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RESEARCH ARTICLE

Oral administration of the flavonoid myricitrin prevents dextran sulfate sodium-induced experimental colitis in mice through modulation of PI3K/Akt signaling pathway

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Scope: We investigated the protective effect of the flavonoid myricitrin in dextran sulfate sodium (DSS) induced colitis as promising candidate for the treatment of ulcerative colitis which is considered an important worldwide public health problem.

Methods and results: Male CD1 mice were provided with a solution of filtered water containing 3% w/v DSS ad libitum over a 5-day period followed by 2 days with normal drinking water. Myricitrin was administered orally, once a day, at the doses 1, 3, and 10 mg/kg of body weight. At the end of day 7th, the animals were euthanized and the colonic tissue was collected to be analyzed by RT-PCR, immunohistochemistry and Western blot. Our results showed that oral treatment with myricitrin exerts consistent anti-inflammatory action in DSS-induced acute colitis in mice by the inhibition of the Akt/phosphatidylinositol-3 kinase-dependent phosphorylation. Consequently, the phosphorylation of mitogen-activated protein kinases (MAPK) p38, extracellular signal-regulated protein kinase (ERK1/2), and c-Jun *N*-terminal kinase and of the nuclear factor B (NF-κB) was reduced and prevented an increase in the cytokines/chemokines levels.

Conclusion: Together, these data reveal that the anti-inflammatory effect of myricitrin in DSS-induced colitis in mice is likely associated with its ability to prevent the activation of upstream kinases, such as phosphatidylinositol-3 kinase-dependent Akt, NF-κB, and mitogen-activated protein kinase.

Keywords:
DSS-induced colitis / MAPK / Myricitrin / NF-κB / PI3K/Akt pathway

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1 Introduction

Inflammatory bowel diseases (IBD) are a group of chronic inflammatory diseases divided into two major forms, ulcerative colitis (UC) and Crohn's disease [1]. The IBD are characterized by an impairment in the mucosal integrity due to the extent of leukocytes infiltration and the

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Abbreviations: **Akt**, protein kinase C-related serine/threonine kinase; **COX-2**, cyclooxygenase-2; **DSS**, dextran sulfate sodium; **ERK**, extracellular signal-regulated protein kinase; **IBD**, inflammatory bowel diseases; **JNK**, c-Jun *N*-terminal kinases; **KC**, keratinocyte-derived chemokine; **MAPK**, mitogen-activated protein kinase; **MPO**, myeloperoxidase; **NF-κB**, nuclear factor-κB; **NOS2**, nitric oxide synthase 2; **PI3K**, phosphatidylinositol-3 kinase; **PKC**, protein kinase C; **TNF-α**, tumor necrosis factor-α; **UC**, ulcerative colitis

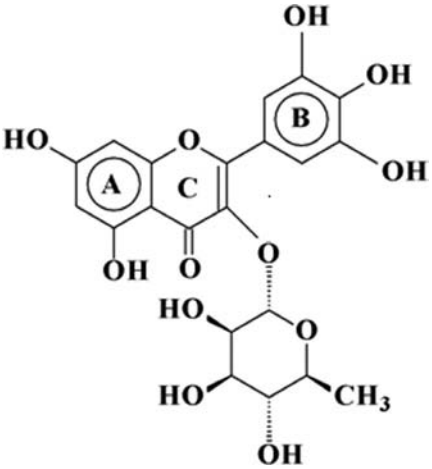

Figure 1. Molecular structure of myricitrin.

release of proinflammatory mediators, such as cytokines, tumor necrosis factor-α (TNF-α), IL-1β and, chemokines, keratinocyte-derived chemokine (CXCL1/KC) [2]. The intracellular pathways activated in IBD include mitogen-activated protein kinase (MAPK), PI-3K, protein kinase C-related serine/threonine kinase (Akt) (also known as protein kinase B or PKB), and nuclear factor-κB (NF-κB) [3]. Of interest, dietary polyphenols are known to modulate the inflammatory process by decreasing the production and expression of proinflammatory cytokines/chemokines due to the inhibition of transcription factors, including NF-κB and AP-1, and modulation of MAPKs [4]. In addition, there are numerous preclinical and some clinical studies suggesting that most naturally occurring flavonoids might present potential therapeutic properties for the treatment of chronic disorders including IBD, mainly by regulating the expression of inflammatory mediators through the inhibition of transcription factors and kinases, such as NF-κB and MAPKs [5–8].

The flavonoid myricitin (Fig. 1) belongs to the flavonol subgroup and is found in the leaves of *Eugenia uniflora* [9]. Previous studies have shown that myricitrin has antioxidant activity in vitro [10], anxiolytic effect with no signs of sedation [11], antipsychotic-like effects in mice [12], and antinociceptive action in acute and persistent pain in mice [13, 14]. In recent studies, myricitrin prevented proinflammatory TNF-α production in a macrophage cell line, Raw 264.7 cells [15], which may represent a putative mechanism for the anti-inflammatory activity of myricitrin. The mechanisms by which myricitrin exerts its pharmacological properties have already been suggested in different experimental models, both in vivo and in vitro, and seem to be associated with inhibition of different subtypes of protein kinase C (PKC), such as PKC-α and -ε [13], inhibition of phospho-p38 MAPK signaling [16], nitric oxide synthase 2 (NOS2), cyclooxygenase-2 (COX-2), TNF-α [17], and myeloperoxidase (MPO) [18].

Taking into account the anti-inflammatory properties of flavonoids, including their effects in experimental colitis models [6, 7], these compounds are becoming attractive molecules for treating inflammatory disorders. The present study investigated the anti-inflammatory effect of myricitrin against dextran sulfate sodium (DSS) induced colitis in mice. Our data show that myricitrin exhibited pronounced anti-inflammatory effect by mechanisms that are related to the inhibition of the upstream kinases phosphatidylinositol-3 kinase (PI3K) dependent Akt, which then modulates the activity of MAPK and NF-κB. Consequently, there was a reduction of PKC-ε, IL-6, TNF-α, KC, and COX-2 overexpression in colonic tissue and, lately, a reduction of neutrophil migration to the inflammatory site.

2 Materials and methods

2.1 Animals

Experiments were conducted using male CD1 mice (from 8 to 10 weeks of age, 25–35 g) obtained from the Laboratory of Experimental Pharmacology at the Universidade Federal de Santa Catarina (UFSC). Mice were housed at 22 ± 1°C under a light/dark cycle (12/12h) with access to food and water ad libitum. Experiments were performed during the light phase of the cycle. All procedures used in the present study followed the Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23) and were approved by the Animal Ethics Committee of the Universidade Federal de Santa Catarina (CEUA-UFSC, protocol number PP00496).

2.2 Drugs and reagents

Myricitrin was isolated from the leaves of the plant *E. uniflora* at the Department of Chemistry, UFSC, Brazil by using a procedure similar to that described previously [19]. Mirycitrin was identified by spectral analyses (RMN-1H) and (RMN-13C) and by comparison with the published spectra, as previously described [20]. The flavonoid myricitrin used in this study presented purity greater than 98%. Myricitrin was dissolved in 5% Tween-80 and then diluted in saline solution (0.9% NaCl). DSS (M.W. 36 000–50 000 kDa) was obtained from MP Biochemicals (Solon, OH, USA). Duoset kits for mouse IL-6 and CXCL1/KC were obtained from R&D Systems (Minneapolis, MN, USA). Monoclonal mouse anti-phospho-p65 NF-κB, monoclonal rabbit anti-phospho-Akt, polyclonal goat anti-phospho-extracellular signal-regulated protein kinase (ERK) 1/2, monoclonal mouse β-actin antibody, and polyclonal anti-COX-2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal rabbit anti-phospho-p38 (Thr 182), monoclonal mouse anti-phospho-c-Jun *N*-terminal kinases (JNK) (G-7), and polyclonal rabbit anti-novel PKC-ε (C-15) were purchased from Santa Cruz Biotechnology. Inc. (Santa Cruz, CA, USA).

Secondary polyclonal antibody anti-rabbit, anti-mouse, and anti-goat were purchased from DakoCytomation (Carpinteria, CA, USA). Trizol, moloney murine leukemia virus (M-MLV) reverse transcriptase and streptavidin–Horseradish Peroxidase (HRP) reagent were purchased from Invitrogen (Carlsbad, CA, USA). Primers and probes for mouse TNF-α (Mm00443258_m1), IL-6 (Mm99999064_m1), prostaglandin 2 (COX-2) (Mm01307334_g1), NOS2 (Mm 01309898_m1), glyceraldehyde 3-phosphate dehydrogenase (NM_008084.2) and TaqManH Universal Polymerase Chain Reaction (PCR) Master Mix Kit were purchased from Applied Biosystems (Foster City, CA, USA).

2.3 DSS-induced colitis and pharmacological treatments

This model of colitis was employed as previously described [21, 22] and consisted of adding DSS 3% w/v to the animals' drinking water. Water (with or without DSS) and foods were provided ad libitum. The treatment was performed for 5 days, followed by 2 days with DSS-free water. The DSS solution was replaced daily and the consumption measured. At the end of 7 days, the animals were euthanized. The control group received only normal drinking water ad libitum during the whole experiment. The treated group received myricitrin at different doses (1, 3, and 10 mg/kg), orally by gavage (p.o.), once a day. The treatment started 1 h before administration of DSS and followed until the seventh day. The animals that received myricitrin were sacrificed 3 h after the last administration of myricitrin. Control groups received vehicle solution p.o., once a day, from day 0 to 7.

2.4 Monitoring DSS-induced colitis

Animals were examined daily from day 0 to 7 for body weight change and disease activity index (DAI) as described previously [23, 24]. Briefly, DAI was determined by the sum of scores given for: body weight loss (scored as: 0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, over 20%), stool consistency (scored as: 0, well-formed pellets; 2, loose stools; 4, diarrhea) and presence or absence of fecal blood (scored as: 0, negative hemoccult test; 2, positive hemoccult test; 4, gross bleeding).

2.5 Macroscopic damage assessment of DSS-induced colitis

After 7 days, animals were sacrificed and the colons were collected. Colons were measured for: weight (stools were removed), consistency of the stool found within of tissue, gross macroscopic appearance and length (measured 1 cm above the anus to the top of the cecum), as previously described [25]. Macroscopic damage was assessed by the sum of given scores: stool consistency (0, normal well-formed fecal pellets; 1, loosely shaped moist pellets; 2, amorphous, moist, sticky pellets; 3, diarrhea; plus 1 for presence of blood in stool), colon damage (0, no inflammation; 1, reddening, mild inflammation; 2, moderate or more widely distributed inflammation; 3, severe and/or extensively distributed inflammation), colon weight loss (0, for $\leq$ 5%; 1, for 5–14%; 2, for 15–24%; 3, for 25–35%; 4, for $\geq$ 35%) and colon length shortening (0, for $\leq$5%; 1, for 5–14%; 2, for 15–24%; 3, for 25–35%; 4, for $\geq$ 35%), with a maximum total score of 15 [25].

2.6 Microscopic damage assessment of DSS-induced colitis

For analysis of microscopic damage, immediately after colon collection, the distal portion was excised and fixed in a 4% w/v formaldehyde solution for 72 h and then transferred to 70% ethanol. The colon samples were embedded in paraffin, sectioned transversally (4 μm of thickness), mounted on glass slides, deparaffinized, and stained with hematoxylin and eosin. Samples were analyzed by light microscopy and scored as described previously [26, 27].

2.7 Western blot analysis

Equal amounts of protein for each sample (35 μg) were loaded per lane and electrophoretically separated using 10% denaturing SDS-PAGE. Afterward, the proteins were transferred to nitrocellulose membranes using a Mini Trans-Blot Cell System (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's protocol. Western blot analysis was carried out using monoclonal rabbit anti-phospho-Akt (1:1000), and incubated overnight. Following washing, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:10 000). The immunocomplexes were visualized using SuperSignal West Femto Chemiluminescent Substrate Detection System (Thermo Fischer Scientific, Rockford, IL, USA) and densitometric values were normalized using monoclonal mouse β-actin antibody (1:500). Protein levels were quantified by optical density using Image-J Software and expressed as the ratio to β-actin represented by arbitrary units.

2.8 Immunohistochemical analysis

Immunohistochemical analysis was performed according to methods previously described [28]. Paraffin-embedded slices of colon tissue (4 μm) were incubated with primary antibodies: monoclonal mouse anti-phospho-p65 NF-kB (1:50), polyclonal goat anti-phospho-ERK1/2 (1:300), polyclonal anti-COX-2 (1:50), polyclonal rabbit anti-phospho-p38 (Thr 182; 1:100), monoclonal mouse anti-phospho-JNK (G-7; 1:300), and polyclonal rabbit anti-nPKC-ε (C-15; 1:100). After overnight incubation at 4°C with primary antibodies, the slices were washed with PBS and incubated

with the secondary antibody Envision Plus (ready to use) for 1 h at room temperature. After incubation with the appropriate biotinylated secondary antibody, the sections were developed with 3,3′-diaminobenzidine (DakoCytomation, Glostrup, Denmark) in a chromogen solution and counter-stained with Harris hematoxylin. Images of colon sections were acquired using a Sight DS-5M-L1 digital camera connected to an Eclipse 50i light microscope (both from Nikon, Melville, NY, USA) and image acquisition software (Qcapture Pro 5.1; QImaging, Surrey, BC, Canada). All image (five ocular fields per section, —six to eight animals per group) were captured and a threshold optical density that best discriminated staining from the background was performed using NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD, USA). For immunohistochemistry analysis, total pixel intensity was determined and data are expressed as optical density (OD).

2.9 RNA extraction and quantification by real-time PCR

Total RNA was extracted from the colon segment using the Trizol protocol (Invitrogen). The total concentration of RNA was determined by NanoDropTM 1100 (NanoDrop Technologies, Wilmington, DE, USA). A reverse transcription assay was performed as described in the M-MLV Reverse Transcriptase protocol according to the manufacturer's instructions. cDNA (300 ng) was amplified in duplicate using TaqMan Universal PCR Master Mix Kit with specific TaqMan Gene Expression target genes, the 3′ quencher MGB and FAM-labeled probes for mouse TNF-α (Mm00443258_m1), IL-6 (Mm99999064_m1), COX-2 (Mm01307334_g1), NOS2 (Mm 01309898_m1), and glyceraldehyde 3-phosphate dehydrogenase (NM_008084.2), which was used as an endogenous control for normalization. The PCR reactions were performed in a 96-well Optical Reaction Plate (Applied Biosystems). The parameters for the thermocycler were: 50°C for 2 min, 95°C for 10 min, 50 cycles of 95°C for 15 s, and 60°C for 1 min. Expression of the target genes was calibrated against conditions found in control animals, i.e. mice that received only vehicle: 5% Tween-80 in saline 0.9% NaCl.

2.10 Determination of cytokine levels

For the determination of cytokine levels, the colon segments were homogenized in phosphate buffer containing 0.05% Tween-20, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA, and 20 UI aprotinin A. The homogenate was centrifuged at 3000 × *g* for 10 min, and the supernatant was used to determine the levels of IL-6 and CXCL1/KC using ELISA. The amount of protein in each sample was measured using the Lowry method [29].

2.11 Statistical analysis

All analyses were conducted using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as means ± SEM of—six to eight mice/group. Statistical analysis was performed using Kruskal–Wallis followed by Dunn's test for nonparametric data, and, one-way ANOVA followed by Newman–Keuls test was used for parametric data. Differences with $p \leq 0.05$ were considered to be statistically significant.

3 Results

3.1 Preventive treatment with myricitrin ameliorates DSS-induced acute colitis

The oral administration with DSS for 7 days induced acute colitis characterized by the presence of blood in the stools, marked diarrhea, and body-weight loss in mice, which resulted in a significant increase in the DAI from day 2 onward when compared to healthy mice (vehicle group) (Fig. 2A and B). In order to determine a possible dose-dependent effect of myricitrin, the compound was administered by oral route at three different doses: 1, 3, and 10 mg/kg. Interestingly, the treatment with 3 and 10 mg/kg of myricitrin significantly reduced the DAI score, from day 2 onward (Fig. 2A and B). The consumption of water was measured during the whole experiment and there were no significant differences between groups (data not shown). The macroscopic analysis of the colon showed that DSS administration resulted in marked inflammation associated with hyperemia, ulceration, and bowel wall thickening, leading to an increase in the macroscopic colon damage score and body-weight loss (Fig. 2C and D). Oral treatment with myricitrin consistently reduced the macroscopic damage score when the flavonoid was used at the two higher doses (Fig. 2D). It has already been demonstrated that colon length is inversely associated with the severity of DSS-induced colitis [21]. The significant shortening of the colon length was observed in DSS-induced acute colitis, which was protected by preventive treatment with myricitrin at the three doses administered (Fig. 2E and F). The treatment with myricitrin (10 mg/kg) alone (in the absence of DSS-induced colitis) did not affect the DAI score, body-weight change, colon length, or macroscopic damage, when compared to the control mice group (vehicle group) (data not shown).

As the dose of myricitrin at 10 mg/kg was more effective when assessed in all parameters analyzed, this dose was used in all subsequent experiments to investigate the mechanism underlying its anti-inflammatory action.

3.2 Myricitrin reduces microscopic colon damage in DSS-induced acute colitis

The histological and morphological characteristics of the colons were carried out after hematoxylin and eosin staining

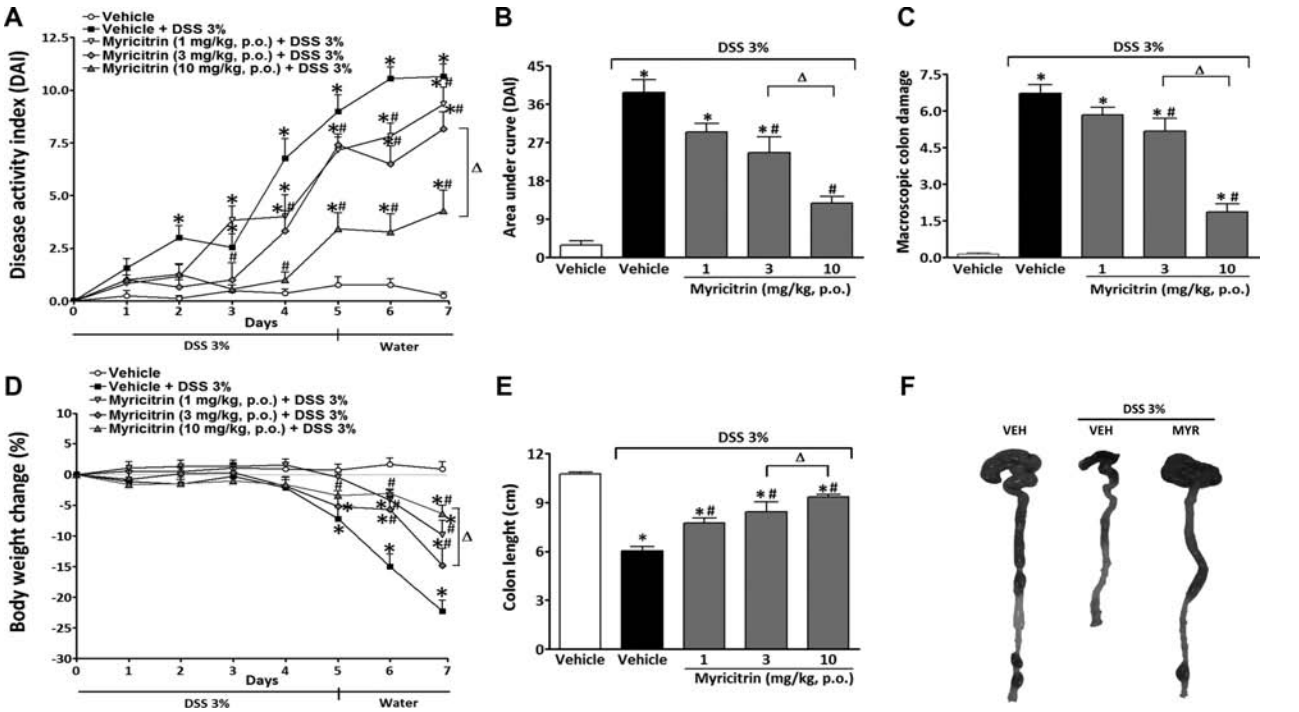

Figure 2. Myricitrin ameliorates DSS-induced acute colitis. Preventive oral treatment with myricitrin improved the DAI score (A and B), prevented the macroscopic colon damage (C), reduced body weight loss (D), and enhanced colon length (E), compared with mice from the DSS group. Representative photographs of colons from vehicle group (VEH), DSS-treated mice (DSS), and preventive myricitrin-treated mice (MYR, 10 mg/kg, p.o.) (F). Data are reported as means ± SEM of six to eight mice per group. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus DSS treated group; Δ = significant among groups.

and the representative results and microscopic score are shown in Fig. 3A–G. In control mice (vehicle group), the colons presented a typical morphology of normal crypts, abundant goblet cells, a small number of lamina propria mononuclear cells, no thickening of the mucosa, and complete absence of ulcerations or crypt abscesses (Fig. 3A and D). On the other hand, the DSS-treated mice presented a severe epithelial damage with extensive cellular infiltration into the lamina propria and colon mucosa, depletion of the goblet cells, mucosa thickening, and complete destruction of the architecture (Fig. 3B and E), resulting in a high microscopic damage score (Fig. 3G). Relevantly, we observed that oral treatment with myricitrin (10 mg/kg) completely blocked the inflammatory cell infiltration with minimal loss of epithelial cells (Fig. 3C and F), which resulted in a very low microscopic damage score, when compared with colons from mice treated with DSS (Fig. 3G).

3.3 Myricitrin reduces Akt, MAPK, and NF-κB phosphorylation in DSS-induced colitis

In order to determine whether myricitrin was able to modulate the PI3k/Akt signaling pathway in DSS-induced colitis model, we assessed the amount of p-Akt (a marker of PI3K activation) in colonic tissue of DSS-treated mice. As shown in Fig. 4, there was a significant increase in Akt phosphorylation in the colonic tissue of DSS-induced mice and it was prevented when the animals were treated with myricitrin (10 mg/kg). The present results suggest that myricitrin exerts its anti-inflammatory effect by inhibiting the PI3k/Akt signaling pathway.

The Akt downstream cascade, MAPK, was also investigated and the results are presented in Fig. 5. Low levels of phospho-p38 (A), phospho-JNK (E), and phospho-ERK (I) were detected in the vehicle-treated mouse colon tissue, but these values were markedly increased when assessed 7 days following DSS administration (Fig. 5B, F, and J, respectively). The same result for phospho-JNK in the colonic tissue could be observed when analyzed by Western blot method (Supporting Information, Fig. 1). Of note, the pretreatment with myricitrin (10 mg/kg p.o.) significantly inhibited the phosphorylation of p38 (Fig. 5C and D), JNK (Fig. 5G and H), and, ERK1/2 (Fig. 5K and L) in the colonic tissue following DSS-induced colitis.

The treatment with DSS induced pronounced phosphorylation of NF-κB (Fig. 6B and D), when compared with control group (Fig. 6A and D). Oral treatment with myricitrin (10 mg/kg) significantly reduced the phosphorylation of NF-κB in the colon tissue (Fig. 6C and D).

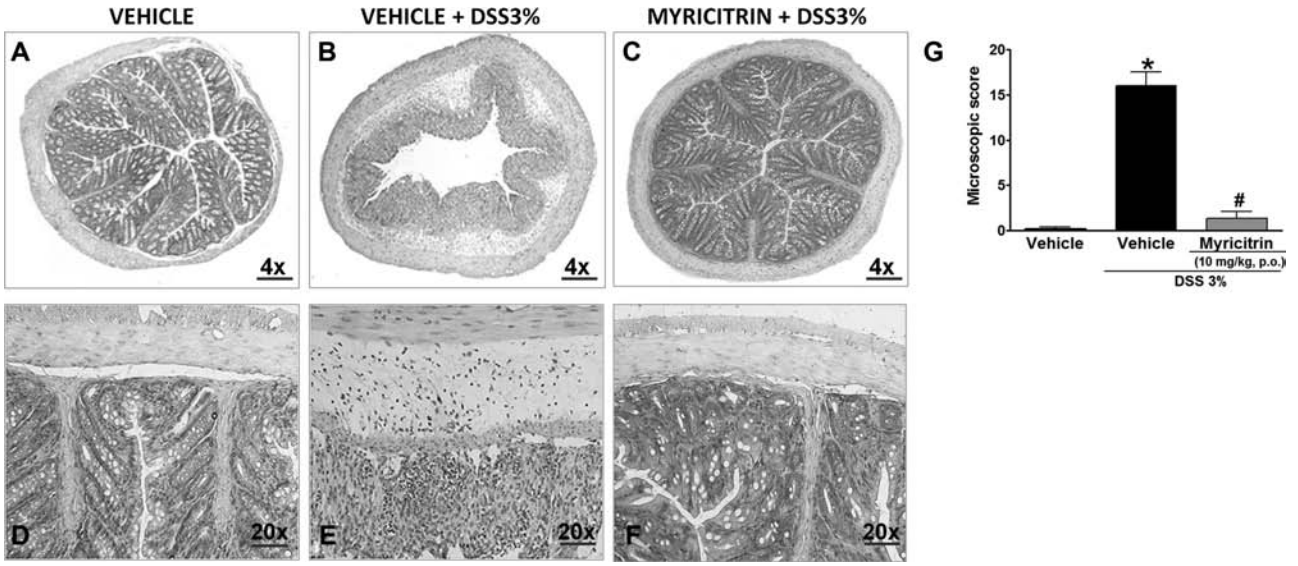

Figure 3. Myricitrin reduces microscopic colon damage in DSS-induced acute colitis. Representative paraffin sections of colons from control mice (A and D), DSS plus vehicle-treated (B and E), and DSS plus myricitrin-treated mice (C and F), stained with hematoxylin and eosin with original magnification, × 4 (A–C) and × 20 (D–F). The treatment with myricitrin (10 mg/kg, p.o.) decreased the microscopic damage score in mice colonic tissue (G). Data are reported as means ± SEM ($n = 5$ to 7 mice per group). *$p < 0.05$ versus control group; #$p < 0.05$ versus DSS-treated group.

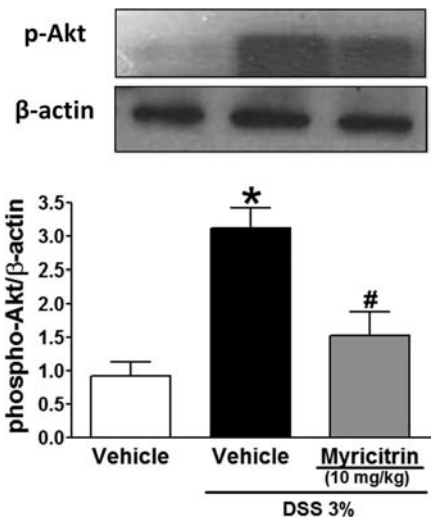

Figure 4. Myricitrin reduces Akt phosphorylation in colonic tissue of DSS-induced mice. Western blot gel and quantification of the ratio between Akt protein band and β-actin protein band in colonic tissue of DSS-induced mice. The treatment with myricitrin (10 mg/kg) decreased the Akt phosphorylation in colonic tissue from mice treated with DSS. Each column represents the mean ± SEM ($n = 5$ to 7 mice per group). *$p < 0.05$ versus control group; #$p < 0.05$ versus DSS-treated group.

3.4 Effects of myricitrin on protein kinase C (PKC) epsilon expression

Because PKC-ε has a key role in inflammatory and pain signaling transduction [30–32], we assessed the expression of PKC-ε in the colonic tissue. There was a significant increase for PKC-ε immunostaining in the colon of DSS-treated mice when compared to control mice group (Fig. 7A and B). Notably, the oral treatment with myricitrin (10 mg/kg) consistently prevented the increase of PKC-ε in colonic tissue following DSS-induced colitis (Fig. 7C and D).

3.5 Myricitrin reduces mRNA and protein expression of inflammatory enzymes in DSS-induced colitis

Using real-time PCR and immunohistochemistry assays, we investigated whether the anti-inflammatory effects of myricitrin were also linked to a decrease in enzymes expression in colon tissue. As shown in Fig. 8, only low levels of both NOS2 (Fig. 8A) and COX-2 (Fig. 8B–D) were detected in tissue of control mice, but these values were markedly increased, following DSS administration (Fig. 8 A–C and E). Relevantly, the treatment with myricitrin (10 mg/kg, p.o.) significantly inhibited the upregulation of mRNA expression of NOS2 (Fig. 8A). In addition, myricitrin also significantly reduced mRNA (Fig. 8B) and protein level (Fig. 8C and F) of COX-2 in the colon tissue after DSS administration.

3.6 Myricitrin reduces the levels of cytokines/chemokines in DSS-induced colitis

As shown in Fig. 9, treatment with DSS significantly increased the levels of IL-6 (Fig. 9A) and CXCL1/KC (Fig. 9B), as well as the mRNA expression of TNF-α (Fig. 9C) and IL-6 (Fig. 9D) in the colonic tissue. Interestingly, oral treatment

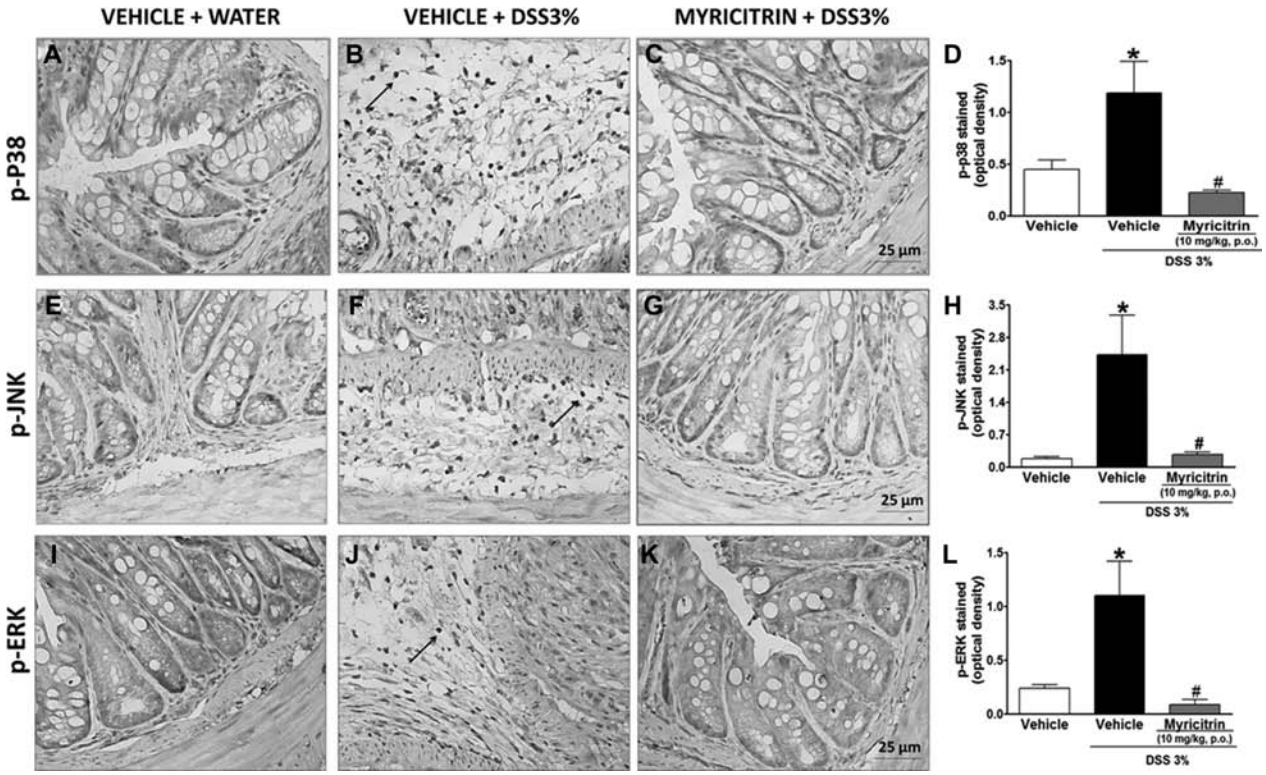

Figure 5. Myricitrin reduces colonic MAPK phosphorylation in DSS-induced colitis. Representative immunohistochemistry sections of phospho-p38 (A–C), phospho-JNK (E–G) and phospho-ERK (I–K) in colonic tissues from mice treated with (A, E, and I) vehicle, (B, F, and J) DSS alone, or (C, G, and K) DSS plus myricitrin (10 mg/kg, p.o.). Treatment with myricitrin (10 mg/kg, p.o.) significantly reduced phospho-p38 (C), phosphor-JNK (G) and phospho-ERK (K) immunostaining when compared to DSS group (B, F, and J, respectively). (D, H, and L) Quantitative histrogram of the immunostaining for phospho-p38 (D), phospho-JNK (H), and phospho-ERK (L) in colon tissue. Scale bar corresponds to 25 μm and applies throughout. Data are reported as means ± SEM of six to eight mice per group. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus DSS-treated group. Arrow indicates the positive staining.

with myricitrin (10 mg/kg) significantly inhibited both the IL-6 and CXCL1/KC levels (Fig. 9A and B) and the expression of mRNA for both, TNF-α and IL-6 (Fig. 9C and D). Such data suggest that the anti-inflammatory action of myricitrin is likely to be associated with its ability to inhibit the neutrophil influx, as well as, release and expression of proinflammatory cytokines/chemokines in the colonic tissue after DSS administration.

4 Discussion

IBD is a chronic inflammatory disorder of the gastrointestinal tract and represents an important worldwide public health problem [1]. The current therapy used for the management of human IBD is very expensive and, mainly, is not effective in all patients. In addition, the chronic use of these therapies is frequently associated with undesirable side

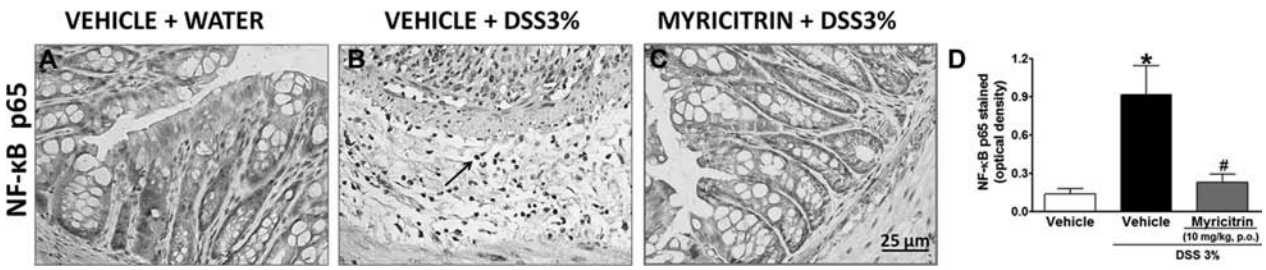

Figure 6. Myricitrin inhibits NF-kB phosphorylation induced by DSS in colonic tissue. Representative sections of p65 NF-κB immunohistochemistry in colonic tissues from mice treated with (A) vehicle (B) DSS alone or (C) DSS plus myricitrin (10 mg/kg, p.o.). (D) Quantitative histogram for phosphorylated NF-κB immunostaining. Scale bar corresponds to 25 μm and applies throughout. Data are reported as means ± SEM of six to eight mice per group. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus DSS-treated group. Arrow indicates the positive staining.

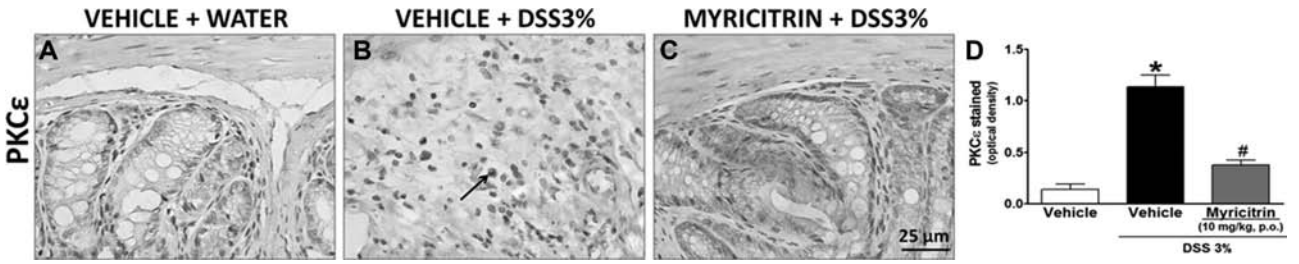

Figure 7. Myricitrin reduces the expression of PKC-ε in colonic tissue. Representative immunohistochemistry sections of colonic tissues from mice treated with (A) vehicle, (B) DSS alone, or (C) DSS plus myricitrin (10 mg/kg, p.o.). Scale bar corresponds to 25 μm and applies throughout. Oral treatment with myricitrin (10 mg/kg, once a day) significantly reduced PKC-ε (C) immunostaining level when compared to DSS group (B). (D) Quantitative histogram for PKC-ε immunostaining in colon tissue. Data are reported as means $\pm$ SEM ($n = 5$ to 7 mice per group). $^{*}p < 0.05$ versus the vehicle group; $^{\#}p < 0.05$ versus DSS-treated group. Arrow indicates the positive staining.

effects [33]. Therefore, effective and safe new therapies for the management of IBD are needed. In this context, several studies have been performed to demonstrate the preventive and therapeutic effect of polyphenols in animal models of IBD [34]. However, just few reports have demonstrated the sequential signaling cascade triggered by polyphenols to counteract inflammation in UC [35, 36]. In addition, little structural difference changes the ability of the flavonoids to affect a specific pathway [5]. Therefore, it is not possible to assume that flavonoids from the same subfamily will behave identically. Taking this into account, we selected the flavonoid myricitrin to evaluate its effect in a mouse model of UC because this flavonoid has demonstrated potent anti-inflammatory properties against acute and chronic inflammation by mechanisms dependent on the inhibition of kinases phosphorylation in vivo [13, 14, 16]. In this paper, we reported that myricitrin consistently ameliorated the inflammatory symptoms associated with DSS-induced colitis. We followed the putative proteins that were modulated by myricitrin and found that their protective effect was clearly related to inhibition of upstream kinase, PI3K-dependent Akt phosphorylation and, consequently, MAPKs phosphorylation

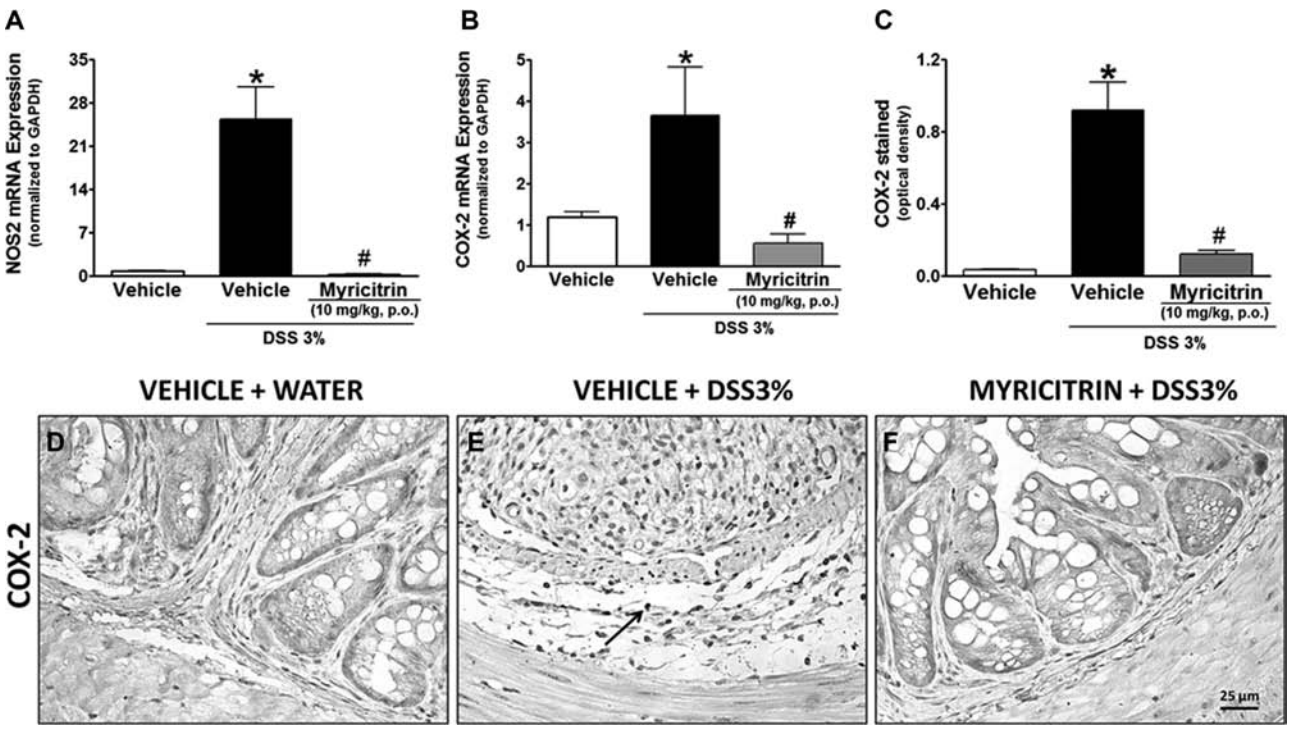

Figure 8. Myricitrin reduces the expression of inflammatory enzymes in DSS-induced colitis. mRNA expression of NOS2 and COX-2 (A and B) by real-time PCR. Data were normalized to the relative amount of glyceraldehyde 3-phosphate dehydrogenase mRNA. (C) Histogram representation for COX-2 immunostaining in colon tissue. Immunohistochemistry of COX-2 levels in (D) control, (E) DSS vehicle, and (F) DSS -myricitrin (10 mg/kg) treated group. Scale bar corresponds to 25 μm and applies throughout. The oral treatment with myricitrin (10 mg/kg) reduced the colonic protein levels and/or expression of NOS-2 and COX-2. Data are reported as means $\pm$ SEM ($n = 5$ to 7 mice per group). $^{*}p < 0.05$ versus the vehicle group; $^{\#}p < 0.05$ versus DSS-treated group. Arrow indicates the positive staining.

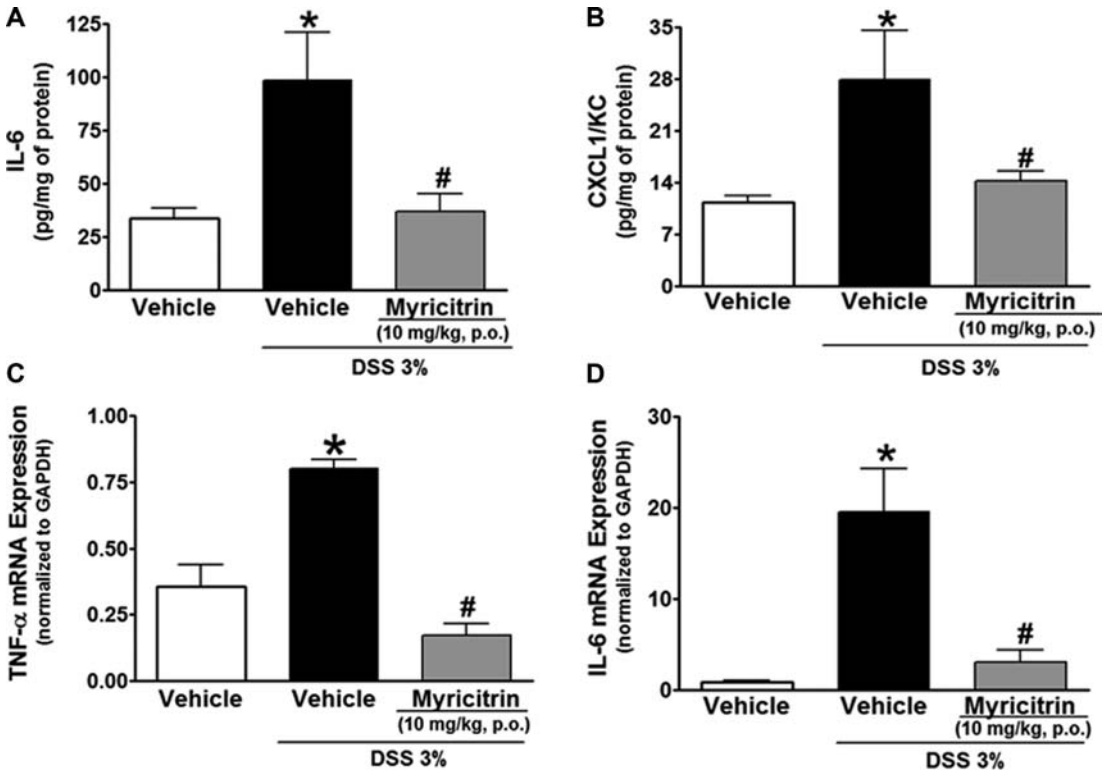

Figure 9. Myricitrin reduces the levels of cytokines/chemokines in DSS-induced colitis. Protein levels of (A) IL-6 and (B) CXCL1/KC quantified by ELISA assay and mRNA expression for (C) TNF-α and (D) IL-6 measured by real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase mRNA was used to normalize the relative amount of mRNA. The oral treatment with myricitrin (10 mg/kg) reduced the colonic protein levels and/or expression of IL-6, CXCL1/KC, and TNF-α. Data are reported as means ± SEM ($n = 5$ to 7 mice per group). *$p < 0.05$ versus the vehicle group; #$p < 0.05$ versus DSS-treated group.

(JNK, ERK1/2, and p38) and the transcriptional factor NF-κB. The inhibition of this signaling cascade brought about a reduction in PKC-ε, COX-2, NOS2, CXCL-1/KC, IL-6, TNF-α overexpression and neutrophils migration in the colon tissue. All these mediators are involved in chronic IBD and therefore, myricitrin might be a promising therapy in advanced intestinal chronic inflammation. However, additional models for chronic bowel disease, i.e. a longer DSS treatment or IL10-$^{-/-}$-induced chronic colitis, are necessary to confirm it.

The PI3K-dependent Akt phosphorylation is an early event for the inflammatory signaling in UC [37]. The Akt is a serine/threonine kinase, also known as protein kinase B and is the direct downstream target of PI3K [38, 39]. In fact, the inhibition of PI3K by wortmaninn decreases Akt phosphorylation and colon damage in UC [37]. In addition, Selvaraj et al. [40] described that a transient activation of Akt is followed by ERK1/2 activation in human monocytes. Our results demonstrated an increase in Akt, ERK1/2, JNK, and p38 MAPK phosphorylation in the colonic tissue of mice treated with DSS. The treatment with myricitrin caused inhibition in the phosphorylation of Akt and all the three MAPKs tested. Therefore, we hypothesize that myricitrin exerts an inhibitory effect in an upstream protein from this cascade. Indeed, myricitrin has been reported to inhibit phosphoinositide 3-kinase (PI3K), Akt phosphorylation [13, 41, 42], and PKC translocation to the membrane [13].

It is now well recognized that the expression of most inflammatory cell mediators in some inflammatory responses is critically dependent on the activation of MAPK [43, 44], whose activation is regulated by PKC [31]. Of interest, the exacerbated activation of MAPKs has been associated with the development of IBD representing an important target for the development of new anti-inflammatory therapy [44, 45]. The phosphorylation of MAPKs generates a positive feedback loop in inflammation since it activates transcriptional factors, such as, NF-κB, that regulates the gene transcription of proinflammatory cytokines [45–47]. Moreover, NF-κB phosphorylation plays a critical role in most immune and inflammatory processes including the etiology of IBD [48]. The activation of NF-κB by different stimuli leads to the induction of numerous genes encoding proinflammatory cytokines and chemokines, and inducible enzymes, such as COX-2 and NOS2 to promote the recruitment and activation of immune cells [49]. The reported anti-inflammatory properties of some plant-derived flavonoids have been associated with their ability to modulate NF-κB activation and translocation to the cell nucleus [5, 6, 17]. Results from the present study corroborate these findings by demonstrating that oral treatment with

myricitrin consistently prevented the translocation of p65, a subunit of NF-κB, into the nucleus. In addition, myricitrin decreased COX-2 and NOS expression in colon tissue. It has been noticed that the expression levels of COX-2 and NOS2 are increased in patients with UC [50, 51]. Of note, earlier studies have shown that flavonoids including apigenin, luteolin, kaempferol, myricetin, rutin, and genistein, can downregulate the production of NO by regulating the expression and activation of the NOS2 enzyme [52, 53], and, are able to decrease PGE2 production in vitro [54]. Our data corroborate these previous studies by showing that the reduction in colitis by myricitrin is associated with a significant inhibition of both NOS2 and COX-2 expression in the colon tissue. Beside a reduction in the expression of proinflammatory enzymes, myricitrin exerts a direct inhibition of MPO both in vivo and in vitro [14, 18]. These mechanisms may explain, at least in part, the anti-inflammatory effect of myricitrin in the DSS-induced colitis experimental model.

The inflammatory degree in colon tissue was confirmed by an upregulation of PKC-ε. The PKC-ε isoform, is a calcium-independent activated protein that modulates many crucial cell functions, including proliferation, differentiation, and survival [31] and might be responsible for the non-resolution of inflammation. In the present study, we observed that the oral treatment with myricitrin significantly reduced the PKC-ε immunostaining in colonic tissue, an effect that may explain the pronounced anti-inflammatory property of myricitrin in DSS-induced colitis. Studies carried out in both, human IBD [55] and animal model of colitis [56, 57] have shown a positive relationship between increased neutrophil migration to the inflammatory site and the exacerbation of the disease. For instance, the inflammatory chemokine CXCL1/KC plays a crucial role in regulating the neutrophil migration to the colonic tissue [21]. In agreement with these observations, the present results showed that the treatment with myricitrin reduced the chemokine CXCL1/KC levels in colonic tissue, an effect that may account for the reduction of neutrophil influx into the inflammatory site, diminished colon damage, and amelioration of colon inflammation when compared with the group treated with DSS.

The upregulation of proinflammatory cytokines, TNF-α and IL-6, is also a hallmark of inflammation in IBD, Crohn's disease, and UC [58, 59]. For this reason, the regulation of these proinflammatory cytokines production has been extensively studied and even considered as a novel therapeutic target for the treatment of IBD [59, 60]. In our hands, myicitrin protected against the upregulation of both IL-6 and TNF-α, leading to the reduction of inflammatory damage to the colon. Taking into account the above data, we can infer that myricitrin modulates neutrophil activation and, in turn, regulates the cytokine production and expression, resulting in less damage to the colon. Noteworthy, the aglycone myricetin but not myricitrin inhibited TNF-α expression in LPS-activated murine macrophage (data not shown). This suggests that the aglycone is the main candidate to permeate cell to directly inhibit inflammatory pathways. In agreement to this view, experiments using the glycoside flavonoids, quercetrin and myricitrin and, their respective aglycones, quercetin and myricetin, have reported that only the aglycone form is active in vitro [7, 35]. In addition, myricetin is much more potent than myricitrin to inhibit MPO activity [18]. If on one hand myricetin and quercetin permeate the cell and inhibit inflammatory pathways, on the other hand, they present a very low potency in vivo when administered orally [7, 35, 61] probably by undergoing a rapid degradation in the digestive tract, as already described for the majority of glycoside flavonoids [62, 63].

In conclusion, our results demonstrate that the plant-derived flavonoid, myricitrin, when given orally, exerts an anti-inflammatory action in DSS-induced acute colitis in mice. The anti-inflammatory properties of myricitrin were associated to the inhibition of upstream kinases, PI3K-dependente Akt phosphorylation and, consequently, MAPKs and NF-κB phosphorylation. As a result, the overexpression of PKC-ε, COX-2, NOS2, CXCL-1/KC, IL-6, TNF-α and neutrophils migration were inhibited, which ameliorate micro and macroscopic parameters in DSS-induced colitis. Importantly, upstream proteins from inflammatory pathways are interesting targets to counteract tissue damage related to inflammation because it avoids the amplification of signal in inflammation and increase the potency of the purposed medicine. Therefore, myricitrin might constitute a promising molecule for the treatment of IBD.

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