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Analgesic and Anti-Inflammatory Bioactivities of Eburicoic Acid and Dehydroeburicoic Acid Isolated from *Antrodia camphorata* on the Inflammatory Mediator Expression in Mice

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ABSTRACT: Eburicoic acid (**TR1**) and dehydroeburicoic acid (**TR2**), an active ingredient from *Antrodia camphorata* (AC) solid-state culture, were evaluated for analgesic and anti-inflammatory effects. Treatment with **TR1** and **TR2** significantly inhibited a number of acetic acid-induced writhing responses and formalin-induced pain in the late phase. In the anti-inflammatory test, **TR1** and **TR2** decreased paw edema at the fourth and fifth hour after λ-carrageenan (Carr) administration and increased the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the paw edema tissue. We also demonstrated that **TR1** and **TR2** significantly attenuated the malondialdehyde (MDA), nitric oxide (NO), tumor necrosis factor (TNF-α), and interleukin-1β (IL-1β) levels in either edema paw or serum at the fifth hour after Carr injection. Western blotting revealed that **TR1** and **TR2** decreased Carr-induced inducible nitric oxide synthase (iNOS) and cycloxyclase (COX-2) expressions at the fifth hour in paw edema. Treatment with **TR1** and **TR2** also diminished neutrophil infiltration into the paw edema at the fifth hour. The present study suggests that the anti-inflammatory mechanisms of **TR1** and **TR2** might be related to the decrease of inflammatory cytokines and an increase of antioxidant enzyme activity.

KEYWORDS: *Antrodia camphorata, eburicoic acid, dehydroeburicoic acid, anti-inflammation, MDA, NO, TNF-α*

INTRODUCTION

Antrodia cinnamomea (AC; Polyporaceae, Aphyllophorales) is a parasitic microorganism found on the wall of the inner cavity of *Cinnamomum kanehirai* Hay. It has been used for the treatment of food and drug intoxication, diarrhea, abdominal pain, hypertension, skin itching, and cancer.[1] Because of the potential pharmaceutical value of its biologically active ingredients, the fruiting bodies of AC are regarded as one of the healthy treasure troves of Taiwan. The fruiting body and cultured mycelia of AC contain fatty acids, lignans, phenylderivatives, sesquiterpenes, steroids, and triterpenoids.[2] The triterpenoids, a large and structurally diverse group of natural products derived from squalene or related acyclic 30-carbon precursors, are uniquely abundant in AC, especially in its fruiting bodies. The large group of natural products displays well over 100 distinct skeletons and has well-characterized biological activities.[3] Some researchers have purified a number of triterpenoids from fruiting bodies and cultured mycelia of AC and evaluated their immunostimulatory and anti-inflammatory activity.[4,5] Eburicoid acid (**TR1**) was isolated from *Fomes pinicola*,[6] *Lenzites thermophila*,[7] and AC.[8] Dehydroeburicoic acid (**TR2**) was isolated from *Poria cocos*[9] and AC.[8] **TR2** had inhibitory activity against human 5-hydroxytryptamine 3A (5-HT (3A)) receptor channel activity,[9] induced calcium- and calpain-dependent necrosis in human U87MG glioblastomas,[10] and exhibited significant selective cytotoxic effects to cancer cells.[11]

Many scientific papers have reported that the inflammatory effect induced by Carr could be associated with free radical formation. Free radical, prostaglandin, and NO will be released when administrating with Carr for 1−5 h. The edema effect was raised to maximum at the 3rd hour, and its MDA production was due to free radical attack of the plasma membrane.[12] Thus, the inflammatory effect would result in the accumulation of MDA.[13] Therefore, in this article we examined the analgesic effects of **TR1** and **TR2** on nociception induced by acetic acid and formalin. We also evaluated the anti-inflammatory effects of **TR1** and **TR2** on paw edema induced by Carr in mice, and we detected the levels of MDA, NO, TNF-α, IL-1β, iNOS, and COX-2 in either paw edema or serum. Also, the activities of

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CAT, SOD, and GPx in the edema paw at the fifth hour after Carr injection were investigated to understand the relationship between the anti-inflammatory mechanism of **TR1** and **TR2** and antioxidant enzymes.

MATERIALS AND METHODS

Chemicals. Acetic acid was purchased from Merck (Darmstadt, Germany). λ-Carrageenan (Carr) (type IV) and indomethacin (Indo) were obtained from Sigma (St. Louis, MO, USA). Formalin was purchased from Nihon Shiyaku Industries (Japan). TNF-α and IL-1β were purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, and anti-β-actin antibody (Santa Cruz, USA) 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, USA).

Plant Material. The solid culture of the mix of mycelium and a little of the fruiting bodies of *A. camphorata* were identified and provided by Well Shine Biotechnology Development, Taipei, Taiwan. A voucher speciment was deposited at Well Shine Biotechnology Department Co. Ltd. The preparative processes were all proprietary.

Isolation and Determination of the Active Compound. Freeze-dried powder of AC of the submerged whole broth (1.6 kg) was extracted three 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 HPLC. Eburicoic acid (**TR1**) and dehydroeburicoic acid (**TR2**) (5.4 g) (Figure 1) was eluted with 10% EtOAc in hexane and

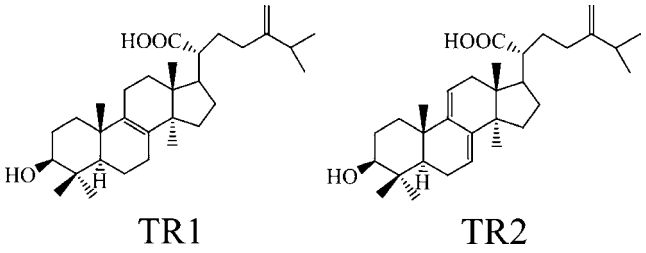

Figure 1. Chemical structure of eburicoid acid (**TR1**) and dehydroeburicoic acid (**TR2**).

recrystallization with EtOH.[8,10] The yield of TR1 and TR2 was obtained about 0.1% and 0.2% (w/w). The purities of **TR1** and **TR2** were higher than 99%.

TR1. ^{1}H NMR (300 MHz, pyridine-d_5): δ 3.41 (1H, br t, J = 7.6 Hz, H-3), 1.00 (3H, s, H-18), 1.06 (3H, s, H-19), 2.63 (1H, td, J = 2.4, 10.6 Hz, H-20), 2.27 (1H, m, H-25), 1.00(3H, H-26 or H-27), 1.01 (3H, H-27 or H-26), 4.87 (1H, br s, H-28a), 4.91 (1H, br s, H-28b), 1.05 (3H, s, H-29), 1.22 (3H, s, H-30), 1.00 (3H, s, H-31)

TR2. ^{1}H NMR (300 MHz, pyridine-d_5): δ 1.90 (2H, m, H-2), 3.43 (1H, t, J = 7.5 Hz, H-3), 1.26 (1H, H-5), 2.16 (2H, H-6), 5.61 (1H, br s, H-7), 5.36 (1H, d, J = 5.1 Hz, H-11), 2.50 (1H, H-12α), 2.33 (1H, H-12β), 0.99 (3H, s, H-18), 1.19 (3H, s, H-19), 2.64 (1H, td, J = 11.0, 3.0 Hz, H-20), 2.29 (1H, H-25), 1.02 (3H, d, J = 3.0 Hz, H-26 or H-27), 1.00 (3H, d, J = 3.0 Hz, H-27 or H-26), 4.88 (1H, br s, H-28α), 4.92 (1H, br s, H-28β), 1.11 (3H, s, H-29), 1.05 (6H, s, H-30, 31)

Animals. Six to eight week male ICR mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ± 1 °C and a relative humidity of 55 ± 5% with a 12 h dark-light cycle for at least 2 weeks before the experiment. They were given food and water ad libitum. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. All tests were conducted under the guidelines of the International Association for the Study of Pain.

After a 2-week adaptation period, male ICR mice (18−25 g) were randomly assigned to five groups (n = 6) for acetic acid-induced writhing (1%, 0.l mL/10 g i.p.) and formalin-induced licking (5%, 20 μL/per mice i.p.) experiments. These experiments include a pathological model group (received acetic acid or formalin), a positive control (acetic acid or formalin + Indo), and **TR1**- and **TR2**-administered groups (acetic acid or formalin + **TR1** or **TR2**). In the Carr-induced edema experiment, there were 6 groups (n = 6) of animals. The control group receives normal saline (i.p.). The other five groups included a Carr-treated, a positive control (Carr + Indo), and **TR1**- and **TR2**-administered groups (Carr + **TR1** or **TR2**).

Acetic Acid-Induced Writhing Response. The test was performed as described by Chang et al.[14] Writhing was induced by an intraperitoneal (i.p.) injection of 0.1 mL/10 g acetic acid solution (10 mL/kg). Positive control animals were pretreated with Indo (10 mg/kg, i.p.) 25 min before acetic acid. Each **TR1**- and **TR2**-administered group was pretreated with 1 mg/kg, 5 mg/kg, or 10 mg/kg i.p. 25 min before acetic acid. Five minutes after the i.p. injection of acetic acid, the number of writhing and stretching events was recorded.

Formalin Test. The antinociceptive activity of the drugs was determined using the formalin test.[14] Twenty microliters of 5% formalin was injected into the dorsal surface of mice for 30 min after the administration of **TR1** and **TR2** (1, 5, and 10 mg/kg, i.p.), or Indo (10 mg/kg, i.p.). 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 post-formalin injection is referred to as the early phase and the period between 15 and 40 min 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 the determination of anti-inflammatory activity.[15] Animals were i.p. treated with **TR1** or **TR2** (1, 5, and 10 mg/kg), Indo (10 mg/kg), or normal saline 30 min prior to the injection of 1% Carr (50 μL) into 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 was the volume of the right hind paw after Carr treatment, and b was the volume of the right hind paw before Carr treatment. Indo was used as a positive control. After 5 h, the animals were sacrificed, and the Carr-induced edema feet were dissected and stored at −80 °C. Also, blood was withdrawn and kept at −80 °C.

In the secondary experiment, the right hind paw tissue and paw edema tissue were taken at the fifth hour. The right hind paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline four times its volume and homogenized at 4 °C. Then the homogenate was centrifuged at 12,000g for 5 min. The supernatant was obtained and stored at −20 °C for MDA assays. The whole paw edema tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline one times its volume and homogenized at 4 °C. Then the homogenate was centrifuged at 12,000g for 5 min. The supernatant was obtained and stored at −20 °C for the antioxidant enzyme (CAT, SOD, and GPx) activity assays. The protein concentration of the sample was determined by the Bradford dye binding assay (Bio-Rad, Hercules, CA).

MDA Assay. Lipid peroxidation levels were evaluated by the method of thiobarbituric acid reactive substances (TBARS).[15] Briefly, paw edema tissue was homogenized in ice-cold KCl (1.15%), and the homogenate was mixed with 1% H_3PO_4 and 0.67% thiobarbituric acid (TBA) solution. After heating the mixture for 45 min at 100 °C, n-butanol was added, followed by vigorous vortexing and centrifugation at 3,000g for 15 min. The amount of MDA formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm against a reagent blank.

Measurement of Serum TNF-α and IL-1β by an Enzyme-Linked Immunosorbent Assay (ELISA). Serum levels of TNF-α and IL-1β were determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the

manufacturer's instructions. TNF-α and IL-1β were determined from a standard curve. The concentrations were expressed as pg/mL.

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.[14] Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10,000*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, Orleans Drive, Sunnyvale, CA). By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

Antioxidant Enzyme Activity Measurements. The following biochemical parameters were analyzed to check the antioxidant enzyme activity in paw edema by the methods given below.

Total SOD activity was determined by the inhibition of cytochrome *c* reduction.[16] 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.[17] In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity in cytosol was determined according to the method of Paglia and Valentine.[18] The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2), and the absorbance at 340 nm was measured. The activity was calculated by using a calibration curve of GPx established from bovine whole blood. A linear relationship between the activity (unit/mL) of GPx and the reduction of NADPH absorbance at 340 nm was found, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute.

Western Blot Analysis. Soft tissues were removed from individual mouse paws and homogenized in a solution containing 10 mM CHAPS, 1 mM phenylmethylsulfonyl 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 30 μg of protein from the supernatants was then separated on 10% sodium dodecylsulphate–polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Following transfer, the membrane was blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline–Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, and 0.1% Tween 20). The membranes were then incubated with mouse monoclonal anti-iNOS or anti-COX-2 antibody in 5% skim milk in TBST for 2 h at room temperature. The membranes were washed three times with TBST at room temperature and then incubated with a 1:2000 dilution of antimouse IgG secondary antibody conjugated to horseradish peroxidase (Sigma, St Louis, MO, U.S.A.) 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) by 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 5 μm) were deparaffinized with xylene and stained with H & E stain. All samples were observed and photographed with Nikon microscopy. Three to five tissue slices were randomly chosen from Carr, Indo, and **TR1**- or **TR2**-treated (10 mg/kg) groups. Histological examination of these tissue slices under microscopes revealed an excessive inflammatory response with massive infiltration of neutrophils (polymorphonuclear leukocytes [PMNs]). The numbers of neutrophils were counted in each scope (400×), and thereafter, we obtained their average count from 5 scopes for every tissue slice.

Statistical Analysis. Data are expressed as 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 TR1 and TR2 on Acetic-Induced Writhing Response. The cumulative amount of abdominal stretching correlated with the level of acetic acid-induced pain (Figure 2A). **TR1** and **TR2** treatment (1 mg/kg) significantly inhibited the number of writhing events in comparison with that in the normal controls ($p < 0.05$). **TR1** and **TR2** (5 or 10 mg/kg)

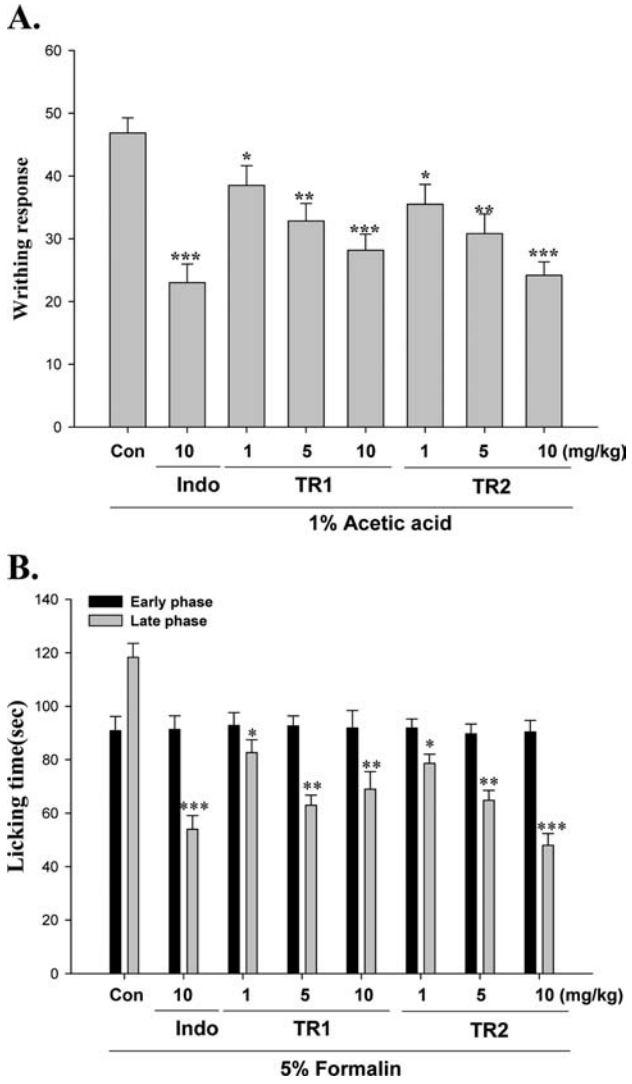

Figure 2. Analagesic effects of **TR1**, **TR2**, and indomethacin (Indo) on acetic acid-induced writhing response (A) and on the early phase and late phase in formalin test (B) in mice. Each value represents the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared with the pathological model group (Con) (one-way ANOVA followed by Scheffe's multiple range test).

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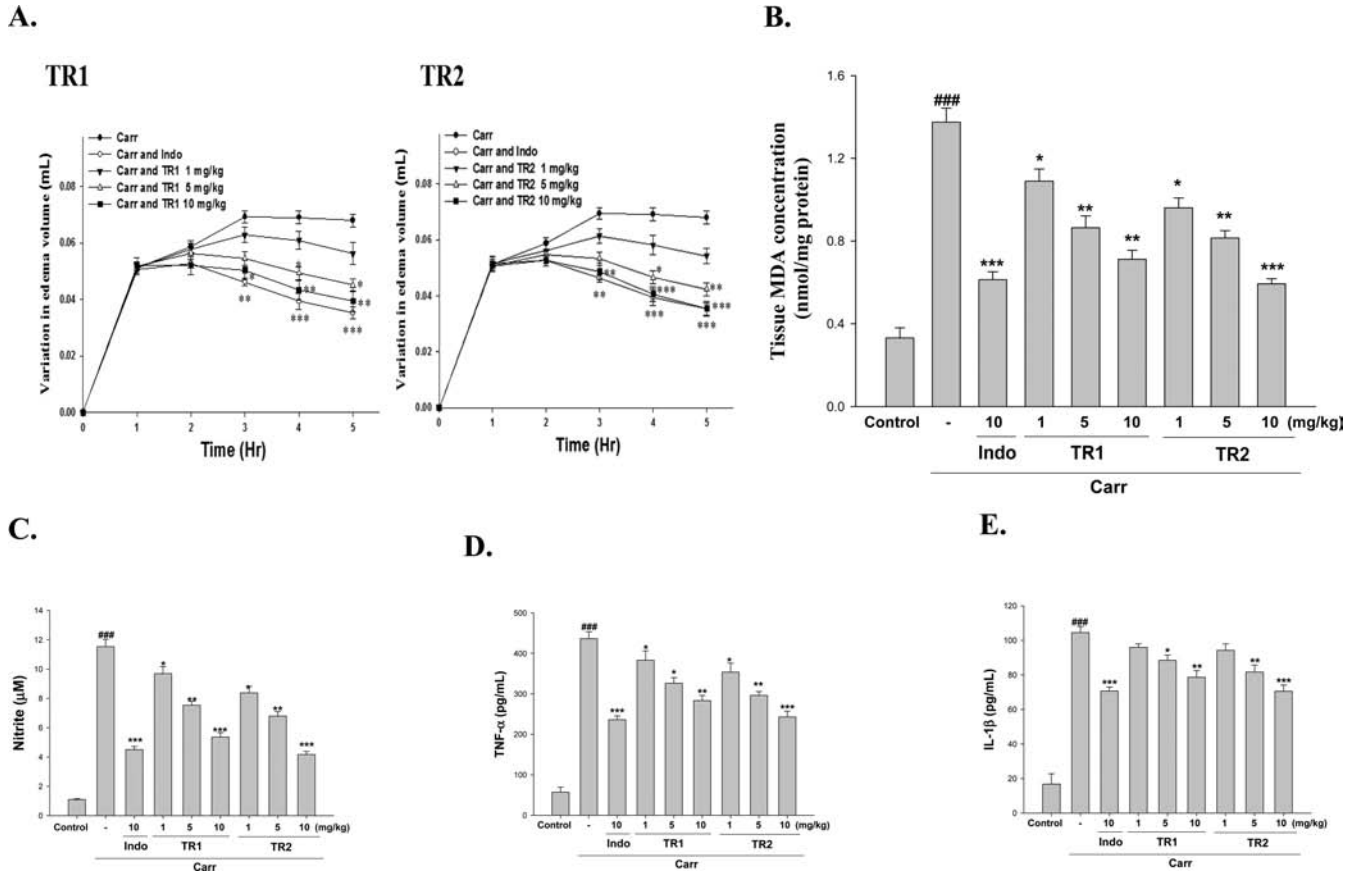

Figure 3. Effects of **TR1**, **TR2**, and Indo on hind paw edema induced by λ-carrageenan (Carr) in mice (A), the tissue MDA concentration of foot in mice (B), Carr-induced NO (C), TNF-α (D), and IL-1β (E) concentrations of serum at the 5th hour in mice. Each value represents the mean ± SEM. $^{###}p < 0.001$ as compared with the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

further reduced the number of writhing events ($p < 0.01$ or $p < 0.001$), but Indo (10 mg/kg) demonstrates much more inhibition than **TR1** and **TR2**.

Formalin Test. **TR1** and **TR2** (1 mg/kg) significantly ($p < 0.05$) inhibited formalin-induced pain in the late phase (Figure 2B); however, it did not show any inhibition in the early phase. The positive control Indo (10 mg/kg), **TR1**, and **TR2** also significantly ($p < 0.01$ or $p < 0.001$) inhibited the formalin-induced pain in the late phase.

Effects of TR1 and TR2 on Carr-Induced Mouse Paw Edema. In this study, we used Carr-induced edema because this model is widely employed for screening the effects of anti-inflammatory drugs. Carr-induced paw edema is shown in Figure 3A. **TR1** and **TR2** (10 mg/kg) significantly inhibited ($p < 0.01$ or $p < 0.001$) the development of paw edema induced by Carr (10 mg/kg) at the fourth hour and the fifth hour after the treatment. With **TR1** and **TR2** at the concentration of 10 mg/kg, the levels of edema volume were decreased to 38.78% and 45.21% of that observed in the Carr alone group. Indo (10 mg/kg) significantly decreased the Carr induced paw edema at the fourth and the fifth hours after the treatment ($p < 0.001$).

Effects of TR1 and TR2 on MDA Level. The MDA level increased significantly in the edema paw at the fifth hour after Carr injection ($p < 0.001$). However, the MDA level was decreased significantly by treatment with **TR1** and **TR2** (10 mg/kg) ($p < 0.01$ or $p < 0.001$), as well as 10 mg/kg Indo (Figure 3B). In the 10 mg/kg, the inhibition mouse MDA levels of **TR1**, **TR2**, and Indo compared with those of the Carr group are 48.18%, 56.93%, and 55.47%, respectively.

Effects of TR1 and TR2 on the TNF-α and IL-1β Levels. The TNF-α and IL-1β levels increased significantly in serum at the fifth hour post-Carr injection ($p < 0.001$). However, **TR1** and **TR2** (10 mg/kg) decreased the TNF-α and IL-1β levels in serum at the fifth hour after Carr injection ($p < 0.01$ or $p < 0.001$), as well as 10 mg/kg Indo (Figure 3C and D). The inhibition of **TR1**, **TR2**, and Indo at 10 mg/kg on the mice serum TNF-α level compared with that in the Carr group is 35.18%, 45.02%, and 45.89%, respectively. The inhibition of **TR1**, **TR2**, and Indo at 10 mg/kg on the mice serum IL-1β level compared with that in the Carr group is 24.81%, 32.49%, and 32.37%.

Effects of TR1 and TR2 on NO Level. In Figure 3D, the NO level increased significantly in the edema serum at the fifth hour post-Carr injection ($p < 0.001$). **TR1** and **TR2** (10 mg/kg) significantly decreased the serum NO level ($p < 0.01$ or $p < 0.001$). Meanwhile, in the 10 mg/kg, the inhibition mice serum NO levels of **TR1**, **TR2**, and Indo could be compared with the Carr group and are 53.64%, 62.76%, and 60.67%. The inhibitory potency was similar to that of Indo (10 mg/kg) at the fifth hour after induction.

Effects of TR1 and TR2 on Activities of Antioxidant Enzymes. At the fifth hour after the intrapaw injection of Carr, paw edema tissues were also analyzed for biochemical parameters such as CAT, SOD, and GPx activities. Carr decreased the activities of CAT, SOD, and GPx in paw edema

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by 48.72%, 43.95%, and 35.21%, respectively, in comparison to the control group. In the 10 mg/kg, **TR1** and **TR2** could increase the activities of CAT about 151.62% and 160.73%, SOD about 153.85% and 161.27%, and GPx about 138.34% and 143.56%, respectively, of that observed in the Carr alone group. Indo also exhibited increased effects in the activities of CAT (174.23%), SOD (163.58%), and GPx (145.92%) in comparison to that in the Carr group ($P < 0.001$) (Table 1). These data implied that the protective effects of **TR1** and **TR2** might be attributed to its elevation in the antioxidant enzyme activities of Carr-induced mice.

Table 1. Effects of TR1, TR2, and Indo on Changes in CAT, SOD, and GPx Activities Were Studied on Carr-Induced Mouse Paw Edema (5th Hour)[a]

groups	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
control	6.36 ± 0.41	25.48 ± 0.58	22.18 ± 1.86
Carr	3.26 ± 0.17	14.28 ± 0.37	14.37 ± 0.92
Carr + Indo	5.68 ± 0.13[d]	23.36 ± 0.55[d]	20.97 ± 0.72[d]
Carr + **TR1** (1 mg/kg)	3.92 ± 0.25	16.12 ± 0.49	15.76 ± 1.07
Carr + **TR2** (1 mg/kg)	4.34 ± 0.22[b]	17.53 ± 0.34[b]	16.49 ± 0.66[b]
Carr + **TR1** (5 mg/kg)	4.13 ± 0.43[b]	18.23 ± 0.56[b]	17.34 ± 0.64[b]
Carr + **TR2** (5 mg/kg)	4.76 ± 0.25[c]	20.21 ± 0.48[c]	18.71 ± 0.61[c]
Carr + **TR1** (10 mg/kg)	4.91 ± 0.33[c]	21.97 ± 0.38[c]	19.88 ± 0.47[c]
Carr + **TR2** (10 mg/kg)	5.42 ± 0.45[d]	23.03 ± 0.33[d]	20.63 ± 0.80[c]

[a]Each value represents the mean ± SEM. [b]$p < 0.05$ as compared with the Carr (λ-carrageenan) group (one-way ANOVA followed by Scheffe's multiple range test). [c]$p < 0.01$ as compared with the Carr (λ-carrageenan) group (one-way ANOVA followed by Scheffe's multiple range test). [d]$p < 0.001$ as compared with the Carr (λ-carrageenan) group (one-way ANOVA followed by Scheffe's multiple range test).

Effects of TR1 and TR2 on Carr-Induced iNOS and COX-2 Protein Expressions in Mouse Paw Edema. In order to investigate whether the inhibition of NO production was due to a modulated iNOS and COX-2 protein level, the effect of **TR1** and **TR2** on iNOS and COX-2 protein expression were studied by Western blot. The results showed that injecting **TR1** and **TR2** (10 mg/kg) into the Carr-induced group for 5 h inhibited iNOS and COX-2 protein expression in mouse paw edema (Figure 4A). β-Actin was used in the same blot as an internal control. The intensity of protein bands was analyzed and showed an average of 62.6% and 61.2% down-regulation of iNOS and COX-2 protein after treatment with **TR1** at 10 mg/kg compared with that in the Carr-induced alone group (Figure 4B). The protein expression showed an average of 69.8% and 73.4% down-regulation of iNOS and COX-2 protein after the treatment with **TR2** at 10 mg/kg compared with that in the Carr-induced alone group (Figure 4B). The down-regulation of iNOS and COX-2 activity of **TR1** and **TR2** (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 **TR1** or **TR2** (10 mg/kg) showed a reduction in Carr-induced inflammatory response. Actually, inflammatory cells were reduced in number and confined to areas 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 hypoderm connective tissue was not damaged (Figure 5A). Neutrophils were noticed to be increased with Carr treatment ($P < 0.001$), as Indo, **TR1**, and **TR2** (10 mg/kg) could significantly decrease the neutrophils numbers as compared to the Carr-treated group ($P < 0.01$ or $P < 0.001$) (Figure 5B).

DISCUSSION

We have evaluated the putative analgesic and anti-inflammatory activities of **TR1** and **TR2** to clarify the pain and inflammation relieving effects. Two different analgesic testing methods were 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 nonspecific (e.g., anticholinergic, antihistaminic, and other agents also show activity in the test), it is widely used for analgesic screening.[19] In our study, we found that **TR1** and **TR2** (1, 5, and 10 mg/kg) exhibited antinociceptive effects in acetic acid-induced writhing response. This effect may be due to the inhibition of the synthesis of the arachidonic acid metabolites.[20]

The formalin test is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The formalin test produced a distinct biphasic response, and different analgesics may act differently in the early and late phases of this test. Centrally acting drugs such as opioids inhibit both phases equally, but peripherally acting drugs such as aspirin, indomethacin, and dexamethasone only inhibit the late phase. The late phase seems to be an inflammatory response with inflammatory pain that can be inhibited by anti-inflammatory drugs.[21] The inhibitory effect of **TR1** and **TR2** on the nociceptive response in the late phase of the formalin test suggested that the antinociceptive effect of **TR1** and **TR2** could be due to its peripheral action.

Carr-induced mouse paw edema is a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the antiedematous effect of natural products. Carr is known to be devoid of apparent systemic effects, and it is nonantigenic but offers a reproducible model for anti-inflammatory agent evaluation. The development of edema in the paw of the mice after injecting the phlogistic agent is believed to be a biphasic mechanism,[22] of which the first 1−2 h is due to the release of histamine or serotonin, while the second phase of edema formation is due to the released prostaglandins/protease and lysosome, which peak at the 3th hour.[23] Statistical analysis revealed that **TR1**, **TR2**, and Indo significantly inhibited the development of edema at the fifth hour after treatment ($p < 0.01$ or $p < 0.001$). They both showed anti-inflammatory effects in Carr-induced mouse paw edema. In addition, the classification of antinociceptive drugs is usually based on their mechanism of action either on the central nervous system or on the peripheral nervous system.[24]

NO acts as a neuromediator with many physiological functions, including the formation of memory, coordination between neuronal activity and blood flow, and modulation of pain.[25] In the studies of the mechanism of inflammation, the L-arginine−NO pathway has been proposed to play an important role in the Carr-induced inflammatory response.[26] In our study, the level of NO was decreased significantly by treatment with 1, 5, and 10 mg/kg **TR1** and **TR2**. We suggest the mechanism of

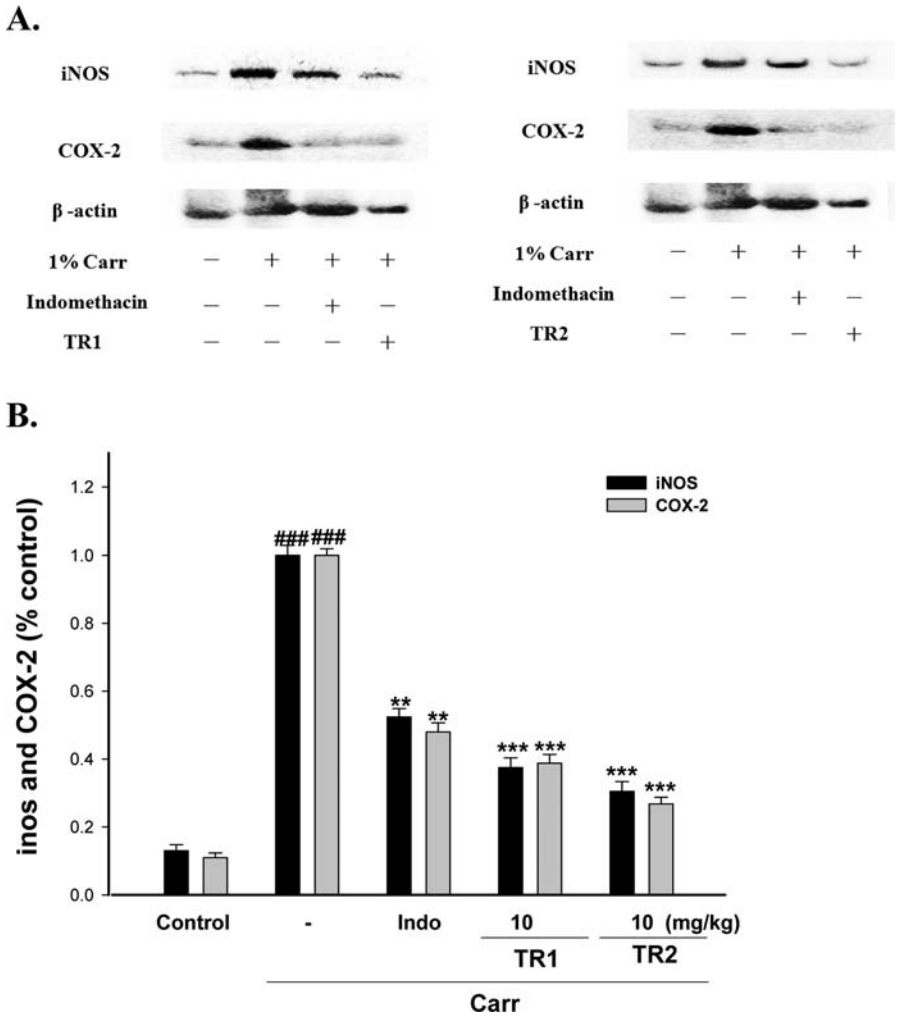

Figure 4. Inhibition of iNOS and COX-2 protein expressions by **TR1** and **TR2** induced by Carr of foot at the 5th hour in mice. Tissues suspended were then prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2. β-Actin was used as an internal control. (A) A representative 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. Each value represents the mean ± SEM. $^{###}p < 0.001$ as compared with the control group. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

anti-inflammatory **TR1** and **TR2** may be through the L-arginine−NO pathway since **TR1** and **TR2** significantly inhibit the NO production.

The proinflammatory cytokines such as TNF-α and IL-1 are small secreted proteins, which mediate and regulate immunity and inflammation. TNF-α and IL-1β are mediators of Carr-induced inflammatory incapacitation and are 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.[27] In this study, we found that **TR1** and **TR2** significantly decreased the TNF-α and IL-1β levels in serum after Carr injection by treatment with 5 and 10 mg/kg **TR1** and **TR2**.

The injection of Carr is a well-established model for the induction of localized and resolving inflammation, where neutrophil leukocyte-driven reactions reach an early peak.[28] It has been shown that neutrophil leukocytes are mobilized within 1 h and are then gradually replaced by monocytes/macrophages and that the attenuation of synovial leukocyte infiltration directly influences the intensity of inflammatory reaction.[29] The Carr-induced inflammatory response also 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. MDA production is due to free radical attack of the plasma membrane.[30] Thus, an inflammatory effect would result in the accumulation of MDA. GSH is a known oxyradical scavenger. Enhancing the level of GSH is conducive toward the reduction of MDA production. Endogenous GSH plays an important role against Carr-induced local inflammation.[31,32] In this study, there are significantly increases in CAT, SOD, and GPx activities with **TR1** and **TR2** treatment. Furthermore, there are significant decreases in MDA levels with **TR1** and **TR2** treatment. We assume the suppression of MDA production is probably due to the increase of CAT, SOD, and GPx activities.

The wild-type fruiting body of AC is well known as an effective and expensive folk remedy for many diseases. Anti-inflammatory activity of methanol extracts from AC has been suggested to contribute to the preventive or therapeutic candidate for the treatment of inflammatory disorders through suppression of both the inducible NO and COX-2 expression in vivo and in vitro.[33,34] Antrocamphin A, antcin A, and antcin B from AC exhibited potent inhibition against *N*-formyl-

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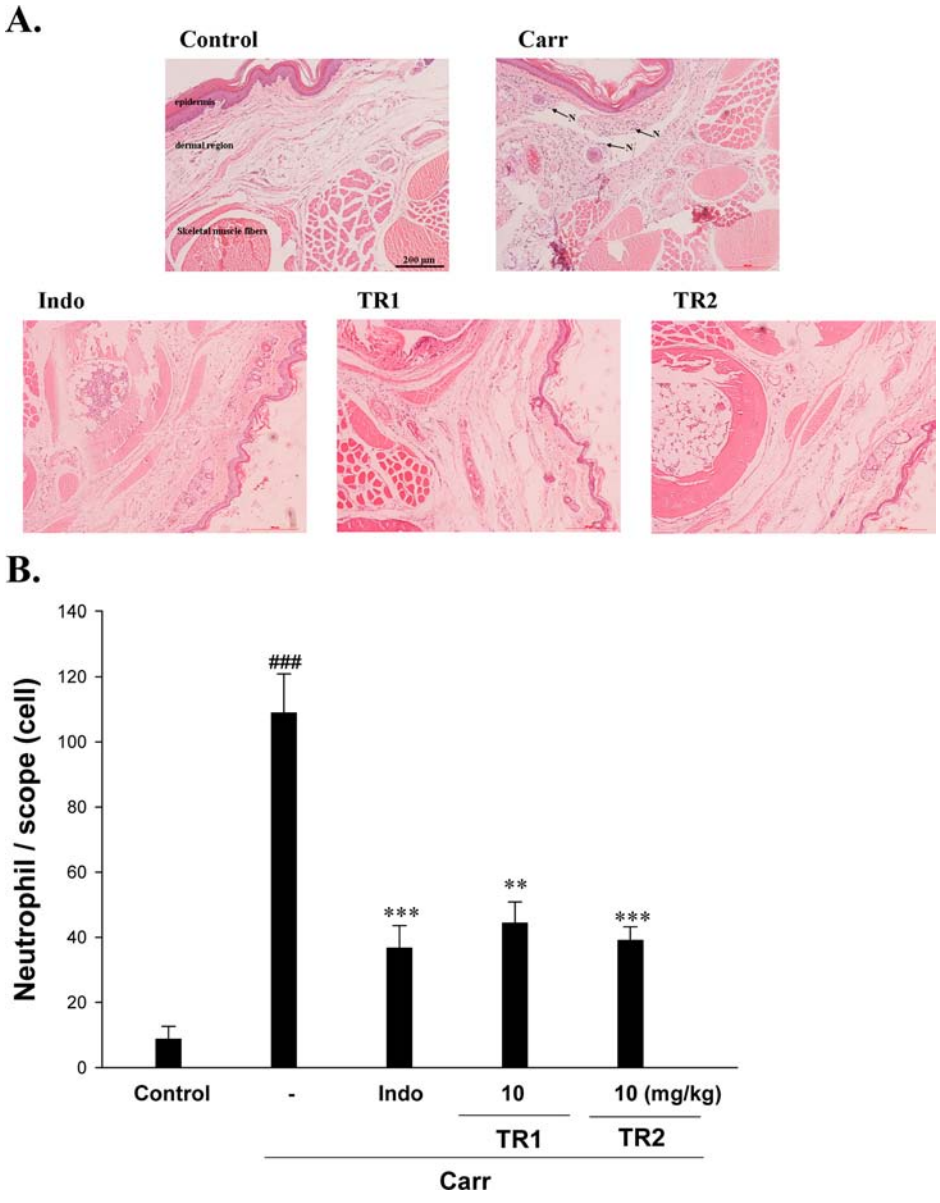

Figure 5. Representative light micrographs of mouse hind footpad H&E stained (A) to reveal hemorrhage, edema, and inflammatory cell infiltration in control mice. Carr-treated mice demonstrate hemorrhage with moderately extravascular red blood cells and large amounts of inflammatory leukocyte, mainly neutrophil, infiltration in the subdermis interstitial tissue of mice. Mice given indomethacin (Indo) (10 mg/kg) before Carr **TR1** and **TR2** significantly show morphological alterations (100×) and the numbers of neutrophils in each scope (400×) compared to subcutaneous injection of Carr only are shown in panel B. $^{###}p < 0.001$ as compared with the control group. $^{**}P < 0.01$ and $^{***}p < 0.001$ compared with the Carr group. Scale bar = 200 μm. The infiltrating cells were predominantly neutrophils (N; arrows).

methionyl-leucyl-phenylalanine (fMLP)-induced superoxide production.[35] In this study, there are significant decreases in iNOS and COX-2 activities with **TR1** and **TR2** treatment. Furthermore, there are significant decreases in NO level with **TR1** and **TR2** treatment. We assume the suppression of NO production is probably due to the decreased iNOS and COX-2 activities.

In conclusion, these results suggested that **TR1** and **TR2** possessed analgesic and anti-inflammatory effects. The anti-inflammatory mechanism of **TR1** and **TR2** may be related to iNOS and associated with the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). **TR1** and **TR2** may be used as pharmacological agents in the prevention or treatment of disease in which free radical formation is a pathogenic factor.

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Notes

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