



**UNIVERSIDADE ESTADUAL DE FEIRA DE
SANTANA
PROGRAMA DE PÓS-GRADUAÇÃO EM
BIOTECNOLOGIA**



POLLYANA DE SOUZA SIQUEIRA LIMA

**EFEITO DO COMPLEXO DE INCLUSÃO CONTENDO
BETA-CICLODEXTRINA E ÓLEO ESSENCIAL DE *LIPPIA
GRATA* (VERBENACEAE) NA NOCICEPÇÃO OROFACIAL
EM MODELOS EXPERIMENTAIS**

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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia, da Universidade Estadual de Feira de Santana como requisito parcial para obtenção do título de Mestre em Biotecnologia.

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Co-orientador: Prof. Dr. Lucindo José Quintans Júnior

Feira de Santana, BA
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“Até aqui nos ajudou o Senhor” (I Sam 7:12)

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RESUMO

O tratamento da dor orofacial representa um importante desafio para a medicina moderna. Desta forma, novas propostas terapêuticas têm buscado nos produtos naturais opções farmacológicas para esta nosologia. No presente estudo, foi preparado e caracterizado fisicoquimicamente, um complexo de β -ciclodextrina incorporado com o óleo essencial das folhas de *Lippia grata* (β -CD/OE) buscando avaliar sua possível ação antinociceptiva em modelos animais de dor orofacial. O óleo essencial (OE) foi obtido por hidrodestilação em aparato de Clevenger e os compostos marjoritários foram identificados por CG-MS. A análise fitoquímica do óleo demonstrou a presença de cânfora (28,69%), (E)-cariofileno (11,47%), biciclogermacreno (10,22%), cânfeno (9,45%) e borneol (7,64%) como principais componentes. Após estes resultados preliminares, este óleo foi então complexado em β -ciclodextrina e a análise fisicoquímica realizada. Os resultados da DSC e TG/DTG demonstraram que produtos preparados por métodos de coevaporação foram capazes de incorporar grandes quantidades de óleo essencial. No difractograma de raios X, foi mostrado que o complexo entre OE e β -CD foi formado. Camundongos machos Swiss foram pré-tratados com β -CD/OE (6, 12 ou 24 mg/kg, *per os*, gavagem, v.o.), morfina (5 mg/kg, i.p.), ou veículo (água destilada, v.o.), 90 min. antes do tratamento com formalina (20 μ l, 2%), capsaicina (20 μ l, 2,5 μ g), ou glutamato (40 μ l, 25 μ M) no lábio superior direito. Os dados obtidos foram avaliados por análise de variância de uma via (ANOVA), seguido pelo teste de Tukey. Os resultados demonstraram que o tratamento v.o. com β -CD/OE foi capaz de reduzir ($p < 0,05$ ou $p < 0,001$) o comportamento nociceptivo de coçar a face em ambas as fases do teste da formalina. Camundongos tratados com β -CD/OE foram também ($p < 0,05$ ou $p < 0,001$) protegidos contra a nocicepção induzida por capsaicina e glutamato. Para avaliar a ação no sistema nervoso central (SNC), noventa minutos após o tratamento, os camundongos foram perfundidos, os cérebros coletados, crioprotetidos, cortados num criostato e submetidos a um protocolo de imunofluorescência para proteína Fos. O protocolo de imunofluorescência demonstrou que o complexo de β -CD/OE ativou ($p < 0,05$; $p < 0,01$ ou $p < 0,001$) o córtex motor, o *locus ceruleus*, o núcleo magno da rafe e a substância cinzenta periaquedutal do SNC. Esses efeitos não alteraram, nas doses testadas, a coordenação motora dos camundongos no teste rota-rod. Os resultados sugerem que o tratamento com β -CD/OE induz perfil analgésico em modelos experimentais de nocicepção orofacial, sendo um complexo farmacológico promissor no tratamento da dor orofacial.

Palavras chave: *Lippia grata*. Óleo essencial. Ciclodextrina. Dor. Proteína Fos.

ABSTRACT

The treatment of orofacial pain remains a major challenge for modern medicine. So, new therapeutic proposals has sought in natural products pharmacological options for this nosology. In the current study, it was prepared and physicochemically characterized a β -cyclodextrin complex containing *L. grata* leaf essential oil (β -CD/EO) in order to investigate their possible antinociceptive activity in animal models of orofacial pain. The essential oil from the leaves of *Lippia grata* (EO) was obtained by hydrodistillation in a Clevenger-type apparatus and the main compounds identified was made through CG-MS. The phytochemical analysis of EO demonstred the presence of camphor (28,69%), (E)-caryophyllene (11,47%), bicyclogermacrene (10,22%), camphene (9,45%) and borneol (7,64%) as the main compounds. Following these preliminary results, the inclusion complex of β -cyclodextrin and EO (β -CD/EO) was prepared by slurry procedure (SC). It was taken physicochemical characterization. The results of DSC and TG/DTG showed that the products prepared by SC methods were able to incorporate greater amounts of EO. In the X-ray diffractogram, it was showed that complex between EO and β -CD was formed. Male Swiss mice were pretreated with β -CD/EO (6, 12 or 24 mg/kg, *per os*, gavage, p.o.), morphine (5 mg/kg, i.p.), or vehicle (distilled water, p.o.), 90 min. before treatment with formalin (20 μ l, 2%), capsaicin (20 μ l, 2,5 μ g), or glutamate (40 μ l, 25 μ M) into the right upper lip. The obtained data were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's test. The results demonstrated that p.o. treatment with β -CD/EO was ($p < 0,05$ or $p < 0,001$) capable in reducing the nociceptive face-rubbing behavior in both phases of the formalin test. β -CD/EO-treated mice were also ($p < 0,05$ or $p < 0,001$) protected against nociception induced by capsaicin and glutamate. For the action in the central nervous system (CNS), ninety minutes after the treatment, the mice were perfused, the brains collected, crioprotected, cut in a criostate and submitted to an imunofluorescence protocol for Fos protein. The imunofluorescence protocol demonstrated that the β -CD/EO significantly activated ($p < 0,05$; $p < 0,01$ or $p < 0,001$) the motor cortex, the *locus ceruleus*, the nucleus raphe magnus and the periaqueductal gray of the CNS. These effects did not alter, in tested doses, the motor coordination of mice in the rota-rod test. These results suggest that treatment with β -CD/EO induces an analgesic profile in experimental models of orofacial nociception, promising pharmacologic complex in treatment of orofacial pain.

Keywords: *Lippia grata*. Essential oil. Cyclodextrin. Pain. Fos protein.

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INTRODUÇÃO

1 INTRODUÇÃO

Segundo a Associação Internacional para o Estudo da Dor (IASP - *International Association for the Study of Pain*), “a dor é uma experiência sensorial e emocional desagradável que é associada a lesões reais ou potenciais, ou descrita em termos que sugerem tal lesão” (MERSKEY et al., 1979). As dores orofaciais apresentam elevada casuística e constitui-se uma das maiores causas de sofrimento humano, comprometendo a qualidade de vida, e tornando-se por isso, um relevante problema social e médico (BOTTEGA; FONTANA, 2010).

A dor orofacial é derivada de muitos tecidos diferenciados como meninges, córnea, polpa dentária, mucosa oral e nasal e articulação temporomandibular, apresentando várias características fisiológicas únicas quando comparadas ao sistema nociceptivo espinal. Devido a estas considerações, não é surpresa que o diagnóstico acurado e o tratamento efetivo das condições dolorosas representam um problema de saúde significativa (HARGREAVES, 2011).

O tratamento da dor continua a ser um importante desafio para a medicina moderna (HARGREAVES, 2011). Opióides, anticonvulsantes e drogas anti-inflamatórias não-esteroidais (AINES) continuam sendo as principais opções farmacológicas usadas para aliviar dores agudas e crônicas, inclusive orofaciais (MIRANDA et al., 2009). No entanto, infelizmente, o uso de opióides e AINES é limitado por suas reações adversas. Neste contexto, pesquisas recentes que visam à descoberta de novas drogas analgésicas têm sido realizadas usando produtos naturais como alternativas terapêuticas para estas condições dolorosas, almejando um aumento da eficácia e redução de tais efeitos colaterais (GUIMARÃES et al., 2012; VENÂNCIO et al., 2011). Um último grande lançamento inovador do setor farmacêutico para o tratamento da dor foi o Ziconotide (Prialt®), que é um produto natural extraído do molusco *Conus magus*, que produz suas propriedades farmacológicas através do bloqueio de canais de Ca^{+2} ativados por voltagem (LI; VEDERAS, 2009).

Grande parte destes produtos naturais que vem sendo pesquisados, já tem sido amplamente utilizada na medicina popular. Um exemplo de planta medicinal com aplicação terapêutica e vasto uso popular, é o gênero *Lippia* da família Verbenaceae, que inclui aproximadamente 250 espécies de arbustos e árvores pequenas, principalmente distribuídas nas Américas Central e do Sul e África Tropical (TERBLANCHÉ; KORNELIUS, 1996).

O gênero *Lippia* tem sido descrito em vários trabalhos científicos pelo seu uso na medicina tradicional e efeitos farmacológicos, dentre estes podem ser destacados os efeitos analgésicos, anti-inflamatórios, anestésicos locais, ansiolíticos e sedativos (ABENA et al., 2003; AHMED et al., 2004; BOTELHO; CARVALHO; MONTENEGRO, 2007; GIRÃO et al., 2003; GURGEL DO VALE et al., 2002; PASCUAL et al, 2001). Estas atividades têm sido atribuídas aos terpenóides, principalmente monoterpenos e sesquiterpenos, presentes nos óleos essenciais das espécies deste gênero.

Uma espécie do gênero *Lippia* pouco estudada no aspecto químico e farmacológico é a *L. grata* Schauer. Esta espécie é um arbusto encontrado no semiárido baiano, e de acordo com Craveiro et al. (1981) a espécie *L. grata* possui atividade antiespasmódica, sendo esta atribuída à presença dos monoterpenos majoritários: o timol e o carvacrol. A estes monoterpenos isolados uma ampla variedade de atividades biológicas tem sido atribuída, tais como atividade analgésica, anti-inflamatória, antioxidante, antiespasmódica, ansiolítica e depressora do sistema nervoso central (ANAMURA et al., 1988; ANGELES-LOPEZ et al., 2010; BEER et al., 2007; GUIMARÃES et al., 2010; GUIMARÃES et al., 2012; HOTTA et al., 2010; MASTELIC et al., 2008; MOHAMMADI et al., 2001; WAGNER; WIERERM, 1986).

Diante das evidências científicas relacionadas ao gênero *Lippia* e considerando a escassez de estudos relacionados a esta espécie, propusemo-nos a investigar o efeito antinociceptivo do óleo essencial de folhas de *L. grata* complexado em β -ciclodextrina (β -CD/OE), já que estes carboidratos têm sido empregados como agentes de encapsulação para aumentar a solubilidade, estabilidade, biodisponibilidade de monoterpenos e óleos essenciais (QUINTANS ET AL., 2013; QUINTANS JÚNIOR et al., 2013; PRAGADHEESH et al., 2013; SERAFINI et al. 2012; MENEZES et al. 2012).

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OBJETIVOS

2 OBJETIVOS

2.1 OBJETIVO GERAL

- ▶ Investigar o efeito antinociceptivo do óleo essencial de folhas de *L. grata* complexado em β -ciclodextrina (β -CD/EO) usando protocolos experimentais de dor orofacial.

2.2 OBJETIVOS ESPECÍFICOS

- ▶ Realizar um levantamento bibliográfico (revisão sistemática) acerca das espécies de *Lippia* com atividade analgésica, bem como seus componentes majoritários e possíveis mecanismos de ação;
- ▶ Extrair e identificar os constituintes do óleo essencial de *Lippia grata* através de CG-MS;
- ▶ Complexar o óleo essencial de *L. grata* em β -ciclodextrina;
- ▶ Caracterizar o complexo formado por análise térmica e difração de raios-X;
- ▶ Investigar as propriedades farmacológicas do β -CD/OE em protocolos de nocicepção orofacial;
- ▶ Investigar possível envolvimento de vias centrais na atividade analgésica do complexo

CAPÍTULO 1
ATIVIDADE ANALGÉSICA DO GÊNERO *LIPPIA* –
UMA REVISÃO SISTEMÁTICA

3 ATIVIDADE ANALGÉSICA DO GÊNERO *Lippia* – UMA REVISÃO SISTEMÁTICA

RESUMO

Muitas pessoas utilizam plantas com ação analgésica para aliviar a dor e inflamação embora a maioria delas não tenha comprovação de tais propriedades. Plantas do gênero *Lippia* (Verbenaceae) são amplamente usadas pela população para diferentes tipos de dor dentre elas, dor de estômago, dor vesicular e dores abdominais. Apesar de seu uso, não há revisão alguma sobre atividade analgésica de plantas deste gênero. Então, uma pesquisa com os termos *Lippia*, atividade analgésica e atividade antinoceptiva foram utilizados para buscar artigos em inglês nas bases de dados MEDLINE-Pubmed, EMBASE e LILACS. Doze artigos foram encontrados. Por esta razão, o objetivo desta revisão sistemática foi resumir brevemente o conhecimento atual sobre os efeitos analgésicos deste gênero como um possível candidato para o tratamento das condições dolorosas.

ABSTRACT

Many people use the analgesic plants to relieve pain and inflammation though most of them have not yet been proved to possess such properties. Plants of the *Lippia* genus (Verbenaceae) are widely used in the population for different types of pain, such as stomach ache, vesicle pain, and abdominal pains. Despite its use, there are no reviews on the analgesic activity of plants of this genus. So, a search terms *Lippia*, analgesic activity, antinociceptive activity, were used to retrieve English language articles in MEDLINE-PubMed, EMBASE and LILACS. Twelve papers were found. For this reason, the aim of the present systematic review is to briefly summarize the current knowledge about the analgesic effects of this genus as possible candidates for the treatment of painful conditions.

3.1 INTRODUÇÃO

O gênero *Lippia*, pertencente à família *Verbenaceae*, compreende cerca de 250 espécies herbáceas, arbustos e pequenas árvores amplamente distribuídas em toda a América Central e do Sul, bem como na África tropical (TERBLANCHÉ; KORNELIUS, 1996, AGUIAR ; COSTA, 2005). Tais espécies estão distribuídas nas regiões áridas do sudoeste dos

EUA, nas florestas tropicais decíduas da América Central e nos campos rupestres e cerrados do Brasil, regiões de altos índices de endemismos. Estende-se até o Uruguai e região central da Argentina. Na África, sua ocorrência também coincide com centros de alto endemismo, na região leste, associada às montanhas mais altas e picos alpinos (SALIMENA, 2000).

Estima-se que o Brasil abrigue entre 70–75% das espécies conhecidas (ARTHUR et al., 2011) representado por cerca de 120 espécies conspícuas por sua aparência durante seu curto período de florescência e sua fragrância geralmente forte e agradável (BEZERRA et al., 1981). Tais espécies têm seus principais centros de diversidade específicos localizados na Cadeia do Espinhaço, em Minas Gerais, e na Chapada Diamantina, na Bahia (SALIMENA, 2002).

A utilização deste gênero é bastante ampla e vai desde o uso no preparo de alimentos, principalmente como temperos, até na medicina tradicional, onde tem sido atribuídas atividades: analgésica, anti-inflamatória, antipirética, antihipertensiva, antimicrobiana, bem como ações benéficas sobre as desordens gastrointestinais, menstruais e respiratórias (PASCUAL et al., 2001).

Diante da grande relevância medicinal e econômica desse gênero, inclusive com espécies como *L. alba*, *L. sidoides* e *L. gracilis* incluídas na primeira lista da Central de Medicamentos (CEME), extinto órgão do Ministério da Saúde do Brasil, que selecionava plantas medicinais a serem investigadas quanto ao efeito farmacológico e toxicológico e à composição química (FERREIRA et al., 1998). Mais recentemente, em 2009, o Governo Brasileiro criou a Relação Nacional de Plantas Medicinais de Interesse ao SUS (*Renisus*), uma lista de espécies vegetais já utilizadas na medicina tradicional que apresentam potencial para gerar produtos de interesse ao SUS (Sistema Único de Saúde), na qual inseriu a *L. sidoides* devido suas propriedades farmacológicas (MINISTÉRIO DA SAÚDE, 2009).

Dentre os efeitos medicinais relatados para o gênero *Lippia*, pode-se destacar o efeito analgésico como importante ação farmacológica, visto que a dor é um sintoma bastante frequente em diversas condições patológicas. Nesse sentido, as plantas desse gênero são amplamente usadas para tratamento de diferentes tipos de dor, incluindo a dor estomacal, vesicular (*L. alba*), e abdominal (*L. javanica*) (HUTCHINGS E VAN STADEN, 1994). Além disto, alguns estudos relatam atividades antinociceptiva e antiinflamatória de *L. sidoides* (GIRÃO et al., 2003; BOTELHO et al., 2007), *L. multiflora* (ABENA et al., 2003) e *L. nodiflora* (AHMED et al., 2004).

Neste sentido, como não há descrito na literatura nenhuma revisão sistemática sobre as propriedades analgésicas deste importante gênero, o presente estudo buscou na literatura os

principais trabalhos científicos que avaliaram este perfil em espécies de *Lippia* em protocolos experimentais.

3.2 MÉTODOS

A revisão sistemática da literatura foi realizada seguindo às orientações preconizadas pelo Transparent Reporting of Systematic Reviews and Meta-Analyses (PRISMA statement; MOHER et al., 2009).

3.2.1 Estratégia de busca

Três bases de dados digitais foram usadas para buscar estudos que satisfizessem o objetivo da pesquisa. Estes incluíram a MEDLINE-PubMed (National Library of Medicine, Washington, DC), EMBASE (Excerpta Medical Banco de Dados Elsevier) e LILACS (Caribe em Ciências da Saúde da América Latina e). A busca nas bases de dados foram realizadas no período incluindo até 30 de agosto de 2013.

A estratégia de busca estruturada foi delineada para incluir qualquer estudo publicado que avaliasse a ação antinociceptiva das espécies de *Lippia*. As citações foram limitadas a estudos em modelos animais. Trabalhos adicionais foram incluídos nesse estudo após análise de todas as referências dos artigos selecionados. Não houve contato com pesquisadores e/ou tentativas para identificar dados não publicados.

3.2.2 Seleção dos estudos

Todos os títulos eletrônicos encontrados, resumos selecionados e textos completos de artigos foram revisados por um mínimo de dois revisores (JSSQ, PSSS, RSSB) de forma independente. Discrepâncias sobre inclusão/exclusão de estudos foram resolvidas com uma reunião de consenso. Foram aplicados os seguintes critérios de inclusão: os estudos de avaliação antinociceptiva das espécies de *Lippia*. Os estudos em humanos, revisões da literatura, editoriais / cartas e relatos de casos foram excluídos.

3.2.3 Extração de dados

Os dados foram extraídos por um revisor, utilizando formulários padronizados e foram verificados quanto à completude e precisão por um segundo revisor. Informações

extraídas incluíram dados sobre as espécies de *Lippia*, componentes majoritários, dose e via de tratamento, modelos animais e mecanismos de ação.

3.3 RESULTADOS

Um total de 1031 resumos e citações foi identificado eletronicamente para a revisão. Após remoção dos artigos duplicados e triados quanto aos títulos e resumos relevantes, 30 artigos foram submetidos à leitura na íntegra. No final 12 artigos preencheram os critérios de inclusão/exclusão estabelecidos previamente (Fig. 1).

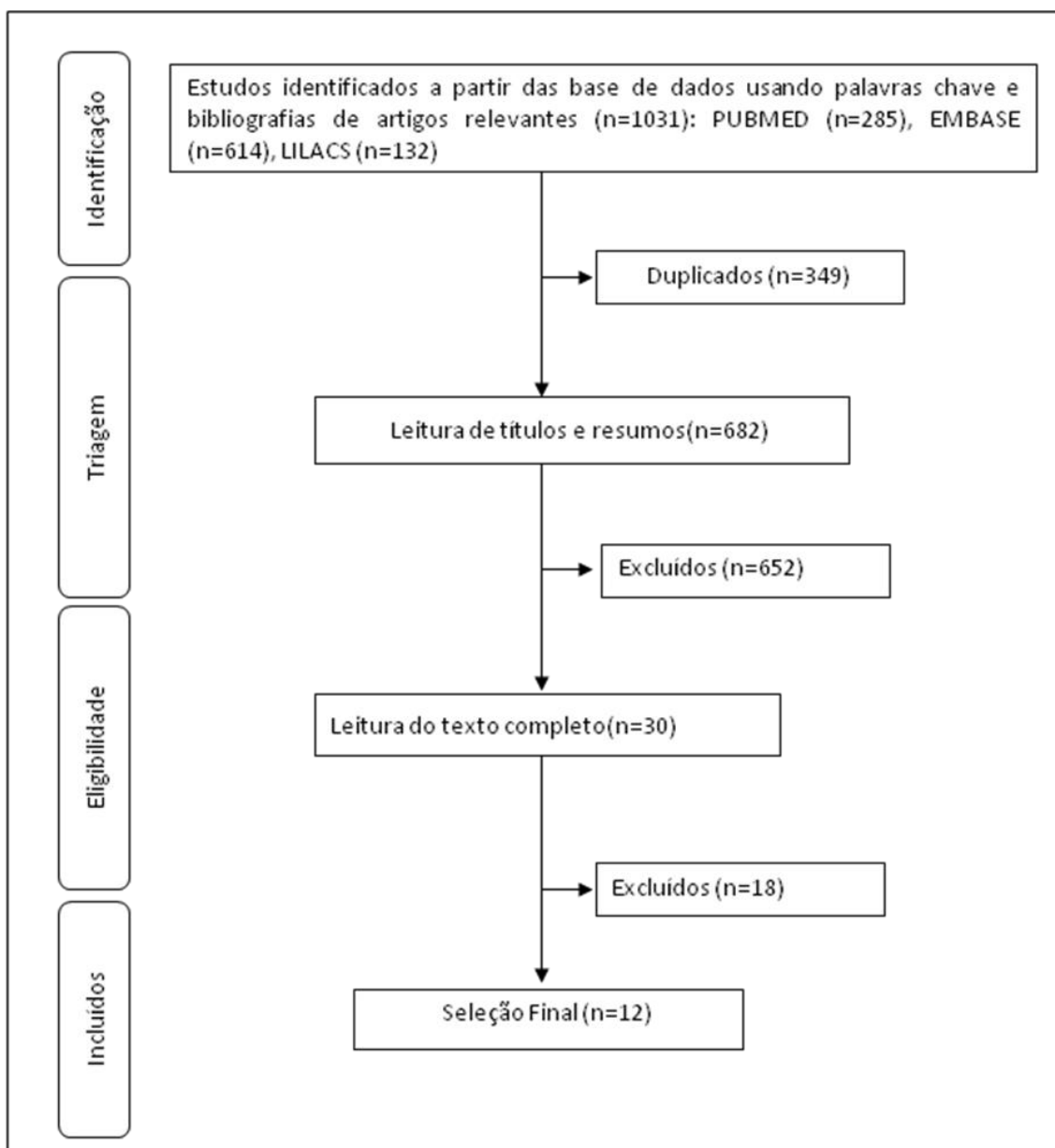


Figura 1: Pesquisa e seleção dos artigos

3.3.1 Descrição de estudos incluídos

Em relação às espécies de *Lippia* estudadas, os trabalhos encontrados descrevem 7 diferentes espécies estudadas: *L. citriodora*, *L. adoensis*, *L. multiflora*, *L. nodiflora*, *L. gracilis*, *L. alba* e *L. geminata*. Em poucos estudos foi realizada uma extensa triagem fitoquímica para descrever os componentes majoritários, entretanto, os mais citados foram os terpenóides (carvacrol, p-cimeno, o-cimeno e timol) e o fenilpropanóide (verbacoside). Alguns estudos evidenciaram a classe química prevalente tais como flavonóides, ácido fenólico, alcalóides, fitoesteróides (Tabela 1).

A maioria dos estudos foi realizada a partir do extrato da planta (70%): extrato aquoso, etanólico, metanólico, clorofórmico e éter de petróleo, 20% dos estudos avaliaram o óleo essencial e apenas um estudo avaliou a substância isolada (verbacoside).

Em relação aos modelos animais utilizados para avaliação antinociceptiva, 41% dos estudos utilizaram o teste das contorções abdominais induzidas pelo ácido acético, 23% o teste da placa quente, 18% o teste da retirada da cauda, 14% o teste da formalina e apenas um estudo investigou a ação em dois modelos de dor crônica: constrição do nervo ciático e injeção intra-articular de monoiodoacetato sódico.

Tabela 1: Características dos estudos incluídos

Autores, ano, País	Espécie (s) de <i>Lippia</i>	Componente majoritário	Tipo de extração	Dose (s) mg/kg (via)	Animais	Modelos de Nocicepção	Mecanismos de ação
Isacchi et al., 2011, Itália	<i>L. citriodora</i>	Verbascoside	Isolado	100 - 600 mg/kg v.o. i.p.	Ratos machos albinos SD	CCI MIAS	Não necessita de sistema opióide para reverter hiperalgesia
Makonnen et al., 2003, Etiópia	<i>L. adoensis</i>	Ácidos fenólicos flavonóides, glicosídeos, açúcares	EA, EE	400, 600 e 800 mg/kg v.o.	Camundongos machos albinos	Teste da placa quente Teste de retirada da cauda Clipagem da cauda	NR
Abena et al., 2003, Congo	<i>L. multiflora</i> Moldenke	<i>p</i> -cimeno timol	OE	2, 4 e 8 ml/kg v.o.	Ratos machos <i>Wistar</i> Camundongos <i>Swiss</i>	Contorções abdominais induzidas pelo AA	NR
Ahmed et al., 2003, Bangladesh	<i>L. nodiflora</i> Linn.	NR	EM	250 e 500 mg/kg v.o.	Camundongos <i>Swiss</i> Albino	Contorções abdominais induzidas pelo AA	Modo de ação poderia envolver um mecanismo periférico.
Guilhon et al., 2011, Brasil	<i>L. gracilis</i> Schauer	Carvacrol <i>o</i> -cimeno	OE	10, 30 e 100 mg/kg v.o.	Camundongos machos Balb/C	Contorções abdominais induzidas pelo AA Teste da formalina; Teste da placa quente	Participação da via do óxido nítrico (NO) no efeito anti-hiperalgésico. Envolvimento dos receptores colinérgicos (atropina) e sistema opióide (naloxona)
Mendes et al., 2010, Brasil	<i>L. gracilis</i> Schauer	Timol <i>p</i> -cimeno	OE	50–200 mg/kg; v.o.	Camundongos <i>Swiss</i>	Contorções abdominais induzidas pelo AA	NR
Haldar et al., 2012, Índia	<i>L. alba</i> (Mill.) N.E. Brown	fitosterol, flavonóides, carboidratos, alcalóides	EP, EC, EE, EA	500 mg/kg. v.o. 460 mg/kg. v.o.	Ratos adultos machos <i>Wistar</i> Albino	Teste de retirada da cauda (Imersão da cauda)	Possivelmente envolve flavonóides que reduziram a avaliabilidade das prostaglandinas.
Debella et al., 2003, Etiópia	<i>L. adoensis</i>	NR	EA, EE, EM	400, 600 and 880 mg/kg v.o.	Camundongos Albino machos	Contorções abdominais induzidas pelo AA	NR

Guimarães et al., 2011, Brasil	<i>L. gracilis</i> Schauer	NR	NR	100, 200, 400 mg/kg/ v.o.	Camundongos Swiss machos	Contorções abdominais induzidas pelo AA Teste da formalina; Teste da placa quente	NR
Forestieri et al., 1996, Itália	<i>L. geminata</i> <i>L. nodiflora</i>	NR	Decocção EP, EE, EA	0,5 mg/kg v.o.		Contorções abdominais induzidas pelo AA Teste da placa quente	NR
Vianna et al., 1998	<i>L. alba</i>	NR	OE		Camundongos	Contorções abdominais induzidas pelo AA Teste da formalina; Teste da placa quente	NR
Costa et al., 1989, Brasil	<i>L. alba</i>	NR	EE	1g/kg v.o.	Camundongos Albino fêmeas	Contorções abdominais induzidas pelo AA Teste da formalina; Teste de retirada da placa	NR

Definição das abreviações: NR = não registrado, EA = Extrato aquoso, EE = Extrato etanólico, EM = Extrato metanólico, OE = Óleo Essencial, EP = éter petróleo/benzina, EC = extrato clorofórmio, AA = ácido acético, CCI = injúria crônica por constrição, MIAS= monoiodoacetato sódico; v.o. = via oral; i.p.=, via intraperitoneal

3.4 DISCUSSÃO

A busca por novas propostas terapêuticas para o tratamento de distúrbios e/ou condições dolorosas continua sendo um dos maiores desafios da medicina moderna (JONES E BACKONJA, 2013). Neste sentido, os produtos naturais são responsáveis por cerca de 40% das novas entidades químicas utilizadas como princípios ativos de medicamentos liberados pelo FDA (*Food and Drug Administration*) nas últimas duas décadas, inclusive sendo a fonte do último medicamento inovador para o tratamento da dor com o Ziconotide (Prialt), um peptídeo extraído de um caracol (LI E VEDERAS, 2009).

Dentre as espécies de *Lippia* descritas neste estudo a *L. citriodora*, popularmente conhecida como limão verbena para grupos indígenas da América do Sul (QUIRANTES-PINÉ et al., 2013), é bastante utilizada na forma de infusão como antiespasmódico, antipirético, sedativo e apresentando propriedades digestivas (CARNAT et al. 1999; PASCUAL et al. 2001; VALENTAO et al. 1999). Segundo Quirantes-Piné e colaboradores (2009) as folhas da *L. citriodora* contêm um grande número de compostos polares como fenilpropanóides, flavonóides, ácidos fenólicos, iridóides, glicosídeos, sendo os verbacosídeos os mais abundantes (BILIA et al., 2008).

A literatura descreve algumas propriedades para esse composto, tais como atividade anti-inflamatória (DEEPAK; HANDA, 2000; DIAZ et al., 2004; HAUSMANN et al., 2007), antimicrobiana (AVILA et al., 1999), antitumoral (OHNO et al, 2002) e antioxidante (LIU et al., 2003; SICILIANO et al., 2005). Segundo Herbert et al., (1991) o verbacoside é um potente inibidor da enzima proteína quinase C (PKC) em animais, e a ativação desta enzima está envolvida em respostas celulares de sensibilização de nociceptores e processos inflamatórios (HASMEDA et al., 1999). Assim esse efeito pode estar relacionado com a ação analgésica desta substância (ISACCHI et al., 2011).

L. multiflora popularmente conhecida como “chá de Gambia”, é uma espécie amplamente usada como infusão na África. Tradicionalmente, suas folhas são usadas como uma bebida quente (chá), para febre, distúrbios gastrintestinais, enterite, tosses, constipações e como laxante (ADESINA et al., 1993). O óleo essencial isolado a partir de suas folhas e flores contém muitos constituintes voláteis incluindo: linalol, geraniol, terpineol, cânfora, neralidol, α -e β -pineno, limoneno e timol (ABENA et al., 2003, OLADIMEJI et al. , 2001; VALENTIN et al., 1995).

Óleos essenciais extraídos da *L. gracillis* (‘alecrim-de-tabuleiro’) também expressam como principais componentes os monoterpenos p-cimeno, γ -terpineno e variável teor de

carvacrol e/ou timol (PESSOA et al., 2005, SILVA et al. 2008; NEVES et al., 2008; MENDES et al., 2010; TELES et al., 2010)

Estudos evidenciam que o timol modula canais de Na⁺ voltagem-dependentes (HAESLER et al., 2002) e canais de K⁺ (ELLIOT; ELLIOT, 1997), receptores gaba-aminobutíricos (MOHAMMADI et al., 2001), α e β -adrenérgico (BEER et al., 2007) e ainda estão relacionados a síntese de prostaglandina (ANAMURA et al., 1988), o que pode contribuir para o controle de sensações dolorosas.

Em relação ao p-cimeno, sua atividade antinociceptiva foi avaliada em testes de contorções induzidas por ácido acético, teste da formalina, placa quente, bem como análise de efeito anti-inflamatório (BONJARDIM et al., 2012). Além disso, Santana et al. (2011), relacionaram o efeito antinociceptivo ao sistema opióide. Esses monoterpenos encontrados no óleo essencial desta espécie como constituintes majoritários, podem estar relacionados à atividade analgésica descrita por Abena et al, (2003) e Mendes et al., (2010).

O efeito antinociceptivo do carvacrol foi avaliado em diferentes modelos animais, os quais podem estar relacionados à ação deste monoterpeno no recrutamento de neutrófilos, liberação de TNF- α e NO, e conseqüente diminuição da produção de metabólitos inflamatórios/hipernociceptivos, provavelmente PGE₂ ou outros (GUIMARÃES et al., 2012). Em contrapartida, Cavalcante Melo et al. (2012) afirmam que sua atividade antinociceptiva não está relacionada ao sistema opióide, ou a inibição da via do óxido nítrico.

A *L. nodiflora* é encontrada em lugares úmidos, em grande parte da Índia, tradicionalmente usada como antibacteriano e diurético (PASCUAL et al., 2001). Alguns estudos fitoquímicos demonstraram que esta espécie é rica em flavonóides, flavonas (tais como os glicosídeos, lippiflorin α e β , nodiflorin α e β), alcalóides, (BINA et al., 2007; FRANCISCO et al., 1987). Segundo Balamurugan e Ignacimuthu (2012) o extrato metanólico revela a presença de esteróides, saponinas, cumarinas, quinonas, taninos e flavonóides.

A *L. alba* conhecida popularmente como “cidreira”, é uma planta nativa da América do Sul presente também na América Central, habita praticamente todas as regiões do Brasil (TAVARES et al., 2005). A composição de seu óleo essencial apresenta variação quantitativa e qualitativa, levando à separação em quimiotipos (MATOS et al., 1996; FRIGHETO et al., 1998; ZOGHBI et al., 1998), justificando suas diferentes atividades farmacológicas (MATOS, 1996; CORRÊA, 1992).

Atividades analgésicas e anti-inflamatórias foram demonstradas (AGUIAR, 2006; COSTA et al., 1989) e alguns estudos associam essa ação à presença de flavonóides (luteonin, apigenin e naringenin) e monoterpenos (carvona, b-mirceno, citral, limoneno e linalol).

Segundo Guimarães et al., (2013) os monoterpenos são excelentes candidatos para o desenvolvimento de novas drogas para o tratamento da dor.

Entre os modelos animais estudados estão ácido acético, teste da formalina, teste da placa quente, teste da retirada da cauda. Em geral, estes testes são usados para uma triagem inicial para avaliação da atividade analgésica. No teste das contorções abdominais, o ácido acético induz a liberação de mediadores que estimulam os neurônios nociceptivos sensíveis aos anti-inflamatórios não esteroidais e narcóticos. Portanto, apesar desse teste ser uma boa ferramenta nos ensaios farmacológicos, possui baixa especificidade (COLLIER et al., 1968).

O teste da formalina possui duas fases distintas, que caracterizam tipos diferentes de dor (HUNSKAAR; HOLE, 1987). Considerando as propriedades diferentes das fases inicial e tardia, esse teste além de avaliar substâncias antinociceptivas, também pode indicar possíveis mecanismos envolvidos (SHIBATA et al. 1989). Analgésicos opióides inibem ambas as fases, embora a segunda fase seja mais sensível. Por outro lado, AINES suprimem apenas a segunda fase do teste (HUNSKAAR; HOLE, 1987; SHIBATA et al., 1989; MALMBERG; YAKSH, 1992; JOURDAN et al., 1997).

No que concerne aos testes de retirada da cauda e placa quente, nos quais os animais são expostos ao estímulo térmico, em geral a resposta nestes testes pode envolver estruturas neurais superiores, dessa forma tais métodos identificam principalmente analgésicos centrais (JENSEN; YAKSH, 1986; LE BARS et al. 2001).

3.5 CONCLUSÃO

Diante do exposto, apesar de várias espécies de *Lippia* apresentarem atividade analgésica, a maioria dos estudos não explorou os mecanismos responsáveis pelos efeitos observados, não identificou os mediadores inflamatórios envolvidos nos processos e não tentou elucidar os mecanismos responsáveis pelas atividades antinociceptivas apresentadas.

Além do mais, é sabido que os extratos de planta exibem significantes propriedades terapêuticas devido aos efeitos combinados dos vários metabólitos secundários (FERRO, 2006); porém segundo McKeena (1996), os estudos com compostos isolados apresentam maiores vantagens, uma vez que, compostos isolados de fontes vegetais, podem ser empregados como ferramentas na identificação de mecanismos de ação, além de fornecerem moldes estruturais para a obtenção de substâncias sintéticas.

Portanto, apesar dos mecanismos de ação não estarem completamente elucidados, quer pelos modelos principalmente inespecíficos, quer pelos extratos avaliados, há fortes indícios

de que os constituintes dos extratos e óleos essenciais das espécies de *Lippia* são candidatos promissores ao alívio da dor.

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CAPÍTULO 2

**β-CYCLODEXTRIN COMPLEX CONTAINING *LIPPIA*
GRATA LEAF ESSENTIAL OIL REDUCES OROFACIAL
NOCICEPTION IN MICE - EVIDENCE OF POSSIBLE
INVOLVEMENT OF DESCENDING INHIBITORY PAIN
MODULATION PATHWAY**

4 β -CYCLODEXTRIN COMPLEX CONTAINING *LIPPIA GRATA* LEAF ESSENTIAL OIL REDUCES OROFACIAL NOCICEPTION IN MICE - EVIDENCE OF POSSIBLE INVOLVEMENT OF DESCENDING INHIBITORY PAIN MODULATION PATHWAY

Running title: *Lippia grata* leaf essential oil complexed in β -cyclodextrin reduces orofacial nociception

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ABSTRACT

The treatment of orofacial pain remains a major challenge for modern medicine. Thus, we prepared and physicochemical characterized a new β -cyclodextrin complex containing *L. grata* leaf essential oil (β -CD/EO) in order to investigate their possible antinociceptive activity in animal models of orofacial pain. The results of DSC and TG/DTG showed that the products prepared by SC method were able to incorporate greater amounts of EO. In the X-ray diffractogram, it was showed that complex between EO and β -CD was formed. Male Swiss mice were pretreated with β -CD/EO (6, 12 or 24 mg/kg, *per os*, gavage, p.o.), morphine

(5 mg/kg, i.p.), or vehicle (distilled water, p.o.), 1h before treatment with formalin (20 μ l, 2%), capsaicin (20 μ l, 2.5 μ g), or glutamate (40 μ l, 25 μ M) into the right upper lip. Our results demonstrated that p.o. treatment with β -CD/EO was significantly ($p < 0.05$ or $p < 0.001$) capable in reducing the nociceptive face-rubbing behavior in both phases of the formalin test. β -CD/EO-treated mice were also significantly ($p < 0.05$ or $p < 0.001$) protected against nociception induced by capsaicin and glutamate. For the action in the central nervous system (CNS), ninety minutes after the treatment, the mice were perfused, the brains collected, crioprotected, cut in a criostate and submitted to an immunofluorescence protocol for Fos protein. The immunofluorescence protocol demonstrated that the β -CD/EO significantly activated ($p < 0.05$; $p < 0.01$ or $p < 0.001$) the motor cortex, the *locus ceruleus*, the nucleus raphe magnus and the periaqueductal gray of the CNS. These effects apparently did not alter, in tested doses, the motor coordination of mice in the rota-rod test. Our results propose that β -CD/EO might present an important draft of drug to the study of new compounds for the treatment of orofacial pain.

Keywords: *Lippia grata*, essential oil, cyclodextrin, pain, cFos, periaqueductal gray.

4.1 INTRODUCTION

Pain in the oral and craniofacial system represents a major medical and social problem [1]. Orofacial pain often constitutes a challenging diagnostic problem that can be complicated by psychosocial factors and typically requires multidisciplinary treatment approaches [2]. The management of pain continues to be a major challenge for medicine. Opioids, anticonvulsants and non-steroidal anti-inflammatory drugs (NSAIDs) are the main agents used to relieve acute and chronic pain [3]. Unfortunately, the use of opioids and NSAIDs are limited by side effects.

A new approach to discover new analgesic drugs has been performed using medicinal plants and its secondary metabolites as therapeutic alternatives for painful disorders [4, 5, 6] such as orofacial pain, including promising studies being developed by our group with essential oils and monoterpenes: citronellal [7], *Ocimum basilicum* essential oil and (-)-linalool [8], p-cymene [9] and citronellol [10]. However, low water solubility, slow dissolution rate, instability, and short half-life are limitations for essential oils and monoterpenes therapeutic applications.

Several approaches have been used to improve therapeutic properties of essential oils and monoterpenes, including the employment of drug-delivery systems [11-13]. In this

regard, β -cyclodextrins (β -CD) have been widely used to prepare inclusion complexes with essential oils and monoterpenes, to improve stability and solubility, modify the release of the drugs, and turn liquid substances into stable and free flowing powders [11,14]. Additionally, inclusion of β -CD turns liquid essential oils into water dispersible and easy-to-handle powders and allows control of their volatility [15]. Recently, our group demonstrated that β -CD inclusion could improve the pharmacological profile of p-cymene, a constituent of a number of essential oils, most commonly the oil of cumin and thyme [16].

The genus *Lippia* (Verbenaceae) mainly distributed throughout the South and Central Americas and tropical Africa [17] is widely used in popular medicine showing several traditional uses and pharmacological activities. There are approximately 200 species and some of them are used as an analgesic and anti-inflammatory remedies [18]. The species *Lippia grata* Schauer is a native bush of the semi-arid area of Northeastern Brazil, the antispasmodic activities of leaf essential oil (EO) obtained from *L. grata* were demonstrated and its pharmacological effects were attributed of the presence of carvacrol and thymol, two monoterpenes [19,20]. However, no work using EO to investigate analgesic profile and other pharmacological studies has been found. Likely, some studies have attempted improve the pharmacological profile of *Lippia* species essential oils with β -CD complex inclusion [11, 15].

Thus, we investigated the antinociceptive effect of β -cyclodextrin complex containing *L. grata* leaf essential oil (β -CD/EO) using orofacial pain induced by -formalin, -capsaicin and -glutamate protocols in mice, and to investigate the central nervous system (CNS) areas involved in this response.

4.2 MATERIAL AND METHODS

4.2.1 Plant material and essential oil (EO)

The fresh leaves of *L. grata* was collected in June 2011 in Capim Grosso, Bahia, in northeastern 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 from 200 g, during 3h, of air dried leaves in a Clevenger apparatus in duplicate. The samples obtained in each extraction were dried over anhydrous sodium sulphate. The essentials oils were stored in sealed glass containers covered with aluminum foil and kept under refrigeration at -10°C until used. The

essential oil (EO) content was determined in mL of oil per 100 g of leaves on a dry weight basis. The identification of the components of the essential oil was performed by CG/MS and CG/FID.

4.2.2 Identification of essential oil constituents

The essential oils were analyzed by GC/MS on a Shimadzu QP5050A (Shimadzu Corporation, Kyoto, Japan) system equipped with a AOC-20i autosampler under the following conditions: J&W Scientific DB-5MS fused silica capillary column (30 m x 0.25 mm i.d., x 0.25 μ m film thickness, composed of 5%-phenyl-95%-methylpolysiloxane) operating in electron impact mode at 70 eV. Helium (99.999%) as the carrier gas at a constant flow of 1.2 mL/min. The injection volume was 0.5 μ L (split ratio of 1:100) and the injector temperature was 250°C and the ion-source temperature was 280°C. The oven temperature was programmed at 50°C (isothermal for 1.5 min), with an increase of 4°C/min to 200°C, then 10°C/min to 300°C, ending with a 10 min isothermal at 300°C. Mass spectra were taken at 70 eV with a scan interval of 0.5 s and fragments from 40 to 500 Da.

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID) using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) instrument under the following operational conditions: capillary ZB-5M5 column (5%-phenyl-arylene-95%- methylpolysiloxane fused silica capillary column 30 m x 0.25 mm i.d. x 0.25 μ m film thickness), under same conditions reported for the CG-MS. Quantification of each constituent was estimated by area normalization (%). Compound concentrations were calculated from the CG peak areas and they were arranged in order of CG elution.

Identification of individual components of the essential oils was performed by computerized matching of the acquired mass spectra with those stored in WILEY8, NIST107 and NIST21 mass spectral library of the GC/MS data system. Retention indices (RI) for all compounds were determined according to literature as previously described (Adams, 2007).

4.2.3 Preparation of inclusion complex

Inclusion complex of β -CD/EO was prepared by slurry procedure. Slurry complexation (SC) was carried out by the addition of water to a beaker containing 1.135 g of β -CD (3:4, v/w). 152 mg of EO, which is equal to about a 1:1 (molar guest: host ratio) (based on camphor molecular weight), were added to the slurry and stirred for 36 h by a magnetic

stirring device operating at 400 rpm (Quimis Q 261A21, Brazil). A mechanical mixture was prepared by the addition of EO to an agate mortar containing powdered β -CD under manual agitation as benchmark. The EO/ β -CD mass ratio was maintained as described for inclusion complex preparation and the mechanical mixture was stored in airtight glass containers.

4.2.4 Physicochemical characterization

4.2.4.1 Thermal analysis

Differential Scanning Calorimeter (DSC) curves were obtained in a DSC-50 cell (Shimadzu) using aluminium crucibles with about 2 mg of samples, under dynamic nitrogen atmosphere ($50 \text{ mL}\cdot\text{min}^{-1}$) and heating rate of $10^\circ\text{C}\cdot\text{min}^{-1}$ in the temperature range from 25 to 600°C . The DSC cell was calibrated with indium (m.p. 156.6°C ; $\Delta H_{\text{fus.}} = 28.54 \text{ J}\cdot\text{g}^{-1}$) and zinc (m.p. 419.6°C). Thermogravimetry/Derivative Thermogravimetry (TG/DTG) curves were obtained with a thermobalance model TGA 50 (Shimadzu) in the temperature range of 25 - 900°C , using platinum crucibles with ~ 3 mg of samples, under dynamic nitrogen atmosphere ($50 \text{ mL}\cdot\text{min}^{-1}$) and heating rate of $10^\circ\text{C}\cdot\text{min}^{-1}$. The TG/DTG was calibrated with calcium oxalate monohydrate, conforming to ASTM standard.

4.2.4.2 X-ray diffraction

Powder X-Ray diffractometer results were obtained on a Rigaku with a tube of $\text{CuK}\alpha$, in the range of 3 - 65° (2θ) and 1s of pass time, using the powder XRD (X-ray diffraction) method.

4.2.4.2 Scanning electron microscopy

The β -CD, physical mixture (PM), and slurry complex (SC) were mounted on aluminum stubs, coated with a thin layer of gold and visualized with a JEOL Model JSM-6360-LV scanning electron microscope at an accelerated voltage of 20 kV.

4.2.5 Pharmacological Procedures

4.2.5.1 Animals

In pharmacological protocols we used adult (3-month-old) male albino Swiss mice (27-35 g), which were randomly housed in appropriate cages at $21 \pm 2^\circ\text{C}$ with a 12-hr light/dark cycle (light from 06:00 to 18:00) with free access to food (Purina[®], Brazil) and tap water. All experiments were carried out between 09:00 am and 16:00 pm in a quiet room. All experiments involving the behavioral analysis were carried out by the same visual observer and in a double-blind manner. Experimental protocols were approved by the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS # 57/11). All efforts were made to minimize the number of animals used and their discomfort.

4.2.5.2 Formalin test

Orofacial nociception was induced in mice by injection (s.c.) of 20 μl of 2% formalin into the right upper limb (perinasal area) [21]. This volume and the concentration percentage of formalin were selected from pilot studies that showed a nociceptive-related biphasic behavioral response (face-rubbing) of great intensity at periods of 0 to 5 minutes (first phase) and 15 to 40 minutes (second phase). Nociception was quantified at these periods by measuring the time (seconds) that the animal spent face-rubbing in the injected area with its fore or hindpaws. To assess the effect of the test drug, groups of mice (n=6, per group), were pretreated with vehicle (distilled water), β -CD/EO (6, 12 or 24 mg/kg, p.o.) or MOR (5 mg/kg; i.p.) ninety minutes before the local injection of formalin.

4.2.5.3 Glutamate-and capsaicin-induced nociception

The orofacial pain was induced by glutamate or capsaicin in mice as previously described by Quintans-Júnior et al. [7]. Mice (n=6, per group) were injected with 40 μl of glutamate (25mM) or capsaicin (20 μL , 2.5 μg) subcutaneously into the right upper lip (perinasal area), using a 27 gauze needle. Capsaicin was dissolved in ethanol, dimethyl sulfoxide and distilled water (1:1:8). In pilot studies, rodents manifested pain-related face-rubbing behavior following the injection of capsaicin with a high intensity at 10-20 min period. Therefore, pain quantification was performed at this period measuring the time (s) that

the animals spent face-rubbing the injected area with fore - or hindpaws. β -CD/EO (6, 12 or 24mg/kg, p.o.) or vehicle (distilled water) was given to animals as described in the formalin test 90 min before the local injection of algogen (glutamate or capsaicin) [7]. Morphine (5 mg/kg, i.p.), administered 1.5 h before the algogen, was included as a positive control. An additional group received a similar volume of capsaicin vehicle (data no shown).

4.2.5.4 Evaluation of the motor activity

To investigate if the treatments could influence the motor activity of the animals and consequently impair the assessment of the nociceptive behavior in experimental models, the motor activity was evaluated in a Rota-rod apparatus [7]. Initially, the mice that were able to remain on the Rota-rod apparatus (AVS[®], Brazil) longer than 180 s (7 rpm) were selected 24 h before the test. The selected animals were then divided into five groups (n=6, per group) and treated p.o. with vehicle, β -CD/EO (6, 12 or 24 mg/kg, p.o.), and diazepam (3 mg/kg, i.p.). 1h, 1.5h and 2h later, each animal was tested on the Rota-rod apparatus and the time (s) remained on the bar for up to 180 s.

4.2.5.5 Immunofluorescence

To evaluate the action of the test drug in the central nervous system (CNS), we following the method described by Brito et al [10]. Ninety minutes after the injection of β -CD/EO (6, 12 or 24 mg/kg; p.o.) or vehicle (distilled water), the animals (n=6, per group) were perfused and the brains were collected and crioprotected for immunofluorescence processing to Fos protein expression. The time for realization of immunofluorescence protocol was grounded in the study of Barr [22].

Frozen serial transverse sections (20 μ m) of all of the brain were collected on gelatinized glass slides. The tissue sections were stored at -80°C until use. The sections were washed with phosphate buffer (0.01M) saline isotonic (PBS) 5 times for 5 minutes and were incubated with 0.1 M glycine in PBS for 10 minutes. Non-specific protein binding was blocked by incubation of the sections for 30 minutes in a solution containing 2% BSA. After that, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (1:2000). Afterwards, the sections were incubated for two hours with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (1:2000). The cover slip was mounted with Fluoromount G. 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 minutes [10].

4.2.5.6 Acquisition and analyses of images

Pictures from Fos positive brain areas were acquired for each animal with an Axioskop 2 plus, Carl Zeiss, Germany. The brain regions were classified according to Paxinus and Watsu Atlas, 1997. Neurons were counted by the free software Image J[®] (National Institute of Health) using a plug-in (written by authors) that uses the same level of label intensity to select and count the Fos positive cells.

4.2.6 Statistical analysis

The data obtained were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered to be statistically significant when $p < 0.05$. All statistical analyses were done using the software GraphPad Prism[®] 5.0 (GraphPad Prism Software Inc., SanDiego, CA, USA).

4.2.7 Chemicals

Glutamate, capsaicin, formalin, Tween 80, β -cyclodextrin, fluoromount G, glycine and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA). Morphine (MOR) and Diazepam (DZP) was purchased from Cristália (Brazil). Rabbit anti-Fos and donkey anti-rabbit Alexa Fluor 594 were obtained from Santa Cruz Biotechnology (USA).

4.3 RESULTS

When GC-MS analysis were performed, a mixture of terpenoids was recorded, being camphene (11.3%) camphor (27.2%), borneol (6.0%), (E)-caryophyllene (11.6%) and bicyclogermacrene (9.4%), we as the main compounds in the EO (Table 1).

Table 1- Chemical composition of *Lippia grata* essential leaf essential oil

TR (min)	component ^a	(%) ^b	RI exp. ^c	identification ^d
7.500	α -pinene	2.4	931	RI, MS, Std
8.042	Camphene	11.3	948	RI, MS, Std
8.800	Sabinene	0.3	971	RI, MS
8.967	β -pinene	1.1	976	RI, MS, Std
9.367	Myrcene	2.0	988	RI, MS, Std
10.625	p-cimene	1.7	1023	RI, MS, Std
10.800	Limonene	4.2	1028	RI, MS, Std
10.917	1,8-cineole	3.2	1031	RI, MS, Std
11.867	γ -terpinene	1.3	1057	RI, MS, Std
12.883	Terpinolene	1.2	1084	RI, MS
13.425	Linalool	0.4	1099	RI, MS, Std
15.250	Camphor	27.2	1147	RI, MS, Std
16.167	Borneol	6.0	1171	RI, MS, Std
18.267	Methyl thymol	0.4	1228	RI, MS
20.775	Carvacrol	3.6	1297	RI, MS, Std
23.542	α -copaene	1.3	1376	RI, MS
24.008	β -elemene	0.9	1389	RI, MS
25.050	E-caryophyllene	11.6	1420	RI, MS, Std
25.675	Aromadendrene	0.7	1439	RI, MS
26.242	α -humulene	1.0	1456	RI, MS
26.383	Allo-aromadendrene	0.6	1460	RI, MS
27.092	γ -muurolene	1.6	1481	RI, MS
27.558	Biciclogermacrene	9.4	1495	RI, MS
28.267	δ -cadinene	0.7	1518	RI, MS
30.133	Spathulenol	1.6	1577	RI, M
30.308	Caryophyllene oxide	0.7	1583	RI, MS, Std
Total		96.4		

^aCompounds are listed in order of their elution from DB-5MS ^bFID percentage ^cRI = retention indices as determined on DB-5MS using homologous series of C9-19 n-alkanes. ^dMethod of identification: MS, by comparison of the mass spectrum with those of the computer mass libraries; RI, by comparison of RI with those from the literature; Std, by injection of an authentic sample (present in our laboratory-made data base)

Thermal analyses of the EO/ β -CD revealed the formation of a complex. The DSC curve of EO shows an endothermic peak at nearly 123°C corresponding to its volatilization. As it can be seen in Fig. 1, the curve corresponding to EO/ β -CD complex did not show a sharp endothermic peak in the range of the volatilization of the pure compound (123°C). The disappearance of this event is due to its encapsulation in the host β -CD.

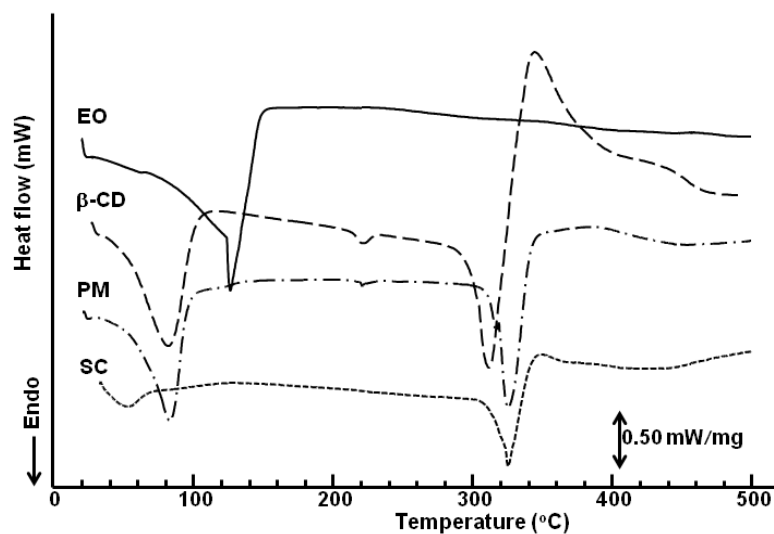


Fig 1. DSC curves of EO, β -CD, physical mixture (PM), and slurry complex (SC) in dynamic nitrogen atmosphere ($100 \text{ mL}\cdot\text{min}^{-1}$) and heat rate $10^\circ\text{C}\cdot\text{min}^{-1}$.

Table 2 lists the mass losses obtained by TG/DTG curves calculated from specific intervals for each material studied in the present work

Table 2- Mass losses for *Lippia grata* essential oil (EO), β -CD, physical mixture and *Lippia grata*/ β -CD complexes and moisture contents obtained by Karl Fisher method

	Mass loss/%			
	1 st step	2 nd step	3 rd step	4 th step
EO	98.50 [#]	1.50	-	-
β -CD	12.51 [*]	2.19	72.88 ^{**}	10.76 ^{***}
Physical Mixture (PM)	23.96 [*]	2.96 ⁺⁺	63.65 ^{**}	10.18 ^{***}
Slurry complex (SC)	10.14 ⁺	6.08 ⁺⁺	75.67 ^{**}	10.57 ^{***}

[#]percentage of the EO evaporates up to 200°C ; ^{*}percentage of water releasing up to 120°C ; ⁺mass loss related to evaporation of the EO and the water release up to 140°C ; ⁺⁺mass loss probably attributed to EO release in the interval from 120 to 270°C ; ^{**}thermal decomposition in the interval from 270 to 365°C ; ^{***}elemental carbon formation due to sample carbonization in the interval from 365 to 900°C .

The X-ray diffraction patterns of solid samples were performed (Fig. 2). As shown in Fig. 2, some sharp peaks at the diffraction angle of 2θ 4.76, 6.12, 9.02, 10.72, 13.98 and 20.70 are present in the X-ray diffractogram of β -CD powder (Fig. 2) and it suggests that the

powder is present as a crystalline material. The β -CD crystallinity peaks were still detectable in the physical mixture with EO (Fig. 2), however, these crystallinity peaks originally in the β -CD sample modified.

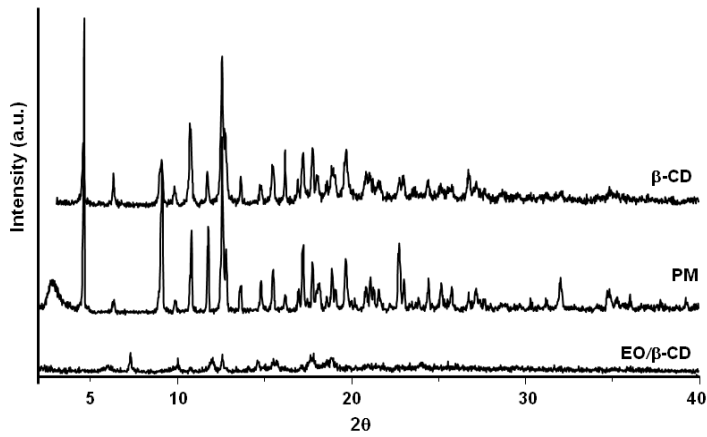


Fig. 2. X-ray diffraction of β -CD, PM and EO/ β -CD.

As showed in Figure 3, oral treatment of β -CD/EO induced a significant antinociceptive effect ($p < 0.001$) with different potencies either at phase I and phase II on the orofacial formalin test.

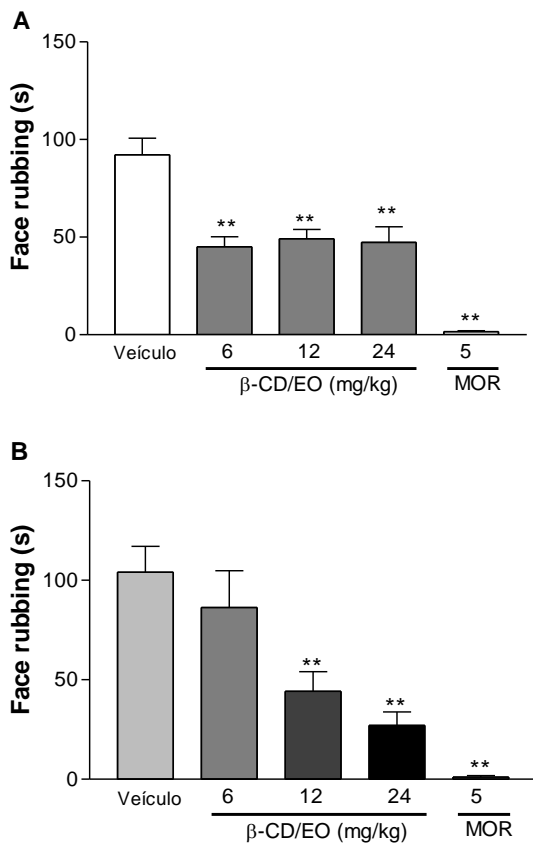


Fig. 3. Effects of β -CD/EO on formalin-induced orofacial nociceptive behavior. Vehicle (control), β -CD/EO (6, 12, and 24 mg/kg, p.o.) or MOR (5 mg/kg, i.p.) were administered 90 minutes before formalin injection. (A) First phase (0–5 min.) and (B) second phase (15–40 min.) of the formalin test. Values represent mean \pm S.E.M. (n = 6, per group). *p < 0.05 or **p < 0.001 versus control (one-way ANOVA followed by Tukey's test).

As β -CD/EO was given in higher doses, the face-rubbing behavior induced by glutamate decreased significantly (p<0.001) when compared with the control group (vehicle) (Figure 4).

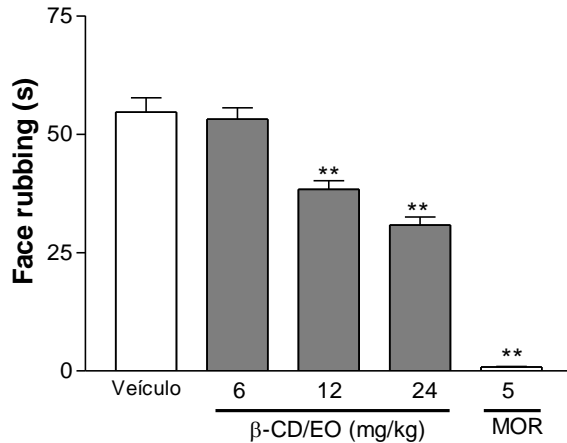


Fig. 4. Effects of β -CD/EO on glutamate-induced orofacial nociceptive behavior. Vehicle (control), β -CD/EO (6, 12, and 24 mg/kg, p.o.) or MOR (5 mg/kg, i.p.) were administered 1.5h before glutamate injection. Values represent mean \pm S.E.M. (n = 6, per group). *p < 0.05 or **p < 0.001 versus control (one-way ANOVA followed by Tukey's test).

The β -CD/EO, in all doses, significantly (p<0.05 or p<0.001) reduced the face-rubbing behavior induced by administration of capsaicin (Figure 5)

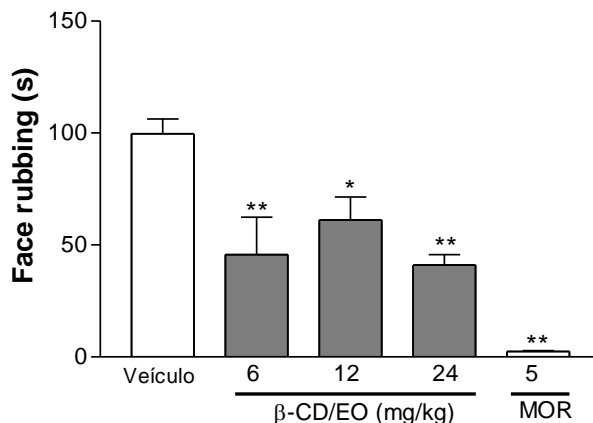


Fig. 5. Effects of β -CD/EO on capsaicin-induced orofacial nociceptive behaviour. Vehicle (control), β -CD/EO (6, 12, and 24 mg/kg, p.o.) or MOR (5 mg/kg, i.p.) were administered 1.5h before capsaicin injection. Values represent mean \pm S.E.M. (n = 6, per group). *p < 0.05 or **p < 0.001 versus control (one-way ANOVA followed by Tukey's test).

When Fos protein labeled by immunofluorescence was performed to evaluate possible Central Nervous System (CNS) action of β -CD/EO, showed a significantly ($p < 0.05$ or $p < 0.001$) activation of the motor cortex, *locus ceruleus* (LC), nucleus raphe magnus (NRP) and periaqueductal gray (PAG), when compared to the control group (vehicle) (As showed in Figure 6 and 7).

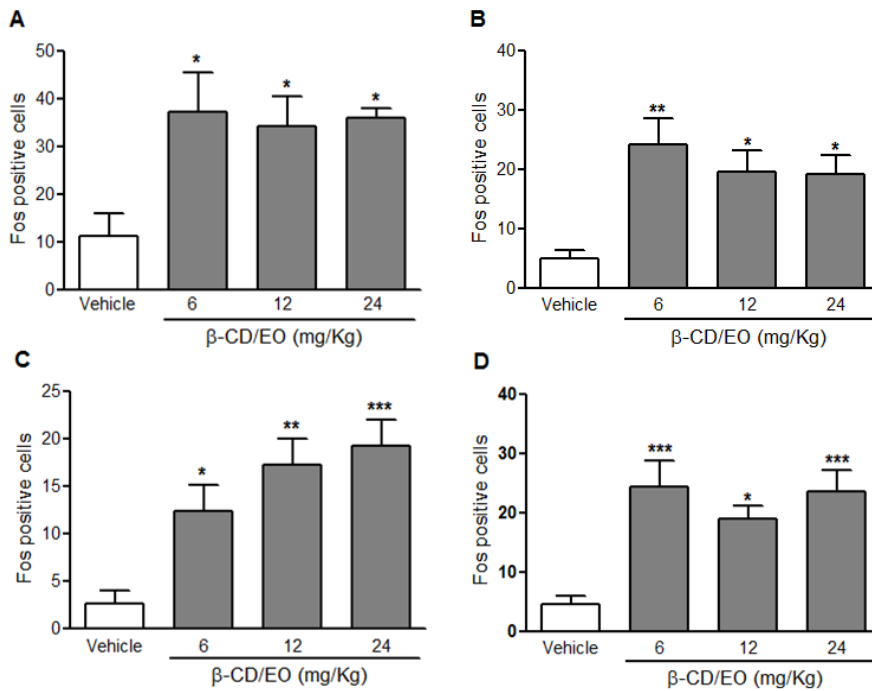


Fig. 6. Neurons Fos positive in the motor cortex (A), *locus ceruleus* (B), nucleus raphe magnus (C) and periaqueductal grey (D). Vehicle (control) or β -CD/EO (6, 12 and 24 mg/kg) were administered orally 1.5h before perfusion. Values represent mean \pm S.E.M. (n=6, per group). * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ versus control (one-way ANOVA followed by Tukey's test).

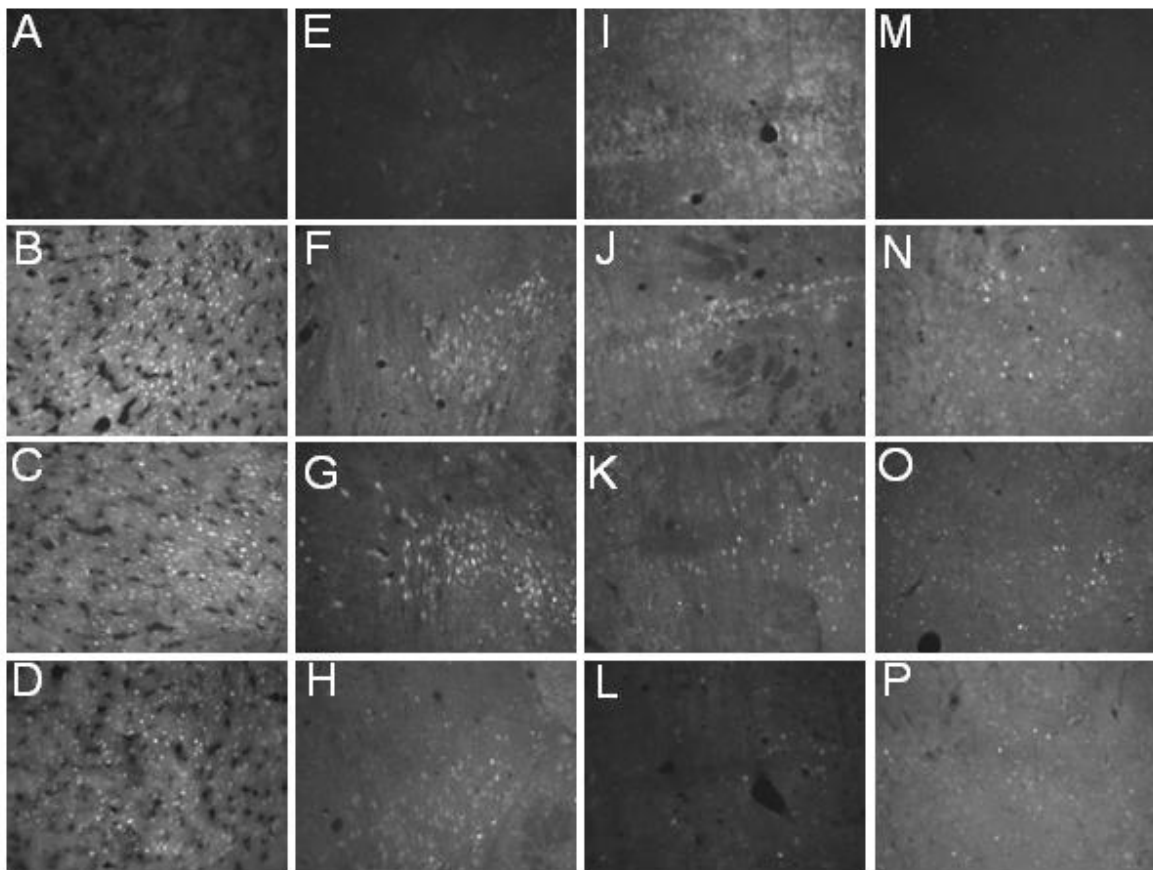


Fig. 7. Immunofluorescence for Fos protein in the neurons of the motor cortex (A, B, C, D), locus ceruleus (E, F, G, H), nucleus raphe magnus (I, J, K, L) and periaqueductal grey (M, N, O, P). Vehicle (control) or β -CD/EO (6, 12 or 24 mg/kg) were administered orally 1.5h before perfusion.

β -CD/EO-treated mice did not indicate any significant motor performance alterations in rota-rod apparatus (Figure 8).

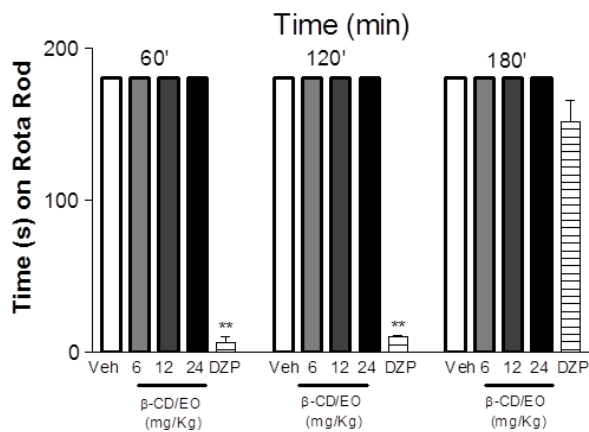


Fig. 8. Time(s) on the Rota-rod observed in mice after treatment with vehicle (control), β -CD/EO (6, 12, 24 mg/kg, p.o.) or diazepam (DZP, 1.5 mg/kg, i.p.). The motor response was recorded for the following 180 sec. after drug treatment. Statistical differences *versus* control group were calculated using ANOVA, followed by Tukey' test (n = 6, per group). *p < 0.001

4.4 DISCUSSION

In our opinion, the most interesting finding of this study is that the orally administration of β -CD/EO produced a strong antinociceptive activity in orofacial nociceptive behavior induced by -formalin, -glutamate and -capsaicin in mice, and assess evidence for CNS involvement in analgesic profile. Inclusion complexation of EO with β -CD was important to improve water solubility and pharmacologic effects and it can be related with presence of terpenes, such as camphor, borneol and (E)-caryophyllene.

The results GC-MS analysis in this study are different from previous findings by Craveiro et al. [23] that showed mainly monoterpenes: γ -terpinene (14.4%) p-cymene (22.2%), carvacrol (20%) and thymol (18,8%).

By data analysis obtained by TG/DTG curves, it can be seen that the major fraction of EO ($\Delta m = 98.50\%$) evaporates up to 200°C . As observed in TG/DTG (omitting figure), the mass loss (%) of free β -CD at the heating rate of $10^{\circ}\text{C}\cdot\text{min}^{-1}$ can be divided into three consecutive processes. The sample in process a is in the temperature range from 25 to 125°C with a mass loss of 12.51% , which is due to the release of water molecules from the outside and/or inside of β -CD cavity. Subsequently, the sample is kept in a very wide temperature range from 125 to 270°C . Then the sample undergoes a rapid melting/decomposition ($\Delta m = 72.88\%$, 270 to 391°C). During continuous heating, the sample is carbonized and incinerated ($\Delta m = 10.76\%$, $T = 391$ to 800°C).

The curve of the PM was a superposition of the guest and host curves, which indicates a lower evidence of inclusion and significant interaction between the host and guest molecules. Two overlapping steps were exhibited as causing 23.96% of mass loss, related to the evaporation of the EO and the water release from the β -CD up to 120°C . The curve of the complex prepared by slurry method (SC) showed a water/EO loss event from r.t. up to 125°C . In the interval from 125 to 275°C , a gradual mass loss (6.08%) was recorded and can be attributed to EO release.

The inclusion complex of EO with β -CD (1:1) show undefined, broad, diffuse peaks of low intensities in the X-ray diffraction. This feature indicates the formation of a significant amount of amorphous material. This phenomenon suggested that an inclusion complex between EO and β -CD was formed.

Scanning electron microscopy (MEV) was used to assess the microscopic aspects of the β -CD, of the physical mixture and of the complex formation. Pure β -CD appears as

crystalline particles of different sizes without a definite shape. The electron microscopic pictures showed that the physical appearance and size of the complexes formed were different. There were drastic changes in particle shapes and original morphologies of the inclusion complex product. The complexation between EO and β -CD appeared as agglomerates. In contrast, the particle shapes and morphologies of the corresponding physical mixture were similar to those β -CD. These differences are due to the stacking interactions, which appear in the case of natural cyclodextrins (cyclodextrin hydrate) in compared to the cyclodextrin complexes.

Recently, Marreto et al [11] demonstrated that the inclusion of volatile oils with β -CD can be applied to protect essential oils against oxidation, heat and light degradation, evaporation, and moisture. In this process, every volatile constituent (guest) is tightly held within the cyclodextrin molecule (host), which offers an effective protection against the damaging effects of the environment [24]. Besides, inclusion with cyclodextrins turns liquid essential oils into water dispersible and easy-to-handle powders [13]. Another innovative approach to improve pharmacological and chemical properties of natural products or derivatives is incorporates them in controlled drug delivery systems [25-27]. Thus, some effort are being developed by Brazilian research groups on developing drug delivery systems and them incorporation with medicines seeking a clinical applicability safely and effectively [25-28].

The formalin test is based on a chemical stimulus and induces a tissue damage that mimics acute post-injury pain in humans. The biphasic component of formalin-induced nociception reflects different underlying mechanisms. The first phase is related to the direct chemical stimulation of nociceptive nerve endings, which reflects centrally mediated pain with the release of substance P [29,30] and the second phase depends on a combination of inputs from nociceptive afferents, due to the release of excitatory amino acids, PGE₂, nitric oxide (NO) and tachykinin, kinins among other peptides [21,31]. It has been reported that the development of hyperalgesia due to the injection of formalin involves the glutamatergic system, mainly NMDA receptors [31].

It is well established that glutamate is involved in the transmission of nociceptive signals from the peripheral nervous system to the dorsal horn of the spinal cord. Moreover, it has been reported that the glutamate injection evoked pronounced nociceptive responses, which are mediated by neuropeptides (Substance P) released from C fibres and activate of glutamate receptors (e.g. NMDA). This can stimulate the production of a variety of

intracellular second messengers such as NO, and pro-inflammatory cytokines such as TNF- α and IL-1 β , which act synergistically in the excitation of the neurons [32].

The ability of camphor, mainly EO-compound, to modulate sensations of warmth in humans has been attributed to its ability to activate TRPV3 [33]. TRPV3 is expressed in keratinocytes, the dorsal root ganglia, brain and spinal cord [34]. It has been implicated in hyperalgesia, inflamed tissues and possibly skin sensitization. Vogt-Eisele et al. [34] demonstrated that some monoterpenes (as camphor) activated TRPV3 receptors. As β -CD/EO, in all doses, significantly ($p < 0.05$ or $p < 0.001$) reduced the face-rubbing behavior induced by administration of capsaicin (Figure 5), it is possible that antinociceptive effects may be related to the vanilloid receptors family.

It is likely that experimental studies have been demonstrated that terpenoids (as camphor and β -caryophyllene) have analgesic and anti-inflammatory properties [4,35,36]. Waning et al. [37] demonstrated that the capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1), which plays an important role in pain transduction, is one of the Ca^{+2} influx channels involved in cell migration. It was also shown that TRPV1 activation increases the expression of the TNF receptor 1 (TNFR1) by a ROS-dependent mechanism [38]. Camphor inhibited several related TRP channels, including ankyrin-repeat TRP 1 (TRPA1) [35, 37]. Additionally, β -caryophyllene is a Food and Drug Administration (FDA) approved food additive and is ingested daily with food. It is the first dietary cannabinoid [39]. Moreover, β -caryophyllene act in CB2 receptors and activation of CB2 receptors can produces directly antinociceptive response, by causing the release of mediators from non-neuronal cells that alter the responsiveness of primary afferent neurons to noxious stimuli [36]. Liu et al. [40] recently demonstrated that inhibition of I κ B α -NF- κ B and translocation signaling pathway, as well as the modulation of intracellular ROS and iNOS/NO pathway may be involved with an analgesic and anti-inflammatory profile demonstrated by borneol. So, it is possible that main compounds of EO contributed in synergic way to produce analgesic profile.

The action of β -CD/EO in Central Nervous System (CNS) by Fos protein labeled by immunofluorescence showed activation of the motor cortex, *locus ceruleus* (LC), nucleus raphe magnus (NRP) and periaqueductal gray (PAG), when compared to the control group (vehicle).

Following the report of Reynolds [41], the PAG was rapidly established as being important to descend inhibition of spinal nociceptive processing and also as a site where opioids, when microinjected directly into the PAG, replicated the inhibitory effects of

electrical stimulation [42]. It also became clear that stimulation in widespread sites in the brain, including the sensory cortex, thalamus, hypothalamus, midbrain, pons and spinal cord, similarly produced inhibitory effects on spinal nociceptive processing. Additionally, NRP produces an important role in the PAG area and it is stimulated, can produce descending inhibitory of pain effects from the PAG [42].

Wilson-Poe et al. [43] has demonstrated that activation of CB1 receptors by agonist HU-210 into the PAG enhances the analgesic effect of opioids. These findings are particularly interesting because they suggest long lasting and bidirectional enhancement of antinociception between opioids and cannabinoids [44]. Additionally, Katsuyama et al. [45] recently described that possible involvement of peripheral cannabinoid and opioid receptors produced by β -caryophyllene in antinociceptive response. Thus, it is seems reasonable to propose that the β -CD/EO may be acting by via peripheral and central.

Despite the fact that β -CD/EO increase significantly Fos protein marking in motor cortex CNS area, β -CD/EO-treated mice did not indicate any significant motor performance alterations in rota-rod apparatus. However, as previously demonstrated by Viisanen and Pertovaara [46, 47], the electrical stimulation of the motor cortex has been shown to induce spinal antinociception in the spinal nerve-ligated neuropathic model in rats involving the rostro ventro medial medulla and LC [46,47].

The findings of this study corroborate with other important research that seek improve some intrinsic characteristics of natural products, as essential oils or related compounds. Thus, the incorporation of these compounds in drug delivery systems (such as cyclodextrins, ie.) seems to be one inventive way to improve their plasma half-life, effectiveness and, mainly, to manage side-effects and/or toxicity. Recently, Botelho et al developing active targeting systems, evaluating the efficacy and toxicity for novel delivery systems via transdermal and transmucosal in short and long-term clinical [28,48], which it can be a tool for new applicability. In fact, the research with these new entities (natural products and related compounds) should be undertaken with the prospect to clinical applicability of the medium or short term and new active targeting systems could be an approach for this applicability.

4.5 CONCLUSION

Together, our results give a preliminary and not yet described indication of an antinociceptive effect of β -CD/EO on orofacial pain models in mice. Antinociceptive profile might be linked to the presence of some terpenoids, such as camphor, borneol and β -

caryophyllene, and to the activation of the motor cortex, NRP and PAG (cerebral areas involved in pain modulation). So, the present investigation supported the use of β -CD/EO as a formulation with therapeutic potential for management of orofacial pain. Further studies will continue to define the possible clinical uses of this new agent.

Acknowledgments

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Conflict of interest: The authors declare no conflict of interest.

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CONCLUSÃO

5 CONCLUSÃO

- ▶ Considerando o gênero *Lippia*, a atividade analgésica foi evidenciada em poucos estudos, os quais podem ser considerados bastante preliminares uma vez que, em geral foram realizados em testes inespecíficos e com poucos estudos de mecanismos de ação;
- ▶ No óleo, extraído por hidrodestilação e analisado por GC-MS, foi observada uma mistura de terpenóides, sendo cânfora (27,2%), (E)-cariofileno (11,6%) canfeno (11,3%), biciclogermacreno (9,4%) e borneol (6,0%) os principais compostos do OE de *Lippia grata*.
- ▶ O óleo essencial de folhas de *L. grata* foi complexado em β -ciclodextrina (β -CD/OE) através do método de coevaporação.
- ▶ O complexo foi caracterizado através de análises térmicas cujos comportamentos na curva de calorimetria diferencial exploratória (DSC) e nas perdas de massa calculadas a partir dos intervalos do complexo submetido à termogravimetria e termogravimetria diferenciada sugerem a sua formação. Além disto, os picos observados através da difração de raio-X também indicam tal formação.
- ▶ O complexo formado β -CD/OE demonstrou efeito antinociceptivo com doses baixas de 6mg/kg, 12 mg/kg e 24 mg/kg em protocolos experimentais de dor orofacial, realizado em camundongos; demonstrando que o processo da complexação contribui de forma significativa para melhorar a biodisponibilidade do óleo.
- ▶ O perfil antinociceptivo pode estar ligado à presença de alguns terpenóides, como cânfora e β -cariofileno, bem como, com a ativação de áreas cerebrais envolvidas na modulação da dor como: o córtex motor, o núcleo magno da rafe e a substância cinzenta periaquedutal.

PERSPECTIVAS

6 PERSPECTIVAS

O tratamento com o complexo em β -ciclodextrina do óleo essencial de *Lippia grata* Schauer (β -CD/EO) mostrou-se efetivo em modelos de nocicepção em camundongos, ativando áreas cerebrais envolvidas na modulação da dor. Entretanto, como é comum no desenvolvimento de um projeto de mestrado novas perguntas surgiram a partir dos nossos resultados e que nortearão o grupo de pesquisa em Biotecnologia e Inovação Terapêutica em novas etapas deste estudo, a saber:

- Estudo da toxicidade aguda e crônica da *Lippia grata* seguindo os critérios preconizados pela ANVISA (Agência Nacional de Vigilância Sanitária);
- Avaliação do envolvimento de sistemas de neurotransmissão no efeito antinociceptivo do β -CD/EO, tais como opioidérgico, glutamatérgico, serotoninérgico e GABAérgico;
- Avaliação farmacológica do β -CD/EO em modelos crônicos de nocicepção;
- Patenteamento e possível negociação com o setor produtivo do protótipo do invento, desde que seja assegurada sua eficácia e segurança terapêutica para posteriores estudos clínicos.

ANEXOS

**ANEXO A: Protocolo de Aprovação do Comitê de Ética em Pesquisa Animal da
Universidade Federal de Sergipe (CEPA/UFS)**



UNIVERSIDADE FEDERAL DE SERGIPE
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COORDENAÇÃO DE PESQUISA
COMITÊ DE ÉTICA EM PESQUISA COM ANIMAIS (CEPA)

DECLARAÇÃO

Declaro, para os devidos fins, que o Projeto de Pesquisa intitulado **“ESTUDO DO EFEITO DO GEL DENTAL INCORPORADO COM NANOPARTÍCULAS DO ÓLEO ESSENCIAL DE LIPPIA MICROPHYLLA NA DOENÇA PERIODONTAL EXPERIMENTAL E NA DOR OROFACIAL”**, sob coordenação do **Prof. Dr. LUCINDO JOSÉ QUINTANS JÚNIOR** (protocolo **CEPA 57/2011**) foi aprovado pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal de Sergipe, em reunião realizada dia 08/09/2011.

São Cristóvão, 09 de setembro de 2011.

Prof.^a. Dr.^a. Flávia Teixeira Silva
Presidente do CEPA/UFS

ANEXO B – Trabalhos apresentados em congressos relacionados ao tema da dissertação

Resumo apresentado no 43rd International Symposium on essential Oils (ISEO 2012)

43rd International Symposium on Essential Oils (ISEO2012) Posters | 103

P 119. Phytochemical screening and antinociceptive activity of *Lippia microphylla* CHAM. (Verbenaceae) leaf essential oil in rodents

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Keywords: *Lippia microphylla*; Monoterpenes; Camphor; (E)-Caryophyllene; Pain

Lippia microphylla Cham. (Verbenaceae) is a medicinal plant known in Brazilian Northeast as 'alecrim-de-tabuleiro' and it is a plant used popularly to treat heart disease, inflammatory and painful disorders. However, there is little information about *L. microphylla*'s biological properties. Thus, we investigated the antinociceptive effect of the essential oil obtained from the leaves of *L. microphylla* (LEO) in mice. LEO was obtained by hydrodistillation in a Clevenger-type apparatus using 1200 g of dried leaves. The identification of the components was made through comparison of substance mass spectrum with the database of the GC-MS, literature and retention index. Male mice (28-30g) were pretreated with LEO (25, 50 or 100 mg/kg, orally route, *per os*, p.o.), morphine (5 mg/kg, i.p.) or vehicle (distilled water + Tween 80 0.2%), before acetic acid- (0.85%) (writhing reflex) or formalin (20 µl of 1%)-induced nociception tests. The motor coordination was also evaluated using Rota rod (8 rpm, 180 s). Experimental protocols and procedures were approved by the Universidade Federal de Sergipe Animal Care and Use Committee (CEPA/UFS # 26/09). The obtained data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. The phytochemical analysis of LEO demonstrated the presence of camphor (28.698%), (E)-caryophyllene (11.47%), bicyclogermacrene (10.22%), camphene (9.45%) and borneol (7.64) and as the main compounds. LEO pre-treatments inhibited writhing ($p < 0.001$) in the acetic acid test, 25 (17.0 ± 1.5), 50 (15.5 ± 1.9) or 100 (10.1 ± 1.8) mg/Kg, respectively, when compared to control group (28.5 ± 2.9). In the formalin test first phase only the doses of 25 (42.2 ± 5.9), 50 (41.3 ± 4.9) or 100 (35.2 ± 3.4) mg/Kg had promoted a reduction in the time spend licking the paw with $p < 0.01$ or $p < 0.001$, when compared to control group (64.7 ± 4.3). In addition, in the second phase all doses reduce the time ($p < 0.001$) at doses 25 (72.6 ± 8.6), 50 (41.8 ± 5.0) or 100 (27.2 ± 4.1) mg/Kg, respectively, when compared to control group (136.5 ± 8.9). Such results were unlikely to be provoked by motor abnormality (data not shown). Our results suggest that *L. microphylla* leaf essential oil modulates inflammatory and central nociception and might represent important tool for management and/or treatment of painful conditions.

Acknowledgments: Financial Support by FAPITEC/SE/Brazil, CNPQ/Brazil, CAPES/Brazil.

5 to 8 September 2012, Lisbon, Portugal

Certificado de Apresentação no Experimental Biology – 2013, Boston, MA, USA. *Os resumos foram publicados num número especial do The FASEB Journal.*



EB 2013
Experimental Biology
BOSTON

April 20-24, 2013
Boston Convention & Exhibition Center

CERTIFICATE OF ATTENDANCE

This certifies that the following person participated and attended the Experimental Biology (EB) Meeting at the Boston Convention & Exhibition Center in Boston, MA over the dates of April 20 - 24, 2013.

Pollyana de Souza Siqueira Lima
Name

UEFS
Affiliation

Pharmaceutical Sciences: Formulation and Delivery / A179
Session Title/Board Number

Monday April 22, 2013 / 12:30PM- 2:30PM
Date of Session/Time of Session


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Basic & Clinical Pharmacology & Toxicology

manuscript: 4034

beta-cyclodextrin complex containing Lippia grata leaf essential oil
reduces orofacial nociception in mice - evidence of possible involvement
of descending inhibitory pain modulation pathway

Date submitted: 2013-08-30

Decision date: 2013-09-03

Decision: Accept

Decision letter:

Dear Professor

It is a pleasure to inform you that the above manuscript is acceptable for
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Decision and reviewer reports

ANEXO D – Pedido de Patente junto ao INPI

<Uso exclusivo do INPI>

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Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. **Depositante (71):**

1.1 Nome: UNIVERSIDADE FEDERAL DE SERGIPE
1.2 Qualificação: INSTITUIÇÃO PÚBLICA DE ENSINO
1.3 CNPJ/CPF: 13031547000104
1.4 Endereço Completo: AV. MARECHAL RONDON, S/N, CONJ. ROSA ELZE, SÃO CRISTÓVÃO-SE
1.5 CEP: 49100000
1.6 Telefone: 79 21056865 1.7 Fax:
1.8 E-mail: reitor@ufs.br

continua em folha anexa

2. **Natureza:** Invenção Modelo de Utilidade Certificado de Adição

3. **Título da Invenção ou Modelo de Utilidade (54):**
COMPLEXOS DE INCLUSÃO CONTENDO ÓLEO ESSENCIAL DE LIPPIA GRATA E B-CICLODEXTRINA PARA O TRATAMENTO DE DOR E INFLAMAÇÃO E MÉTODO DE OBTENÇÃO DOS MESMOS.

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4. **Pedido de Divisão: do pedido N°** **Data de Depósito:**

5. **Prioridade:** Interna (66) Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

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