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3,5-Dicaffeoyl-4-malonylquinic acid reduced oxidative stress and inflammation in a experimental model of inflammatory bowel disease

ROSANNA DI PAOLA[1], EMANUELA ESPOSITO[1,2], EMANUELA MAZZON[1], ROCCO CAMINITI[3], ROBERTO DAL TOSO[4], GIOVANNA PRESSI[4] & SALVATORE COZZOCREA[1,5]

[1]*IRCCS Centro Neurolesi 'Bonino-Pulejo', Messina, Italy,* [2]*Department of Experimental Pharmacology, University of Naples 'Federico II', Napoli, Italy,* [3]*Department of Human Pathology, School of Medicine, University of Messina, Italy,* [4]*I.R.B. srl Altavilla Vicentina Italy, and* [5]*Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Italy*

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Abstract

Aim: The aim of the present study was to examine the effects of 3,5-dicaffeoyl-4-malonylquinic acid (CA1), extract from Centella Asiatica, in rats subjected to experimental colitis.

Results: Colitis was induced in rats by intracolonic instillation of dinitrobenzene sulphonic acid (DNBS). CA1 was administered daily orally (0.2 or 2 mg/kg). Four days after DNBS administration, treatment with CA1 significantly reduced the appearance of diarrhoea and the loss of body weight. This was associated with a significant reduction in colonic MPO activity. CA1 also reduced NF-κB activation, the pro-inflammatory cytokines release, the appearance of I-NOS, nitrotyrosine, PARP and proMMP-9 and -2 activity in the colon and reduced the up-regulation of ICAM-1 and the expression of P-Selectin.

Conclusions: The results of this study suggested that administration of CA1 may be beneficial for treatment of inflammatory bowel disease.

Keywords: *3,5-Dicaffeoyl-4-malonylquinic acid, colitis, neutrophil infiltration, NF-κB*

Introduction

Caffeoylquinic acid derivatives (CQAs) with diversity and extensive distribution in the plant kingdom are important bioactive components in some Chinese medicines, such as Flos Lonicerae, Herba Erigerontis and Fructus Xanthii. The compounds of this category were well known as potential antioxidants [1–4]. Furthermore, they also possessed significant anti-tumour [5], hepatoprotective [6,7], anti-inflammatory [8], antimicrobial activities [9], anti-mutagenicity [10] and so on [11].

Yonathan et al. [12] showed the anti-inflammatory and anti-nociceptive activities of Cheilanthes farinosa (Forsk.) Kaulf (Adianthaceae), a fern used in many parts of Ethiopia to treat inflammatory skin disorders, using *in vivo* models of inflammation and pain.

Also *Centella asiatica* is listed in the official Chinese Pharmacopeia, and is used as an anti-pyretic, diuretic, to treat ulcers, eczemas and trauma [13]. It is a small perennial plant, belonging to the Apiaceae family, used in traditional Indian medicine to treat various pathologies.

Recent studies have shown *Centella Asiatica* may have a positive effect on the circulatory system and may improve blood flow throughout the body by strengthening the veins and capillaries [14].

Belcaro et al. [15,16] describes that triterpenes of *Centella asiatica* are effective in counteracting chronic venous and microcirculation insufficiency.

Correspondence: Salvatore Cuzzocrea, Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Torre Biologica, Policlinico Universitario Via C. Valeria, Gazzi, 98100 Messina Italy. Tel: (39) 090 2213644. Fax: (39) 090 2213300. Email: salvator@unime.it

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Centella Asiatica is also quoted in the treatment of phlebitis [17] as well as leg cramps, swelling of the legs and heaviness or tingling in the legs (particularly useful for bedridden people) [18] and for treatment of high blood pressure [15].

The major constituents are triterpene saponins, mainly asiaticoside, sapogenin asiatic acid, madecassoside and madecassic acid [19]. The aqueous extract of CA possesses antioxidant, cognitive-enhancing and anti-epileptic properties.

It was shown that *Centella asiatica* induces levels of antioxidants in wounds and newly formed tissue, including superoxide dismutase, glutathione peroxidase, vitamin E and vitamin C. Moreover, *Centella asiatica* improves collagen formation and angiogenesis [20].

Recent study confirms this data showing that CaE functions as an antioxidant *in vitro*, scavenging free radicals, reducing lipid peroxidation and protecting against DNA damage, modulating components of the oxidative stress response that has been implicated in the neurodegenerative changes that occur with Alzheimer's disease [21].

By *Centella asiatica* was extracted a significantly active compound, the 3,5-di-O-caffeoyl 4-malonylquinic acid, together with chlorogenic acid [22], 1,5-di-O-caffeoylquinic acid [23], 3,4-di-*O*-caffeoylquinic acid [22], 4,5-di-O-caffeoylquinic acid [22] and also the flavonoids, kaempferol, quercetin, kaempferol, 3-O-β-D-glucuronide, quercetin-3-O-β-D-glucuronide and the saponin, asiatico side [24].

The objectives of this study were to evaluate the anti-inflammatory activities and the antioxidant power of CA extract in an animal model of colitis.

Inflammatory bowel disease (IBD) encompasses several chronic inflammatory conditions, most significantly ulcerative colitis (UC) and Crohn's disease (CD).

The main difference between Crohn's disease and UC is the location and nature of the inflammatory changes. Crohn's can affect any part of the gastrointestinal tract, from mouth to anus (skip lesions), although a majority of the cases start in the terminal ileum. Ulcerative colitis, in contrast, is restricted to the colon and the rectum [25].

As many as 1.4 million persons in the US and 2.2 million persons in Europe suffer from these diseases. Previously noted racial and ethnic differences seem to be narrowing. Differences in incidence across age, time and geographic region suggest that environmental factors significantly modify the expression of Crohn's disease and ulcerative colitis.

However, IBS remains a complex and inadequately understood disease. The aetiologies of these diseases still remain unknown, although immunological, infective and genetic factors begin to emerge as candidate mechanisms underlying disturbed sensory and motor function in this syndrome.

Several genes have been implicated in the aetiology of CD, the most prominent of which are the NOD2/CARD15 located on chromosome 16 [26,27], the OCTN1 gene located on chromosome 5 [28] and the DLG5 gene located on chromosome 10 [29]. The TLR4 gene has recently been implicated in CD but is not associated with the chromosomal region previously linked to CD.

Although several possible mechanisms for a bacterial aetiology in the development of CD have been proposed: (1) an immune response to a specific pathogen resulting in intestinal inflammation [30]; (2) alterations in normal bacterial content of the intestinal tract [31]; (3) a defective mucosal barrier and overwhelming exposure to resident bacteria and their antigens and endotoxins [32]; and (4) alterations to the intestinal immune response [30].

Oxidative stress is thought to play a significant role in the pathogenesis of inflammatory bowel disease, including CD. Endogenous antioxidants such as superoxide dismutase (SOD), glutathione and catalase are normally able to counteract oxidative stress in the intestinal mucosa. However, inflammation increases the demand for these important antioxidants and results in an imbalance between pro-oxidants and antioxidants, with subsequent mucosal damage [33].

These immunological, infective and genetic mechanisms may help to aid a better understanding of symptom generation and to develop novel pharmacological drugs for this common condition.

Many animal models of inflammatory bowel disease (IBD) have been developed over the past 30 years and are widely used to investigate the pathogenesis of intestinal inflammation and as models in which novel therapeutic regimens can be tested.

Among the most commonly used animal models for chronic colitis is the 'hapten model'. This model involves a single, intracolonic administration of a hapten, trinitrobenzenesulphonic acid (TNBS), dissolved in a vehicle of 30–50% (vol/vol) ethanol. This treatment results in the formation of colonic ulcers that persist for up to 8 weeks. Since Wallace et al. [34] suggested that 2,4-dinitrobenzene sulphonic acid (DNBS or oxazolone), which is readily available from a number of commercial sources, is as effective as TNBS for inducing chronic colitis in the rat, in our experimental model, the colitis was induced by the intracolonic instillation of this haptenizing agent, that quickly develops an acute inflammatory disease, considered important to prevent the development of IBD at an early stage.

Materials and methods

Reagents

Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA).

All other reagents and compounds used were purchased from Sigma Chemical Company (St. Louis, MO).

Extraction of 3,5-dicaffeoyl-4-malonylquinic acid from cell cultures of Centella asiatica

Calluses grown on solid medium (GAMBORG B5 with 1% agar supplemented with 20 g/l sucrose, 1 g/l plant peptone, 1 mg/l kinetin, 1 mg/l naphthalenacetic acid and 0.2 mg/l indolacetic acid at pH 6.5) and subjected to subculture for at least 3 months, are used to inoculate 10 flasks of 3 L volume, with the final medium content of 1 L for each flask.

Fermentation is monitored by means of samples taken sequentially over time and is terminated upon reaching an age of 14 days. The biomass is combined and filtered through nylon mesh with a porosity of 100 μm.

The filtrate is discarded and the cells suspended in an equal volume of ethanol supplemented with 0.5 g/L ascorbic acid and 2 g/L citric acid.

The suspension is homogenized using an Ultra Turrax and then filtered. The cell residue is extracted twice further with 96% ethanol. The combined filtrates contain 9.5 g of 3,5-dicaffeoyl-4-malonylquinic acid. All the ethanol is removed from the filtrates and the aqueous residue extracted twice with ethyl acetate.

The ethyl acetate extracts are anhydrated over sodium sulphate, filtered and dried by reduced pressure distillation; 17.5 g of a pale yellow coloured crude extract, containing 9.3 g of 3,5-dicaffeoyl-4-malonylquinic acid, is obtained. In order to obtain purer material, the extract is loaded onto an RPC18 column in 15% acetonitrile in water and the column eluted isocratically. A chromatographic peak is obtained which, following removal of the organic solvent by distillation, and the aqueous phase by lyophilization, leaves a residue of 7.1 g of pure 3,5-dicaffeoyl-4-malonylquinic acid, suitable for structural analysis and experimental use (Figure 1).

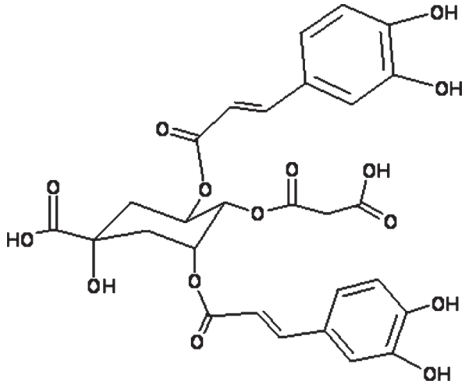

Figure 1. The chromatogram of *Centella asiatica* extract, containing 3,5-dicaffeoyl-4-malonylquinic acid, at 330 nm.

Table I. The High pressure liquid chromatography (HPLC) analysis gradient

T (min)	solv. B(%)
0	20
5	30
7	40
10	40
15	20

HPLC analysis method

High pressure liquid chromatography (HPLC) analysis is performed using a Phenomenex 4.6 × 150 mm 3 μm C-18 column, with the following eluent mixtures:

- Solvent A: 0.1% H_3PO_4 in H_2O, supplemented with 0.5% solvent B
- Solvent B: 0.1% H_3PO_4 in CH_3CN, supplemented with 0.5% solvent A.
- Flow rate: 1 ml/min

The gradient used has been as shown in Table I Quantification of the compound has been performed at 330 nm (Table II).

Table II. Retention time of the compound.

Product	*RT* (min)
3,5-dicaffeoyl-4-malonylquinic acid	7.9

Animals

Male Sprague-Dawley rats (300–350 g, Charles River, Milan, Italy) were placed single in cages with wire-net floors in a room with controlled environment, (temperature 24–25°C, humidity 70–75%, lighting regimen of 12L/12D) and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986). Experiments followed a protocol approved by the local animal Ethics Committee and the Local Government.

Experimental group

Animals were randomly divided into six groups (n = 10 for each group):

1. Sham + Vehicle group: Vehicle solution (saline) was administered daily orally.
2. Sham + 3,5-dicaffeoyl-4-malonylquinic acid (2 mg/kg): CA extract was administered daily orally.
3. Sham + 3,5-dicaffeoyl-4-malonylquinic acid (0.2 mg/kg): CA extract was administered daily orally.

4. DNBS + Vehicle: Vehicle was administered daily orally and animals were sacrificed on day 4 after administration of DNBS.
5. DNBS + 3,5-dicaffeoyl-4-malonylquinic acid (2 mg/kg): CA extract was administered as by gavage at 2 mg/kg every 24 h, starting from 3 h after the administration of DNBS.
6. DNBS + 3,5-dicaffeoyl-4-malonylquinic acid (0.2 mg/kg) CA extract was administered as by gavage at 0.2 mg/kg every 24 h, starting from 3 h after the administration of DNBS.

The doses of 3,5-dicaffeoyl-4-malonylquinic acid (0.2 and 2 mg/kg) used here were based on previous dose–response and time-course studies by our laboratory.

Induction of experimental colitis

Colitis was induced by using a technique of acid-induced colonic damage as described previously. Briefly, in fasted rats lightly anaesthetized with isoflurane, a 3.5 F catheter was inserted into the colon via the anus until approximately the splenic flexure (8 cm from the anus). 2,4,6-dinitrobenzene sulphonic acid (DNBS; 25 mg/rat) was dissolved in 50% ethanol (total volume, 0.8 ml) and deposited into the colon. Thereafter, the animals were kept for 15 min in a Trendelenburg position to avoid reflux. After colitis and sham colitis induction, the animals were observed for 3 days. On day 4, the animals were weighed and anaesthetized with chloralium hydrate (400 mg/kg, intraperitoneally) and the abdomen was opened by a midline incision. The entire colon was removed, isolated from surrounding tissues, opened along the anti-mesenteric border, rinsed, weighed and processed for histology and immunohistochemistry.

Evaluation of colon damage

After its removal, the entire colon was gently rinsed with saline solution, opened by a longitudinal incision and immediately examined under a microscope. Colon damage (macroscopic damage score) was evaluated and scored by two independent observers as described previously according to the following criteria: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers with no 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 centimetre of ulceration beyond an initial 2 cm.

Optical microscopy

After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid), entire colon samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Thereafter, 7-μm sections were deparaffinized with xylene, stained with haematoxylin-eosin and observed in a Dialux 22 Leitz (Wetzlar, Germany) microscope. In order to have a quantification (quantitative estimation) of colon damage, each section ($n = 6$ for each animals) was scored by two independent observers blinded to the experimental protocol. The following morphological criteria were considered: score 0, no damage; score 1 (mild), focal epithelial oedema and necrosis; score 2 (moderate), diffuse swelling and necrosis of the villi; score 3 (severe), necrosis with presence of neutrophil infiltrate in the submucosa; score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and haemorrhage.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined. At the specified time following the intracolonic injection of DNBS, colon tissues were obtained and weighed. Each piece of tissue was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20 000 × g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37°C and was expressed in mU per gram weight of wet tissue.

Immunohistochemical localization of ICAM-1, P-Selectin, iNOS, nitrotyrosine, PAR, TNF-α and IL-1β

At 4 days after DNBS administration, colon tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 7 μm 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 min. The sections were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with (1) purified goat polyclonal antibody directed towards P-Selectin which reacts with rats; (2) purified hamster anti-mouse ICAM-1 (CD54) (1:500 in PBS, w/v); (3) anti- TNF-α polyclonal

antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v); (4) anti-IL-1β antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v); (5) anti- PAR antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v); 6) anti-nitrotyrosine antibody; or (7) anti-iNOS antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v). Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). Immunocytochemistry photographs ($n = 5$) were assessed by densitometry using Imaging Densitometer (Axio-Vision, Zeiss, Milan, Italy) and a computer program. In particular the densitometry analysis was carried out in sections in which the colon was orientated in order to observe all the mucosa villi. In this type of section, it is possible to evaluate the presence/absence or the alteration of the distribution pattern. Therefore, the densitometry data obtained represent all these differences.

Western blot analysis for IκB-α, phospho-NF-κBp65 (serine 536), NF-κBp65

Cytosolic and nuclear extracts were prepared as previously described [35] with slight modifications. Briefly, colon tissues from each rat were suspended in extraction Buffer A (0,32 M sucrose, 10 mM TRIS-HCl pH 7.4, 1 mM ethyleneglycol-*bis* (β-aminoethyl)-N,N,N′,N′-tetraacetic acid [EGTA], 2 mM ethylenediaminetetraacetic acid [EDTA], 5 mM NaN_3, 10 mM 2-mercaptoethanol, 50 mM NaF, 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, 1 mM sodium orthovanadate), homogenized at the highest setting for 2 min and centrifuged at 1000 × g for 10 min, 4°C. The supernatant, representing the cytosolic fraction, were stored at –80°C. The pellets, containing enriched nuclei, were washed with Buffer A containing 0.6% (v/v) Nonidet P40 and centrifuged (1000 × *g*, 5 min, 4°C) and 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, 0,2 mM sodium orthovanadate. After centrifugation, 30 min at 15 000 × g at 4°C, the supernatants containing the nuclear protein were stored at –80°C.

The levels of IκB-α and phospho-NF-κBp65 (serine 536) were quantified in cytosolic fraction from colon tissue collected at 4 days after DNBS administration by Western blot analysis. Nuclear fractions were incubated with anti-NF-κB p65 (1:500; Santa Cruz Biotechnology). Proteins were transferred onto nitrocellulose membranes. The filters were blocked with 1x PBS, 5% (w/v) non-fat dried milk for 40 min at room temperature and subsequently probed with specific mAbs IκB-α (Santa Cruz Biotechnology, 1:1000) or phospho-NF-κB p65 (serine 536) (Cell Signaling,1:1000) in 1× PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 at 4°C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The immune complexes were visualized using the SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Milan, Italy).

To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β-actin protein (1:10 000 Sigma-Aldrich Corp.). The relative expression of the protein bands of P65 ser536 (65 kDa) IκB-α (~ 37 kDa) and phospho NF-κB (75 kDa) was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM) were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO-RAD USA).

Gelatin zymography

The activity of gelatinases (A or matrix metalloproteinase type 2 MMP-2 and B or MMP-9) activity was measured as previously described [36] in colonic homogenates. Briefly, colonic samples were homogenized, mixed with electrophoresis loading buffer and subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with copolymerized gelatin (0.2%; Sigma Chemical Co, St. Louis, MO). After electrophoresis, the gels were washed and gently shaken in three consecutive washings in 2.5% Triton X-100 solution to remove SDS. The gel slabs were then incubated at 37°C overnight in development buffer (50 mmol/L Tris HCl, 200 mmol/L NaCl, 10 mmol/L CaCl2 and 1 μmol/L ZnCl2, pH = 7.5) and subsequently stained with 0.5% Coomassie Blue. After intensive destaining (4% methanol with 8% acetic acid), proteolysis areas appeared as clear bands against a blue background. Molecular weight markers were used as standards. To measure the activities of the detected enzymes, zymograms were read using a ScanJet scanner. The intensities of the separate bands were analysed using Gel measurement software. Quantitative evaluation of both surface and intensity of lysis bands, on the basis of grey levels, were compared relative to non-treated control wells and expressed as 'relative expression' of gelatinolytic activity.

Data analysis

All values in the figures and text are expressed as mean ± standard error (SEM) of the mean of *n* observations. For the *in vivo* studies *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results

were analysed by one-way ANOVA followed by a Bonferroni *post-hoc* test for multiple comparisons. A p-value less than 0.05 was considered significant.

Results

Effects of CA extract treatment on the degree of colitis

No histological alteration was observed in the colon tissue from sham-treated rats (Figures 2A, see macroscopic score 2H; 2E, see histological score 2I). Four days after intra-colonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon and rectum showed presence of mucosal congestion, erosion and haemorrhagic ulcerations (Figure 2B, see macroscopic score 2H). The histopathological features included a transmural necrosis and oedema and a diffuse leukocyte cellular infiltrate in the sub-mucosa of colon section from DNBS-treated rats (Figure 2F, see histological score 2I).

The treatment with 3,5-dicaffeoyl-4-malonylquinic acid significantly reduced in a dose-dependent manner the extent and severity of the macroscopic (Figures 2C and D, respectively, see macroscopic score 2H) and histological signs of colon injury (Figure 2G, see histological score 2I). Four days after colitis induced by DNBS treatment, all rats had diarrhoea and a significant reduction in body weight (compared with the sham groups of rats) (Figure 2L). 3,5-dicaffeoyl-4-malonylquinic acid treatment resulted in a significant reduction of loss of body weight induced by DNBS-administration in rats (Figure 2L).

Effects of CA extract treatment on NF-κB activation in colon tissues

By Western Blot analysis, we evaluated IκB-α, phospho-p65 and NF-κB p65 expression after DNBS administration. A basal level of IκB-α was detected in the colon tissues from sham-treated rats, whereas in

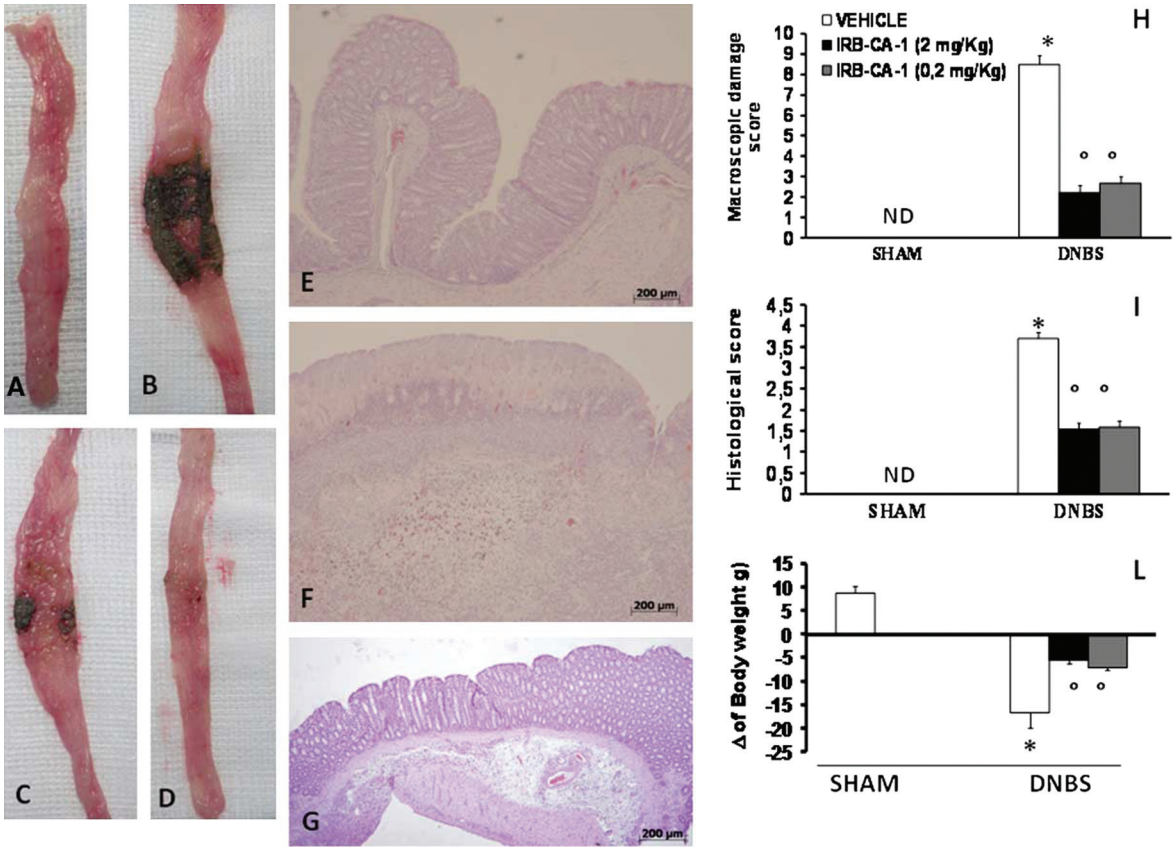

Figure 2. Effect of CA extract treatment on colon injury and body weight changes after DNBS intracolonic administration. Colon tissues from sham-treated rats (A), colon tissues from DNBS-treated rats at 4 days post-DNBS administration (B) and the colon tissues collected from DNBS-treated rats which have received 3,5-dicaffeoyl-4-malonylquinic acid at 2 mg/kg (C) or at 0.2 mg/kg (D) treatment. No histological alteration was observed in the colon section from sham-treated rats (E). Mucosal injury was produced after DNBS administration characterized by absence of epithelium and a massive mucosal and submucosal infiltration of inflammatory cells (F). Treatment with 3,5-dicaffeoyl-4-malonylquinic acid at 2 mg/kg (G) corrected in a dose-dependent manner the disturbances in morphology associated with DNBS administration. The macroscopic damage score and the histological score (H, I) was made by two independent observers. Figure is representative of all the animals in each group. Treatment with CA at 2 mg/kg or at 0.2 mg/kg significantly reduced in a dose-dependent manner the increase in body weight loss (L). Data are means ± SEM of 10 rats for each group. $^{*}p < 0.01$ vs SHAM; $^{\circ}p < 0.01$ vs DNBS.

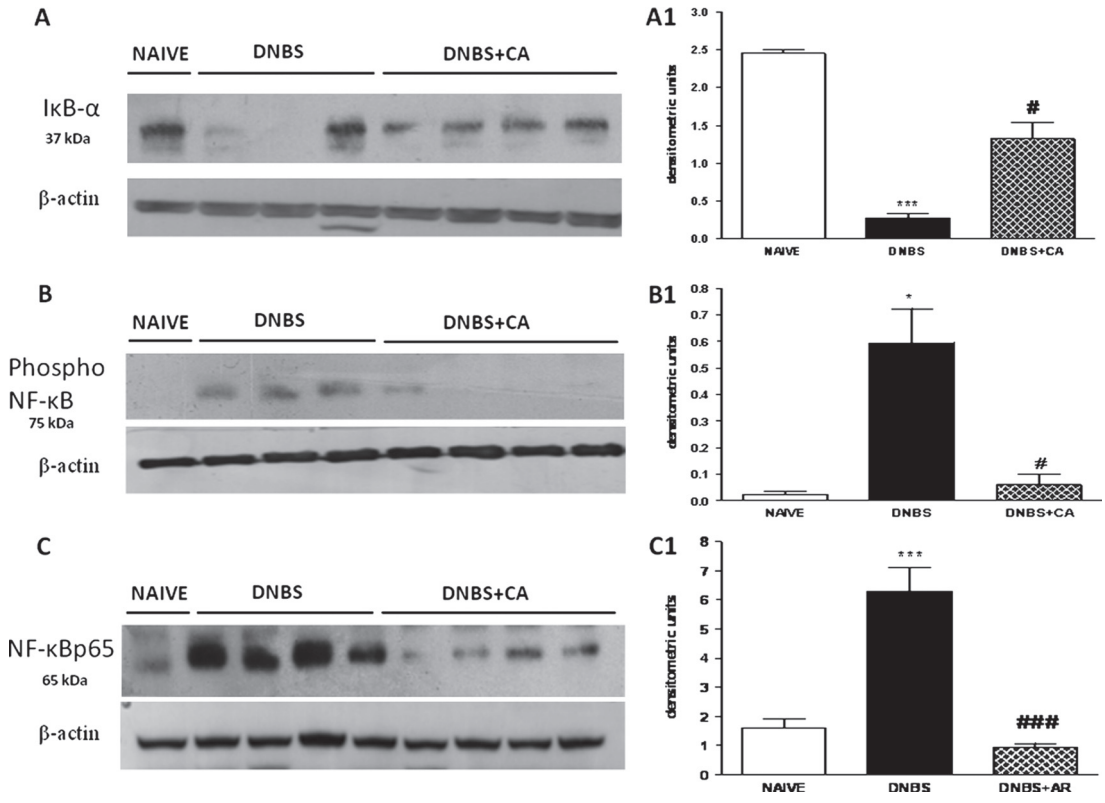

Figure 3. Effects of CA extract treatment on IκB-α degradation, phosphorylation of Ser536 on the NF-κB subunit p65, total NF-κB p65. By Western Blot analysis, a basal level of IκB-α was detected in the colon tissues from sham-treated animals, whereas IκB-α levels were substantially reduced in DNBS-treated rats. CA (2 mg/kg) treatment prevented the DNBS-induced IκB-α degradation (A). In addition, DNBS administration caused a significant increase in the phosphorylation of Ser536 at 4 days (B) and in nuclear NF-kB p65 (C) compared to the sham-treated rats. CA (2 mg/kg) treatment significantly reduced the phosphorylation of p65 on Ser536 (B) and NF-κB p65 levels (C). A representative blot of lysates obtained from three/four animals per group is shown and densitometric analysis of all animals is reported (n = 5 rats from each group). The relative expression of the protein bands from three separated experiments was standardized for densitometric analysis to β-actin or lamin levels and reported in A1, B1, C1. $^{*}p < 0.01$ vs SHAM; $^{\circ}p < 0.01$ vs DNBS.

DNBS-treated rats IκB-α levels were substantially reduced (Figures 3A and A1). Significant reduced levels of IκB-α degradation were observed in the colon tissues collected from 3,5-dicaffeoyl-4-malonylquinic acid (2 mg/kg) treated rats (Figures 3A and A1).

Furthermore, DNBS administration caused a significant increase in the phosphorylation of p65 on Ser536 in the colon tissues from DNBS-treated rats and a significant reduction of p65 phosphorylation in the colon tissues from DNBS-treated rats which have received 3,5-dicaffeoyl-4-malonylquinic acid (Figures 3B and B1). Moreover, NF-κB p65 sub-unit presence in the nuclear fractions from colon tissue was also significantly increased after DNBS instillation compared to the sham-treated rats (Figures 3C and C1). A significant reduction of the NF-κB p65 nuclear levels was observed in the tissues from CA-treated rats (Figures 3C and C1).

Effects of CA extract treatment on matrix metalloproteinases expression in colon tissues

To assess whether colon injury is associated with the alterations in activity and expression of secreted MMP-9 and -2, rats were sacrificed at 4 days after DNBS administration and colon were subjected to zymography and Western blot analysis, respectively, as described in Materials and methods. A representative zymography and its densitometric analysis (Figure 4A) shows both secreted MMP-9 and -2 activities. Pro-MMP-2 activity is observed in colonic extracts prepared from sham-treated rats (Figure 4A, see densitometry analysis 4B). Administration of DNBS (Figure 4A) significantly increased MMP-2 (both pro and active see densitometry analysis 4B and C, respectively) activity as compared with sham animals, whereas pro and active MMP-9 activity was moderately elevated (Figure 4A, see densitometry analysis 4D). 3,5-dicaffeoyl-4-malonylquinic acid (2 mg/kg) treatment attenuated the degree of activation of pro-MMP-2 and MMP-9 activity (Figure 4A, see densitometry analysis 4D).

The low availability of active MMP-9 seen in the zymographies may be due to its high level of instability and the removal of active enzyme during the washing of specimens, as it has been previously suggested.

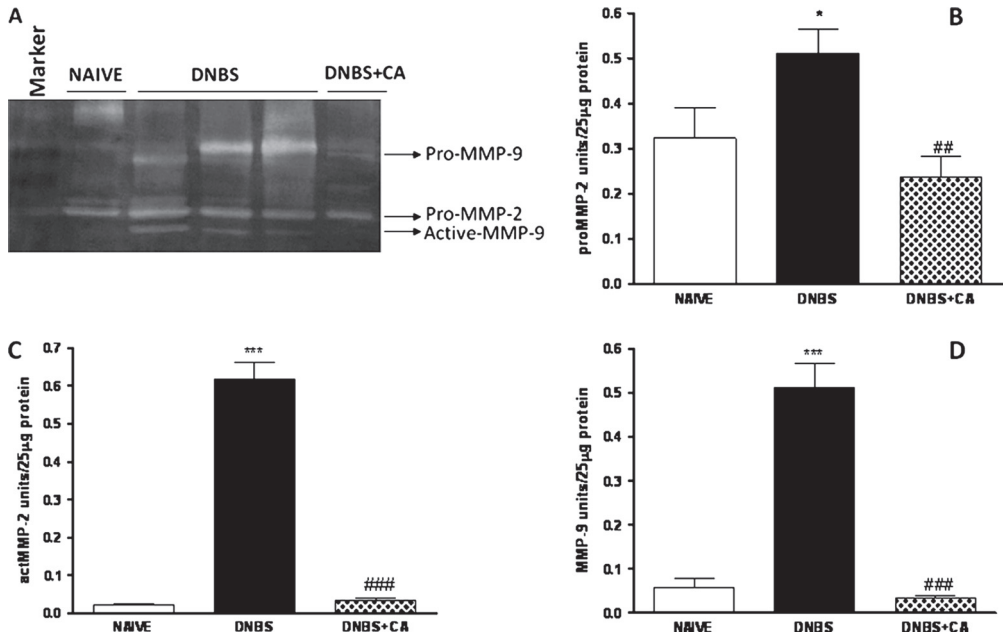

Figure 4. Effects of CA extract treatment on MMP-2 and MMP-9 activity by zymogram. Pro-MMP-2 and pro-MMP-9 activity was observed in colon tissues extracts prepared from sham-treated rats (A, B, D). In contrast to sham-treated rats, colon tissues extracts from DNBS-treated rats showed an induction of pro-MMP-2 (A and B) and pro-MMP-9 (A and D). Active form of MMP-2 and MMP-9 was also evidenced (A, C and E). CA (2 mg/kg) treatment reduced the degree of activation of pro-MMP-2 and pro-MMP-9 activity as well as active form of MMP-2 and MMP-9 (A, C and D). This figure is representative of at least three experiments performed on different experimental days. $^{*}p < 0.01$ vs SHAM; $^{\circ}p < 0.01$ vs DNBS.

Effects of CA extract treatment on production and expression of TNF-α and IL-1β after DNBS administration

To test whether 3,5-dicaffeoyl-4-malonylquinic acid treatment modulates the secretion of cytokines, we analysed the colon levels of TNF-α and IL-1β. A positive staining for TNF-α (Figure 5β, see densitometry analysis 5G) and for IL-1β (Figure 5E, see densitometry analysis 5H) was mainly localized in the infiltrated inflammatory cells in damaged tissues. The treatment with 3,5-dicaffeoyl-4-malonylquinic acid 2 mg/kg reduced the staining for TNF-α (Figure 5C, see densitometry analysis 5G) and for IL-1β (Figure 5F, see densitometry analysis 5H) in the colon tissues collected from DNBS-treated rats. No positive staining for TNF-α and IL-1β was observed in the colon tissue from sham-treated rats (Figures 5A–D, see densitometry analysis 5G–H).

Effects of CA extract treatment on ICAM-1 and P-Selectin expression and neutrophils infiltration

Animals DNBS-treated showed an increase in myeloperoxidase activity, an indicator of the neutrophils accumulation in the colon (Figure 6I). On the contrary, 3,5-dicaffeoyl-4-malonylquinic acid treatment significantly reduced the degree of MPO activity and, consequently, of PMN infiltration in inflamed colon (Figure 6I). Moreover, we evaluated the intestinal expression of ICAM-1 and P-Selectin by immunohistochemical staining. Positive staining for ICAM-1 (Figure 6B, see densitometry analysis 6G) and for P-Selectin (Figure 6E, see densitometry analysis 4H) was substantially increased in the vessels of the lamina propria and sub-mucosa as well as in epithelial cells of injured colon and in infiltrated inflammatory cells in damaged tissues from DNBS-treated rats. The treatment with 3,5-dicaffeoyl-4-malonylquinic acid at 2 mg/kg reduced the staining for ICAM-I (Figure 6C, see densitometry analysis 6G) and for P-Selectin (Figure 6F, see densitometry analysis 6H) in the colon tissues collected from DNBS-treated rats. No positive staining for ICAM-I and for P-Selectin was observed in the colon tissue from sham-treated rats (Figures 6A–D, see densitometry analysis 6G and H).

Effects of CA extract treatment on iNOS expression

Positive staining for iNOS (Figure 7B, see densitometry analysis 7D) was substantially increased in the epithelial cells of injured colon and in infiltrated inflammatory cells in damaged tissues from DNBS-treated rats. The treatment with 3,5-dicaffeoyl-4-malonylquinic acid at 2 mg/kg reduced the staining for iNOS (Figure 7C, see densitometry analysis 7D) in the colon tissues collected from DNBS-treated rats. No positive staining for iNOS (Figure 7A) was

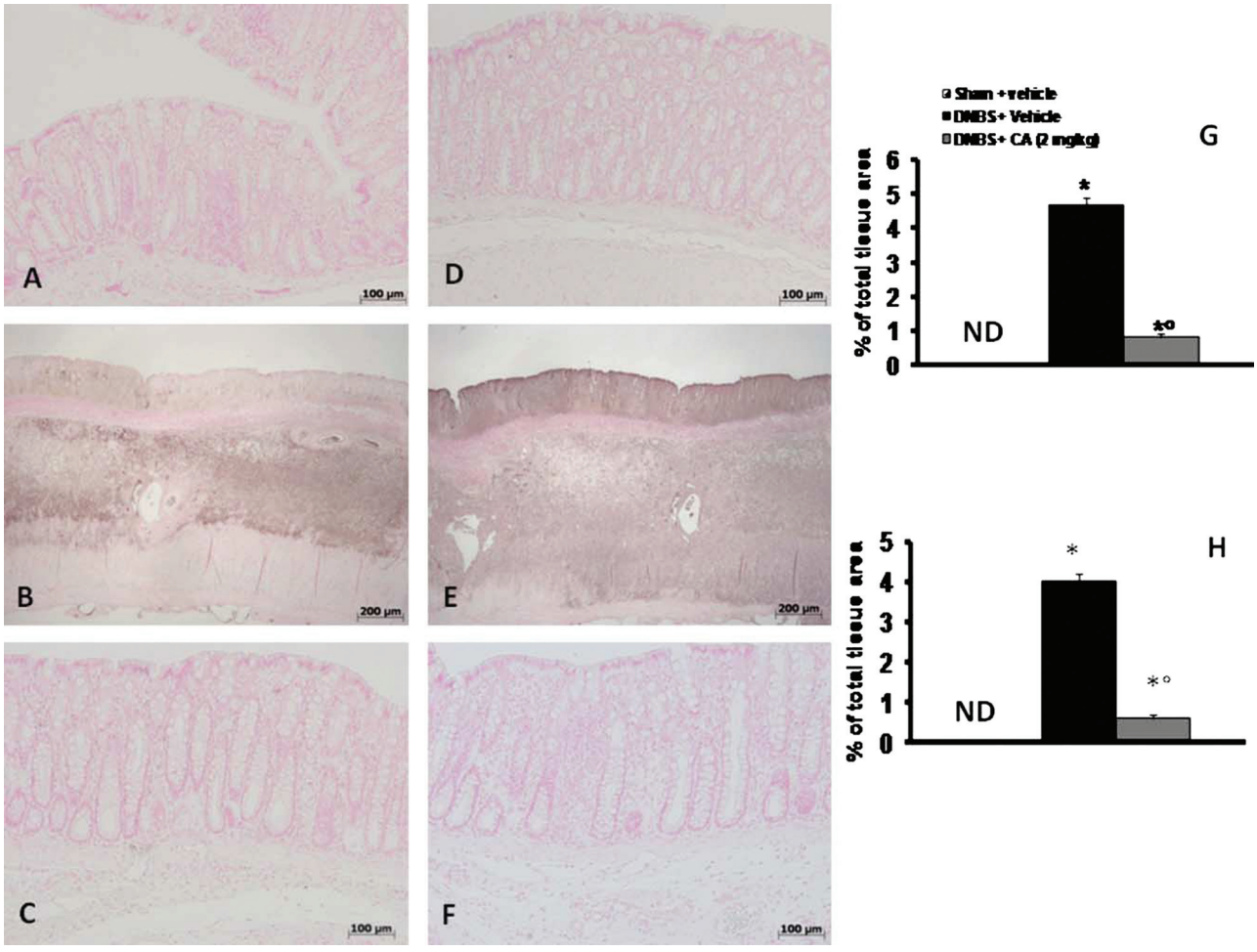

Figure 5. Effect of CA extract treatment on colon levels and immunohistochemical localization of TNF-α and IL-1β. Immunohistochemical analysis for TNF-α and IL-1β (A, D) show positive staining localized in the inflammatory cells in the injured area from DNBS-treated animals. The intensity of the positive staining for TNF-α and IL-1β (B, E) was markedly reduced in tissue section obtained from DNBS-treated animals which have been treated with CA (2 mg/kg). Densitometry analysis of immunohistochemistry photographs (n = 5 photos from each sample collected from all rats in each experimental group) for TNF-α and IL-1β (C, F) from colon tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. Moreover treatment with CA at 2 mg/kg or at 0.2 mg/kg significantly reduced in a dose-dependent manner the increase of cytokine production (G, H) in the colon tissue after DNBS administration. This figure is representative of at least three experiments performed on different experimental days. $^{*}p < 0.01$ vs SHAM; $^{\circ}p < 0.01$ vs DNBS.

observed in the colon tissues collected from sham-treated rats.

Effects of CA extract treatment on nitrotyrosine formation and PAR formation

Four days after DNBS administration, nitrotyrosine was measured by immunohistochemical analysis. Colon sections obtained from DNBS-treated rats exhibited positive staining for nitrotyrosine (Figure 8B, see densitometry analysis 8G). The positive staining was mainly localized in inflammatory cells as well as around the vessels. 3,5-dicaffeoyl-4-malonylquinic acid treatment (2 mg/kg) reduced the degree of positive staining for nitrotyrosine (Figure 8C, see densitometry analysis 8G) in the colon. No positive staining for nitrotyrosine (Figure 8A) was observed in the colon tissues collected from sham-treated rats. In our study, immunohistochemistry for PAR, as an indicator of *in vivo* PARP activation, revealed the occurrence of positive staining for PAR localized in nuclei of inflammatory cells in the colon tissues from DNBS-treated rats (Figure 8E, see densitometry analysis 8H). 3,5-dicaffeoyl-4-malonylquinic acid treatment (2 mg/kg) reduced the degree of positive staining for PAR (Figure 8F, see densitometry analysis 8H) in the colon.

Discussion

Intestinal inflammation is accompanied by excessive production of reactive oxygen and nitrogen radical species, such as superoxide (O_2^{-}), nitric oxide (NO·), peroxynitrite (ONOO·$^{-}$) and hydroxyl radicals (·OH)

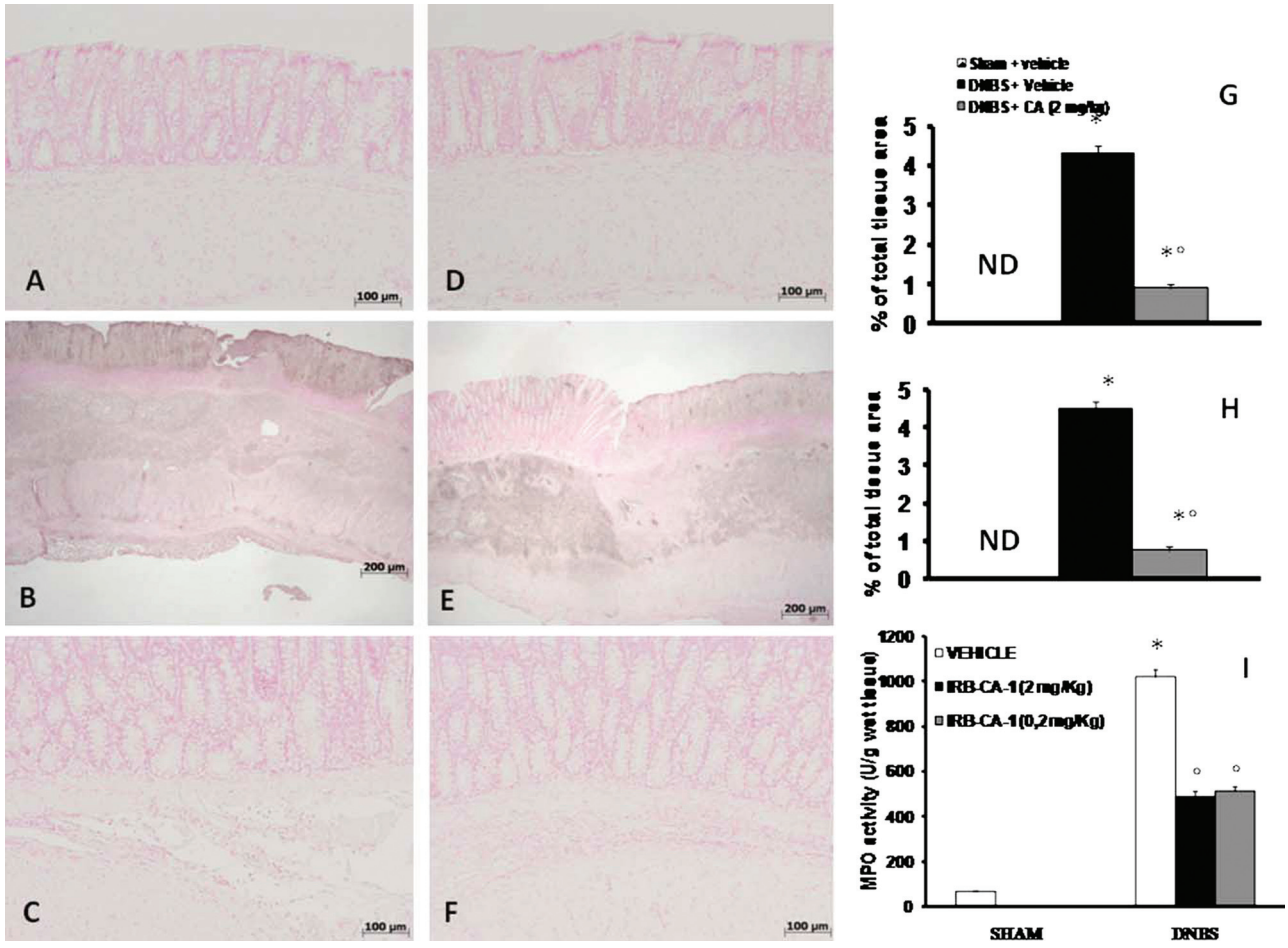

Figure 6. Effects of CA extract treatment on immunohistochemical localization of ICAM-I, P-Selectin and on polymorphonuclear leukocyte infiltration in the colon. Immunohistochemical analysis for ICAM-1 and P-Selectin (A, D) show positive staining mainly localized on endothelial cells and in the inflammatory cells in the injured area from DNBS-treated rats. The intensity of the positive staining for ICAM-1 and P-Selectin (B, E) was markedly reduced in tissue section obtained from DNBS-treated rats which have been treated with CA (2 mg/kg). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all rats in each experimental group) for ICAM-1 and P-Selectin (C, F) from colon tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. Myeloperoxidase (MPO) activity (G) was significantly increased in DNBS-treated rats in comparison to sham-treated rats. Treatment with CA at 2 mg/kg or at 0.2 mg/kg significantly reduced in a dose-dependent manner the colon MPO activity. This figure is representative of at least three experiments performed on different experimental days. $^{*}p < 0.01$ vs SHAM; $^{\circ}p < 0.01$ vs DNBS.

[37–40] because of the massive infiltration of polymorphonuclear and mononuclear leukocytes which may produce large amounts of free radicals [41–44]. Large numbers of peripheral neutrophils [45] producing oxygen-derived free radicals [46] migrate into the intestinal wall of inflammatory bowel disease patients. In addition, nitric oxide (NO·) overproduction, due to the expression of the inducible isoform of NO·synthase (iNOS), plays an important role in several animal models of inflammation [47]. Inflammatory bowel disease patients show high levels of nitrite (metabolite of NO· in water) and increased iNOS activity [39,48]: inhibition of iNOS activity exerts beneficial effects in animal models of experimental colitis [49,50]. NO-induced damage is believed to be mediated, at least in part, by peroxynitrite ($ONOO^{\cdot -}$), a highly reactive oxidant produced by the combination of O_2^- and NO· at rates approaching the diffusion limit [49,51]. $ONOO^{\cdot -}$ can induce cytotoxicity by initiating lipid peroxidation, inactivating various enzymes (mitochondrial respiratory enzymes and membrane pumps) [52] and by depleting glutathione [53]. Peroxynitrite can also cause DNA damage [54] resulting in the activation of the nuclear enzyme poly (ADP-ribose) synthetase and poly (ADP-ribose) synthase-driven cell death [55]. Furthermore, $ONOO^{\cdot -}$ inhibits the activity of the endogenous superoxide dismutase enzymes, contributing to an increased $\cdot O_2^-$ production [56].

Rich amounts of chlorogenic acid (CHA) are present in coffee beverages, blueberries, apples, cider and some vegetables [57]. Those people habitually drinking coffee may consume 300–700 mg of CHA

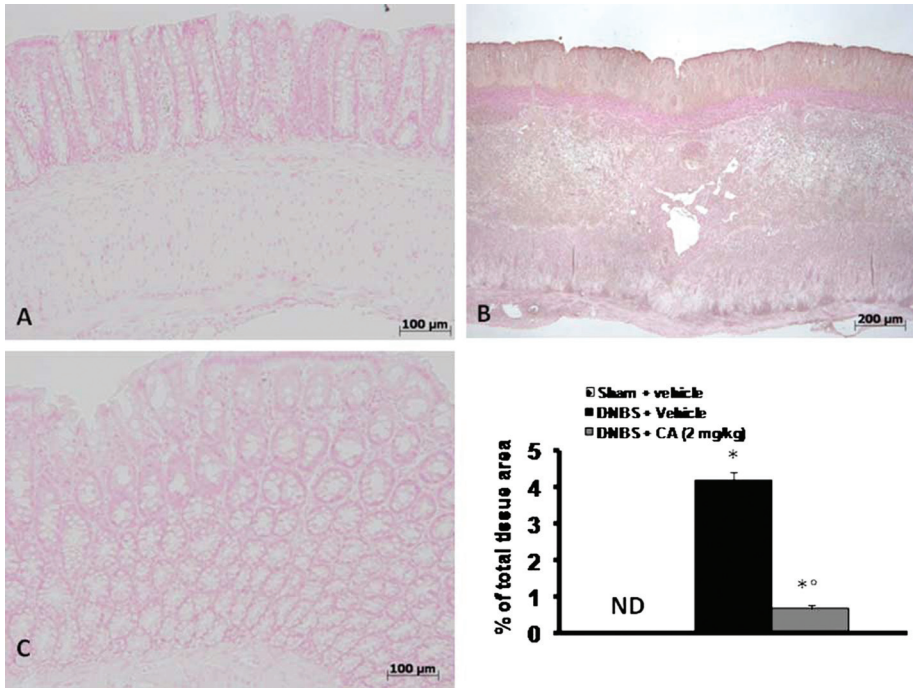

Figure 7. Effects of CA extract treatment on immunohistochemical localization of iNOS in the colon. No positive staining for iNOS was observed in the colon tissues from sham-treated rats (A). Immunohistochemical analysis for iNOS (B) show positive staining mainly localized in the inflammatory cells in the injured area from DNBS-treated rats. The intensity of the positive staining for iNOS (C) was markedly reduced in tissue section obtained from DNBS-treated rats which have been treated with CA (2 mg/kg). Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all rats in each experimental group) for iNOS (D) from colon tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days. $^{*}p < 0.01$ vs SHAM; $^{\circ}p < 0.01$ vs DNBS.

daily [57]. Its polyphenol structure endows CHA with potent anti-oxidative and free radical scavenging abilities [58]. These abilities enable CHA to decrease DNA damage [59] and to inhibit the oxidation and peroxidation of low-density lipoproteins [60–62] *in vitro*, suggesting its benefits in preventing cardiovascular disease.

Centella asiatica (CA), of which the principal component extracted is the 3,5-dicaffeoyl-4-malonylquinic acid, have been used for treatment of various illnesses. The main use of *Centella asiatica* is in wound healing and promoting healthy tissue. It is used to prevent the formation of scar tissue as well as to treat leprous ulcers. It is also used to treat stomach and duodenal ulcers, but the mechanisms of action remain largely unknown. So, in this study, we wanted to test the antioxidant properties of 3,5-dicaffeoyl-4-malonylquinic acid, in the physiopathogenesis of inflammatory bowel disease.

One of the key regulators in this disease was identified as the nuclear transcription factor kappaB (NF-kappaB). Its activation is markedly induced in IBD patients and through its ability to promote the expression of various pro-inflammatory genes, NF-kappaB strongly influences the course of mucosal inflammation [63].

We report here that DNBS caused a significant increase in the phosphorylation of NF-κB p65 on Ser536 in the colon tissues at 4 days, whereas treatment with CA significantly reduced this phosphorylation. Moreover, we also demonstrate that the CA inhibited IκB-α degradation. The exact mechanisms by which CA extract suppress NF-κB activation in inflammation are not completely known. However, there are *in vitro* studies which have clearly demonstrated that asiatic acid, isolated from the leaves of CA, down-regulated in a dose-dependent manner, NF-kappaB activation via suppression of IKK and MAP kinase (p38, ERK1/2 and JNK) phosphorylation in RAW 264.7 cells [64].

Matrix metalloproteinases (MMP) play an important role in pathogenesis of inflammatory bowel disease (IBD). Two known gelatinases, MMP-2 and MMP-9, are upregulated during IBD. Epithelial-derived MMP-9 is an important mediator of tissue injury in colitis, whereas MMP-2 protects against tissue damage and maintains gut barrier function [65].

It has been demonstrated that Matrix metalloproteinases expression is partly controlled by NF-κB [66], in fact an NF-KB binding site is present in the promoter of the MMP-9 gene and an NF-κB-like element in the promoter of the MMP-1 gene [67].

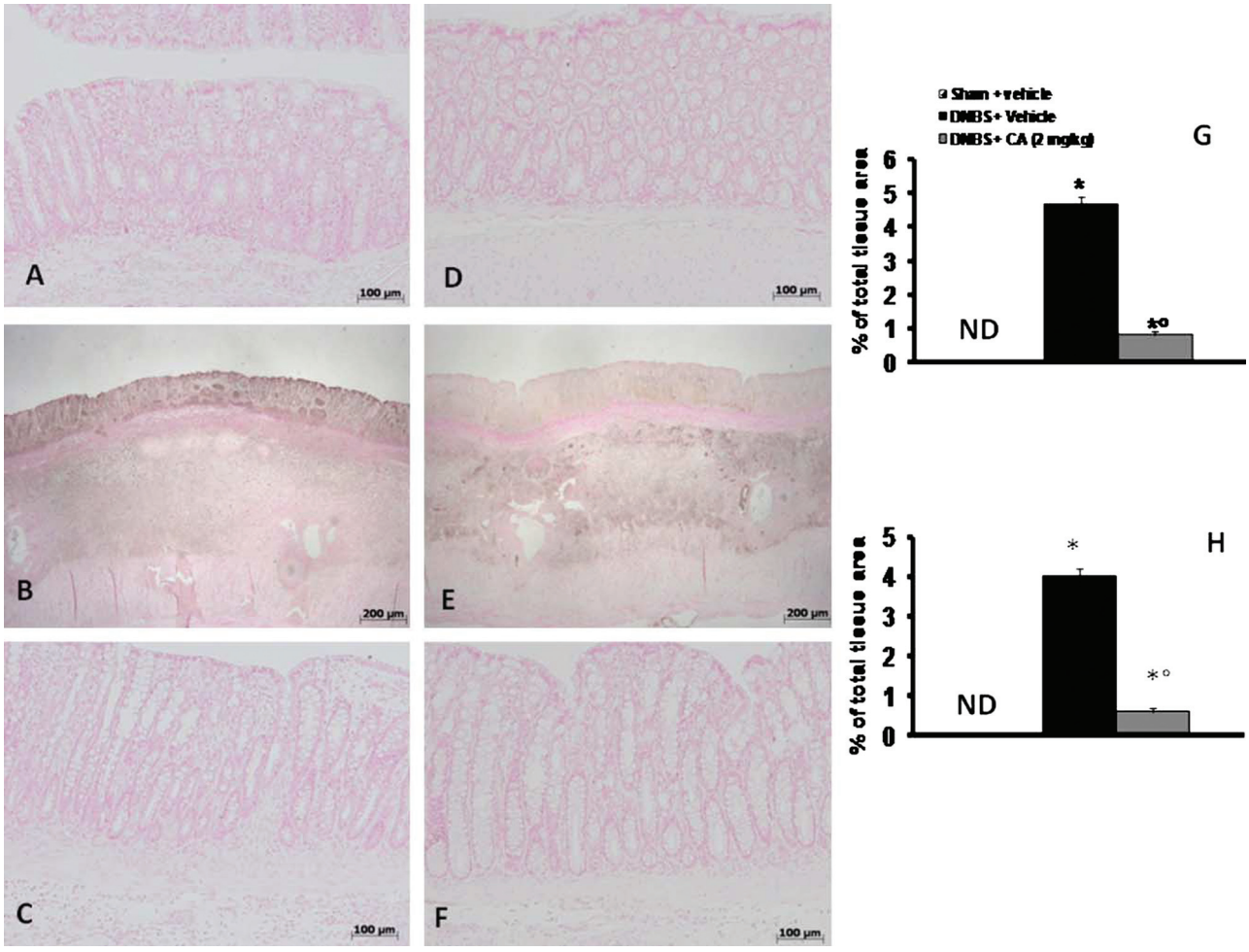

Figure 8. Effects of CA extract treatment on nitrotyrosine and PAR formation and lipid peroxidation. Sections obtained from DNBS-treated rats demonstrate positive staining for nitrotyrosine and PAR (A, D) mainly localized in inflammatory cells. CA (2 mg/kg) treatment reduced the degree of positive staining for nitrotyrosine and PAR (B, E) in the colon tissues. Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all rats in each experimental group) for nitrotyrosine and PAR (C, F) from colon tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. In addition, a significant increase in thiobarbituric acid-reactant substances (G) were observed in the colon tissues collected at 4 days after DNBS administration when compared with sham-treated rats. Thiobarbituric acid-reactant substances were significantly attenuated in a dose-dependent manner by CA treatment (G). Figures are representative of at least three experiments performed on different experimental days. Data are means ± SE means of 10 rats for each group. $^{*}p < 0.01$ vs SHAM; $^{\circ}p < 0.01$ vs DNBS.

Furthermore, inhibition of NF-κB (nuclear factor κB) activation blocked MMP-2 production and activity, indicating the involvement of NF-κB, a ubiquitous transcription factor playing a central role in the differentiation, proliferation and malignant transformation [68].

We hypothesized that the protective effect of 3,5-dicaffeoyl-4-malonylquinic acid in this model could be also related to MMP-2, especially to MMP-9 reduction by suppression of NF-κB activation.

MMPs are intimately involved in the regulation of the activities of cytokines and cytokine receptors [69].

Tumour necrosis factor (TNF-α) and interleukin-1 (IL-1β) are the most important cytokines present in colon tissues and involved in the pathogenesis of colitis [70]. We confirm here the expression of TNF-α and IL-1β in the colon tissue of DNBS-treated rats. Treatment with 3,5-dicaffeoyl-4-malonylquinic acid reduced TNF-α and IL-1β levels, as demonstrated in a previous study *in vitro* [64].

As already mentioned elsewhere, there is consistent evidence in the literature that during acute and chronic colitis the sustained production of pro-inflammatory cytokines leads to the up-regulation of adhesion molecules. These adhesion molecules play a key role in the adherence and infiltration of mononuclear and poly-morphonuclear leukocytes to endothelial cells, maintaining the chronic inflammation into the cecal and colonic interstitium [71].

In the present study, we have confirmed that DNBS administration induced the appearance of P-Selectin on the endothelial vascular wall and

up-regulated the surface expression of ICAM-1 on endothelial cells. Treatment with 3,5-dicaffeoyl-4-malonylquinic acid abolished the expression of P-Selectin and the up-regulation of ICAM-1 without effecting constitutive levels of ICAM-1 on endothelial cells. These results demonstrate that CA extract may interrupt the interaction of neutrophils and endothelial cells both at the early rolling phase mediated by P-Selectin and at the late firm adhesion phase mediated by ICAM-1. The absence of an increased expression of the adhesion molecule in the colon tissue of DNBS-treated administered 3,5-dicaffeoyl-4-malonylquinic acid correlated with the reduction of leukocyte infiltration as assessed by the specific granulocyte enzyme MPO and with the attenuation of the colon tissue damage as evaluated by histological examination.

Altered peripheral neutrophil function is a feature of IBD that may contribute to the chronicity and extra-gastrointestinal manifestations of this disease [72].

So, the activated neutrophils produce reactive oxygen and nitrogen species within intestinal mucosa, which induce oxidative stress. Reactive oxygen species (ROS) are highly reactive. When they are generated close to cell membranes, possibly by intestinal epithelial cells, they induce oxidative stress and oxidized membrane phospholipids (lipid peroxidation), which may continue in the form of a chain reaction [73].

It has been said that nitric oxide (NO) and reactive oxygen species (ROS) are important mediators in the pathogenesis of inflammatory bowel disease (IBD). NO in IBD can either be harmful or protective. NO can react with superoxide anions ($O2^{\bullet -}$) yielding the toxic oxidizing agent peroxynitrite (ONOO-). Peroxynitrite induces nitration of tyrosine residues (nitrotyrosine) leading to changes of protein structure and function. Enhanced formation of NO by iNOS may contribute to the inflammatory process associated with inflammatory bowel disease (IBD). The effects of Centella asiatica water extract (CaE) and its active constituent, asiaticoside (AC), on the expression and activity of inducible nitric oxide synthase (iNOS) during gastric ulcer healing in rats were investigated [74].

This study demonstrates that 3,5-dicaffeoyl-4-malonylquinic acid attenuates the expression of iNOS in the colon tissues in DNBS-treated rats.

This reduction in the expression of iNOS by 3,5-dicaffeoyl-4-malonylquinic acid may contribute to the attenuation of nitrotyrosine formation and lipid peroxidation in the colon tissues in DNBS-treated animals. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation 'footprint' of peroxynitrite [52]. There is, however, recent evidence that certain other reactions can also induce tyrosine nitration, e.g. reaction of nitrite with hypoclorous acid and the reaction of MPO with hydrogen peroxide can lead to the formation of nitrotyrosine [75].

Increased nitrotyrosine staining is therefore considered as an indicator of 'increased nitrosative stress' rather than a specific marker of the generation of peroxynitrite. Moreover, ROS cause DNA single-strand damage, leading to poly (ADP ribose) synthetase activation and cell death [55]. Some evidence exists to support the possible role of poly (ADP ribose) synthetase activation in the inflammatory process [76]. As shown in this study 3,5-dicaffeoyl-4-malonylquinic acid treatment reduced poly (ADP ribose) synthetase immunoreactions, an effect that might account for the overall protective action of CA.

Taken together, the data presented in the present study demonstrates that 3,5-dicaffeoyl-4-malonylquinic acid treatment reduced the development of colitis injury via a number of distinct mechanisms. We speculate that CA extract may be useful in the treatment of conditions associated with inflammation. These observations could suggest new therapeutic approaches of therapy with CA extract in inflammatory colon diseases.

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