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Anti-inflammatory effects of Piper mollicomum Kunth inflorescence extract and isolated compounds

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Abstract

The anti-inflammatory activity of *Piper mollicomum* Kunth, traditionally used for various ailments, was analysed by examining its ethanol inflorescence extract and isolated compounds. The extract was analyzed for its composition, yielding three compounds (C1, C2, and C3) identified by NMR and GC-MS. *In vitro*, the effects on cytokine (TNF, IL-6, IL-1β and IL-10) and nitric oxide (NO) levels, and neutrophil chemotaxis were assessed in LPS-stimulated macrophages (RAW 264.7) macrophages and neutrophils. The efferocytosis assay was performed as one of parameters of inflammation resolution. The extract anti-inflammatory activity was also tested using a hypersensitivity model induced by LPS (100 ng/paw) allied to the analysis of TNF and IL-1β levels in inflamed tissue. Both the extract and its compounds significantly reduced pro-inflammatory cytokine and NO production, inhibited neutrophil migration, and promoted efferocytosis of apoptotic neutrophils. The extract also alleviated LPS-induced hypersensitivity in mice, without affecting post-incisional pain. *Piper mollicomum* extracts and its isolated compounds exhibit promising anti-inflammatory properties by modulating key inflammatory mediators. These findings suggest their potential for therapeutic use in managing inflammatory diseases, warranting further investigation into their mechanisms of action and clinical applications.

Introduction

Inflammation is a physiological body response to tissue damage or cell stressto restore its homeostasis. This response involves a complex process of cellular and molecular interactions, resulting in the cardinal signs of inflammation (Ciaccia 2011; Ansar and Ghosh 2016). A successful acute inflammatory process is one that occurs in a controlled manner and resolves inflammation by eliminating the harmful stimulus, followed by a phase of resolution and repair. However, when resolution fails in the presence of ongoing injury, it leads to chronic inflammation, for exemple, autoimmune diseases, metabolic disorders, and cancer, among others (Nathan and Ding, 2010; Serhan, 2017).

The inflammatory response induced by microorganisms starts with the activation of pattern recognition receptors on cell surfaces, especially resident macrophages and dendritic cells. Then, inflammatory pathways lead the production and release of chemical mediators from plasma and resident cells in the injured or infected tissue, as well as cellular recruitment(Fullerton and Gilroy, 2016). Different cell types are activated in this process and are responsible for the release of important inflammatory mediators, such as cytokines, chemokines, anaflotoxins, among others. Macrophages play significant role in the inflammatory response by inducing acute phase responses, endothelial activation, and leukocyte recruitment. These inflammatory mediators also contribute to local reactions, such as increased permeability, leukocyte activation, chemokine production, and systemic inflammation reactions such as fever, leukocytosis, and acute phase protein production(Delgado-Rizo et al. 2017; Medzhitov, 2008) .

Studies have highlighted the importance of natural medicines for prevention, treatment, and health promotion due to their rich source of biologically active compounds. Research focusing on new

treatments for managing the inflammatory process has shown significant biological activity from extracts and isolated compounds (Bashir Dar et al. 2016; Newman and Cragg, 2020; Nunes et al. 2019).

Species belonging to the Piperaceae family have been distinguished for their promising bioactive compounds. Piperaceae is among the largest plant families among basal angiosperms, distributed across tropical and subtropical regions, comprising approximately 3000 species divided into four genera: Zippelia, Manekia, Piper, and Peperomia (Santos et al. 2012). The genus Piper has great importance within the family, comprising the largest number of species. Known for their rapid growth as shrubs, they are utilized in traditional medicine for treating various ailments due to their diverse secondary metabolites, acting as antiseptics, insecticides, antimicrobials, antifungals, and anti-inflammatories(Guerrini et al. 2009; Lago et al. 2005; Parmar et al. 1997).

Piper mollicomum Kunth, known as pariparoba, jaguarandi, jaborandi, or jaborandi-manso, is a shrub found in Brazil and some Latin American countries, commonly found in moist, sunny, and shaded areas. This species is used in folk medicine to treat stomach disorders and gonorrhea, toothaches, and is also applied as a hair tonic Despite its popular medicinal use, pharmacological data on this species are still limited. In the phytochemical analysis studies carried out by Lago and colaborators (2007) using methanol extract of *P. mollicomum* leaves isolated and identified two chromenes methyl, 2,2-dimethyl-*2H*-chromene-6-carboxylate and methyl 8-hydroxy-2,2-dimethyl-*2H*-chromene-6-carboxylate, and the dihydrochalcone 2',6'-dihydroxy-4'-methoxychalcone. Silva and collaborators (2018) have isolated from dichloromethane extract of leaves, stem and roots piperamides tembamide, tembamide acetate and riparin.

Additionally, essential oils have been found to contain compounds such as arylpropanoids myristicin, α-phellandrene, linalool, β-phellandrene, dillapiole, apiol, eupatoriocromene, and E-nerolidol (Ramos et al. 2020).

There are reports of antifungal activity from compounds isolated from leaf extracts of *P. mollicomum* (Da Silva et al. 2018), and antimicrobial activity from essential oil, leaf extracts (Cordova et al. 2010; Da et al. 2016) and stem extracts of *P. mollicomum* (Da et al. 2016).

Given the widespread use of this plant and existing literature on the pharmacological potential of metabolites isolated from other Piper species, this study aimed to explore the anti-inflammatory activity several extracts obtained from the *P. mollicomum* inflorescence and the isolated compounds.

Material and Methods

Plant Material

P. mollicomum Kunth was obtained in Blumenau, Santa Catarina, Brazil (latitude: 26°97'69.66"S, longitude: 49°06'24.31"W, altitude: 200 m) in September 2017. Prof. Andre Luis de Gasper identified the vegetal material (curator of the Herbarium Dr. Roberto Miguel Klein at the Universidade Regional de

Blumenau). Specimen samples are deposited in this Herbarium (# 41603) and registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN, # a5d9853).

Plant extraction

Inflorescences of *P. mollicomum* were dried at a constant temperature with air circulation for 7 days. Subsequently, the plant material was pulverized and mixed with ethanol 95 °GL at a plant-to-solvent ratio of 1:20 (w/v) under dynamic maceration at room temperature for 4 hours using a magnetic stirrer (Marte model ASF8). The extract was then concentrated using a rotary evaporator with a maximum temperature of 50°C and left at room temperature until the solvent was completely evaporated.

Column chromatography (CC): Approximately 250 grams of Silica gel 60 (0.063-0.2 mm / 70-230-400 mesh ASTM), of the brand Macherey-Nagel, packed with hexane, were used in a column of four centimeters in diameter. The inflorescences extract (10 g) was solubilized in acetone, added silica until formation of tablet and then added to the column to be subjected to chromatography process. The initial mobile phase was hexane. The polarity was gradually increased with the addition of ethyl acetate (95: 5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, 50:50, 40:60, 20:80, 0: 100). Subsequently, ethyl acetate with methanol was added, with a gradual increase in polarity. In the CC procedure, 80 fractions of 100 mL each were obtained and evaluated, and then pooled according to TLC analysis.

Gas Chromatography – Mass Spectrometry (GC-MS)

The fractions obtained from the column carried out with the extract of the inflorescences were solubilized in dichloromethane (20 mg/mL) and subjected to analysis by GC-MS (Shimadzu®, QP2010 S). An Rtx-1 capillary column, 100% dimethyl polysiloxane (30 m x 0.25 mm x 0.10 µm) was used for the procedure. The injection mode used was splitless, with an injection temperature of 300°C. The initial column temperature was 80°C for 1 minute, then heating rate was 5°C/min to 120°C, 20°C/min to 300°C and finally the heating speed was 25°C/min up to 310°C remaining for 10 minutes. Helium gas was used as a carrier, maintaining a constant flow rate of 0.9 mL/min, with an injection volume of 10 µL. The mass detector was used in scan mode, with m/z ratio from 29 to 500 g/mol, EI interface at 70 eV. After obtaining the chromatogram, the analysis of the percentage area of the peaks was carried out, as well as the mass spectra through comparison with those existing in the NIST-8.0 library and/or by comparison with literature data. These conditions were previously established in our laboratories.

^{1}H (300 MHz) and ^{13}C (75.5 MHz) nuclear magnetic resonance (NMR) spectra were obtained on a Varian Gemini AC 300 Spectrometer with $CDCl_3$ as solvent and tetramethyl silane (TMS) as internal standard (purity 99.8% + 0.05% TMS) of Cambridge Isotope Laboratories Inc. Chemical shifts have been recorded in dimensionless values (ppm) indicating the sign as singlet (s), doublet (d), triplet (t).

Thin layer chromatography (TLC) analysis - Fractions obtained from column were solubilized in acetone, applied to the chromatoplates (silica gel 60 F234 (230–400 mesh) and then eluted in a hexane: ethyl acetate mobile phase in different concentrations.

Animals

Male Swiss mice (20-30g) were used for some experiments, and they were raised in the Central Animal House of Universidade do Vale do Itajaí (UNIVALI). The animals were housed in cages (n = 6) with individual filtered air flow, with enriched environment with controlled temperature (22 ± 1ºC), humidity (40–60%), and a cycle of 12-hour light and 12-hour dark. They were provided with food and filtered water *ad libitum*. They stayed at a local vivarium for 5 days prior to the testes for acclimatization. For the neutrophil collection and *in vivo* experiment, the animals were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.) to minimize suffering. All procedures were performed following the guidelines of the Brazilian Society of Laboratory Animal Science. The study was approved by the local ethics committee (CEUA/UNIVALI # 022/22–02/03/2022).

Macrophages cell culture and viability

Murine macrophages (RAW 264.7 cells; Cell Bank of Rio de Janeiro, Brazil) were cultured in DMEM high-glucose (10% FBS, 100 µg/L streptomycin, 100 IU/mL penicillin) and kept at 37°C in a 5% CO_2 atmosphere. The cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, as described by Denizot et al. (1986). In brief, RAW 264.7 cells were plated in 96-well plates (5×10^5 cells/well) and ater 24 h the cells were treated with *P. mollicomum* (1, 10, or 100 µg/mL; diluted in the assay culture medium with a final concentration of 0.05% DMSO for all *in vitro* assays) and DMSO was used as a positive control for cell death at a final concentration of 10%. Optic density was measured at 560 nm.

LPS-stimulated macrophages

The macrophages (RAW 264.7 cells; 1×10^6 cells per well) were incubated with LPS (5 µg/mL) or vehicle. *P. mollicomum* extract (1, 10, or 100 µg/mL) or isolated compounds (0.1, 1, or 10 µM) were added immediately after the LPS treatment and then incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. The culture supernatant was used for mediators analysis.

Neutrophil Isolation

For the neutrophils isolation, male Swiss mice received an intraperitoneal (i.p.) injection of 3 mL of PBS solution containing 1% sterile oyster glycogen and the exudate was collected 4 h later under anesthesia, as described by Benvenutti et al. (2021). The cell viability (98%) was assessed using the Trypan blue exclusion test.

In vitro neutrophil chemotaxis

This method of chemotaxis using solidified agarose gel was previously described by Nelson and Quie (1975). Three wells were performed in the gel, one in the centre of the plate and two other wells localized in the right and left side of the center well, with a distance of 2.5 mm from the centre. The neutrophils (1×10^7 cells/mL) obtained from the mouse pritonium previously treated with the vehicle, the *P. mollicomum* extract (100 µg/mL for 1 hour) or isolated compounds (0.1 µM for 1 hour). In the central perforation, 10 µL of chemotactic factor (fMLP/0.1 µM) was added. The plates were incubated at 37°C with 5% CO_2 for 4 hours. After incubation, neutrophils were counted under a microscope. Quantification was performed from the margin of the peripheral perforations toward the chemotactic agent (central perforation). The experiment was performed in quadruplicate, and the results were expressed as the number of neutrophils per field. As a positive control for cell migration, neutrophils not treated with the extract or compounds were used in the peripheral perforations, and chemotactic factor (fMLP/0.1 µM) in the central perforation.

Efferocytosis Assay

Senescent neutrophils were previously obtained from neutrophil collection using the oyster glycogen method and cultured for 20 hours at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with 2% FBS to induce apoptosis. Murine macrophages previously isolated from the mouse medullary cavity were seeded (5×10^6 cells/well) onto a round glass coverslip placed inside the 24-well plate and incubated for 2 h at 37°C with 5% CO_2 for macrophage adherence. Then the wells were washed three times with sterile PBS to remove non-adherent cells. The cells were treated with *P. mollicomum* extract (100 µg/mL) or isolated compounds (0.1 µM) diluted in DMEM, and 1 hour later, the apoptotic neutrophils (1×10^6 cells) were added to each well for 20 minutes to allow efferocytosis. The coverslips were washed once with PBS to remove excess senescent neutrophils and stained with modified May Grumwald-Giemsa staining solution. The dried coverslips were then inverted and glued to glass slides with Canada balsam. One hundred macrophages were observed, and the phagocytosis of senescent neutrophils was expressed as a percentage. The supernatant was collected for cytokine analysis (IL-10 and TNF) and the coverslips were stained with Giemsa and analyzed under a microscope with 1000x of magnification (Broering et al. 2019).

Cytokine and Nitrite Analysis

The cytokine levels were assessed in the supernatants of the cell cultures according to the manufacturer's guidelines (DuoSet R&D Systems – Minneapolis, MN, USA). Nitric oxide (NO) was indirectly measured by quantifying its metabolite, nitrite (NO_2^-), using the Griess reaction method (Giustarini et al. 2008).

LPS-induced hypersensitivity

The inflammatory hypersensitivity was induced injecting 10 µL of LPS (100 ng/paw) into the right hindpaw plantar surface 1 h after the respective treatments. Mice were divided into 4 goups: control group that received vehicle as treatment (saline 0.9%, 10 mL/kg p.o) and *P. mollicomum* groups treated with three different doses of the extract (10, 30 or 100 mg/kg, p.o.). Before the experiments, the mice were assessed for their baseline mechanical withdrawal threshold using von Frey filaments. Following this, measurements were taken at 1, 2, 4, 6, and 24 h after the LPS injection (Calil et al. 2014). The mice were then euthanized at the end of the experiments.

Post incisional pain model

Mice were previously anaesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and the asepsis of their right hindpaw were performed with iodinated alcohol. A longitudinal incision of 5 mm was performed using a n.11 scalpel blade reaching the skin and plantar fascia, following the method described by (Pogatzki and Raja 2003). The skin was then sutured with a nylon suture 5.0. Mice received the treatment with the *P. mollicomum* extract (30 mg/kg, p.o.), twice per day, and the mechanical hypersensitivity was assessed using the von Frey filaments 1, 2, 4, 6 and 24 h after the first treatment. As previously described, the mechanical withdrawal threshold of the animals was obtained before the surgery, representing the basal measurement.

Mechanical sensitivity evaluation

The mice mechanical sensitivity was assesseded using the von Frey filaments, as described by(Cobos et al. 2012). The animals were individually placed into Plexiglas compartments (9 X 7 X 11 cm) on raised wire platforms. The plantar surface of the right hindpaw was stimulated 10 times with the von Frey 0.6 g (VFH, Stoelting, Chicago, USA). After that, animals that presented withdrawal response frequency less than 50% were stimulated again with higher strength filaments, to achieve withdrawal threshold response frequency 50% or higher. Animals with or more then 50% of hindpaw withdrawal response with the filament 0.6 g were then stimulated with filaments of lower weight (0.07, 0.16 e 0.4 g) until obtain at least 50% of withdrawal response.

Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The percentages of inhibition were individually calculated and expressed as mean ± SEM, with baseline values taken into account. Statistical comparisons between groups were performed using Student's t-test, one-way analysis of variance (ANOVA) followed by Tukey’s or Dunnett’s post-hoc test, or two-way ANOVA followed by Bonferroni’s post-hoc test, as appropriate. A p-value of < 0.05 was considered significant.

Results

Phytochemical profile

The inflorescence extract of *P. mollicomum* (EEBI) was obtained by static maceration with ethanol, yielding 9.42%. Conventional chromatographic procedures from the extract resulted in the isolation of three entities identified as 8-hydroxy-2,2-dimethyl-2H-1-chromene-6-carboxylate **(C1)**, 2', 6'-dihydroxy-4'-methoxy dihydrochalcone **(C2)**, and 2', 4-dihydroxy-4', 6'-dimethoxy-dihydrochalcone **(C3)**. The structures were elucidated based on spectrometric evidence using ^{1}H and ^{13}C NMR and GC-MS.

Compound **C1** was obtained from fraction 20–22 (0.202 g) of the column. It was eluted with hexane: ethyl acetate (85:15, v/v), as a brown solid soluble in acetone and identified as 8-hydroxy-2,2-dimethyl-2H-1-chromene-6-carboxylate.

^{1}H NMR ($CDCl_3$ 300 MHz) δ ppm (m; *J* Hz) 7.48 (d; 1.8; H-7), 7.32 (d; 1.8; H-5), 6.35 (d; 10; H-4), 5.66 (d; 10; H3), 3.87 (s, $COOCH_3$), 1,48 (s, H-9/H-10). **^{13}C NMR** ($CDCl_3$ 75.5 MHz) δ ppm 166.8 (C = O), 144.1 (C-8), 143.5 (C-6), 130.8 (C-5), 122.7 (C-8a), 121.7 (C-3), 120.5 (C-4a), 119.9 (C-7), 116.3 (C-4), 78.4 (C-2), 51.9 ($COOCH_3$), 28.3 (C-9/C-10). **CG/MS** m/z 234 (M^+, 18%), 219 (100%). Data NMR and MS are according to the literature (Da Silva et al. 2018; Orjala et al. 1994).

Compound **C2** was obtained from fraction 34–37 (0.418 g). It was eluted with hexane: ethyl acetate (85:15, v/v), as a dark green solid soluble in acetone and identified as 2', 6'-dihydroxy-4'-methoxy dihydrochalcone.

^{1}H NMR ($CDCl_3$ 300 MHz) δ ppm (m; *J* Hz) 14.04 (s; OH), 7.26 (m; H-2/H-6; H-5/H-3; H-4), 6.02 (s; H-3'), 5.98 (s; H-5'), 3.30 (m; H-α), 2.98 (m; H-β). **^{13}C NMR** (CDCl3 75.5 MHz) δ ppm 204.7 (C = O); 167.6 (C-4'), 163.9 (C-2'/C-6'), 153.9 (C-4); 133.6 (C-1), 129.5 (C-2/C-6), 115.2 (C-3/C-5), 105.7 (C-1'); 92.2 (C-3'/C-5'); 55.5 (OCH_3); 46.0 (C-α); 29.8 (C-β). **CG/MS** m/z 302 (M^+, 21%); 181 (100%); 154 (54%); 120 (25%). Data NMR and MS are according to the literature (Hermoso et al. 2003).

Compound **C3** was obtained from fraction 28–29 (0.086g). It was eluted with hexane: ethyl acetate (80:20, v/v), presented as a green solid soluble in acetone and identified as 2', 4-dihydroxy-4', 6'-dimethoxy-dihydrochalcone.

^{1}H NMR ($CDCl_3$ 300 MHz) δ ppm (m; *J* Hz) 14.07 (s; OH), 7.09 (d; 8.2; H-2/H-6), 6.77 (d; 8.2; H-5/H-3), 6.01 (s; H-3'), 5.96 (s; H-5'), 3.80 (s, OCH_3), 3.27 (t; 5.7; H-α), 2.91 (t; 5.97; H-β). **^{13}C NMR** ($CDCl_3$ 75,5 MHz) δ ppm 204.5 (C = O), 167.1 (C-4'), 163.4 (C-2'/C-6'), 141.7 (C-1),128.8 (C-3/C-5), 128.4 (C-2/C-6), 125.9 (C-4), 105.5 (C-1'), 96.5 (C-3'/C-5'), 55.6 (OCH_3), 45.6 (C-α), 29.0 (C-β). **CG/MS** m/z 272 (M^+, 20%); 167 (100%); 140 (30%); 91 (10%). Data NMR and MS are according to the literature (Da Silva et al. 2018; Hermoso et al. 2003). RMN and CG/MS data are available in the supplementary material.

P. mollicomum extract and the isolated compounds reduced the secretion of inflammatory mediators by macrophages

To assess the effects of the extract on the production of inflammatory mediators (TNF, IL-6, IL-1β, and NO_2^- levels), the supernatant from the macrophage cultures was analyzed. Data depicted in Fig. 1 demonstrate that LPS enhanced the inflammatory-mediators lvels in the supernatant of macrophages. Treatment with *P. mollicomum* extract (1, 10, or 100 µg/mL) significantly reduced NO_2^- production by macrophages, with the 100 µg/mL group showing an inhibition of 82.43 ± 3.74% compared to the LPS-stimulated macrophages (Fig. 1). Additionally, treatment with *P. mollicomum* at 100 µg/mL significantly reduced TNF (41.38 ± 1.59%), IL-6 (81.06 ± 3.38%), and IL-1β (36.06 ± 4.5%) levels compared to LPS-stimulated macrophages (Fig. 1C-E). Furthermore, the extract did not affect macrophage viability, as shown in Fig. 1A.

The isolated compounds C1, C2, and C3 significantly reduced NO_2^- production in macrophages at all evaluated concentrations (0.1, 1 and 10 µM) (Fig. 3). C1 exhibited an IC_{50} of 0.31 µM (0.03–3.06), while C3 showed an IC_{50} of 3.77 µM (2.88–4.94). For C2, it was not possible to determine the IC_{50}. The IC_{50} values were calculated based on the effects observed in the NO_2^- evaluation. Notably, even at the lowest concentration (0.1 µM), a significant reduction in TNF, IL-6, and IL-1β levels was observed in macrophages stimulated with LPS (Fig. 3A-C). None of the compounds affected the cell viability (Fig. 2).

P. mollicomum extract and isolated compounds increase the efferocytosis

P. mollicomum extract (100 µg/mL) showed a slight enhancement in the efferocytosis activity, as demonstrated in Fig. 4A. Furthermore, the extract did not reduce TNF (Fig. 4B) or IL-10 (Fig. 4C) levels in the supernatant samples. On the other hand, all isolated compounds increased the efferocytosis process (Fig. 5A). C2 and C3 demonstrated the ability of reducing TNF levels (Fig. 5B), while all compounds increased IL-10 levels in the supernatant samples, as presented in Fig. 5C.

P. mollicomum extract and isolated compounds modulates neutrophil migration

Figure 6 illustrates the effects of *P. mollicomum* extract on neutrophil migration, reducing the chemotaxis, with a migration inhibition rate of 27.16 ± 1.84% at a concentration of 100 µg/mL (Fig. 6A), when compared to the control group (treated with vehicle). Likewise, compounds C1, C2, and C3 also decreased neutrophil chemotaxis, with inhibition rates of 32.16 ± 3.13%, 34.69 ± 1.92%, and 27.46 ± 1.88%, respectively (Fig. 6B).

P. mollicomum extract reduces the inflammatory hypersensitivity in mice

Figure 7 presents the results obtained with subcutaneous LPS injection into the plantar surface of mouse´s the right hindpaw. As observed, control group that was injected with LPS and received vehicle as treatment had their mechanical withdrawal thresholds reduced significantly when compared with their basal withdrawal threshold. Mice pre-treated with the *P. mollicomum* extract had the mechanical hypersensitivity significantly reduced for up to 6 h after the LPS injection. This result could be seen qualitatively with the percentage of response frequency (Fig. 7A) and quantitatively with the mechanical withdrawal threshold (Fig. 7B). Furthermore, the extract was able to reduce the increase in the cytokines

TNF (Fig. 7C) and IL-1β (Fig. 7D) levels in the spinal cord of mice injected with LPS. However, when mice with post incisional pain was evaluated, no interference in the mechanical hypersensitivity could be seen for the group treated with the *P. mollicomum* extract (Fig. 7E and F).

Discussion

This study explores the anti-inflammatory potential of *P. mollicomum* inflorescence extract and its isolated entities by assessing their influence on several steps of the inflammatory process. While inflammation is essential for tissue repair and homeostasis, its dysregulation can result in chronic inflammation and tissue damage (Pacienza et al. 2019). The inflammatory process involves several components, including afferent fibres activation, neutrophils and monocytes migration and activation, the production of chemical mediators, and, eventually, its resolution, all of which are tightly controlled by cellular and molecular mechanisms (Ramos and Oehler, 2024). Our findings demonstrate that *P. mollicomum* inflorescence extract and its isolated compounds can modulate these processes, offering valuable insights into their anti-inflammatory properties.

In fact, the obtained data underscore the significant anti-inflammatory effects of extracts and isolated entities obtained from the *P. mollicomum* inflorescences. The *in vitro* treatment with the extract and the isolated compounds C1, C2, and C3 demonstrated notable effects on LPS-stimulated macrophages in lowest concentration (0.1 μM), reducing the production of key inflammatory mediators. This provides strong evidence for the therapeutic promise of these natural products in managing inflammation.

Compounds C1, C2, and C3 significantly reduced NO_2^- production in macrophages at all evaluated concentrations (0.1, 1, and 10 μM), without affecting cell viability. Compound C1, identified as 8-hydroxy-2,2-dimethyl-2H-1-chromen-6-methyl carboxylate, exhibited the strongest effect, with an IC_{50} of 0.31 μM (0.03–3.06), aligning with reports of chromenes such as Sargachromanol D (Heo et al. 2014) and reinforcing the potential of this scaffold as an anti-inflammatory agent. Among the dihydrochalcones, compound C3 (2',4'-dihydroxy-4',6'-dimethoxy-dihydrochalcone) showed moderate potency, with an IC_{50} of 3.77 μM (2.88–4.94), while for C2 (2',6'-dihydroxy-4'-methoxy-dihydrochalcone) it was not possible to determine an IC_{50}, despite its marked reduction of NO, TNF, IL-6, and IL-1β levels, even at the lowest concentration tested (0.1 μM).

The structural comparison between C2 and C3 suggests that substitution patterns on the aromatic rings critically modulate their activity. In the A-ring, C2 contains a hydroxyl group at C-2' and a methoxy group at C-4', whereas C3 bears hydroxyls at C-2' and C-4', together with additional methoxy substituents at C-4' and C-6'. These subtle differences may explain the higher potency of C2 in reducing pro-inflammatory cytokines, despite the absence of a defined IC_{50}, while C3, although less effective against cytokine release, displayed a measurable inhibitory concentration for NO. Collectively, these findings highlight a structure–activity relationship (SAR) in which the presence and relative positioning of hydroxyl and methoxy groups play a pivotal role in the anti-inflammatory effects of dihydrochalcones

The anti-inflammatory activity of chalcones and dihydrochalcones are well-documented, with numerous studies highlighting their ability to inhibit inflammatory mediators. For example, chalcones have been reported to suppress the production of nitrite and prostaglandin E2 (PGE2) in LPS-stimulated RAW 264.7, supporting our results (Mohd Faudzi et al. 2020; Salehi et al. 2021). There are reports that dihydrochalcone reduces the production of important cytokines, such as IL-6, IL-10, and TNF and promotes a significant decrease in the expression levels of cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB) (Erdem et al. 2024; He et al. 2024). Taken together, these findings reinforce the therapeutic potential of these compounds for managing inflammatory conditions.

NO is a critical pro-inflammatory mediator, primarily produced by iNOS in macrophages (Szliszka et al. 2011). The observed reduction in NO production by the compounds and extracts may be probably attributed to the inhibition of iNOS, a mechanism reported in other chalcones (Emam et al. 2021; Jiwrajka et al. 2016; Kim et al. 2007a; Mead et al. 2012). Given the harmful effects of excess NO and its role in tissue damage, the inhibition of NO production presents a valuable therapeutic strategy for managing inflammation (Chen et al. 2020; Cinelli et al. 2020).

The inflorescence extracts of *P. mollicomum* demonstrated significant anti-inflammatory activity by reducing the *in vitro* levels of NO, TNF, IL-6, and IL-1β in the supernatant of LPS-stimulated macrophages without exhibiting relevant cellular toxicity at tested concentrations. LPS activation of Toll-like receptor 4 (TRL-4) on macrophages, dendritic cells, and neutrophils triggers inflammatory pathways, primarily through NF-κB activation, leading to the production of inflammatory mediators (Naegelen et al. 2015; Taniguchi and Karin, 2018). One hypothesis is that the observed reductions may be associated with the inhibition of NF-κB, a critical transcription factor involved in cytokine production

Similar activities have been reported for other Piper genus extracts, including the methanolic extract of *P. attenuatum* (Kim et al. 2017) and *P. cubeba* extract, which were shown to suppress NO production, iNOS expression, and NF-κB activation while reducing inflammatory mediators such as TNF, IL-6, and IL-1β, supporting our results (Qomaladewi et al. 2019).

Additionally, we evaluated the chemotactic response of neutrophils to the chemotactic agent fMLP using the agarose migration technique. These results indicated that the inflorescence extract and its isolated compounds C1, C2, and C3 reduced fMLP-induced neutrophil chemotaxis, suggesting that they might affect the expression or affinity of fMLP receptors, or the intracellular signaling pathways induced by fMLP. Formyl peptides like fMLP are crucial in defense mechanisms against microbial infection and tissue damage, binding to formyl peptide receptors (FPR1) expressed in various cells, including phagocytic leukocytes (He and Ye, 2017). Upon binding, fMLP activates signaling pathways that regulate ROS expression, leukocyte adhesion, transmigration, and phagocytosis (Porro et al. 2019).

Chronic tissue damage can evolve into persistent inflammation, making the resolution of inflammation crucial for tissue recovery and homeostasis. Efferocytosis, the phagocytosis of apoptotic cells, is essential for resolving acute inflammation. This process promotes the phenotypic shift from M1 to M2 macrophages, increasing anti-inflammatory cytokine production, and inhibiting pro-inflammatory

cytokines. The clearance of apoptotic cells is essential for the maintenance of tissue homeostasis. Accordingly, defective efferocytosis underlies a growing list of chronic inflammatory diseases (Yunna et al. 2020). Our study found that the extract and compounds C1, C2, and C3 enhanced efferocytosis. Although the extract did not significantly alter TNF or IL-10 production, compounds C2 and C3 decreased TNF production, and all compounds increased IL-10 production, which is linked to an effective efferocytosis. The *P. mollicomum* inflorescence extract also presented *in vivo* activity, reducing the mechanical hypersensitivity induced by the intraplantar injection of LPS in mice. It is well known that local injection of LPS induces mechanical hypersensitivity dependent on TLR4/MyD88 signaling pathway. The recruited neutrophils and macrophages contribute to inflammatory hypersensitivity by producing and releasing numerous mediators including prostanoids, cytokines, NO, among others (Fujiwara and Kobayashi, 2005). Considering the *in vitro* results obtained with macrophages, we can suggest that the extract interference with the LPS-induced inflammation also corroborates for the reduction in pain hypersensitivity, reinforced by the results of reduced levels of TNF and IL-1β in the spinal cord tissue.

On the other hand, the extract did not interfere with the post incisional hypersensitivity in mice (Fuller et al. 2023) presented in their review article the mechanisms involved in the post incisional pain model in mice and rats and there is an important neurogenic component for the acute phase of this process, including glutamate receptors, calcium channels, and purinergic receptors. Chen and coworkers (2015) demonstrated that a TLR-4 inhibitor could reduce the post incisional pain when administrated intrathecally, by interfering with the DRG p38 and IL-1β activity. Comparing the results obtained in both models, it seems that the extract presents anti-inflammatory activity that culminate in the anti-hypersensitive effects observed in LPS-induced pain (Chen et al. 2015).

Conclusion

In conclusion, the inflorescence extract and isolated compounds from *P. mollicomum* demonstrate notable anti-inflammatory properties by regulating key mediators and pathways associated with inflammation. These findings underscore the therapeutic potential of these natural products for the management of inflammation and subsidy further studies to elucidate their mechanisms of action and explore potential clinical applications.

Declarations

Author's Contribution: JCPH: Writing – original draft, Methodology. LB: Writing – original draft, Methodology. PMN: Methodology. RN: Methodology. KCSF: Methodology. JSRS: Methodology. CRV: Methodology. OMSG: Methodology. LCD: Methodology. RMLS: Resources, Methodology, Writing – review & editing. AM: Resources, Methodology, supervision, Writing – review & editing. NLMQ: Resources, Methodology, supervision, Writing – review & editing. JRS: Project administration, Resources, Methodology, supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Figures

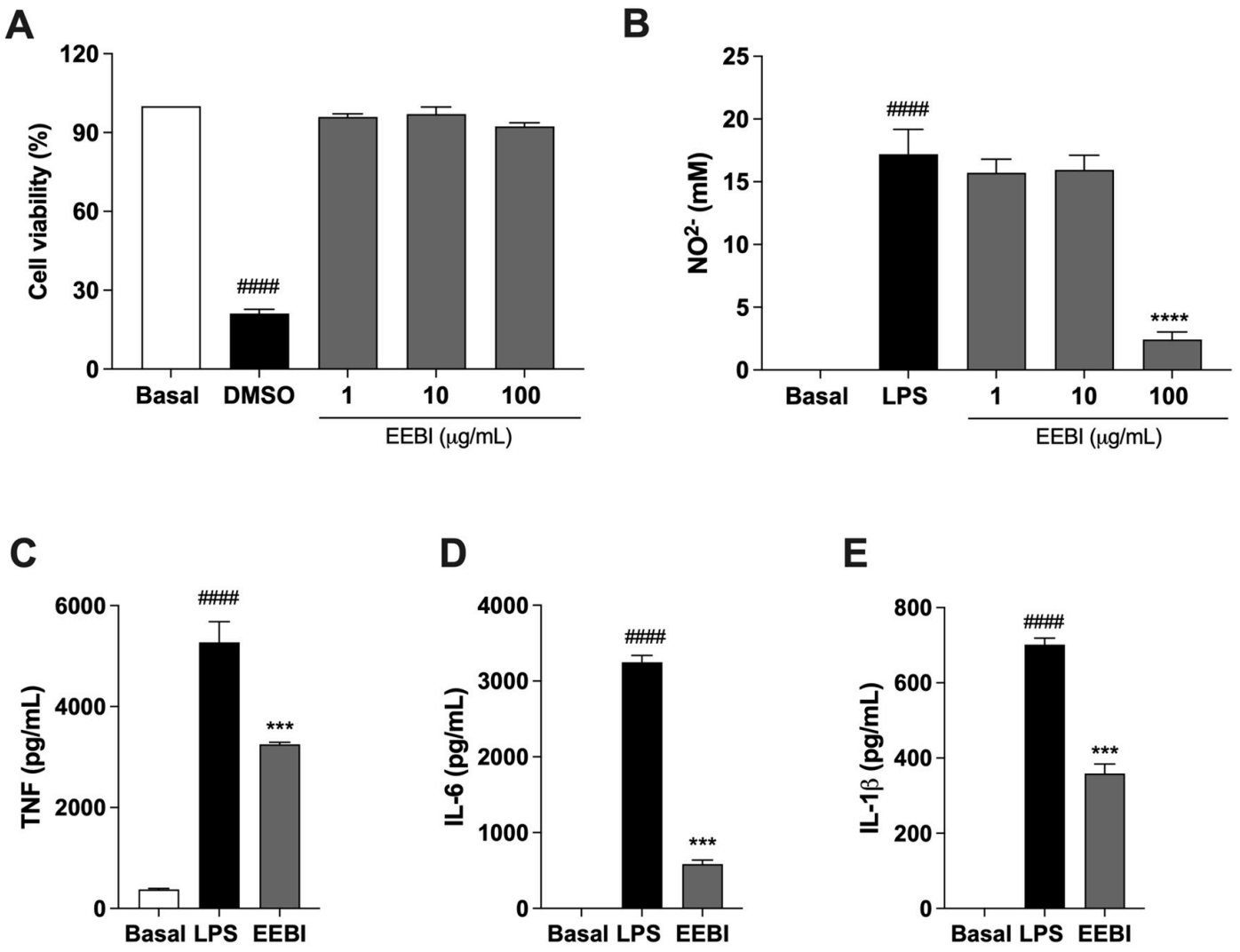


Figure 1

The murine macrophages strain RAW 264.7 was obtained from the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro, RJ, Brazil) and incubated (5×10^5 cells per well for MTT and 1×10^6 cells per well for nitrite and cytokines) in the presence or absence of LPS (5 µg/ml) together with the dry extract of *P. mollicomum* inflorescences (EEBI) (1, 10 or 100 µg/ml). Cell viability by MTT (A). Quantification of NO_2^- (B) was performed using the Griess reaction. The concentration of TNF (C), IL-6 (D), and IL-1β (E) levels were determined by the ELISA method according to the manufacturer's standards. The assays were performed in quadruplicates and values express the means ± S.E.M. where ***p<0.001 and ****p<0.0001 vs LPS group. #### p <0.0001 significantly different from the baseline group (one-way ANOVA followed by Tukey's post hoc test)

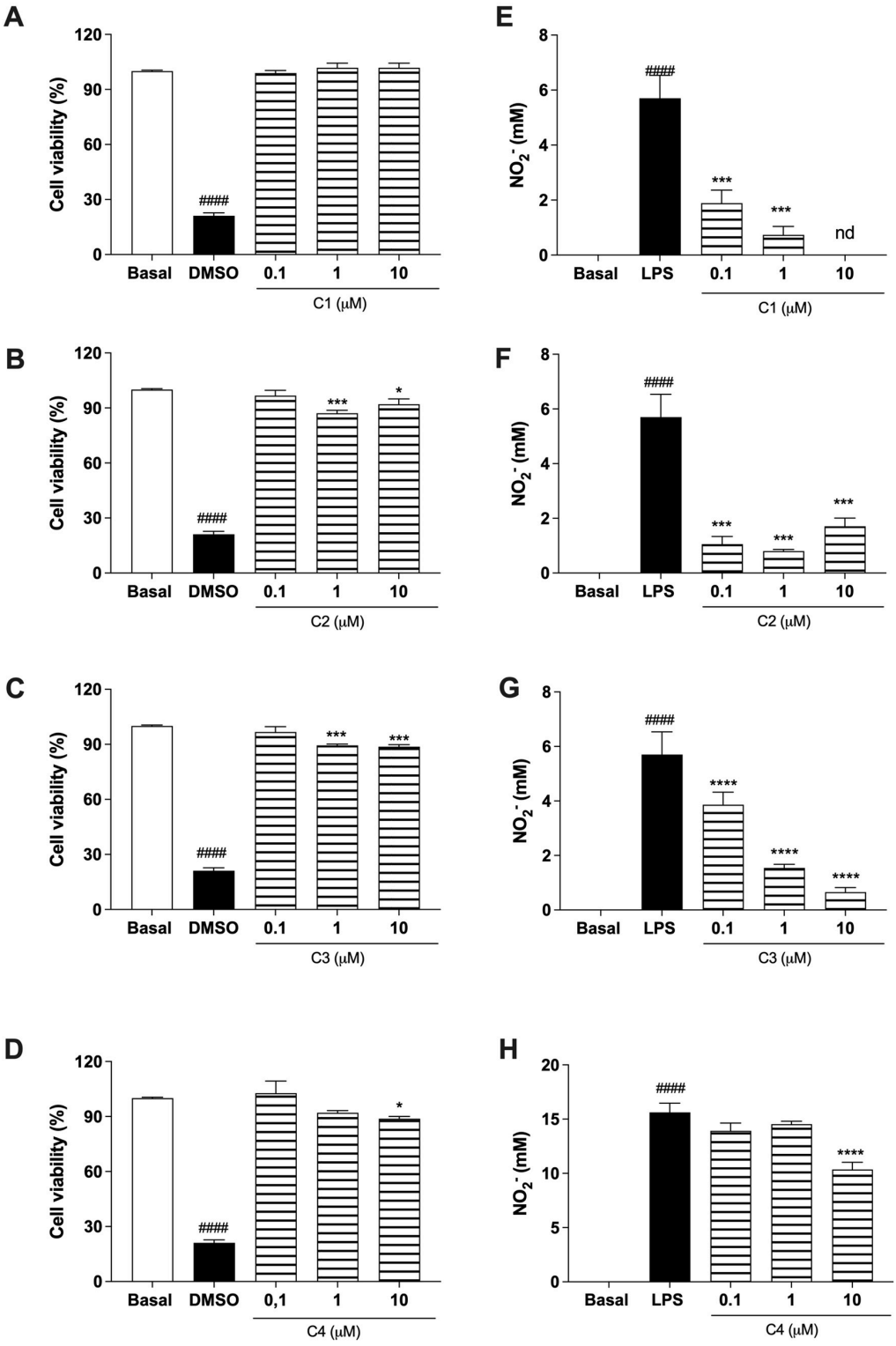


Figure 2

The murine macrophages strain RAW 264.7 was obtained from the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro, RJ, Brazil) and incubated (1×10^6 cells per well) in the presence or absence of LPS (5 μg/mL) together with C1, C2 or C3 (0.1, 1 or 10 μM). Cell viability by MTT (A, B and C). Quantification of NO_2^- (D, E and F) was performed using the Griess reaction. The assays were performed in quadruplicates and values express the means ± S.E.M. where ***p<0.001 and ****p<0.0001 vs LPS group. #### p

<0.0001 significantly different from the baseline group (one-way ANOVA followed by Tukey's post hoc test).

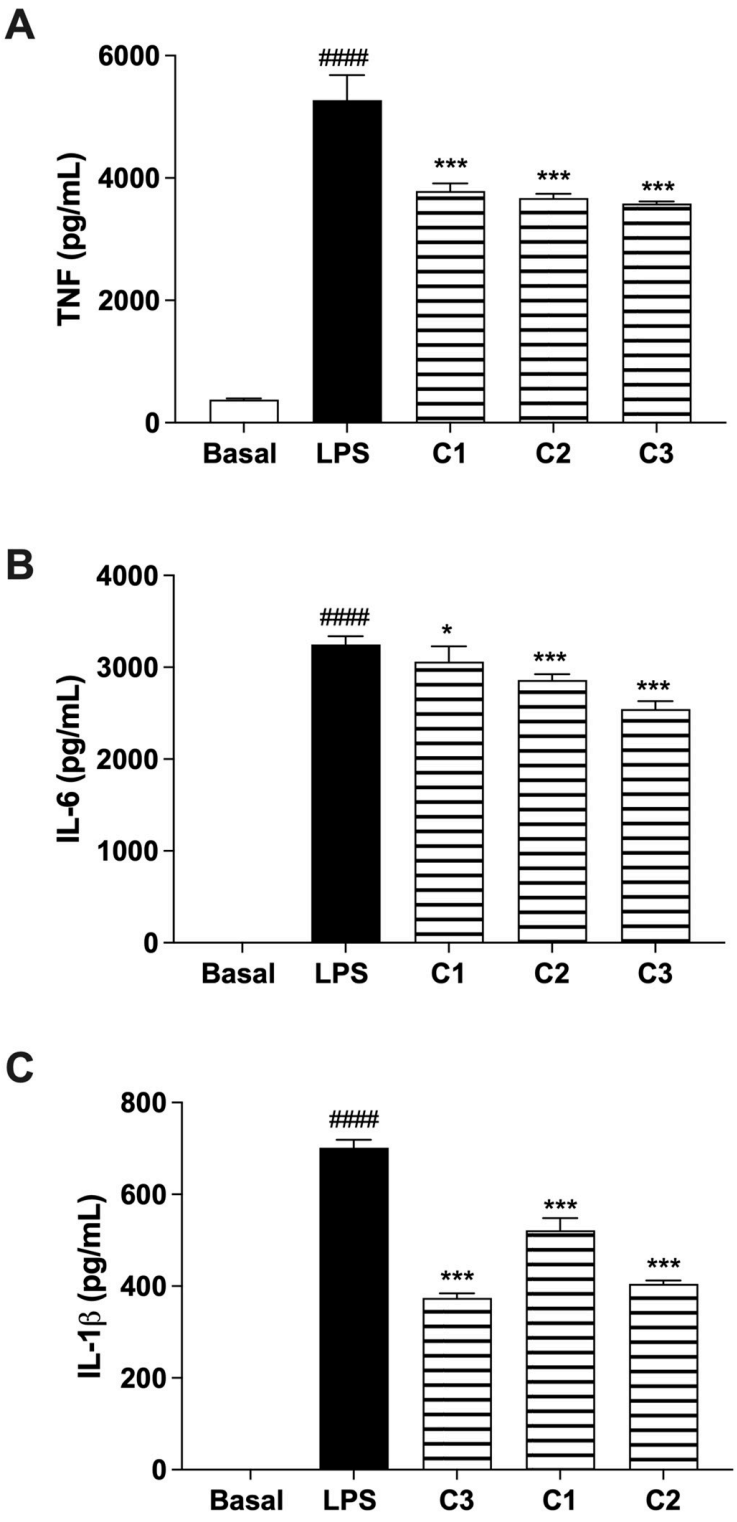


Figure 3

The murine macrophages strain RAW 264.7 was obtained from the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro, RJ, Brazil) and incubated (1×10^6 cells per well) in the presence or absence of LPS (5 µg/ml) together with C1, C2 or C3 (0.1 µM). The concentration of TNF (A), IL-6 (B), and IL-1β (C) levels

were determined by the ELISA method according to the manufacturer's standards. The assays were performed in quadruplicates and values express the means ± S.E.M. where *p<0.05 and ***p<0.001 vs LPS group. #### p <0.0001 significantly different from the baseline group (one-way ANOVA followed by Tukey's post hoc test)

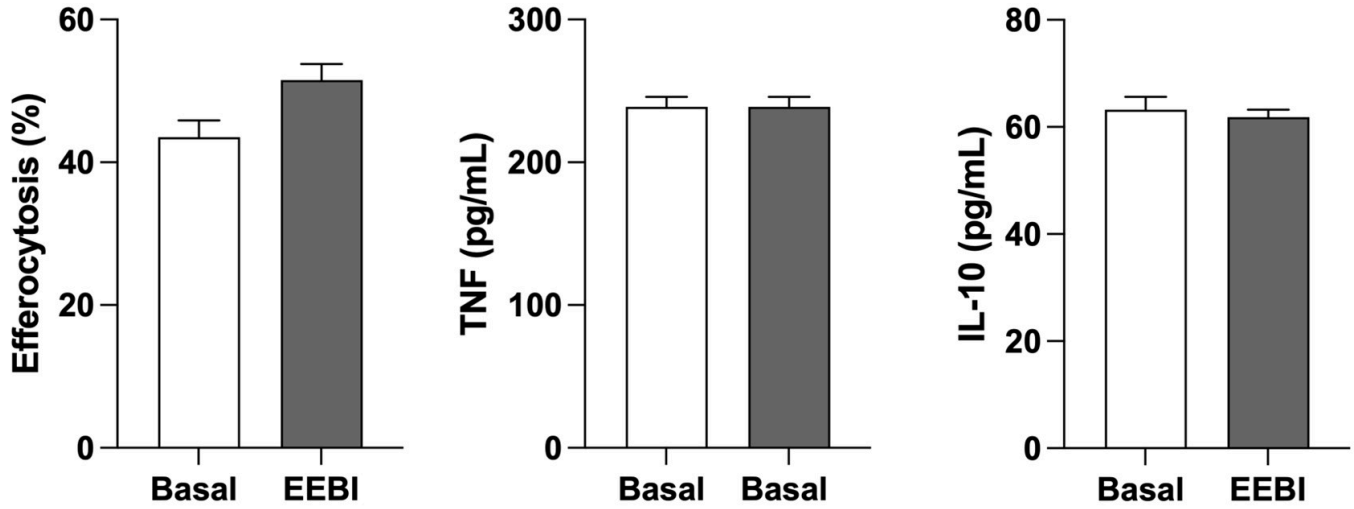


Figure 4

Neutrophils were obtained from the peritoneal cavity of Swiss mice (oyster glycogen 1%). For the *in vitro* efferocytosis assay, a cell suspension of neutrophils (1×10^6 cells per well) was incubated for 20 hours to reach the senescence state. Macrophages were obtained through bone marrow washing and placed in culture on round coverslips for adherence. Macrophages were then treated or not with *P. mollicomum* inflorescences extract (100 µg/ml) and senescent neutrophils were added. After incubation, the percentage of efferocytosis was evaluated (A) and the values express the means ± S.E.M. of tests . In the supernatant of cells TNF (B) and IL-10 (C) levels were determined by the ELISA method according to the manufacturer's standards. Assays were performed in quadruplicates and the values express the means ± S.E.M.

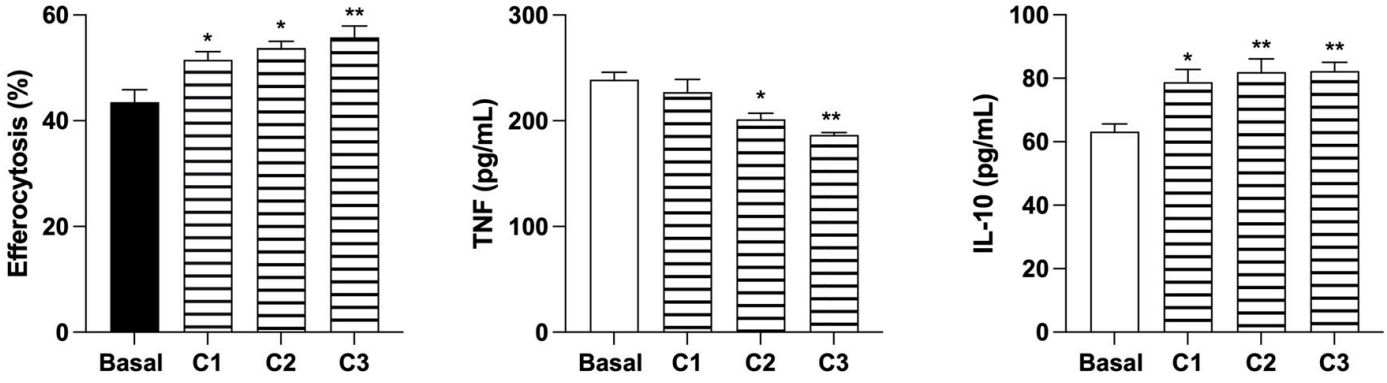


Figure 5

Neutrophils were obtained from the peritoneal cavity of Swiss mice (oyster glycogen 1%). For the *in vitro* efferocytosis assay, a cell suspension of neutrophils (1×10^6 cells per well) was incubated for 20 hours to reach the senescence state. Macrophages were obtained through bone marrow washing and placed in culture on round coverslips for adherence. Macrophages were then treated or not with C1, C2 or C3 (0.1 µM) and senescent neutrophils were added. After incubation, the percentage of efferocytosis was evaluated (A) and TNF (B) and IL-10 (C) levels were determined by the ELISA method according to the manufacturer's standards in the supernatant of treated cells. Tests were conducted in quadruplicates and the values express the means ± S.E.M. where *p<0.05 and **p<0.01 vs Basal group (one-way ANOVA followed by Tukey's post hoc test).

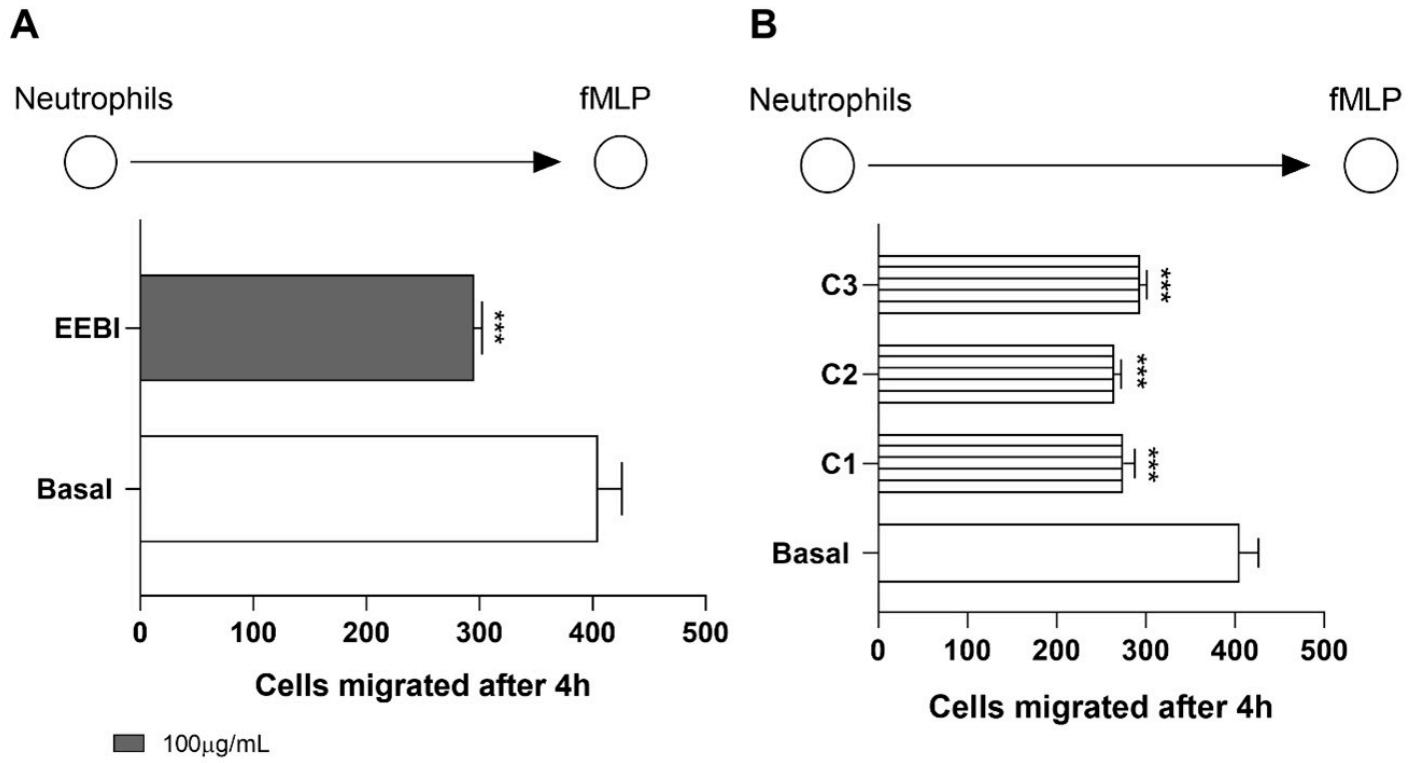


Figure 6

Neutrophils were obtained from the peritoneal cavity of Swiss mice (1% oyster glycogen). For the chemotaxis assay neutrophil cell suspension (1×10^7 cells per well) was incubated with different concentrations of *P. mollicomum* inflorescences extract (100 µg/ml) (A); C1, C2 or C3 (0.1 µM) (B). Cells were counted using inverted microscopy at 20 × magnification. Quantification was made from the margin of the peripheral perforations towards the chemotactic agent (central perforation). The results were expressed as number of neutrophils/field. Tests were performed in quadruplicates and values express the mean ± S.E.M of tests performed and ***$p < 0.001$ significantly different from basal group (one way ANOVA followed by Turkey's post hoc test)

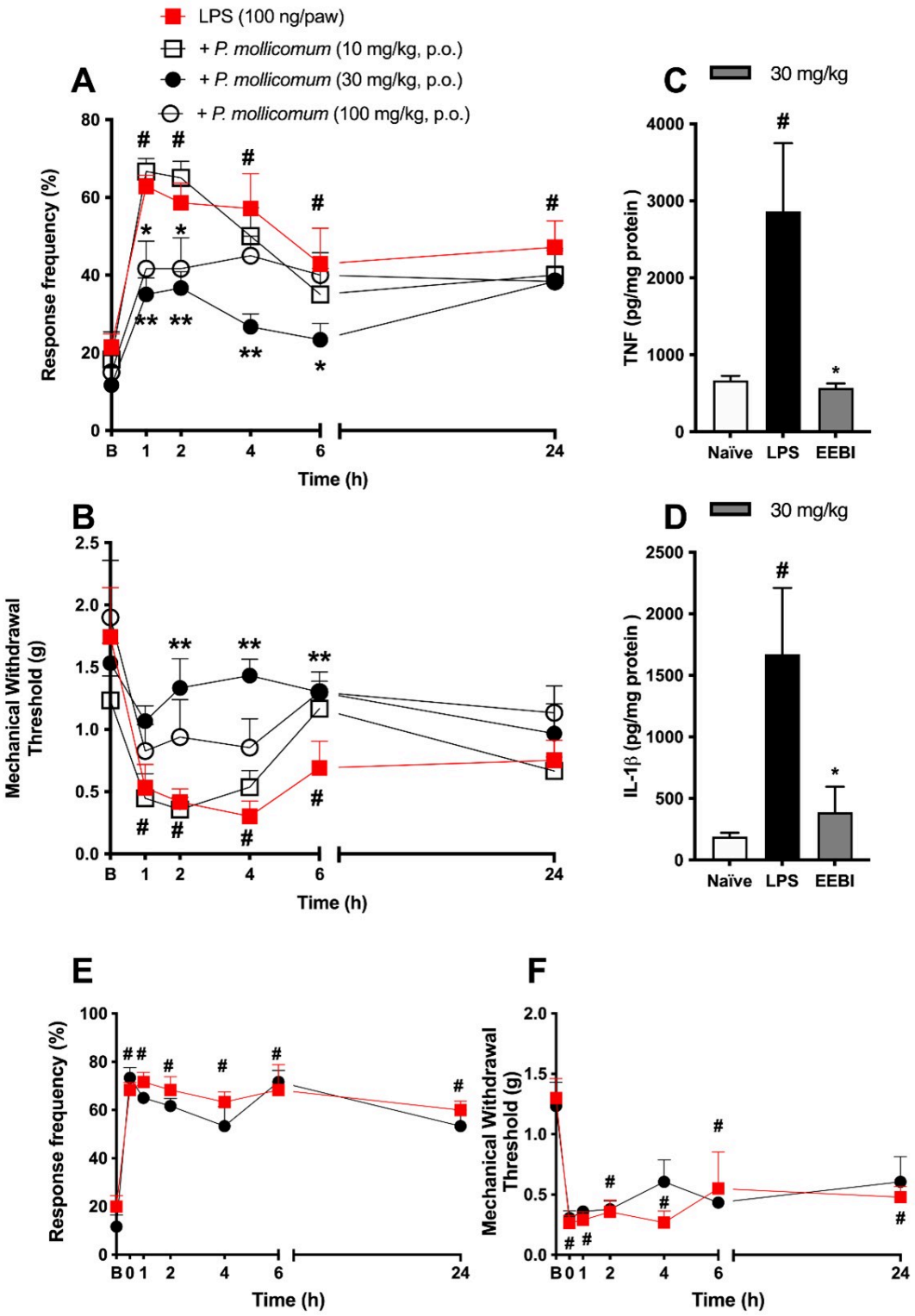


Figure 7

Effect of *P. mollicomum* extract on the mechanical hypersensitivity induced by (A and B) LPS (100 ng/paw) in mice, and the cytokines (C) TNF and (D) IL-1 β levels evaluated in the spinal cord tissuof mice treated or not with the extract (30 mg/kg, p.o.). E and F represents the mechanical hypersensitivity of mice with incisional pain receiving or not treatment with the extract of *P. mollicomum*(30 mg/kg, p.o.) twice a day, starting the treatment 24 h after the surgery. Experiments were conducted with 6 animals

per group and values express the mean ± S.E.M. *$p < 0.05$ and **$p < 0.01$ significantly different from LPS group. #$p < 0.05$ significantly different from basal (naïve) group (one way ANOVA followed by Turkey's post hoc test).

Supplementary Files

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