Sclareol exhibits antiinflammatory activity in both lipopolysaccharide-stimulated macrophages and the λ-carrageenan-induced paw edema model

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Sclareol is a natural fragrance compound and being widely used in the cosmetic and food industries. The lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and the λ-carrageenan (Carr)-induced edema paw models were applied to examine the antiinflammatory property of Sclareol (**1**) and its possible molecular mechanisms. Experimental results demonstrated that **1** inhibited cell growth, nitric oxide (NO) production, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins expression in LPS-stimulated macrophages. Compound **1** also reduced paw edema, tissue content of NO, tumor necrosis factor-alpha (TNF-α) and malondialdehyde (MDA), iNOS and COX-2 proteins expression, and neutrophil infiltration within the tissues by Carr stimulation. The present study suggested that the antiinflammatory mechanisms of **1** might be related to the decrease of the inflammatory cytokine and the increase of antioxidant enzymes activity, which will subsequently reduce inflammatory responses.

The acute inflammatory response is a series of local cellular and vascular responses that occurs immediately following tissue damage, and this complex biological response is a protective mechanism of organisms to remove the injurious stimuli, such as pathogens, irritants or physical injury, from the tissues and to initiate the healing process. However, chronic inflammation has been reported to involve in the development of several diseased conditions or disorders such as Alzheimer disease,[1] asthma,[2] atherosclerosis,[3] autoimmune diseases,[4] cancers[5] and rheumatoid arthritis,[6] which may lead to progressive destruction of the tissue, fibrosis, and necrosis.[7, 8]

Numerous molecules have been mentioned to contribute the local tissue destruction during chronic inflammation.[9-11] Of these, inducible nitric oxide synthase (iNOS), a member of the NOS protein family, catalyzes the formation of nitric oxide (NO) from L-arginine.[12] NO can activate guanylate cyclase to induce smooth muscle relaxation in the normal physiological condition. High-output NO produced by the activated macrophage via iNOS has been found to play a major role as antimicrobial molecule.[13] However, highly level of NO have the opportunity to react with superoxide resulting in peroxynitrite formation and cell toxicity, which are found to play important roles in inflammation and carcinogenesis. The expression of COX-2 (cyclooxygenase 2) has also been mentioned to implicate the response for the prostaglandin biosynthesis involved in inflammation and pain, and clinical application of highly selective inhibitors of COX-2 has been demonstrated to provide effective antiinflammatory activity with marked reduction in

gastrointestinal toxicity as compared to traditional NSAIDs (non-steroidal antiinflammatory drugs).[14] Similarly, TNF-α (tumor necrosis factor-alpha), an endotoxin-induced glycoprotein, is a critical modulator of host immune response to infection, but inappropriate or excessive production can be harmful. Receiving anti-TNF-α antibody and oral administration of soluble TNF receptors have been demonstrated to control the inflammatory conditions.[11]

Compound **1** (labd-14-ene-8,13-diol) belongs to the member of labdane type diterpenes and first isolated from the plant *Salvia sclarea* L.(Lamiaceae), whose natural fragrance ingredient was widely applied in both cosmetic and food industries. Several studies demonstrated that **1** has been identified as a biologically active molecule to provide the cytotoxic or cytostatic effects against numerous human cancer cell lines.[15-18] Noori *et al.* noticed that **1** modulated the immune response through shifting cytokines pattern in the splenocytes obtained from intra-tumorally injected mice.[19] Although this report implicated that **1** might has potential activity to regulate the inflammatory response, the possible pharmacological mechanisms are unclear until now. Thereby, the present study designed both in vitro and in vivo study to examine whether **1** has potential effects against inflammatory response in the lipopolysaccharide (LPS)-stimulated macrophages and the λ-carrageenan-induced edema paw model, and to clarify its possible molecular mechanisms, which will help us to further evaluate the clinical therapeutic potential of **1** on antiinflammation.

RESULTS AND DISCUSSION

Effect of Sclareol (1) on Cell Viability of Macrophage. The growth regulation of **1** on macrophages cell viability was examined by a MTT assay (Figure S1, Supporting Information). The cells were pre-treated with **1** at the concentrations (0, 1, 5 and 10 μg/mL) for 1 h and then co-incubated with 100 ng/mL of LPS for further 24 h. The results indicated that 100 ng/mL of LPS did not change cell viability of macrophages. Similarly, the cell viability of macrophages also did not be influenced even if treating the highest concentration (10 μg/mL) of **1** with presence of LPS. However, several studies reported that **1** can markedly reduce the cell viability of numerous human leukemic cell lines at a range of IC_{50} value from 6.0 to 24.2 μg/mL,[15, 18] inhibit human breast cancer cells at a range of IC_{50} value from 7.0 to 11.6 μg/mL,[17, 18] and suppress human colon cancer cells (HCT116) at 10.6 μg/mL of IC_{50} value.[18] In the normal cell such as peripheral blood mononuclear (PBMC) cells, **1** still exhibited its growth inhibition at IC_{50} value of 10.7 μg/mL.[18]

Inhibitory Effect of Sclareol (1) on LPS-Stimulated NO, iNOS and COX-2. Various concentration (0, 1, 5 and 10 μg/mL) of **1** were used on macrophages to test whether **1** can reverse LPS-induced dramatically accumulation of NO (Figure 1). The results revealed that 100 ng/mL LPS can evidently increase NO production as compared with control group ($p < 0.001$),

and this effect can be markedly suppressed in a dose-dependent manner by pre-treatment of **1** (1, 5 and 10 μg/mL) as compared to those in LPS treated only group. Additionally, **1** (0, 1, 5 and 10 μg/mL) was also tested on macrophages to examine whether it can reduce protein expression of inflammation-associated molecules triggered by LPS (Figure 2). The experimental results suggested that 100 ng/mL of LPS can significantly stimulated protein expression of iNOS and COX-2 ($p < 0.001$), and pre-treatment of **1** at concentration of 5 and 10 μg/mL can obviously down-regulate expression of these LPS-stimulated proteins as compared to LPS treated only group ($p < 0.01$ and $p < 0.001$). It has been mentioned that **1** can increase expression of interferon-gamma (INF-γ) and decrease interleukin-4 (IL-4) in the splenocytes isolated from intra-tumorally injected mice.[19] Chan and Riches demonstrated that co-incubation of LPS and INF-γ can induce both TNF-α and IL-1β production, which up-regulated protein expression of iNOS *via* activating ERK and JNK MAPK pathways in the RAW 264.7γNO(−) cells that do not produce NO with IFN-γ stimulation alone.[20] Roach *et al.* also reported that co-treatment of LPS and INF-γ will increase both transcriptional and translational levels of iNOS in the macrophages.[21] Moreover, the up-regulatory effect of INF-γ on the expression of iNOS also has been identified in aortic smooth muscle cells, epithelial cells, and saphenous vein endothelium.[22-24] In human foreskin fibroblasts, IFN-γ has been reported to suppress COX-2 promoter activity.[25] On the other hand, IL-4 has been evidenced to down-regulate

IFN-γ-inducible iNOS transcription in the murine macrophage cell line RAW264.7.[26] Cui *et al.* also noticed that IL-4 has the capacity to inhibit COX-2 mRNA transcription in human non-small cell lung cancer and follicular dendritic cells.[27, 28] In contrast to the regulation of IFN-γ, Guo *et al.* noticed that IL-4 played a critical role for mRNA stabilization of iNOS in primary human airway epithelial cells stimulated by IFN-γ.[29] To date, it is still unknown whether **1** can modulate the expression of INF-γ and IL-4 in the macrophages. In the present study, experimental data suggested that **1** can markedly decrease LPS-stimulated iNOS expression and then reduce NO production in the macrophages.

Inhibitory Effect of Sclareol (1) on Carr-Induced Paw Edema. Carr-induced paw edema model was used to evaluate the in vivo antiinflammatory effect of **1** (Figure 3). The results showed that Carr injection will stimulate local inflammation and then induce edema of paw tissues. Indo, a common clinical NSAID, was used as positive control to indicate that pre-treatment of 10 mg/kg Indo can effectively reduce paw edema after 3 h Carr stimulation ($p < 0.001$). Similarly, pre-treatment of 10 mg/kg of **1** can also markedly attenuate paw edema after 3 h Carr stimulation, as same as the result of Indo + Carr group ($p < 0.01$). Sclareol belongs to a diterpene alcohol that exhibits hormone-like property similar to that of estrogen according to the structural similarity. It has been proved that the estrogen receptor ligands possess antiinflammatory activity with potential use in the treatment of inflammatory diseases.[30] Thereby,

sclareol may act via the activation of the ligand-induced estrogen-like pathway to exhibit its antiinflammatory effect.

Inhibitory Effect of Sclareol (1) on NO, TNF-α and MDA within Carr-Induced Edema Paw. In Figure 4A, the NO level increased significantly in the edema serum at the 5 h after Carr injection ($p < 0.001$), which can be markedly reversed by **1** as concentration more than 1 mg/kg ($p < 0.01$). Likewise, both TNF-α and MDA level were increased significantly in the edema paw at the 5 h after Carr injection ($p < 0.001$), and this effect was decreased significantly by treatment with **1** as well as 10 mg/kg Indo (Figures 4B and 4C). The Carr test is highly sensitive to non-steroidal antiinflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies.[31] It is well known that the third phase of the edema-induced by Carr, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction found that the injection of Carr into the rat 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.[32] In the present study, statistical analysis revealed that 10 mg/kg of Indo and 10 mg/kg of **1** significantly inhibited the development of edema 3 h after treatment ($p < 0.001$ or $p < 0.01$) (Figure 3). L-arginine–NO pathway has been proposed to play an important role in the Carr-induced inflammatory response,[33] and the expression of the inducible isoform of NO

synthase has been proposed as an important mediator of inflammation.[34] The results confirm that Carr-induced paw edema model results in the production of NO, and the level of NO was decreased significantly by treatment with 1, 5, and 10 mg/kg of **1** (Figure 4A). These results suggest the antiinflammatory mechanism of **1** may be through the L-arginine−NO pathway because **1** significantly inhibits the NO production. TNF-α is also 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.[35] In this study, the results showed that **1** obviously decreased the level of serum TNF-α after Carr injection by treatment with 5 and 10 mg/kg of **1** (Figure 4B).

Inhibitory Effect of Sclareol (1) on iNOS and COX-2 within Carr-Induced Edema Paw. Experimental results showed that **1** (10 mg/kg) can obviously inhibit ($p < 0.001$) both iNOS and COX-2 proteins expression in edema paw as compared to Carr-treated alone group (Figure 5). The experiments showed an average of 69.6 % and 70.3 % down-regulation of iNOS and COX-2 protein, respectively, after treatment with **1** at 10 mg/kg compared with the Carr-induced alone (Figure 5B). In addition, the protein expression showed an average of 61.5 % and 58.1 % down-regulation of iNOS and COX-2 protein after treatment with Indo at 10 mg/kg compared with the Carr-induced alone (Figure 5B). The potency of **1** (10 mg/kg) on down-regulating the expression of iNOS and COX-2 proteins was similar to that of Indo (10 mg/kg).

Inhibitory Effect of Sclareol (1) on neutrophils infiltration within Carr-Induced Edema Paw. In Figure S2 (Supporting Information), neutrophils increased with Carr treatment ($p < 0.001$). As Indo and **1** (10 mg/kg) could significantly decrease the neutrophils numbers as compared to the Carr-treated group ($p < 0.001$). The Carr-induced inflammatory response has been linked to neutrophils infiltration and the production of neutrophils-derived free radicals as well as the release of other neutrophils-derived mediators.[35] It has been demonstrate that free radical and NO will be released when administrating with Carr, and increasing free radical might attack plasma membrane and result in the accumulation of MDA. The present study demonstrated that 10 mg/kg of **1** the same as Indo can markedly decrease neutrophils infiltration and accumulation of MDA within edema paw after Carr treatment (Figures S2 and 5C).

In Vivo Regulatory Effect of Sclareol (1) on the Activity of Antioxidative Enzymes. At 5h after the intrapaw injection of Carr, liver tissues were analyzed for the biochemical parameters such as CAT, SOD, and GPx activities (Table 1). CAT, SOD, and GPx activities in liver tissue were decreased significantly by Carr administration, which were increased significantly after treated with 5 mg/kg of **1** ($p > 0.05$ or $p < 0.01$) and 10 mg/kg Indo ($p < 0.01$). Glutathione (GSH) plays an important role against Carr-induced local inflammation,[36] and endogenous GSH can reduce MDA production. In the present study, increases of CAT, SOD, and GPx activities were found in the group with **1** treatment (Figure 4C and Table 1). Thereby, the suppression of MDA

production is probably due to the increases of CAT, SOD, and GPx activities. Besides, it has been also demonstrated that sclareol (**1**) possess the antioxidant activity in vitro,[37] whose effect may be an alternative explanation for the reduced MDA found in hepatic tissue.

Conclusions. These results suggested that the antiinflammatory mechanism of **1** may be related to the inhibitions of iNOS and COX-2, and it is associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). Based on reported bioactivities above, it might be partially explained why **1** can exhibit the antiinflammatory effect in the LPS-stimulated macrophages and the Carr-induced paw edema model. Compound **1** may be used as a pharmacological agent or hormone-like ligand in the prevention or treatment of inflammatory diseases.

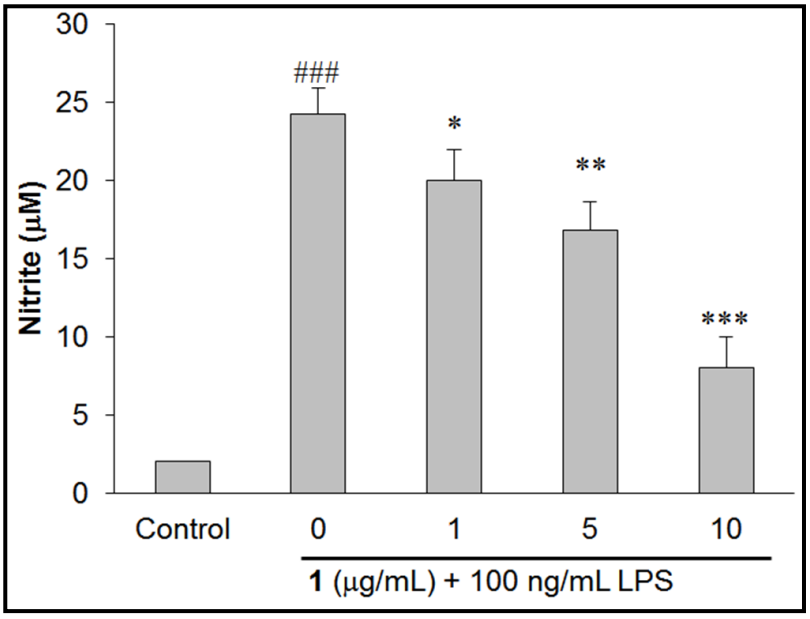

Figure 1. Sclareol (**1**) reduced LPS-induced NO production in macrophages. ### $p < 0.001$ compared with control group (normal saline treated only). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to LPS treated alone group.

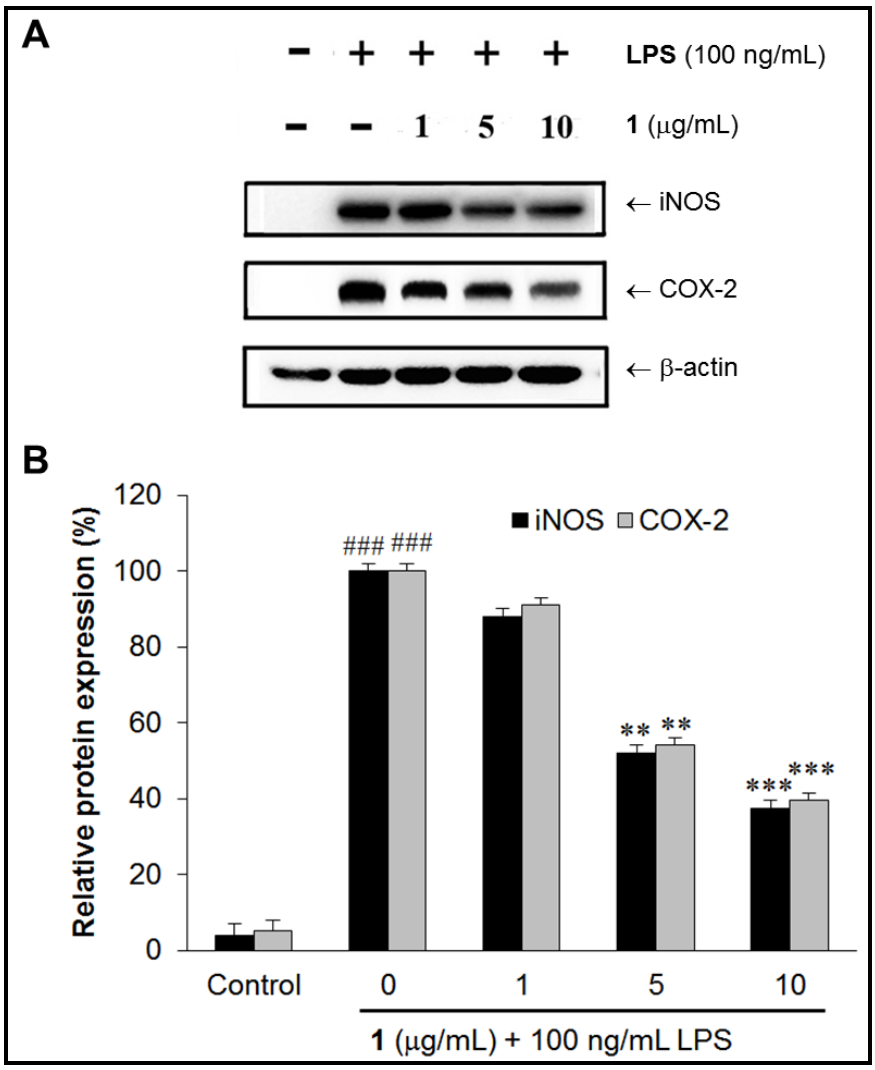

Figure 2. Sclareol (**1**) inhibits LPS-stimulated expression of inflammation-associated proteins in macrophages. (A) Western blot showing expression of iNOS and COX-2 proteins. The bar chart indicated the relative protein expression of iNOS and COX-2 in macrophages after treatments (B). ### $p < 0.001$ compared with control group (normal saline treated only). ** $p < 0.01$ and *** $p < 0.001$ compared with LPS treated only group.

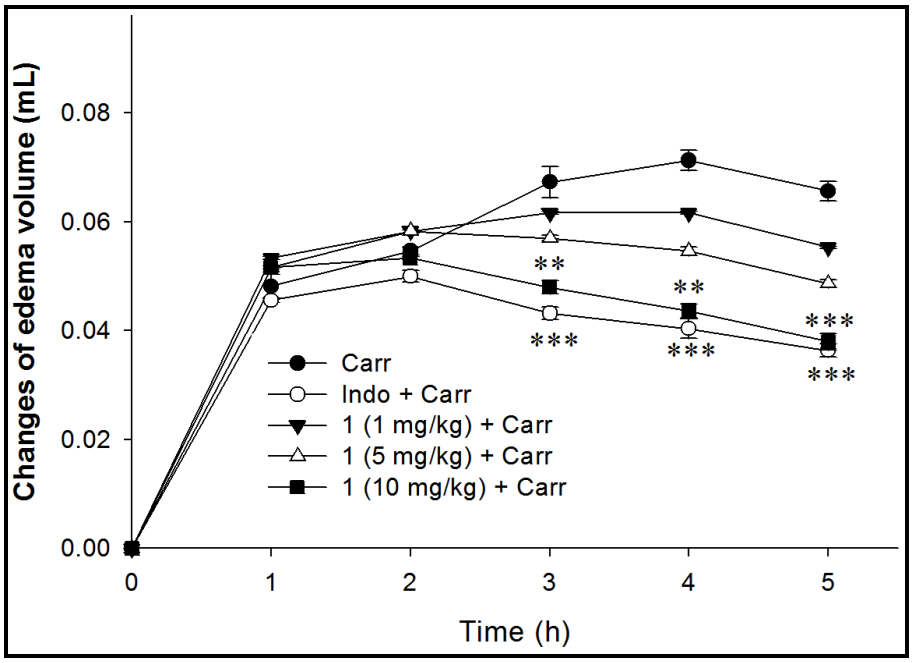

Figure 3. Sclareol (**1**) reduced mice hind paw edema induced by λ-carrageenan (Carr). ** $p < 0.01$ and *** $p < 0.001$ compared with the Carr group. Carr, 1% λ-carrageenan; Indo, 10 mg/kg indomethacin.

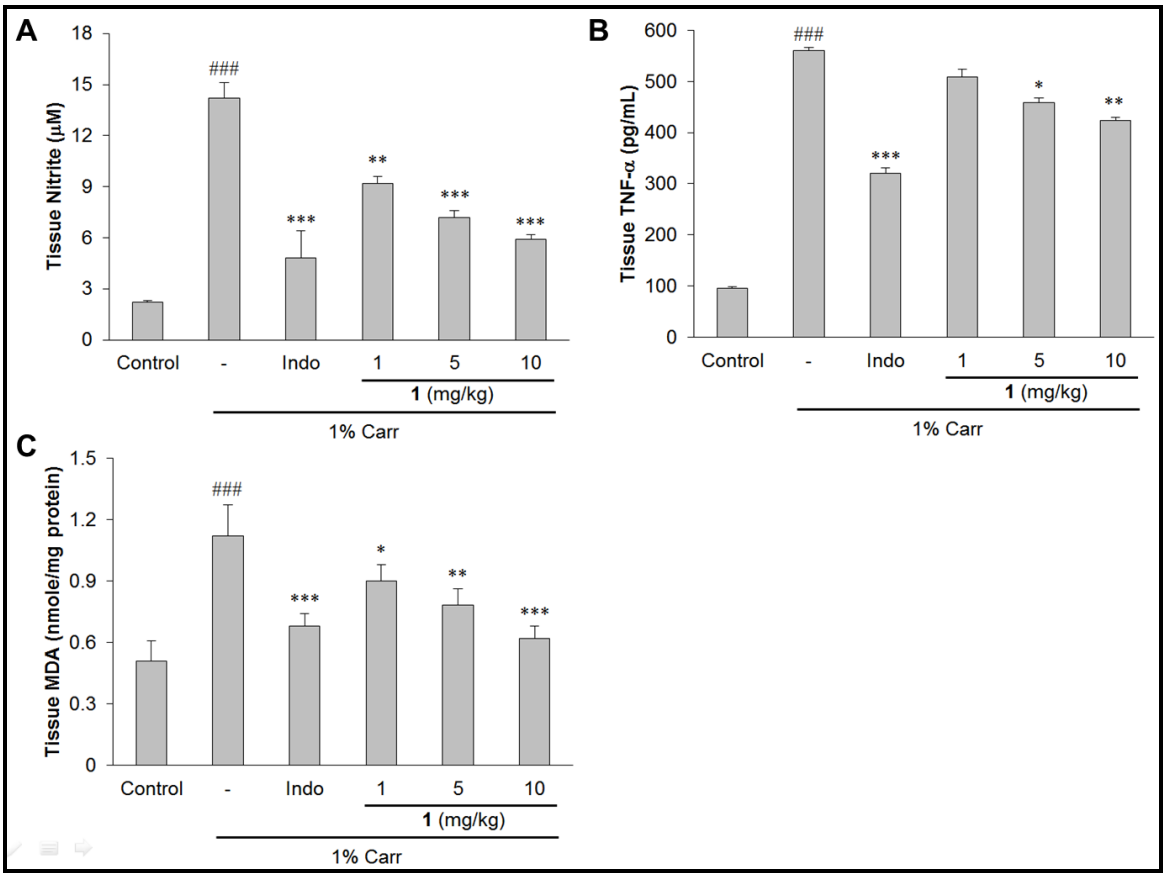

Figure 4. Sclareol (**1**) inhibits tissue content of NO (A), TNF-α (B) and MDA (C) in Carr-induced paw edema model. ### p <0.001 compared with control group (normal saline treated only). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the Carr group. Indo, 10 mg/kg indomethacin.

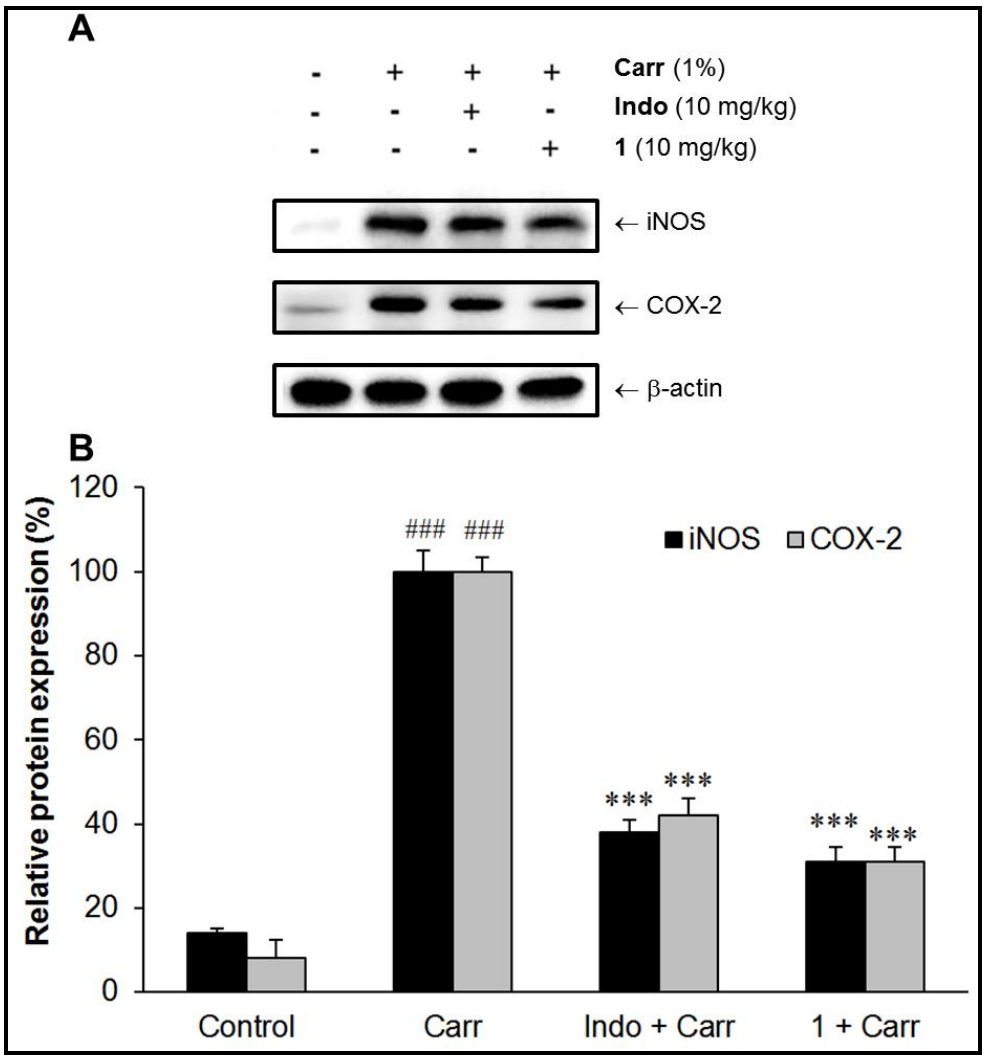

Figure 5. Sclareol (**1**) reduced expression of inflammation-associated proteins in edema paw induced by Carr. (A) Western blot showing protein expression of iNOS and COX-2. The bar chart indicated the relative protein expression of iNOS and COX-2 within the paw tissue after treatments (B). ### $p < 0.001$ compared with control group (normal saline treated only). *** $p < 0.001$ compared with Carr treated only group. Carr, 1% λ-carrageenan; Indo, 10 mg/kg indomethacin.

Table 1. Regulation of sclareol (**1**) on the enzyme activities of catalase, superoxide dismutase and glutathione peroxidase in mouse liver.

Groups	Catalase	Glutathione peroxidase	Superoxide dismutase
Control	5.3 ± 0.2	24.4 ± 0.3	3.4 ± 0.1
Carr	3.6 ± 0.3$^{###}$	15.6 ± 0.3$^{###}$	1.9 ± 0.2$^{###}$
Indo + Carr	4.7 ± 0.2**	22.5± 0.6**	2.8 ± 0.2**
1 (1 mg/kg) + Carr	3.8 ± 0.3*	17.2 ± 0.5*	2.0 ± 0.3
1 (5 mg/kg) + Carr	4.4 ± 0.2**	19.8 ± 0.4**	2.6 ± 0.4*
1 (10 mg/kg) + Carr	5.0 ± 0.5***	22.9 ± 0.6**	2.8 ± 0.2**

Activity unit of enzymes is presented as U/mg protein. Carr, 1% λ-carrageenan; Indo, 10 mg/kg indomethacin. ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the Carr group.

EXPERIMENTAL SECTION

Chemicals. Sclareol (**1**), lipopolysaccharide (LPS) from Escherichia coli (serotype 0127:B8), carrageenan (Carr), indomethacin (Indo) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). TNF-α was purchased from Biosource International Inc (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, anti-β-actin antibody (Santa Cruz Biotechnology,

CA, USA) and a protein assay kit (Bio-Rad Lab, Watford, Herts, UK) were obtained as indicated. Polyvinylidene fluoride (PVDF) membrane (Immobilon-P®) was obtained from Millipore Corp (Bedford, MA, USA).

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 culture dishes containing Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) in a CO_2 incubator (5% CO_2 in air) at 37℃ and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+} ions.

Mice Model of Carr-Induced Paw Edema. Twenty-four male ICR mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals housed in Plexiglas cages with free access to food and water, and maintained at a constant temperature of 22 ± 1℃ and relative humidity of 55 ± 5 % with a photocycle of 12-h light/dark. The 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.[38] After a 2-week adaptation period, the mice (about 18-25 g) were randomly assigned to four groups (n = 6) for further experiments. The control group receives normal saline,

and the other three groups include a Carr alone, Carr + Indo (a positive control), and **1** administered groups (**1**+ Carr). The Carr-induced hind paw edema model was used for determination of antiinflammatory activity.[39] Animals were treated with normal saline, Indo or **1** (1, 5 and 10 mg/kg) with intraperitoneal injection, 30 min prior to injection of 1% Carr (50 μL) in the plantar side of right hind paws of the mice. The paw volume was measured immediately 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 is the volume of the right hind paw after Carr treatment, and B is the volume of the right hind paw before Carr treatment. Finally, the animals were sacrificed and all of right hind paw were dissected and stored at -80 ℃. Also, blood were withdrawn and kept at -80 ℃.

MTT Cell Viability Assay. Macrophages (2×10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were pre-treated with several concentrations (0, 1, 5 and 10 μg/mL) of **1** for 1 h and then co-stimulated with 100 ng/mL of LPS for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for 2 h at 37℃, and then the medium was discarded and 100 μL of dimethyl sulfoxide (DMSO) was added. After 30 min incubation, absorbance at 570 nm was read

using a microplate reader.

Measurement of Nitric Oxide/Nitrite. NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined according to previous study.[39] The cells were pre-incubated with **1** (0, 1, 5 and 10 μg/mL) for 1 hr and then co-treated with 100 ng/mL LPS at 37℃ for 24 h. Subsequently, 100 μL of each collected culture medium 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. The absorbance of mixture was measured at 540 nm with a Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale, CA). Homogenized tissue samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 mg/mL) to a final concentration of 15 mg/mL. After centrifugation at 10,000 ×g for 5 min at room temperature, 100 μL of supernatant was applied into a microtiter plate, followed by 100 μL 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 by absorbance at 540 nm.

Western Blot Analysis. The stimulated macrophages were washed with PBS and lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1mM sodium orthovanadate, 1mM EGTA, 10mM sodium fluoride, 1mM sodium pyrophosphate, 20 mM Tris buffer (pH 7.9), 100 mM

β-glycerophosphate, 137 mM sodium chloride, 5 mM EDTA and one protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)] on ice for 1 h, followed by centrifugation at 12,000 ×g for 30 min at 4℃. Soft tissues were removed from individual mice paws and homogenized in a solution containing 10 mM CHAPS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg/mL, aprotinin, 1 μM pepstatin and 10 μM leupeptin. The homogenates were centrifuged at 12,000 ×g for 20 min, and the supernatant was collected for Western blot analysis. Protein concentration was measured by the Bio-Rad protein assay kit with bovine serum albumin as a standard. About 30 μg of protein from the supernatants was then separated on 10% sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes. After transfer, the membrane was blocked for 2 h at room temperature with 5% skim milk in TBST buffer (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 antibody in 5% skim milk in TBST buffer 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 anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich) 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® ECL reagent (Amersham International, Buckinghamshire, UK). The results of Western blot analysis were quantified by measuring the

relative intensity compared to the control using Kodak Molecular Imaging Software Ver.4.0.5 (Eastman Kodak Company, Rochester, NY, USA) and represented in the relative intensities. The results for iNOS and COX-2 were normalized to the band density of internal control (β-actin), and the relative proteins expression were calculated according to the values of LPS treated alone group as 100%.

Malondialdehyde Assay. Malondialdehyde (MDA) from Carr-induced edema foot was evaluated by the thiobarbituric acid reacting substance (TRARS) method.[39] Briefly, MDA reacted with thiobarbituric acid in the acidic high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

Measurement of Serum TNF-α. Serum levels of TNF-α were determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. The concentration of serum TNF-α was presented as pg/mL and determined according to the regression equation of the standard curve.

Measurement of Antioxidant Enzymes Activity. The following biochemical parameters were analyzed to check the hepatoprotective activity of **1** by the methods given below. Total superoxidase dismutase (SOD) activity was determined by the inhibition of cytochrome c reduction.[40] 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%. Total catalase (CAT) activity was measured according to previous study.[41] In brief, the reduction of 10 mM hydrogen peroxide in 20 mM of phosphate buffer (pH 7.0) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total glutathione peroxidase (GPx) activity in cytosol was determined according to Paglia and Valentine's method.[42] 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. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute.

Statistical Analysis. Data are expressed as mean ± standard error of the mean (SEM). Statistical evaluation was carried out by one-way analysis of variance (ANOVA, Scheffe's post-hoc test). A value of $p < 0.05$ was regarded as being statistically significant.

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SUPPORTING INFORMATION

The experimental procedure and pathological examination regarding the effect of sclareol (**1**) on neutrophil infiltration within Carr-induced edema paw were provided online.

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