Janaina Kieling Fröhlich

DESENVOLVIMENTO E AVALIAÇÃO *IN VITRO* E *IN VIVO* DE SISTEMAS DE LIBERAÇÃO NANOEMULSIONADOS CONTENDO A FRAÇÃO DICLOROMETANO DE *Jatropha isabellei* MÜLL. ARG. PARA O TRATAMENTO DA ARTRITE

Tese de doutorado apresentada ao Programa de Pós-Graduação em Farmácia da Universidade Federal de Santa Catarina como requisito parcial à obtenção do grau de doutora em Farmácia.

Orientadora: Prof[a]. Dr[a]. Elenara Lemos Senna.

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"Desenvolvimento e avaliação in vitro e in vivo de sistemas de liberação nanoemulsionados contendo a fração diclorometano de Jatropha isabellei Müll. Arg. para o tratamento da artrite"

POR

Janaina Kieling Fröhlich

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À minha família. Aos meus pais Paulo e Marta e meu irmão Gabriel, pelo apoio e amor incondicional. Ao meu noivo Guilherme pelo carinho, atenção e por me ajudar a superar as dificuldades encontradas. Amo muito vocês!

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Quem acredita sempre alcança...

RESUMO

As partes subterrâneas de *Jatropha isabellei* Müll. Arg. (Euphorbiaceae) têm sido utilizadas popularmente para o tratamento de diferentes tipos de doenças reumáticas, especialmente a artrite. O extrato hidroalcoólico apresentou atividade anti-inflamatória e antinociceptiva em modelo de artrite gotosa, em ratos e presume-se que estas atividades possam ocorrer devido à presença de terpenos neste extrato. Assim, uma fração diclorometano, que concentra os compostos terpênicos, foi preparada a partir das partes subterrâneas de *J. isabellei* para ser testada em modelo de artrite aguda em ratos. As atividades antinociceptiva e anti-inflamatória da fração diclorometano de *J. isabellei* (FD_{Ji}) foram avaliadas através do tempo de elevação de pata (TEP) e do diâmetro articular (DA) de ratos sensibilizados com a injeção intra-articular de carragenina (300 µg). Quando administrada pela via oral, a FD_{Ji} (100 e 200 mg/kg) exibiu uma redução significativa do TEP. O efeito da dose de 200 mg/kg foi similar ao apresentado pela dexametasona (10 mg/kg) e indometacina (5 mg/kg). A administração intravenosa da FD_{Ji} (10, 25 e 50 mg/kg) também exibiu uma redução significativa do TEP. A FD_{Ji} preveniu parcialmente a formação do edema após os tratamentos pelas vias oral (50, 100 e 200 mg/kg) ou intravenosa (10 mg/kg). A eficácia terapêutica observada após a administração intravenosa indica que os constituintes da FD_{Ji} apresentam uma absorção oral limitada, a qual pode ser explicada, em parte, pela baixa solubilidade dos compostos terpênicos nos fluídos biológicos. Ambas as atividades antinociceptiva e anti-inflamatória foram atribuídas ao diterpeno jatrofona. Este diterpeno foi isolado e sua estrutura elucidada através dos deslocamentos de RMN H^1 e NoE, bem como pelas técnicas de correlação heteronuclear bidimensionais (HSQC e HMBC) e espectroscopia de massas. Um método rápido e simples de cromatografia líquida ultra-rápida de fase reversa, acoplado a detector de arranjo diodos, foi desenvolvido e validado para a quantificação de jatrofona na FD_{Ji}. O método foi considerado linear, específico, preciso e exato. O diterpeno jatrofona foi encontrado na concentração de 89,68 ± 1,56 µg por miligrama de fração, sendo proposto como um marcador químico desta fração. A análise por cromatografia gasosa acoplado a

detector de ionização de chama (CG-FID) evidenciou a presença majoritária dos ácidos graxos aráquico ou eicosanóico (C20:0), linoleico (C18:2), oleico (C18:1), e heneicosanóico (C21:0). Com a finalidade de superar a baixa solubilidade aquosa da FD_{Ji} e visando aumentar sua absorção oral, carreadores lipídicos nanoestruturados (CLN) com e sem a fração diclorometano (CLN_{Ji} e CLN_{branco}) foram desenvolvidos. Os CLN apresentaram formato esférico e uma distribuição monodispersa, com tamanhos médios variando de 180 até 200 nm e valores de potencial zeta em torno de -18 mV. A eficiência de encapsulação foi de 90,4 % para a jatrofona. Os CLN_{Ji} e CLN_{branco} armazenados à 4 °C não apresentaram alteração do tamanho médio de partícula e do potencial zeta durante 120 dias e o conteúdo de jatrofona permaneceu inalterado durante 60 dias. A estabilidade dos CLN_{Ji} e CLN_{branco} armazenados à 4 °C também foi confirmada pelos estudos de estabilidade acelerada utilizando o LUMiSizer e apresentaram valores médios de índice de estabilidade de 0,083 e 0,536, respectivamente. No fluido gástrico que simula o estado de jejum (FaSSGF) a liberação da jatrofona a partir dos CLN_{Ji} alcançou 38,81% em duas horas, enquanto que a fração livre liberou apenas 23,4% no mesmo período. A liberação da jatrofona a partir dos CLN_{Ji} também foi mais rápida do que a resultante da FD_{Ji} livre no fluido intestinal que simula o estado alimentado (FeSSIF-old) após 8 horas, alcançando 104,2% e 61,0%, respectivamente. No fluido intestinal que simula o estado de jejum (FaSSIF-V2) a liberação da jatrofona a partir dos CLN_{Ji} e da FD_{Ji} livre foi similar, apresentando valores médios de 86,6% e 69,3%, respectivamente. Quando testados em modelo de artrite aguda em ratos, o tratamento com os CLN_{Ji} apresentou propriedades antinociceptivas após a administração oral na dose de 50 mg/kg, enquanto que para a FD_{Ji} livre este efeito não foi observado. Estes resultados sugerem um aumento na absorção oral dos constituintes da FD_{Ji} devido a sua incorporação em CLN, melhorando a sua atividade farmacológica. Além disso, neste estudo foram desenvolvidos CLN_{Ji} peguilados, para serem administrados pela via intravenosa, com a finalidade de obter um sistema de liberação de fármacos que libere os constituintes terpênicos nas articulações inflamadas. A peguilação foi obtida com a incorporação do polímero DSPE-PEG_{2000} em diferentes

concentrações (15, 30, 60 e 90 mg). CLN_{branco} apresentaram tamanho médio de partícula entre 174,7 e 185,9 nm, enquanto que os CLN_{Ji} exibiram uma diminuição do tamanho de partícula com a adição do DSPE-PEG_{2000} de 185,2 (sem polímero) para 158,3; 144,6; 148,9 e 147,1 nm (com 15, 30, 60 3 90 mg do polímero, respectivamente). Todos os CLN apresentaram distribuição monodispersa e forma esférica. O potencial zeta dos CLN_{branco} e CLN_{Ji} variou de -24,7 até -53,9 mV e de -25,1 até -48,0 mV, respectivamente, aumentando (em módulo) com o aumento da quantidade do DSPE-PEG_{2000}. A eficiência de encapsulação da jatrofona foi maior que 89,2 % para todas as formulações. O estudo de estabilidade foi realizado usando o equipamento LUMiSizer e demonstrou que o aumento da quantidade de DSPE-PEG_{2000} e a presença da FD_{Ji} tornaram as formulações mais estáveis. O ensaio de hemocompatibilidade dos CLN mostrou que os CLN_{branco} não foram hemolíticos em nenhuma das concentrações testadas enquanto que os CLN_{Ji} causaram a lise das hemácias somente nas concentrações mais altas testadas. Os CLN não apresentaram efeito citotóxico contra macrófagos J774 e as formulações contendo 60 e 90 mg de DSPE-PEG_{2000} exibiram uma significativa redução da captação fagocítica. O perfil de liberação da jatrofona a partir dos CLN_{Ji} contendo 90 mg de DSPE-PEG_{2000} foi similar ao apresentado pelos CLN_{Ji} sem o polímero. As atividades antinociceptiva e antiedematogênica dos CLN_{Ji} foram demonstradas por um modelo de artrite induzida pela carragenina em ratos e os efeitos comparados com o apresentado pela FD_{Ji} livre quando ambos foram administrados pela via intravenosa na dose de 10 mg/kg. Em especial, o efeito antiedematogênico dos CLN_{Ji} contendo 90 mg de DSPE-PEG_{2000} foram melhores do que os apresentados pela fração livre, provavelmente devido a uma modificação no perfil farmacocinético dos constituintes terpênicos da FD_{Ji}.

Palavras-chave: *Jatropha isabellei*, jatrofona, artrite, atividades antinociceptiva e antiedematogênica, UFLC-DAD, ácidos graxos, carreadores lipídicos nanoestruturados, nanopartículas peguiladas, estabilidade, estudo de liberação, ensaio de hemólise, viabilidade celular, captura por macrófagos.

ABSTRACT

The underground parts of *Jatropha isabellei* Müll. Arg. (Euphorbiaceae) have been used popularly to treat different types of rheumatic diseases, especially arthritis. The hidroalcoholic extract showed anti-inflamatory and antinociceptive activities in a rat model of gouty arthritis and this activity is supposed to be due the presence of terpenes in this extract. So, a dichloromethane fraction, which concentrates the terpenic compounds, was prepared from the underground parts of *J. isabellei* (DF_{Ji}) to be tested in an acute arthritis model in rats. The anti-inflammatory and antinociceptive activities of the *J. isabellei* dichloromethane fraction (DF_{Ji}) were evaluated through assessment of paw elevation time (PET) and articular diameter (AD) of rats stimulated with an intra-articular injection of carrageenan (300 µg). Given by oral route DF_{Ji} (100 and 200 mg/kg) produced significant reduction of PET. The effect produced by 200 mg/kg dose was similar to dexamethasone (10 mg/kg) and indomethacin (5 mg/kg). DF_{Ji} (10, 25 and 50 mg/kg) administered intravenously also produced a significant reduction of PET. DF_{Ji} partially prevented the edema formation either after oral (50, 100 and 200 mg/kg) or intravenous (10 mg/kg) treatments. The higher therapeutic effectiveness observed after intravenous administration indicated that the DF_{Ji} constituents display limited oral absorption, which may be explained, in part, by the low solubility of the terpenic compounds in the biological fluids. Both antinociceptive and anti-inflammatory activities were discussed to be due to the diterpene jatrophone. This diterpene was isolated and its structure was elucidated by ^{1}H NMR shifts and NoE as well as 2D long-range heteronuclear correlations (HSQC and HMBC) and mass spectroscopy. A fast and simple reverse phase UFLC-DAD method was developed and validated for the determination of jatrophone in the DF_{Ji} The method was considered linear, specific, precise, and accurate. The diterpene jatrophone was found at a concentration of 89.68 ± 1.56 µg per milligram of fraction being proposed as a chemical marker of this one. The analysis of gas-chromatography coupled with flame ionization detector (GC-FID) of the DF_{Ji} evidenced the presence of arachidic acid (C20:0), linoleic acid (C18:2), oleic acid (C18:1), and heneicosanoic acid (C21:0), as major fatty acids. In order to

overcome the poor water solubility of the DF_{Ji} and considering increase its absorption by oral route, unloaded and loaded DF_{Ji}-lipid nanocarriers were developed (LNC_{Ji} and LNC_{blank}).The developed lipid nanocarriers displayed spherical shape and monodisperse size distribution, with mean sizes ranging from 180 to 200 nm and zeta potential values of around –18 mV. The entrapment efficiency was 90.4% for jatrophone. LNC_{Ji} and LNC_{blank} stored at 4 °C did not exhibit mean size and zeta potential changes during 120 days and the jatrophone content of LNC_{Ji} was unchanged during 60 days. The stability of the LNC_{Ji} and LNC_{blank} stored at 4°C was also confirmed by the accelerated stability studies using LUMiSizer and presented mean values of 0.083 and 0.536 of instability index, respectively. In fasted state simulate gastric fluid (FaSSGF), the release of jatrophone from LNC_{Ji} reached 38.81% in two hours, while the free fraction released only 23.4% in the same period. The release of jatrophone from LNC_{Ji} was also faster than that obtained for the free DF_{Ji} in fed state simulate intestinal fluid (FeSSIF-old) after 8 hours, reaching 104.2% and 61.0%, respectively. In the fasted state simulate intestinal fluid (FaSSIF-V2) the release of jatrophone from LNC_{Ji} and the free DF_{Ji} was similar exhibiting mean values of 86.6% and 69.3%, respectively. When tested in an acute arthritis model the $LNC_{Ji,}$ but not the free fraction, exhibited antinociceptive properties after the oral administration at dose of 50 mg/kg. These results suggested that an increase in the oral absorption of the DF_{Ji} constituents occurred by carrying it in the LNC, improving its pharmacological activity. In this study, DF_{Ji}-loaded pegylated lipid nanocarriers intended for intravenous administration were developed aiming to obtain drug delivery systems that could release the terpenic constituents preferentially into the inflamed joints. The pegylation was obtained with the addition of the polymer DSPE-PEG_{2000} at different concentrations (15, 30, 60 e 90 mg). LNC_{blank} presented a mean particle size between 174.7 e 185.9 nm, while the LNC_{Ji} exhibited a decrease in the mean particle size with the DSPE-PEG_{2000} addition from 185.2 (without polymer) to 158.3; 144.6; 148.9 and 147.1 nm (with 15, 30, 60 and 90 mg of polymer, respectively). All the LNC presented a monodisperse distribution and spherical shape. The zeta potential of LNC_{blank} and LNC_{Ji} changed from -24.7 to -53.9 mV and from -25.1 to -48.0 mV respectively, increasing (in

module) with the amount of DSPE-PEG_{2000}. The entrapment efficiency was higher than 89.2 % for all the developed formulations. The stability study was performed using the LUMiSizer equipment and revealed that the increase in DSPE-PEG_{2000} amount and the presence of the $DF_{Ji,}$ making the formulations more stable. The hemocompatibility assay showed that the LNC_{blank} were not hemolytic at any tested concentrations, while the LNC_{Ji} provoked the lysis only at higher tested concentrations. The LNC did not present cytotoxic effects against J774 macrophages and the formulations containing 60 and 90 mg of DSPE-PEG_{2000} exhibited a significant reduction of the phagocytic uptake. The jatrophone released profile from LNC_{Ji} containing 90 mg of DSPE-PEG_{2000} was similar to that presented by LNC_{Ji} without the polymer. The antinociceptive and antiedematogenic effects from LNC_{Ji} were demonstrated in a carrageenan-induced acute arthritis model in rats with the effects compared to that presented by free DF_{Ji} when both were administered intravenously at dose of 10 mg/kg. In special, the antiedematogenic effect of LNC_{Ji} containing 90 mg of DSPE-PEG_{2000} was better than the free fraction, probably due to a modification in the pharmacokinetic profile of terpenic constituents of DF_{Ji}.

Key-words: *Jatropha isabellei*, jatrophone, arthritis, antinociceptive and antiedematogenic effects, UFLC-DAD, fatty acids, lipid nanocarriers, pegylated nanoparticles, stability study, drug release study, hemolysis assay, cell viability, macrophage uptake.

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CAPÍTULO 3

Manuscrito 3: Preparation, characterization and in vivo evaluation of the antinociceptive and anti-inflammatory activities of intravenously-administered PEG-coated lipid nanoparticles containing terpenic constituents of *Jatropha isabellei*

LISTA DE ABREVIATURAS E SIGLAS

AAA – Acetyl Aleuritolic Acid / Ácido acetil aleuritólico
AD / DA – Articular Diameter / Diâmetro articular
ANOVA – Análise de Variância
DE – Dissolution Efficiency / Eficiência de Dissolução
DF_{Ji} / FD_{Ji} – Dichloromethane Fraction from *Jatropha isabellei* / Fração Diclorometano de *Jatropha isabellei*
DMSO – Dimethyl Sulphoxide/ Dimetilsulfóxido
$DSPE\text{-}PEG_{2000}$ - Distearoylphosphatidylethanolamine – polyethylene glycol / Diesteroil fosfatidil etanolamina - polietilenoglicol
EE – Entrapment Efficiency / Eficiência de Encapsulação
EPR - Enhanced Permeability and Retention / Melhora da permeabilidade e retenção
FaSSGF – Fasted State Simulate Gastric Fluid / Fluído gástrico que simula o estado de jejum
FaSSIF – Fasted State Simulate Intestinal Fluid / Fluído intestinal que simula o estado de jejum
FeSSIF – Fed State Simulate Intestinal Fluid / Fluído intestinal que simula o estado alimentado
GC-FID / CG-FID – Gas-chromatography coupled with Flame Ionization Detector / Cromatografia gasosa acoplada à detector de ionização em chamas
HR – Hemolysis Rate / Taxa de Hemólise
ICH – International Conference of Harmonization / Conferência Internacional de Harmonização
LNC – Lipid nanocarriers / Carreadores lipídicos nanoestruturados ou Nanocarreadores lipídicos
LNC_{blank} / CLN_{branco} – Lipid nanocarriers without dichloromethane fraction from *J. isabellei* / Carreadores lipídicos nanoestruturados sem a fração diclorometano de *J. isabellei*
LNC_{Ji} / CLN_{Ji} – Lipid nanocarriers containing dichloromethane fraction from *J.isabellei* / Carreadores lipídicos nanoestruturados contendo a fração diclorometano de *J. isabellei*
LOD – Detection Limit / Limite de Detecção
LOQ – Quantification Limit / Limite de Quantificação

NMR / RMN – Nuclear Magnetic Ressonance / Ressonância Magnética Nuclear
HMBC – Heteronuclear Multiple Bond Correlation / Correlação Heteronuclear de Ligações Múltiplas
HSQC - Heteronuclear Single Quantum Correlation / Correlação Heteronuclear de Quantum Único
NoE – Nuclear Overhauser Effect / Efeito Nuclear Overhauser
PBS – Phosphate Buffer Saline / Tampão fosfato salino
PDI – Polydispersity Index / Índice de polidispersão
PEG – Polyethylene Glycol / Polietilenoglicol
PET / TEP – Paw Elevation Time / Tempo de Elevação de Pata
RES – Reticuloendothelial System / Sistema Reticuloendotelial
RSD – Relative Standard Deviation / Desvio Padrão Relativo
s.d. – Standard Deviation / Desvio Padrão
S.E.M – System Error of Mean / Erro Sistemático da Média
TGI – Trato Gastrointestinal
UFLC-DAD – Ultra Fast Liquid Chromatography with Diode Array Detector / Cromatografia líquida ultra-rápida com detector de arranjo diodos.

SUMÁRIO

INTRODUÇÃO

As doenças reumáticas compreendem um conjunto de diferentes doenças que afetam as articulações, os tendões, os ligamentos, os ossos e os músculos. Dentre estas doenças estão incluídas as artrites, que afetam uma ou mais articulações e são caracterizadas por dor, inchaço, rigidez articular e limitação dos movimentos, estando entre as maiores causas de incapacidade para o trabalho e para as atividades cotidianas (EBERHARDT et al., 2007; ALLAIRE et al., 2008; SOKKA et al., 2010; CDC, 2015). De acordo com a Organização Mundial de Saúde (OMS), as doenças reumáticas representam cerca de metade da prevalência das doenças crônicas em pessoas com idade superior a 50 anos. Com o envelhecimento da população, é previsível um aumento significativo na sua prevalência nos próximos anos, o que certamente causará impacto econômico nos sistemas de saúde do mundo todo (LAIRES; GOUVEIA; BRANCO, 2012).

As formas mais comuns de artrite incluem a artrite reumática e a osteoartrite. Ainda de grande importância é a artrite gotosa, conhecida por ser a forma mais dolorosa. Os medicamentos utilizados para o tratamento das artrites têm a finalidade de aliviar a dor, o desconforto do inchaço e das complicações associadas à doença. Entretanto, os pronunciados efeitos colaterais e a intolerância aos fármacos comumente utilizados dificultam a adesão dos pacientes ao tratamento e muitas vezes o risco-benefício precisa ser avaliado (CICONELLI, 2005). Assim, a busca por novos fármacos que permitam o tratamento efetivo da doença com a promessa de melhores resultados é persistente e novas alternativas estão sendo estudadas, como as plantas que possuem seu uso popular recomendado para artrite.

A fitoterapia é uma terapêutica que se caracteriza pelo tratamento de doenças através do uso de plantas medicinais em suas diferentes preparações, sem a utilização de substâncias ativas isoladas (ARAÚJO et al., 2007). O ser humano utiliza as espécies vegetais para aliviar ou tratar suas enfermidades desde a antiguidade, sendo que população traz consigo conhecimentos ancestrais sobre o uso de plantas medicinais (ELDIN; DUNFORD, 2001), as quais são amplamente utilizadas em práticas tradicionais como medicamentos caseiros, processo conhecido como medicina popular.

No Brasil, a rica diversidade cultural e étnica aliada a grande biodiversidade vegetal (em torno de 15-20% do total mundial) resultou em um acúmulo considerável de conhecimentos populares sobre manejo e uso de plantas medicinais (BRASIL, 2006). A medicina tradicional e o uso popular descrito para as plantas têm grande relevância para a pesquisa científica, que tem como finalidade comprovar a ação farmacológica, a identidade botânica, os compostos ativos, bem como avaliar a ausência de toxicidade, dados estes que podem levar ao desenvolvimento de um novo medicamento fitoterápico. Dentro do contexto das regulamentações do registro de medicamentos no Brasil, o registro de produtos fitoterápicos foi normatizado em 1995, e em 1996 a 10ª Conferência Nacional de Saúde recomendou a incorporação, no SUS, das práticas de saúde como fitoterapia, acupuntura e homeopatia, contemplando as terapias alternativas e práticas populares. Todavia, a inserção das plantas no SUS foi concretizada somente em 2006 através da aprovação da Política Nacional de Plantas Medicinais e Fitoterápicos pelo decreto 5.813 de 22 de junho de 2006 (BRASIL, 2006).

Uma pesquisa realizada nos Estados Unidos mostrou que aproximadamente 90 % dos pacientes que possuem algum tipo de artrite fazem uso de terapias alternativas no tratamento da doença, como o uso dos medicamentos a base de plantas. As razões citadas para a escolha desta opção incluem a falta de eficácia dos medicamentos convencionais, os efeitos adversos e o fácil acesso aos fitoterápicos (ERNST, 2011). Este panorama também tem sido mostrado no Brasil em diversos estudos que relatam o uso de uma variedade de plantas oriundas da fauna brasileira para o tratamento de doenças reumáticas (ROSA; MACHADO, 2007).

A espécie *Jatropha isabellei* é conhecida na medicina popular Paraguaia como “yagua rova” e a infusão ou decocção das partes subterrâneas são utilizados para tratar doenças reumáticas, como as artrites, em especial a artrite gotosa (BASUALDO; ZARDINI; ORTIZ, 1991; RIVEROS et al., 2009). No estado do Rio Grande do Sul, a planta é conhecida como “turubiti” ou “mamoneiro-do-campo” e é utilizada popularmente para dores nas costas (FRÖHLICH et al., 2013). De fato, um estudo utilizando o extrato bruto obtido a partir das partes

subterrâneas de *J. isabellei* evidenciou efeitos antinociceptivos e anti-edematogênicos moderados em modelo de artrite gotosa induzida pela injeção intra-articular de cristais de urato em ratos, após administração oral (SILVA et al., 2013). Entre os compostos identificados nesta planta estão o triterpeno ácido acetilaleuritólico, o sesquiterpeno ácido ciperenoico e os diterpenos jatrofona, jatrofolona A e B, e 9β,13α-dihidroxi-isabelliona, os quais foram obtidos a partir das soluções extrativas em éter de petróleo e acetato de etila dos rizomas da mesma (PERTINO et al., 2007a). Ainda, a fração diclorometano obtida a partir do extrato hidroetanólico apresentou os compostos ácido acetilaleuritólico e uma mistura binária de sitosterol glicosilado e estigmasterol (FRÖHLICH et al., 2013).

Considerando que compostos terpênicos são reconhecidos por apresentar atividade anti-inflamatória (SULTANA; SAIFY, 2012), a utilização da fração diclorometano das partes subterrâneas de *J. isabellei* parece ser promissora para o tratamento da artrite. Entretanto, esta fração caracteriza-se por apresentar compostos essencialmente lipofílicos, sendo esperado que tais compostos apresentem limitada solubilidade nos fluidos biológicos, o que pode levar a uma reduzida biodisponibilidade, quando administrados oralmente. Neste caso, a incorporação da fração em carreadores lipídicos permite a sua veiculação na forma de dispersões aquosas nanométricas, possibilitando uma melhora da absorção dos compostos lipofílicos. Além disso, a incorporação da fração diclorometano em nanocarreadores lipídicos torna viável a sua administração pela via intravenosa e sua eficácia terapêutica pode ser beneficiada pelo efeito de permeação e retenção aumentados dos tecidos inflamados (efeito EPR), com o consequente aumento da concentração do fármaco nestes tecidos.

Com o passar dos anos, grandes avanços tem ocorrido no desenvolvimento de novos sistemas de liberação de ativos de origem vegetal. Uma grande variedade de formulações, como nanopartículas poliméricas, nanocápsulas, lipossomas, fitossomas, nanoemulsões e microesferas, têm sido descritas utilizando compostos bioativos e extratos de plantas. Estas formulações possuem inúmeras vantagens para os compostos fitoterápicos, incluindo o aumento da solubilidade e biodisponibilidade, redução da toxicidade, melhora do efeito

farmacológico e da estabilidade física e química, liberação sustentada e proteção contra degradação química e física, quando comparado com os derivados vegetais em preparações simplificadas (SARAF, 2010). Entre estes sistemas destacam-se as nanoemulsões, as quais são sistemas emulsionados, transparentes ou translúcidos, cineticamente estáveis, apresentando diâmetro médio entre 50 e 200 nm. Tais sistemas são capazes de incorporar grandes quantidades de fármacos hidrofóbicos e, devido ao seu elevado grau de dispersão, se depositam uniformemente nos tecidos e mucosas oferecendo uma elevada superfície de contato para absorção, melhorando desta forma a sua biodisponibilidade (BOUCHEMAL et al., 2004; ALMEIDA; TEIXEIRA; KOESTER, 2008).

Assim, considerando a potencial aplicação de *J. isabellei* para o tratamento das doenças articulares, este trabalho tem como objetivo desenvolver nanoemulsões contendo a fração diclorometano das partes subterrâneas da planta, e avaliar e comparar o efeito farmacológico das formulações após administração pela via oral e intravenosa.

Objetivos

Objetivo geral

Desenvolver sistemas nanoemulsionados contendo a fração diclorometano de *Jatropha isabellei* visando o tratamento da artrite.

Objetivos específicos

1. Preparar o extrato hidroetanólico das partes subterrâneas de *Jatropha isabellei* e obter a fração diclorometano.
2. Caracterizar biologicamente a fração diclorometano de *J. isabellei* quanto a sua atividade anti-inflamatória e antinociceptiva, em modelo animal de artrite aguda induzida por carragenina, quando administrada pelas vias oral e intravenosa.
3. Isolar um constituinte da fração diclorometano de *J. isabellei* para ser utilizado como o marcador químico da fração e caracterizá-lo por técnicas espectroscópicas.
4. Desenvolver e validar metodologia analítica por CLAE para caracterizar quimicamente a fração diclorometano de *J. isabellei*.
5. Caracterizar a fração diclorometano de *J. isabellei* quanto aos seus constituintes graxos por CG-FID.
6. Preparar carreadores lipídicos nanoestruturados contendo a fração diclorometano de *J. isabellei* para administração oral e intravenosa utilizando a técnica de emulsificação espontânea e caracterizar as formulações quanto ao teor, eficiência de encapsulação, tamanho, índice de polidispersão, potencial zeta, morfologia e estabilidade.
7. Avaliar a ação hemolítica dos carreadores lipídicos nanoestruturados desenvolvidos para a administração intravenosa, bem como, sua captura por macrófagos J774 e a viabilidade celular destas formulações.
8. Avaliar o perfil de liberação do marcador químico da fração diclorometano a partir dos carreadores lipídicos nanoestruturados.

9. Avaliar a atividade anti-inflamatória e antinociceptiva das formulações contendo a fração diclorometano de *J. isabellei* em modelo experimental de artrite quando administrada pelas vias oral e intravenosa.

REVISÃO DA LITERATURA

1 DOENÇAS REUMÁTICAS: ARTRITE

As doenças reumáticas estão entre as doenças mais antigas reconhecidas, entretanto, sua classificação é difícil pela ausência de evidências etiológicas para a maioria das doenças e devido à heterogeneidade nas apresentações clínicas. Tipicamente, são caracterizadas por dor, inchaço e rigidez articular, podendo acometer uma ou mais articulações. O termo reumatismo é utilizado para designar um grupo de doenças que afetam as articulações, tendões, ligamentos, ossos e músculos e não tem um limite claro, pois mais de 100 diferentes condições estão classificadas como doenças reumáticas, incluindo as doenças autoimunes como lúpus eritematoso e escleroderma, osteoporose, dores nas costas, fibromialgia, tendinite e as artrites (SANGHA, 2000; CDC, 2015).

A artrite é uma doença incapacitante oriunda da inflamação de uma ou mais articulações, podendo levar à infiltração de células inflamatórias, à hiperplasia sinovial em decorrência da proliferação dos sinoviócitos, ao edema articular, à destruição da cartilagem e à erosão óssea, com nova formação e estreitamento do espaço articular (BENDELE et al., 1999). Com base nos dados do National Health Interview Survey (NHIS), no período de 2010-2012, 52,5 milhões (22,7%) de adultos com idade de 18 anos ou mais tiveram o diagnóstico médico de artrite, enquanto que 22,7 milhões (9,8% ou 43,2% dos que têm artrite) relataram limitações das atividades devido à artrite, nos Estados Unidos. A alta prevalência da artrite foi observada entre adultos com mais de 65 anos (49,7%), com doenças cardíacas (49,0%) e diabetes (47,3%). A prevalência da artrite também foi maior entre homens do que mulheres, entre brancos e negros do que hispânicos e asiáticos, entre aqueles com menos escolaridade, entre os obesos e com sobrepeso e entre os que não seguem as recomendações de atividades físicas (CDC, 2013). No Brasil, cerca de 31% da população sofre algum tipo de doença crônica, sendo que as doenças reumáticas ocupam o terceiro lugar, com cerca de 6% da população, atrás apenas da hipertensão e asma. Conforme a Pesquisa Nacional de Saúde, realizada pelo Instituto Brasileiro de Geografia e Estatística (IBGE), no ano de 2013 foram diagnosticados 9.382 novos casos

de artrite ou reumatismo no país, dos quais 6.986 são mulheres e 2.396 são homens (IBGE, 2013). As formas mais comuns de artrite são a artrite reumática e a osteoartrite. Outro tipo de artrite de grande importância é a artrite gotosa, conhecida por ser uma das mais dolorosas (SANTOS, 2016).

1.1 ARTRITE REUMATOIDE

A artrite reumatoide é uma doença autoimune inflamatória, crônica e incapacitante que afeta as articulações do corpo revestidas pela membrana sinovial, a qual é responsável pela nutrição e lubrificação da cartilagem e dos ossos articulares. A artrite reumatoide caracteriza-se pela dor, vermelhidão, rigidez articular e edema decorrente do processo inflamatório crônico da membrana sinovial (sinovite), que leva à destruição da articulação devido à erosão da cartilagem e do osso, causando deformidades. Geralmente apresenta-se simétrica, ou seja, afeta múltiplas articulações periféricas em ambos os lados do corpo. Manifestações clínicas associadas à artrite reumatoide podem incluir nódulos subcutâneos, vasculite, pericardite, nódulos pulmonares, fibrose intestinal, mononeurite múltipla, episclerite e esclerite. Nos exames laboratoriais, a maioria dos pacientes com artrite reumatoide apresenta níveis séricos elevados do autoanticorpo fator reumatoide e peptídeo citrulinado (SANGHA, 2000; ASQUITH et al., 2009; SCOTT; WOLFE; HUIZINGA, 2010).

A etiologia da artrite reumatoide é desconhecida, mas acredita-se que muitos fatores genéticos e ambientais (especialmente o fumo) predispõem à doença, levando a uma perturbação do sistema imune com subsequente inflamação crônica (SCOTT; WOLFE; HUIZINGA, 2010). Além do aparecimento das dores devido à inflamação, a incapacidade de trabalhar é um problema frequente para os pacientes que desenvolvem a artrite reumatoide. Em torno de 20-30 % pacientes se tornam permanentemente incapacitados a trabalhar durante os primeiros 2-3 anos da doença. A incapacidade de trabalhar está associada principalmente a parâmetros como a demanda física do paciente (movimento das articulações) e a idade, sendo que geralmente o paciente não volta ao trabalho (SOKKA, 2003).

1.2 OSTEOARTRITE

A osteoartrite é uma doença crônica e degenerativa das articulações que afeta a cartilagem articular e o osso subcondral. Caracteriza-se pela perda progressiva da cartilagem com a formação de osteófitos marginais e o aumento da espessura do envelope ósseo (esclerose óssea). Os tecidos moles e as estruturas em torno da articulação também são afetados. Estas estruturas incluem o líquido sinovial, que pode apresentar infiltrados inflamatórios, os ligamentos que se tornam frouxos e os músculos que podem ficar enfraquecidos (FELSON et al., 2000). A dor, a rigidez articular, o edema, as limitações funcionais e a perda da qualidade de vida são os primeiros sintomas associadas à osteoartrite (SANGHA, 2000). Em sua patologia podem ser verificadas alterações radiológicas, como a presença de osteófitos, o estreitamento do espaço articular e a esclerose óssea (CDC, 2015).

Alguns fatores de risco para o desenvolvimento da osteoartrite incluem a idade, ser do sexo feminino, a presença de lesões nas articulações, obesidade, fraqueza muscular, estresse mecânico excessivo, deficiência de estrogênio, alta densidade óssea, deficiência de vitamina D e os fatores genéticos (FELSON et al., 2000; ARDEN; NEVITT, 2006; BUSIJA et al., 2010). A osteoartrite é a segunda mais frequente causa de dor, perda de função e incapacidade de trabalhar de adultos americanos com mais de 50 anos de idade e contabiliza mais internações hospitalares do que a artrite reumatoide (ARDEN; NEVITT, 2006)

1.3 ARTRITE GOTOSA

A artrite gotosa é caracterizada por uma reação inflamatória intensa que é desencadeada pela precipitação de cristais de urato monossódico dentro das articulações (BIEBER; TERKELTAUB, 2004; RICHETTE; BARDIN, 2010). Evidências clínicas sugerem que a artrite gotosa esteja fortemente associada com a síndrome metabólica (que se caracteriza pela obesidade, resistência à insulina, hipertensão e dislipidemias). Outros fatores de risco para o desenvolvimento de gota incluem: ser do sexo masculino, ser da raça negra, dietas alimentares ricas em proteínas (carne e frutos do mar), insuficiência renal,

envelhecimento, uso de alguns medicamentos (principalmente diuréticos), consumo de álcool e hiperuricemia prolongada (BIEBER; TERKELTAUB, 2004; CHOI, 2005; CHOI, 2006; SAAG; CHOI, 2006).

A artrite gotosa aguda inicia frequentemente com uma articulação afetada nos membros inferiores (85-90% dos casos), maior dedo do pé (50% dos casos), pés, tornozelos e joelhos. O primeiro ataque raramente é poliarticular (3-14% dos casos) e não costuma afetar quadris e ombros, podendo ser autolimitado em um período de 7-10 dias. O início da crise geralmente é abrupto deixando a articulação eritematosa, quente, inchada e sensível. A dor é intensa e comumente afeta as atividades diárias. Os ataques subsequentes frequentemente duram mais que os primeiros e afetam várias articulações, se propagando pelos membros superiores, como punhos, braços, cotovelos e mãos. Nos idosos a artrite gotosa pode coexistir com a osteoartrite e os ataques poliarticulares são comuns (RICHETTE; BARDIN, 2010; GRASSI; DE ANGELIS, 2012).

A gota aguda, quando não tratada, pode se tornar crônica, a qual é caracterizada pela destruição poliarticular crônica com baixo grau de inflamação articular, deformidade, danos permanentes à articulação e desenvolvimento de tofos. Os tofos são depósitos de cristais de urato que ocorrem nos tecidos subcutâneos, sendo o cotovelo, a região aquiliana, mãos e pavilhão auricular os locais mais frequentes. Costumam aparecer alguns anos após às crises recorrentes de gota, sendo que em 12% dos pacientes os tofos aparecem em cinco anos e em 55%, após vinte anos de doença não tratada. Seu desenvolvimento está associado aos níveis séricos e à duração da hiperuricemia, sendo que a sua formação progride silenciosamente, podendo ser muito mais rápida em pacientes tratados com diuréticos ou com doenças renais (EMMERSON, 1996; RICHETTE; BARDIN, 2010; GRASSI; DE ANGELIS, 2012). Outras manifestações da artrite gotosa incluem paniculite gotosa, nefropatias e doenças cardiovasculares. Além disso, o estado físico dos pacientes se torna prejudicado pela impossibilidade de realizar esportes e atividades físicas devido às comorbidades relacionadas à doença (GRASSI; DE ANGELIS, 2012).

1.4 DIAGNÓSTICO E TRATAMENTO

O diagnóstico da artrite geralmente é feito através do histórico do paciente, exames físicos, raio-X e exames de sangue. Somente a artrite gotosa tem um diagnóstico definitivo por meio da identificação dos cristais de urato nas articulações e pela presença de tofos (RICHETTE; BARDIN, 2010; CDC, 2015).

O tratamento das artrites não inflamatórias, como a osteoartrite, consiste na administração de medicamentos analgésicos, assim como no manejo não farmacológico, incluindo a prática de atividades físicas, a perda de peso (em casos de sobrepeso) e o gerenciamento da dor através do autoconhecimento. Para casos de artrites inflamatórias, como a artrite reumática, o tratamento é o mesmo, porém medicamentos anti-inflamatórios também são administrados, como corticosteroides e anti-inflamatórios não esteroidais, assim como fármacos antireumáticos modificadores da doença (metotrexato) e fármacos de origem biotecnológica, como etanercepte e infliximabe (BRATS, 2012; CDC, 2015).

O tratamento da artrite gotosa consiste no alívio da dor do ataque agudo, assim como na prevenção das complicações associadas com a deposição crônica de cristais de urato nos tecidos. Para a prevenção utiliza-se a colchicina, capaz de inibir a infiltração leucocitária para dentro da articulação, fármacos capazes de inibir a síntese do ácido úrico, como o alopurinol, ou que aumentam a sua excreção renal, os denominados agentes uricosúricos, como a probenecida, sulfimpirazona e a benzobromarona (SILVA, 2006). Entretanto, a terapia medicamentosa da gota apresenta efeitos colaterais e muitas vezes os fármacos não são tolerados pelo organismo, dificultando a adesão ao tratamento.

Devido à existência de pacientes que apresentam dificuldade no gerenciamento das artrites, os efeitos colaterais dos medicamentos e os limitados avanços no tratamento desta doença, a busca por novos fármacos que permitam o tratamento efetivo da doença com a promessa de melhores resultados persiste entre os pesquisadores. Novas alternativas terapêuticas estão sendo estudadas, como as plantas medicinais, dentre as quais algumas já são conhecidas pelo seu uso popular para artrite.

2 *Jatropha isabellei* MÜLL. ARG.

Jatropha isabellei Müll. Arg. (sinônimos *Jatropha antisyphilitica; Jatropha grossypiifolia*) (Figura 1) é uma planta pertencente à família Euphorbiaceae, a qual é constituída por 290 gêneros, distribuídos principalmente em regiões tropicais e subtropicais, sendo muito bem representada na flora brasileira (WEBERLING; SCHWANTES, 1986; JOLY, 1998). O gênero *Jatropha* é um dos maiores desta família, contendo de 175 a 200 espécies conhecidas. *Jatropha* deriva da palavra grega *jatrós* que significa médico, e *trophé* que quer dizer alimento. Trata-se de um gênero com plantas do tipo arbusto e árvores resistentes à seca, sendo amplamente distribuídas na América do Sul e Central, África, Índia e sudoeste da Ásia (KUMAR; SHARMA, 2008). Este gênero é reconhecido por possuir um grande número de substâncias bioativas, principalmente diterpenos e triterpenos (ZHANG et al., 2009; DEVAPPA; MAKKAR; BECKER, 2011).

J. isabellei é conhecida na medicina popular Paraguaia como “yagua rova” e a infusão ou decocção das partes subterrâneas é utilizada para tratar doenças reumáticas, como a artrite, sendo recomendada também como digestivo e abortivo (BASUALDO; ZARDINI; ORTIZ, 1991; RIVEROS et al., 2009). No estado do Rio Grande do Sul, a planta é conhecida como “turubiti” ou “mamoneiro-do-campo” e é utilizada popularmente para dores nas costas (FRÖHLICH et al., 2013).

Figura 1. Partes subterrâneas (esquerda) e partes aéreas (direita) de *Jatropha isabellei* Müll Arg. em seu local de coleta.

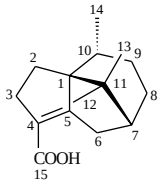
(Fonte: arquivo pessoal)

Alguns estudos têm sido realizados visando identificar os constituintes presentes nas partes subterrâneas de *J. isabellei*. A combinação das frações de éter de petróleo e acetato de etila revelaram a presença do triterpeno ácido acetil aleuritólico, do sesquiterpeno ácido ciperenoico e dos diterpenos jatrofona, jatrofolonas A e B, 9β,13α-dihidroxiisabelliona, além de um monoterpeno 1,4 epoxi -*p*- mentan-2-ol (Figura 2) (PERTINO et al., 2007a).

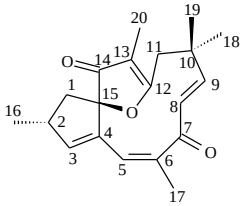
A fração acetato de etila das partes subterrâneas de *J. isabellei* apresentou quantidades significativas de compostos fenólicos, flavonoides e taninos condensados, apresentando atividade antioxidante similar à do ácido ascórbico e inibição significativa da produção de substâncias reativas ao ácido tiobarbitúrico (TBARS) em homogeneizados de cérebro de ratos. A fração diclorometano também inibiu significativamente a produção de TBARS e apresentou atividade antifúngica contra *Sporothrix schenkii* e *Fusarium proliferatum* (concentração inibitória mínima, CIM, 250 µg/mL) e antimicrobiana contra bactérias do gênero *Micrococcus* (CIM 250 µg/mL) no método da microdiluição em caldo. O isolamento dos triterpenos ácido acetil

aleuritólico e de uma mistura binária de sitosterol 3-*O*-β-D-glicosídeo e estigmasterol (Figura 3) foi relatado a partir da fração diclorometano (FRÖHLICH et al., 2013).

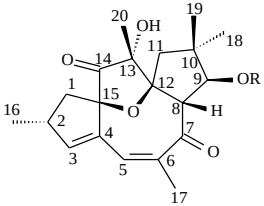
Figura 2. Terpenos isolados de *J. isabellei*. (1) Ácido ciperenoico; (2) Jatrofona; (3) (a) 9β-13α-Dihidroxiisabelliona e (b) 13α-Hidroxi-9β-acetoxiisabelliona, seu derivado acetilado; (4) 1,4 Epoxi -*p*- mentan-2-ol; (5) (a) Jatrofolona A, (b) Jatrofolona B; (6) ácido acetil aleuritólico.



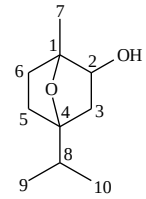
(1)



(2)



(3) R: H (a)
R: Ac (b)



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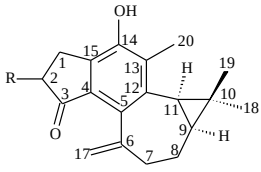
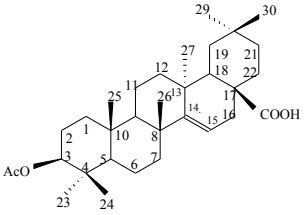


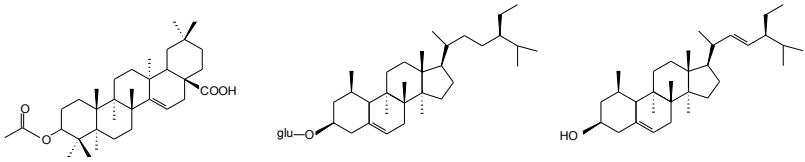
(5) R: βCH_3, αH(a)
R: αCH_3, βH(b)



(6)

(Adaptado de Pertino et al, 2007a)

Figura 3. Terpenos isolados da fração diclorometano das partes subterrâneas de *J. isabellei*: (7) Ácido acetil aleuritólico, (8) Sitosterol 3-*O*-β-D-glicosídeo, (9) Estigmasterol.



(7) (8) (9)

(Fonte: Fröhlich et al, 2013)

A atividade anti-edematogênica e antinociceptiva do extrato hidroetanólico (70% v/v) de *J. isabellei* foi avaliada em modelo de artrite gotosa induzida pela injeção de cristais de urato na articulação. Neste ensaio, uma hora antes da injeção dos cristais de urato monossódico (MSU), os ratos wistar utilizados no experimento foram divididos em quatro grupos e tratados pela via oral com o veículo, a colchicina (30 mg/kg), o extrato hidroetanólico da planta (300 mg/kg) ou com a fração de alcaloides (0,15 mg/kg). Na avaliação da nocicepção, o extrato hidroetanólico de *J. isabellei* (300 mg/kg) foi capaz de reverter completamente a alodínia mecânica e reduzir a hiperalgesia térmica (68 ± 20%) após 2 horas da injeção dos cristais de MSU. A colchicina, utilizada como controle positivo, provocou a redução destes parâmetros em 74 ± 21% e 79 ± 14%, respectivamente. O extrato hidroetanólico de *J. isabellei* também foi capaz de reduzir o edema em 35 ± 8% e 24 ± 3% após 2 e 4 horas, respectivamente, enquanto a colchicina impediu a formação do edema em 44 ± 14% em 2 horas. Com a finalidade de descartar um possível efeito diurético da planta, o qual poderia estar originando um quadro de hipouricemia e originando resultados falsos-positivos, os níveis de ácido úrico, uréia e creatinina foram dosados e verificou-se que, mesmo com a administração do extrato bruto de *J. isabellei* os valores das dosagens bioquímicas mantiveram-se inalterados. Assim,

sugere-se que as atividades anti-inflamatória e antinociceptiva não ocorrem pela inibição da xantina oxidase nem por alterações nos níveis de ácido úrico. Finalmente, o extrato hidroetanólico de *J. isabellei* também foi capaz de inibir a infiltração leucocitária em percentuais comparáveis aos da colchicina. Uma fração purificada de alcaloides (0,15 mg/kg) foi capaz de prevenir a alodínia mecânica (83 ± 12 % de prevenção), a hiperalgesia térmica (43 ± 6 % de prevenção), o edema da articulação (61 ± 11 % de prevenção) e a infiltração de leucócitos polimorfonucleares (46 ± 5 % de prevenção) no tempo de 2 horas após a administração dos cristais, de maneira similar ao extrato hidroetanólico e a colchicina (SILVA et al., 2013).

Os estudos relatados acima evidenciaram que o extrato hidroetanólico de *J. isabellei* apresenta efeitos antinociceptivos e anti-edematogênicos em modelo animal para artrite gotosa sem causar hipouricemia (SILVA et al., 2013). Contudo, sugere-se que os terpenos, concentrados na fração diclorometano e com reconhecida atividade anti-inflamatória, contribuíram de forma majoritária para estes resultados. Assim, o emprego dessa fração rica em terpenos no desenvolvimento de novas formulações com atividade anti-inflamatória parece ser promissor.

3 TERPENOS

Os terpenos são metabólitos secundários de plantas amplamente estudados e que apresentam uma ampla diversidade estrutural e inúmeras atividades biológicas. Apesar desta ampla diversidade estrutural, tais compostos possuem uma origem biossintética comum que se inicia com a síntese da unidade de isopreno (cinco carbonos - isopenteno) a partir de uma molécula de acetil-CoA e outra de acetoacetil-CoA, seguida da adição de grupamentos fosfato pela via do mevalonato, originando o isopentenil difosfato ou isopentenil pirofosfato (comumente designado IPP ou isopreno ativo). O IPP é, portanto, a unidade básica na formação dos terpenos, sendo utilizado em uma sequência de reações para a produção de precursores de diferentes classes de terpenos (Figura 4) (MCGARVEY; CROTEAU, 1995; CROTEAU; KUTCHAN; LEWIS, 2000; DEGENHARDT; KÖLLNER; GERSHENZON, 2009).

A fusão repetitiva de unidades do isopreno (IPP) dá origem a numerosos esqueletos estruturais que se organizam seguindo a regra do isopreno. Assim, os terpenos são classificados segundo o número de unidades de isopreno que contêm. Os terpenos menores apresentam uma única unidade de isopreno e são denominados hemiterpenos (C_5), enquanto os terpenos formados por duas unidades de isopreno são denominados monoterpenos (C_{10}) e são constituintes dos óleos essenciais de ervas e essências de flores. A união de três unidades de isopreno dá origem aos sesquiterpenos (C_{15}), os quais também são constituintes de óleos essenciais e desempenham função de anti-herbivoria nas plantas, como por exemplo, as fitoalexinas. Os diterpenos (C_{20}) são formados por quatro unidades de isopreno e incluem o fitol e o hormônio giberelina, bem como metabólitos importantes farmacologicamente como o taxol, utilizado no tratamento anticâncer. Os triterpenos (C_{30}) são formados pela união de duas cadeias de sesquiterpenos (C_{15}) e formam uma grande classe de moléculas que incluem componentes formadores de membrana e de ceras. A última classe de terpenos é formada pelos tetraterpenos (C_{40}), conhecidos como carotenoides, que dão pigmentos às plantas (MCGARVEY; CROTEAU, 1995;

CROTEAU; KUTCHAN; LEWIS, 2000). Neste trabalho a atenção será voltada para a classe dos di- e triterpenos que estão presentes de forma majoritária na fração diclorometano de *J. isabellei*.

3.1 OS DITERPENOS

Os diferentes esqueletos diterpênicos oriundos da fusão de quatro unidades de isopreno podem ser acíclicos ou policíclicos e possuir diferentes grupos funcionais, o que prediz as suas características físico-químicas. Estudos têm demonstrado que os diterpenos identificados a partir de diferentes espécies de plantas do gênero *Jatropha* apresentam atividades biológicas como: antitumoral, citotóxica, molusquicida, inseticida, fungicida e anti-inflamatória (DEVAPPA; MAKKAR; BECKER, 2011).

Apesar de alguns trabalhos relatarem atividade anti-inflamatória para estes compostos (FERNANDEZ et al., 2001; PAIVA et al., 2002), esta atividade ainda não está descrita para os diterpenos isolados das partes subterrâneas de *J. isabellei*. Entretanto, merece destaque a capacidade de inibição da proliferação de linfócitos da jatrofona, a qual pode estar relacionada com um possível mecanismo de ação anti-inflamatória, conforme mostrado por Moraes e colaboradores (1996). Nesse estudo o diterpeno jatrofona inibiu de maneira dose-dependente a proliferação estimulada de linfócitos humanos e de ratos, indicando que este composto bloqueia uma etapa comum da resposta proliferativa. Com estes resultados os autores sugeriram que estava ocorrendo inibição da proteína quinase C (PKC). Sendo assim, a jatrofona poderia agir atenuando os efeitos da resposta inflamatória pela diminuição da amplificação da resposta.

Martini e colaboradores (2000) mostraram que a jatrofona inibe a ligação do [^{3}H]-glutamato, em modelo de membrana de tecido cerebral em ratos. Considerando que o glutamato é um dos mais importantes neurotransmissores envolvidos na transmissão da dor, este resultado indicou que este parâmetro pode estar envolvido na ação antinociceptiva deste composto. Outras atividades descritas incluem a gastroprotetora, sendo mostrado que a jatrofona reduz as lesões gástricas induzidas por

ácido clorídrico em 88 a 93%, e citotóxica frente aos fibroblastos e a células permanentes do epitélio gástrico humano (AGS)(PERTINO et al., 2007a), além das atividades molusquicida (SANTOS; SANT'ANA, 1999), antiprotozoária (SCHMEDA-HIRSCHMANN et al., 1996; HADI et al., 2013), antiproliferativa contra fibroblastos, células de adenocarcinoma gástrico, células de câncer de pulmão, carcinoma de bexiga e de leucemia (THEODULOZ et al., 2009), relaxante sobre a musculatura lisa (CALIXTO; SANT'ANA, 1990; DUARTE; SANT'ANA; CALIXTO, 1992; SILVA; BRUM; CALIXTO, 1995), inibitória da agregação plaquetária (DUTRA et al., 1996) e antitumoral (KUPCHAN et al., 1970; KUPCHAN et al., 1976; TAYLOR et al., 1983).

Outros diterpenos presentes na *J. isabellei* incluem as jatrofolonas A e B, as quais são isômeros α e β do C-16 da molécula. Ambos isômeros apresentaram efeito gastroprotetor sobre as lesões induzidas com ácido clorídrico e etanol em ratos, reduzindo as lesões em 54% e 83-91%, respectivamente, na dose de 100 mg/kg, o que evidencia a relevância da estereoquímica na atividade destes compostos. O efeito desta diferença estrutural também foi verificado na atividade citotóxica. Enquanto a jatrofolona B não foi citotóxica (IC_{50} > 1000 μM) contra fibroblastos e células permanentes do epitélio gástrico humano (AGS), a jatrofolona A apresentou uma toxicidade considerável contra as células AGS, com IC_{50} de 49μM (PERTINO et al., 2007a). A jatrofolona B também exibiu uma atividade antiproliferativa contra todas as linhagens de células tumorais testadas (fibroblastos, células de adenocarcinoma gástrico, células de câncer de pulmão, carcinoma de bexiga e de leucemia), enquanto a jatrofolona A não foi ativa contra estas células (THEODULOZ et al., 2009). Outros estudos mostram que uma mistura das jatrofolonas A e B apresentou atividade molusquicida (SANTOS; SANT'ANA, 1999) e a jatrofolona A teve efeito contra *Plasmodium falciparum* (SUTTHIVAIYAKIT et al., 2009).

3.2 OS TRITERPENOS ESTEROIDAIS E NÃO-ESTEROIDAIS

Os triterpenos possuem as unidades de isopreno distribuídas na forma de ciclos sendo organizados em 6-6-6-6-5 ou 6-6-6-6-6 pentaciclos e 6-6-6-5 tetraciclos (XU; FAZIO; MATSUDA, 2004). Os triterpenos pentacíclicos originam-se a partir da biossíntese pela via do mevalonato e os triterpenos tetracíclicos ou fitoesteroides são considerados triterpenos modificados, pois são oriundos da ciclização do esqualeno e do cicloartenol e possuem uma estrutura similar ao ciclopentanoperidrofenantreno (DEWICK, 2002; AČIMOVIČ; ROZMAN, 2013). Muitas atividades biológicas são descritas para a classe dos triterpenos como atividade antiviral, antimicrobiana, antitumoral, imunomoduladora e principalmente anti-inflamatória (RÍOS, 2010).

Estudos relatam atividade anti-inflamatória e antinociceptiva para os triterpenos isolados das partes subterrâneas de *J. isabellei*. O ácido acetil aleuritólico (AAA) mostrou ser capaz de inibir significativamente o edema de pata induzido pela carragenina, dextrana e histamina em 47,7; 28,5 e 40,5%, respectivamente, quando administrado pela via oral na dose de 50 mg/kg, revelando seu potencial anti-inflamatório. O AAA também se mostrou eficaz em modelo de nocicepção periférica, reduzindo as contrações induzidas pela injeção intraperitoneal de ácido acético 1% em 60,4%, (PERAZZO et al., 2007).

A atividade anti-inflamatória também foi demonstrada para o β-sitosterol que apresentou potente redução do edema induzido pela carragenina, de modo similar à hidrocortisona e oxifembutazona (GUPTA et al., 1980) e ao ibuprofeno (NIRMAL et al., 2012) quando administrado pela via intraperitoneal. Este ativo mostrou-se seguro com DL_{50} > 3 g/kg (i.p.) para camundongos e dose ulcerogênica de 600 mg/kg (i.p.) em ratos (GUPTA et al., 1980). Outro estudo mostrou a atividade anti-inflamatória do β-sitosterol (14 mg/kg) através da significativa redução do granuloma induzido pelo adjuvante completo de Freund (PARK et al., 2001). O efeito antinociceptivo deste ativo também foi demonstrado em vários trabalhos. Em modelo de contração induzida por ácido acético em camundongos, o β-sitosterol e sua forma glicosilada (100 mg/kg, i.p.) provocaram a

redução do número de contrações em 70 e 73%, respectivamente. No teste da placa quente, pode-se observar um aumento na tolerância à dor de 300 e 157%, respectivamente, enquanto que o controle positivo ácido mefenâmico, apresentou um aumento de 171% (VILLASEÑOR et al., 2002). Este efeito também foi observado por Nirmal e colaboradores (2012) em que o β-sitosterol (5, 10 e 20 mg/kg i.p.) mostrou uma atividade dose-dependente comparável ao fármaco padrão pentazocina (10 mg/kg). No mesmo estudo este ativo também demonstrou inibir as contrações induzidas por ácido acético de maneira dose-dependente e de forma similar ao paracetamol (50 mg/kg i.p.).

O estigmasterol provocou a redução do edema agudo e crônico induzido pelo TPA (12-0-tetradecanoilforbolacetato) em cerca de 40 %, quando aplicado topicamente em orelha de rato (0,5 mg/orelha), exibindo, portanto, significativa atividade anti-inflamatória tópica (GARCÍA et al., 1999). A atividade anti-inflamatória do estigmasterol e do acetato de estigmasterol também foi evidenciada por Santos e colaboradores (1995). Nesse trabalho foi mostrado que estes compostos inibem tanto a fase neurogênica (primeira fase) quanto a fase inflamatória (segunda fase) nas doses de 10 a 100 mg/kg (i.p.), em modelo de dor e inflamação induzido por formalina em ratos. A atividade antinociceptiva do estigmasterol e seu éster acetato também foi demonstrada por estes autores após administração intraperitoneal nas doses de 3 a 100 mg/kg, em modelo de nocicepção induzida por ácido acético em ratos, e pela via oral nas doses de 50-200 mg/kg, em modelo de nocicepção induzida por ácido acético e formalina em ratos.

Como pode ser visto, inúmeros trabalhos se referem à atividade anti-inflamatória e antinociceptiva dos triterpenos, os quais foram encontrados na fração diclorometano de *J. isabellei.*

4 CARREADORES COLOIDAIS DE FÁRMACOS

Um dos grandes desafios da indústria farmacêutica é o fato de muitos fármacos potencialmente ativos apresentarem caráter lipofílico, exibindo baixa solubilidade nos fluidos biológicos. Esta característica resulta em uma baixa

biodisponibilidade do fármaco quando administrado pela via oral, grandes variações na concentração plasmática e na dependência da administração de alimentos para que ocorra a absorção por esta via. Por outro lado, a administração intravenosa destes fármacos é muitas vezes inviável ou então requer o uso de excipientes potencialmente tóxicos por esta via (MÄDER; MEHNERT, 2005). Neste sentido, o uso de nanocarreadores tem sido uma estratégia bastante explorada, pois eles permitem veicular fármacos lipofílicos em dispersões aquosas. Dependendo da aplicação específica e dos objetivos requeridos, os nanocarreadores também permitem o direcionamento de fármacos para órgãos, tecidos e/ou receptores específicos, a redução da dose e dos efeitos colaterais, o controle da liberação do fármaco ao longo do tempo, o aumento da permeabilidade através das diversas barreiras anatômicas e o aumento da estabilidade química nos meios ambiente e fisiológico (MEHNERT; MÄDER, 2001; MÄDER; MEHNERT, 2005; MULLAICHARAM, 2011).

Os carreadores coloidais de fármacos são caracterizados pelo seu tamanho submicrométrico, mas podem diferir quanto a sua estrutura e composição, dando origem a sistemas poliméricos (nanocápsulas e nanoesferas) ou lipídicos (nanoemulsões, lipossomas, micelas, entre outros). Fármacos podem encontrar-se adsorvidos, encapsulados ou dissolvidos no carreador. Estes carreadores também diferem em termos de estabilidade termodinâmica, capacidade de encapsulação e tipo de aplicação, podendo ser utilizados para o diagnóstico e o tratamento de diversas doenças, por diferentes vias de administração (MÄDER; MEHNERT, 2005).

Os órgãos específicos podem ser alcançados pelos nanocarreadores por mecanismos ativos ou passivos. A primeira estratégia consiste no direcionamento da partícula para o alvo através da conjugação desta com ligantes específicos como anticorpos, ácido fólico e peptídeos. Esta estratégia também pode ser alcançada por meio da manipulação de estímulos físicos, como pH, temperatura e magnetismo (WILCZEWSKA et al., 2012). A vetorização passiva é alcançada principalmente quando as nanopartículas são administradas na circulação sistêmica, onde, após etapa de opsonização e captura pelos macrófagos, tendem a se acumular em órgãos do sistema

reticuloendotelial (SER) como baço e fígado (GREF et al., 1994). Esta tendência natural de vetorização passiva pode ser uma estratégia quando os órgãos-alvo da terapia medicamentosa são os mesmos acumuladores das células do SER, entretanto, para a ação em outros órgãos é desejável que as partículas não sejam capturadas, evitando assim a diminuição da sua disponibilidade. Outra forma de vetorização passiva para nanopartículas administradas sistemicamente é o resultado do aumento da retenção e da permeabilidade vascular, conhecido como efeito EPR – *Enhanced Permeability and Retention*. Este efeito ocorre em tecidos inflamados e tumorais devido a um desequilíbrio na expressão e ativação dos fatores pró- e anti-angiogênicos, levando a uma angiogênese descontrolada nos tecidos com a formação de uma camada endotelial descontínua e com fenestras, o que facilita a permeabilidade das nanopartículas e a liberação do fármaco no local. Adicionalmente, uma drenagem linfática insuficiente também pode ocorrer nos tecidos tumorais devido à linfangiogênese anormal e a compressão dos vasos linfáticos devido ao crescimento do tumor, fazendo com que aumente a permanência dos fluidos intersticiais no local e consequentemente das nanopartículas que alcançaram o tumor, aumentando o tempo para a liberação dos fármacos (WILCZEWSKA et al., 2012; NEHOFF et al., 2014).

4.1 CARREADORES LIPÍDICOS NANOESTRUTURADOS: NANOEMULSÕES

Nanoemulsões são dispersões coloidais cineticamente estáveis, constituídas de gotículas de óleo de tamanho nanométrico dispersas em uma fase aquosa externa e estabilizadas por tensoativos. O diâmetro médio das gotículas varia de 50 até 1000 nm, sendo que usualmente o tamanho das gotículas está entre 100 e 500 nm (TADROS et al., 2004; LOVELYN; ATTAMA, 2011). O reduzido tamanho da gota de óleo e o movimento Browniano próprio das dispersões coloidais fazem com que a ação da gravidade seja superada, reduzindo assim, os fenômenos de sedimentação ou cremação, floculação e a coalescência, fazendo com que o sistema permaneça disperso (TADROS et al., 2004).

A fase interna oleosa das nanoemulsões serve como reservatório para os fármacos lipofílicos, onde estes se encontram solubilizados, apesar de poderem estar adsorvidos na interface da nanoestrutura. Os óleos mais amplamente utilizados incluem ácidos graxos saturados e insaturados, triglicerídeos de cadeia média e longa e óleos vegetais, como óleo de soja, oliva e rícino (CHEN et al., 2011; BRUXEL et al., 2012).

A estabilização das nanoemulsões é feita pela escolha de um sistema tensoativo adequado. Tensoativos de origem natural como as lecitinas de ovo e de soja são amplamente empregadas no desenvolvimento de nanoemulsões por serem biocompatíveis e biodegradáveis. Em geral, a lecitina é combinada com outros tensoativos sintéticos não-iônicos, como os poloxâmeros e ésteres do sorbitano etoxilados (Tweens). O uso de tensoativos não-iônicos confere estabilidade devido a um efeito estérico, que impede que as gotas coalesçam. Tensoativos aniônicos, como o ácido oleico (carga negativa) e lipídeos catiônicos como a estearilamina e a oleilamina apesar de serem mais tóxicos, podem ser adicionados para conferir carga à superfície da partícula garantindo uma estabilização eletrostática pelo aumento do potencial zeta (BRUXEL et al., 2012).

A fase aquosa externa pode ser constituída de reguladores de pH e agentes de tonicidade. Agentes conservantes também podem ser adicionados à fase aquosa quando esta é vulnerável à contaminação. Os agentes de tonicidade são adicionados em formulações de uso parenteral para a manutenção da osmolaridade próxima à sanguínea, o que pode ser alcançado com a adição de cloreto de sódio, dextrose, sorbitol e com glicerol, constituinte mais frequentemente utilizado nas formulações parenterais. O ajuste do pH é realizado de acordo com a via de aplicação da nanoemulsão e pode ser feito com soluções diluídas de hidróxido de sódio ou ácido clorídrico (KLANG; BENITA, 1998).

Os métodos usados para preparar as nanoemulsões podem ser classificados em de alta e baixa energia. Os métodos de alta energia incluem homogeneização de alta pressão, microfluidização e homogeneização ultrassônica e requerem equipamentos específicos para a obtenção dos diâmetros reduzidos das partículas. Estes métodos são muito eficazes na redução do tamanho das gotas, entretanto, podem levar à

degradação de fármacos lábeis e de macromoléculas como as proteínas, pela geração de calor durante a operação de emulsificação (FLOURY; DESRUMAUX; LEGRAND, 2002; KUHN; CUNHA, 2012).

Os métodos de baixa energia mais comumente relatados são o método da temperatura de inversão de fases e o método da emulsificação espontânea. O método da temperatura de inversão de fases se aplica a emulsões que contém tensoativos cuja solubilidade depende da temperatura, tais como os tensoativos não-iônicos polietoxilados. Em temperaturas acima da temperatura de inversão de fases, estes tensoativos tornam-se mais lipofílicos, devido à desidratação das cadeias de polioxietileno, estabilizando emulsões água em óleo. Com o resfriamento do sistema emulsionado e consequente aumento da solubilidade aquosa destes tensoativos, ocorre a inversão de fases e formação de emulsões óleo em água de tamanho de gota reduzida e mais estáveis (IZQUIERDO et al., 2002).

A emulsificação espontânea é um método simples e não requer equipamentos sofisticados. Nesse método a nanoemulsão é obtida como resultado da mistura de duas soluções constituídas de solventes que são perfeitamente miscíveis entre si, em temperatura ambiente e com agitação magnética. Uma destas soluções é a fase aquosa que contém um tensoativo hidrofílico e a outra a fase oleosa, que contém um óleo e um tensoativo lipofílico, em um solvente orgânico, geralmente etanol ou acetona. Com a adição da fase orgânica na fase aquosa ocorre a rápida difusão do solvente orgânico, originando uma grande turbulência na interface óleo/água, denominado efeito Maragoni, que resulta na formação de nanogotas. O solvente orgânico é posteriormente removido por evaporação sob pressão reduzida para gerar dispersões aquosas. A quantidade de solvente orgânico e a sua miscibilidade com a água, a velocidade de agitação, a viscosidade do óleo, o tipo de tensoativo utilizado são alguns dos fatores que afetam as propriedades finais da nanoemulsão formada (BOUCHEMAL et al., 2004; ALMEIDA; TEIXEIRA; KOESTER, 2008; ANTON; VANDAMME, 2009).

4.2 ADMINISTRAÇÃO ORAL DE NANOEMULSÕES

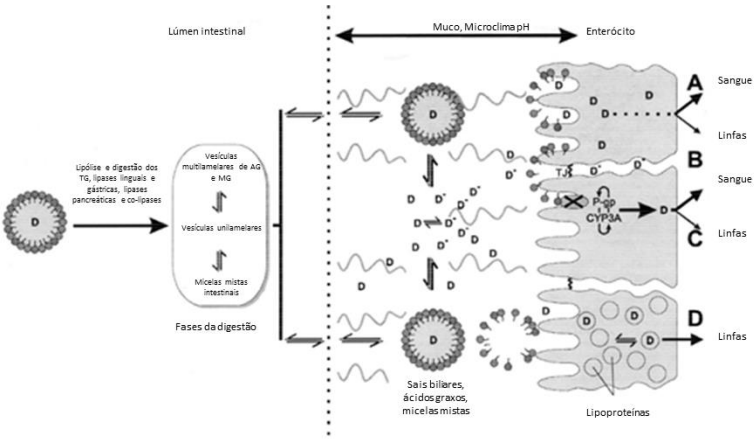
A via oral é a rota preferencial para a administração de fármacos pela facilidade de administração, minimização dos erros de dosagem e pela ausência de dor, apresentando maior aceitação e adesão do paciente. Entretanto, a administração de fármacos lipofílicos pela via oral é prejudicada em função da sua baixa solubilidade nos fluídos do trato gastrointestinal (TGI), o que reduz sua biodisponibilidade. Desta forma, muitos estudos são voltados ao desenvolvimento de formulações que visem a melhora da absorção e da biodisponibilidade dos fármacos lipofílicos e que resultem na redução da dose a ser administrada. Uma estratégia que tem se mostrado promissora consiste na veiculação de fármacos em formulações lipídicas, tais como as nanoemulsões.

Os componentes lipídicos são de grande importância por estimular as respostas fisiológicas para a absorção de fármacos lipofílicos ou pouco solúveis em água. A ingestão de uma refeição rica em gorduras resulta no estímulo das secreções biliar e pancreática, no prolongamento do tempo de residência no trato gastrointestinal, no estimulo do transporte linfático, alterações na circulação sanguínea mesentérica e hepática, aumento da permeabilidade da parede intestinal e redução do metabolismo e da atividade de efluxo, aspectos que contribuem significativamente para a melhora da biodisponibilidade dos compostos ativos lipofílicos (WAGNER et al., 2001; KALANTZI et al., 2006; PORTER et al., 2008; CHAKRABORTY et al., 2009). Embora a natureza e a quantidade dos lipídeos existentes em uma refeição e na formulação sejam diferentes, as formulações lipídicas podem reduzir as limitações de uma dissolução lenta e incompleta dos compostos lipofílicos e proporcionar a formação de vesículas, micelas e lipoproteínas, as quais funcionam como carreadores de fármacos e possibilitam a absorção destes (CHAKRABORTY et al., 2009).

Os possíveis mecanismos de absorção dos compostos lipofílicos a partir das vesículas, micelas e lipoproteínas formadas após a digestão dos lipídeos das formulações incluem: o aumento na fluidez da membrana facilitando a absorção transcelular (A), abertura das junções oclusivas para permitir o transporte paracelular (B), a inibição da glicoproteína P e do

citocromo P450 para aumentar a concentração intracelular do fármaco e o seu tempo de residência (C) e o estímulo para a produção de mais lipoproteínas (quilomicron) (D) (Figura 4) (O'DRISCOLL, 2002; PORTER; TREVASKIS; CHARMAN, 2007).

Figura 4. Mecanismos de transporte de fármacos lipofílicos do lúmen intestinal até as circulações sistêmica ou linfática. (A) aumento da fluidez da membrana facilitando a absorção transcelular; (B) abertura das junções oclusivas permitindo o transporte intercelular; (C) inibição da glicoproteína P e do citocromo P450 aumentando a concentração intracelular do fármaco e seu tempo de residência; (D) estímulo da produção de lipoproteínas.



(Adaptado de O'Driscoll, 2002).

Uma vez absorvidos pelos enterócitos, os fármacos atingem a circulação sistêmica, através da veia porta, ou a circulação linfática, dependendo do tamanho da estrutura coloidal na qual o fármaco está inserido (vesícula, micela ou lipoproteínas). Diferente dos capilares sanguíneos, as junções intercelulares entre as células endoteliais dos capilares linfáticos são mais abertas, assim, as partículas coloidais como lipoproteínas (quilomícrons; diâmetro 200 – 800 nm) que são

grandes demais para os capilares sanguíneos são captados pelos capilares linfáticos. O fluído linfático é esvaziado através do duto torácico, dentro da veia subclávica, evitando assim, o metabolismo de primeira passagem. Esta via é considerada uma importante via de absorção para fármacos lipofílicos, melhorando assim a sua biodisponibilidade (O'DRISCOLL, 2002; PORTER; TREVASKIS; CHARMAN, 2007; CHAKRABORTY et al., 2009; FRICKER et al., 2010).

Adicionalmente, estudos têm demonstrado que o tamanho das nanopartículas desempenha um papel fundamental na taxa de absorção dos fármacos, pois influencia na adesão e interação com enterócitos. Devido ao reduzido tamanho das partículas e alto grau de dispersibilidade, estas podem se aderir parede do trato gastrointestinal (bioadesão) ou entrar nos espaços intercelulares, aumentando a área de contato do fármaco com a mucosa e residência no trato gastrointestinal, o que também irá resultar na melhora da biodisponibilidade (NICOLAOS et al., 2003; WIN; FENG, 2005).

4.3 ADMINISTRAÇÃO INTRAVENOSA DE NANOEMULSÕES

As formulações intravenosas fornecem uma rápida liberação do fármaco, o que é fundamental em casos de emergência médica ou quando nenhuma outra via para a administração é possível. Entretanto, a solubilização de fármacos lipofílicos para a administração parenteral representa um grande desafio para a indústria farmacêutica. Diversas estratégias são descritas na literatura para esta finalidade, porém, limitações relacionadas à biocompatibilidade dos adjuvantes empregados, aos relatos de dor e de possível precipitação dos fármacos durante a administração, podem ser considerados fatores limitantes do uso destas abordagens (BRUXEL et al., 2012). Neste contexto, as nanoemulsões como sistemas carreadores de fármacos têm se mostrado promissoras por serem sistemas biodegradáveis e biocompatíveis.

A capacidade das nanoemulsões de solubilizar grande quantidade de fármacos lipofílicos, junto com a sua habilidade de proteger os fármacos da hidrólise e degradação enzimática, faz delas veículos ideais para a administração parenteral. Além disso, existe a possibilidade da redução da frequência de

injeções durante o período da terapia medicamentosa, pois as nanoemulsões podem garantir a liberação prolongada e sustentada por longos períodos de tempo (LOVELYN; ATTAMA, 2011; PRAKASH; THIAGARAJAN, 2011).

Contudo, a eliminação de um carreador coloidal do organismo acontece de maneira muito rápida, pois estes são reconhecidos e captados pelo sistema fagocítico mononuclear, também conhecido como sistema reticuloendotelial (SRE). Ao entrarem na circulação sanguínea as nanopartículas sofrem o processo de opsonização, em que as proteínas opsoninas se ligam à superfície lipofílica das partículas, tornando-as visíveis às células fagocíticas (principalmente macrófagos). Após a opsonização ocorre a fagocitose das partículas, com consequente acúmulo das mesmas nos órgãos ricos em macrófagos, ou ainda a sua excreção pelo sistema renal (OWENS; PEPPAS, 2006).

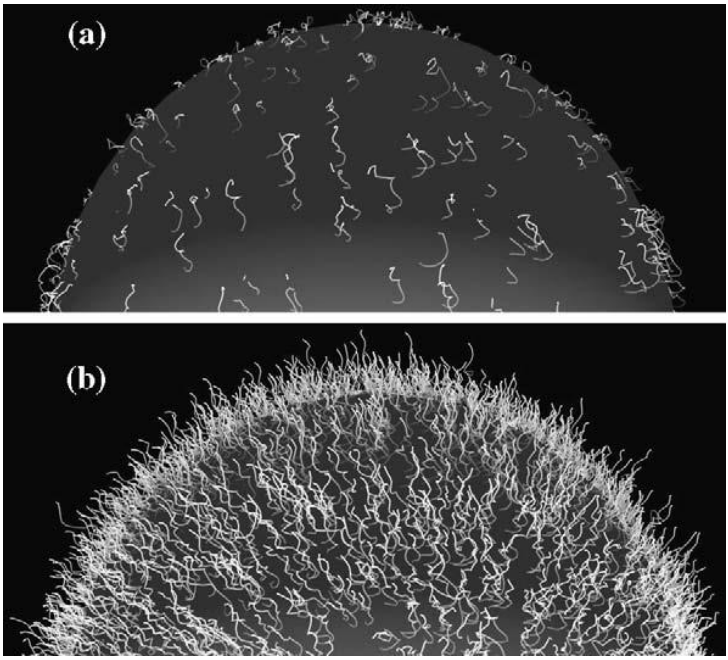
Para superar estas limitações, algumas alternativas para camuflar as nanopartículas do SRE têm sido desenvolvidas. Uma delas consiste na adsorção de polímeros hidrofílicos à superfície das nanopartículas como, por exemplo, o polietilenoglicol (PEG). O PEG ainda pode ser utilizado acoplado a um segmento núcleo hidrofóbico, como por exemplo, a diesteroilfosfatidiletanolamina (DSPE). Este polímero (DSPE-PEG) é biocompatível, possui característica anfifílica e pode encapsular, carrear e vetorizar fármacos lipofílicos através da circulação sanguínea, sendo amplamente utilizado no preparo de sistemas coloidais de fármacos (WANG et al., 2012)

Os polímeros hidrofílicos conseguem impedir que ocorra o processo de opsonização, evitando a captação das nanopartículas pelo SER, fazendo com que estas permaneçam mais tempo na circulação sanguínea (LIU; LIU, 1995). Estas partículas são conhecidas como furtivas (do inglês, *stealth*) e podem alcançar outros órgãos, além daqueles ricos em células fagocitárias (OWENS; PEPPAS, 2006).

Para que se consiga alterar o perfil farmacocinético dos fármacos lipofílicos administrados pela via intravenosa com o uso de nanocarreadores é necessário otimizar a formulação, no que diz respeito à peguilação das partículas. Parâmetros como densidade do polímero e comprimento das cadeias de PEG utilizadas são determinantes para prevenir a opsonização (GREF

et al., 2000). Partículas com densidade superficial de PEG mais alta apresentam mobilidade restrita das cadeias do polímero (*brush-like configuration*) resultando na diminuição da opsonização e no aumento do tempo de permanência na circulação sanguínea, enquanto que uma baixa densidade superficial do polímero (*mushroom-like configuration*) faz com que haja um aumento da mobilidade entre as cadeias peguiladas, facilitando a opsonização (Figura 6). O comprimento das cadeias de PEG tem mostrado interferir em menor proporção na captação das nanopartículas pelos macrófagos, havendo controvérsias na literatura sobre qual o melhor comprimento de cadeia a ser utilizado (PEG 1000, PEG 2000, PEG 5000 ou PEG 10000), entretanto, o mais indicado é que as cadeias de PEG tenham massa molar de 2000 ou mais (DUNN et al., 1994; LIU; LIU, 1995; OWENS; PEPPAS, 2006). Nanoemulsões contendo o composto danazol e os polímeros DSPE-PEG2000, 5000 e 10000 mostraram-se significativamente furtivas em relação às respectivas formulações não peguiladas. Além disso, a diminuição da captura pelos macrófagos foi proporcional ao aumento da quantidade dos polímeros DSPE-PEG2000 e 5000 nas formulações, sendo que o uso do polímero DSPE-PEG5000 resultou na formulação com melhores propriedades furtivas do estudo (DEVALAPALLY et al., 2013). O uso do PEG nas partículas também resulta em melhoras no perfil farmacocinético das formulações, como por exemplo, as nanoemulsões de prostaglandina E1 (PGE1) formuladas com o polímero DSPE-PEG2000 que apresentaram uma melhora do perfil farmacocinético de 1,47 e 5,98 vezes nos parâmetros de área sob a curva e tempo de meia-vida, respectivamente, se comparada à respectiva formulação comercial (CHENG et al., 2016).

Figura 5. Esquema das configurações do PEG na superfície das partículas: (a) *mushroom-like configuration* e (b) *brush-like configuration.*



(Fonte: Owens, Peppas, 2006).

CAPÍTULO 1

Manuscrito 1: Antinociceptive and anti-inflammatory activities of the dichloromethane fraction from *Jatropha isabellei* and isolation and quantitative determination of jatrophone by UFLC-DAD

Antinociceptive and anti-inflammatory activities of the dichloromethane fraction from *Jatropha isabellei* and isolation and quantitative determination of jatrophone by UFLC-DAD

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Abstract

Context: Jatropha isabellei Müll. Arg. (Euphorbiaceae) has been used in the traditional medicine to treat arthritis.

Objective: To evaluate the anti-inflammatory and antinociceptive activities of the dichloromethane fraction (DF_{Ji}) from underground parts of *J. isabellei*, and to develop and validate an UFLC-DAD method to quantify the diterpene jatrophone.

Materials and methods: Anti-inflammatory and antinociceptive activities of the DF_{ji} were determined by an acute arthritis model through assessment of the paw elevation time (PET) and articular diameter (AD) of orally (50, 100 and 200 mg/kg) and intravenously (10, 25 and 50 mg/kg) treated Wistar rats. The isolation of jatrophone was confirmed by spectroscopic techniques. A UFLC-DAD method was developed and validated according to linearity, limits of detection and quantification, specificity, accuracy, and precision.

Results: The oral and intravenous administrations of the DF_{Ji} at doses of 100 and 200 mg/kg and 10, 25, and 50 mg/kg, respectively, were able to significantly reduce the PET. The DF_{Ji} prevented the formation of paw edema in orally and intravenously treated rats at doses of 50, 100 and 200, and 10 mg/kg, respectively. The UFLC-DAD method allowed the quantification of jatrophone content in the DF_{Ji} which was found to be around 90 µg per mg of fraction.

Discussion and conclusion: The DF_{Ji} displayed potent antinociceptive and antiedematogenic activities, which were associated with the presence of terpenic compounds in this fraction, in particular the jatrophone. These results indicated that the DF_{Ji} represents a promising plant product for the development of herbal medicines for the arthritis treatment.

Keywords: *J. isabellei*, arthritis, inflammation, pain, jatrophone, UFLC-DAD.

1. Introduction

Arthritis is a disabling condition that affects millions of people worldwide. It is a form of joint disorder that involves inflammation of one or more joints with infiltration of inflammatory cells, synovial hyperplasia, cartilage destruction, bone erosion, narrowing of the joint space and ankylosis of the joint (BENDELE et al., 1999). The major complaint by individuals who have arthritis is joint pain, which is due to an inflammation that occurs around the joint, and is associated with muscle weakness, loss of flexibility, joint stiffness, fatigue, loss of quality of life and others symptoms (LEE, 2013). The most common forms of arthritic conditions are osteoarthritis, rheumatoid arthritis, and gout, being the latter responsible for the worst episodes of acute pain and can lead to the development of chronic and tophaceous gouty arthritis and renal damage (CANNELLA; MIKULS, 2005). There is no available cure for these arthritic conditions and the treatment options may include physical therapy, lifestyle changes (including exercise and weight control), and medications. The pharmacological treatment for arthritis includes the administration of analgesics, corticosteroids, disease-modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and biological drugs (NEGREI et al., 2016). However, besides presenting several adverse effects, many patients are refractory to these drugs, making medication adherence difficult. These drawbacks have stimulated research on the arthritis treatment and plant constituents (RATES, 2001; KHANNA et al., 2007; GHOSH et al., 2016).

Jatropha isabellei Müll. Arg. (Euphorbiaceae) is a shrub with red-violet inflorescences known in the Paraguayan and Brazilian folk medicine as “yagua rova”, “turubiti” and “mamoneiro do campo” (BASUALDO; ZARDINI; ORTIZ, 1991; RIVEROS et al., 2009; FRÖHLICH et al., 2013). The infusion or decoction obtained from the underground parts of *J. isabellei* has been popularly used to treat different types of arthritis (BASUALDO; ZARDINI; ORTIZ, 1991). In fact, the antinociceptive and anti-inflammatory properties of the crude extract were evidenced in a rat gout model induced by sodium monourate (MSU) crystals. Although the crude extract was able to prevent the mechanical allodynia, thermal hyperalgesia, edema, and neutrophil infiltration

induced by intra-articular MSU injection, it was not able to alter the uric acid levels increased by potassium oxanate (SILVA et al., 2013). Also, the crude extract of *J. isabellei* was able to inhibit the xanthine oxidase activity *in vitro* only at high concentrations. These results suggested that neither the xanthine oxidase inhibition nor the decrease of uric acid blood levels are implicated in the antinociceptive and antiedematogenic effects verified for *J. isabellei* (SILVA et al., 2013).

Many studies have been carried out to elucidate the chemical composition of the underground parts of *J. isabellei*. The combined petroleum ether and ethyl acetate extracts obtained from rhizomes of this plant have been found to include triterpene acetyl aleuritolic acid, sesquiterpene cyperenoic acid, and diterpenes jatrophone and jatropholones A and B, besides a monoterpene and a firstly related diterpene named 9β,13α-dihydroxyisabellione (PERTINO et al., 2007). Also, acetyl aleuritolic acid and a binary mixture of sitosterol-3-*O*-β-D-glucoside and stigmasterol were identified from the dichloromethane fraction obtained from the underground parts of this plant (FRÖHLICH et al., 2013).

In general, terpenes are recognized for having anti-inflammatory and analgesic properties and therefore have been considered as potential candidates for new drugs intended to control painful syndromes and inflammatory diseases (SULTANA; SAIFY, 2012; GUIMARÃES; SERAFINI; QUINTANS-JÚNIOR, 2014). Since these bioactive compounds are identified as the major constituents in the *J. isabellei* dichloromethane fraction, and given the popular use of this plant to treat arthritis, the aim of this study was to evaluate the anti-inflammatory and analgesic activities of the dichloromethane fraction after oral and intravenous administration in an acute arthritis model induced by carrageenan in rats. Additionally, considering that no analytical methods have been reported to chemically characterize the *J. isabellei* dichloromethane fraction, an important constituent of this fraction was identified and isolated, and an analytical methodology of ultra-fast liquid chromatography with diode array detection (UFLC-DAD) was developed and validated to quantitatively determine this compound.

2. Materials and methods

2.1. Plant collection and extraction

J. isabellei was collected in the municipality of Cacequi (State of Rio Grande do Sul, Brazil, coordinates: 29°53'01" S and 54°49'30" W) in May of 2008. An exsiccate was archived in the herbarium of the Biology Department at the Federal University of Santa Maria (SMDB 11816). The underground parts were dried at room temperature and powdered in a knife mill. The powder was macerated with 70% (v/v; plant: solvent ratio 1:3 w/v) ethanol for ten days at room temperature. After filtration, the ethanol was evaporated under reduced pressure and this dispersion was partitioned with dichloromethane to obtain its respective fraction which was further taken to dryness under reduced pressure, resulting in the dichloromethane fraction (DF_{Ji}, yield 3.7%).

2.2. Drugs and reagents

The dichloromethane fraction from *J. isabellei* (DF_{Ji}) was resuspended in a mixture composed of dimethyl sulphoxide (DMSO), polyethylene glycol 400 (PEG 400), and phosphate buffered saline (PBS), pH 7.4 (5:47.5:47.5 v/v) for oral administration (p.o.) in rats. For intravenous administration, the fraction was dissolved in a mixture composed of DMSO, polysorbate 80, and saline solution (5:4:91 v/v). A combination of kappa and lambda carrageenan was purchased from BDH Chemicals Ltd. (UK). Dexamethasone and indomethacin were purchased from Deg (Brazil), and colchicine from Sigma-Aldrich (USA). The jatrophone isolation from the dichloromethane fraction required the use of Silica Gel 60 and Silica Gel 60 F254 coated plates, which were purchased from Merck (Germany). Analytical grades hexane, acetone, dichloromethane, ethanol and methanol were purchased from Vetec (Brazil). Acetonitrile HPLC grade was purchased from Panreac (Spain). The ultrapure water utilized in the UFLC analyses was obtained using a Milli-Q purification system (Millipore, USA).

2.3. Antinociceptive and anti-inflammatory activities in a carrageenan-induced arthritis model of the DF_{Ji}

2.3.1. Animals

The experiments were conducted in accordance with the NIH guide for the care and use of laboratory animals (NIH), the ethical guidelines of the International Association for the Study of Pain (IASP, 1983), and approved by the local committee for ethical use of animals (P00723/CEUA-UFSC). All experiments were performed using adult male Wistar rats weighing 250 – 300 g. The animals were housed under a controlled temperature (21 ± 2°C) on a 12 h light/dark cycle with standard lab chow and water *ad libitum* until the experimental sessions. The animals were acclimatized into the experimental room for at least 30 min before the experiments.

2.3.2. Carrageenan-induced articular incapacitation in rats

Articular incapacitation was induced by the injection of 300 µg of carrageenan (solubilized in 50 µL sterile 0.9% saline) into the right knee joint of the rats. In this assay, the animals were stimulated to walk on a revolving steel cylinder (constant speed of 3 rpm) wearing metallic gaiters in their hind paws. The right paw gaiter was connected to a computer system that counted the total duration of no contact on the cylinder surface during the one minute test period. This paw elevation time (PET), in seconds, was taken as an estimate of nociception (TONUSSI; FERREIRA, 1992). Two hours after the carrageenan injection the animals were treated with the DF_{Ji} either orally (gavage) (50, 100 or 200 mg/kg) or intravenously by gingival vein punction (OLIVEIRA; SILVA; TONUSSI, 2009) (0.1, 1, 10, 25 or 50mg/kg). The PET was evaluated in the third hour and hourly until the sixth hour, and presented as an average of these time points.

Oral dexamethasone (10 mg/kg), colchicine (30 mg/kg) and indomethacin (5 mg/kg) were used as positive controls and their effects were compared to that of the dichloromethane fraction. Dexamethasone (1 mg/kg) was also used as a positive control in the intravenously treated group. Negative control groups were treated only with the vehicle used.

2.3.3. Edema measurement

The articular diameter (AD) was used to quantify the inflammatory edema induced by carrageenan and it was taken by measuring the medio-lateral axis (in mm) of the knee-joint, using a micrometer at three consecutive arbitrary points in a proximo-distal direction. The AD measured just before carrageenan injection was subtracted from AD values taken hourly from 3rd to 6th h, just after the incapacitation measurement, and presented as an average of these time points.

2.3.4. Statistical analysis

The sample size for incapacitation and articular edema were estimated using a statistical power test, and a minimum of 6 animals were used for both parameters. Data were expressed as mean ± S.E.M or mean ± s.d. Statistical significance between groups was calculated by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison test or Student's test when appropriate. Only p values lower than 0.05 ($p < 0.05$) were considered significant.

2.4. Isolation and identification of jatrophone in the DF_{Ji}

Approximately 1 g of the dichloromethane fraction from the underground parts of *J. isabellei* was submitted to column chromatography on silica gel 60 (70 g) and eluted with different proportions of hexane:acetone (from 95:5 to 0:100) and acetone:methanol (from 90:10 to 30:70). This procedure resulted in sub-fractions that were analyzed by thin layer chromatography (TLC) and grouped based on similarity of their chromatographic profiles after revelation with sulfuric anisaldehyde/100°C for 2 min. The characterization of the isolated compound **2** was performed by 1D and 2D nuclear magnetic resonance experiments in a Bruker Ascend 600 equipment, and by HRMS (APPI-QTof) using a Bruker micrOTOF QII. The UV/VIS spectrum of the isolated compound was recorded using an SPD-M20 DAD UV/VIS detector, during the UFLC analysis.

2.5. Determination of jatrophone in the DF_{Ji} by UFLC-DAD

2.5.1. Chromatographic conditions

The chromatographic analyses were performed on a UFLC-DAD system (Shimadzu, Japan) equipped with a LC-20AD binary pump, an SIL-20AC HT auto-sampler, a CTO-20A forced air-circulation-type column oven, an SPD-M20 photo diode array UV/VIS detector, and the software LC Solution 1.2 (Shimadzu, Tokyo, Japan). The analyses were carried out in reversed phase mode using a Phenomenex Luna C18 column (150 mm x 4.6 mm x 5 µm) and a mobile phase consisted of acetonitrile and water, filtered prior to use through 0.45 µm polyvinylidene flouride (PVDF) and a regenerated cellulose (RC) membrane filter, respectively. In order to determine the jatrophone concentration in the $DF_{Ji,}$ the mobile phase was eluted at a flow rate of 1.0 mL/min using the following gradient program: 53-65% acetonitrile from 0-12 min, and 65-75% acetonitrile from 12-15 min. After this time, rebalancing was performed to restore the system and column to the initial mobile phase condition prior to the next injection. The total runtime was 23 min. The injection volume of the samples was 20 µL and the detection of jatrophone was monitored at 280 nm, according to its maximum absorption.

2.5.2. Preparation of the samples and standard solutions

Jatrophone, identified as a possible chemical marker of the DF_{Ji}, was used as an external standard. Working standard solutions of this diterpene were prepared by dissolving it in acetonitrile to obtain a concentration of 100 µg/mL. In order to quantitatively determine jatrophone, the DF_{Ji} was dissolved in acetonitrile at concentrations of 0.5 mg/mL. All standard solutions and samples were filtered through a 0.45 µm PVDF membrane filter before the UFLC injection.

2.5.3. Validation of the UFLC method

The UFLC method was validated according to the International Conference on Harmonization (ICH) and the ANVISA guidance, and included the parameters of linearity, limits

of detection (LOD) and quantification (LOQ), specificity, accuracy, and precision (BRAZIL, 2003; ICH, 2005). The linearity of the analytical method was assessed by constructing calibration curves for jatrophone, in triplicate, after analyzing eight jatrophone standard solutions at concentrations ranging from 1.0 to 100.0 µg/mL, in three different days. The linearity of the method was evaluated by calculating the linear regression coefficient using the least square method. The LOD and LOQ were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively, based on the standard deviation of the y-intercept of the regression curves (ICH, 2005). In order to determine repeatability (intra-day precision), an analysis of the DF_{Ji} was performed in sextuplicate at one level concentration in a single day. The intermediate precision (inter-day precision) was determined through a single analysis of the DF_{Ji} in sextuplicate for another two consecutive days. The results were expressed as relative standard deviation (%RSD). The accuracy of the method was determined through an analyte recovery test by spiking a dichloromethane fraction sample with standard solutions of jatrophone at three levels (low, medium and high). Specificity was confirmed by the peak purity index obtained directly from the spectral analysis report.

3. Results

3.1. Antinociceptive and anti-inflammatory activities of the DF_{Ji}

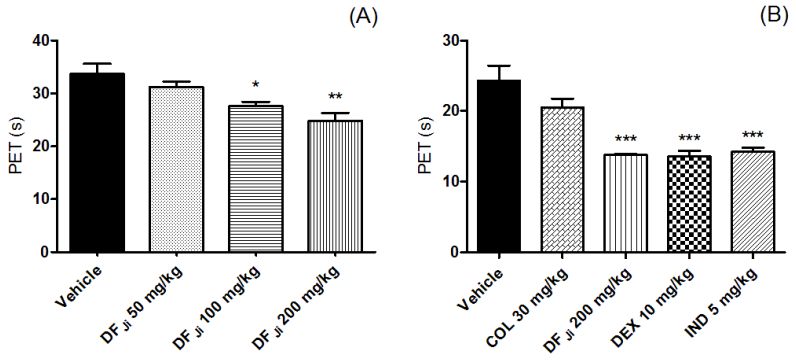
3.1.1. Effect of oral administration of the DF_{Ji} on rat paw elevation time

Animals treated only with vehicle p.o., 2 h after carrageenan injection (negative control group), showed an average PET value of 33.7 ± 1.8 s. The DF_{Ji} was administered orally at doses of 50, 100, and 200 mg/kg. The higher doses produced average PET values of 27.5 ± 0.9 s ($p < 0.05$) and 24.8 ± 1.4 s ($p < 0.01$), which were significantly different from those of the negative control group (Figure 1a).

The effect obtained with the administration of 200 mg/kg DF_{Ji} on PET was then compared with three drugs commonly used for arthritis; dexamethasone (10 mg/kg), indomethacin (5 mg/kg),

and colchicine (30 mg/kg) (Figure 1b). The incapacitation reversal produced by the DF_{Ji} (200 mg/kg, $p > 0.001$) was similar to the effects observed with dexamethasone and indomethacin treatments. Colchicine did not reduce the PET under these conditions. The average PET values were 24.3 ± 2.1, 13.8 ± 0.1, 13.5 ± 0.8, 14.2 ± 0.5, and 20.5 ± 1.2 s, for vehicle, dichloromethane fraction, dexamethasone, indomethacin, and colchicine, respectively (Figure 1b).

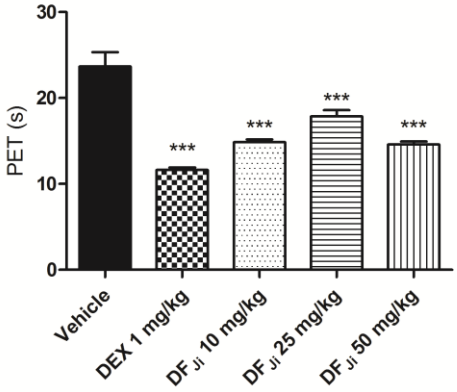
Figure 1. (A) Effect of the *J. isabellei* dichloromethane fraction (DF_{Ji}) 50, 100 and 200 mg/kg on paw elevation time (PET) after oral administration. (B) Effect of oral administration of the *J. isabellei* dichloromethane fraction (200 mg/kg), indomethacin (IND, 5 mg/kg), colchicine (COL, 30 mg/kg) and dexamethasone (DEX, 10 mg/kg) on paw elevation time (PET). The animals were treated 2 hours after the intra-articular carrageenan injection (300 µg/knee). The negative control group received only the vehicle (DMSO: PEG 400: PBS 5:47.5:47.5). *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ represents a significant difference compared with the negative control group. The statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test.



3.1.2. Effect of intravenous administration of the DF_{Ji} on rat paw elevation time

By the intravenous route, DF_{Ji} also produced a significant reduction of incapacitation, but with nearly 4-fold lower doses. The averaged PET values were 14.8 ± 0.3 s, 17.8 ± 0.7 s, and 14.5 ± 0.4 s ($p < 0.001$) for the doses of 10 mg/kg, 25 mg/kg, and 50 mg/kg, respectively (Figure 2). Vehicle treated animals showed an averaged PET of 23.7 ± 1.6 s. Intravenous administration of dexamethasone at a dose of 1 mg/kg (positive control group) caused a decrease of incapacitation (11.6 ± 0.3 s), similar to that produced after DF_{Ji} intravenous administration. The administration of the DF_{Ji} at doses of 1 mg/kg and 0.1 mg/kg did not produce an effect on PET (data not shown).

Figure 2. Effect of intravenous administration of the *J. isabellei* dichloromethane fraction (10, 25 and 50 mg/kg) and dexamethasone (DEX, 1 mg/kg) on paw elevation time (PET). The animals were treated 2 hours after the intra-articular carrageenan injection (300 µg/knee). The negative control group received the vehicle (DMSO: polysorbate 80: saline solution 5:4:91). ***$p < 0.001$ represents a significant difference compared with the negative control group. The statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test.



3.1.3. Effect of the oral administration of the DF_{Ji} on the rat paw edema

A single 300 µg intra-articular injection of carrageenan produced a progressive increase in the rat knee-joint diameter, indicating the development of edema. Oral treatment with the DF_{Ji}, at doses of 50, 100, and 200 mg/kg partially prevented the average edema (Figure 3a). This edematogenic response was insensitive to orally administered indomethacin (5 mg/kg) and colchicine (30 mg/kg), while dexamethasone (10 mg/kg) was able to significantly reduce the articular diameter ($p < 0.001$). There was no statistical difference in the articular diameter between the groups that received the DF_{Ji} and dexamethasone (Figure 3b).

3.1.4. Effect of the intravenous administration of the DF_{Ji} on rat paw edema

By intravenous route, the DF_{Ji}, was able to prevent the increase of the articular diameter only at dose of 10 mg/kg ($p < 0.001$). No statistical difference was found in the paw diameter between the group receiving the DF_{Ji} and the group receiving dexamethasone at doses of 10 mg/kg and 1mg/kg, respectively (Figure 4). Similarly to what was verified for the PET values, the intravenous administration of the DF_{Ji} at doses of 1 and 0.1 mg/kg did not have an effect on edema (data not shown).

Figure 3. Effect of oral administration of the *J. isabellei* dichloromethane fraction (DF_{Ji}) on the rat paw edema. (A) Articular diameter (AD) of animals receiving different doses of the DF_{Ji}. (B) Articular diameter of animals receiving DF_{Ji} (200 mg/kg) and the positive controls indomethacin (IND, 5 mg/kg), colchicine (COL, 30 mg/kg), and dexamethasone (DEX, 10 mg/kg). The animals were treated 2 hours after the intra-articular carrageenan injection (300 µg/knee). The negative control group received the vehicle (DMSO: PEG 400: PBS 5:47.5:47.5). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ represents a significant difference compared with the negative control group on the day of the experiment. The statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test.

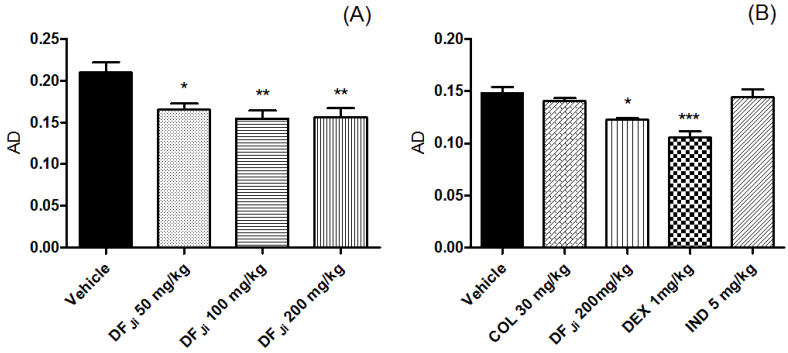
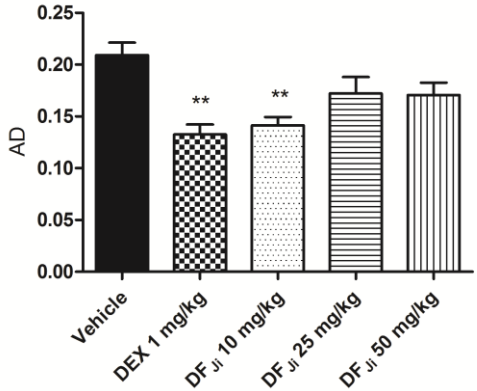


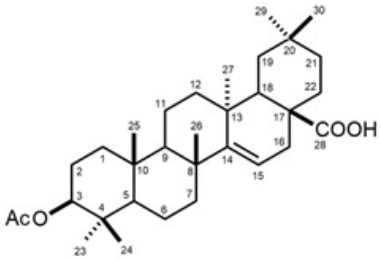
Figure 4. Effect of intravenous administration of the *J. isabellei* dichloromethane fraction (DF_{Ji}) and dexamethasone (DEX, 1 mg/kg) on rat paw edema. The animals were treated 2 hours after the intra-articular carrageenan injection (300 µg/knee). The negative control group received the vehicle (DMSO: polysorbate 80: saline solution 5:4:91). ***p* < 0.01 represent a significant difference compared with the negative control group. The statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test.



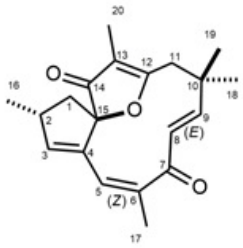
3.2. Isolation and identification of the diterpene jatrophone from the DF_{Ji}

The dichloromethane fraction was submitted to column chromatography and gave the sub-fractions 19-20 and 24-25 which, after precipitation with hexane, resulted in the compound **1** (2.4 mg) and **2** (5.0 mg), respectively (Figure 5).

Figure 5. Structure of acetyl aleuritolic acid (1) and jatrophone (2) isolated from DF_{Ji}.



1



2

Compound **1** was identified as acetyl aleuritolic acid by matching its retention factor value obtained in the TLC analysis with that obtained for the standard compound. This triterpene was previously isolated from *J. isabellei* of combined petroleum ether and ethyl acetate fractions by Pertino et al. (2007b) and from the dichloromethane fraction by Fröhlich et al. (2013).

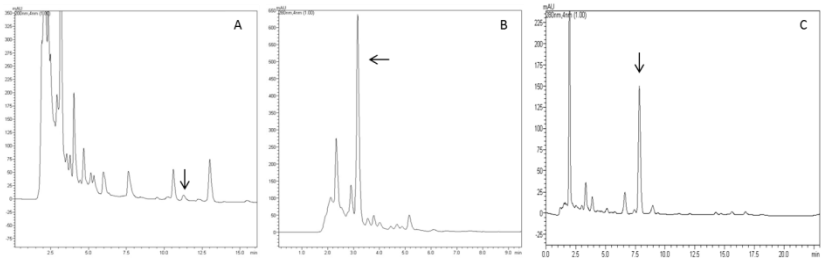
Compound **2** was identified as jatrophone and was obtained as colorless crystals (5.00 mg). ^{1}H NMR (acetone-d_6, 600 MHz) assignments of jatrophone were as follows: δ H (ppm) 2.15 (1H, dd, *J*=13.6; 5.9 Hz, H-1a), 1,74 (1H, dd, *J*=13.6; 7.8 Hz, H-1b), 2.92-2.99 (1H, m, H-2), 5.68 (1H, m, H-3), 5.71(1H, m, H-5), 5.91 (1H, d, *J*=16.3 Hz, H- 8), 6.58 (1H, d, *J*=16.3 Hz, H-9), 3.04 (1H, d, *J*=15.1 Hz, H-11a), 2.47 (1H, dd, *J*=15.1; 0.8 Hz, H-11b), 1.08 (3H, d, *J*=7.1 Hz, H-16), 1.83 (3H, d, *J*=1.6 Hz, H-17), 1.25 (3H, s, H-18), 1.37 (3H, s, H-19) and 1.67 (3H, d, *J*=0.8 Hz, H-20). ^{13}C NMR (acetone-d_6, 150 MHz) were as follows: δ C (ppm) 43.2 (C-1), 39.3 (C-2), 147.1 (C-3), 139.2 (C-4), 123.9 (C-5), 143.6 (C-6), 201.7 (C-7),129.4 (C-8), 160.3 (C-9), 37.5 (C-10), 41.6 (C-11), 184.4 (C-12), 113.1 (C-13), 203.6 (C-14), 100.3 (C-15), 19.7 (C-16), 21.1 (C-17), 30.6 (C-18), 27.4 (C-19) and 6.4 (C-20). The ^{13}C NMR shifts as well as the 2D long-range heteronuclear correlations (HMBC) observed for compound **2** are in agreement with the data previously reported for the diterpene jatrophone (GOULART et al., 1993; BATISTA et al., 2014), although, recent studies have demonstrated results in which

chemical shifts for carbons C-3 and C-5 of this compound were inverted (FERNANDES et al., 2013; SAHIDIN, 2013). The mass spectrum indicated a molecular weight of 313.17980 *m/*z $[M+H]^+$, with 0.06 ppm of error (calcd for $C_{20}H_{24}O_3H^+$, $[M+H]^+$: 313.17982 *m/*z). The UV/VIS spectrum obtained in acetonitrile showed a λ_{max} of 280 nm.

3.3. Development and validation of the UFLC-DAD method

For the development of the analytical method by UFLC-DAD, several conditions were previously tested, including different proportions of acetonitrile:water as eluent and flow rates from 0.8 to 1.0 mL/min. A first separation of the terpenic compounds was carried out using a C18 column and a mobile phase gradient starting with 90% of acetonitrile and 10% of water, followed by an isocratic condition with 100% of acetonitrile with a flow rate of 0.8 mL/min. Using these conditions, it was possible to separate acetyl aleuritolic acid, which showed retention time of 10.44 min (Figure 6a). The presence of acetyl aleuritolic was verified by comparing the retention times and by spiking the sample with the triterpene before the UFLC analysis. On the other hand, the presence of a large peak displaying retention time of 3.06 min was visualized in this UFLC chromatogram, when the detector was set at 280 nm (Figure 6b). The identification of this compound was performed by mass analysis in a mass spectrophotometer (Bruker micro TOF-QII, source type APPI, operating in positive mode) coupled to the LC system. The result indicated that this peak corresponded to the diterpene jatrophone. Since it appeared to be an important compound of this fraction, the chromatographic conditions were changed in order to obtain a better resolution of this peak in order to use this compound as a possible chemical marker of the DF_{Ji}. To obtain a better separation of jatrophone, the mobile phase was eluted at flow rate of 1.0 mL/min in a gradient mode starting from 53:47 acetonitrile:water, which was increased to 65% acetonitrile over 12 min and then to 75% acetonitrile over 15 min of analysis. In such conditions, the total run time was 23 min and jatrophone retention time was 7.8 min (Figure 6c).

Figure 6. UFLC-DAD chromatograms obtained for DF_{Ji}. (A) Detection at 200 nm, indicating the acetyl aleuritolic acid, (B) detection at 280 nm, enlarged, indicating the diterpene jatrophone, (C) detection at 280 nm exhibiting the jatrophone compound with a good resolution.



The method developed to quantify jatrophone in the DF_{Ji} was validated according to the ICH and the ANVISA guidelines (BRAZIL, 2003; ICH, 2005). The calibration curve (y = 41010 x – 270.76) obtained for jatrophone showed a correlation coefficient (r^2) of 0.9997, indicating that the method is linear over the concentration range from 1.0 to 100.0 µg/mL. The lowest amount of jatrophone, which could be detected (LOD) and quantitatively determined (LOQ), was 0.04 µg/mL and 0.15 µg/mL, respectively. Measurements of intra- and inter-day were used to determine the precision of the method and evaluated by the relative standard deviation (RSD%). The intra-day and inter-day precision analyses indicated RSD of 1.73% and 1.94%, respectively. The accuracy of the UFLC method was assessed by the recovery data. The recovery values obtained for the jatrophone-spiked dichloromethane fraction were higher than 90% for the three levels evaluated (Table 1), and this result can be considered acceptable for the analysis of the compounds in a complex matrix as a plant extract. The peak purity index found was 0.999948, indicating that jatrophone is clearly separated from any interfering peak, demonstrating the specificity of the developed method (ICH, 2005).

Table 1. Recovery values (%) obtained for the evaluation of the accuracy of the method.

Constituent	Spiked (µg/mL)	Found (µg/mL) (s.d.)*	Recovery (%)
Jatrophone	5.45	5.86 (0.20)	107.65
	27.25	26.19 (0.09)	96.13
	81.75	81.38 (0.43)	99.54

* n=3, triplicate injection

Considering the results described above, the UFLC method was found to be linear, specific, precise, and accurate to determine jatrophone in the *Jatropha isabellei* dichloromethane fraction. After analysis, the results indicated the presence of 89.68 ± 1.55 µg of jatrophone per milligram of extract, which correspond a concentration of 8.97% (w/w) of this diterpene in the dichloromethane fraction.

4. Discussion

In this study, the effects of the orally and intravenously administered dichloromethane fraction from *J. isabellei* (DF_{Ji}) for articular pain and edema were evaluated using the hind paw elevation time and knee diameter of rats sensitized with an intra-articular injection of carrageenan. The dichloromethane fraction reduced both incapacitation and articular edema after oral and intravenous administration, indicating that the chemical constituents in this fraction demonstrated antinociceptive and antiedematogenic properties. Only the DF_{Ji} and dexamethasone were effective in reducing both parameters by both administration routes.

Previous studies have addressed the likely anti-inflammatory and antinociceptive mechanisms of the terpenoid constituents found in the $DF_{Ji.}$ The diterpene jatrophone, which may be considered as a possible chemical marker of the DF_{Ji},

has previously shown to inhibit lymphocyte proliferation, presumably through inhibition of the protein kinase C (PKC) pathway, which in turn mediates a number of intracellular signaling pathways involved in the pathogenesis of inflammation (MORAES; RUMJANEK; CALIXTO, 1996). Other constituents previously isolated from the DF_{Ji}, as sitosterol and acetyl aleuritolic acid, also have demonstrated an anti-inflammatory effect on edema induced by carrageenan (PERAZZO et al., 2007; BHALKE; PAL, 2012). On the other hand, the lack of an antiedematogenic effect with the higher doses of DF_{Ji} after intravenous administration may be explained, at least in part, by a vasodilating effect of jatrophone. The vasodilating effect of this drug was demonstrated in the portal vein and aorta of the rats and it was attributed to the inhibition of a PKC-dependent mechanism (SILVA; BRUM; CALIXTO, 1995), as well as the inhibition of Ca^{2+} influx and activation of K^{+} channels (DUARTE; SANT'ANA; CALIXTO, 1992). This vasodilation could be attenuating its antiedematogenic effect. Furthermore, jatrophone was able to inhibit the [^{3}H] glutamate binding in a dose-dependent way, which also supported an antinociceptive effect of this compound (MARTINI et al., 2000).

The effective oral dose of the DF_{Ji} was about 20 and 5 times higher than the effective intravenous dose for the antinociceptive and antiedematogenic effect, respectively. These findings are supposed to be due to the incomplete absorption of the chemical constituents of the dichloromethane fraction after oral administration. Many factors affect the oral absorption of drugs, including the anatomical and physiological characteristics of the gastrointestinal tract and the physicochemical properties of the drug. In this case, the lower pharmacological effectiveness of the orally administered DF_{Ji} could be explained in part by the low water solubility of the terpenes and phytosterols present in this fraction (ROSSI et al., 2010; THOPPIL; BISHAYEE, 2011; DUCHATEAU et al., 2012). Thus, formulations that allow the increase of the solubility of the chemical constituents of the DF_{Ji} in the biologic fluids should be developed to overcome their limited oral absorption and to take advantage of the beneficial pharmacological properties of this herbal medicine to treat arthritis.

The selection of chemical markers is very important for the quality control of herbal medicines since their content should be determined at various stages of the development and manufacturing of a product. Some of these stages comprise authentication and differentiation of species, harvest of the best quality raw materials, quality evaluation of intermediates and finished products, stability assessment, and detection of toxic compounds (LI et al., 2008). The diterpene jatrophone was isolated from the DF_{Ji} and quantified by UFLC-DAD method. Because a large amount of this compound was found in this fraction and considering its contribution for the anti-arthritic activity, it was supposed that jatrophone could be used as a chemical marker of the DF_{Ji}. Additionally, this diterpene could be determined by using a UFLC-DAD method, in which the chromatographic conditions allowed to obtain a satisfactory separation of the jatrophone peak from the other constituents of the dichloromethane fraction in a short time and with minimal tail. The chromatograms exhibited a good baseline resolution and the method was successfully validated, according to the results presented above. Taking into account the results obtained in this study, the development of new dosage forms from the *J. isabellei* dichloromethane fraction may be considered as promising research to be undertaken.

5. Conclusion

The dichloromethane fraction from *J. isabellei* Müll Arg. was able to produce potent antinociceptive and antiedematogenic effects in a model of acute arthritis in rats, when administered by both oral and intravenous routes, being nearly as effective as dexamethasone in reducing inflammatory incapacitation and edema. This effect may be related to the presence of terpenes in this fraction, especially the diterpene jatrophone. The pharmacological effectiveness was higher after intravenous administration, indicating that the chemical compounds from DF_{Ji} exhibits limited absorption in the gastrointestinal tract. The UFLC-DAD analytical methodology developed in this study allowed to quantify the jatrophone in the DF_{Ji}, which was suggested to be used as a chemical marker to guide the development of drug dosage forms from this fraction. Thus, the DF_{Ji} may represent a

promising plant product for the development of herbal medicines for the treatment of arthritis.

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Declaration of Interest

The authors report no conflicts of interest.

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CAPÍTULO 2

Manuscrito 2: Development and *in vitro* and *in vivo* evaluation of lipid-based nanocarriers containing *Jatropha isabellei* dichloromethane fraction aiming the oral treatment of arthritic diseases

Development and *in vitro* and *in vivo* evaluation of lipid-based nanocarriers containing *Jatropha isabellei* dichloromethane fraction aiming the oral treatment of arthritic diseases

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Abstract

In this study, the dichloromethane fraction from underground parts of *Jatropha isabellei* (DF_{Ji}) was used to prepare lipid nanocarriers (LNC). Firstly, the fatty acids composition of the DF_{Ji} was characterized by gas-chromatography and evidenced the presence of arachidic acid (C20:0), linoleic acid (C18:2), oleic acid (C18:1), and heneicosanoic acid (C21:0), as major fatty acids. The developed lipid nanocarriers displayed spherical shape and monodisperse size distribution, with mean sizes ranging from 180 to 200 nm and zeta potential values of around –18 mV. High value of entrapment efficiency (> 90%) was obtained for jatrophone, the chemical marker of the DF_{Ji}. LNC_{Ji} and LNC_{blank} stored at 4 °C did not exhibit mean size and zeta potential changes during 120 days and the jatrophone content was unchanged during 60 days. The stability of LNC_{Ji} stored at 4°C was also confirmed by the accelerated stability studies using LUMiSizer. In fasted state simulate gastric fluid (FaSSGF), the release of jatrophone from LNC_{Ji} reached 38.81% in two hours, while the free fraction released only 23.4% in the same period. The release of jatrophone from LNC_{Ji} was also faster than that obtained for the free DF_{Ji} in FeSSIF-old, but was similar in FaSSIF-V2. When tested in an acute arthritis model, the LNC_{Ji}, but not the free fraction, exhibited antinociceptive properties after oral administration at dose of 50 mg/kg. These results suggested that an increase in the oral absorption of the DF_{Ji} constituents occurred by carrying it in LNC, improving its pharmacological activity.

Keywords: *Jatropha isabellei*; lipid nanocarriers; jatrophone; stability study; drug release; antinociceptive activity; carrageenan-induced arthritis model.

1. Introduction

Arthritis is a painful and disabling condition, which involves inflammation of one or more joints with infiltration of inflammatory cells, synovial hyperplasia, cartilage destruction, bone erosion, narrowing of the joint space and ankylosis of the joint (BENDELE et al., 1999). The most common forms of arthritic conditions are osteoarthritis, rheumatoid arthritis, and gout, which, in turn, is responsible for the worst episodes of acute pain (CANNELLA; MIKULS, 2005). The pharmacological treatment of arthritis consists in to reduce joint pain and inflammation or slow down the disease progression by administration of drugs as analgesics, corticosteroids, disease-modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and biological drugs (NEGREI et al., 2016). However, these drugs exhibit severe adverse effects, making patient compliance difficult. This shortcoming have stimulated the research on arthritis treatment, and a great variety of active compounds for developing new medicines can be found in plants (RATES, 2001; KHANNA et al., 2007; GHOSH et al., 2016).

Jatropha isabellei (Müll. Arg.) is a shrub with red-violet inflorescences that belongs to Euphorbiaceae family and its underground parts have been popularly used to treat arthritis, when its dried slices are administered after decoction (BASUALDO; ZARDINI; ORTIZ, 1991). Indeed, antinociceptive and anti-inflammatory properties were demonstrated for the hydro alcoholic extract (70% v/v) obtained from the underground parts of *J. isabellei,* in a rat gout model induced by sodium monourate (MSU) crystals, although it has not been able to alter the uric acid levels increased by potassium oxonate (SILVA et al., 2013). The undergrounds parts of this plant have been chemically characterized by presence of terpenic compounds. In particular, diterpene jatrophone was found as an important constituent of the dichloromethane fraction (DF_{Ji}) obtained from hydro alcoholic extract of *J. isabellei*, besides minor amounts of acetyl aleuritolic acid, and a binary mixture of sitosterol-3-*O*-β-D-glucoside and stigmasterol (FRÖHLICH et al., 2013). In a carrageenan-induced acute arthritis model in rats, the oral and intravenous administration of the dichloromethane fraction was able to significantly reduce the rat paw elevation time (PET) and to

partially prevent the edema formation, after intra-articular carrageenan injection, evidencing both antinociceptive and anti-inflammatory effects of this fraction. In this study, the pharmacological effectiveness was found to be higher after administration by the intravenous route than by oral route, indicating that chemical compounds from DF_{Ji} exhibits limited absorption in the gastrointestinal tract (results submitted elsewhere).

Thus, despite its beneficial pharmacological properties of $DF_{Ji,}$ it is constituted by lipophilic compounds and display low water solubility. To overcome this drawback, lipid-based formulations might be considered an interesting approach to administer the DF_{Ji} by oral route. The ability of lipids to enhance the bioavailability of lipophilic drugs has been ascribed to several mechanisms, including (1) the improvement of the solubility of drugs inside the micelles, mixed micelles, and vesicles formed by stimulating the secretion of bile salts and phospholipids; (2) reduction of the TGI transit, which increases the time available for drug dissolution; (3) interaction of the lipids with enterocyte-based transporters, thereby potentially changing drug uptake, efflux, and the formation of metabolites within the enterocyte; and (4) stimulation of the intestinal lymphatic transport for the highly lipophilic drugs, which can reduce the first-pass drug metabolism in the liver (PORTER; CHARMAN, 2001; PORTER; TREVASKIS; CHARMAN, 2007).

Colloidal lipid carriers, as nanoemulsions, solid lipid nanoparticles and lipid nanocapsules, are formed by different kinds of lipids and surfactants and exhibit different structures, but they present as a common characteristic their particle size below 1 µm. The reduced size of the particles may offer the additional advantage to these lipid formulations of presenting the drug in a dissolved form with a large surface area for drug absorption, which results in a more uniform and reproducible bioavailability (CHAKRABORTY et al., 2009). Moreover, the entrapped drug substances can be protected against degradation by gastrointestinal fluids, while drug absorption through the gastrointestinal epithelium or lymphatic transport can be enhanced (CAI et al., 2010). Finally, lipid carriers present the advantage of low toxicity due to its composition be similar to

physiological lipids, if compared to polymeric nanoparticles (HEURTAULT et al., 2003).

Considering the above mentioned, this paper describes the preparation and characterization of lipid nanocarriers containing the dichloromethane fraction from underground parts of *J. isabellei*, with the aim to improve the oral absorption of the bioactive compounds, with particular interest for the treatment of arthritic conditions. Additionally, *in vitro* drug release, lipid nanocarrier stability, and *in vivo* pharmacological studies are described.

2. Materials and methods

2.1. Materials

Medium chain triglyceride oil (MCT, Ritamollient CCT) was acquired from Brasquim (Brazil). Hydrogenated soybean lecithin (LIPOID S100, 97.5% phosphatidylcholine) and egg lecithin (Lipoid E80, 80% phosphatidylcholine) were purchased from Lipid Ingredients & Technology (Brazil). Sodium lauryl sulfate and anhydrous sodium sulfate were acquired from Vetec (Brazil). Polyethylene glycol 400 (PEG 400) was purchased from Synth (Brazil). Sodium hydroxide and sodium chloride were purchased from Alphatec (Brazil). Polysorbate 80 (Tween 80), sodium taurocholate hydrate, pepsin from porcine gastric mucosa, supelco 37-component fatty acid methyl ester (FAME) mix, and maleic acid were acquired from Sigma-Aldrich (USA). Anhydrous potassium carbonate and acid chloride were purchase from Fluka (USA). Combination of kappa/lambda carrageenan was purchased from BDH Chemicals Ltd. (UK). Dexamethasone was purchased from Deg (Brazil). HPLC and MS grades acetonitrile were acquired from Panreac, Spain and Tedia, USA, respectively, and heptane GC grade from Sigma Aldrich, USA. Analytical grade dichloromethane, dimethyl sulphoxide, acetic acid, acetone and ethanol were acquired from Vetec (Brazil). Water utilized in the HPLC analyses was obtained using a Milli-Q purification system (Millipore, USA).

2.2. Plant collection and preparation of dichloromethane fraction

J. isabellei was collected in the municipality of Cacequi (State of Rio Grande do Sul, Brazil, coordinates: 29°53'01" S and 54°49'30" W) in May of 2008. An exsiccate was archived in the herbarium of the Biology Department at the Federal University of Santa Maria (SMDB 11816). The underground parts were dried at room temperature and powdered in a knife mill. The powder was macerated with 70% (v/v; plant: solvent ratio 1:3 w/v) ethanol for ten days at room temperature. After filtration, the ethanol was evaporated under reduced pressure and this dispersion was partitioned with dichloromethane to obtain its respective fraction which was further taken to dryness under reduced pressure, resulting in the dichloromethane fraction (DF_{Ji}, yield 3.7%).

2.3. Analysis of DF_{Ji} fatty acids by gas chromatography-flame ionization detection (GC-FID)

2.3.1. Sample preparation

For analyses of the fatty acid methyl esters (FAME), the dichloromethane fraction was subjected to methylation reaction. Briefly, an exactly weighted amount of the dichloromethane fraction was dissolved in 2 mL of heptane. Then, 3 mL of a 10% (v/v) chloride acid solution was added to this heptane solution and the mixture was placed in a water-bath at 90 ^{0}C for two hours. After this time, the sample was cooled in an ice bath and then 1 mL of heptane and 10 mL of a 6% anhydrous potassium carbonate (K_2CO_3) solution were added. After shaking, the sample was centrifuged at 500 x *g* for 5 minutes. The supernatant was treated with anhydrous sodium sulfate and kept at rest for 1 hour (CHRISTIE, 1993). The sample was analyzed by GC-FID as described below.

2.3.2. Chromatographic conditions

The analyses were carried on an Agilent 7820A gas chromatography system equipped with a flame-ionization detector. A CP-Sil 88 fused silica capillary column (100 m X 0.25

mm ID. X film thickness 0.20 µm) was used to separate the compounds. Injector and detector temperature were kept at 250 ºC and the injection volume was 1.0 µL with split ratio of 1:50. Hydrogen was used as carrier gas with constant flow of 1.0 mL/min and nitrogen as an auxiliary gas (30mL/min). The oven temperature was set at 45 ºC for 4 min and initially increased to 175 ºC at 13 ºC/min, held for 27 min, and after increased by 4 ºC/min to 215 ºC and held for 35 min (CRUZ-HERNANDEZ et al., 2007). Peaks were identified by comparison of their retention times with those of a Supelco 37-component fatty acid methyl ester (FAME) mix.

2.4. Preparation of lipid nanocarriers

Lipid nanocarriers containing dichloromethane fraction from *J. isabellei* (LNC_{Ji}) were prepared using the spontaneous emulsification method (BOUCHEMAL et al., 2004). Briefly, an organic phase containing 100 mg of medium chain triglyceride oil (MCT), 10 mg of soybean lecithin and 100 mg of *J. isabellei* dichloromethane fraction in 10 mL of acetone:ethanol (7:3, v/v) was slowly added to 50 mL of an aqueous phase containing 0.5% of polysorbate 80, under vigorous magnetic stirring. After, the organic solvents were removed by evaporation under reduced pressure and the colloidal dispersions were concentrated to a final volume of 10 mL. Unloaded lipid nanocarries (LNC_{blank}) were prepared without the DF_{Ji} using the same conditions. All formulations were prepared in triplicate.

2.5. Characterization of lipid nanocarriers

2.5.1. Particle size and zeta potential

The mean particle diameter and zeta potential were determined by dynamic light scattering and laser-Doppler anemometry, respectively, using a Zetasizer Nano Series (Malvern Instruments, UK). The measurements were performed at 25 °C after appropriate dilution of the samples in ultrapure water (MilliQ, Millipore, USA). Each size analysis lasted 100 s and was performed with and angle detection of 173°. For measurement of zeta potential, the diluted samples were placed

in the electrophoretic cell, where a potential of ± 150 mV was established. The zeta potential values were calculated as mean of electrophoretic mobility values using Smoluchowsky's equation.

2.5.2 pH measurements

The pH of LNC dispersions was determined using a pH meter (Oakton pH 5 Acorn Series, USA), previously calibrated with buffer solutions pH 4.0 and 7.0.

2.5.3. Transmission electron microscopy

The morphology of the LNC_{Ji} (diluted 500 x in ultrapure water) was examined by transmission electron microscopy (TEM) using a JEOL JEM-1011 microscope (Tokyo, Japan), with an acceleration voltage of 100 kV. The nanoparticles suspensions were dropped onto carbon-coated copper grids and visualized stained with 1% phosphotungstic acid.

2.5.4. Determination of jatrophone concentration in the lipid nanocarriers by UFLC-DAD

Instrumentation and chromatographic conditions

The ultra-fast liquid chromatography was performed using a Shimadzu UFLC system (Japan) equipped with a LC-20AD binary pump, a SIL-20AC HT auto-sampler, a CTO-20A forced air-circulation-type column oven, a SPD-M20 photo diode array UV/VIS detector, and software LC Solution 1.2 (Shimadzu, Tokyo, Japan). The analyses were carried out in reversed phase mode using a Phenomenex Luna C18 column (150 mm x 4.6 mm x 5 µm) maintained at 30 ^{o}C. The mobile phase was eluted at flow rate of 1.0 mL/min using gradient elution as follows: firstly a gradient of 53:47 acetonitrile: water increased to 65% acetonitrile over 12 min of analyze and after 75% acetonitrile over 15 min. After this time the column was set to initial condition in 3 min and rebalanced under this condition for 4 min. The total runtime was 23 min and the jatrophone retention time was 7.8 min. The injection volume of the samples was 20 µL and the detection of

jatrophone was monitored at 280 nm, according its maximum absorption. This UFLC method was previously validated according the parameters of linearity, specificity, accuracy, precision, and determination of detection and quantification limits (LOD and LOQ) (results submitted elsewhere). According to the chromatograms obtained for unloaded and DF_{Ji}-loaded lipid nanocarriers, this method also was specific for determining jatrophone in the lipid nanocarriers.

Determination of jatrophone content and entrapment efficiency

For UFLC analysis, the LNC_{Ji} were prepared at 0.5 mg/ml of DF_{Ji} in acetonitrile. Then the samples were filtered through a 0.45 µm PVDF membrane (Merck Millipore) and were injected in the chromatograph. The total concentration of jatrophone in the colloidal dispersions was expressed in µg/mL of colloidal suspension.

Entrapment efficiency (%) was estimated as being the difference between the total concentration of jatrophone found in the lipid nanocarriers and the concentration found in the ultrafiltrate obtained after ultrafiltration/centrifugation procedure (5000 rpm/ 10 min), using an ultrafiltration device (Amicon Ultra with Ultracel-100 membrane, 100 kDa MWL, Millipore, USA).

2.6. Stability studies

2.6.1. Short-term stability study

A short-term stability of the unloaded and DF_{Ji}-loaded lipid nanocarriers was carried out after storing the formulations at 4º C or 25º C. The stability of the lipid nanocarriers was evaluated according to their parameters of mean particle size, polydispersity index, zeta potential (during 120 days), and jatrophone content (60 days), as described above.

2.6.2. Accelerated stability studies

The accelerated stability of LNC_{Ji} and LNC_{blank} stored at 4 °C was investigated using an analytical centrifuge LUMiSizer (L.U.M. GmbH, Germany), that consists of a centrifugal rotor with

12 optical cells, and a light source (pulsed near-infrared light-emitting 880 nm diode and a light sensor). This equipment allows the measurement of the intensity of the transmitted near-infrared light as a function of time and position over the entire sample length, while the samples are simultaneously subjected to centrifugal force, providing information about instability phenomena, such as sedimentation, flocculation or creaming. For the analysis, 400 µL of each sample was placed into polycarbonate optical cells and subjected to rotation at 4.000 rpm for 7.650 s, with temperature of 25 °C, and interval time of 30 s according Yuan et al (2013). The samples were analyzed in triplicate without prior dilution. The results were displayed as a space- and time-related transmission profiles over the sample and instability index.

2.7. *In vitro* drug release studies

2.7.1. Release media

The release studies were carried out in biorelevant media. Fasted state simulated gastric fluid (FaSSGF) and the fasted state simulated intestinal fluid (FaSSIF-V2) proposed by Vertzoni et al (2005) and Jantratid et al (2008) were used for simulating the fast gastric and intestinal environments, respectively. The fed state simulated intestinal fluid (FeSSIF 'old') proposed by Wagner et al (2012) was employed for simulating intestinal fed state conditions. Each medium was added of 0.2 % of sodium lauryl sulfate to obtain *sink* conditions (Table 1).

Table 1. Dissolution media simulating gastric (FaSSGF) and intestinal (FaSSIF-V2) fasted state conditions, and intestinal fed (FeSSIF 'old') state conditions.

Composition	FaSSGF	FaSSIF-V2	FeSSIF 'old'
pH	1.6	6.8	5.0
Sodium taurocholate	80 µM	3 mM	15 mM
Lecithin from egg	20 µM	0.2 mM	3.75 mM
Pepsin	0.1 mg/mL	-	-
Sodium chloride	34.2 mM	68.62 mM	203.2 mM
Maleic acid	-	19.12 mM	-
Sodium hydroxide	-	34.8 mM	101 mM
Hydrochloric acid	qs. pH 1.6	-	-
Acetic acid	-	-	144.1 mM
Sodium lauril sulfate	0.2 %	0.2 %	0.2 %

2.7.2. Determination of jatrophone in the dissolution media by UFLC-DAD

The ultra-fast liquid chromatography was performed using the Shimadzu UFLC using the same chromatographic conditions described above. Calibration curves for jatrophone in the different release media were prepared over the concentration range from 0.1 or 0.3 to 15.0 µg/mL. The UFLC method found to be specific to determine jatrophone in the biorelevant media. The calibration

curves of jatrophone indicated the UFLC method was linear over the tested concentration range (R^2 > 0.99). LOD values were 0.02, 0.07, and 0.03 µg/mL, and LOQ values were 0.07, 0.22, and 0.10 µg/mL for FaSSGF, FaSSIF-V2, and FeSSIF 'old' media, respectively, indicating the method was enough sensible to quantitate the released drug.

2.7.3. Experimental

The release studies were carried out using dialysis bag method (LUO et al., 2006). For the experiment, an aliquot of the DF_{Ji}-loaded lipid nanocarrier or the free DF_{Ji} were placed into a dialysis bag (MWCO 14,000, Sigma Aldrich, USA). The dialysis bags were placed into a dissolution apparatus (USP 2, Varian, USA) containing 200 mL of medium per vessel. The release medium was maintained at 37 °C under mechanical stirring at 75 rpm. Sampling times were 0.5, 0.75, 1 and 2 hours for FaSSGF and 0.5, 0.75, 1, 2, 4, 6 and 8 hours for FaSSIF-V2 and FeSSIF 'old'. At each time point, the release medium was immediately replaced with fresh medium. The withdrawn samples were diluted with acetonitrile, frozen and then filtered through 0.45 µm PVDF membranes and analyzed by UFLC. All dissolution experiments were performed in triplicate. The cumulative percentage of jatrophone released in each medium (%) was plotted against time (h). Dissolution efficiency (DE%) was calculated from the area under the dissolution profile and expressed as the percentage of the area of the rectangle described by 100% dissolution within the same period of time. The difference (f1) and similarity (f2) factors were also used to compare the release profiles (PETRO; EROS; CSOKA, 2012).

2.8. Pharmacological activity of LNC_{Ji} in a carrageenan-induced arthritis model in rats

2.8.1 Animals and drugs

All experiments were performed using adult male Wistar rats weighing 250–300 g. The animals were housed under a controlled temperature (21 ± 2°C) on a 12 h light/12h dark cycle with standard lab chow and water *ad libitum* until the

experimental sessions. The animals were acclimatized into the experimental room for at least 30 minutes before the experiments. The experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain (IASP, 1983) and approved by the local committee for the ethical use of animals (P00723/CEUA-UFSC). The DF_{Ji} was resuspended in a mixture composed of dimethyl sulfoxide (DMSO), polyethylene glycol 400 (PEG 400), and phosphate buffered saline (PBS), pH 7.4 (5:47.5:47.5 v/v) for oral administration (p.o.) in rats.

2.8.2. Carrageenan-induced articular incapacitation in rats

Articular incapacitation was induced by the injection of 300 µg of carrageenan (solubilized in 50 µL sterile 0.9% saline) into the right knee joint of rats. In this assay, the animals were stimulated to walk on a revolving steel cylinder (constant speed of 3 rpm) wearing metallic gaiters in the hind paws. The right gaiter was connected to a computer system that counts the time spent with the paw lifted from the cylinder surface during one minute test period. This paw elevation time (PET), in seconds, was taken as an estimate of nociception (TONUSSI; FERREIRA, 1992). Two hours after carrageenan injection the animals were treated with DF_{Ji} orally (gavage) (50 mg/kg) or LNC_{Ji} (50 mg/kg). PET was evaluated hourly from the third to the sixth hour, and expressed as an average of these time points. Oral dexamethasone (10 mg/kg) was used as positive control and the negative control groups were treated with the vehicle or LNC_{blank}.

2.8.3. Edema measurement

The articular diameter (AD) was used to quantify the inflammatory edema induced by carrageenan and it was taken by measuring the medio-lateral axis (in mm) of the knee-joint, using a micrometer, at three consecutive arbitrary points in a proximo-distal direction. The AD measured just before carrageenan injection was subtracted from AD values taken hourly from the 3rd to the 6th hour, just after the incapacitation measurements, and presented as an average of these time points.

2.9. Statistical analyzes

Data were expressed as mean ± standard deviation (s.d.) or relative standard deviation (RSD) and mean ± S.E.M. The statistical significance was assessed by t- test or one-way analysis of variance (ANOVA) followed by post-hoc Tukey or Dunnett's Multiple Comparison test. P values lower than 0.05 ($p < 0.05$) were considered indicative of significance. The sample size for incapacitation and articular edema were estimated using a statistical power test, and a minimum of six animals were used for both parameters.

3. Results and discussion

3.1. Analysis of fatty acids by GC-FID

Previous studies concerning the chemical characterization of the dichloromethane fraction obtained from the underground parts of *J. isabellei* revealed this extract is constituted mainly by terpenic coumpounds, in which the jatrophone was found to be the an important diterpene, making around 9 wt% of the extract (results submitted elsewhere). Considering dichloromethane is a non polar solvent that has been used to extract lipids and fatty acids from plants (CEQUIER-SÁNCHEZ et al., 2008) and due to the fact the concentration of these compounds could to impact the preparation of lipid nanocarriers, the fatty acid composition of this fraction was determined by CG-FID. The analyses of dichloromethane fraction showed the presence of 30 compounds of which 9 compounds were identified, representing 50.49% of the total fatty acid composition. The major fatty acids found in the dichloromethane fraction were arachidic acid (20:0; 19.82%), linoleic acid (C18:2; 12.64%), oleic acid (C18:1; 8.05%), and heneicosanoic acid (21:0; 7.06%), followed by small amounts of linolelaidic acid (C18:2), tridecanoic acid (C13:0), myristic acid (C14:0), lauric acid (12:0) and capric acid (10:0). The presence of these fatty acids was also related in the oil seeds of *Jatropha* genus plants, which have been studied for biodiesel production (BERCHMANS; HIRATA, 2008; AKBAR et al., 2009). Here, the characterization of the dichloromethane fraction in terms of fatty acid composition will be useful for the development of lipid drug

delivery systems, since this feature may affect the physicochemical properties and stability of the final drug products.

3.2. Preparation and characterization of lipid nanocarriers

In this study, unloaded and DF_{Ji}-loaded lipid nanocarriers were prepared by the spontaneous emulsification method, which consists in a low energy method, where the very fast diffusion rate of the water-miscible organic solvent towards to the aqueous phase leads to the spontaneous formation of droplets in submicron-size range. The physicochemical and drug loading properties of the lipid nanocarriers obtained in this study are summarized in Table 2. Both unloaded and DF_{Ji}-loaded lipid nanocarriers displayed mean size ranging from 180 to 200 nm and polydispersity index (PDI) lower than 0.20, indicating the formation of colloidal dispersions with monodisperse size distribution. The lipid nanocarriers exhibited negative surface charge, with zeta potential values of -19.6 mV and -18.8 mV for LNC_{Ji} and LNC_{blank}, respectively. The zeta potential values are similar for LNC containing dichloromethane fraction and without fraction, thus, the presence of the fraction seems not affect the zeta potential of LNC. The negative values may be caused by the presence of small amounts of acidic lipids, such as phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol in the soybean lecithin which are preferentially located on the LNC surface (SCHUH; BRUXEL; TEIXEIRA, 2014). In general, it has been accepted that zeta potential values higher than |30| mV are required for electrostatic stabilization of colloidal dispersions. However, non-ionic surfactants, as the polysorbate 80, are able to stabilize nanodispersions by steric effect, instead electrostatic stabilization (KRONBERG et al., 1990). The pH of the formulations was 4.13 ± 0.1 and 5.25 ± 0.2 for LNC_{Ji} and LNC_{blank}, respectively.

Jatrophone was used as chemical marker to monitor the encapsulation of the DF_{Ji} in the lipid nanocarriers, since it was found as an important constituent of this fraction. The entrapment efficiency obtained for the chemical marker was higher than 90%, which may be explained by the high lipophilicity this bioactive

compound and, therefore, by its high affinity for the internal phase of the colloidal dispersion.

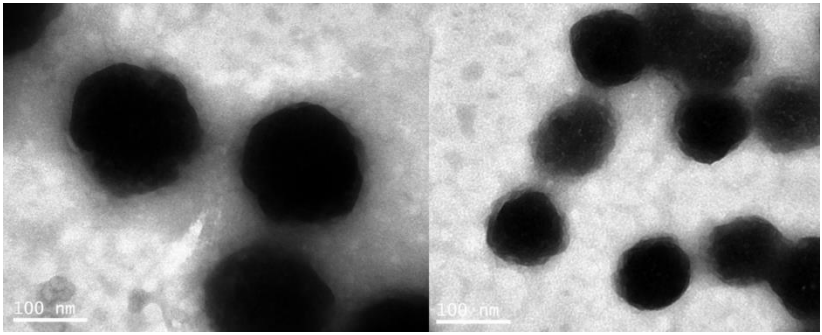
Table 2. Physicochemical and loading properties of the lipid nanocarriers.

	Mean size (nm)(PDI)[1]	**Zeta potential (mV)**	**pH**	**Drug content (µg/mL)***	**EE (%)[2] (s.d.)**
LNC_{blank}	191.1± 4.6 (0.15)	-18.8 ± 4.4	5.25 ± 0.2	-	-
LNC_{Ji}	182.9 ± 2.7 (0.09)	-19.6 ± 1.8	4.13 ± 0.1	867.2 ± 11.1	90.4 ± 0.9

[1]Polydispersity index, [2]Entrapment efficiency. *µg of jatrophone per mL of LNC_{Ji}.

The transmission electron microscopy images of LNC_{Ji} negatively stained with phosphotungstic acid exhibited spherical particles with size around 100 - 200 nm (Figure 1).

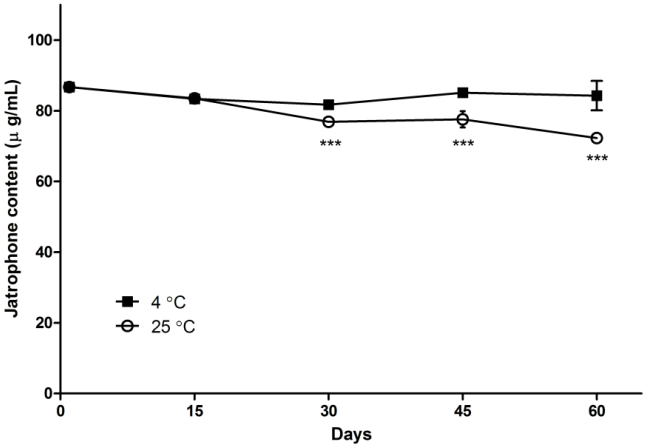
Figure 1. TEM images of LNC_{Ji} negatively stained with 1% phosphotungstic acid.



3.3. Stability studies

To evaluate the short-term stability of the LNC_{Ji}, the jatrophone content was determined during two months, after storing the colloidal dispersions at 25 ± 2°C and 4 ± 2°C temperatures. The formulations stored at 4°C did not have the content of jatrophone changed during 60 days. On the other hand, LNC_{Ji} stored at 25°C showed a significant ($p < 0.001$) decrease on jatrophone content after a 30, 45 and 60 day-storage period when the jatrophone content reduced from 86.7 ± 1.1 µg/mL to 76.8 ± 0.01, 77.6 ± 2.2 and 72.3 ± 0.2 µg/mL, respectively (Figure 2).

Figure 2. Jatrophone content (± s.d.) in the LNC_{Ji} after storing at 25 ± 2°C and 4 °C ± 2°C. ***p < 0.001 when compared to initial jatrophone content (n=3). The statistical analysis was performed using one-way ANOVA followed by Tukey *post hoc* test.



The particle size and zeta potential of the lipid nanocarriers were monitored during three months after storing the formulations at the same temperatures. No changes in the mean size and polydispersity index (PDI) of the unloaded and DF_{Ji}-loaded lipid nanocarriers were observed, when the colloidal dispersions were stored at 4 ± 2°C during 120 days (Figure 3A, B). The zeta potential also remained unchanged for both LNC_{blank} and LNC_{Ji} during this time (Figure 3C). On the other hand, the lipid nanocarriers were not stable when stored at 25 ± 2°C. LNC_{blank} stored at 25°C remained monodisperse only up to 30th day. In the 45th day, the mean particle size of LNC_{blank} reached 258.6 nm with a mean PDI of 0.420 (Figure 3A,B) and a visible but reversible creaming could be seen. It was not possible to carry out the measurement of the particle size from the 60th day of storage, since the formulations exhibited a complete phase separation. LNC_{Ji} displayed a monodisperse system only up to 15th day, exhibiting an increase in the mean particle size and PDI

from 182.9 to 216.0 nm and from 0.092 and 0.144, respectively. At the 30th and 45th days LNC_{Ji} exhibited polydisperse systems with PDI values reaching 1.0 (Figure 3B). A complete and irreversible phase separation could be verified at 60th day. A slight increase in the zeta potential values of the lipid nanocarriers was observed after 45 day-storage period at 25°C, reaching values of -22.5 mV and -23.0 mV for LNC_{blank} and LNC_{Ji}, respectively (Figure 3C).

The high stability of LNC_{Ji} when stored at 4°C was also confirmed by the accelerated stability studies using LUMiSizer. The figure 4 illustrates the representative light transmission profiles averaged over the height of the sample for unloaded (LNC_{blank}) and dichloromethane fraction-loaded lipid nanocarriers (LNC_{Ji}). The light transmission profiles of the LNC_{blank} revealed a clarification area – increase of the light transmission – at the bottom of the cuvette, possible due to initial creaming phenomenon. The colloidal dispersion stability could also be described by the instability index values. In this assay, the instability index obtained for LNC_{blank} was 0.536 ± 0.083, while LNC_{Ji} exhibited an instability index of 0.083 ± 0.004. The lower instability index obtained for LNC_{Ji} indicated the presence of dichloromethane fraction contributes to the physical stabilization the colloidal dispersion, probably due to the presence of lipid compounds as fatty acids in its composition.

Figure 3. (A) Particle size (± s.d.), (B) PDI values (± s.d.) and zeta potential values (± s.d.) obtained for LNC_{blank} (circles) and LNC_{Ji} (squares) stored at 25 ± 2°C (open symbols) and 4 ± 2°C (closed symbols) temperatures (n=3).

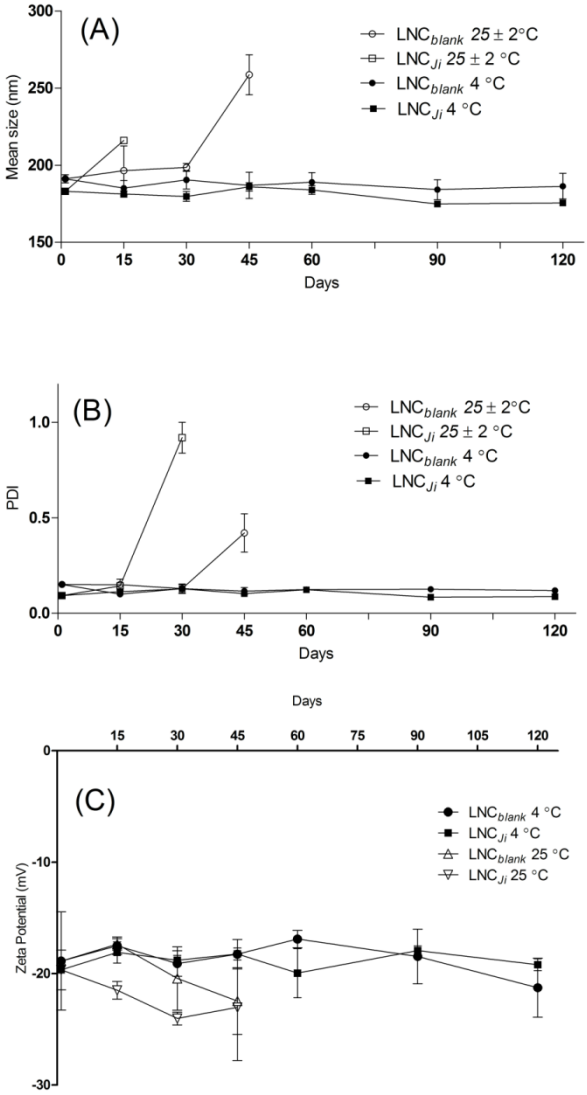


Figure 4. Light transmission profiles through the cell of samples, stored at 4 °C, subjected to centrifugation at 4.000 rpm. (A) LNC_{blank} and (B) LNC_{Ji}.

3.4. *In vitro* drug release studies

It is well known that two of the main process that influence the absorption of orally administered drugs are the dissolution of the drug in the biological fluids at the site of absorption and the transport of the drug through the gastrointestinal mucosa to reach the systemic blood circulation (WAGNER et al., 2012). In this way, drug release studies in biorelevant media constitute an interesting approach to predict *in vivo* performance of lipid oral dosage forms, since these media better simulate the

physiological conditions in the stomach and small intestine. Since jatrophone was found to be as an important compound of DF_{Ji}-loaded lipid nanocarriers, the release studies were carried out by the measuring the concentration of this diterpene in the FaSSGF, FaSSIF-V2 and FeSSIF 'old' media. The jatrophone solubility was previously determined in each media and que amount used for these studies followed the *sink* conditions. In this study, the release profile of jatrophone from LNC_{Ji} was compared with that of the free drug obtained after dispersion the dichloromethane fraction directly in the release media.

The jatrophone release profiles are shown in Figure 5. In FaSSGF, the release of jatrophone from LNC_{Ji} reached 38.81% in two hours, while only 23.4% of this diterpene was released when the free fraction was tested, in the same time. The cumulative percentages of released drug were 69.3% and 86.6 % in FaSSIF-V2, and 61.0% and 104.2% in FeSSIF-'old", for the free fraction and LNC_{Ji}, respectively. In order to compare the jatrophone release profiles obtained for LNC_{Ji} and free DF_{Ji}, the difference (f1) and similarity (f2) factors, as well as the dissolution efficiency values (DE%) were calculated (Table 3). For releasing profiles to be considered similar, f1 values should be between 0 and 15 whereas f2 values should be between 50 and 100 (PETRO; EROS; CSOKA, 2012). Although the FDA endorses both equations of difference (f1) and similarity (f2) factors as acceptable methods for dissolution profile comparison, the f2 equation has been preferred to compare release profiles (O'HARA et al., 1998). So, taking into account the f2 values showed in Table 3, the release profile of jatrophone from LNC_{Ji} and from free fraction were different in FaSSGF and FeSSIF, but they were similar in FaSSIF. This result was confirmed by the dissolution efficiency values (DE%), since they were demonstrated to be statistically different only when release studies were carried out in FaSSGF and FeSSIF ($p < 0.001$) (Table 3).

In this study, the ability of LNC in to carry the DF_{Ji} in a very fine aqueous dispersion, increasing the release rate of a lipophilic drug as jatrophone was clearly demonstrated in the Figure 5. The differences found in the jatrophone release rate was a reflex not only of the pH, buffer capacity, and osmolarity of the biorelevant media, but also of the presence of physiological

surface active species such as bile salts and phospholipids. In particular for lipophilic drugs, as those belonging to the class II of BCS, dissolution behavior has been demonstrated to be strongly influenced by the presence of these natural surfactants (GALIA et al., 1998). In this study, the percent of jatrophone released from LNC_{Ji} after 2 h in FaSSGF was almost twice higher than that released from the free fraction. In FeSSIF-'old', the large amount of emulsifying components of media (15 mM sodium taurocholate and 3.75 mM of egg lecithin) contributed to the higher solubilization of the jatrophone, allowing the increase in the jatrophone release rate, especially from LNC, in which the amount of released drug was around 40% higher than that verified for the free fraction. On the other hand, in FaSSIF-V2, the similarity between the release profiles may be explained by the solubility of jatrophone in this media, due to a combination between the presence of lecithin and sodium taurocholate and the pH, which favored the jatrophone solubilization from the free fraction.

Figure 5. *In vitro* jatrophone release (± SD) from LNC_{Ji} and from free fraction in fasted state simulate gastric fluid (FaSSGF) (A), fasted state simulate intestinal fluid (FaSSIF) (B) and fed state simulate intestinal fluid (FeSSIF) (C) at 37 °C (n=3).

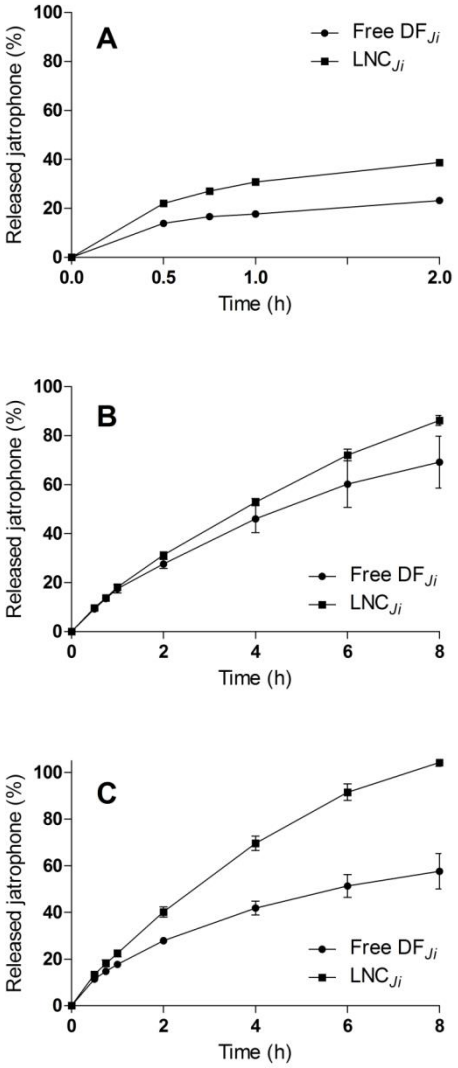


Table 3. Difference (f1), similarity (f2) and dissolution efficiency (DE,%) values calculated from the jatrophone release profiles.

	FaSSGF		**FaSSIF-V2**		**FeSSIF-'old'**	
	Free DF_{Ji}	LCN_{Ji}	Free DF_{Ji}	LCN_{Ji}	Free DF_{Ji}	LCN_{Ji}
DE (%)	16.0* ± 0.5	26.8* ± 0.7	42.6 ± 5.6	50.2 ± 1.3	38.1$^{\#}$ ± 3.0	63.8$^{\#}$ ± 2.4
f1	66.41		17.34		58.97	
f2	47.27		53.07		29.95	

* and $^{\#}$ represented statistical difference at $p < 0.001$.

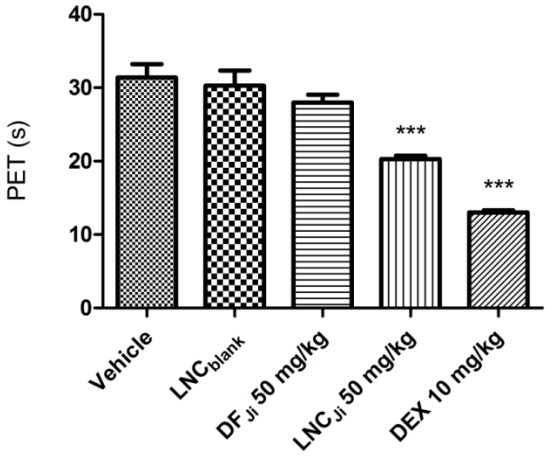
3.5. Pharmacological activity of LNC_{Ji} in a carrageenan-induced arthritis model in rats

The effect of oral administration of free DF_{Ji} and DF_{Ji}-loaded LNC at dose of 50 mg/kg is demonstrated in Figure 6. Animals that received only the vehicle p.o. or LNC_{blank} p.o., two hours after knee-joint carrageenan injection (negative control groups), exhibited an averaged PET values increasing from 9.4 ± 0.4 and 9.7 ± 0.7 s at basal levels to 31.4 ± 1.8 s and 30.3 ± 2.0 s, respectively (Figure 6). The animals receiving the free DF_{Ji} did not exhibit a significant reduction in averaged PET value, when compared to the negative control groups, remaining at 27.9 ± 1.0 s. On the other hand, when the animals were treated with LNC_{Ji} at the same dose, a significant reduction in the averaged PET values was verified, reaching 20.2 ± 0.5 s ($p < 0.001$). The effect obtained with the administration of DF_{Ji} on PET was compared with the oral administration of dexamethasone (10 mg/kg), a drug commonly used to treat the arthritis. The administration of this drug caused a significant reduction in the averaged PET values to

13.0 ± 0.3 s, when compared with the negative controls group. These findings could be explained, at least in part, by the ability of LNC_{Ji} to increase the oral absorption of the dichloromethane fraction constituents that are solubilized into the oil phase of the colloidal dispersions, especially the diterpene jatrophone, which is thought to be related to the antinociceptive properties due their capacity to inhibit the [^{3}H] glutamate binding in a dose-dependent way (MARTINI et al., 2000). These results are in accordance with the *in vitro* release studies that indicated a faster release rate of jatrophone from LNC_{Ji} in the biorelevant media, pointing to that the dissolution of the DF_{Ji} constituents is a rate-limiting step to their absorption in the gastrointestinal tract.

In contrast, the oral administration of LNC_{Ji} was not able to significantly reduce the knee-joint edema in rats, when compared with the free DF_{Ji} administered at the same dose (data not shown). In this case, the increased absorption of DF_{Ji} could also be reverting the antiedematogenic effect, due to a reported vasodilating effect of jatrophone. Such vasodilating effect of jatrophone was demonstrated on portal vein and aorta and it was related to the inhibition of PKC dependent mechanisms (SILVA; BRUM; CALIXTO, 1995), and also to the blockade of the Ca^{2+} influx through voltage-sensitive channels and activation of K^+ channels (DUARTE; SANT'ANA; CALIXTO, 1992), respectively. Considering the results of the *in vivo* studies, the beneficial effect observed in the popular use of the *J. isabellei* to treat arthritis may at least in part related to the antinociceptive properties of terpene compounds from this plant. In this case, the encapsulation of the DF_{Ji} into LNCs would allow to increase the absorption of the lipophilic constituents of these fraction, which presents a great potential to became a new herbal medicine for the treatment of the arthritic conditions.

Figure 6. Effect of the LNC_{Ji} and DF_{Ji} on paw elevation time (PET) after oral administration. The animals were treated 2 hours after the intra-articular carrageenan injection (300 µg/knee). The negative control group received the vehicle DMSO: PEG 400: PBS (5:47.5:47.5) or LNC_{blank}. Dexamethasone (DEX, 10 mg/kg, p.o.) was used as positive control. ***$p < 0.001$ represents a significant difference compared with the vehicle and LNC_{blank}. The statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test.



4. Conclusion

Lipid nanocarriers containing dichloromethane fraction from underground parts of *J. isabellei* were successfully obtained by the use of the spontaneous emulsification method and exhibiting spherical shape, monodisperse size distribution, and high entrapment efficiency of jatrophone. The stability of the lipid nanocarriers was dependent on the storage temperature and the high stability of LNC at 4 °C was confirmed by an accelerated stability study. The studies of drug release in biorelevant medium showed that the jatrophone was released from LNC in FaSSFG and FeSSIF-old at a faster rate, when compared with the free DF_{Ji}. Besides, the pharmacological studies in a carrageenan-

induced arthritis model demonstrated that the oral administration of LNC_{Ji} at dose of 50 mg/kg exhibits antinociceptive properties, reducing significantly the PET values, differently of the free DF_{Ji} that was not able to reduce the PET values. The results indicated that carrying DF_{Ji} in LNC promotes the oral absorption of the drug, improving its therapeutic efficacy and making it a promising herbal medicine to treat arthritis.

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Declaration of interest

The authors report no conflicts of interest.

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CAPÍTULO 3

Manuscrito 3: Preparation, characterization and in vivo evaluation of the antinociceptive and anti-inflammatory activities of intravenously-administered PEG-coated lipid nanoparticles containing terpenic constituents of *Jatropha isabellei*

Preparation, characterization and in vivo evaluation of the antinociceptive and anti-inflammatory activities of intravenously-administered PEG-coated lipid nanoparticles containing terpenic constituents of *Jatropha isabellei*

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Abstract

Jatropha isabellei (Müll Arg) is a shrub with red-violet inflorescences, which its underground parts have been commonly used in the popular medicine to treat different rheumatic diseases. In fact, the dichloromethane fraction obtained from underground parts of *Jatropha isabellei* (DF_{Ji}) has been demonstrated antinociceptive and anti-inflammatory activities, due to the presence of terpenic constituents, mainly jatrophone, which making it a promisor raw material to obtain new herbal medicines. In this study, DF_{Ji}-loaded lipid nanocarriers intended for intravenous administration were developed, aiming to obtain drug delivery systems that could to release the terpenic constituents preferentially to the inflamed joints and compare its effective dose with that presented by oral route. The lipid nanocarriers were prepared with differents amounts of DSPE PEG_{2000} and characterized according to its size and surface properties, jatrophone release rate, hemolysis rate, and macrophage uptake. The antinociceptive and antiedematogenic effects of LNC_{Ji} were demonstrated in a carrageenan-induced arthritis model in rats. The results revealed that the addition of DSPE-PEG_{2000} to the formulations increased both the physical stability and hemocompatibility of the nanocarriers and reduced the macrophage uptake. In special, the antiedematogenic effect of LNC_{Ji}-DSPE-PEG 2000_{90} was higher than the free fraction, probably due to a modification in the pharmacokinetic profile of terpenic constituents of DF_{Ji}.

Keywords: *Jatropha isabellei*, pegylated lipid nanocarriers, hemocompatibility, macrophage uptake, drug release studies, antinociceptive and anti-inflammatory activities.

1. Introduction

Arthritis is an inflammation disorder that affects one or more joints, in which the major complaint by affected individuals is joint pain (BENDELE et al., 1999; LEE, 2013). The most common forms of arthritic conditions are osteoarthritis, rheumatoid arthritis, and gout, being the latter responsible for the worst episodes of acute pain (CANNELLA; MIKULS, 2005). The pharmacological treatment for arthritic diseases includes the administration of analgesics, corticosteroids, disease-modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and biological drugs (NEGREI et al., 2016). However, these drugs cause severe adverse effects, which may lead to the non-compliance of the patient with drug therapy.

Traditional medicine using plants have been practiced worldwide for treatment of arthritis, especially in developing countries. The popular use of plants for treating arthritic conditions have stimulated the investigation of anti-arthritic activity and the development of many herbal medicines for such purpose (CHOUDHARY et al., 2015). *Jatropha isabellei* (Müll Arg) is a shrub with red-violet inflorescences that belongs to Euphorbiaceae family, in which its underground parts have been commonly used in the Paraguayan and Brazilian folk medicine to treat different rheumatic diseases (BASUALDO; ZARDINI; ORTIZ, 1991; RIVEROS et al., 2009; FRÖHLICH et al., 2013). In fact, in a carrageenan-induced acute arthritis model in rats, the dichloromethane fraction (DF_{Ji}) obtained from underground parts of *J. isabellei* was able to significantly reduce the rat paw elevation time and to partially prevent the rat knee-joint edema, after oral and intravenous administration, evidencing the antinociceptive and anti-inflammatory properties of this plant (data submitted elsewhere). The diterpene jatrophone was identified as an important constituent of the DF_{Ji}, besides minor amounts of acetyl aleuritolic acid and a binary mixture of sitosterol-3-O-β-D-glucoside and stigmasterol (FRÖHLICH et al., 2013). On the other hand, the pharmacological effectiveness was significantly higher in rats receiving the intravenous dose, indicating that the chemical compounds from the DF_{Ji} exhibits limited absorption in the gastrointestinal tract (data submitted elsewhere).

Lipid-based nanocarriers are one of the most explored approaches to deliver hydrophobic drugs into the body. They are nanosized drug delivery systems composed of solid and/or liquid lipids with ability to associate drugs, increasing drug bioavailability, besides of using excipients with inherent biocompatibility and low toxicity (PURI et al., 2009). In a previous study, we have demonstrated that the oral administration of DF_{Ji}-loaded lipid-based nanocarriers at dose of 50 mg/kg, but not the free fraction, led to a significantly reduction of the paw elevation time of rats, three to six hours after carrageenan intra-articular injection (data submitted elsewhere). This result highlighted the beneficial properties of lipid nanocarriers in to increase the oral absorption and therapeutic efficacy of the active constituents of the DF_{Ji}.

On the other hand, one important property of nanocarriers when they are systemically administered is its ability to extravasated into inflamed tissues, in which the permeability of the vasculature is often higher than those encountered in normal tissues (PHAM, 2011). This approach, known as passive targeting by enhanced permeation and retention effect (EPR effect), has been successfully used to increase the concentration of anti-inflammatory drugs in the joints, increasing the therapeutic index and reducing the adverse effects (PHAM, 2011; HUA, 2013; DOLATI et al., 2016). However, one fact that can limit the systemic exposure of the nanoparticles includes their rapid clearance from the blood by the phagocytic cells of the reticuloendothelial system (RES). In particular, the macrophages have the ability to recognize opsonin proteins that bind onto the surface of the hydrophobic nanoparticles, removing the particles from the bloodstream within seconds after intravenous administration (GREF et al., 1994). One of the most successful approaches for reducing the macrophage uptake of the nanoparticles to prolong its circulation time consists in to modify their surface with polyethylene glycol (PEG). The purpose of decorating the particle surface with PEG chains is to create a more hydrophilic barrier in order to block the adhesion of opsonins present in the blood. Using this approach, the nanoparticles can remain invisible or stealth to phagocytic cells, extending the residence time of drugs in the blood (OWENS; PEPPAS, 2006; ALAYOUBI et al., 2013). Then, considering the

above mentioned, the aim of this study is to evaluate the anti-inflammatory and antinociceptive properties of DF_{Ji}-loaded lipid nanocarriers after intravenous administration. For that, pegylated lipid nanocarriers were prepared and characterized according to its size and surface properties, jatrophone release rate, hemolysis rate, and macrophage uptake. The pharmacological activity of free and nanoencapsulated DF_{Ji} was evaluated in the carrageenan-induced acute arthritis model in rats.

2. Materials and methods

2.1. Materials

Medium chain triglyceride oil (MCT, Ritamollient CCT) was acquired from Brasquim (Brazil). Hydrogenated soybean lecithin (Lipoid S100, 97.5% phosphatidylcholine) and disteroyl phosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG_{2000}, Lipoid PE 18:0/18:0-PEG 2000) were purchased from Lipid Ingredients & Technology (Brazil). Polysorbate 80 (Tween 80) was acquired from Sigma-Aldrich (USA). Glycerol was acquired from Neon (Brazil). A combination of kappa and lambda carrageenan was purchased from BDH Chemicals Ltd. (UK). Dexamethasone was purchased from Deg (Brazil). HPLC grade acetonitrile was acquired from Panreac (Spain). The ultrapure water used for HPLC analyses was obtained using a Milli-Q water system (Millipore, USA). All other solvents and reagents were analytical grade and were used as received.

2.2. Plant collection and preparation of dichloromethane fraction

J. isabellei was collected in the municipality of Cacequi (State of Rio Grande do Sul, Brazil, coordinates: 29°53'01" S and 54°49'30" W) in May of 2008. An exsiccate was archived in the herbarium of the Biology Department at the Federal University of Santa Maria (SMDB 11816). The underground parts were dried at room temperature and powdered in a knife mill. The powder was macerated with 70% (v/v; plant: solvent ratio 1:3 w/v) ethanol for ten days at room temperature. After filtration, the ethanol was evaporated under reduced pressure and this dispersion was partitioned with dichloromethane to obtain its respective fraction

which was further taken to dryness under reduced pressure, resulting in the dichloromethane fraction (DF_{Ji}, yield 3.7%).

2.3. Preparation of the lipid nanocarriers

Lipid nanocarriers containing dichloromethane fraction from *J. isabellei* (LNC_{Ji}) were prepared using the spontaneous emulsification method (BOUCHEMAL et al., 2004). Briefly, an organic phase containing different amounts of DSPE-PEG_{2000} (0, 15, 30, 60 or 90 mg), 100 mg of medium chain triglyceride oil (MCT), 10 mg of soybean lecithin, and 100 mg of DF_{Ji} in 10 mL of acetone:ethanol (7:3, v/v) was slowly added to an aqueous phase (50 mL) containing 0.1% (w/v) of polysorbate 80 and 0.5% (w/v) of glycerol under vigorous magnetic stirring. After, the organic solvents were removed by evaporation under reduced pressure and the colloidal dispersions were concentrated up to a final volume of 10 mL. Unloaded lipid nanocarries (LNC_{blank}) were prepared in the same manner. For macrophage uptake assays, 1 mg of Nile Red (NR) was added to the organic phase of the formulations to obtain fluorescent unloaded (LNC_{blank}-NR) and DF_{Ji}-loaded lipid nanocarriers (LNC_{Ji}-NR). All formulations were prepared in triplicate.

2.4. Physicochemical and morphological characterization of the lipid nanocarriers

The mean particle diameter and zeta potential were determined by dynamic light scattering and laser-Doppler anemometry, respectively, using a Zetasizer Nano Series (Malvern Instruments, UK). The measurements were performed at 25 °C after appropriate dilution of the samples in ultrapure water (Milli-Q, Millipore, USA). Each size analysis lasted 100 s and was performed with and angle detection of 173°. For measurement of zeta potential, the samples were placed electrophoretic cells where a potential of ±150 mV was applied. The zeta potential values were calculated from the mean electrophoretic mobility values using the Smoluchowsky's equation.

The pH of the lipid nanocarriers was determined on an Acorn® pH 5 pH meter (Oakton Instruments, USA), previously calibrated with pH 4.0 and 7.0 buffer solutions.

The morphology of the lipid nanocarriers was examined using a JEM-1011 transmission electron microscope (Jeol, Tokyo, Japan), with an acceleration voltage of 100 kV. The samples were diluted in ultrapure water, dropped onto carbon-coated copper grids and stained with 1% phosphotungstic acid.

2.5. Determination of jatrophone content and entrapment efficiency

The jatrophone content of the LNC_{Ji} was determined using an ultra-fast liquid chromatography method with diode array detection (UFLC-DAD). The experiments were performed on a Shimadzu UFLC-DAD system equipped with a LC-20AD binary pump, an SIL-20AC HT auto-sampler, a CTO-20A forced air-circulation-type column oven, an SPD-M20 photo diode array UV/VIS detector, and the software LC Solution 1.2 (Shimadzu, Tokyo, Japan). The analyses were carried out in reversed phase mode using a Phenomenex Luna C18 column (150 mm x 4.6 mm x 5 µm). The mobile phase consisted of acetonitrile and water, which was eluted at flow rate of 1.0 mL/min using the following gradient program: 53-65% acetonitrile from 0-12 min, and 65-75% acetonitrile from 12-15 min. After this time, the rebalancing was performed to restore the system and column to the initial mobile phase condition prior to the next injection. The total runtime was 23 min. The injection volume was 20 µL and the detection of jatrophone was monitored at 280 nm, according its maximum absorption. The jatrophone retention time was 7.8 min. The UFLC method was previously validated according to the parameters of linearity, specificity, accuracy, precision, and detection and quantification limits (LOD and LOQ), according to the ICH (2005). The calibration graph of jatrophone was linear over the range from 1.0 to 100.0 µg/mL (r^2 = 0.9997). The LOQ and LOD were 0.15 and 0.04 µg/mL, respectively, indicating the method was sufficiently sensitive to determine the jatrophone content in the lipid nanocarrier dispersions. The intra- and inter-day relative standard deviation values, as well as the recovery values,

satisfied the acceptance criteria for precision and accuracy in this study (data submitted elsewhere).

For UFLC analyses, each LNC_{Ji} formulation was dissolved up to volume with acetonitrile. The resulting solutions were filtered through 0.45 µm PVDF membrane (Merck Millipore) and injected in the chromatograph. The total concentration of jatrophone in the colloidal dispersions was expressed in µg/mL. The entrapment efficiency (%) was estimated as being the difference between the total concentration of jatrophone found in the lipid nanocarrier dispersions and the concentration found in the ultrafiltrate obtained after ultrafiltration/centrifugation (5000 rpm/ 10 min) procedure, using an Amicon Centrifugal Filter Device with Ultracel-100 membrane (100 kDa NMWL, Millipore, USA).

2.6. Accelerated stability studies

The accelerated stability of unloaded and DF_{Ji}-loaded LNC prepared with different amounts of DSPE-PEG 2000 was investigated using an analytical centrifuge LUMiSizer (L.U.M. GmbH, Germany), that consists of a centrifugal rotor with 12 optical cells, and a light source (pulsed near-infrared light-emitting 880 nm diode and a light sensor). For the analysis, 400 µL of each sample was placed into polycarbonate optical cells and subjected to rotation at 4.000 rpm for 7.650 s, with temperature of 25 °C, and interval time of 30 s according Yuan et al (2013). The samples were analyzed in triplicate without prior dilution. The results were displayed as a space- and time-related transmission profiles over the sample and instability index.

2.7. *In vitro* hemolysis assay

The *in vitro* hemolysis assay was performed as described by Wang et al (2009), with modifications. In brief, 50 µL of the erythrocyte dispersion was added to 950 µL of saline solution containing different concentrations of pegylated LNC_{Ji}: 50, 100, 250, 500 and 750 µg/mL, calculated according the content of dichloromethane fraction. For LNC_{blank} were used the correspondent volumes. The samples were incubated at 37 °C with gentle tumbling of the test tubes for 1 h. After, the samples

were centrifuged at 6.200 rpm for 10 min to remove the intact erythrocytes. Then, the absorbance of the supernatant was measured at 450 nm to determine the percentage of cells undergoing hemolysis. Saline solution and distilled water were employed as negative (0% lysis) and positive (100% lysis) controls, respectively. The hemolysis rate (HR) was calculated according to the equation HR (%) = $(D_t - D_{nc})/(D_{pc} - D_{nc}) \times 100$, where D_t, D_{nc}, and D_{pc} are the absorbance of the tested sample, negative control and positive control, respectively (ZHANG et al., 2007). The experiments were performed in triplicate.

2.8. Cell viability and macrophage uptake assays

2.8.1. Culture of macrophages

Murine macrophage-like J774 cells obtained originally from Rio de Janeiro Cell Bank (UFRJ, RJ, Brazil) were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL streptomicin, 2 mM glutamine and 10 mM HEPES (pH 7.4) (complete DMEM). Cells were maintained in a CO_2 incubator (HF 212UV, Axiom, Germany) in humidified atmosphere containing 5% CO_2, at 37 °C.

2.8.2 Evaluation of cell viability by the MTT assay

In vitro cell viability was assessed using the MTT assay (MOSMANN, 1983). For this assay, the J774 cells were plated at 4.0×10^5 cells per well in 200 µL of complete DMEM and incubated overnight at 37 °C with 5% CO_2 for adherence. In the next day, the cells were washed with sterile PBS (pH 7.2) to remove non-adherent cells, and then 200 µL of DMEM and 1 µL of LNC_{blank}-NR or LNC_{JI}-NR with different amounts of DSPE-PEG_{2000} were added to wells. The cells were further incubated at 37 °C for 1h in CO_2 incubator. After this time, the supernatant was discarded and the cells were washed with PBS to remove the non-phagocytosed LNC. MTT (5 mg/mL in DMEM) was then added to each well and incubated for 1 h at 37 °C in CO_2 incubator. After careful removal the medium, 300 µL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the

formazan crystals. Control groups were plated with cell culture medium and MTT reagent only. The measurement of absorbance was determined at 540 nm using plate reader (TECAN Infinite 2000, Switzerland) and then the percentage of viable cells was calculated in relation to the control-group. All assays were performed in triplicate.

2.8.3. Macrophage uptake studies

Macrophage uptake of the LNC_{JI}-NR prepared with different amounts of DSPE-PEG_{2000} was investigates by flow cytometry upon incubation with J774 cells. The cells (1.0 x 10^6 cells/well) were distributed in 24-well plates and maintained overnight in complete DMEM for adherence. After, the cells were washed with sterile PBS (pH 7.2) to remove non-adherent cells, and then 200 µL of DMEM and 1 µL of each lipid nanocarrier dispersion were added to different wells. Adherent cells were further incubated at 37 °C for 1 h with 5% CO_2. After this time, the cells were washed with PBS and detached from the plate with a PBS solution containing 0.5 mM of EDTA, and then resuspended in PBS containing 1% fetal bovine serum. The events were acquired in a flow cytometer (FACSCanto II™, Becton Dickinson Immunocytometry Systems) and the data were analyzed using Flowing Software. For visualization of the J774 cell uptake by fluorescence microscopy, LNC_{JI}-NR prepared without or with DSPE-PEG_{2000} were incubated with 4×10^5 cells in DMEM for 1h at 37 °C with 5% CO_2. After this time, the culture medium was removed and the cells were washed with PBS. The fluorescence of cells was observed using a fluorescence microscope (Leica Microsystems DMI 3000B, Germany). Images of the cells were acquired at the excitation and emission wavelengths of 530 and 635 nm, respectively, using the LAS AF Lite® software.

2.9. *In vitro* jatrophone release studies

The release studies were carried out using the dialysis bag method (LUO et al., 2006). For the experiments, an aliquot of DF_{JI}-loaded LNC prepared without (LNC_{JI}) or with 90 mg of DSPE-PEG_{2000} (LNC_{JI}-DSPE-PEG 2000_{90}) was transferred to dialysis bags (MWCO 14,000, Sigma Aldrich, USA). The dialysis

bags were placed into a dissolution apparatus (USP 2, Varian, Brazil) containing 200 mL of phosphate buffer pH 7.4 added of 2% of sodium lauryl sulfate per vessel. The release medium was maintained at 37^{o}C under mechanical stirring at 75 rpm. Samples of the release medium were withdrawn after 0.25, 0.5, 0.75, 1, 2, 4 and 6 hours, with the release medium being immediately replaced with fresh medium. The samples were diluted with acetonitrile, filtered through 0.45 µm PVDF membranes, and analyzed by a UFLC-DAD method, using the same chromatographic conditions described above. The calibration curve for jatrophone in phosphate buffer pH 7.4 was linear over the concentration ranging from 0.3 to 15.0 µg/mL (r^2 = 0.9987). The LOQ and LOD were 0.1 and 0.3 µg/mL, respectively, indicating the method was sufficiently sensitive to determine the jatrophone concentration in release medium. The experiments were carried in triplicate. The cumulative percent of jatrophone released (%) was plotted against time (h). Dissolution efficiency (DE%) was calculated from the area under the dissolution profile and expressed as the percentage of the area of the rectangle described by 100% dissolution within the same period of time.

2.10. Evaluation of *in vivo* antinociceptive and anti-inflammatory activities

2.10.1 Animals

All experiments were performed using adult male Wistar rats weighing 250–300g. The animals were housed under a controlled temperature (21 ± 2°C) on a 12h light/dark cycle with standard lab chow and water *ad libitum* until the experimental sessions. The animals were acclimatized into the experimental room for at least 30 minutes before the experiments. The experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain (IASP, 1983) and approved by the local committee for the ethical use of animals (P00723/CEUA-UFSC).

2.10.2. Carrageenan-induced articular incapacitation in rats

Articular incapacitation was induced by the injection of 300 µg of carrageenan (solubilized in 50 µL sterile 0.9% saline) into the right knee joint of rats. In this assay, the animals were stimulated to walk on a revolving steel cylinder (constant speed of 3 rpm) wearing metallic gaiters in the hind paws. The right paw gaiter was connected to a computer system that counted the total duration of no contact on the cylinder surface during the one minute test period. This paw elevation time (PET), in seconds, was taken as an estimate of nociception (TONUSSI; FERREIRA, 1992). Two hours after carrageenan injection the animals were treated with 10 mg/kg of the free DF_{Ji} or 10 mg/kg of LNC_{Ji} containing 90 mg of DSPE-PEG_{2000} (LNC_{Ji}-DSPE-PEG 2000_{90}), intravenously by gingival vein punction (OLIVEIRA; SILVA; TONUSSI, 2009). The PET was evaluated from the third hour to the sixth hour, and presented as an average of these time points. Intravenous dexamethasone (1 mg/kg) was used as positive control, and vehicle and LNC_{blank} were used as negative control groups.

2.10.3. Edema measurement

The articular diameter (AD) was used to quantify the inflammatory edema induced by carrageenan and it was taken by measuring the medio-lateral axis (in mm) of the knee-joint, using a micrometer, at three consecutive arbitrary points in a proximo-distal direction. The AD measured just before carrageenan injection was subtracted from AD values taken hourly from the third to the sixty hour, just after the incapacitation measurement, and presented as an average of these time points.

2.11. Statistical analysis

Data were expressed as mean ± standard deviation (s.d.) or relative standard deviation (RSD) and mean ± S.E.M. The statistical significance was assessed by t- test or one-way analysis of variance (ANOVA) followed by post-hoc Tukey or Dunnett's Multiple Comparison test. P values lower than 0.05 ($p < 0.05$) were considered indicative of significance. The sample size

for incapacitation test and articular edema were estimated using a statistical power test, and a minimum of six animals were used for both parameters

3. Results and discussion

3.1. Characterization of lipid nanocarriers

In this study, pegylated lipid nanocarriers containing the DF_{Ji} were prepared with the aim to obtain a drug delivery system that could release the terpenic constituents of this fraction preferentially into the inflamed joints. For that, different amounts of DSPE-PEG_{2000} (15, 30, 60 and 90 mg) were added in the organic phase of the formulations. DSPE-PEG_{2000} is an amphiphilic polymer which has been approved by the Food and Drug Administration for medical applications and has been widely used in the preparation of stealthy liposomes, polymeric, and lipid nanoparticle (WANG et al., 2012). In this study, pegylated lipid nanocarriers were successfully prepared by the spontaneous emulsification technique (BOUCHEMAL et al., 2004). The size, zeta potential, polydispersity index (PDI), and pH of the formulations are given in Table 1. Unloaded lipid nanocarriers (LNC_{blank}) exhibited mean particle size ranging from174.7 to 185.9 nm and no change in the particle size was observed with the addition of DSPE-PEG_{2000} to the formulations. In contrast, the particle size of DF_{Ji}-loaded lipid nanocarriers decreased with the addition of DSPE-$PEG_{2000,}$ presenting mean particle size ranging from 185.2 to 144.4 nm. All formulations displayed a monodisperse size distribution as it was observed by the low values of polydispersity index (< 0.2) (Table 1).

The lipid nanocarriers exhibited pH ranging from 3.95 to 5.28, with more acidic colloidal dispersions obtained in the presence of the DF_{Ji}. The zeta potential values ranged from -24.7 to -55.1 mV (Table 1). The presence of DF_{Ji} into the nanocarriers did not seem to affect the zeta potential of the particles, as it can be seen by comparing LNC_{Ji} and LNC_{blank} prepared without DSPE-PEG_{2000}, and the negative charge of the lipid nanocarrier surface was probably originated from the acidic lipids, such as phosphatidylinositol, phosphatidylserine, and

phosphatidylglycerol, which are present in small quantities in the soybean lecithin (SCHUH; BRUXEL; TEIXEIRA, 2014). On the other hand, LNC_{blank} and LNC_{Ji} prepared with DSPE-PEG_{2000} demonstrated a negative charge surface, in which the mean zeta potential values progressively increased (in module) with the increase of the polymer concentration in the formulations. This is in accordance with previous studies showing that DSPE-PEG_{2000} contributes to the negative surface charge of liposomes (WOODLE et al., 1992). Similar results were found by Kandadi et al (2011) and Lim et al (2002) during the development of indinavir-loaded pegylated submicron lipid emulsions and solid lipid nanoparticles loaded with retinoic acid.

Table 1. Size, PDI, zeta potential and pH obtained for unloaded and DF_{Ji}-loaded LNC.

	Mean size (nm) (s.d.)*	**PDI[1] (s.d.)***	**Zeta Potential (mV) (s.d)***	**pH (s.d.)***
LNC_{blank}	181.5 (2.6)	0.113 (0.013)	-24.7 (1.3)	5.07 (0.05)
LNC_{blank} DSPE-PEG 2000_{15}	175.3 (5.2)	0.148 (0.002)	-44.6 (1.2)	5.05 (0.04)
LNC_{blank} DSPE-PEG 2000_{30}	185.9 (4.3)	0.177 (0.007)	-49.9 (0.5)	5.18 (0.18)
LNC_{blank} DSPE-PEG 2000_{60}	174.7 (4.0)	0.138 (0.018)	-55.1 (2.5)	5.28 (0.10)
LNC_{blank} DSPE-PEG 2000_{90}	181.8 (5.7)	0.148 (0.015)	-53.9 (0.5)	5.08 (0.08)
LNC_{Ji}	185.2 (7.4)	0.097 (0.017)	-25.1 (0.8)	3.98 (0.07)
LNC_{Ji} DSPE-PEG 2000_{15}	158.3 (7.5)	0.119 (0.019)	-38.3 (1.9)	3.95 (0.07)
LNC_{Ji} DSPE-PEG 2000_{30}	144.6 (9.3)	0.143 (0.023)	-43.3 (1.1)	4.05 (0.05)
LNC_{Ji} DSPE-PEG 2000_{60}	148.9 (6.1)	0.141 (0.018)	-47.0 (1.3)	4.20 (0.03)
LNC_{Ji} DSPE-PEG 2000_{90}	147.1 (5.7)	0.117 (0.010)	-48.0 (1.8)	4.21 (0.04)

*standard deviation, n=3; [1]Polydispersity index.

In previous studies, we have identified the diterpene jatrophone as an important constituent of the DF_{Ji}, therefore, this compound was used as chemical marker in the encapsulation and the release studies. The results obtained in the evaluation of the drug content and entrapment efficiency for jatrophone in the LNC_{Ji} are presented in Table 2. As can be seen in Table 2, the jatrophone content was about 80-90 µg/mL and almost drug was found associated to the nanocarrier. This result may be explained by the high lipophilicity of dichloromethane fraction, and therefore, by the higher affinity of the DF_{Ji} constituents for the particles than for the external aqueous phase.

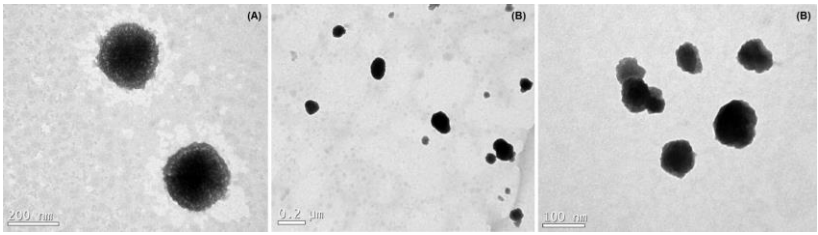
Table 2. Drug content and entrapment efficiency of jatrophone from LNC_{Ji}.

	Drug content (µg/mL)[#](s.d.)*	**Entrapment efficiency (%)(s.d.)***
LNC_{Ji}	866.1 (11.0)	92.2 (0.08)
LNC_{Ji} DSPE-PEG 2000_{15}	855.1 (42.3)	91.5 (0.06)
LNC_{Ji} DSPE-PEG 2000_{30}	859.3 (12.4)	90.0 (0.12)
LNC_{Ji} DSPE-PEG 2000_{60}	848.5 (22.5)	89.4 (0.09)
LNC_{Ji} DSPE-PEG 2000_{90}	838.8 (22.8)	89.2 (0.03)

*standard deviation, n=3. [#] µg of jatrophone per mL of LNC_{Ji}.

The transmission electron microscopy images (Figure 1) of LNC_{Ji} prepared without DSPE-PEG_{2000} (A) and containing 90 mg (B) of this polymer exhibited spherical particles with size of around 100 to 200 nm, agreeing with the size results obtained by dynamic light scattering.

Figure 1. TEM images of LNC_{Ji}: (A) without DSPE-PEG_{2000} and (B) containing 90 mg of DSPE-PEG_{2000}, negatively stained with 1% phosphotungstic acid.



3.2. Accelerated stability studies

Accelerated stability studies were carried out using the analytical centrifugation method. The use of the analytical centrifuge allows to measure of the intensity of the transmitted near-infrared light as a function of time and position over the entire sample length, while the samples are simultaneously subjected to centrifugal force, providing information about instability phenomena, such as sedimentation, flocculation or creaming (YUAN et al., 2013). The representative transmission profiles of the lipid nanocarrier formulations are presented in the Figure 2. Figure 3 illustrates the instability index obtained for LNC_{blank} and LNC_{Ji} prepared with different amounts of DSPE-PEG_{2000}.

The transmission profiles of the LNC_{blank} (Figure 2, left) revealed a clarification area (increase of the light transmission) at the bottom of the cuvette, possible due to an initial creaming phenomenon. The clarification area becomes small with increasing amounts of DSPE-PEG_{2000} at the formulations, indicating the addition of the pegylated phospholipid to the formulations contributes to the colloidal dispersion stabilization. For LNC_{Ji} (Figure 2, right), the clarification process is practically imperceptible, demonstrating an additional effect on the colloid stabilization provided by the presence of the DF_{Ji}.

The instability index is a dimensionless number between 0 and 1, which correspond the clarification at a certain time, divided by the maximum clarification. This value, in turn,

measures the increase in transmission due to sedimentation or creaming, where “0” means more stability of formulations with no changes under test conditions, and 1 means greater instability of the formulations, showing different phenomena of instability, represented in transmission profiles (MENDES et al., 2016). The decrease of the index stability values from 0.692 ± 0.04 to 0.390 ± 0.05 after DSPE-PEG_{2000} addition to LNC_{blank} confirmed that the presence of this pegylated lipid increases the colloidal dispersion stability. Comparing the values of instability index of LNC_{blank} and LNC_{Ji}, both without DSPE-PEG_{2000}, (0.692 ± 0.04 and 0.020 ± 0.01, respectively) is possible to state that the presence of DF_{Ji} in the LNC also contributes to the stabilization of the nanocarrier formulations, possibly due to the presence of fatty acids in this fraction. All formulations of DF_{Ji}-loaded lipid nanocarriers prepared with and without DSPE-PEG_{2000} presented values of instability index lower than 0.05. These results indicate a good stability against phase separation, especially when the LNC were stored at 4 °C.

Figure 2. Representative transmission profiles obtained after centrifugation of unloaded (left) and DF_{Ji}-loaded LNCs (right): (A) without DSPE-PEG_{2000} and with (B) 15 mg and (C) 90 mg of DSPE-PEG_{2000}.

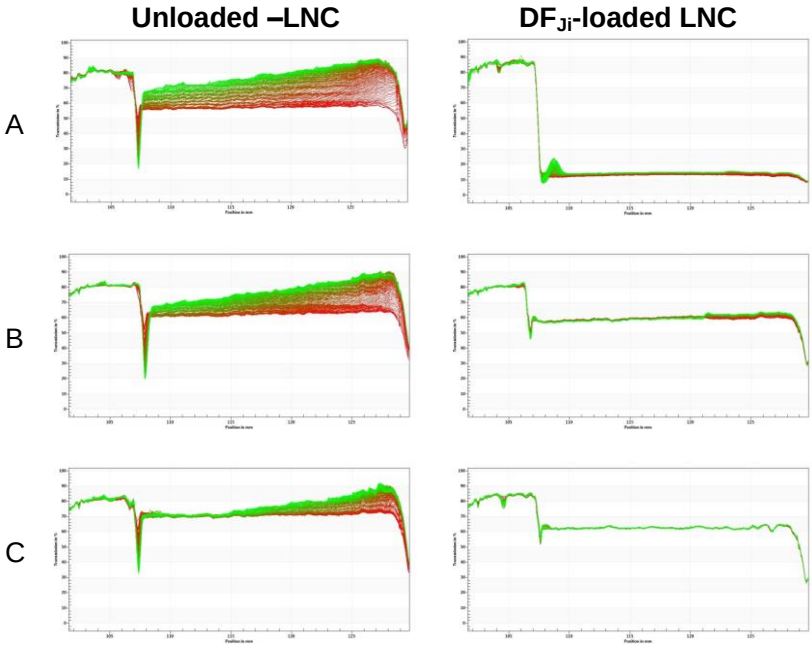
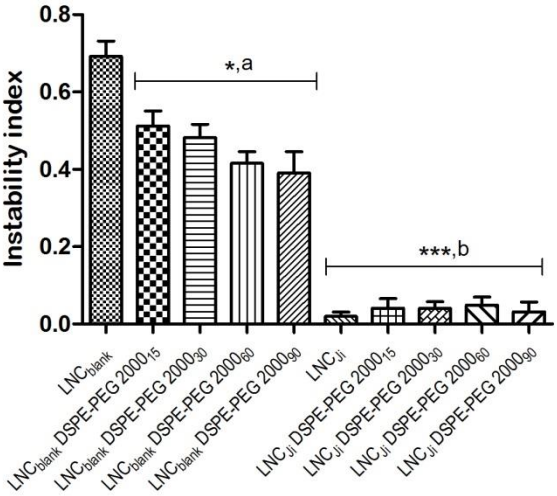


Figure 3. Instability index obtained for LNC_{blank} and LNC_{Ji} containing different amounts of DSPE-PEG_{2000}. * and *** represent significance at $p < 0.05$ and $p < 0.001$, respectively, in relation to LNC_{blank} without DSPE-PEG_{2000}. 'a' and 'b' represent significance at $p < 0.001$ between the LNC_{blank} and the respective LNC_{Ji} considering the different amounts of DSPE-PEG_{2000}. The statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test.



3.3. Erythrocyte hemolysis

Studies of hemocompatibility of drug nanocarriers are very important when the formulations are intended for intravenous administration. Measuring the hemolytic effect of nanoparticles has been suggested as a part of *in vitro* toxicity screen, since it allow to predict the membrane damage caused by such formulations (SHARMA; MADHUNAPANTULA; ROBERTSON, 2012). In this study, the hemocompatibility of the nanocarriers was evaluated in terms of hemolysis using human blood. The results are demonstrated in Figure 4.

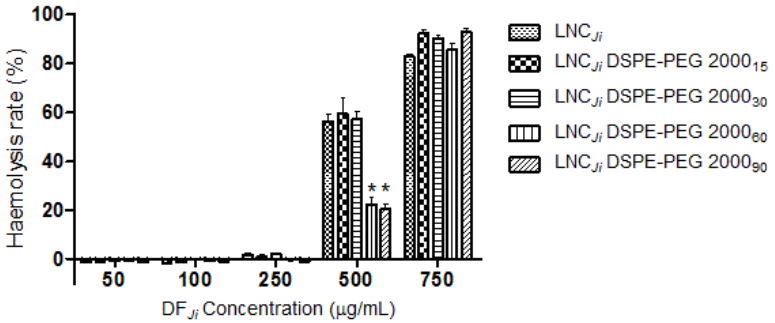
The incubation of LNC_{blank} (all tested concentrations) with the erythrocyte dispersion for 1 h resulted in hemolysis ratios

lower than 1%, which may consider this formulations as hemocompatible, according to the ISO/TR 7406 (hemolysis ratio lower than 5% is the critical safe hemolytic ratio)(data not shown). So, it was possible to conclude that the concentration of phospholipids (lecithin) and emulsifiers (polysorbate 80) used in the LNCs were not toxic to the erythrocytes.

When DF_{Ji}-loaded LNCs were added to the erythrocyte dispersion at DF_{Ji} concentrations of 50, 100, and 250 µg/mL, the hemolysis ratios remained lower than 2%, regardless of the DSPE-PEG_{2000} concentration in the lipid nanocarrier formulations. In contrast, when concentrations of 500 and 750 µg/mL of DF_{Ji} were tested, the rupture of the red blood cells occurred and the hemoglobin was released into the extracellular environment. With 500 µg/mL of dichloromethane fraction, the hemolysis rate reached 56.4 ± 2.6, 59.3 ± 6.4 and 57.3 ± 2.7% for LNC_{Ji} prepared without and with 15 and 30 mg of DSPE-PEG_{2000}, respectively. The hemolysis rate were lower for the lipid nanocarriers prepared with 60 and 90 mg of DSPE-PEG_{2000} at DF_{Ji} concentration of 500 µg/mL, with values of 22.3 ± 3.2 and 20.6 ± 1.7%, respectively. In this case, the presence of PEG chains onto the LNC surface appeared to prevent the interactions of the nanoparticles with the erythrocytes, most likely due to the dense brush conformation assumed by the PEG chains at the particle surface, protecting the nanodroplets from contact with erythrocyte surface and avoiding the cell rupture (ALAYOUBI et al., 2013). For LNC_{Ji} prepared with 750 µg/mL of dichloromethane fraction, the hemolysis rate ranged from 82.8 ± 0.9 to 92.6 ± 1.8%, regardless of the DSPE-PEG_{2000} concentration.

The hemolytic effect of DF_{Ji} may be related to the presence of sitosterol-3-O-β-D-glucoside, a steroidal saponin, in this fraction (FRÖHLICH et al., 2013). Although the exact mechanism of the rupture of erythrocyte membrane by saponins is not yet clearly understood, it was found to be correlated with their amphiphilic properties (GAUTHIER et al., 2009). In particular, steroidal and triterpene saponins with a single sugar chain at C3 have demonstrated strong hemolytic activity (HARUNA et al., 1995). Additionally, compounds like jatropholone A and jatrophone exhibited cytotoxic effects against permanent human epithelial gastric cell line (AGS) (PERTINO et al., 2007a; 2007b) and maybe contributing to this hemolytic effect.

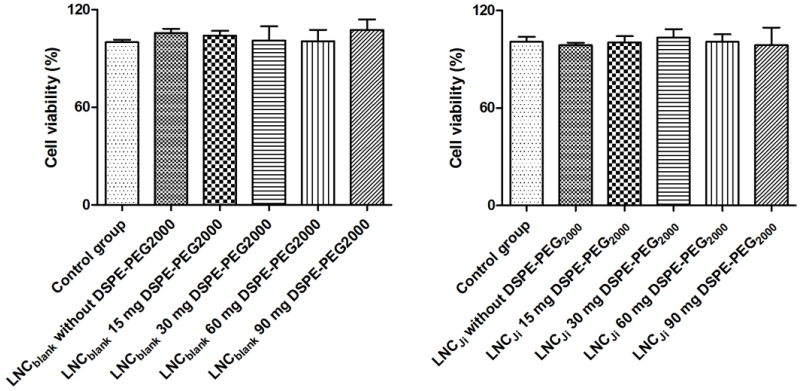
Figure 4. Haemolysis rate of LNC_{Ji} prepared with different amounts of DSPE-PEG_{2000} and as a function of the DF_{Ji} concentration. * represent significance at $p < 0.05$, compared to LNC_{Ji} prepared without DSPE-PEG_{2000} and to LNC_{Ji} prepared with 15 and 30 mg of DSPE-PEG_{2000} and 500 µg/mL of DF_{Ji}. The statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test.



3.4. Macrophage uptake

Firstly, the macrophage viability was assessed by MTT assay in order to investigate possible cytotoxic effects of the formulations. As shown in Figure 5, the nile red-loaded LNC_{blank} and nile red-loaded LNC_{Ji}, both prepared with different amounts of DSPE-PEG_{2000}, did not exhibit cytotoxic effect on J774 cells. This experiment validated the macrophage uptake assay (see below), since the cells were not damaged by the formulation constituents.

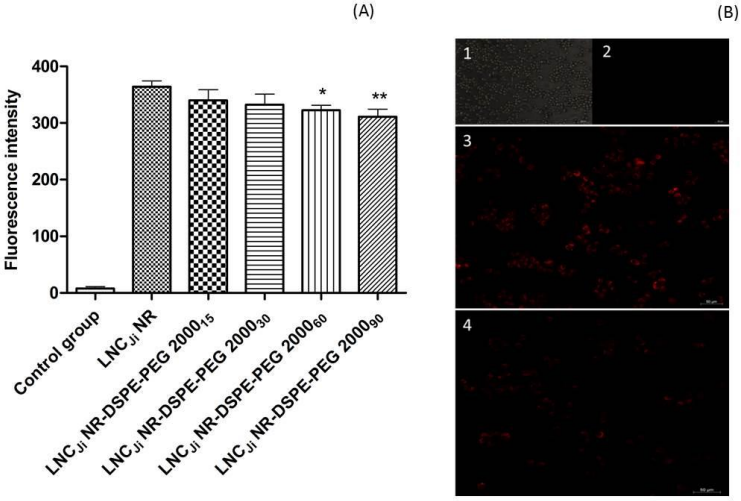
Figure 5. Cell viability (%) obtained after incubation of J774 macrophage cell line with NR-loaded LNC_{blank} (left) and NR-loaded LNC_{Ji} (right), prepared with different amounts of DSPE-PEG_{2000}. Control groups were plated only with cell culture medium and MTT reagent.



The fluorescence intensity measured by flow cytometry and the images obtained by fluorescence microscopy after 1 h incubation of the J744 cells with lipid nanocarriers are shown in Figure 6. All cells incubated with LNC_{Ji}-NR exhibited a red fluorescence as an indicative of the macrophage uptake of the particles (Figure 6a). The red fluorescence intensity decreased with the increase of the amount of DSPE-PEG_{2000} in the formulations, reaching significant values when 60 and 90 mg of this pegylated lipid was incorporated in the lipid nanocarriers ($p < 0.05$ and $p < 0.01$, respectively). For these formulations (LNC_{Ji}-DSPE PEG 2000_{60} and LNC_{Ji}-DSPE PEG 2000_{90}), the higher PEG chain density at the particle surface was able to reduce the interactions of the particles with the J774 cells, probably due to the steric barrier that prevented the protein adsorption and macrophage recognition of particles. This particle property may be useful to extend the residence time of drugs in the blood with more probability to reach the inflamed articulations to produce the therapeutic effect. The fluorescence images confirmed the smaller intracellular accumulation of LNC_{Ji}-NR containing 90 mg

of DSPE-PEG_{2000}, compared to the LNC_{Ji}-NR prepared without DSPE-PEG_{2000} (Figure 6b).

Figure 6. (A) Fluorescence intensity measured by flow cytometry of J774 cells after incubation with LNC_{Ji}-NR containing different amounts of DSPE-PEG_{2000}. *$p < 0.05$ and **$p < 0.01$ compared to LNC_{Ji} prepared without DSPE-PEG_{2000}. The statistical analysis was performed using *t* test. (B) Images of fluorescence microscopy: 1) J774 cell at bright field, 2) Control group, 3) LNC_{Ji}-NR without DSPE-PEG_{2000}, 4) LNC_{Ji}-NR containing 90 mg DSPE-PEG_{2000}.

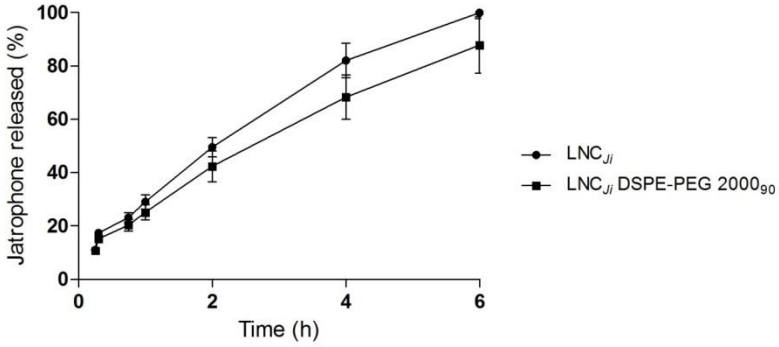


3.5. *In vitro* jatrophone release

The jatrophone release profiles from the lipid nanocarriers prepared without and with 90 mg of DSPE-PEG_{2000} are demonstrated in Figure 7. As can be seen in this figure, 99.9% and 87.8% of jatrophone was release from LNC_{Ji} (without DSPE-PEG 2000) and LNC_{Ji}-DSPE PEG 2000_{90}, respectively, after 6 h

in phosphate buffer pH 7.4. The jatrophone release profiles were compared by calculating the dissolution efficiency (DE%), which were estimated to be 61.55 ± 3.7 and 52.47 ± 6.4 for LNC_{Ji} (without DSPE-PEG_{2000}) and LNC_{Ji}-DSPE PEG 2000_{90}, respectively. The DE% values were not statistically significant ($p > 0.05$), indicating the presence of DSPE-PEG_{2000} chains in the LNC_{Ji} did not affect the jatrophone release rate.

Figure 7. *In vitro* jatrophone release (± SD) from LNC_{Ji} and LNC_{Ji}-DSPE-PEG 2000_{90} in phosphate buffer pH 7.4 at 37 °C (n=3).

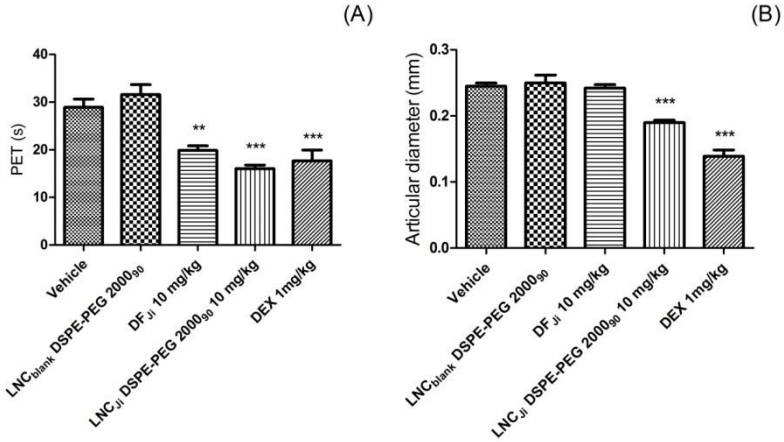


3.6. *In vivo* antinociceptive and anti-inflammatory activities

The antinociceptive and anti-inflammatory activities of LNC_{Ji} containing 90 mg DSPE-PEG_{2000} was evaluated in a carrageenan-induced arthritis model in rats. Negative control groups that received only the vehicle or LNC_{blank} by intravenous route, two hours after knee-joint carrageenan injection, exhibited an averaged PET values increasing from 9.4 ± 0.4 s at basal levels to 28.9 ± 1.7 s and 31.6 ± 2.0 s, respectively (Figure 8). The animals receiving the LNC_{Ji}-DSPE-$PEG2000_{90}$ and free DF_{Ji} at dose of 10 mg/kg exhibited a significant reduction in averaged PET values ($p < 0.001$ and $p < 0.01$, respectively) in the same way that dexamethasone at dose of 1 mg/kg, which was used as

positive control (Figure 8a). The antinociceptive effect of LNC_{Ji} and free DF_{Ji} may be attributed to the presence of the diterpene jatrophone in the dichloromethane fraction. This diterpene was already reported to inhibit the [^{3}H] glutamate binding in a dose-dependent way, a major mediator in the nociceptive transmission (MARTINI et al., 2000). On the other hand, the antiedematogenic effect was observed when the lipid nanocarrier, but not the free fraction, was administered at dose of 10 mg/kg ($p < 0.001$) (Figure 8b). The antiedematogenic effect is also supposed to be related to jatrophone. This diterpene has previously shown to inhibit lymphocyte proliferation, presumably through inhibition of the protein kinase C (PKC) pathway, which in turn, mediates a number of intracellular signaling pathways involved in the pathogenesis of inflammation (MORAES; RUMJANEK; CALIXTO, 1996). These results evidenced the beneficial properties of DF_{Ji}-loaded lipid nanocarriers to treat the symptoms related to arthritis. Besides to allow the administration of a lipophilic fraction by intravenous route using only biocompatible excipients, the increase of the blood half-life of terpenic constituents and preferential reaching of these drugs in the inflamed joints may be occurred, as indicated by the higher antiedematogenic activity of LNC_{Ji}-DSPE-PEG 2000_{90}.

Figure 8. Effect of the LNC_{Ji}-DSPE-PEG 2000_{90} and free DF_{Ji} on paw elevation time (PET) (A) and edema (B) after intravenous administration. The animals were treated 2 hours after intra-articular carrageenan injection (300 µg/knee). The negative control group received the vehicle DMSO: polysorbate 80: saline solution (5:4:91) or LNC_{blank}-DSPE-PEG $2000_{90.}$ Dexamethasone (DEX, 1 mg/kg, i.v.) was used as positive control. ** $p < 0.01$ and *** $p < 0.001$ represents a significant difference compared with the vehicle and LNC_{blank}. The statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test.



4. Conclusions

Unloaded and DF_{Ji}-loaded lipid nanocarriers containing different amounts of DSPE-PEG_{2000} were successfully prepared by spontaneous emulsification method. The accelerated stability revealed that both the increase of the DSPE-PEG_{2000} amount and the presence of the dichloromethane fraction contribute for the physical stabilization of the colloidal dispersions. The hemolysis assay revealed that LNC_{blank} did not cause the lysis of cell membranes, however, hemolysis rate was dependent on the DF_{Ji}

concentration, being this result attributed to the presence of steroidal saponin or diterpenes jatropholone A and jatrophone in the dichloromethane fraction. None of developed formulations affected the cell viability, and LNC_{Ji} containing 60 and 90 mg of $DSPE\text{-}PEG_{2000}$ were significantly lesser uptaked by J774 macrophage cells. The release studies demonstrated that jatrophone release rate was not affect by the presence of the $DSPE\text{-}PEG_{2000}$. LNC_{Ji}-DSPE-PEG 2000_{90} was tested in carrageenan-induced arthritis model and exhibited antinociceptive effects similar to that dexamethasone. In special, the antiedematogenic effect of LNC_{Ji}-DSPE-PEG 2000_{90} was higher than the free fraction, indicating the nanoencapsulation may have altered the blood half-life and/or distribution of terpenic constituents of DF_{Ji} after intravenous administration.

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Declaration of interest

The authors report no conflicts of interest.

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DISCUSSÃO GERAL

A fração diclorometano obtida a partir de extratos de plantas é conhecida por concentrar compostos terpênicos. Para a espécie *Jatropha isabellei* esta fração foi obtida a partir do extrato hidroalcoólico (70%, v/v) das partes subterrâneas por partição líquido-líquido, seguida da evaporação do solvente a pressão reduzida, resultando numa fração seca. Esta fração foi avaliada por cromatografia líquida ultra-rápida, acoplada à espectrômetro de massas, através da qual foi possível identificar um grande pico, o qual apresentou massa compatível com o diterpeno jatrofona, que foi proposto como o marcador químico desta fração. Este constituinte já havia sido relatado para a espécie *J. isabellei* (PERTINO et al., 2007a) e foi novamente isolado, pela técnica de cromatografia em coluna, e caracterizado, por técnicas espectroscópicas, para ser utilizado como substância de referência durante a validação da metodologia analítica por UFLC. O método de UFLC desenvolvido permitiu a caracterização do diterpeno jatrofona na fração diclorometano através de uma eluição rápida e simples, possibilitando a validação do método quanto aos critérios de linearidade, limites de detecção e quantificação, especificidade, exatidão e precisão. Esta metodologia analítica também foi utilizada para a caracterização do diterpeno jatrofona nas formulações desenvolvidas neste estudo.

A caracterização farmacológica da fração diclorometano foi realizada após sua administração pelas vias oral e intravenosa através de ensaios em um modelo de artrite aguda, o qual foi escolhido devido os relatos do uso popular da planta em doenças reumáticas (BASUALDO; ZARDINI; ORTIZ, 1991). A via oral foi escolhida para ser testada nos ensaios farmacológicos por ser a forma de administração de fármacos mais conveniente e aceita pelos pacientes, enquanto que a via intravenosa foi escolhida a fim de comparar as doses eficazes e a biodisponibilidade da fração diclorometano em relação à via oral. Além disso, como o objetivo deste trabalho foi a incorporação da fração diclorometano em nanocarreadores lipídicos e a comparação do seu efeito com o da fração livre, a escolha da via oral se deu pela possibilidade de melhora da absorção e da eficácia da fração diclorometano quando administrada sob a forma de formulações lipídicas nanoestruturadas se comparada à fração livre. Já a escolha da via intravenosa consiste em explorar

o efeito de permeabilidade e retenção aumentadas (efeito EPR - *Enhanced Permeability and Retention*) dos tecidos inflamados na artrite e assim melhorar a eficácia da fração diclorometano na artrite através da liberação dos compostos terpênicos diretamente na articulação inflamada.

Os estudos farmacológicos realizados com a fração diclorometano livre demonstraram que por ambas as vias de administração a fração mostrou-se ativa na redução do edema e do tempo de elevação de pata, ou seja, apresentando efeitos anti-inflamatórios e antinociceptivos, os quais foram atribuídos à presença dos terpenos na fração, em especial ao diterpeno jatrofona. Entretanto, como esperado, a fração diclorometano apresentou maior eficácia quando administrada pela via intravenosa se comparada à via oral, o que sugere uma reduzida absorção dos terpenos pelo trato gastrointestinal, provavelmente devido sua elevada lipofilicidade e baixa solubilidade nos fluídos do trato gastrointestinal.

A baixa solubilidade aquosa de constituintes ativos lipofílicos representa um dos maiores obstáculos para o desenvolvimento de novos medicamentos e aproximadamente 40% dos candidatos a novos fármacos apresentam esta característica (LIPINSKI, 2002; CHEN et al., 2011) Assim, uma estratégia utilizada para melhorar a absorção dos terpenos pelo trato gastrointestinal foi a incorporação da fração diclorometano em carreadores lipídicos nanoestruturados. Estes veículos de fármacos são reconhecidos por propiciarem uma melhora na absorção e na biodisponibilidade oral dos ativos lipofílicos por meio da solubilização e proteção de tais substâncias (SINGH; VINGKAR, 2008; VYAS; SHAHIWALA; AMIJI, 2008), o que representa um grande avanço para a eficácia destes fármacos quando administrados oralmente.

Inicialmente, para o desenvolvimento dos carreadores lipídicos nanoestruturados foi avaliado o conteúdo de ácidos graxos da fração diclorometano, pois as suas características podem afetar as propriedades físico-químicas e a estabilidade das formulações porque são responsáveis por auxiliar na formação da fase interna oleosa das nanogotículas. Para isto, foi utilizada a técnica de cromatografia gasosa que revelou a presença majoritária de ácidos graxos saturados na fração.

Os carreadores lipídicos nanoestruturados contendo a fração diclorometano (CLN_{Ji}) foram desenvolvidos pelo método da emulsificação espontânea e resultaram em formulações com elevada porcentagem de eficiência de encapsulação do marcador químico jatrofona, potencial zeta negativo, tamanho médio de partícula em torno de 182 nm, formato esférico e índice de polidispersão menor que 0,1, apresentando uma distribuição de tamanho monodispersa. As formulações brancas (CLN_{branco}) apresentaram características semelhantes, assim, a presença da fração diclorometano pareceu não afetar as características morfológicas e físico-químicas das partículas. Além disso, sugere-se que a adição da fração diclorometano na formulação dos nanocarreadores lipídicos possa ter contribuído para o aumento do número de partículas, uma vez que o tamanho e o volume das partículas não foram aumentados.

No estudo de estabilidade, os CLN_{Ji} armazenados a 4°C apresentaram-se bastante estáveis e para confirmação foi realizado o estudo de estabilidade acelerada com o equipamento LuMiSizer que consegue medir os fenômenos de instabilidade das formulações quando submetidas à centrifugação. Através deste estudo foi possível verificar que os CLN_{Ji} permaneceram bem estáveis durante o processo de centrifugação e que a presença da fração diclorometano influenciou na estabilidade das partículas, tornando-as mais estáveis se comparado com as formulações brancas (índice de estabilidade médio de 0,536 e 0,083, respectivamente). Isto se deve, provavelmente, devido à presença dos ácidos graxos na fração que ajudam a formar a fase interna oleosa das partículas, tornando-as mais estáveis. Apenas para os CLN_{branco} foi verificada uma região de clarificação no fundo da cubeta (aumento da porcentagem de transmissão), entretanto, não foi possível identificar o fenômeno de cremação durante o tempo de centrifugação testado. A cremação é um fenômeno de instabilidade comum para as formulações lipídicas e se caracteriza pela diminuição da porcentagem de transmissão no topo da cubeta (YUAN et al., 2013).

Para avaliar a liberação da jatrofona dos CLN_{Ji} desenvolvidos para a administração oral, foram empregados os meios biorrelevantes que simulam o estado de jejum gástrico e intestinal e o estado alimentado intestinal (FaSSGF, FaSSIF e

FeSSIF, respectivamente). Estes resultados foram avaliados em relação aos fatores de diferença e similaridade e também em relação a eficiência de dissolução, os quais demonstram a liberação significativa do ativo jatrofona a partir dos carreadores lipídicos nos meios gástrico jejum e intestinal do estado alimentado em relação à fração livre. Nestes dois meios, e em especial no meio gástrico do jejum, a solubilidade da fração diclorometano livre é mais baixa, demonstrando assim, a importância dos nanocarreadores lipídicos na veiculação da fração e melhora da liberação do ativo jatrofona. Estes resultados são considerados importantes para predizer o desempenho *in vivo* das formulações de via oral porque podem fornecer informações sobre o local de absorção da jatrofona.

As condições de uma liberação e solubilidade aumentadas propiciadas para a fração diclorometano quando veiculadas aos carreadores lipídicos nanoestruturados refletiram na eficácia da fração quando administrada pela via oral aos ratos wistar em modelo de artrite aguda. Este ensaio revelou que as formulações lipídicas contendo a fração diclorometano foram mais eficazes na redução do tempo de elevação de pata se comparadas à fração livre, quando ambas foram testadas na mesma dose. Isto se deve, possivelmente, à maior solubilização do ativo jatrofona nos fluídos biológicos, facilitando sua absorção e contribuindo para o efeito antinociceptivo. Da mesma forma que o aumento da absorção do ativo jatrofona contribuiu para a redução da dor, este também pode ter levado ao aumento da ação vasorelaxante deste ativo, atenuando seu efeito anti-inflamatório, que não foi observado durante este experimento.

A melhora do efeito e da biodisponibilidade oral de substâncias lipofílicas quando veiculadas às nanopartículas lipídicas tem sido demonstrada para fármacos de diferentes classes farmacêuticas, como a vimpocetina (LUO et al., 2006), paclitaxel (PANDITA et al., 2011) e sinvastatina (ZHANG et al., 2010). Assim, sugere-se para estudos futuros que a farmacocinética da jatrofona também possa ser determinada.

Quando administrada pela via intravenosa a fração diclorometano foi efetiva no tratamento da dor e do edema em doses menores do que pela via oral, entretanto, devido a sua alta lipofilicidade e baixa solubilidade em água, foi necessário o uso de co-solventes como dimetilsulfóxido e polisorbato 80 para a

solubilização da fração e administração por esta via. Entretanto, o uso de co-solventes para a solubilização de fármacos apresenta várias desvantagens como a possível precipitação do fármaco, dor no local da injeção e hemólise (DATE; NAGARSENKER, 2008). Assim, uma alternativa para a administração da fração diclorometano pela via intravenosa é através da incorporação em nanocarreadores lipídicos, os quais tem a capacidade de incorporar o fármaco lipofílico na fase interna oleosa e ser veiculados em meio aquoso (fase externa).

Contudo, um fato que pode limitar a distribuição sistêmica dos nanocarreadores lipídicos é a sua rápida remoção do sangue pelos macrófagos do sistema reticuloendotelial, que ocorre através do reconhecimento das opsoninas que se ligam à superfície das partículas (OWENS; PEPPAS, 2006). Uma maneira de superar este problema e prolongar o tempo de circulação das partículas no sangue é através da peguilação das partículas, o que faz com que estas assumam características furtivas frente à opsonização e aos macrófagos (GREF et al., 2000).

Assim, carreadores lipídicos contendo a fração diclorometano (CLN_{Ji}) foram preparados pelo método da emulsificação espontânea contendo diferentes quantidades de DSPE-PEG_{2000} (0, 15, 30 60 e 90 mg) com a finalidade de otimizar as formulações para que sejam furtivas, ou seja, escapem da captura dos macrófagos. Tendo em vista a administração intravenosa, estas formulações também foram adicionadas de 2,5% de glicerol na fase externa aquosa para garantir isotonia (FRONZA; CAMPOS; TEIXEIRA, 2004). As respectivas formulações brancas (CLN_{branco}) também foram preparadas nestas condições. Os carreadores lipídicos desenvolvidos apresentaram índice de polidispersão menor que 0,2, indicando a formação de populações monodispersas.

Para as formulações dos CLN_{Ji} foi possível verificar uma elevada porcentagem de eficiência de encapsulação do ativo jatrofona, devido a grande lipofilicidade da fração diclorometano e também a redução do tamanho das partículas com o aumento da quantidade de DSPE-PEG_{2000}. Uma possível justificativa pode ser devido ao surgimento de uma interação mais coesa entre os lipídeos da fração diclorometano e a parte apolar da molécula do co-polímero DSPE-PEG_{2000}, que é formada por uma cadeia de

18 carbonos (18:0) orientados para a parte interna oleosa da partícula, resultando em tamanhos menores. O potencial zeta foi negativo para todos os CLN e aumentou (em módulo) com o aumento da quantidade do DSPE-PEG_{2000} devido ao grupamento fosfato carregado negativamente da molécula do polímero.

Os CLN_{Ji} desenvolvidos para a administração intravenosa apresentaram-se esféricos e com superfície levemente irregular, sendo observado, nas imagens de microscopia eletrônica, uma região interna com maior densidade eletrônica se comparada com o seu entorno.

Os CLN formulados para a administração intravenosa apresentaram-se extremamente estáveis quanto submetidos à centrifugação no LUMiSizer com índices de estabilidade menores que 0,05 para todas as formulações dos CLN_{Ji}. Já para os CLN_{branco} os valores variaram de 0,692 até 0,390, diminuindo com o aumento da quantidade de DSPE-PEG_{2000}. Os perfis de transmissão dos CLN_{branco} revelam uma área de clarificação no fundo da cubeta mas sem apresentar uma redução da porcentagem de transmissão no topo, ou seja, durante o tempo de centrifugação não pode ser visualizada qualquer tipo de cremação nas formulações brancas. Além disso, esta região de clarificação é diminuída com o aumento da quantidade de DSPE-PEG_{2000}, mostrando que a presença do copolímero influencia na estabilidade das partículas. Já para os CLN_{Ji} os perfis de transmissão são muito semelhantes entre as formulações, sem indicativos de mudança da porcentagem de transmissão ao longo da cubeta, ou seja, sem demonstrar quaisquer fenômeno de instabilidade das formulações. Neste estudo mais uma vez foi possível verificar que a fração diclorometano contribui claramente para a estabilidade das partículas, provavelmente devido ao seu conteúdo de ácidos graxos e que a presença do polímero DSPE-PEG_{2000} nas formulações também contribui tornando-as mais estáveis.

Tendo em vista a via intravenosa, os CLN foram avaliados com relação à sua biocompatibilidade em relação às hemácias. Foi verificado que nenhuma das formulações brancas apresentou qualquer atividade hemolítica, ou seja, a quantidade de tensoativos da formulação não está sendo prejudicial à membrana das hemácias. Já as formulações contendo a fração diclorometano apresentaram hemólise nas concentrações mais

altas testadas (500 e 750 µg/mL), provavelmente pela presença de saponinas na fração diclorometano, como o sitosterol 3-O-β-D-glicosídeo (daucosterol) e talvez pela presença em alta concentração dos terpenos jatrofolona A e jatrofona, que apresentaram efeitos citotóxicos sobre células permanentes do epitélio gástrico humano e fibroblastos (PERTINO et al., 2007a; 2007b; FRÖHLICH et al., 2013). Contudo, ainda pode ser verificado que a presença do polímero DSPE-PEG_{2000} auxilia na redução dos danos à membrana das hemácias devido ao impedimento estérico que exerce em torno da partícula impedindo a aproximação destas com as hemácias (ALAYOUBI et al., 2013).

Os estudos de viabilidade celular mostraram que as formulações lipídicas desenvolvidas não foram citotóxicas contra as células de macrófagos J774 e assim foi possível realizar os ensaios de captura. Estes ensaios mostraram que as formulações contendo 60 e 90 mg de DSPE-PEG_{2000} mostraram-se significativamente furtivas contra a fagocitose dos macrófagos, provavelmente pelo fato de apresentarem uma combinação de densidade e de comprimento de cadeias peguiladas ideais para desempenhar o efeito estérico e de barreira em torno das partículas capaz de reduzir ou até evitar o processo de opsonização e de fagocitose.

Com o objetivo de avaliar o efeito da presença do copolímero DSPE-PEG_{2000} sobre a superfície das partículas, foi realizado o ensaio de liberação da jatrofona a partir dos CLN_{Ji} sem o polímero e contendo 90 mg. A partir dos dados de eficiência de dissolução foi possível verificar que a presença do polímero não retarda a liberação do ativo, o que é ideal para estas formulações uma vez que foram testadas em modelo de artrite aguda.

Assim, tendo em vista a dose eficaz utilizada nos estudos farmacológicos iniciais da fração diclorometano quando administrada pela via intravenosa, conhecendo a dose limite dos CLN_{Ji} que pode ser administrada para que não haja hemólise nos animais e sabendo a quantidade de DSPE-PEG_{2000} necessária para que as partículas tenham capacidade furtiva frente aos macrófagos sem afetar a liberação da jatrofona, os CLN_{Ji} contendo 90 mg de DSPE-PEG_{2000} foram escolhidas para serem

testadas em modelo de artrite aguda e ter seu efeito comparado com o da fração diclorometano livre na mesma dose.

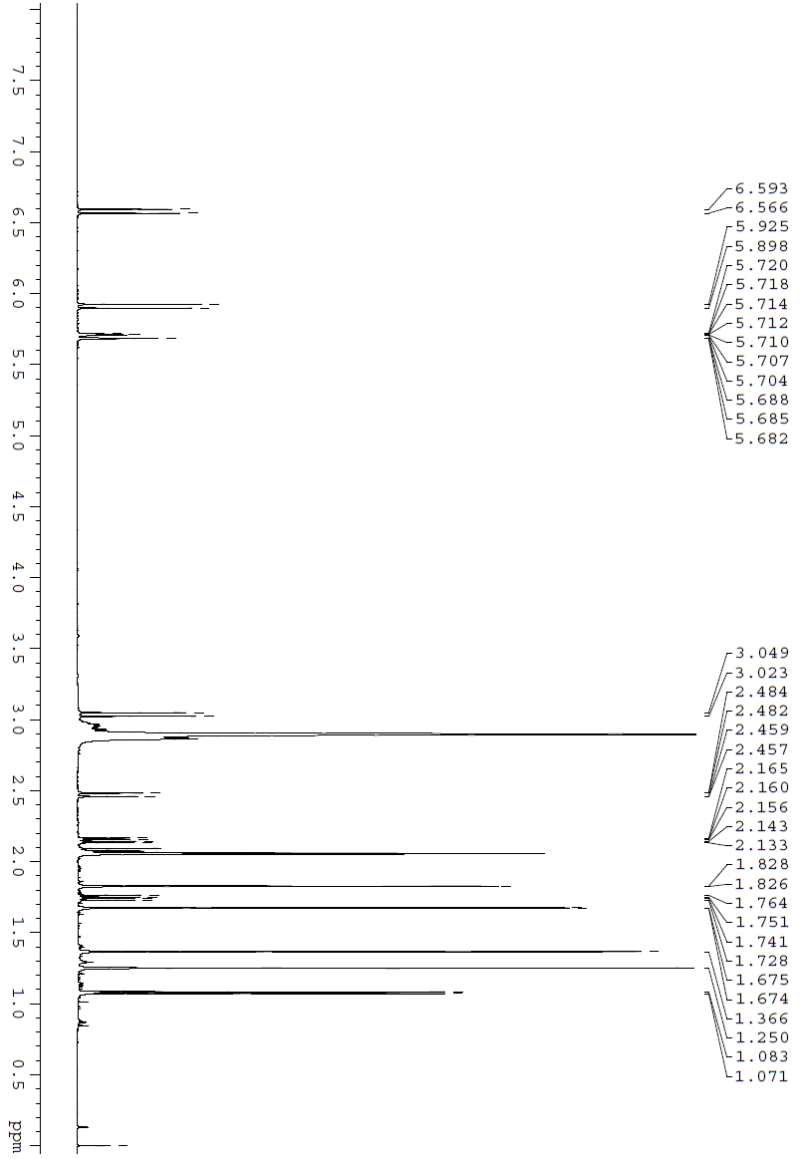
O estudo farmacológico realizado com ratos Wistar em modelo de artrite aguda mostrou que quando veiculada aos CLN a fração diclorometano é mais eficaz no tratamento da dor e do edema se comparada à fração livre. Isto ocorre devido à capacidade dos CLN de liberar a jatrofona e os demais terpenos da fração diretamente na articulação inflamada pelo aumento da permeabilidade da vasculatura destes tecidos (efeito EPR) sem ser rapidamente removidos da circulação sistêmica pelos macrófagos do SER. Além disso, a fração diclorometano foi administrada de forma segura aos animais, sob o ponto de vista farmacotécnico, pois está dispersa em meio aquoso e isotônico.

Tendo em vista os resultados apresentados neste trabalho, pode-se concluir que a fração diclorometano foi incorporada com sucesso nos carreadores lipídicos nanoestruturados e que quando veiculada nestas formulações possui uma boa eficácia terapêutica para o tratamento da artrite. O diterpeno jatrofona, ao qual foram atribuídos os efeitos anti-inflamatório e antinociceptivo, possui poucos mecanismos farmacológicos de dor e inflamação estudados, assim, devido a importância deste ativo e dos resultados obtidos, estudos sobre o mecanismo de ação, toxicológicos e sobre o perfil farmacocinético merecem ser aprofundados.

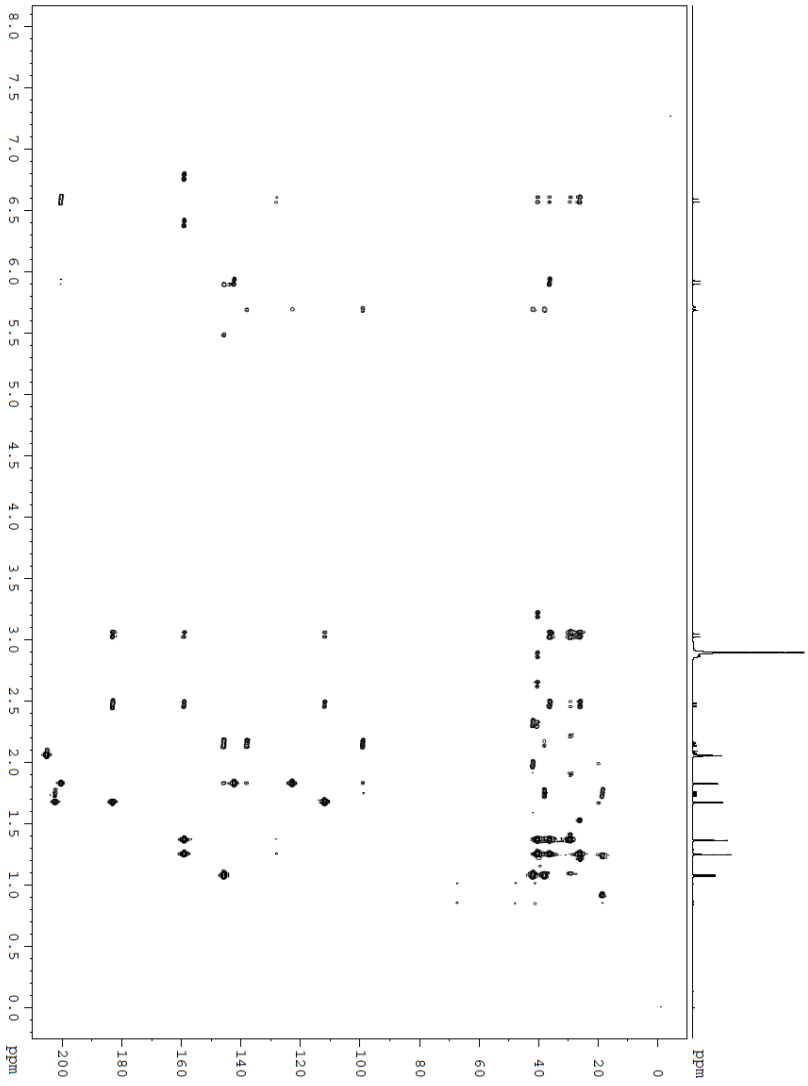
Assim, este trabalho é um passo que abre muitas alternativas de estudos sobre a espécie *J. isabellei* e o diterpeno jatrofona para o desenvolvimento de um medicamento fitoterápico ou simplesmente, para o uso da jatrofona na sua forma isolada, no tratamento da artrite.

ANEXOS

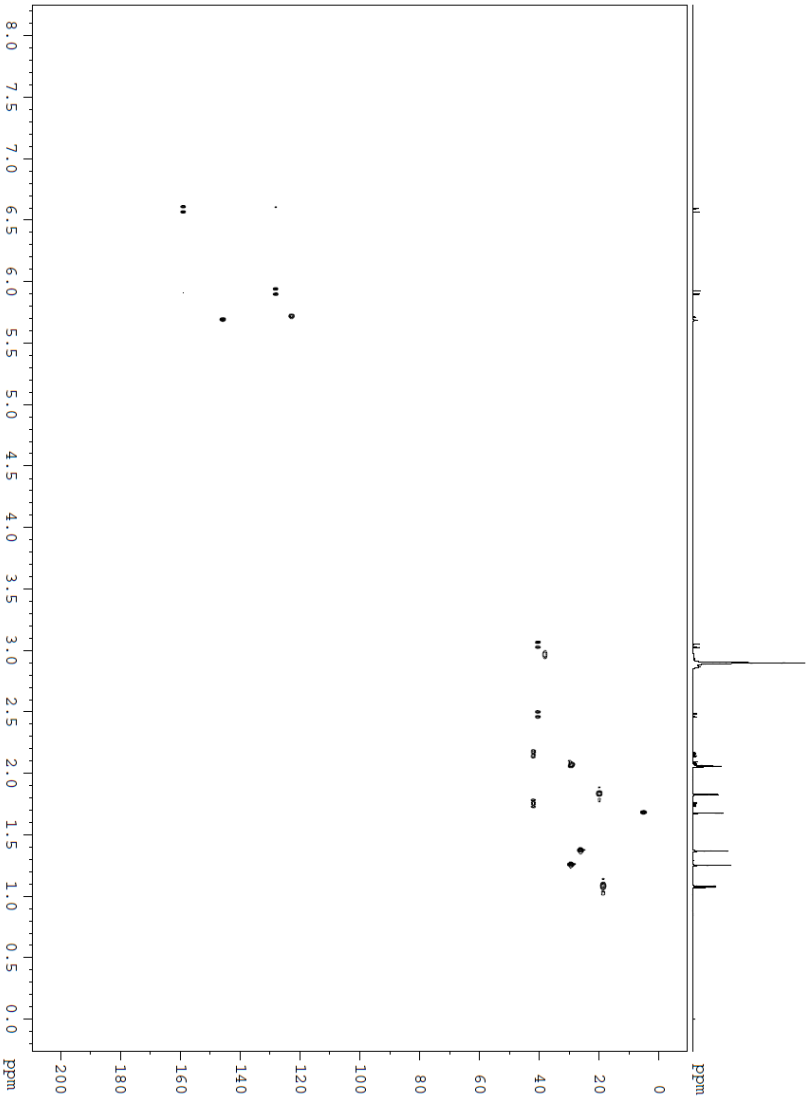
Anexo 1. Espectro de RMN H^1 (acetona-d_6, 600 MHz) do diterpeno jatrofona.



Anexo 2. Mapa de correlação HMBC do diterpeno jatrofona.

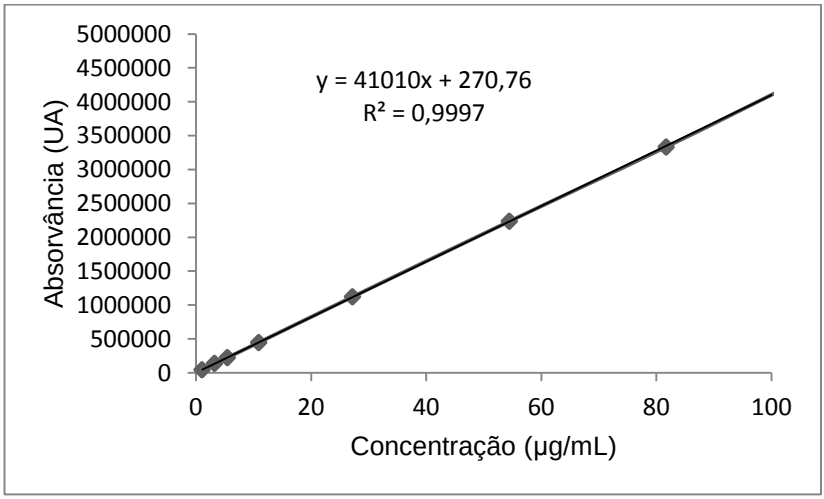


Anexo 3. Mapa de correlação HSQC do diterpeno jatrofona.

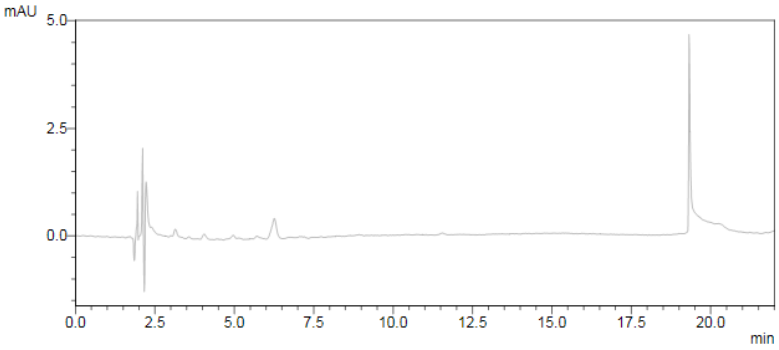


Anexo 4. Dados da validação da metodologia analítica por UFLC-DAD.

4.1. Curva de calibração.



4.2. Especificidade.



4.3. Precisão interdia.

	Absorbâncias (UA)	Concentração (µg/ml)	CV%
Dia 1	1886606	90,99299271	1,94%
	1885320	90,93095867	
	1888945	91,10582133	
	1887166	91,02000598	
	1885680	90,94832435	
	1885423	90,93592719	
Dia 2	1837875	87,00715857	
	1840437	87,12846452	
	1839811	87,09882458	
	1840091	87,11208206	
	1838220	87,02349368	
	1838028	87,01440283	
Dia 3	2041654	90,17698228	
	2039676	90,08960522	
	2042452	90,21223350	
	2041477	90,16916341	
	2042484	90,21364708	
	2039901	90,09954447	

4.4. Precisão intradia.

	Absorbâncias (UA)	Concentração (µg/ml)	CV%
Manhã	1907098	87,48192064	1,73%
	1911311	87,67520576	
	1912866	87,74654646	
	1912558	87,73241595	
	1908167	87,53096450	
	1911878	87,70121873	
Tarde	2071577	91,25085009	
	2071990	91,26904470	
	2070038	91,18304985	
	2070702	91,21230220	
	2072166	91,27679833	
	2073742	91,34627265	
Noite	2041654	90,17698228	
	2039676	90,08960522	
	2042452	90,21223350	
	2041477	90,16916341	
	2042484	90,21364708	
	2039901	90,09954447	

4.5. Exatidão.

Ativo	Teor (µg/mL)	Dosado (µg/mL) (d.p.)*	Recuperação (%)
Jatrofona	5.45	5.86 (0.20)	107.65
	27.25	26.19 (0.09)	96.13
	81.75	81.38 (0.43)	99.54

*desvio padrão.

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