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FILMES DE POLISSACARÍDEOS NATURAIS PARA A VEICULAÇÃO CUTÂNEA DE NANOCÁPSULAS DE SILIBININA NO TRATAMENTO DA DERMATITE ATÓPICA

Santa Maria, RS
2022

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

Orientadora: Profa. Dra. Letícia Cruz

Santa Maria, RS
2022

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Mailine Gehrcke

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Santa Maria, RS
2022

Dedico esta tese à minha família, especialmente aos meus pais, com todo meu amor.

"...E eu só quero agradecer por ter vocês, pra acompanhar minhas loucuras, me deixar bem mais segura daquilo que eu posso ser... Por me amarem com a mesma intensidade e por serem, de verdade, a melhor família que eu pudesse ganhar..."

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RESUMO

FILMES DE POLISSACARÍDEOS NATURAIS PARA A VEICULAÇÃO CUTÂNEA DE NANOCÁPSULAS DE SILIBININA NO TRATAMENTO DA DERMATITE ATÓPICA

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ORIENTADORA: Letícia Cruz

Os tratamentos atuais para dermatite atópica apresentam restrições devido à eficácia limitada e a efeitos adversos marcantes, havendo uma necessidade do desenvolvimento de alternativas terapêuticas. Assim, este estudo objetivou associar as potencialidades das nanocápsulas com as vantagens proporcionadas pelos filmes poliméricos, a fim de desenvolver uma nova formulação tópica contendo silibinina (SB), um flavonoide antioxidante e anti-inflamatório, para o tratamento da dermatite atópica. As suspensões de nanocápsulas foram preparadas pelo método de deposição interfacial do polímero pré-formado, utilizando etilcelulose e triglicerídeos de cadeia média, e demonstraram tamanho nanométrico, índice de polidispersão abaixo de 0,2, potencial zeta negativo, pH ácido, teor e eficiência de encapsulação de cerca de 100 %. Posteriormente, estas suspensões foram incorporadas em filmes de goma gelana pelo método de deposição/evaporação do solvente, utilizando glicerol como plastificante. Com fins comparativos foram também preparados filmes veículo e contendo o flavonoide não-nanoencapsulado. Os filmes desenvolvidos apresentaram-se finos, transparentes, flexíveis e com capacidade de intumescimento maior que 100 %, permanecendo intactos após 24 h em contato com o tampão pH 7,4. O potencial de irritação destes filmes foi avaliado pelo teste da membrana cório-alantóide que evidenciou a biocompatibilidade das formulações. Ainda, em comparação a filmes contendo a SB livre, os filmes de base nanotecnológica apresentaram maior propriedade oclusiva e melhor estabilidade durante o armazenamento em temperatura ambiente. O ensaio de liberação *in vitro* mostrou que os filmes contendo o flavonoide nanoencapsulado tiveram um perfil de liberação controlada, o que está de acordo com o perfil de permeação cutânea obtido. Apesar do controle de liberação, foi possível a quantificação de quantidades terapêuticas da SB nas camadas alvo para o tratamento de doenças da pele (epiderme e derme). Em uma segunda etapa do trabalho foi demonstrada a viabilidade de produção de filmes bicamada constituídos por goma gelana/pullulan utilizando o mesmo método de preparo. Para isto, uma dispersão de pullulan foi vertida sobre a camada de goma gelana e nanocápsulas parcialmente seca. Os filmes bicamada demonstraram microestrutura em duas camadas bem definidas, a qual foi avaliada por microscopia eletrônica de varredura. Ainda, a adição da camada de pullulan foi capaz de conferir bioadesividade e menor rigidez aos filmes, bem como aumentou em cerca de duas vezes o potencial oclusivo destes, sem alterar os perfis de liberação e permeação cutânea da SB. A performance antioxidante dos filmes bicamada foi avaliada utilizando o radical ABTS, que evidenciou a alta capacidade sequestrante do flavonoide. Foi também demonstrado que os filmes bicamada são hemo/biocompatíveis através de um ensaio *in vitro* de hemólise por contato direto. Por fim, foi avaliada a performance biológica dos filmes bicamada em um modelo animal de dermatite atópica induzida por dinitroclorobenzeno. A associação de nanocápsulas de SB em filmes bicamada de goma gelana/pullulan atenuou as lesões do tipo dermatite nos camundongos, bem como modulou parâmetros oxidativos e inflamatórios. Como conclusão, a combinação de nanocápsulas em filmes de goma gelana possibilita maior estabilidade e é capaz de controlar a liberação/permeação cutânea da SB. Ainda, no contexto da aplicação cutânea, as características destes filmes podem ser aprimoradas através da adição de uma camada de pullulan, sendo esta uma nova e promissora estratégia para o tratamento da dermatite atópica.

Palavras-chave: Nanopartículas. Silibinina. Filmes. Pullulan. Goma gelana. Eczemas.

ABSTRACT

NATURAL POLYSACCHARIDE FILMS FOR THE CUTANEOUS RELEASE OF SILIBININ NANOCAPSULES IN THE TREATMENT OF ATOPIC DERMATITIS

AUTHOR: Mailine Gehrcke
ADVISOR: Letícia Cruz

Current treatments for atopic dermatitis have restrictions due to the limited efficacy and pronounced adverse effects, and there is a need to develop therapeutic alternatives. Thus, this study aimed to associate the potential of nanocapsules with the advantages provided by polymeric films, in order to develop a new topical formulation containing silibinin (SB), an antioxidant and anti-inflammatory flavonoid, for atopic dermatitis treatment. The nanocapsule suspensions were prepared by the interfacial deposition method of the preformed polymer, using ethylcellulose and medium chain triglycerides, and showed nanometric size, polydispersity index below 0.2, negative zeta potential, acidic pH, SB content and encapsulation efficiency of about 100 %. Subsequently, these suspensions were incorporated into gellan gum films by the solvent deposition/evaporation method, using glycerol as plasticizer. For comparative purposes, vehicle films and films containing the non-nanoencapsulated flavonoid were also prepared. The developed films were thin, transparent, flexible and with swelling capacity greater than 100 %, remaining intact after 24 h in contact with pH 7.4 buffer. The film's irritation potential was evaluated by the chorio-allantoic membrane test, which showed the formulations biocompatibility. Also, compared to films containing free SB, nano-based films showed greater occlusive property and better stability during storage at room temperature. The *in vitro* release assay showed that the films containing the nanoencapsulated flavonoid had a controlled release profile, which is in agreement with the cutaneous permeation profile obtained. Despite the controlled release, it was possible to quantify therapeutic amounts of SB in the target layers for the skin diseases treatment (epidermis and dermis). In a second stage of the work, the feasibility of producing bilayer films of gellan gum/pullulan using the same preparation method was demonstrated. For this, a pullulan aqueous dispersion was cast onto the partially dried gellan gum layer containing nanocapsules. The bilayer films demonstrated a well-defined two-layer microstructure, which was evaluated by scanning electron microscopy. Furthermore, the addition of the pullulan layer was able to give bioadhesive and less stiff characteristics to the films, as well as increasing their occlusive potential by about 2 times, without altering the profiles of release and cutaneous permeation of SB. The antioxidant performance of the bilayer films containing silibinin nanocapsules was evaluated using the ABTS radical, which showed the high scavenger capacity of this flavonoid. It was also demonstrated that bilayer films are hemo/biocompatible through a direct contact hemolysis assay. Finally, the biological performance of bilayer films was evaluated in an animal model of dinitrochlorobenzene-induced atopic dermatitis. The association of SB nanocapsules into gellan/pullulan gum bilayer films attenuated dermatitis-like lesions in mice, as well as modulating oxidative and inflammatory parameters. In conclusion, the incorporation of nanocapsules into gellan gum films provides greater stability and is able to control the skin release/permeation of SB. Also, in the context of cutaneous application, the characteristics of these films can be improved by adding a pullulan layer, which is a novel and promising strategy for atopic dermatitis treatment.

Keywords: Nanoparticles. Silibinin. Films. Pullulan. Gellan gum. Eczemas.

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1 INTRODUÇÃO

A dermatite atópica é uma doença crônica da pele, que apresenta como característica principal a barreira cutânea alterada, aumentando assim a sensibilização a substâncias químicas e alérgenas e provocando um aumento na perda de água transepidérmica. Assim, a pele destes pacientes apresenta ressecamento e prurido, os quais, juntamente com o ato de coçar o local, levam ao surgimento de escoriações, gerando um ciclo vicioso, onde a restauração da integridade cutânea é dificultada. Embora existam diferentes alternativas terapêuticas na clínica (BAYLET et al., 2021; MALIK; HEITMILLER, 2017; RERKNIMITR et al., 2017), tais tratamentos estão associados à baixa penetração cutânea dos ativos ou a efeitos adversos sistêmicos marcantes (MANCUSO et al., 2021; PAREKH et al., 2021).

Nesse sentido, a busca por novas substâncias ativas e novos sistemas de entrega de fármacos para o tratamento da dermatite atópica ainda é relevante. A silibinina (SB) é um composto natural polifenólico que vem demonstrando ser uma alternativa terapêutica para o tratamento de uma vasta gama de doenças inflamatórias cutâneas, incluindo dermatoses, devido ao seu potencial antioxidante, anti-inflamatório e imunomodulador (CASTELLANETA et al., 2016; RAJ et al., 2020; RIGON et al., 2019; SAMANTA et al., 2016; SHROTRIYA; VIDHATE; SHUKLA, 2017). No entanto, a SB apresenta limitada solubilidade em diferentes solventes e é bastante susceptível a processos de oxidação (BIJAK, 2017), tornando-a um desafio para o desenvolvimento de formulações de aplicação cutânea.

Deste modo, a combinação deste flavonoide em nanocápsulas é vantajosa, pois estes sistemas apresentam alta eficiência de encapsulação de ativos lipofílicos, controlando a liberação e aumentando a solubilidade e estabilidade destes (JAIN; THAREJA, 2019; MORA-HUERTAS; FESSI; ELAISSARI, 2010). Ainda, no caso específico da dermatite atópica, estas nanoestruturas podem melhorar a retenção de substâncias ativas nas camadas viáveis da pele, favorecendo seu efeito terapêutico local, com redução de efeitos sistêmicos (BADIHI et al., 2020).

Assim como as nanoestruturas, filmes poliméricos vêm sendo considerados uma alternativa promissora no tratamento da dermatite atópica, pois são capazes de se aderir ao tecido de forma a proteger a área lesionada e melhorar a hidratação da pele (ALVES et al., 2016; JEONG et al., 2019; VOSS et al., 2020). Para a produção de filmes para o tratamento de patologias cutâneas, os polissacarídeos naturais, como goma gelana e pullulan, têm se mostrado excelentes candidatos, por serem biocompatíveis e atóxicos (HAMIDI et al., 2022; OSMA; FROELICH; TASAREK, 2014). Enquanto a goma gelana forma filmes ultrafinos, com resistência física e mecânica e com alta capacidade de intumescimento (ARIFAH et al., 2019; MAHMOOD et al., 2021; SEBRI; AMIN, 2016), o pullulan forma filmes elásticos, bioadesivos

e de rápida dissolução (CERVI et al., 2021, 2022; JEONG et al., 2019). As características filmogênicas destes polissacarídeos tornam promissora a avaliação da associação dos mesmos em um filme para o tratamento da dermatite atópica. Tal associação é mais vantajosa quando realizada em camadas separadas, produzindo filmes bicamada, pois neste tipo de filme consegue-se preservar as características filmogênicas de cada polímero em sua respectiva camada (NETO et al., 2019; PEREDA et al., 2011).

Portanto, devido: a) à necessidade de novos tratamentos para a dermatite atópica; b) aos efeitos terapêuticos da SB e as suas limitações; c) às potencialidades das nanocápsulas e dos filmes poliméricos; d) à utilização da goma gelana e do pullulan como agentes filmógenos, esta tese foi delineada no sentindo de associar este flavonoide em nanocápsulas poliméricas, de forma a facilitar a sua incorporação em filmes mono e bicamada de goma gelana e goma gelana/pullulan, respectivamente, almejando propor uma formulação para aplicação cutânea que se sobressaia como uma nova alternativa terapêutica no tratamento desta doença inflamatória de pele. Na sequência, alguns assuntos serão abordados de maneira mais aprofundada, a fim de dar um embasamento teórico para esta tese.

1.1 SILIBININA

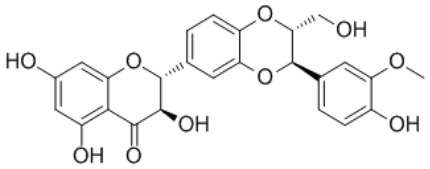
Ativos fitoquímicos têm sido amplamente estudados para a prevenção e tratamento de diferentes doenças de pele. Dentre estes fitoquímicos está a SB, um flavonóide (ou flavoglicano) obtido a partir do extrato seco das sementes da planta *Silybum marianum*, o qual é denominado silimarina (AHMAD et al., 2015; SINGH e AGARWAL, 2002). Este extrato seco é composto de aproximadamente 80 % de flavonoides polifenólicos e 20 % de uma fração química diversa. A porção polifenólica é constituída basicamente por SB, isosilibinina, dehidrosilibinina, silicristina, silidianina e taxifolin, sendo a SB (CAS No. 22888-70-6) o componente mais abundante deste complexo, dando origem aos efeitos terapêuticos do extrato silimarina (SINGH e AGARWAL, 2002; GAZAK et al., 2007).

Tanto a silimarina quanto a SB são tradicionalmente conhecidas por uma potente ação em doenças hepáticas (AHMAD et al., 2015). No entanto, atualmente estão emergindo na literatura científica devido às suas múltiplas atividades benéficas que vão além da ação hepatoprotetora, incluindo a prevenção e tratamento de diferentes patologias cutâneas como: câncer de pele (RAJ et al., 2020), feridas cutâneas (SAMANTA et al., 2016) e dermatite de contato (RIGON et al., 2019; SHROTRIYA; VIDHATE; SHUKLA, 2017). Tais ações estão relacionadas, principalmente, aos efeitos antioxidante e anti-inflamatório da SB. O efeito

antioxidante deste flavonoide se dá pela sua contribuição para as defesas antioxidantes de diferentes maneiras, tais como: a) eliminação direta de radicais; b) redução da formação de radicais, através da inibição de enzimas responsáveis pela produção destes (xantina oxidase e NADPH oxidase); c) participação na manutenção do status redox ideal da célula, preservando e/ou potencializando uma gama de enzimas antioxidantes (superóxido dismutase, catalase e glutationa peroxidase) e de antioxidantes não-enzimáticos (PYSZKOVÁ et al., 2016; SURAI, 2015). Já o efeito anti-inflamatório da SB se dá pela modulação dos níveis de citocinas pró-inflamatórias, como IL-10 e TNF-α (GAZAK et al., 2007). Além disso, há evidências de que a SB também possui propriedades imunomoduladoras e polariza as respostas imunes Th1/Th2 através de modificações funcionais de células dendríticas (CASTELLANETA et al., 2016).

Apesar das propriedades benéficas da SB, ela possui peso molecular de 482,44 g/mol, e apresenta uma estrutura polifenólica com características apolares (Figura 1). Devido a esta estrutura, este polifenol é insolúvel em água, possuindo pouca solubilidade em solventes próticos (etanol e metanol) e alta solubilidade em solventes apróticos (acetona, dimetilsulfóxido, tetraidrofurano e dimetilformamida) (BIJAK, 2017). Assim, objetivando melhorar a solubilidade e a biodisponibilidade da SB tem sido proposta a associação da SB com diferentes sistemas nanoestruturados na literatura (EL-SAMALIGY; AFIFI; MAHMOUD, 2006; XU et al., 2013; GOHULKUMAR et al., 2014; SADIQ; RASSOL, 2014; MARCHIORI et al., 2017a).

Figura 1. Estrutura molecular da SB.



Fonte: próprio autor.

Esta associação da SB em nanocarreadores tem sido proposta também para permitir a sua aplicação no tratamento de doenças inflamatórias cutâneas, como no estudo de Shrotriya, Vidhate, Shukla (2017), os quais desenvolveram géis de Carbopol® 940 contendo nanopartículas lipídicas sólidas de SB para o tratamento dermatite de contato. O gel contendo SB nanoencapsulada desenvolvido mostrou características promissoras para a aplicação cutânea nesta patologia, como melhora do potencial oclusivo do semissólido, controle de

liberação e aumento na retenção cutânea do flavonoide. Estas características contribuíram para a melhor performance *in vivo* (maior redução do edema e menor perda de água cutânea) do semissólido de base nanotecnológica em comparação ao semissólido de SB não-nanoencapsulada, utilizando um modelo animal de dermatite de contato induzida por DNCB.

Nosso grupo de pesquisa também vem se dedicando ao desenvolvimento de formulações de base nanotecnológica para a aplicação cutânea da SB. Primeiramente, Marchiori e colaboradores (2017a) desenvolveram suspensões de nanocápsulas contendo a associação SB e óleo de romã, as quais foram capazes de controlar a liberação do flavonoide e de proteger as células dos efeitos tóxicos causados pelos ativos isolados. Em um outro estudo, foi desenvolvido um hidrogel, a partir da goma gelana, contendo estas mesmas nanocápsulas de SB e óleo de romã, o qual foi eficaz frente a exposição à radiação UVB e ainda, aumentou a solubilidade aparente em água da SB (MARCHIORI et al., 2017b). Na sequência, Rigon e colaboradores (2019) desenvolveram hidrogéis bioadesivos de Pemulen® TR2 contendo nanocápsulas de SB e óleo de romã, os quais demonstraram características adequadas para a aplicação cutânea. Além disso, o hidrogel contendo estas nanocápsulas demonstrou efeitos promissores em um modelo animal de dermatite de contato irritativa, induzida por óleo de cróton, apresentando redução de edema e de infiltração celular semelhante ao controle positivo dexametasona. Todos estes estudos ressaltam as vantagens da associação da SB em sistemas nanoestruturados, como nanocápsulas, os quais facilitam a incorporação deste flavonoide em formulações de uso dermatológico e melhoram seu efeito terapêutico em doenças inflamatórias cutâneas.

Além de sistemas nanoestruturados, alguns estudos também demonstram a incorporação da SB em materiais poliméricos para utilização na área biomédica como, por exemplo, o estudo de Yang e colaboradores (2019) que desenvolveram membranas de polissulfona contendo SB visando obter um material antioxidante e hemocompatível para aplicação em hemodiálise (YANG et al., 2019). Ainda, existem relatos da incorporação da SB em *scaffolds*, como nos trabalhos de Kapadnis e Shrotriya (2019) e de Leena, Vairamani e Selvamurugan (2017) que incorporaram a SB livre em *scaffolds* de óxido de polietileno visando a regeneração cutânea e a SB associada a nanopartículas de quitosana em *scaffolds* de alginato e gelatina visando a regeneração óssea, respectivamente (KAPADNIS; SHROTRIYA, 2019; LEENA; VAIRAMANI; SELVAMURUGAN, 2017).

Com relação a filmes poliméricos, até o presente momento, não foram encontrados relatos da associação da SB, livre ou nanoencapsulada nesta forma farmacêutica sólida visando o tratamento de patologias inflamatórias cutâneas. Existem estudos apenas que envolvem a

combinação do extrato seco silimarina em filmes de alginato, os quais foram desenvolvidos contendo este extrato livre (GHELEJLU; ESMAIILI; ALMASI, 2016) e nanoencapsulado (LEE et al., 2017) visando a proteção de alimentos frente a processos de oxidação e contaminação bacteriana.

1.2 ESTRUTURA E COMPOSIÇÃO DA PELE, MECANISMOS DE PERMEAÇÃO/PENETRAÇÃO CUTÂNEA E FORMULAÇÕES DE USO TÓPICO

A pele é o maior órgão do corpo humano e, estruturalmente, está organizada em três diferentes camadas: epiderme, derme e hipoderme (Figura 2). A epiderme, constituída por um tecido epitelial de revestimento pavimentoso queratinizado, é a camada mais superficial da pele e é composta por duas camadas, o estrato córneo (EC) e a epiderme viável. O EC é a camada mais externa da epiderme, sendo constituído por corneócitos (queratinócitos), que são células mortas, anucleadas e ricas em queratina, os quais estão imersos em uma matriz lipídica composta principalmente por ceramidas, colesterol e ácidos graxos (BARONI et al., 2012). Já a epiderme viável é formada por células em constante atividade proliferativa e onde são produzidos os queratinócitos, produtores de queratina, os quais são constantemente empurrados até o EC onde se tornam anucleados e mais achatados. Além dos queratinócitos, a epiderme é constituída ainda por melanócitos, responsáveis pela produção de melanina, pelas células de Langerhans, presente em toda a epiderme e responsáveis pela defesa imunológica da pele (macrófago tecidual), e pelas células de Merckel, presente em toda a epiderme e com terminações nervosas e sensoriais, atuando assim como um mecanorreceptor (KHAVKIN; ELLIS, 2011; MANN et al., 2012).

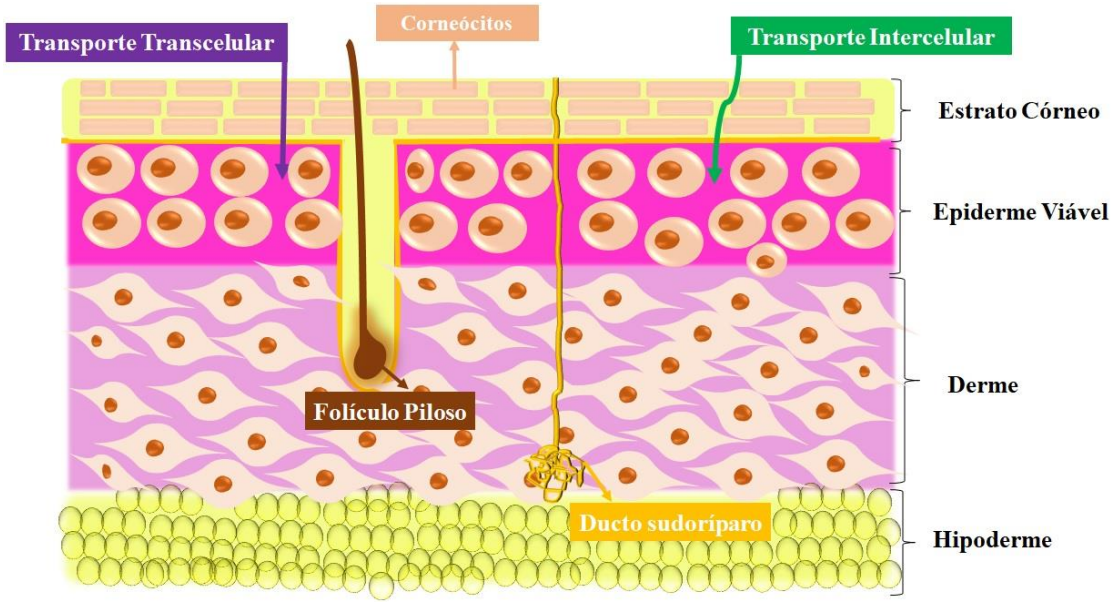
Abaixo da epiderme encontra-se a derme, a qual se apresenta como uma camada de sustentação. Nesta camada são encontradas fibras elásticas, responsáveis pela elasticidade da pele, além dos capilares sanguíneos, vasos linfáticos, nervos e terminações nervosas. Ainda, estão presentes as estruturas derivadas da epiderme, os chamados apêndices cutâneos, tais como os folículos pilosos, as glândulas sebáceas e sudoríparas. As principais células presentes na derme são os fibroblastos, os quais são responsáveis pela produção das fibras de colágeno, elastina e da matriz extracelular (substância fundamental). Ainda, nesta camada cutânea são encontradas células dendríticas dérmicas e células T (MANN et al., 2012). A última camada da pele e, portanto, a mais profunda, é a hipoderme, também chamada de tecido subcutâneo. Esta camada é formada por tecido conjuntivo frouxo e por adipócitos, conferindo reserva de energia, isolamento térmico e proteção mecânica (BARONI et al., 2012).

Esta estrutura complexa da pele faz com que ela desempenhe um papel importante de proteção, termorregulação e defesa imune. Por estar na interface com o meio externo, a pele está constantemente sujeita a estímulos, tais como patógenos e agentes mecânicos, químicos e físicos (MANN et al., 2012). A principal barreira física para a entrada de substâncias químicas e patógenos é o EC, devido a sua composição e a organização. Assim, a manutenção desta função de barreira do EC é de importância crítica, onde o rompimento da mesma pode desencadear uma série de processos imunológicos, inflamatórios e oxidativos (BIELFELDT et al., 2022). Além de dificultar a entrada de agentes agressores, o EC também evita a perda excessiva de água através da pele (BARONI et al., 2012).

O tratamento de doenças inflamatórias cutâneas é realizado, preferencialmente, com formulações de uso tópico. Estas, são selecionadas devido serem de fácil aceitação pelo paciente, com aplicação direta na lesão. Assim, exercem seus efeitos específicos no local da aplicação, reduzindo os efeitos adversos sistêmicos e evitando o metabolismo de primeira passagem (IQBAL et al., 2018). No entanto, o desenvolvimento deste tipo de formulação é bastante desafiador. Ao delinear uma estratégia de tratamento tópico de doenças imuno-inflamatórias da pele deve-se garantir que a substância ativa penetre no EC em pele íntegra, a fim de controlar os processos inflamatórios e imunológicos que ocorrem na epiderme e derme, bem como proteger este tecido de danos oxidativos (GUPTA; AGRAWAL; VYAS, 2012).

Assim, ao administrar uma formulação sobre a pele, primeiramente ocorre a partição do fármaco para o EC, seguido da sua difusão, devido ao gradiente de concentração, para a epiderme e derme, com ou sem absorção sistêmica (BIELFELDT et al., 2022). Conceitualmente, é importante diferenciar a terminologia referente aos processos de permeação e penetração cutânea. Diz-se que há penetração cutânea quando a substância ativa atravessa a barreira exercida pelo EC, e que há permeação cutânea quando a mesma migra de uma camada para outra (OECD, 2004). Em pele íntegra, a penetração de substâncias pode ocorrer pela via intercelular (ao longo de bicamadas lipídicas), transcelular (através dos corneócitos) ou parafolicular (IQBAL et al., 2018). A Figura 2 ilustra as possíveis vias de penetração/permeação de substâncias através da pele. Estas vias vão depender da lipofilicidade e do peso molecular de cada substância. Além de atravessar o EC, o fármaco pode entrar no tecido viável através dos folículos pilosos e suas glândulas sebáceas e das glândulas sudoríparas. Esta via de penetração utilizando apêndices cutâneos é utilizada principalmente por moléculas que se difundem lentamente ou que apresentam alta massa molecular (BIELFELDT et al., 2022).

Figura 2 - Representação esquemática das possíveis vias de penetração/permeação de substâncias através da pele íntegra.



Fonte: Próprio autor.

Tendo em vista os mecanismos de penetração/permeação cutânea, muitos fatores podem interferir neste processo, como a condição da pele, a idade e a espessura e a natureza do EC conforme a região do corpo (DANCIK; BIGLIARDI; BIGLIARDI-QI, 2015). Ainda, características físico-químicas do fármaco e a da base dermatológica que o veicula precisam ser levadas em consideração, pois deve haver um adequado coeficiente de partição do ativo entre o seu veículo e o EC e, posteriormente, entre o EC e a epiderme viável (BIELFELDT et al., 2022). Somado a isso a estabilidade dos ativos frente a agentes ambientais como luz, calor e ar pode contribuir para que concentrações sub-terapêuticas atinjam a epiderme viável e a derme (CUCÉ, NETO, 2001; DIANZANI et al., 2014).

Com relação à condição da pele, sabe-se que patologias que acometem a composição, organização e hidratação do tecido cutâneo alteram a permeabilidade de substâncias através do mesmo (DANCIK; BIGLIARDI; BIGLIARDI-QI, 2015). Uma das patologias cutâneas em que há comprometimento da barreira física exercida pelo EC, levando a um aumento da perda transepidérmica de água e da permeabilidade de substâncias através da pele, é a dermatite atópica. No caso específico desta doença imuno-inflamatória cutânea, o desenvolvimento de formulações tópicas para tratá-la é ainda mais desafiador, pois, dependendo do estágio e gravidade da doença, a barreira cutânea pode estar totalmente comprometida ou em processo de restauração (FULLERTON, 2007). Nesse sentido, estratégias de formulação baseadas em

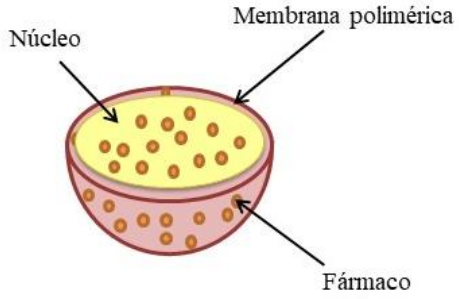
nanotecnologia têm sido sugeridas para superar esses desafios. No capítulo 1 desta tese está detalhada a fisiopatologia que envolve a dermatite atópica, bem como seus tratamentos atualmente disponíveis na clínica e o avanço nas pesquisas envolvendo sistemas nanoestruturados para o tratamento tópico desta patologia crônica da pele.

1.3 NANOCÁPSULAS POLIMÉRICAS: CARACTERÍSTICAS E DESENVOLVIMENTO

As nanopartículas poliméricas tem sido o propósito de inúmeros estudos visando a terapia de diferentes patologias cutâneas (FERREIRA et al., 2019; FLORES et al., 2015; PEGORARO et al., 2017; SARI et al., 2020). Em linhas gerais, esses sistemas nanoestruturados proporcionam vantagens à substância ativa encapsulada como proteção físico-química, modulação da liberação, melhora na sua solubilidade, além de diminuir a toxicidade durante a aplicação (FRANK et al., 2015; JAIN; THAREJA, 2019). Ainda, quando aplicadas cutaneamente, as nanopartículas poliméricas tendem a manter a sua integridade estrutural, o que pode ocasionar um depósito sobre a superfície da camada córnea, ou ainda em sulcos ou poros (ABDEL-MOTTALEB; LAMPRECHT, 2016; POHLMANN et al., 2016).

Dentre as nanopartículas poliméricas destacam-se as nanocápsulas (Figura 3), as quais podem ser compostas por um núcleo oleoso circundado por uma parede polimérica, onde o fármaco pode estar dissolvido no núcleo e/ou adsorvido no material polimérico (MORA-HUERTAS; FESSI; ELAISSARI, 2010). Estas nanoestruturas podem ainda ser classificadas como convencionais ou de núcleo lipídico, as quais se diferenciam pela composição do núcleo. As nanocápsulas de núcleo lipídico possuem um lipídeo sólido, como o monoestearato de sorbitano, disperso em um lipídeo líquido (FIEL et al., 2011; JAGER et al., 2009).

Figura 3 – Representação esquemática da estrutura de uma nanocápsula polimérica.



Fonte: próprio autor.

O principal método de preparo das nanocápsulas é a deposição interfacial do polímero pré-formado. Esta técnica consiste em uma fase orgânica contendo o polímero, o óleo, um solvente miscível com a água, como, por exemplo, a acetona, juntamente com um estabilizante de baixo EHL e o princípio ativo, sob agitação magnética e temperatura controlada por um determinado período de tempo. Após, esta fase é lentamente vertida em uma solução aquosa, contendo um tensoativo de elevado EHL, sob agitação magnética. Neste momento, por meio da emulsificação espontânea, há a formação das vesículas e o polímero precipita na interface dos dois componentes imiscíveis (água e óleo) com a retirada do solvente orgânico por evaporação à pressão reduzida (FESSI et al., 1989). Após o preparo, é realizada a caracterização físico-química destas suspensões através da sua análise morfológica, distribuição do tamanho de partícula, potencial zeta, pH, teor e eficiência de encapsulação, bem como a análise do perfil e cinética de liberação, entre outras avaliações que forem necessárias dependendo do objetivo a ser alcançado com o desenvolvimento desta suspensão nanoestruturada (JAIN; THAREJA, 2019).

Com relação aos seus constituintes, o material polimérico para o desenvolvimento de nanocápsulas deve ser biocompatível, podendo ou não ser biodegradável, e ter sua origem natural, semissintética ou sintética (FRANK et al., 2015). Dentre os polímeros utilizados para o preparo de nanopartículas poliméricas está a etilcelulose, que tem demonstrado ser interessante para a aplicação tópica destes sistemas, por possuir um perfil de liberação controlado e maior retenção nas camadas da pele, minimizando o alcance do ativo à circulação sistêmica (RIGON et al., 2019; SAHU et al., 2013). Outro constituinte importante no preparo das nanocápsulas é o óleo, o qual compõe o núcleo destes sistemas, possuindo funções importantes, tais como solubilização e controle da liberação da substância ativa, bem como aumento da estabilidade da mesma (FRANK et al., 2015; GEHRCKE et al., 2017, 2018; MATTIAZZI et al., 2019). Diversos tipos de óleos são utilizados no preparo de nanocápsulas, entre eles, destacam-se os triglicerídeos de cadeia média. Este óleo é amplamente utilizado devido a sua capacidade de solubilizar diversos tipos de fármacos, e também, devido a sua compatibilidade com diferentes polímeros (MORA-HUERTAS; FESSI; ELAISSARI, 2010).

As nanocápsulas são obtidas na forma de suspensão aquosa, o que possibilita a incorporação de ativos lipofílicos associados a estes nanossistemas em bases dermatológicas hidrofílicas, as quais são bem aceitas pelos pacientes. No entanto, a baixa viscosidade destas suspensões dificulta a sua aplicação direta sobre a superfície da pele, sendo comum o desenvolvimento de uma forma farmacêutica que viabilize esta aplicação. Neste sentido, em sua grande maioria, os trabalhos demonstram a incorporação de tais suspensões em hidrogéis

(CARDOSO et al., 2019; CONTRI et al., 2014; FERREIRA et al., 2019; MARCHIORI et al., 2017b; SARI et al., 2020). No entanto, a incorporação de nanopartículas poliméricas em formas sólidas, como filmes poliméricos, tem se mostrado uma vantajosa e promissora alternativa no tratamento de desordens cutâneas. A tabela 1 resume as pesquisas encontradas na literatura que envolvem a incorporação de nanopartículas poliméricas em filmes objetivando a aplicação cutânea. Estes estudos, em sua maioria, elucidam as vantagens da combinação de nanopartículas e filmes, com controle de liberação da substância ativa e melhora do efeito terapêutico proposto.

Tabela 1. Filmes poliméricos contendo sistemas nanoestruturados para a aplicação sobre a pele

Nanoestrutura	**Ativo**	**Agente filmógeno**	**Aplicação**	**Referência**
Nanocápsulas de Eudragit RS 100	Óleo de rosa mosqueta	Quitosana	Dermatológica	(CONTRI et al., 2016)
Nanopartícula de ácido hialurônico	Vitamina E	Alginato	Cicatrização	(PEREIRA et al., 2016)
Nanopartícula de quitosana	Cefazolina	Alginato e pectina	Cicatrização	(SHAHZAD et al., 2019)
Nanopartícula de PLGA	Gentamicina	Pullulan	Cicatrização	(DHAL e MISHRA, 2020)
Nanocápsulas de Eudragit RS 100	Óleo de romã	Pullulan	Dermatite atópica	(CERVI et al., 2021)
Nanopartícula de zeína	Naringenina	Gelatina e goma gelana oxidada	Cicatrização	(GHORBANI; HASSANI; RAEISI, 2022)

1.4 FILMES POLIMÉRICOS COMO PLATAFORMA DE ADMINISTRAÇÃO CUTÂNEA DE FÁRMACOS

1.4.1 Conceitos, aplicações e desenvolvimento

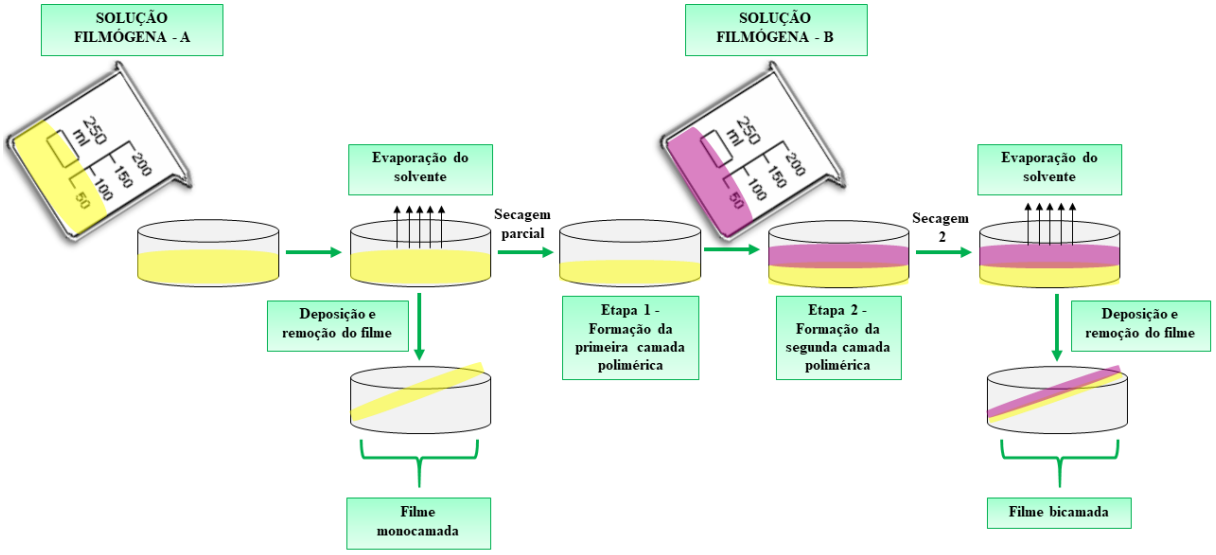
Os filmes poliméricos vêm ganhando popularidade na área farmacêutica como uma nova forma de administração de fármacos, uma vez que apresentam potencial para diferentes aplicações (KARKI et al., 2016). No que diz respeito à aplicação cutânea, os filmes poliméricos apresentam diversas vantagens quando comparados a formulações líquidas e semissólidas, tais como: dosagem mais precisa, fácil e conveniente aplicação e controle de liberação, evitando múltiplas aplicações (PADULA et al., 2019; PEREIRA et al., 2016; SARUNYA et al., 2018). Ademais, os filmes poliméricos possuem a característica de se aderir aos tecidos lesionados, atuando com uma barreira à infecção bacteriana, bem como muitos materiais são capazes de absorver o exsudato formado em alguns tipos de lesões cutâneas (MALI et al., 2018).

Frente a isso, filmes e membranas ativas têm se mostrado promissores para utilização em diferentes patologias da pele, como feridas cutâneas (NASCIMENTO et al., 2020; PAGANO et al., 2019), queimaduras (BAOYONG et al., 2010; PEREIRA et al., 2014) e úlceras diabéticas (HUSSAIN et al., 2017). Ainda, filmes têm sido desenvolvidos para o tratamento da dermatite atópica, por serem de fácil aplicação, por controlarem a liberação de ativos, proporcionando um efeito terapêutico prolongado e de baixa absorção sistêmica, e por atuarem como uma barreira física à pele irritada (ALVES et al., 2016; BOUTHILLETTE et al., 2019; JEONG et al., 2019; VOSS et al., 2020).

Os filmes podem ser produzidos a partir de um único agente formador de filme ou a partir de uma mistura destes. Esta mistura de polímeros pode ser feita tanto na forma de blendas poliméricas, quanto a partir da produção de filmes multicamadas (KARKI et al., 2016). Especificamente, os filmes em bicamada são uma opção promissora para melhorar suas propriedades gerais, complementando as propriedades filmogênicas de cada polímero e aproveitando as suas melhores características isoladamente (NETO et al., 2019). Ao contrário das blendas, os filmes bicamada apresentam uma estrutura heterogênea onde as propriedades intrínsecas de cada agente filmógeno são preservadas na camada correspondente do filme. Dessa forma, esta combinação pode melhorar as propriedades mecânicas, óticas e de barreira física quando comparadas com os filmes produzidos com os polímeros isolados ou com blendas destes polímeros (FERREIRA et al., 2016; KHWALDIA et al., 2020; NETO et al., 2019; PEREDA et al., 2011).

Em relação ao preparo de filmes poliméricos, existem diferentes métodos de produção, tais como deposição/evaporação do solvente, extrusão a quente e a impressão 3D (KARKI et al., 2016). O método de deposição/evaporação do solvente (*solvent casting*) é o mais comumente utilizado, pela sua facilidade, praticidade e baixo custo. Este método de preparo envolve a solubilização/dispersão do polímero, bem como dos demais componentes da formulação, em um solvente ou mistura de solventes adequados, formando um sistema relativamente viscoso. Esta solução ou dispersão é então vertida sobre um suporte, geralmente placas de *petri* ou de silicone, o qual é levado para uma estufa para que o solvente evapore. Depois que todo o solvente foi evaporado, o filme seco pode ser retirado do suporte (DESAI, 2017). Através desta técnica, é possível produzir filmes monocamada, utilizando apenas um agente filmógeno ou uma mistura de polímeros em uma mesma camada, e filmes multicamadas, combinando diferentes soluções filmogênicas em diferentes camadas. Para este último caso, as camadas poliméricas posteriores são depositadas sobre a primeira camada parcialmente seca, sendo então a placa de *petri* novamente levada para secagem em estufa, permanecendo até a formação final do filme seco (NETO et al., 2019). A Figura 4 esquematiza o processo de preparo de filmes em mono e bicamada utilizando a técnica de evaporação do solvente em uma e duas etapas, respectivamente.

Figura 4 – Esquematização do preparo de filmes bicamada utilizando o método de deposição/evaporação do solvente.



Fonte: próprio autor.

Após a produção dos filmes poliméricos é fundamental realizar a caracterização destes materiais a fim de garantir que possuam características compatíveis com a aplicação proposta. Objetivando a aplicação cutânea, estes materiais devem ser finos, flexíveis e com sensorial agradável, bem como permitir a liberação do ativo associado, garantindo assim a aceitabilidade pelo paciente e o efeito terapêutico (KARKI et al., 2016). Os filmes poliméricos podem ser avaliados quanto à espessura e variação de peso, os quais se correlacionam diretamente com a dosagem de ativo, e variam de acordo com o tipo e quantidade dos componentes presentes no filme. Filmes com espessura uniforme na faixa de 5-200 μm podem fornecer uma dose precisa e uma boa absorção do ativo, além de melhorar o conforto durante a aplicação. Esta pode ser medida com o auxílio de um micrômetro ou paquímetro digital, ou através de técnicas de microscopia (RAJARAM; LAXMAN, 2017).

Para filmes que contenham substâncias ativas incorporadas, é de extrema importância a avaliação do teor e da homogeneidade do ativo, para garantir que este se manteve estável durante o processo de preparo, bem como se distribui de forma homogênea ao longo do filme produzido (KARKI et al., 2016). Além disso, a molhabilidade e o índice de intumescimento do filme devem ser avaliados, uma vez que podem impactar diretamente no tempo de desintegração e dissolução do filme, influenciando na liberação do ativo (KARKI et al., 2016; RAJARAM; LAXMAN, 2017). Ainda, o índice de intumescimento também está diretamente relacionado com a capacidade do filme produzido em absorver fluidos, característica importante para a aplicação deste em lesões cutâneas exsudativas (SHAHZAD et al., 2019).

As propriedades mecânicas são também relevantes, pois estes devem ser resistentes à ruptura e flexíveis, para que possam se adaptar às possíveis deformações durante seu manuseio e sua aplicação (MORALES; MCCONVILLE, 2011). Estas geralmente se baseiam em metodologias como a ASTM D882, da Sociedade Americana para Testes e Materiais, podendo ser avaliadas em termos de resistência à dobradura, força de tensão, módulo de Young e percentual de alongamento que correspondem à flexibilidade, resistência, rigidez e plasticidade do filme (ASTM, 2002). A resistência à dobradura determina a flexibilidade do filme através da avaliação da quantidade de vezes que é possível dobrá-lo no mesmo lugar em um ângulo de 180° sem rachar ou quebrar (WASILEWSKA; WINNICKA, 2019). Filmes que podem ser dobrados até 300 vezes sem rachar ou quebrar possuem excelente flexibilidade (MUKHERJEE; BHARATH, 2013).

Ensaios de tensão-deformação são normalmente utilizados, onde se mede a variação no comprimento como função da carga (F) aplicada. A tensão é definida como a resistência interna de um material a uma força externa aplicada, por unidade de área, enquanto a deformação é

definida como a variação de uma dimensão qualquer desse corpo, por unidade da mesma dimensão, quando submetido a um esforço qualquer (KARKI et al., 2016). Assim, neste tipo de ensaio, é permitido que o material sofra deformação em uma taxa constante, onde o estresse máximo necessário para rompê-lo corresponde à força de tensão (IRFAN et al., 2016). Quanto maior a força de tensão, ou seja, a força aplicada necessária para romper o filme, mais resistente é o material (KARKI et al., 2016).

O módulo de Young ou módulo elástico determina a rigidez do filme, indicando sua resistência à deformação. Este pode ser obtido através da inclinação de uma curva tensão×deformação, sendo representado como a razão entre a tensão aplicada e a deformação do material, na região de deformação elástica. Dessa forma, quanto maior a força de tensão apresentada, maior será o valor do módulo de Young, indicando que o material é mais firme, rígido e pouco flexível (WASILEWSKA; WINNICKA, 2019).

Tendo em vista as importantes características que os filmes de aplicação cutânea devem possuir, a escolha dos componentes da formulação deve ser bastante criteriosa, uma vez que afetam as propriedades mecânicas e físico-químicas dos filmes. Em linhas gerais, os filmes poliméricos são preparados a partir de um polímero e solventes, sendo facultativa a adição de plastificantes (HASSAN et al., 2018; KARKI et al., 2016). Plastificantes são um grupo de compostos auxiliares que tem como objetivo melhorar a performance de diferentes formas farmacêuticas, aumentando a flexibilidade e diminuindo a fragilidade do filme (LIEW; TAN; PEH, 2014). Quando incorporados em uma matriz polimérica, tais compostos interagem com as cadeias do polímero reduzindo as forças intermoleculares deste e aumentando a mobilidade entre as cadeias poliméricas (KHATRI et al., 2018). Os plastificantes devem ser compatíveis com o polímero de escolha, sendo substâncias como PEG 200 e 400, sorbitol e glicerol alguns dos mais utilizados (LIEW; TAN; PEH, 2014; SOTHORNVIT; KROCHTA, 2001). No presente trabalho foi utilizado o glicerol como plastificante, devido à sua compatibilidade com a matriz utilizada. Este poliol possui características hidrofílicas, sendo altamente miscível em água. Devido ao seu pequeno tamanho molecular, ele é capaz de se posicionar entre as cadeias do polímero formador de filme, interagindo com este e aumentando a mobilidade molecular e, consequentemente, a hidrofilicidade e a flexibilidade dos filmes plastificados (KHATRI et al., 2018; WATANABE et al., 2021).

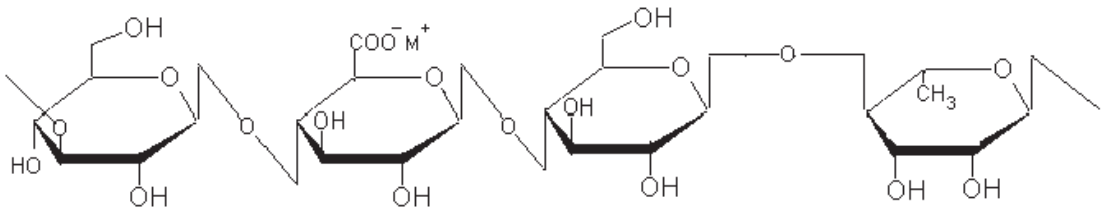
Com relação aos polímeros utilizados no desenvolvimento de filmes, os mesmos devem ser atóxicos, não-irritantes e compatíveis com a via de administração escolhida (TIWARI; UMASHANKAR; DAMODHARAN, 2018). Os polímeros podem ser naturais ou sintéticos, sendo os hidrofílicos os de escolha na área farmacêutica devido a não utilização de solventes

orgânicos e por apresentarem boas propriedades mecânicas (HASSAN et al., 2018; KARKI et al., 2016).

1.4.2 Goma gelana e pullulan como polímeros formadores de filme

Os polissacarídeos naturais vêm ganhando destaque no desenvolvimento de filmes devido à versatilidade de uso e suas características biocompatíveis e biodegradáveis. Dentre tais polissacarídeos está a goma gelana (Figura 5), a qual tem sido estudada tanto visando sua aplicação na indústria de alimentos, quanto na área farmacêutica e biomédica. Esta goma é um polissacarídeo extracelular obtido pela fermentação aeróbia da bactéria *Pseudomonas elodea*. Sua estrutura constitui-se de um esqueleto aniônico linear, composto de unidades repetidas de α-L-ramnose, β-D-glucose e β-D-glucoronato, na proporção molar de 1:2:1 (HISHAMUDDIN; RAZALI; AMIN, 2022). A forma de ocorrência natural da goma gelana apresenta dois substituintes acila (L-glicerila e acetila), os quais são removidos por hidrólise alcalina dando origem à gelana desacetilada, denominada "low-acetyl" ou "low-acyl". O interesse depositado nesta goma, na área acadêmica e industrial, se deve ao fato de ser um polímero biocompatível, biodegradável e não tóxico, com propriedades espessante, gelificante e formadora de filme (OSMALEK; FROELICH; TASAREK, 2014).

Figura 5 - Estrutura química da goma gelana.



Fonte: próprio autor.

Estudos demonstram que a goma gelana possui potencial como polímero formador de filme em concentrações de 1 a 3%. A espessura dos filmes produzidos por esta goma varia de 10 a 50 μm, sendo considerados filmes ultrafinos (CHANG et al., 2010; PAOLICELLI et al., 2018). No entanto, a gelana sozinha forma filmes extremamente rígidos e quebradiços, sendo necessária a adição de plastificante em sua composição (YANG; PAULSON, 2000). Neste sentido, diferentes plastificantes já foram estudados a fim de melhorar as propriedades

mecânicas dos filmes produzidos com esta goma, sendo relatado que apenas o glicerol foi capaz de atuar como um bom plastificante, produzindo filmes transparentes, flexíveis e resistentes a uma concentração mínima de 50 % em relação ao peso final do filme seco (YANG; PAULSON, 2000).

O desenvolvimento de filmes a partir da goma gelana já foi relatado em diferentes abordagens tais como, embalagem protetora de alimentos (MIRÓN-MÉRIDA et al., 2019), regeneração óssea (CHANG et al., 2010) e tratamento local para câncer oral (TSAI et al., 2018). Há poucos estudos visando a utilização de filmes de goma gelana para a veiculação de substâncias através da pele. Os poucos estudos encontrados demonstram que este filme é promissor como material para este fim, uma vez que possui alta capacidade de intumescimento e baixa transmissão de vapor de água, influenciando na absorção de exsudato e hidratação da pele, respectivamente (ARIFAH et al., 2019; ISMAIL et al., 2019; SEBRI; AMIN, 2016).

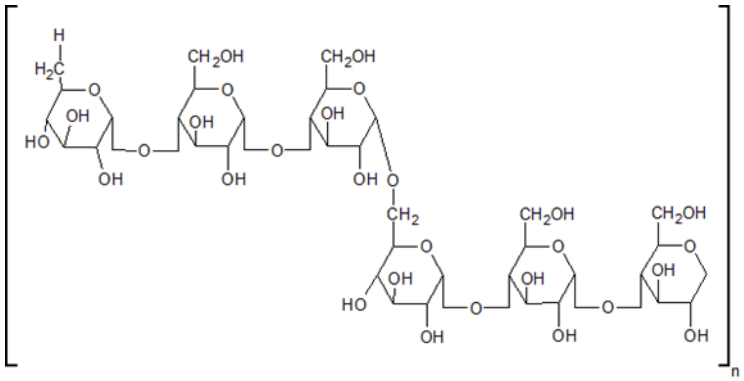
Em sua maioria, os estudos de aplicação sobre a pele de filmes de goma gelana visam o tratamento de feridas cutâneas. Filmes de goma gelana contendo óleo de coco e mel de Manuka foram capazes de acelerar o processo de cicatrização e aumentar a hidratação da pele, proporcionando menor dor no local da lesão em ratos (MUKTAR, 2018). Nanotubos de dióxido de titânio foram incorporados a filmes de goma gelana visando uma ação antimicrobiana e cicatrizante (ARIFAH et al., 2019; RAZALI et al., 2020). Mais recentemente, Mahmood e colaboradores (2021) associaram o ofloxacino com óleo de melaleuca e óleo de lavanda em filmes de goma gelana pelo método de gelificação ionotrópica, visando o tratamento de feridas cutâneas. Os filmes produzidos foram transparentes, flexíveis e exibiram liberação controlada, bem como apresentaram atividade antioxidante (avaliada através do ensaio de eliminação do radical DPPH) e antibacteriana (avaliada *in vitro* frente a bactérias gram positivas e negativas). Ainda, os filmes contendo ofloxacino e óleos essenciais apresentaram rápida contração de feridas de espessura total, a qual foi avaliada em modelo animal.

Bali e Salve (2020) estudaram a utilização de filmes de goma gelana para liberação transdérmica da rasagilina associada a nanopartículas de poli(ácido lático-co-ácido glicólico) (PLGA). Tais filmes se mostraram promissores para o tratamento da doença de Parkinson, uma vez que aumentaram a biodisponibilidade do fármaco e apresentaram uma maior eficiência no direcionamento do mesmo para o cérebro (BALI; SALVE, 2020).

Outro polissacarídeo natural bastante utilizado na produção de filmes é o pullulan, o qual caracteriza-se por ser um polissacarídeo não iônico e linear, composto por unidades repetidas de D-glicose conectadas por uma sequência periódica de ligações glicosídicas (Figura 6). Por causa de sua estrutura molecular, é muito solúvel em água, mesmo em altas

concentrações (HAMIDI et al., 2022). Assim como a goma gelana, o pullulan vem se destacando no desenvolvimento de filmes para aplicação farmacêutica e biomédica, principalmente para o preparo de filmes orodispersíveis, devido ao seu perfil de rápida dissolução (CERVI et al., 2022; PRAJAPATI et al., 2018; SHAHZAD et al., 2020).

Figura 6 – Estrutura química do pullulan.



Fonte: próprio autor.

Além disso, este polissacarídeo natural tem ganhado destaque no preparo de filmes para uso cutâneo. Li e colaboradores (2018) desenvolveram filmes de pullulan copolimerizado com ácido hialurônico para cicatrização de feridas, os quais não apresentaram citotoxicidade *in vitro*, foram biocompatíveis, não irritantes e apresentaram ação cicatrizante *in vivo*. Também objetivando o tratamento de feridas cutâneas, Dhal e Mishra (2020) desenvolveram filmes de pullulan contendo gentamicina encapsulada em nanopartículas de PLGA. Estes filmes de base nanotecnológica proporcionaram uma liberação lenta e gradual da gentamicina, bem como apresentaram efeito antimicrobiano e proporcionaram o crescimento de células de fibroblasto *in vitro*. Ainda foram capazes de acelerar o processo de cicatrização *in vivo*, em comparação a um creme comercial de gentamicina.

Filmes de pullulan também já foram produzidos visando o tratamento da dermatite atópica. Jeong e colaboradores (2019) desenvolveram filmes de pullulan, pela técnica de deposição do solvente, contendo ou não o extrato de *Rhus verniciflua*, e avaliaram a ação destes em um modelo animal de dermatite atópica neonatal induzida por capsaicina. Os animais foram avaliados quanto ao escore de gravidade da dermatite, espessura epidérmica, infiltração de mastócitos e atividade da mieloperoxidase sérica. O filme de pullulan sozinho se mostrou capaz de melhorar a aparência da pele com dermatite e atenuar todos os parâmetros avaliados. Os

resultados obtidos podem estar relacionados à barreira física contra arranhões excessivos e a proteção frente à perda de água transepidérmica. No entanto, ao adicionar o extrato de *Rhus verniciflua*, todos os efeitos foram potencializados. Assim, os autores sugerem que a combinação pullulan + *Rhus verniciflua* possui uma dupla função, combinando os efeitos físicos do pullulan com o efeito farmacológico do extrato.

Também visando o tratamento da dermatite atópica, recentemente, nosso grupo de pesquisa desenvolveu filmes de pullulan contendo nanoemulsões e nanocápsulas de óleo de romã, os quais demonstraram propriedades compatíveis com a aplicação cutânea, como flexibilidade, potencial oclusivo e característica não-irritante. Tais filmes foram avaliados em modelo *in vivo* de dermatite atópica induzida por DNCB, no qual foi evidenciado que o filme contendo nanocápsulas de óleo de romã era promissor para o tratamento desta patologia, uma vez que foi capaz de atenuar a lesão cutânea e o comportamento hipernociceptivo mecânico induzido pela exposição ao agente irritante, bem como modulou os parâmetros inflamatórios e de estresse oxidativo avaliados, se sobressaindo aos resultados obtidos para o óleo em sua forma livre ou associado a nanoelmulsões (CERVI et al., 2021).

Tendo em vista os estudos que evidenciam a goma gelana e o pullulan como materiais promissores para a veiculação de substâncias ativas, nanoencapsuladas ou não, no tratamento de doenças da pele, surge a hipótese de que a combinação destes polissacarídeos naturais em filmes bicamada possa representar uma nova e promissora forma farmacêutica. É válido mencionar que não foram encontrados estudos que combinem a utilização de goma gelana e pullulan em filmes. Há relatos na literatura apenas da associação em filmes bicamada da goma gelana com polímeros como o ágar (ZHAI et al., 2020) e a gelatina (YANG et al., 2021), e de filmes de pullulan em bicamada utilizando caseinato de sódio (KRISTO; BILIADERIS; ZAMPRAKA, 2007). No entanto, em tais trabalhos os filmes em bicamada são desenvolvidos visando a aplicação como embalagem protetora de alimentos, ressaltando ainda mais o caráter inovador da proposta desta tese.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Desenvolver filmes monocamada de goma gelana e bicamada de goma gelana e pullulan contendo nanocápsulas de silibinina, visando propor uma nova formulação de aplicação cutânea para o tratamento da dermatite atópica.

2.2 OBJETIVOS ESPECÍFICOS

- Preparar suspensões de nanocápsulas de silibinina, utilizando triglicerídeos de cadeia média como núcleo oleoso;
- Realizar a caracterização das suspensões de nanocápsulas quanto ao teor de silibinina, pH, eficiência de encapsulação, tamanho de partícula, índice de polidispersão e potencial zeta;
- Produzir filmes monocamada de goma gelana contendo a silibinina nanoencapsulada e em sua forma livre;
- Produzir filmes bicamada contendo nanocápsulas de silibinina, utilizando o pullulan como agente formador da segunda camada polimérica;
- Caracterizar os filmes poliméricos mono e bicamada quanto à uniformidade de peso, espessura e conteúdo de silibinina;
- Realizar a avaliação morfológica das nanoestruturas e dos filmes poliméricos desenvolvidos através de microscopia eletrônica de varredura;
- Determinar o índice de intumescimento dos filmes mono e bicamada produzidos;
- Avaliar as propriedades mecânicas dos filmes, bem como determinar a resistência à dobradura dos mesmos;
- Determinar *in vitro* o perfil de liberação e a permeação/retenção cutânea da silibinina a partir dos filmes produzidos;
- Testar *in vitro* o potencial oclusivo e bioadesivo dos filmes poliméricos;
- Avaliar o possível efeito irritativo e a hemocompatibilidade dos filmes produzidos;
- Avaliar *in vivo* o efeito dos filmes produzidos frente a um modelo de dermatite atópica induzida por 2,4-dinitroclorobenzeno.

3 DESENVOLVIMENTO

O desenvolvimento desta tese está apresentado na forma de três capítulos, nos quais estão apresentados os métodos e resultados da presente tese. O capítulo 1 foi redigido na forma de capítulo de livro, que está sendo editado pela Apple Academic Press. O capítulo 2 consiste no artigo 1, o qual foi publicado na revista Materials Science & Engineering: C e, portanto, encontra-se no formato de versão publicada. No capítulo 3 consta o manuscrito 1, o qual foi redigido na forma de artigo científico e será submetido em revista internacional indexada.

Em anexo a esta tese, consta o certificado de aprovação da Comissão de Ética para o Uso de Animais da Universidade Federal de Pelotas-RS, bem como a permissão para uso do artigo científico no corpo da tese.

3.1 CAPÍTULO 1: NANOCARREADORES PARA O TRATAMENTO DA DERMATITE ATÓPICA

Capítulo de livro em edição, o qual fará parte do livro "Novel Nanocarriers For Skin Diseases: Advances and Applications" a ser publicado pela Apple Academic Press.

CHAPTER X

NANOCARRIERS FOR ATOPIC DERMATITIS TREATMENT

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ABSTRACT

Atopic dermatitis is a chronic and immune inflammatory skin disease that has a complex pathophysiology initiated by the skin barrier impairment. Although it is incurable, the treatment of this eczema is based on the control of its clinical signs (itching, dehydration, papules and wounds formation, edema). However, in addition to the lack of a single treatment, the pharmacological therapy for atopic skin is associated with limited efficacy and many adverse effects. The drawbacks of current therapies lead to the development of novel therapeutic strategies such as nanotechnology-based formulations. Different types of nanocarriers can be designed to transport active substances, enhancing their therapeutic efficacy and reducing their adverse effects. This chapter first introduces what pathophysiology of atopic dermatitis involves and the current treatments available on the market. Then, with numerous findings reported, it dwells on nanocarriers and their advancements in atopic dermatitis. Similarly, nano-based dosage forms are highlighted shedding light on both nanocarriers skin penetration and animal models for screening potential nanoformulations. But, it is important to mention that although there is progress in nanotechnology-based approaches to treat atopic dermatitis, more confirmatory *in vivo* studies about their effectiveness and toxicity are encouraged.

KEYWORDS: Atopic dermatitis, Nanostructures, Topical therapy.

X.1. ATOPIC DERMATITIS

Atopic dermatitis (AD) or atopic eczema is a chronic and non-contagious inflammatory cutaneous disease, characterized by intense itching. In addition, this eczema is associated with clinical features that include papules and plaques, along with xerosis and keratosis, involving the face, scalp, and extremity areas. Thus, the patients skin presents redness with or without wounds and exudation (Malik and Heitmiller, 2017; Rerknimitr et al. 2017). This eczema is part of the atopic triad (asthma, allergic rhinitis and dermatitis) and affects up to 20% of children and 1–3% of adults globally (Misery et al. 2019).

The pathophysiology of AD is a complex interrelationship among genetic, environmental, immunological, pharmacological and psychological factors, as well as skin barrier dysfunction (Malik and Heitmiller, 2017). Besides, this pathology can present heterogeneous manifestations with severity ranges from mild, moderate to severe. The diagnosis can be assessed using Eczema Area Severity Index (EASI) which defines the lesions extent in each body region and then a lesion in each of these areas is evaluated for formation of the erythema, edema or papules, excoriation and lichenification. Another severity score used is the Scoring AD (SCORAD) which is based on the extent and severity of the lesion and the presence of subjective symptoms, such as pruritus and sleeplessness (Rullo et al. 2008).

The skin has a complex structure, playing an important role in protection, thermoregulation and immune response and is constantly subject to stimuli, such as pathogens and mechanical, chemical and physical agents. The main physical barrier to the entry of chemicals and pathogens is the *stratum corneum*, the outermost epidermis layer formed by corneocytes, dead cells, and keratin, which are immersed in a lipid matrix composed mainly by ceramides, cholesterol and fatty acids (Wickett and Visscher, 2006). This lipid organization prevents excessive water loss through the skin. The skin barrier disruption in the AD may be related to changes in the corneocytes structural proteins expression and the lipids composition present in the *stratum corneum* (Yang et al. 2020).

Corneocytes are composed of densely cross-linked proteins such as filaggrin, loricrin and involucrin. Filaggrin is crucial for keratin alignment and its metabolites integrate the natural moisturizing factor which is essential for appropriate *stratum corneum* hydration (Wickett and Visscher, 2006). Filaggrin genes mutations are highly associated with AD development (Yang et al. 2020). Regarding lipid disorders, studies show that individuals with AD have a long chains reduction of lipids, an increase of unsaturated free fatty acids and a decrease in the ceramide levels. The ceramides reduction is due to an increased pH and high epidermal serine protease activity (Smeden and Bouwstra, 2016).

The allergens penetration through the skin leads to loss of tissue homeostasis. Dendritic cells induce the differentiation of naive CD4(+) T cells into T helper 2 (Th2) cells which secret Th2 cytokines (IL-4, IL-5, and IL-13). These cytokines stimulate the differentiation of B cells to IgE-producing plasma cells and the eosinophils activation. In addition to eosinophils activation, AD is also characterized by the infiltration of other inflammatory cells, such as lymphocytes, macrophages and mast cells (Malik and Heitmiller, 2017; Rerknimitr et al. 2017; Yang et al. 2020). Some authors categorize the AD into extrinsic and intrinsic types. Extrinsic or allergic type has high serum levels of total IgE with presence of specific IgE for environmental and food allergens. On the other hand, the intrinsic type has lower expression of specific IgE and Th2 cytokines, but presents higher expression of interferon-γ (IFN-γ) (Bieber 2017; Tokura, 2010).

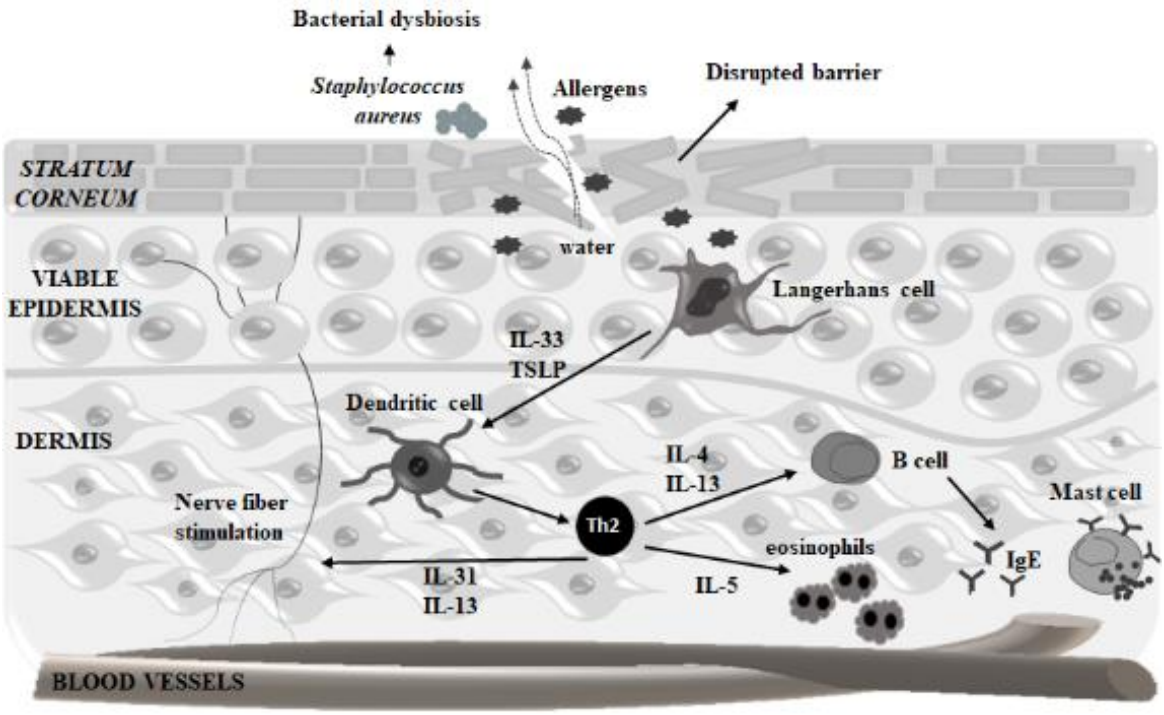

Figure X.1 Barrier impairment and immunological contributing to the pathogenesis of atopic dermatitis (skin structure not drawn to scale).

Pruritus in AD may be related to the release of mediators, such as thymic stromal lymphopoietin (TSLP), IL-13, and IL-31, which stimulate nerve fibers (Rerknimitr et al. 2017). During scratching the skin barrier is frequently removed and a constant release of cytokines occurs. This process is known as the vicious cycle of AD. The itch-scratch cycle is crucial to progression and inflicts a substantial psychosocial burden on patients (Malik and Heitmiller,

2017; Rerknimitr et al. 2017; Sibbald and Drucker, 2017). Moreover, in atopic skin is observed an abnormal microbial colonization with pathogenic organisms, mainly *Staphylococcus aureus*, which exacerbates the immune response. It is estimated that more than 90% of AD lesions are colonized with this pathogen (Malik and Heitmiller, 2017).

In addition to inflammatory/immunological modulation, reactive oxygen species (ROS) have been reported as important contributors to the pathogenesis of AD (Bertino et al. 2020). ROS damage epidermal keratinocytes through alterations in cellular DNA and/or through lipid peroxidation of cellular structures. Furthermore, enzymatic antioxidants such as superoxide dismutase, superoxide reductase, catalase, and the entire glutathione system may be reduced, while malondialdehyde levels may be increased. Thus, the oxidative stress contribute to exacerbation of cutaneous inflammation and disruption of skin barrier function, as well as enables infections by pathogens (Ji and Li, 2016).

X.2.CURRENT TREATMENTS

AD is not yet curable and presents a great individual clinical variability making its management difficult. The severity ranges of the AD generally determine the choice of therapy and its treatment is based on pruritus minimization and achieve long-term disease control. Prevention of skin dryness and contact with irritating agents are the primary approach that is achieved (Ghosalkar et al. 2022; Singh et al. 2022). The use of products composed by emollients, humectants and occlusive substances is necessary to preserve skin hydration and restore the integrity of the skin barrier (Mancuso et al. 2021).

The anti-inflammatory topical therapy is the first-line treatment for AD. Topical corticosteroids are the main options for acute flares, and they differ in potency. The choice of which one to use depends on the disease severity and the application site. Topical calcineurin inhibitors are used as second-line treatment in patients with inadequate response to topical corticosteroids (Tier et al. 2021). Patients with severe AD refractory to the habitual topical treatment can be medicated with systemic drugs, such as oral immunosuppressive drugs and biological therapy (Simpson et al. 2017). Topical and systemic approaches most frequently used in AD and their respective mechanisms of action are shown in table X.1.

Other therapies may be beneficial for the treatment of AD. Ultraviolet (UV) based phototherapy (UVB, narrow-band UVB or UVA) reduces inflammation and induces apoptosis in pathogenic cells. First-generation oral antihistamines can be used to control the pruritus associated sleep disturbances. Antibiotics are used for the treatment of infected lesions associated with AD (Berger et al. 2014; Singh et al. 2022)

Table X.1 Current drugs for AD treatment.

Treatment type	Class	Drug	Mechanism of action	Reference
Topical	Calcineurin Inhibitors	Tacrolimus, Pimecrolimus	It inhibits calcineurin- dependent T-cell activation, blocking the production of proinflammatory cytokines and mediators	(Carroll and Fleischer, 2015; Huang and Xu, 2015)
	Corticosteroids	Clobetasol propionate, Halobetasol propionate, Betamethasone dipropionate, Mometasone furoate, Hydrocortisone valerate, Triamcinolone acetonide	It suppresses inflammatory cytokines and immune cells	(Ghosalkar et al. 2022)
Systemic	Immunosuppressants	Cyclosporin	It inhibits calcineurin blocking transcription of IL-2 and decreases activation of T cells	(Schmitt, Schmitt and Meurer, 2007)
		Azathioprine	It is a 6-mercaptopurine analog. It inhibits purine synthesis, hindering cellular metabolism and preventing mitosis mainly in leukocytes	(Meggitt and Reynolds, 2001)
		Mycophenolate mofetil	It inhibits lymphocyte activation by monophosphate dehydrogenase reversible inhibition	(Neuber et al. 2000)
		Methotrexate	It is a folic acid antagonist. It inhibits dihydrofolate reductase, blocking division and lymphocyte proliferation	(Deo et al.2014)
	Biologic	Dupilumab	It is human monoclonal antibody that inhibits IL-4 receptor α, a common part of IL-4 and IL-13 receptors, blocking IL-4 and IL-13 signaling	(Ferreira and Torres, 2018)

However, conventional topical and systemic treatments present drawbacks. Topical corticosteroids can cause rashes, rosacea-like eruptions and systemic effects (Ghosalkar et al. 2022), while topical calcineurin inhibitors are associated to burning, pain and greater incidence of viral infection (Huang and Xu, 2015). Oral immunosuppressive drugs suffer from poor target-specific effect, low bioavailability, and pronounced side effects (Hemrajani et al. 2022). In consequence, patient satisfaction and compliance to therapy are often poor among the individuals affected by AD. Thus, novel drug delivery systems have been studied in the last years to alter the landscape of therapeutic options for AD management.

X.3.DRUG DELIVERY NANOCARRIERS

Nanocarriers development have been offered new opportunities in designing of drug delivery strategies for cutaneous disorders management, including AD (Ramanunny et al. 2021). Over the past few decades there has been extensive research demonstrating the effectiveness of classical drug-loaded nanocarriers (corticosteroids, immunosuppressants and antihistamines) to treat AD. The main benefits provided by the nanoencapsulation of traditional drugs are in terms of increased bioavailability and improved skin permeability, which enables dose reduction and, consequently, diminishes side effects (Alam et al. 2013; Badihi et al. 2020; Hussain et al. 2014; Li et al. 2012; Wang et al. 2019). However, research involving the nanoencapsulation of non-traditional drugs (drug repositioning) and novel therapeutic molecules to treat AD have increased (Cervi et al. 2021; Espinoza et al. 2020; Lin et al. 2021; Weber et al. 2018).

Most studies focus on the development of novel nanocarriers for topical application, including micro and nanoemulsions, liposomes, ethosomes, transfersomes, solid lipid nanoparticles, nanostructured lipid carriers, polymeric micelles, nanofibers, polymeric nanoparticles and inorganic nanoparticles (Fig.X.2). In fact, in addition to being the first-line treatment for AD, topical therapy is more advantageous than systemic administration because of the direct contact with the site of action. Moreover, specifically for AD, the formulation components can contribute to restore the skin barrier (Paiva-Santos et al. 2022). The choice of the ideal nanocarrier and its components should be based on the encapsulated drug characteristics and the objective to be achieved. In topical treatment, the type and the composition of the nanocarrier may influence the drug skin permeation (Fig.X.3) (Roberts et al. 2017). In the following sections of this chapter is approached an overview of the several nanocarriers along with their applications for AD treatment.

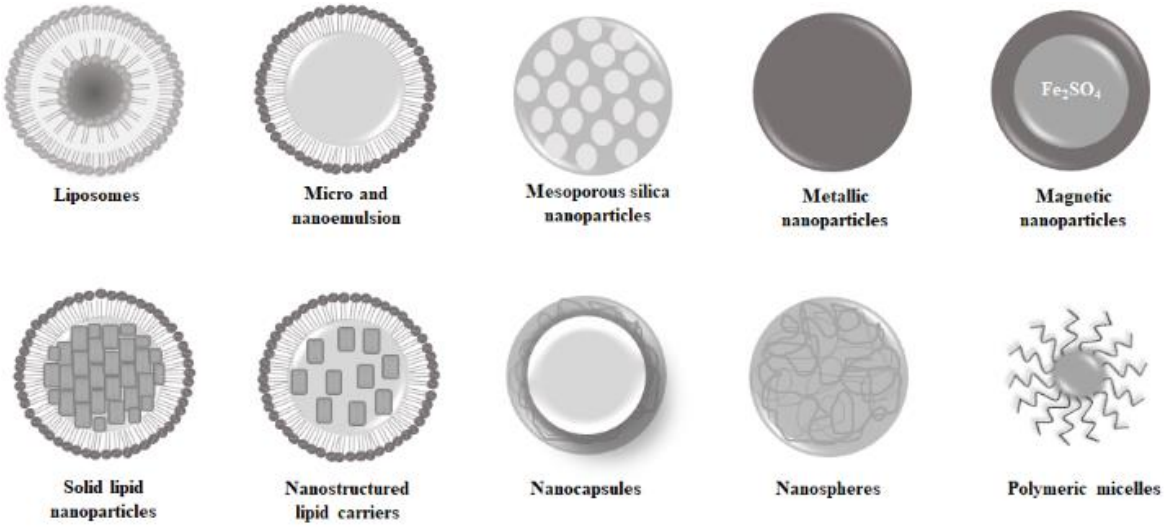

Figure X.2 Representative figures of nanocarriers investigated for drug delivery in atopic dermatitis treatment.

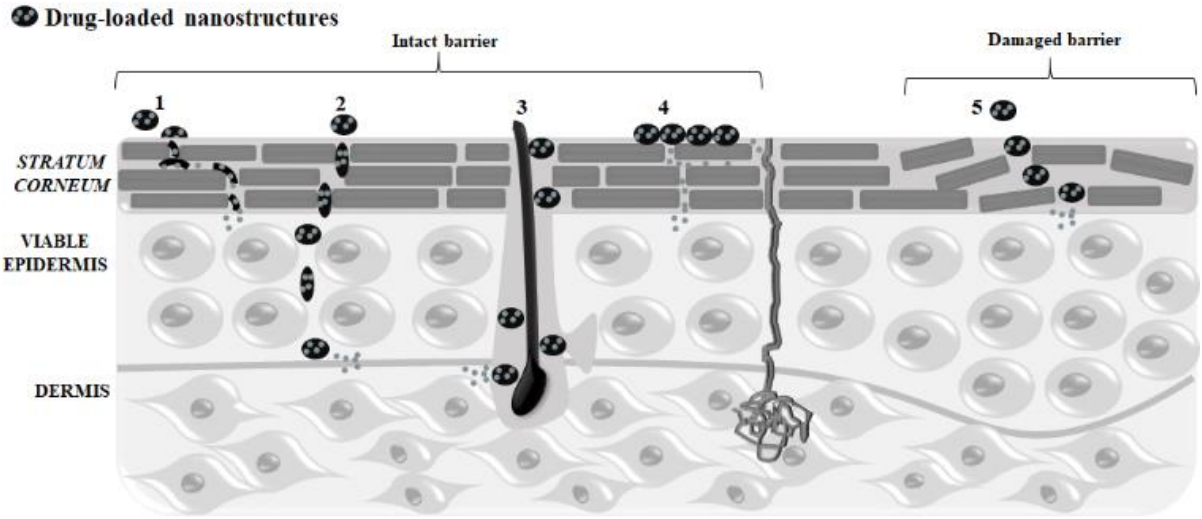

Figure X.3 Main penetration routes of nanocarrier-based formulations on skin (skin structure not drawn to scale). 1) Nanocarrier fusion with lipid structure of stratum corneum (e.g. liposomes, nanoemulsions); 2) Nanocarrier intact penetration across skin (flexible nanocarriers; e.g. ethosomes, transferomes); 3) Increased penetration by nanocarrier accumulation in hair follicles (e.g. polymeric nanoparticles); 4) Skin nanoparticles deposition with improved occlusion and penetration (e.g. lipid nanoparticles); 5) Increased penetration across inflamed skin.

X.3.1 Lipid-based nanostructures

X.3.1.1 Vesicular systems

Lipid-based vesicular carriers have been extensively studied for AD treatment due to their advantageous characteristics as low toxicity, biodegradability and low cost. The amphiphilic structure of nanovesicles enables encapsulation of both hydrophobic and hydrophilic drugs. In addition, the vesicles composition influences in their physicochemical properties, stability and efficacy (Kushwaha et al. 2021; Paiva-santos et al. 2021; Souto et al. 2021). Different vesicular systems have been studied for AD treatment such as liposomes, ethosomes, niosomes and transferosomes.

Conventional liposomes are hollow vesicles composed of phospholipids bilayer surrounding an aqueous core with similar composition of the biological membranes. Besides, liposomes may include other lipids, such as cardiolipin, cholesterol or sphingomyelin which may affect stability, membrane fluidity or degree of hydration (Kushwaha et al. 2021). Liposomes were developed to reduce the side effects of betamethasone valerate and diflucortolone valerate, medium and high potency topical corticosteroids, respectively. The vesicles were prepared by thin-film hydration method using Phospholipon® 90G and cholesterol for lipid bilayer formation and with only 10% of corticosteroid concentrations used in commercial creams. Despite the lower drug concentration, the *in vitro* skin permeation and *in vivo* therapeutic efficacy studies (2,4-dinitrofluorobenzene-induced AD of rats) showed that the liposomal formulations had amounts of drug retained in the skin and values of skin hydration similar to conventional formulations for both corticosteroids used, suggesting a possible side effects minimization (Eroğlu et al. 2016).

X_L-DNA-loaded liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) and cholesterol were produced by emulsion transfer method and presented a mean diameter of 115 nm, a narrow size range and a negative zeta potential. Transdermal delivery was assessed in rat back skin fragments mounted in a Franz cell system using fluorescein-labeled X_L-DNA.Skin samples observed under confocal microscopy revealed that non-nanoencapsulated X_L-DNA remained only on the skin surface while X_L-DNA-loaded liposomes were distributed in the epidermal and dermal layers. Besides, the efficacy of liposomes was evaluated using a NC/Nga mouse model stimulated with house dust mite plus 2,4-dinitrochlorobenzene. The results clearly showed that X_L-DNA encapsulated into liposomes allows a Th1/Th2 balance, reducing the levels of GATA3, IL-4 and IL-5 (cell transcription factor and Th2 cytokines, respectively) and increasing

the expression of T-bet (Th1 cell transcription factor) and IFN-γ, being an effective immunomodulatory formulation for the treatment of AD (Yang et al. 2018).

In both studies, an increase in permeation of active substances was found when associated with liposomes, which may have increased therapeutic efficacy. Liposomes can be fused with the *stratum corneum* releasing the drug in deeper layers of the skin. However, liposomes do not cross the *stratum corneum* intact (Kushwaha et al. 2021). To improve drug penetration, liposomes were modified by adding edge activators or single chain surfactant, being designated flexible or elastic or highly deformable liposomes (transfersomes). This unilamellar vesicle is more effective because its flexibility permits the vesicles to squeeze themselves penetrating through the *stratum corneum* in their intact form, reaching deeper skin layers (Souto et al. 2021).

Increasing liposome flexibility may be beneficial in the treatment of AD. Cetirizine dihydrochloride-loaded transferosomes composed of Phospholipon® 90G phospholipid, stearylamine and edge activator (sodium deoxycholate, Tween® 80 or Span® 80) were prepared by the thin film hydration method. Among tested edge activators, 10% Span® 80 was selected for the elastic vesicles development because presented maximum drug entrapment efficiency. This optimized formulation was compared to conventional liposomes prepared using 10% cholesterol. It was showed that conventional liposomes are reduced in size by half when subjected to extrusion to assess their degree of deformability, while elastic vesicles maintained their size close to their initial value, proving their flexibility. Notably, the transfersomes increased skin permeation rates of cetirizine by almost two-fold. Besides, the developed formulation reduced the itch score and the dermal eosinophil count compared to conventional cetirizine cream in AD model induced by topical treatment with oxazolone on female BALB/c mice (Goindi et al. 2013).

Skin permeation and consequently the therapeutic effect of elastic liposomes may be improved by the permeation enhancers addition. Kang and colleagues developed conventional and elastic liposomes associated with the TAT peptide (transactivator of transcription) as a permeation enhancer for oregonin delivery, a natural and hydrophilic substance. The addition of the peptide did not change the initial physicochemical characteristics of the vesicles (average diameter of 130-140 nm, negative zeta potential and loading efficiency of around 50%), as well as maintained the flexibility of the elastic liposomes. However, TAT peptide increased the permeation flux of about 20% and showed a greater reduction in the lesion scores and inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 levels (Kang et al. 2010). The same research group in another study developed elastic liposomes for the taxifolin glycoside skin

delivery conjugated with Pep-1 peptide, a type of penetrating peptide. This formulation presented enhanced drug skin permeation (*in vitro*), as well as improved skin hydration and reduced IL-4 and IgE blood levels in AD-induced by 2,4,6-trinitro-1-chrolobenzene on NC/Nga mice (Kang et al. 2010).

Ethosomes also emerged in the investigation of novel techniques to improve cutaneous delivery. Ethosomes display a phospholipid bilayer and a high ethanol concentration (20–45%) as an alternative to cholesterol. The ethanol presence makes these vesicles smaller and with higher entrapment efficiency, deformability, fluidity and stability than liposomes. In addition, it improves the extension and efficiency of skin penetration (Paiva-santos et al. 2021). Studies have shown promising performances of ethosomal formulations designed for dermal delivery of drugs in AD treatment. In one of these works, ethosomes containing the immunosuppressant tacrolimus were prepared from Lipoid® S100 and 30% ethanol *versus* traditional liposomes containing only Lipoid® S100. In addition to reducing the nanovesicles size, the presence of ethanol increased the drug encapsulation efficiency. Moreover, ethosomes showed increased tacrolimus permeation through *stratum corneum*, reaching the target of AD (Li et al. 2012). In another study involving an immunosuppressant drug, Carreras and co-workers compared the skin permeation of four novel lipid vesicles containing cyclosporine A: liposomes (formulated with Phospholipon®90G and cholesterol), transfersomes 1 (prepared with Phospholipon®90G and Tween® 80), transfersomes 2 (containing Phospholipon®90G and Tween® 20 and D-limonene) and ethosomes (formulated with Phospholipon® 90G and ethanol). Ethosomes were the most flexibles among the produced nanovesicles, reducing only a 14% of their initial size after extrusion. The cyclosporine A permeation using human heat-separated epidermis (HHSE) was ordered as follows: ethosomes > transfersomes 1 > transfersomes 2. No cyclosporine A diffusion was detected from conventional liposomes (Carreras et al. 2020).

In recent studies conducted by Kumar and team, ethosomes were formulated for the AD management varying the concentrations of phosphatidylcholine and ethanol, for skin delivery of piperine or tea tree oil. In both studies, the entrapment efficiency was increased with the increasein phosphatidylcholine and ethanol concentrations, while the size was increased with the increasein phosphatidylcholine concentration and reduced with the increase in ethanol concentration. The optimized piperine-loaded ethosomal dispersion was formulated with 3% phosphatidylcholine and 40% ethanol, which presented size of 318.1 nm and entrapment efficiency of 74.30%. The optimized tea tree oil-loaded ethosomes were produced with 3% phosphatidylcholine and 30% ethanol and presented size and entrapment efficiency of 333.6 nm and 76.19%, respectively. These ethosomal formulations were incorporated into creams and

showed higher deposition in the epidermis and dermis in comparison to their respective conventional creams. Moreover, the drug-loaded ethosomal creams were more effective than their conventional creams, reducing the inflammatory response in 2,4-dinitrochlorobenzene-induced female BALB/c mice model (Kumar et al. 2021a, 2021b).

Binary ethosomes has also been developed for topical drug delivery in AD treatment. The vesicle dispersion was produced by adding another alcohol type to the classical ethosome dispersion, such as propylene glycol and isopropyl alcohol (Abdulbaqi et al. 2016). Akhtar and colleagues developed binary ethosomes with ethanol plus propylene glycol aiming to improve the penetration of topical corticosteroids into deeper epidermis to treat atopic skin. In the first study, the authors explored the role of binary solvents in topical drug delivery of triamcinolone acetonide. A reduction in vesicle size was observed with the increase in both propylene glycol and ethanol concentrations, suggesting an improved bilayer flexibility (Akhtar et al. 2017). Besides, binary ethosomes presented higher entrapment efficiency and permeated amount of triamcinolone acetonide through the skin than conventional ethosomes. In the second study, the use of β-cycloamylose as penetration enhancer in binary ethosomes (β-cycloethosomes) was investigated in comparison to reference ethosomes (without β-cycloamylose) for fluocinolone acetonide topical delivery. β-cycloethosomes showed maximum *in vitro* permeability and presented a deeper uniform penetration of fluorescent dye deep into the epidermis, suggesting a synergistic effect exerted by binary ethosomes and β-cycloamylose (Akhtar et al. 2018).

The drug systemic absorption may be reduced and its residence time in the upper skin layers (*stratum corneum* and epidermis) may be improved by niosomes development. This vesicular carrier is composed by cholesterol and non-ionic surfactants, as alternatives to phospholipids (Limongi et al. 2021). In addition to skin retention, niosomes may prevent water loss from the skin and replenish lost cutaneous lipids, improving the skin barrier. Considering this, a comparative study between levocetirizine loaded polymeric nanoparticles and niosomal formulation was conducted by Pal and co-workers. Niosomes were prepared by thin film hydration method with Span® 20, Tween® 40 and cholesterol, while chitosan nanoparticles were prepared by ionic gelation method. Both nanocarriers exhibited particle size around 380 nm, but niosomes presented higher entrapment efficiency than nanoparticles. Besides, it was observed higher skin retention percentage of levocetirizine provided by niosomes. In the *in vivo* test, levocetirizine niosomes reduced the erythema score and scratching frequency in 2,4-dinitrochlorobenzene-induced AD animal model (Pal et al. 2021).

Phytosomes have been developed to improve the effectiveness of natural actives, including for AD treatment. Phytosomes are formed by bound between phospholipids of the

lipid membrane and phytomolecules (Limongi et al. 2021). The effects of centella asiatica phytosome were examined by using phthalic anhydride-induced AD mouse model. This phytosome inhibited infiltration of inflammatory cells and decreased the hyperkeratosis and proinflammatory cytokines release such as IL-1β and IgE (Ho et al. 2018). In a registry study, Togni and co-workers evaluated the effects of curcumin phytosome (Meriva®) as supplementary management of patients affected by AD. After 90 days, it was observed a better response in the group that received standard management (commercially available moisturizers and ceramide creams) plus phytosomes, with reduction of clinical manifestations such as itch, eczema, dryness, loss of sensitivity, and edema, as well as reduction in oxidative stress and improvement in skin oxygenation and hydration (Togni et al. 2019).

X.3.1.2 Lipid nanoparticles

As well as vesicular nanocarriers, lipid nanoparticles present low toxicity and improved drug skin permeability, being widely studied for the topical treatment of AD. These nanoparticles adhere to the skin forming a lipid film, presenting occlusive properties, as well as being able to provide drug slow release. However, these carriers are restricted to the encapsulation of lipophilic substances (Sengar et al. 2018). Lipid nanoparticles comprise solid lipid nanoparticles and nanostructured lipid carriers.

Solid lipid nanoparticles are composed by solid lipids at room temperature which are involved by surfactants in aqueous environment (Roberts et al. 2017). Hydroxyzine solid lipid nanoparticles were developed to enhance drug skin permeation and to improve its antidermatitis effect. Different formulations were prepared by the double emulsification method varying the lipid type (cholesterol or glyceryl dibehenate), soybean lecithin amount and Tween® 80 concentration. The optimized formulation presented negative zeta potential, average diameter of 111 nm and encapsulation efficiency of 75%. Besides, it presented higher values of rate transdermal flux and permeability coefficient, reaching a cumulative percent of permeating drug of 75% after 12 h. Moreover, 2,4-dinitrochlorobenzene-inducedAD mice model showed the potential of gels containing hydroxyzine solid lipid nanoparticles to reduce scratching behavior, IL-4 and substance P levels, as well as to improve hypertrophy of epidermis (El-telbany et al. 2021).

Aiming to develop cyclosporine A-loaded solid lipid nanoparticles for topical AD treatment, the formulations were prepared using the hot ultrasonication method and were composed of Softisan® 649 and Tween® 80. After preparation, the nanoparticles presented sizes of around 200 nm, negative zeta potential and encapsulation efficiency of 88%. Then, lipid

nanoparticles were freeze-dried, resulting in an oleogel formation. This oleogel presented *in vitro*drug controlled permeation profile in comparison to lipid nanoparticles in aqueous dispersion, indicating its potentiality fortopical administration (Silva et al. 2020).

In another study approaching the nanoencapsulation of an immunosuppressant drug in solid lipid nanoparticles, Pople and Singh (2010) optimized a formulation containing glyceryl trimyristate, polysorbate 80 and sorbitan monooleate for tacrolimus dermal targeting with reduced adverse effects. This formulation presented superior drug release profile, skin penetration and accumulation in comparison to the marketed product. Skin visualization by confocal laser scanning microscopy confirmed that lipid nanoparticles targeted into deeper cutaneous layers. Moreover, no skin irritations were observed in the Draize test, while the reference ointment presented irritation signs (Pople and Singh, 2010). In a subsequent study, the same investigators evaluated the dermato pharmacokinetics, biodistribution, efficacy and safety of tacrolimus-loaded lipid nanoparticles. The results showed that lipid nanoparticles presented a bioavailability 3.02-fold higher than reference tacrolimus. Biodistribution evaluation showed the nanoparticles localization at the target-skin-area without spreading to other body organs. The *in vivo* efficacy and toxicity studies showed that tacrolimus-loaded lipid nanoparticles reduced inflammatory atopic-like skin lesions and presented no evident toxicity, representing an efficient alternative for AD management (Pople and Singh 2012).

The therapeutic effectiveness of tetrahydrocurcumin-loaded solid lipid nanoparticles-based gels was investigated by Saini et al. in atopic skin treatment. These particles were prepared bymicroemulsification method usingTween® 80, Phospholipon® 90G and Compritol® 888 ATO and presented mean size of 109 nm. *Ex vivo* and *in vivo* hydration tests showed that solid lipid nanoparticles enhanced skin hydration and achieved C_{max} greater and T_{max} earlier than non-nanoencapsulated tetrahydrocurcumin gel in both epidermis and dermis. Confocal laser scanning microscopy proved an improved drug penetration through the skin reaching cutaneous deeper layers. In addition, tetrahydrocurcumin-loaded solid lipid nanoparticle reduced the levels of inflammatory cytokines and presented a complete healing in 2,4-dinitrochlorobenzene-inducedAD mice model (Saini et al. 2022).

Nanostructured lipid carriers are the second generation of lipid nanoparticles and are composed by a mixture of solid and liquid lipids (Roberts et al. 2017).Usually, this lipid nanocarrier has higher encapsulation efficiency and greater stability than solid lipid nanoparticles due to the presence of liquid lipids in the particles core making it less ordered and with lower melting point, preventing recrystallization of solid lipids (Man et al. 2007).

A skin care formulation containing microsilver complexed with nanostructured lipid carriers was developed. The nanocarriers were composed by cetylpalmitate, Inutec® SP 1, hemp seed oil and TegoCare® 450 and presented a size of about 200 nm. The final formulation was applied to the skin of volunteers with AD for 3 weeks, twice daily. *In vivo* studies revealed the potential of microsilver-nanostructured lipid carriers to minimize the symptoms of AD (Keck and Schwabe, 2009). In a further study, these microsilver-nanostructured lipid carriers showed a pronounced antimicrobial activity against *Staphylococcus aureus* strain. Moreover, erythema and edema induced by 2,4-dinitro-1-fluorobenzene was attenuated in an AD mice model (Keck et al. 2014).

For topical treatments a high skin retention with low drug penetration is desired. For this, an ointment-based nanostructured lipid carrier for betamethasone dipropionate topical application was produced with Precirol® ATO5, oleic oil and Tween® 80 by melt emulsification method. It was observed that drug permeation and skin retention increase with enhancing Tween® 80 and oleic acid concentrations in the formulation. The optimized formulation presented tissue distribution as follow: skin > muscle > blood and no skin irritation (Kong et al. 2016).

X.3.1.3 Micro and Nanoemulsions

Micro and nanoemulsions are colloidal dispersions composed by an oily phase (oil blend or single oil plus lipophilic surfactants) and an aqueous phase (surfactants, co-surfactant and other components). Generally, these colloidal dispersions are non-irritating and have high drug skin permeation and drug-loading capacity (Dinshaw et al. 2021; Nastiti et al. 2017). Although micro and nanoemulsions present many similarities, they also have differences that allow their identification and classification. Microemulsions are formed spontaneously when the precise amounts of immiscible liquids are mixed with surfactants at the specific conditions of pressure and temperature. These nanosystems present thermodynamic stability with droplet size usually below 100 nm, and are visually transparent or translucent (Mariyate and Bera, 2022). On the other hand, nanoemulsions are kinetically stable with droplet in the size range 100-600 nm, presenting milky appearance (Bouchemal et al. 2004).

The oily components of nanoemulsions may offer benefits for the treatment of AD. Negatively and positively charged nanoemulsions were formulated for topical administration of *stratum corneum* lipids (ceramide 3B, ceramide 3, palmitic acid, cholesterol and vitamin E). Negative nanoemulsion was prepared with myristic acid and positive nanoemulsion with phytosphingosine. Both nanoemulsions presented size of about 200 nm. A clinical study carried

out on healthy female volunteers showed that positively charged nanoemulsion significantly increased skin hydration and elasticity than negatively charged nanoemulsion (Yilmaz and Borchert, 2006). Another study reported the evaluation of the skin hydration capacity of nanoemulsions composed of rice bran oil, castor oil and surfactants sorbitan oleate/PEG-30 on volunteers with normal and diseased skin types. Nanoemulsions were classified as non-irritating in the HET-CAM assay. The topical nanoemulsion application on skin of volunteers increased the hydration, oiliness and maintained the skin pH values (Bernardi et al. 2011). Moreover, nanoemulsions containing linseed oil were prepared by ultrasonic emulsification method using isopropyl myristate, DL-alpha tocopherol acetate, isodecyl neopentanoate, undecyl alcohol, Labrafil® M1944, Transcutol® HP and capric/caprylic triglyceride. Linseed oil nanoemulsion presented a mean droplet size of about 99 nm and negative zeta potential and no mutagenic effect was observed on strains of *Salmonella typhimurium*. *In silico* evaluations showed that this nanoemulsion may be an effective strategy to treat AD because ω-3 fatty acids in linseed oil did not show allergen properties and presented appropriate skin permeability values (Kildaci et al. 2021).

Considering drug association, clobetasol propionate-loaded nanoemulsions were prepared by aqueous phase titration method, using eucalyptus oil, ethyl alcohol and Tween® 20. The efficacy of this formulation was evaluated against nickel-induced dermatitis. The results showed an increased NTPDase activity in lymphocytes by clobetasol nanoemulsions, which is responsible for the adenosine triphosphate hydrolysis, reducing the inflammatory processes (Alam et al. 2013).

In a recent study, nanoemulsion based gels of cetirizine, a second-generation antihistamine, were developed to avoid the drug side effects and to enhance skin permeation. The formulations were prepared by water titration method and their optimized composition consisted of palm oil and Cremophor® RH40/ethanol. Cetirizine-loaded nanoemulsion presented higher permeability parameters through skin than free cetirizine. Moreover, in a clinical study it was observed a significant decrease in the degree of wheals and itching in urticarial patients treated with cetirizine-loaded nanoemulsion compared with free cetirizine after 4 weeks of treatment (Wang et al. 2019).

Moreover, the effectiveness of an optimized pioglitazone-loaded nanoemulsion composed by Capryol® 90, Labrasol® and Transcutol® P was evaluated on AD model using oxazolone-sensitized mice. A high retention of pioglitazone was found in the skin without achieve receptor compartment. Pioglitazone nanoemulsions reduced oxazolone-induced skin lesions, prevented transepidermal water loss and suppressed pro-inflammatory cytokines (TNF-

α, IL-6, and IL-17). Moreover, atomic force microscopy demonstrated a reversal of skin rigidification caused by treatment with nanoemulsions (Espinoza et al. 2020).

Similarly to what happen to nanoemulsions, microemulsions also stand out as an interesting strategy for topical drug delivery. Due to their reduced droplet size, microemulsions were developed to increase the penetration and to reduce the local side effects (burning sensation and irritation) of tacrolimus. Menthol/camphor eutectic oil solution was chosen as the oily phase because both compounds provide cool sensation, reducing pruritus and pain, as well as presenting skin penetration enhancing effects. Besides, Cremophor® EL and Transcutol® P were used as the surfactant and co-surfactant, respectively. Tacrolimus-loaded microemulsions presented an average droplet size of 26.59 nm enhanced 2-fold the percutaneous delivery of drug in comparison to the commercial ointment. In addition, microemulsions potentiated the therapeutic effect of tacrolimus, reducing the ear thickness, the scratch frequency and the IgE levels, as well as restoring the skin barrier (Wang et al. 2019).

X.3.2 Polymer-based nanostructures

X.3.2.1 Polymeric nanoparticles

Polymeric nanoparticles have gained considerable attention in the last few years for AD treatment. These polymeric nanocarriers generally are obtained with size smaller than 500 nm and include both nanospheres and nanocapsules. Nanocapsules are reservoir-type nanoparticles presenting an aqueous or oily core surrounded by a polymeric layer. In this nanosystem, the drug may be adsorbed onto the polymeric shell and/or dissolved in the core. In turn, nanospheres are matrix-type nanoparticles where drugs can be entrapped or adsorbed onto their surface (Mora-Huertas et al. 2010; Pohlmann et al. 2016; Quintanar-Guerrero et al. 1998). Several biodegradable polymers, with natural or synthetic origin, such as chitosan, poly(lactide-co-glycolide) (PLGA), poly-ε-caprolactone (PCL) can be applied in the formulation of nanoparticles (Abdel-mottaleb and Lamprecht, 2016). However, polymers of class of copolymers of poly(ethylacrylate, methyl-methacrylate and chlorotrimethyl-ammonioethyl methacrylate), such as Eudragit® RS100 has aroused interest for topical formulations due to their bioadhesive potential (Cervi et al. 2021; Prado et al. 2021).

The most accepted cutaneous permeation mechanism for polymeric nanoparticles is their deposition on *stratum corneum* surface and accumulation in the furrows and cavities of hair follicles after skin application. This accumulation generates a depot effect with high drug concentrations into skin tissue and subsequent release in a sustained manner into deep skin layers (Abdel-mottaleb and Lamprecht, 2016; Pohlmann et al. 2016). Polymeric nanoparticles

have a negligible penetration across the *stratum corneum* in intact form. However, in diseased skin, the nanoparticle absorption into epidermal and dermal layers can be significantly enhanced (Kahraman et al. 2017). Try and co-workers evaluated the size influence of polymeric nanoparticles on their penetration through atopic skin. PLGA nanoparticles linked to fluoresceinamine were produced with two sizes (70 nm and 300 nm) and their cutaneous distributions in both mice and pig skins with oxazolone-induced AD were compared to healthy skin. Regardless of size, nanoparticles remained on *stratum corneum* after topical application on both pig and mice healthy skins. On the other hand, in excised biopsies of atopic skins, the lower sized-nanoparticles presented greater penetration depth with fluorescence intensity 5-fold higher than nanoparticles with 300nm. The diffusion of smaller nanoparticles through the inflamed skin can be due to the alteration of the skin barrier, with accumulation of interstitial fluid in cutaneous tissue and spatial rearrangement of viable epidermis cells (Try et al. 2016).

The sustained skin release of drugs from polymeric nanoparticles favor a localized effect with reduced blood absorption and systemic side effects (Kahraman et al. 2017). To reduce the transcutaneous absorption of hydrocortisone, a low-potency corticosteroid, chitosan nanoparticles were produced via ionic gelation. Hydrocortisone-loaded nanoparticles showed a reduction in flux and permeability coefficient across the full thickness skin, as well as an increase in the drug accumulation in the epidermis and dermis. Besides, *in vivo* studies using a model similar to AD induced by 2,4 dinitrofluorobenzene revealed that the treatment with hydrocortisone-containing chitosan nanoparticles prevented transepidermal water loss and reduced the dermatitis index and erythema formation, showing anti-inflammatory and antifibrotic activities (Hussain et al. 2013). In another study, these same authors explored the immunomodulatory effects of 214 nm sized chitosan nanoparticles containing hydrocortisone. It was possible to observe a release suppression of cytokines involved in the dermatitis pathology such as IL-4, IL-5, IL-6, IL-13, IL-12, IFN-γ, and TNF-α in both serum and skin homogenates. Furthermore, histological findings indicated that hydrocortisone-loaded nanoparticles maintained the integrity of elastic connective tissues by inhibiting fibroblast infiltration and fragmentation of elastic fibers (Hussain et al. 2014).

To obtain additional benefits for AD treatment, co-encapsulation of hydrocortisone and hydroxytyrosol into chitosan nanoparticles has been the focus of several studies involving their development and both pre-clinical and clinical evaluations. Hydroxytyrosol is a potent antimicrobial and antioxidant and was added to the formulation to improve the efficacy against AD. Co-loaded nanoparticles were prepared by ionic cross-linking, and the optimized formulation showed about 235 nm and positive zeta potential. Notably, nanoparticles reduced

the permeability and increased the skin retention (epidermis and dermis) for both hydroxytyrosol and hydrocortisone. This greater cutaneous retention may have favored the better performance observed for the nanoformulation in an animal model of AD (controlled transepidermal water loss, decreased erythema, dermatitis index reduction) (Hussain et al. 2013). Further, *in vivo* studies showed that chitosan nanoparticles penetrated 2.46-fold deeper in the skin than the commercial formulation without spreading to the surrounding tissues. Besides, dermal pharmacokinetic showed a higher C_{max} for nanoparticles than the commercial formulation. This nanoformulation also inhibited the *in vitro* growth of *Staphylococcus aureus*and *Escherichia coli* strains, and presented no evidence of toxicity in repeated dermal application (Siddique et al. 2016). Also, co-loaded nanoparticles presented LD_{50} 100-fold higher than the normal human dose of hydrocortisone and did not cause skin irritation and adverse effects after repeated dose in albino Wistar rats (Siddique et al. 2015).

Following, hydrocortisone and hydroxytyrosol co-loaded chitosan nanoparticles were successfully upscaled by ionic gelation method using overhead spinning-disc mixer, without changing its physicochemical characteristics. Besides, the co-loaded chitosan nanoparticles 28-day application showed no signs of local irritation, redness and toxicity on healthy human skin, as well as the blood hematology, blood biochemistry, and adrenal cortico-thyroid hormone level did not indicate systemic toxicity in female Malaysian volunteers (Siddique et al. 2019). The clinical efficacy of co-loaded chitosan nanoparticles was demonstrated in a double-blind, randomized controlled study which had subjects of mild to moderate AD (EASI score of <18). The 6-weeks treatment with the nanoformulation provided a transepidermal water loss and erythema intensity decreased while melanin level was not significant reduced. In addition, no changes were found in the liver enzymes, fasting blood sugar and serum cortisol level in the nanoparticle-treated patients. In contrast, in the treatment with hydrocortisone commercial formulation the transepidermal water loss was not reduced as with co-loaded chitosan nanoparticles, and an increased blood cortisol level was observed in the group treated with the commercial formulation (Siddique et al. 2021). This set of studies demonstrated that hydrocortisone and hydroxytyrosol-loaded chitosan nanoparticles are superior to the hydrocortisone commercial formulation and did not reach the systemic circulation, preventing side effects.

In a different strategy to improve the solubility and skin permeation of tacrolimus, this drug was associated to nicotinamide into chitosan nanoparticles. The addition of nicotinamide, a water-soluble derivative of vitamin B3 and hydrotropic agent, improved the entrapment efficiency of tacrolimus and decreased the nanocarriers particle size. Besides, *in vitro* and *in*

vivo permeation studies showed a synergistic effect between chitosan nanoparticles and nicotinamide. The total tacrolimus amount retained in the skin was ordered as follows: nanoparticles containing nicotinamide and tacrolimus association > tacrolimus and nicotinamide solution > commercial formulation ≈ tacrolimus suspension. Likewise, *in vivo* evaluations demonstrated that tacrolimus and nicotinamide association into chitosan nanoparticles presented higher efficacy in ameliorating the AD symptoms than commercial formulation and tacrolimus+nicotinamide solution, reducing epidermal thickness, mast cell accumulation and IgE levels at approximately one-third the dose of commercial formulation in 2,4-dinitrochlorobenzene-induced AD-like clinical symptoms on BABL/c mice (Yu et al. 2018).

Another promising approach to treat AD includes coating nanoparticles with hyaluronic acid, which can provide sustained release and improved dermal targeting to drugs. Pandey and co-workers developed betamethasone valerate-loaded chitosan nanoparticles coated with hyaluronic acid. These nanoparticles presented average diameter around 300 nm, positive zeta potential and entrapment efficiency around 86%. Besides, coated nanoparticles provided a more controlled release profile to betamethasone than non-coated nanoparticles. Furthermore, the drug release from coated-nanoparticles was pH-triggered, showing higher rates of drug released at pH 5.5 than at pH 7.4. Because of its mucoadhesive properties the coating with hyaluronic acid provided lower betamethasone permeation flux and higher drug deposition in the epidermis and the dermis than non-coated nanoparticles (Pandey et al. 2019). In another study, hyaluronic acid-coated chitosan nanoparticles with size of about 223 nm were produced for tacrolimus delivery. In addition to controlling release and improving skin retention, AD induced by 2,4-dinitroflourobenzene on NC/Nga mice was mitigated by the topical treatment with these nanoparticles, being the anti-dermatitis efficacy more pronounced for coated nanoparticles (Zhuo et al. 2018).

Non-pharmacological therapies such as skin regenerators also have their effectiveness improved by nanoencapsulation. Chitosan-coated PLGA nanoparticles loaded with ceramide were developed and presented mean diameter of 211 nm and controlled release profile, which was pH and temperature dependent. Additionally, the nanoparticles were labeled with fluorescein isothiocyanate for skin penetration observation. As required for correct ceramide action, nanoparticles penetrated into *stratum corneum* accumulating in the epidermis without reaching the dermis. *In vivo* studies were performed by application of 10% sodium dodecyl sulfate on rats with 4-week old, simulating AD in infants, children and adolescents. The

treatment with coated-nanoparticles containing ceramides regenerated quickly the epidermis, and presented filaggrin filaments well-dispersed (Jung et al. 2015).

Also, the encapsulation of natural substances into matrix-type polymeric nanoparticles has been reported. Dictamnine, a chinese herbal medicine widely used on skin inflammations, was encapsulated into PLGA nanoparticles by antisolvent flash precipitation method. Dictamnine-loaded nanoparticles were spherical and presented approximately 186 nm, encapsulation efficiency of about 93% and controlled release. The nanoparticles notably improved the apparent epidermal regeneration, presented the CD3+ and CD45+ cell infiltration and the proinflammatory component COX-2 reduced in oxazolone-induced AD on mouse. In addition, the atopic mouse skin treated with free or nanoencapsulated dictamnine blended with fluorescent dye was evaluated in intravital two-photon microscopy and proved that nanoencapsulated dictamnine reached the dermal tissue layer while free drug showed a sparse fluorescence on the superficial epidermis and most fluorescence was found on residual hairs. Moreover, dictamnine-loaded nanoparticles significantly reduced TSLP, IL-1β and TNF-α expression in comparison to free dictamnine (Lin et al. 2021).

Nanocapsules are also advantageous for drugs targeting the skin immune system, providing a localized effect. Different cyclosporine A-loaded PLGA nanocapsules were prepared by varying the oil core Permeation studies showed that nanocapsules increased the cyclosporine A penetration through the skin, without reaching the receptor compartment. Besides, nanoencapsulated cyclosporine A presented higher accumulation in *stratum corneum* and epidermis than dermis. Because the higher drug encapsulation efficiency, the oleic:labrafil oil core was selected for efficacy studies. The reduction of cell proliferation and IL-2 secretion and suppression of pro-inflammatory cytokines were shown in *in vitro* and *ex vivo* studies, respectively. Moreover, cyclosporine A-loaded nanocapsules meliorated cutaneous inflammatory reactions induced by ovalbumin on BALB/c mice, reducing the allergen-specific immune responses of serum ovalbumin-specific IgE and the Th2 cytokines, as well as preserved skin integrity (Badihi et al. 2020).

Moreover, considering that AD is an inflammatory skin disease, non-steroidal anti-inflammatories have also been studied to treat this eczema. In this sense, Weber and colleagues associated meloxicam with PCL and Miglyol® 810 nanocapsules, aiming to improve the drug solubility and to investigate the effects of the nanoformulation in mitigating the signs of AD. Nanocapsule suspensions presented negative zeta potential and particle diameter of about 247 nm. In 2,4-dinitrochlorobenzene-induced dermatitis model, meloxicam-loaded nanocapsules reduced skin severity scores, scratching behavior, edema and myeloperoxidase activity in the

ears of mice. Contrary, unloaded nanocapsules and non-nanoencapsulated meloxicam presented no effect in these parameters (Weber et al. 2018).

Recently, films containing pomegranate oil nanocapsules were evaluated in AD-like skin lesions induced by 2,4-dinitrochlorobenzene. Nanocapsules were prepared by the interfacial precipitation of preformed polymer method using Eudragit® RS100 as polymer and presented size around 181 nm. In the *in vivo* study, films containing the nanocapsules showed an oil pure-likereduction in dermatitis severity, while pomegranate oil nanoemulsions film had no effect in this parameter. In the biochemical analysis of the excised skin, only the nanocapsules formulation showed a reduction in reactive species and myeloperoxidase activity and an increase in superoxide dismutase activity. The better *in vivo* performance observed for the film containing nanocapsules was suggested to be related to the bioadhesive characteristic of Eudragit® RS100, providing higher oil residence time in the skin tissue, which increased and prolonged its effect (Cervi et al. 2021).

Although topical therapy is the focus of studies involving nanocarriers for AD treatment, the systemic administration of nanocapsules has also been evaluated. Tacrolimus-loaded PCL nanocapsule suspensions were prepared by interfacial deposition of preformed polymer method using medium-chain triglycerides as oil core and presented drug encapsulation efficiency of 99.39%, mean diameter of 226 nm and controlled release profile. The efficacy of subcutaneously administered nanoformulations was demonstrated in AD model induced by topical application of 12-O-tetradecanoylphorbol-13-acetate on ear of mice. Tacrolimus-loaded nanocapsules demonstrated better inflammation control than the pure drug, with a marked reduction in serum fibrinogen, inflammatory infiltrate and in the epidermis thickness of the ear (Camargo et al. 2021).

X.3.2.2 Polymeric micelles

In recent years, polymeric micelles have emerged as promising drug delivery nanocarriers. The use of amphiphilic polymers above the critical micellar concentration enables micelles formation through molecular self-assembly resulting in a structure formed by a hydrophobic core and hydrophilic shell. Because of their self-assemble formation, these micelles present an easier preparation and a higher scale-up feasibility than lipid-based nanocarriers and polymeric nanoparticles (Ghezzi et al. 2021). Besides, the unique characteristics of polymeric micelles allows the formation of smaller sized particles (10-200 nm) providing a more efficient cellular internalization. In addition, the lipophilic core gives

adequate solubilization of hydrophobic drugs, while the hydrophilic shell provides a prolonged blood circulation time (Pepić, Lovrić, and Filipović-Grčić, 2013).

Transdermal micelles were designed to improve hydrocortisone absorption through the skin and to provide a safe treatment for AD with reduced side effects. Micelles were produced with monomethyl poly(ethylene glycol)-poly(ε-caprolactone) by thin-film hydration method. The optimized formulation with drug ratio of 10% presented mean diameter of 25.3 nm and significantly increased the hydrocortisone permeation through the skin at a low dose, presenting 9-fold increase in the drug cumulative quantity transferred compared with hydrocortisone cream. The skin penetration was confirmed by fluorescent images of tissue sections in which was visualized that the micelles helped to transport the encapsulated dye to the epidermis and dermis, while non-encapsulated dye reached in lower intensity the epidermis without reaching the dermis. Additionally, the nanoformulation presented higher reduction in inflammatory response (epidermal thickness, cell infiltration and level of IL-6) and lower systemic effects than hydrocortisone cream on a mouse model of AD induced by 1-fluoro-2,4-dinitrobenzene (Yuan et al. 2020).

The AD treatment with a localized effect and minimal systemic absorption can also be achieved with a correct management of nanocarrier size based on the choice of formulation constituents and their proportions. Assem and co-workers (2019) developed mixed micelles with Pluronic® L121/Pluronic® F127 to carry beclomethasone dipropionate. The micelles presented size around 120 nm and caused a higher local accumulation efficiency (amount retained versus amount permeated) than micelles prepared with Pluronic® L121/Poloxamer P84 mixture with 48 nm of size. The micelle with highest dermal deposition was incorporated into hydroxypropyl methylcellulose gels which have increased even more the drug local accumulation efficiency. In addition, Pluronic® L121/Pluronic® F127 micelles into gels were almost 40 times higher deposited into skin than the commercial formula (Beclozone®) causing a faster and efficacious healing of skin tissue as evaluated on sub-chronic dermatitis induced by 12-otetradecanoylphorbol 13-acetate in Wister rats (Assem et al. 2019).

X.3.2.3 Nanofibers

Polymeric nanofibers are an interesting class of nanomaterials composed by fibers with diameters in sub-micrometer range and produced using synthetic or natural polymers (Hu et al. 2014). Nanofibers present large surface area, high porosity and small pore size. These unique features provide a superior mechanical properties and favor high skin adhesion compared with any other material (Kamble et al. 2017).

In recent studies, nanofibers have been considered as drug carriers for AD treatment. Nanofibers containing tacrolimus dispersed into self-microemulsifying drug delivery system (SMEDDS) were optimized by electrospinning process using different ratios of Poly vinyl alcohol and SMEDDS. The optimized nanofiber formulation consisted of 90:10 ratio due to its tacrolimus sustained release. In 2,4-dinitrochlorobenzene-induced AD model, nanofibers showed a thinning of the cornified layer of epidermis similar to tacrolimus commercial oinment (Protopic®). However, nanofibrous membranes were applied every 48 h, while Protopic® was applied daily, proving the benefits of sustained drug release (Shams et al. 2021). The association of a non-traditional drug into nanofibers for dermal and transdermal delivery have also been reported. Pioglitazone-loaded polyvinylpyrrolidone nanofibers were produced by electrospinning method in comparison to a casted film. Polyvinylpyrrolidone-based nanofibers showed a high swelling degree which led to faster release of pioglitazone from nanofibers than from the casted film. Moreover, the higher surface area of nanofibers structure led to a greater skin permeation of pioglitazone than the casted film (Obaidat et al. 2022).

X.3.3 Inorganic nanocarriers

Inorganic-based nanocarriers have been investigated for skin disease treatment due to their large surface area, biocompatibility, and stability, as well as by their unique features as optical and superparamagnetic properties. For skin application, the maininorganic nanocarriers are: a) metallic nanoparticles (iron oxide, gold and silver and titanium dioxide and zinc oxide); b) mineral-based nanoparticles (natural clay minerals, layered double hydroxides, mesoporous silica); c) quantum dots (Sa et al. 2018). For the treatment of AD, some studies have demonstrated the benefits of inorganic nanoparticles, as metallic nanoparticles and mesoporous silica nanoparticles.

Mesoporous silica nanoparticles presented tunable size (50–200 nm) and shape. Pore diameter and volume can influence the drug loading and the release profile. Additionally, mesoporous silica nanoparticles can have their surface chemically functionalized through by different organic functional groups (Vallet-Regí et al. 2018). Aiming to improve solubility and skin retention of tacrolimus for AD control, a mesoporous silica nanoparticle-based gel was produced. Nanoparticles were synthesized and functionalized with amino or phosphonate groups using sol-gel technique. The optimized formulation consisted of phosphonate functionalized mesoporous silica nanoparticles which presented size in the 300-350 nm range and increased by 7 folds the tacrolimus solubility. Also, these nanoparticles significantly improved the drug amount retained into skin, which may have contributed for the good

performance on 1-fluoro-2,4-dinitrobenzene induced dermatitis in Balb/c mice. Tacrolimus-loaded nanoparticles reduced the mice ear thickness and the hyperkeratosis as well as presented low degree of inflammatory infiltration cells. Moreover, the formulation reduced acanthosis formation contrary to observed for free tacrolimus treatment in the irritation test (Parekh et al. 2021).

Iron oxide nanoparticles are iron-based magnetic materials, such as maghemite, magnetite and hematite. Maghemite nanoparticles are preferred for biomedical applications because their stable, biocompatible and non-toxic characteristics (Łazarczyk et al. 2019). For AD management, Lee and co-workers (2021) produced maghemite nanoparticles using various Fe2+: Fe3+ molar ratios (1:1, 1:2 and 2:3) by co-precipitation from ferrous/ferric salts which presented size around 16 nm. *In vitro* antioxidant assay showed that the 1:2 ratio presented higher2,2-difenil-1-picrilhidrazil scavenging activity than other ratios. In addition, topical application of maghemite nanoparticles presented anti-inflammatory and immunomodulatory effects dependents on the proportion of Fe3+ which were evaluated in *Dermatophagoides farinae* extract/2,4-dinitrochlorobenzene-sensitized BALB/c mice. The greatest proportions of Fe3+ showed better inhibition ofTh1, Th2 cytokines and inflammation-related proteins (COX-2, iNOS, and TNF-α) (Lee et al. 2021). Following, this same research group coated maghemite nanoparticles with hederagenin, a natural compound with anti-inflammatory effects. These nanoparticles presented higher *in vivo* efficacy than both non-coated maghemite nanoparticles and ceramide in AD-like lesions. Besides, only nanoparticles with hederagenin ameliorated the enlarged lymph nodes demonstrating the immunomodulatory effect of this novel formulation (Lee et al. 2022).

In another study, Ilves and colleagues compared the skin penetration and biological effects of zinc oxide nanoparticles and pure zinc oxide on mechanically damaged mouse skin with or without ovalbumin and staphylococcal enterotoxin B sensitization. The nanoparticles presented increased skin penetration in both damaged and allergic skin, as well as presented a greater reduction in the Th2-type cytokines expression than pure zinc oxide. However, zinc oxide nanoparticles presented an aggravation of IgE-antibody secretion, signaling an alert for the use of sunscreen containing these nanoparticles in patients with allergic skin (Ilves et al. 2014).

Indeed, some inorganic nanomaterials can exhibit significant immunomodulatory effects to penetrate or damaged skin (Yoshioka et al. 2017). Some studies report the aggravation of atopic effects after skin exposure with certain inorganic nanomaterials. For example, the reduction of amorphous silica nanoparticles from 1000 nm to 30 nm increased the ear thickness

and induced total-IgE, IL-18 and TSLP production after their intradermal co-administration with mite allergen extract using the same animal model (Hirai et al. 2012). Also, contrary to what was observed for 100 nm silver nanoparticles, 5 nm nanoparticles induced the ROS generation and granule release in mast cell *in vitro*, as well as increased the skin lesions and IgE serum levels after their co-administration with mite *in vivo* (Kang et al. 2017).

X.4. FINAL DOSAGE FORMS CONTAINING NANOSTRUCTURES

As previously commented, nanocarriers for topical and/or transdermal drug delivery have been extensively studied in AD management. However, most of these nanocarriers are obtained as aqueous dispersions which makes it difficult for the formulation to remain on the skin and complicates accurate dose administration Thus, for effective topical delivery in AD treatment, the incorporation of nanocarriers into appropriate dosage forms has been reported (Eroğlu et al. 2016; Kang et al. 2010; Kong et al. 2016; Pople and Singh, 2010). However, the development of formulations as vehicle for nanocarriers is complex. The appropriate topical dosage form must consider the flexibility of nanocarriers and solubility of both nanosystem and drug encapsulated (Pohlmann et al. 2016). Additionally, the integrity of nanocarriers after their incorporation into a final formulation needs to be evaluated, as well as the vehicle must attend to specific needs of the diseased skin (Boisgard et al. 2017; Stefanov and Andonova, 2021). In AD, the vehicle interaction with the compromised skin barrier may promote its restoration, presenting therapeutic value (Danby et al. 2022).

One of the most explored and promising strategies to increase the contact and residence time of the nanocarriers in the skin surface is to incorporate them in semisolid vehicles such as hydrogels(Liu et al. 2020; Pohlmann et al. 2016). In this case, the water generally used to form the hydrogel is partially or completely substituted by the nanocarriers aqueous dispersion (Pohlmann et al. 2016). Gel formulations are suitable for all AD lesions (Danby et al. 2022). Nano-based hydrogels have been produced for AD treatment using bioadhesive gelling agents of natural (e.g., chitosan), and or synthetic (e.g., carbomers) origin. Table X.2 summarizes the nanocarrier type and gelling agents used as final formulation for AD treatment.

The gelling agent type may influence the accumulation of the drug on skin. Aiming to develop an innovative nano-based topical formulation to act as vehicle for AD and psoriasis treatment, Boisgard and co-workers tested five semisolid formulations as vehicle for poly(lactic acid) (PLA) nanoparticles: 1) the viscosity modulator Avicel® RC-591; 2) the oil-in water emulsion base Pentravan®; 3) the gel-forming thickening agent Lutrol® F127; 4) the thickening-emulsifying Sepineo™ P600; and 5) the carrageenan gel polymer Viscarin® GP 209F. The

spreadability and rheological behavior showed that only Avicel®, Pentravan® and Viscarin®presented adequate spreadability (threshold > 10 cm^2). However, Pentravan® formulation destabilized the nanoparticle structure, as observed by electron microscopy. Avicel® and Viscarin® formulations were evaluated in *in vivo* studies carried out in mice for 8 consecutive days and no irritation was observed on mouse skin. In addition, the incorporation of PLA-nanoparticles into Avicel® or Viscarin® semisolids decreased dye skin penetration while did not modify dye retention through mouse inflamed skin, suggesting a local delivery without systemic absorption (Boisgard et al. 2017).

Table X.2 Hydrogels containing nanocarriers for AD treatment.

Gelling agent	Nanocarrier type	Reference
Chitosan	Liposomes	(Eroğlu et al. 2016)
Hydroxpropyl Methylcellulose	Polymeric micelles	(Assem et al. 2019)
Carboxymethylcellulose Sodium	Liposomes	(Kang et al. 2010)
Carbopol 934	Solid lipid nanoparticle, Niosomes, Ethosomes	(Akhtar et al. 2017; Pal et al. 202; Saini et al. 2022)
Carbopol 980	Solid lipid nanoparticle	(Pople and Singh, 2010)
Carbopol 940	Microemulsions, Polymeric nanoparticles	(Wang et al. 2019; Weber et al. 2018)
Carbopol 941	Polymeric micelles	(Yuan et al. 2020)

Besides hydrogels, emulsions, such as creams, have also been used as vehicle for nanocarriers in AD treatment. In this semisolid, nanocarriers may added to pre-formulated base cream (Kang et al. 2010; Keck et al. 2014; Kumar et al. 2021b, 2021a; Siddique et al. 2015; Yilmaz and Borchert, 2006). The cream composition may influence the drug release and *in vivo* performance. Katas and colleagues evaluated the skin permeation of hydrocortisone-loaded chitosan nanoparticles incorporated into two different creams, QV and aqueous cream. QV-cream is composed of higher contents of light liquid paraffin, white soft paraffin, and glycerol than the aqueous cream. Besides, this hydrophobic cream contains natural oils and other lipids which improve their occlusive and bioadhesive properties. The researchers observed that aqueous cream presented a sustained release while nanoencapsulated hydrocortisone was quickly released from QV-cream, probably due to the better hydrocortisone dissolution in the hydrophobic base and to glycerol presence (Katas et al. 2012). Although no differences were

observed in immunological parameters between different tested creams, the QV-based nanoformulation was more effective in the dermatitis index reduction (Hussain et al. 2014).

Besides, the ointment may be a convenient strategy for incorporation of lipid nanoparticles containing hydrophobic drugs. It was demonstrated that W/O ointment was more appropriate for the topical use of betamethasone dipropionate-loaded nanostructured lipid carriers than carbopol emulgel in AD control. The ointment increased the skin retention and decreased skin penetration amount of betamethasone dipropionate-loaded nanostructured lipid carriers. This probably happened due to the high affinity of both ointment and hydrophobic drug with the *stratum corneum* lipids, inducing retention. In contrast, the more hydrophilic feature of the carbopol reduces the drug affinity with the vehicle, increasing its skin penetration which may increase the adverse effects induced by systemic absorption (Kong et al. 2016).

Another dosage form that has been studied as vehicle for the treatment of skin diseases is polymeric films. They can be composed by natural or synthetic polymers, being a solid and flexible dosage form, which may promote a longer residence time at the action site. In contrast with semisolid formulations, polymeric films are less associated to easy removal, uncertainty of the dose applied and sticky sensation during administration (Karki et al. 2016). Besides, polymeric films have shown promising benefits for the incorporation of aqueous formulations such as nanoparticles, improving their stability and assisting in the drug sustained release (Dhal and Mishra, 2020; Gehrcke et al. 2021; Shahzad et al. 2019). For AD treatment, films have showed important features such as controlled release, high swelling behavior and occlusive properties (Alves et al. 2016; Jeong et al. 2019; Voss et al. 2020). When associated to nanoparticles or nanoemulsions for application on atopic skin, the *in vitro* occlusive properties of polymeric films increased and in the *in vivo* performance they proved to be a promising vehicle for nanocarriers (Cervi et al. 2021), encouraging further studies with this association.

X.5. PERFORMANCE EVALUATION OF FORMULATIONS IN ATOPIC DERMATITIS

X.5.1 Skin permeation/penetration evaluation

Both drug physicochemical properties and formulation type influence in skin retention and permeation of active substances. Besides, the skin conditions (health or damaged) can also alter the skin retention and permeation, especially when are attended by skin barrier disruption (see Fig. X.3) (Vogt et al. 2016). It is anticipated that the drug permeation in atopic skin is greater than healthy skin which can result in systemic toxicity, being an aggravating factor mainly for transdermal formulations. Besides, for local AD treatment, it must be ensured that

the active substance will reach the deeper layers of the epidermis and the dermis to exert its therapeutic effect by controlling the immunoinflammatory effects of the disease (Fang et al. 2016). Therefore, it is essential to evaluate this parameter in different skin conditions in the early stage of development of nano-based formulations since nanocarriers modulate the dermal retention/penetration/permeation of drugs across the skin.

The effect of cutaneous barrier disrupted simulating atopic skin condition on drug-loaded nanocarriers penetration may evaluated using skin biopsy after *in vivo* induction of the damage and allergic response in animal model (Ilves et al. 2014; Try et al. 2016). However, *in vitro* mechanical damage of *stratum corneum* is a well-established method to obtain an impaired skin barrier simulating atopic skin. The mechanical damage can be performed by tape-stripping technique in which the skin impairment is achieved by the *stratum corneum* removing using tape pieces (Fullerton, 2007). The mechanical skin barrier disruption by abrasion can also be used. In this case, a sponge with an aluminum coating is used to remove part of *stratum corneum* by smooth motion over the skin surface (Schlupp et al. 2014). Both mechanical skin barrier disruption showed to be a suitable alternative method to predict the drug permeation/penetration across the atopic skin and may serve as screening for topical nano-based formulations.

X.5.2 Animal models of atopic dermatitis

In addition to achieve in therapeutic concentrations the target layer of skin, the anti-dermatitis efficacy of drug-loaded nanocarriers must be evaluated. A suitable model of human AD should present both relevant cytokine networks and epidermal impairment. However, to date no single animal model simulate these two aspects (Ewald et al. 2017). Although the skin structure and pathologic mechanism of AD are different between murine and human species, animal models for preclinical evaluation of new formulations for AD are widely used. The murine models are considered easy to manipulate, low cost, and have the availability of genetically manipulated strains (Gilhar et al. 2021).

Actually, there are three murine models for AD evaluation: 1) spontaneous mutation; 2) transgenic mice; 3) epicutaneous sensitization (Jin et al. 2009). In the first model, an inbred mouse strain (Nc/Nga mice) present skin barrier abnormalities, development atopic-dermatitis-like lesion spontaneously after allergens exposure, such as oxazolone, 2,4-dinitrochlorobenzene and mites (*Dermatophagoides farina*) (Matsumoto et al. 2004; Yamada et al. 2016). In this model is possible to observe scratching behavior, skin dryness and erythematous and erosive lesions with hemorrhage occurrence. Besides, skin histological examination shows infiltration of mast cells and eosinophils and hyperkeratose and hyperplasia. Increased IgE production and

induction of Th2 responses are also observed in this model (Matsuda et al. 1997). On the order hand, the second model uses transgenic mice overexpressing a single gene, such as IL-4, IL-31, TSLP, caspase-1 or apolipoprotein C1. According to transgenic mouse chosen, a factor associated with AD will be observed, contradicting the complexity of human AD. Besides, this model is time-consuming, and therefore it is not used much (Jin et al. 2009).

The third model involves hapten-induced micewhich are commonly used to produce AD-like lesions. In this model multiple challenges with haptens on the skin of hairless mice were carried out, usually BALB/c or C57BL/6 mice strains, over a prolonged period. The haptens more used to induce AD-like lesions are ovalbumin, dinitrofluorobenzene, oxazolone, dinitrochlorobenzene, mite allergen and exotoxins of *Staphylococcus aureus* (Gilhar et al. 2021). The challenge with these haptens induces Th2 immune response with mast cell and eosinophil infiltrations. Besides, ovalbumin-sensitized mice may present specific IgE and IgG1 antibody responses (Man et al. 2007).

X.6. CONCLUSION

The knowledge of pathophysiology of AD reveals the complex and heterogeneous nature of this eczema with numerous aspects to consider for its treatment. Traditional treatments for atopic eczema include topical and systemic therapeutic agents, which are used as single or combined therapy depending on the injury severity. However, both topical and systemic treatments present limited effectiveness and relevant side effects. Additionally, to date does not exist a unique formulation that combines immunoinflammatory effects with skin barrier recovery effects. All these aspects make the treatment of patients difficult and result in low adherence to the proposed therapy. To overcome limitations and challenges of traditional treatment, drug delivery systems based on nanotechnology have shown a promising strategy for AD management.

This chapter reported numerous research have shown the benefits of nanocarriers for atopic eczema treatment. The studies conducted demonstrated the superiority of nano-based formulations to penetrate the skin and to improve the immune response while having little side effects as compared to classical topical treatment of AD. The action and selectivity into inflamed skin may influenced by both type and physicochemical features (size, charge and flexibility) of nanocarriers. Besides, depending on the nanocarrier composition it is possible to obtain a synergistic effect of high hydration and recovery of the skin barrier with immunoinflammatory effects. In addition to nanocarriers composition, the vehicle used to facilitate their application on the skin may also influence the final biological effect, and the

correct development and evaluation of this final formulation is important. However, although studies demonstrate a positive potential of nanocarriers for the treatment of AD, more in-depth research is still needed. There is a lack of investigation regarding the large-scale production and stability of these nanocarriers. Also, there is still a need for a better understanding of the penetration of nanocarriers through atopic skin, as well as their effects on the skin after repeated applications. Moreover, existing animal models for the preclinical evaluation of new formulations in AD do not involve the complexity and phenotypic variability as occurs in humans. Thus, further clinical studies in different patients are required to investigate the effects of nanocarriers in preventing atopic eczema progression. Despite these shortcomings, nano-based formulations demonstrated excellent results and represent a promising future option in AD treatment.

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3.2 CAPÍTULO 2: DESENVOLVIMENTO DE NANOCÁPSULAS DE SILIBININA E DE FILMES DE GOMA GELANA PARA VEICULAÇÃO TÓPICA DESTES SISTEMAS NANOESTRUTURADOS

3.2.1 Estudos de pré-formulação

A SB tem sido estudada na prevenção e tratamento de diferentes patologias que acometem a pele. No entanto, sua limitada solubilidade prejudica o desenvolvimento de formulações dermatológicas. Para contornar essa questão, foram selecionadas as nanocápsulas como uma estratégia tecnológica que proporcione a incorporação deste flavonoide lipofílico em filmes poliméricos hidrofílicos. Assim, primeiramente foram desenvolvidas suspensões de nanocápsulas de triglicerídeos de cadeia média contendo SB, através do método de deposição interfacial do polímero pré-formado. Estas foram preparadas inicialmente utilizando três diferentes polímeros, o Eudragit® RS 100, a poli (ε-caprolactona) e a etilcelulose, a fim de verificar a interação e performance destas diferentes composições poliméricas em filmes de goma gelana. Os resultados da caracterização físico-química das suspensões de nanocápsulas preparadas com Eudragit® RS 100 e poli (ε-caprolactona) contendo SB são demonstrados na tabela 2, enquanto os resultados obtidos para as nanocápsulas de etilcelulose estão descritos no item 3.2.2 deste capítulo (artigo 1).

Tabela 2 - Caracterização físico-química das suspensões de nanocápsulas produzidas com Eudragit® RS 100 e poli (ε-caprolactona).

	NC EUD SB	NC PCL SB
Teor de SB (%)	98,68 ± 1,09	103,01 ± 0,45
pH	4,15 ± 0,12	5,68 ± 0,10
Diâmetro médio (nm)	155 ± 8	221 ± 7
Índice de Polidispersão	0,11 ± 0,01	0,21 ± 0,03
Potencial Zeta (mV)	+8,45 ± 1,51	-9,10 ± 5,05

Os resultados acima demonstram a adequada obtenção de nanocápsulas de Eudragit® RS 100 e poli (ε-caprolactona) contendo SB. Assim como as produzidas com etilcelulose (artigo 1), estas formulações apresentaram-se como um líquido branco com aspecto homogêneo, teor de SB próximo ao teórico, pH adequado para aplicação cutânea, tamanho nanométrico e baixo índice de polidispersão, corroborando outros trabalhos que produziram nanocápsulas a partir

destes polímeros (CHASSOT et al., 2015; GEHRCKE et al., 2017, 2018; MARCHIORI et al., 2017a).

Em paralelo ao desenvolvimento das suspensões de nanocápsulas, foram também realizados testes para o desenvolvimento de filmes de goma gelana, através do método de evaporação do solvente. A escolha da goma gelana como agente filmógeno foi feita com base em uma tendência mundial de se buscar opções terapêuticas naturais, bem como devido as suas promissoras características físico-químicas para aplicação cutânea. A presente goma forma filmes em concentrações de 1 a 3 %, a partir da sua dispersão inicial em água a uma temperatura mínima de 60 °C, para evitar a sua gelificação (PAOLICELLI et al., 2018). Assim, neste estudo optou-se pela temperatura de 80 °C, por ser bem relatada na literatura para o preparo de filmes de goma gelana e por proporcionar uma dispersão mais rápida desta goma (ARIFAH et al., 2019; CHANG et al., 2010; LEE; CHEN; TSAO, 2010). Ainda, a concentração de 1 % de goma foi escolhida, uma vez que concentrações superiores formam soluções muito viscosas, dificultando a sua deposição sobre a placa de *petri*.

Após a definição da temperatura e concentração de preparo, foram realizados testes para definir o melhor plastificante para estes filmes, uma vez que sem plastificante a goma gelana forma filmes extremamente rígidos que não são removíveis da placa (YANG; PAULSON, 2000). Os plastificantes testados foram o glicerol e sorbitol, nas concentrações de 0,5, 1, 2 e 4 %. Os filmes contendo as concentrações de 0,5 e 1 %, de ambos os plastificantes, não foram facilmente removidos da placa de *petri*, inviabilizando o seu preparo. Já as concentrações de 2 e 4 % de sorbitol formaram filmes que foram facilmente removidos da placa, porém ainda eram rígidos e rachavam durante o manuseio. As concentrações de 2 e 4 % de glicerol, formaram filmes facilmente removíveis da placa, sendo a concentração de 4 % a aparentemente mais promissora para administração cutânea, por ser mais maleável, podendo se adaptar melhor ao local de aplicação. Estes achados corroboram estudos que verificaram que o glicerol é o plastificante mais adequado para a produção de filmes de goma gelana (YANG; PAULSON, 2000; YANG; PAULSON; NICKERSON, 2010).

Como próxima etapa do trabalho, foi verificada a viabilidade de incorporar as nanocápsulas desenvolvidas, como os três diferentes polímeros, no filme de goma gelana otimizado. Para isto, a fim de evitar uma longa exposição dos nanossistemas à alta temperatura de preparo, primeiramente a goma gelana foi dispersa em água a 80 °C por 2 h. Após esta dispersão se tornar clara, foi adicionado o plastificante. Em seguida, esta dispersão foi retirada do aquecimento e misturada a 10 mL das suspensões de nanocápsulas, a fim de obter uma concentração final de goma gelana de 1 %, e então vertida em placa de *petri* para ser levada à

estufa para secagem. Os filmes foram formados após cerca de 24 h de secagem a 40 °C. No entanto, foi possível completar as etapas de formação do filme apenas para as nanocápsulas de etilcelulose. Ao misturar a dispersão de goma gelana a suspensões de Eudragit® RS 100 ocorria uma separação de fases, não sendo possível verter esta mistura nas placas. Já os filmes contendo nanocápsulas de poli (ε-caprolactona) apresentaram aglomerados, sendo não homogêneos após a secagem e, portanto, inadequados para posterior investigação. Frente a isso, este estudo foi conduzido apenas com as nanocápsulas de etilcelulose, as quais foram incorporadas com sucesso em filmes de goma gelana, com recuperação total de seu tamanho após extração dos filmes.

3.2.2 Artigo 1 - Incorporação de nanocápsulas em filmes de goma gelana: uma estratégia para aumentar a estabilidade e prolongar a liberação cutânea da silibinina

Incorporation of nanocapsules into gellan gum films: a strategy to improve the stability and prolong the cutaneous release of silibinin

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Incorporation of nanocapsules into gellan gum films: A strategy to improve the stability and prolong the cutaneous release of silibinin

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ABSTRACT

This study aimed to develop gellan gum films containing silibinin-loaded nanocapsules as a novel approach for cutaneous administration of this flavonoid. The nanocapsule suspensions were prepared and presented mean size around 140 nm with homogenous distribution, negative zeta potential and silibinin encapsulation efficiency close to 100%. Then, these suspensions were converted into gellan gum films by solvent casting method. The films were transparent, flexible and maintained the gellan gum hydrophilicity. Nanocapsules provided the silibinin homogenous distribution in the films and prolonged its release, as well as improved the gellan gum occlusion potential. Besides, the nanosuspensions conversion into films improved the silibinin stability. Additionally, the nano-based films presented a swelling index 1.5 times higher than films containing non-nanoencapsulated silibinin. Microscopic analysis evidenced the homogeneous surface of the nano-based films, while films containing non-nanoencapsulated silibinin presented small cracks. The *in vitro* skin permeation profile confirmed the silibinin gradual release from the nano-based films and its greater retention in the dermis when the skin is damaged. Finally, the formulations presented no irritant effect in the HET-CAM assay. Therefore, the conversion of silibinin-loaded nanocapsule suspensions into films might be considered a promising platform for skin delivery of this flavonoid.

1. Introduction

In the last years, nanocapsules (NC) have been extensively studied as a drug delivery system due to their ability to protect drugs against degradation and to improve their therapeutic efficacy. Besides, these systems may control skin release and permeation of different drugs, targeting the active substance to specific skin layers and preventing their systemic absorption [1–3]. NC are obtained as aqueous suspensions in which the particles have core-shell architecture. The core is usually oily and the shell is composed by a polymer which acts as a membrane controlling the drug release [4,5]. This structure allows the retention and solubilization of lipophilic substances inside the particles resulting in a better incorporation of them into hydrophilic bases for cutaneous administration [6,7].

Silibinin (SB) is an example of active substance whose incorporation in hydrophilic dermatological bases is limited. This compound is the main and most biologically active flavonoid found in silymarin, an extract from *Silybum marianum* seeds. SB is known for its potent hepatoprotective action [8] and most recently, it has been considered a potential agent to treat different cutaneous diseases such as dermatitis [9] and wounds [10] due to its antioxidant and anti-inflammatory actions. In order to circumvent the SB limitations previous studies of our research group have showed its association into pomegranate oil NC [11] with subsequent conversion into hydrogels, which effectively reduced skin damage induced by UVB radiation [12] or croton oil [9] in mice.

Although it is a promising approach, the use of semisolid formulations, such as hydrogels, is associated with some limitations, such as low skin contact time and easy removal as well as the uncertainty of the dose applied [13,14]. Moreover, the main NC disadvantage is their limited stability when stored in an aqueous medium [15]. Thus, the high water content of hydrogels may favor this instability, as well as may allow the drug hydrolysis or crystallization. In this sense, polymeric films have been studied as an alternative to overcome the hydrogels limitations [14]. Films may enhance the stability of nanoencapsulated substances since reduce drug release from nanocarriers

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due to its solid nature [16], preventing drug degradation. It is important to mention that, in contrast to hydrogels, there are still few studies associating polymeric nanoparticles to films for cutaneous applications [17–19], which reinforces the relevance of further studies on this association.

Natural polymers, such as polysaccharides, with film-forming properties have received special attention in recent years [20,21]. Among these natural polymers is the gellan gum (GG), an anionic, linear and biodegradable polysaccharide produced by *Pseudomonas elodea* [22]. Nowadays, GG films have been studied in tissue engineering [23], anti-adhesion barrier [24], oral cancer treatment [25] and for wound healing purposes [26,27]. Considering these promising advantages, the aim of this study was to use GG to convert SB-loaded NC aqueous suspensions into films in order to propose a new and stable approach to skin delivery for this lipophilic flavonoid. There are some reports about the application of films containing silymarin-loaded nanoparticles for antioxidant and antibacterial food packaging [28,29]. However, to the best of our knowledge, no studies reporting the association of isolated SB (free or nanoencapsulated) to GG films were found.

2. Materials and methods

2.1. Materials

Silibinin (98% purity), Span 80® (sorbitan monooleate) and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (São Paulo, Brazil). Ethylcelullose was donated by Colorcon (Cotia, Brazil). Medium chain triglycerides and Tween 80® (polysorbate 80) were bought from Delaware (Porto Alegre, Brazil). Gellan gum low acyl (Kelcogel®) was donated by CP Kelco (Georgia, USA). All other solvents and reagents were analytical grade and used as received.

2.2. Methods

2.2.1. Analytical method

The quantitative analyses were performed according Marchiori et al. [11]. For this, a LC-10A HPLC system (Shimadzu, Japan) equipped with a SIL-20A HT valve sample automatic injector, an UV-VIS SPD-M20A detector, a LC-20AT pump, CBM-20A system controller, a guard column and a Kinetex C_{18} Phenomenex column (250 mm × 4.60 mm, 5 μm; 110 Å) were used. The mobile phase consisted of water pH 3.5 and acetonitrile (60:40, v/v) at isocratic flow rate (1.0 mL/min). The detection wavelength used for SB was 288 nm and the column was kept at room temperature. For studies of SB skin permeation and *in vitro* release the injection volume was 50 μL and the method proved to be linear with regression coefficient (r^2) of 0.998 (range 0.25 to 15 μg/mL), precise, accurate, and specific. For the other quantitative analyses, the injection volume was 20 μL and the method was also linear in the range of 2.5 to 25 μg/mL (r^2 = 0.999), precise, accurate, and specific.

2.2.2. Preparation and characterization of nanocapsule suspensions

SB loaded-NC were prepared using interfacial deposition of preformed polymer method [11]. For this, an organic phase containing ethylcelullose (0.25 g), Span 80® (0.1925 g), medium chain triglycerides (0.75 g), SB (25 mg) and acetone (68 mL) was kept under moderate magnetic stirring at 40 °C. After 60 min, this organic phase was injected into 132 mL of a Tween 80® aqueous dispersion (0.1925 g), under magnetic stirring at room temperature. After 10 min, the acetone and part of the water were eliminated by evaporation under reduced pressure to achieve a 10 mL final volume, corresponding to 2.5 mg/mL of SB. This formulation was named NC-SB. For comparison purposes, formulations without SB were also prepared (NC-B).

The granulometric distribution profiles of NC were determined by laser diffraction (Mastersizer® 3000E, Malvern Instruments, UK). For this, the formulations were directly inserted into the equipment sampling apparatus, without previous treatment, until laser obscuration reached 15%. Distilled water was used as dispersing phase. The refractive index of the dispersed phase was 1.47. The NC-SB and NC-B mean particle sizes and polydispersity index (PDI) were determined by photon correlation spectroscopy (PCS) using a Zetasizer® Nano-ZS ZEN 3600 model (Malvern Instruments, UK). In the same equipment, zeta potential was measured by microeletrophoresis. pH values were determined by immersing the electrode of a potentiometer (Model pH 21, Hanna Instruments, Brazil) in the aqueous suspensions.

The total SB content was measured after dissolving an aliquot of formulation in 10 mL of methanol, followed by sonication for 5 min to extract the compound. After this step, the sample was filtered through a 0.45 μm membrane and injected into the HPLC system. The SB encapsulation efficiency was evaluated by ultrafiltration/centrifugation technique. For this, an aliquot of NC-SB was placed in a 10,000 MW centrifugal filter device (Amicon® Ultra, Millipore) and centrifuged at 7000 rpm for 10 min. The ultrafiltrate was analyzed by HPLC method. The encapsulation efficiency (EE %) was calculated by the difference between total and non-nanoencapsulated SB concentrations, determined in the nanostructures and in the ultrafiltrate, respectively.

2.2.3. Preparation of polymeric films

The films were developed in accordance with the solvent casting technique [14]. The polymer dispersion was prepared by adding 0.25 g of GG into 15 mL distilled water at 80 °C for 2 h under constant mechanical stirring. After the complete GG dispersion, 1 g of glycerol was added as plasticizer. Then, 10 mL of NC suspension or non-nanoencapsulated SB solution were added in the filmogenic dispersion under constant stirring for 5 min. This mixture was poured in Petri dishes (90 × 13 mm) and dried at 40 °C for 24 h. The non-nanoencapsulated SB (25 mg) solution was prepared through compound solubilization in 2.5 mL of DMSO followed by the addition of 7.5 mL of water. Films containing nanoencapsulated and non-nanoencapsulated SB were named NC SB GG and SB GG, respectively. For comparison purposes, films containing unloaded NC (NC B GG) or DMSO (DMSO GG) solution were also prepared. Besides, GG films without non-nanoencapsulated SB or NC were used as comparison and were named vehicle.

2.2.4. Films transparency

The films transparency was observed using a spectrophotometer (UV-1800 UV Spectrophotometer, Shimadzu, Japan). For this, the ultraviolet-visible spectra of all the produced films were recorded at wavelength 200–800 nm.

2.2.5. Homogeneity of thickness, weight and SB content of gellan gum films

In order to evaluate the thickness homogeneity, the films (n = 3) were prepared for each formulation and then five measurements were performed on each film (four at the corner and one in the middle) using a digital micrometer. The mean values of thickness were calculated and expressed as μm. For the determinations of weight and SB content homogeneity, the films (n = 3) were cut into three fragments of 1.0 cm^2 each. These fragments were subjected to individually weighing and then the SB content was determined. The SB content in each fragment was quantified by compound extraction in methanol and subjecting it to stirring for 20 min followed sonication for 20 min. The samples were filtered (0.45 μm) and analyzed by HPLC method. The mean values of weight and SB content were calculated and expressed as mg/cm^2 and $\mu g/cm^2$, respectively.

2.2.6. Folding endurance

Folding endurance was determined by repeatedly folding the film at the same place until it 300 times (n = 3). After this, the films were evaluated as to groove formation or breakage.

2.2.7. Swelling index

A film of each formulation was cut into fragments of 2 cm × 2 cm

and weighed (W_d). Then, the fragments were added in a flask containing 50 mL of phosphate buffer pH 7.4 at 37 °C. After 24 h, the films were removed from flask and the water excess was removed followed by weighing of the hydrated film (W_s). The swelling index was calculated following Eq. (1).

$$\text{Swelling index} = [(W_s - W_d)/W_d] \times 100 \quad (1)$$

where: W_s is the weight of the film after swelling and W_d is the weight of the dried film.

2.2.8. Residual water content

The films were cut into fragments of 2 cm × 2 cm and then placed in the oven at 60 °C. These fragments were weighed after regular time intervals until the weight after drying becomes constant. The water content of the films was determined following Eq. (2).

$$\text{Moisture content} = [(W_d - W_i)/W_i] \times 100 \quad (2)$$

where: W_d is the weight of the films after drying and W_i is the initial weight of the films.

2.2.9. Mechanical properties

Mechanical properties in terms of stress and strain were determined using a universal testing machine (Emic, São José dos Pinhais, Brazil), according to ASTM-D882-02 [30]. Film strips with dimensions of 60 mm × 45 mm were individually attached to the machine probe and a tensile load was applied at a crosshead speed of 50 mm/min until the film broke. The stress and strain were measured when the film broke. The Young's modulus was calculated from the slope of the linear part of the stress-strain curve, while the strain was calculated as shown in Eq. (3).

$$\text{Strain (\%)} = [(L_b - L_i)/L_i] \times 100 \quad (3)$$

where: L_b is the length of the film at the time of breakage and L_o is the initial length of the film.

2.2.10. Water contact angle

In order to determine the films hydrophobicity, contact angle of water droplets on the film surface was examined using a goniometer (Drop Shape Analysis, DAS 30S model, Kruss; Hamburg, Germany). The samples were cut into 2 cm × 2 cm fragments and then 11 μL distilled water were added to the surface of these specimens using a microsyringe. Images were taken using a digital camera and the calculation of the contact angle was done as the angle between the tangent line on the droplet in the point of contact and the line drawn along the surface of the film using a software (model DSA4).

2.2.11. Nanocapsules mean particle size from films

Mean particle size and polydispersity index of NC in the films were evaluated by PCS method. For this, the film fragments (0.1 g) were dispersed in 50 mL of ultrapure water. The samples were subjected to stirring for 2 h before the analysis, for NC extraction from the films.

2.2.12. Morphology

Surface morphology of NC and films was analyzed by scanning electron microscopy (SEM). NC suspensions were previously lyophilized using lactose (cryoprotectant), and the films were cut into fragments and viewed as such. The samples were gold sputtered and subsequently analyzed using an accelerating voltage of 10 and 15 kV (scanning microscope JSM 6360).

2.2.13. Evaluation of gellan gum films stability

The stability of films containing nanoencapsulated or non-nanoencapsulated SB was evaluated considering the influences of time and the storage condition regarding the weight and SB content variation. The film fragments (1 cm^2) were submitted to two different storage conditions: refrigeration (2–8 °C) and room temperature (25 °C). At predetermined time periods, samples were weighed and the SB content in each fragment was quantified by HPLC method. For comparison purposes, NC suspensions were submitted to the same conditions to estimate their stability.

2.2.14. In vitro SB release

The *in vitro* SB release from films was conducted through vertical Franz diffusion cells, at 32 ± 0.5 °C (n = 3). The area for diffusion was 3.14 cm^2 and dialysis membrane (10,000 Da, Sigma Aldrich) was fitted between donor and receptor compartments. Two receptor media were assayed separately: the first medium was composed by phosphate buffer pH 7.4:ethanol (70:30) to reach the sink condition, and the second one corresponded to phosphate buffer pH 7.4, in order to use a medium closer to the physiological condition. The films (1 cm^2) corresponding to 430 μg SB were fitted on the membrane surface. At predetermined periods (1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 h), 300 μL of the receptor medium were withdrawn and replaced by the same volume of fresh medium. The amount of SB released was determined using the HPLC conditions previously mentioned (Section 2.2.1).

To verify the release mechanism, the experimental data were analyzed according to the Higuchi equation, $Q_t = K_H t^{0.5}$, where Q_t is the amount of drug released in time t, and K_H is the release rate constant for the Higuchi model.

2.2.15. Occlusion test

The *in vitro* occlusion test was carried out according to Pereira et al. [19]. Briefly, a 100 mL flask was filled with 50 mL water and sealed with a cellulose acetate filter. Then, the films (n = 3) were applied on the filter and stored at 32 °C for 48 h. At predetermined time points (0, 6, 24 and 48 h) the flask was weighed, and the water loss determined. A flask without film was used as negative control. The occlusion factor was calculated according to Eq. (4).

$$\text{Occlusion factor} = [(A - B)/A] \times 100 \quad (4)$$

where: A is the water loss of the negative control and B is the water loss in film presence.

2.2.16. Skin permeation/penetration study

For this study, skin samples were obtained from a healthy female patient who had undergone abdominal plastic surgery. The subcutaneous fatty tissue was removed and the skin was stored at −80 °C until use. The protocol was approved by the committee for research with humans of Universidade Federal de Santa Maria - RS with no identifying data (CAEE: 27168719.4.0000.5346).

This experiment was carried out for two different skin conditions (injured and non-injured). To simulate the injured condition, the *stratum corneum* of each skin fragment was removed using 18 pieces of adhesive tape before films application, according to Cardoso et al. [1].

Permeation/penetration study was carried out using Franz cells (n = 6) with a 3.14 cm^2 area of contact with the membrane and a 6 mL of capacity for the receptor medium. The receptor medium was phosphate buffer pH 7.40 and was maintained under magnetic stirring at 32 ± 0.5 °C. Circular skin fragments (injured and non-injured) were fitted between donor and receptor compartments with the dermis in contact with the receptor medium. The films (1 cm^2), corresponding to approximately 430 μg SB, were fitted on the skin surface.

After 24 h, the films were gently removed from the skin surface, the skin was carefully removed and the receptor media was collected. For non-injured skin, the *stratum corneum* was removed by the tape stripping technique using 18 pieces of adhesive tape (3 M Durex®). These tapes were placed in test tubes containing methanol and were subjected to vortexing (2 min) and sonication (30 min). The samples were filtered (0.45 μm) and analyzed by HPLC. For both conditions (injured and non-injured) the epidermis was separated from the dermis by heating the skin in ultrapure water at 60 °C for 45 s. Then, the epidermis was removed using a spatula and the dermis was cut into small pieces.

Epidermis and dermis were placed in different test tubes containing methanol and subjected to vortexing (2 min) followed by sonication (30 min). No interference of any substance of the human skin or adhesive tapes was observed in the chromatographic run, as evaluated by a specificity test.

2.2.17. Evaluation of formulations irritant potential

The biocompatibility of formulations (NC suspensions, films, non-nanoencapsulated SB and DMSO solution) was studied using Hen's Egg Test -Chorioallantoic Membrane (HET-CAM [31]. For this, fertilized chicken eggs were obtained from Languiru (Teutonia, Brazil). The non-nanoencapsulated SB and NC (300 μL) were applied for 20 s. Then, the membrane was carefully washed with NaCl 0.9% solution and analyzed for hemorrhage, lysis and coagulation of blood vessels for 5 min. The films (1 cm^2) were placed on the membrane and observed for 5 min. The positive and negative controls were NaOH and NaCl 0.9%, respectively.

2.2.18. Statistical analyses

Formulations were prepared and analyzed in triplicate and the results were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). GraphPad Prism version 6 was used for the analyses of variance (ANOVA) one and two way followed by post-hoc Tukey's. Statistical differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Preparation and characterization of nanocapsule suspensions

After preparation, the NC suspensions presented homogeneous and milky appearance characteristic of colloidal systems without precipitate formation. The results of characterization of NC suspensions are shown in the Table 1. The granulometric profile of the NC suspensions by laser diffractometry demonstrated that 90% of the particles were lower than 450 nm for NC-SB and NC-B. Besides, volume-weighted mean diameters ($D_{4,3}$) were below 340 nm with SPAN values less than 2.0 for both formulations.

PCS analysis showed that both formulations (NC SB and NC B) presented mean diameter and PDI below 140 nm and 0.2, respectively. However, in both laser diffractometry and PCS methods the NC SB presented a lower size than NC B ($p < 0.05$). Supplementary data (Supplementary Fig. 1) show the images obtained in the analysis by SEM in which is possible to visualize the presence of spherical particles at the lactose (cryoprotectant) surface. Concerning the zeta potential, it was negative for the formulations. Moreover, the SB content into NC was close to the theoretical value (1.0 mg/mL) and encapsulation efficiency was 98%.

Table 1
Results of nanocapsule suspensions characterization.

	NC SB	NC B
SB content (%)	101.68 ± 1.09	–
pH	5.49 ± 0.12	6.06 ± 0.21
Size (nm)	118 ± 7*	133 ± 5*
Polydispersity index	0.14 ± 0.01	0.13 ± 0.04
Zeta potential (mV)	−11.48 ± 2.34	−9.88 ± 5.12
$D_{(4,3)}$ (nm)	213 ± 4*	338 ± 2*
$D_{(90\%)}$ (nm)	314 ± 1*	442 ± 10*
Span	1.16 ± 0.45	1.34 ± 0.98

The results are expressed by mean with SD of triplicate. Asterisks denote the significant difference (*) $p < 0.05$ by unpaired Student's *t*-test between NC SB and NC B.

3.2. Preparation and characterization of gellan gum films

After drying, all the films had a homogeneous appearance. The films containing NC were slightly whitish in appearance in comparison to films without the NC (Fig. 1). Besides, in order to verify the films transparency, UV–visible spectra were recorded. The films without NC presented transmittance values close to 100% in the rage of 400–800 nm. On the other hand, due to the NC presence, NC SB GG demonstrated lower transmittance value (64%).

Table 2 shows the results of films characterization. All the films were thin with thickness of 20 μm. Besides, the film fragments (1 cm^2) were homogeneous (SD < 2 mg) in weight for the different films produced. No significant difference was observed for weight and thickness comparing NC SB GG and SB GG with the respective controls NC B GG and DMSO GG (data not shown). In relation to SB content, the film fragments presented around 430 μg/cm^2 which represents 90% of theoretical value (477 μg/cm^2). Besides, the films containing SB nanoencapsulated showed a lower SD value in comparison to those prepared with non-nanoencapsulated SB, suggesting better flavonoid homogeneity in those films. Moreover, the PCS analyzes showed that NC maintained its size after the incorporation into the films, showing mean particle size of 120 nm (Table 2 and Supplementary Fig. 2). The residual solvent content in the films was 16.57 ± 0.73% for vehicle (neat GG) and 17.53 ± 3.88% for films containing NC.

3.3. Swelling index and water contact angle

The swelling capacity and water contact angle were investigated to evaluate the hydration and hydrophilicity of the films, respectively, and the data are presented in Table 2. The results showed that the vehicle film displayed the highest values of swelling and non-nanoencapsulated SB films (SB GG) presented the lower values. The films with NC (NC SB GG) presented intermediate values, uptaking of about 1.5 times more buffer than films containing SB dissolved in DMSO. Regarding the contact angle, all films developed presented values lower than 15°, indicating high hydrophilicity.

3.4. Folding endurance and mechanical properties

The GG films flexibility was measured with respect to their folding endurance. As the result, all the films remained intact after > 300 folding repeats.

The results of stress and strain are presented in Table 2. In comparison to neat GG (vehicle), both SB GG and NC SB GG films significantly reduced the stress and strain values ($p < 0.05$). However, NC SB GG films presented intermediate values in the mechanical characteristics showing higher values of stress and strain than non-nanoencapsulated SB ($p < 0.05$). Besides, the values of young's modulus obtained by plotting the stress-strain curve (Supplementary material Fig. 3) were 67 ± 1 MPa, 64 ± 2 MPa and 65 ± 3 MPa for NC SB GG, SB GG and vehicle, respectively, without significant difference among them ($p > 0.05$).

3.5. Morphology of gellan gum films

SEM analysis carried out to characterize the morphology and homogeneity of the GG films (Fig. 2). The vehicle film (Fig. 2A) and NC B GG (Fig. 2B) showed a smooth and continuous surface without apparent difference between them. This result affirms miscibility between NC and GG in the films. However, films containing DMSO SB solution presented irregularities and some small cracks (Fig. 2C).

3.6. Stability evaluation

At predetermined intervals, fragments (1 cm^2) of SB GG and NC SB GG, stored under refrigeration (2–8 °C) or at room temperature, were

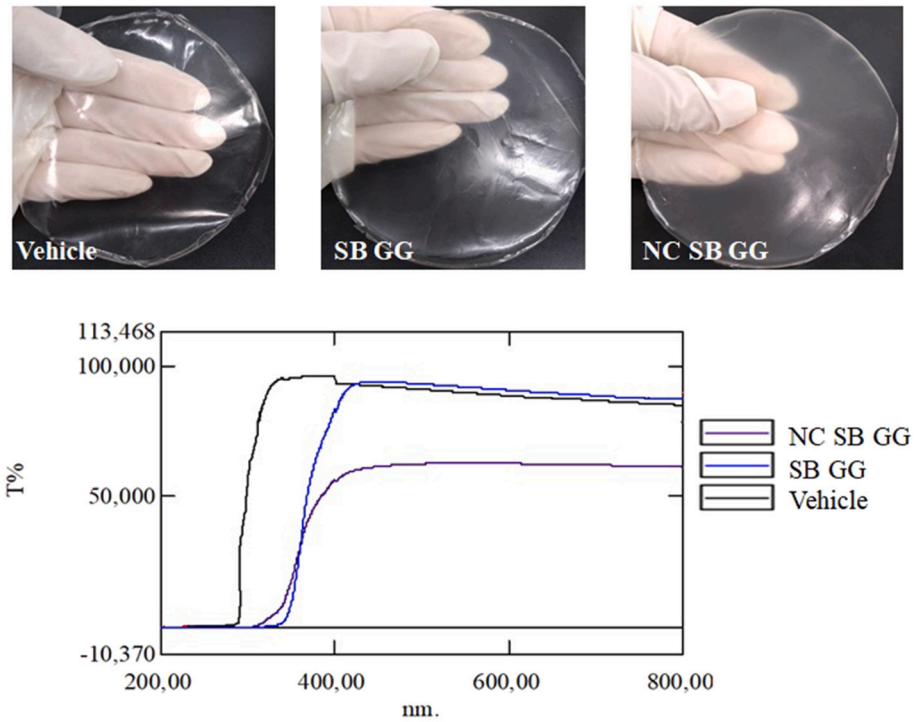

Fig. 1. Macroscopic appearance and UV–Vis spectra of developed films.

Table 2
Results of films characterization.

	SB GG	NC SB GG	GG
Drug content homogeneity (μg/cm^2)	423.68 ± 29.12	435.07 ± 5.86	–
Thickness (μm)	20 ± 2	19 ± 2	20 ± 4
Weight homogeneity (mg/cm^2)	68.3 ± 1.4	46.2 ± 0.5	30.1 ± 0.9
Size (nm)	–	120 ± 4	778 ± 28
Polydispersity index	–	0.25 ± 0.02	0.57 ± 0.10
Swelling index (%)	70.25 ± 9.69	105.11 ± 2.40	156.50 ± 7.13
Water contact angle	12.70 ± 2.55	14.03 ± 2.00	14.80 ± 2.80
Stress (MPa)	2.04 ± 0.23*	3.70 ± 0.26*#	6.96 ± 0.98
Strain (%)	3.54 ± 0.21*	4.71 ± 0.37*#	7.92 ± 0.39

The results are expressed by mean with SD of triplicate. Asterisks denote the significant difference (*) $p < 0.05$ by unpaired Student's *t*-test between Vehicle and NC SB GG or SB GG. Sharp denote the significant difference (#) $p < 0.05$ by unpaired Student's *t*-test between NC SB GG and SB GG.

withdrawn and analyzed in order to determine SB content, weight and particle size. Concerning the SB content of the films stored under refrigeration, both SB GG and NC SB GG were stables throughout the study period (Fig. 3A). However, at room temperature (Fig. 3B) only NC SB GG remained stable for 120 days. In this condition, the film containing non-nanoencapsulated SB had its content reduced to 357.98 ± 10.79 μg/cm^2 which represents about 15% degradation.

No change in film weight was found for NC SB GG and SB GG over time when stored at room temperature or under refrigeration (data not shown). Besides, the NC SB GG film presented particle sizes of 128 ± 5 nm and 123 ± 9 nm when stored at room temperature or under refrigeration, respectively, demonstrating nanostructure maintenance.

On the other hand, NC aqueous suspensions (NC SB) were unstable in both conditions evaluated. After just 15 days of storage, the SB content in the suspensions reduced for 65.91 ± 15.01% at refrigeration and for 59.44 ± 10.95% at room temperature, demonstrating the influence of the water presence in the SB stability.

3.7. In vitro release study

The SB release from GG films (non-nanoencapsulated and nanoencapsulated) are presented in Fig. 4. In sink condition (phosphate buffer pH 7.4 and ethanol, 70:30), non-nanoencapsulated SB was totally released in 5 h of experiment, while SB nanoencapsulated showed an average release of 18.53 ± 1.84% in 24 h of experiment. In phosphate buffer pH 7.4, it was also observed that when the SB was nanoencapsulated, a lower flavonoid amount reached the receptor medium. The percentage of SB released in this condition was 33.35 ± 0.03% for SB GG and 5.07 ± 0.72% for NC SB GG. Thus, both experimental conditions proved that nanoencapsulation promotes sustained release of SB from GG films.

Many drug delivery systems composed by ethylcellulose, the polymeric wall of NC, have been reported to show a Higuchi release profile, that is, the release mechanism happens through the diffusion of the drug through the system. Thus, the release data were analyzed according to this equation showing a good correlation with this model (> 0.98) and the release is therefore mostly diffusion controlled.

3.8. Occlusion test

The Fig. 5 shows the occlusion factor of the developed films. As can be observed, the nano-based films presented occlusion factor of 25.34 ± 3.40% in 6 h of experiment and this value remained constant until the end of the assay. On the other hand, non-nanoencapsulated SB and vehicle films presented significantly lower occlusion factor values ($p < 0.05$).

3.9. Skin permeation

The skin permeation of SB (non-nanoencapsulated or nanoencapsulated) from the films was carried out using non-injured and injured human skin as the membrane in Franz diffusion cells. The total SB retained on the non-injured and injured skin is shown in Fig. 6. In both conditions, the SB retention in the skin was lower for NC SB GG compared with SB GG film ($p < 0.05$). The SB amount retained in the

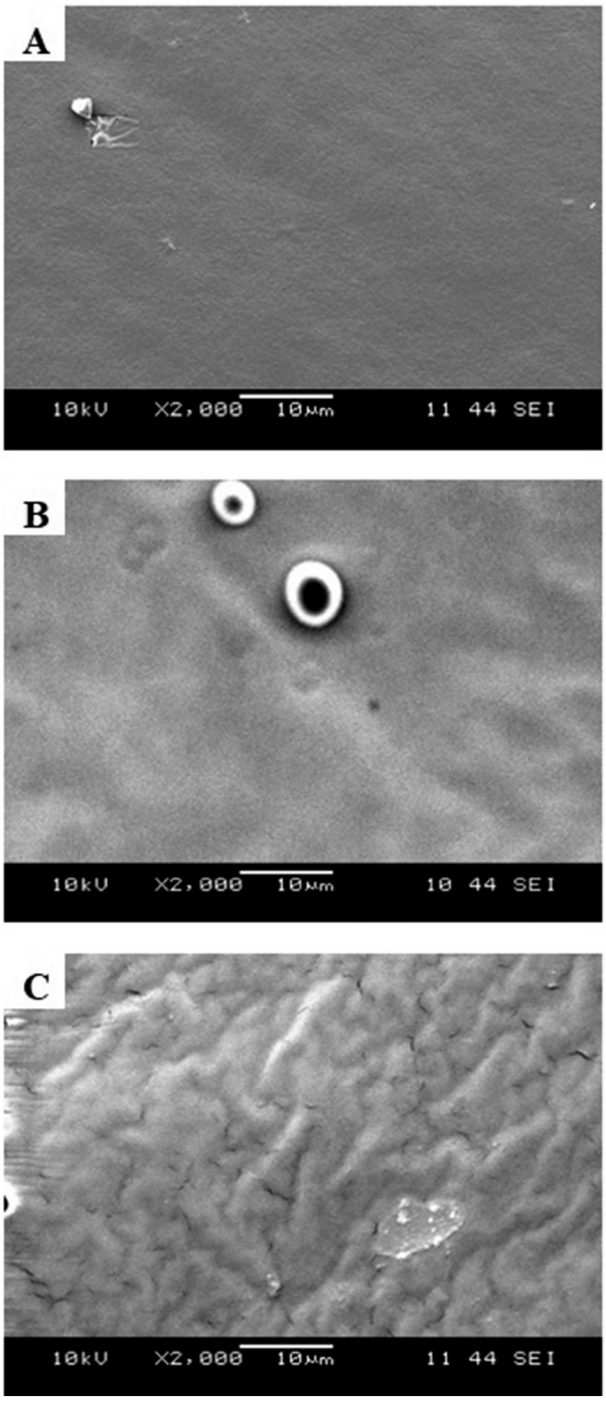

Fig. 2. SEM images of A) vehicle film; B) NC SB GG; C) SB GG.

non-injured skin was 307.9 ± 71.43 μg/g of skin for SB GG and 96.91 ± 7.14 μg/g of skin for NC SB GG film. Besides, as expected, after removal of the *stratum corneum* (injured skin), significant differences in the amount of SB reaching the skin were observed for SB GG (1316.02 ± 117.61 μg/g of skin) and NC SB GG (153.50 ± 3.71 μg/g of skin) after 24 h of experiment ($p < 0.05$). Moreover, regardless the SB form (non-nanoencapsulated or nanoencapsulated) or the skin condition (non-injured and injured) this flavonoid did not reach the receptor medium, compartment that represents the blood circulation.

The Fig. 7 shows the distribution percentage of SB in different layers of skin after 24 h of experiment for non-injured and injured human skin. Regarding non-injured skin (Fig. 7A), the distribution in the layers for both non-nanoencapsulated and nanoencapsulated SB can be ordered as follows: *stratum corneum* > epidermis > dermis. Besides, greater retention in the *stratum corneum* was found for nanoencapsulated SB (91.11 ± 1.33%) in comparison to non-nanoencapsulated SB film (83.05 ± 1.00%) ($p < 0.05$). On the other hand, in the epidermis it was observed higher percentages ($p < 0.05$) of SB for SB GG (13.33 ± 1.03%) than NC SB GG film (6.94 ± 1.47%). No significant difference was observed in the dermis for the different films.

In the injured skin (Fig. 7B), non-nanoencapsulated SB (98.51 ± 0.32%) also provided higher flavonoid amount in the epidermis in comparison to nanoencapsulated SB film (91.21 ± 1.30%) ($p < 0.05$). However, in relation to dermis, the nanoencapsulated SB reached this layer more than its non-nanoencapsulated form ($p > 0.05$). The compound percentage on the dermis was 1.20 ± 0.32% for non-nanoencapsulated SB and 6.27 ± 0.37% for nanoencapsulated SB.

3.10. Irritant potential of formulations

The irritant potential of NC suspensions and films was investigated using the HET-CAM model. As shown in Fig. 8, it was observed irritation only for the positive control (0.1 M NaOH), suggesting that all the formulations might be well-tolerated and safe for cutaneous use.

4. Discussion

The inclusion of lipophilic substances such as SB in hydrophilic matrices is challenging. However, NC are carriers known for their ability to increase the apparent solubility of lipophilic drugs in aqueous media. However, these nanocarriers may be unstable in formulations with a high water content, and drug release may occur with consequent hydrolysis [16]. In this sense, the conversion of SB-loaded NC into GG films is an alternative to incorporate this lipophilic flavonoid in a hydrophilic matrix without using organic solvents to its solubilization, as well as preventing its degradation due to the low amount of water present in films.

Firstly, NC suspensions were prepared by interfacial deposition of preformed polymer method using ethylcellulose and medium chain triglycerides as polymer and oil, respectively. After preparation, these suspensions presented characteristics compatible with NC used for drug delivery such as size between 100 and 300 nm, narrow size distribution and absence of micrometric population [4,5]. However, the unloaded NC size was lower than SB-loaded NC suggesting that the SB may also act as a surfactant at the interface with water. In fact, Lee et al. [29] also observed nanoparticles formation with smaller size distribution after adding silymarin extract to the formulation. The authors reported that the flavonoids present in this extract, such as silibinin, have an amphiphilic character and act as weak surfactants, reducing the surface tension [29]. In addition, NC showed high encapsulation efficiency and slightly acid pH values, corroborating the data obtained by Marchiori et al. for SB-loaded pomegranate oil NC suspensions [11]. Besides, the suspensions presented negative zeta potential due to the negative density of charge of ethylcellulose, the polymeric membrane of NC, which is in accordance to previous studies of our group [11,32].

In the next step, NC suspensions were incorporated into GG films by solvent casting method. The gellan gum forms films in the concentration range of 1 to 3%. However, concentrations above 2% have high viscosity and cannot be cast homogeneously in the plate besides forming thicker films [33]. Thus, GG at 1% was chose as the optimal concentration to produce homogeneous thin films. The neat GG forms very hard brittle films due to the formation of strong interactions among the chains of this gum after the solvent evaporation. Thus, GG films were plasticized with glycerol at 4%. Previous studies indicated that glycerol is a better plasticizer for GG films than other commonly used plasticizers such as PEG 400 and sorbitol because it forms more stretchable and transparent films [33–35]. After drying, the films presented water residual content around 17% which is suitable considering

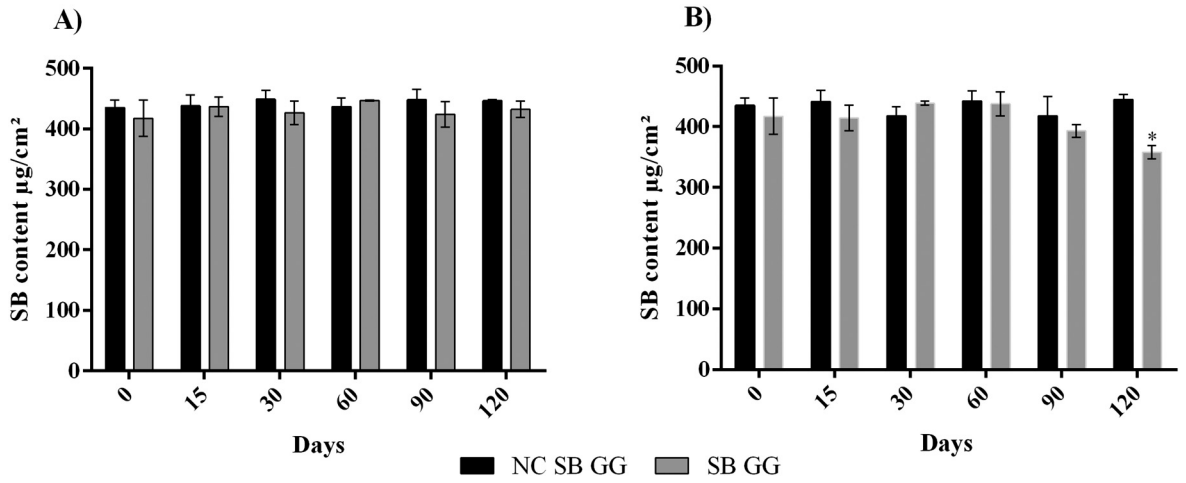

Fig. 3. Stability evaluation of gellan gum films containing non-nanoencapsulated or nanoencapsulated SB stored under refrigeration (A) or at room temperature (B). The results are expressed as mean ± SD and the statistical analysis was carried out by one-way ANOVA analysis followed by Tukey's test.
(*) $p < 0.05$. Significant differences between initial time (0 day) and final time (120 days).

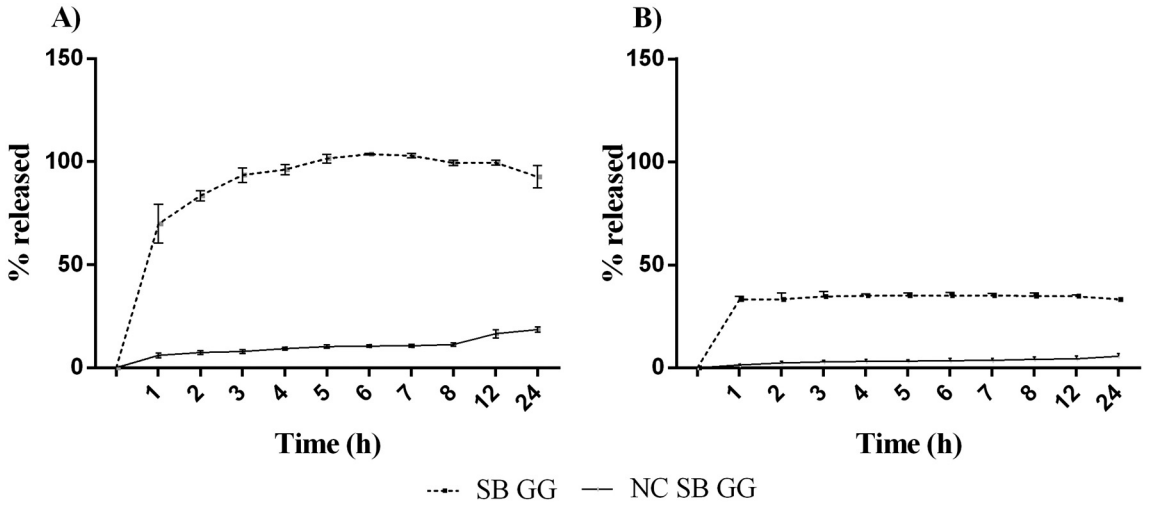

Fig. 4. *In vitro* SB release profile from gellan gum films containing non-nanoencapsulated (SB GG) and nanoencapsulated SB (NC SB GG). (A) phosphate buffer pH 7.4 and ethanol (70:30) and (B) phosphate buffer pH 7.4. The results are expressed as mean ± SEM of three independent experiments.

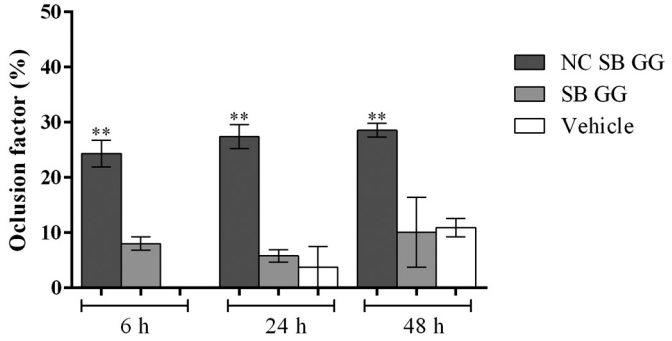

Fig. 5. Occlusion factor of vehicle film and films containing SB-loaded nanocapsules (NC SB GG) or non-nanoencapsulated SB (SB GG). The results are expressed as mean ± SD and the statistical analysis was performed by one-way ANOVA analysis followed by Tukey's test.
(*) $p < 0.05$. Significant differences between NC SB GG and SB GG or vehicle film.

the preparation method [14,33]. Besides, as observed in UV–Vis transmittance spectra, the films without NC exhibited higher transmittance as compared to nano-based films. This result could be explained by the ability of these nanocarriers to scatter UV light, due to their colloidal size [4]. However, all the films were transparent since presented transmittance values higher than 50%.

The physical and mechanical evaluations, such as thickness and flexibility, of the films are important parameters concerning the cutaneous administration. These characteristics are related to the dose accuracy of active substance and to the comfortable feeling on the skin [36]. Regardless of their composition, the films presented homogeneous weight and thickness demonstrating that their formation occurs evenly on the plate. All the films produced in this study were considered thin since presented thickness values around 20 µm [14], corroborating previously published data for GG films [23,25,26]. Besides, the films of this study may be considered highly flexible since they exhibit folding endurance value greater than 300 [14].

Concerning the stress and strain values, these were dependent on films composition. The films containing nanoencapsulated or non-nanoencapsulated SB reduced these two parameters in comparison to vehicle film (neat GG). Our result is in accordance to other studies which reported that the addition of substances into GG films modify its

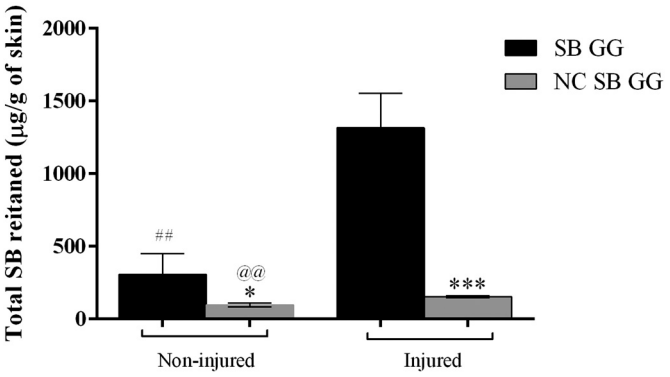

Fig. 6. Cumulative amount of SB in the non-injured and injured skin after 24 h of incubation with gellan gum films containing non-nanoencapsulated (SB GG) or nanoencapsulated SB (NC SB GG) (n = 6). The results are expressed as mean ± SEM and the statistical analysis was performed by Student's *t*-test.
(*) $p < 0.05$. Significant differences between SB GG and NC SB GG in the same condition (non-injured or injured).
(#) $p < 0.05$. Significant differences between compound quantified in the non-injured and injured skin from SB GG.
(@) $p < 0.05$. Significant differences between compound quantified in the non-injured and injured skin from NC SB GG.

structure and mechanical properties. This is probably due to a modification in the interaction between the chains of the polymer matrix with glycerol, promoting a decrease in the stress and elongation [25,37,38]. However, NC SB GG presented intermediate values of stress and strain, demonstrating superior flexibility than SB GG. This result is in accordance with other studies: the inclusion of NC into starch films provided higher values of strain than non-nanoencapsulated lycopene [39] and bixin [40], explained by the hydrophobicity of these substances which may promote discontinuities in the polymer matrix due to their immiscibility in films. Indeed, SB has poor water solubility [8] requiring prior solubilization in DMSO for its incorporation into the films. Despite DMSO to be miscible with water, GG is insoluble in organic solvents [22]. Thus, both SB and DMSO may contribute for formation of irregular films with lower elongation.

SEM images showed that films containing non-nanoencapsulated SB presented some discontinuities and cracks, corroborating the previous observations related to the mechanical properties and content homogeneity of this film. The lower miscibility between the two phases leads to an interruption of polymer matrix continuity and cracks formation after drying. On the other hand, no apparent visual differences were found between the vehicle and nano-based films, which presented a uniform structure and better mechanical properties As suggested in a previous study, the miscibility of the components in the filmogenic dispersion may improve the films flexibility [41]. In addition, our results agree with other studies that showed cracks in SEM images and weak mechanical properties in films with non-homogeneous drug distribution due to the low components miscibility [39,40].

Surprisingly, it was not possible to observe by SEM the NC in the films surface. Machado et al. also found no differences between films with or without nanoparticles, suggesting these particles were intimately and uniformly embedded in the polymeric network [16]. However, in our study, the presence of NC in the films was confirmed by PCS analysis which showed similar size to that obtained initially for the liquid suspensions.

In relation to SB content homogeneity, nanoencapsulation seems to provide a more homogeneous distribution of this flavonoid in the films since they presented lower SD values. This result can be explained by the SB apparent solubility enhance provided by the nanostructures. This observation is corroborated by the study carried out by Lee et al. [29] that also observed a better solubility for nanoencapsulated silymarin in alginate films [29]. However, non-nanoencapsulated and nanoparticulated SB films presented a total compound content around 90% which is in accordance to the film production process. It is reported that a suitable active substance content for films is in the range of 85–115% [14]. Furthermore, in the stability study, both films containing non-nanoencapsulated and nanoencapsulated SB were stable when stored under refrigeration. On the other hand, only NC SB GG was stable at room temperature demonstrating that NC protected the flavonoid from temperature variation. Besides, the aqueous NC suspensions demonstrated a decrease in SB content within 15 days, demonstrating that the films, as a solid pharmaceutical dosage form, proved to be a promising way to stabilize SB-loaded NC.

The contact angle analysis is used to characterize the surface hydrophilicity of the materials. All the films produced in this study presented contact angle around 15° indicating high films hydrophilicity since values bellow 90° characterize hydrophilic polymers [41]. Corroborating GG hydrophilicity, the films swelled when in contact with pH 7.4 phosphate buffer. This result is in accordance to literature data which demonstrate the high GG swelling capacity [33]. However, this characteristic was reduced by the addition of NC or SB solution in the

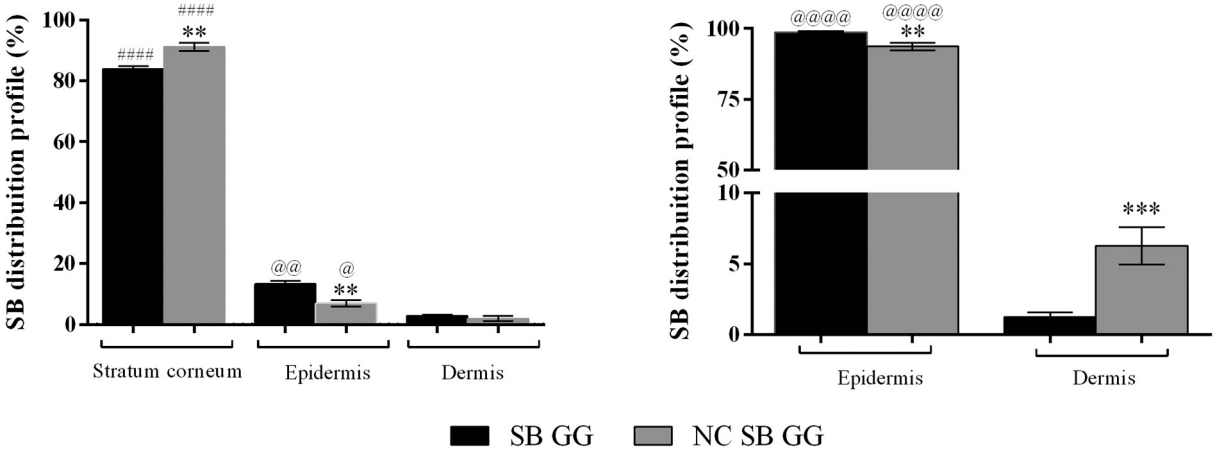

Fig. 7. Percentage of SB distribution in the different skin layers after 24 h of incubation with gellan gum films containing non-nanoencapsulated (SB GG) or nanoencapsulated SB (NC SB GG) (n = 6). (A) Non-injured skin and (B) injured skin. The results are expressed as mean ± SEM and the statistical differences were evaluated by one-way ANOVA analysis followed by Tukey's test.
(*) $p < 0.05$. Significant differences between SB GG and NC SB GG in the skin layers.
(#) $p < 0.05$. Significant differences between stratum corneum and epidermis in the non-injured skin.
(@) $p < 0.05$. Significant differences between epidermis and dermis in the non-injured skin or injured skin.

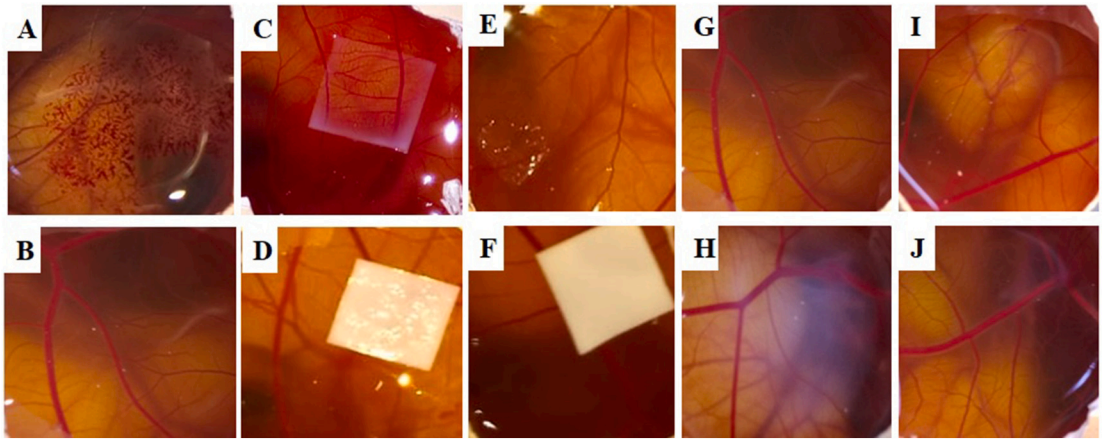

Fig. 8. Irritancy potential evaluation of the formulations in the HET-CAM test. A) Positive control; B) Negative control; C) SB GG; D) NC SB GG; E) GG; F) NC B GG; G) Free SB solution; H) NC SB suspension; I) DMSO solution; J) NC B.

films. The swelling mechanism is based on the polymer chains relaxation after immersion in aqueous medium, resulting in the exposure of hydrophilic groups following by the network expansion [14]. Thus, when nanoparticles or SB solution are added in the films an increase in the compactness of GG structure may occur, resulting in lower expansion. Similar observation was reported by Sebri et al. after the inclusion of ibuprofen in GG films [38]. However, films containing NC presented 1.5 times more absorption than SB GG. Besides, after 24 h in contact with the buffer, the films remain intact. These results suggest that when in contact with an exudative skin injury, such as wounds, the films may absorb this liquid without dissolving, acting as a physical barrier to the injury.

The SB release from GG films was performed in Franz cells using a dialysis membrane as barrier. Regardless the release medium (pH 7.4 phosphate buffer and ethanol 70:30 or pH 7.4 phosphate buffer), it was observed a controlled release of SB from NC. This result could be attributed to ethylcellulose, a polymer with hydrophobic characteristics and known by its controlled release properties [11,32]. Subsequently, it was evaluated the occlusion potential of GG films and it was possible to observe an improvement in the occlusion factor with the NC addition. This result is in accordance to previous studies, in which it was found that nanoparticles are able to increase the occlusive potential of topical dosage forms due to their reduced size [42,43]. These two characteristics are beneficial for cutaneous administration since allows the SB gradual release leading to a less frequent films replacement, as well as maintains the skin moisture providing less pain in the injury and accelerating the skin regeneration process [20,21,36].

The most important function of skin is to protect the organism of the entrance of exogenous substances. This function is mainly related to the *stratum corneum* presence which is constituted by a complex lipid composition limiting the permeation of topical drugs through the skin [3,44]. However, this barrier may be impaired in some skin lesions. Thus, in this study the SB permeation from films was evaluated in intact and injured skin. A superficial skin lesion was simulated by *stratum corneum* removal before the experiment [1].

The results of *in vitro* permeation obtained in this study showed that the SB amount retained in the skin is lower for nano-based films than films containing the non-nanoencapsulated compound. This result can be explained by the slower release observed for the films containing SB-loaded NC. Besides, after *stratum corneum* removal, the cumulative amount of SB in the skin was increased 4.5-fold for the non-nanoencapsulated flavonoid and 1.5 times for nanoencapsulated SB. The low influence of *stratum corneum* presence observed for SB NC confirms the controlled release provided by NC. Thus, a lower concentration of released SB is available on the skin surface to permeate through its layers. Therefore, the presence or absence of the *stratum corneum* as a barrier was not a crucial factor for this permeation. This found is in accordance with previous reports in which it was observed a reduction in drug penetration/permeation by nanostructures due to the slower release provided by these systems [1,2]. Moreover, the DMSO, which is present in films containing the non-nanoencapsulated SB may act as a permeation enhancer [45].

In the SB relative distribution profile in the different layers of the non-injured skin, it was possible to observe that NC tends to be more retained in the *stratum corneum* than the non-nanoencapsulated compound. Consequently, smaller amounts of SB were found for this formulation in the viable epidermis. Similar results were observed by Rigon et al. for SB-loaded pomegranate oil NC incorporated into hydrogels [9]. In fact, polymeric nanoparticles accumulate in the *stratum corneum*, which acts as a reservoir for them, and the gradual drug release to the other skin layers occurs [1,2]. On the other hand, in the injured skin it was observed that nanoencapsulated SB was 5-fold more retained in the dermis than its non-nanoencapsulated form. Such behavior is an important feature for wound healing since this layer has the cells required for the healing process [44].

Regarding film biocompatibility evaluation, CAM is a suitable model to study signs of irritation due to the presence of arteries, veins and capillaries in its structure. [46,47]. Our results clearly showed the absence of vasodilatation and hemorrhage when CAM were put in contact with the films, suggesting that they are well-tolerated and safe for cutaneous use.

5. Conclusions

The technological conversion of SB-loaded NC aqueous suspensions into polymeric films represents another approach to explore the therapeutic applications of nanoparticles. The present research showed the feasibility of preparing SB-loaded NC using medium chain triglycerides as oily core, which were successfully incorporated into a GG film. These films provided the stabilization of SB content for a longer time, as well as the NC protected this flavonoid from degradation at room temperature. Besides, GG films presented suitable characteristics such as flexibility, thin and homogeneous thickness, transparency and adequate swelling index. In addition, NC provided occlusion to GG films and better SB homogeneity and miscibility in this film as well as being able to control the release of this flavonoid and to increase its delivery in the dermis. Moreover, the films showed no irritant potential in the HET-CAM test. Therefore, the developed nano-based GG films congregate several appropriate features for an optimal SB cutaneous delivery to treat skin disorders especially wounds.

CRediT authorship contribution statement

Mailine Gehrcke: conceptualization, investigation, formal analysis, visualization, writing - original draft. **Taíne de Bastos Brum:** conceptualization, investigation, formal analysis, visualization. **Lucas Saldanha da Rosa:** investigation; formal analysis, visualization. **Bruna Dias Ilha:** investigation, formal analysis, visualization. **Fabio Zovico Maxnuck Soares:** conceptualization, supervision, visualization, writing - review & editing. **Letícia Cruz:** conceptualization, supervision, visualization, writing - review & editing, project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2020.111624.

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SUPPLEMENTARY MATERIAL

Incorporation of nanocapsules into gellan gum films: a strategy to improve the stability and prolong the cutaneous release of silibinin

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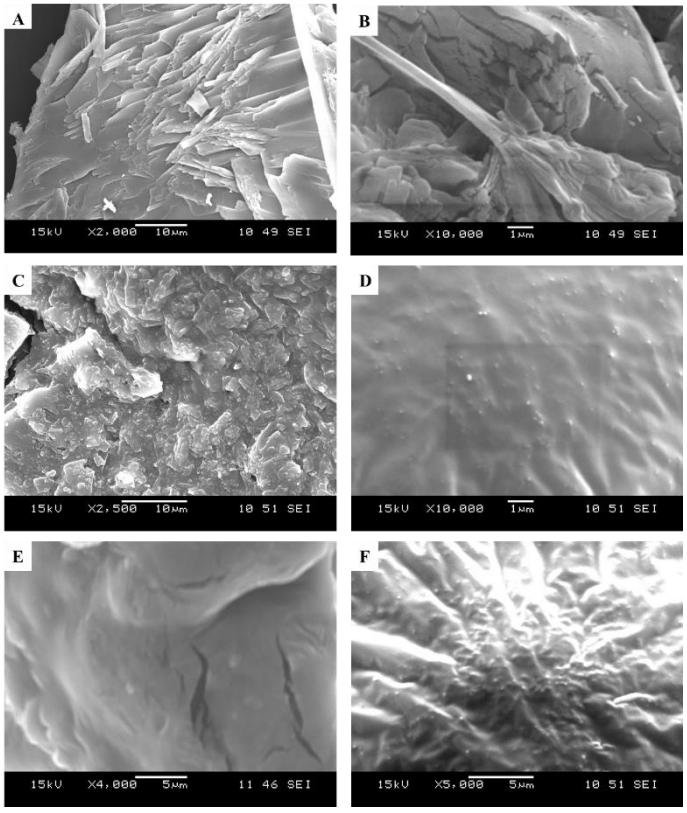
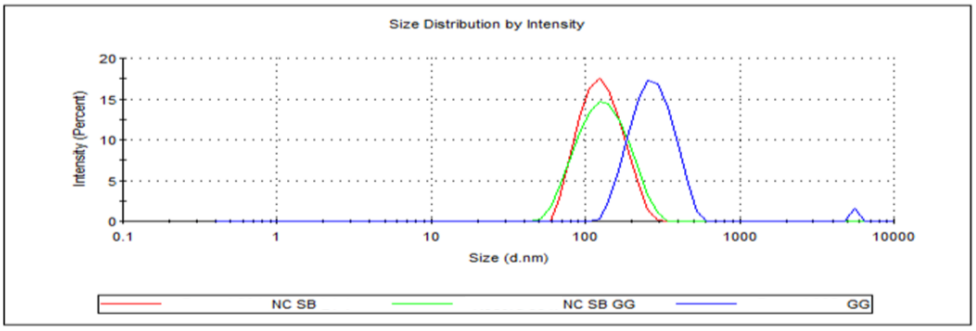
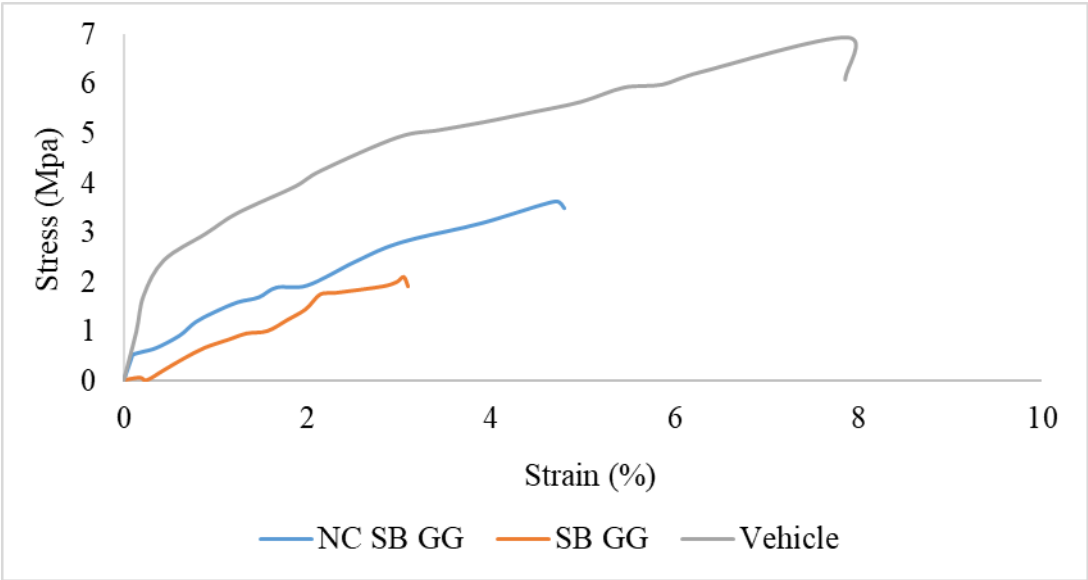


Supplementary figure 1. SEM images of A) Lactose (magnification = 2,000); B) Lactose (magnification = 10,000); C) NC SB (magnification = 2,500); D) NC SB (magnification = 10,000); E) NC B (magnification = 4,000); F) NC B (magnification = 5,000).



Supplementary figure 2. Size distribution of nanocapsule suspensions (NC SB), nanocapsules incorporated into gellan gum films (NC SB GG), and vehicle film (GG).



Supplementary figure 3. Stress–strain curves obtained from gellan gum film

3.3 DESENVOLVIMENTO DE FILMES BICAMADA DE GOMA GELANA E PULLULAN CONTENDO NANOCÁPSULAS DE SILIBININA VISANDO O TRATAMENTO DA DERMATITE ATÓPICA

3.3.1 Estudos de pré-formulação

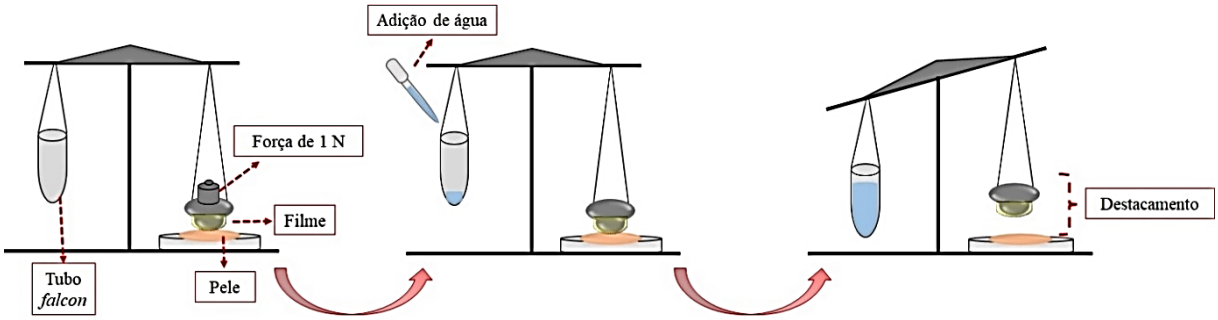
Tendo em vista os resultados promissores dos filmes de goma gelana como veículo para nanocápsulas de SB, este terceiro capítulo visou a associação deste filme com o pullulan. O interesse nesta associação surgiu com base em resultados prévios do nosso grupo de pesquisa obtidos para filmes de pullulan, como rápida dissolução, elasticidade, potencial oclusivo maior que 50 % e mucoadesão, sendo promissor tanto para o tratamento da dermatite atópica, quanto para o tratamento de doença fúngica vaginal (CERVI et al., 2021, 2022). Assim, hipotetizou-se que a combinação da goma gelana e do pullulan em um filme para a aplicação tópica de nanocápsulas de SB demonstraria características mais aprimoradas, beneficiando o tratamento da dermatite atópica.

Frente a isso, foi proposta a combinação da goma gelana e do pullulan em um filme bicamada, a fim de criar uma microestrutura que preservasse as propriedades físico-químicas naturais destes polissacarídeos isolados em suas respectivas camadas, como já demonstrado na literatura para filmes bicamada produzidos com outros polímeros (FERREIRA et al., 2016; NETO et al., 2019; PEREDA et al., 2011). Para isto, foram testadas 3 concentrações de pullulan para a formação da segunda camada polimérica, 1,5, 3 e 6 %, sendo a solução deste polissacarídeo vertida sobre a camada de goma gelana. Todos os filmes foram facilmente removidos da placa de *petri*, apresentaram característica macroscópica homogênea e foram transparentes.

Ao desenvolver uma formulação de uso tópico tem-se o objetivo de que esta fique sobre o local de ação desejado por tempo suficiente para executar seu efeito terapêutico. No caso específico dos filmes poliméricos, é importante que estes permaneçam aderidos ao tecido, evitando a utilização de fitas que possuem cola em sua composição, especialmente em lesões que comprometem a barreira cutânea da pele (PAGANO et al., 2019). Desse modo, como *screening* para escolha da melhor concentração de pullulan para a produção dos filmes bicamada foi utilizado o potencial bioadesivo dos filmes produzidos, o qual foi avaliado utilizando o método da balança de dois pratos modificado (GUPTA; GARG; KHAR, 1992; OSMARI et al., 2020), conforme figura 7 e metodologia descrita no manuscrito 1. Como resultado do teste de bioadesão, a inclusão do pullulan como segunda camada polimérica em

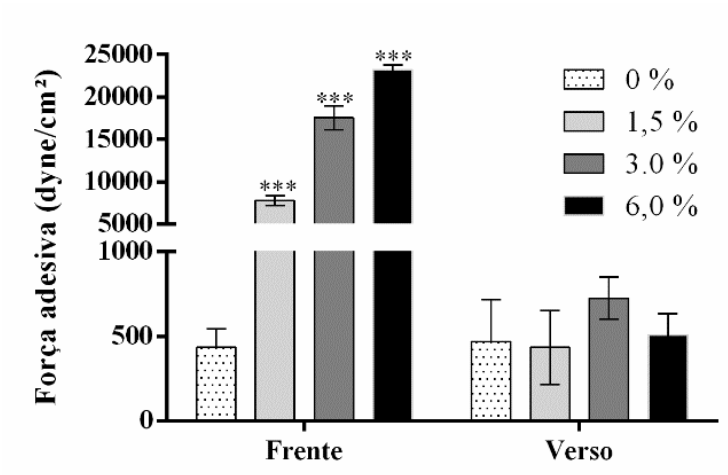
filmes de goma gelana aumentou consideravelmente a bioadesão destes filmes, sendo esta dependente de sua concentração (Figura 8). Assim como a bioadesão, a espessura dos filmes foi dependente da concentração do pullulan, sendo a de 6 % a com o maior valor de espessura, atingindo valores de 96 ± 5 µm. Tendo em vista que a espessura pode impactar na liberação da substância ativa, bem como do sensorial do filme e aceitabilidade pelos pacientes (KARKI et al., 2016), os filmes produzidos na concentração de 6 % foram excluídos dos testes posteriores. Deste modo, a concentração de 3 % foi escolhida para dar continuidade ao trabalho.

Figura 7 – Figura representativa da avaliação da bioadesão dos filmes desenvolvidos.



Fonte: Próprio autor.

Figura 8 - Avaliação da bioadesão dos filmes monocamada de goma gelana (0 %) e bicamada de goma gelana/pullulan (1,5, 3 e 6 % de pullulan).

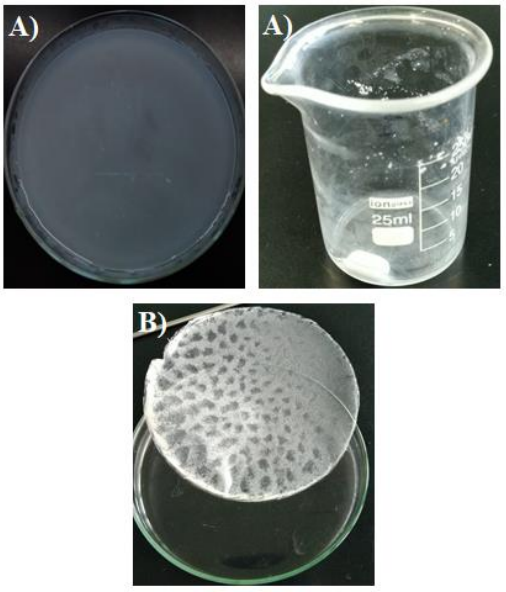


Os resultados são expressos como média ± desvio padrão (n=3). A avaliação foi realizada em ambos os lados do filme, sendo o verso do filme considerado aquele que estava em contato com a placa de *petri* durante o preparo e a frente do filme aquela que não estava em contato com a placa de *petri.* *** p <0,001 mostram diferença significativa das diferentes concentrações de pullulan quando comparado ao filme monocamada de goma gelana (ANOVA duas vias seguida por pós-teste de Tukey).

É válido mencionar que, inicialmente, foram também realizados testes utilizando blendas poliméricas da goma gelana com pullulan em diferentes proporções (1:1; 2:1; 1:2; 1:3), as quais formaram filmes transparentes, facilmente removíveis da placa de *Petri* e maleáveis. No entanto, não foi possível evidenciar qualquer melhora na bioadesão destes filmes, hipotetizando-se que a interação entre a mistura polimérica poderia não estar favorecendo a manutenção das características bioadesivas do pullulan.

Ademais, é importante destacar também que não foi possível a produção de filmes bicamada contendo a SB em sua forma livre, pois a camada de pullulan não se formou de maneira adequada sobre a superfície da primeira camada do filme, provavelmente devido a presença do DMSO. Foram realizadas inúmeras tentativas de solubilizar a SB em etanol, glicerol e propilenoglicol, bem como com o uso de tensoativos, como o polissorbato 80, a fim de se obter um filme comparativo aos que continham nanoestruturas. No entanto, a SB apresentou baixa solubilidade nestes solventes. Já em testes realizados com acetona, apesar da SB solubilizar facilmente neste solvente, ocorria precipitação ao adicioná-la à dispersão filmogênica de goma gelana (Figura 9A). Ainda, em testes realizados através de um preparo de uma nanodispersão deste flavonoide, apesar desta se manter aparentemente solúvel nestas condições, mesmo após a adição da goma gelana, durante o período de secagem havia precipitação do flavonoide no filme (Figura 9B).

Figura 9 – Preparo de filmes bicama contendo SB livre previamente solubilizada em aceta (A) ou associada à nanodispersão (B).



3.3.2 Manuscrito 1 - Novo filme bicamada de pullulan/goma gelana como veículo para nanocápsulas contendo silibinina no tratamento tópico da dermatite atópica

Novel pullulan/gellan gum bilayer film as vehicle for silibinin-loaded nanocapsules in topical treatment of atopic dermatitis

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Abstract

In this study a novel gellan gum/pullulan bilayer film associated with silibinin-loaded nanocapsules was developed for topical treatment of AD-like lesions induced by 2,4-dinitrochlorobenzene (DNCB). The bilayer films were produced by applying a pullulan layer on a gellan gum layer incorporated with silibinin nanocapsules by two-step solvent casting method. The bilayer formation was confirmed by microscopic analysis. Evaluations with human skin showed that pullullan imparts bioadhesitvity for the bilayer films. Despite nano-based film being less swellable and bioadhesive than the vehicle film, the presence of nanocapsules increased the occlusion factor of bilayer films almost 2-fold, being considered the most suitable for AD treatment. *In vitro* studies showed that the nano-based film presented a slow silibinin release and high affinity for cutaneous tissue. Besides, this film presented high scavenger capacity and non-hemolytic property. In the *in vivo* study, interestingly, the treatments with bilayer films with or without nanocapsules attenuated the scratching behavior and the ear edema induced by DNCB exposure to mice. However, the nano-based film containing silibinin modulated the inflammatory and oxidative parameters in a similar or more pronounced way than silibinin solution and vehicle bilayer films, as well as than hydrocortisone, a classical drug used in the clinical practice. In conclusion, these data lead us to believe that itself gellan gum/pullulan bilayer film might attenuate the effects induced by DNCB, acting together with silibinin-loaded nanocapsules, which protected the skin from oxidative damage caused by the hapten, improving the therapeutic effect in this AD-model.

Keywords: Nanocapsules, films, silibinin, pullulan, gellan gum, atopic dermatitis.

1. Introduction

Atopic dermatitis (AD), a chronic inflammatory skin condition, results from a complex interaction among the genetic predisposition, the environmental factors, as well as the dysfunctions in the skin barrier and in the permeability of allergens and pathogens agents (MALIK AND HEITMILLER, 2017). These pathological mechanisms could play synergistically to maintain the clinical symptoms of AD, including pruritus, eczematous lesions, remodeling of the skin surface and generalized skin dryness due to the constant inflammation (MALIK AND HEITMILLER, 2017; RERKNIMITR ET AL. 2017). The current treatments for AD are based on skin barrier recovery and the use of corticosteroids and topical and systemic immunosuppressant, which are used as unique or combined therapy. However, these pharmacological treatments present limited effectiveness and relevant side effects (APPHEMRAJANI et al., 2022; GHOSALKAR; PRABHA; PADMINI, 2022; MANCUSO et al., 2021).

In this context, the attempts to planning and developing a new, safe, and effective therapeutic strategy for AD have been receiving notable priority. Several studies have been demonstrated the safety and the effectiveness of naturally occurring substances in the AD treatment and thereby, their arise as an interesting option to overcome conventional issues of the available pharmacological treatment (Wu et al., 2021). Silibinin (SB), the main biologically active flavonoid extracted from *Silybum marianum* seeds, is well recognized for its potent antioxidant and anti-inflammatory properties (LIU et al., 2021; SONG et al., 2017). This flavonoid has been widely studied to prevent and to treat inflammatory skin disorders, including dermatoses (CASTELLANETA et al., 2016; RAJ et al., 2020; RIGON et al., 2019; SAMANTA et al., 2016; SHROTRIYA; VIDHATE; SHUKLA, 2017). However, SB is poor soluble in water and its solubilization in others solvents is limited (ATRUX-TALLAU et al., 2010). Few studies have explored the development of formulations that allow the application of this flavonoid on the skin. The existent studies involve the use of nanotechnology to improve the SB solubility and performance against skin diseases (MARCHIORI et al., 2017; RIGON et al., 2019; SHROTRIYA; VIDHATE; SHUKLA, 2017).

Moreover, drug delivery systems based on nanotechnology have shown a promising strategy for AD management due to the nanostructures's properties to achieve enhanced skin penetration and retention, release control, and reduced side effects (ALAM et al., 2013; BADIHI et al., 2020; HUSSAIN et al., 2014; LI et al., 2012; WANG et al., 2019). Among these nanostructured systems stand out the nanocapsules (NC) which have a core/shell structure

constituted by oil and polymer, respectively (JAIN; THAREJA, 2019; MORA-HUERTAS; FESSI; ELAISSARI, 2010). This structural organization favors the encapsulation of lipophilic substances into NC, increasing the solubility and therapeutic efficacy of these substances (FERREIRA et al., 2019; MARCHIORI et al., 2017; RIGON et al., 2019).

In this same way, polymeric films present many advantages to treat inflammatory skin diseases, such as AD, due to their potential to adhere to the skin providing protection and hydration for this tissue (ALVES et al., 2016; CERVI et al., 2021; JEONG et al., 2019). For the films development aiming cutaneous use, natural polysaccharides have shown to be promising candidates due to low toxicity, biocompatibility and biodegradability (KARKI et al., 2016). Among such polysaccharides is gellan gum, which has been shown to be suitable for the formation of ultrathin films with physical and mechanical resistance and with swelling capacity, being promising for protecting the area and absorbing exudate of lesions (ARIFAH et al., 2019; ISMAIL et al., 2019; SEBRI; AMIN, 2016). Another natural polysaccharide that has attracted the interest of researchers is pullulan, which forms elastic, bioadhesive and fast-dissolving films (CERVI et al., 2021, 2022). In addition, it was suggested by Jeong and co-workers that pullulan may present a physical action against AD (JEONG et al., 2019).

Studies have reported the bilayer films as an advantageous alternative to polymeric blends for cutaneous delivery. These films are able to preserve the intrinsic characteristics of each film-forming agent in it respective polymeric layer, improving the film properties (CONTARDI et al., 2019; NETO et al., 2019). Besides, combining nanostructures into polymeric films for cutaneous delivery renders a dosage form that can be administered over the affected skin, promoting a physical barrier for injured skin, and allowing a lower frequency of administration, which improves the therapeutic efficacy (DHAL; MISHRA, 2020; SHAHZAD et al., 2019).

Also, NC have shown a promising alternative to incorporate lipophilic substances into hydrophilic films (CERVI et al., 2021). In a previous study, we demonstrate the promising advantages of SB encapsulation into NCs followed by their incorporation into gellan gum films for cutaneous delivery. The developed films presented physical and mechanical resistance and fluid absorption capacity. Besides, due to the high SB lipophilicity, the nanoencapsulation was fundamental to guarantee it dosage homogeneity into gellan gum film. Also, the NC association into the gellan gum film provided increased SB stability, as well as controlled release and improved skin permeation to the dermis(GEHRCKE et al., 2021). These results plus to the attractive pullulan films characteristics stimulated our curiosity about combining them in a novel bilayer film to treat AD.

The objective of the present study was to develop a bilayer film composed by gellan gum layer incorporated with SB-loaded NC as the bottom side and with a pullulan layer as the top side, as well as to evaluate the film potential against AD-like skin lesions. This novel nano-based bilayer film was engineered such that the distinct layers provide their respective beneficial features for AD treatment.

2. Materials and methods

Materials

SB (98% purity), Span® 80 (sorbitan monooleate) were obtained from Sigma Aldrich (São Paulo, Brazil). Ethylcelullose was donated by Colorcon (Cotia, Brazil). Medium chain triglycerides and Tween® 80 (polysorbate 80) were bought from Delaware (Porto Alegre, Brazil). Gellan gum (Kelcogel®) and pullulan were donated by CP Kelco (Georgia, USA) and Hayashibara, respectively. 2,4-dinitrochlorobenzene (DNCB) was purchased from Sigma (St. Louis, MO, USA) and it was used as an inductor of atopic dermatitis (AD)-like lesions. The hydrocortisone ointment (HC) was obtained commercially and it was used as a reference drug. All other solvents and reagents were analytical grade and used as received.

Methods

2.1 Preparation of nanocapsule suspensions and bilayer films

SB-loaded NC were prepared by the interfacial deposition of preformed polymer method (FESSI et al., 1989), at a concentration of 2.5 mg/mL as described previously (GEHRCKE et al., 2021). The organic phase was composed by SB (0.025 g), ethylcellulose (0.25 g), Span® 80 (0.1925 g), medium chain triglycerides (0.75 g) and acetone (68 mL). The aqueous phase was composed of water (132 mL) and Tween® 80 (0.1925 g). Then, the organic phase was added under magnetic stirring into aqueous phase followed by solvent evaporation under reduced pressure to achieve a 10 mL final volume. Unloaded NC suspensions were prepared using this same protocol, omitting SB. After preparation, NC suspensions presented particle diameter of 115 ± 3 and 134 ± 5 nm for SB-loaded NC and unloaded NC, respectively. The polydispersity index was below 0.2 and the zeta potential was around -10 mV for both NC

suspensions. Besides, the content of SB was 98.9 %, being suitable for incorporation into the films.

The bilayer films were produced by two-step solvent casting method. For this, firstly, the gellan gum dispersion was prepared by dispersing 0.25 g of this gum in 15 mL distilled water while heating at 80 °C, under magnetic stirring for 2 h. After, an amount of glycerol (1 g) was added to this dispersion. Subsequently, the mixture was removed from the heating and 10 mL of water or NC suspension were added to gellan gum dispersion to produce the first layer of the vehicle film or nano-based films, respectively. After, this mixture was instantly poured into a Petri dish (90 × 13 mm) and was partially dried at 40 °C for 15 h. Then, a water solution containing 3 % (w/v) of pullulan and 0.5 % (w/v) of glycerol was prepared at room temperature and under magnetic stirring for 30 min. After complete pullulan solubilization, this solution was poured on the surface of the first layer and dried at 40 °C for 24 h. The bilayer films were named BF NC SB, BF NC B and BF vehicle for films produced with nanoencapsulated SB, unloaded NC and control film, respectively.

2.2 Scanning Electron Microscopy

The structure of the bilayer films was evaluated by scanning electron microscopy (SEM) (JEOL JSM 6360, Japan). To visualize the layers, the films were cryofractured in order to analyze the sides sections after fracture. To carry out the analysis, the samples were previously placed on a double–sided adhesive carbon tape, mounted on the sample slab and coated with gold (Denton Vaccum II, 100 Å) under reduced pressure. The samples were subsequently analyzed using an accelerating voltage of 10 kV. This analysis was also performed for a monolayer vehicle film (MF vehicle) which was produced containing only the layer of gellan gum and served as a control.

2.3 Bilayer films characterization

The bilayer films were characterized by homogeneity of SB content, thickness, moisture, nanometric size maintenance and swelling index. For thickness measurement, films (n=3) were prepared for each formulation and then five measurements were performed on each film (four in the corner and one in the middle). Mean thickness values were calculated and expressed in μm. For homogeneity of SB content, the films (n=3) were cut into three fragments of 1 cm × 1 cm each. The SB content in each fragment was quantified by extracting the

phytochemical in methanol, subjecting it to stirring for 20 min followed by sonication for another 20 min. Samples were filtered (0.45 μm) and analyzed by high performance liquid chromatography (HPLC), using a guard column and a Kinetex C_{18} Phenomenex column (250 mm × 4.60 mm, 5 μm; 110 Å) at room temperature. The mobile phase consisted of water pH 3.5 and acetonitrile (60:40, v/v) at isocratic flow rate (1.0 mL/min) and the detection wavelength used for SB was 288 nm, as described previously (GEHRCKE et al., 2021). The mean values of the SB content were calculated and expressed in μg/cm², and the content (%) was calculated in relation to the theoretical amount of SB present in the film.

The particle size and the polydispersity index of the NC after their incorporation into films were evaluated by photon correlation spectroscopy (PCS) (ZetaSizer, Malvern). For this, film fragments (0.1 g) were dispersed in 50 mL of ultrapure water (500x dilution). The nanostructures were extracted from the films under magnetic stirring for 2 h before analysis. For moisture assessment, the films were cut into 2 cm × 2 cm fragments and later placed in an oven at 60 °C (SHAHZAD et al., 2019). These fragments were weighed after regular time intervals until the weight became constant. The residual water content in the films was determined following equation 1.

$$\textit{Moisture content} = [(W_d - W_i) / W_i] \times 100 \qquad \text{Eq (1)}$$

Where: W_d is the weight of the films after drying and W_i is the initial weight of the films.

To evaluate the swelling index, the films were cut into 2 cm × 2 cm pieces and weighed (Wd). Then, these fragments were placed in beakers containing 50 mL of pH 7.4 phosphate buffer at 37 °C for 24 h (SHAHZAD et al., 2019). Afterwards, the films were removed from contact with the buffer and dried with absorbent paper, and the hydrated fragment was weighed (Ws). The swelling index was calculated following equation (2).

$$\textit{Swelling index} = [(W_s - W_d) / W_d] \times 100 \qquad \text{Eq (2)}$$

Where: W_s is the weight of the film after swelling and W_d is the weight of the dried film.

2.4 Folding endurance and mechanical properties

Folding endurance was determined by repeatedly folding the films in the same place up to 300 times (*n*=3). Then, the films were evaluated for groove formation or breakage. The mechanical properties in terms of tensile strength, deformation and Young's modulus was determined using the universal testing machine (Emic, São José dos Pinhais, Brazil), according to ASTM-D882-02 standards (ASTM, 2002). For this, film samples measuring 60 mm × 45 mm were individually fixed on the machine probe and a tensile load was applied at an initial separation of 4 cm and 50 mm/min of the cross-head speed. The maximum deformation suffered by the film was determined by the percentage change in the length of the sample in relation to its original size. The tensile strength was determined by the ratio of the force needed to rupture the film and the cross-sectional area of the strip, whereas the young's modulus was calculated by the ratio of stress and strain values.

2.5 In vitro studies

2.5.1 Occlusion test

The *in vitro* occlusion test was carried out according to our previous protocol (GEHRCKE et al., 2021). For this, a 100 mL capacity beaker containing 50 mL of water was sealed with a cellulose acetate filter (90 mm, Sterlitech, USA), which was subsequently covered with the bilayer films (*n*=3). The films were applied with the pullulan layer in contact with the filter paper. Then, the beakers were stored at 32 °C and at predetermined times (0, 6, 24 and 48 h) they were weighed for the water loss determination. A film-free beaker was used as a negative control and the occlusion factor was calculated according to equation 3.

$$\text{Occlusion factor} = [(A - B) / A] \times 100 \qquad \text{Eq (3)}$$

Where: A is the water loss of the negative control and B is the water loss in film presence.

2.5.2 Skin preparation

Human skin fragments were obtained from healthy female patients undergoing abdominoplasty surgery. Subcutaneous fat was removed and the skin was stored at -4 °C (freezer) until use. Two different skin conditions were obtained. The first condition was the whole skin (intact cutaneous tissue), with the presence of *stratum corneum*, epidermis and dermis. The second condition was skin with an impaired barrier in which the *stratum corneum* was removed by a successive tape-stripping procedure (SCHLUPP et al., 2014) using 18 pieces of adhesive tape. The protocol was approved by the research committee with humans from the Universidade Federal de Santa Maria - RS without identifying data (CAEE: 27168719.4.0000.5346).

2.5.3 Bioadhesive strength

To carry out the experiment, an adapted apparatus was used composed of two balanced arms (OSMARI et al., 2020). A plastic frame was connected to one of these arms under which the films were fixed. The skin (intact or injured) was fixed on a glass plate under the frame. The contact between the films and the skin fragment occurred by applying a weight of 1 N for 60 s. Afterwards, water was added at a constant speed of 1 drop/s in an opposite side plastic tube until the separation between skin and film occurred.

All analyzes were performed in triplicate and the volume of water used was measured in a graduated cylinder. Both the top layer (gellan gum layer) and the botton layer (pullulan layer) of the bilayer films were analyzed. The bioadhesive strength was calculated using equation 4 and the result was expressed in dyne/cm².

$$\text{Bioadhesive strength (dyne/cm}^2) = (V \times G) / A \qquad \text{Eq (4)}$$

Where: V = amount of water (g) required for the detachment between the sample and the tissue; G = acceleration of gravity (980 cm/s²); A = area of exposed tissue (cm^2).

2.5.4 SB release and skin permeation/penetration study

The *in vitro* SB release and skin permeation/penetration from films was conducted through vertical Franz diffusion cells with diffusion area of 3.14 cm^2. The receptor medium used in the assays was phosphate buffer pH 7.4 at 32 ± 0.5 °C. The films (1 cm × 1 cm)

corresponding to 440 μg of SB were placed on a dialysis membrane (10,000 Da, Sigma Aldrich) or on the skin surface with the pullulan layer in contact with the donator medium.

For the release study, at predetermined periods (1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 h), a volume of 300 μL of the receptor medium was removed and replaced by the same volume of medium fresh. The amount of SB released was determined using HPLC method described in the section 2.3. For the skin permeation study, skin samples were obtained and treated as described in the section 2.3. This experiment was performed using intact (separating the skin layers only at the end of 24 h) and injured skin (without *stratum corneum*). The circular skin fragments were placed between the donor and recipient compartments with the dermis in contact with the recipient medium. After 24 h, the films were gently removed from the skin surface, the skin was carefully removed and the receptor medium was collected. For intact skin, the *stratum corneum* was removed using 18 pieces of adhesive tape. For both intact and injured skin, the epidermis was separated from the dermis by heating the skin in ultrapure water at 60 °C for 45 s, followed by removing the epidermis with a spatula. The strips containing the *stratum corneum*, and the epidermis and dermis fragments were placed in different test tubes containing methanol and vortexed for 2 min followed by an ultrasound bath for another 30 min. The SB content in the different skin layers and in the receptor medium was determined by HPLC (section 2.3).

2.5.5 Free radical scavenging activity

The antioxidant effect of the film containing nanoencapsulated SB was evaluated through the ability to scavenge the synthetic radical 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$), as previously described by Yang et al (2019) (YANG et al., 2019), with some modifications. First, an $ABTS^+$ solution was prepared by reacting the ABTS stock solution (7 mM) with sodium persulfate (140 mM), 12 h before the assay (final $ABTS^+$ concentration 42.7 Mm). Bilayer films were cut into 0.5 cm × 0.5 cm pieces and added to tubes containing 1 ml of $ABTS^+$ solution. The tubes were mixed by inversion and incubated at room temperature for 30 min. An $ABTS^+$ solution was kept under the same reaction conditions and was used as a negative control. Blank samples containing the film fragments and water were also prepared. After incubation, the films were removed and the absorbance of solution was measured at 734 nm (UV-1800 Spectrophotometer, Shimadzu, Japan). The experiment was carried out in triplicate. Percentage radical scavenging capacity was calculated using the equation 5.

$$SC\ \% = 100\frac{(AbsA - AbsB)\ x\ 100}{\text{AbsC}}$$

Eq (5)

where: SC %: Scavenging capacity in percentage; Abs: sample absorbance; Abb: blank absorbance; Abc: negative control absorbance.

2.5.6 Hemocompatibility Evaluation of formulations

The films hemocompatibility was evaluated by direct contact test according to Standard Practice for Assessment of Hemolytic Properties of Materials (ASTM, 2020), with some modifications. For this purpose, anti-coagulated blood (9 parts of blood to 1 part of citrate) was collected from a healthy human volunteer. Then, 2 mL of the anticoagulated blood was centrifuged at 2000 rpm for 5 min, followed by discarding the plasma. The resulting pellet was washed with saline solution 3 times to completely remove plasma and obtain only erythrocytes. Afterwards, the erythrocytes were resuspended in saline and at a final concentration of 10 % (v/v). Film fragments measuring 0.5 cm × 0.5 cm were inserted into microtubes containing 700 μL of saline solution and allowed to equilibrate for about 1 h. Afterwards, 100 μL of resuspended erythrocytes were added to the tubes. Positive and negative hemolysis controls were prepared with water and saline, respectively. In addition, blanks were prepared containing the film fragments and water. The tubes were then incubated for 1 h at 37 °C. After incubation, all samples were centrifuged at 2000 rpm for 5 min and the absorbance of the supernatant was measured spectrophotometrically at 540 nm (UV-1800 Spectrophotometer, Shimadzu, Japan). The percentage of hemolysis was calculated according to equation 6. This protocol was approved by research committee with humans from the Universidade Federal de Santa Maria - RS (CAEE: 27168719.4.0000.5346).

$$\%\ \text{of hemolysis} = \left(\frac{\text{AbA-AbB}}{\text{AbC}} \times 100\right)$$

Eq (6)

where: AbA: sample absorbance; AbB: blank absorbance; AbC: positive control absorbance.

2.6 In vivo study

2.6.1 Animals

Female BALB-c mice (6-8 weeks old) were housed in a separate animal room at controlled temperature (22 ± 2 °C), under a 12/12-h light/dark cycle (the lights were turned on at 07:00 AM), with free access to standard diet and water. The experimental study was conducted according to the Ethical Research Committee of the Federal University of Pelotas, Rio Grande do Sul, Brazil and registered under the number CEEA 23357-2018/ 140-2019. The number of animals used was the minimum necessary to evaluate the consistent effects of the treatments and every effort was made to minimize their suffering.

2.6.2 Experimental design

The experimental design of this study is illustrated in Figure 1. The allergen sensitization and challenge induced by DNCB lead to the development of skin lesions similar to those of AD, as previously described by Chan et al (2013). The dorsal skin of each mouse was shaved to remove all hair from the area. In the sensitization phase, it was applied 200 µL of 0.5 v/v-% DNCB dissolved in acetone/olive oil (3:1 ratio) on the shaved area in the first three days of the experimental protocol. These animals were also challenged by applying 20 µL and 200 µl of 1.0 v/v-% DNCB on the right ear and the dorsal skin, respectively, on days 14, 17, 20, 23, 26 and 29 of the experimental protocol.

In order to evaluate the effects of free SB or bilayer films treatments on the AD-like skin lesions, mice were randomly divided into seven experimental groups (n = 7 animals/group): normal control mice were exposed to the vehicle containing acetone/olive oil (3:1) and AD-induced mice were sensitized and challenged with DNCB. All other experimental groups were sensitized and challenged with DNCB, as well as received the following treatments: the Free SB (500 µL); the bilayer vehicle film (BF vehicle) (2.5 H x 2.5 L); the bilayer film containing NC without SB (BF NC B) (2.5 H x 2.5 L), the bilayer film containing nanoencapsulated SB (BF NC SB) (2.5 H x 2.5 L) or 1% of hydrocortisone (HC) (0.5 g), as a comparative drug commonly prescribed for the AD treatment. The Free SB solution was prepared by dissolving the SB in 10 mL of acetone/olive oil (3:1).

The treatments mentioned above were applied in the dorsal region of mice and secured with a bandage starting on day 14 of the experimental protocol. At the same days of mice were challenged with DNCB (14, 17, 20, 23, 26 and 29), the films were changed. The animals were

monitored in order to ensure that the films were not removed from the application site. The treatment schedule was based on previous studies that used the same animal model and assessed the pharmacological action of films formulations (VOSS et al., 2018; WEBER et al., 2018).

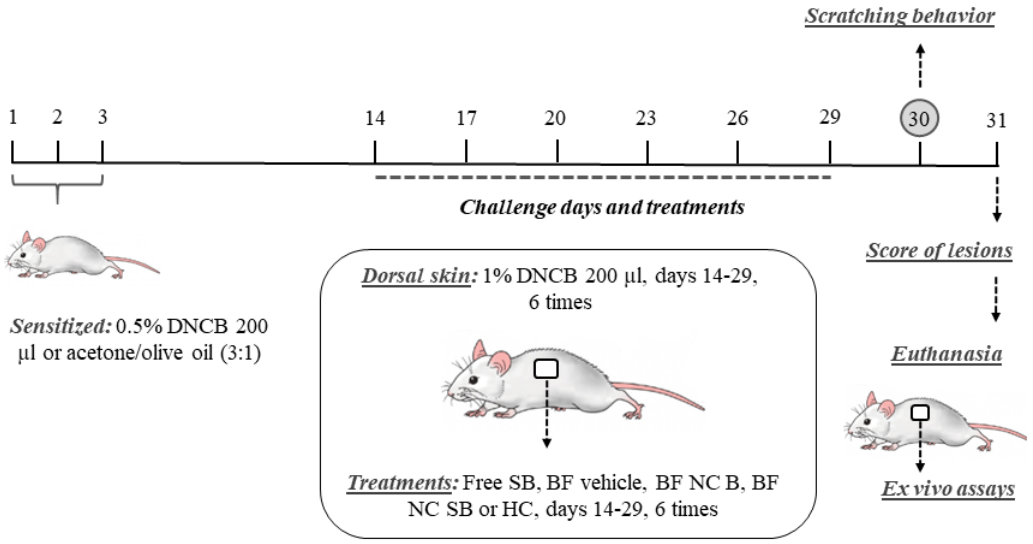

Fig. 1. Schematic representation of the experimental design of this study. DNCB (2, 4-dinitrochlorobenzene); Free SB (free silibinin solution); BF Vehicle (bilayer vehicle film); BF NC B (bilayer film containing unloaded nanocapsules); BF NC SB (bilayer film containing nanoencapsulated silibinin); HC (hydrocortisone).

Followed the last treatment, on day 30 of the experimental protocol, the scratching behavior, one of the hallmark AD-like behaviors, was evaluated in the animals. Twenty-four hours later (day 31), the clinical skin severity scores were determined to assess the manifestations of the AD-like signs in mice. After that, the animals were euthanized by inhalation of isoflurane anesthetic. After that, the samples of dorsal skin of each mouse were rapidly dissected, weighted and frozen at -20 °C to further biochemical analyses. In addition, both ears and the spleen were collected to determine the ear edema and the spleen index, respectively.

2.6.3 AD-like clinical signs

2.6.3.1 Scratching behavior

The scratching behavior, one of the classic AD-like behavior, was evaluated on day 30 of the experimental protocol. The time that mice spent rubbing the dorsal skin, ears and nose

with their hind paws was measured and recorded for 30 minutes (KIM et al., 2014). The data was expressed in seconds (s).

2.6.3.2 Clinical skin severity scores

On day 31 of the experimental protocol, the dorsal skin of mice was photographed and the skin severity scores were assessed according to the method described by Park and Oh [(2014)]. The characteristic signs of skin lesions were classified as: (1) pruritus/itching, (2) erythema/hemorrhage, (3) edema, (4) excoriation/erosion and (5) scaling/dryness. The above-mentioned signs were ranked as: 0 (no signs), 1 (mild), 2 (moderate) and 3 (severe).

2.6.4 Evaluation of the inflammation markers

2.6.4.1 Ear swelling

On day 31 of the experimental protocol, the animals were euthanized and both ears were cut at the base and weighted on the analytical balance. The ear swelling was measured by the difference between the samples of the DNCB-treated ear (right) and the control ear (left). The results were expressed in mg.

2.6.4.2 NOx content

The samples of dorsal skin were homogenized in $ZnSO_4$ (200 mM) and acetonitrile (96%). The homogenates were then centrifuged at 14,000 rpm for 30 minutes at 4ºC, and the supernatant was collected for the NOx assay. The accumulation of nitrite in the supernatant, an indicator of NO oxidation, was assessed by Griess reaction (GREEN et al., 1982). Briefly, the NOx content was estimated in a medium containing 2% vanadium chloride (in 5% HCl), 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2% sulfanilamide (in 5% HCl). After incubation at 37 ºC for 1h, the color reaction was measured spectrophotometrically at $\lambda = 540$ nm. The concentration of nitrite/nitrate in the supernatant was determined from a sodium nitrite standard curve and expressed as nmol NOx/g of tissue.

2.6.5 Evaluation of the immune function

2.6.5.1 Spleen index

On day 31, mice were sacrificed and the spleen were harvested and weighted on the analytical balance to calculate its relative weight trough the formula: *Spleen (g)/ Body weight (g)*. The results were expressed as spleen index.

2.6.6 Evaluation of the oxidative stress markers

2.6.6.1 Tissue preparation

To elucidate the involvement of oxidative stress, the samples of dorsal skin were homogenized (1:10, w/v) in 50 mM Tris-HCl at pH 7.4. The homogenates were centrifuged at 2500 rpm for 10 minutes and the low-speed supernatant (S_1) was used to determine the thiobarbituric acid reactive species (TBARS) and non-protein thiol (NPSH) levels as well as the catalase (CAT) activity.

2.6.6.2 Protein concentration

The protein concentration was estimated according to the method described by Bradford (1976), using a bovine serum albumin (1 mg/mL) as a standard. The color reaction was measured spectrophotometrically at $\lambda = 595$ nm.

2.6.6.3 TBARS levels

TBARS assay was performed to indirectly determine the malondialdehyde (MDA) levels, an important lipid peroxidation marker. As previously described by OHKAWA et al (1979), MDA reacts with 2-thiobarbituric acid (TBA) under acidic conditions and high temperatures to yield the chromogen. The S_1 aliquots were incubated with 0.8% TBA, acetic acid buffer (pH 3.4) and 8.1% sodium dodecyl sulfate (SDS) for 2 h at 95 ºC. The color reaction was measured at $\lambda = 532$ nm and the results were expressed as nmol of MDA/mg of protein, respectively.

2.6.6.4 NPSH levels

The NPSH, a non-enzymatic antioxidant defense, was determined by Ellman's method (ELLMAN, 1959). Briefly, S_1 was mixed (1:1) with 10% trichloroacetic acid. After centrifugation (3000 rpm for 10 minutes), an aliquot of supernatant containing free SH-groups was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The color reaction was measured at $\lambda = 412$ nm and NPSH levels were expressed as nmol of NPSH/g tissue.

2.6.6.5 CAT activity

CAT activity was spectrophotometrically determined by monitoring the H_2O_2 consumption at $\lambda = 240$ nm, as previously described (AEBI, 1984). In a medium containing an aliquot of S_1 and 50 mM potassium phosphate buffer pH 7.0, the enzymatic reaction was started by adding the substrate H_2O_2 (0.3 mM). The enzymatic activity was expressed as Units (1U decomposes 1 mmol of H_2O_2 per minute at pH 7.0 and 25 °C)/mg protein.

2.7 Statistical analysis

Formulations were prepared and analyzed in triplicate and the results were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). A Gaussian distribution was tested using D'Agostino normality test. For data considered parametric, a one or two-way analysis of variance (ANOVA) followed by *post-hoc* Tukey's test was performed to compare the significant difference among the experimental groups. All statistical analyses were performed using GraphPad Prism software (version 8.0, USA). Values of $p<0.05$ were considered statistically significant.

3. Results

3.1 Bilayer films presented suitable physicochemical characteristics

The bilayer films were prepared using gellan gum as the first polymeric layer forming agent and pullulan as the second layer. The prepared films presented a macroscopically homogeneous appearance. Besides, the BF NC SB was slightly whitish due the nanostructure

presence (Figure 2). Figure 2A shows the images obtained from the side sections of the films at a magnification of 300×. The bilayer formation was clearly observed, without visualization of detachment between the different layers, confirming their adhesion. Besides, the interface between the layers of vehicle film was slightly delimited, which was probably due to the high affinity existing between the gellan gum and pullulan. Whereas, for the films containing the NC, it was observed an irregular interface, probably due to the partial nanoparticles migration from the gellan gum layer to the pullulan layer during the process of the second layer formation. However, it was not possible to observe the presence of these nanostructures in the different polymeric layers at higher magnification (Figures 2B and 2C), indicating that the nanostructures are intimately embedded between the chains of the film-forming polymers.

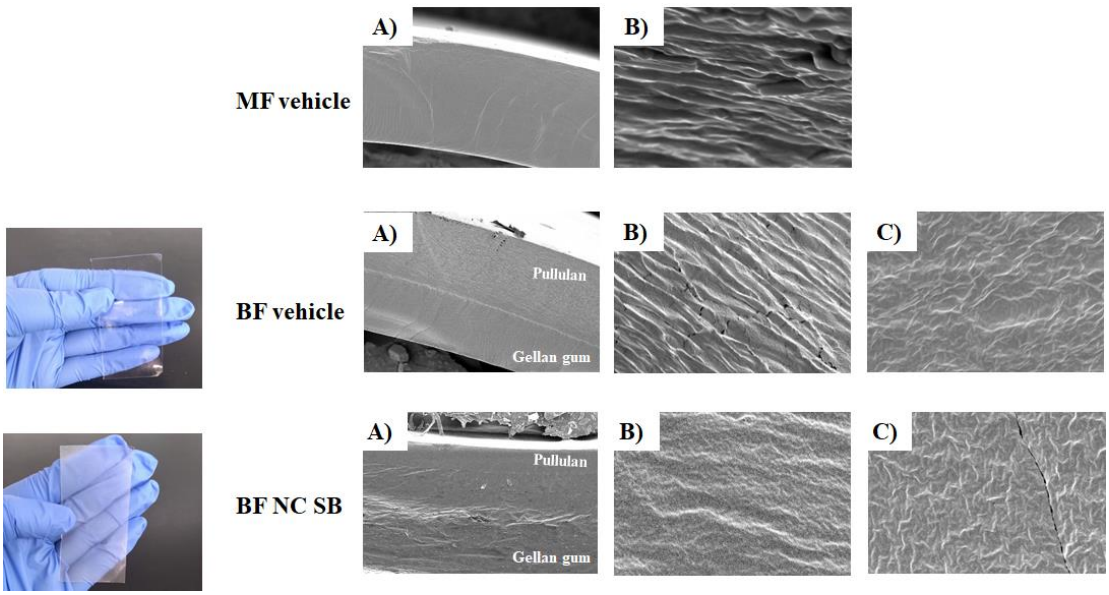

Fig. 2. Macroscopic appearance and comparative SEM images of the side sections of monolayer vehicle film (MF vehicle), bilayer vehicle film (BF vehicle) and bilayer film containing silibinin-loaded nanocapsules (BF NC SB) with magnification of 300× (A) and magnification of 5,000× on the gellan layer (B) and pullulan layer (C).

Then, bilayer films were examined for thickness, moisture, content homogeneity, swelling index and mechanical properties (Table 1). Analysis of the homogeneity of dosage and thickness were performed to ensure the consistency of dosage and film of film formation along the extended area. We found that the films had a homogeneous thickness with values close to 40 µm, as well as the film fragments presented around 440 µg/cm² of SB, which represents about 92.25 % of theoretical value (477 µg/cm²).

Table 1.

Results of bilayer films characterization.

	BF NC SB	BF NC B	BF Vehicle
Drug content homogeneity (µg/cm²)	440.61 ± 5.21	-	-
Thickness (µm)	43± 7	42 ± 5	40 ± 5
Size (nm)	117 ± 11	137 ± 15	514 ± 143
Polydispersity index	0.28 ± 0.05	0.24 ± 0.05	0.47 ± 0.11
Swelling index (%)	106.95 ± 2.56*	101.02 ± 6.45*	134.42 ± 4.78
Moisture (%)	13.89 ± 2.01	12.83 ± 1.07	14.83 ± 2.64
Tensile strength (MPa)	1.09 ± 0.03*	1.13 ± 0.09*	0.51 ± 0.05
Elongation (%)	4.93 ± 0.45 *	4.61 ± 0.18*	7.69 ± 0.15
Young's modulus (MPa)	27.25± 5.95 *	24.25± 6.57 *	7.25± 5.95

The results are expressed by mean with SD of triplicate. Asterisks denote the significant difference (*) $p<0.05$ by paired Student's t test between BF vehicle and BF NC SB or BF NC B.

After NC incorporation into bilayer film, it was observed that their nanometric size was maintained. In addition, this inclusion reduced the swelling index and deformation values of the film, as well as increased the tensile strength and Young's Modulus values ($p<0.05$). The folding endurance test was manually measured in which no film showed formation of cracks or breaks after being folded in the same place for 300 times, suggesting adequate flexibility.

3.2 Nanocapsules improved the occlusive potential of bilayer film

The result of the occlusion test is presented in table 2. The occlusion factor of nano-based films was significantly higher than BF vehicle at all analyzed time points ($p<0.001$), indicating that the incorporation of NC into the polymeric matrix increased the capacity of water retention by the film. Besides, no significant difference ($p>0.05$) was observed between films containing unloaded and the corresponding SB-loaded NC, suggesting that flavonoid encapsulation did not change the occlusion factor.

Table 2.
Occlusion factor of bilayer films.

Formulation	Occlusion factor (%)		
	6 h	24 h	48 h
BF Vehicle	20.18 ± 3.66**	28.18 ± 2.05**	29.45 ± 1.47**
BF NC SB	51.90 ± 2.01	50.16 ± 1.84	55.78 ± 2.35
BF NC B	49.50 ± 4.31	52.30 ± 6.09	50.26 ± 2.45

The results are expressed by mean with SD of triplicate. Asterisks denote the significant difference by One-way ANOVA followed by the Tukey's test. (**) $p<0.01$ between BF vehicle and BF NC SB or BF NC B.

3.3 Pullulan layer confers bioadhesion to the film

Figure 3 shows the results obtained after evaluating the bioadhesive strength of bilayer films using two skin conditions: full thickness and superficial lesion. In both skin conditions evaluated, the films showed higher values of bioadhesive strength when evaluated with the pullulan layer in contact with the skin surface ($p<0.001$). Bioadhesive strength values were reduced after the NC incorporation into films ($p<0.001$). The skin condition studied also influenced the films bioadhesion, presenting a reduction in the values of 17554 ± 1399 to 11753 ± 528 and of 10934 ± 699 to 7230 ± 863 dyne/cm^2 for BF vehicle and BF NC SB, respectively.

3.4 The BF NC SB slowly releases SB and retains it in the skin

The SB release from the films is represented by the cumulative release of substance per area as a function of time in hours (Figure 4A). The BF NC SB film presented a slow release over the period of 24 h, reaching 7.14 ± 1.34 % of SB released.

In relation to the skin permeation of SB from the bilayer film, the total SB retained (Figure 4B) on the uninjured was less than injured skin ($p<0.01$). Besides, regardless the skin condition (uninjured and injured) the SB was not detected in the receptor medium. The figures 4C and 4D show the distribution percentage of SB in different layers of skin after 24 hours of experiment for uninjured and injured human skin, respectively. In both skin conditions, SB penetrated the cutaneous tissue in quantifiable drug amounts. In uninjured skin, the flavonoid accumulated preferably in the *stratum corneum*. For the injured skin a higher SB amount in the epidermis in comparison to dermis was observed ($p<0.001$).

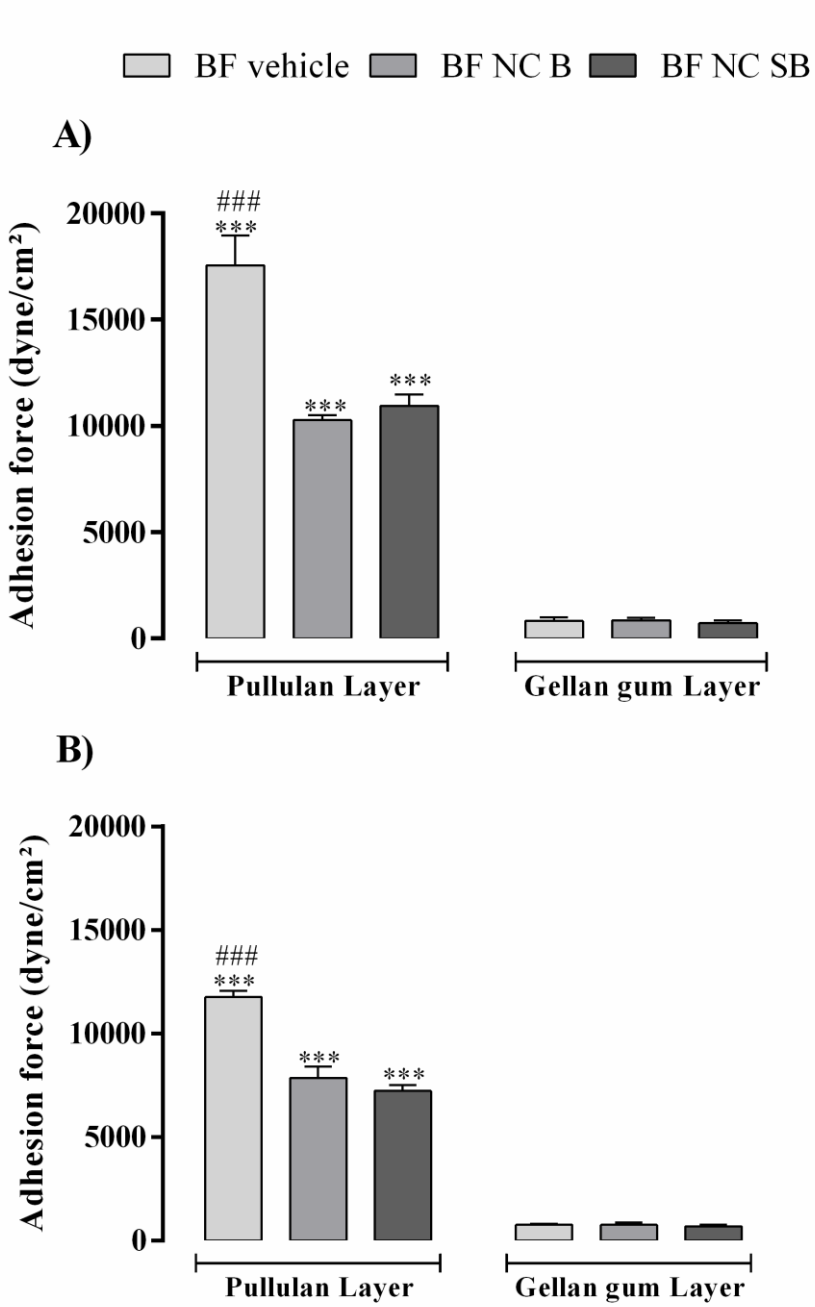

Fig. 3. *In vitro* bioadhesion evaluation using the non-injured (A) and injured (B) skin of bilayer vehicle film (BF vehicle) and films containing nanocapsules with silibinin (BF NC SB) or without (BF NC B). Each column represents the mean ± SD. The films were evaluated on both sides (pullulan layer and gellan gum layer). (***) $p<0.001$ significant differences between pullulan layer and gellan gum layer, (###) $p<0.001$ significant differences between BF vehicle and BF NC SB or BF NC B (Two-way ANOVA followed by Tukey's test).

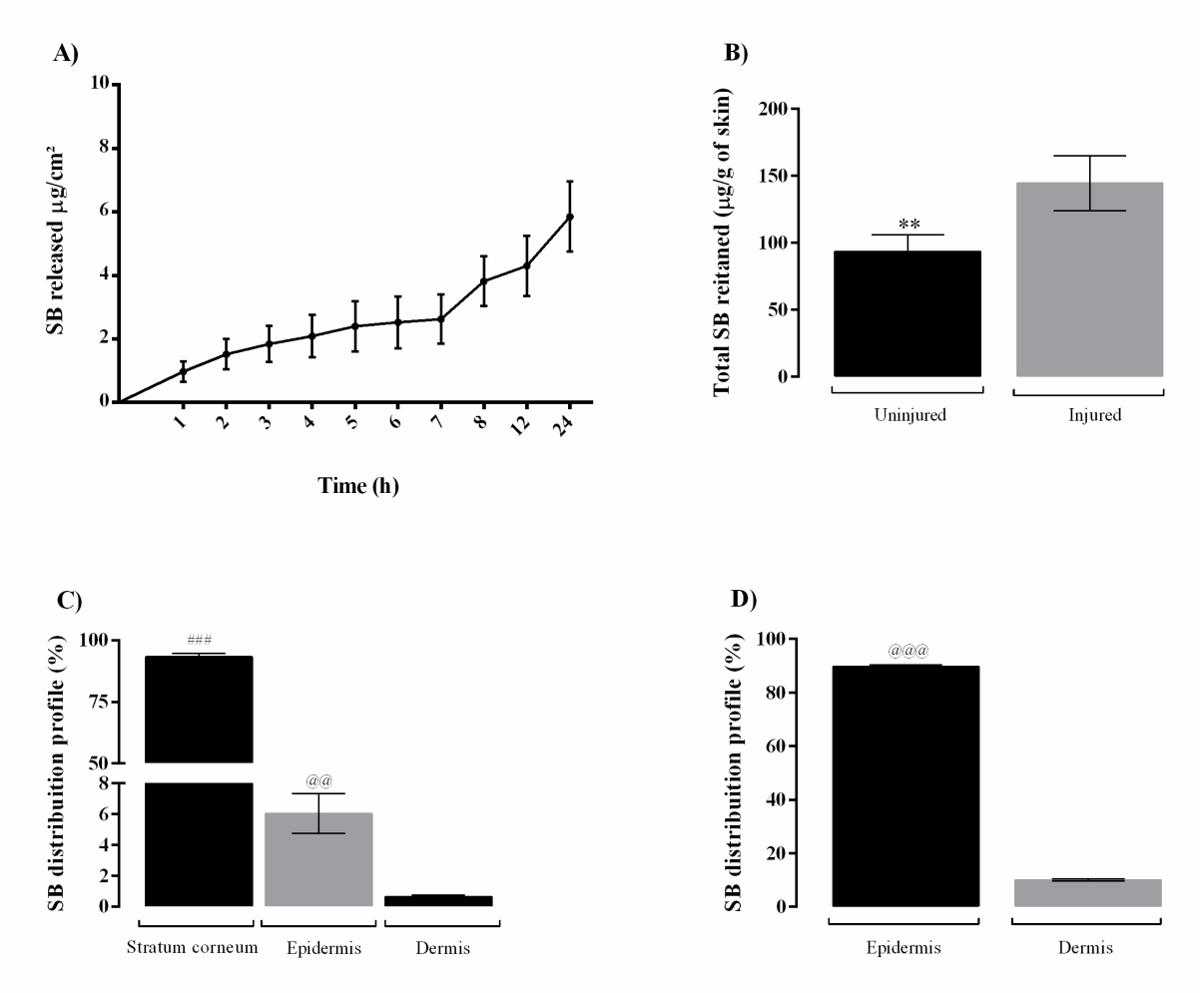

Fig. 4. *In vitro* release profile and skin permeation of silibinin from nano-based bilayer film. A) Cumulative amount of released silibinin from film in phosphate buffer receptor medium pH 7.4 at 32.0 °C (n=3); B) Cumulative amount of retained silibinin in the uninjured and injured skin after 24 h of incubation (n=6); C) Percentage of silibinin distribution in the different non-injured skin layers; D) Percentage of silibinin distribution in the different injured skin layers. All the results are expressed as mean ± SEM. (**) $p<0.01$ significant differences between cumulative amount of SB in the non-injured and injured skin, (###) $p<0.001$ significant differences between SB quantified in the *stratum corneum* and epidermis in the non-injured skin, (@@) $p<0.01$ and (@@@) $p<0.001$ significant differences between compound quantified in the epidermis and dermis in the non-injured or injured skin.

3.5 The BF NC SB neutralized the ABTS$^+$ radical

Figure 5 shows radical scavenging activity of SB-loaded NC bilayer film in comparison to vehicle and bilayer film containing unloaded NC. Corroborating the antioxidant activity of SB, the BF NC SB presented high radical scavenging activity (about 100 %). Both BF vehicle and BF NC B had a low influence on the neutralization of the ABTS$^+$ radical, demonstrating that there is no false positive in the test performed.

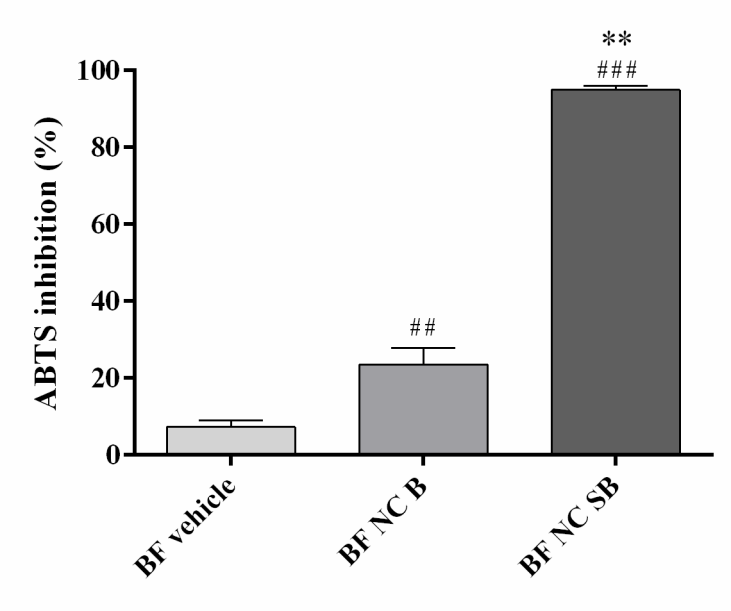

Fig. 5. Percentage of ABTS radical inhibition by films. Each column represents the mean ± SD. (**) $p<0.01$ significant differences between bilayer film containing nanocapsules with (BF NC SB) or without (BF NC B) silibinin, (##) $p<0.01$ and (###) $p<0.001$ significant differences between BF NC SB or BF NC B and BF vehicle (One-way ANOVA followed by the Tukey's test).

3.6 Bilayer films are hemocompatible

The bilayer films were evaluated for their potential to cause lysis in human erythrocytes. The percentage of hemolysis was 0.73±0.05 %, 0.56±0.23 % and 0.61±0.11 % for vehicle film, film containing unloaded NC and film containing SB-loaded NC, respectively. All these values are similar to the hemolytic percentage of saline solution (0.70±0.09 %), indicating a good blood compatibility of produced films.

3.7 The BF NC SB treatment attenuated the AD-like clinical signs induced by DNCB in mice

The figure 6 depicts the effect of free SB or BF NC SB treatments on the severity of the skin lesions and the scratching behavior in mice.

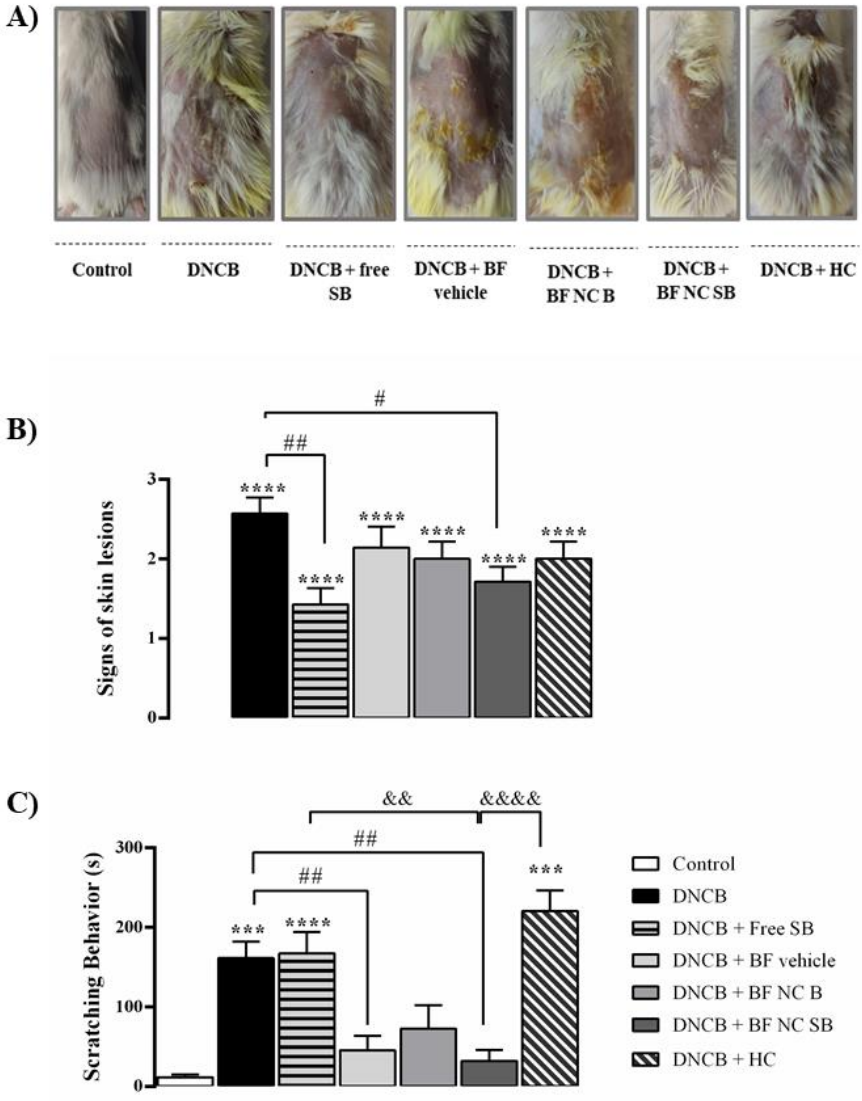

Figure 6: Effect of the different treatments on AD-like clinical signs induced by DNCB in mice. (A) Images of the skin lesions from the groups of mice taken on the last day of the experiment (day 31). (B) Score of the skin lesions. (C) Scratching time evaluated on day 30 of the experimental protocol. Each column represents the mean ± SEM of 7 mice per group. (****) $p<0.0001$ and (***) $p<0.001$ compared with the control group, (##) $p<0.01$ and (#) $p<0.05$ compared with the DNCB group, (&&&&) $p<0.0001$ and (&&) $p<0.01$ compared with the BF NC SB group (One-way ANOVA followed by the Tukey's test).

Our data demonstrated that all the animals exposed to DNCB exhibited AD-like clinical signs, represented by an increase in the clinical skin severity score when compared with the control group ($p<0.0001$). The free SB or the BF NC SB treatments markedly decreased the characteristics AD-like signs in the DNCB-exposed mice. In turn, the BF vehicle, the BF NC B and the HC did not alter the severity of lesions induced by DNCB.

The results demonstrated that the DNCB-exposed mice exhibited an increase in the scratching time when compared with the control group ($p<0.001$). In contrast, both free SB and HC treatments did not alter the scratching behavior induced by DNCB in mice ($p>0.05$). Despite the treatment with BF NC B had no statistical difference on scratching time when compared to DNCB- or vehicle-treated mice ($p>0.05$), the repeated applications of BF vehicle or BF NC SB reduced this typical AD-like behavior in the DNCB group ($p<0.01$). In fact, there is a statistically significant difference among the BF NC SB, HC, and free SB treatments, suggesting that the topical application of BF NC SB was more effective to reduce the scratching behavior in DNCB treated mice than the free SB ($p<0.01$) or HC ($p<0.0001$).

3.8 The BF NC SB treatment suppressed the ear swelling induced by DNCB in mice

The figure 7 illustrates the effect of free SB as well as all formulations containing or not SB on the development of ear swelling induced by DNCB in mice. The results evidenced that the DNCB substantially increased the ear swelling when compared with the control group ($p<0.0001$), whereas the topical application of BF vehicle, BF NC B and BF NC SB reduced the ear swelling induced by DNCB in mice ($p<0.01$). On the other side, the treatments with free SB or HC had no statistical difference on ear swelling when compared to vehicle or DNCB exposed mice ($p>0.05$).

3.9 The BF NC SB treatment did not alter the spleen index after DNCB exposure in mice

As shown in figure 8, the DNCB-exposed mice significantly increased the spleen index in comparison with the control group ($p<0.001$). Only the HC treatment attenuated the splenomegaly induced by DNCB in mice ($p<0.0001$). No statistically significant difference was evidenced in the spleen index after the topical applications of BF vehicle, the BF NC B, and the BF NC SB in the dorsal skin of mice when compared to DNCB or control groups ($p>0.05$)

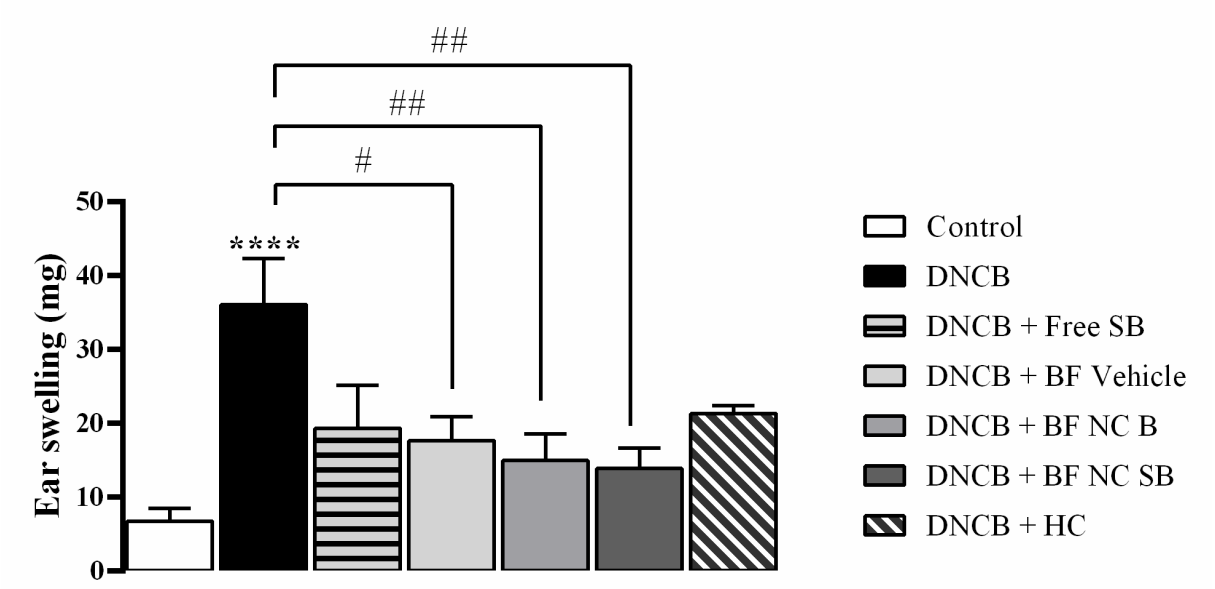

Figure 7: Effect of the different treatments on the ear swelling induced by DNCB in mice. The ear swelling was assessed on day 31 of the experimental protocol. Each column represents the mean ± SEM of 7 mice per group. (****) $p<0.0001$ compared with the control group, (##) $p<0.01$ and (#) $p<0.05$ compared with the DNCB group (One-way ANOVA followed by the Tukey's test).

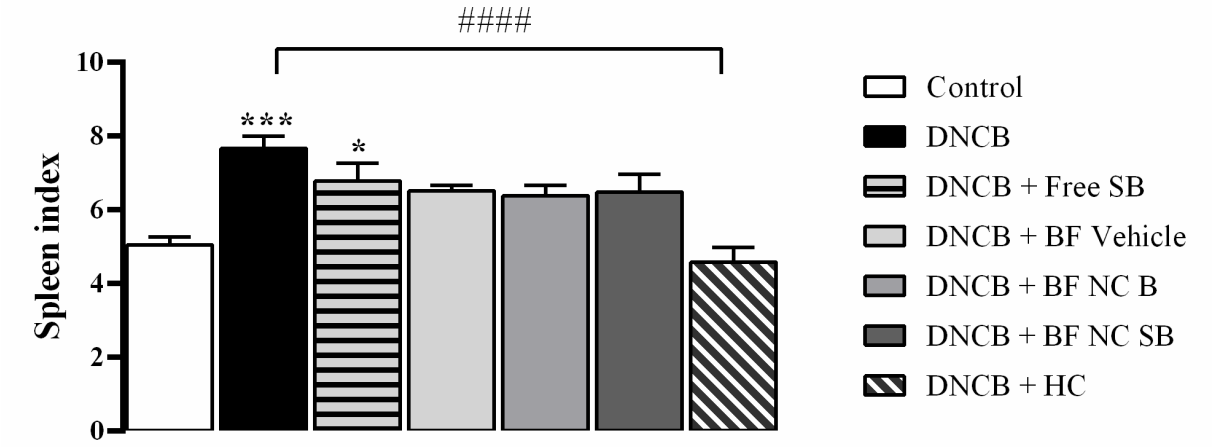

Figure 8: Effect of the different treatments on the spleen index. This parameter was assessed on day 31 of the experimental protocol. Each column represents the mean ± SEM of 7 mice per group. (***) $p<0.001$ and (*) $p<0.05$ compared with the control group, (####) $p<0.0001$ compared with the DNCB group (One-way ANOVA followed by the Tukey's test).

3.10 The BF NC SB treatment modulated some markers of oxidative stress and inflammation in the dorsal skin of mice exposed to DNCB

The dorsal skin of DNCB-treated mice exhibited a significant excessive production of NOx levels when compared with the control group ($p<0.0001$), as shown in Figure 9. The topical applications of free SB, BF NC SB and HC reduced the NOx levels in the dorsal skin of DNCB exposed mice ($p<0.01$) whereas the treatments with BF vehicle or BF NC B had no statistical difference on the NOx levels in the dorsal skin of mice when compared to vehicle or DNCB groups ($p>0.05$).

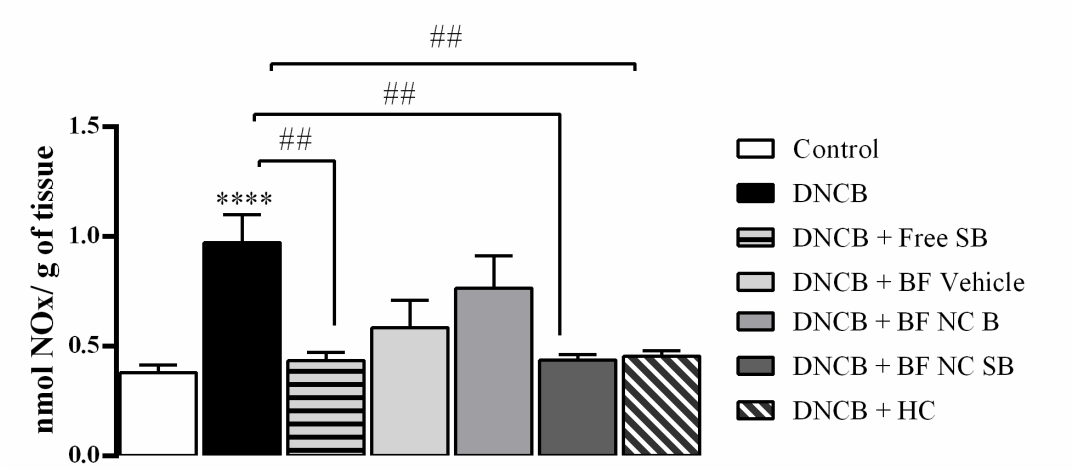

Figure 9: Effect of the different treatments on the NOx levels in the dorsal skin of mice exposed to DNCB. Each column represents the mean ± SEM of 7 mice per group. (****) $p<0.0001$ compared with the control group and (##) $p<0.01$ compared with the DNCB group (One-way ANOVA followed by the Tukey's test).

The figure 10 summarizes the results regarding the effect of the film formulations in some oxidative stress parameters in the dorsal skin of mice exposed to DNCB. In relation to the control group, the DNCB exposure led to an enhancement of TBARS levels in the dorsal skin of mice ($p<0.05$). In turn, the treatments with BF vehicle, BF NC B, as well as BF NC SB reduced the TBARS levels in animals exposed to DNCB ($p<0.0001$). The topical applications of free SB or HC did not alter the TBARS levels in the dorsal skin when compared to vehicle or DNCB groups ($p>0.05$). In this line, the BF NC SB treatment was statistically more effective in reducing the TBARS levels in the dorsal skin of mice exposed to DNCB than the free SB or HC ($p<0.01$) (figure 10A).

Regarding the non-enzymatic antioxidant defenses, the repeated applications of BF NC SB increased the levels of NPSH in the dorsal skin of mice in relation to the control group ($p<0.05$) whereas the statistical analysis also revealed similar NPSH levels in the dorsal skin of mice among all other experimental groups ($p>0.05$) (figure 10B). Moreover, the animals exposed to DNCB presented an inhibition of CAT activity in the dorsal skin when compared with the control group ($p<0.0001$). All the formulations tested, as well as the positive control (HC) were unable to restore the CAT activity at the control levels in the dorsal skin of DNCB-treated mice ($p>0.05$) (figure 10C).

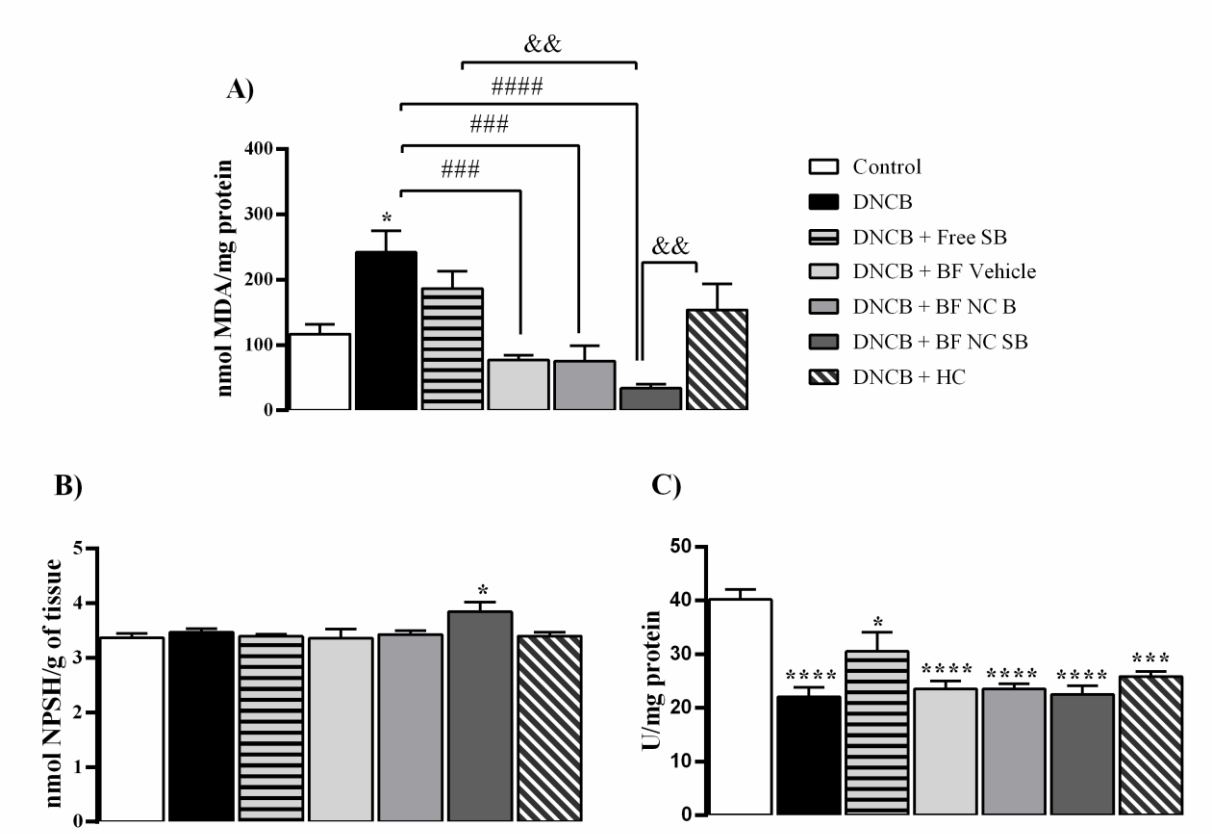

Figure 10: Effect of the different treatments on the levels of TBARS (A) and of NPSH (B) as well as on the CAT activity (C) in the dorsal skin of mice exposed to DNCB. Each column represents the mean ± SEM of 7 mice per group. (****) $p<0.0001$, (***) $p<0.001$ and (*) $p<0.05$ compared with the control group, (###) $p<0.001$ and (##) $p<0.01$ and (#) $p<0.05$ compared with the DNCB group, (&&) $p<0.01$ compared with the BF NC SB group (One-way ANOVA followed by the Tukey's test).

4. Discussion

Aiming to suppress the clinical AD symptoms, this study was designed such that the immediate layer composed by pullulan could provide an adhesion to the cutaneous tissue, whilst a gellan gum layer containing SB-loaded NC provides a therapeutic effect in sustained manner. For this, firstly, the bilayer films were produced by applying a second pullulan layer on top of the first layer composed by gellan gum incorporated with SB-loaded NC using two-step solvent casting method. Micrographs of side sections of produced films exhibited clearly the formation of two polymeric layers, which seem to be adhered to each other, without gap signals.

After drying, is important to assure the homogeneity of the final formulation, since during this process may be observed agglomeration or sedimentation of solid particles or air bubbles on the surface, leading to homogeneity problems (KARKI et al., 2016). The bilayer films produced presented a thin and homogeneous thickness, which is required for cutaneous application. In addition, the SB content was in the range of 85 to 115% recommended for polymeric films (DIXIT; PUTHLI, 2009), as well as this flavonoid was evenly distributed throughout the film as demonstrated by the content uniformity test. The residual water content in the films was less than 15 % for all films, corroborating the previous reports for films produced by the same technique (GEHRCKE et al., 2021; MAHMOOD et al., 2021). All these evaluations indicate that the casting solvent method employed to produce the bilayer films was successful.

In the SEM analysis, although a difference was observed between the microstructure of vehicle film and of nano-based film, it was not possible to visualize the NC. However, PCS analysis indicated the nanometric size maintenance of particles in the final dosage form, suggesting that the NC are intimately inserted between the polymeric chains. The nanoparticles presence in the polymeric film network has already been suggested in others researches involving polymeric films containing nanostructured systems (CERVI et al., 2021; MACHADO et al., 2016). This insertion may restrict the free movement of polymer chains (RAZALI et al., 2020). In fact, our results demonstrated that the films containing NC showed higher young's modulus values and lower swelling index than vehicle film, indicating a reduction of both mobility and relaxation of the polymeric chains, making the film stiffer and less susceptible to penetration by fluids. However, all the produced bilayer films showed fluid absorption capacity, as well as remained intact after 24 h in contact with the buffer, suggesting that they are a resistant material for the exudative lesions treatment. This result may be due to the presence of the gellan gum layer in the film, which forms swellable and resistant films,

providing replaced less frequently in the lesion site (ARIFAH et al., 2019; ISMAIL et al., 2019; SEBRI; AMIN, 2016). Moreover, the young's modulus obtained for the developed films have values within of the young's modulus range of skin, which can vary between 0.02 and 57 MPa (EVANS et al., 2013).

The formation of two distinct layers in the film was also confirmed through the bioadhesion test, in which the layer composed by pullulan presented values of bioadhesive strength greater than the layer composed by gellan gum. In fact, pullulan-based formulations have already been reported in the literature with bioadhesive properties (CERVI et al., 2022; LIMA et al., 2017). The bioadhesion can contribute to an intimate contact of the pharmaceutical dosage form with the skin, increasing the residence time at the action site and decreasing the administration frequency. Besides, the use of bioadhesive films may reduce the use of adhesive tapes for their application, which are constantly associated with greater skin irritation and pain (PAGANO et al., 2019). Considering that AD is characterized by a loss of skin barrier function, mainly due to a damage to the lipids of the *stratum corneum* (KIM; LEUNG, 2018), the bioadhesion was also evaluated using an injured skin model (without *stratum corneum*). The films bioadhesive potential was lower when in contact with the injured skin. Similar results were observed for hydrogels containing β-caryophyllene nanoemulsions (PARISOTTO-PETERLE et al., 2020). Besides, the bioadhesion values of nano-based films were lower than vehicle film in both uninjured and injured skin conditions. In fact, it was observed an irregular interface in the SEM images for film containing NC. This result reinforces our argument that NC migrate from the gellan gum layer to the pullulan layer during the film formation.

Distinct theories are used to describe the bioadhesion. Among these theories is adsorption, in which the bioadhesiveness between a substance and a tissue results from van der Waals, hydrophobic or hydrogen bond interactions (MANSURI et al., 2016). The hydrophilic groups of pullulan may be preferentially oriented towards the inside of the film interacting with glycerol used as plasticizers, as showed in others studies involving hydrophilic polymers-based films (PAGANO et al., 2019, 2020). Thus, the film surface may become slightly more hydrophobic, favoring its interaction with lipophilic surface of *stratum corneum* in uninjured skin. However, simulating a skin damage condition through *stratum corneum* removal occurs viable epidermis exposure, which is less lipophilic than *stratum corneum*, reducing the pullulan interaction. This greater bioadhesion in the intact skin than in the injured skin can be beneficial, since during the film peeling from skin, the lesion area will not be more damaged. In addition, the NC inclusion in polymeric chains may be masking the chemical groups in pullulan that interact with the skin, resulting in lower bioadhesion. In fact, in others studies were also

observed a bioadhesive reduction of hydrophilic polymers after inclusion of solid substances into films (PAGANO et al., 2019; TIMUR et al., 2019).

The skin barrier impairment in patients with AD leads to the transepidermal water loss, as well as the drug permeability barrier is diminished in these patients, increasing the risk of systemic drug absorption (DANBY et al., 2022). The *in vivo* skin hydration can be correlated with *in vitro* occlusion factor in a linear form. In other words, the greater the occlusion factor, the greater the cutaneous hydration observed *in vivo* (MONTENEGRO et al., 2017). Our results confirm the higher occlusive effect provided by nanostructures, as previously reported in other studies (CERVI et al., 2021; PEREIRA et al., 2016). This result points out that nano-based films are promising in the design of novel treatments intended for improving skin hydration.

Regarding permeation study, in the intact skin, the BF NC SB providing a SB deposition in higher amounts in the *stratum corneum* followed by its delivery on epidermis and dermis. This find is in line with the cutaneous permeation mechanism observed for polymeric nanoparticles reported in the literature (ABDEL-MOTTALEB and LAMPRECHT, 2016; POHLMANN et al. 2016). As expected, after removing the main barrier to substances penetration across the skin, an increase in the SB accumulation in the epidermis and dermis was observed. However, despite the SB amount increased in the injured skin, no amount of this flavonoid was detected in the receptor medium. This affinity for cutaneous is advantageous for AD treatment because favors a site-specific therapeutic response, without systemic absorption (HUSSAIN et al. 2013). In addition, *in vitro* release test evidenced a sustained SB release from the nano-based film. This controlled release may favor the SB delivery locally in the skin in a well-controlled manner, allowing a less frequent film replacement and avoiding greater local irritation and the risk of injured area contamination (HUSSAIN et al., 2013a; SHAHZAD et al., 2019).

Previously published data showed that gellan gum films had young's modulus about three times greater and occlusion factor about twice lower than bilayer films produced (GEHRCKE et al., 2021). Besides, this same gellan gum film presented SB amount released from nano-based film around 5 % in 24 h under the same conditions, as well as similar SB distribution profile in the different skin layers to that found for bilayer films. The improvement of young's modulus and occlusive properties observed here are in line with described in the literature for bilayer films. This type of film presents heterogeneous structures, taking advantage of the best characteristics of each individual polymer, and thus, improving the physical and mechanical properties of the final film (NETO et al., 2019). Besides, pullulan is highly hydrophilic, forming a fast-dissolving film (CERVI et al., 2022). Thus, it is possible to

infer that the pullulan layer addition confers an increase in bioadhesion and occlusion values, without altering the release and skin permeation profile of SB.

Flavonoids have antioxidant and anti-inflammatory effects which may ameliorate signs of allergic diseases, including AD (WU et al., 2021). Besides, studies describe that the beneficial effects of SB in skin pathologies are mainly due to its action in preventing the generation of oxidative stress. Thus, a preliminary *in vitro* evaluation of the SB antioxidant effect from the nano-based bilayer films produced was performed. The results showed a high capacity of BF NC SB to neutralize the $ABTS^{+}$ radical, corroborating others studies that evaluated the antioxidant effect of this flavonoid using this same synthetic radical (KHELIFI et al., 2020; YANG et al., 2019). Next, the hemocompatibility of the developed bilayer films was determined in order to assess their safety when in contact with red blood cells, since AD lesions may bleed. Our results showed a hemolytic index less than 1 % for all the bilayer films. According to the Standard Practice for Assessment of Hemolytic Properties of Materials, materials that cause 0-2 % hemolysis are considered non-hemolytic and safe (ASTM, 2020).

Given the *in vitro* results obtained, it was performed a pre-clinical study to evidence the therapeutic efficacy of bilayer films against AD using hapten-induced mice. AD is characterized by itchy, red and swollen skin due to chronic inflammation and immune dysregulation. Multiple challenges with haptens, such as DNCB, into the skin of mice mimics the ongoing maintenance and the progression of AD. Thereby, the epicutaneous sensitizer DNCB is commonly used to induce a chemical animal model of dermatitis (KABASHIMA; NOMURA, 2017). The present study reinforces that DNCB model reproduced the typical AD-like signs, as evidenced by an increase in the severity of skin lesions and the scratching behavior. Considered a typical AD symptom, scratching could be responsible for trigger a physical injury to epidermis, as well as aggravate the inflammatory processes. In this line, it is recognized as an essential and a skin specific behavior related to itching (TANG et al., 2020; XU et al., 2018).

Immunologically, it has been reported that haptens exposure for an extend period evoke primarily the release of T helper 1 (Th1) cytokines that shift to a delayed chronic Th2-dominated inflammatory response, that it is similar to human AD. In this context, the immunological and inflammatory process is closely implicated in the pathogenesis of AD (SAINI and PANSARE, 2021; WU et al., 2021). Some studies established that the local inflammation and the skin irritation induced by the external application of DNCB could also result in ear edema, probably due to an increase in the vascular permeability (KIM et al., 2014; WEBER et al., 2018). As expected, our results showed that repeated DNCB exposure promoted

a marked ear swelling, as well as an enhancement in the spleen index and the NOx levels in the dorsal skin of mice. Interestingly, the spleen index, known as a peripheral immune organ, may be reflected the stimulation of immunological responses by activating T-lymphocytes (TANG et al., 2020). Besides, it is well known that cytokines may lead to an excessive production of NO which aggravate the inflammatory response and consequently sustain the local tissue injury (FAN et al., 2021; ORITA et al., 2011).

Previous studies have shown that melanocytes and keratinocytes, some types of skin cells, generates RS (PELLE et al., 2005; RODRIGUEZ et al., 2013). Similarly, the immune cells response and the release of inflammatory mediators are often associated with an increase in the production of oxidative molecules and reactive free radicals (STEULLET et al., 2016). Indeed, an overproduction of NO levels lead to the development of oxidative stress that is also involved as a pathological aspect of AD (LI et al., 2016; WINK et al., 2011). In agreement with those findings, our results showed that repeated DNCB challenges inhibited the CAT activity and increased the TBARS levels, indicating the establishment of oxidative stress in the dorsal skin of mice. Indeed, these data reaffirm that the multifaceted interactions among the inflammatory mediators, the immune cells and the oxidative stress favor the maintenance of skin lesion like-AD.

Although topical corticosteroids are usually recommended as the standard anti-inflammatory treatment to alleviate the severe sings related to AD, their long-term use results in the manifestation of innumerous adverse effects, such as skin atrophy, purpura, dyspigmentation and declined immune function (BARNES; KAYA; ROLLASON, 2015). Consistent with these findings, our results revealed that mice treated with HC exhibited skin lesions like-AD, scratching behavior, a reduction in the spleen index and in the NOx levels on the local injury in mice. This data agrees with the fact that the long-term steroids use lead to the development of skin thinning, cracking or bleeding, as well as, immunosuppression (SLATER; MORRELL, 2015).

All the bilayer films presented a significant reduction of scratching behavior, ear edema and TBARS levels whereas the other treatments did not reduce these parameters. It is known that the skin hydration is related to reduction of pruritus and skin barrier recovery, reducing the AD aggravation (DANBY et al., 2022). Consistent with this information, the *in vitro* results showed the bioadhesive and occlusive potential of films, suggesting that they may adhere to skin, improving it hydration. Similar result was obtained by Jeong and co-workers in which pullulan films with and without *Rhus verniciflua* extract ameliorated the AD-like sings as epidermal thickness and reduced the cell infiltration, suggesting that pullulan itself suppress

AD development (JEONG et al., 2019). In fact, the pullulan layer was applied in contact with mice skin surface to act with bioadhesive function, while the gellan gum layer could act as external barrier against aggression and as vehicle for SB-loaded NC. In addition, both pullulan and gellan gum layers were plasticized with glycerol, a humectant used in dermatological formulations. Beyond to act as humectant, glycerol appears to accelerate the skin barrier repair after its disruption (ATRUX-TALLAU et al., 2010; DANBY et al., 2022). Thus, we speculate that bilayer films components may be promoting a physical barrier against scratches and other sensitizing agents, improving the hydration and restoring the skin's barrier function.

Moreover, the current study demonstrated that free SB, BF NC SB and HC treatment reduced the NOx levels in the dorsal skin of mice after DNCB exposure. Considering the involvement of NO in inflammatory conditions, we speculated that a decrease in the NOx levels might trigger, at least in part, a decrease in vascular permeability as well as a lower production of cytokines and prostaglandins, thus decreasing the severity of the inflammatory process on the local injury (McDANIEL et al., 1996). NO, a highly reactive free radical, also contribute to oxidative damage that occur by lipid peroxidation and oxidation of proteins and thiols (LUPERCHIO et al.,1996; WINK et al., 2011).

SB potentiates the action of a range of antioxidant enzymes and molecules, including CAT and glutathione (PYSZKOVÁ et al., 2016; SURAI, 2015). In our study, although CAT levels were not altered by treatment with SB formulations, the BF NC SB treatment increased the levels of NPSH in the dorsal skin. Tripeptide glutathione (GSH), the major non-protein thiol quantified in the NPSH assay, plays an essential role in the cellular redox homeostasis, protecting organelles from oxidative damage and inflammatory cascade (WINTERBOURN, 2016). In this sense, our findings suggest that the BF NC SB enhanced the GSH production which led to an increase in the NPSH levels in an attempt to counteract the lipid peroxidation and the oxidative damage in the dorsal skin of DNCB exposed mice.

Our *in vivo* results confirmed the benefits of SB nanoencapsulation since topical applications of free SB alleviated the AD-like signs and NOx levels in mice whereas the BF NC SB decreased the incidence of the skin lesions score, the scratching behavior, the ear swelling and oxidative parameters. We believe that NC could increase the residence time of SB in the cutaneous tissue, and thus, exert a better antioxidant performance. In line with these findings, previous studies reported the promising anti-inflammatory effect of hydrogels containing nanoencapsulated SB in a model of contact dermatitis induced by croton oil (RIGON et al., 2019) or DNCB (SHROTRIYA; VIDHATE; SHUKLA, 2017). In addition, specifically for AD, the better performance of polymeric nanoparticles in attenuating the inflammatory and

immunological effects of the disease is well documented in the literature when compared to non-nanoencapsulated substances (HUSSAIN et al., 2013b; LIN et al., 2021). Recently our research group demonstrated that pullulan films incorporated with pomegranate seed oil NC presented better biological effects than this free vegetable oil or associated into nanoemulsions against the same mice model of AD used here, highlighting the superiority of NC formulations (CERVI et al., 2021).

Collectively the results obtained in *in vitro* and *in vivo* evaluations suggest that bilayer films containing SB-loaded NC may exert a physical barrier action in AD treatment, as cutaneous bioadhesion, prevention of skin dryness, protection of lesions and ability to absorb exudates, followed by therapeutic action, with sustained and localized SB release and improved antioxidant effect. In this sense, the results obtained here encourage further investigations involving pullulan/gellan gum bilayer film as vehicle for AD control, as well as the combination of NC into films to improve the therapeutic performance. Furthermore, it is important to mention that, to our knowledge, this is the first study where a bilayer film based on gellan gum and pullulan containing nanostructures was produced, characterized and assessed against the AD injuries in a pre-clinical study.

5. Conclusions

This study demonstrated a facile prepare of gellan gum/pullulan bilayer, where the top pullulan layer was able to ensure a good bioadhesion to the skin while the botton gellan gum layer has swelling capacity and acts as a vehicle for the release of SB-loaded NC. The *in vitro* studies showed that nano-based film presented higher occlusion factor. Besides, this film released SB in a slow and gradual manner and allowed the retention of this flavonoid in the skin tissue. Also, SB-loaded NC incorporated into bilayer films presented high scavenger capacity and did not present hemolytic degree. The *in vivo* study provided evidence that topical applications of BF NC SB attenuated the AD-like skin lesions, the scratching behavior and the ear edema by modulating some markers related to redox signaling and the inflammatory process. Interestingly, the bilayer film without SB presence attenuated the scratching behavior, the ear edema and TBARS levels, suggesting that the novel bilayer vehicle film might also provide benefits to treat atopic skin. It should be noted that the efficacy of BF NC SB treatment on behavioral and biochemical parameters was similar or better than the free SB solution, the classical treatment (HC) or bilayer films without SB. In this scenario, our data reinforce that the combination of NC and films could be applied to enhance the performance of antioxidant

substances in skin disorders treatment. Particularly, we highlighted that the gellan gum/pullulan bilayer film containing SB-loaded NC might combine skin protection and hydration effects with improved anti-inflammatory and antioxidant effects, being a promising and potential therapeutic alternative for AD treatment.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Mailine Gehrcke: Methodology, Data curation, Formal analysis, Investigation, Writing original draft, Writing – review & editing, Visualization, Conceptualization. **Carolina Cristovão Martins:** Methodology, Data curation, Conceptualization, Writing – review & editing. **Taíne de Bastos Brum:** Methodology, Data curation, Investigation, Writing – review & editing. **Lucas Saldanha da Rosa:** Methodology, Writing – review & editing. **Fabio Zovico Maxnuck Soares:** Methodology, Conceptualization, Writing – review & editing. **Cristiane Luchese:** Data curation, Writing – review & editing. **Ethel Antunes Wilhelm:** Data curation, Writing – review & editing. **Letícia Cruz:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

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4 DISCUSSÃO GERAL

A crescente busca por novas plataformas de administração tópica de fármacos é justificada pelas limitações intrínsecas das formas farmacêuticas disponíveis clinicamente, dentre as quais destacam-se as aplicações mais de uma vez ao dia e toxicidade local ou sistêmica dos fármacos utilizados. Como forma de contornar esta questão, a nanotecnologia tem demonstrado ser uma estratégia eficaz, por ser capaz de promover o controle de liberação e aumento da retenção cutânea de substâncias ativas, contribuindo para uma melhor performance terapêutica e redução de efeitos adversos (PAREKH et al., 2021). Ainda, a fim de viabilizar a aplicação dos sistemas nanoestruturados sobre a pele, diferentes formas farmacêuticas têm sido exploradas, as quais, dependendo da composição, podem contribuir para a melhora da aparência da pele (BOISGARD et al., 2017; HUSSAIN et al., 2014). No caso específico da dermatite atópica, formulações com componentes bioadesivos, oclusivos e/ou umectantes podem atuar de forma a restaurar a barreira cutânea, a qual está alterada nesta doença, aumentando a hidratação das camadas profundas da pele e reduzindo a progressão dos processos oxidativos, inflamatórios e imunológicos desenvolvidos no curso da doença (DANBY et al., 2022). Assim, a combinação de nanoestruturas em filmes poliméricos surge como uma promissora estratégia no tratamento da dermatite atópica, pois une as potencialidades da nanotecnologia com as vantagens dos filmes em proporcionar fácil aplicação, adesão à pele de forma a protegê-la e aumentar a sua hidratação. Dessa forma, o **capítulo 1** trouxe uma detalhada revisão de estudos prévios envolvendo os benefícios das nanoestruturas para o tratamento da dermatite atópica, bem como uma reflexão a respeito de estratégias que possam viabilizar a administração tópica destas, e que também contribuam no tratamento da pele atópica.

Somado ao desenvolvimento de novos sistemas de administração de fármacos, a busca por novas moléculas terapêuticas para tratar doenças de pele também vem sendo alvo de diversas pesquisas. Dentre a novas moléculas estudadas estão as com características antioxidantes, como a SB, uma flavoglicana que além de atenuar processos oxidativos, possui potencial anti-inflamatório e imunomodulador (CASTELLANETA et al., 2016; SAMANTA et al., 2016; SHROTRIYA; VIDHATE; SHUKLA, 2017). A SB é uma molécula que tem sido beneficiada pela nanotecnologia devido a sua limitada solubilidade e estabilidade. Identificando estes benefícios, nosso grupo de pesquisa vem se dedicando em estudar a associação deste polifenol em formulações de base nanotecnológica para tratar doenças inflamatórias cutâneas, sendo o pioneiro na incorporação da SB em nanocápsulas poliméricas. Nestes estudos do nosso grupo, a SB foi encapsulada nestes sistemas associada ao óleo de romã, o qual é rico em substâncias antioxidantes e anti-inflamatórias (MARCHIORI et al., 2017a, 2017b; RIGON et al., 2019). Dessa forma, torna-se relevante estudar um óleo quimicamente inerte para o

desenvolvimento de nanocápsulas contendo SB, a fim de melhor elucidar as suas propriedades quando associada a este nanocarreador.

Frente a isso, para o desenvolvimento desta tese, primeiramente foi verificada a viabilidade de associar a SB a nanocápsulas de triglicerídeos de cadeia média utilizando três diferentes polímeros: 1) etilicelulose, polímero utilizado nos trabalhos anteriormente publicados em nosso grupo; 2) Eudragit® RS 100, polímero catiônico e com propriedade bioadesiva; 3) poli (ε-caprolactona), polímero aniônico e com natureza biodegradável. O primeiro conjunto de resultados da presente tese demonstrou que as suspensões de nanocápsulas produzidas com os diferentes polímeros apresentaram características compatíveis com sistemas nanoestruturados produzidos com os seus respectivos polímeros, demonstrando a compatibilidade entre a SB e os constituintes utilizados para o preparo das suspensões de nanocápsulas.

Na sequência, buscou-se estudar a incorporação das nanocápsulas de SB desenvolvidas em filmes poliméricos, como uma nova plataforma de administração cutânea do nanocarreador. Para o desenvolvimento dos filmes, foi proposta a utilização da goma gelana como agente filmógeno, por ser um polissacarídeo natural, atóxico e com promissora característica formadora de filme para uso cutâneo (ARIFAH et al., 2019; MAHMOOD et al., 2021; RAZALI et al., 2020). Nos estudos de pré-formulação foi demonstrada, de forma qualitativa, a escolha do tipo de plastificante e sua concentração, e a escolha da melhor concentração de goma gelana para dar continuidade ao estudo. Com base nos testes realizados, os filmes foram preparados utilizando goma gelana na concentração de 1 % e glicerol na concentração de 4 %. Ainda, com o intuito de verificar as potencialidades da nanoencapsulação da SB, este flavonoide também foi incorporado a filmes de goma gelana na sua forma livre. Para obtenção desta formulação foi requerida a adição de DMSO (2,5 mL). No entanto, a incorporação das nanocápsulas nos filmes de goma gelana só foi bem-sucedida para aquelas preparadas com etilcelulose. Para as nanocápsulas preparadas com Eudragit® RS 100, parecia ocorrer uma desestabilização do nanossistema ao misturá-lo com a dispersão de goma gelana, possivelmente pela diferença das cargas catiônicas do Eudragit e aniônica da goma. Já para as nanocápsulas preparadas com poli (ε-caprolactona), possivelmente ocorriam incompatibilidades com os constituintes do filme (goma e glicerol), pois, após a secagem, eram vistos grumos poliméricos, provavelmente devido à aglomeração das partículas ou desestruturação do nanocarreador. Deste modo, no **artigo 1** desta tese foram detalhados o desenvolvimento de filmes de goma gelana contendo nanocápsulas de etilcelulose e triglicerídeos de cadeia média.

Um filme polimérico para administração de fármacos deve possuir características compatíveis com a aplicação pretendida. Em geral, os filmes devem possuir flexibilidade, maciez e elasticidade para serem manuseados e aplicados facilmente. No entanto, estas características devem ser controladas, uma vez que filmes muito elásticos podem apresentar variações de dose durante corte ou manuseio (KARKI et al., 2016). Ainda, os filmes devem possuir boa estabilidade e apresentar peso, espessura e conteúdo de fármaco uniformes, tanto a cada lote de preparo, quanto em diferentes pontos de um mesmo filme (WASILEWSKA; WINNICKA, 2019). Dessa forma, os filmes preparados no **artigo 1** desta tese foram cuidadosamente caracterizados, demonstrando possuir adequada flexibilidade, transparência, espessura fina e homogênea, capacidade de intumescimento e alta resistência a fluidos biológicos, sendo promissores para atuar como barreira física sobre a pele lesionada, principalmente quando há formação de exsudatos. Ainda, para analisar se a SB estava homogeneamente distribuída ao longo da forma farmacêutica, foi realizada a quantificação desta em 3 pontos diferentes do filme, em 3 diferentes lotes de filme. Como resultado, o filme contendo a SB nanoencapsulada demonstrou uma distribuição mais homogênea deste flavonoide lipofílico ao longo do filme, quando comparado ao filme de SB na sua forma livre.

A combinação de nanocápsulas com filmes de goma gelana proporcionou a formação de uma forma farmacêutica sólida com potencial oclusivo, estável durante o armazenamento e de liberação controlada. Esta última, foi avaliada em um ensaio de liberação utilizando células de *Franz* e como meios de liberação foram escolhidos o tampão fosfato pH 7,4, a fim de simular condições fisiológicas, e o mesmo tampão acrescido de etanol (70:30) para aumentar a solubilidade da SB no meio e atender a condição *sink*. A utilização do etanol para a manutenção da condição *sink* já foi descrita por outros autores (MELERO et al., 2014; MARCHIORI et al., 2017b; RIGON et al., 2019). O pH 7,4 foi escolhido tendo em vista que quando há comprometimento da barreira cutânea ocorre uma tendência à alcalinização do local acometido, principalmente quando há extravasamento sanguíneo ou formação de exsudatos (DEVORE et al., 2020; HÜLPÜSCH et al., 2020; SCHMID-WENDTNER; KORTING, 2006; SHAHZAD et al., 2019; TANG et al., 2021).

Ainda no contexto da administração cutânea, estudou-se a distribuição da SB nas camadas da pele. O método para quantificação da SB na pele foi linear (r=0,998) e específico, ou seja, não houve interferência dos componentes da pele nem da fita adesiva empregada no *tape stripping*. Durante a fase preliminar do estudo de permeação cutânea, foram testados diferentes tempos de contato entre o filme e a pele (dado não mostrado). Em 12 h de ensaio, foi verificada uma quantidade cerca de 3 vezes menor de SB nanoencapsulada na epiderme do que

a encontrada no experimento de 24 horas. Além disso, não foi quantificada nenhuma quantidade de SB (livre ou nanoencapsulada) na derme, sugerindo que há uma liberação gradual do flavonoide para camadas mais profundas da pele. Dessa forma, objetivando uma redução na frequência de aplicação dos filmes, optou-se por conduzir o experimento em 24 horas, uma vez que não é recomendado conduzir este experimento por períodos mais longos porque a viabilidade da pele pode estar comprometida (MAN; HOSKINS, 2020).

Definido o tempo, este experimento foi realizado em dois tipos de condições de pele, íntegra (espessura total) e lesionada (remoção do EC), a fim de simular a penetração da SB através da pele com a função de barreira comprometida. Corroborando com o mecanismo de penetração cutânea de nanocápsulas, em pele íntegra foi demonstrado que a nanoencapsulação da SB promoveu maior retenção desta no EC, podendo assim permitir uma liberação gradual do ativo para as camadas subsequentes do tecido, como já foi observado em trabalhos anteriores (CARDOSO et al., 2019; RIGON et al., 2019). Já para a pele lesionada, foi evidenciado uma menor influência da remoção do EC na permeação da SB nanoencapsulada em relação à SB livre, corroborando a liberação controlada observada para este filme. Ao contrário da SB livre, a qual possui uma rápida liberação e disponibilidade para atingir as camadas cutâneas, o que pode acarretar aplicações mais frequentes e irritação local. Ainda, em pele lesionada a SB nanoencapsulada atingiu maiores concentrações na derme, possivelmente pela melhora da solubilidade deste ativo. Este comportamento de liberação controlada, bem como acúmulo na epiderme e derme é requerido para o tratamento de doenças inflamatórias da pele, uma vez que são nestas camadas da pele que são iniciados os processos de liberação de mediadores inflamatórios e a regeneração tecidual (BADIHI et al., 2020; SIDDIQUE et al., 2015).

Ademais, os filmes de goma gelana produzidos foram não-irritantes quando avaliados utilizando o modelo de Membrana Corioalantóide (HET-CAM). Este ensaio surgiu como uma alternativa que respeita a política dos 3 Rs (*replacement* – substituição; *reduction* – redução; *refinement* - refinamento), sendo aplicado inicialmente para testes de irritação ocular, mas que vem sendo utilizado também para outras membranas biológicas, como pele e mucosas (CERVI et al., 2021; OSMARI et al., 2020).

O tratamento tópico ideal para doenças onde há perda ou alteração na integridade cutânea, como na dermatite atópica, além de ser capaz de reduzir a resposta inflamatória e imunológica, deve incluir a manutenção da hidratação cutânea, a remoção de exsudato excessivo formado em lesões mais graves, proteção física do local irritado frente a abrasão, capacidade de remoção sem causar mais irritação, impermeabilidade a patógenos, conforto e aplicações menos frequentes (BOUTHILLETTE et al., 2019; DANBY et al., 2022). Diante

disso, os filmes compostos por goma gelana e nanocápsulas de SB demonstravam características promissoras para o tratamento desta doença. Porém, os resultados obtidos para filmes de pullulan em nosso grupo de pesquisa (CERVI et al., 2021, 2022) estimularam o interesse em associar os filmes desenvolvidos no **artigo 1** com as potencialidades deste outro polissacarídeo.

Dessa forma, o **manuscrito 1** demonstrou que a associação do pullulan com a goma gelana em filmes bicamada resultou na formação de um filme homogêneo em espessura e conteúdo de SB, com duas camadas bem definidas quando visualizadas por microscopia eletrônica de varredura. Esta estrutura bicamada pode ter favorecido a permanência das características individuais de cada polissacarídeo, como observado no resultado de bioadesão, no qual a camada composta por pullulan apresentava valores significativamente maiores de força bioadesiva que a camada de goma gelana. Na literatura, até o presente momento, não foram encontrados relatos na literatura sobre a bioadesão de filmes de goma gelana na pele. No entanto, já foi relatado que a mucoadesão da goma gelana em pastilhas de mucina é reduzida pela adição do glicerol, plastificante necessário para a obtenção de filmes mais flexíveis (PAOLICELLI et al., 2018), e utilizado em nosso estudo. Assim, a inclusão do pullulan pode facilitar a adesão dos filmes sobre a pele, bem como evitar a utilização de fitas adesivas para a fixação, as quais estão relacionadas a estética desagradável, desconforto, dor e maior irritação (proveniente da destruição do EC) durante a remoção (PAGANO et al., 2019).

Além de conferir bioadesão, a adição da camada de pullulan proporcionou um aumento também no fator de oclusão destes (cerca de 2 vezes mais). Formulações com melhor perfil oclusivo podem prevenir a perda de água transepidérmica, favorecendo a hidratação das camadas mais profundas da pele, melhorando as consequências da pele seca observada na dermatite atópica (DANBY et al., 2022). Ainda, os filmes bicamada apresentaram uma redução em sua rigidez (menores valores de módulo de Young) quando comparados a filmes monocamada de goma gelana, sugerindo que os filmes bicamada assumem as características de elasticidade do pullulan, como observado em outros estudos que produziram filmes em bicamada de caseinato de sódio e pullulan (KRISTO; BILIADERIS; ZAMPRAKA, 2007; KRISTO; BILIADERIS, 2006). Esta redução na rigidez do filme pode proporcionar com que este se adapte melhor ao local de aplicação, aumentando a aceitabilidade pelos pacientes.

Com relação ao intumescimento dos filmes produzidos no **manuscrito 1**, pode-se notar que este foi menor para os filmes bicamada em relação ao encontrado para os filmes produzidos no **artigo 1**. Este resultado pode estar relacionado a alta hidrofilicidade de filmes de pullulan (CERVI et al., 2022), assim, quando em contato com um líquido aquoso, pode estar ocorrendo

sua rápida dissolução. Esta rápida dissolução também pode estar relacionada a não influência da adição deste polissacarídeo no perfil de liberação e na permeação/retenção cutânea da SB. Neste sentido, é importante notar que a formação de uma segunda camada composta de pullulan sobre os filmes de goma gelana é capaz de melhorar as características bioadesivas, oclusivas e elásticas deste filme, sem demonstrar uma barreira para a difusão da SB.

Além do aumento da permeabilidade de substâncias, já foi relatado na literatura que a remoção do EC, simulando uma lesão superficial, pode modificar a bioadesão de formulações na pele (PARISOTTO-PETERLE et al., 2020). Neste viés, a bioadesão dos filmes bicamada desenvolvidos foi também avaliada em pele sem EC, com a camada de pullulan em contato com a pele, simulando a aplicação pretendida. Como já discutido, houve uma redução na bioadesão do filme bicamada, a qual provavelmente se deve a uma redução da interação entre o pullulan e uma superfície menos hidrofóbica (epiderme viável). No entanto, apesar desta redução, os resultados de bioadesão em pele íntegra e lesionada foram considerados satisfatórios tendo em vista a aplicação pretendida.

Posteriormente, foi realizada uma avaliação preliminar do efeito antioxidante da SB e da biocompatibilidade dos filmes bicamada produzidos, utilizando ensaios *in vitro*. A ação antioxidante foi avaliada através do ensaio ABTS, o qual foi escolhido por ser simples, rápido, sensível e reprodutível (GULCIN, 2020). Ainda, este ensaio tem sido bastante utilizado para a avaliação antioxidante de compostos associados ou não a nanoestruturas ou a filmes (MATTIAZZI et al., 2019; YANG et al., 2019). O radical ABTS é oxidado previamente com a adição de persulfato de potássio, formando seu cátion-radical, o qual possui uma coloração verde-azulada. Assim, a capacidade antioxidante de determinada amostra é avaliada através da neutralização do cátion ABTS+, por meio de doação de elétrons ou de átomos de hidrogênio, provocando a perda de coloração do meio reacional (GULCIN, 2020). Neste ensaio foi possível observar o potencial da SB em neutralizar o cátion-radical, corroborando estudos que relatam a ação sequestrante de radicais da SB avaliada por métodos semelhantes (MARCHIORI et al., 2017a; YANG et al., 2019). A alta capacidade de neutralização do radical observada se deve à estrutura polifenólica da SB, sendo a hidroxila ligada ao carbono 20 do anel aromático “E” da sua estrutura química o primeiro e principal grupamento envolvido no processo redox, a partir do qual ocorre então a transferência de átomos de hidrogênio (PYSZKOVÁ et al., 2016).

A biocompatibilidade dos filmes bicamada foi avaliada através de um ensaio de hemólise, tendo em vista que na dermatite atópica pode haver a formação de escoriações e eritema, com ou sem extravasamento de sangue (TOKURA, 2010). Assim, se torna fundamental garantir que a formulação que poderá estar em contato direto com estas lesões seja

bio/hemocompatível. O ensaio de hemólise é preconizado pela Sociedade Americana para Testes e Materiais (American Society for Testing and Materials – ASTM) para a avaliação da biocompatibilidade de materiais para a saúde que possam entrar em contato com o sangue. A hemólise de um material pode ser avaliada por diferentes técnicas, sendo a por contato direto a escolhida neste estudo. Nenhum dos filmes testados apresentou grau de hemólise significativo, sendo considerados hemocompatíveis e seguros.

Os resultados *in vitro* obtidos no **manuscrito 1** encorajaram a realização de um estudo *in vivo* para evidenciar os efeitos biológicos dos filmes bicamada desenvolvidos em um modelo animal de dermatite atópica. Esta doença crônica da pele apresenta uma alta heterogeneidade e uma fisiopatologia complexa, sendo difícil reproduzir em modelos animais a complexidade da doença tal qual como ocorre em humanos (EWALD et al., 2017). No entanto, os modelos animais são uma ferramenta de pesquisa pré-clínica indispensável para testar novas formulações para a dermatite atópica. Existem 3 modelos animais bem aceitos para o teste de novas abordagens terapêuticas para esta doença da pele: mutação espontânea, sensibilização epicutânea e camundongos transgênicos (JIN et al., 2009). Nesta tese foi utilizado o modelo de sensibilização epicutânea com hapteno, para o qual foi utilizado como agente sensibilizador o DNCB, o qual, após múltiplos desafios epicutâneos, produzem lesões na pele semelhantes a dermatite atópica.

Para evidenciar o efeito terapêutico das formulações desenvolvidas no **manuscrito 1**, o tratamento dos camundongos com os filmes bicamada foi comparado a tratamentos com uma solução de SB e com um fármaco utilizado na prática clínica, a hidrocortisona 1 %. A SB foi aplicada em solução, pois, como já mencionado, não foi possível a produção de filmes bicamada com este flavonoide sem a sua associação em nanocápsulas. Para isto, ela foi solubilizada no mesmo veículo utilizado para o DNCB (uma mistura de acetona e óleo de oliva), afim de descartar qualquer influência do solubilizante na atenuação ou agravamento das lesões.

Quando se observa a performance dos diferentes tratamentos no modelo animal de dermatite atópica, é possível visualizar que o filme bicamada veículo, por si só, é capaz de reduzir o comportamento de coçar, o edema de orelha e a peroxidação lipídica. Já ao adicionar a SB nanoencapsulada a este filme, além da atenuação destes parâmetros, percebe-se uma redução no escore da dermatite e melhora na proteção antioxidante, sendo estas ações similar e superior a SB em solução, respectivamente. Assim, estes resultados sugerem que o efeito antioxidante da SB é potencializado pela sua encapsulação em nanocápsulas, bem como o efeito antidermatite é melhorado pela proteção física da pele e absorção de exsudatos conferidas pela goma gelana, e pela bioadesão e maior oclusão conferidas pelo pullulan, proporcionando um

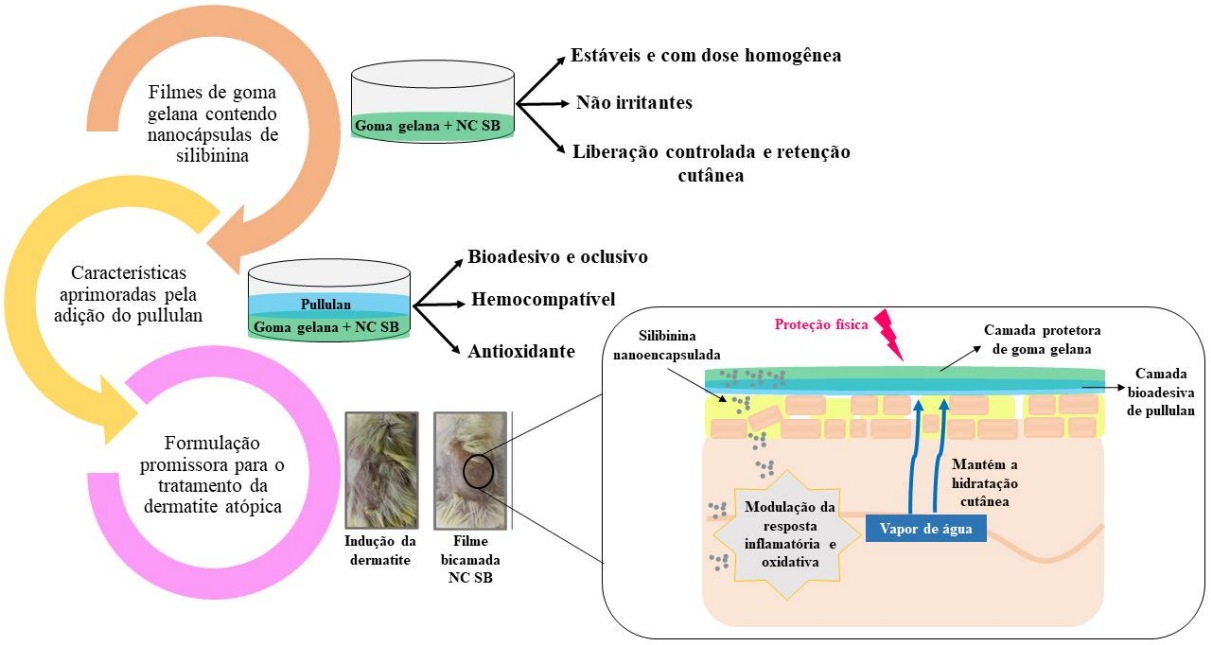
tratamento mais eficaz. Além das particularidades conferidas pela combinação destes polissacarídeos em um filme, acredita-se que o glicerol, presente como plastificante, esteja exercendo sua ação de umectação e restauração cutânea, como já demonstrado anteriormente (ATRUX-TALLAU et al., 2010). Outros estudos na literatura já demonstraram também os efeitos benéficos de filmes no tratamento da dermatite atópica, os quais não atuam apenas como veículo para substâncias, mas também possuem papel importante na atenuação dos sinais clínicos desta dermatose (JEONG et al., 2019; VOSS et al., 2020).

Outro ponto a destacar a respeito da avaliação *in vivo*, é a ineficácia do tratamento com hidrocortisona nos parâmetros acima mencionados (prurido e redução do escore da dermatite). Em nosso estudo, este fármaco atenuou apenas os níveis de óxido nítrico, semelhante a SB livre ou nanoencapsulada, e reduziu o índice esplênico, corroborando sua ação anti-inflamatória e imunossupressora. Possivelmente, a hidrocortisona não tenha sido eficaz em reduzir o escore da dermatite e prurido devido ao seu uso prolongado neste estudo, levando a sua toxicidade (SLATER; MORRELL, 2015). Ainda, o intervalo de aplicações tópica realizado no modelo animal utilizado em nosso estudo (a cada 3 dias) pode ter contribuído para a ineficácia do medicamento referência em reduzir as lesões da dermatite. Na clínica, recomenda-se a aplicação da pomada de hidrocortisona diariamente, a cada 12 horas, uma vez que esta é classificada como de um glicocorticoide de baixa potência. A nossa hipótese de toxicidade sistêmica e posologia inadequada está de acordo com um estudo prévio que avaliou o desempenho de filmes de gelatina e amido contendo hidrocortisona em atenuar lesões de dermatite atópica utilizando o mesmo modelo e técnicas empregados nesta tese. Os pesquisadores observaram que os filmes desenvolvidos apresentaram uma liberação controlada da hidrocortisona. Nos resultados *in vivo*, foi possível observar que os filmes reduziram a toxicidade sistêmica do corticoide, atenuando o prurido e melhorando significativamente as lesões semelhantes à dermatite quando comparados à pomada comercial de hidrocortisona (VOSS et al., 2020).

Diante do que foi discutido, pode-se refletir que os resultados *in vitro* e *in vivo* obtidos para os filmes bicamada contendo nanocápsulas de SB apresentam características vantajosas para o tratamento da dermatite atópica. Ainda, o filme bicamada composto por goma gelana e pullulan pode ser uma alternativa promissora em comparação com outras tecnologias propostas para a veiculação de substâncias no manejo da dermatite atópica, pois: a) é produzido utilizando polissacarídeos biocompatíveis, produzindo filmes não hemolíticos; b) é obtido facilmente através de técnicas simples de preparo; c) possui fácil aplicação e adesão à pele; d) pode atuar como um curativo, servindo como uma barreira física capaz de impedir o contato da lesão com o meio externo; e) pode absorver o exsudato formado em lesões mais severas.

Desse modo, os resultados demonstrados na presente tese servem como degrau para futuros e mais aprofundados estudos, tanto sobre os efeitos promissores apresentados para as nanocápsulas contendo a SB, ressaltando que essa formulação viabiliza a incorporação deste flavonoide lipofílico em filmes, como também sobre os efeitos proporcionados pelos filmes inovadores aqui produzidos. A figura 10 resume os resultados encontrados nesta tese.

Figura 10 – Ilustração dos principais achados da tese (*graphical abstract*).



5 CONCLUSÃO GERAL

As nanocápsulas desenvolvidas neste estudo permitiram a incorporação da SB em filmes de goma gelana, contornando as limitações físico-químicas e prevenindo a degradação do flavonoide ao longo do armazenamento, viabilizando assim sua administração tópica. Ainda, a combinação da goma gelana e pullulan em camadas poliméricas distintas proporciona um filme com características aprimoradas para uso sobre a pele, sendo que esta associação foi demonstrada pela primeira vez. Além disso, uma detalhada caracterização dos filmes produzidos foi realizada, demonstrando a viabilidade dos mesmos para uso cutâneo, até mesmo em lesões exsudativas e com sangramento. Por fim, a incorporação de nanocápsulas de SB em filmes bicamada de goma gelana e pullulan representa uma formulação única e inovadora, a qual é capaz tanto de atenuar as respostas oxidativas e inflamatórias da pele, quanto de protege-la de danos externos, acelerando a reparação da barreira cutânea, e possuindo assim, elevado potencial no tratamento da dermatite atópica.

6 PERSPECTIVAS

Com base no conjunto de resultados obtidos nesta tese sugere-se, para estudos futuros, a determinação de parâmetros biométricos na pele de voluntários humanos, como pH, grau de hidratação e grau de perda de água transepidérimica, tanto em voluntários saudáveis quanto em voluntários com dermatite atópica, a fim de verificar o efeito dos filmes desenvolvidos na manutenção da barreira cutânea. Além disso, é importante também avaliar a segurança biológica *in vivo* dos filmes produzidos, a fim de garantir que as múltiplas aplicações da forma farmacêutica sobre a pele não acarretarão em toxicidade local ou sistêmica. Por fim, este trabalho abre perspectivas para outros estudos utilizando os filmes produzidos como veículo para outras substâncias ativas no tratamento de diferentes doenças da pele.

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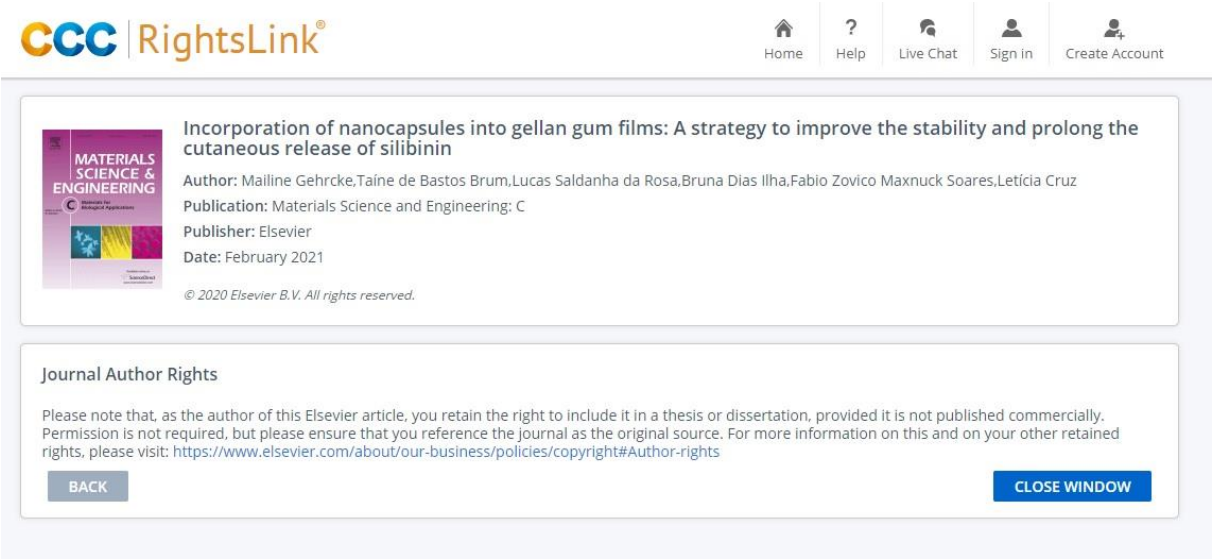
ANEXOS

ANEXO A. Autorização para reprodução do artigo científico: “Incorporation of nanocapsules into gellan gum films: A strategy to improve the stability and prolong the cutaneous release of silibinin”. Publicado na Materials Science and Engineering: C.



ANEXO B. Certificado de aprovação do projeto realizado com animais experimentais pela comissão de ética no uso de animais da Universidade Federal de Pelotas.

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **140/2019/CEEA/REITORIA**
PROCESSO Nº 23110.046760/2019-30

Certificado

Certificamos que a solicitação de **adendo** à proposta intitulada **" AVALIAÇÃO DO EFEITO DE COMPOSTOS SINTÉTICOS INÉDITOS NA DERMATITE ATÓPICA INDUZIDA POR 2,4-DINITROCLOROBENZENO EM CAMUNDONGOS " (CEEA 23357-2018),** registrada com o n° 23110.046760/2019-30**,** sob a responsabilidade de **Ethel Antunes Wilhelm** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de **13 de novembro de 2019**.

Solicitação: Acréscimo de 200 camundongos BALB/c, fêmeas, com 60 dias.

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M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Documento assinado eletronicamente por **ANELIZE DE OLIVEIRA CAMPELLO FELIX**, **Médico Veterinário**, em 18/11/2019, às 15:20, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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