UNIVERSIDADE REGIONAL DO CARIRI – URCA
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE – CCBS
PROGRAMA DE PÓS-GRADUAÇÃO EM QUÍMICA BIOLÓGICA – PPQB

ROGER HENRIQUE SOUSA DA COSTA

ATIVIDADE ANTIBACTERIANA E ANTINOCICEPTIVA DO ESTRAGOL E DO COMPLEXO DE INCLUSÃO ESTRAGOL / β-CICLODEXTRINA

Crato – CE
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Tese apresentada ao Programa de Pós-Graduação em Química Biológica, da Universidade Regional do Cariri – URCA, como requisito para a obtenção do Título de Doutor em Química Biológica.

Orientador: Prof. Dr. Irwin Rose Alencar de Menezes

Coorientadora: Dra. Anita Oliveira Brito Pereira Bezerra Martins

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Tese defendida e aprovada em: 05 de outubro de 2022

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Dedico este trabalho a Deus, a minha esposa Rachel, a meus filhos Victor e Gustavo, aos meus pais e a todas as pessoas que cooperaram e contribuíram para o êxito desse nosso trabalho.

Primeiramente a Deus, por me conceder força, saúde e sabedoria para conquistar meus sonhos, mesmo nos momentos mais difíceis.

À minha família, por toda a segurança e acolhimento durante essa jornada.

Em especial, agradeço ao meu orientador Prof. Dr. Irwin Rose Alencar de Menezes, pelos ensinamentos e pela oportunidade de fazer parte de sua equipe tão dedicada.

À minha coorientadora Dra. Anita Oliveira, sempre me apoiando com seus ensinamentos e conselhos.

À minha banca de defesa e qualificação pelas valiosas considerações.

Aos amigos e companheiros de jornada durante esse doutorado: Renata Pessoa, Lucas Yuri, Maria Rayane, Lucas Batista, Isabel Sousa, Tarcísio, Eduardo, Cícero Júnior, Gabriely Silva, Isis Oliveira, e a todos do LFQM, eu agradeço a vocês por toda dedicação e apoio. A Andressa Guedes, Luiz Jardelino e Gyllyandeson Delmondes, pelo companheirismo nesse grande percurso.

A todos os professores do Programa de Pós-graduação em Química Biológica, pela dedicação para com os alunos. Aos professores Henrique Douglas e Francisco da Cunha pelos ensinamentos e disponibilidade a qualquer tempo.

Agradeço a Universidade Regional do Cariri (URCA), por me oferecer essa oportunidade e me acolher nessa caminhada.

A Agência de Defesa Agropecuária do Estado do Ceará – ADAGRI, onde trabalho desde 2009, por apoiar seus servidores e me permitir o fechamento de mais este ciclo de aperfeiçoamento profissional.

Aos Laboratórios: LFQM, LMBM, LFPN, LPPN, pelas parcerias, e as agências financiadoras: Coordenação de Aperfeiçoamento de Pessoal do Nível Superior (CAPES), Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo auxílio financeiro tão importante para o desenvolvimento da nossa pesquisa.

“Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente”. (Roger Von Oech).

RESUMO

Estragol (Es) é um dos constituintes mais representativos dos óleos essenciais de plantas aromáticas do gênero *Croton*, *Artemisia*, *Ocimum*, *Illicium* e *Foeniculum*, apresentando propriedades farmacológicas de interesse, porém limitações devido à baixa solubilidade em água e volatilidade. A complexação de substâncias como o estragol em β-Ciclodextrina (β-CD) se apresenta como alternativa para aumentar a solubilidade, estabilidade e biodisponibilidade. O objetivo deste estudo foi investigar a atividade antibacteriana e antinociceptiva do estragol livre (Es) e complexado em β-CD (Es/CD), buscando desvendar seus possíveis mecanismos de ação. A atividade antibacteriana do Es foi avaliada frente as cepas de bactérias, *Staphylococcus aureus* 1199B e *S. aureus* K2068, que possuem respectivamente o mecanismo de bomba de efluxo NorA e MepA. Para avaliar a reversão da ação da bomba de efluxo foram usadas concentrações crescentes de norfloxacina, ciprofloxacina e do brometo de etídio, um indicador de bomba de efluxo e inibidores padrões de bomba, CCCP e clorpromazina, em presença da concentração subinibitória do Es. Foi observado que o Es modulou positivamente a norfloxacina e o brometo de etídio frente a cepa de *S. aureus* 1199B e que também reduziu a CIM do brometo de etídio frente a cepa de *S. aureus* K2068. Estes resultados sugerem que o Es atua na bomba de efluxo, desta forma, revertendo o perfil de resistência das cepas testadas. Em relação aos ensaios *in vivo*, inicialmente, foi realizado o estudo de toxicidade oral aguda em camundongos Swiss tratados com Es e Es/CD, sendo calculada com dose 625 mg/kg/v.o., não apresentando sinais clínicos de toxicidade, de acordo com os parâmetros avaliados, e nem mortalidade. A dose de 60 mg/kg/v.o. do Es e Es/CD foi selecionada para avaliação dos efeitos sobre sistema nervoso central (SNC), não apresentando alterações sobre os parâmetros avaliados do SNC. Es e Es/CD mostraram efeito antinociceptivo nas doses 60, 30 e 15 mg/kg/ v.o. para os ensaios de: contorções abdominais, teste da formalina, teste da placa quente, teste de retirada de cauda, e ensaio de hipernocicepção mecânica por pressão, propondo o envolvimento das vias farmacofisiológicas: via do óxido nítrico, glutamatérgica, monofosfato de guanosina e vaniloide. Os resultados sugerem que o Es e o Es/CD apresentam um potencial antibacteriano e antinociceptivo promissor, e que o encapsulamento do Es em β-CD aperfeiçoa as suas propriedades farmacológicas.

Palavras-chave: estragol; nanoencapsulamento; infecção bacteriana; dor.

IMPORTÂNCIA/RELEVÂNCIA PARA A SOCIEDADE

A humanidade desde de tempos remotos utiliza produtos naturais através de chás e raizadas, por exemplo, para combater e tratar doenças como infecções e dores. O estragol é um dos constituintes mais encontrado e isolado, principalmente em plantas aromáticas, como por exemplo na canela de cunhã, no estragão, no manjericão e na erva-doce. A pergunta chave desse estudo é: Será que o estragol teria a capacidade de combater infecções e tratar os sintomas da dor? Apresentaremos nessa tese que o monoterpeno estragol é capaz de aumentar o efeito de antibióticos para combater infecções causadas por bactérias resistentes e que ele isolado e associado a molécula de ciclodextrina apresenta atividade analgésica, sem causar danos às pessoas que por ventura viessem a utilizá-los nas doses recomendadas. Considerando que a ciência está sempre em busca de novos medicamentos para tratar as doenças, o estragol isolado e associado a ciclodextrina poderiam ser uma alternativa no desenvolvimento de novos antibacterianos e novos analgésicos.

ABSTRACT

Estragole (Es) is one of the most representative constituents of the essential oils of aromatic plants of the genus *Croton*, *Artemisia*, *Ocimum*, *Illicium* and *Foeniculum*, presenting interesting pharmacological properties, but limitations due to low water solubility and volatility. In this sense, the complexation of substances such as estragole in β-Cyclodextrin (β-CD) is presented as an alternative to increasing solubility, stability and bioavailability. This study aimed to investigate the effect of free estragole (Es) and estragole complexed in β-CD (Es/CD) on antibacterial and antinociceptive actions, to unveil their possible mechanisms of action. The antibacterial activity of Es was evaluated against two strains of bacteria, *Staphylococcus aureus* 1199B and *S. aureus* K2068, carrying NorA and MepA efflux pumps, respectively. To evaluate the reversal of efflux pump action, increasing concentrations of norfloxacin, ciprofloxacin and ethidium bromide, an efflux pump indicator, and standard pump inhibitors, CCCP and chlorpromazine, were used in the presence of a sub-inhibitory concentration of Es. It was observed that Es positively modulated norfloxacin and ethidium bromide against the *S. aureus* strain 1199B and that it also reduced the MIC of ethidium bromide against the *S. aureus* strain K2068. These results suggest that Es interferes with the efflux pump, thus reversing the resistance profile of the tested strains. Regarding the *in vivo* tests, initially, an acute oral toxicity study was carried out in Swiss mice treated with Es and Es/CD, which is calculated at a dose of 625 mg/kg, showing no clinical signs of toxicity, according to the parameters evaluated, and no mortality. The dose of 60 mg/kg of Es and Es/CD was selected to evaluate the effects on the central nervous system (CNS), showing no alterations in the evaluated parameters of the CNS. Es and Es/CD showed an antinociceptive effect at doses of 60, 30 and 15 mg/kg for abdominal writhing test, formalin test, hot plate test, tail-flick test, and mechanical pressure hypernociception test, proposing the involvement of pharmacophysiological pathways: nitric oxide, glutamatergic, guanosine monophosphate and vanilloid pathways. The results suggest that Es and Es/CD have promising antibacterial and antinociceptive potential and demonstrate that encapsulation of Es in β-CD improves its pharmacological properties.

Keywords: Estragole; nanoencapsulation; bacterial infection; pain.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

BHI – Brain Heart Infusion

β-CD –β-Ciclodextrina (β-CD)

CCCP – Carbonylcyanide 3-chlorophenylhydrazone

CIM– Concentração inibitória mínima

CIM/8 – Concentrações subinibitórias

CPZ – Chlorpromazine

DMSO – Dimetilsulfóxido

DSC – Calorimetria Exploratória Diferencial

DTG – Termogravimetria derivada

Es– Estragol

Es/CD – Complexo de inclusão estragol/ β-Ciclodextrina

FTIR – Espectroscopia de infravermelho por transformada de Fourie

GMPc– Monofosfato cíclico de guanosina

IBE – Inibidor de Bomba de efluxo

MDR– Multidrug resistance

MepA – Gene que expressa a proteína de efluxo de mesmo nome

MES – Multidrug efflux systems

MFS– Major facilitator superfamily

MEV – Microscopia Eletrônica de Varredura

MRSA – *Staphylococcus aureus* resistente à meticilina

NorA – Gene que codifica a proteína de efluxo de mesmo nome

OECD – Organização para a Cooperação e Desenvolvimento Econômico

SA – *Staphylococcus aureus*

SDS – *Sodium dodecyl sulfate*

TG – Termogravimetria/Termogravimetria derivada

SUMÁRIO

INTRODUÇÃO GERAL

1.1 OBJETIVOS E QUESTIONAMENTOS

As infecções acompanham os humanos desde tempos imemoriais e se desenvolvem quando bactéria, seja da microbiota ou não penetra no corpo, ou coloniza a pele, ou outros tecidos. Estas infecções provocam grande impacto à saúde pública a nível mundial, e, para solucionar a crise causada pelo surgimento contínuo de patógenos resistentes, existe uma grande necessidade de descoberta e desenvolvimento de novos antimicrobianos. Diante disso a crescente ocorrência de resistência bacteriana estimula as pesquisas de novas abordagens na busca por novos agentes não apenas antibacterianos, mas que possuam uma ação eficaz frente os mecanismos de resistência expressos pela bactéria como as bombas de efluxo (AHMAD; MALAK; ABULREESH, 2021). Clinicamente, as infecções, principalmente as bacterianas, frequentemente causam sinais de dor e inflamação, necessitando muitas vezes, terapias com dois ou mais grupos de substâncias no seu processo terapêutico, entre elas antibióticos e anti-inflamatórios com efeitos analgésicos.

O processo inflamatório se caracteriza pela presença de sinais clássicos como: dor, calor, rubor, edema, perda da função do órgão ou lesão tecidual (KIM; DEUTSCHMAN, 2000; CHOI; CHA; JEON, 2012). Dentre estes sinais, a dor é um sinal decorrente de estímulos nocivos, que tenta restabelecer a homeostase, ativando reações com a finalidade de suprimir a etiologia da dor e, por fim, restaurar ou limitar as lesões que se instalaram (LENT, 2001). Em alguns casos pode auxiliar na sobrevivência de humanos e animais, exercendo função de alerta para o organismo, protegendo-o de uma lesão ou ameaça iminente, mas é uma sensação desagradável, presente desde o nascimento dos indivíduos até a sua morte, associada, principalmente a diversos processos inflamatórios, trauma tecidual e várias doenças, provocando desconforto e alterações comportamentais, sendo necessário o seu tratamento (CALVINO; GRILO, 2006).

Buscar a comprovação dos efeitos farmacológicos de substâncias bioativas é muito importante para o desenvolvimento de novos fármacos, mesmo diante da crescente disponibilidade de medicamentos no mercado farmacêutico, ainda é necessário continuar as pesquisas por agentes que apresentem maior biodisponibilidade, melhor estabilidade e solubilidade, menos efeitos colaterais ou toxicidade e uma favorável relação custo-benefício. Vale ressaltar que aproximadamente metade dos medicamentos atualmente disponíveis no mercado mundial de fármacos é direta ou indiretamente derivada de produtos naturais (ROBINSON; ZHANG, 2011), considerando assim, conhecer a medicina popular e o uso de

plantas medicinais representa uma estratégia de fundamental importância para a descoberta e desenvolvimento de novos agentes terapêuticos.

Dentre os produtos naturais com efeitos farmacológicos os óleos essenciais têm sido amplamente utilizados por serem uma mistura complexa de compostos e possuírem em sua constituição uma variedade de moléculas voláteis como terpenos e terpenóides, componentes aromáticos derivados de fenol e componentes alifáticos passíveis de investigação (CHOUHAN; SHARMA; GULERIA, 2017).

Os terpenos são os constituintes químicos de maior ocorrência nos óleos essenciais, compostos apenas de átomos de carbono, hidrogênio e oxigênio, sua estrutura básica consiste em várias unidades repetidas de isopreno (C5H8), são classificados em grupos de acordo com o número de carbonos que possuem e com o número de unidades isoprênicas formadoras em: hemiterpenos, monoterpenos, sesquiterpenos, diterpenos e politerpenos (MEWALAL et al., 2017).

Dentre estes, os monoterpenos são os constituintes mais representativos dos óleos essenciais, chegando a 90% dos mesmos, além disso, já demonstraram possuírem várias atividades farmacológicas e biológicas (SALAKHUTDINOV; VOLCHO; YAROVAYA, 2017).

Entretanto, esta classe, apresenta baixa solubilidade em água e significativa volatilidade que dificultam sua incorporação nas formulações em sistemas terapêuticos (CHEN, Y. et al., 2015). Compostos bioativos, como é o caso do monoterpeno estragol, nanoencapsulados ou complexados em β-Ciclodextrina (β-CD) podem representar uma alternativa, considerando que essa complexação pode aumentar a estabilidade da substância ativa, isso porque esse encapsulamento protege o fármaco de interações indesejáveis. A utilização de substâncias isoladas ou nanoencapsuladas, cujo estudo possa levar a descoberta de novos fármacos, com menores custos e melhor eficácia terapêutica, pode ser determinante para melhorar a qualidade de vida de pacientes com distúrbios da dor e processos infecciosos.

O desenvolvimento de novos protótipos farmacológicos a partir do estragol (Es) e do complexo de inclusão estragol/β-Ciclodextrina (Es/CD) pode resultar um impacto econômico significativo no meio social, além de possibilitar conhecimentos inéditos em relação ao seu potencial farmacológico. Neste sentido, investigar e validar cientificamente as ações do estragol para o efeito antinociceptivo e contra bactérias resistentes a antibióticos é de suma importância para contribuir para o avanço do estado arte na descoberta e desenvolvimento de compostos com potencial terapêutico. Portanto, este estudo justifica-se por buscar e fornecer informação cientifica da atividade antinociceptiva e antibacteriana do estragol e do complexo de inclusão estragol/β-Ciclodextrina, a partir de estudos *in vitro* e pré-clínicos em modelos animais.

Considerando o potencial promissor dos monoterpenos, sobretudo do estragol, a justificativa desta proposta também considera as dificuldades terapêuticas no tratamento da dor e das comorbidades causadas por infecções bacterianas, buscando descrever uma importante opção para o tratamento dessas condições e promover uma investigação da eficácia do estragol livre e complexado, tendo em vista o desenvolvimento efetivo e seguro de um fármaco para o tratamento dessas condições dolorosas e infecciosas.

1.2 ESTRATÉGIAS DE PESQUISA

A atividade antibacteriana do Es foi avaliada frente a duas cepas de bactérias possuidoras de mecanismo de bomba de efluxo, que são *Staphylococcus aureus* 1199B e *S. aureus* K2068, que possuem respectivamente a bomba de efluxo NorA e MepA. Para isso seguiu-se a metodologia proposta por *Clinical and Laboratory Standards Institute* (CLSI, 2015) com modificações, e para avaliar a reversão da bomba de efluxo foram usadas concentrações subinibitórias (CIM/8) do estragol e dos inibidores padrões de bomba, Carbonylcyanide 3-chlorophenylhydrazone (CCCP) e Clorpromazina e foi verificado se eles conseguiam modular a ação da Norfloxacina, Ciprofloxacina e do brometo de etídio, um indicador de bomba de efluxo.

A determinação da Concentração Inibitória Mínima - CIM foi realizada de acordo com a metodologia de microdiluição em caldo, em placa de 96 poços proposta pela CLSI(2015), com adaptações (TINTINO et al., 2017). Este método é bastante utilizado por ser rápido, eficaz e economicamente viável, fornecendo dados quantitativos confiáveis (NUH et al., 2020).

Outra opção foi utilizar também o método proposto pela CLSI(2015), para avaliar a capacidade do estragol a candidato a um possível inibidor de bomba de efluxo (IBE), analisando a sua capacidade de potencializar as CIMs dos antimicrobianos utilizados. Geralmente nesses ensaios os resultados são expressos pela observação da potencialização da atividade dos antimicrobianos ou como redução da concentração inibitória mínima (CIM) em concentrações específicas (LAMUT et al., 2019). Neste ensaio, a inibição da bomba de efluxo foi testada utilizando uma Concentração Subinibitória (CIM / 8) do estragol e utilizando os Inibidores da Bomba de Efluxo (IBE) para verificar o efeito nas bombas testadas OLIVEIRA-TINTINO et al., 2018).

Os protocolos e técnicas disponíveis para a avaliação da atividade antinociceptiva de moléculas bioativas são diversos, porém deve ser considerado sempre a confiabilidade e os custos que cada um proporciona, além de optar por modelos com o máximo de reprodutibilidade e confiança (FERREIRA et al., 2019). Para tanto, neste estudo foram usados modelos animais

que se assemelham aos distúrbios nociceptivos em humanos. Alguns modelos experimentais foram padronizados e representam métodos importantes na investigação de agentes biológicos para a terapêutica dos distúrbios da dor. A investigação da nocicepção tem posição de destaque nesses modelos, tendo em vista que a dor é um dos principais motivos de incapacidade gerada pela maioria das doenças (LEITE, 2010; ROCHA et al., 2007).

Foram utilizados no presente trabalho protocolos clássicos de nocicepção, como o teste da formalina, contorções abdominais induzidas por ácido acético, teste da placa quente (*hot plate*), teste de retirada de cauda (*tail-flick*), e o ensaio de hipernocicepção mecânica por pressão (*Von Frey*) para avaliação de uma possível atividade antinociceptiva do Es e do Es/β-CD. Sarmento Neto (2015) realizou um levantamento bibliográfico onde foram encontrados trabalhos com ensaios pré-clínicos envolvendo óleos essenciais de 36 espécies vegetais com atividade antinociceptiva, sendo os principais modelos de nocicepção o teste de contorções abdominais, formalina, placa quente e retirada da cauda (tail-flick).

Iniciando pelo teste de formalina, que é um método de avaliação comportamental muito utilizado para investigar o potencial antinociceptivo de diversos agentes, permitindo a avaliação de dois tipos diferentes de dor: uma de primeira fase, chamada de neurogênica relacionada a estimulação química direta de nociceptores e uma de segunda fase, chamada de fase inflamatória associada a liberação de vários mediadores inflamatórios (HUNSKAAR; HOLE, 1987), seguido do ensaio de contorções abdominais induzidas por ácido acético, que é um modelo de nocicepção química, considerado sensível para a avaliação de drogas com atividades analgésicas, sendo um protocolo geral, não seletivo (COUTO et al., 2011).

O ensaio de placa quente é considerado diferente, sendo mais específico para avaliação de agentes analgésicos com efeito sobre o sistema nervoso central, já que apenas drogas analgésicas com efeitos centrais conseguem aumentar o tempo de resposta do animal na placa, considerando que esse modelo utiliza o efeito do aumento da temperatura como estímulo nociceptivo (SILVA et al., 2013), enquanto que, o teste de *Von Frey* é um método de escolha para avaliação da sensibilidade ao estímulo mecânico, que permite avaliar o aumento da sensibilidade do nociceptor a estímulos inócuos (LE BARS; GOZARIU; CADDEN, 2001).

A investigação dos mecanismos e possíveis vias sinalizadoras envolvidas na resposta antinociceptiva do Es e do Es/CD foi realizado verificando-se a participação das seguintes vias e sistemas: opioide, colinérgico, óxido nítrico, α-2 adrenérgico, dopaminérgico, adenosinérgica, glutamatérgica, monofosfato cíclico de guanosina (GMPc), e vanilóide. Estas são as vias mais influentes nos processos álgicos e, em muitas delas havendo substâncias já utilizadas na terapêutica (ABED et al., 2022).

A análise estatística dos resultados foi planejada concomitante ao desenho do estudo e o programa estatístico utilizado foi o GraphPad Prisma 9.0. Os dados foram analisados pelo modelo de análise de variância (ANOVA) de uma ou duas vias conforme o caso. ANOVA é um dos métodos estatísticos mais comuns usados em ensaios pré-clínicos (ABAN; GEORGE, 2015). Assim, para os ensaios foi utilizado o ANOVA de uma ou duas vias seguidas pelo teste de Tukey, sendo os valores expressos como média ± erro padrão da média (E.P.M.) e diferenças a partir de $p<0.05$ sendo consideradas significativas.

1.3 ESTRUTURA DA TESE

A presente tese discorrerá sobre as atividades biológicas do estragol (Es) e do seu complexo de inclusão em β-CD (Es/CD) através de análises microbiológicas para a investigação das atividades antibacterianas e modelos experimentais de nocicepção realizados em animais. Portanto, este trabalho foi estruturado em forma de capítulos, que ao final, possuirá quatro capítulos, que descrevem informações acerca do monoterpeno estragol, principais vantagens do seu nanoencapsulamento em β-ciclodextrina, bem como seus efeitos sobre SNC, toxicidade, atividade antinociceptiva e antibacteriana relacionada à reversão das bombas de efluxo e os respectivos modelos experimentais utilizados para alcançar os resultados. Conforme já descrito na primeira parte deste trabalho, foi descrita as informações sobre os questionamentos que estimularam e motivaram o seu desenvolvimento, apresentando a necessidade do desenvolvimento de pesquisas que busquem novos protótipos farmacológicos com menos riscos e efeitos adversos para humanos e animais.

O capítulo I aborda a fundamentação teórica, embasando a discussão sobre os questionamentos levantados, servindo de alicerce para a elaboração dos capítulos seguintes. Neste também serão abordadas informações pertinentes sobre a importância dos produtos naturais na busca de novos medicamentos, as características dos monoterpenos, em especial do estragol, abordando suas atividades biológicas, características de bactérias resistentes a antibióticos e nocicepção, além de outros tópicos relevantes que contribuem positivamente para o desenvolvimento da pesquisa.

O Capítulo II é um artigo científico, que foi publicado na revista *Archives of Microbiology* (IF=1.8 - 2019/Citescore 3.3) intitulado *Evaluation of antibacterial activity and reversal of the* NorA and MepA *efflux pump of estragole* against *Staphylococcus aureus* bacteria.

O Capítulo III é um manuscrito, intitulado *Action Mechanisms Involved in The Antinociceptive Effect of Estragole and its β-Cyclodextrin Inclusion Complex in Animal*

Models, submetido na revista *Plants* (IF: 4,658). Esse manuscrito aborda a atividade antinociceptiva do estragol e do seu complexo nos modelos clássicos de dor, bem como avaliação dos mecanismos de ação envolvidos nessa atividade.

As considerações finais estão descritas no Capítulo IV, nele serão apresentadas as evidências produzidas durante o desenvolvimento da tese, as conclusões com base nos resultados, a relevância desse trabalho para a ciência e a sociedade, além da possibilidade de futuras investigações envolvendo o objeto em estudo.

CAPÍTULO 1: FUNDAMENTAÇÃO TEÓRICA

As infecções acompanham os humanos desde tempos imemoriais e se desenvolvem quando bactéria, seja da microbiota ou não, vírus, fungo, helmintos ou protozoário penetram no corpo, ou coloniza a pele, ou outros tecidos. Estas infecções, principalmente as bacterianas, frequentemente causam sinais clínicos de dor e inflamação, necessitando muitas vezes, terapias com dois ou mais grupos de substâncias no seu processo terapêutico, entre elas antibióticos e anti-inflamatórios com efeitos analgésicos. Esta abordagem politerapêutica pode ser questionável, especialmente, em decorrências de efeitos sinérgicos ou antagonismo farmacocinéticos ou farmacodinâmicos que podem acarretar significativa modificação da eficácia ou aumento de efeitos colaterais, principalmente em pacientes com funções hepáticas e/ou renais prejudicadas, aumentando o risco de abandono da terapia (ZHANG et al., 2017). Essa tendência negativa resulta em aumento do número de hospitalizações, falha mais frequente do tratamento e persistência de microrganismos resistentes (ASOKKUMAR; RAMACHANDRAN, 2020; CHRISTAKI; MARCOU; TOFARIDES, 2020). Entre as bactérias, *Staphylococcus aureus* (SA) tem grande relevância clínica por ser uma das bactérias mais comuns encontradas em quadros infecciosos.

1.1 *Staphylococcus aureus*

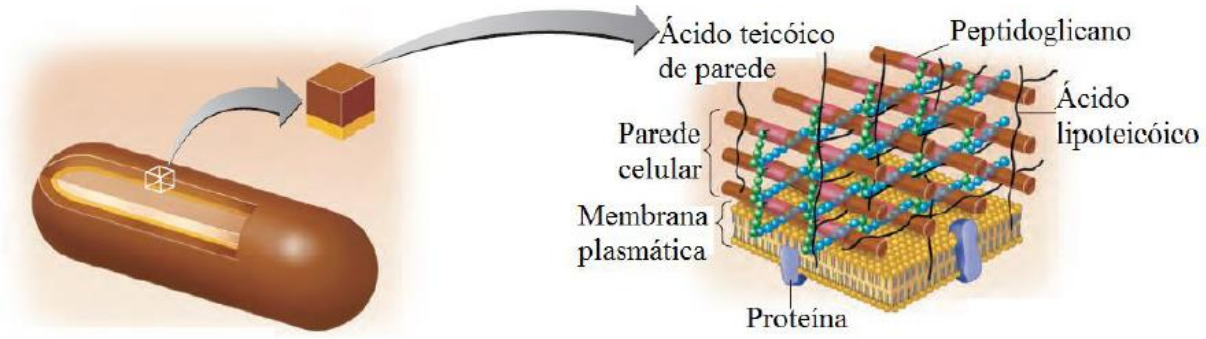
Staphylococcaceae é a família a qual pertence o gênero *Staphylococcus*, o qual possui 33 espécies e oito subespécies, sendo que 17 delas fazem parte da microbiota natural da pele humana e outras regiões anatômicas. Dentre essas espécies, destaca-se *Staphylococcus aureus* (SA) por ser a de maior interesse médico, em particular devido às infecções nosocomiais, e por estar relacionada a diversos subtipos de infecções (CASSETTARI; STRABELLI; MEDEIROS, 2005; ONDUSKO; NOLT, 2018).

As características fenotípicas mais importantes do *S. aureus* incluem: micro-organismo anaeróbico facultativo, crescimento por fermentação ou respiração aeróbica; imóvel e não esporulado; considerados Gram positivos, em formato de cocos formadores de colônias; a fermentação da glicose produz principalmente ácido lático; fermenta manitol; são coagulase e catalase positivos; forma colônia de coloração amarela ou dourada em ágar (KLOOS; MUSSELWHITE, 1975). Além disso, apresentam resistência ao calor e toleram altas concentrações de sal (ONDUSKO; NOLT, 2018; SANTOS et al., 2007).

A parede celular dessa bactéria é uma camada resistente, amorfa, com cerca de 20 a 40 nm de espessura, que protege a membrana celular e o citoplasma, suportando a alta pressão osmótica interna dos estafilococos (PASQUINA-LEMONCHE et al., 2020; SHOCKMAN;

BARREN, 1983), também apresenta diversas moléculas que são antigênicas, capazes de induzir a resposta do sistema imune, com destaque o ácido teicóico, ácido lipoteicóico, proteína A, adesinas e com menos frequência a cápsula (KNOX; WICKEN, 1973; LUTZ et al., 2003).

Figura 1. Parede celular de bactéria Gram-positiva.



Fonte: adaptado de Tortora, Funke e Case (2012).

Quanto ao grau de patogenicidade, ou seja, a capacidade desse agente biológico em causar doenças, o *S. aureus* é responsável pelas infecções mais comuns, incluindo: bacteremia, abcessos cutâneos, síndrome da pele escaldada, síndrome do choque tóxico, intoxicação alimentar, infecções pulmonares como pneumonia e empiema, artrite séptica, infecções de próteses ósseas, meningite, gastroenterite, infecções do trato urinário e peritonite decorrente da diálise peritoneal continua (TONG et al., 2015).

S. aureus é um patógeno de importância crescente, não apenas por ser responsável por causar uma variedade de infecções e manifestações clínicas, mas também devido ao aumento da sua resistência aos antibióticos (TONG et al., 2015; KOURTIS et al., 2019). O tratamento contra essas infecções continua sendo um desafio, devido ao surgimento de cepas resistentes a vários medicamentos, como a cepa de *S. aureus* resistente à meticilina (MRSA) (LEE et al., 2018; TURNER et al., 2019). Dessa forma, muitos estudos têm como foco o desenvolvimento e caracterização de diversos agentes que, além de possuírem ação antibacteriana, possam driblar a resistência de *S. aureus*.

1.2 RESISTÊNCIA BACTERIANA

A resistência aos antibióticos pode ser compreendida, em análise bioquímica, como sendo a incapacidade de um antibiótico de atingir seu alvo microbiano, em uma concentração adequada para inibir a atividade do alvo. O surgimento da resistência aos antibióticos é promovido pela liberação de antibióticos no meio ambiente; seu uso excessivo e indiscriminado

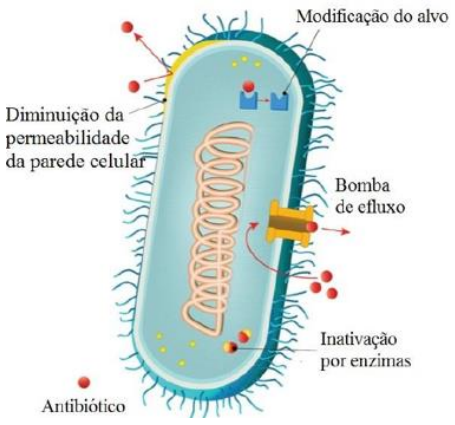
por automedicação, com uso em infecções de origem não bacterianas, como resfriado se outras infecções virais; aplicação de antibióticos sem o devido acompanhamento em animais, propagando a seleção de bactérias resistentes, através de múltiplas rotas de transferência como a água, alimentos e aplicação de adubo no solo (MARSHALL; LEVY, 2011; CHRISTAKI; MARCOU; TOFARIDES, 2020).

A capacidade dos micro-organismos resistirem à ação de diferentes fármacos pode ocorrer de duas formas distintas: resistência intrínseca ou adquirida. A forma intrínseca deve-se às características funcionais ou estruturais que já são inerentes da própria bactéria. Mecanismos intrínsecos são codificados por cromossomos e incluem as enzimas inativadoras de antibióticos, bombas de efluxo inespecíficas e barreira da permeabilidade, envolvendo composição da membrana para bactérias Gram-positivas e Gram-negativas. Os mecanismos intrínsecos conferem uma resistência mais baixa, todavia em hospedeiros com sistema imunológico comprometido, as bactérias podem se tornar patógenos oportunistas (COX; WRIGHT, 2013; PONTES et al., 2018).

Os mecanismos de resistência adquiridos são obtidos por transferência horizontal de genes, em elementos móveis como plasmídeos. Neste grupo, também estão inclusos os mecanismos de inativação por enzimas, que modificam o antibiótico ou seu alvo (VAN HOEK et al., 2011). Como os elementos de transferência de genes podem ser facilmente disseminados entre os microorganismos, estes mecanismos representam um grande risco no avanço rápido e descontrolado da resistência bacteriana (DANTAS; SOMMER, 2012; MARTÍNEZ, 2018).

Nesse contexto, os mecanismos de resistência podem se agrupar nas seguintes categorias gerais: mecanismos de modificação, desvio e proteção do alvo; modificação ou degradação de antibióticos; e prevenção de acesso ao alvo, por efluxo ou por diminuição da permeabilidade da parede celular (BLAIR et al., 2015; PETERSON; KAUR, 2018) (Figura 2).

Figura 2. Mecanismos de resistência bacteriana aos antibióticos



Fonte: adaptado de Nicolsen e Stang (2019).

1.3 BOMBAS DE EFLUXO

Os transportadores integrais de membrana são conhecidos como bombas de efluxo de drogas, sendo considerados um dos mecanismos de resistência bacteriana aos antibióticos e biocidas (ALCALDE-RICO et al., 2020). Foram descritas a primeira vez por McMurry e colaboradores, em cepas de *E. coli* resistentes à tetraciclina (MCMURRY; PETRUCCI; LEVY, 1980).

A ocorrência da exposição da bactéria aos antibióticos e biocidas, é desencadeada um conjunto de reações que levam ao aumento da codificação de um grupo de proteínas transportadoras de membrana responsáveis pelo efluxo, amplamente conhecidas por sistema de efluxo de múltiplas drogas, do inglês *multidrug efflux systems* (MES). Este sistema fornece várias vias de efluxo, funcionando de maneira cooperativa e é responsável pelo fenótipo de resistência a múltiplas drogas, do inglês *multidrug resistance* (MDR) (NICHOLS et al., 2011; DU et al., 2018).

As bombas de efluxo podem ser codificadas geneticamente no cromossomo central bacteriano, como é o caso das bombas de efluxo NorA (família MFS) e MepA (família MATE) ou codificadas no plasmídeo, como nas bombas MsrA (superfamília ABC), QacA e QacB (superfamília MFS) e QacC (família SMR) (HASSANZADEH et al., 2020).

As bombas de efluxo são imprescindíveis para as bactérias tanto na fisiologia como na defesa bacteriana, sendo responsáveis por exportar substratos com estruturas diversas incluindo quinolonas como norfloxacina, ciprofloxacina e ácido nalidíxico; tetraciclina e cloranfenicol; detergentes como Triton X-100 e dodecilsulfato de sódio (sodium dodecylsulfate, SDS); corantes como brometo de etídio e acriflavina; e desinfetantes (biocidas) como cetrimida e triclosan (ALVAREZ-ORTEGA; OLIVARES; MARTÍNEZ, 2013; BLANCO et al., 2016; PU et al., 2016; HASSANZADEH et al., 2020).

1.3.1 BOMBA DE EFLUXO NorA

A NorA é uma bomba de efluxo multidrogas e um dos sistemas de efluxo mais estudados no *S. aureus*. É codificada pelo gene cromossômico NorA, descrito pela primeira vez em um isolado resistente à fluoroquinolona e coletado pela primeira vez em 1986 em um hospital japonês(UBUKATA; ITOH-YAMASHITA; KONNO, 1989), pertence à família *Major facilitator super family* (MFS) de bombas de efluxo, está localizada na membrana citoplasmática e é uma proteína de 388 aminoácidos que compreende 12 segmentos

transmembranares (YOSHIDA et al., 1990) e é capaz de extrudar uma série de compostos estruturalmente e quimicamente diferentes, como por exemplo, corantes como o brometo de etídio e biocidas, além de compostos lipofílicos e monocíclicos como cetrimida, cloreto de benzalcônio, brometo de tetrafenilfosfônio e acriflavina (KAATZ; SEO, 1995; KAATZ; SEO; RUBLE, 1993). Portanto, é bastante reconhecida por causar resistência moderada aos antibióticos da classe de fluoroquinolonas hidrofílicas como norfloxacina e ciprofloxacina(NG; TRUCKSIS; HOOPER, 1994).

Promover a inibição da bomba de efluxo NorA mostra-se uma estratégia interessante para a reversão da resistência bacteriana à antibióticos de uso clínico, com estudos *in vitro* mostrando diversas substâncias capazes de promover este tipo de bloqueio, seja tais compostos, sintéticos ou naturais (MUNIZ et al., 2021), seja extratos ou substâncias isoladas (PEREIRA DA CRUZ et al., 2020; RIBEIRO et al., 2019).

As bombas de efluxo pertencentes à família MFS, como é o caso da NorA, usam a força próton-motriz, ou seja, o gradiente de prótons através da membrana como fonte de energia para o transporte de antibióticos para o meio extracelular, realizando o antiporte droga/H^+ (NG; TRUCKSIS; HOOPER, 1994; PALAZZOTTI et al., 2019). Sendo assim, o mecanismo de um inibidor potencial dessa proteína deverá agir, dentre outras formas, inibindo essa fonte de energia, como é o caso do protonóforo carbonilcianeto m-clorofenil-hidrazona (CCCP), que altera a permeabilidade da membrana ao $H^{+,}$ dissipando a gradiente de prótons e o potencial da membrana, atuando como um inibidor do efluxo de brometo de etídio e norfloxacina mediado por NorA (KAATZ; SEO; RUBLE, 1993; NG; TRUCKSIS; HOOPER, 1994; PALAZZOTTI et al., 2019).

A NorA foi identificada em cepas de *S. aureus* como SA-1199-3, SA-K1904,SA-K2361 e SA-K3092(SCHINDLER; JACINTO; KAATZ, 2013). Porém, as cepas mais estudadas que expressam NorA, consistem nas cepas SA-1199, considerada do tipo selvagem e que expressa NorA de forma induzível e a sua mutante ou derivada SA-1199B que super expressa essa proteína de forma constitutiva (KAATZ; SEO; RUBLE, 1991, 1993).

1.3.2 BOMBA DE EFLUXO MepA

A MepA é uma bomba de efluxo codificada pelo gene cromossômico MepA, sendo considerada o primeiro transportador multidrogas da família MATE a ser descrito em *S. aureus*, conferindo o seu fenótipo de resistência a cepa SA-K2068 (KAATZ; MCALEESE; SEO, 2005; CASTELLANO et al., 2021) É uma proteína que possui 451 aminoácidos e 12 segmentos transmembranares. O gene MepA está integrado no operon mepRAB (KAATZ; MCALEESE;

SEO, 2005). Sua fonte de energia para o transporte de substrato é o gradiente de prótons, através do antiporte exclusivo dos íons sódio (Na^+), tendo em vista que pertencem à família MATE, cujas proteínas atuam por esse mecanismo, porém diferente dessas utilizam também o gradiente de H^+ (HE et al., 2004; KURODA; TSUCHIYA, 2009; MORITA et al., 2000; MIYAUCHI et al., 2017).

A MepA atua em vários substratos, dentre eles as fluoroquinolonas hidrofílicas (ciprofloxacina, norfloxacina; fluoroquinolonas) hidrofóbicas (moxifloxacina, sparfloxacina), glicilciclinas como tigeciclina; compostos de amônio quaternário (cloreto de benzalcônio, dequalinium, tetrafenilfosfônio, cetrimida, pentamidina) e corantes como brometo de etídio. Contudo, é importante ressaltar que de todos esses substratos as fluoroquinolonas, ciprofloxacina e norfloxacina demonstraram ser substratos fracos de MepA(KAATZ; MCALEESE; SEO, 2005; KAATZ; DEMARCO; SEO, 2006; ROCHA et al., 2021).

1.4 NOCICEPÇÃO E DOR

O processo inflamatório é caracterizado por apresentar alguns sinais clássicos como: calor, rubor, dor, edema, perda da função do órgão ou lesão tecidual(CHOI; CHA; JEON, 2012; KIM; DEUTSCHMAN, 2000). Desses a dor é um sinal, consequência de estímulos nocivos, que tenta devolver a homeostase nos tecidos envolvidos, ativando reações com a finalidade de suprimir a etiologia da dor e, por fim, limitar ou recuperar as lesões e agravos(LENT, 2001). Entretanto, a nocicepção é um processo sensorial estimulado por meio da sensibilização e ativação de receptores, chamados nociceptores(PATEL, 2010; TEIXEIRA, 2009).

Os animais utilizados na pesquisa científica não apresentam a capacidade de expressar verbalmente a dor, portanto, nesses modelos não são avaliadas a percepção da dor, mas sim a percepção de nocicepção, pois as definições de dor e analgesia são atribuídas apenas para humanos. Animais, quando submetidos ao estímulo nociceptivo, desenvolvem respostas comportamentais, motoras e fisiológicas, sendo estas usadas na avaliação da resposta nociceptiva (LAPA, 2008). O que ocorre de fato é uma manifestação comportamental, com poucas diferenças, frente a estímulos dolorosos mecânicos, térmicos ou químicos em relação ao limiar de dor (LUNA, 2006).

Nocicepção (do latim, nocere, “doer”) se refere ao processo sensorial estimulado. Entretanto, a dor faz referência à percepção de uma sensação ou sentimento possível de ser descrito como dor, que pode variar entre dolorosa, irritativa, persistente, pulsátil ou intolerável. Portanto, nocicepção e dor são distintas em aspectos, de tal forma que uma pessoa com lesões teciduais pode ou não apresentar o comportamento de dor, enquanto, a nocicepção pode

desencadear a dor, aparente ou não, como, por exemplo: um indivíduo pode ter uma sensação de dor sem efeito nociceptivo evidente (PATEL, 2010). As lesões teciduais ou neuronais sensibilizam os nociceptores, promovendo um mecanismo nociceptivo periférico ou central (ROCHA et al., 2007).

A sensação de dor pode ser considerada subjetiva, se iniciando após uma experiência emocionalmente desagradável, e considerando o nível de intensidade dos componentes da dor, esses sofrem influência das características relacionadas ao estímulo (CALVINO; GRILO, 2006). Quanto a sua classificação, pode ser definida a partir do tipo de lesão e mediadores envolvidos, como: nociceptiva (estimulação excessiva dos nociceptores); neurogênica (lesão do tecido neuronal); neuropática (disfunção de um nervo); e psicogênica (relacionada a fatores psicológicos). Com relação ao tipo, ela pode ser aguda, por estar associada à ativação local de nociceptores a partir de uma lesão tecidual, podendo desaparecer de forma rápida, enquanto a dor crônica é consequência de uma lesão ou doença que persiste e ultrapassa o tempo de recuperação do organismo, ou seja, é de longa duração, e pode levar ao desenvolvimento de sofrimento e incapacidade (MILLAN, 2002).

A dor pode ser considerada um problema mundial de saúde, que só tem aumentado ao longo do tempo, em razão das dificuldades enfrentadas no tratamento, como restrição no número de profissionais, recursos inadequados, baixo conhecimento e falta de prioridade dos governos. Outrora, a dor era compreendida como um componente inevitável da vida, que os seres humanos só podiam influenciar parcialmente conforme a sua etiologia considerada possivelmente sobrenatural, e que somente muito tempo depois, se desenvolveu um conceito fisiológico sobre o controle da dor. Pesquisas, já realizadas, contribuíram para esclarecer os possíveis mecanismos da dor e facilitar o desenvolvimento de novas estratégias para o tratamento, como por exemplo, os fármacos opioides são bastante úteis no tratamento da dor aguda, neuropática e oncológica (WHITE; STEIN, 2010).

Dentre as várias estratégias para o tratamento da dor, visando minimizar as sensações desagradáveis por ela provocadas, além dos analgésicos opioides, as principais classes de fármacos utilizadas são os anti-inflamatórios não-esteroidais (AINES) e os corticosteroides, porém, apesar dessas alternativas, o manejo farmacológico da dor traz por muitas vezes, efeitos colaterais indesejáveis, problemática essa que desperta o interesse na descoberta de novas drogas que possam vir a oferecer menos efeitos colaterais, incentivando assim o desenvolvimento de novas pesquisas (DO AMARAL et al., 2007).

Nesse contexto as plantas medicinais, associadas ao uso na medicina popular, tem se destacado em estudos desenvolvidos, a fim de buscar formas diversificadas de terapia para atenuar a dor (ALMEIDA; NAVARRO; BARBOSA-FILHO, 2001). Os diversos constituintes

isolados de produtos naturais fornecem uma variedade de estruturas químicas, com atividade biológica, capazes de servirem como modelos para novos antibióticos (ATANASOV et al., 2021). Em se tratando de produtos naturais extraídos de plantas medicinais, os óleos essenciais se configuram como uma mistura complexa de compostos, sendo os terpenos como os principais constituintes desses óleos essenciais (CHOUHAN; SHARMA; GULERIA, 2017).

1.5 MONOTERPENOS

Os terpenos são hidrocarbonetos classificados de acordo com o número de unidades isoprênicas formadoras em: hemiterpenos (C5, 1 unidade isoprênica), monoterpenos (C10, 2 unidades isoprênicas), sesquiterpenos (C15, 3 unidades isoprênicas), diterpenos (C20, 4 unidades isoprênicas) e politerpenos (C5)n (AHARONI; JONGSMA; BOUWMEESTER, 2005).

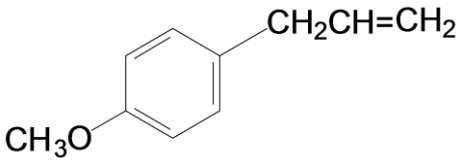
Quimicamente os monoterpenos são caracterizados por possuírem 10 átomos de carbono e duas unidades de isopreno. Apresentam grande variabilidade de hidrocarbonetos, álcoois, aldeídos e outros oxigenados e englobam coletivamente muitos não apenas funcionais, mas isômeros posicionais e geométricos (KHOSRAVI et al., 2018).

São considerados como uma das maiores famílias de produtos naturais e percussores importantes para a produção biotecnológica (OZ et al., 2015; PROBST, 2012). Por sua vez, os monoterpenos são difundidos na natureza, principalmente nas plantas ricas em óleos essenciais, em que representam a classe majoritária na composição destes óleos, constituindo 90% dos mesmos, e possuem várias atividades farmacológicas descritas (QUINTANS et al., 2019), como analgésica, anti-inflamatória, antidepressiva, anticonvulsivante (DA SILVA et al., 2014, 2015), além de antioxidantes, antitumorais, cardioprotetores, neuroprotetores, entre outros (JAKARIA et al., 2018).

1.5.1 ESTRAGOL (4-ALLYLANISOLE)

O monoterpeno estragol (Figura 3), quimicamente identificado como 1- metoxi-alil-benzeno ou 4-metoxialilbenzeno, apresenta fórmula química $C_{10}H_{12}O$ e peso molecular 148,2 g/mol, é capaz de formar misturas azeotrópicas em água e apresenta solubilidade conhecida em álcool e clorofórmio (SMITH et al., 2002).

Figura. 3. Estrutura química do Estragol.



Fonte: adaptado de VINCENZI et al., 2000

O estragol caracteriza-se por ser um éter, possui a nomenclatura de 4-2-(propenil) metoxibenzeno, e possui alguns sinônimos como metil chavicol, p-alilanisol, chavicol metiléter e 4-metoxialilbenzeno (VINCENZI et al., 2000).

É um composto orgânico natural usado como um aditivo, agente aromatizante ou fragrância em uma variedade de produtos alimentícios, de limpeza e cosméticos, bem como um remédio herbal, agente antimicrobiano contra a microflora alimentar tolerante a ácidos, e na produção de óleo de anis sintético (BRISTOL, 2011; PURUSHOTHAMAN et al., 2018).

O estragol já é amplamente utilizado na indústria de alimentos e bebidas como aromatizante e, também nos perfumes, sabões e detergentes. Sendo um constituinte químico importante dos óleos essenciais de muitas plantas aromáticas, tais como *Croton zehntneri* Pax e Hoffm. (Euphorbiaceae), *Artemisia dracunculus* (Asteraceae), *Ocimum basilicum* (Lamiaceae), *Pimpinella anisum* (Apiaceae), *Illicium anisatum* (Illiciaceae) e *Foeniculum vulgare* (Apiaceae)(DE VINCENZI et al., 2000; DA COSTA et al., 2008; SILVA-COMAR et al., 2014; MURÁRIKOVÁ et al., 2017).

Ensaios experimentais *in vivo* e *in vitro* demonstraram que o estragol tem muitos efeitos biológicos, incluindo atividades antioxidantes e antimicrobianas (contra *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* e *Salmonella enterica*) (FRIEDMAN; HENIKA; MANDRELL, 2002; MORAIS et al., 2006), outras atividades farmacológicas são relatadas como ansiolíticas (COSENTINO; NORTE; LAZARINI, 2004), para induzir a contração do músculo esquelético e para relaxar o íleo e músculos lisos vasculares (COELHO-DE-SOUZA et al., 1997; SOARES et al., 2007). Além dessas atividades, o estragol demonstrou possuir atividade anti-inflamatória, que é farmacologicamente potente e é eficaz em doses orais menores do que aquelas consideradas tóxicas (PONTE et al., 2012; RODRIGUES et al., 2016).

O estragol é um fenilpropanóide e, algumas substâncias dessa classe apresentam efeitos anestésicos locais, pois bloqueiam a excitabilidade predominantemente através de efeitos inibitórios diretos na ativação da condutância do canal de Na^+ (RAVENS; WETTWER; HÁLA, 2004; CHO et al., 2008; MOREIRA-LOBO et al., 2010; JOCA et al., 2012), e também bloqueia a excitabilidade do nervo ciático de ratos (LEAL-CARDOSO et al., 2004).

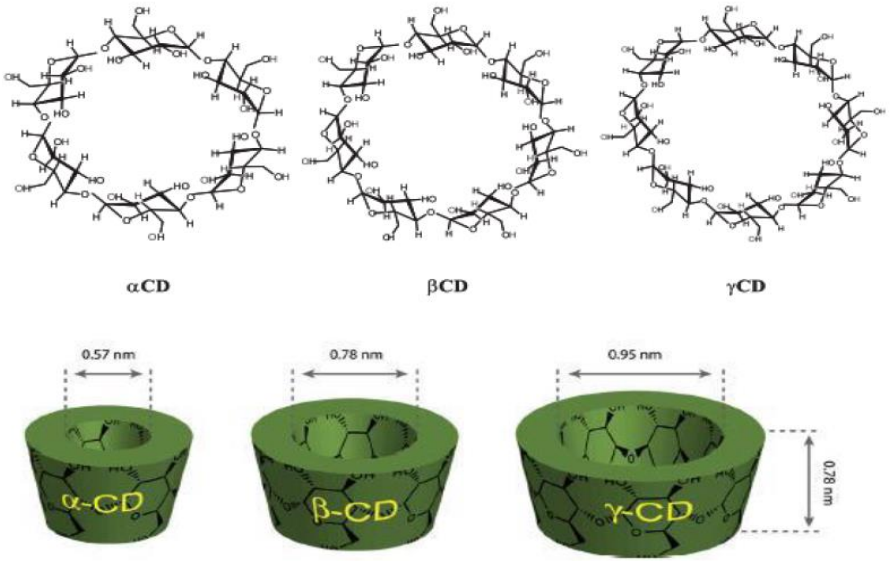
Em relação a toxicidade o estragol é genotóxico e carcinogênico em roedores em dosagens de 300 mg/kg/v.o. por quatro semanas (DRINKWATER et al., 1976; MILLER et al., 1983; NING et al., 2017; ISHII et al., 2017), todavia, Ding et al. (2015) sugerem que o estragol provoque danos ao DNA de células hepáticas apenas em doses maiores (600 e 1000 mg/kg).

Esse monoterpeno apresenta limitações devido à baixa solubilidade em água e volatilidade. Como alternativa esses compostos podem ser complexados em β-Ciclodextrina (β-CD), considerando que esse nanoencapsulamento pode aumentar a estabilidade e a biodisponibilidade da substância ativa e reduzir as limitações (L SANTOS et al., 2017; SITHOLE et al., 2018).

1.6 CICLODEXTRINAS

Ciclodextrinas (CDs) são oligossacarídeos cíclicos, obtidos a partir da degradação enzimática do amido, através da enzima ciclodextrina glicosiltransferase, compostos por unidades de glicose unidas através de ligações α-1,4 (JUN et al., 2007). As mais conhecidas e utilizadas são as naturais, que contêm seis, sete ou oito unidades de glicose, denominadas: α-ciclodextrinas, β-ciclodextrinas e γ-ciclodextrinas (CANNAVÀ et al., 2008; CARNEIRO et al., 2019) que são diferenciadas pelo diâmetro de suas cavidades (CIOBANU; LANDY; FOURMENTIN, 2013) (Figura 4) e outras características como: capacidade de inclusão, pureza, solubilidade, toxicidade e preço (CUNHA-FILHO; SÁ-BARRETO, 2009).

Figura 4 - Estruturas moleculares das ciclodextrinas (α, β e γ).



Fonte: Adaptado de Ciobanu; Landy; Fourmentin, 2013; Leyva et al., 2004

As CDs são substâncias importantes na indústria farmacêutica, sendo amplamente usadas pelas vantagens em aumentar a solubilidade, estabilidade e biodisponibilidade de várias

drogas. Pesquisas apontam que esses oligossacarídeos são facilmente excretados do organismo, o que torna mais eficaz em se tratando da farmacocinética e são encontrados em uma variedade de produtos das linhas alimentícias e farmacêuticas (KURKOV; LOFTSSON, 2013). A α-ciclodextrina é comumente empregada em complexação de hidrocarbonetos alifáticos e gases, a γ- ciclodextrina com moléculas maiores e orgânicas, e a β-ciclodextrina com moléculas menores e aromáticas, incluindo os derivados de terpenos (CHALLA et al., 2005).

As propriedades físico-químicas, que mais se destacam das CDs, são a capacidade de solubilizar-se em meio aquoso e simultaneamente encapsular no interior da sua cavidade moléculas hidrofóbicas (CUNHA-FILHO; SÁ-BARRETO, 2009) melhorando a biodisponibilidade de algumas substâncias (L SANTOS et al., 2017; SITHOLE et al., 2018), o que está associado a solubilidade, dissolução e permeabilidade promovidos de forma indireta por ela ao fármaco complexado, uma vez facilitando a transferência de moléculas hidrofóbicas hóspedes em solução até as membranas celulares lipofílicas facilitando a sua absorção (BRITO; JÚNIOR; SANTOS, 2004).

As β-CDs por serem de fácil obtenção, possuir cavidade com tamanho adequado e preço acessível são as mais utilizadas para complexação de substâncias, com ênfase aos estudos envolvendo atividades farmacológicas, especialmente de terpenos (CHALLA et al., 2005; QUINTANS-JÚNIOR et al., 2013; NASCIMENTO et al., 2014; COSTA et al., 2019).

Para agentes anti-inflamatórios e antimicrobianos, por exemplo, a nanoencapsulação em β-Ciclodextrina pode aumentar a concentração do fármaco na região desejada, além de induzir uma liberação lenta e controlada (LOPES et al., 2015; MARTINS et al., 2020).

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CAPÍTULO 2

Artigo 1: **Evaluation of antibacterial activity and reversal of the NorA and MepA efflux pump of estragole against *Staphylococcus aureus* bacteria**– publicação na revista Archives of Microbiology. Fator de impacto (IF=1,8). Qualis em Ciências Biológicas II – B2.

Archives of Microbiology
https://doi.org/10.1007/s00203-021-02347-x

ORIGINAL PAPER

Evaluation of antibacterial activity and reversal of the NorA and MepA efflux pump of estragole against *Staphylococcus aureus* bacteria

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Received: 14 February 2021 / Revised: 21 April 2021 / Accepted: 22 April 2021

Abstract
The antibacterial activity of the monoterpene estragole was evaluated against two strains of bacteria with an efflux pump mechanism, which are *Staphylococcus aureus* 1199B and *S. aureus* K2068, which have a NorA and MepA pump, respectively. For that, the methodology proposed by CLSI with modifications was followed, and to evaluate the reversal of the efflux pump, subinhibitory concentrations (MIC/8) of estragole and standard pump inhibitors, CCCP and Chlorpromazine were used and it was verified whether they managed to modulate the action of Norfloxacin, Ciprofloxacin and Ethidium Bromide, an indicator of an efflux pump. It was observed that estragole positively modulated norfloxacin and ethidium bromide against the strain of *S. aureus* 1199B and that it also managed to reduce the MIC of ethidium bromide against the strain of *S. aureus* K2068. In the non-clinical acute toxicity tests with estragole, the animals treated with the dose of 625 mg/kg/v.o. showed no clinical signs of toxicity, according to the parameters evaluated. These results are promising, since it places estragole as a possible inhibitor of the efflux pump, thus managing to inhibit this mechanism of action in the strains tested.

Keywords Efflux pump · Terpenes · Bacterial resistance · Estragole

Introduction

The resistance of microorganisms to antibiotics is a particularly important public health problem. The steady increase in diseases that are caused by resistant multi-drug microorganisms has also increased dramatically worldwide. One of the causes that has contributed to this increase is the indiscriminate and wrong use of antibiotics, as well as self-medication, which contributes to the increase of these resistant strains, which leads to a significant increase in mortality rates due to diseases caused by bacteria (WHO 2014; Blaskovich et al. 2017; Cheesman et al. 2017; Laxminarayan 2014; dos Santos et al. 2018).

Staphylococcus aureus is a Gram-positive bacteria, whose size can vary from 0.5 µm to 1.5 µm in diameter, characterized by being a commensal microorganism, which causes several infections, as it has the ability to acquire resistance in front many classes of antibacterial, such as the quinolone classes. It is an immobile bacteria, which has no ability to produce spores, has positive catalase and coagulase and negative oxidase, and can exist commensally with humans, as a colonizer or pathogen (Harris et al.

Communicated by Erko Stackebrandt.

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Published online: 03 May 2021

Evaluation of antibacterial activity and reversal of the NorA and MepA efflux pump of estragole against *Staphylococcus aureus* bacteria

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Abstract

The antibacterial activity of the monoterpene estragole was evaluated against two strains of bacteria with an efflux pump mechanism, which are *Staphylococcus aureus* 1199B and *S. aureus* K2068, which have a NorA and MepA pump, respectively.For that, the methodology proposed by CLSI with modifications was followed, and to evaluate the reversal of the efflux pump, subinhibitory concentrations (MIC / 8) of estragole and standard pump inhibitors, CCCP and Chlorpromazine were used and it was verified whether they managed to modulate the action of Norfloxacin, Ciprofloxacin and Ethidium Bromide, an indicator of an efflux pump. It was observed that estragole positively modulated norfloxacin and ethidium bromide against the strain of *S. aureus* 1199B and that it also managed to reduce the MIC of ethidium bromide

against the strain of *S. aureus* K2068.These results are promising, since it places estragole as a possible inhibitor of the efflux pump, thus managing to inhibit this mechanism of action in the strains tested

Keywords: Efflux pump; Terpenes; Bacterial resistance; Estragole.

1. Introduction

The resistance of microorganisms to antibiotics is a particularly important public health problem. The steady increase in diseases that are caused by resistant multi-drug microorganisms has also increased dramatically worldwide. One of the causes that has contributed to this increase is the indiscriminate and wrong use of antibiotics, as well as self-medication, which contributes to the increase of these resistant strains, which leads to a significant increase in mortality rates due to diseases caused by bacteria (OMS, 2014; BLASKOVICH; BUTLER, 2017; CHEESMAN et al., 2017; LAXMINARAYAN, 2014; BRUSSELAERS; VOGELAERS; BLOT, 2011; DOS SANTOS et al., 2018).

It is a Gram-positive bacteria, whose size can vary from 0.5 µm *Staphylococcus aureus* to 1.5 µm in diameter, characterized by being a commensal microorganism, which causes several infections, as it has the ability to acquire resistance in front many classes of antibacterial, such as the quinolone classes. It is an immobile bacteria, which has no ability to produce spores, has positive catalase and coagulase and negative oxidase, and can exist commensally with humans, as a colonizer or pathogen (HARRIS; FOSTER and RICHARDS, 2002; CHUA et al., 2014; QUINN, 2011; NEMEGHAIRE et al., 2014).

Several resistance mechanisms are attributed to *S. aureus*, including the efflux pump mechanism, which besides being involved in resistance, also contributes to the overexpression of resistance genes, thus contributing to the virulence and formation of biofilms (UHLEMANN et al., 2014; HASSANZADEH et al., 2020). The knowledge about this mechanism, motivates the search for products that are able to inhibit them, known as Efflux Pump Inhibitors (EPI), which may recover the action of antibiotics used today. Plants are a rich source of compounds that modify antibiotic activity. Essential oils are complex natural systems and have several compounds, including terpenes (MATIAS et al., 2016; GIBBONS, 2005; EDRIES, 2007).

Terpenes are hydrocarbons classified according to the number of isoprene forming units, thus they can be hemiterpenes (C5, 1 isoprene unit), monoterpenes (C10, 2 isoprene units), sesquiterpenes (C15, 3 isoprene units), diterpenes (C20, 4 isoprene units) and polyterpenes (C5)n (AHARONI et al., 2005).Monoterpenes are widespread in nature, especially in plants rich in essential oils, in which they represent the majority class in their composition, constituting 90%, have several pharmacological activities described, such as analgesic, anti-inflammatory, antidepressant, anticonvulsant, antimicrobial, among others(SILVA, et al., 2015; SILVA, et al., 2014; NOGUEIRA NETO et al., 2013).

Estragole (1-methoxy-allyl-benzene or 4-methoxyalyl-benzene) is a monoterpene widely used in the food and beverage industry as a flavoring agent and in perfumes, soaps and detergents. It is an important chemical constituent of the essential oils of many aromatic plants (DE VINCENZI et al., 2000; COSTA et al., 2008; SILVA-COMAR et al., 2014). Experimental in vivo and in vitro tests have shown that estragole has many biological effects, including antioxidant and antimicrobial activities (DE MORAIS et al., 2006; FRIEDMAN, HENIKA; MANDRELL, 2002).

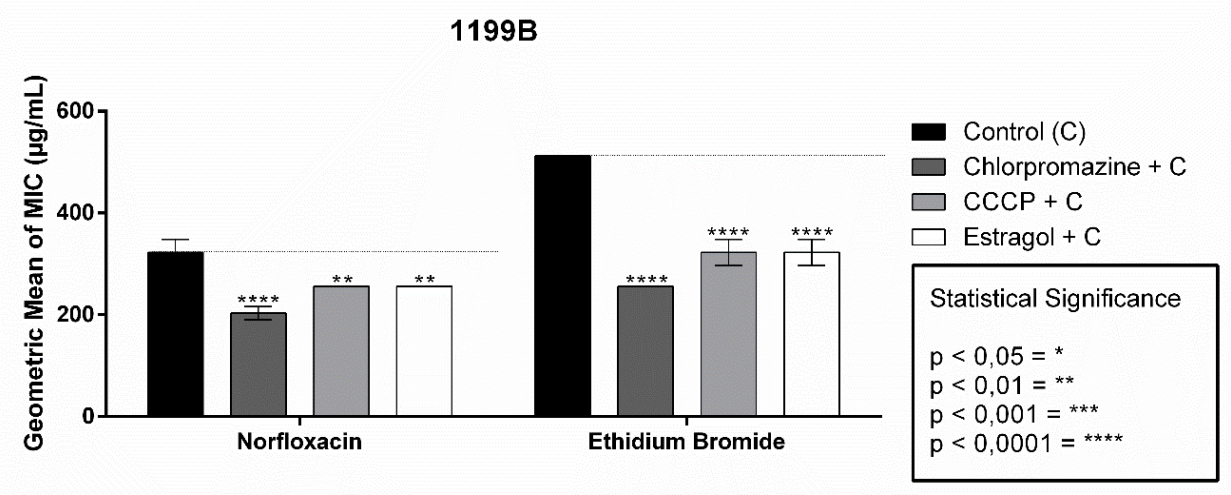
In view of the above, this study aims to evaluate the antibacterial activity and the ability of estragole to reverse the efflux resistance mechanism of *Staphylococcus aureus* strains carrying the NorA and MepA pumps.

2. Results

It was observed that estragole was able to reduce the MIC of the antibiotic Norfloxacin, thus causing synergism, reducing the value of the antibiotic from 322.54 µg/mL to 256 µg/mL. Similar results were found for the pump inhibitors CCCP and Chlorpromazine, thus suggesting that the product may be acting on the bacteria pump mechanism.

Regarding ethidium bromide, a reduction in MIC was also observed when associated with the monoterpene estragole, thus showing the presence of an efflux pump in the bacterium and that the product is acting on it, because the mechanism by which the bacteria expels the substrate to the extracellular medium it is through the efflux pump. Standard inhibitors also reduced the bromide MIC, with the result of estragole, similar to that of chlorpromazine, as can be seen in Figure 1.

Figure 1: Evaluation of estragole activity against the *Staphylococcus aureus* 1199B strain carrying the NorA efflux pump.

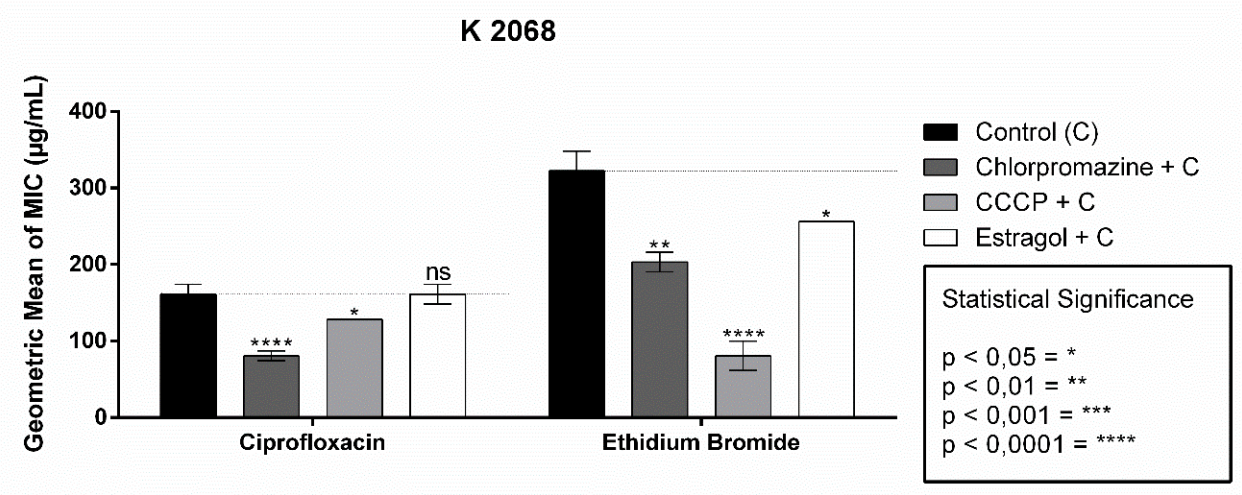


For the strain of the bacterium *S. aureus* K2068, carrying the MepA pump, estragole did not present statistically relevant results when used with the antibiotic Ciprofloxacin, without altering its result.

The efflux pump inhibitors, CCCP and chlorpromazine, were able to reduce the MIC of the antibiotic from 161.27 µg/mL to 128 µg/mL and to 80.63 µg/mL, respectively.

When tested in association with ethidium bromide, the terpene studied in its subinhibitory concentration (MIC/8) was able to reduce the bromide MIC from 322.54 to 256 µg/mL, CCCP and Chlorpromazine also reduced the bromide MIC, thus resulting in synergism. It can then be seen that estragole acts on the efflux pump mechanism of this bacteria, as shown in Figure 2.

Figure 2: Evaluation of estragole activity against the *Staphylococcus aureus* K2068 strain carrying the MepA efflux pump.



In study of Oliveira et al 2016 show by cytotoxicity assay using HepG2, HeLa cell lines and murine peritoneal macrophages that estragole demonstred the dose-dependent inhibition of cell growth with 2280, 2684, and 267 µg /ml, respectively. When analyzing the in vivo acute non-clinical toxicity of estragole, it can be observed that animals treated with the substance in a dose of 2000 mg/kg/p.o. presented deaths and signs of toxicity, according to the parameters evaluated in the table by Malone and Robichaud (Malone and Robichaud 1962). The group of animals treated at a dose of 625 mg/kg/p.o. did not present clinical signs of toxicity, according to the evaluated parameters, nor animal mortality.

3. Discussion

The efflux pump present in bacteria, as in the *Staphylococcus aureus* strain, is a proton pump that depends on driving force, as for example we can mention the NorA efflux pump present in SA1199B. MepA, on the other hand, belongs to the MATE family, which are extrusion pumps for multidrug and toxins (HOLLER et al., 2012; KAATZ; MCALEESE; SEO, 2005; GUILHELMELLI et al., 2013).

Ethidium bromide is a substrate widely used in several experiments in the search for new efflux pump inhibitors and has been used as a model for proving an efflux pump in bacteria (VIVEIROS et al., 2008; DOS SANTOS et al., 2018; OLIVEIRA et al., 2020).

Coelho and collaborators (2016) in their study analyzed the antibacterial activity of some terpenes, including estragole, against the *S. aureus* 1199B bacterium strain, showing as a result that monoterpene reduced the MIC of norfloxacin and ethidium bromide, data that corroborate with the present study. Bezerra et al. (2020)

evaluated the effect of estragole against *S. aureus* RN4220, which also has an efflux pump. Estragole showed synergism when associated with erythromycin, thus reducing its MIC when combined. However, in association with ethidium bromide, it resulted in antagonism, data that do not corroborate our work in dealing with ethidium bromide, as it had its MIC reduced both against *S. aureus* 1199B and against K2068.

Evaluating the activity of eugenol, a monoterpene, against *S. aureus* 1199B, a reduction in MIC of norfloxacin was observed (MUNIZ et al., 2021). Coutinho et al. (2010) evaluated the antibacterial activity of the essential oil of *C. zehntreri*, which contains estragole in its composition, and it was seen that it can modulate the action of norfloxacin.

Silveira et al. (2020), evaluated the activity of thymol and carvacrol, both monoterpernes as well as estragole, in the reversal of the efflux pump mechanism present in *S. aureus* IS-58. Thymol reduced the MIC of tetracycline but had no effect on ethidium bromide and carvacrol resulted in antagonism when associated with the antibiotic and also did not cause changes when associated with ethidium bromide.

Some studies have shown that products of natural origin, such as plants, essential oils and substances isolated from them, have the ability to inhibit the efflux pump mechanism. Due to the lipophilicity of the monoterpenes they preferentially move to the bacterial membrane, thus interacting with polysaccharides, phospholipids and fatty acids, which can alter the membrane's permeability (LIMAVERDE et al., 2017).

Several authors have evaluated the toxicity of estragole in different models. Villarini et al. (2014) evaluated the toxicity of estragole in the human hepatoma cell line HepG2, in addition to in vitro cytotoxic assays, genotoxic activities, among others, it was observed that there was no induction of DNA damage or cellular apoptosis in experimental conditions. In another study, the monoterpernoid estragole was isolated from the essential oil of *Croton zehntneri* (Euphorbiaceae) and its antimicrobial and cytotoxic effect was analyzed, where estragole showed toxicity in the model of *Artemia saline* with LC50 values of 4.54 µg.mL−1 and LC90 of 8.47 µg.mL−1 (Andrade et al. 2015). HepaRG cells, HepG2, primary rat hepatocytes and CHO cells were exposed to estragole at a concentration of 50 µM and DNA adduct formation was quantified after exposure and after a repair period, where an inefficient DNA repair was observed in HepaRG cells and primary hepatocytes from rats (Yang et al. 2020).

3. Materials and methods

3.1 Materials

The monoterpene used in the work was estragole. Chlorpromazine (CPZ), carbonyl-m-chlorophenyl hydrazone cyanide (CCCP) and ethidium bromide were obtained from Sigma Aldrich Co. Ltd.

The antibiotics (Ciprofloxacin and Norfloxacin) and estragole were dissolved in dimethylsulfoxide (DMSO) and then in sterile water. CPZ and ethidium bromide solutions were dissolved in distilled and sterile water, kept protected from light. The CCCP was dissolved in a 1:1 methanol/water solution. The final concentration of all compounds was 1024 µg/mL.

The culture media used in the work were Brain Heart Infusion (BHI)-Agar (BHI, Acumedia Manufacturers Inc.), prepared according to the manufacturer and Brain Heart Infusion (BHI)-broth (BHI, Acumedia Manufacturers Inc.) prepared at a concentration of 10%.

3.2 Bacterial Strains

The strains of *S. aureus* used were SA1199B (overexpressed NorA) and SAK2068 (which expresses MepA). The strains were provided by the Prof. S. Gibbons (University of London), being kept on blood agar (Laboratórios Difco Ltda., Brazil) and, before the experiments, they were grown for 24 h at 37 °C in solid Brain Heart Infusion (BHI)-Agar (BHI, Acumedia Manufacturers Inc.).

Determination of Minimum Inhibitory Concentration (MIC).

To determine the Minimum Inhibitory Concentration (MIC), the CLSI methodology (2015) with modifications was used.

Evaluation of NorA and MepA efflux pump inhibition.

The inhibition of the Efflux Pump was tested using a Sub-inhibitory Concentration (CIM/8) of the estragole and using the Effluent Pump Inhibitors (EPI) to verify the effect on the tested pumps, following the methodology proposed by OLIVEIRA-TINTINO et al., 2018.

3.3 Acute non-clinical toxicity of estragole

Acute toxicity studies were performed with Swiss mice, described by OECD 425 (Guideline 2001) with some modifications. The animals were randomly divided in

to groups (*n* = 3) subjected to fasting (8–10 h) of solids and free access to water. The control group received H2O (0.1 mL/10 g/v.o.) and the groups were treated with estragole, at doses of 2000 mg/kg/v.o. and 625 mg/kg/v.o. The animals were observed at 30, 60, 120, 180 and 240 min after treatment and every 24 h for 14 days for clinical signs of toxicity or mortality (Malone & Robichaud 1962).

3.4 Statistical analysis

The data were analyzed using a two-way ANOVA test, using the geometric mean of the triplicates as the central data and the Standard Deviation, using the statistical program GraphPad Prism 5.0. Then, a post hoc Bonferroni test was performed (where $p < 0.05$ and $p < 0.0001$ are considered significant and $p > 0.05$ is not significant).

4. Conclusion

Estragole was able to synergistically modulate the action of norfloxacin and ethidium bromide against the bacterial strain *Staphylococcus aureus* 1199B, carrying a NorA pump, and to reduce the MIC of ethidium bromide against *S. aureus* K2068, carrying an efflux pump MepA, thus behaving as a possible efflux pump inhibitor.

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CAPÍTULO 3 - Manuscrito 1: **Action Mechanisms Involved in The Antinociceptive Effect of Estragole and its *β*-Cyclodextrin Inclusion Complex in Animal Models -** Submetido na revista *Plants* – Fator de impacto: 4,658. Qualis Ciências Biológicas II: A1

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Action Mechanisms Involved in The Antinociceptive Effect of Estragole and its *β*-Cyclodextrin Inclusion Complex in Animal Models

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Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Plants* **2022**, *11*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

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Abstract: (1) Background: Estragole is a monoterpene found in the essential oils of several aromatic plants, which has several pharmacological activities. The aim of this study was to evaluate the antinociceptive effect of estragole (Es) and its β-cyclodextrins inclusion complex (Es/CD). (2) Methods: The effects of Es and Es/CD on the central nervous system (CNS) were evaluated through open field and rota-rod assays, and the antinociceptive effect in formalin models, abdominal writhing induced by acetic acid, hot plate, tail flick test and plantar mechanical hyperalgesia. (3) Results: Es and Es/CD showed no alterations on the CNS evaluated parameters and the results suggested there was an antinociceptive action in the formalin, abdominal writhing, hot plate, tail flick tests and plantar mechanical hyperalgesia, proposing the involvement of the nitric oxide, glutamatergic signaling pathways, cyclic guanosine monophosphate and vanilloid pathways. (4) Conclusion: The results suggest that Es and Es/CD have a promising antinociceptive potential as a possible alternative for the pharmacological treatment of pain, also showing that the encapsulation of Es in β-cyclodextrins probably improves its pharmacological properties, since the complexation process involves much lower amounts of the compound, contributing to better bioavailability and a lower probability of adverse effect development.

Keywords: estragole; monoterpene; nociception; cyclodextrins.

1. Introduction

Pain is a signal resulting from noxious stimuli, which tries to restore homeostasis in the affected tissues, activating reactions with the purpose of suppressing the etiology and, ultimately, limiting or recovering the lesions and disorders [1]. It is also considered an adaptive response aimed to maintain the integrity of the organism in the presence of stimuli that can generate tissue damage [2].

However, nociception is a sensory process stimulated through sensitization and activation of receptors, called nociceptors [3,4]. Pain and nociception are considered different terms; however, pain cannot occur without nociception, a process that involves the detection of stimuli that can be harmful, with consequent defensive and immediate reflex behavior [5]. The search for new drugs as therapeutic alternatives for pain treatment is a continuous one [6]. Most new drugs available on the market originate from natural products or compounds derived from natural products, especially those extracted from plants [7].

Terpenes are considered as one of the largest families of natural products and important precursors for biotechnological production [8,9] and are regarded as one of the largest classes of secondary metabolites [10]. The monoterpenes that are part of the terpene group are widespread in nature, especially in plants rich in essential oils, where they represent the main class regarding the composition of these oils, constituting 90% of them and showing several pharmacological activities [11], such as anti-inflammatory, antioxidant, antitumor, cardioprotective, and neuroprotective effects, among others [12].

Estragole (Es) is one of the most representative monoterpenes of the essential oils of aromatic plants of the genera *Croton, Artemisia, Ocimum, Illicium* and *Foeniculum*, showing antioxidant and antimicrobial pharmacological properties [13], anti-inflammatory activity, being pharmacologically potent and effective in oral doses lower than those considered to be toxic [14,15].

However, estragole, like most terpenoids, has limitations for its technological application due to its high volatility, instability to light, heat, as well as instability in the presence of oxygen, in addition to low aqueous solubility [16]. Therefore, to minimize these limitations, techniques of monoterpene inclusion in cyclodextrin molecules have been used, which is already common in the pharmaceutical industry [17], seeking to improve the bioavailability of these substances through encapsulation [18,19].

The aim of the present study was to assess the antinociceptive activity of estragole and its β-CD complex, as well as to investigate the possible signaling pathways involved in the antinociceptive response in animal models.

2. Results

2.1. Characterization of Inclusion Complexes

2.1.1. Es/CD Infrared Spectroscopy Measurement

Attenuated total reflection infrared spectra (ATR-FTIR) showed common and specific bands of Es/CD, β-cyclodextrin and estragol, shown in Figure 1-A/B/C. This characterization allowed us to obtain important information to confirm the identity of the analyzed substances, as in the identification of functional groups. Based on the analysis of the absorbance spectrum, characteristic bands of the estragole were identified; the mode evidenced at 3001cm^{-1} correspond to the stretching of single bonds between carbon and hydrogen (C-H) that belong to the

aromatic ring and weak bands in the region of 2800-2900cm^{-1} may indicate axial stretching of the CH group bond. In the range of 1600-1400 cm^{-1} are located the vibrational modes of double bond (C=C) of the ring; in the infrared spectrum of the estragole, these vibrations were identified at 1608cm^{-1} and 1506cm^{-1}. The high intensity band observed at 1242 cm^{-1} is a single bond vibration between C-O-C and the wavenumber of 808 cm^{-1} is attributed to an angular deformation of the aromatic ring.

The *β*-cyclodextrin absorption spectrum showed characteristic bands of this substance, and it is possible to observe in the wavenumber 3359 cm^{-1} a typical stretching broad band of the hydroxyl group (O-H); the band evidenced at 2926 cm^{-1} is a stretching of the (C-H). The wavenumber 1642 cm^{-1} is an angular deformation between H-O-H, and in the region of 1260-1000 cm^{-1} are the stretching vibrations of the carbon and hydrogen bond groups; in the spectrum this vibrational mode was observed in the bands at 1154 cm^{-1} (C-O stretching) and at 1032 cm^{-1} (C-O-C stretching).

In the Es/CD complex spectrum, characteristic peaks and a strong similarity with the β-CD spectrum were observed. In particular, the bands 3359, 2926, 1153 and 1029 cm^{-1} were very evident in the formed complex, with emphasis also on the bands 1511 cm^{-1} and 1238 cm^{-1}, characteristic of the estragole, which showed a reduction in intensity when compared to the isolated spectrum (1505 cm^{-1} and 1242 cm^{-1}, respectively), which had a small wavenumber shift, suggesting that these bonds are possibly involved in the formation of these complexes.

2.2. Evaluation of Estragole and Es/CD on the CNS

2.2.1 Rota-rod Test

In the Rota-rod assay, after the treatment with Es and Es/CD at a dose of 60 mg/kg, the animals did not show characteristics of motor discoordination when compared to the control group, as the number of falls and permanence on the bar were similar.

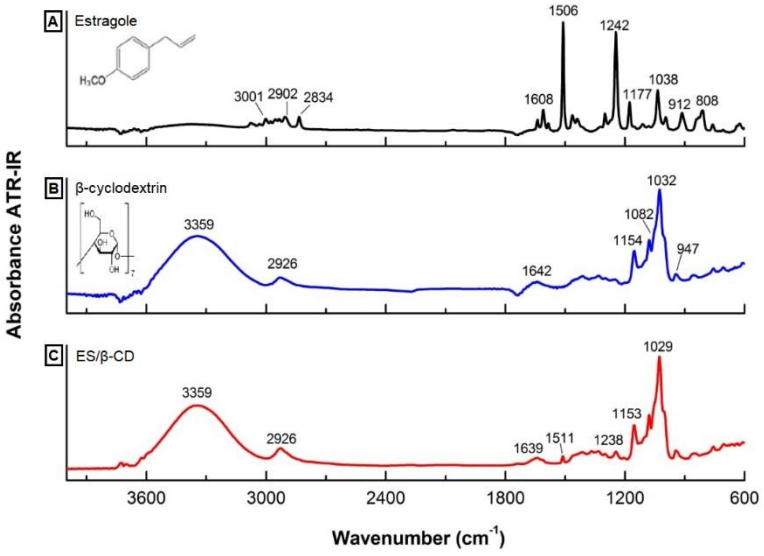

Figure 1. Spectrum in the infrared region: (A) estragole; (B) β-cyclodextrin; (C) Es/CD.

2.2.2. Open Field Test

Treatment with Es and Es/CD at a dose of 60 mg/kg did not reduce the number of behaviors related to rearing (lifting up), grooming (self-cleaning) and number of crossings. In other words, it did not change the

behavioral reactions, since there were no significant differences between the control, estragole and Es/CD groups at the dose of 60 mg/kg.

2.3. Evaluation of Peripheral and Central Antinociceptive Action of Es and Es/CD

2.3.1. Abdominal Writhing Induced by 0.6% Acetic Acid

In the abdominal writhing test, the groups treated with Es and Es/CD at doses of 60, 30 and 15 mg/kg showed a reduction of 89.89; 72.48 and 79.22%; and 82.03; 82.03 and 91.02% respectively, when compared to the negative control (Figure 1A, 1B).

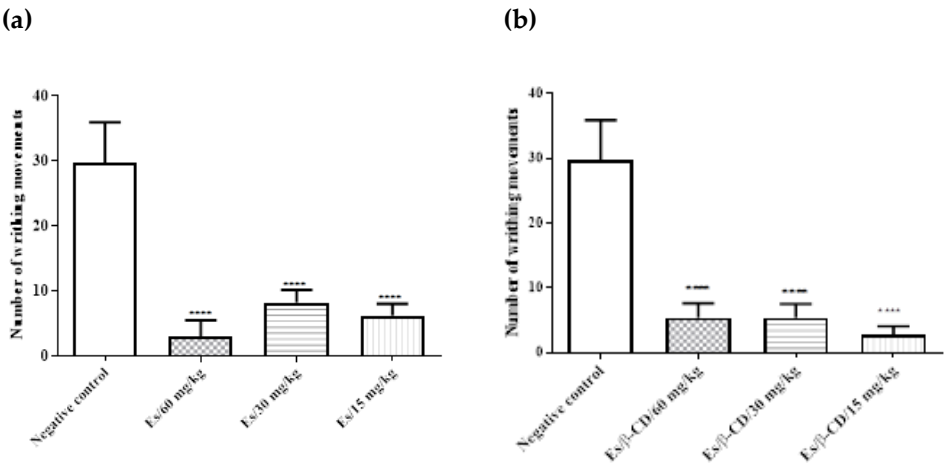

Figure 2. Antinociceptive effect of estragole and Es/CD (60, 30 and 15 mg/kg) in the abdominal writhing test induced by acetic acid. Antinociceptive effect of estragole and Es/β-CD (60, 30 and 15 mg/kg) in the abdominal writhing test induced by acetic acid, for (6=n/group). The values show the arithmetic mean ± S.E.M (Standard Error of the Mean). One-way ANOVA followed by Tukey's test. (****p<0.0001, when compared to the negative control group).

2.3.2 2.5% Formalin Test

In the evaluation of the first phase of the Formalin assay, all the doses treated with the estragole and the Es/CD (60, 30 and 15 mg/kg) showed a significant reduction in paw licking time compared to the control group, at 51.53; 45.46; 53.41%, and 50.72; 45.46 and 54.56%, respectively (Figure 2-A, B). In the evaluation of the second phase, all groups treated with Estragole and Es/CD (60, 30 and 15 mg/kg) showed a reduction in paw licking time of 66.94; 60.42 and 63.75%, and 45.16; 68.53 and 78.49%, respectively compared to the negative control group (Figure 2- C, D).

2.3.3 Hot Plate Test

In the hot plate test, treatments with Es (60, 30 and 15 mg/kg. p.o.) significantly increased the animals' permanence time on the plate by 99.8; 99.79; and 99.8% respectively (Figure 3A). Es/CD (60, 30 and 15 mg/kg) showed an increase of 99.82; 99.78; and 99.77% (Figure 3B), respectively, when compared to the control group in the time interval of 30 to 180min.

2.3.4 Tail Flick Test

In the tail flick test, treatments with Es (60, 30 and 15 mg/kg. p.o.) significantly increased the tail flick time at 99.83; 99.85; and 99.83% (Figure 3A). The treatments with Es/CD (60, 30 and 15 mg/kg. p.o.), promoted an increase of 99.85; 99.86 and 99.84% (Figure 3B), respectively, when compared to the control group in the time interval of 30 to 180min.

2.3.4 Mechanical hypernociception pressure test - Von frey

In the Von Frey assay, treatments with Es (60, 30 and 15 mg/kg), by oral route, significantly reduced the pain threshold at 67.30; 67.92 and 74.29% and with Es/β-CD (60, 30 and 15 mg/kg), at 43.47; 85.68 and 93.81%, respectively. Thus, demonstrating the antinociceptive activity of estragole and Es/β-CD when compared to the control group in the interval and 1, 2, 3, 4 and 24h after the formalin injection.

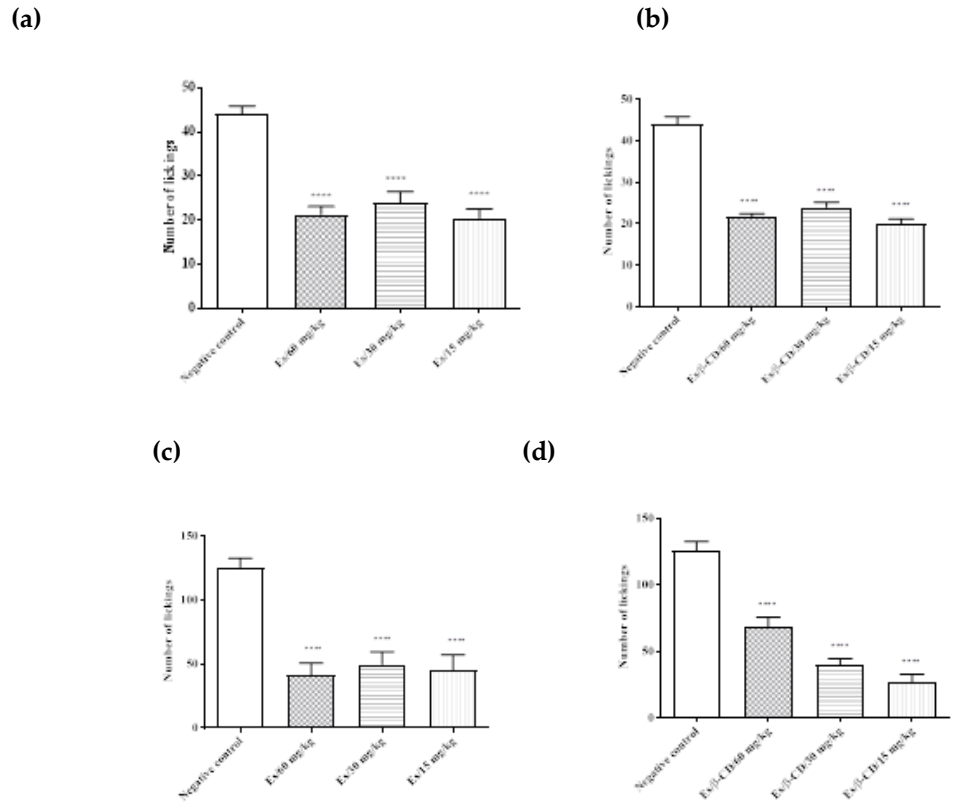

Figure 3. Antinociceptive effect of Es/CD and Es/C (60, 30 and 15 mg/kg) in the formalin test in the neurogenic (A and B) and inflammatory (C and D) phases. Antinociceptive effect of estragole and Es/β-CD (60, 30 and 15 mg/kg) in the Formalin test in the Neurogenic and Inflammatory phases, for (6=n/groups). Values show the mean ± S.E.M (Standard Error of the Mean). One-way ANOVA followed by Tukey's test (*p<0.05; ***p<0.001; ****p<0.0001, when compared to the negative control group). (A – neurogenic phase) (B – inflammatory phase).

(a) **(b)**

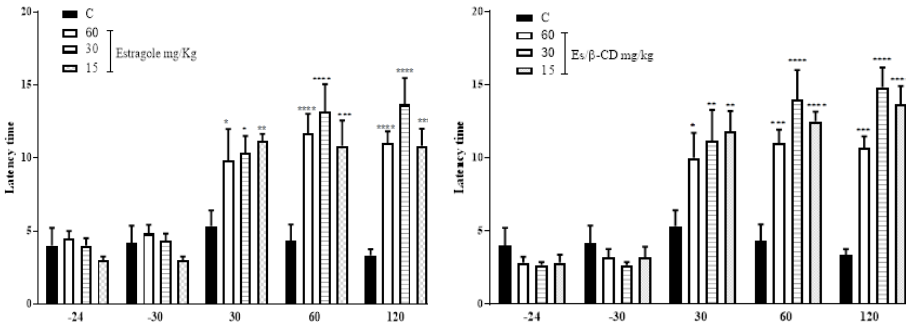

Figure 4. Antinociceptive effect of estragole and Es/CD (60, 30 and 15 mg/kg), in the Hot plate test. Antinociceptive effect of estragole and Es/β-CD in the hot plate test, for (6=n/groups). Values show the mean ± S.E.M (Standard Error of the Mean). Two-way ANOVA followed by Tukey's test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, when compared to the negative control group). (A – Es values 60 30 and 15 mg/kg. p.o.) (B – Es/β-CD values 60, 30 and 15 mg/kg. p.o.).

(a) **(b)**

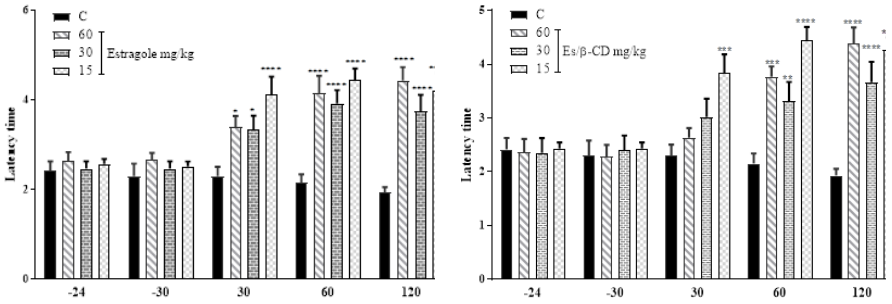

Figure 5. Antinociceptive effect of estragole and Es/CD (60, 30 and 15 mg/kg), in the tail flick test. Antinociceptive effect of estragole and Es/β-CD in the tail flick test, for (6=n/groups). Values show the mean ± S.E.M (Standard Error of the Mean). Two-way ANOVA followed by Tukey's test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, when compared to the negative control group). (A – Es values 60 30 and 15 mg/kg. p.o.) (B – Es/β-CD values 60, 30 and 15 mg/kg. p.o.).

(a) **(b)**

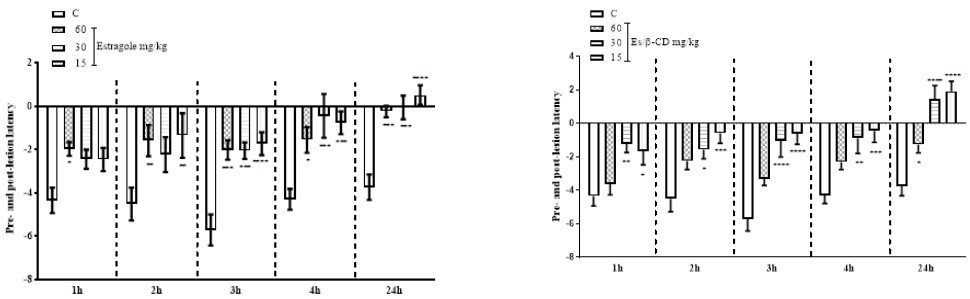

Figure 6. Antinociceptive effect of estragole and Es/CD (60, 30 and 15 mg/kg) in the von Frey assay. Antinociceptive effect of estragole and Es/β-CD in the von Frey test, for (6=n/groups). Values show the mean ± S.E.M (Standard Error of the Mean). Two-way ANOVA followed by Tukey's test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, when compared to the negative control group). (A – Es values 60 30 and 15 mg/kg. p.o.) (B – Es/β-CD 60 30 and 15 mg/kg. p.o.).

2.4. Evaluation of Pain Signaling Pathways and their Interactions in the Antinociceptive Response of Estragole and Es/CD, (Opioid, Cholinergic, Nitric Oxide, A-Adrenergic, Dopaminergic, Adenosinergic, Glutamatergic, Cyclic Guanosine Monophosphate (cGMP), and Vanilloid Pathways).

2.4.1. Action on the Opioid System

Treatments with Es and Es/CD, (v. o.) and morphine (pathway-specific agonist), (s.c.), significantly reduced the paw-licking time of animals after intraplantar formalin injection (0-5min) at 59.45; 68.10 and 97.30% respectively when compared to the negative control group. There was no statistically significant difference when the animals in the Es and Es/CD groups received pre-treatment with naloxone (a specific inhibitor of the opioid pathway), thus suggesting that both Es and Es/CD do not act on the opioid signaling pathway.

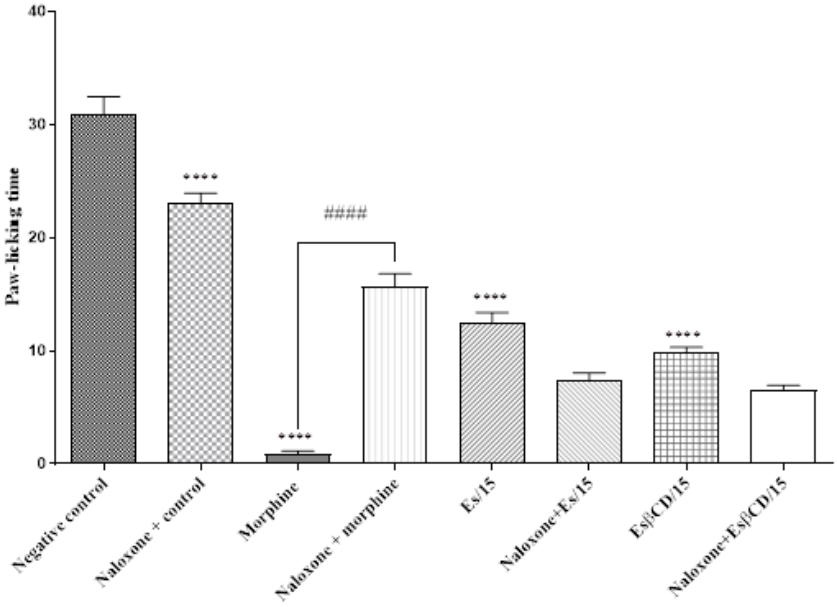

Figure 7. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the opioid signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the opioid signaling pathway, for (6=n/group). Values show the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (****p<0.0001, when compared to the negative control group). (####p<0.0001, when comparing the antagonist + agonist vs. agonist.

2.4.2. Action on the Cholinergic System

Treatments with Es and Es/CD (p.o.) and acetylcholine (pathway-specific agonist) (s.c.) significantly reduced the paw-licking time of animals after intraplantar formalin injection (0-5min) at 38.52; 52.39 and 66.68%, respectively, when compared to the negative control group.

However, there was no statistical difference when the animals in the estragole and Es/CD groups received pre-treatment with atropine (specific inhibitor of the cholinergic pathway), thus suggesting that both estragole and Es/CD are not acting on the cholinergic system signaling pathway.

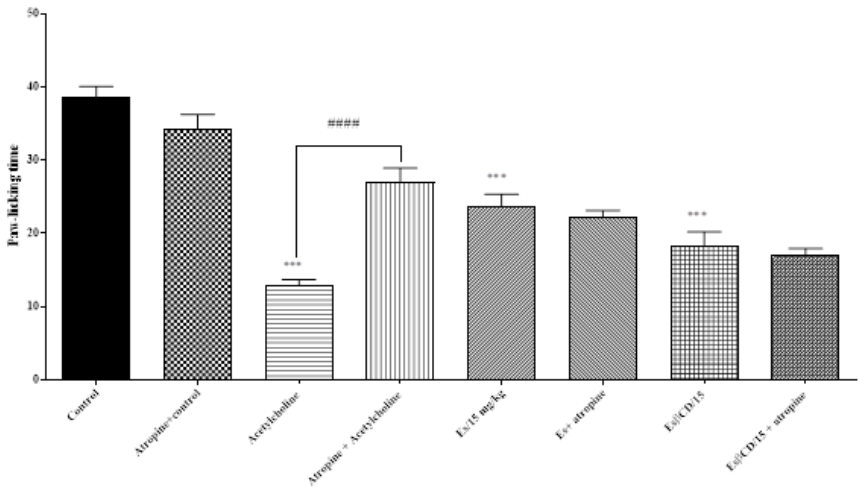

Figure 8. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the cholinergic system signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the signaling pathway of the cholinergic system, for (6=n/group). Values show the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (****$p<0.0001$, when compared to the negative control group). ####$p<0.0001$, when comparing the antagonist + agonist vs. agonist).

2.4.3. Action on the Nitric Oxide Pathway

The treatments with Es and Es/CD (15 mg/kg), by oral route and L-NOARG (pathway agonist), intraperitoneally, significantly reduced the paw-licking time of the animals, after the intraplantar injection of formalin (0 -5min) at 64.97; 67.29 and 63.59% respectively when compared to the negative control group.

However, when evaluating the animals of the Es and Es/CD groups, which received the pre-treatment with L-Arginine (pathway inhibitor), a process of reversal of the antinociceptive effect was observed, suggesting that both Es and Es/CD act on the nitric oxide signaling pathway.

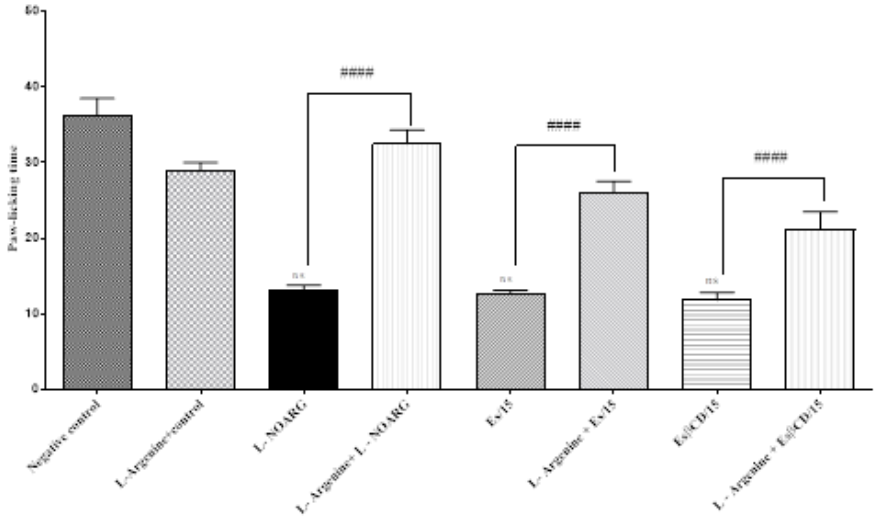

Figure 9: Antinociceptive response of estragole and Es/CD (15 mg/kg) in the nitric oxide signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the nitric oxide signaling pathway, for (6=n/group). Values show the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (****p<0.0001, when compared to the negative control group). (####p<0.0001, when comparing antagonist + agonist vs. agonist; estragole alone vs. atropine + estragole; Es/β-CD alone vs. atropine + Es/β-CD.

2.4.4. α-2 Adrenergic Receptor Activity

Treatments with oral Es and Es/CD and intraperitoneal clonidine (pathway-specific agonist) significantly reduced the animals' paw-licking time after the intraplantar formalin injection (0-5min) at 55.10; 51.68 and 90.69%, respectively, when compared to the negative control group.

However, when evaluated, there was no statistical difference in the animals from the Es and Es/β-CD groups, which received pre-treatment with yohimbine (a specific inhibitor of the opioid pathway). This result suggests that both Es and Es/CD are not acting on the α-2 adrenergic system signaling pathway.

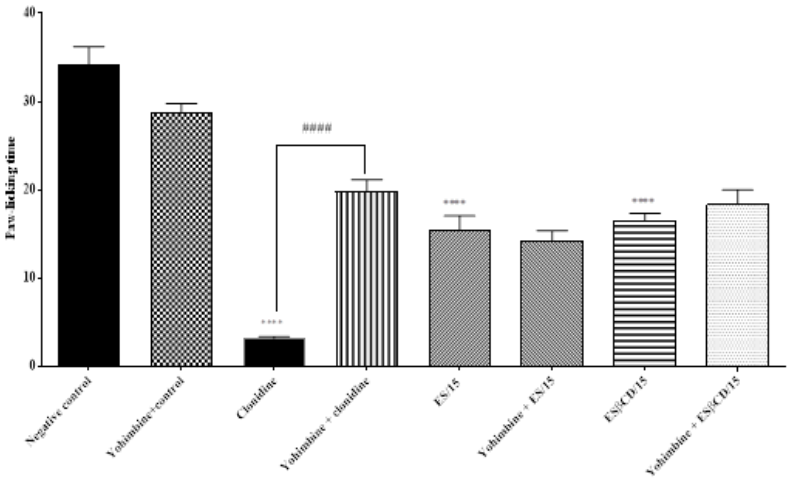

Figure 10. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the α-2 adrenergic system signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg), in the α-2 adrenergic system signaling pathway, for (6=n/group). Values show the mean ± S.E.M

(Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (****p<0.0001, when compared to the negative control group). (####p<0.0001, when comparing antagonist + agonist vs. agonist).

2.4.5. Action on the Dopaminergic System

The treatments with Es and Es/CD (15 mg/kg), through the oral route, significantly reduced the paw-licking time of the animals, after the intraplantar injection of formalin (0-5min) at 66.34 and 48.08% respectively when compared to the negative control group.

However, there was no statistical difference when the animals in the Es and Es/CD groups received pre-treatment with haloperidol (non-specific inhibitor of the pathway), thus suggesting that both estragole and Es/CD do not have any activity in the dopaminergic signaling pathway.

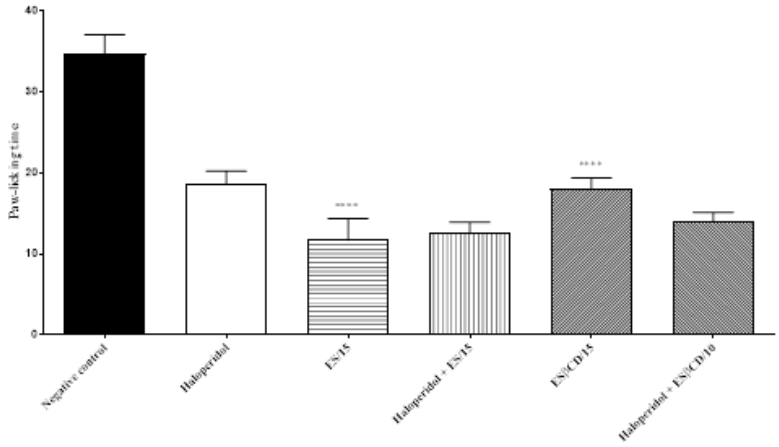

Figure 11. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the dopaminergic signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the dopaminergic system signaling pathway, for (6=n/group). Values show the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (****p<0.0001, when compared to the negative control group). (####p<0.0001, when comparing antagonist + agonist vs. agonist).

2.4.6. Action on the Adenosinergic System

Treatments with Es and Es/CD (15 mg/kg/p.o.) significantly reduced the paw-licking time of animals after the intraplantar formalin injection (0-5min) at 63.03 and 49.47%, respectively, when compared to the negative control group.

There was no statistical difference when the animals in the Es and Es/CD groups received pretreatment with caffeine (non-specific inhibitor of the pathway), thus suggesting that both Es and Es/CD do not have any activity on the adenosinergic signaling pathway.

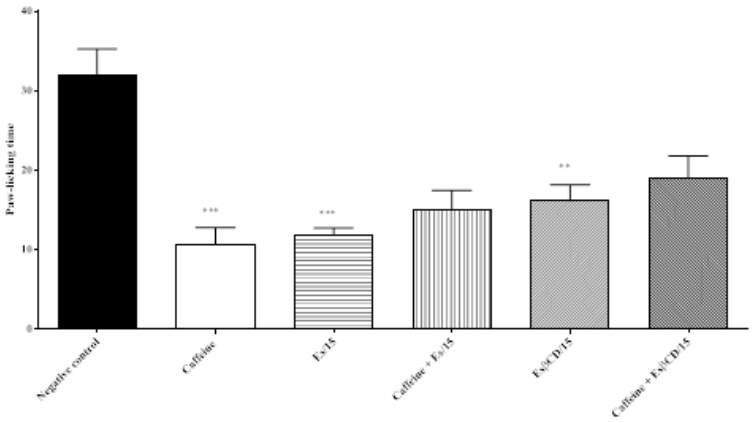

Figure 12. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the adenosinergic signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the adenosinergic signaling pathway, for (6=n/group). Values show the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (****p<0.0001; ***p<0.001, when compared to the negative control group).

2.4.7. Action on the Glutamatergic Signaling Pathway

Treatments with Es and Es/CD (15 mg/kg), through the oral route, significantly reduced the paw-licking time of the animals, after the intraplantar injection of glutamate (an inflammatory agent specific to the glutamatergic pathway), at 57,21 and 46.74%, respectively, when compared to the negative control group. These results demonstrate the significant antinociceptive effect of the compounds on the glutamatergic signaling pathway.

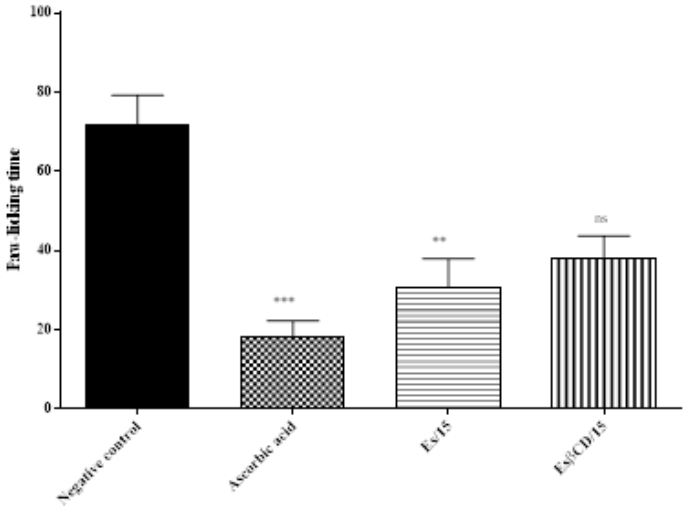

Figure 13. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the glutamatergic signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the glutamatergic signaling pathway, for (6=n/group). Values show as the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (**p<0.01; ***p<0.001; ****p<0.0001, when compared to the negative control group).

2.4.8. Action in the Cyclic Guanosine Monophosphate (cGMP) Signaling Pathway

The treatments with Es and Es/CD (15 mg/kg), through the oral route, and methylene blue, (i.p.), significantly reduced the paw-licking time of the animals, after the intraplantar injection of glutamate (specific inflammatory agent of the glutamatergic pathway), (0-15min) at 24.70 and 97.32%, respectively, when compared to the negative control group.

Based on the results, it can be observed that there was no statistical difference in relation to the animals in the Es and Es/CD groups that received the pre-treatment with methylene blue (pathway-specific inhibitor), thus suggesting that estragole has an effect on the cyclic guanosine monophosphate signaling pathway.

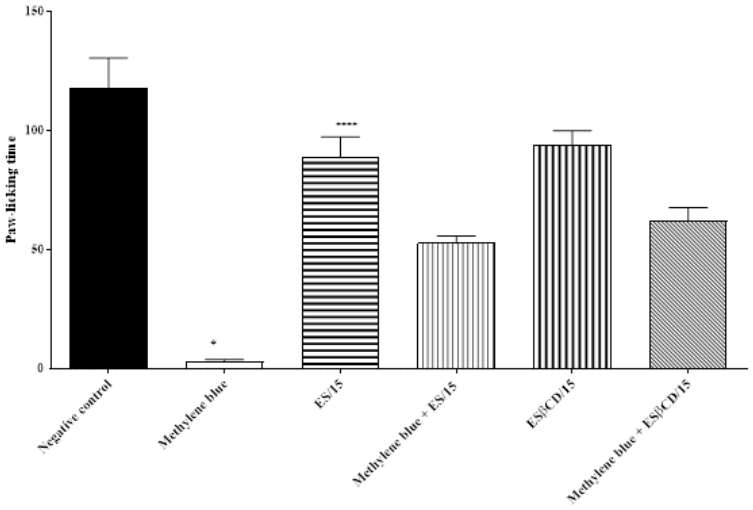

Figure 14. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the cyclic guanosine monophosphate (cGMP) signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the cyclic guanosine monophosphate (cGMP) pathway, for (6=n/group). Values show the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (*$p<0.05$; ****$p<0.0001$, when compared to the negative control group).

2.4.9. Action on the Vanilloid System

Treatments with Es and Es/CD (15 mg/kg), through the oral route and ruthenium red (non-specific TRP antagonist) intraperitoneally, significantly reduced the paw-licking time of the animals after intraplantar capsaicin injection (specific inflammatory agent of the vanilloid pathway), (0-5min) at 50.75; 36.94 and 100%, respectively, when compared to the negative control group. These results demonstrate the significant antinociceptive effect of the tested substances and prove their action on the vanilloid pathway.

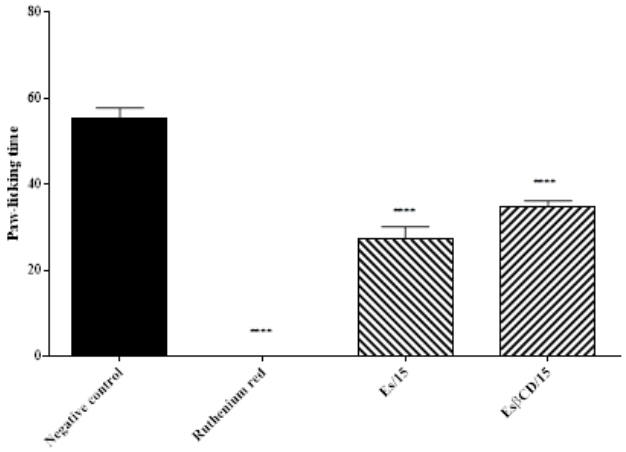

Figure 15. Antinociceptive response of estragole and Es/CD (15 mg/kg) in the vanilloid signaling pathway. Antinociceptive response of estragole and Es/β-CD (15 mg/kg) in the vanilloid signaling pathway, for (6=n/group). Values show the mean ± S.E.M (Standard Error of the Mean). One-way (ANOVA) followed by Tukey's test (***p<0.001; ****p<0.0001, when compared to the negative control group).

3. Discussion

The present study, for the first time, provides a detailed analysis of the antinociceptive effect of Es and Es/CD, as well as the pain signaling pathways involved in pain response in animal models. The physicochemical analysis of Es/CD using the FT-IR technique proved to be effective, allowing the confirmation of the Es/β-CD complexation through the displacement of the bands, which showed common and characteristic peaks of Es, as well as β-CD, with a strong similarity (Figure 1-A/B/C).

In a study carried out by Fonseca et al., (2019) , it was also possible to confirm the evidence of the inclusion complex formation between Es and β-CD through the ATR-FTIR method, clearly demonstrating the encapsulation of Es with β-CD, suggesting that complexation with these excipients is an effective method to improve the stability of substances.

In the models used to evaluate the effect of Es and Es/CD on the central nervous system, the treatments did not promote any changes in the evaluated parameters. Thus, the data presented herein suggest that the nociceptive actions of Es and Es/CD do not seem to be associated with unspecific central actions, as they did not significantly influence behaviors related to motor coordination (rota-rod), the number of crossings, self-cleaning (grooming) and vertical exploration of the mice (open field test). This places Es among the compounds that are favorable to a possible therapeutic application for the treatment of pain.

Regarding the antinociceptive effect, treatments with Es and Es/CD significantly reduced the number of abdominal writhing movements at all tested doses. However, Es/CD showed a more significant action than Es alone in this model. Based on the data presented for Es/CD, it was possible to observe that the lowest doses of the complex showed the best results, which confirms the more robust antinociceptive effect of the complex inclusion when related to isolated monoterpenes. The inclusion of substances in cyclodextrins has been studied, as they act to increase the solubility, permeability and chemical stability of several volatile compounds and products [21].

The formalin test is a classic model for investigating the potential of analgesic substances, being characterized in two phases. The first phase (neurogenic pain) is associated with stimulation of type C and Aδ afferent fibers and promotes the release of excitatory amino acids, substance P, nitric oxide, and others. The second phase (inflammatory pain) involves the release of several pro-inflammatory chemical mediators, which include histamine, serotonin, bradykinin and prostaglandins [22].

Pre-treatment with Es and Es/CD at all doses in the formalin test showed an antinociceptive effect in both phases. In the study by Rodrigues et al., (2016) pre-treatment with estragole significantly reduced the acute and chronic inflammatory process, with this action being attributed to the inhibition of chemical mediators, vascular permeability and leukocyte migration. It is worth noting that the antinociceptive effect of Es and Es/CD in the formalin test corroborates the abdominal writhing test in this study, where a more significant effect was observed with Es/CD at its lowest dose when compared to Es alone.

The types of nociceptive stimuli (electrical, thermal, mechanical, or chemical) that have been used in different pain models are likely to more closely mimic acute clinical pain that affect supraspinal and spinal components [23]. In the tail flick, hot plate, and von Frey tests that act at the level of central receptors, it was possible to observe the action of Es and Es/CD in the reduction of pain perception caused by the stimuli generated in each assay. These results corroborate the effect observed in the writhing and formalin tests, suggesting that Es and Es/CD have centrally-acting antinociceptive activity. Recent previous studies have demonstrated the antinociceptive activity of monoterpenes, including 1,8-cineole [24] and geraniol [25].

From the confirmation of the antinociceptive action of Es and Es/CD, this study focused on investigating the involved signaling pathways using the lowest effective dose of the previous assays: 15 mg/kg of Es and Es/CD. The analyzed pathways were: opioid, cholinergic, nitric oxide, α2 adrenergic, dopaminergic, adenosinergic, glutamatergic, cyclic guanosine monophosphate and vanilloid pathways. Based on the tests, both Es and Es/CD demonstrated activity in the systems: nitric oxide, glutamatergic, guanosine monophosphate and vanilloid pathways.

As for the L-arginine/nitric oxide/cGMP pathway, Es and Es/CD had their antinociceptive effects reversed when associated with the pathway antagonist (L-arginine), demonstrating a possible participation in the antinociceptive effect of both substances. In inflammatory pain, NO is derived from migrating cells, such as neutrophils [26]. In the present study, Es and Es/CD may possibly be acting on the recruitment of neutrophils, and their antinociceptive action can be explained by the modulation of the migration of these cells, causing a reduction in tissue damage and, consequently, in nociception.

Regarding the glutamatergic system, Es and Es/CD showed a good response in reducing paw-licking time when compared to the control and ascorbic acid, suggesting a possible participation of Es and Es/CD compounds in the tested pathway. Glutamate receptors are located in the central and peripheral nervous system, being involved in the sensation and transmission of pain [27]. Ascorbic acid promotes extracellular glutamate accumulation, involving glutamate uptake inhibition; it also increases the activity of NMDA receptors, promoting a decrease in glutamate-stimulated levels [28].

Transient receptor potential (TRP) channels constitute a large family of ion channels capable of being activated in different ways; the TRPV subfamily has six members that can be broadly divided into low

selectivity cation channels and channels that show high selectivity to Ca2+ [29,30]. Ruthenium red is a non-competitive antagonist with the capacity to block transmembrane and mitochondrial Ca2+ sequestration and inhibits capsaicin-mediated excitatory effects on sensory neurons and peripheral nociceptors; this fact justifies its antagonistic effect on capsaicin-induced nociceptive response [31].

As for the vanilloid system, treatments with Es and Es/CD significantly reduced the paw-licking time of the animals after the intraplantar capsaicin injection, demonstrating the significant antinociceptive effect of the tested substances and proving their possible participation in the vanilloid pathway.

4. Materials and Methods

4.1. Assessed Substances and Drugs

The substances were obtained from Sigma-Aldrich (St. Louis, MO, USA). All substances were prepared immediately before oral, intraperitoneal, intraplantar and subcutaneous administration, according to the animal weight (0.1 mL/10 g of body mass) and specific protocols.

4.2. Preparation of Inclusion Complexes in β-cyclodextrins (β-CD)

The β-CD inclusion complex was prepared by the co-evaporation and precipitation method, according to the procedures described by [32], with modifications. Approximately 7g of β-CD were dissolved in 20 mL of distilled water and acetone (3:1 v/v) at 40°C for 30 min. Then, the estragole in hydro-acetone solutions of β-CD was added, with continuous stirring for 1h at 300 rpm at 37°C. After that, the mixture was sonicated for 10 minutes at 4°C to decrease particle size and incubated for 12h at 4°C. Afterwards, the mixture was left to stir on the magnetic stirrer for 36 hours at 35°C at 300 rpm, and after the stirring time, the mixture was left for 24h in the freezer. The samples were lyophilized under vacuum at 40°C for 36h.

4.3. Characterization of the Inclusion Complexes

4.3.1. Es/CD Infrared Spectroscopy Measurement

The chemical characterization of the estragole inclusion complex in β-cyclodextrin (Es/CD), as well as of the β-cyclodextrin (β-CD) and estragole alone was carried out using the infrared spectroscopy technique. The attenuated total reflection Fourier transform infrared (ATR-FTIR) absorbance spectra were obtained using an Agilent spectrometer, model CARY 660 FT-IR, with the results being processed using the software for infrared spectroscopy (OriginPro v.8.5). The ATR - FTIR spectrum was recorded at room temperature, with a spectral resolution of 4 cm^{-1} and performing 32 scans in the wave number region from 4000 cm^{-1} to 600 cm^{-1}.

4.4. Animals

Male and female Swiss mice (*Mus musculus*) weighing between 20-30 g, obtained from the Animal Containment Unit of Universidade Regional do Cariri, were used in the study. The animals were housed with food and water *ad libitum* (Labina, Purina, Brazil) in a room with a controlled temperature of 24 ± 2°C, with a 12-h light/dark cycle. Prior to the experiments, the animals were kept in the Laboratory of Pharmacology and Molecular Chemistry (LFQM) of Universidade Regional do Cariri - URCA - for a period of 24h for acclimatization. The study was carried out in accordance with the recommendations of the National Council for the Control of Animal Experimentation (CONCEA)

and the protocols were approved by the Ethics Committee on Animal Use of the Universidade Regional do Cariri (CEUA N. 358/2019-2).

4.5. In Vivo Assay

To evaluate the antinociceptive effect, the following protocols were performed: abdominal writhing test induced by 0.6% acetic acid, 2.5% formalin test, hot plate test, tail flick test, plantar mechanical hyperalgesia test– Von Frey test. The animals were divided into groups (n=6) and treated with H_2O (0.1 mL/10 g/p.o.), Es and Es/CD (60, 30 and 15 mg/kg/p.o.) in the formalin screening tests and abdominal writhing, hot plate, tail flick and von Frey; for the other tests, to evaluate the pain signaling pathways, the lowest effective dose of Es and Es/CD was used, being it 15 mg/kg/p.o., defined from previous screening protocols. To evaluate the pain signaling involved in the antinociceptive response, the opioid, cholinergic, nitric oxide, α–adrenergic 2, dopaminergic, adenosinergic, glutamatergic, cyclic guanosine monophosphate and vanilloid pathways were investigated.

4.6. Assessment of Estragole and Es/CD on the CNS

The evaluation of the influence of Es and Es/CD on the central nervous system was performed only with the highest effective dose of 60 mg/kg using the open field and rota-rod assays.

4.6.1. Open Field

Swiss mice (n=6) were treated with H_2O (0.1 mL/10 g/p.o.), Es and Es/CD (60 mg/kg/p.o.). After 1 h (p.o.) the animals were individually placed in the open field for a period of 5 min, where their horizontal exploration behaviors (number of crossings), self-cleaning behavior (grooming) and vertical exploration (rearing) were recorded [33].

4.6.2. Rota-rod

Swiss mice (n = 6) were selected and pre-trained with up to 3 sessions (1 min) 24h before the treatment. The selected animals were divided into groups and treated respectively with H_2O (0.1 mL/10 g/p.o.), Es and Es/CD (60 mg/kg/p.o.), and after 1h (p.o.) the animals were placed on the rota-rod device for 1 min (16 rpm) and the number of falls was recorded [34].

4.7. Evaluation of Peripheral and Central Antinociceptive Action of Es and Es/CD

4.7.1. Abdominal Writhing Induced by Acetic Acid (0.6%)

Swiss mice (n = 6) were treated with H_2O (0.1 mL/10 g/p.o.), Es and Es/CD (60, 30 and 15 mg/kg). One hour (p.o.) after the treatments, the animals received glacial acetic acid PA (0.6%/0.1 mL/10 g/i.p.) diluted in water for injection. After the administration of acetic acid, the animals were placed under individual transparent glass funnels for 30min and the number of abdominal writhing movements was cumulatively quantified and characterized by the contraction and rotation of the abdomen, followed by the extension of one or both hind legs [35].

4.7.2. 2,5% Formalin Test

Swiss mice (n = 6) were treated with H_2O (0.1 mL/10 g/p.o.), Es and Es/CD (60, 30 and 15 mg/kg). After 1 h (p.o.), the animals were injected with 20 μL of formalin (2.5%) in the right hind paw (subplantar space) and placed individually immediately afterwards under an inverted glass funnel, next to a mirror to facilitate the observation. The time was

recorded in seconds, in which the animal licked, continued licking or biting the injected paw ("licking time') during the first phase, attributed to the neurogenic phase (0-5 min.) and the second characterized as the inflammatory phase (15-30min) [36].

4.7.3. Hot Plate Test

The Swiss mice (n = 6) were individually placed on a hot plate (52–54°C ± 0.5°C) and after obtaining two baseline values at 24h and 30 min before the test, the mice were treated (p.o.) according to the groups: H_2O (0.1 ml/10 g/p.o.), Es and Es/CD (60, 30 and 15 mg/kg). Subsequently, the response was evaluated 30, 60, 120 and 180 min after treatment administration, with the maximum contact time of the animal with the hot plate being held at 15s (baseline cutoff time) and 30s (test cutoff time) to avoid paw injuries. The nociceptive response was characterized by shaking the hind paws, licking or lifting the paw, or jumping on the plate [37]. To compare the effects over time, the percentage of each group was calculated using the average effect observed at times 30 up to 180min.

4.7.4. Tail Flick Test

Swiss mice (n = 6) were divided into 4 groups and placed in an apparatus containing a thermal light source and a mouse tail holder. Soon after that, two baseline values were obtained, 24h and 30 min before the test. Afterwards, the mice were treated (p.o.) according to the groups: H_2O (0.1 mL/10 g/p.o.), Es and Es/CD (60, 30 and 15 mg/kg). At the moment when the tail was placed in the apparatus, the noxious heat source was activated and the timer started, and the time until tail withdrawal was evaluated at 30, 60, 120 and 180min after the treatments. A maximum latency period of 15 seconds was established to avoid tissue damage [38]. The results were expressed as mean ± standard error of mean of the latency time.

4.7.5. Plantar Mechanical Hyperalgesia Test - Von Frey

Swiss mice (n = 6) were divided into 4 groups treated with H_2O (0.1 mL/10 g/p.o.), Es and Es/CD (60, 30 and 15 mg/kg). After 60 min (p.o.) of the treatments, all animals were injected with 20 μL of formalin (2.5%) in the right hind paw. The animals were placed individually in glass boxes on an elevated surface and covered with wire mesh. Stimuli were applied with the von Frey anesthesiometer filament to the injected plantar surface. The device records the sufficient force in grams to remove the paw in contact with the filament [39]. Positive responses were those in which the animal performed withdrawal movements, followed by shivering after the mechanical stimulation. All tests were performed after obtaining two baseline measurements (with an interval of 24 h) [40]. After the formalin injection, each animal was evaluated after 1, 2, 3, 4 and 24h. The results were expressed as the mean of the difference between the baseline value and that after the formalin injection (Δ), in the aforementioned time periods [41,42].

4.8. Evaluation of Pain Signaling Pathways and Their Interactions in the Antinociceptive Response of Es And Es/CD (Opioid, Cholinergic, Nitric Oxide, A-Adrenergic, Dopaminergic, Adenosynergic, Glutamatergic, Cyclic Guanosine Monophosphate (Cgmp), And Vanilloid Pathways)

4.8.1. Action on the Opioid System

Swiss mice (n = 6) were divided into 8 groups, so that the first 4 groups were treated with H_2O (0.1 mL/10 g/p.o.), opioid agonist - morphine (5 mg/kg s.c.), Es and Es/CD (15 mg/kg/p.o.) respectively, whereas the remaining 4 groups received naloxone - opioid antagonist (4 mg/kg i.p.) 15 min before the treatment. After 1h (p.o.) or 30min (s.c.) of the treatments, the animals were evaluated through the 2.5% formalin-induced nociception test (0-5 min.) [35].

4.8.2. Action on the Cholinergic System

Swiss mice (n = 6) were divided into 8 groups, where the first 4 groups were treated with H_2O (0.1 mL/10 g/p.o.), acetylcholine (cholinergic agonist - 1 mg/kg i.p.), Es or Es/CD (15 mg/kg/p.o.), while the other 4 groups were pre-treated with atropine (non-selective cholinergic antagonist 1 mg/kg/i.p.) 15 min before treatment with Es or Es/CD. Subsequently, 1h (p.o.) or 30 min (i.p.) after the treatment, the animals were evaluated through the 2.5% formalin test (0–5 min) [43].

4.8.3. Action on the Nitric Oxide Pathway

Swiss mice (n = 6) were divided into 8 groups, where the first 4 groups were treated with H_2O (0.1 mL/10 g/p.o.), L-NG-nitroarginine (L-NOARG); 75 mg/kg , i.p.), Es and Es/CD (15 mg/kg/p.o.), while the other 4 groups were pre-treated with L-Arginine (NOS substrate 600 mg/kg/i.p.), 15 min before treatment with Es or Un/CD. Then, 1h (p.o.) or 30 min (i.p.) after treatment, the animals were evaluated through the 2.5% formalin-induced nociception test (0-5min) [44].

4.8.4. α-2 Adrenergic Receptor Activity

Swiss mice (n = 6) were divided into 8 groups, where the first 4 groups were treated with H_2O (0.1 mL/10 g/p.o.), clonidine (α-2 agonist 0.1 mg/kg/ i.p.), Es and Es/CD (15 mg/kg/p.o.), while the other 4 groups received yohimbine (α-2 antagonist - 0.15 mg/kg i.p.) 15 min before treatment with Es or Es/CD. Then, 1 h (p.o.) or 30 min (i.p.) after treatment the animals were evaluated in relation to the 2.5% formalin test (0-5 min) [45].

4.8.5. Action on the Dopaminergic System

Swiss mice (n = 6) were divided into 6 groups: the first 4 groups were treated with H_2O (0.1 mL/10 g/p.o.), haloperidol (non-selective dopamine receptor antagonist 2 mg/kg i.p.), Es and Es/CD (15 mg/kg/p.o.), while the other 2 groups were pre-treated with haloperidol 15 min before treatment with Es or Es/CD. Then, 1h (p.o.) or 30 min (i.p.) after treatment, the animals were evaluated in relation to the 2.5% formalin test (0–5 min) [46].

2.8.6. Action on the Adenosinergic System

Swiss mice (n = 6) were divided into 6 groups: the first 4 groups were treated with H_2O (0.1 mL/10 g/p.o.), caffeine (10 mg/kg i.p.), Es and Es/CD (15 mg /kg/p.o), while the other 2 groups were pre-treated with caffeine 15 min before treatment with Es or Es/CD. Then, 1h (p.o.) or 30 min (i.p.) after treatment the animals were evaluated in relation to the 2.5% formalin test (0–5 min) [47].

2.8.7. Action on the Glutamatergic Signaling Pathway

Swiss mice (n = 6) were divided into 4 groups treated with H_2O (0.1 mL/10 g/p.o.), ascorbic acid (NMDA receptor antagonist 100 mg/kg i.p.), Es and ES/β-CD (15 mg/kg/p.o.). Then, 1h (p.o.) or 30 min (i.p.) after

treatment the animals were analyzed for 15 min in relation to the nociception induced by the intraplantar injection of 20 μl of buffered glutamate at 20 μmoL/paw, where the time the animal spent licking the paw was considered a parameter suggestive of pain [48].

2.8.8. Action on the Cyclic Guanosine Monophosphate (cGMP) Signaling Pathway

To verify the involvement of cyclic guanosine monophosphate in the antinociception caused by the compounds under study, the animals were pre-treated with methylene blue (20 mg/kg/i.p.), a guanylate cyclase inhibitor, 15 min before the administration of Es and Es/CD (15 mg/kg/p.o.). In addition, other groups were treated with H_2O alone (0.1 mL/10 g/p.o.), Es or Es/CD (15 mg/kg/p.o.) or methylene blue (20 mg/kg, i.p.). The nociceptive response was evaluated after 1h (p.o.) or 15 min (i.p.) of the treatments, through the intraplantar injection of 20 μL of formalin solution and the paw licking time (0-5 min) was quantified [49].

2.8.9. Action on the Vanilloid System

Swiss mice (n = 6) were divided into 4 groups, which were treated with H_2O (0.1 mL/10 g/p.o.), ruthenium red (non-selective TRP antagonist 3 mg/kg i.p.) [50], Es and ES/β-CD (15 mg/kg/p.o.). Subsequently, 1h (p.o.) or 30min (i.p.) after treatment the animals were analyzed for 5 min in relation to the nociception induced by the intraplantar injection of 20 μl of capsaicin (TRPV1 receptor agonist) at 5.2 nmol/paw [51] where the time (seconds) during which the animal spent licking the paw was considered as behavior suggestive of pain.

2.9. Statistical Analysis

The results are presented as mean ± standard error of the mean (S.E.M), evaluated by one-way and two-way analysis of variance (ANOVA), using Tukey's multiple comparison tests, and the calculations were performed using the statistical software GraphPad Prism (version 9.0), according to the values obtained in the tests. For all analyses, $p < 0.05$ was considered significant.

5. Conclusions

The present study showed that, regarding the activity on the central nervous system, estragole (Es) and estragole/β-cyclodextrin (Es/CD) did not alter exploratory or motor coordination activity, suggesting they do not have a depressant or excitatory action. in the CNS. As for the antinociceptive effect, Es and Es/CD showed central and peripheral antinociceptive activity in the abdominal writhing model induced by acetic acid, formalin test, hot plate, tail flick and von Frey assay, with involvement of the nitric oxide, glutamatergic, guanosine monophosphate and vanilloid pathways. Importantly, Es/CD showed significant effects at lower doses than Es alone. The data of the present study suggest that Es and Es/CD may contribute to the formulation of new compounds for the treatment of pain.

Author Contributions: R.H.S.C. and J.E.R., Writing – original draft; R.T.P., L.Y.S.S. and I.S.A., Methodology; H.D.M.C. and I.R.A.M., Resources; A.G.R.B and A.R. Visualization; M.R.C.O., F.L.A.B. and G.A.D., Data curation, A.O.B.P.B.M., A.R., P.A.M.F., C.C., J.R.J., S.A.A., S.W., M.F.A., M.S. and I.R.A.M., Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Finance code 001).

Data Availability Statement: The data presented in this study are available in the article.

Acknowledgments: The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through the Large Research Groups Project under grant number (RGP.2/58/43).

Conflicts of Interest: The authors declare no conflict of interest.

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CAPÍTULO 4: CONSIDERAÇÕES FINAIS

O estragol (1-metoxi-alil-benzeno, 4-metoxialilbenzeno, metil chavicol, p-alilanisol ou chavicol metiléter) é um monoterpeno amplamente utilizado na indústria de alimentos e bebidas como aromatizante e também nos perfumes, sabões e detergentes, sendo um constituinte químico importante dos óleos essenciais de muitas plantas aromáticas. Ensaios experimentais *in vivo* e *in vitro*, encontrados na literatura, demonstraram que o estragol tem muitos efeitos biológicos, incluindo atividades antioxidantes, antimicrobianas, antifúngicas, ansiolítica, antiedematogênica e anti-inflamatória.

No presente estudo foi observado que o estragol conseguiu reduzir a CIM do antibiótico Norfloxacina, causando desta forma um sinergismo, reduzindo a concentração do antibiótico. Resultados semelhantes foram encontrados para os inibidores de bomba CCCP e Clorpromazina, podendo sugerir desta forma que o produto pode estar agindo no mecanismo de bomba NorA do *Staphylococcus aureus*1199B.

Em relação ao Brometo de Etídio, também foi observada a redução da CIM quando associado ao estragol, evidenciando desta forma a presença de bomba de efluxo NorA na bactéria e que o produto está agindo na mesma, pois o mecanismo pelo qual a bactéria expele o substrato para o meio extracelular é através da bomba de efluxo NorA. Os inibidores padrões, também reduziram a CIM do brometo, sendo o resultado do estragol, semelhante ao da Clorpromazina.

Para a cepa de bactéria *S. aureus* K2068, portadora da bomba MepA, o estragol não apresentou resultados estatisticamente relevantes quando utilizados com o antibiótico Ciprofloxacina, desta forma não alterando seu resultado, entretanto os inibidores de bomba de efluxo, CCCP e clorpromazina, foram capazes de reduzir a CIM do antibiótico.

Quando testado em associação com o Brometo de Etídio, o terpeno estudado em sua concentração subinibitória (CIM/8) foi capaz de reduzir a CIM do brometo. E como a CCCP e Clorpromazina também reduziram a CIM do brometo, desta forma resultando em um sinergismo. Pode-se observar então que o estragol agiu no mecanismo de bomba de efluxo MepA presente nessa bactéria.

A complexação do estragol teve como objetivo melhorar a biodisponibilidade, solubilidade e estabilidade da molécula. Além disso, reduzir os efeitos colaterais e citotoxicidade e prolongar o tempo de efeito das substâncias, visto que a complexação melhora algumas características farmacocinéticas o que reduz a necessidade de usar grandes quantidades do princípio ativo.

Os estudos de toxicidade oral aguda do Es e Es/CD foram realizados em camundongos *Swiss*. Os animais tratados com a dose de 2000 mg/kg/v.o., apresentaram óbitos e sinais de toxicidade, de acordo com os parâmetros avaliados, no entanto o grupo de animais tratados com Es e Es/β-CD na dose 625 mg/kg/v.o., não apresentaram sinais clínicos de toxicidade, de acordo com os parâmetros avaliados, e nem mortalidade de animais. Com esses resultados, as doses foram selecionadas para a investigação da atividade antinociceptiva, considerando o limite igual ou inferior a 10% da DL_{50}, como preconiza a OECD, (2008) (Organização para a Cooperação e Desenvolvimento Econômico), sendo essas 60, 30 e 15 mg/kg/v.o.

Quanto a avaliação do efeito do Es e do Es/CD sobre o SNC no ensaio de *Rota-rod* os animais não apresentaram características de descoordenação motora em comparação ao grupo controle, o número de quedas e de permanência na barra foram semelhantes. E no Teste do campo aberto também não houve alteração nas reações comportamentais entre os grupos controle.

Neste estudo, o estragol e seu complexo em β-CD apresentaram atividade antinociceptiva quando avaliados através de modelos clássicos de nocicepção como teste de formalina, o de contorções abdominais por ácido acético, teste de placa quente (*Hot plate*), teste de retirada de cauda (*Tail Flick*) e teste de hipernocicepção mecânica por pressão (*Von Frey*), com determinação das possíveis vias de sinalização envolvidas (óxido nítrico, glutamatérgica, monofosfato cíclico de guanosina e vanilóide) e exclusão de efeito depressor sobre SNC. Estatisticamente não houve diferença entre os grupos tratados com Es e ES/CD, no entanto os resultados obtidos com o grupo Es/CD tornam-se mais interessantes do que os encontrados com o Es, já que o processo de complexação envolve quantidades bem inferiores de estragol, demonstrando assim um aperfeiçoamento das propriedades farmacológicas do Es pelo processo de nanoencapsulamento em ciclodextrinas.

4.1 CONCLUSÕES GERAIS

O monoterpeno estragol estudado isoladamente, foi capaz de modular sinergicamente a ação da norfloxacina e do brometo de etídio frente a cepa bacteriana *Staphylococcus aureus* 1199B portadora de bomba NorA e de reduzir a CIM do brometo de etídio frente a *S. aureus* K2068, portadora de bomba de efluxo MepA, desta forma comportando-se como um possível inibidor de bomba de efluxo.

A avaliação do efeito do Es e Es/β-CD na dose de 60 mg/kg/v.o. sobre o sistema nervoso central dos animais não provocou alterações na coordenação motora e nem alterações nas reações comportamentais.

Os compostos Es e Es/CD também apresentaram uma promissora atividade antinociceptiva nos modelos clássicos avaliados, reduzindo os estímulos nociceptivos na fase neurogênica e na fase inflamatória, com prováveis vias de sinalização determinadas.

4.2 PERSPECTIVAS DE INVESTIGAÇÕES FUTURAS

O desenvolvimento de novos protótipos com atividades farmacológicas antibacterianas e antinociceptivas a partir do estragol e do Complexo de inclusão Estragol / β-Ciclodextrina pode trazer benefícios significativos para a sociedade, pois além de possibilitar conhecimentos inéditos em relação ao seu potencial farmacológico, podem contribuir com informações e resultados que podem ser utilizados na validação científica sobre o seu uso com eficácia e segurança em futuros estudos clínicos.

Em relação à atividade antibacteriana poderão ser realizados ensaios de dinâmica molecular para investigar os mecanismos de ação do Es e do Es/ β-CD, utilizando a membrana bacteriana como modelo alvo da ação indireta desses terpenos na inibição das bombas de efluxo NorA e MepA.

Pretendemos desenvolver ensaios de PCR em Tempo Real, com o objetivo de verificar a capacidade do Es e do Es/ β-CD de inibirem as proteínas de efluxo NorA e MepA através da inibição da expressão gênica.

Também planejamos obter imagens de microscopia eletrônica de varredura (MEV), principalmente nas membranas bacterianas, com o objetivo de verificar possíveis danos nas estruturas bacterianas.

Ainda está previsto a caracterização físico-química dos complexos de inclusão obtidos por co-evaporação por meio das técnicas de Calorimetria Exploratória Diferencial (DSC) em atmosfera inerte e oxidativa, Termogravimetria/Termogravimetria derivada (TG/DTG), Microscopia Eletrônica De Varredura (MEV) e Karl Fischer, mas infelizmente o laboratório parceiro da Universidade Federal de Sergipe esteve com suas atividades suspensas devido a pandemia causada pelo Sars-Cov-2. Seguimos aguardando a possibilidade de realizá-las.

Vamos envolver a instituição na qual trabalho, através da diretoria de sanidade animal da Agência de Defesa Agropecuária do Estado do Ceará – ADAGRI em parceria com a URCA, buscando novas alternativas, através da pesquisa, para melhorar o controle e erradicação de doenças de notificação obrigatória que causam tantos prejuízos aos nossos pecuaristas e criadores.

Devemos continuar as atividades de pesquisa buscando a colaboração, parceria e apoio de instituições externas a URCA e dar oportunidade a outros profissionais e estudantes de se envolverem nas atividades, estimulando o interesse científico dessas pessoas.

ANEXO I− AUTORIZAÇÃO DA COMISSÃO DE ÉTICA E USO DE ANIMAIS − PARA O DESENVOLVIMENTO DAPESQUISA

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Declaração

Declaro para devidos fins, que o projeto intitulado "Atividade antinociceptiva do estragol e do complexo de inclusão estragol/β-ciclodextrina em modelos animais"- processo **000358/2019.2** foi **APROVADO** pela Comissão de Experimentação e Uso de animais-CEUA-URCA .

Roseli Barbosa

Coordenadora do CEUA-URCA

Crato-04-12-2020

ANEXO II–PRODUÇÃO CIENTÍFICA

Biologia (2022) 77:1667–1674
https://doi.org/10.1007/s11756-021-00862-2

ORIGINAL ARTICLE

Acaricide activity of the *Ximenia americana* L. (Olacaceae) stem bark hydroethanolic extract against *Rhipicephalus* (*Boophilus*) *microplus*

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Received: 2 February 2021 / Accepted: 15 July 2021 / Published online: 23 August 2021

Abstract

Ximenia americana L. is popularly known as a plum or wild plum, which belongs to the Olacaceae family. *Rhipicephalus* (*Boophilus*) *microplus* represents one of the most important bovine ectoparasites that causes damage to the livestock sector. The objective of this study was to evaluate the acaricide effect of the *X. americana* stem bark hydroethanolic extract (EHXA) against *R.* (*B.*) *microplus* and its possible modulatory action when in association with cypermethrin, through the adult immersion test. The *in vitro* acaricide activity of the extract over *R.* (*B.*) *microplus* engorged females was tested using concentrations ranging from 5 to 80 mg mL^{-1}, which resulted in mortalities between 13.34 and 100 %, with a posture index (PI) between 0.038 and 0.226 and a posture inhibition index (PII) between 0.87 and 83.33 %. The modulatory effect of the EHXA (1.58 mg mL^{-1}) resulted in mortality between 0 and 46.6 %, with a posture index of 0.221 to 0.139 and a posture inhibition index between 3.01 and 38.49 %. The acaricide activity of the EHXA and its action on oviposition was demonstrated for the first time in this study, demonstrating the potential use of this extract as an auxiliary therapy in *R.* (*B.*) *microplus* control.

Keywords *Rhipicephalus* (*Boophilus*) *microplus* · *Ximenia americana* L. · Acaricide

Introduction

Rhipicephalus (*Boophilus*) *microplus* (Canestrini, 1888) (Acari: Ixodidae) is an ectoparasite responsible for causing great economic losses to cattle breeding worldwide, especially in herds in tropical and subtropical climate regions (Rodríguez-Vivas et al. 2004). In Brazil, it is estimated that losses caused by the bovine tick are $3.24 billion (Grisi et al. 2014). This parasite is responsible for the transmission of herd diseases, such as cattle tick fever, caused by agents from the

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Folia Microbiologica
https://doi.org/10.1007/s12223-021-00945-2

ORIGINAL ARTICLE

Evaluation of the antifungal activity of α, β, and δ-damascone and inclusion complexes in β-cyclodextrin against *Candida* spp

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Received: 19 September 2021 / Accepted: 22 December 2021

Abstract

Due to the increase in fungal resistance to existing drugs, a need exists to search for new antifungals. This study aimed to evaluate the antifungal activity of α, β, and δ-damascone and inclusion complexes with β-cyclodextrin against different Candida spp. The inclusion complex of β-damascone was prepared by the co-evaporation method using three molar proportions (1:1; 2:1; 3:1 (βDA-βCD)) and analyzed using Fourier transform infrared spectroscopy (FTIR). Standard *Candida albicans* (CA INCQS 40,006), *Candida krusei* (CK INCQS 40,095), and *Candida tropicalis* (CT INCQS 40,042) strains were used to evaluate antifungal activity. The substances were tested individually or in association with fluconazole (FCZ). The IC_{50} and cell viability curve constructions were performed using the microdilution method. The minimum fungicidal concentration (MFC) was determined by the subculture method in a solid medium. The α, β, and δ-DA isolated or in combination with fluconazole (FCZ) showed significant antifungal activity. β-damascone showed effective complexation in the three molar proportions assayed; however, none of the inclusion complexes was demonstrated clinically significant effects against the fungal tested. Then, all compounds have shown promising antifungal activities; however, in vivo assays are necessary to have therapeutical application in the future.

Introduction

Infections related to the *Candida* genus occur with great frequency in human hosts (Lopes Colombo and Guimarães 2003). Despite being part of the organism's microbiota, species belonging to this genus are considered opportunistic pathogens, since these infections occur when there is an imbalance in defense mechanisms (Barbedo and Sgarbi 2010), and are responsible for high rates of morbidity and mortality (Campion et al. 2015).

Candida albicans is among the species that cause the greatest number of infections. However, infections by other species belonging to this genus have become increasingly more common (Arendrup and Patterson 2017). In this sense, the most significant species, in terms of clinical and epidemiological aspects, are as follows: *C. albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* (Vieira and Santos 2017).

Regarding the treatment of fungal infections, four classes of medications are used: azoles (which include fluconazole, itraconazole, izavulconazole, posaconazole, and voriconazole), polyenes (conventional amphotericin B, as well as lipid formulations), echinocandin (anidulafungin, caspofungin, micafungin), and flucytosine, which is a pyrimidine analogue (Arendrup and Patterson 2017). However, these microorganisms have acquired resistance to these drugs (Kullberg and Arendrup 2016; Arendrup and Patterson 2017), where this is considered an increasing aggravating factor (Campion et al. 2015) and studies are needed to try to understand the cellular and molecular mechanisms that lead to resistance to antifungals (Vieira and Santos 2017). Therefore, due to the few antifungals that are available and their adverse side effects, further studies in search of new antifungal drugs as alternatives, which are more efficient

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Bioorg. Med. Chem. Lett. 50 (2021) 128334

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Evaluation of Elaiophylin extracted from *Streptomyces hygroscopicus* as a potential inhibitor of the NorA efflux protein in *Staphylococcus aureus*: An *in vitro* and *in silico* approach

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ARTICLE INFO

Keywords:
Antibiotics
Bacterial resistance
Natural products
Efflux pump
NorA

ABSTRACT

Compounds capable of inhibiting the efflux pump mechanism are a promising alternative against bacterial resistance because, when combined with antibiotics, they can increase the effectiveness of these drugs by inhibiting active efflux. Elaiophylin, derived from *Streptomyces hygroscopicus*, is a natural antibiotic that exhibits a variety of biological activities, including antibacterial activity. However, its potential as an inhibitor of the bacterial efflux mechanism has not been investigated. This study evaluated the ability of Elaiophylin to inhibit the NorA efflux pump in *Staphylococcus aureus* strains. Therefore, tests were performed to obtain the Minimum Inhibitory Concentration (MIC) and to verify the ability of Elaiophylin to potentiate the MIC of the antibiotic Norfloxacin and Ethidium Bromide (EtBr), known substrates of NorA efflux. Real-time PCR and molecular docking assays were also performed to assess the potential of Elaiophylin against NorA. The strains SA-1199 (wild type) and SA-1199B (NorA over-expressed) of *S. aureus* were used for this study. The results showed that Elaiophylin significantly decreased the MIC of Norfloxacin and EtBr, increasing the activity of these substrates against *S. aureus*, which carries the efflux protein NorA. However, Elaiophylin provided a non-significant reduction in *norA* gene expression, however, molecular docking demonstrated a high binding affinity between Elaiophylin and NorA efflux protein, indicating that Elaiophylin can act as a potential NorA in *S. aureus*.

Staphylococcus aureus is a causative agent of several infectious diseases in several species, including human beings. Examples of such pathologies include wound-related infections, endocarditis, osteomyelitis, pneumonia, toxic shock syndrome, food poisoning, carbuncles, and boils.[1] The pathogenic potential of *S. aureus* is further aggravated by the frequent acquisition of resistance to antibiotics for clinical use and other compounds with antimicrobial activity, such as antiseptics, quaternary ammonium compounds and preservatives.[2,3]

This resistance is attributed to mechanisms such as drug target changes, antibiotic inactivation enzymes, reduced membrane permeability and active efflux,[4] with active efflux being one of the main modulators of Multiple Drug Resistance (MDR) in *S. aureus*, acting as a primary response to harmful compounds that expel toxic substances to the outside of the bacterial membrane.[5]

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https://doi.org/10.1016/j.bmcl.2021.128334
Received 30 April 2021; Received in revised form 13 August 2021; Accepted 16 August 2021
Available online 20 August 2021
0960-894X/

Biologia (2021) 76:1275–1283
https://doi.org/10.2478/s11756-020-00672-y

ORIGINAL ARTICLE

Toxicity of methyl eugenol against *Drosophila melanogaster* and its myorelaxant activity in bronchioles isolated from *Sus scrofa domesticus*

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Received: 22 September 2020 / Accepted: 28 December 2020 / Published online: 13 January 2021

Abstract
This study aimed to evaluate the toxic action of methyl eugenol against *Drosophila melanogaster* and to evaluate the myorelaxant effect of this constituent on bronchioles isolated from swine from the *Sus scrofa domesticus* species. The toxicity assay was performed using the fumigation method while myorelaxant activity was assessed by the administration of increasing and cumulative concentrations (1–1000 μM) to swine bronchial rings under 1 gF tension in an organ bath precontracted with 60 mM potassium chloride (KCl) and 10 μM acetylcholine (ACh). The results showed that methyl eugenol possessed insecticidal action (LC_{50} 0.116 μg/mL of air). The compound showed a myorelaxant activity over bronchioles (IC_{50} of 25.50±14.93 μM and 12.79±9.57 μM for KCl and ACh, respectively). This study serves as starting point for further detailed studies focusing on both the insecticidal and bronchodilatory activity of methyl eugenol.

Keywords Alternative methods · Methyl eugenol · Toxicity · Myorelaxant

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
EOs	Essential oils
IC_{50}	Concentration of the substance that is capable of producing 50% of the maximum inhibitory effect.
KCl	Potassium chloride
LC_{50}	Lethal concentration to cause 50% mortality in a population
PET	Polypropylene terephthalate
TM	Modified Tyrode
3 Rs	Replacement, reduction, refinement

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Introduction

Natural products derived from plants such as essential oils (EOs) or their isolated constituents are widely investigated for their toxic potential against insects, such as disease vectors (Chellappandian et al. 2018; Silva et al. 2018) agricultural pests (Scopel et al. 2018; Mercês et al. 2018) urban and structural pests (Gaire et al. 2019; Gaire et al. 2020; Perry and Choe 2020).

Chemically, the greater majority of volatile oil constituents have a terpenoid or phenylpropanoid structure (Simões et al. 2017). Methyl eugenol, a compound analogous to eugenol, is among the phenylpropanoid compounds present in the chemical composition of many plants, for example *Croton nepetaefolius* (Lima et al. 2013), *Asarum heterotropoides* (Perumalsamy et al. 2009), *Peperomia hispidula* (Arrieta et al. 2018), and other aromatic plant species (Lima et al. 2000).

Among the biological properties of methyl eugenol, its potential to exert a repellent action (Du et al. 2014), fumigant toxicity (Liu et al. 2013), as well as its toxic effects on adult (Huang et al. 2002) and nymph insects (Gaire et al. 2017) stand out. In addition to an insecticidal activity, studies indicate that methyl eugenol possesses pharmacological properties such as myorelaxant effects (Lima et al. 2000),

Food Chemistry 337 (2021) 127776

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

In vitro and *in silico* inhibitory effects of synthetic and natural eugenol derivatives against the NorA efflux pump in *Staphylococcus aureus*

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ARTICLE INFO

Keywords:
Staphylococcus aureus
Efflux pump inhibitors
Antibiotic resistance
Eugenol derivatives

ABSTRACT

Staphylococcus aureus is a Gram-positive bacterium responsible for a number of diseases and has demonstrated resistance to conventional antibiotics. This study aimed to evaluate the antibacterial activity of eugenol and its derivatives allylbenzene, 4-allylanisole, isoeugenol and 4-allyl-2,6-dimethoxyphenol against the *S. aureus* NorA efflux pump (EP) in association with norfloxacin and ethidium bromide. The antibacterial activity of the compounds was assessed using the broth microdilution method to determine the minimum inhibitory concentration (MIC). A reduction in the MIC of ethidium bromide (a substrate for several efflux pumps) or norfloxacin was used as a parameter of EP inhibition. Molecular modeling studies were used to predict the 3D structure and analyze the interaction of selected compounds with the binding pocket of the NorA efflux pump. Except for 4-allylanisole and allylbenzene, the compounds presented clinically effective antibacterial activity. When associated with norfloxacin against the SA 11998 strain, 4-allyl-2,6-dimethoxyphenol eugenol and isoeugenol caused significant reduction in the MIC of the antibiotic, demonstrating synergistic effects. Similar effects were observed when 4-allyl-2,6-dimethoxyphenol, allylbenzene and isoeugenol were associated with ethidium bromide. Together, these findings indicate a potential inhibition of the NorA pump by eugenol and its derivatives. This *in vitro* evidence was corroborated by docking results demonstrating favorable interactions between 4-allyl-2,6-dimetoxyphenol and the NorA pump mediated by hydrogen bonds and hydrophobic interactions. In conclusion, eugenol derivatives have the potential to be used in antibacterial drug development in strains carrying the NorA efflux pump.

1. Introduction

Antibiotic resistance is currently a major worldwide public health problem (OMS, 2014). The indiscriminate use of antibiotics has been directly correlated with the emergence of strains resistant to multiple drugs, which have a significant impact on the mortality rates for bacterial diseases. *Staphylococcus* is a genus of Gram-positive bacteria with remarkable pathogenicity mechanisms (Dos Santos et al., 2018), among which *Staphylococcus aureus* stands out for its notable virulence, capacity of colonization and development of resistance to multiple drugs. Accordingly, this pathogen has been recognized as the causative agent of a great variety of diseases, such as endocarditis, severe necrotizing pneumonia, toxic shock syndrome, bacteremia and cutaneous infections (Chambers & Deleo, 2009).

Antibiotic resistance can result from either intrinsic or acquired mechanisms associated with the development of mutations and horizontal gene transfer leading to one of the following mechanisms: changes in the active site of antibiotics; changes in membrane

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https://doi.org/10.1016/j.foodchem.2020.127776
Received 7 November 2019; Received in revised form 4 July 2020; Accepted 2 August 2020
Available online 05 August 2020
0308-8146/

molecules

MDPI

Article

Myorelaxant Effect of the *Dysphania ambrosioides* Essential Oil on *Sus scrofa domesticus* Coronary Artery and Its Toxicity in the *Drosophila melanogaster* Model

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Citation: De Lacerda Neto, L.J.; Ramos, A.G.B.; da Silva, R.E.R.; Pereira-de-Morais, L.; Silva, F.M.; da Costa, R.H.; Dantas, L.B.R.; da Costa, J.G.M.; Coutinho, H.D.M.; Kowalska, G.; et al. Myorelaxant Effect of the *Dysphania ambrosioides* Essential Oil on *Sus scrofa domesticus* Coronary Artery and Its Toxicity in the *Drosophila melanogaster* Model. *Molecules* 2021, *26*, 2041. https://doi.org/10.3390/molecules26072041

Academic Editor: Vincenzo De Feo

Received: 13 March 2021
Accepted: 26 March 2021
Published: 2 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Purpose: Alternative methods for the use of animals in research have gained increasing importance, due to assessments evaluating the real need for their use and the development of legislation that regulates the subject. The principle of the 3R's (replacement, reduction and refinement) has been an important reference, such that in vitro, ex vivo and cord replacement methods have achieved a prominent place in research. Methods: Therefore, due to successful results from studies developed with these methods, the present study aimed to evaluate the myorelaxant effect of the *Dysphania ambrosioides* essential oil (EODa) using a *Sus scrofa domesticus* coronary artery model, and the toxicity of both the *Dysphania ambrosioides* essential oil and its major constituent, α-terpinene, against *Drosophila melanogaster* in toxicity and negative geotaxis assays. Results: The EODa relaxed the smooth muscle of swine coronary arteries precontracted with K^+ and 5-HT in assays using *Sus scrofa domesticus* coronary arteries. The toxicity results presented LC_{50} values of 1.546 mg/mL and 2.282 mg/mL for the EODa and α-terpinene, respectively, thus showing the EODa and α-terpinene presented toxicity to these dipterans, with the EODa being more toxic. Conclusions: Moreover, the results reveal the possibility of using the EODa in vascular disease studies since it promoted the relaxation of the Sus scrofa domesticus coronary smooth muscle.

Keywords: essential oil; natural product; myorelaxative activity; toxicity

1. Introduction

The use of animals in essential research has been constantly questioned in terms of the principles of the 3R's (replacement, reduction and refinement), thus creating the need for reflection in the number of animals used in research (reduction), the possibility of replacing vertebrate animals with invertebrates, embryos, microorganisms, organs or isolated tissues, in addition to guaranteeing the quality of experiments (refinement), where

Comparative Immunology, Microbiology and Infectious Diseases 64 (2019) 40–46

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Ximenia americana L. enhances the antibiotic activity and inhibit the development of kinetoplastid parasites

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ARTICLE INFO

Keywords:
Leishmania
Trypanosoma
Ximenia americana
Pseudomonas aeruginosa
Staphylococcus aureus

ABSTRACT

Objective: The objective of this work was evaluate the cytotoxic, leishmanicidal and trypanocidal activity, as well as to evaluate its antimicrobial and modulatory activity in association with different antibiotics of the hydroethanolic extract of the *Ximenia Americana* stem bark (EBXA).

Method: *In vitro* tests against *Trypanosoma cruzi*, *Leishmania* sp. and cytotoxicity were performed. The evaluation of the antibacterial and bacterial resistance modulatory effect was given by the microdilution method.

Results: The chemical profile show different classes of compounds with significant presence of quercetrin and caffeic acid. The EBXA demonstrated activity only in the concentration of 1 000 μg/mL against the *L. infantum* and *L. braziliensis* promastigotes, causing mortality percentage of 40.66 and 27.62%, respectively. The extract presented a significant toxicity only in the concentration of 1 000 μg/mL, causing a mortality of 55.42% of fibroblasts. The antibacterial activity of the EBXA demonstrated a MIC value ≥ 1 024 μg/mL against all the tested bacteria. However, in the modulation assay with EBXA in association with different antibiotics the extract had a synergistic effect against *S. aureus* strains when associated with norfloxacin.

Conclusion: The results of this investigation demonstrate for the first time the chemical composition of the hydroethanolic extract of the *Ximenia Americana* stem bark, your potential antiparasitic and modulatory effect. The low cytotoxic and biological potential against *S. aureus* open therapeutic perspectives against leishmaniasis and bacterial infections.

1. Introduction

"Neglected diseases" is a term used to refer to a group of diseases affecting mainly poor and developing countries, favoring the maintenance of inequality and social exclusion. For the public health, this group of diseases is a big problem, as they can cause physical and cognitive deficits in the individual; as well as impacting the epidemiological profile. In Brazil, we can cite as examples of neglected diseases, in special, leishmaniasis and Chagas' disease [1].

Leishmaniasis is an infectious disease, which is considered zoonotic, caused by protozoan of the genus *Leishmania* and is transmitted to the definitive host by the bite of an infected female sand-fly; it possesses a wide distribution around the world, from Asia to America. According to Palatnik-de-Sousa and Day (2011), leishmaniasis is an endemic disease in 88 countries around the world, of which 60 per cent of disease foci are in well-defined areas of Bangladesh, India and Nepal, with an annual register of 1 million to 1.5 million cases [2]. From 1980 to 2005, Brazil recorded 59,129 cases of visceral leishmaniosis, 82.5%, it has a

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https://doi.org/10.1016/j.cimid.2019.02.007
Received 24 March 2018; Received in revised form 5 February 2019; Accepted 12 February 2019

ANAIS da Academia Cearense de Ciências, v. 4, n. 1, p. 40-47, 2020.
www.aceci.com.br

Artigo Científico/ *Scientific Article*

Bronchodilator Activity of the Essential Oil from *Lippia sidoides* in Bronchial Isolated from Swine *Sus scrofa domesticus*

Luís Pereira-de-Morais*, Andressa de Alencar Silva, Renata Evaristo Rodrigues da Silva, Carla Mikevely de Sena Bastos, Isaac Moura Araújo, Roger Henrique Sousa da Costa, Francisco Assis Bezerra da Cunha, José Henrique Leal-Cardoso, Roseli Barbosa

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Pereira-de-Morais, L.; Silva, A. A.; Silva, R. E. R.; Bastos, C. M. S.; Araújo, I. M.; Costa, R. H. S.; Cunha, F. A. B.; Leal-Cardoso, J. H. & Barbosa, R. Bronchodilator Activity of the essential oil from *Lippia sidoides* in bronchial isolated from swine *Sus scrofa domesticus*. ANAIS da Academia Cearense de Ciências, v. 4, n. 1, p.40-47, 2020.

ABSTRACT

Natural products have molecules with therapeutic properties. Among the natural products, the plant *Lippia sidoides* Cham (Verbenaceae), popularly called Rosemary Pepper, is used in folk medicine as antiseptic agent. Some studies have shown that *L. sidoides* promotes electrophysiological changes in the smooth muscle of the airways of rats. However, the effect of *L. sidoides* on pig airways has not yet been demonstrated. In the literature it has been described that rodent and pig tissues respond differently to drugs, and that pig tissue is very similar to human tissue, being an alternative model with less ethical limitations, since pigs are slaughtered for human consumption. The aim of this study was to investigate the effect of *L. sidoides* essential oil (EOLs) on the bronchial respiratory smooth muscle isolated from *Sus scrofa domesticus* pigs. To evaluate the effect of EOLs, increasing and cumulative concentrations of 1-3000 µg/mL were administered in bronchial rings, previously contracted and maintained in presence of KCl (60 mM) or acetylcholine (ACh; 10 µM). It was observed that the EOLs relaxed, in a concentration-dependent manner, the bronchial preparations pre-contracted by KCl (60 mM), having their maximum efficiency in the concentration of 1000 µg/mL. For the contraction induced by ACh, adding at a concentration of 3000 µg/mL, the EOLs relaxed only about 30 % of the contraction. The EOLs have greater pharmacological potency in electromechanical coupling, with a bronchodilator effect, being a potential therapeutic agent with action on the respiratory tract. The effectiveness of the method chosen for the development of the research was also evidenced.

Additional keywords: Pigs; respiratory smooth muscle; organ bath; *L. sidoides*; essential oil.

Food and Chemical Toxicology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

HPLC profile and antiedematogenic activity of *Ximenia americana* L. (Olacaceae) in mice models of skin inflammation

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ARTICLE INFO

Keywords:
Ximenia americana
Skin inflammation
Antiedematogenic activity
Natural products

ABSTRACT

The aim of this study was to evaluate the anti-edematogenic activity of X. americana L. (HEXA) hydromethanolic extract in ear edema models (acute and chronic) induced by croton oil and by different phlogistic agents (arachidonic acid, capsaicin, phenol and histamine), identifying the possible anti-edematogenic mechanism. HEXA demonstrated a significant anti-edematogenic effect at concentrations of 100–500 μg/ear in ear edema induced by croton oil with higher inhibition of edema of 39.37. However, the concentrations of 100 and 200 μg/ear were taken as a standard, demonstrating the effect in the chronic model induced by croton oil with inhibition of 61.62% and 48.74%. In the AA-induced ear edema model, HEXA showed inhibition of: 24.45% and 32.31%; capsaicin inhibition of 72.72% and 47.57%; phenol inhibition of 34% and 20.1%; and histamine inhibition of 31.8% and 21.62%. Then, the results were showed that HEXA demonstrated an anti-edematogenic effect in acute and chronic inflammation models, demonstrating a probable mechanism of action by the inhibition or modulation of key mediators of the inflammatory process. The chemical profile and presence of flavonoids guaranteeing a profile of activity similar to natural drugs that act or modulate the production of mediators of inflammations.

1. Introduction

The skin is an important barrier between the body and the external environment, playing a key role in protection and regulation of the body homeostasis. However, this protective barrier is constantly exposed to different noxious stimuli, such as pathogens, ultraviolet rays, oxidative stress (and reactive oxygen species (ROS)), and various mechanical, physical or chemical stimuli; and therefore, it works as an interface for triggering local inflammatory responses (Elias, 2007).

Exposure to these noxious stimuli, as well as mounting inadequate or misdirected immune responses that lead to the production of specific cytokines, may be critical for the pathogenesis of skin inflammatory conditions, such as psoriasis and dermatitis (Kupper and Fuhlbrigge, 2004; Song et al., 2008; Esser et al., 2012). These events result in the production and release of several inflammatory mediators, including the vasoactive amines (histamine and serotonin), metabolites of the arachidonic acid (prostaglandins, thromboxanes, leukotrienes and platelet activating factor (PAF)), bradykinin, nitric oxide, neuropeptides and cytokines (Coutinho et al., 2009).

Corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) and histamine receptor antagonists are widely used to treat inflammatory conditions because these drugs modulate essential steps in the cascade of production or action of inflammatory mediators. Nevertheless, there are some conditions under which these drugs are not effective, and in addition, they cause significant side effects, justifying the search for new drugs to treat inflammatory and allergic

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https://doi.org/10.1016/j.fct.2018.04.061
Received 3 March 2018; Received in revised form 14 April 2018; Accepted 18 April 2018

Original Article | Published: 27 May 2021

Antinociceptive Effect of Volatile Oils from *Ocimum basilicum* Flowers on Adult Zebrafish

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Revista Brasileira de Farmacognosia (2021) | Cite this article

Abstract

The species *Ocimum basilicum* L., Lamiaceae, is popular for culinary purposes and medicinal use as a larvicide, repellent, antifungal, and antimicrobial agent. Therefore, the aim of the present study is to evaluate the chemical composition and antinociceptive effect of the volatile oil of *O. basilicum* flowers using adult zebrafish (*Danio rerio*) (n = 6/group) treated orally (20 µl) with volatile oil (0.25, 1.25, or 2.5 mg/ml; 20 µl) or vehicle (0.9% NaCl, 20 µl). The volatile oil of the *O. basilicum* flowers was obtained by hydrodistillation and analyzed by GC–MS, and the antinociceptive action is evaluated by different stimuli using motor parameters. The analysis of the chemical profile identified fourteen components with linalool (1) as a major chemical constituent (56.37%). The oral administration of volatile oil did not show any acute toxicity or behavior effects in all tested doses. The volatile oil has a pharmacological potential for the treatment of acute pain by modulation of opioid system, *N*-methyl-D-aspartate receptors (glutamatergic receptor), and the transient receptor potential vanilloid subtype 1 and acid-sensing ion channels. Together, these data provide support for analgesic properties of the volatile oil and contribute to suggest that the adult zebrafish model presents the cheapest, cost-effective pharmacological alternative for the discovery of novel analgesics.