UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

NANOEMULSÕES DE ÓLEO DE COPAÍBA (*Copaifera multijuga* HAYNE): DESENVOLVIMENTO TECNOLÓGICO, ESTUDO DE PERMEAÇÃO CUTÂNEA E AVALIAÇÃO DAS ATIVIDADES ANTI-INFLAMATÓRIA E LEISHMANICIDA TÓPICAS.

LETICIA GROLLI LUCCA

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Nanoemulsões de óleo de copaíba (*Copaifera multijuga* Hayne): desenvolvimento tecnológico, estudo de permeação cutânea e avaliação das atividades anti-inflamatória e leishmanicida tópicas.

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Orientadora: Profa. Dra. Letícia Scherer Koester

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Profa Dr Karina Paese
Universidade Federal do Rio Grande do Sul (UFRGS)

Profa Dr Nadia Maria Volpato
Universidade Federal do Rio Grande do Sul (UFRGS)

Prof Dr Pedro Roosevelt Torres Romão
Universidade Federal de Ciências Básicas da Saúde de Porto Alegre (UFCSPA)

Lucca, Leticia Grolli
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RESUMO

O óleo de copaíba é um produto natural encontrado principalmente na região amazônica, onde é utilizado na medicina popular como tratamento para inflamações e como cicatrizante. A espécie *Copaifera multijuga* Hayne demonstrou um potencial efeito anti-inflamatório em relação a outras espécies de *Copaífera* L., tendo como principal responsável o composto majoritário β-cariofileno. Nosso grupo de pesquisa vem estudando a veiculação deste óleo em nanoemulsões e desenvolveu uma formulação que contém uma elevada proporção de óleo de copaíba no núcleo oleoso (20 % w/v), sem prejuízo da estabilidade do sistema. A partir desta formulação, seguiram-se os estudos de avaliação da permeação cutânea, de otimização da formulação e de avaliação de atividade anti-inflamatória, apresentados neste trabalho. Um método em cromatógrafo a gás acoplado à espectrômetro de massas no modo *headspace* (HS-CG/EM) foi validado a fim de analisar β-cariofileno em mostras de pele provenientes do teste de permeação cutânea com nanoemulsões de óleo de copaíba. O método mostrou-se específico, linear, preciso e exato. O teste de permeação cutânea demonstrou que apenas com a nanoemulsão é possível detectar β-cariofileno na camada da derme, enquanto que, com o óleo, somente no estrato córneo. A seguir, foram testados dois tensoativos catiônicos na formulação da nanoemulsão para verificar se a carga positiva na interface da gotícula poderia promover a permeação cutânea do β-cariofileno. O tensoativo brometo de cetiltrimetilamônio provou ser mais eficiente em concentrações consideradas seguras para o uso tópico, revertendo o potencial zeta para valores adequados, sem interferir no tamanho de gotícula e índice de polidispersão. O tensoativo oleilamina também reverteu o potencial zeta, porém somente em concentrações muito elevadas, podendo ser consideradas tóxicas. O teste de permeação cutânea demonstrou que a incorporação de tensoativos catiônicos aumenta a retenção de β-cariofileno na epiderme em três vezes, enquanto que, na derme, não há diferença estatística entre as formulações aniônica e catiônica. Após, as nanoemulsões escolhidas foram incorporadas em hidrogéis de Carbopol®, Natrosol® e quitosana com vistas ao espessamento e

adequação da viscosidade ao uso tópico. O hidrogel com quitosana apresentou-se instável, com aumento de tamanho de gotícula e índice de polidispersão, apesar de não ter afetado o potencial zeta. Os hidrogéis de Carbopol® e Natrosol® apresentaram bons resultados de caracterização físico-química, porém somente o hidrogel de Natrosol® foi escolhido para os estudos de permeação cutânea e atividade anti-inflamatória *in vivo*, devido ao seu caráter neutro. No estudo de permeação cutânea das nanoemulsões incorporadas em hidrogel, houve um aumento na retenção de β-cariofileno na derme em relação às nanoemulsões, e houve a facilitação de permeação até o fluido receptor. Por fim, o estudo da atividade *in vivo* das formulações selecionadas demonstrou que o óleo de copaíba apresenta atividade anti-inflamatória e que a sua incorporação em nanoemulsões aumenta este efeito. No entanto, ambas nanoemulsões, tanto negativamente quanto positivamente carregadas, apresentaram resultado semelhante para a inibição do edema. Quando compara-se a permeação cutânea das formulações verifica-se que na derme não há diferença estatística, o que pode justificar a semelhança do grau de inibição da inflamação no teste *in vivo*. Em relação à atividade das nanoemulsões incorporadas em hidrogéis, pode-se verificar que no teste de edema de pata apenas a formulação carregada positivamente teve um efeito mais pronunciado, enquanto no edema de orelha as formulações obtiveram um perfil equivalente ao controle cetoprofeno, porém não potencializaram o efeito do óleo. Quanto ao estudo com os parasitos causadores da Leishmaniose, os testes *in vitro* mostraram que os tratamentos (óleo de copaíba, β-cariofileno e suas nanoemulsões) foram mais eficazes contra as espécies *L. major* e *L. donovani* em comparação às espécies *L. amazonensis* e *L. braziliensis*. O teste *in vivo* mostrou que todos os tratamentos foram capazes de reduzir a área da ferida dos camundongos infectados com *L. major*. No entanto, eles não conseguiram recuperar totalmente os animais.

Palavras-chave: Óleo de copaíba; nanoemulsão; hidrogel; anti-inflamatório; permeação cutânea; leishmaniose

ABSTRACT

Copaiba oil nanoemulsions: technological development, skin permission study and evaluation of topical anti-inflammatory and leishmanicidal activities.

Copaiba oil is a natural product found especially in the Amazon region, where it is used as treatment for inflammations and as wound healer in the popular medicin. The species *Copaifera multijuga* Hayne showed a potential anti-inflammatory effect in relation to other *Copaifera* L. species, mainly due to its major compound, β-caryophyllene. Our research group studied the oil incorporation in nanoemulsions and developed a formulation containing a high copaiba oil proportion in the oil core (20% w/v) without loss of system stability. Based in this nanoemulsion, we present in this study the skin permeation, the formulation optimization and the anti-inflammatory activity. A method in gas chromatograph coupled with mass spectrometer in headspace mode (HS-GC/MS) was validated to analyze β-caryophyllene in skin samples from the skin permeation assay. The method proved to be specific, linear, precise and accurate. Skin permeation test showed that only with the nanoemulsion is possible to detect β-caryophyllene in the dermis layer, while with the oil, only in the stratum corneum. After, two cationic surfactants were tested in the nanoemulsion to prove if the positive charge on the droplet interface could promote β-caryophyllene permeation. Cetyltrimethylammonium bromide proved to be more effective at concentrations considered safe for topical use, reversing the zeta potential to suitable values, without interfering with droplet size and polydispersity index. Oleylamine also reversed the zeta potential, but only at very high concentrations, that may be considered toxic. Skin permeation test showed that the incorporation of cationic surfactants increases β-caryophyllene retention in the epidermis by three fold, whereas in the dermis, there is no statistical difference between the cationic and anionic nanoemulsions. Afterward, the chosen nanoemulsions were incorporated in Carbopol®, Natrosol® and chitosan hydrogels in order to adjust the viscosity for topical use. Chitosan hydrogel presented instability, with an increase in droplet size and polydispersity index, although it has not affected the zeta potential.

Carbopol® and Natrosol® hydrogels showed good results in physicochemical characterization, but only Natrosol® hydrogel was chosen for the following skin permeation and anti-inflammatory activity *in vivo* studies due to its neutral character. In nanoemulsions thickened-hydrogel skin permeation study, there was an increase in the β-caryophyllene retention in the dermis compared to nanoemulsions, and promoted permeation to the receptor fluid. Finally, *in vivo* anti-inflammatory activity from selected formulations showed that the copaiba oil has anti-inflammatory activity and that its incorporation into nanoemulsions increases this effect. However, both negative and positively charged nanoemulsions, showed similar results for inhibition of edema. When the nanoemulsions' skin permeation is compared, it is found that in the dermis there is no statistical difference, which may explain the similarity degree of inflammation inhibition in the *in vivo* test. Regarding to the activity of the nanoemulsions incorporated in hydrogels, it can be verified that in paw edema assay, only the positively charged formulation had a more pronounced effect, whereas in the ear edema the formulations obtained a profile equivalent to the control, ketoprofen, but did not potentiate the effect of the oil. As for the study with the parasites causing Leishmaniasis, *in vitro* tests showed that the treatments (copaiba oil, β-caryophyllene and their nanoemulsions) were more effective against *L. major* and *L. donovani* compared to *L. amazonensis* and *L. braziliensis*. *In vivo* assay showed that all treatments were able to reduce the wound area of mice infected with *L. major*. However, they were unable to fully recover the animals from the disease.

Keywords: copaiba oil, nanoemulsion, hydrogel, anti-inflammatory, skin permeation, leishmaniasis

SUMÁRIO

INTRODUÇÃO

INTRODUÇÃO

Inúmeras espécies de plantas medicinais são utilizadas diariamente pela população brasileira para o tratamento de doenças inflamatórias (JÚNIOR et al., 2011). Dentre estas plantas encontra-se a família das copaíferas. O óleo-resina retirado do tronco destas plantas é utilizado popularmente na região amazônica para processos inflamatórios das vias urinárias, das vias respiratórias e da pele (VEIGA-JUNIOR; PINTO, 2002). Alguns estudos já comprovaram que este óleo possui atividade anti-inflamatória (BASILE et al., 1988; VEIGA-JUNIOR et al., 2001, 2006, 2007; CARVALHO et al., 2005; GOMES et al., 2007, 2010; GELMINI et al., 2013), a qual é atribuída principalmente aos componentes majoritários do óleo, tais como o sesquiterpeno β-cariofileno, porém, sua textura e caráter untuoso o tornam pouco atrativo ao uso *in natura* por via tópica.

Nosso grupo de pesquisa vem estudando o desenvolvimento de nanoemulsões a base de óleo de copaíba na perspectiva de melhorar a ação anti-inflamatória tópica do óleo de copaíba visto que sistemas nanoestruturados podem penetrar até a derme (ALVES et al., 2007; PROW et al., 2011), região da pele onde acontecem os fenômenos típicos da inflamação.

Em um primeiro trabalho, desenvolvido por Dias e colaboradores (2012) foi realizada a otimização de um método de microextração em fase sólida (SPME) do β-cariofileno no modo *headspace* e validação de um método indicativo de estabilidade por cromatografia gasosa (CG) acoplada a detector de ionização de chama. Esse método foi utilizado para o doseamento e acompanhamento da estabilidade das formulações preparadas subsequentemente (DIAS et al., 2014) por dois métodos, homogeneização à alta pressão e emulsificação espontânea. A partir desse estudo de formulação, que empregou um planejamento fatorial fracionado 2^{4-1}, foi selecionado o método de homogeneização à alta pressão para produzir as nanoemulsões e a composição que se mostrou mais adequada continha 20% (p/v) de óleo de copaíba, 10% (p/v) de triglicerídeos de cadeia média (com a finalidade de fixar o óleo volátil), 3% (p/v) de tensoativo lipofílico (Span 80®) e 1% (p/v) de tensoativo hidrofílico (Tween 20®).

No presente trabalho pretende-se, portanto, dar continuidade aos trabalhos realizados, visando ao estudo da atividade anti-inflamatória das nanoemulsões contendo óleo de copaíba e também a sua incorporação em formas farmacêuticas semissólidas, como os hidrogéis. Os resultados obtidos até então apontavam para a necessidade de: (i) validar uma metodologia bioanalítica sensível e que permita a quantificação do marcador em todas as camadas da pele e no fluido aceptor, durante os estudos de permeação cutânea em pele de orelha suína em células de difusão de Franz; (ii) avaliar a influência da incorporação de tensoativo catiônico na formulação, assim como o emprego de outras estratégias farmacotécnicas, tais como a incorporação em hidrogéis, na penetração cutânea do marcador e (iii) investigar atividade anti-inflamatória *in vivo* do óleo de copaíba associados às nanoemulsões. Outra perspectiva de estudo traçada, e também inédita, (iv) foi a investigação acerca da atividade leishmanicida do óleo extraído desta espécie e deste nanoemulsionado, já que alguns dados na literatura sugerem a possível atividade leishmanicida deste óleo, porém sem muita profundidade.

Na leishmaniose cutânea, a derme também se apresenta como a camada de interesse, pois é nela que os parasitos encontram-se internalizados por macrófagos (SILVEIRA et al., 2004). Tendo em vista que neste trabalho buscou-se aumentar a penetração dos compostos (avaliados através de seu principal marcador, o β-cariofileno) na derme, e que o óleo de copaíba apresenta uma promissora atividade leishmanicida (SANTOS et al., 2008; DOS SANTOS et al., 2011, 2012), despertou-se o interesse em estudar de maneira mais aprofundada esta atividade para as formulações desenvolvidas. Mais recentemente, foi demonstrado que o β-cariofileno isolado apresenta promissora atividade leishmanicida (SOARES et al., 2013). Estes achados, somados ao fato de que a leishmaniose cutânea é uma doença que afeta a população mundial, especialmente em países pouco desenvolvidos como o Brasil (WHO, 2014), abriram uma última pespectiva de estudo desta tese, que foi o estudo da atividade leishmanicida *in vitro* das nanoemulsões à base de óleo de copaíba.

OBJETIVOS

OBJETIVO GERAL

O objetivo geral desta tese foi desenvolver uma nova nanoemulsão de óleo de copaíba a partir de uma formulação otimizada, além de uma formulação semissólida, e avaliar as atividades anti-inflamatória (em modelos *in vivo*) e leishmanicida (em modelos *in vitro* e *in vivo*) das mesmas.

OBJETIVOS ESPECÍFICOS

- Validar um método bioanalítico para determinação do componente majoritário do óleo de copaíba, β-cariofileno, em amostras de pele de orelha de porco e de fluido aceptor dos estudos em células de Franz, empregando-se cromatógrafo a gás acoplado a espectrômetro de massas, no modo *headspace*;

- Realizar estudos de formulação com tensoativos catiônicos a fim de incrementar a permeação cutânea do composto majoritário do óleo a partir de nanoemulsões e hidrogéis derivados;

- Investigar o perfil de permeação cutânea *in vitro* do óleo de copaíba a partir das nanoemulsões selecionadas, incorporadas e não incorporadas a veículos semissólidos (hidrogéis), através do modelo da pele de orelha suína em células de difusão de Franz;

- Avaliar a atividade anti-edematogênica *in vivo* do óleo de copaíba associado às nanoemulsões, incorporadas ou não aos hidrogéis, através do modelo de edema de orelha de camundongo e de edema de pata de rato.

- Escrever um artigo de revisão da literatura sobre a utilização de óleos essenciais em pesquisas que utilizam modelos *in vivo* de inflamação, descrevendo os principais modelos usados para estudar a inflamação aguda em animais.

- Avaliar a atividade leishmanicida *in vitro* do óleo de copaíba e da nanoemulsão otimizada frente a diferentes espécies de *Leishmania* bem como a atividade *in vivo* em modelo de leishmaniose cutânea induzida por *Leishmania major*.

REVISÃO DA LITERATURA

ÓLEO DE COPAIBA

O gênero *Copaífera* L. abrange diversas espécies nativas do continente americano. Estas árvores, cuja altura pode chegar a 40 metros e que vivem até 400 anos, são facilmente encontradas nas regiões amazônica e centro-oeste do Brasil e são de grande interesse comercial, tanto pela extração do seu óleo quanto pela sua madeira (VEIGA-JUNIOR; PINTO, 2002; TAPPIN et al., 2004).

O óleo-resina extraído do tronco das copaíferas é constituído de uma parte resinosa, contendo principalmente diterpenos, e uma parte volátil, contendo principalmente sesquiterpenos. A retirada do óleo pode ser feita de diversas maneiras. Muitas delas prejudicam a árvore e podem levar à sua morte, mas a prática de coleta menos agressiva e menos prejudicial se dá pela inserção de um trado de metal no tronco da árvore. Após a remoção do trado, o óleo é coletado a partir do orifício produzido que é facilmente fechado após a coleta. O óleo extraído é utilizado sem a necessidade de purificação (CASCON; GILBERT, 2000; SILVA MEDEIROS, DA; VIEIRA, 2008; PIERI et al., 2009).

O óleo de copaíba é muito utilizado tradicionalmente na região amazônica onde é facilmente encontrado em mercados populares do local, sendo administrado tanto pela via tópica quanto pela via oral (LEANDRO et al., 2012). Os seus principais efeitos farmacológicos descritos na medicina popular são a atividade anti-inflamatória e a atividade cicatrizante. Alguns estudos científicos vêm comprovando estas hipóteses, descrevendo a eficácia do óleo tanto *in vivo* quanto *in vitro* (BASILE et al., 1988; VEIGA-JUNIOR et al., 2001; CARVALHO et al., 2005; GOMES et al., 2007; SILVA et al., 2009; GELMINI et al., 2013; DIAS et al., 2014; SANTIAGO et al., 2015).

Atualmente, existem diversas pesquisas na literatura com óleo de copaíba, onde foram verificadas outras atividades, tais como antimicrobiana (PIERI et al., 2012; SANTOS et al., 2012; MORELLI et al., 2015), antitumoral (GOMES et al., 2008), leishmanicida (SANTOS et al., 2012; GUPTA et al., 2015), antisséptica (BOTELHO et al., 2014), hepatoprotetora (HENRIQUES et al., 2014), gastroprotetora (PAIVA et al., 1998), antinociceptiva (GOMES et al., 2007).

β-cariofileno

Os constituintes do óleo de copaíba podem variar muito quanto a espécie de *Copaifera* estudada (CASCON; GILBERT, 2000; VEIGA-JUNIOR et al., 2007). Normalmente, utiliza-se o β-cariofileno como marcador deste óleo, já que ele está presente em maior ou menor quantidade em todos os óleos de copaíba já descritos na literatura (LEANDRO et al., 2012). O β-cariofileno é um sesquiterpeno bicíclico e, além do óleo de copaíba, ocorre naturalmente nos óleos essenciais de outras plantas como *Eugenia caryophyllata* L. e *Cannabis sativa* L. Devido ao seu odor característico, é também utilizado na indústria de perfumes e flavorizantes, sendo descrito na lista de substâncias flavorizantes autorizados na Europa (SARPIETRO et al., 2015).

Na literatura científica, o β-cariofileno já foi descrito como anti-inflamatório (FERNANDES et al., 2007; GERTSCH et al., 2008; KLAUKE et al., 2014), e no tratamento de enfermidades relacionadas à inflamação, tais como na antinocicepção (GHELARDINI et al., 2001; PAULA-FREIRE et al., 2014) na prevenção da colite (CHO et al., 2007) e no tratamento da endometriose (ABBAS et al., 2013). Além disso, foi demonstrado que o β-cariofileno é um ligante seletivo e agonista não-psicoativo dos receptores canabinóides CB_2, apresentando-se como uma potencial estratégia no tratamento da inflamação, dor, aterosclerose e osteoporose (GERTSCH et al., 2008).

Dentre os estudos tecnológicos e analíticos realizados com esta molécula podem-se citar os trabalhos de Liu et al. (2013) e Dias et al. (2012). A complexação com β-ciclodextrina, descrita por Liu e colaboradores (2013), teve como objetivo diminuir a volatilização e aumentar a solubilidade em água, tendo sido avaliada a

sua dissolução *in vitro* e a sua biodisponibilidade *in vivo*. Neste estudo, o complexo de inclusão foi produzido com sucesso, apresentando rápida liberação no teste de dissolução e uma melhora na biodisponibilidade oral comparado à molécula não complexada.

Dias e colaboradores (2012) desenvolveram e validaram um método para quantificação do β-cariofileno em nanoemulsões contendo óleo de copaiba, que utiliza microextração em fase sólida (SPME) e cromatografia gasosa (CG). Este trabalho descreve a otimização dos parâmetros de extração do β-cariofileno das formulações preparadas à base de óleo de copaíba por HS-SPME-CG/FID, utilizando planejamento fatorial Box–Behnken 3^3. Melhores níveis de β-cariofileno foram encontrados quando a extração foi feita sem adição de NaCl, à temperatura de 45°C e após 20 minutos de exposição da fibra de SPME. O método analítico de doseamento do β-cariofileno em nanoemulsões mostrou-se linear, preciso, exato e robusto e nos testes de degradação forçada (hidrólise ácida e básica e exposição à oxidação, temperatura e luz) observou-se efeito parcialmente protetor das nanoemulsões sobre a degradação do β-cariofileno frente às condições de hidrólise ácida, oxidação, temperatura e luz.

VIA CUTÂNEA

A pele é uma barreira biológica que impede a entrada de agentes exógenos químicos, físicos e microrganismos e que protege o organismo contra a perda de água (JUNQUEIRA; CARNEIRO, 1995). Este órgão é dividido basicamente em duas camadas: epiderme e derme (JAIN et al., 2014).

O estrato córneo é a camada mais externa da epiderme e atua propriamente como a barreira da pele, sendo composto por corneócitos envolvidos em uma matriz lipídica rica em proteínas. O estrato córneo representa a etapa limitante no transporte de fármacos pela pele que pode ocorrer basicamente por três vias: transcelular, intercelular e transfolicular (PROW et al., 2011). Na derme, camada abaixo da epiderme, encontram-se as terminações nervosas, vasos sanguíneos e as fibras elásticas que dão sustentação à pele (CEVC; VIERL, 2010). Nesta camada

é onde ocorrem as condições inflamatórias que acometem a pele além das doenças de fundo alérgico (JAIN et al., 2014).

A hipoderme, apesar de ser constituída de células da mesma origem da derme, não faz parte da pele. Esta camada serve de sustentação e conexão com os órgãos subjacentes e é onde se encontram a gordura subcutânea e a base das glândulas sebáceas e dos folículos capilares (JUNQUEIRA; CARNEIRO, 1995; JAIN et al., 2014).

O interesse pela via cutânea vem aumentando nos últimos anos, principalmente para terapias de uso local, como para analgesia e atividade anti-inflamatória, mas também para liberação de substâncias na circulação (LEITE-SILVA et al., 2012). A via tópica é uma alternativa para a administração de fármacos, permitindo uma entrega sustentada para a corrente circulatória e evitando efeitos adversos que provém da administração oral ou parenteral. Entretanto, existem desafios para esta via de administração, já que o estrato córneo forma uma barreira micrométrica que regula a passagem de agentes externos, tornando-se, assim, um passo limitante da penetração cutânea (CROSERA et al., 2009; CEVC; VIERL, 2010; PROW et al., 2011; BOLZINGER et al., 2012).

Permeação cutânea

O termo geral absorção cutânea descreve o processo da passagem de substâncias através da pele, o qual pode ser dividido em três etapas: penetração cutânea (entrada de uma substância na pele, mais especificamente a passagem pelo estrato córneo), permeação cutânea (passagem de uma substância pelas camadas da pele) e absorção cutânea (chegada de uma substância no sistema circulatório como nos linfonodos e vasos sanguíneos) (BARTOSOVA; BAJGAR, 2012).

A absorção cutânea de fármacos depende tanto da estrutura da pele quanto das características da molécula estudada e do veículo em que esta está incorporada. Em relação à pele, os fatores que influenciam a penetração de substâncias são a integridade do tecido (já que em certas condições o estrato córneo fica danificado, tais como em infecções, inflamações, dermatites, psoríase, queimaduras, baixa

umidade no ambiente, danos físicos) e também a região corporal e densidade de folículos pilosos (BAROLI, 2010; LEITE-SILVA et al., 2012).

Quanto à molécula a ser estudada, os fatores que influenciam sua capacidade de penetrar a barreira da pele são: pK_a (apenas moléculas não ionizadas penetram a pele), coeficiente de partição óleo/água (moléculas lipofílicas penetram o estrato córneo facilmente, mas tem dificuldade de passar pelas outras camadas), massa molecular e coeficiente de difusão. Além disso, a composição e as características físico-químicas do veículo também influenciam no processo de absorção cutânea e devem ser levadas em consideração em estudos de permeação (BAROLI, 2010; LEITE-SILVA et al., 2012)

A maioria dos trabalhos na literatura vem apresentando estudos de permeação cutânea *in vitro* utilizando, principalmente, pele de porco que é similar à pele humana (SCHMOOK et al., 2001; BARBERO; FRASCH, 2009). A pele humana também é utilizada, porém em menor escala, pois sua disponibilidade é muito menor e pode apresentar variabilidades como gênero, idade e local anatômico de origem do doador (BARBERO; FRASCH, 2009). Também existem peles de origem sintética, obtidas a partir de pele de animal liofilizada, através da engenharia de tecidos (modelo de epiderme humana reconstruída: EpiSkin®, EpiDerm®, SkinEthic®) ou através de modelos de pele artificial (membrana de polidimetilsiloxano, células PAMPA modificadas com ceramidas, etc) (FLATEN et al., 2015).

A metodologia mais empregada para a avaliação da permeação cutânea *in vitro* é feita em células de difusão de Franz estática, onde pode-se modificar parâmetros como temperatura, dose aplicada, constituição do fluido aceptor e área de difusão (GODIN; TOUITOU, 2007). Normalmente a temperatura utilizada neste teste a fim de mimetizar a temperatura da pele humana é de 32°C ± 1°C. O fluido aceptor é usualmente constituído de tampão fosfato ou solução salina adicionado ou não de uma quantidade limitada de solvente orgânico (etanol, metanol, isopropanol) ou tensoativos (polissorbato, laurilsulfato de sódio) que facilitam a solubilização de substâncias lipofílicas e garantem as condições Sink (FDA, 1997; BARTOSOVA; BAJGAR, 2012).

Ao final do experimento as camadas da pele podem ser facilmente separadas utilizando-se técnicas simples. O estrato córneo é separado pela técnica do *tape-stripping* e a epiderme é separada da derme com bisturi (podendo ser submetida a temperatura de até 60 °C) (FDA, 1997; ESCOBAR-CHÁVEZ et al., 2008; KLANG et al., 2012).

INFLAMAÇÃO

Em geral, a inflamação é uma resposta à infecção, lesão ou exposição a partículas externas, levando ao extravasamento local de células sanguíneas e fluidos. Normalmente, é descrita pelos quatro sinais clássicos: calor, dor, vermelhidão e inchaço (RYAN; MAJNO, 1977; FULLERTON; GILROY, 2016).

A primeira resposta ao à lesão é intermediada por macrófagos teciduais, mastócitos e outras células residentes, levando à produção de mediadores inflamatórios, tais como histamina, prostaglandinas e leucotrienos, que, por consequênicia, induzem o aumento da permeabilidade vascular e migração de células (neutrófilos e monócitos). Os neutrófilos liberam espécies reativas de oxigênio e nitrogênio (ERO e ERN) que podem danificar tanto o patógeno quanto o tecido não inflamado. Os monócitos iniciam o reparo tecidual, removendo células mortas no tecido lesado. A infiltração de células também é mediada por citosinas, tais como interleucinas (IL-6, IL-8, IL-12, IL-15), interferon-gama (IFN-γ) e fator de necrose tumoral-alfa (TNF-α) estimulados pela regulação nuclear através da ativação de NF-*k*B. Se a lesão continuar, a fase aguda da inflamação é alterada para um estado crônico, levando a formação de granuloma e de tecido linfático terciário (RYAN; MAJNO, 1977; MEDZHITOV, 2008; ASHLEY et al., 2012).

O tratamento mais comumente utilizado hoje é feito através do uso de anti-inflamatórios não-esteroides (AINEs). O mecanismo de ação destes fármacos se dá pela inibição das formas 1 e 2 da enzima cicloxigenase (COX-1 e COX-2), que, por consequência, inibe a produção de prostaglandinas. A COX-1 é responsável, entre outras funções, pela proteção gastrointestinal e a COX-2, por outro lado, é a responsável pelo processo inflamatório nas células do corpo. Como a maioria dos

AINEs é administrada por via oral, o principal efeito colateral são os distúrbios gastrointestinais, podendo chegar a hemorragias e úlceras gástricas (RANG et al., 2007).

Visto que o tratamento oral com anti-inflamatórios pode levar a sérios distúrbios, o tratamento tópico com estes fármacos vem apresentando grande interesse pelos usuários, já que a apresenta apenas efeito local, sem ou com raros efeitos adversos sistêmicos (HAROUTIUNIAN et al., 2010). Além disso, o tratamento com anti-inflamatórios tópicos diminui a dose administrada e também a interação com outros fármacos quando comparado com a administração pela via oral (RAZA et al., 2014). Na literatura, encontram-se diversos trabalhos abordando o uso tópico de anti-inflamatórios, tanto com fármacos já conhecidos como com produtos extraídos de plantas (MCPHERSON; CIMINO, 2013; MO et al., 2013; OLIVEIRA et al., 2013; VERAS et al., 2013; RAZA et al., 2014).

Existem diversos métodos para avaliar a atividade anti-inflamatória tópica, que podem ser divididos entre inflamação aguda e crônica. Os modelos agudos são normalmente induzidos por agentes flogísticos que promovem a liberação de mediadores inflamatórios, enquanto os modelos crônicos são induzidos pela implantação subcutânea de corpos estranhos, que promovem a formação de granulomas. Dentre os modelos de inflamação aguda pode-se citar o edema em pata de rato (induzido por carragenina, histamina ou formalina) e o edema em orelha de camundongo (induzido por xileno, óleo de cróton, ácido araquidônico, capsaisina ou fenol). Dentre os modelos de inflamação crônica pode-se citar o granuloma em ratos induzido por *pellet* de algodão (CARVALHO et al., 2005; SARAIVA et al., 2011; EDDOUKS et al., 2012).

Uma abordagem mais detalhadas desse tema com foco nos óleos essenciais e no óleo de copaiba em especial estão apresentados na forma de um artigo de revisão, apresentado no Capítulo I.

Atividade anti-inflamatória do óleo de copaiba

A espécie *Copaifera multijuga* Hayne vem demonstrando um potencial efeito anti-inflamatório em relação a outras espécies de *Copaífera*. Veiga Junior e

colaboradores (2007) fizeram um estudo comparativo entre três espécies (*Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke e *Copaifera multijuga* Hayne) em relação à composição química de seus óleos e o efeito anti-inflamatório no modelo de pleurisia em camundongos. Neste trabalho, foi verificado que a espécie *Copaifera multijuga* Hayne apresentou um efeito mais potente de inibição da pleurisia na menor dose utilizada (100 mg/Kg), com diminuição no acúmulo de leucócitos totais e neutrófilos.

Gomes et al. (2010) e Veiga Junior et al. (2006) verificaram e compararam a atividade anti-inflamatória do óleo extraído de *Copaifera multijuga* Hayne e de suas frações em hexano, clorofórmio/diclorometano e metanol. Estas frações têm por objetivo separar componentes representantes das três classes de moléculas encontradas no óleo de copaíba: sesquiterpenos, sesquiterpenos oxigenados e diterpenos, respectivamente. Em relação à atividade anti-inflamatória, estes estudos demonstraram que as frações com hexano e clorofórmio/diclorometano tendem a apresentar resultados melhores ou muito semelhantes ao do óleo puro, já que nelas estão presentes componentes com efeito anti-inflamatório proeminente, como β-cariofileno, α-humuleno e óxido de cariofileno.

NANOEMULSÕES

Nanoemulsões são sistemas heterogêneos compostos por gotículas de óleo estabilizadas por surfactantes dispersas em um meio aquoso. O tamanho de gotícula considerado para descrever uma nanoemulsão diverge muito entre os trabalhos encontrados na literatura, podendo variar entre 50 e 500 nm. Para serem utilizadas pela via tópica na área farmacêutica podemos considerar uma variação entre 100 e 300 nm, o qual vai depender da quantidade de núcleo oleoso e tensoativos, concentração de fármaco e técnica de preparação (SONNEVILLE-AUBRUN et al., 2004; GUTIÉRREZ et al., 2008; LOVELYN; ATTAMA, 2011; BURGUERA; BURGUERA, 2012; SUTRADHAR; AMIN, 2013; WU et al., 2013). A formação de uma nanoemulsão se dá pela combinação de uma fase oleosa, uma fase aquosa, uma mistura de tensoativos que compõe a interface óleo/água e pela presença de uma força mecânica (MASON et al., 2006).

A escolha da composição e da concentração de fase oleosa é de extrema importância para a formação da nanoemulsão, assim como a solubilidade de fármacos nesta fase. Pode-se utilizar tanto óleos de origem natural quanto sintética ou uma mistura destes, sendo que a concentração de núcleo oleoso varia entre 5 e 30% (SHAH et al., 2010).

A mistura de tensoativos também é importante para a formação de nanoemulsões, já que fará a estabilização da interface óleo/água no produto final. O principal aspecto para a escolha dos tensoativos é a sua toxicidade e a sua estabilidade. Existem muitas opções de tensoativos, tanto de origem natural como a lecitina (de ovo ou de soja), quanto sintéticos como o polisorbato e o monooleato de sorbitano (FLOYD, 1999; SONNEVILLE-AUBRUN et al., 2004).

As nanoemulsões podem ser produzidas de diversas maneiras, dependendo da substância ativa e excipientes a serem utilizados. Atualmente, os métodos mais citados na literatura são a homogeneização a alta pressão e a microfluidização, mas também pode-se citar a ultra-sonicação e a emulsificação espontânea (TADROS et al., 2004; ALMEIDA et al., 2008).

O método de homogeneização a alta pressão mostra-se muito vantajoso em relação aos outros, pois tem fácil escalonamento, curto tempo de produção e não utiliza solventes orgânicos. A sua única desvantagem seria a necessidade da compra de um equipamento específico para realizar a produção da nanoemulsão (PARDEIKE et al., 2009). Ainda, este método vem apresentando ótimos resultados no que se refere à veiculação de altas concentrações de fase oleosa em nanoemulsões, como descrito no estudo de Müller e colaboradores (2012), o qual relata a produção de nanoemulsões contendo até 40% de núcleo oleoso com baixo índice de polidispersão e tamanho de gotícula na faixa de 150 a 250 nm.

Após a sua produção, as nanoemulsões devem ser caracterizadas quanto as suas propriedades físicas, químicas e físico-químicas. Uma caracterização completa pode incluir: inspeção visual (presença de fenômenos como coalescência, floculação, separação da fase oleosa, mudança de coloração), doseamento da substância ativa, determinação do pH, tamanho de gotícula, carga

de superfície ou potencial zeta, índice de polidispersão, densidade, viscosidade e morfologia (FLOYD, 1999; THAKUR et al., 2013).

Nanoemulsões de uso tópico

As nanoemulsões têm sido utilizadas em pesquisas recentes com a finalidade de aperfeiçoar o desempenho terapêutico de diversos fármacos lipofílicos, pois aumentam a sua solubilidade aparente, taxa de dissolução e permeação em membranas biológicas (FATHI et al., 2012; THAKUR et al., 2013). Além disso, as nanoemulsões podem ser utilizadas em todas as vias de administração e mostram-se muito vantajosas para tratamentos na via tópica, já que apresentam baixa irritabilidade, alto poder de penetração e alta capacidade de veicular grandes quantidades de fármacos (SONNEVILLE-AUBRUN et al., 2004; TADROS et al., 2004; MOU et al., 2008; MÜLLER et al., 2012).

Visto que muitos trabalhos demonstram o potencial das nanoemulsões na absorção pela pele deve-se levar em consideração alguns fatores que podem influenciar o processo de permeação cutânea desses carreadores O reduzido tamanho de gotícula das nanoemulsões promove um aumento na superfície de contato com a pele, facilitando a sua entrada pelo estrato córneo, podendo formar reservatórios nesta camada e liberar lentamente os fármacos até as outras camadas. Além disso, acredita-se que uma carga de superfície positiva na gotícula da nanoemulsão pode interagir mais fortemente com as cargas negativas presentes no estrato córneo e facilitar a liberação de fármacos pela pele (YILMAZ; BORCHERT, 2006; SHAH et al., 2010; ABOLMAALI et al., 2011; SUTRADHAR; AMIN, 2013; THAKUR et al., 2013).

No que se refere ao uso tópico de nanoemulsões, diversos autores relatam as vantagens da administração cutânea destes carreadores, tanto para fármacos já conhecidos (ALAM et al., 2013; LAI et al., 2013; SANDIG et al., 2013; CIURLIZZA et al., 2014; KIM et al., 2014; YU et al., 2014), quanto para produtos naturais (BIDONE et al., 2014; DOMÍNGUEZ-VILLEGAS et al., 2014; LU et al., 2014). Em relação a fármacos anti-inflamatórios, as nanoemulsões já foram descritas como carreadores de nimesulida, celecoxibe, meloxicam, ibuprofeno,

aceclofenaco, diclofenaco, flurbiprofeno, indometacina, cetoprofeno, hidrocortisona e piroxicam (SHAKEEL et al., 2010).

Nosso grupo de pesquisa também já estudou o desenvolvimento de nanoemulsões contendo o óleo de copaíba (DIAS et al., 2014). Neste trabalho foi verificada a composição da nanoemulsão através de um desenho experimental, do tipo planejamento fatorial fracionado 2^{4-1}, onde variou-se a concentração dos tensoativos Span 80® e Tween 20®, a concentração de óleo de copaíba, a presença de um óleo fixo (triglicerídeos de cadeia média) e também a diferença entre dois métodos de preparação: homogeneização a alta pressão e emulsificação espontânea. Como resposta, foram verificados parâmetros de caracterização físico-química (tamanho de gotícula, potencial zeta e índice de polidispersão) e teor de β-cariofileno.

Os resultados obtidos demonstraram que o método de homogeneização à alta pressão mostrou-se mais vantajoso para produzir nanoemulsões com óleo de copaíba e que a composição mais adequada da nanoemulsão contém 20% de óleo de copaíba, 10% de TCM, 3% Span 80® e 1% de Tween 20®. Após 90 dias de armazenamento a 4 e 25 °C, a temperatura de 4°C manteve as nanoemulsões com menos sinais de instabilidade e com menor perda da fração volátil do óleo de copaíba. Além disso, o emprego de TCM na formulação como óleo fixo provou ser uma boa estratégia para fixar a fração volátil do óleo de copaíba durante a preparação e o período de armazenamento. Assim, foi comprovado que este sistema permite que uma grande quantidade de óleo de copaíba seja veiculada e que, por consequência, uma elevada quantidade de β-cariofileno esteja disponível para exercer ação farmacológica (DIAS et al., 2014).

HIDROGÉIS

Hidrogéis são polímeros com a habilidade de intumescer na presença de soluções aquosas. A estrutura do polímero é capaz de reter grandes quantidades de água, porém, devido a presença de *cross-links*, eles são insolúveis nos solventes utilizados. As redes formadas por estes polímeros são reticuladas por ligações químicas ou outras forças coesivas, tais como interações iônicas, ligações de

hidrogênio ou interações hidrofóbicas (KIN et al., 1992; PEPPAS, 2000; HAMIDI et al., 2008).

Uma ampla gama de composições poliméricas tem sido utilizada na produção de hidrogéis, as quais podem ser classificadas baseadas em diversas características destes polímeros, tais como natureza dos grupos laterais (aniônicos, catiônicos e neutros) e origem (sintéticos e naturais) (HOFFMAN, 2002; HAMIDI et al., 2008).

Dentre os hidrogéis utilizados em preparações farmacêuticas podemos citar os derivados do ácido poliacrílico, como o Carbopol® (polímeros sintéticos de natureza aniônica), os derivados de celulose como a hidroxietilcelulose (polímero semi-sintético de natureza não-iônica) e a quitosana (polímero de origem natural de natureza catiônica). Visto que existem muitas opções para o desenvolvimento de formulações tópicas utilizando-se hidrogéis, a escolha deve basear-se em requisitos como a estabilidade do produto final, liberação e eficácia da substância ativa.

A forma farmacêutica hidrogel é a uma interessante escolha para a administração tópica de compostos ativos, uma vez que apresenta um elevado teor de água, torna-se facilmente lavável e não deixa um aspecto gorduroso na pele, promovendo, assim, uma maior aceitação pelo usuário (LEONARDI et al., 2005). Além disso, a formulação de uso tópico contendo um sistema nanoemulsionado não deve apresentar maior afinidade pelo veículo do que pela pele. Assim, a incorporação em uma base hidrofílica tende a eliminar esse problema aumentando, também, a liberação dos componentes contidos na matriz do hidrogel (ALVES et al., 2007; BABOOTA et al., 2007; MOU et al., 2008).

Alguns estudos recentes vêm demonstrando o potencial da incorporação de nanoemulsões em hidrogéis relatando o aumento da permeação de compostos ativos através da pele (KARRI et al., 2015; KOOP et al., 2015; SAMPATHI et al., 2015). A fim de entender os mecanismos que regem a permeação cutânea de ativos pela pele, Khurana e colaboradores (2013) estudaram a interação de uma nanoemulsão de meloxicam incorporada em hidrogel de Carbopol® 940. Neste trabalho, os autores notaram uma modificação na estrutura dos lipídios intercelulares do estrato córneo após o ensaio de permeação cutânea com a

formulação, sugerindo que a extração destes lipídios seja um importante mecanismo no aumento da passagem de ativos pela pele.

É importante ressaltar o estudo reológico de formulações semissólidas para o uso tópico, já que este parâmetro influencia no processo da aplicação na pele, assim como a influência da temperatura, do tempo e da incorporação de nanocarreadores (BECK et al., 2011). Dependendo das características de fluxo e viscosidade, os materiais podem ser divididos entre fluidos não-Newtonianos e Newtonianos. Os fluidos Newtonianos tem viscosidade constante independente do cisalhamento aplicado (em uma dada temperatura), enquanto os fluidos não-Newtonianos mudam de viscosidade com o aumento da taxa de cisalhamento (ALLEN et al., 2011).

Segundo as características de deformação esses sistemas podem ser classificados em três grupos: pseudoplásticos, plásticos ou dilatantes. O comportamento mais comum para os hidrogéis é o pseudoplástico, pois quando a taxa de cisalhamento é aplicada há uma reestruturação nas cadeias poliméricas lineares no sentido da força aplicada. Assim, no reograma isso é apresentado como uma diminuição da viscosidade à medida que a tensão ou força de cisalhamento aumenta (NETZ; ORTEGA, 2002).

Para realizar a medida de viscosidade podem-se utilizar os viscosímetros do tipo Ostwald e de Höppler (para fluidos Newtonianos) e os viscosímetros rotatórios (para fluidos não-Newtonianos) (NETZ; ORTEGA, 2002). Um método complementar para a caracterização da viscosidade de formulações semissólidas de aplicação tópica é a determinação da espalhabilidade, característica que está profundamente ligada à aplicação da formulação no local de ação. O método usado para determinar a espalhabilidade é realizado pela sobreposição de placas sobre a amostra, visualizando-se o diâmetro atingido pela formulação (BORGHETTI; KNORST, 2006).

Normalmente, veículos semissólidos como os hidrogéis apresentam um fluxo não-Newtoniano, ao contrário das nanoemulsões que são fluidos Newtonianos, apresentando baixa viscosidade, próxima à da água. Assim, é importante avaliar a

influência da incorporação destes sistemas em hidrogéis em relação aos aspectos reológicos como viscosidade e espalhabilidade.

LEISHMANIOSE

A leishmaniose é uma doença endêmica em países de clima tropical e é a segunda doença parasitária mais prevalente no mundo após a malária (KUMAR, 2013). Apesar de apresentar uma estimativa de vinte a quarenta mil mortes por ano, essa doença pertence ao grupo das doenças negligenciada, já que a maioria dos afetados são pessoas pobres em países subdesenvolvidos (SAVOIA, 2015). De acordo com a Organização Mundial da Saúde, existem 2 milhões de novos casos todos os anos e 350 mil pessoas consideradas em risco (WHO, 2010). A doença é transmitida pelo flebotomíneo, ou mosquito-palha (gêneros *Lutzomya* e *Phlebotomus),* infectado com o parasito *Leishmania* spp, e pode levar a três tipos de manifestações clínicas: leishmaniose cutânea (LC), localizada ou difusa, leishmaniose mucocutânea (LMC) e leishmaniose visceral (LV) (SAVOIA, 2015). No continente americano, a leishmaniose cutânea também é conhecida como leishmaniose tegumentar americana (LTA).

Existem diversas espécies de *Leishmania* que podem causar a leishmaniose e suas formas de manifestação clínica no ser humano (Tabela 1). Durante seu ciclo de vida, o parasito pode apresentar-se em duas formas: promastigota e amastigota. O vetor inocula a forma promasigota na pele do hospedeiro, que é fagocitada por macrófagos, onde se transforma na forma amastigota. No macrófago, o parasito se multiplica e infecta outros macrófagos, por consequência. Na leishmaniose cutânea, a infecção limita-se à pele e sistema linfático, podendo chegar em mucosas, como na boca, nariz e faringe (caracterizando a forma mucocutânea da doença, LMC). Já a forma visceral (LV), atinge órgãos internos vitais e pode ser fatal se não tratada (BAILEY; LOCKWOOD, 2007; PACE, 2014).

Além de humanos, o parasito da leishmaniose também utiliza outros mamíferos (domésticos e silvestres) como hospedeiros, como cachorros, gatos, cavalos, roedores e marsupiais (SAVOIA, 2015). Cachorros domésticos são hospedeiros importantes principalmente em áreas urbanas e contribuem na cadeia

epidemiológica já que podem permanecer assintomáticos por longos períodos de tempo, contribuindo para a transmissão e manutenção da doença (DINIZ et al., 2008).

Tabela 1. Principais espécies causadoras de leishmaniose, suas localizações geográficas e manifestações clínicas (REITHINGER et al., 2007; PACE, 2014).

Espécie de *Leishmania*	Localização	Manifestação clínica
L. aethiopica	Etiópia, Quênia	Cutânea (localizada e difusa), mucocutânea
L. amazonensis	América do Sul	Cutânea (localizada e difusa)
L. braziliensis	América do Sul, partes da América Central, México	Cutânea (localizada), mucocutânea
L. donovani	África, centro e sudoeste asiático	Cutânea (localizada), visceral
L. gyyanensis	América do Sul	Cutânea (localizada), mucocutânea
L. infantum/L. chagasi	Europa, norte da África, América central e América do Sul	Cutânea (localizada), visceral
L. major	Ásia central, norte e leste da África, Oriente-médio	Cutânea (localizada)
L. mexicana	América Central, México, Estados Unidos	Cutânea (localizada e difusa), mucocutânea
L. panamensis	América do Sul (norte) e América Central (sul)	Cutânea (localizada), mucocutânea
L. peruviana	Peru	Cutânea (localizada), mucocutânea
L. tropica	Centro e sudoeste asiático, norte da África e Oriente-médio	Cutânea (localizada)

No Brasil, a leishmaniose está presente em todos as regiões, mais prevalentemente nas regiões norte e nordeste, e vem avançando das zonas rurais e florestais, principalmente devido ao desmatamento, mudanças climáticas e à migração de famílias para as zonas urbanas (LINDOSO; LINDOSO, 2009;

DAWIT et al., 2013). Segundo o Ministério da Saúde, foram reportados no Sistema de Informação de Agravos de Notificação (Sinan) cerca de 20.000 casos confirmados de LTA e 3.000 de LV no país entre os anos de 2000 e 2013 (BRASIL. MINISTERIO DA SAUDE, 2015). As principais espécies de *Leishmania* causadoras da LTA no Brasil são *L. braziliensis, L. guyanensis, L. amazonensis, L. naiffi, L. lainsoni, L. shawi* e *L. lindenbergi* (GUERRA et al., 2015). Os caso de LV no país são causados principalmente pela *L. infantum/chagasi* (LINDOSO; LINDOSO, 2009).

Tratamentos para a leishmaniose

O tratamento de primeira escolha para a leishmaniose é feito com antimoniais pentavalentes, como o antimoniato de meglumina (Glucantime®, Aventis, França), e estibogluconato de sódio (Pentosan®, GSK, Reino Unido). Seu mecanismo de ação ainda é pouco conhecido, mas sabe-se que são absorvidos pelos macrófagos, onde se transformam na forma ativa da molécula, que interfere em processos energéticos do parasito (SILVA-JARDIM et al., 2014). O tratamento com esses medicamentos pode ser administrado pelas vias intramuscular ou intravenosa para tratamentos contra a forma visceral e também intralesional para a forma cutânea da doença (MINODIER; PAROLA, 2007). No entanto, os antimoniais pentavalentes apresentam efeitos colaterais severos (mialgia, problemas cardíacos e renais, dor abdominal, dor de cabeça, etc), requerem longo tempo de tratamento e já apresentam casos de resistência (AMEEN, 2010).

Outros medicamentos utilizados no tratamento da leishmaniose são a pentamidina, a anfotericina B e a miltefosina. A pentamidina é utilizada como segunda escolha em pacientes refratários ou intolerantes ao tratamento com antimoniais. Pode ser utilizada para todas as formas da doença pela via intramuscular ou por infusão intravenosa, porém apresenta efeitos adversos que limitam seu uso, como diabetes *mellitus*, hipoglicemia, toxicidade cardíaca, hepática e renal. O seu mecanismo de ação também não está bem esclarecido, mas parece estar relacionado com danos na mitocôndria do parasito (SILVA-JARDIM et al., 2014).

A anfotericina B, apesar de ser um antifúngico, é considerada um leishmanicida de segunda geração e é utilizada em casos onde os antimoniais pentavalentes falharam. Seu mecanismo de ação se dá pela ligação com os esteróis da membrana celular do parasito, levando à morte celular. Devido a sua toxicidade e efeitos adversos gerados pela administração da anfotericina B, formulações lipídicas (lipossoma, dispersão coloidal e complexo lipídico) contendo este fármaco são atualmente utilizadas no tratamento da leishmaniose, resolvendo problemas como urticária e insuficiência renal. Porém são medicamentos caros, o que pode prejudicar o tratamento em países mais pobres (CHÁVEZ-FUMAGALLI et al., 2015).

A miltefosina foi o primeiro leishmanicida oral a ser produzido e pode ser utilizado em todas as formas clínicas de leishmaniose. No entanto, apresenta limitações como alto custo e efeitos adversos como náusea, vômitos, toxicidade hepática e renal e teratogenicidade. Seu mecanismo de ação se dá pela inibição da biosíntese de fosfolipídios e esteróis (KEVRIC et al., 2015; SAVOIA, 2015).

Atividade leishmanicida do óleo de copaíba

A atividade leishmanicida do óleo de copaíba já foi demonstrada em alguns trabalhos publicados na literatura, entretanto os resultados são muito variáveis, dada a quantidade de espécies existentes da planta e de suas características individuais. Os testes avaliam principalmente a atividade *in vitro* do óleo nas formas promatigota e amastigota axênica do parasito além da forma mais interessante ao tratamento da doença, a forma amastigota intramacrofágica. A Tabela 2 apresenta os valores de CI_{50} (concentração inibitória de 50% dos parasitas) encontrados nos trabalhos citados, bem como valores de CC_{50} (concentração citotóxida para 50% das células) para os macrófagos não infectados.

Santos e colaboradores (2008) descreveram a atividade leishmanicida para o óleo de nove espécies de *Copaifera* encontradas no Brasil (*C. reticulata, C. martii, C. cearensis, C. paupera, C. langsdorfii, C. officinalis, C. multijuga* e *C. lucens*) em *Leishmania amazonensis*. Dentre todas as espécies, a que melhor apresentou atividade na forma promastigota foi a amostra de *C. reticulata* (IC_{50} de

5,0 µg/mL). Já nas formas amastigota axênica e amastigota intracelular esta amostra apresentou valores maiores de IC_{50} (15,0 e 20,0 µg/mL, respectivamente). Em seguida, o mesmo grupo de pesquisa (SANTOS et al., 2011) testou o efeito do tratamento oral, tópico ou subcutâneo do óleo de copaíba (*Copaifera martii*, em formulação semissólida ou não) em modelo de leishmaniose cutânea (*Leishmania amazonensis*) em pata de camundongos BALB/c. O tratamento oral conjugado ao tratamento tópico demonstrou ser eficaz na diminuição da lesão quando comparado aos controles positivo e negativo. Entretanto, os tratamentos tópico e subcutâneo não foram capazes de fazer o mesmo. Além disso, após o tratamento com o óleo de *Copaifera reticulata* Ducke, mudanças importantes na morfologia dos parasitos foram verificadas em microscópio eletrônico, como modificação no formato das células e aumento do tamanho da mitocôndria (SANTOS et al, 2012).

Em trabalho mais recente, Gupta e colaboradores (2015) produziram uma nanoemulsão contendo óleo de copaíba e anfotericina B, obtendo valores baixos de IC_{50} nas formas amastigotas intramacrofágicas de *Leishmania donovani*, valores ainda menores do que os apresentados nos trabalhos anteriores, tanto para a nanoemulsão contendo o óleo quanto para o óleo puro. Ainda, os autores afirmam que os resultados obtidos decorrem do efeito sinérgico entre o óleo e a anfotericina B, porém não foram feitos testes de sinergismo para confirmar esta afirmação.

Já foi demonstrado também o efeito de alguns dos componentes majoritários do óleo de copaíba, como o β-cariofileno e ácidos diterpênicos isolados a partir do óleo. Soares e colaboradores (2013) compararam amostras de óleo de copaíba ricas no sesquiterpeno β-cariofileno, duas frações (uma rica em sesquiterpenos e uma rica em diterpenos) e o padrão de β-cariofileno. Neste trabalho, as amostras apresentam valores baixos de IC_{50} na forma amastigota intramacrofágica do parasito *Leishmania amazonensis,* com valores 20 vezes menores comparados aos apresentados por Santos et al. (2008). Tanto a fração rica em sesquiterpenos quanto o β-cariofileno apresentaram atividade leishmanicida similar ao óleo na forma amastigota. Já a fração rica em diterpenos foi ativa apenas na concentração mais alta testada.

Tabela 2. Valores de CI_{50} do óleo de copaíba encontrados para diferentes formas do parasito causador da leishmaniose nos diferentes trabalhos na literatura.

Referência	Espécie de *Leishmania*	Espécie de *Copaifera*	Forma estudada	CI_{50} (µg/mL)	CC_{50} (µg/mL)
Santos et al., 2008	*L. amazonensis*	*C. reticulata*	Promastigota	5,00	37,50
			Amastigota axênica	15,00	
			Amastigota intramacrofágica	20,00	
Soares et al., 2013	*L. amazonensis*	Amostras comerciais, sem especificação	Amastigota intramacrofágica	2,30 – 2,90	50,00
Gupta et al., 2015	*L. donovani*	Amostra comercial, sem especificação	Amastigota intramacrofágica	Óleo: 0,38 NE-óleo: 0,33 NE-óleo-AB: 0,018	20,00

NE-óleo: Nanoemulsão contendo óleo de copaíba; NE-óleo-AB: Nanoemulsão contendo óleo de copaíba e anfotericina B

Santos e colaboradores (2013) isolaram 6 ácidos diterpênicos de óleos de copaíba e testados na formas promastigota e amastigota de *Leishmania amazonensis*. Os diterpenos mais ativos contra as formas promastigotas foram o ácido hidroxicopálico e o metilcopalato (IC_{50} de 2,5 e 6,0 µg/mL, respectivamente) e contra as formas amastigotas foram o ácido pinifolico e o ácido kaurenoico (IC_{50} de 4,0 e 3,5 µg/mL, respectivamente). Além disso, foram visualisadas em microscópio eletrônico alterações estruturais após o tratamento com ácido

hidroxicopálico, tais como ruptura da membrana plasmática, perda de conteúdo celular e alterações na membrana flagelar.

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CAPÍTULO I

Manuscrito do artigo de revisão a ser submetido à publicação

In vivo acute anti-inflammatory activity of essential oils

*Leticia G. Lucca, Letícia S. Koester**

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

** Correspondence author:*
Letícia Scherer Koester
Mailing address: Avenida Ipiranga 2752, Laboratório 606, 90610-000, Porto Alegre/RS, Brasil
Office telephone: +55 51 33085278
Fax number: +55 51 33085243
E-mail: leticia.koester@ufrgs.br

Abstract

In the last few years, there was an increase in the search for new and alternative strategies for inflammation treatment. Many studies present natural products as an alternative treatment, searching for scientific proof from natural medicines used in popular or traditional medicines. Besides their function on plants (insect attraction and repellency), essential oils have also pharmacologic effect, such as antibacterial, antifungal, antimutagenic, antiviral, antiprotozoal, antioxidant, anti-inflammatory and antidiabetic. In this review, we describe the most used *in vivo* acute inflammation assays and the studies that present essential oil anti-inflammatory activity *in vivo*. Essential oil from plants from Asteraceae, Burseraceae, Boraginaceae, Cupressaceae, Euphorbiaceae, Lamiaceae, Lauraceae, Leguminosae, Myrtaceae, Piperaceae, Poaceae, Rutaceae, Umbelliferae, Verbenaceae and Zingiberaceae families were described as anti-inflammatory *in vivo*. Five acute inflammation models were used in the papers disclosed in this work: paw end ear edema, pleurisy, peritonitis and air pouch. The most common model was paw edema, especially due to the facility to perform. Many studies describe the search for inflammatory mediators, such as cytokines, prostaglandine E2 and nitric oxide, which could explain the mechanism of action.

Keywords*:* essential oil, *in vivo* models, acute inflammation, edema, pleurisy, peritonitis, air pouch

1. Introduction

In the last few years, there was an increase in the search for new and alternative strategies for inflammation treatment. Many studies present natural products as an alternative treatment, searching for scientific proof from natural medicines used in popular or traditional medicines.[1]

It is well known in the literature that essential oils are secondary metabolites that present important physiological effects on plants, such as insect attraction and repellency.[2] Nevertheless, essential oils are widely used in folk medicine around the world for many syndromes and are also used as flavoring and fragrance,

expressing an important role as a raw materials in cosmetic and chemical industries.[3] In general, they are a complex mixture of low molecular weight substances, with lipophilic and volatile characteristics, that can be synthetized in the plant in oil cells, secretion ducts or cavities and in glandular hairs.[4,5]

Essential oils are extracted using different techniques such as steam distillation, hydrodistilation, supercritical fluid extraction, microwave oven extraction or cold pressing (for peels from citrus fruits).[6] Each technique and the part of the plant used to extract the essential oil can provide different composition in the final product.[3] For example, the oil extracted from bitter orange tree (*Citrus aurantium* L., Rutaceae) has different composition depending on the part (zest, leaves and flowers) that it is extracted.[7]

Besides their function on plants, essential oils have also pharmacologic effect, such as antibacterial, antifungal, antimutagenic, antiviral, antiprotozoal, antioxidant, anti-inflammatory and antidiabetic. Frequently, the major components in essential oils, such as phenylpropanoids and terpenoids, are the responsible for these activities. However, it is not often that isolated components produce a better activity as the essential oil it is extracted from, indicating that the presence of a complex mixture of components present an additive or even a synergic effect.[8–11]

Terpenes are substances that derive from isoprene units, which, in turn, are derived from mevalonic acid on aromatic plants.[12] They present a diverse chemical class, containing hydrocarbons and oxygenated substances, with number of carbons varying from 10 to 40 units.[13] Normally, monoterpenes (C_{10}) and sesquiterpenes (C_{15}) are the terpenoids found in essential oils.[14] Terpenes with more than 20 carbon units are not volatile like mono- and sesquiterpenes and are mostly found in plant resins and other organisms (marine algae, mollusks, insects, etc).[15]

In general, inflammation is a response to infection, injury or exposure to external particles, leading to local extravasation of blood cells and fluid.[16] Normally it is described by four signs: heat, pain, redness and swelling.[17] The first response to the injury is intermediated by local macrophages and mast cells, which leads to production of inflammatory mediators (histamine, prostaglandins and leukotrienes), increase vascular permeability and release of effector cells

(neutrophils and monocytes). Neutrophils release reactive oxygen and nitrogen species (ROS and RNS) that can damage the pathogen whereas monocytes initiate tissue repair, removing dead cells in the injured tissue. Cell infiltration is also mediated by cytokines such as interleukins (IL-6, IL-8, IL-10, IL-12, IL-15), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) stimulated by nuclear regulation through NF-κB activation. If the injury continues, the inflammation acute phase is changed to a chronic state, leading to granuloma and tertiary lymphoid tissue formation.[18,19]

In this paper, we describe a literature review on essential oils' anti-inflammatory activity, as well as the most used *in vivo* tests to assess this type of action.

2. *In vivo* acute inflammation models

There are several animal models to study the inflammatory response, both in acute and chronic levels. Most papers researching essential oils as treatment present acute models, such as paw edema, ear edema, pleurisy and peritonitis. I*n vivo* acute inflammation models are important to discover mechanisms of action and present reliable and constant results for new drugs.

Table 1 summarizes the main models found in the literature for *in vivo* acute anti-inflammatory assay. It is important to highlight that edema models alone do not show the anti-inflammatory effect, and assays to search cytokines or histopathological analyses in the samples can prove this effect. Each model present its advantages and disadvantages, and have its peculiarities.

Nevertheless, it seems that carrageenan is the "first-choice" irritant agent for most models used in the literature.

2.1 Paw edema

The most common *in vivo* inflammation model is the paw edema, which is used to test new ant-inflammatory drugs or to discover the mechanism of action involved in inflammation.[20] It is normally performed in rats, but can also be performed in mice. Many phlogistic agents can be used in this assay, such as carrageenan,

dextran, bradykinin, substance P, histamine, egg albumin, arachidonic acid and formalin (Table 1).

Carrageenan is the main agent used as irritant. It produces the principal signs of inflammation right after it is applied in the paw (edema, erythema and hyperalgesia) and can last for maximum five hours. [21] Biochemically, the injection of carrageenan releases initially (first phase, up to 3 hours after the injection) histamine and 5-hydroxytryptamine (5-HT), which increase vascular permeability, followed by cell infiltration (predominantly neutrophils), release of arachidonic acid products (such as prostaglandins) and increase in COX-2 levels (second phase, from 3 to 5 hours).[20,22–24]

Carrageenan is applied subcutaneously, 0.1 mL at 1% (w/v) concentration, in the right hind paw of the animal and, for comparison, the other hind paw receives the same volume of saline solution. It is important to highlight that all animals must be sedated to receive the carrageenan injection. The results are calculated by the swelling of the inflamed paw, and there are three methods that can be used: paw circumference using a cotton thread, paw thickness using a caliper (digital or not) or paw volume using a plethysmometry. After the end of the assay the paws can be collected and examined by imunohistochemistry or by quantification of specific inflammatory mediators, such as cytokines and chemokines.[25]

2.2 Ear edema

Another common model is the ear edema. It can be performed with different irritants (Table 1) and provides a quick response with consistent results to test anti-inflammatory drugs.[26] It is the first-choice method to study topical administration of drugs and can mimic atopic dermatitis, for exemple.[27] In research papers, the most used phlogistic agents are croton oil, 12-O-tetradecanoylphorbol acetate (TPA) and arachidonic acid. Arachidonic acid-induced edema involves both cycloxygenase and 5-lipoxygenase pathways of inflammation (production of prostaglandins and leukotrienes, respectively), which are very important to describe the mechanism of action of anti-inflammatory drugs (specially to NSAIDs).[27,28] Croton oil and its sub-product TPA-induced ear edema, differently

from arachidonic acid, provoke histamine, serotonin and prostaglandins release, on which COX inhibitors are more effective.[29]

Normally in this test, the irritant is applied topically in the ear surface. To evaluate anti-edematogenic effect, ears are cut in a 6-8 mm disk and weighted to reveal the response. Ears can also be used to search for inflammatory mediators and confirm the anti-inflammatory effect.[25]

2.3 Pleurisy

The pleural cavity irritation presents a good and reliable acute inflammation model, since researchers can easily find inflammatory cell migration (especially neutrophils, which, normally, are not found in the cavity), fluid extravasation and biochemical mediators for inflammation. In this method, the irritant agent (Table 1) is injected to the pleural cavity and, in the end of the experiment, the exudate formed in the cavity is collected to perform inflammatory cell count and biochemical parameters analysis.[30,31]

2.4 Peritonitis

The injection of phlogistic agents to the peritoneum is a simple model that can complement the edema assays previously described, by producing an exudate with inflammatory mediators and migratory cells.[17] Briefly, the irritant (Table 1) is administrated by intraperitoneal route and, at times determined by the researcher, animals can be sacrificed to proceed the exudate collection.[32]

2.5 Air pouch

Subcutaneous air pouch *in vivo* model can be used to study both acute and chronic inflammation process. Basically, sterile air is injected to the intra-scapular area in the animal's back. The irritant agent, such as carrageenan, is injected to the pouch to form the inflammatory response (normally 6 days after pouch formation), releasing inflammatory mediators, cell migration and exudate production.[33]

Table 1. Most common *in vivo* assays to test anti-inflammatory effect in rats and mice.

In vivo assay	Phlogistic agent	Measurement	Specie
Paw edema	Carrageenan Dextran Bradykinin Substance P Histamine Egg albumin Arachidonic acid Formalin	Paw volume Paw circumference Paw thickness	Rat/Mouse
Ear edema	12-O-tetradecanoylphorbol acetate Arachidonic acid Croton oil Carrageenan Capsaicin Xylene Zymosan	Ear weight Ear thickness	Rat/Mouse
Pleurisy	Carrageenan Bradykinin Zymosan	Pleural exudate recovery and cytological analysis	Rat
Peritonitis	Carrageenan Lipopolysaccharide Zymosan	Cytological analysis from peritoneum cells	Mouse
Air pouch	Subcutaneous injection of air and phlogistic agent (carrageenan)	Cytological analysis in air pouch	Rat/Mouse

Table 2. Essential oils and *in vivo* models used to describe their anti-inflammatory activity.

Plant material	Extraction method	Major components	*In vivo* anti-inflammatory assay	Administration route	Dose tested	Reference
ASTERACEAE						
Vanillosmopsis arborea (bark)	Steam distillation	α-Bisabolol, α-cadinol, elemicine, β-bisabolene	Croton oil, arachidonic acid, capsaicin, phenol or histamine-induced ear edema	Topical	50, 100 mg/ml	Leite *et al*., 2011[34]
BURSERACEAE						
Protium heptaphyllum (trunk resin), *P. strumosum*, *P. grandifolium*, *P. lewellyni*, *P. hebetatum* (leaves)	Hydrodistillation	Terpinolene, cymene, caryophyllene, limonene	Zymozan or LPS-induced pleurisy	Oral	100 mg/kg	Siani *et al*., 1999[35]
Boswellia carterii (frankinsence)	ND	α-Pinene, linalool, 1-octanol	Xylene-induced ear edema; formalin-induced paw edema	Topical	ND	Li *et al*, 2016[36]
BORAGINACEAE						
Cordia verbenacea (leaves)	Hydrodistillation	α-humulene and trans-caryophyllene	Carrageenan, bradykinin, substance P, histamine or PAF-induced paw edema; carrageenan-induced pleurisy; carrageenan-induced air pouch	Oral	150, 300, 600 mg/kg	Passos *et al*., 2007[37]
CUPRESSACEAE						
Chamaecyparis obtusa (leaves)	Steam distillation	Bornyl acetate, D-limonene, myrcene	Formalin-induced paw edema	Intraperitoneal	5, 10 mg/kg	Park *et al*., 2015[38]

Tetraclinis articulata (aerial parts)	Hydrodistillation	Bornyl acetate, camphor, α-pinene	Carrageenan-induced paw edema	Oral	100, 200 mg/kg	El Jemli *et al.*, 2016[39]
EUPHORBIACEAE						
Croton argyrophyllus (leaves)	Hydrodistillation	Bicyclogermacrene, spathulenol, (E)-caryophyllene, β-elemene	Carrageenan-induced paw edema; carrageenan-induced peritonitis	Oral	10, 30, 100 mg/kg	Ramos *et al.*, 2013[40]
LAMIACEAE						
Lavandula angustifolia (leaves)	Hydrodistillation	1,8-Cineole, borneol, camphor	Carrageenan-induced paw edema	Oral	200 mg/kg	Hajhashemi *et al.*, 2003[41]
Rosmarinus officinalis (leaves)	Steam distillation	Myrcene, 1,8-cineol, 1-*O*-menthen-8-ol	Carrageenan-induced paw edema; carrageenan-induced pleurisy	Oral	125-750 mg/kg	Takaki *et al.*, 2008[42]
Nepeta cataria (leaves)	Hydrodistillation	Trans-trans nepetalactone, trans-cis nepetalactone	Carrageenan-induced paw edema	Intraperitoneal	0.5 µl/kg	Ricci *et al.*, 2010[43]
Nepeta pogonosperma (aerial parts)	Hydrodistillation	1,8-cineole and 4aα,7α,7aβ-nepetalactone	Formalin-induced paw edema	Intraperitoneal	50, 100, 200 mg/kg	Ali *et al.*, 2012[44]
Lavandula augustifolia (ND)	Commercial sample	Linalol, linalyl acetate	Carrageenan-induced pleurisy; croton oil-induced ear edema	Oral and topical	0.6 mg/kg (oral) 50µl/ear (topical)	Da Silva *et al.*, 2015[45]
Stachys lavandulifolia (aerial parts)	Hydrodistillation	(-)-α-Bisabolol, bicyclogermacrene, δ-cadinene, spathulenol	Carrageenan-induced pleurisy	Oral	50 mg/kg	Barreto *et al.*, 2016[46]

Ocimum basilicum (leaves)	Steam distillation	Estragole, linalool, 1,8-cineol	Carrageenan, dextran, histamine, arachidonic acid-induced paw edema; carrageenan-induced peritonitis	Oral	10, 25, 50,100 mg/kg	Rodrigues *et al*., 2016[47]
Pogostemon cablin (leaves)	ND	Patchoulol, α-bulnesene, α-guaiene, seychellene, α-patchoulene	Zymosan-induced peritonitis	Oral	50,100,200, 300 mg/kg	Silva-Filho *et al;*, 2016[48]
Hyptis martiusii Benth (leaves)	Hydrodistillation	1,8-cineole, δ-carene, camphor	Croton oil-induced ear edema; carrageenan-induced paw edema	Oral and topical	50, 75, 100 mg/kg	Barbosa *et al.*, 2017[49]
Ocimum basilicum (ND)	ND	ND	Carrageenan, dextran, histamine, arachidonic acid-induced paw edema; carrageenan-induced peritonitis	Oral	5, 10 mg/kg	Rodrigues *et al*., 2017[50]
LAURACEAE						
Cinnamomum insularimontanum (fruit)	Hydrodistillation	Citral, citronellal, citronellol, α-pinene	Croton oil-induced ear edema	Topical	100 µg/ear	Lin *et al*., 2008[52]
Ocotea quixos (calices)	Steam distillation	Trans-cinnamaldehyde, methyl cinnamate	Carrageenan-induced paw edema	Oral	10, 30, 100 mg/kg	Ballabeni *et al.*, 2010[53]
Cinnamomum cassia (twigs)	Hydrodistillation	(E)-Cinnamaldehyde	Carrageenan-induced paw edema	Oral	15, 30, 60 mg/kg	Sun *et al*, 2016[51]
LEGUMINOSAE						

Copaifera L. (oilresin)	Trunk exudation	β-bisabollene, β-caryophyllene, β-cubellene.	Carrageenan-induced paw edema	Oral	0.7, 0.98, 1.37, 1.92, 2.96 ml/kg	Basile *et al.*, 1988[56]
Copaifera L. (oilresin)	Comercial samples	α-bergamotene, β-caryophyllene, α-aromadendrene	Carrageenan or bradykinin-induced paw edema	Intraperitoneal	ND	Veiga-Junior *et al.*, 2001[57]
Copaifera duckei (oilresin)	Trunk exudation	β-Bisabolene, α-bergamotene, β-caryophyllene	Carrageenan-induced paw edema; croton oil-induced ear edema	Topical	517, 1035, 1802 mg/kg	Carvalho *et al.*, 2005[55]
Copaifera multijuga (oilresin)	Trunk exudation	β-Caryophyllene, humulene, α-copaene, calerene	Carrageenan or bradykinin-induced paw edema	Intraperitoneal	30 mg/kg	Veiga-Junior *et al.*, 2006[59]
Copaifera cearensis, C. reticulata, C. multijuga (oilresin)	Trunk exudation	β-caryophyllene, α-humulene, α-copaene	Zymosan-induced pleurisy	Oral	100, 200, 400 mg/kg	Veiga-Junior *et al.*, 2007[54]
Copaifera multijuga (oilresin)	Trunk exudation	ND	Carrageenan, histamine or serotonin–induced paw edema	Oral	150 mg/kg	Gomes *et al.*, 2010[60]
Copaifera multijuga (oilresin)	Trunk exudation	β-caryophyllene, α-copaene, β-bisabolene, α-trans-bergamotene	Carrageenan-induced pleurisy	Oral	100, 200 mg/kg	Kobayashi *et al.*, 2011[58]
MYRTACEAE						
Eucalyptus citriodora, E. tereticornis, E. globulus (leaves)	Steam distillation	ND	Carrageenan or dextran-induced paw edema; carrageenan-induced peritonitis	Subcutaneous	10, 100 mg/kg	Silva *et al.*, 2003[61]

PIPERACEAE						
Peperomia serpens (whole plant)	Steam distillation	(E)-Nerolidol, ledol, α-humulene	Carrageenan or dextran-induced paw edema; croton oil-induced ear edema; carrageenan-induced peritonitis	Oral	188.8 mg/kg	Pinheiro *et al.*, 2011[62]
Piper aleyreanum (leaves and stems)	Steam distillation	Caryophyllene oxide, β-pinene, spathulenol, camphene	Carrageenan-induced pleurisy	Oral	1-100 mg/kg	Lima et al., 2012[63]
Piper vicosanum (leaves)	Hydrodistillation	Limonene, γ-elemene, α-alaskene	Carrageenan-induced pleurisy; carrageenan-induced paw edema	Oral	100, 300 mg/kg	Brait *et al*., 2015[65]
Piper glabratum (leaves)	ND	β-Pinene, longiborneol, α-pinene, caryophyllene	Carrageenan-induced paw edema; carrageenan-induced pleurisy	Oral	10,100,300, 700 mg/kg	Branquinho *et al*, 2017[64]
POACEAE						
Cymbopogon flexuosus (leaves)	Steam distillation	ND	Carrageenan-induced paw edema	Oral	50, 100,200 mg/kg	Chandrashekar *et al.*, 2010[66]
Cymbopogon validus (leaves and flowers)	Hydrodistillation	Artemisia ketone, linalool, northujane, verbenone	Egg albumin-induced paw edema	Oral	ND	Rungqu *et al*., 2016[67]
RUTACEAE						
Citrus latifolia (fruit)	Steam distillation	Limonene	Zymosan-induced peritonitis	Oral	125, 250, 500 mg/kg	Kummer *et al.*, 2013[68]

Citrus limon, C. latifolia, C. aurantifolia, C. limonia (fruit peel)	Hydrodistillation	Limonene, γ-terpinene	Carrageenan-induced air pouch	Oral	10, 30, 100 mg/kg	Amorim *et al.*, 2016[69]
UMBELLIFERAE						
Heracleum persicum (fruit)	Hydrodistillation	Hexyl butyrate, octyl acetate, hexyl 2-methylbutanoate, hexyl isobutyrate	Carrageenan-induced paw edema	Oral and intraperitoneal	50-500 mg/kg	Hajhashemi *et al*., 2009[74]
Angelica sinensis (roots)	Hydrodistillation	(Z)-Ligustilide, (Z)-butylidenephthalide	Carrageenan-induced paw edema	Oral	0.088, 0.176, 0.352 ml/kg	Yao *et al*., 2015[72]
Angelica sinensis (ND)	Hydrodistillation	ND	LPS-induced peritonitis	Oral	0.176 ml/kg	Li *et al*., 2016[71]
Angelica dahurica (roots)	Supercritical fluid	Dodecyl alcohol, elemene hexadecanoic acid, α-pinene	Xylene-induced ear edema; carrageenan-induced paw edema	Oral	100, 200 mg/kg	Wang *et al*., 2016[70]
Angelica sinensis (roots)	Hydrodistillation	ND	Carrageenan-induced paw edema	Oral	0.176 ml/kg	Zhong *et al*., 2016[73]
VERBENACEAE						
Lippia sidoides (leaves)	Steam distillation	Thymol and *E*-caryophyllene	TPA-induced ear edema	Topical	1, 10 mg/ear	Monteiro *et al*., 2007[77]
Lippia gracilis (leaves)	Hydrodistillation	Thymol, *p*-cymene, methyl thymol, carvacrol	Carrageenan-induced paw edema; carrageenan-induced peritonitis	Oral	50, 100, 200mg/kg	Mendes *et al*., 2010[76]

Lippia gracilis (leaves)	Hydrodistillation	Carvacrol, *o*-cymene, γ-terpinene, β-caryophyllene	Carrageenan-induced air pouch	Oral	10, 30, 100 mg/kg	Guilhon *et al.*, 2011[75]
Lippia sidoides (leaves)	Hydrodistillation	Thymol, ρ-cymene, ethyl-methyl carvacrol	Croton oil, arachidonic acid, phenol or histamine-induced ear edema	Topical	100 mg/ml	Veras *et al.*, 2013[78]
ZINGIBERACEAE						
Zingiber cassumunar (rhizome)	Steam distillation	Sabinene, Terpinen-4-ol, γ-terpinene, α-terpinene, DMPBD	Carrageenan-induced paw edema	Topical	3-24 mg/paw	Pongprayoon *et al.*, 1996[79]
Curcumae wenyujin (ND), *Chrysanthemum indicum* (capitulum), *Pogostemon cablin* (ND)	Supercritical fluid and commercial sample	ND	Carrageenan-induced paw edema	Oral	42.5, 85, 170 mg/kg	Su *et al.*, 2012[80]

ND: not described; LPS: lipopolysaccharide; TPA: 12-O-tetradecanoylphorbol 13-acetate; PAF: platelet-activating factor; DMPBD: (E)-1-(3,4-dimethoxyphenyl) butadiene.

3. Anti-inflammatory activity from essential oils

Natural products are reported in the literature as important sources to treat inflammation-related diseases, especially the ones used in folk medicine. Essential oils are natural products with highly promising anti-inflammatory effect. Table 2 summarizes all studies described in this section.

3.1 Asteraceae

Vanillosmopsis arborea Baker is a Brazilian plant, which produces an essential oil rich in bisabolol, a terpene with anti-inflammatory properties, widely used in cosmetics.[14] Leite et al. (2011)[34] tested the topical administration of this oil (50 and 100 mg/ml doses) on mice ear edema induced by different inflammatory agents (*i.e*. croton oil, arachidonic acid, capsaicin, phenol and histamine).

On croton oil and arachidonic acid models, the smaller dose was able to decrease edema formation, while the higher dose had no or not significant effect. On the ears irritated with phenol, the treatment presented a decrease on edema in a dose-dependent mode. In contrast, both doses were not effective to decrease edema on capsaicin and histamine models. These results could explain the mechanism of action, since croton oil, arachidonic acid and phenol present similar inflammation pathways (release of cytokines, inflammatory cells migration, release of histamine and serotonin and synthesis of inflammatory eicosanoids by cyclooxygenase and lipoxygenase enzymes), and capsaicin and histamine present other.

3.2 Burseraceae

In the Burseraceae family, the frankincense from *Boswellia carterii* is also a popular medicine in the traditional Chinese medicine used as an analgesic. Li *et al* (2016)[36] studied the effect of the essential oil extracted from the frankincense in this plant in two edema models: ear edema induced by xylene and paw edema induced by formalin. In both assays, topical administration of the oil reduced swelling. However, there was no research for inflammatory cytokines that could explain the mechanism of action.

Another plant genus from the Burseraceae family was studied by Siani *et al* (1999)[35]. Authors researched the anti-inflammatory effect from the essential oil of four species of *Protium* on pleurisy induced by LPS and zymosan. The oral treatment in the pleurisy induced by zymosan reduced protein extravasion to the cavity for three species, but did not change the number of leucocytes cells counted. Regarding the treatment for the pleurisy induced by LPS, only one specie (*P. hebetatum* Daly) could reduce all inflammatory cells (leukocyte, mononuclear cells and neutrophils) count. Authors showed that the plants studied have promising anti-inflammatory effect, but did not present a mechanism of action for them.

3.3 Boraginaceae

Cordia verbenacea is a plant used on folk medicine in Brazil. Passos and co-workers (2007)[81] verified its essential oil effect in several inflammatory models. First, the oral treatment reduced significantly the edema in rat and mice paws induced by carrageenan

(300 and 600 mg/kg for mice and 600 mg/kg for rats). On rat paw edema, the treatment also reduced myeloperoxidase (MPO) activity, which indicates indirectly the neutrophil content in the paw tissue and TNF-α, which is an important cytokine release on inflammatory response. Next, the oil was similarly able to reduce edema induced by several inflammatory mediators that participate on carrageenan-induced edema such as bradykinin, histamine, substance P, and platelet-activating factor. In addition, authors verified that the oral administration of the major compounds found in the oil (α-humulene and trans-caryophyllene at 50 mg/kg) were also effective on paw edema reduction. In order to confirm anti-inflammatory activity, *C. verbenacea* oil was submitted to carrageenan-induced pleurisy and the carrageenan-induced air pouch models. On the first one, the oil reduced volume of pleural exudate and migration of polymorphonuclear cells, but was not effective on mononuclear cells levels. On the second one, treatment reduced MPO activity, indicating reduction on neutrophil migration to the air pouch cavity. Altogether, results indicate the potential anti-inflammatory activity from *C. verbenacea* essential oil by interfering on cytokines production such as TNF-α.

3.4 Cupressaceae

El Jemli *et al.* (2016)[39] researched the oral anti-inflammatory effect from essential oil of *Tetraclinis articulata* L, which is an important popular anti-inflammatory in the Maghreb area. To assess anti-inflammatory effect, authors used paw edema induced by carrageenan (chemical stimuli) and trauma (mechanical stimuli, performed by weight drop on paw

dorsum). Results showed reduction on paw edema on both model on a dose-dependent manner. In both cases, essential oil reduced edema after 3 hours, which could explain its mechanism of action. Authors hypothesize that *T. articulata* L. essential oil act by inhibiting the synthesis of COX products, such as prostaglandins, cytokines and nitric oxide, which are consistent to the oil effect on the time-course of the experiment.

Chamaecyparis obtusa, another plant from Cupressaceae family, has been studied with respect to its anti-inflammatory response after an antinociceptive assay. Park *et al.* (2015)[38] verified the production of cytokines (TNF-α and IL-1β) and expression of pro-inflammatory enzymes (iNOS and COX) in paw tissue of formalin-induced edema. Animals intraperitonealy treated with *C. obtusa* essential oil presented dose-dependent reduction on TNF-α and IL-1β levels and inhibition of enzymes expressions. Authors suggest that the antinociceptive effect may be related to anti-inflammatory process caused by the oil treatment.

3.5 Euphorbiaceae

Croton argyrophyllus was studied by Ramos *et al.* (2013)[40] due to its traditional use on folk medicine in Brazil. In this paper, authors describe the anti-inflammatory assess of oral treatment of C. *argyrophyllus* essential oil on carrageenan-induced rat paw edema and carrageenan-induced peritonitis in mice. On the first model, the oil reduced edema and MPO activity on paw tissue and, on the second, it reduced leucocyte and

polymorphonuclear cell migration to the cavity, indicating the probable anti-inflammatory activity.

3.6 Lamiaceae

Lamiaceae family comprises several genera and has many plants that produce essential oils. *Hyptis martiusii* Benth is a plant commonly found in Brazilian flora, with interesting pharmacological properties due to its use in folk medicine. Barbosa *et al.* (2017)[49] studied the oral treatment on mice ear edema induced by single and multiple doses of croton oil and on rat paw edema induced by carrageenan. Results were not as positive as expected, since any dose studied reduced edema on croton oil model and only the higher dose reduced edema on carrageenan model.

Lavandula angustifolia is a well-known aromatic plant, used in different parts of the world in perfumes/cosmetics and as popular medicine. Two research papers studied the anti-inflammatory effect of this plant essential oil *in vivo*. Hajhashemi *et al.* (2003)[41] compared the antiedematogenic activity from *L. angustifolia* essential oil and hydroalcoholic extract on carrageenan-induced rat paw edema. The oil, rich in 1,8-cineole, reduced the edema at 200 mg/kg dose, while the extract could not even with extremely high doses (4000 mg/kg). Authors did not include the extract's composition, however they conclude that, since only the oil had potential anti-inflammatory effect, it must be its major substance the responsible for the activity. More recently, Da Silva *et al.* (2015)[45] used the essential oil from *L. angustifolia* on two different acute inflammation models: croton oil induced mice ear

edema and carrageenan-induced pleurisy in rats. Differently from the previous study described, this oil contained linalool as major component. It significantly reduced all responses in pleurisy model (exudate volume, protein concentration on exudate, leucocyte and polymorphonuclear cells count) and edema in ear edema model (in this case, in the same degree as dexamethasone).

Nepeta genus is commonly studied due to its popular use. *N. cataria* L., also known as catnip, produced an essential oil rich in nepetalactone isomers, which was tested against carrageenan-induced mice paw edema.[43] *Nepeta pogonosperma* Jamzad et Assadi produced an oil rich in 1,8-cineole and nepetalactone, which as tested on formalin-induced rat paw edema.[44] Even though dose and route of administration were different, on both papers, authors found that the oils significantly inhibited edema formation after 60 minutes. Ali and co-workers (2012)[44] suggest that this could be explained by the similar composition found in the oils.

Ocimum basilicum, also from Lamiaceae family, was studied in two reaseach papers, in with the oil was tested against mice paw edema induced by carrageenan, dextran, histamine and arachidonic acid and against peritonitis induced by carrageenan in mice.[47,50] The first article describes the use of an essential oil rich in estragole (61%), which ws also tested on anti-inflammatory assays.[47] Essential oil dose-dependently reduced edema induced by carrageenan and dextran, but only the higher dose (100 mg/kg) had significant results. On histamine and arachidonic acid-induced edema, the oil had a small antiedematogenic effect (the dose tested was 50 mg/kg) and estragole had even smaller response (the dose tested

was 30 mg/kg). On the other hand, 50 mg/kg essential oil dose had significant effect on peritonitis assay, similar to dexamethasone, the control drug. Estragole was not as effective, but reduced protein extravasation and cell migration to peritoneal cavity.

On the second study, *O. basilicum* essential oil was complexed with β-cyclodextrin.[50] Authors treated animal with 10 mg/kg dose, which was not effective on the previous study, and verified that the technological approach influenced on anti-inflammatory response, increasing the essential oil effect.

Pogostemon cablin (patchouli) essential oil is commonly used in cosmetic, yet it has pharmacological activities already described in the literature. Silva-Filho *et al*. (2016)[48] described the effect of patchouli oil on zymozan-induced peritonitis in rats. Orally treated animal with 50, 100, 200 or 300 mg/kg doses had reduction on leucocyte migration to the peritoneal cavity and nitric oxide production on inflammatory exudate. Mechanis of action was not confirmed, but it could be related to inhibition of inflammatory cells recruitment and consequently reduction on inflammation mediators (cytokines and nitric oxide, for example).

Another common household plant, *Rosmarinus officinalis* L., was studied by Takaki *et al.* (2008) against carrageenan-induced rat paw edema and rat pleurisy.[42] The oral administration of *R. officinalis* essential oil reduced significantly the edema in all doses tested (250, 500, and 750 mg/kg) and pleural exudate production on the higher dose (500 mg/kg), indicating a potential anti-inflammatory effect.

Barreto and co-workers (2016)[46] researched the effect from *Stachys lavandulifolia* var. *lavandulifolia* essential oil and its major component, (-)-α-bisabolol, on carrageenan-induced pleurisy in mice. Both oil and isolated compound (50 mg/kg) reduced significantly leucocyte migration to pleural cavity and TNF-α production. However, only the oil was effective to inhibit IL-1β production. Considering the results, authors suggest that the anti-inflammatory effect from *S. lavandulifolia* essential oil is related to cytokine inhibiton, while (-)-α-bisabolol could inhibit other inflammatory mediators.

3.7 Lauraceae

The genus *Cinnamomum*, from the Lauraceae family, present aromatical plants with potential anti-inflammatory activity. *Cinnamomum insularimontanum* Hayata fruits produced an essential oil rich in citral. Both oil and major component were tested for its anti-edematogenic effect in mice ear edema induced by croton oil.[52] In addition, authors studied the oil inhibition of inflammatory mediators *in vitro* (nitric oxide, TNF-α and inflammatory proteins quantification in LPS-stimulated macrophages).

In vivo assay showed that both samples inhibit, in a dose-dependent manner, the edema formation. *In vitro* anti-inflammatory tests showed that samples inhibited NO formation, also in a dose-dependent mode. In this test, citral showed even better results compared to the oil, thus authors tested the compound on its inhibition of TNF-α. Citral showed a concentration-dependent inhibition of TNF-α. To confirm the mechanism of action, authors tested citral influence on proteins expression on LPS-stimulated macrophages. Western blot

analysis showed that citral inhibited iNOS and NF-κB levels, but not COX-2. Thus anti-inflammatory mechanism of action of citral, and consequently from *C. insularimontanum* essential oil, could be due to inhibiton of NF-κB pathway.

Cinnamomum cassia Presl twigs produced an essential oil rich in (E)-cinnamaldehyde with anti-edematogenic effect on mice paw edema stimulated with carrageenan.[51] Authors confirmed the anti-inflammatory effect by testing in vivo samples on levels of TNF-α, IL-1β, NO, PGE_2 and COX-2 and iNOS protein expression. All doses inhibited the pro-inflammatory mediators significantly, compared to the non-treated group, as well for the protein expression, leading to a conclusion that this oil acts by inhibiting the COX-2 pathway.

Ecuadorian sample from *Ocotea quixos* (Lam.) Kosterm, also from Lauraceae family, produced an essential oil rich in trans-cinnamaldehyde, known for its anti-inflammatory properties *in vitro*.[82] In this study, Ballabeni and co-workers (2010)[53] researched the anti-inflammatory effect from *O. quixos* essential oil and its major constituents (trans-cinnamaldehyde and methylcinnamate) both *in vitro* and *in vivo*. In vitro assays demonstrated that the oil was effective on reducing nitric oxide (NO) and COX-2 expression in LPS-stimulated macrophages, however only tran-cinnamaldehyde could do the same on NO production (methylcinnamate was not effective). Still, the oil and its components could reduce cAMP concentration on forskolin-stimulated human neuroblastoma cells, although the oil was more effective than the isolated substances.

Oral treatment on carrageenan-induced rat paw edema reduced significantly the paw swelling for the oil and tran-cinnamaldehyde, but not for methylcinnamate. Altogether, results suggest that the oil and its major component present anti-inflammatory activity but futher studies are necessary to predict a mechanism of action.

3.8 Leguminosae

From the Leguminosae family, the genus *Copaifera* is well known to produce an oil that has anti-inflammatory properties. By tapping the trunk of these trees, an oilresin rich in diterpenes (resinous part) and sesquiterpenes (essential oil) can be extracted, without killing the plant.[83] The first study was carried out by Basile *et al* (1988)[56] with an oilresin with no species identification. Authors verified that the oral administration reduced the edema caused by carrageenan in the rat paw in a dose-dependent manner, but did not present a mechanism of action.

Carvalho *et al* (2005)[55] researched the effect of the oilresin extracted from *Copaifera duckei* dwyer on two acute inflammation models: carrageenan-induced paw edema and croton oil-induced ear edema. The oilresin was applied topically on the animals' paw and ear on the doses of 517 mg/kg, 1035 mg/kg and 1802 mg/kg. On the first model, only the higher dose significantly reduced the edema on the paw and on the second, all doses reduced edema on the ear. Authors did not studied the difference on biochemical parameters, thus there is no possible mechanism of action. However, they conclude that the activity is probably due to the presence of terpenes and their synergistic effect on the models.

Since copaiba oil is a popular medicine in the Amazonian rain forest area, it is widely sold in popular markets. However, it rarely presents a quality control or even species identification, leading to probable adulteration. Veiga-Junior *et al.* (2001)[57] studied the composition and antiedematogenic effect from eigth commercial samples acquired in different popular markets in Brazil. On bradikynin and carrageenan-induced paw edemas, only three samples were effective, probable due to their composition that presented similar concentration of sesquiterpenes α-bergamotene, β-caryophyllene and α-aromadendrene. Other samples presented small or no effect. Only one sample presented an altered chemical profile, which could indicate an adulteration.

Copaifera multijuga is the most studied specie on the *Copaifera* genus. Veiga-Junior and co-workers[59] evaluated the antiedematologenic effect from oilresin samples of *Copaifera multijuga* and from its organic fractions. In this study, authors found that chemical variation could interfere on the pharmacological effect, since the oil and fractions containing a higher amount of β-caryophyllene showed an increase activity on bradykinin-induced paw edema. Gomes and co-workers (2010)[60] produced three fractions from the oil of *Copaifera multijuga* (in hexane, chloroform and methanol). The most apolar fractions presented higher antiedematogenic activity on rat paw edema induced by carrageenan, histamine or serotonin. The crude oil also presented a significant edema reduction, however it was not as high as the fractions. Authors suggest that the mechanism of action could be due to receptor blockage on the mediators studied (histamine and serotonin) by many constituents of the oil, in a synergistic way.

Kobayashi *et al.* (2011)[58] studied the effect of oral administration on rats with carrageenan-induced pleurisy. Both doses studied reduced significantly the leucocyte and polymorphonuclear cells migration to pleural exudate, however there was no significant changes on leucocyte, lymphocyte, neutrophil and monocyte levels on blood samples. In another research on *Copaifera* species and pleurisy, Veiga-Junior *et al.* (2007)[54] demonstrated that the oral administration of *Copaifera multijuga* Hayne oil could inhibit leucocyte accumulation on the smaller dose (100 mg/kg) and also neutrophil migration with all tested doses (100, 200 and 400 mg/kg). Both papers describe similar results, even though the phlogistic agent used were different (carrageenan and zymosan), indicating that the oil from *C. multijuga* is a potent anti-inflammatory. In addition, authors suppose that this activity is related to the amount of β-caryophyllene in both samples, which has already been described as an anti-inflammatory agent by itself.[84–89]

3.9 Myrtaceae

Eucalyptus genus present a significant importance on the treatment of respiratory diseases such as cold and flu, as analgesic, anti-inflammatory and antipyretic remedy. Silva and co-workers (2003)[61] described the effect of three *Eucalyptus* species (*Eucalyptus citriodora, Eucalyptus tereticornis*, *Eucalyptus globulus*) on *in vivo* inflammation models of edema and peritonitis.

Rat paw edema was induced by two agents (carrageenan or dextran) and peritonitis was induced by carrageenan. On the first model, animals were treated with 10 and 100 mg/kg

essential oil dose, while on the second only with 100 mg/kg. Concerning the paw method, only the higher dose could inhibit edema during the 4 hours of experiment (for both phlogistic agents). On rat peritonitis, all samples reduced significantly the neutrophil migration to the cavity after 3 hours. *E. tereticornis* exhibited the best response between the species tested, especially for dextran-induced edema and peritonitis.

Due to lack of oil composition information, it was difficult for authors to conclude which components could influence on *Eucalyptus* anti-inflammatory activity; however they could hypothesize that neutrophils are linked to the mechanism of action.

3.10 Piperaceae

Peperomia serpens (Sw.) Loud commonly grow in host trees of the Amazonian rain forest and is used by locals to treat inflammatory symptoms. Pinheiro *et al.* (2011)[62] extracted an essential oil rich in the sesquiterpenes (*E*)-nerolidol and ledol to study anti-inflammatory activity on carrageenan or dextran-induced rat paw edema, croton oil-induced mice ear edema and carrageenan-induced rat peritonitis. The oral treatment on edema models significantly reduced paw and ear swelling. Since carrageenan, dextran and croton oil produce different inflammation pathways and the oil was active against all irritants, authors evaluated activity in inflammatory cell migration (mechanism of action hypothesis) on peritonitis model. In this assay, essential oil treatment reduced leucocyte and neutrophil migrations in the same level as the control drug, dexamethasone, confirming anti-

inflammatory activity. Mechanism of action is not clear, yet it can be related to release of inflammatory mediator that increase cell migration.

The *Piper* genus is the most common among the 14 genera in Piperaceae family. They grow in tropical regions and have been used in folk medicine for different purposes. Lima and co-workers (2012)[63] studied the effect of oral treatment of *Piper aleyreanum* C.DC essential oil in mice with carrageenan-induced pleurisy. Doses of 3, 10 and 100 mg/kg were able to reduce leucocyte and neutrophil infiltration, compared to the non-treated control. Brait *et al.* (2015)[65] studied the effect oral administration of *Piper vicosanum* essential oil on carrageenan-induced rat paw edema and rat pleurisy (100 and 300 mg/kg). The oil was active in both models, in a dose-dependent manner. In a most recent study, Branquinho *et al.* (2017)[64] verified the activity from *Piper glabratum* essential oil on carrageenan-induced mice paw edema and mice pleurisy (10, 100, 300 and 700 mg/kg). On paw edema, only the higher doses were effective to reduce the swelling and on pleurisy model, again, only the higher doses reduced leucocyte migration (and only the 700 mg/kg dose was able to reduce protein extravasation).

Altogether, results indicate that Piper species present anti-inflammatory activity, each one in different degrees of effect. This could be explained by their composition similarities.

3.11 Poaceae

Plants from the *Cymbopogon* genus appear to be important essential oil producers on the Poaceae family, with more than 180 species around the world. Essential oils extracted from

C. flexuosus[66] leaves and *C. validus*[67] leaves and flowers inhibited, by oral treatment, rat paw edema induced by carrageenan and egg albumin, respectively. Unfortunately, information on dose and oil composition are missing on both studies, thus impairing their comparison.

3.12 Rutaceae

The pericarp in the fruits from *Citrus* genus (Rutaceae) produces essential oil used in many industries, and can also present pharmacological activity. Amorim *et al* (2016) studied the essential oil from four Citrus species (*C. limon, C. latifolia, C. aurantifolia, C. limonia*) on air pouch model with carrageenan as cell infiltration agent.[69] In this study, authors found that *C. limon* and *C. limonia* presented the best results on anti-inflammatory effect, with reduction of cell infiltration and cytokines (TNF-α, IL-1β and IFN-δ) production. These two species presented high amounts of limonene, different from the other two species, which could explain the difference on the anti-inflammatory effect.

In another study, limonene was isolated from the essential oil extracted from the fruits of *Citrus latifolia* Tanaka and was administrated orally to animals with zymosan-induced peritonitis.[68] In this study, authors describe the decrease of leucocyte infiltration on the peritoneal exudate on the 500 mg/kg dose. This dose also inhibited the production of TNF-α on the exudate, but did not interfered on IL-10 levels. Although these two papers used

different in vivo models and Citrus species, they both found that this genus presents a promisor material to treat inflammatory related diseases.

3.13 Umbelliferae

From the Umbelliferae family, *Angelica sinensis*, also known as danggui, in China, is a popular medicine in the traditional Chinese medicine and has been studied a few times for its anti-inflammatory effect. Li *et al* (2016)[71] studied the effect of oral treatment with the essential oil from this plant on peritonitis induced by LPS in mice. After 8 hours of experiment, blood was collected to verify the levels of several biochemical parameters (TNF-α, IL-1β, IL-6, IL-10, HIS, 5-HT, NO, iNOS, COX-2, PGE_2). The treatment was able to reduce significantly the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), the inflammatory mediators (HIS, 5-HT, PGE2 and NO) and the inflammation-related enzymes (iNOS and COX-2) and promote the production of anti-inflammatory cytokine IL-10.

Yao *et al* (2015)[72] and Zhong *et al* (2016)[73] also studied *Angelica sinensis* essential oil, however, they used the rat paw edema as anti-inflammatory *in vivo* model. Both searched for inflammatory cytokines, using metabolomics to investigate the oil mechanism of action. In these studies, essential oils (crude or in different previous preparation methods before the hydrodistillation process) regulated different inflammation pathways, with high influence in the arachidonic acid and glycine networks.

Another plant from the Umbelliferae family, *Heracleum persicum*, produced an essential oil with potential anti-inflammatory effect, widely used as a popular medicine in Iran.

Hajhashemi *et al* (2009)[74] studied the effect of oral administration of the essential oil and a hydroalcoolic extract from *Heracleum persicum* in rat paw edema model. Both samples decreased the swelling in the paw, however there are not any other suggestions about the difference on biochemical parameters that indicate inflammation decrease.

3.14 Verbenaceae

Lippia genus comprises a significant amount of plants that present potential pharmacological effects, since they are used as popular medicine.[90] A few studies have been published on the anti-inflammatory effect produced by species of *Lippia* essential oil.

Monteiro *et al.* (2007)[77] verified that topical administration of *L. sidoides* essential oil (1 and 10 mg/ear) on TPA-induced ear edema in mice, reduced significantly the ear swelling (45 and 35%, respectively). Since thymol was the oil's major component (66%), authors suggest that it is the responsible for the antiedematogenic response, but do not present a mechanism of action.

In another paper, Veras and co-workers (2013)[78] studied the topical administration of *L. sidoides* essential oil on mice ear edema caused by several irritants (croton oil, arachidonic acid, phenol or histamine). Similarly, this oil showed a high amount of thymol (84%) and an effective response to arachidonic acid pathway models (arachidonic acid and phenol as irritant agents), such as TPA. However, with the other two irritants, the oil was incapable of reducing edema, suggesting that this product do not work on lipooxygenase pathways such as corticosteroids, but on cyclooxygenase pathway, such as AINEs.

Mendes and co-workers (2010)[76] researched the effect of oral treatment of *L. gracilis* essential oil in animals with paw edema and peritonitis induced by carrageenan. On the first model, only the higher dose (200 mg/kg) presented antiedematogenic effect. On the second, all doses reduced leucicyte recruitment to the peritoneal cavity. This oil was also rich in thymol, which could explain partially the oil efficacy.

Contrasting to these findings, Guilhon et al. (2011)[75] extracted an essential oil of *L. gracilis* poor in thymol, but rich in carvacrol. However, it was highly efficient to reduce pouch exudate production, leucocyte migration, inflammatory mediator production (NO, TNF-α, INF-γ and PGE_2) and iNOS expression, clearly indicating anti-inflammatory properties on the oil. Suggested mechanism of action is through blockage of NO pathway. Clearly, it could be concluded that the effect in *Lippia* essential oils is not related to a single substance, but a synergistic effect of major and minor compounds.

3.15 Zingiberaceae

Antiedematogenic effect from rhizome essential oil of *Zingiber cassumunar* Roxb. (phlai oil) and its major components (*i.e*. sabinene, terpinen-4-ol, γ-terpinene, α-terpinene and DMPBD) was tested on carrageenan rat paw edema. Topical administration of the essential oil had dose-dependent response on edema inhibition. Concerning the major components, DMPBD and terpinen-4-ol were the most active substance with edema inhibition in all

doses tested (0.03, 0.3, 3 and 6 mg/paw). α-Terpinene showed edema inhibition only on the higher dose (6 mg/paw) and the other two components did not present any effect.

Traditional Chinese medicine has been a resource on natural products research. Su *et al.* (2012)[80] studied the essential oils from three plants from three different families, which are used in a traditional medicine recipe: *Curcumae wenyujin* (Zingiberaceae), *Chrysanthemum indicum* (Compositae) and *Pogostemon cablin* (Lamiaceae). Authors tested the mixture on rat paw edema induced by carrageenan, verifying edema inhibition and levels of PGE_2, IL-1β, TNF-α and NO levels. Oral treatment suppressed dose-dependently paw edema (up to 70% at the higher dose), reduced PGE_2 and IL-1β levels (only at 170 mg/kg), but had no influence on TNF-α and NO concentration. Considering the results, authors could not affirm exactly what is CPZ's mechanism of action, yet they suggest that it do not involve COX pathways such as NSAIDs.

4. Discussion

The ability to produce essential oil is distributed in all plant kingdom. However there are certain families that stand out and comprise the plants with the most important essential oils used in the industry and commercial fields. Some of the families are Acoraceae, Apiaceae, Asteraceae, Cupressaceae, Geraniaceae, Illiciaceae, Lamiaceae, Lauraceae, Liliopsida, Magnoliopsida, Myristicaceae, Myrtaceae, Poaceae, Rosaceae, Rosopsida, Santalaceae and Zingiberaceae[91]. In this review, we described plants from some of these families and others, known mostly from folk medicine.

Essential oils have been studied for a long time due to its pharmacological properties. In this review, we found that the anti-inflammatory effect from essential oils are studied since 1980's decade, where simple tests were performed (such as rat paw edema), with growing interest over the years. More recently, studies began to present specific tests, in order to truly identify the anti-inflammatory effect and mechanism of action, searching for inflammatory mediators such as interleukines, TNF-α, INF-γ, PGE_2, and NO.

One interesting data retrieved from this research is the origin of the papers. More than half (52%) were produced in Brazil, which can be explained by the vast flora found in the county and the fact that popular medicine is a rich bland of different cultures and native plants[92].

Acute anti-inflammatory *in vivo* assays include paw and ear edema, pleurisy, peritonitis and air pouch. In most papers, paw edema is the chosen tests, due to advantages such as simplicity to perform and great amount of inflammatory agents available (with different costs). In this review, for instance, thirty-two papers describe the use of paw edema. However, even though there are many studies using the same method, sometimes for the same essential oil, the results are different. Indeed, many factor can influence on the response, such as the essential oil composition, the administration route, the dose, or even the vast amount of biochemical mediators that can be studied.

5. Conclusions

In conclusion, essential oils present a growing interest for their anti-inflammatory effect and have been studied for the last three decades. In this review essential oil from plants

from Asteraceae, Burseraceae, Boraginaceae, Cupressaceae, Euphorbiaceae, Lamiaceae, Lauraceae, Leguminosae, Myrtaceae, Piperaceae, Poaceae, Rutaceae, Umbelliferae, Verbenaceae and Zingiberaceae families were described as anti-inflammatory. Five acute inflammation models were used in the papers disclosed in this work: paw end ear edema, pleurisy, peritonitis and air pouch. The most common model was paw edema, especially due to the facility to perform. Many studies describe the search for inflammatory mediators, such as interleukines, TNF-α, INF-γ, PGE_2, and NO, which could explain the mechanism of action.

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CAPÍTULO II

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Determination of β-caryophyllene skin permeation/retention from crude copaiba oil (*Copaifera multijuga* Hayne) and respective oil-based nanoemulsion using a novel HS-GC/MS method

Letícia G. Lucca[a], Sheila Porto de Matos[a], Bruna Tassi Borille[a], Daiane de O. Dias[a], Helder F. Teixeira[a], Valdir F. Veiga Jr.[b], Renata P. Limberger[a], Letícia S. Koester[a*]

[a] *Pharmacy College, Federal University of Rio Grande do Sul, Porto Alegre - RS, Brazil.*

[b] *Department of Chemistry, Federal University of Amazonas, Manaus - AM, Brazil.*

** Correspondence author: +55-51-33085278, leticia.koester@ufrgs.br (L.S. Koester)*

Abstract

Copaiba oil is largely used in the Amazonian region for the treatment of inflammation, and recent studies demonstrated that one of the major components of the oil, β-caryophyllene (CAR), is a potent anti-inflammatory. The nanoemulsification of this oleoresin, which has unctuous character, converts it in a more acceptable hydrophilic formulation and may improve CAR penetration through the skin due to the small droplet size and the high contact surface afforded by the nanoemulsions. This paper describes the validation of a novel, sensitive, practical and solvent free method that uses gas chromatography in headspace mode coupled with mass spectrometry to evaluate the skin permeation/retention of CAR from the crude copaiba oil and its nanoemulsion. Our results show that the bioanalytic method was fully validated, demonstrating linearity ($r^2 > 0.99$), specificity (no peaks co-eluting with CAR retention time), precision (RSD < 15%) and accuracy (recovery > 90%) within the accepted parameters and that the copaiba oil nanoemulsion presented a better skin penetration compared to the crude oil, with CAR achieving the most profound layer of the skin, the dermis.

Keywords: *Copaifera multijuga* Hayne, β-caryophyllene, skin permeation, HS-GC/MS

1. Introduction

The genus *Copaifera* L. includes different species and occurs mainly in the Brazilian Amazonian region. Copaiba oil is an oleoresin extracted from the trunk of the *Copaifera* tree and is widely used in popular medicine in that region for different purposes, such as anti-inflammatory, antitumoral and antimicrobial [1,2]. The anti-inflammatory activity was attributed to β-caryophyllene (CAR), since in the study of Veiga Junior *et al.* [1], the oil extracted from the specie *C. multijuga* Hayne presented the highest amount of this compound and exhibited the highest activity. Also, recent studies proposed the mechanism of action from the oil and from β-caryophyllene. According to Gomes *et al* [3], the oil extracted from *Copaifera multijuga* act similarly as anti-inflammatory compounds

(inhibiting histaminergic and serotoninergic pathways) and presents antinociceptive effect (possibly mediated by opioid receptors). Furthermore, according to Gertsch *et al* [4], β-caryophyllene is a selective agonist to the peripheral cannabinoid receptor, CB_2, which is related to the treatment of pain and inflammation.

Nanoemulsions present penetration-enhancing ability and have already proved to be advantageous for the administration of anti-inflammatory drugs [5,6,7]. In this context, the feasibility of developing a stable nanoemulsion with copaiba oil was recently investigated by our research group [8]. This formulation is intended for topical treatment of locally inflamed skin.

The volatile character of the oleoresin imposes difficulties in terms of developing both a stable formulation and an analytical method for skin permeation/retention studies. To the best of our knowledge, there is only one report of GC/MS analytical method validation for volatile compounds in a skin permeation assay [9]. In that study, the authors report the full validation of a method for sesquiterpenes lactones assay in *Arnica* formulations and the determination of its penetration profile in porcine ear skin.

Therefore, our main objective in this study was to describe the validation of a bioanalytical gas chromatography coupled with mass spectroscopy method to analyze CAR in the samples from pig ear skin permeation assays carried out with the crude copaiba oil and its nanoemulsion formulation.

2. Materials and methods

2.1 Chemical and reagents

The oleoresin from *C. multijuga* Hayne was collected in the Ducke Forest Reserve of the Instituto Nacional de Pesquisas da Amazônia (INPA) at Manaus, Amazonas State (Brazil), and the exsiccate was deposited at the INPA herbarium. It was extracted by an usual method, artificial exudation, described by Veiga Junior *et al.* [1] and characterized by gas chromatography coupled with mass spectrometry (GC/MS), which confirmed the presence of 42% (w/w) of β-caryophyllene. β-caryophyllene reference standard, Span 80® and Tween

20® were obtained from Sigma–Aldrich (St. Louis, MO, USA). Medium Chain Triglycerides (MCT) was kindly donated by Lipoid GmbH (Ludwigshaten, Germany). Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, Billerica, USA). All other chemicals or reagents were of analytical grade.

2.2 Instrumentations and chromatographic conditions

The samples were analyzed using a gas chromatograph 5975C (Agilent Technologies, United States of America), consisting of a split/splitless injector port and a mass spectrometer detector. The injection was made in the splitless mode. The GC system was equipped with a DB-5 column (30 m x 0.25 mm x 0.25 mm). The carrier gas was helium (1.0 mL/min). The oven temperature was programmed from 60 °C for 3 min with an increase of 40 °C/min, to 300 °C, finalizing the chromatographic run at 9 minutes. Injector, transfer line (interface), source and quadrupole temperatures were set at 220 ºC, 300 °C, 230 °C and 150 °C respectively. The mass detector was operated with electron impact system at 70 eV. The signal was recorded and processed with GC/MS Data Analysis Software.

2.3 Determination of β-caryophyllene in the samples

The analysis of the main marker of copaiba oil, β-caryophyllene (CAR), was determined by headspace (HS) in a gas chromatograph coupled with mass spectrometer (GC/MS). The GC equipment was coupled with headspace sample preparation system (CTC Analytics Combipal, Basel, Switzerland). The samples were placed into a 10 mL glass vial and transferred for the heating station for 10 minutes at 50 ºC. After 10 minutes, 1.0 mL of the volatiles in the vial was aspirated into a 1.0 mL syringe and introduced in the GC port. 10 mL clear glass vials with magnetic 18 mm screw caps with septa (Agilent Technologies, United States of America) were used in this paper.

2.4 Method validation

The method was validated for CAR assay in skin samples. The study included tests of specificity, linearity, limits of detection and quantification, precision, accuracy and matrix effect [10,11]. A methanol solution of porcine ear skin was used as the skin matrix for the bioanalytical method validation.

2.4.1 Specificity

The specificity was assessed comparing the CAR peak retention time with blank samples containing the porcine skin methanol solution, the tapes used in the tape-stripping method and the receptor fluid.

2.4.2 Linearity, limits of detection and quantification

For linearity experiments, solutions of CAR were prepared in the range of 0.14 – 0.68 µg/mL in 3 different days. Linearity was evaluated by calculating the regression line using the least squares method. Using the calibration curve data, detection (LOD) and quantification (LOQ) limits were determined based on the standard deviation of the response and the slope.

2.4.3 Precision and accuracy

Precision was assessed by analyzing samples of methanol skin extract spiked with three CAR concentrations (0.14, 0.45, 0.68 µg/mL), in the same day (intra-day) and in three different days (inter-day). Results for precision are shown as relative standard deviation (R.S.D.). Accuracy of the method was determined by recovering CAR from the skin extract, at three different levels (0.14; 0.45 and 0.68 µg/mL). Accuracy results are shown as percentage of CAR recovered from the skin samples.

2.4.4 Matrix effect

According to Niessen *et al.* [12] the percentage of matrix effect (%ME) can be assessed by calculating the difference between the peak area of a medium concentration in the

calibration curve with and without the skin matrix (skin methanol solution). In this paper, matrix effect was calculated comparing the peak areas of CAR methanol solution (0.45 µg/mL) with and without the skin methanol extract.

2.5 Preparation and characterization of copaiba oil nanoemulsion

Briefly, the oily phase (20% w/w copaiba oil; 10% w/w MCT; 3% w/w Span 80®) and the aqueous phase (1% w/w Tween 20® and q.s.p. water) were mixed under magnetic stirring (5 minutes at room temperature) to form a coarse emulsion. In order to gradually decrease the droplet size, this coarse emulsion was subjected to high-pressure homogenization (EmulsiFlex-C3®, Avestin, Canada) at 750 bar for 6 cycles, producing the nanoemulsion, as optimized by Dias *et al.* [8].

After preparation, the nanoemulsion was characterized for droplet size, polydispersity index, zeta potential and CAR content. Droplet size and polydispersity index were measured in triplicate by dynamic light scattering after dilution of 10 µL of the nanoemulsion in 10 mL of purified water (Zetasizer Nanoseries ZN90, Malvern Instruments, Worcestershire, UK). The zeta potential value was measured in triplicate by laser Doppler velocimetry using the same instrument, after dilution of 10 µL of the nanoemulsion in 10 mL in NaCl (1 mM) ultra-filtered in 0.45 µM filter. CAR content was determined as described by Dias *et al.* [13] using a 0.45 µg/mL nanoemulsion solution in water.

2.6 Permeation/retention studies

Permeation/retention studies were performed using porcine ear skin (n=10) in Franz-type diffusion cell equipment (Dist, Florianópolis, Brazil).

Porcine ear was obtained from a local slaughterhouse and the skin was excised from the outer part of the pig's ear using a scalpel. The hair and fat excess from the extracted skin were removed with scissors and the thickness was measured with a thickness gauge

(Mitutoyo Corporation, Kanagawa, Japan). Only skin cuts in the range of 0.90 and 1.10 millimeters were used in the experiments.

Receptor fluid was a mixture of ethanol and phosphate buffered saline (PBS) pH 7.4 (50:50). The optimum amount of ethanol was verified in a CAR solubility test, in which only 50% of ethanol was able to maintain sink conditions. The receptor compartment was kept at 32 ºC ± 1 ºC and under magnetic stirring during the entire experiment. The formulations were placed directly in the porcine ear skin in the donator compartment. 500 µL of the nanoemulsion or oleoresin were used to perform the experiment. An aliquot of the receptor fluid of 1 mL was collected every 2 hours from the beginning of the experiment to verify the permeation of β-caryophyllene through the skin. After 8 hours of experiment, the skin was removed from the equipment. The excess of formulation/oleoresin was removed from its surface using Milli-Q water. The excess of skin, which was not in contact with the formulation/oleoresin, was cut off and the tape-stripping method was used to remove the stratum corneum [14] from the viable skin. The epidermis was separated from the dermis with a scalpel and both were weighted in analytical scale [15,16]. The tapes, the epidermis, the dermis and the fluid aliquots were frozen (-20 ºC) for posterior analysis in HS-GC/MS. The maximum freezing time for all the samples before their analysis was seven days. All samples (receptor fluid, tapes, epidermis and dermis) were directly placed in the headspace vials without any addition of solvents and the determination of β-caryophyllene was performed according to item 2.3. The results for the receptor fluid and the tapes were expressed as µg/mL and for the epidermis and dermis as µg/g.

2.8 Statistical analysis

Results in this paper are shown as mean ± standard deviation. ANOVA test was performed to verify the difference in the permeation study. p values less than 0.05 ($*p < 0.05$) were significant.

3. Results and discussion

3.1 Method validation

Chromatograms from each blank sample of porcine skin methanol solution, the tapes used in the tape-stripping method and the receptor fluid used in the specificity assay were compared with a CAR methanol solution (Figure 1). The results indicated that there were no interferents in the CAR peak (7.126 minutes), demonstrating that the method is specific for samples containing CAR. The mass fragmentation of the CAR peak indicated the absence of impurities in CAR samples, presenting the usual peaks m/z 93, m/z 133 and m/z 204 (Figure 2) [17]. In addition, a previously validated stability indicating method using GC coupled with flame ionization detector (FID) demonstrated that the nanoemulsion components did not interfere in the CAR analysis [13]. It is important to reinforce the idea new method validation using HS and GC/MS. By modifying the detector from flame ionization detector to a mass detector, we were able to quantify higher amounts of CAR in the permeation samples, characterizing a more sensible response to that experiment.

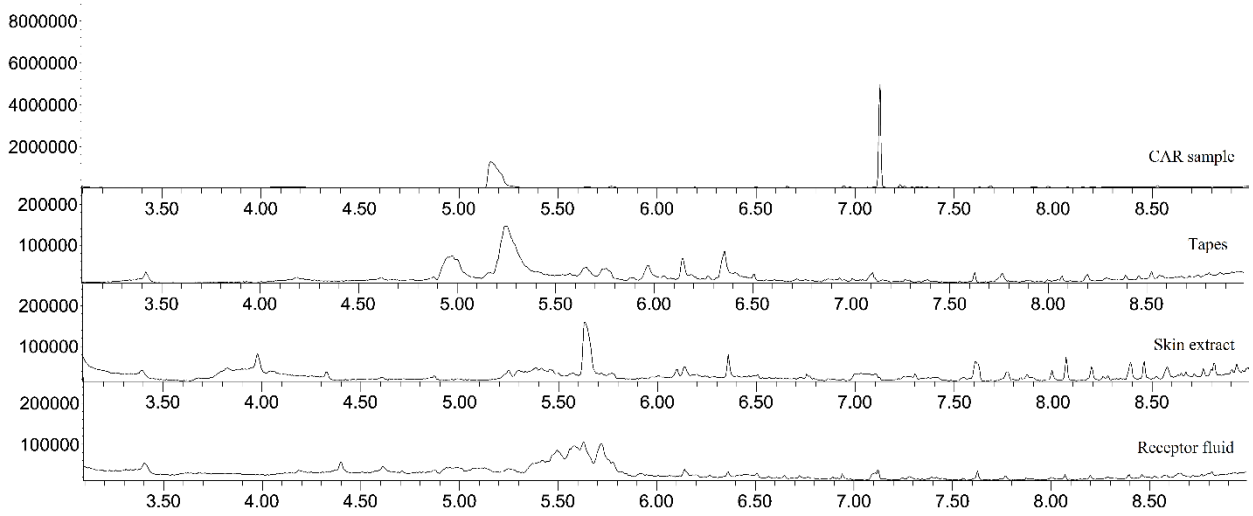

Figure 1. Specificity for CAR sample compared to skin extract, receptor fluid and tapes from the tape-stripping test. CAR purity 99,5%.

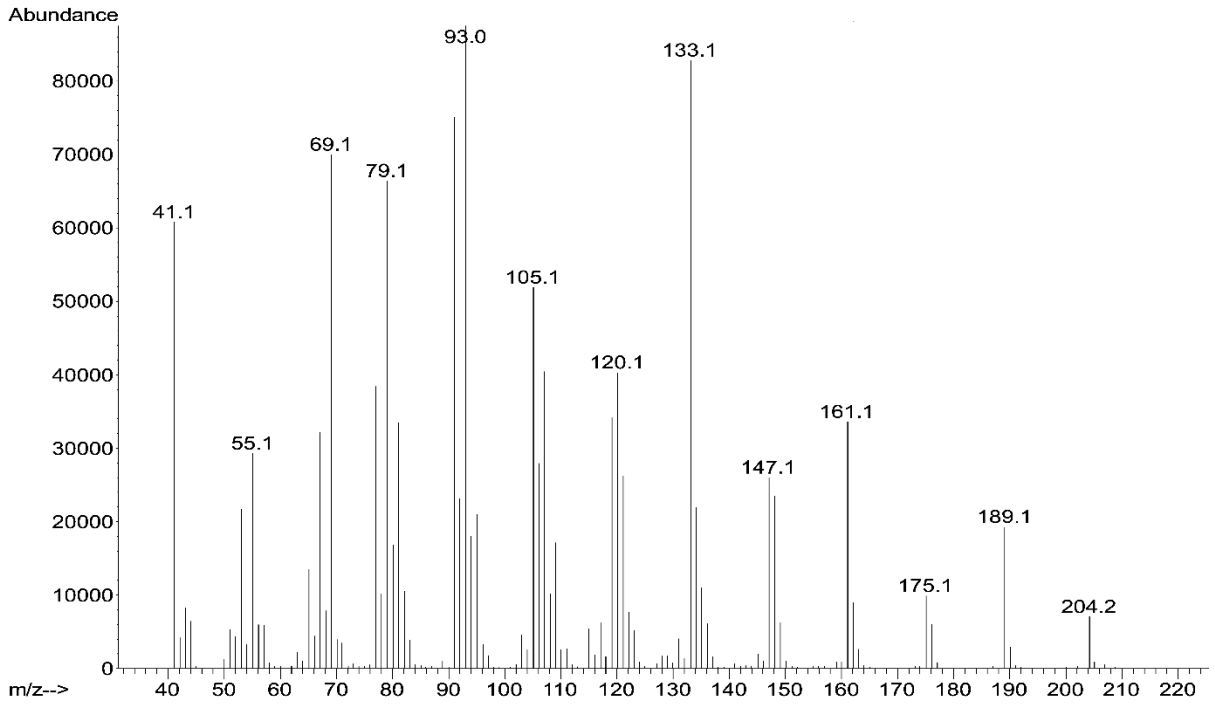

Figure 2. CAR fragmentation spectrum.

Linearity was evaluated with and without the presence of methanol skin extract. The method demonstrated to be linear in the range of 0.14 - 0.68 µg/mL for both conditions and the calibration equations showed excellent correlation coefficient ($r^2 > 0.99$), highly significant for the method ($p < 0.05$). The absence of a constant systematic error was confirmed since the confidence intervals included zero. The LOD and LOQ limits for the assay containing skin extract were 0.07 and 0.14 µg/mL, respectively. The low values of LOD and LOQ indicated the sensitivity of the proposed method.

Precision was assessed considering repeatability and intermediary precision at three CAR concentrations (0.14, 0.45 and 0.68 µg/mL) on three different days. All results presented R.S.D. values lower than 15.0 % (Table 1), which were considered satisfactory for bioanalytical methods, indicating that the method was precise for the analysis of CAR. The accuracy of the method was assessed considering the recovery of CAR from the skin methanol extract. Table 2 shows the average percentages of recovery in the range of 94.47–96.98%, demonstrating the accuracy of the proposed method.

The percentage of matrix affect calculated for CAR in the skin matrix was 8.4%, which can be considered not significant, according to Walorczyk [18]. Also, the absence of matrix affect can be observed by the high values of CAR recovery.

Table 1. Results for intra-day precision and intermediary precision for β-caryophyllene (CAR).

CAR Concentration (µg/mL)	Intra-day precision (R.S.D. %)			Inter-day precision (R.S.D. %)
	Day 1	Day 2	Day 3	
0.14	1.37	10.18	9.81	7.66
0.45	4.08	7.08	10.94	7.12
0.68	8.05	0.98	9.17	11.29

R.S.D. = Relative Standard Deviation

Table 2. Recovery of β-caryophyllene (CAR) added to skin methanol extract

Theoretical concentration (µg/mL)	Experimental concentration (µg/mL ± SD)	%
0.14	0.1357 ± 0.0104	96.98
0.45	0.4249 ± 0.0303	94.44
0.68	0.6790 ± 0.0731	95.21

SD = Standard Deviation

3.2 Nanoemulsion characterization

In a previous study [8], the amount of copaiba oil, medium chain triglycerides (MCT), Span 80® and Tween 20® were optimized to produce a nanoemulsion that would contain the maximum amount of copaiba oil (20 %, w/w), and consequently, it would contain the maximum amount of CAR.

After preparation, the nanoemulsion was characterized for droplet size, polydispersity index, zeta potential and CAR content. The nanoemulsion used in the skin permeation experiments presented a droplet size of 253.9 ± 2.74 nm, a polydispersity index of 0.058 ± 0.02, a zeta potential of -31.3 ± 0.36 mV and a β-caryophyllene content of 100.8 ± 0.01 %. All the results obtained were in agreement with the formulation optimized in a previous study [8].

3.3 Permeation/retention studies

Table 3 shows the results of CAR skin permeation and retention profile from the copaiba oil and respective nanoemulsion, including the penetrated percentage of CAR in the skin layers. For the crude copaiba oil, CAR was only found in the stratum corneum layer, but not in the epidermis, dermis or receptor fluid, indicating no penetration or permeation in the skin.

On the other hand, for the copaiba oil-based nanoemulsion, CAR was detected in the stratum corneum, epidermis and dermis, implying that the nanoemulsion facilitated the penetration of CAR through the skin, even though its concentration in the experiment with the crude oil was five times higher than in the experiment with the nanoemulsion (which contains 20% of this oleoresin in its composition). However, CAR was not detected in the receptor fluid for the nanoemulsion formulation either, which could represent that the nanoemulsion did not enable the permeation through the skin.

According to the literature, it is suggested that lipophilic drugs (with high partition-coefficient), such as some anti-inflammatory, always find an obstacle to pass through the skin and remain retained in the lipid layer (particularly the stratum corneum) since the

epidermis and dermis layers have more hidrofilic characteristics [19]. Moreover, nanoemulsions seem to facilitate the penetration of actives in the skin since they present a small droplet size, a large surface area and also have a tendency to interact with inflamed tissues demonstrating a great advantage in topical administration and incorporation of a lipophilic anti-inflammatory drug [7,20,21,22].

Table 3. Results for the skin permeation/retention profile of β-caryophyllene (CAR) from crude copaiba oil and oil-based nanoemulsion. Stratum corneum unit is µg/mL, dermis and epidermis unit is µg/g. Results are also shown as percentage of CAR in skin layers.

Sample	Unit	Crude copaiba oil	Copaiba oil nanoemulsion
Stratum corneum	µg/mL	1.349 ± 0.749 [a]	0.197 ± 0.067 [a]
	%	0.0002 ± 0.0001	0.0001 ± 0.00006
Epidermis	µg/g	ND	153.025 ± 79.314 [b]
	%	ND	0,041 ± 0,015
Dermis	µg/g	ND	19.226 ± 14.182 [b]
	%	ND	0.037 ± 0.014
Receptor fluid	µg/mL	ND	ND
	%	ND	ND

ND = Not Detected; Same letters indicate that the values are statistically different ($p < 0.05$)

4. Conclusion

Therefore, a solvent free gas chromatography coupled with mass spectrometry method was fully validated to determine β-caryophyllene in skin samples, demonstrating that it was specific, linear, precise and accurate. Also, the nanoemulsion produced showed great skin

penetration profile compared with the crude copaiba oil, indicating that it facilitated the penetration of copaiba oil and its major component, β-caryophyllene, through the skin up to the most profound layer, the dermis.

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CAPÍTULO III

Manuscrito publicado no periódico *Journal of Biomedical Nanotechnology*

Nanoemulsification potentiates *in vivo* antiedematogenic effect of copaiba oil

Leticia G. Lucca[a], Sheila P. de Matos[a], Cristiane B. de Mattos[a], Helder F. Teixeira[a], Renata P. Limberger[a], Valdir F. Veiga Jr.[b], Bibiana V. de Araújo[a], Letícia S. Koester[a*]

[a] Pharmaceutical Sciences Graduation Program, Pharmacy College, Federal University of Rio Grande do Sul, 2752 Ipiranga Avenue, Porto Alegre-RS, Brazil.

[b] Chemistry Department, Federal University of Amazonas, 6200 General Rodrigo Octávio Avenue, Manaus-AM, Brazil.

** Correspondence author:*

Letícia Scherer Koester

Phone: +55 51 33085278

E-mail: leticia.koester@ufrgs.br

Abstract

Copaiba oil is a natural product obtained from the trunk of *Copaifera* trees. This oil-resin is used in folk medicine in Amazonia as an anti-inflammatory, antiparasitary and antimicrobial. β-caryophyllene, a major component in copaiba oil, had its anti-inflammatory effect studied in recent papers and is used as a copaiba biomarker. In the present study, we developed positively charged copaiba oil nanoemulsions (PCN), with cetyltrimethylammonium bromide and oleylamine, and compared to a negatively charged nanoemulsion (NCN) concerning skin permeation and *in vivo* antiedematogenic effect. Results show that skin permeation with the PCN increased three fold β-caryophyllene retention in the epidermis, and also in the receptor fluid compared to the NCN. *In vivo* tests were performed in mouse ear edema induced by arachidonic acid and in rat paw edema induced by formalin. In mouse ear edema, NCN and PCN promoted an edema inhibition (33 %) with statistically equal effect ($p > 0.05$) to the positive control, ketoprofen (44 %). In rat paw edema, both nanoemulsions presented antiedematogenic effect (edema inhibition above 60%) similar to the positive control. Copaiba oil also exhibited edema inhibition, but the nanoemulsification process led to an increased effect to the oil.

Key words: *Copaifera multijuga*; skin permeation; β-caryophyllene; rat paw edema; mouse ear edema.

1. Introduction

Copaiba oil is an oil-resin exuded from the trunk of the trees of several *Copaifera* species. It is used as a popular medicine in the Amazon rainforest area, in Brazil, especially to treat inflammatory related diseases, as antimicrobial, antiparasitary and also for wound healing.[1] This oil-resin is composed mainly by terpenes, and each species presents a different profile of these substances.[2] *Copaifera multijuga* Hayne, the species used in this paper, produces an oil-resin rich in sesquiterpenes, especially β-caryophyllene. Different authors described the potential of both copaiba oil and β-caryophyllene as anti-inflammatory.[3-11]

Nanoemulsions are heterogeneous systems composed of oil droplets stabilized by surfactants dispersed in an aqueous medium, reaching droplet sizes from 100 nm to 500 nm. This systems are suitable carriers for topical use, since their small droplet size and large surface area can facilitate skin penetration of substances.[12] Also, they present low skin irritability and high drug-loading capacity, especially for lipophilic compounds.[13]

Recently, our research group described the optimization of a copaiba oil nanoemulsion and a new method to analyze its major component, β-caryophyllene, in skin permeation samples.[14-15] In our findings, nanoemulsions were appropriate carriers to load copaiba oil, arriving in a dosage form with 30% oily core (20% copaiba oil and 10% fixed oil), using the method of high pressure homogenization.[14] Furthermore, a method to detect the previously mentioned β-caryophyllene in skin samples was validated, by means of a method in gas chromatography coupled with mass spectrometer. In that study, it was found that the copaiba oil nanoemulsification facilitated the skin permeation of β-caryophyllene down to the dermis, the deepest layer of the skin.[15]

In order to enhance the permeation of molecules through the skin using nanostructures, data in the literature suggests that nanoparticles with cationic surface could improve the passage in this barrier, since these positive charges could interact with the negative charges in the stratum corneum and open an access through the skin.[16-22] Furthermore, in a topical anti-inflammatory treatment, the dermis is the layer of interest, and the positive surface charge in a nanoparticle could lead to a higher retention there.[16]

The previously optimized nanoemulsion containing copaiba oil presents a negative surface charge, which was credited to the resinous acid components present in the oil-resin.[14] Therefore, the aims in this study were to develop a copaiba oil nanoemulsion containing a positive surface charge using cationic surfactants, to evaluate the influence of this positive charge on β-caryophyllene skin permeation and its *in vivo* antiedematogenic effect.

2. Materials and methods

2.1. Chemicals and reagents

Crude copaiba oil was obtained from *Copaifera multijuga* Hayne tree in the Ducke Forest Reserve of the Instituto Nacional de Pesquisas da Amazônia (INPA) at Manaus, Amazonas State, Brazil (S 2°57'43'', W 59°55'38'', 120 m). Chromatographic characterization from the oil-resin confirmed the presence of 41.3% of β-caryophyllene, the major component. β-caryophyllene reference standard, arachidonic acid, Span 80®, Tween 20®, ketoprofen reference standard, cetyltrimethylammonium bromide (CTAB) and oleylamine (OA) were obtained from Sigma–Aldrich (St. Louis, USA). OA consisted of a mixture of 70% oleylamine and 30% of other fatty amines. Lipoid GmbH (Ludwigshaten, Germany) kindly donated Medium Chain Triglycerides (MCT). Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, USA). All other chemicals or reagents were of analytical grade.

2.2. Copaiba oil positively charged nanoemulsions

In order to produce a positively charged nanoemulsion (PCN) two cationic surfactants were used: CTAB and OA. Formulations were based in a previously optimized nanoemulsion, which is negatively charged (NCN).[14] Formulations are described in Table 1.

All formulations were produced according to the method described by Dias et al (2014).[14] To prepare the formulations F1 to F4, OA was added to the oily phase, while in formulations F5 to F8, CTAB was added to the aqueous phase. Briefly, aqueous and oily phases were mixed separately under magnetic stirring at room temperature. After complete solubilization of the phases' components, aqueous phase was poured into the oily phase to form a coarse emulsion. After five minutes under magnetic stirring at room temperature, the emulsion was subjected to high-pressure homogenization (EmulsiFlex-C3, Avestin, Canada) at 750 bar for 6 cycles, without any previous downsizing step. After production, all samples were kept under 4°C refrigeration.

After preparation, all formulations were submitted to an evaluation concerning zeta potential, polydispersity index and droplet size. Zeta potential was measured by laser

Doppler velocimetry. Droplet size and polydispersity index were measured by dynamic light scattering with 1.45 refractive index value. All analysis were made in triplicate in Zetasizer Nanoseries ZN90 (Malvern Instruments, United Kingdom) by diluting 10 µL of the nanoemulsion in 10 mL NaCl 1.0 mM ultra-filtered in 0.22 µm filter.

Table 1. Positively charged nanoemulsion formulations composition.

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
Copaiba oil (%)	20	20	20	20	20	20	20	20
MCT (%)	10	10	10	10	10	10	10	10
Span 80™ (%)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
OA (%)	0.2	0.4	1.0	2.0	--	--	--	--
CTAB (%)	--	--	--	--	0.25	0.5	0.75	1.0
Tween 20™ (%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Water q.s. (mL)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

OA: oleylamine; CTAB: cetyltrimethylammonium bromide. OA concentrations are corrected to 70% of OA.

2.3. *In vitro* skin permeation studies

After choosing the positively charged nanoemulsions, *in vitro* permeation studies were conducted according to a previous paper.[15] The assay was performed in Franz diffusion cell apparatus (Dist, Brazil) with 12 ml receptor fluid compartment and 1.5 cm diameter in acceptor compartment (part in contact with the skin), using porcine ear skin as membrane (mean skin thickness was 1.0 ± 0.2 mm). The receptor fluid consisted of a mixture of phosphate buffer saline (PBS) pH 7.4 and ethanol in a 1:1 proportion, to obtain a previously determined sink condition.[15] After 8 hours, the skin was cleaned with purified water and its layers were separated. Stratum corneum was removed using the tape-stripping method (15 tapes).[23] With the help of a scalpel, epidermis was scraped from the dermis. Permeation studies were performed with the positively and negatively charged nanoemulsions (n=6).

2.4. Chromatographic analyses

Samples from the permeation studies were analyzed by gas chromatograph (GC) coupled with mass spectrometer (7890A/5975C, Agilent Technologies, USA) according to a previous study.[15] All samples were prepared using headspace mode in CombiPal Autosampler (CTC Analytics AG, Switzerland) set at 50 ºC for 10 minutes. Samples (epidermis, dermis and tapes for each cell) were placed in vials and analyzed separately. Injection was made in splitless mode.

GC system was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 mm). Carrier gas was ultrapure helium (1.0 mL/min). Oven temperature was programmed from 60 ºC for 3 min with an increase of 40 ºC/min, to 300 ºC, finalizing the chromatographic run at 9 min. Injector, transfer line (interface), source and quadrupole temperatures were set at 220 ºC, 300 ºC, 230 ºC and 150 ºC respectively. Mass detector was operated with an electron impact system at 70 eV. The signal was recorded and processed with GC/MS Data Analysis Software.

Also, β-caryophyllene content was determined in the formulations used in the skin permeation experiment according to Dias et al (2012).[24]

2.5. Animals

Adult male Swiss mice (30-40 g) were provided by Bioterio Central from Federal University of Pelotas. Adult male Wistar rats (100-200 g) were provided by CREAL (Centro de Reprodução e Experimentação de Animais de Laboratório). All animals were maintained under standard conditions (22 ± 1 °C at 40-60% relative humidity and 12 hours light-dark cycle). All animals had free access to food and water. Mice were sacrificed by cervical dislocation and rats were sacrificed by intraperitoneal propofol injection (30 mg/kg). This study was approved by Animal Use Ethics Committee at Federal University of Rio Grande do Sul (protocol number 25866).

2.6. Arachidonic acid-induced mice ear edema

Groups of five mice were treated topically with copaiba oil, nanoemulsion formulations or ketoprofen (positive control) in the posterior and anterior part of the right ear. Left ear did not receive any treatment and served as a control for each animal. After one hour, edema was induced by topical application of arachidonic acid (solution in ethanol, 0.2 mg/µL) at 2 mg/ear (10 µL) only in the right ear. Negative control group received only the vehicle (ethanol) in the right ear. Positive control group received a ketoprofen solution at 4 mg/ear (in acetone solution, 10 µL).

After the dose-response curve using 100, 200 and 400 mg/kg copaiba oil doses, 200 mg/kg dose was chosen to perform the experiment with the respective nanoemulsion (data not shown). Since the nanoemulsion presents 20% of copaiba oil, the volume of nanoemulsion used was 5 fold that of the copaiba oil volume. Treatments and arachidonic acid were applied using an automatic pipette (20 and 100 µL).

Ear edema was measured one hour after the inflammation induction in the right ear, using a thickness gauge (Mitutoyo Corporation, Japan). The right ear weight of each mouse in each group was also used as an edema measurement. Edema inhibition (EI) was calculated comparing the thickness difference of the right and left ear from the groups to the thickness difference of the right and left ear of the control group according to Eq. (1), using the medium thickness value for each group.

$$\text{EI}\ (\%) = \left[1 - \left(\frac{REt - LEt}{REc - LEc}\right)\right] * 100 \qquad (1)$$

Where REt is the thickness of the treated group right ear, REc is the thickness of the control group right ear, LEt is the thickness of the treated group left ear and LEc is the thickness of the control group left ear.

2.7. Formalin-induced rat paw edema

Edema was induced by intraplantar injection of formalin. Each group (n=5) received the treatment (copaiba oil or nanoemulsion) in the right hind paw one hour before the edema induction.

Positive control group received a ketoprofen solution at 4.0 mg/paw (in acetone). After the dose-response curve using 100, 200 and 400 mg/kg doses, a 200 mg/kg copaiba oil concentration was chosen to perform the experiment with the respective nanoemulsion (data not shown). Negative control group did not receive treatment.

Before the edema induction, animals were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg) and xylasine (25 mg/kg). Formalin solution (100 µL, 10% v/v in saline) was injected in the right hind paw, while the left paw received the same amount of vehicle, saline (NaCl 0.9%).

Paw volume was measured after four hours using a plethysmometer (Ugo Basile, Italy). Edema was measured by the difference between right hind paw volume and the basal volume for each animal. Edema inhibition (EI) was measured by the percentage of edema comparing the volume of the paw in the times for the groups to the volume of the control group as shown in Eq. (2), using the medium volume value for each group.

$$\text{EI (\%)} = \left[1 - \left(\frac{RPt}{RPc}\right)\right] * 100 \quad (2)$$

Where RPt is the volume of the treated right paw and RPc is the volume of the control right paw.

2.8. Statistical analysis

Statistical analysis in the *in vivo* tests were performed by one-way ANOVA methodology followed by Tukey's tests with a significance level of $P < 0.05$.

3. Results and discussion

The main goal in the modification of the previously optimized copaiba oil nanoemulsion was to produce a positively charged nanoemulsion, in order to increase β-caryophyllene penetration to the dermis, skin layer of interest for topical anti-inflammatory activity.[14]

CTAB is a quaternary ammonium compound used in the cosmetic industry as a cationic surfactant and as antimicrobial preservative. Its safe concentration to use in cosmetics is between 0.1 and 1.0% (2.74 – 27.40 mM), although it can reach 10% (274 mM) for seborrheic dermatitis formulations.[25] OA is a long chain primary amine with one unsaturation and is used as surfactant and, more recently, as a precursor/stabilizer of nanoparticles.[26] According to Greim et al (1998), OA's LD_{50} in rats is 200-2000 mg/kg, but it presents skin and eye irritating characteristics.[27]

According to Zhang et al (2015), CTAB and OA can cause a decrease in cell viability in high concentrations after 2 hours (above 100 mM) and 24 hours (above 30 mM).[26] In the present study, we used CTAB concentrations in the range accepted and they proved to be enough to reduce droplet size. However, for OA formulations, only concentrations above 35 mM were able to reduce droplet size and to increase/reverse zeta potential.

Concerning zeta potential (Fig. 1), only F1 and F5 presented a negative value, which means that the cationic surfactants concentrations used were not adequate to this kind of formulation. Other formulations presented an increase in the zeta potential value according to the increase in the surfactant concentration.

Only F4 and F6-F8 presented an adequate value for zeta potential (22 to 50 mV). Even though F6-F8 formulation presented zeta potential values slightly below the value considered ideal, 30 mV, they did not present signs of coalescence or flocculation and were considered suitable to our research.[28,29] F2 and F3 presented a low zeta potential value, indicating a probable future instability in the system.

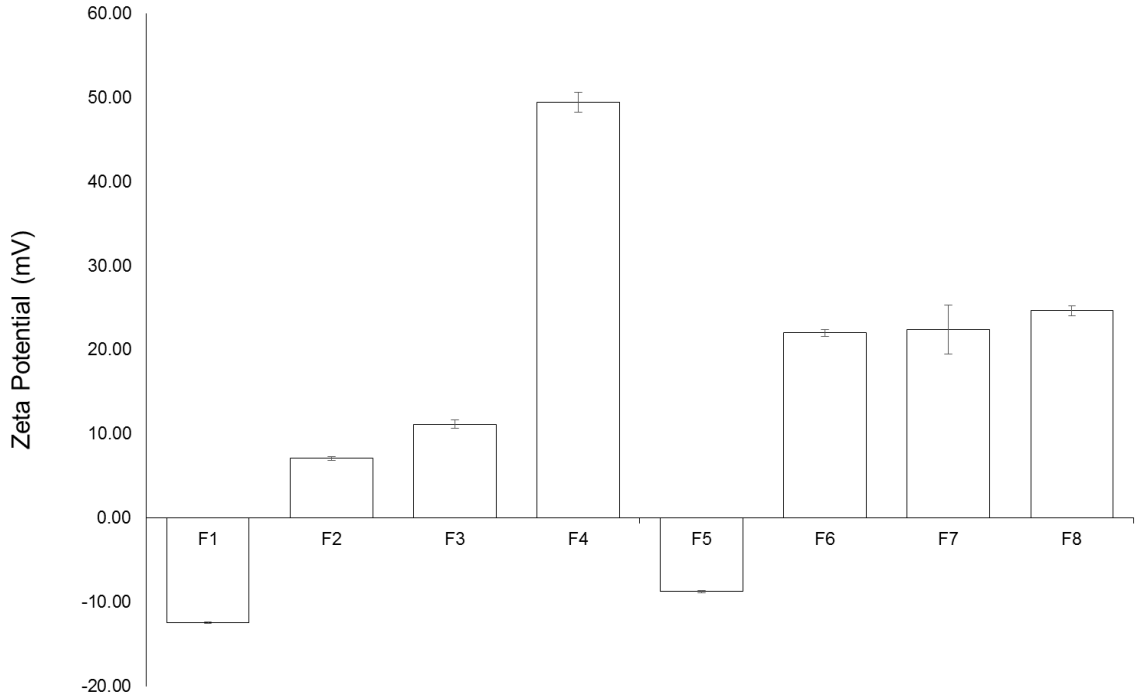

Figure 1. Nanoemulsions zeta potential (mV).

As stated by Martini et al (2008), there are two phenomena that can justify the increase of zeta potential in a nanoparticle droplet interface: an alkaline environment or an interface saturation by the cationic surfactant.[30] Furthermore, they can explain the presence of a plateau or a constant increase in the values. Our results show that the increase of CTAB concentration did not influence in the zeta potential values, hence it suggests that there is a saturation of cationic molecules in the droplet surface, which is common for quaternary amines.[31]

On the other hand, OA is a primary amine and its increase lead to a progressive increase in potential zeta values, indicating that the medium pH (in this case acid, due to resinous acids in copaiba oil) influenced in the zeta potential. In acid pH, OA is protonated and to reverse and increase zeta potential it is necessary to increase the medium pH to a more alkaline state.[32,33] In this case, the nanoemulsion medium is slightly acid (around pH 5.0), thus the

saturation hypothesis can be applied here too, but higher concentrations of OA were not added since they would be toxic.

Concerning droplet size, formulations F1-F3 and F5 presented elevated droplet size and polydispersity index values (Fig. 2). This results indicate that these formulations are unstable, probably because of droplet aggregation due to their low zeta potential value.[19] Droplet sizes between 100 nm and 500 nm are considered usual for topical nanoemulsions.[18,34] However, in this study, we selected nanoemulsions between 200 nm and 250 nm, considering that the amount of oil used could not produce nanoemulsions with smaller size than those cited.[35]

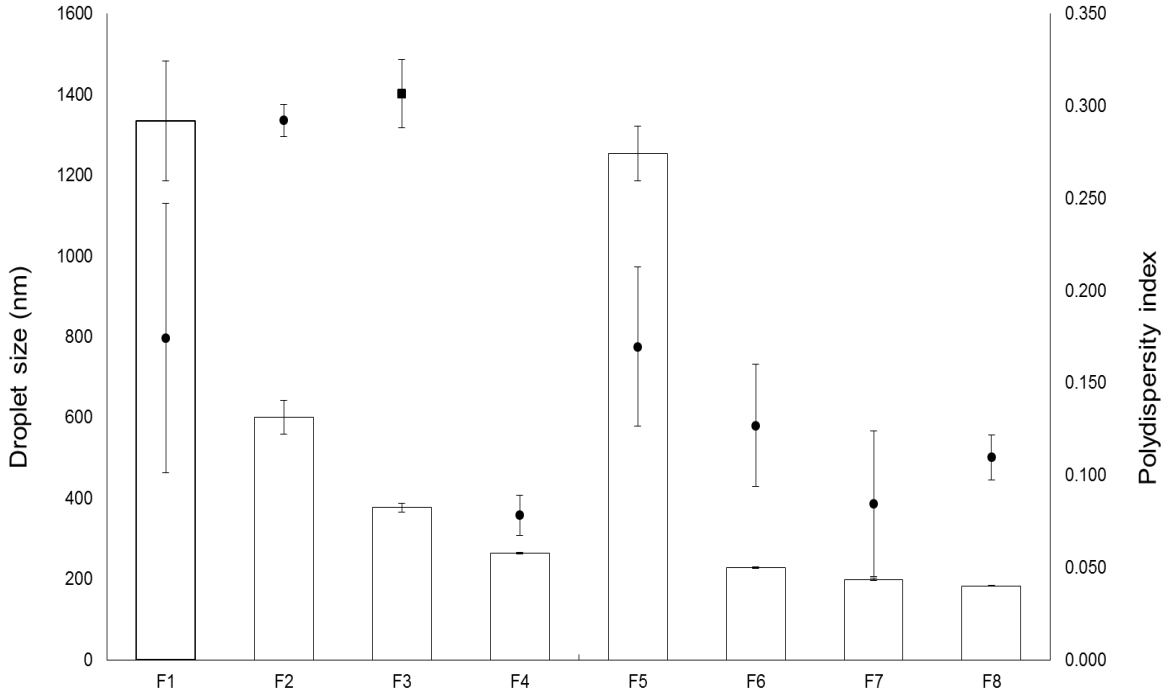

Figure 2. Formulations droplet size (bars) and polydispersity index (black points).

Based on the results obtained in this section, formulations F4 and F7, containing 70.0 and 21.0 mM of OA and CTAB respectively, were chosen to perform the following skin permeation experiments. They presented a similar droplet size and polydispersity index to the negatively charged nanoemulsion previously optimized (250.6 ± 2.1 nm and 0.08 ±

0.03, respectively).[14] In addition, CTAB nanoemulsions presented a stable zeta potential between the high concentrations, and we have chosen F7, which presented a good physicochemical profile in a smaller CTAB concentration.

Table 2 shows the results for the skin permeation experiment comparing the negatively charged nanoemulsion with the two positively charged nanoemulsions, for each cationic surfactant selected (F4 and F7). All formulations presented a higher retention in the epidermis layer, followed by the dermis, stratum corneum and then small or not present permeation to the receptor fluid.

Table 2. Permeation assay results. Results are shown as mean ± standard deviation.

	Nanoemulsions		
Skin layers	NCN	PCN-OA (F4)	PCN-CTAB (F7)
Stratum corneum (µg/mL)	0.18 ± 0.12	0.44 ± 0.27	0.12 ± 0.07
Epidermis (µg/g)	293.44 ± 133.15	1091.98 ± 350.01	887.21 ± 193.99
Dermis (µg/g)	17.85 ± 10.93	21.13 ± 8.64	15.47 ± 8.65
Receptor fluid (µg/mL)	ND	0.29 ± 0.19	0.11 ± 0.09

NCN: negatively charged nanoemulsion; PCN-OA: positively charged nanoemulsion with oleylamine; PCN-CTAB: positively charged nanoemulsion with cetyltrimethylammonium bromide; ND: not detected.

Many factors regarding nanoparticles influence skin retention/permeation, such as surface charge and droplet size.[18,29,36-38] Our results suggest that the positive charge in the nanoemulsion could promote skin permeation, caused by a higher interaction with the stratum corneum corneocytes, increasing β-caryophyllene content in epidermis in 3.72 and

3.02 folds for PCN-OA (F4) and PCN-CTAB (F7) respectively compared to NCN.[29,39] Furthermore, there was an increase of β-caryophyllene in the receptor fluid when PCNs were tested, while β-caryophyllene was not detected in this compartment when NCN was applied over the skin. However, in the dermis, the layer of interest, there was no increase in β-caryophyllene retention. Further studies are necessary to understand why no difference in dermis retention was observed even though a higer permeation down to the receptor fluid is supposed to be related to a higher retention in the epidermis, as observed. Nevertheless, it is worth mentioning that the results represent a "snapshot" after 8 hours of *in vitro* permeation experiment, and the epidermis could behave as a "reservoir" to supply more β-caryophyllene to dermis.

Even though the formulation containing OA presented higher values in all the skin layers and in the receptor fluid, these values were not statistically different ($p > 0.05$) from values found for CTAB-containing formulation. Hence, F7 was chosen to perform the following experiments. Furthermore, CTAB concentration used is considered safer to use topically and is already used as an excipient in topical formulations in the cosmetic industry.[25]

β-caryophyllene content was determined for NCN and PCN-CTAB (F7), since they were chosen to perform *in vivo* experiments. NCN presented 102.13 ± 0.07% and PCN-CTAB (F7) presented 106.73 ± 0.08% of β-caryophyllene.

Arachidonic acid is involved in the cyclooxygenase (COX) and lipoxygenase (LOX) inflammation pathways and its topical administration leads to immediate vasodilatation and erythema.[40] Mouse ear edema was induced in the right ear by topical application of arachidonic acid (0.2 mg/µL, in ethanol).

Table 3 shows the edema measured by ear thickness (mm) and ear weight (mg) and edema inhibition (%).

Concerning the antiedematogenic effect measured using ear thickness results, both nanoemulsions (NCN and PCN-CTAB) presented an equal antiedematogenic profile after 60 minutes, reducing significantly ($p < 0.05$) ear edema (compared to the negative control group). They also presented a statistically equal result to copaiba oil ($p > 0.05$). However,

when compared to the positive control, ketoprofen, only the nanoemulsions presented a statistically equal result ($p > 0.05$).

Table 3. Edema measured by mouse ear thickness (mm) and weight (mg) for the treatments with ketoprofen, copaiba oil, NCN, PCN-CTAB (F7) and blank nanoemulsion formulations (n=5). Data shown as mean (standard deviation).

Groups	Thickness (mm)	Weight (mg)	Edema Inhibition (%)
Control	0.48 (0.03)	11.20 (0.69)	-
Copaiba oil	0.35 (0.03) [a]	4.66 (0.66) [a]	25.0
Ketoprofen	0.27 (0.02) [a]	1.56 (0.35) [a]	44.0
PCN-CTAB (F7)	0.32 (0.05) [a b]	6.12 (2.90) [a]	33.0
NCN	0.32 (0.02) [a b]	2.40 (0.91) [a c]	33.0
PCN-Blank	0.36 (0.04)	9.12 (2.05)	24.0
NCN-Blank	0.41 (0.01)	7.30 (0.52)	24.0

[a] Statistically different ($p < 0.05$), compared to control (non-treated group); [b,c] Statistically equal ($p > 0.05$) to ketoprofen.

As long as ear weight was measured, only NCN did not present statistically difference ($p > 0.05$) from the positive control, ketoprofen. It is noteworthy that weight measurements presented higher Relative Standard Deviation (RSD) when compared to thickness measures (6.0 to 15.0 % for thickness and 6.0 to 47.0 % for weight), specially for the result with PCN. In addition, weight measurements were more challenging to assess during the experimental stage, due to difficulties in cutting the ear skin in an equal size to all samples or in the real affected area of the ear. Thus, weight results were used as complementary data to thickness results, but the last ones were used to calculate edema inhibition.

Since arachidonic acid is a precursor from prostaglandins and leukotrienes produced by COX and LOX enzymes, the inhibitor of this pathways acts like non-steroidal drugs, such

as ketoprofen. In addition, arachidonic acid can cause mast cells degranulation, leading to histamine release.[40-43] Hence, copaiba oil could behave as a non-steroidal anti-inflammatory and the nanoemulsification process can enhance this effect, although the presence of a cationic surfactant did not exhibit an influence in this process.

Rat paw edema was induced by formalin 10% (v/v). According to Lee and Jeong (2002), this concentration is ideal to promote prominent edema formation and inflammation induction in rats, while lower concentrations (below 5%) present only behavior response.[44] Formalin induces a biphasic inflammation event. The initial phase (up to 5 minutes) mediators like substance P and bradykinin are released while in the second phase, histamine, serotonin, prostaglandins, and bradykinin are involved.[45]

In addition, the rat paw model could indicate a more profound topical action from the copaiba oil nanoemulsions, since the model is considered to increase systemic circulation achievement through the dermis, also resembling the process of arthritis.[46,47] Our result shows that both copaiba oil nanoemulsions (NCN and PCN-CTAB) had a similar antiedematogenic effect in this model, suggesting that the oil compounds can reach the dermis, which corroborates with the permeation assay, where β-caryophyllene reached this layer from all formulations in the same range.

Table 4 presents the paw edema (mL) measured 4 hours after the edema induction and edema inhibition for each treatment.

As can be seen, both copaiba oil nanoemulsions (NCN and PCN-CTAB) presented a high antiedematogenic effect not different to the positive control, ketoprofen ($p > 0.05$), but higher than the crude oil. As expected, blank samples, which did not contain copaiba oil, did not present high edema inhibition. Also, copaiba oil was statistically equal ($p > 0.05$) to the non-treated group (control).

Copaiba oil presented anti-inflammatory activity in previous studies, both *in vivo* and *in vitro.*[2,4,5,48,49] Gomes et al (2010) demonstrated that different copaiba oil *(Copaifera multijuga* Hayne) fractions presented antiedematogenic activity through inhibition of peripheral serotonin and histamine receptors in rat paw edema induced by carrageenan,

histamine and serotonin.[5] Also, Veiga Junior et al (2007) concluded that *Copaifera multijuga* Hayne presented a higher anti-inflammatory effect compared to other species (*Copaifera cearensis* Huber ex Ducke and *Copaifera reticulata* Ducke), probably due to its high β-caryophyllene content.[4]

Table 4. Edema (mL) in rat paw 4h after treatment with ketoprofen, copaiba oil, NCN, PCN-CTAB (F7) and blank nanoemulsion formulations. Data shown as mean (standard deviation).

Groups	Edema (mL)	Edema Inhibition (%)
Control	2.53 (1.29)	-
Copaiba oil	2.20 (0.97)	13.0
Ketoprofen	0.82 (0.71) [a]	67.0
PCN-CTAB (F7)	0.58 (0.42) [a b]	77.0
NCN	0.64 (0.57) [a b]	75.0
PCN-Blanc	2.00 (1.17)	21.0
NCN-Blanc	1.93 (0.96)	24.0

[a] Statistical difference from control group ($p < 0.05$); [b] do not present statistic difference ($p > 0.05$) to ketoprofen.

Our findings confirm that copaiba oil presents an antiedematogenic effect, which is an inflammation symptom. Furthermore, the nanoemulsification process increases this effect, in a smaller dose compared to the data shown in the literature. Carvalho et al (2005) described the topical anti-inflammatory effect of copaiba oil extracted from *Copaifera duckei* Dwyer in very high doses (517, 1035 and 1802 mg/kg), reaching an edema inhibition of 18% only for the higher dose in the rat paw edema assay.[2] In that paper, the oil-resin used presented only 5% of β-caryophyllene, while our sample presents 41.3% of β-caryophyllene. This could point out the importance of this sesquiterpene and its probable responsibility for the *in vivo* activity in the copaiba oil.

The presence of a cationic surfactant did not present an increased effect in the antiedematogenic activity, since the results obtained were not statistically different ($p > 0.05$) between the formulations and between the positive control, ketoprofen.

4. Conclusion

In this paper, we compared copaiba oil nanoemulsion with different surface charge: one positive and one negative. Concerning skin permeation, our results suggest that the positive charge increased β-caryophyllene retention in the epidermis, but not in the dermis. Furthermore, positively charged nanoemulsion increased permeation through the skin, arriving in the receptor compartment. In mouse ear edema, negatively charged nanoemulsion presented an increased activity compared to the positively charged one and was statistically equal to the positive control, ketoprofen. Rat paw edema showed that both formulations (positively and negatively charged) presented a similar antiedematogenic profile and did not differ from the positive control, ketoprofen. In both tests, crude copaiba oil presented a smaller edema inhibition compared to the nanoemulsion treatments. Therefore, this study showed for the first time that the nanoemulsification of copaiba oil potentiates its antiedematogenic effect and that the positive charge influenced β-caryophyllene skin retention profile but not *in vivo* pharmacological response.

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CAPÍTULO IV

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Anti-inflammatory effect from a hydrogel containing nanoemulsified copaiba oil (*Copaifera multijuga* Hayne)

Leticia G. Lucca[a], Sheila P. de Matos[a], Tainá Kreutz[a], Helder F. Teixeira[a], Valdir F. Veiga Jr.[b], Bibiana V. de Araújo[a], Renata P. Limberger[a], Letícia S. Koester[a*]

[a] *Pharmaceutical Sciences Graduation Program, Federal University of Rio Grande do Sul, Avenida Ipiranga 2752, 90610-000, Porto Alegre - RS, Brazil.*

[b] *Chemistry Departament, Federal University of Amazonas, Av. Gal. Rodrigo Octávio, 6.200, 69.079-000, Manaus - AM, Brazil.*

** Correspondence author:*

Letícia Scherer Koester

Mailing address: Avenida Ipiranga 2752, Laboratório 606, 90610-000, Porto Alegre/RS, Brasil

Office telephone: +55 51 33085278

Fax number: +55 51 33085243

E-mail: leticia.koester@ufrgs.br

Abstract

Copaiba oil is used as a popular medicine in the Amazonian forest region, especially due to its anti-inflammatory properties. In this paper, we describe the formulation of hydrogel containing copaiba oil nanoemulsions (with positive and negative charges), its skin permeation and its anti-inflammatory activity in two *in vivo* models: mouse ear edema and rat paw edema. Three hydrogels were tested (Carbopol®, hydroxyethylcellulose and chitosan), but only Carbopol® and hydroxyethylcellulose hydrogels presented good stability and did not interfere with the nanoemulsions droplet size and polydispersity index. In skin permeation assay, both formulations, positively charged nanoemulsion (PCN) and negatively charged nanoemulsion (NCN), presented a high retention in epidermis (9.76 ± 2.65 µg/g and 7.91 ± 2.46 µg/cm², respectively) followed by a smaller retention in the dermis (2.43 ± 0.91 µg/cm² and 1,95 ± 0.56 µg/cm², respectively). They also presented permeation to the receptor fluid (0.67 ± 0.22 µg/cm² and 1.80 ± 0.85 µg/cm², respectively). In addition, anti-inflammatory effect was observed to NCN and PCN with edema inhibitions of 69% and 67% in mouse ear edema and 32% and 72% in rat paw edema, respectively. Histological cuts showed the decrease of inflammatory factors, such as dermis and epidermis hyperplasia and inflammatory cells infiltration, confirming the anti-inflammatory effect from both copaiba oil nanoemulsions incorporated in hydrogel.

Key-words: Hydrogel; *Copaifera multijuga* Hayne; inflammation; mouse ear edema; rat paw edema.

Introduction

Essential oils are used in many areas, such as in the cosmetic and perfume industries and also as a popular medicine, specially due to their antimicrobial properties [1,2]. Copaiba oil is extracted from the trunk of *Copaifera* trees and represents a great commercial product, as well as a renewable source of natural therapy in the Amazonian region popular medicine,

where it is used as anti-inflammatory, anti-septic and wound healer, both by oral and topical routes [3].

Copaifera multijuga Hayne is a common species of *Copaifera* tree in the Amazon rain forest, Brazil [4]. Its oilresin is composed basically by sesquiterpenes (hydrogenated and oxygenated) and diterpenes and has been described as a potent anti-inflammatory, even when compared to other *Copaifera* species, specially due to its high β-caryophyllene concentration[3]. β-caryophyllene is a sesquiterpene and has been also studied due to its anti-inflammatory effects [5,6].

Recently, studies involving copaiba oil and nanoemulsions have been published by our research group, including the development of a nanoemulsion [7] and a method to detect the major component β-caryophyllene in nanoemulsions and skin samples [8,9]. Also we described the potentialization of copaiba oil anti-edematologic effect when incorporated into nanoemulsions [10].

However, this dosage form has very low viscosity to be applied to the skin and its incorporation into a hydrogel can afford a better therapeutic compliance. Moreover, hydrogels are aqueous formulations with wet and pleasant touch sensing properties, which do not present affinity for oil droplets or lipophilic compounds [11,12]. In this way, it is hypothesized that the incorporation of copaiba oil nanoemulsion into a hydrogel may enhance the permeation of its major compound, β-caryophyllene through the skin.

Thus, the aim of this study is to incorporate copaiba oil nanoemulsions in different hydrogel polymers and to evaluate the influence of its thickening effect on β-caryophyllene skin permeation and on the anti-inflammatory effect *in vivo*. This paper shows for the first time the production of a copaiba oil semi-solid dosage form that can be used in the skin and its pharmacological activity.

Materials and Methods

Materials

β-caryophyllene reference standard, arachidonic acid, Span 80™, Tween 20™, cetyltrimethylammonium bromide (CTAB), chitosan (CHI), Natrosol™ or hydroxyethylcellulose (HEC) and Carbopol 980™ (CARB) were purchased from Sigma–Aldrich (St. Louis, USA). Medium chain triglycerides (MCT) was purchased from Delaware (Porto Alegre, Brazil). Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, Billerica, USA). All other chemicals or reagents were of analytical grade.

Copaiba oil was extracted from *Copaiferamultijuga* Hayne trunk in Ducke Forest Reserve from InstitutoNacional de Pesquisas da Amazônia (INPA) at Manaus, Amazonas state, Brazil(S 2°57'43'', W 59°55'38'', 120 m).

Preparation of copaiba oil nanoemulsion and hydrogels

Nanoemulsions containing copaiba oil were prepared according to a previous study [10]. Table 1 describes the positively charged nanoemulsion (PCN) and negatively charged nanoemulsion (NCN) formulations.

First, aqueous (water and Tween 20™ or CTAB) and oily phases (copaiba oil, MCT and Span 80™) were mixed separately. After, the aqueous phase was poured in the oily phase, under magnetic stirring, to form a coarse emulsion. This coarse emulsion was submitted to high-pressure homogenization (Emulsiflex-C3, Avestin, Canada) for 6 cicles at 750 bar. All steps were performed under room temperature.

Hydrogels were formed by mixing the polymer powder with the nanoemulsion. HEC hydrogel (2%) was left to swell overnight, CARB hydrogel (0.5%) was formed by adding triethanolamine and CHI hydrogel (3%) was formed by adding acetic acid.

Blank nanoemulsions were prepared without copaiba oil (only MCT up to 30 % w/w). Blank hydrogel was prepared with water instead of nanoemulsion.

After preparation, all samples were analyzed according to their droplet size, polydispersity index, and zeta potential. β-caryophyllene content was analyzed in the hydrogels used on

in vivo experiments and during one year (for stability purposes), by a previously validated method [8].Morphological analysis from PNC and NCN incorporated in hydrogel was performed using a scanning electron microscope (SEM) with a TM3000 (Hitachi High Technologies America, Illinois, USA).

Table 1. Copaiba oil nanoemulsions.

Composition	**PCN**	**NCN**
Copaiba oil (%)	20.0	20.0
MCT (%)	10.0	10.0
Span 80™ (%)	3.0	3.0
Tween 20™ (%)	1.0	1.0
CTAB (%)	0.75	-
Water q.s. (%)	100	100

PCN: positively charged nanoemulsion; NCN: negatively charged nanoemulsion; MCT:,medium chain triglycerides; CTAB: cetyltrimethylammonium bromide.

Rheological study

The hydrogel chosen to perform skin permeation and *in vivo* tests was evaluated for its rheological profile using a Brookfield Rotational Viscosimeter, model DV–II+ (Brookfield Engineering Laboratories, Middleboro, USA). 20 g of formulation was placed in a container suitable for the equipment, at rotational speed 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 3.0 and 5.0 rpm with spindle 29. Results are shown as shear stress (Pa) *vs* shear rate (s^{-1}).

In vitro skin permeation

Skin permeation assay (n= 5) was performed in Franz diffusion cell apparatus according to a previous study [9]. Full thickness porcine ear skin was used as membrane. Previously

from use, the fat tissue and the hair were removed from the outer part of the ear. Receptor fluid consisted in a mixture of phosphate buffer saline pH 7.4 and ethanol (1:1). After 8 hours, the skin was cleaned with ultrapure water to remove formulation excess and skin layers were separated. Stratum corneum was separated using the tape-stripping method. Epidermis was separated from dermis using a scalpel. A 1 mL aliquot from the receptor fluid was also collected after 8 hours of study.

All samples were placed in headspace vials to perform analysis in gas chromatograph coupled with mass spectrometer (5975C, Agilent Technologies, USA), using a previously validated method [9].Samples were prepared using headspace mode in CombiPAL Autosampler (CTC Analytics AG, Basel, Switzerland) set at 50ºC for 10 minutes.

Animals

Adult male Swiss mice (30-40g) were provided by Bioterio Central from Universidade Federal de Pelotas. Adult male Wistar rats (100-200g) were provided by CREAL (Centro de Reprodução e Experimentação de Animais de Laboratório). All animals were maintained under standard conditions (22 ± 1°C at 40-60% relative humidity and 12 hours light-dark cycle). All animals had free access to food and water. Mice were sacrificed by cervical dislocation and rats were euthanized by intraperitoneal propofol injection (30 mg/Kg). The Animal Use Ethics Committee from Federal University of Rio Grande do Sul approved this study (protocol number: 25866).

Arachidonic acid-induced mouse ear edema

Groups of five mice were treated with copaiba oil or hydrogel formulation in the posterior and anterior part of the right ear. The left ear did not receive any treatment, as a control for each animal. After one hour, the edema was induced by topical application of arachidonic acid (solution in ethanol, 0.2 mg/µL) at 2 mg/ear (10 µL) only in the right ear.

Positive control group received a ketoprofen solution at 4 mg/ear (in acetone solution, 10 µL). 200 mg/Kg copaiba oil concentration was chosen [10] to perform the experiment with

the respective hydrogel. Control group received only the vehicle (ethanol) in the right ear. Since the nanoemulsion presents 20% of copaiba oil, the concentration used with the final dosage form treatment was 1000 mg/Kg (5 fold the copaiba oil dose). Treatments were applied with automatic semi-solid pipette (100 µl) and arachidonic acid was applied using an automatic pipette (20 µL).

Ear edema was measured after 1 hour, using a thickness gauge (Mitutoyo Corporation, Kanagawa, Japan). After the sacrifice, 6 mm^2 fragment of both ears was removed and weighted. Edema was measured by the ear thickness in the groups' right ear. The weight difference between the right and the left ear of each rat in each group was also used as an edema measurement. Edema inhibition percentage (EI%) was calculated comparing only the weight difference of the right and left ear for the groups to the weight difference of the right and left ear of the control group according to Equation (1)

$$EI\ (\%) = [1 - \left(\frac{REt - LEt}{REc - LEc}\right)] * 100 \quad (1)$$

Where REt is the weight of the treated right ear, REc is the weight of the control right ear, LEt is the weight of the treated left ear and LEc is the weight of the control left ear.

Formalin-induced rat paw edema

Each group (n = 5) received the treatment (copaiba oil or hydrogel) in the right hind paw one hour before the edema induction. 200 mg/kg copaiba oil concentration was chosen to perform the experiment with the respective hydrogel [10]. Positive control group received a ketoprofen solution at 4 mg/paw (in acetone). Negative control group did not receive treatment.

Before the edema induction, animals were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg) and xylasine (25 mg/kg) mixture. Formalin solution (100 µL, 10% v/v in saline) was injected in the right hind paw, while the left paw received the same amount of vehicle, saline (NaCl 0.9%).

Paw volume was measured after four hours using a plethysmometer (UgoBasile, Varese, Italy). Edema was measured by paw volume (mL) in the groups' right hind paw. Edema inhibition (EI) was measured by the percentage of edema comparing the volume of the paw in the measurement times for the groups to the volume of the control group (Equation (2)).

$$\text{EI (\%)} = \left[1 - \left(\frac{RPt}{RPc}\right)\right] * 100 \quad (2)$$

Where RPt is the volume of the treated right paw and RPc is the volume of the control right paw.

Histological analysis

For histological examination, samples of mice ear and rat right rind paw were collected from *in vivo* experiments and stored in a solution of formaldehyde at 37% in PBS pH 7.2. Histological cuts were stained with hematoxylin-eosin and visualized in optical microscope.

Statistical analysis

Statistical difference on skin permeation and *in vivo* assays were calculated by one-way ANOVA followed by Tukey test. For *in vivo* assays, statistical difference was calculated by one-way ANOVA followed by Holm-Sidak method (rat paw edema) and Tukey test (mice ear edema). Values with *P* smaller than 0.05 were considered significant. SigmaSTAT® software was used to analyze the statistics.

Results and Discussion

Copaiba oil characterization

Composition characterization in gas chromatograph coupled with mass spectrometer (GC/MS) demonstrated the presence of 41.2% of β-caryophyllene, representing the major sesquiterpene in the oilresin. Other major sesquiterpenes were α-copaene (7.1%), α-

humulene (6.9%) and caryophyllene oxide (1.3%). This composition is normally found in copaiba oils [13]. It can be modified depending on time of the year it is collected, presence of rain before the extraction, presence of injury caused by insects or fungi, variation on soil nutrient and light exposure [14].

Characterization of copaiba oil nanoemulsion and respective hydrogels

Three different polymers were tested to increase the nanoemulsions viscosity: CARB (anionic polymer), HEC (non-ionic polymer) and CHI (cationic polymer). All hydrogel formulations presented good zeta potential (ZP), above |30| mV (Table 2). When CARB was used as polymer, ZP presented negative values, even with the cationic nanoemulsion and when CHI was used, ZP presented cationic values, even with the anionic nanoemulsion. Since HEC is a non-ionic polymer, ZP in the formulation was given by the nanoemulsion surface charge.

Table 2. Nanoemulsions and hydrogels physicochemical characterization.

	ZP (mV)	DS (nm)	PDI
NCN	-17.00 ± 0.96	247.53 ± 1.46	0.051 ± 0.041
NCN-CARB	-45.77 ± 0.47[a]	267.20 ± 3.86	0.117 ± 0.023
NCN-HEC	-42.53 ± 0.40[a]	271.63 ± 4.62[a]	0.237 ± 0.036 [a]
NCN-CHI	54.87 ± 0.29[a]	524.33 ± 12.75[a]	0.489 ± 0.049[a]
PCN	22.43 ± 2.90	198.83 ± 2.69	0.085 ± 0.039
PCN-CARB	-51.53 ± 1.52[b]	192.13 ± 4.86	0.087 ± 0.062
PCN-HEC	34.57 ± 1.36[b]	223.67 ± 3.54[b]	0.175 ± 0.015
PCN-CHI	44.83 ± 0.74[b]	412.80 ± 10.59[b]	0.562 ± 0.058[b]

Letter [a] indicates statistical difference ($p<0.05$) between results found with hydrogel-thickened NCN formulations compared to NCN non-thickened; Letter [b] indicates statistical difference ($p<0.05$) between results found with hydrogel-thickened PCN formulations compared to PCN non-thickened. PCN: positively charged nanoemulsion; NCN: negatively charged nanoemulsion; CARB: Carbopol®; HEC; hydroxyethylcellulose; CHI: chitosan; ZP: zeta potential; DS: droplet size; PDI: polydispersity index.

Even though ZP values were considered good and this parameter can indicate nanoemulsion stability when above 30 mV (in modulus) [15], droplet size (DS) and polydispersity index (PDI) values showed that, when CHI was used as hydrogel, both formulations presented an increase in these parameters. An increase in PDI could imply that the droplets are aggregating and forming a bigger droplet, which could explain the increase in DS.

Souto *et al.* [16] also found that the incorporation of chitosan hydrogel in nanoparticles can destabilize the formulation, leading to an increase in DS and PDI. This can be explained by the presence of acetic acid to form the hydrogel, the interaction between the nanoemulsion surface charge and polar groups from chitosan and also from the instability around zero charge point when ZP is reversed. Moreover, when hydrogel formulations are compared to the nanoemulsions, there is an increase in ZP and DS, also verified by other authors [16–18], which can be explained by polymer adsorption on nanoemulsion droplet surface.

HEC hydrogel was chosen to continue the studies, since it presented good characterization parameters for both nanoemulsions, due to its neutral character. Figure 1 shows SEM image for NCN-HEC and PCN-HEC, where the polymeric network organization can be seen for both formulations.

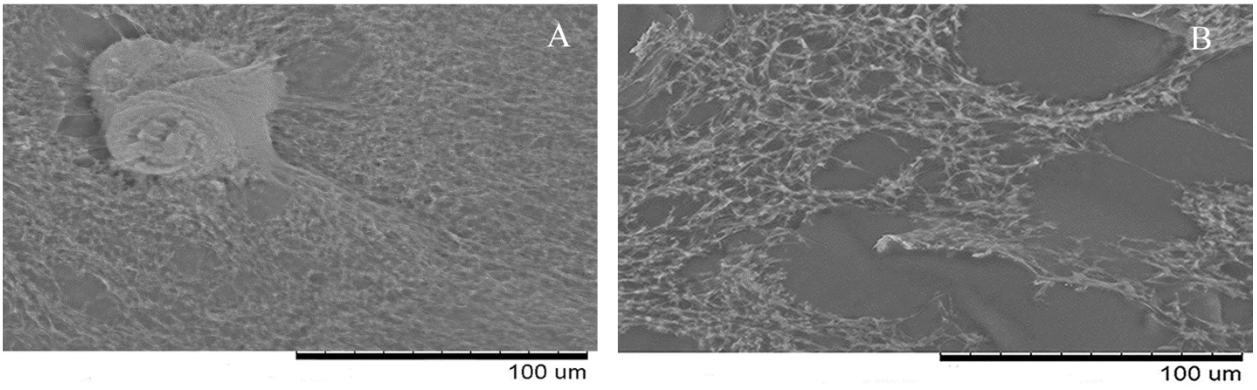

Figure 1. SEM images for NCN (A) and PCN (B) HEC hydrogel.

Hydrogel-based nanoemulsions rheological profile

Figure 2 demonstrates the rheological profile comparing NCN-HEC, PCN-HEC and Blank-HEC hydrogels. As can be seen in the rheogram, HEC hydrogels (2%) containing or not copaiba oil nanoemulsions presented non-Newtonian flow, since the relation between shear stress and shear rate is not linear [19]. Among non-Newtonian fluids, there are three behaviors that can occur: plastic, pseudoplastic or dilatant. According to our results, the hydrogel produced shows pseudoplastic characteristics. In addition, they do not present any thixotropic behavior, as both ascendant and descendant curves are overlapping. Figure 3 shows the viscosity profile from NCN-HEC, PCN-HEC and Blank-HEC hydrogels. As observed, nanoemulsions influenced in the viscosity behavior of HEC hydrogel, given that the control hydrogel (Blank-HEC) presented lower viscosity values compared to HEC-loaded nanoemulsions. That can be explained by the nanoemulsions' higher viscosity compared to the water viscosity.

Long-term storage stability

Regarding the formulations' stability study (Table 3), during the one-year monitoring, values for ZP, DS, PDI and β-caryophyllene content slowly changed. For both hydrogels, ZP and β-caryophyllene content values decreased and DS and PDI values increased, indicating a probable instability after 12 months. Nevertheless all values stayed in an acceptable range during the first 6 months. It is worth mentioning that both formulations were kept under 4°C temperature and covered from light, which can explain the smaller loss in content when compared to other studies in the literature [20–22]. In addition, there was no phase separation, presence of fungi contamination or other instability indicative aspects, during the time studied.

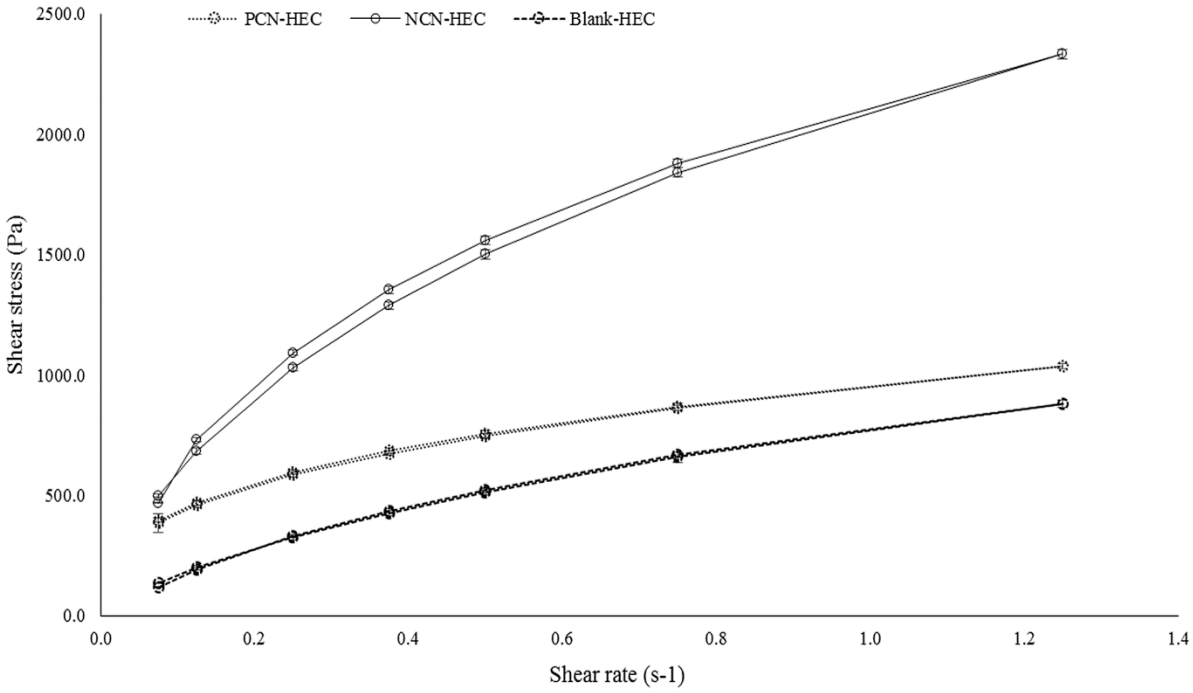

Figure 2. Rheological profile from copaiba oil nanoemulsions thickened with HEC polymer (n=3).

In vitro skin permeation

Table 4 describes the skin permeation/retention profile for copaiba oil nanoemulsions incorporated in hydroxyethylcellulose hydrogels. Results are shown as β-caryophyllene (the major sesquiterpene in copaiba oil) content in skin layers and receptor fluid. After 8 hours, β-caryophyllene was found in the receptor fluid for both formulations, characterizing skin permeation. It was also found in great amount in the epidermis layer, followed by the dermis and the stratum corneum in a smaller amount. In comparison with the nanoemulsion permeation profile reported in a previous study [10], there is a higher permeation when the hydrogel is used, since for the nanoemulsion there was not β-caryophyllene detected in the receptor fluid.

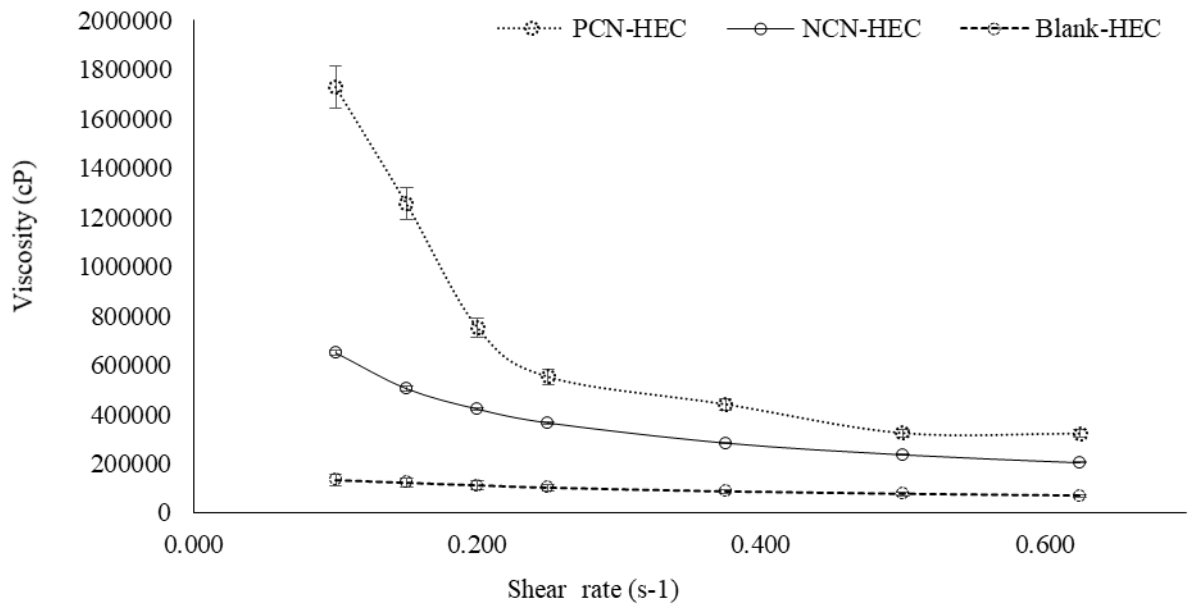

Figure 3. Viscosity profile from copaiba oil nanoemulsions thickened with HEC polymer (n=3).

The higher β-caryophyllene permeation with the hydrogel-loaded nanoemulsion can provide evidence that this formulation is suitable for the purpose of topical application in an anti-inflammatory therapy, indicating that the nanoemulsion was released from the gel matrix and that the hydrogel did not present affinity to it when in contact to the skin. Since the nanoemulsion has small droplet size and high superficial area, it is supposed to penetrate the stratum corneum, permeate through the epidermis (or establish a type of reservoir in this layer) and reach the dermis and the receptor fluid, which mimics the deeper layers in the skin [23,24]. In addition, many factor could explain why the addition of a hydrogel to the formulation could improve the nanoemulsions' skin permeation such as occlusion, viscosity and hydration of the site, which can increase the partitioning of the stratum corneum layer and enable the penetration [25,26].

Table 3. Stability characterization from hydrogels containing positively and negatively charged nanoemulsions in zero, six and twelve months.

			NCN-HEC			PCN-HEC
	T0	***T6***	***T12***	***T0***	***T6***	***T12***
Zeta potential (mV)	-45.8 ± 0.80	-32.7 ± 0.20 *	-25.4 ± 4.10*	22.2 ± 5.20	37.2 ± 3.20*	15.27 ± 4.42
Droplet size (nm)	280.10 ± 4.30	284.53 ± 11.21	280.00 ± 12.77	258.70 ± 5.20	302.97 ± 7.42*	333.47 ± 3.84*
Polydispersity index	0.079 ± 0.023	0.082 ± 0.017	0.149 ± 0.050	0.258 ± 0.010	0.229 ± 0.030	0.317 ± 0.044*
Content (%)	97.10 ± 0.07	101.77 ± 0.07*	82.22 ±0.06*	105.38 ± 0.08	105.32 ± 0.08	97.78 ± 0.03*

T0: time zero; T6: time six months; T12: time twelve months; NCN-HEC: negatively charged nanoemulsion thickened in hydroxyethylcellulose hydrogel; PCN-HE: positively charged nanoemulsion thickened in hydroxyethylcellulose hydrogel.
* Statistically different from time T0.

Table 4. Skin permeation results.

	PCN-HEC	NCN-HEC
Stratum corneum (µg/cm²)	0.09 ± 0.07	0.18 ± 0.17
Epidermis (µg/cm²)	9.76 ± 2.65	7.91 ± 2.46
Dermis (µg/cm²)	2.43 ± 0.91	1.95 ± 0.56
Receptor fluid (µg/cm²)	0.67 ± 0.22	1.80 ± 0.85

PCN-HEC: positively charged nanoemulsion thickened hydrogel; NCN-HEC: negatively charged nanoemulsion thickened hydrogel.

In vivo anti-inflammatory activity

Two *in vivo* models demonstrated the topical anti-inflammatory potential effect from copaiba oil nanoemulsion incorporated in hydrogel: mouse ear edema and rat paw edema. Mouse ear edema was induced by topical administration of arachidonic acid (2 mg/ear) which is involved in the cyclooxygenase (COX) and lipoxygenase (LOX) inflammation pathways and its topical administration leads to immediate vasodilatation and erythema [27]. Figure 4 shows the result 60 minutes after ear inflammation induction.

As can be seen, ketoprofen, crude copaiba oil and its nanoemulsions incorporated in hydrogels significantly inhibited the edema when compared to the control ($p < 0.05$). However, when compared to the positive control, ketoprofen, the hydrogels with nanoemulsions (NCN-HEC and PCN-HEC) and copaiba oil were statistically equivalent ($p > 0.05$). Blank hydrogel, as expected, did not present anti-edematogenic effect. Edema inhibition values for ketoprofen, NCN-HEC, PCN-HEC and copaiba oil were 86%, 69%, 67% and 58%, respectively. Thus, both formulations had an equivalent profile compared to ketoprofen, however they did not change the effect of the crude oil.

Since the formulations and the oil inhibited the arachidonic acid induced inflammation, copaiba oil could be involved in the inhibition of COX and LOX pathways, like non-steroidal anti-inflammatories.

Rat paw edema was induced by intraplantar administration of formalin (10%). It is well known that formalin causes a biphasic edema response. The first phase (normally up to 5 minutes after induction) releases substance P and bradykinin. In this phase, it is considered to cause a neuropathic-kind pain. In the second phase, histamine, serotonin, prostaglandins and bradykinin are involved, producing inflammatory response [28].

Figure 5 presents the results for rat paw edema. Statistically, ketoprofen and PCN-HEC were different to the negative control ($p < 0.05$), indicating their anti-edematogenic activity. Copaiba oil, NCN-HEC and blank formulation were statistically equal to the control ($p > 0.05$). Edema inhibition values for ketoprofen, NCN-HEC, PCN-HEC and copaiba oil were 67%, 32%, 72% and 13%, respectively. In this case, the formulation could improve the effect of the oil, corroborating with the permeation profile and indicating that the positive surface charge has an important role and can enable skin permeation.

It is important to highlight that the oil produced a smaller edema inhibition, which can be correlated to its permeation profile through the skin. In previous studies, we found that the oil stays in the stratum cornenum, without any β-caryophyllene retention in the dermis and epidermis, unlike the nanoemulsions containing the oil [9,10].

In both experiments, ketoprofen, a blank formulation and non-treated animals were used as control. Ketoprofen is a non-steroidal anti-inflammatory drug (NSAID) widely used to treat rheumatoid arthritis and other inflammatory diseases [29], and was used in this study as a positive control for the anti-inflammatory effect. The dose was 4 mg/paw (paw edema) and 4 mg/ear (ear edema) is normally used in anti-inflammatory assays and was described previously [30,31]. In order to evaluate if the hydrogel could perform an anti-inflammatory effect, there was also a hydrogel control (Blank-HEC), which consisted in a formulation containing only the polymer (hydroxyethylcellulose and water).

Concerning histological examination, rat paw edema assay (Fig. 6) showed the presence of epidermis hyperplasia, inflammatory cell infiltration and vasodilation in the non-treated control. In mice ear edema, histological examination (Fig. 7) showed the presence of dermis and epidermis hyperplasia and inflammatory cell infiltration in non-treated control. Treatments showed a decrease in these factors, demonstrating the anti-inflammatory effect for both models.

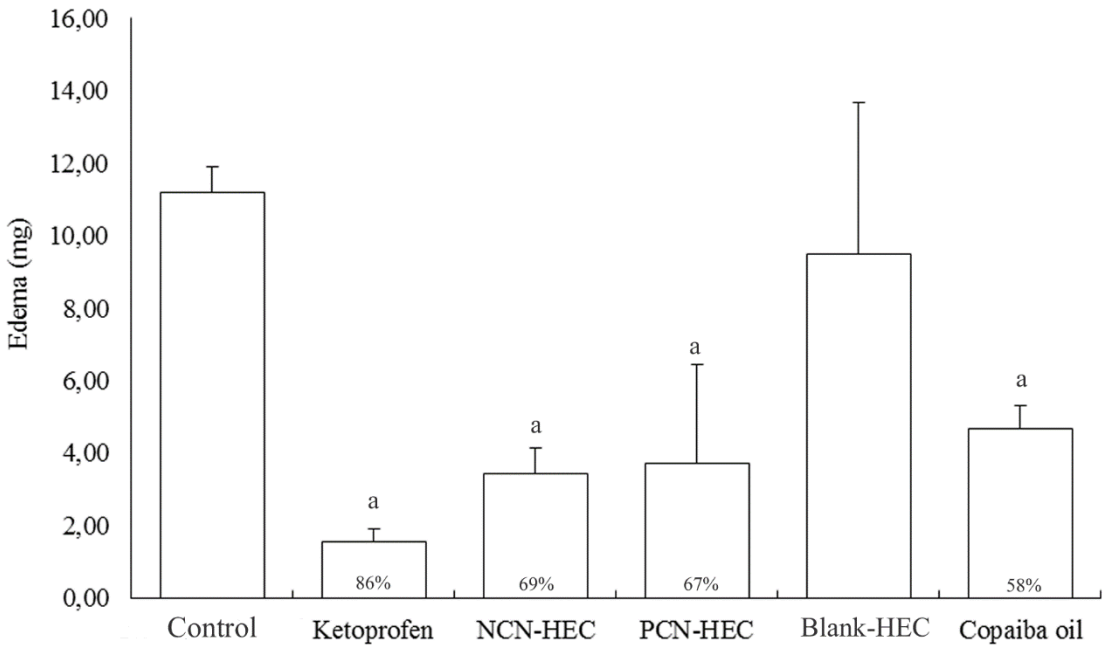

Figure 4. Arachidonic acid induced-mouse ear edema measured by ear weight (mg). Edema inhibition percentages were placed inside the bars. [a] Statistically different from control ($p < 0.05$).

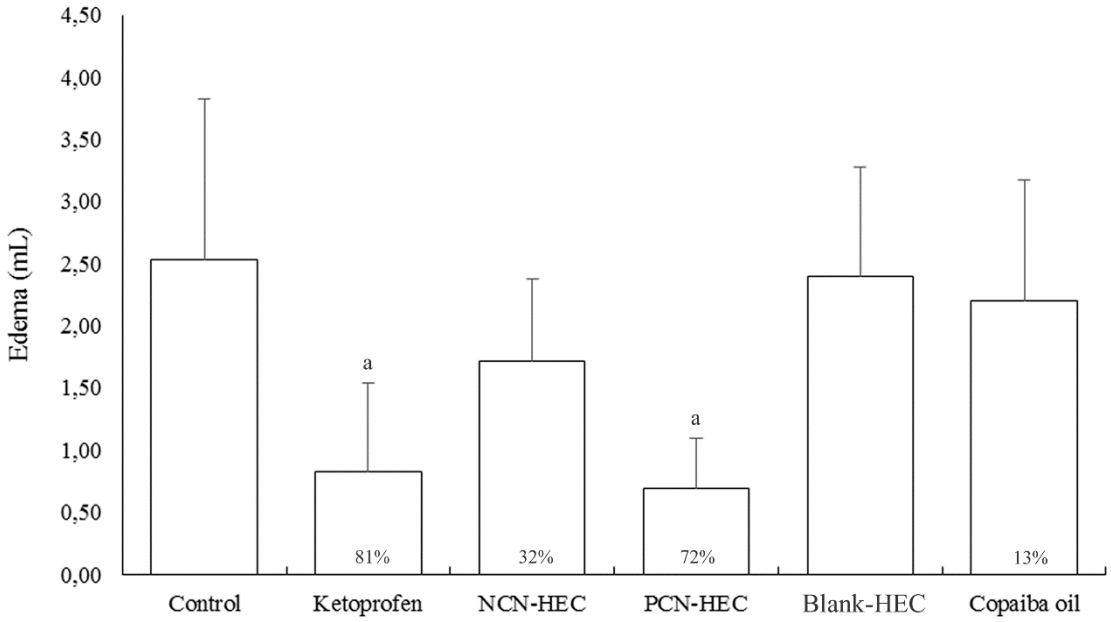

Figure 5. Rat paw edema induced by formalin 10%. Edema inhibition percentages were placed inside the bars. [a] Statistically different from control ($p < 0.05$).

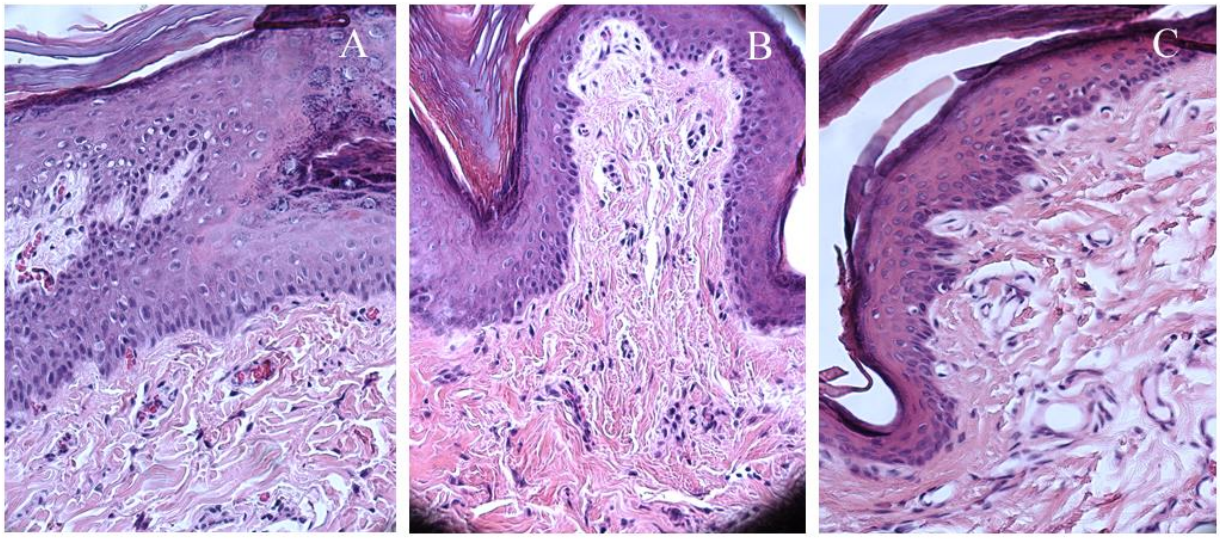

Figure 6. Photomicrographs from transversal cuts of right rat paws after topical formalin administration, stained with hematoxylin-eosin and examined in optical microscope with 40x magnification. (A) Control; (B) PCN-HEC; (C) NCN-HEC.

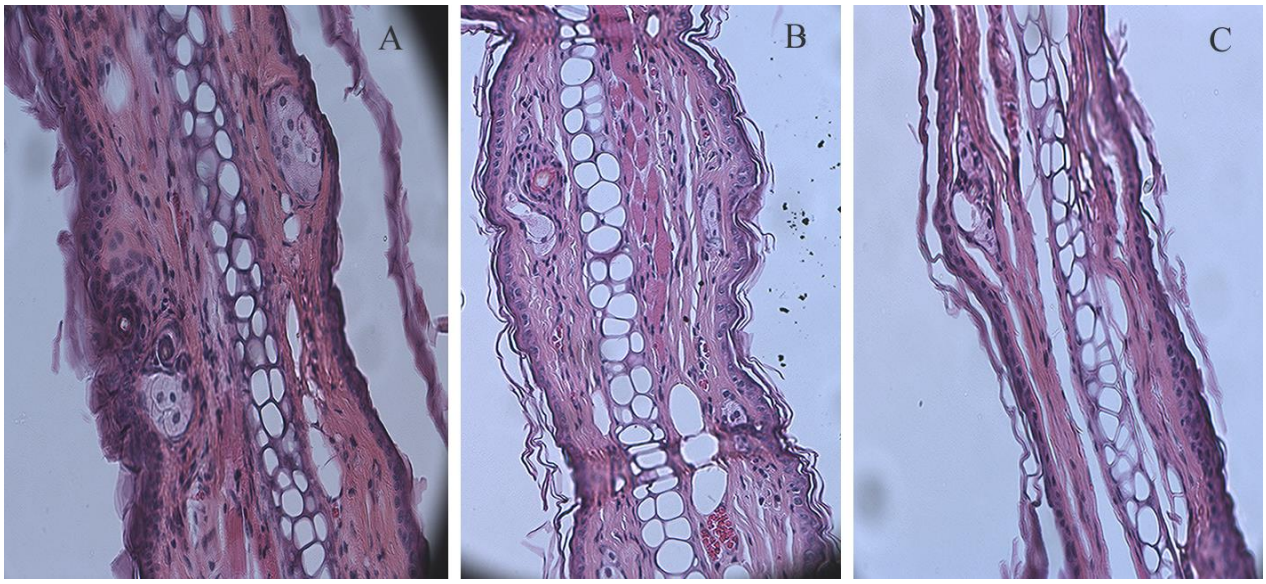

Figure 7. Photomicrographs from transversal cuts of mice ears after arachidonic acid topical application, stained with hematoxylin-eosin and examined in optical microscope with 40x magnification. (A) Non-treated control; (B) NCN-HEC; (C) PCN-HEC.

CONCLUSIONS

In this paper, we described the incorporation of copaiba oil nanoemulsions (positive and negatively charged) in different hydrogel polymers. The best hydrogel that did not interfere with nanoemulsions' droplet size and polydispersity index was the one formed by hydroxyethylcellulose (HEC), which remained stable for a 12-month stability study and was chosen to perform skin permeation and *in vivo* experiments. Concerning skin permeation, for both formulations it was possible to detect β-caryophyllene in the most profound skin layer (dermis) and in the receptor fluid, characterizing skin permeation. In mouse ear edema, both formulations presented similar anti-edematologic profile, presenting high edema inhibition and statistically similar to ketoprofen ($p < 0.05$). In rat paw edema, both formulations presented anti-edematologic effect, although the negatively charged nanoemulsion presented a smaller edema inhibition compared to the positively charged one.

In both *in vivo* edema studies, it was possible to visualize by histological cuts a decrease in epidermis hyperplasia, inflammatory cell infiltration and vasodilation, demonstrating the anti-inflammatory activity from both treatments.

Acknowledgments

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CAPÍTULO V

Manuscrito a ser submetido ao periódico *Antimicrobial Agents and Chemotherapy*

Copaiba oil and β-caryophyllene nanoemulsions anti-leishmanial activity

Leticia G. Lucca[a b], Valdir F. Veiga-Junior[c], Helder F. Teixeira[a], Leticia S. Koester[a], Philippe M. Loiseau[b], Sandrine Cojean[b]

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil [a]; Université Paris-Sud, UMR 8076 Chimiothérapie Antiparasitaire, Châtenay-Malabry, France [b]; Departamento de Quíımica, Instituto de Ciências Exatas, Universidade Federal do Amazonas, Manaus, Brazil [c].

** Corresponding author:*
Sandrine Cojean
Chimiothérapie Antiparasitaire, UMR 8076 CNRS, Faculté de Pharmacie, Université Paris-Sud, rue J. B. Clément, 92296 Châtenay- Malabry Cedex, France.
E-mail: sandrine.cojean@u-psud.fr
Tel.: +33 1 46 83 55 53
Fax: +33 1 46 83 55 57

Abstract

Leishmaniasis is an endemic disease in tropical countries and is the second most prevalent parasitic disease in the world after malaria.. The first-choice treatment for leishmaniasis, both cutaneous and visceral types, are pentavalent antimonials, however, this group of drugs presents severe side effects, require long treatment time and already present cases of resistance in some parts of the world. Copaiba oil (*Copaifera multijuga* Hayne) is a natural product exuded from the trunk of the *Copaifera* tree and present β-caryophyllene as its major component and biomarker. In this paper, we describe the formulation of copaiba and β-caryophyllene nanoemulsions, their *in vitro* activity against four Leishmania strains (*L. amazonensis, L. braziliensis, L. donovani* and *L. major*), cytotoxicity against non-infected macrophage cells and *in vivo* effect on *L. major* infected mice. *In vitro* tests showed that treatments were more effective on Old World *Leishmania* strains *(L. major* and *L. donovani*) compared to New World *Leishmania* strains (*L. amazonensis* and *L. braziliensis*). Against intracellular amastigotes, β-caryophyllene and β-caryophyllene-loaded nanoemulsion were the most active treatments, with small IC_{50} values (below 4 µg/ml). In vivo assay showed that all treatments were able to reduce wound area against *L. major* infected mice. However, they were not able to totally recover the treated animal from the infection.

Key-words: *Copaifera multijuga*; β-caryophyllene; nanoemulsion; cutaneous leishmaniasis; *Leishmania major*

Introduction

Leishmaniasis is an endemic disease in tropical countries and is the second most prevalent parasitic disease in the world after malaria (1). Although leishmaniasis presents an estimate of twenty to forty thousand deaths per year, it belongs to the group of neglected diseases, since the most affected are poor people in underdeveloped countries (2). According to the

World Health Organization, there are 2 million new cases every year and 350,000 people considered at risk (3).

Leishmaniasis is transmitted by a sandfly (*Phlebotomus* sp. in the Old World and *Lutzomyia* sp. in the New World) carrying the parasite *Leishmania* which is responsible for the three clinical forms of the disease (cutaneous, mucocutaneous and visceral) depending on the species (4). The first-choice treatment for leishmaniasis, both cutaneous and visceral types, are pentavalent antimonials, however, this group of drugs presents severe side effects (myalgia, heart and kidney toxicity, abdominal pain, headache, etc.), require long treatment time and already present cases of resistance in some parts of the world (5, 6). Other treatments include pentamidine, paromomycin, amphotericine B and miltefosine, nevertheless they have limited activity against some strains and can also cause several side effects (7).

Copaiba oil is a natural product exuded from the trunk of the *Copaifera* tree, which is found in South America. It presents a viscous aspect with yellowish color and is widely used in the Amazonian rain forest region, in Brazil, as a popular medicine to treat different inflammatory and parasitic diseases and for wound healing, both by oral or topical routes (8).

Currently, there are many studies about copaiba in the literature, including studies of anti-leishmanial activity (9–11). However, the results in these studies are variable, given the amount of existing plant species and their individual characteristics, such as major components. The tests evaluate the activity of the oil, especially *in vitro,* with promastigote, axenic amastigote and intramacrophagic amastigote forms. Studies *in vivo* were performed once in the rat paw infection model (12). Other studies include anti-inflammatory activity (13–16), antimicrobial (17–20) and larvicidal (21, 22). Furthermore, the major component from this oil, β-caryophyllene, has also been studied regarding its leishmanicidal activity, showing low IC_{50} values and similar to copaiba oil (23).

Due to the lack of effective treatments that can be used for all *Leishmania* strains, it is important to search for new treatment alternatives, such as natural products. Therefore, the

main goal of this study is to evaluate the activity *in vitro* of copaiba oil extracted from *Copaifera multijuga* Hayne, its major component (β-caryophyllene) and nanoemulsions produced with these components in different species of *Leishmania* and the *in vivo* activity against topical leishmaniasis caused by *Leishmania major*.

Materials and methods

Plant material. Copaiba oil was extracted from a natural population of *Copaifera multijuga* Hayne located in the Ducke Forest Reserve (S 2°57'43'', W 59°55'38'', 120 m), Manaus City (Amazonas State, Brazil). Voucher samples from this population were deposited at the herbarium of National Institute of Amazonian Research (INPA). The oilresin was obtained by the usual method of artificial exudation, which consists in a non-aggressive incision in the trunk with a metal auger, 1 meter above ground level (8, 24). The oilresin was characterized by gas chromatography coupled with mass spectrometry (GC-MS) which revealed a 39% β-caryophyllene concentration.

Copaiba oil and β-caryophyllene nanoemulsions. Nanoemulsions were produced as optimized by Dias et al. (25). Briefly, the oily phase (20% w/w copaiba oil or β-caryophyllene; 10% w/w MCT; 3% w/w Span 80®) and the aqueous phase (1% w/w Tween 20® and water) were mixed under magnetic stirring (5 minutes at room temperature) to form a coarse emulsion. In order to gradually decrease the droplet size, this coarse emulsion was subjected to high-pressure homogenization at 750 bar for 6 cycles. Blank nanoemulsion was also produced with just MCT in the oily phase.

After preparation, the nanoemulsion was characterized for droplet size, polydispersity index, zeta potential. Droplet size and polydispersity index were measured in triplicate by dynamic light scattering after dilution of 10 μL of the nanoemulsion in 10 mL of purified water (Zetasizer Nanoseries ZN90, Malvern Instruments, Worcestershire, UK). Zeta potential value was measured in triplicate by laser Doppler velocimetry using the same

instrument, after dilution of 10 µL of the nanoemulsion in 10 mL in NaCl (1 mM) ultra-filtered in 0.45 µM filter.

Parasite strains and culture. *Leishmania amazonensis* (MHOM/BR/73/M2269), *Leishmania donovani* (MHOM/ET/67/HU3), *Leishmania braziliensis* and *Leishmania major* were used to test the *in vitro* activity from copaiba oil, β-caryophyllene and their nanoemulsions. Promastigote forms were grown in M199 supplemented with 40 mM HEPES, 0.1 mM adenosine, 0.02 mM hemin and 10% heat-inactivated fetal bovine serum, in a 25°C incubator and protected from light.

***In vitro* evaluation on axenic amastigote form.** Differentiation of promastigotes into axenic amastigotes was achieved by dilution of 1 x 10^6 promastigotes in the previously mentioned medium supplemented with 2mM of $MgCl_2$ 1M and 2mM $CaCl_2$ 1M. All the experiments were performed with parasites in their logarithmic phase of growth, at 37°C in a 5% CO_2 incubator and protected from light.

Axenic amastigotes were seeded into 96 well plates and treated with copaiba oil, β-caryophyllene or their nanoemulsions using the serial dilution method. Parasites viability was assessed using the SYBR® Green I (Invitrogen, France). Cells were lysed by 3 cycles of freezing and thawing mixed to 20 µl of lysis buffer (NaCl 100 mM, Tris HCl pH 8 10 mM, EDTA pH 8 25 mM, SDS 0.5%, Proteinase K 0.1 mg/ml). Then, 25 µL of lysed solution of each well was added to 25 µL of SYBR® Green I solution in a 96 wells qPCR plate. Fluorescence was measured with Realplex^2 Mastercycler (Eppendorf, France). Fluorescence obtained was compared to those from the range obtained with parasite, infected cell and non-infected cell (26). Miltefosine (hexadecylphosphocholine or HePC) was used as negative growth control. The results are expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a 72 hours incubation period.

***In vitro* evaluation on intramacrophagic amastigotes.** Macrophages were infected with 1 x 10^6 *Leishmania* parasites in a 96 well plate. After 12 hours, infected macrophages were treated with copaiba oil, β-caryophyllene or their nanoemulsions using the serial dilution method. This test was performed at 37°C, in a 5% CO_2 incubator and protected from light. The viability of the amastigotes into macrophages was assessed using the previous mentioned method after 72 hours incubation time.

Macrophage cell culture and *in vitro* evaluation of macrophage viability. Macrophage cell line RAW 264.7 was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum. RAW 264.7 cells were seeded into a 96-well plate at a density of 5 × 10^3 cells/well in 100 µl of DMEM and treated with copaiba oil, β-caryophyllene and their nanoemulsion using the serial dilution method. The viability of RAW264.7 macrophages was determined after 72h incubation in a 5% CO_2 incubator at 37 °C by SYBR® Green as described previously.

Animals. Female Balb/c mice weighting approximately 20 g were kept under temperature control (25 ± 1 °C), at a 12 h light–dark cycle and with free access to water and food.

Infection. Animals were infected in the base of the tail with 1 x 10^6 promastigotes of *Leishmania major* Villanova. Parasites were inoculated subcutaneously in a volume of 100 µl.

Treatment. After the development of the lesion in the inoculation site (approximately 20 days after infection), animals were separated in 7 groups (n=8): (a) non treated group; (b) miltefosine group; (c) copaiba oil group; (d) β-caryophyllene group; (e) copaiba oil nanoemulsion group; (f) β-caryophyllene group; (g) blank nanoemulsion group. Miltefosine group was treated by intraperitoneal injection with a dose of 0.98 µM of miltefosine in 100 µl, every day. The other groups were treated by topical application of

copaiba oil, β-caryophyllene and their nanoemulsions every day with a dose of 100 µg/ml, directly to the wound. Treatment lasted 12 days.

Treatment evaluation. During the treatment, lesion size was measured in all animals and their general condition was observed every day. After the end of treatment period, animals were sacrificed and a sample of the skin lesion, blood, spleen and liver were collected from all animals.

qPCR analysis was performed with samples of blood and skin tissue. DNA extraction was performed according to DNeasy Blood & Tissue Kit (Qiagen, France). After the extraction, Cytochrome B (CytB) levels were quantified with SensiFAST SYBR NoROX Kit (Bioline, France) and qPCR assay was performed on Realplex^2 Mastercycler (Eppendorf, France). Temperature program was set as 5 min at 25°C, 60 min at 42°C and 15 min at 70°C.

Statistical analysis. All statistical analysis were performed with ANOVA followed by Tukey's test. $P < 0.5$ was considered significant.

RESULTS

Nanoemulsion production. Nanoemulsions produced with copaiba oil (NECOP) and caryophyllene (NECAR) were characterized for their droplet size, zeta potential and polidispersity index. NECOP presented a droplet size of 215.2 ± 10.1 nm, polydispersity index of 0.101 ± 0.007 and zeta potential of -19.1 ± 0.5 mV. NECAR presented a droplet size of 225.0 ± 1.8 nm, polydispersity index of 0.072 ± 0.031 and zeta potential of -16.9 ± 0.7 mV.

***In vitro* evaluation on axenic amastigote form.** Table 1 presents the results for the *in vitro* evaluation in axenic amastigote form from four *Leishmania* strains in contact with copaiba oil, β-caryophyllene and their nanoemulsions. For *L. donovani* and *L. major* nanoemulsions presented smaller IC_{50}.

Macrophage viability *in vitro* assay. Table 2 presents the results for macrophage viability when in contact with copaiba oil, caryophyllene and their nanoemulsion. CAR, NECAR and NECOP presented high values of CC_{50}. COP presented a smaller value, but it is higher than the IC_{50} presented in infected macrophages.

***In vitro* evaluation on intramacrophagic amastigotes.** Table 3 presents the results for the evaluation of the treatments (copaiba oil, caryophyllene and their nanoemulsions) in contact with *Leishmania* infected macrophages *in vitro*. In addition, the selectivity index (SI), presented in this table, and was calculated according to the values obtained from the LD_{50} values obtained from the macrophage viability assay.

***In vivo* activity.** During the 12 days treatment period, no animal presented signal of secondary infections. However, the control group presented signals of stress caused by progression of infection. Animals treated with β-caryophyllene showed hair loss on the site of the leasion, which was reduced by the nanoemulsion-loaded β-caryophyllene treatment. Figure 1 presents the effect of treatments.

Discussion

Copaiba oil and caryophyllene nanoemulsions presented a satisfactory characterization, with low droplet size and polydispersity index and high zeta potential, similar to results previously found by Dias et al (25), without presence of instability such as coalescence and phase separation. These results indicate that the system is stable, with low probability of droplet aggregation and suitable for topical application (27).

Copaiba oil, β-caryophyllene and their nanoemulsions were tested against four *Leishmania* strains: *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania donovani* and *Leishmania major*. As can be seen in Table 1, nanoemulsions presented better response to Old World *Leishmania* strains (*L. major* and *L. donovani*), with smaller values when

compared to the crude products. This could indicate that the nanoemulsification process potentiated the effect. However, upon looking to New World *Leishmania* strains (*L. amazonensis*, *L. braziliensis*), neither copaiba oil and β-caryophyllene nor their nanoemulsions presented an expressive activity.

Dos Santos et al (28) studied the effect of several species of *Copaifera* on *Leishmania amazonensis*. Authors showed that *Copaifera reticulata* was the most active specie with an IC_{50} of 15 µg/ml in amastigote forms of *L. amazonensis*, similar to IC_{50} results found in the present study. In addition, *C. reticulata* presented a β-caryophyllene concentration similar to *C. multijuga* used in the present study (40%), which could indicate that this sesquiterpene is essential for copaiba oil activity. It is important to highlight that crude copaiba oil presented statistical different ($p<0.05$) IC_{50} values when compared to β-caryophyllene for all strains, indicating that copaiba oil activity is due to a synergistic effect from all terpenes in the complex matrix.

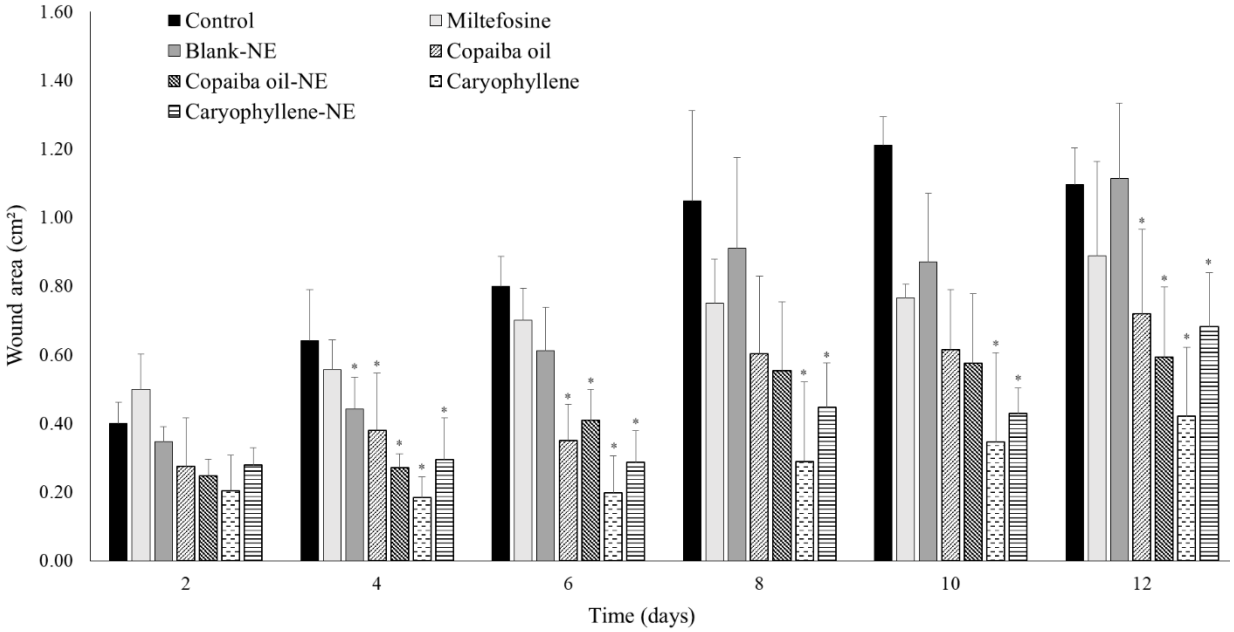

Figure 1. Progression of wound area during the 12 days of treatment (topical for formulations and intraperitoneal for miltefosine). * Statistically different from non-treated control.

Table 1. IC_{50} for copaiba oil, copaiba oil nanoemulsion, caryophyllene, caryophyllene nanoemulsion and miltefosine in amastigote forms of *L. amazonensis*, *L. braziliensis*, *L. donovani* and *L. major* (n=3).

		L. amazonensis	***L. braziliensis***	***L. donovani***	***L. major***
µg/ml	**COP**	15.19 ± 1.86	24.64 ± 1.90	11.04 ± 0.42	10.74 ± 1.67
	NECOP	11.77 ± 0.75	30.46 ± 3.62	7.96 ± 0.67	6.12 ± 0.36
	CAR	22.97 ± 3.02	45.26 ± 4.82	24.19 ± 2.28	25.30 ± 3.44
	NECAR	52.26 ± 2.73	114.56 ± 11.12	10.32 ± 0.39	11.57 ± 0.23
µM	**HePC**	5.77 ± 0.28	4.35 ± 0.27	8.09 ± 1.79	3.01 ± 0.11

COP: copaiba oil; NECOP: copaiba oil nanoemulsion; CAR: caryophyllene; NECAR: caryophyllene nanoemulsion; HePC: miltefosine.

Table 2. CC_{50} values for non-infected macrophages.

COP (n=3)	15.14 ± 1.63 µg/ml
NECOP (n=3)	122.39 ± 12.09 µg/ml
CAR (n=3)	65.28 ± 5.72 µg/ml
NECAR (n=3)	31.35 ± 3.07 µg/ml

COP: copaiba oil; NECOP: copaiba oil nanoemulsion; CAR: caryophyllene; NECAR: caryophyllene nanoemulsion.

Table 3. IC_{50} and SI values for copaiba oil, copaiba oil nanoemulsion, caryophyllene and caryophyllene nanoemulsion in macrophages infected with *L. amazonensis*, *L. braziliensis*, *L. donovani* or *L. major* (n=3).

		L. amazonensis	*SI*	***L. braziliensis***	*SI*	***L. donovani***	*SI*	***L. major***	*SI*
µg/ml	**COP**	18.59 ± 0.83	*0.8*	20.88 ± 2.16	*0.7*	0.83 ± 0.07	*18.2*	0.81 ± 0.04	*16.7*
	NECOP	33.78 ± 2.47	*3.6*	1.72 ± 0.33	*71.2*	3.84 ± 0.4	*31.6*	0.81 ± 0.04	*151.1*
	CAR	60.95 ± 3.49	*1.1*	50.83 ± 9.20	*1.3*	3.88 ± 0.3	*16.8*	0.92 ± 0.08	*70.9*
	NECAR	0.97 ± 0.09	*32.3*	1.89 ± 0.17	*16.6*	1.94 ± 0.1	*16.1*	0.96 ± 0.06	*32.6*

COP: copaiba oil; NECOP: copaiba oil nanoemulsion; CAR: caryophyllene; NECAR: caryophyllene nanoemulsion; HePC: miltefosine.

Copaiba oil, caryophyllene and their nanoemulsions were tested against non-infected macrophages in order to verify their cytotoxicity in these cells (Table 2). Results showed that copaiba oil nanoemulsion presented a higher cytotoxicity concentration value (CC_{50}) when compared to the crude product, indicating that the nanoemulsification process can protect the cell against copaiba toxicity.

Opposite to that, β-caryophyllene-loaded nanoemulsion presented a smaller CC_{50} when compared to the crude product. However, both concentrations were significantly higher than IC_{50} values found for *Leishmania*-infected macrophages. β-Caryophyllene is a natural compound found in many essential oils, in plants used as food (*Origanum vulgare* L., *Cinnamomum* spp. and *Piper nigrum* L.) and in fragrances (29, 30). Soares et al (23) showed that macrophages were viable up to 50 µg/ml β-caryophyllene concentration, similar to the results found in this study. Schmitt et al (31) verified that β-caryophyllene was not toxic up to 700 mg/kg in subchronic toxicity tests in rats.

Table 3 shows the results for IC_{50} on intramacrophagic amastigotes and respective selectivity index (SI). Again, it is possible to verify that crude products and their nanoemulsions had better performance on Old World strains, with high selectivity index, above the threshold suggested in the literature (>5) (32). β-Caryophyllene-loaded nanoemulsion presented small IC_{50} values in all four *Leishmania* strains. For *Leishmania amazonensis* and *Leishmania braziliensis* there is a significant decrease on IC_{50} (63 and 27 times, respectively) and significant increase on the selectivity index (29 and 13 times respectively), representing a perspective on the search for new drugs to treat cutaneous leishmaniasis.

Many studies have shown that drug delivery using nanosystems for leishmaniasis treatment can improve drug efficacy, decrease toxicity, target the release of the drugs and prolong the exposition of the parasite to the drug (33, 34). For example, different nanoparticles are described for drugs already used in leishmaniasis treatment such as Amphotericin B (11, 35–38), primaquine (39), meglumine (40), but also for natural products (41, 42) and metal oxides (43, 33).

In vivo assay with *Leishmania major* infected mice showed that all treatments (copaiba oil, β-caryophyllene and nanoemulsions) were able to reduce lesion area, when compared to the non-treated control. These results corroborate with *in vitro* tests, in which all treatments showed small IC_{50} values. Crude β-caryophyllene presented the best results on lesion reduction.

Dos Santos and co-workers (12) reported the effect of oral and topical treatment of copaiba oil (*Copaifera martii*) on *Leishmania amazonensis* infected mice (footpad method). Authors found that the oral treatment was more effective than topical one. In addition, simultaneous oral and topical treatment was also effective on lesion reduction. However, total recovery from the infection was not reported.

Conclusion

This is the first report of the use of copaiba oil, β-caryophyllene and their nanoemulsions on *in vitro* and *in vivo* treatment of leishmaniasis. In this paper, nanoemulsions presented small droplet size, narrow size distribution, and good zeta potential values. *In vitro* tests showed that treatments were more effective on Old World *Leishmania* strains *(L. major* and *L. donovani*) compared to New World *Leishmania* strains (*L. amazonensis* and *L. braziliensis*). Against intracellular amastigotes, β-caryophyllene and β-caryophyllene-loaded nanoemulsion were the most active treatments, with small IC_{50} values (below 4 µg/ml). In vivo assay showed that all treatments were able to reduce wound area against *L. major* infected mice. However, they were not able to totally recover the treated animal from the infection. Thus, copaiba oil and β-caryophyllene represent a novel perspective to treat *Leishmania* and, as perspective, could be deeply studied using other administration routes against topical and visceral leishmaniasis.

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DISCUSSÃO GERAL

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O óleo de copaíba vem sendo estudado nos últimos anos devido ao seu grande interesse pela medicina popular da região amazônica e também por representar uma fonte renovável de um remédio natural neste local (LEANDRO et al., 2012). Popularmente o óleo de copaíba é utilizado como anti-inflamatório (tanto pela via oral quanto tópica), antisséptico e cicatrizante (VEIGA-JUNIOR et al., 2006), mas alguns trabalhos já tem demonstrado seu potencial como antimicrobiano (ALMEIDA VAUCHER, DE et al., 2015; MORELLI et al., 2015), antiparasitário (SILVEIRA et al., 2004; SANTOS et al., 2008, 2011, 2012), larvicida (TRINDADE et al., 2013; RODRIGUES et al., 2014), antineoplásico (GOMES et al., 2008), etc.

Entretanto, o maior interesse no estudo do óleo de copaíba está em seu potencial anti-inflamatório. Alguns estudos já demonstraram que o óleo e até mesmo frações enriquecidas com os sesquiterpenos e diterpenos majoritários do óleo tem a capacidade de diminuir processos inflamatórios causados por diferentes agentes (BASILE et al., 1988; VEIGA-JUNIOR et al., 2001, 2006, 2007; CARVALHO et al., 2005; GOMES et al., 2010).

Nosso grupo de pesquisa vem estudando o desenvolvimento tecnológico de nanoemulsões contendo o óleo de copaíba extraído da espécie *Copaifera multijuga* Hayne que, segundo Veiga-Junior e colaboradores (2007), apresentou melhor atividade anti-inflamatória quando comparada a outras espécies de *Copaifera.* A fim de verificar este potencial pela via tópica, foi desenvolvida e otimizada uma nanoemulsão contendo de óleo de copaíba (DIAS et al., 2014), a qual foi estudada quanto a sua permeação cutânea em comparação com o óleo puro, empregando-se um método validado em cromatógrafo a gás acoplado a espectrômetro de massas (CG/EM) (LUCCA et al., 2015).

O método em CG/EM no modo *headspace* permite que as amostras de pele recolhidas no teste de permeação cutânea (fitas do *tape-stripping*, epiderme e derme) sejam colocadas diretamente no *vial* submetido a temperatura e agitação que volatiliza os compostos de interesse. Neste caso, o composto majoritário do óleo de copaíba é o β-cariofileno, tendo

sido validado um método para a sua análise. O método, original, desenvolvido no âmbito desta tese, demonstrou-se linear, preciso, exato e específico.

Além disso, o resultado da permeação cutânea demonstrou que a nanoemulsão contendo o óleo consegue promover a penetração do β-cariofileno até a camada da derme (camada mais profunda), enquanto que, com óleo, o composto não ultrapassa o estrato córneo (camada mais superficial). No entanto, a formulação não conseguiu promover a permeação da substância até o fluido receptor, o qual mimetiza a chegada até a circulação e tecidos mais profundos.

A fim de verificar se modificações na carga de superfície e modificação da viscosidade poderiam facilitar ou aumentar a retenção/permeação de β-cariofileno a partir das nanoemulsões de óleo de copaíba, foi testada a adição de dois tensoativos catiônicos à nanoemulsão previamente otimizada e, após, a sua incorporação em uma forma farmacêutica semissólida.

A incorporação dos tensoativos catiônicos oleilamina (OA) e brometo de cetiltrimetilamônio (CTAB) reverteu o potencial zeta da nanoemulsão para uma carga positiva. Entretanto, a concentração de OA que conseguiu reverter foi bastante elevada (acima de 35 mM), enquanto as concentrações de CTAB que reverteram a carga são consideradas baixas e seguras para a utilização em formulações tópicas (ROWE et al., 2009). A OA é uma amina primária que é influenciada pelo pH do meio. Como o pH das nanoemulsões contendo óleo de copaíba apresenta-se ácido (cerca de 3), provavelmente pela presença de ácidos resinosos na interface óleo/água (DIAS et al., 2014), a molécula do tensoativo se apresenta ionizada e isto influencia na reversão do potencial zeta. Como o CTAB é uma amina quaternária, a única explicação para a reversão da carga é a sua saturação na superfície da gotícula (RABINOVICH-GUILATT et al., 2004; MARTINI et al., 2008).

O estudo de permeação/retenção cutânea das formulações contendo OA e CTAB demonstrou que a presença da carga positiva na gotícula da nanoemulsão consegue aumentar a concentração de β-cariofileno na epiderme em cerca de duaz vezes a

concentração obtida para a nanoemulsão carregada negativamente (NCN), mas não aumenta sua concentração da derme, como era almejado. Por outro lado, o β-cariofileno foi quantificado no fluido receptor para as nanoemulsões carregadas positivamente (NCP), o que demonstra uma influência também da carga da partícula na permeação através da pele.

É importante ressaltar que o estudo de permeação/retenção cutânea foi realizado até 8 horas, ou seja, as concentrações obtidas mostram um perfil alcançado após esse tempo de exposição da formulação à pele. Esse período foi estabelecido com base no fato de que após 8 horas torna-se difícil manter a integridade da pele e também porque de certa forma mimetiza o tempo de exposição usual a uma formulação tópica. Com base nisso, esse aumento da retenção do β-cariofileno na epiderme deve ser visto como um resultado positivo, visto que esta camada pode representar um "reservatório" para a camada subsequente.

Como as nanoemulsões são sistemas de baixa viscosidade, a incorporação em veículos que promovam o aumento da viscosidade também foi estudado. Foram escolhidos três polímeros para a formação de hidrogéis contendo as nanoemulsões selecionadas, NCN e CNP-CTAB: Carbopol 980®, Natrosol® e quitosana

Os três polímeros apresentam caraterísticas diferentes, já que o Carbopol® apresenta carga aniônica, o Natrosol® é um polímero não iônico e a quitosana tem carga positiva. A incorporação em gel de quitosana levou a uma instabilidade das nanoemulsões, aumentando seu tamanho de gotícula e seu índice de polidispersão, apensar de continuar apresentando um potencial zeta elevado. Tanto os hidrogéis de Carbopol® quanto de Natrosol® mostraram-se eficientes na incorporação nas nanoemulsões, porém somente o gel de Natrosol®, devido ao se caráter neutro foi selecionado para dar continuidade aos estudos de permeação cutânea e atividade anti-inflamatória *in vivo*.

Quanto a permeação das nanoemulsões incorporadas em hidrogel, pode-se verificar que houve um aumento na retenção de β-cariofileno na derme em relação ao encontrado para as nanoemulsões, e que houve a facilitação de permeação até o fluido receptor. Porém, não há diferença estatística entre as duas formulações, indicando que a carga não influencia

na penetração na pele e sim outro fatores, como oclusão, viscosidade e hidratação do local devido à quantidade de água presente na formulação (AL-SUBAIE et al., 2015). Todos os resultados do estudo de permeação cutânea (formulações nanoemulsionadas e semissólidas) estão dispostos na Tabela 1 para fins de comparação.

Tabela 1. Resultados do estudo de permeação cutânea para as formulações desenvolvidas nesta tese e o óleo de copaíba.

	OC	**NCN**	**NCP**	**NCN-HEC**	**NCP-HEC**
Estrato córneo (µg/cm²)	0,57 ± 0,26	0,07 ± 0,05	0,05 ± 0,03	0.18 ± 0.17	0.09 ± 0.07
Epiderme (µg/cm²)	ND	3,53 ± 0,97	6,63 ± 2,74	7.91 ± 2.46	9.76 ± 2.65
Derme (µg/cm²)	ND	1,06 ± 0,39	1,45 ± 0,83	1.95 ± 0.56	2.43 ± 0.91
Fluido receptor (µg/cm²)	ND	ND	0,32 ± 0,32	1.80 ± 0.85	0.67 ± 0.22

OC: óleo de copaíba; NCN: nanoemulsão carregada negativamente; NCP: nanoemulsão carregada positivamente; NCN-HEC: nanoemulsão carregada negativamente incorporada em hidrogel de hidroxietilcelulose; NCP-HEC: nanoemulsão carregada positivamente incorporada em hidrogel de hidroxietilcelulose; ND: não detectado.

Finalmente, o estudo da atividade *in vivo* das formulações selecionadas demonstrou que o óleo de copaíba apresenta atividade anti-inflamatória e que a sua incorporação em nanoemulsões aumenta este efeito, já que estas apresentaram maior percentagem de inibição de edema tanto no modelo de edema de pata de rato quanto no modelo de edema de orelha de camundongo. O estudo desenvolvido neste trabalho foi o primeiro da literatura

a demonstrar a potencialização da atividade anti-inflamatória do óleo de copaiba a partir de sua nanoemulsificação, com ou sem espessamento em hidrogel, bem como a correlação dessa atividade com os resultados da retenção/permeação do β-cariofileno nas camadas da pele.

No entanto, ambas nanoemulsões, tanto negativa quanto positivamente carregadas, apresentaram resultado semelhante para a inibição do edema. Quando compara-se a permeação cutânea das formulações verifica-se que na derme não há diferença estatística, o que pode justificar a semelhança na inibição da inflamação no teste *in vivo*. Uma hipótese para explicar tal efeito seria a saturação da camada da derme, que pode apresentar um limite na concentração de substâncias que permeiam até ela.

Em relação à atividade das formulações incorporadas em hidrogel, pode-se verificar que a incorporação das nanoemulsões de óleo de copaíba em hidrogel também favoreceu o efeito anti-inflamatório tópico do óleo no modelo de edema de pata, especialmente para a formulação carregada positivamente. Quando ao resultado do modelo de edema de orelha, as formulações tiveram um perfil equivalente ao controle cetoprofeno, porém não modificaram o efeito do óleo, que também apresentou valores altos de inibição de edema.

Como descrito anteriormente, o óleo de copaíba, bem como seu componente majoritário, β-cariofileno, já foram descritos na literatura como leishmanicidas frente à espécie *Leishmania amazonenses,* causadora da forma cutânea da doença (SANTOS et al., 2008, 2011, 2012). Ainda, Gupta e colaboradores (GUPTA et al., 2015) descrevem que este óleo fornece um efeito sinérgico ao efeito da anfotericina B frente à *Leishmania donovani*, quando incorporados em uma nanoemulsão. Neste trabalho, foi descrito, de forma inovadora, a avaliação das nanoemulsões contendo o óleo de copaíba de uma espécie determinada (*Copaifera multijuga*) e de β-cariofileno no tratamento de quatro espécies de *Leishmania* (*L. amasonensis*, *L. braziliensis*, *L. donovani* e *L. major*).

Frente às formas amastigotas intracelulares, tanto o óleo e β-cariofileno quanto as suas formulações foram efetivas para as espécies *L. donovani* e *L. major*. Contra as espécies *L. amasonensis* e *L. braziliensis* apenas β-cariofileno puro e as nanoemulsões foram efetivos,

apresentando baixos valores de IC_{50} (concentração inibitória de 50% da população). Além disso, todos os tratamentos apresentaram baixa citotoxicidade frente a células de macrófagos não infectados.

No teste *in vivo* com camundongos infectados com *Leishmania major* observou-se que todos os tratamentos (óleo de copaíba, β-cariofileno e nanoemulsões) foram capazes de reduzir a área da lesão, em comparação com o controle não tratado. Estes resultados corroboram com testes *in vitro*, nos quais todos os tratamentos apresentaram valores de CI_{50} pequenos. Porém nenhum animal apresentou recuperação total da doença, o que pode estar relacionado com a dificuldade dos tratamentos acessarem o local da infecção devido à formação de uma crosta muito espessa. Além disso, são necessários outros testes para verificar a quantidade de parasitos no local da infecção e confirmar a redução da carga parasitária.

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CONCLUSÕES

CONCLUSÕES

Através do estudo realizado pode-se concluir que:

- O método bioanalítico validado em cromatógrafo a gás acoplado a espectrômetro de massas no modo *headspace* mostrou-se linear, exato, preciso e específico para a quantificação de β-cariofileno em amostras de pele submetidas ao teste de permeação cutânea de nanoemulsões de óleo de copaíba (*Copaifera multijuga* Hayne);

- A permeação cutânea comparando o óleo de copaíba puro e as nanoemulsões contendo o óleo demonstrou que o β-cariofileno fica retido no estrato córneo quando aplicou-se o óleo puro, enquanto que, a partir da nanoemulsão, ele atinge a derme;

- O estudo de formulação de nanoemulsões de óleo de copaíba com carga de superfície positiva através da incorporação de tensoativos catiônicos a uma formulação previamente otimizada demonstrou que:

  (i) a adição de oleilamina somente mudou o potencial zeta da formulação quando em altas concentrações;

  (ii) o tensoativo brometo de cetiltrimetilamônio conseguiu inverter a carga de superfície das nanoemulsões em concentrações aceitáveis para o uso tópico;

  (iii) a retenção cutânea do β-cariofileno a partir das formulações positivamente carregadas aumentou em três vezes na camada da epiderme (em relação a nanoemulsão aniônica), mas na derme não houve mudança estatisticamente significativa;

- A incorporação das nanoemulsões (negativa e positivamente carregadas) em três tipos de hidrogéis revelou que:

(i) os polímeros iônicos (carbômero e quitosana) promovem a inversão do potencial zeta de nanoemulsões com carga de superfície contrária à do polímero;

(ii) o valor do potencial zeta aumenta com a incorporação em hidrogéis, levando a crer que os polímeros podem adsorver na superfície da nanoemulsão;

(iii) a incorporação em quitosana aumenta a instabilidade das formulações, aumentando o tamanho de gotícula e índice de polidispersão;

- A permeação cutânea e a retenção epidérmica do β-cariofileno a partir das nanoemulsões catiônica e aniônica incorporadas em hidrogel de hidroxietilcelulose foi aumentada em 2 e 1,5 vezes, respectivamente, em relação às nanoemulsão original, mas não ocasionou alteração na derme;

- O estudo da atividade *in vivo* das formulações revelou que a nanoemulsificação do óleo de copaíba leva a um aumento no efeito anti-inflamatório tópico, o que corrobora a hipótese de que as nanoemulsões facilitam a penetração dos componentes do óleo na pele;

- A incorporação das nanoemulsões de óleo de copaíba em hidrogel também favoreceu o efeito anti-inflamatório tópico do óleo no modelo de edema de pata, especialmente para a formulação carregada positivamente. Quanto ao resultado do modelo de edema de orelha, as formulações tiveram um perfil equivalente ao controle cetoprofeno, porém não modificaram o efeito do oleo;

- Quanto à atividade do óleo de copaíba, β-cariofileno e das formulações nanoemulsionadas frente ao parasito causador da Leishmaniose pode-se concluir que:

(i) Os testes *in vitro* mostraram que os tratamentos foram mais eficazes nas espécies de *Leishmania* do "Velho Mundo" (*L. major* e *L. donovani*) em comparação com as do "Novo Mundo" (*L. amazonensis* e *L. braziliensis*);

(ii) Contra as formas amastigotas intracelulares, o β-cariofileno e a nanoemulsão carregada com β- cariofileno foram os tratamentos mais ativos, com baixos valores de IC_{50} (abaixo de 4 μg/mL);

(iii) O teste *in vivo* mostrou que todos os tratamentos foram capazes de reduzir a área da ferida dos camundongos infectados com *L. major*. No entanto, eles não conseguiram recuperar totalmente os animais.

ANEXOS

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Determination of β-caryophyllene skin permeation/retention from crude copaiba oil (*Copaifera multijuga* Hayne) and respective oil-based nanoemulsion using a novel HS-GC/MS method

Letícia G. Lucca[a], Sheila Porto de Matos[a], Bruna Tassi Borille[a], Daiane de O. Dias[a], Helder F. Teixeira[a], Valdir F. Veiga Jr.[b], Renata P. Limberger[a], Letícia S. Koester[a,*]

[a] Pharmacy College, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Department of Chemistry, Federal University of Amazonas, Manaus, AM, Brazil

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ABSTRACT

Copaiba oil is largely used in the Amazonian region for the treatment of inflammation, and recent studies demonstrated that one of the major components of the oil, β-caryophyllene (CAR), is a potent anti-inflammatory. The nanoemulsification of this oleoresin, which has unctuous character, converts it in a more acceptable hydrophilic formulation and may improve CAR penetration through the skin due to the small droplet size and the high contact surface afforded by the nanoemulsions. This paper describes the validation of a novel, sensitive, practical and solvent free method that uses gas chromatography in headspace mode coupled with mass spectrometry to evaluate the skin permeation/retention of CAR from the crude copaiba oil and its nanoemulsion. Our results show that the bioanalytic method was fully validated, demonstrating linearity ($r^2 > 0.99$), specificity (no peaks co-eluting with CAR retention time), precision (RSD < 15%) and accuracy (recovery > 90%) within the accepted parameters and that the copaiba oil nanoemulsion presented a better skin penetration compared to the crude oil, with CAR achieving the most profound layer of the skin, the dermis.

1. Introduction

The genus *Copaifera* L. includes different species and occurs mainly in the Brazilian Amazonian region. Copaiba oil is an oleoresin extracted from the trunk of the *Copaifera* tree and is widely used in popular medicine in that region for different purposes, such as anti-inflammatory, antitumoral and antimicrobial [1,2]. The anti-inflammatory activity was attributed to β-caryophyllene (CAR), since in the study of Veiga Junior et al. [1], the oil extracted from the species *C. multijuga* Hayne presented the highest amount of this compound and exhibited the highest activity. Also, recent studies proposed the mechanism of action from the oil and from β-caryophyllene. According to Gomes et al. [3], the oil extracted from *Copaifera multijuga* act similarly as anti-inflammatory compounds (inhibiting histaminergic and serotoninergic pathways) and presents antinociceptive effect (possibly mediated by opioid receptors). Furthermore, according to Gertsch et al. [4], β-caryophyllene is a selective agonist to the peripheral cannabinoid receptor, CB_2, which is related to the treatment of pain and inflammation.

Nanoemulsions present penetration-enhancing ability and have already proved to be advantageous for the administration of anti-inflammatory drugs [5–7]. In this context, the feasibility of developing a stable nanoemulsion with copaiba oil was recently investigated by our research group [8]. This formulation is intended for topical treatment of locally inflamed skin.

The volatile character of the oleoresin imposes difficulties in terms of developing both a stable formulation and an analytical method for skin permeation/retention studies. To the best of our knowledge, there is only one report of GC/MS analytical method validation for volatile compounds in a skin permeation assay [9]. In that study, the authors report the full validation of a method for sesquiterpenes lactones assay in *Arnica* formulations and the determination of its penetration profile in porcine ear skin.

Therefore, our main objective in this study was to describe the validation of a bioanalytical gas chromatography coupled with mass spectroscopy method to analyze CAR in the samples from pig ear skin permeation assays carried out with the crude copaiba oil and its nanoemulsion formulation.

* Corresponding author. Tel.: +55 51 33085278; fax: +55 51 3308 5437.
E-mail address: leticia.koester@ufrgs.br (L.S. Koester).

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2. Materials and methods

2.1. Chemical and reagents

The oleoresin from *C. multijuga* Hayne was collected in the Ducke Forest Reserve of the Instituto Nacional de Pesquisas da Amazônia (INPA) at Manaus, Amazonas State (Brazil), and the exsiccate was deposited at the INPA herbarium. It was extracted by an usual method, artificial exudation, described by Veiga Junior et al. [1] and characterized by gas chromatography coupled with mass spectrometry (GC/MS), which confirmed the presence of 42% (w/w) of β-caryophyllene. β-Caryophyllene reference standard, Span 80® and Tween 20® were obtained from Sigma–Aldrich (St. Louis, MO, USA). Medium Chain Triglycerides (MCT) was kindly donated by Lipoid GmbH (Ludwigshaten, Germany). Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, Billerica, Massachusetts, USA). All other chemicals or reagents were of analytical grade.

2.2. Instrumentations and chromatographic conditions

The samples were analyzed using a gas chromatograph 5975C (Agilent Technologies, Santa Clara, California, USA), equipped with a CombiPAL Autosampler (Basel, Switzerland), consisting of a split/splitless injector port and a mass spectrometer detector. The injection was made in the splitless mode. The GC system was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 mm). The carrier gas was helium (1.0 mL/min). The oven temperature was programmed from 60 °C for 3 min with an increase of 40 °C/min, to 300 °C, finalizing the chromatographic run at 9 min. Injector, transfer line (interface), source and quadrupole temperatures were set at 220 °C, 300 °C, 230 °C and 150 °C respectively. The mass detector was operated with electron impact system at 70 eV. The signal was recorded and processed with GC/MS Data Analysis Software.

2.3. Determination of β-caryophyllene in the samples

The analysis of the main marker of copaiba oil, β-caryophyllene (CAR), was determined by headspace (HS) in a gas chromatograph coupled with mass spectrometer (GC/MS). The GC equipment was coupled with headspace sample preparation system (CTC Analytics CombiPAL, Basel, Switzerland). The samples were placed into a 10 mL glass vial and transferred for the heating station for 10 min at 50 °C. After 10 min, 1.0 mL of the volatiles in the vial was aspirated into a 1.0 mL syringe and introduced in the GC port. 10 mL clear glass vials with magnetic 18 mm screw caps with septa (Agilent Technologies, United States of America) were used in this paper.

2.4. Method validation

The method was validated for CAR assay in skin samples. The study included tests of specificity, linearity, limits of detection and quantification, precision, accuracy and matrix effect [10,11]. A methanol solution of porcine ear skin was used as the skin matrix for the bioanalytical method validation.

2.4.1. Specificity

The specificity was assessed comparing the CAR peak retention time with blank samples containing the porcine skin methanol solution, the tapes used in the tape-stripping method and the receptor fluid.

2.4.2. Linearity, limits of detection and quantification

For linearity experiments, solutions of CAR were prepared in the range of 0.14–0.68 μg/mL in 3 different days. Linearity was evaluated by calculating the regression line using the least squares method. Using the calibration curve data, detection (LOD) and quantification (LOQ) limits were determined based on the standard deviation of the response and the slope.

2.4.3. Precision and accuracy

Precision was assessed by analyzing samples of methanol skin extract spiked with three CAR concentrations (0.14, 0.45, 0.68 μg/mL), in the same day (intra-day) and in three different days (inter-day). Results for precision are shown as relative standard deviation (R.S.D.). Accuracy of the method was determined by recovering CAR from the skin extract, at three different levels (0.14; 0.45 and 0.68 μg/mL). Accuracy results are shown as percentage of CAR recovered from the skin samples.

2.4.4. Matrix effect

According to Niessen et al. [12] the percentage of matrix effect (%ME) can be assessed by calculating the difference between the peak area of a medium concentration in the calibration curve with and without the skin matrix (skin methanol solution). In this paper, matrix effect was calculated comparing the peak areas of CAR methanol solution (0.45 μg/mL) with and without the skin methanol extract.

2.5. Preparation and characterization of copaiba oil nanoemulsion

Briefly, the oily phase (20% w/w copaiba oil; 10% w/w MCT; 3% w/w Span 80®) and the aqueous phase (1% w/w Tween 20® and q.s.p. water) were mixed under magnetic stirring (5 min at room temperature) to form a coarse emulsion. In order to gradually decrease the droplet size, this coarse emulsion was subjected to high-pressure homogenization (EmulsiFlex-C3®, Avestin, Canada) at 750 bar for six cycles, producing the nanoemulsion, as optimized by Dias et al. [8].

After preparation, the nanoemulsion was characterized for droplet size, polydispersity index, zeta potential and CAR content. Droplet size and polydispersity index were measured in triplicate by dynamic light scattering after dilution of 10 μL of the nanoemulsion in 10 mL of purified water (Zetasizer Nanoseries ZN90, Malvern Instruments, Worcestershire, UK). The zeta potential value was measured in triplicate by laser Doppler velocimetry using the same instrument, after dilution of 10 μL of the nanoemulsion in 10 mL in NaCl (1 mM) ultra-filtered in 0.45 μM filter. CAR content was determined as described by Dias et al. [13] using a 0.45 μg/mL nanoemulsion solution in water.

2.6. Permeation/retention studies

Permeation/retention studies were performed using porcine ear skin (n = 10) in Franz-type diffusion cell equipment (Dist, Florianópolis, Brazil).

Porcine ear was obtained from a local slaughterhouse and the skin was excised from the outer part of the pig's ear using a scalpel. The hair and fat excess from the extracted skin were removed with scissors and the thickness was measured with a thickness gauge (Mitutoyo Corporation, Kanagawa, Japan). Only skin cuts in the range of 0.90–1.10 mm were used in the experiments.

Receptor fluid was a mixture of ethanol and phosphate buffered saline (PBS) pH 7.4. The optimum amount of ethanol was verified in a CAR solubility test, in which a CAR excess was added to a mixture of PBS and ethanol in different proportions (30:70, 40:60 and 50:50), in order to verify the *sink* conditions for the skin permeation experiment. The samples were kept under magnetic stirring for 24 h. The test was carried out in triplicate. After 24 h the samples were centrifuged for 15 min. An aliquot of 20 μL from the aqueous phase (bottom phase) was diluted in a 10 mL volumetric flask with water. The final solution was analyzed by gas chromatography

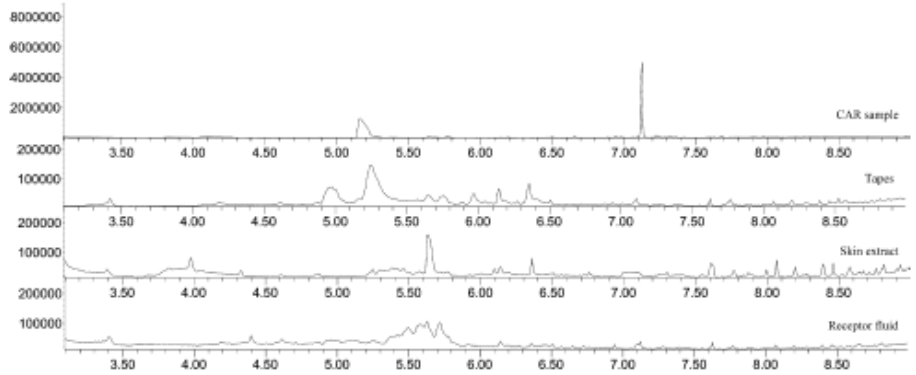

Fig. 1. Specificity for CAR sample compared to skin extract, receptor fluid and tapes from the tape-stripping test. CAR purity 99.5%.

according to item 2.3 and CAR solubility was calculated comparing a CAR standard solution area to the CAR area in the samples in the same concentration.

The receptor compartment was kept at 32 °C ± 1 °C and under magnetic stirring during the entire experiment. Formulations were placed directly on the porcine ear skin in the donator compartment, 500 μL of the nanoemulsion or oleoresin were used to perform the experiment. An aliquot of the receptor fluid of 1 mL was collected every 2 h from the beginning of the experiment to verify the permeation of β-caryophyllene through the skin. After 8 h of experiment, the skin was removed from the equipment. The excess of formulation/oleoresin was removed from its surface using Milli-Q water. The excess of skin, which was not in contact with the formulation/oleoresin, was cut off and the tape-stripping method was used to remove the stratum corneum [14] from the viable skin. The epidermis was separated from the dermis with a scalpel and both were weighted in analytical scale [15–17]. The tapes, the epidermis, the dermis and the fluid aliquots were frozen (−20 °C) for posterior analysis in HS-GC/MS. The maximum freezing time for all the samples before their analysis was 7 days. All samples (receptor fluid, tapes, epidermis and dermis) were directly placed in the headspace vials without any addition of solvents and the determination of β-caryophyllene was performed according to item 2.3. The results for the receptor fluid and the tapes were expressed as μg/mL and for the epidermis and dermis as μg/g.

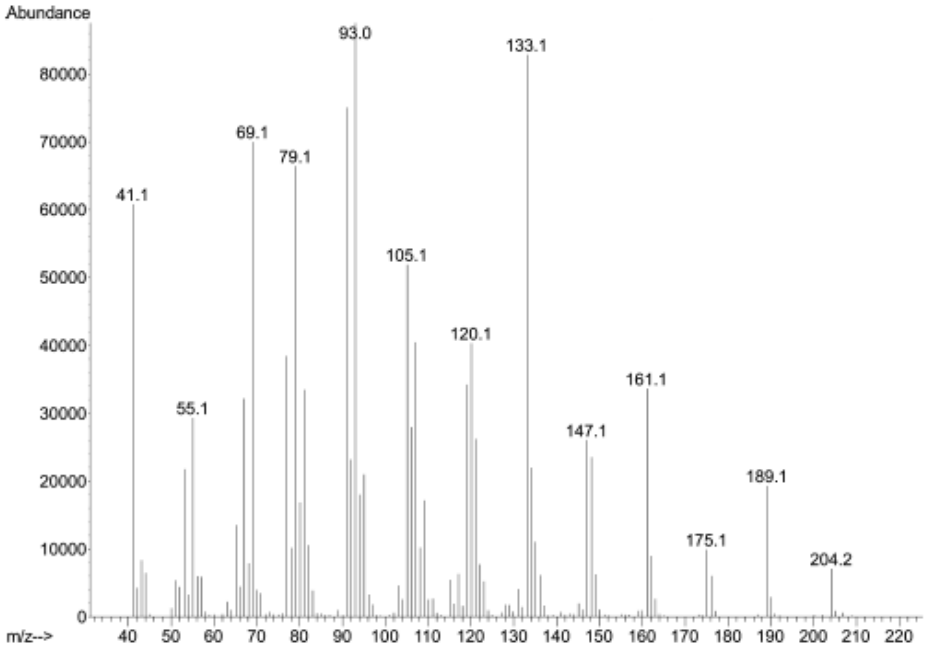

Fig. 2. CAR fragmentation spectrum.

Table 1
Results for intra-day precision and intermediary precision for β-caryophyllene (CAR).

CAR concentration (μg/mL)	Intra-day precision (R.S.D. %)			Inter-day precision (R.S.D. %)
	Day 1	Day 2	Day 3	
0.14	1.37	10.18	9.81	7.66
0.45	4.08	7.08	10.94	7.12
0.68	8.05	0.98	9.17	11.29

R.S.D. = relative standard deviation.

2.7. Statistical analysis

Results in this paper are shown as mean ± standard deviation. ANOVA test was performed to verify the difference in the permeation study. p values less than 0.05 ($^*p < 0.05$) were significant.

3. Results and discussion

3.1. Method validation

Chromatograms from each blank sample of porcine skin methanol solution, the tapes used in the tape-stripping method and the receptor fluid used in the specificity assay were compared with a CAR methanol solution (Fig. 1). The results indicated that there were no interferents in the CAR peak (7.126 min), demonstrating that the method is specific for samples containing CAR. The mass fragmentation of the CAR peak indicated the absence of impurities in CAR samples, presenting the usual peaks m/z 93, m/z 133 and m/z 204 (Fig. 2) [18]. In addition, a previously validated stability indicating method using GC coupled with flame ionization detector (FID) demonstrated that the nanoemulsion components did not interfere in the CAR analysis [13]. It is worth emphasizing that the use of HS-GC/MS instead of HS-SPME-GC/FID [13] improved our results, since the mass detector proved more sensible for our samples. Moreover, the use of headspace extraction without solid phase microextraction (SPME) step decreases the expenses and the time of analysis. Productivity gains were also achieved by the use of CombiPAL autosampler, what is important in an experiment with so many samples as a skin permeation/retention study.

Linearity was evaluated with and without the presence of methanol skin extract. The method demonstrated to be linear in the range of 0.14–0.68 μg/mL for both conditions and the calibration equations showed excellent correlation coefficient ($r^2 > 0.99$), highly significant for the method ($p < 0.05$). The absence of a constant systematic error was confirmed since the confidence intervals included zero. The LOD and LOQ limits for the assay containing skin extract were 0.07 and 0.14 μg/mL, respectively. The low values of LOD and LOQ indicated the sensitivity of the proposed method.

Precision was assessed considering repeatability and intermediary precision at three CAR concentrations (0.14, 0.45 and 0.68 μg/mL) on 3 different days. All results presented R.S.D. values lower than 15.0% (Table 1), which were considered satisfactory for bioanalytical methods, indicating that the method was precise for the analysis of CAR. The accuracy of the method was assessed considering the recovery of CAR from the skin methanol extract. Table 2 shows the average percentages of recovery in the range of 94.47–96.98%, demonstrating the accuracy of the proposed method.

The percentage of matrix affect calculated for CAR in the skin matrix was 8.4%, which can be considered not significant, according to Walorczyk [19]. Also, the absence of matrix affect can be observed by the high values of CAR recovery.

Table 2
Recovery of β-caryophyllene (CAR) added to skin methanol extract.

Theoretical concentration (μg/mL)	Experimental concentration (μg/mL ± SD)	%
0.14	0.1357 ± 0.0104	96.98
0.45	0.4249 ± 0.0303	94.44
0.68	0.6790 ± 0.0731	95.21

SD = standard deviation.

3.2. Nanoemulsion characterization

In a previous study [8], the amount of copaiba oil, medium chain triglycerides (MCT), Span 80® and Tween 20® was optimized to produce a nanoemulsion that would contain the maximum amount of copaiba oil (20%, w/w), and consequently, it would contain the maximum amount of CAR.

After preparation, the nanoemulsion was characterized for droplet size, polydispersity index, zeta potential and CAR content. The nanoemulsion used in the skin permeation experiments presented a droplet size of 253.9 ± 2.74 nm, a polydispersity index of 0.058 ± 0.02, a zeta potential of −31.3 ± 0.36 mV and a β-caryophyllene content of 100.8 ± 0.01%. All the results obtained were in agreement with the formulation optimized in a previous study [8].

3.3. Permeation/retention studies

In order to keep the *sink* conditions in the test, three ethanol proportions were studied in the receptor fluid. In the 50% ethanol proportion, CAR was visibly homogenized during the test, showing a solubility of 4.26 mg/g. The other ethanol proportions (30% and 40%) presented smaller values of solubility (3.52 and 2.58 mg/g), but still capable of guaranteeing *sink* conditions. Nevertheless, during pilot experiments with these proportions, whitish solutions with perceptible oil droplets were formed, that could visibly demonstrate that CAR was not solubilizing in the receptor fluid sample in those ethanol proportions. Thus, a 50% ethanol proportion was chosen to perform the experiments, as already observed in other studies with low aqueous soluble drugs [20,21].

Table 3 shows the results of CAR skin permeation and retention profile from the copaiba oil and respective nanoemulsion, including the percentage of CAR found in each sample relative to the total amount applied on top of the skin. For the crude copaiba oil, CAR was only found in the stratum corneum layer, but not in the epidermis, dermis or receptor fluid, indicating no penetration or permeation in the skin.

Table 3
Results for the skin permeation/retention profile of β-caryophyllene (CAR) from crude copaiba oil and oil-based nanoemulsion. Stratum corneum unit is μg/mL, dermis and epidermis units are μg/g. Results are also shown as percentage of CAR in skin layers relative to total amount applied on top of skin.

Sample	Unit	Crude copaiba oil	Copaiba oil nanoemulsion
Stratum corneum	μg/mL	1.349 ± 0.749 [a]	0.197 ± 0.067 [a]
	%	0.0002 ± 0.0001	0.0001 ± 0.00006
Epidermis	μg/g	ND	153.025 ± 79.314 [b]
	%	ND	0.041 ± 0.015
Dermis	μg/g	ND	19.226 ± 14.182 [b]
	%	ND	0.037 ± 0.014
Receptor fluid	μg/ml	ND	ND
	%	ND	ND

ND = not detected.
Same letters indicate that the values are statistically different ($p < 0.05$).

On the other hand, for the copaiba oil-based nanoemulsion, CAR was detected in the stratum corneum, epidermis and dermis, implying that the nanoemulsion facilitated the penetration of CAR through the skin, even though its concentration in the experiment with the crude oil was five times higher than in the experiment with the nanoemulsion (which contains 20% of this oleoresin in its composition). However, CAR was not detected in the receptor fluid for the nanoemulsion formulation either, which could represent that the nanoemulsion did not enable the permeation through the skin.

According to the literature, it is suggested that lipophilic drugs (with high partition-coefficient), such as some anti-inflammatory, always find an obstacle to pass through the skin and remain retained in the lipid layer (particularly the stratum corneum) since the epidermis and dermis layers have more hydrophilic characteristics [22]. Moreover, nanoemulsions seem to facilitate the penetration of actives in the skin since they present a small droplet size, a large surface area and also have a tendency to interact with inflamed tissues demonstrating a great advantage in topical administration and incorporation of a lipophilic anti-inflammatory drug [7,23–25].

4. Conclusion

Therefore, a solvent free gas chromatography coupled with mass spectrometry method was fully validated to determine β-caryophyllene in skin samples, demonstrating that it was specific, linear, precise and accurate. Also, the nanoemulsion produced showed great skin penetration profile compared with the crude copaiba oil, indicating that it facilitated the penetration of copaiba oil and its major component, β-caryophyllene, through the skin up to the most profound layer, the dermis.

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Article

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Nanoemulsification Potentiates *In Vivo* Antiedematogenic Effect of Copaiba Oil

Letícia G. Lucca[1], Sheila P. de Matos[1], Cristiane B. de Mattos[1], Helder F. Teixeira[1], Renata P. Limberger[1], Valdir F. Veiga Jr.[2], Bibiana V. de Araújo[1], and Letícia S. Koester[1,*]

[1] *Pharmaceutical Sciences Graduation Program, Pharmacy College, Federal University of Rio Grande do Sul, 2752 Ipiranga Avenue, Porto Alegre-RS, Brazil*
[2] *Chemistry Department, Federal University of Amazonas, 6200 General Rodrigo Octávio Avenue, Manaus-AM, Brazil*

Copaiba oil is a natural product obtained from the trunk of *Copaifera* trees. This oil-resin is used in folk medicine in Amazonia as an anti-inflammatory, antiparasitary and antimicrobial. β-caryophyllene, a major component in copaiba oil, had its anti-inflammatory effect studied in recent papers and is used as a copaiba biomarker. In the present study, we developed positively charged copaiba oil nanoemulsions (PCN), with cetyltrimethylammonium bromide and oleylamine, and compared to a negatively charged nanoemulsion (NCN) concerning skin permeation and *in vivo* antiedematogenic effect. Results show that skin permeation with the PCN increased three fold β-caryophyllene retention in the epidermis, and also in the receptor fluid compared to the NCN. *In vivo* tests were performed in mouse ear edema induced by arachidonic acid and in rat paw edema induced by formalin. In mouse ear edema, NCN and PCN promoted an edema inhibition (33%) with statistically equal effect ($p > 0.05$) to the positive control, ketoprofen (44%). In rat paw edema, both nanoemulsions presented antiedematogenic effect (edema inhibition above 60%) similar to the positive control. Copaiba oil also exhibited edema inhibition, but the nanoemulsification process led to an increased effect to the oil.

KEYWORDS: *Copaifera multijuga, Skin Permeation, β-Caryophyllene, Rat Paw Edema, Mouse Ear Edema.*

INTRODUCTION

Copaiba oil is an oil-resin exuded from the trunk of the trees of several *Copaifera* species. It is used as a popular medicine in the Amazon rainforest area, in Brazil, especially to treat inflammatory related diseases, as antimicrobial, antiparasitary and also for wound healing.[1] This oil-resin is composed mainly by terpenes, and each species presents a different profile of these substances.[2] *Copaifera multijuga* Hayne, the species used in this paper, produces an oil-resin rich in sesquiterpenes, especially β-caryophyllene. Different authors described the potential of both copaiba oil and β-caryophyllene as anti-inflammatory.[3–11]

Nanoemulsions are heterogeneous systems composed of oil droplets stabilized by surfactants dispersed in an aqueous medium, reaching droplet sizes from 100 nm to 500 nm. This systems are suitable carriers for topical use, since their small droplet size and large surface area can facilitate skin penetration of substances.[12] Also, they present low skin irritability and high drug-loading capacity, especially for lipophilic compounds.[13]

Recently, our research group described the optimization of a copaiba oil nanoemulsion and a new method to analyze its major component, β-caryophyllene, in skin permeation samples.[14,15] In our findings, nanoemulsions were appropriate carriers to load copaiba oil, arriving in a dosage form with 30% oily core (20% copaiba oil and 10% fixed oil), using the method of high pressure homogenization.[14] Furthermore, a method to detect the previously mentioned β-caryophyllene in skin samples was validated, by means of a method in gas chromatography coupled with mass spectrometer. In that study, it was found that the copaiba oil nanoemulsification facilitated the skin permeation of β-caryophyllene down to the dermis, the deepest layer of the skin.[15]

In order to enhance the permeation of molecules through the skin using nanostructures, data in the literature suggests that nanoparticles with cationic surface could

*Author to whom correspondence should be addressed.
Email: leticia.koester@ufrgs.br
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improve the passage in this barrier, since these positive charges could interact with the negative charges in the stratum corneum and open an access through the skin.[16–22] Furthermore, in a topical anti-inflammatory treatment, the dermis is the layer of interest, and the positive surface charge in a nanoparticle could lead to a higher retention there.[16]

The previously optimized nanoemulsion containing copaiba oil presents a negative surface charge, which was credited to the resinous acid components present in the oil-resin.[14] Therefore, the aims in this study were to develop a copaiba oil nanoemulsion containing a positive surface charge using cationic surfactants, to evaluate the influence of this positive charge on β-caryophyllene skin permeation and its *in vivo* antiedematogenic effect.

MATERIALS AND METHODS

Chemicals and Reagents

Crude copaiba oil was obtained from *Copaifera multijuga* Hayne tree in the Ducke Forest Reserve of the Instituto Nacional de Pesquisas da Amazônia (INPA) at Manaus, Amazonas State, Brazil (S 2°57′43″, W 59°55′38″, 120 m). Chromatographic characterization from the oil-resin confirmed the presence of 41.3% of β-caryophyllene, the major component. β-caryophyllene reference standard, arachidonic acid, Span 80®, Tween 20®, ketoprofen reference standard, cetyltrimethylammonium bromide (CTAB) and oleylamine (OA) were obtained from Sigma–Aldrich (St. Louis, USA). OA consisted of a mixture of 70% oleylamine and 30% of other fatty amines. Lipoid GmbH (Ludwigshaten, Germany) kindly donated Medium Chain Triglycerides (MCT). Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, USA). All other chemicals or reagents were of analytical grade.

Copaiba Oil Positively Charged Nanoemulsions

In order to produce a positively charged nanoemulsion (PCN) two cationic surfactants were used: CTAB and OA. Formulations were based in a previously optimized nanoemulsion, which is negatively charged (NCN).[14] Formulations are described in Table I.

Table I. Positively charged nanoemulsion formulations composition.

	F1	F2	F3	F4	F5	F6	F7	F8
Copaiba oil (%)	20	20	20	20	20	20	20	20
MCT (%)	10	10	10	10	10	10	10	10
Span 80™ (%)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
OA (%)	0.2	0.4	1.0	2.0	–	–	–	–
CTAB (%)	–	–	–	–	0.25	0.5	0.75	1.0
Tween 20™ (%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Water q.s. (mL)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

Notes: OA: oleylamine; CTAB: cetyltrimethylammonium bromide. OA concentrations are corrected to 70% of OA.

All formulations were produced according to the method described by Dias et al.[14] To prepare the formulations F1 to F4, OA was added to the oily phase, while in formulations F5 to F8, CTAB was added to the aqueous phase. Briefly, aqueous and oily phases were mixed separately under magnetic stirring at room temperature. After complete solubilization of the phases' components, aqueous phase was poured into the oily phase to form a coarse emulsion. After five minutes under magnetic stirring at room temperature, the emulsion was subjected to high-pressure homogenization (EmulsiFlex-C3, Avestin, Canada) at 750 bar for 6 cycles, without any previous downsizing step. After production, all samples were kept under 4 °C refrigeration.

After preparation, all formulations were submitted to an evaluation concerning zeta potential, polydispersity index and droplet size. Zeta potential was measured by laser Doppler velocimetry. Droplet size and polydispersity index were measured by dynamic light scattering with 1.45 refractive index value. All analysis were made in triplicate in Zetasizer Nanoseries ZN90 (Malvern Instruments, United Kingdom) by diluting 10 μL of the nanoemulsion in 10 mL NaCl 1.0 mM ultra-filtered in 0.22 μm filter.

In Vitro Skin Permeation Studies

After choosing the positively charged nanoemulsions, *in vitro* permeation studies were conducted according to a previous paper.[15] The assay was performed in Franz diffusion cell apparatus (Dist, Brazil) with 12 ml receptor fluid compartment and 1.5 cm diameter in acceptor compartment (part in contact with the skin), using porcine ear skin as membrane (mean skin thickness was 1.0 ± 0.2 mm). The receptor fluid consisted of a mixture of phosphate buffer saline (PBS) pH 7.4 and ethanol in a 1:1 proportion, to obtain a previously determined sink condition.[15] After 8 hours, the skin was cleaned with purified water and its layers were separated. Stratum corneum was removed using the tape-stripping method (15 tapes).[23] With the help of a scalpel, epidermis was scraped from the dermis. Permeation studies were performed with the positively and negatively charged nanoemulsions ($n = 6$).

Chromatographic Analyses

Samples from the permeation studies were analyzed by gas chromatograph (GC) coupled with mass spectrometer (7890A/5975C, Agilent Technologies, USA) according to a previous study.[15] All samples were prepared using headspace mode in CombiPal Autosampler (CTC Analytics AG, Switzerland) set at 50 °C for 10 minutes. Samples (epidermis, dermis and tapes for each cell) were placed in vials and analyzed separately. Injection was made in splitless mode.

GC system was equipped with a DB-5 column (30 m × 0.25 mm × 0.25 mm). Carrier gas was ultrapure helium (1.0 mL/min). Oven temperature was programmed from 60 °C for 3 min with an increase of 40 °C/min, to 300 °C, finalizing the chromatographic run at 9 min. Injector,

transfer line (interface), source and quadrupole temperatures were set at 220 °C, 300 °C, 230 °C and 150 °C respectively. Mass detector was operated with an electron impact system at 70 eV. The signal was recorded and processed with GC/MS Data Analysis Software.

Also, β-caryophyllene content was determined in the formulations used in the skin permeation experiment according to Dias et al.[24]

Animals

Adult male Swiss mice (30–40 g) were provided by Bioterio Central from Federal University of Pelotas. Adult male Wistar rats (100–200 g) were provided by CREAL (Centro de Reprodução e Experimentação de Animais de Laboratório). All animals were maintained under standard conditions (22 ± 1 °C at 40–60% relative humidity and 12 hours light-dark cycle). All animals had free access to food and water. Mice were sacrificed by cervical dislocation and rats were sacrificed by intraperitoneal propofol injection (30 mg/kg). This study was approved by Animal Use Ethics Committee at Federal University of Rio Grande do Sul (protocol number 25866).

Arachidonic Acid-Induced Mice Ear Edema

Groups of five mice were treated topically with copaiba oil, nanoemulsion formulations or ketoprofen (positive control) in the posterior and anterior part of the right ear. Left ear did not receive any treatment and served as a control for each animal. After one hour, edema was induced by topical application of arachidonic acid (solution in ethanol, 0.2 mg/μL) at 2 mg/ear (10 μL) only in the right ear. Negative control group received only the vehicle (ethanol) in the right ear. Positive control group received a ketoprofen solution at 4 mg/ear (in acetone solution, 10 μL).

After the dose-response curve using 100, 200 and 400 mg/kg copaiba oil doses, 200 mg/kg dose was chosen to perform the experiment with the respective nanoemulsion (data not shown). Since the nanoemulsion presents 20% of copaiba oil, the volume of nanoemulsion used was 5 fold that of the copaiba oil volume. Treatments and arachidonic acid were applied using an automatic pipette (20 and 100 μL).

Ear edema was measured one hour after the inflammation induction in the right ear, using a thickness gauge (Mitutoyo Corporation, Japan). The right ear weight of each mouse in each group was also used as an edema measurement. Edema inhibition (EI) was calculated comparing the thickness difference of the right and left ear from the groups to the thickness difference of the right and left ear of the control group according to Eq. (1), using the medium thickness value for each group.

$$\text{EI}\ (\%) = \left[1 - \left(\frac{\text{REt} - \text{LEt}}{\text{REc} - \text{LEc}}\right)\right] * 100 \qquad (1)$$

Where REt is the thickness of the treated group right ear, REc is the thickness of the control group right ear, LEt is the thickness of the treated group left ear and LEc is the thickness of the control group left ear.

Formalin-Induced Rat Paw Edema

Edema was induced by intraplantar injection of formalin. Each group ($n = 5$) received the treatment (copaiba oil or nanoemulsion) in the right hind paw one hour before the edema induction.

Positive control group received a ketoprofen solution at 4.0 mg/paw (in acetone). After the dose-response curve using 100, 200 and 400 mg/kg doses, a 200 mg/kg copaiba oil concentration was chosen to perform the experiment with the respective nanoemulsion (data not shown). Negative control group did not receive treatment.

Before the edema induction, animals were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg) and xylasine (25 mg/kg). Formalin solution (100 μL, 10% v/v in saline) was injected in the right hind paw, while the left paw received the same amount of vehicle, saline (NaCl 0.9%).

Paw volume was measured after four hours using a plethysmometer (Ugo Basile, Italy). Edema was measured by the difference between right hind paw volume and the basal volume for each animal. Edema inhibition (EI) was measured by the percentage of edema comparing the volume of the paw in the times for the groups to the volume of the control group as shown in Eq. (2), using the medium volume value for each group.

$$\text{EI}\ (\%) = \left[1 - \left(\frac{\text{RPt}}{\text{RPc}}\right)\right] * 100 \qquad (2)$$

Where RPt is the volume of the treated right paw and RPc is the volume of the control right paw.

Statistical Analysis

Statistical analysis in the *in vivo* tests were performed by one-way ANOVA methodology followed by Tukey's tests with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

The main goal in the modification of the previously optimized copaiba oil nanoemulsion was to produce a positively charged nanoemulsion, in order to increase β-caryophyllene penetration to the dermis, skin layer of interest for topical anti-inflammatory activity.[14]

CTAB is a quaternary ammonium compound used in the cosmetic industry as a cationic surfactant and as antimicrobial preservative. Its safe concentration to use in cosmetics is between 0.1 and 1.0% (2.74–27.40 mM), although it can reach 10% (274 mM) for seborrheic dermatitis formulations.[25] OA is a long chain primary amine with one unsaturation and is used as surfactant and, more recently, as a precursor/stabilizer of nanoparticles.[26] According to

Greim et al.,[27] OA's LD_{50} in rats is 200–2000 mg/kg, but it presents skin and eye irritating characteristics.[27]

According to Zhang et al.,[26] CTAB and OA can cause a decrease in cell viability in high concentrations after 2 hours (above 100 mM) and 24 hours (above 30 mM).[26] In the present study, we used CTAB concentrations in the range accepted and they proved to be enough to reduce droplet size. However, for OA formulations, only concentrations above 35 mM were able to reduce droplet size and to increase/reverse zeta potential.

Concerning zeta potential (Fig. 1), only F1 and F5 presented a negative value, which means that the cationic surfactants concentrations used were not adequate to this kind of formulation. Other formulations presented an increase in the zeta potential value according to the increase in the surfactant concentration.

Only F4 and F6–F8 presented an adequate value for zeta potential (22 to 50 mV). Even though F6–F8 formulation presented zeta potential values slightly below the value considered ideal, 30 mV, they did not present signs of coalescence or flocculation and were considered suitable to our research.[28,29] F2 and F3 presented a low zeta potential value, indicating a probable future instability in the system.

As stated by Martini et al.,[30] there are two phenomena that can justify the increase of zeta potential in a nanoparticle droplet interface: an alkaline environment or an interface saturation by the cationic surfactant.[30] Furthermore, they can explain the presence of a plateau or a constant increase in the values. Our results show that the increase of CTAB concentration did not influence in the zeta potential values, hence it suggests that there is a saturation of cationic molecules in the droplet surface, which is common for quaternary amines.[31]

On the other hand, OA is a primary amine and its increase lead to a progressive increase in potential zeta values, indicating that the medium pH (in this case acid, due to resinous acids in copaiba oil) influenced in the zeta potential. In acid pH, OA is protonated and to reverse and increase zeta potential it is necessary to increase the medium pH to a more alkaline state.[32,33] In this case, the nanoemulsion medium is slightly acid (around pH 5.0), thus the saturation hypothesis can be applied here too, but higher concentrations of OA were not added since they would be toxic.

Concerning droplet size, formulations F1–F3 and F5 presented elevated droplet size and polydispersity index values (Fig. 2). This results indicate that these formulations are unstable, probably because of droplet aggregation due to their low zeta potential value.[19] Droplet sizes between 100 nm and 500 nm are considered usual for topical nanoemulsions.[18,34] However, in this study, we selected nanoemulsions between 200 nm and 250 nm, considering that the amount of oil used could not produce nanoemulsions with smaller size than those cited.[35]

Based on the results obtained in this section, formulations F4 and F7, containing 70.0 and 21.0 mM of OA and CTAB respectively, were chosen to perform the following skin permeation experiments. They presented a similar droplet size and polydispersity index to the negatively charged nanoemulsion previously optimized (250.6 ± 2.1 nm and 0.08 ± 0.03, respectively).[14] In addition, CTAB nanoemulsions presented a stable zeta potential between the high concentrations, and we have

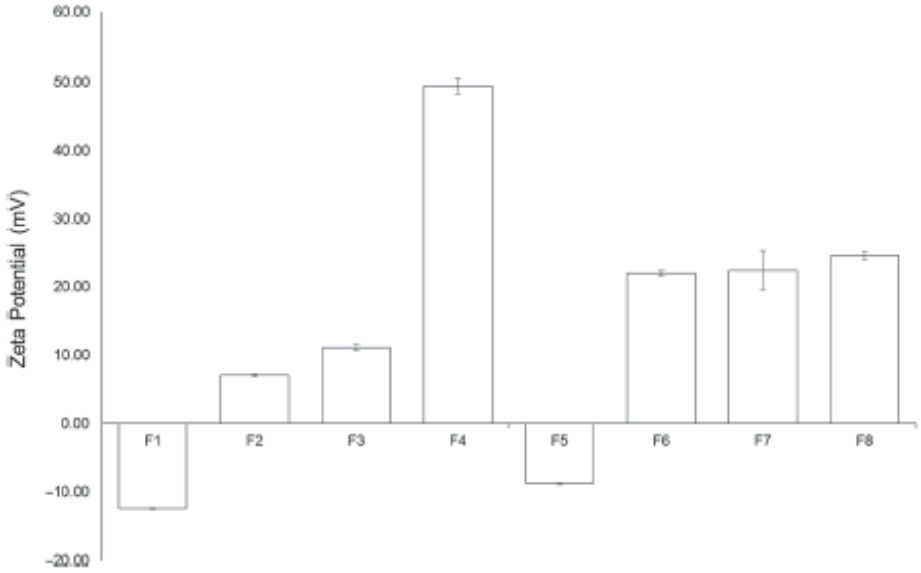

Figure 1. **Nanoemulsions zeta potential (mV).**

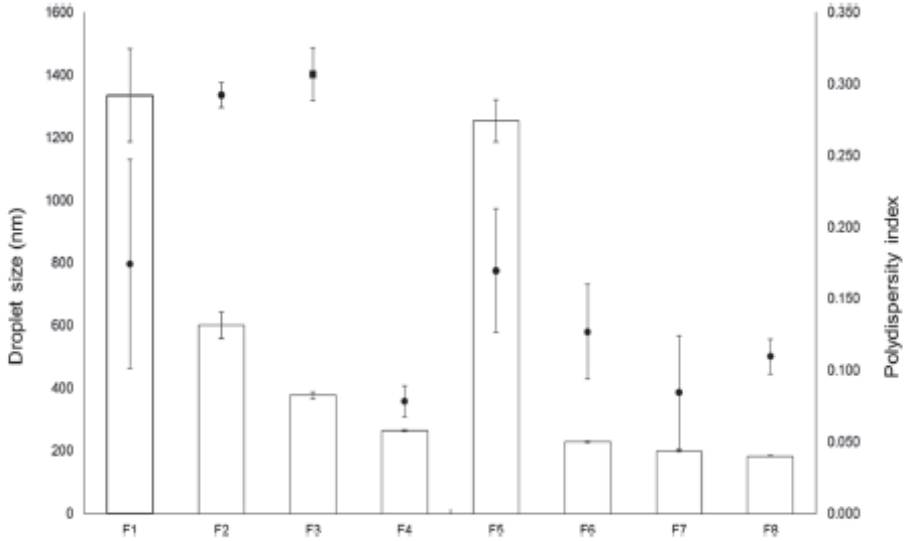

Figure 2. Formulations droplet size (bars) and polydispersity index (black points).

chosen F7, which presented a good physicochemical profile in a smaller CTAB concentration.

Table II shows the results for the skin permeation experiment comparing the negatively charged nanoemulsion with the two positively charged nanoemulsions, for each cationic surfactant selected (F4 and F7). All formulations presented a higher retention in the epidermis layer, followed by the dermis, stratum corneum and then small or not present permeation to the receptor fluid.

Many factors regarding nanoparticles influence skin retention/permeation, such as surface charge and droplet size.[18, 29, 36–38] Our results suggest that the positive charge in the nanoemulsion could promote skin permeation, caused by a higher interaction with the stratum corneum corneocytes, increasing β-caryophyllene content in epidermis in 3.72 and 3.02 folds for PCN-OA (F4) and PCN-CTAB (F7) respectively compared to NCN.[29, 39] Furthermore, there was an increase of β-caryophyllene in the receptor fluid when PCNs were tested, while β-caryophyllene was not detected in this compartment when NCN was applied over the skin. However, in the dermis, the layer of interest, there was no increase in β-caryophyllene retention. Further studies are necessary to understand why no difference in dermis retention was observed even though a higher permeation down to the receptor fluid is supposed to be related to a higher retention in the epidermis, as observed. Nevertheless, it is worth mentioning that the results represent a "snapshot" after 8 hours of *in vitro* permeation experiment, and the epidermis could behave as a "reservoir" to supply more β-caryophyllene to dermis.

Table II. Permeation assay results. Results are shown as mean ± standard deviation.

	Nanoemulsions		
Skin layers	NCN	PCN-OA (F4)	PCN-CTAB (F7)
Stratum corneum (μg/mL)	0.18 ± 0.12	0.44 ± 0.27	0.12 ± 0.07
Epidermis (μg/g)	293.44 ± 133.15	1091.98 ± 350.01	887.21 ± 193.99
Dermis (μg/g)	17.85 ± 10.93	21.13 ± 8.64	15.47 ± 8.65
Receptor fluid (μg/mL)	ND	0.29 ± 0.19	0.11 ± 0.09

Notes: NCN: negatively charged nanoemulsion; PCN-OA: positively charged nanoemulsion with oleylamine; PCN-CTAB: positively charged nanoemulsion with cetyltrimethylammonium bromide; ND: not detected.

Even though the formulation containing OA presented higher values in all the skin layers and in the receptor fluid, these values were not statistically different ($p > 0.05$) from values found for CTAB-containing formulation. Hence, F7 was chosen to perform the following experiments. Furthermore, CTAB concentration used is considered safer to use topically and is already used as an excipient in topical formulations in the cosmetic industry.[25]

β-caryophyllene content was determined for NCN and PCN-CTAB (F7), since they were chosen to perform *in vivo* experiments. NCN presented 102.13 ± 0.07% and PCN-CTAB (F7) presented 106.73 ± 0.08% of β-caryophyllene.

Arachidonic acid is involved in the cyclooxygenase (COX) and lipoxygenase (LOX) inflammation pathways and its topical administration leads to immediate vasodilatation and erythema.[40] Mouse ear edema was induced

Table III. Edema measured by mouse ear thickness (mm) and weight (mg) for the treatments with ketoprofen, copaiba oil, NCN, PCN-CTAB (F7) and blank nanoemulsion formulations ($n = 5$). Data shown as mean (standard deviation).

Groups	Thickness (mm)	Weight (mg)	Edema inhibition (%)
Control	0.48 (0.03)	11.20 (0.69)	–
Copaiba oil	0.35 (0.03)[a]	4.66 (0.66)[a]	25.0
Ketoprofen	0.27 (0.02)[a]	1.56 (0.35)[a]	44.0
PCN-CTAB (F7)	0.32 (0.05)[a,b]	6.12 (2.90)[a]	33.0
NCN	0.32 (0.02)[a,b]	2.40 (0.91)[a,c]	33.0
PCN-blank	0.36 (0.04)	9.12 (2.05)	24.0
NCN-blank	0.41 (0.01)	7.30 (0.52)	24.0

Notes: [a]Statistically different ($p < 0.05$), compared to control (non-treated group); [b,c]statistically equal ($p > 0.05$) to ketoprofen.

in the right ear by topical application of arachidonic acid (0.2 mg/μL, in ethanol).

Table III shows the edema measured by ear thickness (mm) and ear weight (mg) and edema inhibition (%).

Concerning the antiedematogenic effect measured using ear thickness results, both nanoemulsions (NCN and PCN-CTAB) presented an equal antiedematogenic profile after 60 minutes, reducing significantly ($p < 0.05$) ear edema (compared to the negative control group). They also presented a statistically equal result to copaiba oil ($p > 0.05$). However, when compared to the positive control, ketoprofen, only the nanoemulsions presented a statistically equal result ($p > 0.05$).

As long as ear weight was measured, only NCN did not present statistically difference ($p > 0.05$) from the positive control, ketoprofen. It is noteworthy that weight measurements presented higher Relative Standard Deviation (RSD) when compared to thickness measures (6.0 to 15.0% for thickness and 6.0 to 47.0% for weight), specially for the result with PCN. In addition, weight measurements were more challenging to assess during the experimental stage, due to difficulties in cutting the ear skin in an equal size to all samples or in the real affected area of the ear. Thus, weight results were used as complementary data to thickness results, but the last ones were used to calculate edema inhibition.

Since arachidonic acid is a precursor from prostaglandins and leukotrienes produced by COX and LOX enzymes, the inhibitor of this pathways acts like non-steroidal drugs, such as ketoprofen. In addition, arachidonic acid can cause mast cells degranulation, leading to histamine release.[40–43] Hence, copaiba oil could behave as a non-steroidal anti-inflammatory and the nanoemulsification process can enhance this effect, although the presence of a cationic surfactant did not exhibit an influence in this process.

Rat paw edema was induced by formalin 10% (v/v). According to Lee and Jeong,[44] this concentration is ideal to promote prominent edema formation and inflammation induction in rats, while lower concentrations (below 5%) present only behavior response.[44] Formalin induces a biphasic inflammation event. The initial phase (up to 5 minutes) mediators like substance P and bradykinin are released while in the second phase, histamine, serotonin, prostaglandins, and bradykinin are involved.[45]

In addition, the rat paw model could indicate a more profound topical action from the copaiba oil nanoemulsions, since the model is considered to increase systemic circulation achievement through the dermis, also resembling the process of arthritis.[46,47] Our result shows that both copaiba oil nanoemulsions (NCN and PCN-CTAB) had a similar antiedematogenic effect in this model, suggesting that the oil compounds can reach the dermis, which corroborates with the permeation assay, where β-caryophyllene reached this layer from all formulations in the same range.

Table IV presents the paw edema (mL) measured 4 hours after the edema induction and edema inhibition for each treatment.

As can be seen, both copaiba oil nanoemulsions (NCN and PCN-CTAB) presented a high antiedematogenic effect not different to the positive control, ketoprofen ($p > 0.05$), but higher than the crude oil. As expected, blank samples, which did not contain copaiba oil, did not present high edema inhibition. Also, copaiba oil was statistically equal ($p > 0.05$) to the non-treated group (control).

Copaiba oil presented anti-inflammatory activity in previous studies, both *in vivo* and *in vitro*.[2,4,5,48,49] Gomes et al.[6] demonstrated that different copaiba oil (*Copaifera multijuga* Hayne) fractions presented antiedematogenic activity through inhibition of peripheral serotonin and histamine receptors in rat paw edema induced by carrageenan, histamine and serotonin.[5] Also, Veiga-Junior et al.[5] concluded that *Copaifera multijuga* Hayne presented a higher anti-inflammatory effect compared to other species (*Copaifera cearensis* Huber ex Ducke and *Copaifera reticulata* Ducke), probably due to its high β-caryophyllene content.[4]

Our findings confirm that copaiba oil presents an antiedematogenic effect, which is an inflammation symptom. Furthermore, the nanoemulsification process

Table IV. Edema (mL) in rat paw 4 h after treatment with ketoprofen, copaiba oil, NCN, PCN-CTAB (F7) and blank nanoemulsion formulations. Data shown as mean (standard deviation).

Groups	Edema (mL)	Edema inhibition (%)
Control	2.53 (1.29)	–
Copaiba oil	2.20 (0.97)	13.0
Ketoprofen	0.82 (0.71)[a]	67.0
PCN-CTAB (F7)	0.58 (0.42)[a,b]	77.0
NCN	0.64 (0.57)[a,b]	75.0
PCN-blanc	2.00 (1.17)	21.0
NCN-blanc	1.93 (0.96)	24.0

Notes: [a]Statistical difference from control group ($p < 0.05$); [b]do not present statistic difference ($p > 0.05$) to ketoprofen.

increases this effect, in a smaller dose compared to the data shown in the literature. Carvalho et al.[3] described the topical anti-inflammatory effect of copaiba oil extracted from *Copaifera duckei* Dwyer in very high doses (517, 1035 and 1802 mg/kg), reaching an edema inhibition of 18% only for the higher dose in the rat paw edema assay.[2] In that paper, the oil-resin used presented only 5% of β-caryophyllene, while our sample presents 41.3% of β-caryophyllene. This could point out the importance of this sesquiterpene and its probable responsibility for the *in vivo* activity in the copaiba oil.

The presence of a cationic surfactant did not present an increased effect in the antiedematogenic activity, since the results obtained were not statistically different ($p > 0.05$) between the formulations and between the positive control, ketoprofen.

CONCLUSION

In this paper, we compared copaiba oil nanoemulsion with different surface charge: one positive and one negative. Concerning skin permeation, our results suggest that the positive charge increased β-caryophyllene retention in the epidermis, but not in the dermis. Furthermore, positively charged nanoemulsion increased permeation through the skin, arriving in the receptor compartment. In mouse ear edema, negatively charged nanoemulsion presented an increased activity compared to the positively charged one and was statistically equal to the positive control, ketoprofen. Rat paw edema showed that both formulations (positively and negatively charged) presented a similar antiedematogenic profile and did not differ from the positive control, ketoprofen. In both tests, crude copaiba oil presented a smaller edema inhibition compared to the nanoemulsion treatments. Therefore, this study showed for the first time that the nanoemulsification of copaiba oil potentiates its antiedematogenic effect and that the positive charge influenced β-caryophyllene skin retention profile but not *in vivo* pharmacological response.

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Research Article

Anti-inflammatory Effect from a Hydrogel Containing Nanoemulsified Copaiba oil (*Copaifera multijuga* Hayne)

Letícia G. Lucca,[1] Sheila P. de Matos,[1] Tainá Kreutz,[1] Helder F. Teixeira,[1] Valdir F. VeigaJr.,[2] Bibiana V. de Araújo,[1] Renata P. Limberger,[1] and Letícia S. Koester[1,3]

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Abstract. Copaiba oil is used as a popular medicine in the Amazonian forest region, especially due to its anti-inflammatory properties. In this paper, we describe the formulation of hydrogel containing copaiba oil nanoemulsions (with positive and negative charges), its skin permeation, and its anti-inflammatory activity in two *in vivo* models: mouse ear edema and rat paw edema. Three hydrogels were tested (Carbopol®, hydroxyethylcellulose and chitosan), but only Carbopol® and hydroxyethylcellulose hydrogels presented good stability and did not interfere with the nanoemulsions droplet size and polydispersity index. In skin permeation assay, both formulations, positively charged nanoemulsion (PCN) and negatively charged nanoemulsion (NCN), presented a high retention in epidermis (9.76 ± 2.65 μg/g and 7.91 ± 2.46 μg/cm^2, respectively) followed by a smaller retention in the dermis (2.43 ± 0.91 and 1.95 ± 0.56 μg/cm^2, respectively). They also presented permeation to the receptor fluid (0.67 ± 0.22 and 1.80 ± 0.85 μg/cm^2, respectively). In addition, anti-inflammatory effect was observed to NCN and PCN with edema inhibitions of 69 and 67% in mouse ear edema and 32 and 72% in rat paw edema, respectively. Histological cuts showed the decrease of inflammatory factors, such as dermis and epidermis hyperplasia and inflammatory cells infiltration, confirming the anti-inflammatory effect from both copaiba oil nanoemulsions incorporated in hydrogel.

KEY WORDS: hydrogel; *Copaifera multijuga* Hayne; inflammation; mouse ear edema; rat paw edema.

INTRODUCTION

Essential oils are used in many areas, such as in the cosmetic and perfume industries and also as a popular medicine, especially due to their anti-microbial properties (1,2). Copaiba oil is extracted from the trunk of *Copaifera* trees and represents a great commercial product, as well as a renewable source of natural therapy in the Amazonian region popular medicine, where it is used as anti-inflammatory, antiseptic, and wound healer, both by oral and topical routes (3).

Copaifera multijuga Hayne is a common species of *Copaifera* tree in the Amazon rain forest, Brazil (4). Its oilresin is composed basically by sesquiterpenes (hydrogenated and oxygenated) and diterpenes and has been described as a potent anti-inflammatory, even when compared to other *Copaifera* species, especially due to its high β-caryophyllene concentration (3). β-Caryophyllene is a sesquiterpene and has been also studied due to its anti-inflammatory effects (5,6).

Recently, studies involving copaiba oil and nanoemulsions have been published by our research group, including the development of a nanoemulsion (7) and a method to detect the major component β-caryophyllene in nanoemulsions and skin samples (8,9). Also, we described the potentialization of copaiba oil anti-edematologic effect when incorporated into nanoemulsions (10).

However, this dosage form has very low viscosity to be applied to the skin and its incorporation into a hydrogel can afford a better therapeutic compliance. Moreover, hydrogels are aqueous formulations with wet and pleasant touch sensing properties, which do not present affinity for oil droplets or lipophilic compounds (11,12). In this way, it is hypothesized that the incorporation of copaiba oil nanoemulsion into a hydrogel may enhance the permeation of its major compound, β-caryophyllene, through the skin.

Thus, the aim of this study is to incorporate copaiba oil nanoemulsions in different hydrogel polymers and to evaluate the influence of its thickening effect on β-caryophyllene skin permeation and on the anti-inflammatory effect *in vivo*. This paper shows for the first time the production of a copaiba oil

[1] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Avenida Ipiranga 2752, Laboratório 606, Porto Alegre, RS 90610-000, Brazil.
[2] Departamento de Química, Universidade Federal do Amazonas, Av. Gal. Rodrigo Octávio, 6.200, Manaus, AM 69079-000, Brazil.
[3] To whom correspondence should be addressed. (e-mail: leticia.koester@ufrgs.br)

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semi-solid dosage form that can be used in the skin and its pharmacological activity.

MATERIALS AND METHODS

Materials

β-Caryophyllene reference standard, arachidonic acid, Span 80™, Tween 20™, cetyltrimethylammonium bromide (CTAB), chitosan (CHI), Natrosol™ or hydroxyethylcellulose (HEC), and Carbopol 980™ (CARB) were purchased from Sigma-Aldrich (St. Louis, USA). Medium chain triglycerides (MCT) were purchased from Delaware (Porto Alegre, Brazil). Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, Billerica, USA). All other chemicals or reagents were of analytical grade.

Copaiba oil was extracted from *C. multijuga* Hayne trunk in Ducke Forest Reserve from Instituto Nacional de Pesquisas da Amazônia (INPA) at Manaus, Amazonas state, Brazil (2° 57′ 43″ S, 59° 55′ 38″ W, 120 m).

Preparation of Copaiba Oil Nanoemulsion and Hydrogels

Nanoemulsions containing copaiba oil were prepared according to a previous study (10). Table I describes the positively charged nanoemulsion (PCN) and negatively charged nanoemulsion (NCN) formulations.

First, aqueous (water and Tween 20™ or CTAB) and oily phases (copaiba oil, MCT and Span 80™) were mixed separately. After, the aqueous phase was poured in the oily phase, under magnetic stirring, to form a coarse emulsion. This coarse emulsion was submitted to high-pressure homogenization (Emulsiflex-C3, Avestin, Canada) for 6 cycles at 750 bar. All steps were performed under room temperature.

Hydrogels were formed by mixing the polymer powder with the nanoemulsion. HEC hydrogel (2%) was left to swell overnight, CARB hydrogel (0.5%) was formed by adding triethanolamine, and CHI hydrogel (3%) was formed by adding acetic acid.

Blank nanoemulsions were prepared without copaiba oil (only MCT up to 30% *w/w*). Blank hydrogel was prepared with water instead of nanoemulsion.

After preparation, all samples were analyzed according to their droplet size, polydispersity index, and zeta potential. β-Caryophyllene content was analyzed in the hydrogels used on *in vivo* experiments and during 1 year (for stability purposes) by a previously validated method (8). Morphological analysis from PNC and NCN incorporated in hydrogel was performed using a scanning electron microscope (SEM) with a TM3000 (Hitachi High Technologies America, Illinois, USA).

Table I. Copaiba oil nanoemulsions

Composition	PCN	NCN
Copaiba oil (%)	20.0	20.0
MCT (%)	10.0	10.0
Span 80™ (%)	3.0	3.0
Tween 20™ (%)	1.0	1.0
CTAB (%)	0.75	–
Water q.s. (%)	100	100

PCN positively charged nanoemulsion, *NCN* negatively charged nanoemulsion, *MCT* medium chain triglycerides, *CTAB* cetyltrimethylammonium bromide

Rheological Study

The hydrogel chosen to perform skin permeation and *in vivo* tests was evaluated for its rheological profile using a Brookfield Rotational Viscometer, model DV-II+ (Brookfield Engineering Laboratories, Middleboro, USA). Twenty grams of formulation was placed in a container suitable for the equipment, at rotational speed 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 3.0, and 5.0 rpm with spindle 29. Results are shown as shear stress (Pa) *vs* shear rate (/s).

In Vitro Skin Permeation

Skin permeation assay ($n = 5$) was performed in Franz diffusion cell apparatus according to a previous study (9). Full thickness porcine ear skin was used as membrane. Previously from use, the fat tissue and the hair were removed from the outer part of the ear. Receptor fluid consisted in a mixture of phosphate buffer saline pH 7.4 and ethanol (1:1). After 8 h, the skin was cleaned with ultrapure water to remove formulation excess and skin layers were separated. Stratum corneum was separated using the tape-stripping method. Epidermis was separated from dermis using a scalpel. A 1-mL aliquot from the receptor fluid was also collected after 8 h of study.

All samples were placed in headspace vials to perform analysis in gas chromatograph coupled with mass spectrometer (5975C, Agilent Technologies, USA), using a previously validated method (9). Samples were prepared using headspace mode in CombiPAL Autosampler (CTC Analytics AG, Basel, Switzerland) set at 50°C for 10 min.

Animals

Adult male Swiss mice (30–40 g) were provided by Bioterio Central from Universidade Federal de Pelotas. Adult male Wistar rats (100–200 g) were provided by CREAL (Centro de Reprodução e Experimentação de Animais de Laboratório). All animals were maintained under standard conditions (22 ± 1°C at 40–60% relative humidity and 12 h light-dark cycle). All animals had free access to food and water. Mice were sacrificed by cervical dislocation and rats were euthanized by intraperitoneal propofol injection (30 mg/kg). The Animal Use Ethics Committee from Federal University of Rio Grande do Sul approved this study (protocol number: 25866).

Arachidonic Acid-Induced Mouse Ear Edema

Groups of five mice were treated with copaiba oil or hydrogel formulation in the posterior and anterior part of the right ear. The left ear did not receive any treatment, as a control for each animal. After 1 h, the edema was induced by topical application of arachidonic acid (solution in ethanol, 0.2 mg/μL) at 2 mg/ear (10 μL) only in the right ear.

Positive control group received a ketoprofen solution at 4 mg/ear (in acetone solution, 10 μL). Two hundred milligrams

Table II. Nanoemulsions and hydrogels physicochemical characterization

	ZP (mV)	DS (nm)	PDI
NCN	− 17.00 ± 0.96	247.53 ± 1.46	0.051 ± 0.041
NCN-CARB	− 45.77 ± 0.47[a]	267.20 ± 3.86	0.117 ± 0.023
NCN-HEC	− 42.53 ± 0.40[a]	271.63 ± 4.62[b]	0.237 ± 0.036[c]
NCN-CHI	54.87 ± 0.29[a]	524.33 ± 12.75[b]	0.489 ± 0.049[c]
PCN	22.43 ± 2.90	198.83 ± 2.69	0.085 ± 0.039
PCN-CARB	− 51.53 ± 1.52[d]	192.13 ± 4.86	0.087 ± 0.062
PCN-HEC	34.57 ± 1.36[d]	223.67 ± 3.54[e]	0.175 ± 0.015
PCN-CHI	44.83 ± 0.74[d]	412.80 ± 10.59[e]	0.562 ± 0.058[f]

PCN positively charged nanoemulsion, *NCN* negatively charged nanoemulsion, *CARB* Carbopol®, *HEC* hydroxyethylcellulose, *CHI* chitosan, *ZP* zeta potential, *DS* droplet size, *PDI* polydispersity index
[a] ZP different from NCN ($p < 0.05$)
[b] DS different from NCN ($p < 0.05$)
[c] PDI different from NCN ($p < 0.05$)
[d] ZP different from PCN ($p < 0.05$)
[e] DS different from PCN ($p < 0.05$)
[f] PDI different from PCN ($p < 0.05$)

per kilogram copaiba oil concentration was chosen (10) to perform the experiment with the respective hydrogel. Control group received only the vehicle (ethanol) in the right ear. Since the nanoemulsion presents 20% of copaiba oil, the concentration used with the final dosage form treatment was 1000 mg/kg (5-fold the copaiba oil dose). Treatments were applied with automatic semi-solid pipette (100 µL), and arachidonic acid was applied using an automatic pipette (20 µL).

Ear edema was measured after 1 h, using a thickness gauge (Mitutoyo Corporation, Kanagawa, Japan). After the sacrifice, 6 mm^2 fragment of both ears was removed and weighted. Edema was measured by the ear thickness in the groups' right ear. The weight difference between the right and the left ear of each rat in each group was also used as an edema measurement. Edema inhibition percentage (EI%) was calculated comparing only the weight difference of the right and left ear for the groups to the weight difference of the right and left ear of the control group according to Eq. (1)

$$EI(\%) = \left[1-\left(\frac{REt-LEt}{REc-LEc}\right)\right] \times 100 \quad (1)$$

where REt is the weight of the treated right ear, REc is the weight of the control right ear, LEt is the weight of the treated left ear, and LEc is the weight of the control left ear.

Formalin-Induced Rat Paw Edema

Each group ($n = 5$) received the treatment (copaiba oil or hydrogel) in the right hind paw 1 h before the edema induction. Two hundred milligrams per kilogram copaiba oil concentration was chosen to perform the experiment with the respective hydrogel (10). Positive control group received a ketoprofen solution at 4 mg/paw (in acetone). Negative control group did not receive treatment.

Before the edema induction, animals were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg) and xylasine (25 mg/kg) mixture. Formalin solution (100 µL, 10% *v/v* in saline) was injected in the right hind paw, while the left paw received the same amount of vehicle, saline (NaCl 0.9%).

Paw volume was measured after 4 h using a plethysmometer (UgoBasile, Varese, Italy). Edema was measured by paw volume (mL) in the groups' right hind paw. Edema inhibition (EI) was measured by the percentage of edema comparing the volume of the paw in the measurement times for the groups to the volume of the control group (Eq. (2)).

$$EI(\%) = \left[1-\left(\frac{RPt}{RPc}\right)\right] \times 100 \quad (2)$$

where RPt is the volume of the treated right paw, and RPc is the volume of the control right paw.

Histological Analysis

For histological examination, samples of mice ear and rat right rind paw were collected from *in vivo* experiments and stored in a solution of formaldehyde at 37% in PBS pH 7.2. Histological cuts were stained with hematoxylin-eosin and visualized in optical microscope.

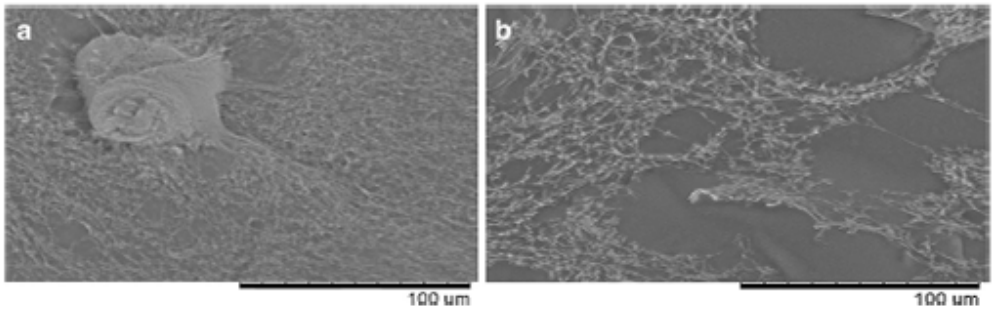

Fig. 1. SEM images for NCN (**a**) and PCN (**b**) HEC hydrogel

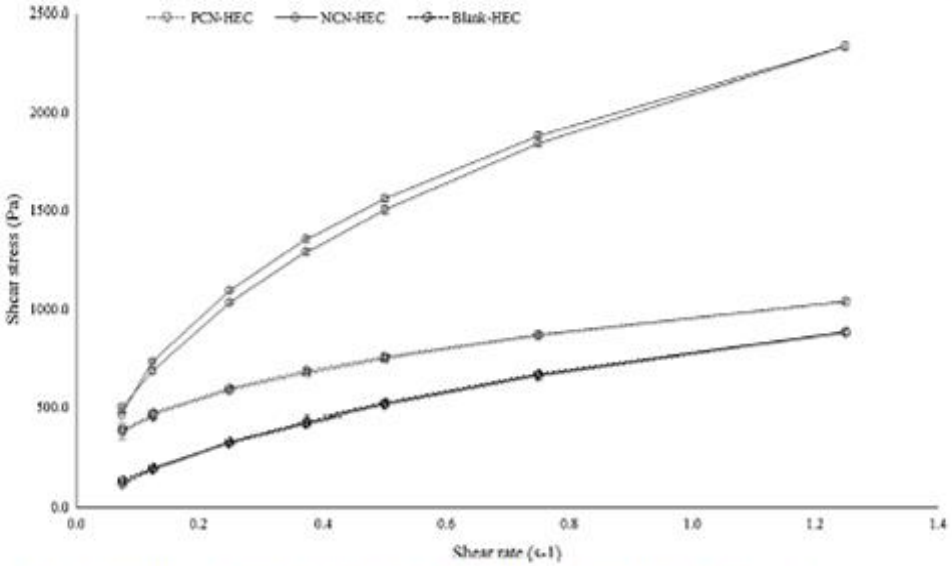

Fig. 2. Rheological profile from copaiba oil nanoemulsions thickened with HEC polymer ($n = 3$)

Statistical Analysis

Statistical difference on skin permeation and *in vivo* assays were calculated by one-way ANOVA followed by Tukey's test. For *in vivo* assays, statistical difference was calculated by one-way ANOVA followed by Holm-Sidak method (rat paw edema) and Tukey's test (mice ear edema). Values with P smaller than 0.05 were considered significant. SigmaSTAT® software was used to analyze the statistics.

RESULTS AND DISCUSSION

Copaiba Oil Characterization

Composition characterization in gas chromatograph coupled with mass spectrometer (GC/MS) demonstrated the presence of 41.2% of β-caryophyllene, representing the major sesquiterpene in the oilresin. Other major sesquiterpenes were α-copaene (7.1%), α-humulene (6.9%), and caryophyllene oxide (1.3%). This composition is normally found in copaiba oils (13). It can be modified depending on time of the year it is collected, presence of rain before the extraction, presence of injury caused by insects or fungi, variation on soil nutrient, and light exposure (14).

Characterization of Copaiba Oil Nanoemulsion and Respective Hydrogels

Three different polymers were tested to increase the nanoemulsions viscosity: CARB (anionic polymer), HEC (non-ionic polymer), and CHI (cationic polymer). All hydrogel formulations presented good zeta potential (ZP), above 130| mV (Table II). When CARB was used as polymer, ZP presented negative values, even with the cationic nanoemulsion and when CHI was used; ZP presented cationic values, even with the anionic nanoemulsion. Since HEC is a non-ionic polymer, ZP in the formulation was given by the nanoemulsion surface charge.

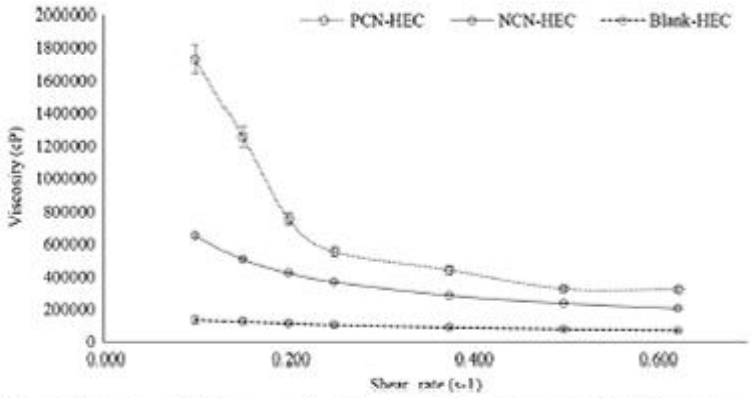

Fig. 3. Viscosity profile from copaiba oil nanoemulsions thickened with HEC polymer ($n = 3$)

Table III. Stability characterization from hydrogels containing positively and negatively charged nanoemulsions in 0, 6, and 12 months

	NCN-HEC			PCN-HEC		
	T0	T6	T12	T0	T6	T12
Zeta potential (mV)	− 45.8 ± 0.80	− 32.7 ± 0.20*	− 25.4 ± 4.10*	22.2 ± 5.20	37.2 ± 3.20*	15.27 ± 4.42
Droplet size (nm)	280.10 ± 4.30	284.53 ± 11.21	280.00 ± 12.77	258.70 ± 5.20	302.97 ± 7.42*	333.47 ± 3.84*
Polydispersity index	0.079 ± 0.023	0.082 ± 0.017	0.149 ± 0.050	0.258 ± 0.010	0.229 ± 0.030	0.317 ± 0.044*
Content (%)	97.10 ± 0.07	101.77 ± 0.07*	82.22 ± 0.06 *	105.38 ± 0.08	105.32 ± 0.08	97.78 ± 0.03*

T0 time zero, *T6* time 6 months, *T12* time 12 months, *NCN-HEC* negatively charged nanoemulsion thickened in hydroxyethylcellulose hydrogel, *PCN-HE* positively charged nanoemulsion thickened in hydroxyethylcellulose hydrogel
*Statistically different from time T0

Even though ZP values were considered good and this parameter can indicate nanoemulsion stability when above 30 mV (in modulus) (15), droplet size (DS) and polydispersity index (PDI) values showed that, when CHI was used as hydrogel, both formulations presented an increase in these parameters. An increase in PDI could imply that the droplets are aggregating and forming a bigger droplet, which could explain the increase in DS.

Souto *et al.* (16) also found that the incorporation of chitosan hydrogel in nanoparticles can destabilize the formulation, leading to an increase in DS and PDI. This can be explained by the presence of acetic acid to form the hydrogel, the interaction between the nanoemulsion surface charge and the polar groups from chitosan and also from the instability around zero charge point when ZP is reversed. Moreover, when hydrogel formulations are compared to the nanoemulsions, there is an increase in ZP and DS, also verified by other authors (16–18), which can be explained by polymer adsorption on nanoemulsion droplet surface.

HEC hydrogel was chosen to continue the studies, since it presented good characterization parameters for both nanoemulsions, due to its neutral character. Figure 1 shows SEM image for NCN-HEC and PCN-HEC, where the polymeric network organization can be seen for both formulations.

Hydrogel-Based Nanoemulsions Rheological Profile

Figure 2 demonstrates the rheological profile comparing NCN-HEC, PCN-HEC, and Blank-HEC hydrogels. As can be seen in the rheogram, HEC hydrogels (2%) containing or not copaiba oil nanoemulsions presented non-Newtonian flow, since the relation between shear stress and shear rate is not linear (19). Among non-Newtonian fluids, there are three behaviors that can occur: plastic, pseudoplastic, or dilatant. According to our results, the hydrogel produced shows pseudoplastic characteristics. In addition, they do not present any thixotropic behavior, as both ascendant and descendant curves are overlapping. Figure 3 shows the viscosity profile from NCN-HEC, PCN-HEC, and Blank-HEC hydrogels. As observed, nanoemulsions were influenced in the viscosity behavior of HEC hydrogel, given that the control hydrogel (Blank-HEC) presented lower viscosity values compared to HEC-loaded nanoemulsions. That can be explained by the nanoemulsions' higher viscosity compared to the water viscosity.

Table IV. Skin permeation results

	PCN-HEC	NCN-HEC
Stratum corneum (μg/cm^2)	0.09 ± 0.07	0.18 ± 0.17
Epidermis (μg/cm^2)	9.76 ± 2.65	7.91 ± 2.46
Dermis (μg/cm^2)	2.43 ± 0.91	1.95 ± 0.56
Receptor fluid (μg/cm^2)	0.67 ± 0.22	1.80 ± 0.85

PCN-HEC positively charged nanoemulsion thickened hydrogel, *NCN-HEC* negatively charged nanoemulsion thickened hydrogel

Long-Term Storage Stability

Regarding the formulations' stability study (Table III), during the 1-year monitoring, values for ZP, DS, PDI, and β-caryophyllene content slowly changed. For both hydrogels, ZP and β-caryophyllene content values decreased and DS and PDI values increased, indicating a probable instability after 12 months. Nevertheless, all values stayed in an acceptable range during the first 6 months. It is worth mentioning that both formulations were kept under 4°C temperature and covered from light, which can explain the smaller loss in content when compared to other studies in the literature (20–22). In addition, there was no phase separation, presence of fungi contamination, or other instability-indicative aspects during the time studied.

In Vitro Skin Permeation

Table IV describes the skin permeation/retention profile for copaiba oil nanoemulsions incorporated in hydroxyethylcellulose hydrogels. Results are shown as β-caryophyllene (the major sesquiterpene in copaiba oil) content in skin layers and receptor fluid. After 8 h, β-caryophyllene was found in the receptor fluid for both formulations, characterizing skin permeation. It was also found in great amount in the epidermis layer, followed by the dermis and the stratum corneum in a smaller amount. In comparison with the nanoemulsion permeation profile reported in a previous study (10), there is a higher permeation

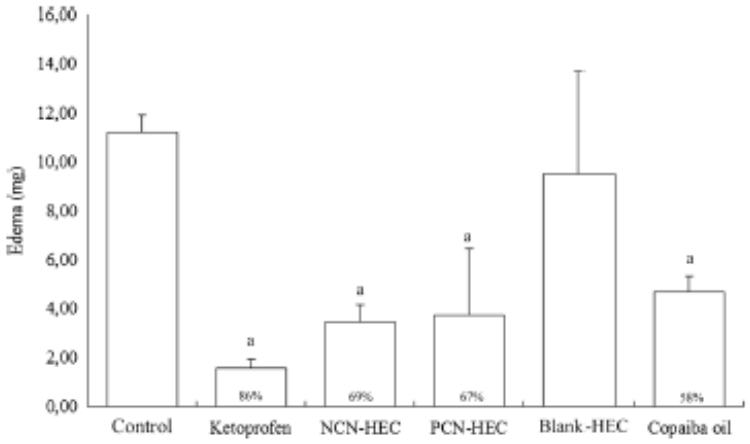

Fig. 4. Arachidonic acid induced-mouse ear edema measured by ear weight (mg). Edema inhibition percentages were placed inside the bars. Superscript letter "a" indicates statistically different from control ($p < 0.05$)

when the hydrogel is used, since for the nanoemulsion there was no β-caryophyllene detected in the receptor fluid.

The higher β-caryophyllene permeation with the hydrogel-loaded nanoemulsion can provide evidence that this formulation is suitable for the purpose of topical application in an anti-inflammatory therapy, indicating that the nanoemulsion was released from the gel matrix and that the hydrogel did not present affinity to it when in contact to the skin. Since the nanoemulsion has small droplet size and high superficial area, it is supposed to penetrate the stratum corneum, permeate through the epidermis (or establish a type of reservoir in this layer), and reach the dermis and the receptor fluid, which mimics the deeper layers in the skin (23,24). In addition, many factors could explain why the addition of a hydrogel to the formulation could improve the nanoemulsions' skin permeation such as occlusion, viscosity, and hydration of the site, which can increase the partitioning of the stratum corneum layer and enable the penetration (25,26).

In Vivo Anti-Inflammatory Activity

Two *in vivo* models demonstrated the topical anti-inflammatory potential effect from copaiba oil nanoemulsion incorporated in hydrogel: mouse ear edema and rat paw edema.

Mouse ear edema was induced by topical administration of arachidonic acid (2 mg/ear) which is involved in the cyclooxygenase (COX) and lipoxygenase (LOX) inflammation pathways, and its topical administration leads to immediate vasodilatation and erythema (27). Figure 4 shows the result 60 min after ear inflammation induction.

As can be seen, ketoprofen, crude copaiba oil, and its nanoemulsions incorporated in hydrogels significantly inhibited the edema when compared to the control ($p < 0.05$). However, when compared to the positive control, ketoprofen, the hydrogels with nanoemulsions (NCN-HEC and PCN-HEC), and copaiba oil were statistically equivalent ($p > 0.05$). Blank hydrogel, as expected, did not present anti-edematogenic effect. Edema inhibition values for ketoprofen,

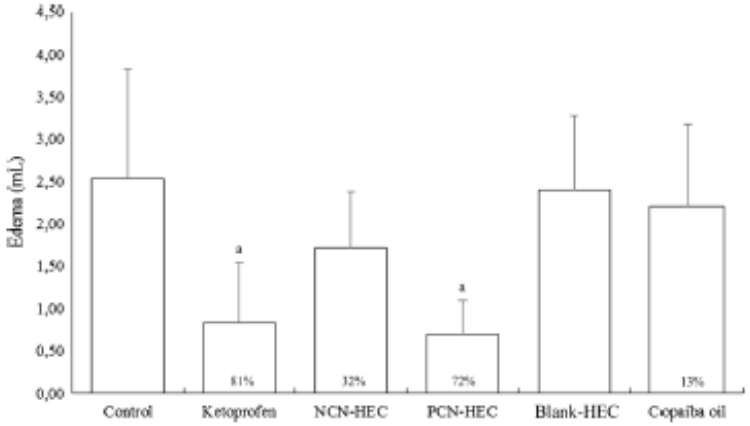

Fig. 5. Rat paw edema induced by formalin 10%. Edema inhibition percentages were placed inside the bars. Superscript letter "a" indicates statistically different from control ($p < 0.05$)

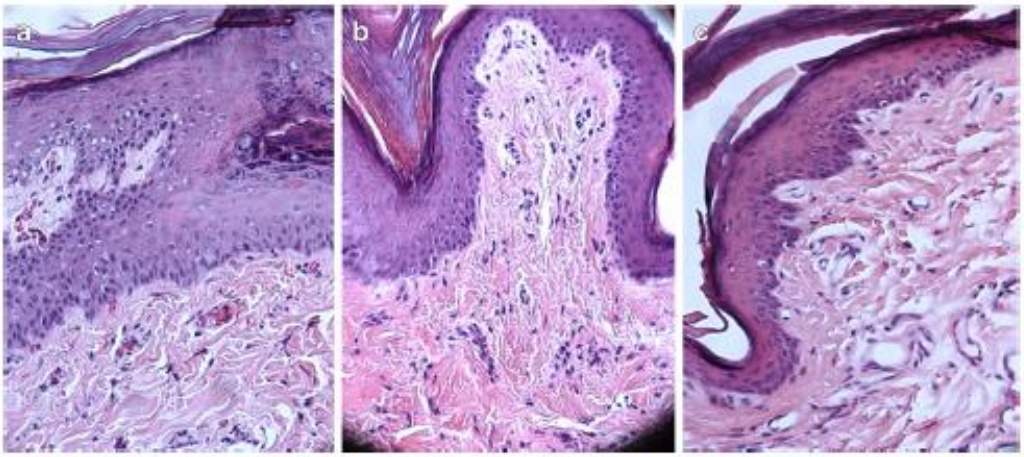

Fig. 6. Photomicrographs from transversal cuts of right rat paws after topical formalin administration, stained with hematoxylin-eosin and examined in optical microscope with × 40 magnification. **a** Control. **b** PCN-HEC. **c** NCN-HEC

NCN-HEC, PCN-HEC, and copaiba oil were 86, 69, 67, and 58%, respectively. Thus, both formulations had an equivalent profile compared to ketoprofen; however, they did not change the effect of the crude oil.

Since the formulations and the oil inhibited the arachidonic acid induced inflammation, copaiba oil could be involved in the inhibition of COX and LOX pathways, like non-steroidal anti-inflammatories.

Rat paw edema was induced by intraplantar administration of formalin (10%). It is well known that formalin causes a biphasic edema response. The first phase (normally up to 5 min after induction) releases substance P and bradykinin. In this phase, it is considered to cause a neuropathic-kind pain. In the second phase, histamine, serotonin, prostaglandins, and bradykinin are involved, producing inflammatory response (28).

Figure 5 presents the results for rat paw edema. Statistically, ketoprofen and PCN-HEC were different to the negative control ($p < 0.05$), indicating their anti-edematogenic activity. Copaiba oil, NCN-HEC, and blank formulation were statistically equal to the control ($p > 0.05$). Edema inhibition values for ketoprofen, NCN-HEC, PCN-HEC, and copaiba oil were 67, 32, 72, and 13%, respectively. In this case, the formulation could improve the effect of the oil, corroborating with the permeation profile and indicating that the positive surface charge has an important role and can enable skin permeation.

It is important to highlight that the oil produced a smaller edema inhibition, which can be correlated to its permeation profile through the skin. In previous studies, we found that the oil stays in the stratum corneum, without any β-caryophyllene retention in the dermis and epidermis, unlike the nanoemulsions containing the oil (9,10).

In both experiments, ketoprofen, a blank formulation and non-treated animals were used as control. Ketoprofen is a non-steroidal anti-inflammatory drug (NSAID) widely used

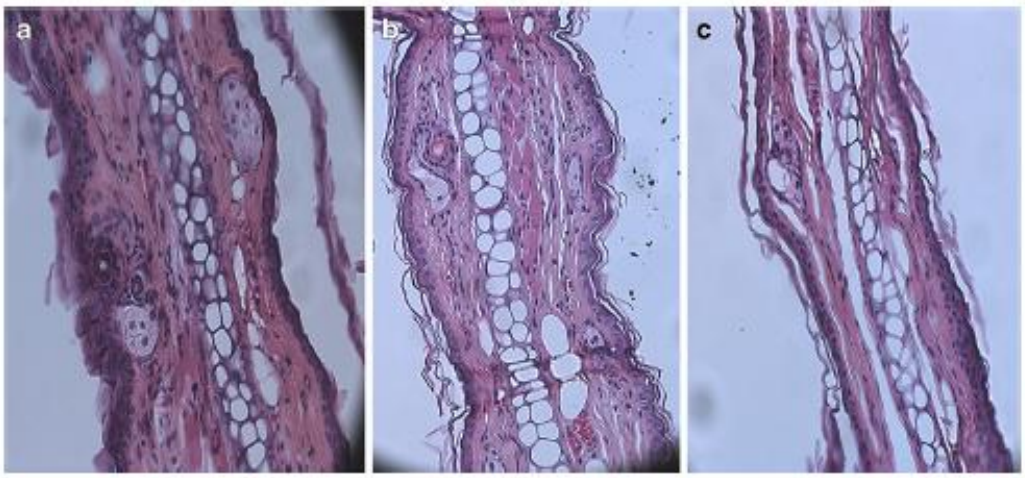

Fig. 7. Photomicrographs from transversal cuts of mice ears after arachidonic acid topical application, stained with hematoxylin-eosin and examined in optical microscope with × 40 magnification. **a** Non-treated control. **b** NCN-HEC. **c** PCN-HEC

to treat rheumatoid arthritis and other inflammatory diseases (29) and was used in this study as a positive control for the anti-inflammatory effect. The dose was 4 mg/paw (paw edema) and 4 mg/ear (ear edema) is normally used in anti-inflammatory assays and was described previously (30,31). In order to evaluate if the hydrogel could perform an anti-inflammatory effect, there was also a hydrogel control (Blank-HEC), which consisted in a formulation containing only the polymer (hydroxyethylcellulose and water).

Concerning histological examination, rat paw edema assay (Fig. 6) showed the presence of epidermis hyperplasia, inflammatory cell infiltration, and vasodilation in the non-treated control. In mice ear edema, histological examination (Fig. 7) showed the presence of dermis and epidermis hyperplasia and inflammatory cell infiltration in non-treated control. Treatments showed a decrease in these factors, demonstrating the anti-inflammatory effect for both models.

CONCLUSIONS

In this paper, we described the incorporation of copaiba oil nanoemulsions (positive and negatively charged) in different hydrogel polymers. The best hydrogel that did not interfere with nanoemulsions' droplet size and polydispersity index was the one formed by hydroxyethylcellulose (HEC), which remained stable for a 12-month stability study and was chosen to perform skin permeation and *in vivo* experiments. Concerning skin permeation, for both formulations, it was possible to detect β-caryophyllene in the most profound skin layer (dermis) and in the receptor fluid, characterizing skin permeation. In mouse ear edema, both formulations presented similar anti-edematologic profile, presenting high edema inhibition and statistically similar to ketoprofen ($p < 0.05$). In rat paw edema, only PCN-HEC formulation presented anti-edematologic effect equal to the positive control, indicating the important hole of the positive charge on β-caryophyllene permeation and edema inhibition. In both *in vivo* edema studies, it was possible to visualize by histological cuts a decrease in epidermis hyperplasia, inflammatory cell infiltration, and vasodilation, demonstrating the anti-inflammatory activity from both treatments.

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COMPLIANCE WITH ETHICAL STANDARDS

The Animal Use Ethics Committee from Federal University of Rio Grande do Sul approved this study (protocol number: 25866).

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