UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

AVALIAÇÃO DA ATIVIDADE ANTINOCICEPTIVA DO ÓLEO ESSENCIAL DE *Annona vepretorum* Mart. (Annonaceae) E DO COMPLEXO DE INCLUSÃO DO OCIMENO COM β-CICLODEXTRINA EM MODELO EXPERIMENTAL DE DOR ONCOLÓGICA

MARIANA GAMA E SILVA

RECIFE-PE
2021

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Tese de doutorado apresentada ao Programa de Pós-graduação da Rede Nordeste de Biotecnologia (RENORBIO), como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Prof.° Dr. Jackson Roberto Guedes da Silva Almeida.

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Tese defendida e aprovada em 26 de fevereiro de 2021

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RESUMO

As plantas medicinais e os seus metabólitos secundários representam uma importante fonte para o desenvolvimento de novas opções terapêuticas mais eficazes e seguras para o manejo da dor crônica, incluindo a dor associada ao câncer. Nesse sentido, o objetivo central desse estudo foi avaliar a atividade antinociceptiva do óleo essencial de *Annona vepretorum* (Av-OE) e do (*E*)-β-ocimeno complexado e não complexado em β-ciclodextrina (β-CD) em modelo experimental de dor oncológica. O primeiro capítulo do trabalho traz uma visão geral sobre a química dos monoterpenos e os sistemas de liberação de fármacos aplicados a monoterpenos com potencial analgésico. O segundo capítulo fornece evidências de que o tratamento por via oral com Av-OE (50 e 100 mg/kg) foi capaz de atenuar o processo de hiperalgesia e/ou alodinia induzidos pela presença do tumor nos camundongos. Além disso, observou-se que os animais tratados com a dose de 100 mg/kg apresentaram diminuição do edema/tumor de pata no 12º dia de observação (0,37 ± 0,02 mL). O último capítulo descreve inicialmente o processo de obtenção e caracterização físico-química do complexo de inclusão do (*E*)-β-ocimeno em β-CD (ocimeno/β-CD), cujos resultados sugeriram a adequada complexação. A triagem de toxicidade demonstrou que houve uma diminuição da citotoxicidade do ocimeno/β-CD diante de linhagens de células tumorais (CI_{50} > 5 µg/mL), linhagem celular não tumoral (CI_{50} > 5 µg/mL) e de larvas de *Artemia salina* (CL_{50} > 1000 µg/mL) se comparado ao ocimeno não complexado. Contrariamente, ocimeno/β-CD mostrou maior potencial hemolítico diante de eritrócitos de camundongos Swiss (CH_{50} = 134,3 ± 3,61 µg/mL). Estimou-se um valor de dose letal 50% (DL_{50}) superior a 300 mg/kg quando avaliada a toxicidade aguda *in vivo* do ocimeno livre e complexado. A administração de ocimeno não complexado ou ocimeno/β-CD (25 e 50 mg/kg) por via oral em camundongos portadores de tumor sólido do sarcoma 180 na pata direita traseira foi capaz de reduzir a dor oncológica e o volume tumoral, com possível participação na modulação da resposta inflamatória, já que houve aumento significativo dos níveis da citocina anti-inflamatória IL-10 no soro dos animais tratados com ocimeno (15,98 ± 4,04 pg/mL) quando comparado com o grupo sham (6,73 ± 0,43 pg/mL). A avaliação dos parâmetros hematológicos sugeriu menor toxicidade do ocimeno quando complexado. Dessa forma, o tratamento com o óleo essencial de *A. vepretorum* ou com o complexo de inclusão contendo (*E*)-β-ocimeno e β-CD apresenta atividade antinociceptiva em camundongos portadores de tumor sólido de sarcoma 180.

Palavras-chave: Câncer. Dor. Produtos Naturais. Monoterpenos. Biotecnologia.

ABSTRACT

Medicinal plants and their secondary metabolites represent an important source for the development of new, more effective, and safer therapeutic options for pain management, including pain associated with cancer. In this sense, the central objective of this study was to evaluate the antinociceptive activity of the essential oil of *Annona vepretorum* (Av-OE) and of the (*E*)-β-ocimene free and complexed in β-cyclodextrin (β-CD) in an experimental model of cancer pain. The first chapter of the work provides an overview of the chemistry of monoterpenes and drug delivery systems applied to monoterpenes with analgesic potential. The second chapter provides evidence that oral treatment with Av-OE (50 and 100 mg/kg) was able to attenuate the process of hyperalgesia and/or allodynia induced by the presence of tumor in the mice. In addition, it was observed that the animals treated with the dose of 100 mg/kg showed a reduction in edema/paw tumor on the 12th day of observation (0.37 ± 0.02 mL). The last chapter initially describes the process of obtaining and physicochemical characterization of the ocimene inclusion complex in β-CD (ocimene/β-CD), whose results suggested the adequate complexation. It was also demonstrated that there was a decrease in the cytotoxicity of ocimene/β-CD against tumor cell lines (CI_{50} > 5 µg/mL), non-tumor cell line (CI_{50} > 5 µg/mL) and *Artemia salina* larvae (CL_{50} > 1000 µg/mL) when compared to free ocimene. In contrast, ocimene/β-CD showed greater hemolytic potential in the presence of erythrocytes from Swiss mice (CH_{50} = 134.3 ± 3.61 µg/mL). A lethal dose 50% (LD_{50}) value greater than 300 mg/kg was estimated when assessing the acute *in vivo* toxicity of free and complexed ocimene. The administration of free ocimene or ocimene/β-CD (25 and 50 mg/kg) orally in mice with a solid tumor of sarcoma 180 in the right hind paw was able to reduce cancer pain and tumor volume, with possible participation in the modulation of the inflammatory response, since there was a significant increase in the levels of the anti-inflammatory cytokine IL-10 in the serum of animals treated with ocimene (15.98 ± 4.04 pg/mL) when compared with the sham group (6.73 ± 0.43 pg/mL). The evaluation of hematological parameters suggested less toxicity of the ocimene when complexed. Thus, treatment with the essential oil of *A. vepretorum* or with the inclusion complex containing (*E*)-β-ocimene and β-CD has antinociceptive activity in mice with solid sarcoma tumor 180.

Keywords: Cancer. Pain. Natural Products. Monoterpenes. Biotechnology.

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LISTA DE ABREVIATURAS E SIGLAS

ASIC	Canal iônico sensível a ácido
ATP	Adenosina trifosfato
Av-EO	*Annona vepretorum essential oil*
Av-OE	Óleo essencial de *Annona vepretorum*
CB_2	Receptor canabinoide tipo 2
CGRP	Peptídeo relacionado com o gene da calcitonina
DMAPP	*Dimethylallyl diphosphate*
DMSO	*Dimethyl sulfoxide*
DRG	Gânglio da raiz dorsal
EP	Receptor de prostaglandina E
ET	Endotelina
ET_AR	Receptore de endotelina-A
FPP	*Farnesyl diphosphate*
GC-MS	*Gas chromatography–mass spectrometry*
GGPP	*Geranylgeranyl diphosphate*
GliR	Receptor de glicina
GPP	*Geranyl diphosphate*
HDA	Histona desacetilase
HMG-CoA	*3-hydroxy-3-methylglutaryl-CoA*
HMGR	*HMG-CoA reductase*
HMGS	*HMG-CoA synthase*
HP-β-CD	*Hydroxypropyl-β-cyclodextrin*
IASP	Associação Internacional para o Estudo da Dor
IL-1β	Interleucina 1 beta
IPP	*Isopentenyl diphosphate*
mGlu1/5	receptores metabotrópicos de glutamato do grupo I
MK	*Mevalonate kinase*
MVA	*Mevalonate*
NGF	Fator de crescimento neural
NMDA	N-metil-D-aspartato
NSAIDs	*Non-steroidal anti-inflammatory drugs*
P_2X_3	Receptor purinérgico sensível ao ATP

PBS	*Phosphate buffered saline*
PCL	*Polycaprolactone*
PG	Prostaglandina
PGE_2	Prostaglandina E2
PLA	*Poly-l-lactic acid*
PLGA	*Poly(lactic-co-glycolic acid)*
PPMK	*Phosphomevalonate kinase*
PVA	*Polyvinyl alcohol*
RANTES	Citocina Regulada sob Ativação, Expressa e Secretada por Células T Normais
RM-β-CD	*Randomly methylated-β-cyclodextrin*
ROS	Espécies reativas de oxigênio
SBE-β-CD	*Sulfobutylether-β-cyclodextrin*
SNC	Sistema Nervoso Central
TGF-β	Fator de transformação do crescimento β
TNF-α	Fator de necrose tumoral α
TrKA	Receptor tirosina-quinase A
VEGF	Fator de crescimento endotelial vascular
VR1	Receptor vaniloide tipo 1
α-CD	α-ciclodextrina, *α-cyclodextrin*
β-CD	β-ciclodextrina, *β-cyclodextrin*
γ-CD	γ-ciclodextrina, γ-*cyclodextrin*

SUMÁRIO

1 INTRODUÇÃO

De acordo com a Associação Internacional para o Estudo da Dor (IASP), o termo dor pode ser entendido como uma experiência sensitiva e emocional desagradável, associada, ou semelhante àquela associada, a uma lesão tecidual real ou potencial (RAJA et al., 2020). A dor acomete cerca de 55% dos pacientes em tratamento contra o câncer e é o sintoma mais frequente no estágio avançado da doença ou em metástase, representando impacto negativo na qualidade de vida dos pacientes (VAN DEN BEUKEN-VAN et al., 2016). A etiologia da dor oncológica pode ser multifatorial e está relacionada principalmente a presença do próprio tumor, devido a compressão de tecidos ou nervos periféricos ou infiltração e metástase das células tumorais. Além disso, a dor pode estar relacionada aos métodos de diagnóstico e/ou ao tratamento, como procedimentos cirúrgicos, quimioterapia e radioterapia (AMERICAN CANCER SOCIETY, 2015).

O tratamento farmacológico da dor associada ao câncer é baseado no método denominado de escada analgésica, desenvolvido pela Organização Mundial da Saúde (OMS). A escada analgésica original consistia na organização e padronização do tratamento analgésico conforme uma escada de três degraus de acordo com a intensidade da dor que o paciente apresenta, incluindo o uso de fármacos não opioides, opioides fracos, opioides fortes e fármacos adjuvantes. Mais recentemente foi adicionado um quarto degrau, incluindo procedimentos intervencionistas e minimamente invasivos (ANEKAR; CASCELLA, 2020). Entretanto, esses fármacos exibem reações adversas que acabam limitando a sua utilização, o que tem estimulado a busca por novas alternativas terapêuticas que promovam não apenas o controle da dor oncológica, mas também a redução do crescimento tumoral e da ocorrência de metástases.

Nesse sentido, os produtos naturais têm merecido especial atenção, pois mesmo diante do grande desenvolvimento da síntese orgânica e de novos processos biotecnológicos, estima-se que cerca de 50% de todos os medicamentos aprovados no período entre 1980 e 2014 foram obtidos a partir de fontes naturais (NEWMAN; CRAGG, 2016). Isso porque a maioria dos princípios ativos obtidos a partir das plantas são metabólitos secundários biossintetizados por elas que funcionam como um mecanismo de defesa para atuar em alvos específicos moleculares de seus predadores, mas que também podem alcançar alvos terapêuticos de doenças humanas (FERREIRA; PINTO, 2010).

Os monoterpenos são compostos sintetizados principalmente por plantas aromáticas e pertencem à classe de metabólitos secundários conhecidos como terpenoides. São considerados os principais constituintes químicos dos óleos essenciais (DHIFI et al., 2016) e apresentam várias propriedades terapêuticas, incluindo atividade analgésica (GOUVEIA et al., 2017). O monoterpeno (*E*)-β-ocimeno é um dos constituintes químicos majoritários do óleo essencial das folhas de *Annona vepretorum* Mart. (Annonaceae), espécie vegetal endêmica do bioma Caatinga, e pode estar contribuindo para seus efeitos farmacológicos (COSTA et al., 2012; MEIRA et al., 2015; BOMFIM et al., 2016). Entretanto, apesar do seu potencial medicinal, os monoterpenos exibem solubilidade aquosa muito baixa, alta volatilidade e instabilidade diante de luz e oxigênio, o que pode limitar seu uso (KFOURY et al., 2014). Recentemente, estudos têm demonstrado que as propriedades físico-químicas e o perfil farmacológico dos monoterpenos melhoram quando complexados em β-ciclodextrina (LIMA et al., 2016; CARNEIRO et al., 2019; GANDHI et al., 2020).

Diante da busca por novas alternativas terapêuticas para o manejo da dor oncológica e do potencial farmacológico de *A. vepretorum* o objetivo desse estudo é avaliar a atividade antinociceptiva do óleo essencial de *A. vepretorum* e do (*E*)-β-ocimeno complexado e não complexado em β-ciclodextrina (β-CD) em modelo experimental de dor oncológica induzida pelo sarcoma 180.

O presente estudo está estruturado em 3 capítulos. O primeiro consiste em uma visão geral sobre a química e os principais sistemas de liberação de fármacos aplicados a monoterpenos com atividade analgésica. O segundo capítulo relata o efeito antinociceptivo do óleo essencial das folhas de *A. vepretorum* em camundongos portadores de sarcoma 180. O último capítulo demonstra o método de obtenção, caracterização físico-química, triagem toxicológica e a investigação do efeito do (*E*)-β-ocimeno livre e complexado no referido modelo experimental de dor oncológica.

2 REVISÃO DE LITERATURA

2.1 Dor oncológica

A dor é um dos sintomas mais comuns em pacientes com câncer e diminui a qualidade de vida desses indivíduos. Ela pode ser descrita como uma experiência multifatorial que envolve vias de neurotransmissão além de fatores emocionais, ambientais e cognitivos. Os três mecanismos subjacentes à fisiopatologia da dor são nociceptivos, neuropáticos e psicogênicos (NATIONAL CANCER INSTITUTE, 2016).

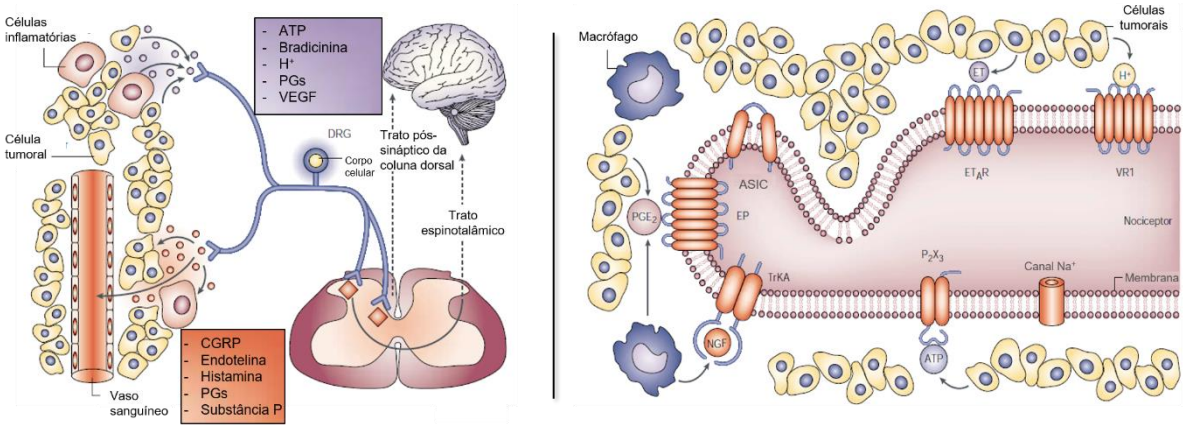
A dor nociceptiva é o resultado do dano inicial aos tecidos ou nervos periféricos. A informação é então conduzida por meio da ativação das vias sensoriais, usando fibras nervosas especializados (fibras sensoriais do tipo C e A-delta), para a medula espinhal que, após a integração dos estímulos recebidos, leva o sinal aos centros superiores do Sistema Nervoso Central (SNC) (BASBAUM et al., 2009; GRACE et al., 2014).

Conforme mostrado na Figura 1, as células cancerosas, células do sistema imunológico e células do estroma presentes no microambiente tumoral secretam várias substâncias, como as prostaglandinas, endotelinas, fator de necrose tumoral α (TNF-α), interleucina-1 (IL-1) e 6 (IL-6), fator de transformação do crescimento β (TGF-β), fator de crescimento derivado de plaquetas e íons hidrogênios (H^+), os quais podem promover a estimulação ou sensibilização dos nociceptores. Nesse sentido, os nociceptores são terminações nervosas livres de neurônios aferentes primários cuja função principal é detectar e converter estímulos ambientais reconhecidos como prejudiciais em sinais eletroquímicos que são transmitidos ao SNC. Isso é possível graças a presença de diferentes tipos de receptores, como o receptor vaniloide tipo 1 (VR1), receptores de endotelina-A (ET_AR), receptores de prostaglandinas e receptor tirosina cinase (MANTYH, et al., 2002; BROWN; RAMIREZ, 2015).

A ativação do nociceptor provoca a liberação de neurotransmissores, como o peptídeo relacionado com o gene da calcitonina (CGRP), endotelina, histamina, glutamato e substância P no corno dorsal da medula espinhal e o sinal é transmitido para os centros superiores do SNC por pelo menos duas vias principais, o trato espinotalâmico e a coluna dorsal. Como resultado de todo esse processo, ocorrem mudanças neuroquímicas no SNC, como hipertrofia de astrócitos, diminuição da expressão dos transportadores de recaptação de glutamato, provocando um aumento dos níveis extracelulares desse neurotransmissor, excitotoxicidade do SNC e regulação positiva da dinorfina, um peptídeo pró-hiperalgésico da classe dos opioides. Consequentemente, os neurônios da medula espinhal que normalmente seriam ativados apenas

por estímulos nocivos podem ser ativados por estímulos que normalmente seriam não-nocivos, evento conhecido como sensibilização central (MANTYH, et al., 2002).

Figura 1 - Mecanismo de nocicepção induzida por células tumorais. ASIC: canal iônico sensível a ácido; PGs: prostaglandinas; VEGF: fator de crescimento endotelial vascular; CGRP: peptídeo relacionado com o gene da calcitonina; VR1: receptor vaniloide tipo 1; ATP: adenosina trifosfato; DRG: gânglio da raiz dorsal; EP: receptor de prostaglandina E; ET_AR: receptor de endotelina-A; P_2X_3: receptor purinérgico sensível ao ATP; TrKA: receptor tirosina-quinase A; PGE_2: prostaglandina E2; NGF: fator de crescimento neural; ET: endotelina.



Fonte: Adaptado de Mantyh e colaboradores (2002).

A dor neuropática é provocada por lesões no sistema nervoso periférico e/ou central. No paciente oncológico pode ser um resultado dos efeitos diretos da presença do tumor, que comprime ou infiltra nervos, bem como do tratamento (DWORKIN et al., 2003). Vincristina e paclitaxel, drogas comumente usadas em protocolos clínicos para o tratamento do câncer, interferem diretamente na polimerização da tubulina, comprometendo o transporte axonal de fatores tróficos, levando à degeneração dos neurônios sensoriais e consequentemente à liberação de citocinas pró-inflamatórias que sensibilizam diretamente os neurônios aferentes primários, caracterizando neuropatia periférica, o tipo mais comum de dor em pacientes com câncer (BRIGO et al., 2012; LAPOINTE et al., 2013). A alodinia, dor diante de estímulos geralmente não nocivos, e a hiperalgesia, aumento da sensação dolorosa diante de um estímulo que normalmente provoca dor, são fenômenos frequentes na dor neuropática (DWORKIN, 2003; SCHESTATSKY, 2008).

O estado emocional do paciente oncológico também pode contribuir para a sua experiência dolorosa. Quando as queixas parecerem desproporcionais ao estímulo subjacente à dor, é importante avaliar, dentre outros fatores, a angústia psicológica e existencial que pode estar associadas à doença. Dessa forma, o manejo da dor oncológica pode incluir, além do

tratamento farmacológico, cuidados psicossociais e espirituais. É importante mencionar que o objetivo do controle da dor é reduzi-la a um nível que permita uma qualidade de vida aceitável para o paciente. Além disso, deve-se considerar que o benefício do alívio da dor esteja equilibrado com o risco de efeitos adversos, dependência e overdose que podem resultar em depressão respiratória (NATIONAL CANCER INSTITUTE, 2019).

Uma escada de gerenciamento da dor associada ao câncer foi estabelecida pela OMS para servir como um guia geral para manejo desse sintoma. A escada analgésica inclui o uso de analgésicos não-opioides, como o paracetamol e os anti-inflamatórios não esteroidais, para dores fracas; opioides fracos, como a codeína, para dores moderadas ou mais intensas; opioides fortes, como a morfina, hidromorfona, oxicodona, fentanil e metadona, para dores severas (OMS, 2018). Os anticonvulsivantes (carbamazepina) e antidepressivos (amitriptilina e venlafaxina) podem ser utilizados como adjuvantes no tratamento da dor oncológica para aumentar a analgesia; os corticosteroides (dexametasona, metilprednisolona e prednisolona) podem ser úteis em reduzir o edema peritumoral nos tumores do cérebro, fígado ou torácicos (BRUERA; KIM, 2003).

2.2 Modelos animais para avaliação da dor oncológica

Modelos animais experimentais são importantes para melhor conhecer os mecanismos básicos que atuam no surgimento e manutenção da dor associada ao câncer e oferecem uma oportunidade para o desenvolvimento de estratégias de tratamento mais eficazes. Nos últimos anos, uma variedade de modelos animais surgiu para o estudo da nocicepção relacionada ao tumor, como o modelo de dor associada ao câncer ósseo, dor relacionada à quimioterapia, dor induzida por tumor na pata traseira e dor devido à invasão do tumor (PACHARINSAK; BEITZ, 2008; CURRIE et al., 2013).

A dor óssea relacionada ao tumor é o tipo de dor mais comum em pacientes com câncer avançado. Isso ocorre principalmente porque uma variedade de neoplasias malignas, como câncer de mama, próstata e pulmão, tem uma propensão notável à metástase óssea, mas a dor pode surgir também de tumores ósseos primários (PACHARINSAK; BEITZ, 2008; GROND et al., 1996). Dessa forma, os modelos de dor oncológica relatados mais frequentemente na literatura são de dor óssea, principalmente relacionada ao crescimento de tumores na medula óssea (CURRIE et al., 2013). Outros modelos de nocicepção oncológica em animais mimetizam a dor causada pela invasão tumoral dos nervos periféricos, a dor produzida pela neuropatia

periférica relacionada à quimioterapia e modelos associados a tumores que ocorrem espontaneamente em animais (PACHARINSAK; BEITZ, 2008).

A maioria dos modelos animais de dor oncológica é desenvolvida em roedores, principalmente ratos (SLOSKY; LARGENT-MILNES; VANDERAH, 2015). Especialmente nos modelos de dor associada ao câncer ósseo os ratos apresentam algumas vantagens quando comparados aos camundongos, como o tamanho do animal que, por possuir ossos maiores, facilita a inoculação intramedular das células tumorais (IANNACCONE; JACOB, 2015).

A escolha do sexo é outro fator importante a ser considerado na avaliação de parâmetros comportamentais relacionados à nocicepção oncológica (SLOSKY; LARGENT-MILNES; VANDERAH, 2015). Estudos demonstraram que as fêmeas são mais sensíveis a estímulos químicos, térmicos e elétricos e apresentam diferenças no sistema opioide endógeno em comparação aos machos. Além disso, existem evidências de que os níveis hormonais influenciam nas respostas à dor (WIESENFELD-HALLIN, 2005; FILLINGIM et al., 2009).

Grande parte das pesquisas para avaliação da nocicepção oncológica utiliza a aplicação de estímulos térmicos ou mecânicos nos animais para observar comportamentos relacionados à dor. A sensibilidade ao estímulo mecânico pode ser avaliada pelo limiar de retirada da pata após estimulação com um analgesímetro digital ou filamentos de von Frey e pelo recuo ou proteção induzida por palpação. A avaliação da nocicepção diante de um estímulo térmico pode ser realizada nos animais por meio um teste de placa quente ou sensibilidade térmica ao calor radiante ou por meio do teste de acetona a frio ou placa a frio. Parâmetros relacionados com dor espontânea e dor relacionada ao movimento também podem ser avaliados em modelos experimentais de dor oncológica. Além disso, existe um interesse crescente na investigação de dor espontânea em protocolos de avaliação comportamental em modelos de dor relacionados a tumores, devido à importância clínica desse problema (PACHARINSAK; BEITZ, 2008; CURRIE et al., 2013).

2.3 Avaliação farmacológica pré-clínica de produtos naturais em protocolos experimentais de dor oncológica

Devido aos efeitos adversos associados à farmacoterapia convencional utilizada na dor associada ao câncer, faz-se necessário buscar novos compostos bioativos que proporcionem a redução da dor oncológica e que apresentem um bom perfil de segurança. Os produtos naturais continuam desempenhando um papel importante nesse processo de descoberta e desenvolvimento de novos fármacos, principalmente devido à grande diversidade química que

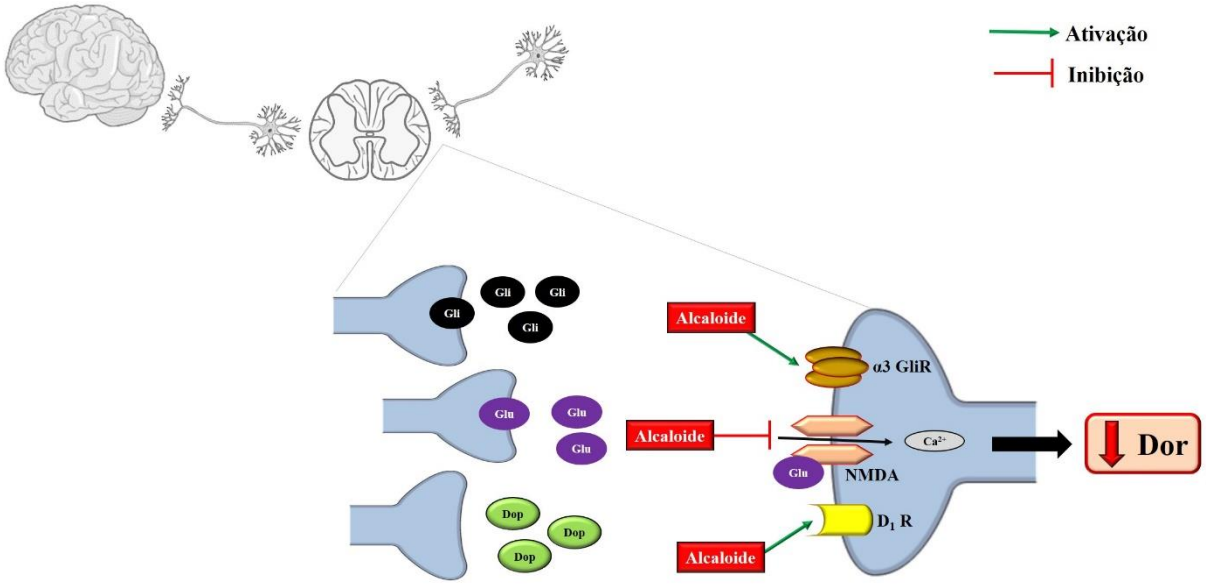
apresentam. Estima-se que no período entre 1980 e 2014, dos 1211 medicamentos aprovados, 619 tiveram origem natural, a partir de fontes vegetais, animais, marinhas e microbianas (SIDDIQUI et al., 2014; NEWMAN; CRAGG, 2016).

Muitos estudos pré-clínicos descritos na literatura evidenciam a atividade analgésica de extratos vegetais, formulações à base de ervas medicinais e metabólitos secundários como alcaloides, flavonoides, terpenoides, quinonas e cumarinas em modelos experimentais de nocicepção oncológica, conforme descrito a seguir (ZHANG et al., 2013; GUIMARÃES et al., 2014; GUO et al., 2014; HANG et al., 2014; CALIXTO-CAMPOS et al., 2015; DAI et al., 2017; HU et al., 2017; JIANG et al., 2017).

Alcaloides são compostos orgânicos de baixo peso molecular, derivados do metabolismo de aminoácidos e com nitrogênio na sua estrutura química. Vinte etapas enzimáticas podem estar presentes na biossíntese de alcaloides e essas vias complexas permitem a diversidade de núcleos e propriedades biológicas exibidas por esses compostos, destacando-se o potencial anticâncer, anticolinérgico, antimalárico, antibacteriano, antifúngico, anti-helmíntico, cardiotônico, anticonvulsivante, anti-hiperglicêmico, anti-inflamatório e analgésico (KAUR; ARORA, 2015).

Zhang e colaboradores (2013) observaram que o alcaloide gelsemina promoveu atividade antinociceptiva em modelo de dor associada a câncer ósseo possivelmente por meio da ativação da subunidade α3 do receptor de glicina (α3 GliR). Sinapses glicinérgicas mediam a neurotransmissão inibitória rápida principalmente na medula espinhal e tronco cerebral, controlando uma variedade de funções motoras e sensoriais, incluindo o processamento da dor. *Levo*-coridalmina, um alcaloide presente na espécie *Corydalis yanhusuo* W.T. Wang, bloqueou os receptores N-metil-D-aspartato (NMDA) e os receptores metabotrópicos de glutamato do grupo I (mGlu1/5) na medula espinhal de ratos com câncer ósseo, promovendo a atenuação da nocicepção associada ao tumor. Sabe-se que a ativação desses receptores contribui para a transmissão sináptica excitatória e sensibilização central, devido ao aumento das concentrações citoplasmáticas de íons cálcio (DAI et al., 2017). Já a ativação de receptores dopaminérgicos pareceu estar envolvida com o mecanismo de ação antinociceptiva do alcaloide *levo*-tetrahidropalmatina em modelo de dor neuropática associada ao tratamento quimioterápico (GUO et al., 2014). A Figura 2 apresenta um resumo dos principais mecanismos de ação analgésica de alcaloides avaliados em modelos experimentais de dor oncológica.

Figura 2 - Possíveis mecanismos de ação analgésica de alcaloides avaliados em modelos experimentais pré-clínicos de dor oncológica. Gli: glicina; Glu: glutamato; Dop: dopamina; GliR: receptor de glicina; NMDAR: receptor NMDA; NMDA: N-metil-D-Aspartato; $D_{1,2}R$: receptor de dopamina tipo 1 ou 2; Ca^{2+}: íon cálcio.



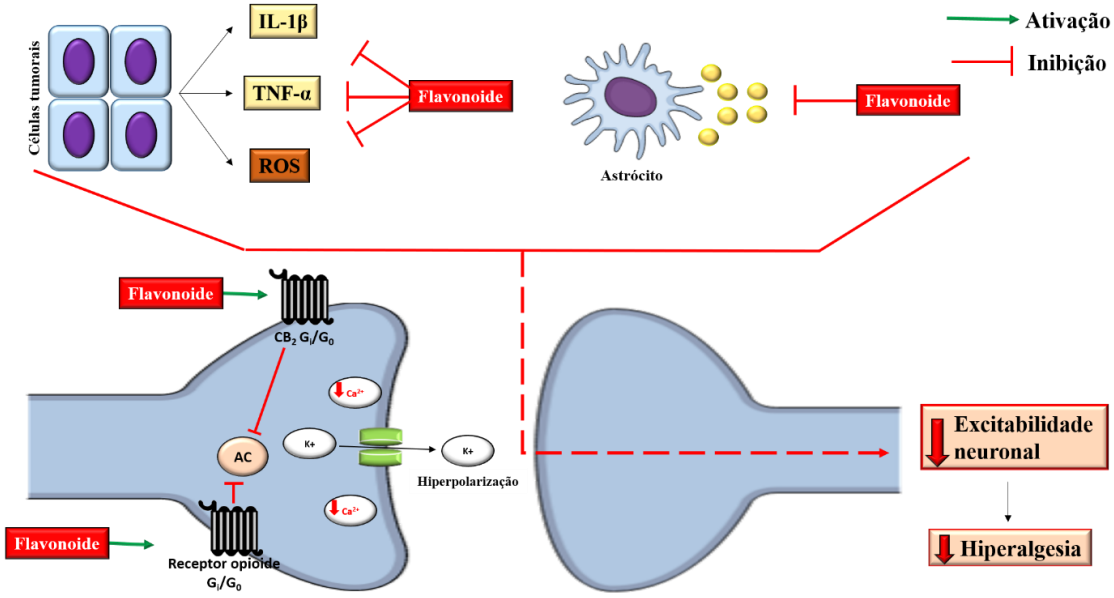
Fonte: Autoria própria.

Os flavonoides são um grupo de compostos naturais encontrados em vegetais, biossintetizados a partir da via dos fenilpropanoides e constituem uma importante classe de polifenóis. A maioria dos flavonoides possui 15 átomos de carbono no seu núcleo fundamental, constituído por duas fenilas ligadas por uma cadeia de três carbonos entre elas (SIMÕES et al., 2010). Mais de 6.000 estruturas diferentes dessa classe já foram identificadas até o momento. Esses metabólitos secundários geralmente apresentam-se oxigenados e muitos são conjugados com açúcar. Nas plantas, são utilizados como fator de crescimento, pigmentos e mecanismo de defesa contra o ataque de insetos, microrganismos e radiação ultravioleta (UV) (FERREYRA; RIU; CASATI, 2012; PANCHE; DIWAN; CHANDRA, 2016). Muitos estudos já demostraram, dentre outros, o potencial antioxidante, anticâncer, fotoprotetor, hipolipemiante, anti-inflamatório e analgésico dos flavonoides (QUINTANS et al., 2014; PANCHE; DIWAN; CHANDRA, 2016).

No que se refere a atividade antinoceptiva, Calixto-Campos e colaboradores (2015) demonstraram que o tratamento com o flavonoide quercetina promoveu efeito anti-hiperalgésico em camundongos portadores de tumor sólido na pata possivelmente por meio de diferentes mecanismos, como redução da produção das citocinas hiperalgésicas interleucina-1β (IL-1β) e fator de necrose tumoral α (TNF-α), recrutamento de neutrófilos e redução do estresse

oxidativo. Por sua vez, o flavonoide morina possivelmente é capaz de promover a ativação dos receptores canabinoides do tipo 2 (CB2) na medula espinhal e a supressão da ativação de astrócitos (JIANG et al., 2017), conforme esquematizado na Figura 3.

Figura 3 - Possíveis mecanismos de ação analgésica de flavonoides avaliados em modelos experimentais pré-clínicos de dor oncológica. IL-1β: interleucina 1 beta; TNF-α: fator de necrose tumoral alfa; ROS: espécies reativas de oxigênio; CB_2: receptor canabinoide tipo 2; AC: adenilil ciclase; Ca^{2+}: íon cálcio; K^+: íon potássio.



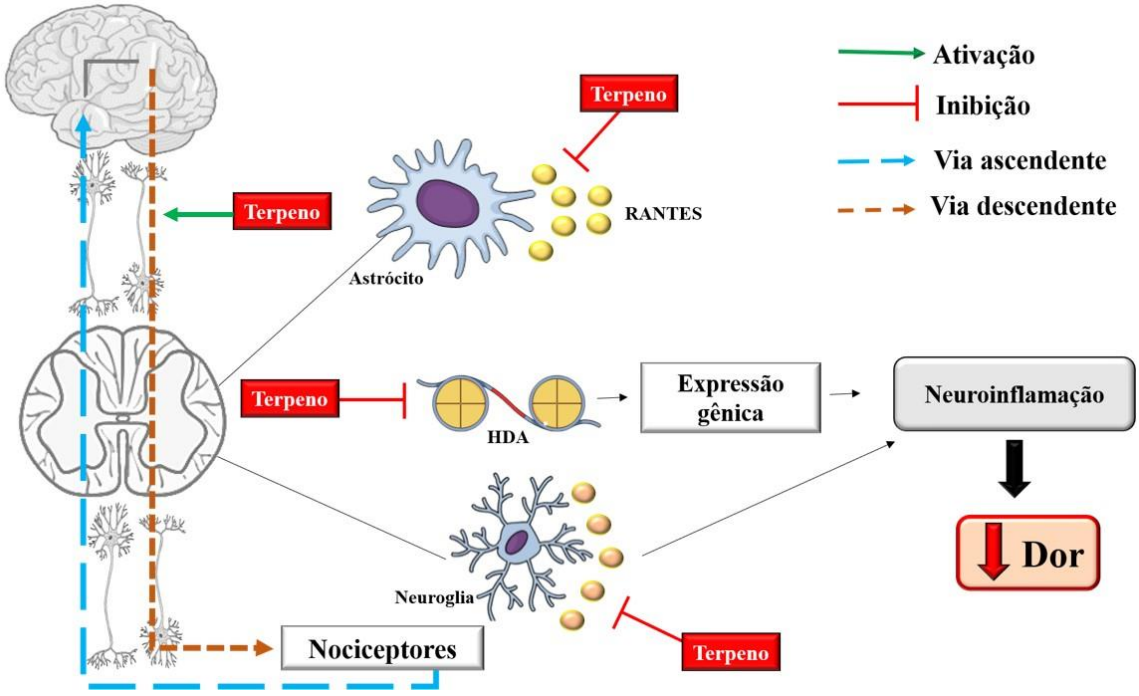
Fonte: Autoria própria.

Os terpenoides, por sua vez, constituem uma classe diversificada de substâncias vegetais com uma gama de propriedades biológicas, tais quais antimicrobiana, anticâncer, hepatoprotetora, anti-inflamatória e antinociceptiva (THOLL, 2015; LUDWICZUK; SKALICKA-WO´ZNIAK; GEORGIEV, 2017). São produzidos a partir da condensação de unidades de isopreno, o qual, por sua vez, origina-se a partir do ácido mevalônico. Os monoterpenos são os compostos terpênicos mais frequentes nos óleos voláteis (cerca de 90%) e podem ser divididos em três subgrupos: acíclicos, monocíclicos e bicíclicos. Dentro desses subgrupos há ainda outras classificações: hidrocarbonetos insaturados, álcoois, aldeídos ou cetonas, lactonas e tropolonas (SIMÕES et al., 2010).

Estudos demonstram que os terpenos também podem inibir a dor associada ao câncer por diversos mecanismos (Figura 4). A modulação da via descendente inibitória da dor, por exemplo, parece estar envolvida com a atividade analgésica do carvacrol (GUIMARÃES et al., 2014). O diterpeno triptolide demonstrou promover ação analgésica por meio da inibição

espinhal de RANTES (citocina Regulada sob Ativação, Expressa e Secretada por Células T Normais) (HANG et al., 2014), inibição da regulação positiva de histona desacetilases nas células da glia no corno dorsal espinhal e bloqueio da neuroinflamação induzida pela ativação glial (HU et al., 2017).

Figura 4 - Possíveis mecanismos de ação analgésica de terpenos avaliados em modelos experimentais pré-clínicos de dor oncológica. RANTES: citocina Regulada sob Ativação, Expressa e Secretada por Células T Normais; HDA: histona desacetilase.



Fonte: Autoria própria.

2.4 (*E*)-β-ocimeno: monoterpeno presente no óleo essencial das folhas de *Annona vepreoturm* Mart.

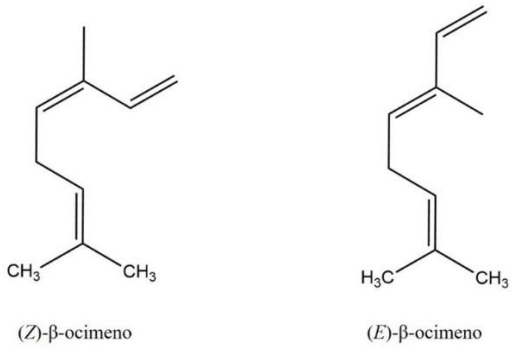
A região Nordeste do Brasil, cujo bioma Caatinga é o principal ecossistema, apresenta uma rica diversidade de plantas e o interesse pelo estudo dos usos tradicionais dessas espécies vegetais e seus produtos têm aumentado progressivamente (AGRA et al., 2007). Pesquisa realizada por Agra e colaboradores (2008) sobre as plantas conhecidas pelos usos etnomedicinais nessa região revelou um total de 650 espécies, das quais cerca de 126 foram referidas pela sua utilização popular. No entanto, grande parte delas ainda não foi estudada quanto aos seus constituintes químicos e/ou atividades biológicas.

A. vepretorum, pertence à família Annonaceae e ao gênero Annona, *e* é uma espécie vegetal endêmica do bioma Caatinga, onde recebe o nome popular de “pinha-da-caatinga”

(MAAS; LOBÃO; RAINER, 2015). Essa planta possui indicação popular contra picadas de abelhas e é conhecida como um anti-inflamatório natural (COSTA et al., 2011). Estudos realizados com extrato etanólico das folhas de *A. vepretorum* relataram principalmente a presença de alcaloides e descreveram o seu potencial efeito sedativo, antinociceptivo, anti-inflamatório, antioxidante, citotóxico e antimicrobiano (DINIZ et al., 2013; ALMEIDA et al., 2014; SILVA et al., 2015; SILVA et al., 2016). Das suas cascas isolou-se diterpenos, os quais apresentaram elevada atividade citotóxica (DUTRA et al., 2014). O óleo essencial das folhas de *A. vepretorum*, com composição predominante de monoterpenos e sesquiterpenos, também tem apresentado diversas atividades farmacológicas, tais quais citotóxica, antitumoral, antimicrobiana, antinociceptiva, anti-inflamatória, tripanocida e antimalárica (COSTA et al., 2012; SILVA, 2013; MEIRA et al., 2015; BOMFIM et al., 2016).

β-ocimeno (3,7-dimetil-1,3,6-octatrieno) é um dos componentes mais abundantes dos óleos voláteis que são emitidos pelas folhas em resposta a danos mecânicos ou provocados por herbívoros (FALDT et al., 2003). Além disso, é comumente presente em muitos aromas florais (KNUDSEN; TOLLSTEN; BERGSTROM, 1993). Esse monoterpeno apresenta fórmula química $C_{10}H_{16}$ e possui dois estereoisômeros, *cis*- e *trans*-β-ocimeno (ou (*Z*)- e (*E*)-β-ocimeno, respectivamente), conforme mostrado na figura 5, sendo o isômero *trans* mais comum e mais abundantemente emitido em aromas florais (FARRÉ-ARMENGOL et al., 2017). (*E*)-β-ocimeno é um dos constituintes químicos majoritários do óleo essencial das folhas de *A. vepretorum* (COSTA et al., 2012; ARAÚJO, 2013; BOMFIM et al., 2016; DINIZ et al., 2019) e apresenta múltiplas funções nos vegetais, conferindo resistência a patógenos, resistência a altas temperaturas, atuando na interação planta-planta e na atração de agente polinizadores (KNUDSEN; TOLLSTEN; BERGSTROM, 1993; DUDAREVA et al., 2003; FALDT et al., 2003; FARRÉ-ARMENGOL et al., 2017; KANG et al., 2018; XIAO et al., 2020). Além disso, ocimeno demonstrou atividade antifúngica contra cepas de *Candida albicans* quando testado isoladamente ou em associação com fluconazol (THAKRE et al., 2016).

Figura 5 – Estrutura química dos estereoisômeros do β-ocimeno, (*Z*)-β-ocimeno e (*E*)-β-ocimeno.



(*Z*)-β-ocimeno (*E*)-β-ocimeno

Fonte: Autoria própria.

Esses achados demonstram que ocimeno apresenta potencial biológico e terapêutico a ser explorado. Entretanto, assim como muitos outros compostos bioativos obtidos a partir de plantas, possui aplicação limitada como produto farmacêutico devido às suas características físico-químicas, necessitando de tecnologias farmacêuticas que sejam capazes de garantir sua integridade estrutural e sua bioatividade. Nesse cenário, sistemas de liberação de fármacos foram formulados e estão sendo investigados para a administração de metabólitos secundários não polares, incluindo as ciclodextrinas (CDs) (LIMA et al., 2016).

2.5 Ciclodextrinas

As CDs são oligossacarídeos cíclicos em forma de cone truncado compostos principalmente por seis, sete ou oito monômeros de D-glicose unidos por ligações α-1,4-glicosídicas, conhecidas respectivamente como α-ciclodextrina (α-CD), β-ciclodextrina (β-CD) e γ-ciclodextrina (γ-CD) (SZEJTLI, 1998; SHARMA; BALDI, 2016). São moléculas relativamente grandes com grupos hidroxila primários e secundários voltados para superfície externa o que garante certa hidrofilia e uma cavidade central lipofílica. O resultado dessa conformação permite que as CDs formem complexos de inclusão com várias moléculas hidrofóbicas (DEL VALLE, 2004; RASHEED et al., 2008; REN et al., 2016).

Dentre as CDs naturais, a β-CD é a mais utilizada por apresentar produção simples, de baixo custo e acessível, além de hospedar moléculas de massa molecular entre 100 e 400 g/mol, faixa de massa molecular da maioria das moléculas de interesse (WANG et al., 2011; REN et al., 2016).

Dentre as propriedades conferidas pela complexação em CDs estão o aumento da solubilidade, aumento da biodisponibilidade farmacológica, diminuição da volatilidade, como

também redução da irritação grastrointestinal dos farmácos. Além disso, as CDs atuam no controle das propriedades de volatilidade e sublimação e mascaramento de sabores e odores desagradáveis (DEL VALLE, 2004; MARTÍN; OSTOS; ÂNGULO, 2017; DINIZ et al., 2018). Sobre o incremento da solubilidade em meio aquoso, destaca-se a capacidade de formar complexos de inclusão reversíveis com moléculas apolares, além de serem substâncias cristalinas, homogêneas e não higroscópicas (GIDWANI; VYAS, 2015).

Devido as propriedades citadas anteriormente, as CDs são amplamente utilizadas em diversas áreas como indústria farmacêutica, agroquímica, cosméticos, alimentos e bebidas (RASHEED et al., 2008; LIMA et al., 2016). No âmbito farmacêutico as CDs estão presentes em mais de 35 medicamentos (como o Maxsulid®, cujo princípio ativo é a nimesulida e o Cicladol®, cujo princípio ativo é o piroxicam) (OLIVEIRA et al., 2015) e apresentam-se como alternativa para carrear moléculas com características físico-químicas e biofarmacêuticas indesejáveis, aumentando o potencial terapêutico dos fármacos, reduzindo sua decomposição e atuando na liberação controlada nos tecidos (DEL VALLE, 2004; GIDWANI; VYAS, 2015).

A α-CD, β-CD e γ-CD são consideradas CDs naturais e ainda podem ser modificadas por meio de reações como aminações e esterificações. Assim, os derivados de CDs, presentes em mais de 1/3 de todos os medicamentos que contêm CD, conferem menor toxicidade e melhor desempenho aquoso quando comparados às CDs naturais (KURKOV; LOFTSSON, 2013; DINIZ et al., 2018).

3 CAPÍTULO 1

Chemistry and delivery systems applied to monoterpenes with analgesic potential: An overview

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Chemistry and delivery systems applied to monoterpenes with analgesic potential: An overview

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Abstract

Monoterpenes are compounds belonging to the class of secondary metabolites known as terpenoids that are present in essential oils obtained from medicinal and aromatic plants. They have aroused much interest due to the several biological activities that they present, including analgesic activity. However, these compounds exhibit extremely low aqueous solubility which impair their oral absorption. This fact encouraged several investigations about delivery systems that could improve the therapeutic properties of monoterpenes and potentialize its clinical application. Thus, this paper summarizes the main preclinical pain assessment models, chemistry of monoterpenes and main drug delivery systems applied to monoterpenes with analgesic potential by extensively literature search on several scientific databases and Google. Results of the investigation shown that the structural variety of the monoterpenes permits a diversity of biological activities, among them analgesic potential demonstrated in diverse animal models of pain, such as: abdominal writhing, formalin test, hot plate test, randall-selitto's test, von frey test, tail-flick test, orofacial pain, chronic muscle pain and cancer pain models. In general, the pharmacological activity of these secondary metabolites is increased when applied a delivery system. Most of the studies to improve oral absorption of monoterpenes evaluated the complexation with β-cyclodextrin and demonstrated the involvement of central pathways in their action mechanisms, such as opioidergic, serotoninergic and GABAergic system, vanilloid receptor type 1 (TRPV1) modulation and descending-pain inhibitory mechanisms. It has also been reported that monoterpenes can be used as adjuvant to increase oral absorption of drugs already used in clinical practice, such as ibuprofen. In addition to the oral route, these chemical constituents demonstrated application in transdermal delivery system as permeation enhancer or active ingredient for pain management. However, in despite the variety of preclinical studies found in the literature, it was also observed a scarcity of pharmacokinetic data, which could difficult the translation of these studies to justify later clinical trials. In this way, monoterpenes demonstrate remarkable analgesic properties and applicability in drug delivery systems, which improves their physicochemical and pharmacological characteristics, and encourages further investigation.

Keywords: Natural products, essential oil, monoterpenes, chemistry, drug delivery, pain.

1. Introduction

Pain is a pathological condition with multifactorial etiology that involves neurotransmission, emotion, environment, and cognitive factors. Most of the time, pain does not occur in isolation, but rather accompanies most diseases functioning as an important clinical sign. Despite the prevalence and impact of pain, conventional therapies sometimes have several adverse effects that limit the use of certain medications, characterizing pain management as a major challenge for physicians and patients [1-4].

In the search for new pharmacological therapies safer and effective, the natural products have shown pharmacological potential for the treatment of various diseases. In this context, the essential oils have aroused much interest due to the various biological activities that presents including a potential therapeutic for the disorders related to pain, reducing nociception in preclinical pharmacological models besides already participating in some commercial formulations, such as Acheflan® [5-7].

The monoterpenes are among the main constituents of the essential oils obtained of plant species. Considering the development of new drugs from monoterpenes, several investigations are being conducted to improve the solubility of these compounds and reduce the number of doses and toxic effects. Among the main alternatives to enable the use of monoterpenes is use of drug delivery systems which allows the incorporation of active principles without compromising its pharmacological activity, guaranteeing adhesion to the treatment and safety of the patient [8,9].

Therefore, this paper aims to carry out an overview of chemistry and delivery systems applied to monoterpenes with analgesic potential by extensively literature search on several scientific databases and Google.

2. Pain Disorders and Experimental Models

The exposure of the individual to potentially harmful stimuli induce unpleasant sensation whose processed information can be differentiated as physiological or pathological pain. While physiological pain induces protective responses to the organism, pathological pain involves abnormal discomfort and sensitivity in the patient's clinical symptoms [10,11].

The physiological component of pain is the nociception which encompasses the processes of transduction, transmission, and modulation of neural signals generated in response to an external harmful stimulus. However, most of the time the potentially harmful stimulus is not transient and may be associated with other factors, such as inflammation and nerve injury, promoting several alterations changes in the processing of harmful information and

characterizing the types of pain. Pathological pain can be classified as inflammatory pain (involving somatic or visceral structures) or neuropathic (involving lesions of the nervous system). It can also be classified according to the time of duration of pain and classified as acute pain (recent) or chronic pain (long duration) [12-15].

Considering the complexity of the mechanisms involved in the pathophysiology of pain and the need for the incessant search for new and safer drugs it is necessary the use of experimental pharmacological models. In this perspective, many pharmacological models were developed considering the harmful and persistence of the stimulus, besides of the ability to differentiate the involvement of different pathways (central or peripheral) in the pharmacological activity of the chemical under study [16,17]. The main experimental models used to evaluate acute and chronic pain in preclinical studies are described in Table 1.

INSERT TABLE 1

The treatment of pain is still a great challenge for medicine. Among the most used drugs we include opioids, antidepressants, anticonvulsants, and non-steroidal anti-inflammatory drugs (NSAIDs), these classes being the most prescribed for the relief of acute and chronic pain. Despite the great therapeutic arsenal available there is still a low adherence to conventional treatments, mainly resulting from the diversity of side effects caused using these drugs such as constipation and nausea, caused using opioids, and gastric ulceration, caused by the long-term use of NSAIDs [29,30].

Therefore, it is necessary to develop new drugs with fewer adverse effects, decreasing the discontinuation of the treatment. Based on this problem, in recent years, natural products of plant origin have been constantly studied by the diversity of biologically active chemical compounds in an attempt to put on the market safer and more effective drugs [31].

3. Monoterpenes: Chemistry and Analgesic Potential

Monoterpenes are compounds synthesized by plants belonging to the class of secondary metabolites known as terpenoids. These are mainly found in aromatic plants, being considered the main chemical constituents of essential oils extracted from leaves, flowers, fruits, stem barks and even roots. In general, essential oils are complex mixtures of volatile, lipophilic, usually odoriferous, and liquid substances. For this reason, essential oils can also be called volatile oils, ethereal oils, or essences. Its main characteristic is the volatility, which differ from the fixed oils, mixtures of lipid substances usually obtained from seeds [32].

Due to their physicochemical properties and organoleptic characteristics, essential oils are widely used in the food, cosmetic and pharmaceutical industries. They are used as condiments and flavorings for foods, or as essences for perfumery and hygiene products. Moreover, vegetable drugs rich in volatile oils are employed *in natura* for the preparation of infusions or in the form of simple galenic preparations [33,34]. In addition, many essential oils are used in function of their pharmacological properties, such as antioxidant [35], anti-inflammatory [36], antimicrobial [37] gastroprotective [38], anticancer [39] and analgesic [7] activities.

The chemical composition of essential oils includes terpene hydrocarbons, simple and terpene alcohols, aldehydes, ketones, phenols, esters, ethers, oxides, peroxides, furans, organic acids, lactones and coumarins. These compounds may be present in different concentrations, but usually one of them is the major compound [32]. In fact, most of the essential oils consists of terpenoids or phenylpropanoid derivatives, with terpenoids predominating, mainly monoterpenes and sesquiterpenes. Terpenoids represent a wide variety of substances whose biosynthetic origin derives from isoprene, formed from mevalonic acid pathway [40,41]. Most often, the skeletons of the terpenoids are formed by the condensation of a variable number of isoprene units, giving rise to monoterpenes (10 carbons), sesquiterpenes (15 carbons), diterpenes (20 carbons) and triterpenes (30 carbons), according to shown in Figure 1.

INSERT FIGURE 1

Monoterpenes are composed of 10 carbon units and can be divided into three subgroups: acyclic, monocyclic, and bicyclic. In each of these subgroups there are further classifications: unsaturated hydrocarbons, aldehydes, ketones, alcohols, lactones, tropolones, epoxides, etc. Numerous substances are classified into each of these groups, characterized by about 200 different types of skeletons. The number of known terpene compounds exceeds 8,000, and about 150 different monoterpenes have been described [42,43].

Like other terpenoids, the synthesis of monoterpenes is often linked to the mevalonate pathway in plants. Initially, the condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is catalyzed by the enzyme HMG-CoA synthase (HMGS). Then, HMG-CoA reductase (HMGR) catalyzes reductive deacylation of HMG-CoA to mevalonate (MVA) via mevalonate and use two equivalents of NADPH as reductant. Subsequently, mevalonate kinase (MK) catalyzes the ATP-dependent phosphorylation of mevalonate to mevalonate 5-phosphate, then mevalonate-5-diphosphate is formed by the action

of phosphomevalonate kinase (PPMK). Finally, these reactions lead up to formations of the biogenic isoprene units: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Figure 2). It is important to note that IPP and DMAPP units can also be biosynthesized through a non-mevalonate pathway, known as deoxyxylulose phosphate pathway, occurring in higher plants chloroplasts [44,45].

INSERT FIGURE 2

Many polyprenyl diphosphate synthase enzymes are relatively non-specific and catalyze the head-to-tail condensation of DMAPP with IPP to originate a geranyl diphosphate (GPP) unit (Figure 1). The condensation of GPP units in farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) is what guarantees the formation of terpenes of different structural skeletons (sesquiterpenes, diterpenes, triterpenes, etc.) [44]. However, the structural variety of the terpenes, especially of the monoterpenes, occurs in the final stages of biosynthesis through the cyclization reaction of the phosphate precursor catalyzed by monoterpene synthases and the functionalization of the carbon skeletons by the enzymatic complexes of the cytochrome P_{450} [46,47].

In fact, the varieties of monoterpene chemical structures guarantee a broad spectrum of biological activities to these compounds. The literature indicates that monoterpenes present antimicrobial [48], antitumor [49], gastroprotective [50], hypotensive [51], anti-inflammatory [52] and antioxidant [53], activities. Because of their lipophilicity, monoterpenes can cross the blood-brain barrier and act on the central nervous system, which accounts for the vast number of published studies on the anxiolytic, sedative, anticonvulsant, and analgesic effects [54,55].

In recent years, monoterpenes have been specially studied as new candidates for analgesic drugs. They act by modulating the production and release of neurotransmitters involved in the processes of pain or even through direct interaction with specific receptors, blocking painful stimuli [56]. In addition, monoterpenes can also modulate signaling pathways involved in inflammatory processes, reducing the intensity of symptoms observed in experimental models *in vivo,* including inflammatory pain [57,52].

Guimarães et al. (2013) performed a systematic review that describes all monoterpenes evaluated in experimental animal models of pain between 1990 and 2012. In all, 27 different compounds were identified as analgesic agents [58]. In Figure 3, we show the chemical structures of the monoterpenes evaluated in experimental models of pain.

INSERT FIGURE 3

Among the monoterpenes investigated, linalool is considered the most studied for the treatment of pain disorders. It is an acyclic monoterpene alcohol that has two enantiomeric forms usually found in aromatic plants: (–)-linalool and (+)-linalool. In addition, its derivative linalyl acetate also exhibits antinociceptive activity and is commonly found in aromatic plants such as "lavender". Pharmacological investigations have reported that (–)-linalool is a multi-target molecule, reducing the nociceptive response in animals through the modulation of cholinergic, dopaminergic, adenosinergic, glutamatergic and GABAergic pathways [59,60]. Moreover, the antinociceptive effect of linalool in inflammatory pain models has been related to the reduction of nitric oxide synthesis as well as its ability to prevent lipid peroxidation [61-65].

In addition to linalool, many other monoterpenes have been reported as analgesic agents, such as carvacrol, limonene, citronellal, myrcene, thymol, α-terpineol, carvone, *p*-cymene, menthol, α-pinene, β-pinene and 1,8-cineol [58]. However, these compounds have some common physicochemical characteristics that hinder their pharmacological effect after oral administration. They have low solubility and are rapidly metabolized, which decreases its bioavailability and duration of action. In this sense, research groups have been devoted to the development of delivery systems that improve the solubility profile of these compounds as well as their stability in the bloodstream, allowing them to be absorbed and distributed appropriately to achieve a maximal therapeutic effect [66,67].

4. Drug Delivery Systems

Drug delivery relates to the approaches, formulations, technologies, and systems for administering a pharmaceutical compound to achieve a therapeutic effect on the disease [68,69]. Through these systems, it is possible to increase the efficacy and safety by controlling the rate, time, and place of release of drugs in the body. This process comprises the administration of the therapeutic product, the release of the active constituents by the product, and the following transport of the active ingredients across the biological membranes to the site of action [70]. In addition, drug delivery technology permits the use of alternate routes for the administration of drugs, such as the use of oral route rather than injectable, which can promote more compliance and convenience to the patient [71].

The drug-delivery systems can facilitate the delivery of small compounds as well as large molecules for example peptides, nucleic acids, polymers, and therapeutic agents with low

solubility in water obtained from natural or synthetic sources [72]. Diverse types of delivery systems have been successfully applied which includes the use of lipid-based nanoparticles such as liposomes, solid-lipid nanoparticles, micelles and niosomes, polymeric nanoparticles such as chitosan and atelocollagen dendrimers, carbon nanotubes, metal-based nanoparticles, quantum dots and silica nanoparticles (Figure 4) [73].

INSERT FIGURE 4

Regarding natural products, in general, the combination with polymeric nanoparticles has been the most tested [74]. Polymer nanoparticles are particles of fewer 1 μm diameter which can be obtained from natural or synthetic polymers. Among these, synthetic polymers are most used because natural polymers vary in purity, and often require crosslinking that could denature the embedded drug. Poly (lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly-l-lactic acid (PLA), polycaprolactone (PCL), and chitosan are the polymers most applied in these delivery systems because of their biocompatibility, biodegradability, and the fact that they are easy to functionalize [75].

Liposomes are small artificial vesicles of spherical shape composed of a lipid bilayer. They can be created from cholesterol and natural non-toxic phospholipids and form a closed structure surrounding an internal aqueous phase. Due to their size, hydrophobic and hydrophilic character and biocompatibility, liposomes are convenient systems for drug delivery. Liposome properties vary significantly with lipid components, surface charge, size, and the technique of preparation [76]. Already the micelles composed of phospholipids only have one layer that internally have a hydrophobic core and externally a hydrophilic surface [77]. Hendradi et al. (2003) showed the promisor anti-inflammatory effect of mixed micelle formulations including monoterpenes on the transdermal delivery of diclofenac [78].

Solid-lipid nanoparticles are colloidal carriers established as an alternative system to the existing traditional carriers such as emulsions, liposomes, and polymeric nanoparticles. This system consists in a submicron-sized lipid emulsion where the liquid lipid (oil) has been replaced by a solid lipid. Solid-lipid nanoparticles present important advantages such as small size, large surface area, high drug loading and the interaction of phases at the interfaces. These properties confer to this system the potential to improve performance of pharmaceuticals, nutraceuticals, and other materials [79]. Encapsulation of the monoterpene linalool in solid-lipid nanoparticles demonstrated an enhancement of antitumor activity both *in vitro* and *in vivo* [80].

Dendrimers present nanometric size, radially symmetric structure with well-defined, homogeneous and monodisperse structure that has a typically symmetric core, an inner shell, and an outer shell. Their three typical macromolecular architectural classes are widely known to produce rather polydisperse products of diverse molecular weights. A diversity of dendrimers exists, and each has biological properties such as polyvalence, self-assembling, electrostatic interactions, chemical stability, low cytotoxicity, and solubility [81]. These characteristics mentioned previously give dendrimers the possibility of being harnessed as effective carriers of many pharmaceuticals [82].

Inorganic nanoparticles show low toxicity and are hydrophilic, biocompatible, and highly stable when compared to organic materials [83]. Inorganic nanoparticles present exclusive physicochemical properties such as high surface area per unit volume, their optical and magnetic uniqueness, and the capacity to be functionalized with a great number of ligands to increase their affinity towards target molecules. In this way, among the greatest advantages of the combination of inorganic nanoparticles with drugs are the extensive availability, rich functionality, good biocompatibility, potential capability of targeted delivery and controlled release of carried drugs, which allows the reduction of systemic side effects and a higher efficiency of the therapeutic molecule [84,85].

5. Drug Delivery Systems Applied to Analgesic Monoterpenes

As described previously, monoterpenes have aroused much interest due to the various biological activities that presents including analgesic activity, as summarized in a review of [58]. However, these compounds exhibit extremely low aqueous solubility which hinders their oral absorption and extensive pre-systemic metabolism, which may be responsible for the unfavorable pharmacokinetics of the molecule. In this way, several methods have been used to improve the therapeutic properties of non-polar natural compounds such as monoterpenes, mainly the employment of drug-delivery systems such as cyclodextrins [86].

Natural cyclodextrins presents a hydrophobic cavity that permits a formation of an inclusion complex with a wide variety of non-polar compounds and alters the physicochemical and biological characteristics of guest molecules. These changes may improve the therapeutic properties of drugs by diminishing their decomposition before they enter tissues and by varying how they enter tissue. The most common native cyclodextrins are cyclic oligosaccharides composed of 6 (α-), 7 (β-) or 8 (γ-) glucose units linked by α-1,4 glycosidic bonds [87], as shown in Figure 5. Amongst these, β-cyclodextrin is the most used for complexation because

presents a perfect cavity size, efficient drug complexation and loading, availability, and relatively low cost [88].

FIGURE 5

Some alterations in natural cyclodextrins have been employed to improve their inclusion capacity and to enhance the physicochemical and biopharmaceutical characteristics of the complexed drug. For this, the cyclodextrins have been structurally tailored with several charged groups, hydrophilic segments, hydrophobic moieties, or both hydrophilic and hydrophobic units. Furthermore, another strategy has been the conjugation of cyclodextrins in polymers of various architectures [89]. Hydroxypropyl-β-cyclodextrin (HP-β-CD), randomly methylated-β-cyclodextrin (RM-β-CD), and sulfobutylether-β-cyclodextrin (SBE-β-CD) are mostly preferred for complexation. In relation to polymerized cyclodextrins, some examples are soluble anionic β-cyclodextrin polymer, soluble γ-cyclodextrin polymer, and epichlorohydrin β-cyclodextrin polymer [88].

Several studies published with delivery systems applied to monoterpenes with analgesic activity involve the formation of a complex with β-cyclodextrin. These studies evaluated the inclusion complex in animal models of pain using Swiss mice as presented in Table 2 and described below.

INSERT TABLE 2

Araújo-Filho et al. (2017) demonstrated that the monoterpene *D*-limonene complexed with β-cyclodextrin presented anti-hyperalgesic action superior to its free form in chronic musculoskeletal pain model. It was suggested a possible action of d-limonene/β-cyclodextrin complex in the dorsal horn of the spinal cord since pretreatment with the complex produced a decreased in the expression of Fos protein. Pretreatment with flumazenil (GABA receptor antagonist) was able to reverse the antinociceptive effect of the complex, suggesting the involvement of GABAergic receptors in the antinociceptive effect. In addition, *D*-limonene/β-cyclodextrin complex showed to have antinociceptive activity when the noxious agent used was capsaicin, an agonist for transient receptor potential cation channel subfamily V member 1 (TRPV1) channels, reinforcing the hypothesis that *D*-limonene also acts on this type of receptors [95].

Orally administrated carvacrol/β-cyclodextrin complex was able to reduce hyperalgesia, spontaneous and palpation-induced nociception and promote an increased duration of effect against sarcoma 180 cancer pain. However, pure carvacrol at the dose of 50 mg/kg did not cause significant changes in nociceptive responses. The mechanism of action involved in the activity of carvacrol/β-cyclodextrin complex has not been investigated [92]. In another study, carvacrol/β-cyclodextrin complex was tested in orofacial pain model. The complex reduced the nociceptive during the two phases of the formalin test, whereas free carvacrol did not produce the reduction in face-rubbing behavior in the initial phase and produced a little inhibition in the second phase of formalin test. Carvacrol and carvacrol/β-cyclodextrin showed a significant reduction against nociception caused by capsaicin and glutamate injection, suggesting the involvement of vanilloid and glutamate systems in the analgesic action. In addition, the participation of the opioid system in the antinociceptive effect of carvacrol/β-cyclodextrin was suggested due the effect of complex was reversed by pretreatment with naloxone, a non-selective opioid antagonist [94].

A study with (-)-linalool suggested that their complexation in cyclodextrin improved analgesic profile of this monoterpene in an animal model of chronic non-inflammatory muscle pain with a probable involvement of descending pain pathways, showed by a significant intensification in the number of Fos-positive cells observed in the periaqueductal gray (PAG), nucleus raphe magnus (NRM), locus coeruleus (LC) [9].

Quintans-Júnior et al. (2013) also demonstrated that the inclusion complexes, (-)-linalool/β-cyclodextrin, significantly improved the antinociceptive activity when compared with (-)-linalool alone, without promote any motor abnormality. The results suggested the involvement of TRPV1 and ionotropic glutamatergic-dependent mechanisms in the analgesic effect, considering that the complex induced a significant inhibition of nociception in glutamate- and capsaicin-induced nociception model [91].

Results obtained by Oliveira et al. (2016) showed that the α-terpineol/β-cyclodextrin presented a lasting effect when compared with the free compound and caused a significant inhibition of the mechanical hyperalgesia in animal model of non-inflammatory chronic muscle pain. This effect was probably evoked by the descending inhibitory pain system, specifically by opioid and serotoninergic receptors since analgesic effect was reversed by the systemic administration of naloxone and ondansetron (antagonist of 5-HT_3 receptors) [93].

Quintans et al. (2013) demonstrated that the acute treatment with *p*-cymene/β-cyclodextrin complex caused a reduction of nociceptive behavior and prolonged the duration

of analgesic effect on chemical and thermal nociceptive pain models without promote any motor abnormality [90].

The citronellal/β-cyclodextrin complex, when administrated orally, also induced an increase of the duration in anti-hyperalgesic effect against chronic muscle pain. In addition, the complex reduced mechanical hyperalgesia on all days of treatment, without changing muscle strength. The observed effects are probably mediated by the activation of the descending inhibitory pathway, with a possible interaction of the glutamate receptors and activation of ventrolateral area of periaqueductal gray (PAG) and rostroventromedular area (RVM) [26].

These results showed that the complexation of monoterpenes in cyclodextrin efficiently improve their pharmacological activity when administered by oral route against chronic muscle pain, cancer pain and orofacial pain models. Most of these studies include the analysis of mechanism of action and suggested that the effects of monoterpenes involved mainly central pathways, such as opioidergic, serotoninergic, GABAergic, modulation of TRPV1, reduction of Fos protein expression and descending-pain inhibitory mechanisms. However, the pharmacokinetic parameters were not evaluated to demonstrate the advantages of the complexation, which hinders the translatability of animal research. Preliminary trials with monoterpenes have also demonstrated the potential of these chemical constituents in increasing the oral bioavailability of poorly water-soluble drugs already used in pain management. Results obtained by Yong et al. (2005) showed that an ibuprofen-loaded preparation contained menthol and poloxamer would be useful to deliver ibuprofen in a form that permits fast absorption in the initial phase, leading to better absorption in rats [96].

In addition, several investigations have demonstrated chemical constituents of essential oils like terpenes, can be used as skin penetration enhancer for transdermal drug delivery [97]. Transdermal delivery of drugs should be a recognize alternative to conventional dosage forms, which presents important advantages such as controlled delivery, patient compliance, noninvasiveness, and reduced side effects. However, the major problem of delivery of drugs through skin is the less permeability to high molecular weight and polar drugs. An alternative is including chemicals in the formulations that improve the permeability of skin for these drugs and permit larger therapeutic levels. Studies have been realized in effort to develop effective and non-toxic enhancers from natural products and synthetic chemicals. In this sense, terpenes have aroused much interest since they are generally considered safe and have fewer irritant properties related to others skin penetration enhancers [98-100]. Monoterpenes have been used for a long time in topical drug delivery systems, mainly transdermal dosage forms, acting as skin permeation enhancers. Moreover, the pharmacological potential of these compounds has

also been explored when applied in transdermal systems, including in pain management [101,98,102].

Arunkumar et al. (2015) evaluated the effect of chemical penetration enhancer geraniol, l-menthol, and thymol on transdermal iontophoretic delivery of diclofenac sodium. They observed that geraniol followed by l-menthol significantly improved the iontophoretic transport of diclofenac sodium from phosphate buffer saline across porcine ear skin [103]. Geraniol is an acyclic monoterpene that presents a trans-conformation with two double bonds [104]. This chemical constituent is essentially an unsaturated primary alcohol that occurs in liquid form at room temperature. Due to its structure, acyclic terpenes possess the capacity to disrupt the intercellular lipids localized in the stratum corneum by virtue of the definitive hydrocarbon tail with a polar head [103]. l-menthol, on the other hand, is a monocyclic monoterpene which present a phenolic alcoholic group that occurs as a solid at room temperature [104]. In this way, one of the factors that permits that geraniol demonstrate better efficacy is the fact that liquid terpenes form fewer hydrogen bonds than solid terpenes which makes it easier to pass through the lipid within the stratum corneum [98].

Moreover, it is described in the literature that in general the success of terpenes as chemical permeation enhancers is related to the physicochemical characteristics of the drug and the terpene [105]. Kang et al. (2007) concluded that terpenes which have one or combinations of the specific properties associated to the level of hydrophobicity, phase (liquid state), appearance of specific functional groups (ester or aldehyde but not acid), and chemical types (not a triterpene or tetraterpene) may be better enhancers for drug permeation through skin [98]. Generally, terpenes that present polar functional group promote the permeation of hydrophilic permeants while lipophilic terpenes can be provide better penetration enhancement for lipophilic permeants [105]. In this context, l-menthol was described to increase skin permeation of hydrophilic compounds such as morphine *in vitro* as described by Morimoto et al. (1993) [106]. In this study, the effect of l-menthol as a chemical enhancer on the skin permeation of morphine was evaluated using the *in vitro* diffusion cell technique and removed hairless rat skin. The results demonstrated that the use of a combination of l-menthol with ethanol showed greater penetration enhancement which suggested that the high activity was due to both the action of 1-menthol against the stratum corneum barrier and the effect of ethanol against the viable skin layer beneath it [106]. The effect of combination of cyclic monoterpenes, such as carvone and limonene epoxide, and ethanol on percutaneous absorption of diclofenac sodium was also described in other studies [107,108].

Glycyrrhetinic acid and paeoniflorin, a pentacyclic triterpenoid and a monoterpene glycoside, respectively, are the main active ingredients in Chinese peony Liquorice Decoction, a widely used Traditional Chinese Medicine. These compounds were produced to transdermal patches and administered in dysmenorrhea mice. The results showed that glycyrrhetinic acid with paeoniflorin could attenuate pain and can be adequate for topical spasmolysis and anti-inflammatory treatment [109].

This presented data evidenced the promisor pharmacological activity of monoterpenes and its application in delivery systems.

6. Conclusion

The monoterpenes exhibit a variety of biological activities, which can be justified by its varied chemical structure. However, their low solubility limits oral absorption and consequently their bioavailability. The use of delivery systems has been an alternative to improve the pharmacokinetics of such compounds. Regarding analgesic activity, it was observed that the complexation with β-cyclodextrin is the most used technique, promoting an increase in the pharmacological activity of monoterpenes in different pre-clinical models of pain when administered by oral route. It was also observed that the monoterpenes have a potential to be used as an adjuvant to improve the oral bioavailability of other drugs. Moreover, these chemical constituents can also be incorporated into transdermal delivery systems where they can act as a penetration enhancer or active ingredient in pain management.

Further research is needed to complement *in vivo* existing studies, which in most cases do not present pharmacokinetic data. Furthermore, is important to test others delivery systems that can be applied to monoterpenes by oral route, since most existing studies involves the incorporation into cyclodextrin.

Author contributions

Mariana Gama e Silva: conceptualization, investigation, writing – original draft and revision. Érica Martins de Lavor: investigation, writing – original draft. Raimundo Gonçalves de Oliveira Júnior: conceptualization, investigation, methodology, writing – original draft, designed the figures. Juliane Cabral Silva: investigation, writing – original draft. Roxana Braga de Andrade Teles: revision. Ana Paula de Oliveira: designed the figures, revision. Angela Caroline Lima Amorim dos Santos: revision. Jackson Roberto Guedes da Silva Almeida: supervision, project administration, funding acquisition, revision.

Conflict of interest

The authors declare no conflict of interest.

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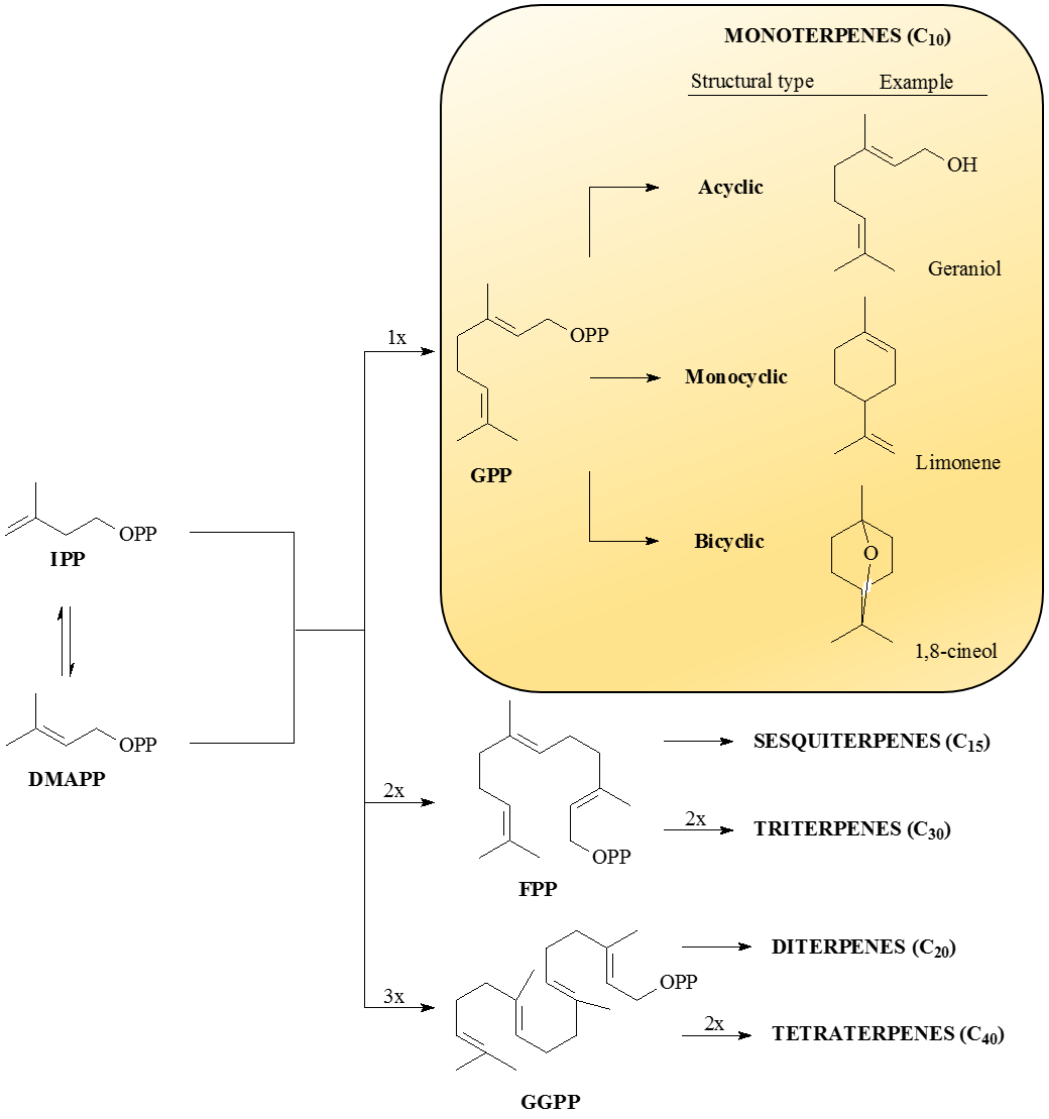

Figure 1. Overview of terpenoids biosynthesis from isoprenoid units (IPP and DMAPP). Abbreviations: IPP (isopentenyl diphosphate), DMAPP (dimethylallyl diphosphate), GPP (geranyl diphosphate), FPP (farnesyl diphosphate), GGPP (geranylgeranyl diphosphate). Adapted from Mahmoud and Croteau [41].

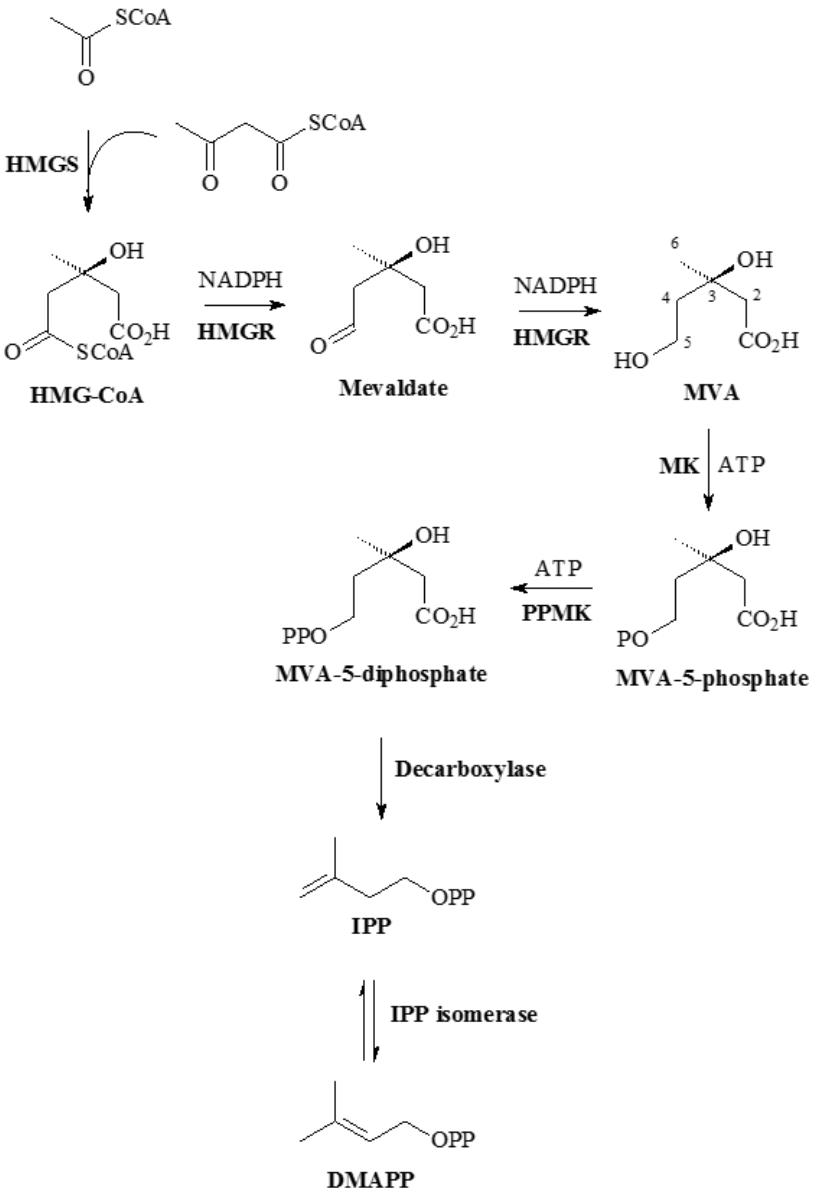

Figure 2. Overview of mevalonate pathway. Abbreviations: HMG-CoA (3-hydroxy-3-methylglutaryl-CoA), HMGS (HMG-CoA synthase), HMGR (HMG-CoA reductase), MVA (mevalonate), MK (mevalonate kinase), PPKM (phosphomevalonate kinase), IPP (isopentenyl diphosphate), DMAPP (dimethylallyl diphosphate). Adapted from Dewick [44].

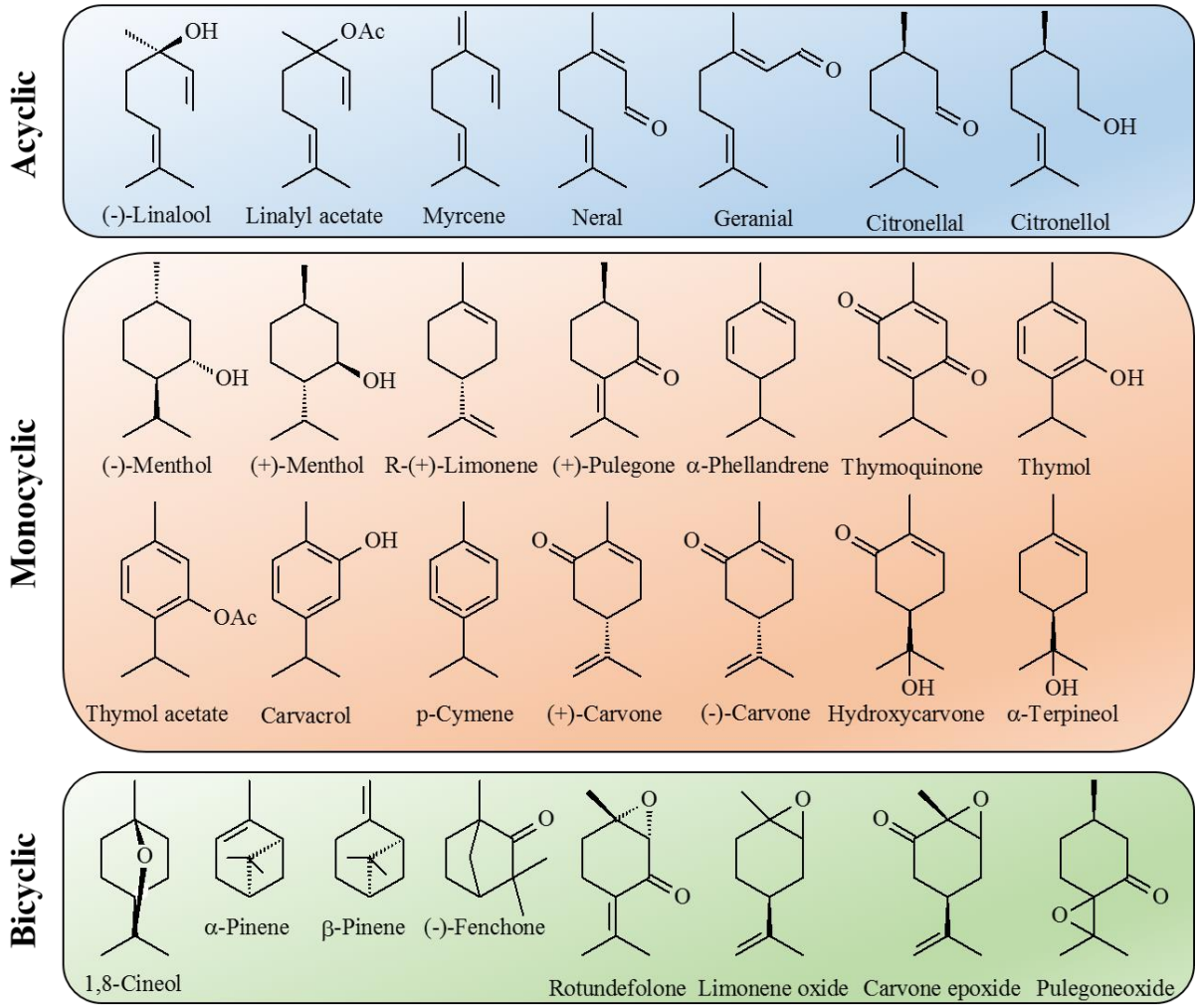

Figure 3. Monoterpenes with antinociceptive activity presented in experimental models *in vivo*. Adapted from Guimarães *et al*. [58].

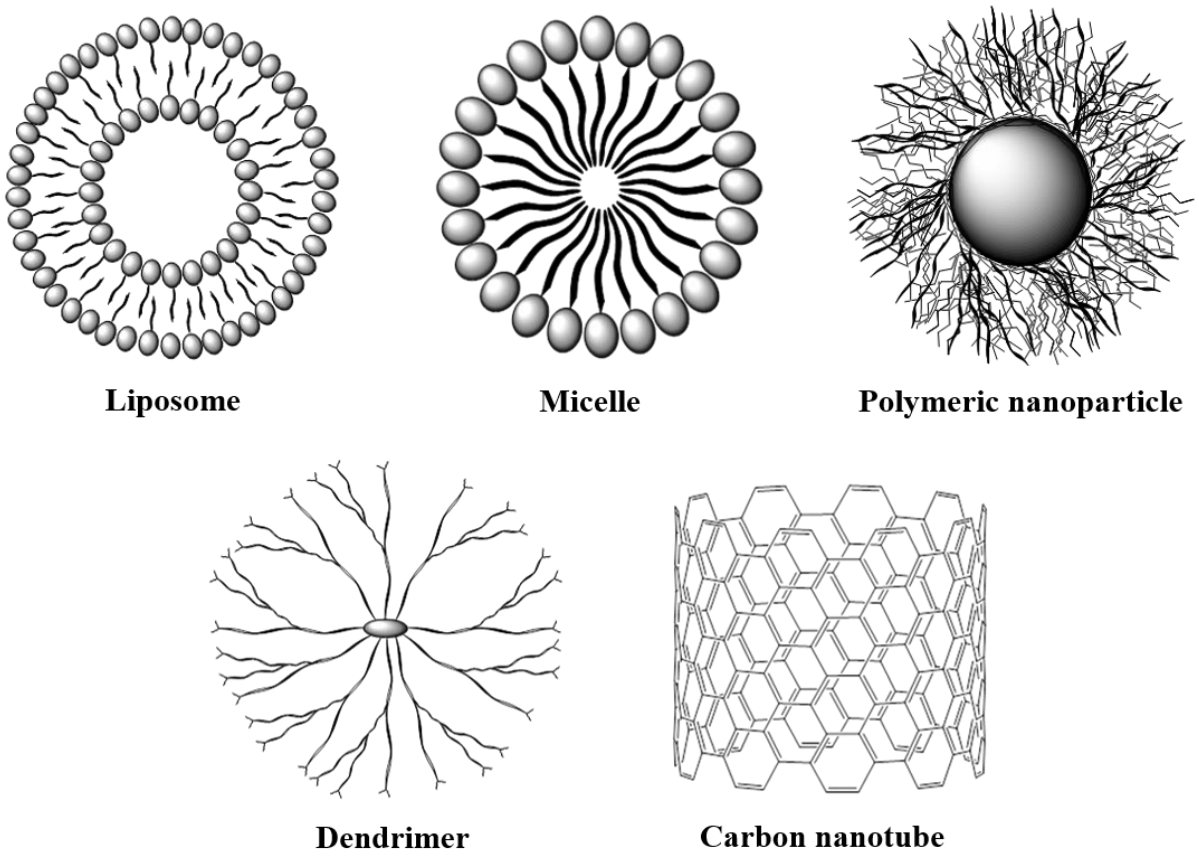

Figure 4. Schematic representations of main delivery systems applied to natural products.

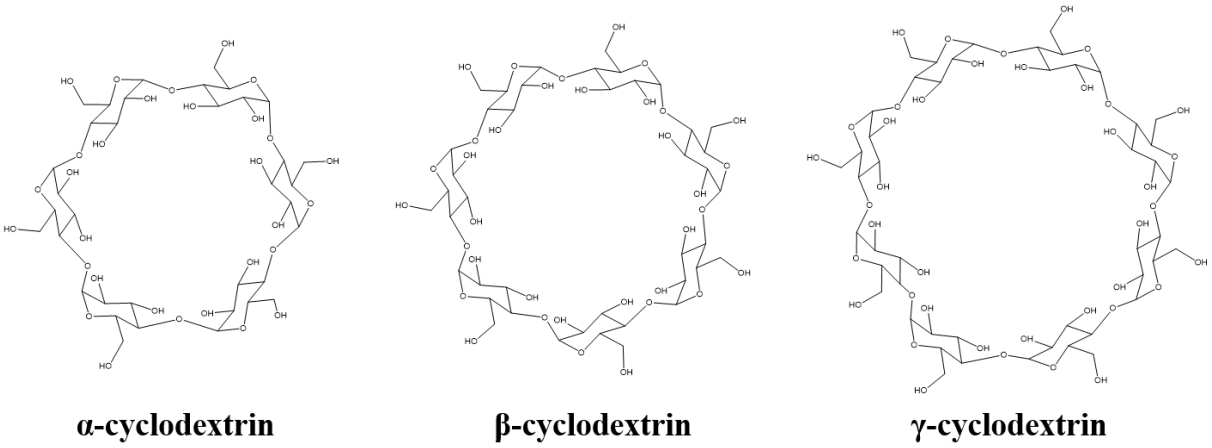

Figure 5. Structures of natural cyclodextrins, the delivery system most used in preclinical studies of monoterpenes with analgesic potential.

Table 1. Main pharmacological models for evaluating antinociceptive activity in preclinical studies.

Classification		Pharmacological model	Nociceptive agent	Parameters evaluated in experimental tests	References
Duration of pain	**Pathophysiological mechanism**				
Acute pain	Visceral pain	Abdominal writhing	Acetic acid	Number of abdominal writhing	[18]
Acute pain	Inflammatory or nociceptive pain	Formalin test	Formalin solution	Duration of paw licking	[19]
Acute pain	Nociceptive pain	Hot plate test	Thermal stimulus	Latency time to lick paws or jump	[20]
Acute pain	Nociceptive pain	Randall-Selitto's test	Mechanical stimulus	Mechanical hyperalgesia	[21]
Acute pain	Nociceptive pain	Von frey test	Mechanical stimulus	Thermal hyperalgesia	[22]
Acute pain	Neuropatic pain	Orofacial pain	Formalin, glutamate, capsaicin	Face rubbing	[23,24]
Acute pain	Nociceptive pain	Tail-flick test	Thermal stimulus	Tail withdrawal movement	[25]
Chronic pain	Nociceptive pain	Chronic muscle pain	Mechanical and thermal stimulus	Mechanical hyperalgesia, thermal hyperalgesia, muscular strength	[26]
Chronic and acute pain	Nociceptive or Neuropatic pain	Cancer pain	Tumor cells and mechanical stimulus	Mechanical hyperalgesia, thermal hyperalgesia, spontaneous and palpation-induced nociception, movement-evoked pain, and grip strength meter.	[16,27,28]

Table 2. Characteristics of pre-clinical studies that evaluate the complexation of monoterpenes with analgesic potential in β-cyclodextrin.

Monoterpene	Animal	Dose, route	Drug Delivery System	Pain model	Methods	Results	References
p-Cymene	Swiss mice	20 or 40 mg/kg, p.o	β-cyclodextrin	Nociception	Acetic acid writhing test; hot-plate; carrageenan-induced paw edema and rota-rod	The p-cymene or p-cymene/β-CD complex (40 mg/kg, p.o.) caused a reduction of nociceptive behavior and prolonged the duration of analgesic effect on acetic acid-induced writhes and hot plate test. In the rota-rod test, there was no change in motor performance. The *p*-cymene/β-CD complex (40 mg/kg, p.o.) could reduce the edema formation induced by carrageenan.	[90]
(-)-Linalool	Swiss mice	20 or 40 mg/kg, p.o	β-cyclodextrin	Nociception	Acetic acid writhing test; formalin-, glutamate- and capsaicin-induced nociception; hot plate; open-field; rota-rod and peritonitis induced by carrageenan	The (-)-linalool and (-)-linalool/β-CD complex had antinociceptive effect in all the chemical- and heat-induced mice models with the involvement of both peripheral and central antinociceptive mechanisms. Possible involvement of TRPV1 and ionotropic glutamatergic-dependent mechanisms in the analgesic effect. In peritonitis test there was a reduction of total leucocyte migration and TNF-α levels in peritoneal fluid. The results were unlikely to be provoked by any motor abnormality.	[91]
(-)-Linalool	Swiss mice	25 mg/kg, p.o	β-cyclodextrin	Chronic muscle pain	Von Frey; rota-rod; grip strength meter and Fos immunofluorescence	The (-)-linalool and (-)-linalool/β-CD complex reduced the mechanical hyperalgesia and activated neurons of the locus coeruleus, nucleus raphe magnus, and periaqueductal gray areas. There was no change in the force and motor performance.	[9]
Carvacrol	Swiss mice	12.5, 25 or 50 mg/kg, p.o	β-cyclodextrin	Sarcoma 180 cancer pain	Von Frey; spontaneous and palpation-induced nociception; movement-evoked pain and grip strength meter	Carvacrol/β-CD complex (50 mg/kg, p.o.) was able to reduce the hyperalgesia during 24 h, unlike the free carvacrol (100 mg/kg, p.o.), which promoted effects until 9 h. Administration on alternate days of carvacrol/β-CD (12.5–50 mg/kg, p.o.) reduced hyperalgesia, spontaneous and palpation-induced nociception. Carvacrol (50 mg/kg) did not cause significant changes in nociceptive responses. There was no change the grip strength of animals.	[92]
α-Terpineol	Swiss mice	25, 50 or 100 mg/kg, p.o	β-cyclodextrin	Non-inflammatory chronic muscle pain	Von Frey; rota-rot; grip strength meter and antagonism assessment (naloxone and ondasentron)	α-Terpineol/β-CD, at all doses tested, produced a significant decrease in the mechanical hyperalgesia, without causing any alteration in the force and in motor performance. This analgesic effect was reversed by the systemic administration of naloxone or ondansetron.	[93]
Citronelal	Swiss mice	50 mg/kg, p.o	β-cyclodextrin	Chronic muscle pain	Von Frey; grip strength meter and Fos immunofluorescence	Citronelal/β-CD complex reduced mechanical hyperalgesia on all days of treatment, without changing muscle strength. Periaqueductal gray and rostroventromedular area showed significant increase in the Fos protein expression while in the spinal cord, there was a reduction.	[26]
Carvacrol	Swiss mice	10 or 20 mg/kg, p.o	β-cyclodextrin	Orofacial pain	Formalin-, glutamate- and capsaicin-induced nociception; participation of transient receptor potential vanilloid system and opioid system in nociception; rota-rod and grip strength meter	Carcacrol/β-CD reduced the nociceptive during the two phases of the formalin test, whereas carvacrol did not produced the reduction in face-rubbing behavior in the initial phase. Carvacrol and carvacrol/β-CD showed a significant reduction against nociception caused by capsaicin or glutamate injection. The possible mechanism through which the Carvacrol and Carcacrol/β-CD exerts its analgesic action might be on the opioid, vanilloid and glutamate systems. There was no change in the force and motor performance.	[94]
D-Limonene	Swiss mice	50 mg/kg, p.o	β-cyclodextrin	Chronic musculoskeletal pain	Von Frey; hot plate; tail flick; capsaicin-induced nociception; grip strength meter and Fos immunofluorescence	D-limonene/β-CD produced a longer lasting analgesic profile when compared with uncomplexed limonene. The antihyperalgesic profile seems to act by involvement of descending pain-inhibitory mechanisms since the limonene reduced the expression of Fos protein in the dorsal horn of the spinal cord. The effects of limonene on TRPV1 and GABAA targets, at least in part, may be contributing to its anti-hyperalgesic effect. There was no change the grip strength of animals.	[95]

p.o – oral; β-CD - β-cyclodextrin; TNF-α - tumor necrosis factor alpha; TRPV1 - transient receptor potential vanilloid 1; GABAA - type A γ-aminobutyric acid receptor.

4 CAPÍTULO 2

Evaluation of the antinociceptive activity of *Annona vepretorum* Mart. (Annonaceae) essential oil in sarcoma 180-bearing mice

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Evaluation of the antinociceptive activity of *Annona vepretorum* Mart. (Annonaceae) essential oil in sarcoma 180-bearing mice

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Short running title: Antinociceptive activity of *Annona vepretorum*

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Abstract

The drugs currently used to cancer pain management have many side effects, which makes treatment more difficult and further reduces the quality of life of cancer patients. In this scenario, natural products have shown promise in the search for new safer and more effective therapeutic options. In previous studies, the essential oil from the leaves of *Annona vepretorum* (Av-EO) demonstrated antioxidant, cytotoxic, antinociceptive, anti-inflammatory and antitumor activity, in addition to the presence of mono and sesquiterpene compounds in its chemical composition. Thus, the objective of this study was to evaluate the effect of Av-EO in an experimental model of nociception induced by sarcoma 180 in mice. The results of the von Frey test indicated that Av-EO (50 mg/kg) was able to reverse the initial phase of mechanical hyperalgesia induced by sarcoma 180. In the hot plate test, treatment with Av-EO (50 mg/kg) promoted an increase in the latency time of animals exposed to painful stimulus on all days of evaluation and Av-EO (100 mg/kg) until the 8th day of evaluation when compared with the negative control, indicating possible involvement of central action mechanisms in the antinociceptive activity of the oil. The administration of Av-EO (50 and 100 mg/kg) also reduced significantly spontaneous and palpation-induced nociception. The monitoring of paw volume showed that animals that received the dose of 100 mg/kg of Av-EO showed a significant decrease on the 12th day of evaluation when compared to the negative control group. Thus, these results provide preliminary evidence that Av-EO may be useful in the search for new therapeutic alternatives for cancer pain management and encourage further studies to elucidate the mechanism of action and identification of the chemical constituents responsible for the pharmacological activity of this natural product.

Keywords: Natural Products. *Annona.* Sarcoma 180. Cancer pain.

Avaliação da atividade antinociceptiva do óleo essencial de *Annona vepretorum* Mart. (Annonaceae) em camundongos portadores de sarcoma 180

Resumo

Os fármacos atualmente utilizados para manejo da dor associada ao câncer apresentam muitos efeitos colaterais, o que dificulta o tratamento e diminui ainda mais a qualidade de vida dos pacientes oncológicos. Nesse cenário, os produtos naturais têm se mostrado promissores na busca por novas opções terapêuticas mais seguras e eficazes. Em estudos prévios, o óleo essencial das folhas de *Annona vepretorum* (Av-OE) demonstrou atividade antioxidante, citotóxica, antinociceptiva, anti-inflamatória e antitumoral, além da presença de compostos mono e sesquiterpênicos em sua composição química. Dessa forma, o objetivo desse estudo foi avaliar o efeito do Av-OE em modelo experimental de nocicepção induzida pelo sarcoma 180 em camundongos. Os resultados do teste de von Frey indicaram que Av-OE (50 mg/kg) foi capaz de reverter a fase inicial da hiperalgesia mecânica induzida pelo sarcoma 180. No teste da placa quente, o tratamento com Av-OE (50 mg/kg) promoveu aumento do tempo de latência dos animais expostos ao estímulo doloroso em todos os dias de avaliação e Av-OE (100 mg/kg) até o dia 8 de avaliação quando comparados com o controle negativo, indicando possível envolvimento de mecanismos de ação central na atividade antinociceptiva do óleo. A administração de Av-OE (50 e 100 mg/kg) também reduziu significativamente a nocicepção espontânea e induzida por palpação. O monitoramento do volume da pata mostrou que os animais que receberam a dose de 100 mg/kg de Av-OE apresentaram uma diminuição significativa no 12º dia de avaliação quando comparados com o grupo controle negativo. Dessa forma, esses resultados fornecem evidências preliminares de que Av-OE pode ser útil na busca por novas alternativas terapêuticas para manejo da dor oncológica e encorajam estudos

posteriores para elucidação do mecanismo de ação e identificação dos constituintes químicos responsáveis pela atividade farmacológica desse produto natural.

Palavras-chave: Produtos Naturais. *Annona*. Sarcoma 180. Dor oncológica.

1. Introduction

Pain is one of the most common symptoms in cancer patients and greatly reduces the quality of life. Cancer pain is mainly related to tumor presence, due to compression of peripheral tissues or nerves or infiltration and metastasis of tumor cells. In addition, pain may be related to methods of diagnosis and/or treatment, such as surgical procedures, chemotherapy, and radiotherapy (American Cancer Society, 2019). Pain affects about 20 to 50% of patients undergoing cancer therapy and more than 80% of patients in advanced disease stage (Neufeld et al., 2017).

Cancer pain management is based on the patient's pain intensity and includes non-opioid drugs, weak opioids, strong opioids, and adjuvant drugs (WHO, 1966). However, these drugs exhibit several side effects that limit the use and stimulate the search for new therapeutic alternatives that promote not only the control of cancer pain, but also the reduction of tumor growth and the reduction of the occurrence of metastases. In this context, natural products have a great potential to contribute to the discovery of antinociceptive and antitumor drugs, since it is estimated that in the last thirty years the percentage of new structures, originated or inspired by natural products, increased by 50% (Katza and Baltz, 2016).

Annona vepretorum is an endemic species in Brazil, found in the Caatinga biome and popularly known as "araticum" and "pinha da Caatinga". The essential oil obtained from the leaves of *A. vepretorum* has a predominant composition of monoterpenes and sesquiterpenes. Previous studies have evidenced its pharmacological activity, with emphasis on cytotoxicity against tumor cell lines and antioxidant properties (Costa et al., 2012; Meira et al., 2015; Bomfim et al., 2016).

Thus, the objective of this study was to evaluate the antinociceptive effect of the essential oil of *A. vepretorum* (Av-EO) leaves in a murine experimental model of cancer pain induced by sarcoma 180.

2. Material and Methods

2.1 Extraction of the essential oil

The leaves of *A. vepretorum* were collected in the city of Petrolina, State of Pernambuco, Brazil, in April 2015, at the experimental farm of the Federal University of Vale do São Francisco (coordinates: 09°19′38″S,40°33′01″W). A voucher specimen (#18350) was deposited at the Herbarium Vale do São Francisco at the same university and the project was registered in National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) (Register # A4AB302).

To obtain the essential oil (Av-EO) the fresh leaves of *A. vepretorum* (941 g) were powdered and subjected to the hydrodistillation procedure for 2 hours in a Clevenger type apparatus. The essential oil obtained (3 ml) was stored in a refrigerator at a temperature of 4 °C until the use.

2.3 Animals

Female adult (8 to 10 weeks of age) albino Swiss mice (30 to 40 g) were used in this study. They were randomly housed in appropriate cages containing six animals in each (n=6), at 22 ± 2 ºC, on a 12 h light/dark cycle (lights on from 07:00 AM to 07:00 PM) and with free access to food and water. Experimental protocols and procedures were approved by the Animal Care and Use Committee at the Federal University of Vale do São Francisco under the number 0003/241017.

2.4 Induction of tumor and treatments

Sarcoma 180 tumor cells were obtained from the peritoneal cavity of Swiss mice after seven days of implantation and cell viability was evaluated using trypan blue. A suspension

containing 10^6 cells per 25 µL of physiologic solution was administered in the right hind paw of mice for development of the solid tumor (day 0). The sham group was composed of animals that were not inoculated with the tumor cells, receiving only physiologic solution in the right hind paw (Guimarães et al., 2014, adapted). The animals were divided into five groups with six animals each (n=6) and treated daily from the 2nd until the 12th day after tumor induction. The sham and negative control groups received physiologic solution orally (p.o.), the positive control group received 10 mg/kg of morphine intraperitoneally (i.p.) and the other groups were treated with Av-EO at the doses of 50 and 100 mg/kg (p.o.), respectively.

2.5 Antinociceptive assays

The mechanical hyperalgesia test was conducted before tumor implantation (day 0) and on day 2 after tumor induction. The hot plate test was performed on days 2, 4, 6, 8, 10 and 12 after tumor inoculation. Behavior tests (evaluation of paw use, spontaneous and palpation-induced nociception) were performed on days 3, 5, 7, 9 and 11 after tumor implantation. All tests were performed 30 minutes after the administration of morphine (10 mg/kg, i.p.) and 1 hour after the administration of Av-EO (50 and 100 mg/kg, p.o.).

2.5.1 Mechanical hyperalgesia test

The mice were acclimated for ten minutes and then were subjected to mechanical stimulation through gradual increase in pressure on the plantar surface of tumor-bearing paw using an electronic anesthesiometer (model: EFF-301, Insight®, Ribeirão Preto, São Paulo, Brazil) adapted with a polypropylene tip. This stimulus causes a production of a paw response characterized as flinch, an indication of hyperalgesia. The intensity of hypernociception was quantified as the variation in pressure obtained through the average of three values, expressed

in grams (strength), observed before tumor induction (baseline) and 48 hours after inoculation of sarcoma 180 cells (Cunha et al., 2005; Dziubina, 2019, adapted).

2.5.2 Hot plate test

In the hot plate test the mice were placed on a heated metal plate (model: EFF-361, Insight®, Ribeirão Preto, São Paulo, Brazil) (55 ± 0.5 °C) and evaluated for the time they remained until react to the thermal stimulus with the behavior of jumping, lifting, or licking the paws (Kuraishi et al., 1983; Ramos et al., 2020).

2.5.3 Evaluation of paw use

The mice were acclimatized in a mirrored box for 10 minutes and then evaluated for another 10 minutes regarding the use of the paw with tumor through the scale: 0 = complete lack of use of the limb, 1 = partial use of the limb, 2 = limping and withdrawing the limb, 3 = limping, 4 = walking normally (Sabino et al., 2003, adapted).

2.5.4 Spontaneous and palpation-induced nociception test

The mice were placed in mirrored boxes and acclimated for 10 minutes. After that time, the animals were observed for 10 minutes and the number of flinching and licking paw behaviors were quantified. Then, the animals were submitted to non-harmful palpation of the paw with tumor for 2 minutes and the number of licks and flinches was quantified for 5 minutes (Sabino et al., 2003, adapted).

2.6 Evaluation of tumor/paw volume

The assessment of paw volume was performed on days 2, 4, 6, 8, 10 and 12 after tumor inoculation, using an image plethysmometer (model: EFF-370, Insight®, Ribeirão Preto, São

Paulo, Brazil). For this, 30 minutes or 1 hour after the treatments, the paw with the tumor was immersed in a solution and three independent measurements were recorded (Guimarães et al., 2014, adapted).

2.7 Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed by Tukey´s test. Kruskal Wallis followed by Dunn's post-test was performed for the analysis of hind paw use. Differences were considered significant when $p<0.05$. All data were analyzed in the Graph Pad Prism software version 7.0.

3. Results

The von Frey apparatus was used to show whether tumor cell inoculation induced hyperalgesia and to investigate the initial effect of treatment with Av-EO in sarcoma 180-bearing mice. For that, before the tumor induction, the animals were evaluated to obtain the baseline values of their pain thresholds and 48 hours after tumor transplantation, a second analysis was performed. As expected, there was no statistically significant difference in mechanical hyperalgesia before tumor implantation (Figure 1A). After 48 h of tumor cell inoculation, there was a significant decrease ($p<0.05$) of the nociceptive threshold in the negative control group (6.03 ± 0.39 g) and Av-EO (100 mg/kg) (7.62 ± 0.92g) when compared to the healthy group (sham) (12.01 ± 0.51), proving that the presence of the tumor alters the nociceptive sensitivity. However, there was no significant difference in the nociceptive threshold after treatment with morphine (13.49 ± 1.44 g) and Av-EO (50 mg/kg) (13.31 ± 0.72 g) when compared to sham group, in addition, these groups, when compared to negative group (10.9 ± 0.65), had a significant increase ($p <0.05$) in latency time (Figure 1B).

INSERT FIGURE 1

In the hot plate test, there was observed a statistically significant ($p<0.05$) increase in latency time on days 2, 4, 6, 8 10 and 12 after treatment with morphine (10 mg/kg) and Av-EO (50 mg/kg) when compared to negative control. Treatment with Av-EO (100 mg/kg) promoted an increase in the latency time of animals exposed to painful stimulus until the 8th day of evaluation.

INSERT FIGURE 2

The use of paw with tumor was evaluated using the following scale: 0 = complete lack of limb use, 1 = partial non-use of the limb, 2 = limping and withdrawing the limb, 3 = limping, 4 = walking normally. A significant increase ($p<0.05$) in the use of the paw was observed after treatment with morphine (3.7 ± 0.4), Av-EO 50 mg/kg (2.4 ± 0.7) and 100 mg/kg (2.6 ± 0.8) when compared to negative control group (1.3 ± 0.4). The results also indicated that treatment with Av-EO was able to reduce the hyperalgesic stimulus of sarcoma 180, improving the use of the paw with tumor. Results are shown in Figure 3.

INSERT FIGURE 3

Treatment with Av-EO (50 and 100 mg/kg) and morphine were also able to significantly ($p<0.05$) reduce spontaneous and palpation-induced nociception in the animals with sarcoma 180. This can be seen in Table 1, that shows the mean number of licks and flinches from the five days of analysis.

INSERT TABLE 1

The assessment of paw volume was performed on days 2, 4, 6, 8, 10 and 12 after tumor induction using a plethysmometer. Results of this test are presented in Figure 4 and shown than from the 4nd day of analysis there was a significant increase ($p<0.05$) in animals paw volume in all experimental groups when compared to the healthy control (sham). However, only on the 12th day of observation the animals treated with Av-EO (100 mg/kg) showed a statistically significant decrease ($p<0.05$) in the paw volume (0.37 ± 0.02 ml) when compared to the

negative control group (0.55 ± 0.05 ml). Animals treated with morphine (10 mg/kg) (0.52 ± 0.03 ml) and Av-EO (50 mg/kg) (0.48 ± 0.02 ml) did not showed this paw volume reduction.

INSERT FIGURE 4

4. Discussion

The chemical composition of the essential oil of *A. vepretorum* leaves is predominantly of monoterpenes and sesquiterpenes, which confers various biological activities, such as cytotoxic, antitumor (Bomfim et al., 2016), trypanocidal (Costa et al., 2012), antimalarial (Costa et al., 2012; Meira et al., 2015) and antimicrobial (Meira et al., 2015). A previous study carried out by our research group identified (*E*)-β-ocimene (42.59%), bicyclogermacrene (18.81%), germacrene D (12.19%) and limonene (10.02%) as major constituents of the oil sample tested in this paper and evidenced its anxiolytic, sedative, antiepileptic and antidepressant effects in mice (Diniz et al., 2019).

In view of the chemical and biological potential of *A. vepretorum*, this study aimed to evaluate the antinociceptive activity of Av-EO in an experimental model of cancer pain induced by sarcoma 180 in mice, thus mimicking the types of pain commonly presented by patients with neoplasms, such as hyperalgesia, spontaneous nociception, and allodynia (Allegri et al., 2012). In the von Frey test, treatment with Av-EO (50 mg/kg) promoted a reduction of mechanical hyperalgesia when administered 48 hours after tumor implantation (Figure 1B). Hyperalgesia is due to the lowering of the sensitivity threshold of Aδ and C fibers. Sensitization of these fibers can be generated by chemical mediators released by tumor cells or by polymorphonuclear cells found in the peripheral regions of sarcoma 180 (Sato et al., 2005), which increase the excitability of nociceptive neurons, thus increasing the probability of triggering action potentials in response to a stimulus, a characteristic phenomenon of hyperalgesia (Mendes, 2017).

In the hot plate test, it was observed that the treatment with Av-EO (50 and 100 mg/kg) was able to increase the nociceptive threshold (Figure 2). The hot plate test consists of exposing the animal to a hot surface, for thermal stimulation, with the objective of evaluate analgesic activity mediated by central mechanisms, this pathway being constantly used in the clinical management of cancer pain (Shi et al., 2011; Almeida-Júnior, 2019). Thus, it can be suggested that central mechanisms of action may be involved with the antinociceptive activity of Av-EO, by blocking thermal receptors or by inhibiting the stimulus of afferent nervous system. Previous studies carried out with monoterpenes, such as *p*-cymene, indicated that this substance can modulate the opioid receptors non-selectively, promoting G protein activation and triggering intracellular signals that promote increased potassium conductance and calcium conductance inhibition, thus generating cellular hyperpolarization and reducing pain (Bonjardim et al., 2012; Pathan and Williams, 2012; de Santana et al., 2015).

Another parameter evaluated was the use of the limb with a tumor using a paw use score, assessment of spontaneous nociception and palpation-induced nociception. Results showed that the first day of analysis was the only one in which all groups presented a score of 4 (walking normally). Over the days the group that did not receive treatment showed a significant decrease in the use of the paw, while in the groups treated with morphine and Av-OE this decrease was not so drastic. The spontaneous nociception behavioral test assesses the painful process related to tumor growth without being subjected to any stimulus. Palpation-induced nociception, on the other hand, assesses the allodynia of animals with a sarcoma 180 implant when analyzing the response to non-harmful palpation (Santos, 2019).

From the results of monitoring paw volume, it was possible to correlate the pattern of development of the sarcoma 180 (Figure 4) with the reduction of the capacity of use of the limb with the tumor of all the analyzed animals, mainly in the negative control group, which received only the vehicle. The results also indicated that the treatment with Av-EO, at doses of 50 and

100 mg/kg, was able to increase the nociceptive threshold, maintaining an average of 3 (hobbling) on the paw use scale proposed by Sabino, et al., 2003, similar to observed after treatment with morphine (Figure 3). In the evaluation of spontaneous and palpation-induced nociception (Table 1), it was observed that animals in the groups treated with Av-EO (50 and 100 mg/kg) and morphine also had a significantly ($p<0.05$) lower mean of flinches and paw licking when compared to the negative control group, suggesting that treatment with the oil could attenuate the process of hyperalgesia and allodynia caused by the tumor.

As previously described, there was an increase in the paw volume of the animals throughout the experiment. However, on the last day of treatment the animals that received Av-EO (100 mg/kg) presented a significant ($p<0.05$) decrease in paw volume (0.37 ± 0.02 ml) when compared with the negative control group (0.55 ± 0.05 ml) (Figure 4). This reduction in paw volume of these animals can be justified due to the anti-tumor action of Av-EO as well as the anti-inflammatory effect detected in previous studies with this plant species. Bomfim and collaborators indicated that the cytotoxic and anti-tumor activity of Av-EO is linked to its chemical composition predominantly of sesquiterpenes and monoterpenes (Bomfim et al., 2016), since these compounds are lipophilic and can easily cross cytoplasmic membranes, disrupting the layers of polysaccharides, fatty acids and phospholipids which make up the lipid bilayer (Tundis et al., 2017).

In conclusion, the results of this study indicate the antinociceptive effect of Av-EO on the experimental model of cancer pain induced by sarcoma 180 in mice. In addition, is suggested a possible involvement of central mechanisms of action in the antinociceptive activity of oil, requiring further detailed studies.

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FIGURE LEGENDS

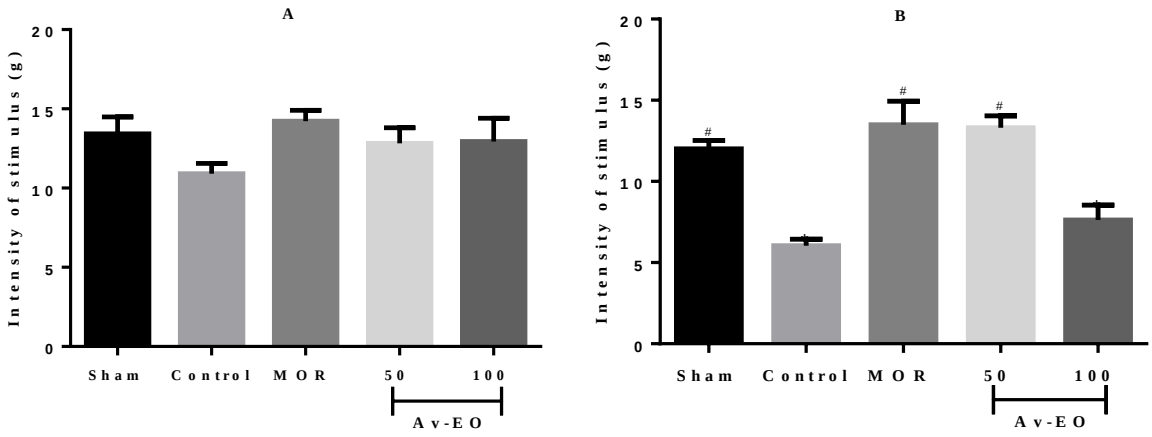
Figure 1. Effect of Av-EO (50 mg/kg and 100 mg/kg) on the mechanical hyperalgesia before tumor induction (A) and 48 hours after inoculation of sarcoma 180 cells in the hind paw of mice (B). Values are expressed as mean ± S.E.M, n = 6. $^{*}p < 0.05$ compared to sham group and $^{\#}p < 0.05$ compared to negative control group by ANOVA followed by Tukey´s test. Av-EO: *Annona vepretorum* essential oil. Control: negative control. MOR: morphine (10 mg/kg).

Figure 2. Effect of Av-EO (50 mg/kg and 100 mg/kg) on the thermal hyperalgesia evaluated on days 2, 4, 6, 8, 10 and 12 after inoculation of sarcoma 180 cells in the hind paw of mice. Values are expressed as mean ± S.E.M, n = 6. $^{*}p < 0.05$ compared to sham group and $^{\#}p < 0.05$ compared to negative control group by ANOVA followed by Tukey´s test. Av-EO: *Annona vepretorum* essential oil. Control: negative control. MOR: morphine (10 mg/kg).

Figure 3. Effect of Av-EO (50 mg/kg and 100 mg/kg) on the paw use score evaluated on days 3, 5, 7, 9 and 11 after inoculation of sarcoma 180 cells in the hind paw of mice. Values are expressed as mean ± S.E.M, n = 6. $^{\#}p < 0.05$ compared to negative control group by Kruskal-Wallis followed by Dunn´s test. Av-EO: *Annona vepretorum* essential oil. Control: negative control. MOR: morphine (10 mg/kg).

Figure 4. Effect of Av-EO (50 mg/kg and 100 mg/kg) on paw/tumor volume evaluated on days 2, 4, 6, 8, 10 and 12 after inoculation of sarcoma 180 cells in the hind paw of mice. Values are expressed as mean ± S.E.M, n = 6. $^{*}p < 0.05$ compared to sham group and $^{\#}p < 0.05$ compared to negative control group by ANOVA followed by Tukey´s test. Av-EO: *Annona vepretorum* essential oil. Sham: healthy control. Control: negative control. MOR: morphine (10 mg/kg).

Figure 1.


A
Intensity of stimulus (g)
20
15
10
5
0
Sham
Control
MOR
50
100
Av-EO
B
Intensity of stimulus (g)
20
15
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Control
MOR
50
100
Av-EO

Figure 2.

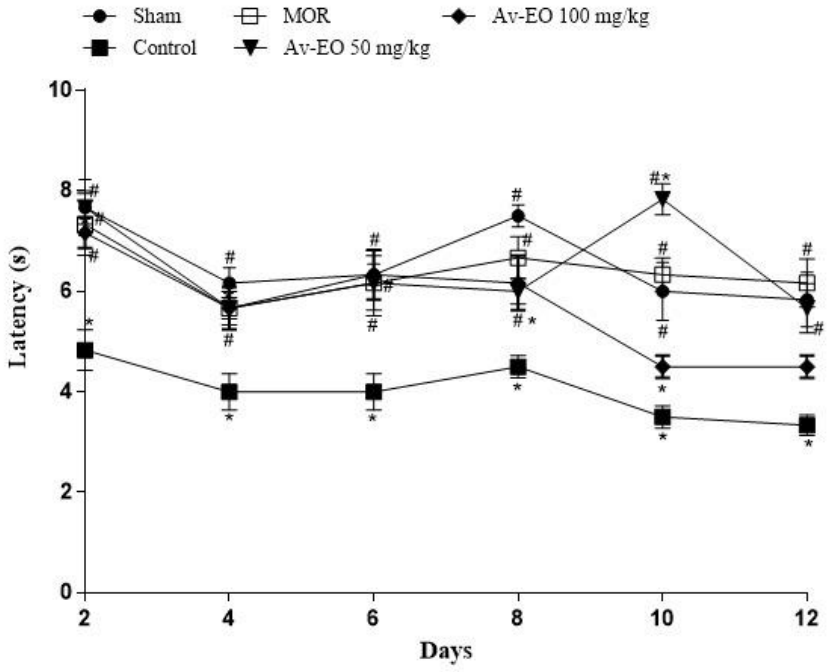
Sham
MOR
Av-EO 100 mg/kg
Control
Av-EO 50 mg/kg
Latency (s)
Days
0
2
4
6
8
10
12

Figure 3.

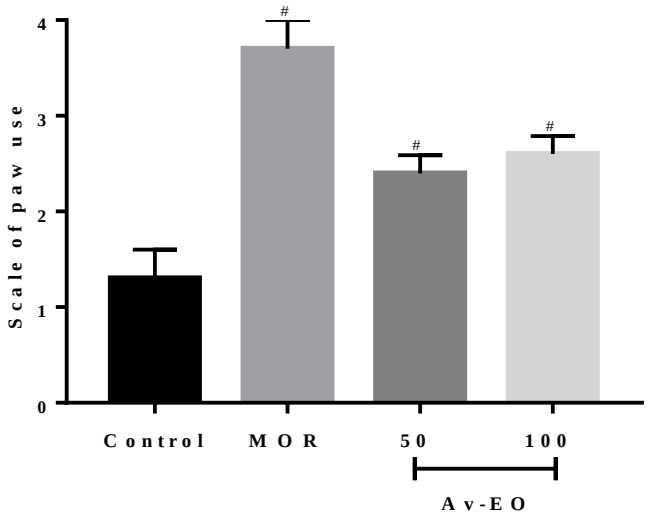

Figure 4.

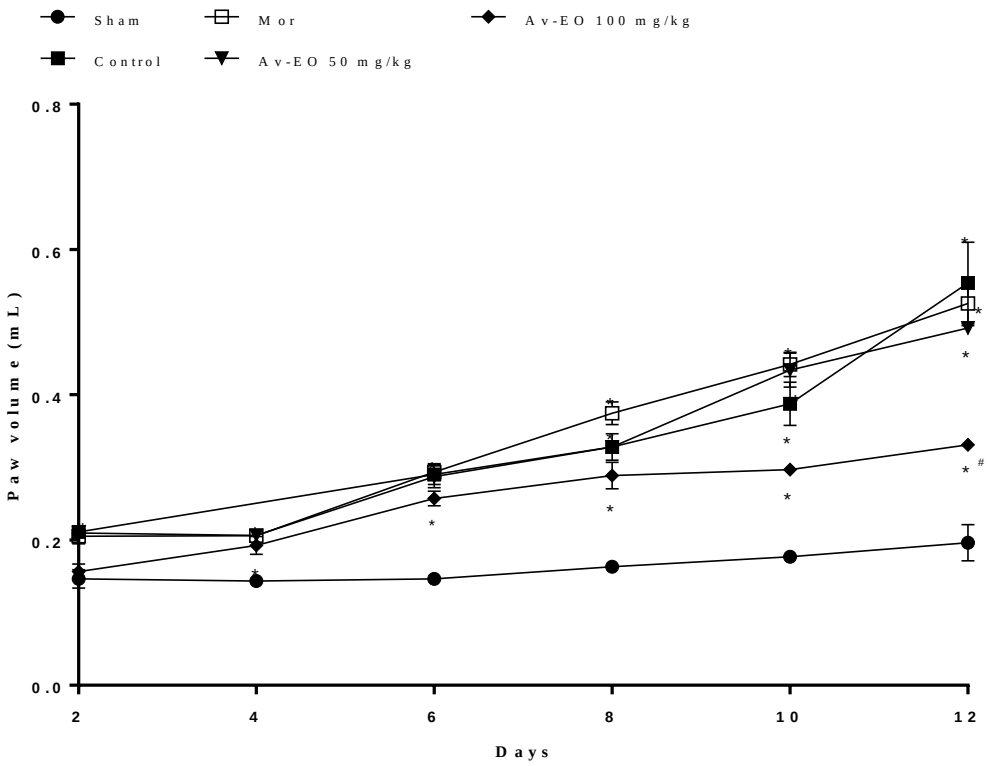
Sham
Mor
Av-EO 100 mg/kg
Control
Av-EO 50 mg/kg
Paw volume (mL)
0.0
0.2
0.4
0.6
0.8
2
4
6
8
10
12
Days
*
#

TABLE LEGENDS

Table 1. Effect of Av-EO (50 mg/kg and 100 mg/kg) on spontaneous and induced nociception on days 3, 5, 7, 9 and 11 after inoculation of sarcoma 180 cells in the hind paw of mice. Values are expressed as mean ± S.E.M, n = 6. $^{\#}p < 0.05$ compared to negative control group by Kruskal-Wallis followed by Dunn´s test. Av-EO: *Annona vepretorum* essential oil. Control: negative control. MOR: morphine (10 mg/kg).

Table 1.

Spontaneous nociception				
	Control	**Mor**	**Av-EO (50 mg/kg)**	**Av-EO (100 mg/kg)**
Limbs	15 ± 0.76	8 ± 0.17$^{\#}$	10 ± 0.30$^{\#}$	8 ± 0.22$^{\#}$
Flinches	18 ± 0.19	4 ± 0.05$^{\#}$	11 ± 0.03$^{\#}$	8 ± 0.05$^{\#}$
Palpation-induced nociception				
	Control	**Mor**	**Av-EO (50 mg/kg)**	**Av-OE (100 mg/kg)**
Limbs	18 ± 0.71	8 ± 0.18$^{\#}$	11 ± 0.25$^{\#}$	11 ± 0.24$^{\#}$
Flinches	18 ± 0.09	4 ± 0.06$^{\#}$	10 ± 0.06$^{\#}$	9 ± 0.04$^{\#}$

5 CAPÍTULO 3

Preparation, characterization and evaluation of toxicity and analgesic activity of inclusion complex of β-ocimene with β-cyclodextrin and free β-ocimene in an experimental mice cancer pain model

Artigo submetido ao periódico (comprovante de submissão em anexo):

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Preparation, characterization and evaluation of toxicity and analgesic activity of inclusion complex of β-ocimene with β-cyclodextrin and free β-ocimene in an experimental mice cancer pain model

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Running title: Toxicity and analgesic activity of ocimene.

Keywords: β-cyclodextrin, β-ocimene, Monoterpene, Toxicity, Cancer pain.

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Abstract

Cancer pain affects most oncological patients. Preclinical studies have shown that natural products have promising activity in neoplasm-related pain management. This work evaluated the toxicity and analgesic activity of β-ocimene, and β-ocimene complexed with β-cyclodextrin (β-CD) in the model of cancer pain induced by sarcoma 180 cells. A total of 71 female *Swiss* mice (*Mus musculus*) were used. The inclusion complex was prepared by freeze-drying method and characterized by scanning electron microscopy, Fourier-transform infrared spectroscopy and nuclear magnetic resonance. Only free β-ocimene presented cytotoxicity against tumor cell lines and non-tumor cell line at tested concentrations. β-ocimene and β-ocimene/β-CD complex were toxic in *Artemia salina* and hemolysis assay. Acute toxicity was assessed against Swiss mice and the results demonstrated that the oral LD_{50} was estimated to be greater than 300 mg/kg. Administration of β-ocimene/β-CD complex in tumor bearing-mice promoted a significant ($p < 0.05$) decrease in mechanical and thermal hyperalgesia and in tumor volume in determinate days of observation, when compared to the negative control or free ocimene groups. The data suggests that the complex was efficiently obtained, exhibits cytotoxic activity, and can be an alternative to enable the application of β-ocimene in the development of new options for pain management.

Introduction

Monoterpenes are components of essential oils, whose biological and pharmacological properties, such as antimicrobial, antioxidant, anti-inflammatory, antitumor and antinociceptive activity are already well established [1, 2]. (*E*)-β-ocimene is a monoterpene found in different concentrations in the most varied plant species and presents potential medicinal activity [2]. However, monoterpenes, in general, have physico-chemical characteristics that limit their applications and use, among which, high volatility, low water solubility and strong instability against light and oxygen [1, 3].

In this way, high performance drug transport systems have gained notoriety in recent years that, especially cyclodextrins. Cyclodextrins are crystalline and chemically stable molecules, constituted by cyclic oligosaccharides that act by promoting, through complexation, an increase in the solubility of monoterpenes in water, in addition to providing protection against oxidative and thermal damage, maintaining the pharmacological profile, as well as increasing the safety, increasing the bioavailability, and reducing possible side and toxic effects [4-7]. β-CD, in turn, is classified as one of the most common and present in pharmaceutical formulations [8].

Thus, the objective of this study was to obtain and characterize an inclusion complex of β-ocimene with β-cyclodextrin and evaluate the toxicological profile, analgesic potential, and effect on tumor/paw volume of β-ocimene alone and β-ocimene/β-CD complex in a mice cancer pain model.

Materials and Methods

Chemicals

Ocimene (mixture of isomers, stabilized, ≥ 90%), β-CD, and trypan blue were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Morphine was purchased from Cristália (São Paulo, São Paulo, Brazil). Inclusion complex was prepared with MiliQ water. D_2O was purchased from Tedia® Brasil and doxorubicin was purchased from Glenmark®.

GC-MS analyses of ocimene

Hydrocarbons standard (C_9-C_{40}) and isomers mixture were individually analyzed using a Shimadzu gas chromatograph mass spectrometer QP-2010 model, equipped with SH-RTx-5MS (30 m x 0.25 mm x 0.25 um, Shimadzu) column and EI-MS of 70 eV. For thus, the samples (1.0 µL) were injected into equipment with follow operating conditions: split mode ratio 1:40;

injector and detector temperature 240 ºC; column temperature 60 ºC-240 ºC at 3 ºC/min and helium as carrier gas at flow rate 1.3 mL/min. Then, the percentage of each isomer was calculated using area normalization method and the mass spectra of each peak were compared NIST 08 and WILEY 7 libraries data for compounds identification.

Preparation of β-CD complex

Inclusion complex was obtained as previous described by Yallapu; Jaggi and Chauhan, with some adaptations [9]. β-CD was dissolved in ultrapure water and ocimene was dissolved in ethanol (1:1 molar ratio in equivalent of (*E*)-β-isomer). These solutions were put on the stirrer at room temperature for 24 h until completely solubilized. Then, the final solution was freeze dried at -46 °C, 91 x 10^{-3} mBar and the resulting solid was stored at 4 °C until further use. The total of recovery was obtained according to equation: Total recovery (%) = [Recovered powder / Initial (β-CD + Ocimene)] x 100

A physical mixture (PM) was obtained by manual agitation of β-CD and β-ocimene (1:1 molar ratio) in closed container.

Determination of β-ocimene loading in β-CD

The amount of β-ocimene adsorbed on the surface of β-CD was determined based in the methodology described by Menezes and collaborators with some adaptations [10]. β-ocimene/β-CD inclusion complex (20 mg) was shaken with 2.0 mL of chloroform for 20 min and then filtered to extract the adsorbed ocimene on the surface of the cyclodextrin. A standard solution of β-ocimene was prepared by dissolving 20 mg of the compound in 2.0 ml of chloroform. Thus, the materials, supernatant and standard β-ocimene solution, were analyzed by gas chromatography/mass spectrometry (GC-MS), using an Agilent gas chromatograph model GC 7820A equipped with HP – 5ms (30 m x 0.25 mm x 0.25 µm) column, coupled to mass spectrometer quadrupole type Agilent MSD 5977E model. For analysis, 1.0 µL of samples were injected into equipment using split mode ratio 1:40, helium as carrier gas at 1.1 mL/min and injector temperature at 240 ºC. The process was carried out by adjusting the oven temperature 100-280 ºC, through increment in temperature as follow: 100 ºC for 5 minutes, increase temperature until 250 ºC at 10º C/min and constant during 5 min, follow increase temperature until 280 ºC at 10º C/min and constant during 15 min. In EI-MS, 70 eV were used as energy ionization and fragments analyzed at 781 m/s in 30 – 500 Da. The content (mg) of β-ocimene adsorbed in complex surface was calculated in relation a total area of β-ocimene standard, where total area corresponds to 20 mg and supernatant area to a fraction of this total.

The content of β-ocimene in β-CD cavity was calculated by difference between initial content of β-ocimene and adsorbed. The complexation rate was calculated using follow equation:

$$\%\ complexation = \left[\frac{(mg\ initial\ ocimene - mg\ of\ internal\ ociemene)}{mg\ initial\ ocimene}\right] x\ 100$$

Scanning electron microscopy (SEM)

The samples were mounted on aluminum stubs, metallized with gold powder for 300 s and morphologically analyzed with a Tescan VEGA3 scanning microscope, at an accelerate voltage of 10 kV.

Fourier-transform infrared spectroscopy (FTIR) analysis

The dried products were analyzed in an infrared spectrophotometer (PerkinElmer Spectrum, Version 10.4.00), in the region from 4000 to 650 cm^{-1} and using potassium bromide pellet method.

Nuclear magnetic resonance (NMR) analysis

The ^{1}H NMR analysis were conducted in a Bruker Avance III™ 400 MHz spectrometer operating at 9.4 Tesla. The samples were prepared in D_20 and transferred to 5 mm diameter NMR tubes. The experiments were performed at 288 K using the residual water peak (4.80 ppm) as a reference to report chemical shifts values.

Cells

Tumor cell lines PC-3 (human prostate cancer), MDA-MB-231 (human breast cancer), HL-60 (human promyelocytic leukemia) and non-tumor cell line L929 (mouse fibroblasts) were donated by National Cancer Institute (USA). Cells were cultured in RPMI-1640 (L929 was cultured in DMEM) medium supplemented with 10% fetal bovine serum and 1% of antibiotics. The cell lines were maintained at 37 °C in atmosphere with 5% CO_2. Sarcoma 180 tumor cells, obtained from the Laboratory of Immunology at the Federal University of São Francisco Valley (Brazil), were maintained in the peritoneal cavity of Swiss mice.

Cytotoxic activity on tumor and non-tumor cells

Cells were seeded in 96-well plates at the concentrations of 0.1 x 10^6 cell/mL for PC-3 and MDA-MB-23 lines, 0.3 x 10^6 cell/mL for HL-60 and 0.7 x 10^6 cell/mL for L929. Then,

cells were treated with different concentrations (0.08–5 μg/mL) of β-ocimene, β-ocimene/β-CD complex or doxorubicin solubilized in DMSO and allowed to incubation for 72 h in an oven at 5% CO_2 at 37 °C. At the end of this time, cells were centrifuged and the supernatant was removed. Subsequently, 100 μl of MTT solution (tetrazolium salt) was added, and the plates were incubated for 3 h. The absorbance at 570 nm was measured using a multiplate reader after dissolution of the precipitate with 100 μL of pure DMSO. The IC_{50} was calculated using a non-linear regression and all experiments were performed in three replicates.

Cytotoxic activity on *Artemia salina*

The cytotoxicity of the samples on *Artemia salina* was evaluated according to the method described by Meyer and collaborators with some adaptations [11]. Encysted eggs of the brine shrimp (20 mg) were incubated in salt water (38 g/L) for 48 h. After hatching, 10 nauplii were transferred with a Pasteur pipette to tubes containing the test samples. β-CD, β-ocimene and β-ocimene/β-CD complex were evaluated in triplicate (1-1000 μg/mL) dissolved in 1% DMSO and with each tube having a final volume of 5 mL. The negative control contained 1% DMSO without sample. They were incubated at a temperature of 25 °C, under constant illumination for 24 h. After this time, the number of survivors was counted, and the Medium Lethal Concentrations (LC_{50}) values were obtained considering the concentration versus percentage lethality.

Hemolysis assay

In vitro hemolytic effect of β-CD, ocimene and inclusion complex was performed according to the methodology described by Kang and collaborators with some adaptations [12]. A volume of 2 mL of whole blood was collected by cardiac puncture in Swiss mice (*Mus musculus*) and added in an EDTA containing tube. Phosphate buffered saline (PBS, pH 7.4) was added to the whole blood to complete the final volume of 10 mL and this mixture was centrifuged at 3,000 rpm for 5 minutes. The supernatant plasma was removed, and the process was repeated two more times. Precipitated erythrocytes were resuspended in PBS to obtain the 1% erythrocyte suspension used in this test. Then, 1 mL of the samples, dissolved in 1% DMSO, were added to 1 mL of the suspension of red blood cells for final sample concentration ranging from 1.95-500 μg/mL, in triplicate. Positive control was prepared by incubating erythrocytes with 1% Triton X-100 in PBS and negative control was prepared by incubating erythrocytes with PBS alone. The tubes were gently agitated for 60 minutes and then centrifuged at 3,000

rpm for 5 minutes. The supernatant was removed, and its absorbance was determined at 540 nm to measure the concentration that produces 50% hemolysis (HC_{50}).

Animals

A total of 71 female *Swiss* mice (*Mus musculus*), 6-8 months and 24-39 g, were obtained from Federal University of São Francisco Valley. The animals were randomly housed in polypropylene cages at 22-25 °C, with free access to food (commercial pellets) and water, kept under a 12 h light/dark cycle (lights on at 7:00 a.m.) and adapted for a week before the tests. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [13]. Experimental protocols were previously approved by the Ethics Committee on Animal Use of this institution, under the number 0003/241017.

Acute oral toxicity test

Evaluation of acute oral toxicity was performed with 15 female *Swiss* mice (*Mus musculus*) randomly separated into groups with 3 animals according to guide 423 of the Organization for Economic Cooperation and Development [14]. The animals were fasted for 4 hours before the test. Negative control group (n=3) was orally (p.o.) administrated with distilled water and 1% Tween 80 by gavage, while the other groups were treated with β-ocimene (300 mg/kg, p.o., n=3) or β-ocimene/β-CD complex (300 mg/kg, p.o., n=3) dissolved in distilled water and 1% Tween 80 in a single dose. Subsequently, the animals were subjected to a behavioral analysis at times 30 min, 1, 2 and 4 h after administration and thereafter daily, until the fourteenth day to observe possible signs of toxicity of the tested samples [15]. The body weight of each animal was measured on the first day and at the end of the experiment, and the body weight gain was calculated using the mean difference between final and initial weight. At the end of the observation period, the animals were euthanized, their organs (liver, kidneys, heart, spleen, lungs, and stomach) were macroscopically evaluated for changes in texture and coloration, removed and weighed to calculate the relative values of organ weights (g/100 g body weight). After this first step, the process was repeated with the administration of vehicle (negative control group, p.o., n=3) or free β-ocimene at a dose of 2000 mg/kg, p.o. (n=3).

Tumor implantation and treatment of animal

Sarcoma 180 cells were collected from the peritoneal cavity of maintenance animal and counted in a Neubauer chamber using 1% Trypan blue to determine the percentage of viable cells. Fifty-six animals were randomly divided into seven experimental groups and a suspension

of these tumor cells containing 10^6 cells in 25 µL was administered subcutaneously in the hind paw of mice, except in the healthy control group (sham group), which was inoculated with physiological solution. The treatment was started 48 h after tumor induction, once a day until 12^{th} day as described follow: Groups I (healthy control, n=8) and II (negative control, only induced, n=8) were treated with vehicle (0.9% saline, p.o.); Group III (reference drug, n=8) was treated with morphine (10 mg/kg) intraperitoneally (i.p.); Group IV was treated with free β-ocimene (25 mg/kg p.o., n=8); Group V was treated with free β-ocimene (50 mg/kg p.o., n=8); Group VI was treated with the inclusion complex (25 mg/kg, p.o., n=8) and Group VII was treated with the inclusion complex (50 mg/kg, p.o., n=8). Evaluation of mechanical and thermal hyperalgesia and paw/tumor volume were performed after the treatments from the 2^{nd} to 12^{th} days following tumor inoculation, on alternate days. This methodology was adapted from Guimarães and collaborators [16] and Calixto-Campos and collaborators [17].

Mechanical hyperalgesia

Mice were evaluated for sensitivity to mechanical stimulation with an electronic analgesiometer (von Frey, Insight®, Ribeirão Preto, São Paulo, Brazil), which consists in a pressure transducer adapted to a digital force counter. The animals were acclimated in acrylic boxes with wire grid floors for 10 minutes before the test. A linearly increasing pressure was applied to the central region of the hind paw until a response characterized as a flinch was produced, an indication of hyperalgesia. Three measurements were performed on each animal and the pressure intensity exerted on the paw was automatically recorded and expressed in grams (g).

Thermal hyperalgesia

Animals were placed individually on a hot plate (Insight®, Ribeirão Preto, São Paulo, Brazil) heated to 55 ± 0.5 ºC until they react to the thermal stimulus with the behavior of jumping or lick the paws. This time interval was expressed as thermal threshold.

Measurement of paw/tumor volume

Paw thickness/tumor growth was evaluated using a plethysmometer (Insight®, Ribeirão Preto, São Paulo, Brazil). In this equipment the volume is measured by the displacement of the water column by immersing the hind paw of the animal containing the tumor to the height of the malleolus.

Systemic toxicological evaluation

Animals weight was evaluated daily. On the 12th day, after behavioral analysis, the animals were anesthetized, and their organs (liver, kidneys, heart, lungs, spleen, thymus, and stomach) were macroscopically observed and weighed to determine the relative weight (g/100 g body weight). Blood was collected by cardiac puncture. Hematological analyses were performed using an automated analyzer (Hematoclin 2.8 vet, Bioclin®) and biochemical serum dosages of liver transaminases were performed using commercial kits (Bioclin®).

Micronucleus assay

Following euthanasia of the animals, as described above, both femurs were removed, and bone marrow cells were collected by washing with 1 mL fetal bovine serum in each femur. The collected material was resuspended several times until a homogeneous suspension was obtained. Cell suspension was centrifuged at 1000 rpm for 5 minutes, the supernatant was discarded, and pellet was resuspended in 500 µL of fetal bovine serum and then a small amount was placed on a slide. After drying, the material was fixed with absolute methanol for 5 min and stained with Leishman solution. The slides were examined using an optical microscope at 1000 x magnification to determine the number of micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes in mice marrow [18].

Cytokine measurement

Serum samples from four (n = 4 from each group) randomized mice were used as representative of the group for cytokine and nitric oxide (NO) dosage. Serum samples of sham group, negative control group and animals treated with free β-ocimene (50 mg/kg) were collected for cytokine quantification using the BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 kit (Becton Dickinson Biosciences, USA) for simultaneous detection of interleukins (IL-2, IL-4, IL-6, IL-10 and IL-17A), tumor necrosis factor (TNF-α) and interferon-gamma (IFN-γ). Assays were performed according to the manufacturer's instructions and data were acquired on the BD Accuri C6 cytometer. Seven standard curves of individual cytokines (0-5000 pg/mL) were generated in each assay. The detection range was between 2 and 5000 pg/mL. Results were analyzed using BD Accuri C6 software (BD Biosciences).

Nitric oxide dosage

Nitric oxide production was determined by the nitrite (NO_2^-) concentration present in serum of sham group, negative control group and animals treated with free β-ocimene (50 mg/kg) using the Griees reagent. For this, 200 µL of samples was collected, deproteinized with

a 1% zinc sulfate solution for 15 min and subjected to centrifugation (3000 rpm/15 min), after which the supernatant was separated, and 100 µL was added to each well in a 96-well plate followed by the addition of 100 µl Griess reagent. After incubation for 15 minutes at room temperature, the absorbance of the sample was measured by spectrophotometer with the 500 nm filter. Results were expressed in µM NO_2^- according to the $NaNO_3$ (sodium nitrite) standard curve of known concentrations.

Statistical analysis

Results are expressed means ± S.E.M. or S.D. Differences between experimental groups were compared using Student's t-test and differences were considered statistically significant when $p < 0.05$. Data were analyzed using GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA, EUA).

Results

Preparation of inclusion complexes

It was observed that there was 31.79% of β-ocimene in isomers mixture. For inclusion complex, the total recovery of β-ocimene/β-CD complex was 62.15% and the inclusion ratio of β-ocimene in cyclodextrin cavity was 89.48%.

Characterization of inclusion complex by SEM

Figure 1 shows SEM images obtained for pure cyclodextrin (a), physical mixture (b) and β-ocimene/β-CD inclusion complex (c and d). For cyclodextrin it was possible to observe the presence of structures with rectangular shape, which were also seen in the physical mixture, suggesting that the complexation does not occur by simply mixing of the compounds. In contrast, for the inclusion complex a drastic alteration in the morphology and size of the material were observed, suggesting an interaction between the ocimene and the cyclodextrin and formation of the inclusion complex.

INSERT FIGURE 1

Characterization of inclusion complex by FTIR

Figure 2 shows FTIR spectra of β-ocimene (a), β-CD (b), physical mixture (c) and β-ocimene/β-CD inclusion complex (d), respectively. The FT-IR spectrum of β-CD showed intense absorption bands at 3770 - 3013 cm^{-1} (for O–H stretching vibrations), 3013 - 2800 cm^{-1} (for C-H sp^3 stretching vibrations), 1648 cm^{-1} (for H–O–H bending), 1160 cm^{-1} (for C–O stretching vibration) and 1038 cm^{-1} (C–O–C stretching vibration). The FT-IR spectrum of β-ocimene showed prominent absorption bands of asymmetric stretching vibration of $=CH_2$ (3091 cm^{-1}), of C–H sp^3 stretching (3076 - 2781 cm^{-1}). The very intense peak at 1642 cm^{-1} is attributed to C=C stretching vibration of the allyl group. The double peak at 1451–1379 cm^{-1} may be assigned to the stretching $–CH_2–$ group and strong peaks at 989 and 904 cm^{-1} to $–CH=CH_2$ [19, 20]. The FTIR of physical mixture showed similarity with both isolated compounds spectra while inclusion complex spectra shoed strong differences as disappearance of characteristic peaks from β-ocimene.

INSERT FIGURE 2

Characterization of inclusion complex by NMR

As shown in Table 1, the ^1H NMR analysis of the inclusion complex detected greater shifts in protons H-3 (0.113 ppm) and H-5 (-0.1155 ppm), which are located within the cavity of cyclodextrin. The hydrogens on the outer surface of cyclodextrin (H-1, H-2, H-4 and H-6) showed minimal shifts.

INSERT TABLE 1

Cytotoxic activity on tumor and non-tumor cells

The cytotoxic activity of β-ocimene and β-ocimene/β-CD complex was conducted according to the MTT assay. Table 2 shows the obtained IC_{50} values. β-ocimene presented an IC_{50} value of 1.01 against HL-60 and 3.33 μg/mL against MDA-MB-231 tumor cell line, while β-ocimene/β-CD complex showed no cytotoxicity at the tested concentrations. For the L929 cell line, β-ocimene presented an IC_{50} value of 1.48 μg/mL. Doxorubicin was used as positive control and showed IC_{50} values of 0.01, 0.44 and 1.72 μg/mL for the HL-60, PC-3 and L929 cell lines, respectively.

INSERT TABLE 2

Cytotoxic activity on *Artemia salina*

The results of artemicidal cytotoxic assay demonstrated that exposure to β-CD produced no deaths (LC_{50} >1000 μg/mL). β-ocimene and β-ocimene/β-CD complex treatment showed LC_{50} values of 91.96 ± 36.95 and 123.4 ± 17.36 μg/mL, respectively, which did not present a statistically significant difference.

Hemolysis assay

Like observed in the test with brine shrimp, treatment with β-CD showed no cytotoxic effect at evaluated concentrations (HC_{50} > 500 μg/mL). On the other hand, treatment with β-ocimene and β-ocimene/β-CD showed HC_{50} values of 286.7 ± 2.12 and 134.3 ± 3.61, respectively, indicating a higher hemolytic activity of complexed ocimene when compared to free substance ($p < 0.05$).

Acute oral toxicity test

Treatment with the acute dose of β-ocimene and β-ocimene/β-CD complex (300 mg/kg) did not cause death of the animals and no signs of toxicity in the first 4 hours of observation and 14 days thereafter. There was no significant variation in body weight of treated mice compared to the negative control group (Table 3).

INSERT TABLE 3

No alterations were observed on macroscopic examination of vital organs such as liver, kidneys, heart, spleen, lungs, and stomach. Organ index was calculated and is presented in the Table 4. A statistically significant ($p < 0.05$) decrease in liver index was observed in animals treated with β-ocimene and β-ocimene/β-CD when compared to the negative control group. When the test was conducted with 2000 mg/kg of β-ocimene all animals died within the first 4 hours of observation.

INSERT TABLE 4

Mechanical hyperalgesia

As shown in Figure 3, the inoculation of tumor cells in the animals of negative control group promoted a significant decrease of mechanical stimulus sensitivity threshold from the 6th day when compared with healthy control group (sham group). Morphine showed significant ($p < 0.05$) antinociceptive activity from the 2nd day. On day 6, treatment of animals with free β-

ocimene (25 mg/kg) was able to significantly ($p < 0.05$) reduce the intensity of sarcoma 180-induced hypernociception when compared with negative control group. On the 12th day a significant decrease ($p < 0.05$) of mechanical hyperalgesia was observed in the groups treated with free β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD inclusion complex (50 mg/kg) when compared with negative control group.

INSERT FIGURE 3

Thermal hyperalgesia

In the results of this test (Figure 4), it was observed that treatment with free β-ocimene (25 and 50 mg/kg) reduced thermal nociception only on the 12th day when compared to the negative control group ($p < 0.05$).

The treatment with β-ocimene/β-CD complex (25 and 50 mg/kg) showed a significant increase ($p < 0.05$) in the nociceptive threshold on the 4th day and 12th day when compared to negative control group. In addition, on the 4th day, treatment with β-ocimene/β-CD complex (50 mg/kg) also reduced nociception when compared to free β-ocimene (50 mg/kg) ($p < 0.05$).

INSERT FIGURE 4

Measurement of paw/tumor volume

Paw volume measurements (Figure 5) showed that the inoculation of tumor cells in the negative control group animals promoted a significant increase ($p < 0.05$) of paw volume, starting on the 4th day until the 8th day of observation, when compared to the sham group. The administration of morphine did not promote significant changes in tumor volume. Treatment with free β-ocimene (50 mg/kg) was able to significantly ($p < 0.05$) reduce tumor volume on the 8th and 10th days of observation when compared to negative control group. The results also demonstrated that administration of the inclusion complex (50 mg/kg) promoted a statistically significant ($p < 0.05$) decrease in paw volume on the 6th and 10th days after tumor inoculation.

INSERT FIGURE 5

Systemic toxicological evaluation

No significant changes in body weight were observed in experimental groups ($p > 0.05$, data not shown). Regarding the relative organ weight, there was a statistically significant change ($p < 0.05$) in the liver and lungs of morphine-treated animals, in the heart of morphine and ocimene-treated animals (25 mg/kg) and in the spleen of all experimental groups compared to the healthy control group (sham) (Table 5).

INSERT TABLE 5

Regarding leukocyte evaluation, animals treated with β-ocimene/β-CD (50 mg/kg) showed an increase in the percentage of granulocytes compared to the negative control group (Table 6).

INSERT TABLE 6

Hematological analysis showed a significant decrease ($p < 0.05$) in the number of Red Blood Cells (RBC) and hematocrit in the morphine and β-ocimene (25 mg/kg) treated groups when compared to the sham group. Mean Corpuscular Hemoglobin (MCH) value was decreased ($p < 0.05$) in the morphine-treated group when compared to the sham and negative groups. Treatment with β-ocimene (50 mg/kg) promoted an increase ($p < 0.05$) in MCV value when compared to sham group and an increase ($p < 0.05$) in Red Cell Distribution Width (RDW) value when compared to sham and negative groups (Table 7).

INSERT TABLE 7

Biochemical analysis of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are presented in Table 8. Results showed that treatment with β-ocimene at a dose of 25 mg/kg caused a statistically significant ($p < 0.05$) decrease in ALT value when compared to negative control group. Treatment with β-ocimene at a dose of 50 mg/kg also promoted a significant ($p < 0.05$) decrease in ALT value compared to sham and negative groups. No statistically significant change ($p < 0.05$) in AST value was identified.

INSERT TABLE 8

No statistically significant changes ($p < 0.05$) were observed in the micronucleus assay (data not shown). Results of cytokine and nitric oxide dosage showed that there was a significant increase ($p < 0.05$) in IL-10 serum levels of mice treated with β-ocimene (50 mg/kg) when compared to the negative control group. In addition, a significant ($p < 0.05$) decrease in IL-17 levels was observed in the negative control group when compared to the sham group (Table 9).

INSERT TABLE 9

Discussion

Monoterpenes present several therapeutic properties, including analgesic activity [21]. However, these compounds exhibit extremely low aqueous solubility and high volatility which limit their use [1]. Recently studies have been demonstrated that the pharmacological profile and physicochemical properties of monoterpenes are improved when they are complexed in β-CD [6].

Various well-known methods are used for inclusion complexes formation and among these freeze-drying is the most efficient technique to thermosensitive compounds like the monoterpene β-ocimene [22, 23]. The high inclusion rate (89.48%) can be explained through chemical interactions between host and guest. The torus-shaped of β-CD have an interior cavity suitable for guests with molecular weights between 200 and 800 g/mol and with hydrophobic character [24, 25]. Thus, β-CD can form inclusion complexes with various molecules though the hydrogen bonds, van de Walls force and hydrophobic interactions. In this way, the small molecular weight (136.23 g/mol) and nature hydrophobic of the monoterpene β-ocimene, favor hydrophobic interactions and inclusion complex formation with β-CD [24-26].

Several of analytical techniques can be used to ascertain the formation of β-CD inclusion complexes, such as scanning electron microscopy (SEM), infrared spectroscopy (IR), and nuclear magnetic resonance spectroscopy (NMR). The combination of informations generated by these techniques or others is able to prove whether β-CD complexes were satisfactorily formed [23]. In SEM images of cyclodextrin and physical mixture the presence of structures with rectangular shape, suggesting that the complexation does not occur by simply mixing of the compounds. In contrast, for the inclusion complex a drastic alteration in the morphology and size of the material was observed, suggesting an interaction between the ocimene and the cyclodextrin and formation of the inclusion complex [10, 24]. The obtention of inclusion complex was confirmed still by FTIR and NMR ^{1}H.

FTIR is a useful technique to confirm the formation of inclusion complexes once that modifications or bands disappearances indicate the formation of new materials [10, 24]. The FTIR spectrum of β-ocimene/β-CD inclusion complex was similar to β-CD spectrum, however, it presents marked differences when compared to the ocimene spectrum. In inclusion complex spectrum the disappearance of characteristic peaks from ocimene at 2.731-3.090 cm^{-1} (C-H sp^3 stretching), very intense peak at 1642 cm^{-1} (C=C stretching vibration), of the double peak at 1451–1379 cm^{-1} of -CH_2- group stretching and of the double peak at 989 and 904 cm^{-1} [20], suggest that the ocimene enter in the cavity of β-CD [10, 22, 24].

The NMR technique is one of the most used for the characterization of inclusion complexes. NMR provides direct evidence of the inclusion of a guest molecule inside the CD cavity, which can be identified by comparing the chemical shifts of the free guest molecule and the CD with those of its complex. In general, the 3 (H-3) and 5 (H-5) hydrogens, located in the inner cavity of the cyclodextrin macrocyclic ring, are most affected by complexation, due to the entry and accommodation of the guest molecule in that cavity [27].

In present study, the shifts in H-3 and H-5 allow us once again to suggest that the β-ocimene molecule lies within the cyclodextrin cavity. The data allow suggesting still that the methyl groups of isopropyl portion are near an H-5, causing a protector effect and shifts in its protons for protected region of spectrum. In addition, double bonds as presents in allyl group have electronegativity able of attracting the electronic cloud of nearby nuclei causing unprotection and increased chemical shifts explain thus, the positive shift observed to H-3 [19, 20].

This study showed that free ocimene present significant cytotoxic activity against tumor cell lines. According to the literature, pure substances with $IC_{50} < 4$ µg/mL may be considered promising [28]. Moreover, free β-ocimene was also cytotoxic to non-tumor cells suggesting low selectivity. This monoterpene is among the major chemical constituents of essential oils of plant species such as *Annona vepretorum* Mart. (Annonaceae) and may be contributing to its pharmacological activities [29-31]. β -ocimene/β-CD complex showed no cytotoxicity at the tested concentrations, which can be explained by the decrease in reactivity due to the stabilization of the host molecule in the cyclodextrin cavity [32].

Results of *Artemia salina* lethality test demonstrated that treatment with pure β-CD did not induce deaths in the maximum concentration tested. According to Meyer and collaborators samples with LC_{50} values above 1000 µg/mL, as β-CD, are considered non-toxic [11]. On the other hand, β-ocimene and inclusion complex showed toxicity on *Artemia salina* with no statistically significant difference between these two samples.

In contrast to low cytotoxicity against tumor and non-tumor cell lines, β-ocimene/β-CD inclusion complex demonstrated a significant hemolytic activity. Natural products or drugs can cause hemolysis by a variety of mechanisms, from dissolving or increasing the permeability of cell membranes to complete cell lysis. The methods to determine the hemolytic activity evaluate the damage caused by the sample to the erythrocyte membrane, which when lysed release hemoglobin in the medium [33].

Regarding acute toxicity in mice, the current study revealed there was no mortality and signs of toxicity observed in animals treated with β-ocimene and β-ocimene/β-CD at the dose of 300 mg/kg. Following this first step, the OECD 423 Guidelines recommends the exposure of three more animals to the dose of 2000 mg/kg; nevertheless, this dose was administered only for free β-ocimene and caused the death of all animals. Thus, the oral LD_{50} was estimated to be greater than 300 mg/kg in mice (category 4) [14].

The analgesic activity of monoterpenes complexed in β-CD has been demonstrated in several studies reported in literature, in which the complexation not only maintains or improves the pharmacological profile of this chemical compounds, but also increases the solubility, improves stability, increases bioavailability, and decreases toxicity [16, 34-40]. In this paper, β-ocimene and β-ocimene/β-CD complex significantly reduced nociception induced by Sarcoma180 on the 4^{th}, 6^{th} day or 12^{th} days. In addition, the treatment promoted a decrease in tumor volume until the 10th day. It can be suggested that the formulation with β-cyclodextrin is beneficial and can increase the pharmacological potency of the ocimene, since the evaluated dose of the complex had less ocimene (rate of inclusion of ocimene in cyclodextrin was around 89%) obtaining still activity comparable or superior to the free ocimene and reducing the toxicity of this molecule. Moreover, in some days of observation, the free β-ocimene and/or the inclusion complex showed analgesic efficacy like morphine.

The mechanism of action of these natural products may be related to antioxidant and anti-inflammatory activity, reducing the nociceptive threshold by reducing NO, modulating cytokines and arachidonic acid derivatives through cyclooxygenase (COX) inhibition. As cancer pain has a direct relationship with the inflammatory process, natural products with this activity promote reduction of painful entries in the central pain pathways and contribute to cancer pain control [16, 21, 41]. Our study did not show any change in the NO dosage but suggested that ocimene may modulate the release of anti-inflammatory cytokines, such as IL-10. However, further studies should be carried out to investigate the mechanism of action of antinociceptive activity of this monoterpene in experimental models of pain in cancer.

When evaluating systemic toxicity after treatment, it was observed that the spleen of the animals of all experimental groups had significantly higher relative weight when compared to the healthy group. This may occur because of the presence of the tumor that induces a leukemoid reaction, characterized by increased peripheral blood granulocyte count and splenomegaly [42].

The analysis of the erythrogram indicated a significant reduction ($p < 0.05$) in the number of red blood cells and hematocrit in the group treated with free β-ocimene (25 mg/ kg), indicating a correlation with the hemolytic activity as presented in this study. Moreover, in malignant neoplasias occur abnormalities in blood cells and/or in its content, which are due to treatment and/or tumor presence. These abnormalities are responsible for the development of characteristic clinical manifestations of anemia, which are often exacerbated by treatment [43, 44]. No changes in the erythrogram were observed in the groups treated with the β-ocimene/β-CD inclusion complex, suggesting a reduction in the toxicity profile of the β-ocimene when complexed. Biochemical analysis of serum aminotransferases (AST and ALT) does not suggest alterations in hepatic function.

Conclusion

The present study indicates that the inclusion complex between β-ocimene and β-CD was efficiently obtained. Free β-ocimene demonstrated cytotoxic activity in tumor and non-tumor cells while the complex was not cytotoxic at evaluated concentrations. In addition, β-ocimene and β-ocimene/β-CD demonstrated cytotoxic activity against *Artemia salina* and mice erythrocytes. The acute toxicity test estimated an oral LD_{50} greater than 300 mg/kg. Regarding analgesic activity, β-ocimene/β-CD complex reduced the nociception induced by sarcoma 180 in some days of treatment and reduced the tumor volume when compared with negative or free β-ocimene groups.

Thus, complexation with β-CD may be an alternative for the pharmacological application of this monoterpene, as it preserves its therapeutic activity, reduce toxicity, and minimizes physico-chemical inconveniences.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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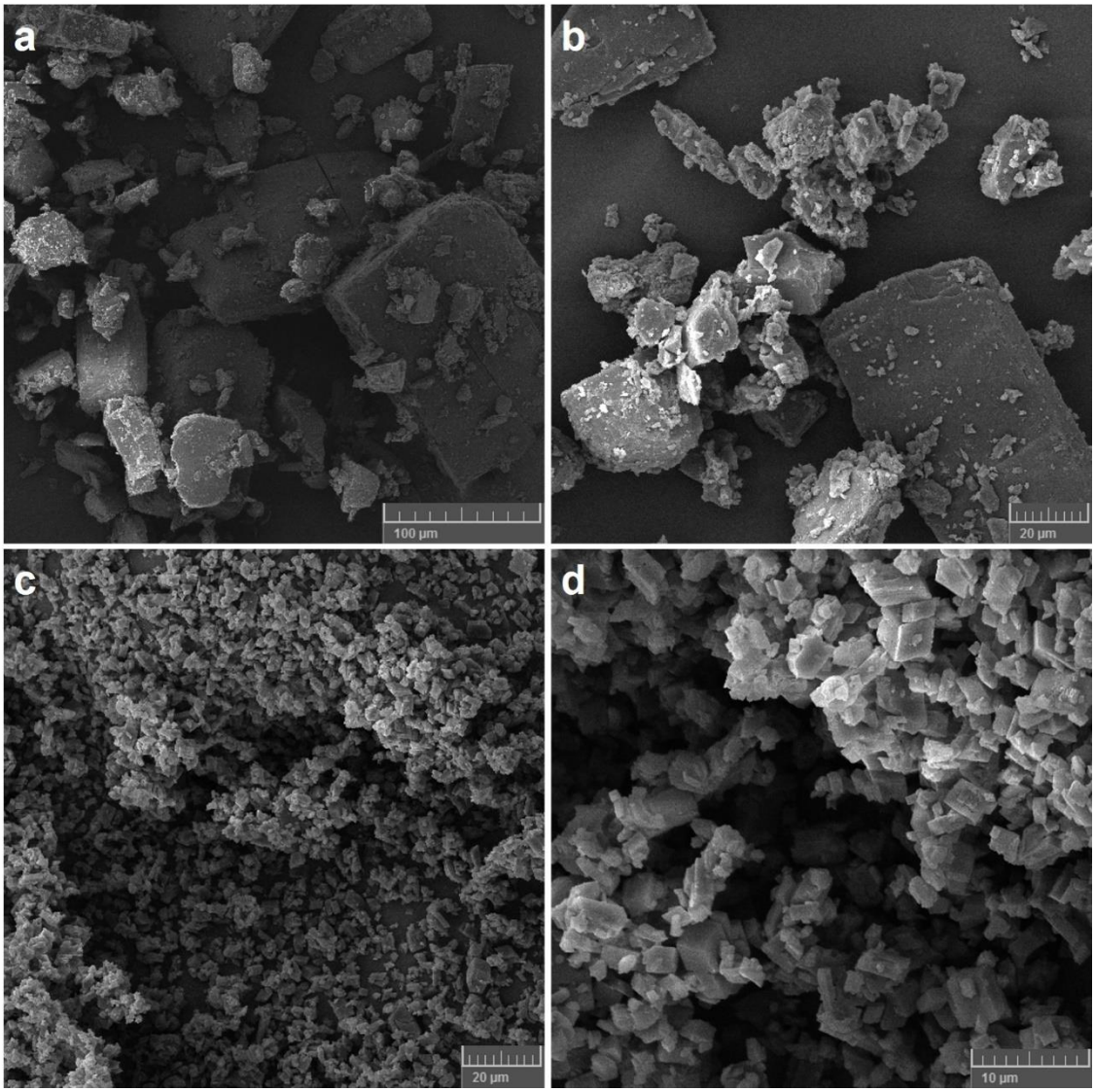

Figure 1. Scanning electron microscopy (SEM) photographs of different materials. a: β-CD (x 400); b: Physical mixture (PM) (x 1000); c: β-ocimene/β-CD inclusion complex (x 1000); d: β-ocimene/β-CD inclusion complex (x 3000).

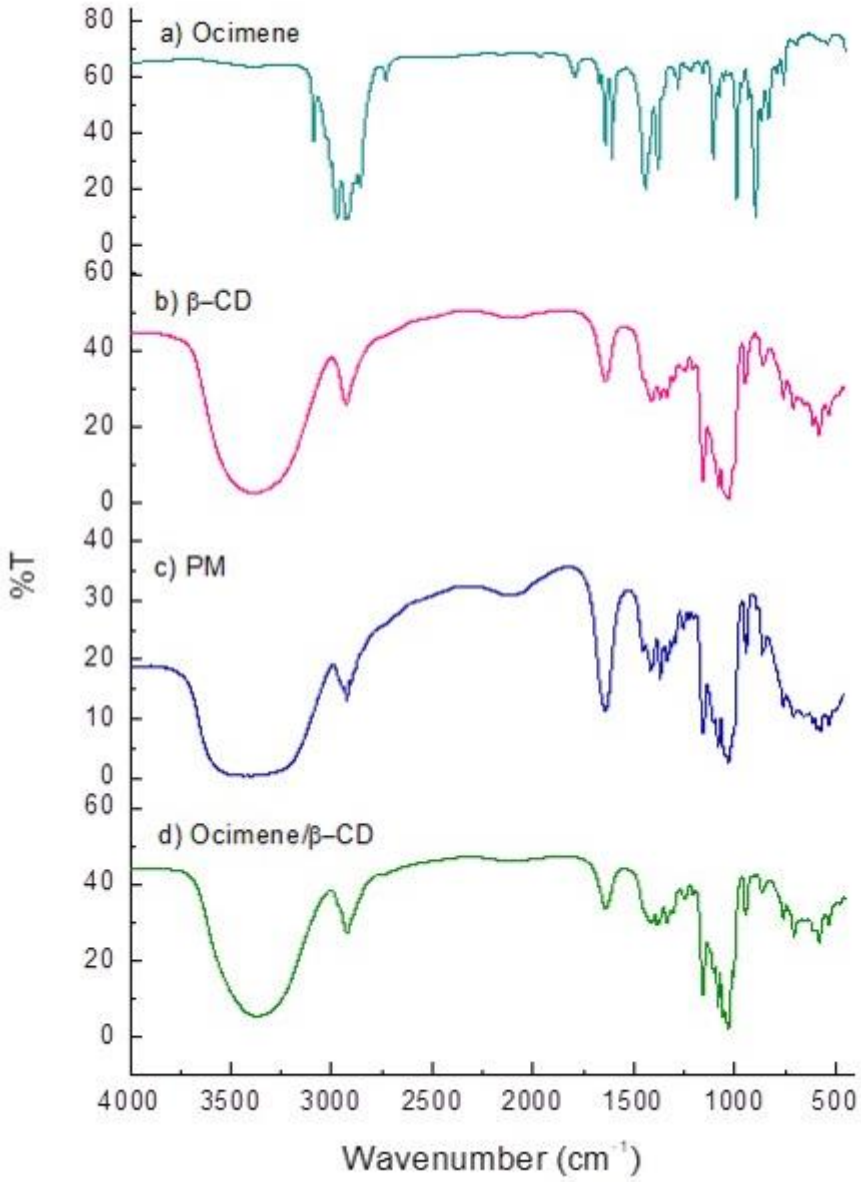

Figure 2. Fourier transform–infrared (FT-IR) spectra of different materials. a: β-ocimene; b: β-cyclodextrin; c: Physical mixture (PM); d: β-ocimene/β-CD inclusion complex.

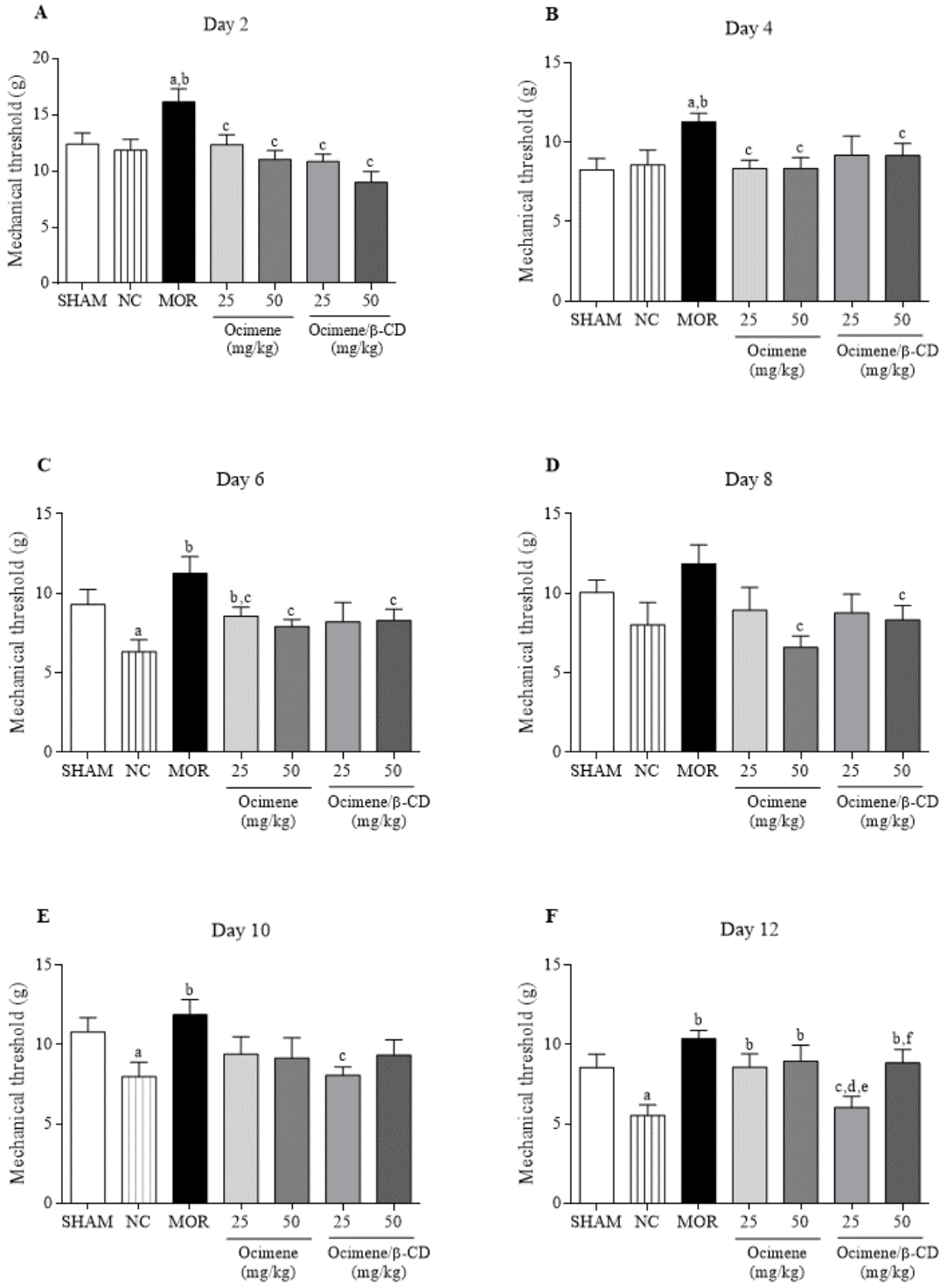

Figure 3. Effect of β-ocimene and β-ocimene/β-CD inclusion complex on the mechanical hyperalgesia induced by sarcoma 180 in mice. The animals were treated with vehicle (sham group and negative control group), β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD complex (25 and 50 mg/kg) from the 2nd to 12th days following tumor inoculation and evaluated for mechanical hyperalgesia on alternate days. n = 8. $^{a}p < 0.05$ compared to sham group, $^{b}p < 0.05$ compared to negative control group, $^{c}p < 0.05$ compared to morphine treated group, $^{d}p < 0.05$ compared to free β-ocimene (25 mg/kg) treated group, $^{e}p < 0.05$ compared to free β-ocimene (50 mg/kg) treated group and $^{f}p < 0.05$ compared to β-ocimene/β-CD (25 mg/kg) treated group by Student's t test. NC: Negative Control. MOR: Morphine.

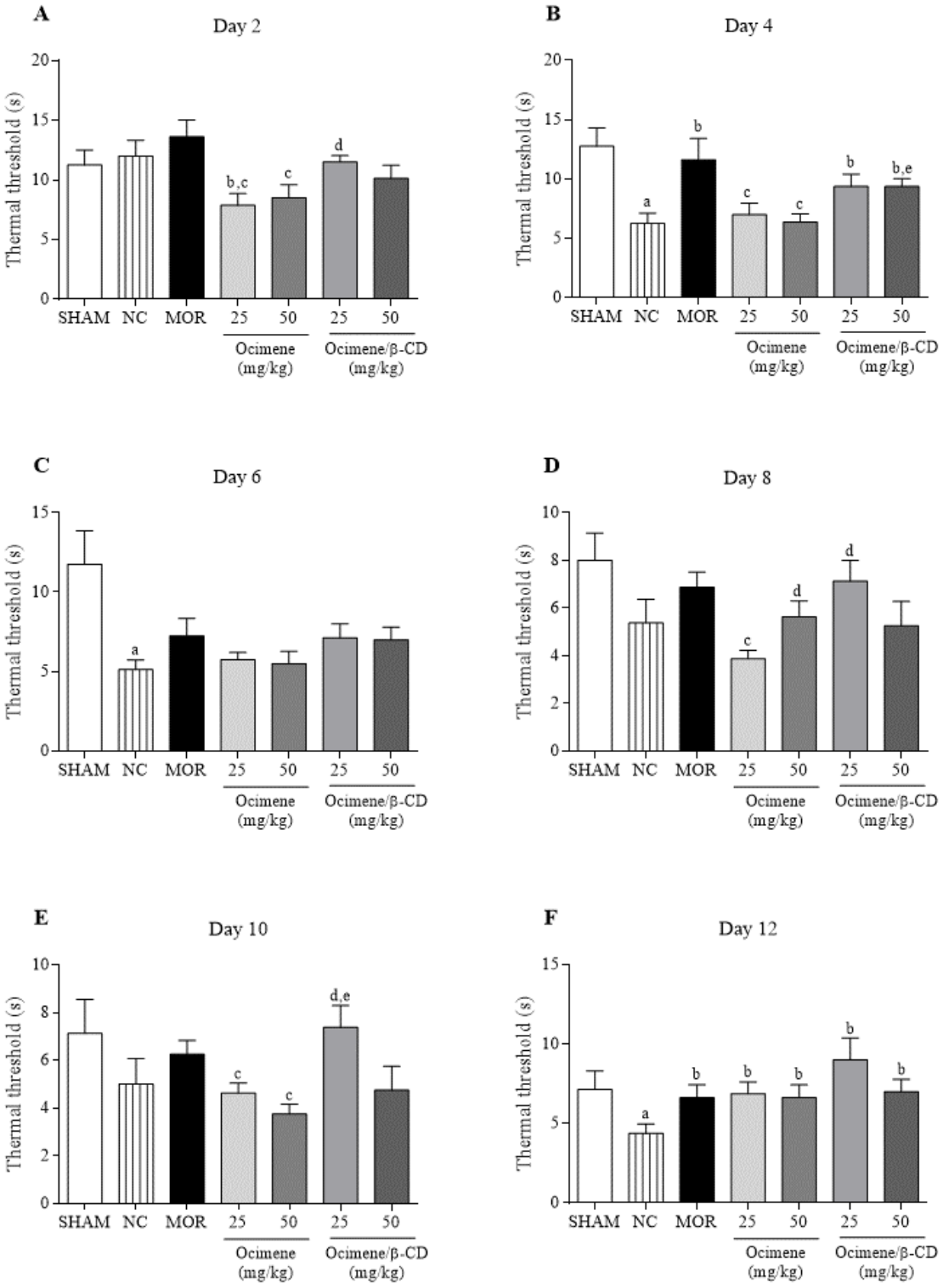

Figure 4. Effect of β-ocimene and β-ocimene/β-CD inclusion complex on the thermal hyperalgesia induced by sarcoma 180 in mice. The animals were treated with vehicle (sham group and negative control group), β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD complex (25 and 50 mg/kg) from the 2nd to 12th days following tumor inoculation and evaluated for thermal hyperalgesia on alternate days. n = 8. [a]$p < 0.05$ compared to sham group, [b]$p < 0.05$ compared to negative control group, [c]$p < 0.05$ compared to morphine treated group, [d]$p < 0.05$ compared to free β-ocimene (25 mg/kg) treated group and [e]$p < 0.05$ compared to free β-ocimene (50 mg/kg) treated group by Student's t test. NC: Negative Control. MOR: Morphine.

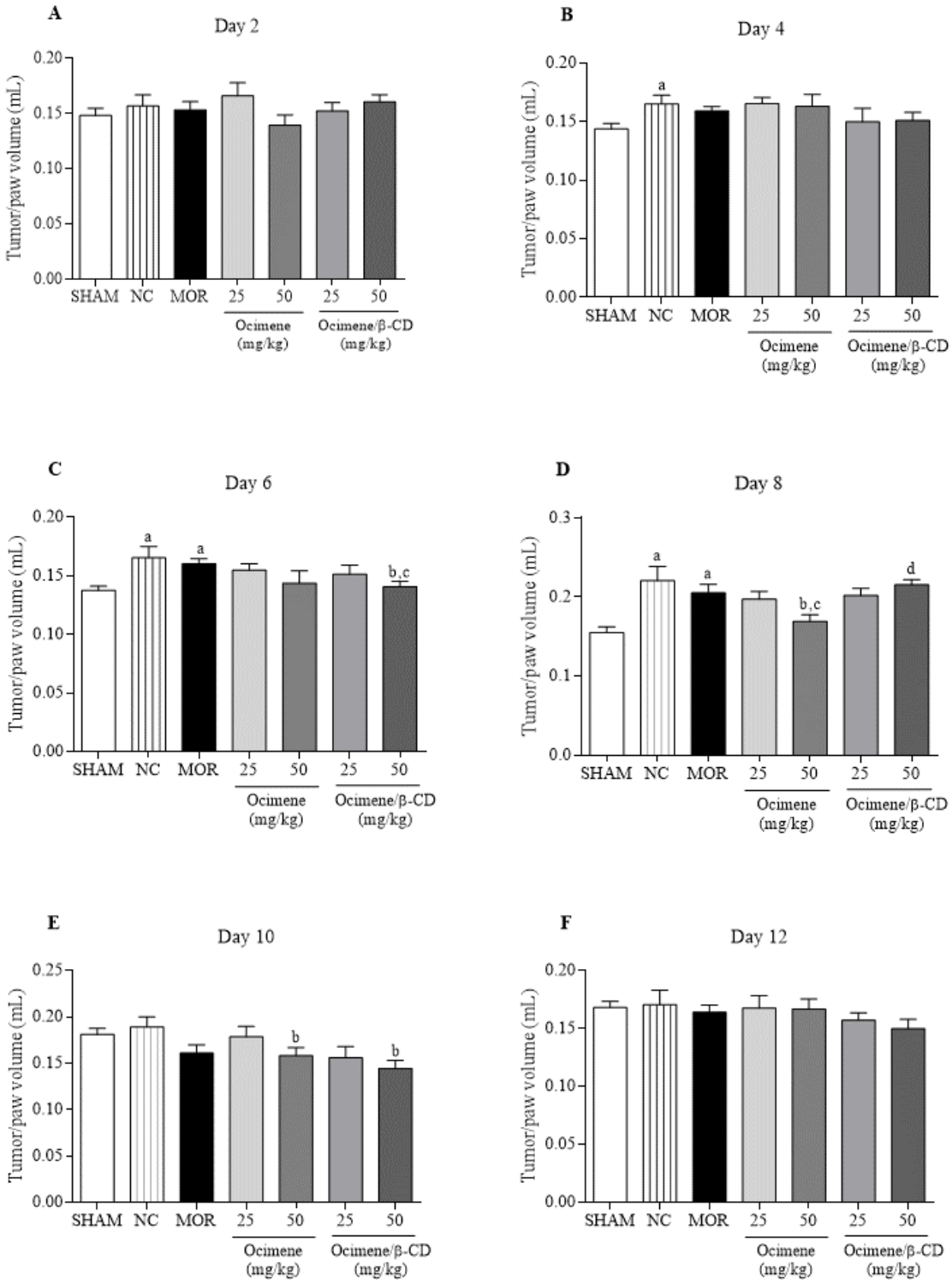

Figure 5. Effect of β-ocimene and β-ocimene/β-CD inclusion complex on paw/tumor volume of sarcoma 180-bearing mice. The animals were treated with vehicle (sham group and negative control group), β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD complex (25 and 50 mg/kg) from the 2nd to 12th days following tumor inoculation and evaluated for paw/tumor volume on alternate days. n = 8. [a]$p < 0.05$ compared to sham group, [b]$p < 0.05$ compared to negative control group, [c]$p < 0.05$ compared to morphine treated group and [d]$p < 0.05$ compared to free β-ocimene (50 mg/kg) treated group by Student's t test. NC: Negative Control. MOR: Morphine.

Table 1. Chemical shifts for the protons of β-CD and β-ocimene/β-CD complex obtained by ^{1}H NMR analysis.

	$\delta_{\beta\text{-CD}}$	$\delta_{\text{Ocimene}/\beta\text{-CD}}$	$\Delta\delta$
H-1	4.9726	4.9779	0.0053
H-2	3.4900	3.4956	0.0056
H-3	3.7621	3.8751	0.113
H-4	3.5500	3.5555	0.0055
H-5	3.8733	3.7578	-0.1155
H-6	3.7834	3.7843	0.0009

Values are expressed in ppm. $\Delta\delta = \delta_{\text{Ocimene}/\beta\text{-CD}} - \delta_{\beta\text{-CD}}$

Table 2. *In vitro* cytotoxic activity of β-ocimene, β-ocimene/β-CD complex and doxorubicin against human tumor cell lines (HL-60, PC-3 and MDA-MB-231) and murine non-tumor cell line (L929).

	IC_{50} µg/mL (Interval)*			
	HL-60	**PC-3**	**MDA-MB-231**	**L929**
Ocimene	1.01 (0.94 – 1.10)	>5	3.33 (3.12 – 3.56)	1.48 (1.20 – 1.81)
Ocimene/β-CD	>5	>5	>5	>5
Doxorubicin	0.01 (0.005-0.01)	0.44 (0.34-0.54)	-	1.72 (1.58-1.87)

* Half-maximal inhibitory concentration (IC_{50}) values with a 95% confidence interval obtained by nonlinear regression from two independent experiments, measured using MTT assay after 72-hr incubation. Doxorubicin was used as a positive control. HL-60: human promyelocytic leukaemia, PC-3: human prostate cancer, MDA-MB-231: human breast cancer, L929: Mouse fibroblasts.

Table 3. Effect of acute treatment with free β-ocimene and β-ocimene/β-CD (300 mg/kg, p.o.) on body weight gain of mice after 14 days of observation. Body weight gain was calculated using the mean difference between final and initial weight.

Group	Body weight gain (g)
Negative control	3.00 ± 0.57
Ocimene	3.66 ± 0.88
Ocimene/β-CD	1.33 ± 0.88

Values are expressed as mean ± S.E.M. n = 3.

Table 4. Effects of ocimene and ocimene/β-CD (300 mg/kg, p.o.) treatment on organ indices (g/100g body weight).

Group	Liver	Kidneys	Heart	Spleen	Lungs	Stomach
Negative control	5.68 ± 0.03	1.24 ± 0.10	0.50 ± 0.02	0.44 ± 0.04	0.64 ± 0.02	0.82 ± 0.06
Ocimene	5.09 ± 0.09*	1.20 ± 0.03	0.46 ± 0.01	0.41 ± 0.02	0.62 ± 0.02	0.94 ± 0.07
Ocimene/β-CD	5.01 ± 0.11*	1.21 ± 0.03	0.55 ± 0.02	0.50 ± 0.06	0.60 ± 0.03	0.96 ± 0.09

Values are expressed as mean ± S.E.M. n = 3. *$p < 0,05$ when compared with the negative control group by Student's t test.

Table 5. Effect of free β-ocimene and β-ocimene/β-CD complex on mice organ index (g/100g body weight). The animals were treated with vehicle (sham group and negative control group), β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD complex (25 and 50 mg/kg) from the 2nd to 12th days following tumor inoculation.

Group	Liver	Kidneys	Heart	Spleen	Lungs	Stomach	Thymus
Sham	5.12 ± 0.18	1.10 ± 0.04	0.50 ± 0.03	0.44 ± 0.02	0.63 ± 0.01	1.64 ± 0.08	0.25 ± 0.03
Negative control	5.45 ± 0.15	1.14 ± 0.02	0.43 ± 0.02	0.65 ± 0.01[a]	0.63 ± 0.03	1.62 ± 0.09	0.31 ± 0.02
Morphine	5.78 ± 0.14[a]	1.15 ± 0.03	0.41 ± 0.00[a]	0.74 ± 0.03[a]	0.70 ± 0.02[a]	1.54 ± 0.10	0.31 ± 0.03
Ocimene (25 mg/kg)	5.29 ± 0.11	1.17 ± 0.04	0.41 ± 0.01[a]	0.64 ± 0.03[a]	0.66 ± 0.02	1.61 ± 0.18	0.30 ± 0.03
Ocimene (50 mg/kg)	5.32 ± 0.20	1.12 ± 0.04	0.45 ± 0.00	0.64 ± 0.01[a]	0.67 ± 0.03	1.54 ± 0.08	0.32 ± 0.04
Ocimene/β-CD (25 mg/kg)	4.85 ± 0.12[b]	1.07 ± 0.03	0.45 ± 0.01	0.60 ± 0.02[a]	0.65 ± 0.03	1.57 ± 0.10	0.22 ± 0.02
Ocimene/β-CD (50 mg/kg)	5.13 ± 0.17	1.14 ± 0.05	0.43 ± 0.01	0.83 ± 0.08[a]	0.69 ± 0.02	1.56 ± 0.09	0.39 ± 0.07

Values are expressed as mean ± S.E.M. n = 8. [a]$p < 0.05$ when compared with the sham group and [b]$p < 0.05$ when compared with the negative control group by Student's t test.

Table 6. Effect of free β-ocimene and β-ocimene/β-CD complex on mice hematological parameters (total leukocytes and differential count of leukocytes). The animals were treated with vehicle (sham group and negative control group), β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD complex (25 and 50 mg/kg) from the 2nd to 12th days following tumor inoculation.

Group	Total leukocytes (10^3 mm^{-3})	Differential count of leukocytes (%)		
		Lymphocytes	Monocytes	Granulocytes
Sham	10.18 ± 1.08	84.45 ± 1.24	2.36 ± 0.14	13.19 ± 1.10
Negative control	10.28 ± 1.16	84.08 ± 0.91	2.65 ± 0.15	13.28 ± 0.77
Morphine	7.77 ± 1.01	74.49 ± 6.51	4.95 ± 1.73	20.56 ± 4.79
Ocimene (25 mg/kg)	7.63 ± 0.82	84.69 ± 0.43	2.63 ± 0.10	12.68 ± 0.35
Ocimene (50 mg/kg)	9.27 ± 1.53	79.48 ± 6.29	3.12 ± 0.75	17.40 ± 5.54
Ocimene/β-CD (25 mg/kg)	9.90 ± 1.70	85.46 ± 0.49	2.38 ± 0.18	12.16 ± 0.33
Ocimene/β-CD (50 mg/kg)	8.00 ± 0.84	79.75 ± 2.02	2.88 ± 0.21	17.37 ± 1.82[b]

Values are expressed as mean ± S.E.M. n = 8. [b]$p < 0.05$ when compared with the negative control group by Student's t test.

Table 7. Effect of free β-ocimene and β-ocimene/β-CD complex on mice hematological parameters (RBC, hemoglobin, hematocrit, MCV, MCH, MCHC and RDW). The animals were treated with vehicle (sham group and negative control group), β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD complex (25 and 50 mg/kg) from the 2nd to 12th days following tumor inoculation.

Group	RBC (10^6 mm^{-3})	Hemoglobin (g/dL)	Hematocrit (%)	MCV (fm^3)	MCH (pg)	MCHC (g/dL)	RDW (%)
Sham	9.38 ± 0.32	16.16 ± 0.62	45.64 ± 1.64	48.66 ± 0.18	21.09 ± 4.01	35.38 ± 0.55	13.24 ± 0.25
Negative control	8.95 ± 0.50	15.85 ± 0.96	43.94 ± 2.70	49.08 ± 0.36	21.59 ± 3.97	36.04 ± 0.18	13.71 ± 0.33
Morphine	8.55 ± 0.15[a]	14.80 ± 0.26	40.86 ± 0.76[a]	47.81 ± 0.19[a,b]	17.26 ± 0.09	36.19 ± 0.09	13.99 ± 0.26
Ocimene (25 mg/kg)	8.47 ± 0.13[a]	14.93 ± 0.24	41.21 ± 0.57[a]	48.71 ± 0.32	17.55 ± 0.12	36.16 ± 0.11	13.83 ± 0.16
Ocimene (50 mg/kg)	8.33 ± 0.67	14.98 ± 1.25	41.40 ± 3.39	49.70 ± 0.38[a]	17.86 ± 0.17	36.06 ± 0.16	14.75 ± 0.18[a,b]
Ocimene/β-CD (25 mg/kg)	8.77 ± 0.14	15.39 ± 0.31	42.41 ± 0.87	48.40 ± 0.43	17.49 ± 0.15	34.81 ± 1.50	13.40 ± 0.23
Ocimene/β-CD (50 mg/kg)	8.33 ± 0.43	14.67 ± 0.74	41.00 ± 2.16	49.25 ± 0.59	17.55 ± 0.22	35.75 ± 0.26	14.78 ± 0.48

Values are expressed as mean ± S.E.M. n = 8. [a]$p < 0.05$ when compared with the sham group and [b]$p < 0.05$ when compared with the negative control group by Student's t test.

Table 8. Effect of free β-ocimene and β-ocimene/β-CD complex on mice biochemical parameters. The animals were treated with vehicle (sham group and negative control group), β-ocimene (25 and 50 mg/kg) and β-ocimene/β-CD complex (25 and 50 mg/kg) from the 2nd to 12th days following tumor inoculation.

Group	ALT	AST
Sham	21.28 ± 3.95	72.78 ± 8.15
Negative control	34.33 ± 8.69	79.16 ± 11.09
Morphine	11.67 ± 6.02	76.41 ± 12.72
Ocimene (25 mg/kg)	12.78 ± 2.09b	84.96 ± 21.32
Ocimene (50 mg/kg)	11.78 ± 1.38[a,b]	75.83 ± 10.15
Ocimene/β-CD (25 mg/kg)	18.94 ± 3.86	83.29 ± 14.86
Ocimene/β-CD (50 mg/kg)	15.89 ± 4.40	79.52 ± 6.87

Values are expressed as mean ± S.E.M. n = 8. [a]$p < 0.05$ when compared with the sham group and [b]$p < 0.05$ when compared with the negative control group by Student's t test.

Table 9. Effect of free β-ocimene on cytokine and nitric oxide dosage. The animals were treated with vehicle (sham group and negative control group) and β-ocimene (50 mg/kg) from the 2nd to 12th days following tumor inoculation.

Group	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-17A (pg/ml)	IFN-γ (pg/ml)	TNF-α (pg/ml)	Nitric oxide (µM)
Sham	7.87 ± 0.17	9.52 ± 0.26	6.83 ± 0.36	6.73 ± 0.43	8.63 ± 0.20	6.58 ± 0.55	9.60 ± 1.19	0.02 ± 0.00
Negative control	9.35 ± 1.89	10.39 ± 1.17	6.66 ± 0.27	5.69 ± 0.25	8.17 ± 0.01	6.57 ± 0.58	8.14 ± 0.37	0.01 ± 0.00
Ocimene	7.62 ± 0.12	11.57 ± 1.64	8.41 ± 1.20	15.98 ± 4.04[a]	10.00 ± 2.03	7.16 ± 0.60	11.29 ± 2.01	0.02 ± 0.00

Values are expressed as mean ± S.E.M. n = 4. [b]$p < 0.05$ when compared with the negative control group by Student's t test.

6 CONCLUSÃO GERAL

A partir desse estudo foi possível evidenciar que a variedade estrutural dos monoterpenos permite uma diversidade de atividades biológicas, dentre elas o potencial analgésico demonstrado em diferentes modelos animais de dor. Os resultados demonstraram ainda que as características físico-químicas e as atividades farmacológicas desses metabólitos secundários são melhoradas quando aplicado um sistema de liberação de fármacos. A maioria dos trabalhos encontrados na literatura com o objetivo de melhorar a absorção oral de monoterpenos avaliou a complexação com β-ciclodextrina e sugeriu que eles promovem analgesia principalmente por meio de mecanismos de ação central. Além da via oral, os monoterpenos demonstraram aplicação em sistemas de liberação transdérmica de fármacos, como promotores de permeação ou atuando como principal ativo para o controle da dor. Também foi relatado que esses compostos podem ser utilizados como adjuvantes para aumentar a absorção oral de medicamentos já utilizados na prática clínica.

Tendo em vista o potencial farmacológico dos monoterpenos e os resultados promissores da avaliação antinociceptiva do Av-OE em modelo experimental de dor oncológica, realizou-se o estudo do β-ocimeno e o desenvolvimento do seu complexo de inclusão com β-CD por meio do método de liofilização. Os resultados sugeriram que a complexação aconteceu, conforme observado pelos resultados do RMN, FTIR e MEV, e elevada taxa de complexação.

Por meio da realização dos protocolos experimentais, pode-se verificar que o ocimeno livre e o complexo ocimeno/β-CD (25 e 50 mg/kg) foram capazes de reduzir a nocicepção e o volume/edema tumoral provocados pela inoculação das células do sarcoma 180 em camundongos. Este efeito, pelo menos em parte, pode estar relacionado com a modulação de mediadores químicos da inflamação, como a citocina anti-inflamatória IL-10. Vale ressaltar que o complexo ocimeno/β-CD apresentou menor toxicidade durante o tratamento quando comparado ao ocimeno na forma livre.

Dessa forma, o presente estudo leva a sugerir que o óleo essencial das folhas de *A. vepretorum* apresenta atividade antinociceptiva diante do modelo experimental de dor oncológica induzida pelo sarcoma 180 e que o (*E*)-β-ocimeno pode estar contribuindo para a essa atividade. Além disso, a complexação com β-CD pode melhorar o perfil farmacológico e toxicológico desse monoterpeno.

7 PERSPECTIVAS

A partir das informações reunidas nesse trabalho espera-se que o óleo essencial das folhas de *A. vepretorum* e o ocimeno apresentem potencial para o desenvolvimento de novas alternativas terapêuticas para o manejo da dor oncológica. Para isso, são necessários ainda estudos mais aprofundados sobre o seu mecanismo de ação e os sistemas biológicos envolvidos com a atividade antinociceptiva. Estudos farmacocinéticos também precisam ser incluídos nessa fase de estudos pré-clínicos.

Os dados de triagem toxicológica do ocimeno realizada nesse estudo sugerem que esse monoterpeno apresenta DL_{50} maior do que 300 mg/kg, entretanto são necessários dados de exposição mais prolongada a essa substância, como a realização de testes de toxicidade subcrônica e crônica.

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ANEXO A - Declaração da comissão de ética no uso de animais (CEUA) da UNIVASF

MINISTÉRIO DA EDUCAÇÃO
MINISTÉRIO DE CIÊNCIA, TECNOLOGIA E INOVAÇÃO
UNIVERSIDADE FEDERAL DO VALE DO SÃO FRANCISCO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de autorização

Certificamos que a proposta intitulada: "**EFEITO DO COMPLEXO DE INCLUSÃO CONTENDO O ÓLEO ESSENCIAL DAS FOLHAS DE *Annona vepretorum* MART. (ANNONACEAE) EM β-CICLODEXTRINA E DE EXTRATOS OBTIDOS DAS SEMENTES EM MODELO EXPERIMENTAL DE DOR ONCOLÓGICA**", registrada com o nº **0003/241017**, sob a responsabilidade de **Jackson Roberto Guedes da Silva Almeida** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Federal do Vale do São Francisco - UNIVASF, em 24/10/2017.

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	01/11/2017 a 31/11/2020
Espécie/linhagem/raça	*Mus musculus*
Nº de animais	212
Peso/Idade	30-40g / 8 a 10 semanas
Sexo	M 25; F 187
Origem	Biotério da UNIVASF - Campus Ciências Agrárias

Em: 25/10/2017

Dra. Andréa Vieira Colombo
Coordenadora da Comissão de Ética da UNIVASF

ANEXO B – Comprovante de submissão do artigo 1

Biomedicine & Pharmacotherapy

Chemistry and delivery systems applied to monoterpenes with analgesic potential: An overview

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Natural products; essential oil; monoterpenes; chemistry; drug delivery; pain
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Manuscript Region of Origin:	South America
Abstract:	Monoterpenes are compounds belonging to the class of secondary metabolites known as terpenoids that are present in essential oils obtained from medicinal and aromatic plants. They have aroused much interest due to the several biological activities that they present, including analgesic activity. However, these compounds exhibit extremely low aqueous solubility which impair their oral absorption. This fact encouraged several investigations about delivery systems that could improve the therapeutic properties of monoterpenes and potentialize its clinical application. Thus, this paper summarizes the main preclinical pain assessment models, chemistry of monoterpenes and main drug delivery systems applied to monoterpenes with analgesic potential by extensively literature search on several scientific databases and Google. Results of the investigation shown that the structural variety of the monoterpenes permits a diversity of biological activities, among them analgesic potential demonstrated in diverse animal models of pain, such as: abdominal writhing, formalin test, hot plate test, randall-selitto's test, von frey test, tail-flick test, orofacial pain, chronic muscle pain and cancer pain models. In general, the pharmacological activity of these secondary metabolites is increased when applied a delivery system. Most of the studies to improve oral absorption of monoterpenes evaluated the complexation with β-cyclodextrin and demonstrated the involvement of central pathways in their action mechanisms, such as opioidergic, serotoninergic and GABAergic system, vanilloid receptor type 1 (TRPV1) modulation and descending-pain inhibitory mechanisms. It has also been reported that monoterpenes can be used as adjuvant to increase oral absorption of drugs already used in clinical practice, such as ibuprofen. In addition to the oral route, these chemical constituents demonstrated application in transdermal delivery system as permeation enhancer or active ingredient for pain management. However, in despite the variety of preclinical studies found in the literature, it was also observed a scarcity of pharmacokinetic data, which could difficult the translation of these studies to justify later clinical trials. In this way, monoterpenes demonstrate remarkable analgesic properties and applicability in drug delivery systems, which improves their physicochemical and pharmacological characteristics, and encourages further investigation.

ANEXO C – Comprovante de submissão do artigo 2

Zimbra **jackson.guedes@univasf.edu.br**

[BJB] Agradecimento pela submissão

De : Rogério Pessa <noreply.ojs2@scielo.org> qui, 28 de jan de 2021 13:25
Assunto : [BJB] Agradecimento pela submissão
Para : Prof. Jackson Roberto Guedes da Silva Almeida <jackson.guedes@univasf.edu.br>

Prof. Jackson Roberto Guedes da Silva Almeida:

Obrigado por submeter o manuscrito, "Evaluation of the antinociceptive activity of Annona vepretorum Mart. (Annonaceae) essential oil in sarcoma 180-bearing mice: Antinociceptive activity of Annona vepretorum" ao periódico Brazilian Journal of Biology. Com o sistema de gerenciamento de periódicos on-line que estamos usando, você poderá acompanhar seu progresso através do processo editorial efetuando login no site do periódico:

URL da Submissão:
https://submission.scielo.br/index.php/bjb/authorDashboard/submission/248081
Usuário: jackson_guedes

Se você tiver alguma dúvida, entre em contato conosco. Agradecemos por considerar este periódico para publicar o seu trabalho.

Rogério Pessa

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ANEXO D – Comprovante de submissão do artigo 3

Pharmacology Research & Perspectives

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Submission Confirmation

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Pharmacology Research & Perspectives

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Title
Preparation, characterization and evaluation of toxicity and analgesic activity of inclusion complex of β-ocimene with β-cyclodextrin and free β-ocimene in an experimental mice cancer pain model

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