Effects of *Myrcia pubipetala* Miq (Myrtaceae) extract on innate inflammatory response

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Abstract

Abstract

Myrcia is a genus widespread in South America with species presenting anti-inflammatory properties. We investigated the anti-inflammatory activity of crude hydroalcoholic extract of *Myrcia pubipetala* leaves (CHE-MP). LPS-induced inflammatory mediator levels (cytokines by ELISA; and NO release by Griess reaction) and adhesion molecule expression (CD62L and CD18, by flow-cytometry) were assessed in neutrophils or macrophages treated with different concentrations of CHE-MP. The *in vivo* effects of the (CHE-MP) were evaluated in the air-pouch model of carrageenan-induced inflammation in mice. *In vitro*, the CHE-MP significantly reduced NO, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF) levels in the exudate and the supernatant culture. CHE-MP did not present cytotoxicity and modulated the percentage of positive neutrophils for CD18 and its expression *per* cell, without modifying the expression of CD49. Taken together, the data demonstrate that CHE-MP presents a potential activity on innate inflammatory based on the modulation of neutrophil and macrophage functions.

Keywords: *Myrcia pubipetala*, innate response, inflammation, Myrtaceae, macrophages, neutrophil.

1. Experimental

1.1 Plant material

Leaves from *M. pubipetala* were collected in Blumenau, Santa Catarina, Brazil (26º90'63'' S, 49º08'01" W) in August 2016. The identification was made by André Luís de Gasper. A voucher specimen from this plant was deposited in the Dr. Roberto Miguel Klein Herbarium (FURB, http://furb.jbrj.gov.br, 34168).

The collected material was dried at room temperature and grounded. The sample was extracted by maceration in 70% ethyl alcohol for seven days. After filtration, the procedure was repeated once more. The extracts resulting from the two macerations were pooled and concentrated with a rotary evaporator under reduced pressure until complete drying to yield crude hydroalcoholic extract (CHE-MP).

1.1.1 Preliminary phytochemical analysis

In order to identify classes of compounds present in the CHE-MP, qualitative colorimetric assays were carried out according to Urbain (2008). The classes of compounds investigated were phenols, tannins, leucoanthocyanidins, catechins, flavanones, flavonols, flavonols, xanthones, steroids, and triterpenes, saponins, resins, alkaloids, quaternary bases, quinones, enthralls and coumarins.

1.1.2 Phytochemical analysis by HPLC-ESI MS/MS

CHE-MP was analyzed by HPLC-ESI-MS/MS (High-Performance Liquid chromatography-tandem Mass Spectrometry with Electrospray Ionization) in the LABEC (Capillary Electrophoresis Laboratory) at the Federal University of Santa Catarina (UFSC), according to Siebert et al. (2020). Analyses were conducted with a Phenomenex® Synergi 4 µ Polar-RP 80A (150 mm× 2 mm ID, particle size of 4 µm) at 30 °C. The eluents were formed by mixing solvents A (MeOH/H2O in the ratio of 95:5, v v−1) and B (H2O ultrapure/formic acid (0.1%) as follows: 1st stage – 10% solvent A and 90% B (isocratic mode) for 5 min; 2nd

stage – linear gradient of solvents A and B (from 10 to 90% of A) for 2 min; 3rd stage – 90% solvent A and 10% B (isocratic mode) for 3 min; 4th stage – linear gradient of solvents A and B (from 90 to 10% of A) for 7 min with a flow rate of 250 μL min−1 for the mobile phase. For the analysis, an aliquot of 50 mg of EAE was resuspended in 5 mL of HCl at pH 2. This 5 mL were extracted three times with 2 mL of ethyl ether each, which were then combined. After drying the combined extract, it was stored sealed at −20 °C. To perform the analysis, the dried material was dissolved in 1 mL of MeOH and centrifuged at 12,000 rpm for 120 s. Three parts of the supernatant were added to 7 parts of ultra-pure water and the injected volume was 5 μL. For the identification of compounds, 47 standard phenolic compounds (4-aminobenzoic acid, 4-methyl-umbelliferone, 4-hydroxymethylbenzoic acid, p-anisic acid, caffeic acid, cinnamic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, mandelic acid, methoxyphenylacetic acid, p-coumaric acid, rosmarinic acid, salicylic acid, sinapic acid, syringic acid, vanillic acid, apigenin, aromadendrin, carnosol, catechin, chrysin, coniferaldehyde, epicatechin, epigallocatechin, epigallocatechin-gallate eriodictyol, scopoletin, fustin, galangin, hispidulin, isoquercetrin, kaempferol, myricetrin, naringenin, naringin, pinocembrin, protocatechuic acid, quercetin, resveratrol, rutin, sinapaldehyde, syringaldehyde, taxifolin, umbelliferone, and vanillin) diluted in methanol (1 mg/L) were analyzed under the same conditions as described above. For the quantitative analysis of the identified compounds, the area of each peak was interpolated into calibration curves performed with the identified standards ($r2 > 0.98$), under the same conditions of analysis. The experiments were performed in duplicate. The liquid chromatograph was coupled to a mass spectrometer with an electrospray ionization source using a negative ionization mode with the following source parameters: ion spray interface at 400°C; ion spray voltage of 4,500 V; curtain gas, 10 psi; nebulizer gas, 45 psi; auxiliary gas, 45 psi; collision gas, medium. The Analyst® (version 1.5.1) software was

used for recording and processing the data. Pairs of ions were monitored in MRM (Multiple Reaction Monitoring) modes.

To perform the analysis, an aliquot of 50 mg of CHE-MP was resuspended in 5 mL of a 0.01 mol/L HCl solution (pH 2.0). This solution was washed twice with 5 mL of ethyl ether, the organic phases being combined and evaporated to dryness. After drying, the organic extract was stored at -20°C until the moment of analysis. At analysis, the dried material was resuspended in 1 mL methanol and centrifuged (x 12,000 rpm) for 120 seconds. Three parts of the supernatant were added to seven parts of ultra-purified water and the volume of solution injected was 5 µL.

For the quantitative analysis of the identified compounds, the area of each peak was interpolated into a calibration curve performed with the identified standards (0.02 to 6 mg/L, 19 points, R2 > 0.98). Injections were performed in duplicate.

1.1.3 Total Phenolic Content

The content of total phenolic compounds was determined using the Folin-Ciocalteau reagent, according to the method described by Anagnostopoulou et al. (2006). To the extract solution at a concentration of 1,000 ppm in methanol, 5.0 mL of distilled water, 0.25 mL of Folin-Ciocalteau reactive, and 1.0 mL of saturated sodium carbonate solution (Na_2CO_3) were added. After 1 hour of rest, the absorbances were determined in a spectrophotometer at 725 nm. As a blank, a solution without the presence of the sample, prepared as described above, was used. The concentration of total phenols was estimated by interpolation with a calibration curve constructed with standard solutions of gallic acid at concentrations from 25 to 300 µg/mL, diluted in methanol. The linear fit was confirmed by the coefficient of determination of the straight-line equation ($y = 0.0013x + 0.0074$, $R2 = 0.9981$). The content of total phenolic compounds was expressed in mg of gallic acid per g of dry extract or fraction (mgAG/g).

1.1.4 Determination of Flavonoid Content

The determination of the flavonoid content was performed as described by Woisky and Salatino (1998). An aliquot of 0.5 mL of a crude extract solution and fractions at a concentration of 1,000 ppm diluted in ethanol, were added to 2.5 mL of ethanol and 0.5 mL of an aluminum chloride solution ($AlCl_3$, 2 %). After 1 hour, the absorbances of the solutions were determined in a spectrophotometer at 415 nm, using ethanol as a blank. To calculate the flavonoid content in the crude extract and fractions of the studied species, a calibration curve was constructed using the flavonoid quercetin at concentrations from 6.25 to 100 µg mL^{-1} as a standard. The linear fit was confirmed by the coefficient of determination of the straight-line equation ($y = 0.0021x – 0.029$, $R^2 = 0.9993$). Flavonoid content was expressed in mg of quercetin per g of dry extract or fraction (mgQUE/g).

1.1.5 Evaluation of antioxidant activity by DPPH free radical scavenging

The determination of the antioxidant activity using the free radical DPPH (2,2diphenylpicryl-hydrazyl) was based on the method described by Cavin et al. (1998). A 0.004% DPPH solution was added to samples diluted in methanol at different concentrations. After 30 minutes, the absorbances of the solutions were determined in a spectrophotometer at 517 nm. The result was expressed as % inhibition of control discoloration without the presence of the sample. To determine the 50% inhibitory concentration (IC_{50}), the values obtained were plotted as a % decrease in absorbance, in relation to the control, as a function of the logarithm of the concentration of the test sample.

1.2 Animals

Experiments were performed on male Swiss mice, 6-8 weeks old, (n = 6). All animals were obtained from the Central Animal Facility of the Universidade Regional de Blumenau (FURB), climate-controlled room at 22 ± 2 °C, under a light/dark (12:12 h) cycle, with water

and food *ad libitum*. All experiments were approved by the local ethics committee (CEUA/FURB, 009/20).

1.2.1In vitro anti-inflammatory activity

The evaluation of the *in vitro* anti-inflammatory activity of the extract was performed on neutrophils collected from Swiss mice and RAW 264.7 (macrophage cell line from the American Type Culture Collection (ATCC)).

1.2.2 Obtaining neutrophils migrated to the peritoneum

To obtain neutrophils, 3 mL of oyster glycogen solution (1 %, ip.) was injected to stimulate the recruitment of leukocytes into the mine peritoneal cavity. After 4 hours, animals were anesthetized and exsanguinated by sectioning the carotid artery. Then, 3 mL of PBS were injected into the peritoneal cavity and the cells were removed with a Pasteur pipette. The cellular suspensions were centrifugated (600 g, 15 min, 4 ºC) and the formed pellet was resuspended in 1 mL of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% Fetal Bovine Serum (FBS). The total quantification of neutrophils was done in the Neubauer chamber. A total of 1 x 10^6 neutrophils were added per well in 96-well culture plates, with a final volume completed to 300 µL with RPMI medium, to obtain the cell culture used in the assays described below.

1.2.3 Lipopolysaccharide-stimulated macrophage

Macrophages (RAW 264.7 cells, 1x 10^5 cells per well) were incubated with RPMI medium (10% FBS) in the presence or absence of lipopolysaccharide (LPS; 5 µg/mL) and simultaneously treated with CHE-MP (1, 10 or 100 µg/mL) for 18 h, at 37°C in a 5% CO_2. Then, the supernatant was collected for the subsequent cytokine and nitrite (NO_2^-) levels analysis.

1.2.4 Cell Viability

RAW 264.7 cells and neutrophils were plated (1x 105 cells per well) in 96-well microplates (TTP) and maintained at 37 °C with 5 % CO_2. The cells were treated with 10 µL of CHE-MP (1, 10, or 100 µg/mL). As a positive cytotoxicity control, 10 % dimethyl sulfoxide (DMSO) was used. RAW 264.7 cell viability was revealed by the MTT reduction assay (3-(4,5-Dimethylthiazol-2- yl) -2,5-Diphenyltetrazolium Br). After 21 hours of incubation at 37 °C with 5 % CO2, 10 µL of MTT (5 mg/mL) were added and the plate was kept for another 3 hours at 37 °C and 5 % CO2. In the end, the supernatant was removed, and for the dissolution of the formazan crystals formed by the reduction of MTT, 100 µL of DMSO was added. The optical density (OD) of each well was determined in a microplate spectrophotometer at 570 nm (Asxys Expert Plus, Eugendorf, Salzburg, AUSTRIA) (Denizot; Lang, 1986). The results in OD performed in quadruplicate were analyzed after eliminating the migration background of the cells of each experiment, using the percentage of migration as an indicator (Florão et al. 2007). The neutrophil viability was revealed by Trypan Blue. After 18 h of incubation, 10 µL of neutrophil suspension from each well was mixed with 10 µL of 0.4% trypan blue solution. The suspension (10 µL) was placed in the Neubauer chamber, 200 cells were counted, and the percentage of viable cells was calculated.

1.2.5 Cytokine and nitrite analysis

The levels of tumor necrosis factor (TNF), interleukin (IL)-6, nitrite (NO_2^-), and IL-1β were analyzed in the supernatants obtained from the RAW 264.7 cell culture or in exudate samples from the air pouch model. The assays were performed according to the manufacturer's instructions (DuoSet R&D Systems – Minneapolis, MN, USA), and the results were expressed in pg/mL. NO was indirectly quantified by the formation of its metabolite nitrite (NO_2^-) using the Griess reaction (Green et al. 1982).

1.2.6 Evaluation of adhesion molecules

To evaluate the effect of CHE-MP on the expression of CD62L and CD49 proteins, a flow cytometry assay was performed. Neutrophils were incubated in the presence and absence of the extract and stimulated or not with LPS (5 µg/mL, 1 h). Subsequently, the cells were washed with PBS and incubated with monoclonal antibodies anti-CD62L conjugated with phycoerythrin (PE) or anti-CD49 with Allophycocyanin (APC) at 4 °C for 20 minutes in dark. Cells were analyzed on Accuri C6 (BD Bioscience), and data from 10,000 events were obtained. The results were expressed as mean fluorescence intensity (MFI) and percentage of expression per cell.

1.3 In vivo anti-inflammatory evaluation

1.3.1 Leukocyte migration: Air pouch model

Air pouches were produced on the back of the mice as previously described by Sedgwick and Lees (1986) and Jain and Parmar (2011). Briefly, the animals were orally treated by gavage with CHE-MP at doses of 3, 30, or 300 mg/kg, indomethacin (30 mg/kg, positive control), or vehicle (PBS; 10 mL/kg). After 1 h, carrageenan (1%; 3 mL/cavity) was injected directly into the air pouch chamber. Four hours later, the cavity was washed with 3 mL of PBS and the inflammatory infiltrate was collected for analysis. Total and differential cell counts were performed, and the rest of the exudate was kept for future analyses of cytokines.

The air pouch lining tissue was collected for histological analysis and preserved in formaldehyde solution until the blades were made. The preparation consisted of the insertion of the tissue fragment into a cassette. The sample was submitted for a 1 h dehydration process in increasing concentrations of ethanol (70%, 96%, and absolute ethanol). Subsequently, each sample was placed in xylol and then, in a paraffin bath. After this process, the cassette with the sample was placed in a microtome where serial sections of 3 or 4 µm were made. The sections were placed on slides and stained with hematoxylin and eosin. Samples were then analyzed by optic microscopy (4, 10, and 40 X).

1.4 Statistical Analysis

The obtained results were expressed as mean ± standard error of the mean (SEM) and statistically analyzed by analysis of variance with multiple comparisons (ANOVA) and, when necessary, the Tukey- Kramer or Dunnett post hoc test was used. The data were analyzed using the GraphPadPrism® program, assuming $p < 0.05$ as statistically significant.

2. Discussion Supplementary

After CHE-MP obtention, the quantification by the Folin-Ciocalteau measured a significant quantity of phenolic compounds. These phenolic compounds were identified by HPLC-ESI-MS/MS (Table S1). Among them were some compounds with anti-inflammatory activity reported as Salicylic acid (Rainsford; Whitehouse, 1980; Munir et al. 2020), gallic acid (Bensaad et al. 2017; Bai et al. 2021), *p*-coumaric (Kheiry et al. 2019), chlorogenic acid (Miao, Xiang, 2019) and isoquercetin (Rogerio et al. 2007; Li et al. 2016). This phytochemical composition can effort to the CHE-MP a greater antioxidant activity, which can collaborate for inflammatory process minimization (Randjelovic et al. 2012).

CHE-MP treatment was able to inhibit the NO production by macrophage in all tested concentrations, especially in the high concentration, which was chosen to perform the following *in vitro* experiments. During inflammation, activated macrophages and neutrophils produce excessive inflammatory mediators including NO and PGE2, as well as inflammatory cytokines such as TNF, IL-1β, and IL-6 (Liew; Kubes, 2019). Previous studies demonstrated that some *Myrtaceae* species can reduce the inflammatory process (Cascaes et al. 2015), as *Plinia edulis* leaves (Azevedo et al. 2016), *Eugenia stipitate* (Araújo et al. 2019), and *M. ovata* (Santos et al. 2014).

At the first moment of the inflammatory process occurs hemodynamic changes or production of inflammatory mediators, which increases the CD62L expression on the

leukocyte's membrane (Liew; Kubes, 2019). After the activation process, CD62L is cleaved by ADAM-17, allowing the activation of CD18, which is responsible for the steady adhesion of leukocytes to the endothelium (Petri; Sanz, 2018). CHE-MP also modulates the expression of the adhesion molecule CD18.

These anti-inflammatory effects may be due to phenolic compounds in CHE-MP, polyphenols are one of the major classes of naturally occurring compounds and show multiple biological effects. Phenolic compounds such as phenolic acids and flavonoids have demonstrated important anti-inflammatory activity (Mutha et al. 2021). Studies have shown that phenolic compounds reduced the translocation of the transcription factor NF-κB, necessary for the translation of inflammatory mediators such as cytokines (Baena; Salinas, 2015). Flavonoids have demonstrated a decreasing proinflammatory cytokine, PGE2, and NO levels (Shin et al. 2009).

To elucidate the mechanism throughout which CHE-MP impairs the leukocyte migration, the air pouch model was used to evaluate the *in vivo* effects. The CHE-MP reduced the thickness of the air pouches tissue, oedema, number of leukocytes into lesion site, neutrophil migration, the volume of exudate and the inflammatory mediators when compared to the control group. Altogether, the data herein obtained allow us to suggest that CHE-MP presents anti-inflammatory properties, and these findings emphasize the importance of bioprospection of natural resource on inflammatory process.

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Declaration of Interest statement

The authors declare no conflicts of interest.

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TABLE AND FIGURE LEGENDS

Table S1: Chromatographic, spectrometric and quantitative data of phenolic compounds identified on the CHE-MP of *M. pubipetala* leaves by HPLC-ESI-MS/MS.

Phenolic Compound	Rt (min)*	Calculated mass (g)	Experimental mass (M-H)	MS/MS (*m/z*)	Phenolic content**($\mu g\ g^{-1}$)
Gallic acid	2.79	170.12	168.83	122.70	4.17 ± 2.11
Protocatecuic acid	9.10	154.12	152.92	109.00	1.59 ± 0.55
Vanillin	9.44	198.17	196.94	136.00	0.89 ± 0.34
Salicylic acid	10.58	138.12	136.85	90.11	13.15 ± 3.64
Chlorogenic acid	10.58	354.31	353.15	191.00	0.87 ± 0.25
p-Coumaric acid	12.19	164.05	162.92	119.00	1.36 ± 0.55
Isoquercetin	12.34	464.38	465.10	303.10	0.80 ± 0.51
Aromadendrin	13.81	288.25	287.01	125.00	2.48 ± 0.99

*Rt, retention time; **Concentrations are shown as means ± standard deviation

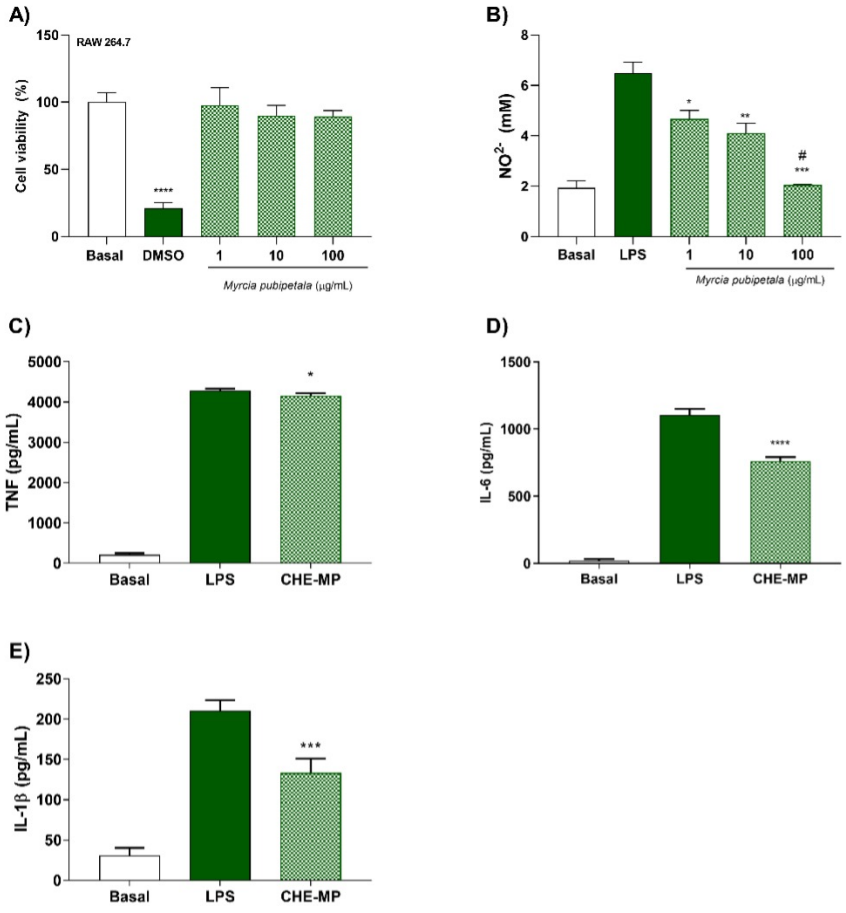

Figure S1. Effects of CHE-MP on cell viability and inflammatory profile of RAW 264.7 stimulated by LPS. (A) Cell viability of CHE-MP-treated cells was accessed by MTT method. (B) RAW 264.7 was incubated in the presence of LPS (5 µg/mL) and CHE-MP (1, 10 or 100 µg/mL) and the nitrite quantification was performed by the Griess reaction. (C) Quantification of TNF, (D) IL-6, and (E) IL-1ß was determined by ELISA. Data is expressed as a mean ± S.E.M. (n=6). Statistical analysis was performed using one-way ANOVA followed by Dunett post hoc test. *p <0.05, **p <0.01, *** p < 0.001, and **** p < 0.0001 vs LPS. #p <0.05 vs CHE-MP at 10 µg/mL.

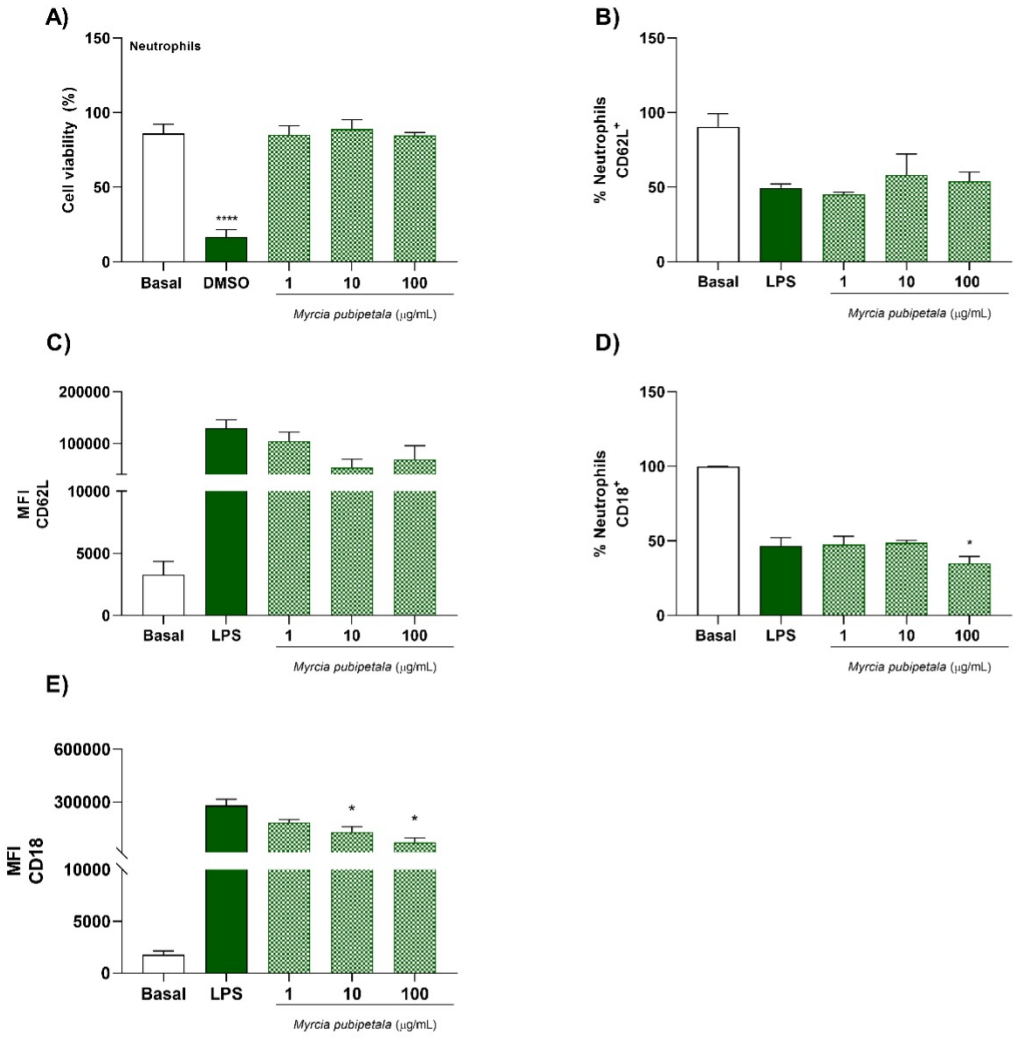

Figure S2. Effects of CHE-MP on cell viability and adhesion molecule expression by peritoneal neutrophils. (A) Cell viability of CHE-MP-treated neutrophils was accessed by trypan blue staining. To evaluate the adhesion molecules expression neutrophils (1 x 10^6) were incubated in the presence or absence of LPS (5 µg/mL) along with CHE-MP (1, 10 or 100 µg/mL). Quantification of adhesion molecules expression was assessed by labelling with CD62L (B-C) and CD18 (D-E) antibodies by flow cytometry. Data is expressed as a mean ± S.E.M. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. *p <0.05 vs LPS.

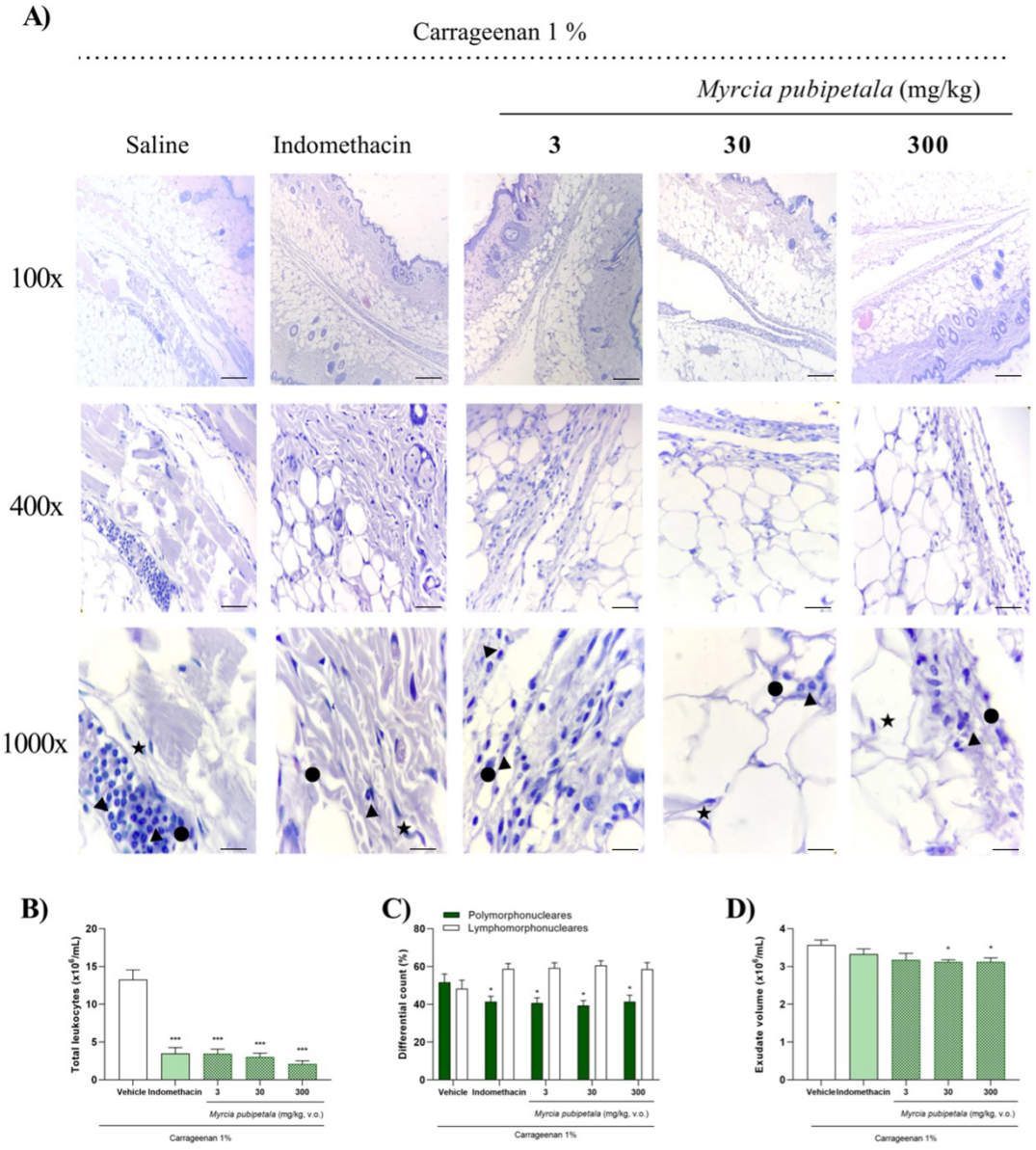

Figure S3. Leukocyte migration to the inflamed subcutaneous tissue of *Swiss* mice. The animals received oral treatments (CHE-MP, vehicle, or indomethacin) 1 hour before the injection of 1 mL of 1 % carrageenan into the pouch. The inflammatory infiltrate lavage was collected 4 hours after the carrageenan injection. (A**)** Images were observed at 100, 400 and 1000x. Caption: ▲ Polymorphonuclear ● Macrophages ★ Cell core; (B) Total leukocytes was

performed in a Neubauer chamber by optical microscopy; (C) Differential count: differential count was performed under optical microscopy with the smear stained by May-Grünwald-Giemsa, differentiating polymorphonuclear and mononuclear cells; (D) Total exudate volume of exudate inside the air pouch was quantified. Values express the mean ± S.E.M. of assays performed with cells obtained from 6 animals in each group. *$p<0.05$ and ***$p<0.001$ vs vehicle group. Scale bar = 100 μm

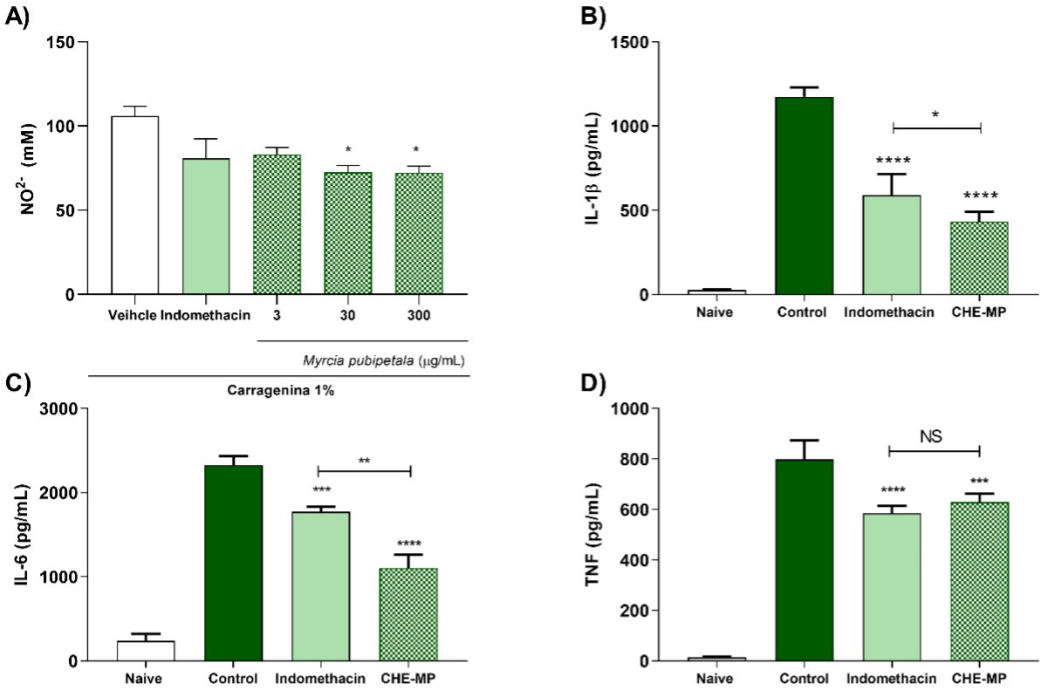

Figure S4. Chemical mediators of the exudate from air pouch lavage. The animals received oral treatments (CHE-MP, vehicle, and indomethacin) one hour before the injection of 1 mL of 1 % carrageenan in the formed compartment; the inflammatory infiltrate lavage was collected 4 hours after the injection of carrageenan into the air pouch. The (A) NO_2^- measurement was performed by the Griess reaction, whereas the (B) IL-1β, (C), IL-6, (D) TNF were quantified in the samples from animals treated with CHE-MP at 300 mg/mL. The chemical mediators were measured by ELISA. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; vs control group.