Oleuropein Protects against Dextran Sodium Sulfate-Induced Chronic Colitis in Mice

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Oleuropein Ameliorates Acute Colitis in Mice

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ABSTRACT: Oleuropein, the major secoiridoid in olive tree leaves, possesses a wide range of health promoting properties. It has recently been shown to exhibit anti-inflammatory activity. We have evaluated the effect of oleuropein on dextran sulfate sodium (DSS)-induced experimental colitis in mice in order to provide insight into its mechanisms of action. Oral administration of oleuropein notably attenuated the extent and severity of acute colitis while reducing neutrophil infiltration; production of NO, IL-1β, IL-6, and TNF-α; expression of iNOS, COX-2, and MMP-9; and the translocation of the NF-κB p65 subunit to the nucleus in colon tissue. In LPS-stimulated peritoneal macrophages, the oleuropein metabolite, hydroxytyrosol, was shown to inhibit NO production, iNOS expression, NF-κB p65 subunit translocation, mRNA expression, and the release of IL-1β, IL-6, and TNF-α. These results suggest that the effect of oleuropein on DSS-induced colitis is associated with a decrease in the production of interleukins and expression of proteins, principally through reduction of NF-κB activation.

KEYWORDS: *oleuropein, dextran sulfate sodium, colitis, rutin, hydroxytyrosol*

INTRODUCTION

Human intake of naturally occurring phenolics is associated with a reduced risk of certain diseases because of their effects as antioxidant, anti-inflammatory, antiapoptotic and anti-cell proliferator agents. Oleuropein (3,4-dihydroxyphenylethanol-elenolic acid glucoside) is the main secoiridoid of the leaves (up to 6−9% of dry matter) and unprocessed olive drupes of *Olea europaea* (Oleaceae), however, the most abundant phenols in extra virgin olive oil and table olives are the nonpolar oleuropein- and ligstroside-aglycons, formed by enzymatic removal of glucose, and the polar compounds hydroxytyrosol and tyrosol, end products of their hydrolysis.[1] Olive tree leaves have been used in the human diet as an extract, herbal tea or a powder for its potential beneficial effects.[2] Oleuropein is not absorbed in the small intestine, but undergoes rapid degradation in the large intestine to yield hydroxytyrosol.[3,4] Oleuropein and its metabolite hydroxytyrosol have been reported as powerful antioxidant, antiangiogenic and anticancer agents.[5,6] An olive oil polyphenolic extract containing oleuropein and hydroxytyrosol exerted chemopreventive effects in the large intestine by inhibiting p38/CREB and cyclooxygenase (COX)-2 expression, signaling pathways responsible for colorectal cancer development.[7] Oleuropein inhibited proliferation of MCF-7 breast cancer cells by interfering with extracellular regulated kinase (Erk)1/2 activation,[8] and exerted antiproliferative and anti-apoptotic effects on human colorectal cancer cells SW620 with a mechanism no fatty acid synthase mediated, a key anabolic enzyme in colon carcinoma development.[9] Oleuropein has also demonstrated anti-inflammatory effect, by inhibiting interleukin (IL)-1β and prostaglandin (PG)E_2 release in human whole blood cultures,[10] and its aglycon caused a significant reduction on IL-1β and tumor necrosis factor (TNF)-α production, expression of adhesion molecules and nitric oxide (NO) synthesis in a mouse carrageenan-induced pleurisy model.[11]

Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory disorder with unknown etiology, which includes ulcerative colitis (UC) and Crohn disease (CD). Ulceration and inflammation of the intestinal mucosa with leukocyte infiltration are the common histological changes associated with IBD. One of the most important signaling pathways implicated in IBD is the activation of nuclear factor (NF)-κB,[12] generating increased levels of inflammatory mediators, such as IL-6, IL-1β and TNF-α, and COX-2, iNOS and metalloproteinase (MMP) expression. Different animal models of experimental colitis have been developed to study the molecular and cellular mechanisms of inflammation and immunological disease; one of these is the dextran sulfate sodium (DSS) induced colitis model in rodents, that induces colonic inflammation with clinical and histological similarity to human UC and is frequently used to evaluate the effect of novel anti-inflammatory drugs. Depending on the doses of DSS and the duration of experiment, the inflammatory process can be acute or chronic, progressing to colorectal cancer. Oral DSS administration in the drinking water induces a colon inflammation accompanied by diarrhea, rectal bleeding, body weight loss and shortening of the colorectum.[13] Although increasing evidence has come light to the mechanisms of the inflammation and immune responses in the IBD process and a number of therapeutic approaches targeting inflammatory mediators implicated are available, new treatment approaches are still necessary.

A growing body of evidence indicates that polyphenols can modulate intestinal inflammation,[14] supporting these types of plant secondary metabolites as promising candidates among

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natural preventive/protective treatments of IBD. Although a number of studies have investigated the effects of phenolic compounds on intestinal inflammation, as pure molecules or as plant extracts, little information is available in the literature concerning the efficacy of oleuropein. The aim of the present study is to assess the efficacy of oleuropein on DSS-induced colitis model in mice, therefore to gain insight into the mechanism of action and pharmacological value of oleuropein as regards the inflammatory mediators involved in IBD, histological analyses have been carried out along with an analysis of the different proinflammatory cytokines important for exacerbation of IBD, and iNOS, COX-2, MMP-9 expression and p65 translocation to the nucleus found in colonic tissue affected by DSS.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all chemicals including oleuropein and biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Baker (Deventer, Holland). Dextran sulfate sodium (36−50 kDa) was purchased from MP Biomedical (France). Specific antibodies for β-actin and MMP-9 were also obtained from Sigma-Aldrich, and antibodies recognizing iNOS and COX-2 were purchased from Millipore (Billerica, MA, USA) and Cayman (Ann Arbor, MI, USA), respectively. Anti-p65 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ELISA kits for cytokines were purchased from eBioscience (San Diego, CA, USA).

Animals. All animal care and experimental protocols were approved by the Institutional Ethics Committee of the University of Valencia, Spain. Female BALB/c mice (Harlan Interfauna Iberica, Barcelona, Spain), 6−8 weeks of age weighing 18−20 g were acclimatized under a 12 h light/dark cycle at 22 °C and 60% humidity, for 7 days before the experiments, and fed with a standard laboratory rodent diet and water ad libitum.

Isolation and Culture of Mouse Peritoneal Macrophages. Two milliliters of 3% thioglycolate broth was injected into peritoneal cavities of BALB/c mice. After 4 days, peritoneal macrophages were harvested by washing with 10 mL of phosphate-buffered saline (PBS), centrifuged at 240*g* for 5 min at 4 °C, seeded onto a 6-well plate at a density of 1.5×10^6 cells per well in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) and then cultured at 37 °C under a humidified atmosphere of 5% CO_2.[15] After incubation for 2 h, macrophages were treated with the vehicle alone or various concentrations of oleuropein or hydroxytyrosol (50 μM and 100 μM) for 1 h and then stimulated with lipopolysaccharide (LPS, 1 μg mL^{-1}) for 24 h. Following incubation, cells were scraped and the cytosolic and nuclear protein fractions were extracted with the aid of the Proteojet cytosolic and nuclear extraction kit (Fermentas, Thermo Scientific, Waltham, MA, USA), used according to the manufacturer's instructions. The protein concentration of samples was determined by Bradford's method. Proteins and cell-free supernatants collected were stored at −80 °C until use. The cytotoxicity of compounds on cells was performed spectrophotometrically at 490 nm by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.[16] The percentage of viability was calculated as the relation of viable cell number/total cell number × 100.

Induction of Dextran Sulfate Sodium (DSS) Colitis and Treatment. Acute colitis was induced by supplementing drinking water with 5% (w/v) DSS for 7 days.[17] The animals were randomly assigned to one of four treatment groups: control (mice received drinking water and control diet), DSS group (mice received 5% DSS in drinking water and control diet), DSS+O group (mice received 5% DSS in drinking water and diet supplemented with 1% oleuropein) and DSS+R group (mice received 5% DSS in drinking water and diet supplemented with 2% rutin). The diet was prepared as described previously,[18] by mixing powdered food with products (1% oleuropein and 2% rutin, final content). When it was completely homogenized, water (0.65 mL g^{-1} of diet) and microwave-melted agar (0.2% final content in the diet) were added and mixed. The food was dried for 24 h and divided into portions based on an estimated consumption of 4 g of dry matter/mouse/day. Mice were sacrificed at day 8 by cervical dislocation and their colons removed. Three colon samples were prepared for histological analysis as described later, and the rest were rinsed with cold PBS, blotted dry and weighed and their length was measured to determine the weight/length ratio. Then, samples were frozen immediately in liquid nitrogen and stored at −80 °C until use.

Disease Activity Index (DAI). DAI was used to assess the severity of colitis (Table 1). Three times a week, mice were checked by monitoring body weight. At the end of the experiment, stool consistency (presence of diarrhea) and gross rectal bleeding (measured by visible fecal blood and macroscopic examination of the anus) were evaluated.

Table 1. Scoring System To Calculate the Disease Activity Index (DAI)[a]

score	weight loss	stool consistency	visible blood in feces
0	none	normal	none
1	1−5%		
2	6−10%	loose	slight bleeding
3	11−20%		
4	>20%	diarrhea	gross bleeding

[a]DAI value is calculated as the sum of scores of weight loss, stool consistency and blood in feces.

Histological Analysis. Colons were cut along the vertical axis and rolled up in a spiral starting from the distal (duodenal) margin. Then, they were fixed in 4% buffered formaldehyde, paraffin embedded, sectioned and stained with hematoxylin and eosin. Severity of colitis was assessed using a histological disease score. Eight randomly selected fields (magnification ×100) in each section were observed and graded as above in a blinded fashion by an expert pathologist. The mean in each section was calculated by scoring the grades in eight fields (Table 2).

Table 2. Histologic Scoring System for DSS-Induced Colitis[a]

score	description
	Severity of Inflammation
0	none
1	mild
2	moderate
3	severe
	Extent of Inflammation
0	none
1	mucosa
2	mucosa and submucosa
3	transmural
	Crypt Damage
0	none
1	1/3 damaged
2	2/3 damaged
3	crypts lost, surface epithelium present
4	crypts and surface epithelium lost

[a]Scores were calculated by adding the score for the three parameters, giving a maximum score of 10.

The pathological manifestations were graded as follows: severity of inflammation (0 = none, 1= mild, 2 = moderate, 3 = severe), extent of inflammation (0 = none, 1 = mild, 2 = moderate, 3 = severe) and crypt damage (0 = none, 1 = 1/3 damaged, 2 = 2/3 damaged, 3 = crypt lost, surface epithelium present, 4 = crypt lost and surface epithelium lost). The overall histological score was the sum of the three evaluations (maximal score of 10).

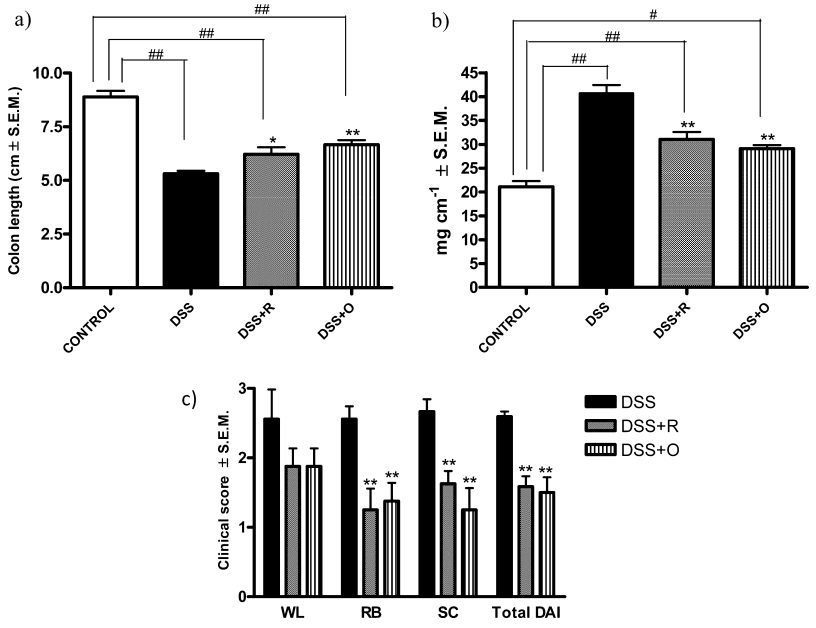

Figure 1. Effects of oleuropein in dextran sulfate sodium (DSS)-induced colitis in mice. Colitis was induced by drinking 5% DSS water for 7 days. Mice were sacrificed at day 8, and colons were harvested. (a) Colon lengths in each group measured at the end of the experiment (day 8). (b) Weight:length ratio. (c) Disease activity index (DAI) scored at day 8 using the following parameters: weight loss (WL), rectal bledding (RB) and stool consistency (SC). Each bar chart shows the mean ± SEM for at least four independent experiments (n = 10 animals for each experiment). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the DSS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different between the control group and the groups treated; using one-way ANOVA following by Dunnett's t test.

Colonic Tissue Culture. Colon tissue was washed with RPMI-1640 medium (Invitrogen, Langley, OK, USA), supplemented with 2% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Then, tissues were cut into small pieces (1 cm approximately), placed in a 24-well culture plate with RPMI-1640 medium containing 10% FBS and antibiotics, and incubated in 5% CO_2-air-humidified atmosphere at 37 °C for 24 h.[19]

Determination of NO Production. NO concentration in the colonic tissue culture media and macrophage-free supernatants was measured at 570 nm using a Labsystems Multiskan MCC/340 plate reader (Helsinki, Finland) by means of the Griess reagent.[20]

Interleukin-1β, Interleukin-6 and Tumor Necrosis Factor-α Production. The colons were thawed, grounded to powder in a mortar, weighed and then suspended (20% w/v) in PBS containing 0.1% Nonidet NP-40 buffer, homogenized, sonicated for 30 s and centrifuged at 20000g for 10 min at 4 °C. Supernatants were collected and assayed, as well as those from macrophage-free supernatants, for IL-1β, IL-6 and TNF-α by use of specific enzyme immunoassay kits, used according to the manufacturer's instructions. The amount of cytokines was expressed as pg mL^{-1} per mg of protein.

Measurement of Myeloperoxidase (MPO) Activity in Colonic Tissue. The colons were thawed, grounded to powder in a mortar, weighed and then suspended (10% w/v) in 80 mM sodium phosphate buffer (pH = 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), sonicated for 30 s and centrifuged at 11000g for 20 min at 4 °C. The assay mixture was performed in a 96-well plate, adding 30 μL of supernatant, 100 μL of PBS, 85 μL of 0.22 M sodium phosphate buffer (pH = 5.4) and 15 μL of 0.017% H_2O_2. The reaction was started by adding 20 μL of 18.4 mM of tetramethylbenzidine dissolved in 8% dimethylformamide and incubating at 37 °C for 3 min. The reaction was stopped by the addition of 30 μL of sodium acetate 1.46 M (pH = 3) on ice, and MPO activity was determined spectrophotometrically at 630 nm. MPO activity was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 unit g^{-1} of tissue.[21]

Cytosolic and Nuclear Protein Extraction. Proteins from powdered tissue samples were extracted by using a buffer A, containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin and 1 μg mL^{-1} pepstatin, pH = 7.9. After that, the samples were sonicated, added 0.5% Nonidet NP-40 solution and incubated on ice 45 min. The lysates were centrifuged at 2500g for 10 min at 4 °C, and the supernatants, corresponding to cytosolic fraction, were collected and stored at −80 °C. After that, pellets were resuspended with 500 μL of buffer B containing 20 mM HEPES, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, and 1 μg mL^{-1} pepstatin A (pH = 7.9). Samples were incubated 30 min on ice and centrifuged at 20000g for 15 min, and supernatants, corresponding to nuclear fraction, were collected and stored at −80 °C.[15] The protein concentration of samples was determined by Bradford's method.

Western Blot Analysis. Equal amounts of protein (25 μg) were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Amersham, GE Healthcare, Buckinghamshire, England, U.K.). The membranes were then blocked with PBS−0.05% Tween 20 containing 6% w/v defatted milk for 2 h. For COX-2, iNOS, MMP-9, the membranes were incubated with the respective polyclonal antibodies (1:1000 dilution); for NF-κB p65, the membranes were incubated with anti-p65 polyclonal antibody (1:500, Santa Cruz); for β-actin, the membranes were incubated with anti-β-actin polyclonal antibody (1:12000 dilution, Sigma-Aldrich). The blots were washed and incubated with peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat immunoglobulin G (1/20000) (Cayman). Finally, the immunoreactive bands were visualized with the aid of an enhanced chemiluminiscence system (Millipore). To unify Western blot densitometry results in the processed images, data from the DSS group (or, in the case of peritoneal macrophages, from the LPS-stimulated cell group) were taken as reference and assigned the value 100. Relative percentages of the other groups were then calculated and represented graphically.[15]

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Peritoneal macrophages (5×10^5) were pretreated with oleuropein and hydroxytyrosol (50 μM and 100 μM) for 1 h and then stimulated with LPS (1 μg mL^{-1}) for 4 h. Cells were collected and total RNA was extracted with the aid of RNeasy mini spin columns (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. The concentration of the extracted RNA

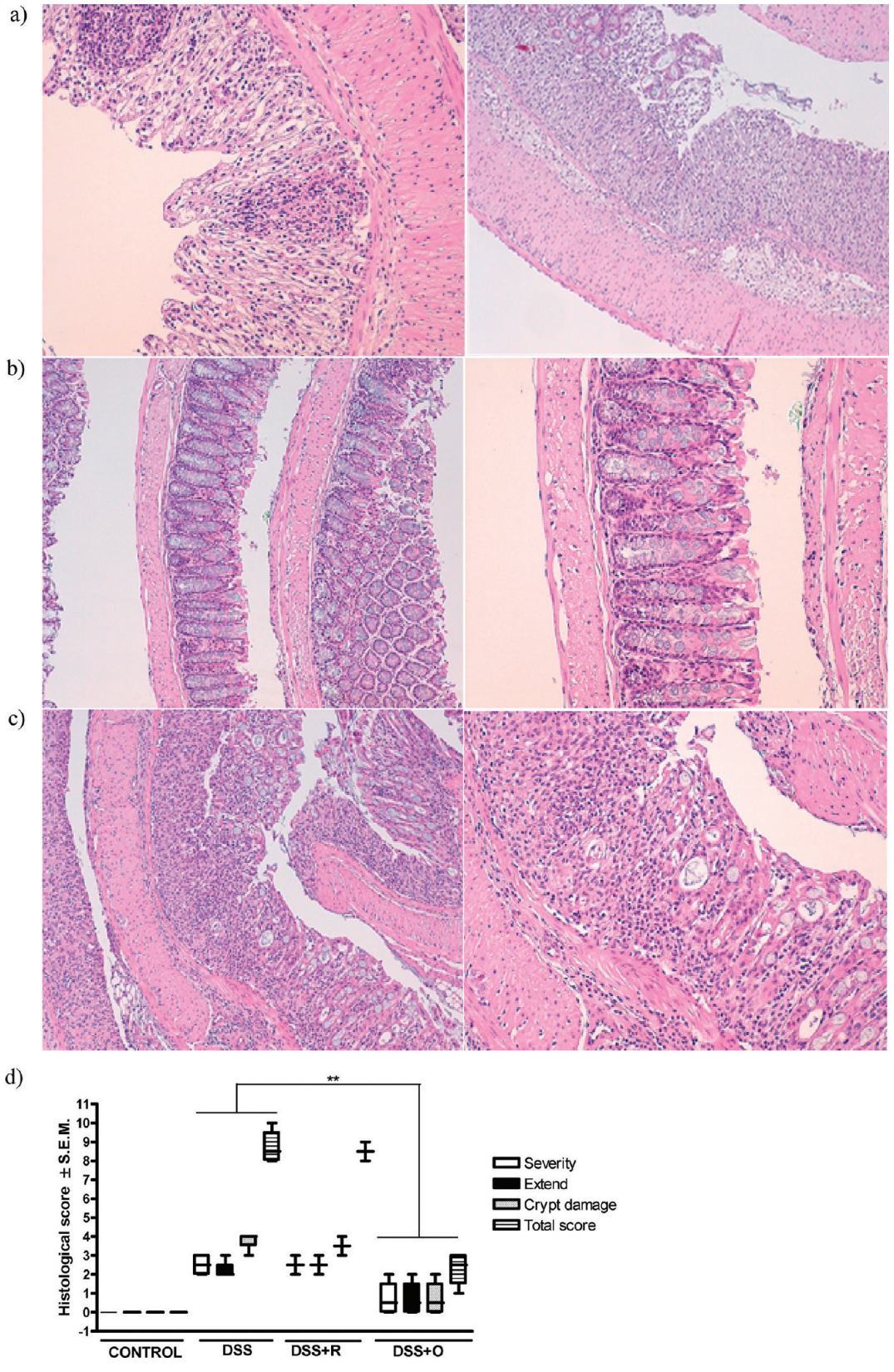

Figure 2. Effects of oleuropein on histological manifestation in dextran sulfate sodium (DSS)-induced colitis in mice. Hematoxylin and eosin staining images of representative colons are shown at the same magnification (100x). (a) DSS. (b) DSS+O. (c) DSS+R. (d) Histological scores were evaluated. Each bar chart represents the mean ± SEM for at least four independent experiments (n = 10 animals for each experiment). *P < 0.05, **P < 0.01, significantly different from the DSS group; using one-way ANOVA following by Dunnett's t test.

was calculated by measuring the optical density at 260 nm. The ratio of the optical density at 260 nm to that at 280 nm was always higher than 1.8. Aliquots of 500 ng of RNA were transformed to first strand complementary DNA (cDNA), and 1 μL of the resulting cDNA was

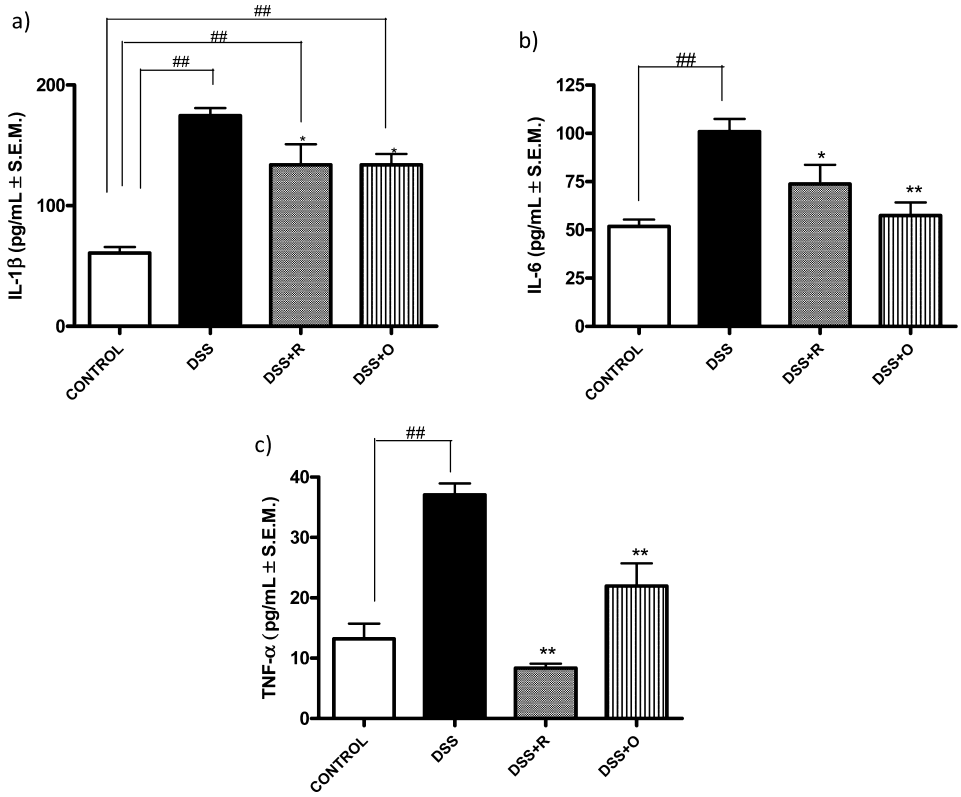

Figure 3. Effects of oleuropein on cytokine levels in dextran sulfate sodium (DSS)-induced colitis in mice. Mice were sacrificed at day 8, and harvested colon tissues were powdered in a mortar with liquid nitrogen. The cytokine levels in colonic homogenates were determined by ELISA. The amount of cytokines was expressed as pg mL^{-1} per mg of protein. (a) IL-1β levels. (b) IL-6 levels. (c) TNF-α levels. Each bar chart represents mean ± SEM for at least 3 independent experiments (n = 7 animals for each experiment). *P < 0.05, **P < 0.01, significantly different from the DSS group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, significantly different between the control group and the groups treated; using one-way ANOVA following by Dunnett's t test.

mixed with 12.5 μL of Red Taq mix and 1 μM primers (Invitrogen) and completed to 25 μL with RNase free water of IL-6 (sense, 5′-ATGCTGGTGACAACC ACGGCC-3′; antisense, 5′-GGCATAACG-CACTAGGTTTGCCGA-3′), TNF-α (sense, 5′-AGCCCACGTCG-TAGCAAACCAC-3′; antisense, 5′-TAGACGTGCCCGGACT CCGC′), IL-1β (sense, 5′-GCTGGAGAGTGTGGATCCCAAGCA-3′; antisense, 5′-AGCGACCTGTCTTGGCCGAGG-3′) and β-actin (sense, 5′-GCAGAGCAAGAGAGGCATCC-3′; antisense, 5′-CTGTGGT-GGTGAAGCTGTAG-3′). The thermocycler conditions were 94 °C for 1 min, with an annealing temperature of 60 °C for 1 min and an elongation temperature of 72 °C for 1 min for the first 30 cycles, followed by an elongation temperature of 72 °C for 10 min. After the reaction was completed, the amplified product was removed from the tubes and run on 2% agarose gel in TAE buffer (4.84 g L^{-1} Tris base, 1.144 mL L^{-1} glacial acetic acid, 2 mL L^{-1} EDTA 0.5 mM, pH = 8.5).[20]

Statistical Analysis. The results are expressed as the mean ± standard error values. Statistical significance was determined with a one-way analysis of variance (ANOVA) and Dunnett's t-test for multiple comparisons.

Software. Images for all Western blot and RT-PCR experiments were acquired with the image analysis system LAS-3000 mini (Fujifilm, Tokyo, Japan). Digital images were processed and band density measurements were made with the aid of a Multi Gauge V3.0 software package (Fujifilm).

RESULTS

Effect of Oleuropein on DSS-Induced Colitis. Water consumption and diet were checked three times a week; no differences among the various experimental groups were observed. Mice given 5% DSS in drinking water for 7 days developed colitis symptoms without mortality. Animals in the treatment groups consumed either 2.4 g of oleuropein kg^{-1} or 3.7 g of rutin kg^{-1} daily, which in humans is equivalent to 194.0 mg kg^{-1} or 302.3 mg kg^{-1}, respectively, according to the human equivalent formula.[22] Having established that consuming these amounts daily for 7 days did not affect the viability of the mice, we proceeded to examine the effects on various manifestations of acute DSS-induced colitis, including body weight loss, diarrhea, bloody stools, edematous inflammation in the colon, and shortening of the colon.[13] Weight loss was monitored at 0, 4, and 8 days; diarrhea and blood in stools were both monitored on day 8. Treatment with DSS dramatically reduced colon length, increased colon wet weight caused by edema, and produced severe diarrhea and rectal bleeding in comparison with mice that received regular drinking water (control). Treatment with oleuropein or the reference drug rutin significantly reduced colon shortening and the mg/cm ratio; however, they were only mildly effective in preventing body weight loss. Overall, the DAI score, an indicator of the severity of intestinal inflammation, which was 0 in control mice, was significantly lower in mice treated with oleuropein or rutin than in DSS mice (Figure 1).

Histological Evaluation. Histological analyses of the samples from the DSS-treated mice showed typical inflammatory changes in colonic architecture, including mucosal ulceration, crypt damage, edema, and cell infiltration into mucosal tissue (Figure 2a). Compared to those taken from DSS mice, colon tissue sections from mice fed with oleuropein exhibited far fewer infiltrating cells, a significantly lower degree of mucosal injury, and less edema (Figure 2b). Rutin produced no significant effect, although inflammatory cell infiltration, edema, and crypt damage were all slightly attenuated (Figure 2c). The total histological score determined as indicated in Table 2 is given in Figure 2d.

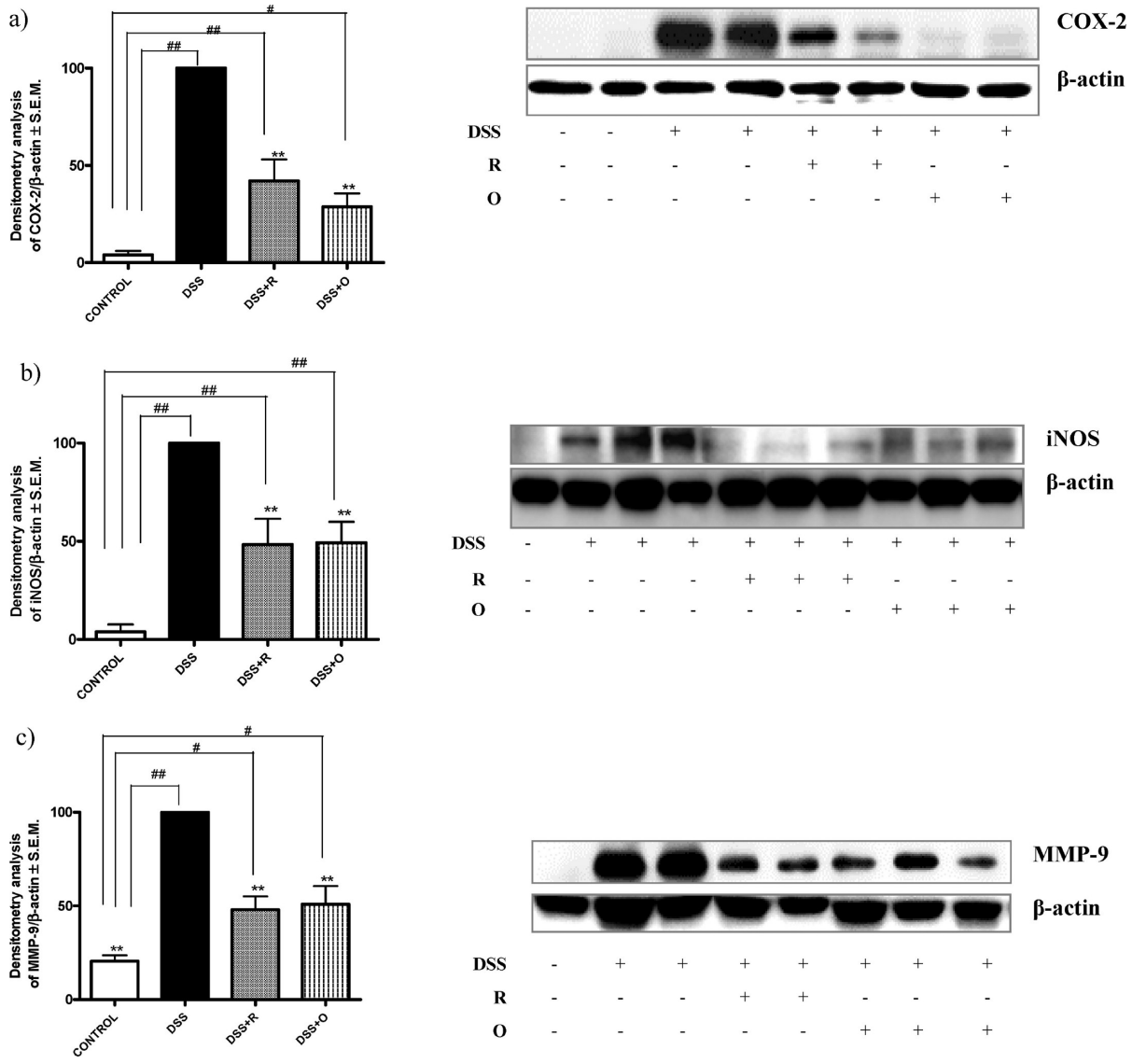

Figure 4. Effects of oleuropein on cyclooxygenase-2 (COX-2) (a), inducible nitric oxide synthase (iNOS) (b), metalloproteinase (MMP)-9 (c) and expressions in DSS-induced colitis in mice. Mice were sacrificed at day 8, harvested colon tissues were powdered in a mortar with liquid nitrogen and tissue proteins were extracted. A representative Western blot of each experiment is shown. The expressions of COX-2, iNOS, and MMP-9 are determined by densitometry and normalized to β-actin content for each sample. The histograms represent the data derived from the Western blots following densitometry analysis and considering the DSS group as having 100% expression. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the DSS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different between the control group and the groups treated; using one-way ANOVA following by Dunnett's t test ($n = 7$ animals for each experiment).

Oleuropein Suppressed NO Release in Colon Culture Supernatants. NO is a common inflammatory mediator involved in the pathogenesis of IBD. At day 8, we detected an increase of nitrite levels, an indicator of NO secretion, in the colon culture supernatants of the DSS group with regard to the control group (25-fold compared to control). These levels were significantly reduced in mice fed with oleuropein or rutin (90% and 100% inhibition, respectively, $^{**}P < 0.01$).

Effect of Oleuropein on MPO Activity in Colon Tissues. MPO, an enzyme produced mainly by polymorphonuclear leukocytes, is a marker of neutrophil infiltration in tissue. After being administered DSS for 7 days, mice showed a significant increase in MPO activity (5-fold compared to control), which was drastically reduced in oleuropein-fed mice (90% inhibition, $^{**}P < 0.01$). Rutin produced no effect on MPO activity.

Oleuropein Reduced IL-1β, IL-6 and TNF-α Production in Colon Tissue. Colonic IL-1β, IL-6 and TNF-α are proinflammatory cytokines that play a critical role in the development of IBD. After 7 days of DSS administration, IL-1β, IL-6 and TNF-α levels in colonic tissue increased markedly in DSS-treated mice in comparison with the control group. Treatment with oleuropein significantly suppressed IL-1β (Figure 3a), IL-6 (Figure 3b), and TNF-α (Figure 3c) levels by 37%, 88%, and 60%, respectively. Rutin also attenuated IL-1β, IL-6 and TNF-α levels by 37%, 30% and 90%, respectively (Figure 3).

Oleuropein Decreased Inflammatory Protein Expression in DSS Induced Colitis. Western blot analyses of colonic tissue from the DSS group showed a marked increase in the expression of COX-2 (Figure 4a) and iNOS (Figure 4b). This effect was reversed in mice treated with oleuropein (71% and 50% inhibition, respectively), with similar results for mice treated

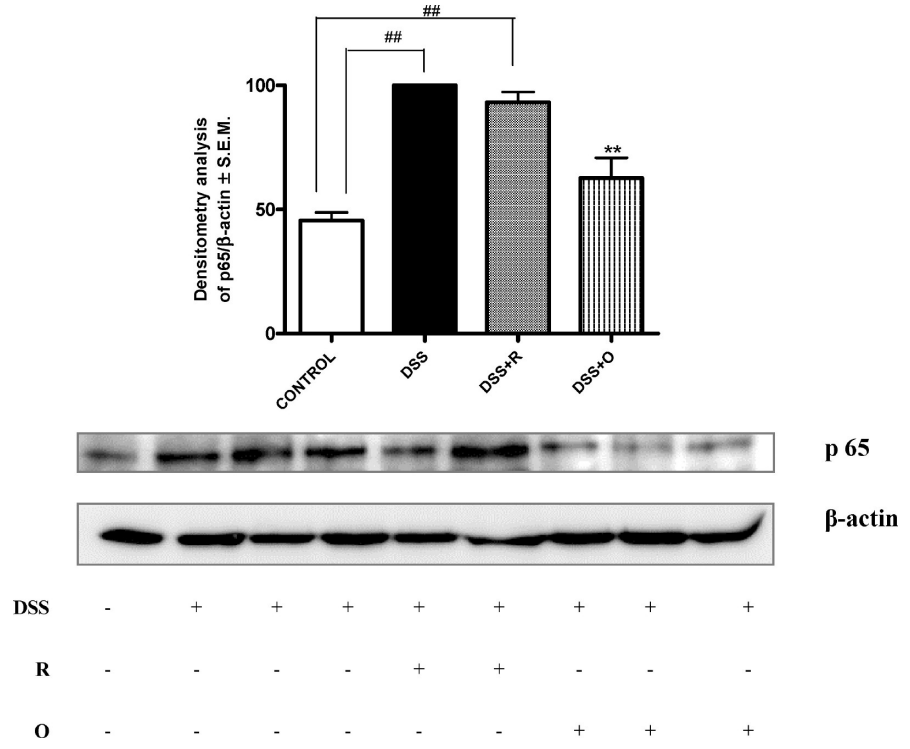

Figure 5. Effects of oleuropein on p65 expression DSS-induced colitis in mice. Mice were sacrificed at day 8, colon tissues were powdered in a mortar with liquid nitrogen and tissue nuclear proteins were extracted. A representative Western blot of the experiment is shown. The expressions of p65 is determined by densitometry and normalized to β-actin content for each sample. The histograms represent the data derived from the Western blots following densitometry analysis and considering the DSS group as having 100% expression. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the DSS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different between the control group and the groups treated; using one-way ANOVA following by Dunnett's t test (n = 7 animals for each experiment).

with rutin (63% and 51% inhibition, respectively) (Figure 4). MMPs have been shown to play a crucial role in the pathogenesis of IBD, increasing mucosal proteolysis and generating inflammation. The most abundant MMP expressed in patients with UC is MMP-9.[23] Both oleuropein and rutin significantly attenuated this increment by 62% and 65%, respectively (Figure 4c).

Oleuropein Inhibited p65 Subunit Translocation to Nucleus. Activation of NF-κB leads to the gene expression of inflammatory cytokines and other mediators involved in the pathogenesis of IBD such as COX-2 and iNOS. Upon stimulation, the functional form of NF-κB, the inactive p50/p65 heterodimer, which forms a complex with the inhibitory protein IκBα in the cytoplasm, is rapidly phosphorylated by IκB kinase and proteolytically degraded, allowing translocation of p50/p65 to the nucleus. Inhibition of NF-κB activation has thus been proposed as an anti-inflammatory strategy in IBD. Using Western blot analysis, we detected an increase in p65 subunit levels in nuclear proteins obtained from DSS mice. Interestingly, oleuropein treatment significantly reduced DSS-induced NF-κB nuclear translocation by 40% whereas rutin exerted no effect on NF-κB p65 in the nuclear extract of colon tissue (Figure 5).

Effect of Oleuropein and Its Metabolite Hydroxytyrosol on Several Proinflammatory Mediators in Lipopolysaccharide Stimulated-Mouse Peritoneal Macrophages. After having demonstrated that neither oleuropein nor hydroxytyrosol at 100 μM exhibited any cytotoxicity on mouse peritoneal macrophages in the MTT test (data not shown), we tested the effects of these compounds on the expression and release of several proinflammatory proteins. As shown in Figure 6, levels of IL-1β, IL-6, and TNF-α in peritoneal macrophages increased markedly after stimulation with LPS. Only hydroxytyrosol treatment (100 μM) 1 h before stimulation significantly decreased the production of IL-1β (72%), IL-6 (50%), and TNF-α (33%). It also reduced iNOS expression by 72% (Figure 7a), and the subsequent production of NO was inhibited by 87%. Hydroxytyrosol treatment also led to a significant reduction of NF-κB p65 in the nuclear extract of LPS-stimulated cells in a concentration dependent manner (Figure 7b). To determine whether the inhibitory effect of oleuropein and hydroxytyrosol on cytokine production occurred through the inhibition of the corresponding gene expression, mRNA expression of IL-6, TNF-α, and IL-1β was analyzed with the aid of semiquantitative RT-PCR. As shown in Figure 8, IL-6, TNF-α and IL-1β were undetectable in non-stimulated cells. However, upon stimulation with LPS, the protein expression of these cytokines showed a marked increase. Hydroxytyrosol at 100 μM significantly reduced IL-6 and IL-1β gene mRNA expression by 25% and 21%, respectively.

DISCUSSION

Ulcerative colitis induced by DSS is characterized by an increased mucosal infiltration of inflammatory cells, which activated, release proinflammatory cytokines, induce increased expression of iNOS, COX-2 and nuclear transcription factor NF-κB. These changes impair the mucous barrier and cause swelling, erosions, ulcers and hemorrhage leading to destructive damage of intestinal mucosa. In this study, we have demonstrated that oleuropein orally administered at a dose of 2.4 g kg^{-1} is effective for attenuating DSS-induced colitis in mice via inhibition of proinflammatory protein expression and release as well as by acting on NF-κB signaling. The severity of the colitis has

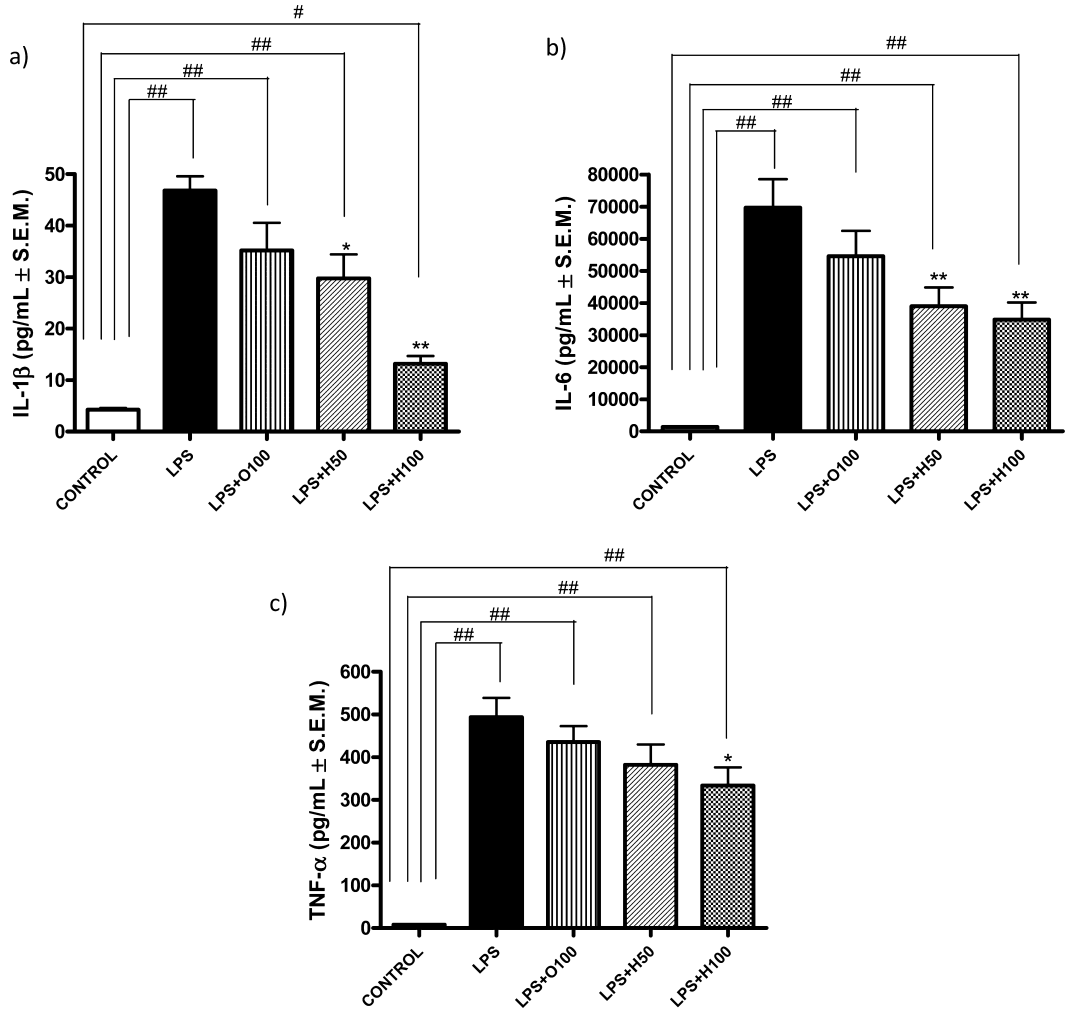

Figure 6. Effects of oleuropein and hydroxytyrosol on cytokine levels in lipopolysaccharide (LPS)-stimulated peritoneal macrophages. Peritoneal macrophages from mice intraperitoneally injected with 3% thioglycolate broth 4 days before were seeded onto a 6-well plate at a density of 1.5×10^6 cells/well in DMEM. After incubation for 2 h, macrophages were treated with the vehicle (medium) or oleuropein or hydroxytyrosol (50 μM and 100 μM) for 1 h and then stimulated with LPS (1 μg mL^{-1}) for 24 h. Following incubation, the cytokine levels in cell-free supernatants were determined by ELISA and expressed as pg mL^{-1}. (a) IL-1β levels. (b) IL-6 levels. (c) TNF-α levels. Each bar chart represents mean ± SEM for at least 3 independent experiments (n = 12). *P < 0.05, **P < 0.01, significantly different from the control group; using one-way ANOVA following by Dunnett's t test.

been determined according to various morphological, histological and biochemical parameters, and oleuropein has been shown to protect against this model of colitis since it decreased the extension of inflammation and enhanced integrity of the epithelium as observed on histologic assessment.

DSS-induced inflammation is controlled by an array of pro- and anti-inflammatory cytokines which normally recruits inflammatory cells to the colitic tissue. Our results exhibit a marked increased of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in colitic tissue that are inhibited by oleuropein. There is an amount of data to show that cytokines can increase MMP production. MMPs are implicated in tissue remodeling and destruction associated with several inflammatory conditions, the predominant proteinases being expressed in the gut mucosa during active IBD. Indeed, both an increased activity and expression of MMPs in colonic tissues of patients with IBD have been found. Moreover, of the MMPs, the secreted gelatinase MMP-9 is upregulated in both animal models of colitis such as DSS-induced colitis and human IBD, and several studies have indicated that DSS-induced colitis is markedly attenuated in animals containing a targeted deletion of the MMP-9 gene.[24,25] Thus, strategies to inhibit MMP-9 may be of potential therapeutic benefit. In addition, neutrophil infiltration is considered a key histological characteristic of acute activity of the disease, so concomitantly, the activity of myeloperoxidase is enhanced, and these cells mainly secrete MMP-9. Our data show that oleuropein not only markedly attenuated neutrophil accumulation but also inhibited MMP-9 expression in colitic tissue. This result is in agreement with a previous report indicating that oleuropein aglycon in vitro prevented the stimulation of MMP-9 expression and secretion in TNF-α treated THP-1 cells due to impaired NF-κB signaling.[26]

Among the principal intracellular signal transduction cascades involved in intestinal inflammation, NF-κB plays a crucial role as a key regulator of inflammatory gene expression. When activated, NF-κB translocates into the nucleus to regulate the expression of genes encoding cytokines such as IL-1β, IL-6 and TNF-α, inflammatory enzymes particularly iNOS and COX-2, adhesion molecules, immunoregulatory proteins and acute phase response proteins.[14] Recent investigations have established a close interrelationship between both the inhibition of NF-κB and the reduction of cytokine production and the

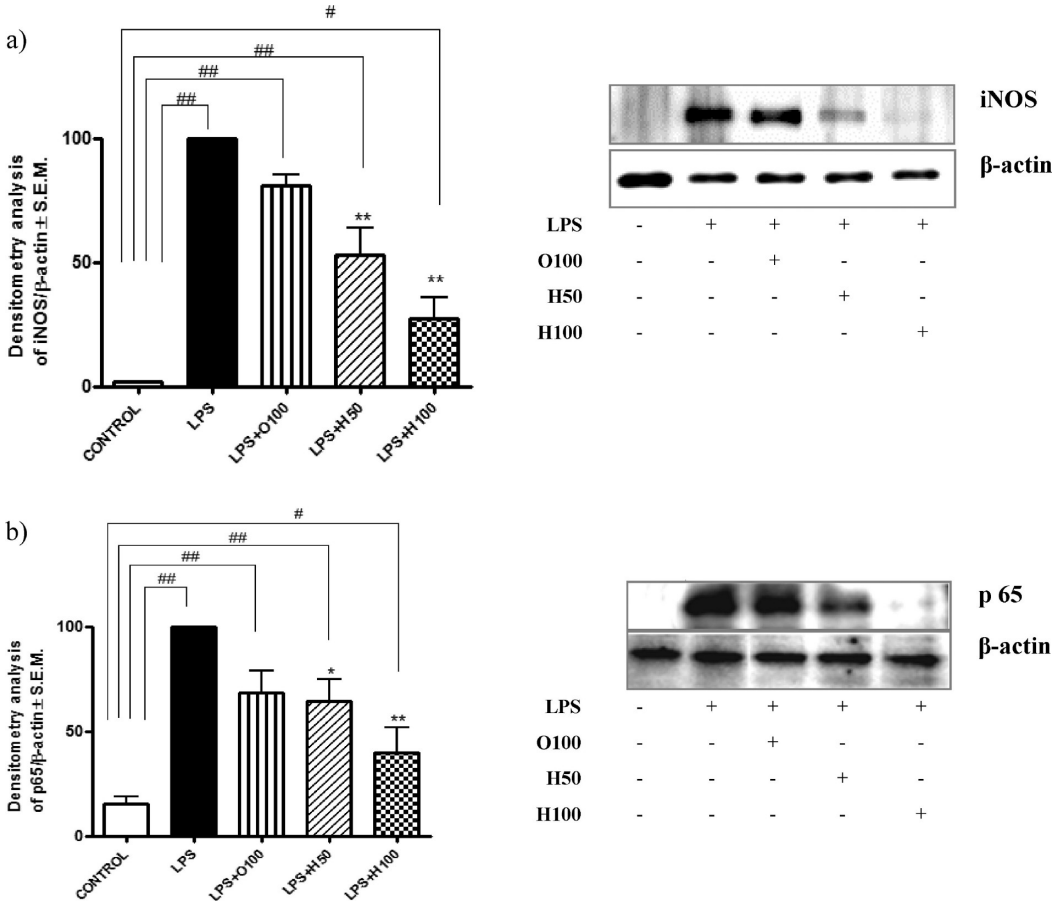

Figure 7. Effects of hydroxytyrosol on inducible nitric oxide synthase (iNOS) (a) and p65 (b) expressions in lipopolysaccharide (LPS)-stimulated peritoneal macrophages. Peritoneal macrophages from mice intraperitoneally injected with 3% thioglycolate broth 4 days before were seeded onto a 6-well plate at a density of 1.5×10^6 cells/well in DMEM. After incubation for 2 h, macrophages were treated with the vehicle (medium) or hydroxytyrosol (50 μM and 100 μM) for 1 h and then stimulated with LPS (1 μg mL^{-1}) for 24 h. Following incubation, cells were scraped and proteins were extracted with a protein extraction kit. The expressions of iNOS and p65 are determined by densitometry and normalized to β-actin content for each sample. The histograms represent the data derived from the Western blots following densitometry analysis and considering the control group as having 100% expression. *$P < 0.05$, **$P < 0.01$, significantly different from the control group; using one-way ANOVA following by Dunnett's t test ($n = 12$).

attenuation of IBD. In this context, oleuropein manifest a clear reduction on NF-κB p65 nuclear translocation in the colon tissue. This inhibitory effect may partly justify the decrease observed in the cytokine production.

In ulcerative colitis, cytokines induce expression of iNOS leading to an increase of NO synthesis, which causes development of inflammatory processes and ulcerogenesis. Development of colitis is accompanied not only by the activation of the NO-synthase system but also by the increased expression of COX-2. Despite the pivotal role of both iNOS and COX-2 in the pathogenesis of colitis, their relative contributions in intestinal inflammation remain controversial. Although iNOS is an excellent marker of intestinal inflammation, it has been claimed to be associated to both exacerbation and protection of colitis. In this regard, both deterioration of trinitrobenzene sulfonic acid colitis and improvement of DSS colitis in mice with deletion of iNOS have been reported.[27] In the present study, we have identified large increases in iNOS expression in DSS-induced colitis and found that oleuropein treatment of the mice significantly reduce iNOS levels. These findings are consistent with a previous report indicating a marked attenuation of colitis in iNOS-deficient mice in other experimental models[27] suggesting that the reduction in iNOS with oleuropein is likely contributing to its benefit in this model. On the other hand, animal models of colitis using COX-2 knockout mice and COX inhibitors indicate that COX-2 has a protective role against colon inflammation.[28] However, contrary results were observed using celecoxib, which caused a substantial reduction of the degree of colonic injury. In this context, results obtained by Sklyarov et al.[29] provide evidence of the marked cytoprotective effects of the combination of both iNOS and COX-2 inactivation under conditions of ulcerative colitis, thus suggesting the development of drugs capable of combined blockage of iNOS and COX-2 as a new approach for the treatment of ulcerative colitis. In the current study, a clear reduction of COX-2 was also observed in the colonic tissue of mice treated with oleuropein, so this inhibition could display additive character to its effect on the inflammation process.

We also investigated the effect of oleuropein and its active metabolite hydroxytyrosol on peritoneal LPS-stimulated macrophages. In contrast to the strong inhibitory effects shown in the in vivo experiments, oleuropein exerts negligible activity in vitro. However, hydroxytyrosol in a concentration dependent manner is shown to inhibit significantly iNOS expression, accompanied by a reduction of NO production and p65 expression as well as cytokine production. Furthermore, we observe a modest decrease in IL-1β, IL-6 and TNF-α mRNA expression when cells were exposed to hydroxytyrosol. Our findings shows that the oral treatment of mice with oleuropein

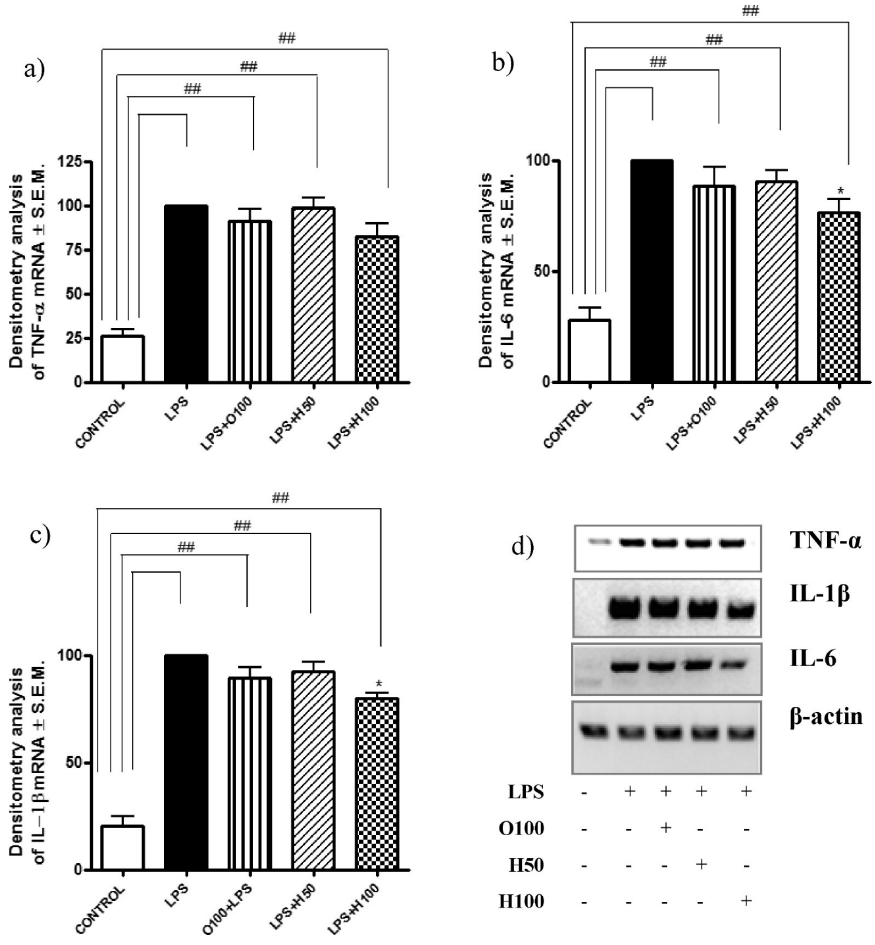

Figure 8. Effects of oleuropein and hydroxytyrosol on TNF-α (a), IL-6 (b), and IL-1β (c) gene mRNA expression in lipopolysaccharide (LPS)-stimulated peritoneal macrophages. Peritoneal macrophages from mice intraperitoneally injected with 3% thioglycolate broth 4 days before were seeded onto a 6-well plate at a density of 1.5×10^6 cells/well in DMEM. After incubation for 2 h, macrophages were treated with the vehicle (medium) or oleuropein or hydroxytyrosol (50 μM and 100 μM) for 1 h and then stimulated with LPS (1 μg mL^{-1}) for 4 h. Following incubation, cells were collected and total RNA was extracted with the aid of RNA extraction kit. Expression of TNF-α (b), IL-6 (c), and IL-1β (d) genes are measured using RT-PCR, determined by densitometry and normalized to β-actin content for each sample. The histograms represent the data derived from the RT-PCR following densitometry analysis and considering the control group as having 100% expression. *$P < 0.05$, **$P < 0.01$, significantly different from the control group; using one-way ANOVA following by Dunnett's t test ($n = 12$).

improve DSS-induced colitis and provide support for hydroxytyrosol as an anti-inflammatory agent in vitro.

Collectively, our results clearly indicate that oleuropein effectively has a protective role in the development of ulcerative colitis in a DSS model of colitis in mice. This beneficial effect is correlated with a better DAI index, a reduction of proinflammatory cytokine levels and a reduction of COX-2, iNOS and MMP-9 protein expression in the colonic tissue, at least in part, via inhibition of NF-κB activity.

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ABBREVIATIONS USED

ADP, adenosine diphosphate; COX-2, cyclooxygenase-2; cAMP, cyclic adenosine monophosphate; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-kappa B; IL, interleukin; PGE_2, prostaglandin E_2; TNF, tumor necrosis factor; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn disease; DSS, dextran sulfate sodium; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; PBS, phosphate buffer saline; FBS, fetal bovine serum; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAI, disease activity index; NO, nitric oxide; MPO, myeloperoxidase; HTAB, hexadecyltrimethylammonium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; cDNA, complementary deoxyribonucleic acid; ANOVA, one-way analysis of variance; RT-PCR, reverse transcription-polymerase chain reaction

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