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Analgesic Effects and Mechanisms of Anti-inflammation of Taraxeren-3-one from *Diospyros maritima* in Mice

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ABSTRACT: This study investigated the analgesic effects of taraxeren-3-one, which is an ingredient from *Diospyros maritima* (DM), using the models of acetic acid-induced writhing response and the formalin test, and its anti-inflammatory effects using the model of λ-carrageenan (Carr)-induced paw edema. Treatment of male ICR mice with taraxeren-3-one inhibited the numbers of writhing response and formalin-induced pain in the late phase, significantly. In the anti-inflammatory test, taraxeren-3-one decreased paw edema at 4 and 5 h after Carr administration and increased the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) in the liver tissue at 5 h after Carr injection. Taraxeren-3-one affects malondialdehyde (MDA), nitric oxide (NO), and tumor necrosis factor-α (TNF-α) levels from both the edema paw and serum at 5 h after Carr injection. Western blotting revealed that taraxeren-3-one decreased Carr-induced inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions. These anti-inflammatory mechanisms of taraxeren-3-one might be related to the decrease in the level of MDA in the edema paw via increasing the activities of SOD, CAT, GPx, and GSH in the liver. Also, taraxeren-3-one could affect the production of NO and TNF-α and, therefore, affect the anti-inflammatory effects.

KEYWORDS: Chinese herb, taraxeren-3-one, anti-inflammation, analgesic, MDA, NO, TNF-α

INTRODUCTION

The fruits and stems of *Diospyros maritima* (DM; Ebenaceae) are traditional Chinese medicines in Taiwan. They have been used for the treatment of abdominal pain, rheumatic arthralgia, detumescence, and fever. The fruits of DM contain naphthoquinone derivatives, 6-(1-ethoxyethyl)-plumbagin, ethylidene-3,3′-biplumbagin, ethylidene-3,6′-biplumbagin, isozeylanone, and 3,3′-biplumbagin.[1] The stems of DM contain phenolic acid, bis(6-hydroxy-2,3,4-trimethoxyphen-1-yl)methane, butylmethyl succinate, epi-isoshinanolone, and 5,7-dihydroxy-2-methylchromanone.[2] Study of both cytotoxicity and antimicrobial assay of DM bark was reported.[3] Stems of DM were evaluated for in vitro cytotoxicity in four cancer cell lines.[4] However, little information is available on the analgesic and anti-inflammatory effects of taraxeren-3-one.

Some researchers have demonstrated that the inflammatory effect induced by λ-carrageenan (Carr) could be associated with free radicals. Free radical, prostaglandin, and NO would be released when Carr was administered for 1−6 h.[5] The edema effect was raised to maximum at 3 h,[6] and malondialdehyde (MDA) production of tissue was due to free radical attack on the plasma membrane.[7] Thus, an inflammatory effect would result in the accumulation of MDA. Therefore, in this paper we examined the analgesic effects of taraxeren-3-one on nociception induced by acetic acid and formalin. We also evaluated the anti-inflammatory effects of taraxeren-3-one on paw edema induced by Carr in mice. We detected the levels of MDA, nitric oxide (NO), and tumor necrosis factor-α (TNF-α) in either paw edema or serum. Also, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) in the liver at 5 h after Carr injection were investigated along with the relationship between the anti-inflammatory mechanism of taraxeren-3-one and antioxidant enzymes.

MATERIALS AND METHODS

Chemicals. λ-Carrageenan (Carr), indomethacin (Indo), Griess reagent, and other chemicals were purchased from Sigma-Aldrich Chemical Co. Formalin was purchased from Nihon Shiyaku Industry Ltd. TNF-α concentration was quantified using a commercial ELISA (Biosource International Inc., Camarillo, CA).

Plant Material. The stems of *D. maritima* Blume (Ebenaceae) was collected in September 1992 on Lin-Ko, Taiwan. They were identified

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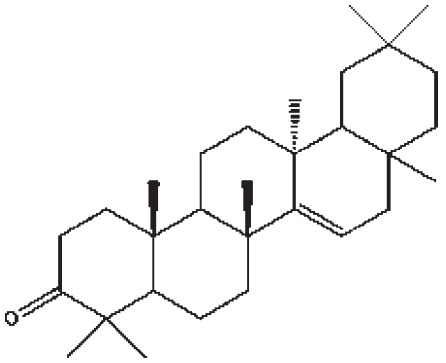

Figure 1. Chemical structure of taraxeren-3-one.

and authenticated by Dr. Yuan-Shiun Chang, Professor, School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University. A voucher specimen (no. 00393) is deposited at the National Research Insitute of Chinese Medicine.

Extraction and Isolation. The dried stems of *D. maritima* (16 kg) were an exhaustive extraction completed with ethanol. The crude ethanol syrup was extracted five times with hexane. The *n*-hexane extract (125 g) was chromatographed on a silica gel column (1.7 kg) with *n*-hexane/EtOAc (5:1) to give six fractions (each 2 L), fractions 1−6. The bioactive fraction 2 was further separated by column chromatography on silica gel (600 g) eluting with *n*-hexane, *n*-hexane/EtOAc (20:1), *n*-hexane/EtOAc (10:1), *n*-hexane/EtOAc (5:1), *n*-hexane/EtOAc (1:1), *n*-hexane/EtOAc (1:2), and EtOAc to yield 10 fractions, fractions 2-1−2-10 (each 1 L). Taraxeren-3-one was obtained from fractions 4−6 (54 mg) (Figure 1).

Taraxeren-3-one. The IR spectrum (KBr) of taraxeren-3-one exhibited absorption bands at 1715, 3050, 1640, and 810 cm^{-1}. The EI-MS (70 eV) showed the molecular ion at m/z 424 $[M]^+$ (61.3) ($C_{30}H_{48}O$). The spectrum also displayed other major fragment ions at m/z 409 $[M - Me]^+$ (27.4), 300 (100), 285 (52.3), 218 (22.8), 204 (85.6), 189 (18.7), and 133 (48.7). The ^1H NMR spectrum ($CDCl_3$, 500 MHz) of taraxeren-3-one displayed six singles of eight methyl groups at δ 0.81 (3H, s), 0.89 (6H, s), 0.93 (3H, s), 1.05 (3H, s), 1.06 (6H, s), and 1.12 (3H, s) and one olefinic proton at δ 5.54 (J = 8.1, 3.2 Hz), which are assigned to the eight tertiary methyl groups of the pentacyclic triterpenoid skeleton containing one olefinic group.[4]

Animals. Imprinting control region (ICR; 6−8 weeks old, male) mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ± 1 °C and relative humidity of 55 ± 5% with 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*. The placebo groups were given 0.1 mL/10 g saline intraperitoneally (ip) using a bent blunted 27-gauge needle connected to a 1 mL syringe. All tests were conducted under the guidelines of the International Association for the Study of Pain.[8] Taraxeren-3-one (5, 10, and 20 mg/kg) and Indo (10 mg/kg) were dissolved in 0.5% sodium carboxymethyl cellulose (CMC) suspension.

Acetic Acid-Induced Writhing Response. After a 2 week adaptation period, male ICR mice (18−25 g) were randomly assigned to five groups (n = 8). These included a normal and a positive control and taraxeren-3-one-administered groups. Control mice received normal saline. Positive control animals were pretreated with Indo (10 mg/kg, ip) 20 min before acetic acid (0.1 mL/10 g). Each taraxeren-3-one administered group was pretreated with 5, 10, and 20 mg/kg per os (po) 60 min before acetic acid (0.1 mL/10 g). Five minutes after the ip injection of acetic acid, the number of writhings during the following 10 min was counted.[9,10]

Formalin Test. The antinociceptive activity of the drugs was determined using the formalin test described by Dubuisson and Dennis.[11] Male ICR mice (18−25 g) were randomly assigned to five groups (n = 8). These include a normal and a positive control group and taraxeren-3-one-administered groups. The normal control group received only drugless vehicle (0.1 mL/10 g). Taraxeren-3-one (5, 10, and 20 mg/kg, po) and Indo (10 mg/kg, ip) were suspended in Tween 80 plus 0.9% (w/v) saline solution and administered ip in a volume of 0.1 mL/10 g. One hour before testing, the animal was placed in a standard cage (30 cm × 12 cm × 13 cm) that served as an observation chamber. Taraxeren-3-one (5, 10, and 20 mg/kg, po) was administered 60 min before formalin injection. Indomethacin (10 mg/kg, ip) was administered 30 min before formalin injection. The control group received the same volume of saline po. Twenty microlitres of 5.0% formalin was injected into the dorsal surface of the right hind paw. The mice were observed for 40 min after the injection of formalin, and the amount of time spent licking the injected hindpaw was recorded. The first 5 min after 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 stopwatch. The activity was recorded in 5 min intervals.

λ-Carrageenan-Induced Edema. The anti-inflammatory activity of taraxeren-3-one was determined by the Carr-induced edema test in the hind paws of mice. Male ICR mice (eight per group, 18−25 g) were fasted for 24 h before the experiment with free access to water. Fifty microliters of a 1% suspension of Carr in saline was prepared 30 min before each experiment and was injected into the plantar side of right hind paws of the mice. Taraxeren-3-one and Indo were suspended in Tween-80 plus 0.9% (w/v) saline solution. The final concentration of Tween-80 did not exceed 5% and did not cause any detectable inflammation. After 2 h, taraxeren-3-one at doses of 5, 10, and 20 mg/kg was administered po, and after 90 min, Indo was administered ip at a dose of 10 mg/kg before the Carr treatment. 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.[12] After 5 h, the animals were sacrified; the Carr-induced edema paws were dissected and stored at −80 °C. Blood samples were withdrawn and kept at −80 °C.

Therefore, the right hind paw tissue and liver tissue were taken at 5 h. The right hind paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline 4 times its volume and homogenized at 4 °C. Then the homogenate was centrifuged at 12000g for 5 min. The supernatant was obtained and stored in a −20 °C refrigerator for MDA assays. The whole liver tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline 1 times its volume and homogenized at 4 °C. Then the homogenate was centrifuged at 12000g for 5 min. The supernatant was obtained and stored in the refrigerator at −20 °C for the antioxidant enzyme (CAT, SOD, GPx, and GSH) activity assays. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

MDA Assay. MDA was evaluated by the thiobarbituric acid reacting substances (TRARS) 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.

Determination of NO. The production of NO was assessed indirectly by measuring the nitrite levels in plasma and paw edema tissue determined by a calorimetric method based on the Griess reaction.[14] Plasma and paw edema tissue samples were diluted 4 times

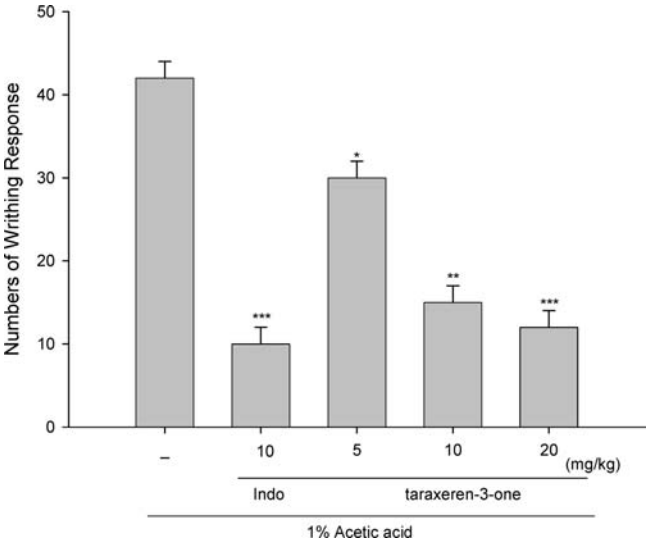

Figure 2. Analgesic effects of taraxeren-3-one and indomethacin (Indo) on acetic acid-induced writhing response in mice. Each value represents as the mean ± SEM. (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.001$ as compared with the only acetic acid induced group (one-way ANOVA followed by Scheffe's multiple-range test).

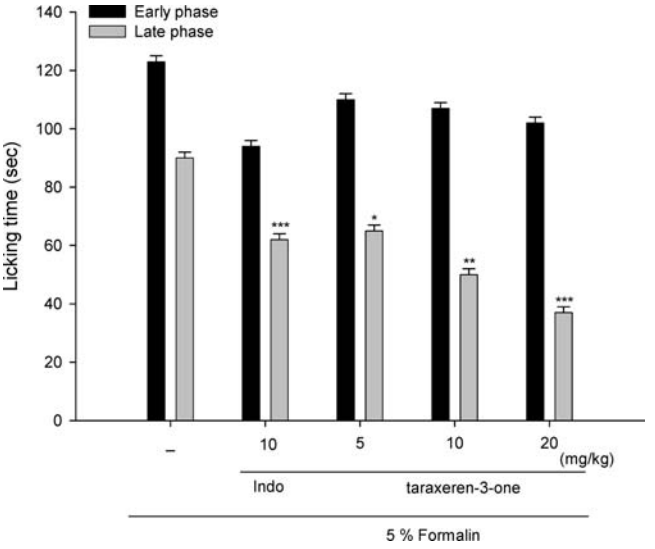

Figure 3. Effects of taraxeren-3-one and indomethacin (Indo) on the early and late phases in the formalin test in mice. Each value represents the mean ± SEM. (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.001$ as compared with the control (Con) group (one-way ANOVA followed by Scheffe's multiple-range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

with distilled water (v/v) and deproteinized by adding a $^1/_{20}$ 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 MicroReader (Hyperion, Inc., Miami, FL). Nitrite was quantified by using sodium nitrate as a standard curve.

Measurement of Serum and Paw Tissue TNF-α by ELISA. Paw tissue and serum levels of TNF-α were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA). according to the manufacturer's instruction. TNF-α was determined from a standard curve for the combination of these cytokines. The concentrations were expressed as picograms per milliliter.[15]

Protein Lysate Preparation and Western Blot Analysis of iNOS and COX-2. Total protein was extracted with a radioimmunoprecipitation assay buffer (RIPA) solution at −20 °C overnight. We used bovine serum albumin (BSA) as a protein standard to calculate equal total cellular protein amounts. Protein samples (30 μg) were resolved by denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) using standard methods and then were transferred to PVDF membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies (iNOS, COX-2, and β-actin) at 4 °C overnight, washed three times with PBST, and incubated for 1 h at 37 °C with horseradish peroxidase conjugated secondary antibodies. The membranes were washed three times, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The results of Western blot analysis were quantified by measuring the relative intensity compared to the control using Kodak Molecular Imaging software and represented in the relative intensities.

Antioxidant Enzyme Activity Measurements. The following biochemical parameters were analyzed to check the hepatoprotective activity of ECH by the methods given below. Total SOD activity was determined by the inhibition of cytochrome *c* reduction.[16] The

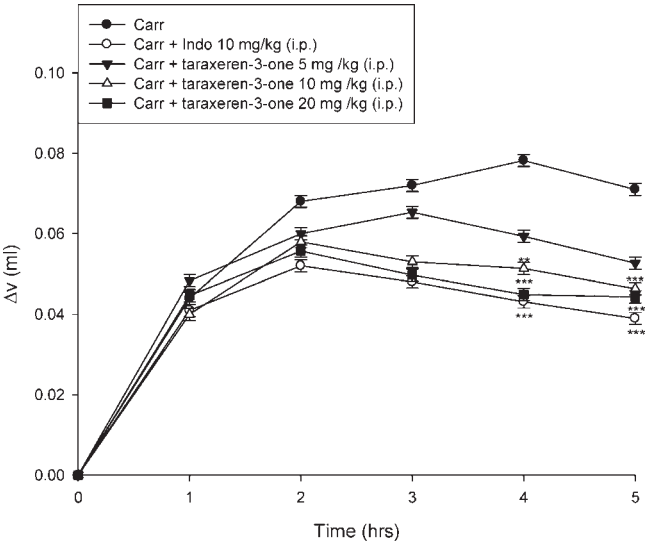

Figure 4. Effects of taraxeren-3-one and indomethacin (Indo) on hind paw edema induced by λ-carrageenan in mice. Each value represents the mean ± SEM. (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.001$ as compared with the λ-carrageenan (Carr) group (one-way ANOVA followed by Scheffe's multiple-range test).

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 estimation was based on that of Aebi.[17] In brief, the reduction of 10 mM H_2O_2 in 20 mM phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated by using a 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 as previously reported.[18] The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2), and

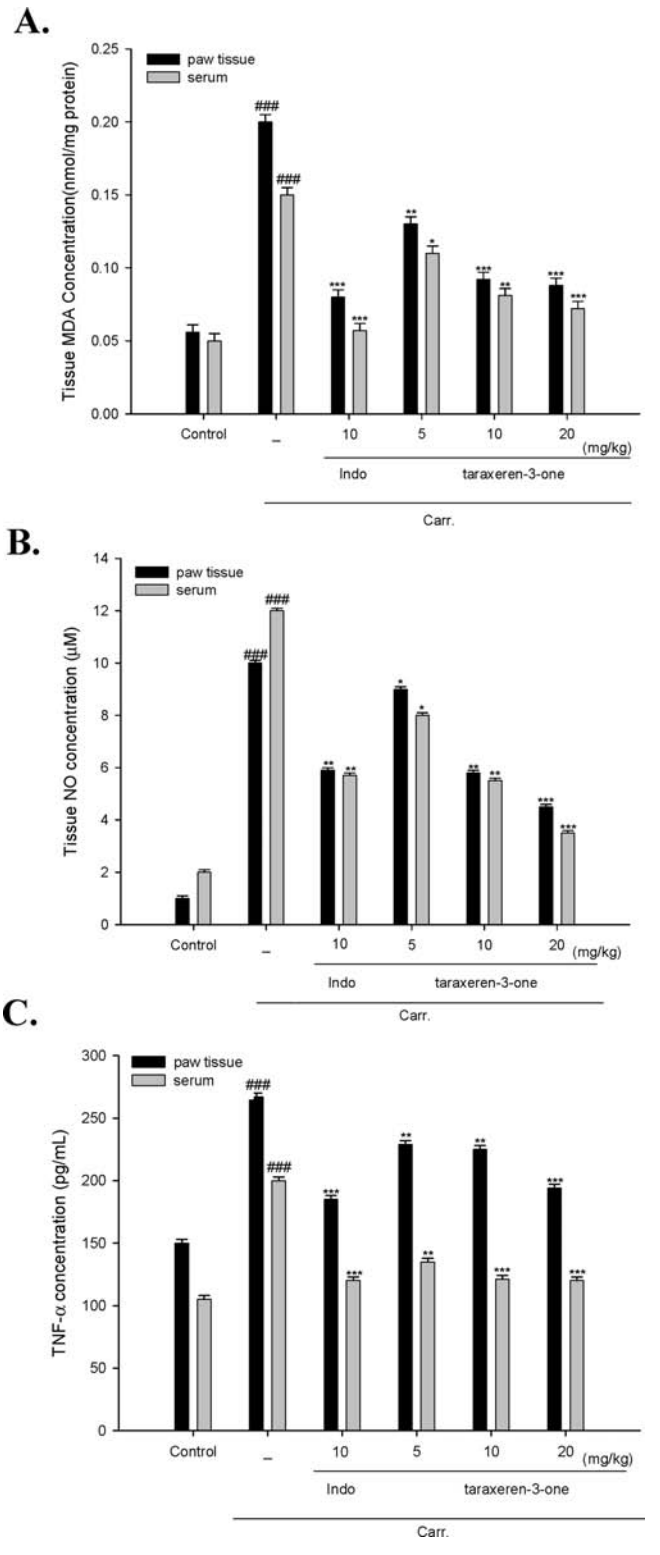

Figure 5. Effects of taraxeren-3-one and indomethacin (Indo) on carrageenan (Carr)-induced MDA (A), NO (B), and TNF-α (C) concentrations of edema paw and serum detected at 5 h in mice. Each value represents the mean ± SEM. (###) compared with sample of control group. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple-range test).

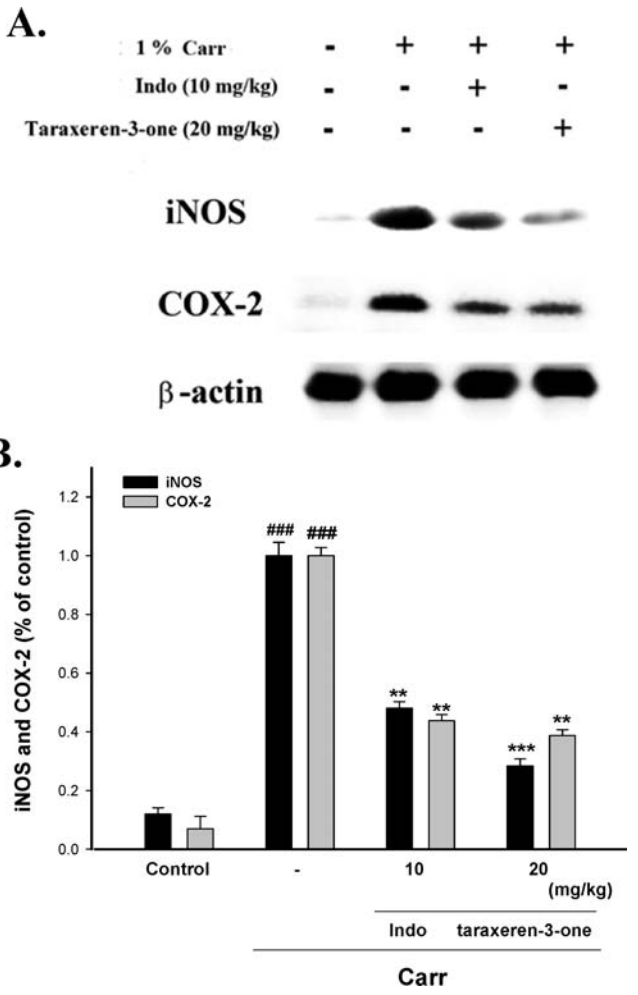

Figure 6. Inhibition of iNOS and COX-2 protein expression by taraxeren-3-one induced by Carr of foot at 5 h in mice: (A) representative Western blot from two separate experiments; (B) relative iNOS and COX-2 protein levels calculated with reference to a Carr-injected mouse. Tissues suspended were prepared and subjected to Western blotting using an antibody specific for iNOS and COX-2. β-Actin was used as an internal control. (###) compared with sample of control group. The data are presented as the mean ± SD for three different experiments performed in triplicate. (***) $p < 0.001$ compared with the Carr-alone group.

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. Hepatic GSH level was determined according to the method of Davis et al.[19] with slight modifications. Briefly, 720 μL of liver homogenate in 200 mM Tris buffer (pH 7.2) was diluted to 1440 μL with the same buffer. Five percent TCA (160 μL) was added and mixed thoroughly. The samples were then centrifuged at 10000*g* for 5 min at 4 °C. Ellman's reagent (DTNB solution) (660 μL) was added to the supernatant (330 μL). Finally, the absorbance was recorded at 405 nm.

Histological Examination. For histological examination, biopsies of paws were taken 5 h following the intraplantar injection of Carr. The tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical). Sections (thickness = 7 μm) were deparaffinized with xylene and stained with trichromic Van Gieson. All samples were observed and photographed with a BH2 Olympus microscope. Histological examination of these tissue slices revealed an excessive inflammatory response with massive infiltration of neutrophils [ploymorphonuclear leukocytes

Table 1. Effects of Taraxeren-3-one and Indomethacin (Indo) on Liver SOD, CAT, GPx, and GSH Activities in Mice[a]

group	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GSH (U/mg protein)
control	98.00 ± 0.01	0.72 ± 0.02	10.01 ± 0.21	5.01 ± 0.15
Carr	51.04 ± 0.02###	0.21 ± 0.01###	3.25 ± 0.03###	2.52 ± 0.01###
Carr + Indo	82.14 ± 0.16	0.53 ± 0.22	7.35 ± 0.06	3.49 ± 0.12***
Carr + taraxeren-3-one (5 mg/kg)	72.13 ± 0.09	0.41 ± 0.43*	6.79 ± 0.01*	3.01 ± 0.32*
Carr + taraxeren-3-one (10 mg/kg)	80.02 ± 1.08*	0.45 ± 0.52**	7.16 ± 0.28**	3.35 ± 0.02**
Carr + taraxeren-3-one (20 mg/kg)	81.04 ± 1.13**	0.49 ± 0.31***	7.19 ± 0.63***	3.81 ± 0.42***

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

(PMNs)] by microscopy. The numbers of neutrophils were counted in each scope (400×), and thereafter their average count was obtained from five scopes of 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 (*) $p < 0.05$, (**) < 0.01, and (***) $p < 0.001$.

RESULTS

Effects of Taraxeren-3-one on Acetic-Induced Writhing Response. The cumulative amount of abdominal stretching correlated with the level of acetic acid induced pain (Figure 2). Taraxeren-3-one treatment (10 mg/kg) significantly inhibited the number of writhings in comparison with the normal controls ($p < 0.01$). Taraxeren-3-one (20 mg/kg) further inhibited the number of writhings ($p < 0.001$) as well as Indo (10 mg/kg).

Formalin Test. Taraxeren-3-one (20 mg/kg) significantly ($p < 0.001$) inhibited formalin-induced pain in the late phase (Figure 3). However, it did not show any inhibition in the early phase. The positive control Indo (10 mg/kg) also significantly ($p < 0.001$) inhibited the formalin-induced pain in the late phase.

Effects of Taraxeren-3-one on λ-Carrageenan-Induced Mice Paw Edema. As shown in Figure 4, Carr induced paw edema. Taraxeren-3-one (20 mg/kg) significantly inhibited ($p < 0.001$) the development of paw edema induced by Carr after 3 and 4 h of treatment. Indo (10 mg/kg) significantly decreased the Carr-induced paw edema after 3 and 4 h of treatment ($p < 0.001$).

Effects of Taraxeren-3-one on MDA Level Measurements. Figure 5A indicates taraxeren-3-one (10 mg/kg) decreased the MDA level in the edema paw and serum at 5 h after Carr injection ($p < 0.01$ or $p < 0.001$), and taraxeren-3-one (20 mg/kg) decreased the MDA level in the edema paw and serum at 5 h after Carr injection ($p < 0.001$).

Effects of Taraxeren-3-one on NO Measurement. Taraxeren-3-one (5, 10, and 20 mg/kg) decreased the NO level from the edema paw and serum at 5 h after Carr injection. Taraxeren-3-one (10 mg/kg) significantly decreased the edema paw and serum NO level ($p < 0.01$). However, taraxeren-3-one (20 mg/kg) decreased the edema paw and serum NO level ($p < 0.001$) (Figure 5B).

Effects of Taraxeren-3-one on TNF-α Measurement. Taraxeren-3-one (10 mg/kg) decreased the TNF-α level in paw tissue and serum at 5 h after Carr injection ($p < 0.01$), and taraxeren-3-one (20 mg/kg) decreased the TNF-α level at 5 h after Carr injection ($p < 0.001$) (Figure 5C).

Effects of Taraxeren-3-one 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 effects of taraxeren-3-one on iNOS and COX-2 protein expression were studied by Western blot. The results showed that injection of taraxeren-3-one (20 mg/kg) inhibited Carr-induced iNOS and COX-2 protein expression in mouse paw edema at 5 h (Figure 6A). The intensity of protein bands was analyzed using Kodak Quantity software in three independent experiments and showed averages of 72.4 and 61.3% down-regulation of iNOS and COX-2 protein, respectively, after treatment with taraxeren-3-one compared with the Carr-induced alone (Figure 6B). In addition, protein expression showed averages of 52.8 and 56.2% down-regulation of iNOS and COX-2 protein after treatment with Indo at 10.0 mg/kg compared with Carr-induced alone. The down-regulation of iNOS and COX-2 activity of taraxeren-3-one (20 mg/kg) was better than that of Indo (10.0 mg/kg).

Effects of Taraxeren-3-one on Activities of Antioxidant Enzymes. At 5 h following the intrapaw injection of Carr, liver tissues were also analyzed for biochemical parameters such as SOD, CAT, GPx, and GSH activities (Table 1). SOD, CAT, GPx, and GSH activities in liver tissue were decreased significantly by Carr administration. SOD, CAT, GPx, and GSH activities were increased significantly after treatment with 20 mg/kg taraxeren-3-one and 10 mg/kg Indo ($p < 0.01$ or $p < 0.001$) (Table 1).

Histological Examination. Paw biopsies of control 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 the extract, at a dose of 20 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 hypoderm connective tissue was not damaged (Figure 7).

DISCUSSION

We have evaluated the putative analgesic and anti-inflammatory activities of taraxeren-3-one 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.[20] In our study, we found that taraxeren-3-one (10 and 20 mg/kg) exhibited an antinociceptive effect in acetic acid-induced writhing response (Figure 2.). This effect may be due to inhibition of the synthesis of the arachidonic acid metabolites.[21]

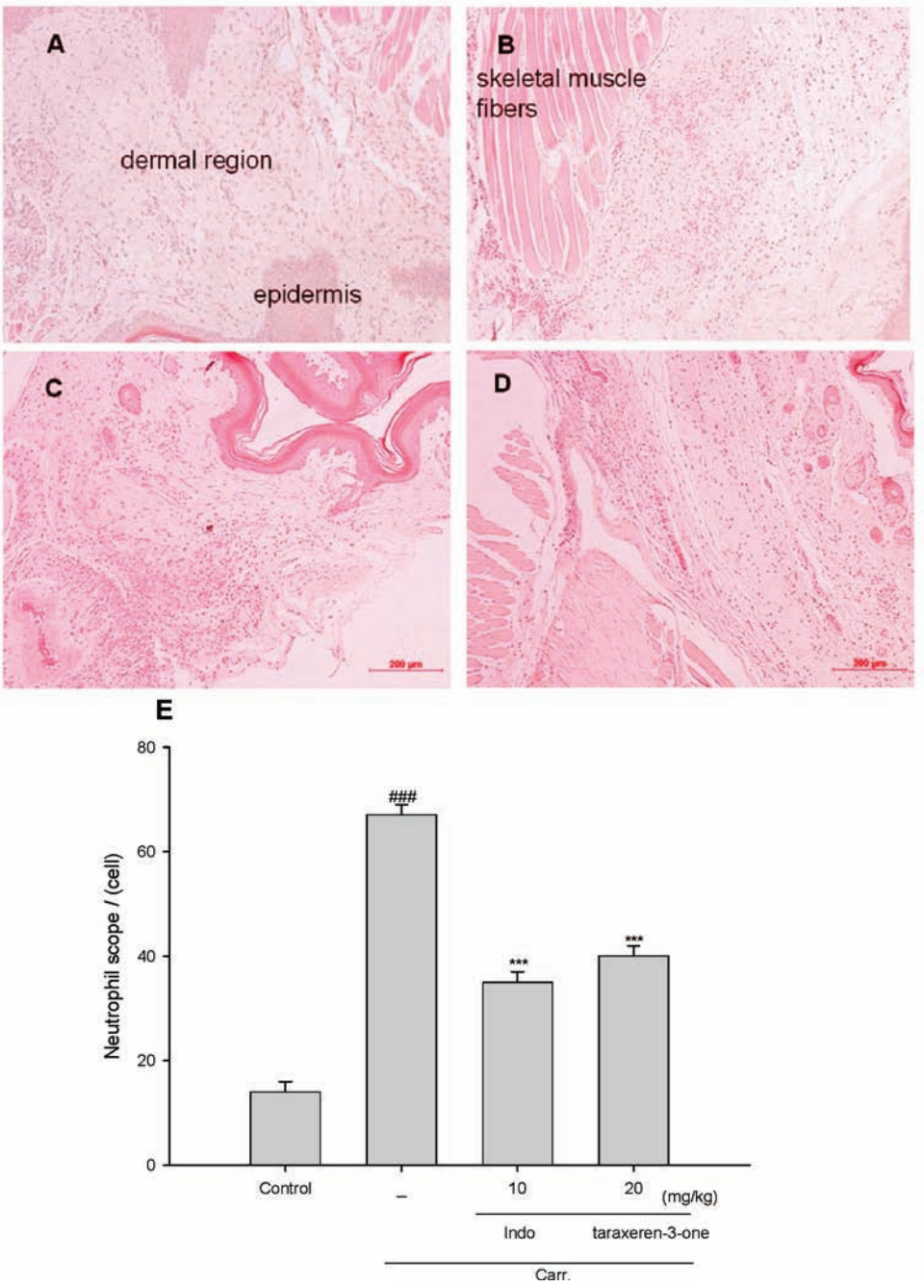

Figure 7. Histological appearance of the mouse hind footpad after a subcutaneous injection with 0.9% saline (control group) or carrageenan stained with H&E stain: (A) control mice, showing the normal appearance of dermis and subdermis without any significant lesion; (B) hemorrhage with moderately extravascular red blood cell and large amount of inflammatory leukocyte mainly neutrophil infiltration in the subdermis interstitial tissue of mice following subcutaneous injection of Carr only (detail of the subdermis layer shows enlargement of the interstitial space caused by edema with exudate fluid); (C) indomethacin (Indo) significantly reduced the level of hemorrhage, edema, and inflammatory cell infiltration compared to subcutaneous injection of Carr only; (D) taraxeren-3-one showed significant morphological alterations compared to subcutaneous injection of Carr only (100×); (E) numbers of neutrophils were counted in each scope (400×) and their average count obtained from five scopes of every tissue slice. (###) $p < 0.001$ as compared with the control group; (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ as compared with the Carr group.

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.[22] Centrally acting drugs such as opioids inhibit both phases equally,[20] but peripherally acting drugs such as aspirin, Indo, and dexamethasone inhibit only the late phase. The inhibitory effect of taraxeren-3-one on the nociceptive response in the late phase of the formalin test suggested that the antinociceptive effect of taraxeren-3-one could be due to its peripherial action (Figure 3).

The Carr test is highly sensitive to nonsteroidal anti-inflammatory drugs and has long been accepted as a useful phlogistic

tool for investigating new drug therapies.[23] The degree of swelling of the Carr-injected paws was maximal 3 h after injection. Statistical analysis revealed that taraxeren-3-one and Indo significantly inhibited the development of edema 4 h after treatment ($p < 0.001$) (Figure 4). They both showed anti-inflammatory effects in Carr-induced mice edema paw. It is well-known that the third hour of Carr-induced edema, when the edema reaches its highest volume, is characterized by the injection of Carr into the rat paw inducing the liberation of bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates.[24,25] Besides, in the Carr-induced rat paw edema model, the production of prostanoids is through the serum expression of COX-2 by a positive feedback mechanism.[26] Therefore, it is suggested that the action mechanism of taraxeren-3-one may be related to prostaglandin synthesis inhibition, as described for the anti-inflammatory mechanism of Indo in the inhibition of the inflammatory process induced by Carr.[27] 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.[28]

In 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.[29] Our present results also confirm that the level of NO production increased in the Carr-induced paw edema model. The expression of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation.[30] In our study, the level of NO was decreased significantly by treatment with 10 and 20 mg/kg taraxeren-3-one. We suggest the anti-inflammatory mechanism of taraxeren-3-one may be through the L-arginine—NO pathway because taraxeren-3-one significantly inhibits NO production (Figure 5A).

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.[31] 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 play an important role in the maintenance of long-lasting nociceptive response.[32] In this study, we found taraxeren-3-one decreased the TNF-α level in serum after Carr injection (Figure 5C).

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.[33] Some research had demonstrated that the inflammatory effect induced by Carr is associated with free radicals. Free radicals, prostaglandin, and NO will be released when Carr is administered for 1—6 h.[5] The edema effect was raised to the maximum at the third hour.[6] Janero et al. demonstrated that MDA production is due to free radical attack on plasma membrane.[7] Thus, the inflammatory effect would result in the accumulation of MDA. GSH is a known oxyradical scavenger. Enhancing the level of GSH is conducive toward reducing MDA production. Cuzzocrea suggested that endogenous GSH plays an important role against Carr-induced local inflammation.[34] In this study, there were significant increases in SOD, CAT, GPx, and GSH activities with taraxeren-3-one treatment (Table 1). Furthermore, there was a significant decrease in MDA level with taraxeren-3-one treatment (Figure 6). The result indicated that the suppression of MDA production is probably due to increases of SOD, CAT, GPx, and GSH activities.

In conclusion, these results suggest that taraxeren-3-one possesses analgesic and anti-inflammatory effects. The anti-inflammatory mechanism of taraxeren-3-one may be related to iNOS and COX-2 activity,[35] and it is associated with the increase in the activities of antioxidant enzymes (SOD, CAT, GPx, and GSH). Taraxeren-3-one may be used as a pharmacological agent in the prevention or treatment of disease in which free radical formation is a pathogenic factor.

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