UNIVERSIDADE FEDERAL DE SERGIPE

PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA

MESTRADO EM CIÊNCIAS FARMACÊUTICAS

SÍNTESE DO PROPIONATO DE CARVACROL E ESTUDO DE SUAS PROPRIEDADES ANTI-HIPERALGÉSICA E ANTI-INFLAMATÓRIA EM PROTOCOLOS EXPERIMENTAIS

Marilia Trindade de Santana Souza

São Cristóvão-SE

2014

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Dissertação apresentada ao Núcleo de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal de Sergipe como requisito parcial à obtenção do grau de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Lucindo José Quintans Júnior

Co-orientador: Prof. Dr. Sócrates Cabral de H. Cavalcanti

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Aprovada em: _____/_____/_____

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PARECER

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RESUMO

SANTANA, M.T. **SÍNTESE DO PROPIONATO DE CARVACROL E ESTUDO DE SUAS PROPRIEDADES ANTI-HIPERALGÉSICA E ANTI-INFLAMATÓRIA EM PROTOCOLOS EXPERIMENTAIS** Dissertação de Mestrado em Ciências Farmacêuticas, Universidade Federal de Sergipe, 2014.

Os terpenos são compostos naturais obtidos do metabolismo secundário das plantas. Apesar de apresentar efeitos farmacológicos, modificações estruturais realizadas no seu esqueleto podem levar o aumentando de suas atividades farmacológicas e atenuar os efeitos toxicológicos. Neste contexto, insere-se o carvacrol, um monoterpeno fenólico, presente em óleos essenciais de plantas pertencentes à família Labiatae. Estudos comprovam a atividade farmacológica deste monoterpeno. No entanto, modificações estruturais podem diminuir a dose efetiva deste composto. Desta forma, no presente estudo realizamos uma extensa revisão sistemática que avaliou a atividade anti-inflamatória de terpenos que sofreram modificações em sua estrutura, através de síntese. Adicionalmente, sintetizar o propionato de carvacrol (CP), a partir do carvacrol, e avaliar seus possíveis efeitos antinocicepivo, anti-hiperalgésico e anti-inflamatório. Para construir a revisão, foi realizada a busca nas bases de dados Scopus, PubMed e Embase, utilizando os descritores agentes anti-inflamatórios, terpenos e relação estrutura atividade. Já para a parte experimental, foram utilizados camundongos *Swiss* machos (25-35 g) com 2 a 3 meses de idade. Os animais foram divididos em grupos e foram tratados com CP (25, 50 e 100 mg/kg), veículo (solução salina 0,9% + Tween 80 0,2%) ou droga padrão, por via intraperitoneal (i.p.). O efeito antinociceptivo foi avaliado utilizando o protocolo de formalina (1%) e o teste da placa quente. A hiperalgesia mecânica foi avaliada após a administração dos agentes álgicos carragenina (CG; 300 µg/pata), fator de necrose tumoral-α (TNF-α; 100 pg/pata), prostaglandina E_2 (PGE_2; 100 ηg/pata) ou dopamina (DA; 30 µg/pata) utilizando o analgesímetro digital Von Frey. Na avaliação do efeito anti-inflamatório utilizou-se o teste de pleurisia e edema de pata induzido por CG (1%) em pletismômetro digital. A citotoxicidade foi avaliada através do método colorimétrico MTT. Os protocolos experimentais foram aprovados pelo comitê de ética da UFS (CEPA/UFS: 35/12). Os resultados foram expressos como média ± erro padrão da média e as diferenças entre os grupos foram analisadas por meio do teste de variância ANOVA, uma via ou duas vias, seguido pelo teste de Tukey ou Bonferroni. Valores de $p < 0,05$ foram considerados estatisticamente significantes. Na revisão sistemática foram encontrados 27 artigos sobre modificação estrutural de terpenos e atividade anti-inflamatória. Na parte experimental, a administração do CP produziu uma redução significativa ($p < 0,01$ ou 0,001) no teste da nocicepção induzida por formalina, em ambas as fases do teste. No teste da placa quente, o tempo de reação aumentou significativamente nas doses de 50 e 100 mg/kg ($p < 0,05$; 0,01 ou 0,001). O CP também foi capaz de inibir o desenvolvimento da hiperalgesia mecânica induzida por todos os agentes testados ($p < 0,05$; 0,01 ou 0,001). Na avaliação da atividade anti-inflamatória, o tratamento com CP causou uma diminuição significativa ($p < 0,001$) no número total de leucócitos, diminuindo os níveis de TNF-α ($p < 0,001$), IL-1β ($p < 0,05$) e extravasamento de proteínas ($p < 0,01$). Além disso, o edema de pata induzido por CG também foi inibido pelo CP ($p < 0,05$; 0,01 ou 0,001). Desta forma, conclui-se que o CP possui atividade antinociceptiva, anti-hiperalgésica e anti-inflamatória, provavelmente por inibição de citocinas. Dessa maneira, a modificação estrutural em terpeno pode ser uma alternativa interessante para obtenção de moléculas com propriedades farmacológicas.

Palavras-chaves: terpeno, modificação estrutural, carvacrol, propionato de carvacrol, inflamação, hiperalgesia.

ABSTRACT

SANTANA, M.T. **CARVACROL PROPIONATE SYNTHESIS AND STUDY OF ITS ANTI-HYPERALGESIC AND ANTI-INFLAMMATORY PROPERTIES IN EXPERIMENTAL PROTOCOLS.** Dissertação de Mestrado em Ciências Farmacêuticas, Universidade Federal de Sergipe, 2013.

Terpenes are naturally occurring compounds obtained from the plants secondary metabolism. Despite presenting pharmacological effects, structural changes within their skeleton may increasing their pharmacological activity and attenuate the toxicological effects. Carvacrol is a phenolic monoterpene present in essential oils from plants belonging to the Labiatae family. Studies have demonstrated that carvacrol has anti-inflammatory activity. However, sctructural changes may reduce the effective dose of this monoterpene. Thus, in this study, we conducted an extensive systematic review evaluating the anti-inflammatory activity of terpenes that suffered an alteration in its structure through synthesis and semi-synthesis, synthesize the carvacrol propionate (CP) from the carvacrol and evaluate its potential antinociceptive, anti-hyperalgesic and anti-inflammatory effects. To build the revision, it was made the search in Scopus, Embase and PubMed databases, using the descriptors anti-inflammatory agents, terpenes and structure activity relationship. In the experimental part, it was used Male Swiss mice (25-35 g) with 2 to 3 months age. The animals were divided in groups and were treated with CP (25, 50 and 100 mg/kg), vehicle (saline solution 0.9% + Tween 80 0.2%) or standard drug, intraperitoneally (i.p.). The antinociceptive effect was evaluated through the formalin (1%) protocol and the hot plate test. The mechanical hyperalgesia was evaluated through the algic agents injection: carrageenan (CG; 300 μg/paw), tumor necrosis factor-α (TNF-α; 100 pg/paw), prostaglandin E2 (PGE_2; 100 ng/paw) or dopamine (DA; 30 μg/paw) using a digital analgesimeter (von Frey). To assess the anti-inflammatory effect, it was used the pleurisy and paw edema induced by GC (1 %) in digital plethysmometer. The cytotoxicity of CP was evaluated by the MTT colorimetric method. The experimental protocols were approved by the UFS ethics committee (CEPA/UFS: 35/12). The results are expressed as mean ± SEM and differences between groups were analyzed by one-way or two-way ANOVA test followed by Tukey or Bonferroni tests. Values of $p < 0.05$ were considered statistically significant. In systematic review, 27 papers were found concerning about terpenes structural modification and the evaluation of their anti-inflammatory activity. In the experimental part, the administration of CP produced a significant decrease ($p < 0.01$ and 0.001) in the test formalin-induced nociceptive in both phases of the test. In the hot plate test, the reaction time increased significantly at doses 50 and 100 mg/kg ($p < 0.05$, 0.01 and 0.001). CP inhibited the development of mechanical hyperalgesic induced by all agents tested ($p < 0.05$, 0.01 and 0.001). In the evaluation of anti-inflammatory activity, the treatment with CP was able to decrease significantly the leukocyte recruitment ($p < 0.001$), the TNF-α ($p < 0.001$), the IL-1β ($p < 0.05$) and protein leakage ($p < 0.01$). In addition, the paw edema induced by CG in mice was inhibited significantly by CP ($p < 0.05$, 0.01 and 0.001). Thus, it is concluded that the CP attenuates nociception, mechanical hyperalgesia and inflammation, through an inhibition of cytokines. Therefore, structural modification terpene can be an interesting alternative for obtaining molecules with pharmacological properties.

Key-words: terpene, structural modification, carvacrol, carvacrol propionate, inflammation, hyperalgesia.

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LISTA DE ABREVIATURAS

AINES – Anti-inflamatório não esteroidais

AMPc – Adenosina monofosfato cíclico

Cg – Carragenina

COX – Enzima ciclo-oxigenase

CP – Propionato de Carvacrol

DA - Dopamina

DIP – Dipirona

IL – Interleucina

IND – Indometacina

MOR – Morfina

MTT - Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

NF-kB – Fator Nuclear Kappa B

NO – Óxido Nítrico

PGE_2 - Prostaglandina E_2

PPAR – Receptor ativados por proliferador de peroxisomo

TNF-α – Fator de Necrose Tumoral alfa

1.0 INTRODUÇÃO

1.0 INTRODUÇÃO

O processo inflamatório é a resposta do organismo a diferentes estímulos, incluindo danos mecânicos, físicos, químicos e biológicos (Gregory *et al*., 2008). De forma controlada, é uma resposta benéfica que protege o organismo contra os agentes invasores, uma vez que atenua uma infecção contribuindo até o retorno da homeostase (Cotran *et al.,* 2006). No entanto, devido à resistência do patógeno, a inflamação pode se tornar crônica, levando a um aumento do número de mediadores inflamatórios que conduz a efeito nocivo ao organismo (Medzhitov, 2008).

As características do processo inflamatório incluem uma complexa cascata de eventos bioquímicos e celulares, que envolve extravasamento de líquido, migração celular, produção de mediadores pró-inflamatórios e sensibilização de nociceptores (Becker, 1983). Estas por sua vez, geram a sintomatologia característica da inflamação, conhecida pelos cinco sinais cardinais: eritema, calor, rubor, dor e a perda da função (Medzhitov, 2008).

O aumento da sensibilidade a estímulos dolorosos, conhecido como hiperalgesia, é uma característica marcante da inflamação. Mediadores inflamatórios, liberados por células inflamatórias como, tais como, citocinas (Interleucina-1β, Fator de Necrose Tumoral-α) estimulam a produção de metabólitos da enzima ciclo-oxigenase (COX) e aminas simpatomiméticas. Estes contribuem para aumentar a sensibilização dos receptores nociceptivos (Woolf e Ma, 2007).

Provavelmente, devido à interação destes mediadores a canais iônicos de membrana, tipo voltagem-dependente ou receptores da membrana acoplados a proteínas regulatórias denominadas de proteínas G (Ferreira, 1995). Ambos receptores quando ativados elevam as concentrações de adenosina monofosfato cíclico (AMPc) e cálcio intracelular, contribuindo para diminuição do limiar de excitabilidade neural (Rocha *et al.,* 2007).

Atualmente, o manejo terapêutico para condições inflamatórias e dolorosas, foca na cascata da inflamação (Carvalho e Lemônica, 1998). A primeira escolha para o tratamento inclui os anti-inflamatórios não esteroidais (AINES), que são fármacos inibidores da COX, consequentemente bloqueiam a formação de mediadores finais, tais como, prostaglandinas (PGE_2), os

medicamentos desta classe incluem a aspirina, indometacina, diclofenaco (Rao *et al.,* 2003). A segunda opção de tratamento é impedir o desenvolvimento da hiperalgesia, através do mecanismo de dessensibilização, consequentemente restaurando o limiar do nociceptor, pode-se destacar a morfina e a dipirona (Rodrigues e Duarte, 2000; Reis e Rocha, 2006).

No entanto, estes medicamentos provocam efeitos adversos como, lesão gástrica, nefrotoxidade, náuseas e efeito tolerância (Vane *et al.,* 1998; Furlan *et al.,* 2006). Logo, existe a necessidade clínica para a procura de novas drogas anti-inflamatórias. Esta busca se dá através de melhorias das práticas em investigações pré-clínicas e o refinamento em modelos animais que mimetizem as condições inflamatórias (Knowles, 2013).

Dessa maneira, alternativas farmacológicas que apresentem alta eficácia no tratamento e menos efeitos indesejáveis são necessárias (Wang *et al*., 2013). Em resposta à demanda de novos medicamentos para o tratamento da dor inflamatória, os produtos naturais e derivados representam uma ferramenta farmacológica de extrema importância. Uma vez que apresentam uma grande diversidade e complexidade de estrutura química, o que não é visto nos compostos puramente sintéticos. Por isso, é de extrema importância a descoberta de novos fármacos para o tratamento de diversas doenças que acometem a população (Gautam e Jachak, 2009).

Além disso, de acordo com estudo de Porto *et al.,* (2009), a modificação estrutural realizada em produtos naturais originando uma nova molécula pode apresentar atividades promissoras, visto como uma forma interessante de obtenção de novas estruturas, com a possibilidade de aprimoramento da sua atividade.

Logo, o estudo da relação estrutura-atividade de produtos naturais é considerado, atualmente, uma ferramenta fundamental no planejamento de novos protótipos de fármacos (Vechia *et al*., 2009). O fato é que, uma pequena modificação na estrutura pode conduzir a uma alteração na atividade biológica (Guha, 2012), permitindo que químicos possam realizar substituições especificas que melhorem as propriedades da molécula, como lipofilicidade, esta que contribui com a biodisponibilidade da droga no organismo (Martin *et al*., 2002).

Dessa maneira, realizar modificações na estrutura de um composto ativo, pode aumentar a sua eficácia e também a seletividade, diminuindo a toxicidade. Portanto, o interesse da comunidade científica pelos produtos naturais tem como objetivo, descobrir novas entidades químicas ativas, passíveis de modificações que representem potencialidades terapêuticas, contribuindo assim para a prevenção e/ou tratamento de determinadas doenças (Dias *et al.,* 2012).

Muitos fármacos disponíveis atualmente foram obtidos sinteticamente, baseados em estruturas naturais ativas (Bauer e Brönstrup, 2013). Em se tratando do efeito analgésico, um grande exemplo de sucesso terapêutico na modificação estrutural de um composto natural é o ácido acetilsalicílico, primeiro produto sintético para fins terapêuticos, obtido a partir de um glicosídeo natural, salicina, identificado como princípio ativo de *Salix* sp (Barreiro e Bozani, 2009).

A morfina também merece destaque, uma vez que também foi originalmente isolada da *Papaver somniferum,* e inspirou a descoberta posterior dos derivados 4-fenil-piperidínicos, a meperidina, destacando-se pela reduzida propriedade indutora de tolerância quando comparada ao produto natural morfina (Barreiro e Manssour, 2008).

Dentro deste contexto, os monoterpenos, representantes de uma classe de compostos químicos chamados de terpenos, constituintes dos óleos essenciais de plantas, são ricos em substâncias químicas com atividade biológica (Barbosa-Filho *et al.,* 2006). Apesar de possuir uma estrutura simples, uma vez que apresentam duas unidades isoprênicas, apresentam diversas atividades biológicas (Las Heras *et al.,* 2003)

Por isso, sua importância para a comunidade cientifica, já que existem diversos estudos que comprovam seu efeito farmacológico (Quintans-Júnior *et al.,* 2010; Batista *et al.,* 2010; Riella *et al*., 2012). Dentre os monoterpenos, pode-se destacar o carvacrol, presente em óleos essenciais de plantas pertencentes à família Labiatae. Nos últimos anos, estudos comprovam que o carvacrol tem efeito anti-inflamatório provavelmente por inibição de mediadores como PGE_2, IL-1β e TNF-α. (Guimarães *et al.*, 2012; Lima *et al.,* 2013). Também já foi comprovada a inibição da enzima ciclo-oxigenase-2

(Landa *et al.,* 2009), além de estimular os receptores ativados por proliferador de peroxisomo (PPAR) (Hotta *et al.,* 2010). No entanto, os estudos demonstram a atividade deste monoterpeno em doses relativamente altas.

Por isso, como alternativa de diminuir a dose efetiva de compostos ativos, modificações estruturais podem ser propostas, para a obtenção de uma nova molécula ativa (Carvalho *et al.,* 2003). Alguns estudos já comprovam a eficácia de modificações estruturais em plantas medicinais, obtendo derivados sintéticos (Newman *et al.,* 2003). Podem ser citados a di-hidrocarvona, um derivado sintético da carvona que apresentou propriedade anti-inflamatória (De Souza *et al.,* 2010) e antinociceptiva (Oliveira *et al.,* 2008). Análogos sintéticos da rotundifolona demonstraram efeito antinociceptivo significativo (De Sousa *et al*., 2007).

Uma estrutura interessante, que contribui com potencial efeito anti-inflamatório é a classe dos propionatos, visto que na clínica já se utilizam o proprionato de clobetasol, propionato de fluticasona e dipropionato de betametasona para condições inflamatórias (Menter *et al.*, 2012; Ynson *et al.*, 2013). No entanto, como são anti-inflamatórios esteroidais, o seu uso é limitado, devido às reações adversas associadas (Stuetz *et al*., 2001).

Baseado na literatura, e levando-se em consideração que a modificação estrutural de produtos naturais é uma fonte importante para a obtenção de moléculas biologicamente ativas, uma vez que diversos medicamentos utilizados no tratamento de várias doenças são oriundos desta forma. Estudos científicos voltados à análise da efetividade das modificações estruturais em plantas medicinais são escassos, tornam-se necessárias pesquisas voltadas para a descoberta de uma nova estrutura com potencial terapêutico.

Dessa maneira, visando atender a necessidade no desenvolvimento de fármacos anti-inflamatórios com menores efeitos colaterais e considerando a possibilidade de modificações estruturais em monoterpenos resultarem em novas entidades químicas com propriedade analgésicas (De Souza *et al.,* 2007), este trabalho teve como foco realizar uma revisão sistemática, buscando verificar se, modificação estrutural em terpenos melhora a atividade anti-inflamatória. Adicionalmente sintetizar o propionato de carvacrol (CP) e avaliar os possíveis efeitos analgésico e anti-inflamatório deste composto.

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2.0 OBJETIVOS

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2.1 GERAL

Sintetizar e determinar a estrutura do propionato do carvacrol (CP) e avaliar seus efeitos antinociceptivo, anti-hiperalgésico e anti-inflamatório em protocolos experimentais.

2.2. ESPECÍFICOS

Realizar um levantamento bibliográfico buscando a construção de uma revisão sistemática sobre a relação estrutura atividade de terpenos com efeito anti-inflamatório;

Sintetizar e determinar a estrutura do CP;

Avaliar a ação antinociceptiva do CP;

Verificar a atividade do CP na hiperalgesia mecânica induzida por diversos agentes;

Avaliar o efeito do CP em modelos inflamatórios e quantificar a produção de mediadores pró-inflamatórios;

Analizar traços de citotoxicidade do CP;

Verificar a possível interferência do CP sobre a coordenação motora dos animais.

3.0 DESENVOLVIMENTO

3.1 CAPÍTULO 1

STRUCTURE-ACTIVITY RELATIONSHIP OF TERPENES WITH ANTI-INFLAMMATORY PROFILE – A SYSTEMATIC REVIEW

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Structure–Activity Relationship of Terpenes with Anti-Inflammatory Profile – A Systematic Review

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Abstract: Inflammation is a complex biological response that in spite of having available treatments, their side effects limit their usefulness. Because of this, natural products have been the subject of incessant studies, among which the class of terpenes stands out. They have been the source of study for the development of anti-inflammatory drugs, once their chemical diversity is well suited to provide skeleton for future anti-inflammatory drugs. This systematic review reports the studies present in the literature that evaluate the anti-inflammatory activity of terpenes suffering any change in their structures, assessing whether these changes also brought changes in their effects. The search terms anti-inflammatory agents, terpenes, and structure-activity relationship were used to retrieve English language articles in SCOPUS, PUBMED and EMBASE published between January 2002 and August 2013. Twenty-seven papers were found concerning the structural modification of terpenes with the evaluation of anti-inflammatory activity. The data reviewed here suggest that modified terpenes are an interesting tool for the development of new anti-inflammatory drugs.

The word 'inflammation' comes from the Latin *inflammare* (to set on fire), and it is defined as a complex biological response of vascular tissues against aggressive agents, involving a cascade of biochemical events comprising the local vascular system, the immune system and different cell types found in the injured tissue [1,2].

For the treatment of various inflammatory diseases, the non-steroidal anti-inflammatory drugs (NSAIDs) are most widely prescribed, but the gastrointestinal, renal and cardiovascular toxicity associated with common NSAIDs limits their usefulness [3]. Because of this, the potential therapeutic evaluation of the medicinal plants has been the subject of incessant studies, which have proved pharmacological actions, such as the anti-inflammatory, of some plants and their constituents, including the terpenes [4] .

Terpenes, which make up a very large family of natural products, contain more than 50,000 structurally diverse compounds, which are categorized by a number of C_5 isoprene units [5]. Terpenes have been described as having important biological activities, such as analgesic [6,7], anticonvulsant [8] and cardiovascular [9]. Additionally, anti-inflammatory activity of some terpenes is described in the literature, such as β-caryophyllene [10], citral [11], α-pinene [12], citronellal [13], limonene [14] and surgiol [15].

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Despite the existing technology in organic chemistry for the synthesis of a new drug, the natural products, including terpenes, serve as a source of raw material for innovative drug discovery [16], once the chemical diversity of terpenes is well suited to provide skeleton for future drugs [17]. Thus, in an attempt to improve the efficacy/safety profile of new anti-inflammatory drugs, including those of natural origin, the structure–activity relationship has been extensively studied, taking into account up-to-date knowledge on the mechanism of inflammation [18,19]. Despite its importance, there are no reviews on the anti-inflammatory activity of structurally modified terpenes.

In this context, this study aimed to analyse, through a systematic review, the studies present in the literature that evaluate the anti-inflammatory activity of terpenes that suffered any change in their structures, assessing whether these changes also brought changes in their effects.

Materials and Methods

A systematic review was carried out through a literature search performed in August 2013 and included articles published over a period of 10 years (January 2002 to August 2013). This literature search was performed through specialized databases (PUBMED, SCOPUS and EMBASE) using different combinations of the following keywords: terpenes, anti-inflammatory agents, and structure-activity relationship. Manuscript selection was based on the inclusion criteria: articles published in English and articles with keywords in the title, abstract or full text, as well as studies with isolated terpenes for further structural modifications. Studies conducted with structure–activity relationship of terpenes isolated from plants were excluded.

STRUCTURE-ACTIVITY RELATIONSHIP OF TERPENES WITH ANTI-INFLAMMATORY PROFILE – A SYSTEMATIC REVIEW

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Abstract: Inflammation is a complex biological response that has no treatment without side effects. Because of this, natural products have been the subject of incessant studies, among which the class of terpenes stands out. They have been the source of study for the development of anti-inflammatory drugs, once their chemical diversity is well suited to provide skeleton for future anti-inflammatory drugs. This systematic review reports the studies present in the literature that evaluate the anti-inflammatory activity of terpenoids suffering any change in their structures, assessing whether these changes also brought changes in their effects. The search terms anti-inflammatory agents, terpenes, structure-activity relationship were used to retrieve English language articles in SCOPUS, PUBMED and EMBASE published between January 2002 and August 2013. Twenty-seven papers were found concerning the structural modification of terpenes with evaluation of the anti-inflammatory activity. The data reviewed here suggest that modified terpenes are an interesting tool for the development of new anti-inflammatory drugs.

Keywords: Anti-inflammatory agents, terpenes, structure-activity relationship.

INTRODUCTION

The word ‘inflammation’ comes from the Latin *inflammare* (to set on fire) and it is defined as a complex biological response of vascular tissues against aggressive agents, involving a cascade of biochemical events comprising the local vascular system, the immune system and different cell types found in the injured tissue [1,2].

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In this context, the present study aimed to analyze, through a systematic review, the studies present in the literature that evaluate the anti-inflammatory activity of terpenoids that suffered any change in their structures, assessing whether these changes also brought changes in their effects.

METHODS

A systematic review was carried out through a literature search performed in August 2013 and included articles published over a period of 10 years (January 2002 to August 2013). This literature search was performed through specialized databases (PUBMED, SCOPUS and EMBASE) using different combinations of the following keywords: terpenes, anti-inflammatory agents, structure-activity relationship. The manuscript selection was based on the inclusion criteria: articles published in English and articles with keywords in the title, abstract or full text, as well as studies with isolated terpenes for further structural modifications. Articles conducted with structure-activity relationship isolated from plants were excluded.

For the selection of the manuscripts, two independent investigators (MTSS and LJQJ) first selected the articles according to the title, then to the abstract and then through an analysis of the full-text publication. Any disagreement was resolved through a consensus between them. The resulting articles were manually reviewed with the goal of identifying and excluding the works that did not fit the criteria described above.

RESULTS AND DISCUSSION

This review searched for structural modifications in terpenes which enhanced the anti-inflammatory activity in the last ten years. The primary search identified 762 articles, with 445 from PUBMED, 13 from SCOPUS and 304 from EMBASE. However, out of this total, 7 were indexed in two or more databases and were considered only once, resulting in 755

articles or referred to studies. After the initial screening of the titles, abstracts, full text and time of publication, 27 articles were selected and the others did not meet the inclusion criteria (n = 728). We excluded studies that evaluated the structure-activity relationship of only compounds isolated from plants or papers that were not within the limits of the year (January 2002 and August 2013). A flowchart illustrating the progressive study selection and numbers at each stage is shown in the Figure 1.

Structural modification of natural products showed promising activities that must be seen as an interesting source of new structures, with the possibility of presenting a better biological activity [20]. Table 1 and Fig. 2 show the chemical modification and pharmacological aspects of the terpenes identified by this systematic review.

It was possible to verify that structural changes in terpene compounds are common in order to improve the anti-inflammatory activity. The most used protocol was *in vitro* tests, with only a few *in vivo* tests and topical administration.

Monoterpene

Isoegomaketone (IK)

This monoterpene is the main essential oil component of *Perilla frutescens*. In an attempt to enhance the activity, we proposed a chemical modification in the IK, focusing on the aromatic heterocyclic ring. It was carried out to improve the suppressive effects on the production of NO, MCP-1 and IL-6, important mediators in the inflammatory process, which were evaluated through the regulation of the NF-κB and AP-1 transcriptional activation [21].

The IK and its five derivatives were able shown to inhibit the NO, MCP-1 and IL-6 formation by the LPS-induced inflammatory responses, that it was investigated in RAW 264.7 mouse macrophage cell line. Besides, it would inhibit the expression of these genes through the suppression of NF-κB or AP-1 activation. Among the synthesized derivatives, we could

check that the introduction of a methyl group at the 5-position furan ring in the IK improved threefold the inhibitory activities towards NO and MCP-1 production. Furthermore, a significant suppression of NF-κB and AP-1 DNA binding activities was shown for this derivative [21].

Sesquiterpene lactones

Pseudoguaianolides, psilostachyin, parthenin and coronopilin are sesquiterpenes lactones, in other words, have 3 isoprene units merged into a lactone ring. These compounds are found in the species *Parthenium hysterophorus*, *Ambrosia psilostachya*, *Parthenium hysterophorus*, respectively. The modifications of these sesquiterpenes form the type: acetylation at C-1 and, subsequently, inserted a propionate and butyrate group. The parthenin generates a library of analogues of the type: δ-valerolactones, spirolactone, azaspiro lactones and butenolide. These were evaluated as to their anti-inflammatory potential through in vitro TNF-α, IL-1β and IL-6 expression in murine neutrophils [22].

Chib et al [22], interestingly, found that the structural modification improved the activity of parent molecules, since azaspiro lactones and butenolide analogues displayed maximum inhibitory effect on TNF-α cytokine secretion. Moreover, they suppressed the extracellular IL-1β expression level in LPS-activated neutrophils at dose level of 1 µg/ml and also suppressed the extracellular IL-6 expression at dose level of 1 µg/ml, even though the inhibition of expression was not significant.

Despite of the fact that the α-methylene-γ-lactones are required for the activity of sesquiterpene lactones, other steric requisites must be fulfilled [23]. In this case, the insertion of the azaspiro and butenolide contributed to improve the anti-inflammatory activity.

Parthenolide

Another type of sesquiterpene lactone present in the species *Tanacetum parthenium* is the parthenolide. Changes in the parthenolide skeleton comprised compounds with different structure types, such as: guaianolides, pseudoguaianolide, germacrolides, melampolides, heliangolides and 4,5-dihydrogermacranolides. These changes were proposed by Neukirch and collaborators [24] in order to improve the IL-8 chemotaxis.

Once this modification occurred, the bicyclic compounds derived from acidic treatment of parthenolide inhibited the chemotaxis more than did the parthenolide substrate. In fact, the modest structural changes have marked influence on the migration of neutrophils [25], demonstrating the α-methylene γ-lactone plays an important role in anti-inflammatory effect.

However, we did not discard the addition of another structure, since the simple α-methylene-γ-lactones caused minimal anti-inflammatory activity, which means that for pharmacological activity, other steric requisites must be considered [23].

Sesquiterpene hydroquinones/quinones

Bolinaquinone

Bolinaquinone is a hydroquinones/quinones sesquiterpene belonging to one class of marine sponge metabolites, which have received considerable attention due to their varied biological activities [26]. Aiming at the improvement of the bolinaquinone activity, we proposed structural changes of the basic molecule variations in the aromatic system and evaluated the inhibition of PGE_2 production in the LPS-treated RAW 264.7 cells [27].

Remarkably, the (4A) inhibitor showed good ability in reducing LPS-induced PGE_2 release with potency degrees better than the parent compound, the bolinaquinone.

Curiously, the analogues lack the methyl spacer; in other words, variations in the aromatic system directly attached to the quinone ring were inactive compounds, which

suggested that the linker between the hydrophobic pocket and quinone ring is essential for the activity [27].

Avarol

Avarol is a marine sesquiterpenoid hydroquinone with interesting pharmacological properties. Because it is a molecule able of modifications, Amigó and collaborators [28] undertook the synthesis of the avarol ester derivatives, avarol oxidation and amino derivatives. We evaluated as potential antipsoriatic agents by the inhibition of superoxide generation in activated human neutrophils or reduction of cell proliferation and the PGE_2 generation in the cultured human keratinocyte HaCaT cell line.

According to Amigó and collaborators [28], the Avarol-3'-thiosalicylate (5A) showed better anti-inflammatory properties as antioxidant and inhibitor of PGE_2 release compared with the avarol. This result, in summary, could be related to the presence of a thiosalicylic function at the hydroquinone moiety, which could act through cyclooxygenase (COX) inhibition, in a manner similar to nonsteroidal anti-inflammatory drugs (NSAIDs) [28]. Furthermore, (5A) derivative inhibits both *in vivo* and *in vitro* mediators related to the inflammatory response. Its action mechanism is related to the inhibition of NF-kB activation and can be mediated by the down-regulation of intracellular signal-transduction pathways influenced by ROS, TNF-α and arachidonic acid metabolism [29].

Siphonodictyal

Siphonodictyal is a sesquiterpene belonging to the hydroquinone sesquiterpene class. Laube and collaborators [30] demonstrated the similar structural synthesis of the sesquiterpene quinones and hydroquinones from the siphonodictyal tested for their anti-inflammatory activities.

It was found that cyclohexadienone and sesquiterpene *o*-benzoquinone derivatives showed a very good inhibition of 3α-hydroxysteroid dehydrogenase (3α-HSD), comparable with the indomethacin [30]. The 3α-HSD is a key enzyme in the inflammatory cascade involved in the glucocorticoids metabolism. Thus, it is inhibited by the major nonsteroidal and steroidal agent types [31]. Therefore, the 3α-HSD inhibition can be used in an assay in search for anti-inflammatory drugs.

Abscisic acid

The abscisic acid (ABA) is a phytohormone sesquiterpene. It has been showed that it stimulates several functions of human granulocytes phagocytosis, reactive oxygen species, nitric oxide production and chemotaxis. Aiming to improve its activity, Grozio and collaborators [32] synthesized an ABA analog compound, the (7A), evaluating its anti-inflammatory properties on *in vitro* human granulocytes and monocytes through its ability to compete with ABA for binding to cell membranes and to the recently identified human ABA receptor.

Grozio et al [32] showed that ABA analog has higher affinity than ABA for binding to granulocyte membranes and inhibiting chemotaxis, phagocytosis, ROS and PGE_2 production by human granulocytes.

Diterpenes

Andrographolide

Andrographolide is a bicyclic diterpenoid lactone isolated from the *Andrographis paniculata* (Burm. f) leaves. The novel synthesis of derivatives from andrographolide to screen for more effective anti-inflammatory drugs has been studied for many years [33-35].

The synthesis derivated in the isoandrographolide and 12-hydroxy-14-dehydroandrographolide, and was evaluated as inhibitory activity of IL-6 and TNF-α expression in mouse macrophages. The andrographolide derivative presented cytokines inhibitory effect, being better than the andrographolide. On the other hand, the compound with 12-hydroxy-14-dehydroandrographolide structure, having aryl moiety C-12, showed the best inhibitory activity [33].

Suebsasana and collaborators [34] presented the andrographolide effect on writhing test and carrageenan-induced paw edema. The animals were treated with andrographolide derivatives and 12-hydroxy-14-dehydroandrographolide at dose of 4 mg/kg intraperitoneally. Andrographolide derivatives and 12-hydroxy-14-dehydroandrographolide presented better anti-inflammatory and analgesic effects compared with the parent compound.

Although previous studies indicated that the derivatives 12-hydroxy-14 dehydroandrographolide are the most potent, Dai and collaborators [35] proposed a different modification: introducing the group 15-alkylidene structure of andrographolides. Hence, to investigate whether these compounds display inflammatory properties, dimethylbenzene-induced mouse ear edema was used, as well as rat paw edema model induced by egg albumin. Thus, it was possible to determine that 15-alkylidene structure presented anti-inflammatory properties, probably due to the inhibition of serum iNOS activity and PGE_2. In summary, the study demonstrated that the introduction of the *p*-chlorobenzylidene group in the C-15 presented better anti-inflammatory effects, probably inhibiting PGE_2, inhibition of iNOS activity and the remarkable diminution of NO production.

Hispanolone

The labdane diterpenoid hispanolone was first isolated from *Ballota hispanica*. Previous studies of hispanolone and the structurally related diterpene hispanolone has

revealed the anti-inflammatory activity and a very low former cytotoxicity [36]. Thus, the hispanolone and galeopsin biological activities were proposed with a series of nine hispanolone derivatives as potential anti-inflammatory agents [37].

The data presented in this study demonstrate that two labdane diterpenoids of the series tested, (11A) and (11B), have potent anti-inflammatory activity due to the inhibition of the NO and PGE_2 production in LPS-stimulated macrophages, probably on account of the inhibition of NOS-2 and COX-2 expression. These effects are mediated by the inhibition of IKK activity, which results in stabilization of the NF-κB /IκB complex and inhibition of the NF-κB nuclear translocation.

This study corroborates with potential anti-inflammatory actions of semisynthetic labdane derivatives and the mechanisms involved. Only studies demonstrating biological activities by the hispanolone have been identified [38].

Ent-kaurene

Ent-kaurene diterpenes are known to have interesting biological properties, some of these compounds have been found to be cytotoxic against several cancer cell lines [39]. Thus, we proposed the development of potential anti-inflammatory agents for the preparation and evaluation of anti-inflammatory activity of kaurene derivatives [40].

Hueso-Falcón and collaborators [40] synthesized 63 derivatives. Some derivatives had no effect, however, other demonstrated consistent cytotoxicity by MTT assay. Only three of these analog compounds, (12A), (12B) and (12C), showed the most potent anti-inflammatory effect. The existence of a carboxylic acid seems to play an important role for NO inhibitory activity and cell survival, since it is present in the three mentioned active compounds [40].

Therefore, the activity of these compounds may be at least in part due to its NF-kB inhibitory activity. In addition to the inhibitory effects on NO production, these compounds

were able to inhibit several cytokines involved in the inflammatory response after LPS stimulation, such as IL-6, IL-1b, TNF-α and IFN-γ.

Pseudopterosin

The pseudopterosins (PsA) is a diterpene glycoside class isolated from the marine octocoral *Pseudopterogorgia elisabethae* [41]. They are quite simple molecules structurally, consisting of a tricyclic hydrocarbon core possessing four stereocenters, and a sugar, that is appended directly to one of the rings [42].

Zhong and collaborators [43] proposed the insertion of a methyl group between *C*-glycoside and the PsA, assessing their anti-inflammatory effect from the phorbol myristate acetate (PMA) induced inflammation in mouse ears. This paper demonstrated that this new molecule inhibits phorbol myristate acetate (PMA) induced inflammation in mouse ears in a dose-dependent manner, despite it was not significantly greater than the PsA. Furthermore, the *C*-glycoside is an effective binding agent toward adenosine receptors A_{2A} and A_3.

Flachsmann and collaborators [44] reported the synthesis and *in vivo* anti-inflammatory activity of a pseudopterosin analogues series. These ones were tested for their ability to reduce PMA-induced mouse ear edema.

Structural modifications included the substitution degree of the hexahydrophenalene core, different relative carbon configurations as well as variations of the sugar moiety, and the site of glycosidation was performed. All compounds, except for one, proved to be active in the mouse-ear assay and not professionally potency statistically differences could be identified among the compounds. This compound presented of ketone which may contribute to their ineffectiveness [44].

Acanthoic acid

Acanthoic acid is a novel pimarane-type diterpene that was first isolated from the *Acanthopanax koreanum* Nakai (Araliaceae) root bark. Studies revealed that acanthoic acid suppresses the production of IL-1β and TNF-α, being orally active and having no significant toxicity in a rodent model of chronic inflammation [45].

Inspired by the medicinal potential of acanthoic acid, researchers sought to develop structural modifications aiming to improve beyond the biological effects of the parent molecule.

Lam and collaborators [46] synthesized acanthoic acid analogues and evaluated these compounds as a TNF-α modulators. These analogues differ from acanthoic acid in the conformation or composition of the rigid tricyclic core. Between the synthesized analogs, the compound (15A), which features connection of methyl ester with the C-4 inhibited up to 99% of TNF-α production, inhibition of IL-1β and IL-6 at concentrations in which it was not cytotoxic corroborating studies. Suh and collaborators [45] revealed that C-4 modification provides the enhanced *in vitro* activities.

Another study reports syntheses of acanthoic acid analog and their *in vivo* activities as anti-inflammatory agents, according to Suh and collaborators [47]. The changes proposed in this paper occurred at C-4. Suh and collaborators [45], as well as and Lam and collaborators [46], confirmed that these changes enhance the anti-inflammatory effect.

It is demonstrated that some analogs exhibited good inhibitory activities in *in vitro* assays and in NO and COX inhibition, showing that the *in vivo* effect was compound (15B). Thus, the acanthoic acid analogs exhibited anti-inflammatory effects by regulation mechanisms of pro-inflammatory cytokine and transcription factors as well as iNOS inhibition [47].

As previously shown, the length of the linker between C-4 and the terminal carboxyl group plays an important role for the anti-inflammatory effects of the acanthoic acid analogs.

Lee and collaborators [48] described the synthesis of the C-4-chain modified acanthoic acid analogs as well as the evaluation of their inhibitory activities in NO generation in Raw 264.7 cells.

The C-4-chain length plays an important role for the NO inhibitory activity of the acanthoic acid analogs. The C-4 extension, in the two carbon homologations, improved the activity when the compound (15C) exhibited the most potent activity. That also suggests that the presence of double bond in the C-4-chain is beneficial to improve NO inhibitory activity [48].

Quinopimaric acid

Quinopimaric acid is derived from the levopimaric acids. These are abietane diterpenoids, an important class of natural products which have been used as enantiomerically pure starting materials for the production of highly effective drugs [49].

With this objective, the quinopimaric acid synthetic transformations and the evaluation of their anti-inflammatory activity from carrageenan were reported [49]. In this study, it was demonstrated that quinopimaric acid derivatives (16A), (16B), (16C), (16D) and (16E) possessed higher anti-inflammatory activities than did diclofenac. Corroborating with this, it was showed that quinopimaric and 3'-chloroquinopimaric acid possess anti-inflammatory activity [50].

Triterpenes

Esculentoside

Esculentoside A (EsA) is a kind of triterpene saponin isolated from the *Phytolacca esculenta* root. Studies reported that EsA inhibits inflammatory mediators secretion such as tumor necrosis factor (TNF), interleukins (IL-1β and IL-6) and prostaglandin E_2 in several

cell types [51]. However, haemolytic activity is the main toxicity of EsA, which needs to be overcome.

Aiming to optimize the EsA structure and explore its structure-activity relationship in order to seek the derivatives with increased biological activity and lower toxicity, so, the EsA structural modifications was proposed seeking to improve anti-inflammatory profile and reduces haemolytic effect [52].

The conversion of the C-28 carboxylic acid into an amide affected its inhibitory activity towards COX-2 and haemolytic activity. Since the most active compound was the derivative (17A), that corroborates with the study which related the EsA derivatives showed higher inhibitory effects on LPS-induced NO production and lower haemolytic activities than EsA [53].

Glycyrrhizin

Glycyrrhizin (GL) is a triterpene glycoside extracted from the *Glycyrrhiza glabra* root, consisting of glycyrrhetic acid (GA), a pentacyclic triterpene and two molecules of glucuronic acid at the C-3 position [54]. The chemical modification of glycyrrhetinic enhances the biological activity of this terpene [54]. Thus, Matsui and collaborators [55] investigated the structure-activity relation of GL derivatives about the inhibitory effect of the chemokine production on IL-8 and eotaxin 1.

Structural changes occurred at C-11, C-18 and C-30 positions of GL. Notably, GL-modified compounds, homo-30-OH-GL (18A) and hetero-30-OHGL (18B) are presumed to be good with their inhibitory activity against both IL-8 and eotaxin 1 production. Corroborating the results, Baltina and collaborators [54] demonstrated that changes of this kind enhance the biological effect of GL, in addition to alcoholic triterpenoids, which are in general more active than acidic ones [56].

Dammarane-type

A new novel triterpene naturally occurring compound based on the dammarane skeleton is (17α)-23-(*E*)-dammara-20,23-diene-3β,25-diol, which has been elucidated in the Palmyrah palm *(Borassus flabellifer)* [57]. It presents very promising immunosuppressive profile *in vitro* and *in vivo*. Based on the promising biological properties, we investigated the influence of the configuration of the (C-17) substituent about the anti-inflammatory effect [58].

This way, we identified compounds which are more thermodynamically stable and with a synthetically better accessible C-17β configuration, particularly (19A), which exhibits better *in vivo* activity from allergic contact dermatitis

Lupane

Lupane is pentacyclic triterpenoid, biosynthetically derived from the cyclization of squalene and a vast class of natural products. Its structural diversity includes a wide array of functional groups, including the betulin and its betulinic and betulonic acids derivatives [59].

Targeting the investigation of the biological activities of lupane, Reyes and collaborators [60] isolated 19 lupane triterpenes from the *Maytenus cuzcoina* root and bark, and the *Maytenus chiapensis* leaves, synthesizing the betulin analogues and rigidenol from the acetylation reaction. Then, they investigated their pharmacological activity as inhibitors of NO and PGE_2 production in macrophages.

Reyes and collaborators [60] concluded that the acetylation of betulin C-28 increases the potency and reduces the cytotoxicity of this compound. Also, the acetylation of rigidenol at C-11 (20A) or the chlorination at C-30 (20B) increases the potency of the compound. That was expected because Huguet and collaborators [56] assumed that lupane derivatives are more active when they are present in the carboxyl groups.

Betulinic acid

Betulinic acid, a pentacyclic triterpene discovered in 1995, is a compound isolated from various plants widespread in tropical regions (e.g., *Tryphyllum peltatum, Ancistrocladus heyneaus, Zizyphus joazeiro, Diospyoros leucomelas, Tetracera boliviana* and *Syzygium formosanum*) [61, 62]. It was reported that the combination of enone functionalities with cyano and carboxyl groups in ring A and an enone functionality in ring C is an essential structural feature for high potency in various bioassays related to inflammation [63].

The inhibitory activities of new synthetic triterpenoids on NO production induced by IFN-γ in mouse macrophages were evaluated, and the compounds which have the group dinitrile are much more potent than other derivatives. The 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) derivative, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, derivated of oleanolic acid, showed high inhibitory activity against the production of NO in mouse macrophages, about 100 times more potent than the CDDO. Several of these compounds presented *in vivo* anti-inflammatory activity, i.p. or p.o., against peritoneal inflammation induced by thioglycollate and IFN-γ [63].

Honda and collaborators [64] showed that several new semi-synthetic betulinic acid analogues display highly potent anti-inflammatory activity *in vitro*. Moreover, the compound (22A) was highly and orally active *in vivo*. In the inhibition of NO production in RAW 264.7, cells stimulated with interferon-γ and induction of the anti-inflammatory, cytoprotective enzyme, heme oxygenase-1 in the liver (*in vivo*), the compounds with a cyano enone functionality in ring A were highly active. A similar effect was showed for the CDDO, whereas betulinic acid was inactive. The new analogue (22A), oral dosing of 2 μM, presented significantly more potency *in vivo* than both betulinic acid and the oleanolic acid analogue, CDDO [64].

Recently, it was demonstrated that betulinic acid (20 and 40 mg/kg) reduced the paw edema at 3, 4 and 5 h after λ-carrageenan administration by detecting the levels of cyclooxygenase-2 (COX-2), nitric oxide (NO), tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and malondialdehyde (MDA) in the tissue [65], which confirms the results obtained from betulinic acid analogues that were described previously [64].

Novel tricyclic compounds having acetylene groups at C-8a and cyano enones in rings A and C are a novel class of potent anti-inflammatory, cytoprotective, growth-suppressive and pro-apoptotic compound shave. Some C-8a functionalized analogues using new tricycles as starting materials were used and evaluated as to their potency for inhibition of NO production in RAW 264.7 cells stimulated with interferon-γ. The compounds with acetylene groups were the most potent *in vitro* and *in vivo* bioassays as anti-inflammatory [66].

Thus, carboxyl, methoxycarbonyl, and nitrile groups at C-2 enhanced activity, while hydroxyl, aminocarbonyl, methoxy, chloride and bromide groups decreased it. For some analogues, triterpenoids bearing C-28 in the carboxyl group were more potent than C-28 methyl; esters, but for other similar activity or even less potent activities, were observed when C-28 was carboxylic acid [67].

Faradiol

Faradiol is a monoester pentacyclic triterpenoid obtained from *Calendula officinalis* L. flowers [68]. With the goal to improve the anti-inflammatory activity of faradiol, Neukirch and collaborators [69] proposed a modification of the chemical groups of the monoester or the introduction of new functional groups. Selective chemical modifications, such as changes to the ester function at C-3 (ring A), the free OH group at C-16 (ring D) and the C=C bond in ring. It was proved that the substitution of methyl groups at C-30 to alcohol (23A) or to aldehyde (23B) markedly improved the anti-inflammatory potency of faradiol [69].

Boswellic acids

Boswellic acids (BA's) are triterpenoid pentacyclic acids isolated from *Boswellia carterii*. Several studies reported that the biological activity can highlight the inhibitory activity of 5-lipoxygenase, the key enzyme of leukotriene biosynthesis [70]. Henkel and collaborators [71] proposed modifications to BA's of type C-3 (OH or acetoxy group) and C-11 (oxo moiety present or absent) position, yielding 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA), 3-*O*-acetyl-β-boswellic acid (Aβ-BA), 11-keto-β-boswellic acid (KBA) and β-boswellic acid (β-BA). For assessing the anti-inflammatory effect of BA's derivatives, the LPS activities and of iNOS expression were verified.

Polar residues were found in C-3 position and the absence of the 11-keto group are structural determinants required for the inhibition of LPS activity and LPS-induced iNOS expression inhibition without significantly affecting cell viability up to 10 μM [71]. Setting a paradox, Siddiqui and collaborators [72] found that this dual inhibitory action on the inflammatory process is unique to BA's. Of these BA's derivates, 3-acetyl-11-keto-β-boswellic acid (AKBA) is the most potent inhibitor of 5-LO, an enzyme responsible for inflammation.

The need to find drugs which can effectively attenuate inflammation led the researchers to search drugs derived from structural changes in terpenes. This review shows terpenes, as betulinic acid and andrographolide that suffered some structural changes, getting more active compounds than the parent molecule. The importance of the terpenes structural change in the search for an effective drug led the researchers to discover docetaxel, a derivative of taxol (diterpene) more potent against cancer cells. That leads us to believe that the terpenes modification is an interesting tool for the discovery of a drug with a good anti-inflammatory effect.

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Figure 1. Flowchart of included studies.

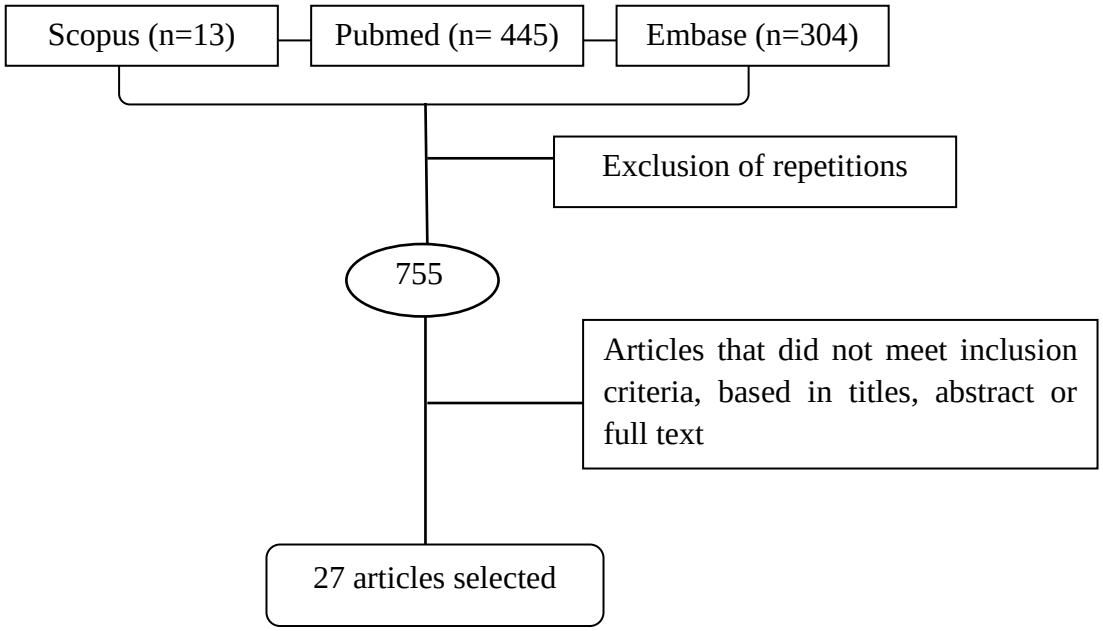
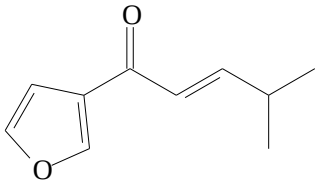
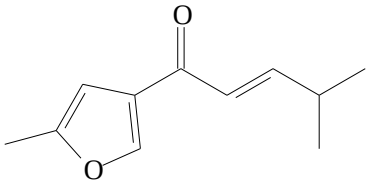


Figure 2: Structures of terpenes derivatives.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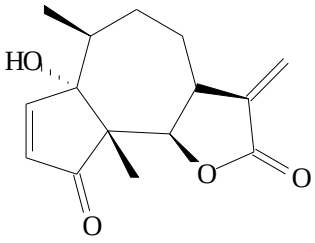	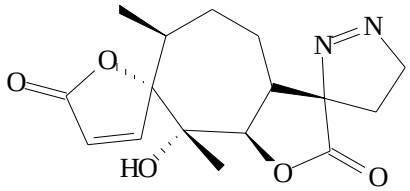
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	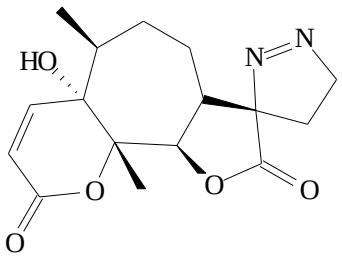
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	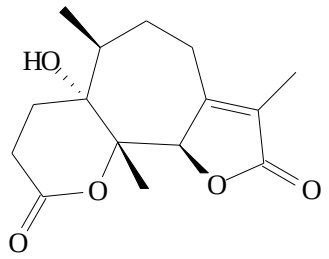
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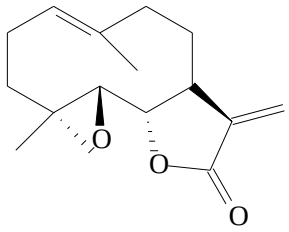	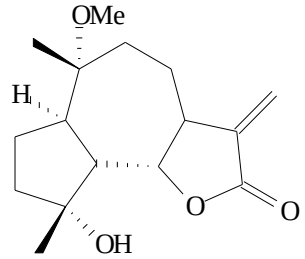
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	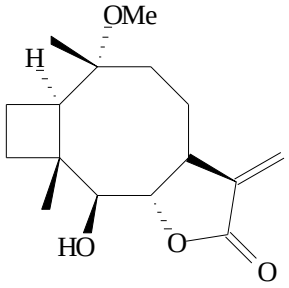
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	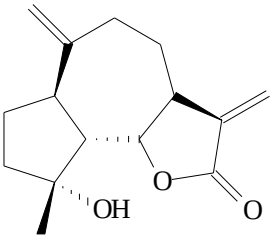
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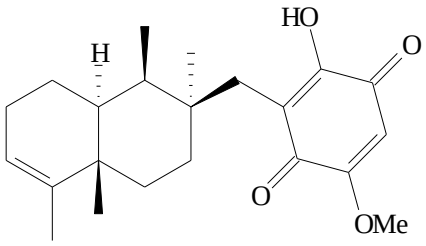	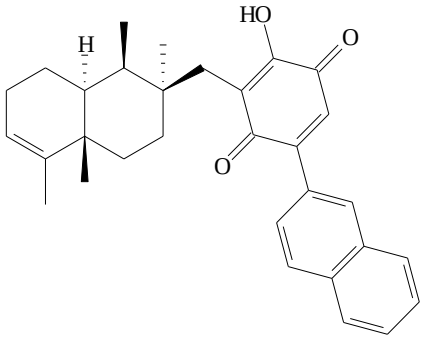
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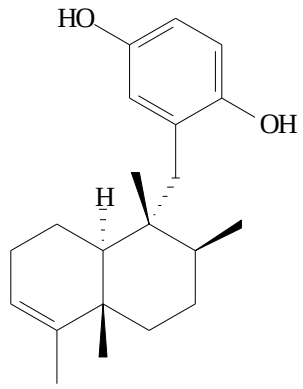	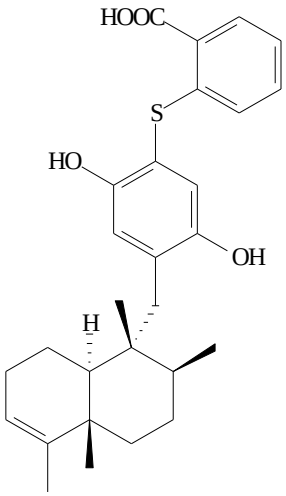
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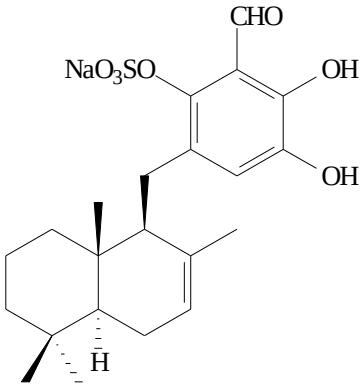	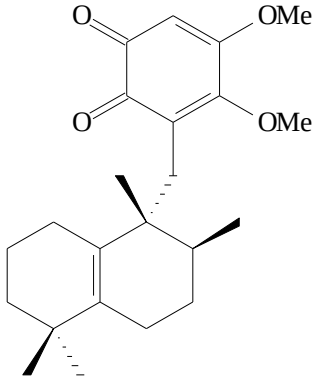
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	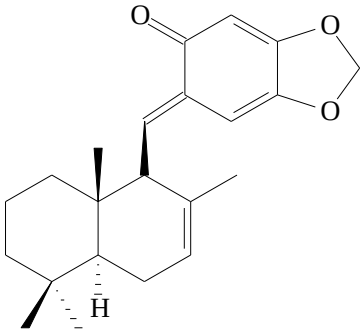
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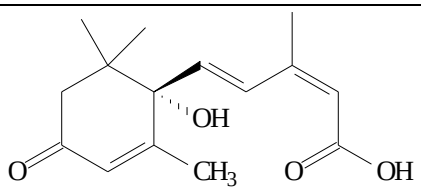	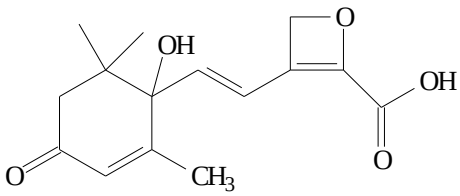
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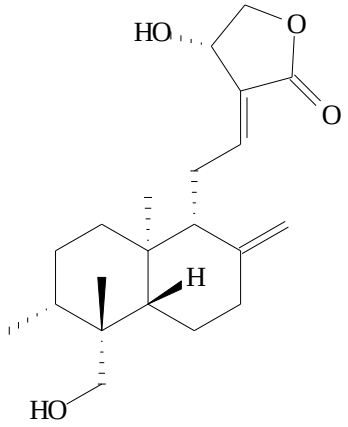	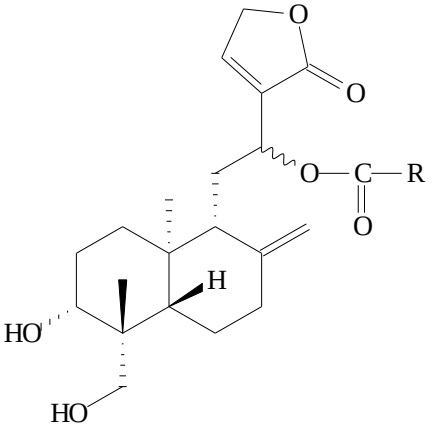
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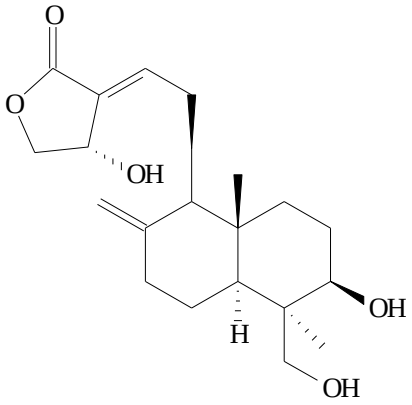	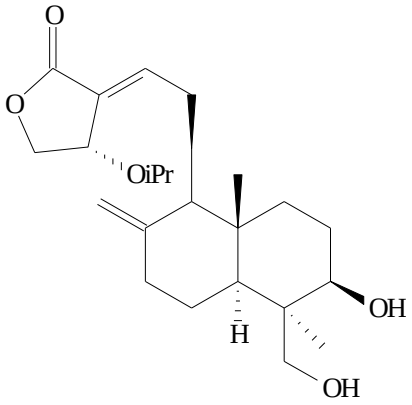
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	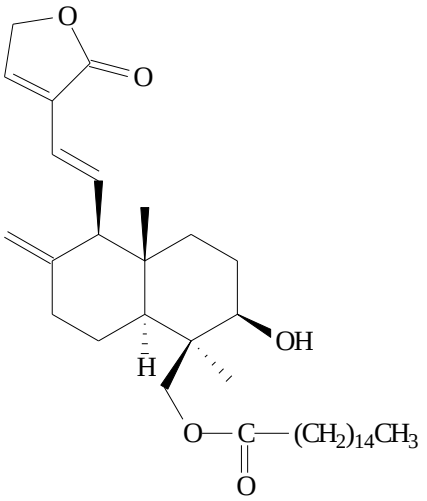
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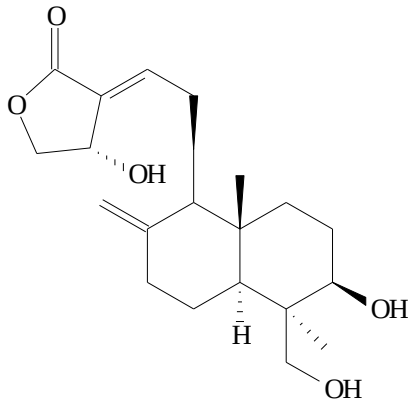	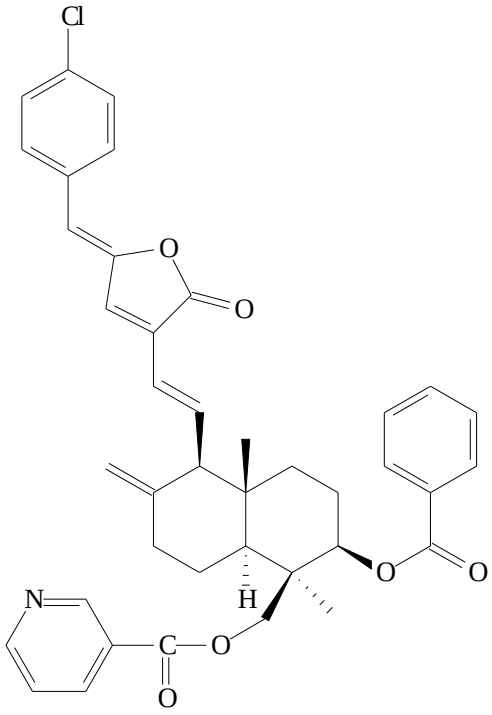
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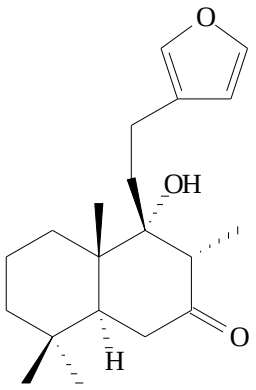	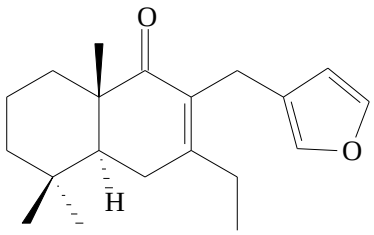
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	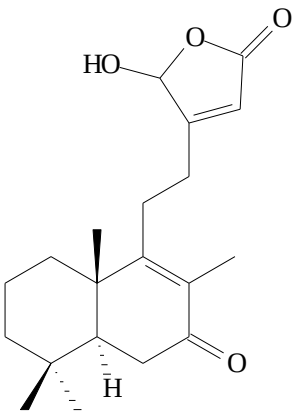
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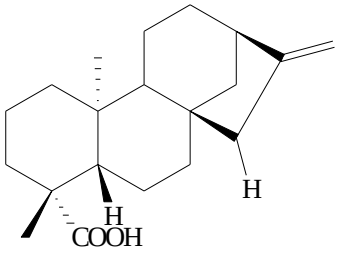	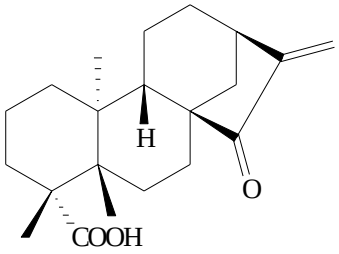
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	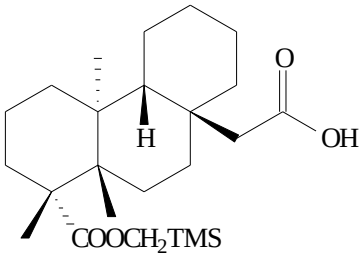
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	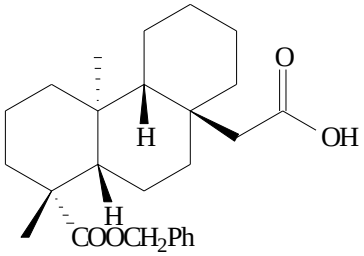
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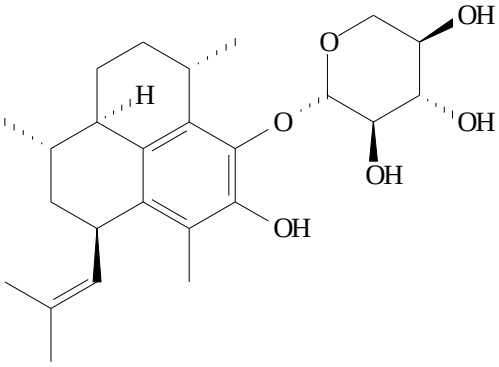	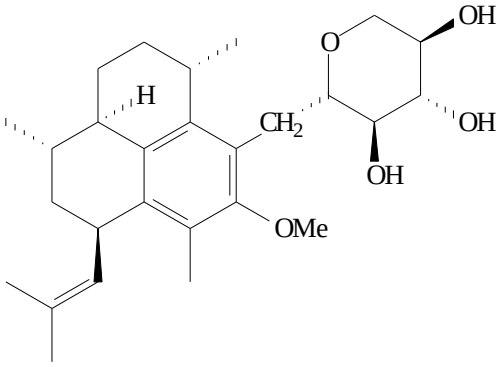
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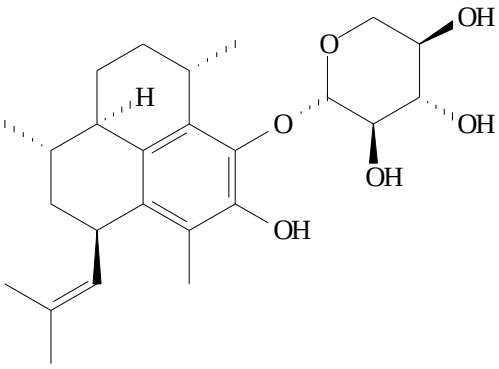	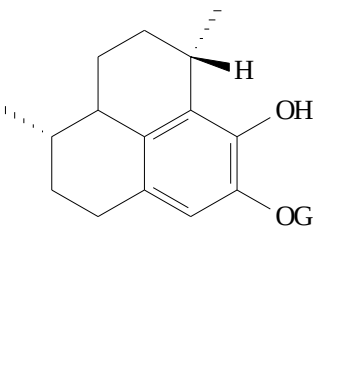
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	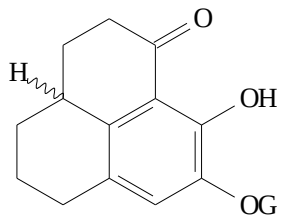
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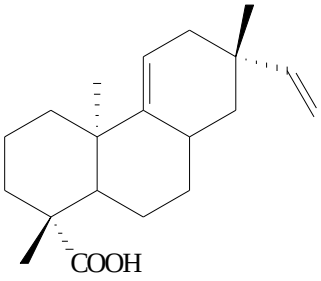	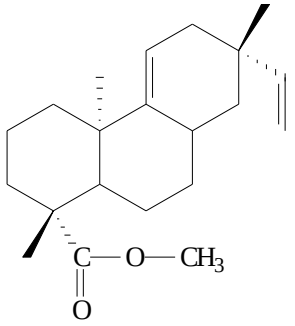
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	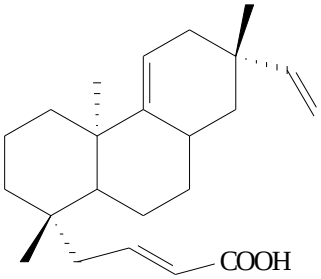
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	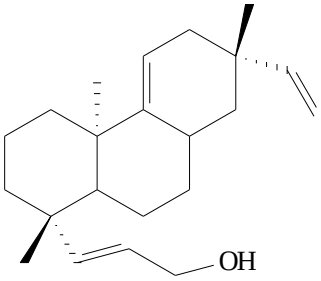
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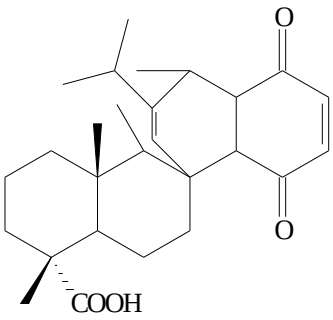	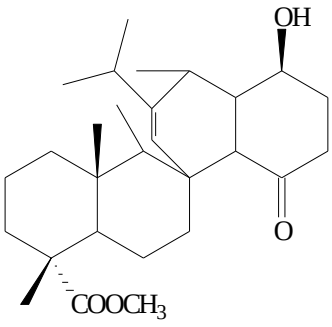
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	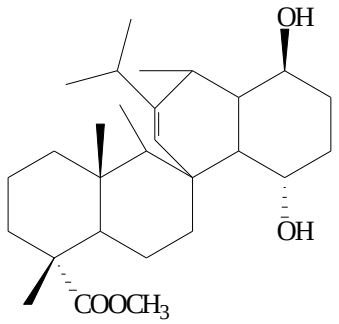
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	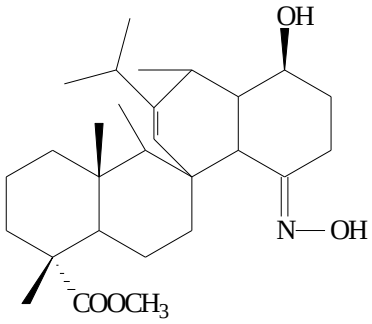
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	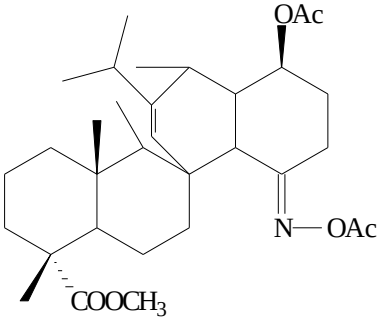
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	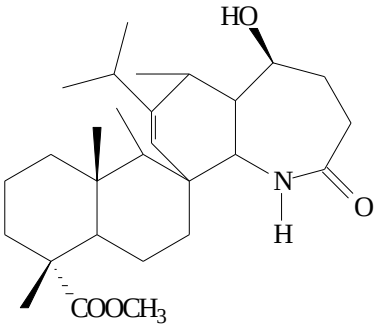
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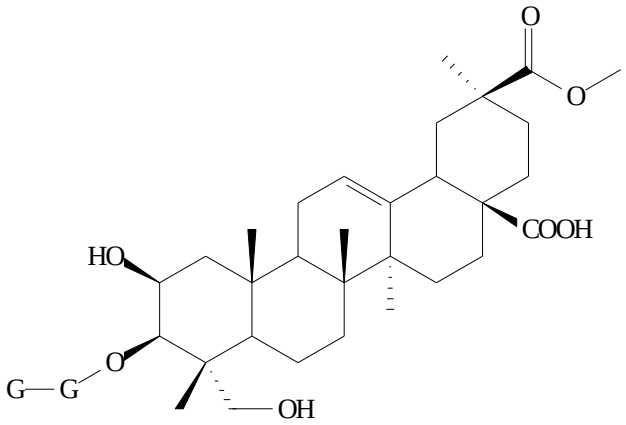	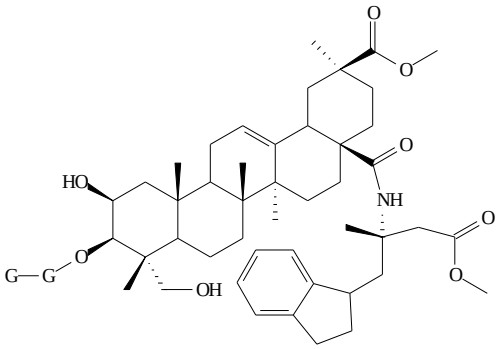
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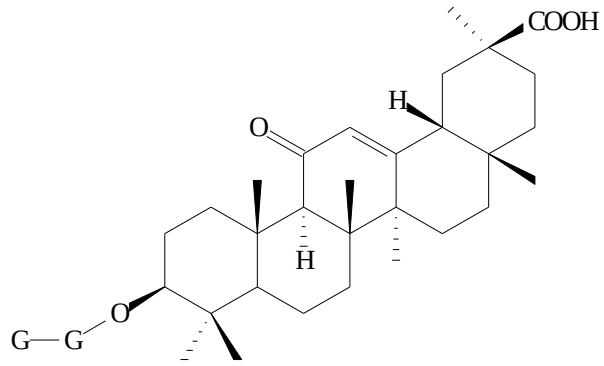	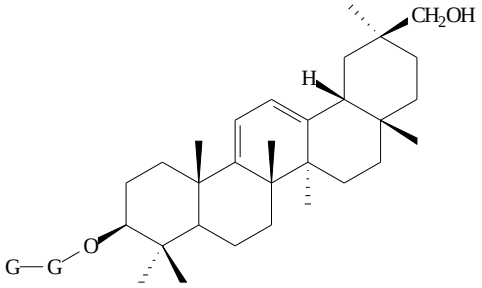
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	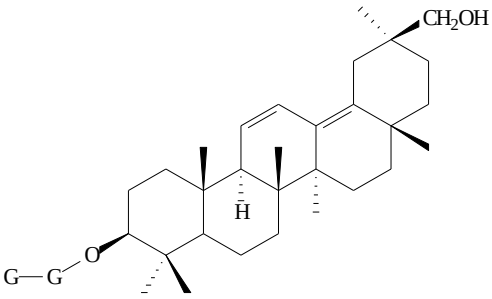
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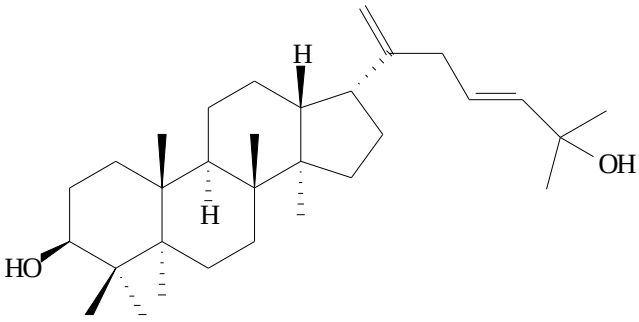
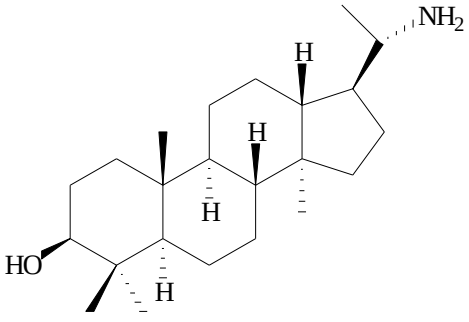
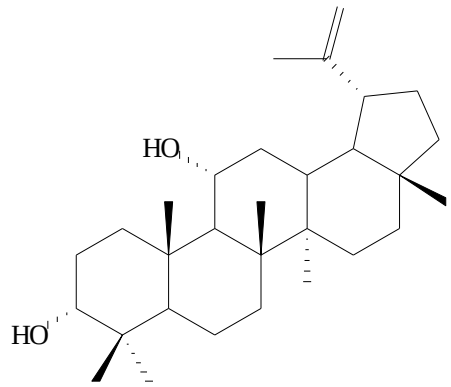
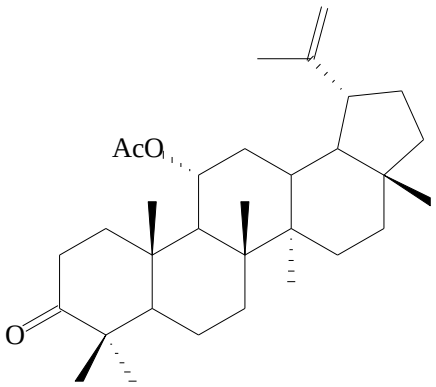
	
(19)	**(19A)**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	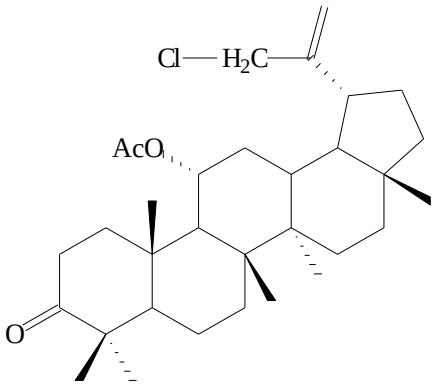
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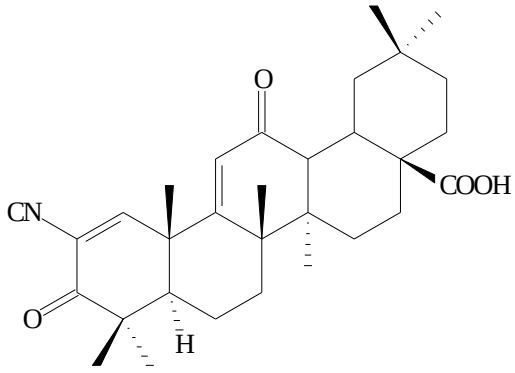	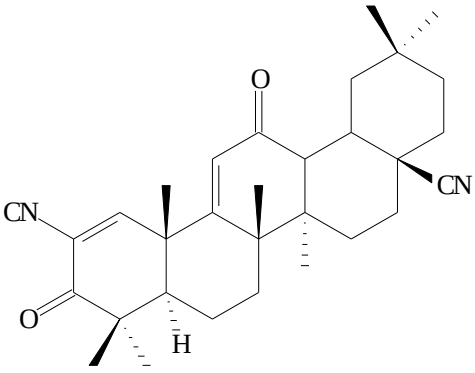
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	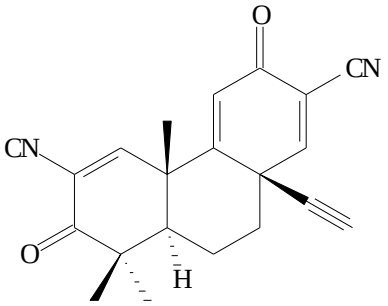
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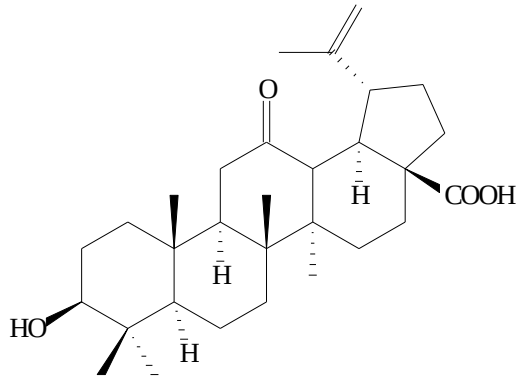	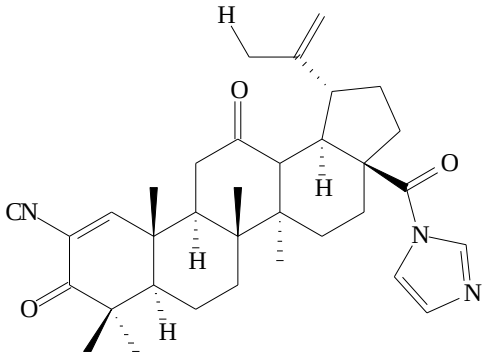
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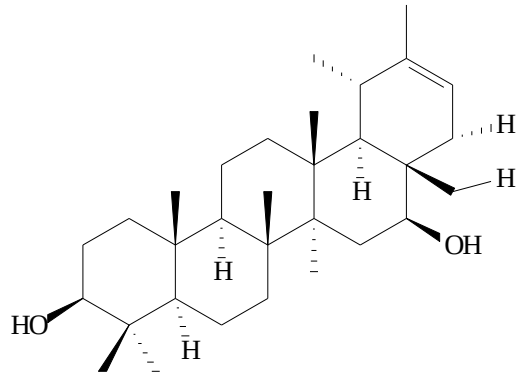	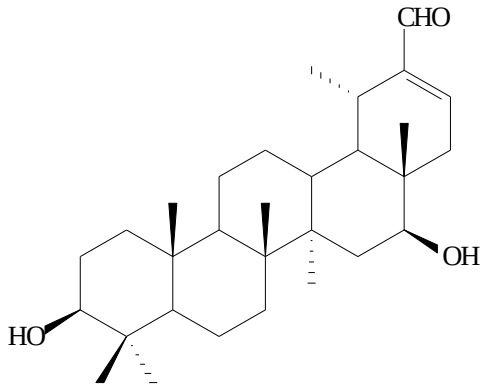
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	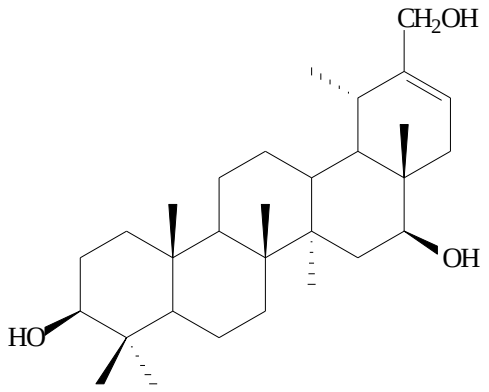
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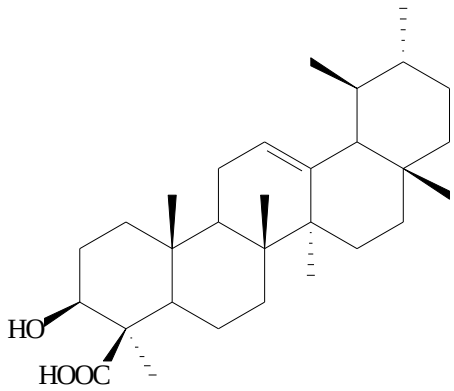
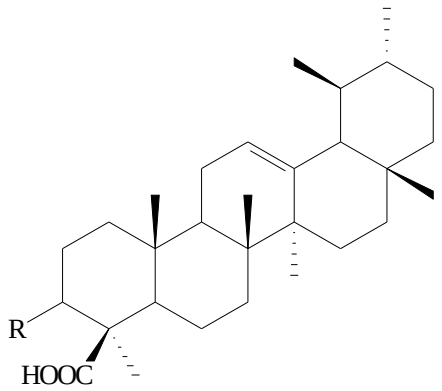
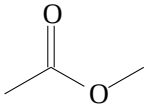
	
(**24**)	(**24A**) R= 

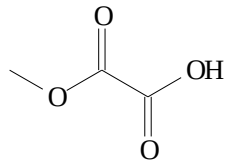
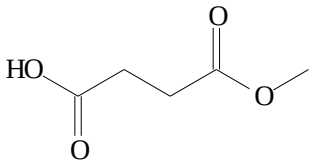
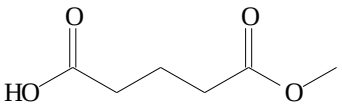
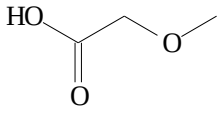
	(**24B**) R= 
	(**24C**) R= 
	(**24D**) R= 
	(**25E**) R= 

Table 1. Description of the modification chemical of the terpenes and pharmacological aspects of the studies included in systematic review.

Ref	Terpene	Source	Methods used	DE/CE	Route	Animal/Cell	Result	Country
Park et al., 2011	Isoegomaketone (**1**)	Isolated from *Perilla frutescens*	Me NO; Me MCP-1; Me IL-6; LA NF-kB; LA AP-1			RAW 264.7 cells	(1A)	Korea
Chib et al., 2011	Parthenin (**2**)	Isolated from *Parthenium hysterophorus*	Me TNF-α; Me IL-1β; Me IL-6	1 µg/ml		Ne Murine	(2A) (2B) (2C)	India
Neukirch et al., 2003	Parthenolide (**3**)		Me IL-8	10 pg/ml 1 ng/ml 100 ng/ml 10 µg/ml		Ne Human	(3A) (3B) (3C)	Italy
Petronzi et al., 2010	Bolinaquinone (**4**)		Me PGE_2			RAW 264.7 cells	(4A)	Italy
Amigó et al., 2004	Avarol (**5**)	Isolated from *Dysidea avara*	Me PGE_2	5 µM		CHK HaCaT cell line	(5A)	Spain
Laube et al., 2009	Siphonodictyal (**6**)	Synthesis	Ac 3α-HSD; Pr ROS			Gr	(6A) (6B)	Germany
Grozio et al., 2011	Abscisic acid (**7**)		Me PGE_2; Me MCP-1; Pr ROS; CBHG	0.5 nM; 1 nM; 10 nM; 100 nM; 1 µM; 5 nM		Gr and Mo Human	(7A)	Italy

Table 1 (Continued)

Ref	Terpene	Obtainment	Methods used	DE/CE	Route	Animal/Cell	Result	Country
Li et al., 2007	Andrographolide (**8**)		Me TNF-α; Me IL-6	20 μM		J774A.1 cells	(8A) (8B) (8C)	China
Suebsasana et al., 2009	Andrographolide (**9**)	Isolated from *A. paniculata*	EPICg; WT	4 mg/kg	i.p.	Mice and Rats SD	(9A) (9B)	Thailand
Dai et al., 2011	Andrographolide (**10**)	Furen Medicines Group, Pharmaceutical Co., Ltd.	EEIDd; EPIEA; Pr NO; Ac iNOS	0.45 mmol/kg 0.9 mmol/kg 1.35 mmol/kg	i.g.	Mice Kunming and Rats SD	(10A)	China
Girón et al., 2008	Hispanolone (**11**)	Isolated from *Ballota hispanica*	Sy NO; In NOS-2; In COX-2; Ex IL-6; Ex mRNA; Me TNF-α; EEITPA; Ac NF-κB; Ac MAPK; Ac IKK	1 μM 10 μM 20 μM 50 μM 0.25 mg/ear 0.5 mg/ear 1 mg/ear	a.t.	RAW 264.7 cell and Swiss mice	(11A) (11B)	Spain
Hueso-Falcón et al., 2011	*Ent*-kaurene (**12**)	Synthesis	Pr NO; Ex NOS-2; Ex mRNA; Ac NF-kB; Me IL-6	1 μM 5 μM 10 μM 25 μM 50 μM		RAW 264.7 cell	(12A) (12B) (12C)	Spain
Zhong et al., 2008	Pseudopterosin (**13**)		EEIPMA; BAR A_{2A} and A_3	17 μg/ear	a.t.	Mice	(13A)	USA

Table 1 (Continued)

Ref	Terpene	Obtainment	Methods used	DE/CE	Route	Animal/Cell	Result	Country
Flachsmann et al., 2010	Pseudopterosin (**14**)		EEIPMA	25 µg/ear	a.t.	Mice	(14A) (14B)	USA
Lam et al., 2003	Acanthoic acid (**15**)	Isolated from *Perilla frutescens*	Me TNF-α			HPBMC cells	(15A)	USA
Suh et al., 2004	Acanthoic acid (**15**)	Isolated from *Perilla frutescens*	In COX-2; In NO; AICFA	5 mg/kg 15 mg/kg 25 mg/kg	i.p.	Raw 264.7 cells and Rats	(15B)	South Korea
Lee et al., 2005	Acanthoic acid (**15**)		Me NO			Raw 264.7 cells	(15C)	Korea
Kazakova et al., 2010	Quinopimaric Acid (**16**)		EPICg	50 mg/kg 100 mg/kg	i.g.	Rats	(16A) (16B) (16C) (16D) (16E)	Russia
Wu et al., 2007	Esculentoside (**17**)		Ex hCOX-2	10 µM		sf-9 cells	(17A)	China
Matsui et al., 2004	Glycyrrhizin (**18**)	Isolated from *Glycyrrhiza uralensis*	Me IL-8; Me eotaxin 1; Ex IL-8; Ex eotoxin 1 Ex mRNA	1 µg/ml 10 µg/ml 30 µg/ml 100 µg/ml		HFL-1 cells	(18A) (18B)	Japan
Scholz et al., 2004	Dammarane-type (**19**)	Syntesis	ACD	0.1 M	a.t.	Mice	(19A)	Austria

Table 1 (Continued)

Ref	Terpene	Obtainment	Methods used	DE/CE	Route	Animal/Cell	Result	Country
Reyes et al., 2006	Lupane (**20**)	Isolated from *Maytenus cuzcoina*	Pr NO; Pr PGE_2	5 µM 10 µM		RAW 264.7 cell	(20A) (20B)	Spain
Honda et al., 2002	CDDO (**21**)		Pr NO			Ma	(21A)	USA
Honda et al., 2007	CDDO (**21**)		Pr NO			RAW 264.7 cells	(21B)	USA
Honda et al., 2006	Betulinic acid (**22**)		Pr NO			RAW 264.7 cells	(22A)	USA
Neukirch et al., 2005	Faradiol (**23**)		EEIC		a.t.	Mice	(23A) (23B)	Italy
Henkel et al., 2012	Boswellic acids (**24**)	Isolated from *Boswellia sp*	Ex iNOS	10 µM		RAW 264.7 cells	(24A) (24B) (24C) (24D) (24E)	Germany

Methods abbreviations: ME, Measurement; LA, Luciferase assay; Ac, Activity; Pr, Production; CBHG, Competition Binding on Human Granulocytes; EPICg, Carrageenan Induced Paw Edema; WT, Writhing Test; EEIDd, Dimethylbenzene Induced Ear Edema; EPIEA, Egg Albumin Induced Paw Edema; Sy, Synthesis; In, Induction, Ex, expression; EEITPA, Tetradecanoylphorbol-13-Acetate Induced Ear edema; EEIPMA, Phorbol Myristate Acetate Induced Ear Edema, BAR, Bind to Adenosine Receptors, AICFA, Arthritis Induced by Freund's Complete Adjuvant; ACD, Allergic Contact Dermatitis; EEIC, Croton oil Induced Ear Edema; MCP-1, Monocyte Chemoattractant Protein 1; ROS, reactive oxygen species; 3α-HSD, 3α-Hydroxysteroid Dehydrogenase.
Abbreviations of administration routes: a.t., Administration Topically; i.g., Intragastrically; i.p., Intraperitoneally.
Abbreviations of animal/cell: Ne, Neutrophils, CHK, Cultured Human Keratinocyte; Gr, Granulocytes; Mo, Monocytes; MA, Macrophages; SD, Sprague Dawley; HPBMC cells, Human Peripheral Blood Mononuclear; HFL-1 cells, Human Fetal Lung Fibroblastos; Sf, *Spodoptera frugiperda*.

3.2 CAPÍTULO 2

SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF CARVACROL PROPIONATE

Artigo publicado ao periódico:

Inflammation

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Synthesis and pharmacological evaluation of carvacrol propionate

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Abstract

This study aimed at synthesizing the carvacrol propionate (CP) and evaluating its pharmacological profile. CP was obtained from carvacrol and propionyl chloride through an esterification reaction. Male Swiss mice were treated with CP (25, 50 or 100 mg/kg). We evaluated the analgesic effect, mechanical hyperalgesia and anti-inflammatory effect. Pretreatment with CP inhibited ($p < 0.01$ and 0.001) the formalin-induced nociception in both phases. CP inhibited ($p < 0.05$, 0.01 and 0.001) the development of mechanical hyperalgesia. CP was able to decrease the leukocyte recruitment ($p < 0.001$) and the amount of TNF-α ($p < 0.001$), IL-1β ($p < 0.05$) and protein leakage ($p < 0.01$) into the pleural cavity. In addition, the paw edema was inhibited by CP ($p < 0.05$, 0.01 and 0.001). The CP attenuates nociception, mechanical hyperalgesia and inflammation, through an inhibition of cytokines.

Key-words: Terpene, carvacrol propionate, hyperalgesia, inflammation, pain.

1.0 Introduction

The inflammatory response is an important cause of painful conditions, resulting from tissue injury. The tissue damage occurs due to the accumulation of various cell types, such as masts, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes and fibroblasts [1]. These cells produce a variety of mediators, such as neurotrophic factors, neuropeptides, prostanoids and kinins which, by acting on their own receptors, contribute to alter the firing pattern of the primary sensory neurons, leading to inflammatory pain. Those are peripheral sensitization changes in the chemical environment of the nerve fiber [2].

Currently, there are two approaches often used as therapeutic management for the inflammatory pain. The first clinical alternative and the most widely used is the Non-steroidal anti-inflammatory drugs (NSAIDs), which block the formation of pro-inflammatory mediators, reducing the inflammatory pain by inhibiting the cyclooxygenases (COX-1 and COX-2), as, for example, the aspirin, indomethacin and ketoprofen [3]. The second treatment option is the desensitization of nociceptors through the stimulation of expression of potassium channels, which hyperpolarize the cell, decreasing the established hyperalgesia, such as opioid drugs [4].

However, new strategies for treating the inflammatory pain are needed, once the current treatment is limited because of side effects and tolerance [5, 6]. Thus, great effort has been expended on the development of drugs for the treatment of inflammation.

In this context, natural products are employed worldwide in folk medicine to treat different painful and inflammatory conditions [7, 8]. Plants, fungi, marine organisms and bacteria are the source of potentially active chemical substances, being considered as raw materials, i.e, the starting point for the discovery of new

pharmacologically active molecules [9-11]. Most drugs used in pre-clinical or clinical studies are of natural origin and have been developed from these structural changes [12]. The structural modification of natural products showed promising activities that must be seen as an interesting source of new structures, with the possibility of presenting an important biological activity [13].

Within the natural products, we can highlight the monoterpenes, main chemical constituents of the plant essential oils with anti-inflammatory properties. Recently, Guimarães et al. [8] suggested that monoterpenes are possible candidates for the treatment of painful conditions. These results were corroborated by De Cassia da Silveira e Sá et al. [7], who identified 32 monoterpenes with anti-inflammatory activity, such as menthol, citral, (±)-citronellal, (+)-limonene, thymol, carvacrol, linalylacetate and linalool, among others.

Aiming to improve the biological activity, the research has modified the structure of monoterpenes, through specific chemical reactions, resulting on derivatives [14]. Studies have shown the importance of these chemical modifications. Hydroxy-di-hydrocarvone, which is a synthetic derivative of carvone, possesses anti-inflammatory [15] and antinociceptive activity [16]. Carvone or active analogs inhibit nerve excitability in accordance with different chemical structures [17]. According to De Sousa et al. [18], monoterpenes properly derivatized enable results on new analgesic drugs. For example, proprionate of carvacrol (CP), which is a monoterpene derivative obtained by the esterification of carvacrol. Although its synthesis is known, there is only one study demonstrating its antimicrobial activity [19]. Hence, it is necessary to conduct studies to evaluate the pharmacological activity of carvacrol propionate in models of nociception, hyperalgesia and inflammation.

2.0 Materials and Methods

2.1 Drugs and reagents

Carrageenan (CG), tumor necrosis factor-alpha (TNF-α), prostaglandins-E2 ($PGE_{2)}$, dopamine (DA), cremophor, carvacrol, propionylchloride, ethylenediaminetetraacetic acid (EDTA), Griess reagent, Türk solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Saint Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) for mouse´s quantitative determination of TNF-α and IL-1β was obtained from BD-Bioscience Pharmingen (San Diego, CA, USA). Indomethacin and dipyrone were obtained from União Química (São Paulo, Brazil). Diazepam (DZP) was purchased from Cristália (São Paulo, Brazil). Ethyl acetate, hexane and triethylamine were obtained from Vetec (Rio de Janeiro, Brazil).

The CP was dissolved in 0.9% saline and 0.2% cremophor, used as an emulsion, for pharmacological experiments. The other substances were solubilized with distilled water or saline. In these protocols, the agents were injected intraperitoneally (i.p.) at volumes of 0.1 mL/10 g. All doses and route of administration of the CP were chosen according to Quintans-Júnior et al. [20] and Guimarães et al. [21].

2.2 Synthesis and characterization of carvacrol propionate (CP)

CP was prepared from carvacrol using the method of Dolly and Barba [22] and characterized by ^{1}H and ^{13}C nuclear magnetic resonance, mass spectrometry and infrared spectroscopy.

To obtain the CP, carvacrol (5.15 mL; 33.33 mmol) dissolved in THF was added to propionyl chloride (4.62 mL; 50 mmol) in THF to form the ester derivative in the

presence of triethylamine (5.07mL; 50 mmol). The reaction was stirred for 2 h at room temperature. The reaction mixture was concentrated under vacuum, diluted with water and extracted with dichloromethane. The organic layer was washed with water, and dried over Na_2SO_4. The solvent was distilled off and the residue purified through silica gel column chromatography (Hex:EtOAc, 99:1) yielding CP 72.81% as a yellowish oil. The compound obtained was characterized by ^{1}H and ^{13}C NMR, mass spectrometry and infrared spectroscopy.

NMR data were recorded on a Bruker DRX400 spectrometer using CDCl3 as solvent and tetramethylsilane (TMS) as an internal standard, and the chemical shifts are reported in ppm (δ). Coupling constants (J) are reported in hertz (Hz). The abbreviations used are *s* (singlet), *d* (doublet), *t* (triplet), *q* (quadruplet), *sept* (septuplet). FT-IR was recorded on a Perkin Elmer Spectrum BX FT-IR System. Mass spectra were recorded on a Shimadzu GCMS-QP2010S Gas Chromatograph Mass Spectrometer (equipped with an AOC-20S auto sampler).

2.3 Animals

Adult (approximately 3 months old) male Swiss mice (28-32 g) were randomly housed in appropriate cages at 21 ± 2°C on a 12 h light/dark cycle (lights on 06:00 a.m. to 6:00 p.m.), with free access to food (Purina®, Brazil) and tap water. All experiments were carried out between 09:00 a.m. and 16:00 p.m. in a quiet room. All nociceptive, hyperalgesia and inflammatory tests were carried out by the same visual observer, double-blinded and all efforts were made to minimize both the number of animals and any discomfort inflicted upon them. Experimental protocols were approved by the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS #

35/12) and handling procedures were in accordance with the International Council for Laboratory Animal Science (ICLAS) and National Institute of Health (NIH).

2.4 Formalin induced nociception

The formalin test was carried out as described by Hunskaar and Hole [23]. The animals were treated with the vehicle (saline + cremophor 0.2%), CP (25, 50, and 100 mg/kg, i.p.) or morphine (3 mg/kg, i.p.) 30 min before the formalin injection. Formalin (1%; 20 μL) was injected into the dorsal surface of the right hind paw using a microsyringe with a 26-gauge needle. These mice were individually placed in a transparent plexiglass cage observation chamber (25 cm × 15 cm × 15 cm). The amount of time spent licking the injected paw was indicative of pain. The number of lickings from 0-5 min (first phase) and 15-30 min (second phase) was counted after the injection of formalin.

2.5 Hot-plate test

The hot-plate test was used according to Kuraishi et al. [24]. The animals were placed on an aluminum plate that was adapted to a water bath at 55 ± 0.5°C. The reaction time was noted by observing the licking of the hind paws at basal, 0.5, 1.0, 1.5, and 2.0 h after i.p. administration of vehicle, CP or morphine to different groups of 6 mice.

2.6 Hyperalgesia induced by CG, TNF-a, PGE_2 and dopamine

This study was performed according to Cunha et al. [25] and Villarreal et al. [26]. Mice were divided into five groups (n = 6, per group), which were treated with vehicle (saline + cremophor 0.2% v/v, i.p.), CP (25, 50 or 100 mg/kg, i.p.),

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 hyperalgesia was evaluated at 30, 60, 120 and 180 min after the injection of algogen agents.

2.7 Measurement of mechanical hyperalgesia

Mechanical hyperalgesia was tested in mice as reported by Cunha et al. [25]. In a quiet room, 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®, Ribeirão Preto, 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 the treatments with vehicle, CP or control drugs, and at selected times after the injection of the nociceptive agents. The protocol was carried out blindly, where the researcher who performed the measures did not know which group the animal belonged to. The results are presented as the Δ withdrawal threshold (g), calculated by the difference between the values obtained after the treatment and before the treatment [25].

2.8 Carrageenan-induced pleurisy

Pleurisy was induced by intrathoracic (i.t.) injection of CG (300 μg; 0.1 mL) diluted in sterile saline. Control animals received the same volume of vehicle. The animals were pretreated as described above 30 min before the injection of the inflammatory agent. Four hours after stimulation, the animals were sacrificed in a CO_2 chamber; the pleural cavities were opened and washed with 1 mL of PBS (1x) containing EDTA (10 mM). Total leukocyte counts collected in the pleural lavage were performed on a Neubauer chamber under an optical microscope. The samples were diluted (40x) in Türk solution. The differential leukocyte analysis was performed under a light microscope with immersion oil objective in cytocentrifuged smears colored with May-Grunwald-Giemsa, on which 100 cells per slide were counted. The amounts of TNF-α and IL-1β produced in the pleural cavity were assessed 4 h after injection of CG. The recovered pleural lavage was centrifuged at 770 *xg* for 10 min. TNF-α and IL-1β were quantified on supernatant free of cells through enzyme immunoassay (ELISA) using matched antibody pairs from R&D Systems (Minneapolis, MN, USA; Quantikine), according to the manufacturer's instructions. The measurement of total protein was held collecting the fluids recovered from the pleural cavity of the animals, which were centrifuged for 10 min at 1.500 ×*g*, and the total protein content was quantified in the supernatant, at 540 nm, using the Bradford reagent.

2.9 MTT cell viability assay

The cytotoxic effect of CP on macrophages was determined using the MTT assay method according to Mosmann [27]. Murine peritoneal macrophages (2.5×10^5 cells) were treated with CP at concentrations ranging from 1.0 μg/mL to 500.0 μg/mL and were further cultured in RPMI-1640 supplemented with 10% FBS for 24 h.

Thereafter, the medium was replaced with fresh RPMI containing 5 mg/mL of MTT. After additional 4 h of incubation at 37°C, the supernatant was discharged and DMSO solution (150 μL/well) was added to each culture plate. After 15 min of incubation at room temperature, absorbance of solubilized MTT formazan product was spectrophotometrically measured at 540 nm. Five individual wells were assayed per treatment and percentage of viability was determined in relation to controls [(absorbance of treated cells/absorbance of untreated cells) x 100].

2.10 Measurement of paw edema

The effect of CP on edema formation caused by the intraplantar injection of CG was analyzed according to the method previously reported by Levy [28]. The animals were divided into five groups (n = 6, per group) and treated as described above. Right paw volume was measured by the dislocation of the water column of a plethysmometer before (time zero) and at 1, 2, 3, 4, 5 and 6 h after subplantar injection of 40 μL of CG (1%). Paw edema was expressed (in milliliter) as the difference between the volume of the paw after and before CG injection. The area under the curve (AUC [0–240 min]; in milliliter per minute) was also calculated using the trapezoidal rule.

2.11 Spontaneous locomotor activity

Mice were divided into five groups (n = 6, per group) and treated with vehicle, CP or diazepam (1.5 mg/kg; i.p.). The spontaneous locomotor activity was assessed in a cage activity (50×50×50 cm) at 0.5, 1, and 2 h after the treatment [29].

2.12 Evaluation of the motor activity

Initially, mice able to remain on the rota-rod apparatus (AVS®, Brazil) longer than 180 sec (7 rpm) were selected 24 h before the test [30]. Then, the selected animals were divided into five groups (n = 6, per group) and treated intraperitoneally as described above. Each animal was tested on the rota-rod and the time (sec) that they remained on the bar for up to 180 s was recorded after 30, 60, and 120 min of the treatment.

2.13 Statistical analysis

Data were evaluated using GraphPad Prism Software Inc. (San Diego, California, USA) version 5.0. Formalin, hot-plate, pleurisy and MTT tests, as well as the evaluation of the motor through the one-way analysis of variance (ANOVA) were followed by Tukey's test. While mechanical hyperalgesia and edema of paw the data obtained were evaluated by the two-way analysis of variance (ANOVA) to compare the groups and doses at all times. If a significant interaction between the factors evaluated (treatment and time) was detected, Bonferroni´s post-test was used. The results are presented as mean ± SEM. In all cases, the differences were considered significant if $p < 0.05$.

3.0 Results

3.1 Synthesis and characterization of propionate carvacrol (CP)

The synthesis resulted in the formation of CP (Fig. 1) yielding 72.81% as clear oil and the characterization is in agreement with previous literature data [19].

IR (film, cm^{-1}) 1760 (C=O). ^{1}H RMN (400 MHz, $CDCl_3$) δ 7.10 (*d*, 1H, *J* = 7.8 Hz, Ar-H), 6.98 (*d*, 1H, *J* = 7.8 Hz, Ar-H), 6.85 (*s*, 1H, Ar-H), 2.84 (*sept*, 1H, *J* = 6.8 Hz, C<u>H</u>$(CH_3)_2$), 2.56 (*q*, 2H, *J* = 7.6 Hz, CO-C<u>H</u>$_2$-CH_3), 2.10 (*s*, 3H, Ar-CH_3), 1.25 (*t*, 3H, *J*

= 7.5 Hz, CO-CH_2-C<u>H</u>$_3$), 1.21 (*d*, 6H, *J* = 6.9 Hz, CH(C<u>H</u>$_3$)$_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.5, 149.3, 147.9, 130.8, 127.1, 124.0, 119.7, 33.6, 27.6, 23.9, 15.7, 9.2. MS (EI) *m/z* $[M]^+$ 206.

3.2 Effect of CP on formalin-induced nociception

In the test of nociception induced by formalin, CP (at all doses) and morphine reduced significantly ($p < 0.001$ and $p < 0.01$) the licking time in the neurogenic phase (0-5 min). In the inflammatory phase (15-30 min), treatment with CP, at all doses, reduced significantly ($p < 0.001$) the licking time (Fig. 2A, B).

3.3 Effect of CP on hot-plate test

When tested in the central antinociceptive model (hot-plate model), the pre-treatment with CP resulted in significant antinociceptive activity in doses 50 and 100 mg/kg. At 30 min after oral administration, CP doses resulted in significant activity with $p < 0.05$ (100 mg/kg) and $p < 0.001$ (50 mg/kg). Similarly, we observed, 60 min after the oral administration, a significant antinociceptive effect ($p < 0.01$ and $p < 0.05$) for the doses of 50 and 100 mg/kg, respectively. After 90 and 120 min, the antinociceptive effect was also significant, at doses of 50 and 100 mg/kg, with $p < 0.001$ and $p < 0.05$ for 90 min after treatment, and $p < 0.001$ for 120 min after treatment. The effect of morphine, as expected, was significant at $p < 0.001$ all times observed (Table 1).

3.4 Effect of CP on the CG-induced mechanical hyperalgesia

Treatment with CP (25, 50, or 100 mg/kg; i.p.) 30 min before CG administration exhibited a significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$) reduction of the mechanical

hyperalgesia induced by CG; except for (25 mg/kg) in the time of 180 min, when compared with animals of the control group that received only vehicle (Fig. 3A).

3.5 Effect of CP on the TNF-α induced mechanical hyperalgesia

The inhibitory effect of CP on the mechanical hyperalgesia induced by TNF-α is shown in Figure 3B. CP (25, 50, or 100 mg/kg) reduced significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) mechanical hyperalgesia induced by TNF-α, at all doses and time, except for the lowest dose (25 mg/kg) in the time of 180 min when compared with animals of the vehicle group (Fig. 3B).

3.6 Effects of CP on the PGE_2-induced mice paw mechanical hyperalgesia

The nociception was significantly reduced ($p < 0.001$) by dipyrone (60 mg/kg; ip) at all times. However, CP showed a significant reduction in the doses 25, 50 and 100 mg/kg, with $p < 0.001$ (Fig. 3C).

3.7 Effect of CP on the DA-induced mechanical hyperalgesia

Figure 3D shows the inhibitory effect of CP on the mechanical hyperalgesia induced by DA. Dipyrone showed reduction in nociception at all times with $p < 0.001$. CP at the time of 0.5 h showed no positive effect. However, 1 h after the treatment with the CP, the dose of 25, 50 and 100 mg/kg showed a significant decrease ($p < 0.001$). Furthermore at the time of 2 h, doses of 25, 50 and 100 mg/kg significantly reduced mechanical hyperalgesia induced by DA when compared with animals of the vehicle group ($p < 0.001$, $p<0.05$ and $p < 0.001$), respectively. However, at the last observation time, all doses are significantly efficient ($p < 0.01$ and $p < 0.001$) in reduction of mechanical hyperalgesia induced by DA.

3.8 Effect of CP on carrageenan-induced pleurisy

All doses of CP (25, 50 and 100 mg/kg) were able to suppress significantly ($p < 0.001$) the recruitment of leukocytes to the mouse´s pleural cavity; similar results were obtained with the positive control, indomethacin, as shown in Fig. 4A. Pretreatment with CP significantly reduced ($p < 0.001$ and $p < 0.05$), in all doses, the migration of neutrophils, as shown in Fig. 4B. This inhibition is not related to cytotoxicity, since the CP, at concentrations of 1, 10, 100 and 250 μg/mL, did not change the morphological profile of polymorphonuclear cells in the MTT protocol of cell viability assay. Only the concentration 500 μg/ml presented a profile, as shown in Fig. 5. Moreover, when we evaluated inflammatory mediators, CP (25, 50, and 100 mg/kg) also significantly decreased the levels of TNF-α ($p < 0.001$) and IL-1β ($p < 0.01$ and $p < 0.05$) in the pleural exudates collected at 4 h after carrageenan injection (Fig. 6A, B). The same occurred with vascular leakage, once the CP, at doses of 25 and 100 mg/kg, significantly decreased ($p < 0.01$) the number of proteins in plasma (Fig. 6C).

3.9 Effect of CP on Measurement of paw edema

As shown in Fig. 7A, CG injection increased mice paw volumes. Additionally, treatment with CP significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) decreased the edema. At 50 and 100 mg/kg, CP, as well as indomethacin (10 mg/kg), was able to maintain reduction of the edema during the six-hour evaluation period. CP percentages of inhibition, based on the AUC values, were 26.4%, 49.8% ($p < 0.01$), and 56.6% ($p < 0.001$) for 25, 50, and 100 mg/kg, respectively, while indomethacin showed an inhibition of 55.3% ($p < 0.001$) (Fig. 7B).

3.10 Effect of CP on spontaneous locomotor activity and Rota Rod

The effect of CP in the animal coordination was tested through the spontaneous locomotor activity and the rota rod. In either test, it has been proved that the CP does not alter the coordination of the animals, unlike DZP, which altered the ambulation (number of crossings) and the ability to stay on the rota rod in the times of 0.5, 1, and 2 h after the treatment (data not shown).

4.0 Discussion

This study aims at evaluating the analgesic and anti-inflammatory effects of a synthetic drug, obtained through an esterification reaction of the monoterpene carvacrol. In recent years, studies have showed that carvacrol has anti-inflammatory effect probably due to the inhibition of mediators such as PGE_2, IL-1β and TNF-α [21, 31]. However, in these studies, carvacrol at lower doses seemed to be ineffective. Thus, the structural modification in carvacrol could improve the action of this monoterpene.

Chemical modification of carvacrol monoterpenoids to ester derivatives has already been performed to evaluate the antimicrobial and antifungal activity [19, 32]. However, the antinociceptive, hyperalgesic and anti-inflammatory activities of CP have not yet been studied. Therefore, carvacrol was used as a starting material for the synthesis of CP according to the literature with some modifications [22]. Formation of CP was confirmed by the ultraviolet spectrum, mass spectra and nuclear magnetic resonance as previously reported by Mathela et al. [19].

As no literature data regarding CP antinociceptive activity were found, the first experimental protocol conducted to evaluate the effect of CP was nociception tests induced by formalin and hot plate, in mice, protocols widely used in the literature. The test of formalin-induced nociception involves a continuous and moderate pain from the

injured tissue, such feature distinguishes it from other existing tests of nociception [33]. Two phases are present in the test, namely the initial phase, which seems to be related to direct activation by neurogenic stimulation of C fibers, mediated by substance P, and late phase, which depends on the activation of nociceptive afferent neurons as well as the release of Prostaglandin E_2, nitric oxide (NO), tachykinins, kinins and other inflammatory mediators [23, 34].

Previous studies prove that the formalin is an important agonist of channels in this family of receptors, transient-receptor-potential subfamily 1 (TRPA1) [35], besides the involvement of glutamatergic receptors AMPA and NMDA receptors in the acute phase in the late phase of the test nociception induced by formalin. The activation of these receptors linked to glutamatergic pathway implies a probable interaction with the nociceptive pathway [36]. Such information suggests as a possible mechanism for the antinociceptive action of CP acting on the TRPA1 receptors, NMDA and AMPA receptors, since this compound had a significant effect in both phases of the test.

After application of a thermal stimulus, Aβ nerve fibers are activated and the information is carried to the brain. When this same thermal stimulus presents an noxious aspect shall, it activates the nerve fibers Aδ and C and the information is carried to the brain [1], as occurs in the hot-plate test, which makes it suitable for the screening of substances with analgesic activity center [37].

The VR1 receptor, present on nerve fiber Aδ and C may be activated when the thermal exposure is at approximately 43°C and which, consequently, leads to the opening of calcium channels [38, 39]. One possible explanation for the increase in the time response in the hot-plate test in the animals treated with CP is the activation of this receptor. Carvacrol, only at the highest dose (100mg/kg), showed a central analgesic effect [40]. Thus, the obtained result indicates that the modification in the structure of

carvacrol contributed to the analgesic activity, since the CP had an effect at doses of 50 and 100 mg/kg. However, molecular studies could further elucidate this mechanism.

Hyperalgesia induced by injection of carrageenan, in animal models, is widely used for evaluating new antihyperalgesic drugs in rodents. The CG, in animal models, stimulates various cell types, particularly the resident and migratory cells to produce a cascade of cytokines [41]. The first cytokine released is the TNF-α, which triggers the release of IL-1β and keratinocyte-derived chemokine (KC) responsible for the synthesis stimulation of prostaglandins and the release of the sympathetic amines, respectively [42]. These final mediators will act on the nerve endings, but specifically on metabotropic receptors to trigger the activation of second messenger pathways leading to a decrease in cellular excitability threshold [43]. In this state, nociceptor activation and impulse transmission by the primary nociceptive neurons are facilitated; in response to that, the animals withdraw the paw with a force which is lower than the baseline threshold.

In this protocol, the CP, at all doses, increased the animal sensitivity threshold, as it happened with indomethacin, a cyclooxygenase inhibitor. Such effect can be related to a possible inhibition of cytokine cascade. This inhibition may occur at the level of the enzyme cyclooxygenase. Similarly, carvacrol inhibits the enzyme cyclooxygenase-2 [44] and in larger doses (50 and 100 mg/kg) also has anti-hyperalgesic effect [21, 40].

The TNF-α is further associated with the development of inflammatory pain since it interacts with target cells through high-affinity membrane receptors, such as TNF receptor Type 1 (TNFR1 or p55) and Type 2 (TNFR2 or p75) [45], stimulating the secretion of IL-1β [46] and consequently, inducing the expression of COX-2, responsible for various prostanoid biosynthesis, as PGE_2 [47].

It was shown that the hyperalgesia induced by the injection of TNF-α was reduced with administration of CP, at all doses. This reduction was also demonstrated with indomethacin. Such results corroborate the idea of a possible COX-level inhibition, without, however, ruling out a possible interaction at the level of receptor. Especially with the receptor TNFR1, according to Sommer et al. [48] and Verri et al. [45] TNF-α interacts with TNFR1 and triggers the hyperalgesic cascade.

Nevertheless, another hypothesis that was verified to evaluate the possible mechanism of action of CP involves the blockade of sensitization or activation of the nociceptor through the evaluation of its effect on hyperalgesia induced by PGE_2 and DA. These inflammatory mediators induce hyperalgesia by activating mainly receptors present in nociceptor membranes, EP2 and D1, respectively, triggering their sensitization [49]. By increasing the concentration of cAMP as well as the PKA signaling pathways and/or PKC [50], which in turn catalyze phosphorylation reactions resistant to sodium channels [51], phosphorylation of these channels changes the conductance that increases neuronal excitability, thereby contributing to the induction of inflammatory hyperalgesia [52].

As CP inhibited the hyperalgesia induced by PGE_2 and DA, we are led to believe that there is a possible involvement with the receptors present on neuronal membranes (EP2 and D1). This action may even relate to structural modifications made to the structure of carvacrol, since carvacrol was not able to inhibit hyperalgesia induced by these agents [21]. The inhibition of hyperalgesia was also seen with the positive control, dipyrone. One of the proposed mechanisms for dipyrone is in the activation of arginine-NO-cGMP-channel ATP-sensitive K^+, which induces desensitization of peripheral nociceptors [53].

According to Cunha et al. [54], during the inflammatory process, neutrophils actively participate in the hyperalgesic cascade activation with the induction training of final mediators. Effects of hyperalgesic cytokines depend on neutrophil migration and the ability of these cells to release direct-acting mediators such as PGE_2.

Therefore, the blockade of neutrophil migration could be a target for the development of new drugs, not only anti-inflammatory but analgesic as well. For this reason, and to better investigate the anti-inflammatory and anti-hyperalgesic potential of CP, we performed a cell migration test through carrageenan-induced pleurisy. The results allowed us to detect a marked inhibitory effect of CP on neutrophil and mononuclear cell migration, without altering the morphological profile of these cells, what rules out the possibility of cytotoxicity.

CP has anti-inflammatory and anti-hyperalgesic properties since it reduces neuronal excitability threshold and also inhibits the migration of neutrophils, thereby reducing the inflammatory pain. Since the participation of neutrophils in this process has been extensively studied, the pronociceptive action of neutrophils was first suggested almost 35 years ago [55] and since then, several studies have reported the importance of neutrophils in the pathogenesis of inflammatory pain [56-59].

Considering that cytokines, TNF-α and IL-1β, play key roles in inflammatory processes, they stimulate the recruitment of neutrophils and monocytes to the sites of infection and activate these cells to eradicate microorganisms [60]. Although there is evidence to support a direct action of these cytokines on nociceptors, their primary contribution to pain hypersensitivity results from potentiation of the inflammatory response and increased production of algesic agents such as prostaglandins, bradykinin, and extracellular protons [1].

In this way, there is the need to quantify these cytokines after an inflammatory process induced by carrageenan. Corroborating previous results, CP decreased the levels of TNF and IL-1, what leads us to believe that CP has a satisfactory anti-inflammatory effect, since these cytokines are important in severe inflammatory conditions.

In addition to the characteristics of the inflammatory processes mentioned above, such as cell migration, cytokine release has also extravasation of plasma fluid, rich in proteins. This parameter was also evaluated and CP was effective against plasma extravasation in dose of 25 and 100 mg/kg. Therefore, CP has satisfactory anti-inflammatory effect, since it has decreased the essential factors in inflammatory process.

The model of paw edema induced by carrageenan is widely used by the scientific community with the goal of potential drug discovery with anti-inflammatory activity. The carrageenan induces a biphasic response. In the first hours after administration of the agent, the edema is mediated by the early release of histamine and serotonin followed by the release of kinin and finally through the release of bradykinin and prostaglandins (PGs) [61, 62]. According to the result of our study, CP, in doses of 50 and 100 mg/kg, was able to effectively inhibit the edema throughout the observation period, suggesting that CP inhibits different chemical mediators of inflammation.

Interestingly, the anti-inflammatory nature of CP was similar with to carvacrol as described by Guimarães et al. [21], which leads us to believe that the addition of a propionyl group does not alter the anti-inflammatory activity of carvacrol. However, in respect to its action on neural stimulation, as demonstrated in protocols in hyperalgesia induced by PGE_2 and DA, the addition in this group had to be limited since carvacrol had no positive effect positive in this protocol, according to Guimarães et al. [21].

As shown by Passos et al. [63], many terpenoids have activity on the central nervous system (CNS) due to the inhibitory effect on the CNS or muscle relaxation. Thus, these activities could reduce the motor coordination of animals and invalidate the results obtained for the CP. Therefore, it was necessary to evaluate the effect of CP on the CNS.

As shown, the CP did not alter the spontaneous movement and coordination of animals, which leads one to believe that the CP has no inhibitory effect on the CNS. Since mobility is a function of the degree of excitability of the central nervous system and a decrease of this parameter is suggestive of a depressive activity [64], the animals have remained on the rotating bar during the time set, discarding thus the possibility of a myorelaxing effect [65].

Thus, it can be concluded that CP is effective as an analgesic and anti-inflammatory compound in various pain models, probably mediated via inhibition of peripheral mediators (as TNF-α and IL-1β synthesis) as well as central inhibitory mechanisms. Nevertheless, further studies are necessary to understand the precise mechanisms of action of CP on inflammatory pain.

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FIGURES

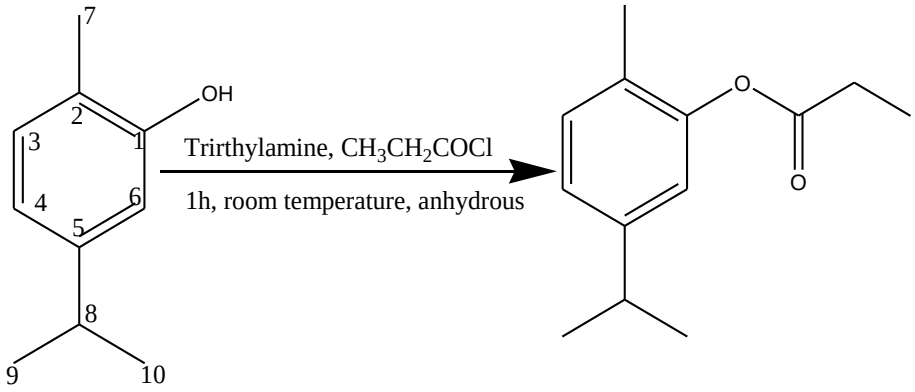


Figure 1. Sythesis reaction of the carvacrol propionate (CP) from the reagents carvacrol, triethylamine and propionile chloride.

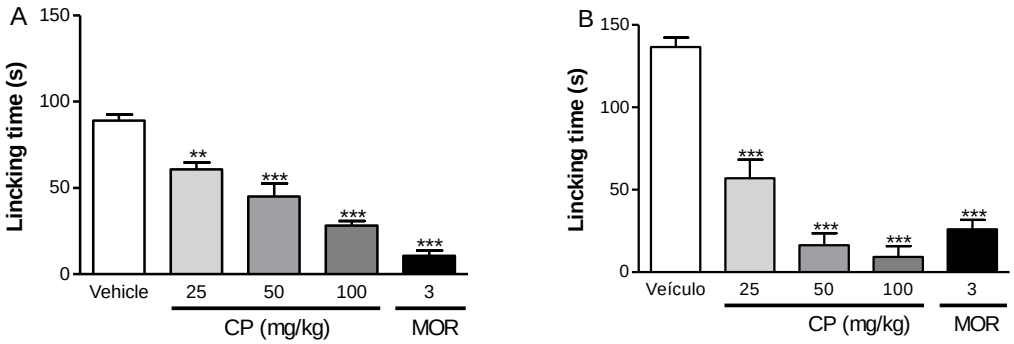


Figure 2. Effects of carvacrol proprionate (CP; 25, 50 or 100 mg/kg, i.p.) or morphine (MOR, 3 mg/kg; i.p.) on formalin-induced nociceptive behavior were administered intraperitoneally 0.5 hr before formalina injection. (panel A) First phase (0-5 min.) and (panel B) second phase (15-30 min.) of the formalin test. Values represent mean ± S.E.M. (n = 6, per group). **p < 0.01 and ***p < 0.001 versus control (one-way ANOVA followed by Tukey'stest).

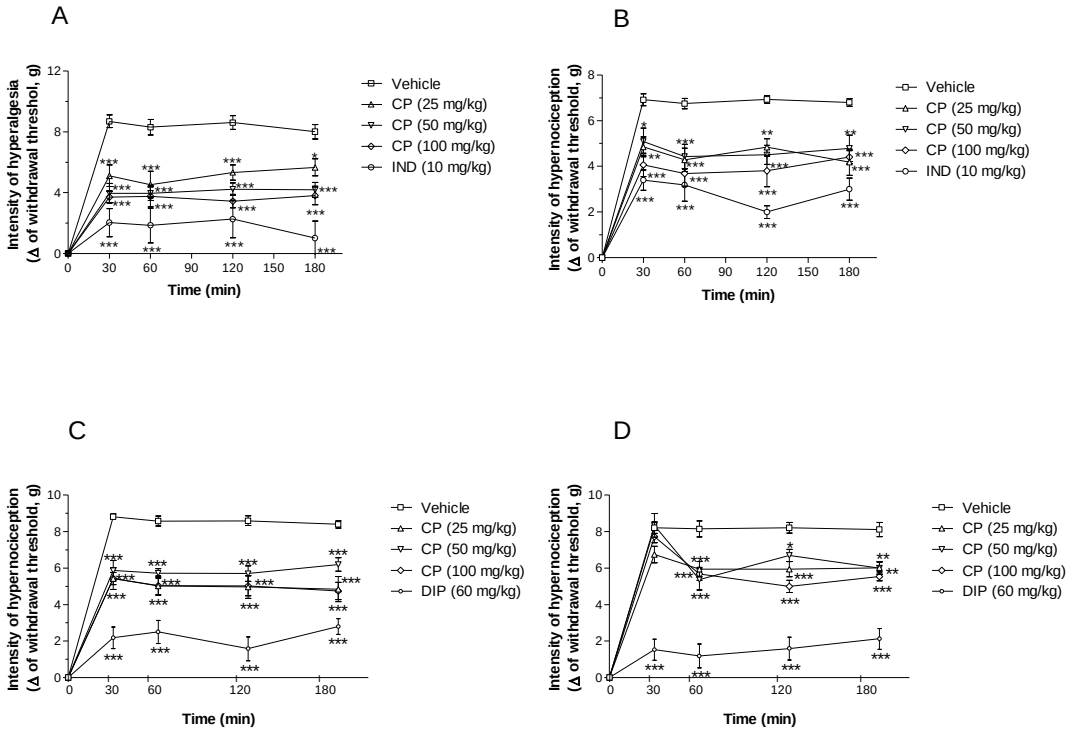


Figure 3. Effect of acute administration of vehicle, carvacrol propionate (CP; 25, 50 or 100 mg/kg, i.p.), indomethacin (IND, 10 mg/kg, i.p.) or dipyrone (DIP, 60 mg/kg, i.p.) on mechanical hypernociception induced by carrageenan (A), TNF-α (B), PGE_2 (C) and dopamine (D). Each point represents the mean ± S.E.M. of the paw withdrawal threshold (in grams) to tactile stimulation of the left hind paw. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control group (two-way-ANOVA followed by Bonferroni).

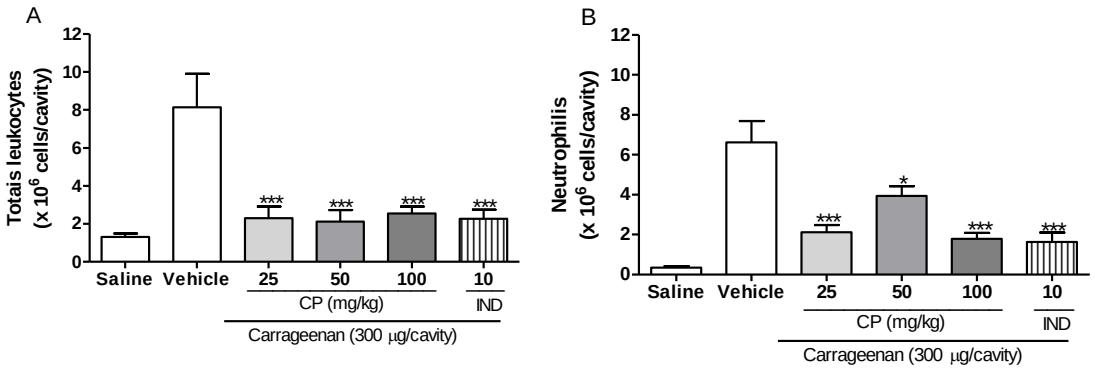


Figure 4. Effect of acute administration of vehicle, carvacrol propionate (CP; 25, 50 or 100 mg/kg, i.p.) or indomethacin (IND, 10 mg/kg; i.p.) on the inflammation by carrageenan in mice pleurisy. The analyses were performed 4 h after carrageenan injection (300 μg/cavity) to evaluate the recruitment of total leukocytes (A), neutrophils (B). 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 test).

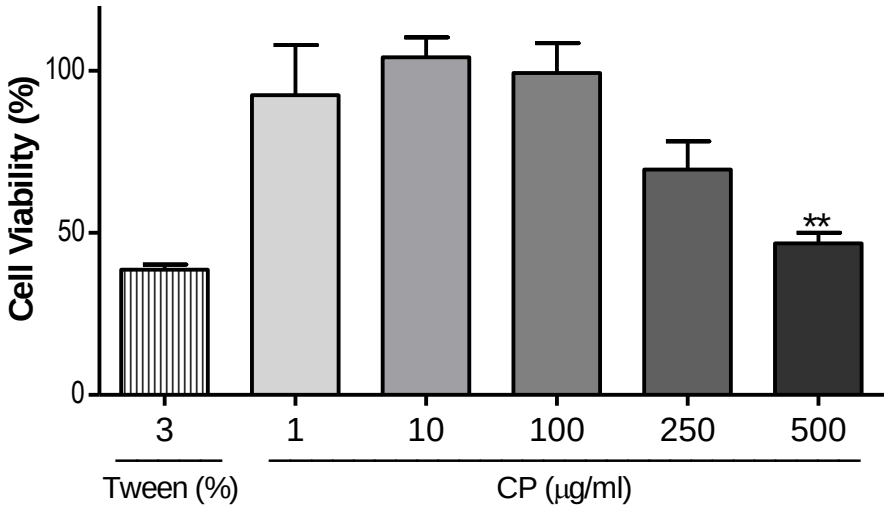


Figure 5. Effect of vehicle, carvacrol propionate (CP; 1, 10, 100, 250 or 500 μg/mL, in vitro) on murine peritoneal macrophages (2.5×10^5 cells). The percentage of viability was determined in relation to controls. Data were expressed as mean ± SEM. ** $p < 0.01$ compared with the control group (vehicle) (ANOVA followed by Tukey test).

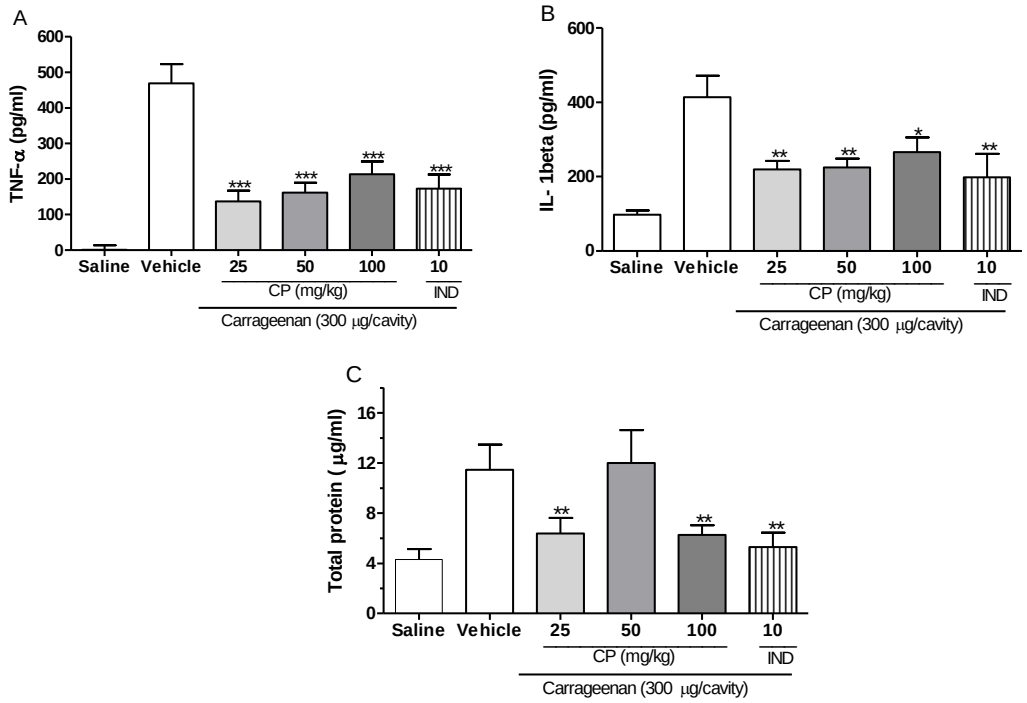


Figure 6. Effect of acute administration of vehicle, carvacrol propionate (CP; 25, 50 or 100 mg/kg, i.p.) or indomethacin (IND, 10 mg/kg; i.p.) on the inflammation by carrageenan in mice pleurisy. The analyses were performed 4 h after carrageenan injection (300 μg/cavity) to evaluate to assess tumor necrosis factor-alpha (TNF-α) (A), and interleukin-1β (IL-1β) levels (B), and total protein (C). 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 test).

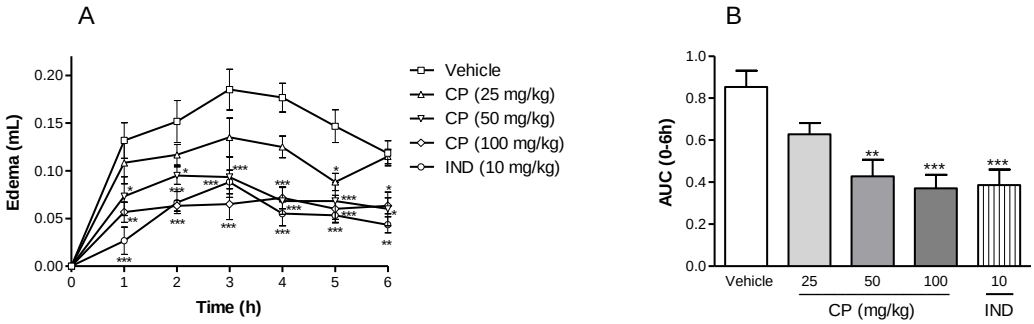


Figure 7. Effect of acute administration of vehicle, carvacrol proprionate (CP; 25, 50 or 100 mg/kg, i.p.) or indomethacin (IND, 10 mg/kg; i.p.) on edema induced by carrageenan. Each point represents the mean±SEM of the paw volume (in milliliter, panel A) or the area under curve (AUC) from 0 to 6 h (panel B). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control group (two-way-ANOVA followed by Bonferroni – panel A and ANOVA followed by Tukey test – panel B).

Table 1. Effect of CP (25, 50, or 100 mg/kg; i.p.) or MOR (3.0 mg/kg; i.p.) on the hot plate test in mice.

Treatment	Dose (mg/kg)	Reaction time (licking of the hind paws) (s)[a]				
		Basal	**0.5h**	**1h**	**1.5h**	**2h**
Vehicle	-	7.0 ± 0.68	8.7 ± 0.42	7.7 ± 0.33	6.7 ± 0.21	5.7 ± 0.71
CP	25	7.3 ± 0.67	10.0 ± 0.58	10.8 ± 1.25	9.5 ± 0.96	10.0 ± 0.52
CP	50	8.0 ± 0.45	14.2 ± 1.3[d]	16.5 ± 2.3[c]	15.7 ± 2.0[d]	16.7 ± 1.8[d]
CP	100	7.8 ± 0.70	12.2 ± 0.5[b]	13.8 ± 1.3[b]	12.3 ± 1.1[b]	15.5 ± 1.5[d]
MOR	3	7.6 ± 1.9	30.0 ± 0.0[d]	29.5 ± 0.4[d]	29.0 ± 0.9[d]	22.7 ± 4.9[d]

Values are the mean ± SEM (n = 6, per group)

[a] Values represent mean ± S.E.M.

[b] $p < 0.05$ as compared with control (vehicle) (ANOVA followed by Tukey test).

[c] $p < 0.01$ as compared with control (vehicle) (ANOVA followed by Tukey test).

[d] $p < 0.001$ as compared with control (vehicle) (ANOVA followed by Tukey test).

4.0 CONCLUSÃO

4.0 CONSIDERAÇÕES FINAIS

Tendo em vista os resultados obtidos no presente estudo, pode-se concluir:

CAPÍTULO 1

- Modificação estrutural em terpenos representa uma ferramenta farmacológica para a descoberta de drogas com ação anti-inflamatória;

CAPÍTULO 2

- O propionato de carvacrol foi sintetizado e identificado;
- Apresenta ação antinociceptiva, sendo capaz de reduzir a nocicepção em roedores;
- Tem efeito anti-hiperalgésico, já que inibe a cascata hiperalgésica;
- Possui efeito anti-inflamatório, provavelmente mediado pela inibição de citocinas pró-inflamatórias, a exemplo do TNF-α e IL-1β;
- Não apresenta citotoxicidade celular;
- Nas doses utilizadas não induz qualquer alteração na coordenação motora dos animais.

DISSERTAÇÃO

- Os dados apresentados no presente estudo nos permitem sugerir que a semi-síntese de monoterpenos pode ser útil para a descoberta de drogas com possível ação anti-inflamatória.
- Novas metodologias podem ser propostas para melhor caracterizar o mecanismo exato do CP.

ANEXOS

Anexo 1: PROTOCOLO DE APROVAÇÃO NO COMITÊ DE ÉTICA EM PESQUISA ANIMAL DA UNIVERSIDADE FEDERAL DE SERGIPE

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 **"SÍNTESE DO PROPIONATO DE CARVACROL E ESTUDO PRÉ-CLÍNICO DO SEU POTENCIAL ANTI-HIPERNOCICEPTIVO E ANTI-INFLAMATÓRIO", sob coordenação do Prof. Dr. Lucindo José Quintans Júnior** (protocolo **CEPA 35/2012**) 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 2: CERTIFICADO DE HONRA AO MÉRITO

FeSBE Regional 2012

CERTIFICADO DE HONRA AO MÉRITO

Certificamos que

A FeSBE tem a grande satisfação de conferir este certificado de Honra ao Mérito pela brilhante apresentação do trabalho **10.037** - PROPIONATO DE CARVACROL PRODUZ EFEITO ANTI-HIPERNOCICEPTIVO E ANTI-INFLAMATÓRIO de autoria Santana, M. T., SILVA, V. B., CAVALCANTI, S. C. H., OLIVEIRA, M. G. B., FERRO, J. N. S., BARRETO, E. O., BONJARDIM, L. R., QUINTANS-JÚNIOR, L. J. - Universidade Federal de Sergipe - UFS, Universidade Federal de Alagoas – UFAL na

VII Reunião Regional da Federação de Sociedades de Biologia Experimental-FeSBE, realizada no Centro de Convenções Ruth Cardoso, Maceió – AL, de 31 de maio a 02 de junho de 2012.

Comissão Organizadora

Ministério da Educação · BRASIL · CNPq 60 ANOS · FIOCRUZ · FAPEAL · ALAGOAS · Estácio | FAL

Anexo 3: CERTIFICADO DE HONRA AO MÉRITO

Anexo 4: ACEITE DO PERIÓDICO INFLAMMATION

---------- Forwarded message ----------
From: **Inflammation** <jonalyn.blando@springer.com>
Date: 2014-03-12 11:42 GMT-03:00
Subject: IFLA-D-14-00086R1 - accepted, needs copy editing
To: Lucindo Quintans-Júnior <lucindojr@gmail.com>

Dear Dr Lucindo Quintans-Júnior,

We are pleased to inform you that your manuscript, "Synthesis and pharmacological evaluation of carvacrol propionate" has been accepted for publication in Inflammation pending correction of the language of the manuscript.

To improve the quality of the manuscript and render it worthy of publication in Inflammation we recommend you take advantage of a commercial copyediting service. Information about such a service is available at: http://www.prof-editing.com/

Once the copyediting of the manuscript has been completed please resubmit the manuscript at: http://ifla.edmgr.com/.

Your username is: lucindo
Your password is: rotte2008

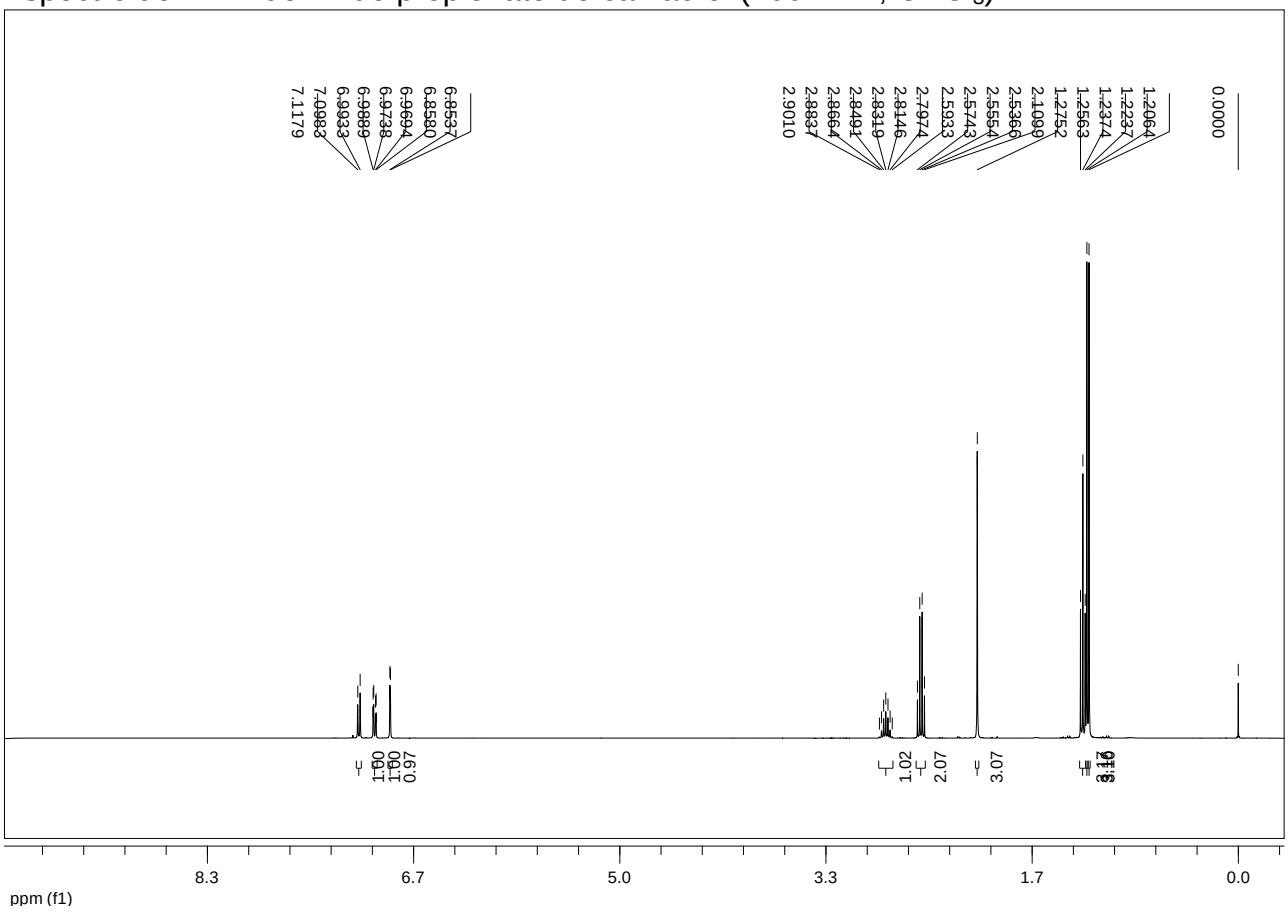
For queries regarding your accepted paper, please visit the journal homepage by clicking on "Journal overview" from the toolbar.

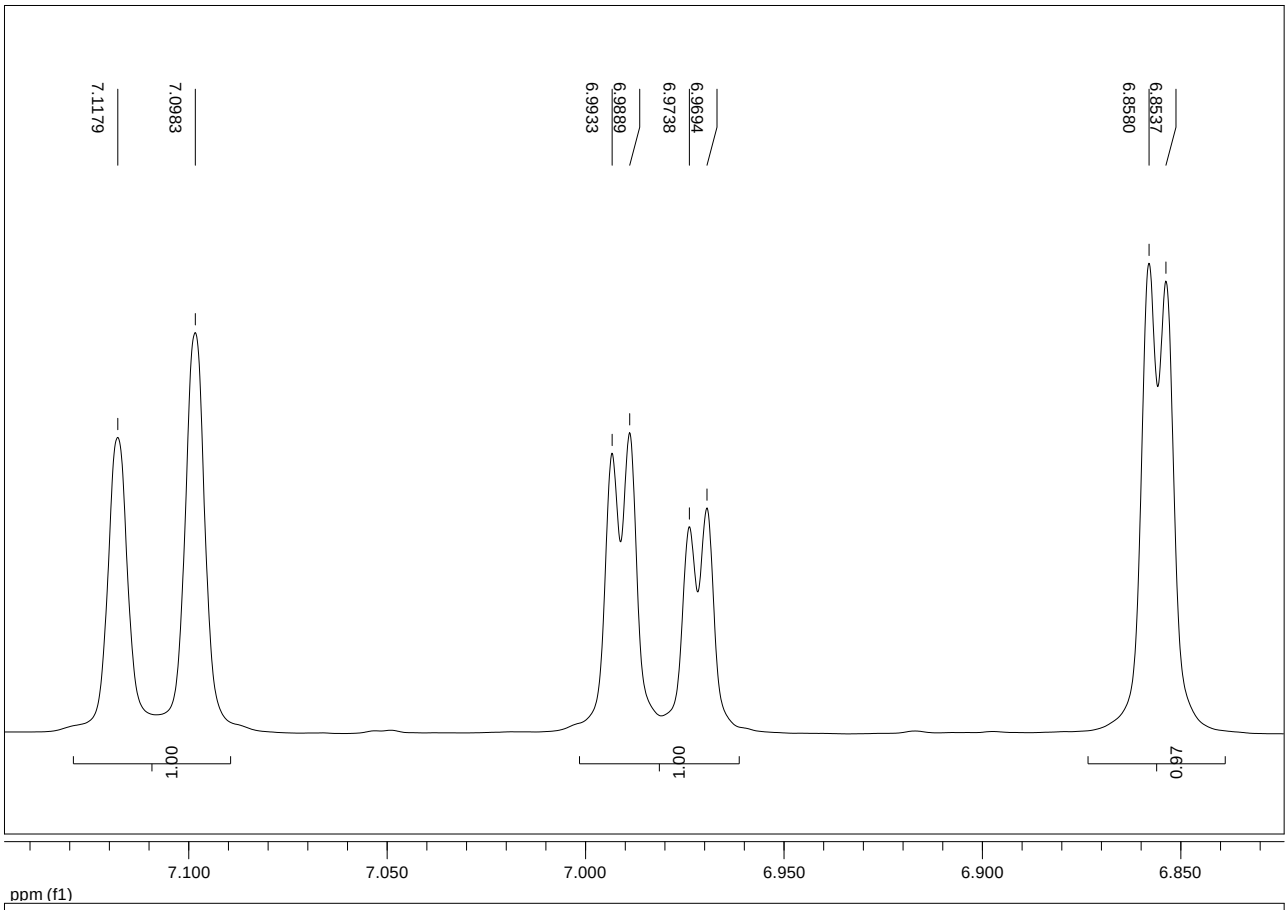
Please remember to always include your manuscript number, IFLA-D-14-00086R1, whenever inquiring about your manuscript. Thank you.

With best regards,
Bruce N Cronstein

ANEXO 5: ESPECTROS DE RMN DO PROPIONATO DE CARVAROL

Espectro de RMN de ^{1}H do propionato de carvacrol (400 MHz, $CDCl_3$)

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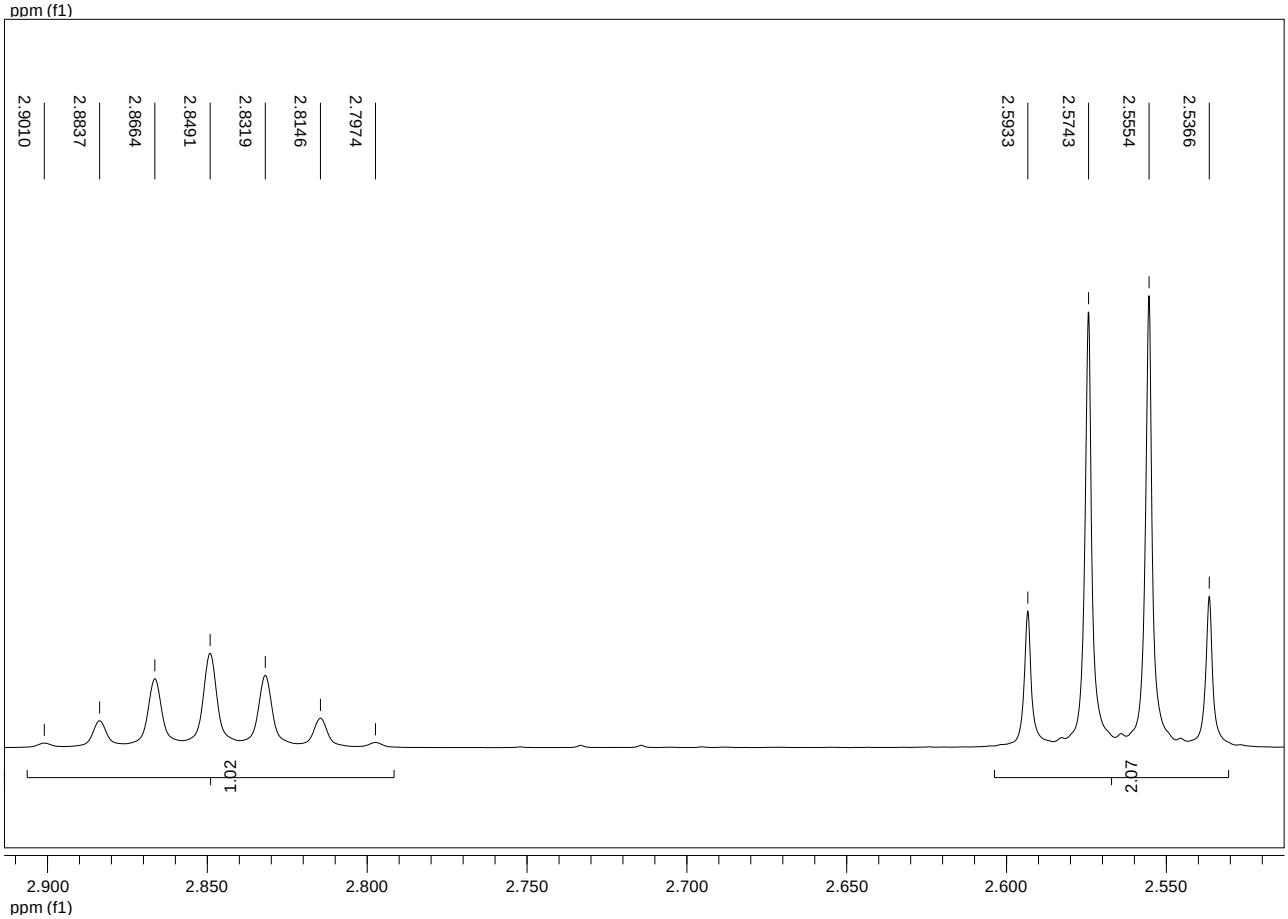
7.0983
6.9933
6.9889
6.9738
6.9694
6.8580
6.8537
1.00
1.00
0.97
7.100
7.050
7.000
6.950
6.900
6.850
ppm (f1)


2.9010
2.8837
2.8664
2.8491
2.8319
2.8146
2.7974
2.5933
2.5743
2.5554
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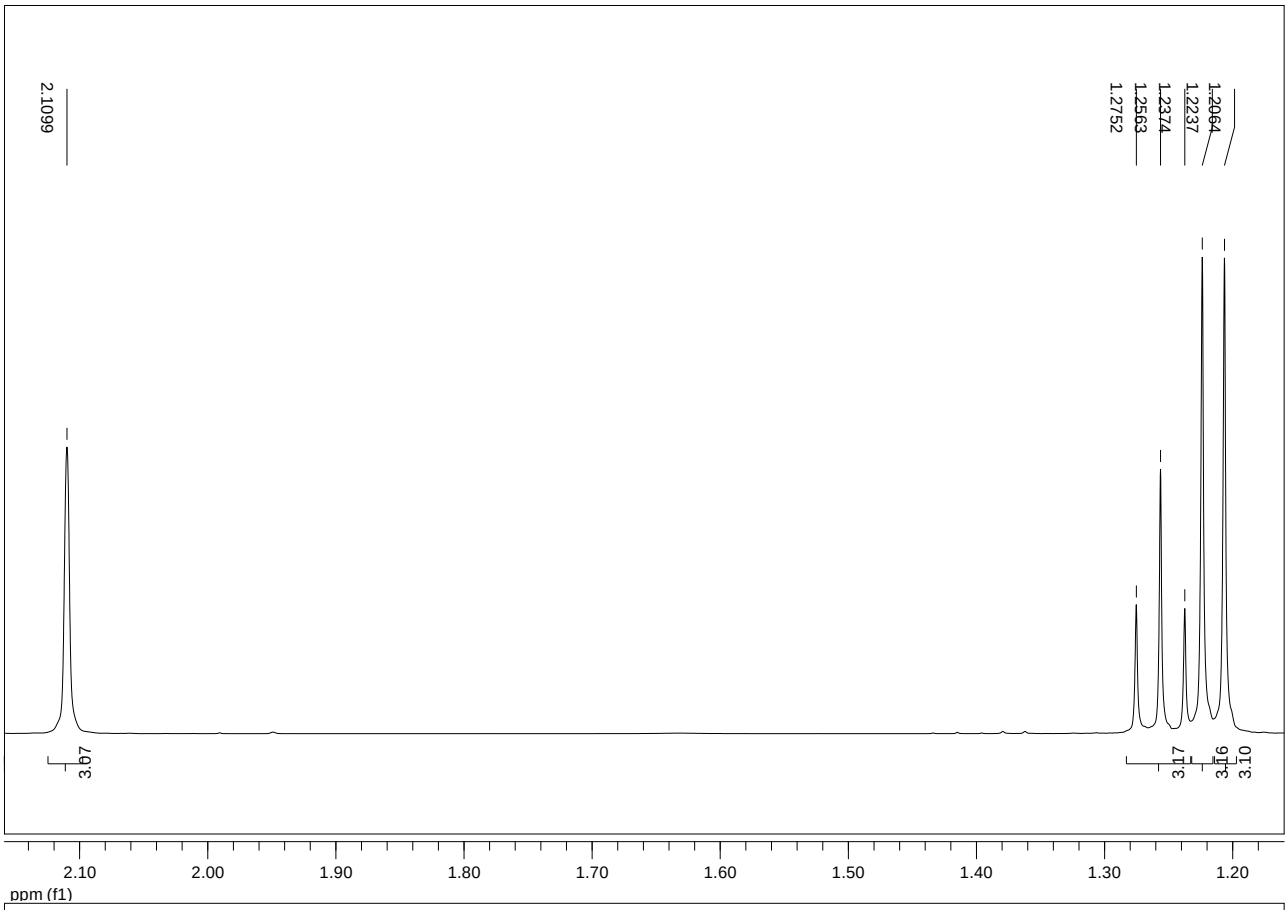
2.700
2.650
2.600
2.550
ppm (f1)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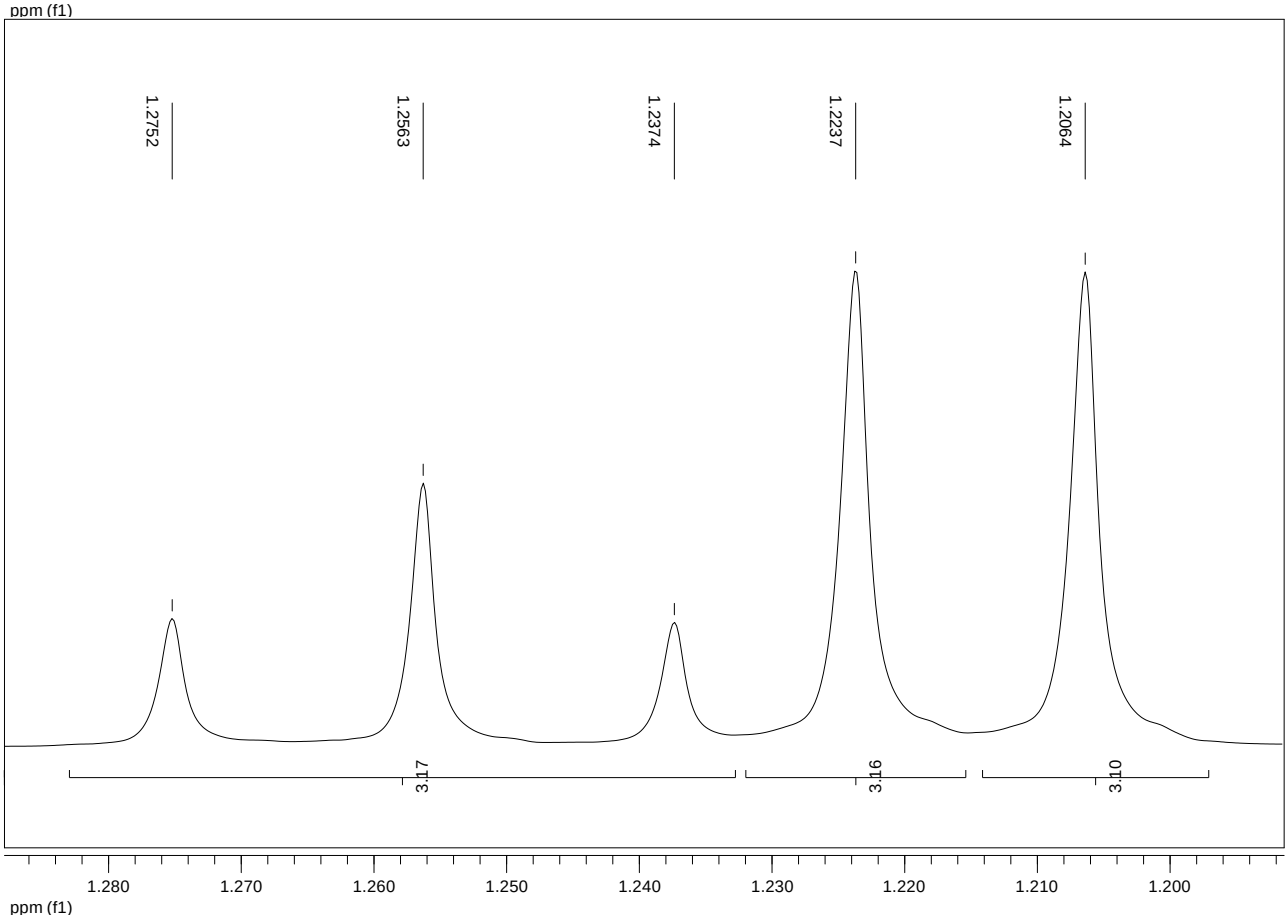
1.2563
1.2374
1.2237
1.2064
3.07
3.17
3.16
3.10
2.10
2.00
1.90
1.80
1.70
1.60
1.50
1.40
1.30
1.20
ppm (f1)


1.2752
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1.2064
3.17
3.16
3.10
1.280
1.270
1.260
1.250
1.240
1.230
1.220
1.210
1.200
ppm (f1)

Espectro de RMN de ^{13}C do propionato de carvacrol (100 MHz, $CDCl_3$)