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POLLYANA DE SOUZA SIQUEIRA LIMA

EFEITO ANTI-HIPERALGÉSICO DO ÓLEO ESSENCIAL DE *LIPPIA GRATA* LIVRE E COMPLEXADO EM β-CICLODEXTRINA EM MODELOS ANIMAIS DE DOR CRÔNICA NÃO INFLAMATÓRIA E DOR NEUROPÁTICA

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Estadual de Feira de Santana como requisito parcial à obtenção do grau de Doutor em Biotecnologia.

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Aos que agradeço, também dedico

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os velozes nem sempre vencem a corrida;
os fortes nem sempre triunfam na guerra;
os sábios nem sempre têm comida;
os prudentes nem sempre são ricos;
os instruídos nem sempre tem prestígio;
pois o tempo e o acaso afetam a todos.
(Eclesiastes 9:11)

RESUMO

EFEITO ANTI-HIPERALGÉSICO DO ÓLEO ESSENCIAL DE *LIPPIA GRATA* LIVRE E COMPLEXADO EM β-CICLODEXTRINA EM MODELOS ANIMAIS DE DOR CRÔNICA NÃO INFLAMATÓRIA E DOR NEUROPÁTICA**.** Siqueira-Lima, de Souza Pollyana, Universidade Estadual de Feira de Santana, Feira de Santana-BA, 2018.

As dores crônicas associadas a estados não inflamatórios e neuropáticos são prevalentes e debilitantes permanecendo ainda sem um tratamento eficiente e seguro. Para tanto, este estudo foi delineado com o intuito de investigar através de ensaios funcionais e moleculares, o efeito anti-hiperalgésico do óleo essencial de *Lippia grata* livre e complexada em β-ciclodextrina (OEL/β-CD) em modelos animais de dor crônica não inflamatória (fibromialgia) e dor neuropática. Neste estudo foi possível demonstrar fortes evidências experimentais de como a β-ciclodextrina pode agir como um sistema de complexação de drogas seguro e de baixo custo, melhorando as propriedades farmacológicas dos terpenos, transforrnando estes produtos naturais em uma escolha atrativa para uso farmacológico. Ao realizar uma revisão sistemática, selecionando as espécies de *Lippia* com propriedades sobre o sistema nervoso central, este estudo observou que apesar de várias espécies apresentarem atividade analgésica poucos estudos exploraram o mecanismo de ação responsáveis por estes efeitos ou fizeram uma descrição fitoquímica detalhada ou ainda investigaram a toxicidade e/ou segurança terapêutica do uso continuado destas drogas. Apesar disto, os resultados das análises de extratos e óleos foram consistentes com a maioria dos relatos dos estudos etnofarmacológicos reafirmando a importância da medicina popular como guia para tais estudos. Utilizando um modelo de dor muscular não inflamatório, este estudo demonstrou que o OEL/βCD reduziu a hiperalgesia primária e secundária sem alterar a força muscular. Atribuiu estes efeitos ao possível envolvimento de receptores opiodérgicos e serotoninérgicos, corroborando com a hipótese de envolvimento da via descendente inibitória da dor, suportada por estudo *in silico* e pela expressão da proteina Fos no corno dorsal da medula, além da atividade antioxidante demonstrada pelo OEL e OEL/βCD. Ainda foi investigada a ação anti-hiperalgésica mecânica e térmica do OEL e OEL/βCD (24mg/kg) em modelos de dor neuropática (ligação parcial do nervo ciático) e de dor inflamatória persistente (CFA). A migração atenuada de leucócitos associada aos níveis reduzidos de TNF-α e IL-1β observados em modelo de pleurisia podem sugerir a redução de hiperalgesia e edema causados pela injeção intraplantar de CFA observadas após o tratamento oral com OEL e OEL/βCD. Este tratamento também reduziu o desenvolvimento de hiperalgesia mecânica e térmica desencandeada pela ligação parcial do nervo ciático. A redução dos níveis de TNF-α no nervo ciático e na medula bem como de fosforilação de NFκB e PKA nestas mesmos tecidos sugerem uma correlação positiva entre a ação do óleo e a redução do efeito álgico destes mediadores. O OEL (24mg/kg) e OEL/βCD/ (24mg/kg) ainda inibiram a nocicepção desencandeada pela injeção plantar de cinamaldeído (agonista do TRPA1) e mentol (agonista do TRPM8). O tratamento oral agudo e prolongado com OEL e OEL-βCD (24 mg/kg) não alterou a atividade motora dos animais. Os resultados citados indicam que o OEL e o OEL/βCD (24mg/kg) podem ser potencialmente interessantes para o desenvolvimento de drogas clinicamente relevantes para o tratamento das desordens dolorosas crônicas. **Palavras-chave:** dor crônica, dor não inflamatória, dor neuropática, óleo essencial, β-ciclodextrina, *Lippia grata*

ABSTRACT

ANTI-HIPERALGESIC EFFECT OF *LIPPIA GRATA* ESSENTIAL OIL FREE AND COMPLEXED IN β-CYCLODEXTRIN IN ANIMAL MODELS OF NON-INFLAMMATORY CHRONIC PAIN AND NEUROPATHIC PAIN Siqueira-Lima, de Souza Pollyana, Universidade Estadual de Feira de Santana, Feira de Santana-BA, 2018.

Chronic pain associated with non-inflammatory and neuropathic states is prevalent and debilitating, and still remains without an efficient and safe treatment. For this purpose, this study was designed to investigate the antihyperalgesic effect of free and complexed *Lippia grata* essential oil on β-cyclodextrin (OEL/βCD) in animal models of chronic non-inflammatory pain (fibromyalgia) and neuropathic pain**.** In this study, it was possible to demonstrate strong experimental evidence of how β-cyclodextrin can act as a safe and low cost drug complexation system, improving the pharmacological properties of terpenes, transforming these natural products into an attractive choice for pharmacological use. In a systematic review, selecting the species of Lippia with properties on the central nervous system, this study observed that although several species present analgesic activity few studies have explored the mechanism of action responsible for these effects or have made a detailed phytochemical description or even investigated the toxicity and/or therapeutic safety of continued use of these drugs. Despite this, the results of the extracts and oils analyzes were consistent with most reports of ethnopharmacological studies reaffirming the importance of folk medicine as a guide for such studies. Using a non-inflammatory muscle pain model, this study demonstrated that OEL/βCD reduced primary and secondary hyperalgesia without altering muscle strength. It attributed these effects to the possible involvement of opiodergic and serotonergic receptors, corroborating the hypothesis of involvement of the pain inhibitory descending pathway supported by *in silico* study and the expression of the Fos protein in the dorsal horn of the medulla, in addition to the antioxidant activity demonstrated by OEL and OEL/βCD. The mechanical and thermal anti-hyperalgesic action of OEL and OEL/βCD (24mg / kg) in neuropathic pain (partial sciatic nerve ligation) and persistent inflammatory pain (CFA) models was also investigated. The attenuated migration of leukocytes associated with reduced levels of TNF-α and IL-1β observed in the pleurisy model may suggest the reduction of hyperalgesia and edema caused by the intraplantar injection of CFA observed after oral treatment with OEL and OEL/βCD. This treatment also reduced the development of mechanical and thermal hyperalgesia unleashed by the partial sciatic nerve ligation. Reduction of TNF-α levels in the sciatic nerve and medulla as well as phosphorylation of NFκB and PKA in these same tissues suggest a positive correlation between the action of the oil and the reduction of the algic effect of these mediators. The OEL (24mg/kg) and OEL/βCD (24mg/kg) did not inhibit the intraplantar injection-induced nociception of cinnamaldehyde (TRPA1 agonist) and menthol (TRPM8 agonist). The acute and prolonged oral treatment OEL and OEL-βCD (24 mg / kg) did not alter the motor activity of the animals. The results indicated that OEL/βCD (24mg/kg) may be potentially interesting for the development of drugs clinically relevant for the treatment of chronic pain disorders. **Key words**: chronic pain, non-inflammatory pain, neuropathic pain, essential oil, β-cyclodextrin, *Lippia grata*

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INTRODUÇÃO

1 INTRODUÇÃO

A dor crônica é o problema de saúde humana mais prevalente, afetando mais de um quarto da população mundial e aumenta sua incidência à medida que a população envelhece (MOGIL, 2012). Esta desordem é caracterizada por uma dor significativa, duradoura além do tempo de reparo tecidual, normalmente por meses ou até anos, portanto, de caráter contínuo ou recorrente (WOLFE et al., 1990). Esses tipos de síndromes dolorosas são comuns em indivíduos com doença cardíaca, acidente vascular cerebral, diabetes, herpes zoster e câncer (ERNST et al., 2015). A dor crônica é um problema de saúde pública mundial e que tem custos sociais e econômicos elevados. Em levantamento realizado pelo *Institute of Medicine Report*, nos Estados Unidos, denominado de "aliviando a dor na América", a dor é responsável por despesas em saúde em torno de US$ 560-635 bilhões de dólares (GASKIN E RICHARD, 2012). No Reino Unido afeta mais de 20% da população sendo a maior parte na população economicamente ativa (KELLEHER et al., 2017). No Brasil este tipo de registro é escasso e muitas vezes inconsistente, contudo, em um estudo realizado com pacientes com dor crônica no Estado de São Paulo, verificou-se que 94,9% apresentava comprometimento da atividade profissional, gerando prejuízos sociais e econômicos (KRELING et al., 2006).

Dentre as dores crônicas, as dores chamadas de "disfuncionais" são provavelmente as de menor conhecimento neurofisiológico, pior prognóstico de tratamento devido a refratariedade dos tratamentos existentes e menor adesão terapêutica (NAKAGURA, 2015). A síndrome da fibromialgia (FM), uma modalidade de dor crônica, é caracterizada por dor generalizada, hipersensibilidade, rigidez matinal, distúrbio do sono e fadiga pronunciada (HSU et al., 2011). O critério diagnóstico proposto pelo Colégio Americano de Reumatologia (ACR) inclui a dor difusa em conjunto com sensibilidade à palpação de 11 ou mais dos 18 *tender points* especificados (WOLFE et al., 2011). Apesar de ser considerada uma síndrome reumatológica não inflamatória, várias evidências tem mostrado que citocinas pró-inflamatórias como IL-6 e TNF-α estão elevadas no líquido cefalorraquidiano de pacientes com FM (TSILIONI et al., 2016; BOISSONEAULT et al., 2017).

Outra dor crônica que é igualmente considerada um problema de saúde mundial é a dor neuropática (DN). Tal condição é determinada como consequência direta de uma lesão ou doença que afete o sistema somatossensorial (MIRANDA et al., 2016). A DN é uma entidade complexa e de difícil tratamento, sendo considerada um dos maiores desafios da medicina moderna (KISSIN, 2010). A DN é um tipo de dor que costuma ter um grande impacto na vida de quem é acometido. Em comparação com outros tipos de dor, costuma ser mais intensa

,estar associada à incapacidade, e apresentar uma considerável diminuição na qualidade de vida (ZHANG et al., 2014). Estudos com a população em geral usando instrumentos validados de triagem observaram que 7-8% dos adultos têm dor crônica com características de DN (BENNETT et al., 2012).

Tais lesões tornam-se cada vez mais frequentes na rotina dos atendimentos de urgência dos hospitais em consequência do aumento da violência urbana, dos acidentes de trânsito, acidentes profissionais e domésticos (DE SÁ et al., 2004; MAZZER et al., 2006), estando entre os problemas neurológicos mais comuns. Apesar disso, a terapêutica atual tem se mostrado pouco efetiva para a maioria dos pacientes acometidos (ZOCHODNE, 2008).

Apesar da complexidade desta disfunção e do número significativo de pacientes acometidos, poucas terapias e intervenções estão disponíveis para deter ou reverter o dano que lhes estão associados e, principalmente, sintomas como a dor crônica (ZOCHODNE, 2008). O tratamento farmacológico da DN baseia-se em modular os mediadores inflamatórios relacionados à lesão do nervo (por exemplo como TNF-alfa) ou no bloqueio das vias centrais algésicas através do uso de fármacos opióides ou correlatos, bem como o uso de outras drogas como estabilizadores de membrana (QUINTANS et al., 2014). Contudo, os eventos adversos relacionados ao uso dos medicamentos e as baixas taxa adesão são limitadores do sucesso do tratamento (ZOCHODNE, 2008).

De forma semelhante, o tratamento da FM é complexo e envolve formas de tratamento heterogêneas devido as comorbidades relacionadas, tais como ansiedade, insônia, estresse, fadiga, entre outras. De acordo com Menzies et al. (2016), apesar da descoberta de novas drogas ou do reposicionamento de fármacos na última década com o uso da pregabalina, duloxetina e milnacipran, o tratamento farmacológico da FM continua a usar abordagens que são baseadas no perfil de dor central da doença, no entanto, não há um consenso sobre a escolha ideal e a sequência do tratamento. Outro aspecto importante é a disponibilidade de um número muito limitado de modelos experimentais, incluindo modelos animais, que mimetizem a FM ou os principais sintomas e, consequentemente, o número de estudos farmacológicos direcionados à busca de novas opções terapêuticas é insipiente na literatura.

Portanto, o tratamento farmacológico tanto da da FM como da DN continua a ser um desafio para medicina atual, bem como o desenvolvimento de novas propostas terapêuticas. De fato, as doenças crônicas apresentam altas taxas de abandono do esquema terapêutico, sendo importante o estudo de sistemas mais modernos de administração de fármacos que com um número menor de intervenções farmacológicas e/ou que diminuam a irritabilidade gástrica possam ter: eficácia, segurança, diminuição dos efeitos adversos e, consequentemente, adesão

terapêutica (GOLDENBERG et al., 1996; GASKELL et al., 2014; OLIVEIRA et al., 2017). Apesar da grande diversidade sintética derivada do desenvolvimento de química combinatória e alto rendimento, os produtos naturais continuam sendo elementos extremamente importantes das farmacopeias e estão relacionados com pelo menos 1/3 das novas drogas aprovadas pelo *Food and Drug Administration* (FDA) (QUINTANS et al., 2014; OLIVEIRA et al., 2017; PINA et al., 2017).

Os princípios ativos extraídos de plantas medicinais continuam despertando o interesse científico e econômico, em virtude da grande diversidade de compostos com propriedades farmacológicas, das quais se destacam as atividades analgésica e anti-inflamatória, e do desenvolvimento de novos fármacos (CARLINI, 2003). Dutra et al. (2016) demonstraram que vários estudos pré-clínicos e clínicos com algumas plantas medicinais brasileiras, selecionadas em diferentes áreas de interesse, vêm sistematicamente sendo realizados por grupos de pesquisa no Brasil e no exterior. Os autores destacam ainda, o crescente mercado brasileiro de produtos à base de plantas, e os esforços dos pesquisadores brasileiros para desenvolver novos fitomedicamentos.

Neste contexto, dentre os produtos naturais com propriedades terapêuticas e que fornecem novas entidades químicas promissoras destacam-se dentre outras, as plantas aromáticas ricas em óleos essenciais (BAKKALI et al., 2008). Os óleos essenciais (OEs) são originados do metabolismo secundário das plantas e possuem composição química complexa, destacando-se a presença de terpenos e fenilpropanoides (GONÇALVES et al., 2003). De acordo com De Sousa (2011), Guimarães et al. (2013; 2014) e Gouveia et al. (2017) os terpenoides são metabólitos secundários de grande interesse pela indústria farmacêutica principalmente no estudo de compostos com potencial emprego como analgésicos e anti-inflamatórios.

Paralelamente, o desenvolvimento de novas formulações farmacêuticas tende a alterar, em breve, o conceito atual de medicamento (IGBAL et al., 2016). Assim, têm surgido nos últimos anos, diversos sistemas de administração de fármacos com a finalidade de modelar a cinética de liberação, melhorar a absorção, aumentar a estabilidade do fármaco ou vetorizá-lo para uma determinada população celular (JANES et al., 2001; BREWSTER et al., 2008). Esses sistemas terapêuticos surgiram da necessidade de minimizar os problemas que se prendem com a administração das formas farmacêuticas tradicionais e, por exemplo, melhorar as propriedades fisicoquímicas e farmacológicas de moléculas apolares (KURKOV e LOFTSSON, 2013; SIQUEIRA-LIMA et al., 2016).

O uso de ciclodextrinas (CDs) em aplicações farmacêuticas envolvendo solubilização e melhorias das propriedades farmacológicas especialmente de fármacos com baixa polaridade tem se expandido exponencialmente a cada década, desde a descobertas das primeiras ciclodextrinas (CDs), isoladas por Villiers, em 1891, a partir de produtos de degradação de amido (GUEDES et al., 2008). As ciclodextrinas (CDs) são formadas por unidades de glicopiranose unidas por ligações α (1-4), possuindo uma estrutura rígida em forma de um cone truncado, onde os grupos OH secundários ligados aos carbonos C-2 e C-3 ocupam a base de maior diâmetro do tronco, enquanto as hidroxilas primárias ligadas ao carbono C-6 localizam-se na base menor do tronco (CHALLA et al., 2005). Atualmente, as CDs são utilizadas para melhorar algumas características físico-químicas de alguns fármacos, nutracêuticos (termo não técnico para junção que resulta da combinação dos termos "nutrição" e "farmacêutica") e/ou cosméticos (MARQUES, 2010). Os compostos lipofílicos, tais como os óleos essenciais e seus metabólitos, quando incorporados às CDs, aumentam sua solubilidade em água, estabilidade e eficácia farmacológicas (MARRETO et al., 2008; SERAFINI et al., 2012; QUINTANS et al., 2013; QUINTANS-JÚNIOR et al., 2013). Ainda alguns estudos têm demonstrado que complexos de inclusão contendo OEs ou monoterpenos e CDs podem aumentar a meia vida plasmática, bem como a eficácia farmacológica, quando comparado com os monoterpenos isolados, em modelos experimentais de analgesia e inflamação (BRITO et al., 2015; OLIVEIRA et al., 2015; SIQUEIRA-LIMA et al., 2016).

O gênero *Lippia* (Verbenaceae) inclui aproximadamente 200 espécies entre ervas, arbustos e pequenas árvores. As espécies estão distribuídas principalmente em regiões tropicais e sub-tropicais, com destaque para países das Américas do Sul e Central e em alguns países da África (TERBLANCHE´ e KORNELIUS, 1996). Espécies do gênero *Lippia* são usadas principalmente para o tratamento de distúrbios gastrointestinais, respiratórios e como analgésicos e anti-inflamatórios. Geralmente, o óleo essencial ou os compostos fenólicos (flavonoides) desses extratos de plantas são descritos como princípios ativos (PASCUAL et al., 2001).

Dentre as espécies de *Lippia* que estão sendo estudadas destaca-se a *L. grata* Schauer, uma planta aromática, conhecida popularmente como “alecrim-serrote”, amplamente distribuída no Nordeste Brasileiro, principalmente no Semi-Árido dos Estados da Bahia e Sergipe (PASCUAL et al., 2001). As propriedades espasmolíticas e anti-inflamatória do óleo essencial obtido das folhas de *L. grata* (OEL) foram demonstradas previamente, e esses efeitos foram atribuídos à presença de carvacrol e timol, dois monoterpenos fenólicos (SOUZA BRITO e SOUZA BRITO, 1993). De acordo com Viana et al (1981), a *L. grata* é

utilizada na medicina tradicional de alguns estados do Nordeste Brasileiro para o tratamento de distúrbios dolorosos e inflamatórios.

Recentemente, nosso grupo demonstrou efeito antinociceptivo do OEL/βCD em modelos de dor orofacial, mediado por mecanismos centrais e periféricos, com o provável envolvimento do sistema glutamatérgico e inibição de citocinas pró-inflamatórias, como TNF-α (SIQUEIRA-LIMA et al., 2013). Neste contexto em que as dores crônicas são condições de difícil tratamento, que acarretam episódios dolorosos prolongados, considerando ainda a existência de poucos tratamentos farmacológicos que produzam uma melhor condição clínica aos pacientes, sem a produção de reações adversas consideráveis, torna-se desafiador o desenvolvimento de novas preparações farmacêuticas utilizando plantas medicinais e/ou seus metabólitos secundários que possuam aplicabilidade na hiperalgesia não inflamatória experimental (fibromialgia experimental) e na dor neuropática (lesão parcial do nervo ciático).

OBJETIVOS

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar o efeito anti-hiperalgésico do óleo essencial de *Lippia grata* livre e complexado em β-ciclodextrina (OEL/β-CD) em modelo animal de dor crônica não inflamatória (fibromialgia) e dor neuropática.

2.2 OBJETIVOS ESPECÍFICOS

Realizar revisão da literatura sobre os possíveis benefícios da inclusão dos compostos terpênicos em ciclodextrinas e suas atividades farmacológicas;

Realizar revisão sistemática dos efeitos de plantas do gênero *Lippia* sobre o sistema nervoso central (SNC);

Avaliar os efeitos do β-CD/OEL sobre a hiperalgesia mecânica em modelo animal de dor crônica musculoesquelética caracterizando seu possível mecanismo farmacológico;

Avaliar o efeito do tratamento oral agudo e sub-crônico com OEL e OEL/β-CD sobre a hiperalgesia e edema em modelo animal de dor inflamatória persistente e de dor neuropática.

Verificar o possível efeito do OEL e OEL/β-CD sobre a atividade motora de roedores.

CAPÍTULO 1

Central nervous system and analgesic profiles of *Lippia* genus

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Abstract: Many people use medicinal plants to relieve disorders related to the central nervous system, such as depression, epilepsy, anxiety and pain, even though the effectiveness of most of them has not yet been proven through scientific studies. Plants of the *Lippia* genus,Verbenaceae, are widely used in ethnobotany as a food, for seasoning and in antiseptic remedies. They are also marketed and used for the treatment of different types of pain, including stomachache, abdominal pain and headache, as well as being used as sedatives, anxiolytics and anticonvulsants. Despite their widespread use, there are no reviews on the central nervous system profile of plants of this genus. Therefore, the databases Medline-PubMed, Embase, Scopus and Web of Science were searched using the terms *Lippia* and biologic activity. Thirty-five papers were found. Eleven species of Lippia showed central nervous system activity, with leaves and the aerial parts of plants being the most commonly used, especially in aqueous and ethanol extracts or volatile oil. The species are composed mainly of terpenoids and phenylpropanoids, including polyketides, flavonoids and in less quantity some alkaloids. Although several species of *Lippia* present analgesic activity, most studies have not explored the mechanisms responsible for this effect, however, there is some evidence that volatile oils and constituents of the extracts may be responsible for the relief of some CNS disorders, but the effects on pain modulation seem to be the most exploited so far.

Key words: Verbenaceae, medicinal plants, pain, CNS disorders, inflammation

Introduction

The genus *Lippia* belongs to the family Verbenaceae and comprises about 250 herbaceous species of shrubs and is widely distributed all over Central and South America, as well as tropical Africa (Terblanché and Kornelius, 1996; Aguiar and Costa, 2005). The species are distributed in the arid regions of the southwestern United States of America, in the deciduous tropical forests of Central America and in the tropical savannas ('cerrados') of Brazil, which are the regions with high indexes of endemism (Salimena, 2002). Among the prominent examples we can highlight the *L. origanoides*, which is popularly known as 'oregano' in Mexico, and is recognized in the Mexican Pharmacopoeia as a substitute for common 'oregano' (*Origanum vulgare*). It is, therefore, widely used as a condiment in the kitchen and in the preparation of several dishes (Oliveira et al., 2006). The dried and milled leaves of some *Lippia* sp., or the flowers and fruits of this genus have been used as a substitute for *Thymus vulgaris* (another species known as 'oregano') in spice mixtures for pizzas and meats (Lorenzi and Matos, 2002; Santoro et al., 2007).

Brazil is considered to have the largest number of known species (Arthur et al., 2011) represented by species conspicuous by their appearance during the short flowering phase and also by their generally strong and pleasant fragrance (Bezerra et al., 1981). These features make the use of this genus very widespread, ranging from in food preparation as a spice/herb, in cosmetics, as well as in traditional medicine due to it being linked to a range of analgesic, anti-inflammatory, antipyretic, antihypertensive and antimicrobial properties, as well as having beneficial actions in relation to gastrointestinal conditions, menstrual symptoms, pain, migraine and respiratory disorders (Pascual et al., 2001). Moreover, the *Lippia* genus has shown to be of relative economic importance due to the different uses of its volatile oils and the many medicinal uses of different species (Salimena, 2002), including their importance for veterinary medicine and agriculture (Soares and Tavares-Dias, 2013).

Due the great medicinal and economic importance of plants and their wide distribution across the regions of the country, the Brazilian government produced the National List of Medicinal Plants Aimed by the Public Health System (SUS – *Sistema Único de Saúde*), a list of vegetal species already used in traditional medicine which have potential to generate products that could be relevant to the Public Health System. *L. origanoides* was included in this list due to its pharmacological properties and its possible use in the development of new pharmaceutical products, including medicaments (herbal medicines) and adjuvants (Ministério da Saúde, 2009).

Among the biological effects reported for the genus *Lippia,* its central nervous system properties are highlighted by a number of studies (Bezerra et al., 1981; Pascual et al., 2001; Mamun-Or-Rashid et al., 2013). As the genus includes many aromatic plants rich in volatile oils their pharmacological properties are commonly attributed to these oils. They are largely comprised of terpene compounds which have already been shown to have clinical applicability and are part of various drugs (Craveiro et al., 1988; Guimarães et al., 2013; 2014; Gouveia et al., 2017).

This study aims to examine research in relation to the use of species of the *Lippia* genus directed to conditions related to the CNS. This will hopefully help to promote improvements in methodological and theoretical methods; identify trends, overlaps and gaps in research; as well as clarifying and summarizing the main existing works. Other studies of this nature have been described in the literature, however, there has been no systematic review focused on the correlation between the pharmacological effects and the chemical composition of the plants and their influence on the CNS (Terblanché and Kornelius, 1996; Pascual et al., 2001; Catalan and De Lampasona, 2002; Hennebelle et al., 2006; 2008, Oliveira et al., 2006; Ombito et al., 2015).

Many studies just describe the use of plants of this genus in traditional medicine, often with contradictory results, or only their use in food or as raw material). Therefore, considering the importance of this plant genus and the absence of systematic reviews of its pharmacological importance through preclinical studies, we carried out this extensive systematic survey in order to support translational studies and/or new preclinical studies.

Search strategy

Four digital databases, Medline-PubMed, Embase, Scopus and Web of Science were used to search for studies that met the inclusion criteria: preclinical animal-model studies of CNS pharmacological studies of *Lippia* species. The database search was performed in the period up to March 30, 2018 using the MesH and free search terms *Lippia* and biologic activity. The search strategy structure was designed to include any study published that assessed the pharmacological pre-clinical profile of the *Lippia* species. The search was limited to animal-model studies. There was no contact with researchers and/or attempts to identify non-published data.

All the electronic titles found, selected abstracts and complete texts from articles were revised independently by at least two reviewers (JSSQ, PSSL). Discrepancies over the inclusion/exclusion of studies were solved with a consensus meeting. Studies in humans,

literature reviews, editorials/letters, case reports, and isolated substances were excluded. The information extracted included data on the *Lippia* species, the part of the plant used, their main compounds, type of animal used, model of study and key findings.

Outcomes

A total of 3817 abstracts and citations were electronically identified in the first search. After the exclusion of duplicate articles and the triage of relevant titles and abstracts, 776 titles were included in our list for analysis, trying to identify studies that evaluated *Lippia* that met our inclusion criteria. Thirty five articles met the inclusion/exclusion criteria previously established and were included in our review .

The search of the databases showed that studies related to the genus *Lippia* included a broad range of species, with 27 different ones being the subject of research for different purposes This alone confirms the importance of the ethnopharmacological study as a basis for initiating preclinical studies. The areas of interest found in the articles are mainly phytochemical studies, CNS disorders, pain and inflammation. Our survey identified the following nine plant species as being the subject of research in studies: *L. alba, L. multiflora, L. gracilis, L. grata, L. origanoides, L. graveolens, , L. geminata, L. origanoides,* and *L. adoensis.* So, the number of species studied is still very small in relation to the number of species of the genus, which reinforces our argument that more studies of this important genus are required.

We found that the parts of the plants mainly used in the experimental protocols in the studies were leaves and aerial parts, particularly as aqueous and ethanol extracts or volatile oils. The part of the plants used is similar to that found in ethnopharmacological studies, which shows that they are primarily used in infusions that require the leaves and aerial parts (Oliveira, 2004; Hennebelle et al., 2008; De Carvalho Nilo Bitu et al., 2015). As we expected, due to the presence of many aromatic plants in the genus which are used for the treatment of diseases, studies with volatile oil, mainly terpenoids and phenylpropanoids, with some polyketides and in less quantity some alkaloids, predominate in our survey (36%) (De Sousa, 2011).

Many VO mainly comprising terpenes whose various activities and mechanisms of action have already been well described in the literature were found. The studies identified do not state if the presence of terpenes influenced the selection of these mixtures (VO) for pharmacological evaluation by the authors. However, we hypothesize that this may well be the case given the number of studies which support the idea that VO are pharmacologically

interesting because of the presence of terpenes (Guimarães et al., 2013; Lillehei and Halcon, 2014; De Cássia da Silveira e Sá et al., 2017). Studies investigating its applicability in diseases that cause pain, inflammation, oxidative stress imbalance and CNS disturbances are common (Dobetsberger and Buchbauer, 2011; El Hadi et al., 2013; Ali et al., 2015). Moreover, terpenes has demonstrated strong bioactivity on the modulation of cytokines and in the inflammatory process, central nervous system activity, pain and nerve sensitization, among other interesting pharmacological targets (González-Burgos and Gómez-Serranillos, 2012; Quintans et al., 2019; Santos et al., 2019).

Most of the studies performed an extensive phytochemical analysis to describe the main compounds (terpenoids: carvacrol, *p*-cymene, *o*-cymene, thymol and *E*-caryophyllene, and others chemical classes, such as flavonoids, phenolic acid, and alkaloids) (Table 1).

Terpenes and terpenoids are the primary constituents of the VO of many types of medicinal plants and flowers. They are derived biosynthetically from units of isoprene, which has the molecular formula C_5H_8. Terpenes are chemical entities having low molecular weight and usually low water solubility. They can penetrate the blood-barrier and produce their effects, anxiolytic, sedative and anticonvulsant, on the CNS (Quintans-Júnior et al., 2008; De Sousa, 2011). The articles found in our review highlighted action on the CNS through the GABAergic pathways, which corroborated the pharmacological evidence of the anxiolytic, sedative, myorelaxant and anticonvulsant properties (Heldwein et al., 2012; Razavi et al., 2017).

Moreover, drugs that enhance GABA-mediated inhibitory transmission, and subsequently affect neuronal repetitive firing, can be of relevance in alleviating several painful syndromes, since they can produce a membrane stabilizing effect on sensory neurons and/or enhance intrinsic analgesic responses (Jasmin et al., 2004; Enna and McCarson, 2006). This evidence may help to explain the fact that most of the studies presented in Table 1 (64%) are studies of the possible effect of plants of this genus on pain management (or simple screening for analgesic drugs, which was the most common type of study described). Obviously, the fact that previous ethnopharmacological surveys had described the great concentration of terpenes or flavonoids in the *Lippia* genus and their analgesic and anti-inflammatory properties was important in encouraging many research groups to explore this genus (Siqueira-Lima et al., 2014). Additionally, VO and extracts from *Lippia* genus with antioxidant properties, which modulate the inflammatory process by reducing the production of proinflammatory cytokines (such as IL1-β, TNF-α and others) and act on neurotransmission systems that participate in descending pain-inhibitory mechanisms

corroborate this search more directed by the researchers (Leyva-López et al., 2016; Siqueira-Lima et al., 2014; 2017).

Our review provides evidence that the *Lippia* genus is rich in flavonoid compounds, at least in the species studied here, the most commonly described being extracts rich in polyphenols (mainly flavonoids) and naringenin, apigenin, nodifloretin A, nodiflorin A, nodifloridin A and others (Table 1). Flavonoids are a class of plant polyphenols that are consumed in the human diet via vegetables, fruits, cereals, spices, and other plant-based products (Pandey and Rizvi, 2009; Jaeger et al., 2017). Flavonoids are probably one of the most important NP due to the potent biological molecules and are already described and used in clinical practice for the treatment of various diseases, as they have a range off antioxidant, anti-inflammatory, analgesic, anxiolytic and anticonvulsant properties (Diniz et al., 2015; Nijveldt et al., 2001).

Lippia species

Lippia alba (Mill.) N.E.Br. ex Britton & P.Wilson, popularly known as “cidreira” (in the south and southeast of Brazil) and “Basula” (in Hindi, in India), is a plant which is present in Central and South America, being recorded in all regions of Brazil (Tavares et al., 2005). *Lippia alba* is a fast growing plant with a mounding habit and round lavender-like blossoms (Haldar et al., 2012). The main pharmacological studies, arising from folk use, found it had varied activities including cardiovascular (Gazola et al., 2004), anticonvulsant (Soares, 2001), sedative, analgesic, bronchodilator (Carvalho et al., 2018) antioxidant and anti-inflammatory (Viana, 1998; Zétola et al., 2002; Hennebelle et al., 2008; Haldar et al., 2012; Hatano et al., 2012), as well as antiulcerogenic effects (Pascual et al., 2001).

The composition of its VO presents quantitative and qualitative variation, leading to its separation into chemotypes according to its major components (De Abreu Matos et al., 1996; Frighetto et al., 1998; Zoghbi et al., 1998; Hennebelle et al., 2008). In Brazil, there are at least three major chemotypes of *L. alba* with a large variation in some terpenoids, especially citral, carvone and linalool; therefore having different pharmacological effects (De Abreu Matos et al., 1996; Yamamoto, 2006; Linde et al., 2016).

The remarkable action of this plant species on the CNS was characterized by the presence of nine articles demonstrating biological activities typically involving the central pathways, including analgesic (with a central component), sedative, anticonvulsant and anxiolytic effects. Viana et al. (1998) compared the analgesic and anti-inflammatory effects of the VO from the leaves of two chemotypes: “citral” (type I) and “carvone” (type II). The

antinociceptive effect was more consistent and marked in the type II, but this effect was not reversed by naloxone (an opioid antagonist). A central analgesic effect was also evident with type I (rich in citral). Interestingly, Gonçalves et al. (2008) and Quintans-Junior et al. (2011) demonstrated the central analgesic effects of carvone and citral with no involvement of the opioid system, but with possible participation of blocking Na^{+}-channels and the GABAergic system, respectively. In addition, the anti-nociceptive action of citral was found to involve significant activation of the 5-HT_{2A} serotonin receptor (Nishijima et al., 2014). Sousa et al. (2015) showed that VO of *Lippia alba* and its main constituent citral block the excitability of rat sciatic nerves.

Haldar et al. (2012) reported that flavonoids in an aqueous extract of *L. alba.* have analgesic and anti-inflammatory effects, which they attributed to the presence of polyphenol compounds that inhibited the enzyme cyclooxygenase and subsequently inhibited prostaglandin synthesis.

The most striking CNS effects for this plant are sedative, anticonvulsant and anxiolytic. The anticonvulsant activity of *L. alba* (type I, "citral") has been demonstrated in classic screening tests for new antiepileptic drugs (Neto et al., 2009). Citral is a sedative and anticonvulsant compound that produces its effects through , at least in part, the involvement of the GABAergic system, but its effect on the stabilization of the neuronal membrane and the blockade of families of ion channels act synergistically (Stotz et al., 2008; Quintans-Júnior et al., 2010).

The anxiolytic effect studied elucidate a possible GABAergic action and it is believed that this action is related to the presence of non-volatile substances (phenylpropanoids, flavonoids and/or inositols), and also to volatile terpenoids (myrcene, citral, limonene and carvone), which have been previously shown to have anticonvulsant and anxiolytic properties (Viana et al., 2000; Zétola et al., 2002; Neto et al., 2009; Zhu et al., 2014).

In fact, much of the pharmacological evidence relating to *Lippia* genus, including *L. alba*, is strongly associated with the presence of terpenes (mainly monoterpenes) with remarkable action on the CNS (Passos et al., 2009; Guimarães et al., 2013; 2014; Quintans-Júnior et al., 2013; Pina et al., 2017; Habtemariam, 2018).

Lippia multiflora Moldenke, popularly known as "chá-de-gambia", is a species widely used as an infusion in Africa. Traditionally, its leaves are used as a hot beverage (tea) to treat fever, gastrointestinal disturbances, enteritis, and coughing (Adesina et al., 1995). The VO isolated from its leaves and flowers contains *p*-cymene, thymol and carvacrol (Abena et al., 2003), which have been attributed analgesic and antipyretic activities. Iwalewa et al. (2007)

demonstrated a decrease in the plasma level of both nitric oxide (NO) and malondialdehyde (MDA) that was closely associated with the anti-inflammatory and analgesic activities produced by VO.

p-Cymene and carvacrol produce an analgesic effect by the involvement of descending pain-inhibitory mechanisms; by inhibition of pro-inflammatory cytokines (such as IL1β, TNF-α, IL-4, TGF-β, and IL-17), by enhancement of anti-inflammatory cytokines (IL-10), and by involvement of the opioid system (Lima et al., 2013; Guimarães et al., 2014; De Santana et al., 2015; Kianmehr et al., 2016). Thus, these terpenes seem to be key to the effects of VO from *L. multiflora*.

The pharmacological activity of the aqueous extract of *L. multiflora* was assessed by Abena et al. (1998) who concluded that it had tranquilizer and analgesic profiles. However, comparing the extract and VO to confirm the previous activities, the study suggested that the crude extract produced more muscle relaxant effects and the VO was more analgesic (Abena et al., 2001). The phytochemical screening of its crude extract demonstrated the remarkable presence of alkaloids, tannins, flavonoids and saponins (Valentin et al., 1995; Oladimeji et al., 2001). Although not an outstanding feature of the crude extract, the significant analgesic and anti-inflammatory effects were attributed to the presence of alkaloids (Jigam et al., 2009).

Moreover, some of the most important monoterpenes found abundantly in *L. multiflora* volatile oil inhibited allergic inflammation by the modulation of inflammatory cytokines (Lima et al., 2013; Pina et al., 2018), which are synthesized within the CNS by glial cells and neurons, and have modulatory functions on these same cells via interactions with specific cell-surface receptors contributing, at least in part, to the central effects of terpenes and similar compounds (Benveniste, 1998; Cho et al., 2017).

Lippia gracilis Schauer (“alecrim-da-chapada”) is a shrubby aromatic species that is distributed in the Brazilian Northeast with a high occurrence in the states of Bahia, Segipe and Piauí. It is probably one of the most popular *Lippia* species in the Brazilian Northeast due to its medicinal properties and food application (Albuquerque et al., 2012). The VO of *L. gracilis* is composed mainly of mono- and sesquiterpenes, and the main compounds are *p*-cymene, *γ*-terpinene, carvacrol and thymol (Pessoa et al., 2005; Neves et al., 2008; Silva et al., 2008; Mendes et al., 2010; Teles et al., 2010; Guilhon et al., 2011).

As already described here and reinforced in the excellent reviews published by De Sousa (2011), De Cássia Da Silveira E Sá et al. (2013) and Guimarães et al. (2013; 2014) the analgesic and anti-inflammatory profiles produced by the VO are attributed to the presence of

terpenes. They act by inhibiting inflammatory mediators, such as cytokines, and reducing neuronal excitability in certain CNS areas.

Studies point out that thymol (another important compound found in VO) modulates voltage-dependent Na^+-channels (Haeseler et al., 2002), K^+-channels (Elliott and Elliott, 1997), GABA A receptors (Mohammadi et al., 2001), α and β-adrenergic receptors (Beer et al., 2007), as well as being related to prostaglandin synthesis (Anamura et al., 1988), which together may contribute to the control of painful sensations produced by this terpene.

The antinociceptive profile of *p*-cymene, the main compound from *L. gracilis* VO, was assessed in animal models of pain in respect of contortions induced by acetic acid, the formalin test and hot-plate test.; It has also been reported to have an anti-inflammatory effect (Bonjardim et al., 2012). Moreover, Santana et al. (2011) reported an antinociceptive effect through the opioid system which corroborates other studies that describe the effects of this terpenoid on the CNS, due to, among other factors, its antioxidant profile (De Oliveira et al., 2012; 2015).

Furthermore, the analgesic profile of carvacrol has already been described consistently in a number of papers and patents for new drugs or pharmaceutical products (Guimarães et al., 2014; Suntres et al., 2015; Oliveira et al., 2016). The effects of the VO of *L. gracilis* are attributed to its anti-inflammatory actions, rather than to its profile on the CNS. Carvacrol is able to block the recruitment of neutrophils, to reduce the release of IL-1β, TNF-α and NO, and enhance levels of IL-10, resulting in a decrease in the production of inflammatory factors and block hyperalgesic behavior (Guimarães et al., 2010; 2012; Lima et al., 2013; Pina et al., 2017). It was also able to regulate COX-2 expression through its agonistic effect in PPARγ (Hotta et al., 2010). Controversially, some authors attribute the effect on the opioid system, but these data are contradictory in different articles and no evidence of direct involvement of the opioid system in the analgesic effect of carvacrol has been found (Guimarães et al., 2010; Cavalcante Melo et al., 2012).

Additionally, the monoterpenes found in the VO are extensively described as analgesic and its lipophilic characteristics and molecular size facilitates both its passage through the blood brain barrier, to produce local actions such as in relation to oxidative balance, to manage the production of inflammatory factors (such as cytokines) and to directly block ion channels (Abena et al., 2003; Mendes et al., 2010; González-Burgos and Gómez-Serranillos, 2012; Guimarães et al., 2013; 2014; Gouveia et al., 2017; Pina et al., 2017).

Guilhon et al. (2011) also investigated the mechanism of action producing the analgesic behavior of the VO of *L. gracilis.* The authors clearly indicated the involvement of

cholinergic receptors in this process (as atropine inhibited the antinociceptive effect) and the involvement of the opioid system (by antagonism produced by naloxone). However, the different chemotypes may elicit significantly different biological responses as reported by Mendes et al. (2010) (thymol - major component) and Guilhon et al. (2011) (carvacrol - major component). Chemotypes of *Lippia* species with their different chemical profiles are equally interesting for comparative study, since the difference in concentrations of the major compounds is an area that should be better explored by drug manufacturers.

Another monoterpene presents in these oils, the γ-terpinene, was evaluated by De Brito Passos et al. (2015); the authors demonstrated that γ-terpinene antinociception was inhibited in the presence of naloxone, glibencamide, atropine and mecamylamine, suggesting that this antinociceptive effect in models of chemical nociception was produced through the cholinergic and opioid systems. These effects reflect the previously described pharmacological profile of the major terpenes of VO which has a more complex, complete and therapeutic action than the isolated terpenes.

The therapeutic effects on the CNS of thymol and carvacrol (the main compounds of VO from *L. gracilis*) are related (directly or indirectly) to their anti-inflammatory and antioxidant properties, being difficult to dissociate from it (Suntres et al., 2015; Parsaei et al., 2016). Carvacrol and thymol have potent antioxidant potential, and probably exert a protective action against free radicals, as well as inhibiting superoxide and superoxide-derived reactive species. This is an attractive strategy to control the peripheral and central sensitization associated with several painful states or to improve the neuronal functions of neurotransmission systems in the control of anxiety, depression as well as reducing the *status epilepticus* (defined as continuous convulsions lasting more than 30 min). Additionally, terpenes seem to act directly as antioxidants through free radical scavenging mechanisms and/or as indirect antioxidants by enhancing antioxidant status (enzymatic and non-enzymatic) (González-Burgos and Gómez-Serranillos, 2012). Thus, the characteristics of these two monoterpenes associated with the other terpenes present in the VO from *L. gracilis* (or other VO from *Lippia* sp. rich in terpenes) must be acting synergistically to produce their main biological properties.

Lippia origanoides Kunth is popularly known in Brazil as “alecrim-pimenta” (“pepper-rosemary”), and is native to the northeastern region of Brazil and north of the state of Minas Gerais (Brazil). This species was called *L. sidoides*, but recently it was renamed as *L. origanoides* which has made it difficult to search for articles in the bibliographic databases, however, it remains an attractive species for pharmacological study. In folk medicine, this

aromatic species is used as an antiseptic and antimicrobial (Veras et al., 2017) and is usually applied topically on the skin, mucous membranes, mouth, and throat, or used for vaginal washings (De Oliveira et al., 2014).

Similarly to other *Lippia* species, the crude extract of *L. origanoides* presented antinociceptive activity, mainly in screening tests such as acetic acid-induced writhing and formalin tests, but not in tests involving a greater participation of the CNS component, such as the tail flick test (de Morais et al., 2016). This profile, more oriented to the anti-inflammatory properties seems to be related to the presence of polyphenols in the extract (Lima et al., 2016). The major constituents of the VO of *L. origanoides* are *p*-cymene, thymol and myrcene and demonstrated an analgesic profile in chemical and thermal pain in pre-clinical models. The activation of the opioidergic system appears to play a crucial role in the observed analgesic profile produced by the VO (Marçal et al., 2006). As suggested for other species of *Lippia,* the presence of *p*-cymene (Santana et al., 2011; Quintans-Júnior et al., 2013) and myrcene may involve the mediation of endogenous opioids and α-adrenoreceptors (Rao et al., 1990), as well as an increase in cGMP mediated by stimulation of the arginine-NO-cGMP (Duarte et al., 1992). De Morais et al. (2016) suggested that the chemical composition of VO of *L. origanoides* is variable (depending on the chemotype), therefore this should drive the main biological potentialities, being a pivotal factor for the beginning of the study with this species

Recently, some new approaches using nanotechnology and encapsulation of drugs have shown promising results following the incorporation of different VO, including some from the *Lippia* species (Quintans-Júnior et al., 2016; 2017; Siqueira-Lima et al., 2017). For example, Botelho et al. (2016) demonstrated that a nanostructured thymol gel (the main compound obtained from *L. origanoides*) was able to provide a significant MPO decreasing in gingiva tissue confirming it to be effective in reducing gingival inflammation in this model. The authors reported that this reduction in the inflammatory process (with the reduction of pro-inflammatory cytokines) contributed to the reduction of pain.

Lippia grata Schauer is a native bush of the semi-arid area of Northeastern Brazil and is used in folk medicine to treat pain and inflammation, but is poorly described in the scientific literature with few reports, especially in relation to its pharmacological effects (O'Leary et al., 2012). The leaf VO demonstrated antispasmodic activities attributed to the presence of carvacrol and thymol (Craveiro et al., 1981; Santos et al., 2011).

Siqueira-Lima et al. (2014) identified a very different phytochemical profile of the VO of *L. grata* than that described by Craveiro et al. (1981), using gas chromatography–mass

spectrometry (CG/MS) analysis, they demonstrated the main compounds of this VO to be: camphor, *E*-caryophyllene, camphene, and bicyclogermacrene. Different chemotypes of Verbenaceae (mainly from the *Lippia* genus) can produce different VO phytochemical profiles and, in addition, the time of the year and the place where the botanical specimen was collected can affect this profile. Consistent techniques in the collection and identification of VO are essential for their standardization from an industrial perspective (Craveiro et al., 1981; Tavares et al., 2005). In fact, identifying individual chemotypes is essential in choosing the VO that is most appropriate to the aim of the study. Studies that are guided with support from chemistry professionals who are able to identify these chemotypes are more likely to be successful in their assessment of the CNS properties of *Lippia* species.

The effect of OE on orofacial pain in animal models was evaluated because of the common clinical challenges for orofacial pain management. The authors used an approach involving VO complexed with β-cyclodextrin (β-CD) (used to improve the water solubility and bioavailability of VO). Cyclodextrins have been shown to be an important tool for improving the analgesic effect of OE (Siqueira-Lima et al., 2014; 2016). The use of β-CD in this case helped the VO to produce a stronger antinociceptive activity. The authors demonstrated the involvement of both descending pain-inhibitory mechanisms and CNS areas that contribute to controlling pain, such as the periaqueductal gray (PAG), *Locus coruleus*, rostral ventromedial medulla (RVM) and the nucleus raphe magnus, in the attenuation of orofacial pain by VO. These CNS areas appear to be modulated whenever terpenes and/or VO act on the descending pain suppression pathway (Nascimento et al., 2014; Quintans-Júnior et al., 2016; 2017; Araújo-Filho et al., 2017; Santos et al., 2018). Therefore, the chemical characteristics of terpenes present in VO from *Lippia* species seems to be pivotal for the variability of effects produced by them on the CNS. Their ability to easily pass through the blood-brain barrier (which seems to be common to most terpenes) makes them attractive targets to explore in various central disturbances (Kam et al., 2012).

Moreover, Vogt-Eisele et al. (2007) demonstrated that some monoterpenes (such as camphor) activated TRPV3 receptors, which have been implicated in hyperalgesia, inflamed tissues and possibly skin sensitization, and inhibited several related TRP channels, including ankyrin-repeat TRP 1 (TRPA1) (Waning et al., 2007; Xu, 2005). Another terpene, β-caryophyllene, acts on CB2 receptors whose activation can produce a direct antinociceptive response by causing the release of mediators from non-neuronal cells that alter the responsiveness of primary afferent neurons to noxious stimuli (Ibrahim et al., 2005). β-Caryophyllene is one of the terpenes with a profile acting on CNS areas that modulate the

descending pain suppression pathway, at least when evaluated in a chronic non-inflammatory widespread pain animal model (a rodent fibromyalgia-like model) (Quintans-Júnior et al., 2016; 2018). Recently, VO of *L. grata* complexed with βCD enhanced the pharmacological efficacy of the VO and produced a longer-lasting analgesic activity. The presence of β-caryophyllene was considered by the authors to play a key role in the pharmacological effect (Siqueira-Lima et al., 2017). The authors pointed out that the VO was antagonized by naloxone and partially antagonized by methysergide, but was not antagonized by yohimbine, thus suggesting that the anti-hyperalgesic effect produced by VO is related to the opioid and serotonergic systems. These features of VO are essential for the development of new proposals for the management of chronic pain, especially in relation to 'dysfunctional pain' which are neglected by the drugs currently used. (Nagakura, 2015; Oliveira et al., 2017).

Lippia adoensis Hochst. was cited in two Ethiopian studies whose objective was to screen for the analgesic properties of this and other plants in an attempt to validate their traditional uses. With local names such as "kessie" or "kusaye", this shrub is found in different regions of Ethiopia at an altitude between 1.600-2.200m above sea level (Debella et al., 2003; Makonnen et al., 2003). Pre-clinical studies using screening tests to assess analgesic effects, such as the acetic acid-induced abdominal constrictions (Debella et al., 2003) and tail flick, hot plate and tail-pinch tests (Makonnen et al., 2003) have revealed that the extract produced an analgesic profile. The presence of phenolic compounds as major chemical constituents may contribute to the analgesic effect, however, the authors themselves acknowledge that the mechanisms of analgesia produced by the extracts need to be investigated further.

Final comments

Our review discussed *Lippia* species being investigated in pre-clinical animal studies that showed significant medicinal properties in relation to the CNS and that could be important in the control of pain. We chose this approach due to the wide spectrum of plants of this genus that are used for medicinal purposes. However, clinical studies are very rare making systematic reviews very difficult. We therefore chose a more fruitful approach, searching for preclinical studies. We imagine that in the near future it will be possible to carry out systematic reviews of the results of clinical studies, as government institutions (as is happening more in Brazil) start to support the development of herbal medicines from plant species such as *L. origanoides* (formerly known as *L. sidoides*). Translational studies are

urgently required to validate the biological effects found in preclinical studies and especially to corroborate the widespread use of these traditional medicinal plants.

Although several species of *Lippia* present activities on the CNS, the central analgesic effects are the most commonly described. However, most studies have not explored the mechanisms responsible for the effects observed and also have not identified the inflammatory mechanisms involved in the processes, the participation of specific neurotransmission systems or the CNS regions involved. Therefore, the majority of studies carried out are speculative in their conclusions about CNS effects, as there are few studies with a molecular approach and with a deep phytochemical study of the species. Thus, this is an essential problem that needs to be solved if research is to translate these results into clinical studies with humans.

Another worrying aspect of the pharmacological studies made using *Lippia* genus is that there are few preclinical reports with chronic models that explore toxicity and/or the therapeutic safety of the continuous use of these drugs (extracts or VO). Our survey did not find any in the searched databases, although there may be some but this was not a focus of our review. This represents a gap in knowledge that needs to be filled.

Furthermore, it is known that significant therapeutic properties of plant extracts are due to the combined effects of several secondary metabolites. However, in our opinion, studies with isolated compounds present some important advantages, as isolated compounds from natural sources can be employed as tools in the identification of action mechanisms and can also provide structural molds to obtain synthetic substances, though the capacity of the synergistic effect that usually seems to happen with the use of extracts or VO will be lost. Interestingly, the results with the extracts and the VO were consistent with the majority of ethnopharmacological studies, which corroborated the importance of folk medicine as a kind of guide for preclinical studies. Obviously, a consistent prior phytochemical study, knowledge of the pharmacological properties of major components and a guided scientific approach are key in the study of any natural product seeking to minimize possible false positive results. Moreover, studies with species of the genus *Lippia* need to evaluate its effects in chronic disease models and in long-term treatment in repeated doses.

A modern approach that is still little found in studies with *Lippia* species is the use of pharmaceutical technology, such as nanotechnology, complexation of drugs (such as cyclodextrins) or incorporation in polymers. The traditional approach of testing nonstandard extracts or complex mixtures (as VO) may be a limitation in looking for more modern preparations using pharmaceutical technology. These scientific barriers need to be urgently

overcome, seeking formulations that guarantee better pharmacological effects, low toxicity and greater effectiveness.

Although the action mechanisms are not completely understood (in most studies), either because of the mainly unspecific animal models used or due to the extracts evaluated (non-standardized and without specific chemical markers) the findings of the articles presented here strongly suggest that *Lippia* species are clinically promising and that its uses in folk medicine are rational and appear to produce important clinical effects (since preclinical studies corroborate these effects). Therefore, there is evidence that the constituents of the extracts and VOs are candidates for the relief of some CNS disorders, such as anxiety and perhaps can act as adjuvant in the treatment of the seizures of epileptic patients, as well as for the management of painful conditions.

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Table 1 – Studies on CNS and analgesic profiles of *Lippia* genus

Authors, year, Country	Extract and part of the plant	Majority compounds	Models of study	Animals (strain/sex)	Dose (mg/kg)/route	Key findings
Lippia alba						
Costa et al., 1989, Brazil	Ethanolic extract /leaves	NR	Analgesic	Mice (*Swiss*/F)	50 mg/ml *p.o*.	Significant analgesic effect in screening test
Viana et al., 1998, Brazil	EO/leaves	**I** citral **II** carvone	Analgesic	Mice (*Swiss*/F)	0.5, 1, 2, 10/ *i.p*. and *p.o*.	Both chemotypes present analgesic effects and only EO I was reversed by the opioid antagonist (naloxone)
Vale et al., 1999, Brazil	EO/leaves	**I** citral (55.1%) β-myrcene (10.5%) **II** citral (63.0%) limonene (23.2%) **III** carvone (54.7%) limonene (12.1%)	Anxiolytic	Mice (*Swiss*/M)	25=200/*i.p*.	Anxiolytic effect from three chemotypes. However, a more potent activity was presented by EO II which showed a significant effect with a lower dose
Viana et al., 2000 Brazil	EO/leaves	β-myrcene citral limonene	Anticonvulsant	Mice (*Swiss*/F)	100, 200, 400/ *i.p*.; *p.o.*	The constituents of the EO present a pharmacological profile similar to that shown by DZP-like drugs and are responsible, at least in part, for the anticonvulsant effect
Zétola et al., 2002 Brazil	Ethanolic extract /leaves	Flavonoid	PTB-induced sleep Anticonvulsant	Mice (*Swiss*/M)	200/*p.o*.	Extracted in ethanol 80% (v/v), presents sedative and myorelaxant effects and this presents the highest flavonoid content.
Neto et al., 2009 Brazil	Ethanolic extract /leaves	**I** linalool (77.95%) **II** geranial (33.49%) myrtenyl acetate (23.4%)	Anticonvulsant	Mice	300/*i.p*.	Anticonvulsant properties might be correlated to the presence of a complex of non-volatile substances phenylpropanoids, flavonoids and/or inositols, and also to

		III geranial (35.98%) myrtenyl acetate (25.58%)		(*Swiss*/M)		the volatile terpenoids which have been previously validated as anticonvulsants.
Hatano et al., 2012, Brazil	EO/leaves	carvone (54.17%) limonene (23.13%)	Anxiolytic	Rats (*Wistar*/M)	25/ *i.p.*	Repeated treatment exerts anxiolytic-like effects
Heldwein et al., 2012, Brazil	EO/leaves	linalool (59.66%) 1,8-cineole (9.11%)	Potentiation with BDZ/ Reversal of anesthetic effects	Fishes (Silver catfish/-)	50, 100, 300 µl/l	Anaesthetic effects of the EO were reversed sooner by flumazenil, suggesting the involvement of the GABAergic system
Haldar et al., 2012, India	PELA,CELA, EELA, AELA/leaves	phytosterol, alkaloid, flavonoid, phenolic compound, saponin	Analgesic	Rats (*Wistar*/M)	460, 500/ *p.o*.	AELA has a potent analgesic effect probably due to the presence of flavonoids in its composition
L. multiflora						
Abena, et al., 1998, Congo	aqueous extract/leaves	NR	Analgesic	Rats (*Wistar*/F,M)	200, 400, 600/ *i.p*. or *p.o*.	Possess tranquilizer and analgesic activities similar to Diazepam
Abena et al., 2001, Congo	aqueous extract and EO/leaves	NR	Spontaneous motor activity PBT-induced sleep Analgesic	Rat (*Wistar*/F,M)	2 ml/kg *p.o*.	The results confirm the tranquillizer and analgesic activities and reveal that the crude extract would be more of a muscle relaxant and the volatile oil more an analgesic
Abena et al., 2003, Congo	EO/leaves	*p*-cymene (41.1%) thymol (19.0%) thymylacetate (14.2%)	Analgesic	Mice (*Swiss*/M)	2, 4 and 8 ml/kg *p.o.*	Monoterpenes, as the major constituents of volatile oil of *L. multiflora,* possesses analgesic and antipyretic activities
Jigam et al., 2009, Nigeria	crude extract /leaves	alkaloids, flavonoids, tannins, saponins, glycosides, volatile oils	Analgesic	Mice (*Swiss*/F,M)	200, 400/ *i.p.*	Significant analgesic activity by leaf extracts at the two doses used.
Iwalewa et al.,	EO/leaves	NR	Analgesic	Rats	1.2-4.8/	The lippia oil formulation exhibited a significant dose-

2007, Nigeria	(emulsion)			(*Wistar*/M)	*i.p.*	dependent analgesic effect, showing peripheral and central activities. Level of the antioxidant markers as possible mechanism of this activity.
Bassoueka et al., 2015, France	aqueous extracts/leaves	alkaloids, flavonoids, steroids, tannins	Strychnine-induced convulsion	Rats (Wistar/F,M)	400, 800/*p.o.*	The extract of Lippia multiflora has no significant effect on the parameters studied
L. gracilis						
Mendes et al., 2010, Brazil	EO/leaves	thymol (32.68%) *p*-cymene (17.82%) methyl thymol (10.83%)	Analgesic	Mice (*Swiss*/M)	50, 100, 200/ p.o.	The EO inhibited acid-acetic writhing.
Guilhon et al, 2011, Brazil	EO/leaves	carvacrol (44.43%) *o*-cymene (9.42%) *γ*-terpinene (9.16%)	Analgesic	Mice (Balb-c/ M)	10, 30, 100/p.o.	Antinociceptive effect (not reversed by Naloxone) could potentially be mediated by cholinergic receptors and the nitric oxide pathway.
Guimarães et al., 2012, Brazil	methanolic extract/leaves	Naringenin	Analgesic	Mice (*Swiss*/M)	100, 200, 400/p.o.	Methanolic extract has a therapeutic potential for painful conditions.
Lippia grata						
Siqueira-Lima et al., 2014, Brazil	OE/leaves	camphor (27.2%) *E*-caryophyllene (11.6%) camphene (11.3%) bicyclogermacrene (9.4%)	Analgesic	Mice (*Swiss*/M)	6,12,24/*p.o.*	EO was capable of reducing the nociceptive face-rubbing in capsaicin, glutamate and both phases of the formalin test. The immunofluorescence protocol demonstrated that the βCD-EO activated important areas in the CNS
Siqueira-Lima et al., 2017, Brazil	OE/leaves	camphor (27.2%) *E*-caryophyllene (11.6%) camphene (11.3%) bicyclogermacrene (9.4%)	Analgesic	Mice (*Swiss*/M)	6,12,24/*p.o.*	Decreased paw withdrawal and muscle threshold. The OE was shown to affect the opioidergic and serotoninergic pathways. Fos protein immunofluorescence showed decreased expression in the dorsal horn of the spinal cord.

						Docking study showed interaction energies with the alpha-adrenergic and μOpioid receptors.
Lippia origanoides						
Marçal et al., 2006, Brazil	OE/leaves	*p*-cymene (26.8%) thymol (21.9%) myrcene (12.8%)	Analgesic	Mice (*Swiss*/M)	25-400/*s.c*.	The activation of the opioidergic system appears to play a crucial role in the observed antinociceptive effect.
De Morais et al., 2016, Brazil	ethanol extract/leaves	isoborneol (14.66%), bornyl acetate (11.86%), *α*-humulene (11.23%), *α*-fenchene (9.32%), 1.8-cineole (7.05%),	Analgesic	Mice (*Swiss*/M)	100, 300, 1000/*p.o.*	Crude ethanol extract presented antinociceptive and anti-inflammatory activities
Oliveira et al., 2014, Brazil	hydroethanolic extract/aerial parts	NR	Analgesic	Mice (*Swiss*/M)	10, 30, 100/*p.o*.	Analgesic activity
Lippia graveolens						
González-Trujano et al., 2017, Mexico	aqueous extract/leaves	thymol (33.4%) *m*-cymen-8-ol (16.37%) methyl salicylate (10.48%) carvacrol (6.75%)	Anxiolytic	Mice (CD-1/M)	1, 3, 10, 30/*i.p*.	Exerts anxiolytic-like activity involving many kinds of constituents, mainly the terpenoids and flavonoids
L. geminate						
Forestieri et al., 1996, Italy	petroleum ether, ethanolic/aqueous extracts/leaves	flavonoids, saponins, tannins, alkaloids, sesquiterpenes, sterols	Analgesic	Mice (*Swiss*/M,F)	0.5/*p.o*.	Ethanol extract of L. germinata showed a significant analgesic activity
L. adoensis						

Makonnen et al., 2003, Ethiopia	Aqueous and ethanolic extracts/leaves	phenolic acids, flavonoids, glycosides	Analgesic	Mice (in-house bred/M)	400, 600, 800/*p.o*.	Dose-dependent analgesia produced by both aqueous and ethanol extracts.
Debella et al., 2003, Ethiopia	aqueous and ethanolic extracts/leaves	phenolic compounds	Analgesic	Mice (in-house bred/M)	400, 600, 800/*p.o*.	Water extract seems to be slightly more potent at low dose
Debell et al., 2005, Ethiopia	aqueous and ethanolic extracts/leaves	phenolic compounds	Inducing pyrexia	Mice (in-house bred/M)	50, 100, 200/*p.o*.	Aqueous extract was found to have more potent antipyretic effect than the ethanol extract.

Abbreviations: Volatile oil (VO); petroleum ether extracts (PELA); chloroform extracts (CELA); ethanol extracts (EELA) and aqueous extract (AELA); Not related (NR); Pentobarbital (PBT); Benzodiazepine (BDZ), Diazepam (DZP); Male (M); Female (F), intraperitoneal (*i.p*.), orally (*p.o*.)

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CAPÍTULO 2

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Review

Inclusion of terpenes in cyclodextrins: Preparation, characterization and pharmacological approaches

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ABSTRACT

Terpenes constitute the largest class of natural products and are important resources for the pharmaceutical, food and cosmetics industries. However, due to their low water solubility and poor bioavailability there has been a search for compounds that could improve their physicochemical properties. Cyclodextrins (natural and derived) have been proposed for this role and have been complexed with different types of terpenes. This complexation has been demonstrated by using analytical techniques for characterizing complexes such as DSC, NMR, XRD, FTIR, and TGA. The formation of inclusion complexes has been able to improve drug characteristics such as bioavailability, solubility and stability; and to enhance biological activity and efficacy. This review shows strong experimental evidence that cyclodextrins improve the pharmacological properties of terpenes, and therefore need to be recognized as being possible targets for clinical use.

Contents

Abbreviations: CDs, cyclodextrins; α-CD, alpha cyclodextrin; β-CD, beta cyclodextrin; γ-CD, gamma cyclodextrin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DSC, differential scanning calorimetry; DTG, derivative thermogravimetry; FDA, Food and Drug Administration; FTIR, Fourier transform infrared spectroscopy; GA-18β, 18β glycyrrhetinic acid; GRAS, generally recognized as safe; HepG-2, human hepatic cancer cell line; HPβCD, 2 hydroxypropyl-β-cyclodextrin; HPγCD, 2 hydroxypropyl-γ-cyclodextrin; NMR, nuclear magnetic resonance; NP, natural products; RAMEB, randomly methylated β-cyclodextrin; RYSMEB, low methylated β-cyclodextrin; SBEβCD, sulfobutyl ether β-cyclodextrin; TG, thermogravimetric; TGA, thermogravimetry; XRC, X-ray crystallography; XRD, X-ray diffraction.

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Inclusion of Terpenes in Cyclodextrins: Preparation, Characterization and Pharmacological Approaches

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Contents

Abbreviations

CDs – cyclodextrins

α-CD – alpha cyclodextrin

β-CD – beta cyclodextrin

γ-CD – gamma cyclodextrin

DPPH – 2,2-diphenyl-1-picrylhydrazyl

DSC - differential scanning calorimetry

DTG - derivative thermogravimetry

FDA – Food and Drug Administration

FTIR - Fourier transform infrared spectroscopy

GA-18β - 18β glycyrrhetinic acid

GRAS - generally recognized as safe

HepG-2 – human hepatic cancer cell line

HPβCD – 2 hydroxypropyl-β-cyclodextrin

HPγCD – 2 hydroxypropyl-γ-cyclodextrin

NMR – nuclear magnetic resonance

NP – natural products

RAMEB - randomly methylated β-cyclodextrin

RYSMEB – low methylated β-cyclodextrin

SBEβCD – sulfobutyl ether β-cyclodextrin

TG –thermogravimetric

TGA - thermogravimetry

XRC - X-ray crystallography

XRD - X-ray diffraction

Highlights

Cyclodextrin-terpene complexes are promising host-guest systems

Insolubility in water and a short life-time limit the therapeutic use of terpenes

CD inclusion complexes enhance the bioavailability, pharmacological efficacy and safety of terpenes

Abstract

Terpenes constitute the largest class of natural products and are important resources for the pharmaceutical, food and cosmetics industries. However, due to their low water solubility and poor bioavailability there has been a search for compounds that could improve their physicochemical properties. Cyclodextrins (natural and derived) have been proposed for this role and have been complexed with different types of terpenes. This complexation has been demonstrated by using analytical techniques for characterizing complexes such as DSC, NMR, XRD, FTIR, and TGA. The formation of inclusion complexes has been able to improve drug characteristics such as bioavailability, solubility and stability; and to enhance biological activity and efficacy. This review shows strong experimental evidence that cyclodextrins improve the pharmacological properties of terpenes, and therefore need to be recognized as being possible targets for clinical use.

Keywords: natural products, terpenes, cyclodextrins, pharmaceutical technology

1. Introduction

Terpenoids constitute the most abundant group of secondary metabolites in higher plants with around 30,000 compounds (Connolly & Hill, 1991; Moraes, Mescher & Tumlinson, 2001). It is a diverse family of natural products (NP) derived from C5 isoprene units joined in a head-to-tail fashion. They represent a class of NP that provide a great number of possible solutions to different human-health issues (Liu et al., 2014b).

Terpenes have significant economic value due to their widespread use in a range of industries including the pharmaceutical, food, and cosmetics sectors where they can be found as food additives, flavorings, in fragrances and spices, drug excipients (to enhance penetration of the skin) and in many other uses (Guimaraes, Serafini & Quintans-Junior, 2014; Guimaraes, Quintans & Quintans Júnior, 2013). There is a wide range of possible therapeutic pharmacological uses for terpenes including as: anticancer drugs, analgesics, anti-inflammatories, neuroprotective agents, immune modulators and in wound healing (Ali et al., 2013; Araujo-Filho, Quintans-Junior, Barreto, Almeida, Barreto & Quintans, 2016; Barreto et al., 2014; Guimaraes et al., 2015; Guimaraes, Quintans & Quintans Júnior, 2013; Rodrigues, Sieglitz & Bernardes, 2016; Quintans, Antoniolli, Almeida, Santana-Filho & Quintans-Junior, 2014)Although, there is no current data on the exact value of terpenes in the pharmaceutical market, the sales of terpene-based pharmaceuticals in 2002 were around US $12 billion (Wang, Tang & Bidigare, 2005). Terpenes can be found in many examples of where companies are in the early stages of commercializing technology or when developing new products (Erickson, Nelson & Winters, 2012), and terpene use across a range of industries shows significant annual growth, particularly in the pharmaceutical sector (Ahad et al., 2009; Guimaraes, Serafini & Quintans-Junior, 2014).

The pharmaceutical and cosmetic industries have been increasingly using drug carrier systems, such as cyclodextrins (CDs), in order to enhance the physico-chemical and pharmacological profiles of non-polar drugs, while also improving side effect profiles. The complexation of various drugs with CDs has improved their analgesic, anti-cancer and anti-inflammatory profiles, in both clinical and preclinical studies (Brito, Araujo, Quintans, Sluka & Quintans-Junior, 2015; Gidwani & Vyas, 2015; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015; Williamson, Menzies, Nair, Tutuncu & Lipworth, 2009). The growing use of CDs with terpenes to make them more bioavailable to pharmacological application has been noteworthy (Oliveira et al., 2015; Guimaraes et al., 2015). However, there are no reviews that summarize the main technological, chemical and pharmacological aspects of terpene complexation with CDs.

In this review, an overview was achieved by considering the pre-clinical, and clinical approaches of the published works on complexes between CDs and terpenes, summarizing the existing data on preparation methods and conducting an analysis and appraisal of the evidence on the results of complexation in pharmaceutical applications. The review was based on articles published in the period up to, and including, 2016. The search strategy followed the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (see supplementary material) (Moher, Liberati, Tetzlaff & Altman, 2009).

2. Terpenes

Terpenes have been isolated from living organisms, mainly plants (Singh & Sharma, 2015). These compounds are also found in bacteria (Yamada et al., 2015), fungi (Lin et al., 2014), insects - such as termites or butterflies (la Cruz, Santos Júnior, Rezende, Alves, Cancello & Rocha, 2014) and marine organisms (Hegazy et al., 2015).

In general, terpenes represent a heterogeneous group of chemical substances constructed from hydrocarbons. They consist of the combination of several 5-carbon-base units called isoprenes (C_5H_8), mainly in a head-to-tail linkage. Oxidation in the carbon skeletons, cyclizations, proton and methyl removal or shifts can lead to variations in their structures, giving rise to modified terpenes (terpenoids), with other functional groups such as alcohols, aldehydes, or ketones (Dewick, 2002; Singh & Sharma, 2015; Bach, 2010). Marine terpenes include halides, dichloroimines and isonitriles which are very rare in terrestrial plants (Hegazy et al., 2015).

Terpenes are classified according to the number of isoprene units as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes (up C_{40}) (Figure 1) (Ashour, Wink & Gershenzon, 2010; Dewick, 2002). Hemiterpenes are the simplest terpenes and less than a hundred compounds are known as isoprene and the oxygenated derivatives prenol, tiglic acid, angelic acid and isovaleric acid. Monoterpenes and sesquiterpenes are the major compounds in essential oils and occur in different skeletons: acyclic (myrcene and farnesol), monocyclic (terpinolene and α-bisabolene) and bicyclic (α-pinene and β-caryophyllene) (Singh & Sharma, 2015). Structures with heteroatoms such as oxygen (α- terpineol and artemisinin) and chloride (longifone and acanthene A) can also be found (Ashour, Wink & Gershenzon, 2010). Tricyclic rings have been reported such as α-santonin. Iridoids are also a kind of bicyclic monoterpenoid in origin, with a cyclopentane ring fused to a six-membered oxygen heterocycle, as in genipin (Nagatoshi, Terasaka, Nagatsu & Mizukami, 2011). Sesterpenes are rare, while diterpenes and triterpenes have a great structural diversity, from acyclic

compounds (teprenone sapelenin G) to complex polycyclic rings, such as triptolide and azadirachtin, with a high pattern of oxygenation (Hanson, 2009; Hill & Connolly, 2015).

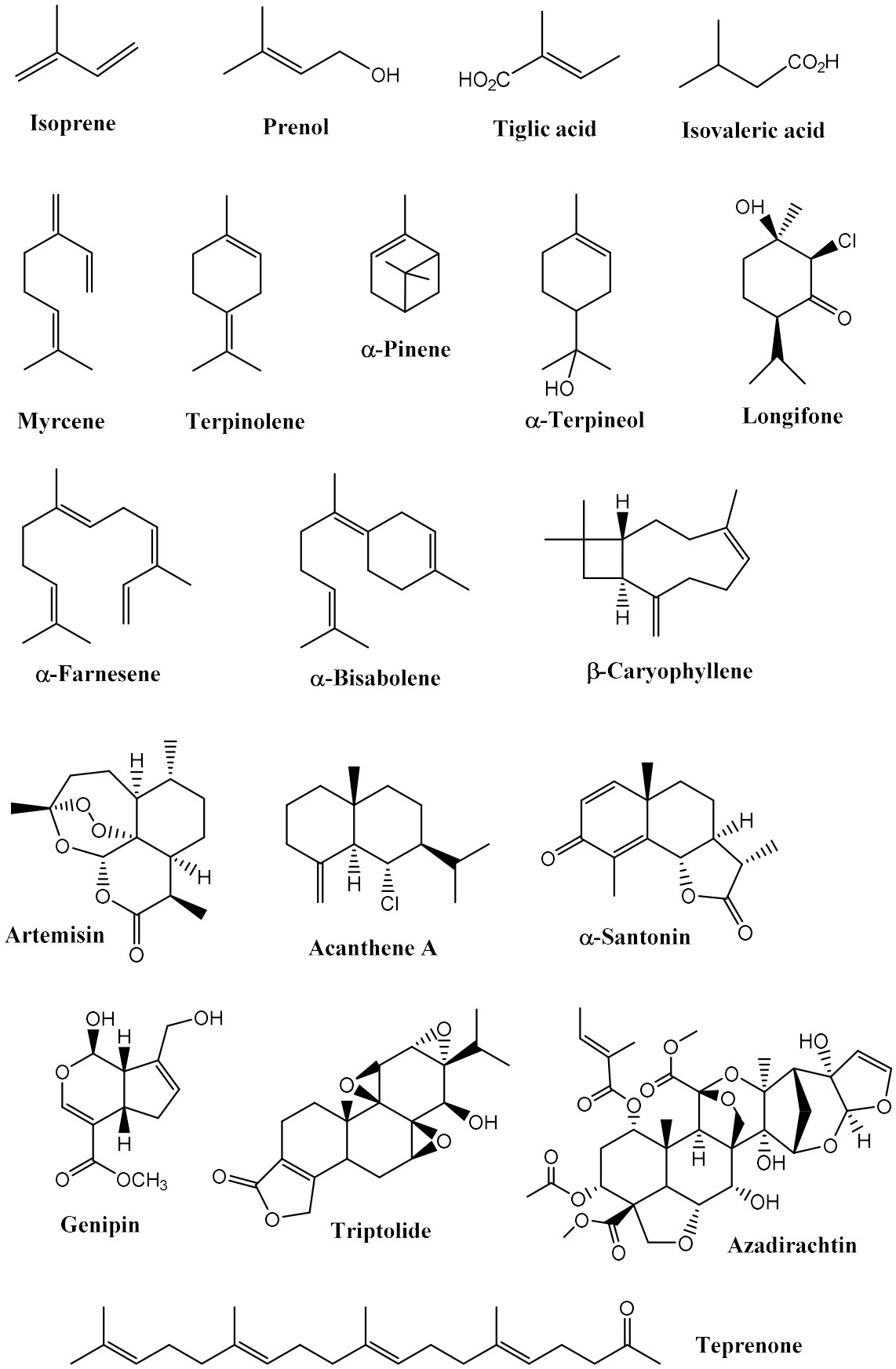

Figure 1 – Structural formulas and names of some representative terpenes

Natural product chemists have long marveled at the structural diversity of terpenes and speculated on their biosynthetic basis. In higher plants, the isoprene units of isopentenyl diphosphate and its isomer dimethylallyl diphosphate are the precursors of terpenes. These compounds can be synthesized via the mevalonic acid (MVA) pathway in the cytosol/endoplasmic reticulum, and they provide the precursors mainly for sesquiterpenoids and **triterpenoids.** The 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Croteau, Kutchan & Lewis, 2000), is responsible for the precursors of mono-, di, and tetraterpenes (Chappell, 1995; Gershenzon & Kreis, 1999).

The wealth of terpene carbon skeletons can be attributed to an enzyme class known as the terpene synthases. These catalysts convert the acyclic prenyl diphosphates and squalene into a multitude of cyclic and acyclic forms. The chief causes of terpene diversity are the large number of different terpene synthases and the fact that some terpene synthases produce multiple products (more information about this can be assessed in the excellent review published by (Degenhardt, Kollner & Gershenzon, 2009).

3. Cyclodextrins (CDs)

Currently, CDs can be found in over 35 commercially available drug products, including tablets, parenteral solutions, eye drops, ointments, and suppositories (Loftsson & Brewster, 2010; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015). Over the last two decades, cyclodextrins, a family of macrocyclic oligosaccharides, have received growing attention in the pharmaceutical field. Natural and modified CDs have been studied and some have gained Food and Drug Administration (FDA) approval or have been accredited as being “generally recognized as safe” (GRAS) (Lakkakula & Macedo Krause, 2014). Due to their properties of forming host-guest type inclusion complexes, CDs and their synthetic derivatives exhibit a wide range of utilities in different areas, such as in pharmaceuticals, in drug delivery systems, in cosmetics, in food and nutrition, in textiles, and in the chemical industry (Loftsson, 2002; Marques, 2010a; Roux, Perly & Djedaini-Pilard, 2007). This capability has led to extensive investigations into cyclodextrin applications in several different areas, with the purpose of overcoming certain limitations (Brito, Araujo, Quintans, Sluka & Quintans-Junior, 2015; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015; Sharma & Baldi, 2016; Varca, Andreo-Filho, Lopes & Ferraz, 2010).

The formation of inclusion complexes increases the guest’s *in vivo* stability against hydrolysis, oxidation, decomposition, and dehydration, as well as increasing their bioavailability (Brito, Araujo, Quintans, Sluka & Quintans-Junior, 2015; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015; Loftsson & Duchene, 2007).

CDs have frequently been used as solubilizing and stabilizing agents in pharmaceutical product development (Brewster & Loftsson, 2007). Complexation may prolong and increase the duration and the intensity of a drug's effects (Guimaraes et al., 2015; Nascimento et al., 2015; Nascimento et al., 2014; Quintans et al., 2013b) and provide enhanced drug delivery through biological membranes (Yallapu, Jaggi & Chauhan, 2010). This aforesaid complexation reduces side effects, such as gastrointestinal and ocular irritation, eliminates unpleasant odors or tastes, prevents drug-additive interactions (Polyakov, Leshina, Konovalova, Hand & Kispert, 2004), and turns liquid substances into stable and free flowing powders (Marreto et al., 2008; Waleczek, Marques, Hempel & Schmidt, 2003). Additionally, CDs are the preferred agent for the complexation of drugs in the pharmaceutical industry, because of their low price and high rates of production (Tsai, Tsai, Wu & Tsai, 2010).

3.1. Types of CD

Produced from starch by an enzymatic conversion, CDs are "naturally" (this process is done commercially) occurring cyclical oligosaccharides (Chepulis & Francis, 2012), composed of glucopyranose units linked by α-1,4-glycosidic bonds to form macrocycles. According to the number of glucopyranose units, the native CDs (Figure 2) can be classified as α-CD, β-CD and γ-CD, having six, seven and eight glucopyranose units respectively (Crini, 2014). They can be represented as a truncated cone structure with a hydrophobic cavity at their center and with a hydrophilic outer surface. Their structure enables the enclosure of highly hydrophobic molecules (guest) inside their hydrophobic cavity (host), constituting a true molecular complexation (Pinho, Grootveld, Soares & Henriques, 2014a).

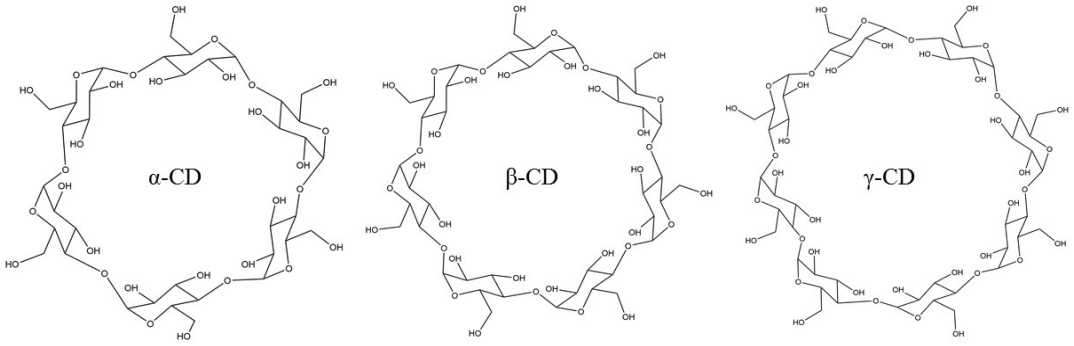

Figure 2 - Molecular structure of native cyclodextrins (ChemBioDraw Ultra 13.0 software). Adapted from (Pinho, Grootveld, Soares & Henriques, 2014a).

The choice of the suitable CD depends on the guest size because of the steric factors (Menezes et al., 2015) and the administration route of interest. With the aim of improving β-CD solubility, new CDs such as 2,6-Di-O-methyl-β-CD (DIMEB), hydroxipropil-β-cyclodextrin (HP-β-CD), randomly methylated-β-cyclodextrin (RAMEB), and sulfobutyl ether β-cyclodextrin (SBE-β-CD), were synthesized. Native and modified CDs, particularly HP-β-CD and sulfobutylether-γ-cyclodextrin (SBEγCD), are widely used as excipients in nonclinical safety studies because of their excellent physicochemical and biological properties and limited/reversible toxicity, particularly when dosed orally, but also by the intravenous (i.v.) route (Rosseels, Delaunois, Hanon, Guillaume, Martin & van den Dobbelsteen, 2013). Typically, hydrophilic CDs such as HP-β-CD, one of the β-CD derivatives, are considered toxicity free at moderate oral and intravenous doses (Liao et al., 2016).

3.2 Restrictions of CDs application and possible risks

Despite the fact that CDs may be considered as GRAS by the FDA and have recently been approved as an additive by the European Union (Arima, Hayashi, Higashi & Motoyama, 2015) they are not free of toxicity, especially when used in improper administration routes. Thus, CD toxic effects can be minimized by an appropriate choice of the CD or its derivative for the administration route (Szejtli, 1998). All toxic studies have evidenced that orally administered CDs are practically non-toxic, due to the lack of absorption from the gastrointestinal tract (Irie & Uekama, 1997). CDs are hardly absorbed throughout the gastrointestinal tract and are degraded by bacterial enzymes in the colon where they are treated as glucopyranose. This feature can be exploited for drug release into the colon through the drug's combination with CDs, which functions as a prodrug medication. As a result, the drug is only released in the colon after its connection to the CDs has been degraded. The drug can, therefore, act locally without any extensive systemic absorption, which is often responsible for the adverse effects of drugs (Hirayama, Minami & Uekama, 1996; Minami, Hirayama & Uekama, 1998; Uekama, Minami & Hirayama, 1997).

CDs for an enteric preparation can be classified as a time-controlled release agent, since the drug is preferentially released in the intestinal tract (Rasheed & Kumar, 2008). This drug-guest system seems to increase bioavailability, and consequently, is able to improve the therapeutic profiles of volatile compounds such as monoterpenes (Pinho, Grootveld, Soares & Henriques, 2014b; Marques, 2010a). Corroborating this approach, an intravenous dose of glaucocalyxin A, a diterpene, was made possible due to the use of an inclusion complex with SBE-β-CD, demonstrating water solubility significantly higher than that of β-CD. Additionally, it did not exhibit the nephrotoxicity associated with β-CD and provided a protective effect against drug-induced cytotoxicity. This resulted in a better solubility and a sustained function of the drug's delivery (Ren, Jing, Chen, Miao

& Wei, 2014). In fact, choosing the best CDs for pharmacological applications depends upon the chemical characteristics of the non-polar compound that is being complexed. If the CD is a wildcard, by improving the drug's biological activity, it minimizes any side effects.

In our investigation, the characteristics of the studies described in relation to the formation of the inclusion complexes (Table 1), were examined in order to determine the efficiency of the complexation process.

The naturally occurring β-CD was present in most of the studies (76%), of which 40% studied only the β-CD complex, and 35% studied one or more branches of the β-CD complex. The derivate CDs improved their toxicological profiles due a chemical modification of their hydroxyl groups. of the natural CDs. Thus, many authors employed modified CDs, such as: M-β-CD (methyl-β-cyclodextrin), HP-β-CD (hydroxypropyl-β-cyclodextrin), HP-γ-CD (hydroxypropyl-γ-cyclodextrin), and SBE-β-CD (sulfobutylether-β-cyclodextrin), which all offer a high tolerance in the human body and open new doors for the development of improved, more efficient injectable formulations (Kurkov & Loftsson, 2013). The HP-β-CD complex appeared in 23% of the works and is a very interesting β-CD derivation with improved water solubility (>70wt %). Furthermore, due to hydroxypropyl substitution, the process of complexation with HP-β-CD was much facilitated when compared with the native β-CD. In contrast, only a few studies chose α- and γ- CDs to complex the natural products, probably related to the high cost.

The primary routes of administration were orally and intraperitoneally. The main positive effects that were observed, and that were due to the use of inclusion complexes were: an improvement in bioavailability (37%), better solubility (34%), an enhancement of biological activity (23%), an enhanced efficacy (15%), and increased stability (15%). Furthermore, it was observed that there were notable reductions in side effects and cytotoxicity, and a prolonged time effect.

Table 1 - Characteristics of the studies that described the formation of the inclusion complexes

Reference	Classification Substance	Type of CD*	Method of preparation	Evidence of complexation		
				Characterization techniques**	Ratio of complexation (guest-host: G-H)	General aspects described
Monoterpenes						
(Donze & Coleman, 1993), France	(-)-borneol, terpineol, (+)-camphor, (-)-carvone , geraniol , (+)-linalool, cineole, (-)-fenchone, (+)-isomenthol, citral, thymol, (-)-menthone, (+)-menthol, o-cresol, eugenol, (+)-limonene, (-)-bornyl acetate, anethol, (+)-camphene, (-)-pinene, myrcene	β-CD	Co-precipitation	NMR	1:1	Physical properties of 21 substances were not determinants alone complexation.
(Yoshii, Furuta, Yasunishi & Hirano, 1994), Japan	(+)-limonene	α-CD β-CD γ-CD	Micro-aqueous	Crystal analysis	1:1, 2:1 and 2:3	Low water content in the cavity of CD because replace by D-Limonene caused through ethanol effect.
(Moeder, O'Brien, Thompson & Bicker, 1996),	(+) and (-)-α-pinene, (+) and (-)-β-pinene, (+) and (-)-limonene, (+) and (-)-camphene	β-CD α-CD	Not reported	Stoichiometric coefficients of complexation,	1:1 and 1:2	A difference in apparent formation constants was only observed when a 1:2 terpene-CD complex was formed.

USA				Apparent formation constants		
(Eddaoudi, Coleman & Baszkin, 1997), France	borneol, (-)-carvone, (+)-carvone, (-)-menthol, (+)-menthol,	DIMET-β-CD	Not reported	Surface Potential, Orthogonal Dipoles, Molecular modeling	Not reported	The direction of surface potential determined changes in the packing density of the CDs.
(Ajisaka, Hara, Mikuni, Hara & Hashimoto, 2000), Japan	(+)-limonene, terpinolene, geraniol, nerol, α-terpineol, citral, (+)-citronellal, (-)-perillaldehyde, (-)-carvone and menthone	β-CD, G2-β-CD GUG-β-CD	Co-precipitation	GC	1:1	G2 and GUG-modified βCDs showed greater stabilizing ability for terpenes than unmodified βCD.
(Demian, 2000), USA	linalool, carvone, thymol anethole, cinnamic aldehyde cinnamyl acetate carvone, linalool, methyl salicylate, thymol	HP-β-CD	Not reported	UV/VIS	1:2	The inclusion complex showed quantitative structure–property relationship involving steric and hydrophobicity parameters.
(Asztemborska, Bielejewska, Duszczyk & Sybilska, 2000), Poland	(+) and (-)-camphor	α-CD β-CD	Not reported	G-L, HPLC	1:1 and 1:2	Only α-CD recognized enantiomers of camphor, but both CDs formed inclusion complex.

(Silva, Empis & Teixeira-Dias, 2002), Portugal	Carvone	β-CD	Co-evaporation	NMR	1:1	The chiral discrimination against carvone enantiomers led to different water content and structures in the inclusion compounds.
(Yu et al., 2003), France	Camphor	α-CD β-CD γ-CD	Recrystallization	Phase-solubility, NMR, Circular dichroism	1:2	The techniques showed complexation of camphor with all CDs.
(Dodziuk, Nowinski, Kozminski & Dolgonos, 2003), Poland	camphor	α-CD	Not reported	NMR	1:1	NMR showed that is impossible to determinate the binding constant for the camphor 1:2 complex.
(Asztemborska, Sybilska, Nowakowski & Perez, 2003), Poland	(+) and (-)-α-pinene, (+) and (-)-β-pinene, citronellene, citronellal menthone, pulegone, fenchone, linalool, neomenthol, α-terpineol, isopinocampheol, (+) and (-)-limonene, (-)-α-phellandrene, (+) and (-)-camphene, carvone, terpinen-4-ol	α-CD	Not reported	GC	1:1 and 1:2	Some terpenes were complexed in α-CD cavity, others not complexed because remarkable enantioselectivity.
(Liu et al., 2004), China	cyclopentanol, cyclohexanol, cycloheptanol, cyclooctanol), (±)-	β –CD	Not reported	NMR, ITC, ROESY	1:1	The binding modes revealed that the enhanced molecular and chiral recognition ability was mainly

	borneol, and (±)-camphor	6P-β-CD 6F-β-CD 6N-β-CD 6B-β-CD 6C-β-CD 6H-β-CD				attributed to the stronger induced-fit interaction between modified β-CDs and guest molecules.
(Locci, Lai, Piras, Marongiu & Lai, 2004), Italy	carvacrol, thymol, and eugenol	β-CD	Supercritical Carbon Dioxide	NMR	Not reported	Successfully complexation by the used method.
(Skorka, Asztemborska & Zukowski, 2005), Poland	(+)- and (−)-β-pinene, (+) and (−)-fenchone, (+)-and (−)-menthone, (+)- and (−)-pulegone, (+)- and (−)- α-pinene, (+)- and (−)-limonene, (+)- and (−)-isomenthone	α-CD β-CD	Not reported	Distribution constants, stability constants of inclusion complexes, Thermodynamic parameters, Absolute enantioselectivity, Enthalpy–entropy compensation	1:1 and 1:2	Complexation processes were enthalpy-driven and α-CD can be a better host molecule for monoterpenes guests.
	linalool	HP-β-CD	Co-evaporation	NMR, HPLC,	1:1 and 1:2	

(Numanoglu, Sen, Tarimci, Kartal, Koo & Onyuksel, 2007), Turkey				Circular Dichroism		The inclusion complex with HP-β-CD indeed occurred, increased solubility and stability.
(Liu, Zhang & Chen, 2007), China	borneol and camphor	MTD-β-CD MAD-β-CD MUD-β-CD	Synthesis of three nucleobase-modified β-CD	ITC, Circular Dichroism, NMR, Molecular modeling	1:1	The interaction of borneol with nucleobase-modified β-CD occurred preferably in the enthalpy of the system as to the camphor was the entropy.
(Mourtzinos, Kalogeropoulos, Papadakis, Konstantinou & Karathanos, 2008), Greece	thymol and geraniol	β-CD	Freeze-drying	GC-MS, DSC	1:1	Complexation of terpenes with higher water solubility and the inclusion complexes were protected from oxidation.
(Moon et al., 2008), Korea	Pulegone	β-CD γ-CD	Co-precipitation	UV/VIS, NMR, FTIR, TGA	1:1	Pulegone formed inclusion complexes with both CDs and substantially stabilized in aqueous solution as well as solid state.
	(+)- and (-)-camphor	α-CD	Co-precipitation	XRC, molecular	1:1	The dimers formed inclusion complexes with α-CD

(Kokkinou, Tsorteki, Karpusas, Papakyriakou, Bethanis & Mentzafos, 2010), Greece				dynamics simulations		and the carbonyl oxygen of the dimers switched between different hydrogen bonding partners.
(Rungsardthong Ruktanonchai, Srinuanchai, Saesoo, Sramala, Puttipipatkhachorn & Soottitantawat, 2011), Thailand	Citral	α-CD, β-CD and HP-β-CD	Freeze-drying	Computer modeling, complexation efficiency, XRD, DSC	1:1 and 1:2	β-CD was the best candidate for to complex citral and the inclusion complex was functioned as excellent controlled release system.
(Rukmani & Sundrarajan, 2012), India	Thymol	β-CD	Loading of thymol on ungrafted and β-CD grafted fabric	UV/VIS, FTIR, HPLC	Not reported	UV-visible and FTIR spectral studies showed the presence of thymol complexed in β-CD on the fabrics.
(Menezes et al.,	Geraniol	β-CD	Paste and slurry complex	DSC, TGA, KF FTIR, SEM	Not reported	The complexation occurred preferably through paste complex.

2012), Brazil						
(Su et al., 2012a), China	Borneol	β-CD	Ultrasound	GC, DSC, XRD, FTIR	1:1, 1:4, 1:6, and 1:8	The stability and aqueous solubility of terpene were significantly increased by inclusion with *β*-CD.
(Su et al., 2012b), China	(+)-borneol	HP-β-CD	Ultrasound	DSC, XRD, FTIR	1:6	(+)-borneol formed inclusion complexes with HP-β-CD and had stability against heat and light.
(Serafini et al., 2012), Brazil	p-cymene	β-CD	Slurry and paste methods	DSC, TGA/DTG, FTIR, KF, SEM	1:1	p-cymene was efficiently complexed with β-CD by paste method.
(Ceborska, Szwed & Suwinska, 2013), Poland	Isopulegol	β-CD	Co-precipitation	HPLC, XRC	1:1	The more stable complexes with β-CD crystal structures were obtained showing the formation of molecular capsules forming molecular container.
(Ceborska, Asztemborska, Luboradzki & Lipkowski,	camphene and fenchene	β-CD	Co-precipitation	NMR, GC, XRC	1:1	The 1:1 complexes of both (+/−) camphene and fenchene with β-CD were obtained with stability constant calculated.

2013), Poland						
(Ciobanu, Landy & Fourmentin, 2013), France	α-pinene, β-pinene, camphene, eucalyptol, limonene, linalool, p-cymene, myrcene, menthone, menthol, trans-anethole, pulegone and camphor	α-CD β-CD γ-CD HP-β-CD RAMEB CRYSMEB	Static headspace gas chromatography	Determination of the stability constant, Complexation efficiency	1:1	α-CD and γ-CD gave generally lower stability constants than β-CDs.
(Christoforides, Mentzafos & Bethanis, 2014)	(+) and (-)- borneol	α-CD β-CD	Co-precipitation	XRC	2:1	Crystallographic analysis showed that no significant differences concerning the inclusion geometry and crystal packing were observed between the inclusion complexes of the borneol enantiomers with the same host CD
(Menezes et al., 2014), Brazil	(-)-linalool	β-CD	Paste and Slurry complex	DSC, TGA/DTG, FTIR, SEM, XRD, GC/MS	1:0.86 and 1:1	The inclusion complex was totally formed through slurry method.
(Nowakowski & Ejchart, 2014), Poland	(+) and (-)-fenchone	α-CD	Not reported	NMR	1:2	Stoichiometry and sequential association constants was determined for diastereomeric complexes of fenchone enantiomers with α-CD

(Kayaci, Sen, Durgun & Uyar, 2014), Turkey	Geraniol	α-CD, β-CD and γ-CD	Co-precipitation	XRD, TGA, NMR, Computer modeling	0.78:1, 0.9:1 and 1:1	The geraniol was complexed preferably with γ-CD because larger dimension of cavity.
(Nieddu et al., 2014a), Italy	Thymol	β-CD	Sealed-heating and co-precipitation	Complexation efficiency, Thymol release, DSC	Not reported	β-CD accelerated the *in vivo* thymol absorption rate compared with the free drug.
(Kfoury, Auezova, Fourmentin & Greige-Gerges, 2014b), France	eucalyptol, geraniol, limonene, linalool, α-pinene, β-pinene, pulegone and thymol	HP-β-CD	Freeze drying	FTIR, DSC, Complexation efficiency	1:1	All the monoterpenes were complexed with successfully by hydrophobic interactions driving forces.
(Kfoury, Balan, Landy, Nistor & Fourmentin, 2015), France	β-caryophyllene, cis-ocimene, trans-ocimene, sabinene hydrate (thujanol), γ-terpinene and α-terpineol	α-CD β-CD γ-CD HP-β-CD RAMEB CRYSMEB	Static headspace-GC	Retention studies, Determination of formation constants, UV/VIS, Molecular modeling,	1:1	The inclusion stability depended mainly on geometric fit between CD cavity and the encapsulated compound.
(Guimaraes et al., 2015), Brazil	Carvacrol	β-CD	Slurry complex	DSC TGA/DTG	Not reported	Slurry complexation method showed a better inclusion profile of the carvacrol than physical mixture.

				SEM		
(Zhu, Feng, Xiao, Zhou & Niu, 2015), Hungary	Citral	MCT-β-CD	Freeze-drying	TEM, SEM, FTIR, TGA, Molecular mechanics calculations	1:1	The inclusion complexes were obtained with loading capacity was about 8.96 %.
(Xiao, Feng, Zhu & Niu, 2016), China	Citral	MCT-β-CD	Freeze-drying and after grafted Fabrics	UV/VIS, DLS, DSC, TGA, XRD, FTIR, SEM	1:1	The results indicated that the loading capacity is about 9%, which is close to the theoretical load. Its mean particle size is about 200–300 nm in a narrow distribution.
(Moreira et al., 2016)	(-)-β-pinene	β-CD	Physical mixture and slurry complex	DSC, TGA/DTG, FTIR, XRD, docking, SEM	1:1	The physicochemical characterization showed (-)-β-pinene/β-CD complex formation.
(Dos Passos Menezes et al., 2016)	Limonene	α-CD β-CD	Physical mixture, paste and slurry complex	TGA/DTG, FTIR, XRD, GCMS, molecular modeling, NMR	1:1	Total inclusion complexes were not formed, but interactions outside of the cavity of CDs were observed through the PC and SC methods by the *in silico* data.
Sesquiterpenes						

(Usuda, Endo, Nagase, Tomono & Ueda, 2000), Japan	Artemisinin	α-CD β-CD γ-CD HP-β-CD SBE-β-CD DM-β-CD PM-β-CD G1-β-CD G2-β-CD	Heating in a sealed container	UV/VIS, Phase solubility	Not reported	The addition of CDs enabled the solubilization of artemisinin.
(Wong & Yuen, 2001), Malaysia	Artemisinin	β-CD γ-CD	Slurry complex	Not reported	Not reported	Complexes exhibited a higher rate and extent of artemisinin absorption compared to the reference preparation.
(Illapakurthy, Sabnis, Avery, Avery & Wyandt, 2003), USA	artemisinin, artether, aihydroartemisinin, aeoxoartemisinin	HP-β-CD	Not reported	Phase-Solubility, Molecular Modeling	1:1	The molecular modeling technique seemed to offer a better correlation in terms of inclusion modes and docking scores for the treatment of inclusion complexes.

(Wong & Yuen, 2003a), Malaysia	Artemisinin	α-CD β-CD γ-CD	Slurry complex	DSC, Phase solubility, Dissolution	1:1	The complexation process had negative enthalpy and occurred spontaneously. Furthermore, the dissolution profiles were increased in rate and extent.
(Waleczek, Marques, Hempel & Schmidt, 2003), Germany	(-)-α-bisabolol camomile oil	β-CD	Co-precipitation	Phase solubility, GC, n-hexane-washing, Leitz hot-stage, Microscopy, Molecular modeling	2:1	An excess amount of β-CD improved the stability of the complexes leading to a higher inclusion rate in a 2:1 complex.
(Hartell, Hicks, Bhattacharjee, Koser, Carvalho & Van Hamont, 2004), USA	artelinic acid and artesunic acid	β-CD	Not reported	UV/VIS, NMR, Molecular modeling	1:1, 2:1, 2:2 and 3:1	Combined NMR and molecular modeling study clearly demonstrates that both artelinic acid and artesunic acid will form complexes with natural βCD.
(Illapakurthy, Wyandt & Stodghill, 2005), USA	artemisinin and naproxen	HP-β-CD	Co-precipitation	ITC	Not reported	The complexation was primarily driven by enthalpy with entropic assistance at all temperatures studied.

(Ziémons et al., 2007), Belgium	tagitinin C	β-CD γ-CD DIMEB	Not reported	UV/VIS, Electrochemical determination, HPLC, NMR, ROESY, molecular modeling	1:1	Tagitinin C formed inclusion complexes with the three forms of CDs and the complexation with 2,6-Di-O-methyl-β-CD was the most power.
(Ansari, Iqbal & Sunderland, 2009), Pakistan	dihydroartemisinin	HP-β-CD	Co-precipitation	DSC, FTIR, XRD	1:1	HP-β-CD complexation with recrystallized dihydroartemisinin increased its solubility and stability.
(Yang, Lin, Chen & Liu, 2009), China	artemether	HP-β-CD	Slurry complex	NMR, Phase-solubility, XRD, DSC, TGA	1:1	The inclusion complex was more stable and could enhance the water solubility of artemether.
(Shah & Mashru, 2010), India	Artemether	β-CD	Kneading	Phase solubility, FTIR, DSC, XRD, SEM, Dissolution	Not reported	CD molecules could be used to increasing the hydrophilicity of the drug and decreasing the retention of bitter drug molecules on the tongue's surface.

(Ansari, Batty, Iqbal & Sunderland, 2011), Pakistan	Dihydroartemisinin	HP-β-CD	Co-evaporation	XRD, DSC	1:1	Dihydroartemisinin-HP-β-CD complexes exhibited a complete amorphous state, as verified by XRD patterns and DSC thermograms.
(Kakran, Sahoo, Li & Judeh, 2011), Singapore	Artemisinin	β-CD	Co-evaporation	SEM, FTIR, XRD, DSC	1:1, 1:2 and 1:4	The complex formed showed a significantly faster dissolution rates than that of the free artemisinin.
(Liu et al., 2013), China	β –caryophyllene	β-CD	Co-precipitation	UV/VIS, DTA, FTIR	6:1	β –Caryophyllene/ β-CD delivery system was able to improve its absorption in rats.
(Ghasemali et al., 2013), Iran	Helenalin	β-CD	Freeze drying	SEM, Complexation efficiency FTIR	Not reported	β-CD could be superior carrier for this kind of hydrophobic agent.
(Jiang et al., 2014), China	Artesunate	β-CD	Not reported	NMR, HRMS, XRD, TGA	1:1	The inclusion complex was formed by bound covalently. Furthermore, its aqueous solubility of was 26–45 times better than free artesunate.
(Quintans-Júnior et al., 2016)	β-caryophyllene	β-CD	Physical mixture and slurry complex	DSC, TGA/DTG, FTIR, XRD, SEM	1:1	The characterization tests indicated that β-caryophyllene were efficiently incorporated into βCD

Diterpenes						
(Correa, Melo, de Carvalho, de Azevedo, Duran & Haun, 2005), Brazil	trans-dehydrocrotonin	β-CD	Co-evaporation	XRD, DSC, SEM, TGA	1:2	Formed inclusion complex and decrease in the cytotoxicity of complexed dehydrocrotonin compared to free dehydrocrotonin.
(Yuexian, Junfen & Chuan, 2005), China	tanshinone IIA and tanshinone I	β-CD	Coprecipitation	NMR, FTIR, DSC, UV/VIS	1:1	The inclusion process of CD-tanshinone was a simultaneous and enthalpy-controlled process and it satisfied the law of enthalpy–entropy compensation.
(Ling, Rui & Hua, 2007), China	tanshinone IIA	HP-β-CD	Co-evaporation	Phase solubility, determination of tanshinone IIA, Dissolution	1:1	The inclusion complex demonstrated was much faster dissolution ratio, increased solubility and decreased in crystallinity.
(Yan et al., 2008), China	Oridonin	HP-β-CD	Freeze-drying	DSC, XRD, FTIR, NMR	1:1	The solubility of oridonin was greatly enhanced by forming inclusion complex and this had no significant effect on the i.v. pharmacokinetics in rats.

(Saruta, Fukami, Furuishi, Suzuki & Tomono, 2010), Japan	Teprenone	α-CD β-CD γ-CD	Kneading	TGA, XRD, FTIR, NMR	1:2	The inclusion complex formed with showed improved photostability.
(Liu, Zhao, Li & Liu, 2011), China	Oridonin	β-CD	Not reported	NMR, ROESY	1:1	1:1 stoichiometry complexes were formed between three oridonin derivatives and CDs.
(Danel et al., 2011), France	Triptolide	α-CD β-CD γ-CD HP-α-CD HP-β-CD, HP-γ-CD CM-βCD amino-βCD	Not reported	ACE, ITC, NMR	1:1	Both HP-βCD and amino-βCD displayed interesting binding capacities.
(Ren, Jing, Chen, Miao & Wei, 2014), China	glaucocalyxin A	SBE-β-CD	Co-evaporation	UV/VIS, DSC, FTIR, XRD, NMR	1:1	Glaucocalyxin A formed inclusion complexes, significantly improved the solubility and dissolution behavior with sustained delivery of the drug.
Triterpenes						

(Rode et al., 2003), Germany	Sericoside	γ-CD HP-γ-CD M-β-CD HP-β-CD	Co-evaporation	NMR, XRD, TGA, DSC	1:2, 1:2.2 and 1:2.6	The inclusion complex improved the solubility of sericoside and only complexes with γ-CD were stable.
(Guo, Zhang, Song, Wang, Liu & Liu, 2003), China	oleanolic acid	α-CD β-CD γ-CD	Freeze-drying	Solubility study and ESI/MS	1:1 and 2:1	Direct correlation between stability and binding forces of complexes in solution and gas phases.
(Liu, Chen, Chen & Lin, 2005), China	Azadirachtin	β -CD DM-β-CD TM-β-CD HP-β-CD	Co-precipitation	XRD, TGA, DTA, DSC, UV/VIS, NMR	1:1 and 2:1	The binding behavior was mainly dependent on the individual structural features of host and guest.
(Shen, Yang, Wu & Ma, 2005), China	Gossypol	β-CD	Prepared under microwave Irradiation	UV/VIS, FTIR, NMR, DSC, TGA	1:2	Gossypol formed inclusion complexes with β-CD by the mentioned technique.
(Shen, Ying, Yang & Wu, 2006), China	Gossypol	β-CD	Prepared under microwave Irradiation	UV/VIS, Fluorescence spectral, SEM, XRD	Not reported	The formation of the inclusion complex could be proposed as a new formulation to optimize its pharmacological profile.

(Soica et al., 2012), Romania	Betulin	GCDG	Kneading	SEM, DSC	Not reported	The disappearance of the sublimation peak of botulin was direct evidence of the formation of a real inclusion complex.
(Liu, Zhang, Wang, Sun & Diao, 2013), China	ilexgenin A	CDP	Co-precipitation	NMR, FTIR, UV/VIS	2:1	Ilexgenin A formed inclusion complexes with CDP, water-soluble host CDP remarkably improved the aqueous solubility of IGA and might prevent obesity.
(Liu et al., 2014a), China	Pedunculoside	β-CD	Co-evaporation	FTIR, UV/VIS, NMR, XRD, TGA	2:1	All the techniques confirmed the complexation.
(Soica et al., 2014b), Romania	betulinic acid	GCDG	Kneading	SEM, DSC, XRD,	Not reported	The techniques evidenced the complexation.
(Izutani, Kanaori & Oda, 2014), Japan	glycyrrhizic acid	α-CD, β-CD and γ-CD	Not reported	DLS, ITC, NMR	Not reported	The binding of glycyrrhizic acid to γ-CD was higher than that to β-CD, however, in both cases, the binding was mostly due to hydrophobic interactions with dehydration.

Iridoids						
(Lu, Zhang, Tao, Ji & Wang, 2009), China	Genipin	β-CD	Freeze drying	FTIR, UV/VIS, DSC, XRD, Phase solubility, stability, Pharmacokinetic	1:1	The concentrations of genipin decreased showed significant influence of pH on aqueous solution.
(Zhang, Meng, Cui & Song, 2011), China	Genipin	HP-β-CD	Freeze drying	NMR, FTIR, DSC, XRD, Dissolution,	1:1	The inclusion complex exhibited higher solubility and superior dissolution when compared to genipin alone.

* 2,3,6-partially methylated-β-CD (PM-β-CD), 2,6-Di-O-methyl-β-CD (DIMEB), 3 Mono-(6-thy-6-deoxy)-β-CD (MTDβCD), 6-O-(4-bromophenyl)-β-CD(6B-β-CD), 6-O-(4-chlorophenyl)]-β-CD (6C-β-CD), 6-O-(4-formylphenyl)-β-CD (6F-β-CD, 6-O-(4-nitrophenyl)-β-CD (6N-β-CD), 6-O-(4-hydroxybenzoyl)-β-CD (6H-β-CD), 6-O-phenyl-β-CD (6P-β-CD), 6-O-α-(4-O-α-D-glucuronyl)-D-glucosyl-βCD (GUG-βCD), 6-O-α-maltosyl-β-CD (G2-β-CD), carboxymethyl-β-cyclodextrin (CM-βCD), dimethyl-β-cyclodextrin (DM-β-CD), glucosyl- β-CD (G1-β-CD), heptakistert-butyldimethylsilyl-β-cyclodextrin (DIMETβCD), hydroxipropil-β-cyclodextrin (HP-β-CD), low methylated-β-cyclodextrin (CRYSMEB), maltosyl-β-CD (G2-β-CD), methylated-β-cyclodextrin (M-β-CD), mono(6-ade-6-deoxy)-β-CD (MADβCD), mono(6-ura-6-deoxy)-β-CD (MUDβCD), monochlorotriazine-β-cyclodextrin (MCT-β-CD), octakis-[6-deoxy-6-(2-sulfanyl ethanesulfonate)]-γ-CD (GCDG), permethyl-β-cyclodextrin (TMβCD), polymer β-cyclodextrin (CDP), randomly methylated-β-cyclodextrin (RAMEB), sulfobutyl ether β-cyclodextrin (SBE-β-CD).

** Affinity capillary electrophoresis (ACE), Derivate thermogravimetry (DTG), Differential scanning calorimetry (DSC), Differential thermal analysis (DTA), Dynamic light Scattering (DLS), Electrospray ionization (ESI), Fourier transform infrared spectroscopy (FTIR), Gas-chromatography (GC), Gas Chromatography/Mass Spectrometry (GCMS), Gas-liquid-chromatography (G-L), High performance liquid chromatography (HPLC), High Resolution Mass Spectrometry (HRMS), Isothermal Titration Calorimetry (ITC), Karl Fisher analysis (KF), Mass spectrometric (MS), Nuclear magnetic resonance (NMR), Rotating-frame Overhauser Spectroscopy (ROESY), Scanning electronic microscopy (SEM), Thermogravimetry (TGA), Transmission electron microscopy (TEM), Ultraviolet–visible spectroscopy (UV/VIS), X-ray crystallography (XRC), X-ray diffraction (XRD).

3.3 Inclusion methods and complexation efficiency

The effectiveness of CD applications is related to the amount of the guest that is included in the cavity. This percentage depends on both the stability constant and the host and guest concentrations. The strength of the host-guest complexation depends mainly on the polarity and the geometric accommodation between the CD cavity and the guest molecule. These parameters play a critical role in determining the stoichiometry and the formation constant (Kf) of the inclusion complexes (Kfoury, Auezova, Fourmentin & Greige-Gerges, 2014a; Marques, 2010b). Nieddu et al. (2014a) proposed the following non-conventional method of preparation for the inclusion complex and observed that it showed a very good loading efficiency.

The inclusion complex that was prepared by sealed-heating, using a 1:1 molar ratio between the two components, was able to increase the dissolution rate of thymol, a phenol monoterpene, which dissolved slowly in gastrointestinal-simulated fluid. This effect is due to the well-known consequence of β-CD acting as a solubilizer of substances that are poorly water soluble (Nieddu et al., 2014b). It is possible that this method of preparation can produce a better complexation ratio. In addition, Rungsardthong Ruktanonchai et al. (2011) studied citral, a monoterpene that is present in the essential oils of citrus fruits, and its complexation with CDs by a freeze-drying method. The obtained total complexation was with a 1:1 and a 1:2 molar ratio. Menezes et al. (2014) demonstrated a partial complexation for a paste method and a total complexation for a slurry method (1:0.86 and 1:1, respectively). In these cases, the efficiency of a complexation depends upon the affinity and the size of drug, as well as the type of CD and method of preparation (Valle, 2004).

Another complexation procedure is the solid-state mechanochemical method, which has been developed through dynamic interdisciplinary research, and has been described in the literature in studies, including those with natural products (Dushkin, 2010; Voinovich, Perissutti, Grassi, Passerini & Bigotto, 2009). The main advantages of this method are its speed, simplicity, cost-effectiveness, easy scalability and the reduction of waste and solvent use (when required), which makes it an attractive option for several application fields (Rinaldi, Binello, Stolle, Curini & Cravotto, 2015; Cravotto, Garella, Carnaroglio, Gaudino & Rosati, 2012). The method consists of grinding treatments that may dramatically enhance the solubility and bioavailability of poorly water-soluble drugs (Xie, Yang, Shi, Zhu, Su & Wang, 2013) due to particle size

reduction, higher surface area and porosity. Ultrafine milling is a straightforward technique based on impact forces and friction effects, which result in large amounts of mechanical energy that usually cause structural changes, and even chemical reactions including the enhancement of molecular interaction and complex formation. Moreover, characteristics such as solubility and dissolution rate are improved because of the reduced particle size (Bragagni, Maestrelli & Mura, 2010; Iwata et al., 2009).

These characteristics of CDs are interesting in relation to complex compounds, such as terpenes, that present many limitations due to their low solubility in an aqueous medium (Quintans-Júnior et al., 2016; Siqueira-Lima et al., 2014). CDs are usually able to enhance the bioavailability of insoluble compounds by increasing their drug solubility, their dissolution, and their drug permeability (Rasheed & Kumar, 2008). These effects corroborate previous findings that CDs can improve various aspects of non-polar compounds (Pinho, Grootveld, Soares & Henriques, 2014a). However, this does not appear to be feasible for all non-polar compounds due to the different complexation rates of major compounds. A better complexation with CDs is usually found with compounds and/or active substances from essential oils (Menezes et al., 2015; Pinho, Grootveld, Soares & Henriques, 2014a; Siqueira-Lima et al., 2014).

The biomedical applications of CDs are extremely attractive due to their low toxicity and their low immunogenicity, and thus, they create a wide possibility for complexing different drugs (Irie & Uekama, 1997; Zhang & Ma, 2013). However, CDs do not readily permeate biological membranes due to their chemical structure, their molecular weight, and their very low octanol/water partition coefficient (Loftsson, Sigfusson, Sigurdsson & Masson, 2003). Only the free form of the drug, which was in equilibrium with the drug/CD complexes, was capable of penetrating the lipophilic membranes. Thus, the physicochemical properties of the drug (e.g., its solubility in water), the composition of the drug's formulation (e.g., aqueous or non-aqueous), and the physiological composition of the membrane barrier (e.g., the presence of an aqueous diffusion layer), will determine whether the CDs will enhance or will hamper the drug's delivery through a biological membrane (Loftsson, Masson & Brewster, 2004). This seems to be quite advantageous for natural non-polar compounds such as terpenes (Brito, Araujo, Quintans, Sluka & Quintans-Junior, 2015; Guimaraes et al., 2015; Marques, 2010a; Pinho, Grootveld, Soares & Henriques, 2014a; Quintans et al., 2013b).

3.4 Evidence of Complexation

Some authors have reported that the relatively hydrophobic cavity of a CD can accommodate non-polar molecules (such as terpenes) to form inclusion complexes (Marques, 2010a; Pinho, Grootveld, Soares & Henriques, 2014a).

According to Ciobanu, Landy and Fourmentin (2013), the complexation behavior of several types of CDs, with 13 monoterpenes focused on by the authors, suggested that β-CD had better complexation efficiency than did α-CD and γ-CD because the cavity size is suitable for small molecules. Indeed, β-CD might complex between 80% and 99% of the volatile flavor compounds that are present in food. Moreover, the complexation efficiency of native β-CD is close to that of the modified β-CDs (HP β-CD, RAMEB, and CRYSMEB). However, the solubility of modified β-CDs is greatly enhanced when compared to native β-CDs, and higher complexation efficiencies might be obtained.

It is interesting to note that measurements of complexation efficiency should be carried out in order to determine the drug complexation percentage in the cavity of the CDs, as well to establish whether the complexation was full or partial, based on the host:guest ratio. This kind of a technique was only performed in 7% of the studies analyzed, using HPLC, UV/vis and GC/MS. These analyses allow the level of drug entrapment in the final product as well as the host:guest ratio to be measured. Haloci et al (2014) in a study of the essential oil of *Satureja montana* reported the probable reasons for material loss during the complexation procedure can be attributed to the oil, CD and complex dissipation. There are several factors which may contribute to the loss of oil such as retention of the oil in the solution after forming the complex, equilibrium of flavors between the liquid and the complexed state, evaporation of surface oil during the long complexation process and evaporation during the drying stage. The loss of the CD and complex powder can be mainly attributed to their solubility in water (Haloci, Toska, Shkreli, Goci, Vertuani & Manfredini, 2014).

3.4.1. Spectroscopic characterization of the complexes

The analytical techniques of characterization that are required to assess the formation of a drug-CD inclusion complex are not a simple task. It often requires the use of different analytical methods, whose results have to be combined and examined together, since each method explores a particular feature of the inclusion complex (Mura, 2014). As shown in Table 1, the most used techniques for the characterization of inclusion complexes are DSC (40%), NMR (37%), XRD (34%), FTIR (33%), and TGA (23%).

Other techniques, such as DLS, were only used in 2.75% of the papers (i.e. in two papers). The researchers were not determining the particle size. This information is very important to explain phenomena of dissolution and the phase solubility analysis.

All of these techniques are very important in order to determine the inclusion complex formation. In general, all of the techniques that were used for the characterization were employed together, because one technique alone cannot provide comprehensive data on complex formation, unless the technique is NMR. 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 (Van Axel Castelli et al., 2008). Such evidence is based on the fact that, if an inclusion is present, then the physical or chemical environment is affected by the hydrogen elements at the internal surface of the cavity (H3 and H5 of the CD) and the included guest molecule (Tsao et al., 2011). Another interesting and little used technique was molecular modeling. It was only performed in 10% of the publications. This technique provides theoretical data for the complexation, and whether it can occur or not, based on the geometry and the steric factors of the molecules under study. The deformation energy ($E_{deformation}$) is determined by the difference between the energy of the partners of the complex at their respective equilibrium geometry and their energy at the complexed geometry (Menezes et al., 2016). The interaction energy ($E_{interaction}$) is defined as the difference between the energy of the complex and the sum of the energies of both partners at their complexed geometry, as described by Ziémons et al. (2007). This technique is useful before a study because it is a theoretical method which can estimate if complex formation is possible or not, thereby avoiding wasting time and money on a study that will not yield satisfactory results.

4. Inclusion Complexes – CDs and Terpenes

Several studies have demonstrated the pharmaceutical or pharmacological benefits of a cyclodextrin inclusion complex with natural products (Ceborska, 2014; Porte, Porte & Oliveira, 2014), but we found no reviews that could be useful for developing new approaches to improving CD-based host-guest complexes. We therefore conducted this review in order to assess the evidence for improvements in the pharmacological effect of natural products when included in CDs.

The use of some plant-derived compounds, such as terpenoids, have found some restrictions in the pharmaceutical marketplace, since they have limited water solubility, poor bioavailability, and can be affected by many factors, including temperature, pH and light. Therefore, the integrity of bioactive molecules needs to be protected, and it is necessary to use a formulation that can deliver them to physiological targets without losing any bioactivity (Pinho, Grootveld, Soares & Henriques, 2014a). Finding ways to enhance the absorption and the bioavailability of these compounds is one of the major challenges that the pharmaceutical industry presently faces (Aburahma, 2015; Munin & Edwards-Levy, 2011).

Several approaches have been explored in order to improve the therapeutic properties of non-polar natural compounds, including the employment of drug-delivery systems such as liposomes, nanoparticles or nanogels, surfactants, and CDs (Guimaraes et al., 2015; Menezes et al., 2015; Quintans-Junior et al., 2013; Siqueira-Lima et al., 2014). Cyclodextrins (CDs) seem to have a key role to play when they are used to improve the chemical and pharmacological properties of non-polar drugs (Brito, Araujo, Quintans, Sluka & Quintans-Junior, 2015; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015).

4.1 Biological Activities (Therapeutic activity)

In the studies in this review, terpenes have been shown to exhibit a wide range of biological activities on the human body; as an analgesic, an anti-inflammatory, an anticonvulsant, an antimicrobial, an antibacterial, an antitumoral, and in lipid metabolism. The effects of β-CD were also assessed in respect of their biological activities (Tables 2, 3 and 4).

4.2 Pharmacokinetics

Brito et al. (2015) and Oliveira et al. (2015) have recently reported that terpenes complexed with CDs have improved bioavailability, solubility, and a reduced effective dose, thereby having better pharmacological efficacy. These are probably the most promising benefits of this type of a drug as they are mainly analgesic drugs. However, the authors have noted that there are few pharmacokinetic studies that focus on the drug's potential and its usefulness in the development of new clinical drug candidates.

Ineffectiveness and toxicity are considered to be the major reasons for the failure of new drugs, so pharmacokinetics has had a great impact on these undesirable effects (Rosa et

al., 2014). Pharmacokinetic studies, investigating drug concentration-effect relationships, efficacy and safety have usually been conducted in animal models, in order to predict the clinical setting for the drug (Cunha-Filho & Sá-Barreto, 2007; Singh, 2006). The determination of pharmacokinetic parameters and the understanding of the biological mechanisms underlying these important parameters is vitally important for supporting the planning of the clinical stage (Amore, Gibbs & Emery, 2010). In respect of pharmacokinetic studies with CD inclusion complexes containing terpenes, or related compounds, we found few references in the literature and those studies we did identify were often at an early stage.

Table 2 - Details of the included animal studies

References	Substance	CD type*	Animals (Strain)	Sex	Dose, Concentration or Quantity (route)	Activity	Improved characteristics
Monoterpenes							
(Quintans-Junior et al., 2013), Brazil	(-)-linalool	β-CD	Mouse (S)	M	20 and 40 mg/kg (p.o.)	Analgesic	Enhanced biological activity
(Quintans et al., 2013a), Brazil	p-cymene	β-CD	Mouse (S)	M	20 or 40 mg/kg, (p.o.)	Analgesic and anti-inflammatory	The inclusion complexes prolongeds the time action possible increased solubility and stability
(Lins et al., 2014), Brazil	Geraniol	β-CD	Mouse (S)	-	50 - 200 mg/kg (i.p.)	Anticonvulsant effect	The inclusion complex enhanced efficacy and allows a reduction in the dose
(Nascimento et al., 2014), Brazil	(-)-linalool	β-CD	Mouse (S)	M	25 mg/kg (p.o.)	Analgesic	The inclusion complex prolongs the time-effect by possibly increase stability and solubility
(Nieddu et al.,	Thymol	β-CD	Pig	-	0.03 -0.05 g/kg (p.o.)	Pharmacokinetic studies Free thymol	Solubility, dissolution rate Prolonged GI transit time of Thymol

2014a), Italy						concentration.	
(Guimaraes et al., 2015), Brazil	Carvacrol	β-CD	Mouse (S)	M	50 mg/kg (p.o.)	Analgesic	The inclusion complex prolongs the time-effect by possibly increase stability and solubility
(Moreira et al., 2016)	(-)-β-pinene	β-CD	Rat (W)	M	200 mg/kg (p.o.)	Antihypertensive Effect	The complex, but not βP alone, promoted hypotension at 36 and 48 hours after administration
Sesquiterpenes							
(Yang, Lin, Chen & Liu, 2009), China	Artemether	HP-β-CD	Rat (SD)	M	10.8 mg/kg (p.o.)	Pharmacokinetics study	Inclusion complex had a much higher rate and extent of bioavailability compared to artmether suspension.
(Ansari, Batty, Iqbal & Sunderland, 2011), Australia	dihydroartemisinin (DHA)	HP-β-CD	Mouse (S)	-	100 mg/kg (i.p.)	Pharmacokinetics study	Improvement of DHA bioavailability
	β –caryophyllene	β-CD	Rat (SD)	M,F	50 mg/kg (p.o.)	Pharmacokinetics study	Inclusion complex significantly increased

(Liu et al., 2013), China							the oral bioavailability in rats (about 2.6 times) comparing to drug free
(Quintans-Júnior et al., 2016)	β-caryophyllene	β-CD	Mouse (S)	M	10 or 20 mg/kg (p.o.)	Analgesic	β-caryophyllene -βCD, at all doses tested, produced a significant reduction on mechanical hyperalgesia and a significant increase in muscle withdrawal thresholds, without producing any alteration in force.
Diterpenes							
(Michaelis, Cinatl, Vogel, Pouckova, Driever & Cinatl, 2001), Germany	Aphidicolin	γ-CD	Mouse (CD-1nude)	F	7.5 or 15 mg/kg (i.p)	Antitumor activity	No observed
(Yan et al., 2008), China	Oridonin	HP-β-CD	Rat (W) and Mouse (Kunming)	M	10 mg/kg, (i.v.)	Pharmacokinetics study	HP-β-CD enhanced the solubility of oridonin moreover had no significant effect on the pharmacokinetics in rats, while it changed the tissue distributions in mice to some extent

(Pan et al., 2008), China	Cryptotanshinone	HP-β-CD	Rats (SD) Dogs (B)	F	60 mg/Kg (i.p.) 53.4 mg/Kg- (i.p.)	Pharmacokinetics study	The bioavailability of the complex has been improved significantly as compared with the free compound.
(Ren, Jing, Chen, Miao & Wei, 2014), China	glaucocalyxin A	SBE-β-CD	Rat (SD)	M	10 mg/kg (i.v.)	Pharmacokinetics study	Inclusion complex was retained for a longer duration compared with the free glaucocalyxin A
Triterpenes							
(Soica et al., 2012), Romania	Betulin	GCDG	Mouse (C57BL/6J)	M	20 mg/kg (i.p)	Antitumor activity	An increase in hydrosolubility demonstrates an important improvement for Betulin antitumor activity
(Liu, Zhang, Wang, Sun & Diao, 2013), China	ilexgenin A	β-CD polymer	Mouse (C57BL/6J)	-	15 – 60 mg/kg (p.o)	lipid metabolism	The relative potencies of inclusion complex compared to drug free were increased about 2–3 fold.
(Liu et al., 2014a), China	Pedunculoside	β-CD	Mouse (ICR)	M,F	20, 45, 90 mg/kg (p.o)	Anti-inflammatory	PE–CDP showed safety and acted as anti-inflammatory more effectively, possibly because CDP contributes to the absorption of PE–CDP in vivo.

(Soica et al., 2014a), Romania	betulinic acid	GCDG	Mouse (C57BL/6J)	F	100 mg/kg (i.p)	Antitumor activity	The application of the water-soluble complex was necessary to obtain a clear parenteral formulation
Iridoids							
(Lu, Zhang, Tao, Ji & Wang, 2009), China	Genipin	β-CD	Mouse (Kunming)	M	10 mg/kg, (i.v.)	Pharmacokinetics study	The inclusion complex of GP–β-CD had longer and better bioavailability.
(Zhang, Meng, Cui & Song, 2011), China	Genipin	HP-β-CD	Rat (SD)	M	50 mg/kg (p.o.)	Intestinal absorption	The inclusion complex improved the absorption of genipin by both enhancing solubility and inhibiting the Pgp efflux pump.

* hydroxipropil-β-cyclodextrin (HP-β-CD), octakis-[6-deoxy-6-(2-sulfanyl ethanesulfonate)]-γ-CD (GCDG), sulfobutyl ether β-cyclodextrin (SBE-β-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD)

Table 3 – Details of the included *in vitro* studies

References	Substance	CD type*	Strain/Cell line	Concentration or Quantity	Activity	Improved characteristics
Monoterpenes						
(Liang, Yuan,	carvacrol, eugenol,	α-CD	ATCC 6538	50 mg	Antimicrobial	Solubility and efficacy

Vriesekoop & Lv, 2012), China	linalool and 2-pentanoylfuran	β-CD HP-β-CD	ATCC 6633 ATCC 8099 ATCC 9763			
(Rukmani & Sundrarajan, 2012)	Thymol	β-CD	ATCC 10536 ATCC 11632	-	Antibacterial property	Durability of the antibacterial property (thymol in β-CD cannot be excluded from the inclusion complex during washing).
Sesquiterpenes						
(Ghasemali et al., 2013), Iran	Henalin	β-CD	T47D	1,3 – 1,5 nM	Cytotoxicity Assay	Inclusion complex is more effective and kill some more breast cancer cells. Besides the results showed effect in low dosage and fewer side effects of the complexed
(Jiang et al., 2014), China	Artesunate	β-CD	HCT116, LOVO, SW480 and HT-29	0.5 – 60 µmol/L	Cytotoxicity Assay	Inclusion complex exhibited higher cytotoxicity than drug free.
Diterpenes						
(Michaelis, Cinatl, Vogel, Pouckova, Driever &	Aphidicolin	γ-CD	IMR-32 UKF-NB-2 and UKF-NB-3	2 mg/ml	Cytotoxicity Assay	The antitumoral action of inclusion complex is about 2-fold lower than that of the free drug

Cinatl, 2001), Germany (Correa, Melo, de Carvalho, de Azevedo, Duran & Haun, 2005), Brazil	Dehydrocrotonin	β-CD	Hepatocytes V79	25 – 500 µM	Cytotoxicity Assay	Decrease in the cytotoxicity of inclusion complexed compared to free drug
(Ling, Rui & Hua, 2007), China	tanshinone IIA	HP-β-CD	Rats (SD)-intestine	250#250 µg/mL	Intestinal Absorption	The inclusion complex improved the intestinal absorption of tanshinone as a consequence of the increased solubility and decrease in crystallinity caused by complexation.
Triterpenes						
(Soica et al., 2012), Romania	Betulin	GCDG	B164A5	10 mM	Cytotoxicity Assay	The stable complex augmented the properties of the active compound.
(Soica et al., 2014b), Romania	betulinic acid	GCDG	B164A5	10 mM	Cytotoxicity Assay	An increase in hydrosolubility demonstrates a better therapeutic outcome might likely be due to a superior distribution at the tumor level

*

Table 4 – Details of the included clinical studies

References	Substance	CD type*	Population	Concentration or Quantity	Activity	Improved characteristics
Sesquiterpenes						
(Wong & Yuen, 2001), Malaysia	Artemisinin	β-CD γ-CD	12 healthy human volunteers (22 - 44 years old)	250 mg in 150 ml of water	Pharmacokinetics analysis	Inclusion complexes achieved much higher plasma drug levels, thus indicating a higher rate and extent of artemisinin absorption.
(Shah & Mashru, 2010), India	Artemether	β-CD	20 volunteers	1 g of CD and artemether was dispersed in 50 mL of water for 15 s.	Gustatory sensation test	The complete bitter taste masking of ARM was achieved with improved in vitro drug release in physical mixture. because it was further incorporated into a reconstitutable dry suspension.

- β-cyclodextrin (β-CD), γ- cyclodextrin (γ-CD), hydroxipropil-β-cyclodextrin (HP-β-CD), octakis-[6-deoxy-6-(2-sulfanyl ethanesulfonate)]-γ-CD (GCDG), sulfobutyl ether β-cyclodextrin (SBE-β-CD), α- cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ- cyclodextrin (γ-CD)

A number of pharmacokinetic studies evaluated animal models and highlighted the potential benefits of terpenes with CDs. Yan et al. (2008) suggested that the complex of oridonin and HP-β-CD approximately behaved as a true solution in plasma. The lower drug concentration they found in the heart, kidneys, and spleen in their study can be explained by the equilibrium mechanism of the CD drug after administration, highlighting its possible beneficial use in the clinical application of oridonin. Assessing the mean plasma concentration *versus* the time profiles of free β-caryophyllene and β-caryophyllene/CD, after oral administration a comparison of the pharmacokinetic parameters indicated that β-caryophyllene/CD had an earlier T_{max} and a higher C_{max}, suggesting a better bioavailability of about 2.6 times (Liu et al., 2013). Importantly, the complexation of β-caryophyllene and β-caryophyllene/CD improved the pharmacological aspects when compared with terpene alone. This effect seems to be related to an increased bioavailability of the active compound (Quintans-Júnior et al., 2016). Lu et al. (2009) found that the relative bioavailability of the inclusion complex of genipin/β-CD compared to free genipin was 305.3%. Corroborating this the apparent bioavailability of artemether following a complexation with HP-β-CD was found to be 181% when compared to an artemether suspension, indicating that the complex had a much higher rate and extent of bioavailability compared to artemether suspension (Yang, Lin, Chen & Liu, 2009).

In an important clinical study with twelve healthy male volunteers that was conducted to evaluate the absorption of artemisinin, an anti-malarial drug and a sesquiterpene lactone, it was found that when complexed with the two forms of natural CDs, in comparison with a normal commercially available preparation the complexes exhibited a more rapid dissolution, and hence, absorption. Consequently, this enhanced the therapeutic effectiveness of artemisinin (Wong & Yuen, 2003b). These results were also played out in others studies using the derivatives of dihydroartemisinin (DHA), which in a complexation with HP-β-CD, equally improved the pharmacokinetic parameters when compared with DHA alone (Ansari, Batty, Iqbal & Sunderland, 2011). Artesunate in β-CD conjugates with a cytotoxicity against three types of human colon cancer cell lines (Jiang et al., 2014).

4.3 Pharmacological Improvement

Among the substances whose chemical properties can be improved by CDs are essentials oils and their main compounds, such as terpenes and phenylpropanoids (Guimaraes, Serafini & Quintans-Junior, 2014; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015). The pharmacologically beneficial effects that are produced by forming CD-lipophilic drugs seem to improve their analgesic and anti-inflammatory profiles (Brito, Araujo, Quintans, Sluka & Quintans-Junior, 2015; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015).

Monoterpenes are commonly able to produce analgesic, antioxidant and anti-inflammatory effects, including their ability to treat chronic pain (Guimaraes, Quintans & Quintans Júnior, 2013; Guimaraes, Serafini & Quintans-Junior, 2014; Quintans, Antoniolli, Almeida, Santana-Filho & Quintans-Junior, 2014). These compounds possess a poor oral absorption due to their extremely low aqueous solubility or their extensive pre-systemic metabolism and this may be responsible for the unfavorable pharmacokinetics of the molecule (Yadav, Suresh, Devi & Yadav, 2009). Some studies have demonstrated an improvement in the oral absorption of terpenes after complexing them with CDs and their derivatives and an increment in the pharmacological efficacy of these compounds.(Guimaraes et al., 2015; Ishida et al., 2013; Nascimento et al., 2015; Nascimento et al., 2014; Quintans et al., 2013b; Quintans-Junior et al., 2013; Siqueira-Lima et al., 2014). However, the articles have little pharmacokinetic and pharmacodynamic information about how CD-complex-drugs improve these particular aspects. Research in this area may be a promising line of approach to understand how monoterpene-CD-complexes enhance the effects and reduce the side effects of these natural products.

Figure 3 provides studies that show chemical and pharmacological characteristics of the β-caryophyllene/β-cyclodextrin complex (BCP-βCD). Hădărugă et al. (2009) demonstrated using molecular modeling and docking studies that the rate of complex formation between BCP and βCD is relatively low, but the stability of the BCP-βCD complex seems to be higher when compared with other terpenes-βCD-complexes, such as caryophyllene-oxide or α-bisabolol. In fact, βCD has an intrinsic ability to form inclusion complexes with terpenes, which was strongly demonstrated in several studies with BCP (Liu et al., 2013; Quintans-Júnior et al., 2016; Hădărugă, Hădărugă, Riviş & Pârvu, 2009), and to enhance pharmacological aspects, mainly bioavailability and

efficacy to produce a lasting therapeutic effect (as can see in Figure 3, B and C) (Liu et al., 2013). Several pre-clinical and clinical studies show that the analgesic effect produced by terpenes can be improved by CDs (Brito, Araujo, Quintans, Sluka & Quintans-Junior, 2015; Oliveira, Guimaraes, Araujo, Quintans, Santos & Quintans-Junior, 2015). We clearly show in the figure 3 (C) that BCP free has a lower analgesic effect in the first phase of the formalin-induced orofacial pain test when compared to BCP-βCD complex (at the same nominal dose). However, it should be noted that within the terpenes-βCD-complex the dose of active ingredient (i.e. BCP) is about 10 times lower than the monoterpene free (Quintans et al., 2013a; Siqueira-Lima et al., 2014). So, βCD is able to improve the therapeutic effects of terpenes with aa smaller nominal dose. As demonstrated in Figure 3 (D) by the expression of Fos protein, the BCP-βCD-complex activated the spinal cord-inhibitory mechanism, producing a lasting anti-hyperalgesic effect in an animal model for fibromyalgia, which seems to be higher than BCP alone (Quintans-Júnior et al., 2016).

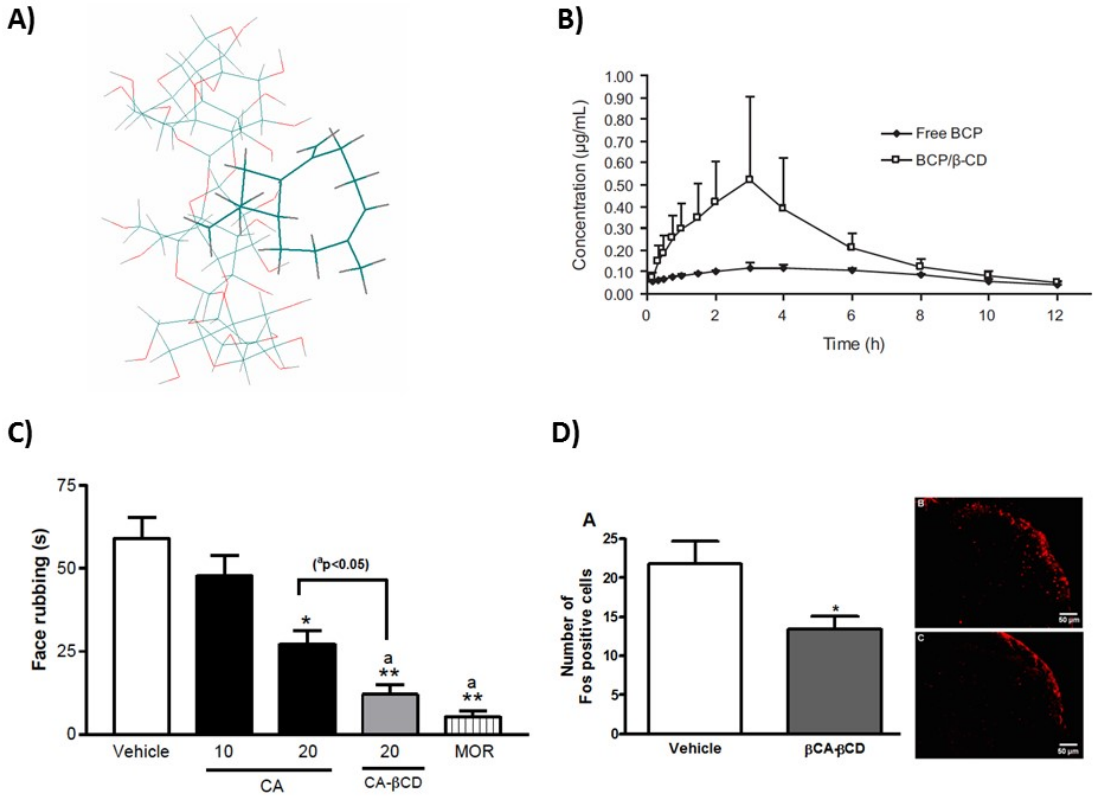

Figure 3 - **A)** Most stable conformation of β-caryophyllene/β-cyclodextrin complex (Adapted from Hădărugă et al., 2009). **B)** Mean plasma concentration-time profile of β-caryophyllene (BCP) after oral administration of free BCP or BCP/β-cyclodextrin (β-

CD) inclusion complex (Adapted from Liu et al., 2013). **C)** Effects of β-caryophyllene (CA) or CA-β-cyclodextrin (CA-βCD) on formalin-induced orofacial nociceptive behavior. Vehicle (control), CA (10 or 20 mg/kg, p.o.) or CA-βCD (20 mg/kg, p.o.) were administered 90 min. before formalin injection (data shown results from first phase, 0–5 min., of the formalin test). Values represent mean + S.E.M. (n = 6 per group). *p<0.05 or **p< 0.001 versus control and ap<0.05 versus CA free (20 mg/kg, p.o.) (one-way ANOVA followed by Tukey's test). (unpublished data) **D)** Fos-positive neurons in the lumbar spinal cord lamina I. Vehicle (control group, C) or β-caryophyllene/β-cyclodextrin (βCP-βCD) (20 mg/kg) were administered orally and, after 90 min, the animals were perfused. Values represent mean ± SEM (n = 6, per group). *p<0.05 vs. control group (unpaired t-test) (Adapted from Quintans-Júnior et al., 2016).

Figure 4 shows the in silico aspect of the possible interaction between limonene (LIM) and β-CD, which it is more stable with a binding energy of −4.54 kcal. The larger cavity of β-CD allows greater efficiency than α-CD (Dos Passos Menezes et al., 2016). The LIM/βCD-complex produced a lasting and extremely significant analgesic effect when compared to LIM alone (Data not shown). Similarly, p-cymene alone produced a fleeting analgesic profile, which lasted only 2 h. However, p-cymene/β-CD-complex corroborates the fact that CDs are the host:guest encapsulate agent which improve the efficacy of non-polar drugs (Figure 4B) (Quintans et al., 2013a). This concept can be better seen in Figure 4C which shows how the carvacrol/β-cyclodextrin-complex produced an analgesic profile 24 h after the oral administration assessed in animal models of cancer pain, an intense type of pain involving a strong inflammatory process (Guimaraes et al., 2015). Thus, it is interesting to hypothesize that the use of monoterpenes complexed with CDs can be a tool to improve effectiveness and bioavailability, moreover they produce a lasting effect which is essential for chronic conditions, such as cancer or chronic pain.

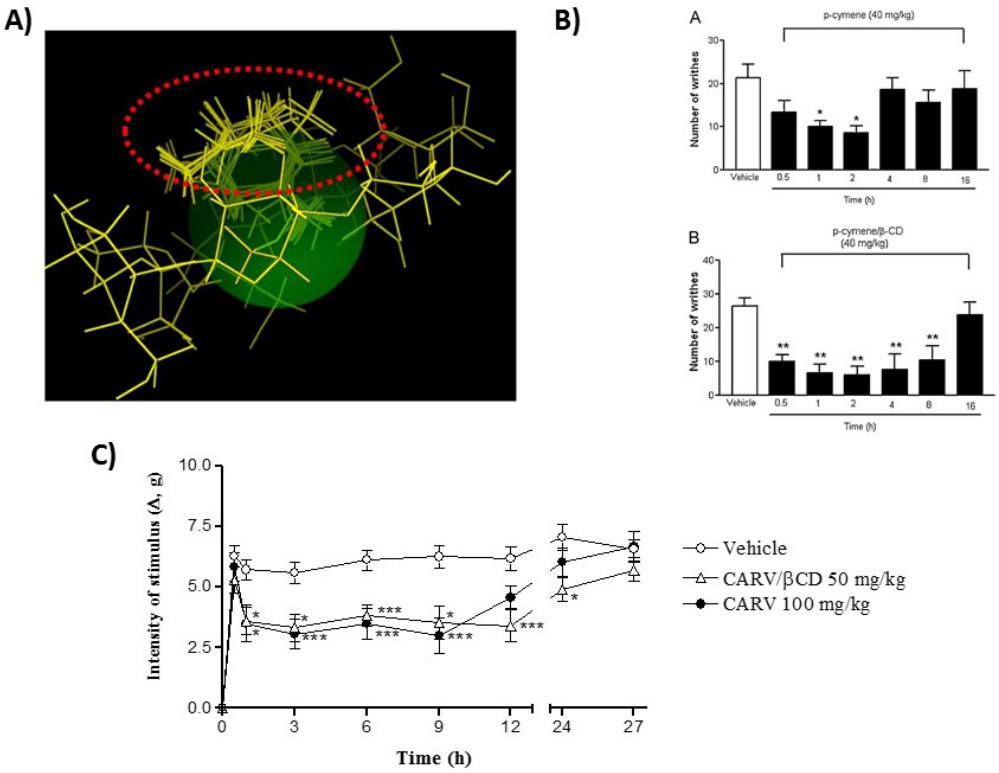

Figure 4 - **A)** Ten possible interactions of LIM and CDs obtained through molecular modeling. The green space represents the CD cavity (Adapted from Menezes et al., 2016). **B)** Time response curve for the antinociceptive effect of (A) p-cymene or (B) p-cymene/β-CD complex on acetic acid-induced writhing response in mice. Writhings were counted over 20 min following i.p. administration of acetic acid (0.65%). p-cymene or p-cymene/β-CD (40 mg/kg) was administered p.o. 0.5, 1, 2, 4, 8 or 16 h before acid acetic injection (0.65%). Control animals received an injection of vehicle by p.o. route. Each column represents mean ± S.E.M. (n = 8, per group). *p<0.05 or **p<0.001 vs. control (ANOVA followed by Tukey's test) (Adapted from Quintans et al., 2013). **C)** Effect of carvacrol/β-cyclodextrin complex (CARV/β-CD) on the mechanical hyperalgesia induced by S180. Time-Effect Curve of CARV/β-CD (50 mg/kg) and CARV (100 mg/kg). *p< 0.05, **p < 0.01 and ***p < 0.001 vs. the control group (vehicle) (ANOVA followed by Tukey's test) (Adapted from Guimaraes et al., 2015).

Within this context, linalool a monoterpene found in the *Ocimum* and *Thymus* species, when complexed with α-CD, β-CD, or HP-β-CD, showed a significant enhancement in its water solubility that was established through specific tests (Pinho, Grootveld, Soares

& Henriques, 2014a). A pretreatment with linalool induced analgesic and anti-inflammatory profiles which were comparatively better than when linalool was used alone in an animal model with fibromyalgia, and when challenged by pro-inflammatory agents (Nascimento et al., 2014; Quintans-Junior et al., 2013). More recently, some studies have shown that complexation between terpenes and CDs can be more pharmacologically activity even with lower molecular doses of active compound (terpene) when compared with terpene non-complexed with CDs (Quintans et al., 2013b; Guimarães et al., 2014; Menezes et al., 2015; Brito et al., 2015; Quintans-Júnior et al., 2016).

One of the most studied pharmacological uses for terpenes has been its anticancer effects. However, problems with toxicity, side effects, and resistance, encouraged the development of new pharmacological tools to improve the properties of the drugs under study (da Rocha, Lopes & Schwartsmann, 2001; Harvey, 2008). Michaelis, Cinatl, Vogel, Pouckova, Driever and Cinatl (2001) studied an aphidicolin (APH), a tetracyclic diterpene, and compared the cytotoxic actions between an APH free treatment and when complexed with γ-CD against neuroblastoma cells. Surprisingly, when *in vitro*, the antitumoral action of APH-CD was about 2-fold lower than that of the APH free treatment. Others studies employed a cytotoxicity assay, as did Ghasemali et al. (2013), who encapsulated a cytotoxic chemotherapeutic agent. They showed that β-CD-helanin complexes inhibited the growth of a T47D breast cancer cell line in a time and dose-dependent manner. Moreover, Soica et al. (2012) and Soica et al. (2014a) assessed the pentacyclic triterpenes betulin and betulinic acid when complexed with γ-CD derivates. They demonstrated that these complexes had a gain in solubility and a beneficial influence in terms of anti-proliferative activity and *in vivo* tumor development, respectively.

On the above, Complexation with cyclodextrins is a well-known procedure which opens up new possibilities for their broad practical application in various fields to improve the biological activities of terpenes in living systems.

5. Conclusion

Although CDs are not able to form complexes with compounds with a high molecular weight and a larger size, they continue to be an innovative drug encapsulation system

which improve the pharmacological aspects of compounds that are able to form host:guest inclusion complexes with CDs. Our systematic review brings data to support the use of CDs as a key tool to improve the pharmaceutical and pharmacological aspects of terpenes and as a safe and low cost approach for turning these widely available natural products into effective drugs. The lack of pharmacokinetic studies, coupled with the limited number of clinical studies, are the main limitations that we found in most of the papers. However, the strong experimental evidence in the articles reviewed shows terpenes to be an attractive choice for pharmacological use. This should be an exciting avenue for new studies to explore different areas for the use of terpenes. For complementary information, we recommend the excellent reviews published by Brito et al., (2015), Kurkov and Loftsson (2013), Marques (2010a), Oliveira et al., (2015), Pinho et al., (2014), and Polyakov and Kispert (2015).

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CAPÍTULO 3

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Original article

Anti-hyperalgesic effect of Lippia grata leaf essential oil complexed with β-cyclodextrin in a chronic musculoskeletal pain animal model: Complemented with a molecular docking and antioxidant screening

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ABSTRACT

Background: Due to its unclear pathophysiology, the pharmacological treatment of fibromyalgia is a challenge for researchers. Studies using medicinal plants, such as those from the genus *Lippia*, complexed with cyclodextrins (CDs) have shown innovative results.
Objective: The present research intended to evaluate the effect of an inclusion complex containing β-cyclodextrin (βCD) inclusion complex with *Lippia grata* (LG) essential oil in a chronic musculoskeletal pain model, its central activity and its possible interaction with neurotransmitters involved in pain.
Methods: After acid saline-induced chronic muscle pain, male mice were evaluated for primary and secondary hyperalgesia and muscle strength. Moreover, an antagonist assay was performed to assess the possible involvement of the opioidergic, serotonergic and noradrenergic pathways. In addition, Fos protein in the spinal cord was assessed, and a docking study and antioxidant assays were performed.
Results: The treatment with LG-βCD, especially in the dose of 24 mg/kg, was able to significantly decrease ($p < 0.05$) the paw withdrawal and muscle threshold. Furthermore, LG-βCD was shown to affect the opioidergic and serotonergic pathways. There were no significant changes in muscle strength. Fos protein immunofluorescence showed a significant decrease in expression in the dorsal horn of the spinal cord. The main compounds of LG showed through the docking study interaction energies with the alpha-adrenergic and μOpioid receptors. In all antioxidant assays, LG exhibited stronger antioxidant activities than LG-βCD.
Conclusion: This study suggested that LG-βCD could be considered as a valuable source for designing new drugs in the treatment of chronic pain, especially musculoskeletal pain.

Abbreviations: 5-HT, 5-hydroxytryptamine; CDs, Cyclodextrins; CNS, Central Nervous System; DLF, dorsolateral funiculus; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; FM, Fibromyalgia; GABA, gamma-aminobutyric acid; LC, *Locus ceruleus*; LG, *L. grata* leaf essential oil; LG-βCD, *L. grata* leaf essential oil complexed β-cyclodextrin; NMR, nucleus raphe magnus; PAG, periaqueductal gray; TRP, transient receptor potential; βCD, β-cyclodextrin; RVM, rostral ventromedial medulla.

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Anti-hyperalgesic effect of β-cyclodextrin complexed with *Lippia grata* leaf essential oil in a chronic musculoskeletal pain animal model: complemented with a molecular docking and antioxidant screening

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Abstract

Background: Due to its unclear pathophysiology, the pharmacological treatment of fibromyalgia is a challenge for researchers. Studies using medicinal plants, such as those from the genus *Lippia,* complexed with cyclodextrins have shown innovative results. **Objective**: The aim of this study was to evaluate the effect of an inclusion complex in a chronic musculoskeletal pain model, its central activity and its possible interaction with neurotransmitters involved in pain. **Method**: After acid saline-induced chronic muscle pain, male mice were evaluated for primary and secondary hyperalgesia and muscle strength. Moreover, an antagonist assay was performed to assess the possible involvement of the opioidergic, serotonergic and noradrenergic pathways. In addition, Fos protein in the spinal cord was assessed, and a docking study and antioxidant assays were performed. **Results**: The treatment with LG-βCD, especially in the dose of 24 mg/kg, was able to significantly decrease ($p<0.05$) the paw withdrawal and muscle threshold. Furthermore, LG-βCD was shown to affect the opioidergic and serotonergic pathways. There were no significant changes in muscle strength. Fos protein immunofluorescence showed a significant decrease in expression in the dorsal horn of the spinal cord. The main compounds of LG showed through the docking study interaction energies with the alpha-adrenergic and μOpioid receptors. In all antioxidant assays, LG exhibited stronger antioxidant activities than LG-βCD. **Conclusion**: This study suggested that LG-βCD could be considered as a valuable source for designing new drugs in the treatment of chronic pain, especially musculoskeletal pain.

Keywords: Terpenes, camphor, caryophyllene, pain, opioid, 5-HT, antioxidant.

Introduction

Fibromyalgia (FM) is present in as much as 2% to 8% of the population (7 times more prevalent in females than males), is characterized by generalized chronic pain, fatigue, stiffness, and is often accompanied by memory problems, and sleep disturbance of variable intensity [7]. FM is the most common rheumatic disease, and has been considered as a 'dysfunctional pain', a type of chronic pain associated with a broad range of clinical disorders (such as irritable bowel syndrome, temporomandibular joint disease and interstitial cystitis, and neuropathic pain), for which treatment is difficult, requiring both pharmacological and non-pharmacological approaches, with an empiric approach to drug therapy focused toward individual symptoms, particularly pain [21, 29].

The effectiveness of current drugs is limited, with many patients discontinuing use due to low clinical efficacy, side-effects and poor ability to minimize the main symptoms [7, 21]. Most of these current medications, at least the majority, can be classified as 'repositioned drugs' (the application of known drugs and compounds to treat new indications), so there has been little 'radical innovation' to develop specific new drugs for FM [2, 29]. The development of new drugs is a challenge for researchers and pharmaceutical companies because its pathophysiology is still unclear, although there is evidence of involvement of a genetic component, an unbalance of neurotransmitters and neuroendocrine , autonomic nervous system (ANS) , and psychophysiological dysfunctions [6, 59].

Progress in the development of new drugs for the treatment of FM seems to lie in a better understanding of its multifaceted pathophysiological pathways and relevant molecular networks [7, 29]. Animal models are an essential tool in the development of new strategies for FM symptom management [40, 51, 53]. However, studies involving models specific to muscle pain are scarce in the literature, although muscle pain is a major clinical problem [43]. The non-inflammatory model mimics painful muscle diseases (such as FM), in which inflammation is not present, so here responds to a variety of analgesics drugs (mainly drugs such as opioid) but not to anti-inflammatory agents [43, 53].

Natural products (such as medicinal plants and secondary metabolites) seem to offer an interesting alternative for the development of new drugs, due to their invaluable role as a source of new molecules that can act by innovative mechanisms of action [24]. In addition, herbal medicines are popular, self-prescribed treatments for rheumatic conditions [13, 30]. Plants rich in terpenes are an important resource for the development and study of analgesic compounds that can act by modulation of the central nervous system (CNS) [9, 14, 15, 32].

The genus *Lippia* (Verbenaceae) includes approximately 200 species of herbs, shrubs and small trees, mainly distributed throughout South and Central America, and the tropical areas of Africa [35]. The species *Lippia grata* Schauer is a native bush found in Northeastern of Brazil, which is used in folk medicine to treat pain and inflammation conditions [56]. Recently, our group demonstrated that *L. grata* leaf essential oil (LG), which is rich in terpenes, produced an orofacial analgesic profile with involvement of the descending pain-inhibitory mechanisms and activation of the nucleus raphe magnus (NRM) and periaqueductal gray (PAG) (the areas of the CNS involved in pain modulation) [48, 49]. Moreover, we have also demonstrated that the pharmacological effects of some essential oils and terpenes can be improved by β-cyclodextrin (βCD) especially in terms of analgesic or anti-inflammatory properties [25, 33, 40, 41, 45, 48].

In the present study, we aimed to assess the effect of *L. grata* leaf essential oil complexed β-cyclodextrin (LG-βCD) in a chronic muscle pain animal model (considered to be an animal model for FM) as well as assessing Fos protein in the spinal cord (by immunohistochemistry) and evaluating any possible interactions with important neurotransmitter pathways in pain modulation via *in vivo* (by pharmacological antagonism) and *in silico* (by a docking study).

Material and Methods

Chemicals

β-cyclodextrin (≥97%, molecular weight: 1134.98 $g.mol^{-1}$), and Tween 80 were purchased from Sigma (USA). Tramadol, in free form without additives, was purchased from Teuto/Pfizer (Anápolis-GO, Brazil). Ketamine and Xylazin were purchased from Cristália (Itabira-SP, Brazil). Morphine, methysergide and yohimbine were obtained from Sigma-Alterich (San Diego, CA, USA).

Preparation of binary mixture of LG with βCD

Preparation and physical-chemical characterization of the *L. grata* leaf essential oil (LG) complexed with β-cyclodextrin (βCD) was carried out beforehand in accordance with [49] the methodologies previously described by our group [28, 41].

Animals

Experimental protocols were performed using male C57BL/6J or male Swiss mice obtained from the Animal Care at the Federal University of Sergipe (UFS/Brazil. The mice were housed in controlled-temperature rooms (22-25°C), under a 12/12 h light-dark cycle, with access to water and food *ad libitum* until use. All behavioral protocols were performed between 8:00 a.m. and 2:00 p.m. Experimental protocols were approved by the Animal Care and Use Committee at CEPA/UFS/Brazil (CEPA/UFS #57/11). All behavior experiments were performed with the examiner blinded to group. All effort was sought to minimize the number of mice used and any discomfort.

Acid saline-induced chronic muscle pain

The experimental procedure to induce chronic widespread non-inflammatory muscle pain (an animal model for FM) followed that previously described by our group [52]. Animals were anesthetized with 2% to 3% isoflurane, and 20 μL of pH 4.0 saline, adjusted with HCL to ± 0.1 pH unit, was injected into the left gastrocnemius muscle. This procedure was performed again 5 days after the first injection. This model produces a bilateral mechanical hyperalgesia lasting for 4 weeks after the second injection as described previously by our group [52].

Mechanical sensitivity of the muscle (primary hyperalgesia)

Mechanical withdrawal thresholds of the muscle were used to assess hyperalgesia of the deep tissue as previously described [16]. The gastrocnemius muscle of the mouse was squeezed with a pair of calibrated force-sensitive tweezers until the mouse withdrew from the stimulus. The force at which the mouse withdrew was measured in millinewtons (mN) and called the muscle withdrawal threshold. Each hindlimb was tested 3 times, and the 3 trials were averaged. A decrease in threshold was interpreted as primary muscle hyperalgesia [10].

Mechanical sensitivity of the paw (secondary hyperalgesia)

Mechanical withdrawal threshold of the paw was measured with von Frey monofilaments (2.44) applied to the plantar surface of the ipsilateral paw 10 times. Five trials of 10 were averaged at each time period. An increase in the number of responses was interpreted as secondary cutaneous hyperalgesia [52].

Pharmacological treatments

After confirming mechanical hyperalgesia 24h after induction of the model, mice (n=8, per group) were treated with LG-βCD (6, 12 or 24 mg/kg; p.o., gavage, p.o.) or vehicle (saline; p.o.) daily for 8 days. 24 hours after treatment, the mechanical hyperalgesia was re-evaluated.

Muscle strength

To assess muscle strength, mice with acid-saline induced muscle pain (n=8, per group) were treated with LG-βCD (6, 12 or 24 mg/kg; p.o.) or vehicle (saline; p.o.). Fore and hindpaw grip strength was tested before, and at the end of the experiment in the same mice that were tested for muscle and paw withdrawal thresholds. Mice were familiarized with the apparatus twice a day for 2 days by performing the grip force task. Mice were pulled by the tail to read grip strength of the fore and hindpaw. An average of three trials was recorded (mN). The animals were evaluated 1h, 2h, 3h and 4h after the treatment.

Antagonism assessment

To assess the possible involvement of the opioidergic, serotonergic and noradrenergic systems, different groups of animals (n=8 per group) were examined to establish if the anti-hyperalgesia produced by LG-βCD was reversed by systemic delivery of naloxone (5 mg/kg; i.p.), methysergide (1.5 mg/kg; i.p.) or yohimbine (2 mg/kg; i.p.). All antagonists were given to animals which had gone through the chronic muscle pain model and received LG-βCD (24 mg/kg; p.o.) or vehicle (p.o.). Withdrawal thresholds of the muscle were tested before and 24h after induction of the acidic saline model, and daily for 5 days. On Day 4, antagonists were injected systemically, and behavior measured 30 minutes after injection.

Immunofluorescence for Fos

To evaluate the involvement in descending pain-inhibitory mechanisms through Fos protein expression in the dorsal horn, animals with formalin induced pain (formalin 2%), as standardized by [5, 45] were treated with LG-βCD (24 mg/kg, p.o.) or vehicle (isotonic saline, p.o), and then perfused. Their spinal cords were collected and cryoprotected for immunofluorescence processing to Fos protein [5, 40, 45]. Frozen serial transverse sections (20 μm) of spinal cords were collected on gelatinized glass slides. The tissue sections were stored at -80 °C until use. The sections were washed with phosphate buffer saline (PBS, 10 mM) 5 times for 5 min and incubated with 0.01 M glycine in PBS for 10 min. Non-specific protein binding was blocked by the incubation of the sections for 30 min in a solution

containing 2% BSA. Then, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (k-25; 1:2000). Subsequently, the sections were incubated for two hours with donkey anti-rabbit Alexa Fluor 555 as secondary antibodies (1:2000). The cover slip was mounted with glycerol solution. As an immunofluorescence control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS 5 times for 5 min.

Acquisition and analyses of images

Pictures from Fos-positive areas were acquired for each animal with an Olympus IX2-ICB (Tokyo, Japan). The brain regions were classified according to the Paxinos and Franklin Atlas [37]. Neurons were counted by the free software Image J (National Institute of Health) using a plug-in that uses the same level of label intensity to select and count the Fos-positive cells [5, 40, 45].

Docking Study

The structures of the receptors were downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The receptors investigated and their respective PDB IDs are: Alpha adrenergic – 1hof, μOpioid – 5C1m and 5HT – 4ib4. For a comparative study, we calculated the energy of formation of the complex obtained between three ligands: camphor, trans-caryophyllene, bicyclogermacrene and the three selected molecular receptors. Using the program Hyperchem v. 8.0.3 (*HyperChem, 2009*), the chemical structures of the ligands were drawn, and their geometries were optimized using MM+ force field [1]. Afterwards, we performed a new geometry optimization based on the semi-empirical method AM1 (Austin Model 1) [8, 11, 22]. The optimized structure was subjected to conformational analyses using the Spartan for Windows 10.0 software. We selected the random search method with 1,000 interactions, 100 cycles of optimization, and 10 conformers of lowest minimum energy. The dihedrals were evaluated by rotation in accordance with the standard (default) conditions of the program, in which the number of simultaneous variations was 1 to 8: acyclic chains were submitted to rotations from 60 to 180° and torsion rings were in the range of 30 to 120°.

The ligand was submitted to molecular docking using the Molegro Virtual Docker v. 6.0.1 (MVD) [54]. All the water compounds were deleted from the receptors and the method was prepared using default parameter settings on the same software: GRID of 15 Å of radius.

The MolDock score [GRID] algorithm was used as the score function, and the search algorithm was MolDock [54].

In vitro Antioxidant Assays

Antioxidant (radical scavenging - DPPH and ABTS), reducing power (CUPRAC and FRAP), phosphomolybdenum and metal chelating (ferrozine method)) activities were determined using the methods previously described by [47]. Antioxidant abilities were expressed as equivalents of trolox and EDTA (for metal chelating).

Statistical analysis

The results were expressed as mean ± S.E.M. Differences between multiple groups were analyzed using one-way analyses of variance (ANOVA) followed by Tukey's test, respectively. In all cases, differences were considered significant if $p<0.05$. The statistical analyses were assessed using the GraphPad Prism 5.0 software (GraphPad Prism Software Inc., San Diego, CA, USA).

Results

Twenty-four hours after induction of the chronic muscle pain model there was a significant decrease in withdrawal threshold of the paw (Figure 1). The treatment with LG-βCD, in doses of 12 and 24 mg/kg, was able to significantly inhibit ($p<0.05$) the mechanical sensitivity after the chronic muscle pain induction when compared to the control. No difference was observed when animals were treated with LG-βCD at a dose of 6 mg/kg.

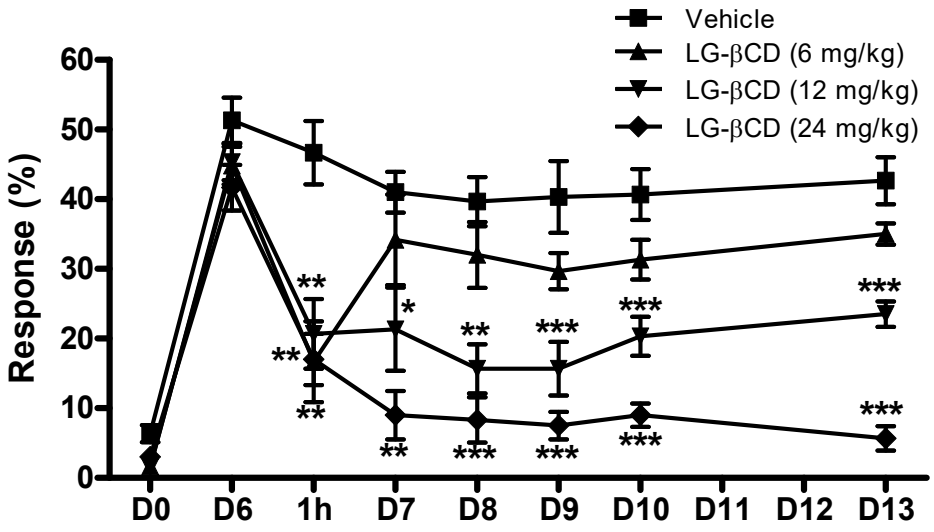

Figure 1. Effect of *L. grata* leaf essential oil complexed with β-cyclodextrin (LG-βCD) (6, 12 or 24 mg/kg; p.o.) or vehicle (saline; p.o.) on mechanical sensitivity induced by acidic saline in mice. Each point represents the mean ± S.E.M (n = 8, per group) of the ipisleteral paw withdrawal threshold. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control group (ANOVA followed by Tukey´s test).

To exclude the possibility of reduction of motor performance typical for some natural products, we performed the grip strength test. No difference was observed in the hind and forepaw muscle force when animals treated with LG-βCD, at all doses, were compared with animals treated with vehicle (Figure 2).

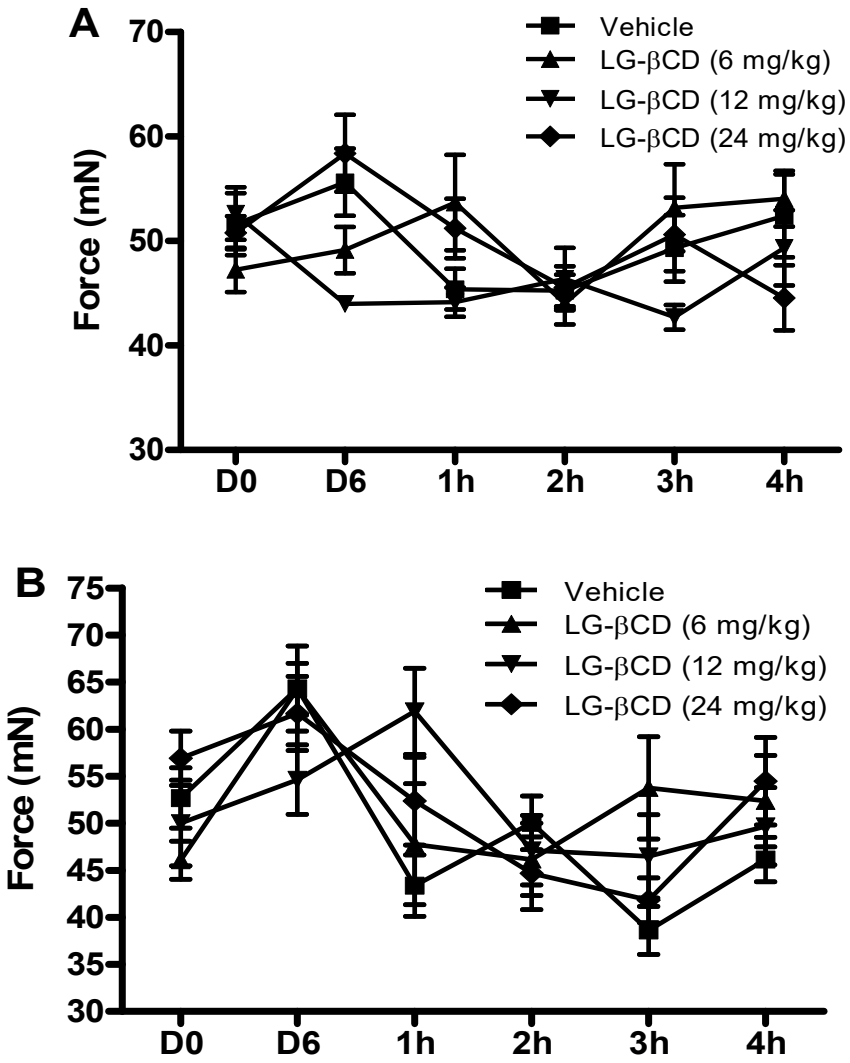

Figure 2. Effect of *L. grata* leaf essential oil complexed with β-cyclodextrin (LG-βCD) (6, 12 or 24 mg/kg; p.o.) or vehicle (saline; p.o.) on the grip strength meter in mice. **A.** Hindpaw. **B.** Forepaw Values are expressed in mean ± S.E.M (n = 8, per group).

Figures 3 and 4 show that the treatment with LG-βCD (24 mg/kg; p.o.) was able to significantly inhibit ($p<0.05$) the muscle threshold after the chronic pain induction when compared to the control. Systemic naloxone and methysergide, which block opioid and serotonin receptors, respectively, significantly decreased the withdrawal threshold of the muscle in animals that received LG-βCD (24 mg/kg; p.o.). On the other hand, yohimbine, which blocks noradrenergic receptors, had a weak effect on the analgesia produced by LG-βCD (24 mg/kg; p.o.).

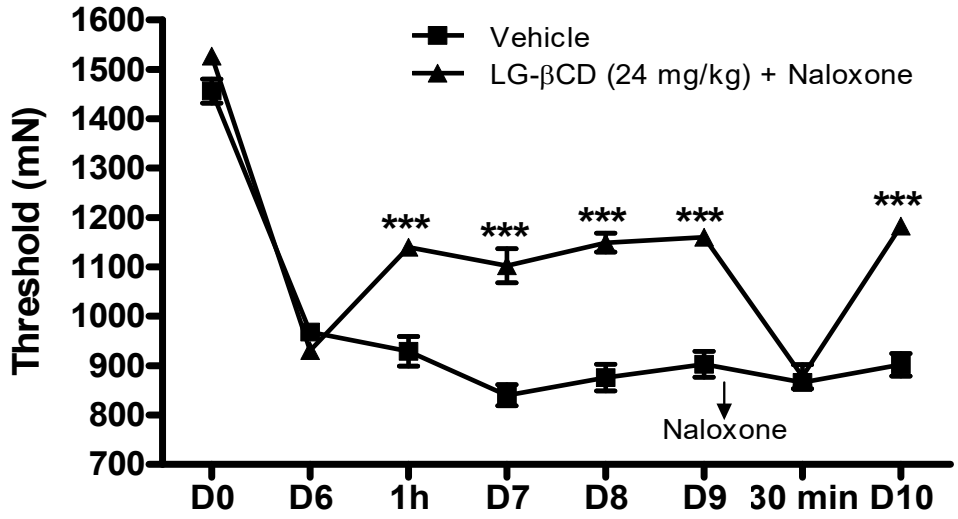

Figure 3. Effect of the pharmacological antagonists naloxone (5 mg/kg; i.p.) on muscle withdrawal thresholds in mice after treatment with *L. grata* leaf essential oil complexed with β-cyclodextrin (LG-βCD) (24 mg/kg; p.o.) or vehicle (saline; p.o.). Each point represents the mean ± S.E.M. of the ipsilateral muscle withdrawal thresholds (n = 8, per group). ***$p < 0.01$ vs. control group (ANOVA followed by Tukey´s test).

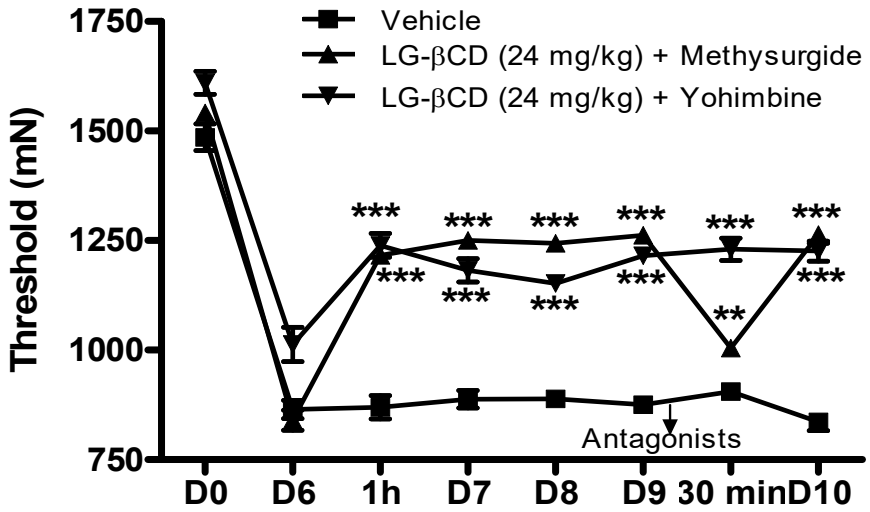

Figure 4. Effect of the pharmacological antagonists methysergide (1.5 mg/kg; i.p.) and yohimbine (2 mg/kg; i.p.) on muscle withdrawal thresholds in mice after treatment with *L. grata* leaf essential oil complexed with β-cyclodextrin (LG-βCD) (24 mg/kg; p.o.) or vehicle (saline; p.o.). Each point represents the mean ± S.E.M. of the ipsilateral muscle withdrawal thresholds (n = 8, per group). **p < 0.01 and ***p < 0.001 vs. control group (ANOVA followed by Tukey´s test).

Moreover, Fos protein immunofluorescence showed a significant decrease in expression (Fos-positive cells) in the dorsal horn of the spinal cord, when compared to the control ($p<0.01$), as demonstrated in Figure 5.

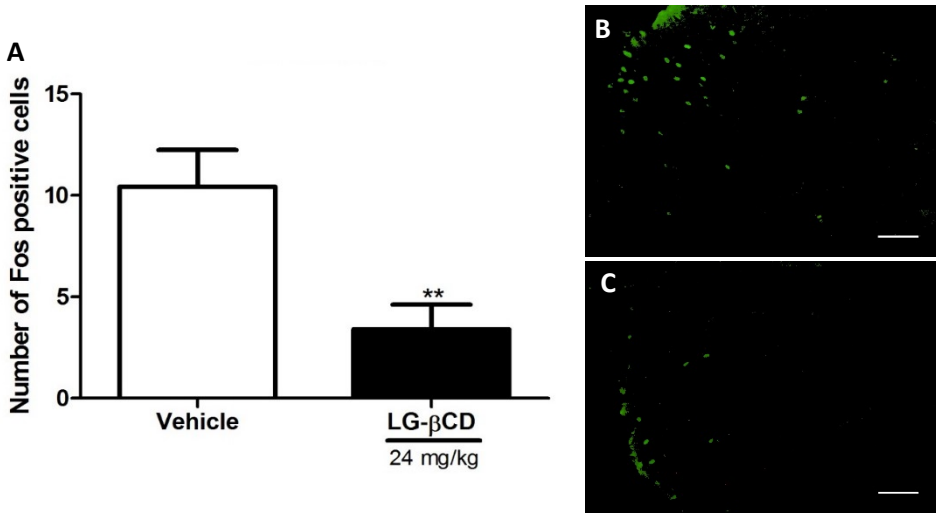

Figure 5. Number of positive Fos cells in the dorsal horn in mice (A). Vehicle (B), or *L. grata* leaf essential oil complexed with β-cyclodextrin (LG-βCD) (24 mg/kg; p.o.) (C) was administered 90 min before the perfusion. Values expressed as mean ±S.E.M. (n = 6 per group). □□p < 0.01 when compared with control (Student's t test). 100 μm.

The docking study demonstrated that camphor, *trans*-caryophyllene (*E*-carophyllene), bicyclogermacrene, the main compounds of LG, have different energies of interaction with the receptors (Figures 6 and 7). Table 1 summarizes the main binding energies showing bicyclogermacrene interaction with the alpha-adrenergic and μ Opioid receptors, while *E*-caryophyllene and camphor bind more strongly to the alpha-adrenergic receptors.

Table 1 - Main secondary metabolites of *Lippia grata* leaf essential oil with respective MolDock energies regarding Alpha adrenergic, μ-opioid (μ-OR) and 5-HT receptors.

Ligant	Alpha adrenergic	μ Opioid	5-HT
Camphor	-382.187 kcal/mol	-38.355 kcal/mol	-51.835 kcal/mol
Trans-carophyllene	-492.335 kcal/mol	-63.201 kcal/mol	-79.742 kcal/mol
Bicyclogermacrene	-487.325 kcal/mol	-78.466 kcal/mol	-92.707 kcal/mol

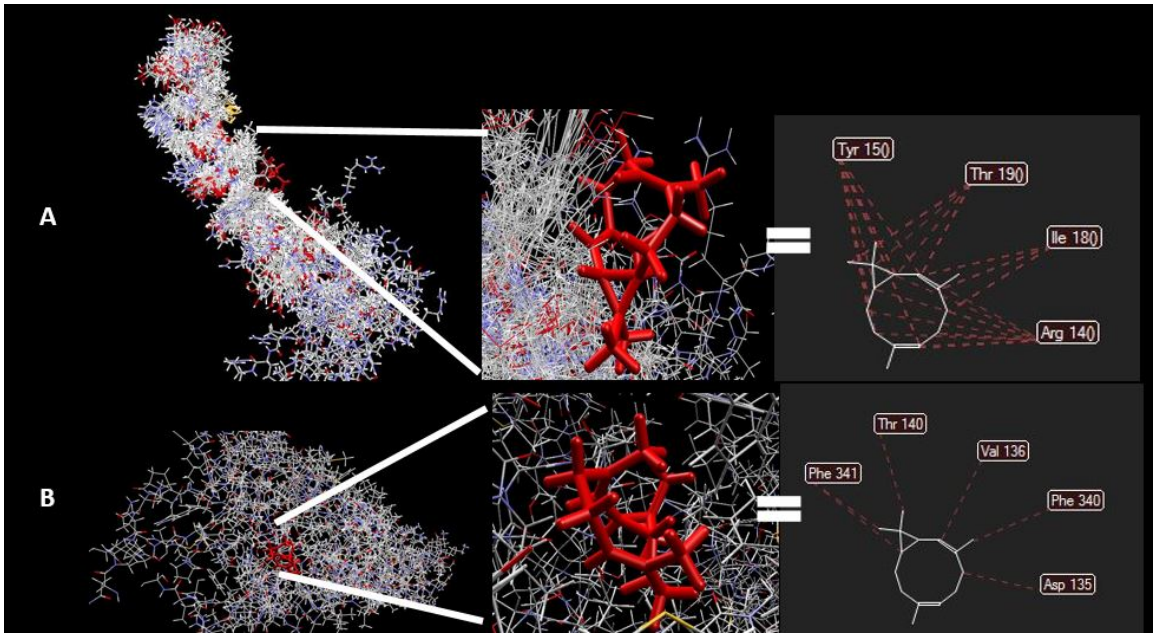

Figure 6. Interactions of the bicyclogermacren with the receptors (A) alpha adrenergic and (B) 5-HT. We observed that bicyclogermacren-alpha adrenergic complex shows steric

interactions with the residues TYR150, THR190, LLE180 and ARG140. The bicyclogermacren-5-HT complex presentes steric interactions with PHE341, THR140, VAL136, PHE340 and ASP135.

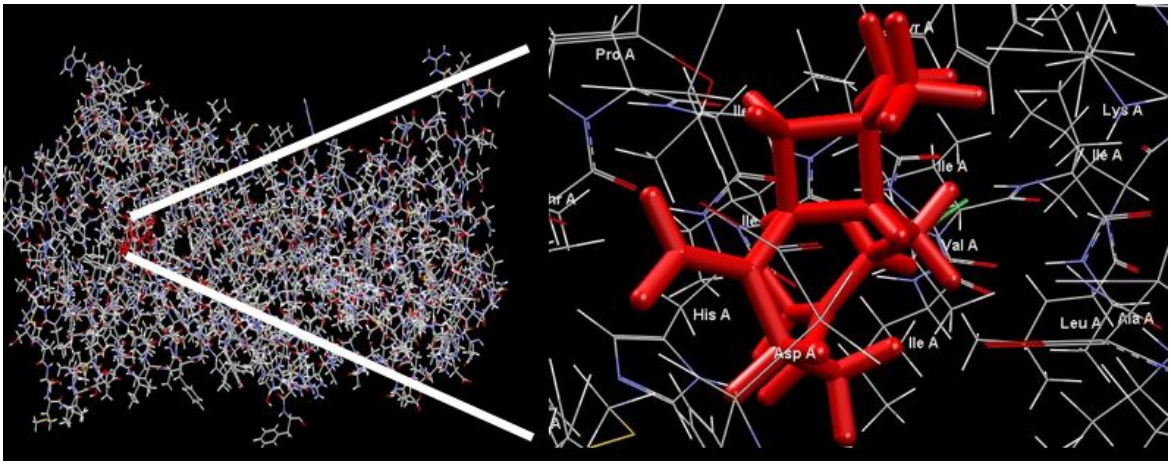

Figure 7. Trans-carophyllene-μ opioid complex.

The results are summarized in Table 2. In all antioxidant assays, LG exhibited stronger antioxidant activities than LG-βCD. For example, the DPPH radical scavenging ability of LG was 33.58 mgTE/g, while this activity was noted as 4.25 mgTE/g in LG-βCD. Similar differences were also determined in ferric and cupric ion reducing abilities.

Table 2 - *In vitro* antioxidant properties of the samples: *L. grata* leaf essential oil (LG) and *L. grata* leaf essential oil complexed with β-cyclodextrin (LG-βCD).

Antioxidant assays	LG	LG-βCD
Phosphomolybdenum assay (mmolTE/g sample)	22.74±1.75*	0.57±0.02
DPPH (mgTE/g sample)	33.58±2.02	4.25±0.17
ABTS (mgTE/g sample)	328.65±0.62	23.28±1.46
CUPRAC (mgTE/g sample)	536.89±10.98	34.06±1.52
FRAP (mgTE/g sample)	268.62±2.47	20.20±0.36
Chelating activity (mgEDTAE/g sample)	8.63±0.10	4.98±0.05

TE: Trolox equivalents; EDTAE: EDTA equivalents. *Data from three repetitions, with mean ± standard deviation.

Discussion

Previous study performed by our research group demonstrated that the inclusion complex LG-βCD or LG alone produced a remarkable analgesic profile in orofacial pain animal models with neuronal activation of CNS areas which are important areas to central pain modulation, such as the *Locus coruleus (LC)*, NRM and PAG. So, this study strongly suggested the involvement of the descending inhibitory pain modulation pathway in the production of analgesia after treatment with LG-βCD [48, 49]. Moreover, βCD enhanced the pharmacological effects of LG by increasing efficacy and producing longer-lasting analgesic activity compared with LG alone [48]. CDs are known to enhance the activity of analgesic and anti-inflammatory drugs [4, 20, 33, 46], including when evaluated in FM experimental protocols [30, 31, 33, 40]. Therefore, we assessed the hypothesis that LG-βCD could produce an anti-hyperalgesic effect in a chronic non-inflammatory muscle pain protocol in mice (considered to be an animal model for fibromyalgia, FM) due its CNS analgesic effects, and also examine its antioxidant effect.

The current study demonstrated that oral administration with LG-βCD (6, 12 or 24 mg/kg, p.o.) also produced outstanding antihyperalgesic activity (significantly reducing the primary and secondary hyperalgesia) ($p<0.05$; $p<0.01$; $p<0.001$) against a chronic non-inflammatory muscle pain model induced by saline acid injection into the gastrocnemius muscle of mice. LG-βCD produced no change in muscle strength, excluding the possibility of a reduction in motor performance typical of some drugs that act in the CNS [38]. These effects occurred with the probable involvement of opioid, and serotonin (5-HT) receptors (at least in part), which suggest its potential applicability in the management of chronic pain, such as FM. These effects may be related to the antioxidant profile demonstrated by LG.

FM is a differentiated pain syndrome, a type of ‘dysfunctional pain’, and there are a limited number of animal models that mimic the major symptoms, mainly widespread pain and the associated symptoms, such as fatigue and anxiety [10]. The non-inflammatory pain model induced by repeated intramuscular (gastrocnemius muscle) saline acid injections in rodents has been suggested as having validity to chronic widespread pain conditions (such as FM) in humans [51, 52, 58]. Morphological evaluation of the gastrocnemius muscle shows no apparent damage or inflammation associated with the administration of the saline acid [10, 52]. Moreover, central mechanisms involving the spinal cord, brainstem and cortex have been associated with development of hyperalgesia in this animal model [55].

Additionally, it is likely that pain modulation results from a descending pain modulatory pathway with inputs feeding into the midbrain PAG area. Neurons within the

NRM and nucleus reticularis gigantocellularis (NRG), which are included within the rostral ventromedial medulla (RVM), have been shown to project to the spinal or medullary dorsal horns to directly or indirectly enhance or diminish nociceptive traffic, changing the experience of pain [34, 57]. Moreover, decreased 5-HT levels and increased substance P and nerve growth factor levels are found in the cerebrospinal fluid of patients with FM [51]. It is well established that there are 5-HT inhibitory pathways to the spinal cord from the brainstem which contribute to analgesia and inhibit dorsal horn neuron activity, so the inhibition or blocking of these 5-HT pathways may facilitate central sensitization producing pain [18, 23, 51]. Moreover, PAG is the primary control center for descending pain modulation, its activation producing enkephalin-releasing neurons that project to the NRM. Thus, 5-HT released from the NRM descends to the dorsal horn of the spinal cord where it forms excitatory connections with the "inhibitory interneurons" located in the Laminae II (*substantia gelatinosa*), producing analgesia [34]. Our current results reinforce this hypothesis by the observed reduction in Fos-positive cells in the dorsal horn after pretreatment with LG-βCD, which suggests the involvement of descending inhibitory pain mechanisms.

Previously, we demonstrated that LG-βCD increased Fos protein expression in the NMR and PAG areas [49], which inhibits pain inputs. Interestingly, we have now shown that pre-treatment with LG-βCD (24 mg/kg, p.o.) decreased Fos protein expression in the dorsal horn ($p<0.01$), strongly suggesting that LG-βCD could be acting through the involvement of descending pain-inhibitory mechanisms: We have also now shown that LG-βCD was antagonized by naloxone (an opioid antagonist) and partially antagonized by methysergide (a $5HT_2$ antagonist and also a 5HT1 agonist) but was not antagonized by yohimbine (an alpha-adrenergic antagonist), important neurotransmitter system related with NRM and PAG areas in pain control which act strongly modulating the descending pain-inhibitory pathway on dorsal horn. Thus, it is reasonable to propose that the anti-hyperalgesic effects produced by LG-βCD administration are related to its actions on the opioid, and serotonergic systems.

In addition, LG has as its main compounds a rich mixture of terpenoids: camphor (28.7%), trans-caryophyllene (11.5%), bicyclogermacrene (10.2%), camphene (9.5%) and borneol (7.6%) [49]. Terpenes are known for their analgesic properties, especially their effects on the CNS, which makes them useful for treatment of neurogenic pain, such as FM [3, 9, 14, 15, 33, 40]. This pharmacological property of terpenes may contribute to the remarkable anti-hyperalgesic effect demonstrated in our study.

Reinforcing this hypothesis, previous studies disclose that *trans*-caryophyllene (*E*-caryophyllene) has attenuated acute and chronic pain in experimental protocols through the

involvement of the opioid and endocannabinoid systems [36]. Borneol and camphor are well-known analgesic compounds described in several patented pharmaceuticals available on the market to treat pain [15]. In addition, borneol has been shown to reduce mechanical hyperalgesia in inflammatory rodent models by enhancing $GABA_A$-mediated GABAergic transmission in the spinal cord [19]. Borneol and camphor are drugs that have affinity for a large number of receptors from the TRP family which are expressed in sensing neurons and primary afferent nociceptors [27]. Moreover, camphene has shown potent central analgesic activity and also an antioxidant profile in TBARS (Thiobarbituric acid reactive substances), and TRAP/TAR (Total reactive antioxidant potential/Total antioxidant reactivity) assays, as well as having the highest scavenging activities against different free radicals, such as hydroxyl and superoxide radicals [39]: these antioxidant properties are associated with modulation of pain in FM patients [17].

To further corroborate the results *in vivo*, we performed *in silico* assessment using a molecular docking approach. Target validation by *in silico* analysis contributed to a theoretical explanation of the drug-receptor relationship, and corroborated the evidence created *in vivo* protocols [12, 42]. We demonstrated that camphor, *E*-caryophyllene, bicyclogermacrene binds to the alpha-adrenergic receptor while bicyclogermacrene binds with moderate energy to the μOpioid and 5-HT receptors (as shown in Table 1). The docking study suggested that *E*-caryophyllene binds with weak energy to the μOpioid receptor, however, the *in vivo* data demonstrated that the interaction between drug-receptor is enough to produces an analgesic effect [36]. Controversially, our docking data suggested a strong binding energy between the major compounds and the alpha-adrenergic receptor but our *in vivo* data (when it was antagonized with yoimbine) did not corroborate this hypothesis. These apparent contradictory results of the possible effect of LG-βCD in the alpha-adrenergic receptor could be explained by the rich mixture of compounds present in the essential oil from *L. grata* which can act synergistically or antagonistically [49, 50]. Moreover, the evidence of the management of descending serotonergic pathways by LG, with the serotonergic neurons projecting from the RVM (rostral ventromedial medulla) through the DLF (dorsolateral funiculus) [55], was corroborated by the effect of oil components on the 5HT receptors (*in silico* data) and more strongly by the antagonism of the analgesic effect by methysergide. So, the docking data suggest that bicyclogermacrene and *E*-caryophyllene seem to act synergistically in favor of anti-hyperalgesic effect.

Seeking to corroborate the hypothesis of LG or LG-βCD having an antioxidant role which could contribute to their anti-hyperalgesic activity, we performed different chemical

assays (*in vitro*) including phosphomolybdenum, free radical scavenging (ABTS and DPPH), reducing power (CUPRAC and FRAP) and metal chelating assays. We used the complexed and non-complexed form of LG because it comes from *in vitro* protocols. LG produced stronger antioxidant activities than LG-βCD. Apparently, the antioxidant effects of EO were decreased with β-CD. [26] It has been reported that the antioxidant abilities of a complex cannot be detected by the test systems because *in vitro* test systems including DPPH, FRAP and CUPRAC measure different properties of the complex. Interestingly, the *in vitro* antioxidant profile presented by LG (although discreetly demonstrated by LG-βCD) corroborates the analgesic data demonstrated in our study, and reinforces this hypothesis.

Together, the present results indicate that LG-βCD produced an anti-hyperalgesic effect, reducing primary and secondary hyperalgesia, in a chronic non-inflammatory muscle pain model in mice without producing a reduction of hind and forepaw muscle force. In addition, the anti-hyperalgesic activity of LG-βCD seems to involve opioid and serotoninergic receptors, corroborating the hypothesis of the possible involvement of descending inhibitory pain modulation systems, which was partially supported by an *in silico* study, and by Fos protein expression in the dorsal horn. The antioxidant effect demonstrated by LG (non-complexed form) corroborated its antihyperalgesic activity. Moreover, these findings reemphasize the importance of new approaches, such as natural products associated with CDs, in the development of innovative tools in the management of chronic pain conditions, such as FM and others types of ‘dysfunctional pain’. Finally, further studies may be directed at characterizing the efficacy and safety of LG-βCD for possible clinical applicability.

List of Abbreviations

5-HT - 5-hydroxytryptamine

CDs – Cyclodextrins

CNS - Central Nervous System

DLF - dorsolateral funiculus

DPPH - 2,2-diphenyl-1-picryl-hydrazyl-hydrate

FM – Fibromyalgia

GABA - gamma-aminobutyric acid

LC - *Locus ceruleus*

LG - *L. grata* leaf essential oil

LG-βCD - *L. grata* leaf essential oil complexed β-cyclodextrin

NMR - nucleus raphe magnus

PAG - periaqueductal gray

TRP - transient receptor potential

βCD - β-cyclodextrin

RVM - rostral ventromedial medulla

Conflict of interest

The authors declare no conflict of interest.

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CAPÍTULO 4

Evidence for the involvement of the PKA pathway and inhibition of voltage gated Ca^{2+} channels in DRG neurons in antihyperalgesic activity produced by the essential oil of *Lippia grata*/β-cyclodextrin complex in rodent neuropathic pain-like models

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Abstract

Neuropathic pain (NP) is a difficult condition to treat because of the modest efficacy of available drugs. New treatments are, therefore, required. In the present study we aimed to investigate the effects of the essential oil from the leaf of *Lippia grata* alone or complexed in β-cyclodextrin (LG or LG-βCD) on persistent inflammatory and neuropathic pain in a mouse model. We also investigated Ca^{2+} currents in rat dorsal root ganglion (DRG) neurons. Male Swiss mice were treated with LG or LG/βCD (24 mg/kg, i.g.) and their effect was evaluated using an acute inflammatory pleurisy model and nociception triggered by intraplantar (i.pl.) injection of an agonist of the TRPA1 and TRPM8 channels. We also tested their effect in two chronic pain models: i.pl. injection of Freund´s Complete Adjuvant and partial sciatic nerve ligation (PSNL). In the pleurisy model, LG reduced the number of leukocytes and the levels of TNF-α and IL-1β. It also inhibited cinnamaldehyde and menthol-induced nociceptive behavior. The pain threshold in mechanical and thermal hyperalgesia was increased and paw edema was decreased in models of inflammatory and neuropathic pain. PSNL increased inflammatory protein contents and LG and LG-βCD restored the the protein contents of TNF-α, NF-κB, and PKA, but not IL-1β and IL-10. LG inhibited voltage gated Ca^{2+} channels from DRG neurons. Our results suggested that LG or LG-βCD produce anti-hyperalgesic effect in chronic pain models through reductions in TNF-α levels and PKA, and inhibited voltage-gated calcium channels and may be innovative therapeutic agents for the management of NP.

Key words: Natural prodcuts, pain, inflammatory pain, dorsal root ganglion, TNF-α, cyclodextrin.

1. Introduction

According to the World Health Organization (WHO), about 20% of the population live with some degree of chronic pain, which is more common in women, older individuals and people with relative deprivation. (Kehlet et al., 2006). Neuropathic pain (NP) is one of the most important types of chronic pain. It is triggered by lesions to the somatosensory nervous system that alter its structure and function (Costigan et al., 2009), causing hyperalgesia, allodynia and spontaneous pain. (Kaulaskar et al., 2012). Because NP does not have a role in protecting individuals against tissue damage it is not simply a symptom, but a disease in itself (Yekkirala et al., 2017) (White et al., 2007). Moreover, NP is one of the most difficult types of chronic pain to treat, due to the limitations of the current therapeutic arsenal and to the lack of understanding of its neurophysiological basis.

Peripheral nerve injury induces the activation of spinal cord glial cells including astrocytes, microglia, and oligodendrocytes (Tsuda et al., 2003; Zarpelon et al., 2016). Once activated, glial cells release pro-inflammatory mediators (such as cytokines) that activate/sensitize nociceptors in the spinal cord, enhancing nociceptive neurotransmission. Cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL-1β), and IL-33, which induce nociceptor sensitization, play a key role in the production of pain commonly experienced by patients with NP (Ji et al., 2013; Zarpelon et al., 2016). This complex interaction between inflammatory mediators and neuronal cells leads to central sensitization, decreases peripheral sensitization and induces spontaneous firing of nociceptors (Julius and Basbaum, 2001; Pinho-Ribeiro et al., 2017).

Additionally, the voltage gated calcium channels (VGCCs) have a pivotal role in the ascending pain pathways, because they are required for the generation and transmission of the pain process, especially in primary afferent neurons (Zamponi et al., 2009). N-type calcium channels are localized to synaptic nerve terminals in laminae 1 and 2 of the dorsal horn where their activation can generate an increase in the release of neurotransmitters (such as glutamate and substance P) that are essential in establishing pain and increasing pain sensitivity (Krarup, 2003). Moreover, calcium channel activation in DRG cells by pro-inflammatory cytokines (such as TNF-α and IL-1β) leads to increased cellular excitability with consequent activation of the ascending pain pathways (James N.Campbell AND Richard A. Meyer, 2006). Anti-TNF-α therapy has been shown to improve axonal regeneration and consequently reduce pain in experimental models of sciatic nerve ligation. Thus, new drugs that block or attenuate the action of VGCCs, or regulate of levels of pro-inflammatory

cytokines (such as TNF-α) may be potentially useful for treating NP and may also help to induce neural regeneration in these patients (Fornasari, 2017; Kato et al., 2010; Pina et al., 2017).

The complexity and multiplicity of its underlying pathophysiological mechanisms has made it difficult to identify tractable targets with broad involvement in NP (Yekkirala et al., 2017). Thus, the first treatment of choice includes available analgesics - nonsteroidal anti-inflammatory drugs, amine reuptake inhibitors, antiepileptic drugs and opioids – which have varying, but typically low levels of analgesic efficacy, and are generally coupled with deleterious effects (Cohen and Mao, 2014; Truini et al., 2003; Woolf and Mannion, 1999; Yekkirala et al., 2017). As current treatments lack efficacy, some researchers have turned their attention to medicinal plants as a source of new active compounds because they are already widely used, especially in relation to the treatment of pain and inflammation (Calixto et al., 2004; De Sousa, 2011; Guimarães et al., 2013).

The plants belonging to the genus *Lippia* (Verbenaceae) comprise more than 250 species, which are widely distributed in most tropical and subtropical countries. This genus is constituted of aromatic plants, rich in essential oils (EO). Their pharmacological properties are attributed to the terpene compounds making up most of the EO which have proven clinical applicability, already being used in various drugs (Craveiro et al., 1981; Guimarães et al., 2013; Pina et al., 2017).

Recently, our research group demonstrated that the essential oil of the species *Lippia grata* (LG) complexed in β-cyclodextrin (β-CD), given orally, presented analgesic-like activity in orofacial pain models and a fibromyalgia-like mice model (Siqueira-Lima et al., 2017, 2014). Despite its proven effect as an antinociceptive agent in acute pain, probably linked to some terpenoids such as camphor, borneol and β-caryophyllene, little is known about the effect of this essential oil on chronic pain such as NP. Thus, we decided to investigate whether the essential oil of *L. grata* leaves complexed in β-cyclodextrin (LG/βCD) produces an anti-hyperalgesic effect in persistent neuropathic (partial sciatic nerve ligation [PSNL]) and inflammatory pain (intraplantar injection of Freund's Complete Adjuvant- CFA) in rodent pain models and also the possible mechanisms underlying any anti-hyperalgesic profile.

2.Material and Methods

2.1. Plant material and essential oil.

The fresh leaves of L. grata were collected in June 2011 in Capim Grosso, Bahia, in north-eastern Brazil (11°19′28.4″S, 40°09′08″W). L. grata was identified by Dr. Tania Silva (Herbarium of The State University of Feira de Santana, UEFS), where a voucher specimen has been deposited (HUEFS 169543). The essential oil was extracted by hydrodistillation in accordance with the methodologies previously described by our group (Siqueira-Lima et al., 2014).

All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SISGEN (Cadastro A0FBC55)

2.2. Preparation of binary mixture of LG with βCD

Preparation and physical-chemical characterization of the *L. grata* (LG) leaf essential oil complexed with β-cyclodextrin (βCD) was carried out in accordance with (Siqueira-Lima et al., 2014) the methodologies previously described by our group (Menezes et al., 2014; Quintans et al., 2013).

2.3. Animals

Experimental protocols were performed using male Swiss mice (28-35 g) and adult male Wistar rats (180-220g) obtained from the Animal Facilities of the Federal University of Sergipe (UFS/Brazil) and the Federal University of Minas Gerais (UFMG/Brazil) respectively. Animals were randomly housed in controlled-temperature rooms (22-24°C), under a 12/12 h light-dark cycle, with access to water and food *ad libitum* until use. All behavioral protocols were performed between 9:00 a.m. and 3:00 p.m. Experimental protocols were approved by the Animal Care and Use Committee at UFS/Brazil (65/15) and UFMG/Brazil (CEUA 149/2015). Rats were used only for the electrophysiological recordings in the dorsal root ganglia (DRG) neurons. The behavioral experiments and animal treatment were performed with the examiner blinded to the group. We made all necessary efforts to minimize the number of rodents used and any discomfort to them.

2.4. Carrageenan-induced pleurisy

Pleurisy was induced in the mice by intrapleural administration of 100µl of 1% (w/v) carrageenan suspension in sterile saline solution (de Oliveira et al., 2012). A specially adapted 13x5 needle was introduced into the right side of the thoracic cavity for injection of the carrageenan solution. The animals were pre-treated with LG or LG-βCD (24 mg/kg, gavage) or vehicle 60 min. before the injection of the inflammatory agent. Four hours after induction

of the pleurisy, the animals were euthanized, and the pleural inflammatory exudate was removed by aspiration through pleural lavage with 1 mL of sterile phosphate buffered saline (PBS) containing ethylenediaminetetraacetic acid (EDTA; 10 mM). The exudate volume was measured, and an aliquot of 50µl was diluted with Turk's solution (1:20). Total leukocytes were counted in a Neubauer chamber, using a light microscope (Vinegar et al., 1973). The fluid obtained from the pleural cavity was collected for further determination of cytokine (TNF-α, IL-1β) levels.

2.5. Pain behavior induced by the intraplantar injection of TRPM8 and TRPA1 agonists

To evaluate the possible involvement of transient receptor potential cation channels (TRP), subfamily A member 1 (TRPA1) and melastatin 8 (TRPM8), on the antinociceptive effect of LG and LG/βCD, mice were submitted to a test using either cinnamaldehyde or menthol, activators of these channels respectively, as previously described by (Córdova et al., 2011). The mice were pretreated with LG, LG/βCD (24 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.) 1 h prior to the injection of 20 µL of cinnamaldehyde (an activator of the TRPA1 channel, 10 nmol/paw), menthol (an activator of the TRPM8 channel, 1.2µmol/paw) or corresponding vehicle in the ventral surface of the right hind paw. The animals were individually placed in an acrylic chamber (9×11×13 cm), and paw licking or biting were recorded for 5 min (cinnamaldehyde), or 20 min (menthol). The time spent licking/biting the injected paw was considered indicative of nociception.

2.6. Complete Freund's adjuvant induced inflammation

Mice received 20 µL of complete Freund's adjuvant (CFA 1 mg/mL of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate; Sigma-Aldrich) in the plantar region of the right hind paw, according to a previously reported method (Bortalanza et al., 2002). The degree of inflammation was evaluated by measuring the volume of paw edema 2 and 4 hours after the CFA injection and mechanical hyperalgesia 24 hours after the CFA injection and then daily for 8 consecutive days.

2.6.1. Plethysmometer test

The volume of each mouse paw was measured (mL) with a plethysmometer (EFF 361; Insight®, Brazil) before (Vo) and after (VT) the CFA injection, as described previously

(Winter et al., 1962). The amount of paw swelling was determined for each mouse and data were represented as paw volume variation (Δ, mL).

2.6.2. Measurement of Mechanical Hypersensitivity

The mice were acclimatized in individual transparent boxes on a wire-mesh platform to allow access to the ventral surface of the hind paws. Their sensitivity to mechanical stimulation generated by the gradual increase of pressure of a manual force transducer (electronic analgesimeter, model EFF-301, Insight®, Brazil), adapted with a polypropylene tip, was evaluated. This test indicates hyperalgesia by automatically recording the pressure intensity needed to evoke a flexion reflex of the hind paw, which is defined as a withdrawal of the paw followed by rapid movements, characterized as flinches (Vivancos et al., 2004).

2.6.3. Thermal hyperalgesia evaluation

For the hot plate test, the mice were placed in the hot plate apparatus (EFF 361; Insight®, Brazil) maintained at 55°C. The first ipsilateral hind paw flexion reflex was considered the nociceptive endpoint. The response latency was recorded before the surgery and at the 10th day post-surgery. The maximum latency (cut-off) was set at 20s to avoid tissue damage (Lemons and Wiley, 2012; Nucci et al., 2012). To obtain data purely derived from the treatment, the inhibition values are presented as the difference between the basal values of vehicle or drug-treated animals and the respective controls.

2.7. Segmental spinal nerve ligation-induced neuropathic pain

Partial sciatic nerve ligation (PSNL) was performed according to the procedure previously described in mice (Malmberg and Basbaum, 1998). Mice were anaesthetized with a premixed solution containing ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Partial ligation of the right sciatic nerve was performed by ligating the distal one-third to one-half of the dorsal portion of the sciatic nerve. In sham-operated mice, the sciatic nerve was exposed without ligation. The wound was closed and covered with 10% povidone-iodine solution. The sham-operated mice received only vehicle (10 mL/kg, i.g.), and the PSNL-operated mice were randomly divided into control and treatment groups, which received vehicle (10 mL/kg, i.g.) or LG and LG/βCD (24 mg/kg, v.o.), respectively, 7 days after surgery. The mechanical hyperalgesia response was recorded before surgery (B), immediately before treatment (0 h), and after treatment (1, 2, 3, 4, 6 and 8 h) to verify the time-course effect of the treatment as

described below. To investigate the effects of repeated treatment on the mechanical hyperalgesia response, LG and LG/βCD (24 mg/kg, i.g.) were administered once daily for 7 consecutive days (from the 8th to 14th day after PSNL), and the effects were examined 1 to 2 h after treatment. After 2 days without treatment, treatment was resumed on the 17th and 18th days after PSNL to assess the development of LG and LG/βCD tolerance.

2.8. Determination of cytokines levels

The levels of TNF-α, IL-1β or IL-10 in sciatic nerve or lumbar spinal cord segments (L4 and L6) were determined at 17 days after PLSN. The samples were homogenized in 150µL of phosphate-buffered saline (PBS 50 mM, pH 7.4) containing EDTA (0.3%), nonidet P 40 (0.5%) and protease inhibitors. The samples were centrifuged at 6000 or 8000 rpm for 10 min at 4 ° C, and the supernatant obtained was used to determine cytokines levels. The protein concentration was measured using the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard protein.

TNF-α, IL-1β and IL-10 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience®) according to the manufacturer's instructions, and colorimetric measurements at 450 nm were made using a microplate reader (ASYS®). The concentration was obtained by interpolation from a standard curve. All results were expressed as picograms (pg) of cytokine per milligram (mg) of total protein.

2.9. Western blot assay

For the preparation of total protein homogenate, the mice were killed by cervical dislocation on the 17th day after PNSL, the spinal cord and the sciatic nerve were removed. For Western blot assay the tissues were homogenized in 150 µl of a lysis solution RIPA contend 1 mM EDTA, 20 mM Tris–HCl, pH 7,5, 150 mM NaCl, 1 mM EGTA, 1% sodium deoxycholate, 1% nonidet 40, containing proteases and phosphatases inhibitors. For the electrophoresis analysis, samples were dissolved in 25% (v/v) of solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 5 min. The protein concentration was determined by the method of Bradford (1976) using BSA as the standard.

Tissue homogenates (50 µg) were separated by SDS-PAGE and transferred to PVDF membranes (BioRad) for 16 h at 25 V in transfer buffer (48 Mm Tris, 39 mM glycine, 20%

methanol). The PVDF membranes were washed for 15 min in Tris–buffered saline with 0.1% Tween-20 (T-TBS; 0.5 M NaCl, 20 Mm Tris, 0.1% Tween-20, pH 7.5), followed by 1 h incubation in blocking solution (T-TBS plus 5% defatted dried milk). After incubation, the blots were washed three times for 15 min with T-TBS, and then incubated overnight at 4 °C in blocking solution containing the following antibodies: anti-PKA (Imuny IM0409) diluted 1:1000; anti PKAcα (Cell Signaling Technology), diluted 1:1000; anti-phospho-NFκB (Ser536) (93H1) (Cell Signaling Technology) diluted 1:1000; anti- NFκBp65 (D14E12) XP (Cell Signaling Technology) diluted 1:1000; and anti-β-actin (Cell Signaling Technology) diluted 1:1000. The blots were then washed three times for 15 min with T-TBS and incubated for 2 h in blocking solution containing peroxidase conjugated anti-rabbit IgG diluted 1:2000. The blots were washed twice again for 15 min with T-TBS. The blots were then developed using a chemiluminescence substrate. Blots were quantified, and optical density values were obtained for the studied proteins. All results were expressed as a relative ratio to β-actin.

2.10. Measurement of motor performance

2.10.1. Evaluation of muscle strength

The hindpaw grip strength evaluation, previously described by Burnes et al. (2008), was used before the experiment as a baseline measurement and after PLSN. The mice were pulled by the tail to measure the grip strength of the hindpaw. The animals were evaluated 3 times and the mean calculated to obtain the absolute strength (g). The mice then received the same pharmacological treatment as previously described.

2.10.2. Rotarod test

In order to discard possible non-specific muscle relaxant or sedative effects of essential oil of Lippia grata, motor performance was evaluated using the rota-rod test (Valerio et al., 2007). The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (AVS Projetos, Ribeirão Preto, SP, Brasil). The bar rotated at a constant speed of 22 rotations per min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with vehicle (saline) or LG or LG-βCD (24 mg/kg, p.o.), and testing was performed 1.5, 3.5 and 5.5 h after treatment. A cut-off time of 120s was used.

2.10. Voltage Clamp

We recorded calcium currents (Ica++) from adult male Wistar rats' dorsal root ganglia (DRG) neurons. We used the patch-clamp technique (Hamill et al., 1981) on neurons that were perfused (1mL/min) and dialyzed with Na^+,K^+-free solutions containing channel blockers to isolate calcium currents from other membrane currents. The bath solution contained (in mM): choline chloride (105), TEA chloride (30), $CaCl_2$ (2), $MgCl_2$ (4) and HEPES (10) and it was adjusted to pH 7.4 with tetraethylammonium hydrochloride. The pipette internal solution was (in mM): CsCl (126), MgATP (1), EDTA (1), EGTA (10), HEPES (10) and it was adjusted to pH 7.2 with CsOH. All experiments were performed at room temperature.

The cells were kept in a holding potential of −80mV and at every 7 seconds we applied depolarizing pulses to 0mV for 200ms. The liquid junction potential was corrected to eliminate the difference of −6mV between the pipette and bath solutions. The sampling rate was 10 kHz.

Pipettes were prepared from glass capillaries (World Precision Instruments, Sarasota, USA) pulled and heat-polished (PP 830 puller and MF-830 microforge, Narishige, Tokyo, Japan) to a tip resistance of 1 to 3 MΩ. Data were acquired using a patch-clamp amplifier (Multiclamp 700B, Axon Instruments, USA) and a digitizer (Digidata 1200 series, Axon Instruments, USA) controlled by the pClamp 8.2 software (Axon Instruments, USA).

The LG essential oil was diluted in DMSO (33% v/v) and added to the bath resulting in the following concentrations: 0.1, 0.3 or 1.0 µL/mL. As control, we used DMSO at its highest concentration.

We recorded calcium current (I_{Ca}) for 1 to 4 minutes, in order to estimate the current rundown. Once the current reached stability we calculated the percentage of inhibition using the last control and the first experimental currents. In some cells we performed multiple drug applications, washing it at different intervals.

2.11. Statistical Analysis

Results were expressed as mean ± S.E.M. The differences between groups were analyzed using one-way or two-way ANOVA, followed by the Tukey test or Bonferroni test, respectively. Values of $p < 0.05$ were considered statistically significant. All statistical analyzes were performed using GraphPad Prism 5.0 software (GraphPad Prism Software Inc., San Diego, CA, USA). The dose response curves of Ica++ inhibition were fitted to a Hill

equation, which we used to calculate IC50. Student's two-tailed T-test was used to analyze the paired data.

3. Results

3.1. Carrageenan-induced pleurisy

All mice that had received carrageenan developed acute pleurisy, producing leucocyte migration (turbid exudate) (Fig. 1A). The administration of carrageenan into the pleural space of mice induced an inflammatory process with a significant increase in total leukocyte count compared with untreated-mice. Mice pretreated with LG as well as LG/βCD showed a significant attenuation of the number of leukocytes within the exudate [$F(3,25)=50.51$; $p=0.0001$)]. In addition, the levels of TNF-α [$F(3,9)=14.67$; $p=0.0008$)] and IL-1β [$F(3,10)=20.16$; $p=0.0001$)] were significantly elevated in the exudate from mice at 4 h after carrageenan administration in the control group. In contrast, the levels of these cytokines were reduced in mice treated with LG and LG/βCD (Fig. 1B and C) compared to the control group ($p< 0.001$).

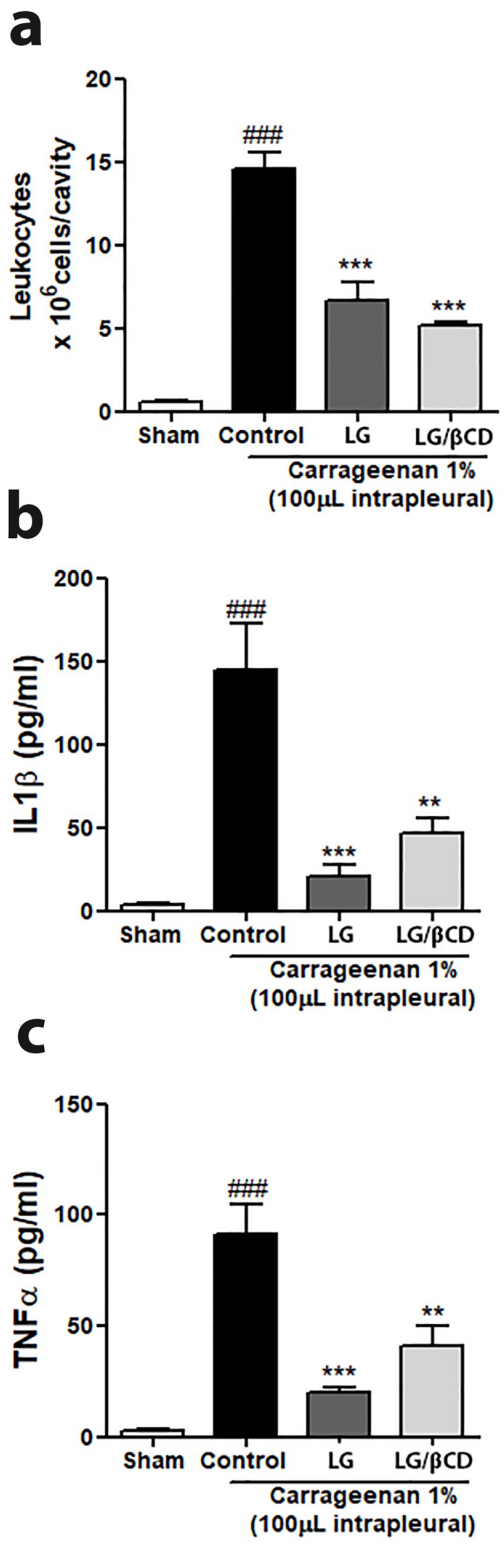

Figure 1. Effect of LG and LG/βCD on the number of PMNs **(A)** and on the levels of pro-inflammatory cytokines TNF-α **(B)** and IL-1β **(C)**. The analyses were made from the exudates at 4 hours after the induction of pleurisy by carragenan injection. Data are reported as means ± SEM of 8 animals. Statistical analysis was performed using a one-way ANOVA

followed by the Tukey's test.**p<0.01, ***p<0.001 vs control group; ###p<0.01 vs sham group.

3.2. Pain induced by TRPA1 and TRPM8 agonists

As shown in Fig.2A, the LG and LG/βCD (24 mg/kg, i.g.) inhibited the nociceptive behavior induced by cinnamaldehyde. Pretreatment with camphor (7.6 mg/kg, s.c.), a nonspecific TRP blocker, inhibited the cinnamaldehyde-induced nociception [F(4,20)=18.65; p=0.0001)]. Intraplantar injection of menthol produced a marked nociception in mice. Previous treatment with LG and LG/βCD (24 mg/kg, i.g.) inhibited menthol-induced nociceptive behavior [F(3,16)=57.05; p=0.0001)] (Fig. 2B).

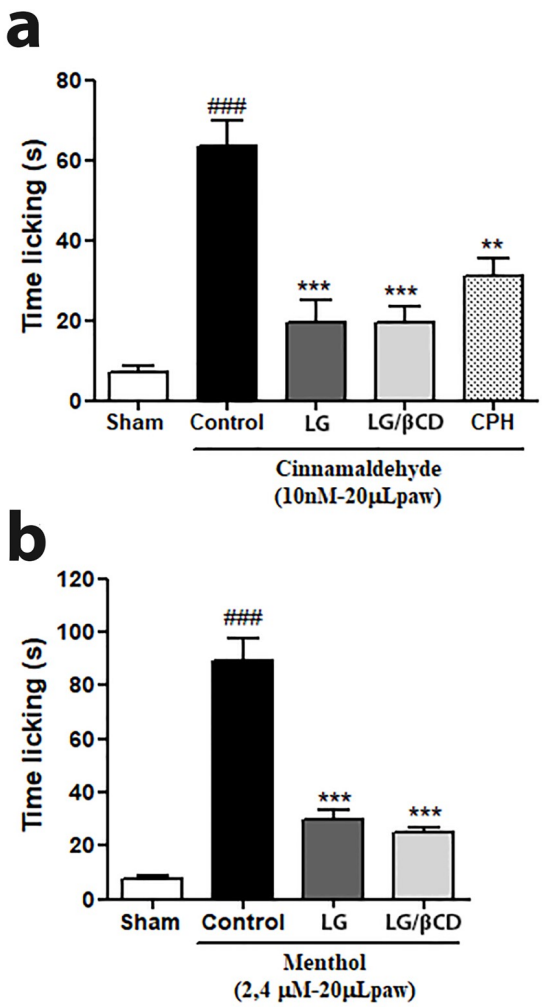

Figure 2. Effect of LG and LG/βCD on the time licking after the intraplantar injection of cinnamaldehyde 10 nM **(A)** and menthol 2,4 µM **(B).** Oral treatment was given 1 hour prior to

injection and the paw licking or biting was recorded for 5 min (cinnamaldehyde) and 20 min (menthol). Data expressed as means ± SEM of 8 animals. Statistical analysis was performed using a one-way ANOVA followed by the Tukey's test.**$p<0.01$, ***$p<0.001$ vs control group; $^{###}p<0.01$ vs sham group.

3.3. CFA-Induced Chronic Inflammatory Pain

To investigate the antinociceptive and anti-inflammatory activity of LG and LG/βCD, a chronic inflammatory model was used. For this, mechanical hypersensitivity was evaluated from day 1 to day 8 after an intraplantar injection of CFA in the mice. As shown in Figure 3A, CFA caused significant mechanical hypersensitivity characterized by a reduced paw withdrawal threshold compared to the control group ($p<0.001$). Oral administration of LG and LG/βCD was able to significantly reverse mechanical hypersensitivity, which lasted up to 6 hours in the LG group ($p<0.001$) and 8 hours in LG/βCD group ($p=0.0001$)). This antihyperalgesic effect was maintained while LG and LG/βCD was orally administered daily, until the 8th day posttreatment ($p=0.0001$). When treatment was interrupted for 2 days, mechanical hypersensitivity in the treated group was reestablished. On the 8th day, the treatment was restarted and LG and LG/βCD were able to reduce mechanical hypersensitivity with a profile similar to the 1st day. These results excluded the possibility of the development of a tolerance effect of LG and LG/βCD. Paw edema was also evaluated after CFA induction. The administration of LG and LG/βCD 60 min before CFA significantly reduced ($p<0.01$) paw edema at 2 and 4h after the CFA injection (Fig. 3B). The results obtained with control groups supported the effects of LG and LG/βCD, since the vehicle (distilled water) had no activity.

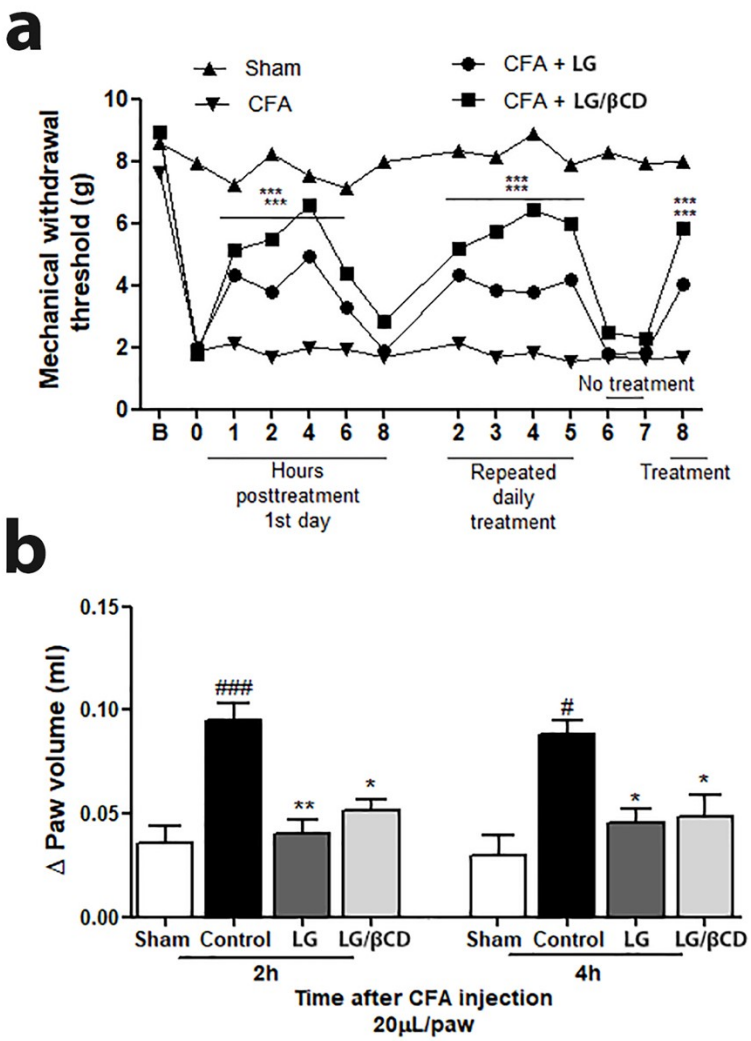

Figure. 3. Effect of LG and LG/βCD on (**A**) mechanical hyperalgesia 24 hours after the CFA injection and daily for 8 consecutive days; and on (**B**) paw edema 2 and 4 hours after the CFA injection. Data are reported as means ± SEM of 8-10 animals. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Bonferroni (**A**) and Tukey's test (**B**). *p<0.05**p<0.01, ***p<0.001 vs control group; $^{\#}$p<0.05, $^{\#\#\#}$p<0.01 vs sham group.

3.4. Segmental spinal nerve ligation-induced neuropathic pain

Partial sciatic nerve ligation produced a marked and long-lasting development of hyperalgesia on the ipsilateral side eight days after PSNL in the control group, and in the experimental groups treated with LG and LG/βCD (24 mg/kg, i.g.). The group that underwent

sham surgery maintained the frequency response at baseline levels throughout the experiment (Figure 4A). Acute oral treatment with the LG and LG/βCD (24mg/kg, p.o.) significantly decreased the paw withdrawal response, which lasted up to 6 hours ($p<0.001$). This antihyperalgesic effect was maintained while LG and LG/βCD were orally administered daily until the 14th day posttreatment ($p < 0.001$). When treatment was interrupted for 2 days, mechanical hyperalgesia in the treated group was reestablished. On the 17th day the treatment was restarted, and LG and LG/βCD were able to reduce mechanical hyperalgesia with an effect profile similar to the 1st day.

Regarding thermal hyperalgesia, the results presented in Figure 4B show that PSNL induced a decrease in paw withdrawal latency to thermal stimulus (heat) compared to baseline. However, pretreatment with LG and LG/βCD (24 mg/kg, i.g.) reduced the thermal hyperalgesia induced by PSNL and the response latency increased compared to the vehicle group ($p=0.0001$).

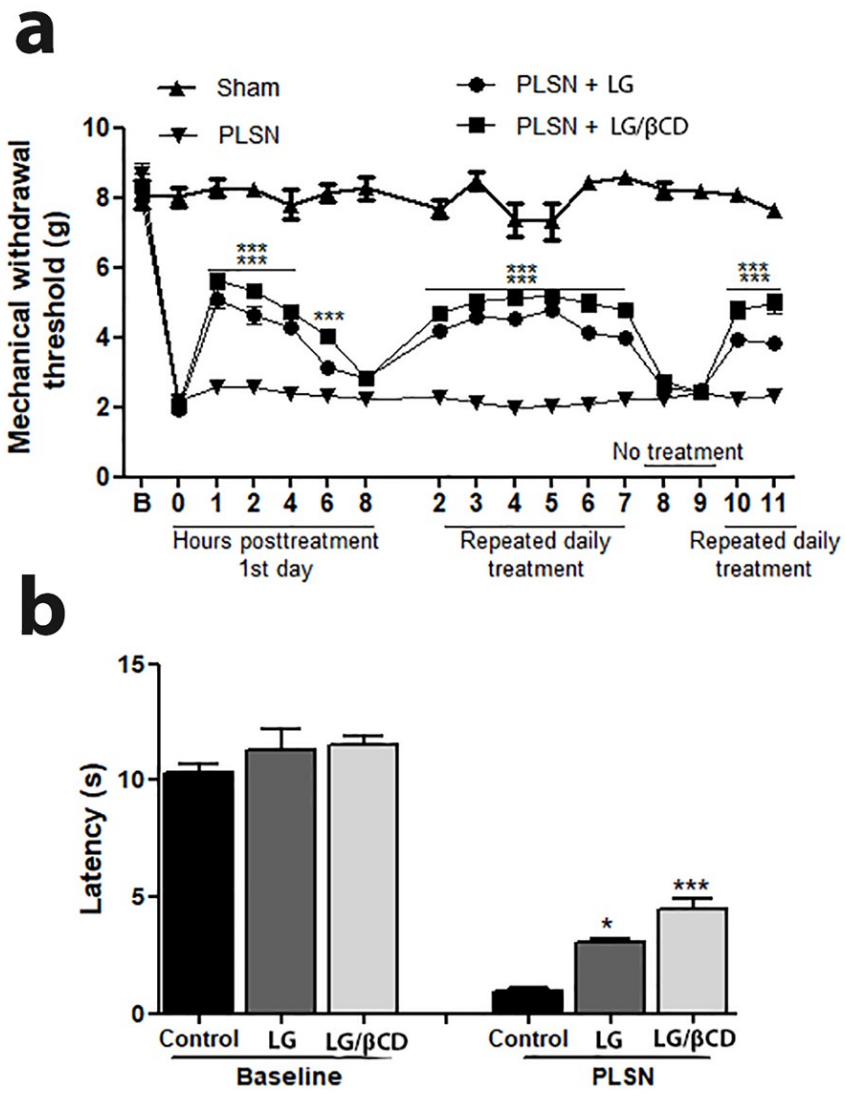

Figure. 4. Effect of LG and LG/βCD on (**A**) mechanical hyperalgesia response after partial sciatic nerve ligation. The measures were recorded before surgery (B), immediately before treatment (0 h), after treatment (1, 2, 3, 4, 6 and 8 h) and daily for 8 days with a two-day break (eighth and ninth day); and on (**B**) the time of latency on the hot plate 1 hour after the oral treatment. Data are reported as means ± SEM of 8-10 animals. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Bonferroni (**A**) and Tukey's test (**B**). *p<0.05, ***p<0.001 vs control group.

3.5. Locomotor activity

Acute and prolonged intragastric treatment with LG and LG-βCD (24 mg/kg) did not alter the locomotor activity of animals in the rotarod and Grip Strength Meter tests, compared with the control group (data not shown).

3.6. Cytokine levels in sciatic nerve and spinal cord

The effects of LG and LG/βCD (24 mg/kg, i.g.) were evaluated on TNF-α, IL-1β and IL-10 levels in the sciatic nerve and spinal cord 17 days after surgery. The results showed that PNSL increased TNF-α and IL-1β ($p < 0.05$) production in the sciatic nerve [$F(3,18)=9.891$; $p=0.0002$)] and spinal cord [$F(3,17)=9.369$; $p=0.0007$)]. In addition, LG and LG/βCD were able to reduce the TNF-α levels in both structures ($p < 0.05$) when compared to the PNSL control group (Figures 5A and D). No difference was found in IL-1β production between the treated-groups and the control group (Figure 5).

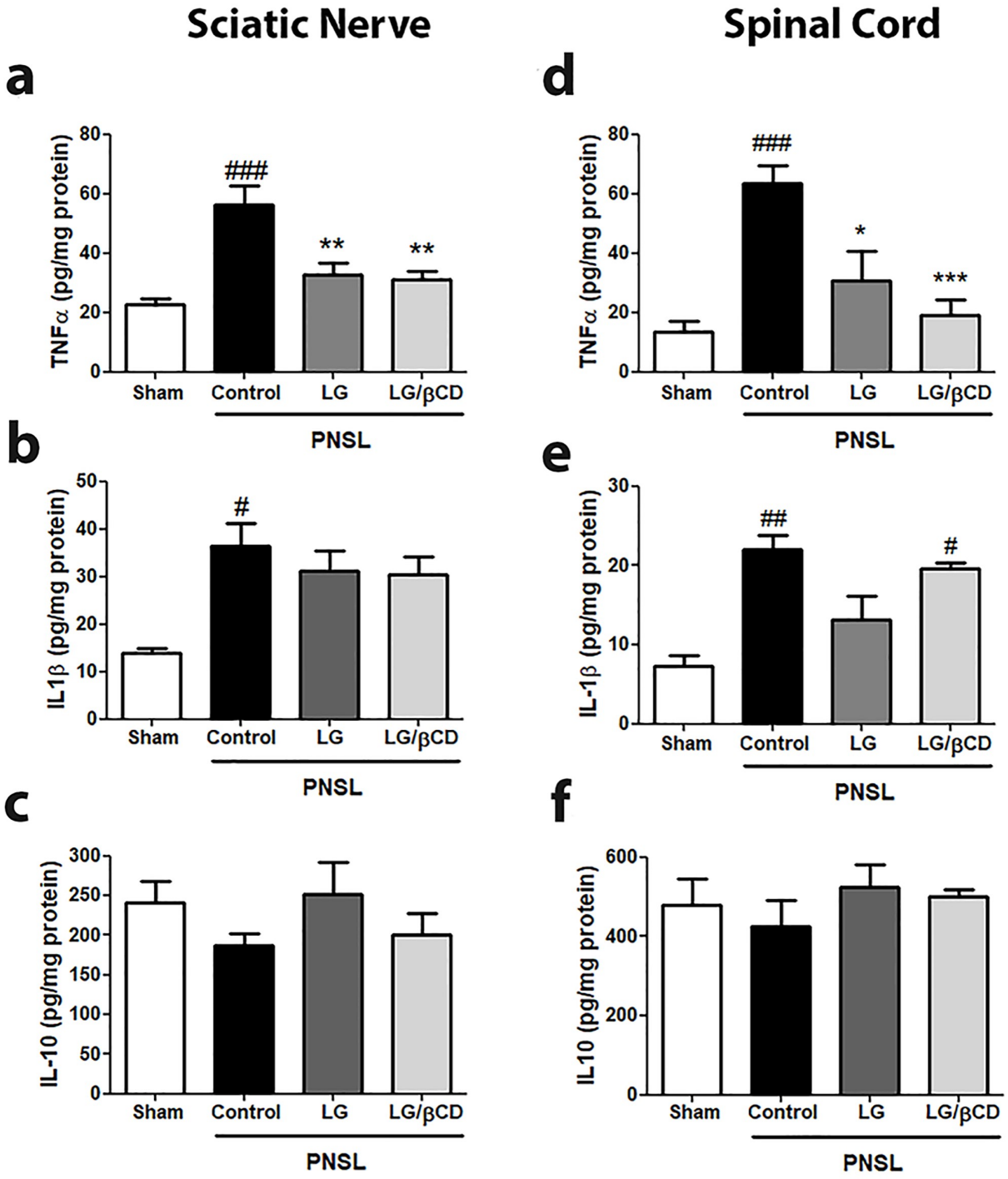

Figure 5. Effect of LG and LG/βCD on the levels of pro-inflammatory cytokines TNF-α (**A** and **D**) and IL-1β (**B** and **E**) and anti-inflammatory cytokine IL-10 (**C** and **F**) on the sciatic nerve and spinal cord, respectively. Data expressed as means ± SEM of 8-10 animals. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Tukey's test. *p<0.05**p<0.01, ***p<0.001 vs control group; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.01 vs sham group.

3.7. NFκB and PKAc-α immunocontent in sciatic nerve and spinal cord

Figure 6A summarizes the effect of LG and LG-βCD (24 mg/kg, i.g) on NFκB activation in sciatic nerve and spinal cord tissue. The results showed that PSNL increased phospho-NFκB (Ser536)/total NFκB immunocontent ratios compared to the sham group in the sciatic nerve [$F(3,16)=11.86$; $p=0.0002$)] and spinal cord [$F(3,17)=4.466$; $p=0.0173$)]. However, LG and LG-βCD presented unaltered phospho-NFκB (Ser536)/total NFκB immunocontent ratios in the sciatic nerve and spinal cord (Figures A, C).

Figure 6B shows the PKAc-α immunocontent assessed by western blot. The results determined that LG and LG-βCD were able to reduce PKAc-α immunocontent compared to the control group in the sciatic nerve [$F(3,12)=11.13$; $p<0.0001$)] and spinal cord [$F(3,16)=7.315$; $p=0.0028$)] (Figures 6 B, C).

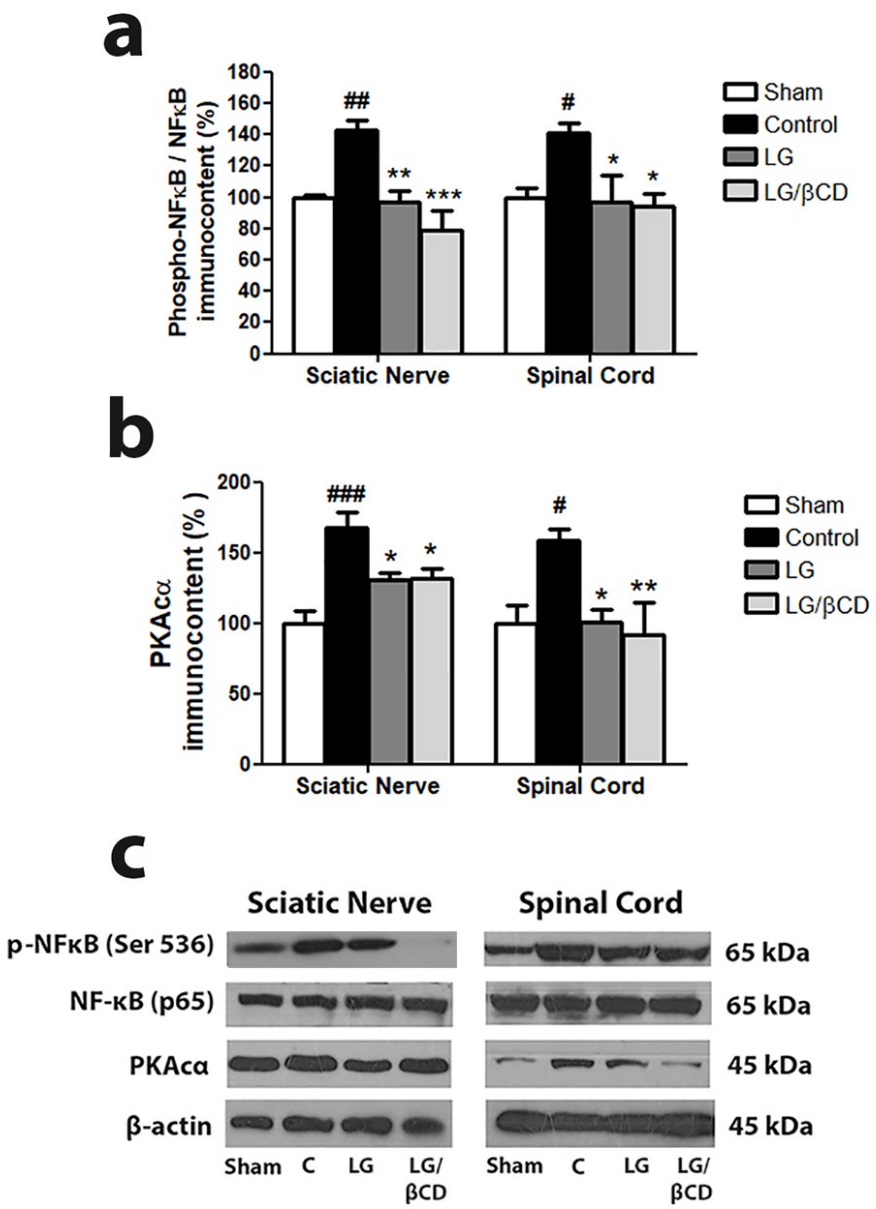

Figure 6. Effect of LG and LG-βCD on phospho-NFκB/ NFκB immunocontent ratio (A) and PKAcα immunocontent (B) in the sciatic nerve and spinal cord of mice submitted to PSNL. Representative blots are shown (C). Data are reported as means ± SEM of 5-6 animals and expressed as percent of sham value. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Tukey's test. *p<0.05**p<0.01, ***p<0.001 vs control group; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.01 vs sham group

3.8. Inhibition of calcium current by LG

Our results show that the essential oil produced an inhibitory effect on calcium current (Ica) with fast onset (Fig 7A and 7B). Perfusion of the *L. grata* at 0.1 µL/mL on DRG neurons decreased the peak of ICa by 24 ± 2% (mean ± SEM, n=5; p<0.05). The sustained component at the end of Ica was inhibited by 62± 8% (n=5; p<0.05). At an intermediate concentration (0.3 µL/mL), peak I_{Ca} decreased 71± 8% (n=5; p<0.05). The peak of I_{Ca} was essentially eliminated (inhibition of 94 ± 3%, n=3; p<0.001) by the essential oil at the highest concentration (1.0 µL/mL). The calculated IC50 was 0.21 µL/mL. The dose-response relationship is shown in figure 7C.

The inhibition caused by *L. grata* was reversible (Figure 7B). After a 70-second wash, we observed a partial recovery of the calcium current.

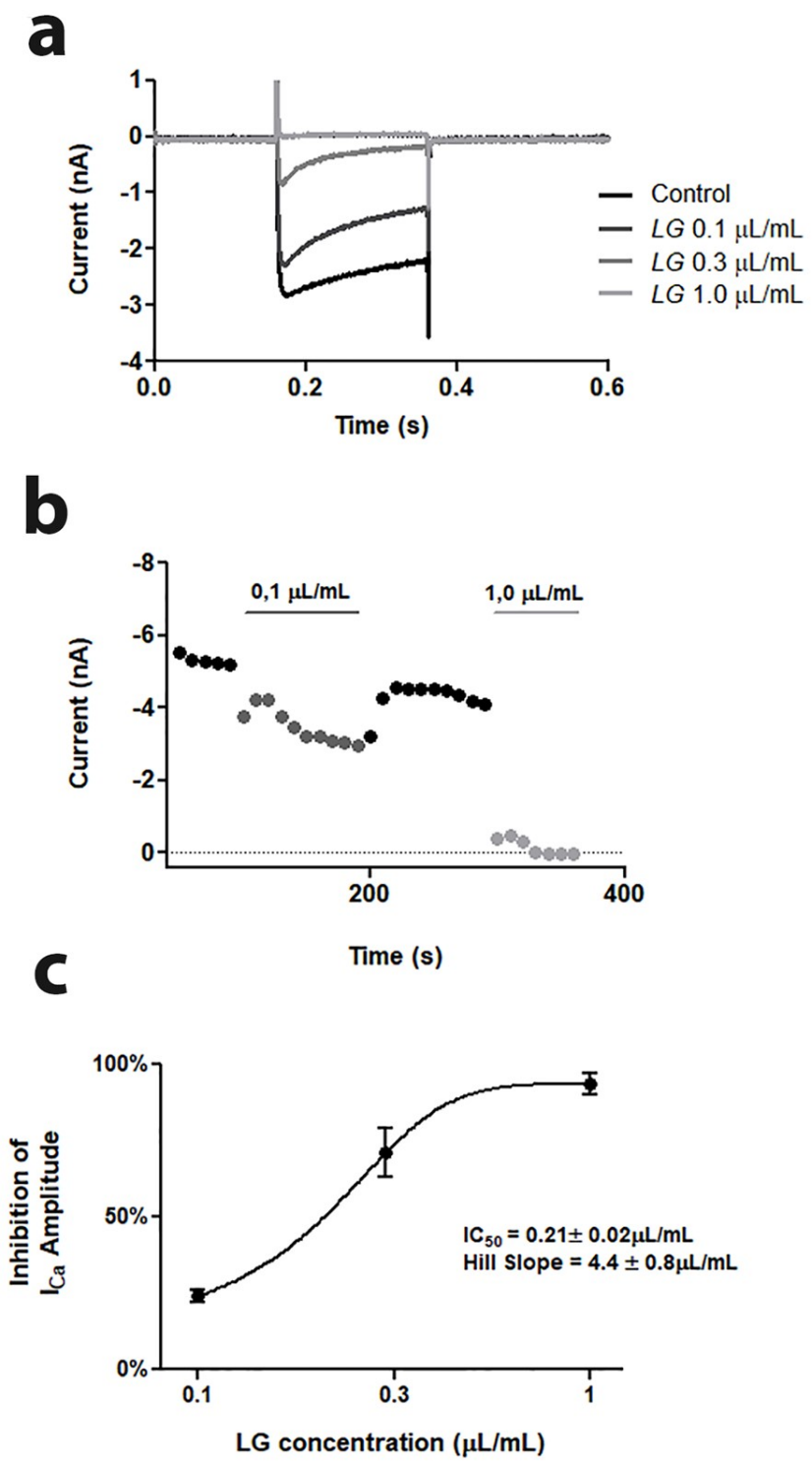

Figure 7. Effect of essential oil of *Lippia grata* on amplitude and sustained component of Ca^{2+} current (I_{Ca}) in dorsal root ganglion neurons from *Wistar* rats. (a) Representative traces of I_{Ca} recorded before and after 7s of oil application at 0.1, 0.3 or 1.0 µL/mL in the same cell.

(b) Time course of peak Ca^{+2} currents showing their inhibition and recovery after washout. **(d)** Dose-response relationship between the essential oil concentration and the inhibition of the Ca^{+2} current amplitude. IC50 was 0.21 ± 0.02 and the Hill slope was 4.4 ± 0.8

4. Discussion

The International Association for the Study of Pain (IASP), defines neuropathic pain as "pain caused by a lesion or disease of the somatosensory system" (Jensen et al., 2011). The most dramatic symptom for NP patients is intense, persistent and often disabling chronic pain, therefore pain management is the main focus of new drugs for the control of NP.

There is strong evidence that both the peripheral nervous system and the central nervous system exert mechanisms that culminate in chronic pain, mainly NP. Tissue damage can result in activation of nociceptors through the release of several mediators, including excitatory amino acids, peptides, protons, lipids and cytokines, which bind to receptors and activate signaling pathways, among these are protein kinases A and C, calcium/calmodulin-dependent protein kinase, and mitogen-activated protein kinases (MAPKs) (Ji and Strichartz, 2004). The essential oil of *L. grata* (rich in camphene, camphor, 1.8-cineole, borneol, β-caryophyllene and bicyclogermacrene) has shown interesting peripheral and central pharmacological activity, notably in the control of different types of pain. Siqueira-Lima et al. (Siqueira-Lima et al., 2014), showed that LG-βCD protected against orofacial nociception induced by formalin, capsaicin and glutamate in animal models. The authors also investigated the effects in a fibromyalgia-like animal model which seem to be related to the opioidergic and serotonergic pathways. In addition, LG-βCD treatment induced a significant decrease in Fos protein expression (Fos-positive cells) in the dorsal horn of the spinal cord, which suggests the possible involvement of descending pain-inhibitory mechanisms in its anti-hyperalgesic profile (Siqueira-Lima et al., 2017).

The present study aimed to understand the possible mechanisms by which the essential oil of *L. grata* leaves induces its analgesic effect in animal models. Our results demonstrated that essential LG and LG-βCD pretreatment reduces inflammatory pain by three main mechanisms: 1) inhibition of leukocyte migration; 2) inhibition of NF-kB activation and hyperalgesic cytokine production; and 3) involvement of the PKA pathway, which could sensitize ions channels such as TRPs (transient receptor potential channels).

We performed the carrageenan induced-pleurisy test to evaluate whether LG and LG/βCD could produce some alteration in the production of pro-inflammatory cytokines

because it is an acute protocol for the screening of possible anti-inflammatory drugs. There are no other reports in the literature of this type of approach to evaluate the effects of LG. Moreover, this test is characterized by leukocyte migration and the release of chemical mediators important in the inflammatory process, notably pro-inflammatory cytokines such as TNF-α and IL-1β (Dhalendra et al., 2013). As shown in Fig. 1, the pretreatment with LG or LG-βCD decreased the number of leucocytes recruitment to the pleural exudate and drastically reduced TNF-α and IL-1β levels. This result corroborated our initial hypothesis that LG has a possible anti-inflammatory effect as suggested by Siqueira-Lima et al. (Siqueira-Lima et al., 2014), and the inhibition of these two cytokines previously shown to be important in the establishment and severity of pain felt by NP. Moreover, the inhibition by LG of these two cytokines previously shown to be important in NP pain is encouraging in terms of new treatment approaches.

The inhibition of pro-inflammatory cytokines is key to the management of inflammatory conditions which are common to chronic pain such as NP (Wen et al., 2011). TNF-α is established as one of the most important cytokines in relation to cellular chemotaxis. (Fröde et al., 2001; Mazzon and Cuzzocrea, 2007) and inflammatory hyperalgesia (Chichorro et al., 2004; Cunha et al., 2005), common processes in patients with NP. Some studies have suggested that TNF-α can act as a molecular marker of the pain process in NP patients and that reducing its levels or silencing its expression are promising targets for new therapeutic approaches (Nwagwu et al., 2016; Ogawa et al., 2014; Xu et al., 2015). Additionally, a recent study reported that overexpression of TNF-α in nociceptive neurons activates a cellular cascade, with subsequent TRPV1 phosphorylation and an increase in pain signaling which seems to be important in long lasting pain (Rozas et al., 2016).

On the basis of these findings, we investigated the effect of LG or LG-βCD on edema and mechanical hyperalgesia induced by CFA. CFA causes a chronic inflammatory response, characterized by mechanical and thermal hyperalgesia and edema. This response is dependent on the severity of the tissue lesion and leads to the production and release of several inflammatory mediators (Levy et al., 2006; Raghavendra et al., 2004). We demonstrated that CFA induced edema and mechanical hyperalgesia was reduced by LG and LG/βCD.

It is worth mentioning that the edema and hyperalgesia associated with inflammation are independent responses (Walker et al., 2001) Brittain et al., 2011). In addition, it is known that mechanical and thermal hyperalgesia observed in the CFA-induced inflammatory pain model were attenuated by TRPV1 gene deletion and activation of the opioid and adenosine A1 receptors (Liao et al., 2017). This result is in line with the results of Siqueira-Lima et

al.(Siqueira-Lima et al., 2017, 2014), who demonstrated a possible effect of LG/βCD on TRPV1 and opioid receptors. Moreover, it is possible to infer that the reduction in edema by LG and LG/βCD may be due to either decreased production or release of pro-inflammatory cytokines as researchers have shown that NSAIDs reduce edema in this way in a does dependent manner (Gris et al., 2014; Mirshafiey et al., 2005).

In an attempt to better understand TNF-α and IL-1β levels after pretreatment with LG or LG/βCD, we assessed it in a partial sciatic nerve ligation of mice (an NP-animal model), since the inflammatory process is an important factor for pathogenesis of NP (Xu and Yaksh, 2011). In peripheral nerve injury, glial cells support neuroinflammation and induce the release of pro-inflammatory mediators, such as cytokines (TNF-α, IL-1β and IL-6) (Kiguchi et al., 2010; Schäfers et al., 2003; Scholz and Woolf, 2007). TNF-α is the key cytokine released during neuroinflammation during NP. Due to its peripheral and central actions it is known as a "pro-neuropathic" cytokine and once released triggers the development of hypersensitivity after nerve injury (Hess et al., 2011; Morioka et al., 2015; Park et al., 2011; Zhang et al., 2010). We demonstrated that LG or LG/βCD were effective in reducing thermal and mechanical hyperalgesia and also in decreasing TNF-α levels (but not IL-1β levels) in the sciatic nerve and spinal cord of mice. This selective effect of modulating TNF-α levels in the injured nerve (which seems to be promising in terms of neuroregeneration) and in the spinal cord (which inhibits the activation of a cascade of factors that culminate in the usual hyperalgesic process in NP patients) is a very exciting finding. It highlights the importance of our results in relation to chronic pain management given that the literature (in animal models) suggests that treatments targeting control of TNF-α levels can partially alleviate NP. This could be a promising new approach in relation to the development of new painkillers to treat NP (Leung and Cahill, 2010a).

The inflammatory environment that sensitizes nociceptors and accompanies pain is transcriptionally regulated, such as with the nuclear factor NF-κB (Haddad, 2007). NF-κB is a nuclear transcription factor that regulates the expression of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α (Grilli et al., 1993; Ji et al., 2017). This factor plays a pivotal role in immune and inflammatory responses (Wright and Christman, 2003; Zhang et al., 2012). NF-κB activation in the spinal cord can be associated with inflammatory pain hypersensitivity (Lee et al., 2004) and induces the degradation of the inhibitor IκBα from the NF-κB-IκBα complex, followed by phosphorylation of NF-κB p65 and its translocation into the nucleus (Baeuerle and Baltimore, 1988). The decrease in phosphorylation of the NF-κB p65 subunit found in LG or LG/βCD-treated animals indicates reduced NF-κB activation,

leading to a decrease in cytokine production in the sciatic nerve and spinal cord. This finding agrees with the lower TNF-α levels found in the LG or LG-βCD treated groups when compared to control animals.

We also demonstrated the involvement of adenylyl cyclase and synthesis of cyclic adenosine monophosphate/protein kinase A (AMPc/ PKA) in the LG or LG/βCD treated animals in the NP-model. Studies have demonstrated that PKA acts downstream of inflammatory mediators and is implicated in the processing of nociception in NP, enhancing nociceptive hypersensitization (Qiu et al., 2014; Simões et al., 2016; Zhu et al., 2014). PKA enhances the activity of sensory neurons, provoking sensitization at the peripheral C-fiber terminals resulting in a reduced nociceptive threshold or at the central terminals leading to increased nociceptive processing at the spinal cord level (Aley and Levine, 1999; Huang et al., 2015; Zhu et al., 2014). This enzyme is involved in cAMP responsive element binding prolonged synaptic plasticity during central sensitization, regulating nociception-related gene expression in nociceptive neurons, such as c-Fos, COX-2, NK-1 (Simonetti et al., 2013; Zhou et al., 2015). In addition, the PKA pathway sensitizes ion channels, such as TRPs, that promote neuronal and nociceptor excitability, leading to pain and hyperalgesia (Rios et al., 2013; Simões et al., 2016). Thus, the inhibition of PKA activity induced by LG or LG/βCD treatment could be causing TRP dephosphorylation and consequently ion channel desensitization, decreasing neuronal excitability and producing a painkiller effect. Therefore, PKA seems to be an important target for the anti-hyperalgesic effect of this essential oil.

Alterations in the normal neurophysiology of the spinal cord and dorsal root ganglion (DRG) play important roles in NP. Wang et al. (2008), showed that the activation of cAMP/PKA in DRG neurons by bradykinin contributes to sensitizing TRPA1. Interestingly, TRPA1 acts an integrator molecule and can respond to the release of inflammatory mediators, allowing the amplification of the inflammatory and nociceptive processes (Meotti et al., 2016). In order to provide more direct evidence of the participation of TRPA1 in the effect of LG or LG-βCD, their antinociceptive effect was investigated in cinnamaldehyde induced-pain in the mice. LG or LG/βCD significantly attenuated cinnamaldehyde-induced nociception, and this analgesic activity could be related to modulation and/or blockade of the TRP receptors (TRPV1 and TRPA1). Camphor, 1,8-cineole and β-caryophyllene are components of LG and are reported to exert analgesic effects, at least in part, through the inhibition of TRPA1 (Liu et al., 2013; Takaishi et al., 2014; Xu, 2005) and activation of TRPM8 (Caceres et al., 2017; Takaishi et al., 2014). Many drugs of natural origin, including essential oil compounds, interact with TRPM8 afferents (as activators or as inactivators) (Bautista et al.,

2007; Calixto et al., 2005; Julius, 2013). Our results are not clear enough to prove the involvement of LG and LG-CD with TRPM8, however, the compounds present in LG such as camphor and 1,8-cineole could possibly be acting on this receptor, thereby preventing its activation.

The expression of TNF-α in the spinal cord and DRG has been implicated in NP establishment, where the over expression of this cytokine is upregulated in satellite cells of the DRG after peripheral nerve injury (Leung and Cahill, 2010b). The mechanical hyperalgesia induced by spinal nerve injury is associated with upregulation of satellite cells and TNF-α in the contralateral DRG (Hatashita et al., 2008; Ohtori et al., 2004). Therefore, the overexpression of TNF-α in the sensory neurons increases the activity of TRPV1-dependent Ca^{2+} influx, resulting in inflammatory sensitization and pain (Rozas et al., 2016). The terpenes (camphor, 1.8-cineole and borneol) present in LG are compounds that commonly act on the TRPA1 receptors and also modulate TNF-α, thereby ameliorating pain (Takaishi et al., 2014; Xu, 2005). Thus, the consistent inhibitory effect on the Ca^{2+} current of DRG neurons produced by LG reinforces the hypothesis that Ca^{2+} and TRP receptors are involved in the pharmacological mechanism of LG and that its major components may be acting synergistically in relation to this.

We compared the biologic effects of pure LG (non-βCD-complexed) and LG/βCD for the first time in “in vivo” protocols and found a significant analgesic effect, although the mechanism of action is not yet clear. However, pure LG has low water solubility (data not shown) and showed reduced effectiveness in the animal models we used that could limit its clinical use, particularly in chronic conditions. However, cyclodextrins (CDs) have been demonstrated to be a useful tool to improve a number of chemical and pharmacological properties of non-polar compounds (Brito et al., 2015; Quintans et al., 2013; Siqueira-Lima et al., 2017). Our findings showed that the use of the inclusion complex improved the analgesic effect of LG, maintaining its action for 2 hours longer than pure LG. Although LG and LG/βCD were used at the same dose (24 mg/kg) it is noteworthy that LG/βCD was prepared using a molar concentration of 1:1, so the effective dose of LG present in the βCD-complex is lower when compared to LG alone. This fact is extremely important because it means the pharmacological effect was obtained with a smaller dose of LG.

5. Conclusion

The essential oil from *L. grata* leaf exerts an outstanding anti-hyperalgesic effect on pain of different origins and with equally diverse pharmacological mechanisms. These results are in line with the use of this medicinal plant in folk medicine for pain relief, and the complexation of its essential oil in βCD was shown to increase its effectiveness. The antihyperalgesic effect of LG is related to its anti-inflammatory profile (especially in mitigating pro-inflammatory cytokines), with the involvement of the PKA pathway and also the inhibition of voltage Ca^{2+} current. Moreover, the inhibition of TNF-α appears to be key to its analgesic profile. Thus, considering the limited number of drugs currently available for the treatment of NP, our results provide a rationale for the development of interventions targeting the study of LG as a potential new tool for chronic pain management in patients with NP, which could lead to better treatments for those affected by this chronic pain in the future.

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Considerações finais

CONSIDERAÇÕES FINAIS

De acordo com os resultados apresentados nesta tese podemos concluir que:

A revisão da literatura demonstrou fortes evidências experimentais de que a β-ciclodextrina age como um sistema de complexação de drogas seguro e de baixo custo, melhorando as propriedades farmacológicas dos terpenos, transformando estes produtos naturais em uma escolha atrativa para uso farmacológico;

A revisão sistemática incluiu várias espécies de *Lippia* com propriedades sobre o sistema nervoso central e com propriedades analgésicas em aboradgens pré-clínicas. Foi observado que poucos estudos exploraram o mecanismo de ação responsável por estes efeitos ou fizeram uma descrição fitoquímica detalhada ou ainda investigaram a toxicidade e/ou segurança terapêutica do uso continuado destas drogas. Apesar disto, os resultados das análises de extratos e óleos foram consistentes com a maioria dos relatos dos estudos etnofarmacológicos reafirmando a importância da medicina popular como guia para tais estudos;

A administração oral do OEL/β-CD foi capaz de reduzir a hiperalgesia mecânica em modelo animal de dor crônica musculoesquelética. Um dos mecanismos de ação pode estar associado à ativação dos receptores das vias opiodérgicas e serotoninérgicas que, juntamente com a redução da expressão da proteína Fos no corno dorsal da medulla, sugerem uma forte ação na via inibitória descendente da dor. A análise de energias de interação dos compostos majoritários aos receptores α-adrenérgicos, μ-opioides e 5HT, através de um estudo *in silico* demonstrou fortes interações da cânfora e do E-cariofileno aos receptors alfa-adrenérgicos, comportamento contrário ao demonstrado *in vivo.* A atividade antioxidante, que foi mais pronunciada no OEL do que no OEL/β-CD, também reforça a ação anti-hiperalgésica;

O tratamento oral agudo e sub-crônico com OEL e OEL/β-CD reduziu a hiperalgesia mecânica e o edema de pata induzidos pela injeção intraplantar de CFA, assim como a hiperalgesia mecânica e térmica induzidas pela ligação parcial do nervo ciático também foram reduzidas após o mesmo tratamento. Estes efeitos podem estar associados à redução da migração de leucócitos, bem como redução da síntese/liberação de mediadores TNF-α e IL-1β, observadas após o tratamento com OEL e OEL/β-CD no modelo de pleurisia. A redução dos níveis de TNF-α , mas não da IL-1β no nervo ciático e na medulla, bem como de

fosforilação de NFκB e PKA nestes mesmos tecidos, reforçam uma correlação positiva entre a ação do óleo e a redução do efeito álgico destes mediadores. O OEL e OEL/β-CD (24mg/kg) ainda inibiram a nocicepção desencandeada pela injeção plantar de cinamaldeído (agonista do TRPA1) e mentol (agonista do TRPM8);

O tratamento oral agudo e sub-crônico com OEL ou OEL/β-CD (24 mg/kg) não alterou a atividade motora dos animais nos modelos de dor inflamatória persistente (CFA) ou dor neuropática (PSNL), bem como não reduziu a força muscular no modelo de dor musculoesquelética não inflamatória.

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