UNIVERSIDADE ESTADUAL DE FEIRA DE SANTANA

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ÉRICA MARTINS DE LAVOR

EFEITO ANTINOCICEPTIVO DO EXTRATO AQUOSO DAS FOLHAS DE *Passiflora cincinnata* Mast. (Passifloraceae) EM CAMUNDONGOS

Feira de Santana-BA
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EFEITO ANTINOCICEPTIVO DO EXTRATO AQUOSO DAS FOLHAS DE *Passiflora cincinnata* Mast. (Passifloraceae) EM CAMUNDONGOS

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

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

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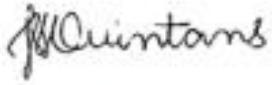

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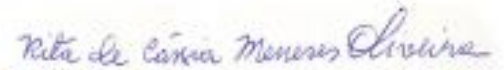

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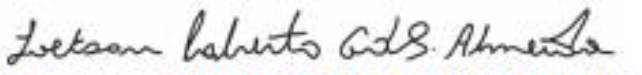

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A Deus e a Nossa Senhora pelas constantes graças. Ao meu filho, meus pais, meu esposo, minha família e aos amigos pelo apoio incondicional.

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A Deus e a Nossa Senhora por me darem a maior das oportunidades: a vida. Por me guiarem em todas as decisões.

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RESUMO

A espécie *Passiflora cincinnata* Mast. é nativa do bioma Caatinga, cujo uso é descrito na medicina tradicional para fins analgésicos e anti-inflamatórios. No entanto, poucos estudos que comprovam seu uso são descritos na literatura. Assim, este trabalho tem como objetivo avaliar a atividade antinociceptiva do extrato aquoso das folhas de *P. cincinnata* (Pc-Aq) por meio de ensaios pré-clínicos em camundongos. Foi realizada a caracterização farmacognóstica da droga vegetal, e a composição química do extrato aquoso (Pc-Aq) foi avaliada por cromatografia líquida de alta eficiência acoplada a detector de arranjo de diodos (CLAE-DAD). A avaliação antinociceptiva do extrato administrado por via oral, nas doses de 100 e 200 mg/kg, foi realizada utilizando modelos químicos *in vivo* (contorções abdominais induzidas por ácido acético e teste de lambida de pata induzida por formalina) e térmicos (teste da placa quente) de nocicepção. O mecanismo de ação foi avaliado por meio do uso de drogas antagonistas dos receptores opioides, de canais para potássio, receptores muscarínicos, serotoninérgicos ($5\text{-}HT_3$) e α-2 adrenérgicos. O teste do rota-rod foi realizado para verificar a possível interferência do tratamento com o extrato no desempenho motor dos animais. Os resultados das análises químicas indicaram a presença dos flavonoides vitexina e isoorientina em Pc-Aq. O tratamento com o extrato reduziu o número de contorções abdominais e diminuiu o tempo de lambida da pata em ambas as fases do teste da formalina ($p < 0,05$). No teste da placa quente, o extrato aumentou o tempo de latência para retirada da pata, indicando redução do comportamento nociceptivo. A avaliação do mecanismo de ação antinociceptiva indicou uma possível ação das vias centrais no efeito do extrato, com participação de receptores opioides, com influência dos canais para potássio e da via adrenérgica α-2, sem alteração significativa do controle motor, avaliado pelo teste do rota-rod. Portanto, este estudo sugere o extrato aquoso das folhas possui ação antinociceptiva mediada por vias centrais, cuja ação pode ser atribuída à presença de flavonoides presentes no extrato.

Palavras-chave: *Passiflora cincinnata*; Flavonoides; Atividade antinociceptiva; Mecanismos de antinocicepção.

ABSTRACT

The species *Passiflora cincinnata* Mast. it is native to the Caatinga biome, whose use is described in traditional medicine for analgesic and anti-inflammatory purposes. However, few studies that prove its use are described in the literature. Thus, this work aims to evaluate the antinociceptive activity of *P. cincinnata* aqueous leaf extract (Pc-Aq) through preclinical assays in mice. The pharmacognostic characterization of the plant drug was performed, and the chemical composition of the aqueous extract (Pc-Aq) was evaluate by high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD). The antinociceptive evaluation of the extract administered orally, at doses of 100 and 200 mg/kg was performed using *in vivo* chemical models (acetic acid-induced abdominal constriction and formalin-induced paw licking test) and thermal (hot plate test) of nociception. The mechanism of action was evaluated with opioid receptor antagonist drugs, potassium channels, TRPV-1, muscarinic, serotonergic (5-HT3) and α-2 adrenergic. The rota-rod test was performed to verify the possible interference of the treatment with the extract in the animal's motor performance. The results of chemical analyzes indicated the presence of the flavonoids vitexin and isoorientin in Pc-Aq. Treatment with the extract reduced the number of abdominal contortions and decreased paw licking time in both phases of the formalin test ($p < 0.05$). In the hot plate test, the extract increased the latency time for paw withdrawal, indicating a reduction in painful behavior. The evaluation of the antinociceptive mechanism indicated a possible action of central pathways in the antinociceptive activity, with participation of opioid receptors with influence of potassium channels and α-2 adrenergic pathway, without significant change in motor control, assessed by the rota-rod test. Therefore, this study suggests that Pc-Aq has an antinociceptive action mediated by central pathways, whose action can be attribute to the presence of flavonoids present in the extract.

Keywords: *Passiflora cincinnata*; Flavonoids; Antinociceptive activity; Mechanisms of antinociception.

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SUMÁRIO

INTRODUÇÃO GERAL

De acordo com a Associação Internacional para o Estudo da Dor (IASP), o termo dor pode ser entendido como uma experiência sensorial e emocional desagradável, diretamente relacionado à lesão tecidual real ou potencial, ou descritas em termos desse tipo de dano, sendo, portanto, carcterizada como uma experiência individal que é influenciada, por diversos fatores, como os biológicos, psicológicos e sociais (LOESER; TREEDE, 2008; RAJA et al., 2020).

Em diversas desordens clínicas, a dor é incluída como sintoma e afeta grande parte da população, alterando a qualidade de vida e constituindo a causa principal de limitação e incapacidade para atividades normais de pessoas, influenciando de forma onerosa, psicossociais e econômicas na qualidade de vida (SBED, 2017). No entanto, destaca-se que a dor se caracteriza como uma experiência emocional complexa, não havendo, portanto, uma relação direta a um estimulo, envolvendo assim um componente sensorial, denominado nocicepção, sendo este último dependente da ativação de receptores específicos (nociceptores) e vias neuroanatômicas que fazem a comunicação entre o Sistema Nervoso Periférico (SNP) e o Sistema Nervoso Central (SNC) (BALIKI, e APKARIAN, 2015).

A classificação da dor leva em consideração a duração, patogênese, localização e causa, sendo a duração da sensação dolorosa o critério mais utilizado, e dessa forma classificando-a em: transitória, aguda ou crônica (LOESER e TREEDE, 2008; SBED, 2017).

Em relação aos mecanismos que explicam a ocorrência da sensação dolorosa, a transdução consiste na ativação dos nociceptores por meio de um estímulo nocivo, seja ele mecânico, térmico ou químico, gerando um potencial de ação. Dessa forma, a transmissão é efetuada pelas vias responsáveis por conduzir o impulso nervoso gerado pelo nociceptor, transmitindo a informação dolorosa para o SNC, e por fim a modulação, que pode ativar vias responsáveis pela supressão da dor gerada pelos próprios nociceptores e suas vias (KLAUMANN et al., 2008).

Alguns nociceptores apresentam sensibilidade a um estímulo específico, enquanto outros são sensíveis a vários tipos de estímulos. Sendo assim, podem ser classificados em quatro classes principais: mecânicos, térmicos, polimodais e silenciosos (FEIN, 2011).

Os nociceptores caracterizados como mecânicos respondem à pressão intensa, enquanto os nociceptores térmicos respondem a temperaturas extremas, sejam elas

quentes (> 45 °C) ou frias (< 5 °C). A maioria dos nociceptores dispersos pelo corpo são classificados como polimodais, uma vez que respondem aos diferentes tipos de estímulos (mecânicos, térmicos e/ou químicos), enquanto que os nociceptores silenciosos são ativados inicialmente por estímulos químicos oriundos do processo inflamatório, respondendo posteriormente a estímulos mecânicos e térmicos, conduzindo informações aos neurônios de ordem superior sobre a lesão tecidual desencadeada por estímulos nocivos (FEIN, 2011).

A condução das vias nociceptivas se dá por meio da ativação dos nociceptores neuronais, por estímulos químicos, mecânicos ou térmicos, promovendo influxo celular de sódio (Na^{+}) e cálcio (Ca^{2+}) os quais geram alterações na permeabilidade membranar (despolarização), deflagrando assim potenciais de ação nos neurônios, que se propagam pela fibra nervosa ascendente até a medula espinhal, e posteriormente para o córtex cerebral, onde serão comandadas e geradas as respostas fisiológicas, emocionais e comportamentais característica da sensação dolorosa (BASBAUM et al., 2008; TRACEY; DICKENSON, 2012).

Os neurônios aferentes primários, estruturalmente apresentam corpo celular, localizados nos gânglios das raízes dorsais da medula espinhal (GRD), do qual parte um prolongamento que se bifurca, e origina um prolongamento central direcionado ao corno dorsal da medula espinhal, e um periférico que percorre os nervos sensitivos até os órgãos periféricos, constituindo assim a fibra sensitiva que se dividem em três grupos: Aβ, Aδ e C. Fisiologicamente, apenas as fibras do tipo C (amielinizada) e Aδ (mielinizada) transmitem a informação nociceptiva, estando, portanto, relacionadas à transdução e condução do estímulo nocivo. Quando um estímulo nociceptivo é aplicado à pele, os nociceptores Aδ são responsáveis pela dor aguda imediata (dor rápida), seguida por uma dor difusa, sendo esta última resultado da ativação dos nociceptores C, cuja condução é mais lenta. Dessa forma, a velocidade de transmissão do estímulo doloroso está diretamente relacionada ao diâmetro dos axônios dos neurônios sensoriais, e se eles são ou não mielinizados (DUBIN; PATAPOUTIAN, 2010; MIDDLETON et al., 2021).

Os eventos que resultam na conexão dos sinais dolorosos envolvem além de nociceptores periféricos, neurônios de projeção por meio de vias ascendentes principais na medula espinhal, áreas de integração no cérebro (localizadas principalmente no tálamo) e projeções corticais (para áreas do córtex somatossensorial primário e secundário, bem como para o córtex insular e cingular). Na medula espinhal ocorre a produção, armazenamento e liberação pelas fibras nervosas sensoriais de

neurotransmissores, como aminoácidos excitatório (glutamato) e neuropeptídeos (substância P), bem como de mediadores inflamatórios (bradicinina, citocinas, eicosanoides, serotonina e histamina), que agem em receptores específicos, alterando o limiar nociceptivo dessas fibras (CALVINO; GRILO, 2006; CURY et al., 2011).

Em resposta à estimulação nociceptiva ascendente, ocorre ativação de vias descendentes de controle da nocicepção, as quais agem sobre os terminais dos nociceptores e nos neurônios medulares, facilitando ou inibindo a liberação de neurotransmissores, como a noradrenalina, serotonina e encefalinas na região do corno dorsal da medula espinhal, modulando assim o sinal nociceptivo. Dessa forma, a sensibilização central altera impulsos periféricos, levando à redução do limiar ou aumento da resposta aos impulsos aferentes, descargas persistentes após estímulos repetidos e ampliação dos campos receptivos dos neurônios do corno dorsal (BASBAUM et al., 2009; LOPES, 2010).

A ativação de uma fibra nociceptiva por um estímulo nocivo leva à liberação de glutamato na sinapse entre o terminal central e o neurônio espinhal. O glutamato, por sua vez, liga-se aos receptores AMPA (Ácido α-amino-3-hidroxi-5-metilisoxazol-4-propiônico) e NMDA (*N*-metil-*D*-aspartato) presentes no neurônio espinhal. Apesar disso, enquanto a ligação aos receptores NMDA não provoca qualquer efeito, devido ao bloqueio pelo íon magnésio (Mg^{+}), a ligação aos receptores AMPA provoca a rápida entrada de íon sódio (Na^{+}) e consequentemente a despolarização da membrana do neurônio espinhal, levando a um potencial de ação, uma transmissão sináptica rápida que finaliza com a inativação dos receptores AMPA e a abertura de canais de potássio sensíveis à voltagem, levando à repolarização da membrana (LOPES, 2010; MIDDLETON et al., 2021).

A despolarização transitória da membrana provoca a abertura de canais para cálcio (Ca^{2+}) controlados por voltagem, com isso, a membrana neuronal tende a ficar despolarizada devido ao aumento da concentração intracelular de Na^{+} e Ca^{2+}. Sendo assim, os canais iônicos dos receptores NMDA deixam de estar bloqueados pelo Mg^{+}, e a sua ativação pelo glutamato liberado pelos terminais centrais das fibras C permite a entrada de grandes quantidades de Ca^{2+}, ativando assim, enzimas fundamentais para as alterações da excitabilidade neuronal (ROCHA et al., 2007; ASHMAWI e FREIRE, 2016).

Após a liberação de aminoácidos excitatórios, peptídeos e neurotrofinas e sua interação com receptores específicos, ocorre a ativação de segundos mensageiros, como:

adenosina monofosfato cíclico (AMPc), proteína quinase dependente de AMPc (PKA), proteína quinase dependente de cálcio (PKC), inositol-1,4,5-trifosfato (IP_3), fosfolipase C (PLC) e fosfolipase A_2 (PLA_2), levando assim, à fosforilação de receptores NMDA (o que promove abertura do canal, por efluxo do íon magnésio), abertura de canais para cálcio e a produção de prostaglandinas e óxido nítrico, que migram em direção à fenda sináptica e promovem a liberação de glutamato, aspartato e substância P, contribuindo para a ampliação do processo álgico e condução para as vias nociceptivas superiores (ROCHA et al.,2007; LOPES, 2010; ASHMAWI e FREIRE, 2016).

Por outro lado, a dor inflamatória decorre da sensibilização dos neurônios nociceptivos sensoriais primários, onde os nociceptores (fibras nervosas tipos Aδ e C), são sensibilizados pela ação de substâncias químicas presentes no tecido lesado, como por exemplo: bradicinina, histamina, serotonina, leucotrieno, substância P, fator de ativação plaquetário (PAF), radicais ácidos (H^+), íons potássio (K^+), prostaglandinas (PG), tromboxanos (TX), interleucinas (IL), fator de necrose tumoral (TNF-α) e AMPc (VERRI et al., 2006; LUMPKIN e CATERINA, 2007).

Quando o estímulo provoca lesão tecidual, há ativação do processo inflamatório, e as células lesionadas liberam enzimas como a fosfolipase A_2, que age na membrana celular e leva a formação da cicloxigenase (COX) que origina as prostaglandinas, que por sua vez desempenham papel fundamental na nocicepção periférica. As prostaglandinas agem modificando receptores transientes vaniloides específicos (TRPV1) acoplados a canais iônicos dependente de ligantes, por meio da ativação da via do AMPc, e das proteínas quinases PKA e PKC, diminuindo o tempo de pós-hiperpolarização da membrana neuronal e consequentemente o limiar de disparo da fibra nervosa, bem como elevando a frequência de potenciais de ação produzidos durante uma estimulação supralimiar, levando assim ao processo de sensibilização periférica (hipersensibilidade) com consequente potencialização da resposta ao estímulo doloroso (LINLEY et al., 2010).

Muitos estudos têm sido desenvolvidos a fim de entender melhor a fisiopatologia da dor e dos processos inflamatórios associados, na busca de desenvolver fármacos mais específicos para cada tipo de dor, e que apresentem ação analgésica e anti-inflamatória, com maior eficácia e segurança, o qual é justificado pelo fato de que a terapia farmacológica ainda é o recurso clínico mais eficaz no tratamento da dor. Contudo, apesar da ampla utilização dos medicamentos na prática clínica, cerca de 40 - 60% dos pacientes não respondem à farmacoterapia convencional, uma vez que alguns tipos de dor podem

ser resistentes a analgésicos comuns (XU et al., 2012; CLAUW; ARNOLD; MCCARBERG, 2011).

O interesse por produtos naturais de origem vegetal, para o desenvolvimento de novos fármacos é cientificamente explorado, uma vez que apresentam um vasto número de compostos químicos biologicamente ativos com diversas propriedades farmacológicas (NEWMANN e CRAGG, 2020). A região Nordeste do Brasil, apresenta uma rica diversidade de espécies vegetais, cujo bioma Caatinga é o seu principal ecossistema. Tendo em vista a diversidade de espécies vegetais desse bioma, a ampla utilização de plantas para fins medicinais por comunidades traicionais e a escassez de estudos científicos sobre os efeitos biológicos de determinadas espécies, torna-se imprescindível estudos que visem demonstrar potencialidades terapêuticas de plantas endêmicas da região.

A família Passifloraceae, com espécies presentes na região Nordeste, possui aproximadamente 20 gêneros e 650 espécies, sendo o gênero *Passiflora* considerado o mais importante, abrangendo aproximadamente 400 espécies. Muitas espécies desse gênero são conhecidas popularmente como maracujá, e são utilizadas na medicina popular devido à grande variedade de fitoconstituintes, que geram efeitos farmacológicos como depressores do sistema nervoso central (SNC), antioxidantes, antinociceptivos, anti-inflamatórios, cicatrizantes, antimicrobianos, hipotensores e efeito hipolipemiante (LORENZI; MATOS, 2002; ZUCOLOTTO et al., 2009; SOUZA et al., 2014; COSTA et al., 2016; LAVOR et al., 2018).

A espécie *Passiflora cincinnata* é encontrada no semiárido brasileiro e vem apresentando interesse por parte dos pesquisadores por apresentar resistência à seca. Popularmente conhecida como maracujá-do-mato, as folhas dessa espécie são utilizadas na medicina popular para regulação dos níveis pressóricos e por suas propriedades anti-inflamatórias, além disso, os frutos são utilizados como calmante e antitussígeno. Tais propriedades podem ser atribuídas à presença de metabólitos secundários presentes nessa espécie, como por exemplo os flavonoides, especialmente os *C*-glicosilados, isoorientina e isovitexina, identificados em várias partes da planta, especialmente nas folhas e talos (LI et al., 2011; YAZBEK et al., 2016; WHOSCHA et al., 2016; LAVOR et al., 2018; LEAL et al., 2020).

Estudos farmacológicos realizados com a espécie *P. cincinnata* demonstraram que o extrato etanólico das partes aéreas apresentam potencial atividade antinociceptiva e anti-inflamatória, cuja atividade farmacológica envolve a participação de mecanismos

centrais no controle da nocicepção, bem como mecanismos periféricos. Estudos envolvendo mecanismo de ação sugerem a participação de receptores opióides e canais para potássio no mecanismo antinociceptivo do extrato, o que pode ser atribuído aos metabólitos secundários já descritos para a espécie (LAVOR et al., 2018; LEAL et al., 2020).

Diante da relevância dos produtos naturais no desenvolvimento de novos compostos bioativos, esse trabalho foi desenvolvido em dois capítulos distintos, onde o capítulo 1 desta tese refere-se ao artigo de revisão que aborda o uso de óleos essenciais em processos inflamatórios crônicos e o potencial terapêutico desses por meio de estudos *in vivo* e *in vitro*, e o capítulo 2 apresenta um estudo experimental de avaliação pré clínica do efeito antinociceptivo do extrato aquoso de *Passiflora cincinnata* e o possível envolvimento de vias centrais na antinocepção induzida pelo extrato aquoso de *P. cincinnata*.

OBJETIVOS

OBJETIVO GERAL

Avaliar o efeito antinociceptivo do extrato aquoso das folhas de *Passiflora cincinnata* Mast. (Passifloraceae) em modelos animais.

OBJETIVOS ESPECÍFICOS

- ✓ Obter e realizar a caracterização farmacognóstica da droga vegetal de *P. cincinnata*, segundo os parâmetros da farmacopeia brasileira;
- ✓ Quantificar marcadores químicos nos extratos por meio de cromatografia líquida de alta eficiência acoplada a detector de arranjo de diodos (CLAE-DAD);
- ✓ Analisar o perfil toxicológico do extrato aquoso das folhas de *P. cincinnata*;
- ✓ Investigar a atividade antinociceptiva do extrato aquoso das folhas de *P. cincinnata*;
- ✓ Investigar o mecanismo de ação envolvido na atividade farmacológica do extrato.

CAPÍTULO 1

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Essential oils and their major compounds in the treatment of chronic inflammation: a review of antioxidant potential in pre-clinical studies and molecular mechanisms

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Abstract

Inflammatory diseases result from the body's response to tissue damage, and if the resolution is not adequate or the stimulus persists, there will be the progression from acute inflammation to chronic inflammation, leading to the development of cancer, neurodegenerative and autoimmune diseases. Due to the complexity of events that occur in inflammation associated with the adverse effects of drugs used in clinical practice, it is necessary to search for new biologically active compounds with anti-inflammatory activity. Among natural products, essential oils (EOs) present promising results in pre-clinical studies, with action in the main mechanisms involved in the pathology of inflammation. The present systematic review summarizes the pharmacological effects of EOs and their compounds in *in vitro* and *in vivo* models for inflammation. The research was conducted in the following databases: PubMed, Scopus, BIREME, Scielo, Open Grey and Science Direct**.** Based on the inclusion criteria, 30 articles were selected and discussed in this review. The studies listed revealed a potential activity of EOs and their compounds for the treatment of inflammatory diseases, especially in chronic inflammatory conditions, with main mechanism involving reduction of reactive oxygen and nitrogen species associated to an elevation of antioxidants enzymes as well as the reduction of the nuclear factor kappa B (NF-kB), reducing the expression of pro-inflammatory cytokines. Thus, this review suggests that the EOs and their major compounds are promising tools for the treatment of chronic inflammation.

1. Introduction

Inflammation is characterized as a normal response to tissue damage caused by several potentially injurious stimuli, induced by biological, chemical, and physical factors [1]. Initially, inflammatory agents elicit an acute inflammatory response which generally promotes complete destruction of the irritants. This type of inflammation persists for a short time and is beneficial for the host [2, 3]. However, if resolution of inflammation is inadequate or the stimulus persists, chronic inflammation occurs, predisposing the host to various diseases including, for example, cancer and neurodegenerative diseases [4-6].

During chronic inflammation, a variety of intracellular signaling pathways are activated, comprising of cell surface receptors, tyrosine kinases and transcription factors, leading to overexpression of proinflammatory genes involved in the development of chronic diseases [7]. Furthermore, the cellular components represented by the mast cells and leukocytes are recruited to the site of the damage, which leads to a "respiratory burst" result of increased oxygen uptake and therefore an increased release and accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) at the site of damage [4-8]. Under physiological conditions, ROS are generated in phagocytes to neutralise the invading organisms, presenting an important role in the host defense mechanism. In contrast to oxidant mechanisms, the organism has endogenous defense antioxidant systems, including for example superoxide dismutase, glutathione peroxidase and catalase. When ROS production is greater than cellular antioxidant capacity, oxidative stress can damage DNA, proteins and lipids [9-12].

A diversity of protein kinases is activated in the inflammatory process, such as members of the Janus-activated kinase (JAK), phosphatidylinositol-3-kinase (PI3K/, AKT) and mitogen-activated protein kinase (MAPK) families to alter cell proliferation. In the chronic inflammatory process, the excessive activation of these signaling pathways

causes also the activation of certain transcription factors, such as NF-κB, signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1α (HIF1-α) and activator protein-1 (AP-1), potentiating the initial inflammatory response. In addition to these factors, the cyclooxygenase enzyme (COX), inducible nitric oxide synthase (iNOS), cytokines, chemokines, have also been reported to play a role in oxidative stress-induced inflammation [7, 13-14].

In recent years, the search for more effective drugs for the treatment of the inflammation with fewer side effects has encouraged researchers to study and develop new drugs. The search for natural products derived from plants is a promising reality, and among the substances with pharmacological potential we can cite the essential oils (EOs).

EOs are liquid mixtures of volatile compounds obtained from aromatic plants, which represent a small fraction of the plant composition [15]. However, they are responsible for providing characteristics that favor their use in the food, cosmetic and pharmaceutical applications. The essential oils have a complex composition, the great majority of the identified components include terpenes (oxygenated or not), predominantly monoterpenes and sesquiterpenes. However, allyl and propenylphenols (phenylpropanoids) also are important components of some essential oils [16-18]. These secondary metabolites have been related as potent antioxidants, free radical scavengers and metal chelators, also presenting antinociceptive, neuroprotective, anticonvulsant and anti-inflammatory properties, reported in pre-clinical studies, characterizing as potential source for the development of new drugs [18-21].

The objective of this review was to relate the use of essential oils correlating its antioxidant effect in the treatment of chronic inflammations.

2. Material and Methods

2.1. Search Strategy. In this review, the specialized databases Pubmed, Science Direct, Scopus, Open Grey, Scielo and BIREME were used for literature search in March and April 2018, using different combinations of the following keywords: essential oils, volatile oils, antioxidants and inflammation. We did not contact investigators, and we have not attempted to identify unpublished data until the date of the search.

2.2. Study Selection. In this step, two independent researchers (J.C.S. and A.W.C.F.) first selected the articles according to the title, abstract and finally through an analysis of the full-text publication. The following inclusion criteria were applied: studies with EOs or their major compounds with anti-inflammatory and antioxidant activity *in vitro* and/or *in vivo*. Studies were excluded according to the following exclusion criteria: review articles, meta-analyses, abstracts, conference proceedings, editorials/letters, case reports and studies in humans and articles published over 20 years ago. Additional papers were included in our study after analyses of all references from the selected articles. In cases of non-consensus, a third independent review was consulted (E.M.L.) for final decision.

2.3. Data Extraction. Data were collected and examined by one reviewer using standardized forms and were checked by a second reviewer. The information extracted from the articles included EOs or their major compounds, cell lines (*in vitro* studies), animal models (*in vivo* studies), doses or concentrations, routes of administration, biochemical assays and molecular mechanisms investigated.

2.4. Methodological Quality Assessment. The risk of bias and quality of pre-clinical *in vivo* studies were performed using an adaptaded checklist [22-24]. This investigation

allowed assessing the methodological quality of the included studies concerning mainly the randomization of the treatment allocation, blinded drug administration, blinded outcome assessment and outcome measurements.

3. Results and Discussion

3.1. Study Selection. The primary search identified 429 articles (200 from Scopus, 18 from Science Direct, 32 from BIREME and 179 from Pubmed). However, 146 manuscripts were indexed in two or more databases and considered only once, resulting in 283 original articles. After an initial screening of titles and abstracts, 192 articles were excluded because they did not meet the inclusion criteria or presented completely different themes from the proposal of this review. After an initial screening of titles and abstracts and a full-text analysis, 27 articles were considered potentially relevant. In addition, 3 articles were included after manual search for data extraction, totalizing 30 final articles included in this systematic review. A flowchart illustrating the progressive study selection and numbers at each stage is shown in Figure 1.

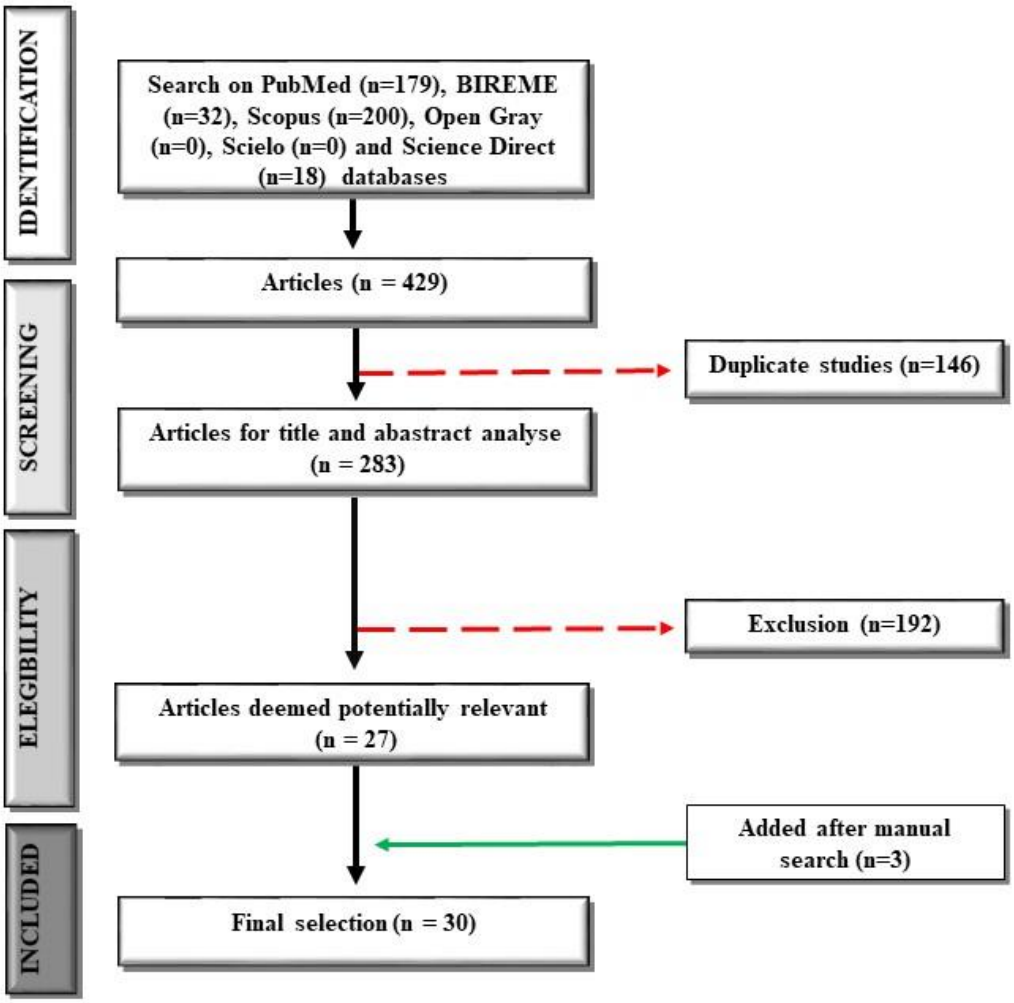

FIGURE 1: Flowchart detailing literature searching and screening.

3.2. Characteristics of Included Studies. The selected final articles were carefully analyzed in relation to the country where the study was conducted, year of publication, family of the studied species and whether the study was carried out with essential oils or substances obtained from them. The Table 1 summarizes the general informations contained in the selected *in vitro* and *in vivo* studies.

TABLE 1: General characteristics of included studies (*in vitro* and *in vivo* reports).

Authors, year, country	**Model**	**Essential oil**	**Major constituents**	**Family**	**Induction of inflammation**	**Type of inflammation**
Tsai et al, 2011, Taiwan [25]	*In vitro*	Essential oils of the aerial parts of *Eucalyptus bridgesiana*, *Cymbopogon martinii*, *Thymus vulgaris*, *Lindernia anagallis*, and *Pelargonium fragrans*	1,8-Cineole Geraniol Thymol *p*-Menthanone (-)-Spathulenol	Myrtaceae Poaceae Lamiaceae Linderniaceae Geraniaceae	Lipopolysaccharide (LPS) from *Escherichia coli* and heat-killed *Propionibacterium acnes*	Inflammation induced by biological agent
Ritter et al, 2013, Brazil [26]	*In vivo*	-	Anethole	-	Complete Freund's Adjuvant	Inflammation induced by biological agent

Jeena et al, 2013, India [27]	*In vivo*	Essential oil of ginger	Zingiberene	Zingiberaceae	Formalin	Inflammation induced by chemical agent
El-Readi et al, 2013, Egypt [28]	*In vitro*	Essential oils from leaves and stems of *Liquidambar styraciflua*	α-Pinene	Altingiaceae	LPS from *Escherichia coli*	Inflammation induced by biological agent
Valente et al, 2013, Portugal [29]	*In vitro*	Essential oils of the aerial parts of *Oenanthe crocata L.*	*β*-Ocimene Sabinene	Apiaceae	LPS from *Escherichia coli* and INF-γ	Inflammation induced by biological agent
Jing et al, 2014, China [30]	*In vitro*	Essential oil of *Patrinia scabiosaefolia*	Caryophyllene oxide	Caprifoliaceae	LPS from *Escherichia coli*	Inflammation induced by biological agent
Destryana et al, 2014, Indonesia [31]	*In vitro*	Essential oil from leaf and branches of *Ocotea quixos*, wood, branches	*trans*-Caryophyllene β-Caryophyllene	Lauraceae Cupressaceae Fabaceae	LPS from *Escherichia coli*	Inflammation induced by biological agent

		and leaves of *Callitris intratropica* and *Copaifera reticulata/langsdorfii* gum-resin	(+)-Calarene			
Shirole et al, 2014, India [32]	*In vitro* and *in vivo*	Essential oil of *Pistacia integerrima*	4-Carvomenthenol	Anacardiaceae	LPS from *Escherichia coli* and ovalbumin	Inflammation induced by biological agent
Patil et al, 2014, India [33]	*In vivo*	Essential oil of *Camellia reticulata* L.	-	Theaceae	Indomethacin	Inflammation induced by chemical agent
Khodabakhsh et al, 2014, Japan [34]	*In vivo*	Essential oil from blossoms of *Citrus aurantium* L.	Linalool	Rutaceae	Cotton pellet - subcutaneous	Inflammation induced by physical agent

Wu et al, 2014, China [35]	*In vivo*	-	Linalool	-	*Pasteurella multocida* intranasal	Inflammation induced by biological agent
Jeena et al, 2014, India [36]	*In vivo*	Essential oil of *Piper nigrum* Linn	Caryophyllene	Piperaceae	Formalin	Inflammation induced by chemical agent
Entok et al, 2014, Turkey [37]	*In vivo*	Essential oil of *Nigella sativa* L.	-	Ranunculaceae	LPS from *Escherichia coli*	Inflammation induced by biological agent
Kazemi, 2015, Iran [38]	*In vitro*	Essential oils *of Achillea millefolium* L., *Anethum graveolens* L., and *Carum copticum* L.	Thymol	Asteraceae Apiaceae	LPS from *Escherichia coli*	Inflammation induced by biological agent

Pinheiro et al, 2015, Brazil [39]	*In vivo*	Essential oil from leaves of *Choisya ternata* Kunth	-	Rutaceae	Dorsal subcutaneous injection of sterile air and carrageenan suspension	Inflammation induced by chemical agent
Kara et al, 2015, Turkey [40]	*In vivo*	-	Carvacrol	-	LPS from *Escherichia coli*	Inflammation induced by biological agent
Allam et al, 2015, Egypt [41]	*In vivo*	Essential oil of thyme	-	Lamiaceae	*Shigella flexneri*	Inflammation induced by biological agent
Shen et al, 2016, China [42]	*In vitro*	Essential oil of calyx of *Hibiscus sabdariffa L.*	*n*-Hexadecanoic acid	Malvaceae	LPS from *Escherichia coli*	Inflammation induced by biological agent

Park et al, 2016, Korea [43]	*In vitro and in vivo*	Essential oil of *Chamaecyparis obtusa*	-	Cupressaceae	*In vitro*: LPS from *Escherichia coli* *In vivo:* Carrageenan-induced paw edema. Thioglycollate-induced peritonitis.	Inflammation induced by biological and chemical agent
Skala et al, 2016, Poland [44]	*In vitro*	Essential oils from roots of *Rhaponticum carthamoides*	Cyperene Aplotaxene	Asteraceae	LPS from *Escherichia coli*	Inflammation induced by biological agent
Zhao et al, 2016, China [45]	*In vivo*	-	Cinnamaldehyde	-	LPS from *Escherichia coli*	Inflammation induced by biological agent
Yu et al, 2016, Brazil [46]	*In vivo*	-	Thymol	-	High-fat-diet-induced	Inflammation induced by chemical agent

					hyperlipidemia and atherosclerosis.	
Kennedy-Feitosa et al, 2016, Brazil [47]	*In vivo*	-	Eucalyptol	-	Exposition to commercial cigarettes	Inflammation induced by chemical agent
Alvarenga et al, 2016, Brazil [48]	*In vivo*	-	Carvacrol	-	Irinotecan	Inflammation induced by chemical agent
Shen et al, 2017, China [49]	*In vitro*	Essential oil from blossoms of *Citrus aurantium*	-	Rutaceae	LPS from *Escherichia coli*	Inflammation induced by biological agent
Liu et al, 2017, China [50]	*In vivo*	-	β-Elemene	-	High-fat-diet-induced	Inflammation induced by chemical agent

					hyperlipidemia and atherosclerosis.	
Leelarungrayu b et al, 2017, Thailand [51]	*In vivo*	Essential oil of *Zingiber cassumunar* Roxb. in niosomes entrapped	Terpinen-4-ol	Zingiberaceae	LPS from *Porphyromonas gingivalis*	Inflammation induced by biological agent
Arigesavan and Sudhandiran, 2017, India [52]	*In vivo*	-	Carvacrol	-	1,2 Dimethylhydrazine (DMH) and dextran sodium sulphate (DSS)	Inflammation induced by chemical agent
Marques et al, 2018, Brazil [53]	*In vitro*	-	l-Carveol, l-carvone, *m*-cymene, valencene and guaiene	-	LPS from *Escherichia coli*	Inflammation induced by biological agent

Pivetta et al, 2018, Brazil [54]	*In vivo*	-	Thymol in nanoparticles from natural lipids	-	Imiquimod	Inflammation induced by chemical agent

Studies were conducted by research groups located in about 13 different countries. Most of the investigations were authored by researchers from Brazil (7 reports, 24.13%), China (6 reports, 20.68%) and India (5 reports, 17.24%).

The largest number of studies found in Brazil is justified by the fact that Brazil has an extremely rich biodiversity, corresponding to approximately 20% of all living species known globally, comprising over 45,000 species of higher plants. In addition, the Brazilian population has a historical tradition in the use of medicinal plants for the treatment of different diseases, including acute and/or chronic inflammation disorders [55] [56]. Another interesting fact is the number of studies conducted in China and India, which may be justified as a reflection of the contribution of Oriental medicine in the search and development for new drugs from natural products. In fact, Traditional Chinese Medicine (TCM) and Ayurveda as major traditional treatment systems used not only in India and China, but also in several countries, contributed to the development of new pharmaceutical products based on plants species [57] [58] [59].

Regarding the number of annual publications, we noted that a large number of articles was published from 2010 to 2015 (12 reports). Only in the last three years, 18 studies (62.02%) were found, suggesting that the involvement of oxidative stress in anti-inflammatory activity of essential oils or their major compounds has attracted the attention of the researchers in diverse regions of the world. These results are graphically presented in figure 2.

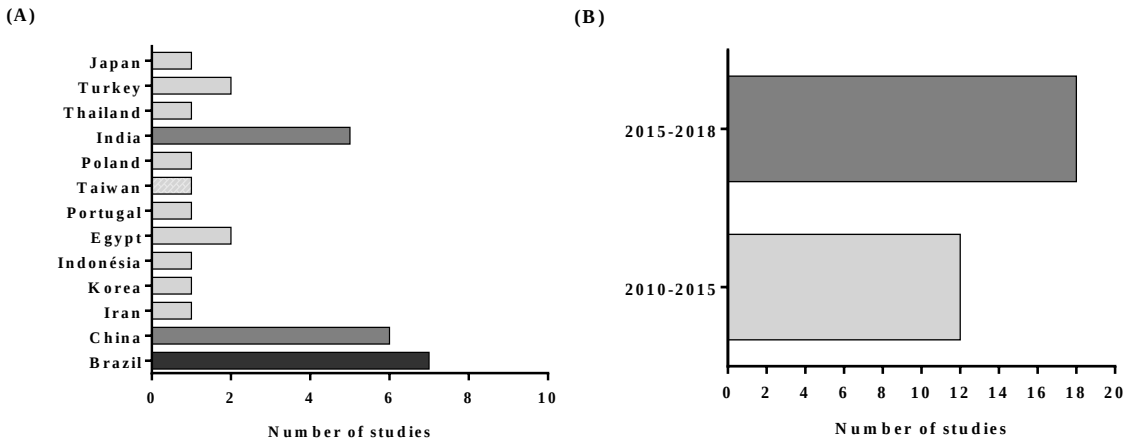

Figure 2: Distribution of the selected studies by country (A) and year of publication (B).

Among the included articles, only 10 (32.25%) corresponded to studies with isolated components of essential oils, demonstrating that reports involving EOs are still predominant in this subject. Of these oils, three studies were reported for species belonging to the Rutaceae family and two studies for the families Zengiberaceae, Apiaceae, Cupresseaceae and Lamiaceae. The other studies correspond to other families reported in Table 1.

As described in Table 1, our review included 2 reports presenting in *vitro* and *in vivo* studies, 9 reports presenting only *in vitro* studies and 19 reports presenting only *in vivo* studies. In the studies reported in this review biochemical and molecular targets were verified by colorimetric and enzymatic assays, biochemical analyzes and techniques such as western blot and immunohistochemistry. These studies base their assays on methodologies using cell culture commonly found in chronic inflammatory processes, such as macrophages, monocytes, astrocytes, and cancer cells, correlating anti-inflammatory results with the antioxidant potential of essential oil or their major components. The evaluated *in vitro* studies parameters and main outcomes are summarized in Table 2 and *in vivo* studies in Table 3.

Table 2. *In vitro* studies involving essential oils, anti-inflammatory and antioxidant activity.

Essential oil and/or majority constituent	**Doses**	**Antioxidant and anti-inflammatory assays**	**Cell line**	**General results and proposed mechanism of action**	**Reference**
Essential oils of the aerial part of *Eucalyptus bridgesiana*, *Cymbopogon martinii*, *Thymus vulgaris*, *Lindernia anagallis*, and *Pelargonium fragrans*	0.01 µg/mL	β-Carotene linoleic acid bleaching test, DPPH radical and nitric oxide scavenging assay 5-LOX inhibition assay Measurement of IL-1β, IL-8, TNF-α	THP-1 (Human mylelomonocytic cell)	Strong antioxidant activity in the tests performed; Inhibition of 5-LOX activity and reduction of IL-1β, IL-8, TNF-α secretion in THP-1 cells	Tsai et al, 2011 [25]

Essential oils of the aerial parts of *Oenanthe crocata L.*, *β*-ocimene or sabinene	EO: 0.08, 0.16, 0.32 µL/mL *β*-ocimene and sabinene: 0.32–1.25 µL/mL	Measurement of NO, Western blot analysis for iNOS, Nitric oxide scavenging activity	RAW 264.7 macrophages	Strong NO scavenging activity and inhibition of iNOS expression; Sabinene exhibited NO scavenging activity only at higher concentrations	Valente et al, 2013 [29]
Essential oils from leaves and stems of *Liquidambar Styraciflua*	1, 10, 100 and 500 µg/mL	5-LOX and PGE_2 inhibition DPPH radical and superoxide scavenging activity	HepG-2 cells	Reduction of DPPH, ($OH^{\bullet}$) and ($O_2^{\bullet}$) radicals Inhibition of 5-LOX and PGE_2	El-Readi et al, 2013 [28]

Essential oil of *Patrinia scabiosaefolia*	50,100,150, 200,250 µg/mL	Measurement of IL-1 and IL-6 DPPH radical scavenging assay	BV-2 cell (microglia)	Inhibition of the production of IL-1 and IL-6; scavenging activity against the DPPH radical	Jing et al,2014 [30]
Essential oil from leaf and branches of *Ocotea quixos*, wood, branches and leaves of *Callitris intratropica* and *Copaifera reticulata/langsdorfi i* gum-resin	5, 10, 20 µg/mL	β-Carotene linoleic acid bleaching test and DPPH radical scavenging assay Measurement of NO production Western blotting analyses for the iNOS and COX-2 and measurement of IL-8, IL-6 and IL-1β	RAW 264.7 macrophages	The EO of *O. quixos* and *C.reticulata* did not possess an antioxidant activity, while Blue Cypress possessed a moderate anti-oxidant activity Only Ocotea suppress the LPS-induced PGE_2 production, LPS-mediated iNOS and COX-2 elevation Suppression of LPS-stimulated IL-8 and IL-1β production in the cells	Destryana et al, 2014 [31]

Essential oils *of Achillea millefolium* L., *Anethum graveolens* L., and *Carum copticum* L.		DPPH radical scavenging and FRAP assay β-Carotene bleaching test Determination of NO production.	RAW 264.7 macrophages	*A. millefolium* had the highest antioxidant activity in all conducted assays and inhibited nitric oxide production	Kazemi, 2015, Iran [38]
Essential oil of calyx of *Hibiscus sabdariffa L.*	25, 50, 100,200,300 µg/mL	Determination of NO production Measurement of cytokines Production (IL-1 and IL-6)	RAW 264.7 macrophages	Inhibition of NF-κB signaling pathways and MAPKs (JNK and ERK1/2) , reduction of NO production and IL-1, IL-6, TNF-α, COX-2 and iNOS	Shen et al, 2016 [42]

		RT-PCR assay of IL-1, IL-6, TNF-α, iNOS and COX-2 mRNA Western blot analyses for the p-JNK, p-ERK1/2 NF-κB and GAPDH			
Essential oil of *Chamaecyparis obtusa*	*In vitro*: 1,10,50 and 100 μg/mL *In vivo*: 5 and 10 mg/kg	*In vitro:* Measurement of NO, IL-1β, TNF-α and IL-6 by levels; Western blot analyse for expression of iNOS and COX-2 *In vivo:* Carrageenan-induced paw	RAW 264.7 macrophages	Decreasing in the number of total cells and suppression of TNF-α, IL-1β and IL-6 levels in peritoneal fluid Suppression of iNOS and COX-2 expression	Park et al, 2016 [43]

		edema and thioglycollate-induced peritonitis			
Essential oils from roots of *Rhaponticum carthamoides*	25, 50, and 100 μg/mL	Measurement of cytokines IL-1β, IL-6, IL-8, IL-10, TNF-α, and GM-CSF and RT-PCR. ROS formation assay using H2DCF-DA.	Human astrocytes	Decreasing the expression of IL-1β, IL-6, and TNF-α and the ROS level	Skala et al, 2016 [44]
Essential oil from blossoms of *Citrus aurantium*	15.625, 31.25,62.5 ,125 and 250 µg/mL	DPPH and ABTS radical scavenging activity	RAW 264.7 macrophages	Did not show scavenging effects on DPPH and ABTS radicals	Shen et al, 2017 [49]

		Determination of morphology and NO production. Quantification of IL-6, TNF-α, and IL-1β Reverse Transcription and PCR-RT for iNOS		Inhibition of NO accumulation and suppression of IL-6, TNF- α, and IL-1β secretion Inhibition of the expression of COX-2 and NF-κB activation Involvement of the inflammation process through MAPK signaling pathways, by inhibiting phosphorylation of JNK (c-Jun N-terminal kinase) and p38	
l-carveol, l-carvone,, *m*-cimene, valencene and guaiene	1, 10 and 100 μM	Protective effect against oxidative damage produced by superoxide anion	RAW 264.7 macrophages	Reduction in TNF-α and IL-1α levels and increasing in the production of IL-10	Marques et al, 2018 [53]

	production ($O_2^{\cdot -}$) and hydrogen peroxide	Guaiene and *m*-cymene inhibited NO production
	Determination of NO production	l-carveol and *m*-cymene significantly inhibited $O_2^{\cdot -}$ production
	Quantification of IL-1α, TNF-α, and IL-10	Terpenes suppressed NF-κB activity
	Activity of NF-kB	

Legend: EO - Essential oil; NO - nitric oxide; ROS- Reactive oxygen species; iNOS - inducible nitric oxide synthase; IL-1β - interleukin-1 beta; DPPH - 2,2-diphenyl-1-picrylhydrazyl radical; LPS – Lipopolysaccharide; M – Male; ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; NF-kB - nuclear factor kappa B, COX-2 – Cicloxygenase; TNF-α - tumor necrosis factor alpha; IL-6 - interleukin-6; IL-8 - interleukin-8; IL-10 - interleukin-10, FRAP- ferric reducing antioxidant power; MPO – myeloperoxidase; PCR-RT- Real-Time Quantitative Polymerase Chain Reaction; H2DCF-DA - 2',7'- hichlorodihydrofluorescein diacetate; 5-LOX – 5, lipoxygenase; PGE2 - prostaglandin E2; MAPK - Mitogen Activated Protein Kinases; GAPDH- Glyceraldehyde 3-phosphate dehydrogenase.

Table 3. *In vivo* studies involving essential oils and antioxidant activity.

Essential oil and/or majority constituent	**Animals (strain/sex), n (per group)**	**Doses, route, administration period**	**Antioxidant assays**	**Experimental model of inflammation**	**General results**	**Reference**
Essential oil of Giger	Mice (Balb/c/), n=3, 5 or 6	10, 50, 100, 250, 500 or 1000 mg/kg (i.p. or p.o.), single dose or 4 days	Lipid peroxidation, SOD and Hydroxyl activity assay DPPH and ABTS radical scavenging and FRAP assay PMA-induced radical generation and dosage of SOD,	Formalin induced chronic inflammation	Scavenged superoxide, DPPH, hydroxyl radicals and lipid peroxidation inhibition Increasing of SOD, GSH and GR enzymes levels in blood and glutathione peroxidase and SOD enzymes in liver Reduction in formalin induced chronic inflammation	Jeena et al, 2013 [27]

			CAT and GSH *in vivo*			
Anethole	Mice (Swiss/M), n=6	125, 250, or 500 mg/kg (p.o.), for until 7 days	MPO activity	Paw edema induced by complete Freund's adjuvant	Inhibition of paw edema on all of the days analyzed Inhibition of MPO activity and reduction of TNF-a, IL-1β and IL-17 levels in acute and persistent inflammation models	Ritter et al, 2013 [26]
Essential oil of *Nigella sativa* L.	Rats (Sprague–Dawley), n=7	500 mg/kg (p.o.), 3 times a 1 day	Determination of SOD, CAT activity, MDA and NO levels	LPS induced inflammation	Increasing of SOD and CAT, and reduction of MDA and NO in lung	Entok et al, 2014 [37]

Essential oil of *Piper nigrum* Linn	Mice (Balb/C), n=5 or 6	10, 50, 100, 250, 500 or 1000 mg/kg (i.p. or p.o.), 5 or 30 days.	Lipid peroxidation, SOD and Hydroxyl activity assay DPPH radical scavenging and FRAP assay PMA-induced radical generation and dosage of SOD, CAT and GSH *In vivo*	Formalin induced chronic inflammation	Scavenged SOD, DPPH, hydroxyl radicals; inhibition of lipid peroxidation *in vitro* Increasing of SOD, GSH enzyme levels in blood of mice and CAT, SOD and GSH enzymes in liver Reduction of chronic inflammation in formalin test	Jeena et al, 2014 [36]

Linalool	Mice (C57BL/6J/M), n= 10	5, 15 or 25 mg/kg (s.c)	ROS and SOD activity assay	*Pasteurella multocida* induced intranasal lung infection/ inflammation	Increasing of nuclear Nrf-2 protein amount and the reduction of SOD expression Reduction of TNF-α and IL-6 levels and decreasing of neutrophil accumulation	Wu et al, 2014 [35]
Essential oil from blossoms of *Citrus aurantium* L.	Rats (Wistar/M), n=8	5, 10, 20, 40 or 80 mg/kg (i.p.) for until 7 days	Measurement of NO	Cotton pellet-induced granuloma	Decreasing of transudate and granuloma formation amount involving the nitric oxide pathway	Khodabakhsh et al, 2014 [34]
Essential oil of *Camellia reticulata* L.	Rats (Wistar/M), n=6	200 or 400 mg/kg (p.o.) for 11 days	Colonic GSH content and lipid	Enterocolitis induced by indomethacin	Decreasing of macroscopic and microscopic score for inflammation	Patil et al, 2014 [33]

			peroxides concentration		Reduction of MPO, lipid peroxidation and increasing of GSH content	
Essential oil of *Pistacia integerrima*	Rats (Sprague-Dawley/F), n=6	5-30 µg/mL, 10, 30 or 100 mg/mL, 7,5, 15 or 30 mg/kg (i.p.)	DPPH radical scavenging, Lipoxygenase activity and Measurement of NO and MPO	LPS and ovalbumin - induced bronchial inflammation	Inhibition of lipoxygenase enzyme and DPPH scavenging activity Anti-allergic activity by inhibiting of mast cell degranulation Reduction of total leucocyte, neutrophils, NO, total protein, albumin levels in bronchoalveolar fluid and	Shirole et al, 2014 [32]

					MPO levels in lung homogenates	
Essential oil of thyme	Rats (Sprague-Dawley/M), n= 25	7,5, 15 or 30 mg/kg (i.p.) for 21 days	FRAP assay	Ulcer-forming induced by *Shigella flexneri strain*	Synergistic activity of thyme oil decreased the inflammation of the lamina propria and decreased the bacterial load in the colon Increasing of total antioxidant capacity time	Allam et al, 2015 [41]
Essential oil from leaves of *Choisya ternata* Kunth	Mice (Webster/M), n= 4, 6, 8 or 10	3-10 or 30 mg/kg (p.o)	NO levels and trapping capacity of anthranilates	Formalin test, Subcutaneous air pouch (SAP) model	Reduction of migration, exudate volume, protein extravased, and reduced levels of NO, TNF-α and IL-1β)	Pinheiro et al, 2015 [30]

Carvacrol	Rats (Sprague-Dawley/F), n=6, 7 or 8	20, 40 or 80 mg/kg (p.o.) for 6 days	MDA and NO levels	LPS induced peritoneal inflammation	Decreasing of levels of TNF-α and IL-6, MDA, NO levels, and arginase activity levels	Kara et al, 2015 [40]
Cinnamaldehyde	Rats (Sprague-Dawley/M), n= 6	30, 60 or 90 (p.o.) 1x/day for 30 days	Determination of intracellular levels of ROS	LPS-induced cardiac dysfunction	Inhibition of cardiac dysfunction, inflammatory infiltration and the levels of TNF-α, IL-1β and IL-6 in LPS stimulated rats by blocking the TLR4, NOX4, MAPK and autophagy signaling pathway	Zhao et al, 2016 [45]
Thymol	Rabbits (M), n= 6	3 or 6 mg/kg (p.o) for 8 weeks	DPPH and ABTS radical scavenging activity and	Inflammatory process in	High antioxidant activity in both tests	Yu et al, 2016 [46]

			measurement of MDA level in serum	aortic intimal thickening	Reduction of TC, TG, LDL-C, and MDA levels Reduction of VCAM-1, MCP-1 levels and pro-inflammatory cytokines IL-1β, IL-6 and TNF-α	
Eucalyptol	Mice (C57BL/6/M), n= 8	1, 3, 10 mg/mL) via inhalation (15 min/daily) for 5 days	NBT assay, SOD and CAT activity Measurement of GSH and TBARS levels	Cigarette smoke exposure	Reduction of IL-1β, IL-6, and TNF-α levels Decreasing of NF-kB expression Reduction of ROS, SOD, CAT, MDA and GSH levels	Kennedy-Feitosa et al, 2016 [47]

					Rare presence of leukocytes in alveolar septa	
Carvacrol	Mice (Swiss/F), n= 5 or 8	25, 75 or 150 mg/kg (i.p.) for 8 days	GSH, MDA, NO levels	Intestinal mucositis induced by CPT-11 chemotherapy	Reduction of TNF-α, IL-1β, and KC levels Decreasing of MPO, NF-κB, COX-2 and oxidative stress (GSH, MDA, and NO levels)	Alvarenga et al, 2016 [48]
β-Elemene	Mice ApoE−/− (C57BL/6/M) , n= 6	Not related	Measurement of eNOS and NO concentrations, ROS assay, enzyme activity SOD, CAT, GPx, GSH and MDA	Atherosclerosis induced by high-fat	Inhibition of atherosclerotic lesion size and increasing of plaques stability Reduction of vascular oxidative stress and preventing	Liu et al, 2017 [50]

					pro-inflammatory cytokine production Improvement in NO levels, expression of eNOS and phosphorylation of eNOSser1177 and Akt	
Carvacrol	Rat (Fischer 344/M), n= 6	50 mg/kg (p.o.) for 7 days before and 7 days, after tumor induction	Antioxidant enzymes activities SOD, CAT, GPx, GR, GSH, vitamin E and vitamin C, NO level and MDA contents	Colitis induced by DMH associated colon cancer	Increasing of SOD, CAT, GSH levels and reduction of LPO, MPO and NO Suppression of pro-inflammatory mediators iNOS and IL-1β Reduction of ulcer size	Arigesavan and Sudhandiran, 2017 [52]

Essential oil of *Zingiber cassumunar* Roxb. in niosomes entrapped	Rats (Wistar/M), n= 5	12.5–400 μg/mL	DPPH radical scavenging	LPS-induced subcutaneous inflammatory assay	Inhibition of DPPH radical and decreasing of skin temperature and blood flow, reducing tissue inflammation process	Leelarungrayub et al, 2017 [51]
Thymol in nanoparticles from natural lipids	Mice (C57B/6/M), n=10 or 12	5 mg/day (p.o.), 15 days	Anthralin induced ear edema model	Imiquimod-induced psoriasis	Improved inflammation and healing, on antharalin model and imiquimod	Pivetta et al, 2018 [54]

Legend : eNOS - nitric oxide synthase; NO - nitric oxide; ROS- Reactive oxygen species; SOD-Superoxide dismutase; CAT – Catalase; GPx - Glutathione peroxidase; GSH – Glutathione; GR - reductase glutathione; MDA- malondialdehyde; DMH - 1, 2-dimethyl hydrazine; LPO - lipid peroxides; iNOS - inducible nitric oxide synthase; IL-1β - interleukin-1 beta; DPPH - 2,2-diphenyl-1-picrylhydrazyl radical; LPS – Lipopolysaccharide; M – Male; ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; TC- total cholesterol; TG – triglycerides; LDL - low density ipoprotein; VCAM-1 - vascular cell adhesion molecule-1; MCP-1 -monocyte chemotactic protein-1 (MCP-1); NBT- Nitroblue tetrazolium; TBARS - thiobarbituric acid; NF-kB - nuclear factor kappa B; MDA – malonaldehyde; CPT-11 - Irinotecan, Camptosar,

Camptothecin-11; COX-2 – Cicloxygenase; TNF-a - tumor necrosis factor alpha; IL-6 - interleukin-6; IL-17 - interleukin-17; FRAP- ferric reducing antioxidant power; MPO – myeloperoxidase; TNBS - trinitrobenzenesulphonic acid; NrF2 - nuclear factor erythroid 2–related factor 2; PMA - phorbol-12-myristate-13-acetate.

3.3. Methodological quality of in vivo studies

Regarding methodological quality, all *in vivo* studies were evaluated using a standardized checklist, as shown in Figure 3. It was observed that all studies described the objectives, outcomes to be measured and main findings obtained. However, none of the included articles reported sample size calculation. In general, doses, routes of administration and frequency of treatment were adequately described. Most of the *in vivo* studies (18 reports, 85.7%) adequately reported the animal allocation, but less than half (10 reports, 47.6%) reported that the animals were randomly housed. In addition, the majority of included studies did not make it clear if the investigators or the outcome assessor were blinded from the treatment used.

The number of animals to be used, randomization and blinding are important steps in preclinical protocols in order to reduce the risk of bias and improve translatability of animal research [60] [61]. In this way, the evaluation of the methodological quality indicated that the majority of *in vivo* studies included in this review present moderate quality, which limits the interpretation of the results.

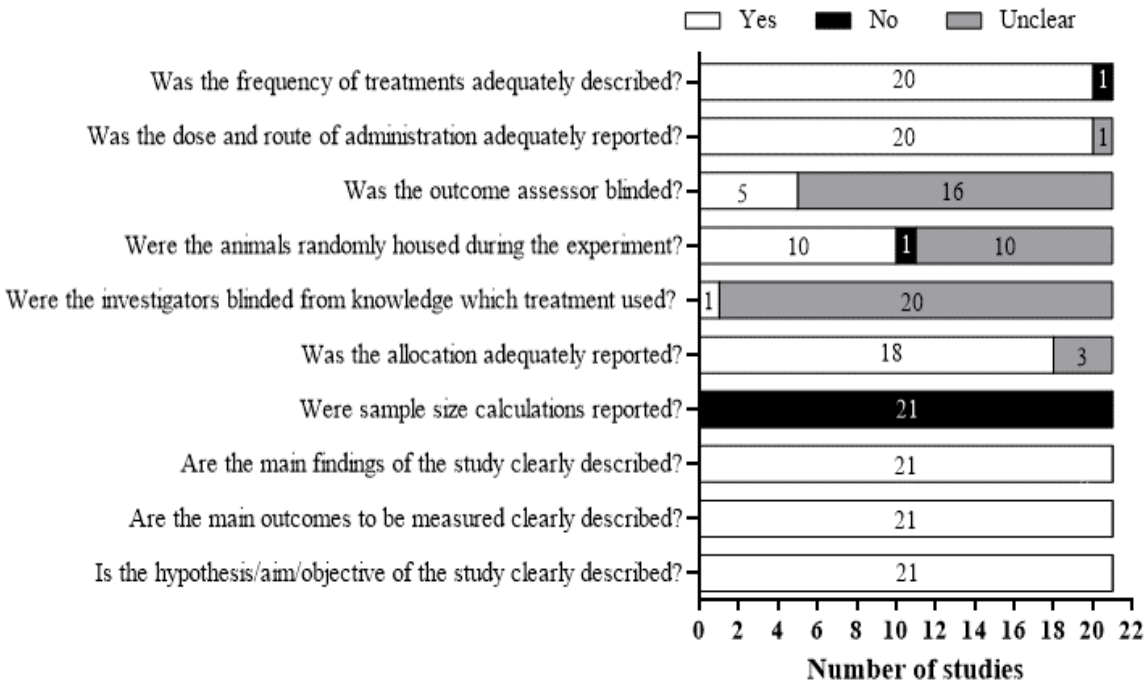

Figure 3: Methodological quality of included *in vivo* studies. White bars indicate the proportion of articles that met each criterion; black bars indicate the proportion of studies that did not and gray bars indicate the proportion of studies with unclear answers.

3.4. In vitro tests of anti-inflammatory activity

Researchers, when proposing to investigate the pharmacological evaluation of substances, initially carry out *in vitro* tests, since these tests present a high reproducibility and function as a trait to evaluate the pharmacological potential of these substances, as for example the anti-inflammatory activity. The assays employed are, in most instances, cell culture techniques, in which the cells receive various stimuli (chemical or biological) that induce the production of mediators involved in inflammatory processes, such as arachidonic acid and cytokines and their metabolites [25] [29] [31] [43].

In the majority of *in vitro* selected papers the anti-inflammatory activity tests employed the macrophage cell line RAW 264.7 activated by LPS [29] [31] [42] [43] [49]. Macrophages play a critical role in the inflammatory process through the production of various cytokines.

When these cells are activated, they express the inflammatory enzymes (iNOS and COX-2) and proinflammatory cytokines (TNF-α and IL-6). However, they also may play an anti-inflammatory role in which they express IL-4, IL-13 or IL-10 cytokines [62] [63] [64].

Other cells participate in the inflammatory process, and have a crucial role in the development of inflammatory diseases. To evaluate this activity, Singh et al. [65] proposed the utilization of the human THP-1 cell, a common model to estimate modulation of monocyte and macrophage activities. Circulating monocytes have the potential to differentiate into tissue macrophages, providing help in the phagocytosis of invading pathogens, reducing tissue aggression by potentially harmful agents [66].

In recent years, inflammatory processes have been correlated to the development of chronic diseases. However, chronic inflammation and cytokine dysfunction are associated with conditions such as cancer progression, cardiovascular disease, diabetes, and neurodegenerative disease [67]. To better study this molecular aspects, inflammatory models using microglial [30] and astrocytes [44] cell line have been used to evaluate the influence of inflammatory processes on the development of neurodegenerative diseases, and tumor cell lines such as HepG2 [28] to evaluate the relationship between the processes inflammatory and malignant neoplasms.

3.5 Animal models in Chronic inflammation

Chronic inflammation is an aggravating factor for tissue damage, commonly present in many chronic diseases, including asthma, obstructive pulmonary disease, neuroinflammatory and autoimmune disorders [68]. For this reason, it is necessary to understand the molecular mechanisms involved in inflammatory process in order to develop new treatment and prevention protocols. Thus, many experimental models have been developed, most often using

mice and rats, in order to correlate the pathophysiology of the disease and to aid in the development of new drugs [69] [70].

Concerning to *in vivo* studies included in this review, EOs were investigated in experimental models of ulcerative enterocolitis, lesions developed by chemotherapeutic agents, peritoneal, subcutaneous, pulmonary and cardiac inflammation induced by biological and chemical agents and atherosclerosis.

In recent years, some reports relate pathogen infection to the development and progression of chronic inflammation. In this systematic review, we found 9 studies reporting inflammatory conditions induced by microorganisms or their components, including LPS from *E. coli, Complete-Freunds, S. flexneri, P. multocida* [23] [32] [35] [37] [40-41] [45] [51]. The LPS is the major cause of endotoxemia and sepsis. Toll like receptor 4 (TLR4) it is the main way to activate the inflammation pathway. Some authors suggest that LPS would stimulate inflammation by stimulating the production of reactive oxygen species, mainly by the production of superoxide anion (O_2^-), these factors activate for example the MAPK pathway that will trigger cellular responses to increase the production of proinflammatory cytokines to evoke the immune system to fight the injury [45] [71].

In addition, administration of LPS or microorganisms induces transcription factor NF-κB for initiating and sustaining inflammatory reactions. In the cell cytoplasm, NF-kB is inactivated by interaction with newly synthesized protein inhibitory κB (IκB), in the TLR4 signaling promoted dissociation of complex iκB- NFkB and translocation of the NF-κB into the nucleus from the cytoplasm to induce gene transcription of the cytokines and chemokines [72].

Another widely used model corresponds to the evaluation of inflammatory bowel disease (IBD), for which several pharmacological models are employed, such as induction of ulcers by *S. flexneri* strains., intestinal mucositis induced by chemotherapy and enterocolitis induced by DMH [41] [48] [52]. These pathologies are characterized by an excessive response of the

immune system of the intestinal mucosa, activating the production and release of inflammatory mediators, such as eicosanoids, cytokines, reactive oxygen species (ROS) and nitrogen. In addition, defense cells such mast cells produced toxic superoxide anions in the inflammatory environment and recruits neutrophils generating excess of ROS, proteolytic enzymes and ROS that contribute to the lipid peroxidation. Furthermore, activated macrophages, neutrophils and mast cells express receptors for IL-1β and iNOS playing an important role in progression or persistence of intestinal lesion [73] [74] [75] .

Involvement of inflammation in the pathogenesis of atherosclerosis is also well documented. Inflammatory cell types as T-cells, monocytes, and neutrophils play major roles in mediating the inflammatory response in atherosclerosis. The deposition of lipid and oxidized low-density lipoprotein contribute to the initial and prolongated inflammatory response, especially in lipids oxidation, which are taken up by macrophages, dendritic cells, and smooth muscle cells to form lipid-laden foam cells. In addition, cells of immune system participate to the inflammatory process producing pro-inflammatory cytokines IL-1 and TNF-α, mediators associated with reactive oxygen species (ROS) and nitric oxide (NO) (in excess) induced-expression of adhesion molecules and potentiate inflammation within the atherosclerotic lesion, which induces the chemoattraction of defense cells [50] [76] [77] [78].

Other experimental models have been well reported to assess chronic inflammation, such as cotton-pellet induced granuloma, subcutaneous air pouch, and formalin test. However, these tests present a low similarity to the previously described models in relation to the ability to resembles specific human inflammations, since they reproduce the general aspects of the chronic inflammatory process [27] [34] [36] [39].

The formalin test is commonly described in acute inflammation tests, however, repeated application was described in the studies of Jeena et al. [27] [36]. The inflammatory process is a result of tissue and functional alterations in the tissue accompanied by the release of

inflammatory mediators such as histamine, prostaglandins, nitric oxide and cytokines. To evaluate this, the authors monitor the reduction of edema and perform dosage of the involved mediators [27] [36] [79] [80].

The granulomatous tissue induced by the subcutaneous cotton implant is a widely used method for the assessment of anti-inflammatory substance in chronic inflammation. This type of inflammation is a result of several infectious, autoimmune, toxic, allergic and neoplastic conditions, characterized by the presence of mononuclear leukocytes, specifically macrophages, which respond to several chemical mediators of cell damage, most often forming multinucleated giant cells. In the injured tissue, some histological patterns are observe, such as edema, neovascularization and early-stage fibrosis [81] [82].

3.5. Role of Antioxidants in chronic Inflammation

Free radical corresponds to a molecule or atom that carries unpaired electrons that makes them highly reactive and unstable, and can cause cell damage. In normal cell metabolism many free radicals are produced, which serve important functions in the signaling of specific pathophysiological pathways, the great majority of these radicals being produced in the mitochondrial metabolism. Examples of these are the hydroxyl radical, the superoxide anion, hydrogen peroxide and organic peroxides. In addition, in the absence or low concentrations of oxygen, excessive lipid peroxidation occurs and mitochondria also generate nitric oxide (NO), which can generate reactive nitrogen species, which can produce other reactive species such as malondialdehyde [83] [84] [85].

In the inflammatory process, defensive cells located in injured regions lead to a "respiratory burst" in the tissue resulting from increased uptake of oxygen and, therefore, increased production and release of ROS in the damaged area. The release of mediators by these

cells associated with the presence of ROS and RNS stimulate signal transduction cascades and alter transcription factors, such as NF-κB, which mediate vital reactions of cellular stress, leading to expression of COX-2, iNOS and proinflammatory cytokines. Metabolites generated in inflammation associated with oxidative stress impair healthy tissue by altering the stroma and surrounding epithelial cells, which after a long period of time can evolve into more serious problems and trigger, for example, carcinogenesis [86] [87] .

In general, the body has an enzymatic system to combat the damage caused by oxidative stress. Three major antioxidants are the first line of defense against oxidative stress: superoxide dismutase, catalase and glutathione peroxidase, being these antioxidants commonly measured in the investigation of the antioxidant activity of natural compounds [88] [89].

SOD enzyme, which converts highly reactive superoxide radicals in hydrogen peroxide (H_2O_2) and molecular oxygen [88], performs a first antioxidant defense in an oxidative stress situation [90]. Catalase also participates in this defense process, catalyzing the conversion of hydrogens peroxide (highly reactive) to water and molecular oxygen, being located mainly in the peroxisomes [91]. Glutathione exists in two forms with different subunits and different active sites. Glutathione peroxidase catalyzes the reduction of H_2O_2 or organic peroxides (ROOH) to water or alcohol by the presence of GSH, which is converted to oxidize glutathione during this reaction. The main function of this latter enzyme is the protection of the polyunsaturated cell membranes [89].

In the articles reported in this study, the authors correlated the antioxidant tests with the anti-inflammatory activity of the essential oils and substances tested. For this, isolated tests of *in vitro* antioxidant activity, such as DPPH, inhibition of β-carotene degradation, ABTS, NO and FRAP tests, were used as initial screening of the pharmacological activity of EOs or isolated substances. Based on the satisfactory results obtained, some anti-inflammatory activity tests were subsequently conducted [25] [28] [30] [31] [32] [41] [49] [51]. In contrast, other studies,

especially *in vivo* studies assessed the anti-inflammatory activity and at the end of the experiment the animals were euthanized and blood collected for serum levels indicative enzymes of oxidative stress. In these models, the analyzes of superoxide dismutase, catalase, glutathione, malondialdehyde, and lipid peroxidation were mostly described [26] [27] [33] [36] [37] [40] [47] [48] [50].

3.6. Essential oils with antioxidant properties in the treatment of chronic inflammation

In view of the wide use of traditional medicine associated to its importance in drug discovery, EOs have been studied and their compounds identified/isolated components due to their diverse pharmacological properties, including the treatment of acute and chronic inflammation justified by their antioxidant properties [17] [18]. EOs are volatile compounds that may contain more than 300 different compounds. The most of chemical constituents are terpenes, especially mono and sesquiterpenes, but some non-terpene compounds biosynthesized by phenylpropanoids pathway can also be present in EOs[15]. Figure 4 shows the major constituents of the EOs reported in this study and Figure 5 shows the isolated constituents with antioxidant activity tested in chronic inflammation models.

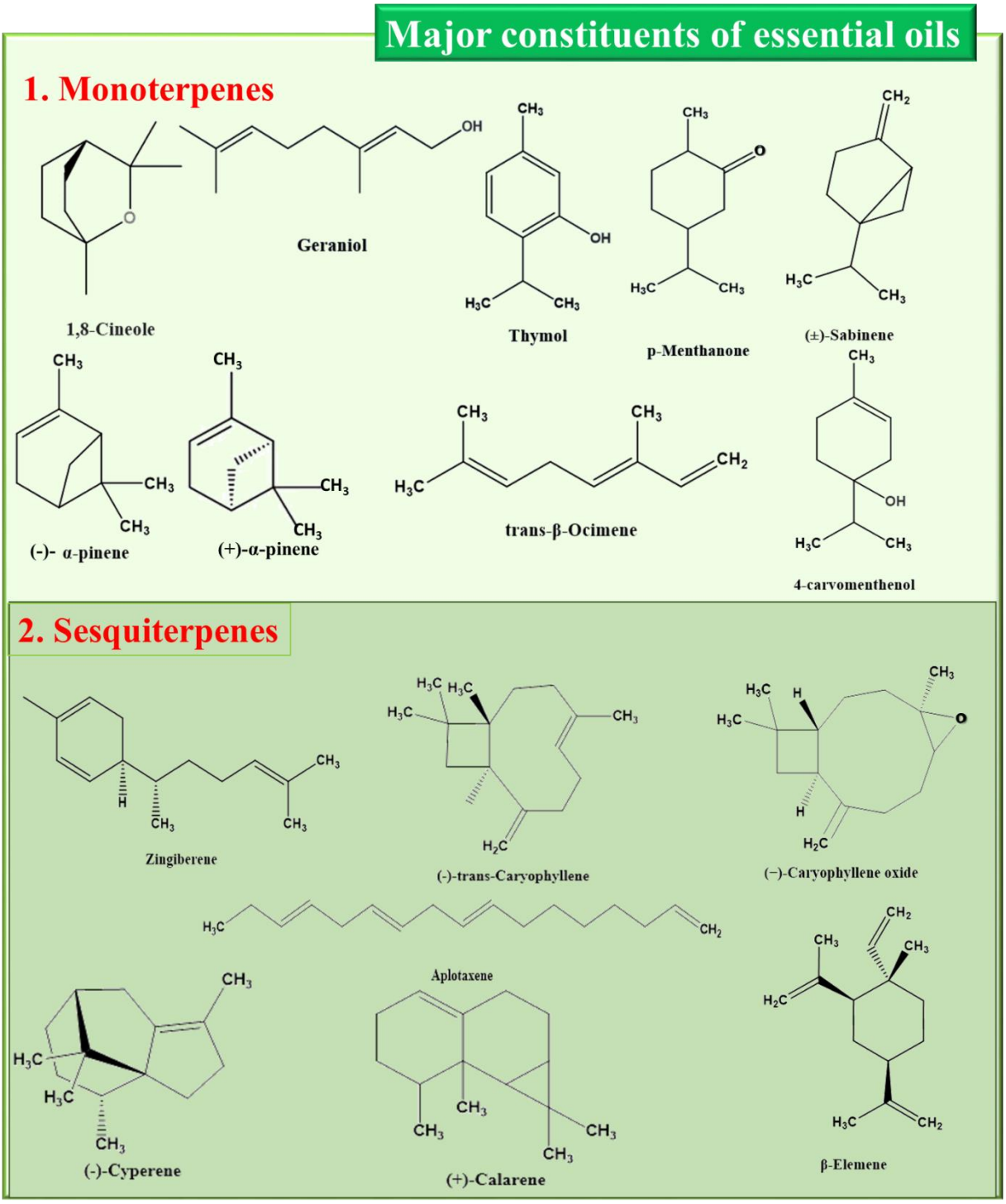

Figure 4: Chemical structure of the major constituents of the essential oils evaluated as antioxidant and anti-inflammatory in chronic inflammation.

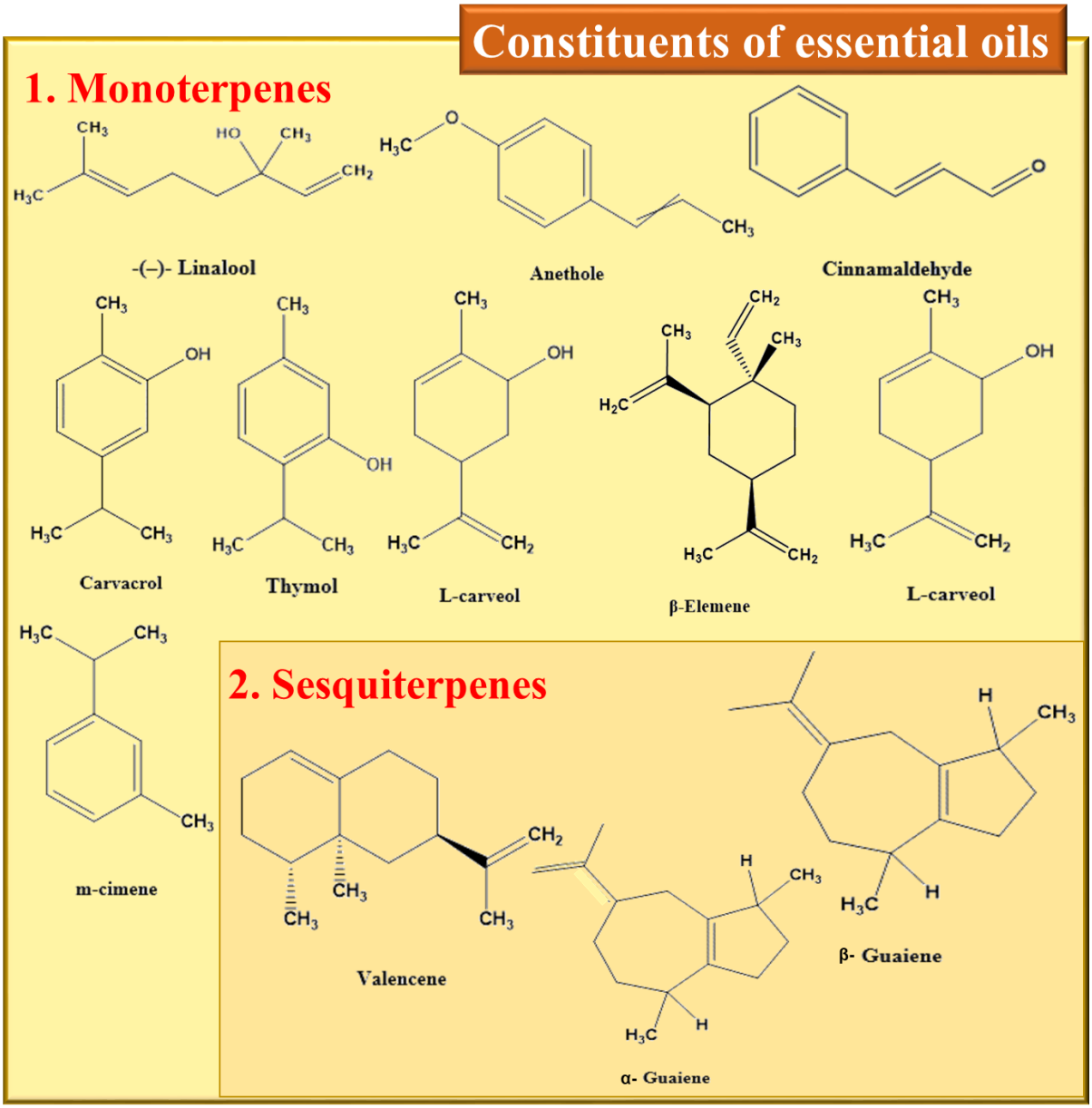

Figure 5: Chemical structure of the constituents isolated of the essential oils evaluated as antioxidant and anti-inflammatory in chronic inflammation.

The anti-inflammatory and antioxidant activities of species and natural compounds were reported in the studies included in this article, where numerous preclinical studies presented promising results. In the experiments using peritoneal macrophages (Raw 264.7, *in vitro*), EOs obtained from *O. crocata, O. quixo, C. intratropica, C. reticulata, H. sabdariffa, C. obtusa* and *C. aurantium* were able to reduce the levels of ROS considerably reducing tissue inflammation

and RNS that cause tissue damage. In addition, EO of *H. sabdariffa* and *C. aurantium* inhibited NF-kB and MAPK signaling and promoted the decrease of the expression of transcription factors for the production of cytokines IL-1β, IL-6 and TNF-α. In addition, these EOs decreased the expression of cyclooxygenase-2 and iNOS enzymes [29] [31] [42] [43] [49].

In vivo tests indicated that the treatment wich Ginger and *P. nigrum* EOs presents ability to sequester superoxide, DPPH and hydroxyl radicals, in addition to inhibiting lipid peroxidation, associated with the reduction of the edema induced by chronic administration of formalin in paw tissue. In this way, the decrease of the inflammatory process occurs due the increase of the activity of the antioxidant enzymes SOD and glutathione [27] [36]. In contrast, *C. aurantium* and *C. ternata* reduced defense cell migration and edema, and reduced levels of nitric oxide in the inflammatory exudate in a granuloma model. In inflammation induced by biological agents such as *E. coli* LPS, treatment with EO of *N. sativa* increased SOD and CAT expression and reducing nitric oxide and malonaldehyde levels [30] [34].

P. integerrima presented potential antiasthmatic activity in pre-clinical studies. This activity is related to inhibition of the degranulation of mast cell and inhibition of 5-LOX, where treatment with EO considerably reduced the number of total leukocytes in bronchoalveolar lavage fluid and pulmonary levels of myeloperoxidase. Associated with this, the plant presented antioxidant potential in the DPPH test, indicating satisfactory results for the treatment of chronic pulmonary diseases with possible involvement of oxidative pathways [32].

Articles that report the pharmacological evaluation of the essential oil of *Z. cassumunar* in encapsulated niosome by therapeutic ultrasound were also found in the searches. The encapsulated niosomes were applied in the skin and subsequently evaluaed using ultrasound therapy to potentiate the anti-inflammatory action of the EO, favoring the absorption by the skin and subsequent action on the inflammation induced by repeated administration of LPS. The anti-inflammatory action of this oil is probably related to the presence of sabinene and

terpinen-4-ol (major compounds) which reduce the expression of NF-kB and interleukin-6. The antioxidant tests indicated antioxidant activity of the EO, that inhibited DPPH radical, demonstrating once again the relation of inflammatory processes and antioxidant mechanisms [51].

In relation to the majority compounds studied, most are classify as monotepenes, such as carvacrol, thymol, L-carveol, L-carvone and m-cymene (Figure 6).

The carvacrol (5-isopropyl-2-methylphenol) is a phenolic monoterpene present in EOs of various species especially the Lamiaceae family, which presented pharmacological potentials, such as anti-oxidant and anti-inflammatory [92] [93]. The compound was the most reported in the studies included in this article, exhibiting activity in preclinical models of inflammatory diseases of the gastrointestinal tract, such as chemotherapy-induced mucositis and DMH-induced colitis. Its pharmacological activity in the mentioned models is a result of reduced expression of NF-kB, COX-2 and iNOS, associated with decreased levels of IL-1β, TNF-α and NO. The treatment was also able to increase the antioxidant enzymes SOD, CAT, MDA and GSH [48] [52].

Anethole (1-methoxy-4-benzene-[1-propenyl]) is an aromatic compound used in the industry, which has antioxidant potential, antibacterial, antifungal and anti-inflammatory [94] [95]. Oral treatment with anethole inhibited Complete Freund Adjuvant induced paw edema, in addition to reducing myeloperoxidase levels, TNF-α, IL-1β and IL-17, thereby reducing the levels of ROS in the injured tissue [26].

Another terpene described in the articles was linalool (3,7-dimethyl-octa-1,6-dien-3-ol), which was investigated to assess its ability to reduce *P. multocida*-induced lung inflammation. Repeated subcutaneous administration of linalool reduced the levels of TNF-α, IL-6 and the number of polymorphs (neutrophils) in lung tissue, associated with an increase in SOD [96] [35]. The eucalyptol (1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane) was also evaluated in a

model of chronic lung inflammation induced by repeated exposure to cigarette smoke, where the treatment reduced the expression of NF-kB and consequently the levels of pro-inflammatory cytokines, promoting the reduction of the presence of leukocytes in the pulmonary alveoli. The levels of the antioxidant enzymes SOD, CAT, MDA and GSH, as well as total ROS were reduced [47].

In the experimental models, cinnamaldehyde, β-elemene and thymol were evaluated in LPS-induced cardiac inflammation or hyperlipidic diet. Cinnamaldehyde possess potent anti-inflammation effect on endotoxemia [96]. Zhao and collaborators [45] showed that cinnamaldehyde inhibited inflammatory infiltration and the levels of TNF-α, IL-1β and IL-6 in LPS stimulated rats by blocking the TLR4 and MAPK pathways, associated with the reduction of ROS levels in cardiac tissue [45]. The β-elemene also showed activity in the cardiac inflammation model. In this evaluation, treatment of Apolipoprotein E (ApoE) knockout mice with β-elemene inhibited atherosclerotic lesions by reducing levels of nitric oxide, cytokines and reduction of oxidative stress indicators, reversed the intracellular ROS production and MAPK signaling activation [50].

Thymol (2-isopropyl-5-methylphenol) was evaluated in two different models, inflammation in aortic intimal and imiquimod induced psoriasis. In first model, the antioxidant tests were evaluated *in vitro* DPPH and ABTS radical scavenging assay, demonstrating high antioxidant activity. The treatment also reduced lipid peroxidation *in vivo*, reducing serum levels of malonaldehyde. In relation to the parameters of anti-inflammatory activity, thymol reduced the expression of vascular adhesion molecules (VCAM), thus reducing leukocyte migration and pro-inflammatory cytokines [46]. Thus, the results suggest that this monoterpene reduced the oxidative stress, the putative mechanism involved in the pathogenesis of endothelial dysfunction, an early key event in the progression of atherosclerosis [97].

Nanoparticules containing thymol were also evaluated, using experimental models that mimics psoriasis. For this, anthralin (1,8-dihydroxy-9-anthrone), a drug used to treat psoriasis was used for inducing inflammation in healthy skin mice and the antioxidant activity was evaluated after exposition to light, generators and oxidative stress events. Thymol in nanoparticles showed better inhibition of edema by reducing inflammatory cells in inflamed tissue when compared to free thymol, indicating that nanoparticles improve anti-inflammatory activity mediated by mechanisms that inhibit the formation of reactive oxygen species [54] [98].

In general, the results of the studies indicated that EOs and/or their compounds presented pharmacological properties through the blockade of the mitogen-activated protein kinases (MAPKs) pathways, blocking NF-κB activation by mechanisms associated to the reduction of oxidative stress, leading to the reduction of production of several pro-inflammatory mediators (Figure 6).

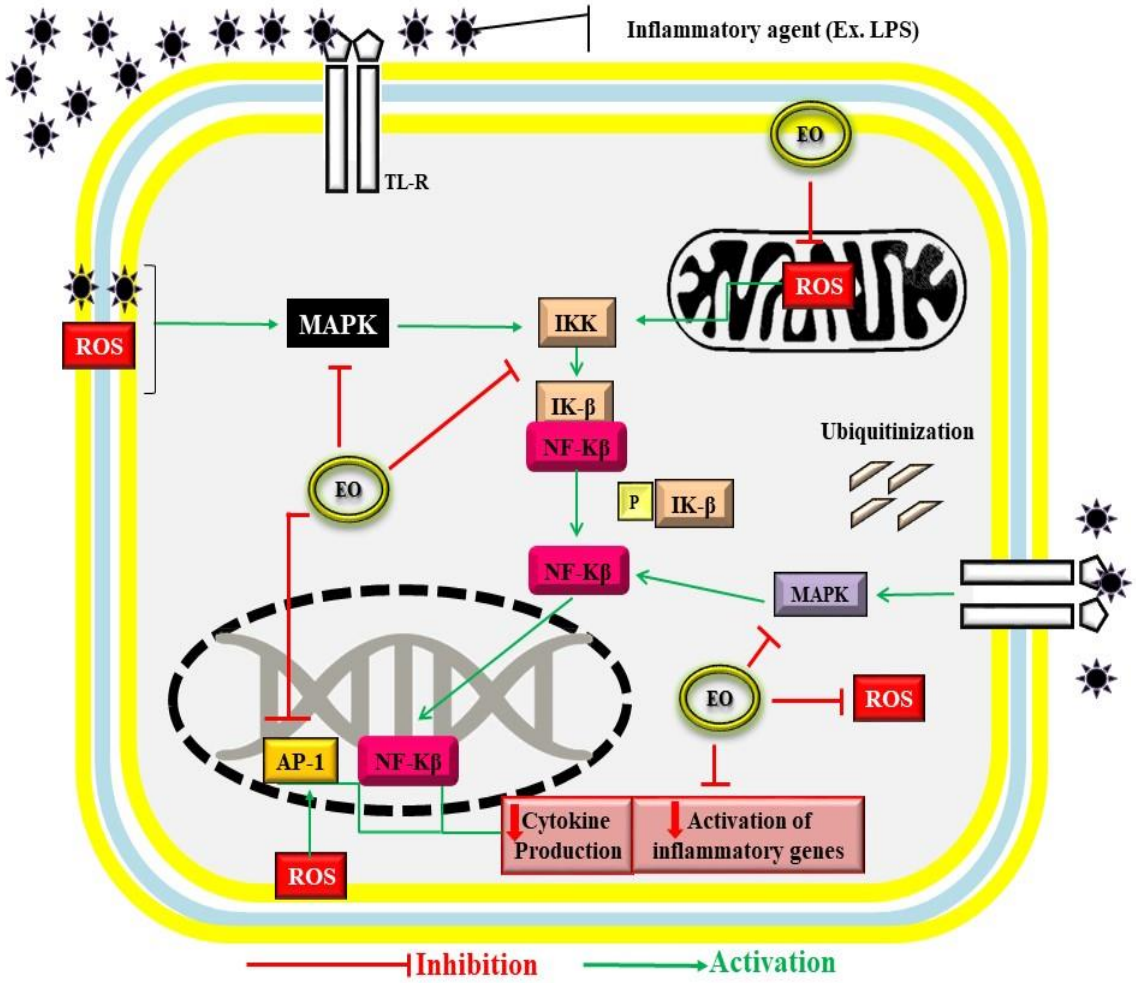

Figure 6: Molecular mechanisms of action of essential oils activity mediating signaling involving inhibition of NF-kB, MAPK and decreased intracellular oxidative stress.

4. Conclusion and Perspectives

This systematic review suggests that EOs and their major compound have a potential for the treatment of inflammatory diseases especially in chronic inflammatory conditions. The main action targets presented in this review for the therapy of chronic inflammations were the reduction of reactive oxygen and nitrogen species, as well as the reduction of the NF-kB reducing the expression of pro-inflammatory cytokines.

In vivo tests reported various models of inflammation that resemble human pathologies, including assessment of their mechanism of action, antioxidant enzyme dosages, and molecular effects of EOs. Regarding the rigor of design and study data included in this review, most of the studies presented moderate quality indicating that some aspects still need to be improved, but in general provide evidence of the anti-inflammatory potential associated with the antioxidant activity of EOs.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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CAPÍTULO 2

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Leaf aqueous extract of *Passiflora cincinnata* Mast. (Passifloraceae) reduces nociception in mice by central mechanisms

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ABSTRACT

Passiflora cincinnata Mast. is native to the Caatinga biome, and its use is described in traditional medicine for analgesic and anti-inflammatory purposes. Thus, this work aims to evaluate the antinociceptive activities of *P. cincinnata* (Pc-Aq) from the leaves through preclinical assays in mice. The pharmacognostic characterization of the vegetal plant was performed, and the chemical composition of the aqueous extract (Pc-Aq) was evaluated by high-performance liquid chromatography coupled to diode array detector (HPLC-DAD). The antinociceptive evaluation of the extract administered orally, at doses of 100 and 200 mg/kg was performed using *in vivo* chemical models (acetic acid-induced abdominal constriction and formalin-induced paw licking test) and thermal (hot plate test) of nociception. The mechanism of action was evaluated through the use of opioid receptor antagonist drugs, ATP-sensitive potassium channels, vanilloid receptor type-1 (TRPV-1), muscarinic, serotonergic (5-HT3) and α-adrenergic receptors. The rota-rod test was performed to verify the possible interference of the extract treatment in the motor performance of the animals. The results of chemical analysis indicated the presence of the flavonoids vitexin and isoorientin in Pc-Aq. The extract treatment reduced the number of abdominal writhings and decreased paw licking time in both phases of the formalin test ($p < 0.05$). In the hot plate test, the extract increased the latency time for paw withdrawal, indicating a reduction in painful behavior. The evaluation of the antinociceptive mechanism indicated a possible action of central pathways in the antinociceptive activity, with participation of opioid receptors, with influence of potassium channels and α-2 adrenergic pathway, without significant change in motor control, assessed by the rota-rod test. Therefore, this study suggests that Pc-Aq has an antinociceptive action mediated by central pathways, whose action can be attributed to the presence of the flavonoids present in the extract.

Keywords: *Passiflora cincinnata*; Flavonoids; Antinociceptive activity; Mechanisms of antinociception.

1. Introduction

Passiflora species (Passifloraceae) are traditionally used in folk medicine for various purposes, including sedative, anxiolytic, diuretic, anti-inflammatory and analgesic effects [1-4]. Previous phytochemical studies indicated the presence of phenolic compounds, cyanogenic glycosides, alkaloids and flavonoids in *Passiflora*, being *C*-glycoside flavonoids the most frequently reported and related to their pharmacological effects [2,5-7].

P. cincinnata is a native Passiflora from the Brazilian northeastern region, popularly known as “maracujá do mato” and “maracujá da Caatinga”. In folk medicine, it is commonly used as sedative and antihypertensive. Preclinical investigations reported antioxidant, antinociceptive and anti-inflammatory activities of ethanolic extracts obtained from leaves and fruit peels [5,8].

HPLC-DAD-MS/MS analysis showed the presence of glycosylated flavonoids in ethanol extracts of *P. cincinnata*, including apigenin derivatives, orientin, isoorientin, vitexin and isovitexin [5].

In recent years, the interest in natural products derived from plants has increased, as well as the prescription of herbal medicines by clinicians. In this perspective, and considering many common pathological conditions, such as chronic pain and inflammation, it is important to consider the development of new drugs with limited adverse effects for the treatment of these conditions.

Previous studies carried out by Lavor et al. [7] indicated the antinociceptive and anti-inflammatory activity of the ethanolic extract of *P. cincinnata* in experimental models. Pursuing the development of a new herbal medicine based on a *P. cincinnata* extract, we describe in this work the pharmacognostic characterization of plant material and the development of an aqueous extract with antinociceptive and anti-inflammatory activity, as well as the possible mechanisms of action involved.

2. Material and methods

2.1. Plant material

P. cincinnata leaves were collected in Uauá, state of Bahia (Coordinates: 9°49′20.82″ S; 39°37′59.34″ W), northeast region of Brazil, in April 2018. Dr. José Alves de Siqueira Filho performed botanical identification, by comparison to a previous voucher specimen (#22870) deposited at Herbarium Vale do São Francisco (HVASF) of the Federal University of Vale do São Francisco (UNIVASF). All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SisGen (Register #ABD9AA7). Plant material was dried in an oven with air circulation at a temperature of 45 °C for 72 h and was later pulverized.

2.2. Pharmacognostic characterization of plant drug

Dried and pulverized leaves of *P. cincinnata* were used to evaluate granulometry and physical-chemical tests were conducted to determine the water loss on drying, granulometry of powders, total ash, acid insoluble ash, sulphated ash, foam index and alcohol extractable substances as recommended by the Brazilian Pharmacopeia 6th Edition [9]. Physico-chemical tests were carried out in triplicate and the results were expressed as mean ± standard deviation.

2.3. Water loss on drying method for moisture determination

To determine water content in the vegetal drug, gravimetric method was used. Dried vegetal drug (2 g) was weighed and later transferred to weighing bottles previously desiccated for 30 minutes in the same conditions to be used in the determination. The weighing bottles were placed in oven at 105 ºC for 2 hours, as specified in the Brazilian Pharmacopoeia [9].

Samples were placed at room temperature in a desiccator and then weighed. The percentage of loss on drying was calculated according to the equation below:

$$(\text{Loss on drying } \%) = (Pu - Os) * 100 / Pa$$

Where: Pu = weight of filter + sample weight (before desiccation); Ps = weight of the filter weighing the sample after drying; Pa = weight of the sample

2.4. Granulometric distribution

To determine powder granulometry, 25 g of the dried and pulverized vegetal drug were used, which were subjected to the passage through a set of five sizes, previously weighed, with mesh diameters of respectively 600, 500, 425, 250, and 150 µm and a collector. This set was subjected to a mechanical vibration for 15 minutes. After this procedure, the sample retained in the upper part of each mesh was weighed, being calculated by the difference in mass of the granulometric sieve (tamis) with the retained powder and the same void [9]. The percentage retained in each tamis was calculated according to the equation below:

$$\% \text{ Retained in tamis} = P1 / P2 \text{ X } 100$$

Where: P1 = weight of the sample retained in each sieve (in grams); P2 = sum of weights retained in each sieve and in the collector (in grams); 100 = percentage factor

2.5. Determination of total ash, acid insoluble and sulfated ash

To determine the total ash content, 3 g of the dried vegetal drug was weighed and pulverized, and the sample was then incinerated gradually to a temperature of 600 ± 25 °C, for a period until all coal was eliminated. The sample was cooled in a desiccator and then weighed. Percentage of total ash was calculated in relation to the weight of the vegetal drug. For acid insoluble ash determination, the residue used was obtained in the previous determination. The residue was heated for 5 minutes with 25 ml of 7% hydrochloric acid and then it was washed with 5 ml of previously heated water. The acid-insoluble residue was filtered and the

subsequently paper filter containing the residue was transferred to the original crucible, which dried on the hot plate and then incinerated at 500 °C until constant weight was obtained [9].

To determine the sulfated ash, 1 g of the vegetal drug was weighed in a crucible and, subsequently, the sample was moistened with concentrated sulfuric acid and carbonized in a Bunsen burner. The sample was again moistened with concentrated sulfuric acid, carbonized, and incinerated with gradual heating up to 800 ºC. The sample was cooled, weighed again, and incinerated for another 15 minutes. The procedure was repeated until the difference between the two successive weighing was not greater than 0.5 mg [9]. The determination of sulfated ash was obtained using the equation below:

$$\% \text{ Sulfated Ashes} = Pr - Pv / Pa \times 100$$

Where: Pr = crucible weight + sample residue; Pv = weight of the empty crucible; Pa = weight of the sample.

2.6. Determination of foam index

To determine this parameter, 1 g of the *P. cincinnata* powder was weighed and transferred to a conical tube containing 50 ml of boiling water, for 30 minutes. Then, the solution was cooled and filtered in a 100 mL volumetric flask. The decoction was obtained and distributed in a successive series from 1 to 10 ml, in 10 test tubes, and the final volume of the liquid was adjusted to 10 ml with distilled water. The tubes were shaken with vertical movements for 15 seconds, with two shakes per second, and left for 15 minutes, after the end of foam height [9]. The foam index was scaled according to the equation:

$$\text{Foam index} = 1000 / A$$

Where: A = volume, in mL, of the decoction used to prepare the dilution in the tube where the foam was observed.

2.7. Determination of alcohol-extractable substances

To determine the content of alcohol-extractable substances, the cold extraction method was used. For this, 4 g of the dried vegetal drug was transferred and macerated with 100 ml of

EtOH (99.9%) for 6 hours, with frequent agitation and then left for rest for 18 hours. The filtered sample was transferred to a crucible and subjected to drying for 6 hours at 105 °C. The sample was then cooled in a desiccator for 30 minutes and weighed [9].

2.8. Plant extract preparation

An ethanol extract was prepared from 10 g of *P. cincinnata*, by exhaustive maceration, using the leaves powder in a 1:10 ratio (plant material: solvent, w/v). This procedure was carried out for 72 hours to obtain the ethanol extract (Pc-EtOH) after solvent evaporation. The extractive solution was concentrated on a rotatory evaporator, generating the crude ethanolic extract of leaves (Pc-EtOH). Aqueous extracts of *P. cincinnata* were obtained from 10 g of leaves powder and 1 L of purified water, during 10 minutes, by two different methods: decoction and infusion. The aqueous extract obtained by decoction was subsequently lyophilized to proceed with toxicological and pharmacological tests.

2.9. Phytochemical screening and identification of chemical constituents by HPLC-DAD

To characterize the chemical composition of Pc-EtOH and Pc-Aq (obtained by decoction), phytochemical screening was performed using TLC plates according to Wagner & Bladt [10]. Both extracts were analyzed by HPLC-DAD to determine the concentration of the analytical markers orientin and vitexin (µg/mg of extract).

Samples were solubilized in methanol (1 mg/ml), filtered (0.22 µm) and then injected into an HPLC-DAD apparatus, using an octadecylsilane column (250 x 4.6 mm, 5 µm, C-18, Agilent®). For the analysis, a Shimadzu® LC-20 chromatograph was used, equipped with a quaternary pump system model LC-20A DVP, degasser model DGU-20A, PDA detector model SPD-20AVP, oven model CTO-20ASVP, automatic injector model SIL-20ADVP and controller model SCL-20AVP and coupled with a diode array detector (DAD). The data

obtained was processed using the software Shimadzu® LC solution 1.0 (Japan). To quantify the flavonoids orientin and vitexin, chemical markers of *Passiflora* species, calibration curves were performed with concentration ranging from 4 to 200 µg/ml vitexin.

Two solutions were used as mobile phase: A (0.1% v/v formic acid) and B (acetonitrile with 0.1% acid formic). Samples were analyzed using a gradient system described in Table 1, with a flow of 0.4 ml/min. Analytical standards and samples were injected in a volume of 10 µL. Detection was performed at a wavelength of 340 nm.

Table 1. Gradient system used in all HPLC-DAD analysis.

	Time (min)	**Solution A (%)**	**Solution B (%)**
Linear gradient	0-40	85-50	15-50
Linear gradient	40-45	69-0	31-100
Isocratic	45-50	0	100
Linear gradient	50-55	0-85	100-15
Isocratic	55-65	85	15

The quantification of these chemical markers in each type of extract was performed using the equation obtained from the calibration curve for vitexin ($y= 37420x - 137628$; $R^2 = 0.9993$). Quantitative results were expressed in vitexin equivalents.

2.10. Pharmacological tests

2.10.1. Animals

Male and female Swiss mice over 6-8 weeks of age were used (30-40 g) animals were divided into groups of ten mice or toxicity tests (n=10, 05 males and 05 females), and in groups

of 06 animals (n=6) for pharmacological tests of antinociceptive activity and evaluation of motor coordination. The animals was manteined kept in a temperature-controlled room at 25 ± 2 °C with a 12/12 h light/dark cycle, with food and water provided *ad libitum*, and were deprived of food for 8 h before experiments. All experimental protocols were approved by Committee on Ethics in Animal Use of Federal University San Francisco Valley (number 0004/130220).

2.10.2. Acetic acid-induced abdominal writhing

Abdominal writhing test was performed according to Collier et al. [11], with slight modifications. For this test, animals were treated with Pc-Aq (100 and 200 mg/kg), or vehicle (saline solution 0.9%) administered orally (p.o., by gavage) 60 minutes before the application of acetic acid 0.9% intraperitoneally (i.p.). Indomethacin (20 mg/kg) and morphine (10 mg/kg) were administered i.p., 30 minutes before the nociceptive stimulus. After the injection of acetic acid, the number of abdominal constrictions (contraction of the abdominal wall, pelvic rotation followed by extension of the posterior limbs) that occurred between 5 and 15 min after the injection was quantified [7,12].

2.10.3. Formalin-induced nociception

Formalin solution (2.0%, 20 µl) was injected into the sub-plantar region of the right hind paw of mice, which were observed for the duration of the paw licking time (in seconds) immediately after the application of formalin for 0-5 minutes (first phase, neurogenic pain) and 15-30 minutes (second phase, inflammatory pain) [7,13-14]. Treatment with vehicle (saline 0.9% p.o.), Pc-Aq (100, 200 mg/kg, p.o.), indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were administered 1 hour (for p.o. treatments) or 30 minutes (for i.p. treatments) before applying the formalin solution to the paw of the animals.

2.10.4. Hot plate-induced nociception

Thermal stimulation is commonly used as an experimental model to investigate the antinociceptive effect of plant extracts, to assess the involvement of central pathways in pharmacological activity. For this reason, 24 h before the experiments, animals were pre-selected, and animals that had a latency time for removing the paw from the preheated plate longer than 20 seconds were excluded of this study. Mice were treated with vehicle (saline 0.9%, p.o.), Pc-Aq (100 and 200 mg/kg, p.o.) or morphine (10 mg/kg, i.p.) and then placed on a hot plate apparatus (55 ± 0.5 °C) after 30, 60, 90 and 120 minutes of treatment. Latency was characterized by time needed for first paw licking or shaking movements. Latency was recorded under the same conditions and a cut-off time of 20 seconds was determined to indicate complete analgesia and to avoid tissue damage [7,15].

2.10.5. Evaluation of the possible mechanisms of action

To assess the possible mechanisms involved in the central antinociceptive action, mice were pre-treated with naloxone (opioid antagonist, 1.5 mg/kg, i.p.), glibenclamide (K^+-ATP channel blocker, 2 mg/kg, i.p.), yohimbine (α-2 adrenergic antagonist, 0.15 mg/kg, i.p.), clonidine (α-2 adrenergic agonist, 0.1 mg/kg, i.p), ondansetron (5-HT_3 serotonergic receptor antagonist, 0.5 mg/kg i.p.), atropine (non-selective muscarinic cholinergic antagonist, 1 mg/kg, i.p.), haloperidol (non-selective dopaminergic antagonist 0.2 mg/kg, i.p.) and ruthenium red (potential non-selective transient receptor antagonist - TRP, 3 mg/kg, i.p.), 30 minutes before Pc-Aq administration (200 mg/kg, v.o.). After a period of 60 minutes, animals were submitted to the formalin test, as previously described, or to the intraplantar administration of capsaicin (2.5 µg) to assess the involvement of the TRP [16-19].

2.10.6. Motor coordination test (rota-rod test)

The rota-rod test (Insight, Brazil) was used to assess possible effects of the treatment with *P. cincinnata* extract on muscle relaxation and motor coordination [20]. 24 hours before experiments, mice were pre-selected based on their ability to remain on the rotating bar for more than 180 s (7 rpm). On the following day, animals were subjected to the test 1 h after the administration of Pc-Aq (100 and 200 mg/kg, p.o.), or vehicle (saline 0.9%) and 30 minutes after the application of diazepam (2.5 mg/kg, i.p.) the time spending on the rotating was recorded with a maximum of three attempts.

2.11. Acute toxicity

In the investigation of acute toxicity, animals received treatments by two different routes of administration: the intended route for administration in humans (oral route) and a parenteral (intraperitoneal - i.p.), as recommended by the *Agência Nacional de Vigilância Sanitária*, in Brazil [21]. Mice were divided into four groups of 6 males and 6 females each (n= 12). Control groups received vehicle (saline 0.9 %) orally or intraperitoneally. Animals were treated with Pc-Aq in a single dose of 2 g/kg by oral (p.o.) or intraperitoneal (i.p.) administration. Then, mice were observed daily for 14 days to evaluate the presence of death and signs of toxicity. In addition, behavioral analysis, body weight, food and water intake were evaluated.

2.12. Statistical analysis

The results were presented as the mean ± standard error of mean (SEM) for 6 animals per group. Statistical comparisons of the data were performed by one-way analysis of variance (ANOVA), followed by Tukey's test or Student´s t-test. In all cases, differences were

considered significant when $p < 0.05$. All statistical analyses were performed using the software GraphPad Prism version 6.01 (GraphPad Prism Software Inc., San Diego, CA, USA).

3. Results

3.1. Pharmacognostic characterization of the vegetal drug

The determination of the loss by drying of the leaves powder of *P. cincinnata* demonstrated a moisture content of 8.08 ± 0.02% for the vegetal drug, therefore being within the ideal moisture content parameters specified in pharmacopeial monographs [9] ranging from 8 to 14%.

After the sizing process, it was possible to observe that the 425 µm mesh retained the largest amount of *P. cincinnata* powder, as shown in Fig. 1, and the particles have an average size of 250 µm (Fig. 2).

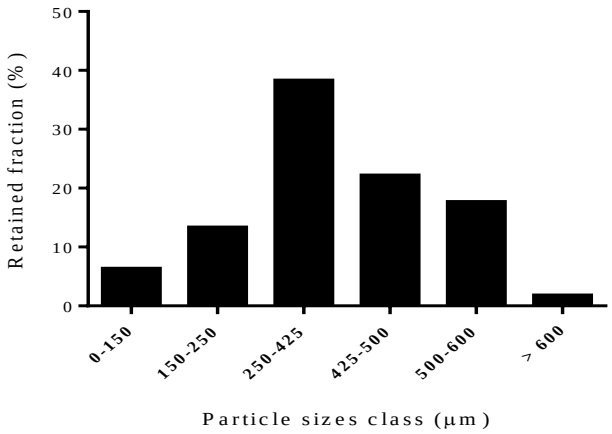

Fig. 1. Particle size distribution histogram of the powder obtained from *P. cincinnata.*

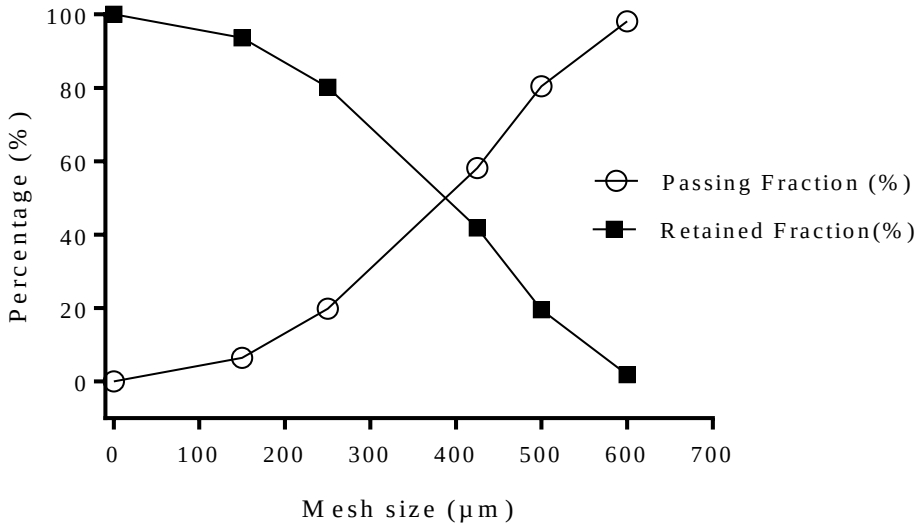

Fig. 2. Graphical determination of the particle size of the powder obtained from *P. cincinnata.*

The results of total ash, insoluble in acid ash and sulfated ash compounds from the vegetal drug are shown in Table 1.

Table 2. Determination of ash contents in samples of *P. cincinnata.*

Species	**Ash**		
	Total	**Insoluble in acid**	**Sulfated**
P. cincinnata	1.39 ± 0.87%	1.42 ± 0.53%	6.98 ± 0.03%

The evaluation of foam index showed that, after 15 minutes of analysis, the foam formation was less than 1 cm in height. Consequently, the foam index obtained was less than 100, demonstrating the negative result for the presence of saponins in the vegetal drug. The analyzes indicated that the content of extractable substances in ethanol for *P. cincinnata* was 8,93 % ± 0,04 %.

3.2. Phytochemical analysis

Preliminary phytochemical analysis indicated a positive reaction for the presence of general anthocyanins, phenolic compounds, coumarins, anthracene derivatives, anthraquinones, flavonoids and terpenes. Flavonoids and terpenes were present in higher intensity, in both extracts, as shown in Table 3.

Table 3. Identification of the main classes of chemical constituents in the ethanolic and aqueous leaves extract of *P. cincinnata*.

Chemical class of metabolites	**Ethanolic extract (Pc-EtOH)**	**Aqueous extract (Pc-Aq)**
Alkaloids	+	-
Anthocyanins	+	+
Anthraquinones	++	+
Coumarins	++	++
Anthracene derivatives	++	++
Flavonoids	++	+++
Lignans	-	-
Mono, sesqui and diterpenes	+++	++
Naphthoquinones	-	-
Saponins	-	-
Condensed tannins	-	-
Hydrolysable tannins	-	-
Steroids and triterpenes	-	-
Xanthines	-	-

Chromatographic analyzes indicated that, in all the extracts prepared, the flavonoids vitexin and orientin are present, whose concentrations are shown in Table 4. However, due to the high concentration of vitexin observed in the decoction, it was decided to continue the tests using this type of preparation. The chromatographic profile of the sample is shown in Fig. 3 and Table 5.

Table 4. Concentration of isoorientin and vitexin in extracts of *P. cincinnata*.

Type of preparation	Vitexin concentration (µg/ml)	Isoorientin concentration (µg/ml)
Ethanolic	25.66 ± 0.44	9.59 ± 0.56
Aqueous - Decoction	74.89 ± 0.12	57.57 ± 0.16
Aqueous – Infusion	25.11 ± 0.11	25.59 ± 0.35

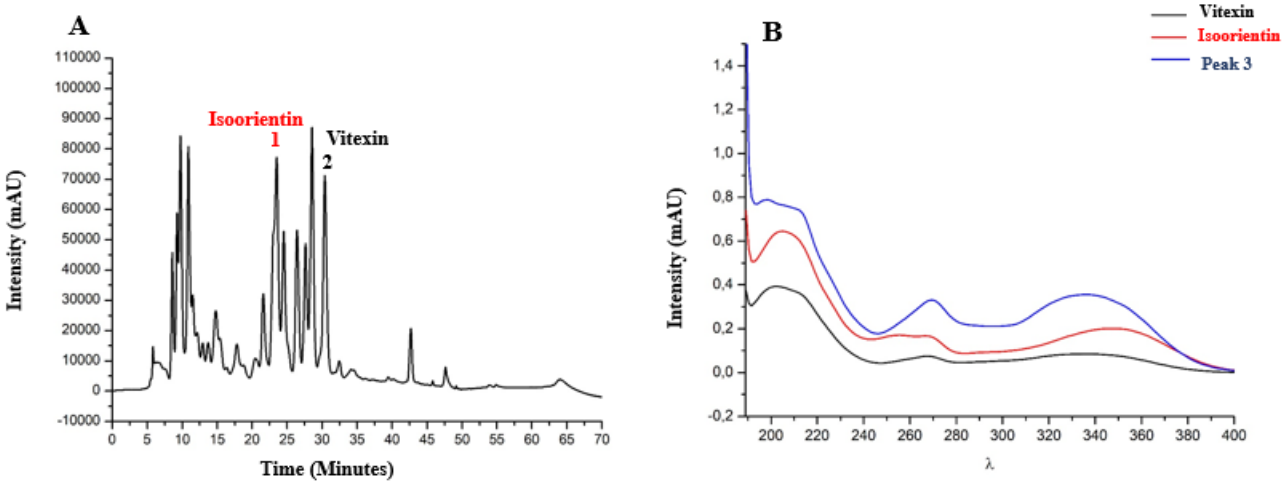

Fig. 3. Chromatogram of the leaf aqueous extract of *P. cincinnata* (Pc-Aq) obtained by HPLC-DAD.

Table 5. Retention times (Rt) and λ_{max} of the peaks detected in the chromatograms of ultraviolet spectra for the flavonoids present in the aqueous leaves extract of *P. cincinnata* (Pc-Aq).

Peaks detected in Pc-Aq	**Rt (min)**	**λ_{max} (nm)**	**Secondary metabolite class**
1	25.02	267/347	Isoorientin
2	29.98	269/336	Vitexin

2.3. Pharmacological tests

2.3.1. Effect of Pc-Aq on the acetic-acid-induced writhing test in mice

As shown in Fig. 4, Pc-Aq treatment significantly reduces ($p < 0.05$) the nociception induced by intraperitoneal administration of acetic acid, inhibiting in 44.11 (10 mg/kg) and 63.23% (200 mg/kg) the number of abdominal writhings when compared to the group that received only vehicle (control group). The reference drugs indomethacin (Indo) and morphine (Mor) reduced the number of contortions in 92.64 and 99.12%, respectively.

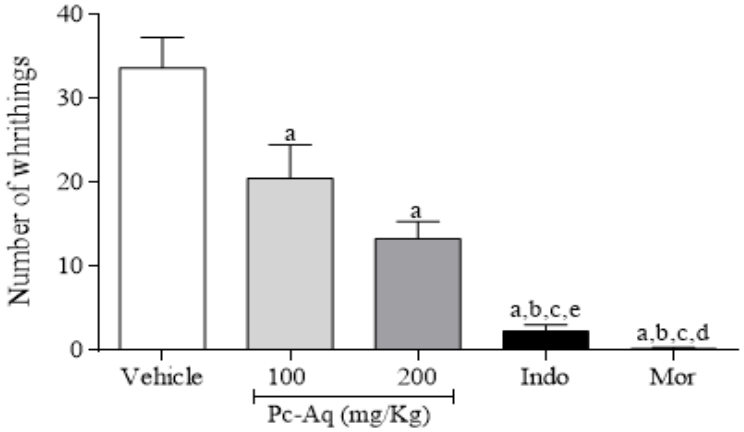

Fig. 4. Effect of leaf aqueous extract of *P. cincinnata* (100 and 200 mg/kg), indomethacin (Indo, 20 mg/kg) and morphine (Mor, 10 mg/kg) in the abdominal writhing test. Values are expressed as mean ± S.E.M (n = 6). [a] $p < 0.05$ compared to the vehicle group; [b] $p < 0.05$, compared to 100 mg/kg of Pc-Aq; [c] $p < 0.05$, compared to the 200 mg/kg Pc-Aq group; [d] $p < 0.05$, compared to the 20 mg/kg of indomethacin group, [e] $p < 0.05$, compared to the 10 mg/kg of Morphine group, by ANOVA followed by Tukey test.

2.3.2. Effect of Pc-Aq on the formalin-induced nociception in mice

The administration of Pc-Aq at doses of 100 and 200 mg/kg reduced ($p < 0.05$) the paw licking time in 53.78% and 64.30%, respectively, in the neurogenic phase (first phase) of the test. In the inflammatory phase (second phase), the reduction was of 68.98% (100 mg/kg) and 59.62% (200 mg/kg), as shown in Fig. 5. In addition, morphine demonstrated significant antinociceptive activity in both phases, whereas indomethacin was effective only in the second phase.

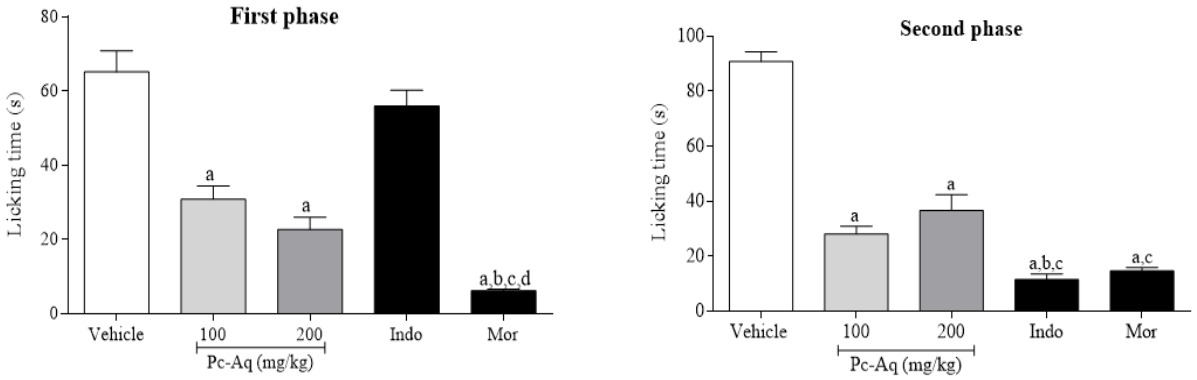

Fig. 5. Effect of leaf aqueous extract of *P. cincinnata* (100 and 200 mg/kg), indomethacin (Indo, 20 mg/kg) and morphine (Mor, 10 mg/kg) in the first and second phases of formalin test. Values are expressed as mean ± S.E.M (n = 6). [a] $p < 0.05$ compared to the vehicle group; [b] $p < 0.05$, compared to 100 mg/kg of Pc-Aq; [c] $p < 0.05$, compared to the 200 mg/kg Pc-Aq group; [d] $p < 0.05$, compared to the 20 mg/Kg of indomethacin group, [e] $p < 0.05$, compared to the 10 mg/Kg of Morphine group, by ANOVA followed by Tukey test.

2.3.3. Effect of Pc-Aq on the hot plate test in mice

In all analyzes (30, 60, 90 and 120 minutes) the leaf aqueous extract of *P. cincinnata* significantly increased ($p < 0.05$) the paw withdrawal latency of the animals from the preheated plate, thus suggesting a possible involvement of central pathways in Pc-Aq antinociceptive activity. The reference drug, morphine, showed antinociceptive activity in the time between 30 and 60 minutes, as shown in Fig. 6.

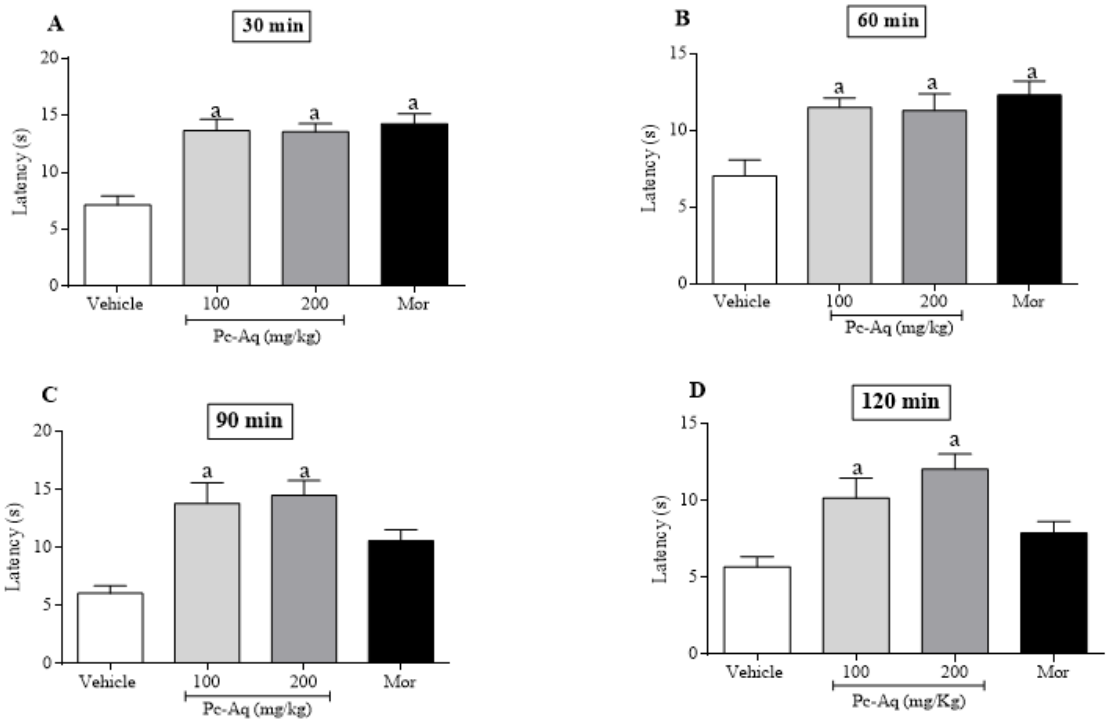

Fig. 6. Effect of leaf aqueous extract of *P. cincinnata* (100 and 200 mg/kg), indomethacin (Indo, 20 mg/kg) and morphine (Mor, 10 mg/kg) in the hot plate test. Values are expressed as mean ± S.E.M (n = 6). [a] $p < 0.05$ compared to the vehicle group, by ANOVA followed by Tukey test.

3.4. Investigation of the antinociceptive mechanism of Pc-Aq

3.4.1. Role of opioid receptors pathway

The evaluation of the involvement of non-selective opioid receptors in the antinociceptive activity of Pc-Aq (Fig. 7) indicated that the pretreatment with naloxone reversed the antinociceptive activity of the extract at the dose of 200 mg/kg ($p < 0.05$) in both first and second phases of the test, thus suggesting the involvement of opioid receptors in antinociceptive activity. The effect of morphine (10 mg/kg) was also reversed by naloxone ($p < 0.05$).

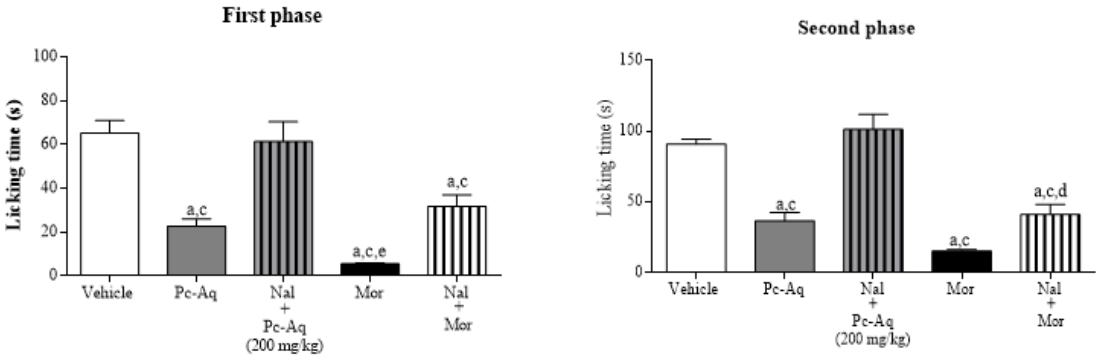

Fig. 7. Effect of pretreatment with naloxone (1.5 mg/kg) on antinociception of leaf aqueous extract of *P. cincinnata* (200 mg/kg) and morphine (Mor, 10 mg/kg), in the first and second phase of formalin-induced nociception. Values are expressed as mean ± S.E.M. (n = 6). ^{a}p <0.05, compared to vehicle group; $^{d}p < 0.05$, compared to the 10 mg/kg morphine group; $^{c}p < 0.05$, compared to the group pretreated with naloxone and subsequently with the Pc-Aq; And $^{e}p < 0.05$, compared to the group pretreated with naloxone and subsequently to morphine, by ANOVA followed by Tukey test.

3.4.2. Role of ATP-sensitive K^+ channels pathway

Fig. 8 shows the involvement of ATP-sensitive K^+ channels in the antinociceptive effect of Pc-Aq. Pretreatment with the K^+-ATP channel blocker, glibenclamide, significantly reversed ($p < 0.05$) the antinociceptive activity of the aqueous extract in both phases of the formalin test. As Pc-Aq, diazoxide, a channel activator for K^+-ATP, significantly inhibited formalin-induced nociception, which was however, reversed in animals pretreated with glibenclamide.

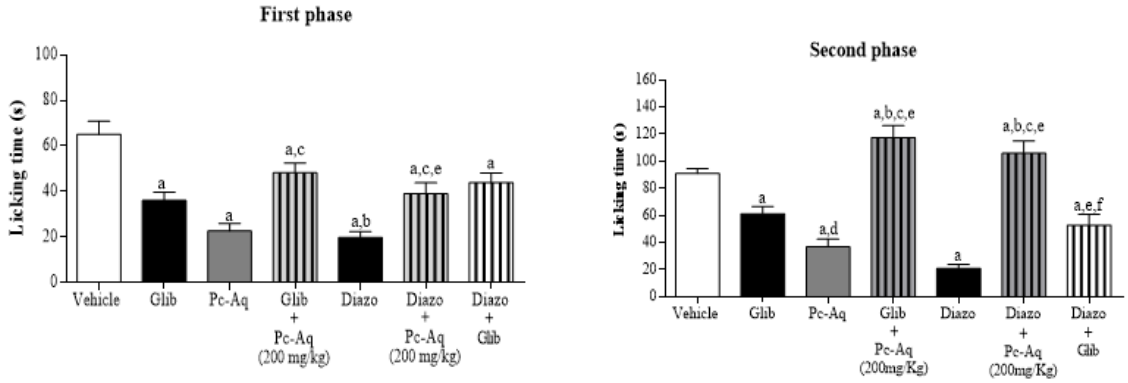

Fig. 8. Effect of pretreatment with glibenclamide (2 mg/kg) and diazoxide (3 mg/kg) on antinociception of leaf aqueous extract of *P. cincinnata* (200 mg/kg) in the first and second phase of formalin-induced nociception. Values are expressed as mean ± S.E.M. (n = 6). [a] $p < 0.05$, compared to vehicle group; [b] $p < 0.05$, compared to the 2 mg/kg Glibenclamide group; [c] $p < 0.05$, compared to the 200 mg/kg Pc-Aq group; [d] $p < 0.05$, compared to the pretreated group with 2 mg/kg of glibenclamide and subsequently with 200 mg/kg of Pc-Aq; [e] $p < 0.05$, compared to the 3 mg/kg Diazoxide group; [f] $p < 0.05$, compared to the pretreated group with diazoxide and subsequently with 200 mg/kg Pc-Aq, by ANOVA followed by Tukey.

3.4.3. Role of α-2 adrenoceptors

In both phases of the formalin test, pretreatment of animals with yohimbine reversed the antinociceptive effect of Pc-Aq with statistical significance ($p < 0.05$), as well as the antinociceptive effect of clonidine, as shown in Fig. 9. Therefore, it is suggested an involvement of α -2 adrenergic pathway in the pharmacological activity of the extract.

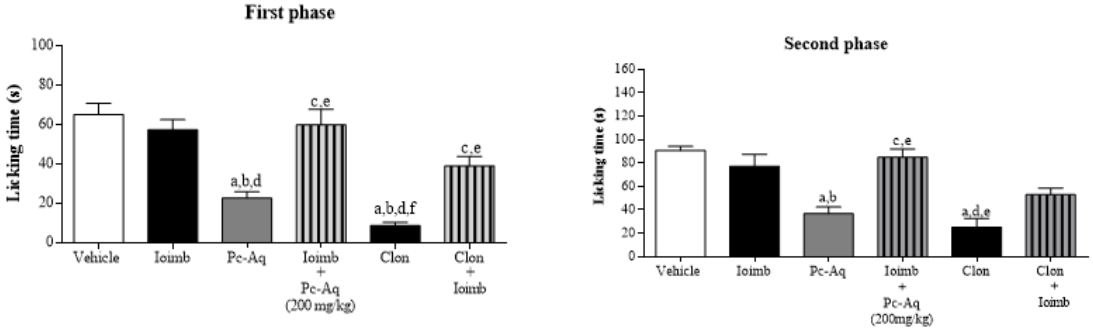

Fig. 9. Effect of pretreatment with yohimbine (0.15 mg/kg) and clonidine (0.1 mg/kg) on antinociception of leaf aqueous extract of *P. cincinnata* (200 mg/kg) in the first and second phase of formalin-induced nociception. Values are expressed as mean ± S.E.M. (n = 6). $^{a}p < 0.05$, compared to vehicle group; $^{b}p < 0.05$, compared to the 0.15 mg/kg yohimbine group; $^{c}p < 0.05$, compared to the 200 mg/kg Pc-Aq group; $^{d}p < 0.05$, compared to the pretreated group with 0.15 mg/kg yohimbine and subsequently with 200 mg/kg of Pc-Aq; $^{e}p < 0.05$, compared to the 0.1 mg/kg clonidine group; $^{f}p < 0.05$, compared to the pretreated group with clonidin and subsequently with yohimbine, by ANOVA followed by Tukey.

3.4.4. Role of muscarinic receptors pathways

The pretreatment with atropine did not reverse the antinociceptive effect of Pc-Aq in neither of the two phases of the formalin test, thus ruling out the possibility that the cholinergic system may be involved in the pharmacological activity of the extract, as shown in Fig. 10.

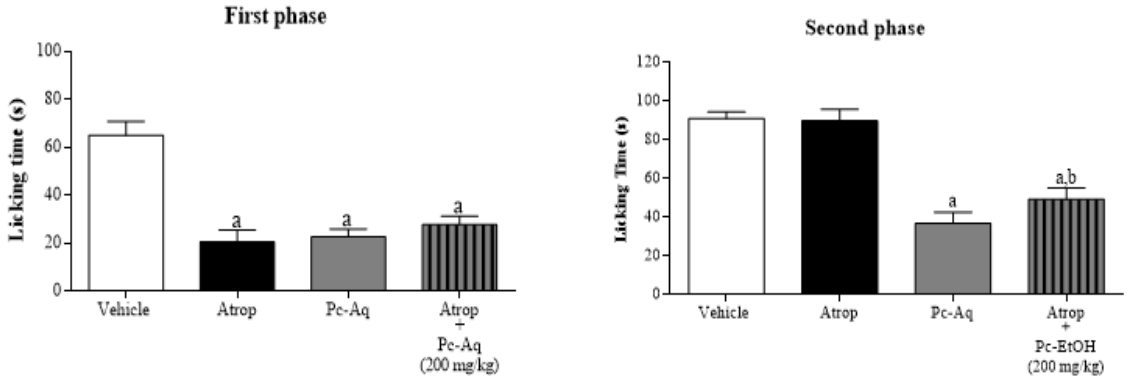

Fig. 10. Effect of pretreatment with atropine (1 mg/kg) in antinociception of leaf aqueous extract of *P. cincinnata* (200 mg/kg) in the first and second phase of formalin-induced nociception. Values are expressed as mean ± S.E.M. (n = 6). $^{a}p < 0.05$, compared to vehicle group; $^{b}p < 0.05$, compared to the 1 mg/kg atropine group, by ANOVA followed by Tukey test.

3.4.5. Role of 5-HT$_3$ serotoninergic receptors pathways

The pretreatment with ondansetron, the 5-HT$_3$ serotonergic receptor antagonist, did not promote any significant change in the antinociceptive effect of Pc-Aq (Fig. 11), suggesting that there is no participation of serotonergic receptors, especially 5-HT$_3$, in the antinociceptive activity of the extract.

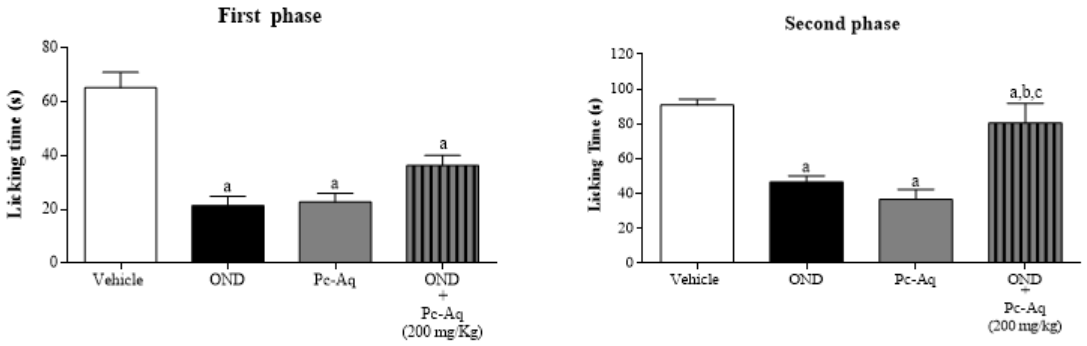

Fig. 11. Effect of pretreatment with ondansetron (0.5 mg/kg) in antinociception of leaf aqueous extract of *P. cincinnata* (200 mg/kg), in the first and second phase of formalin-induced nociception. Values are expressed as mean ± S.E.M. (n = 6). $^{a}p < 0.05$, compared to vehicle group ; $^{b}p < 0.05$, compared with the 0.5 mg/kg ondansetron group; $^{c}p < 0.05$, compared with 200 mg/kg of Pc-Aq, by ANOVA followed by Tukey test.

3.4.6. Role of dopaminergic receptors pathways

The results indicated participation of dopaminergic receptors in the action of Pc-Aq, since pretreatment with Haloperidol (0.2 mg/kg), the dopaminergic receptor antagonist, reversed the antinociceptive effect of Pc-Aq, as shown in Fig. 12.

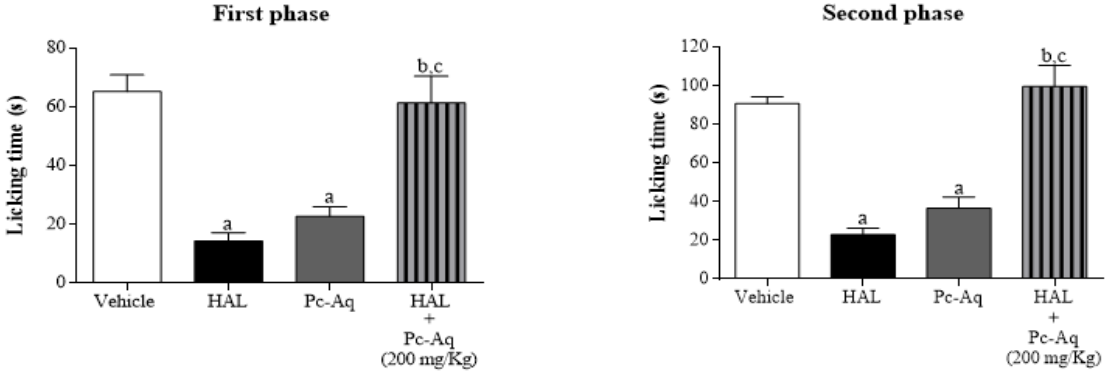

Fig. 12. Effect of pretreatment with haloperidol (0.2 mg/kg) in antinociception of leaf aqueous extract of *P. cincinnata* (200 mg/kg), in the first and second phase of formalin-induced nociception. Values are expressed as mean ± S.E.M. (n = 6). $^{a}p < 0.05$, compared to vehicle group; $^{b}p < 0.05$, compared with the 0.2 mg/kg haloperidol group; $^{c}p < 0.05$, compared with 200 mg/kg of Pc-Aq, by ANOVA followed by Tukey test.

3.4.7. Role of vanilloid transient potential receptor

Pc-Aq treatment was also able to inhibit nociceptive behavior symptoms induced by the injection of capsaicin in the intraplantar region. However, pretreatment with ruthenium red did not reverse the antinociceptive activity of the extract, suggesting that there is no participation of the potential vanilloid disorder (TRPV) receptor system in the antinociception caused by the treatment with the extract against capsaicin injection (Fig. 13).

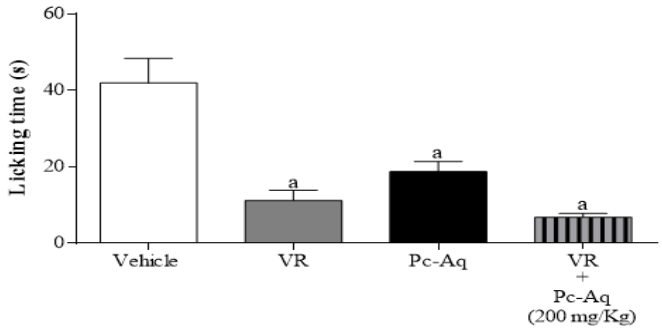

Fig. 13. Effect of pretreatment with ruthenium red (3 mg/kg) in antinociception of aqueous extract of *P. cincinnata* (200 mg/kg) in the capsaicin-induced nociception. Values are expressed as mean ± S.E.M. (n = 6). $^{a}p < 0.05$, compared to vehicle group; by ANOVA followed by Tukey test.

2.4. Effect of Pc-Aq on the motor coordination test

The treatment with Pc-Aq did not alter the motor coordination of the mice at doses tested at the times of 30, 90 and 120 minutes. In contrast, diazepam, a central nervous system depressant, reduced ($p < 0.05$) the time on the rota-rod when compared to the control group, as shown in Fig. 14.

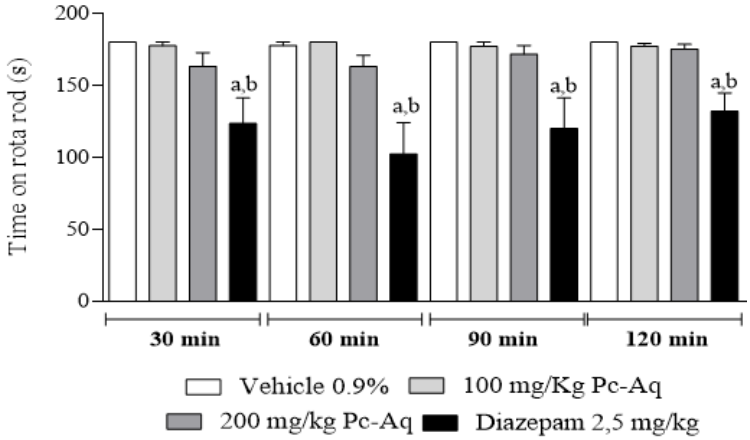

Fig. 14. Effect of aqueous leaves extract of *P. cincinnata* (100 and 200 mg/kg) and diazepam (2.5 mg/kg) in the rota-rod test. Values are expressed as mean ± S.E.M. [a]p <0.05, compared to the vehicle group; [b] p < 0.05, compared to 100 mg/kg of Pc-Aq, by ANOVA followed by Tukey test.

2.5. Acute toxicity

Single-dose administration of the extract did not cause deaths, indicating low toxicity of the extract (LD_{50} > 2 g/kg) when administered by both routes (p.o. and i.p.). In the behavioral analysis, the animals treated with the extract had palpebral ptosis in both administration routes after 30 minutes of analysis and that were not noticed after 90 minutes of application of the extract. The animals did not present significant variation in body weight, water consumption and food intake among the experimental groups (data not shown).

4. Discussion

To obtain the drug registration by ANVISA, the regulatory agency in Brazil, it is necessary to conduct tests that aim to ensure the purity and integrity of the vegetal drug, as well as solvents assessment used in extractive methods, approximate drug:derived purity relationship and integrity of the extract [9]. For this purpose, the determination of the loss by drying of the leaves powder of *P. cincinnata* was initially evaluated, indicating a moisture

content of 8.08 ± 0.02%, therefore being within the ideal moisture content parameters specified in pharmacopeial monographs, ranging from 8 to 14% [9]. Furthermore, low humidity indicates efficiency in the drying process, ensuring the stability of the material, reducing the action of the enzymes that can degrade the chemical constituents of the vegetal drug and the proliferation of microorganisms [22].

After the sizing process, it was possible to observe that the 425 μm mesh retained the largest amount of *P. cincinnata* powder, as shown in Fig. 1, and that the particles have an average size of 250 μm (Fig. 2). According to these results, the vegetal drug can be classified between semi-thin and moderately thick [9]. The determination of total ash is a method to assess the purity of the material under analysis, detecting the excessive presence of non-volatile inorganic substances that may be present as constituents or contaminants in the sample, while acid insoluble ashes refer to the quantification of silica and siliceous constituents in the vegetal drug. Therefore, the results found in the determination of the total ash, sulphated ash and insoluble ash content for the sample of *P. cincinnata* (Table 2) are in accordance with the Brazilian Pharmacopoeia, revealing that the material was subjected to the process of proper collection and processing [23].

Preliminary phytochemical analysis indicated the presence of anthocyanins, phenolic compounds, coumarins, anthracene derivatives, anthraquinones, flavonoids and terpenes in both preparations, the aqueous extract obtained by decoction (Pc-Aq) and the ethanolic extract (Pc-EtOH), as shown in Table 3. By the presence of flavonoids as chemical markers of Passiflora species, HPLC extracts were evaluated to identify and quantify the main chemical markers in *P. cincinnata* extracts (Table 4 and 5, Fig. 3). Both vitexin and isoorientin were quantified in the ethanolic extract and in the aqueous extract, the latter being in greater concentration of the flavonoids. The presence of these metabolites corroborates studies

described in the literature that identify these flavonoids in Passiflora species, such as in *P. alata, P. quadrangularis, P. bogotensis, P. tripartita* var. Mollissima and *P. cincinnata* [5,8,24-29].

In order to evaluate the Pc-Aq safety profile, the acute toxicity test was performed. The animals were evaluated 14 days after single dose administration of 2 g/kg of PC-Aq, orally and intraperitoneally, for determining LD_{50}. The LD_{50} aims to characterize the dose that has a lethal effect in 50% of the animals in a population, providing toxicity data and formulating guidelines for the selection of a safe dose for further pharmacological trials [30].

The administration in a single dose of the extract did not cause deaths, indicating a low toxicity of the extract. Thus, Pc-Aq has an $LD_{50} > 2$ g/kg when administered by both routes tested. Based on this, the oral route was selected to continue the studies, as it has a more convenient administration. Toxicity tests similar to those conducted in this study were performed with *Passiflora alata* species and did not indicate the presence of deaths in groups of animals treated with doses between 600 and 4800 mg/kg of the extract, as well as no signs of toxicity [31]. Recently, Holanda and collaborators [32] carried out acute toxicity tests with the *Passiflora tenuifila* Killip species, in the same dose used in this article (2 g/kg), and it did not cause the death of any of the animals, nor did it cause any nutritional changes related to weight maintenance and behavioral changes during the evaluation time (14 days), indicating a good safety profile, as observed in the tests with Pc-Aq.

For presenting antinociceptive activity in preclinical pain models using tests for pain assessment at central and peripheral levels with promising results [8], the Pc-Aq antinociceptive activity was investigated, as well as the possible mechanism of action involved in the pharmacological activity. In folk medicine, *Passiflora* species are reported for their anti-inflammatory properties, and evaluated in experimental studies as antinociceptive and anti-inflammatory agents [33-37]. Among the studied species, *P. cincinnata* can be cited, and its ethanolic extract showed promising results in preclinical tests [8]. In this study, the treatment

with Pc-Aq obtained by decoction is based on its higher concentration of flavonoid vitexin. Pc-Aq demonstrated significant antinociceptive activity in the acetic acid-induced abdominal writhing test (Fig. 4) at both doses tested. However, despite the significant activity, the test used has low specificity in nociception tests, since the intraperitoneal administration of acetic acid promotes nonspecific nociceptive responses, which promotes the release of mediators involved in different types of pain, such as neurogenic and peripheral. Therefore, drugs with different mechanisms of action may have an action in this test [38].

The nociceptive responses caused by the formalin injection correspond to a biphasic pattern: acute initial phase (first phase) and later (second phase). The first phase of this test is related to chemical stimulation triggered after intraplantar formalin nociceptors application distributed in the afferent C fibers and partly in the Aδ fibers, by promoting the release of excitatory amino acid, substance P and nitric oxide [13-39].

The second phase has an inflammatory component, involving tissue and functional changes in the dorsal horn of the spinal cord and may be accompanied by proinflammatory mediators, such as bradykinin, histamine, and prostaglandins [13-39]. The Pc-Aq presented results in both phases of the formalin test (Fig. 5), suggesting the participation of central and/or peripheral mechanisms in the antinociceptive activity. To confirm the central mechanisms, the hot plate test (thermal stimulation) was performed and the mechanism of action of the Pc-Aq was evaluated.

PC-Aq increased latency in the hot plate test in all analyzes after administration (Fig. 6). These data corroborate the results in the formalin test and the results already described for the species *P. cincinnata* [8], suggesting that the analgesic effect of the extract may involve neurogenic responses, since this test is sensitive and specific for drugs that act by a central mechanism of action, such as opioid analgesics [40]. Therefore, the formalin test was selected to evaluate the possible mechanisms of action involved in the pharmacological effect of Pc-Aq

and the extract at the dose of 200 mg/kg was chosen to be used in these experiments, as it was the dose that showed the best results during nociceptive tests.

Pretreatment with naloxone, glibenclamide and yohimbine reversed the extract's antinociceptive activity (Fig. 7, 8 and 9), indicating that opioid receptors, K^+_{ATP} dependent channels and α-2 adrenergic are involved in the pharmacological effect of the extract.

Initially, the possible mechanism of action of Pc-Aq was investigated in the opioid receptor pathway using naloxone as an opioid antagonist. Opioid receptors are coupled to the Gi/o protein. Once activated, they inhibit the activity of the adenylyl cyclase enzyme, responsible for increasing the intracellular levels of cAMP, thus preventing the entry of calcium into the cell. Concomitantly, K^+_{ATP} channels are opened, causing K^+ efflux, promoting cell hyperpolarization, thus blocking the release of neurotransmitters and the transmission of neuronal pathways by reducing neuronal excitability [41].

The mechanism of antinociception induced by opioid receptors is intrinsically related to the activation of K^+_{ATP} channels, and the influence of these channels on Pc-Aq antinociception using glibenclamide was evaluated. The results (Fig. 8) indicated that glibenclamide does not attenuate pain in either the first or the second phase of the formalin test. However, treatment with sulfonylurea was able to inhibit the antinociceptive effect of the extract in both phases of the formalin test, indicating the participation of this pathway in the antinociception mechanism.

The central administration of agonists for the K^+ channels produces an antinociception and is capable of potentiating the analgesic effect of opioid agonists and α2-adrenergic agonists, as well as the activation of G protein-coupled receptors by agonists, such as α2 adrenoceptors and opioids, can activate K^+ channels. Thus, the involvement of the α2 adrenoceptor pathway in the extract-induced antinociception was evaluated [42-43]. The noradrenergic system participates in the modulation of pain via descending inhibitory. Thus, the activation of α2 receptors by descending noradrenergic pathways promotes an inhibitory regulatory effect in the

modulation of acute pain, mediated by the Gi protein pathway, inhibiting adenylate cyclase, consequently reducing the formation of cAMP and increasing the cell efflux of K^+. Thus, there is a reduction in the release of neurotransmitters, by reduction in the conductance of calcium, in sensory neurons present in the periaqueductal gray substance, in the dorsal horn of the spinal cord, preventing the continuation of the release of substance P and glutamate through the nerve terminals [42-45].

The results (Fig. 9) of this study indicate that the mechanism of action of the extract involves the α2-adrenergic receptors on their antinociceptive effect in both phases of the formalin test, as this result was reversed by pretreatment with yohimbine (non-selective antagonist α2-adrenergic).

Similarly, the involvement of muscarinic, serotonergic and vanilloid type-1 receptors in central and peripheral pain was studied. The results of these tests suggest that pretreatment with the antagonist (atropine – Fig. 10, ondansetron – Fig. 11 and ruthenium red – Fig. 13, respectively) did not significantly reverse the effect of the extract, indicating that the antinociceptive activity is probably not related to those routes.

Finally, the effect of Pc-Aq on the motor coordination of the animals was evaluated through the rota-rod test (Fig. 14). According to the test, no significant changes were observed, suggesting that the extract promotes its antinociceptive effect without interfering in the motor coordination of the animals.

5. Conclusions

From the results presented in this study, it can be concluded that the leaf aqueous extract of *P. cincinnata* presents potential bioactive compounds, especially by the presence of the flavonoid vitexin, detected in the analysis by HPLC-DAD. Regarding the pharmacological effects, PC-Aq presented antinociceptive activity in models of chemical nociception and

thermal nociception. The mechanism of action probably involves central pathways, including the involvement of opioid receptors, ATP-sensitive potassium channels and alpha-2 adrenergic receptors, without interference in the motor coordination of the animals. Thus, the results of this study corroborate the preclinical studies described in the literature, and they also reinforce the traditional use of *P. cincinnata* in the treatment of inflammation and pain, being demonstrated as a promising source in the development of new drugs derivated from plant species.

Authors contributions

EML: Conceptualization, Writing - review & editing. AWCF: Investigation. MGS: Formal analysis.: Writing - review & editing. AEBPL: Investigation. MTMFA: Investigation. JMB: Investigation. ECVP: Investigation. LAR: Writing - review & editing, Data curation, Formal analysis and Supervision. RGOJ: Writing - review & editing. JRGSA: Writing - review & editing, Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no financial interests or personal relationships that may have influenced the development and execution of the present work reported in this article.

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CONCLUSÃO GERAL

O artigo de revisão indicou que os óleos essenciais e seu composto majoritário apresentam potencial terapêutico para o tratamento de doenças inflamatórias, especialmente em condições inflamatórias crônicas, envolvendo mecanismos antioxidantes.

No artigo experimental a obtenção do extrato aquoso e a avaliação farmacognóstica demonstraram que a droga vegetal *P. cincinnata* está de acordo com as especificações da farmacopeia brasileira. Todos os parâmetros avaliados corroboram com o preconizado, demonstrando assim, a qualidade da matéria prima utilizada.

A análise fitoquímica do extrato indicou a presença dos flavonoides vitexina e isorientina, os quais foram identificados por CLAE-DAD.

Com relação ao perfil de segurança, foi observado que o extrato de *P. cincinnata* não provocou mortes nos animais submetidos à avaliação da toxicidade aguda, indicando assim, baixa toxicidade, encorajando a realização dos testes farmacológicos pré-clínicos.

Os ensaios farmacológicos de atividade antinociceptiva, indicaram redução da nocicepção química (contorções abdominais induzidas por ácido acético e teste da formalina) e no de nocicepção térmica (teste da placa quente) nas doses testadas.

Na investigação do provável mecanismo farmacológico da atividade antinociceptiva foi verificado envolvimento de vias centrais no controle da nocicepção, com o envolvimento da via de receptores opioides, canais para potássio dependentes de ATP, e vias α-2 adrenérgicas. Adicionalmente, verificou-se que o extrato, não apresentou incoordenação motora significativa nos animais nas doses testadas.

Os resultados obtidos nesse estudo, sugerem que o extrato de *P. cincinnata* (Pc-Aq) caracteriza-se como um agente promissor, com valor biotecnológico para o tratamento da dor possivelmente envolvendo inibição das vias centrais, no entanto, estudos posteriores com administração subcrônica e crônica do extrato são necessários para confirmação do efeito observado, avaliação do possível efeito tóxico após exposição prolongada e confimação do mecanismo de ação para posteriores estudos pré-clínicos.

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

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

Certificado de autorização

Certificamos que o projeto intitulado: "**DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODO ANALÍTICO PARA OBTENÇÃO DE EXTRATO PADRONIZADO DAS FOLHAS DE PASSIFLORA CINCINNATA MAST. E AVALIAÇÃO DA ATIVIDADE ANTINOCICEPTIVA E ANTI-INFLAMATÓRIA**", registrado com o nº **0004/130220**, sob a responsabilidade de **Érica Martins de Lavor** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Federal do Vale do São Francisco - UNIVASF, em 13/02/2020.

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	Início: 01/05/2020 a 01/02/2022
Espécie/linhagem/raça	*Mus musculus*
Nº de animais	M 444; F 24 = 468
Peso/Idade	30 a 40 g/ 8 a 10 semanas
Sexo	Ambos
Origem	Biotério da UNIVASF, Campus Petrolina-PE, CCA

Em: 18/03/2020

KARINE VIEIRA ANTUNES
Coordenadora da Comissão de Ética no Uso de Animais
CEUA/UNIVASF

ANEXO B – Comprovante de submissão do artigo 2

Chemico-Biological Interactions

Leaf aqueous extract of Passiflora cincinnata Mast. (Passifloraceae) reduces nociception in mice by central mechanisms

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Section/Category:	Natural compounds (incl. toxins and pharmacologically active compounds)
Keywords:	Passiflora cincinnata; Flavonoids; Antinociceptive activity; Mechanisms of antinociception
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Abstract:	Passiflora cincinnata Mast. is native to the Caatinga biome, and its use is described in traditional medicine for analgesic and anti-inflammatory purposes. Thus, this work aims to evaluate the antinociceptive activities of P. cincinnata (Pc-Aq) from the leaves through preclinical assays in mice. The pharmacognostic characterization of the vegetal plant was performed, and the chemical composition of the aqueous extract (Pc-Aq) was evaluated by high-performance liquid chromatography coupled to diode array detector (HPLC-DAD). The antinociceptive evaluation of the extract administered orally, at doses of 100 and 200 mg/kg was performed using in vivo chemical models (acetic acid-induced abdominal constriction and formalin-induced paw licking test) and thermal (hot plate test) of nociception. The mechanism of action was evaluated through the use of opioid receptor antagonist drugs, ATP-sensitive potassium channels, vanilloid receptor type-1 (TRPV-1), muscarinic, serotonergic (5-HT3) and α-adrenergic receptors. The rota-rod test was performed to verify the possible interference of the extract treatment in the motor performance of the animals. The results of chemical analysis indicated the presence of the flavonoids vitexin and isoorientin in Pc-Aq. The extract treatment reduced the number of abdominal writhings and decreased paw licking time in both phases of the formalin test ($p < 0.05$). In the hot plate test, the extract increased the latency time for paw withdrawal, indicating a reduction in painful behavior. The evaluation of the antinociceptive mechanism indicated a possible action of central pathways in the antinociceptive activity, with participation of opioid receptors, with influence of potassium channels and α-2 adrenergic pathway, without significant change in motor control, assessed by the rota-rod test. Therefore, this study suggests that Pc-Aq has an antinociceptive action mediated by central pathways, whose action can be attributed to the presence of the flavonoids present in the extract.

Suggested Reviewers:	Juliane Cabral Silva, Ph.D. Professor, UNCISAL: Universidade Estadual de Ciencias da Saude de Alagoas larbacjuliane@gmail.com She is researcher in pharmacology of natural products.