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# Caffeic Acid Derivatives: *In Vitro* and *In Vivo* Anti-inflammatory Properties

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Caffeic acid and some of its derivatives such as caffeic acid phenetyl ester (CAPE) and octyl caffeate are potent antioxidants which present important anti-inflammatory actions. The present study assessed the *in vitro* and *in vivo* effects of five caffeic acid derivatives (caffeic acid methyl, ethyl, butyl, octyl and benzyl esters) and compared their actions to those of CAPE. In the model of LPS-induced nitric oxide (NO) production in RAW 264.7 macrophages, the pre-incubation of all derivatives inhibited nitrite accumulation on the supernatant of stimulated cells, with mean $IC_{50}$ (μM) values of 21.0, 12.0, 8.4, 2.4, 10.7 and 4.80 for methyl, ethyl, butyl, octyl, benzyl and CAPE, respectively. The effects of caffeic acid derivatives seem to be related to the scavenging of NO, as the compounds prevented SNAP-derived nitrite accumulation and decreased iNOS expression. In addition, butyl, octyl and CAPE derivatives significantly inhibited LPS-induced iNOS expression in RAW 264.7 macrophages. Extending the *in vitro* results, we showed that the pre-treatment of mice with butyl, octyl and CAPE derivatives inhibited carrageenan-induced paw edema and prevented the increase in IL-1β levels in the mouse paw by 30, 24 and 36%, respectively. Butyl, octyl and CAPE derivatives also prevented carrageenan-induced neutrophil influx in the mouse paw by 28, 49 and 31%, respectively. Present results confirm and extend literature data, showing that caffeic acid derivatives exert *in vitro* and *in vivo* anti-inflammatory actions, being their actions mediated, at least in part by the scavenging of NO and their ability to modulate iNOS expression and probably that of other inflammatory mediators.

*Keywords*: Caffeic acid; Macrophage; NO; Mouse; Inflammation; Carrageenan

## INTRODUCTION

Nitric oxide (NO) is produced from L-arginine by three distinct isoforms of the enzyme nitric oxide synthase (NOS). The constitutive isoforms (neuronal and endothelial) are low output enzymes, whereas the inducible isoform (iNOS) is mainly regulated at the transcriptional level and synthesizes higher amounts of NO for longer periods.[1] Despite its important role in maintaining the vascular tone, neurotransmission and immunity, sustained high levels of NO evoke toxic reactions such as DNA deamination, oxidation and/or nitration, lipid peroxidation, etc., resulting in tissue and cell damage.[1–3] There is evidence that iNOS-derived NO mediates a number of pathophysiological events including sepsis, rheumatoid arthritis and psoriasis.[4–6] In such pathologies, therapeutic intervention is required and strategies might include the selective inhibition of the iNOS[7,8] or the scavenging or removal of NO excess.[5] In addition, NO is thought to possess an important pro-inflammatory role in the early stages of inflammation by activating NF-κB[9] and also by increasing the biosynthesis/release of other inflammatory molecules such as ROS and $PGE_2$,[9–12] amplifying the inflammatory response. For instance, administration of NOS inhibitors to rats has been shown to suppress paw edema induced by carrageenan, Freund's adjuvant and fragments of

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the wall of streptococci.[13] Studies using iNOS knockout mice have suggested a pivotal role for iNOS in carrageenan-induced inflammation since mutant mice are resistant to lung injury and pleurisy induced by this agent.[14,15]

Phenolic acid compounds are known to be potent antioxidants, in great part by their radical scavenging activity, although other mechanisms are likely to be involved. Caffeic acid is a non-vitamin phenolic compound, which is largely found in food plants (fruits, vegetables, wine, olive oil, among others) and is therefore present in human plasma in a diet-dependent concentration.[16] Caffeic acid has been extensively studied for its antioxidant properties and has been shown to scavenge a number of reactive species, including DPPH radicals,[17,18] peroxyl[19] and hydroxyl radicals,[20] as well as superoxide anion, peroxynitrite and mutagenic compounds such as nitrosamines.[20–23] Furthermore, caffeic acid has been shown to possess anti-inflammatory properties, since it inhibits 5- and 12-lipooxygenase activity,[24] in addition to inhibiting PKC, PKA and NF-κB activation induced by ceramides in U937 cells.[23,25] These anti-oxidant and anti-inflammatory actions extend to some of the caffeic acid derivatives. In fact, several studies have indicated that caffeic acid phenetyl ester (CAPE), a caffeic acid derivative originally isolated from the honeybee propolis, is able to specifically and potently inhibit NF-κB activation in U937 cells.[26] Furthermore, CAPE is effective in suppressing 5-LOX activity,[27] as well as TPA-induced $PGE_2$ production in human oral epithelial cells.[28] Incubation of RAW macrophages with this caffeic acid derivative inhibits LPS-induced iNOS expression.[29] In addition, CAPE presents anti-oxidant properties, similar to those observed for caffeic acid,[27,30] being able to inhibit lipid peroxidation and xantine oxidase activity,[27,30] as well as to scavenge superoxide anion and DPPH radicals.[30] Importantly, CAPE potently inhibits leukocyte migration and edema formation induced by carrageenan injection in the rat air pouch model and in the rat paw edema model, respectively.[31,32] Finally, in a rat model of vascular injury, the administration of CAPE diminishes COX-2 expression, NF-κB activation, and restenosis.[33] Recently, another caffeic acid derivative has been shown to inhibit iNOS expression induced by LPS/IFN-γ in rat smooth muscle aortic cells.[34]

The available therapeutic arsenal used for the treatment of inflammatory diseases is composed almost solely of non-steroidal anti-inflammatory drugs (NSAIDS) and glucocorticoids. However, these drugs possess severe side effects (e.g. renal, gastric and many other in the case of glucocorticoids) and provide unsatisfactory results in some pathologies.[35] Thus, the need for new anti-inflammatory molecules and the fact that several antioxidant compounds has been shown to exhibit anti-inflammatory activities[36] prompted us to examine the putative anti-inflammatory effects of semi-synthetic caffeic acid and some of its analogues (all of which possessed anti-oxidant properties), comparing their actions with the known caffeic acid derivative CAPE by means of *in vitro* and *in vivo* strategies. Our study also attempted to analyze some of the possible mechanisms underlying the effects of these compounds.

## MATERIALS AND METHODS

### Materials and Reagents

Cell culture plates and flasks were from Costar (New York, USA). Dulbecco's modified Eagle medium (DMEM), fetal calf serum and antibiotics were obtained from GIBCO BRL Life Technologies (Rockville, MD, USA). LPS (serotype O111:B4), carrageenan (lambda IV), cycloheximide, dexamethasone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), CAPE, rabbit polyclonal antibody against iNOS, Trizma™, sucrose, DTT, soybean trypsin inhibitor, PMSF, sodium molibdate, glycerol, $KH_2PO_4$, $MgCl_2$, $CaCl_2$, L-valine, L-citrulline, Dowex resin AG-50, EGTA, L-NIO, L-NAME and aminoguanidine were purchased from Sigma Co. (St. Louis, MO). The Griess reagents sulfanilamide and naphtylethylenediamine were from Merck (Rahway, NJ). The PVDF membrane was from Millipore (Bredford, MA). Reagents for SDS-eletrophoresis and enhanced chemiluminescence assay system, as well as [$^3$H]-L-arginine, were from Amersham Biosciences (São Paulo, Brazil). HRP-labeled anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). SNAP was kindly donated by Prof Jamil Assreuy (Department of Pharmacology, UFSC). Mouse IL-1β ELISA kit was purchased from R&D Systems (Minneapolis, USA). Caffeic acid derivatives were dissolved always on the day of experiments in DMSO at final concentrations under 0.1% (v/v) for *in vitro* assays and equal to 2.0% for *in vivo* studies.

### Caffeic Acid Derivatives

Caffeic acid derivatives were obtained by reaction of caffeic acid purchased from commercial source with an appropriate alcohol in acetyl chloride under reflux (2 h; 60–70°C), according to the methodology previously described.[37,38] The respective products were purified by re-crystallization or chromatographic column over silica gel. All compounds were synthesized in good yields (60–70%) and characterized by $^1$H NMR, IR and microanalyses. The purity grade of these compounds was superior to 95% and

was determined by thin layer chromatography using several solvent systems.

### Animals

Male Swiss mice (20–25 g) were used for *in vivo* experiments. Animals were kept in controlled room temperature (22 ± 1°C) and humidity (60–80%) under a 12:12 h light-dark cycle (lights on 06:00 h) and were acclimatized to the laboratory for at least 1 h before testing, being used only once in each test. Procedures were performed in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals.[39]

### Cell Culture

The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC-Rockville, USA). Cells were cultured in DMEM supplemented with 5% heat-inactivated calf serum, 150 U/ml penicillin, 150 μg/ml streptomycin, and maintained at 37°C in a humidified incubator with 5% $CO_2$.

### Nitrite Assay and Cell Viability

RAW 264.7 cells were plated in 96 wells plates and allowed to reach confluence. For nitrite quantification, macrophages were exposed to different concentrations of one of the following caffeic acid derivatives: caffeic acid methyl ester (6.25–50.00 μM), caffeic acid ethyl ester (6.25–50.00 μM), caffeic acid butyl ester (3.12–50.00 μM), caffeic acid octyl ester (1.56–25.00 μM), caffeic acid benzyl ester (1.56–25.00 μM) or CAPE (1.56–25.00 μM) 30 min before LPS (0.5 μg/ml) stimulation. After 24 h, 100 μl of supernatant were mixed with the same volume of Griess reagent (1.0% sulfanilamide in 5.0% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in water; 1:1, v/v); absorbance of the mixture at 550 nm was determined by an enzyme-linked immunosorbent (ELISA) assay plate reader (EL808, Bio-Tek Instruments, Inc.) and nitrite concentrations were calculated on the basis of standard solutions of sodium nitrite. In another set of experiments, in order to verify whether the compounds were reducing nitrite levels in the culture medium by converting it into nitrate, supernatant samples were firstly incubated with *E. coli* (12 μg/protein/ml) for 3 h at 37°C and then assayed for nitrite with the Griess reaction. Cell viability was determined by the mitochondrial-dependent reduction of MTT to formazan as described by Van de Loosdrecht *et al.*[40]

### SNAP-derived NO Assay

In order to assess whether or not the studied compounds were able to directly interact with NO, we used a cell-free system, where the NO-donor SNAP was the source of NO. For that purpose, SNAP (300 μM) was dissolved in serum-free DMEM and each of the studied caffeic acid derivatives was added at different concentrations. The mixture was then allowed to react for 24 h at 37°C and nitrite was quantified with the Griess reaction.

### NOS Activity

iNOS was obtained following the incubation of RAW 264.7 with LPS (0.5 μg/ml) for 8 h. After the incubation period, the cells were scraped from the tissue culture bottles, centrifuged (2000*g*, 5 min), re-suspended in lysis buffer (320 mM sucrose, 50 mM Tris–HCl, 2 mM DTT, 10 μg/ml soybean-trypsin inhibitor, 10 mg/ml phenylmethylsulphonyl fluoride, 20 mM sodium molybdate, 10% glycerol, pH 7.2) and stored at −70°C until use. On the day of the experiments, cells were lysed by sonication, the lysate was centrifuged (30,000*g*, 30 min) and supernatant was used as the iNOS source. NOS activity was assayed by measuring the conversion of $^3$H-L-arginine to $^3$H-L-citrulline as described by Bredt and Snyder[41] with some modifications. Briefly, 200 μg of protein was incubated with each of the studied caffeic acid derivatives at different concentrations for 30 min at room temperature before the addition of 50 μl of a cocktail containing $^3$H-L-arginine (35 nM) and the following substrates and co-factors: 50.0 mM $KH_2PO_4$; 1.2 mM $MgCl_2$; 0.2 mM $CaCl_2$; 60.0 mM L-valine; 1.2 mM L-citrulline and 1.0 mM DTT, pH 7.2. The mixture was incubated for 1 h at 37°C and the reaction was stopped and free $^3$H-L-arginine was separated from $^3$H-L-citrulline by the addition of 400 μl of a resin, which binds to $^3$H-L-arginine (Dowex AG-50 × 8, $Na^+$ form, 1:1 in water, pH 6.7). After centrifugation (10,000*g*, 3 min, RT), the supernatant (100 μl) was mixed with 1.0 ml of scintillation liquid. $^3$H-L-citrulline was quantified by liquid scintillation counting. For the quantification of calcium-independent NOS (iNOS) activity, EGTA (1.0 mM) was added to the assay buffer. For every sample, a blank sample was run by adding EGTA, L-NIO and aminoguanidine (1.0 mM each) to the assay buffer. Results are expressed as pmol citrulline/min/mg protein.

### Western-blot Analysis

RAW 264.7 cells were cultured in 6 wells plates until confluence and were exposed to the caffeic acid derivatives studied at different concentrations or cycloheximide (1.0 μM) and stimulated with LPS (0.5 μg/ml). After 16 h of incubation, cells were washed, collected in PBS, and centrifuged (1000*g* for 10 min, 4°C). Pellets were re-suspended in lysis buffer (10.0 mM KCl, 0.1 mM EDTA, 1.0 mM DTT,

0.5 mM PMSF, 10.0 mM HEPES, pH 7.6) and allowed to rest for 20 min on ice. Samples were then sonicated for 5 s and centrifuged (12,000*g*, 5 min, 4°C). Supernatants (cytosolic extracts) were used for western-blot assay. Protein concentrations were determined using the Bradford method.[42] Lysates were boiled in equal volumes of loading buffer (125.0 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol) and 3.0 μg of protein were loaded per lane on a 10% polyacrilamide gel. Separated proteins were transferred on to PVDF membranes for 4 h at 300 mA which were blocked overnight at 4°C in TBS-T containing 5% (w/v) defatted milk and incubated with rabbit polyclonal (1/10,000, v/v) anti-iNOS or goat polyclonal anti-actin (1/500, v/v), followed by incubation with peroxidase-conjugated anti-rabbit IgG (1/2000) or peroxidase-conjugated anti-goat IgG (1/5000). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Amersham Biosciences).

### Mouse Paw Oedema

Experiments were performed as previously described.[43] Briefly, animals were pre-treated with caffeic acid butyl ester, caffeic acid octyl ester, CAPE (30.0 mg/kg, i.p., −1 h) or dexamethasone (0.5 mg/kg, s.c., −4 h) and paw edema was induced with an intraplantar (i.pl.) injection of 300 μg of carrageenan dissolved in 50 μl of 0.9% saline solution into the right hindpaw. The left hindpaw received the same volume of 0.9% saline solution and was used as control. Paw volume below the ankle joint was measured with a plethysmometer (Ugo Basile, Milan, Italy) at several time points (30, 60, 120, 240, 360 min. and 1, 2, 3 and 5 days) after carrageenan injection. Paw edema is expressed in μl as the difference between the volumes of right and left paws.

### Measurement of Paw IL-1β Levels and Myeloperoxidase (MPO) Activity

For the quantification of IL-1β levels and myeloperoxidase activity in the subcutaneous tissue of paws, two different groups of mice were treated as described above. After 6 h of carrageenan injection, animals were sacrificed and the tissue of the injected paws removed and kept at −70°C until analysis. IL-1 β measurement was performed according to the method described by Francischi *et al.*[44] Briefly, subcutaneous paw tissue was homogenized in phosphate buffered solution containing Tween 20 (0.05%), PMSF (0.10 mM), benzamethonium chloride (0.10 mM), EDTA (10.00 mM) and aprotinin (2.00 μg/ml). Homogenates were centrifuged (3000*g*, 4°C, 10 min) and IL-1β levels were quantified in supernatant with an ELISA kit according to the instructions of manufacturer.

MPO activity was measured by means of a simple quantitative method for detecting leukocyte sequestration at an inflammatory site. Experiments were conducted according to De Young *et al.*[45] with some modifications. Subcutaneous paw tissue was homogeneized in phosphate buffer 0.08 M (pH 5.4) containing HTAB (0.5%). Paw homogenates were centrifuged (11,000*g*, 4°C, 20 min) and MPO activity was quantified in the supernatant by means of the change in optical density measured at 650 nm using TMB (1.6 mM) and $H_2O_2$ (0.3 mM). Results are expressed as O.D./mg of tissue.

### Statistical Analysis

All the results are presented as the mean ± SEM, except the $IC_{50}$ values (i.e. the concentrations of compounds necessary to inhibit the nitrite production by 50%) that are presented as geometric means accompanied by their respective 95% confidence limits. These values were determined by use of the least squares method for individual experiments. Statistical comparison of the data was performed using ANOVA one-way analysis of variance, followed by Dunnett's test. *P* values less than 0.05 were considered to be significant.

## RESULTS

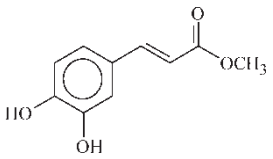
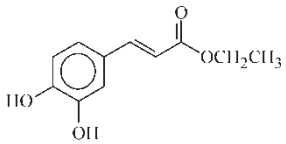
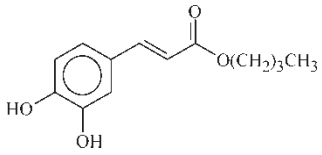
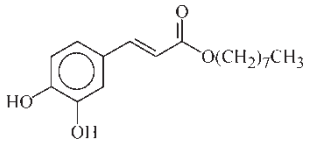
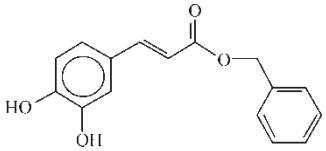
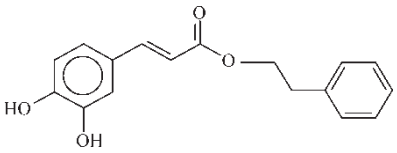
### Effect of Caffeic Acid Derivatives on Nitrite Production in LPS-stimulated RAW 264.7 Cells

Incubation of macrophages with LPS (0.5 μg/ml) for 24 h significantly increased nitrite levels in the culture medium according to the measurement by the Griess reaction. Pre-treatment of RAW macrophages with caffeic acid derivatives, 30 min before stimulation with LPS, reduced nitrite levels in a concentration-dependent manner, with mean $IC_{50}$ values in the μM range (Table I). Apparently, the potency of compounds was proportional to the side chain length. The reduction of nitrite accumulation was not due to interaction of compounds with nitrite or Griess reagents, nor was it due to the conversion of NO and nitrite into nitrate (results not shown). Furthermore, inhibition of nitrite accumulation in the supernatant of the cells was not related to a cytotoxic effect, since compounds do not exhibit cytotoxic effects at concentrations which are near the $IC_{50}$ values (Table II).

### Effect of Caffeic Acid Derivatives on SNAP-derived NO Assay

Incubation of NO donor SNAP (300 μM) in serum-free DMEM for 24 h at 37°C resulted in a marked

TABLE I Chemical structure and $IC_{50}$ ($CI_{95\%}$) values obtained for caffeic acid derivatives on nitrite production in LPS-stimulated RAW cells

| Compound | Nitrite inhibition ($IC_{50}$ ($CI_{95\%}$) μM) | Chemical structure |
|---|---|---|
| Caffeic acid methyl ester | 21.4 (12.8–35.6) |  |
| Caffeic acid ethyl ester | 11.9 (11.0–13.0) |  |
| Caffeic acid butyl ester | 8.4 (7.9–8.9) |  |
| Caffeic acid octyl ester | 2.4 (2.0–3.1) |  |
| Caffeic acid benzyl ester | 10.7 (8.5–13.5) |  |
| Caffeic acid phenethyl ester | 4.8 (2.8–8.4) |  |

Each group represents the mean of 3 experiments.

nitrite increase in the culture medium (51 ± 4 μM) (Fig. 1). In the presence of caffeic acid derivatives, the levels of nitrite were reduced in a significant manner. Inhibitions were: 49 ± 1, 44 ± 1, 44 ± 1, 29 ± 2, 34 ± 3 and 49 ± 4% for methyl (50.00 μM), ethyl (25.00 μM), butyl (25.00 μM), octyl (6.25 μM), benzyl (25.00 μM) and phenethyl (12.50 μM) esters, respectively (Fig. 1).

TABLE II Cell viability of RAW 264.7 cells on treatment of samples

| | Cell viability (% of control) | | | |
|---|---|---|---|---|
| Compound | 50 μM | 25 μM | 12.5 μM | 6.25 μM |
| Caffeic acid methyl ester | 86.6 ± 12.3 | 95.5 ± 4.5 | 100 ± 1 | – |
| Caffeic acid ethyl ester | 86.3 ± 11.7 | 98 ± 2 | 99.5 ± 0.5 | – |
| Caffeic acid butyl ester | 64.6 ± 17.7 | 91.5 ± 8.5 | 98.5 ± 1.5 | – |
| Caffeic acid octyl ester | – | 22.6 ± 2.6** | 84.6 ± 7.4 | 93.3 ± 6.6 |
| Caffeic acid benzyl ester | – | 86.3 ± 7.8 | 97.3 ± 2.6 | 100 ± 1 |
| CAPE | – | 62.3 ± 4.9** | 97 ± 2 | 99 ± 1 |

Each group represents the mean of 3 experiments. $** p < 0.01$.

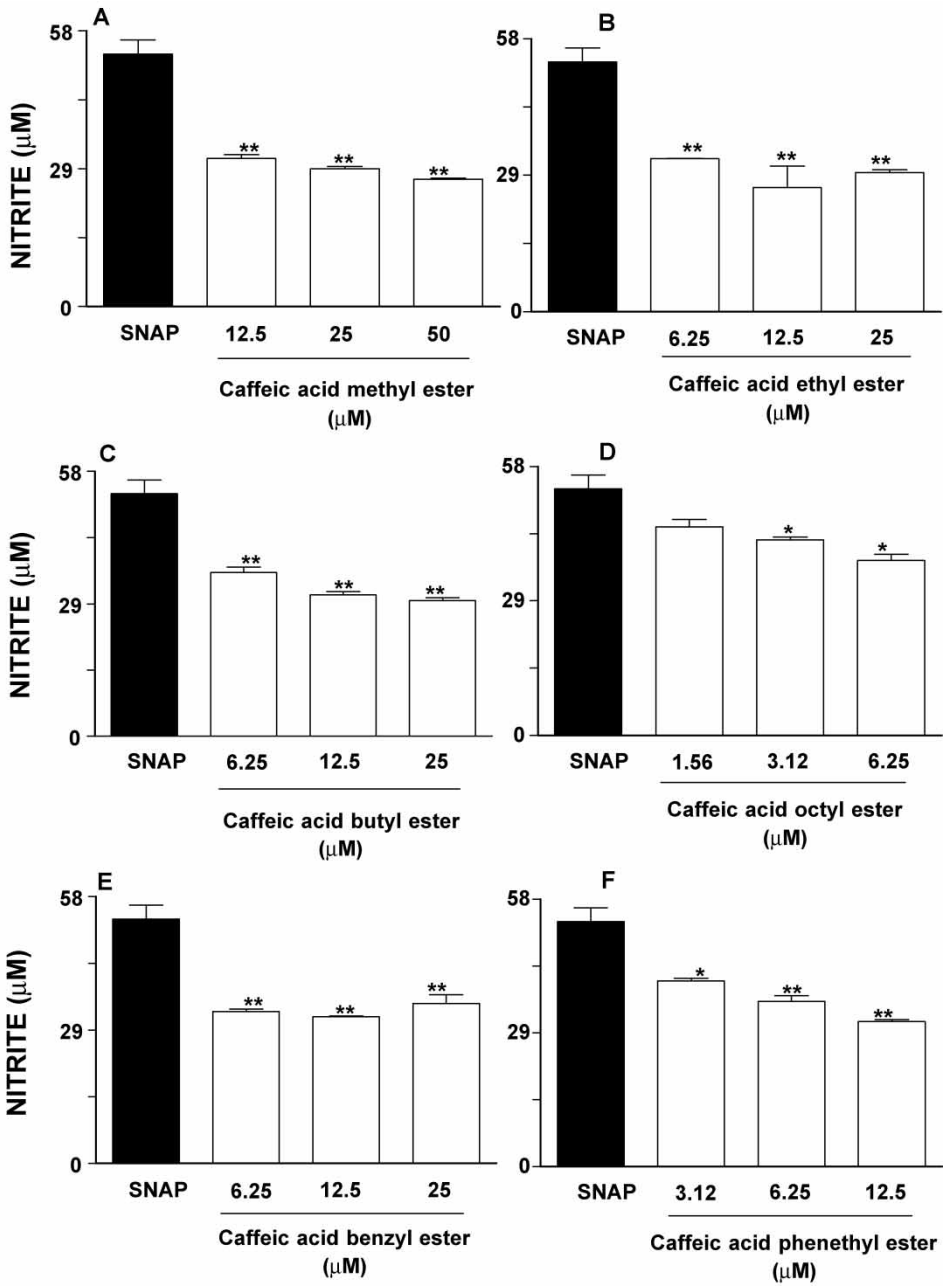



FIGURE 1 Effect of caffeic acid derivatives methyl (A), ethyl (B), butyl (C), octyl (D), benzyl (E) and phenethyl (F) ester on SNAP-derived nitrite accumulation. SNAP solution (300 μM) in DMEM was incubated for 24 h at 37°C in the presence or absence of different concentrations of caffeic acid derivatives. Nitrite accumulation was quantified by Griess reaction as described in the "Material and methods" section. Data is presented as means ± SEM ($N = 3$). Statistically different to SNAP group *$p < 0.05$ and **$p < 0.01$.

### Effect of Caffeic Acid Derivatives on INOS Activity

Incubation of RAW 264.7 cells with LPS (0.5 μg/ml) for 8 h resulted in an accentuated increase of iNOS activity (5.8 pmol of citrulline/min/mg of protein). Incubation of cytoplasmic iNOS protein (e.g. *in vitro*) with caffeic acid derivatives at concentrations in the range of $IC_{50}$ values did not reduce iNOS activity. Instead, caffeic acid derivatives increased it in a significant manner, in contrast to L-NAME (100 μM) that inhibited iNOS activity by 100% (Fig. 2).

### Effect of Caffeic Acid Derivatives on INOS Expression

Unstimulated RAW cells did not express detectable amounts of iNOS protein. However, when exposed to LPS (0.5 μg/ml) for 16 h, iNOS was markedly increased. Pre-incubation (−30 min) of RAW 264.7 cells with caffeic acid butyl, octyl and phenethyl esters (1.56–25.00 μM) as well as with the protein synthesis inhibitor cycloheximide (1.0 μM) resulted in a significant and concentration-dependent inhibition of LPS-induced iNOS expression (Fig. 3).

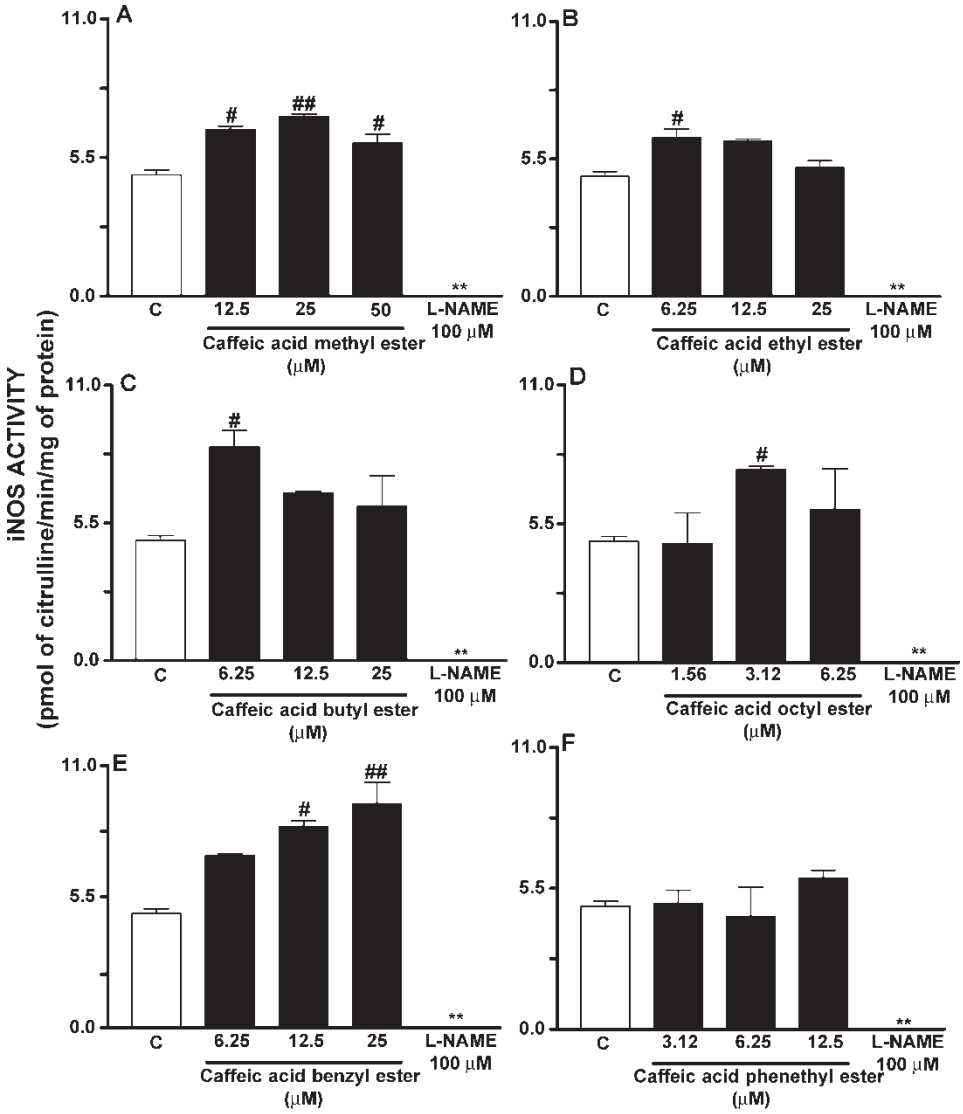



FIGURE 2 Effect of caffeic acid derivatives on *in vitro* iNOS activity. Cytoplasmic iNOS protein was incubated with one of studied compounds at the indicated concentration for 1 h and iNOS activity was measured as citrulline production/min/mg of protein. Data is presented as means ± SEM ($N = 2$). Statistically different of control group $p < 0.05$ and ## or $^{**}p < 0.01$.

### Effect of CAPE or Octyl and Butyl Ester on Carrageenan-induced Paw Edema

In order to extend the results obtained *in vitro*, we chose the three most potent caffeic acid derivatives and tested them in the mouse paw inflammation induced by carrageenan, comparing their actions with those of dexamethasone. Results depicted in Fig. 4 show that: not only CAPE, but also octyl and butyl derivatives, as well as dexamethasone, significantly inhibited the increase in paw volume induced by i.pl. injection of carrageenan. Interestingly, similar to that observed for the glucocorticoid dexamethasone, the treatment with butyl and octyl derivatives displayed a long-lasting profile of action when assessed against the mouse paw edema induced by carrageenan (up to 24 h). In the case of CAPE, a significant inhibition of edematogenic response was observed for up to 72 h (Fig. 4A,B).

### Effect of CAPE or Octyl and Butyl Ester on IL-1β Levels and MPO Activity

IL-1β levels were significantly increased in the subcutaneous paw tissue as assessed 6 h following i.pl. injection of carrageenan (300 μg/paw) in comparison with the saline-injected paw (Fig. 5A). Pre-treatment of the animals with CAPE, caffeic acid octyl and butyl esters (30.0 mg/kg, i.p., −1 h) or dexamethasone (0.5 mg/kg, s.c., −4 h) significantly inhibited (36 ± 3, 24 ± 2, 30 ± 17 and 54 ± 2%, respectively) the increase in IL-1β levels caused by carrageenan injection (Fig. 5A).

MPO activity was markedly increased in carrageenan-injected paw in comparison with the saline-injected paw (Fig. 5B). Pre-treatment of the animals with CAPE, octyl ester, butyl ester (30.0 mg/kg, i.p., −1 h) or with dexamethasone (0.5 mg/kg, s.c., −4 h) significantly inhibited (31 ± 2, 49 ± 1, 28 ± 2 and

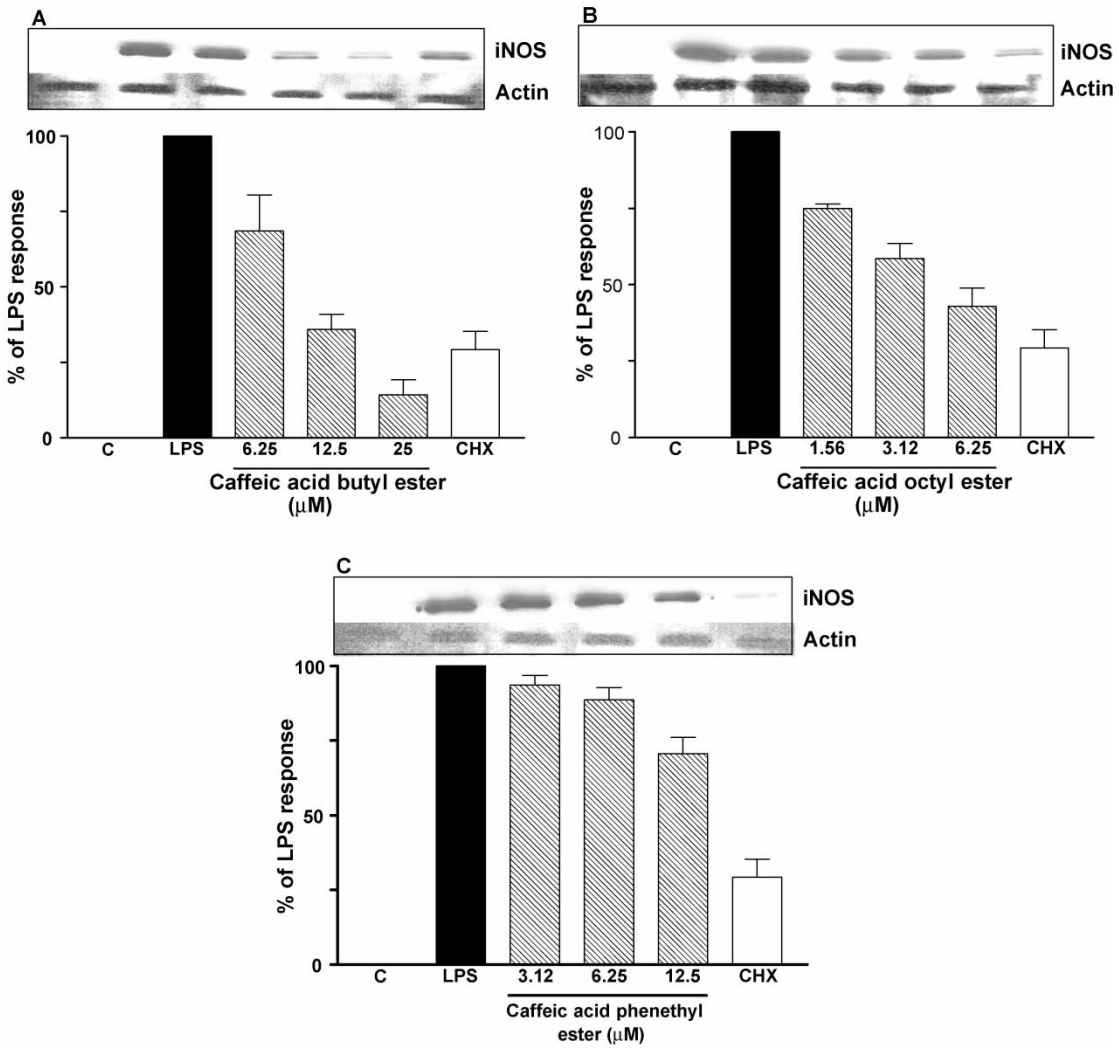



FIGURE 3 Effect of butyl (A), octyl (B) and phenethyl (C) derivatives on LPS-induced iNOS expression in RAW 264.7 macrophages. Cells were pre-incubated (−30 min) with one of the above-mentioned compounds in different doses (1.56–25 μM) or with cycloheximide (CHX: 1 μM) and stimulated with LPS (0.5 μg/ml) for 16 h. Cytosolic proteins (3 μg) were separated on a 10% SDS-PAGE gel and transferred to PVDF membranes. Representative western blot for iNOS and actin expression is shown for each compound. The densitometric evaluation of three independent experiments is depicted below. Lane designations are identical for the blot and the histogram. C = control (resting cells).



38 ± 5%, respectively) the increase in MPO activity induced by carrageenan (Fig. 5B).

## DISCUSSION

In the present work, we have largely extended the literature data by demonstrating the inhibitory effects of CAPE on nitrite production and iNOS expression in RAW 264.7 macrophages *in vitro*, as well as in a model of inflammation evoked by carrageenan in mice *in vivo*. In addition, the present results compare the effects of CAPE with those observed for five additional caffeic acid derivatives in the same models, attempting to further clarify the mechanisms through which they exert their anti-inflammatory properties.

Present data suggests that elongation of the alkyl side chain of the alcoholic part of esters enhances the NO inhibitory activity of the caffeic acid derivatives. Similar results, including very close mean $IC_{50}$ values, have been obtained by Nagaoka *et al.*[46] in the macrophage cell line J774.1. Furthermore, the present results and those obtained by Nagaoka *et al.*[46] indicate that no toxic actions are observed when compounds are tested in concentrations at the range of $IC_{50}$ values, discarding the possibility that the decrease in nitrite levels in RAW 264.7 macrophages supernatant could be related to a cytotoxic effect.

In order to investigate the possible mechanisms involved in the decrease of nitrite levels, three sets of experiments were carried out. The first aimed to clarify whether or not caffeic acid derivatives were

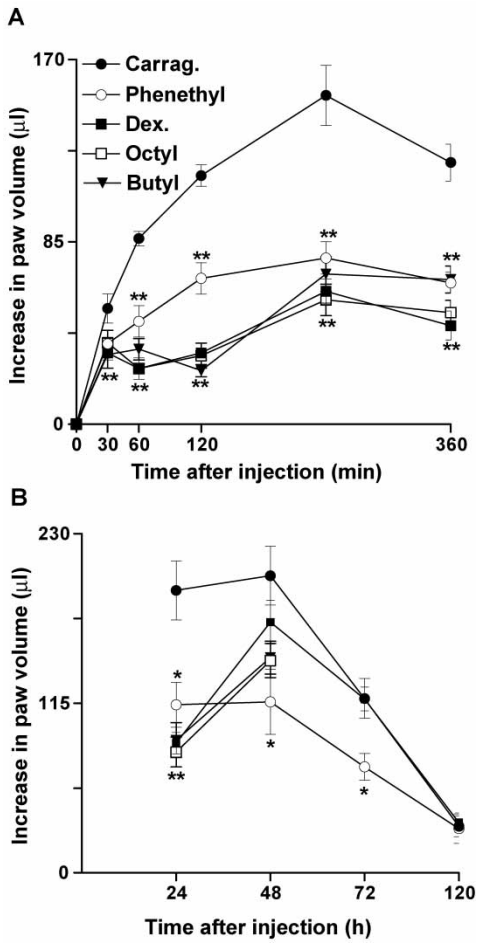



FIGURE 4 Effect of caffeic acid derivatives on carrageenan-induced mouse paw oedema. Animals were pre-treated with phenethyl, octyl or butyl ester derivative (30 mg/kg, i.p., −1 h) or with dexamethasone (Dex.: 0.5 mg/kg, s.c., −4 h) and paw oedema was induced with intraplantar injection of carrageenan (Carrag.: 300 µg/paw). Paw volume was evaluated in a plethysmometer at different time points (panels A and B) after carrageenan injection. Data is presented as means ± SEM ($N = 5$). Statistically different of carrageenan control group *$p < 0.05$ and **$p < 0.01$.

able to directly interact with NO, by means of its scavenging action. For that, the NO donor SNAP was used as the NO source. We showed, for the first time, that studied caffeic acid derivatives do act as NO scavengers, since they largely inhibited nitrite production by SNAP decomposition. Importantly, the decrease in nitrite production is not a consequence of an interference with nitrosothiol chemistry since the compounds also reduced the nitrite levels obtained by decay in nitrite of NO gas bubbled in water (data not shown). Decreased in nitrite levels are not surprising, as caffeic acid possesses a great scavenging ability, being able to scavenge different types of ROS and reactive nitrogen oxide species (RNOS).[16,17,19–21,27,30,47] Likewise, this scavenging ability is shared by some of the caffeic acid derivatives, as previously shown,[18,48] and probably contributes to the anti-inflammatory actions ascribed to these molecules in the literature.[31,32,34,49,50,53]

The second set of experiments sought to determine whether the inhibition of iNOS activity was another mechanism that would account for the caffeic acid derivatives' decrease in nitrite levels in RAW macrophage supernatant. The results show that the compounds did not inhibit iNOS activity. On the contrary, they significantly increased it. These apparently discrepant results can be explained by the previous findings showing that caffeic acid derivatives are NO scavengers. In fact, several reports have shown that NO promotes a negative feedback on iNOS activity by binding to its haem group.[52,53] Furthermore, classical NO scavengers such as haemoglobin have been shown to increase iNOS activity when added to isolated iNOS protein.[53] An alternative explanation for the increased iNOS activity would be that caffeic acid derivatives, being antioxidant compounds, could help to maintain a reductive status in the iNOS assay, protecting enzyme's critical cysteines residues[54] as well as haem iron from oxidation.[55]

Several authors have reported that caffeic acid, CAPE and caffeic acid octyl ester are capable of modulating gene expression. For instance, CAPE has been shown to inhibit iNOS and COX-2 gene expression[28,29] as well as nuclear factor κB activation.[26,31,50] Furthermore, caffeic acid octyl ester, has recently been shown to suppress iNOS gene expression in rat aortic smooth muscle cells stimulated with LPS/IFN-γ.[34] Based on the above-mentioned evidence, we performed the third group of assays, which aimed to analyze the effects of caffeic acid butyl and octyl ester as well as CAPE on LPS-induced iNOS expression in RAW macrophages. As previously described in "Results" section, CAPE as well as butyl and octyl derivatives were able to attenuate LPS-induced iNOS expression in RAW cells, this being the third proposed mechanism that could be involved in the decrease of nitrite levels detected in LPS-stimulated RAW 264.7 cells treated with these caffeic acid derivatives. Thus, even if the unlikely possibility of the increased iNOS activity observed *in vitro* would be an artifact caused by caffeic acid derivatives scavenging and reductive ability, the diminished iNOS expression indicates that at least part of their action is at cellular level. Although the effects of caffeic acid derivatives on NF-κB activation were not investigated in the present work, it is likely that this transcriptional factor is involved in the effects of caffeic acid derivatives on iNOS expression, as NF-κB is an essential transcriptional factor involved in the process of iNOS expression.[56] In fact, previous works have shown that CAPE, as well as caffeic acid octyl ester, are able to inhibit NF-κB activation in other cell types/lines.[26,28,31,34,50]

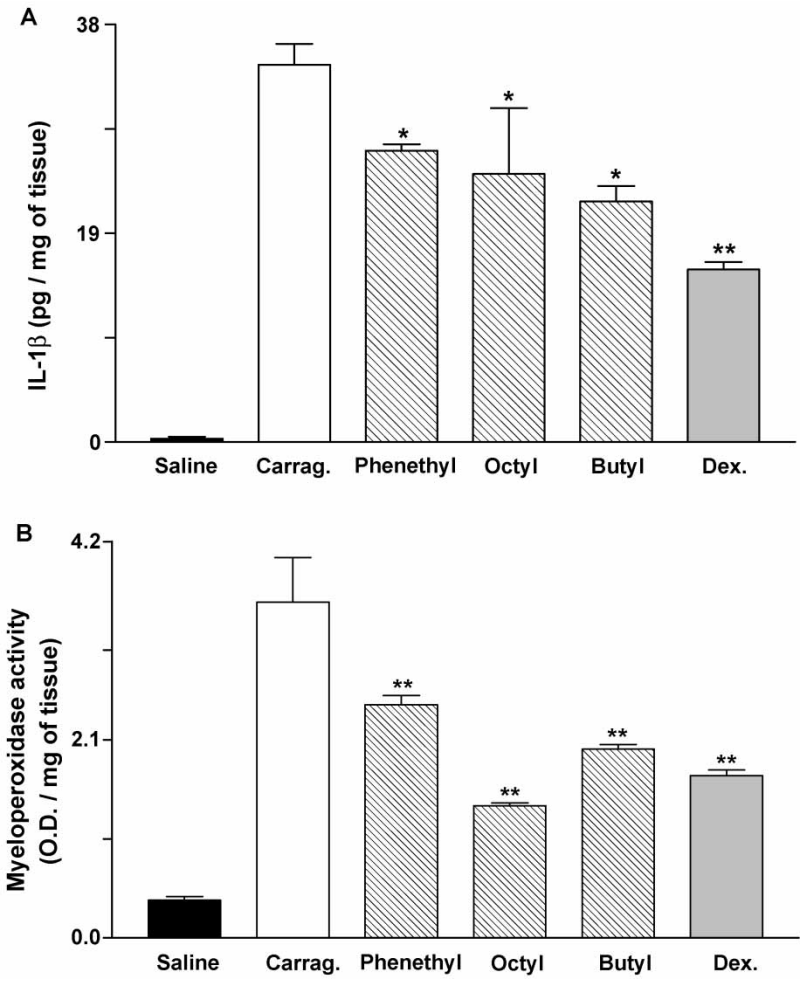



FIGURE 5 Effect of caffeic acid derivatives on carrageenan-induced increase in IL-1 β levels and MPO activity in subcutaneous tissue of paws. Animals were pre-treated with caffeic acid phenethyl, octyl or butyl ester (30 mg/kg, i.p., −1 h) or with dexamethasone (Dex.: 0.5 mg/kg, s.c., −4 h) and received an intraplantar injection of carrageenan (Carrag.: 300 μg/paw). IL-1 β levels were assessed with an ELISA kit (A) and MPO activity assay (B) was performed as described in the "Materials and methods" section. Data is presented as means ± SEM ($N = 5$). Statistically different to carrageenan control group *$p < 0.05$ and **$p < 0.01$.

To further extend the *in vitro* anti-inflammatory effects reported for caffeic acid derivatives in RAW 264.7 macrophage cell line, we tested whether or not the compounds exerted anti-inflammatory effects in a classical model of acute *in vivo* inflammation, namely carrageenan-induced mouse paw edema. This animal model is characterized by a time-dependent increase in paw volume accompanied by neutrophil influx,[57] as well as by the release of various inflammatory mediators (e.g. cytokines, ROS, RNOS and prostanoids) at the injection site.[10,58] In a simplified manner, mouse response to i.pl.-injected carrageenan can be divided into three phases: phase 1, increased release of histamine, serotonin and bradykinin (first 2 h); phase 2, prostanoid dependent phase (2–6 h); and phase 3, a later edema peak occurring between 24 and 72 h after carrageenan injection. Previous work showed that neutrophils in inflammed tissue are substituted by mononuclear cells in that phase.[59] NO is thought to play an important role in carrageenan-induced edema formation, as injection of this phlogistic agent increases $NO_2^-/NO_3^-$ levels at the inflammatory site. Furthermore, this increase in $NO_2^-/NO_3^-$ can be inhibited by L-NMMA or aminoguanidine administration as well as by neurectomy, suggesting the participation of constitutive and induced isoforms of NOS.[10] In addition, early phase mediators (e.g. histamine, serotonin and bradykinin) of the carrageenan edema promote NO release that probably mediates microvasculature dilatation and edema formation. Additional evidence about the involvement of NO in carrageenan-induced paw edema was provided by Handy and Moore.[13] In their work, the authors report that treatment of animals with the non-selective NOS inhibitor L-NAME decreases phases 1 and 2 of carrageenan-induced paw edema, while L-NIL, an iNOS selective inhibitor, decreases phase 2 without affecting phase 1. Based on these findings, authors suggest that constitutive isoforms participate in the early phase of carrageenan edema formation, while the induced

isoform is important for the late phase.[13] The present work shows that CAPE, as well as caffeic acid butyl and octyl esters, possess *in vivo* anti-inflammatory actions, evident by the fact that they consistently inhibited carrageenan-induced mouse paw edema, neutrophil influx and the increase in IL-1β levels. One might suggest that scavenging of NO could be responsible for the inhibition of paw edema observed during the first 2 h following phlogogen injection. This action could prevent the microvascular dilatation induced by histamine, serotonin and bradykinin. Furthermore, the scavenging of the NO produced in the early phase would prevent NF-κB activation by this gas, decreasing the expression of a variety of inflammatory molecules such as chemotactic factors, adhesion molecules and the inflammatory enzymes iNOS and COX-2.

The pre-treatment of animals with octyl and butyl derivatives, and also with CAPE, decreased IL-1β levels in carrageenan-injected paws. It is well known that activated macrophages are the main source of this cytokine following carrageenan stimulation.[60] Once released in inflamed tissue, IL-1β induces the synthesis of various inflammatory mediators, including $PGE_2$, amplifying the inflammatory process. Furthermore, the cytokine IL-1β is able to induce the chemotaxis of neutrophils and monocytes, leading to neutrophil and eosinophil degranulation at the inflammatory site.[61]

The abovementioned caffeic acid derivatives were also able to reduce neutrophil influx to the inflamed paws, possibly as a result, at least in part, of diminished IL-1β levels. In fact, inhibition of neutrophil migration is a very efficient strategy for the control of the inflammatory response, since these cells play a key role in the development and amplification of inflammation by means of ROS and lysosomal enzyme release. Based on the results regarding iNOS expression and other works that show that caffeic acid derivatives are indeed able to modify gene expression, it is tempting to suggest that the synthesis and/or release of many other inflammatory molecules are prevented, resulting in the accentuated inhibition of carrageenan-induced paw edema observed in our work. Kono *et al*.[20] have reported that caffeic acid scavenges superoxide, peroxynitrite and hydroxyl radicals very efficiently. Since peroxynitrite and ROS in a general manner are thought to play an important role in carrageenan-induced inflammation,[58,61] it is also possible that the neutralization of free radicals by means of their scavenging is another mechanism by which caffeic acid derivatives inhibit carrageenan-induced paw edema.

In summary, the present work largely extends the literature data and presents new findings by demonstrating that caffeic acid derivatives, especially octyl, butyl and CAPE, present important *in vitro* and *in vivo* anti-inflammatory properties, and for the first time shows the anti-inflammatory actions of caffeic acid butyl ester.

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