Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

MARCELO FRANCHIN

ATIVIDADE *IN VIVO* E *IN VITRO* DE COMPOSTOS ISOLADOS DE PRÓPOLIS BRASILEIRAS SOBRE A MODULAÇÃO DO PROCESSO INFLAMATÓRIO

IN VIVO AND *IN VITRO* ACTIVITY OF ISOLATED COMPOUNDS FROM BRAZILIAN PROPOLIS ON THE MODULATION OF THE INFLAMMATORY PROCESS

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Tese de doutorado apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutor em Odontologia, na Área de Farmacologia, Anestesiologia e Terapêutica.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dentistry, in Pharmacology, Anesthesiology and Therapeutics area.

Orientador: Prof. Dr. Pedro Luiz Rosalen

Este exemplar corresponde à versão final da tese defendida pelo aluno Marcelo Franchin e orientada pelo Prof. Dr. Pedro Luiz Rosalen

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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RESUMO

O objetivo deste estudo foi avaliar a atividade e os mecanismos de ação dos compostos isolados de própolis brasileiras sobre a modulação do processo inflamatório. As atividades anti-inflamatórias dos compostos vestitol e neovestitol, isolados da própolis vermelha brasileira e do cinamoiloxi-mammeisina (CNM), isolado da geoprópolis de *Melipona scutellaris,* foram avaliadas e os seguintes resultados obtidos: 1) O composto vestitol apresentou atividade inibitória sobre a migração de neutrófilos induzido por LPS ou mBSA, bem como sobre a liberação das quimiocinas CXCL1/KC e CXCL2/MIP-2 *in vivo* e *in vitro*. Foi também observado que o tratamento com vestitol reduziu o rolamento e adesão de leucócitos na microcirculação mesentérica de camundongos, independente da via do óxido nítrico. Quanto à sua atividade em neutrófilos *in vitro*, o vestitol reduziu a quimiotaxia de neutrófilos induzida por CXCL2/MIP-2 e LTB4 e bloqueou o influxo de cálcio nestas células. Não foi constatada alteração da expressão do receptor CXCR2 com o tratamento de vestitol. 2) Em relação ao composto neovestitol, foi verificada que a sua administração reduziu a migração de neutrófilos, o rolamento e adesão de leucócitos e a expressão de ICAM-1 em camundongos desafiados com LPS. No que diz respeito à liberação de TNF-α, CXCL1/KC e CXCL2/MIP-2, não foi constatada atividade do neovestitol sobre estes mediadores inflamatórios. Além disso, o composto não alterou a expressão de ICAM-1 *in vitro*. Por outro lado, foi observado que a administração de um inibidor da óxido nítrico sintetase, aboliu o efeito anti-inflamatório do neovestitol sobre a migração de neutrófilos. No modelo de artrite induzida por colágeno, a administração diária do neovestitol pelo período de 12 dias, reduziu o escore clinico de artrite dos animais, as lesões articulares e a liberação de IL-6. 3) Em relação aos resultados obtidos com o composto CNM, foi observado que a sua administração reduziu a migração de neutrófilos na cavidade peritoneal e articular, bem como a liberação de TNF-α e CXCL2/MIP-2 *in vivo* e *in vitro*. Foi verificado também, que o CNM reduziu a fosforilação das proteínas ERK 1/2, JNK, p38 MAPK e c-jun em macrófagos RAW 264.6. Da mesma forma, o CNM reduziu a ativação de NF-κB, entretanto, não alterou a degradação de IκBα e a translocação de p65 nuclear. Portanto, conclui-se que a própolis brasileira é uma importante fonte de compostos bioativos com potencial biológico e que os compostos isolados delas como vestitol, neovestitol e CNM, são promissores agentes anti-inflamatórios com mecanismos distintos e de interesses

para os desfechos de processos inflamatórios agudos ou crônicos, com possibilidades de uso terapêutico.

Palavras-chave: Cumarinas. Neutrófilos. Macrófagos. Inflamação. Isoflavonas.

ABSTRACT

This study aimed to evaluate the activity and the mechanisms of action of compounds isolated from Brazilian propolis on the modulation of the inflammatory process. The anti-inflammatory activity of vestitol and neovestitol, isolated from Brazilian red propolis and cinnamoyloxy-mammeisin (CNM), isolated from *Melipona scutellaris* geopropolis, was tested and the following results were found: 1) Vestitol had inhibitory activity against LPS- or mBSA-induced neutrophil migration as well as on the release of CXCL1/KC and CXCL2/MIP-2 chemokines *in vivo* and *in vitro*. Treatment with vestitol also reduced leukocyte rolling and adhesion in mice mesenteric microcirculation, independent of nitric oxide pathway. As to *in vitro* activity on neutrophil*s*, vestitol reduced CXCL2/MIP-2 and LTB4-induced neutrophil chemotaxis and blocked the calcium influx in these cells. No change was observed on CXCR2 receptor expression upon pretreatment with vestitol. 2) The administration of neovestitol reduced neutrophil migration, leukocyte rolling and adhesion and ICAM-1 expression in mice challenged with LPS. Despite the release of TNF-α, CXCL1/KC and CXCL2/MIP-2, we found no activity of neovestitol on these inflammatory mediators. Furthermore, this compound did not alter ICAM-1 expression *in vitro*. The administration of a nitric oxide synthase inhibitor abolished the anti-inflammatory effect of neovestitol on neutrophil migration. In the collagen-induced arthritis model daily administration of neovestitol for 12 days reduced arthritis clinical score in the animals, joint injury and IL-6 release. 3) The administration of CNM reduced neutrophil migration into the peritoneal and joint cavity, as well as TNF-α and CXCL2/MIP-2 release *in vivo* and *in vitro*. Concerning its mechanism of action, CNM reduced the phosphorylation of proteins ERK 1/2, JNK, p38 MAPK and c-jun in RAW 264.6 macrophages. Furthermore, it reduced NF-kB activation but did not affect IκBα degradation and p65 nuclear translocation. Thus, we conclude that Brazilian propolis is an important source of active compounds with biological potential. Also, some of its isolated compounds such as vestitol, neovestitol and CNM are promising anti-inflammatory agents with distinct mechanisms of action and potential therapeutic use in acute or chronic inflammatory conditions.

Keywords: Coumarin. Neutrophil. Macrophages. Inflammation. Isoflavones

SUMÁRIO

1 INTRODUÇÃO

Os produtos naturais, historicamente, têm sido utilizados na medicina popular para o tratamento de diferentes doenças. Além disso, apresenta-se como uma grande fonte na descoberta de novos fármacos (Newman et al., 2003). O último levantamento realizado por Newman e Cragg (2012), mostrou que 50% dos novos fármacos descobertos no ano de 2010 foram de produtos naturais ou derivados.

Dentre estes produtos naturais, destaca-se a própolis que é uma mistura de cera e resina coletada por abelhas em diversas fontes vegetais (Silva et al., 2008). Diferentes atividades biológicas foram estudadas e comprovadas para as própolis, que incluem anti-inflamatória, antinociceptiva, antimicrobiana, antiviral e antifúngica (Castaldo e Capasso, 2002). Devido à sua complexidade química, que pode variar de acordo com a região de coleta (Castro et al., 2007), diversos compostos biologicamente ativos foram isolados e identificados de diferentes variedades de própolis. Dentre os compostos, estão o CAPE, (Natarajan et al., 1996), a pinocembrina (Koo et al., 1999), galangina (Jung et al., 2014) e a apigenina (Koo et al., 2003).

As própolis brasileiras coletadas por abelhas *Apis mellifera L.* (Apidae) estão classificadas em 13 tipos (Park et al., 2002; Silva et al., 2008). Dentre as variedades, a própolis tipo 13 (vermelha), proveniente de Maceió, estado de Alagoas, tem se destacado por apresentar promissora atividade anti-inflamatória (Bueno-Silva et al., 2013). Estudos iniciais do nosso grupo de pesquisa, isolaram e identificaram dois compostos pertencentes à classe dos isoflavonóides, sendo eles o vestitol e o neovestitol. Dentre os resultados biológicos obtidos com estes compostos, ambos apresentaram atividade inibitória sobre a migração de neutrófilos, sendo que esta atividade, nunca antes tinha sido relatada para estas substâncias (Bueno-Silva et al., 2013).

Além destes relatos com a própolis vermelha, outra variedade de própolis tem despertado cada vez mais interesse para o nosso grupo de pesquisa. Conhecida como geoprópolis, esta própolis é coletada por abelhas nativas sem ferrão e apresenta, em sua composição, além de resina vegetal, cera e terra (Nates-Parra, 2001; Barth, 2006). Estudos do nosso grupo, demonstraram atividades antimicrobiana, antiproliferativa para células tumorais e gastroprotetora para o extrato etanólico de geoprópolis (Da Cunha et al., 2013; Ribeiro-Junior et al., 2015). Além

disso, estudos com a geoprópolis também mostraram-se promissores quanto à sua atividade anti-inflamatória, onde o extrato apresentou atividade inibitória sobre o rolamento e a adesão de leucócitos na microcirculação mesentérica de camundongos, bem como inibiu a migração de neutrófilos no processo inflamatório. Entretanto, o composto biologicamente ativo responsável por estas importantes atividades não foi isolado e identificado (Franchin et al., 2013).

Assim, com base nestes resultados iniciais apresentados em relação à própolis vermelha e à geoprópolis, mais estudos são necessários para isolar e identificar o composto responsável pela atividade anti-inflamatória da geoprópolis, bem como elucidar os mecanismos de ação dos compostos isolados de ambas as variedades de própolis no processo inflamatório.

Portanto, os objetivos deste trabalho foram: 1) obter os compostos vestitol e neovestitol, dois isoflavonóides identificados na própolis vermelha, bem como identificar e isolar o composto responsável pela atividade anti-inflamatória da geoprópolis de *Melipona scutellaris*. 2) avaliar a atividade dos compostos isolados de própolis brasileiras sobre a modulação do processo inflamatório. 3) elucidar os mecanismos de ação dos compostos isolados sobre a modulação do processo inflamatório.

2 ARTIGOS[1]

2.1 Artigo 1:[2,3]

Vestitol Isolated from Brazilian Red Propolis Inhibits Neutrophils Migration in the Inflammatory Process: Elucidation of the Mechanism of Action

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[1] Este trabalho foi organizado no formato alternativo de tese e está de acordo com o Informativo CCPG/001/2015 (Anexo 1).
[2] Artigo publicado no periódico Journal of Natural Products - DOI: 10.1021/acs.jnatprod.5b00938. Disponível em: http://pubs.acs.org/doi/abs/10.1021/acs.jnatprod.5b00938 (Anexo 6).
[3] Este artigo recebeu "Menção Honrosa" como um dos melhores trabalhos na área de Produtos Naturais apresentado na 30º Reunião Anual da FESBE, set, 2015 (Anexo 4).

ABSTRACT

Vestitol is an isoflavonoid isolated from Brazilian red propolis with potential anti-inflammatory activity. This study investigated the mechanism of action of vestitol on the modulation of neutrophil migration in the inflammatory process. Pre-treatment with vestitol at 1, 3 or 10 mg / kg reduced LPS- or mBSA-induced neutrophil migration and the release of CXCL1/KC and CXCL2/MIP-2 in vivo. Likewise, pretreatment with vestitol at 1, 3 or 10 µM reduced the levels of CXCL1/KC and CXCL2/MIP-2 in macrophage supernatants in vitro. Moreover, the administration of vestitol (10 mg / kg) reduced leukocyte rolling and adherence in the mesenteric microcirculation of mice. The pretreatment with vestitol (10 mg / kg) in $iNOS^{-/-}$ mice did not block its activity concerning neutrophil migration. With regard to the activity of vestitol on neutrophils isolated from the bone marrow of mice, there was a reduction on the chemotaxis of CXCL2/MIP-2 or LTB4-induced neutrophils and on calcium influx after pre-treatment with the compound at 3 µM or 10 µM. There was no change in CXCR2 expression by neutrophils treated with vestitol at 10 µM. These findings demonstrate that vestitol is a promising novel anti-inflammatory agent.

The development of novel anti-inflammatory drugs with few side effects has been one of the major challenges faced by the pharmaceutical industry. Among the current approaches, drugs that inhibit cell migration during the inflammatory process are particularly promising in preclinical and clinical trials.[1,2] This is due to the specific action of these new drugs on target proteins directly related to migration, adhesion and transmigration of neutrophils into the inflammatory site.[3]

Natural products have historically led to discovery of many clinically useful drugs in current therapy.[4] Among these, propolis, a resinous substance collected by bees, has shown various biological activities including anti-inflammatory properties.[5-9]

The 13th type of Brazilian propolis (red propolis) from *Apis mellifera L.* (Apidae) has aroused attention for its biological activities and also due to the presence of compounds with promising pharmacological potential. [10-14] Previous studies demonstrated for the first time that vestitol, an isoflavonoid isolated from red propolis, has promising inhibitory activity on neutrophil migration.[15] Nevertheless, the mechanism of action by which this compound acts in the inflammatory process remains to be elucidated.

Thus, the aim of this study was to evaluate the activity of vestitol on neutrophil migration induced by different inflammatory stimuli, as well as to elucidate its mechanism of action.

RESULTS AND DISCUSSION

Based on previous reports demonstrating the anti-inflammatory activity of vestitol[15], the present study further investigated its mechanism of action. Firstly, the activity of vestitol on neutrophil migration was evaluated in mice challenged intraperitoneally (ip) with lipopolysaccharide (LPS) or methylated bovine serum albumin (mBSA; Figure 1). Neutrophils are the main leukocytes involved in the body defense during acute inflammation.[3] Migration of neutrophils into the inflammatory site involves a series of events that promote their rolling, adhesion and transmigration.[16] However, despite the role of neutrophils in the host defense response, the occurrence of uncontrolled inflammation with high influx of neutrophils is associated with the development of various inflammatory diseases, some of which causing irreversible tissue injury and functional loss.[17,18] In this study, the subcutaneous (sc) administration

of vestitol at the dose of 1, 3 or 10 mg / kg effectively reduced neutrophil migration upon both inflammatory stimuli (Figure 1A and C).

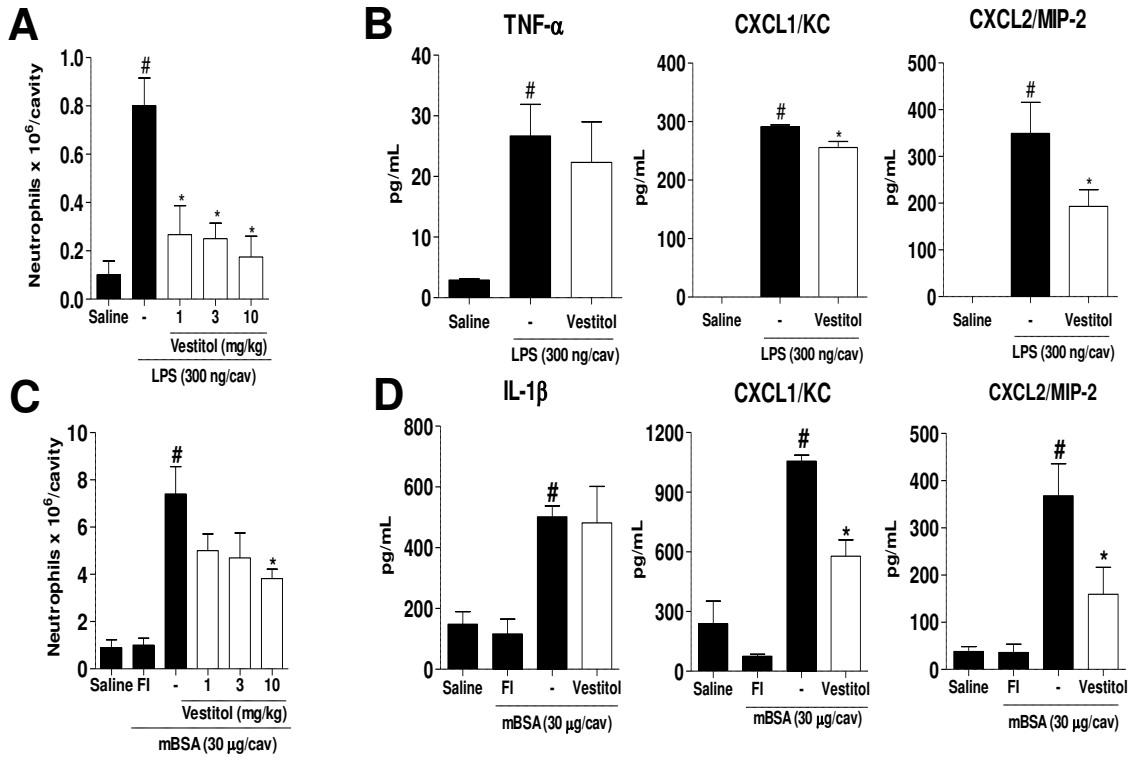


Figure 1. (A) Effect of vestitol on neutrophil migration into the peritoneal cavity induced by LPS (300 ng/cavity). (B) Effect of vestitol (10 mg / kg) on the release of TNF-α (1.5. h), CXCL1/KC and CXCL2/MIP-2 (3 h) in the peritoneal cavity of mice stimulated with LPS (300 ng/cavity). (C) Effect of vestitol on neutrophil migration into the peritoneal cavity induced by mBSA (30 µg/cavity). (D) Effect of vestitol (10 mg / kg) on the release of IL-1β, CXCL1/KC and CXCL2/MIP-2 (3 h) in the peritoneal cavity of mice stimulated with mBSA (30 µg/cavity). The groups were saline, false immunized (FI), LPS or mBSA (treated with vehicle) and vestitol at 1, 3 or 10 mg / kg, sc. Data were expressed as mean ± SEM, with n = 4-5. Symbols indicate statistical difference ($p < 0.05$, Tukey's test). # $p < 0.05$ compared to saline group; * $p < 0.05$ compared to LPS or mBSA group.

Upon determination of the activity of vestitol on the modulation of neutrophil migration, the mechanisms of action underlying such effects were next investigated. Cytokines and chemokines are important mediators that participate in neutrophil migration by inducing the synthesis of inflammatory mediators, or by modulating adhesion molecules (such as selectins and immunoglobulin) on endothelial cells, and through the induction of chemotaxis.[19-21] In this respect, the involvement of vestitol in the modulation of TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 (Figure 1), as well as in the rolling and adhesion of leukocytes in the mesenteric microcirculation of mice

(Figure 2), were examined. According to the results obtained in this study, the administration of vestitol (10 mg / kg) reduced the release of the chemokines CXCL1/KC and CXCL2/MIP-2 (Figure 1B and D) in vivo, but it did not affect TNF-α or IL-1β levels. In addition, vestitol at 10 mg / kg reduced leukocyte rolling and adhesion in the mesenteric microcirculation of mice (Figure 2A and B). Altogether, these findings demonstrate the modulating capacity of vestitol on the production or release of chemokines and on the rolling, adhesion and transmigration of neutrophils in mesenteric venules.

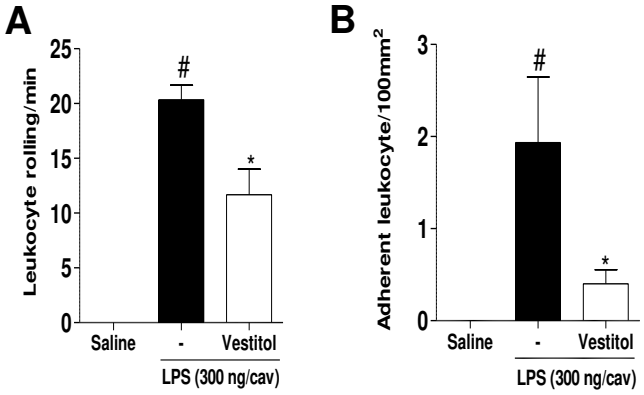


Figure 2. Effect of vestitol on the rolling (A) and adhesion (B) of leukocytes in the mesenteric microcirculation of mice submitted to ip administration of LPS (300 ng/cavity). The groups were saline, LPS (treated with the vehicle) and vestitol at 10 mg / kg sc. Data were expressed as mean ± SEM, with n = 4-5. Symbols indicate statistical difference ($p < 0.05$, Tukey's test). # $p < 0.05$ compared to saline group; * $p < 0.05$ compared to LPS group.

As the results indicate that vestitol reduced the release of the chemokines CXCL1/KC and CXCL2/MIP-2 in vivo, its possible effects on the release of these chemokines were investigated in stimulated macrophages in vitro (Figure 3). Resident macrophages are the main sources of cytokines and proinflammatory chemokines. Moreover, along with their various functions in protective immunity and homeostasis, macrophages contribute to many pathological processes.[22] In the present study, it was observed that vestitol at 1, 3 or 10 μM (Figure 3A) also reduced the levels of CXCL1/KC and CXCL2/MIP-2 in the supernatants of LPS-stimulated macrophages, which confirms the effects observed in vivo. In addition, the active concentration of 10 μM did not affect macrophage viability (Figure 3B). This in vivo and in vitro proven activity of vestitol in the inflammatory process is in line with other studies involving Brazilian propolis extracts and isolated compounds therefrom. One of these studies showed that

Brazilian green propolis extract was effective in reducing the migration of leukocytes (mainly neutrophils) into the peritoneal cavity of mice challenged with carrageenan.[23] Subsequently, the authors isolated and identified Artepellin C from green propolis and also demonstrated its anti-inflammatory activity as being related to the modulation of neutrophil migration.[24] Studies with galangin, a compound found in some varieties of Brazilian propolis, showed its modulatory activity on RAW 264.7 macrophages. However, unlike vestitol, which modulates the chemokines CXCL1/KC and CXCL2/MIP-2, galangin reduces IL-1β and IL-6 levels.[25]

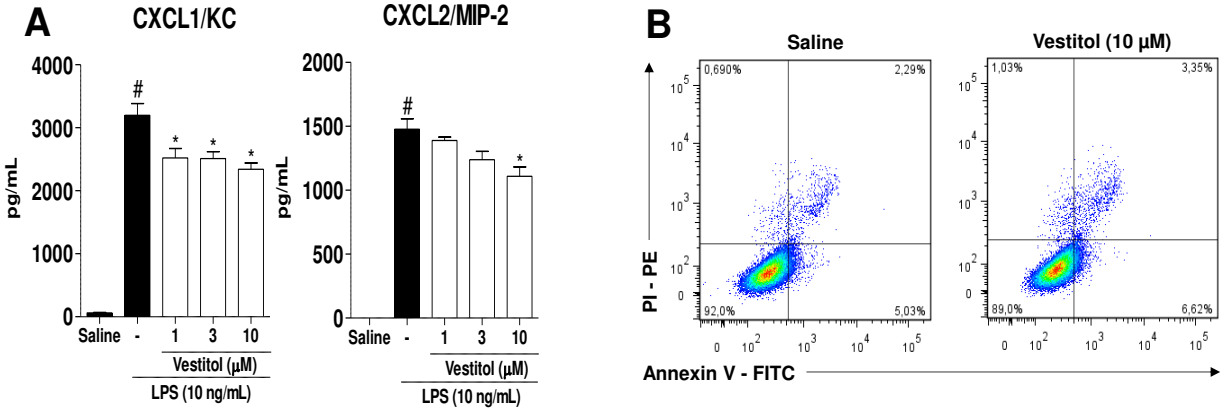


Figure 3. (A) Effect of vestitol on the release of CXCL1/KC and CXCL2/MIP-2 on differentiated macrophages from the bone marrow. (B) Cell viability assay by annexin V and PI on differentiated macrophages from the bone marrow. The groups were saline, LPS (treated with the vehicle) and vestitol at 1, 3 or 10 µM. Data were expressed as mean ± SEM, with n = 4-5. Symbols indicate statistical difference ($p < 0.05$, Tukey's test). # $p < 0.05$ compared to saline group; * $p < 0.05$ compared to LPS group.

The possible involvement of nitric oxide in the reduction of neutrophil migration promoted by vestitol was also evaluated (Figure 4). Several studies have shown that the increase of nitric oxide production in the peritoneum of mice subjected to an inflammatory stimulus is directly related to a reduced level of neutrophil migration into the inflammatory sites. This hypothesis was confirmed by the administration of nitric oxide synthase antagonists (iNOS inhibitors) in animals who had received ip inflammatory stimuli with carrageenan or LPS, resulting in increased neutrophil migration.[26,27] A recent study demonstrated that the bioactive fraction obtained from *Melipona scutellaris* geopropolis extract reduces carrageenan-induced neutrophil migration by increasing the release of nitric oxide into the peritoneum.[28] Thus, iNOS$^{-/-}$ mice were used to assess the involvement of nitric oxide in the anti-inflammatory activity of vestitol. According to the results (Figure 4), at the dose of 10 mg / kg vestitol

reduced neutrophil migration in wild type and iNOS$^{-/-}$ mice, suggesting that its anti-inflammatory activity does not depend on nitric oxide production.

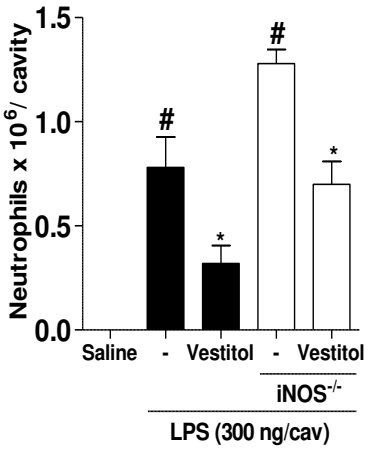


Figure 4. Effect of vestitol on neutrophil migration into the peritoneal cavity induced by LPS (300 ng/cavity) in wild type (WT, black bars) and iNOS knockout (iNOS$^{-/-}$ white bars) mice. The groups were saline, LPS (treated with the vehicle) and vestitol at 10 mg / kg sc. Data were expressed as mean ± SEM, with n = 4-5. Symbols indicate statistical difference ($p < 0.05$, Tukey's test). # $p < 0.05$ compared to saline group; * $p < 0.05$ compared to LPS group.

In addition to the inhibition of neutrophil migration in the inflammatory process through a reduced release of chemokines via resident macrophages, another possible mechanism of action investigated in this study was the direct action of vestitol on neutrophils. During tissue injury and/or infection caused by a microorganism, neutrophils from blood or bone marrow are mobilized and recruited to the inflamed site. Different chemotactic factors are responsible for locomotion of neutrophils in the organism. Among them, fMLP (bacterial product), IL-8 and the lipid mediator LTB4 are of paramount significance.[3] Neutrophil activation upon stimulation of chemotactic receptors induces extracellular calcium influx, promoting the migration via polymerization of actin filaments.[29] Thus, vestitol was tested for its action on the chemotaxis of CXCL2/MIP-2- and LTB4-induced neutrophils isolated from the bone marrow of mice. At the concentrations of 3 and 10 µM, vestitol reduced CXCL2/MIP-2- and LTB4-induced neutrophil chemotaxis (Figure 5A) without changing neutrophil viability (Figure 5B). As to the mechanistic pathway, vestitol (10 µM) reduced the calcium influx in neutrophils stimulated by CXCL2/MIP-2 and LTB4 (Figure 6A and B,

respectively). At 10 µM, vestitol did not alter the expression of CXCR2 in neutrophils (Figure 7).

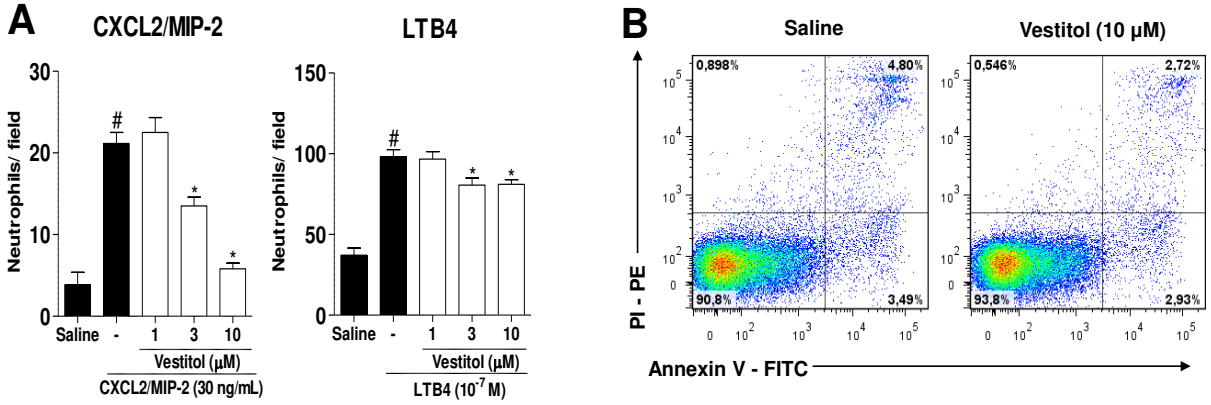


Figure 5. (A) Effect of vestitol on the chemotaxis of neutrophils induced by CXCL2/MIP-2 and LTB4. (B) Cell viability assay by annexin V and PI in isolated neutrophils from the bone marrow of mice. The groups were saline, CXCL2/MIP-2 or LTB4 (treated with the vehicle) and vestitol at 1, 3 or 10 µM. Data were expressed as mean ± SEM, with $n = 3$. Symbols indicate statistical difference ($p < 0.05$, Tukey's test). # $p < 0.05$ compared to saline group; * $p < 0.05$ compared to CXCL2/MIP-2 or LTB4 group.

Given the above, the present study demonstrates that part of vestitol activity on neutrophil migration is related to reduction of calcium influx induced by chemoattractants. A previous study reported the inhibitory effects of propolis extract on the chemotaxis of human polymorphonuclear leukocytes. However, the mechanism of action of the extract and of its isolated bioactive compound remained to be understood.[30] Herein it was demonstrated that in addition to a role in the modulation of chemokine levels by macrophages, vestitol can also act directly on neutrophils and thus reduce chemotactic activity in this cell type. These findings attest the potential of vestitol as a novel substance with anti-inflammatory properties.

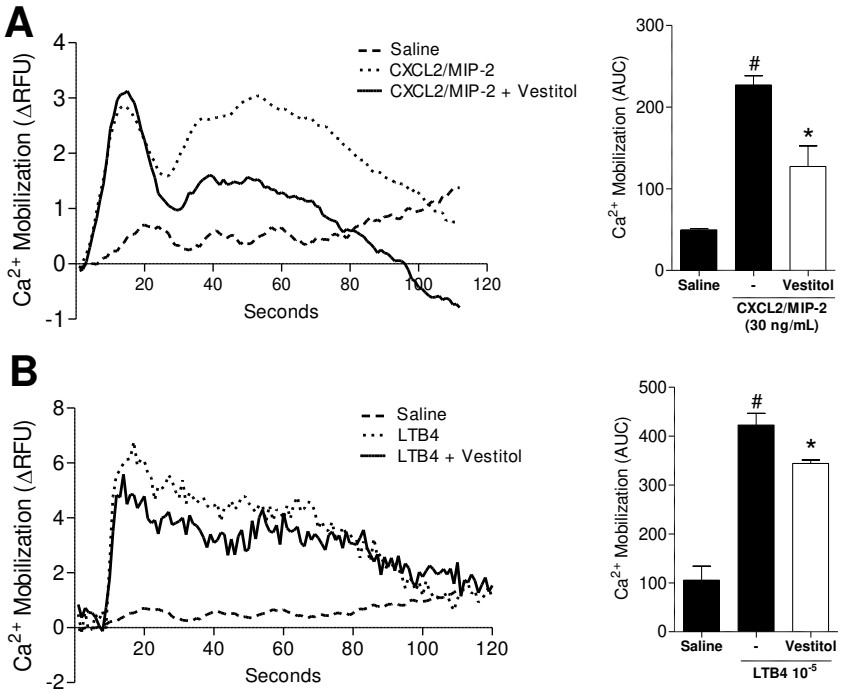


Figure 6. Effect of vestitol on calcium influx induced by CXCL2/MIP-2 (A) and LTB4 (B) in neutrophils. The groups were saline, CXCL2/MIP-2 or LTB4 (treated with the vehicle) and vestitol at 10 µM. Data were expressed as mean ± SEM, with n = 3-4. Symbols indicate statistical difference ($p < 0.05$, Tukey's test). # $p < 0.05$ compared to saline group, * $p < 0.05$ compared to CXCL2/MIP-2 or LTB4 group.

Thus, based on the results it may be concluded that vestitol showed inhibitory activity on neutrophil migration induced by different inflammatory stimuli. Its activity is related to inhibition or decrease of the release of the chemokines CXCL1/KC and CXCL2/MIP-2 by resident macrophages, resulting in diminished rolling and adhesion of leukocytes in the mesenteric microcirculation. Furthermore, it was demonstrated that part of vestitol activity in inhibiting neutrophil migration is also related to decreased chemotaxis via blocking calcium influx. These data show that vestitol is a promising anti-inflammatory agent.

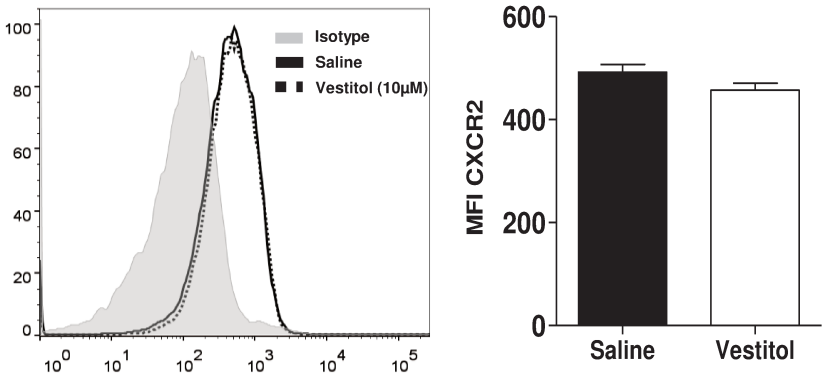


Figure 7. Effect of vestitol on CXCR2 expression by flow cytometry in neutrophils. The groups were saline and vestitol at 10 μM. Data are expressed as mean ± SEM, with n = 3-4 ($p < 0.05$, Tukey's test).

EXPERIMENTAL SECTION

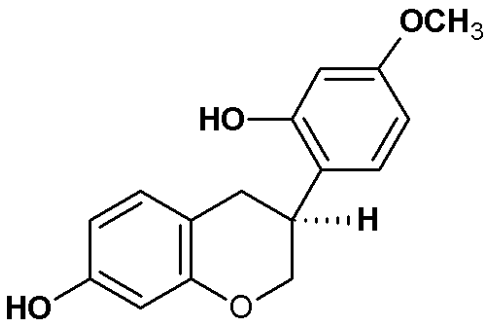
General Experimental Procedures. Lipopolysaccharides from *Escherichia coli* B4 (LPS), methylated bovine serum albumin (mBSA), leukotriene B4 (LTB4), Fluo 3- AM, bovine serum albumin (BSA), RPMI-1640 medium, penicillin, L-glutamine and DMSO were purchased from Sigma (St. Louis, MO, USA); ApoScreen™ Annexin V-FITC Kit was acquired from SouthernBiotech (Birmingham, AL); anti-CXCR2, anti-Ly6G and CXCL2/MIP-2 were purchased from R&D Systems (Minneapolis, MN); and Fetal Bovine Serum was from Gibco (Grand Island, NY). LPS or mBSA was dissolved in saline for ip administration in vivo. CXCL2/MIP-2 and LTB4 were dissolved in 0.01 % RPMI-BSA to stimulate neutrophils in vitro.

Experimental Biological Material. Samples of *Apis mellifera* red propolis were collected in Maceio (9º40' S, 35º41' W), state of Alagoas, northeastern Brazil, during the month of March. The chemical composition of Brazilian propolis extract was evaluated by high performance liquid chromatography on reverse phase (RP-HPLC) (Figure S1). In order to obtain the extract, 64g of propolis was crushed and then added to an aqueous solution of ethanol (80 % v/v in water) at a ratio of 1:12.5 (w/v). The mixture was kept in water bath at 70 °C for 30 min, followed by centrifugation (3000 xg, 5 min at 4 °C), filtration.[15]

RP-HPLC. The extract was ressuspended in methanol and filtered (0.22 μm diameter - Millipore). Twenty microliters of sample was injected in the RP-HPLC system equipped with a Shimadzu ODS-A column (RP-18, 4.6 x 250 mm; particle size 5 μm)

and a photodiode array detector (SPD-M10AVp, Shimadzu Co.). The column was eluted using a linear gradient of water (solvent A) and methanol (solvent B), starting with 40 % (of B), increased to 60 % (45 min). The concentration was then held at 90 % (45-75 min) before decreasing to 30 % of B (75-85 min), using a solvent flow rate of 1 mL/min and a diode array detector. Chromatograms were recorded at 260 nm.[10] The following authentic standards of phenolic acids and flavonoids (Extrasynthese Co.) were examined: formononetin, daidzein, biochanin A, neovestitol, vestitol, catechin, epicatechin, rutin, propyl gallate, ferulic acid and p-coumaric acid.

Extraction and Isolation. The ethanol extract obtained (39 g) was subjected to liquid-liquid fractionation with hexane and chloroform. The chloroform fraction (22 g) was collected, concentrated in rotary evaporator and subjected to separation by dry column chromatography (1 g silica gel *per* column) using a cellulose column (20 cm length and 3 cm inner diameter) and chloroform:ethyl acetate (7:3) as mobile phase. Five subfractions were obtained and the subfraction 4 (2.2 g) was selected for further separation in Sephadex LH-20 gel with methanol as mobile phase. Thus, 100 mg of the subfraction 4 was dissolved in 2 mL of methanol and then fractionated, yielding three subfractions (4.1, 4.2 and 4.3). Fraction 4.2 (200 mg) was purified by semipreparative RP-HPLC 250 x 20 mm column eluted with a gradient starting with methanol/water (65:35) to methanol/water (95:5) in 35 min, flow rate of 3 mL/min, yielding 55 mg of vestitol [(3*S*)-3-(2-hydroxy-4-methoxyphenyl)-3,4-dihydro-2*H*-chromen-7-ol].[15] All steps of the fractionation process were monitored using thin-layer chromatography with anisaldehyde reagent, followed by incubation at 100 °C for 5 min. The identification of the compound was performed by high-resolution mass spectrometry (QTOF-MS/MS) and nuclear magnetic resonance (NMR).[11] Pure vestitol (> 98.0 % purity) was dissolved in 0.1 % DMSO for further use.



Vestitol

Animals. Male SPF (specific-pathogen free) C57BL/6 (WT) or iNOS deficient (iNOS$^{-/-}$) mice weighing 20–22 g were used. The animals were housed at temperatures between 22 and 25 °C, with a light cycle of 12 h light/12 h dark, humidity of 40–60 %, and access to water and food ad libitum. The procedures described were reviewed and approved by the local Animal Research Ethics Committee (CEUA/UNICAMP, process no. 2793-1).

Experimental Procedures to Evaluate LPS-induced Neutrophil Migration in vivo. Mice were pretreated with sc administration of vestitol (1, 3 or 10 mg / kg) 30 min prior to ip injection of LPS (300 ng/cavity). The negative control group received the vehicle. The mice were killed 6 h after the challenge (LPS) and cells from the peritoneal cavity were harvested by washing the cavity with 3 mL of phosphate buffered saline (PBS) containing 1 mM EDTA. The volumes recovered were similar in all experimental groups and equal to approximately 95 % of the injected volume. The total cell count was performed in Neubauer chamber. Smears were prepared using a cytocentrifuge (Cytospin 3; Shandon Lipshaw), stained with Panotic staining kit and the different cells were counted (until 100 cells) using an optical microscope (1000 x). The results are presented as the number of neutrophils per cavity.[27]

Experimental Procedures to Evaluate mBSA-induced Neutrophil Migration in vivo. Mice were immunized by sc injection of an emulsion containing 200 µl of PBS and equal volumes of complete Freud adjuvant (CFA), in which 500 µg of mBSA were dissolved. Seven and fourteen days after the first immunization the animals were administered a reinforcement of the emulsion with incomplete Freud adjuvant (IFA). False-immunized mice (FI) received this same treatment, excepting for the administration of mBSA. Twenty-one days after the first injection, the animals were pretreated with vestitol (1, 3, or 10 mg / kg, sc). Fifteen minutes after administration of vestitol, an ip injection of mBSA (30 µg / cavity) was performed. The mice were sacrificed 6 h after challenge with mBSA, the peritoneal cavity was washed, and total and differential cells counting was performed as described earlier.[31]

Cytokine Assay in vivo. Mice were pretreated with vestitol (10 mg / kg, sc), 30 min prior to injection of LPS (300 ng/cavity) or mBSA (30 µg/cavity). The vehicle was used as the negative control. After 1.5 or 3 h, the animals were killed and their peritoneal fluid was collected as previously described. TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 levels were determined by ELISA using the protocols supplied by the manufacturers (R&D Systems, Inc). The results were expressed as pg/mL.[32]

Intravital Microscopy (Leukocyte Rolling and Adhesion). Mice were pretreated with vestitol (10 mg / kg, sc) 30 min prior to the ip injection of LPS (300 ng/cavity). The vehicle was used as a negative control. After 2 or 4 h since the inflammatory stimulus, the animals were anesthetized and their mesenteric tissue was exposed to assessment by intravital microscopy. The animals were placed on a plate with a thermostat at 37 °C. Post-capillary venules with diameter of 10-18 μm were selected, and the interaction of leukocytes with the luminal surface of the endothelial venule was assessed. Then the number of rolling leukocytes during 10 min was counted. Leukocytes were considered adherent to the endothelium in case they remained stationary for > 30 s. Cells were counted and the images were recorded using five different fields for each animal to avoid variability due to sampling. Calculations were made for each animal.[33,34]

Cytokine Assay in vitro. Total bone marrow cells were obtained from the femur and tibia of the mice; washed and then suspended in RPMI supplemented with 10 % fetal bovine serum (FBS) plus 100 U/mL of penicillin, L-glutamine and 20 % of the supernatant culture of L929. Later on, cells were incubated for 7 days at 37 ºC in 5 % CO_2/95 % atmosphere to complete differentiation of cells into macrophages.[35] Then the macrophages were seeded onto 96-well plates (2 x 10^5 cell/well). Two hours later (after complete adhesion), the cells were pretreated with vestitol at 1, 3 or 10 μM. After 30 min, LPS (10 ng/mL) was added and the plates were immediately incubated at 37 °C, 5 % CO_2, for 4 h. Supernatants were collected and the levels of CXCL1/KC and CXCL2/MIP-2 were determined by ELISA using the manufacturer's protocol (R&D Systems, Inc.). The results were expressed as pg/mL.

Experimental Procedure to Evaluate LPS-induced Neutrophil Migration in iNOS$^{-/-}$ mice. WT or iNOS$^{-/-}$ mice were pretreated with vestitol (10 mg / kg, sc) and 15 min later received ip administration of LPS (300 ng/cavity). After 6 h, the mice were sacrificed, their peritoneal cavity was washed and total and differential cells counting was performed as described earlier.[27]

Isolation of Neutrophils. Total bone marrow cells were obtained from the femur and tibia of the mice. Then cells were washed, suspended in 2 mL of Hanks balanced saline solution and transferred to a centrifuge tube containing 72 % and 65 % of Percoll gradient. After centrifugation (1200 g, 35 min, 18 °C), the band formed between the gradient was collected. Cells were quantified using a Neubauer chamber and the purity

neutrophils (approximately 95 %) was assessed using smear prepared with the aid of a cytocentrifuge.[36]

Neutrophil Chemotaxis in vitro*.* The Boyden chamber (Neuroprobe, Pleasanton, CA, USA) was used in the chemotaxis assay. Neutrophils isolated from the bone marrow of mice were incubated (1 x 10^6 cells/mL) at 37 °C and 5 % CO_2 with vestitol at 1, 3 or 10 µM for 30 min. The stimuli of CXCL2/MIP-2 (30 ng/mL) or LTB4 (10^{-7}) was placed on a single side of the membrane (5-µm porosity; Neuroprobe, Pleasanton, CA, USA). On the other side, 50 µl of a neutrophil suspension was added with or without vestitol. RPMI-BSA (0.01 %) was used as the negative control. Then the membrane was incubated for 60 min under the same conditions described earlier. The filter was removed, washed, subjected to staining by the Panotic kit, and the cells were counted under an optical microscopy (1000x). The results were presented as the average of neutrophils migrated in 5 high-resolution fields (1000x). Each experiment was performed in triplicate. [37]

Calcium Influx. Neutrophils isolated from the bone marrow of mice were incubated (1 x 10^6/mL) for 40 min with Fluo 3-AM at 37 ºC, 5 % CO_2. After that, the neutrophils were incubated for additional 30 min in the presence of vestitol (10 µM). The measurement of calcium influx was carried out on a FlexStation 3 Benchtop Multi-Mode Microplate Reader. CXCL2/MIP-2 (30 ng/mL) or LTB4 (10^{-5}) was put in direct contact with the cells (2x10^5 cells/well) in 96-well black plates using the equipment automatic settings.[38]

CXCR2 Expression. After isolation, neutrophils (1 x 10^6 cells/mL) were incubated with vestitol (10 µM) for 1.5 h. The cells were washed and incubated for 20 min at 4 °C with the antibodies anti-Ly6G conjugated with APC (1:200) and anti-CXCR2 conjugated with PE (1:50). Then, the cell solution was washed and re-suspended in 100 µl of PBS plus 2 % formaldehyde. Cytometric analysis of two-color flow was performed using a BD® FACSVerse.[39]

Cell Viability Assay by Flow Cytometry. Flow cytometry analysis was performed using neutrophils and macrophages isolated from the bone marrow of the mice. Macrophages were cultured in 6-well plates (2 x 10^6 cells/well) at 37 ºC in 5 % CO_2/95 % atmosphere by two hours for complete adhesion. Due to non-adherence in plates, neutrophils were placed in sterile conical tubes at the concentration of 1 x 10^6 cells/well. Both cell lines were pretreated with vestitol (10 µM), then incubated at 37 °C, 5 % CO_2 for 4 h. Subsequently, the cells were washed, suspended in 100 µl of 1x Binding Buffer and incubated for 20 min at 4 °C with anti-annexin V antibody

conjugated with FITC (1:50). Then anti-PI antibody (propidium iodide) at 1:100 was added to the cell solution, and measurements were acquired in a cytometer BD® FACSVerse.

Statistical Analysis. The data were expressed as mean ± standard error of the mean (SEM), and statistical comparisons between groups were carried out using analysis of variance (ANOVA) followed by Tukey's test. Significance was accepted when $p < 0.05$.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Supporting Information

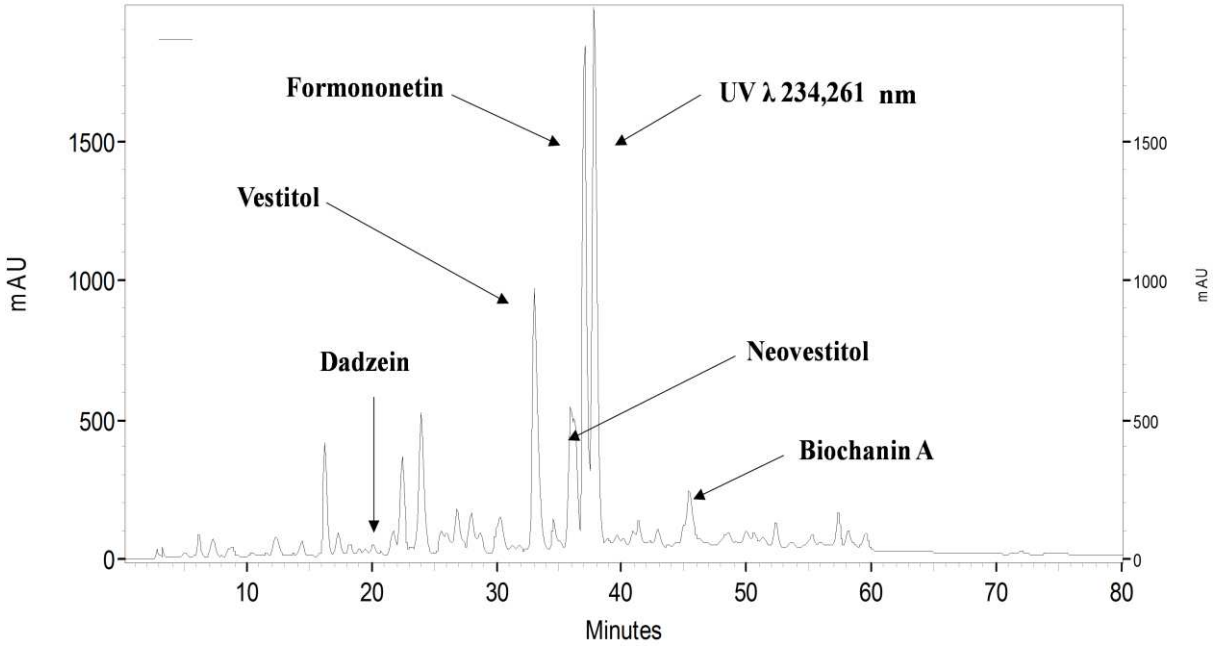
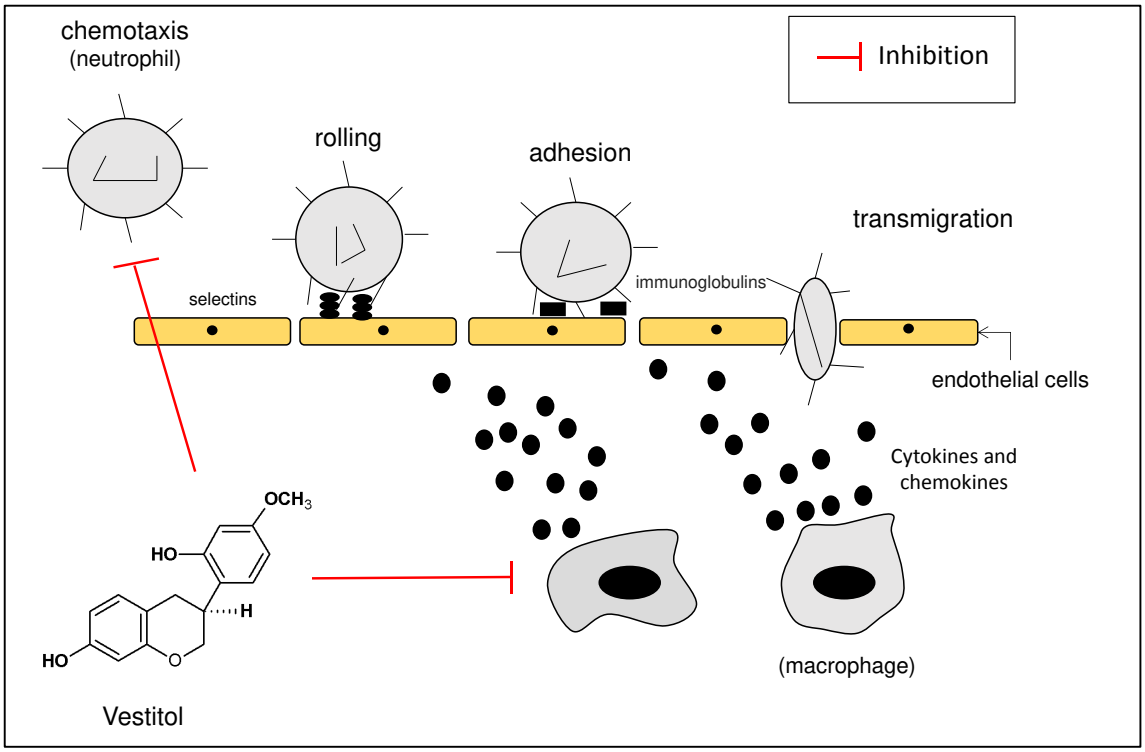


Figure S1. RP-HPLC chromatogram of Brazilian red propolis.

Graphical abstract



2.2 Artigo 2:[4]

Neovestitol, an isoflavonoid isolated from Brazilian red propolis, reduces acute and chronic inflammation: involvement of nitric oxide and IL-6

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Abbreviations

BSA, bovine serum albumin; CFA, complete Freud adjuvant; CXCL1, chemokine ligand 1/KC, keratinocyte chemoattractant; CXCL2, chemokine ligand 2/MIP-2, macrophage inflammatory protein 2; DMSO, dimethyl sulfoxide; IFNγ, interferon gamma; ICAM-1, intercellular adhesion molecule type 1; IL, interleukin; LPS, lipopolysaccharides; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NMR, nuclear magnetic resonance; TNF-α, tumor necrosis factor-alpha.

Keywords: Arthritis, Brazilian red propolis, inflammation, isoflavonoids, neovestitol.

Abstract

Scope

Isoflavonoids have been much studied by science, and different biological activities have been identified. The present study aimed to assess the main mechanisms of action of neovestitol, an isoflavonoid isolated from Brazilian red propolis, in acute and chronic inflammation.

Methods and Results

It was found that neovestitol at 10 mg/kg reduced neutrophil migration, rolling and leukocyte adhesion in the mesenteric microcirculation during acute peritonitis caused by lipopolysaccharide. Likewise, neovestitol reduced the expression of intercellular adhesion molecule type 1 (ICAM-1) *in vivo*. No changes were observed in the levels of TNF, CXCL1/KC and CXCL2/MIP-2 *in vivo* and expression of ICAM-1 *in vitro* with pretreatment with neovestitol. The administration of nitric oxide synthase inhibitor abolished the inhibitory effect of neovestitol in neutrophil migration. No effects of neovestitol were observed on the chemotaxis of neutrophils. It was observed that neovestitol also reduced the clinical score and joint damage in collagen-induced arthritis model. There was no change in the frequency of IL-17-producing CD4+ cells. In addition, pretreatment with neovestitol, reduced the levels of IL-6.

Conclusion

The results demonstrate a potential anti-inflammatory activity of neovestitol, which may be useful for therapeutic purposes and/or as nutraceutical.

1. Introduction

Inflammation comprises a set of events that involve the participation of cytokines, chemokines, lipid mediators, cell adhesion molecules and immune cells. This series of events leads to vasodilation, increased vascular permeability and leukocyte recruitment [1].

The occurrence of acute inflammation is related to a defensive response to infectious agents and is characterized by the presence of neutrophils. On the other hand chronic inflammation is a persistent phenomenon where the presence of macrophages and lymphocytes is more evident [2].

Although it is a host protective response, in some cases exacerbation of inflammation is directly related to tissue damage, thus requiring the use of anti-inflammatory drugs [3]. Furthermore, inflammatory diseases such as rheumatoid arthritis results of chronic inflammation, whose treatment with drugs is still scarce [4].

Propolis has been used in folk medicine for decades for the treatment of various diseases [5]. Furthermore, propolis is also present in cosmetic formulations, toothpastes and food preservatives [6-8]. The Brazilian red propolis, classified as type 13, is known as a new type of Brazilian propolis [9]. Originating from Maceió, northeastern Brazil, this propolis has the peculiar presence of isoflavonoids in its composition. Among the identified isoflavonoids, neovestitol showed antimicrobial, anticaries and antioxidant activity in preliminary studies [10,11]. The anti-inflammatory potential of neovestitol has also been reported [12], however its activity on a chronic inflammation model, as well as the main pathways of action involved on in remain to be elucidated.

Thus, this study aimed to evaluate the activity of neovestitol on acute and chronic inflammation. Moreover, elucidate the main routes of action.

2. Material and methods

2.1. Reagents

Ethanol, hexane, chloroform and ethyl acetate were purchased from Merck. LPS, DMSO, BSA, RPMI-1640 Medium, L-glutamine and penicillin were purchased from Sigma. Type II bovine collagen from MD Biosciences. Apo Screen Annexin V-FITC Kit was purchased from Southern Biotech. CXCL2/MIP-2 from R&D Systems.

Fetal bovine serum from Gibco. Neovestitol was dissolved in 0.1% DMSO in PBS. LPS was dissolved in sterile PBS.

2.2. Extraction and isolation of neovestitol

Samples Brazilian red propolis collected by of *Apis melliferaL.* (Apidae) were obtained in Maceio (9°40′ S, 35°41′ W), state of Alagoas, northeastern Brazil. The chemical profile of red propolis was previously described [9]. Briefly, the propolis extract was prepared in 80 % ethanol (v/v in water). The extract (39 g) was subjected to liquid-liquid fractionation and the chloroform fraction (22 g) was collected, concentrated and fractionated in a dry column using a mixture of chloroform: ethyl acetate at the ratio 7:3 as mobile phase. Subfraction 4 (2.2 g) was subjected to fractionation in Sephadex LH20 using methanol as mobile phase. The subfraction 4.2. (200 mg) was obtained and purified in semipreparative reversed-phase HPLC (Shimadzu) yielding neovestitol (120 mg) (Figure 1) [12]. The identification of neovestitol (2',4'-Dihydroxy-7-methoxyisoflavan) was accomplished by QTOF-MS/MS and NMR [11].

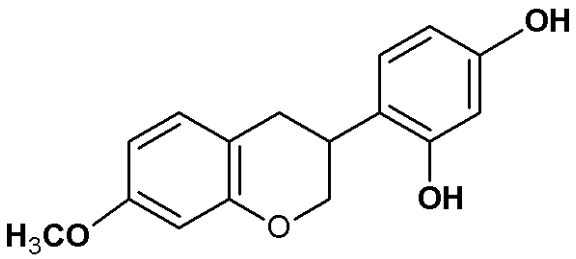

Figure 1. Chemical structure of neovestitol ($C_{16}H_{16}O_4$; 272.29584 g/mol).

2.3. Animals

Specific pathogen-free (SPF) mice, C57BL/6 or DBA-1/J weighing between 20-25 g were housed at temperatures of 22-25 °C, with a light cycle of 12 h light/12 h dark, 40-60% humidity, and with access to water and food *ad libitum*. All procedures involving the use of animals were previously approved by the local ethics committee (CEUA Unicamp, process no. 2793-1).

2.4. Cell culture

bEnd.3 cells, the mice brain cerebrovascular endothelial cell line from the American Type Culture Collection (ATCC), were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 4.4 g of glucose and 100 U/mL of penicillin at 37 °C in 5% CO_2/ 95% O_2 atmosphere.

2.5. Neutrophil migration *in vivo*

Mice were pretreated with neovestitol at the doses of 1, 3 or 10 mg/kg subcutaneously (sc). After 30 min, LPS was administered intraperitoneally (ip) at 300 ng/cavity and then 6 h later animals were killed. The peritoneal cavity of the mice was washed with 3 mL PBS/EDTA (1 mM) and the total cell count was performed in a Neubauer chamber. The differential counting was performed under optical microscopy (1000x magnification) with slides prepared in a cytocentrifuge (Cytospin 4, Shandon, Pittsburgh, PA) following hematologic staining using a commercial kit (Laborclin, Brazil). Results were expressed as the number of neutrophils per cavity [13].

2.6. Quantification of TNF-α, CXCL1/KC and CXCL2/MIP-2 *in vivo*

Mice were pretreated with neovestitol at the dose of 10 mg/kg (sc). After 30 min, LPS was injected (ip) at 300 ng/cavity and, 1.5 h or 3 h later the animals were killed and their peritoneal cavity washed with 3 mL PBS/EDTA. The quantification of TNF-α, CXCL1/KC and CXCL2/MIP-2 was performed by ELISA assay using protocols provided by the manufacturers (R&D Systems). Results were expressed as pg/mL [14].

2.7. Intravital microscopy

Mice were pretreated with neovestitol at 10 mg/kg (sc). After 30 min, LPS was administered (ip) at 300 ng/cavity. The animals were anesthetized with ketamine and xylazine 2 h or 4 h after the LPS stimulus. Their mesentery was exteriorized for observation of the microcirculation (intravital microscope DM 6000, Leica). Leukocyte rolling (2 h after stimulus) was counted during 5 min, and a total of 3 different fields per animal were analyzed. Leukocyte adhesion (4 h after stimulus) was assessed by counting the number of leukocytes adhered to an area of 100 μm^2 on the mesenteric venule. The results of leukocyte rolling were expressed as number of leukocytes rolling/min and the adhesion as number of adhered cells/100 μm^2[15,16].

2.8. Collection of mesenteric tissue and immunofluorescence assay for ICAM-1

Mice were pretreated with neovestitol at the dose of 10 mg/kg (sc). After 30 min, LPS was administered ip at 300 ng/cavity and 4 h later the animals were killed and their mesenteric tissue collected and frozen in Tissue-Tek. Sections of 5 µm of the frozen tissue were made using a cryostat (Leica). Blocking was performed with 1% BSA. The anti-ICAM-1 antibody (BD Bioscience) conjugated to FITC was incubated for 2 h. Hoesch 33342 (Sigma) at 1 µg/mL was used to label the nucleus of the cells. Fluoromount was used for attachment of the cover slip. Fluorescence images were acquired in microscopy Leica [17].

2.9. bEnd.3 cell culture

bEnd.3 cells were cultured in 6-well plates (1 x 10^6 cells/well) at 37 °C and 5% CO_2. Following complete adhesion during 24 h, cells were pretreated with neovestitol at 30 µM and 30 min later, LPS stimulation at 5 µg/mL was performed. Cells were kept for 24 h under conditions previously described [18]. After LPS stimulation cells were manually lysed using RIPA Buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors and, ICAM-1 expression was determined by western blotting assay.

2.10. Western blotting

Protein quantification was performed by the colorimetric method of Coomassie (Bradford) Protein Assay Kit. Homogenate samples were denatured at 100 °C for 10 min and applied into a polyacrylamide 10% gel using Mini-PROTEAN Tetra Cell (Bio-Rad). After the run, the separating gel was transferred to a nitrocellulose membrane. The blocking of nonspecific antigenic sites was carried out by incubation of membranes for 1 hour at room temperature in Tris-buffered saline (TBS) containing 5% (w/v) BSA and 0.1% Tween 20. The ICAM-1 antibody (Santa Cruz) and beta-actin were diluted in Tris-buffered saline (TBS) containing 5% (w/v) BSA and 0.1% Tween 20 and incubated overnight and for 1 hour, respectively. After incubation, the membranes were washed and then incubated with anti-mouse secondary antibody for 1 h at room temperature. For developing, the membranes were incubated with a mixture of reagents of the chemiluminescence kit.

2.11. bEnd.3 viability assay by MTT

bEnd.3 cells were cultured in 96-well plates (1 x 10^5 cells/well) at 37 °C and 5% CO_2. After complete adhesion in 24 h, the cells were pretreated with neovestitol at 30 µM and incubated for 24 h under conditions previously described. The cells were resuspended in RMPI with MTT (1 mg/mL) and incubated for 2 h. Subsequently, the supernatant was removed and 200 µL of ethanol was added. The absorbance was measured at 540 nm using a microplate reader (Flex Station 3 Multi-Mode Microplate Reader).

2.12. Oxide nitric pathway

Mice were pretreated with aminoguanidine at 50 mg/kg (sc) 15 min before the administration of neovestitol at 10 mg/kg sc. After 30 min, LPS was administered (ip) at 300 ng/cavity and then 6 h later the animals were killed. The peritoneal cavity was washed with 3 mL PBS/EDTA (1 mM) and the total and differential cell count was performed as described earlier [13].

2.13. Isolation of neutrophils and chemotaxis *in vitro*

Isolation of neutrophils from the bone marrow of mice was performed by Percoll gradient [19]. Briefly, total cells obtained from the bone marrow (femur and tibia) of the mice, washed and resuspended in 2 mL Hank's buffered salt solution (HBSS, Sigma-Aldrich). Then, cell suspension was transferred to a 15 mL polypropylene sterile tubes (BD Falcon) containing 72% and 65% Percoll gradient and subjected to centrifugation (1200 at 18°C) during 35 min. The band of neutrophil formed between the gradients was collected and, the total number of cells was quantified using a Neubauer chamber. The purity of neutrophils was assessed by differential count following the aforementioned protocol. Approximately 95% of neutrophils was found after isolation.

For chemotaxis, neutrophils were incubated with neovestitol at 0.3, 3 and 30 µM at 37 ° C and 5% CO_2 for 30 min in RPMI-BSA (0.01%). The modified Boyden chamber (Neuroprobe) was used in this assay. Briefly, the CXCL2/MIP-2 stimulus (30 ng/mL) was placed on one side of the membrane (5 µm pore) and 50 µL of a neutrophil suspension at 1 x 10^6 cells/mL were placed on the another side, and incubated under the same conditions earlier described.. After 60 min, the membrane was removed and stained with Panotic Kit and neutrophils counted in an optical microscope (1000X

magnification). Each experiment was performed in triplicate. The results are presented as the average of neutrophils that migrated in 5 fields (resolution 1000 x)[20].

2.14. Neutrophil viability assay by flow cytometry

Neutrophils were incubated for 1.5 h under the conditions previously described with neovestitol at the concentration of 30 µM. For the viability study, the cells were resuspended in 100 µL of 1 x Annexin buffer. Anti-annexin V-FITC antibody (1:50) was added and incubated for 20 min at 4 °C. In addition, the anti-PI antibody (propidium iodide) 1:100 was also added to the solution at the moment of acquisition in a FACSVerse (BD Biosciences) flow cytometer. The analysis was performed using the software "Flow Jo" (TreeStar) [21].

2.15. Arthritis model

2.15.1. Collagen-induced arthritis

Arthritis was induced in DBA-1/J mice by intradermal injection at the base of the tail of an emulsion containing equal volumes of type II bovine collagen and Complete Freud's Adjuvant (CFA) (final volume 50 µL). After 21 days, the mice received a booster injection of type II bovine collagen and CFA. Treatment with neovestitol at the doses of 3 and 10 mg/kg (sc), once a day, was started after the booster injection and remained for 12 days (Figure 2). The control group that did not receive the drug test received the vehicle. The development of experimental arthritis was monitored daily by the clinical score of the animals, as described by [22].

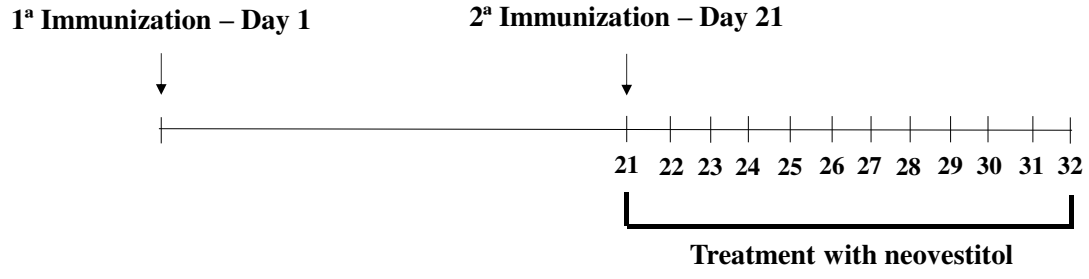

Figure 2. Imunization scheme with type II collagen and CFA and treatment with neovestitol.

2.15.2. Joint histological analysis

At the end of the 12 day-treatment with neovestitol at the doses of 3 and 10 mg/kg, the animals were sacrificed and their femoral-tibial joints were dissected, fixed and stained with hematoxylin and eosin staining (HE).

2.15.3. Collection of draining lymph nodes and analysis of IL-17 by flow cytometry

The inguinal lymph nodes (2 per animal) were collected by surgical procedure, then dissected, macerated in RPMI and filtered through a 100 µm sieve. The cells collected from the lymph nodes were stimulated with 50 ng/mL of phorbol-12-myristate-13-acetate and 500 ng/mL of ionomycin (Sigma) in the presence of GolgiStop (BD Bioscience) for 4 h. Later, the cells were labeled and permeabilized with Perm/fix solutions (eBioscience), labeled with anti-IL-17 and analyzed in FACSVerse (BD Biosciences) flow cytometer using the software "Flow Jo".

2.15.4. Quantification of joint cytokines

Samples from the femorotibial articulation were homogenized, centrifuged and the supernatant frozen at -70°C. The quantification of IL-17, IFNγ,TNF-α and IL-6 was performed using protocols supplied by the manufacturers (R&D Systems). The results were expressed as pg or ng/g tissue.

2.16. Statistical analysis

Data are expressed as means ± standard error of the mean (SEM) and statistical comparison between the groups was performed by analysis of variance (one-way and two-way ANOVA) followed by Tukey's post-test or Bonferroni. Significance was accepted when $P < 0.05$.

3. Results

3.1. Neovestitol reduces neutrophil migration, rolling and adhesion *in vivo* by the nitric oxide pathway

The present study investigated the effects of neovestitol on LPS-induced neutrophil migration, release of inflammatory mediators in the peritoneal cavity, leukocyte adhesion and rolling in the mesenteric microcirculation, nitric oxide pathway and *in vivo* and *in vitro* expression of ICAM-1. According to the results, it was observed

that neovestitol at 3 and 10 mg/kg reduced LPS-induced neutrophil migration ($P < 0.05$, Figure 3A). Regarding the activity of neovestitol at 10 mg/kg on the release of cytokines and chemokines, no changes were observed in the levels of TNF-α, CXCL1/KC and CXCL2/MIP-2 ($P > 0.05$, Figure 3B). As to leukocyte rolling and adhesion, it was found that the administration of neovestitol at the dose of 10 mg/kg reduced leukocyte rolling and adhesion ($P < 0.05$, Figure 3C). Pretreatment with neovestitol at 10 mg/kg did not reduce neutrophil migration in mice pretreated with a nitric oxide synthase inhibitor ($P > 0.05$, Figure 3D). Pretreatment with neovestitol at 10 mg/mL reduce the expression of ICAM-1 *in vivo* ($P < 0.05$, Figure 3E). *In vitro* neovestitol at 30 μM did not reduce the expression of ICAM-1($P > 0.05$, Figure 3F) and also did not change the viability of bEnd.3 cells ($P > 0.05$, Figure S1).

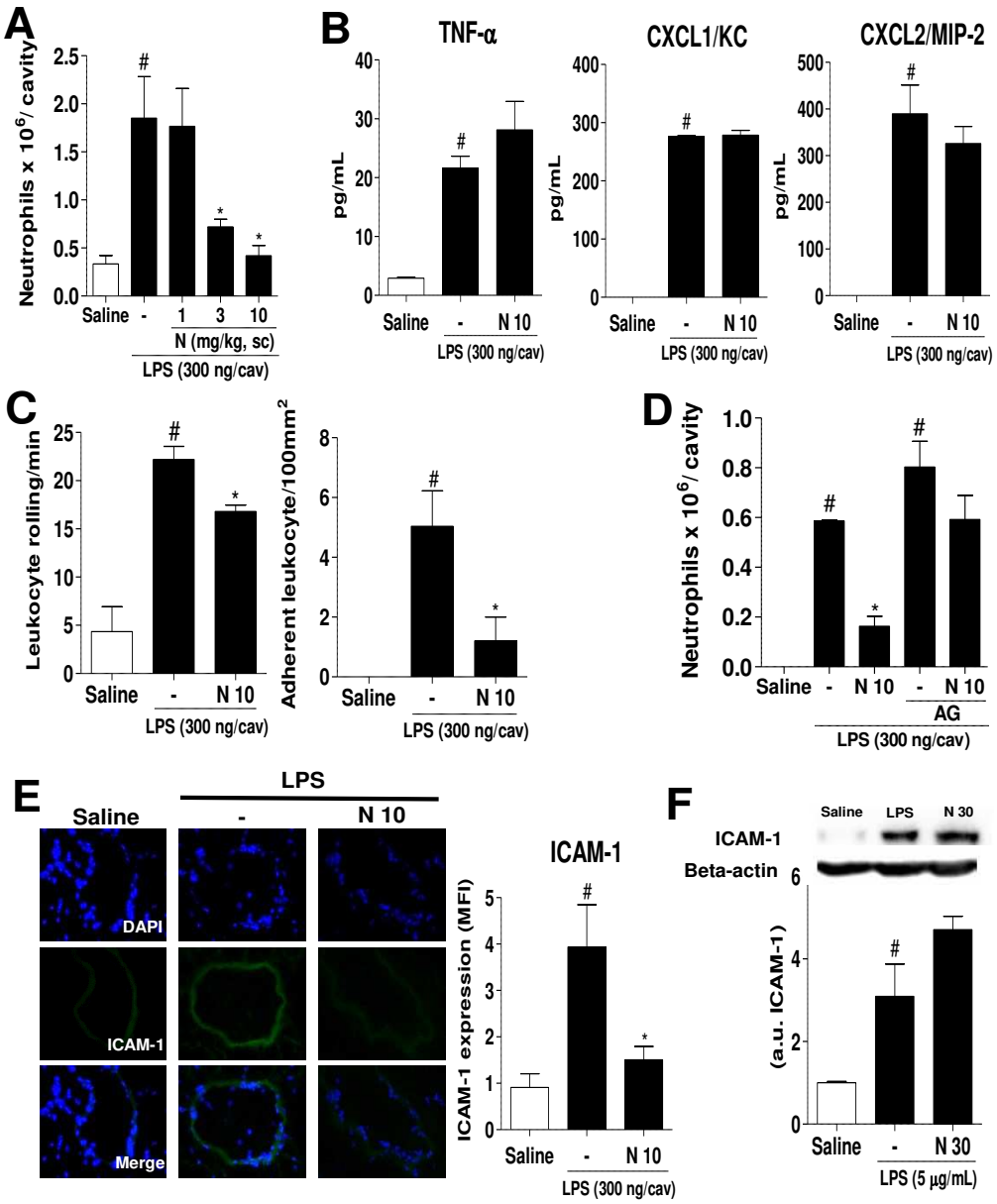

Figure 3. Neovestitol reduces neutrophil migration by a nitric oxide-dependent mechanism. C57BL/6 mice were pre-treated with neovestitol (N) at the doses of 1, 3 or 10 (N 10) mg/kg sc, 30 min before i.p administration of LPS (300 ng/cavity). bEnd.3 cells were pretreated with neovestitol at the concentrations of 30 µM (N 30), 30 min prior to LPS stimulation with 5 µg/mL. (A) Migration of neutrophils into the peritoneal cavity induced by LPS (300 ng/cavity) for 6 h. (B) Levels of TNF-α (1.5. h), CXCL1/KC and CXCL2/MIP-2 (3 h) in the peritoneal cavity of mice stimulated with LPS (300 ng/cavity). (C) rolling and adhesion of leukocytes in the mesenteric microcirculation of mice stimulated with LPS (300 ng/cavity) for 2 or 4 h, respectively. (D) Migration of neutrophils into the peritoneal cavity induced by LPS (300 ng/cavity) for 6 h in mice pretreated with aminoguanidine (50 mg/kg) sc. (E) Expression of ICAM-1 in the mesenteric microcirculation in mice stimulated with LPS (300 ng/cavity) for 4 h. (F) Expression of ICAM-1 in bEnd.3 cells stimulated with LPS (5 µg/mL) for 24 h. The data were expressed as mean ± SEM, with n = 3-5 per group. Symbols indicate statistical difference ($P < 0.05$, Tukey's post-test). # $P < 0.05$ compared to saline group; * $P < 0.05$ compared to LPS group.

3.2. Neovestitol does not affect the chemotaxis of neutrophils *in vitro*

The effects of neovestitol on the chemotaxis of neutrophils induced by CXCL2/MIP-2 and on cellular viability were also investigated. According to the results, it was observed that the pretreatment with neovestitol at the concentrations of 0.3, 3 or 30 uM does not affect neutrophil chemotaxis induced by CXCL2/MIP-2 ($P > 0.05$, Figure 4A). Besides, there was not change in viability of neutrophils during incubation with neovestitol 30 µM ($P > 0.05$, Figure 4B).

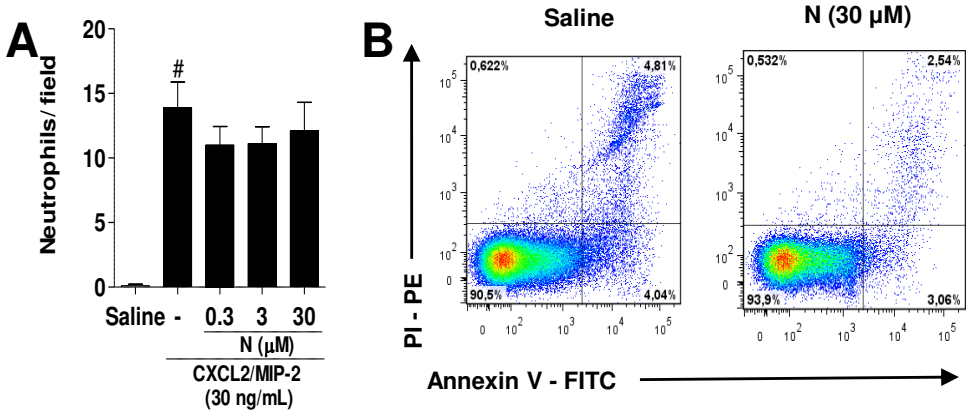

Figure 4. Neovestitol does not affect the chemotaxis of neutrophils induced by CXCL2/MIP-2. Neutrophils were pretreated with neovestitol (N) at the concentrations of 0.3, 3 or 30 µM (N 30 µM), 30 min prior to stimulation with CXCL2/MIP-2 at 30 ng/mL. (A) Chemotaxis of neutrophils induced by CXCL2/MIP-2 (30 ng/mL) was evaluated after 1 h. (B) Neutrophil viability (annexin V and PI) after the incubation with N 30 µM for 1.5 h. The data were expressed as mean ± SEM, with n = 3 per group. Symbols indicate statistical difference ($P < 0.05$, Tukey's post-test). # $P < 0.05$ compared to saline group.

3.3. Neovestitol reduces the clinical score and joint damage in DBA-1/J mice with collagen-induced arthritis

After the verification and elucidation of the major pathways of action of neovestitol on acute inflammation (innate immunity), it was also evaluated its activity on collagen-induced arthritis in DBA-1/J mice. The administration of neovestitol at 10 mg/kg (Figure 5A) reduced the clinical scores of animals with arthritis on days 8 and 12 after the second immunization, compared to the group that received only the challenge ($P < 0.05$). On the other hand, this reduction was not observed at the dose of 3 mg/kg ($P > 0.05$). Regarding the articular lesions after the last day of treatment (day 12), there was a decrease in the articular lesions and cell infiltrate of animals that received the treatment with neovestitol at the dose of 10 mg/kg (Figure 5B).

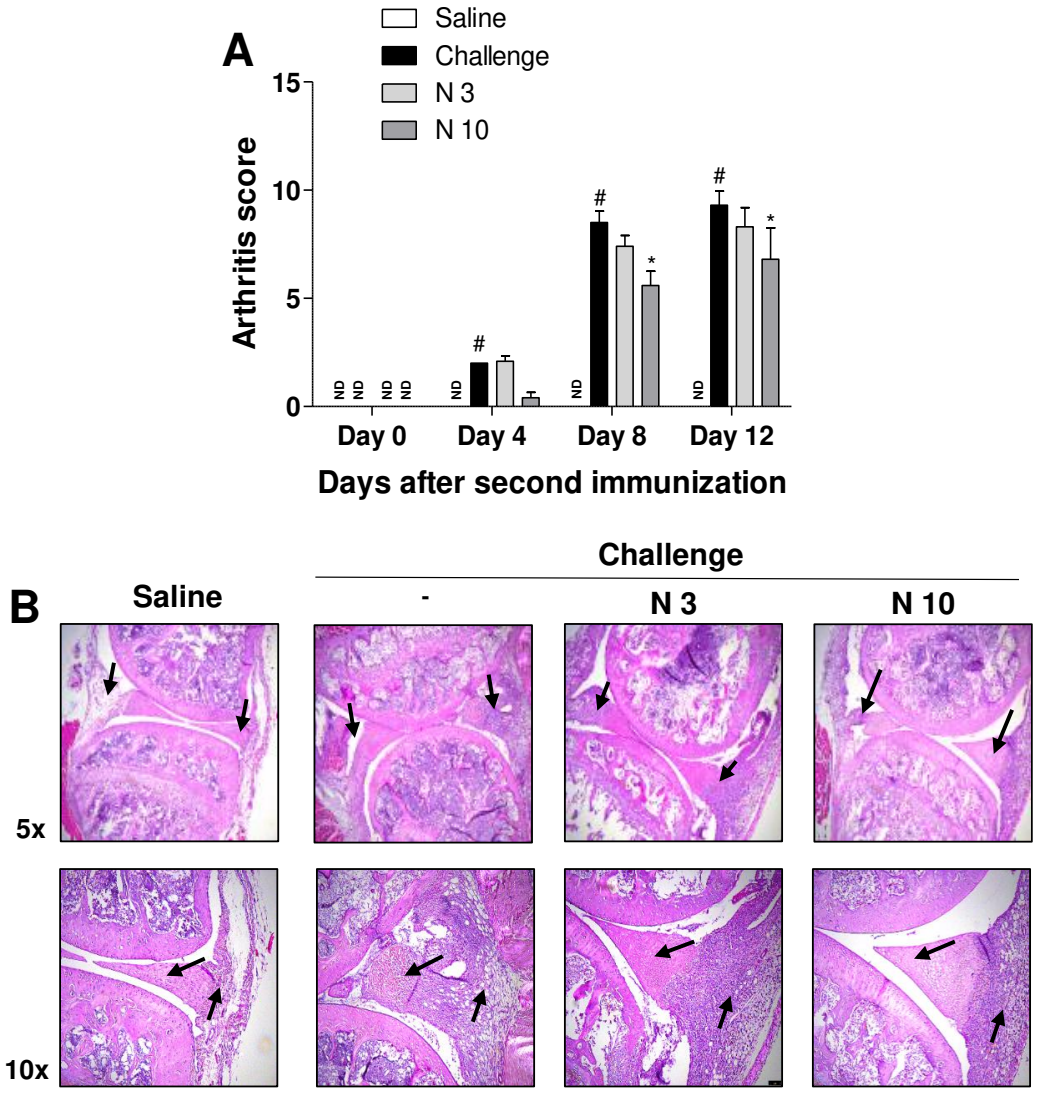

Figure 5. Neovestitol reduces the clinical score and joint damage in DBA1/J mice with arthritis. DBA1/J mice with arthritis were pretreated with neovestitol at the doses of 3 (N 3) or 10 (N 10) mg/kg sc for 12 days. (A) Clinical score on days 0, 4, 8 and 12 after the second injection in the tail with type II collagen and CFA. (B) Histological analysis (5x and 10x magnification, optical microscope) of the joint (HE staining) after 12 days of the second challenge with type II collagen and CFA. The data were expressed as mean ± SEM, with n = 5-6 per group. Symbols indicate statistical difference (P <0.05, Bonferroni test). # P < 0.05 compared to saline group; * P < 0.05 compared to the challenge group. The group of mice showing no increase in clinical scores is presented as “not detected” (ND).

3.4. Neovestitol does not affect the frequency of IL-17-producing TCD4+ cells in draining lymph nodes

On day 12 after the second immunization (last day of treatment), the mice were killed and their draining lymph nodes were collected for analysis of the frequency of IL-17-producing TCD4+ cells (Figure 6). It was found that the

administration of neovestitol at doses of 3 and 10 mg/kg in a 12-day period did not alter the frequency of IL-17-producing CD4+ cells ($P > 0.05$).

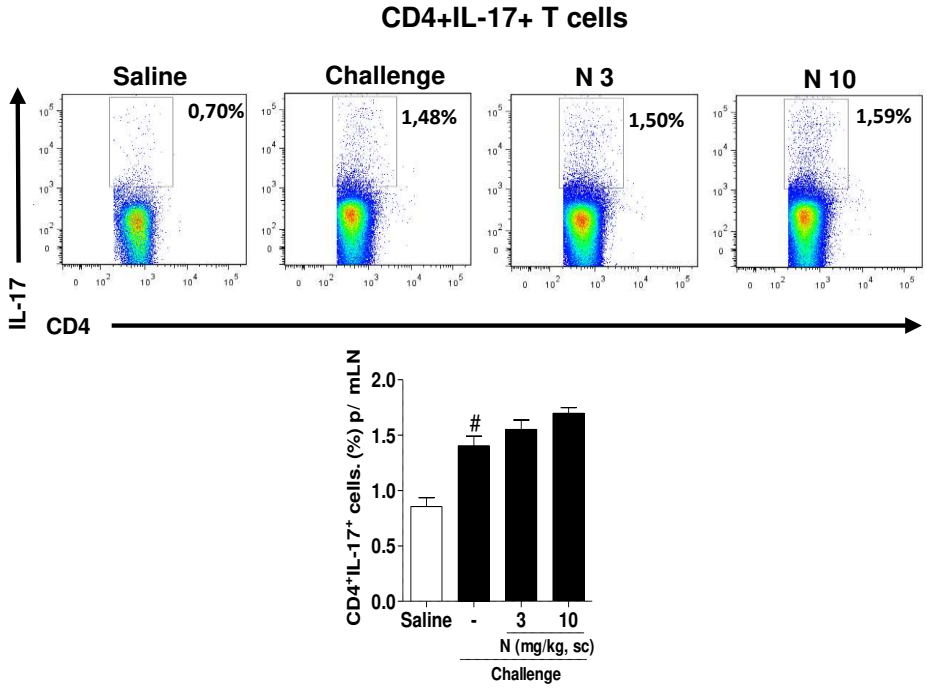

Figure 6. Neovestitol does not change the frequency of IL-17-producing TCD4+ cells. DBA1/J mice with arthritis were treated with neovestitol (N) at the doses of 3 (N 3) or 10 (N 10) mg/kg/day sc for 12 days after the boost. The frequency of IL-17-producing TCD4+ cells from draining lymph nodes 12 days after the second challenge with type II collagen and CFA was calculated. The data were expressed as mean ± SEM, with n = 5-6 per group. Symbols indicate statistical difference ($P < 0.05$, Tukey's post-test). # $P < 0.05$ compared to saline group.

3.5. Neovestitol reduces the release of IL-6

The levels of the cytokines IL-17, IFN-γ, TNF-α and IL-6 in the knee joint of mice (Figure 7) was quantified. It was observed that at the dose of 10 mg/kg, neovestitol reduced the levels of IL-6 after 12 days of treatment ($P < 0.05$). Nevertheless, at 3 and 10 mg/kg it did not affect the levels of the cytokines IL-17, IFN-γ and TNF-α in the joint ($P > 0.05$).

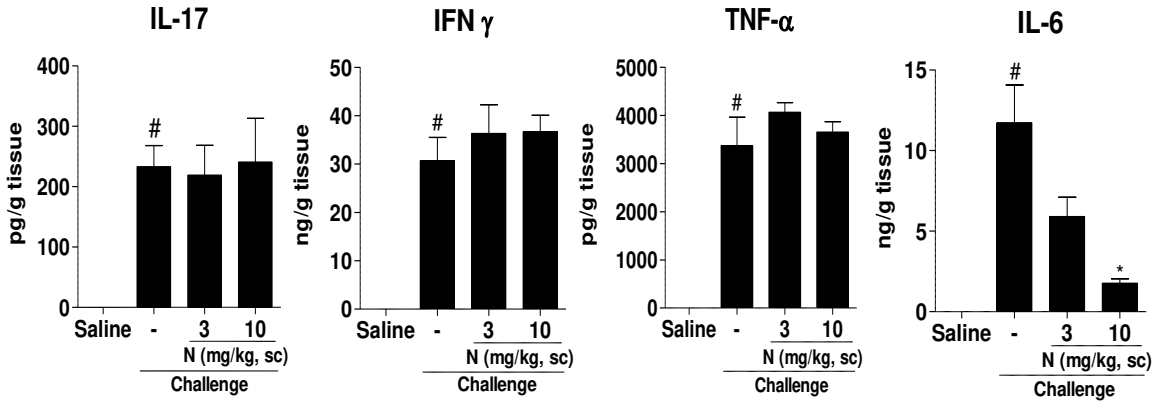

Figure 7. Neovestitol reduces the release of IL-6. DBA1/J mice with arthritis were pretreated with neovestitol (N) at the doses of 3 or 10 mg/kg/day sc for 12 days. The levels of IL-17, IFN-γ, TNF-α and IL-6 in the joint after 12 days of the second challenge with type II collagen and CFA, were measured. The data were expressed as mean ± SEM, with n = 5-6 per group. Symbols indicate statistical difference ($P < 0.05$, Tukey's post-test). # $P < 0.05$ compared to saline group; * $P < 0.05$ compared to the challenge group.

4. Discussion

Studies have been reported the promising potential of isoflavonoids on the modulation of the inflammatory process and have been used them as a functional food or nutraceutical product for many diseases. A study with formononetin, an isoflavonoid also isolated from Brazilian red propolis, showed that administration of this compound in experimental inflammation models reduced carrageenan-induced leukocyte migration and edema formation [23]. In another study, it was found that the genistein, largely found in soy, was able to prevent inflammation in rats. The suppression of pro-inflammatory cytokines production such as TNF-α and IL-1β was one of the observed effects [24]. A study with biochanin A investigated its anti-inflammatory effects on BV2 microglial cells stimulated with LPS. Among the results, there was a reduced production of TNF-α, IL-1β, nitric oxide and reactive oxygen species [25].

In the present study, the sc administration of neovestitol, an isoflavonoid isolated from Brazilian red propolis, reduced LPS-induced neutrophil migration. Inflammation consists in a series of events characterized by the initial influx of neutrophils into the inflammatory focus, and cytokines play a crucial role in this process [26]. Proinflammatory cytokines and chemokines secreted by resident macrophages perform numerous functions, including activation of endothelial cells to express

adhesion proteins and the activation of the leukocyte locomotion. Among these proteins, selectins (L-, P- and E-selectin), promote the interaction of white blood cells that are flowing in the bloodstream to the endothelial cells through a rolling process. Immunoglobulins, including intercellular adhesion molecule type 1 (ICAM-1), are responsible for the strong adhesion of leukocytes with endothelial cells, which allows the transmigration of the cells to the inflamed site [27, 28]. In this study, it was found that the mechanism of action of neovestitol in LPS-induced inflammation is related to the reduced expression of ICAM-1, and its effect do not dependent on the modulation of inflammatory cytokines and chemokines. Studies have shown that nitric oxide plays a crucial role in modulating the adhesion of leukocytes during the inflammatory process. It has been found that the action of nitric oxide in the inflammatory process induced by LPS in the peritoneum of mice is associated with a reduction in ICAM-1 expression in the mesenteric microcirculation of mice [13, 29]. Thus, it is suggested that the ameliorating effects of neovestitol on acute inflammation, at least in part can be associated with the induction of increased nitric oxide levels and the consequent reduction of leukocyte adherence due to the suppression of ICAM-1.

The activity of neovestitol on collagen-induced arthritis in DBA-1/J mice was also evaluated. It was found that administration of neovestitol reduced the clinical scores of animals with arthritis and the levels of IL-6. IL-6 is a cytokine involved in the inflammatory response which participates in neuronal processes and metabolism regulation [30]. It is produced by different cell types, including neutrophils, macrophages, endothelial cells, fibroblasts and dendritic cells. Its action takes place in different cell types. In neutrophils, IL-6 cause increased elastase. In endothelial cells, it increases the expression of ICAM-1 [2]. Studies have been shown a key role of IL-6 in the pathophysiology of rheumatoid arthritis. Among the findings, a high expression of IL-6 was detected in the synovial tissue [31]. Furthermore, the action of IL-6 in the collagen-induced arthritis model demonstrated to be related to Th17 differentiation [32]. In the present study, the administration of neovestitol to animals with arthritis did not change the differentiation of Th17 in inguinal lymph nodes. In addition, it did not affect IL-17 cytokine in the joint of animals with arthritis, although it exerted an inhibitory effect on the release of IL-6. This phenomenon could be explained by the fact that Th17 differentiation occurs early in the immunization process, as a result of the high levels of IL-6 released on the first day after the first immunization [32, 33]. Thus, since the administration of neovestitol was performed on the 21st day once a day after the

second immunization, it is suggested that its activity on IL-6 may be associated with other types of modulation. Studies have shown that the high levels of IL-6 in the synovial fluid are also associated to joint destruction in arthritis [34]. A study found that the blocking of the receptor IL-6R reduces the differentiation of osteoclasts [35]. In this study, it was found that administration of neovestitol reduced the articular lesions of animals with arthritis, suggesting therefore a possible activity on the inhibition of IL-6.

Altogether, the findings of this study suggest a promising activity of neovestitol in the regulation of acute and chronic inflammation. Studies have been shown that the ingestion of food that contains significant amounts of isoflavonoids can bring health benefits in the prevention of numerous chronic diseases, including cardiovascular disease, cancer and obesity [36,37]. Thus, the results obtained with the neovestitol are significant from the nutritional point of view, because this isoflavonoid can be included in the future as a nutraceutical compound to be add in the healthy diet in order to prevent serious chronic degenerative diseases whose etiology is often a subclinical inflammation process. However, further studies are needed to explore the effects of this compound on human health and disease prevention.

Thus, it is concluded that neovestitol from Brazilian propolis showed inhibitory activity on LPS-induced inflammation, and its effects involve the oxide nitric pathway and consequent suppression of ICAM -1. Moreover, neovestitol inhibited the development of collagen-induced arthritis per modulation of IL-6 release. The results demonstrate a potential anti-inflammatory activity of neovestitol, an isoflavonoid that could be useful in the future for therapeutic purposes and/or incorporated as nutraceutical compound.

Author contributions

MF, DC, FVSC, ALLS, MGC and BBS participated in the design of the study, isolation of the neovestitol and the anti-inflammatory tests. SMA participated in the design of the study and interpreted the chemical analysis. TMC participated in the design of the study and the anti-inflammatory tests. PLR participated in the design of the study, interpreted the chemical analysis and the anti-inflammatory tests.

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Conflict of interest statement

Authors declare that there is no conflict of interest.

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Supporting Information

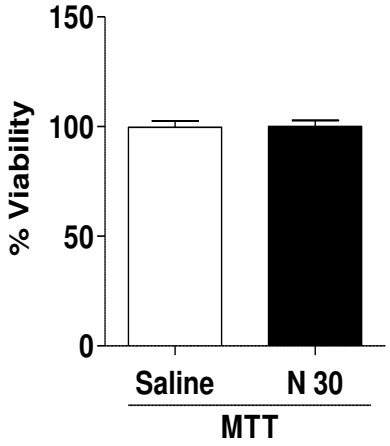
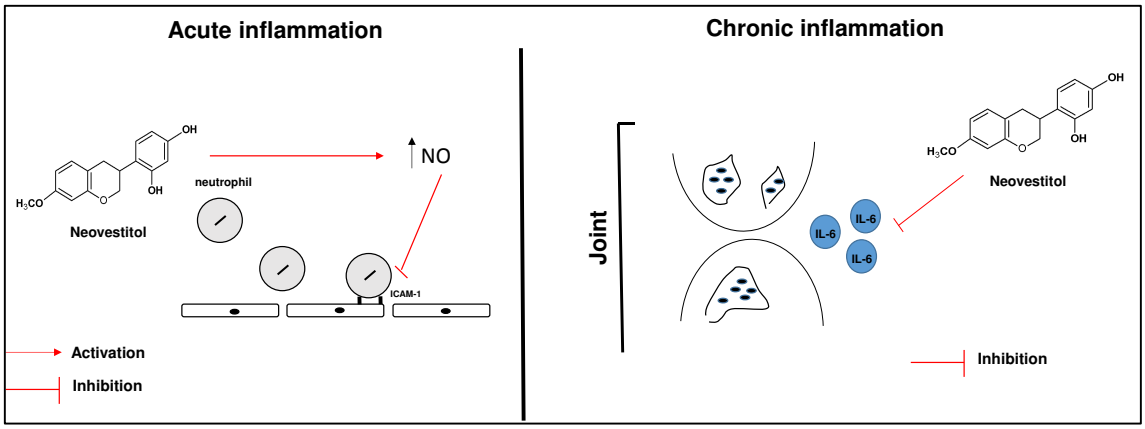


Figure S1. bEnd.3 cell viability (MTT) incubated with neovestitol at 30 μM (N 30) for 24 h. The data were expressed as mean ± SEM, with n = 4-5 per group ($P < 0.05$, Tukey's post-test).

Graphical abstract



2.3 Artigo 3:[5]

Anti-inflammatory activity of cinnamoyloxy-mammeisin, a 4-phenyl coumarin extracted from geopropolis of *Melipona scutellaris*, depends on inhibition of MAP kinases

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Abstract

Chemical compounds belonging to the class of coumarins have promising anti-inflammatory potential. Cinnamoyloxy-mammeisin (CNM) is a 4-phenyl coumarin, which can be isolated from Brazilian geopropolis. To our knowledge its anti-inflammatory activity had never been studied. Therefore, the present study investigated the anti-inflammatory activity of CNM and elucidated its mechanism of action on isolated macrophages. Pretreatment with CNM reduced neutrophil migration into the peritoneal and joint cavity of mice. Likewise, CNM reduced *in vitro* and *in vivo* the release of TNF-α and CXCL2/MIP-2. Regarding the possible molecular mechanism of action, CNM reduced the phosphorylation of proteins ERK 1/2, JNK, p38 MAPK and c-jun in LPS-stimulated macrophages. Pretreatment with CNM also reduced NF-kB activation in RAW 264.7 macrophages stably expressing NF-kB-luciferase reporter gene. On the other hand, it did not alter IκBα degradation or nuclear translocation of p65. Thus, the results of this study demonstrate promising anti-inflammatory activity of CNM and provide an explanation of its mechanism of action in macrophages via inhibition of the MAPK signaling.

Keywords. coumarins; geopropolis; *Melipona scutellaris*; macrophages; inflammation.

1. Introduction

Natural products have been historically used in folk medicine for the treatment of various diseases and are considered a vital source for discovery of novel drugs [1]. A recent survey showed that 50% of the new drugs discovered in 2010 were natural products or their derivatives [2].

Coumarins is a class of naturally-occurring chemical compounds largely studied due its numerous biological activities, such as antibacterials, anti-coagulants, antitumor and anti-inflammatory properties [3] and [4]. Indeed, the coumarin isofraxidin reduces the number of neutrophils and levels of TNF-α, IL-6 and PGE_2 in the bronchoalveolar lavage fluid on lipopolysaccharide-induced acute lung injury [5]. Notwithstanding, nodakenin, another coumarin, also reduced the inflammatory process in the airways of mice [6].

Geopropolis is a type of propolis containing plant resins, wax and characterized by the presence of soil. It can be collected by a native stingless bee species, such as *Melipona scutellaris* [7] and [8]. Recent studies found promising anti-inflammatory activity of geopropolis extract via inhibition of neutrophil migration [7]. Moreover, another study isolated and identified seven coumarin-like compounds with antiproliferative activity against cancer cell lines in geopropolis of *M. scutellaris* [8]. Among these compounds, cinnamoyloxy-mammeisin (CNM), a 4-phenyl coumarin, was found to be the most abundant compound in geopropolis extract. Nevertheless, its activity on inflammatory process has never been studied before. Thus, the present study investigated the anti-inflammatory activity of CNM on peritonitis and arthritis experimental models of inflammation, and elucidated its possible molecular mechanism of action.

2. Materials and methods

2.1. Reagents. Hexane, isopropanol, dichloromethane, ethyl acetate, acetone and methanol were purchased from Merck. RPMI, penicillin, L-glutamine, carrageenan, mBSA, MTT, DMSO, LPS, PG and Triton X-100 were purchased from Sigma. Anti-ERK 1/2, anti-p-ERK 1/2, anti-SAPK/JNK, anti-p-SAPK/JNK, anti-p38 MAPK, anti-p-p38 MAPK, anti- IκBα and β-actin antibodies were purchased from Cell Signaling. Apo

Screen Annexin V-FITC Kit was purchased from Southern Biotech. Fetal bovine serum from Gibco. LPS, PG, carrageenan and mBSA dissolution was performed in sterilized saline solution, while CNM was dissolved in 1% DMSO in 1x PBS.

2.2. Animals. C57BL/6 SPF (specific pathogen-free) male mice weighing between 20-22g were provided by CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brasil). The animals were kept at temperatures of 22-25 °C with food (standard pellet diet) and water *ad libitum*, with controlled light/dark cycle and humidity of 40-60%. The use of animals in this study was previously approved by the ethics committee for animal use (CEUA/UNICAMP, process no. 2793-1).

2.3. Cell culture. Mice macrophage, RAW 264.7 macrophages or RAW 264.7 stably bear the luciferase reporter gene controlled by an NF-κB-sensitive promoter (NF-κB-pLUC) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 2 mM L-glutamine at 37°C in 5% CO_2/95% atmosphere.

2.4. Geopropolis. Samples of *M. scutellaris* geopropolis were collected between June and July 2012 in the city of Entre Rios (12º 22' S, 37º 54' W) in the state of Bahia, Brazil. Samples of *M. scutellaris* bee were deposited in the Bioscience Institute of the Sao Paulo University, in the “Nogueira Neto” Entomological Collection, identified under the voucher number CEPANN 42,863. The present research had authorization for access of genetic heritage components granted by the National Council for Technological and Scientific Development (CNPq), no. 010666/2014-1.

2.5. Extraction and isolation. Extraction and isolation. The isolation and identification of the CNM (Fig. 1) procedures have been previously described [8].

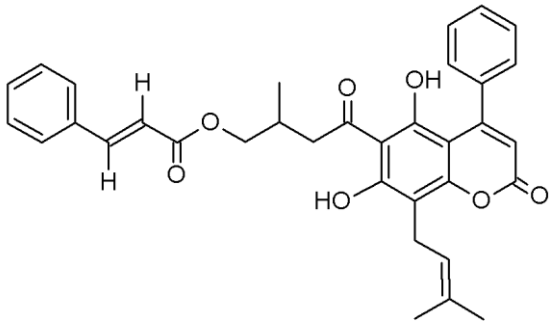

Fig. 1. Chemical structure of CNM isolated from *M. scutellaris* geopropolis.

2.6. Pharmacological protocol *in vivo* and *in vitro*. Mice were pretreated with CNM at 30, 100 or 300 µg/kg sc. After 30 min they received a challenge consisting of i.p. administration of carrageenan (500 µg/cavity) or intra-articular (i.a.) administration of mBSA (30 µg/joint). In the *in vitro* test, RAW 264.7 macrophages were pretreated with CNM at the concentrations of 0.6, 2 or 6 µM 30 min before stimulation with PG (5 µg/mL) or LPS (10 ng/mL). In the *in vivo* and *in vitro* assays, the negative control group received the vehicle used to dissolve CNM, as previously described.

2.7. Migration of neutrophils into the peritoneal cavity. Neutrophil infiltration was determined in the peritoneum 4 h after i.p. injection of carrageenan (500 µg/cavity). Washing of the peritoneal cavity was performed with 3 mL of phosphate buffered saline (PBS) containing EDTA. The fluid was collected (approximately 95% of the injected volume) and total leukocyte count was carried out in a Neubauer chamber. Differential smear was prepared using cytospin (Cytospin 3) and stained with Panotic kit (Laborclin, Brazil). A total of 100 cells were counted using OPICO microscope (x 1000). Results are presented as the number of neutrophils per cavity [9].

2.8. Leukocyte rolling and adhesion in the mesenteric microcirculation. The evaluation of leukocyte rolling in the mesenteric microcirculation was determined 2h after ip stimulation with carrageenan (500 µg/cavity), while adhesion was verified 4h after stimulation. For visualization of leukocytes, the animals were anesthetized with ketamine 90 mg/kg (Dopalen) and xylazine 10 mg/kg (Rompun) and their mesentery exteriorized for observation of the microcirculation (intravital microscope DM 6000, Leica). The rolling of leukocytes was analyzed in three different fields per animal over a period of 5 min. For adhesion, the number of leukocytes adhering to the endothelium

was evaluated over an area of venule of 100 μm^2 (10 micrometers in the tissue corresponds to 3.4 cm on the monitor screen) [10] and [11] (*1, 2*).

2.9. Migration of neutrophils in the joint cavity. Mice were immunized s.c. with an emulsion containing 100 µL of Complete Freud's Adjuvant (CFA), 100 µL of 1x PBS and 500 µg of methylated bovine serum albumin (mBSA). The reinforcement (seven and fourteen days after the first immunization) was administered with Incomplete Freud's Adjuvant (IFA) emulsion and mBSA. On day 21, the challenge was administrated ia with mBSA (30 µg/joint). Six hours later the animals were euthanized, their articular cavity was washed with PBS containing EDTA and total and differential counts were performed as previously described [12].

2.10. Myeloperoxidase activity in the joint tissue. The myeloperoxidase activity *in vivo* was assessed 6 h after stimulation with mBSA (30 µg/joint). Mice were anesthetized and received ip administration of the solution (100 mg/kg) used to determining the activity of myeloperoxidase by bioluminescence (XenoLight Rediject Inflammation Probe). Image acquisition was performed using the IVIS Spectrum System device (Caliper Life Sciences). The results were expressed as bioluminescence intensity (radiance p/sec/cm^2/sr) [13].

2.11. RAW 264.7 culture and collection of the supernatant for quantification of TNF-α and CXCL2/MIP-2. RAW 264.7 macrophages were cultured in 96-well plates (2 x 10^5 cell/well) at 37°C, 5% CO_2 overnight. Four hours after stimulation with PG (5 µg/mL) or LPS (10 ng/mL) the supernatant was collected and stored at -70 ºC.

2.12. Enzyme Linked Immunosorbent Assay (ELISA). The levels of cytokines and chemokines in the peritoneal cavity of the animals were determined 1.5 hour (TNF-α) and 3 h (CXCL1/MIP-2) after ip stimulation with carrageenan at 500 µg/cavity. The levels of TNF-α (1.5 hour) were determined after challenge with mBSA at 30 µg/joint. In the supernatant of RAW 264.7 macrophages, the levels of TNF-α or CXCL1/MIP-2 were determined 4 h after stimulation with PG (5 µg/mL) or LPS (10 ng/mL). ELISA was performed using protocols supplied by the manufacturers (R&D Systems). Results are expressed as pg/mL or pg/mg tissue.

2.13. Viability assay by flow cytometry (annexin V and PI). RAW 264.7 macrophages were grown in 6-well plates (2 x 10^6 cell/well) at 37, 5% CO_2 overnight. After 4 h of incubation with CNM at 6 µM, the cells were washed and resuspended in Annexin buffer. Anti-annexin V-FITC antibody (1:50) was added and incubated at 4 °C for 20 min. Then the anti-PE antibody (propidium iodide, 1: 100) was added and the analysis carried out in a FACSVerse (BD Biosciences) flow cytometer using the software "Flow Jo" [14].

2.14. Cell viability assay by MTT. RAW 264.7 macrophages were cultured in 96-well plates (2 x 10^5 cell/well) at 37°C, 5% CO_2 overnight. After 4 h of incubation with CNM at 6 µM, the supernatant was removed and was added RPMI with MTT (1 mg/mL) and incubated for 2 h. Then the supernatant was removed again and the cells resuspended in 200 µL of absolute ethanol. The absorbance was measured at 540 nm using a microplate reader (Molecular Devices) [15].

2.15. Western blotting. RAW 264.7 macrophages were grown in 6-well plates (2 x 10^6 cell/well) at 37°C, 5% CO_2 and incubated overnight under the conditions described previously. After 30 min of PG stimulation, the cells were lysed with RIPA-containing protease inhibitor (1:25) and phosphatase (1:50). The protein quantification was performed using Bradford reagent (Sigma). Mini-PROTEAN Tetra Cell (Bio-Rad) and gel with 10% polyacrylamide was used for running. Then the gels were transferred to a nitrocellulose membrane and anti-ERK 1/2 primary antibody (1: 1000), anti-p-ERK 1/2 (1: 1000), anti-SAPK/JNK (1: 500), anti-p-SAPK/JNK (1: 500), anti p38 MAPK (1: 500), anti-p-p38 MAPK (1: 500), anti-IκBα (1: 200) and β-actin (1: 5000) were added as internal controls. After overnight incubation at 4 °C, anti-mouse or anti-rabbit secondary antibody (1:10,000) were added and incubated for 1 h. Development of membranes was performed with a mixture of a chemiluminescence kit.

2.16. Immunofluorescence. RAW 264.7 macrophages were cultivated on circular coverslips in 24-well plates (2 x 10^5 cell/well) at 37 °C, 5% CO_2 and incubated overnight under the conditions previously described. After 30 min of PG stimulation, the cells were fixed with 4% paraformaldehyde for 20 min and washed with 1x PBS containing 10 mM glycine. The permeabilization was carried out with Triton X-100 for 30 min and the blocking with 1% BSA for 1 h. Anti-p65 primary antibody 1:100 (Santa Cruz) or

anti-p-c-Jun 1:100 (Cell Signaling) was incubated overnight. The secondary antibody Alexa Fluor 488-conjugated anti-mouse IgG 1:400 (Molecular Probes) was incubated for 1 h. Hoesch 33342 (Sigma) at 1 µg/mL was used as a nucleus marker. The cover slips were fixed on slides with Fluormount (SouthernBiotech) and the images were acquired in the Leica microscope TCS SP5 (Leica Microsystems).

2.17. NF-kB activation assay. RAW 264.7 macrophages stably transfected with the NF-κB-pLUC gene to express luciferase by the transcription factor NF-kB were cultured in 24-well plates (3 x 10^5 cell/well) and incubated overnight under the conditions previously described. The evaluation of NF-kB activation was performed 4 h after challenge with PG (5 µg/mL). The cells were lysed with 50 µL of TNT and an aliquot of the suspension was added along with 25 µL of the Luciferase Assay Reagent containing luciferin (Promega). A microplate reader (FlexStation 3 Multi-Mode Microplate Reader) was used to quantify the luminescence [15] and [16].

2.18. Statistical analysis. Statiscal analysis was done using GraphPad Prism software version 5.03 (GraphPad Software, Inc.). Data were expressed as mean ± SEM. The statistical comparison between groups was performed using analysis of variance (ANOVA) followed by Tukey's post-test. Comparison only between two groups was performed using Student's t-test. Significance was accepted when $P < 0.05$.

Results

3.1. CMN reduces migration, neutrophil adhesion and rolling, and the release of TNF-α and CXCL2/MIP-2 in the peritoneal cavity of mice

First, in order to assess the anti-inflammatory activity of CNM, it was evaluated in a model of carrageenan-induced peritonitis. Pretreatment of mice with CNM (30-300 µg/kg, s.c.) (Fig. 2A) reduced neutrophil infiltration to the peritoneal cavity, induced by carrageenan (i.p.), in a dose dependent manner ($P < 0.05$).

Second, the mechanism by which CNM modulates neutrophil migration was evaluated. We determined the activity of CNM on leukocyte adhesion and rolling in the mesenteric microcirculation of mice, as well as, the levels of TNF-α and CXCL2/MIP-2. According, pretreatment with CNM (300 µg/kg, s.c) reduced leukocyte rolling and

adhesion to mesenteric venules (Fig. 2B) and the release of TNF-α and CXL2/MIP-2 (Fig. 2C) in the peritoneal cavity of mice as compared to the carrageenan group treated with vehicle (P <0.05).

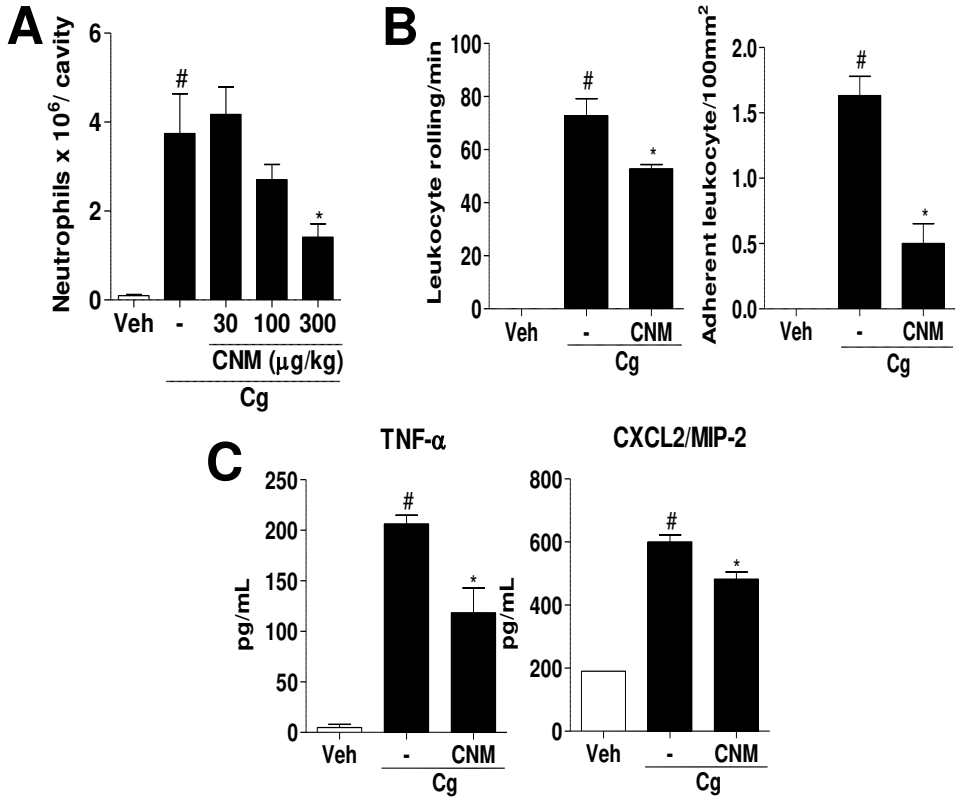

Fig. 2. CNM reduces peritoneal inflammation induced by carrageenan. C57BL/6 mice were pretreated with CNM at the doses of 30, 100 or 300 µg/kg sc or vehicle (veh), 30 min before i.p. administration of carrageenan (Cg) at 500 µg/cavity. (**A**) Migration of neutrophils into the peritoneal cavity 4 h after i.p. injection of Cg. (**B**) Leukocyte rolling and adhesion in the mesenteric microcirculation for 2 or 4 h, respectively, after i.p. injection of Cg in mice pretreated or not with CNM (300 µg/kg). (**C**) TNF-α and CXCL2/MP-2 levels in the peritoneal cavity 1.5 or 3 h after i.p. injection of Cg, respectively, in mice pretreated or not with CNM (300 µg/kg). The data were expressed as the mean ± SEM, with n = 4-5 per group. Symbols indicate statistical difference (P < 0.05, Tukey's post-test). # P < 0.05 compared to vehicle group; * P < 0.05 compared to Cg group.

3.2. CNM reduces neutrophil migration and the release of TNF-α in the joint cavity of mice

Next, we investigated the anti-inflammatory effects of CNM in a model of antigen-induced arthritis. Neutrophil migration, release of TNF-α and activity of myeloperoxidase in the joint cavity of mBSA challenged mice were evaluated. Daily pre-treatment with CNM (100 µg/kg, s.c) (Fig. 3A) for 3 days prior to challenge reduced

neutrophil migration (P <0.05) and the release of TNF-α (Fig. 3B) as compared to the group challenged only with mBSA (P < 0.05). Finally, CNM at 100 µg/kg reduced the localized fluorescence representing the activity of myeloperoxidase (Fig. 3C) in the joint cavity of mice when compared to the group that received the challenge (P < 0.05).

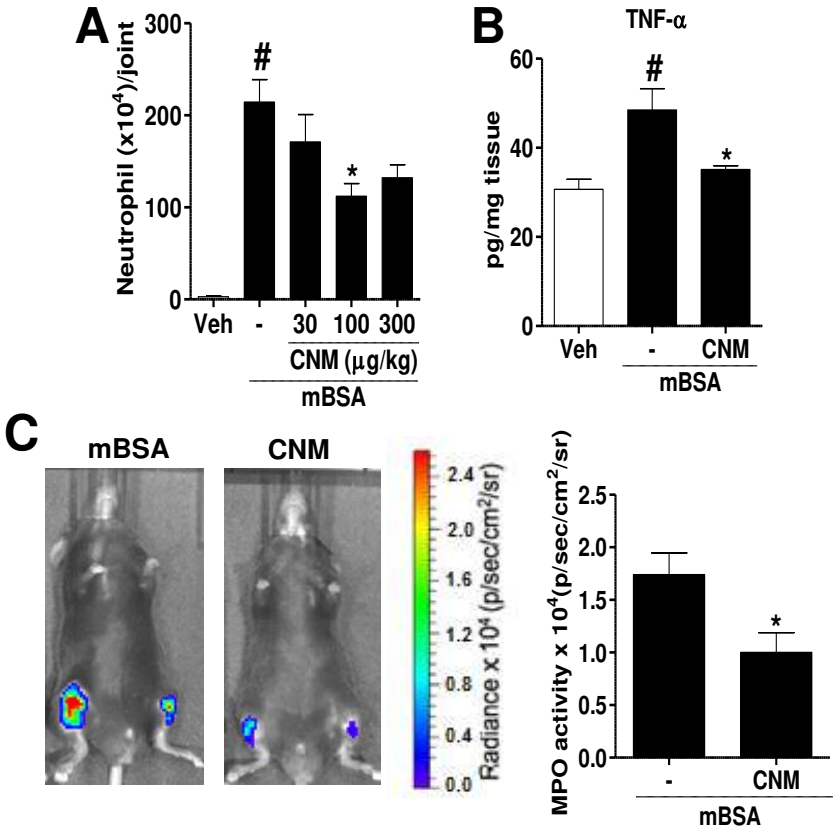

Fig. 3. CNM reduces joint inflammation induced by mBSA. C57BL/6 mice were pre-treated with CNM at the doses of 30, 100 or 300 µg/kg sc or vehicle (veh) 30 min prior to administration of mBSA (30 µg/joint). (**A**) Neutrophil migration in the joint cavity 6 h after ia injection of mBSA. (**B**) TNF-α levels in the joint cavity after 1.5 h of i.a. injection of mBSA in mice pretreated or not with CNM (100 µg/kg). (**C**) Fluorescence intensity 6 h after i.a. injection of mBSA with in vivo imaging system IVIS Spectrum representing the myeloperoxidase (MPO) in mice pretreated or not with CNM (100 µg/kg). The data were expressed as the mean ± SEM, with n=5-6 per group. Symbols indicate statistical difference (P < 0.05, Tukey's or Student's t-tests). # P < 0.05 compared to vehicle group; * P < 0.05 compared to mBSA group.

3.3. CNM reduces the release of TNF-α and CXCL2/MIP-2 in RAW 264.7 macrophages stimulated with LPS or PG

Macrophages is one the major cells involved in the release of inflammatory cytokines and chemokines during acute inflammation [17]. Thus, after determining the activity of CNM on the *in vivo* release of TNF-α and CXCL2/MIP-2, we next investigated

its mechanism of action in RAW 264.7 macrophages. As seen in the results, pretreatment with CNM at the concentrations of 0.6, 2 or 6 µM reduced the release of TNF-α and CXCL2/MIP-2 in macrophages stimulated with peptidoglycan (PG) or lipopolysaccharide (LPS), Fig. 4A and B, respectively ($P < 0.05$). Furthermore, the highest CNM concentration used on *in vitro* (6 µM) experiments showed no citotoxic effect in RAW macrophages as compared to the vehicle group (Fig. 4C and D, apoptosis or MTT assays) ($P < 0.05$).

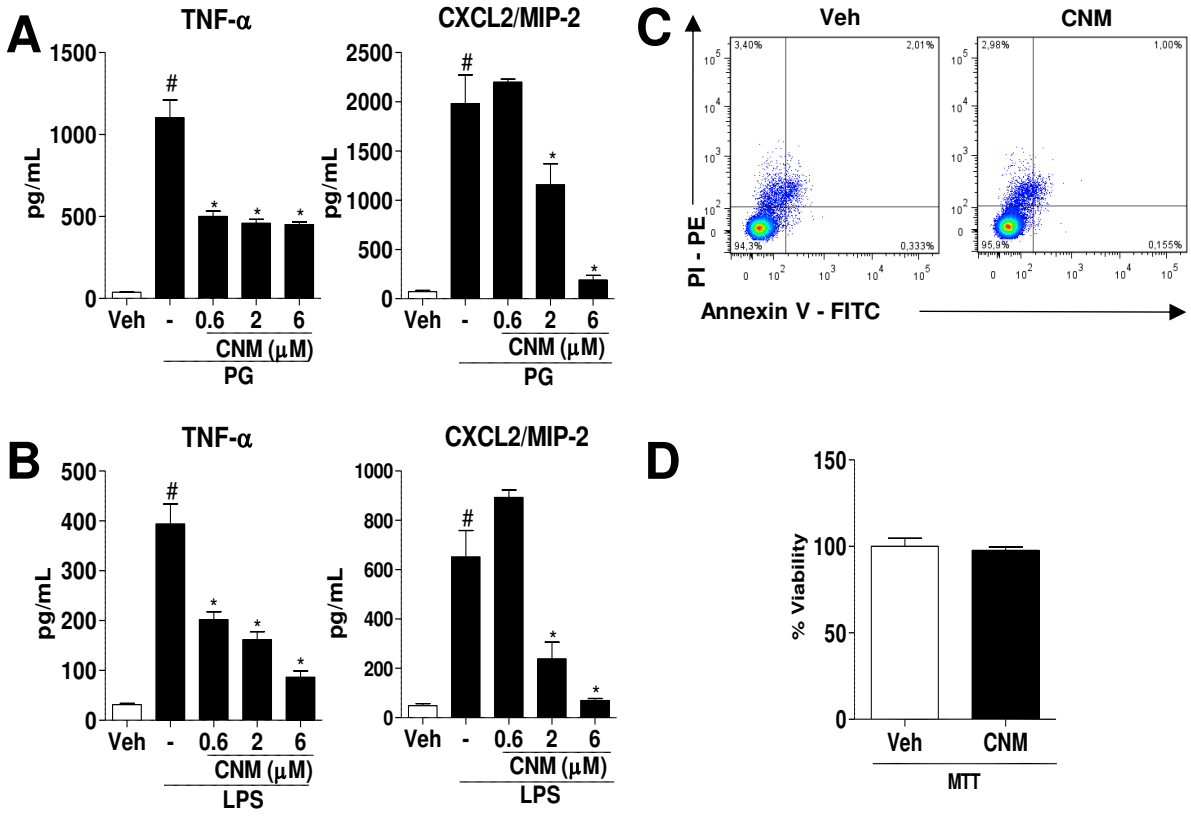

Fig. 4. CNM reduces the release of cytokines from macrophages. RAW 264.7 macrophages were pretreated with CNM at the concentrations of 0.6, 2 or 6 µM or vehicle (veh), 30 min prior to stimulation with PG (5 µg/mL) or LPS (10 ng/mL). (**A, B**) TNF-α and CXCL2/MP-2 levels in the supernatant of RAW 264.7 macrophages stimulated with LPS or PG for 4 h. (**C**) Cell viability using flow cytometry (annexin V and PI) of RAW 264.7 macrophages incubated with CNM 6 µM for 4 h. (**D**) Cell viability by MTT assay of RAW 264.7 macrophages incubated with CNM 6 µM for 4 h. The data were expressed as the mean ± SEM, with n=4-5 per group. Symbols indicate statistical difference ($P < 0.05$, Tukey's or Student's t-tests). # $P < 0.05$ compared to vehicle group; * $P < 0.05$ compared to PG or LPS group.

3.4. CNM reduces MAPK and c-Jun phosphorylation in PG-stimulated RAW 264.7 macrophages

Mitogen-activated protein kinases (MAPKs) play a key role in cellular signaling and are involved in the production of cytokines and chemokines [18]. The mechanism of action of CNM in cell signaling pathways was evaluated using RAW 264.7 macrophages. According to the results, pretreatment with CNM (6 μM) reduced the phosphorylation of MAPKs, including ERK 1/2 (Fig. 5A), SAPK/JNK (Fig. 5B) and p38 MAPK (Fig. 5C), compared to the PG-stimulated group ($P < 0.05$). In addition, pretreatment with CNM at 6 μM (Fig. 5D) reduced the nuclear phosphorylation of c-jun in macrophages as compared to the PG group ($P < 0.05$).

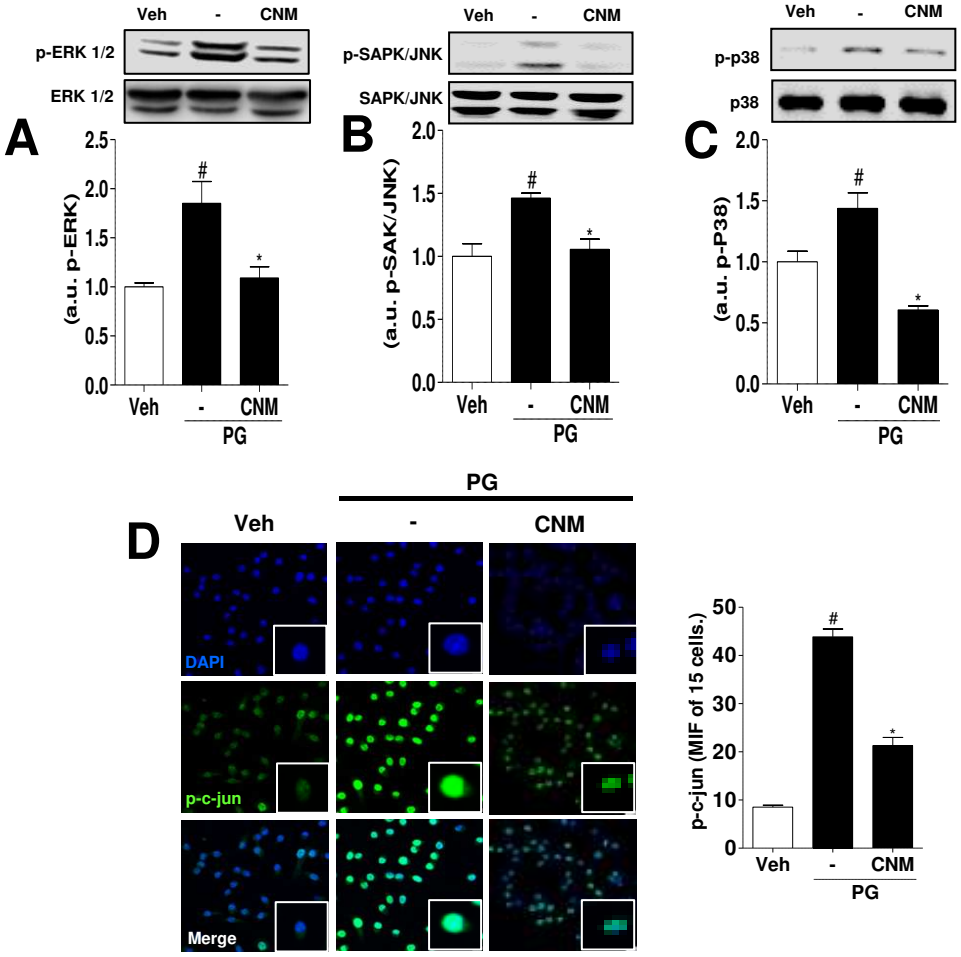

Fig. 5. CNM reduces MAPK and c-Jun phosphorylation in macrophages stimulated. RAW 264.7 macrophages were pretreated with CNM at the concentration of 6 μM or vehicle (veh), 30 min prior to stimulation with PG (5 μg/mL). (**A, B, C**) MAPK phosphorilation (ERK 1/2, SAPK/JNK and p38 MAPK) were evaluated in RAW 264.7 macrophages stimulated with PG for 15 min. (**D**) p-c-Jun was analized in RAW 264.7 macrophages after 30 min of stimulation with PG. The data were expressed as the mean ± SEM, with n=3-4 per group. Symbols indicate statistical difference ($P < 0.05$, Tukey's post-test). #$P < 0.05$ compared to vehicle group; *$P < 0.05$ compared to PG group.

3.5. CNM reduces NF-kB activation in PG-stimulated RAW 264.7 macrophages

The transcription factor NF-kB is the major factor involved on the production of pro-inflammatory mediators, such as cytokines and chemokines [19]. In attempt to identify the activity of CNM on NF-kB activation, it was evaluated in RAW 264.7 macrophages bearing the luciferase gene-containing vector controlled by an NF-κB-activated promoter [15]. It was observed that CNM at 0.6, 2 and 6 μM reduced NF-kB activation compared to PG group ($P < 0.05$), characterized by the reduction in the luminescence emission (Fig. 6A). On the other hand, CNM at 6 μM did not alter IκB-α degradation (Fig. 6B) or nuclear translocation of p65 (Fig. 6C; $P > 0.05$).

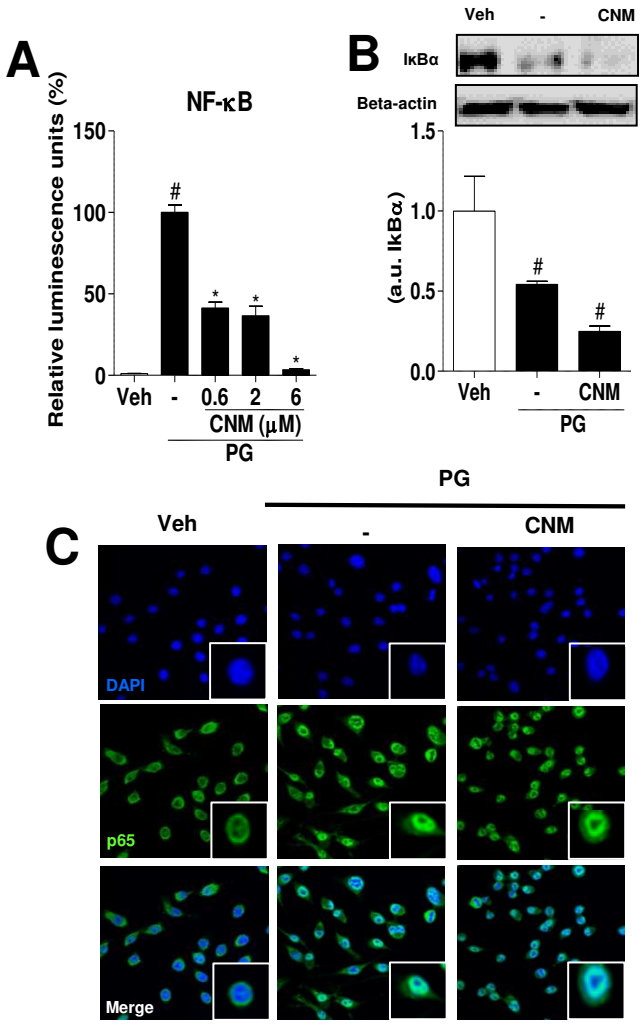

Fig. 6. CNM reduces NF-kB activity but not IkBα degradation or p65 translocation to nucleus. RAW 264.7 macrophages were pretreated with CNM at the concentration of 0.6, 2 or 6 μM or vehicle (veh), 30 min prior to stimulation with PG (5 μg/mL). (**A**) NF-kB activaty was quantified using RAW 264.7 macrophages, stably expressing NF-kB pLUC gene, after stimulation with PG for 4 h. (**B**) IκBα levels were evaluated in RAW 264.7 macrophages stimulated with PG for 30 min, pretreated or not with CNM 6 μM. (**C**) RAW 264.7 macrophages pretreated or not with CNM 6 μM were fixed 30 min after PG stimulation (5 μg/mL) and then p65 (green) and nucleus (blue) were stained. The data were expressed as the mean ± SEM, with n=3-5 per group. Symbols indicate statistical difference ($P < 0.05$, Tukey's post-test). # $P < 0.05$ compared to vehicle group; * $P < 0.05$ compared to PG group.

4. Discussion

Studies have shown that the chemical composition of propolis may vary according to the collection site and type of vegetation in the area where bees collect plant resins [20] and [21]. Due to this fact, many biologically active compounds have

been isolated from Brazilian propolis, such as CAPE, artepelin C, apigenin, *tt*-farnesol and pinocenbrin [22], [23], [24] and [25]. Thus, several studies have been carried out in order to isolate and identify new molecules from Brazilian propolis that can be applied as therapeutic agents [26]. In the study, it was found that CNM obtained from geopropolis of stingless bees (*M. scutellaris*), which had never been described some anti-inflammatory activity, proved to be able to inhibits neutrophil migration into the peritoneal and joint cavity which was associated with a decrease in the production of TNF and CXCL2/MIP-2. Furthermore, CNM seems to act molecularly through inhibition of MAPKs and NF-kB signaling activation on macrophages.

Macrophages are immune cells that play key roles in homeostasis, tissue repair and immunity. Among the functions attributed to macrophages in the immune response are the ability to phagocytize and destroy infectious agents and release inflammatory mediators that contribute to activation of the immune system [17] and [27]. Besides to contribute to the host protective response, macrophages are also involved in the tissue damage and loss of function in multiple pathologies, when the inflammatory response is not adequately controlled. Thus, the excessive release, particularly of reactive oxygen species and nitrogen by macrophages, triggers tissue damage and often contributes to disease progression [17].

Given the above, the signaling pathways involved in the inflammatory activity of macrophages have been investigated as potential targets for development of new drugs [28]. MAPK plays an important role in the activation of multiple inflammatory genes. The presence of receptors expressed on the cell membrane of macrophages or in their cytoplasm when activated in case of pathogenic infections or tissue damage, triggers a chain reaction in which MAPK are also activated [18]. In the present study the activity of CNM on MAPK ERK 1/2, JNK and p38 MAPK, was investigated. ERK 1/2 is an important MAPK that participates in the regulation of cytokine production in macrophages. Its intracellular signaling may occur via post-transcriptional mechanisms via Tpl2/ERK, or transcriptional mechanisms by activation of the AP-1 transcription factor [18], [29] and [30]. Likewise, JNK and p38 MAPK also participate in the regulation of the production of inflammatory mediators in macrophages by activation of AP-1. In the case of JNK, part of its modulatory mechanism is by induction of c- jun phosphorylation [30] and [31]. This study demonstrated that the modulatory effect of CNM on the release of TNF-α and CXCL/2MIP-2 in RAW 264.7 macrophages is associated to the reduced

phosphorylation of ERK 1/2, JNK and p38 MAPK. Furthermore, phosphorylated c-Jun was shown to be diminished, therefore proving the modulation of AP-1 activity.

The transcription factor NF-kB has also been the target of novel anti-inflammatory drugs as part of the innate and adaptive immune response. In addition, NF-kB activity is elevated in various inflammatory diseases, including rheumatoid arthritis [19]. Its activation via TLR receptor involves the cascade reaction by Myd88/IRAK/TRAF6/TAK-1/IKK signaling, followed by IκBα degradation, NF-kB activation and release of cytokines and chemokines inflammatory [19]. Glucocorticoid drugs exert their inhibitory activity on NF-kB through the induction of IκBα [32]. In this study, it was found that CNM reduced NF-kB activation in RAW 264.7 macrophages, stably expressing NF-kB pLUC gene, when stimulated with PG; however, the observed effect was independent from IκBα modulation. Studies showed that the p38 MAPK plays a key role in NF-kB activation, and that p38 MAPK is essential for nuclear p65 phosphorylation via MSK1. This event is necessary for activation of the NF-kB transcription factor [33]. Based on this study, it is suggested that inhibition of p38 MAPK by CNM justifies its activity on the reduction of NF-κB activation.

The data obtained herein corroborate those of other studies investigating the activity of coumarins and derivatives on the MAPK pathway. A recent study showed that the furocoumarin angelicin reduced the levels of TNF-α and IL-6 *in vivo* and *in vitro*. This compound reduced NF-κB (p65) signaling, p38 MAPK and JNK phosphorylation [34]. In another study, imperatorin reduced the inflammatory cytokines TNF-α, IL-6 and IL-1β produced by RAW 264.7 macrophages and the expression of the phosphorylated proteins p38 MAPK and JNK. However, unlike our findings, imperatorin reduced NF-kB translocation to the nucleus by blocking the phosphorylation and degradation of IκBα [35]. Nevertheless, in our study post-trasncriptional modulations of CNM were not evaluated, which could provide reduction of luminescence in NF-kB-pLUC assay without change p65 translocation to nucleus [15].

In addition, to this pioneering study regarding the actions and pharmacological effects of the CNM, it is relevant to mention that the stingless bees (*Melipona scutellaris*), which collect the geopropolis, are native, primitive and vulnerable to extinction in Brazil. Considering the important role of bees in the ecosystem and food production chain, the findings in this study add invaluable scientific value to bees and mainly collaborating for its biology and preservation.

Therefore, we conclude that CNM reduced neutrophil migration in the inflammatory process by inhibiting the release of TNF-α and CXCL2/MIP-2. Furthermore, it was found that the effect of CNM in macrophages is associated to inhibition of the phosphorylation of ERK, JNK and p38 MAPK. These results suggest that CNM from the class of coumarins is a promising agent to combat inflammatory diseases.

Conflict of interest

Authors declare that there is no conflict of interest.

Acknowledgment

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Abbreviations

AP-1, activator protein-1; BSA, bovine serum albumin; CNM, cinnamoyloxy-mammeisin; CXCL2, chemokine ligand 2/MIP-2, macrophage inflammatory protein 2; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinases; IRAK4, interleukin-1 receptor-associated kinase 4; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha IKK1, I kappa B kinase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinases; mBSA, methylated bovine serum albumin; MSK1, mitogen- and stress-activated protein kinase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; MyD88, myeloid differentiation primary response 88; NF-kb, factor nuclear kappa B; PG, peptidoglycan; p38, p38 mitogen-activated protein kinases; SAPK, stress-activated protein kinases/JNK, c-Jun amino-terminal kinases; TAK1, transforming growth factor beta-activated kinase 1; TLR, toll-like receptors; TNF, tumor necrosis factor-alpha; TNT, tris-NaCl-Tween buffer; Tpl2, tumor progression locus 2; TRAF6, tumor necrosis factor receptor-associated factor 6.

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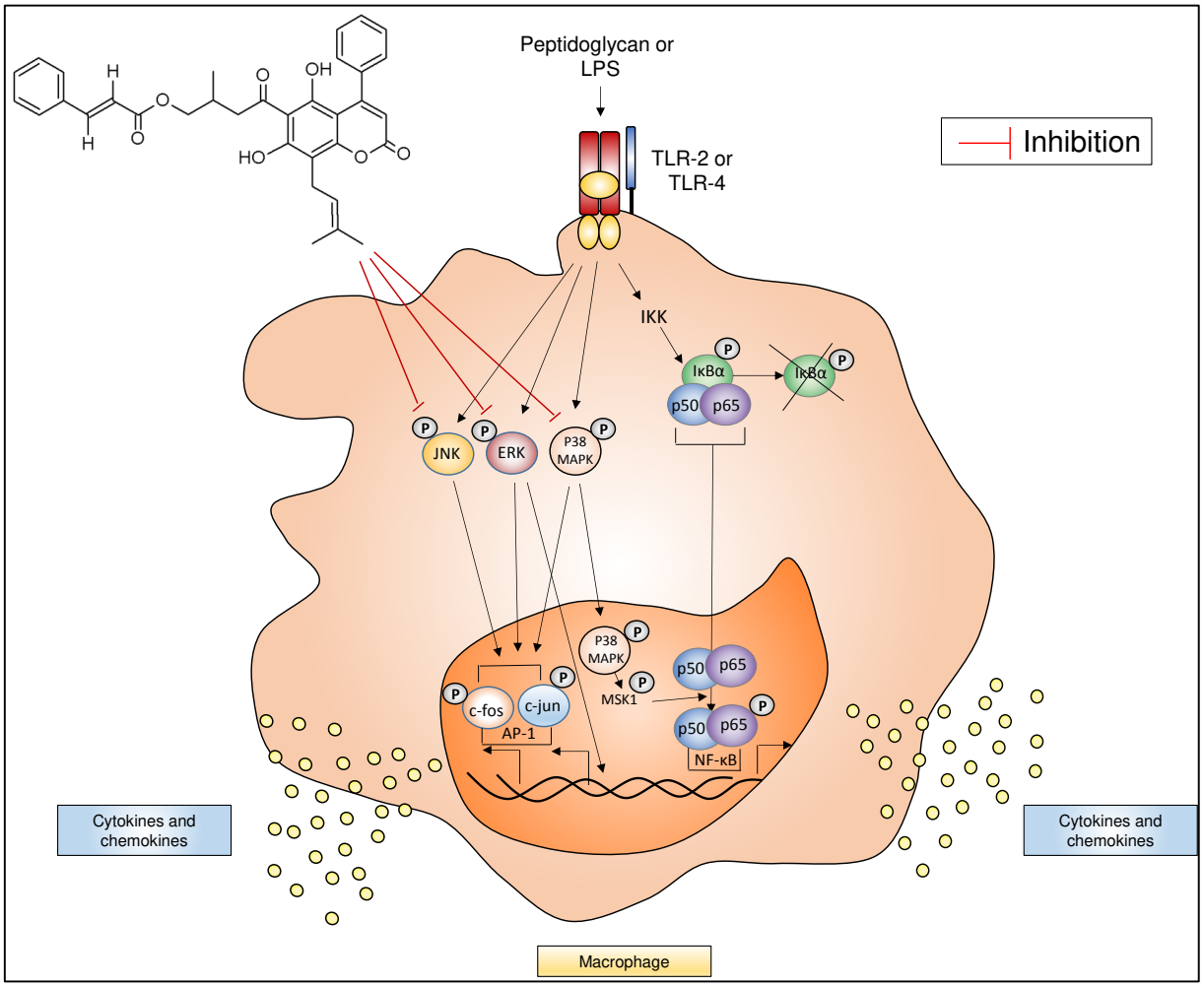
Graphical abstract



3 DISCUSSÃO

Historicamente, as atividades biológicas das própolis brasileiras são amplamente estudadas no mundo todo, e muitos compostos biologicamente ativos já foram isolados e identificados (Huang et al., 2014). Neste estudo foi avaliada a atividade de dois isoflavonóides, isolados da própolis vermelha e de um composto da classe das cumarinas, isolado da geoprópolis de *Melipona scutellaris*.

Inicialmente foi avaliada a atividade anti-inflamatória e elucidado os mecanismos de ação dos compostos vestitol e neovestitol. Os isoflavonóides são compostos que apresentam grande potencial de modulação do processo inflamatório. Dentre alguns estudos que evidenciaram este potencial, destaca-se com o composto formonetina, uma isoflavona isolada da própolis vermelha, onde foi constatado que o seu tratamento em modelos experimentais de inflamação, reduziu a migração de leucócitos induzida por carragenina, bem como a formação de edema (Cavendish et al., 2015). No presente estudo, foi observado que o composto vestitol inibiu a migração de neutrófilos em camundongos estimulados com LPS ou mBSA, sendo a sua atividade relacionada com a redução da liberação das quimiocinas CXCL1/KC e CXCL2/MIP-2. Além disso, o vestitol mostrou-se ativo ao inibir a quimiotaxia de neutrófilos *in vitro*, por meio do bloqueio de influxo de cálcio.

Em relação ao composto neovestitol, também constatou-se atividade anti-inflamatória sobre a migração de neutrófilos em camundongos desafiados com LPS na cavidade peritoneal, entretanto, diferentemente do vestitol, o seu mecanismo de ação foi relacionado com a via do óxido nítrico. No mesmo estudo, devido ao neovestitol ser bastante abundante na própolis vermelha, sua atividade no modelo de artrite também foi avaliada pelo período de 12 dias. Conforme os resultados, foi observado que o neovestitol reduziu o escore clínico, as lesões articulares e a liberação de IL-6 nos animais com artrite induzida por colágeno tipo II.

Posterior aos estudos com os compostos da própolis vermelha, foi realizado um fracionamento bioguiado para atividade anti-inflamatória com a geoprópolis de *Melipona scutellaris*, e então o cinamoiloxi-mammeisina (CNM) foi isolado e identificado. O composto CNM, foi identificado pela primeira vez nas plantas da espécie *Kielmeyera reticulata.* Entretanto, as suas atividades biológicas nunca foram estudadas. Assim com base nos estudos iniciais que demonstraram a atividade

anti-inflamatória da geoprópolis, investigou-se a atividade do CNM sobre a migração de neutrófilos no processo inflamatório. Dentre os resultados obtidos, o CNM inibiu a migração de neutrófilos na cavidade peritoneal e articular, bem como a liberação de TNF-α e CXCL2/MIP-2 *in vivo*.

Para elucidação do mecanismo de ação do CNM, sua atividade foi avaliada em cultura de macrófagos RAW 264.7. Estudos tem demonstrado o efeito de compostos pertencentes a classe das cumarinas sobre a modulação do processo inflamatório, sendo as principais vias de ação, por meio da inibição da liberação de mediadores inflamatório em macrófagos (Guo et al., 2012; Liu et al., 2013). No presente estudo, foi constatado que o CNM inibiu a liberação de TNF-α e CXCL2/MIP-2 *in vitro*, onde o seu mecanismo de ação foi relacionado com a redução da fosforilação das MAPK ERK 1/2, JNK e p38 MAPK. Estes resultados, portanto, demonstram o potencial anti-inflamatório do CNM, nunca antes estudado.

Em acréscimo, a este estudo inédito em relação às ações e efeitos farmacológicos do CNM é relevante mencionar que as abelhas sem ferrão (*Melipona scutellaris*), que coletam a geoprópolis, são insetos nativos, primitivos e em extinção no Brasil. Considerando o importante papel das abelhas no ecossistema e na cadeia de produção de alimento, os achados neste estudo agregam valor científico inestimável as sociedade das abelhas, colaborando, sobretudo para a sua biologia e preservação.

4 CONCLUSÃO

Portanto, conclui-se:

- O composto vestitol apresentou atividade inibitória sobre a migração de neutrófilos induzida por diferentes estímulos inflamatórios. Em relação a suas principais vias de ação, foi verificado que o vestitol reduziu a liberação das quimiocinas CXCL1/KC e CXCL2/MIP-2 em macrófagos, bem como inibiu a quimiotaxia de neutrófilos via bloqueio do influxo de cálcio;

- O composto neovestitol reduziu o influxo de neutrófilos induzido por LPS dependente da via do óxido nítrico. Além disso, foi observado que o tratamento crônico com neovestitol melhorou significativamente os parâmetros clínicos e lesões articulares de animais com artrite induzida por colágeno, sendo o seu efeito relacionado com a redução da liberação de IL-6;

- Em relação ao estudo com a geoprópolis de *Melipona scutellaris*, o composto CNM, pertencente a classe das cumarinas, foi identificado como o bioativo para atividade anti-inflamatória. Nos ensaios biológicos, observou-se que o CNM reduziu a migração de neutrófilos no processo inflamatório, sendo o seu mecanismo de ação relacionado com a inibição da liberação de TNF-α e CXCL2/MIP-2 por supressão da fosforilação de ERK 1/2, JNK e p38 MAPK em macrófagos;

- Estudos futuros com estes três compostos isolados são necessário para que se construa uma identidade terapêutica maior sobre eles, a fim de serem indicados como novos agentes anti-inflamatórios ou protótipos de novos fármacos.

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ANEXOS

Anexo 1 - Informação CCPG/001/2015 que trata do formato padrão das Dissertações de Mestrado e Teses de Doutorado da Unicamp.

INFORMAÇÃO CCPG/001/2015
Substitui Informação CCPG/002/2013
e
Altera a redação da versão aprovada pela CCPG em 17/06/2015).
(Nova redação dos itens; 2, 3 e 4, Inciso I, do Art. 1º)

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade da apresentação do conteúdo das dissertações e teses em formato alternativo ao já estabelecido tradicional, a CCPG resolve:
Art. 1º O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverá obrigatoriamente conter as informações como seguem:
I. Páginas pré-textuais:
1. Primeira folha dando visibilidade à Universidade, à Unidade de defesa, ao autor (a), título da dissertação/tese na língua em que o trabalho foi redigido – português, inglês ou espanhol -, local e data. No caso de tese/dissertação redigida em inglês ou espanhol, além do título original do trabalho, obrigatoriamente, também deverá constar o título em português;

2. Página de rosto dando visibilidade: ao nome do autor; ao título do trabalho; ao número de volumes (quando houver mais de um); ao nível (mestrado ou doutorado); à área de concentração; ao nome do orientador e coorientador; ao local (cidade) e ao ano de depósito. Incluir informação, na parte inferior da página de que o arquivo digital corresponde à versão final da tese/dissertação defendida pelo aluno (nome) e orientada pelo (nome do Orientador).

Nos casos de teses defendidas em Cotutela, logo abaixo do nível e da área de concentração, se houver, deverá ser inserida a informação em português e em inglês ou espanhol de que a tese foi produzida no âmbito de um Acordo de Cotutela firmado entre a Unicamp e a Universidade convenente.
3. Ficha catalográfica (no verso da página de rosto).

Obs. 1) Caso a tese de doutorado seja feita em Cotutela, será necessário informar na ficha catalográfica o fato, o nome da Universidade convenente e os nomes dos orientadores.
Obs. 2) Quando se tratar de Teses e Dissertações financiadas por agências de fomento, os beneficiados deverão fazer referência ao apoio recebido e inserir, no sistema de confecção da Ficha Catalográfica, além do nome da agência, o número do processo pelo qual recebeu o Auxílio;
4. Folha de aprovação, dando visibilidade à Comissão Examinadora com a informação de que a Ata da Defesa, assinada pelos membros da Comissão Examinadora, consta no processo de vida acadêmica do aluno;

5. Dedicatória (opcional);

6. Agradecimento (opcional);

7. Resumo (redigido obrigatoriamente em português, máximo de 500 palavras);

8. Abstract (resumo traduzido para o inglês);

9. Resumo em uma terceira língua (opcional);

10. Lista de ilustrações (opcional);

11. Lista de Tabelas (opcional);
12. Lista de Abreviaturas e Siglas (opcional);

13. Lista de Símbolos (opcional);

14. Sumário.

II. Elementos Textuais: Corpo da dissertação ou tese dividido em tópicos estruturados, segundo as necessidades da área de conhecimento.
III. Elementos Pós-Textuais:
1. Referências;

2. Apêndices;

3. Anexos.

§ 1° Todas as páginas deverão ser contadas; porém, as folhas pré-textuais (da primeira folha até o sumário) não são numeradas. A numeração (contada continuamente) deverá figurar a partir da Introdução até a última folha do trabalho, em algarismos arábicos, no canto superior direito da página.
§ 2° A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; apêndices; anexos.
§ 3° A dissertação ou tese deverá ser redigida em português facultada a redação em inglês ou espanhol, com a concordância simultânea do orientador e orientado, conforme previsão no Regimento Geral da Pós-Graduação da Universidade.
§ 4° A defesa da dissertação ou tese, total ou parcialmente em inglês ou espanhol poderá ser realizada desde que haja concordância explícita (em documento escrito) do orientado, orientador e de todos os membros da comissão examinadora.
§ 5° A dissertação ou tese cujos conteúdos versarem sobre pesquisa envolvendo seres humanos, animais, biossegurança e patrimônio genético, deverá apresentar em anexo os respectivos documentos de aprovação obtidos nas instâncias competentes.
Art. 2° Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ter seu conteúdo apresentado em formato alternativo ao modelo tradicional, observado também o padrão indicado no Art. 1°.

§ 1° É considerado formato alternativo aquele em que as dissertações e teses, obrigatoriamente, apresentem os seguintes capítulos no corpo do trabalho: 1) Introdução; 2) Documentos publicados e/ou a publicar, como: sumário do(s) artigo(s), o(s) artigo(s) propriamente dito(s), sumário de livro(s), capítulo(s) de livro(s), com os dados referentes à publicação e/ou submissão; 3) Discussão (aplicável em casos de dois ou mais documentos e não obrigatória em casos de apenas um documento); 4) Conclusão; 5) Referências.
§ 2° O(s) documento(s) publicado(s) ou a publicar deve(m) ser em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.
§ 3º No caso de documento já publicado, o aluno deverá anexar a autorização da Editora para a sua inclusão na tese/dissertação.
Art. 3° É obrigatória a entrega de mídia contendo o trabalho completo em arquivo único inclusive contendo a Folha de Aprovação, Ficha catalográfica, Apêndices e Anexos. Quando houver cópia impressa, seu conteúdo deve ser fiel ao conteúdo entregue em mídia digital, inclusive quanto à folha de aprovação, ficha de catalogação, apêndice e anexos. O arquivo digital não deve conter chaves que restrinja o acesso. A mídia deve ser identificada com as seguintes informações: a) nome do autor por extenso; b) título e subtítulo do trabalho; c) grau de Defesa; d) Unidade de Defesa; e) data de Defesa; f) endereço de e-mail e telefone para contato; g) identificação do tipo de arquivo.
Parágrafo único. Será necessário entregar, junto com a mídia, uma via do Termo de Autorização para disponibilização da tese ou dissertação em formato digital.

Art. 4° Caso o autor desejar a versão impressa da tese/dissertação, ele será o responsável pela reprodução. As dissertações e teses deverão ser reproduzidas no padrão frente, exceção feita à página que contém a ficha catalográfica. O arquivo do modelo padrão da capa, com formato único, válido tanto para o formato tradicional quanto para o alternativo de tese ou dissertação, com projeto gráfico definido pela PRPG, fornecida pela Universidade, contendo informações relativas à Universidade Estadual de Campinas, ao nível (mestrado ou doutorado), à Unidade e ano de defesa, estará disponível na CPG de cada Unidade.

Art. 5º Quanto à Apresentação, a tese/dissertação deverá ter o seguinte formato, válido tanto para a versão digital quanto impressa:

I – folha tamanho A4 de dimensões 21 x 29,7 cm. É aconselhável o uso do papel branco e tinta de cor preta. A fonte utilizada pode ser escolhida entre Times New Roman, Arial, ou similar, em tamanho 12.

II – espacejamento:

a) entre linhas do texto e referências: espaço 1,5;

b) notas de rodapé e citações textuais longas: espaço simples – uso opcional para Resumo e Abstract;

c) margens:

1) superior: 3,0 cm
2) esquerda: 3,0 cm
3) direita: 2,0 cm
4) inferior: 2,0 cm
5) de parágrafos: 2,0 cm a partir da margem esquerda
6) de citação longa: 4,0 cm a partir da margem esquerda.

Art. 6° Esta Informação entrará em vigor a partir de sua aprovação na CCPG, revogadas as disposições em contrário, principalmente a Informação CCPG 002/2013.

Profa. Dra. Rachel Meneguello
Presidente
Comissão Central de Pós-Graduação
CCPG-PRPG

Anexo 2 - Autorização de acesso e de remessa de componente do patrimônio genético (CGEN/MCTI).

Autorização de Acesso e de Remessa de Componente do Patrimônio Genético O Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, nos termos Deliberação 246/2009, do Conselho de Gestão do Patrimônio Genético, autoriza a instituição identificada no verso deste documento a acessar e remeter componente do Patrimônio Genético com a finalidade de pesquisa científica. Brasília, 15 de Outubro de 2014 Marcelo Marcos Morales Diretor de Ciências Agrárias, Biológicas e da Saúde PO 161/2010	Processo: 010666/2014-1 Validade: 03/11/2014 a 03/11/2016 Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS CNPJ: 460.684.250/0001-33 Pesquisador: Pedro Luiz Rosalen CPF: 030.958.228-80 RG: 11185975 - SSP / SP *Para visualizar a versão digital da Autorização de Acesso e de Remessa de Componente do Patrimônio Genético, V.Sa. poderá utilizar a ferramenta disponibilizada pelo CNPq para esse fim na página http://servicosweb.cnpq.br/visualizador/ e informar o número do protocolo 0260677585930147 para recuperá-la do banco de dados do CNPq*

Anexo 3 - Certificado de aprovação do Comitê de Ética em Pesquisa do Uso de Animais - FOP/UNICAMP.

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto **"Estudo in vivo e in vitro da atividade de compostos isolados de própolis brasileiras sobre a modulação da migração de neutrófilos no processo inflamatório"** (protocolo nº **2793-1**), sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Marcelo Franchin**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **09 de agosto de 2012**.

Campinas, 09 de agosto de 2012.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/ceea/

Anexo 4 - Prêmio concedido pela Federação de Sociedades de Biologia Experimental (FeSBE).

REUNIÃO ANUAL

FeSBE 2015 FEDERAÇÃO DE SOCIEDADES DE BIOLOGIA EXPERIMENTAL

Certificamos que

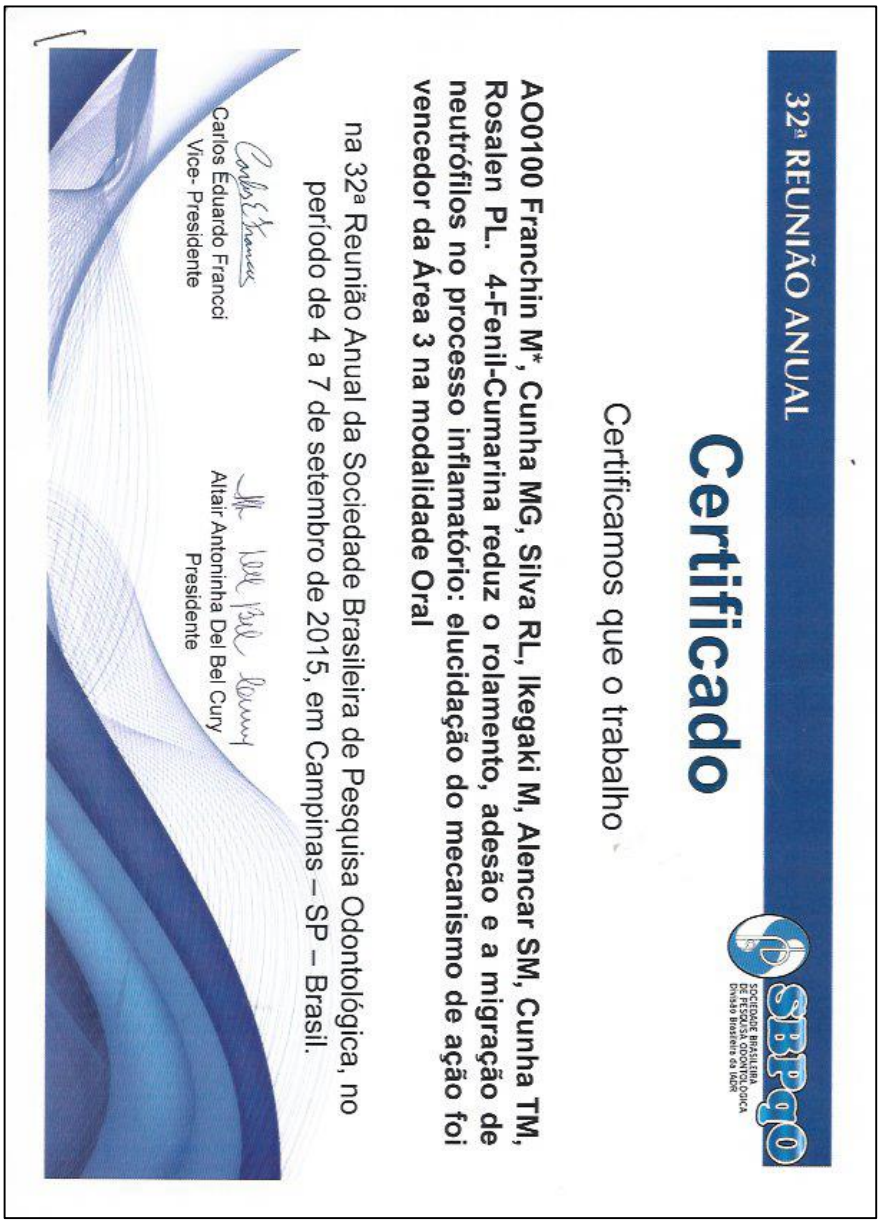
O Trabalho 12.031 VESTITOL REDUZ A MIGRAÇÃO DE NEUTRÓFILOS NO PROCESSO INFLAMATÓRIO: MODULAÇÃO DAS QUIMIOCINAS CXCL1/KC E CXCL2/MIP-2 E DO INFLUXO DE CÁLCIO de autoria Franchin, M. , Castanheira, F. V. S. , Morelo, D. F. C. , Alencar, S. M. , Ikegaki, M. , Cunha, T. M. , Rosalen, P. L. , Ciências Fisiológicas - FOP/UNICAMP Departamento de Farmacologia - FMRP/USP Departamento de Agroindústria, Alimentos e Nutrição - ESALQ/USP Departamento de Alimentos e Medicamentos - UNIFAL, foi contemplado com Menção Honrosa pela brilhante apresentação na

XXX Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, realizada na Faculdade de Medicina da Universidade de São Paulo em São Paulo - SP, de 09 a 12 de setembro de 2015.

Comissão Organizadora

CNPq MEDICINA USP FAPESP FAPERJ

Anexo 5 - Prêmio concedido pela Sociedade Brasileira de Pesquisa Odontológica (SBPqO).



32ª REUNIÃO ANUAL

SBPqO

SOCIEDADE BRASILEIRA DE PESQUISA ODONTOLÓGICA
Divisão Brasileira da IADR

Certificado

Certificamos que o trabalho

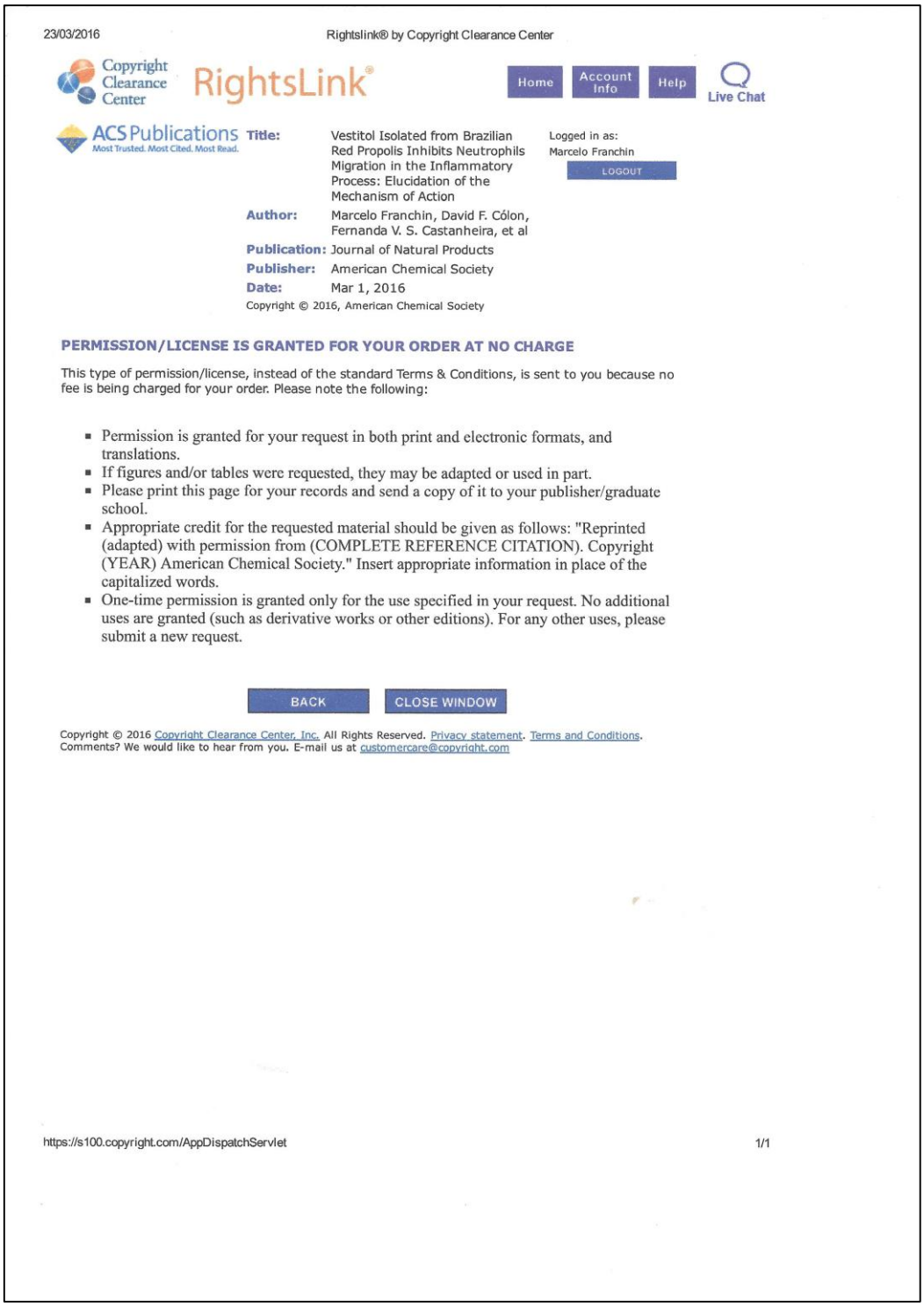
AO0100 Franchin M*, Cunha MG, Silva RL, Ikegaki M, Alencar SM, Cunha TM, Rosalen PL. 4-Fenil-Cumarina reduz o rolamento, adesão e a migração de neutrófilos no processo inflamatório: elucidação do mecanismo de ação foi vencedor da Área 3 na modalidade Oral

na 32ª Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica, no período de 4 a 7 de setembro de 2015, em Campinas – SP – Brasil.

Carlos Eduardo Francci
Vice- Presidente

Altair Antoninha Del Bel Cury
Presidente

Anexo 6 – Autorização da editora para inclusão do artigo “Vestitol isolated from Brazilian red propolis inhibits neutrophils migration in the Inflammatory process: elucidation of the mechanism of action” publicado no periódico Journal of Natural Products.



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Logged in as: Marcelo Franchin LOGOUT

Author: Marcelo Franchin, David F. Cólon, Fernanda V. S. Castanheira, et al

Publication: Journal of Natural Products

Publisher: American Chemical Society

Date: Mar 1, 2016

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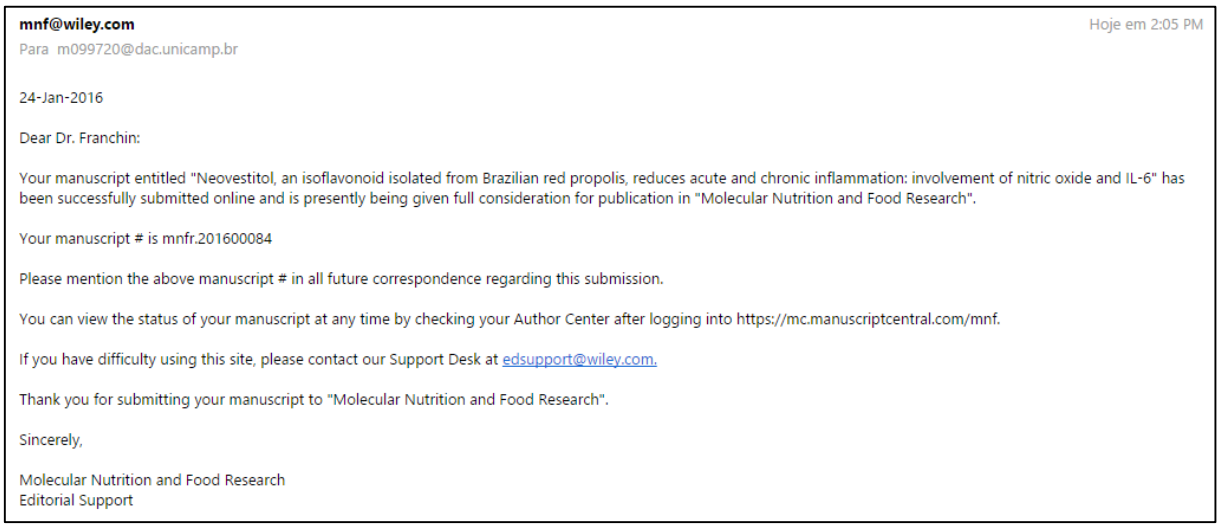
Anexo 7 - Comprovante de submissão do artigo “Neovestitol, an isoflavonoid isolated from Brazilian red propolis, reduces acute and chronic inflammation: involvement of nitric oxide and IL-6” para a revista Molecular Food and Nutrition Research (IF. 4.603).



mnf@wiley.com Hoje em 2:05 PM

Para m099720@dac.unicamp.br

24-Jan-2016

Dear Dr. Franchin:

Your manuscript entitled "Neovestitol, an isoflavonoid isolated from Brazilian red propolis, reduces acute and chronic inflammation: involvement of nitric oxide and IL-6" has been successfully submitted online and is presently being given full consideration for publication in "Molecular Nutrition and Food Research".

Your manuscript # is mnfr.201600084

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