pectin and casein under constant mechanical shaking. Sodium hydroxide (4.0 M) was used to adjust pH to 8.0 $\pm$ 0.1.[43] After complete dispersion, rutin was added at the proportion of 1:5 (drug:polymer). Microcapsules were obtained by slow and gradual reduction of pH from 8.0 $\pm$ 0.1 to 4.0 $\pm$ 0.1 with 1.0 M citric acid. The same methodology was used to prepare microcapsules containing no drug (blank microcapsules). The sample was dried in a lyophilizer (Edwards, model Pirani 501) during 24h.

Rutin (1) quantification in the microcapsules. Samples of 1-loaded microcapsules were dispersed in 10 ml of phosphate buffer pH 6.8 added 5.0% Tween ® 20 and then stirred for 5 min, followed by centrifugation at 3600 rpm for 10 min for the extraction of rutin. The supernatant was filtered through a 0.45 µm nylon syringe filter, and the filtrate was analyzed by DPPH method and quantified spectrophotometrically at 517 nm.[30,44]

Animals and Experimental Protocols. *In vivo* experiments were performed sex matched hairless mice (HRS/J), weighing 20-30 g. The animals were housed in a temperature-controlled room, with access to water and food *ad libitum*. They were housed within cages with a 12 h light and 12 h dark cycles. All experiments were conducted in accordance with National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of the Universidade Estadual de Londrina (registered under the number CEUA 204/13, process number 27025.2013.10). All efforts were made to minimize the number of animals used and their suffering. Hairless mice were randomly designed to different groups with 5 mice each: non-irradiated control group, irradiated control group, irradiated group and treated with topic formulation without drug, irradiated group and treated with topic formulation containing **1**, irradiated group and treated with topic formulation containing microcapsules without drug, irradiated group and treated with topic formulation containing microencapsulated **1**. In the experiments presented in Figures 1-7, mice were treated topically, in dorsal part, with 0.5g of formulation containing 1% of **1**, 12h, 6h, immediately before and 6 h after the beginning of UVB irradiation session.

Irradiation. The UVB source used in the experiments to induce oxidative stress was a Philips TL/12 RS 40W (Medical-Holand) emitting a continuous spectrum between 270 and 400 nm with a peak emission at 313 nm. The lamp was mounted 20 cm above the place where the mice were placed on, resulting in an irradiation of 0.384 mW/cm^2 as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with sensor for UV (SED005) and UVB (SED240). The irradiation dose used for induction of oxidative stress was 4.14 J/cm^2. All groups were irradiated simultaneously. Mice were terminally anesthetized with 1.5% isoflurane 12 h (Figs. 1-4A), 2h (Figs. 4B e 6) or 4h (Figs. 5 e 7) after the UVB exposure, and the full thickness of the dorsal skins was removed. In the tests at 2 h and 4h after the UVB exposure, mice were decapitated immediately after anesthetization and dorsal skin samples were collected. Samples

were stored at -70°C until analysis. The samples collected for verification of cutaneous edema were weighed when removed and were not frozen.[9,11]

Skin Edema. The effect of topical formulation containing microencapsulated **1** on UVB-induced skin edema of hairless mice was measured as an increase in the dorsal skin weight. After dorsal skin removal, a constant area (5 mm diameter) was delimited with the aid of a mold, followed by weighing of this constant area.[9,10] The analysis was obtained by comparing the weight of the skin between groups and the result was expressed in mg of skin.

Analyses of skin proteinase by substrate-embedded enzymography. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) substrate-embedded enzymography was used to detect enzymes with gelatinase activity. Assays were carried out as previously described.[9-11] The total skin of hairless mice (1:4, w/w dilution) were homogenized (T 18 basic, IKA) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.01 M $CaCl_2$ and 1% protease inhibitor cocktail. Whole homogenates were centrifuged at 12000 *g* for 10 min at 4°C twice. The Lowry method was used to measure protein levels in skin homogenates.[45] Aliquots of 50 μL of samples were mixed with 10 μL of 0.1 M Tris-HCl (pH 7.4) containing 20% glycerol, 4% SDS and 0.005% xylene cyanol, and 25 μL of the mixture (40 μg of protein) were taken for electrophoresis in a gel containing 10% acrylamide and 0.025% gelatin. After electrophoresis, the gels were incubated for 1 h with 2.5% Triton X-100 under constant shaking, incubated overnight in 0.05 M Tris-HCl (pH 7.4), 0.01 M $CaCl_2$ and 0.02% sodium azide at 37°C, and stained the following day with brilliant blue R. After destaining in 20% acetic acid, the zone of enzyme activity were analyzed by comparing the groups in the ImageJ Program (NIH, Bethesda, MD, USA).

FRAP assay. The reducing ability of skin sample was determined by FRAP assay.[9,46] The samples of skin (30 mg approximately) were homogenized in 500 μL of KCl (1.15%) using a Tissue-Tearor (Biospec) and centrifuged at 1.000 g for 10 min at 4°C, the supernatant was

employed for measurement the antioxidant capacity of skin. The reaction consists to add the supernatant (30 μL) to the FRAP reagent prepared with 0.3 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM hydroclorid acid and 20 mM ferric chloride. The FRAP reagent was warmed to 37°C for 30 min. The absorvance was determined at 595 nm in a microplate reader (EnSpire, Perkin Elmer). Previously, a curve of trolox (0.01-20 nmol) was prepared and the results are presented as nmol trolox equivalent per mg of skin.

ABTS assay. This assay is based on the inhibition of the absorbance of the radical ABTS. Skin of hairless mice (30 mg approximately) was homogenized in 500 μL of KCl (1.15%) using a Tissue-Tearor (Biospec) and centrifuged at 1.000 *g* for 10 min at 4°C, the supernatant was employed for measurement the antioxidant capacity of skin. The solution of ABTS was prepared with 7 mM of ABTS and 2.45 mM of potassium persulfate diluted with phosphate buffer pH 7.4 to an absorbance of 0.7-0.8 in 730 nm was prepared. The supernatant (7μl) was mixed on ABTS solution and after 6 min the absorvance was determined at 730 nm in a microplate reader (EnSpire, Perkin Elmer).[9,46] Previously, a curve of trolox (0.01-20 nmol) was prepared and the results are presented as nmol trolox equivalent per mg of skin.

Reduced glutathione (GSH) assay. GSH was determined as described previously.[9] Briefly, skin samples were homogenized in 0.02 M EDTA using a Tissue-Tearor (Biospec). Whole homogenates were treated with 50% trichloroacetic acid and were centrifuged twice at 2700 *g* for 10 min at 4°C. The reaction mixture contained 50 μL of sample, 100 μL of 0.4 M Tris and 5 μL DTNB (1.9 mg/mL in methanol). The color developed was read at 405 nm (Multiskan GO, Thermo Scientific). The standard curve was prepared with GSH 5-150 μM. The results are presented as μM of GSH per mg of skin.

Catalase (CAT) assay. The analysis of CAT activity was evaluated by measuring the decay in the concentration of hydrogen peroxide (H_2O_2) and the generation of oxygen as described previously.[47] Skin of hairless mice (100 mg approximately) were homogenized in 500

µL of 0.02 M EDTA using a Tissue-Tearor (Biospec), and centrifuged twice at 2700 *g* for 10 min at 4°C. The reaction mixture contained 10 µL of sample, 160 µL of buffer Tris-HCl 1 M with EDTA 5 mM pH 8,0, 20 µL of deionized water and 20 µL of H_2O_2 200 mM. Measurement of CAT activity was estimated through the difference between the initial reading and the reading conducted 30 seconds after the addition of H_2O_2 at 240 nm in a microplate reader (EnSpire, Perkin Elmer) at 25ºC. The CAT values were expressed as unit of CAT/mg of skin/minute.

Superoxide anion production. The quantitation of superoxide anion ($O_2^{\bullet-}$) production in tissue homogenates (100 mg approximately in 500 µL of 0.02 M EDTA) was performed using the nitroblue tetrazolium assay (NBT).[10] Briefly, 50 µL of homogenates were incubated in 96-well plate for 1 h. The supernatant was carefully removed and the cells were incubated with 100 µL of NBT (1 mg/ml) for 15 min. The supernatant was then carefully removed and the cells were fixed in methanol 100%. Formazan particles were dissolved by adding 120 µL of KOH 2M and 140 µL of dimethylsulfoxide. Reduction of NBT to formazan was measured at 600 nm using a microplate spectrophotometer reader (Asys Expert Plus, Biochrom) and the results are presented as optical density (OD) per 10 mg of skin.

Reverse transcription and quantitative polymerase chain reaction (qPCR). qPCR was performed as previously described.[10] Skin samples were homogenized in trizol reagent, and total RNA was isolated according to manufacturer's directions. The purity of total RNA was measured with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. Reverse transcription of total RNA to cDNA, and qPCR were carried out using GoTaq® 2-Step RT-qPCR System (Promega) and specific primers. qPCR reaction was performed in StepOnePlusTM Real-Time PCR System (Applied Biosystems®). The relative gene expression was measured using the comparative $2^{-(\Delta\Delta Cq)}$ method. The expression of Gapdh mRNA was used as reference control. The primers used were: *gp91phox,* sense: 5'-AGCTATGAGGTGGTGATGTTAGTGG-3', antisense: 5'-

CACAATATTTGTACCAGACAGACTTGAG-3'; pro-IL-1β forward 5'-GAAATGCCACCTTTTGACAGTG-3', reverse 5'- TGGATGCTCTCATCAGGACAG-3'; TNF-α forward 5'- TCTCATCAGTTCTATGGCCC -3', reverse 5'-GGGAGTAGACAAGGTACAAC -3'; IL-10 forward 5'-TCTCATCAGTTCTATGGCCC-3', reverse 5'-GGGAGTAGACAAGGTACAAC-3'; and *Gapdh* sense: 5-ATGACATCAAGAAGGTGGTG-3, Antisense: 5'-CATACCAGGAAATGAGCTTG-3.

Statistical Analysis. The bars in the figures indicate the mean values ± standard error of the mean (SEM) of 5 mice per group per experiment and are representative of two separated experiments. Data were statistically analyzed by one-way ANOVA followed by Tukey's *t* test. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Results were considered significantly different when $p < 0.05$.

Acknowledgment

This work was supported by Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thank Marcelo Tempesta Oliveira and Cristina Aparecida Lopes for their technical support to this research. StepOnePlusTM Real-Time PCR System (Applied Biosystems®) was purchased by FINEP funding (CT-INFRA 01/2013).

REFERENCES

(1) Avila Acevedo, J. G.; Espinosa Gonzalez, A. M.; De Maria y Campos, D. M.; Benitez Flores Jdel, C.; Hernandez Delgado, T.; Flores Maya, S.; Campos Contreras, J.; Munoz Lopez, J. L.; Garcia Bores, A. M. *BMC Complement Altern Med* **2014,** *14*, 281.

(2) Svobodova, A.; Psotova, J.; Walterova, D. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* **2003,** *147*, 137-45.

(3) Esteve-Puig, R.; Gil, R.; Gonzalez-Sanchez, E.; Bech-Serra, J. J.; Grueso, J.; Hernandez-Losa, J.; Moline, T.; Canals, F.; Ferrer, B.; Cortes, J.; Bastian, B.; Ramon, Y. C. S.; Martin-Caballero, J.; Flores, J. M.; Vivancos, A.; Garcia-Patos, V.; Recio, J. A. *PLoS Genet* **2014,** *10*, e1004721.

(4) Choi, K. S.; Kundu, J. K.; Chun, K. S.; Na, H. K.; Surh, Y. J. *Arch Biochem Biophys* **2014,** *559*, 38-45.

(5) Wang, F.; Ge, T.; Gao, Q.; Hu, L.; Yu, J.; Liu, Y. *J Photochem Photobiol B* **2014,** *140*, 205-14.

(6) Saw, C. L.; Yang, A. Y.; Huang, M. T.; Liu, Y.; Lee, J. H.; Khor, T. O.; Su, Z. Y.; Shu, L.; Lu, Y.; Conney, A. H.; Kong, A. N. *Cell Biosci* **2014,** *4*, 39.

(7) Liu, X.; Wang, J. *J. Ethnopharmacol.* **2011**, *133*, 780–787.

(8) Fisher, G. J.; Wang, Z. Q.; Datta, S. C.; Varani, J.; Kang, S.; Voorhees, J. J. *N Engl J Med* **1997,** *337*, 1419-28.

(9) Ivan, A. L.; Campanini, M. Z.; Martinez, R. M.; Ferreira, V. S.; Steffen, V. S.; Vicentini, F. T.; Vilela, F. M.; Martins, F. S.; Zarpelon, A. C.; Cunha, T. M.; Fonseca, M. J.; Baracat, M. M.; Georgetti, S. R.; Verri, W. A., Jr.; Casagrande, R. *J Photochem Photobiol B* **2014,** *138*, 124-33.

(10) Campanini, M. Z.; Pinho-Ribeiro, F. A.; Ivan, A. L.; Ferreira, V. S.; Vilela, F. M.; Vicentini, F. T.; Martinez, R. M.; Zarpelon, A. C.; Fonseca, M. J.; Faria, T. J.; Baracat, M. M.; Verri, W. A., Jr.; Georgetti, S. R.; Casagrande, R. *J Photochem Photobiol B* **2013,** *127*, 153-60.

(11) Casagrande, R.; Georgetti, S. R.; Verri, W. A., Jr.; Dorta, D. J.; dos Santos, A. C.; Fonseca, M. J. *J Photochem Photobiol B* **2006,** *84*, 21-7.

(12) Filip, A.; Clichici, S.; Daicoviciu, D.; Catoi, C.; Bolfa, P.; Postescu, I. D.; Gal, A.; Baldea, I.; Gherman, C.; Muresan, A. *J Physiol Pharmacol* **2011,** *62*, 385-92.

(13) Kim, K. H.; Park, S. J.; Lee, Y. J.; Lee, J. E.; Song, C. H.; Choi, S. H.; Ku, S. K.; Kang, S. J. *Basic Clin Pharmacol Toxicol* **2015,** *116*, 73-86.

(14) Verri, W. A. Jr.; Vicentini, F. T. M. C.; Baracat, M. M.; Georgetti, S. R.; Cardoso, R. D. R.; Cunha, T. M.; Ferreira, S. H.; Cunha, F. Q.; Fonseca, M. J. V.; Casagrande, R. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam. **2012**, *36*, 297-322.

(15) Ansel, H. C.; Popovich, N. G.; Allen Jr, L. V. *Farmacotécnica: formas farmacêuticas e sistemas de liberação de fármacos.* 6.ed. São Paulo: Premier, **2000**, 568.

(16) Baby, A. R.; Haroutiounian-Filho, C. A.; Sarruf, F. D.; Tavante-Júnior, C. R.; Pinto, C. A. S. O.; Zague, V.; Arêas, E. P. G.; Kaneko, T. M.; Velasco, M. V. R. *Braz J Pharm Sci* **2008**, *44*, 233-248.

(17) Gandhi, R.; Lal Kaul, C.; Panchagnula, R. *Pharm Sci Technolo Today* **1999,** *4*, 160-170.

(18) Batheja, P.; Sheihet, L.; Kohn, J.; Singer, A. J.; Michniak-Kohn, B. *J Control Release* **2011,** *149*, 159-67.

(19) Aggarwal, N.; Goindi, S. *Int J Pharm* **2012,** *437*, 277-87.

(20) Aggarwal, N.; Goindi, S.; Khurana, R. *Colloids Surf B Biointerfaces* **2013,** *105*, 158-66.

(21) Manca, M. L.; Zaru, M.; Manconi, M.; Lai, F.; Valenti, D.; Sinico, C.; Fadda, A. M. *Int J Pharm* **2013,** *455*, 66-74.

(22) Liu, D.; Hu, H.; Lin, Z.; Chen, D.; Zhu, Y.; Hou, S.; Shi, X. *J Photochem Photobiol B* **2013,** *127*, 8-17.

(23) Abramovic, Z.; Sustarsic, U.; Teskac, K.; Sentjurc, M.; Kristl, J. *Int J Pharm* **2008,** *359*, 220-7.

(24) Sheihet, L.; Chandra, P.; Batheja, P.; Devore, D.; Kohn, J.; Michniak, B. *Int J Pharm* **2008,** *350*, 312-9.

(25) Vicentini, F. T.; Simi, T. R.; Del Ciampo, J. O.; Wolga, N. O.; Pitol, D. L.; Iyomasa, M. M.; Bentley, M. V.; Fonseca, M. J. *Eur J Pharm Biopharm* **2008,** *69*, 948-57.

(26) Geetha, T.; Kapila, M.; Prakash, O.; Deol, P. K.; Kakkar, V.; Kaur, I. P. *J Drug Target* **2014**, 1–11.

(27) Chen, G.; Li, D.; Jin, Y.; Zhang, W.; Teng, L.; Bunt, C.; Wen, J. *Drug Dev Ind Pharm* **2014,** *40*, 260-5.

(28) Deasy PB. *Drug and The Pharmaceutical Sciences: Microencapsulation and Related Drug Process.* New York: Marcel Dekker, **1984**.

(29) Park, S. N.; Lee, M. H.; Kim, S. J.; Yu, E. R. *Biochem Biophys Res Commun* **2013,** *435*, 361-6.

(30) Casagrande, R.; Georgetti, S. R.; Verri, W. A., Jr.; Borin, M. F.; Lopez, R. F.; Fonseca, M. J. *Int J Pharm* **2007,** *328*, 183-90.

(31) Rieger, M. *Cosmetics & Toiletries* **1999,** *114*, 65-70.

(32) Quan, T.; Qin, Z.; Xia, W.; Shao, Y.; Voorhees, J. J.; Fisher, G. J. *J Investig Dermatol Symp Proc* **2009,** *14*, 20-4.

(33) Wei, H.; Zhang, X.; Wang, Y.; Lebwohl, M. *Cancer Lett* **2002,** *185*, 21-9.

(34) Fonseca, Y. M.; Catini, C. D.; Vicentini, F. T.; Nomizo, A.; Gerlach, R. F.; Fonseca, M. J. *J Ethnopharmacol* **2010,** *127*, 596-601.

(35) Fuchs, J. *Free Radic Biol Med* **1998,** *25*, 848-73.

(36) Vicentini, F. T.; Casagrande, R.; Verri, W. A., Jr.; Georgetti, S. R.; Bentley, M. V.; Fonseca, M. J. *AAPS PharmSciTech* **2008,** *9*, 591-6.

(37) Gutteridge, J. M. *FEBS Lett* **1986,** *201*, 291-5.

(38) Campanini, M. Z.; Custodio, D. L.; Ivan, A. L.; Martins, S. M.; Paranzini, M. J.; Martinez, R. M.; Verri, W. A., Jr.; Vicentini, F. T.; Arakawa, N. S.; Faria, T. J.; Baracat, M. M.; Casagrande, R.; Georgetti, S. R. *AAPS PharmSciTech* **2014,** *15*, 86-95.

(39) Bowie, A.; O'Neill, L. A. *Biochem Pharmacol* **2000,** *59*, 13-23.

(40) Kilpatrick, L. E.; Sun, S.; Li, H.; Vary, T. C.; Korchak, H. M. *J Leukoc Biol* **2010,** *87*, 153-64.

(41) Hasegawa, T.; Shimada, S.; Ishida, H.; Nakashima, M. *PLoS One* **2013,** *8*, e77308.

(42) Piskin, G.; Bos, J. D.; Teunissen, M. B. *Arch Dermatol Res* **2005,** *296*, 339-42.

(43) Baracat, M. M.; Nakagawa, A. M.; Casagrande, R.; Georgetti, S. R.; Verri, W. A. Jr.; de Freitas, O. *AAPS PharmSciTech* **2012**,13, 364-72.

(44) Blois, M. S. *Nature* **1958**, 181, 1199-1200.

(45) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J Biol Chem* **1951,** *193*, 265-75.

(46) Katalinic, V.; Modun, D.; Music, I.; Boban, M. *Comp Biochem Physiol C Toxicol Pharmacol* **2005,** *140*, 47-52.

(47) Aebi, H. *Methods Enzymol* **1984,** *105*, 121-6.

Legends to Figures

Figure 1. Topical formulation containing microencapsulated rutin (**1**) inhibits UVB irradiation-induced skin edema in hairless mice. Skin edema was determined in samples collected 12 h after the end of irradiation. Bars represent means ± SEM of 5 mice per group and are representative of two separated experiments. One-way ANOVA followed by the Tukey's test [*p<0.05 compared to the non-irradiated control group; $^{\#}p$<0.05 compared to the respective control].

Figure 2. Effect of topical formulation containing microencapsulated rutin (**1**) on UVB irradiation-induced increase of MMP-9 activity in hairless mice. The MMP-9 activity was determined in samples collected 12 h after the end of irradiation. Image of gelatin zymography and bars represent means ± SEM of 5 mice per group and are representative of two separated experiments. One-way ANOVA followed by the Tukey's test [*p<0.05 compared to the non-irradiated control group; $^{\#}p$<0.05 compared to the respective control].

Figure 3. Topical formulation containing microencapsulated rutin (**1**) inhibits UVB irradiation-induced FRAP reduction and ABTS scavenge in hairless mice. The FRAP activity (A) and ABTS levels (B) were determined in samples collected 12 h after the end of irradiation. Bars represent means ± SEM of 5 mice per group and are representative of two separated experiments. One-way ANOVA followed by the Tukey's test [*p<0.05 compared to the non-irradiated control group; $^{\#}p$<0.05 compared to the respective control].

Figure 4. Topical formulation containing microencapsulated rutin (**1**) inhibits UVB irradiation-induced GSH and CAT depletion. The GSH levels (A) and CAT activity (B) were determined in samples collected 12 h and 2 h after the end of irradiation, respectively. Bars represent means ±

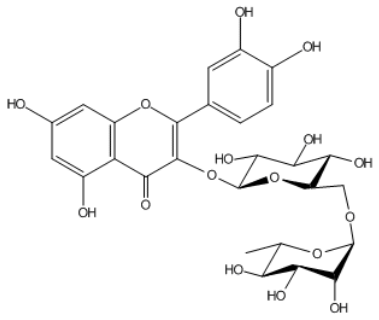
SEM of 5 mice per group and are representative of two separated experiments. One-way ANOVA followed by the Tukey's test [*p<0.05 compared to the non-irradiated control group; $^{\#}p$<0.05 compared to the respective control].

Figure 5. Topical formulation containing microencapsulated rutin (**1**) inhibits UVB irradiation-induced gp91phox mRNA expression. The mRNA expression for gp91phox was determined in samples collected 4 h after the end of irradiation by qPCR. Bars represent means ± SEM of 5 mice per group and are representative of two separated experiments. One-way ANOVA followed by the Tukey's test [*p<0.05 compared to the non-irradiated control group; $^{\#}p$<0.05 compared to the respective control].

Figure 6. Topical formulation containing microencapsulated rutin (**1**) inhibits UVB irradiation-induced superoxide anion production. The nitroblue tetrazolium reduction test was determined in samples collected 2 h after the end of irradiation, respectively. Bars represent means ± SEM of 5 mice per group and are representative of two separated experiments. One-way ANOVA followed by the Tukey's test [*p<0.05 compared to the non-irradiated control group; $^{\#}p$<0.05 compared to the respective control].

Figure 7. Topical formulation containing microencapsulated rutin (**1**) inhibits UVB irradiation-induced TNF-α (A), pro-IL-1β (B) and IL-10 (C) mRNA expression. The mRNA expression for TNF-α, pro-IL-1β and IL-10 were determined in samples collected 4 h after the end of irradiation by qPCR. Bars represent means ± SEM of 5 mice per group and are representative of two separated experiments. One-way ANOVA followed by the Tukey's test [*p<0.05 compared to the non-irradiated control group; $^{\#}p$<0.05 compared to the respective control].

Structure Sheet



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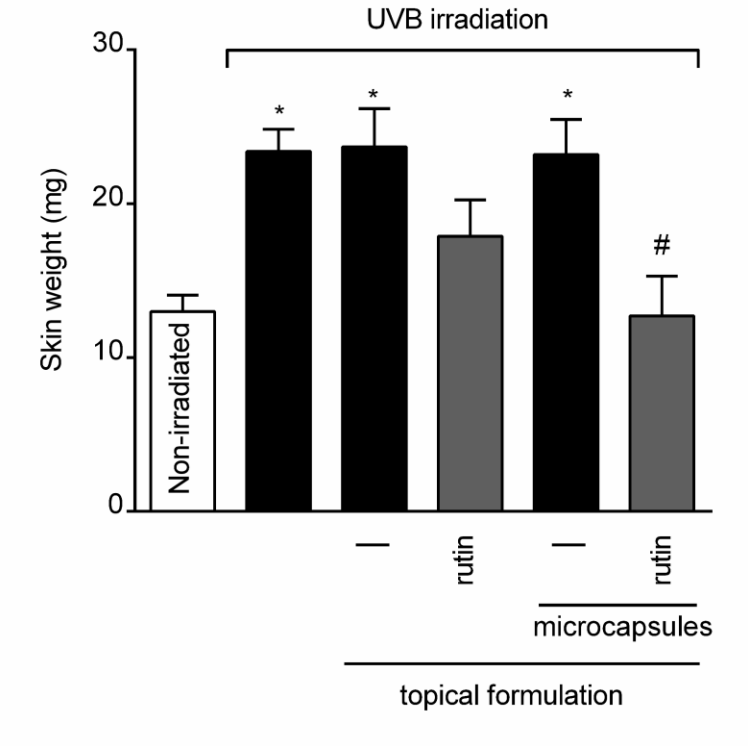

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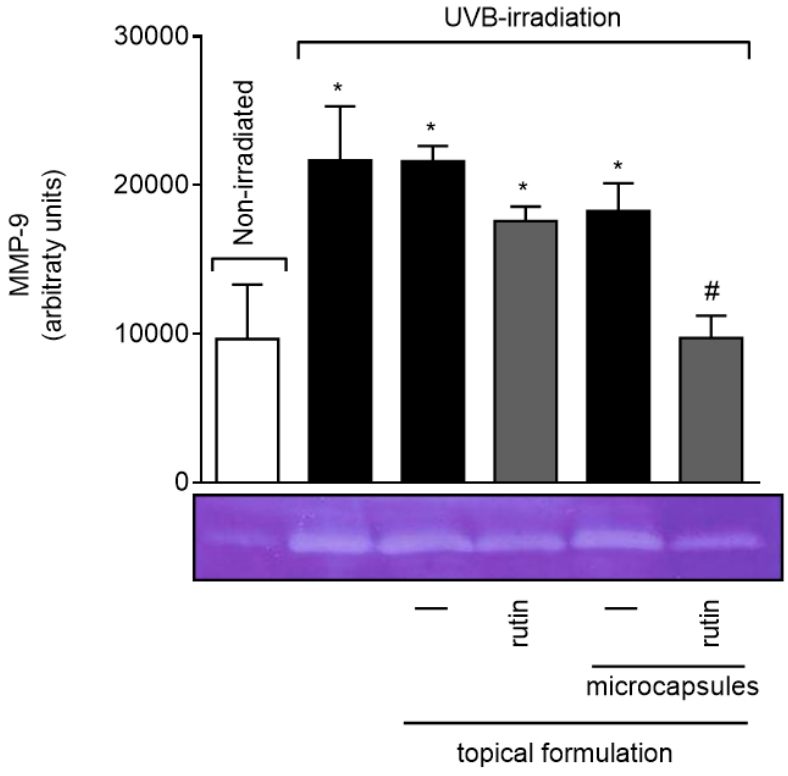

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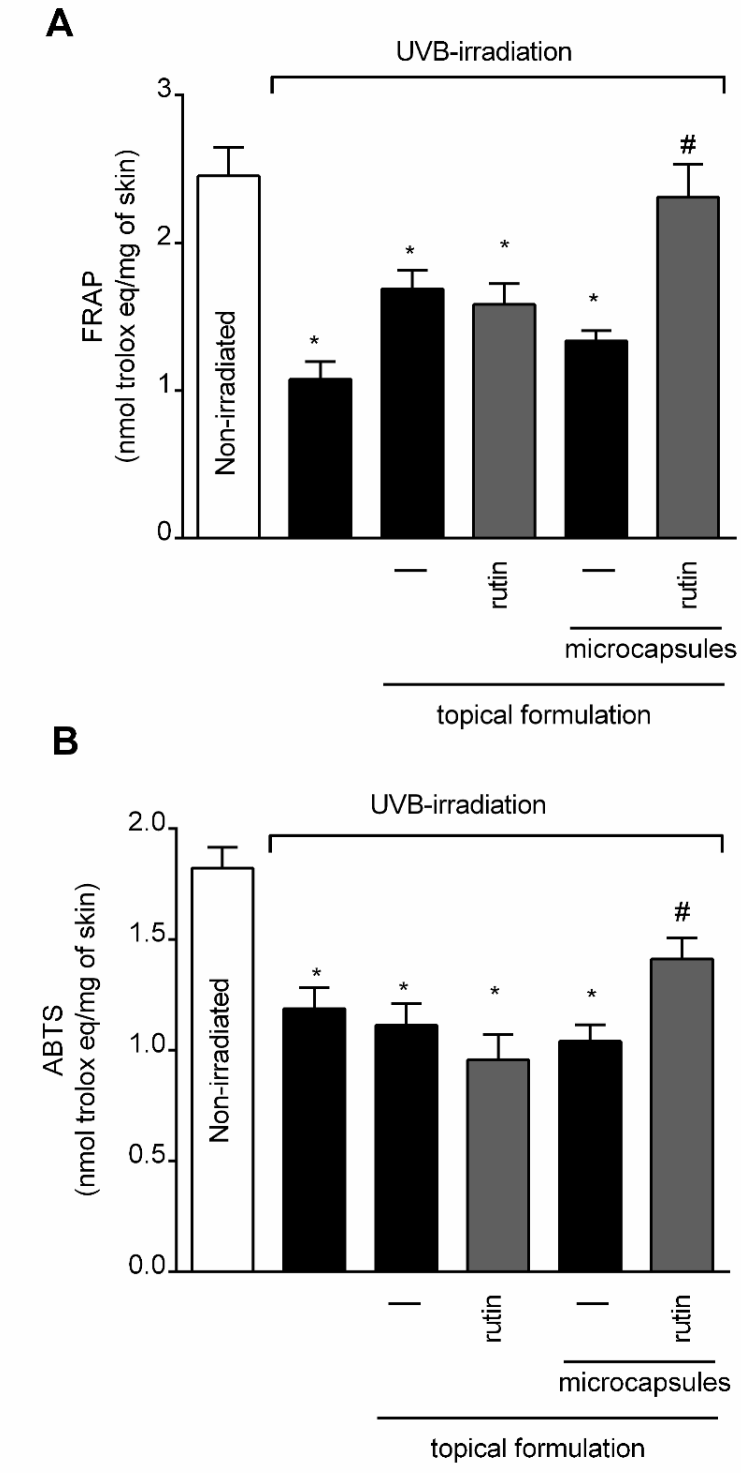
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rutin
rutin
microcapsules
topical formulation
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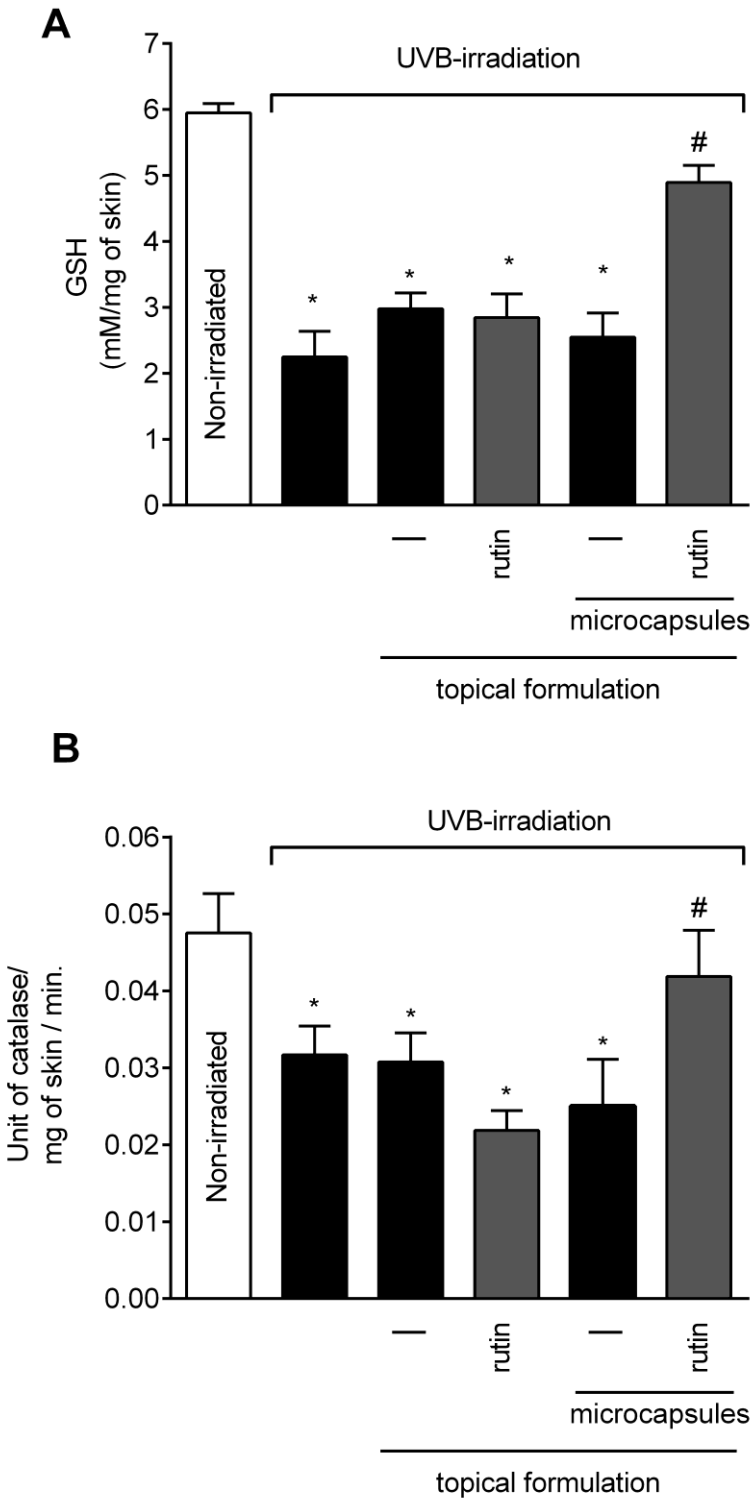
Non-irradiated
rutin
rutin
microcapsules
topical formulation

Figure 4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topical formulation
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Unit of catalase/
mg of skin / min.
Non-irradiated
rutin
rutin
microcapsules
topical formulation

Figure 5

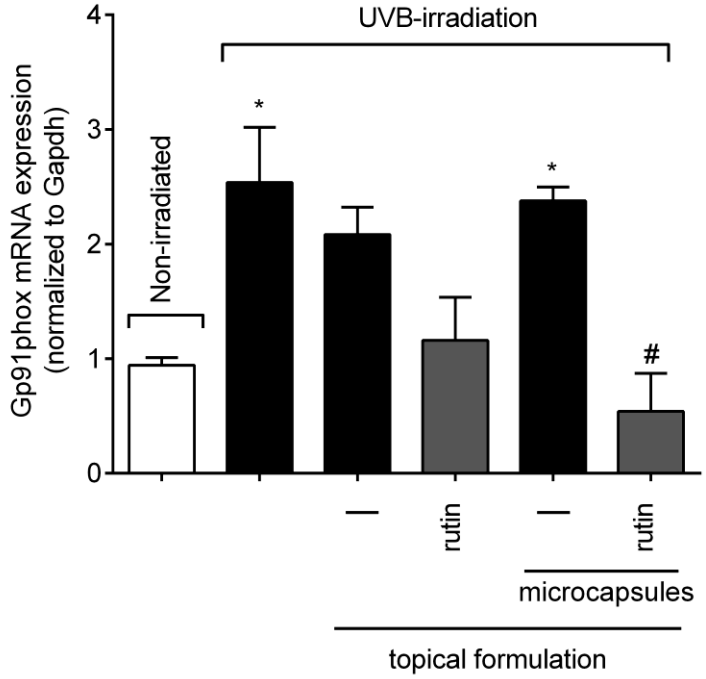


Figure 6

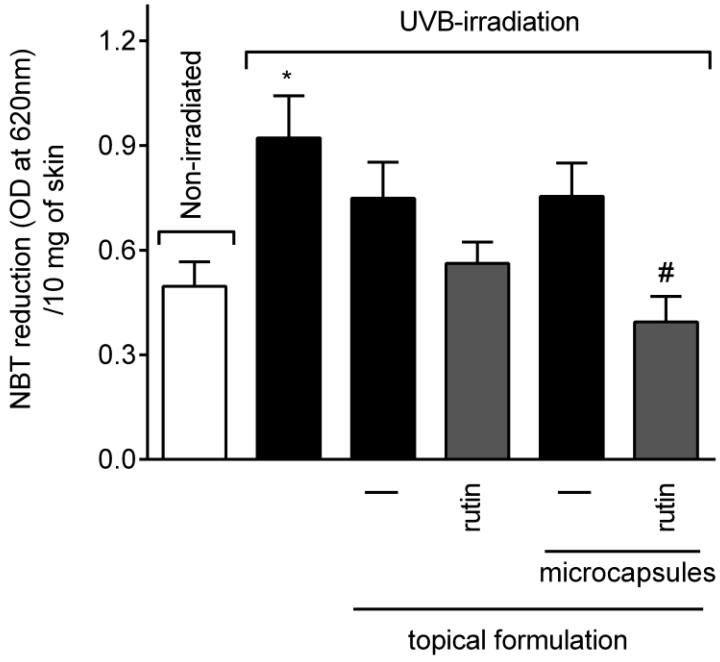


Figure 7

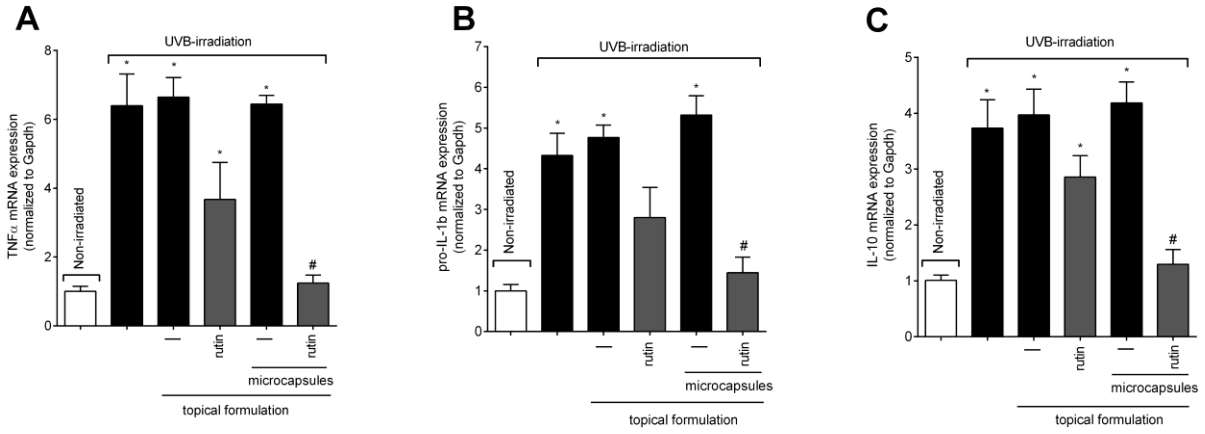
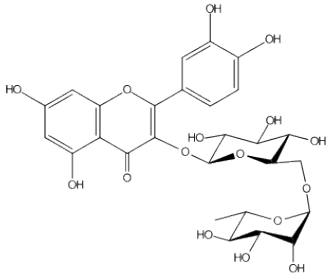

A
UVB-irradiation
TNFα mRNA expression
(normalized to Gapdh)
Non-irradiated
rutin
rutin
microcapsules
topical formulation
B
UVB-irradiation
pro-IL-1b mRNA expression
(normalized to Gapdh)
Non-irradiated
rutin
rutin
microcapsules
topical formulation
C
UVB-irradiation
IL-10 mRNA expression
(normalized to Gapdh)
Non-irradiated
rutin
rutin
microcapsules
topical formulation

Table of Contents Graphic

Topical Formulation Containing Microencapsulated Rutin Flavonoid Reduces Inflammation and Photooxidative Skin Damage Induced by UVB Irradiation.

Daniela C. de Medeiros, Renata M. Martinez, Sandra S. Mizokami, Felipe A. Pinho-Ribeiro, Sandra R. Georgetti, Marcela M. Baracat, Waldiceu A. Verri, Jr. , and Rubia Casagrande.



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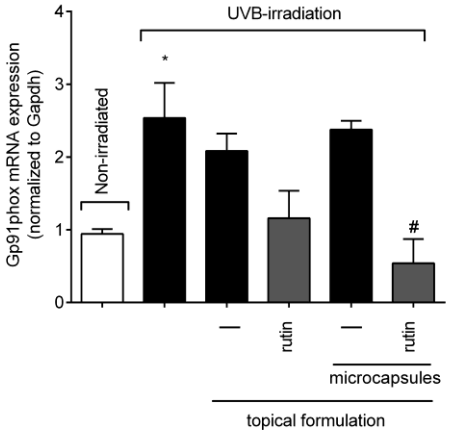

5 CONCLUSÕES GERAIS

No presente estudo, microcápsulas de pectina/caseína contendo FBP ou rutina foram preparadas utilizando o método de coacervação complexa. O processo tecnológico partindo do conjugado caseina/pectina utilizado neste trabalho é de baixo custo, realizado em meio aquoso e em condições brandas, o que significa uma grande vantagem em relação a muitos polímeros disponíveis no mercado. Aliado ao baixo custo está a possibilidade de produção das microcápsulas em grande escala.

Foi desenvolvido e validado um método confiável para quantificação da FBP microencapsulada. Ambas microcápsulas apresentaram forma esférica e distribuição de tamanho uniforme. O teste de dissolução in vitro demonstrou prolongamento da liberação da FBP a partir das microcápsulas comparado com o fármaco não microencapsulado. A FBP possui alto coeficiente de solubilidade no meio de preparação utilizado, característica esta que favorece sua difusão durante o processo de fabricação. Mesmo assim a tecnologia de microencapsulação mostrou-se promissora, porém, alguns fatores devem ser modulados para o desenvolvimento com moléculas hidrossolúveis.

As análises de DSC e difração de raio-X revelaram que a rutina está dispersa em matriz amorfa característica dos polímeros utilizados na preparação das microcápsulas, enquanto a FBP apresentou-se em estado amorfo após a microencapsulação. O prolongamento da liberação e mudança para estado amorfo da FBP mcroencapsulada resultou em uma melhora no efeito anti-inflamatório e analgésico do fármaco em modelo de inflamação na pata induzida por carragenina em camundongos. A presença de fase amorfa nas microcápsulas contendo rutina, mantendo a cristalinidade do fármaco, pode ser responsável pelo aumento da solubilidade da rutina proporcionando melhora da eficácia analgésica do fármaco, no mesmo modelo.

Os resultados obtidos no modelo de inflamação/estresse oxidativo induzido por radiação UVB demonstram que o tratamento com microcápsulas contendo rutina administradas topicamente foi eficaz na melhora dos parâmetros inflamatórios e na manutenção da capacidade antioxidante da pele. Sendo assim, a microencapsulação com pectina/caseína por coacervação complexa é uma estratégia promissora para melhorar os efeitos terapêuticos da FBP e rutina.

REFERÊNCIAS

AEBI, H. Catalase in vitro. **Methods Enzymol**., v. 105, p. 121–126, 1984.

AFAQ, F.; ADHAMI, V. M.; AHMAD, N. Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice. **Toxicology and Applied Pharmacology**, v. 186, p. 28-37, 2003.

AKIMITSU, T.; WHITE, J.A.; CARDEN, D.L.; GUTE, D.C.; KORTHUIS, R.J. Fructose-1,6-diphosphate or adenosine attenuate leukocyte adherence in postischemic skeletal muscle. **Am. J. Physiol**. v. 269, p. 1743-51, 1995.

ANVISA (Agência Nacional de Vigilância Sanitária) – RE 899/2003: Guia para validação de métodos analíticos e bioanalíticos.

ARORA, S.; BUDHIRAJA, R. D. Chitosan-alginate microcapsules of amoxicillin for gastric stability and mucoadhesion. **J. Adv. Pharm. Technol. Res.**, v. 3, n. 1, p. 68-74, 2012.

ARUOMA, O. I. Free radicals, oxidative stress, and antioxidants in human health and disease. **Journal of the American Oil Chemistry Society**, v. 75, n. 2, p. 199-212, 1998.

ATHAR, M.; AN, K. P.; TANG, X.; MOREL, K. D.; KIM, A. L.; KOPELOVICH, L.; BICKERS, D. R. Photoprotective effects of sulindac against ultraviolet B-induced phototoxicity in the skin of SKH-1 hairless mice. **Toxicology and Applied Pharmacology,** v. 195, p. 370-378, 2004.

AULTON, M. E. **Delineamento de formas farmacêuticas**, 2nd edn. Porto Alegre: Artmed, 2005.

AZAMBUJA, A. A.; LUNARDELLI, A.; NUNES, F. B.; GASPARETO, P. B.; DONADIO, M. V.; POLI DE FIGUEIREDO, C. E.; DE OLIVEIRA, J. R. Effect of fructose-1,6-bisphosphate on the nephrotoxicity induced by cisplatin in rats. **Inflammation**, v. 34, n. 1, p. 67-71, 2011.

BARACAT, M. M.; NAKAGAWA, A. M.; FREITAS, L. A. P.; FREITAS, O. Microcapsule Processing in a Spouted Bed. **Can J Chem Eng.**, v. 82, p. 134-41, 2004a.

BARACAT, M. M. Preparação e avaliação de microcápsulas a base de pectina USP. Faculdade de Ciências Farmacêuticas de Ribeirão Preto. **Tese**. 2004b.

BARACAT, M. M.; NAKAGAWA, A. M.; CASAGRANDE, R.; GEORGETTI, S. R.; VERRI, W. A. JR.; DE FREITAS, O. Preparation and characterization of microcapsules based on

biodegradable polymers: pectin/casein complex for controlled drug release systems. **AAPS PharmSciTech.**, v. 13, n. 2, p. 364-72, 2012.

BAILEY, E.; STIRPE, F.; TAYLOR, C. B. Regulation of rat liver pyruvate kinase. The effect of preincubation, pH, copper ions, fructose-1,6-diphosphate and dietary changes on enzyme activity. **Biochem. J**., v. 108, n. 3, p. 427-36, 1968.

BATHIA, N.; DEMMER, T. A.; SHARMA, A. K.; ELCHEVA, I.; SPIEGELMAN, V. S. Role of β-TrCP ubiquitin ligase receptor in UVB mediated responses in skin. **Archives of Biochemistry and Biophysics**, v. 508, p. 178-184, 2011.

BATYCKY, R. P.; HANES, J.; LANGER, R.; EDWARDS, D. A. A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. **J. Pharm. Sci.**, v. 86, n. 12, p. 1464-1477, 1997.

BEAR, W. L.; TEEL, R. W. Effects of citrus flavonoids on the mutagenicity of heterocyclic amines and on cytochrome P450 1A2 activity. **Anticancer Res**, v. 20, n. 5B, p. 3609-14, 2000.

BECHO, J. R. M.; MACHADO, H.; GUERRA, M. O. Rutina – Estrutura, Metabolismo e Potencial Farmacológico. **Revista Interdisciplinar de Estudos Experimentais**, v. 1, p. 21-25, 2009.

BIRT, D. F.; HENDRISCH, S.; WANG, W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. **Pharmacology Therapeut**., v. 90, p. 157-177, 2001.

BLOIS, M. S. Antioxidant determinations by the use of a stable free radical. **Nature**, v. 181, n. 4617, p. 1199-1200, 1958.

CAMPANINI, M. Z.; PINHO-RIBEIRO, F. A.; IVAN, A. L. M.; FERREIRA, V. S.; VILELA, F. M. P.; VICENTINI, F. T. M. C.; MARTINEZ, R. M.; ZARPELON, A. C.; FONSECA, M. J. V.; FARIA, T. J.; BARACAT, M. M.; VERRI, W. A.; GEORGETTI, S. R.; CASAGRANDE, R. Efficacy of topical formulations containing Pimenta pseudocaryophyllus extract against UVB-induced oxidative stress and inflammation in hairless mice. **J. Photochem. Photobiol. B Biol**., v. 127, p. 153–160, 2013.

CARINI, M.; ALDINI, G.; PICCONE, M.; FACINO, R. M. Fluorescent probes as markers of oxidative stress in keratinocyte cell lines following UVB exposure. **Il Farmaco**, v. 55, p. 526-534, 2000.

CASAGRANDE, R.; GEORGETTI, S. R.; VERRI, W. A. Jr; DORTA, D. J.; SANTOS, A. C.; FONSECA, M. J. V. Protective effect of topical formulations containing quercetin against UVB-

induced oxidative stress in hairless mice. **Journal of Photochemistry and Photobiology B:Biology,** v. 84, p. 21-27, 2006.

CASAGRANDE, R.; GEORGETTI, S. R.; VERRI, W. A., JR; BORIN, M. F.; LOPEZ, R. F. V.; FONSECA, M. J. V. In vitro evaluation of quercetin cutaneous absorption from topical formulations and its functional stability by antioxidant activity. **International Journal of Pharmaceutics**, v. 328, p. 183-190, 2007.

CHEN, S.; GONG, J.; LIU, F.; MOHAMMED U. Naturally occurring polyphenolic antioxidants modulate IgE-mediated mast cell activation. **Immunology**, v. 100, n. 4, p. 471-80, 2000.

CHOW, A. H. L.; HO, S. S. S.; TONG, H. H. Y.; MA, H. H. M. Parameters affecting in-liquid drying microencapsulation and release rate of cefaclor. **Int J Pharm**, v. 172, p. 113-25, 1998.

CHUNG, M. I.; GAN, K. H.; LIN, C. N.; KO, F. N.; TENG, C. M. Antiplatelet effects and vasorelaxing action of some constituents of Formosan plants. **J Nat Prod**, v. 56, n 6, p. 929-34, 1993.

CUNHA, T, M,; VERRI, W. A. Jr.; VIVANCOS, G. G.; MOREIRA, I. F.; REIS, S.; PARADA, C. A.; CUNHA, F. Q.; FERREIRA, S. H. An electronic pressure-meter nociception paw test for mice. **Braz J Med Biol Res.**, v. 37, n. 3, p 401–407, 2004.

CUNHA, T, M,; VERRI, W. A. Jr.; SILVA, J. S.; POOLE, S.; CUNHA, F. Q.; FERREIRA, S. H. A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. **PNAS**, v. 102, n. 5, p 1755–1760, 2005.

DAI, C.; WANG, B.; ZHAO, H.; LI, B. Factors affecting protein release from microcapsule prepared by liposome in alginate. **Colloids Surf B Biointerfaces**, v. 42, p. 253-8, 2005.

DE CARVALHO, J. S. Caracterização de filmes de caseína e caseína acilada. Dissertação Mestrado – Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto. 111p. 1996.

DE OLIVEIRA, J. R.; ROSA, J. L.; AMBROSIO, S.; BARTRONS, R. Effect of galactosamine on hepatic carbohydrate metabolism: protective role of fructose 1,6-bisphosphate. **Hepatology**, v. 15, n. 6, p. 1147-1153, 1992.

DE OLIVEIRA, L. M.; PIRES, M. G.; MAGRISSO, A. B.; MUNHOZ, T. P.; ROESLER, R.; DE OLIVEIRA, J. R. Fructose-1,6-bisphosphate inhibits in vitro and ex vivo platelet aggregation

induced by ADP and ameliorates coagulation alterations in experimental sepsis in rats. **J Thromb Thrombolysis**, v. 29, n. 4, p. 387-394, 2010.

DESCHNER, E. E.; RUPERTO, J.; WONG, G.; NEWMARK, H. L. Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. **Carcinogenesis**, v. 12, n. 7, p. 1193-6, 1991.

DI CARLO, G.; AUTORE, G.; IZZO, A. A.; MAIOLINO, P.; MASCOLO, N.; VIOLA, P.; DIURNO, M. V.; CAPASSO, F. Inhibition of intestinal motility and secretion by flavonoids in mice and rats: structure-activity relationships. **J Pharm Pharmacol** , v. 45, n. 12, p. 1054-9, 1993.

DONG, Z. J.; XIA, S. Q.; HUA, S.; HAYAT, K.; ZHANG, X. M.; XU, S. Y. Optimization of cross-linking parameters during production of transglutaminase-hardened spherical multinuclear microcapsules by complex coacervation. **Colloids Surf B Biointerfaces***, v.* 63, p. 41-7, 2008.

FELL, J. T. Gastrointestinal transit of microcapsules. In: DONBROW, M. (ed.) **Microcapsules and nanoparticles in medicine and pharmacy.** Boca Raton, CRC Press, cap. 8, p. 159-165, 1992.

FREITAS, O.; MESTRINER-JR, W.; CONCEIÇÃO, E. C.; BARACAT, M. M.; MORESCHI, P. E. Processo de preparação de complexos biodegradáveis, complexos biodegradáveis assim obtidos e método para avaliação da eficiência mastigatória. **Brazil Patent, PI 0700557-1**, 2007.

FISHER, M. S.; KRIPKE, M. L. Suppressor T lymphocytes control the development of primary skin cancers in UV-irradiated mice. **Science**, v. 216, p. 1133-1134, 1982.

FISHER, G. J.; WANG, Z. Q.; DATTA, S. C.; VARANI, J.; KANG, S.; VOORHEES, J. J. Pathophysiology of premature skin aging induced by ultraviolet light. **N. Engl. J. Med.**, v. 337, p. 1419–1428, 1997.

FONSECA, Y. M.; CATINI, C. D.; VICENTINI, F. T. M. C.; NOMIZO, A.; GERLACH, R. F.; FONSECA, M. J. V. Protective effect of Calendula officinalis extract against UVB-induced oxidative stress in skin: Evaluation of reduced glutathione levels and matrix metalloproteinase secretion. **Journal of Ethnopharmacology**, v. 127, p. 596-601, 2009.

FUCHS, J. Potentials and limitations of the natural antioxidants RRR-alpha-tocopherolL-ascorbic L-ascorbic acid and b-carotene in cutaneous photoprotection**. Free Radical Biology and Medicine**, v. 25, n. 7, p. 848-873, 1998.

FUCHS, J. V.; ZOLLNER, T. M.; KAUFMANN, R.; PODDA, M. Redox-modulated pathways in inflammatory skin diseases. **Free Radical Biology & Medicine**, v. 30, p. 337-353, 2001.

GALAMBOS, L.; STURCHIO, J. L. Pharmaceutical firms and the transition to biotechnology: A study in strategic innovation. **Business History Review**, v. 72, n. 2, p. 250-278, 1998.

GEORGETTI, S. R.; CASAGRANDE, R.; VERRI-JR, W. A.; LOPEZ, R. F. V.; FONSECA, M. J. V. Evaluation of *in vivo* efficacy of topical formulations containing soybean extract. **International Journal of Pharmaceutics**, v. 352, p. 189-196, 2008.

GRUNDY, J. S.; FOSTER, R. T. The nifedipine gastrointestinal system (GITS). Evaluation of pharmaceutical, pharmacokinetics and pharmacological properties. **Clinical Pharmacokinetics**, v. 30, n. 1, p. 28-51, 1996.

GUAZELLI, C. F.; FATTORI, V.; COLOMBO, B. B.; GEORGETTI, S. R.; VICENTINI, F. T.; CASAGRANDE, R.; BARACAT, M. M.; VERRI, W. A. JR. Quercetin-loaded microcapsules ameliorate experimental colitis in mice by anti-inflammatory and antioxidant mechanisms. **J Nat Prod.**, v. 76, n. 2, p. 200-8, 2013.

GUTTERIDGE, J. M. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. **FEBS Lett.**, v. 201, p. 291–295, 1986.

HANCOCK, B. C.; ZOGRAFI, G. Characteristics and significance of the amorphous state in pharmaceutical systems. **J Pharm Sci**. v. 86, n. 1, p. 1-12, 1997.

HECK, D. E.; GERECKE, D. R.; VETRANO, A. M.; LASKIN, J. D. Solar ultraviolet radiation as a trigger of cell signal transduction. **Toxicology and Applied Pharmacology**, v. 195, p. 288-297, 2004.

HERNANDES, J. M. M.; FREITAS, O.; PEREIRA, N. L. The potencial use of casein and casein modified as matrix delivery systems. **Bolletino Chemico Farmaceutico,** v. 137, p. 362-366, 1998.

HOLLMAN, P. C. H.; HERTOG, M. G. L.; KATAK, M. B. Analysis and health effects of flavonoids. **Food Chemistry**, v. 57, n. 1, p. 43-46, 1996.

HUBER, P. C.; ALMEIDA, W. P.; FÁTIMA, A. Glutationa e enzimas relacionadas: papel biológico e importância em processos patológicos. **Química Nova**, Revisão, 2008.

IVAN, A. L. M.; CAMPANINI, M. Z.; MARTINEZ, R. M.; FERREIRA, V. S.; STEFFEN, V. S.; VICENTINI, F. T. M. C.; VILELA, F. M. P.; MARTINS, F. S.; ZARPELON, A. C.; CUNHA, T. M.;

FONSECA, M. J. V; BARACAT, M. M.; GEORGETTI, S. R.; VERRI, W. A.; CASAGRANDE, R. Pyrrolidine dithiocarbamate inhibits UVB-induced skin inflammation and oxidative stress in hairless mice and exhibits antioxidant activity in vitro. **J. Photochem. Photobiol. B.**, v. 138C, p. 124–133, 2014.

JI, L.L. Antioxidant signaling in skeletal muscle: A brief review. **Experimental Gerontology**, v. 42, p. 582-593, 2007.

KATALINIC, V.; MODUN, D.; MUSIC, I.; BOBAN, M. Gender differences in antioxidant capacity of rat tissues determined by 2,2V-azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. **Comp. Biochem. Physiol. C**, v. 140, p. 47-52, 2005.

KILPATRICK, L. E.; SUN, S.; LI, H.; VARY, T. C.; KORCHAK, H. M. Regulation of TNF-induced oxygen radical production in human neutrophils: role of delta-PKC. **Journal of Leukocyte Biology**, v. 87, p. 153-164, 2010.

KIM, K. H.; CHO, S. A.; LIM, J. Y.; LIM, D. G.; MOON, C.; JEONG, S. H. Preparation of microcapsules with the evaluation of physicochemical properties and molecular interaction. **Arch Pharm Res.**, v. 37, n. 12, p. 1570-7, 2014.

KUMAR, V.; ABBAS, A. K.; FAUSTO,N.; ASTER, J.C. **Robbins and Cotran Pathologic Basis of Disease**, 8º edition. Philadelphia: Saunders, 2010.

LEHNINGER, A. L. **Princípios de bioquímica**. São Paulo: Savier, 1984.

LINHARD, R. Biodegradable polymers for controlled release of drugs. In: ROSOFF, M. **Controlled Release of Drugs: Polymers and aggregate systems.** New York: VCH Publisher Inc., p.53-85, 1988.

LOGUERCIO, C.; NARDONE, G.; SICOLO, P.; CUOMO, R.; DEL VECCHIO, C.; BUDILLON, G. Intravenous load of fructose and fructose 1,6-diphosphate: effects on uricemia in patients with nonalcoholic liver disease. **Am. J. Gastroenterol.**, v. 91, n. 3, p. 559-64, 1996.

LORENZO-LAMOZA, M. L.; REMUÑÁN-LÓPEZ, C.; VILA-JATO, J. L.; ALONSO, M. J. Design of microencapsulated chitosan microspheres for colonic drug delivery. **Journal of Controlled Release,** v. 52, n. 1-2, p. 109-118, 1998.

LOWRY, O. H.; ROSE BROUGH, N. J.; FARR, A. L.; RANDALL, R. J. Protein measurement with the folin phenol reagent. **Journal of Biological Chemistry**, v. 193, p. 265-275, 1951.

MAGDALENA, L. C.; TAK YEE, A. W. Reactive oxygen species, cellular redox systems, and apoptosis. **Free Radical Biology and Medicine**, v. 48, n. 6, p. 749-762, 2010.

MANO, E. B.; MENDES, L. C. Os polímeros na composição de alimentos industriais. In: MANO, E. B.; MENDES, L. C. **Introdução a polímeros.** 2. ed. São Paulo: Edgard Blücher, cap. 17, p. 145-157, 1999.

MARKOV, A. K. Hemodynamics and metabolic effects of fructose 1-6 diphosphate in ischemia and shock--experimental and clinical observations. **Ann Emerg Med.**, v. 15, n. 12, p. 1470-1477, 1986.

MARONA, H. R. N.; SCHAPOVAL, E. E. S. A high-performance liquid chromatographic assay for sparfloxacin. **Journal of Pharmaceutical and Biomedical Analysis**, v. 20, p. 413-417, 1999.

MEDEIROS, D. C. **Desenvolvimento de microesferas de diclofenado de sódio de liberação prolongada - Avaliação do potencial de utilização de blendas de acetobutirato de celulose e poloxamers na modulação do perfil de liberação do fármaco.** 129 f. Dissertação (Mestrado em Farmácia) – Universidade Federal de Santa Catarina, Florianópolis. 2003.

MELONI, M.; NICOLAY, J. F. Dynamic monitoring of glutathione redox status in UV-B irradiated reconstituted epidermis: effect of antioxidant activity on skin homeostasis. **Toxicology in vitro**, v. 17, p. 609-613, 2003.

MONTENEGRO, L.; BONINA, F.; RIGANO, L.; GIOGILLI, S.; SIRIGU, S. Protective effect evaluation of free radical scavengers on UVB induced human cutaneous erythema by skin reflectance spectrophotometry. **International Journal Cosmetics Sciences**, v. 17, p. 91-103, 1995.

MORON, M. S.; DEPIERRE, J. W.; MANNERVIK, B. Levels of glutathione, glutathione reductase and glutathione s-transferase activities in rat lung and liver. **Biochimica et Biphysica Acta**, v. 582, p. 67-78, 1979.

PANASIAK, W.; WLEKLIK, M.; ORACZEWSKA, A.; LUCZAK, M. Influence of flavonoids on combined experimental infections with EMC virus and Staphylococcus aureus in mice. **Acta Microbiol Pol**, v. 38, n. 2, p. 185-8, 1989.

PLANAS, M. E.; SANCHEZ, S.; GONZALEZ, P.; DE OLIVEIRA, J. R.; BARTRONS, R. Protective effect of fructose-1,6-bisphosphate against carrageenan induced inflammation. **Eur J Pharmacol.**, v. 237, n. 2-3, p. 251–255, 1993.

PODDA, M.; TRABER, M. G.; WEBER, C.; YAN, L. J.; PACKER, L. UV – irradiation depletes antioxidants and causes oxidative damage in model of human skin. **Free Radical Biology & Medicine**, v. 24, n.1, p. 55-65, 1998.

POSSEBON M. I.; MIZOKAMI, S. S.; CARVALHO, T. T.; ZARPELON, A. C.; HOHMANN, M. S.; STAURENGO-FERRARI, L.; FERRAZ, C. R.; HAYASHIDA, T. H.; DE SOUZA, A. R.; AMBROSIO, S. R.; ARAKAWA, N. S.; CASAGRANDE, R.; VERRI, WA JR. Pimaradienoic acid inhibits inflammatory pain: inhibition of NF-κB activation and cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. **J Nat Prod.** 2014;77(11):2488-96.

QUAN, T.; QIN, Z.; XIA, W.; SHAO, Y.; VOORHEES, J. J.; FISHER, G. J. Matrix-degrading metalloproteinases in photoaging. **Journal of Investigative Dermatology Symposium Proceedings**, v. 1, p. 20-24, 2009.

RATTES, A. L. R.; OLIVEIRA, W. P. Spray drying conditions and encapsulating composition effects on formulation and properties of sodium diclofenac microparticles. **Powder Technology**, v.171, n. 1, p. 7-14, 2007.

RIDLEY, B. L.; O´NEILL, M. A.; MOHNEN, D. Pectins: structure, biosynthesis and oligogalacturonide – related signating. **Phytochemistry**, v. 57, p. 929 – 967, 2001.

RIEGER, M. Participation of metalloproteinases in photoaging. **Cosmetics & Toiletries,** v. 114, p. 65-70, 1999.

ROBBINS, D.; ZHAO, Y. The role of manganese superoxide dismutase in skin cancer. **Enzyme Research**, v. 1, p. 4092-95, 2011.

ROZET, E.; CECCATO, A.; HUBERT, C.; ZIEMONS, E.; OPREAN, R.; RUDAZ, S.; BOULANGER, B.; HUBERT, P. Analysis of recent pharmaceutical regulatory documents on analytical method validation. **Journal of Chromatography A**, v. 1158, p. 111-125, 2007.

RUBINSTEIN, A. Microbially controlled drug delivery to the colon. **Biopharmaceutics & Drug Disposition**, v. 11, n. 6, p. 465-475, 1990.

SAIJA, A.; TOMAINO, A.; TROMBETTA, D.; DE PASQUALE, A.; UCCELLA, N.; BARBUZZI, T.; PAOLINO, D.; BONINA, F. In vitro and in vivo evaluation of caffeic and ferulic acids as topical photoprotective agents. **International Journal of Pharmaceutics**, v. 199, p. 39-47, 2000.

SAWANE, M.; KIDOYA, H.; MURAMATSU, F.; TAKAKURA, N.; KAJIYA, K. Apelin Attenuates UVB-Induced Edema and Inflammation by Promoting Vessel Function. **American Journal of Pathology**, v. 179, n. 6, p. 2691-2697, 2011.

SELLOUM, L.; BOURICHE, H.; TIGRINE, C.; BOUDOUKHA, C.Anti-inflammatory effect of rutin on rat paw oedema, and on neutrophils chemotaxis and degranulation. **Exp Toxic Pathol**., v. 54, p. 313-318, 2003.

SILVA, R. R.; OLIVEIRA, T. T.; NAGEM, T. J.; LEÃO, M. A. Efeito de flavonóides no metabolismo do ácido araquidônico. Revisão. **Medicina-Ribeirão Preto**, n. 35, p. 127-133, 2002.

SRINIVASAN, P.; SABITHA, K. E.; SHYAMALADEVI, C. S. Attenuation of 4-Nitroquinoline 1-oxide induced in vitro lipid peroxidation by green tea polyphenols. **Life Sciences**, v. 80, p. 1080-1086, 2007.

STEENVOORDEN, D. P. T.; van HENEGOUWEN, G. M. J. The use of endogenous antioxidants to improve photoprotection. **Journal of Photochemistry and Photobiology B-Biology**, v. 41, n.1/2, p. 1-10, 1997.

TAVARES, J. R. P.; NASCIMENTO, R. F.; NEVES, M. F. S.; VARGAS, M. E. S.; SILVA, F. O.; ROSA, P. C. P. Cachaças comerciais envelhecidas e sem envelhecer: um perfil quantitativo do grau alcoolico e dos teores de cobre e de açucares. Rev. Bras. Farm., v. 84, n. 3, p. 87-90, 2003.

TOUITOU, E.; GODIN, B. Skin nonpenetrating sunscreens for cosmetic and pharmaceutical formulations. **Clinics in dermatology**, v. 26, p. 375-379, 2008.

VALÉRIO, D. A.; FERREIRA, F. I.; CUNHA, T. M.; ALVES-FILHO, J. C.; LIMA, F. O.; DE OLIVEIRA, J. R.; FERREIRA, S. H.; CUNHA, F. Q.; QUEIROZ, R. H.; VERRI, W. A. JR. Fructose-1,6-bisphosphate reduces inflammatory pain-like behaviour in mice: role of adenosine acting on A1 receptors. **Br J Pharmacol.**, v. 158, n. 2, p. 558-568, 2009.

VERRI, W. A. JR.; CUNHA, T. M.; PARADA, C. A.; POOLE, S.; CUNHA, F. Q.; FERREIRA, S. H. Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? **Pharmacol Ther.**, v. 112, n. 1, p. 116–138, 2006.

VERRI, W. A. JR.; VICENTINI, F. T. M. C.; BARACAT, M. M.; GEORGETTI, S. R.; CARDOSO, R. D. R.; CUNHA, T. M.; FERREIRA, S. H.; CUNHA, F. Q.; FONSECA, M. J. V.;

CASAGRANDE, R. **Studies in Natural Products Chemistry.** In: Atta-ur-Rahman, editor. Elsevier Amsterdam. 2012.

VERRI, W. A. JR.; BARACAT, M. M.; MEDEIROS, D. C.; MIZOKAMI, S.; CASAGRANDE, R.; FREITAS, O.; GEORGETTI, S. R. Sistema microencapsulado contendo rutina para terapéutica analgésica. Depósito de patente: BR 10 2013 015911 5, **INPI – Instituto Nacional de Propriedade Industrial**, 2013.

VICENTINI, T. M. C.; SIMI, T. R. M.; DEL CIAMPO, J. O.; WOLGA, N. O.; PITOL, D. L.; LYOMASA, M. M.; BENTLEY, M. V. L. B.; FONSECA, M. J. V. Quercetin in w/o microemulsion: *In vitro* and *in vivo* skin penetration and efficacy against UVB-induced skin damages evaluated *in vivo*. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 69, p. 948-957, 2008a.

VICENTINI, F. T. M. C.; CASAGRANDE, R.; VERRI-JR, W. A.; GEORGETTI, S. R.; BENTLEY, M. V. L. B.; FONSECA, M. J. V. Quercetin in Lyotropic Liquid Crystalline Formulations: Physical, Chemical and Functional Stability. **AAPS PharmSciTech**, v. 9, p. 591-596, 2008b.

VICENTINI, F. T. M. C.; HE, T.; SHAO, Y; FONSECA, M. J. V; VERRI JR, W. A.; FISHER, G. J.; XU, J. Quercetin inhibits UV irradiation-induced inflammatory cytokine production in primary human keratinocytes by suppressing NF-κB pathway. **Journal of Dermatological Science**, v. 61, p. 162-168, 2011.

WEI, H.; ZHANG, X.; WANG, Y.; LEBWOHL, M. Inhibition of ultraviolet light-induced oxidative events in the skin and internal organs of hairless mice by isoflavone genistein. **Cancer Letters**, v. 185, p. 21-29, 2002.

YANG, J. H.; MAU, J. L.; KO, P. T.; HUANG, L. C. Antioxidant properties of fermented soybean broth. **Food Chemistry**, v. 71, p. 249-254, 2000.

ZARPELON, A. C.; CUNHA, T. M.; ALVES-FILHO, J. C.; PINTO, L. G.; FERREIRA, S. H.; MCINNES, I. B.; XU, D.; LIEW, F. Y.; CUNHA, F. Q.; VERRI, W. A. JR. IL-33/ST2 signalling contributes to carrageenin-imduced innate inflammation and inflammatory pain: role of cytokines, endothelin-1 and prostaglandin E2. **Br J Pharmacol.**, v. 169, p. 90-101, 2013.

ANEXO A: Depósito de patente BR 10 2013 032 152 4

“Sistema microencapsulado contendo D-Frutose-1,6-difosfato para melhora da eficácia analgésica e anti-inflamatória do fármaco.”

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
PROTOCOLO GERAL
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INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Universidade Estadual de Londrina

1.2 Qualificação: Ensino Superior

1.3 CNPJ/CPF: 78.640.489/0001-53

1.4 Endereço Completo: Rod Celso Garcia Cid, PR 445 Km 380, Campus Universitário

1.5 CEP: 86055-900

1.6 Telefone: 43 33715812 1.7 Fax: 43 33715812

1.8 E-mail: aintecpi@uel.br

☒ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

Sistema microencapsulado contendo D-Frutose-1,6-difosfato para melhora da eficácia analgésica e anti-inflamatória do fármaco.

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

☐ continua em folha anexa

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 2/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: Waldiceu Aparecido Verri Junior

6.2 Qualificação: Docente CCB

6.3 CPF: 025.652.899-36

6.4 Endereço Completo: Rua Paranagua 1057, Centro - Londrina - PR

6.5 CEP: 86020-030

6.6 Telefone: 43 3323 4798 6.7 FAX:

6.8 E-mail: waldiceujr@yahoo.com.br

☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐
Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome: Marinete Violin

9.2 CNPJ/CPF: 533643179-68 9.3 API/OAB: 17033-PR

9.4 Endereço Completo: Rod Celso Garcia Cid, PR 445 Km 380, Campus Universitário

9.5 CEP: 86055-900

9.6 Telefone: 43 33715812 9.7 FAX: 43 33715812

9.8 E-mail: violin@uel.br

☐ continua em folha anexa

10. Listagem de sequências biológicas.
Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 3/3
Título do Documento: **Depósito de Pedido de Patente**		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	1
☒	11.2	Procuração.	01
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	09
☒	11.6	Reivindicações.	02
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, 01 por melhor representar a invenção (sujeito à avaliação do INPI).	04
☒	11.8	Resumo.	1
☐	11.9	Listagem de sequências em arquivo eletrônico: nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☒	11.13	Outros (especificar) RG, CPF, Declaração de cessão, Comp de vinculo, Ato de nomeação do Reitor, ANEXO	30

12. Total de folhas anexadas: 48 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Londrina, 13 de novembro de 2013

Local e Data

Assinatura e Carimbo

Marinete Violin
Procuradora Jurídica
OAB/PR 17.639

Anexo 1

1. Depositante (71):

1.1 Nome: Universidade Estadual de São Paulo

1.2 Qualificação: Ensino Superior

1.3 CNPJ/CPF: 63.025.530/0001-04

1.4 Endereço Completo: Av. Brasil, 1971, Jd. Paulista – São Paulo/SP

1.5 CEP: 01431-001

1.6 Telefone: (11) 3091 1578 1.7 Fax:

1.8 E-mail: pidireto@usp.br / Jussara@usp.br

Anexo 2

6. Inventor (72):

6.1 Nome: Marcela Maria Baracat

6.2 Qualificação: Docente

6.3 CPF: 118246918-31

6.4 Endereço Completo: Rua Antônio Pisicchio, 100, casa– Londrina/PR

6.5 CEP: 86050-482

6.6 Telefone: (43) 3334-0799

6.7 FAX: (43) 3371-5812

6.8 E-mail: bacarat@uel.br

6. Inventor (72):

6.1 Nome: Daniela Cristina de Medeiros

6.2 Qualificação: Doutoranda

6.3 CPF: 026791039-80

6.4 Endereço Completo: Rua Delaine Negro, 95, apto subsolo 11, bl D – Londrina, PR

6.5 CEP: 86055-680

6.6 Telefone: (43) 8441-6315

6.7 FAX: (43) 3371-5812

6.8 E-mail: danielamedeiros@hotmail.com

Anexo 3

6. Inventor (72):

6.1 Nome: Sandra Mizokami

6.2 Qualificação: Doutoranda

6.3 CPF: 045525849-01

6.4 Endereço Completo: Av. Juscelino Kubitschek, 2727, apto 303 – Londrina, PR

6.5 CEP: 86010-540

6.6 Telefone: (43) 9910-2575

6.7 FAX: (43) 3371-5812

6.8 E-mail: sandramizokami@hotmail.com

6. Inventor (72):

6.1 Nome: Rubia Casagrande

6.2 Qualificação: Docente

6.3 CPF: 276286868-85

6.4 Endereço Completo: Rua Paranagua, 1057, apto 403 – Londrina/PR

6.5 CEP: 86020-030

6.6 Telefone: (43) 33234798

6.7 FAX: (43) 3371-5812

6.8 E-mail: rubiacasa@yahoo.com.br

Anexo 4

6. Inventor (72):

6.1 Nome: Osvaldo de Freitas

6.2 Qualificação: Docente USP

6.3 CPF: 549732018-04

6.4 Endereço Completo: Rua Maranhão, 1865, Ipiranga – Ribeirão Preto/SP

6.5 CEP: 14055-600

6.6 Telefone: (16) 36331937

6.7 FAX: (43) 3371-5812

6.8 E-mail: ofreitas@fcfrp.usp.br

6. Inventor (72):

6.1 Nome: Sandra Regina Georgetti

6.2 Qualificação: Docente

6.3 CPF: 285977688-51

6.4 Endereço Completo: Rua Fernando de Noronha, 775, apto 701 – Londrina,PR

6.5 CEP: 86020-906

6.6 Telefone: (43) 33231890

6.7 FAX: (43) 3371-5812

6.8 E-mail: sangeorgetti@gmail.com

Anexo 5

6. Inventor (72):

6.1 Nome: Maria Antonia Pedrine Colabone Celligoi

6.2 Qualificação: Docente

6.3 CPF: 040.984.648-10

6.4 Endereço Completo: Av. Gil de Abreu e Souza, 1501 – Londrina/PR

6.5 CEP: 86055-570

6.6 Telefone: (43) 9995 799

6.7 FAX: (43) 3371-5812

6.8 E-mail: macelligoi@uel.br

6. Inventor (72):

6.1 Nome: Josiane Alessandra Vignoli

6.2 Qualificação: Docente

6.3 CPF: 278.450.908-75

6.4 Endereço Completo: Av. José Gabriel de Oliveira, 915, apto 1502/ TII – Londrina/PR

6.5 CEP: 86047-360

6.6 Telefone: (43) 9992 8110

6.7 FAX: (43) 3371-5812

6.8 E-mail: javignoli@uel.br

ANEXO B: Depósito de patente BR 10 2013 015 911 5

“Sistema microencapsulado contendo rutina para terapêutica analgésica.”

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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Título do Documento:		Código: FQ001	Versão: 2
Depósito de Pedido de Patente		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Universidade Estadual de Londrina

1.2 Qualificação: Ensino Superior

1.3 CNPJ/CPF: 78640489/0001-53

1.4 Endereço Completo: Rod Celso Garcia Cid s/n Campus Universitário Londrina/PR

1.5 CEP: 86055-990

1.6 Telefone: 43-33715812 1.7 Fax: 43-33715812

1.8 E-mail: aintecpi@uel.br

☒ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

Sistema microencapsulado contendo rutina para terapêutica analgésica

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponivel)	Data de depósito

☐ continua em folha anexa

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Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: Waldiceu Aparecido Verri Junior

6.2 Qualificação: Docente

6.3 CPF: 025652899-36

6.4 Endereço Completo: Rua Paranaguá 1057, apto 403 Londrina - PR

6.5 CEP: 86020-030

6.6 Telefone: 43-33234798 6.7 FAX: 43-33715812

6.8 E-mail: waldiceujr@yahoo.com.br

☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐
Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome: Marinete Violin

9.2 CNPJ/CPF: 533643179-68 9.3 API/OAB: 17033/PR

9.4 Endereço Completo: Rod. Celso Garcia Cid s/n Campus Universitário Londrina/PR

9.5 CEP: 86055-990

9.6 Telefone: 43-33715812 9.7 FAX: 43-33715812

9.8 E-mail: violin@uel.br

☐ continua em folha anexa

10. Listagem de sequências biológicas.
Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

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Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	1
☒	11.2	Procuração.	1
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	7
☒	11.6	Reivindicações.	2
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, ____ por melhor representar a invenção (sujeito à avaliação do INPI).	3
☒	11.8	Resumo.	1
☐	11.9	Listagem de sequências em arquivo eletrônico: ____ nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☒	11.13	Outros (especificar) CPF, RG, Declaração de cessão, Ato de nomeação do reitor	25

12. Total de folhas anexadas: 40 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Londrina, 04/06/13
Local e Data

Assinatura e Carimbo

Marinete Violin
Procuradora Jurídica
OAB/PR [illegible]

Anexo 1

6. Inventor (72):

6.1 Nome: Marcela Maria Baracat

6.2 Qualificação: Docente

6.3 CPF: 118246918-31

6.4 Endereço Completo: Rua Antônio Pisicchio, 100, casa– Londrina/PR

6.5 CEP: 86050-482

6.6 Telefone: (43) 3334-0799

6.7 FAX: (43) 3371-5812

6.8 E-mail: bacarat@uel.br

6. Inventor (72):

6.1 Nome: Daniela Cristina de Medeiros

6.2 Qualificação: Doutoranda

6.3 CPF: 026791039-80

6.4 Endereço Completo: Rua Delaine Negro, 95, apto subsolo 11, bl D –

Londrina, PR

6.5 CEP: 86055-680

6.6 Telefone: (43) 8441-6315

6.7 FAX: (43) 3371-5812

6.8 E-mail: danielamedeiros@hotmail.com

Anexo 2

6. Inventor (72):

6.1 Nome: Sandra Mizokami

6.2 Qualificação: Doutoranda

6.3 CPF: 045525849-01

6.4 Endereço Completo: Av. Juscelino Kubitschek, 2727, apto 303 – Londrina, PR

6.5 CEP: 86010-540

6.6 Telefone: (43) 9910-2575

6.7 FAX: (43) 3371-5812

6.8 E-mail: sandramizokami@hotmail.com

6. Inventor (72):

6.1 Nome: Rubia Casagrande

6.2 Qualificação: Docente

6.3 CPF: 276286868-85

6.4 Endereço Completo: Rua Paranagua, 1057, apto 403 – Londrina/PR

6.5 CEP: 86020-030

6.6 Telefone: (43) 33234798

6.7 FAX: (43) 3371-5812

6.8 E-mail: rubiacasa@yahoo.com.br

Anexo 3

6. Inventor (72):

6.1 Nome: Sandra Regina Georgetti

6.2 Qualificação: Docente

6.3 CPF: 285977688-51

6.4 Endereço Completo: Rua Fernando de Noronha, 775, apto 701 – Londrina,PR

6.5 CEP: 86020-906

6.6 Telefone: (43) 33231890

6.7 FAX: (43) 3371-5812

6.8 E-mail: sangeorgetti@gmail.com

6. Inventor (72):

6.1 Nome: Osvaldo de Freitas

6.2 Qualificação: Docente da USP

6.3 CPF: 549.732.018-04

6.4 Endereço Completo: Rua Maranhão, 1865, Ipiranga – Ribeirão Preto/SP

6.5 CEP: 14055-600

6.6 Telefone: (16) 3633-1937

6.7 FAX: (43) 3371-5812

6.8 E-mail: sofreitas@fcfrp.usp.br

ANEXO C: Depósito de patente BR 10 2014 020 701 5

“Formulação tópica contendo rutina microencapsulada para redução dos danos cutâneos foto-oxidativos induzidos pela irradiação UVB.”

< Uso exclusivo do INPI >

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Universidade Estadual de Londrina

1.2 Qualificação: Ensino Superior

1.3 CNPJ/CPF: 78.640.489/0001-53

1.4 Endereço Completo: Rod Celso Garcia Cid, PR 445 Km 380, Campus Universitário

1.5 CEP: 86055-900

1.6 Telefone: 43 33715812 1.7 Fax: 43 33715812

1.8 E-mail: aintecpi@uel.br

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

Formulação tópica contendo rutina microencapsulada para redução dos danos cutâneos foto-oxidativos induzidos pela irradiação UVB

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponivel)	Data de depósito

☐ continua em folha anexa

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 2/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: Waldiceu Aparecido Verri Junior

6.2 Qualificação: Docente CCB

6.3 CPF: 025.652.899-36

6.4 Endereço Completo: Rua Paranagua 1057, Centro - Londrina - PR

6.5 CEP: 86020-030

6.6 Telefone: 43 3323 4798 6.7 FAX: 43 3371 5812

6.8 E-mail: waldiceujr@yahoo.com.br

☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐
Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome: Marinete Violin

9.2 CNPJ/CPF: 533643179-68 9.3 API/OAB: 17033-PR

9.4 Endereço Completo: Rod Celso Garcia Cid, PR 445 Km 380, Campus Universitário

9.5 CEP: 86055-900

9.6 Telefone: 43 33715812 9.7 FAX: 43 33715812

9.8 E-mail: violin@uel.br

☐ continua em folha anexa

10. Listagem de sequências biológicas.
Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 3/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	1
☒	11.2	Procuração.	1
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	12
☒	11.6	Reivindicações.	2
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, 1 por melhor representar a invenção (sujeito à avaliação do INPI).	2
☒	11.8	Resumo.	1
☐	11.9	Listagem de sequências em arquivo eletrônico: ____nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☒	11.13	Outros (especificar) RG, CPF, Declaração de cessão, Comp de vinculo, Ato de nomeação do Reitor, anexo formulário.	28

12. Total de folhas anexadas: 43 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Londrina, 29 de julho de 2014
Local e Data

Assinatura e Carimbo
Marinete Violin
Advogada - OAB/PR 17.033

Anexo

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6.1 Nome: Marcela Maria Baracat

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PROCURAÇÃO

Pelo presente instrumento particular de procuração a **UNIVERSIDADE ESTADUAL DE LONDRINA**, pessoa jurídica de direito público interno, constituída sob a forma de autarquia, nos termos da Lei nº 9.663, de 16 de julho de 1991, com sede na cidade de Londrina, Estado do Paraná, no campus universitário, Rodovia Celso Garcia Cid (PR 445), Km. 380, inscrita no CNPJ/MF sob o nº 78.640.489/0001-53, neste ato representada, na forma de seus estatutos, por sua Reitora, Profa. Dra. **Berenice Quinzani Jordão**, brasileira, professora universitária, portadora do RG 6.070.116/SSP/SP, inscrita no CPF nº364.796.169-87, residente e domiciliado nesta cidade de Londrina, Estado do Paraná, constitui sua bastante procuradora a advogada **Marinete Violin**, brasileira, advogada, inscrita na OAB, Seção do Paraná, sob o nº 17033/PR, com endereço à Rodovia Celso Garcia Cid (PR 445), Km. 380, Campus Universitário, CP. 6001, em Londrina, Estado do Paraná, à qual confere os poderes da cláusula ***ad judicia eet extra*, especialmente para atuar perante o INPI - Instituto Nacional de Propriedade Industrial**, no pedido de patente cujo título é **"Formulação tópica contendo rutina microencapsulada para redução dos danos cutâneos foto-oxidativos induzidos pela irradiação UVB"**, podendo, para tanto, firmar todos os requerimentos, petições e documentos necessários, apresentar defesa, requerer providências, ter vista de processos e retirá-los em carga, enfim, propor todas as medidas necessárias para a defesa de quaisquer interesses e direitos relacionados com a referida invenção, inclusive substabelecer.

Londrina, 29 de junho de 2014.

UNIVERSIDADE ESTADUAL DE LONDRINA
Profa. Dra. Berenice Quinzani Jordão
Reitora

INPI Protocolo Geral
Protocolo Interno
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INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/1
Título do Documento: Declaração Negativa de Acesso ao Patrimônio Genético		Código: FQ012	Versão: 01
		Procedimento: DIRPA-PQ013	

Ao Instituto Nacional da Propriedade Industrial:

1. Interessado:

1.1 Nome: Universidade Estadual de Londrina

1.2 Qualificação: Ensino Superior

1.3 CNPJ/CPF: 78.640.489/0001-53

1.4 Endereço Completo: Rod Celso Garcia Cid, PR 445 Km 380, Campus Universitário

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☐ continua em folha anexa

2. Título da Invenção (54):

Formulação tópica contendo rutina microencapsulada para redução dos danos cutâneos foto-oxidativos induzidos pela irradiação UVB

☐ continua em folha anexa

3. Referência:

3.1 Nº Pedido: BR102014020703 5 3.2 Data de Depósito: 22/08/2014

4. Declaração na forma do § 2º do Artigo 3º da Resolução INPI Nº 69/2013:

Declaro ao INPI que o objeto do presente pedido de patente não foi obtido em decorrência de acesso a amostra do patrimônio genético nacional, realizado a partir de 30 de junho de 2000.

5. Procurador (74):

5.1 Nome: Marinete Violin

5.2 CNPJ/CPF: 533643179-68 5.3 API/OAB: 17033-PR

5.4 Endereço Completo: Rod Celso Garcia Cid, PR 445 Km 380, Campus Universitário

5.5 CEP: 86055-900

5.6 Telefone: 43 33715812 5.7 Fax: 43 33715812

5.8 E-mail: violin@uel.br

6. Declaro, sob as penas da Lei, que todas as informações acima prestadas são completas e verdadeiras.

Londrina-PR, 29/07/14
Local e Data

Assinatura e Carimbo
Marinete Violin
Advogada - OAB/PR 17.033

ANEXO D: Guia para autores - Journal of the brazilian chemical society

1. Introduction

The ***Journal of the Brazilian Chemical Society*** (***JBCS***) embraces all aspects of chemistry except education, philosophy and history. It is a medium for reporting selected original and significant contributions to new chemical knowledge. The Journal publishes **Articles, Communications**, **Short Reports**, **Reviews**, **Accounts** and **Letters**.
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Article should be comprehensive and critical accounts of a work in a given area. Although short articles are acceptable, the Editors strongly discourage fragmentation of a substantial body of work into a number of short publications. **Communication** should be restricted to reports of **unusual urgency** and **significance** or **interest**. They should be submitted with a **statement** from the authors as to **why the manuscript meets these criteria**. A manuscript will not be accepted if, in the opinion of the Editors, the principal content has previously been released or published in any other medium. The communication should not exceed 1500 words or occupy more than 3 pages of the Journal. To estimate the length of a communication, an average sized figure is counted as 100 words and separate formulae and lines of a table are counted as 8 words per line, including headings and horizontal rulings. Title, authors' names and literature references are not counted.
Short Report is meant to be a concise terminal report of studies of limited scope. Manuscripts submitted as articles or communications may, in some cases, be accepted as short reports. The standard of quality expected in short reports is the same as in articles.
Review is normally invited by the Editors. However, the Editors welcome suggestions for reviews considered suitable for the Journal. Be aware that the **topics** (items) in the **Reviews** must be **numbered** with Arabic numerals.
In order to help the Editors in the evaluation of the suitability of a proposed Review, the authors should previously submit by e-mail (office@jbcs.sbq.org.br) the following items:

- A synopsis including a brief outline of the Review content;
- At least ten sample references;
- A summary of the lead author's academic career;
- A statement explaining the relevance of the topic to be reviewed and a list of the latest reviews published on the subject, if any;
- If the text is already prepared (with the above items have been considered by the Editors), an invitation for submission will be sent for the author;
- A short Curriculum Vitae (max. 100 words) with photo for each author needs to be added in the end of the main document.

Acceptance of the synopsis does not guarantee publication of the final manuscript.
It is quite common, in Reviews, the reproduction of figures, schemes and photos already published in other works. Even if these materials have been published by the same authors, copyright permissions need to be given by the editorial office.
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In order to help the Editors in the evaluation of the suitability of a proposed Account, authors should previously submit by e-mail (office@jbcs.sbq.org.br) a synopsis considering the following items:

- submission of a focused and readable text, covering current areas of interest for the Chemistry community;
- it is necessary to present topics or summaries of research in an emerging area of Chemistry, covering only the most interesting/significant developments;
- in the conclusion section, the discussion is about possible future approaches of the Account subject;
- a short Curriculum Vitae (max. 100 words) with photo for each author needs to be added in the end of the main document.

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Letter is a medium for the expression of scientific opinions and views normally concerning material published in the Journal, but not for revision/update of the authors' own work. When a **Letter** polemical in nature is accepted, a reply from the implicated parties will be requested for publication alongside the original **Letter**. Contributions in this format are intended to be published as soon as possible. No Abstract is required for letters. They should not exceed one printed page in length.

1.2 Before Beginning the Submission

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Authors should present their materials with the utmost conciseness and clarity. The **Introduction** should clearly and briefly identify, with relevant references, both the nature of the problem under investigation and its background. Extensive reviews of the literature cannot be accepted.

In **Articles** and **Short Reports**, the **Experimental** section may precede or follow the **Results and Discussion** section, but should be separated from it. The addition of a final section at the end of the manuscript, which briefly summarizes the main **Conclusions** of the work, is recommended and needs to be just after the **Results and Discussion** section. **Descriptions of experiments** should be given in sufficient details to enable other researchers to repeat them. The degree of purity of materials should be given, as well as all quantities used. Descriptions of established procedures are unnecessary. Standard techniques and methods used throughout the work should be stated at the beginning of the section in a **Materials and/or Methods** subsection, in the **Experimental** section. Apparatus should be described only if it is non-standard. Commercially available instruments should be referred to by their suppliers and models.

All **new compounds** should be fully characterized, which includes spectroscopic data and elemental analyses. High-resolution mass spectra may substitute for elemental analyses if accompanied by unequivocal proof of sample purity (melting points, copies of NMR spectra, etc.). For compounds prepared in enantiomerically pure or enantiomerically enriched form, specific optical rotation must be given. In cases where enantiomeric excess is determined by chromatographic and/or spectroscopic techniques, copies of the appropriate chromatograms and/or spectra should be included as Supplementary Information upon submission of the manuscript. Data associated with specific compounds should be listed after the name of the compound concerned, followed by the description of the preparation, or else presented in tabular form in the **Results and Discussion** section. All spectra must be included in the **Supplementary Information** (**SI**, see Section 8).

Many theoretical and computational papers use a routine procedure based on a well-documented method, being it semi-empirical or *ab initio*. It is then sufficient to name the particular variant, referring to key papers, in which the method has been developed, to cite the computer program used and to indicate briefly any modification made by the author. Complementary data meant to support the analysis of **Communications** should be included as **Supplementary Information** (SI, see Section 8).

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2. Preparation of Manuscritps

General Overview:

Font: Times New Roman
Font Size: 12
Font Color: Black
Spacing: double spaced
Pages: numbered consecutively

Tables, **Schemes**, **Figures** and **captions**: placed in the text, as close as possible to the first citation.

Figures: numbered with Arabic numerals. For full manuscripts containing material previously published in preliminary form, a copy of the previous communication is required and should be included at the end of the manuscript.

Maps: insert as **Supplementary Information**

Main sections (Introduction, Experimental, Results and Discussion, Conclusion section) of the manuscript should NOT be numbered, EXCEPT for Account and Review.

Supplementary Information (SI): needs to be included at the end of manuscript, after the **Conclusions** section. It should contain RELEVANT and COMPLEMENTARY DATA to those presented in the manuscript. If new compounds are identified or characterized, **all spectra** should be included (see Section 8).

Graphics/Figures/Schemes: send them in the original program FILES: it is important that the files are editable to correct any minor mistake.

Structures in: *.cdx (ChemDraw);
Graphics in: *.opj/org (Origin); *.xls/xlsx (Excel);
Others in: *.cdr (CorelDraw);

We do not accept graphs and chemical structures as image files.

Details:

First Page

- **Graphical Abstract (GA)** (see Section 5)

Second Page

- **Title**
- **Authors' names:** full given name, followed by the middle name initial(s) and then by the full last name. **An asterisk** (*) should follow the name of the corresponding author.
- **Addresses:** Authors are asked to provide full addresses for correspondence. The e-mail address of the corresponding author should be given as a footnote. If the address where the work was carried out is different from the present address of any of the authors, a footnote indicating the current position can be included. Each address should have a correspondent letter. As for instance:

Jailson B. de Andrade,*[,a] Marta V. Andrade[b] and Heloisa L. C. Pinheiro[c]

Third Page

Abstracts: maximum of 150 words for Articles, Accounts and Reviews and 50 words for Short Reports and Communications.
Keywords: a minimum of three and maximum of five. Broad-sense words such as "water" should be avoided.
The text should start from the third page of the manuscript.
Attention: all nomenclature should be consistent, clear, unambiguous and in accordance with the nomenclature rules established by the IUPAC, the International Union of Biochemistry, the Abstracts Service (see Index Guide to Chemical Abstracts, 1987 and http://jbcs.sbq.org.br/iupac.html), the Nomenclature Committee of the American Chemical Society or any other appropriate bodies. Units and symbols should follow IUPAC recommendations. Authors will not be denied any reasonable usage, but if non-SI units are used for critical data or for quantities measured to a high degree of accuracy, final numerical values should also be expressed in SI units.
Be sure that all abbreviations are once specified (as near as possible of their first citation).

3. Language, Style and Format

- **Language**

Only manuscripts written in **English** will be considered. Standard English and American English spellings are allowed but consistency should be maintained within the manuscript.
From now on, all authors are expected to send along with their manuscript a statement from a specialized company (or person), attesting that the text was submitted to **formal English review**. Otherwise, the Editor can, at any time, ask for such procedure to warrant the English precision, conciseness and understanding of the manuscript.

- **Style and Format**
- **Main Sections:** First initial with capital letter, bold, no final full stop. Should not be numbered, except for Reviews and Accounts:
  - **Introduction**
  - **Experimental** (or **Methodology** in case of theoretical and computational papers)
  - **Results and Discussion** or **Results** then **Discussion** (alternatively, Experimental may follow Results and Discussion)
  - **Conclusions**
  - **Supplementary Information** (if you have): include the following text just to mention (not to add graphs and data here) the existence of the supplementary data, see the example:

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

- **Acknowledgments**
- **References**

- **Sub-Sections:** first initial with capital letter, no final full stop. Examples:

Reagents and equipments
X-ray data

- **Formulae (compounds):** should be numbered with bold Arabic numerals.
- **Structural** or **displayed formulae** must be accurately drawn and inserted in the text. All captions should be typed below the structural or displayed formulae, together with it, in the right position.

4. Guidelines for Illustrations

General Size

The authors should think about the illustration size for double column (172 mm) of the journal. But, the font type size of text must be consistent with the illustration since it can be reduced during preparation of the Galley Proof. This is important when choosing symbols for graphics, drawings, charts, photos, etc., be consistent, make your manuscript look nicer: use the same size and same font type in graphics, schemes, etc.

4.1 Graphs and Figures (also see Section 2)

Lines and Lettering: Lines should be black and of an adequate and even thickness. Solid, broke, dotted and dot-dash lines should be used in graphics. Particular care should be taken to ensure that the lines in a spectrum are of adequate thickness. Lettering should not be smaller than 7 pt (Times New Roman) and lines not thinner than 0.5 pt. Lettering and lines should be of uniform density throughout the figures.
Labeled atoms in ORTEP (or any other) diagrams should have atom numbers in parenthesis, e.g., Fe(1), C(44).
Symbols representing physical quantities should be given in italics, e.g., *J* (Hz), δ (ppm), *m/z*, etc.
Units should be expressed in the appropriate form, e.g., g cm^{-3} or mol L^{-1}, rather than g/cm^3 or mol/L (see Section 4.5)
Graphs
- **Scales:** graphs should have only the minimum necessary scale divisions marked by numerals.
- **Axis labels** should use SI units, separated from quantities (see details in the green book http://old.iupac.org/reports/1993/homann/index.html):

For graphs, use slashes in X and Y axes to separate axes names from units. For example: 2θ / degree; Temperature / ^oC; time / min; Size range / mm; Wavenumber / cm^{-1}. Use parentheses only to group a set of units, e.g., Concentration / (mol L^{-1}) ; 10^3 $(T/K)^{-1}$, etc.
Pay close attention to the way decimal values are expressed in English. Employ dots instead of commas.
Figures must have a high quality in order to be well reproduced. Use at least a 900 dpi resolution. If necessary, resize to a smaller size to get higher quality.
Curves should be labeled (a), (b), (c) etc. and further information be given in the figure legend/caption.
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Graphs/Figures should be pasted from their original files (Origin, ChemDraw, Corel etc.) and have an excellent quality. If you have to digitalize (scan) the figures (photos, for instance), choose the following scan options: black & white (B&W), no background and minimum of 300 dpi. If you wish them to be published online in color, send both the colored and B&W versions to the Editorial Office, matching the captions of the figures to accommodate the alternatives. For computer-generated artwork, background or shadings should be avoided.

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Structures should be numbered with bold Arabic numerals, e.g.,**1**, **2**.
All chemical structures included in the manuscript should be drawn using the same letter type (Times New Roman or Arial), size of cyclic groups, size and thickness of chemical bonds, and, the most important, authors should use the same standard throughout the work, including all figures, schemes, etc.
The following organic group abbreviations may be used: Me, Et, ^{n}Pr, ^{n}Bu, ^{s}Bu, ^{t}Bu, Ph, CO^2R, CO^2H, ^{i}PrOH.
One variable univalent substituent is indicated by R. When more than one independent variable general substituent is present, R^1, R^2, R^3, etc. should be used.
A variable metal may be indicated by M and variable ligands by L^1, L^2, L^3 or L1, L2, L3, etc.

4.3 Photographs

Photographs should be highly contrasted, positive and not mounted.
When necessary, the scale should be drawn on the photograph itself and not below.
Color prints are rarely reproduced satisfactorily in black and white. Original B&W photographs are preferred to report experimental results, such as electron micrographs or to illustrate special equipment adaptations.

4.4 Colored Illustrations

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From 2010 onwards, the publication of colored illustrations will be totally free of charge in the ONLINE version of the Journal.
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Black & White (B&W) illustrations are free of charge. If color figures are presented in your Manuscript (Ms), they will automatically be converted into black-and-white (except GA). Color prints rarely reproduce satisfactorily in black and white. Thus, pay attention so that no information is missed because of the conversion. If the authors want to have colored illustrations on the printed version, they will be asked to pay for their cost: the current fee is 250 USD for all figures (remember that in the online version, they are free of charge).

4.5 Tables, Data and Units

Tables
Format your table to give straightforward information to the reader. Do not use shades or bold lettering. Indicate any extra

information as a footnote with letters, e.g., a, b, c, etc. For examples, see any "PDF" files in:http://jbcs.sbq.org.br/forthcoming_papers.asp.

Data

For negative numbers, ions and equations in text and tables use − (negative symbol) instead of - (hyphen). Examples: Cl^-, −0.40, $y = ax - b$.

Units

Use International System Units (SI), e.g., m, s, kg, Pa, mol L^{-1}, etc, separated from quantities with a blank space. Example: 300 K, not 300K. See: http://old.iupac.org/reports/1993/homann/index.html.

Note: Molar (M) is no longer a valid concentration expression for IUPAC; it is suggested mol L^{-1} or mol dm^{-3}, but be consistent throughout your manuscript.

For examples, see any "PDF" files in: http://jbcs.sbq.org.br/forthcoming_papers.asp

5. Graphical Abstract (GA) and Text for GA

Concerning the ***JBCS*** Table of Contents, it is expected from authors careful with their **Graphical Abstract (**GA) proposition. This way, the figure should summarize the content of the manuscript in a concise, pictorial form, designed to capture the attention of a wide readership. The author should present a new figure, using as an idea a key structure, a reaction, an equation, a concept, a graphic, a theorem, etc. It should use colors as much as possible and have an artistic and imaginative idea. Short movies are also welcome (as supplementary information (SI)). It is not acceptable photos of commercial equipment in GA or in the text of the manuscripts.

Pay Attention: the image should have a 900 dpi resolution (*.tiff / *.jpg or any other image file that can be edited and be 8 cm wide and 4 cm high). Along with the GA figure, insert a short explanatory text about it below (three lines at the most). Take a look at our recent publications whose Table of Contents presents Graphical Abstracts (http://jbcs.sbq.org.br). Therefore, be smart to advertise your manuscript: send a beautiful and appealing graphical image.

6. Equations

When writing equations, use the Word editing equation option or any other equation editor. Equation cannot be added in the main text as image format.

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- **Reference numbers**

Reference numbers in the **text** should be typed consecutively as superscripts after punctuation, without parentheses or brackets. Examples:

sodium salicylate,[1-3]

Nishide *et al.*,[4]

by reduction of chromic acid.[4-8,12]

The cited literature should be listed on a separate page (double-spaced) in the same order it appears in the text.

- **Journal Titles**

Journal title abbreviations are those defined in the Chemical Abstracts Service Source Index (see http://www.cas.org/content/references/corejournals). If an authoritative abbreviation for a Journal cannot be located or if the abbreviation is not obvious, the full Journal title should be cited.

- **Style Rules for Year, Volume and Page**

#. Author, A. C.; Author B.; Author C. F.; *Abbreviation of the Journal* **Year**, *Volume*, Page.

1.Author, A. C.; Author, B.; Author, C. F.; *J. Braz. Chem. Soc*. **2010**, *21*, 77.

- Author initials should be separated from each other, e.g., Author, A. C.;

- Use semi-colons to separate different author's names. No "and" is necessary in any case.

- *Journal Abbreviations* should come inItalics: *J. Braz. Chem. Soc.*

- **Years** - bold font: **2010**

- *Volume* - Italic style: *21*

- Page - only the initial page, followed by dot: 77.

Examples:

2. Varma, R. S.; Singh, A. P.; *J. Indian Chem. Soc*. **1990**, *67*, 518.

In case the journal is not easily accessible, the best choice is to quote its Chemical Abstracts number, as follows

3. Provstyanoi, M. V.; Logachev, E. V.; Kochergin, P. M.; Beilis, Y. I.; *Izv. Vyssh. Uchebn. Zadev.; Khim. Khim. Tekhnol.***1976**, *19*, 708. (*CA 85:78051s).*

Pay attention to the connection words in the names, as for instance: da Silva, M. A. or Silva, M. da, as follows:
4. Pinto, A. C.; de Andrade, J. B.; *Quim. Nova* **1999**, *22*, 448.

- **Composite References**

They should be used whenever possible, rather than a series of individual references, without letters (a), (b), (c), etc. Use only a semi-colon to separate them. The style for composite references is as follows:
5. Varela, H.; Torresi, R. M.; *J. Electrochem. Soc*. **2000**, *147*, 665; Lemos, T. L. G.; Andrade, C. H. S.; Guimarães, A. M.; Wolter-Filho, W.; Braz-Filho, R.; *J. Braz. Chem. Soc.* **1996**, *7*, 123; Ângelo, A. C. D.; de Souza, A.; Morgon, N. H.; Sambrano, J. R.; *Quim. Nova* **2001**, *24*, 473.

- **Patents**

They should be identified in the following form. Whenever possible, Chemical Abstracts numbers should be quoted in parentheses:
6. Hashiba, I.; Ando, Y.; Kawakami, I.; Sakota, R.; Nagano, K.; Mori, T.; *Jpn. Kokai Tokkyo Koho 79 73,771* **1979**. *(CA 91:P193174v)*
7. Kadin, S. B.; *US pat. 4,730,004* **1988** *(CA 110:P23729y)*.
8. Eberlin, M. N.; Mendes, M. A.; Sparrapan, R.; Kotiaho, T.; *Br PI 9.604.468-3* **1999**.

- **Books**

9. Cotton, F. A.; Wilkinson, G.; *Advanced Inorganic Chemistry*, 5th ed.; Wiley: New York, USa, 1988.

Chapter in a book: only the main title should be given, with the chapter author's name and the editor´s name after the title (this in italic):
10. Regitz, M. In *Multiple Bonds and Low Coordination in Phosphorus Chemistry*; Regitz, M.; Scherer, O. J., eds.; Georg Thieme Verlag: Stuttgart, Germany, 1990, ch. 2.

- **Software**

11. Sheldrick, G. M.; *SHELXL-93*; *Program for Crystal Structure Refinement*; University of Göttingen, Germany, 1993.

- **Web Pages**

12. http://www.sbq.org.br/jbcs, accessed in June 2013.

- **Unpublished material Reference**

For material **accepted** for publication: in this case, the DOI number should be provided by the authors.
13. Magalhães, U. H.; *J. Braz. Chem. Soc.*, DOI xx.

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8. Supplementary Information (SI)

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Deposition of Crystallographic Data
Prior to the submission of the typescript including crystallographic data, the author(s) should deposit, in the relevant Data Center, the data corresponding to each structure to be reported.
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Data for inorganic compounds should be sent to Fachinformationszentrum Karlsruhe (FIZ) by e-mail: crysdata@FIZ-Karlsruhe.de.

Deposition Codes

The Data Centers will provide deposition codes for each data set, which should be quoted in the typescript under a Supplementary Information heading before the Acknowledgements.

Standard text for CCDC:

Crystallographic data (excluding structure factors) for the structures in this work were deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC XXXXXX. Copies of the data can be obtained, free of charge, via www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033. E-mail: deposit@ccdc.cam.ac.uk.

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8.1.2 The chemical formula should correspond to the complete chemical unit encompassing the crystallographic symmetry, the formula weight, F(000), the absorption coefficient and the measured and calculated densities.

8.1.3 The unit cell parameters with esd's and the X-ray wavelength used.

8.1.4 The crystal system, space group and number of chemical units per cell.

8.1.5 Type of diffractometer used and method of data collection, total number of data collected, number of unique reflections, R(int) value, number of observed reflections with cut-off parameter, use or not of absorption correction, transmission factors.

8.1.6 The final results: R, wR, S and the number of parameters refined; treatment of hydrogen atoms; final peak and hole in the last difference map. Only refinements on F2 will be accepted.

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It must include a labeled diagram of the structure, a list of relevant geometric parameters - interatomic bond distances and angles, torsion angles, hydrogen bond parameters, etc. Data of less important parts of the structure, such as ligand sub-groups (phenyl rings, etc.) should be omitted.

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Whenever a compound is synthesized or identified (new or already known), it is imperative to send all spectral data (data and spectra) as Supplementary Information (SI) along with your submission, at the end of your doc file.

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(ii) Figures/Schemes (just the ones from the main document), including GA image: as jpg, tiff, opj, xls, etc (not as individual doc files or grouped in a doc file).

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Two authors:

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Three or more authors: Huestis MA et al. Monitoring opiate use in substance abuse treatment patients with sweat and urine drug testing. *J Anal Toxicol* 2000; 4(Suppl.3): 509–521.

Article in press:

Ladines CA et al. Impaired renal D1-like and D2-like dopamine receptor interaction in the spontaneously hypertensive rat. *Am J Physiol Regul Integr Comp Physiol* 2008 (in press).

Electronic publication ahead of print:

Teeuwen PHE. Doppler-guided intra-operative fluid management during major abdominal surgery: a systematic review and meta-analysis. *Int J Clin Pract* (accessed 21 November 2007, epub ahead of print).

Online serial:

Margolis PA et al. From concept to application: the impact of a community-wide intervention to improve the delivery of preventive services to children. Pediatrics [online] 2001; 108:e42. www.pediatrics.org/cgi/content/full/108/3/e42 (accessed 20 September 2001).

Corporate author:

The Cardiac Society of Australia and New Zealand . Clinical exercise stress testing. Safety and performance guidelines. *Med J Aust* 1996; 164: 282–284.

Anonymous author:

Anon. Coffee drinking and cancer of the pancreas. *BMJ* 1981; 283: 628.

Author with prefix and/or suffix in their name:

Humphreys Jnr, Sir Robert and Adams T. Reference style in the modern age. *J Bib Cit* 2008; 1: 1–10.

Article not in English:

Sokolov S et al. [Studies of neurotropic activity of new compounds isolated from *Rhodiola rosea* L.] Khim Farm Zh 1985; 19: 1367–1371 [in Russian].

Book references

Book by a single author or group of authors working together as a single author:
Cole MD, Caddy B. *The Analysis of Drugs of Abuse: An instruction manual*, 2nd edn. New York : Ellis Horwood, 1995.
An edited book:
Hoepfner E et al. eds. *Fiedler Encyclopedia of Excipients for Pharmaceuticals*, Cosmetics and Related Areas, 5th edn. Aulendorf: Editio Cantor Verlag, 2002.
An article in an edited book:
Sanders PA. Aerosol packaging of pharmaceuticals. In: Banker GS, Rhodes CT , eds. *Modern Pharmaceutics*. New York : Marcel Dekker, 1979: 591–626.
A book in a series:
Scott RPW. Chromatographic Detectors – *Design, Function, and Operation*. Chromatographic Science Series, 73, Cazes J, ed. New York : Mercel Dekker, 1966.

Other references

Article in conference proceedings:
Dumasia MC et al. LC/MS analysis of intact steroid conjugates: a preliminary study on the quantification of testosterone sulphate in equine urine. In: Auer DE, Houghton E, eds. *Proceedings of the 11th International Conference of Racing Analysts and Veterinarians*. Newmarket : R & W Publications (Newmarket), 1966: 188–194.
Standard:
ISO 9002. *Quality Systems – Model for Quality Assurance in Production, Installation and Servicing Quality Management System*. Geneva : ISO, 1994.
Offline database or publication:
Dictionary of Natural Products. CD-ROM. London : Chapman & Hall/CRC, 2003.
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are complete and final. They have been fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated. Because they are in final form, no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot be cited in the traditional way. They are therefore given a Digital Object Identifier (DOI), which allows the article to be cited and tracked before it is allocated to an issue. After print publication, the DOI remains valid and can continue to be used to cite and access the article.

ANEXO F: Guia para autores – AAPS Pharmscitech

INTRODUCTION

AAPS PharmSciTech (ISSN 1530-9932) is a peer-reviewed online-only journal owned by the American Association of Pharmaceutical Scientists (AAPS). The journal's mission is to disseminate scientific and technical information on drug product design, development, evaluation, and processing to the global pharmaceutical research community. The journal is indexed by PubMed/Medline, Index Medicus, Institute of Scientific Information's Science Citation Index Expanded, and Chem Abstracts.

Editor-in-Chief Robert O. (Bill) Williams III, Ph.D., oversees an international editorial board of leading researchers in the pharmaceutical sciences. Williams is a professor of pharmacy at the University of Texas at Austin, College of Pharmacy.

TYPES OF MANUSCRIPTS

AAPS PharmSciTech publishes the following article types. For examples of published articles, please visit www.PharmaGateway.net.

Reviews, usually by invitation and sometimes organized into theme issues, report on recent advances in pharmaceutical research. Unsolicited reviews are considered only if they are authored by investigators who have demonstrated expertise in the relevant areas.

Mini-reviews discuss a more narrowly focused topic of recent research. Unsolicited mini-reviews are considered only if they are authored by investigators who have demonstrated expertise in the relevant areas.

Original research papers contain innovative, hypothesis-driven research that is supported by sound experimental design, methodology, and data interpretation.

Brief technical notes, normally more limited in scope than original research papers, must be of high quality, general interest, and sufficient importance to warrant publication.

Rapid communications provide a venue for fast-breaking research updates or other news items. The justification for rapid communication should be stated in the cover letter during submission.

Regulatory notes provide a summary of regulatory decisions made and rationale for the regulatory decision made on a product. These notes are typically submitted by invitation only, but authors may propose notes to the editors.

Editorials, commentaries, or summaries are usually published by invitation only. These articles contain topical issues of public and scientific interest.

Meeting reports on AAPS or AAPS-affiliated meetings provide readers with summaries of such meetings, including consensus views. When a meeting report purports to be a consensus report, it may, at the editor-in-chief’s discretion, adhere to the AAPS Guideline for Review of Opinions and Summary Reports, available at www.aaps.org/guidelineopinions. The corresponding author must complete and submit the Corresponding Author’s Consent to Publish form

(www.aaps.org/consentcorr) and all contributing authors must complete and submit the Contributing Author's Consent to Publish form (www.aaps.org/consentcont).

Meeting notices provide readers with information on upcoming AAPS or AAPS-affiliated meetings. They should include the title of the meeting, date, time, location, an outline, description of meeting topics, and a list of invited speakers. If possible, meeting notices should include contact information for the organizers and a URL to the meeting's webpage. Meeting notices should be submitted to the AAPS Editorial Office 2–3 months prior to the meeting.

Letters to the editor may be submitted by readers commenting on articles already published by the journal.

White Papers describe a current problem or issue and propose a solution. White papers written on behalf of AAPS groups are subject to the AAPS Guideline for Review of Opinions and Summary Reports (www.aaps.org/guidelineopinions) at the discretion of the editor-in-chief.

All articles published in the journal will follow the Springer Online First production workflow, enabling publication on the SpringerLink website after receipt of author corrections to page proofs.

MANUSCRIPT SUBMISSION

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SPECIAL FEATURES, APPENDICES, AND SUPPLEMENTARY MATERIAL

These materials can be accommodated and may contain highly interactive features or large databases. All authors are encouraged to take full advantage of the Web-only capabilities of online publishing, including 3-D, video, and interactive graphics. All special features must be created by the author.

Authors who wish to publish electronic supplementary material with their article (Excel files, images, audio/video files, etc.) must submit the supplementary files/materials with their manuscript submission via our online peer review tracking system, Editorial Manager. These files may be submitted as one file or multiple files as desired by the authors. Accepted formats for these files include DOC, DOCX, XLS, XLSX, JPG, PDF, and for videos, MPEG-3 format. If a desired technical feature is in a different format or not covered in the author instructions, please contact the AAPS Editorial Office (AAPSPT@aaps.org) for assistance. Note that supplementary files are not automatically sent to production if the article is accepted. Please therefore note in a cover letter if these materials should be evaluated by reviewers only (not published), or if the supplemental files should be included for review and be published with the article should the manuscript be accepted for publication. If the supplemental files are intended for publication, references using

"supplemental 1," "supplemental 2," etc. should be placed in the appropriate location within the text of the manuscript body.

HYPERTEXT LINKS

Authors may identify URLs for websites that provide the reader with additional information on the topic addressed in the manuscript. Although URLs are an important feature of electronic publishing, authors are encouraged to be selective in their choice of sites to include. Do not include URLs for webpages with newspaper or journal articles that will be removed or archived to another web page. Links to pharmaceutical manufacturers or other sources of product information are acceptable; however, providing a URL to the reader should not be substituted for adequate discussion within the manuscript itself. Do not include links to sites that are not accessible without a password.

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TERMS OF MANUSCRIPT CONSIDERATION

AAPS JOURNALS ETHICS POLICY

The editors-in-chief of the three AAPS journals, Pharmaceutical Research, The AAPS Journal, and AAPS PharmSciTech, along with the AAPS Publications Committee, developed an integrated ethics policy to guide decision-making across the three journals. The document is based on the recommendations on publication ethics policies for medical journals published by the World Association of Medical Editors (WAME), posted at www.wame.org/about/recommendations-on- publication-ethics-policie .

Authors are required to review and adhere to the AAPS Journals Ethics Policy (www.aaps.org/journalsethics) in full prior to submitting manuscripts to AAPS PharmSciTech. Excerpts from the policy are outlined below.

FULL DISCLOSURE

During the manuscript submission process, all authors will be required to confirm that the manuscript has not been previously published in any language anywhere and that it is not under simultaneous consideration by another journal. The journal policy regarding compound disclosure centers on the data presented. If specific data relating to the study compound are reported, then the journal requires identification so that reviewers and readers can judge, based on general principles, whether the data are plausible and internally consistent, and to potentially allow future examination and/or validation of the results and conclusions reported. If however a library of compounds is used to generate correlations, such as in QSAR when the scope and diversity of the compounds are described, then it is possible that the specific chemical compositions of the compounds need not be identified, subject to the discretion of the reviewers and the editor.

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Authors will be required to declare all conflicts of interest (or their absence) during the submission of a manuscript. This conflict declaration includes conflicts or potential conflicts of all listed

authors. If any conflicts are declared, AAPS will publish them with the paper. In cases of doubt, the circumstance should be disclosed so that the editors may assess its significance.

Conflicts may be financial, academic, commercial, political, or personal. Financial interests may include employment, research funding (received or pending), stock or share ownership, patents, payment for lectures or travel, consultancies, nonfinancial support, or any fiduciary interest in a company.

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ETHICS IN ANIMAL AND CLINICAL INVESTIGATIONS

HUMAN SUBJECTS AND CLINICAL TRIALS

AAPS journals require author(s) at the time of manuscript submission to make a statement in the cover letter indicating documented review and approval from a formally constituted review board (Institutional Review Board or ethics committee) for all studies involving people, medical records, and human tissues, per the uniform guidelines from the World Medical Association (www.wma.net/en/30publications/10policies/index.html).

Studies and research using human subjects, medical records, and human tissues (including educational research) must also state this compliance within the Methods section of the paper.

AAPS journals also require that controlled clinical trials must be registered in a publicly available database or the journals will not publish the results of these trials. Manuscripts submitted to the journals must include trial registration information in the cover letter. To register a clinical trial, authors should go to the NIH registry (www.clinicaltrials.gov) or the International Standard Randomized Controlled Trials database (http://isrctn.org). Further information can be obtained from the International Committee of Medical Journal Editors (ICMJE) at www.icmje.org/about-icmje/faqs/clinical-trials-registration.

ANIMAL USE AND ASSURANCES

AAPS journals require author(s) at the time of manuscript submission to make a statement in the cover letter indicating that animal experiments are conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's expectations for animal care and use/ethics committees

(www.aaalac.org/education/module_1.cfm). Investigations using experimental animals (including educational research) must also state this compliance within the Methods section of the paper.

ORIGINALITY OF MANUSCRIPTS

Authors of manuscripts submitted to AAPS are obliged to present accurate representation of the research performed along with an objective discussion of the significance of their findings. The author's submission should be original work that reflects research undertaken with integrity and honesty, and that conforms to ethical practices outlined in the AAPS Journals Ethics Policy. Authors should be willing to reply to any reasonable request from editors, referees, and scientists for materials, methods, or data necessary for verification of the conclusions reported in the paper.

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All submissions will be reviewed anonymously by at least 2 independent reviewers. Authors are encouraged to submit names and email addresses of expert reviewers, but selection remains the prerogative of the editors. Authors should not recommend work colleagues as expert reviewers for their own papers. Authors may include supplementary notes to facilitate the review process. If an accepted paper is cited that has not yet appeared in print and is required for evaluation of the submitted manuscript, authors should provide an electronic version for use by the reviewers.
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MANUSCRIPT ORGANIZATION

Several components of the manuscript must be submitted as individual files within Editorial Manager: cover letter, title page, manuscript body (including references list), individual figure/table files, and the Transfer of Copyright.

(*indicates item is required for all manuscript types, unless otherwise specified)

COVER LETTER

A cover letter is recommended, but not required. Please note, a cover letter is required for a rapid communication submission.

Authors who wish to submit names and email addresses of recommended reviewers for the peer review process may indicate those in the cover letter or during the submission process.

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The title page must be submitted as a separate file and should include:

1. the title of the article,
2. author names with full first name (no degrees),
3. each author's affiliation, and
4. a suggested running head (of less than 50 characters, including spaces).

The affiliation should comprise the department, institution (usually university or company), city, and state (or nation) and should be typed as a footnote to the author's name. For the corresponding author designated to correspond with the AAPS Editorial Office and review proofs, indicate his/her complete mailing address, office/cellular telephone number, fax number, and email address.

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ABSTRACT

Only reviews, mini-reviews, original research articles, rapid communications, and meeting reports require an abstract. The abstract is limited to 250 words or less. For original research articles, the abstract should include a brief (2 to 3 sentence) statement for each of the following sections: Introduction, Materials and Methods, Results, Discussion, and Conclusion, written in paragraph form. All abstracts must be written in one paragraph, with no subheadings, equations, tables, reference citations, or graphics. The abstract must state the significance of the paper and the problem or question being addressed in the first sentence; what you found (include key data numbers); and what you conclude. Tenses should not be mixed in the paper: use the past tense.

*KEYWORDS

Provide a list of no more than 5 key words.

INTRODUCTION

The introduction must define the scope of your paper; articulate the question or hypothesis; summarize relevant work to the study being reported; and inform the reader of the rationale and significance of the study. The literature must be adequately reviewed. Cite the most recent papers that impact directly on your study.

Required for reviews, mini-reviews, original research articles, and meeting reports only.

*MAIN TEXT BODY

Please include continuous line numbers in the manuscript body file.

For original research articles, brief technical notes, and rapid communications, organize the main text as follows:

1. Introduction
2. Materials and Methods
3. Results
4. Discussion
5. Conclusion

Combining results and discussion into a single section is strongly discouraged. The use of subheadings to divide the text is encouraged. Primary, secondary, and tertiary level headings should be clearly defined; do not use numbers or letters in headings.

Results—You should report what you found. Supplement the text with tables and figures.

Discussion—Explain what your findings mean, using the literature in great detail to compare and contrast your data with literature; use the literature as evidence to support your argument. Explain the significance of your results and their practical implications.

Recommended maximum word counts are as follows:

- Reviews, original research articles, meeting reports, and white papers: 5000
- Mini-reviews: 2500
- Brief technical notes and rapid communications: 1200

Use abbreviations sparingly and define them at the first insertion in the text. Define all abbreviations used in tables within the table footnotes. Use the metric system for all measurements. Express metric abbreviations in lowercase letters without periods (cm, mL, sec). Define all symbols used in equations and formulas. When symbols are used extensively, the authors may include a list of all symbols in a table.

Numbers should be reported to reflect the precision of the instrumentation utilized. Calculated numbers, such as means and standard deviations, should be expressed to no more than one significant digit beyond the precision of the instrument. Normally, data reported to more than 3 significant figures should be justified. The precision of the variability (e.g., standard deviation) should not exceed that of the reported mean value.

CONCLUSION

The conclusion should be a brief paragraph, containing 3 to 4 sentences, that summarizes the findings presented. You must state if your findings support your hypothesis.

ACKNOWLEDGEMENTS

Include funding source(s) and other contributions. If the work has been funded by NIH, please provide name(s) of funding institute(s) and grant number(s). This information is required for automatic deposit into PUBMED Central by the publisher.

REFERENCES

References should conform to Vancouver style and be numbered consecutively in the order in which they are cited in the text. Cite in the text by the appropriate Arabic numeral enclosed in

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References to unpublished, peer-reviewed, personal communications, including conference abstracts and papers in preparation or in review, cannot be listed but can be notated parenthetically in the text.

Abbreviations for journal names should conform to those of Vancouver style. The style and punctuation of the references should conform to the following examples:

EXAMPLES BY TYPE

1. Journal article

Smith JJ. The world of science. Am J Sci. 1999;36:234–5.

2. Journal article with DOI (and with page numbers)

O'Mahony S, Rose SL, Chilvers AJ, Ballinger JR, Solanki CK, Barber RW, et al. Finding an optimal method for imaging lymphatic vessels of the upper limb. Eur J Nucl Med Mol Imaging. 2004;31:555– 63. doi:10.1007/s00259-003-1399-3.

3. Journal article by DOI (before issue publication with page numbers)

O'Mahony S, Rose SL, Chilvers AJ, Ballinger JR, Solanki CK, Barber RW, et al. Finding an optimal method for imaging lymphatic vessels of the upper limb. Eur J Nucl Med Mol Imaging. 2004. doi:10.1007/s00259-003-1399-3.

4. Article in electronic journal by DOI (no paginated version)

Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000. doi:10.1007/s801090000086.

5. Journal article in a supplement

Frumin AM, Nussbaum J. Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979; 59 Supple 1:26-32.

6. Book chapter

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251–306.

7. OnlineFirst chapter in a series (without a volume designation but with a DOI)

Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

8. Book, authored

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

9. Online document

Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

10. Online database

Healthwise Knowledgebase. US Pharmacopeia, Rockville. 1998. http://www.healthwise.org. Accessed 21 Sept 1998.

11. Supplementary material/private homepage

Doe J. Title of supplementary material. 2000. http://www.privatehomepage.com. Accessed 22 Feb 2000.

12. University site

Doe, J.: Title of preprint. http://www.uni-heidelberg.de/mydata.html (1999).

13. FTP site

Doe, J.: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999. Accessed 25 Dec 1999.

14. Organization site

ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007. For a full description of the Vancouver reference style, including numerous examples, please access: www.nlm.nih.gov/bsd/uniform_requirements.html

TABLES

Tables must be created in Microsoft Word table format. Tables should be numbered (with Roman numerals) and referred to by number in the text. Center the title above the table and type explanatory

footnotes (indicated by superscript lowercase letters) below the table. Data must be placed in separate cells of the table to prevent text and numbers from shifting when the table is converted for publication on the Internet. Empty cells may be inserted to create spacing. Tables should not duplicate information provided in the text. Instead, tables should be used to provide additional information that illustrates or expands on a specific point the author wishes to make. Each table should be self-explanatory.

FIGURES

AAPS PharmSciTech offers authors the use of color figures in online published manuscripts, free of charge. Figures (as well as photographs, drawings, diagrams, and charts) are to be numbered in

sequential Arabic numerals. The description of each figure should be separated from the manuscript body text and collated in a separate file called “Legend to Figures.” All electronic artwork must be submitted online via our online peer review tracking system, Editorial Manager. Figure files should be submitted in TIFF or EPS format (1200 dpi for line drawings and 300 dpi for half-tones and gray-scale art); however, JPG, GIF, BMP, and PDF files may also be submitted as long as the dpi specifications above are met. Use of a professional graphics program such as Adobe Photoshop to edit and/or save photographs and graphics is highly recommended. Because of quality issues, it is not recommended that images be submitted in DOC, DOCX, PPT, or PPTX format.

Maximum combined count for tables and figures are as follows:

- Reviews, original research articles, meeting reports, and white papers: 10 (suggested)
- Mini-reviews: 6
- Brief technical notes and rapid communications: 6

FOOTNOTES

Footnotes in the manuscript body should be avoided. When their use is absolutely necessary, footnotes should be numbered consecutively using Arabic numerals and should be typed at the bottom of the page to which they refer. Place a line above the footnote, so that it is set off from the text. Use the appropriate superscript numeral for citation in the text.

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ANEXO G: Guia para autores – Journal of Natural Products

Title Page

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

Abstract

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

Introduction

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

Results and Discussion

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

Bolded structural code numbers should only be used for new compounds and for those known compounds for which new biological data or spectroscopic values are being reported. Authors providing manuscripts focusing on the biological properties of two or fewer known natural products have the option of referring to the compound(s) concerned by name, rather than assigning each a bolded numerical code number. Other known compounds should be referred to in the text by name, wherever necessary. Sugar units in glycosides should not be inferred as D or L based solely on NMR data analysis, but should be determined by supporting experimental work such as measurement of their optical rotations following acid hydrolysis or by the preparation of chiral derivatives and comparison with standards using a chromatographic analytical method. If the aglycone of a glycoside is also a new compound, then it should be isolated and its physical

constants and spectroscopic parameters stated. Authors are advised to use correctly the terms "relative and absolute configuration" instead of "relative and absolute stereochemistry". In, for example, a carbocyclic compound, only a stereogenic carbon or a stereogenic element, such as an axis, possesses configuration. Substituents such as methyl groups are either alpha or beta oriented and are **not** alpha or beta configured. Care should be taken not to make erroneous configurational conclusions via NMR NOE associations from ring to side -chain protons of, for example, sterols and tetracyclic triterpenoids. The term "spectral" should be avoided in a structure elucidation discussion, when "spectroscopic" or "spectrometric" are meant instead.

In manuscripts that present results of biological studies with tumor cell lines or animal-based tumor models, authors should pay special attention to the U.S. National Cancer Institute (NIH) guidelines for cancer drug discovery studies. Compounds that suppress the growth of, or kill, isolated tumor cell lines grown in culture should be referred to as either "cytostatic" or "cytotoxic", as appropriate. Only compounds that inhibit the growth of tumors in animal-based models should be called "antitumor". The term "anticancer" should be reserved for compounds that show specific activity in human-based clinical studies (see Suffness, M.; Douros, J. *J. Nat. Prod*. **1982**, *45*, 1–14). Some flexibility in this system is afforded in the description of compounds that show activity in molecular-targeted antitumor assays. Compounds should be compared against a suitable positive control substance and follow accepted guidelines when represented as "active". For example, a cytotoxic pure substance when tested against a cancer cell line would exhibit an IC_{50} value of <10 μM (or 4–5 μg/mL).

Experimental Section

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

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

If the biological material has not been identified as to species, the manuscript will not be considered for publication unless a special protocol has been followed. Thus, a voucher specimen of the organism should be deposited with a recognized taxonomist for the particular group of organisms in question. The taxonomist should then assign to the specimen an identifying number unique to the organism so that any additional collections of the same organism would bear this same number. The number will be retained until the organism is completely identified. The taxonomist should write a brief taxonomic description to be included in the manuscript, which should state how the organism in question relates morphologically to

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Authors who purchase dried "herbal remedies" or other materials from companies must make provision for their proper deposit in a herbarium or other permanent repository, for access by future workers. When a commercially available extract is obtained, the extraction procedure from the organism of origin must be specified. The identification of the extract should be supported by an HPLC trace of known secondary metabolite constituents of the organism, which should be included with the manuscript as Supporting Information.
When physical and spectroscopic data are presented in the body of the manuscript, the following general style must be used (with the various commonly used techniques presented in this same order):

> **Romucosine (1):** colorless needles ($CHCl_3$); mp 152–153 °C; $[\alpha]^{25}_{D}$ –110 (*c* 0.4, $CHCl_3$); UV (EtOH) λ_{max}(log ε) 235 (4.23), 275 (4.18), 292 (sh) (3.52), 325 (3.41) nm; IR (Nujol) ν $_{max}$ 1680, 1040, 920 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ* 8.11 (1H, d, *J* = 7.6 Hz, H-11), 7.54–7.28 (2H, m, H-9, H-10), 7.27 (1H, m, H-8), 6.59 (1H, s, H-3), 6.10, 5.97 (each 1H, d, *J* = 1.5 Hz, OC *H*$_2$O), 4.86 (1H, dd, *J* = 13.7, 4.4 Hz, H-6a), 4.44 (1H, m, H-5a), 3.77 (3H, s, NCOOC*H*$_3$), 3.06 (1H, m, H-7a), 2.99 (1H, m, H-5b), 2.91 (1H, m, H-7b), 2.82 (1H, m, H-4a), 2.61 (1H, m, H-4b); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ* 155.8 (C, N*C*OOCH$_3$), 146.8 (C, C -2), 143.0 (C, C-1), 135.8 (C, C-7a), 130.7 (C, C-11a), 128.7 (CH, C-8), 127.79 (C, C -3a), 127.78 (CH, C-9), 127.2 (CH, C-10), 127.0 (CH, C-11), 125.6 (C, C-3b), 117.3 (C, C-1a), 107.6 (CH, C -3), 100.9 (CH_2, OCH_2O), 52.7 (CH_3, NCOO*C*H$_3$), 51.7 (CH, C-6a), 39.2 (CH_2, C-5), 34.5 (CH_2, C-7), 30.4 (CH_2, C-4); EIMS *m*/*z* 323 $[M]^+$ (98), 308 (28), 292 (5), 262 (20), 248 (21), 236 (81), 235 (100), 206 (17), 178 (27), 88 (17); HREIMS *m*/*z* 323.1152 (calcd for $C_{19}H_{17}NO_4$, 323.1158).

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

Table 1. NMR Spectroscopic Data (400 MHz, C_6D_6) for Aurilides B (**1**) and C (**2**)

	aurilide B (**1**)			aurilide C (**2**)	
position	δ_C, type	δ_H (J in Hz)	HMBC[a]	δ_C	δ_H (J in Hz)
1	170.0, C			170.2	
2	58.9, CH	3.23, m	1, 3, 4, 5	59.6	3.08, m
3	13.8, CH_3	1.21, d (7.1)	1, 2	14.0	1.25, d (7.1)
4	36.1, CH_3	2.63, s	2, 5	36.8	2.55, s
5	172.1, C			172.1	
6	54.3, CH	5.12, dd (9.0, 7.4)	5, 7, 9	54.4	5.15, dd (9.0, 5.0)
7	31.0, CH	1.97, m		32.0	1.98, m
8	20.1, CH_3	1.15, d (7.0)	6, 7, 9	20.4	1.17, d (7.0)
9	17.3, CH_3	1.25, d (7.0)	6, 7, 8	17.5	1.28, d (7.0)
10	169.9, C			170.11	
11	51.8, CH_2	4.40, d (18.0) 3.80, d (18.0)	10, 12, 13	51.9	4.39, d (18.0) 3.80, d (18.0)
12	36.8, CH_3	3.23, s	11, 13	37.1	3.22, s
13	170.0, C			170.14	
14	58.6, CH	5.24, d (10.0)	13, 18, 19, 20	58.7	5.26, d (10.0)
15	33.9, CH	2.48, m	14, 16, 18	34.1	2.49, m
16	27.4, CH_2	1.86, 1.30, m	14, 15, 17	27.6	1.89, 1.30, m
17	12.1, CH_3	1.03, t (7.1)		12.2	1.03, t (6.9)
18	14.8, CH_3	0.85, d (7.0)	15, 16	15.1	0.86, d (7.0)
19	30.7, CH_3	2.88, s	20	30.6	2.85, s
20	173.1, C			173.2	
21	54.7, CH	4.78, dd (8.8, 8.8)	20, 22	54.9	4.75, dd (8.6, 7.5)
22	31.7, CH	1.98, m		31.0	1.95, m
23	18.1, CH_3	0.89, d (6.0)	21, 22, 24	18.9	0.88, d (6.0)
24	20.2, CH_3	0.90, d (6.0)	23	20.3	0.90, d (6.0)
25	170.3, C			170.3	
26	78.5, CH	4.90, d (6.1)	25, 27, 31	80.4	4.54, d (7.5)
27	37.2, CH	2.17, m	26, 30	30.5	2.36, m
28	26.1, CH_2	1.50, 1.14, m	29	18.7	1.00, d (7.0)
29	11.8, CH_3	0.83, t (7.7)	27, 28	18.4	0.88, d (7.0)
30	14.9, CH_3	1.03, d (6.0)	26, 27, 28	169.7	
31	169.3, C			128.3	
32	128.0, C			146.0	7.75, t (9.0)
33	145.3, CH	7.74, t (9.0)	31, 42	30.9	2.14, m
34	30.9, CH_2	2.19, m	32, 33, 42	71.2	3.98, m
35	71.0, CH	3.97, m	34	41.2	2.02, m
36	41.1, CH	2.07, m	43	82.6	5.17, d (11.2)
37	82.5, CH	5.18, d (11.2)	1, 36, 38, 44	132.1	
38	131.4, C			134.6	5.62, t (7.7)
39	134.2, CH	5.61, t (7.7)	37, 44	21.4	1.95, 1.92, m
40	21.4, CH_2	1.95, 1.92, m	38, 39, 41	14.3	0.89, t[b]
41	14.1, CH_3	0.89, t[b]	39, 40	12.8	1.95, s
42	12.7, CH_3	1.95, s	31, 32, 33	10.1	0.66, (7.0)
43	10.2, CH_3	0.64, d (7.0)	35, 36, 37	11.4	1.54, s
44	11.3, CH_3	1.54, s	37, 38, 39		
NH (1)		7.69 brd (9.1)	10		7.66 brd (9.1)
NH (2)		6.75 brd (8.8)	25		6.70 brd (8.8)

[a]HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon.
[b]Signal partially obscured.

The correct format to present elemental analysis data is: anal. C 72.87, H 11.13%, calcd for $C_{37}H_{68}O_6$, C 73.02, H 11.18%. The structures of compounds are expected to be supported by high-resolution mass spectrometry or elemental analysis. Melting point determinations should not be provided for compounds described as "amorphous solids". The unit of concentration to be used for optical rotation measurements is grams per 100 mL. UV extinction coefficient data should be provided as log ϵ values, to two places of decimals. In reporting ^{1}H NMR data of diastereotopic methylene protons, the one at lower field should be listed as the "a" proton and that at the higher field as the "b" proton, as in "H-10a" and "H-10b", respectively. If two proton or carbon signals in an NMR spectrum appear at the same chemical shift but are still distinguishable, an additional decimal place (three for ^{1}H NMR data and two for ^{13}C NMR data) may be used to designate the resonance in question. Carbon-13 NMR data should be reported to the nearest 0.1 ppm with the number of attached protons designated using the C, CH, CH_2, and CH_3 notation.

Acknowledgments

The Acknowledgments section should include credits [initial(s) and last name] for technical assistance, financial support, and other appropriate recognition. During manuscript submission, the submitting author is asked to select funding sources from the list of agencies included in the FundRef Registry http://www.crossref.org/fundref/.

References

References to the literature and all notes, regardless of their nature, should be numbered in order of appearance in the manuscript and cited in the text with superscript numbers. Each reference may have its own citation number, or alternatively, references referring to the same topic may be grouped under a common number using alphabetical subdesignations (e.g., 1a, 1b, 1c, etc.). Each note should be assigned its own number. References and notes should follow the format shown:

(1) Dumdei, E.; Andersen, R. J. *J. Nat. Prod.* **1993**, *56*, 792–794.

(2) Cordell, G. A. *Introduction to Alkaloids: A Biogenetic Approach*; John Wiley & Sons: New York, 1981; p 43.

(3) Pelletier, S. W.; Mody, N. V. In *The Alkaloids*; Rodrigo, R. G. A., Ed.; Academic Press: New York, 1981; Vol. 18, Chapter 2, pp 100–216.

(4) Zheng, G.; Kakisawa, H. *Chin. Sci. Bull.* **1990**, *35*, 1406–1407; *Chem. Abstr.* **1991**, *114*, 43213*m*.

(5) Meyer, B. N. Brine Shrimp Toxicity: Certain Components of *Stapelia*, *Coryphantha*, *Lupinus*, and *Quinoa*. Ph.D. Thesis, Purdue University, West Lafayette, IN, 1983, p 35.

(6) Davis, R. U.S. Patent 5,708,591, 1998.

(7) The biogeographic zone comprising Madiera, the Canary Islands, the Cape Verde Islands, and the Azores.

For additional information on the reference and note format to use, see *The ACS Style Guide*, 3rd ed. (2006) (http://pubs.acs.org/books), available from Oxford University Press, Order Department, 2001 Evans Road, Cary, NC 27513 (http://www.oup.com).

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For IUPAC rules, see:

- *Nomenclature of Inorganic Chemistry, Recommendations, 1990*; Blackwell Scientific Publications: Oxford, England, 1990.
- *A Guide to IUPAC Nomenclature of Organic Compounds, Recommendations, 1993*; Blackwell Scientific Publications: Oxford, England, 1993.
- *Nomenclature of Organic Chemistry, Sections A–F and H*; Pergamon Press: Elmsford, NY, 1979.
- *Compendium of Macromolecular Nomenclature*; Blackwell Scientific Publications: Oxford, England, 1991.
- *Biochemical Nomenclature and Related Documents*, 2nd ed.; Portland Press, Ltd.: London, England, 1992.
- Selected IUPAC recommendations can be found on the Web at http://www.chem.qmw.ac.uk/iupac/iupac.html.
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chain angle	120°
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fixed length	14.4 pt (0.508 cm, 0.2 in.)
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hash spacing	2.5 pt (0.088 cm, 0.0347 in.)

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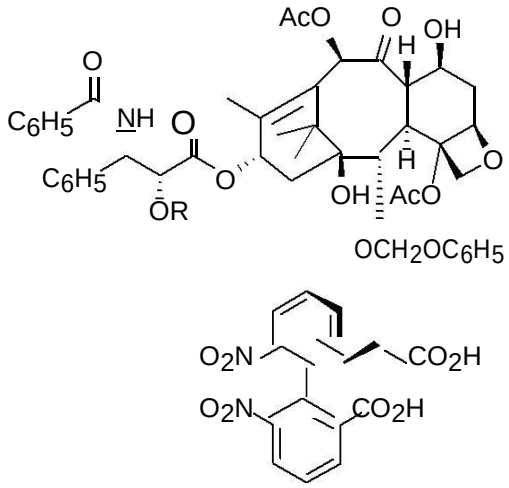
(8)



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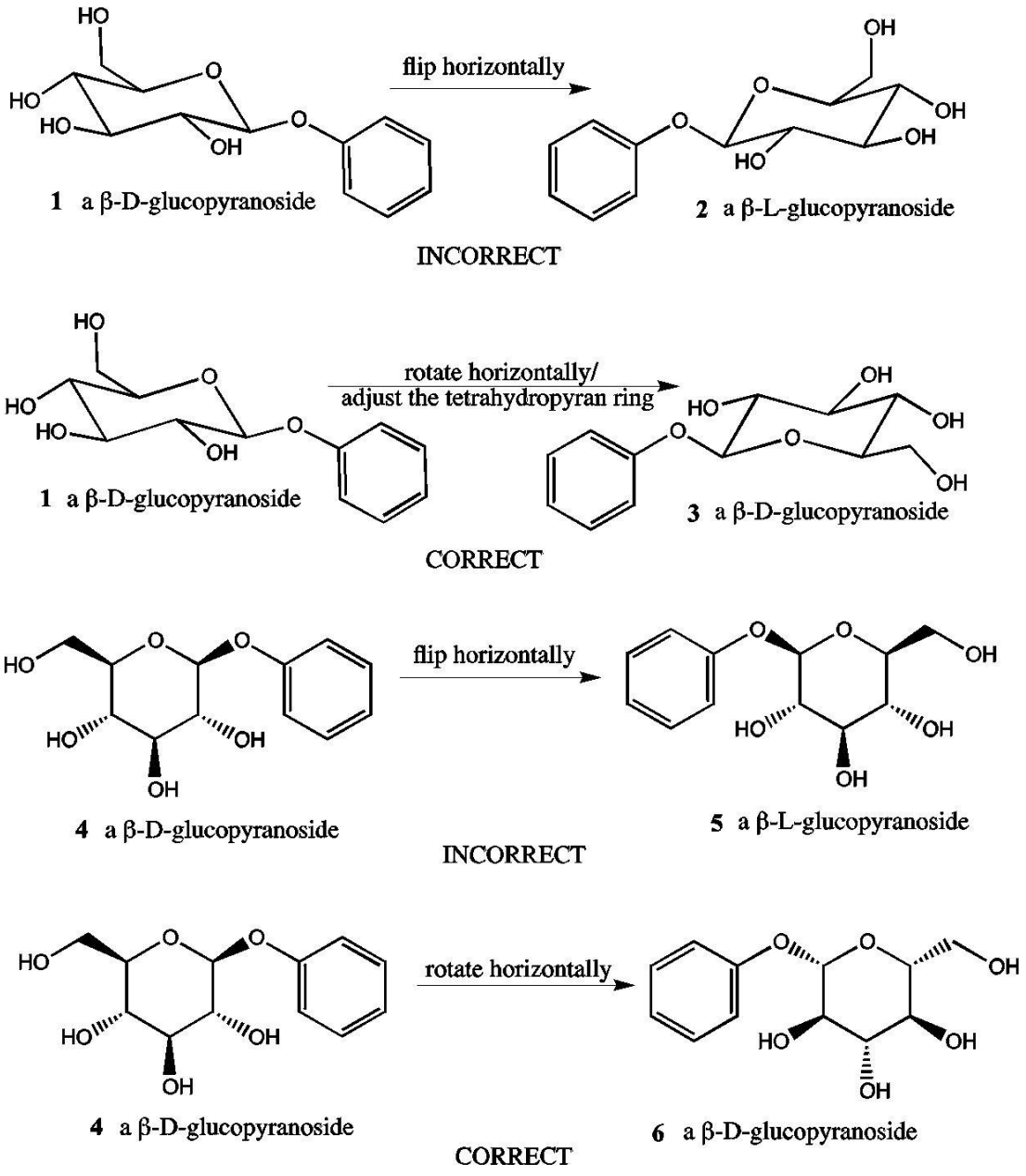

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