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ATIVIDADE ANTINOCICEPTIVA E ANTI-INFLAMATÓRIA DO EXTRATO DAS FOLHAS DE *Aloysia gratissima* OBTIDO POR CO_2 SUPERCRÍTICO

Chapecó – SC, 2019

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Maryelen Alijar Souza

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Prof.ª Dr.ª Liz Giradi Muller

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Marycleu Alijar Souza

Essa dissertação foi julgada adequada para obtenção do grau de Mestre em Ciências Ambientais, APROVADA em sua forma final pelo Programa de Pós-Graduação *stricto sensu* em Ciências Ambientais da Universidade Comunitária da Região de Chapecó.

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RESUMO

Aloysia gratissima é uma planta nativa da América do Sul, com aplicações na medicina popular para uma ampla gama de doenças, como infecções brônquicas, distúrbios pulmonares, distúrbios do sistema nervoso (depressão, ansiedade), entre outros. Entretanto, estudos sobre sua atividade antiinflamatória e antinociceptiva são escassos. O objetivo deste trabalho foi avaliar a atividade anti-inflamatória e antinociceptiva de extratos de folhas de *A. gratissima* obtidas por extração supercrítica (CO_2). As folhas de *A. gratissima* foram extraídas com CO_2 supercrítico a 40, 50 e 60 ° C e 150, 175 e 200 bar por 120 min. Após a extração, a composição química dos extratos foi determinada por cromatografia gasosa acoplada a espectrometria de massas (GC-MS). O perfil antinociceptivo foi avaliado com o extrato de *A. gratíssima* (EAG) obtido a 60 ° C e 200 bar (densidade 724 kg / m3) (administrado por via oral: 1, 10 e 30 mg / kg) utilizando contorções abdominais induzidas por ácido acético e nocicepção induzida por formalina. Os ensaios de campo aberto e rota-rod foram utilizados para avaliar a possível interferência do EAG no desempenho motor de camundongos. O envolvimento do sistema opióide e dos canais de K^+ sensíveis ao ATP no mecanismo de ação do EAG foi avaliado por antagonismos farmacológicos. O teste de edema de pata induzido por carragenina foi usado para avaliar a atividade antiinflamatória do EAG (administrado por via oral na dose de 10 mg / kg). A toxicidade oral aguda do extrato foi investigada de acordo com a diretriz orientadora da OECD 423. Análises químicas mostraram a presença de sesquiterpenos (guaiol, pinocanfona, óxido de cariofileno e espatulenol) como os principais compostos presentes no extrato. EAG em doses de 10 mg / kg e 30 mg / kg reduziu significativamente o número de contorções abdominais em camundongos. No teste da formalina, o EAG reduziu o tempo de lambida da pata nas duas fases do teste e não afetou a atividade locomotora e a coordenação motora dos animais nos testes de campo aberto e rota-rod. O efeito antinociceptivo do EAG foi prevenido pela glibenclamida nas duas fases do teste da formalina, ao contrário do pré-tratamento com naloxona. No teste do edema da pata induzido por carragenina, o EAG reduziu significativamente o edema nas primeiras 3 horas após a injeção de carragenina. No teste de toxicidade, o EAG (2000 mg / kg; po) não causou a morte de nenhum dos camundongos durante os 14 dias de observação e não houve alteração significativa no peso relativo dos animais, no consumo de ração ou no peso significativo dos órgãos dos animais. As folhas de Aloysia livres exibem efeito antinociceptivo mediado por canais de K^+ sensíveis ao ATP, e toxicidade aguda acima de 2000 mg / kg, sendo classificado na categoria de toxicidade 5 de acordo com o Sistema Globalmente Harmonizado de Classificação - OECD 423.

Palavra-chave: A*loysia gratissima*; supercrítico fluído CO_2; antinociceptivo; anti-inflamatório

ABSTRACT

Aloysia gratissima is a plant native to South America, with applications in folk medicine for a wide range of diseases, such as bronchial infections, lung disorders, nervous system disorders (depression, anxiety), among others. However, studies on its antiinflammatory and antinociceptive activity are scarce. The objective of this work was to evaluate the anti-inflammatory and antinociceptive activity of extracts of *A. gratissima* leaves obtained by supercritical extraction (CO_2). The leaves of *A. gratissima* were extracted with supercritical CO_2 at 40, 50 and 60 ° C and 150, 175 and 200 bar for 120 min. After extraction, the chemical composition of the extracts was determined by gas chromatography coupled to mass spectrometry (GC-MS). The antinociceptive profile was evaluated with the extract of *A. gratíssima* (EAG) obtained at 60 ° C and 200 bar (density 724 kg / m3) (administered orally: 1, 10 and 30 mg / kg) using abdominal writhes induced by acetic acid and formalin-induced nociception. The open-field and rota-rod assays were used to evaluate the possible interference of EAG in the motor performance of mice. Involvement of the opioid system and the ATP-sensitive K + channels in the mechanism of action of GAS was assessed by pharmacological antagonisms. The carrageenan-induced paw edema test was used to evaluate the anti-inflammatory activity of EAG (administered orally at a dose of 10 mg / kg). The acute oral toxicity of the extract was investigated according to OECD directive 423. Chemical analyzes showed the presence of sesquiterpenes (guaiol, pinocamphone, caryophyllene oxide and espatulenol) as the main compounds present in the extract. EAG at doses of 10 mg / kg and 30 mg / kg significantly reduced the number of abdominal writhes in mice. In the formalin test, EAG reduced the paw lick time in the two phases of the test and did not affect the locomotor activity and the motor coordination of the animals in the open field and rota-rod tests. The antinociceptive effect of EAG was prevented by glibenclamide in the two phases of the formalin test, unlike naloxone pretreatment. In the carrageenan-induced paw edema test, EAG significantly reduced edema within the first 3 hours after carrageenan injection. In the toxicity test, EAG (2000 mg / kg; po) did not cause the death of any of the mice during the 14 days of observation and there was no significant change in the relative weight of the animals, in feed intake or significant weight changes animal organs. Free Aloysia leaves exhibit antinociceptive effect mediated by ATP-sensitive K + channels, and acute toxicity above 2000 mg / kg, being classified in toxicity category 5 according to the Globally Harmonized System of Classification - OECD 423.

Keywords: *Aloysia gratissima*; supercritical fluid (CO_2) antinociceptive; anti-inflammatory

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AINES – Anti-inflamatórios não esteroidais

ANOVA – Análise de Variância de uma via

CBi – Centro de Bioterismo

CEUA – Comissão de Ética no Uso de Animais

CIONS – Council for International Organizations of Medical Sciences

CO_2 – Dióxido de carbono

CFMV – Conselho Federal de Medicina Veterinaria

CCDR – Experimento composto central rotacional²

EAG – Extrato de *Aloysia gratissima*

FSC – Fluído supercrítico

FSC – CO_2 – Fluído supercrítico com dióxido de carbono

$SCCO_2$ – Fluído supercrítico com dióxido de carbono

GC/MS – Cromatografia gasosa/ Espectrômetro de massas

GAS – Gas antisolvente

INDO – Indometacina

I.P – Intraperitoneal

I.PL – Intraplantar

MS – Ministério da Saúde

OMS – Organização Mundial da Saúde

SBQ – Sociedade Brasileira de Química

SNC – Sistema Nervoso

SUS – Sistema Único de Saúde

UBS – Unidade Básica de Saúde

P.O – Via Oral

SUMÁRIO

CAPITULO I

1. INTRODUÇÃO

O Brasil é o país de maior biodiversidade do planeta que, associada a uma rica diversidade étnica e cultural que detém um valioso conhecimento tradicional associado ao uso de plantas medicinais, tem o potencial necessário para desenvolvimento de pesquisas com resultados em tecnologias e terapêuticas apropriadas (BARRETO et al., 2016).

A eficiência da atividade dos extratos vegetais depende da espécie utilizada, da concentração do princípio ativo presente na planta, da fonte de origem (caule, folha ou sementes, por exemplo), do método de obtenção e da estabilidade dos componentes ativos (KAMEL, 2000; BRUGALLI, 2003).

A biodiversidade brasileira é considerada uma importante fonte de substâncias biologicamente ativas, sendo essas de grande potencial para a produção de novos fármacos (BARREIRO; FRAGA, 1999, PIMENTEL et al., 2015). Os compostos químicos que são encontrados naturalmente podem ser utilizados como base para desenvolvimento de novas moléculas e também como princípios ativos (PIMENTEL et al., 2015). Segundo dados de Newman e Cragg (2016), mais de 50% do arsenal moderno de medicamentos utilizam substâncias e moléculas derivadas de produtos naturais, sendo que menos de 30% são moléculas exclusivamente sintéticas.

A grande variedade de compostos químicos estruturalmente complexos encontrados em diferentes plantas origina-se a partir da produção de metabólitos, que inclui um conjunto de reações químicas catalisadas por enzimas que ocorrem no interior das células. O metabolismo primário está envolvido nos processos básicos do vegetal como a fotossíntese e a respiração, enquanto que o metabolismo secundário está envolvido no processo de defesa do vegetal. Estes compostos bioativos destacam-se na área farmacológica devido aos efeitos produzidos sobre o sistema biológico humano. Como exemplos de metabólitos secundários podem ser citados antocianinas, flavonoides, terpenos, compostos fenólicos e alcaloides (PEREIRA; CARDOSO, 2012). As diversas metodologias existentes para a avaliação e comprovação de bioatividade (antimicrobiana, antioxidante, antineoplásica, antimutagênica, dentre outras) auxiliam na pesquisa e aplicação de tais produtos (SANTOS, 2012).

A família Verbenaceae compreende 31 gêneros, considerando mais importante a *Lippia* e *Aloysia*. O gênero *Aloysia* compreende 30 espécies que possuem registros de distribuição do sul dos Estados Unidos e México até o norte da Patagônia. São caracterizadas por suas

inflorescências aromáticas e algumas espécies conhecidas principalmente por seus óleos essenciais, a *Aloysia* é vastamente utilizada na forma de preparações caseiras, como chás, pois possui propriedades digestiva, diurética, calmante, cardiotônico, estimulante, ansiolítico e antigripais (THOMAS, 2015).

A busca do mercado pela utilização de produtos naturais na indústria alimentícia e farmacêutica que envolva técnicas de processo de engenharia, aplicações bioquímicas e pesquisas tecnológicas, que possam fornecer resultados alternativos as necessidades atuais, as preocupações econômicas e de segurança quanto a toxicidade relacionada aos métodos de extração de substâncias, a obtenção de compostos através de fluidos pressurizados, como o dióxido de carbono (CO_2) e o propano, tem se apresentado como alternativa, tratando-se de processos que utilizam solventes praticamente atóxicos, não inflamáveis e de baixo custo (LANÇAS, 2002; CORSO et al., 2010).

A extração por fluidos supercríticos explora as propriedades do seu composto próximo ao ponto crítico, as suas propriedades termodinâmicas são particularmente sensíveis às alterações de temperatura e pressão. Pequenas modificações na pressão ou temperatura geram mudanças na densidade, e, consequentemente, no poder de solubilização. Além disso, a etapa de separação entre solvente supercrítico e soluto requer apenas uma diminuição na pressão, retomando o estado gasoso do solvente, provocando a separação, diferentemente das técnicas convencionais que requerem novas operações de separação (CORSO, 2008).

Neste contexto o estudo de compostos presentes em matrizes vegetais e sua extração, através do método de fluido supercrítico, é importante para se conhecer melhor as possíveis atividades biológicas *in vivo* utilizando extratos com maior pureza. Não existem relatos na literatura de estudos sobre *A. gratissima* submetida a extração por fluido supercrítico, apenas estudos preliminares por extratos obtidos por solventes orgânicos. Considerando as evidências apontadas pela literatura (VANDRESSEN et al. 2010) sobre o potencial anti-inflamatório da espécie *A. gratissima,* este trabalho teve por objetivo a obtenção de extratos das folhas extrato de *A. gratissima* a partir de extração supercrítica (CO_2), determinar a composição química e avaliar a atividade anti-inflamatória e antinociceptiva.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

O objetivo geral desta pesquisa é a obtenção de extratos a partir das folhas de *Aloysia gratíssima* utilizando o CO_2 supercrítico e investigar da sua atividade anti-inflamatória e antinociceptiva.

1.2.2 Objetivos Específicos

- Obter extratos das folhas de *A. gratissima* utilizando dióxido de carbono supercrítico como solvente;
- Avaliar os efeitos das variáveis temperatura e densidade do fluido sobre o rendimento e composição química dos extratos;
- Identificar e quantificar os componentes químicos presentes nos extratos;
- Avaliar o efeito antinociceptivo do extrato em modelos que utilizam nocicepção química (teste do ácido acético e fase I do teste da formalina) em camundongos;
- Avaliar o possível efeito anti-inflamatório do extrato em modelos que utilizam nocicepção inflamatória (Fase II do teste da formalina e teste da carragenina) em camundongos;
- Avaliar o envolvimento do sistema opioide e de canais de K^+ no mecanismo de ação antinociceptiva do extrato, através de antagonismos farmacológicos, no teste da formalina em camundongos.
- Avaliar o efeito do extrato sobre a atividade locomotora/exploratória e coordenação motora de camundongos utilizando o teste de campo aberto e o teste do Rota Rod;
- Avaliar a toxicidade aguda do extrato através da diretriz 423 da OECD em camundongos.

1.3 FUNDAMENTAÇÃO TEÓRICA

Estão abordados os principais conceitos encontrados e disponíveis na literatura sobre o tema deste trabalho. Destacam-se os testes de atividade anti-inflamatória e antinociceptiva *in vivo,* além das técnicas empregas para extração (detalhadamente, as técnicas de fluido supercrítico) e identificação dos compostos químicos presentes nas folhas de *Aloysia gratissima*.

1.3.1 *Aloysia gratissima* (Gillies & Hook.)

Aloysia gratissima (Gillies & Hook.) popularmente conhecida como alfazema-do-Brasil, erva-de-nossa-senhora, erva santa e garupá, é amplamente distribuída nas Américas, ocorrendo desde os Estados Unidos até a Patagônia (RICCIARDI et al., 2000).

Aloysia gratissima (Verbenaceae) é uma planta aromática distribuída na América do Sul e empregada na medicina popular para o tratamento de distúrbios do sistema nervoso, incluindo a depressão (ZENI, 2013).

Na América, o gênero *Aloysia* é nativa do México ao nordeste da Argentina, e concentra-se na região Sul do Brasil. Esta espécie é um arbusto que pode ter no máximo três metros de altura, com padrão irregular do seu crescimento e pode apresentar espinhos nos ramos. Suas folhas são simples, contrárias, algumas vezes alternadas, inteiras e dentadas, lanceoladas, macias e subcoriáceas. Os brotos são fortes e herbáceos, as flores são brancas, fragrantes, em agrupamentos axilates solitários ou geminados, com floração intensa. A folhagem é persistente e floresce entre a primavera e verão. Esta planta é ornamental, devido à intensidade da floração e ao aroma agradável das flores (RICCIARDI et al., 2000, FRANCO et, al.,2007), como apresentado na Figura 1.1.

Espécies desta família tem despertado interesse tanto pelas suas propriedades biológicas, quanto pelo uso na medicina tradicional. Entre os gêneros mais importantes se encontram *Lippia* e *Aloysia* (RICCO et al., 2010, THOMAS, 2015). Devido às propriedades medicinais, esta espécie vem sendo muito difundida na medicina popular sul-americana (SOUZA et al., 2007).

Figura 1.1. *Aloysia gratissima.*

(Fonte: Rolim, 2016)

Ricciardi et al. (2006), relatam que a composição química do óleo tem como componentes principais β-elemeno, viridiflorol e β-cariofileno. O extrato metanólico de *A. gratissima* tem atividade antioxidante (ROSAS-ROMERO & SAAVEDRA, 2005) e, conforme Vandresen et al. (2010), também possui efeitos antibacteriano e antiedematogênico. Em relação a outras espécies deste gênero, os extratos de *A. polystachya* e *A. virgata* mostraram efeito ansiolítico (MORA et al., 2005; WASOWSKI & MARDER, 2010), enquanto *A. polystachya* apresentou efeito antidepressivo (HELLIÓN-IBARROLA, et al., 2008).

De acordo com Souza & Wiest (2007), no estado do Rio Grande do Sul, *A. gratissima* é utilizada principalmente para dores de cabeça, "problemas de nervos, distúrbios dos sistemas digestivo e respiratório, como gripes e bronquites". No estado de Santa Catarina é utilizado como sedativo e para "tristeza". Na Argentina, também é utilizada para "problemas de nervos" como "levantar el ánimo" e como digestiva (DEL VITTO et al., 1997; ARIAS TOLEDO, 2009; DADÉ et al., 2009; ZENI, 2011).

Em relação ao mecanismo de ação da *A. gratíssima* na neuroproteção e no efeito tipo antidepressivo observados pelo estudo de Zeni et al., (2013), exerce efeitos benéficos através do transporte de glutamato. Ou ainda, poderia estar exercendo um efeito antagônico sobre os receptores NMDA, prevenindo a excitotoxicidade, o desequilíbrio no transporte de glutamato, o excesso de radicais livres e, a morte celular.

A *A. gratíssima* possui agentes antidepressivos envolvendo a participação dos receptores NMDA e a via L-arginina/NO-cGMP, além disso, estudos relatam o aumento da fosforilação de Akt e a redução da expressão de iNOS. Os receptores serotoninérgicos, noradrenérgicos e dopaminérgicos também tem relação com o efeito antidepressivo (ZENI, 2013).

1.3.2 Extração por fluído supercrítico

A crescente preocupação dos consumidores com o uso de substâncias sintéticas na indústria de alimentos e farmacêutica vem desencadeando interesses em pesquisas na área de processamento de biomateriais, associado ao controle da poluição adotado pelos governos e ambientalistas internacionais, têm contribuído para o desenvolvimento de tecnologias limpas (MICHIELIN, 2002; CAPELLETO, 2016).

A extração de compostos de fontes naturais é a aplicação mais estudada dos fluidos supercríticos, sendo um processo flexível, com possibilidade de alteração precisa do poder e seletividade do fluído supercrítico, assim como fácil separação do solvente e extrato, possibilitando a recuperação do solvente pelo ajuste de pressão e/ou temperatura (REVERCHON; DE MARCO, 2006).

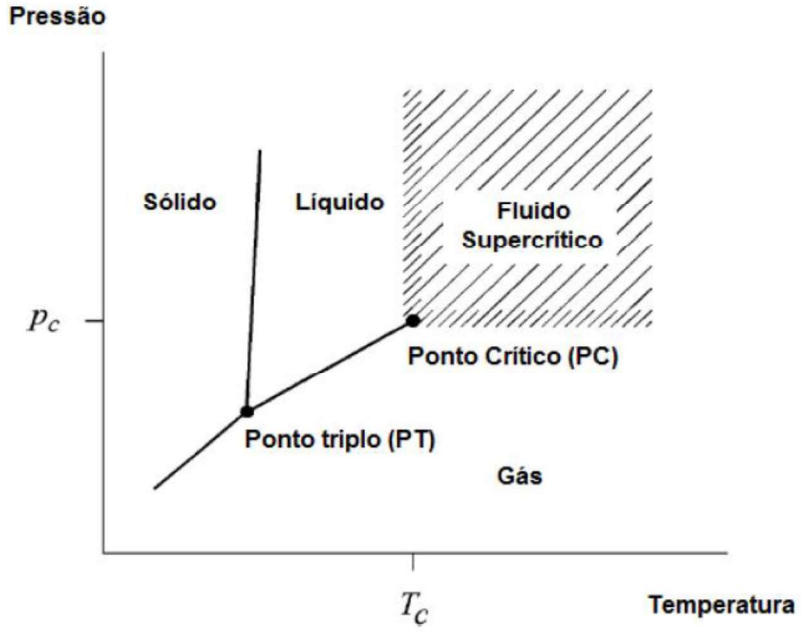
Segundo Mukhopadhyay (2000), a tecnologia que mais obteve crescimento nas últimas décadas, em métodos de extração de produtos naturais é a técnica de Extração com Fluídos Supercríticos. O ponto onde a combinação entre temperatura e pressão tornam indistintas as fases líquida e gasosa de uma substância é chamado de ponto crítico. Assim o fluido supercrítico é definido como uma substância mantida acima de sua temperatura e pressão crítica (ARAUJO, 2006). A temperatura crítica (Tc) é a temperatura mais alta, na qual o gás pode ser convertido em líquido pelo aumento da pressão. A pressão crítica (Pc) é a pressão mais elevada, na qual o líquido pode ser convertido em gás pelo aumento da temperatura do líquido. A área em torno do ponto crítico pode ser chamada de região crítica, abrangendo condições sub e supercrítica (COSTA, 2013).

Caso uma das variáveis de pressão ou temperatura se encontre abaixo do ponto crítico, diz-se que a substância se encontra no estado subcrítico e, quando ambas estão acima dele, o fluido se encontra em estado supercrítico. O diagrama de fases (Figura 1.2) de uma substância pura demonstra a região de temperatura e pressão onde é evidenciado o estado supercrítico.

As propriedades dos fluidos supercríticos são intermediárias entre as do estado líquido e gasoso. A densidade (variável na região supercrítica) é considerada elevada e as boas

solubilidades são semelhantes os do estado líquido. Propriedades como viscosidade baixa, difusividade e compressibilidades altas se assemelham com as do estado gasoso. A difusividade no estado supercrítico é duas vezes maior que do estado líquido em magnitude (BRUNNER, 2005; CORREA, 2016). Uma das propriedades mais úteis afetadas por este procedimento, é a mudança da constante dielétrica da substância. O CO_2 por exemplo, que é apolar em condições normais, em altas pressões tem constante dielétrica similar a solventes polares em condições normais (LANÇAS, 2002).

Figura 1.2. Representação genérica do diagrama de fases de uma substância pura.



(Fonte: Correa, 2016).

O CO_2, principal fluido supercrítico utilizado, possui baixa toxicidade, é quimicamente inerte, não é inflamável, é barato, apresenta possibilidade de ser reutilizado no processo e possui condições supercríticas relativamente brandas (temperatura crítica (Tc) 31,1 °C e pressão crítica (Pc) 73,8 bar (MORANDI et al., 2013).

O dióxido de carbono é gasoso à temperatura e pressão ambiente, o que o torna muito simples a recuperação do extrato livre de solvente. Além disso, as extrações com fluido supercrítico com utilização do dióxido de carbono podem ser conduzidas em temperaturas amenas, o que permite a extração de compostos termolábeis ou facilmente oxidáveis (HERRERO et al., 2010). O dióxido de carbono apresenta propriedades favoráveis de transporte, como a baixa viscosidade, altos coeficientes de difusão e condutividade térmica e entalpia de vaporização adequada, especialmente próxima ao ponto crítico (MANTELL et al,

2013, SOARES, 2015).

A utilização do dióxido de carbono apresenta baixa polaridade que pode ser considerada uma desvantagem quando se objetiva obter compostos mais polares, este problema que pode ser superado utilizando co-solventes (etanol, água, etc.) para mudar a polaridade do fluido e aumentar seu poder de solvatação para os compostos polares de interesse (HERRERO et al, 2010). O estudo de Kraujalis e Venskutonis (2013) concluíram em seu trabalho que a adição de 2-5% de etanol na FSC-CO_2 de esqualeno e tocoferóis, a partir do amaranto, pode aumentar o rendimento em mais de 2 e 3 vezes, respectivamente, e melhorar a capacidade antioxidante dos extratos.

Especificamente, no campo dos extratos vegetais e óleos essenciais o método de extração com fluidos supercríticos tendo dióxido de carbono como solvente é uma alternativa promissora tendo em vista que trata-se de um processo livre de resíduos tóxicos, que não provoca a degradação térmica dos extratos e não requer grandes gastos com energia na eliminação do solvente, principal vantagem quando comparado a processos de extração utilizando solventes convencionais (CAPELETTO et al., 2016; DÍAZ-REINOSO et al., 2006).

1.3.3 Caracterização dos compostos químicos de origem vegetal

A síntese e a acumulação dos compostos químicos nas plantas dependem de vários fatores, sendo os genéticos, ontogênicos, morfogenéticos e ambientais (temperatura, disponibilidade hídrica, altitude, radiação e nutrientes) os mais importantes (VERMA; SHUKLA, 2015).

Os extratos extraídos de espécies vegetais têm em sua composição os metabólitos secundários que são classificados de acordo com a sua origem biossintética em três grupos principais: terpenos, alcaloides e compostos fenólicos. Os terpenos são hidrocarbonetos cíclicos insaturados, com diferentes funções oxigenadas, com subgrupos ligados a um esqueleto carbônico. Os alcaloides são bases orgânicas que possuem um átomo de nitrogênio ligado a uma estrutura cíclica de 5 ou 6 carbonos. Os compostos fenólicos são compostos aromáticos com uma ou mais hidroxilas; a maioria são polifenóis, com grupo hidroxi substituído por um grupo metil ou glicosil (HERBERT 2012, SCAPINELLO et al., 2018).

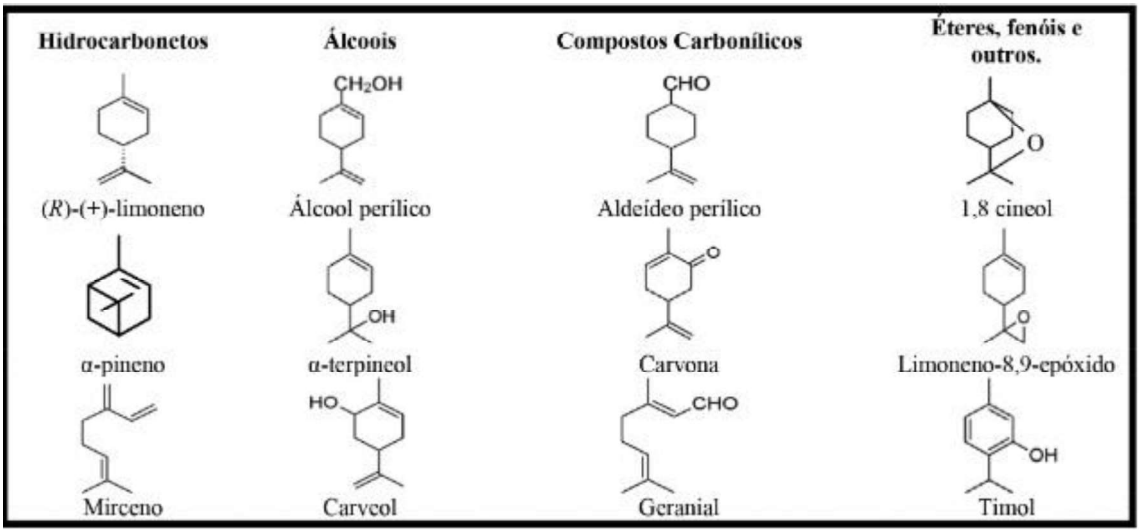
Além disso, os componentes dos extratos podem ser divididos em dois grupos: (i) compostos terpênicos e (ii) compostos aromáticos (PICHERSKY, NOEL, & DUDAREVA, 2006; BAKKALI et al., 2008). Possuem uma composição química complexa de terpenos e fenil propanoides, muitos tendo atividade antimicrobiana (SANTOS, et al., 2013). Os terpenos

encontrados com maior frequência são os monoterpenos e sesquiterpenos, bem como os diterpenos, constituintes minoritários dos óleos essenciais e extratos (CASTRO et al., 2010). Esta diversidade química também pode ser aumentada por modificação durante o processo de extração por ativação térmica de reações químicas.

Os terpenos podem ser definidos como "alcenos naturais", isto é, apresentam uma dupla ligação carbono-carbono sendo caracterizado como um hidrocarboneto insaturado (MCMURRY, 2011), constituem a maior classe de produtos naturais com mais de 55.000 compostos conhecidos (CHANG et al., 2010). Estes produtos químicos são parte do metabolismo secundário espécies vegetais e animais e são derivados de unidades de isopreno (C5), que se unem através de ligações cabeça-cauda por duas vias biossintéticas. As estruturas típicas dos terpenos contêm um esqueleto carbônico representado por (C5)n e são classificados como hemiterpenos (C5), monoterpenos (C10), sesquiterpenos (C15), diterpenos (C20), sesterterpenos (C25), triterpenos (C30) e tetraterpenes (C40) (DEWICK, 2009).

Por outro lado, se um terpeno contém oxigênio, o mesmo é denominado de terpenoide, podendo apresentar diferentes funções químicas, entre as quais: ácidos, álcoois, aldeídos, cetonas, éteres, fenóis (Figura 1.3).

Figure 1.3. Estruturas de metabólicos secundários com presença de terpenos.



(Fonte: Felipe and Bicas, 2017)

Esses compostos são amplamente utilizados na indústria cosmética e alimentícia por suas características antimicrobiana e aromática, enquanto suas outras atividades tais como anti-inflamatórias, analgésicas e antioxidantes os tornam moléculas de grande valor terapêutico (MATOS, 2007; VOGT-EISELE *et al.*, 2007).

Atualmente, os metabólitos secundários, ou ainda, produtos naturais, desempenham importante papel na descoberta de mecanismos para o desenvolvimento de drogas, como por exemplo, a manipulação de vias biossintéticas, visando à produção de substâncias sintéticas com atividades farmacológicas tão eficazes quanto as encontradas naturalmente, buscando a formulação de novos agentes ativos para o tratamento de doenças.

1.3.4 Atividade antinociceptiva e anti-inflamatória

A resposta inflamatória representa um dos mecanismos de defesa do organismo. A palavra inflamação pode ser apropriadamente referida como uma cascata inflamatória, e que consiste de uma longa cadeia de reações e atividades celulares com o intuito de reparar o tecido, em que ocorre uma lesão ou destruição celular, ocorrendo atividades celulares, aumento de arteríolas e vênulas, assim como aumento da permeabilidade vascular e fluxo sanguíneo, como consequência, o acúmulo de líquidos (LAWRENCEETAL., 2002; CHOI, 2012).

A inflamação é originária de uma lesão tecidual, causada por traumas, bactérias, agentes químicos, calor, etc. A lesão celular associada à inflamação atua sobre as membranas celulares, liberando enzimas lisossômicas pelos leucócitos, que consequentemente ocasiona a liberação de ácido araquidônico, a partir de precursores (GUYTON, 2011). A inflamação é caracterizada como uma reação imunitária inata, possuindo três papéis fundamentais contra a infecção nos tecidos lesados: atrair mediadores químicos e células imunitárias para o local; gerar uma barreira física que retarda a disseminação da infecção; promover o reparo tecidual quando a infecção se encontra sob controle (SILVERTHORN, 2010).

Os anti-inflamatórios não esteroides (AINEs) são medicamentos de primeira linha utilizados para reduzir os eventos nocivos associados à inflamação. Esses fármacos exibem eventos adversos importantes, que vão desde irritação gástrica e úlceras até toxicidade hepática e insuficiência renal em administração crônica (KUNANUSORN ET AL., 2009). Essas desvantagens do uso de AINEs podem ser minimizadas quando os mesmos são substituídos por compostos derivados de plantas medicinais relativamente mais seguros e eficientes (KHAN ET AL., 2011). Além disso, as deficiências dos fármacos disponíveis para o tratamento de doenças inflamatórias crônicas, como a artrite, conduziram a descoberta de novos agentes medicinais a partir de fontes vegetais (KHAN ET AL., 2013).

Os modelos animais são utilizados na pesquisa de compostos com atividade antinociceptiva e/ou anti-inflamatória. A investigação da dor pode ser abordada em condições

de normalidade fisiológica ou patológica (CASTRO-COSTA, 2009). Segundo Lapa (2007), os modelos *in vivo* são de grande importância e representam um ponto de partida para a caracterização farmacológica de novos compostos, incluindo aqueles extraídos de plantas e que são utilizados como anti-inflamatórios e analgésicos.

Os animais não apresentam capacidade de comunicar verbalmente a ocorrência da dor, quando são submetidos a um estímulo nocivo, eles exibem respostas comportamentais, que é chamado de comportamento nociceptivo. A nocicepção consiste na recepção dos estímulos pelos receptores que são ativados por diferentes estímulos nocivos (nociceptores) que codificam sinais para fornecer informações ao sistema nervoso central (SNC) da existência do agente causal da dor (NATIONAL RESEARCH COUCIL, 2009).

Entre os modelos animais mais utilizados na investigação pré-clinica de candidatos a fármacos uteis no tratamento da dor e inflamação, pode-se citar o teste de contorções abdominais induzidas por ácido acético, o teste da formalina e o teste do edema de pata induzido por carragenina. As contorções abdominais induzidas por ácido acético em camundongos, um modelo amplamente utilizado de dor visceral, é altamente sensível e útil para rastrear novos fármacos analgésicos (KOSTER, 1959). Este teste é sensível à avaliação de substâncias antinociceptivas e/ou anti-inflamatórias, no entanto, pode ser visto como um modelo geral, não seletivo, para o estudo desses compostos(COUTO et al., 2011), uma vez que a irritação local, produzida pela injeção intraperitoneal do ácido acético provoca a liberação de uma variedade de mediadores, tais como a substância P, bradicininas, prostaglandinas, bem como das citocinas pró-inflamatórias tais como IL-1, IL-6, IL-8 e TNF-α (PINHEIRO et al., 2011).

O teste da formalina é considerado um modelo de dor aguda, é um meio eficaz de avaliar a nocicepção de origem inflamatória e não inflamatória (STEINARANDKJELL, 1987). Durante este teste, os animais apresentam duas fases distintas do comportamento nociceptivo, que provavelmente envolvem diferentes estímulos. A fase aguda, iniciada imediatamente após a injeção de formalina dura cerca de 3 a 5 min. A fase tardia, iniciada 15-20 min após a injeção de formalina, dura cerca de 20 a 30 min. A dor na primeira fase surge devido à estimulação química dos nociceptores. A bradicinina está envolvida na fase inicial, enquanto a histamina, a 5-hidroxitriptamina (5-HT), as prostaglandinas e a bradicinina estão envolvidas na fase tardia (TJOLSEN et al., 1992).

O teste do edema induzido por carragenina tem sido frequentemente utilizado para avaliar o efeito anti-edematogênica de produtos naturais em roedores (THOMAZZI et al., 2010). A resposta inflamatória induzida pela carragenina é caracterizada por ser bifásica, com

a formação de edema resultante da rápida produção de vários mediadores inflamatórios (MENDES et al., 2010). A fase inicial (60-90 minutos após a administração de carragenina) é atribuída a uma liberação de histamina, serotonina e bradicinina, enquanto a fase tardia (4-6 horas após a administração de carragenina) é caracterizada pelo aumento da produção de prostaglandinas (OGONOWSKI et al., 1997). A resposta inflamatória normalmente é quantificada pelo aumento no tamanho da pata (edema) sendo modulada por inibidores de moléculas específicas dentro da cascata inflamatória, como os anti-inflamatórios não-esteroidais (MORRIS, 2003).

Durante o processo da inflamação o uso de produtos naturais pode contribuir no tratamento da dor, podendo representar uma alternativa promissora em reduzir o uso de analgésicos potentes, como os opioides, que apresentam importantes efeitos colaterais (SOUZA et al., 2014).

Opioide é um termo geral usado para identificar qualquer substância, natural ou sintética, cuja ação analgésica é semelhante aos efeitos da morfina e que possuam a naloxona como antagonista (RANG et al., 2008). Os estudos farmacológicos confirmaram, por clonagem dos receptores, que existem três tipos principais de receptores de opioides, chamados de: μ, k e δ que medeiam os principais efeitos farmacológicos dos opioides (WAY et al., 2002;). Esses receptores podem, ainda, ser subdivididos em diferentes subtipos: μ_1, μ_2, k_1, k_2, k_3, δ_1, δ_2 (KRAYCHETE, 2002).

Acredita-se que os receptores μ sejam responsáveis pela maioria dos efeitos analgésicos dos opioides; os receptores δ, provavelmente são mais importantes na periferia, mas também podem contribuir para a analgesia central e os receptores k contribuem para a analgesia no sítio espinhal. Em nível molecular, os receptores opioides se acoplam às proteínas Gi e assim, afetam a regulação dos canais iônicos, modulam o processamento do Ca^{++} e alteram a fosforilação das proteínas (KATZUNG, 2017).

Os opioides agem sobre os neurônios através de duas ações: fechando os canais de Ca^{++} regulados por voltagem nas terminações nervosas pré-sinápticas e, portanto, reduzem a liberação de transmissor; e hiperpolarizam e, assim, inibem neurônios pré e pós-sinápticos através da abertura dos canais de K^+ (GUTSTEIN et al., 1998; KATZUNG, 2017). Esses efeitos sobre a membrana reduzem tanto a excitabilidade neuronal (aumentando a condutância do potássio e provocando hiperpolarização da membrana) quanto a liberação de neurotransmissores (devido à inibição da entrada de cálcio) (NOTTH, 1993; WAY et al., 2002).

A morfina e a maioria de seus análogos exercem seus efeitos analgésicos atuando principalmente nos receptores μ. Os agonistas dos receptores δ também são analgésicos potentes, só que não atravessam a barreira hematoencefálica e por essa razão, precisam ser administrados via intratecal. Já os agonistas k seletivos induzem analgesia mediada principalmente nos locais espinhais. No entanto, em relação aos efeitos neurais, estudos demonstram que os agonistas μ e k exercem efeitos antagônicos, enquanto o primeiro provoca euforia, o segundo causa efeitos disfóricos (GUTSTEIN; AKIL, 2003; MERRER et al, 2009). Os fármacos opioides são frequentemente usados para alívio da dor. Fármacos não opioides, como anti-inflamatórios não esteroides (AINEs), corticosteróides e anticonvulsivantes (que possuem mecanismos antinociceptivos), também são frequentemente usados, particularmente para o alivio da dor neuropática (RODRIGUEZ AND DAVOUDIAN, 2016).

Os canais de K^+ sensíveis à adenosina trifosfato -ATP- (K_{ATP}) pertencem à superfamília dos Kir (SALOMONSSON, 2000). Alguns estudos têm avaliado a atividade dos canais de K^+ na sepse em diferentes espécies, como ratos (SORDI et al., 2010), ovelhas (LANGE et al., 2006), suínos (VANELLI et al., 1995), e também em humanos (MORELLI ORELLI et al., 2007). Busserolles et al., (2016), Tsantoulas et al., (2015) e Bayliss et al., (2008) destacam que os canais de K são importantes no processamento da dor crônica de origem neuropática.

Inúmeros estudos demonstraram que agonistas de vários receptores acoplados à proteína G (como os receptores μ e δ-opioides, adrenoceptores α2, receptores GABAB, etc.) abrem canais específicos de K^+ em neurônios e produzem antinocicepção. Dessa forma, é evidente que a abertura do canal de K^+ está envolvida na antinocicepção induzida por numerosos fármacos. Consequentemente, os canais de K^+ são considerados para o desenvolvimento de novas substâncias antinociceptivas, que abram os mesmos e, interagindo diretamente com os canais de K^+) para reduzir a dor aguda e crônica (OCAÑA et al., 2004)

Os canais de potássio pertencem a família de proteínas da membrana, desempenhando papel essencial aos processos fisiológicos do organismo, entre eles a redução da velocidade cardíaca, a redução da contração muscular, secreção de insulina, entre outros (WICKENDEN, 2002). A variedade dos canais de K^+ sensíveis a neurotransmissores, hormônios e toxinas, faz com que os estudos dos mesmos sejam cada vez mais presentes no desenvolvimento de fármacos com ação analgésica.

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CAPITULO II

Os resultados apresentados neste capítulo estão escritos no formato de artigo científico, intitulado "Supercritical CO_2 extraction from *A. gratissima* leaves and evaluation of anti-inflammatory activity and acute toxicity" que será submetido à revista *Industrial Crops*.

Abstract

Aloysia gratissima is a plant native to South America, with applications in the folk medicine to treat a wide range of diseases such as bronchial infections, lung disorders, nervous system disorders (depression, anxiety), among others. However, scientific studies on its anti-inflammatory activity are scarce. This work aims to evaluate the temperature and pressure parameters in relation to the yield and chemical composition of the supercritical CO_2 extraction of *A. gratissima* leaves (EAG) and then to investigate the anti-inflammatory activity as well as the toxicity of this extract *in vivo*. The leaves of *A. gratissima* were extracted with supercritical CO_2 at 40, 50 and 60 ºC and 150, 175 and 200 bar for 120 min. After extraction, the chemical composition of the extracts was determined by gas chromatography coupled to mass spectrometry (GC-MS). The anti-inflammatory activity was performed with the EAG extract obtained at 60 ° C and 200 bar (density 724 kg/m^3). Additionally, the oral acute toxicity of the EAG was assessed by the OECD guideline 423 (2001). The anti-inflammatory activity was investigated by the carrageenan-induced paw edema. The temperature of 60 ° C and pressure of 200 bar resulted in a better extract yield (4.2%) and temperature of 35.8 ºC and a pressure of 150 bar showed a yield of 0.16% being the lowest that we obtained in extraction. The chemical analysis showed that there was no significative change and/or degradation of the extracts' chemical compounds under the temperature and pressure conditions evaluated of this study. The chemical analysis showed the presence of guaiol, pinocamphone, caryophyllene oxide and spatulenol (sesquiterpenes) as

the main chemical components of the EAG. These compounds may have contributed to the significant reduction of carrageenan-induced paw edema in the first 3 hours after the injury induction. The toxicity test showed that the EAG did not cause death of any mice during the 14 days of observation. There was no significant reduction in the weight of the animals, neither in the food consumption, and there were significant changes in the relative weight of the spleen and adrenal glands of EAG-treated animals. We conclude that *A. gratissima* leaves supercritical extract presents anti-inflammatory activity for a period up to 3 hours and is devoid of acute toxicity (LD50 above 2000 mg/kg, category 5 according to the Harmonized Global Classification System).

Keywords: Supercritical CO_2; *Aloysia gratissima*; anti-inflammatory activity, acute toxicity.

2. 1. Introduction

The species belonging to the Verbanecea family have more than 70% hydrocarbon compounds, of which more than half are sesquiterpenes (Franco, 2007), among the plants belonging to this family, we highlight *Aloysia gratissima*, a native aromatic plant from southern Brazil (Zeni et al., 2011).

A. gratissima is used in folk medicine, being investigated for virucida (Garcia et al., 2018), nematicide (Duschatzky et al., 2004), antioxidant (Zeni et al., 2013a), antidepressants and neuroprotective agents (Zeni et al., 2011) and antibacterial and antiedematogenic (Vandresen et al., 2010).

Inflammation is a reaction of the body to infection or tissue damage. According to Trevisan et al., (2014), inflammation is an important defense mechanism of the host, being characterized by redness, swelling, pain, heat and dysfunction of tissues and organs. Non-steroidal anti-inflammatory drugs (AINEs) are first-line drugs used to reduce the harmful

events associated with inflammation. These drugs exhibit important adverse events, ranging from gastric irritation and ulcers to liver toxicity and chronic renal failure (Kunanusorn et al., 2009). These disadvantages of the use of AINEs can be minimized when they are replaced by relatively safe and efficient medicinal plant derived compounds (Khan et al., 2011). In addition, drug deficiencies available for the treatment of chronic inflammatory diseases, such as arthritis, have led to the discovery of new medicinal agents from plant sources and new technologies (Khan et al., 2013).

Supercritical fluid extraction ($SCCO_2$) appears as an alternative extraction using pressurized fluids, the extraction of plant matrices is the classic, safe and well established application of fluid technology products (Fernandes et al., 2016). Carbon dioxide is usually the most commonly used solvent for $SCCO_2$ extraction because of its properties, carbon dioxide used at mild temperatures provides solvent-free extracts and thermosensitive compounds (de Melo et al., 2014).

We know that *A. gratissima* studies are scarce in anti-inflammatory activity, as well as their toxicity. In this sense, the objective of this work is to investigate the anti-inflammatory and toxicological effects of *A. gratissima* leaves extract obtained by SCCO2 at different temperatures and pressures and the identification of the chemical components present in the extract.

2.2. Materials and methods

2.2.1. Drugs and reagents

Carbon dioxide (99.9% non-liquid purity phase) was purchased from Air Liquide. Dichloromethane (DCM 99.5%). Indomethacin and carrageenan were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2.2. Plant material

Leaves of *A. gratissima* were collected in December 2017 (summer), in the municipality of

Erval Grande, RS, in the south of Brazil (27°23'14.3S, 52°33'49 'W). This region presents a humid subtropical mesothermic climate. The specimens of plant were deposited in the Herbarium of the Community University of Chapecó (Herbário Unochapecó, SC, Brazil) under the accession number UNO 3700. After collection, the leaves were manually separated and dried at room temperature for five days, after being packed in plastic bags, identified and stored at 4 ºC until extraction process.

2.2.3. Extraction of the Aloysia gratissima leaves by $SCCO_2$

The experimental extraction apparatus and procedure have been described in detail in other studies of the research group (Capeletto et al., 2016; Scapinello et al., 2014). The *A. gratissima* leaves were extracted by $SCCO_2$ at a temperature of 40, 50 and 60 °C and pressure of 150, 175 and 200 bar. Approximately 11.03 ± 0.09 g of *A. gratissima* leaves were loaded in the extraction vessel. Then, CO_2 was pumped into bed, which was had two 300 mesh wire disks at both ends and was held in contact with the sample array to allow the system to stabilize at the same condition as the experiment by 30 min. After stabilization the extract from *A. gratissima* was then collected by opening the micrometric valve by 2h of extraction time.

The experimental design used in this study was the CCDR (central composite 2^2), with 3 central points and 4 axial points. In Tables 2.1 and 2.2 we demonstrate the CCDR used.

Table 2.1. Coded and real levels of the independente variable

Independent variables	Level				
	-∞	-1	0	+1	+∞
Temperature (°C)	35.8	40.0	50.0	60.0	65.00
Pressure (bar)	139	150	175	200	210

The ∞ was calculated as a function of the number of independent variables (n = 2), in equation: $\propto = (2^n)^{1/4}$=1,41

Table 2.2. Central planning test of the $SCCO_2$ extraction of leaves of *A. gratissima*

Run	Temperature (ºC)	Pressure (bar)
1	-1 (40)	-1 (150)
2	+1 (60)	-1 (150)
3	-1 (40)	+1 (200)
4	+1 (60)	+1 (200)
5	0 (50)	0 (175)
6	0 (50)	0 (175)
7	0 (50)	0 (175)
8	-1.41 (36)	0 (175)
9	+1.41 (65)	0 (175)
10	0 (50)	-1.41 (139)
11	0 (50)	+1.41 (210)

The variable response was the yield of the extract obtained by $SCCO_2$. The results of the experiment were analyzed according to the software Statistica® 7.0, being analyzed as an analysis of variance (ANOVA) to estimate the statistical parameters, with $p < 0.05$ adopted as significant.

2.2.4. Gas chromatography coupled to mass spectrometry - GC/MS analysis

Extracts of *A. gratissima* (EAG) were analyzed by Agilent GC/MS (7890B) gas chromatography coupled to a quadripolar mass spectrometer (5977A) (Agilent Technologies, Palo Alto, CA, USA). The experimental conditions of the GC/MS system were described by (Scapinello et al., 2018) with some adjustments. Briefly, the system conditions were as follows: Agilent19091S ca-pillary column, dimension: 30 m × 250μm × 0.25μm. The mobile phase flow (carrier gas: He) was adjusted to 1.0 $mL \cdot min^{-1}$. The GC temperature program was

40.0 °C at 4.0 min to 240.0 °C at a rate of 10 °C min^{-1} and up to 300.0 °C at a rate of 40.0 °C·min^{-1} (maintained for 5 min). The injector temperature was 280.0 °C, sample injection volume 1μL, split ratio 1:20. The MS transferline temperature was set to 150.0 °C and the source of ions temperature was set at 230.0 °C. For GC–MS detection, an electron ionization system was used with ionization energy set at 70 eV, and mass range atm/z 40–400. The chemical components present in the extracts were identified by comparison with the equipment library (Agilent P/N G1033A). The relative amounts of each individual component were calculated using their respective peak areas in the chromatogram. To analyze, the extracts were solubilized in dichloromethane.

2.2.5. Animals

Male Swiss mice (25-35 g) were used in the behavioral experiments. Female mice (25-35 g) were used in the toxicity experiments, as recomended by the OECD guideline 423 (2001). The animals were bred at Unochapecó bioterium. A controlled environment kept the animals (22 ± 2 °C) with a light/dark cycle of 12 hours (lights on at 6:00 a.m. to 6:00 p.m.), fed standard laboratory feed and water *ad libitum*. Animal care and experiments were conducted in accordance with the ethical principles of animal research, approved by the Ethics Committee of the Chapecó Regional Community University (Approval number 004-18), in accordance with Brazilian law No. 11794 (Brazil, 2016, 2008) and Council of the European Communities; Directive of 24 November 1986 (86/609/EEC). The animals were fasted for a period of 2 hours (no water restriction) prior to administration of any test substance.

Mice were treated with volumes of 10 mL/kg, according to their weight, by the oral route (p.o.) (by gavage), intraperitoneal (i.p.) and intraplantar (i.pl.) depending on the specific protocol of each experiment. Solubilization of the extract and substances was performed in saline (NaCl 0,9 %) with the aid of 1% Tween 80 (v/v) and ultrasound. The tested doses of the extract were chosen based on the study of Zeni et al. (2013a).

2.2.6. Acute toxicity

The acute toxicity test was based on the Organization for Economic Cooperation and Development (OECD) guideline 423 (2001), which is worldwide recognized as the standard reference tool for testing the toxicity of chemicals

The animals received a single dose of EAG at 2000 mg/kg by gavage (n = 6). The control group (n = 3) was orally treated with a single vehicle administration (0.9% NaCl + 1% Tween 80, 10 $mL \cdot kg^{-1}$). Thereafter, the animals were observed with special attention during the first 4 hours after treatment and daily for 14 days. The occurrence of mice death, and signals such as piloerection, palpebral ptosis, abdominal contortions, locomotion, hypothermia, muscle tone, shacking, hind paw paralysis, salivation, bronchial secretion and seizures were registered. In addition, body weight and food intake were recorded for 14 days. At the end of th experiment, the macroscopic aspect of the organs (liver, kidneys, adrenal glands, spleen, lungs, heart and brain) as well as their relative weight (%) was registered.

2.2.7. Carrageenan-induced paw edema

The animals were injected subcutaneously under the plantar surface of the right hind paw with 20 µL carrageenan (300 µg / paw, in 0.9% NaCl), 1 h after EAG (10 $mg \cdot kg^{-1}$), indomethacin (10 $mg \cdot kg^{-1}$) or vehicle (0.9% NaCl, 1% Tween 80, 10 $mL \cdot kg^{-1}$) administration. The test was performed in accordance to Batista et al. (2016). The left hind paw was injected with saline. The formation of the hind paw edema was described as Δpaw thickness = test paw thickness – basal paw thickness; also, the Δpaw weight was calculated as = inflamed paw weight – non inflamed paw weight. Paw thickness was measured using a caliper (Scapinello et al., 2019)

2.2.8. Statistical analysis

The results are expressed as the mean ± S.E.M. of *n* animals per group. Data were

analyzed by means of one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. The results from food consumption and mice weight gain were analysed by Two-way ANOVA with repeated measures. Data from relative weigh of the organs were analysed by unpaired t-test. Statistical analysis were performed using Graph Pad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA). Values of $p < 0.05$ were considered significant.

2.3. Results and discussion

2.3.1 Extraction and chemical profile

The extraction of the leaves of *A. gratissima* obtained by $SCCO_2$, resulted in the yield of 4.42%, being the greater yield, in temperature of 60ºC and pressure of 200 bar. However, at the lower temperature, 35.8 ºC and 175 bar pressure, the opposite happened, yielding 0.16%, the lowest yield found in our study (Table 2.3).

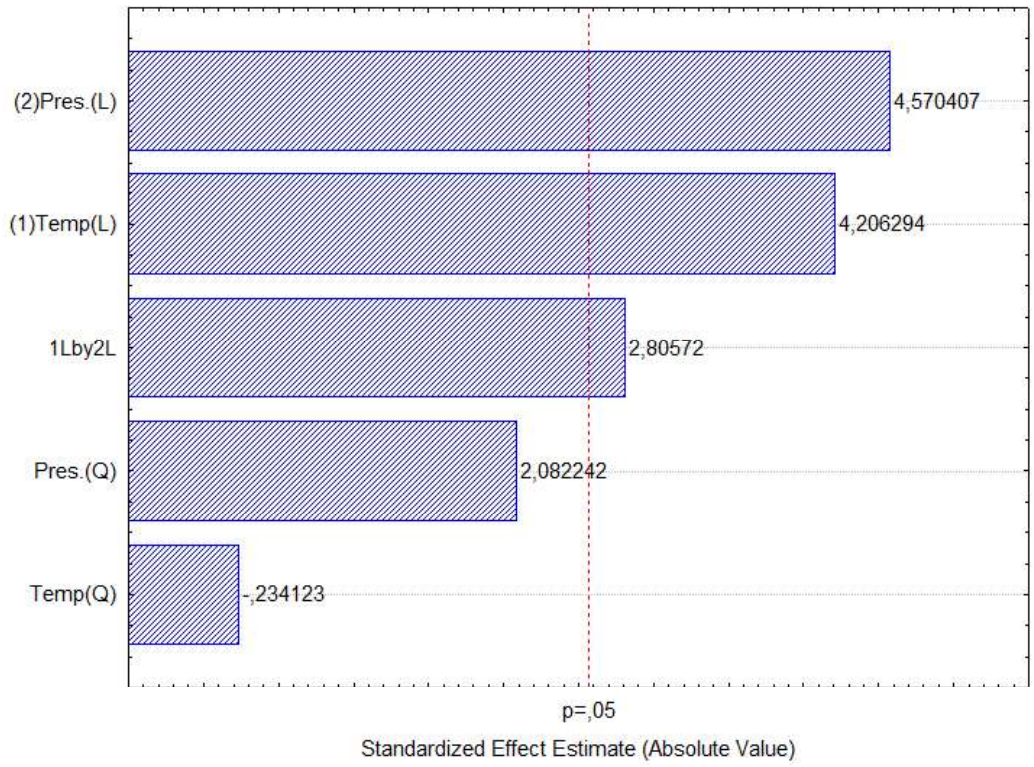
Table 2.3. Results of yields according to the temperatures, pressures and densities studied in the DCSC $SCCO_2$ extraction from EAG.

Run	Temperature (°C)	Pressure (bar)	Density (kg/m³)	Yield (%)
1	40.0	150	748.26	1.13
2	60.0	150	561.86	1.22
3	40.0	200	830.31	1.72
4	60.0	200	694.68	4.42
5	50.0	175	717.99	1.82
6	50.0	175	717.99	1.18
7	50.0	175	717.99	1.49
8	35.8	175	824.64	0.16
9	65.0	175	599.82	2.10
10	50.0	139	621.33	1.25
11	50.0	210	779.04	2.82

From the Pareto graph (Figure 2.1) it was possible to verify that the pressure and temperature were a more influential variable in the process. High binary temperature and pressure. This interaction between pressure and temperature acts positively on yield. It is also

identified when there is a negative effect, when the variables are at the minimum and at the maximum level. The interaction of the variables (1Lby2L) also proves the effect of temperature and pressure on the increase in yield. This effect may be associated with an interaction between energy levels that increases with the effect of CO_2 density and decreases the temperature, resulting in reduced solubility of the extract. Saldaña et al., (1997), which stresses that pressures greater than 190 bar, solubilization behaves with increasing temperature.

Figure 2.1. Pareto chart for the experimental design showing the effect of the temperature and pressure employed in the extraction process.



The highest value was 200 bar and temperature of 60 ° C (Table 2.3). This tendency was also reported by Haloui and Meniai (2017), but with pressures different from ours. The pressure was also determined by other authors as being a more influential variable with no extraction yield (Benelli et al., 2010; NYAN et al., 2010; MICHIELIN et al., 2005).

When the solvent pressure is increased, the CO_2 density increases, reducing the pressure vapor and raising the temperature under the isobaric conditions, indicating a retrograde zone, characterizing the decrease of solubility of the solute in CO_2, which can contribute to a reduction in the extraction efficiency (Scapinello et al., 2014).

Some authors have shown that the effect of pressure and temperature are directly related to the yield of extracts of plant material such as the supercritical method of CO_2. Scapinello, (2014), obtained extracts with supercritical CO_2 of the fruits of *M. Azedarach* in the range of 60ºC and 250 bar, considering its best productivity of 5.40%. Machmudah et al., (2008), using seeds of *Rosa canina* L. (Rosaceae), and that increased productivity with increasing pressure at all temperatures; with higher gains in pressures above 300 bar and higher temperatures.

The identification of the EAG chemical composition obtained by $SCCO_2$ by GC/MS allows us to identify volatile and terpene compounds. The recognition of the chemical compounds is presented in Table 2.4, being found major compounds of EAG, guaiol and pinocanfona, besides the presence of other compounds like pinocarvil, (-) - trans-pinocarvil acetate, γ-elemene, bunesol, caryophyllene, caryophyllene oxide, (-) - spatulenol, myrtenol, isopinocamphone, humulene, in smaller amounts respectively.

Our results support the results obtained by Trovati et al. (1997), in Cleveleng extraction of leaves of *A. gratissima* where the presence of isopinocampona (25.4%), guaiol (12.7%), pinocanfona (7.2%) and biosol (3.7%).

We noticed that there were no significant variations of the extracts compositions according to the pressures and temperatures operated during the research, this can be interconnected with the density of CO_2, that there were no significant changes.

The chemical compound guaiol, is a sesquiterpenoid alcohol, being found in medicinal plants, mainly in cypress and guaiacum woods. It has an aroma similar to the aroma of pine, according to the other terpene compounds. Guaiol has antibacterial and antitumor activities

(Yang et al., 2016). Additionally, guaiol was identified in a hexane extract from another sample of the species that grows in Brazil, along with espatulenol (Silva et al., 2006).

Other authors have identified similar compounds, as well as different proportions of guaiol in *A. gratissima* oils, such as 2.6% (Santos et al., 2013), 11.5% (Santos et al., 2015), 12.5% (Trovati et al., 2009), 6.7% (Benovit et al., 2015) and 6.27% (Franco, 2007). These differences can be attributed to the environmental conditions and the extraction method to obtain the extract or essential oil.

Table 2.4. Identification by GC / MS of the chemical compounds of the $SCCO_2$ extraction of the leaves of *A. gratissima.*

Chemical compound	**Area of each compound (%)**										
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Pinocarvil	3.43	3.11	2.60	3.61	4.17	4.37	3.75	4.55	4.44	4.05	4.04
Pinocamphone	9.36	10.47	8.70	8.89	12.15	12.68	7.34	12.20	12.49	12.23	11.08
Isopinocamphone	3.06	3.30	2.73	2.94	3.82	3.97	2.39	4.32	3.80	3.80	3.54
Myrtenol	3.31	3.13	2.57	2.85	3.95	3.64	4.03	4.25	4.22	3.49	3.89
(-)- trans-pinocarvila acetate	9.57	9.84	9.01	10.47	10.51	9.84	9.21	12.07	10.77	11.25	10.21
Caryophyllene	7.64	6.95	7.01	8.10	7.37	7.76	6.57	8.37	6.83	8.22	7.40
γ – Elemene	1.73	1.33	2.0	2.02	1.80	2.16	1.28	1.22	1.94	1.35	1.69
Humelene	2.83	2.23	2.79	2.61	3.0	2.37	2.98	2.48	2.42	3.20	3.03
(-)- Spathulenol	4.71	4.63	5.22	4.40	6.60	6.68	8.16	6.0	6.27	6.65	6.09
Caryophyllene oxide	3.9	3.82	5.77	6.40	6.67	7.08	8.46	5.69	7.19	7.28	6.78
Guaiol	23.5	21.44	21.16	22.04	29.39	19.99	24.06	20.36	19.63	20.08	20.42
Bunesol	7.77	7.54	8.43	7.91	7.41	6.26	10.46	3.75	8.41	5.03	8.29
Total	80.81	77.79	77.99	82.24	96.84	86.8	88.69	85.26	88.41	86.63	86.46

The second compound quantified in higher quantity was pinocamphone, with a high content of this compound in the EAG. Due to its high content of pinocamphone and isopinocamphone, the EAG can be compared to the extract of *Hyssopus officinalis*, which is widely marketed in the perfumery area (Silva et al., 2007).

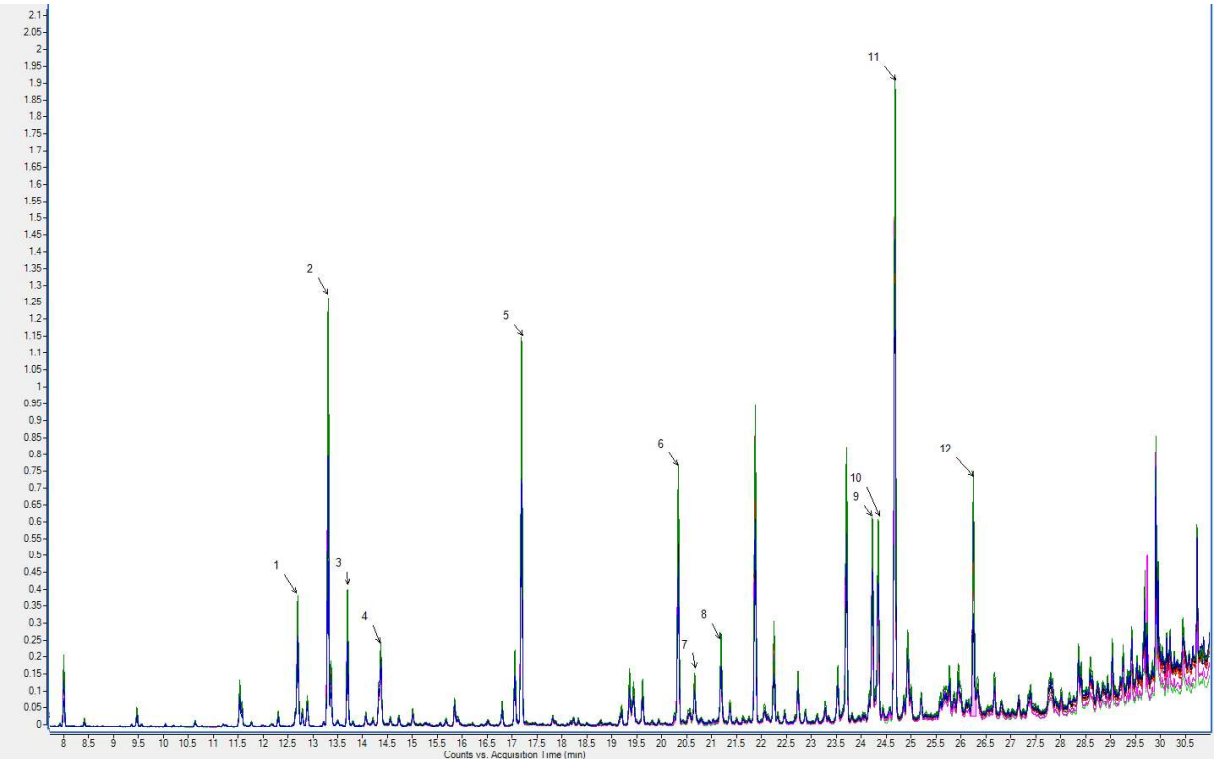
Duschatzky et al. (2004) reported in their study with *A. gratissima* as their main compound the caryophyllene oxide (15.8%) by the Clevenger extraction method. Caryophyllene oxide is linked to plant extracts, being of ecological importance to the plant, and a great value for the perfume industry due to its characteristic aroma, mainly the isomers of caryophyllene and α-humulene (Pereira et al., 2008). In addition, caryophyllene oxide has anticarcinogenic activities (Zheng et al., 1992).

The chemical identification of EAG also reported the presence of spatulenol. Spatulenol is an oxygenated sesquiterpene, which has immunomodulatory and antibacterial activity (Harborne, 2014).

There may be possible degradation of the bioactive compounds during the supercritical process. However, when one observes the chromatogram of the compounds of EAG, it is noted that there was no degradation of its components. (Figure 2.2).

The terpenes are well known for their pharmaceutical properties, such as antimicrobial, anti-inflammatory and antitumor properties (de Cássia da Silveira e Sá et al., 2013). Extracts derived from medicinal and / or aromatic plants are important because they possess many biological effects, mainly anti-inflammatory, antioxidant, antifungal and antibacterial.

Figure 2.2. Chromatogram of the chemical compounds of the EAG constructed by $SCCO_2$. 1: Pinocarvil; 2: Pinocamphone; 3: Isopinocampone; 4: Myrtenol; 5: (-) - trans-pinocarbon acetate; 6: Caryophyllene; 7: γ - Elemene; 8: Humelene; 9: (-) - Spathulenol; 10: Caryophyllene oxide; 11: Guaiol; 12: Bunesol



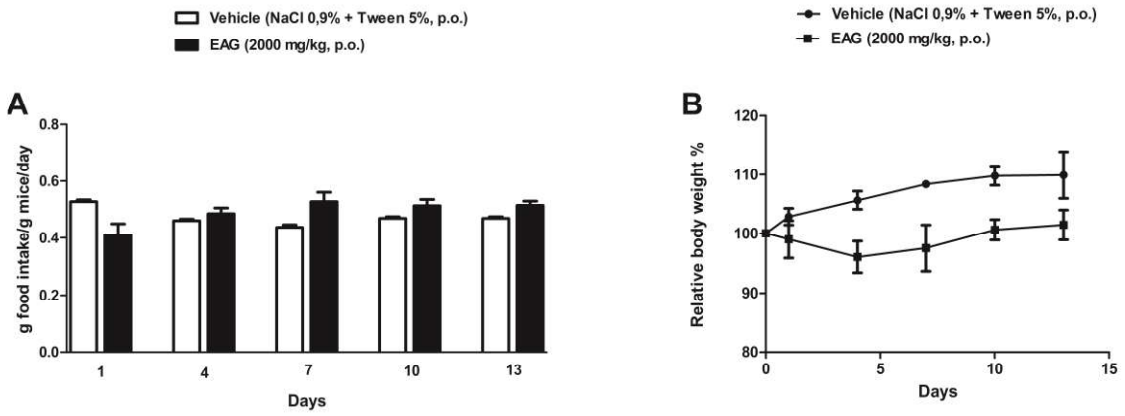
2.3.2. Acute toxicity test

Scientific studies highlight the toxicity, mutagenic and carcinogenic effects of some plants used in alternative medicine (Otang et al., 2014). Acute toxicity studies are important for evaluating the effects of compounds or extracts after a single oral exposure (Parasuraman, 2011). The decrease or increase in body weight of the animals may be associated with toxic effects of substances found in the plant matrix (Kifayatullah et al., 2015) and organ changes may be indicative of damage caused by the test substance (Berenguer-Rivas et al., 2013;

Traesel et al., 2014). In the acute toxicity test in female mice, the dietary changes and relative weight of the animals treated with EAG at 2000 mg·kg^{-1} were evaluated for 14 days (OECD, 2001).

After the administration of the EAG (2000 mg·kg^{-1} p.o.), it was observed an intense sedation of the animals, that lasted 45 minutes. After the recovery, the animals fed normally, with no locomotor changes. There were no changes in food intake (Fig. 2.3A) and in the weight gain (Fig. 2.3B) of animals treated with EAG at 2000 mg·kg^{-1} over the observation period (14 days). Also, no death ocurred due to EAG administration.

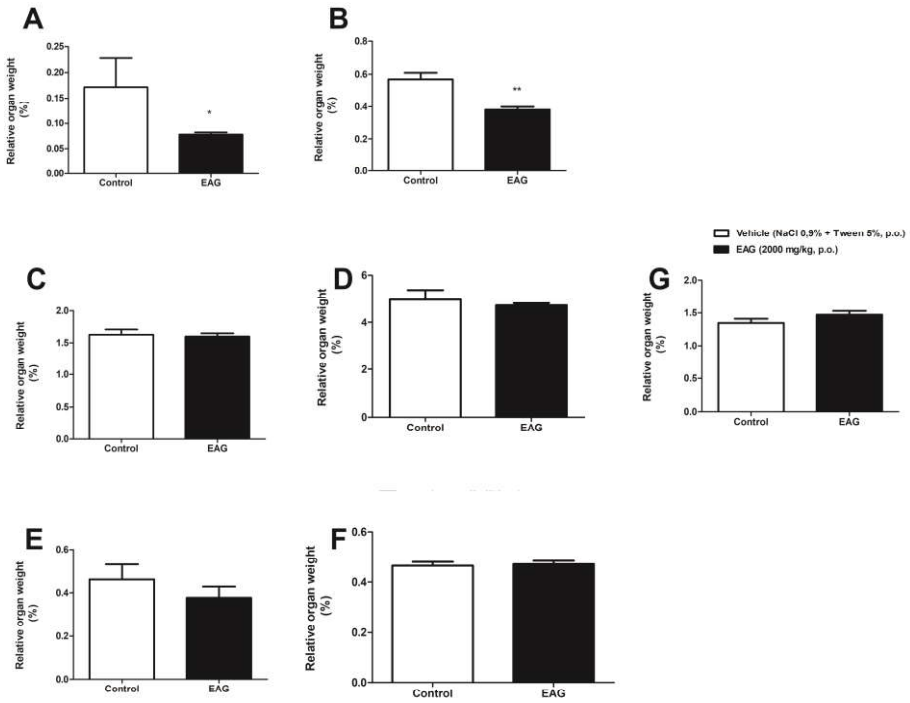
Figure 2.3. Effect of the *A. gratissima* leaves supercritical extract (EAG) acute treatment (2000 mg/kg, p.o.) on mice food intake (A) (g food intake/g mice/day) and relative body weight (%) (B). Data are expressed as mean ± S.E.M. Two-way repeated measures ANOVA.



After the necropsy, the weight of adrenal glands, spleen, brain, liver, thymus, heart and kidneys of vehicle-treated and EAG-treated mice was verified (Figure 2.4). Mice treatment with EAG (2000 mg·kg^{-1}) did not cause changes in weight of the brain, liver, kidneys, lung, heart and thymus. However, there was a significant decrease in the relative weight of the spleen and adrenal glands in EAG-treated mice. This may suggest a sign of toxicity, which

should be further investigated in a repeated dose toxicity test.

Figure 2.4. Effect of *A. gratissima* leaves supercritical extract (EAG) on the relative weight of female Swiss mice organs (%) in the acute oral toxicity test. A: adrenal gland; B: spleen C: brain; D: liver; E: thymus; F: heart and G: kidneys. The mice (n = 3) were orally treated with vehicle (saline + 1% tween 80, 10 ml / kg) or EAG (2000 mg / kg). Unpaired t test, * $p < 0.05$.



Zeni et al. (2013a) studied the toxicological effects of a hydroalcoholic extract of *A. gratissima* and demonstrated that, it induces hepatic toxicity in male mice at 2000 $mg \cdot kg^{-1}$. However, in the present study, we demonstrated the acute toxicity of supercritical *A. gratissima* leaves extract against mice adrenal glands and spleen. The differences between the findings may be due to the gender of mice and the type of extraction used in the experiments. Anyway, both extracts from the same vegetal species have proven to be harmless, since no deaths occurred after their oral administration at 2000 $mg \cdot kg^{-1}$. Our results demonstrate that

the EAG can be included in the Category 5 of the Harmonized Global Classification System (OECD Guideline 423, 2001), since its DL50 is above 2000 mg/kg.

Considering the lack of acute toxicity, we evaluated the anti-inflammatory activity of the EAG.

2.3.3. Carrageenan test

The induction of inflammation by carrageenan is used for the development of new drugs with anti-inflammatory potential (Morris, 2003). Carrageenan is a sulphated polysaccharide, considered as a phlogistic inducer. It evokes an acute inflammatory process, which develops rapidly due to the action of several proinflammatory mediators. Carrageenan elicits hyperalgesia and edema, as well as exacerbated sensitivity to thermal and mechanical stimuli in the inflamed tissue (Batista et al., 2016; Morris, 2003).

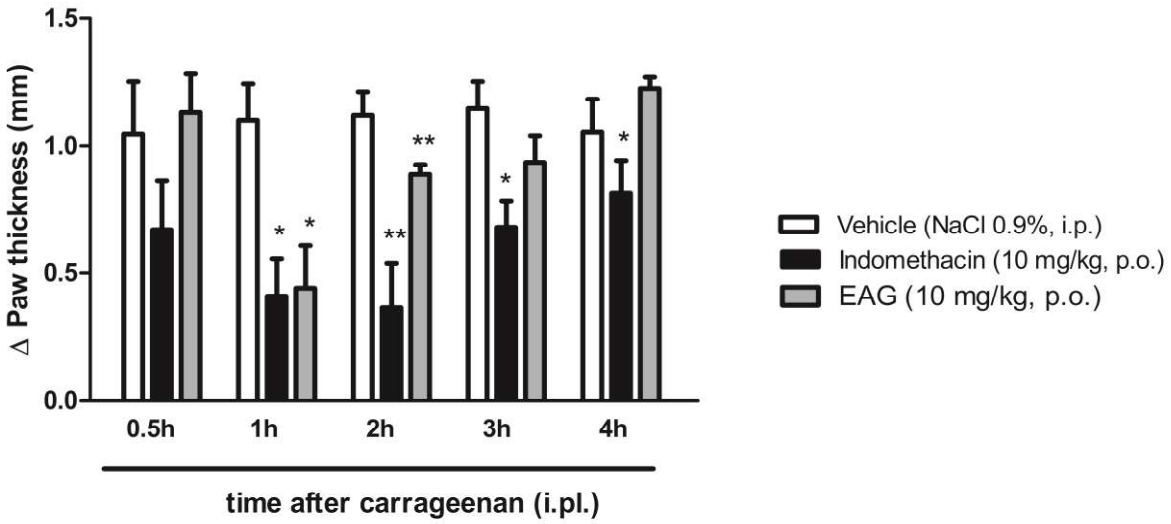
Herein, the EAG (10 $mg \cdot kg^{-1}$) significantly reduced mice paw edema from the 1st hour up to the 3rd hour after carrageenan i.pl. administration. After 3h of inflammation induction, the EAG lost its effects, unlike the positive control, indomethacin. The indomethacin-treated group showed a significant reduction of paw edema during all observation times (Figure 2.5).

According to Carvalho (2011), the paw edema induced by carrageenan involves three steps, which ate related to the involved mediators. The first step (the first 90 minutes of the test) is related to the release of histamine and serotonin, which are responsible for vasodilation and increase in vascular permeability, thus triggering the onset of the inflammatory process. In the second step (90-150 minutes), the activation of kinins begins, which induce increased blood vessel permeability and prostacyclin biosynthesis. The third step (after 150 minutes), involves the increase of prostaglandins synthesis in the inflamed tissue, at this stage there is also polymorphonuclear leukocytes infiltration.

Considering the EAG effects in the carrageenan-induced paw edema test, we may infer that its anti-inflammatory activity is not related to a decrease in prostaglandin synthesis, but to

an inhibition in the release of histamine, serotonin and kinins. This hypothesis deserves further studies.

Figure 2.5. Effect of *A. gratissima* leaves supercritical extract (EAG) on the carrageenan - induced paw edema. Mice were treated with vehicle (saline + 1% tween 80, 10 ml / kg p.o., n = 18), EAG (10 mg / kg p.o., n = 18) or indomethacin (10 mg / kg p.o., n = 18) 1 h before intraplantar (i.pl.) carrageenan administration (300µl / paw). One-way ANOVA followed by Student-Newman-Keuls: * $p < 0.05$ and ** $p < 0.01$, compared to the vehicle treated group. Results expressed as mean ± S.E.M.



At the end of carrageenan-induced paw edema test, the weight of the inflamed (carrageenan) and non-inflamed (saline) paws was assessed. There was no significant difference between the Δpaw weight of indomethacin-treated and EAG-treated animals. However, the Δpaw weight from these groups was significantly ($p<0.01$) lower than the one of vehicle-treated animals (Figure 2.6). These results corroborate the Δpaw thickness (mm) of the EAG and indomethacin-treated animals, which were significantly ($p < 0.05$) lower than the Δpaw thickness (mm) of vehicle-treated mice.

Figure 2.6. Effect of *A. gratissima* leaves supercritical extract (EAG) on the Δpaw weight (g) (= inflamed paw weight (g) – non inflamed paw weight (g)). after euthanasia of mice receiving carrageenan i.pl. (300µl / paw) injection. The mice were treated with vehicle (saline + 1% tween 80, p.o., 10 ml / kg n = 18), EAG (10 mg / kg, p.o., n = 18) or indomenthacin (10 mg / kg p.o., n = 18) 1 h before carrageenan injection. One-way ANOVA, post hoc Student-Newman-Keuls: ** p <0.01 compared to the vehicle treated group. Results expressed as mean ± S.E.M.

Vandresen et al. (2010) demonstrated that the crude aqueous extract of leaves of *A. gratissima* caused a 23.6% reduction in carrageenan-induced ear edema in mice when compared to the animals treated with vehicle, which corroborated with the results found by our group with the reduction of anti-inflammatory effects using EAG.

The compounds spatulenol, β-elemene and cariophyllen oxide are the majoritarian ones found in the essential oil of *C. argyrophyllus*, which reduced carrageenan-induced paw edema at 30 $mg \cdot kg^{-1}$, besides inhibiting the myeloperoxidase activity (Ramos et al., 2013). Indeed, the anti-inflammatory activity of cariophyllen oxide is well stablished (Morris, 2003; Wakte and Shinde, 2010); however, studies about the anti-inflammatory effects of guaiol are scarce.

Therefore, we may suggest that the presence of cariophyllen oxide contribute to the anti-inflammatory activity of the EAG demonstrated in the present study. Also, our study is the first one to demonstrate the *in vivo* anti-inflammatory activity of a extract enriched in guaiol.

2.4. Conclusion

In this study, we demonstrated the effects of supercritical fluid extraction on the leaves of *A. gratissima* (EAG) and the chemical composition of the extract. The temperature and the pressure influenced in the extraction process, there was an increase of yield when high temperature and pressure were combined. Among the EAG chemical constituents of, guaiol, pinocamphone, spatulenol and caryophyllene oxide were the majoritarian ones, which could be associated to the anti-inflammatory activity of EAG in mice. The acute administration of the EAG at 2000 mg/kg did not cause mortality to mice, which demonstrates that it is devoid of acute toxicity.

Declarations of interest: none

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CAPITULO III

Antinociceptive effect and mechanism of action of supercritical CO2 extract of Aloysia gratissima leaves in mice

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Abstract

Aloysia gratissima is a shrub used in Brazilian folk medicine as analgesic and sedative. This work aims to evaluate the antinociceptive effect mechanism of a supercritical CO_2 extract of *A. gratissima* leaves (EAG) in mice. *A. gratissima* leaves were submitted to extraction with supercritical CO_2 (60 ºC, 200 bar, 120 min). The EAG's chemical composition was determined by gas chromatography coupled to mass spectrometry (GC-MS). The GC-MS revealed the presence of sesquiterpenes (guaiol and pinocamphone) in the EAG. The EAG significantly reduced the number of mice abdominal writhes and paw licking time in both phases of the formalin test. The antinociceptive effect of the EAG was prevented by glibenclamide in the formalin test, unlike naloxone pre-treatment. *Aloysia gratissima* leaves present antinociceptive effect, mediated by K^+ channels sensitive to ATP.

***Keywords*:** *Aloysia gratissima*; Verbenaceae; supercritical fluid; antinociceptive; potassium channels; opioid system.

Abbreviations:

ATP – adenosine triphosphate

EAG – supercritical CO_2 extract of Aloysia gratissima leaves;

GC-MS - gas chromatography coupled to mass spectrometry

K^+ - potassium

$SCCO_2$ - Supercritical Carbon Dioxide.

3.1. Introduction

The interest in compounds derived from plant extracts by scientific research is associated with the knowledge of folk medicine. The secondary metabolites present in these natural compounds can have their biological activity evaluated through *in vivo* experimental tests so that their functionalities as drugs, food and cosmetics can be proven. Among the species popularly known for their pharmacological actions, are the ones from *Aloysia* genus, belonging to the family Verbenaceae, native to South America [1].

In Brazil, the species *Aloysia gratissima* (Gillies & Hook) Tronc. is commonly grown as ornamental, being known as "Alfazema do Brasil" [2]. Aerial parts of this plant are used for the treatment of headache, bronchitis, nervous system and digestive disorders [3,4]. The *Aloysia gratissima* essential oil presents more than 70% of hydrocarbons, from which more than a half are sesquiterpenes. This is particularly interesting, since the the pharmacological action of plants is related to the presence of metabolites such as alkaloids, terpenes, flavonoids and phytosterols. [2].

The use of natural products in the food and pharmaceutical industry, which may provide alternative results to current needs, together with the economic and safety concerns regarding

toxicity related to the extraction methods, demonstrate that obtaining compounds through pressurized fluids, such as carbon dioxide and propane, have been presented as alternative, because of the use of non-toxic, non-flammable and low-cost solvents [5].

In this context, the extraction and study of the compounds present in the plant matrices through supercritical fluids is interesting and considering the presence of sesquiterpenes in the *A. gratissima*, it becomes important to investigate, for the first time, the effect of this plant species on animal models of nociception.

The present work aims to evaluate the antinociceptive activity of a supercritical CO_2 extract of *Aloysia gratissima* leaves in models that use different stimuli in mice, as well as to investigate the mechanisms of action involved in its antinociceptive activity.

3.2. **Materials and methods**

3.2.1 Chemical

Carbon dioxide (99.9% non-liquid purity phase) was purchased from Air Liquide. Acidic acid and formaldehyde were purchased from Merck. Ketorolac and glibenclamide were obtained from EMS Sigma Pharma. Naloxone and morphine were purchased from Cristália and indomethacin was obtained from Sigma.

3.2.2. Plant material

A. gratissima leaves were collected in December 2017 (summer), in the municipality of Erval Grande, RS, South Brazil (27°23'14.3S, 52°33’49 'W). This region presents a humid subtropical mesothermic climate. The plants specimen were deposited in the Herbarium of the Community University of Chapecó (Herbário Unochapecó, SC, Brazil) under the accession number UNO 3700. After collection, the leaves were manually separated and dried at room temperature for five days, after being packed in plastic bags, identified and stored at 7 ºC until

extraction process.

3.2.3 Extraction of the Aloysia gratissima leaves by $SCCO_2$

The experimental extraction apparatus and procedure have been described in detail in other studies of the research group [27]. *A. gratissima* leaves were extracted by $SCCO_2$ at 60 °C and 200 bar (density 724 Kg/m^3. Approximately 11.03 ± 0.09 g of *A. gratissima* leaves were loaded in the extraction vessel. Then, CO_2 was pumped into bed, which was had two 300 mesh wire disks at both ends and was held in contact with the sample array to allow the system to stabilize at the same condition as the experiment by 30 min. After stabilization, the extract from *A. gratissima* was then collected by opening the micrometric valve by 2 h of extraction time.

3.2.4 GC/MS analysis

EAG chemical composition was analyzed by Agilent GC/MS (7890B) gas chromatography coupled to a quadripolar mass spectrometer (5977A) (Agilent Technologies, Palo Alto, CA, USA). GC/MS system's experimental condition were described by Scapinello et al. [28] with some modifications. Briefly, the system conditions were: Agilent 19091S capillary column, dimension: 30 m × 250µm × 0.25µm. The mobile phase flow (carrier gas: He) was adjusted to 1.0 mL min^{-1}. The GC temperature program was 40.0 °C at 4 min to 240.0 °C at a rate of 10 °C min^{-1} and up to 300.0 °C at a rate of 40.0 °C min^{-1} (maintained for 5 min). The injector temperature was 280.0 °C, sample injection volume 1 µL, split ratio 1:20. The MS transferline temperature was set to 150.0 °C and the source of ions temperature was set at 230.0 °C. For GC–MS detection, an electron ionization system was used with ionization energy set at 70 eV, and mass range atm/z 40–400. The chemical components present in the extract were identified by comparison with the equipment library (Agilent P/N G1033A). The

relative amounts of each individual component were calculated using their respective peak areas in the chromatogram. The extracts were solubilized in dichloromethane to be analysed.

3.2.5 *In vivo experiments*

3.2.5.1 Animals

Male Swiss mice (25-35 g) bred at Unochapecó bioterium were used in all behavioral experiments. A controlled environment kept the animals (22 ± 2 °C) with a light/dark cycle of 12 hours (lights on at 6:00 a.m. to 6:00 p.m.), fed standard laboratory feed and water *ad libitum*. Animal care and experiments were conducted in accordance with the animal research ethical principles, approved by the Ethics Committee of the university (Approval number 004-18), in accordance with Brazilian law No. 11794 (Brazil, 2016, 2008) and Council of the European Communities; Directive of 24 November 1986 (86/609/EEC). The animals were fasted for a period of 2 hours (no water restriction) prior to administration of any test substance.

Mice were treated with volumes of 10 mL/kg, respectively according to their weight, by the oral route (p.o.) (by gavage), intraperitoneal (i.p.) and intraplantar (i.pl.) according each experiment's specific protocol). Solubilization of the extract and substances was performed in saline (NaCl 0,9 %) with the aid of 1% Tween 80 (v/v) and ultrasound. The extract's tested doses were chosen based on the study of Zeni et al. [1].

3.2.5.2 Acetic acid-induced writhing response

In this test, we observed the number of abdominal writhing evoked by the injection (i.p.) of acetic acid (0.6%, 10 mL/kg) (counted cumulatively over a period of 20 min), according Koster et al., [29]. Mice (n=6-8/group) were treated with EAG (1, 10 or 30 mg/kg, p.o.) or vehicle (0.9% NaCl + 1% Tween 80, p.o., control group) 1 h before the injection (i.p.) of

acetic acid. The positive control was the non-steroid anti-inflammatory indomethacin (10 mg/kg, p.o.). The dose that presented the best result in this experiment was chosen to be used in the other tests of nociception.

3.2.5.3 Open-field test

The open-field test was performed in order to evaluate the possible EAG effects on locomotor and exploratory activities of mice. The experimental protocol was based on the method described by Müller et al. [30]. The animals (n=6/group) orally received EAG's minimal effective dose (10 mg/kg, p.o.) that was able to reduce the nociceptive behavior in the acetic-acid writhing test, 1 h before being exposed to the open-field arena. The control group was treated with vehicle (0.9 % NaCl + 1 % Tween 80, p.o.). The arena consisted in an acrylic box (40 × 30 × 30 cm), with the floor divided into 24 equal squares. After the habituation (5 min), the number of squares crossed with the four paws (crossing), rearings and groomings was recorded for 10 min. The number of fecal bolus was counted after the test.

3.2.5.4 Rota-rod test

The rota-rod test was used to evaluate the EAG effects on mice motor coordination. This test was performed as described by Neves et al. [31] with minor modifications. The apparatus was a cylinder (4 cm of diameter), rotating at 3 rpm. The animals were individually habituated to the apparatus for 5 minutes. Twenty-four hours later, they were trained for 5 minutes and only the ones that were able to stay 90 seconds on the rotating rod were selected for testing. Immediately after, mice (n=7/group) were orally treated with vehicle (NaCl 0,9 % + 1% tween 80), indomethacin (10 mg/kg) or EAG (10 mg/kg). One hour later, mice performance in the rota-rod was evaluated considering the longest time of permanence on the apparatus and the number of falls, in a 5 minutes period.

3.2.5.5 Formalin test

The experimental procedure was similar to the one described by Santos et al. [32]. Briefly, the animals (n=6/group) were orally treated with vehicle (0.9 % NaCl + 1 % Tween 80) or EAG (10 mg/kg) 1 h before the injection of 2 % formalin (20 μL/paw, ipl) in the right hind paw. Indomethacin (administered orally 1 h before the behavioral test, 10 mg/kg) was the positive control. Immediately after the formalin injection, the time spent licking, biting or lifting the injected hind paw (nociceptive behaviour) was registered during the first phase (0-5 min, neurogenic phase) and the second phase (15-30 min, inflammatory phase) of the test.

3.2.5.6 Involvement of the opioid system and K^+ channels sensitive to ATP

In order to assess the opioid system's involvement in the antinociceptive action mechanism of the EAG, mice were pre-treated with naloxone (a non-selective opioid receptor antagonist; 2 mg/kg, i.p.) or vehicle (0.9 % NaCl, i.p.) [33, 34]. After 15 min, the animals (n=4-7/group) were treated with EAG (10 mg/kg, p.o.), morphine (opioid receptor agonist, positive control, 5 mg/kg, s.c.) or vehicle (0.9% NaCl, 1% Tween 80, p.o.).

The involvement of the ATP-sensitive K^+ channels in EAG's antinociceptive effect was evaluated according to Zapata-Morales et al. [35]. Briefly, the animals (n=4-7/group) were pre-treated with glibenclamide (K^+ channels sensitive to ATP blocker; 20 mg/kg, i.p.) or vehicle (0.9 % NaCl, i.p.). The EAG (10 mg/kg, p.o.), ketorolac (non-steroid anti-inflammatory that presents antinociceptive effects mediated by ATP-sensitive K^+ channels [36], positive control, 20 mg/kg, i.p.) or vehicle (0.9% NaCl + 1% Tween 80, p.o.) were administered to the animals 15 min after the pretreatment.

One hour after the treatments, the nociceptive behavior was evaluated in the formalin test,

immediately after the i.pl. injection of formalin 2%.

3.2.6 Statistical analysis

The results of antinociceptive activity and mechanism of action are expressed as the mean ± S.E.M. of *n* animals per group. Data were analyzed by one-way variance (ANOVA) followed by the Student-Newman-Keuls test. Rota-rod test results were analyzed by two-way ANOVA with repeated measures (being the treatment the first factor and the session, second factor, the repeated-measure). Statistical analyses were carried out using Graph Pad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA). Values of p less than 0.05 ($p < 0.05$) were considered significant.

3.3. Results

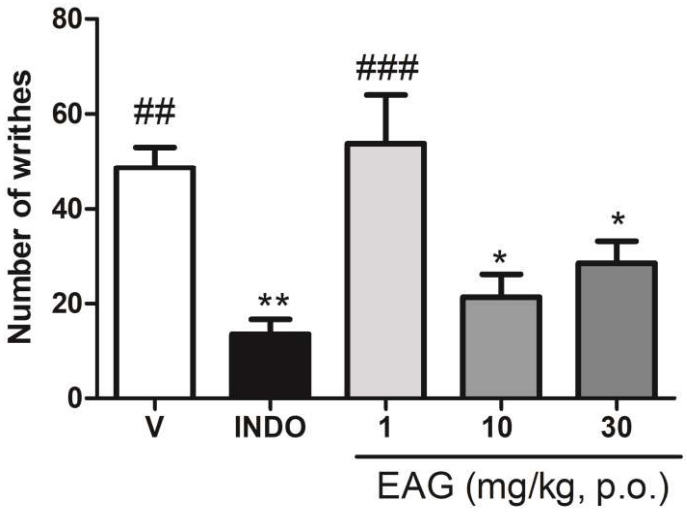
Table **3.1** shows the extract's chemical composition obtained from the extraction process. The GC/MS analysis revealed that several terpene compounds are present in the EAG. The major compounds found in EAG were guaiol (22.04 %) and pinocanfone (10.47 %), in addition to the presence of other compounds such as pinocarvil, (-)-trans-pinocarvilacetate, γ-elemene, bunesol, caryophyllene, caryophyllene oxide, (-)-spathulenol, myrtenol, isopinocamphone, humuleno, in smaller quantities.

Table 3.1. Chemical composition of the *A. gratissima* leaves $SCCO_2$ extract.

Chemical compound	**Area of each compound (%)**
Pinocarvil	3.61
Pinocamphone	10.47
Isopinocamphone	2.94
Myrtenol	2.85
(-)- trans-pinocarvila acetate	8.89
Caryophyllene	8.10
γ – Elemene	2.02
Humelene	2.61
(-)- Spathulenol	4.40
Caryophyllene oxide	6.40
Guaiol	22.04
Bunesol	7.91
Total	**82.24**

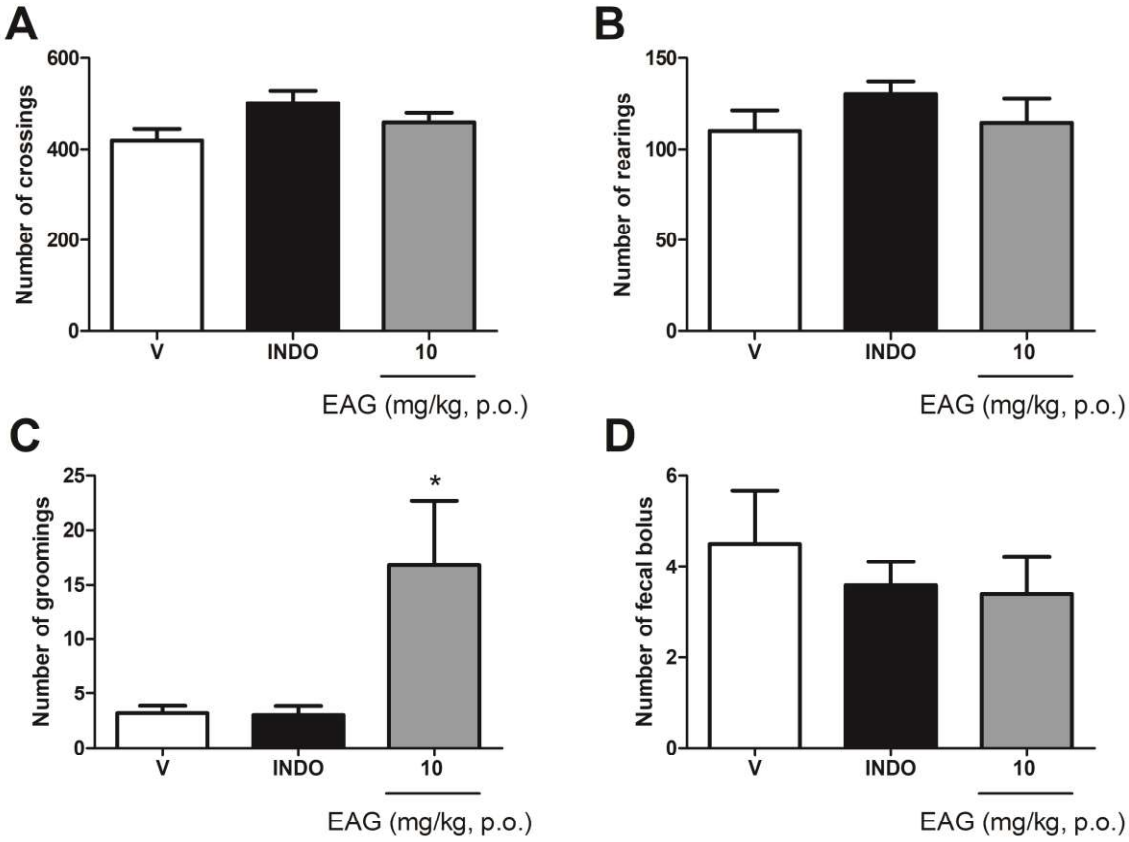
The injection of acetic acid into rodents evokes abdominal contortions, since it induces the peripheral production of several pro-inflammatory mediators. The EAG at 10 and 30 mg/kg ($p < 0.05$) and indomethacin at 10 mg/kg ($p < 0.01$) reduced the number of abdominal writhes induced by the injection of acetic acid when compared to the vehicle group. On the other hand, the EAG at the lowest dose (1 mg/kg) was not effective in reducing the acetic acid-induced writhes (Fig. **3.1**). The abdominal contortion test was applied to investigate the lowest effective dose, in order to continue the other tests.

Figure 3.1. Effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) in the acetic acid-induced abdominal writhing test in mice. V: vehicle treated group (0.9% NaCl + 1% Tween, 10 mL/kg p.o., n = 8). INDO: indomethacin (10 mg kg p.o., n = 6) or EAG (1, 10 and 30 mg/kg p.o., n = 6-8), 1 hour prior to acetic acid administration. Each column represents the mean ± S.E.M. One-way ANOVA followed by the Student-Newman-Keuls test, ** $p < 0.01$ and * $p < 0.05$ different from the vehicle group; ### $P < 0.001$ and ## $P < 0.01$ different from the INDO group.



Considering that a non-specific effect of the EAG on animals' locomotion could influence the results of the antinociceptive tests and, therefore cause false positive or negative results, we investigated the effects of the minimal effective dose of EAG (10 mg/kg, p.o.) in the open field test. Treatment with EAG and indomethacin (10 mg/kg, p.o.) did not alter the numbers of crossings (Fig. **3.2A**), rearings (Fig. **3.2B**) and the number of fecal bolus (Fig. **3.2D**). The grooming number (Fig. **3.2C**) of the EAG-treated animals was significantly higher when compared to the groups treated with indomethacin or vehicle.

Figure 02. Effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) on mice locomotor activity (open field test). A: number of crossings. B: number of rearings. C: number of groomings; D: number of fecal bolus. V: vehicle treated group (0.9% NaCl + 1% Tween, p.o., n = 6). INDO: indomethacin (10 mg/kg, n = 6). EAG (10 mg kg, p.o., n = 6). Each column represents the mean ± S.E.M. One-way ANOVA followed by the Student-Newman-Keuls test, * $p < 0.05$ different from the vehicle-treated and indomethacin-treated groups.



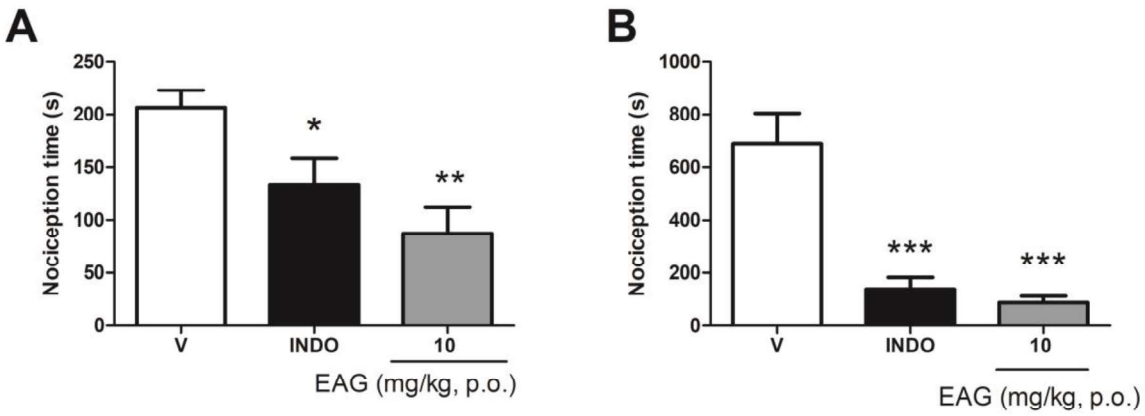
Additionally, the treatment with EAG (10 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.) did not change mice motor coordination, compared to the vehicle group (NaCl 0.9%, p.o.) (Table **3.2**).

Table 3.2. Effect of extraction of *A. gratissima* leaves obtained by $SCCO_2$ on the mice motor coordination (Rota-Rod). The animals (n=7 per group) were treated with vehicle (NaCl 0,9 %, 1% tween 80), indomethacin (10 mg/kg) or extract (10 mg/kg) 1 h before the test. Results expressed as mean ± S.E.M. Two-way ANOVA with repeated measures.

Group	**Lenght of stay (s)**	**Number of falls**
Vehicle	300.0	0.0
Indomethacin	300.0	0.0
EAG 10 mg/kg	299.6 ± 0.3	0.11 ± 0.03

The EAG at 10 mg / kg as well as indomethacin produced antinociception in the first phase ($p<0.01$ and $p<0.05$, respectively) – the neurogenic phase (Fig. **3.3A**) - and in the second phase ($p<0.001$) - inflammatory pain phase (Fig. **3.3B**) of the formalin test.

Figure 3.3. Effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) on the formalin test. Nociceptive behavior was considered as the time (s) of elevation, biting or licking of the paw in the first phase (A: 0-5 min) and second phase (B: 15-30 min) of the test. Mice (n=6 per group) were treated with vehicle (V : NaCl 0.9% + 1% tween 80, 10 ml/kg), Indomethacin (INDO: 10 mg/kg) or EAG (10 mg/kg) 1 hour prior to administration of formalin 2% i.pl. One-way ANOVA, post hoc Student-Newman-Keuls: * $p<0.05$; ** $p<0.01$ and *** $p<0.001$ are different from the vehicle group. Results expressed as mean ± S.E.M.



The investigation of the EAG action mechanism revealed that mice pre-treatment with naloxone did not reverse the EAG antinociceptive activity in the two phases of formalin test (Fig. **3.4A** and **3.4B**), showing that the opioid system is unlikely to be involved in antinociceptive action. However, EAG's effect was prevented by mice pre-treatment with glibenclamide in the two formalin phases (Fig. **3.5A** and **3.5B**), thus suggesting that the K^+ channels sensitive to ATP are involved in the antinociceptive EAG mechanism of action.

Figure 3.4. Effect of mice pre-treatment with naloxone (2 mg/kg, i.p.) on the antinociceptive effect of a supercritical CO_2 extract of Aloysia gratissima leaves (EAG) (10 mg/kg) on the formalin test. Nociceptive behavior in the first phase (A, 0-5 min) or second phase (B, 15-30 min) of the test. Morphine (5 mg/kg, s.c.) was used as positive control. Results expressed as mean ± S.E.M. (n = 4-7 mice/group). One-Way ANOVA post hoc Student-Newman-Keuls test, * P <0.05; ** P <0.01; *** P <0.001 compared to the vehicle plus vehicle-treatedgroup. ## P <0.01 compared to the vehicle plus morphine-treated group; §P<0.05 ; §§P <0.01 compared to the vehicle plus naloxone-treated group and morphine plus naloxone-treated group.

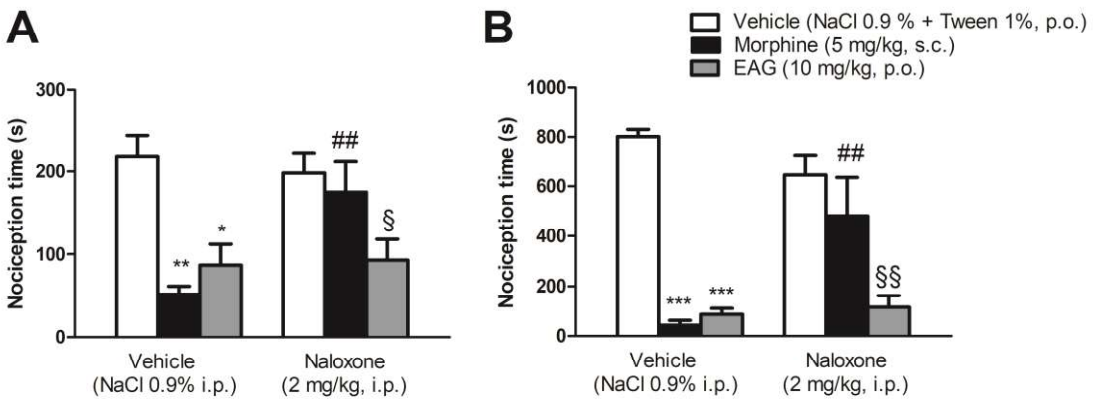
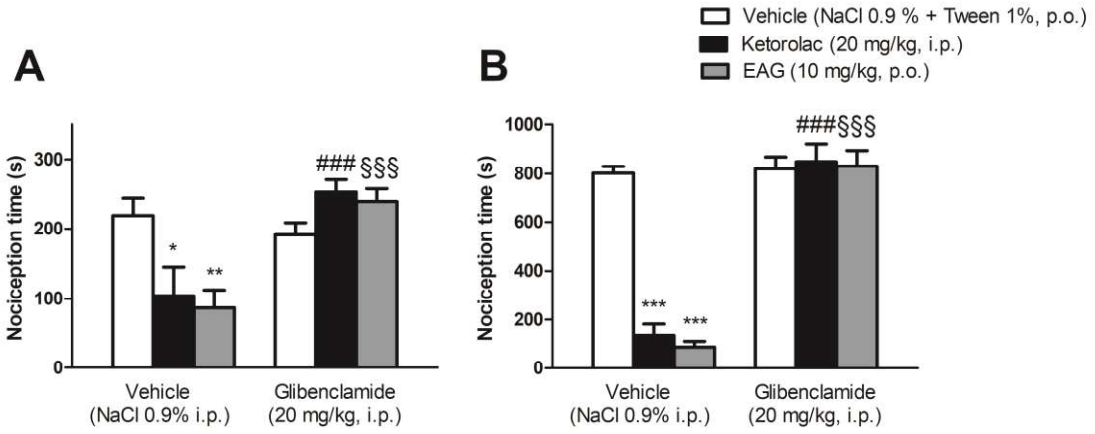


Figure 3.5. Effect of mice pre-treatment with glibenclamide (20 mg/kg, i.p.) on the antinociceptive effect of a supercritical CO_2 extract of *Aloysia gratissima* leaves (EAG) (10 mg/kg, p.o.) on the formalin test. Nociceptive behavior in the first phase (A, 0-5 min) or second phase (B, 15-30 min) of the test. Ketorolac (20 mg/kg, i.p.) was used as positive control. Each column represents the mean ± S.E.M (n = 4-7 mice/group). One-Way ANOVA followed by the Student-Newman-Keuls test, * P <0.05; ** P <0.01; *** P <0.001 compared to vehicle plus vehicle-treated group. ### P <0.001 compared to the vehice plus kerotolac-treated group; §§§ P <0.01vehicle plus EAG-treated group.



3.3 Discussion

The chemical components' solubility obtained in the supercritical fluid is related to the fluid density. When CO_2 is used in the extraction of oils, it demonstrates good solubilization capacity due to low polarity, without altering the chemical composition and the extract produced is free of solvent contamination [6]. The chemical composition of the *A. gratissima* leaves supercritical extract analysed by GC/MS revealed the presence of terpene compounds, being guaiol and pinocanfone the major ones. These compounds are commonly found in species from the *Aloysia* genus, and their antibacterial and antitumor activities have already been demonstrated [7,8]. Terpenes have been known to show a wide range of pharmacological

activities, including effects on the central nervous system, antinociceptive, anti-inflammatory, antimicrobial and antitumor properties [9].

It is known that the acetic acid intraperitoneal injection in rodents evokes abdominal writhing, since it induces the peripheral production of several pro-inflammatory mediators, such as prostaglandins, bradykinin, substance P, prostacyclin and other cytokines, which, therefore, excite the nociceptors in the nerve endings [10]. The protective effect of substances against the noxious chemical stimulus may be an indication for a decreased production of these mediators, thus causing a reduction in the number of writhes [11]. The EAG at 30 mg/kg reduced the abdominal writhing (on average 41%) compared to the vehicle group, while the dose of 10 mg/kg resulted in an average reduction of 56%, both doses being similar to the group that received indomethacin, the positive control. These results may be related to the presence of pinocanfone and guaiol associated to caryophyllene oxide and spathulenol in EAG's chemical composition [12,13].

The acetic acid model is a preliminary test used to screen new analgesic drugs and was applied in our study to investigate the minimal antinociceptive dose of EAG, in order to continue the other tests. The lowest effective dose was used because of lower adverse effect's possibility. In order to investigate EAG's false-positive results in the nociception tests, mice behavior in the open field and rota-rod tests were evaluated [14].

The results obtained in the open field tests demonstrate that EAG (10 mg/kg) did not induce undesirable effects on the animals' locomotor activity and is not sedative or hyperstimulant. The only behavior that changed in comparison to the vehicle group was the number of groomings. Considering that the grooming behavior has been used to measure pharmacologically induced anxiolytic-like effects in rodents [15,16], we may infer that the increase in mice grooming elicited by the EAG might be related to its anxiolytic properties, [3,4].

In order to investigate EAG's effect on mice motor coordination, we used the rota-rod test. The results of indomethacin, vehicle and EAG treated animals were not different between them in the rota-rod test, suggesting that EAG does not impair the motor coordination and, therefore does not induce false-positives in other behavioral tests.

The formalin test aims to explore the analgesic effect of substances through central and peripheral pain mechanism [17]. The antinociceptive activity may occur in two distinct test's phases. In the first phase, neurological pain is induced by the chemical stimulation of afferent sensory fibers, particularly the C fibers, whereas in the late phase, pain is caused by inflammatory mediators' production such as: prostaglandins, histamine, bradykinin, and serotonin [18].

The EAG elicited antinociception both in the first and second phases of the formalin test in mice and therefore, we can suggest that the EAG is effective in the treatment of neurogenic as well as inflammatory pain. Indeed, EAG's antinociceptive and anti-inflammatory activities are probably related to its main constituents. Guaiol and spathulenol's presence may be responsible for the EAG anti-inflammatory effect, as suggested by the study of Apel et al. [19], with the *Myrciaria tenella* leaves extract, enriched in these compounds.

Some authors evaluated the antinocicpetive and anti-inflammatory activities of plant extracts that present the caryophyllene oxide as one of the main constituents. De Oliveira Júnior et al. [20] reported that *Croton conduplicats'* extract obtained by hydrodistillation showed antinociceptive and anti-inflammatory activities, being the caryophyllene and caryophyllene oxide the main constituents of the extract. Caryophyllene oxide is also reported as the main component of *Myrcia pubiflora* DC leaves essential oil, obtained by hydrodistillation, and its antinociceptive and anti-inflammatory activities have been proven in *in vivo* tests [21].

Opioids act at the cellular level by binding to the opioid receptors present throughout the

central nervous system, so the ultimate effect is the reduction of neuronal excitability, resulting in reduced neurotransmission of nociceptive impulses [22]. Pure opioid agonists (such as morphine) have high affinity for opioid receptors. To verify whether opioid receptors mediate EAG's antinociceptive effect, mice were pretreated with naloxone, an opiod antagonist. Naloxone did not prevent the EAG antinociceptive activity in the two phases of the formalin test, suggesting that the opioid system is unlikely to be involved in antinociceptive action. These results are particularly interesting, since there are several limitations to the use of opioid agents, considering their adverse effects, which include sedation, constipation and withdrawal syndrome [23].

Herein, we also investigated the involvement of ATP-sensitive K^+ channels in the mode of EAG EAG's effect, by pretreating mice with glibenclamide (K^+ channel blocker). The ion channel's involvement occurs in the nociception process, and studies support the hypothesis that the opening of the K^+ channels act as a mediator of antinociception, being these channels, such as those sensitive to ATP, particularly involved in the nociceptive responses [24]. Several G protein coupled receptors that stimulate the opening of K^+ channels are involved in antinociception production [24]. Our results demonstrate that the combined administration of glibenclamide (K^+ channel blocker) and EAG resulted in the prevention of the EAG antinociceptive action in the two phases of the formalin test. In this context, our findings strongly suggest that the peripheral antinociceptive action of EAG might be related to the activation of K^+ channels sensitive to ATP. Indeed, the effect of terpenes as activators of K^+ channels sensitive to ATP is well known [25,26]. Corroborating our results, De Oliveira Júnior et al. [20] demonstrated that the antinociceptive *Croton conduplicatus* essential oil's effect, which contains caryophyllene oxide and guaiol, is mediated by K^+ channels sensitive to ATP.

3.5 Conclusion

In this study, we demonstrated for the first time the antinociceptive effects of supercritical extract of *Aloysia gratissima* leaves in mice, which is mediated by ATP-sensitive K^+ channels. These effects may be associated to the presence of several terpenoids that are present in the supercritical CO_2 extract, such as caryophyllene oxide, guaiol, pinocanfone and spathulenol. This study certifies the use of *Aloysia gratissima* by popular medicine as an analgesic.

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Declarations of interest: none

3.6 References

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CAPITULO IV

CONSIDERAÇÕES FINAIS

A utilização de técnicas que auxiliem a busca de compostos bioativos e terapêuticos tem crescido nas últimas décadas e é de suma importância social e econômica. A pesquisa por extratos de plantas nativas, busca ampliar o conhecimento de suas propriedades medicinais.

O método de extração a partir de fluídos supercríticos, tem como intenção substituir a extração convencional (de solventes orgânicos tóxicos), o uso dos fluídos supercríticos mantem as propriedades originais dos compostos químicos das plantas, sem a presença de resíduos contaminantes derivados do solvente.

Este estudo, viabilizou o conhecimento bioativo das folhas de *A. gratíssima* em diferentes temperaturas e pressões supercríticas, além da identificação dos seus constituintes químicos e posteriormente avaliou os efeitos do extrato na atividade antinociceptiva e anti-inflamatória em camundongos Swiss.

Os extratos das folhas de *A. gratíssima* obtidos por fluídos supercrítico CO_2 apresentaram melhores resultados de rendimento na temperatura e pressão de 60ºC e 210 bar. A análise cromatográfica demonstrou que a extração supercrítica não ocasionou variações na composição química do extrato, considerando seus principais constituintes, os terpenos voláteis, sendo o Guaiol e Pinocanfona como majoritários.

Os extratos de *A. gratissima* 10 $mg.kg^{-1}$ apresentaram efeitos antinociceptivos e anti-inflamatórios *in vivo*, além de não interferir na coordenação e atividade locomotora dos camundongos, não apresentando toxicidade aguda oral. Esses efeitos estão corelacionados com os constituintes químicos do gênero Aloysia, onde os seus compostos auxilian na atividade antioxidante, antimicrobiana, antiedpressiva, anti-inflamátoria e atvidade sedativa.

Este estudo comprova a utilização do gênero Aloysia na medicina popular para o tratamento de algumas doenças, principalmente às associadas à dor e à inflamação sem envolver o sistema opioide.

Os extratos de *A. gratíssima* podem contribuir para a comunidade cientifica no âmbito de aprimorar métodos de extração e sua utilização com plantas nativas, buscando aplicações farmacológicas, químicas e alimentícias.