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Analgesic Effects and the Mechanisms of Anti-inflammation of Ergostatrien-3β-ol from *Antrodia camphorata* Submerged Whole Broth in Mice

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Ergostatrien-3β-ol (**ST1**), an active and major ingredient from *Antrodia camphorata* (AC) submerged whole broth was evaluated for the analgesic and anti-inflammatory effects. Treatment of male imprinting control region (ICR) mice with **ST1** (1, 5, and 10 mg/kg) significantly inhibited the numbers of acetic-acid-induced writhing response in 10 min. Also, our result showed that **ST1** (10 mg/kg) significantly inhibited the formalin-induced pain in the late phase ($p < 0.001$). In the anti-inflammatory test, **ST1** (10 mg/kg) decreased the paw edema at 4 and 5 h after λ-carrageenin (Carr) administration and increased the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the liver tissue. We also demonstrated that **ST1** significantly attenuated the malondialdehyde (MDA) level in the edema paw at 5 h after Carr injection. **ST1** (1, 5, and 10 mg/kg) decreased the nitric oxide (NO) levels on both the edema paw and serum level at 5 h after Carr injection. Also, **ST1** (5 and 10 mg/kg) diminished the serum tumor necrosis factor (TNF-α) at 5 h after Carr injection. Western blotting revealed that **ST1** (10 mg/kg) decreased Carr-induced inducible nitric oxide synthase (iNOS), and cycloxyclase (COX-2) expressions at 5 h in the edema paw. An intraperitoneal (ip) injection treatment with **ST1** also diminished neutrophil infiltration into sites of inflammation, as did indomethacin (Indo). The anti-inflammatory mechanisms of **ST1** might be related to the decrease in the level of MDA, iNOS, and COX-2 in the edema paw via increasing the activities of CAT, SOD, and GPx in the liver through the suppression of TNF-α and NO.

KEYWORDS: Chinese herb; ergostatrien-3β-ol; anti-inflammation; analgesic; MDA; NO; TNF-α

INTRODUCTION

The fruiting body of *Antrodia camphorata* (AC; Polyporaceae, Aphyllophorales) is well-known in Taiwan as a traditional Chinese medicine. It has been used for the treatment of food and drug intoxication, diarrhea, abdominal pain, hypertension, skin itching, and cancer (*1*). It is rare and expensive. The fruiting body and cultured mycelia of AC contain fatty acids, lignans, phenyl derivatives, sesquiterpenes, steroids, and triterpenoids (*2*). The submerged fermentation of AC has been developed and commercialized by local companies. In biological studies, the fruiting bodies exhibited immunomodulating (*3*), antioxidative, and hepatoprotective effects (*4*). The fermented culture broth had cytotoxic activity against several tumor cell lines (*1*), and the cultured mycelia showed anti-inflammation (*5*), vasorelaxation (*6*), and cytotoxic activity against several tumor cell lines (*7*) and anti-hepatitis B virus activity (*8*). The filtrate in submerged culture also had protective effects against CCl_4-induced hepatic toxicity and high antioxidant properties (*9*). However, little information is available on the analgesic and anti-inflammatory effects of ergostatrien-3β-ol (**ST1**).

Many scientific papers have reported that the inflammatory effect induced by λ-carrageenin (Carr) could be associated with the free radical. The free radical, prostaglandin, and NO will be released when administrating with Carr for 1−5 h. The edema effect was raised to a maximum at the 3rd h, and its malondialdehyde (MDA) production was due to free-radical attack of the plasma membrane (*10*). Thus, the inflammatory effect would result in the accumulation of MDA. Therefore, in this paper, we examined the analgesic effects of **ST1** on nociception induced by acetic acid and formalin. We also evaluated the anti-inflammatory effects of **ST1** on paw edema induced by Carr in mice, and

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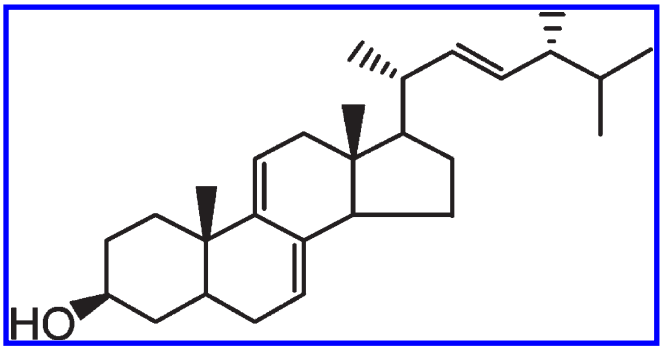

Figure 1. Chemical structure of **ST1**.

we detected the levels of MDA, NO, tumor necrosis factor (TNF-α), inducible nitric oxide synthase (iNOS), and cycloxyclase (COX-2) in either paw edema or serum. Also, the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the liver at 5 h after Carr injection were investigated to understand the relationship between the anti-inflammatory mechanism of **ST1** and antioxidant enzymes.

MATERIALS AND METHODS

Chemicals. Acetic acid was purchased from Merck (Darmstadt, Germany). Carr and indomethacin (Indo) were obtained from Sigma (St. Louis, MO). Formalin was purchased from Nihon Shiyaku Industries (Japan). TNF-α was purchased from Biosource International, Inc. (Camarillo, CA). Anti-iNOS, anti-COX-2, and anti-β-actin antibody (Santa Cruz, CA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) were obtained as indicated. Poly(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA).

Fungus Material. Freeze-dried powder of AC of the submerged whole broth (Batch MZ-247) was provided by the Biotechnology Center of Grape King, Inc., Chung-Li, Taiwan, Republic of China.

Isolation and Determination of the Active Compound. Freeze-dried powder of AC of the submerged whole broth (1.6 kg) was extracted 3 times with methanol (16 L) at room temperature (1 day each). The methanol extract was evaporated *in vacuo* to give a brown residue, which was suspended in H_2O (1 L) and then partitioned (3 times) with 1 L of ethyl acetate. The EtOAc fraction (95 g) was chromatographed on silica gel using mixtures of hexane and EtOAc of increasing polarity as eluents and further purified with high-performance liquid chromatography (HPLC). **ST1** (5.4 g) (**Figure 1**) was eluted with 10% EtOAc in hexane and recrystallization with EtOH (*11*).

ST1. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.61 (s, 3 H, CH_3), 0.80 (d, J = 6.4 Hz, 3H, CH_3), 0.82 (d, J = 6.4 Hz, 3H, CH_3), 0.89 (d, J = 7.2 Hz, 3H, CH_3), 0.92 (s, 3H, CH_3), 1.01 (d, J = 6.4 Hz, 3H, CH_3), 3.61 (m, 1H, CH), 5.14 (dd, J = 15.6, 7.2 Hz, 1H, CH), 5.20 (dd, J = 15.6, 8.0 Hz, 1H, CH), 5.35 (m, 1H, CH), 5.55 (dd, J = 5.6, 2.0 Hz, CH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 12.0, 16.3, 17.6, 19.6, 20.0, 21.1, 23.0, 28.3, 32.0, 33.1, 37.0, 38.4, 39.1, 40.4, 40.8, 42.8, 46.2, 54.6, 55.7, 70.5, 116.3, 119.6, 132.0, 135.6, 139.8, 141.3.

Animals. Male, 6−8-week-old imprinting control region (ICR) mice were obtained from 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 a 12 h dark−light cycle for at least 2 week 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 (*12*).

After a 2 week adaptation period, male ICR mice (18−25 g) were randomly assigned to five groups (n = 6) in acetic-acid-induced writhing [1%, 0.1 mL/10 g intraperitoneal (ip)] and formalin-induced licking (5%, 20 μL/per mice ip) experiments. These include a pathological model group (received acetic acid or formalin), a positive control group (acetic acid or formalin + Indo), and **ST1**-administered groups (acetic acid or formalin + **ST1**). In the Carr-induced edema experiment, there were six groups (n = 6) of the animals in the study. The control group receives normal saline (ip). The other five groups include a Carr-treated group, a positive control group (Carr + Indo), and **ST1**-administered groups (Carr + **ST1**).

Acetic-Acid-Induced Writhing Response. The test was performed as described by Chang et al. (*13*). Writhing was induced by an ip injection of 0.1 mL/10 g of acetic acid solution (10 mL/kg). Positive control animals were pretreated with Indo (10 mg/kg, ip), 25 min before acetic acid. Each **ST1**-administered group was pretreated with 1, 5, or 10 mg/kg ip 25 min before acetic acid. At 5 min after the ip injection of acetic acid, the number of writhing and stretching was recorded.

Formalin Test. The antinociceptive activity of the drugs was determined using the formalin test (*13*). A total of 20 μL of 5% formalin was injected into the dorsal surface of the right hind paw of mice 30 min after ip administration of **ST1** (1, 5, and 10 mg/kg) or Indo. The mice were observed for 30 min after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min after formalin injection is referred to as the early phase, and the period between 15 and 40 min is referred to as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch. The activity was recorded in 5 min intervals.

Carr-Induced Edema. The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity (*13*). Animals were ip-treated with **ST1** (1, 5, and 10 mg/kg), Indo, or normal saline, 30 min prior to injection of 1% Carr (50 μL) in the plantar side of the 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. 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.

Total Protein Assay. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

MDA Assay. MDA from the Carr-induced edema foot was evaluated by the thiobarbituric acid reacting substances (TBARS) method (*13*). 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 Nitric Oxide/Nitrite. NO production was indirectly assessed by measuring the nitrite levels in serum determined by a colorimetric method based on the Griess reaction (*13*). Serum samples were diluted 4 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 10000*g* for 5 min at room temperature, 100 μL of supernatant was applied to a microtiter plate well, followed by 100 μL of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid). After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro Reader (Molecular Devices, Sunnyvale, CA). Using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

Measurement of Serum TNF-α by an Enzyme-Linked Immunosorbent Assay (ELISA). Serum levels of TNF-α were determined using a commercially available ELISA kit (Biosource International, Inc., Camarillo, CA) according to the instructions of the manufacturer. TNF-α was determined from a standard curve. The concentrations were expressed as pg/mL.

Antioxidant Enzyme Activity Measurements. The following biochemical parameters were analyzed to check the hepatoprotective activity of **ST1** by the methods given below.

Total SOD activity was determined by the inhibition of cytochrome *c* reduction (*14*). 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 CAT activity was based on that of Aebi (*15*). In brief, the reduction of 10 mM H_2O_2 in 20 mM phosphate buffer (pH 7.0) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a

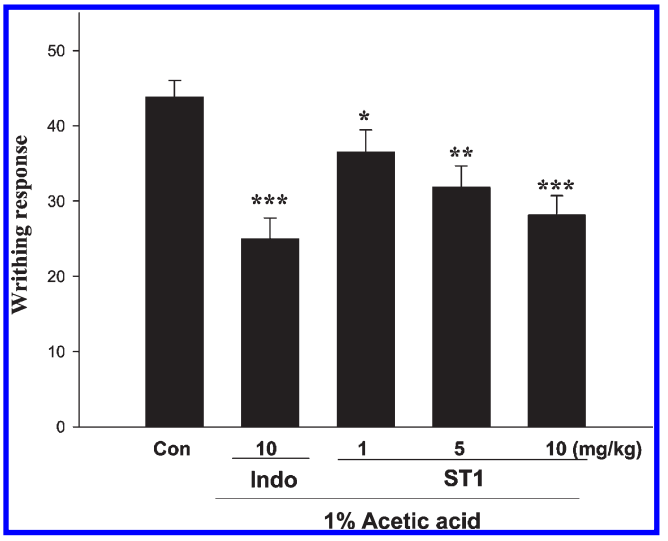

Figure 2. Analagesic effects of **ST1** and Indo on acetic-acid-induced writhing response in mice. Each value represents the mean ± SEM. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared to the pathological model group (Con) (one-way ANOVA followed by Scheffe's multiple range test).

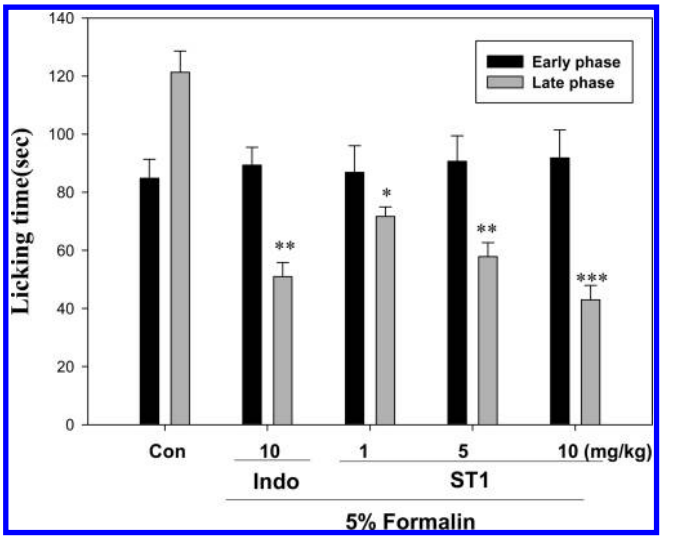

Figure 3. Effects of **ST1** and Indo on the early and late phases in formalin test in mice. Each value represents the mean ± SEM. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared to the pathological model group (Con) (one-way ANOVA followed by Scheffe's multiple range test).

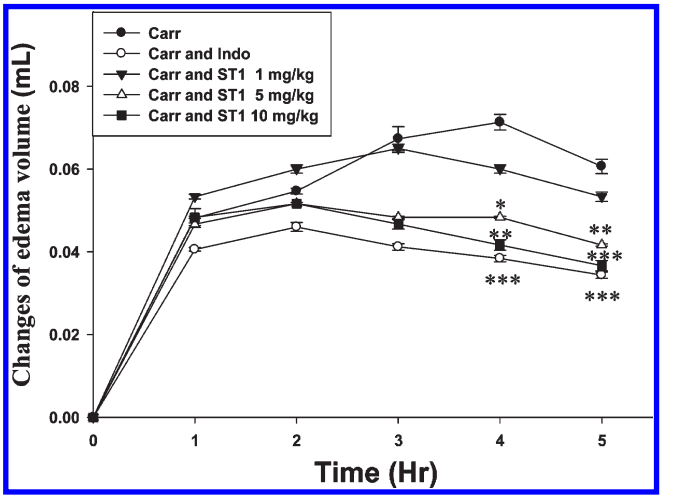

Figure 4. Effects of **ST1** and Indo on hind paw edema induced by Carr in mice. Each value represents the mean ± SEM. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared to the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram of protein per minute. Total GPx activity in cytosol was determined according to Paglia and Valentine's method (*16*). 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 of protein per minute.

Western Blot Analysis of iNOS and COX-2. Soft tissues were removed from individual mice paws and homogenized in a solution containing 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, 1 μM pepstatin, and 10 μM leupeptin. The homogenates were centrifuged at 12000*g* for 20 min, and 30 μg of protein from the supernatants was then separated on 10% sodium dodecyl sulphate−polyacrylamide gel electrophoresis (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 at 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 for 2 h at room temperature. The membranes were washed 3 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, St. Louis, MO) in 2.5% skim milk in TBST for 1 h at room temperature. The membranes were washed 3 times, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). 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 1.85% formaldehyde and 1% acetic acid for 1 week at room temperature, dehydrated by graded ethanol, and embedded in paraffin (Sherwood Medical). Sections (thickness of 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 **ST1**-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 number of neutrophils was counted in each scope (400×), and thereafter, we obtain their average count from five scopes of every tissue slice.

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) followed by Scheffe's multiple range test. Statistical significance is expressed as (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

RESULTS

Effects of ST1 on Acetic-Acid-Induced Writhing Response. The cumulative amount of abdominal stretching correlated with the level of acetic-acid-induced pain (**Figure 2**). **ST1** treatment (1 mg/kg) significantly inhibited the number of writhing in comparison to the normal controls ($p < 0.05$). **ST1** (5 or 10 mg/kg) further reduced the number of writhing ($p < 0.01$ or $p < 0.001$), but Indo (10 mg/kg) demonstrates more inhibition than **ST1**.

Formalin Test. ST1 (1 mg/kg) significantly ($p < 0.05$) inhibited formalin-induced pain in the late phase (**Figure 3**); however, it did

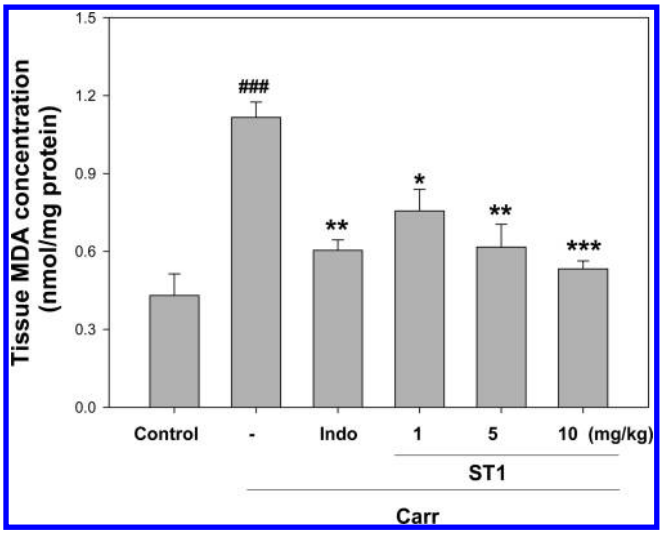

Figure 5. Effects of **ST1** and Indo on the tissue MDA concentration of foot in mice. Each value represents the mean ± SEM. (###) $p < 0.001$ as compared to the control group. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared to the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

not show any inhibition in the early phase. The positive control Indo (5 or 10 mg/kg) also significantly ($p < 0.01$ or $p < 0.001$) inhibited the formalin induced pain in the late phase.

Effects of ST1 on Carr-Induced Mice Paw Edema. Carr-induced paw edema is shown in **Figure 4**. **ST1** (5 or 10 mg/kg) inhibited ($p < 0.01$ or $p < 0.001$) 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 ($p < 0.001$).

Effects of ST1 on the MDA Level. 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 **ST1** (5 mg/kg) ($p < 0.001$), as well as 10 mg/kg Indo (**Figure 5**).

Effects of ST1 on the NO Level. In **Figure 6A**, the NO level increased significantly in the edema serum at 5 h after Carr injection ($p < 0.001$). **ST1** (5 and 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.

Effects of ST1 on the TNF-α Level. The TNF-α level increased significantly in serum at 5 h after Carr injection ($p < 0.001$). However, **ST1** (5 or 10 mg/kg) decreased the TNF-α level in serum at 5 h after Carr injection ($p < 0.05$ or $p < 0.01$), as well as 10 mg/kg Indo (**Figure 6B**).

Effects of ST1 on Activities of Antioxidant Enzymes. At 5 h after the intrapaw injection of Carr, liver tissues were also 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. CAT, SOD, and GPx activities were increased significantly after treatment with 10 mg/kg **ST1** and 10 mg/kg Indo ($p < 0.01$) (**Table 1**).

Effects of ST1 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 **ST1** on iNOS and COX-2 protein expression was studied by western blot. Equal amounts of protein (30 μg/lane) were resolved by SDS−PAGE and then transferred to a nitrocellulose membrane, and iNOS and COX-2 protein was detected using a specific antibody. The results showed that injection of **ST1** (10 mg/kg) on Carr-induced for 5 h inhibited

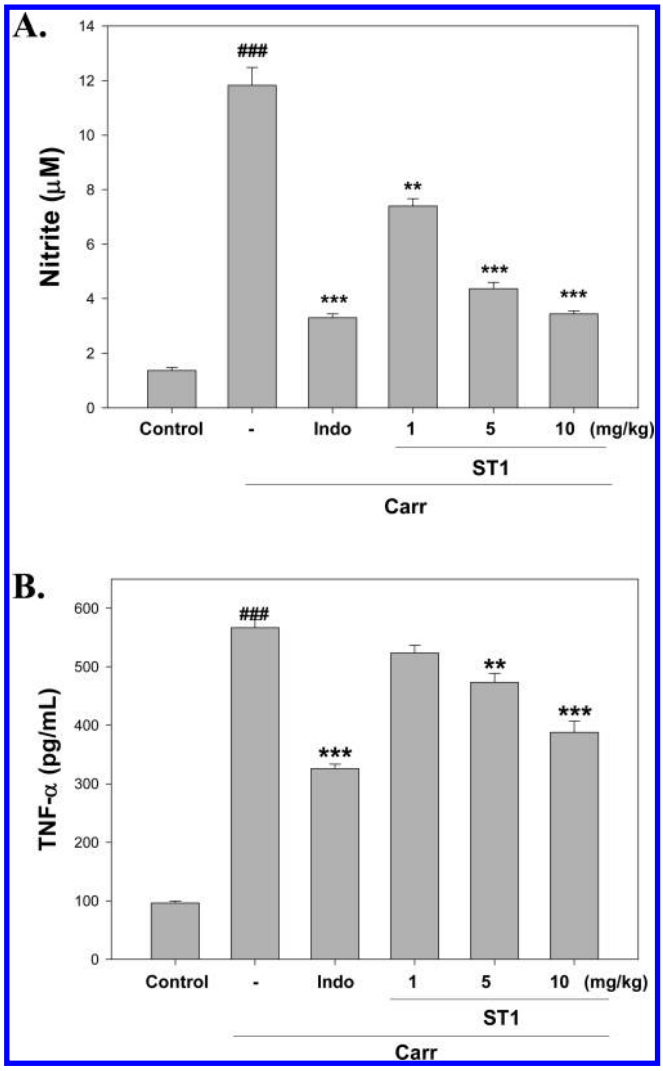

Figure 6. Effects of **ST1** and Indo on Carr-induced (A) NO and (B) TNF-α concentrations of serum at 5 h in mice. Each value represents the mean ± SEM. (###) $p < 0.001$ as compared to the control group. (**) $p < 0.01$ and (***) $p < 0.001$ as compared to the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

iNOS and COX-2 protein expression in mouse paw edema (**Figure 7A**). The detection of β-actin was also performed in the same blot as an internal control. The intensity of protein bands were analyzed using Kodak Quantity software (Molecular Imaging Software System, Kodak) in three independent experiments and showed an average of 67.6 and 63.2% downregulation of iNOS and COX-2 protein, respectively, after treatment with **ST1** at 10 mg/kg compared to Carr-induced alone (**Figure 7B**). In addition, the protein expression showed an average of 35.6 and 43.0% downregulation of iNOS and COX-2 protein after treatment with Indo at 10 mg/kg compared to the Carr-induced alone (**Figure 7B**). The downregulation of iNOS and COX-2 activity of **ST1** (10 mg/kg) was better than Indo (10 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 **ST1** (10 mg/kg) showed a reduction in Carr-induced inflammatory response. Actually, inflammatory cells were reduced in number and 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

Table 1. Effects of **ST1** and Indo on the Liver CAT, SOD, and GPx Activities in Mice[a]

groups	CAT (units/mg of protein)	SOD (units/mg of protein)	GPx (units/mg of protein)
control	4.56 ± 0.47	23.56 ± 0.84	2.18 ± 0.16
Carr	3.26 ± 0.24	15.81 ± 0.87	0.48 ± 0.08
Carr + Indo	3.98 ± 0.16[b]	22.02 ± 0.32[b]	1.54 ± 0.11[b]
Carr + **ST1** (1 mg/kg)	3.63 ± 0.25	19.88 ± 0.49	1.04 ± 0.04
Carr + **ST1** (5 mg/kg)	3.81 ± 0.06[c]	20.45 ± 0.45[c]	1.27 ± 0.14[c]
Carr + **ST1** (10 mg/kg)	4.03 ± 0.08[b]	21.97 ± 0.98[b]	1.62 ± 0.23[b]

[a] Each value represents the mean ± SEM. [b] $p < 0.01$ as compared to the Carr group (one-way ANOVA followed by Scheffe's multiple range test). [c] $p < 0.05$ as compared to the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

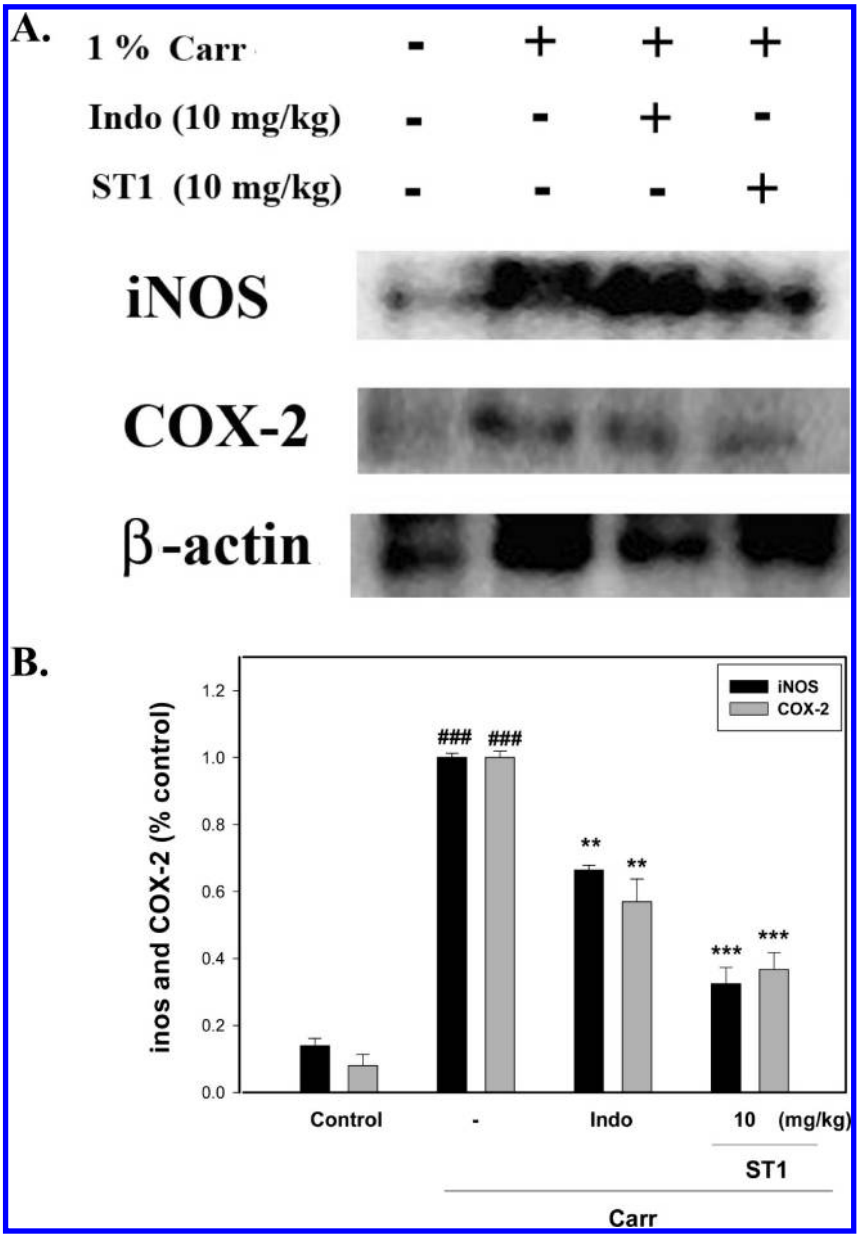

Figure 7. Inhibition of iNOS and COX-2 protein expression by **ST1** induced by Carr in mouse paw edema for 5 h. Tissue suspended were then prepared and subjected to western blotting using an antibody specific for iNOS and COX-2. β-actin was used as an internal control. (A) Representative western blot from two separate experiments is shown. (B) Both relative iNOS and COX-2 protein levels were calculated with reference to Carr-injected mice compared to the control group. Each value represents the mean ± SEM. (###) $p < 0.001$ as compared to the control group. (**) $p < 0.01$ and (***) $p < 0.001$ as compared to the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

hypoderm connective tissue was not damaged (**Figure 8**). Neutrophils increased with Carr treatment ($p < 0.001$). Indo and **ST1** (10 mg/kg) could significantly decrease the neutrophil numbers as compared to the Carr-treated group ($p < 0.01$ or $p < 0.001$) (**Figure 8E**).

DISCUSSION

We have evaluated the putative analgesic and anti-inflammatory activities of **ST1** to clarify the pain- and inflammation-relieving effects. Two different analgesic testing methods were

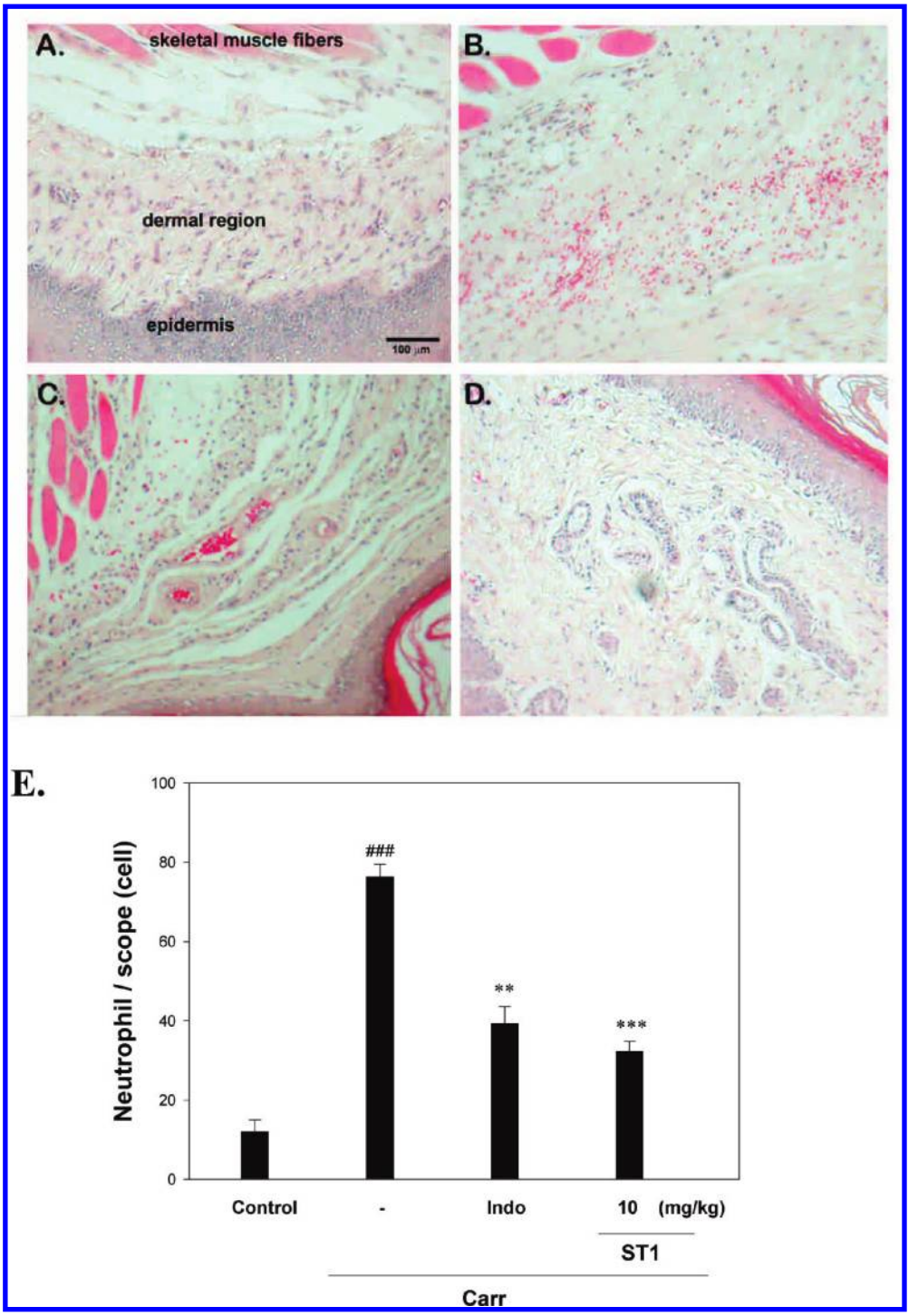

Figure 8. Representative light micrographs of mouse hind footpad H&E stained to reveal hemorrhage, edema, and inflammatory cell infiltration in (A) control mice, (B) Carr-treated mice demonstrating hemorrhage with moderately extravascular red blood cells and a large amount of inflammatory leukocytes mainly neutrophil infiltration in the subdermis interstitial tissue of mice, and (C) mice given Indo (10 mg/kg) before Carr. **ST1** significantly shows (D) morphological alterations (100×) and (E) the number of neutrophils in each scope (400×) compared to subcutaneous injection of Carr only. (###) $p < 0.001$ as compared to the control group. (**) $p < 0.01$ and (***) $p < 0.001$ compared to the Carr group. Scale bar = 100 μm.

employed with the objective of identifying possible peripheral and central effects of the test substances. The acetic writhing test is normally used to study the peripheral analgesic effects of drugs. Although this test is non-specific (e.g., anti-cholinergic, anti-histaminic, and other agents also show activity in the test), it is widely used for analgesic screening (*17*). In our study, we found that **ST1** (1, 5, and 10 mg/kg) exhibited antinociceptive effect in acetic-acid-induced writhing response (**Figure 2**). This effect may be due to the inhibition of the synthesis of the arachidonic acid metabolites (*18*).

In the *in vivo* model of pain, formalin-induced paw pain has been well-established as a valid model for analgesic study. The formalin test produces a distinct biphasic response, and different analgesics may act differently in the early and late phases of this test. Therefore, the test can be used to clarify the possible mechanism of an antinociceptive effect of a proposed analgesic. Centrally acting drugs, such as opioids, inhibit both phases equally, but peripherally acting drugs, such as aspirin, Indo, and dexamethasone, only inhibit the late phase (*19*). The inhibitory

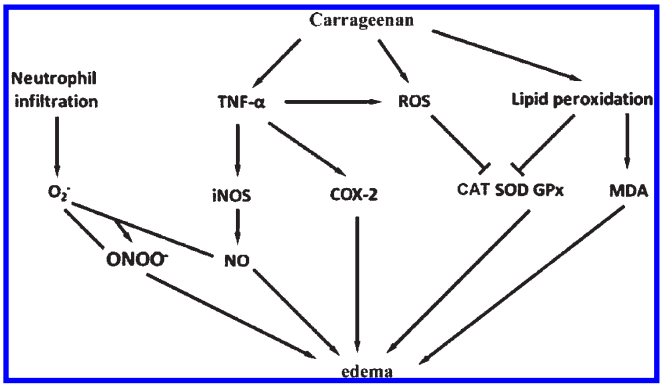

Figure 9. Proposed mechanism of **ST1** in Carr-injected mouse. **ST1** inhibits the production of TNF-α, ROS, and lipid peroxidation, which in turn decreases the MDA level and iNOS and COX-2 activation in the paw edema and increases CAT, SOD, and GPx activities in the liver. MDA, malondialdehyde; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; NO, nitric oxide; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; iNOS, inducible nitric oxide synthase; COX-2, cycloxyclase-2.

effect of **ST1** on the nociceptive response in the late phase of the formalin test suggested that the antinociceptive effect of **ST1** could be due to its peripheral action (**Figure 3**).

The Carr test is highly sensitive to non-steroidal anti-inflammatory drugs and has long been accepted as a useful phlogistic tool for investigating new drug therapies (*20*). The degree of swelling of the Carr-injected paws was maximal at 3 h after injection. Statistical analysis revealed that **ST1** and Indo significantly inhibited the development of edema at 4 h after treatment ($p < 0.01$ or $p < 0.001$) (**Figure 4**). They both showed anti-inflammatory effects in Carr-induced mice edema paw. 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 (*21*) 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. In addition, the classification of antinociceptive drugs is usually based on their mechanism of action on either the central or peripheral nervous systems (*22*).

In the studies of the mechanism on inflammation, the L-arginine−NO pathway has been proposed to play an important role in the Carr-induced inflammatory response (*23*). Our present results also confirm that the Carr-induced paw edema model results in the production of NO (**Figure 9**). The expression of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation (*24*). In our study, the level of NO was decreased significantly by treatment with 1, 5, and 10 mg/kg **ST1**. We suggest that the anti-inflammatory mechanism of **ST1** may be through the L-arginine−NO pathway because **ST1** significantly inhibits NO production (**Figure 6A**).

TNF-α is a major mediator in inflammatory responses, inducing innate immune responses by activating T cells and macrophages and stimulating secretion of other inflammatory cytokines (*25*). Also, TNF-α 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 (*26*). In this study, we found that **ST1** decreased the TNF-α level in serum after Carr injection by treatment with 5 and 10 mg/kg **ST1** significantly (**Figure 6B**).

The wild-type fruiting body of AC is well-known as an effective and expensive folk remedy for many diseases. The anti-inflammatory activity of AC has been suggested to contribute to the prevention of neurodegenerative diseases through suppression of both the inducible NO and COX-2 expression in mouse microglia cell line (*27*). Furthermore, Rao et al. showed that methanol extracts from AC inhibited macrophage-mediated inflammatory mediators, such as NO and TNF-α, in LPS/IFNγ-activated mouse peritoneal macrophages (*28*). The aqueous extract from submerged cultivation mycelium (SCM) dose-dependently (25−100 μg/mL) inhibited the lipopolysaccharide (LPS)-induced NO, TNF-α, interleukin (IL)-1β, and prostaglandin E2 production and iNOS and COX-2 protein expression via NF-κ B pathway in macrophages (*29*). In *in vivo* data, the hexane extract (100, 200, and 400 μg/mL) from SCM of AC has protection from nephritis by suppression of the urine protein and serum blood urea nitrogen levels and decreased the thickness of the kidney glomerular basement membrane in SLE-prone NZB/W F1 mice (*30*). In this study, there are significant decreases in iNOS and COX-2 activities with **ST1** treatment (**Figure 7**). Furthermore, there are significant decreases in the NO level with **ST1** treatment (**Figure 5**). We assume that the suppression of NO production is probably due to decreased iNOS and COX-2 activities.

The Carr-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radicals, as well as the release of other neutrophil-derived mediators (*13*). Some research demonstrates that the inflammatory effect induced by Carr is associated with the free radical. The free radical, prostaglandin, and NO will be released when administrating with Carr for 1−6 h. The edema effect was raised to the maximum at 3 h. MDA production is due to the free radical attack of plasma membrane (*13*). Thus, the inflammatory effect would result in the accumulation of MDA. Glutathione (GSH) is a known oxyradical scavenger. Enhancing the level of GSH conducive toward favor reduces MDA production. Endogenous GSH plays an important role against Carr-induced local inflammation. In this study, there is a significant increase in CAT, SOD, and GPx activities with **ST1** treatment (**Table 1**). Furthermore, there is a significant decrease in the MDA level with **ST1** treatment (**Figure 5**). We assume that the suppression of MDA production is probably due to the increase of CAT, SOD, and GPx activities.

In conclusion, these results suggested that **ST1** possessed analgesic and anti-inflammatory effects. The anti-inflammatory mechanism of **ST1** may be related to iNOS and associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). **ST1** may be used as a pharmacological agent in the prevention or treatment of disease in which free-radical formation is a pathogenic factor.

LITERATURE CITED

(1) Peng, C. C.; Chen, K. C.; Peng, R. Y.; Chyau, C. C.; Su, C. H.; Hsieh-Li, H. M. *Antrodia camphorata* extract induces replicative senescence in superficial TCC, and inhibits the absolute migration capability in invasive bladder carcinoma cells. *J. Ethnopharmacol.* **2007**, *109*, 93–103.

(2) Geethangili, M.; Tzeng, Y. M. Review of pharmacological effects of *Antrodia camphorata* and its bioactive compounds. *Evidence-Based Compl. Altern. Med.* **2009**, DOI: 10.1093/ecam/nep108.

(3) Shen, Y. C.; Wang, Y. H.; Chou, Y. C.; Chen, C. F.; Lin, L. C.; Chang, T. T.; Tien, J. H.; Chou, C. J. Evaluation of the anti-inflammatory activity of zhankuic acids isolated from the fruiting bodies of *Antrodia camphorata*. *Planta Med.* **2004**, *70*, 310–314.

(4) Hsiao, G.; Shen, M. Y.; Lin, K. H.; Lan, M. H.; Wu, L. Y.; Chou, D. S.; Lin, C. H.; Su, C. H.; Sheu, J. R. Antioxidative and hepatoprotective effects of *Antrodia camphorata* extract. *J. Agri. Food Chem*. **2003**, *51*, 3302–3308.
(5) Shen, Y. C.; Chou, C. J.; Wang, Y. H.; Chen, C. F.; Chou, Y. C.; Lu, M. K. Anti-inflammatory activity of the extracts from mycelia of *Antrodia camphorata* cultured with water-soluble fractions from five different *Cinnamomum* species. *FEMS Microbiol. Lett*. **2004**, *231*, 137–143.
(6) Wang, G. J.; Tseng, H. W.; Chou, C. J.; Tsai, T. H.; Chen, C. T.; Lu, M. K. The vasorelaxation of *Antrodia camphorata* mycelia: Involvement of endothelial Ca^{2+}−NO−cGMP pathway. *Life Sci*. **2003**, *73*, 2769–2783.
(7) Yeh, C. T.; Rao, Y. K.; Yao, C. J.; Yeh, C. F.; Li, C. H.; Chuang, S. E.; Luong, J. H.; Lai, G. M.; Tzeng, Y. M. Cytotoxic triterpenes from *Antrodia camphorata* and their mode of action in HT-29 human colon cancer cells. *Cancer Lett*. **2009**, *285*, 73–79.
(8) Lee, I. H.; Huang, R. L.; Chen, C. T.; Chen, H. C.; Hsu, W. C.; Lu, M. K. *Antrodia camphorata* polysaccharides exhibit anti-hepatitis B virus effects. *FEMS Microbiol. Lett*. **2002**, *209*, 63–67.
(9) Song, T. Y.; Yen, G. C. Protective effects of fermented filtrate from *Antrodia camphorata* in submerged culture against CCl_4-induced hepatic toxicity in rats. *J. Agric. Food Chem*. **2003**, *51*, 1571–1577.
(10) Janero, D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biol. Med*. **1990**, *9*, 515–540.
(11) Shao, Y. Y.; Chen, C. C.; Wang, H. Y.; Chiu, H. L.; Hseu, T. H.; Kuo, Y. H. Chemical constituents of *Antrodia camphorata* submerged whole broth. *Nat. Prod. Res*. **2008**, *22*, 1151–1157.
(12) Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **1983**, *16*, 109–110.
(13) Chang, H. Y.; Sheu, M. J.; Yang, C. H.; Leu, Z. C.; Chang, Y. S.; Peng, W. H.; Huang, S. S.; Huang, G. J. Analgesic effects and the mechanisms of anti-inflammation of hispolon in mice. *Evidence-Based Compl. Altern. Med*. **2009**, DOI: 10.1093/ecam/nep027.
(14) Flohe, L.; Otting, F. Superoxide dismutase assays. *Methods Enzymol*. **1984**, *105*, 93–104.
(15) Aebi, H. Catalase *in vitro*. *Methods Enzymol*. **1984**, *105*, 121–126.
(16) Paglia, E. D.; Valentine, W. N. Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidase. *J. Lab. Clin. Med*. **1967**, *70*, 158–169.
(17) Shibata, M.; Ohkubo, T.; Takahashi, H.; Inoki, R. Modified formalin test: Characteristic biphasic pain response. *Pain* **1989**, *38*, 347–352.
(18) Franzotti, E. M.; Santos, C. V.; Rodrigues, H. M.; Mourao, R. H.; Andrade, M. R.; Antoniolli, A. R. Anti-inflammatory, analgesic activity and acute toxicity of *Sida cordifolia* L. (Malva-branca). *J. Ethnopharmacol*. **2000**, *72*, 273–727.
(19) Tjolsen, A.; Berge, O. G.; Hunskaar, S.; Rosland, J. H.; Hole, K. The formalin test: An evaluation of the method. *Pain* **1992**, *51*, 5–17.
(20) Spector, W. G.; Willoughb, D. A. The inflammatory response. *Bacteriol. Rev*. **1963**, *27*, 117–154.
(21) Tohda, C.; Nakayama, N.; Hatanaka, F.; Komatsu, K. Comparison of anti-inflammatory activities of six *Curcuma* rhizomes: A possible curcuminoid-independent pathway mediated by *Curcuma phaeocaulis* extract. *Evidence-Based Compl. Altern. Med*. **2006**, *3*, 255–260.
(22) Salvemini, D.; Wang, Z.; Bourdon, D. M.; Stern, M. K.; Curne, M. G.; Manning, P. T. Evidence of peroxynitrite involvement in the carrageenan induced rat paw edema. *Eur. J. Clin. Pharmacol*. **1996**, *303*, 217–220.
(23) Cuzzocrea, S.; Zingarelli, B.; Calapai, G.; Nava, F.; Caputi, A. P. Zymosan-activated plasma induces paw oedema by nitric oxide and prostaglandin production. *Life Sci*. **1997**, *60*, 215–220.
(24) Pan, M. H.; Lai, C. S.; Dushenkov, S.; Ho, C. T. Modulation of inflammatory genes by natural dietary bioactive compounds. *J. Agric. Food Chem*. **2009**, *57*, 4467–4477.
(25) Yun, K. J.; Koh, D. J.; Kim, S. H.; Park, S. J.; Ryu, J. H.; Kim, D. G.; Lee, J. Y.; Lee, K. T. Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase, cyclooxygase-2, and proinflammatory cytokines expressions via nuclear factor-κB inactivation. *J. Agric. Food Chem*. **2008**, *56*, 10265–10272.
(26) Dawson, J.; Sedgwick, A. D.; Edwards, J. C.; Lees, P. A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int. J. Tissue React*. **1991**, *13*, 171–185.
(27) Chien, S. C.; Chen, M. L.; Kuo, H. T.; Tsai, Y. C.; Lin, B. F.; Kuo, Y. H. Anti-inflammatory activities of new succinic and maleic derivatives from the fruiting body of *Antrodia camphorata*. *J. Agric. Food Chem*. **2008**, *56*, 7017–7022.
(28) Rao, Y. K.; Fang, S. H.; Tzeng, Y. M. Evaluation of the anti-inflammatory and anti-proliferation tumoral cells activities of *Antrodia camphorata*, *Cordyceps sinensis*, and *Cinnamomum osmophloeum* bark extracts. *J. Ethnopharmacol*. **2007**, *114*, 78–85.
(29) Hseu, Y. C.; Wu, F. Y.; Wu, J. J.; Chen, J. Y.; Chang, W. H.; Lu, F. J.; Lai, Y. C.; Yang, H. L. Antiinflammatory potential of *Antrodia camphorata* through inhibition of iNOS, COX-2 and cytokines via the NF-kB pathway. *Int. Immunopharmacol*. **2005**, *5*, 1914–1925.
(30) Chang, J. M.; Lee, Y. R.; Hung, L. M.; Liu, S. Y.; Kuo, M. T.; Wen, W. C.; Chen, P. An extract of *Antrodia camphorata* mycelia attenuates the progression of nephritis in systemic lupus erythematosus-prone NZB/W F1 mice. *Evidence-Based Compl. Altern. Med*. **2008**, DOI: 10.1093/ecam/nen057.

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