

## Inducible Nitric Oxide Synthase and Cyclooxygenase-2 Participate in Anti-inflammatory Activity of Imperatorin from *Glehnia littoralis*

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**ABSTRACT:** In this study, we have investigated the anti-inflammatory effects of imperatorin, a compound isolated from the roots of *Glehnia littoralis*, using a lipopolysaccharide (LPS)-stimulated mouse macrophage (RAW264.7) in vitro and a carrageenan (Carr)-induced mouse paw edema model in vivo. When RAW264.7 macrophages were treated with imperatorin together with LPS, a significant concentration-dependent inhibition of NO production was detected. Western blotting revealed that imperatorin blocked the protein expression of iNOS and cyclooxygenase-2 (COX-2) in LPS-stimulated RAW264.7 macrophages significantly. In the anti-inflammatory test, imperatorin decreased the paw edema at 4 and 5 h after Carr administration and increased the activities of catalase, superoxide dismutase, and glutathione peroxidase in paw edema. We also demonstrated that imperatorin significantly attenuated the malondialdehyde level in the edema paw at the fifth hour after Carr injection. Imperatorin decreased the NO and tumor necrosis factor and prostaglandin E2 levels on serum at 5 h after Carr injection. Western blotting revealed that imperatorin decreased Carr-induced iNOS and COX-2 expressions at 5 h in edema paw. An intraperitoneal injection treatment with imperatorin also diminished neutrophil infiltration into sites of inflammation as did indomethacin. The results suggested that imperatorin had anti-inflammatory effects in LPS-stimulated RAW 264.7 cells and Carr-injected mice, respectively. In addition, inhibition of elevated iNOS and COX-2 protein expression as well as neutrophil infiltration of Carr-injected paws may be involved in the beneficial effects of imperatorin.

**KEYWORDS:** Chinese herb, imperatorin, anti-inflammation, NO, TNF- $\alpha$

### INTRODUCTION

The dried roots and rhizomes of *Glehnia littoralis* (Umbelliferae) have been used in traditional oriental medicine as diaphoretic, antipyretic, and analgesic agents in the Taiwan. Many researcher studies reported that *G. littoralis* has antioxidant, antitumor, anti-amnesic, blood circulation-promoting, immunomodulatory, and antimicrobial activities.<sup>1</sup> Quercetin, isoquercetin, rutin, chlorogenic acid, and caffeic acid have been isolated as the major antioxidative constituents in the underground parts of *G. littoralis*.<sup>2</sup> Imperatorin is the main coumarin in the root of *G. littoralis*. Imperatorin is one of the furanocoumarins. Furanocoumarins have biological functions including antidiabetic, anticonvulsant, and vascular vasodilation functions, increased cell differentiation in osteoblasts, and reduction in liver steatosis.<sup>3</sup> Imperatorin has many medical effects such as anticonvulsant, anti-inflammatory, anti-tumor, antibacterial, and anticoagulant activities.<sup>4,5</sup> However, little information is available on the anti-inflammatory effects of imperatorin.

Many studies on plant-derived anti-inflammatory compounds have investigated the potential inhibitory effects of natural products in an in vitro system, lipopolysaccharide (LPS)-stimulated macrophage. Using this system, bacterial LPS has become one of the best-characterized stimuli used to induce the up-regulation of proinflammatory proteins such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Inducible COX-2 could be responsible for the high prostaglandins observed in much inflammatory pathology.<sup>6</sup> Papers also have reported that an inflammatory effect induced by carrageenan (Carr) could be associated with free radical formation.<sup>7</sup> Free radical, prostaglandin, and nitric oxide (NO) will be released when administering with Carr for 1–5 h. The edema effect was raised to the maximum at 3 h, and its malondialdehyde (MDA) production was

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due to free radical attack plasma membrane.<sup>8</sup> Thus, the inflammatory effect would result in the accumulation of MDA. Therefore, in this paper, we examined the anti-inflammatory effects of imperatorin on LPS-induced RAW264.7 cells and Carr-induced paw edema in mice, and we detected the levels of iNOS and COX-2 in either RAW264.7 cell or paw edema. Also, the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the paw edema at 5 h after Carr injection were investigated to understand the relationship between the anti-inflammatory mechanism of the imperatorin and the antioxidant enzymes.

## MATERIALS AND METHODS

**Chemicals.** LPS (endotoxin from *Escherichia coli*, serotype 0127:B8), Carr (type IV), indomethacin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Tumor necrosis factor (TNF- $\alpha$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were purchased from Biosource International Inc. (Camarillo, CA). Anti-iNOS, anti-COX-2, and anti- $\beta$ -actin antibody (Santa Cruz, United States) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, United Kingdom) were obtained as indicated. Poly-(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA).

**Plant Material.** The root of *G. littoralis* was collected in Tai Chung, Taiwan. The plant material was identified by Muh-Tsuen Gun, formerly a technician of the Department of Botany, National Taiwan University. A voucher specimen has been deposited at the Herbarium of the Department of Botany, National Taiwan University, Taipei, Taiwan.

**Isolation and Determination of the Active Compound.** Air-dried pieces of *G. littoralis* root (12 kg) were extracted three times with acetone (12 L) at room temperature (7 days each time). The acetone extract was evaporated in vacuo to leave a black residue, which was suspended in H<sub>2</sub>O and then partitioned (three times) with 11 L of ethyl acetate (EtOAc). The EtOAc fraction (360 g) was chromatographed on silica gel using *n*-hexane and EtOAc of increasing polarity as an eluent and further purified by high-performance liquid chromatography eluting with *n*-hexane:EtOAc (25:75). Imperatorin (Figure 1A) was eluted with 10% EtOAc in hexane and recrystallized with EtOH.<sup>9</sup>

Imperatorin: C<sub>16</sub>H<sub>14</sub>O<sub>4</sub>, mp 97.5–99 °C. [M] + *m/z* 270.0897. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz): 1.69, 1.71 (each 3H, s), 4.98 (2H, d, *J* = 7.2 Hz), 5.59 (1H, t, *J* = 7.2 Hz), 6.33 (1H, d, *J* = 9.5 Hz, H-3), 6.79 (1H, d, *J* = 2.1 Hz, H-3'), 7.33 (1H, s, H-5), 7.67 (1H, d, *J* = 2.1 Hz, H-2'), 7.73 (1H, d, *J* = 9.5 Hz, H-4). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz): 18.0 (q), 25.7 (q), 70.1 (t), 106.6 (d), 113.1 (d), 114.6 (d), 116.4 (s), 119.7 (d), 125.8 (s), 131.6 (s), 139.6 (s), 143.8 (s), 144.3 (d), 146.6 (d), 148.6 (s), 160.6 (s). UV  $\lambda_{\max}$  ( $\epsilon$ ) nm: 218 (22800), 249 (20600), 301 (10600). IR (KBr) cm<sup>-1</sup>: 1722, 1708 1589, 1150, 838.

**Animals.** Imprinting control region (ICR; 6–8 weeks, male) mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ± 1 °C and relative humidity of 55 ± 5% with 12 h dark–light cycle for at least 2 weeks before the experiment. They were given food and water ad libitum. All experimental procedures were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. In addition, all tests were conducted under the guidelines of the International Association for the Study of Pain.<sup>10</sup>

After a 2 week adaptation period, male ICR mice (18–25 g) were randomly assigned to four groups (*n* = 6) of the animals in the study. The control group received normal saline (intraperitoneal; ip). The other three groups include a Carr-treated, a positive control (Carr + Indo), and imperatorin-administered groups (Carr + imperatorin).

**Cell Culture.** A murine macrophage cell line RAW264.7 (BCRC no. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's modified Eagle's medium (DMEM, Sigma)

supplemented with 10% fetal bovine serum (FBS, Sigma) in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> in air) at 37 °C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free phosphate-buffered saline (DPBS).

**Cell Viability.** Cells (2 × 10<sup>5</sup>) were cultured in a 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then, cells were cultured with imperatorin in the presence of 100 ng/mL LPS for 24 h. After that, the cells were washed twice with DPBS and incubated with 100  $\mu$ L of 0.5 mg/mL MTT for 2 h at 37 °C testing for cell viability. The medium was then discarded, and 100  $\mu$ L of dimethyl sulfoxide (DMSO) was added. After 30 min of incubation, the absorbance at 570 nm was read using a microplate reader (Molecular Devices, Sunnyvale, CA).

**Measurement of NO/Nitrite.** NO production was indirectly assessed by measuring the nitrite levels in the cultured media, and serum was determined by a colorimetric method based on the Griess reaction.<sup>11</sup> The cells were incubated with imperatorin (0, 1, 5, and 10  $\mu$ g/mL) in the presence of LPS (100 ng/mL) at 37 °C for 24 h. Then, cells were dispensed into 96-well plates, and 100  $\mu$ L of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at room temperature for 10 min, and the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices). Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10000g for 5 min at room temperature, 100  $\mu$ L of supernatant was applied to a microtiter plate well, followed by 100  $\mu$ L of Griess reagent. After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured for absorbance at 540 nm.

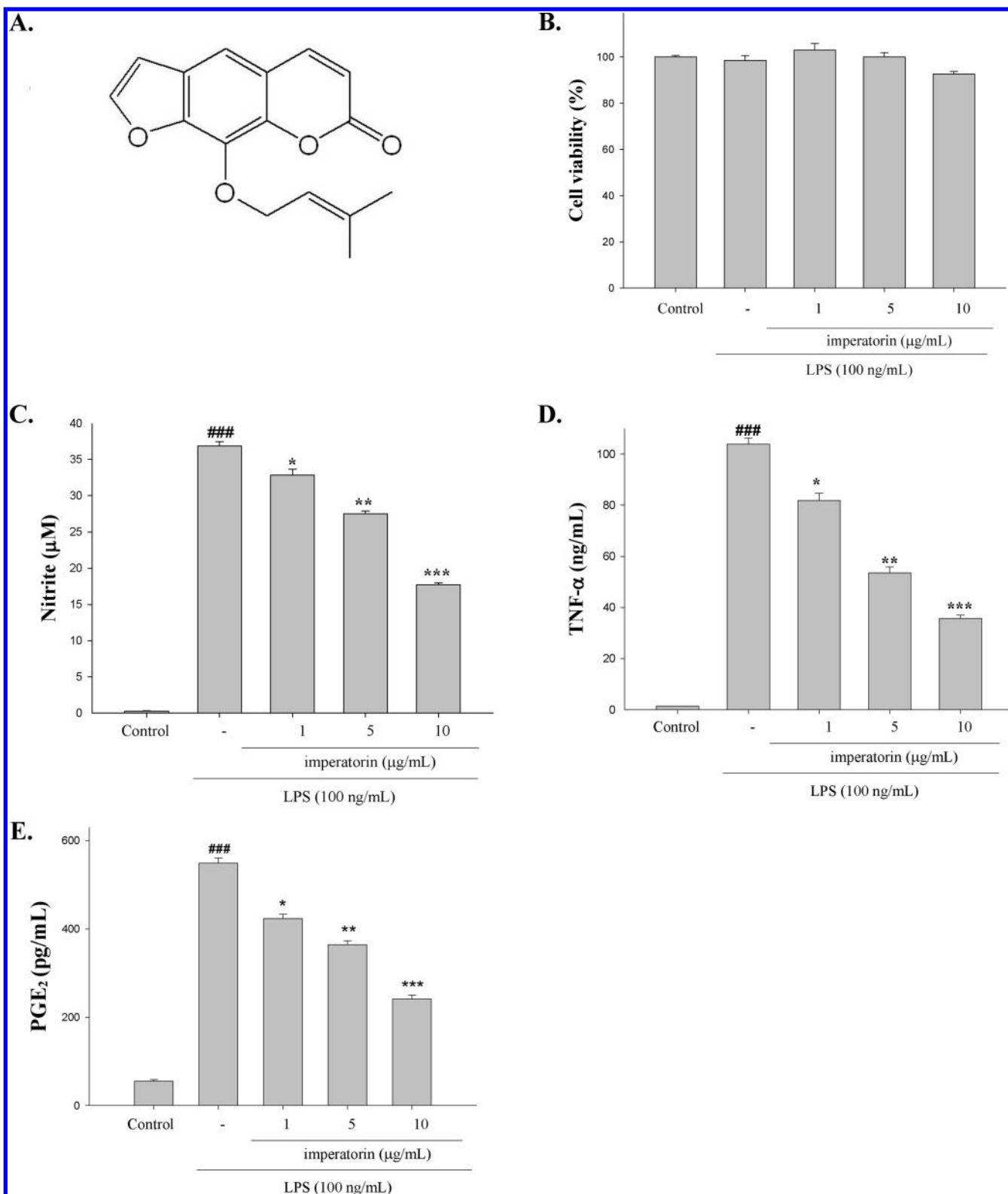
**Carr-Induced Edema.** The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity.<sup>12</sup> Animals were ip treated with imperatorin (1, 5, and 10 mg/kg) (dissolved in 0.5% carboxymethylcellulose), Indo (10 mg/kg), or normal saline, 30 min prior to injection of 1% Carr (50  $\mu$ L) in the plantar side of right hind paws of the mice. The paw volume was measured after Carr injection and at 1, 2, 3, 4, and 5 h intervals after the administration of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio *a/b*, where *a* was the volume of the right hind paw after Carr treatment, and *b* was the volume of the right hind paw before Carr treatment. Indo was used as a positive control. After 5 h, the animals were sacrificed, and the Carr-induced edema feet were dissected and stored at –80 °C. Also, blood was withdrawn and kept at –80 °C.

The right hind paw tissue was taken at 5 h. The right hind paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline four times their volume and homogenized at 4 °C. Then, the homogenate was centrifuged at 12000g for 5 min. The supernatant was obtained and stored at –20 °C for MDA assays. The paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline one time their volume and homogenized at 4 °C. Then, the homogenate was centrifuged at 12000g for 5 min. The supernatant was obtained and stored at –20 °C for the antioxidant enzyme (CAT, SOD, and GPx) activity assays. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

**MDA Assay.** MDA from Carr-induced edema foot was evaluated by the thiobarbituric acid reacting substances (TBARS) method.<sup>13</sup> Briefly, MDA was reacted with thiobarbituric acid in the acidic medium at a high temperature and formed a red complex TBARS. The absorbance of TBARS was determined at 532 nm.

**Measurement of TNF- $\alpha$  and PGE<sub>2</sub> by an Enzyme-Linked Immunosorbent Assay (ELISA).** The levels of TNF- $\alpha$  and PGE<sub>2</sub> were determined using a commercially available ELISA kit (Biosource International Inc.) according to the manufacturer's instruction. TNF- $\alpha$  and PGE<sub>2</sub> were determined from a standard curve.

**Determination of Antioxidant Enzyme Activity in Paw Tissue.** The following biochemical parameters were analyzed to check



**Figure 1.** Chemical structure of imperatorin (A) and the effects of imperatorin on LPS-induced cell viability (B), NO production (C), TNF- $\alpha$  (D), and PGE<sub>2</sub> (E) in LPS-stimulated RAW264.7 cells. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or presence of imperatorin (0, 1, 5, and 10  $\mu\text{g/mL}$ ). Imperatorin was added 1 h before incubation with LPS. The cell viability assay was performed using MTT assay. The nitrite concentration in the medium was determined using Griess reagent. TNF- $\alpha$  and PGE<sub>2</sub> concentrations in the medium were determined using an ELISA kit. The data were presented as means  $\pm$  SDs for three different experiments performed in triplicate. ### $p < 0.001$  control group as compared to LPS-treated group. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  were compared with the LPS-alone group.

the protective activity of imperatorin in the paw tissues by the methods given below. The total SOD activity was determined by the inhibition of

cytochrome *c* reduction.<sup>14</sup> The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system

and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%. The total CAT activity was based on that of Aebi.<sup>15</sup> In brief, the reduction of 10 mM H<sub>2</sub>O<sub>2</sub> in 20 mM phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activities were defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity in cytosol was determined according to Paglia and Valentine's method.<sup>16</sup> The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2), and the absorbance at 340 nm was measured. The activity was calculated by using a calibration curve of GPx established from bovine whole blood. A linear relationship between the activity (unit/mL) of GPx and the reduction of NADPH absorbance at 340 nm was found, and the enzyme activities were defined as nanomoles of NADPH oxidized per milligram protein per minute.

**Protein Lysate Preparation and Western Blot Analysis of iNOS and COX-2.** The stimulated murine macrophage cell line RAW264.7 cells were washed with PBS and lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, 10 mM NaF, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 20 mM Tris buffer (pH 7.9), 100 mM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and one protease inhibitor cocktail tablet (Roche, Indianapolis, IN)] on ice for 1 h, followed by centrifugation at 12000g for 30 min at 4 °C. Soft tissues were removed from individual mice paws and homogenized in a solution containing 10 mM CHAPS [3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, 1 μM pepstatin, and 10 μM leupeptin. The homogenates were centrifuged at 12000g for 20 min, and 30 μg of protein from the supernatants was then separated on 10% sodium dodecylsulphate–polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. After transfer, the membrane was blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline-Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, and 0.1% Tween 20). The membranes were then incubated with mouse monoclonal anti-iNOS or anti-COX-2 (1:1000 dilution) antibody in 5% skim milk in TBST for 2 h at room temperature. The membranes were washed three times with TBST at room temperature and then incubated with a 1:2000 dilution of antimouse IgG secondary antibody conjugated to horseradish peroxidase (Sigma) in 2.5% skim milk in TBST for 1 h at room temperature. The membranes were washed three times, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc, Buckinghamshire, United Kingdom). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging Software (Version 4.0.5, Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.

**Histological Examination.** For histological examination, biopsies of paws were taken 5 h following the interplanetary injection of Carr. The tissue slices were fixed in a solution (1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol, and embedded in paraffin (Sherwood Medical). Sections (thickness 5 μm) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) stain. All samples were observed and photographed with BH-2 Olympus microscopy. Every 3–5 tissue slices were randomly chosen from Carr, Indo, and imperatorin-treated (10 mg/kg) groups. Histological examination of these tissue slices revealed an excessive inflammatory response with massive infiltration of neutrophils [polymorphonuclear leukocytes (PMNs)] by microscopy. The numbers of neutrophils were counted in each scope (400×), and thereafter, we obtained their average count from five scopes of every tissue slice.<sup>6</sup>

**Statistical Analysis.** Experimental results were presented as the mean ± standard deviation (SD) of three parallel measurements. IC<sub>50</sub> values were estimated using a nonlinear regression algorithm (SigmaPlot 8.0; SPSS Inc. Chicago, IL). Data obtained from animal experiments were expressed as mean standard error (±SEM). Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests). Statistical significance is expressed as \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001.

## RESULTS

**Cell Viability and Effect of Imperatorin on LPS-Induced NO Production in Macrophages.** The effect of imperatorin on RAW264.7 cell viability was determined by a MTT assay. Cells cultured with imperatorin at the concentrations (0, 1, 5, and 10 μg/mL) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability (Figure 1B).

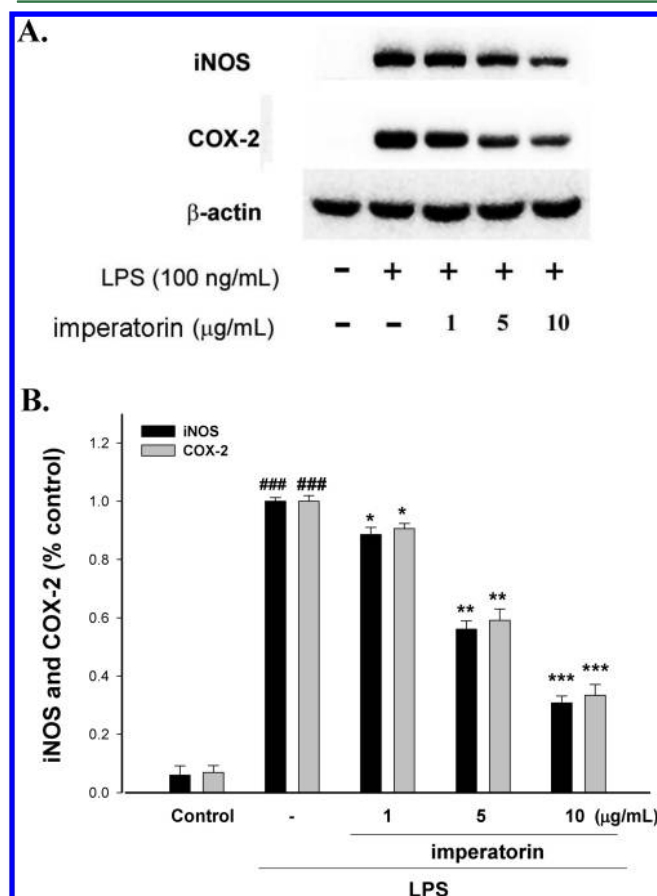
The RAW264.7 cell line is a mouse macrophage cell line used to model macrophage-mediated inflammatory events *in vitro*, and RAW264.7 cells were used to assess the effect of imperatorin on NO synthesis. High levels of NO produced by iNOS have been defined as a cytotoxic molecule in inflammation.<sup>17</sup> Imperatorin did not interfere with the reaction between nitrite and Griess reagents at 10 μg/mL (data not shown). Unstimulated macrophages, after 24 h of incubation in culture medium, produced background levels of nitrite. When RAW264.7 macrophages were treated with different concentrations of imperatorin (1, 5, and 10 μg/mL) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of nitrite production was detected. There was either a significant decrease in the nitrite production of group treated with 1 μg/mL imperatorin (*p* < 0.05) or a highly significant decrease of groups treated, respectively, with 5 and 10 μg/mL of imperatorin when compared with the LPS-alone group (*p* < 0.01 or *p* < 0.001). The IC<sub>50</sub> value for inhibition of nitrite production of imperatorin was about 9.59 ± 0.23 μg/mL (Figure 1C).

TNF-α mediates the production of many other cytokines during inflammation, in particular the production of interleukin-1β (IL-1β) and interleukin-6 (IL-6).<sup>18</sup> We examined the effect of imperatorin on LPS induced up-regulation of TNF-α. A very low amount of TNF-α protein was detected by a specific ELISA for TNF-α in controls (Figure 1D). When RAW264.7 macrophages were treated with different concentrations of imperatorin (1, 5, and 10 μg/mL) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of TNF-α production was detected. There was either a significant decrease in the TNF-α production of group treated with 1 μg/mL imperatorin (*p* < 0.05) or a highly significant decrease of groups treated, respectively, with 5 and 10 μg/mL of imperatorin when compared with the LPS-alone group (*p* < 0.01 or *p* < 0.001). The IC<sub>50</sub> value for inhibition of TNF-α production of imperatorin was about 5.32 ± 0.12 μg/mL (Figure 1D).

PGE<sub>2</sub> represents the most important inflammatory product of COX-2 activity, and it was quantified in cell-free culture supernatant.<sup>18</sup> As shown in Figure 1E, cells were stimulated with LPS alone raise significant amount of PGE<sub>2</sub> in RAW264.7 macrophages. When RAW264.7 macrophages were treated with different concentrations of imperatorin (1, 5, and 10 μg/mL) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of PGE<sub>2</sub> production was detected. The IC<sub>50</sub> value for inhibition of PGE<sub>2</sub> production of imperatorin was about 9.46 ± 0.37 μg/mL.

**Inhibition of LPS-Induced iNOS and COX-2 Protein by Imperatorin.** To investigate whether the inhibition of NO production was due to a decreased iNOS and COX-2 protein level, the effect of imperatorin on iNOS and COX-2 protein expression was studied by immunoblot. The results showed that incubation with imperatorin (0, 1, 5, and 10 μg/mL) in the presence of LPS (100 ng/mL) for 24 h inhibited iNOS and COX-2 proteins expression in mouse macrophage RAW264.7 cells in a dose-dependent manner (Figure 2A). The detection of β-actin was also performed in the same blot as an internal control.

The intensity of protein bands was analyzed using Kodak Quantity software in three independent experiments and showed an average of 69.2 and 66.7% down-regulation of iNOS and COX-2 proteins, respectively, after treatment with imperatorin at 10  $\mu\text{g/mL}$  as compared with the LPS-alone (Figure 2B).



**Figure 2.** Inhibition of iNOS and COX-2 protein expression by imperatorin in LPS-stimulated RAW264.7 cells. Cells were incubated for 24 h with 100 ng/mL of LPS in the absence or the presence of imperatorin (0, 1, 5, and 10  $\mu\text{g/mL}$ ). Imperatorin was added 1 h before incubation with LPS. Lysed cells were then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2.  $\beta$ -Actin was used as an internal control. A representative Western blot from two separated experiments is shown. Relative iNOS and COX-2 protein levels were calculated with reference to a LPS-stimulated culture. The data were presented as means  $\pm$  SDs for three different experiments performed in triplicate. <sup>###</sup> $p < 0.001$  control group as compared to the LPS-treated group. <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ , and <sup>\*\*\*</sup> $p < 0.001$  were compared with the LPS-alone group.

**Effects of Imperatorin on Carr-Induced Mice Paw Edema.** Because imperatorin effectively inhibited iNOS and COX-2 inductions in macrophages, studies were extended to determine whether imperatorin affected acute phase inflammation in mice models. In this study, we used Carr-induced edema because this model is widely employed for screening the effects of anti-inflammatory drugs. Carr-induced paw edema is shown in Figure 3A. Imperatorin (10 mg/kg) inhibited ( $p < 0.001$ ) the development of paw edema induced by Carr after 4 and 5 h of treatment, significantly. Indo (10 mg/kg) significantly decreased the Carr-induced paw edema after 4 and 5 h of treatment ( $p < 0.001$ ). On the contrary, imperatorin showed

a good dose–response activity. Both imperatorin and Indo show a good reduction of mice paw edema  $54.4 \pm 0.8$  and  $47.9 \pm 0.5$  inhibition percentage of volume mice paw at the concentration of 10 mg/kg at 5 h as compared to the control, respectively.

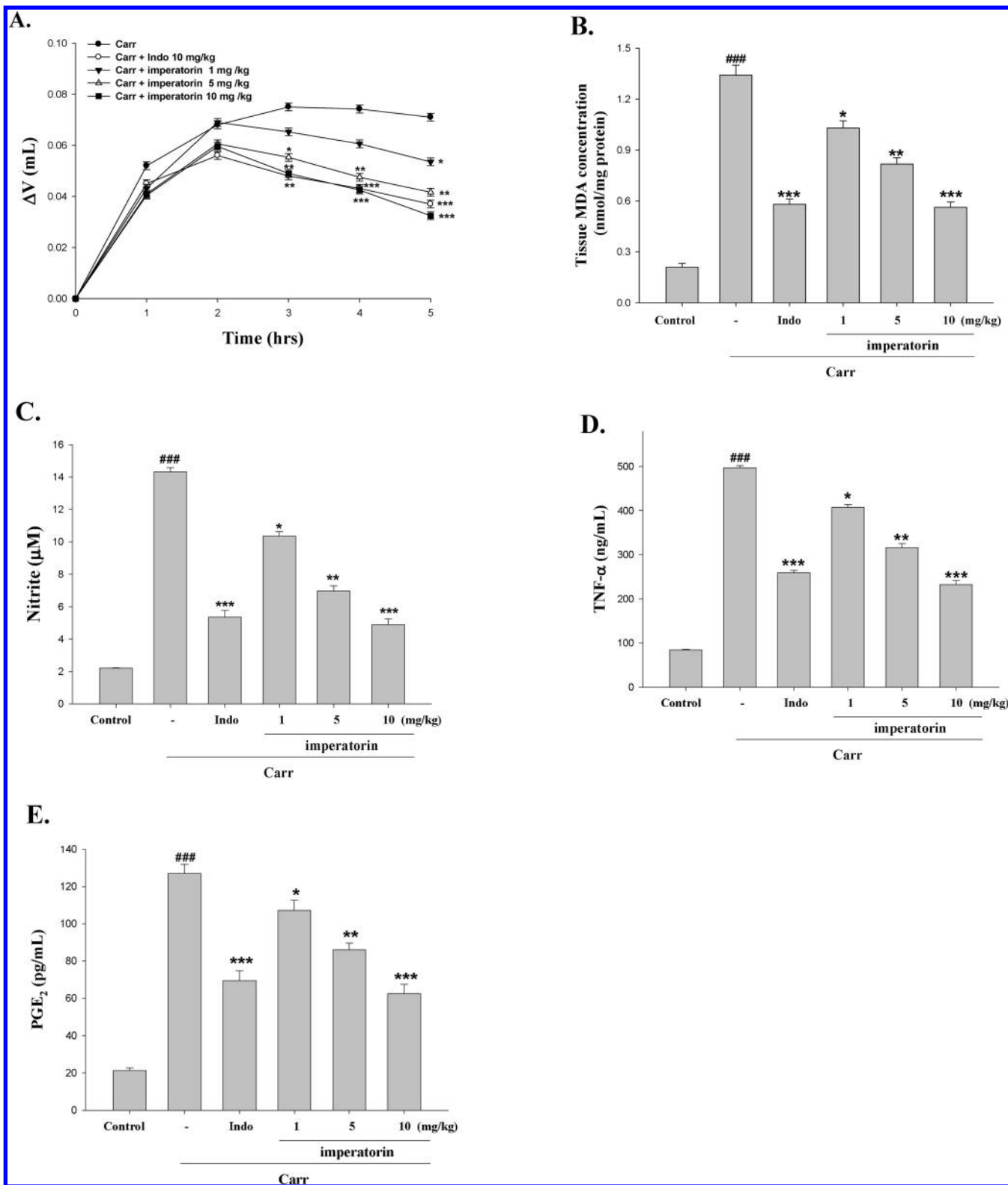
**Effects of Imperatorin on the MDA Level in the Paw Edema.** The MDA level increased significantly in the edema paw at 5 h after Carr injection ( $p < 0.001$ ). However, the MDA level was decreased significantly by treatment with imperatorin (10 mg/kg) ( $p < 0.001$ ) as well as 10 mg/kg Indo (Figure 3B).

**Effects of Imperatorin on the NO, TNF- $\alpha$ , and PGE<sub>2</sub> Levels.** In Figure 3C, the NO level increased significantly in the edema serum at 5 h post-Carr injection ( $p < 0.001$ ). Imperatorin (10 mg/kg) significantly decreased the serum NO level ( $p < 0.001$ ). The inhibitory potency was similar to that of Indo (10 mg/kg) at 5 h after induction. TNF- $\alpha$  and PGE<sub>2</sub> levels increased significantly in serum at 5 h post-Carr injection ( $p < 0.001$ ). However, imperatorin (5 or 10 mg/kg) decreased the TNF- $\alpha$  and PGE<sub>2</sub> levels in serum at 5 h after Carr injection ( $p < 0.01$  or  $p < 0.001$ ) as well as 10 mg/kg Indo (Figure 3D,E).

**Effects of Imperatorin on Activities of Antioxidant Enzymes.** At 5 h after the intrapaw injection of Carr, paw tissues were also analyzed for the biochemical parameters such as CAT, SOD, and GPx activities. CAT, SOD, and GPx activities in paw tissue were decreased significantly by Carr administration. CAT, SOD, and GPx activities were increased significantly after treated with 10 mg/kg imperatorin and 10 mg/kg Indo ( $P < 0.001$ ) (Table 1).

**Effects of Imperatorin on Carr-Induced iNOS and COX-2 Protein Expressions in Mice Paw Edema.** To investigate whether the inhibition of NO production was due to a decreased iNOS and COX-2 protein level, the effect of imperatorin on iNOS and COX-2 proteins expression was studied by Western blot. The results showed that injection of imperatorin (10 mg/kg) on Carr-induced for 5 h inhibited iNOS and COX-2 proteins expression in mouse paw edema (Figure 4A). The intensity of protein bands was analyzed using Kodak Quantity software in three independent experiments and showed an average of 79.8 and 70.3% down-regulation of iNOS and COX-2 protein, respectively, after treatment with imperatorin at 10 mg/kg as compared with the Carr-induced alone (Figure 4B). In addition, the protein expression showed an average of 62.1 and 63.2% down-regulation of iNOS and COX-2 protein after treatment with Indo at 10.0 mg/kg as compared with the Carr-induced alone. The down-regulation of iNOS and COX-2 activity of the imperatorin (10 mg/kg) was better than Indo (10.0 mg/kg).

**Histological Examination.** Paw biopsies of Carr model animals showed marked cellular infiltration in the connective tissue. The infiltrates accumulated between collagen fibers and into intercellular spaces. Paw biopsies of animals treated with imperatorin (10 mg/kg) showed a reduction in Carr-induced inflammatory response. Actually, inflammatory cells were reduced in numbers and were confined to near the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and showed a reduction of intercellular spaces. Moreover, the hypodermal connective tissue was not damaged (Figure 5A). Neutrophil levels were significantly increased with Carr treatment ( $P < 0.01$ ). Indo (10.0 mg/kg) and imperatorin (10 mg/kg) could decrease the neutrophils numbers as compared to the Carr-treated group ( $P < 0.001$ ) (Figure 5B), significantly.



**Figure 3.** Effects of imperatorin and Indo on hind paw edema induced by Carr in mice (A), the tissue MDA concentration of foot in mice (B), Carr-induced NO (C), TNF- $\alpha$  (D), and PGE<sub>2</sub> (E) concentrations of serum at 5 h in mice. Each value represents a mean  $\pm$  SEM. ### $p$  < 0.001 as compared with the control group. \* $p$  < 0.05, \*\* $p$  < 0.01, and \*\*\* $p$  < 0.001 as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

## DISCUSSION

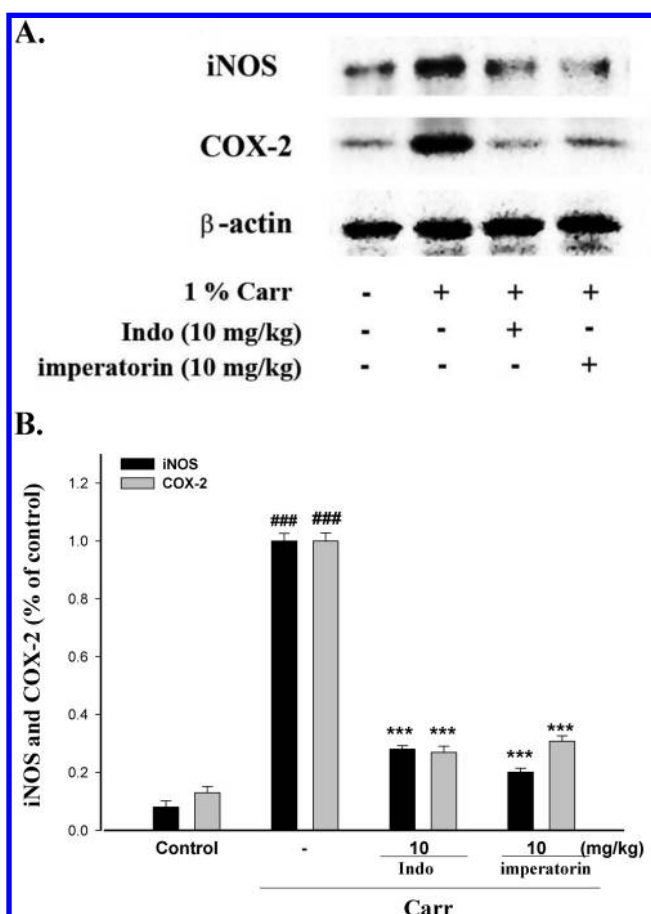
In the present study, we demonstrated anti-inflammatory activities of imperatorin in in vitro and in vivo experimental systems, using LPS-stimulated RAW264.7 macrophages and a

mouse model of topical inflammation, respectively. The inhibitory activities against iNOS and COX-2 as shown in in vitro assays appear to confer on imperatorin, a potent in vivo efficacy in mouse, suggesting its potential therapeutic usage as

**Table 1.** Effects of Imperatorin and Indomethacin (Indo) on Changes in CAT, SOD, and GPx Activities Were Studied on Carr-Induced Mice Paw Edema (5 h)<sup>a</sup>

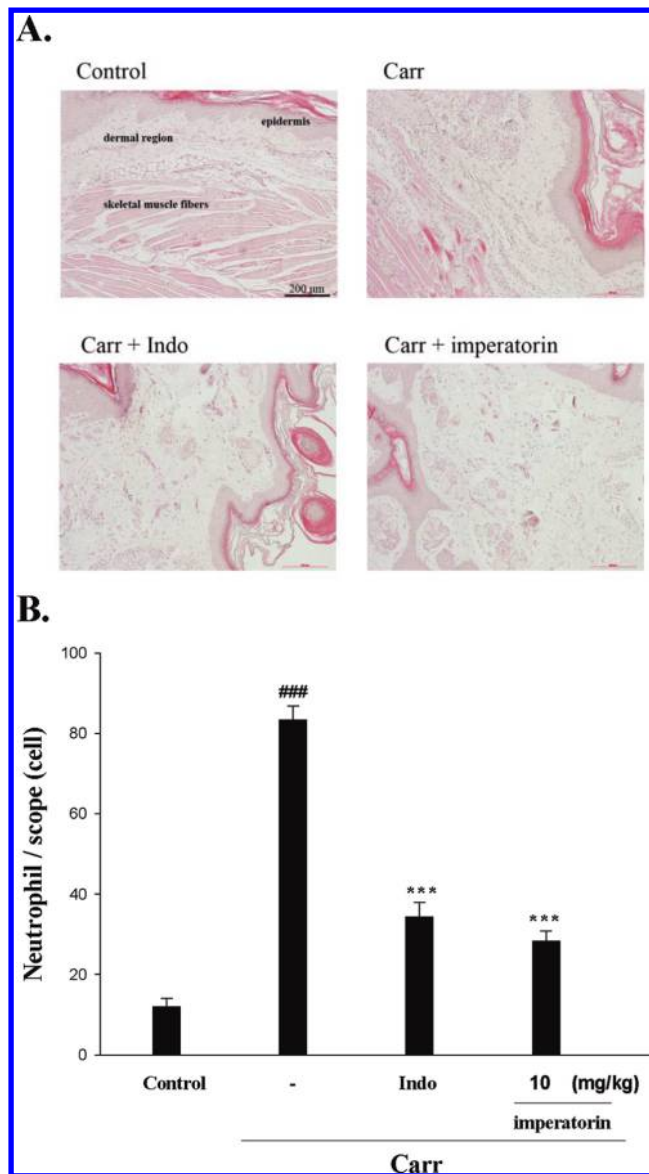
groups	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
control	5.28 ± 0.22	24.38 ± 0.31	23.42 ± 0.13
Carr	3.58 ± 0.31	15.64 ± 0.28	15.86 ± 0.19
Carr + Indo	4.73 ± 0.23**	22.53 ± 0.58**	21.52 ± 0.21**
Carr + imperatorin (1 mg/kg)	3.83 ± 0.28*	17.23 ± 0.45*	16.98 ± 0.26
Carr + imperatorin (5 mg/kg)	4.37 ± 0.24**	19.82 ± 0.35**	18.57 ± 0.39*
Carr + imperatorin (10 mg/kg)	4.98 ± 0.48***	22.93 ± 0.63**	21.83 ± 0.24**

<sup>a</sup>Each value represents a mean ± SEM. \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).



**Figure 4.** Inhibition of iNOS and COX-2 (A) protein expression by imperatorin induced by Carr in the foot at 5 h in mice. Suspended tissue were then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2.  $\beta$ -Actin was used as an internal control. A representative Western blot from two separated experiments is shown. Relative iNOS and COX-2 (B) protein levels were calculated with reference to a Carr-injected mouse. The data were presented as means ± SDs for three different experiments performed in triplicate. ###*p* < 0.001 as compared with the control group. \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

an anti-inflammatory source of health food. LPS is a prototypical endotoxin derived from Gram-negative bacterial membrane and is the initial stimulus leading to induction of



**Figure 5.** Representative light micrographs of mouse hind footpad H&E stained to reveal hemorrhage, edema, and inflammatory cell infiltration in (A) control mice, (B) Carr-treated mice demonstrating hemorrhage with moderately extravascular red blood cells and a large amount of inflammatory leukocyte mainly neutrophils infiltration in the subdermis interstitial tissue of mice, and (C) mice given Indo (10 mg/kg) before Carr. Imperatorin (10 mg/kg) significantly shows (D) morphological alterations (100 $\times$ ) and (E) the numbers of neutrophils in each scope (400 $\times$ ) as compared to subcutaneous injection of Carr only. ###*p* < 0.001 control group as compared to the Carr group. \*\*\**p* < 0.001 as compared with the Carr group. Scale bar = 200  $\mu$ m.

septic shock syndrome. LPS can directly activate macrophages, endothelial cells, and the complement-triggering production of inflammatory mediators, such as NO, TNF- $\alpha$ , interleukins, and leukotrienes.<sup>19</sup> However, few reports have been issued on the anti-inflammatory effect of imperatorin and the mode of action involved. Thus, this study aimed to evaluate the anti-inflammatory effect of imperatorin by screening the effects of imperatorin on LPS-induced pro-inflammatory molecules in vitro and on acute phase inflammation in vivo.

All furanocoumarins, including byakangelicol, oxypeucedanin, and isoimperatorin, had no effects on the inhibition of NO production.<sup>20</sup>

Our study was performed using equally diluted fractions in these conditions; the results indicate that imperatorin had effects on the inhibition of NO production, and imperatorin and phellopterin rather than byakangelicol, oxypeucedanin, and isoimperatorin were the two major compounds responsible for the anti-inflammatory activity in the *Angelica dahurica*.<sup>21</sup> The anti-inflammatory properties of methylene chloride fraction from *G. littoralis* extract (MCF-GLE) may result from the inhibition of pro-inflammatory mediators, such as NO, PGE<sub>2</sub>, TNF- $\alpha$ , and IL-1 $\beta$ , via suppression of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinase-dependent pathways.<sup>22</sup>

Excessive production of NO plays a critical role in the aggravation of circulatory shock and chronic inflammatory diseases, such as septic shock, inflammatory hepatic dysfunctions, inflammatory lung disease, and colitis.<sup>23</sup> As many of these conditions exhibit rapid onset and development, often resulting in the failure of conventional anti-inflammatory therapies and extremely high mortality rates, a simultaneous suppression of NO production pathways, as shown by imperatorin, may satisfy the so far unmet need for control of the rapid progression of the inflammatory process. In vitro models such as macrophage cells or other cell lines are useful materials with a steady high-level production of NO. The mechanisms by which imperatorin inhibits macrophage functions have not been elucidated. Results in vitro showed that imperatorin suppressed LPS-induced production of NO (Figure 1C), the expression of inflammatory protein products such as iNOS and COX-2 (Figure 2A). Examination of the cytotoxicity of imperatorin in RAW264.7 macrophages using MTT assay has indicated that imperatorin even at 10  $\mu$ g/mL did not affect the viability of RAW264.7 cells. Therefore, inhibition of LPS-induced nitrite production by imperatorin was not the result of a possible cytotoxic effect on these cells.

Excess amounts of NO and PGE<sub>2</sub> play a critical role in the aggravation of chronic inflammatory diseases, such as hepatic dysfunction and pulmonary disease. Recently, mounting evidence that in vitro and in vivo have indicated existing cross-talk between the release of NO and prostaglandins (PGs) in the modulation of molecular mechanisms that regulate PGs generating pathway.<sup>24</sup> Scientific papers observed that while the production of both nitrite and PGE<sub>2</sub> was blocked by the NOS inhibitors in mouse macrophages RAW264.7 cells, these inhibitory effects were reversed by coinubation with the precursor of NO synthesis, L-arginine. Furthermore, inhibition of iNOS activity by nonselective NOS inhibitors attenuated the release of NO and PGs simultaneously in LPS-activated macrophages, suggesting that endogenously released NO from macrophages exerted a stimulatory action on enhancing the PGs production. Conversely, it has been shown that COX activation in turn modulates the L-arginine-NO pathway, whereas COX inhibition decreases NOS activity in human platelets.<sup>25</sup> These results are indicative of the cross-talk between NO and PGs pathways.

Carr-induced paw edema is a well-established model of edema formation, which is commonly used for the screening of anti-inflammatory drugs. The intraplantar injection of Carr induces inflammatory responses, including increases in paw volume (edema) and neutrophil infiltration.<sup>26</sup> Recent studies have shown that Carr induced peripheral release of NO as well as that of PGE<sub>2</sub>.<sup>25</sup> NO plays a major role in edema formation in inflammatory responses and tissue injury, and Carr induced the release of TNF- $\alpha$ , which subsequently promotes IL-1 and IL-6 production in the tissue.<sup>27</sup> The degree of swelling of the Carr-injected paws

was maximal 3 h after injection. Statistical analysis revealed that imperatorin and Indo significantly inhibited the development of edema 5 h after treatment ( $p < 0.001$ ) (Figure 3A). The third phase of the edema-induced by Carr, where the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction.<sup>28</sup> It was found that the injection of Carr into the mouse paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates.<sup>29</sup> Our Carr-induced rat paw edema model enabled us to demonstrate the ability of imperatorin to inhibit edema induced by acute inflammation. These results in conjunction with the marked inhibition of LPS-induced NO and TNF- $\alpha$  productions by imperatorin in macrophages imply that the antiedema effects of imperatorin might result from its inhibition of NO and TNF- $\alpha$  syntheses in the peripheral tissues (Figure 3C,D).

The proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  are small secreted proteins, which mediate and regulate immunity and inflammation. The production of TNF- $\alpha$  is crucial for the synergistic induction of NO synthesis in interferon- $\gamma$  (IFN- $\gamma$ ) and/or LPS-stimulated macrophages. TNF- $\alpha$  induces a number of physiological effects including septic shock, inflammation, and cytotoxicity.<sup>30</sup> Also, TNF- $\alpha$  is a mediator of Carr-induced inflammatory incapacitation and is able to induce the further release of kinins and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting nociceptive response.<sup>31</sup> In this study, we found that imperatorin decreased the TNF- $\alpha$  level in LPS-stimulated macrophages or after Carr injection.

The Carr-induced inflammatory response has been linked to neutrophils infiltration and the production of neutrophils-derived free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radicals, as well as the release of other neutrophils-derived mediators.<sup>32</sup> Researchers demonstrated that the inflammatory effect induced by Carr is associated with free radical. Free radical, prostaglandin, and NO will be released when administering with Carr for 1–6 h. MDA production is due to free radical attack plasma membrane. Thus, the inflammatory effect would result in the accumulation of MDA.<sup>11</sup> Glutathione (GSH) acts as a scavenger by scavenging NO and other oxidants. The increased GSH level may favor reduction in MDA production. GSH plays an important role against Carr-induced local inflammation.<sup>32</sup> In this study, there was significantly increased in CAT, SOD, and GPx activities with imperatorin treatment (Table 1). Furthermore, there were significant decreases in the MDA level with imperatorin treatment (Figure 3B). We assume that the suppression of MDA production is probably due to the increases of CAT, SOD, and GPx activities.

In conclusion, these results suggested that imperatorin possessed anti-inflammatory effects. The anti-inflammatory mechanism of imperatorin may be related to iNOS and COX-2, and it is associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). Imperatorin may be used as a pharmacological agent in the prevention or treatment of disease in which free radical formation is a pathogenic factor.

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