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MÔNICA SANTOS DE MELO

EFEITOS ANTIHIPERALGÉSICO E ANTI-INFLAMATÓRIO DA *Kielmeyera rugosa* Choisy (CLUSIACEAE) EM ROEDORES

ARACAJU-SE
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Sergipe como requisito parcial à obtenção do grau de Doutor em Ciências da Saúde.

Orientador: Prof. Dr. Lucindo José Quintans Júnior

Co-orientador: Prof. Dr. Waldecy de Lucca Junior

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DEDICATÓRIA

Dedico este trabalho àquele que, em vida, ensinou-me o verdadeiro sentido da fraternidade: ao meu irmão, Márcio Santos de Melo (in memoriam).

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RESUMO

MELO, Mônica Santos de. **EFEITOS ANTIHIPERALGÉSICO E ANTI-INFLAMATÓRIO DA *Kielmeyera rugosa* CHOISY (CLUSIACEAE) EM ROEDORES**. Tese de Doutorado em Ciências da Saúde, Universidade Federal de Sergipe, Aracaju, 2014.

Diversas plantas do gênero *Kielmeyera,* família Clusiaceae são utilizadas pela população do Nordeste brasileiro na medicina tradicional para o tratamento de diversas doenças, incluindo distúrbios dolorosos e inflamatórios. Um estudo recente demonstrou atividade antitumoral para a *K. rugosa*, no entanto, outras atividades farmacológicas nunca foram estudadas para esta espécie vegetal, por exemplo seu possível perfil analgésico e anti-inflamatório. Dessa forma, o objetivo do trabalho foi avaliar o efeito antihiperalgésico e anti-inflamatório do extrato metanólico obtido do caule de *K. rugosa* (EMKR) em roedores. Foram utilizados camundongos *Swiss* machos (25-35 g), divididos em grupos e submetidos ao tratamento agudo com EMKR (100, 200 e 400 mg/kg; por via oral, v.o.), veículo (solução salina 0,9% + tween 80 0,2%; v.o.) ou droga padrão (i.p.). A hipernocicepção foi avaliada nos tempos 0,5, 1, 2 e 3 h após a administração (i.pl.) de carragenina (CG; 300 µg/pata), Fator de necrose tumoral-α (TNF-α; 100 pg/pata), Prostaglandina E_2 (PGE_2; 100ηg/pata) ou Dopamina (DA; 30 µg/pata), e foram avaliados utilizando-se o analgesímetro digital (Von Frey). Na avaliação da atividade anti-inflamatória foram utilizados dois protocolos, o primeiro de pleurisia induzida por CG (300 µg/cavidade), no qual após 4 horas foram realizadas as contagens total e diferencial de leucócitos, bem como as dosagens dos níveis de TNF-α e IL-1β do lavado pleural. Em outro protocolo realizado para investigar à atividade anti-inflamatória correspondeu ao edema de pata induzida por CG (1%/40µL); o volume da pata foi medido com auxílio do pletismomêtro nos tempos 0-6h após a CG. A citotoxicidade do MEKR foi avaliada através do método colorimétrico do MTT. Para determinar o possível envolvimento de áreas do Sistema Nervoso Central (SNC), os animais foram tratados e, noventa minutos após, foram anestesiados, perfundidos, os cérebros extraídos e cortados em criostato. As secções cerebrais foram submetidas ao protocolo de imunofluorescência para proteína Fos. A coordenação motora do animal foi avaliada através do teste do Rota Rod (7 rpm, 180 s). Os protocolos experimentais foram aprovados pelo comitê de ética da UFS (CEPA/UFS: 102/11) Os resultados foram expressos como média ± erro padrão da média. As diferenças entre os grupos foram analisadas por meio do teste de variância ANOVA, uma via, seguido pelo teste de Tukey. O pré-tratamento com EMKR inibiu significativamente a hiperalgesia induzida pelos agentes álgicos, CG, TNF-α, PGE_2 e DA ($p < 0,001$). O EMKR também foi capaz de inibir o recrutamento leucocitário para a cavidade pleural ($p < 0,01$). Esta inibição leucocitária ocorreu devido à inibição na migração dos neutrófilos. Os níveis das citocinas, TNF-α ($p < 0,01$) e IL-1β ($p < 0,001$) também foram reduzidos quando os animais foram tratados com EMKR. Quanto ao edema, o EMKR diminuiu a formação induzida pela CG ($p < 0,001$). No entanto, o MEKR não apresentou efeito citotóxico ou alteração da coordenação motora dos animais. No teste de imunofluorescência para proteína Fos, o tratamento com MEKR ativou significativamente o bulbo olfatório ($p < 0,01$), córtex piriforme ($p < 0,01$) e a substância cinzenta periaquedutal ($p < 0,001$), áreas do SNC. Os resultados sugerem que o MEKR possui atividade antihiperalgésica e anti-inflamatória provavelmente por ativação de áreas do sistema nervoso central relacionadas com a modulação da dor e por reduzir a produção de citocinas pró-inflamatórias, sem traços de citotoxicidade.

Palavras-chave: Clusiaceae; *Kielemeyera rugosa*; Dor; Inflamação; Proteína Fos, Substância cinzenta periaqueductal.

ABSTRACT

MELO, Mônica Santos de. **ANTI-HYPERALGESIC AND ANTIINFLAMMATORY EFFECTS OF *Kielmeyera rugosa* CHOISY (CLUSIACEAE) IN MICE.** 2014. Tese de Doutorado em Ciências da Saúde, Universidade Federal de Sergipe, Aracaju, 2014.

Plants of the genus Kielmeyera, family Clusiaceae are used by the population of the Northeast of the Brazil in traditional medicine to treat various diseases, such as pain and inflammation diseases. A recent study demonstrated antitumor activity for *K. rugosa*, however, other pharmacological activities have never been studied, for example antihyperalgesic and anti inflammatory activities. Thus, the aim of this study was to evaluate the antihyperalgesic and anti - inflammatory effects of the methanol extract from the stems of *K. rugosa* (MEKR) in rodents. Swiss mice (25-35 g) were divided into groups and subjected to treatment with MEKR (., 100, 200 and 400 mg/kg, oral administration, p.o.) , vehicle (0.9% saline solution + 0.2% Tween 80) or standard drug (i.p.). The hypernociception was evaluated at times 0.5 , 1, 2 , and 3 hours after administration (i.pl.) of carrageenan (CG; 300 mg/paw), tumor necrosis factor-α (TNF-α, 100 pg/paw), Prostaglandin E_2 (PGE_2; 100ηg/pata) or dopamine (DA, 30 µg/paw) using the digital analgesymeter (von Frey). In the evaluation of anti-inflammatory activity two protocols were used, the first induced by GC (300 mg/well) at 4 hours after pleurisy which the full and differential counts were made of leukocytes as well as the dosage levels of TNF-α and IL-1β pleural lavage. In another protocol conducted to investigate the anti-inflammatory activity corresponded to paw edema induced by GC (1 %/40µL), the paw volume was measured with the aid of plethysmometer at 0-6h after CG. The cytotoxicity of MEKR was evaluated by the MTT colorimetric method. To determine the possible involvement of areas of the Central Nervous System (CNS), the animals were treated and ninety minutes, were anesthetized, perfused, the brains extracted and cut into the cryostat. The brain sections were subjected to immunofluorescence protocol for Fos protein. The motor coordination of the animal was assessed by the Rota Rod test (7 rpm, 180 s). The experimental protocols were approved by the ethics committee of the UFS (CEPA/UFS: 102/11) The results were expressed as mean ± standard error of the mean. Differences between groups were analyzed using the ANOVA one way followed by Tukey's test. The acute pretreatment with MEKR significantly inhibited hyperalgesia induced by nociceptive agents, CG, TNF-α, PGE_2 and DA ($p < 0.001$). The MEKR inhibited the leukocyte recruitment into the pleural cavity ($p < 0.01$). This was due to the inhibition of leukocyte migration inhibition of neutrophils. The levels of cytokines, TNF-α ($p < 0.01$) and IL-1β ($p < 0.001$) were also reduced when the animals were treated with MEKR. MEKR decreased edema formation induced by CG ($p < 0.001$). However, MEKR showed no cytotoxic effect or change in motor coordination animals. In the Fos protein immunofluorescence test, MEKR showed that the olfactory bulb ($p < 0.01$), piriform cortex ($p < 0.01$) and periaqueductal gray ($p < 0.001$) for the active significantly of the CNS. Therefore, we conclude that MEKR has antihyperalgesic activity probably by activating the central nervous system areas associated with pain modulation and by reduction the production of proinflammatory cytokines, without traces of cytotoxicity.

Keywords: Clusiaceae, *Kielmeyera rugosa* Choisy, Pain, Inflammation, Fos protein, Periaqueductal gray.

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INTRODUÇÃO

1 INTRODUÇÃO

A dor foi definida pela Associação Internacional para o Estudo da Dor (IASP) como sendo uma experiência sensorial e emocional associada a dano tecidual real ou potencial, ou descrita em termos de tal lesão, a qual pode ser classificada em aguda e crônica (MERSKEY, 1979), afetanto de 10% e 30% na população adulta (BEKKERING et al., 2011; HARKER et al., 2012).

A inflamação gera como sintoma mais comum a dor, sendo resultante de lesão do tecido, uma vez que as células inflamatórias e mediadores estão envolvidos na gênese, persistência e gravidade do sintoma (HADDAD, 2007). Estes mediadores, liberados principalmente por células do sistema imunológico, produzem uma ativação e sensibilização de nociceptores dos neurônios envolvidos na transmissão da entrada nociceptiva (MARCHAND et al., 2005).

Um dos principais tratamentos para a dor é a farmacoterapia. Os analgésicos amplamente utilizados na clínica são classificados em opióides e não-opióides. Os analgésicos não-opióides incluem o paracetamol e os anti-inflamatórios não esteróides (AINEs). Já os opióides, a exemplo da morfina e fármacos correlatos, que geralmente agem como agonistas nos receptores opióides (BECKER, 2010). Entretanto, 40 a 60% dos pacientes não respondem a farmacoterapia atualmente disponível no mercado ou apresentam um número expressivo de reações adversas que limitam seu uso (XU et al., 2012; CLAUW; ARNOLD; MCCARBERG, 2011). Desta forma, a pesquisa por novas propostas terapêuticas para o tratamento dos diversos tipos de dores continua sendo uma constante busca dos pesquisadores em todo o mundo e um dos maiores desafios da medicina (SRINIVASAN et al., 2001).

Nesse sentido, a avaliação do potencial terapêutico de produtos naturais e de seus constituintes tem sido objeto de incessantes estudos (CECHINEL FILHO; YUNES, 1998), com destaque para as plantas medicinais, as quais são utilizadas tanto pelas comunidades tradicionais, como por estudiosos, com o objetivo de obtenção de novos fármacos (COSTA; MAYWORM, 2011), sendo, assim, o objeto de estudo de grupos que trabalham no intuito de validar o seu uso popular e garantir sua efetividade e segurança (MACEDO, 2007).

A *Food and Drug administration* (FDA) e a *European Medicines Agency* (EMA) consideraram, como aptos para a inserção no mercado, 19 medicamentos oriundos de produtos naturais, a exemplo do Dronabinol (Sativex®), Exenatide

(Byetta®) e Romidepsin (Istodax®), indicados no tratamento álgico, diabético e oncológico, respectivamente. (MISHRA, 2011). Já no Brasil, temos como exemplo recente o anti-inflamatório tópico Acheflan®, do Laboratório Aché, produzido a partir do óleo essencial da planta *Cordia verbenacea*, sendo o anti-inflamatório tópico mais prescrito pela classe médica brasileira, correspondendo a cerca de 44% das prescrições médicas, evidenciando-se a importância dos produtos naturais no mercado brasileiro (CALIXTO; SIQUEIRA JUNIOR, 2008).

Os produtos à base de plantas medicinais movimentaram cerca de 30 bilhões de dólares (MELO et al., 2007), o que se justifica pelo fato de 25% dos medicamentos prescritos mundialmente serem de origem vegetal, conforme resultados divulgados pela OMS (CORDEIRO; CHUNG; SACRAMENTO, 2005). Pesquisas realizadas no Brasil mostram que 91,9% da população já fez uso de alguma planta medicinal e cerca de 46% mantêm o cultivo caseiro dessas plantas (ETHUR et al., 2011).

Estudos pré-clínicos têm demonstrado propriedades benéficas das plantas medicinais, especial daquelas comumente encontradas no nordeste brasileiro, tais como os efeitos anti-inflamatório e antinociceptivo do óleo essencial da *Myrcia pubiflora* (ANDRADE et al., 2012), antimicrobiano da *Baccharis dracuncucifolia* D.C. e da *Baccharis uncinella* D.C. (FERRONATTO et al., 2007), antinociceptivo e anti-inflamatório do *Cymbopogon winterianus* (LEITE et al., 2010).

O gênero Kielmeyera (família Clusiaceae) apresenta a maior parte das 47 espécies de ocorrência exclusiva no Brasil (SADDI, 1984). Algumas espécies são popularmente conhecidas como ‘‘pau-santo’’, ‘‘rosa-do-campo’’ e ‘‘malva-do-campo’’ (PINHEIRO et al., 2003). Partes da planta, especialmente as folhas de algumas espécies, são frequentemente utilizadas na medicina popular para o tratamento de várias doenças tropicais, incluindo esquistossomose, leishmaniose e malária, bem como infecções bacterianas e virais (ALVES et al., 2000).

A espécie *Kielmeyera rugosa* Choisy tem sido o foco de avaliação biológica. As xantonas são marcadores quimiotaxonômicos importantes dentro do gênero Kielmeyera (NOGUEIRA et al., 2008) Recentemente, Ribeiro et al. (2012) demonstraram que o extrato de *K. rugosa* possui uma atividade antitumoral significativa. Para este gênero, também se observou que microinjeções no núcleo mediano da rafe de xantonas obtidas a partir da fração diclorometano de *K. coriacea* reduz o tempo de imobilidade no teste da natação forçada, tendo um efeito anti-depressivo em ratos submetidos ao teste (SELA et al., 2010).

Levando-se em consideração a importância das plantas medicinais na atual conjuntura mundial, a necessidade de novas drogas analgésicas com maior potencial terapêutico, maior seletividade e menores efeitos colaterais, e que a espécie *Kielmeyera rugosa* Choisy apresenta-se como uma fonte promissora de bioativos, possuindo, possivelmente, ação sobre o sistema nervoso central, sendo desconhecidas suas possíveis atividades antinociceptiva e anti-inflamatória, assim como seus mecanismos de ação central e periférica, o objetivo do presente estudo foi investigar o efeito do extrato metanólico do caule de *K. rugosa* sobre a hiperalgesia inflamatória induzida por carragenina (CG), fator de necrose tumoral-alfa (TNF-α), a prostaglandina E_2 (PGE_2) e dopamina, assim como seu efeito sobre a liberação de citocinas pró-inflamatórias e sobre a via descendente inibitória da dor.

OBJETIVOS

2. OBJETIVOS

2.1 OBJETIVO GERAL

_ Avaliar os possíveis efeitos antihiperalgésico e anti-inflamatório do EMKR em roedores.

2.2 OBJETIVOS ESPECÍFICOS

- Realizar um levantamento buscando a elaboração de uma revisão sistemática sobre a atividade anti-inflamatória de plantas da família Clusiaceae

- Avaliar o efeito anti-hiperalgésico do EMKR no modelo animal de hipernocicepção induzida por Carragenina, Fator de necrose tumoral alfa, Prostaglandina E_2 e Dopamina;

_ Avaliar o efeito anti-inflamatório do EMKR no modelo animal de pleurisia e edema de pata induzidos por carragenina;

_ Investigar o envolvimento de regiões centrais na possível ação do EMKR;

_ Verificar possíveis alterações motoras induzidas pela administração do EMKR.

DESENVOLVIMENTO

3 DESENVOLVIMENTO

3.1. A systematic review for anti-inflammatory property of *Clusiaceae* family – a pre-clinical approach.

Artigo submetido à BioMed Research International (Fator de Impacto –JCR 2012: 2,88)

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Abstract

Background. Clusiaceae family is extensively used in ethnomedicine for treating a number of disease conditions which include cancer, inflammation and infection. The aim of this review is to report the pharmacological potential of plants of Clusiaceae family with the anti-inflammatory activity. *Methods.* A systematic review about experiments investigating anti-inflammatory activity of Clusiaceae family was carried out by searching bibliographic data bases such as, Medline, Scopus and Embase. In this update, the search terms were "anti-inflammatory agents", “Clusiaceae” and “animals, laboratory”. *Results.* A total of 255 publications with plants this family were identified. From the initial 255 studies, a total of 20 studies were selected for the final analysis. Studies with genus *Allanblackia, Clusia, Garcinia* or *Rheedia* and *Hypericum* showed significant anti-inflammatory activity. The findings include a decrease of total leukocytes, number of neutrophils, total protein concentration, granuloma formation, paw or ear edema formation. Other interesting findings included decreased of the MPO activity, inflammatory mediators such as NF-κB and iNOS expression, PGE_2 and Il-1β levels and a decrease in chronic inflammation. *Conclusion.* The data reported suggests the therapeutic potential of Clusiaceae family for the development of herbal medicine.

1. Introduction

Inflammation is a process that occurs after an infection or tissue injury, characterized by increased post capillary venule permeability to fluid and plasma proteins and polymorphonuclear leukocyte emigration into tissues[1]. The inflammatory response is essential in maintaining homeostasis, however this event may be chronic course, leading to tissue damage due to leukocytosis, fibroplasia, excessive production of cytokines and other mediators[2]. Generally, anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs are effective for temporary relief of symptoms. However, drug-induced severe side effects occur, and most of these treatments are inadequate for chronic use [3].

Many people turn to alternative medicine including traditional plant based remedies for alleviating inflammatory conditions, such as plant-derived extracts or plant derivatives (isolated compounds), by controlling the levels of various inflammatory cytokines or inflammatory mediators [4-6]. The effect of medicinal plants is mediated by multiple targets through multiple active compounds [6, 7]. Although source around the world have made studies on the anti-inflammatory studies from different pathways and aspects and have made substantial progress, further studies are warranted to delineate the inflammation actions in more cogency models, assess the potentials in clinical applications, and make more convenient preparations easy to administrate for patients[8].

In this context, several ethnobotanical studies have reported the bioprospecting surveys on the positive use of Clusiaceae family with pharmacological activity[9]. Consequently, these plant species have received attention from the scientific community for its potential therapeutic capacity. Clusiaceae is a tropical family of trees, shrubs, and herbs comprising approximately 50 genera and 1200 species[10]. Several species of this family are used for medicinal purposes worldwide, as for the treatment of cancer, inflammation and infection. In Brazil, the most described genera are *Kielmeyera* Mart. & Zucc., *Caraipa* Aubl, *Platonia* R.Wight, *Clusia* L., *Rheedia* L. and Calophyllum [3-6].

The Clusiaceae family is a rich source of secondary metabolites, which four major classes of compounds are found: xanthones, coumarins, biflavonoids, and benzophenones, produced by the plants mainly as a defense mechanism[9]. Despite the

importance of this family experimental research on the anti-inflammatory effect with preparations on plants of Clusiaceae family never been reviewed.

Although a lot of important information or clues on the development of inflammation can be obtained from human studies, animal models not only enables us to have a more comprehensive understanding of the inflammation at a molecular level in a controlled manner, but also fulfills the need for drug screening tools. This not only allows a faster and more convenient screening but also serves as an alarm before the presence of cellular or functional lesion. Based on the mechanistic studies, drugs targeting different molecules in the cascade are being developed. In order to evaluate the effect of the drug properly, reliable and appropriate animal models are required. Therefore, in this review, we focus on the animal models of inflammation that researchers have used. So, the aim of our review was to systematically summarize the anti-inflammatory activity of the plants of Clusiaceae family evaluated in animal testing using predefined criteria, seeking to support the therapeutic use of this family.

2. Materials and Methods

2.1. Search Strategy for the Identification of Studies. The following databases were searched: PubMed, Scopus and Embase, for studies reported on animals testing investigating the anti-inflammatory activity of plants of the Clusiaceae family. The electronic databases were assessed between January/2013 – May/2013. Free text searches were performed across each database to combine the terms or key words: “anti-inflamatory agents” and “clusiaceae” and “animals, laboratory”. The general structure of the search strategy was “anti-inflamatory agents” with the following MeSH terms or synonyms: (Anti Inflammatory Agents) OR (Agents, Antiinflammatory) OR (Antiinflammatories) OR (Antiinflammatory Agents) OR (Agents, Anti-Inflammatory) OR (Agents, Anti Inflammatory) OR (Anti-Inflammatories) OR (Anti Inflammatories); “Clusiaceae” MeSH terms or synonyms:(*Psorospermum*) OR (*Psorospermums*) OR (Hypericaceae) OR (Rheedia) OR (Rheedias) OR (Plum, Waika) OR (Plums, Waika) OR (Waika Plum) OR (Waika Plums) OR (*Allanblackia*) OR (*Allanblackias*) OR (*Cratoxylum*) OR (*Cratoxylums*) (Medicinal Plant) OR (Plant,Medicinal) OR (Medicinal Plants) OR (Medicinal Herbs); and “Animals, laboratory” MeSH terms or synonyms were (laboratory animals) OR (animal, laboratory) OR (laboratory animal).

The reference list from each potentially eligible study and relevant review article were checked. The animal experiments were individually checked for inclusion criteria. Two independent researchers screened studies identified using the search strategy for inclusion, first on the basis of title and abstract and of those that were relevant, the full texts were screened for eligibility. Discrepancies between reviewers (MSM and LJQJ) were discussed and resolved by consensus.

2.2. Inclusion and Exclusion Criteria. Preparations of Clusiaceae family administered in the animals for the treatment of inflammation were included. The following selection criteria were used for inclusion of studies in the analysis: animal experiment; outcome measure; published. Only papers in English were included. Papers were excluded if they fulfilled one of the following criteria: (1) Not an original paper (e.g. review or letter etc.); (2) Isolated compounds was combined with plants; (3) Double publication; in case a paper occurred more than one time in one of the databases, only the original manuscript was included; (4) *In vitro* studies and (5) literature reviews. Purely toxicologic, analgesic, antioxidant or other associated terms tests were not included.

2.3. Data extraction items. Items for which data were extracted include: publication year, country of publication, study design, phlogistic agent, animal species, age, control groups, dose, duration, number of animal evaluated in each group, anti-inflammatory effect, outcome measurement tools and author's conclusions.

3. Results and discussion

As illustrated in the flow diagram, of the all unique records identified, only 22 publications met criteria for full-text review. We screened 255 relevant articles, and 233 were excluded, leaving us with 22 full-text eligible articles. Of these, 2 more were excluded (Figure 1).Characteristics of included articles are summarized in Table 1.

From the search, 29 hits were found with different Clusiaceae species reporting one or more of these activities: antinociceptive, anti-inflammatory, and antipyretic activity, gastric and toxicology effects. Some of the reports coincide for a given species, and, therefore, a total of 19 plants were reported to have such activity. However, 11 plants were studied for such activity. In eleven cases, further phytochemical studies

were carried out to find out the active constituent(s).The Clusiaceae plant names mentioned in this review were taken textually from the original sources, whenever they were reported.

The species were able to significantly reduce the inflammatory response in several models with possible involvement of isolated compounds: genus *Allanblackia*: *A. gabonensis*, *A. monticola* Staner L.C.; genus *Clusia*: *C. nemorosa*; genus *Garcinia* or *Rheedia*: *G. brasiliensis*, *G. cambogia*, *G. gardneriana* (Planchon & Triana) Zappi., *G. hanburyi* Hook F., *Rheedia longifolia* Planch & Triana; genus *Hypericum*: *H. androsaemum* L., *H. barbatum*Jacq., *H. canariense* L., *H. empetrifolium* Willd., *H. glandulosum* Ait, *H. hirsutum* L., *H. perforatum* L., *H. reflexum* L. fil., *H. richeri* Vill., *H. rumeliacum* Boiss. subsp. *Apollinis* (Boiss. & Heldr.) Robson & Strid, *H. triquetrifolium* Turra.

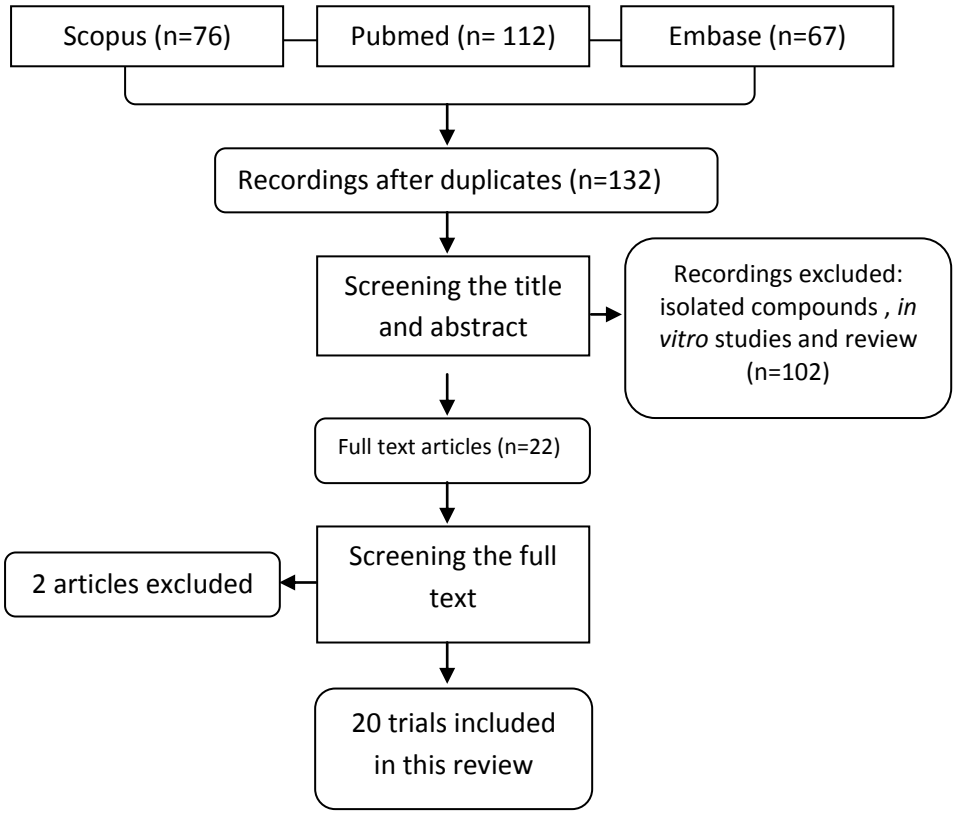

Figure 1: Flow diagram of the literature search

In all studies the minimum information reporting research using animals was included, such as the number and specific characteristics of animals used (including species, strain, sex, and genetic background); details of housing and husbandry; and the experimental and statistical methods.

Since animal models are fundamental tools in biomedical study, as the ones of sharing a high degree homology with humans, mice and rats are commonly used in laboratory tests for better understanding human disorders. As mammals, murine models with drug-induced diseases have been well-established, either for investigating disease pathogenesis and probable mechanisms, or for assessing the effectiveness of diverse candidate instruments and drugs, physically and chemically, which facilitated human health researches[11, 12].

The animals most used were *Swiss* mice, *Wistar* and *Sprague-Dawley* rats of both genders. Animals studied were approximately 3 to 6 weeks of age. Altogether, 12 animal experiments or experimental settings matched inclusion criteria. Often, experiments consisted of several sub-experiments with safety results usually reported globally; accordingly, these sub-experiments are summarized in this review as well. The inflammation tests differed, but in general the tests were based on sensitization with doses of injections of inflammatory agents at different frequencies and for an average duration of hours or days.

Table 1: Description of the anti-inflammatory aspects of the studies included in systematic review

References	Scientific name	Parts used	Animal	Dose	Route	Methods used	Action mechanism
Trovato *et al.*, 2001	*H. empetrifolium*	APME	Wistar rats	100 mg/kg	i.p.	CIPE	Inhibition of PG
Ozturk *et al.*, 2002	*H. triquetrifolium*	DAPE	Wistar rats	25-60mg/kg	i.p.	CIPE	Without mechanism
Rabanal *et al.*, 2005	*H. canariense;H. glandulosum*	BME	Swiss mice	0,25-1 mg/ear	t.a.	TPAIEE	Inhibition AAmetabolism
Salam 2005	*H. perforatum*	Comercial extract	Sprague Dawley rats	50-300 mg/kg	s.c.	CIPE	Inhibition of the liberation of HIS, 5-HT, KN
Sánchez-Mateo *et al.*, 2006	*H. reflexum*	BME	Swiss mice	0.25-1 mg/ear	t.a.	TPAIEE	Inhibition of PLA_2, COX and LOX

Nguemfo *et al.*, 2007	*A. monticola*	SBMCME	Wistar rats	75-300 mg/kg	p.o.	CIPE; HSIPE; AAIPE; DIPE	Inhibition AA metabolism
Savikin *et al.*, 2007	*H. perforatum;H. barbatum;H. hirsutum;H. richeri; H. androsaemum.*	DAPE	Wistar rats	25-200 mg/kg	p.o.	CIPE	Inhibition of NF-κB.
Frutuoso *et al.*, 2007	*R. longifolia*	LAE	Swiss mice; Wistar rats	10-100 mg/kg	p.o.	PILPS	Inhibition neutrophil
Ymele *et al.*, 2013	*A. gabonensis*	SBAE	Wistar rats	100-400 mg/kg	p.o.	CIPE; HSIPE	Reduced liberation and action of His and5-HT; Inhibition AA metabolism
Savikin *et al.*, 2007	*H. perforatum;H. barbatum;H. hirsutum;H. richeri; H. androsaemum.*	DAPE	Wistar rats	25-200 mg/kg	p.o.	CIPE	Inhibition of NF-κB.

Panthong *et al.*, 2007	*G. hanburyi*	BEAE	Sprague-Dawley rats	10-40 mg/kg	p.o.	EPPIEE; CIPE; AAIPE; GGICP	Inhibition of the liberation of His, PG, KN
Castardo *et al.*, 2008	*G. gardneriana*	LHE	Swiss mice	30-300 mg/kg	i.p.	CIPE;HSIPE; TPAIPE; BKIPE; AAIPE MPOAA; SPIPE	Inhibition the activity of neuropeptides and PKC
Galati*et al.*, 2008	*H. rumeliacum*	APME	Wistar rats	50; 70 mg/kg	i.p.	CIPE	Without mechanism
Martins *et al.*, 2008	*G. brasiliensis*	FPO	Wistar rats	100 mg/kg	p.o.	CIPE	Without mechanism
Reis *et al.*, 2009	*G. cambogia*	FPE	Wistar rats;	0,5; 1.0 g/kg	p.o.	CITNBS; MPOAA; $EMPGE_2$	Inhibition COX-2 expression and production PGE_2

Zdunić*et al.*, 2009	*H. perforatum*	FTOE	Wistar rats	1.25 mL/ Kg	p.o.	CIPE	Without mechanism
Paterniti *et al.*, 2010	*H. Perforatum*	ME	Sprague Dawley rats	2 mg/kg		PIL; MPOAA; MVP; CE	Reduces the NF-κB translocation; inhibition the IκB- α degradation; attenuation the expression of iNOS
Suntar *et al.*, 2010	*H. perforatum*	APOOE; APEE	Sprague Dawley rats; Swiss Mice	50-400 mg/kg	p.o.	AcAICP	Without mechanism
Otuki *et al.*, 2011	*G. gardneriana*	LHE; BHE; SHE	Swiss Webster mice	0.01-1 mg⁄ear	t.a.	COIEE; MPOAA	Inflammatory signal transductionpathway not specified
Santa-Cecília *et al.*, 2011	*G. brasiliensis*	LEE	Wistar rats	30-300 mg/kg	p.o.	CIPE; PILPS; GGICP	Inhibition of the liberation of His, 5-HT, BK

Farias *et al.*, 2012	*C. nemorosa*	LHxE	Swiss mice	50-200 mg/kg	i.p.	CIP;MTP; MTNF-α; GGICP	Inhibition of the neutrophilmigration

*Abbreviations of parts used:*APME: aerial parts methanol extract; APEE: aerial parts ethanolic extract; APOOE: aerial parts olive oil extract; BEAE: bark ethyl acetate extract;BHE: bark hydroalcoholic extract; BME: blossom methanol extract; DAPE: dried aerial parts extract; FPE: fruit peel extract; FPO: fruit pee oil; FTOE Flowering tops oil extracts; LAE: leaves aqueous extract; LHE: leaves hydroalcoholic extract; LEE: leaves ethanolic extract; LHxE: leaves hexanic extract;ME: methanolic extract. SBAE: stem bark aqueous extract; SBMCME: stem barks methylene chloride/methanol extract; SHE: seeds hydroalcoholic extract;

Abbreviations of administration routes: i.p.: intraperitoneal; p.o.: oral administration; t.a.: topical administration;

*Abbreviations of methods used:*AAIPE: arachidonic acid-induced paw edema; AcAICP: acetic acid-induced increase in capillary permeability; BKIPE: bradykinin induced paw edema; CE: cytokines expression; CIP: carrageenan-induced pleurisy; CIPE: carrageenan-induced paw edema; CITNBS: colitis induced by 2,4,6-trinitrobenzenesulfonic acid; DIPE; dexrtran-induced paw edema; COIEE: croton oil-induced ear edema; EMPGE2: Evaluation of mucosal PGE_2; EPPIEE: ethyl phenylpropiolateinduced ear edema; GGICP: granulomatous growth induced by cotton pellet;HSIPE: histamine and serotonin induced paw edema; MPOAA: myeloperoxidase activity assay; MTNF-α: measurement of tumor necrosis factor alpha; MTP: measurement of total protein; PEILPS: peritonitis induced by lipopolysaccharide;PIL: peritonitis induced by ligature; PILPS: pleurisy induced by LPS;SPIPE: Substance P induced paw edema; TPAIPE: 12-O-tetradecanoylphorbol 13-acetate induced paw edema.

Abbreviations of action mechanism: AA: arachidonic acid; BK: bradykinin; COX: cycloxigenase;His: histamine;iNOS: inducible nitric oxide synthase; KN: kinins; LOX: lipoxygenase; MVP: measurement of vascular permeability;NF-κB: nuclear factor kappa B; PKC: protein kinase C; PLA2: phospholipase A2;PG: prostaglandin, 5-HT: 5-hydroxytryptamine.

3.1. Plants of genus Allanblackia. Anti-inflammatory effects of *A. gabonensis* stem bark aqueous extract on carrageenan, histamine and serotonin-induced paw edema was assessed. The aqueous extract on serotonin, histamine or carrageenan-induced edema showed a significant inhibition starting from the first hour up to the sixth hour. On paw edema induced by the significant reduction with a maximal inhibition of 56.94% and 40.83%, respectively [13]

Other specie evaluated was *A. monticola* on carrageenan-induced edema and demonstrated that the methylene chloride extract and methylene chloride/methanol extract and its methanol fraction showed maximum reductions showed a maximum inhibition of paw edema. The methylene chloride fraction of *A. monticola* on rat paw edema induced by histamine exhibited a significant reduction of inflammation. The fraction did not reveal an anti-inflammatory activity even at the highest concentration in serotonin test. This same fraction on paw edema induced by arachidonic acid inhibited the paw edema. Although on dextran-induced paw edema the fraction decreased the volume an hour later compared with control groups [14].

Allanblackia gabonensis and *Allanblackia monticola* exhibited significant activity against edematous effect in all the three phases[13, 14], involved in the release of serotonin and histamine, mediated by prostaglandins, cyclooxygenase products, and the phase provided by kinins. Moreover, there is evidence of a possible interaction of *A. gabonensis* extract with the liberation and/or action of endogenous histamine and serotonin, probably mediated by alkaloids, phytosterols, triperpenes and phenolscompoundsisolated, such as xanthones and triterpenes onthe genus *Allanblackia* [15, 16].

For the same animal protocol utilizing histamine and serotonin, *A. monticola*was able to inhibit edema induced by dextran and histamine but not that provoked by serotonin. Probably the result on edema provoked by arachidonic acid indicated that this occurs preferentially by inhibition of lipoxygenase pathway of arachidonate metabolism [14]. Previous studies demonstrated that cytotoxic, anti-inflammatory, antimicrobial, antifungal and HIV inhibitory activities of species this genus are due to many of the secondary metabolites, xanthone derivatives (allanxanthone B,allanxanthone C, rubraxanthone, tovophyllin A, garciniafuran, norcowanin and mangostin), pentacyclic

triterpene (lupeol), saponin (a 3-O-b-D-glucopyranoside of stigmasterol) and phytosterol (stigmasterol) [17, 18].

3.2. Plants of genus Clusia. Hexane extract of leaves from *C. nemorosa* was evaluated on carrageenan-induced pleurisy. The extract caused a significant decrease in total protein extravasations, decreased the volume of the exudates to and inhibited leukocyte migration. A significant reduction in TNF-α concentration was verified in the treated group with hexane extract. The granuloma formation response elicited by subcutaneously implanted cotton pellet was inhibited [19].

Farias et al. 2012 reports these actions to carry out experimental protocols on animals such as the carrageenan-induced pleurisy, a model widely used to investigate the pathophysiology of acute inflammation, and also for evaluating the efficacy of drugs in inflammation[19]. The presence of carrageenan in the pleural cavity attenuates the plasma extravasation by increasing also the amount of total leukocytes especially neutrophils and mononuclear cells. After the fourth time, is also a significant increase in the levels of TNF-α on the site that received the injury with a marked release of histamine and serotonin [20-22]. The parameters involved in the pathological process, the *C. nemorosa* Both acted in significant reduction of leukocyte migration, with emphasis on reducing levels of neutrophils, with proven results in vitro protocols using as the initiator CXCL1. [19].

These results are in agreement with the findings of Ferro et al. (2013), suggesting that the mechanism of *C. nemorosa* may be linked, in part, to inhibition of cycloxygenase and/or lipoxygenase products in inflammatory diseases mediated by peripheral mechanisms [23]. The ratification of the anti-inflammatory effects of this plant occurred with the results obtained in the formalin test, a model of inflammatory pain that has two distinctive phases. The first phase corresponds to neurogenic pain is caused by activation of sensory C-fibers, followed by a second stage which is associated with the development of an inflammatory mediator release[24]. It is established that histamine, serotonin, prostaglandins and bradykinin are involved in the second phase responses [25]. With the findings of this experiment, pelleted better anti-inflammatory properties of *C. nemorosa*, therefore only in the inflammatory phase species under study showed significant results.

Chemical studies carried out with some species belonging to the genus Clusia have demonstrated the presence of many constituents, including polyisoprenylated benzophenones, terpenes, benzoquinone, flavonoids, dihydrophenanthrene derivative, tocotrienolic acids, betulinic acid, kaempferol and sitosterolglucoside [26]. The anti-inflammatory activity this genus can be attributed to these compounds. For betulinic acid, an approach of the action mechanism is attributed to its effect on NF-κB through inhibition of IκB kinase and p65 phosphorylation [27].

3.3. Plants of genus Garcinia. The anti-inflammatory effect of the leaves extract from *G. brasiliensis* on carrageenan-induced rat paw edema or peritonitis induced by lipopolysaccharide or granulomatous tissue growth induced cotton pellet implantation was tested. The leukocyte recruitment at 4 h post-LPS was 27.9%, 51.5% and 55.8% for 30, 100 and 300 mg/kg of the extract, respectively. In the model of chronic inflammation using cotton pellet-induced fibrovascular tissue growth in rats, the extract significantly inhibited the formation of granulation tissues. [28, 29].

One study reported that the hydroalcoholic extract of *G. gardneriana* was evaluated on carrageenan, 12-O-tetradecanoylphorbol-acetate (TPA), or different inflammatory mediators, including bradykinin, substance P, histamine, prostaglandin E_2or arachidonic acid measurement of paw edema. The activity of tissue myeloperoxidase (MPO) was assessed after injection of carrageenan into the mouse right hindpaw. All of the tested extracts from leaves, bark and seeds presented an inhibitory effect on the edema induced by carrageenan. The extract from leaves produced a significant reduction in the mouse paw edema induced by most tested mediators, except for the AA-induced edema. On MPO activity, treatment with extracts from leaves, bark and seeds of *G. gardneriana* significantly prevented the increase in MPO activity induced by carrageenan[30, 31].

Ethyl phenylpropiolate (EPP)-induced ear edema was utilized for testing inflammatory activity topical of the ethyl acetate extract from *G. hanburyi*, which at the dose of 1mg per ear significantly inhibited the edema formation. In the paw edema was produced in rats by either carrageenan or arachidonic acid (AA). The extract markedly reduced the edema formation of the paw induced by carrageenan at all assessment times, however, it is not elicited inhibitory effect on the edema formation of the rat paw induced by AA. For test cotton pellet-induced granuloma formation the ethyl acetate

extract from *G. hanburyi* significantly reduced transudative weight and granuloma formation [32].

The administration of *G. cambogia* extract reduced the length of macroscopically observed lesions at a 1 g/kg dosage in colits, although the MPO activity was significantly reduced by *Garcinia* treatment. The *G cambogia extract* effectively reduced colonic IL-1β expression and was also effective in inhibiting the iNOS colonic expression induced by2,4,6-trinitrobenzene sulfonic acid (TNBS). It was found that the administration of the extract caused a substantial reduction in the COX-2 expression, as well as in the up-regulation of PGE_2 caused by TNBS in the colon [33].

The fruit-peel volatile oil of *G. brasiliensis* was evaluated on the induced gradual edema of rat paw upon application of the inflammatory agent carrageenan. The inflammatory process was inhibited after administration of carrageenan [29].

The leaves aqueous extract of *Rheedia longifolia* inhibited inflammation six hours after the intrathoracic administration of LPS in the pleural wash recovered from LPS-injected mice [34].

These studies for *Garcinia* (*Rheedia*) species revealed the *G. gardneriana* like effective in reducing the edematogenic response. This effect is maybe related to a reduction in the liberation of histamine, serotonin, or bradykinin in local tissue or be dueto the blockage of receptors to these different mediators. This species significantly reduced leukocyte migration and decreased the dry weights of implanted cotton pellets, suggesting the potential to reduce the number of fibroblasts and the synthesis of collagen and mucopolysaccharides, probably by action of the active anti-inflammatory agents [28]. It is suggested that many species of this genus possess anti-inflammatory and analgesic activity in many animal models [32].

Recently, it was showed that 7-epiclusianone, a polyisoprenylated benzophenone naturally found in the fruit of *G. brasiliensi*s or isolated from *G. gardneriana*[35], presents several biological effects, such as antibacterial *in vivo* [36-38]. Volatile oils exhibit of *G. brasiliensis* presented biological activities such as antiviral, antibacterial, and anti-inflammatory properties [29]. The pharmacological study of polyisoprenylated benzophenones has been shown to be of interest due to the wide spectra of activities attributed to its derivates [9, 39].Considering the studies already described and the

polyisoprenylated benzophenones in *Garcinia* species, studies confirmed the probable anti-inflammatory activity of 7-epiclusianone[40].

The species *Rheedia longifolia* inhibits neutrophil accumulation in the pleural cavity of mice, which is indicative of its anti-inflammatory activity. In addition, the aqueous crude extract also shows antinociceptive activity similar to that of an opioid agonist [34]. The *Rheedia* genus is characterized by the presence of triterpenes, steroids, coumaric acid, xanthones, and benzophenones and it is interesting that only the butanol and aqueous fractions inhibited inflammatory nociception, a characteristic of arylpropanoids that is not observed in the dichloromethane and ethyl acetate fractions. The arylpropanoids group may be responsible for the inhibition of neurogenic nociception [41].

3.4. Plants of genus Hypericum. Topical anti-inflammatory activity of the infusion, methanol extract and fractions of the aerial part in blossom of *H. canariense* L. and *H. glandulosum* Ait. in mice were verified in one study. It was observed that all extracts assayed, with the exception of the infusions of both species and the *H. canariense* aqueous fraction, showed a significant inhibition of the TPA-induced ear edema in a dose dependent manner as compared to control. The *H. canariense* methanol extract and *H. glandulosum* butanol fraction at 1mg/ear were the most effective [42].

The anti-inflammatory activity of the total ethanol extracts of *H. perforatum* and some other *Hypericum* species was by using the carrageenan-induced rat paw edema test. The results indicated that all examined extracts*(H. androsaemum, H. hirsutum, H. richeri, H. perforatum* and *H. barbatum*) possessed anti-inflammatory activity, especially the dry extracts of *H. hirsutum* and *H. perforatum*[43]. For the species *H. empetrifolium*, themethanolic extract administered showed a significant anti-edemic effect on carrageenan-induced paw edema in ratsfrom the first hour until the third hour, when the inhibitory effect was greatest[44].

Other member of this genus, *H. rumeliacum* Boiss. subsp.apollinis (Boiss. & Heldr.) Robson & Strid, presented anti-inflammatory activity of the methanol extract in the experimental model of only at a dose of 70 mg/kg. The effect was significant from first to third hour [45].

The effect of an acute administration of *H. perforatum* was verified on carrageenan-induced paw edema. As results, *H. perforatum* dose dependently inhibited

the carrageenan-induced inflammatory edema with maximal effect 1 h after carrageenan injection[46]. Other study for the same species demonstrated that treatment with *H. perforatum* extracts for treatment of active inflammatory periodontal disease, also was demonstrated that *Hypericum* exerts a significant inhibitory effect on plasma extravasation and reduced the degree of bone resorption during periodontitis [47]. Zdunic et al. (2009) investigated the anti-inflammatory activity in rats by administration of *H. perforatum* oil extracts on carrageenan-induced rat paw edema with significantly inhibition by all three tested oil extracts [48]. More recently, Süntar et al. (2010) evaluated on acetic acid-induced increase in capillary permeability of the samples of *H. perforatum* in mice. A dose-dependent inhibitory activity was observed for ethanolic extract up to the dose of 200 mg/kg with the highest inhibitory value of 40.9%, also was exerted by extract and its fractions significant and dose-dependent anti-inflammatory activity [49].

A single study showed the anti-inflammatory activity of the total extract of *H. triquetrifolium* Turra., evaluated by the carrageenan-induced paw edema test in the rat and was able for to inhibit paw swelling dose-dependently after carrageenan injection [50].

Topical anti-inflammatory activity obtained from TPA-induced mice ear edema test was demonstrated for the infusion, methanol extract and different fractions from *H.reflexum*. It was observed that all extracts assayed, with the exception of the infusion and the aqueous fraction, showed a significant inhibition of ear edema in a dose-dependent manner.[51].

These results show that ten studies revealed a decrease in inflammation of species of genus *Hypericum*. Oil extract of *H. perforatum* showed the highest activity probably due to the greatest amount of quercetin and I3,II8-biapigenin, both compounds administered showed anti-inflammatory activity[48]. Moreover, studies demonstrated that quercetin produced an anti-inflammatory effect on the acute inflammatory [52, 53]. More recently, it was suggested the anti-inflammatory effect of *H. perforatum* could interfere with the actions of histamine, serotonin, or kinins and to reduce cells infiltration, mediated by down-regulating adhesion molecules ICAM-1 and P-selectin [54]. Menegazzi et al. (2006) showed that the activity anti-inflammatory of *H. perforatum*might be due to the inhibition of nuclear factor-kappa B and STAT-3

activation [55]. Study *in vitro* evidenced that the flavonoids, such as quercetin, along with pseudohypericin and hyperforin might be the major anti-inflammatory components this species [56], able to inhibit the productionof pro-inflammatory mediators such as prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10).

These researches are according to an investigation of the effect of *H. perforatum* on the NF-κB inflammation factor, conducted by Bork et al.(1999), in which hyperforin provided a potent inhibition of TNFα-induced activation of NF-κB [57].Other important activity for hyperforin is a dual inhibitor of cyclooxygenase-1 and5-lipoxygenase[58]. Moreover, this species attenuated the expression of iNOS in periodontal tissue, which may contribute to the attenuation of the formation of nitrotyrosine, an indication of nitrosative stress [47]. In this context, a combination of several active constituents of *Hypericum* species is the carrier of their anti-inflammatory activity.

The topical treatment *H. canariense* and *H. glandulosum* inhibited TPA-induced ear edema in mice, indicating the presence of active substances endowed with anti-inflammatory activity. The active principle responsible for the anti-inflammatory-like effects of these species is/are, so far, not known, but preliminary phytochemical analysis carried out with the methanol extract of both species revealed the presence of flavonoids, tannins and anthraquinones [59]. In the same topical model of inflammation, *H. reflexum* inhibited the edema probably by the presence of tannins, flavonoids, saponins and anthraquinonesin this species, reported in previous studies [51, 52, 60, 61].

A single study showed the anti-inflammatory effects of *H. barbatum, H. androsaemum, H. richerii, H. hirsutum, H. perforatum* produced significant dose-dependent anti-inflammatory effect which was not correlated with the hypericin content in these extracts. It suggests the involvement of other active substances, besides hypericin, in the anti-inflammatory effect of *Hypericum* species tested [43]. *H. rumeliacum* subsp. apollinis methanol extract administration inhibited the paw edema, and reduced the infiltrates, both between connective fibres and into intercellular spaces [45].

The *H. empetrifolium* Willd. was also reviewed in this study due to significant results in experimental model of inflammation. It has been mentioned above that acute inflammation caused by carrageenan is characterized by a biphasic event and various mediators such as histamine, serotonin, bradykinin and substance P release, and later by

infiltration of PMN cells at the site of inflammation which induces secretion of various pro-inflammatory mediators such as nitric oxide, prostaglandins and cytokines[21, 62]. Within this context, the anti-inflammatory action of *H. empetrifolium* may be related to the inhibition of prostaglandin synthesis.

Some of the studies had insufficiently described methods for detecting this effect or scantily reported the results. The findings include a decrease of total leukocytes, number of neutrophils, total protein concentration, granuloma formation, paw or ear edema formation. Other interesting findings included decreased of the MPO activity, inflammatory mediators such as NF-κB and iNOS expression, PGE_2 and Il-1β levels and a decrease in chronic inflammation.

Conclusion

Taking all results collectively, plants of Clusiaceae family were found to have acceptable anti-inflammatory profiles. The isolation and purification of the chemical constituents from these plants and subsequent evaluation of their pharmacologic effects contributes its anti-inflammatory effect understanding. The data reported suggests the therapeutic potential of this family for the development of herbal medicine. Therefore, this family should attract the interest of researchers for clinical and toxicological studies, as well as for the herbal pharmaceutical industry.

Conflict of interests

The authors declare that they have no competing interest

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ARTIGO 2: **Involvement of cerebral nervous system areas and cytokines on antihypernociceptive and anti-inflammatory activities of *Kielmeyera rugosa* Choisy (Calophyllaceae) in rodents**

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Abstract:

***Kielmeyera rugosa* is a medicinal plant known in Northeastern Brazil as "pau-santo" and is used in the treatment of several tropical diseases such as malaria, schistosomiasis and leishmaniasis. We evaluated antihypernociceptive and anti-inflammatory activities of methanol stem extract of *K. rugosa* (MEKR). The mechanical hypernociception induced by carrageenan and tumor necrosis factor-alpha (TNF-α), prostaglandin E_2 (PGE_2) and dopamine were assessed. We also investigated the anti-inflammatory effect of MEKR on carrageenan-induced pleurisy and mouse paw edema. Ninety minutes after the treatment, the animals were submitted to an imunofluorescence for Fos protein. MEKR (100, 200 and 400 mg/kg; p.o.) inhibited the development of mechanical hypernociception and edema. MEKR significantly decreased TNF-α and interleukin 1β (IL-1β) levels in pleural lavage and suppressed the recruitment of leukocytes. MEKR (1, 10 and 100 mg/mL) did not produce cytotoxicity, determined using the MTT assay in vitro. The locomotor activity was not affected. MEKR activated significantly the bulb olfactory, piriform cortex and periaqueductal gray of the CNS. Our results provide first-time evidence to propose that MEKR attenuates mechanical hypernociception and inflammation, in part, through an activation of CNS areas, mainly the periaqueductal gray and piriform cortex areas.**

Keywords: Kielmeyera rugosa, Calophyllaceae, hypernociception, pain, inflammation, fos.

INTRODUCTION

Pain is the most common reason why individuals seek medical attention, yet the pain sensation is highly necessary to protect the organism from potentially tissue-damaging stimuli. Moreover, pain is one of the classic signs of the inflammatory process, whose treatment represents a major problem due to the use of availablemedications and their side-effect (da Silva *et al.*, 2012; McCurdy and Scully, 2005).

Inflammatory hyperalgesia, commonly associated with hypernociception in animals, is an increased response to a stimulus which is normally painful. (Cunha *et al*., 2008b; Verri *et al.*, 2006). For this, compounds derived from natural products have been utilized since the beginning of time for the treatment of inflammatory pain, as achallenge to reduce side-effects of pain medications currently used (McCurdy and Scully, 2005).

Natural products have been important in the development of modern analgesics. Henceforth, the discovery of new analgesics may also be derived from recent work carried out with plant extracts and compounds (Balunas and Kinghorn, 2005). The genus *Kielmeyera*, family Calophyllaceae, is present in the vast majorityof the 47 species occurring exclusively in Brazil (Pinheiro *et al.*, 2003; Sela *et al.*, 2010). Some species, such as *Kielmeyera coriacea*, are popularly known in Brazil as “pau-santo”, used by the native population of Brazil in the treatment of several tropical diseases such as malaria, schistosomiasis, leishmaniasis and fungal or bacterial infections (Audi *et al.*, 2002).

Considering that this genus is explored in chemical studies of natural products due to its potential use in phytochemical and pharmacological products (Pinheiro *et al.*, 2003), the species *Kielmeyera rugosa* Choisy has been the focus of biological evaluation. Xanthones, besides 4-alkyl and 4-phenylcoumarins, are important chemotaxonomic markers within the genus *Kielmeyera*, in a specific study on the composition parts of *K. rugosa* (Nogueira *et al.*, 2008*)*. Recently, it was demonstrated that the stem extract of *K. rugosa* possesses significant antitumoral activity (Ribeiro *et al.*, 2012). It has also been observed in this genus that microinjections of xanthone from dichloromethane fraction of *K. coriacea* stems in the intra-median raphe nucleus reduce immobility time in the forced swimming test model; it also has an antidepressant effect on rats submitted to the test (Sela *et al.*, 2010). In recent research, extracts from stems of *K. rugosa* showed positive result in a cytotoxic screening and antitumor activity on sarcoma 180 (Oliveira *et al.*, 2013).

In this context, the aim of this study was to investigate the effect of methanol stem extractof *K. rugosa* (MEKR) on inflammatory hypernociception induced bycarrageenan (CG), tumor necrosis factor-alpha (TNF-α),prostaglandin E_2 (PGE_2), and dopamine. In addition, the current studywas designed toclarify the characteristics of pain-associatedneuronal activities by immunofluorescence localization of the c-Fos protein on central nervous system areas.

MATERIALS AND METHODS

Plant material and preparation of extract. Stems of *K. rugosa* were collected in May 2010 from a ‘‘restinga’’ (the vegetation mosaic found on Brazilian coastal sandy plains) near the Pomonga River in the Municipality of Santo Amaro das Brotas, Sergipe State,

Brazil. The species was identified by Dr. Volker Bittrich and Dr. Maria C. E. Amaral, plant taxonomists of the Institute of Biology at the State University of Campinas (UNICAMP). A voucher was registered under the code 206.

The methanolic extract of *K. rugosa* was obtained according to what has been described(Nogueira *et al.*, 2008). The stems (397.4 g) of *K. rugosa* were extracted at room temperature with methanol. The solvent was removed under reduced pressure to give the correspondent crude extract (20.9 g).

LC-grade methanol (Tedia, Fairfield, OH, USA) and formic acid (JT Baker, Philipsburg, PA, USA) were used for HPLC analysis. Deionised water was purified by a Milli-Q system (Millipore, São Paulo, SP, Brazil). All the solvents were filtered through nylon 0.45 μm membranes (MFS) and degassed by ultrasonic bath before use.

High-performance liquid chromatography–photodiode-array detection analysis. HPLC analysis was performed using a Shimadzu-model (Kyoto, Japan)Prominence Liquid Chromatograph equipped with a vacuum degasser (DGU-20A3), autosampler (SIL-10A), two high-pressure pumps (LC-6A) and a SPDM20Avp photodiode array detector (DAD) system coupled with a CBM 20A interface. Data collection was carried out using LC Solution software. Analysis was performed in an analytical PhenomenexKinetex^{TM} C18 column (250 x 4.6 mm i.d., 5 μm of particle diameter, Torrance, CA, USA) with a C18 guard column (4 x 3 mm, 4μm, Phenomenex, Torrance, CA, USA) under the following conditions: flow rate at 0.8 mL/min, injection volume of 20 μL and a mobile phase consisting of 0.1% aqueous formic acid (v/v, A) and methanol (B). The gradient elution for MEKR sample was as follows: 10-30% (B) in 12 min, 30-100% (B) in 70 min, remaining at 100% (B) for 10 min. Photodiode array

detector was set at 254 nm for acquiring chromatogram and ultraviolet spectra were recorded between 200 and 500 nm. Identification was based on comparisons of UV absorption.

A sample of MEKR was dissolved in methanol at a concentration of 2.25 mg/ml, and then was submitted to filtration in a cellulose membrane (pore diameter of 0.45 μm) before HPLC injection.

Drugs and reagents. λ-Carrageenan, TNF-α, PGE_2, dopamine (DA), Tween 80, fluoromount G, glycine and bovine serum albumin (BSA) were purchased from Sigma (USA). Indomethacin and dipyrone were obtained from UniãoQuímica(São Paulo, Brazil). Diazepam (DZP) was purchased from Cristália (Brazil). Rabbit anti-Fos and donkey anti-rabbit Alexa Fluor 594 was obtained fromSanta Cruz Biotechnology (USA).The extract was freshly prepared with 0.9% saline and Tween 80 0.02%(vehicle)for pharmacological experiments. The other substances were solubilized withdistilled water or saline.

Animals. Adult (3-month-old) male albino *Swiss* mice (28-32g) were randomly housed in appropriate cages at 21 ± 2°C on a 12 h light/dark cycle (lights on from 06:00 a.m. to 6:00 p.m.), with free access to food (Purina®, Brazil) and water. All experiments were carried out between 09:00 a.m. and 14:00 p.m. in a quiet room. All hypernociception tests were carried out by the same visual blinded observer. Experimental protocols were approved by the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS 102/11) and handling procedures were inaccordance with the International

Association for the Studyof Pain guidelines for the use of animals in pain research (Zimmermann, 1983).

Hypernociception induced by CG, TNF-a, PGE_2 and dopamine. Mouse paw hypernociception was performed as previously described (Cunha *et al.*, 2004; Vivancos *et al.*, 2004). The mice were divided into five groups (n = 6, per group), which were treated with vehicle (saline + Tween 80 0.02%, v/v, p.o.), MEKR (100, 200 or 400 mg/kg, p.o.), indomethacin (10 mg/kg, i.p.) or dipyrone (60 mg/kg, i.p.). Thirty minutes after treatment, 20 µL of CG (300 µg/paw), PGE_2(100 ng/paw), DA (30 µg/paw) or TNF-α (100 pg/paw) were injected subcutaneously into the subplantar region of the hind paw. The degree of hypernociception was evaluated at 0.5, 1, 2 and 3 h after the injection of hypernociceptive agents.

Measurement of mechanical hypernociception. Mechanical hypernociception was tested in mice as reported by Cunha *et al.* (2004) (Cunha *et al.*, 2004). In a quiet room, the mice were placed in acrylic cages (12 x 10 x 17 cm) with wire grid floors for 15–30 min. before starting the test. This method consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight®, RibeirãoPreto, São Paulo, Brazil) adapted with a polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the withdrawal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was automatically recorded. The intensity of stimulus was obtained by averaging four measurements taken with minimal intervals of 3 min. The animals were tested before

and after treatments.The results are presented as the Δ withdrawal threshold (g), calculated by the difference between the values obtained after the treatments and before the treatment.

Carrageenan-induced pleurisy. Pleurisy was induced by intrathoracic (i.t.) injection of CG (300 μg; 0.1 mL) diluted in sterile saline. Controlanimals received the same volume of vehicle. The animalswere pretreated with MEKR (100, 200 and 400 mg/kg; p.o.) orvehicle (saline + Tween 0.02% v/v; p.o.) 30 min beforeinjection of the inflammatory agent. Four hours after stimulation,the animals were sacrificed in a CO_2 chamber; thepleural cavities were opened and washed with 1 mL of PBS(1×) containing EDTA (10 mM). Total counts of leukocytecollected in the pleural lavage were performed on a Neubauerchamber under an optical microscope. The sampleswere diluted (40×) in Türk solution. The differential leukocyteanalysis was performed under a light microscope with immersion oil objective in cytocentrifuged smears colored with May–Grunwald–Giemsa, where 100 cells per slidewere counted.The amount of TNF-α and IL-1β produced in the pleural cavity were assessed 4 h after injection of CG. The recoveredpleural lavage was centrifuged at 770×g for 10 min. TNF-α and IL-1β were quantified on supernatant free of cells by enzyme immunoassay(ELISA) following the manufacturer's protocol(BD-Bioscience Pharmingen).

Measurement of paw edema. The effect of MEKR on edema formation caused by theintraplantar injection of CG was analyzed according to themethod previously reported (Levy, 1969). A separate group of mice was divided into five groups (n=6, pergroup) that were treated with vehicle (saline + Tween 80 0.02%; p.o.), MEKR (100,

200 and 400 mg/kg; p.o.), or indomethacin (10 mg/kg; i.p.). Right paw volume was measured by the dislocation of the water column of a plethysmometer (Insight®, Brazil) before (time zero) and at 1,2, 3, 4, 5 and 6 h after subplantar injection of 50 μL of CG(1%). Paw edema was expressed (in milliliter)as the difference between the volume of the paw afterand before CG injection. The area under the curve(AUC[0–240 min]; in milliliter per minute) was also calculated using the trapezoidal rule.

MTT cell viability assay. The cytotoxic effect of MEKR on macrophages was determinedusing the MTTassay method according to Mosmann(1983)(Mosmann, 1983). Murine peritoneal macrophages (2.5×105 cells)were treated with MEKR at concentrations ranging from 1 mg/mL to 1000 mg/mL and were later culturedin RPMI-1640 supplemented with 10% FBS for 24 h.Thereafter, the medium was replaced with fresh RPMI containing5 mg/mL of MTT. After additional 4 h of incubationat 37°C, the supernatant was discharged and DMSOsolution (150 μL/well) was added to each culture plate. After15 min of incubation at room temperature, absorbance ofsolubilized MTT formazan product was spectrophotometricallymeasured at 540 nm. Five individual wells were assayedper treatment and percentage of viability was determined inrelation to controls [(absorbance of treated cells/absorbance ofuntreated cells)×100].

Evaluation of the motor activity. In order to investigate the motor activity of the animals treated with MEKR and the consequent impairment of the mechanical hypernociceptionassessment, the motor activity of the animals was evaluated in rota-rod apparatus, according to Dunham and Miya (1957) (Dunham and Miya, 1957)with some modifications. Initially, the mice able to remain on the Rota-rod apparatus (AVS®,

Brazil) longer than 180 s (7 rpm) were selected 24 h before the test. Thirty minutes after the administration of either MEKR (100, 200 and 400mg/kg,p.o.), vehicle (saline + Tween 80 0.02%; p.o.) or diazepam (DZP, 1.5 mg/kg, i.p.), each animal was tested on the Rota-rod apparatus and the time (s) remained on the bar for up to 180 s was recorded at 0.5, 1 and 2 after the administration.

Immunofluorescence. To evaluate the action of the test drug on the central nervous system, ninety minutes after the injection of MEKR (100, 200, or 400 mg/kg; p.o.) or vehicle (Saline + Tween 80 0.02%, p.o.), the animals (n=6, per group) were perfused and the brains were collected and cryoprotectedfor immunofluorescence processing to Fos protein.

Frozen serial transverse sections (20 μm)of all brain were collected on gelatinized glass slides. The tissue sections were stored at –80°C until use. The sections were washed with phosphate buffer (0.01M) saline isotonic (PBS) 5 times for 5 minutes and incubated with 0.1 M glycine in PBS for 10 minutes. Non-specific protein binding was blocked by incubation of the sections for 30 minutes in a solution containing 2% BSA. After that, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (1:2000). Afterwards, the sections were incubated for two hours with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (1:2000). The cover slip was mounted with Fluoromount G. As an immunofluorescence control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS 5 times for 5 minutes.

A striking attribute of Fos is that it israpidly expressed in central neurons after noxious stimuli. As that is a very used way to visualizethe pathways involved in the

integration of noxious input. For this reason, we evaluated the action of the test drug on the central nervous system, ninety minutes after the injection of MEKR (100, 200, or 400 mg/kg; p.o.) or vehicle (Saline + Tween 80 0.02%; p.o.), after injection CG (300 µg/paw), sixty minutes after the treatment.

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

Statistical analysis. Data were evaluated using GraphPad Prism Software Inc. (SanDiego, California, USA) version 5.0, through the analysis of variance(ANOVA) followed by Tukey's test. The results are presented asmean ± SEM. In all cases, the differences were considered significantif $p < 0.05$.

RESULTS

High-performance liquid chromatography–photodiode-array detection analysis. HPLC-DAD analysis of the methanol extract from stems of *K. rugosa* (MEKR) (Fig. 1) revealed the presence of peaks with phenolic compounds-like UV spectra. Based on elution order in C18 column and their UV absorption spectra, peaks 1-3 (*Rt*=11.0 min, *Rt*=21.0 min, and *Rt*=40.8min, respectively) were similar to hydroxybenzoic acids such as vanillic acid, protocatechuic acid and syringic acid, respectively (Sun *et al.*, 2007).

Peaks 4-8 (*Rt*=52.8 min, *Rt*=68.1 min, *Rt*=76.2 min, *Rt*=79.1 min, and *Rt*=81.0 min, respectively) presented UV spectra similar to the one observed for alkyl and phenylcoumarins (Garazd *et al.*, 2003; Scio *et al.*, 2003). Peak 4 (*Rt*=52.8 min)was identified by matching the retention time with that of an authentic sample of the disprorinol A, a 4-propylcoumarin previously isolated from *K. rugosa* (Nogueira *et al.*, 2008).

INSERT FIGURE 1

Effect of MEKR on CG-induced mechanical hypernociception and mouse paw edema. Injection of CG in the subplantar region of the mouse paw induced a marked mechanical hypernociception characterized by an increased sensitivity as the intensity of stimulus was decreased, which remained throughout the 3 h. MEKR demonstrated an anti-hypernociceptive effect in this model, as mice treated with MEKR (100, 200 or 400 mg/kg; p.o.) 0.5 h before CG administration exhibited a significant reduction in mechanical hypernociception induced by CG at all evaluated times, when compared to animals of the control group that received only vehicle (Fig. 2A). These doses produced an effect similar to indomethacin (10 mg/kg). The group of animals that received saline in the subplantar region, instead of CG, did not present any alteration on the threshold of sensitivity to mechanical stimuli (data not shown).

INSERT FIGURE 2

Mouse paw edema induced by CG administration was also evaluated. As shown in Fig. 3, CG injection increased the mouse paw volume from 1 to 6 h after injection, and the treatment of mice with MEKR significantly decreased the edema. The doses of 100, 200 and 400 mg/kg of MEKR were able to maintain the reduction of the edema during the 6-h evaluation period, as did indomethacin. Animals that received only the vehicle of carrageenan (sterile saline) did not present significant alteration in paw volume (data not shown).

INSERT FIGURE 3

Effect of MEKR on TNF-α, dopamine, or PGE_2-induced mechanical hypernociception. The inhibitory effect of MEKR on the mechanical hypernociceptioninduced by TNF-α is shown in Fig. 2B. MEKR (100, 200 and 400 mg/kg, p.o.) was able to reduce mechanical hypernociceptioninduced by TNF-α, when compared to animalsof the vehicle group, similarly to indomethacin.

The MEKR antihypernociceptive effects on PGE_2- and dopamine-induced hypernociception are shown in Fig. 2C and 2D, respectively. Acute treatment with MEKR (100, 200 and 400 mg/kg) can reduce the mechanical hypernociception induced by PGE_2 and dopamine when compared to vehicle group animals.

Effect of MEKR on CG-induced mouse pleurisy. All doses of MEKR were able to significantly suppress the recruitment of leukocytes to the mouse pleural cavity, asshown in Fig. 4A. Pretreatment with MEKR also significantlyattenuated the number of neutrophils (Fig. 4B). MEKR (100, 200 and 400 mg/kg) also significantly reduced

the TNF-α (Fig. 4C) and IL-1β (Fig. 4D) levels in the pleural exudates collected at 4 h after carrageenan injection.

INSERT FIGURE 4

Lack of cytotoxicity effect of MEKR. Increasing concentrations of MEKR (1, 10, 100, 500 and 1000 mg/mL) were unable to cause alteration of murine peritoneal macrophages viability of RPMI control 1 – 100 mg/mL), indicating that MEKR treatment did not significantly affect mitochondrial reduction of MTT to formazan resulting in an undetectable cytotoxic effect, in these concentrations.

INSERT FIGURE 5

Lack of effect of MEKR on motor activity. Figure 6 shows the motor activity of mice treated with different doses of MEKR. In this test, MEKR, in all doses, was unable to cause a significant decrease of ambulation (number of crossings) at 0.5, 1, and 2 h after administration, unlike DZP.

INSERT FIGURE 6

Effect of MEKR on Immunofluorescence. In the olfactory bulb ($p < 0.01$), piriform cortex (100, 400 mg/kg , $p < 0.01$; 200 mg/kg, $p < 0.05$) and in the periaqueductal gray ($p < 0.01$)of the animals brains, the average number of neurons showing Fos protein

was significantly increased by an oral injection of MEKR when compared to control (Figures 7 and 8).

FIGURE 7 AND 8

DISCUSSION

Since the chemical profile of this genus is characterized by the occurrence of phenolic compounds such as xanthones, 4-phenyl and 4-alkylcoumarins (Cruz *et al.*, 2002; Cruz *et al.*, 2001; Garcia Cortez *et al.*, 1998; Nogueira *et al*., 2008; Scio *et al.*, 2003) and based on the results by HPLC-DAD analysis, we suggest that these compounds could be present in the methanolic extract from stems of *K. rugosa*. Further investigations by LC/MS on the chemical composition of *K. rugosa* are ongoing.

The inflammatory hypernociception in mice is mediated by a cascade of cytokines (Cunha *et al.*, 2005). CG induces a concomitant release of TNF-α, which stimulates the subsequent release of interleukin (IL)-6/IL-1β and keratinocyte-derived chemokine (KC/CXCL1), which ultimately induce the synthesis of prostaglandins and the release of sympathetic amines, respectively (Cunha and Ferreira, 2003; Cunha *et al.*, 1992), causing the activation of fiber sensory nerve endings, types Aδ and C, increasing the local flow and vascular permeability by therelease of neurokinin substance P and neurokinin A (Cunha *et al.*, 2005; Nakamura and Ferreira, 1987). This can lead to the inflammatory process resulting in central and peripheral hyperalgesia.

The cytokine cascade begins with TNF-α, whichstimulates two distinct pathways, as previously mentioned: IL-1β, which in turn activates cyclooxygenase to produce prostanoids, and KC/CXCL1 production, which stimulates the release of sympathetic amines (Verri *et al.*, 2006). Prostanoids and sympathetic amines are ultimately responsible for nociceptor sensitization (Cunha *et al.*, 2005). On the basis of these findings, we investigated the possible effect of MEKR on the hypernociception induced by PGE_2 and dopamine.

Studies have shown that the peripheral injection of PGE_2 and sympathomimetic amines, such as dopamine, triggers the activation of nociceptors and transmission impulse by primary nociceptive neurons (de Oliveira *et al.*, 2012; Guimaraes *et al.*, 2012). That effect induces both allodynia and hyperalgesia in response to mechanical stimulation (Ferreira, 1972; Kuhn and Willis, 1973). This nociceptive effect seems to be related to the ability of PGE_2 to sensitize peripheral terminals of small diameter and high threshold, including primary afferent fibers sensitive to thermal, chemical and mechanical stimuli (Kumazawa *et al.*, 1993; Mizumura *et al.*, 1993).

PGE_2 acts on the EP_2 receptors and dopamine acts on the metabotropic-type D_1 receptors. The nociceptive behavior and mechanical allodynia caused by i.pl. PGE_2 are mediated through activation of distinct EP receptors and PK-dependent mechanisms (Kassuya *et al.*, 2007). Therefore, hypernociception elicited by $PGE_2$and dopamine is independent on the production of other inflammatory mediators or recruitment of cellssuch as neutrophils (Cunha *et al.*, 2008a). The fact that MEKR treatment also inhibited dopamine and PGE_2-induced hypernociception implies that either MEKR directly reduces nociceptor sensitization or MEKR can even inducean endogenous mediator through this action, or we cannot exclude the possibility that MEKR interacts even with other types of EP or dopamine receptors.

The injection of carrageenan in mice produces a typical biphasic edema associated with the production of several inflammatory mediators, such as bradykinin, prostaglandins, nitric oxide and cytokines (Henriques *et al.*, 1987; Posadas *et al.*, 2004). It is well accepted that cytokines constitute a link between cellular injuries or immunological recognition and the local or systemic signs of inflammation, e.g. cell migration, edema, fever and hyperalgesia (Dinarello, 2000; Faccioli *et al.*, 1990;

Ferreira *et al.*, 1988). Different cell types, including macrophages, monocytes and glial cells produce IL-1β, which in turn induces the production of other inflammatory mediators involved with cellular recruitment, fever, acute phase protein release and increase of vascular permeability (Dinarello, 1998). We have shown that the doses of 100, 200 and 400 mg/kg of MEKR induced antiedematogenic activity.

The mediators involved in the genesis of inflammatory pain also play an essential role in triggering other inflammatory events, including edema and leukocyte migration (Cunha *et al.*, 2008b). Therefore, the production of cytokines, including TNF-α and IL-1β, in the site of inflammation is essential for the development of inflammatory hypernociception. For this reason, we performed a cell migration assay and measurement of IL-1β and TNF-α by carrageenan-induced pleurisy. Inflammation induced by carrageenan involves cell migration, exudation of plasma and production of mediators such as nitric oxide, prostaglandin E_2, IL-1β, IL-6 and TNF-α (Cunha *et al.*, 2005; Ferreira *et al.*, 1993). These mediators are capableof recruiting leucocytes, such as neutrophils, in various experimentalmodels. Theresults allowed us to detect a marked inhibitory effect of MEKR on neutrophil migration, besides a reduction of TNF-α and IL-1β level in the pleural exudate.

Evidences suggests the role of colorimetric assays using the MTT (methyl-thiazolyl-tetrazolium) for assessment of cytotoxicity, and proliferation studies in cell biology (Berridge *et al.*, 2005; van Meerloo *et al.*, 2011). The concentrations used of MEKR (1–100 mg/mL)did not affect the MTT reduction in murine peritoneal macrophages, indicating a cell viability effect of this extract.

The fact that MEKR induces antihypernociceptive effect in the mechanical hypernociception models suggests that MEKR can block the neural transmission of

pain, like other drugs do, and may induce an analgesia. Moreover, it has been observed that many compounds derived from medicinal plants present a reduction of locomotor activity (Le Bars *et al.*, 2001) by aninhibitory effect on the CNS or by a non-specific musclerelaxation effect (Melo *et al.*, 2011). Thus, these activitiescan reduce the motor coordination response, invalidating thenociceptive behavioral tests (de Sousa *et al.*, 2006). However, relaxing or motor deficit effects were discarded, since MEKR administration, at the therapeutic doses, did not affect the motor performance of the mice, as tested in the rota-rod test.

The expression of immediate early genes, most notably c-fos, has been used to map activation of neural circuits under a variety of experimental conditions. c-Fos is expressed in a variety of brain sites, like in the areas involved in the pain modulation (Barr, 2011), being, the Fos protein, a marker useful for the control of neuronal activities in central pathways of the sensory system, particularly in the nociceptive pathway (Williams *et al.*, 1990). To demonstrate the influence of MEKR in the CNS areas, Fos protein labeled by immunofluorescence was performed, showing a significant activation of the olfactory bulb, piriform cortex and periaqueductal grey.

The piriform cortex (PC) is a three-layered structure in which the principal excitatory neurons are pyramidal cells. One attractive feature of the piriform cortical slice preparation is that functionally distinct inputs from the olfactory bulb (OB) via the lateral olfactory tract. The information arriving through theses distal synapses provides the vast majority of olfactory signals to the cortex that is presumably used for sensory tasks such as odor discrimination and recognition (Bathellier *et al.*, 2009; Suzuki and Bekkers, 2006). The PC, beyond of the olfactory function due to its connections with BO, presents an influence on the aggressive and mating behavior, once this area

receives information from the amygdala and hippocampus and projects their axons to amygdala and hypothalamus. These areas make connection with the brain stem, including the raphe and parabrachial nuclei as well as the periaqueductal gray (PAG), influencing the ascending and descending nociceptive circuits.

The periaqueductal grey (PAG), the most important area of descending pain pathway, is interconnected with the hypothalamus and limbic forebrain structures and also receives direct inputs of spinomesencephalic. The PAG projects to the rostral ventromedial medulla, which in turn sends its output to dorsal horn, inhibiting the I-laminae, an important dorsal horn area involved in the nociception (Heinricher *et al.*, 2009). Pain modulatory drugs such as opioids, serotoninergic and cannabinoids exert central effects in the PAG, and several lines of evidence indicate a central role for prostaglandins in this brain region, what indicates that PAG is an important area involved in the control of inflammatory pain (Breder *et al.*, 1995; Kraft, 2012; Leith *et al.*, 2007; Phillips and Clauw, 2011). Thus, the attenuation of the mechanical hypernociception observed in the present study may be derived from the activation of descending pain pathway and consequent inhibition of spinal cord I-laminae. That can be suggested by the significant activation of the PAG observed in the immunofluorescence protocol.

In summary, the activation of the olfactory bulb and piriform cortex indicates that MEKR influences on the animal behavior and the activation of the PAG suggests the involvement of the central nervous system, more specifically of the pain descending inhibitory pathway, in the action of MEKR on the inflammatory pain observed in the hypernociception and inflammation protocols used in this study.

The exact mechanism through which MEKR exerts its antihypernociception remains to be elucidated. Previously, *Kielmeyera* species showed mainly xanthones and 4-alkyl and 4-phenyl coumarins, which are regarded as the characteristic constituents of plants belonging to this genus. Taechowisan*et al.,* 2005 demonstrated the physiological roles of 5,7-dimethyloxy-4-*p*-methoxylphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin for the development of biologically active substances (Taechowisan *et al.,* 2005). Nevetheless, a previous chemical study of extracts of fruits, leaves and stems of *K. rugosa* were identified on the basis of their spectral data, chemical compounds found in *K. rugosa* resemble those that were previously isolated from the other species of *Kielmeyera,* such as xanthones besides 4-alkyl and 4-phenylcoumarins (Nogueira *et al.,* 2009).

Many coumarin derivatives have special ability to scavenge reactive oxygen species–free radicals and to influence processes involving free-radical injurythey have also been found to possess vasorelaxant(Hoult and Paya, 1996) and anti-inflammatory activity (Fylaktakidou *et al.,* 2004; Nicolaides *et al.,* 2004). Considering that Taechowisan*et al.,* 2006 observed that 5,7-dimethyloxy-4-*p*-methoxylphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin significantly reduced the formation of TNF-α, it is conceivable to suggest that these compounds are responsible for the pharmacological activity presented in this study (Taechowisan *et al.,* 2006).

The present study demonstrates, for the first time, that systemic administration of MEKR, at doses that did not induce any motorperformance alteration, produced consistent antihypernociceptive and anti-inflammatory effects in different models of hyperalgesia and inflammation, probably by interfering of CNS, through stimulation of areas that modulate pain perception or modulation, such as PAG. These effects seem to

be associated with the power of MEKR to inhibit the cytokine cascade generated by carrageenan and/or decrease the production of inflammatory mediators. However, further studies can clarify the exact mechanisms underlying the effects of MEKR.

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FIGURES

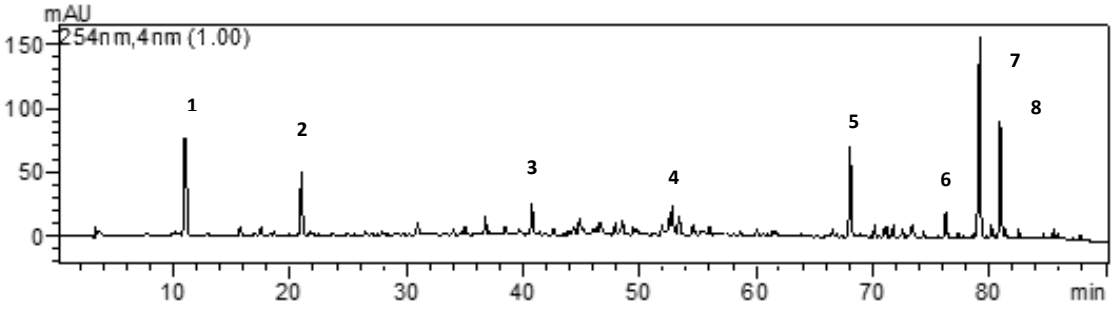

Figure 1. HPLC-DAD chromatogram at 254 nm of methanolic extract from *K. rugosa* stems.

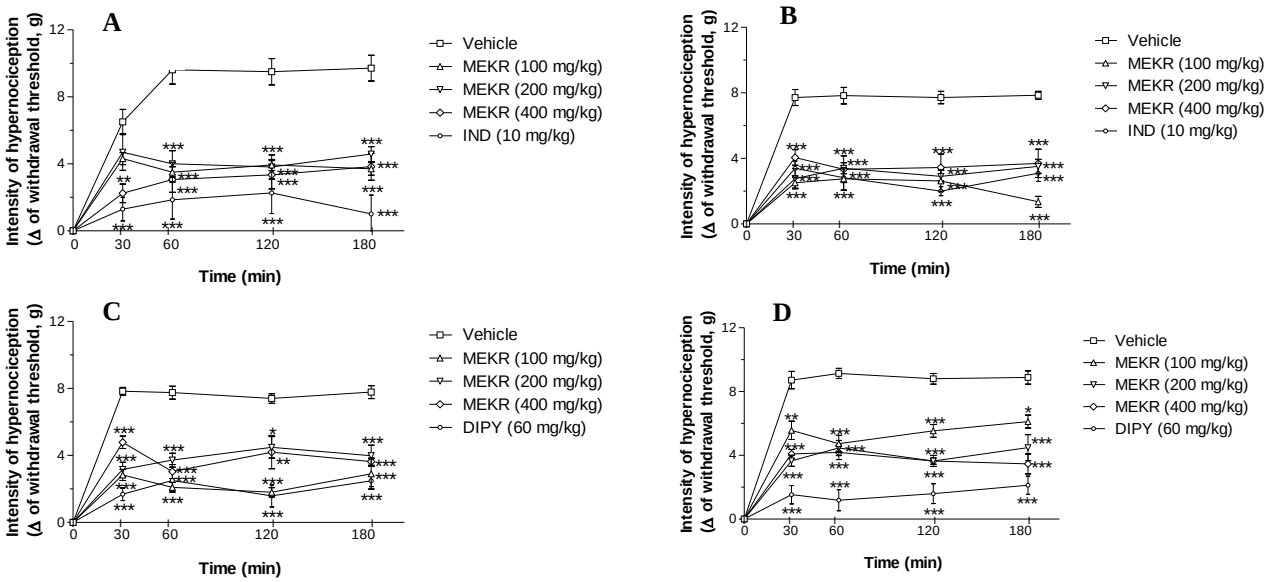

Figure 2. Effect of acute administration of vehicle, methanolic extract of stem of *Kielmeyera rugosa* (MEKR; 100, 200, or 400 mg/kg, p.o.), indomethacin (IND, 10 mg/kg, i.p.) or dipyrone (DIPY, 60 mg/kg, i.p.) on mechanical hypernociception induced by carrageenan (A), TNF-α (B), PGE_2 (C) or dopamine (D). Each point represents the mean ± S.E.M. of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hindpaw. *$p < 0.05$, **$p<0.01$ and ***$p < 0.001$ vs. control group (ANOVA followed by Tukey´s test).

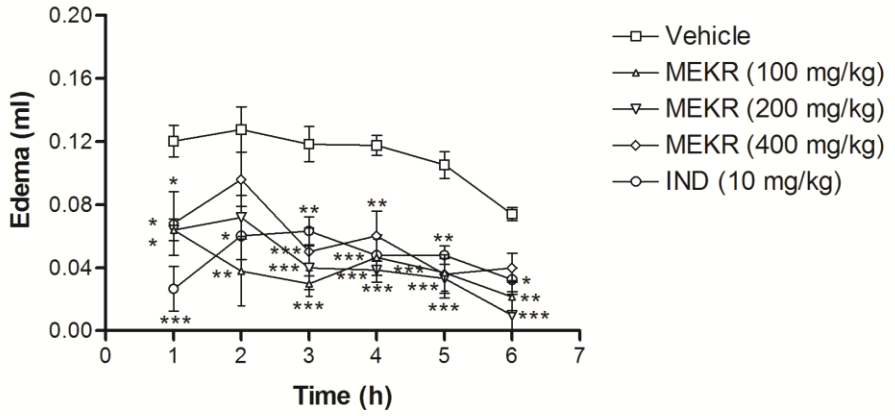

Figure 3. Anti-inflammatory effect of MEKR treatment on carrageenan-induced paw inflammation. Methanolic extract of stem of *Kielmeyera rugosa* (MEKR; 100, 200, or 400 mg/kg, p.o.), saline (control group, p.o.), or indomethacin (IND, 10 mg/kg,i.p.), was administered 6 h before carrageenan. Paw edema measured at 1, 2, 3, 4, 5 and 6 h after the carrageenan injection. Data are expressed as means±S.E.M; *p< 0.05; **p < 0.01; ***p< 0.001 compared to control group (ANOVA followed by Tukey´s test).

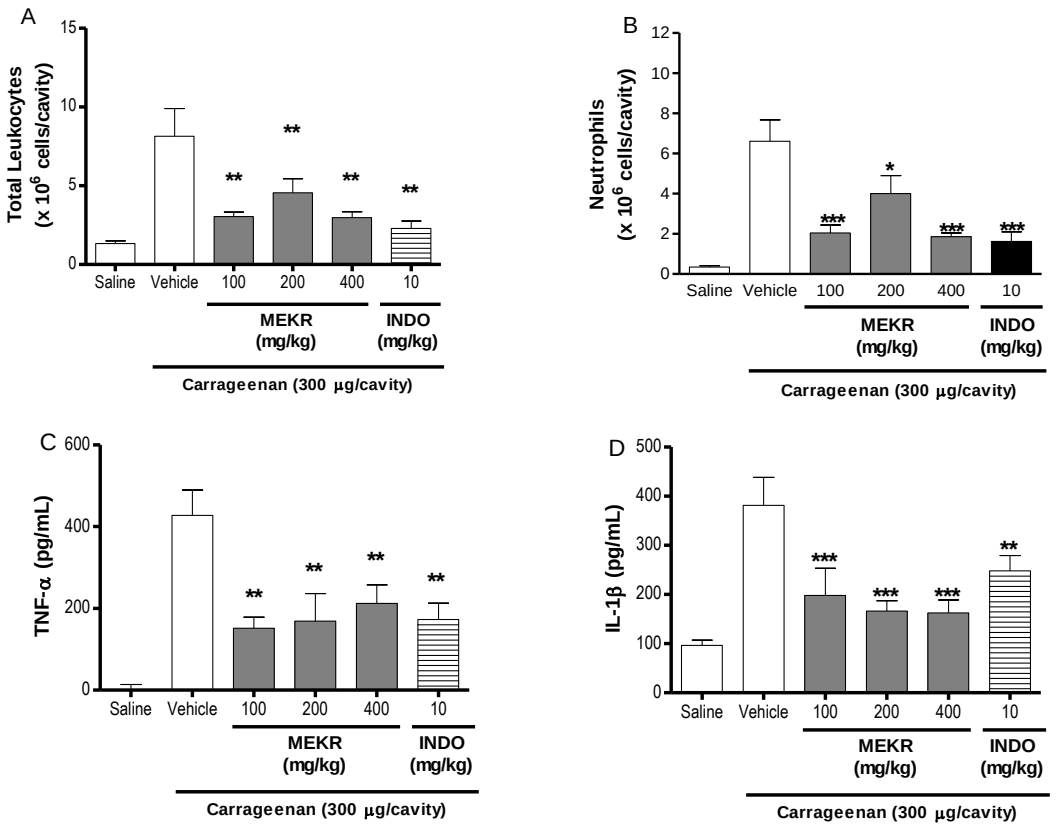

Figure 4. Effect of *Kielmeyera rugosa* (MEKR; 100, 200 and 400 mg/kg; p.o.) or indomethacin (IND, 10 mg/kg;i.p.) on the inflammation by carrageenan in mouse pleurisy. The analyses were performed 4 h after carrageenan injection (300 µg/cavity) to evaluate the recruitment of total leukocytes (A), neutrophils (B), and to assess tumor necrosis factor-alpha (TNF-α) (C) and interleukin-1β (IL-1β) levels (D). Data were expressed as mean±SEM, for a minimum of six animals. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$compared with the control group (vehicle) (ANOVA followed by Tukey´s test).

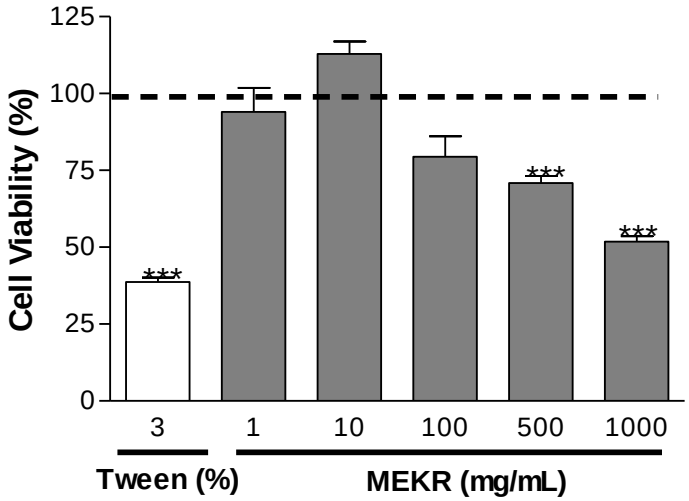

Figure 5. Effect of *Kielmeyera rugosa* (MEKR; 100, 200 and 400 mg/kg; p.o.) or indomethacin (IND, 10 mg/kg; i.p.) on the viability assay by the MTT method for previously MTT-treated cells *in vitro*. Measurementof formazan absorbance in relation to the experimental design, showing mean values±SEM at different times after the first MTT treatment; absorbance of solubilized MTT formazan product was spectrophotometrically measured at 540 nm. *** $p<0.001$ compared with the control group (ANOVA followed by Tukey´s test).

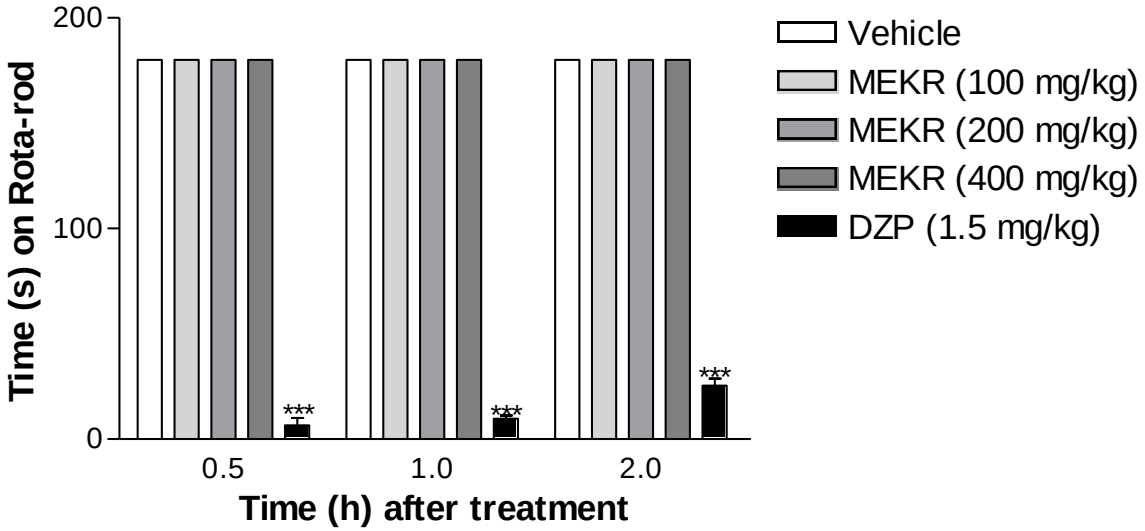

Figure 6. Time (s) on the rota rod observed in mice after i.p. treatment with vehicle (control), *Kielmeyera rugosa* (MEKR; 100, 200 and 400 mg/kg; p.o.) or diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 sec. after drug treatment. Statistical differences *vs* vehicle-treated mice group were calculated using ANOVA, followed by Tukey's test (n = 6, per group), ***$p < 0.001$.

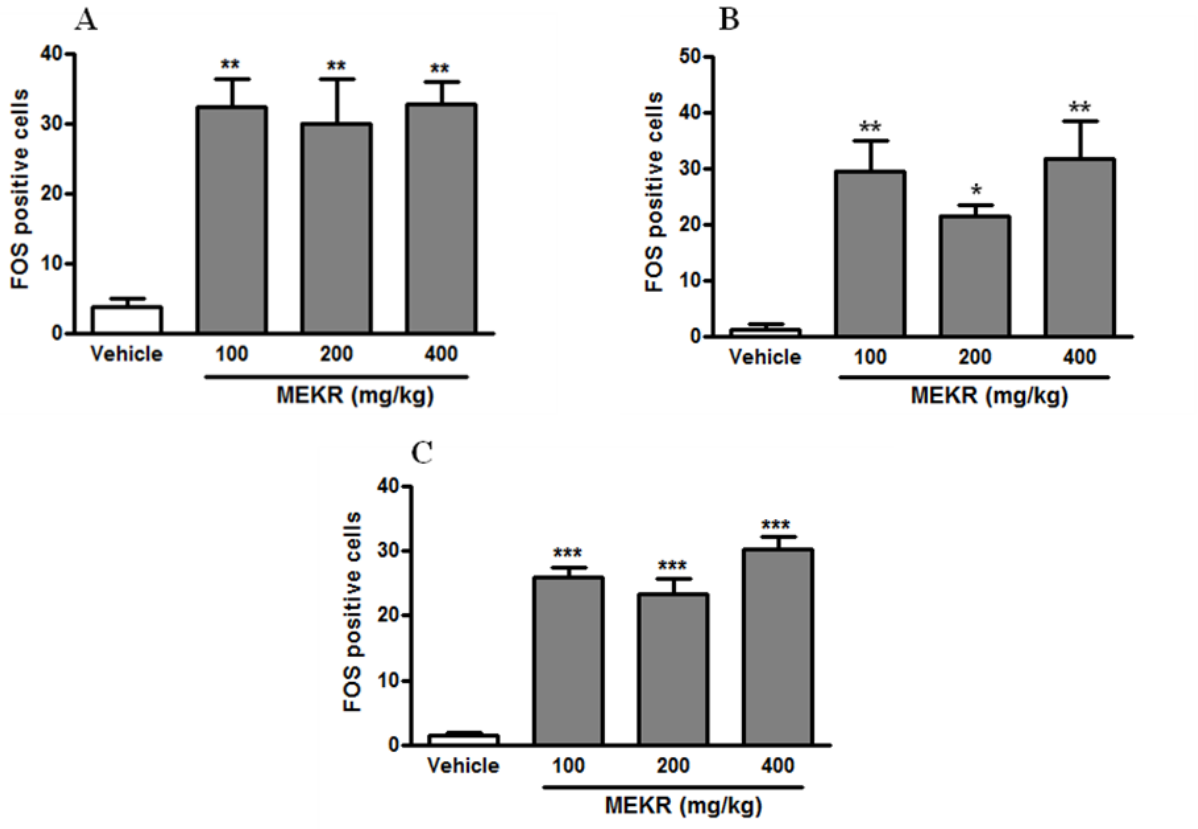

Figure 7. Neurons Fos positive in the bulb olfactory (A), piriform cortex (B) and periaqueductal gray (C). Vehicle (control) or *Kielmeyera rugosa* (MEKR; 100, 200 and 400 mg/kg, p.o.) were administered 1.5 hours before of the perfusion. Values represent in mean ± SEM (n=6, per group). * $p<0.05$, ** $p<0.01$ or *** $p<0.001$ versus control (one-way ANOVA followed by Tukey´s test).

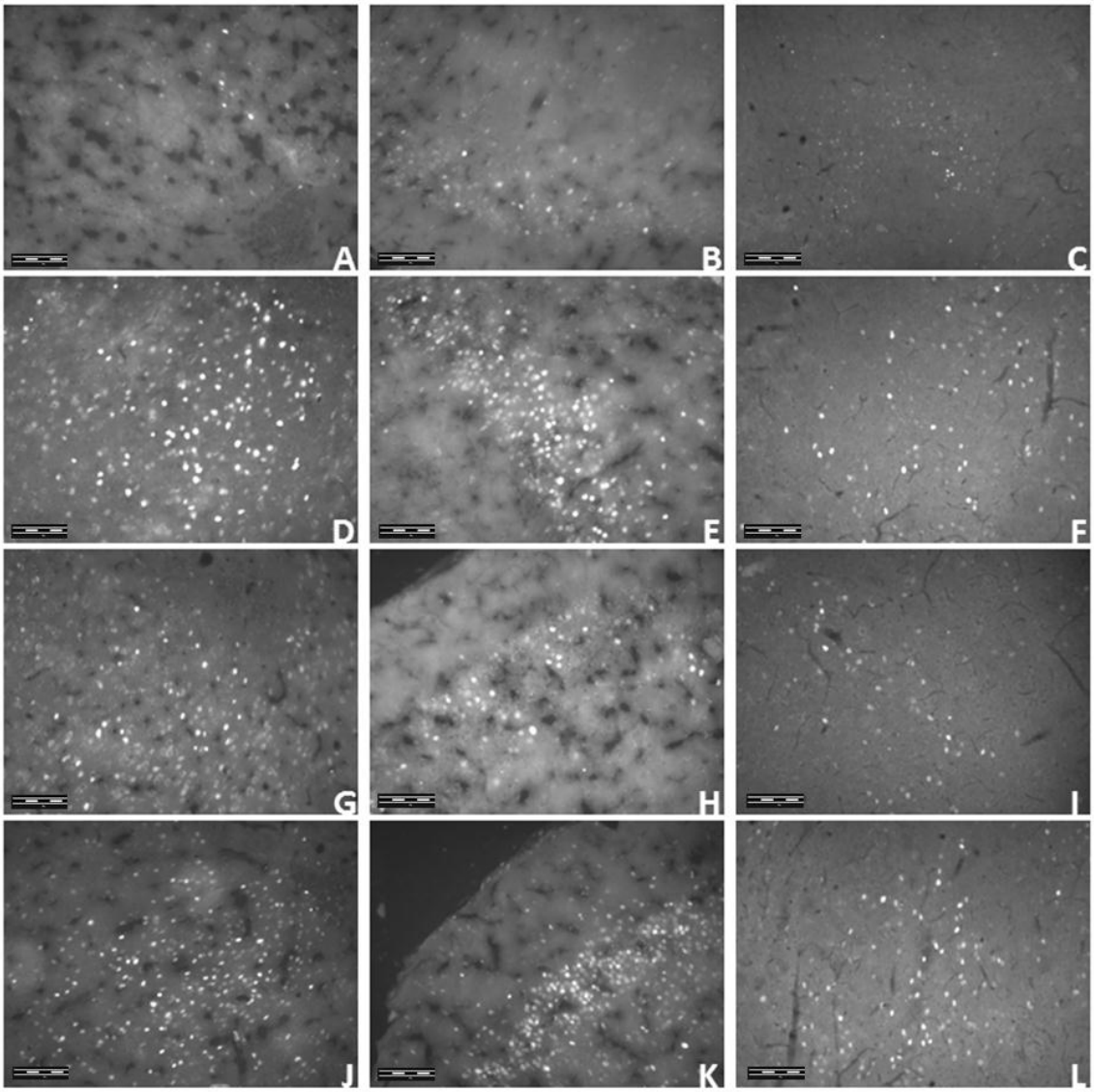

Figure 8. Immunofluorescence for Fos protein in the neurons of the olfactory bulb (A, D, G, J), piriform cortex (B, E, H, K) and periaqueductal gray (C, F, I, L), 1.5 h after the treatment with *Kielmeyera rugosa* (MEKR; 100, 200 and 400 mg/kg; p.o.) or vehicle (control), respectively in the figures of each central area. 20 µm.

CONCLUSÕES

5 CONCLUSÕES

A necessidade urgente de encontrar na medicina alternativa substâncias para o tratamento da inflamação efetivamente levou os pesquisadores a buscar as plantas medicinais como fonte de metabólitos eficazes. A revisão sistemática aqui apresentada reuniu os extratos de plantas da família Clusiaceae de diferentes partes do mundo que apresentam atividade anti-inflamatória, os quais podem ser promissores para pesquisa e utilização como alternativa terapêutica.

Os dados apresentados no presente estudo da atividade anti-inflamatória nos permitem sugerir que o extrato metanólico da planta *Kielmeyera rugosa* Choisy:

Apresenta atividade antihiperalgésica, sendo capaz de reduzir a hipernocicepção mecânica pela indução de vários agentes álgicos;

Possui ação anti-inflamatória, provavelmente mediada pela inibição de citocinas pró-inflamatórias, a exemplo do TNF-α;

Apresenta ação sobre o SNC, especificamente sobre o bulbo olfatório, córtex piriforme, influenciando no comportamento animal, bem como sobre a substância cinzenta periaquedutal (PAG), relacionando sua ação com os mecanismos modulatórios da dor;

Nas doses utilizadas não induz qualquer tipo de alteração motora nos animais;

Em concentrações analisadas neste trabalho não produziu citotoxicidade celular.

Dessa forma, os resultados sugerem que o extrato metanólico do caule de *Kielmeyera rugosa* Choisy pode ser uma alternativa para o estudo e desenvolvimento de preparações farmacêuticas com possível atividade anti-inflamatória.

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ANEXOS

ANEXO A: Protocolo de aprovação do Comitê de Ética em Pesquisa com Animal da Universidade Federal de Sergipe (CEPAUFS)

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

DECLARAÇÃO

Declaro, para os devidos fins, que o Projeto de Pesquisa intitulado **"Desenvolvimento biotecnológico e caracterização físico-química de gel termorreversível do extrato metanólico da kielmeyera rugosa em artrite induzida em roedores", sob coordenação do Prof. Dr. Lucindo José Quintans Júnior** (protocolo **CEPA 102/2011**) foi aprovado pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal de Sergipe, em reunião realizada dia 25/04/2012.

São Cristóvão, 07 de maio de 2012.

Profª. Drª. Flávia Teixeira Silva
Presidente do CEPA/UFS

Cidade Universitária "Prof. Aloísio de Campos"
Jardim Rosa Elze – São Cristóvão – SE
49100-000
Fones: 3212 6661/6606

ANEXO B: Normas do BioMed Research International para submissão do artigo: **A systematic review for anti-inflammatory property of *Clusiaceae* family – a pre-clinical approach.**

Author Guidelines

Submission

Manuscripts should be submitted by one of the authors of the manuscript through the online Manuscript Tracking System. Regardless of the source of the word-processing tool, only electronic PDF (.pdf) or Word (.doc, .docx, .rtf) files can be submitted through the MTS. There is no page limit. Only online submissions are accepted to facilitate rapid publication and minimize administrative costs. Submissions by anyone other than one of the authors will not be accepted. The submitting author takes responsibility for the paper during submission and peer review. If for some technical reason submission through the MTS is not possible, the author can contact bmri@hindawi.com for support.

Terms of Submission

Papers must be submitted on the understanding that they have not been published elsewhere and are not currently under consideration by another journal published by Hindawi or any other publisher. The submitting author is responsible for ensuring that the article's publication has been approved by all the other coauthors. It is also the authors' responsibility to ensure that the articles emanating from a particular institution are submitted with the approval of the necessary institution. Only an acknowledgment from the editorial office officially establishes the date of receipt. Further correspondence and proofs will be sent to the author(s) before publication unless otherwise indicated. It is a condition of submission of a paper that the authors permit editing of the paper for readability. All enquiries concerning the publication of accepted papers should be addressed to bmri@hindawi.com.

Peer Review

All manuscripts are subject to peer review and are expected to meet standards of academic excellence. Submissions will be considered by an editor and “if not rejected right away” by peer-reviewers, whose identities will remain anonymous to the authors.

Microarray Data Submission

Before publication, the microarray data should be deposited in an appropriate database such as Gene Expression Omnibus (GEO) or Array Express, and an entry name or accession number must be included in the manuscript prior to its publication. Microarray data should be MIAME

compliant. During the reviewing process, submitting authors are committed to provide the editor and the reviewers handling his/her manuscript with the login information by which they can access this information in the database.

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Units of Measurement

Units of measurement should be presented simply and concisely using System International (SI) units.

Title and Authorship Information

The following information should be included

Paper title

Full author names

Full institutional mailing addresses

Email addresses

Abstract

The manuscript should contain an abstract. The abstract should be self-contained and citation-free and should not exceed 200 words.

Introduction

This section should be succinct, with no subheadings.

Materials and Methods

This part should contain sufficient detail so that all procedures can be repeated. It can be divided into subsections if several methods are described.

Results and Discussion

This section may each be divided by subheadings or may be combined.

Conclusions

This should clearly explain the main conclusions of the work highlighting its importance and relevance.

Acknowledgments

All acknowledgments (if any) should be included at the very end of the paper before the references and may include supporting grants, presentations, and so forth.

References

Authors are responsible for ensuring that the information in each reference is complete and accurate. All references must be numbered consecutively and citations of references in text should be identified using numbers in square brackets (e.g., “as discussed by Smith [9]”; “as discussed elsewhere [9, 10]”). All references should be cited within the text; otherwise, these references will be automatically removed.

Preparation of Figures

Upon submission of an article, authors are supposed to include all figures and tables in the PDF file of the manuscript. Figures and tables should not be submitted in separate files. If the article is accepted, authors will be asked to provide the source files of the figures. Each figure should be supplied in a separate electronic file. All figures should be cited in the paper in a consecutive order. Figures should be supplied in either vector art formats (Illustrator, EPS, WMF, FreeHand, CorelDraw, PowerPoint, Excel, etc.) or bitmap formats (Photoshop, TIFF, GIF, JPEG, etc.). Bitmap images should be of 300 dpi resolution at least unless the resolution is intentionally set to a lower level for scientific reasons. If a bitmap image has labels, the image and labels should be embedded in separate layers.

Preparation of Tables

Tables should be cited consecutively in the text. Every table must have a descriptive title and if numerical measurements are given, the units should be included in the column heading. Vertical rules should not be used.

Proofs

Corrected proofs must be returned to the publisher within 2-3 days of receipt. The publisher will do everything possible to ensure prompt publication. It will therefore be appreciated if the manuscripts and figures conform from the outset to the style of the journal.

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A competing interest exists when professional judgment concerning the validity of research is influenced by a secondary interest, such as financial gain. We require that our authors reveal any possible conflict of interests in their submitted manuscripts.

If there is no conflict of interests, authors should state that “The author(s) declare(s) that there is no conflict of interests regarding the publication of this paper.”

Clinical Study
When publishing clinical studies, Hindawi aims to comply with the recommendations of the International Committee of Medical Journal Editors (ICMJE) on trials registration. Therefore, authors are requested to register the clinical trial presented in the manuscript in a public trials registry and include the trial registration number at the end of the abstract. Trials initiated after July 1, 2005 must be registered prospectively before patient recruitment has begun. For trials initiated before July 1, 2005, the trial must be registered before submission.

Ethical Guidelines

In any studies that involve experiments on human or animal subjects, the following ethical guidelines must be observed. For any human experiments, all work must be conducted in accordance with the Declaration of Helsinki (1964). Papers describing experimental work on human subjects who carry a risk of harm must include a statement that the experiment was conducted with the understanding and the consent of the human subject, as well as a statement that the responsible Ethical Committee has approved the experiments. In the case of any animal experiments, the authors should provide a full description of any anesthetic and surgical procedure used, as well as evidence that all possible steps were taken to avoid animal suffering at each stage of the experiment.

ANEXO C: Normas do Phytotherapy Research para submissão do artigo: **Central areas and cytokines on activity of *Kielmeyera rugosa* Choisy.**

Author Guidelines

Phytotherapy Research is a monthly, international journal for the publication of original research papers, short communications, reviews and letters on medicinal plant research. Key areas of interest are pharmacology, toxicology, and the clinical applications of herbs and natural products in medicine, from case histories to full clinical trials, including studies of herb-drug interactions and other aspects of the safety of herbal medicines. Papers concerned with the effects of common food ingredients and standardised plant extracts, including commercial products, are particularly welcome, as are mechanistic studies on isolated natural products.

Short communications dealing with the pharmacology and screening of crude or uncharacterised extracts will be considered for publication only if they are clearly of interest to our international readership and are not deemed more suitable for a regional audience.

Phytotherapy Research does not publish agricultural, phytochemical, structure elucidation, quality control or botanical identification papers unless directly pertinent to the pharmacological effects or overall safety of plant based medicines currently in use.

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All papers must be submitted via the online system.

Authors are welcome to submit the names and contact details of up to three suggested reviewers, using the online system. Submission of a manuscript will be held to imply that it contains original unpublished work and is not being submitted for publication elsewhere at the same time.

File types. Preferred formats for the text and tables of your manuscript are *.doc, *.docx and *.rtf. Figures must be provided in *.tiff or *.eps format.

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Manuscript Style. The language of the journal is English. Please ensure that your manuscript has been checked by an English language expert if there is concern for grammatical or other errors. All submissions including book reviews must have a title, be double-line spaced with type no smaller than 12 point, and have a margin of 3cm all round. Tables must be on separate pages after the reference list, and not be incorporated into the main text. Figures should be uploaded as separate Image files.

The title page must list the full title, short title of up to 60 characters and names and affiliations of all authors. Give the full address, including email, telephone and fax, of the author who is to check the proofs.
Include the name(s) of any sponsor(s) of the research contained in the paper, along with grant number(s).
Supply an abstract of up to 200 words for all articles. An abstract is a concise summary of the whole paper, not just the conclusions, and is understandable without reference to the rest of the paper. It should contain no citation to other published work.
Include up to six keywords that describe your paper for indexing purposes.
Authors may suggest up to 3 potential reviewers
A concise introduction is required of the background to the subject, its significance and its relationship to earlier works, with references.
Materials and methods should be presented with clarity and detail. State the original and important findings in the results. Illustrate these with figures or tables where necessary but keep these to a minimum.
Results and discussion may be combined as one section. Discuss the principal conclusions drawn from the results and their important implications.
Convention on biodiversity. Authors must indicate that they have obtained authority to access plant samples (other than freely available commercial crops or herbal products) used for research and that this has been authorised by the appropriate agent of the government of the source country as required under the framework of the United Nations Convention on Biodiversity.

Botanical aspects. Plant materials used must be adequately described using the Latin binomial for the plant, author of the name, plant family, source (e.g. country and region of collection, name of the collector and collection number) means of unambiguous identification (e.g. name and affiliation of expert botanist or the results of comparison with a published monograph and/or authenticated reference specimen). The reference number and place of deposition of a voucher specimen of the plant material must be given. For papers relating to crude plant extracts, the method of extraction and the yield of dried extract as a percentage weight of the starting fresh or dried plant material must also be stated. These should be submitted as short communications (see below).

Experimental procedures. Bioassay data for plant extracts or isolated compounds must be accompanied by data for positive and negative controls. All animal experiments should be conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, the EEC Directive of 1986 (86/609/EEC) or the NIH guide for the care and use of laboratory animals (NIH Publication No. 80-23; revised 1978). The Editors will reject papers if there is any doubt about the suitability of the animal procedures used.

Use Chemical Abstracts nomenclature for chemical names and structures. Use proper or proprietary names with caution. Common acronyms for biomedical names are acceptable but define all others when first mentioned. Define abbreviations when first mentioned and do not use in the title or abstract. Define non-standard units.

Keep acknowledgements brief and place them at the end of the paper.

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