

Cinnamoyloxy-mammeisin Isolated from Geopropolis Attenuates Inflammatory Process by Inhibiting Cytokine Production: Involvement of MAPK, AP-1, and NF- κ B

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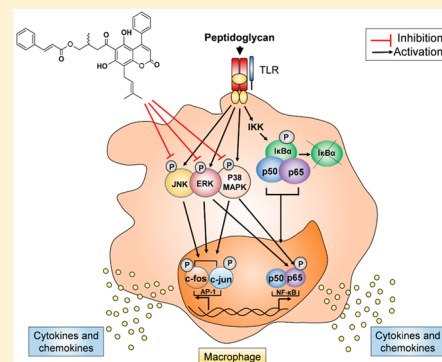
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Supporting Information

ABSTRACT: Chemical compounds belonging to the class of coumarins have promising anti-inflammatory potential. Cinnamoyloxy-mammeisin (CNM) is a 4-phenylcoumarin that can be isolated from Brazilian geopropolis. To our knowledge, its anti-inflammatory activity has 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 the in vitro and in vivo 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 AP-1 (subunit c-jun) in PG-stimulated macrophages. Pretreatment with CNM also reduced NF- κ B activation in RAW 264.7 macrophages stably expressing the NF- κ B-luciferase reporter gene. On the other hand, it did not alter I κ B α 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 MAPK signaling, AP-1, and NF- κ B.



Natural products have been historically used in folk medicine for the treatment of various diseases and are considered a vital source for the 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 are a class of naturally occurring chemical compounds studied due to their numerous biological activities, such as antibacterial, anticoagulant, antitumor, and anti-inflammatory properties.^{3,4} Indeed, the coumarin isofraxidin reduces the number of neutrophils and TNF- α , IL-6, and PGE₂ levels in bronchoalveolar lavage fluid in lipopolysaccharide (LPS)-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 resin and wax and is characterized by the presence of soil. It can be collected by a native stingless bee species, such as *Melipona scutellaris*.^{7–9} Recent studies have 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) (Figure 1), a 4-phenylcoumarin, was found to be the most abundant compound in geopropolis extract. Nevertheless, its activity on the inflammatory process has never been studied.

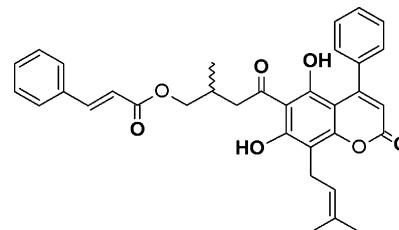


Figure 1. Chemical structure of cinnamoyloxy-mammeisin.

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Thus, the present study investigated the anti-inflammatory activity of CNM on peritonitis and arthritis models of inflammation and further elucidated its possible molecular mechanism of action.

RESULTS AND 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.^{10,11} Due to this fact, many biologically active compounds have been identified and/or isolated from Brazilian propolis, such as caffeic acid phenethyl ester, artemelin C, apigenin, *tt*-farnesol, and pinocembrin.^{12–15} Thus, several studies are carried out to isolate and identify new molecules from Brazilian propolis that could be applied as therapeutic agents.¹⁶ In the present study, we found that the subcutaneous (sc) administration of CNM (300 $\mu\text{g/kg}$) inhibited carrageenan-induced neutrophil migration into the peritoneal cavity of mice (Figure 2A). Further, the

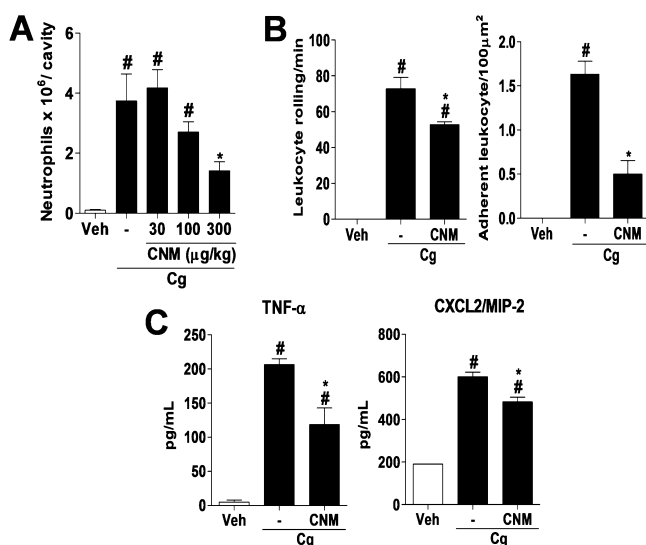


Figure 2. CNM reduces peritoneal inflammation induced by carrageenan. C57BL/6 mice were pretreated with CNM at doses of 30, 100, and 300 $\mu\text{g/kg}$ sc or vehicle (veh), 30 min before ip administration of carrageenan (Cg) at 500 $\mu\text{g/cavity}$. (A) Migration of neutrophils into the peritoneal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

compound inhibited leukocyte rolling and adhesion to mesenteric venules (Figure 2B), which was associated with a decrease in the production of TNF- α and CXCL2/MIP-2 (Figure 2C). Likewise, in a model of antigen-induced arthritis, daily pretreatment with CNM (100 $\mu\text{g/kg}$, sc) (Figure 3A) for 3 days prior to challenge with methylated bovine serum albumin (mBSA) reduced neutrophil migration, release of TNF- α (Figure 3B), and the localized fluorescence representing the activity of myeloperoxidase (Figure 3C) in the joint cavity of mice.

Macrophages are immune cells that play key roles in homeostasis, tissue repair, and immunity. Among the functions

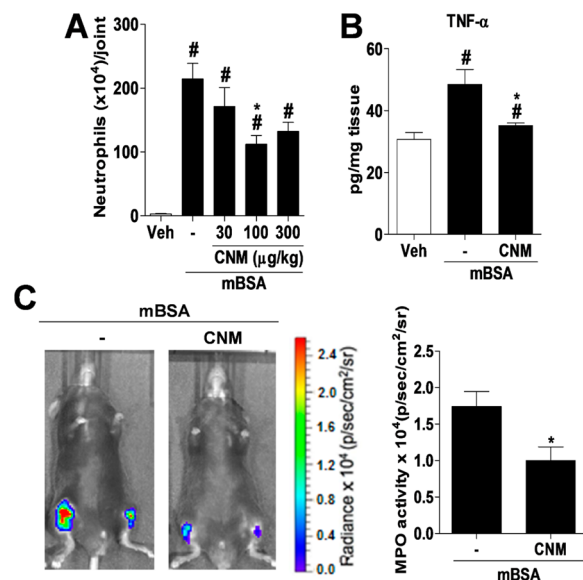


Figure 3. CNM reduces joint inflammation induced by mBSA. C57BL/6 mice were pretreated with CNM at doses of 30, 100, and 300 $\mu\text{g/kg}$ sc or vehicle (veh) 30 min prior to administration of mBSA (30 $\mu\text{g/joint}$). (A) Neutrophil migration in the joint cavity 6 h after intra-articular (ia) injection of mBSA. (B) TNF- α levels in the joint cavity after 1.5 h of ia injection of mBSA in mice pretreated or not with CNM (100 $\mu\text{g/kg}$). (C) Fluorescence intensity 6 h after ia injection of mBSA with the in vivo imaging system IVIS Spectrum representing the myeloperoxidase (MPO) in mice pretreated or not with CNM (100 $\mu\text{g/kg}$). The data are expressed as the mean \pm SEM, with $n = 5$ or 6 per group. Symbols indicate statistical difference ($p < 0.05$, Tukey's or Student's t tests). [#] $p < 0.05$ compared to veh group; ^{*} $p < 0.05$ compared to mBSA (–) group.

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.^{17,18} Besides contributing to the host protective response, macrophages are also involved in tissue damage and loss of function in multiple pathologies in cases where the inflammatory response is not adequately controlled. Thus, the excessive release of reactive oxygen species and nitrogen by macrophages triggers tissue damage and often contributes to disease progression.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 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 LPS, as shown in Figure 4A and B, respectively. Furthermore, the highest CNM concentration used in in vitro (6 μM) experiments showed no cytotoxic effect in RAW macrophages as compared to the vehicle group (Figure 4C and D, annexin V and PI or MTT assays).

Given the above, the signaling pathways involved in the inflammatory activity of macrophages have been investigated as potential targets for the development of new drugs.¹⁹ 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 response to pathogenic infections or tissue damage triggers a chain reaction in which MAPK is also activated.²⁰ In the present study the activity of CNM on MAPK ERK 1/2, JNK, and p38 MAPK was investigated. ERK 1/2 is an

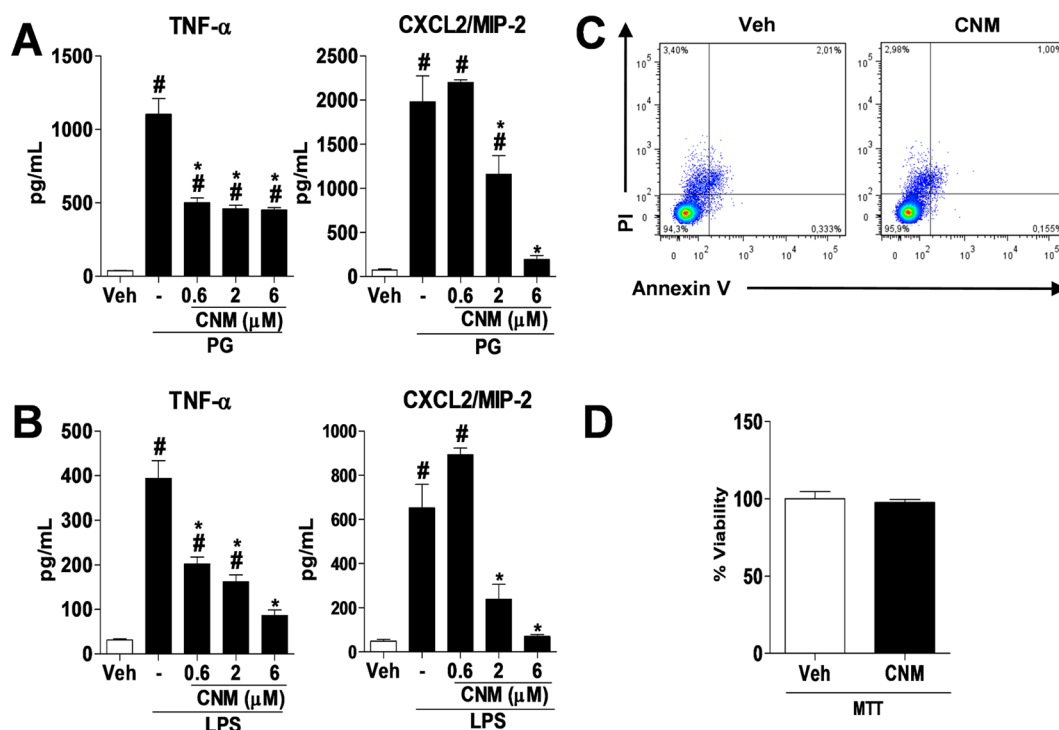


Figure 4. CNM reduces the release of cytokines from macrophages. RAW 264.7 macrophages were pretreated with CNM at concentrations of 0.6, 2, and 6 μM or vehicle (veh), 30 min prior to stimulation with PG (5 $\mu\text{g/mL}$) or LPS (10 ng/mL). (A, B) TNF- α and CXCL2/MIP-2 levels in the supernatant of RAW 264.7 macrophages stimulated with PG or LPS 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 6 μM CNM for 4 h. The data are expressed as the mean \pm SEM, with $n = 3\text{--}5$ per group. Symbols indicate statistical difference ($p < 0.05$, Tukey's or Student's t tests). [#] $p < 0.05$ compared to veh group; ^{*} $p < 0.05$ compared to PG (–) or LPS (–) group.

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 activation of the AP-1 transcription factor.^{20–22} Likewise, JNK and p38 MAPK 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 through induction of c-Jun phosphorylation, a subunit of AP-1.^{22,23} This study demonstrated that the modulatory effect of CNM (6 μM) on the release of TNF- α and CXCL2/MIP-2 in RAW 264.7 macrophages is associated with the reduced phosphorylation of ERK 1/2 (Figure 5A), JNK (Figure 5B), and p38 MAPK (Figure 5C). Furthermore, phosphorylated c-Jun was also reduced by the treatment with CNM (Figure 5D), therefore proving the modulation of AP-1 activity.

The transcription factor NF- κB has also been the target of novel anti-inflammatory drugs as part of the innate and adaptive immune response. In addition, NF- κB activity is elevated in various inflammatory diseases, including rheumatoid arthritis.²⁴ Its activation via toll-like receptors involves the cascade reaction by Myd88/IRAK/TRAF6/TAK-1/IKK signaling, followed by I $\kappa\text{B}\alpha$ degradation, NF- κB activation, and release of inflammatory cytokines and chemokines.²⁴ Glucocorticoid drugs exert their inhibitory activity on NF- κB through the induction of I $\kappa\text{B}\alpha$.²⁵ At 0.6, 2 or 6 μM , CNM reduced NF- κB activation compared to the PG group, characterized by reduced luminescence emission (Figure 6A). On the other hand, CNM at 6 μM did not alter I $\kappa\text{B}\alpha$ degradation (Figure 6B) or nuclear translocation of p65 (Figure 6C). Studies have shown that ERK 1/2 and p38 MAPK play a key role in NF- κB activation and are essential for NF- κB -DNA binding.^{26,27} The

current study suggests that inhibition of ERK 1/2 and p38 MAPK by CNM may cause reduction of NF- κB activation.

The data obtained herein corroborate those of other studies investigating the activity of coumarins and derivatives on the MAPK and NF- κB pathway. A recent study showed that angelicin reduced TNF- α and IL-6 levels in vivo and in vitro.²⁸ This compound reduced NF- κB (p65) signaling, as well as p38 MAPK and JNK phosphorylation.²⁸ In another study, imperatorin reduced the inflammatory cytokines TNF- α , IL-6, and IL-1 β produced by RAW 264.7 macrophages and the phosphorylated proteins p38 MAPK and JNK.²⁹ However, unlike our findings, imperatorin reduced NF- κB translocation to the nucleus by blocking the phosphorylation and degradation of I $\kappa\text{B}\alpha$.²⁹

It is worth noting that this is a pioneer study regarding the actions and pharmacological effects of CNM and that the stingless bees *M. scutellaris* that collect the geopolymer 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 scientific value to bees and underscore the need for their preservation.

In summary, we conclude that CNM reduced neutrophil migration in the inflammatory process by inhibiting the release of TNF- α and CXCL2/MIP-2. Furthermore, we found that the effect of CNM in macrophages is associated with inhibition of ERK 1/2, JNK, and p38 MAPK phosphorylation, AP-1, and NF- κB . These results suggest that CNM could be a promising agent to combat inflammatory diseases.

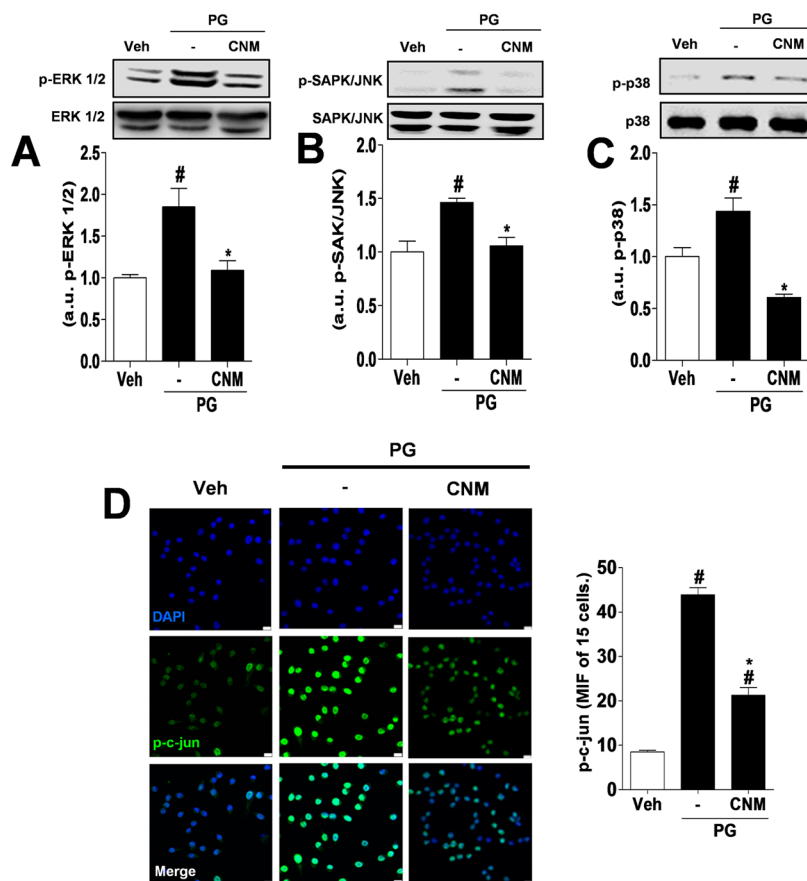


Figure 5. CNM reduces MAPK and c-Jun phosphorylation in stimulated macrophages. RAW 264.7 macrophages were pretreated with CNM (6 μ M) or vehicle (veh), 30 min prior to stimulation with PG (5 μ g/mL). (A, B, C) MAPK phosphorylation (ERK 1/2, SAPK/JNK, and p38 MAPK) was evaluated in RAW 264.7 macrophages stimulated with PG for 15 min. (D) p-c-Jun was analyzed in RAW 264.7 macrophages after 30 min of stimulation with PG. The data are expressed as the mean \pm SEM, with $n = 3$ or 4 per group. Symbols indicate statistical difference ($p < 0.05$, Tukey's post-test). [#] $p < 0.05$ compared to veh group; ^{*} $p < 0.05$ compared to PG (–) group.

EXPERIMENTAL SECTION

General Experimental Procedures. Hexane, 2-propanol, dichloromethane, ethyl acetate, acetone, and methanol were purchased from Merck (Sao Paulo, SP, Brazil). RPMI, penicillin, L-glutamine, carrageenan, mBSA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), LPS, PG, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-ERK 1/2, anti-p-ERK 1/2, anti-SAPK/JNK, anti-p-SAPK/JNK, anti-p38 MAPK, anti-p-p38 MAPK, anti-I κ B α , and Beta-actin antibodies were purchased from Cell Signaling (Danvers, MA, USA). The Apo Screen annexin V-FITC kit was purchased from SouthernBiotech (Birmingham, AL, USA). Fetal bovine serum was from Gibco (Grand Island, NY, USA). LPS, PG, carrageenan, and mBSA dissolution was performed in sterilized saline solution, while CNM was dissolved in 1% DMSO in 1 \times phosphate-buffered saline (PBS).

Experimental Biological Material. Samples of *M. scutellaris* geoproducts were collected between June and July 2012 in the city of Entre Rios (12°22' S, 37°54' W) in the state of Bahia, Brazil. Samples of *M. scutellaris* bees were deposited in the Bioscience Institute of Sao Paulo University, in the "Nogueira Neto" Entomological Collection under the voucher number CEPANN 42.863. The present research received authorization for access to genetic heritage components granted by the National Council for Technological and Scientific Development (CNPq), no. 010666/2014-1.

Extraction and Isolation. The isolation and identification of the CNM were performed as previously described.⁸ The purity of CNM ($\geq 95\%$) was determined by reverse-phase high-performance liquid chromatography (RP-HPLC).

Animals. C57BL/6 SPF (specific pathogen-free) male mice weighing between 20 and 22 g were provided by CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil). The animals were kept at temperatures of 22–25 °C with food (standard pellet diet, Presence) and water ad libitum, with a controlled light/dark cycle and a 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).

Cell Culture. RAW 264.7 macrophages, or RAW 264.7 stably bearing the luciferase reporter gene controlled by an NF- κ B-sensitive promoter (NF- κ B-pLUC) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 2 mM L-glutamine at 37 °C in a 5% CO₂/95% atmosphere.

Pharmacological Protocol in Vivo and in Vitro. Mice were pretreated with CNM at 30, 100, or 300 μ g/kg by sc injection. After 30 min they received a challenge consisting of intraperitoneal (ip) injection of carrageenan (500 μ g/cavity) or intra-articular (ia) injection of mBSA (30 μ g/joint). In the in vitro test, RAW 264.7 macrophages were pretreated with CNM at concentrations of 0.6, 2, or 6 μ M 30 min before stimulation with PG (5 μ g/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.

Migration of Neutrophils into the Peritoneal Cavity. Neutrophil infiltration was determined in the peritoneum 4 h after ip injection of carrageenan (500 μ g/cavity). Washing of the peritoneal cavity was performed with 3 mL of PBS containing EDTA. The fluid was collected (approximately 95% of the injected volume), and total leukocyte counting was carried out in a Neubauer chamber. A differential smear was prepared using a cytospin (Cytospin 4, Thermo Fisher Scientific, Waltham, MA, USA) and stained with Panotich

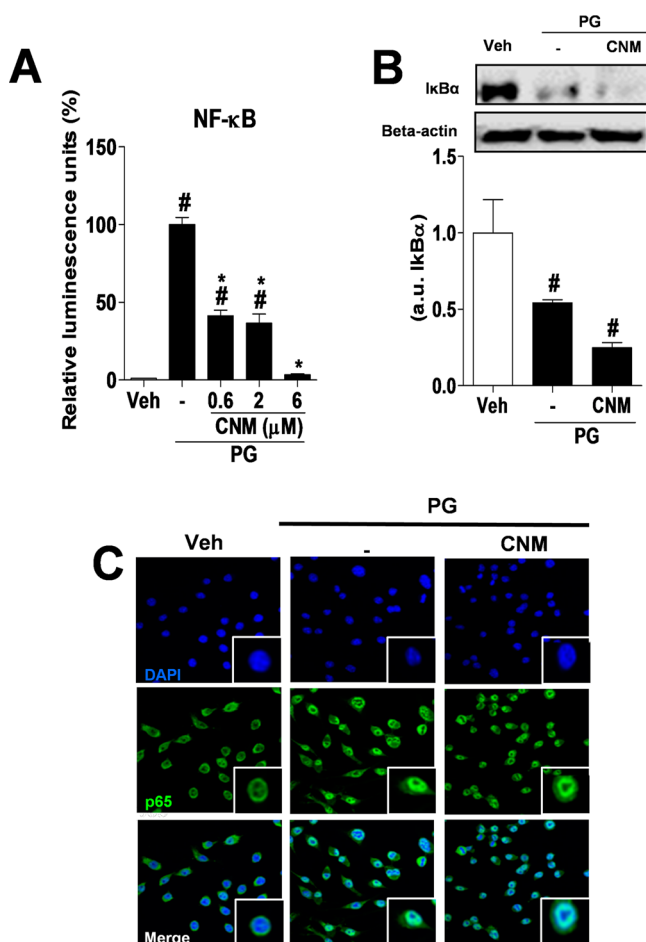


Figure 6. CNM reduces NF- κ B activity but not I κ B α degradation or p65 translocation to the nucleus. RAW 264.7 macrophages were pretreated with CNM at concentrations of 0.6, 2, and 6 μ M or vehicle (veh), 30 min prior to stimulation with PG (5 μ g/mL). (A) NF- κ B activity was quantified using RAW 264.7 macrophages stably expressing the NF- κ B pLUC gene, after stimulation with PG for 4 h. (B) I κ B α levels were evaluated in RAW 264.7 macrophages stimulated with PG for 15 min, pretreated or not with 6 μ M CNM. (C) RAW 264.7 macrophages pretreated or not with 6 μ M CNM were fixed 30 min after PG stimulation (5 μ g/mL), and then p65 (green) and the nucleus (blue) were stained. The data are expressed as the mean \pm SEM, with $n = 3$ –5 per group. Symbols indicate statistical difference ($p < 0.05$, Tukey's post-test). # $p < 0.05$ compared to veh group; * $p < 0.05$ compared to PG (–) group.

Solution Kit (Laborclin, Pinhais, PR, Brazil). A total of 100 cells were counted using an optical microscope (100 \times). Results are presented as the number of neutrophils per cavity.³⁰

Leukocyte Rolling and Adhesion in the Mesenteric Microcirculation. The evaluation of leukocyte rolling in the mesenteric microcirculation was determined 2 h after ip stimulation with carrageenan (500 μ g/cavity), while adhesion was verified 4 h after stimulation. For visualization of leukocytes, the animals were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine, and their mesentery was exteriorized for observation of the microcirculation (intravital microscope DM 6000, Leica Microsystems, Wetzlar, Germany). The rolling of leukocytes was analyzed in three different fields per animal over a period of 10 min. For adhesion, the number of leukocytes adhering to the endothelium was evaluated over a venular area of 100 μ m² (10 μ m in the tissue corresponds to 3.4 cm on the monitor screen).^{31,32}

Migration of Neutrophils in the Joint Cavity. Mice were immunized sc with an emulsion containing 100 μ L of Complete

Freud's Adjuvant, 100 μ L of 1 \times PBS, and 500 μ g of mBSA. The reinforcement (7 and 14 days after the first injection) was administered with Incomplete Freund's Adjuvant emulsion and mBSA. On day 21, the challenge was administered ia with mBSA (30 μ g/joint). Six hours later the animals were euthanized, their articular cavity was washed with PBS containing EDTA, and total and differential leukocyte counts were performed as previously described.³³

Myeloperoxidase Activity in the Joint Tissue. The myeloperoxidase activity in vivo was assessed 6 h after stimulation with mBSA (30 μ g/joint). Mice were anesthetized and received ip administration of the solution (100 mg/kg) used to determine the activity of myeloperoxidase by bioluminescence (XenoLight Rediject Inflammation Probe, PerkinElmer, Inc., Waltham, MA, USA). Image acquisition was performed using the IVIS Spectrum System device (PerkinElmer, Inc., Waltham, MA, USA). The results were expressed as bioluminescence intensity (radiance p/sec/cm²/sr).³⁴

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 (2×10^5 cell/well) at 37 $^{\circ}$ C, 5% CO₂ overnight. Four hours after stimulation with PG (5 μ g/mL) or LPS (10 ng/mL) the supernatant was collected and stored at –70 $^{\circ}$ C.

Enzyme Linked Immunosorbent Assay (ELISA). The levels of cytokines and chemokines in the peritoneal cavity of the animals were determined 1.5 h (TNF- α) and 3 h (CXCL2/MIP-2) after ip stimulation with carrageenan at 500 μ g/cavity. The levels of TNF- α (1.5 h) were determined after challenge with mBSA at 30 μ g/joint. In the supernatant of RAW 264.7 macrophages, the levels of TNF- α or CXCL2/MIP-2 were determined 4 h after stimulation with PG (5 μ g/mL) or LPS (10 ng/mL). ELISA was performed using protocols supplied by the manufacturers (R&D Systems, Minneapolis, MN, USA). Results are expressed as pg/mL or pg/mg tissue.

Viability Assay by Flow Cytometry (Annexin V and Propidium Iodide (PI)). RAW 264.7 macrophages were grown in six-well plates (2×10^6 cells/well) at 37 $^{\circ}$ C, 5% CO₂ overnight. After 4 h of incubation with CNM at 6 μ M, the cells were washed and resuspended in annexin buffer. Anti-annexin V-FITC antibody (1:50) was added and incubated at 4 $^{\circ}$ C for 20 min. Then the anti-PI antibody (1:100) was added, and the analysis carried out in a FACSVerse (BD Biosciences, San Diego, CA, USA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).³⁵ Three to four separate experiments were performed for analysis (annexin and PI), and approximately 10 000 gated events were collected in each analysis.

Cell Viability Assay by MTT. RAW 264.7 macrophages were cultured in 96-well plates (2×10^5 cells/well) at 37 $^{\circ}$ C, 5% CO₂ overnight. After 4 h of incubation with CNM at 6 μ M, the supernatant was removed, RPMI with MTT (1 mg/mL) was added to the plate, and the plate was incubated for 2 h. Then the supernatant was removed again, and the cells were resuspended in 200 μ L of absolute ethanol. The absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western Blotting. RAW 264.7 macrophages were grown in six-well plates (2×10^6 cell/well) at 37 $^{\circ}$ C, 5% CO₂ and incubated overnight. After 15 min of PG stimulation (previously standardized, as shown in Figures S1 and S2), the cells were lysed with RIPA-containing protease inhibitor (1:25) and phosphatase (1:50). The protein quantification was performed using Bradford reagent (Sigma-Aldrich). Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA) and gel with 10% polyacrylamide were 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-I κ B α (1:500), and Beta-actin (1:5000) were added. After overnight incubation at 4 $^{\circ}$ C, anti-mouse or anti-rabbit secondary antibodies (1:10 000) were added and incubated for 1 h. The membranes were washed, and the substrate Luminata (Millipore, Billerica, MA, USA) was added for chemiluminescence revelation using a ChemiDoc XRS apparatus (Bio-Rad) with the software Image Lab 3.0.

Immunofluorescence. RAW 264.7 macrophages were cultivated on circular coverslips in 24-well plates (2×10^5 cell/well) at 37 °C, 5% CO₂ and incubated overnight. After 30 min of PG stimulation, the cells were fixed with 4% paraformaldehyde for 20 min and washed with 1× PBS containing 10 mM glycine. The permeabilization was carried out with Triton X-100 for 30 min, and the blocking with 1% bovine serum albumin (BSA) for 1 h. Anti-p65 primary antibody 1:100 (Santa Cruz Biotechnology, Dallas, TX, USA) 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, Eugene, OR, USA) was incubated for 1 h. Hoechst 33342 (Sigma-Aldrich) at 1 µg/mL was used as a nucleus marker. The coverslips were fixed on slides with Fluormount (SouthernBiotech), and the images were acquired in a TCS SP5 Leica microscope (Leica Microsystems, Wetzlar, Germany).

NF-κB Activation Assay. RAW 264.7 macrophages stably transfected with the NF-κB-pLUC gene to express luciferase by the transcription factor NF-κB were cultured in 24-well plates (3×10^5 cells/well) and incubated overnight. The evaluation of NF-κB activation was performed 4 h after challenge with PG (5 µg/mL). The cells were lysed with 50 µL of Tris-NaCl-Tween buffer, and an aliquot of the suspension was added along with 25 µL of the Luciferase Assay Reagent containing luciferin (Promega Corporation, Madison, WI, USA). A microplate reader (FlexStation 3 Multi-Mode microplate reader, Molecular Devices) was used to quantify the luminescence.^{36,37}

Statistical Analysis. Statistical analysis was done using GraphPad Prism software version 5.03 (San Diego, CA, USA). 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 indicated when *p* < 0.05.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00263.

Additional information (PDF)

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Notes

The authors declare no competing financial interest.

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