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RESEARCH ARTICLE

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Plumericin prevents intestinal inflammation and oxidative stress in vitro and in vivo

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Abstract

Inflammatory bowel diseases (IBDs) are characterized by an inflammatory and oxidative stress condition in the intestinal tissue. In this study, we evaluated the effect of plumericin, one of the main bioactive components of *Himatanthus sucuuba* (Woodson) bark, on intestinal inflammation and oxidative stress, both in vitro and in vivo. The effect of plumericin (0.5-2 μM) in vitro was evaluated in rat intestinal epithelial cells (IEC-6) treated with lipopolysaccharides from *E. coli* (10 μg/mL) plus interferon-γ (10 U/mL). Moreover, a 2,4,6-dinitrobenzene sulfonic acid (DNBS)-induced colitis model was used to evaluate the anti-inflammatory and antioxidant activity of plumericin (3 mg/kg) in vivo. The results showed that plumericin significantly reduces intestinal inflammatory factors such as tumor necrosis factor-α, cyclooxygenase-2 and inducible nitric oxide synthase expression, and nitrotyrosine formation. Plumericin also inhibited nuclear factor-κB translocation, reactive oxygen species (ROS) release, and inflammasome activation. Moreover, plumericin activated the nuclear factor erythroid-derived 2 pathway in IEC-6. Using the DNBS-induced colitis model, a significant reduction in the weight loss and in the development of the macroscopic and histologic signs of colon injury, together with a reduced inflammatory and oxidative stress state, were observed in plumericin-treated mice. These results indicate that plumericin exerts a strong anti-inflammatory and antioxidant activity. Thus, it might be a candidate for the development of a new pharmacologic approach for IBDs treatment.

KEYWORDS

plumericin, intestinal epithelial cells, DNBS-induced colitis, inflammation, oxidative stress

Abbreviations: COX-2, cyclooxygenase-2; DAPI, 40,6-diamine-20-phenylindole dihydrochloride; DNBS, 2,4,6-dinitrobenzene sulfonic acid-induced colitis; DNBS, 2,4,6-dinitrobenzene sulfonic acid; FACSscan, fluorescence-activated cell sorter; H/E, hematoxylin/eosin; H_2DCF-DA, 2,7-dichlorofluorescein-diacetate; HO-1, heme oxygenase 1; i.p., intraperitoneal; IBDs, intestinal inflammatory bowel diseases; IEC-6, intestinal epithelial cell line-6; IFN-γ, interferon-γ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MDA, 1,1,3,3-tetramethoxypropan/99% malondialdehyde bis (dymethylacetal)/99%; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NQO1, NAD(P)H dehydrogenase (quinone 1); Nrf2, nuclear factor erythroid-derived 2; OD, optical density; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α.

Hermann Stuppner and Stefania Marzocco are equally contributed as last authors.

1 | INTRODUCTION

Inflammatory bowel diseases (IBDs) are uncontrolled inflammatory pathologies of the gastrointestinal tract that mainly include ulcerative colitis and Crohn's disease. Even though their etiology remains unknown, environmental factors, as well as infectious, immunological, psychological, and genetic ones, could be the main causes of the IBDs pathogenesis.[1] The intestinal epithelium, which lines the gastrointestinal tract, plays a central role in IBDs by forming a cell barrier between the host and various organisms present in the intestine, which is essential to maintain mucosal homeostasis. In particular, intestinal epithelial cells (IECs) can sense and respond to microbial stimuli to reiforce the barrier function and to participate in the coordination of an appropriate immune response.[2] During IBDs, the integrity of the intestinal layer is damaged and ingested materials and pathogens can cause inflammation by activating the epithelial cells, polymorphonuclear neutrophils, and macrophages to produce inflammatory cytokines and other mediators that further contribute to oxidative stress and perpetuate the inflammatory response in the gut.[3] The main factors involved in the inflammatory cascade during IBDs are cytokines (eg, tumor necrosis factor-α, TNF-α; interferon-γ, IFN-γ; interleukin-6, IL-6), which are also linked to nuclear factor-κB (NF-κB) activation, chemokines, adhesion molecules, and reactive oxygen species (ROS).[4,5] In particular, IBDs are associated with a disequilibrium between ROS and the antioxidant response, giving rise to oxidative stress[6] which is presently considered as potentially critical in the pathogenesis, progression, and severity of IBDs.[7] Thus, the control of the inflammation and the oxidative stress has a primary role in the IBDs treatment. Conventional therapy of IBDs uses anti-inflammatory and immunosuppressive corticosteroids as well as biological drugs. However, the low remission rate and the severe side effects of these therapies are not satisfactory.[8,9] Thus, there is great interest to find new therapeutic strategies for the treatment of IBDs.

Plant-derived natural products significantly contributed to drug discovery in the past and still provide an effective source for lead structure identification.[10] Preparations of the stem bark and latex of the Amazonian tree *Himatanthus sucuuba* (Spruce ex Müll.Arg.) Woodson (Apocynaceae) have been traditionally used in South America as anti-inflammatory, antitumor, analgesic, and antiulcer agents.[11] The spirolactone iridoid plumericin, a major bioactive constituent of *H. sucuuba*, has been shown to exhibit antiparasitic,[12] antimicrobial,[13] and antifungal[14] activities. Moreover, it has been shown to potentlynhibit the NF-κB pathway[15] and to possess antiproliferative properties in the vasculature[16] and it has been characterized as inhibitor of the TNF-α-induced senescence of endothelial cells.[17]

Therefore, in order to evaluate the therapeutical potential of plumericin in IBDs, we investigated the effects of this natural product on intestinal inflammation, oxidative stress, and colon injury, both in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | In vitro studies

2.1.1 | Reagents

Unless otherwise specified, all reagents and compounds were purchased from Sigma Chemicals Company (Sigma).

2.1.2 | Plant material

Plumericin was isolated from the bark of *H. sucuuba*. Powdered bark material (batch number BEL0207) was purchased from Raintree Nutrition, Inc in July 2008. Species identity including a certificate of analysis was provided by the supplier. The identity of the plant material was further confirmed by examining the specimen microscopically. A voucher specimen (No. JR-20080730-A1) has been deposited at the Institute of Pharmacy/Pharmacognosy of the University of Innsbruck. Consequently, the phytochemical work was performed leading to the isolation of plumericin as colorless crystals with a purity ≥95%. Detailed description of the phytochemical work including the isolation and identification of plumericin and other compounds from *H. sucuuba* has previously been provided elsewhere.[18] The compound was stored at −20°C until the realization of the experiments described in this study. At this point, to confirm its stability, the identity and purity of plumericin were verified once again by HPLC-DAD.

2.1.3 | Cell culture

The IEC-6 cell line (CRL-1592) was purchased from the American Type Culture Collection (ATCC). This nontumorigenic cell line, originating from normal rat intestinal crypt cells, was cultured using Dulbecco's modified Eagle's medium (4 g/L glucose) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, and 0.1 U/mL bovine insulin. Cells were used at the 16th-19st passage.

2.1.4 | Cell treatment

IEC-6 cells were plated and, after adhesion, were treated with plumericin (0.5-2 μM) for 1 hour and then co-exposed to plumericin and lipopolysaccharides from *E. coli* (LPS; 10 μg/mL) plus interferon-γ (IFN; 10 U/mL) for different times, based on the mediator to evaluate.

2.1.5 | Antiproliferative activity

Antiproliferative activity was evaluated using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as formerly reported.[19] IEC-6 cells (2.0×10^3 cells/well) were plated on 96-well plates and allowed to adhere for 24 hours at 37°C in a 5% CO_2 atmosphere. Thereafter, the medium was substituted with fresh medium with or without plumericin (0.5-2 μM) and incubated for 24 hours. MTT (5 mg/mL) was then added to the cells. After 3 hours, cells were lysed with 100 μL of a solution containing 50% (v/v) N,N-dimethylformamide, and 20% (w/v) sodium dodecyl sulfate (SDS; pH = 4.5). A microplate spectrophotometer reader (Titertek Multiskan MCC/340-DASIT) was used to measure the optical density (OD) in each well, as we previously reported.[20] The antiproliferative activity was measured as % dead cells = 100 − [(OD treated/OD control) × 100].

2.1.6 | Tumor necrosis factor and interleukin 1β determination

The TNF-α and Interleukin 1β (IL-1β) levels were assessed with an Enzyme-Linked Immuno Sorbent Assay (ELISA). IEC-6 cells were plated into 24-well plates (8.0×10^4 cells/well) and allowed to adhere for 24 hours. Cells were then treated with plumericin, as previously indicated, for 24 hours. Supernatants from IEC-6 cells were then collected and a commercial kit (e-Biocscience) was used to perform the ELISA, according to the manufacturer's instructions (e-Biosciences). Results were expressed as pg/mL as previously reported.[21]

2.1.7 | Measurement of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase (quinone) 1 (NQO1), superoxide dismutase (SOD), and caspase-1 expression and nitrotyrosine formation by cytofluorimetry

IEC-6 cells were plated into 96-well plates (2.0×10^3 cells/well) and treated with plumericin, as previously indicated, for 24 hours. Cells were then collected and washed with phosphate-buffered saline (PBS), followed by the addition of fixing solution. After 20 minutes, IEC-6 cells were incubated in fix perm solution for further 30 minutes. Anti-cyclooxygenase-2 (COX-2; BD Transduction Laboratories), anti-inducible nitric oxide synthase (iNOS; BD Transduction Laboratories), anti-heme oxygenase-1 (HO-1; Santa Cruz Biotechnologies), anti-NAD(P)H dehydrogenase (quinone) 1 (NQO1; Santa Cruz Biotechnologies), anti-superoxide dismutase (SOD; Santa Cruz Biotechnologies), anti-caspase-1 (Abcam), or anti-nitrotyrosine (Merck Millipore) antibodies were then added. After 1 hour, the secondary antibody, in fixing solution, was added to the cells and cell fluorescence was evaluated by a fluorescence-activated cell sorter (FACSscan; Becton Dickinson) and analyzed by Cell Quest software (version 4; Becton Dickinson), as formerly reported.[22]

2.1.8 | Immunofluorescence analysis for NF-κB and the nuclear factor erythroid-derived 2 (Nrf2) by confocal microscopy

IEC-6 cells were plated on coverslips in a 12-well plate (2×10^5 cells/well) and allowed to adhere for 24 hours. After the adhesion, the cellular medium was substituted with fresh medium with or without plumericin (1 μM) and incubated for 1 hour. Thereafter, cells were co-exposed to plumericin and LPS + IFN for 1 hour to evaluate p65-NF-κB subunit and nuclear factor erythroid-derived 2 (Nrf2) nuclear translocation. Cells were then fixed with 4% paraformaldehyde in PBS permeabilized with 0.1% saponin in PBS, and the blocking was performed with bovine serum albumin (BSA) and PBS. Cells were then incubated with rabbit antiphospho p65 NF-κB antibody (SantaCruz Biotechnologies) or rabbit anti-Nrf2 antibody (SantaCruz Biotechnologies) for 1 hour at 37°C. Subsequently, PBS was used to wash the slides and the fluorescein-conjugated (FITC) secondary antibody was added for 1 hour. 4,6-diamidine-2-O-phenylindole dihydrochloride (DAPI) was used for the nuclei identification, as formerly reported.[23] Finally, the coverslips were mounted in mounting medium and images were taken using the laser confocal microscope[24] (Leica TCS SP5, Leica).

2.1.9 | Intracellular ROS release measurement

ROS intracellular production in IEC-6 was evaluated by the probe 2',7'-dichlorofluorescein-diacetate (H_2DCF-DA). The IEC-6 cells were seeded in 24-well plates (8×10^4 cells/well) and allowed to adhere for 24 hours. After cellular treatment with plumericin and LPS + IFN, IEC-6 cells were collected, washed with PBS, and then incubated in PBS containing H_2DCF-DA (10 μM). Cell fluorescence was evaluated after 15 minutes at 37°C using a fluorescence-activated cell sorter (FACSscan; Becton Dickinson) and analyzed by CellQuest software version 4 (Becton Dickinson), as formerly reported.[25]

2.1.10 | Data analysis

Data are reported as mean ± standard error of the mean (SEM) of at least three independent experiments. Each

experiment was conducted in triplicate. Statistical analyses were performed using the variance test. Bonferroni's test was used to make multiple comparisons. A *P*-value of less than .05 was considered significant.

2.2 | In Vivo studies

2.2.1 | Animals

Male CD1 mice (20-25 g, Harlan Nossan) were placed in a controlled environment, maintained on a 12-hours light/dark cycle, and provided with standard rodent chow and water. Water and food were available ad libitum. This study was approved by the University of Messina Review Board for the care of animals. Animal care was in conformity with current legislation of the EU for the protection of animals used for scientific purposes (Directive 2010/63/EU).

2.2.2 | Materials

Unless otherwise stated, all compounds were obtained from Sigma–Aldrich.

2.2.3 | Induction of experimental colitis

Colitis was induced by intrarectal administration of 2, 4, 6-dinitrobenzene sulfonic acid (DNBS, 4 mg *per* mouse) using a modification of the method first described for rats.[26] In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anesthetized by Enflurane. Subsequently, DNBS (4 mg in 100 μL of 50% ethanol/50% saline, v/v) was injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Vehicle alone (100 μL of 50% ethanol/50% saline) was administered in control experiments (sham). Thereafter, the animals were kept for 15 minutes in a Trendelenburg position. After colitis and sham-colitis induction, the animals were observed for 4 days. On day 4, the animals were weighed and anaesthetized with chloral hydrate, and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for histological and biochemical studies. Plumericin (3 mg/kg; 0.2% DMSO; 15) were intraperitoneally (i.p.) administered.

2.2.4 | Experimental groups

Animals were casually divided into several groups (n = 10 for each group):

Sham + vehicle group: Vehicle solution was given by i.p. administration each day for 4 days;

Sham + plumericin (3 mg/kg) group: Plumericin was administered i.p. each day for 4 days (data not shown);

DNBS + vehicle group: DNBS was injected to the mice as described, subsequently, vehicle solution was administered i.p. each day for 4 days; the first dose was injected 1 hour after the injection of DNBS;

DNBS + plumericin (3 mg/kg) group: DNBS was injected to the mice as described, subsequently, plumericin (3 mg/kg) was given i.p. each day for 4 days; the first dose was injected 1 hour after the administration of DNBS.

2.2.5 | Evaluation of colon damage

After 4 days, the entire colon was removed and gently rinsed with saline solution, opened by a longitudinal cut and immediately examined under a microscope. Colon injury (macroscopic damage score) was evaluated and scored by two independent observers as described previously[27] according to the following criteria: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers without significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon; and 5-8, one point is added for each centimeter of ulceration beyond 2 cm.

2.2.6 | Histological examination

For histological analyses, tissues were removed, fixed in 10% buffered formalin phosphate, embedded in paraffin, sectioned, stained with hematoxylin/ eosin (H/E) and studied by using light microscopy connected to an imaging system (AxioVision; Zeiss). The degree of inflammation on microscopic cross sections of the colon was graded on a scale from 0 to 4 where 0 represents no damage, 1 (mild) is focal epithelial edema and necrosis, 2 (moderate) is diffuse swelling and necrosis of the villi, 3 (severe) represents necrosis with the presence of neutrophil infiltrate in the submucosa, and 4 (highly severe) is widespread necrosis with massive neutrophil infiltrate and hemorrhage.[28] The scores from all sections of each colon were averaged to give a final score for each mouse. All histologic analyses were performed in a blinded fashion.

2.2.7 | Measurement of TNF-α and IL-1β levels

TNF-α and IL-1β levels were evaluated in the colon tissues collected 4 days after the DNBS administration. Colon tissues were homogenized as previously described in PBS containing

2 mmol/L of phenyl-methyl sulfonyl fluoride (PMSF, Sigma Chemical Co.; DBA srl.) and tissue TNF-α and IL-1β levels were evaluated. The assay was performed by using a colorimetric commercial kit, that is, DuoSet® ELISA Development System (R&D Systems), as previous described.[27] All TNF-α and IL-1β determinations were performed in duplicate serial dilutions and the means were calculated.

2.2.8 | Western blot analysis of cytosolic and nuclear extracts from colon tissue

Tissue samples from the terminal colon were suspended in Extraction Buffer A containing 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, and 1 mM sodium orthovanadate, homogenized at the highest setting for 2 minutes, and centrifuged at 1000 × g for 10 minutes at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 μM leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation at 15 000 g for 30 minutes at 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. The following primary antibodies were used: anti-IκBα (1:500, Santa Cruz Biotechnology), anti-NF-κB p65 (1:500, Santa Cruz Biotechnology), anti-caspase-1 (1:500, Santa Cruz Biotechnology), anti-Nrf-2 (1:500, Santa Cruz Biotechnology), anti-SOD (1:500, Millipore). They were kept overnight at 4°C in 1 × PBS, 5% (w/v), nonfat-dried milk, and 0.1% Tween-20. Thereafter, membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2.000, Jackson ImmunoResearch) for 1 hour at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against β-actin (1:1.000; Santa Cruz Biotechnology) for the cytosolic fraction and lamin A/C antibody (1:5000; Sigma-Aldrich) for the nuclear fraction. Signals were detected with Enhanced Chemiluminescence Reagent according to the manufacturer's instructions (Super Signal West Pico Chemiluminescent Substrate, Pierce). The relative expression of the protein bands was quantified by densitometry with Bio-Rad ChemiDoc XRS+ software and standardized to β-actin or lamin A/C levels. Images of blot signals (8-bit/600-dpi resolution) were imported to the analysis software (Image Quant TL, v2003).

2.2.9 | Thiobarbituric acid-reactant substances measurement (MDA levels)

Levels of thiobarbituric acid-reactant substances, which are considered good indicators of lipid peroxidation, were determined in the colon tissue 4 days after DNBS injection as previously described.[29] Thiobarbituric acid-reactant substances were calculated by comparison of the OD532 to a standard mixture of 1,1,3,3-tetramethoxypropan/99% malondialdehyde bis(dimethyl acetal)/99% (MDA; Sigma). The absorbance of the supernatant was measured spectrophotometrically at 532 nm.

2.2.10 | Immunohistochemical localization of nitrotyrosine

At day 4 after DNBS administration, colon tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 7 μm long sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 minutes. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 minutes. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 minutes. Endogenous biotin or avidin-binding sites were blocked by sequential incubation for 15 minutes with biotin and avidin (Vector Laboratories), respectively.

Colon tissue sections were incubated overnight with anti-nitrotyrosine antibody (Merck-Millipore, 1:250 in PBS, v/v). Subsequently, they were rinsed with PBS and incubated with peroxidase-conjugated bovine anti-mouse immunoglobulin G (IgG) secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000 Jackson Immuno Research). Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin-conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories). To verify the binding specificity for all antibodies, control slices were incubated with only primary antibody or only secondary antibody. In these controls, any positive staining was detected. Immunohistochemical images were collected using a Zeiss microscope and the Axio Vision software. For graphic display of densitometric analyses, the intensity of positive (brown) staining was measured by computer-assisted color image analysis (Leica QWin V3, UK). The area of immunoreactivity (determined by the number of positive pixels) was expressed as percent of total tissue area (red staining). Replicates for each experimental condition and histochemical staining were obtained from each mouse in all experimental groups. All immunohistochemical analyses were carried out by two observers blinded to the treatment, respectively.

2.2.11 | Statistical evaluation

For the in vivo studies, N represents the number of animals used. In the experiments involving histology or

immunohistochemistry, the figures shown are demonstrative of at least three experiments. The results were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A *P*-value of less than .05 was considered significant.

3 | RESULTS

3.1 | Plumericin does not exert antiproliferative activity in IEC-6

To evaluate the antiproliferative potential of plumericin (0.5-2 μM) on IEC-6, cells were treated with the compound for 24 hours. The obtained results indicate that the plumericin does not have any significant antiproliferative activity on IEC-6 cells (data not shown).

3.2 | Plumericin reduces TNF-α levels as well as COX-2 and iNOS expression in LPS- and IFN-stimulated IEC-6

The effect of plumericin on TNF-α levels in IEC-6 cellular medium was evaluated using an ELISA assay. Our results show that plumericin (0.5-2 μM) significantly inhibits TNF-α release induced by LPS (10 μg/mL) and IFN (10 U/mL) in IEC-6 cellular medium ($P < .01$ vs LPS + IFN; Figure 1A). Under the same experimental conditions, plumericin (0.5-2 μM) also significantly reduced COX-2 ($P < .01$ vs LPS + IFN; Figure 1B) and iNOS ($P < .01$ vs LPS + IFN; Figure 1C) expression at 1 and 2 μM, respectively.

3.3 | Plumericin reduces p65 NF-κB nuclear translocation, intracellular ROS release, and nitrotyrosine formation in LPS- and IFN-stimulated IEC-6

To evaluate NF-κB activation, p65 NF-κB was labeled with a green fluorescent marker. Plumericin alone did not induce p65 nuclear translocation in IEC-6 cells. However, in inflammatory conditions, the tested compound at a concentration of 1 μM inhibited p65 NF-κB nuclear translocation when compared with LPS + IFN treatment (Figure 2A). The antioxidant potential of plumericin (0.5-2 μM) was evaluated by measuring the intracellular ROS production in LPS + IFN-stimulated IEC-6 cells. It was found that plumericin at all tested concentrations significantly inhibits ROS production in IEC-6 cells ($P < .001$ vs LPS + IFN; Figure 2B). Under the same experimental conditions, plumericin (0.5-2 μM) also inhibited nitrotyrosine formation in IEC-6 cells ($P < .05$ vs LPS + IFN; Figure 2C).

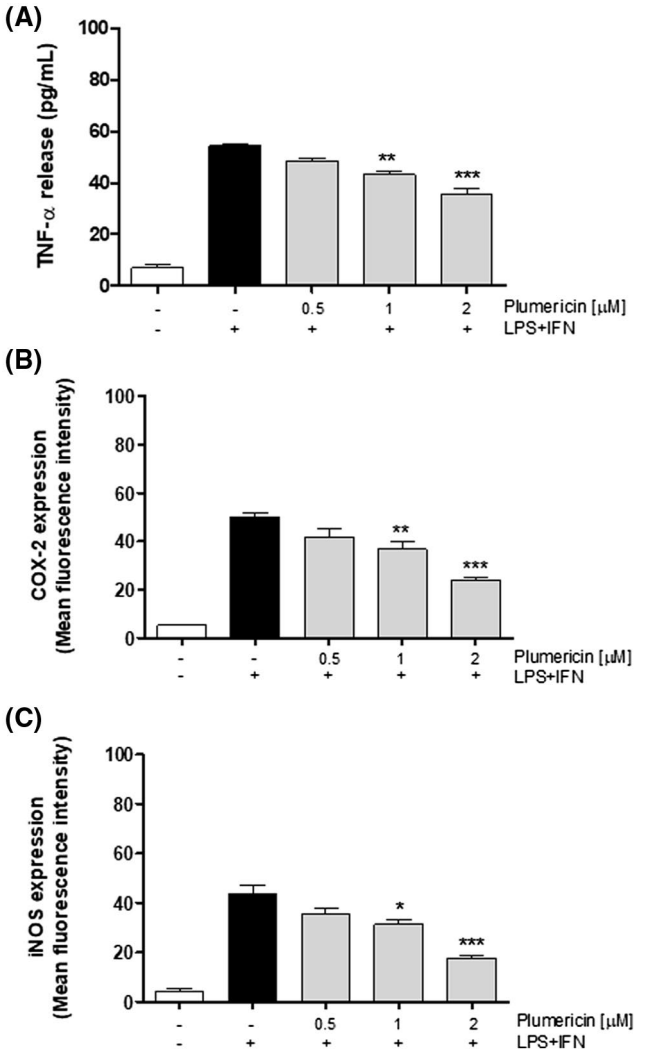

FIGURE 1 Effect of plumericin on tumor necrosis factor-α (TNF-α) release and on cyclooxygenase-2 (COX-2) and iNOS expression in intestinal epithelial cells (IEC-6). The figure shows that plumericin (0.5-2 μM) significantly reduced the release of TNF-α from the cellular medium of IEC-6 cells stimulated by LPS + IFN (A), evaluated by an enzyme-linked immuno sorbent assay assay, and the expression of COX-2 (B) and inducible nitric oxide synthase (iNOS) (C), evaluated by cytofluorimetry. Data are representative of at least three independent experiments (n = 12) and are expressed as pg/mL of TNF-α release or as mean fluorescence intensity. Comparisons were performed using a one-way analysis of variance and multiple comparisons were made by Bonferroni's post test. ***, ** and * indicate $P < .001$, $P < .01$ and $P < .05$, respectively, vs LPS + IFN

3.4 | Plumericin induces Nrf2 activation and HO-1, NQO1, and SOD expression in IEC-6 cells

Oxidative stress is a result of a disequilibrium between prooxidant and antioxidant factors. To assess the antioxidant potential of plumericin, the antioxidant Nrf2 response was evaluated. Nrf2 activation was significantly increased by

plumericin (1 μM) when compared with cells treated with LPS + IFN alone (Figure 3A). Expression of Nrf2-related cytoprotective factors, such as HO-1, NQO1, and SOD, was further increased by plumericin (0.5-2 μM; $P < .05$ vs LPS + IFN; Figure 3B-D), thus supporting its antioxidant potential.

3.5 | Plumericin reduces inflammasome activation in IEC-6 cells

To assess the antinflammatory potential of plumericin on IEC-6 cells, the inflammasome activation was also evaluated. We observed that plumericin (0.5-2 μM), significantly reduced IL-1β production and caspase-1 expression in LPS + IFN-stimulated IEC-6 at 2 and 0.5-2 μM, respectively ($P < .01$ vs LPS + IFN; Figure 4A,B).

3.6 | Plumericin reduces DNBS-induced colon weight, colon length, macroscopic damage, and the degree of colitis

The change in weight is a good and easy way to monitor the health status of mice. The development of colitis leads to weight loss due to the inability to absorb nutrients in the intestine. Thus, the weight of each mouse was measured every day during the course of each experiment. Moreover, four days after the induction of colitis by DNBS treatment, all mice showed diarrhea and a reduction in body weight (compared to the sham groups). Plumericin (3 mg/kg, i.p.) treatment significantly reduced the loss of body weight (Figure 5A). The colon length is another valuable parameter to determine the severity of a colitis. During the course of colitis, the colon is shortened. Compared to the DNBS group, the length of the colon from plumericin-mice was observed to be longer (Figure 5B). Macroscopic examination of colonic mucosa of sham-treated mice showed an intact epithelium (Figure 5C, left panel). Four days after intracolonic administration of DNBS, the colon appeared flaccid and was filled with liquid stool; macroscopic examination of the colon showed mucosal congestion, erosion, and hemorrhagic ulcerations (Figure 5C, central panel). Intraperitoneal administration of plumericin in a dose of 3 mg/kg significantly reduced the degree and severity of the macroscopic damage (Figure 5C, right panel, and Figure 5D).

No histological changes were observed in the colon tissue of the sham-group. Four days after administration of DNBS, the tissue of DNBS-mice was characterized by cellular infiltration, edemas, and the presence of inflammatory lesions. Colon samples from plumericin-treated mice showed a reduction in the severity of histological signs of injury (Figure 5E).

3.7 | Plumericin reduces TNF-α production, IκB-α degradation, and NF-κB p65 translocation

To test whether plumericin modulates the inflammatory process, we analyzed the expression and levels of the pro-inflammatory cytokine TNF-α in the colon. A substantial increase in TNF-α expression was found in the colon tissues collected 4 days after DNBS administration. Plumericin treatment (3 mg/kg; i.p.) reduced in a significant and dose-dependent manner the colon expression of TNF-α ($P < .001$ vs sham; $P < .001$ vs DNBS; Figure 6A). We also evaluated IκB-α degradation and nuclear NF-κB p65 translocation by western blot analysis to investigate the underlying cellular mechanisms whereby treatment with plumericin attenuated the development of DNBS-induced colitis. Basal expression of IκB-α was detected in colon samples from sham-treated animals, whereas IκB-α levels were substantially reduced in colon tissues obtained from vehicle-treated animals 4 days after DNBS injection. Plumericin treatment prevented DNBS-induced IκB-α degradation ($P < .001$ vs sham; $P < .001$ vs DNBS; Figure 6B). Moreover, NF-κB p65 levels in the colon nuclear fractions were substantially increased 4 days after DNBS injection compared to the sham-treated mice. Treatment with plumericin (3 mg/kg; i.p.) reduced the levels of NF-κB p65 ($P < .01$ vs sham; $P < .001$ vs DNBS; Figure 6C).

3.8 | Effects of plumericin on lipid peroxidation, nitrotyrosine and Nrf2 activation, and SOD expression in colitis induced by DNBS

Plasma levels of thiobarbituric acid–reactant substances, which are indicators of lipid peroxidation, increased significantly in mice subjected to DNBS compared with sham-treated mice. Thiobarbituric acid-reactant substance levels were significantly attenuated in DNBS treated with plumericin (3 mg/kg, i.p.; $P < .001$ vs sham; $P < .001$ vs DNBS; Figure 7A).

Four days after DNBS administration, the presence of nitrotyrosine, a specific marker of nitrosative stress, was assessed by immunohistochemical analysis in terminal colon sections to determine the localization of "peroxynitrite formation" and/or other nitrogen derivatives formed during experimental colitis. In colon sections from sham-treated mice, nitrotyrosine could not be detected, but sections from DNBS-injected mice displayed positive nitrotyrosine immunostaining, mainly in inflammatory cells and around vessels. Treatment with plumericin (3 mg/kg, i.p.) reduced the degree of nitrotyrosine immunoreactivity in the colon ($P < .001$ vs sham; $P < .001$ vs DNBS; Figure 7B,C).

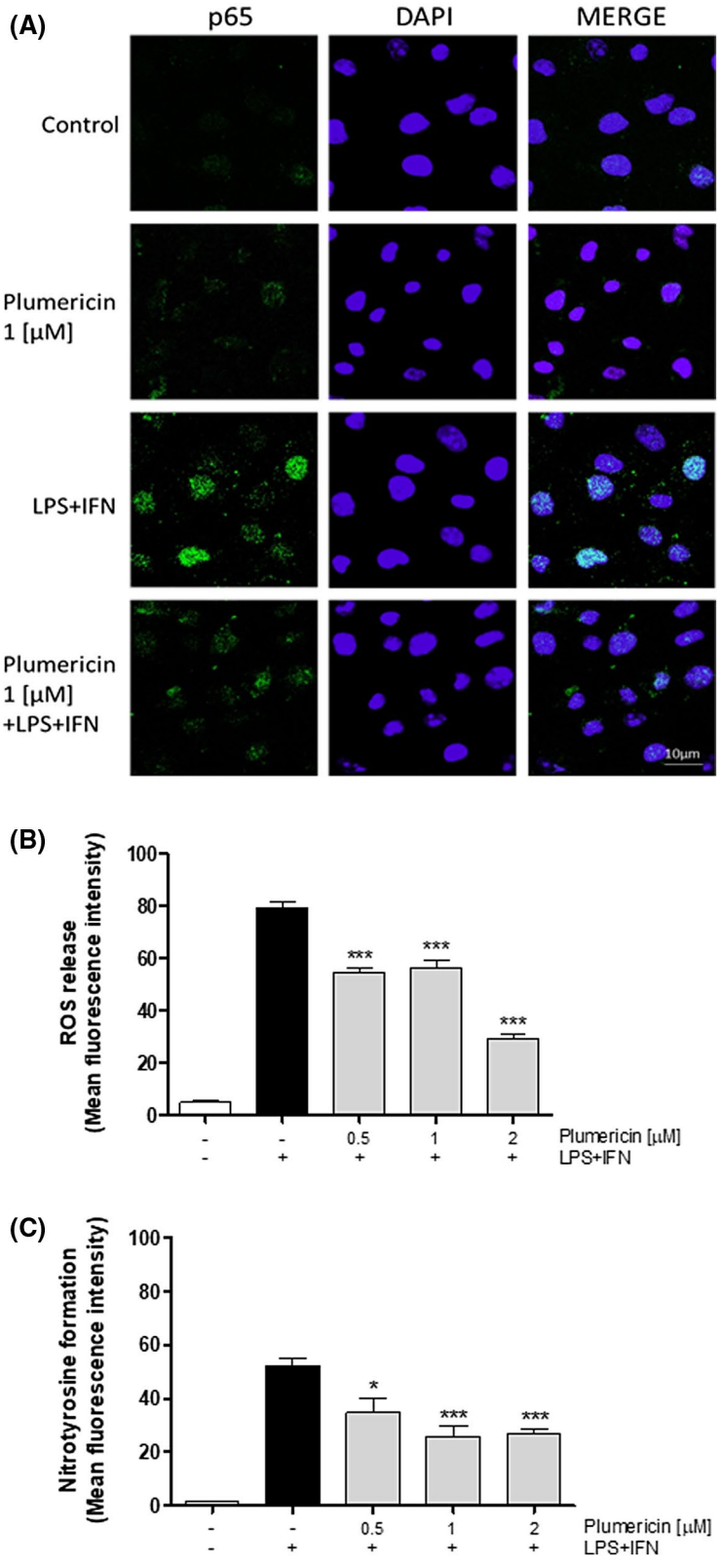

FIGURE 2 Effects of plumericin (1 μM) alone and with LPS + IFN on NF-κB p65 nuclear translocation, evaluated by immunofluorescence analysis. The blue fluorescence identified the nuclei, whereas the green fluorescence indicated the p65 NF-κB subunit (A). Moreover, plumericin (0.5-2 μM) significantly reduced intracellular ROS release ($P < .001$ vs LPS + IFN; (B), evaluated by means of the probe 2',7' dichlorofluorescein-diacetate (H_2DCF-DA), and nitrotyrosine formation ($P < .05$ vs LPS + IFN; (C). Data are representative of at least three independent experiments (n = 12) and are expressed as mean fluorescence intensity. Comparisons were performed using a one-way analysis of variance and multiple comparisons were made by Bonferroni's post test. *** and * indicate $P < .001$ and $P < .05$, respectively, vs LPS + IFN

Since it is well known that ROS play a role in enhancing inflammation, we evaluated by western blot analysis if plumericin in a dose of 3 mg/kg is able to modulate oxidative processes by effecting the Nrf-2 pathway. We found that administration of plumericin at the dose of 3 mg/kg significantly increased Nrf-2 expression. ($P < .001$ vs DNBS; Figure 7D). Furthermore, based on the obtained increment of Nrf-2 expression, we also evaluated SOD expression, an enzyme directly involved in the oxidation stress pathway. In these experiments, a significant increase in SOD expression following treatment with plumericin was observed ($P < .001$ vs DNBS; Figure 7E).

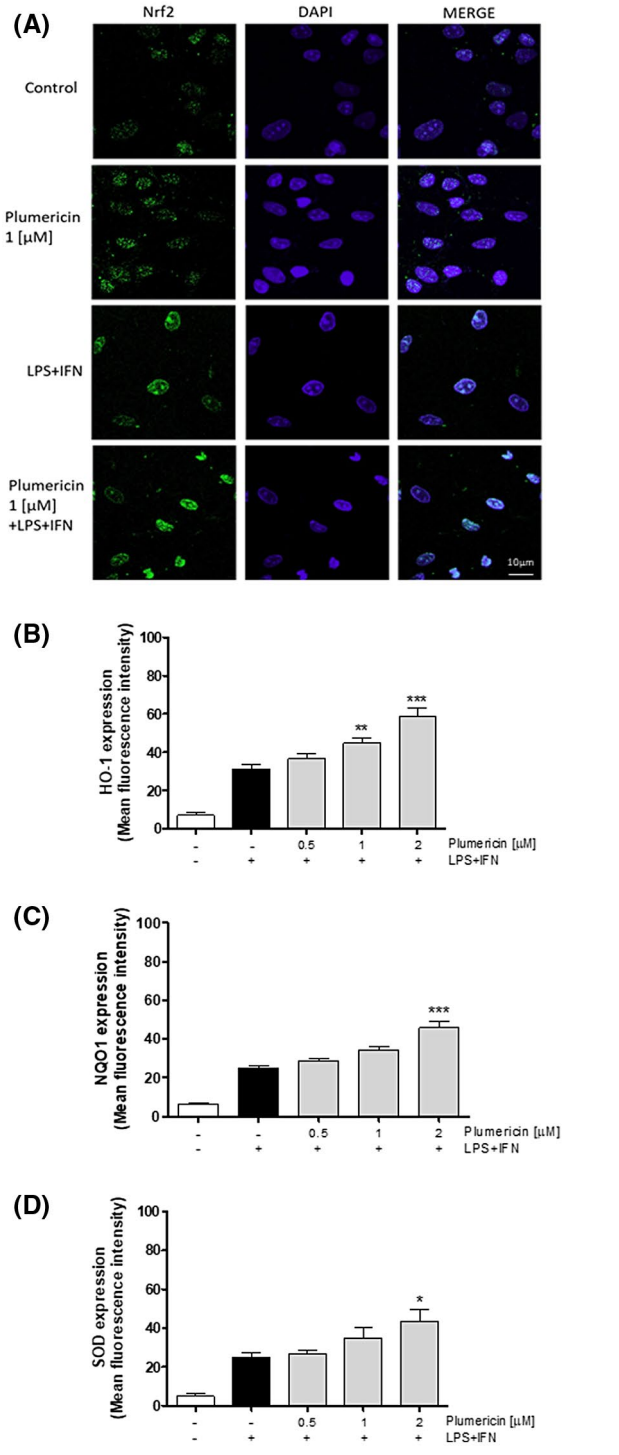

FIGURE 3 Effect of plumericin (1 μM) on Nrf2 activation (A), evaluated by immunofluorescence analysis. The blue fluorescence identified the nuclei, whereas the green fluorescence indicated Nrf2. Moreover, plumericin significantly increased HO-1 expression at 1 and 2 μM (B), and NQO1 (C) and SOD (D) expression at 2 μM in intestinal epithelial cells (IEC-6) cells stimulated with LPS + IFN, evaluated by cytofluorimetry. Data are representative of at least three independent experiments (n = 12) and are expressed as mean fluorescence intensity. Comparisons were performed using a one-way analysis of variance and multiple comparisons were made by Bonferroni's post test. ***, **, and * indicate $P < .001$, $P < .01$, and $P < .05$, respectively, vs LPS + IFN

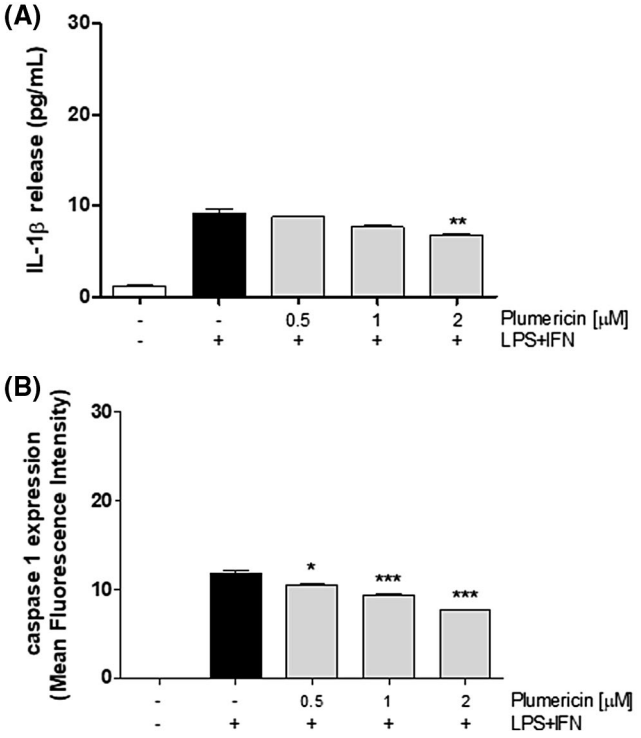

FIGURE 4 Effect of plumericin on inflammasome activation. Plumericin (2 μM) significantly reduced IL-1β release (A) in intestinal epithelial cells (IEC-6) cells during inflammatory conditions, evaluated by an enzyme-linked immuno sorbent assay. Data are representative of at least three independent experiments (n = 12) and are expressed as pg/mL of IL-1β release. Moreover, plumericin (0.5-2 μM) significantly reduced caspase 1 expression (B), evaluated by cytofluorimetry. Data are representative of at least three independent experiments and are expressed as mean fluorescence intensity. Comparisons were performed using a one-way analysis of variance and multiple comparisons were made by Bonferroni's post test. ***, ** and * indicate $P < .001$, $P < .01$, and $P < .05$, respectively, vs LPS + IFN

3.9 | Plumericin reduces inflammasome activation in colon tissues

The colitis caused by DNBS was also characterized by an increase in IL-1β levels and caspase-1 expression. Plumericin treatment (3 mg/kg; i.p.) reduced in a significant and dose-dependent manner the colon expression of IL-1β ($P < .001$ vs sham; $P < .001$ vs DNBS; Figure 8A). Western blot analysis showed an increased expression of caspase-1 in colon tissue collected from DNBS-treated mice compared to sham-treated animals. Treatment with plumericin (3 mg/kg; i.p.) reduced caspase-1 expression (Figure 8B).

4 | DISCUSSION AND CONCLUSION

IBDs are complex pathological conditions that affect millions of people and are a serious health concern in the Western

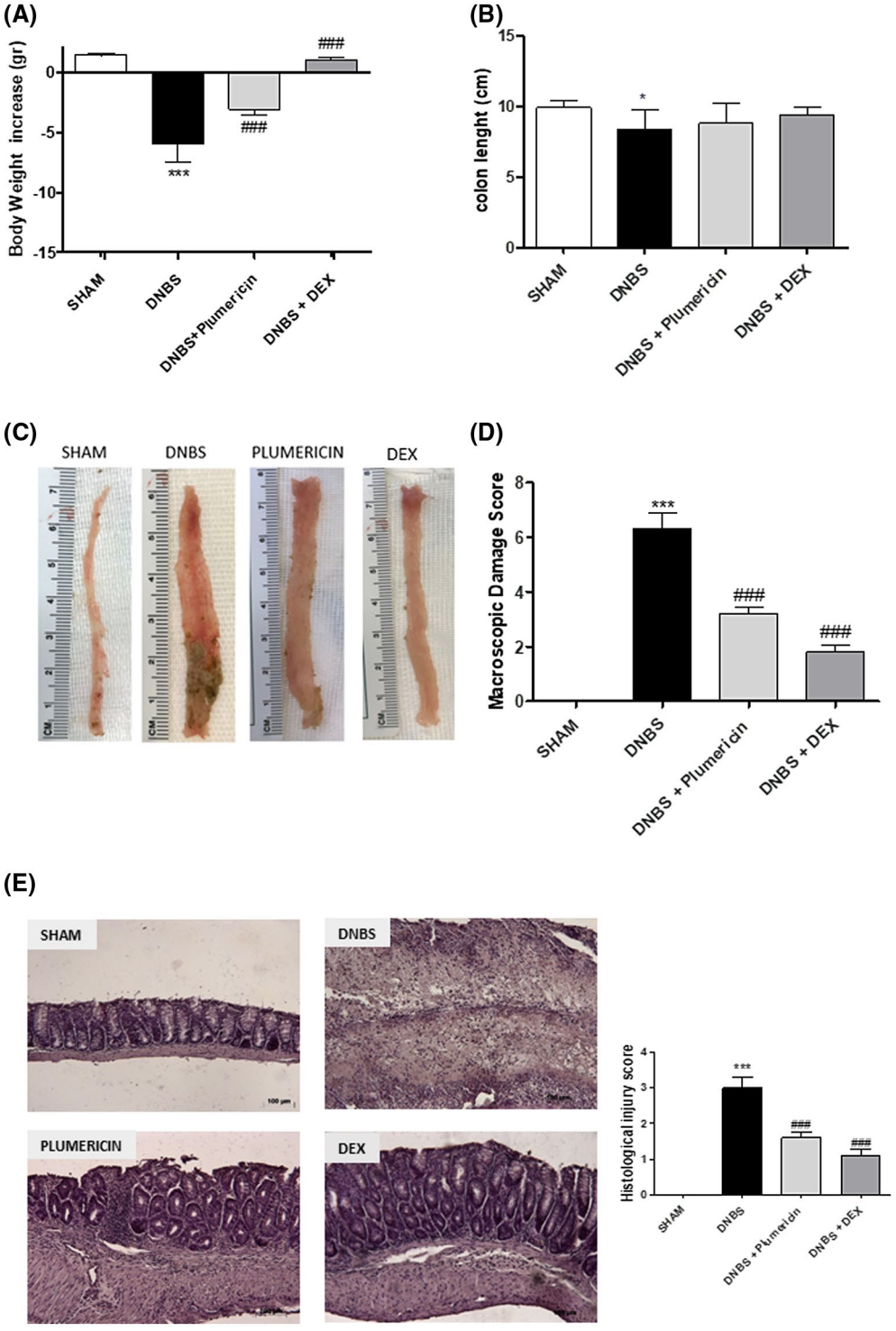

FIGURE 5 Effect of plumericin on macroscopic damage in DNBS-treated mice. Plumericin significantly reduced colon weight and colon length. A reduction in body weight increase and colon length was observed 4 d after DNBS administration. Treatment with plumericin (3 mg/kg) significantly reduced body weight loss (A) and the decrease in colon length (B). DEX (10 mg/kg; positive control) increased body weight loss (A) and colon length (B). Macroscopic inspection showed that colons collected from the DNBS-group showed significative alterations of the normal structure (compared to the control-group; C, left panel) and hemorrhagic ulcerations (C, central panel). Plumericin (3 mg/kg, i.p.) and DEX (10 mg/kg; positive control) significantly reduced the extent and severity of this macroscopic damage (C, right panel); D represents the macroscopic score. Plumericin also reduced the degree of colitis. Histologic alterations were absent in colon tissues from sham-treated mice (E, sham). Colons from the vehicle-group showed necroses and edemas with a diffuse leukocyte cellular infiltrate (E, DNBS). Plumericin (3 mg/kg, i.p.) significantly reduced the histologic signs of colon injury (E, plumericin) as well as showed by DEX-group (positive control). Data are expressed as the mean ± SEM of 10 mice for each group. *** and * denote $P < .001$ and $P < .01$, respectively, vs sham; $^{###}$ denotes $P < .001$ vs DNBS

societies, and they are also on the rise in developing areas.[30,31] They often lead to relapsing bouts and chronic courses, and they can progress to fibrosis, often resulting in pharmacologically unmanageable alterations that need to be resolved, finally, by surgical resections.[32,33] There are currently different

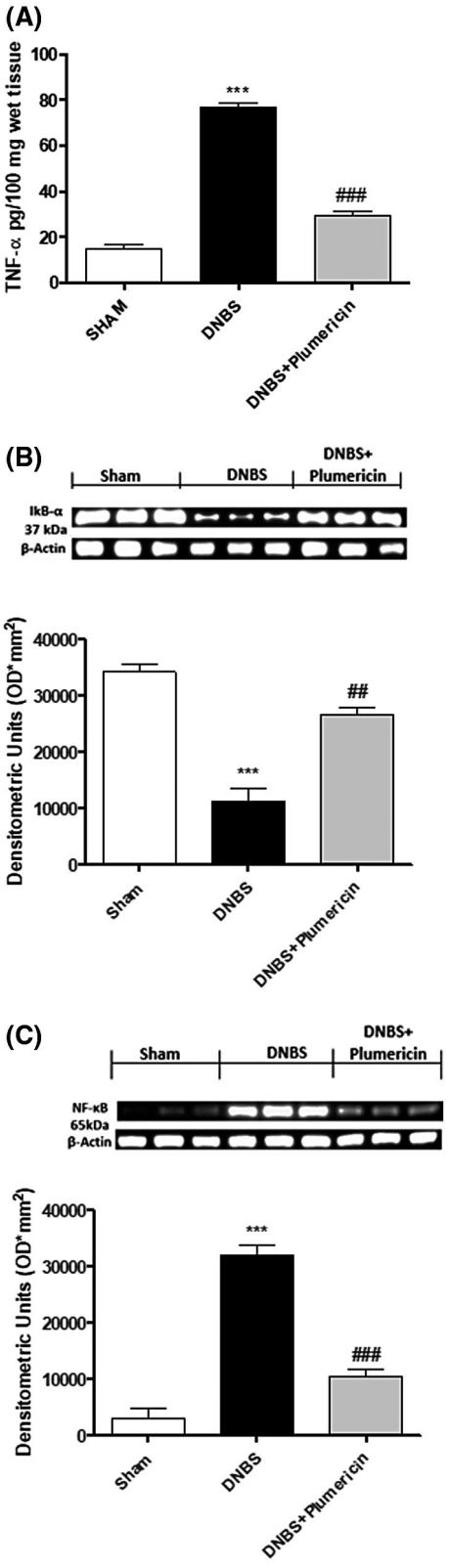

FIGURE 6 Effect of plumericin on cytokine production and NF-κB activation. Treatment with plumericin (3 mg/kg) significantly reduced the levels of tumor necrosis factor-α (TNF-α) in the colon after DNBS administration, evaluated by enzyme-linked immuno sorbent assay (A). Representative western blots showing that plumericin treatment reduced IκB-α degradation (B) and NF-κB p65 translocation (C) compared to the vehicle group. Levels of IκB-α and NF-κB presented in the densitometric analyses of protein bands were normalized for β-actin and laminin, respectively. Data are representative of at least three independent experiments and are expressed as mean ± SEM of 10 mice for each group. A representative blot is shown and densitometric analysis is reported. *** denotes $P < .001$ vs sham; ### and ## denote $P < .001$ and $P < .01$, respectively, vs DNBS

therapeutic strategies for treating IBDs, but they are associated with several adverse effects. Therefore, there is an ongoing search for new therapies that may be beneficial in treating these chronic diseases.[34] In this sense, the interest in the use of plant products for treating IBD has increased,[35,36] as many of them are known to act on different targets of the inflammatory process and protect the tissue microenvironment of oxidative stress. Therefore, this study has evaluated the potential effects of plumericin, an iridoid spironolactone isolated from *H. sucuuba*, on intestinal inflammation. The obtained results indicate that plumericin possesses anti-inflammatory and antioxidant activity in the epithelial cell line IEC-6. LPS-induced release of cytokines might be involved in the deterioration of intestinal inflammation. In particular, studies have revealed that LPS plus IFN-γ stimulated intestinal epithelial cells might release inflammatory cytokines.[21,37,38] One of the major cytokines playing a pivotal role in IBDs is TNF-α. In IECs, it is significantly upregulated by proinflammatory stimuli.[39] Levels of proinflammatory enzymes such as iNOS or COX-2 are also elevated in IBD as a result of the intestinal inflammation. They contribute both to the amplification of the inflammatory response (also *via* TNF-α) and the oxidative stress.[40,41] In our experiments, plumericin significantly inhibited TNF-α release in all tested concentrations (0.5-2 μM) as well as COX-2 and iNOS expression at 1 and 2 μM, respectively. The effect on TNF-α is in accordance with a previous study reporting the effect of plumericin in the inhibition of TNF-α-induced senescence in endothelial cells[17] while here we report for the first time the effects of plumericin on iNOS and COX-2. The NF-κB signaling cascade has been studied well in IBD pathogenesis and is often dysregulated in patients, resulting in aberrant cytokines and chemokines production in the gut. Moreover, it regulates TNF-α, COX-2, and iNOS expression.[42] Our data indicate that plumericin inhibits p65-NF-κB nuclear translocation in IEC-6, thus contributing to a significant reduction in the inflammatory cascade. It has been reported as a potent NF-κB inhibitor in endothelial cells[15] and here we observed this effect also in IECs. Microbial-activated toll-like receptors and TNF-α expressed by IECs stimulate

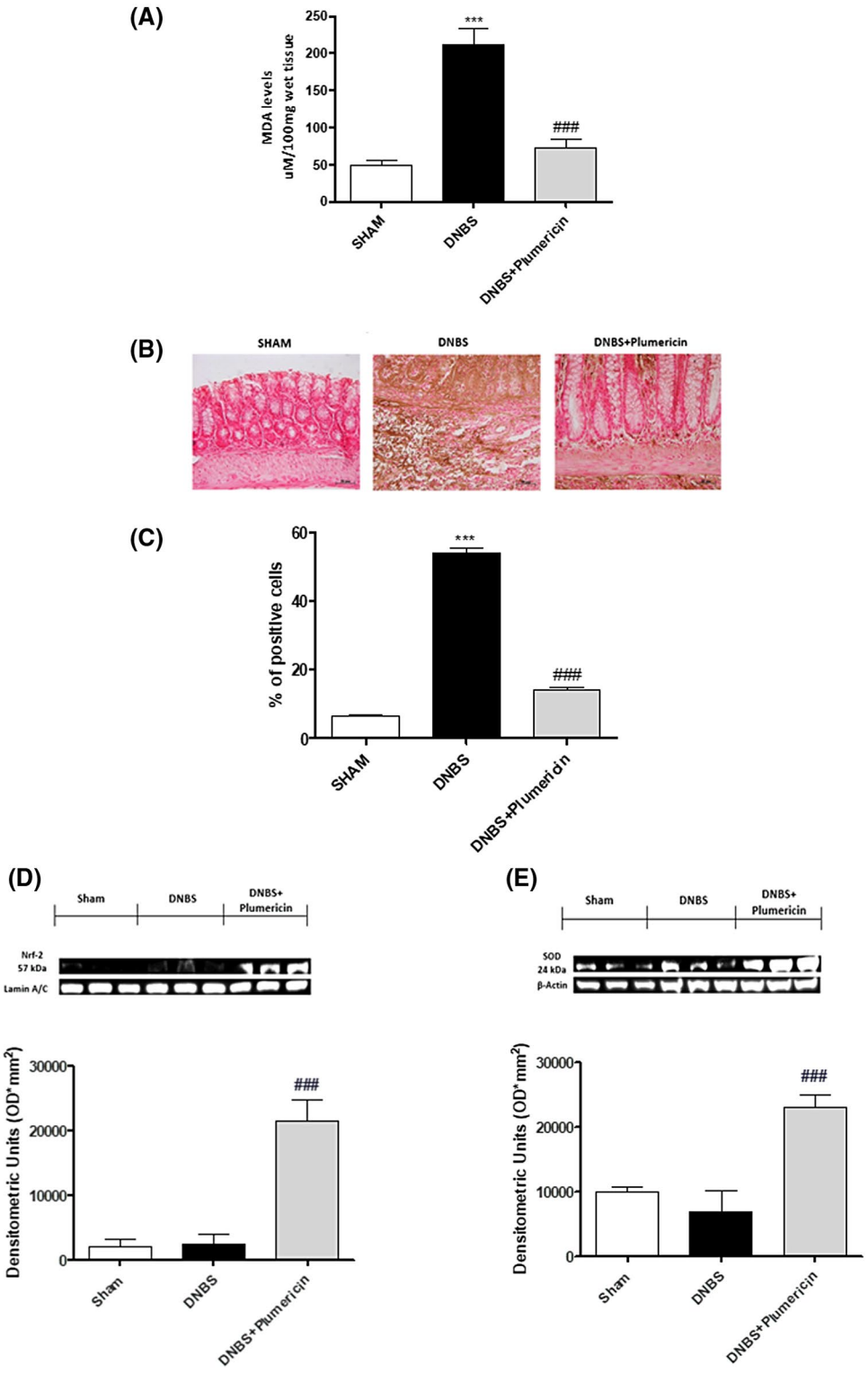

FIGURE 7 Effects of plumericin treatment on lipid peroxidation in colitis induced by DNBS. Levels of MDA, an indicator of lipid peroxidation, were significantly attenuated in the plumericin-group compared to the DNBS-group (A). Immunohistochemical analysis did not show any staining for nitrotyrosine in the sham-group (B). An increase in nitrotyrosine expression was observed in DNBS-injected mice, whereas low nitrotyrosine levels were found in mice treated with plumericin. These data are also visible in the graph showing the percentage of total tissue area (C). Photographs are representative of all animals in each group. Western blot analysis showed that the administration of plumericin (3 mg/kg) significantly increased Nrf-2 expression compared to the vehicle group (D). In the same way, a significant increase in SOD expression was observed in colons collected from plumericin-group-mice compared to DNBS-injected mice (E). Densitometric analysis of protein bands was normalized to the level of β-actin. Data are expressed as the mean ± SEM of 10 mice for each group. *** denotes $P < .001$ vs sham; ### denotes $P < .001$ vs DNBS

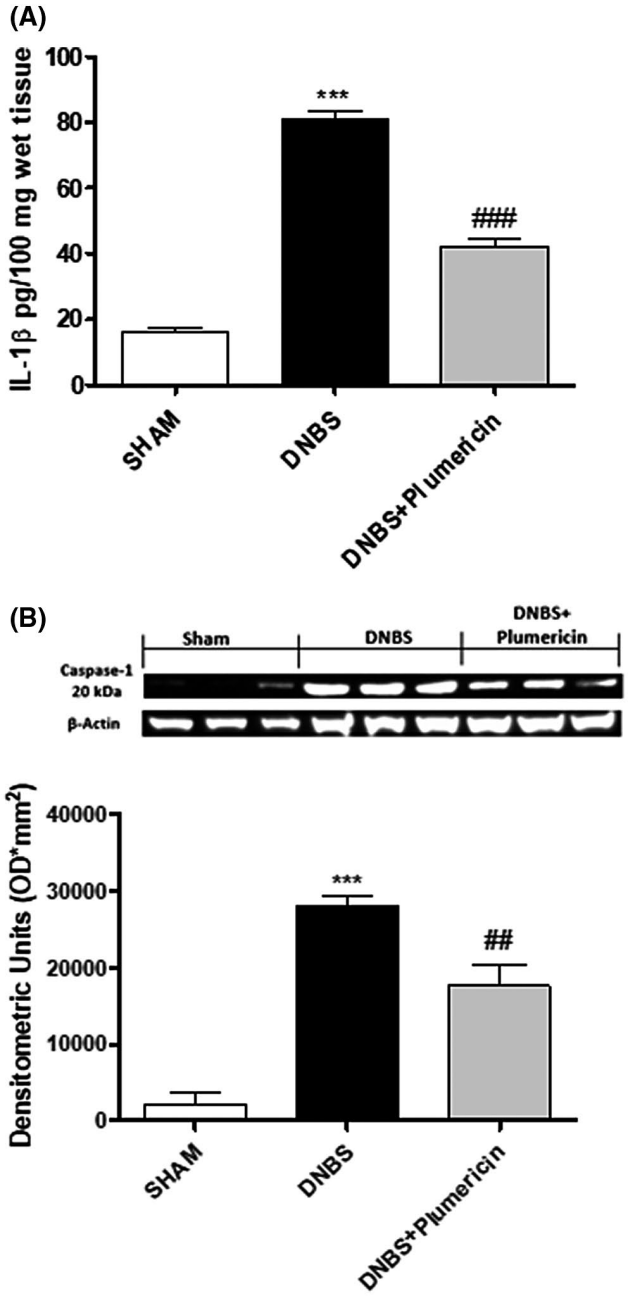

FIGURE 8 Plumericin reduced IL-1β production and caspase-1 expression. Treatment with plumericin (3 mg/kg) significantly reduced the levels of proinflammatory cytokines such as IL-1β in the colon after DNBS administration (A). In addition, caspase-1 levels were significantly increased in the colon tissues of DNBS-injected mice compared to sham mice. Treatment with plumericin significantly reduced caspase-1 expression induced by DNBS injection (B). Levels of caspase-1 presented in the densitometric analysis of protein bands were normalized for β-actin. Data are representative of at least three independent experiments and are expressed as mean ± SEM of 10 mice for each group. A representative blot is shown and densitometric analysis is reported. *** denotes $P < .001$ vs sham; ### and ## denote $P < .001$ and $P < .01$ vs DNBS

downstream NF-κB signaling events, which are also sensitive to oxidative stress. A considerable body of evidence has shown that ROS activate NF-κB, which further amplifies the proinflammatory response.[43,44] Thus, the control of ROS is of primary importance in the treatment of IBD. Our results indicate that plumericin is able to inhibit ROS release and the formation of nitrotyrosine, a marker of nitrosative stress, in IEC-6. These data are in according with the results of a study by Khan et al,[17] who reported the suppressive effect of plumericin on ROS release in HUVECs. In addition, herein we also report that plumericin enhances the antioxidant cellular response by promoting Nrf2 pathway activation. Nrf2 is a key transcription factor that maintains the mucosa homeostasis by suppressing excessive ROS production in IBD. Moreover, Nrf2 negatively regulates the proinflammatory response and mucosal impairment *via* antioxidant mechanisms such as NF-κB inhibition.[1] In this study, we report that plumericin induces Nrf2 activation as well as the production of Nrf2 regulated cytoprotective enzymes such as HO-1, NQO1, and SOD. Accumulating evidence suggests that the inflammasome plays a key role in shaping epithelial response at the host-lumen interface with many inflammasome components highly expressed by IECs.[45] Inflammasome formation and caspase-1 activation lead to the cleavage and the secretion of the active forms of IL-1 cytokines, such as IL-1β and IL-18. Il-1β is a potent proinflammatory cytokine exerting a pleythora of effects, also at intestinal level. Our results indicate that plumericin inhibits the inflammasome activation induced by inflammatory stimuli in IECs. In order to confirm these effects also in an in vivo model, we evaluated the effect of plumericin in a model of DNBS-induced colitis in mice. In plumericin-treated mice, we observe a reduced weight loss and a reduced colon shortening. Plumericin-treated mice were more resistant to DNBS-induced colitis. At the macroscopic and histological levels, in plumericin-treated mice, epithelial disruption as well as other signs of intestinal inflammation were significantly reduced. The inhibition of the inflammatory response by plumericin in DNBS-induced colitis was also confirmed by the observed reduced TNF-α levels and NF-κB activation in intestinal tissues. DNBS-colitis also induces oxidative stress and plumericin on one hand reduced pro-oxidant parameters such as MDA and nitrotyrosine formation, and on the other hand, it increased the antioxidant response in intestinal tissues, inducing Nrf-2 activation and SOD expression. Moreover, also inflammasome activation is inhibited by plumericin in DNBS-induced colitis in mice. Our results obtained both in vitro and in vivo strongly support the beneficial effect of plumericin in IBDs, acting on the inflammatory response and, interestingly, strongly regulating also the oxidative stress response.

Plumericin shares its effects on these pathways with several other electrophilic compounds of high pharmaceutical and nutraceutical interest including dimethylfumarate[46-48] and curcumin,[49,50] which have also been reported to be effective on IBDs.[51,52] This evidence further supports the pharmacological potential of plumericin as an adjuvant in IBDs.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

MS and SH conceived the study; MS and CS designed the experiments; WB and SH provided the test compound; RSF, SR, PAF, DAR, DPR, and AS performed the experiments; MS analyzed the data; RSF and MS wrote the manuscript; DPR and AS reviewed the manuscript; WB, CS, and AG edited the manuscript.

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