ABSTRACT

The purpose of this study was to examine anti-inflammatory effect of ethanolic extract of *Antrodia salmonea* (EAS) in the lipopolysaccharide (LPS)-stimulated RAW246.7 macrophages and the carrageenan (Carr)-induced edema paw model, and to clarify its possible molecular mechanisms. Inhibitory effects of EAS were examined on cells proliferation, nitric oxide (NO) production, expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins, and the activity of antioxidant enzymes. Our data demonstrated that EAS inhibited NO production, and expression of iNOS and COX-2 proteins in LPS-stimulated RAW246.7 cells. EAS can also significantly reduce paw edema, content of NO, TNF-α and malondialdehyde (MDA), expression of iNOS and COX-2 proteins, and neutrophil infiltration within the tissues stimulated by Carr. The anti-inflammatory mechanisms of EAS might be related to the decrease of inflammatory cytokine and increase of antioxidant enzymes activities, which would result in reduction of iNOS, COX-2 and MDA and subsequently inflammatory responses.

Keywords: herbal medicine, anti-inflammation, *Antrodia salmonea*, carrageenan,

INTRODUCTION

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, etc (*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 anti-inflammatory activity with marked reduction in gastrointestinal toxicity as compared to traditional NSAIDs (non-steroidal anti-inflammatory drugs) (*14*). Similarly, tumor necrosis factor-alpha (TNF-α), 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*).

The medical fungus *Antrodia salmonea*, a newly identified species of the genus *Antrodia*, grow on the empty rotten trunk of *Cunninghamia konishii* in Taiwan (*15*). The fruiting body of *A. salmonea* has been used in the food remedy of diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer and is also used as a detoxicant in Taiwan folk medicine (*16*). To date, there were several newly compounds were isolated from the basidiomata of *A. salmonea*, whose *in vitro* studies displayed anti-oxidative effect (*17*) and anti-inflammatory activities in activated inflammatory cells (*18*). Thereby, we designed the *in vivo* study to examine whether the ethanolic extract from fruiting body of *A. salmonea* has potential effects against inflammatory response in the lipopolysaccharide (LPS)-stimulated RAW246.7 cells and the carrageenan (Carr)-induced edema paw model, and to clarify its possible molecular mechanisms, which will help us to further evaluate the clinical therapeutic potential or food remedy of *A. salmonea* on anti-inflammation.

MATERIALS AND METHODS

Chemicals. 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).

Preparation of ethanolic extract of A. salmonea (EAS). The fruiting body of *A. salmonea* was purchased from the Ji pin mushroom store (Nantou, Taiwan), and identified by Drs. Yu-Cheng Dai (Institute of Applied Ecology, Chinese Academy of Science, China) and Sheng-Hua Wu (Department of Botany, National Museum of Natural Science, Taiwan). Dried sample of *A. salmonea* (100 g) was macerated with 1L ethanol for 24 h at room temperature. Filtration and collection of the extract was done three times. The filtrates were collected, concentrated with a vacuum evaporator until the volume was below 10 mL and then freeze-dried. The yield obtained was 4.2 % (w/w).

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 (*19*). 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 EAS administered groups (Carr+ EAS). The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity (*20*). Animals were treated with normal saline, Indo or EAS (12.5, 25, and 50 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. RAW264.7 cells (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 (12.5, 25, 50, 100, and 200 μg/mL) of EAS 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 (*20*). The cells were pre-incubated with EAS (0, 12.5, 25, and 50 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

111 culture medium was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1%
112 naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room
113 temperature for 10 min. The absorbance of mixture was measured at 540 nm with a
114 Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale, CA). Homogenized tissue
115 samples were diluted four times with distilled water and deproteinized by adding 1/20 volume
116 of zinc sulfate (300 mg/mL) to a final concentration of 15 mg/mL. After centrifugation at
117 10,000 ×g for 5 min at room temperature, 100 μL of supernatant was applied into a microtiter
118 plate, followed by 100 μL of Griess reagent. After 10 min of color development at room
119 temperature, the absorbance was measured at 540 nm with a Micro-Reader. By using sodium
120 nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance
121 at 540 nm.

122 **Western blot analysis.** The stimulated RAW264.7 cells were washed with PBS and
123 lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1mM sodium orthovanadate,
124 1mM EGTA, 10mM sodium fluoride, 1mM sodium pyrophosphate, 20 mM Tris buffer (pH
125 7.9), 100 mM β-glycerophosphate, 137 mM sodium chloride, 5 mM EDTA and one protease
126 inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)] on ice for 1 h, followed by
127 centrifugation at 12,000 ×g for 30 min at 4℃. Soft tissues were removed from individual
128 mice paws and homogenized in a solution containing 10 mM CHAPS, 1 mM
129 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 (*20*). 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.

Histological examination. The biopsies of mice hind paws were immediately taken following 5 h treatment with the interplanetary injection of Carr. The tissue slices were fixed in (1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraffin (Sherwood Medical). Tissue sections (5 μm thickness) were deparaffinized with xylene and stained with hematoxylin and eosin for cell counting. All samples were observed and photographed with BH-2 Olympus microscopy. The excessive inflammatory response was illustrated as massive infiltration of ploymorphonuclear leukocytes (PMNs). The observation of tissue slices (3-5 slides) were randomly chosen from every groups, and the number of neutrophils were counted from five scopes (400 ×) on each tissue slice to obtain average value.

HPLC analysis of EAS. HPLC was performed according to the minor modification of

previous studies (*21*,*22*). Before analysis by HPLC, EAS was filtered through a 0.2 µm Millipore filter, and then total volume of 20 µL was loaded into HPLC column. Besides, external standards were prepared as concentration of 100 µg/mL in HPLC grade-methanol and used to calculate the concentration of examined compounds. Reverse phase HPLC was performed on a HITACHI HPLC system (Tokyo, Japan) equipped with HITACHI L-7100 pump, HITACHI L-7400 UV detector and HITACHI L-7200 autosampler. Separations were accomplished on LiChroCART 250-4 C18 HPLC-cartridge (5 µm; Merck, Whitehouse Station, NJ, USA). The separation conditions of HPLC analysis for examined compounds were described in Table 1.

Measurement of antioxidant enzymes activity. The following biochemical parameters were analyzed to check the hepatoprotective activity of EAS by the methods given below. Total superoxidase dismutase (SOD) activity was determined by the inhibition of cytochrome *c* reduction (*23*). 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 (*24*). 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 (*25*). 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 (S.E.M). 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.

RESULTS

Effect of EAS on cell viability of RAW246.7 cells. The growth inhibitory effect of EAS on RAW264.7 cell viability was determined by a MTT assay (Fig. 1). Cells were pre-treated with EAS at the concentrations (0, 12.5, 25, 50, 100, and 200 μg/mL) for 1 h and then co-incubated with 100 ng/mL of LPS for further 24 h. Our results showed that 100 ng/mL of LPS did not change cell viability of RAW246.7 cells. Pre-treatment of EAS at concentration of 100 and 200 μg/mL can significantly inhibit cell viability of RAW264.7 macrophages with presence of LPS. However, Lower concentration of EAS (12.5, 25, and 50 μg/mL) showed no effects in cell viability in the presence of 100 ng/mL LPS incubation for 24 h.

Effect of EAS on LPS-induced NO production in RAW246.7 cells. Various concentration (0, 12.5, 25, and 50 μg/mL) of EAS were used on RAW246.7 cells to test whether EAS can reverse LPS-induced dramatically accumulation of NO (Fig. 2). 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 EAS (25 and 50 μg/mL) as compared to those in LPS treated only group. EAS did not interfere with the reaction between nitrite and Griess reagents at 100 ng/mL (data not shown).

Effect of EAS on LPS-induced iNOS and COX-2 proteins expression in RAW246.7

cells. Pre-incubation of EAS (0, 12.5, 25, and 50 μg/mL) were tested on RAW246.7 cells to examine whether EAS can reduce protein expression of inflammation-associated molecules triggered by LPS (Fig. 3). 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 EAS at concentration of 25 and 50 μg/mL can obviously down-regulate expression of these LPS-stimulated proteins as compared to LPS treated only group ($p < 0.05$ and $p < 0.001$).

Effects of EAS in Carr-induced mice paw edema. Carr-induced paw edema model was used to evaluate the *in vivo* anti-inflammatory effect of EAS (Fig. 4). The results showed that Carr injection will stimulate local inflammation and then induce edema of paw tissues. Indo, a common clinical NSAIDs, was used as positive control to indicate that pre-treatment of 10 mg/kg Indo can effectively reduce paw edema after 3^{th} h Carr stimulation (p<0.01). Similarly, pre-treatment of EAS (25 and 50 mg/kg) can also markedly decrease paw edema after 3^{th} h Carr stimulation, as same as the result of Indo + Carr group (p<0.001).

Effects of EAS on the NO, TNF-α, and MDA Levels. In Fig.5A, the NO level increased significantly in the edema serum at the 5^{th} h after Carr injection (p<0.001), which can be markedly reversed by EAS as concentration more than 12.5 mg/kg (p<0.05), and the inhibitory potency of EAS (50 mg/kg) was similar to that of Indo (10 mg/kg) at 5^{th} h after induction. Likewise, both TNF-α and MDA level were increased significantly in the edema

paw at the 5th h after Carr injection (p<0.001), and this effect was decreased significantly by treatment with EAS as well as 10 mg/kg Indo (Fig.5B and 5C).

Effects of EAS on Carr-induced iNOS and COX-2 proteins expression in edema paw. Our results showed that EAS (50 mg/kg) can obviously inhibit (p<0.001) iNOS and COX-2 proteins expression in edema paw as compared to Carr-treated alone group (Fig. 6). The experiments showed an average of 67.6% and 57.4**%** down-regulation of iNOS and COX-2 protein, respectively, after treatment with EAS at 50 mg/kg compared with the Carr-induced alone (Fig.6B). In addition, the protein expression showed an average of 53.6% and 51.1% down-regulation of iNOS and COX-2 protein after treatment with Indo at 10 mg/kg compared with the Carr-induced alone (Fig. 6B). The potency of EAS (50 mg/kg) on down-regulating the expression of iNOS and COX-2 proteins was similar to that of Indo (10 mg/kg).

Histological examination. Paw biopsies of Carr model animals showed marked cellular infiltration in the connective tissue, and the infiltrates accumulated between collagen fibers and into intercellular spaces (Fig. 7B). Paw biopsies of animals treated with EAS (50 mg/kg) showed a reduction in Carr-induced inflammatory response (Fig. 7D). Actually inflammatory cells were reduced in number and confined to near the vascular areas, and intercellular spaces did not show any cellular infiltrations (Fig.7D). Collagen fibers were regular in shape and showed a reduction of intercellular spaces. Moreover, the hypoderm connective tissue was not damaged (Fig. 7D). In Fig. 7E, neutrophils increased with Carr treatment (p<0.001). As Indo

and EAS (50 mg/kg) could significantly decrease the neutrophils numbers as compared to the Carr-treated group (p<0.001).

Effects of EAS on activities of antioxidant enzymes. At 5th h after the intrapaw injection of Carr, paw tissues were analyzed for the biochemical parameters such as CAT, SOD, and GPx activities (Table 1). CAT, SOD, and GPx activities in paw tissue were decreased significantly by Carr administration. CAT, SOD, and GPx activity were increased significantly after treated with 25 mg/kg EAS ($p < 0.05$) and 10 mg/kg Indo ($p < 0.01$).

Qualification of extraction procedure of EAS. Some of the reference compounds (adenosine and zhankuic acid A) within EAS were identified by HPLC to be as indicator compounds for quality check of extraction procedure of each batch (Fig. 8). Using HPLC quantification, the content of adenosine and zhankuic acid A were calculated to be 16.3 and 11.5 mg/g of EAS, respectively (Table 2).

Discussion

In the present study, we demonstrated anti-inflammatory activities of EAS in both *in vitro* and *in vivo* experimental systems, using LPS-stimulated RAW264.7 macrophages and a mouse model of topical inflammation respectively. Dual inhibitory activities against iNOS and COX-2 as shown in *in vitro* assays appear to confer on EAS a potent *in vivo* efficacy in mouse Carr-induced paw edema, comparable with a potent and well known COX inhibitor,

indomethacin, suggesting its potential therapeutic usage as a novel topical anti-inflammatory source of health food.

Previous studies reported that some bioactive compounds have been identified within extract of the basidiomata of *A. salmonea* and displayed potential with anti-inflammatory effect (*17*,*18*). Our experimental data showed that at least two bioactive compounds, including adenosine and zhankuic acid A, have been identified in the ethanolic extract of fruiting body of *A. salmonea* (Fig. 8). Of these, adenosine levels rise during inflammation and modulate inflammatory responses by the interaction with their receptors (*26*). Activation of A2 and A3 adenosine receptors (AR) has been evidenced to provide anti-inflammatory effects (*27-31*). A3AR is considered to be expressed in macrophage cells (*32*). Lee et al. noticed that a novel A3AR agonist, thio-Cl-IB-MECA, can inhibit the LPS-stimulated expression of pro-inflammatory markers including iNOS, interleukin-1beta (IL-1β), and TNF-α thought suppressing phosphatidylinositol 3-kinase (PI3 kinase)/Akt and NF-kB signaling pathways (*27*). Stimulating A3AR can alter the cytokine milieu by decreasing TNF-α (*33*). Similarly, A3 receptor agonist, IB-MECA, inhibited the production of interleukin-12 and interferon-gamma (IFNγ) and prevented lethality in endotoxemic mice (*31*). It also has been reported that IC51, an adenosine kinase inhibitor, stimulated the extracellular adenosine release and reduced the LPS/ IFNγ-mediated production of NO, and induction of iNOS and TNF-α gene expression (*34*). Bouma et al. evidenced that adenosine acts via A2AR as well as

299 A3AR to inhibit neutrophil degranulation and neutrophil-mediated tissue injury (*35*). Our
300 experimental results showed that EAS significantly reduce serum level of TNF-α (Fig. 5B) as
301 well as the level of iNOS protein (Fig. 3 and 6) and subsequently NO production (Fig. 2 and
302 5A) in *in vitro* and *in vivo* assays, which might be caused by the pharmacological effect of
303 adenosine. On the other hand, zhankuic acid A isolated from *A. camphorate* extract has also
304 been reported to provide anti-inflammatory effect through inhibiting ROS production and
305 firm adhesion of neutrophil in isolated peripheral human neutrophils (*36*), whose
306 pharmacological function might provide one of possible reasons why EAS can decrease
307 serum level of MDA (Fig. 5C), a production of ROS-induced lipid peroxidation.

308 The Carr test is highly sensitive to NSAIDs, and has long been accepted as a useful
309 phlogistic tool for investigating new drug therapies (*37*). It is well known that the third phase
310 of the edema-induced by Carr, in which the edema reaches its highest volume, is
311 characterized by the presence of prostaglandins and other compounds of slow reaction found
312 that the injection of Carr into the rat paw induces the liberation of bradykinin, which later
313 induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the
314 formation of the inflammatory exudates (*38*). In the present study, statistical analysis revealed
315 that 10 mg/kg of Indo and 25 mg/kg of EAS significantly inhibited the development of edema
316 4^{th} h after treatment ($p<0.001$ or $p<0.01$) (Fig. 4). L-arginine–NO pathway has been proposed
317 to play an important role in the Carr-induced inflammatory response (*39*), and the expression

of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation (*40*). Our present 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 12.5, 25, and 50 mg/kg EAS (Fig.5A). We suggest the anti-inflammatory mechanism of EAS may be through the L-arginine–NO pathway because EAS 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 (*41*). In this study, we found that EAS obviously decreased the level of serum TNF-α after Carr injection by treatment with 12.5, 25 and 50 mg/kg EAS (Fig. 5B).

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 (*41*). 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. Our study demonstrated that 50 mg/kg EAS the same as Indo can markedly decrease neutrophils infiltration and accumulation of MDA within edema paw after Carr treatment (Fig. 7 and Fig. 5C). In addition, glutathione (GSH) plays an important role against Carr-induced local inflammation (*42*), and endogenous GSH can reduce MDA production. In the present study, increases of CAT, SOD, and GPx

activities were found in the group with EAS treatment (Fig. 5C and Table 1). Thereby, we assume the suppression of MDA production is probably due to the increases of CAT, SOD, and GPx activities.

In conclusion, these results suggested that the anti-inflammatory mechanism of EAS 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 EAS can exhibit the anti-inflammatory effect in the LPS-stimulated RAW246.7 macrophages and the λ-Carr-induced paw edema model. EAS may be used as a pharmacological agent in the prevention or treatment of disease in which free radical formation in a pathogenic factor.

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