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Main pathways of action of Brazilian red propolis on the modulation of neutrophils migration in the inflammatory process

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ABSTRACT

Background: Brazilian propolis is popularly used as treatment for different diseases including the ones with inflammatory origin. Brazilian red propolis chemical profile and its anti-inflammatory properties were recently described however, its mechanism of action has not been investigated yet.
Aim: Elucidate Brazilian red propolis major pathways of action on the modulation of neutrophil migration during the inflammatory process.
Methods: The ethanolic extract of propolis (EEP) activity was investigated for neutrophil migration into the peritoneal cavity, intravital microscopy (rolling and adhesion of leukocytes), quantification of cytokines TNF-α, IL-1β and chemokines CXCL1/KC, CXCL2/MIP-2, neutrophil chemotaxis induced by CXCL2/MIP-2, calcium influx and CXCR2 expression on neutrophils.
Results: EEP at 10 mg/kg prevented neutrophil migration into peritoneal cavity ($p < 0.05$), reduced leukocyte rolling and adhesion on the mesenteric microcirculation ($p < 0.05$) and inhibited the release TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 ($p < 0.05$). EEP at 0.01, 0.1 and 1 µg/ml reduced the CXCL2/MIP-2-induced neutrophils chemotaxis ($p < 0.05$) without affect cell viability ($p > 0.05$).EEP at 1 µg/ml decreased the calcium influx induced by CXCL2/MIP-2 ($p < 0.05$). On the other hand, none of EEP concentrations tested altered CXCR2 expression by neutrophils ($p > 0.05$).
Conclusion: Brazilian red propolis appears as a promising anti-inflammatory natural product which mechanism seems to be by reducing leukocyte rolling and adhesion; TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 release; CXCL2/MIP-2-induced chemotaxis and calcium influx.

Introduction

Natural products from different sources have been widely used in the folk medicine and several biological properties had been scientifically demonstrated (Newman and Cragg, 2012). Furthermore, numerous studies have sought novel anti-inflammatory drugs that act by selectively blockage of neutrophils influx during inflammatory process (Bueno-Silva et al., 2013a; Franchin et al., 2013; Granica et al., 2015; Li et al., 2013). This fact is understandable due to the crucial role of neutrophils in local tissue damage and also because this episode takes place in different inflammatory diseases (Mackay, 2008).

Brazilian propolis has been investigated around the world and its different ethonopharmacological properties such as antimicrobial, anti-inflammatory, anti-nociceptive, hepato-protective and anti-cancer have been extensively investigated (Bueno-Silva et al., 2015; Bueno-Silva et al., 2013a; Bueno-Silva et al., 2013b; da Cunha et al., 2013; Franchin et al., 2012; Machado et al., 2012). Con-

Abbreviations: ANOVA, Analysis of variance; BRP, Brazilian red propolis; BSA, Bovine serum albumin; CEUA, Committee for Ethics in Animal Research; CXCL1/KC, Chemokine ligand 1; CXCL2/MIP-2, Chemokine ligand 2; CXCR2, Chemokine receptor 2; Dexa, Dexamethasone; DMSO, Dimethylsulphoxide; EDTA, Ethylenediamine tetraacetic acid; EEP, Ethanolic extract of propolis; GC–MS, Gas chromatographic-mass spectrophotometric; IL-1β, Interleukin 1 beta; i.p., Intraperitoneal; PBS, Phosphate buffered saline; PI, Propidium iodide; s.c., Subcutaneous; SPF, Specific-pathogen free; TNF-α, Tumor necrosis factor-alpha.

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sequently, it is used by population as food additive and/or dietary complement in products with beneficial properties to human health and in (bio)cosmetics (de Groot, 2013).

Among them, Brazilian red propolis (BRP) (Silva et al., 2008) had previously demonstrated a potent antimicrobial (Bueno-Silva et al., 2013b), antioxidant (Alencar et al., 2007) and anti-inflammatory activity on neutrophil recruitment (Bueno-Silva et al., 2013a; Lima Cavendish et al., 2015) and on lipopolysaccharide-activated macrophage (Bueno-Silva et al., 2015). It is important to note that BRP was recently divided into, at least, two sub-types: one from Sergipe state and another from Alagoas and Paraíba states (Lopez et al., 2014). Furthering, none of the studies above elucidated the mechanism of action on neutrophils migration.

Thus, the objective of this study was to elucidate the main routes of action of Brazilian red propolis on modulation of neutrophils migration during the inflammatory process.

Material and methods

Drugs and reagents

Ethanol, hexane and chloroform were purchased from Merck (São Paulo, SP, Brazil). Carrageenan, dexamethasone, DMSO, Fluo 3- AM, bovine serum albumin (BSA), RPMI-1640 medium, penicillin and L-glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA); Fetal Bovine Serum was from Gibco (Grand Island, NY, USA); ApoScreen^{TM} Annexin V-FITC Kit was acquired from SouthernBiotech (Birmingham, AL, USA); anti-CXCR2, anti-Ly6G and CXCL2/MIP-2 were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of the ethanolic extract of Brazilian red propolis

A sample of propolis was obtained from Maceio, Alagoas State in the Northeast of Brazil (SL 09.40 and WL 35.41). The propolis sample (64 g) was extracted with ethanol 80% (800 ml) in water bath at 70 °C for 30 min and after filtration; the ethanolic extract of propolis (EEP) yielded 47 g (Bueno-Silva et al., 2013a).

HPLC quantification of BRP compounds

The extract was filtered (0.22 µm diameter Millipore, Billerica, MA, USA) and then 20 µl of sample was injected in the RP-HPLC system equipped with a Shimadzu ODS-A column (RP-18, column size 4.6 × 250 mm; particle size 5 µm) and a photodiode array detector (SPD-M10AVp, Shimadzu Co.). The column was eluted using a linear gradient of water/acetic acid (99.5/0.5 v/v) (solvent A) and methanol (100%) (solvent B), starting with 30% B and increasing to 40% B (15 min), 50% B (30 min), 60% B (45 min), 75% B (65 min), 75% B (85 min), 90% B (95 min), and decreasing to 30% B (105 min), at a solvent flow rate of 0.8 ml/min. Chromatograms were recorded at 260 nm (Alencar et al., 2007). The following authentic standards of phenolic acids and flavonoids (Extrasynthese Co.) were examined: formononetin, daidzein, biochanin A, catechin, epicatechin, rutin, propyl gallate, ferulic acid and *p*-coumaric acid.

Animals

Animals used in every *in vivo* method described below were male, SPF (specific-pathogen free), Balb/c, mice weighing 18–22 g, were purchased from CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil). The mice were maintained in a room with controlled temperature (22 ± 2 °C), 12 h light/12 h dark and humidity of 40–60% with access to water and food *ad libitum*. Groups of *in vivo* experiments were composed by five animals. Dexametasone 2 mg/kg (Sigma-Aldrich, St. Louis, MO, USA) were used as positive control while vehicle DMSO 0.1% as negative control. All experiments were conducted in accordance with the approval of the Institutional Committee for Ethics in Animal Research (CEUA/UNICAMP protocol number: 1484-1) and animals were scarified by cervical dislocation.

Experimental procedure to evaluate neutrophil migration

Neutrophil migration to peritoneal cavity experiment was carried out by EEP (1, 3 or 10 mg/kg) or dexamethasone 2 mg/kg administration by subcutaneous (s.c.) injection, 15 min before the inoculation of inflammatory stimuli by intraperitoneal (i.p.) injection of carrageenan at 500 µg/cavity in naive mice. The vehicle DMSO 0.1% was used as negative control. Mice were killed 4 h after the challenge and the peritoneal cavity cells were harvested by washing the cavity with 3 ml of phosphate buffered saline (PBS) containing EDTA 1 mM. In order to count the total number of cells, a Neubauer chamber was used. Smears were prepared using a Cytospin 4 (Thermo Fisher Scientific, Waltham, MA, USA), stained with Solution Panotic Kit (Laborclin, Pinhais, PR, Brazil) and cells were counted (100 cells total) using an optical microscope (100x). Results were expressed as the number of neutrophils per cavity (Dal Secco et al., 2006).

Intravital microscopy

Based on results of neutrophil migration experiment, we chose EEP 10 mg/kg to further analyze its anti-inflammatory properties. Initially, mice were pretreated with EEP 10 mg/kg and negative control as described before (s.c.) and after 30 min, all groups received the i.p. injection of carrageenan 500 µg/cavity. Leukocytes rolling and adhesion were rated by intravital microscopy after 2 or 4 h of the inflammatory stimulus, as previously described (Baez, 1969; Fortes et al., 1991).

Cytokines

Mice were treated with EEP (10 mg/kg, s.c.) and vehicle as the negative control, 30 min prior the injection of carrageenan 500 µg/cavity. Levels of TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 were determined by ELISA using the protocols supplied by the manufacturers (R&D Systems). The results were expressed as pg/ml (Franchin et al., 2013).

Isolation of neutrophils from mice bone marrow

Isolation of neutrophils from mice bone marrow was performed by Percoll gradient. Total bone marrow cells were taken from femur and tibia of mice. Then cells were washed 2 times, suspended in 2 ml Hanks (Sigma-Aldrich, St. Louis, MO, USA) and transferred to a Falcon tube containing 72% and 65% Percoll gradient. After centrifugation (1200 g, 35 min at 18 °C), the band formed between the gradient was collected and the cells were quantified using a Neubauer chamber. The purity was assessed using smear prepared with the aid of a Cytospin 4 (approximately 95% purity neutrophils) (Boxio et al., 2004).

Cell viability assay by flow cytometry

The flow cytometry analysis was performed on neutrophils isolated from mice bone marrow as described previously. Neutrophils were placed in sterile Eppendorf tubes at concentration of 1×10^6 cells/well pretreated with EEP (0.1; 1; 10 and 100 µg/ml) and incubated at 37 °C, 5% CO2, 1.5 h. Subsequently, the cells were washed,

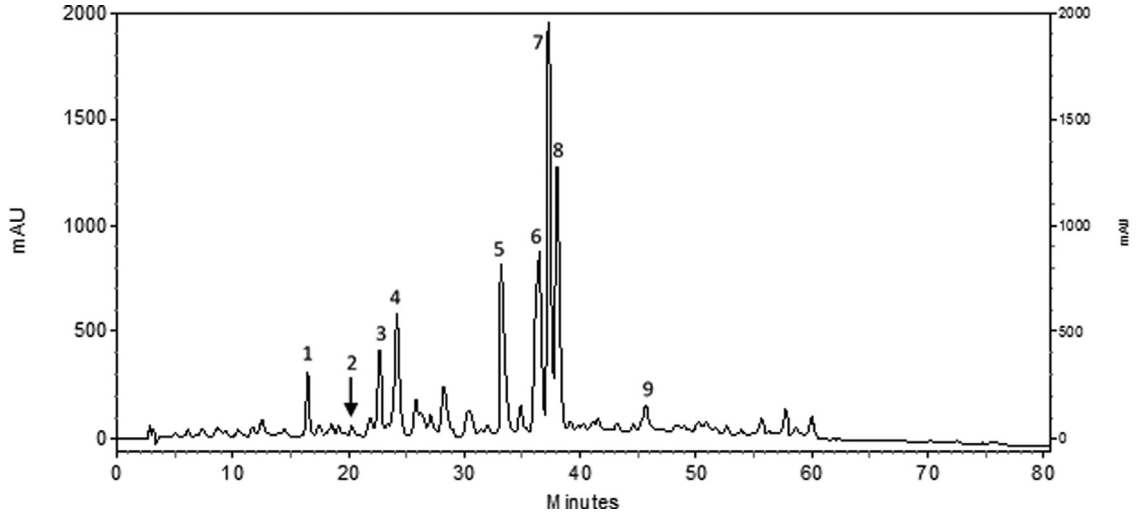

Fig. 1. HPLC chromatograms of ethanolic extract of Brazilian red propolis. 1, UV λ 241, 276, 311 nm; RT = 16.2 min; 2, Daidzein; 3, UV λ 262, 285 nm; RT = 22.4 min; 4, UV λ 249, 290 nm; RT = 23.9 min; 5, UV λ 237, 279 nm; RT = 33.2; 6, UV λ 244, 324 nm; RT = 36.2; 7, Formononetin; 8, UV λ 232, 261 nm; RT = 37.8; 9, Biochanin.

re-suspended in 100 µl of anexin 1x buffer and incubated for 20 min at 4 °C with antibody anti-anexin V conjugated with FITC (1:50). Then it was added to the cell solution, the anti-PI (propidium iodide) antibody at a concentration of 1:100. The reading was performed with FACSVerse (BD Biosciences, San Diego, CA, USA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). (Vermes et al., 1995).

Neutrophil chemotaxis in vitro

For the chemotaxis assay, we used the Boyden chamber (Neuroprobe, Pleasanton, CA, USA), polycarbonate filters (porosity 5 um; Neuroprobe, Pleasanton, CA, USA), CXCL2/MIP-2 (30 ng/ml) as chemotactic stimulus and as negative control, RPMI medium with bovine serum albumin (BSA) at 0.01%. Neutrophils isolated from mice bone marrow were incubated at 37 °C and 5% CO_2 with EEP at 0.01, 0.1 and 1 µg/ml. After 30 min, stimulation was placed on a single side of the membrane and on the other side, 50 µl of a neutrophil suspension (1×10^6 cells/ml). Then, the membrane was incubated under the same conditions already described. After 60 min, the filter was removed, washed, subjected to staining by Solution Panotic Kit and the cells were counted under an optical microscope (1000 x). The results were presented as the average of neutrophils migrated in five fields of high resolution (100 x). Each experiment was performed in triplicate (Da Silva-Santos et al., 2002).

Calcium influx in neutrophils

Based on results of our previous test, neutrophil chemotaxis, we chose to use only EEP 1 µg/ml since this concentration presented better results than others. In this way, analysis of calcium influx was performed on isolated neutrophils from mice bone marrow, as described above. After isolation, cells (1×10^6) were incubated for 40 min with Fluo 3-AM at 37 °C, 5% CO_2. Then, cells were washed 3 times and EEP (1 µg/ml) was incubated for more 30 min with neutrophils. Reading of calcium influx was carried out on FlexStation 3 Multi-Mode microplate reader, Molecular Devices, where the CXCL2/MIP-2 stimulation was added directly into the well of plate through equipment automatic choice (Gao et al., 2007).

Table 1
Quantification of compounds in red propolis by HPLC. LOD, limit of detection; LOQ, limit of quantification. HPLC quantification of BRP compounds.

Compounds	LOD (µg)	LOQ (µg)	Quantity (mg/g)
Daidzein	0.004	0.013	0.40 ± 0.02
Formononetin	0.013	0.039	24.45 ± 0.07
Biochanin A	0.012	0.035	0.95 ± 0.03

CXCR2 neutrophils expression in vitro

The flow cytometry analysis was performed on isolated neutrophils from mice bone marrow. After isolation, cells were incubated with EEP at a concentration of 1 µg/ml for 1.5 h. Subsequently, cells were washed and incubated for 30 min at 4 °C with the antibodies anti-Ly6G conjugated with APC (1:200) and anti-CXCR2 conjugated with PE (1:50). Then, the cell solution was washed with 2 ml FACS buffer and re-suspended in 100 µl of PBS 2% formalin. The reading was performed with FACSVerse (BD Biosciences, San Diego, CA, USA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA) (Rios-Santos et al., 2007).

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and statistical comparisons among groups were made using analysis of variance (ANOVA) followed by Tukey test. Significance was accepted when the p value was ≤ 0.05.

Results

HPLC quantification of BRP compounds

The results of the chemical profile of BRP are shown in Fig. 1. Formononetin was identified as the major compound, representing 24.45 mg/g of EEP (Table 1). Values are expressed as means of triplicate analyses. In addition, Daidzein and Biochanin A were also identified as minor compounds.

Neutrophil migration into the peritoneal cavity

EEP (10 mg/kg) significantly reduced the influx of neutrophils in the peritoneal cavity compared with carrageenan group (Fig. 2,

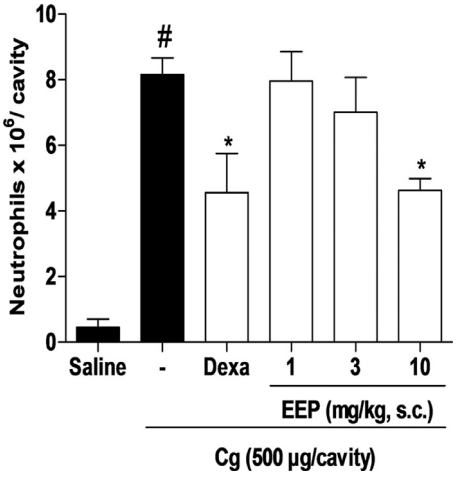

Fig. 2. Inhibitory effects of ethanolic extract of propolis (EEP) on the neutrophils migration into the peritoneal cavity induced by carrageenan (Cg). The neutrophil migration was determined 4 h after the injection of carrageenan 500 µg/cavity. Mice were previously treated with vehicle (saline or carrageenan), EEP 1, 3 or 10 mg/kg or dexamethasone 2 mg/kg. The results are expressed as mean ± S.E.M., $n = 5$. The symbol # indicates statistical difference compared to saline group. The symbol * indicates statistical difference compared to carrageenan group (one-way ANOVA followed by Tukey test, $p < 0.05$).

$p < 0.05$). Besides, EEP were as effective as Dexamethasone 2 mg/kg with no statistical difference between groups ($p > 0.05$).

Intravital microscopy

Red propolis activity on leukocyte rolling and adhesion at the endothelial cells was evaluated using intravital microscopy assay. EEP at 10 mg/kg reduced significantly the leukocyte rolling (Fig. 3A) and adhesion (Fig. 3B) in mesenteric microcirculation of mice submitted to intraperitoneal injection of carrageenan ($p < 0.05$).

Quantification of TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2

TNF-α, IL-1β, CXCL1/KC e CXCL2/MIP-2 levels were significantly reduced by EEP (10 mg/kg) treatment when compared to carrageenan group ($p < 0.05$) (Fig. 4A–D).

Cell viability

EEP (0.1 and 1 µg/ml) treatment during one hour did not affect neutrophils viability ($p > 0.05$) while 10 and 100 µg /ml showed up toxic for neutrophils ($p < 0.05$), when statistically compared to the vehicle group (Fig. 5).

Neutrophil chemotaxis in vitro

The EEP activity on the CXCL2/MIP-2-induced neutrophils chemotaxis is presented in Fig 6. EEP 0.01, 0.1 and 1 µg/ml statistically reduced chemotaxis when compared to the group stimulated only with CXCL2/MIP-2 ($p < 0.05$).

Calcium influx in neutrophils in vitro

Since EEP was able to reduced neutrophil chemotaxis (Fig. 6), we evaluated neutrophils calcium influx under CXCL2/MIP-2 stimulation. In this way, EEP at 1 µg/ml reduced the neutrophils calcium influx under CXCL2/MIP-2 stimulation when statistically compared to the group stimulated only CXCL2/MIP-2 ($p < 0.05$) (Fig. 7).

CXCR2 expression

Furthermore, we also evaluated EEP activity on CXCR2 expression in neutrophils isolated from mice bone marrow (Fig. 8). EEP was not able to change the CXCR2 receptor expression in neutrophils, when statistically compared to vehicle group ($p > 0.05$).

Discussion

Inflammation is a sequence of vascular and cellular events due to involvement and activation of chemical mediators (Mackay, 2008). Migration of neutrophils to the inflammatory focus is resultant of the release of chemotactic mediators, lipid mediators, cytokines and chemokines (Sadik and Luster, 2012). In this way, the present study evaluated the major pathways of action of Brazilian red propolis on modulation of neutrophil migration during the inflammatory process.

The TNF-α is considered an important cytokine related to inflammatory process since studies have shown its critical role on the modulation of neutrophil migration. One of TNF-α actions, that stimulate neutrophil migration, is to increase adhesion molecules expression by endothelial cells. There is a directly association between these molecules with the neutrophil adhesion, rolling

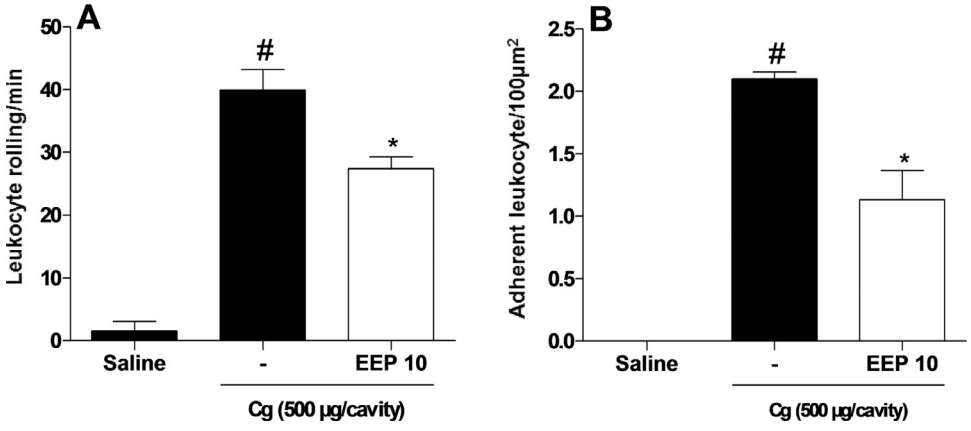

Fig. 3. Inhibitory effects of ethanolic extract of propolis (EEP) on rolling (A) and adhesion (B) of leukocytes as assessed by intravital microscopy in mesenteric microcirculation of mice, 2 and 4 h after i.p. injection of carrageenan (Cg). Mice were previously treated with vehicle (saline or carrageenan) or EEP at 10 mg/kg followed by carrageenan (500 µg/cavity) injection. The results are expressed as mean ± S.E.M., $n = 5$. The symbol # indicates statistical difference compared to saline group. The symbol * indicates statistical difference compared to carrageenan group (one-way ANOVA followed by Tukey test, $p < 0.05$).

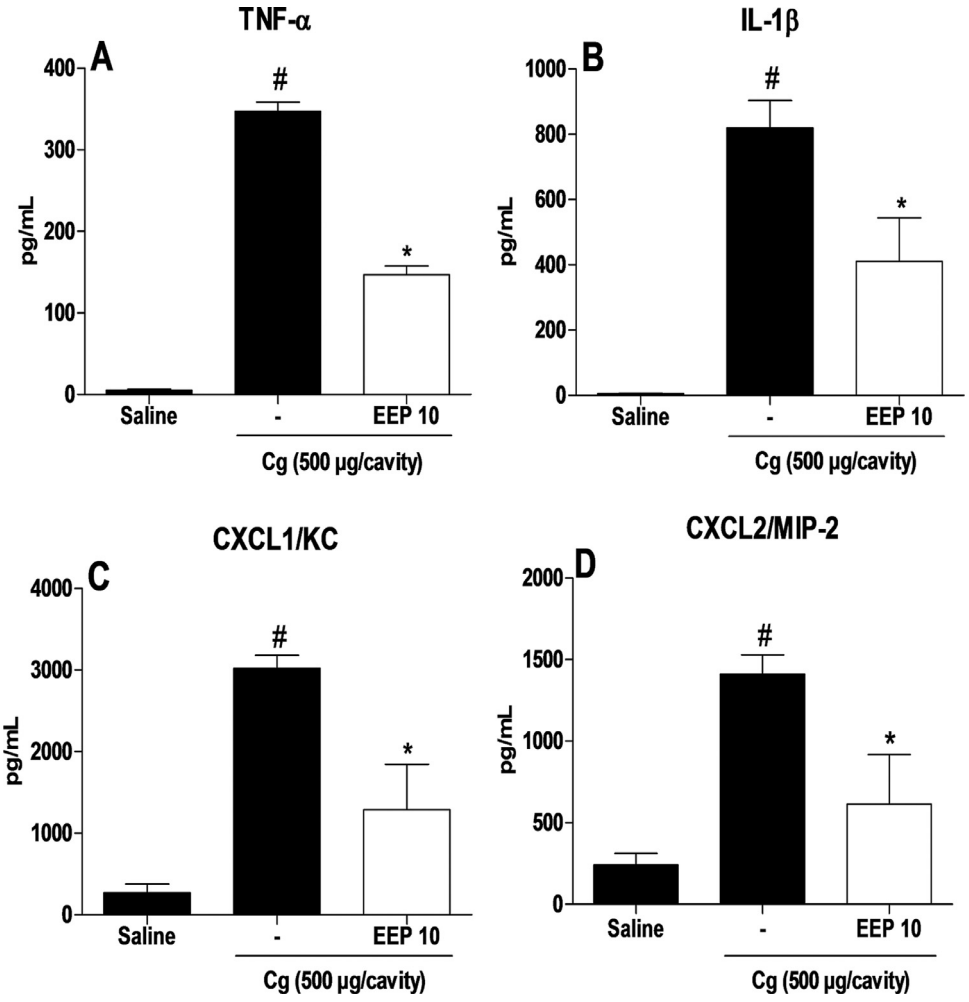

Fig. 4. Inhibitory effects of ethanolic extract of propolis (EEP) on release TNF-α (A), IL-1β (B), CXCL1/KC (C), and CXCL2/MIP-2 (D), in mice subjected to carrageenan (Cg) intraperitoneal injection. Mice were previously treated with vehicle (saline or carrageenan) or EEP at 10 mg/kg followed by carrageenan (500 µg/cavity) injection. The results were expressed as mean ± S.E.M., $n = 5$. The symbol # indicates statistical difference compared to saline group. The symbol $*$ indicates statistical difference compared to carrageenan group (one-way ANOVA followed by Tukey test, $p < 0.05$).

and transmigration during the inflammatory process (Moelants et al., 2013; Sadik and Luster, 2012). In addition, TNF-α modulation showed promising results in slowing the progression of some inflammatory diseases like rheumatoid arthritis (Armuzzi et al., 2014; Moelants et al., 2013). Thus, TNF-α inhibition could be considered as one of BRP mechanisms of action since it may be involved in neutrophils transmigration.

In this way, while TNF-α is related to neutrophils transmigration, IL-1β is involved not only in inflammatory events (eg. fever, hyperalgesia, lymphocyte responses, degenerative changes in joints, bone marrow cell number increases and neutrophil migration) but also in several inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and gout. In addition, IL-1β increases inflammatory cells recruitment in different inflammatory models including peritoneal cavity (Dinarello, 2011; Witko-Sarsat et al., 2000). Since BRP reduced its release, seems that BRP could be useful to control injuries observed in the inflammatory diseases cited above.

The migration of neutrophils during inflammation process is also dependent of many factors despite TNF-α and IL-1-β. These factors act directly on the neutrophils, attracting those cells into the inflammatory focus. One example, is the chemokine receptor CXCR2 which is activated by chemokine CXCL2/MIP-2 and then, stimulates the calcium influx on neutrophils (Wagner and Roth, 2000; Wu, 2005). Besides its chemotactic effects on neutrophils, CXCL1/KC and CXCL2/MIP-2 chemokines have crucial role to stimulate the expression of adhesion molecules on the mice mesenteric microcirculation, leading to increased leukocyte rolling and adhesion (Zhang et al., 2001). Thus, we suggested that the EEP inhibition of neutrophil migration might be related to reduction of CXCL1/KC and CXCL2/MIP-2 release and consequently the decrease of neutrophil chemotaxis, as well as bearing, adhesion and transmigration into the inflammatory focus. Moreover, another EEP mechanism observed in the present study was the direct blockage of calcium influx in neutrophils what in turn decreased the neutrophil chemotaxis, since calcium is necessary for chemotaxis (Steinckwich et al., 2007). Therefore, BRP may be useful to control intense neutrophil migration of inflammatory disease.

Taken together, these results corroborate our previous findings that Brazilian red propolis modulates neutrophils migration (Bueno-Silva et al., 2013a). Furthering, the present manuscript is noteworthy because it was the first to elucidated BRP mechanisms of neutrophil migration inhibition. Since BRP has a complex chemical profile, it is necessary to identify the compounds responsible for the BRP anti-inflammatory property. In this way, BRP used in this study has its chemical profile confirmed by HPLC and

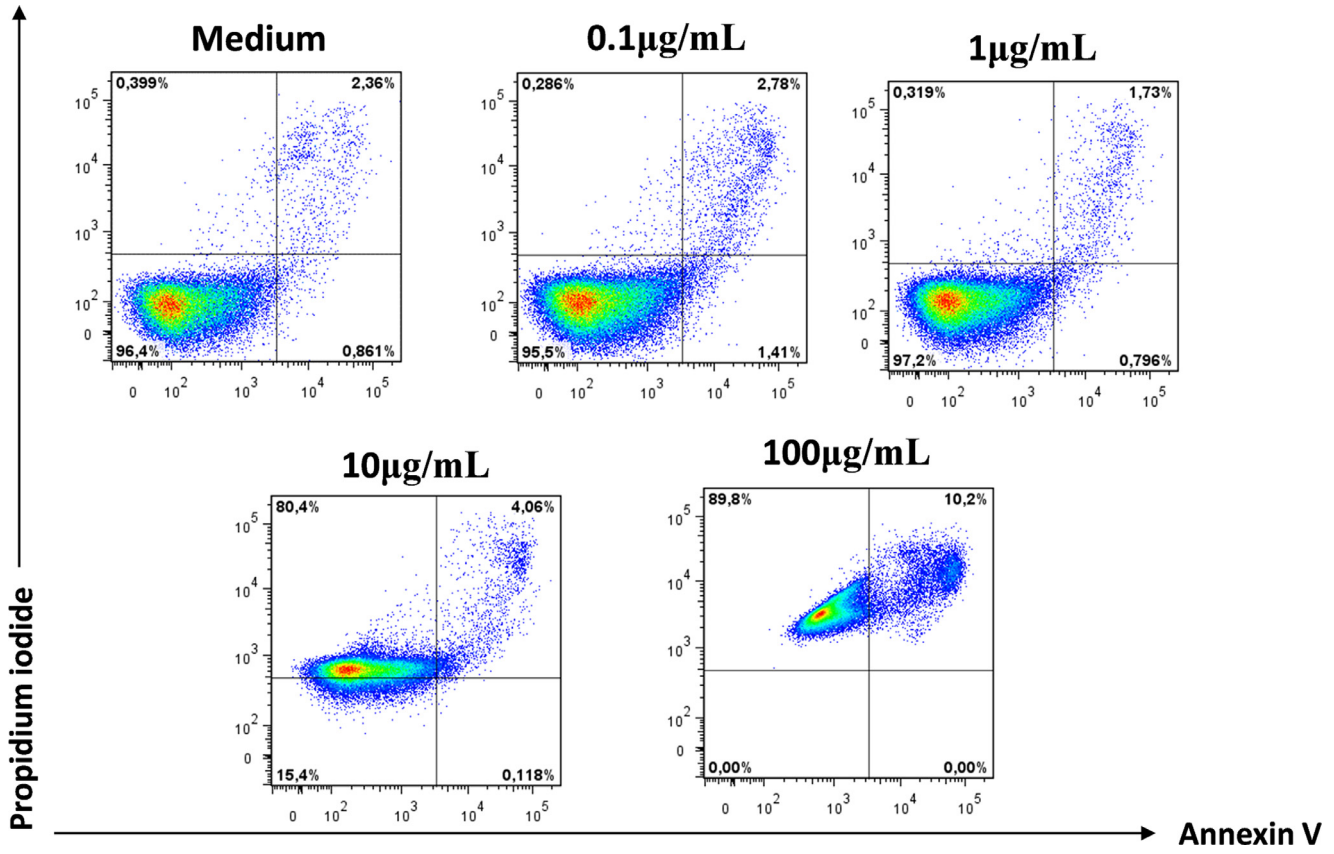

Fig. 5. Effects of ethanolic extract of propolis (EEP) on viability of isolated neutrophils from the bone marrow of mice. Neutrophils were previously treated with vehicle (medium) or EEP at 0.1, 1, 10 or 100 µg/ml (one-way ANOVA Followed by Tukey test, $p < 0.05$, $n = 4$).

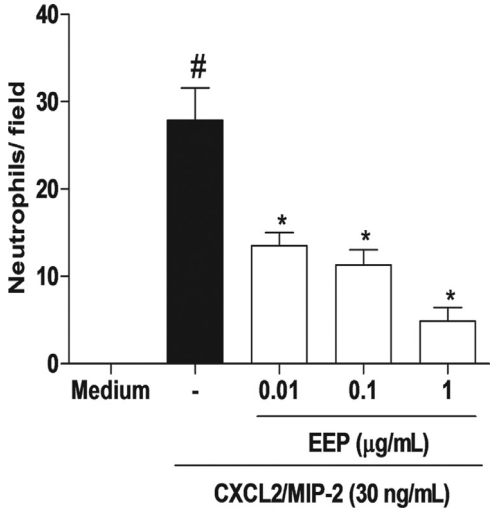

Fig. 6. Inhibitory effects of ethanolic extract of propolis (EEP) in neutrophil chemotaxis *in vitro*. Neutrophils were previously treated with vehicle (medium or CXCL2/MIP-2) or EEP at 0.01, 0.1 or 1 µg/ml. The results were expressed as mean ± S.E.M., $n = 3$. The symbol # indicates statistical difference compared to medium group. The symbol * indicates statistical difference compared to CXCL2/MIP-2 group (one-way ANOVA followed by Tukey test, $p < 0.05$).

Formononetin was the most abundant compound (Table 1). Formononetin already have its antinociceptive and anti-inflammatory effects demonstrated by Lima Cavendish et al. (2015) what corroborates the present manuscript.

Additionally, our research group previously identified and isolated neovestitol and vestitol from BRP. It was demonstrated that both compounds reduced neutrophils migration (Bueno-Silva et al., 2013a) and also has anti-caries activity (Bueno-Silva et al., 2013b). Recently, Franchin et al. (2016) demonstrated that vestitol reduced CXCL1/KC and CXCL2/MIP-2 release and also calcium influx in neutrophils. In this way, vestitol and BRP seems to have the same anti-inflammatory mechanisms of action and then, vestitol, although it is not the most abundant compound, may be considered as one of the BRP biomarkers. Moreover, different varieties of Brazilian propolis resulted in isolation and identification of different bioactive compounds with anti-inflammatory potential, such as Artepelin C (Paulino et al., 2008) and CAPE (Borrelli et al., 2002). Thus, the value of Brazilian propolis including the red type is well recognized in the literature as a promising source of new bioactive molecules with potential anti-inflammatory.

Therefore, we conclude that the Brazilian red propolis was effective in decrease the neutrophils influx to inflammatory site, acting through reduced TNF-α, IL-1β, CXCL1/KC and CXCL2/MIP-2 release and also reduced neutrophils chemotaxis by blocking calcium influx. These results suggest the red propolis as a promising anti-inflammatory natural product.

Conflict of interest

Authors declare that there is no conflict of interest

Acknowledgments

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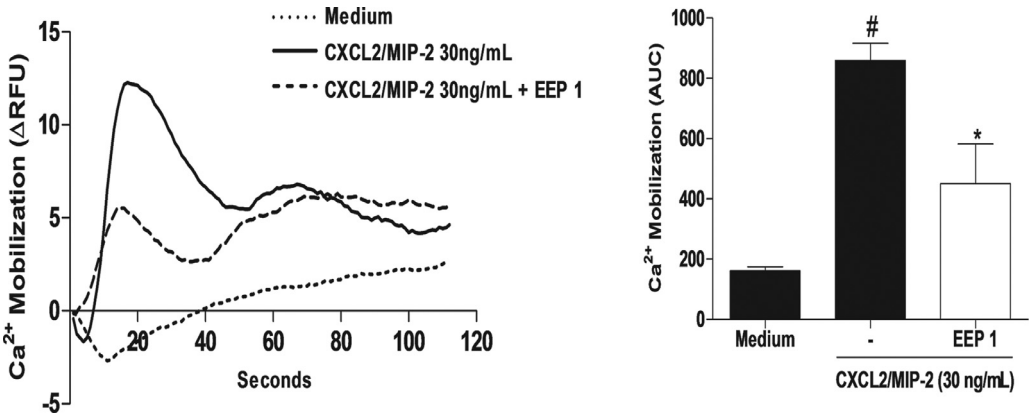

Fig. 7. Inhibitory effects of ethanolic extract of propolis (EEP) on neutrophils calcium influx isolated from the mice bone marrow. Neutrophils were previously treated with vehicle (medium) or EEP 1 μg/ml. The results were expressed as mean ± S.E.M., $n = 4$. The symbol # indicates statistical difference compared to medium group. The symbol * indicates statistical difference compared to CXCL2/MIP-2 group (one-way ANOVA followed by Tukey test, $p < 0.05$).

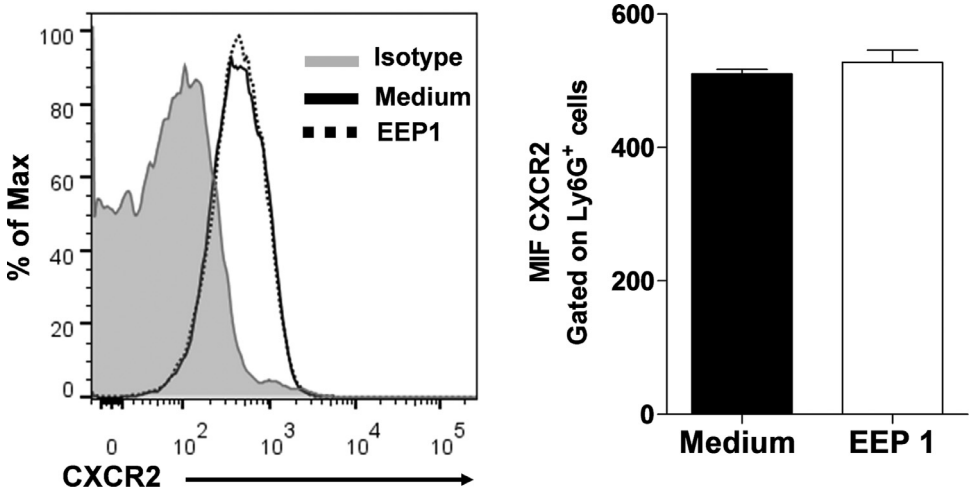

Fig. 8. Effects of ethanolic extract of propolis (EEP) in the CXCR2 receptor expression in neutrophils isolated from the bone marrow of mice. Neutrophils previously treated with vehicle (medium) or EEP at 1 μg/ml. The results were expressed as mean ± S.E.M., $n = 4$ (one-way ANOVA followed by Tukey test, $p < 0.05$).

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