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Phenolic Substances from *Phagnalon rupestre* Protect against 2,4,6-Trinitrochlorobenzene-Induced Contact Hypersensitivity

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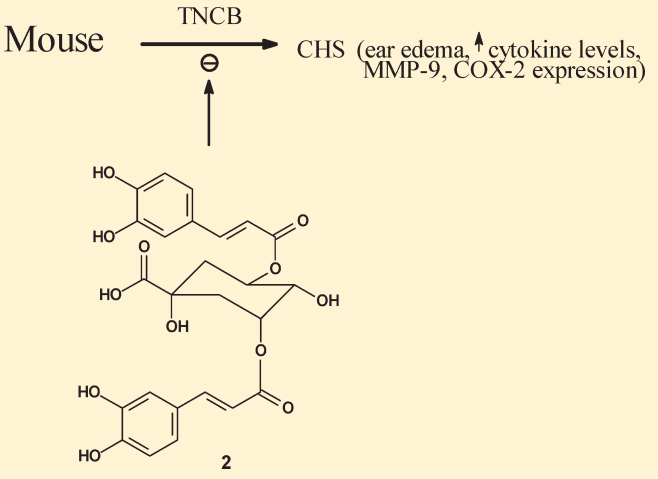
ABSTRACT: 2-Isoprenylhydroquinone-1-glucoside (**1**), 3,5-dicaffeoylquinic acid (**2**), and 3,5-dicaffeoylquinic acid methyl ester (**3**), isolated from *Phagnalon rupestre*, improved the contact hypersensitivity response to 2,4,6-trinitrochlorobenzene in mice. These phenolics reduced ear swelling and IL-1β content by 50% 24 h after challenge; in addition, **2** inhibited tumor necrosis factor-α by 53%. All three compounds also reduced interleukin-2 content by 50% 72 h after challenge. Both **2** and **3** inhibited metalloproteinase-9 levels in the skin lesions by 66% and 41%, respectively, and lowered cyclooxygenase-2 expression by 44% and 49%, respectively, at 24 h. Moreover, **2** was effective against atopic dermatitis induced by repeated application of 2,4,6-trinitrochlorobenzene; it attenuated edema by over 40% from day 7 and inhibited inflammatory cell infiltration by 44% at day 22. In addition, **1**−**3** reduced metalloproteinase-9 expression in a dose-dependent manner in macrophages RAW 264.7 stimulated with lipopolysaccharide. Thus, compounds **2** and **3** were found to exhibit a greater activity against contact hypersensitivity than **1**.



Plant-derived phenolics have long been considered potential chemoprotective agents, against not only cardiovascular disease and cancer but also many other disease conditions. These compounds increasingly are receiving attention as constituent dietary supplements for controlling inflammation, along with other potential health benefits. Despite their potential as antioxidants, it has become clear that their chemopreventive actions also involve the ability to exert a number of nonantioxidant effects. These include a potential to regulate signaling pathways[1] and gene expression involved in cell proliferation, cell-cycle control, and apoptosis.[2−5]

One of the most evident proliferative inflammatory conditions is contact hypersensitivity (CHS), recognized as a classical T-cell-mediated delayed immune response. Initial contact of the skin with a hapten mediates the rapid production of NF-κB-dependent proinflammatory cytokines including TNF-α and IL-1β, which induce maturation of Langerhans cells and emigration from the skin through the draining of the lymphatic channels into the lymph nodes, where hapten-specific T-cells are primed. The T-cells become activated and expand after the first sensitization. Subsequent challenge with the hapten induces an inflammatory reaction by recruiting more inflammatory cells to the hapten-treated area and by releasing inflammatory cytokines, resulting in the characteristic swelling that peaks 24−48 h after challenge and then quickly resolves.

Another related, although quite different in origin, human skin disease is atopic dermatitis (AD), which is characterized by a chronic, relapsing inflammatory dermatitis with immunological disturbance and eczematous skin lesions. While the animal models of skin inflammation designed to mimic atopic dermatitis do not completely reproduce the pathology, they are important research tools for developing new therapeutic approaches. Thus, repeated application of 2,4,6-trinitrochlorobenzene (TNCB) at two-day intervals for three to four weeks results in a site-restricted shift in the time course of antigen-specific hypersensitivity responses from a typical delayed-type to an immediate-type hypersensitivity followed by a late reaction, a phenomenon often seen in skin lesions of AD patients.[6]

We recently reported the anti-inflammatory and antiallergic properties[7−9] of three phenolic substances: 2-isoprenylhydroquinone-1-glucoside (**1**), 3,5-dicaffeoylquinic acid (**2**), and 3,5-dicaffeoylquinic acid methyl ester (**3**), isolated from *Phagnalon rupestre* DC. (Asteraceae).[10,11] The purpose of the present study was to evaluate the efficacy of these phenolics in two mouse models of dermatitis induced by single and repeated application of TNCB. Assays include assessments of ear thickness, inflammatory cell infiltration, and cytokine production in the skin lesion. The effect of these compounds on metalloproteinase-9 (MMP-9) expression in macrophages

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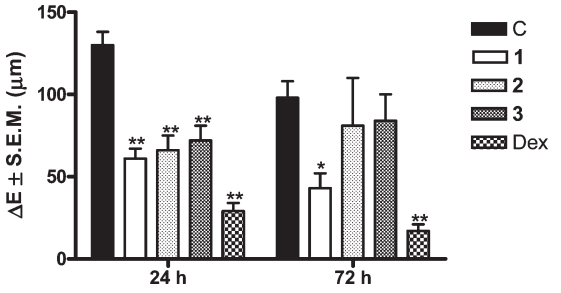


Figure 1. Effects of test compounds (**1**−**3**, 0.5 mg/ear) and dexamethasone (0.025 mg/ear) on CHS ear thickness induced by TNCB, measured 24 and 72 h after challenge. Compounds were topically applied to both ears at 30 min and 24 and 48 h after challenge. Increase in ear thickness is expressed as mean ± SEM of at least six different animals. Statistically significant difference with respect to the control is expressed as $^{**}p < 0.01$, $^{*}p < 0.05$ (Dunnett's *t* test).

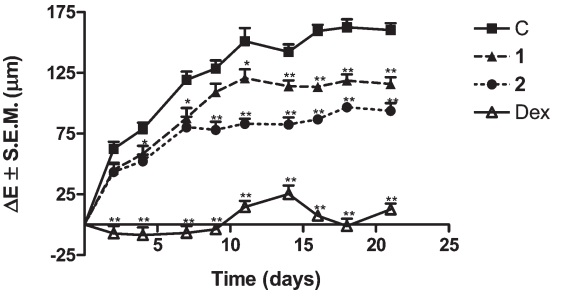
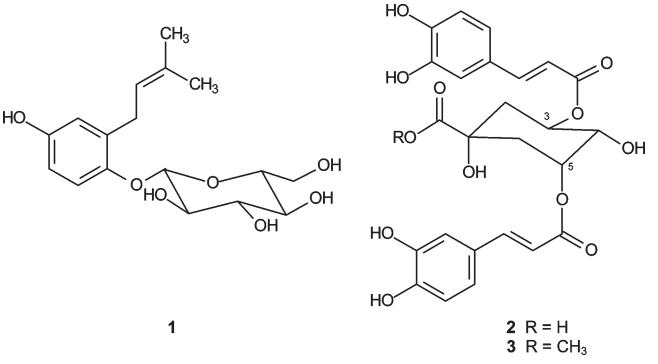


Figure 2. Effects of **1** and **2** (0.5 mg/ear) and dexamethasone (0.025 mg/ear) on the time course of ear thickness induced by repeated application of TNCB. Compounds were applied topically to both surfaces of both ears three times per week, through days 0−21, 30 min after each challenge. Ear thickness was measured before each challenge on days 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21. Increase in ear thickness is expressed as mean ± SEM of at least eight different animals. Statistically significant difference with respect to the control is expressed as $^{**}p < 0.01$, $^{*}p < 0.05$ (Dunnett's *t* test).

stimulated with lipopolysaccharide (LPS) has also been investigated.



RESULTS AND DISCUSSION

The effects of **1**−**3** were assessed on the CHS response to TNCB in mice by determining changes in ear swelling and cytokine production. According to previous studies on CHS induced by TNCB,[12] the edema reaches a maximum at 24 h and then decreases until 72 h. We thus measured ear thickness

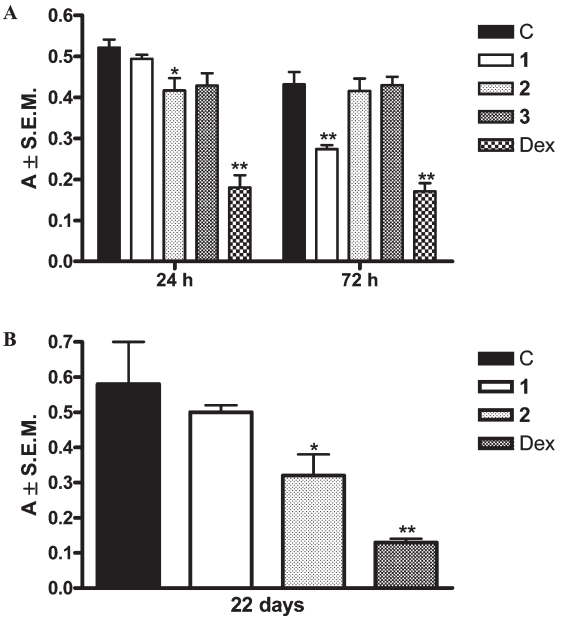


Figure 3. Effects of test compounds (**1**−**3**, 0.5 mg/ear) and dexamethasone (0.025 mg/ear) on neutrophil accumulation, assessed by MPO activity in ear samples. (A) Effects of **1**−**3** on MPO activity in the ear tissue at 24 and 72 h after the challenge with TNCB. (B) Effects of **1** and **2** on the MPO activity in the ear tissue at day 22 after repeated application of TNCB. Each column with a vertical bar represents the mean ± SEM of at least five ear samples. Statistically significant difference with respect to the control is expressed as $^{**}p < 0.01$, $^{*}p < 0.05$ (Dunnett's *t* test).

immediately before sensitization and then 24 and 72 h after elicitation with TNCB. All three phenolics significantly reduced ear thickness at 24 h after challenge, showing edema inhibition percentages of 53%, 50%, and 45%, respectively. Compound **1** remained effective at 72 h (50% edema inhibition). In contrast, the reference compound dexamethasone almost completely abolished edema (78% and 83% inhibition at 24 and 72 h, respectively) (Figure 1).

The effects of **1** and **2** were also evaluated on atopic dermatitis induced by repeated application of TNCB at two-day intervals for three to four weeks. As shown in Figure 2, repeated application of TNCB induced an increase of ear thickness that reached a plateau at day 11, at which point it was approximately two and a half times greater than on day 2. Compound **2** reduced ear thickness at every time point significantly and inhibited edema by more than 40% from day 7 onward. The effect of compound **1** was lower, inhibiting edema by nearly 30% from day 11. Repeated treatment with dexamethasone significantly inhibited ear thickness.

The level of myeloperoxidase (MPO) activity, a quantitative index of neutrophil infiltration into the skin, was measured 24 and 72 h after the single application of TNCB on acute CHS (Figure 3A) and on day 22 after repeated application of TNCB (Figure 3B). In the former protocol, **2** produced a weak but significant inhibition of MPO activity (20%) at 24 h, whereas **1** produced a notable inhibition (43%) at 72 h. Dexamethasone inhibited the infiltration by approximately 65%. After repeated application of TNCB, only **2** significantly inhibited MPO by 44% on day 22. The reference compound dexamethasone significantly reduced MPO activity by 78%.

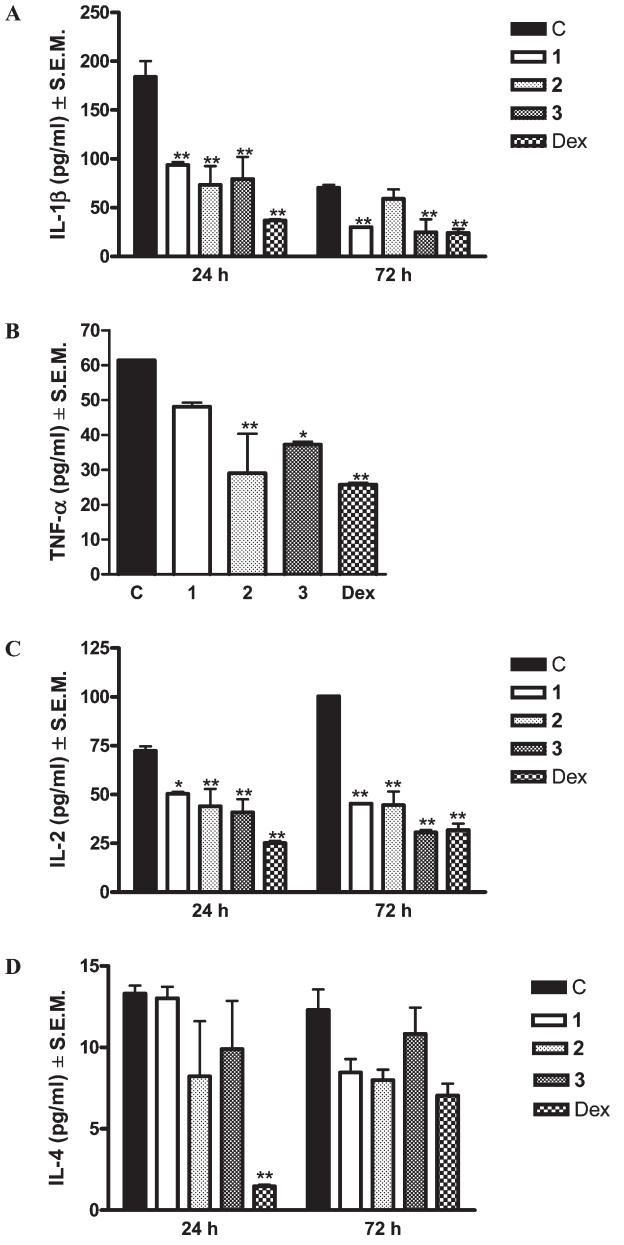


Figure 4. Effects of test compounds (**1**−**3**, 0.5 mg/ear) and dexamethasone (0.025 mg/ear) on cytokine production in CHS induced by TNCB 24 and 72 h after challenge. (A) Effect of test compounds on IL-1β production. (B) Effect of test compounds on TNF-α production at 24 h. (C) Effect of test compounds on IL-2 production. (D) Effect of test compounds on IL-4 production. Values represent production of cytokines in pg/mL and are expressed as mean ± SEM of at least four different animals. Statistically significant difference with respect to the control is expressed as $^{**}p < 0.01$, $^{*}p < 0.05$ (Dunnett's t test).

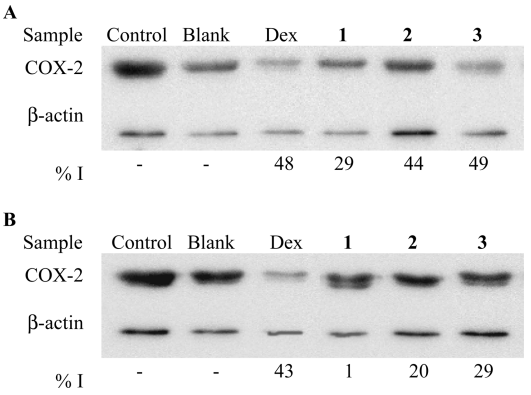


Figure 5. Effects of test compounds (**1**−**3**, 0.5 mg/ear) and dexamethasone (0.025 mg/ear) on COX-2 expression in the ear tissue of mice at 24 h (A) and 72 h (B) after the challenge with TNCB. Total protein extracts were subjected to Western blot analysis with COX-2-specific antibody. COX content for each sample was determined with the aid of densitometry and normalized to β-actin. Percentages of inhibition were calculated with respect to the control group. Figures are representative of three similar experiments performed with similar results.

Experimental studies have revealed that a critical event during the development of cutaneous immune responses is the mobilization and migration of epidermal Langerhans cells, which are regulated by at least two independent cytokine signals provided by IL-1β and TNF-α.[13] Thus, ear tissue was collected 24 and 72 h after the single application of TNCB to evaluate the cytokine production measured with the aid of ELISA in the skin lesions after treatment with each compound. A notable increase in IL-1β and TNF-α levels at 24 h, which was strongly attenuated later, was observed in tissue samples from naive mice treated with TNCB. This is in agreement with published data showing that activation of TNF and IL-1 pathways occurs during the initiation of immediate/early phase immune reactions and leads to a cascade of other changes.[14] The three phenolics significantly decreased IL-1β content by over 50% at 24 h, with **1** and **3** diminishing levels of this cytokine by approximately 60% at 72 h (Figure 4A). At 24 h, compounds **2** and **3** inhibited TNF-α content by 53% and 39%, respectively (Figure 4B). In contrast to the levels observed for these cytokines, the IL-2 levels in the control group were higher at 72 h than at 24 h. In this case, the effect of the phenolics consisted of merely maintaining the production of IL-2, which in fact resulted in a 2-fold inhibition at 72 h (Figure 4C). This is an interesting result because IL-2 is considered to be the primary T-cell growth factor promoting the activation and proliferation of T-cells. It is well-known that IL-4 also plays an important role, particularly in allergic diseases, and that its overexpression in the skin causes increases in the number of Langerhans cells and mast cells and focal deposition of collagen. However, we observed no appreciable amount of this cytokine in the control group, nor did the test compounds exhibit any significant activity, only slightly reducing the already poor increase of IL-4 production observed (Figure 4D). Dexamethasone inhibited the cytokine content by over 50%.

These results suggest that the phenolics used may act as suppressors of T-lymphocyte activation by reducing pro-inflammatory cytokine production. In this context, it should be noted that previous studies have indicated that the effect of these compounds on dinitrofluorobenzene (DNFB) or oxazolone-induced CHS was associated with a decrease in the production of IL-1β.[7]

Transcription factor NF-κB regulates the expression of many genes involved in immune responses and inflammation; for example, it activates the genes that encode pro-inflammatory cytokines such as IL-1β and TNF-α. Our results are consistent with recent in vitro studies conducted by our group indicating that compound **2** inhibits NF-κB activation.[15]

In contrast to previous findings of a prominent up-regulation iNOS gene in the skin samples of nitrohalobenzene-induced CHS,[14]

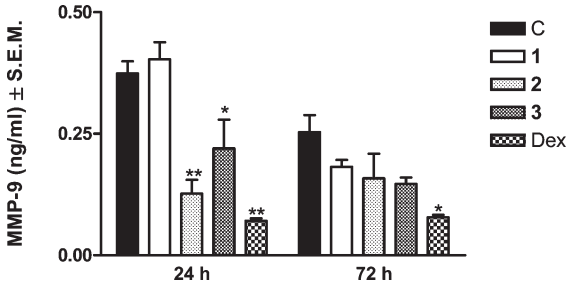


Figure 6. Effects of test compounds (**1**−**3**, 0.5 mg/ear) and dexamethasone (0.025 mg/ear) on MMP-9 production in CHS induced by TNCB 24 and 72 h after challenge. Values represent production of MMP-9 in ng/mL and are expressed as means ± SEM of at least four different animals. Statistically significant difference with respect to the control is expressed as $^{**}p < 0.01$, $^{*}p < 0.05$ (Dunnett's *t* test).

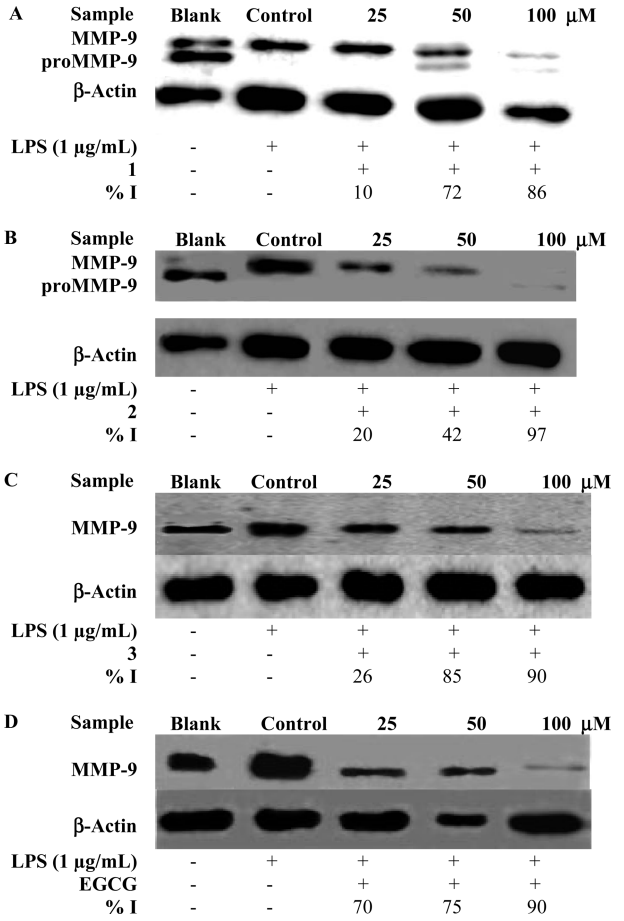


Figure 7. Effects of the test compounds (**1**−**3**) and EGCG, used as reference compound, on MMP-9 expression in stimulated RAW 264.7 macrophages. Cells were stimulated with LPS (1 μg/mL), and the compounds were tested at 25, 50, and 100 μM. Total protein extracts were subjected to Western blot analysis with MMP-9-specific antibody. MMP-9 content for each sample was determined with the aid of densitometry and normalized to β-actin. Percentages of inhibition were calculated with respect to the control group. Figures are representative of three similar experiments performed with similar results.

no iNOS expression was observed (data not shown). Indeed, it was found recently that the 3,5-dicaffeoylquinic acid esters failed to inhibit iNOS expression in CHS induced by DNFB or oxazolone.[7] However, as shown in Figure 5, compounds **2** and **3** reduced the expression of COX-2 by 44% and 49%, respectively, at 24 h after TNCB challenge (Figure 5A). This effect was weaker at 72 h (20% and 29% inhibition, respectively) (Figure 5B). Dexamethasone inhibited COX-2 expression by 48% and 43% at 24 and 72 h, respectively.

Extracellular matrix degradation is known to be involved in lymphocyte infiltration to sites of inflammation, with the gelatinase subfamily of matrix metalloproteinases (comprising MMP-2 and MMP-9) produced by inflammatory cells playing a central role in the migration and invasion of T-lymphocytes in immune-mediated diseases.[16] Compounds **2** and **3** reduced significantly MMP-9 activity in the skin lesions at 24 h (inhibition percentages of 66% and 41%, respectively) (Figure 6). Dexamethasone inhibited the activity by 81% and 69% at 24 and 72 h, respectively.

According to a previous report indicating that activated T-lymphocytes released MMP-9,[17] one of the possible mechanisms of dicaffeoylquinic acid derivatives for controlling the CHS reaction may be the reduction of MMP-9 levels in the skin. In addition, the in vitro studies demonstrated that the induction of MMP-9 expression by LPS-stimulated RAW 264.7 macrophages was inhibited in a dose-dependent manner by the phenolics tested (Figure 7). The reference compound epigallocatechin gallate (EGCG) completely abolished MMP-9 expression at 100 μM (Figure 7). Unlike its role in the case of cytokines, the implication of NF-κB is uncertain here. According to Woo et al.,[18] LPS signaling to MMP-9 expression in macrophages is associated with a ROS-p38 kinase-AP-1 cascade rather than mediated through a NF-κB pathway, especially given the fact that its inhibitor, pyrrolidine dithiocarbamate, does not suppress MMP-9 induction.

Taken together, our results indicate that the 3,5-dicaffeoylquinic acid esters, **2** and **3**, previously determined as antioxidants and inhibitors of peroxynitrite reactivity,[19−21] effectively reduced the swelling, COX-2 expression, and the levels of cytokines and MMP-9 in inflamed mouse ears 24 h after challenge with a single application of TNCB. Moreover, esterification of the carboxyl group at C-1 impaired protection against CHS. Although the isoprenylhydroquinone **1**, a nonantioxidant inhibitor of peroxynitrite-mediated tyrosine nitration,[21] was found to produce a clear reduction in swelling and maintained its effects 72 h after challenge, its activity on cytokines and MMP-9 was not noticeable in comparison to those of the dicaffeoylquinic acid derivatives tested.

In conclusion, we have demonstrated that these three phenolics isolated from *P. rupestre* modulate the immune response in the skin after exposure to the contact allergen TNCB, at least partly via reduction of ear swelling and cytokine production, although with different ranges of activity. Compound **2** was found to be particularly effective in inhibiting most of the inflammation parameters. These findings enhance our understanding of the effects of these phenolics on CHS.

EXPERIMENTAL SECTION

Test Compounds. 2-Isoprenylhydroquinone-1-glucoside (1-*O*-glucopyranosyl-1,4-dihydroxy-2-dimethylallylbenzene, **1**), 3,5-dicaffeoylquinic acid [(1*S*,3*R*,4*S*,5*R*)-1,3,4,5-tetrahydroxy-1-carboxycyclohexane 3,5-di-3-(3,4-dihydroxyphenyl)propenoate, **2**], and 3,5-dicaffeoylquinic acid methyl ester (**3**) were obtained and identified from a bioactive extract of the Mediterranean shrub *Phagnalon rupestre*.[10,11] Briefly, the EtOAc-soluble fraction from the methanolic extract of the aerial parts was chromatographed over Sephadex LH-20 with MeOH to

yield 12 fractions. The fourth fraction was purified by VLC on Si gel 60, and the fraction eluted with CH_2Cl_2−MeOH (9:1) was further rechromatographed on RP-18 to yield **1**. The ninth fraction was chromatographed on Si gel 60, and the fraction eluted with CH_2Cl_2−MeOH (95:5) was purified on RP-18 to yield **2** and **3**. They were identified on the basis of their NMR and mass spectra.

Unless otherwise specified, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), materials for cell culture were purchased from Sarstedt (Nümbrecht, Germany), and media and reagents were supplied by Gibco (Langley, VA). Epigallocatechin gallate and dexamethasone were used as reference compounds.

Cell Culture. Murine macrophages (RAW 264.7) were cultured in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in an incubator. Cells were removed from the tissue culture flask with a cell scraper and cultured in dishes at a density of 2×10^5 cells/mL, and when subconfluent, the medium was substituted by DMEM supplemented with 0.5% FBS. Cells were treated with test compounds (25−100 μM) or vehicle (phosphate-buffered saline, PBS) and 1 h later stimulated with 1 μg/mL of lipopolysaccharide (Sigma-Aldrich). After 24 h, cells were harvested in 100 μL of ice-cold lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, 25 mM Tris−HCl, and EDTA-free protease inhibitor cocktail tablets). The mixture was sonicated (one cycle in 10 s) in a Branson Sonifier 150, and the proteins were measured by means of the Bradford method with BSA as a standard.[8] Finally, immunoblotting with anti-MMP-9 antibody was performed.

Animals. Female BALB/c mice (Harlan Interfauna Ibérica, Sant Feliu de Codines, Spain) were used at 7−9 weeks of age, randomly distributed into groups of eight animals, housed at 22 ± 3 °C in plastic cages under a 12 h light/darkness cycle, and fed with a standard laboratory rodent diet and water ad libitum. The protocol was designed according to the guidelines established by the European Union on Animal Care (Directive 86/609/EEC) and approved by the Animal Ethical Committee of the University of Valencia.

Determination of Contact Hypersensitivity Induced by 2,4,6-Trinitrochlorobenzene. The animals were sensitized by a topical application of 100 μL of 7% TNCB dissolved in acetone−olive oil (4:1) onto their shaved abdomens on day 0. Six days after, the mice were challenged by painting each of their ear surfaces with 10 μL of 1% TNCB in acetone. Test compounds (0.5 mg/ear, 20 μL), dissolved in EtOH−H_2O (8:2), and the standard drug dexamethasone (0.025 mg/ear, 20 μL) in acetone, were applied topically to both ears at 30 min and 24 and 48 h after challenge. Ear thickness was measured with a digital micrometer (Series 293 Mitutoyo, Kawasaki, Japan) 24 and 72 h after challenge. The edema was measured as the difference between the thickness of the naive and the inflamed ears. Inhibition percentages were calculated by subtracting the mean edema value of each group of animals from that of the control group. After measuring ear thickness at 24 and 72 h, four animals from each group were randomly euthanized by cervical dislocation, and then ear samples taken after pinnae excision.[17]

A third of the ear samples was homogenized in a buffer solution (10 mM HEPES pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.8%Triton X-100, 1 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride, and protease inhibitor cocktail from Roche Diagnostics, Mannheim, Germany), sonicated (3 × 10 s), and centrifuged at 14 000 rpm for 15 min at 4 °C. Supernatants were analyzed for protein content with Bradford reagent and frozen at −80 °C. A second third of the ear samples was homogenized in 50 mM phosphate buffer saline containing 0.5% w/v hexadecyl-trimethylammonium bromide for the MPO assay, and then the homogenates were centrifuged for 20 min at 10 300 rpm. Each supernatant was stored at −80 °C until the assay. The final third of the ear samples was homogenized in a buffer solution containing 10 mM HEPES pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenates were kept in ice for 15 min, centrifuged at 5000 rpm for 5 min at 4 °C, and frozen at −80 °C.

Determination of Dermatitis Caused by Repeated TNCB Application. A parallel study was carried out with mice that were sensitized and repeatedly elicited on the same skin site with TNCB, as previously described.[12,22] BALB/c mice were sensitized with 10 μL of 0.3% TNCB dissolved in acetone on day −7 on the inner surface of both ears, and then 10 μL of 0.3% TNCB solution or acetone was repeatedly applied to the inner surface of both ears three times per week, through days 0−21. The ear thickness was measured before each challenge on days 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21. Test compounds (0.5 mg/ear, 20 μL), dissolved in EtOH−H_2O (8:2), and dexamethasone (0.025 mg/ear, 20 μL), dissolved in acetone, were applied topically to both surfaces of both ears three times per week, through days 0−21, 30 min after each challenge. The edema was measured as described above. On day 22, animals were euthanized by cervical dislocation, and then ear samples taken after pinnae excision. Ear samples were divided and homogenized in different buffers, as described above, and the supernatants were stored at −80 °C until the assays were performed.

Assay of Myeloperoxidase Activity in Mouse Ear. To measure MPO activity, the method of De Young et al.[23] was used with modifications as described. Aliquots of 30 μL of the supernatant were added to wells containing 100 μL of Dulbecco's PBS, 85 μL of sodium phosphate buffer (0.22 M; pH 5.4), and 15 μL of 0.017% H_2O_2. The reaction was started by adding 20 μL of 18.4 mM tetramethylbenzidine in 8% aqueous dimethylformamide, and after 3 min at 37 °C, it was stopped with 30 μL of 1.46 M NaOAc in AcOH (pH 3.0) on ice. Enzyme activity was determined colorimetrically using a Labsystem Multiscan MCC/340 plate reader set to measure absorbance at 630 nm.

Western Blot Analysis. Equal amounts of proteins were separated on 8% or 10% SDS-PAGE and transferred onto a nitrocellulose membrane for 90 min at 125 mA. The membrane was subsequently blocked in PBS containing 0.5% BSA, 1% polyvinylpyrrolidone-10, 1% PEG, 0.2% Tween 20, and 10 mM NaF or in PBS-Tween 20 containing 3% defatted milk at room temperature for 2 h. The membranes were incubated with anti-COX-2 (1:2000 dilution) (Cayman, Ann Arbor, MI) and anti-MMP-9 (1:1000 dilution) polyclonal antibodies. The blots were then washed and incubated with peroxidase-conjugate goat anti-rabbit immunoglobulin G (1:12 000 dilution). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, GE Healthcare) with a LAS 3000 mini-system (Fujifilm, Tokyo, Japan). Western blot quantification was carried out with Multi Gauge software (Fujifilm). For β-actin, which was used as an internal control to normalize for differences in protein amounts among different samples, the membranes were incubated with anti-β-actin (1:10 000 dilution). The basal value (blank) was then subtracted from that of each treated sample.

Measurement of Cytokines in Mouse Ear. IL-1β, IL-2, IL-4, and TNF-α (eBioscience, San Diego, CA) and MMP-9 (GE Healthcare) were quantified in the homogenates from the excised ears. Each assay was performed in triplicate according to the manufacturer's instructions.

Statistical Analysis. Data were expressed as means ± SEM. Statistical analysis was performed with a one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test for multiple comparisons. In comparisons with the control group, values of p less than 0.05 were considered to be statistically significant. Inhibition percentages (%*I*) were calculated from the differences between the test compound-treated group and the control group.

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