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Anti-inflammatory properties of bergenin in mice

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ABSTRACT

Inflammation is a protective biological reaction against infection and has undesirable effects. Although bergenin (BG), a C-glycoside derivative of 4-O-methyl gallic acid, has many pharmacological properties, no study has demonstrated the activity of BG in inflammatory mediators, such as histamine and prostaglandin E_2 (PGE_2). The present study investigated the anti-inflammatory activity of BG obtained from *Peltophorum dubium* Taub, in edema models induced by carrageenan and inflammatory mediators in Swiss mice. The assessment of its toxicity was also conducted. The evaluations of this study indicated that the pre-treatment with BG (25 mg/kg) significantly reduced edema induced by carrageenan, compound 48/80, histamine, and PGE_2 ($p < 0.05$). Also, pre-treatment with BG inhibited the recruitment of leukocytes and neutrophils, reducing adhesion and rolling of these cells, myeloperoxidase (MPO) activity, malondialdehyde (MDA) levels, and vascular permeability. Treatment with BG (2,000 mg/kg) showed no toxic signs in hippocratic screening, weight of animals, water and feed intake, vital organs weight and histopathological evaluation, and hematological and biochemical parameters. In conclusion, these results show that the BG obtained from *P. dubium* Taub has anti-inflammatory activity against different models of inflammation, reducing neutrophil migration and damage caused by oxidative stress and lipid peroxidation. BG did not present toxic effects in the evaluated parameters.

INTRODUCTION

Inflammation is a protective biological response against infection, injury, or damage. This protective mechanism eliminates aggressive agents, regenerates and repairs tissues (Taams, 2018). When the body is unable to control inflammation adequately, a chronic inflammatory disease is triggered (Daham *et al.*, 2014; Herenius *et al.*, 2013; Rielland *et al.*, 2014; Singh *et al.*, 2013; Taams, 2018; Zygmunt *et al.*, 2014). The chronic inflammatory disease involves the overproduction of various inflammatory mediators, such as vasoactive amines, increases blood flow and vascular permeability, liberates proinflammatory cytokines from macrophages, complement system cascade, and leukocyte migration, and generates reactive oxygen species (ROS). All of these events result in tissue and DNA damage (Ashour *et al.*, 2018; Silva *et al.*, 2017).

Among the classes of drugs used to treat inflammation, non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used (Ferlazzo *et al.*, 2016; Marino *et al.*, 2015). These drugs exhibit their actions mainly by the inhibition of cyclooxygenases (COX- 1 and COX-2), which are involved in the production of prostaglandins, prostacyclins, and thromboxanes. The use of NSAIDs is closely related to the development of diseases, such as gastrointestinal disorders, hemorrhages, and renal disorders, becoming less recommended in chronic treatment (Akram *et al.*, 2016; Cao *et al.*, 2016; Intini *et al.*, 2017; Perkins *et al.*, 2015; Silva *et al.*, 2017; Yang *et al.*, 2018). NSAIDs are also associated with severe adverse effects and have been used to treat inflammatory disorders to date, despite their high cost (Ferlazzo *et al.*, 2016; Sullivan *et al.*, 2018). Due to the complications associated with the use of NSAIDs, new anti-inflammatories with better cost-effectiveness and low toxicity became a therapeutic need.

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The use of secondary metabolites from nature has been presented as a promising new therapy. When compared to NSAIDs, secondary metabolites are shown to be more effective and safer for use in pathological conditions of inflammation and chronic pain (Almeida *et al.*, 2017; Fukumitsu *et al.*, 2016). The use of new molecules from natural products, such as monoterpenes, polyphenolic compounds, carotenoids, and flavonoids, has been heavily explored, and their anti-inflammatory potential has been demonstrated (Almeida *et al.*, 2017; Chen *et al.*, 2014; Gomes *et al.*, 2017; Jang *et al.*, 2014; Pérez *et al.*, 2014; Silva *et al.*, 2017; Vasconcelos *et al.*, 2017). Among the flavonoids are c-glycoside molecules, which have been widely studied as therapeutic agents for the treatment of various diseases, including inflammatory disorders (Chen *et al.*, 2014; Thao *et al.*, 2016; Wang *et al.*, 2015).

Bergenin (BG) is a C-glycoside derivative of 4-O-methyl gallic acid (Bajracharya, 2015) extracted from roots of the plant *Peltophorum dubium* Taub. BG is also a major constituent of ethanol extracted from the roots of this plant (Oliveira *et al.*, 2015). *Peltophorum dubium* belongs to the family of legumes (Fabaceae) and the subfamily Caesalpinioideae. BG shows physicochemical properties and the structural formula (2R, 3S, 4S, 4aR, 10bS) -3,4,8,10- tetrahydroxy-2- (hydroxymethyl) -9-methoxy-3,4,4a, 10b-tetrahydro-2H-pyrano [c] isochromen-6-one ($C_{14}H_{16}O_9$), as shown in Figure 1 (Bajracharya, 2015; Oliveira *et al.*, 2015).

The studies have shown that the BG has anti-inflammatory properties, being able to selectively inhibit COX-2 *in vitro* (Nunomura *et al.*, 2009), besides having an important role in the reduction of inflammatory pain (Oliveira *et al.*, 2011), chronic bronchitis (Ren *et al.*, 2016), and Lipopolysaccharid (LPS)-induced mastitis, on *in vivo* models (Gao *et al.*, 2015). However, no studies have demonstrated the role of BG in classical models of inflammation and its action on inflammatory mediators as histamine and PGE$_2$.

Therefore, the present study evaluated the possible anti-inflammatory effects of BG in mice and assessed if toxicological effects are associated with the administration of this compound.

MATERIALS AND METHODS

Isolation of BG from *P. dubium* (spreng.) Taub

The peels of the *P. dubium* Taub roots found at the Ondina Campus of the Federal University of Bahia, Salvador—BA, Brazil, were collected. A sample was placed in the herbarium Alexandre

Figure 1. The molecular structure of bergenin. (A) Chemical structure of bergenin (own authorship). (B) ORTEP projection of bergenin, determined and analyzed by X-ray diffraction. Adapted from Oliveira *et al.* (2017).

Leal da Costa, from the Institute of Biology of UFBA, under cataloging number 69237. Isolation and purification of BG from material obtained was performed according to Oliveira *et al.* (2017).

Drugs and reagents

λ-Carrageenan (CG), indomethacin (INDO), compound 48/80, histamine, and PGE$_2$ were purchased from Sigma-Aldrich (Saint Louis, MO). Heparin was provided by Merck, from Sao Paulo, Brazil. Saline solution sterile 0.9% (w/v) NaCl (SAL) was used to dilute all the drugs. All other chemicals used in the experiment had degree analytical and obtained from the standard commercial suppliers.

Animals

Male Swiss mice (25–30 g) from the Federal University of Piauí were used in the experiment. These animals remained in an environment with the temperature of 22°C in a 12 hours light/dark cycle. The experimental groups contained five or six animals. Animal treatments and surgical approaches proceeded according to the National Institute of Health (Bethesda, MD). The experimental protocol used was approved by the ethics committee of the Federal University of Piauí with the protocol number, 220/16**.**

Anti-inflammatory assays

Effects of BG in carrageenan-induced paw edema

The pre-treatment of the mice was performed with BG (5, 25, 50, 100 mg/kg, i.p.) or INDO (10 g/kg, i.p.), 30 minutes before the administration of 50 µl carrageenan (500 µg/paw) on the right hind paw. A 0.9% sterile saline solution was administered to the negative control group. The paw volume of all mice was measured before and after the administration of inflammatory stimulus, as described by Offergeld *et al.* (1992).

Effects of BG in paw edema induced by compound 48/80, histamine and PGE2

The pre-treatment of the animals was carried out from the injection of BG (25 mg/kg, i.p.), INDO (10 mg/kg, i.p.), or saline (0.9%, *w/v*, NaCl-negative control). After 30 minutes, it was performed administration of 50-µL histamine (100 µg/paw), compound 48/80 (12 µg/paw), or PGE2 (3 nmol/paw), in the right hind paw, for the induction of edema, or of 0.9% saline in the negative control group. The experiment was carried out following the methodology used by Chintalwar *et al.* (1999) and Maling *et al.* (1974).

Carrageenan-induced peritonitis

First, was utilized saline (0.9%), INDO 10 mg/kg, or BG 25 mg/kg for pre-treatment of mice. Thirty minutes later, carrageenan was injected into the animals (250 µl, 500 µg/cavity). The protocol was followed according to Silva *et al.* (2017). The results are presented as leukocyte or neutrophil number per milliliter of peritoneal exudate.

Intravital microscopy of leukocytes

Intravital microscopy was used to evaluate the rolling and adhesion of leukocytes in the mesenteric microcirculation, as previously described (Fortes *et al.*, 1991; Silva *et al.*, 2015).
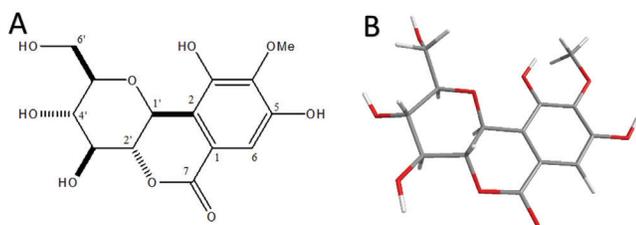
The induction protocol was the one that was used in the peritonitis model; however, the anesthesia with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) was performed in mice in the third hour. The mesenteric tissue was then externalized so that microscopic analysis could be performed *in situ.*

Myeloperoxidase concentration

Myeloperoxidase (MPO) activity of the peritoneal exudate was analyzed according to the method described previously (Bradley *et al.*, 1982). The results are expressed as units of MPO per milliliter of peritoneal exudate (UMPO/ml).

Malondialdehyde concentration

The Malondialdehyde (MDA) concentration was measured according to a method previously described by Uchiyama and Mihara (1978). The butanolic phase was read at 535 and 520 nm. The difference between the two measurements was determined. The MDA values are expressed as nmol MDA/g tissue.

Vascular permeability assay

Animals were pretreated with saline (0.9%), INDO (10 mg/kg), or BG (25 mg/kg). Carrageenan (250 μl, 500 μg/cavity) was then administered 30 minutes following pre-treatment. The vascular permeability was then verified by measuring the extravasation of Evans blue. The reading of the samples was performed with spectrophotometer at 620 nm (Silva *et al.*, 2013).

Acute toxicity study of BG

The toxicity of bergenin was determined according to the parameters of the Organization for Economic Co-operation and Development (Guideline 423/2001) (OECD, 2001). The dose of 2,000 mg/kg BG was administered, while the control group received only saline. The animals' clinical parameters were analyzed for 14 days, followed by hematological, biochemical, and histological analysis.

Statistical analysis

Results are expressed as mean ± standard error of the mean (±SEM). GraphPad Prism statistical software version 6.0 (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. The variables presented normal distribution and differences between the values for groups were evaluated using analysis of variance and Student–Newman–Keuls *post hoc* test. The Kruskal Wallis nonparametric test, followed by Dunn's test was used for histopathological evaluation. Differences between groups were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Anti-inflammatory assays

Effects of BG on carrageenan-induced paw edema

In this research, the action and anti-inflammatory mechanisms of BG was elucidated, since this flavanoid demonstrates great potential in the reduction of cytokines and other agents involved in the inflammatory disorders (Gao *et al.*, 2015; Oliveira *et al.*, 2011). For this was evaluated the action of BG on carrageenan-induced paw edema, a model widely used in studies to assess the anti-inflammatory activity of new compounds with pharmacological potential (Fezai *et al.*, 2013; Trevisan *et al.*, 2014).

Carrageenan promotes the formation of edema, and its effects are related to the release of inflammatory mediators that increase vascular permeability and leukocyte migration (Huang *et al.*, 2011). This agent triggers in its first phase (1–2 hours after CG administration) an increase in vascular permeability resulting from the release of histamine and serotonin from the mast cells (Maling *et al.*, 1974; Silva *et al.*, 2017). The second phase of GC response (3–4 hours) is characterized by an inflammatory infiltrate, bradykinin release, PGE_2, cytokines (e.g., interleukin 1 beta (IL-1β), tumor necrosis factor -Alpha (TNF-α), and interleukin 10 (IL-10)), and nitric oxide (Medzhitov, 2008; Silva *et al.*, 2017). In this study, paw edema significantly increased 1 hour after carrageenan administration (0.046 ± 0.008 ml), with peak edema observed at the third hour (0.100 ± 0.007 ml), followed by a decline by the fourth hour, as shown in Figure 2A and C. The dose of 25 mg/kg BG had significant ($p < 0.05$) effects on reducing carrageenan-induced paw edema at the third hour (0.024 ± 0.008 ml, with 76% inhibition); this was also observed with INDO (0.032 ± 0.009 ml, inhibition of 68%).

Because the dose of 25 mg/kg of BG presented the best effect among all concentrations used in the study, it was selected for use in the next trials. The present research showed that the BG administration promoted the reduction of paw edema and corroborated with the results presented by Gao *et al.* (2015), in which BG acted as an anti-inflammatory agent in mastitis induced by LPS. Our results are similar to those presented by Oliveira *et al.* (2011) in which BG isolated from *Cenostigma gardnerianum* Tul. (Leguminosae) demonstrated anti-inflammatory activity in Complete Freund's adjuvant-induced paw inflammation. However, in the model of edema used to perform the anti-inflammatory screening of BG in the present study the best dose was 25 mg/kg, while Oliveira *et al.* (2011) used higher doses in the measurement of the anti-inflammatory capacity of BG, given
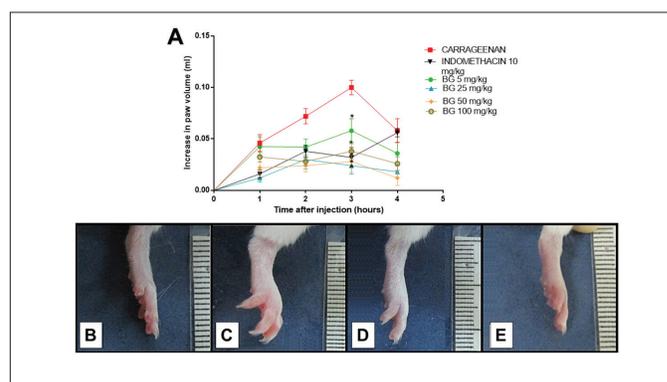

Figure 2. Bergenin decreases carrageenan-induced paw edema. Mice were pretreated with saline (0.9%), bergenin (5, 25, 50, and 100 mg/kg, i.p.), or INDO (10 mg/kg, i.p.). After 30 minutes, 500 μg/paw of carrageenan was administered. (A) Each line represents the mean ± SEM (five to six mice). Photographs of the paw of mice showing normal volume (B), increased volume with carrageenan (C), decreased volume with pretreatment with BG (D), and INDO (E). Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. *$p < 0.05$ compared to the carrageenan group.

the fact that doses of 50 and 100 mg/kg were more effective in the inflammatory pain model.

Effects of BG on paw edema induced by compound 48/80, histamine, and PGE2

As shown, BG had a better impact on the second phase of edema formation induced by carrageenan. This finding suggests that its anti-inflammatory action is related to the modulation of inflammatory mediators, cytokines, and decreased neutrophil infiltrate. Because of this result, we evaluated the mechanisms of BG in anti-edematogenic activity through the administration of inflammatory mediators, compound 48/80, histamine, and PGE_2.

Compound 48/80 has already been well-established as a potent inducer of mast cell degranulation. This polymer can cause rapid release of inflammatory substances as histamine and other mediators such as serotonin, leukotrienes, and a variety of cytokines related to the inflammatory process and the formation of osmotic edema (Jang *et al.*, 2014; Maling *et al.*, 1974; Nishikawa and Kitani, 2008; Silva *et al.*, 2014). Histamine is considered as a potent vasoactive mediator, and its release has been associated with vasodilation and increased vascular permeability, allowing the extravasation of plasma proteins and intravascular fluid to the interstitium. These processes then lead to the consequent formation of edema (Chimona *et al.*, 2008; Kalokasidis *et al.*, 2009; Kim *et al.*, 2005; Nunes-Neto *et al.*, 2017).

Still according to the literature data cited above, our results (Fig. 3) demonstrate the compound 48/80-induced edema (0.086 ± 0.013 ml, Fig. 3A) and histamine-induced edema (0.056 ± 0.005 ml, Fig. 3B). The edemas peak appear 30 minutes after of 48/80 and histamine administration, declining its action after 60 minutes of its administration, corroborating with others studies (Silva *et al.*, 2015; 2017). Pre-treatment with BG (25 mg/kg) significantly inhibited ($p < 0.05$) edema induced by the compound 48/80 (83.72% inhibition), and histamine-induced paw edema (28.57% inhibition) at the time when the agent caused a greater volume of edema in the paw. Indomethacin, the standard drug used, also significantly ($p < 0.05$) inhibited the paw edema induced by the inflammatory mediators used. In addition to confirming the anti-edematogenic effects, these results suggest that the BG influences in the release and/or action of inflammatory mediators. BG was able to inhibit the paw edema caused by the compound 48/80, which may be acting in the preservation of the mast cells membrane, preventing the degranulation of these cells and consequent release of vasoactive amines, such as histamine (Maling *et al.*, 1974; Nishikawa and Kitani, 2008; Silva *et al.*, 2014). Besides, BG also inhibited histamine-induced edema, showing the modulation of BG in this agent related to the formation of edema, which is related to the increase of vasodilation and vascular permeability, an important inflammatory response (Kalokasidis *et al.*, 2009; Nunes-Neto *et al.*, 2017). The literature reports that other flavonoids are involved both in inhibiting the release of histamine by mast cells and in their antihistaminic activity, inhibiting the effects caused by the direct administration of this vasoactive amine, which corroborates with our findings (Bhatia and Paliwal, 2014; Singh *et al.*, 2015; Wilson *et al.*, 1951).

PGE_2 is a relevant mediator to trigger vascular changes, being important for increased prostaglandin production and maintenance of the inflammatory process (Buadonpri *et al.*, 2009).

In this study, PGE2-induced edema presented a peak 30 minutes after its administration (0.070 ± 0.006 ml, Fig. 3C), corroborating with the literature (Silva *et al.*, 2013; 2015), and BG reduced this edema (60% inhibition). This effect may indicate that BG possibly acts on the activity or site of action of PGE_2 since other flavonoids act by suppressing the expression of the PGE_2 receptor, EP1, in its biological effects (Jin *et al.*, 2012; Kiraly *et al.*, 2016). Besides, this potent vasodilator is related to the production of chemoattractants, such as proinflammatory cytokines (TNF-α and IL-1β), related to the migration of leukocytes to the site of inflammation (Herrmann *et al.*, 1990). Indomethacin also significantly ($p < 0.05$) inhibited this edema, corroborating with the literature data (Silva *et al.*, 2017; Sousa-Neto *et al.*, 2018).

BG extracted from *Mallotus philippenensis* has potent activity in the inhibition of inflammatory mediators, as it has been documented in the study by Shah *et al.* (2012), in which BG inhibited the action of nitric oxide and TNF-α in macrophages. The study by Gao *et al.* (2015) also demonstrated its potent activity where, in addition to the mediators above, BG also inhibited the expression of IL-1β and IL-6.

Rolling, adhesion, and migration of inflammatory cells to the injured site

The displacement of the leukocytes, in particular, the neutrophils, is an important phenomenon for the line of defence of the host, where the infiltration in injured tissues is a hallmark signs of acute inflammatory response. Thus, we evaluated the migration of leukocytes and neutrophils to the peritoneal cavity and the possible mechanism of interaction in cell recruitment in carrageenan-induced leukocyte rolling and adhesion in mesenteric postcapillary venules (Barth *et al.*, 2016; Cano *et al.*, 2016; Fayngerts *et al.*, 2017; Muller, 2014; Pietrosimone and Liu, 2015). This procedure was based on the potential reduction of paw edema induced by inflammatory mediators by BG, mainly by the modulation of PGE_2, involved in the production of chemoattractants.
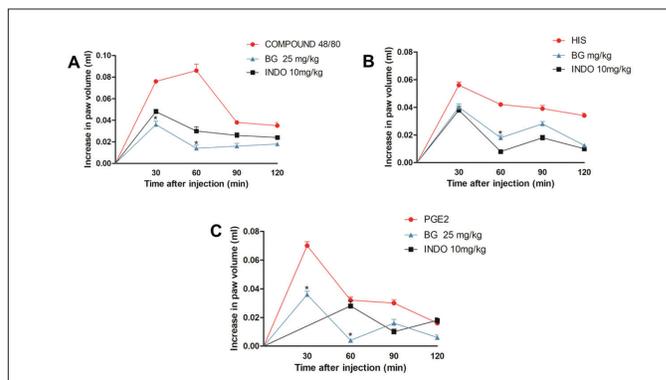

Figure 3. Bergenin decreases paw edema induced by various mediators. Mice were pretreated with saline (0.9%), bergenin (25 mg/kg, i.p.), or INDO (10 mg/kg, i.p.). After 30 minutes, paw edema was induced by compound 48/80 (12 µg/paw—A), histamine (100 µg/paw—B), or PGE_2 (3 nmol/paw—C). Each line represents the mean ± SEM (five to six mice). Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. #$p < 0.01$ compared to the saline group, *$p < 0.05$ compared to the control group.

Corroborating with the literature, carrageenan administration increased the total leukocyte and neutrophil count in the peritoneal lavage ($19.300 \pm 1.084 \ 10^3$ cells/ml; $8.185 \pm 0.833 \ 10^3$ cells/ml, respectively), when compared to the control group ($8.170 \pm 1.084 \ 10^3$ cells/ml; $0.9366 \pm 0.1975 \ 10^3$ cells/ml, respectively), as shown in Figure 4. The pre-treatment with BG significantly decreased the leukocyte ($8.890 \pm 2.099 \ 10^3$ cells/ml, Fig. 4A) and neutrophil count ($4.828 \pm 0.595 \ 10^3$ cells/ml, Fig. 4B) 4 hours after the carrageenan treatment. Pre-treatment with INDO also caused a reduction ($p < 0.05$). These results corroborate with other types of flavonoids that reduce cell migration in the carrageenan-induced peritonitis model (Furtado *et al.*, 2016; Mitra *et al.*, 2015; Rosa *et al.*, 2016). Also, BG extracted from *Mallotus japonicus* was also observed to inhibit the production of proinflammatory cytokines (Takahashi *et al.*, 2003) that are directly related to neutrophil recruitment.

Carrageenan administration also promoted a significant increase ($p < 0.001$; Fig. 5) in leukocyte rolling and adhesion to the endothelium of mesenteric vessels in intravital microscopy (10.250 ± 1.031 leukocyte rolling/minute and 1.650 ± 0.184 adherent cells/100 mm^2, respectively) when compared to the negative control group (1.600 ± 0.509 leukocyte rolling/minute and 0.000 ± 0.089 adherent cells/100 mm^2, respectively). However, the administration of BG (25 mg/kg) significantly reduced ($p < 0.001$) the leukocyte rolling (Fig. 5A) (4.00 ± 0.707 leukocyte rolling/minute) and adherence (Fig. 5B) (0.700 ± 0.089 adherent cells/100 μm^2). BG, when significantly inhibits leukocyte rolling and adhesion, corroborates with edema measurements and leukocyte counts, in agreement with research that showed that BG inhibits cell migration in chronic bronchitis (Ren *et al.*, 2016). These results indicate that BG may also be involved in the modulation of mechanisms related to the regulation of endothelial adhesion, which deserves further investigated. Other flavonoids are also involved with the transendothelial leukocyte migration (Herr *et al.*, 2014; Werner *et al.*, 2015).

Thus, from the analysis of these results, the study suggests that BG decreases the recruitment, bearing, and adhesions of leukocytes in the vascular endothelium. This decrease is probably due to the modulation of inflammatory mediators, histamine, and
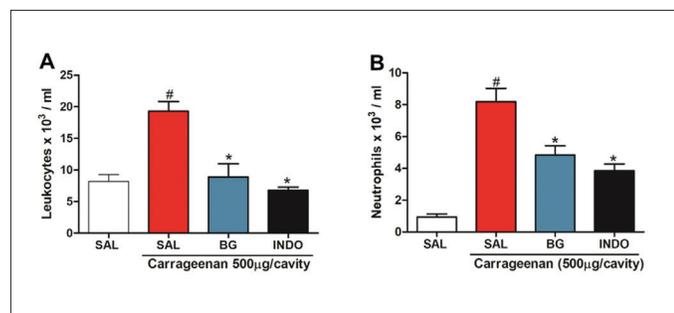
PGE$_2$, related to the production of chemoattractant cytokines, increased blood vessel dilation, and vascular permeability, reducing the number of leukocytes, mainly neutrophils, chemoattractant by pro-inflammatory cytokines, which decreases the neutrophil bearing and adhesion to the blood vessels, and their migration to the inflammatory site.

Effect of BG on myeloperoxidase activity

Carrageenan administration produced a significant increase in myeloperoxidase activity (12.550 ± 2.840 U/mg of peritoneal exudate, $p < 0.05$), an important marker of neutrophil infiltration. Pre-treatment with BG (25 mg/kg) significantly reduced MPO levels (3.951 ± 1.529 U/mg of peritoneal exudate, $p < 0.05$), and was similar to the 0.9% saline control group (3.280 ± 1.992 U/mg of peritoneal exudate) as shown in Figure 6A. MPO is a peroxidase enzyme that is most abundantly expressed in neutrophilic granulocytes (Uchiyama and Mihara, 1978). In this study, it was observed that BG reduced the levels of MPO that were increased with the administration of carrageenan, confirming inhibition of neutrophil migration to the inflamed tissue. Other studies performed with BG isolated from the herb *Saxifraga*
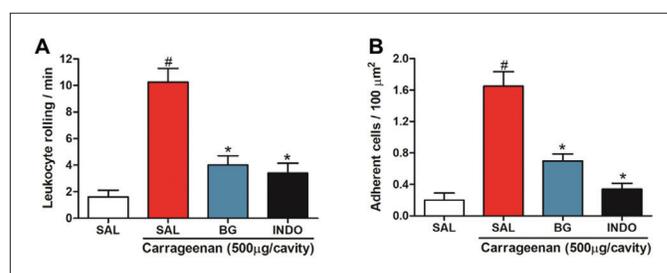
Figure 5. Bergenin reduces leukocyte-endothelial interactions. Mice were pretreated with saline (0.9%), bergenin (25 mg/kg, i.p.), or INDO (10 mg/kg, i.p.). After 30 minutes, 500 μg/cavity of carrageenan was administered. (A) The leukocyte rolling is expressed as the number of passing leukocytes per minute. (B) The number of adherent leukocytes is expressed as the number of adherent cells/100 μm^2. Each bar represents the mean ± SEM (five to six mice). Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. #$p < 0.001$ compared to the saline group, *$p < 0.001$ compared to the control group.

Figure 4. Bergenin reduces cell migration in carrageenan-induced peritonitis. Mice were pretreated with saline (0.9%), bergenin (25 mg/kg, i.p.) or INDO (10 mg/kg, i.p.). After 30 minutes, 500 μg/cavity of carrageenan was administered. Neutrophil migration was evaluated after 4 hours. (A) Total counts and (B) differential counts. Each bar is the mean ± SEM (five to six mice). Results were analyzed with one-way analysis of variance and Newman–Keuls test. #$p < 0.01$ compared to the saline and *$p < 0.05$ compared to the control group.
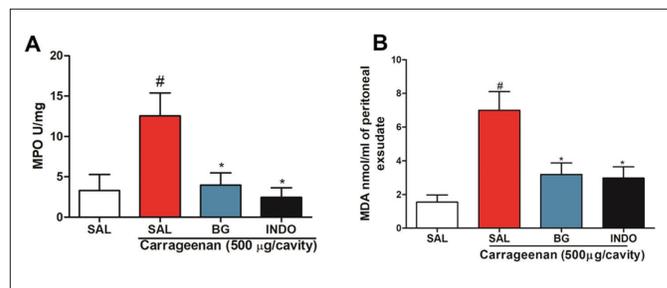
Figure 6. Bergenin decreases MPO activity and MDA levels. Mice were pretreated with saline (0.9%), bergenin (25 mg/kg, i.p.), or INDO (10 mg/kg, i.p.). After 30 minutes, 500 μg/paw of carrageenan was administered. Each bar represents the mean ± SEM of values of MPO activity (A) and MDA levels (B) (five to six mice). Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. (A) #$p < 0.05$ compared to the saline group, *$p < 0.05$ compared to the control group. (B) #$p < 0.01$ compared to the saline group, *$p < 0.01$ compared to the control group.

stolonifera Curt (Hu-Er-Cao) have already demonstrated the potential of BG in reducing MPO levels (Wang *et al.*, 2018; Yang *et al.*, 2017). Therefore, our results demonstrate the protective effect of BG against the infiltration of inflammatory cells.

Effects of BG on malondialdehyde levels

Neutrophilic infiltration into inflamed tissues, a result of the administration of carrageenan, is one of the characteristics of the acute innate inflammatory response, which increases ROS production (Odobasic *et al.*, 2016; Pietrosimone and Liu, 2015). These radicals cause peroxidation of the lipid components of the cell membrane, generating the formation of lipid peroxides, which are metabolized via the oxidation to malondialdehyde. This oxidation reacts with groups of cellular proteins, resulting in metabolic disorders of the cells, tissue damage, and exacerbated inflammation (Kwiecien *et al.*, 2014; Odobasic *et al.*, 2016; Pietrosimone and Liu, 2015). In this way, malondialdehyde is one of the main biomarkers of the oxidative stress.

Likewise, our results showed a significant increase ($p < 0.01$) in MDA levels in the carrageenan group (7.000 ± 1.106 nmol/ml of peritoneal exudate) when compared to the saline group (1.548 ± 0.429 nmol/ml of peritoneal exudate). This present study showed that pre-treatment with BG reduced the migration of neutrophils, we evaluated if this would cause a reduction of lipid peroxidation, since oxidative stress is mainly a result of neutrophilic infiltration. The MDA levels were significantly reduced ($p < 0.01$) after pre-treatment with BG (3.190 ± 0.685 nmol/ml of peritoneal exudate), as can be seen in those pretreated with INDO (2.976 ± 0.671 nmol/ml of peritoneal exudate), in Figure 6B. Thus, the study suggests that BG decrease lipid peroxidation, possibly by reducing the oxidative stress caused by neutrophil migration.

Effects of BG on the vascular permeability test

Vascular permeability, another parameter evaluated, is induced by inflammatory mediators, such as histamine and PGE_2. Vessel permeability facilitates the delivery of antibodies and soluble acute phase proteins to the inflammatory site (Wilhelm, 1973). Here, we evaluated this inflammatory process using Evans blue dye, which binds to plasma proteins, mainly to albumin. In the carrageenan group, there was an increase in vascular permeability (70.030 ± 7.283 µg/ml exudate), when compared to the saline control group (10.420 ± 3.592 µg/ml exudate), as demonstrated in Figure 7. The animals pretreated with BG presented a decrease in vascular permeability that was induced by carrageenan (37.390 ± 2.466 µg/ml exudate); a similar result was obtained using the standard drug, INDO (36.860 ± 4.269 µg/ml exudate). These results corroborate with the decrease in histamine edema as it is directly related to increased vasodilation (Brito *et al.*, 1997; Gros *et al.*, 2015; Sancilio, 1969). Accordingly, we can infer that BG acts by decreasing vascular permeability by inhibiting the release of inflammatory mediators.

Also, one of the factors that facilitate transendothelial migration is the increase in vascular permeability (Aghajanian *et al.*, 2008). BG decreased this parameter and may be one of the reasons why in our trials this flavanoid decreased the bearing and adhesion of the leukocytes in the mesenteric vessels observed in intravital microscopy.

Toxicological evaluation

BG at the concentration of 2,000 mg/kg showed no toxic signs in the treated mice. All animals showed normalities in the parameters: general activity, motility, heart rate, piloerection, exophthalmia, licking paws, snout, tail bite, convulsion, tail erection, tail tremor, sedation, analgesia, anesthesia and loss of reflex (data not shown), and survived throughout the experiment. No significant difference was observed concerning body weight after 14 days of the administration (Table 1). Hematological and biochemical analyses were performed for plasma levels of aspartate transaminase (AST), alanine transaminase (ALT), total direct and indirect bilirubin, liver function mediators, urea and creatinine, indicators of renal function, glucose, potassium, sodium, chlorine, and calcium. No statistical difference was observed between these parameters (Table 2).

Regarding hematology (erythrogram, leukogram, and plaque), no change was observed (Table 3). In the evaluation of the organs, spleen, kidneys, heart, and liver, no abnormalities were found in the tests or difference in their mean weights in the treated groups (Table 1). Similarly, histological analysis of spleen,
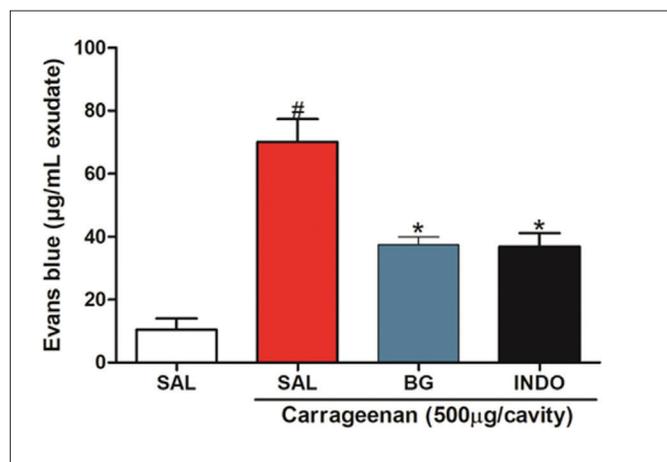

Figure 7. Bergenin acts by decreasing vascular permeability as determined by Evans blue test. Mice were pretreated with saline (0.9%), bergenin (25 mg/kg, i.p.), or INDO (10 mg/kg, i.p.). After 30 minutes, 500 µg/cavity of carrageenan was administered. Thirty minutes before euthanasia, a treatment with Evans blue (50 mg/kg in PBS 10×) via ocular was performed. Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. #$p < 0.001$ compared to the saline group, *$p < 0.001$ compared to the control group.

Table 1. Effect of bergenin on body weight (g) and organ weight after 14 consecutive days.

	Body weight before (g)	Body weight after (g)	Spleen (g)	Kidneys (g)	Heart (g)	Liver (g)
Saline	24 ± 1.15	26 ± 1.15	0.28 ± 0.15	0.30 ± 0.02	0.14 ± 0.01	1.38 ± 0.16
BG 2,000 mg/kg	21.30 ± 1.76	22.66 ± 1.33	0.12 ± 0.01	0.28 ± 0.02	0.13 ± 0.01	1.07 ± 0.10

Values are expressed as mean ± SEM. Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. *$p < 0.05$ compared to the saline group.

Table 3. Effect of bergenin on hematological analyses after 14 consecutive days.

Hematological analyses	Saline	BG 2,000 mg/kg
No of red blood cells (×10⁶/mm³)	5.65 ± 3.6	5.1 ± 1.53
Hematocrit (%)	40.66 ± 0.3	40.66 ± 0.66
Platelets (10⁵/mm³)	8.22 ± 1.21	7.34 ± 0.95
Hemoglobin (g/dl)	16.83 ± 0.18	15.32 ± 1.18
MCV (fl)	72.49 ± 4.07	79.80 ± 2.30
MCH (pg)	30.05 ± 2.01	30.05 2.28
MCHC (%)	41.43 ± 0.78	37.96 ± 3.42
Leukocytes (×10³/mm³)	6.66 ± 0.85	5.51 ± 1.36
Lymphocytes (%)	70.60 ± 4.37	70.00 ± 3.78
Monocytes (%)	0.66 ± 0.33	0.33 ± 0.33
Neutrophils (%)	25.66 ± 4.25	27.66 ± 3.75
Eosinophils (%)	3 ± 0.57	2.00 ± 0.57

Values are expressed as mean ± SEM. Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. *$p < 0.05$ compared to the saline group.

Table 2. Effect of bergenin on biochemical analyses of plasma after 14 consecutive days.

Analyse bichemical	Salina	BG 2,000 mg/kg
Creatinine (mg/dl)	0.23 ± 0.033	0.20 ± 0.00
Urea (mg/dl)	61.68 ± 3.14	42.10 ± 3.80
Total bilirubin (mg/dl)	4.17 ± 0.18	6.06 ± 0.12
Direct bilirubin (mg/dl)	2.78 ± 0.30	5.12 ± 0.31
Indirect bilirubin (mg/dl)	1.39 ± 0.33	0.94 ± 0.19
AST (U/l)	2 ± 0	2 ± 0
ALT (U/l)	65.77 ± 19.23	56.08 ± 6.85
Glucose (mg/dl)	85.68 ± 11.73	42.93 ± 10.73
Potassium (mmol/l)	70.89 ± 3.41	71.91 ± 3.70
Sodium (mmol/l)	151.09 ± 1.46	154.13 ± 5.88
Chlorine (mmol/l)	118.45 ± 2.46	116.16 ± 0.74
Calcium (mmol/l)	0.02 ± 0.0	0.02 ± 0.00

Values are expressed as mean ± SEM. Results were analyzed using one-way analysis of variance followed by Newman–Keuls test. *$p < 0.05$ compared to the saline group.

kidneys, heart, and liver also showed no histopathological changes between groups (data not shown).

CONCLUSION

Our study demonstrated that the BG showed anti-inflammatory activity against different mediators of inflammation. BG also reduced vascular bearing, adhesion, and neutrophil migration, and consequently, minimized the damage caused by oxidative stress and lipid peroxidation. BG did not present toxic effects in the evaluated tests, evidencing its potential as an active ingredient for new drugs. However, it is necessary to conduct more detailed studies on mechanisms of action for BG.

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CONFLICT OF INTEREST

All authors of this study declare that there are no financial or commercial conflicts of interest.

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