Running Title: Analgesic and Anti-inflammatory activities of Taraxeren-3-one

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

TIEN-NING CHANG [†], SHYH-SHYUN HUANG [†], YUAN-SHIUN CHANG[†], ChI-I CHANG [#], HSIN-LING YANG [‡], JENG-SHYAN DENG[Δ], YUEH-HSIUNG KUO [†, §, ∥, ⊥, *] , GUAN-JHONG HUANG [†, *]

[†]School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung 404, Taiwan

[#] Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung 912, Taiwan

[‡] Institute of Nurition, China Medical University, Taichung 404, Taiwan

[Δ]Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan

[§] Department of Chemistry, National Taiwan University, Taipei 106, Taiwan

[∥] Agricultural Biotechnology Research Center, Academic Sinica, Taipei 115, Taiwan.

[⊥]Tsuzuki Institute for Traditional Medicine, China Medical University, Taichung 404, Taiwan

[*]**Corresponding author**

Dr. Kuo Yueh-Hsiung

Tel.: +886 2 3366-1671; Fax: +886 2 2362-6359

E-mail address: yhkuo@ntu.edu.tw

Dr. Guan-Jhong Huang

Telephone: +886- 4- 2205-3366. Ext: 5508. Fax: +886- 4-2208-3362

E-mail address: gjhuang@mail.cmu.edu.tw

In this study, we have investigated the analgesic effects of the taraxeren-3-one which is an ingredient from *Diospyros maritima* (DM) using the models of acetic acid-induced writhing response and the formalin test, the anti-inflammatory effects of taraxeren-3-one using model of λ-carrageenan (Carr)-induced paw edema. Treatment of male ICR mice with taraxeren-3-one inhibited the numbers of writhing response and the formalin-induced pain in the late phase, significantly. In the anti-inflammatory test, taraxeren-3-one decreased the paw edema at the 4^{th} and 5^{th} 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 the 5^{th} h after Carr injection. Taraxeren-3-one affects the malondialdehyde (MDA), nitric oxide (NO), tumor necrosis factor-α (TNF-α) levels from both the edema paw and serum at the 5^{th} 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 fruit or stem of *Diospyros maritima* (DM; Ebenaceae) are a sort of traditional Chinese medicine in Taiwan. It has 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, 3,3'-biplumbagin (*1*). The stems of DM contain phenolic acid, bis(6-hydroxy-2,3,4-trimethoxylphen-1-yl)methane, butylmethyl succinate, *epi*-Isoshinanolone, 5,7-dihydroxy-2-methylchomanone (*2*). Study of both cytotoxicity and antimicrobial assay of DM bark was reported (*3*). Stems of DM were evaluated for *in vitro* cytotoxicity in 4 cancer cell lines (*4*). However, little information is available on the analgesic and anti-inflammatory effects of taraxeren-3-one.

Some researches demonstrated that inflammatory effect induced by Carr could be associated with free radical. Free radical, prostaglandin and NO would be released when administrating with Carr for 1-6 h (*5*). The edema effect was raised to maximum at the 3th h (*6*) and MDA production of tissue was due to free radical attack plasma

membrane (*7*). Thus, 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. And we detected the levels of MDA, NO and TNF-α in either paw edema or serum. Also, the activities of SOD, CAT, GPx and GSH in the liver at the 5^{th} h after Carr injection was investigated 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 stem of *D. maritima* Blume (Ebenaceae) was collected in September 1992 on Lin-Ko, Taiwan. They were identified 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 6 fractions (each 21), fr. 1-6. The bioactive fr. 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 ten fractions, fr. 2-1 to 2-10 (each 1L). Taraxeren-3-one was obtained from fr. 4-6 (54 mg) (Fig. 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), 133(48.7). The ^{1}H-NMR spectrum ($CDCl_3$, 500MHz) 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),

105 1.05(3H, s), 1.06(6H, s), 1.12(3H, s) and one olefinic proton at δ 5.54 (*J*=8.1, 3.2 Hz),
106 which are assigned to the eight tertiary methyl groups of the pentacyclic triterpenoid
107 skeleton containing one olefinic group(*4*).
108
109 **Animals.** Imprinting control region (ICR; 6-8 weeks male) mice were obtained from
110 the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a
111 constant temperature of 22±1℃, relative humidity 55 ± 5% with 12 h dark-light cycle
112 for at least 2 weeks before the experiment. They were given food and water *ad libitum*.
113 All experimental procedures were performed according to the NIH Guide for the Care
114 and Use of Laboratory Animals. The placebo groups were given 0.1 mL/10 g saline
115 intraperitoneally using a bent blunted 27-gauge needle connected to a 1 mL syringe.
116 All tests were conducted under the guidelines of the International Association for the
117 Study of Pain (*8*). Taraxeren-3-one (5 mg/kg, 10 mg/kg, and 20 mg/kg) and Indo (10
118 mg/kg) which were dissolved in 0.5% sodium carboxyl methyl cellulose (CMC)
119 suspension.
120
121 **Acetic Acid-Induced Writhing Response.** After a 2-week adaptation period, male
122 ICR mice (18-25 g) were randomly assigned to five groups (n=8). These include a
123 normal and a positive control, and taraxeren-3-one administered groups. Control mice

124 received normal saline. Positive control animals were pretreated with Indo (10 mg/kg,
125 i.p.) 20 min before acetic acid (0.1 mL/10 g). Each taraxeren-3-one administered
126 group was pretreated with 5 mg/kg, 10 mg/kg, and 20 mg/kg p.o. 60 min before acetic
127 acid (0.1 mL/10 g). Five minutes after the i.p. injection of acetic acid, the number of
128 writhings during the following 10 min was counted (*9, 10*).
129
130 **Formalin Test.** The antinociceptive activity of the drugs was determined using the
131 formalin test described by Dubuisson and Dennis (*11*). Male ICR mice (18-25 g) were
132 randomly assigned to five groups (n = 8). These include a normal and a positive
133 control group, and taraxeren-3-one administered groups. The normal control group
134 received only drugless vehicle (0.1 mL/10 g). Taraxeren-3-one (5 mg/kg, 10 mg/kg
135 and 20 mg/kg, p.o.) and Indo (10 mg/kg, i.p.) were suspended in tween 80 plus 0.9%
136 (w/v) saline solution and administered i.p. in a volume of (0.1 mL/10 g). One hour
137 before testing, the animal was placed in a standard cage (30 cm×12 cm×13 cm) that
138 served as an observation chamber. Taraxeren-3-one (5 mg/kg, 10 mg/kg and 20 mg/kg,
139 p.o.) was administered 60 min before formalin injection. Indomethacin (10 mg/kg, i.p.)
140 was administered 30 min before formalin injection. The control group received the
141 same volume of saline by oral administration. Twenty microlitres of 5.0% formalin
142 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 post formalin injection is referred to as the early phase and the period between 15 min 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.

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 microlitres of a 1% suspension of Carr in saline was prepared 30 mins before each experiment and was injected into the plantar side of right hindpaws of the mice. Taraxeren-3-one and indomethacin 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 hrs, taraxeren-3-one at the doses of 5, 10 and 20 mg/kg were administered orally, and after 90 min, Indo was administered intra-peritoneally 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 ℃. Blood samples were withdrawn and kept at -80 ℃.

Therefore, the right hind paw tissue and liver tissue were taken at the 5 h. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately placed in cold normal saline four times their volume and homogenized at 4 ℃. Then the homogenate was centrifuged at 12,000×*g* for 5 min. The supernatant was obtained and stored at −20 ℃ refrigerator for MDA assays. The whole liver tissue was rinsed in ice-cold normal saline, and immediately placed in cold normal saline one time their volume and homogenized at 4 ℃. Then the homogenate was centrifuged at 12,000*g* for 5 min. The supernatant was obtained and stored in the refrigerator at −20 ℃ for the antioxidant enzymes (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 four times with distilled water (*v*/*v*) 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 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., FL, USA). 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 pg/mL (*15*).

Protein Lysate Preparation and Western Blot Analysis of iNOS and COX-2. Total protein was extracted with a RIPA solution (radioimmuno-precipitation assay buffer) at -20℃ overnight. We used BSA (bovine serum albumin) 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℃ overnight, washed three times with PBST, and incubated for 1 h at 37 ℃ 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 Enzymes Activity Measurements. The following biochemical parameters were analyzed to check the hepatoprotective activity of ECH by the methods given below. Total superoxide dismutase (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 catalase (CAT) activity estimation 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 by 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 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 the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute. 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. 5% TCA (160 µL) was

238 added and mixed thoroughly. The samples were then centrifuged at 10,000 × *g* for 5
239 min at 4 ℃. Ellman's reagent (DTNB solution) (660 µL) was added to the supernatant
240 (330 µL). Finally the absorbance was recorded at 405 nm.
241
242 **Histological examination.** For histological examination, biopsies of paws were taken
243 5 h following the intraplantar injection of Carr. The tissue slices were fixed in Dietric
244 solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room
245 temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood
246 Medical). Sections (thickness 7 µm) were deparaffinized with xylene and stained with
247 trichromic Van Gieson. All samples were observed and photographed with BH2
248 Olympus microscopy. Histological examination of these tissue slices revealed an
249 excessive inflammatory response with massive infiltration of neutrophils
250 [ploymorphonuclear leukocytes (PMNs)] by microscopy. The numbers of neutrophils
251 were counted in each scope (400 x) and thereafter obtained their average count from 5
252 scopes of every tissue slice.
253
254 **Statistical analysis.** Data are expressed as mean ± S.E.M. Statistical evaluation was
255 carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple
256 range test). Statistical significance is expressed as $*p < 0.05$, $**p < 0.01$, $***p <$
257 0.001.
258

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 comparision 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. In Figure 5A, we indicated taraxeren-3-one (10 mg/kg) decreased the MDA level in the edema paw and serum at the fifth hour 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 the fifth hour 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 the fifth 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 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 the fifth hour after Carr injection ($p < 0.01$). And taraxeren-3-one (20 mg/kg) decreased the TNF-α level at the fifth hour 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

297 production was due to a decreased iNOS and COX-2 protein level, the effect of
298 taraxeren-3-one on iNOS and COX-2 proteins expression were studied by Western
299 blot. The results showed that injection of taraxeren-3-one (20 mg/kg) on Carr-induced
300 for 5 h inhibited iNOS and COX-2 proteins expression in mouse paw edema (Figure
301 6A). The intensity of protein bands were analyzed using Kodak Quantity software in
302 three independent experiments and showed an average of 72.4% and 61.3%
303 down-regulation of iNOS and COX-2 protein, respectively, after treatment with
304 taraxeren-3-one compared with the Carr-induced alone (Figure 6B). In addition, the
305 protein expression showed an average of 52.8% and 56.2% down-regulation of iNOS
306 and COX-2 protein after treatment with Indo at 10.0 mg/kg compared with the
307 Carr-induced alone. The down-regulation of iNOS and COX-2 activity of
308 taraxeren-3-one (20 mg/kg) was better than Indo (10.0 mg/kg).
309
310 **Effects of Taraxeren-3-one on Activities of Antioxidant Enzymes.** At the fifth hour
311 following the intrapaw injection of Carr, liver tissues were also analysed for the
312 biochemical parameters such as SOD, CAT, GPx and GSH activities (Table 1). SOD,
313 CAT, GPx and GSH activities in liver tissue was decreased significantly by Carr
314 administration. SOD, CAT, GPx and GSH activities was increased significantly after
315 treated with 20 mg/kg taraxeren-3-one and 10 mg/kg Indo (P<0.01 or P<0.001)

316 (Table 1).

317

318 **Histological Examination.** Paw biopsies of control animals showed marked cellular
319 infiltration in the connective tissue. The infiltrates accumulated between collagen
320 fibers and into intercellular spaces. Paw biopsies of animals treated with the extract, at
321 a dose of 20 mg/kg, showed a reduction in inflammatory response Carr-induced.
322 Actually inflammatory cells were reduced in number and confined to near the
323 vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen
324 fibers were regular in shape and showed a reduction of intercellular spaces. Moreover
325 the hypoderm connective tissue was not damaged (Figure 7).

326

327 ## DISCUSSION

328 We have evaluated the putative analgesic and anti-inflammatory activities of
329 taraxeren-3-one to clarify the pain and inflammation relieving effects. Two different
330 analgesic testing methods were employed with the objective of identifying possible
331 peripheral and central effects of the test substances. The acetic writhing test is
332 normally used to study the peripheral analgesic effects of drugs. Although this test is
333 nonspecific (e.g., anticholinergic, antihistaminic and other agents also show activity in
334 the test), it is widely used for analgesic screening (*20*). In our study, we found that

335 taraxeren-3-one (10 and 20 mg/kg) exhibited antinociceptive effect in acetic
336 acid-induced writhing response (Figure 2.). This effect may be due to inhibition of the
337 synthesis of the arachidonic acid metabolites (*21*).

338 The *in vivo* model of pain, formalin-induced paw pain has been well established
339 as a valid model for analgesic study. The formalin test produces a distinct biphasic
340 response and different analgesics may act differently in the early and late phases of
341 this test. Therefore, the test can be used to clarify the possible mechanism of an
342 anti-nociceptive effect of a proposed analgesic (*22*). Centrally acting drugs such as
343 opioids inhibit both phases equally (*20*), but peripherally acting drugs such as aspirin,
344 Indo and dexamethasone only inhibit the late phase. The inhibitory effect of
345 taraxeren-3-one on the nociceptive response in the late phase of the formalin test
346 suggested that the anti-nociceptive effect of taraxeren-3-one could be due to its
347 peripherial action (Figure 3).

348 The Carr test is highly sensitive to nonsteroidal anti-inflammatory drugs, and has
349 long been accepted as a useful phlogistic tool for investigating new drug therapies
350 (*23*). The degree of swelling of the Carr-injected paws was maximal 3 h after injection.
351 Statistical analysis revealed that taraxeren-3-one and Indo significantly inhibited the
352 development of edema 4 h after treatment ($p<0.001$) (Figure 4). They both showed
353 anti-inflammatory effects in Carr-induced mice edema paw. It is well known that the

354 3th h of the Carr-induced edema, in which the edema reaches its highest volume, is
355 characterized by the injection of Carr into the rat paw induces the liberation of
356 bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids,
357 which are responsible for the formation of the inflammatory exudates (*24*, *25*).
358 Besides, in the Carr-induced rat paw edema model, the production of prostanoids has
359 been through the serum expression of COX-2 by a positive feedback mechanism (*26*).
360 Therefore, it is suggested that the action mechanism of taraxeren-3-one may be related
361 to prostaglandin synthesis inhibition, as described for the anti-inflammatory
362 mechanism of Indo in the inhibition of the inflammatory process induced by Carr (*27*).
363 In addition, the classification of antinociceptive drugs is usually based on their
364 mechanism of action either on the central nervous system or on the peripheral nervous
365 system (*28*).

366 In the studies of mechanism on the inflammation, L-arginine–NO pathway has
367 been proposed to play an important role in the Carr -induced inflammatory response
368 (*29*). Our present results also confirm that the level of NO production increased in the
369 Carr-induced paw edema model. The expression of the inducible isoform of NO
370 synthase has been proposed as an important mediator of inflammation (*30*). In our
371 study, the level of NO was decreased significantly by treatment with 10 and 20 mg/kg
372 taraxeren-3-one. We suggest the mechanism of anti-inflammatory of taraxeren-3-one

373 may be through the L-arginine–NO pathway since taraxeren-3-one significantly
374 inhibits the NO production (Fig. 5A).

375 TNF-α is a major mediator in inflammatory responses, inducing innate immune
376 responses by activating T cells and macrophages, and stimulating secretion of other
377 inflammatory cytokines (*31*). Also, TNF-α is a mediator of Carr-induced
378 inflammatory incapacitation, and is able to induce the further release of kinins and
379 leukotrienes, which is suggested to have an important role in the maintenance of
380 long-lasting nociceptive response (*32*). In this study, we found taraxeren-3-one
381 decreased the TNF-α level in serum after Carr injection (Figure 5C).

382 The Carr-induced inflammatory response has been linked to neutrophil infiltration
383 and the production of neutrophil derived free radicals, such as hydrogen peroxide,
384 superoxide and hydroxyl radicals, as well as the release of other neutrophil derived
385 mediators (*33*). Some researches demonstrate that inflammatory effect induced by
386 Carr is associated with free radical. Free radical, prostaglandin and NO will be
387 released when administrating with Carr for 1–6 h (*5*). The edema effect was raised to
388 the maximum at the third hour (*6*). Janero *et al.*, demonstrated that MDA production
389 is due to free radical attack plasma membrane (*7*). Thus, inflammatory effect would
390 result in the accumulation of MDA. GSH is a known oxyradical scavenger. Enhancing
391 the level of GSH conducive toward reduces MDA the production. Cuzzocrea

suggested that endogenous GSH plays an important role against Carr-induced local inflammation (*34*). In this study, there is significantly increased in SOD, CAT, GPx, and GSH activities with taraxeren-3-one treatment (Table 1). Furthermore, there is 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 the increases of SOD, CAT, GPx, and GSH activities.

In conclusion, these results suggested that taraxeren-3-one possessed analgesic and anti-inflammatory effects. The anti-inflammatory mechanism of taraxeren-3-one may be related to iNOS and COX2 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 in a pathogenic factor.

ACKNOWLEDGEMENTS

The authors want to thank the financial supports from the National Science Council (NSC 97-2313-B-039 -001 -MY3), China Medical University (CMU) (CMU96-171, CMU99-S-29, CCM-P99-RD-042, and CMU99-COL-10) and Taiwan Department of Heath Clinical Trial and Research Center of Excellence

410 (DOH100-TD-B-111-004) and the Cancer Research Center of Excellence
411 (DOH100-TD-C-111-005). The authors would like to thank Dr Jeffrey Conrad for
412 critically reading the manuscript.

413

414 **LITERATURE CITED**

415 1.Higa, M.; Noha, N.; Yokaryo, H.; Ogihara, K.; Yogi, S. Three new naphthoquinone
416 derivatives from *Diospyros maritima* Blume. *Chem. Pharm. Bull (Tokyo).* **2002,** *50*,
417 590-593.
418 2. Chang, C. I.; Chen, C. R.; Chiu, H. L.; Kuo, Y. H. Chemical constituents from the
419 stems of *Diospyros maritima*. *Molecules.* **2009**, *14*, 5281-5288.
420 3. Gu, J. Q. ; Graf, T. N. ; Chai, H. B. ; Mi, Q. ; Kardono, L. B. ; Setyowati, F. M.;
421 Ismail, R.; Riswan, S.; Farnsworth, N. R.; Cordell, G. A.; Pezzuto, J. M.; Swanson,
422 S. M.; Kroll, D. J.; Falkinham, J. O. 3rd.; Wall, M. E.; Wani, M. C.; Kinghorn, A. D.;
423 Oberlies, N. H. Cytotoxic and antimicrobial constituents of the bark of *Diospyros*
424 *maritima* collected in two geographical locations in Indonesia. *J. Nat. Prod.* **2004**,
425 *67*, 1156-1161.
426 4. Kuo, Y. H.; Chang, C. I.; Li, S. Y.; Chou, C. J. ; Chen, C. F. ; Kuo, Y. H. ; Lee, K. H.
427 Cytotoxic constituents from the stems of *Diospyros maritima*. *Planta Med.* **1997**, *63*,
428 363-365.

5. Dudhgaonkar, S. P.; Tandan, S. K.; Bhat, A. S.; Jadhav, S. H.; Kumar, D. Synergistic anti-inflammatory interaction between meloxicam and aminoguanidine hydrocholide in carrageenan-induced acute inflammation in rats. *Life Sci.* **2006**, *78*, 1044–1058.

6. Kirkova, M.; Kassabova, T.; Russanov; E. *In vivo* effects of indomethacin-I. Activity of antioxidant enzymes and lipid peroxidation. *Gen. Pharmacol.* **1992**, *23*, 503–507.

7. Janero, D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* **1990**, *9*, 515–540.

8. Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **1983**, *16*, 109-110.

9. Koster, R.; Anderson, M.; DeBeer, E. J. Acetic acid for analgesic screening. *Fed. Proc.* **1959**, *18*, 418-420.

10. Taber, R. I.; Greenhouse, D. D.; Rendell, J. K.; Irwin, S. Agonist and antagonist interactions of opioids on acetic acid induced abdominal stretching in mice. *J. Pharmacol. Exp. Ther.* **1969**, *169*, 29-37.

11. Dubuisson, D.; Dennis, S. G. The formalin test: a quantitative study of the analgesic effects of morphine meperidine, and brain stem stimulation in rats and

448 cats. *Pain* **1977**, *4*, 161-174.

449 12. Mascolo, N; Jain, R.; Jain, S. C.; Capasso, F. Ethnopharmacologic investigation of

450 ginger (Zingiber officinale). *J. Ethnopharmacol* **1989**, *27*, 129-40.

451 13. Tatum, V. L.; Changchit, C.; Chow, C. K. Measurement of malondialdehyde by

452 high performance liquid chromatography with fluorescence detection. *Lipids* **1990**,

453 *25*, 226–229.

454 14. Moshage, H.; Kok, B.; Huizenga, J.R.; Jansen, P.L. M. Nitrite and Nitrate

455 Determinations in Plasma: A Critical Evaluation. *Clin. Chem.* **1995**, *41*, 892-896.

456 15. Shiratoria, K.; Ohgamia, K. Ilievaa, I.; Jina, X. H.; Koyamab, Y.; Miyashitac, K.;

457 Yoshida, K.; Kasea, S.; Ohnoa, S. Effects of fucoxanthin on

458 lipopolysaccharide-induced inflammation in vitro and in vivo. *Exp. Eye Res.* **2005**,

459 *81*, 422–428.

460 16. Flohe, L.; Otting, F. Superoxide dismutase assays. *Methods Enzymol.* **1984**, 105,

461 93-104.

462 17. Aebi, H. Catalase *in vitro. Methods Enzymol.* **1984**, *105*, 121-126.

463 18. Paglia, D. E.; Valentine, W. N. Studies on the quantitive and qualitative

464 characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **1967**, *70*,

465 158-169.

466 19. Davis, M. H.; Birt, D. F.; Schnell, R. C. Direct enzymatic assay for reduced and

oxidized glutathione. *J. Pharmacol Methods.* **1984**, *12*, 191-194.

20. Shibata, M.; Ohkubo, T.; Takahashi, H.; Inoki, R. Modified formalin test: characteristic biphasic pain response. *Pain* **1989**, *38*, 347-352.

21. 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*, 273–277.

22. 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.

23. Just, M. J.; Recio, M. C.; Giner, R. M.; Cuellar, M. J.; Manez, S.; Bilia, A. R.; Ríos, J. L. Antiinflammatory activity of unusual lupine saponins from *Bupleurum fruticescens*. *Planta Med.* **1998**, *64*, 404-407.

24. Spector, W. G.; Willoughb, D. A. The inflammatory response. *Bacteriol. Rev.* **1963**, *27*, 117–154.

25. Ueno, A.; Naraba, H.; Ikeda, Y.; Ushikubi, F.; Murata, T.; Naramiya, S.; Ohishi, S. Intrinsic prostacyclin contributes to exudation induced by bradikinin or carrageenan: a study on the paw edemainduced in ip-receptordeficient mice. *Life Sci.* **2000**, *66*, 155-160.

26. Nantel, F.; Denis, D.; Gordon, R.; Northey, A.; Cirino, M.; Metters, K. M.; Chan, C. C. Distribution and regulation of cyclooxygenase-2 in carrageenan induced

inflammation. *Br. J. Pharmacol.* **1999**, *128*, 853–859.

27. Di, R. M.; Papadimitrion, J. P.; Willoughby, D. A. Studies of the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J. Pathol.* **1971**, *104*, 15–29.

28. Planas, E.; Sanchez, S.; Rodriguez, L.; Pol, O.; Puig, M. M. Antinociceptive/anti-edema effects of liposomal morphine during acute inflammation of the rat paw. *Pharmacol.* **2000**, *60*, 121–127.

29. Salvemini, D.; Wang, Z.; Bourdon, D. M.; Stern, M. K.; Curne, M. G.; Manning, P. T. Evidence of peroxynitrite involvement in the carrageenaninduced rat paw edema. *Eur. J. Clin. Pharmacol.* **1996**, *303*, 217–220.

30. Cuzzocrea, S.; Zingarelli, B.; Calapai, G. ; Nava, F. ; Caputi, A. P. Zymosanactivated plasma induces paw oedema by nitric oxide and prostaglandin production. *Life Sci.* **1997**, *60*, 215–220.

31. Beutler, B.; Cerami, A. The biology of cachectin/TNF-α primary mediator of the host response. *Annu. Rev. Immunol.* **1989**, *7*, 625–655.

32.Tonussi, C. R.; Ferreira, S. H.; Tumour necrosis factor-alpha mediates arrageenin-induced knee-joint incapacitation and also triggers overt nociception in previously inflamed rat knee-joints. *Pain* **1999**, *82*, 81-87.

33. 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.

34. Cuzzocrea, S.; Costantino, G.; Zingarelli, B.; Mazzon, E.; Micali, A.; Caputi, A. P. The protective role of endogenous glutathione in carrageenaninduced pleurisy in the rat. *Eur. J. Pharmacol.* **1999**, *372*, 187–197.

35. Bonnie, S. P.; Ying, Y. K.; Tsui, Y. C.; Yeuk, C. L. Anti-oxidative and anti-inflammatory activities of two different species of a Chinese herb I-Tiao-Gung. *Life Sci.* **2005**, *77*, 2830–2839.

FIGURE LEGENDS

Figure 1. Chemical structure of taraxeren-3-one

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

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

553

554 Figure 5. Effects of taraxeren-3-one and indomethacin (Indo) on carrageenan
555 (Carr)-induced MDA (A), NO (B), and TNF-α (C) concentrations of edema
556 paw and serum were detected at 5rd h in mice. Each value represents as
557 mean ± S.E.M. ###compared with sample of control group. *$p < 0.05$, **$p <$
558 0.01 and ***$p < 0.001$ as compared with the Carr group (one-way ANOVA
559 followed by Scheffe's multiple range test).

560

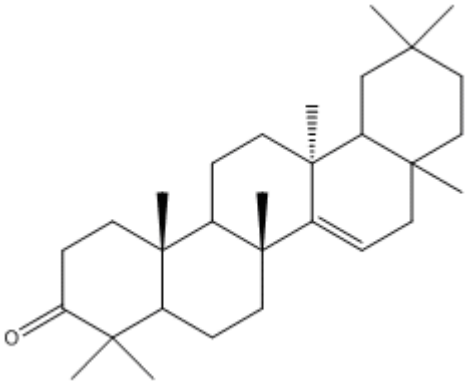
561 Figure 6. Inhibition of iNOS and COX-2 protein expression by taraxeren-3-one
562 induced by Carr of foot at 5^{th} hour in mice. Tissue suspended were then
563 prepared and subjected to Western blotting using an antibody specific for
564 iNOS and COX-2. β-actin was used as an internal control. (A) A
565 representative Western blot from two separate experiments is shown. (B)
566 Relative iNOS and COX-2 protein levels were calculated with reference to a
567 Carr-injected mouse. ###compared with sample of control group. The data
568 were presented as mean ± S.D. for three different experiments performed in
569 triplicate. ***$p < 0.001$ were compared with Carr-alone group.

570

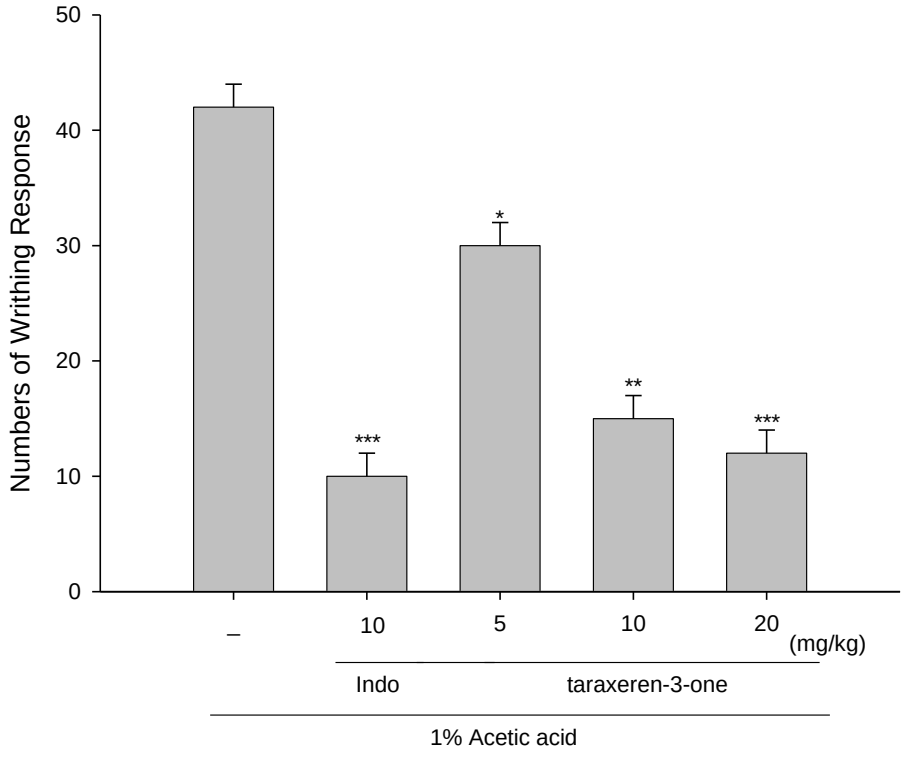
571 Figure 7. Histological appearance of the mouse hind footpad after a subcutaneous

injection with 0.9% saline (Control group) or carrageenan stained with H&E stain. (A) Control mice: show the normal appearance of dermis and subdermis without any significantly lesion. (B) Hemorrhage with moderately extravascular red blood cell and large amount of inflammatory leucocyte mainly neutrophil infiltration in the subdermis interstitial tissue of mice following the subcutaneous injection of Carr only. Moreover, detail of the subdermis layer show enlargement of the interstitial space caused by edema with exudate fluid. (C) Indomethacin (Indo) significantly reduces the level of hemorrhage, edema and inflammatory cell infiltration compared to subcutaneous injection of Carr only. (D) Taraxeren-3-one significantly show morphological alterations compared to subcutaneous injection of Carr only (100×). (E) The numbers of neutrophils were counted in each scope (400×) and obtain their average count 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.

Figure. 1.



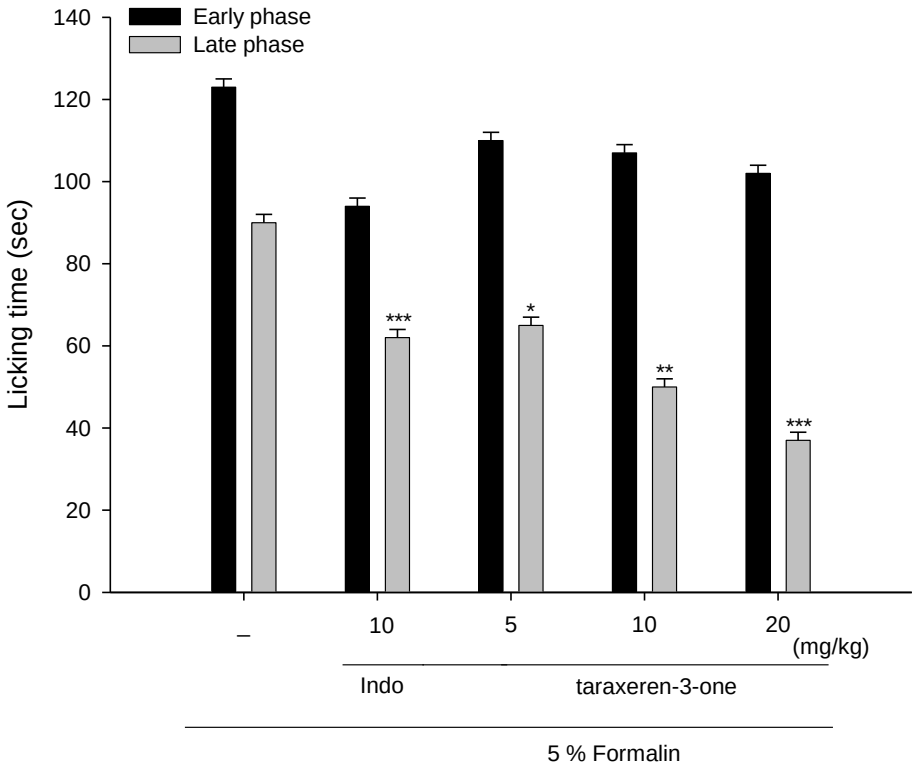
605 **Figure. 2.**



606

607

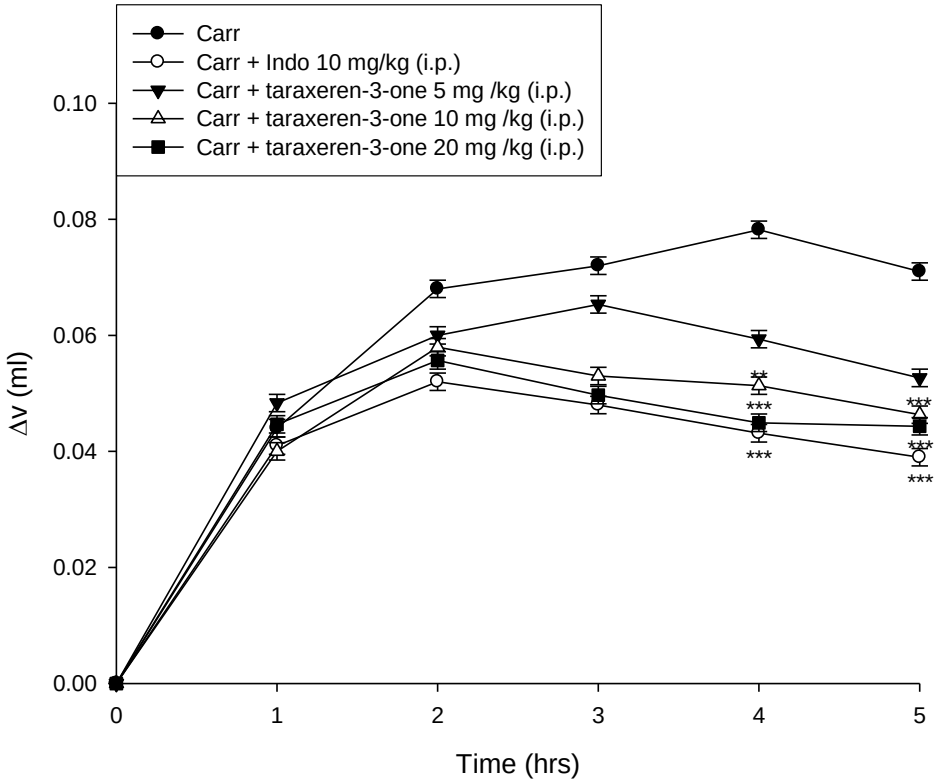
608 **Figure 3.**



609

610

611 **Figure 4.**



612

613

614

615

616

617

618

619

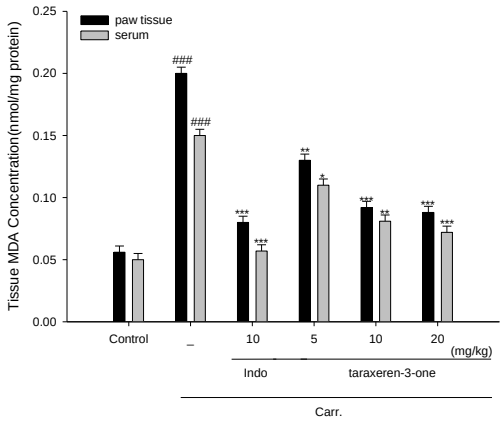
620

621

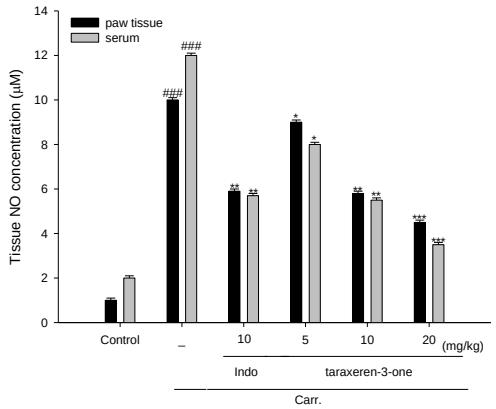
622

Figure 5.

A.


paw tissue
serum
Tissue MDA Concentration(nmol/mg protein)
0.25
0.20
0.15
0.10
0.05
0.00
###
###
***
***
**
*
***
**
***
***
Control
_
10
5
10
20
(mg/kg)
Indo
taraxeren-3-one
Carr.

B.


paw tissue
serum
Tissue NO concentration (μM)
14
12
10
8
6
4
2
0
###
###
**
**
*
*
**
**
***
***
Control
_
10
5
10
20
(mg/kg)
Indo
taraxeren-3-one
Carr.

C.

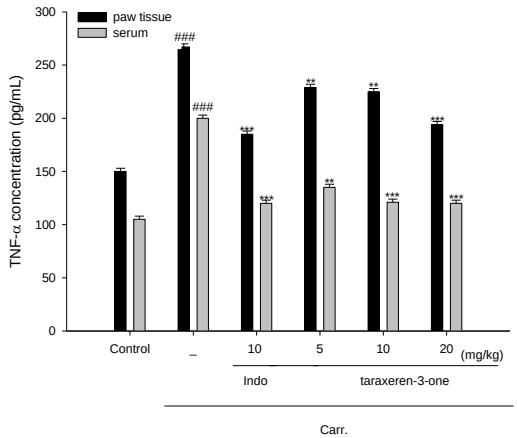
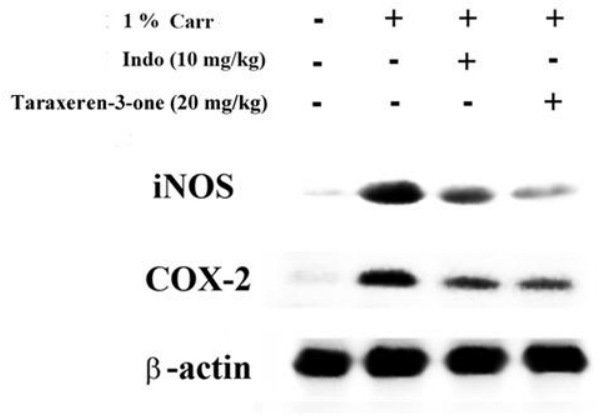

paw tissue
serum
TNF-α concentration (pg/mL)
300
250
200
150
100
50
0
###
###
***
***
**
**
**
**
***
***
Control
_
10
5
10
20
(mg/kg)
Indo
taraxeren-3-one
Carr.

Figure 6.

A.


1 % Carr - + + +
Indo (10 mg/kg) - - + -
Taraxeren-3-one (20 mg/kg) - - - +
iNOS
COX-2
β-actin

B.

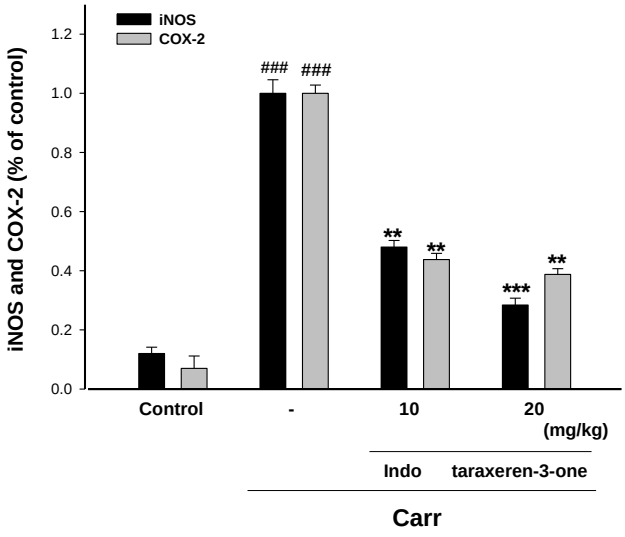
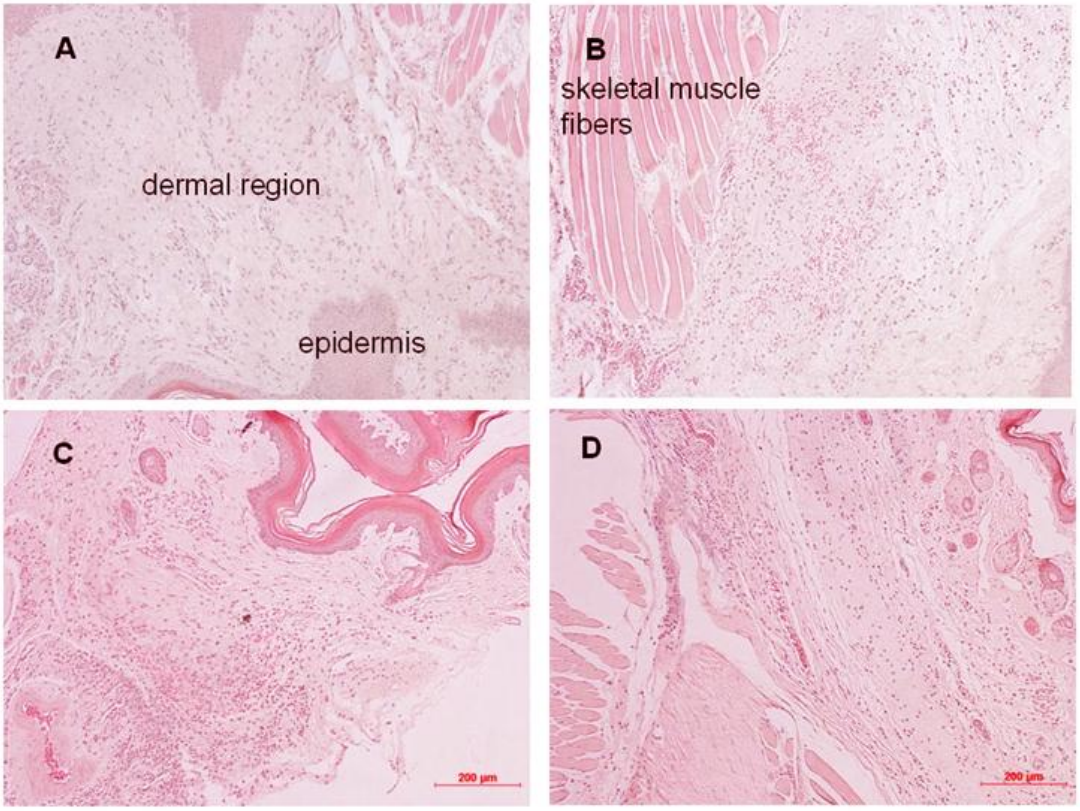
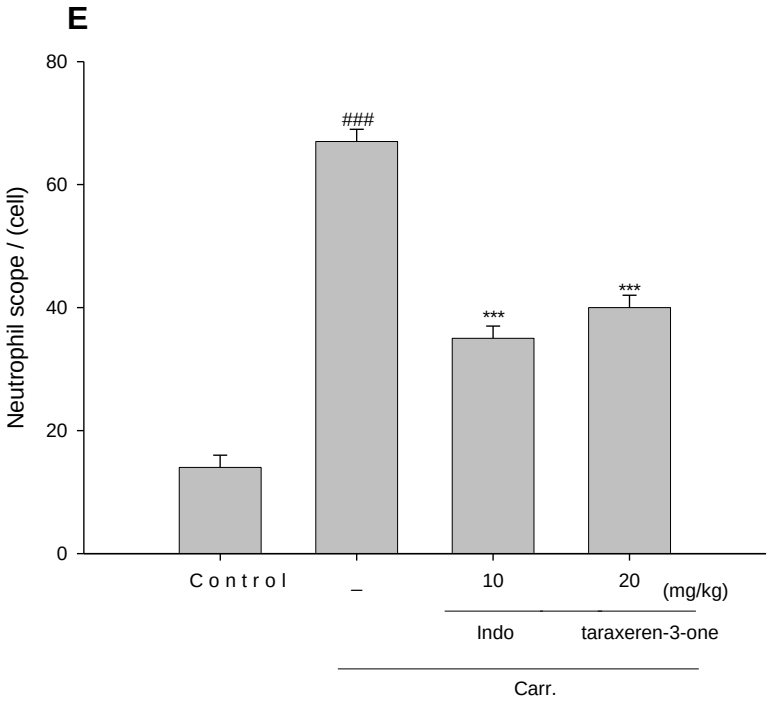

iNOS
COX-2
iNOS and COX-2 (% of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
###
###
**
**
***
**
Control
-
10
20
(mg/kg)
Indo
taraxeren-3-one
Carr

Figure 7.


A
dermal region
epidermis
B
skeletal muscle
fibers
C
200 µm
D
200 µm


E
Neutrophil scope / (cell)
80
60
40
20
0
###
***
***
Control
–
10
20
(mg/kg)
Indo
taraxeren-3-one
Carr.

643 **Table 1.** Effects of taraxeren-3-one and indomethacin (Indo) on the liver SOD, CAT, GPx and GSH activities in mice.

Groups	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***

644 Each value represents as mean ± S.E.M. $^{###}$ $p < 0.001$ as compared with control group,

645 * $p < 0.05$ and ** $p < 0.01$ as compared with the Carr (λ-carrageenan) group (one-way ANOVA followed by Scheffe's multiple range test).

646