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Research Article

Polyphenol metabolites from colonic microbiota exert anti-inflammatory activity on different inflammation models

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The polyphenols in fruits and vegetables may be partly responsible for the health-promoting effects attributed to fruit and vegetable intake. Although their properties have been relatively well studied, the activity of their metabolites, produced after ingestion, has been poorly investigated. Thus, the aim of this work was to study the potential anti-inflammatory effect of 18 polyphenol metabolites, derived from colon microbiota. They were screened by measuring prostaglandin E_2 (PGE_2) production by CCD-18 colon fibroblast cells stimulated with IL-1β. Metabolites that inhibited more than 50% PGE_2 production were hydrocaffeic (HCAF), dihydroxyphenyl acetic (dOHPA), and hydroferulic acid (HFER), that subsequently were tested with the writhing and paw pressure test in rodents where all three compounds showed an anti-inflammatory effect. The effect of HCAF administered orally (50 mg/kg) was also tested in the dextran sodium sulfate (DSS)-induced colitis model. Weight loss and fecal water content were more pronounced in DSS rats than in DSS-HCAF treated rats. HCAF treatment diminished the expression of the cytokines IL-1β, IL-8, and TNF-α, reduced malonyldialdehyde (MDA) levels and oxidative DNA damage (measured as 8-oxo-2′-deoxyguanosine levels) in distal colon mucosa. These results indicate that HCAF, dOHPA, and HFER have anti-inflammatory activity *in vitro* and *in vivo*.

Keywords: Hydrocaffeic acid / Inflammation / MDA / 8-oxodG / Polyphenol metabolite

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1 Introduction

Polyphenols are secondary plant metabolites ubiquitously distributed in fruits and vegetables and are always present in a balanced, healthy diet. Fruit and vegetables have a health-promoting effect, but the mechanism of their protective action is not clearly understood. It has been suggested that polyphenols may be partially responsible for the beneficial effects and polyphenol activity has been studied in many cell culture and animal models. While polyphenols are metabolized by gut bacteria and mammalian tissues, the effect of polyphenol metabolites has been studied much less. It is possible that some of their activities might actually be mediated by their metabolites in addition or in place of the original compound present in food. Russell *et al*. [1] have suggested that the microbiota metabolism of polyphenolic compounds determines the action of these compounds by inhibiting prostanoid production. Hydrocaffeic acid (HCAF), hydroferulic acid (HFER), and 3,4-dihydroxyphenyl acetic acid (dOHPA) are all polyphenol metabolites derived from the colon microbiota, which are found at high concentration in plasma, excreted urine, and fecal water after consumption of coffee or an omnivorous diet including fruits and vegetables [2, 3]. HCAF has potent antioxidant activity in endothelial cells [4], an anti-proliferative effect on cancer colon cells [5] and protects keratinocytes from UV irradiation, reducing the expression of IL-6 and IL-8 [6]. The polyphenol metabolite dOHPA has been studied in connection with dopamine metabolism [7], but not as

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Abbreviations: DSS, dextran sodium sulfate; **IBD,** inflammatory bowel disease; **8-oxodG,** 8-oxo-2′-deoxyguanosine; **ROS,** reactive oxygen species

a colonic microbiota metabolite. Gao *et al*. [8] have demonstrated its anti-proliferative activity on colon cancer cell line HCT-116. The activity of HFER is still not documented.

Chronic inflammatory conditions are present in many human pathological processes, and it is possible that consumption of fruits and vegetables (and the associated polyphenols) might ameliorate inflammation [9 – 11]. Inflammatory bowel diseases (IBD) affect millions of individuals in the western world alone [12] and are influenced by environmental factors and diet. Although the etiology and pathogenesis of IBD is not yet clear, an imbalance of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) seem to play a important role in modulating inflammation [13] and therefore an anti-TNF-α antibody has been used in therapy [14].

The aim of the present study was to investigate the effects of polyphenol metabolites on inflammation in experimental cell and animal models. To study their activity on intestinal inflammation we used the rat colitis model after the administration of dextran sodium sulfate (DSS) p.o. which can induce a syndrome similar to IBD with diarrhea, rectal bleeding, weight loss, multiple erosions, abscesses [15], and variation in Th1 and Th2 cytokine profile [16].

2 Materials and methods

2.1 Chemicals

3-hydroxybenzoic acid (3OHB), 3,4-dihydroxybenzoic acid (3,4dOHB), 4-hydroxy-3-methoxybenzoic acid (OHMB), 4-hydroxy-3-methoxyphenylacetic acid (OHMPA), 3,4-dihydroxyphenylacetic acid (dOHPA), hydrocaffeic acid (HCAF), hydroferulic acid (HFER), and ferulic (FE) were from Fluka (Milan, Italy), 4-hydroxybenzoic acid (4OHB), phenylacetic acid (PA), 3-hydroxyphenylacetic acid (3OHPA), 4-hydroxyphenylacetic acid (4OHPA), hippuric acid (HIPU), 3-hydroxyhippuric (OHHIPU), 3-phenylpropionic acid (3PP), 3-hydroxyphenylpropionic acid (3OHPP), 4-hydroxyphenylpropionic acid (4OHPP), and hydrocinnamic acid-d_9 were purchased from Aldrich (Sigma, Milan, Italy). The compound 5-(3-hydroxyphenyl) valeric acid (OHPVAL) was synthesized by Professor Menichetti, Department of Organic Chemistry, University of Florence, Italy. Formic acid, ACN, and methanol were of LC-MS grade supplied by Merck (Darmstadt, Germany). DSS (Mw = 36 000 – 50 000) was from ICN Biomedicals (Segrate, Milan, Italy). Nuclease P1, alkaline phosphatase, RNAse, proteinase K, 2′-deoxyguanosine (dG), 8-oxo-2′-deoxyguanosine (8-oxodG), 2,4-dinitrophenylhydrazine (DNPH), and 1,1,3,3-tetraethoxypropane (TEP), indomethacin, and acetylsalicylic acid were purchased from Sigma (Sigma Chemical Co., Milan, Italy). EIA prostaglandin-kit was obtained from Cayman Chem (San Diego, CA, USA).

2.2 Anti-inflammatory *in vitro* screening assay

The CCD18-Co human colon fibroblast cell line (ATCC number CRL-1459) was grown in Eagle's MEM containing 2 mM glutamine, 0.1 mM non essential amino acids, 1 mM sodium pyruvate, 100 Units/mL penicillin, and 100 g/mL streptomycin and supplemented with 10% fetal bovine serum (FBS). The cell line was maintained at 37°C in a 95% oxygen 5% CO_2 atmosphere. Screening for anti-inflammatory activity was performed according to Salvemini *et al*. [17] with modifications. Cells were seeded at 5000 cell/well in 96-well plates and after 24 h medium was replaced with fresh medium containing 0.1% FBS for another 24 h. Cells were then treated with different polyphenol metabolites (100 μM) and co-stimulated with 1 ng/mL of IL-1β (Calbiochem, Milan, Italy) for 18 h. The medium was finally removed for PGE_2 assay and cells were fixed with methanol/acetic acid 3:1 v/v for crystal violet assay [18], in order to detect any cytotoxic effect of compounds. PGE_2 levels in cell media were measured using an immunoenzymatic method (EIA kit, Cayman Chem, San Diego, CA, USA) according to the manufacturer's specifications. A dilution of 1:50 was used.

2.3 Animals

Male Fischer 344 rats (175 – 200 g), male Wistar rats (200 – 250 g) were purchased from Nossan (Correzzana, Milan, Italy) and male Swiss albino mice (25 – 30 g) were from Morini (San Polo D'Enza, Reggio Emilia Italy). All the procedures adopted were carried out in agreement with the European Union Regulations on the Care and Use of Laboratory Animals (OJ of ECL 358/1, 12/18/1986) and the experiments were conducted according to Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/1992).

2.4 Writhing test

The writhing test was performed according to Koster *et al*. [19]. Male Swiss albino mice (25 – 30 g) were injected i.p. with a 0.6% aqueous solution of acetic acid (10 mL/kg). The number of stretching movements was counted for 10 min, starting 6 min after acetic acid injection. Treatments (HCAF, dOHPA, and HFER) were administered i.p. at the dose of 30 mg/kg on the opposite abdominal side, 14 min before administration of the acetic acid solution. Indomethacin at 10 mg/kg and acetylsalicylic acid at 800 mg/kg were administered as anti-inflammatory positive controls. Animals were euthanized after the experiments.

2.5 Paw pressure test

The nociceptive threshold was determined with an analgesimeter (Ugo Basile, Varese, Italy) according to the method described by Leighton *et al*. [20]. Threshold pres-

sure was measured before and 30, 45, and 60 min after treatment. The pre-tested rats (male Wistar) which scored below 30 g or over 90 g were rejected. An arbitrary cut-off value of 160 g was adopted. To induce an inflammatory process in the rat, carrageenan (0.1 mL, 1%) was administered s.c. in one paw 4 h before each test. HCAF, dOHPA, and HFER were administered i.p. at 30 mg/kg 30, 45, and 60 min before performing the test. Animals were euthanized after the experiments.

2.6 Experimental diet and colitis induction

Fischer 344 4-wk old male rats were acclimatized for one week and then randomly divided into three experimental groups (6 rats *per* group). The control group was fed with a AIN 76 diet, prepared using components purchased from Piccioni (Gessate, Milan, Italy) and drinking water ad libitum for 18 days. Rats in the DSS group were fed as the control and received 4% DSS dissolved in drinking water *ad libitum* for 4 days; rats were sacrificed 4 days after completion of DSS administration (recovery time). The HCAF group received also the AIN 76 diet where 50 mg/kg/day of HCAF were added. A pre-treatment of 10 days with HCAF diet was done before initiation of 4% DSS treatment. When a 4-day 4% DSS treatment was initiated, HCAF diet was co-administered. After the end of DSS treatment, HCAF was administered for another 4 days (recovery time) and then the rats were sacrificed. Weight, health conditions, and water consumption of each rat was monitored every day. The Human Equivalent Dose (HED) for the administered dose of 50 mg/kg HCAF is 7.25 mg/kg HCAF for a human of 70 kg according to the formula: HED = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$ [21].

2.7 Fecal water content

Feces were immediately collected after deposition and then weighed and dried in a de-humidified oven at 65°C until there was no weight variation for 24 h. The percent of fecal water content was calculated as the difference between the wet and dry weights of the pellet and expressed as percentage.

2.8 Determination of HCAF in feces by LC-MS

Feces (0.3 g) of the 10th day of administration of test compounds were weighted and after adding 8 μg of hydrocinnamic acid-d_9 as internal standard, were homogenized with an Ultraturrax T-25 in 5 mL of water/HCl (99.9:0.1) and centrifuged at 4.500 × *g per* 5 min. Supernatant was acidified with HCl (0.2% final concentration v/v) and loaded onto a SPE 200 mg Strata C18-E cartridge (Phenomenex Italia, Bologna, Italy). Compounds were eluted with 1 mL of methanol and 20 μL were injected for analysis. The LC system was a PerkinElmer Series 200 system (PerkinElmer, Italy) consisting of a binary-pump and autosampler. Samples were separated onto a RP C_{18} Phenomenex Luna column (150 × 4.60 mm^2, 5 μm particle size). The mobile phases were MS grade water (solvent A) and ACN/water 97:3 v/v (solvent B), both containing 0.1% formic acid, at a flow rate of 1 mL/min. The gradient started with 10% of B during 3 min to reach 80% B at 13 min, 80% B at 14 min, and 90% of B at 15 min; chromatographic run time was 30 min *per* injection. A mass spectrometer API 365 triple quadrupole was used for MS detection. The mass spectrometer was operated in negative-ion, multiple reaction monitoring (MRM) in negative ion mode. The temperature and flow rate of the turbo gas was adjusted to 250°C and 8 L/min, respectively. The ionization voltage was set to –4000 V. Nitrogen was used as curtain gas, nebulizing gas, and collision gas, with flow settings of 6, 7, and 3, respectively. Collision energy was 26 V. Singly charged precursor-product ion (MS/MS) transitions were monitored at *m*/*z* 181 → 137, 181 → 59, for HCAF and 158 → 158 → 114 for hydrocinnamic acid-d_9 (internal standard). The dwell time was 200 ms for all compounds. For quantification, a standard curve of HCAF and internal standard was done in blank fecal samples. The percentage recovery was used to correct the concentrations in fecal samples.

2.9 RT-PCR

Total RNA was isolated from rat distal colon mucosa using the Rneasy midi kit (Qiagen, Milan, Italy). For first-strand cDNA synthesis 1 μg of RNA from each sample was reverse-transcribed using 100 Units RT superscript II and 1 × random hexamers (Roche, Monza, Italy). The PCR reaction was carried out on aliquots of the cDNA preparation for each gene as previously described [22]. The PCR products were separated onto 1.5% agarose gel w/v. The amplified products were photographed and the intensity of the bands was analyzed with Quantity-One software (Bio-Rad, Segrate, Milan, Italy). For each target gene the relative amount of mRNA was calculated in the sample using β-actin co-amplified as internal standard. Primer sequences for TNF-α and IFN-γ were from Bradesi *et al*. [23]. β-Actin and IL1-β were from Tsune *et al*. [24] COX-1 was from Vogiagis *et al*. [25] and IL-8 from Kim *et al*. [26].

2.10 PGE_2 assay

PGE_2 levels in distal colon mucosa homogenates were measured using an immunoenzymatic method (EIA kit, Cayman Chem, San Diego, CA, USA) according to the manufacturer's specifications. Samples of distal colon mucosa (10 mg) were homogenized in 1 mL of 0.1 M phosphate pH 7.4 containing 1mM EDTA and 10 μM indomethacin and centrifuged at 9000 × *g per* 20 min. A dilution of sample supernatant 1:50 was used.

2.11 Determination of 8-oxodG in DNA from colon mucosa

An oxidized form of guanine, 8-oxodG, is generated by reactive oxygen species (ROS) and is widely used as a marker of oxidative DNA damage [27]. DNA was isolated according to a standard published procedure [28] and aliquots of 100 μg of DNA were hydrolyzed with 10 μL of nuclease P1 (1100 Units/mL) for 2 h and 14 μL of alkaline phosphatase (750 Units/mL) for 30 min. The hydrolyzed mixture was filtered using Micropure-EZ enzyme remover (Amicon, Millipore, Milan, Italy) and 50 μg were injected into the HPLC for 8-oxodG determination The nucleosides were separated with a C18 RP column (Supelco, Milan, Italy, 5 μm, ID 0.46×25 cm^2). The levels of 8-oxodG were detected using an ESA Coulochem II electrochemical detector in line with a UV detector. The 8-oxodG levels were expressed as the ratio of 8-oxodG/dG $\times 10^{-6}$.

2.12 Malonyldialdehyde (MDA) levels in colonic tissue

MDA levels were determined according to the method of Mateos *et al*. [29] with some modifications. Colon tissue (30 mg) was homogenized in 500 μL of Trizma base buffer pH 7.4 containing 0.2 M sucrose and 5 mM DTT in an Ultraturrax T-25. Homogenate was centrifuged at $10\,000 \times g$ for 30 min at 4°C and 250 μL of supernatant were submitted to alkaline hydrolysis with 50 μL of 6 M NaOH for 30 min at 60°C. Protein was then precipitated with 30% trichloroacetic acid followed by centrifugation at $2800 \times g$ for 10 min. A volume of 250 μL supernatant was mixed with 25 μL of 5 mM DNPH and incubated for 30 min at room temperature, protected from light. The reaction mixture (200 μL) was injected into an HPLC equipped with an LC-10 AD Shimadzu pump, a UV – Vis Perkin Elmer LC 290 detector and with a Water Spherisorb ODS-2 column (4.6×150 mm^2 ID, 5 μm particle size).

2.13 Statistical analysis

All analyses were carried out using SPSS® 14.0 software. All data were expressed as the mean ± SD of independent measurements. Statistical significance was determined by Student's *t*-test or by one-way ANOVA analysis, using $p < 0.05$ as the level of significance.

3 Results

3.1 *In vitro* screening anti-inflammatory activity of polyphenol metabolites

On the basis of the work of Déprez *et al*. [30], Gonthier *et al*. [31], and Rios *et al*. [32], we selected 18 polyphenol metabolites for screening. To avoid interference by growth inhibition or cytotoxic effects, we checked cell growth and cytotoxicity with the crystal violet assay at the end of the experiment. We used a concentration of 100 μM of each compound and an incubation time of 24 h. None of the metabolites tested, affected growth or cytotoxicity (data not shown). From all tested compounds, the metabolites that significantly inhibited ($p < 0.05$) PGE_2 production were dOHPA, 3OH-HIPU, 3OHPP, 4OHPP, HCAF, FER, and HFER (see Table 1). Metabolites that inhibited PGE_2 production (HCAF, HFER, and dOHPA) more than 50% were used to do a dose-response curve (0.1 – 100 μM) (Fig. 1).

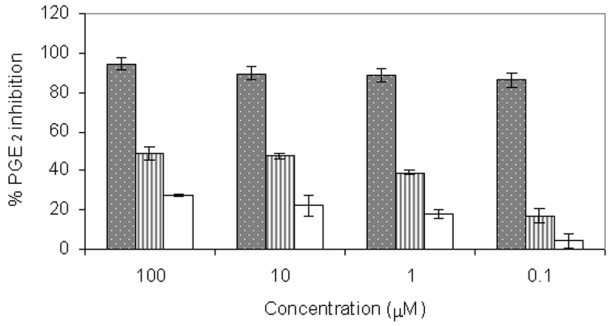


Figure 1. Dose-response effect of HCAF (▩), HFER (▥), and dOHPA (□) on PGE_2 production by CCD18 cells stimulated with 1 ng/mL of IL-1β for 18 h. Data shown are mean ± SD, $n = 3$.

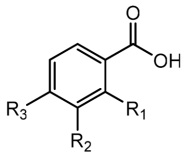
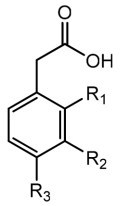
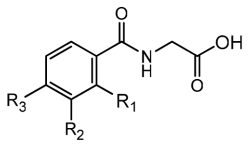
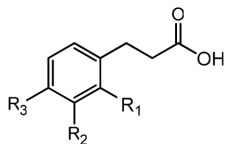
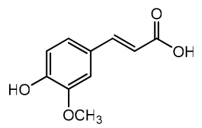
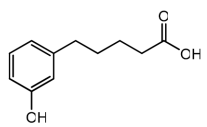
3.2 Anti-inflammatory effects on the writhing test

The three selected polyphenol metabolites (HCAF, HFER, and dOHPA) administered i.p. at a dose of 30 mg/kg caused a significant decrease ($p < 0.05$) in the number of abdominal contractions (writhings) induced by diluted acetic acid administration i.p. in mice, a classic test of anti-nociceptive and anti-inflammatory activity (Table 2). The number of writhings was reduced by 27, 35, and 40% by HFER, HCAF, and dOHPA, respectively. Common anti-inflammatory drugs (indomethacin and sodium salicilate) were used as positive controls.

3.3 Effect on carrageenan-induced hyperalgesia in rats

Carrageenan-induced hyperalgesia is another classic test for measuring the activity of anti-inflammatory analgesic compounds. The maximum effect of the compounds tested was observed 45 min after the subcutaneous administration of 30 mg/kg of HCAF, HFER, and dOHPA. As shown in Table 3, HCAF, HFER, and dOHPA significantly increased the tolerance to the applied pressure of the inflamed paw. HCAF was the most potent of the polyphenol metabolites tested.

Table 1. Percentage of inhibition in PGE_2 production by polyphenol metabolites

Polyphenol metabolites (100 μM)		Percentage inhibition in PGE_2 production
3-Hydroxybenzoic acid (3OHB) R1=H; R2=OH; R3=H 4-Hydroxybenzoic acid (4OHB) R1=H; R2=H; R3=OH 3,4-Dihydroxybenzoic acid (protocatechuic acid, 3,4dOHB) R1=H; R2=OH; R3=OH 4-Hydroxy-3-methoxybenzoic acid (vanillic acid, OHMB) R1=H; R2=OCH3; R3=OH		9 (6, 14) 8 (6, 11) 17 (3, 31) 7 (4, 10)
Phenylacetic acid (PA) 3-Hydroxyphenylacetic acid (3OHPA) R1=H; R2=OH; R3=H 4-Hydroxyphenylacetic acid (4OHPA) R1=H; R2=H; R3=OH 3,4-Dihydroxyphenylacetic acid (homoprotocatechuic acid, dOH-PA) R1=H; R2=OH; R3=OH 4-Hydroxy-3-methoxyphenylacetic acid (homovanillic acid, 4OHM-PA) R1=H; R2=OCH_3; R3=OH		16 (7, 26) 3 (0, 8) 0 (−5, 0) 61 (50, 73)[a)] 0 (−4, 4)
Hippuric acid (HIPU) 3-Hydroxyhippuric acid (3OHHIPU) R1=H; R2=OH; R3=H		0 (−11, 6) 34 (29, 38)[a)]
3-Phenylpropionic acid (3PP) 3-Hydroxyphenylpropionic acid (phloretic acid, 3OHPP)		21 (8, 37) 41 (35, 49)[a)]
R1=H; R2=OH; R3=H 4-Hydroxyphenylpropionic acid (hydrocinnamic acid, 4OHPP) R1=H; R2=H; R3=OH 3,4-Dihydroxyphenylpropionic acid (hydrocaffeic acid, HCAF) R1=H; R2=OH; R3=OH 3-(4-Hydroxy-3-methoxyphenyl) propionic acid (hydroferulic acid, HFER) R1=H; R2=OCH_3; R3=OH		33 (26, 44)[a)] 94 (92, 95)[a)] 35 (25, 45)[a)]
trans-4-Hydroxy-3-methoxycinnamic acid (ferulic acid, FER)		71 (63, 77)[a)]
5-(3-hydroxyphenyl)valeric acid (OHPVAL)		9 (2, 16

a) Significant *versus* control $p < 0.05$ $n = 3$. Values are mean followed by smaller and greater value.

3.4 Effect of HCAF on DSS colonic inflammation

Control and DSS-treated rats did not vary their water intake (17.5 ± 4.5 mL, 17.1 ± 3.6 mL, and 15.9 ± 1.6 mL, in control, DSS, and HCAF groups, respectively) during DSS administration in our experimental protocol. Nevertheless, DSS induced a significant reduction in body weight and increased fecal water content, indicating the presence of colitis in these animals. Body weight gain media was 20.6 ± 7.2 g for control rats, which decreased to 3.8 ± 4.8 g for DSS group. HCAF at 50 mg/kg counteracted this effect

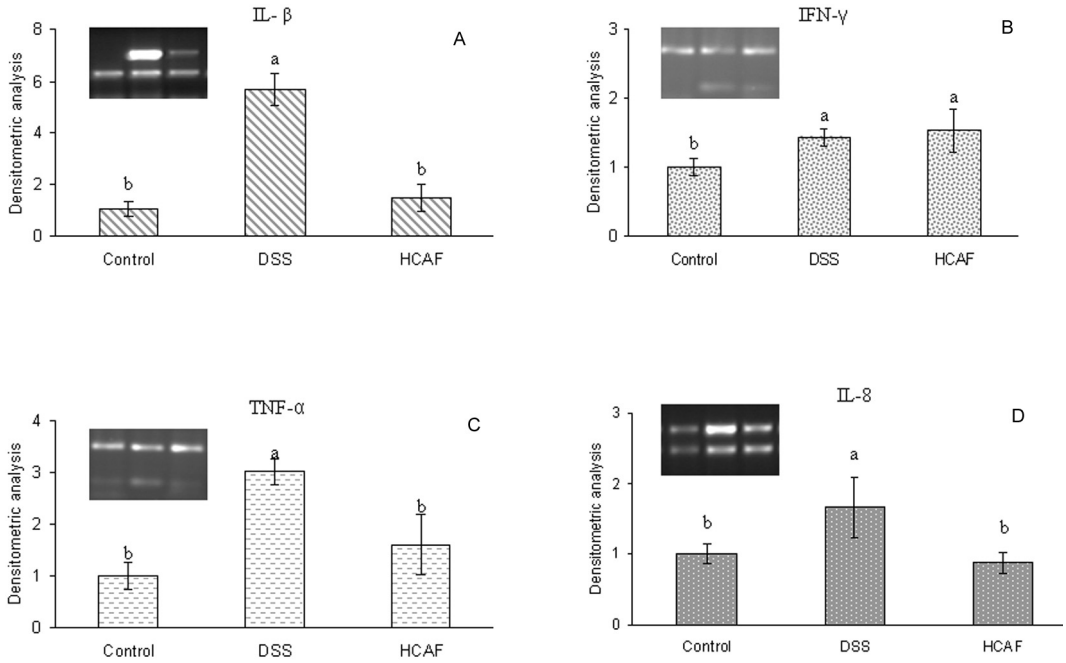


Figure 2. Expression of IL-1β (A), IFN-γ (B), TNF-α (C), and IL-8 (D) measured by RT-PCR in distal colon mucosa of control, DSS, and DSS-HCAF treated rats (upper lines in 2A and 2D, lower lines 2B and 2C). β-actin co-amplified as internal standard (a) significantly different from control group, (b) significantly different from DSS treated group. $n = 6$. Arbitrary units.

Table 2. Effect of dOHPA, HCAF, and HFER on mouse-writhing test

Treatment	*n* mice	*n* Abdominal constrictions
Saline	10	46.4 ± 2.2
dOHPA 30 mg/kg	10	27.4 ± 1.9[a)]
HCAF 30 mg/kg	10	30.3 ± 1.5[a)]
HFER 30 mg/kg	10	34.0 ± 2.1[a)]
Acetylsalicylic acid 800 mg/kg	10	18.1 ± 3.3[a)]
Indomethacin 10 mg/kg	10	13.7 ± 2.4[a)]

a) $p < 0.05$ *versus* saline-treated mice. $n = 10$.

and rats belonging to HCAF group had a body weight gain of 10.3 ± 1.2 g. Fecal water content increased from 28.1 ± 6.7% (control value) to 56 ± 1.1% upon DSS treatment. This increase was prevented by HCAF treatment (28.6 ± 2.8%).

Using RT-PCR, we measured the level of expression of classical inflammation mediators (IL-1β, IFN-γ, TNF-α, and IL-8 cytokines) in the distal colonic mucosa of these animals. DSS induced a significant increase ($p < 0.05$) in IL-1β, IFN-γ, TNF-α, and IL-8 in the colonic mucosa (Fig. 2A – D). Treatment with HCAF significantly reduced the expression of all these cytokines ($p < 0.05$) except IFN-γ. We also observed an increase in COX-2 expression, but HCAF treatment did not vary with this parameter (Fig. 3B). COX-1 expression was down-regulated by DSS and similarly not influenced by HCAF (Fig. 3A). Also PGE_2 mucosal levels were increased by DSS from 500 ± 89 (control levels) to 3590 ± 1019 but did not vary significantly after HCAF treatment (5297 ± 1796) (mean ± SD).

Treatment with DSS increased by three-fold the levels of lipid peroxidation measured as MDA levels in the colon mucosa (Table 4). MDA levels were significantly attenuated by HCAF treatment. As shown in Table 4, oxidized DNA levels measured as the ratio of 8-oxodG to $dG \times 10^{-6}$, were higher in the mucosa of rats treated with DSS ($p < 0.001$). HCAF was able to inhibit DNA oxidation damage induced by DSS ($p < 0.001$).

3.5 HCAF content in feces

Feces of the 10th day after 50 mg/kg HCAF ingestion were analyzed by LC-MS/MS. HCAF content in feces, after feeding for 10 days, was very homogenous varying between 4.5 and 5.5 μM in individual animals. The analysis of feces showed the presence of the $[M-H]^-$ ion corresponding to the ingested compound HCAF (*m*/*z* 181) that produced two daughter ions at *m*/*z* 139 and 59 (Fig. 4). No HCAF was found in control feces.

4 Discussion

Our results demonstrate that some microbiota-derived polyphenol metabolites, found in fecal water of humans con-

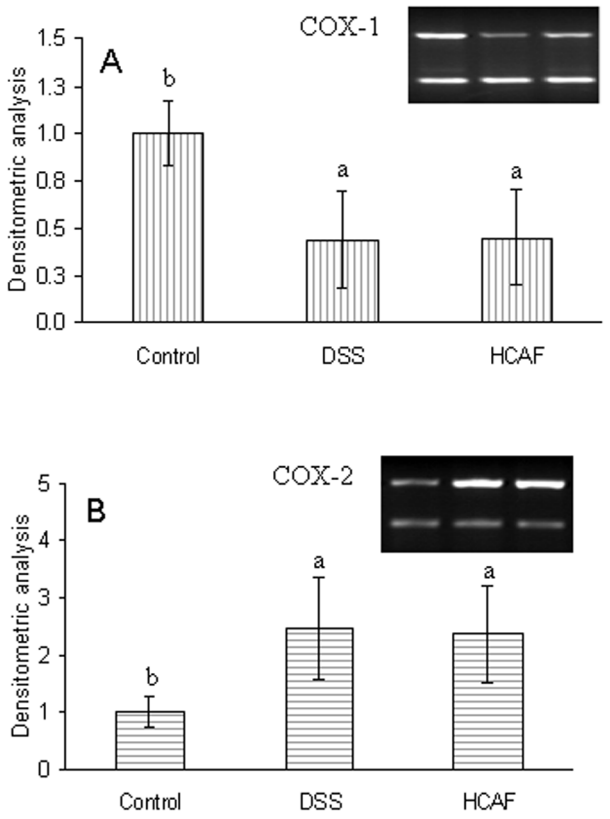


Figure 3. COX-1 (A) and COX-2 (B) expression measured by RT-PCR in distal colon mucosa of control, DSS, and DSS-HCAF treated rats. β-actin co-amplified as internal standard (lower lines) (a) significantly different from control group, (b) significantly different from DSS treated group $p < 0.05$ $n = 6$. Arbitrary units.

suming a balanced diet [3], have an anti-inflammatory action in different inflammation models.

Anti-inflammatory effects are documented in the literature for some polyphenols and derivatives as caffeic acid phenethyl ester [33, 34] and ferulic acid, a possible precursor of HFER, that inhibits macrophage inflammatory protein-2 (MIP-2) and TNF-α levels in macrophages stimulated with LPS [35]. Ferulic acid derivatives have also been shown to have suppressive effect on COX-2 promoter activity on human colon DLD-1 cancer cells [36] and ferulic acid dimer inhibits COX-2 expression in LPS stimulated macrophages [37].

The screening of 18 polyphenol metabolites presented in this paper show that eight of them were able to decrease PGE_2 production *in vitro*. This is the first time that an anti-inflammatory effect has been attributed to many of these compounds. The dose-effect curve done for HCAF showed that even at low dose (0.1 μM) it exerted a potent effect on PGE_2 production inhibition. The 0.1 μM concentration is not so far from the real levels that HCAF reaches in human

Table 3. Effect of HFER, HCAF, and dOHPA on hyperalgesia induced by carrageenan in rat paw pressure test. I.p. injection of 1% carrageenan was performed 3 h before test. Compounds were administered 45 min before test

	Paw pressure (*g*)	
Treatment	Pre-test	45 min
Saline	72.0 ± 1.5	23.5 ± 3.0
HFER 30 mg/kg	72.0 ± 1.7	45.5 ± 1.8[a)]
HCAF 30 mg/kg	72.0 ± 2.2	54.0 ± 1.9[a)]
dOHPA 30 mg/kg	70.0 ± 3.2	46.5 ± 1.5[a)]
Indomethacin 10 mg/kg	69.5 ± 3.1	55.3 ± 4[a)]

a) $p < 0.05$ *versus* Carrageenan-treated rats. $n = 4$.

Table 4. MDA levels expressed as nmol MDA/mg protein and oxidative DNA damage measured as the ratio of 8-oxodG to $dG \times 10^{-6}$ in the colon of control, DSS-, and DSS-HCAF-treated rats

	MDA levels (nmol/mg protein)	8-OxodG × 10^{-6}dG
CONTROL	1.17 ± 0.42[a)]	2.06 ± 0.62[a)]
DSS	3.45 ± 0.72[b)]	9.74 ± 2.18[b)]
HCAF 50 mg/kg	1.26 ± 0.55[b)]	2.06 ± 0.62[a, b)]

$p < 0.05$ for MDA determination and $p < 0.01$ for DNA damage $n = 6$.
a) Significantly different from DSS-treated groups.
b) Significantly different from control group.

plasma (66 nM) after the ingestion of 55 g of cooked artichoke [38].

Of the eight compounds having *in vitro* activity we selected three (HCAF, dOHPA, and HFER) for further *in vivo* testing. When they were administered intra-peritoneally at 30 mg/kg they all decreased the number of abdominal constrictions and increased the weight tolerance in the paw-pressure test, indicating systemic anti-inflammatory action. Our observations are in agreement with previous data on related compounds. For example, vanillic acid has inhibitory activity in arachidonic acid-induced ear edema and in the writhing test [39]. Chlorogenic acid (one of the nutritional precursor of these compounds) inhibits carrageenin-induced paw edema and also the number of flinches during the late phase in the formalin-induced pain test [40].

Of the three compounds with *in vivo* anti-inflammatory activity, we selected HCAF for *in vivo* testing on colitis in rats. The levels of HCAF in rat feces detected after oral administration of 50 mg/kg of HCAF acid were similar to those found (5.2 μM) in human fecal water content when subjects followed a diet without restrictions [3]. The low quantity of HCAF found in feces of rats fed with 50 mg/kg is probably due to the fact that HCAF is mainly absorbed in the stomach or the upper part of the small intestine [41] and

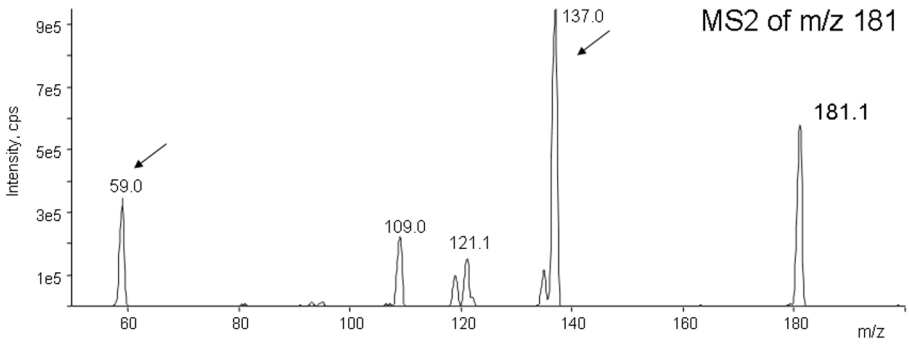


Figure 4. Chromatogram of MS2 (negative ion mode) of HCAF found in feces collected after 10 days of 50 mg/kg HCAF administration. Arrows indicate daughter ions.

only a small part of the administered HCAF reaches the colon.

After HCAF administration the colonic inflammation induced by DSS was clearly inhibited. In fact, HCAF considerably decreased both body weight loss and stool water content after DSS administration. When the cytokine profile was studied, we found that IL-1β and TNF-α mRNA levels were increased by DSS. These cytokines are elevated during colonic inflammation, mainly in the lamina propia containing mononuclear cells [42]. Treatment with HCAF seemed able to down-regulate the expression of both cytokines. These results are in agreement with previous data showing down-regulation of these cytokines in rats fed with phytosteryl ferulates, a possible precursor of HCAF [43]. IL-8 is up-regulated in patients with ulcerative colitis [44] and has been correlated with colorectal carcinogenesis [45]. In our experiments IL-8 was decreased by HCAF treatment almost to control levels. Similar effects of HCAF have been observed in human keratinocytes after UV exposure [8].

Although COX-1 is a constitutively expressed enzyme, its expression in the DSS group was significantly decreased and HCAF treatment was not able to reverse this effect. This might be explained by erosion of the crypt epithelium where this enzyme is located. Similar observations were obtained by Tessner *et al*. [46] and Martin *et al*. [47]. COX-2 expression was increased in DSS but HCAF did not correct this overexpression. This might explain why PGE_2 levels, increased by DSS, were not reduced by HCAF. In contrast, in our *in vitro* experiments, HCAF inhibited the production of PGE_2, an effect that was not observed in the colonic mucosa inflammed after DSS. In these different *in vitro* and *in vivo* models, inflammation is probably regulated by different mediators and pathways. We suggest that HCAF inhibits leukotriene biosynthesis since hydroxyeicosatetraenoic acids (HETEs) enhance IL-1 mediated COX-2 expression in human colonic myofibroblasts [48] and caffeic acid is a 5-lipooxygenase inhibitor [49]. However, we have no direct data on the activity of HCAF on these pathways.

Surprisingly the anti-inflammatory effect of HCAF on DSS colitis was not mediated by inhibition in PGE_2 production. This requires further investigation since *in vitro* HCAF clearly inhibited PGE_2 production. The inherent complexity of physiological conditions *in vivo* including food matrix interactions could be involved in this apparent contradiction. The role of PGE_2 in colon inflammation is controversial. Whereas inhibition in PGE_2 production is generally considered beneficial in inflammation control [50], exogenously applied PGE_2 reduces mucosal inflammatory cytokines and epithelial erosion after DSS [51, 46]. Moreover, endogenous PGE_2 heals small intestinal lesions [52] and the application of EP4 (a stimulator of PGE_2 receptor), suppresses DSS induced colitis [53]. It is also known that gastric and colonic lesions are induced by COX-1 and COX-2 inhibitors [54, 55]. Therefore, it is possible that anti-inflammatory activity on the colon can be exerted through routes not involving the down-regulation of COX-2 or a decrease in PGE_2 concentration in the colonic mucosa. For instance, caffeic acid has shown a protective effect down-regulating iNOS expression via inhibition in NF-κβ activation in neuroinflammation [56].

ROS are also important mediators in colonic inflammation [57] where the antioxidant status is compromised [58]. ROS induces the oxidation of proteins, lipids, and DNA [59, 60]. Guanine is the most easily oxidized base in DNA, having the lowest oxidation potential of the four canonical bases [61]. An oxidized form of guanine is 8-oxodG that is generated by ROS and is widely used as a marker of oxidative DNA damage [27, 62]. DSS treatment enhances ROS levels [63] and free radicals induce oxidative DNA damage in colonic mucosa [64]. Our results show that DSS increased the lipid peroxidation level, measured as MDA and oxidized DNA (as ratio 8-oxodG/dG). Increased oxidation damage and lipid peroxidation were diminished by HCAF treatment, either by direct antioxidant activity or by its ability to increase eNOS expression and activity [4].

In conclusion, this study demonstrates that metabolites derived from polyphenols (HCAF, dOHPA, and HFER) through the metabolism of colon microbiota, are able to inhibit inflammation. HCAF inhibits DSS-induced colitis lipid peroxidation and DNA damage in colon mucosa, down-regulating fundamental cytokines involved in the inflammatory process (TNF-α, IL-1β, and IL-8).

Our results suggest that foods containing significant HCAF precursors (procyanidins, hydroxycinnamic acid derivatives, *etc*.) such as artichoke, cocoa, apples, strawberries, *etc*., could exert anti-inflammatory activity and reduce intestinal inflammation in humans. If these observations are confirmed in clinical trials, polyphenol-rich foods or phenolic acid supplements may have potential therapeutic and/or preventive applications in IBD.

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