Research Article

Analgesic and Anti-Inflammatory Activities of Methanol Extract of Cissus repens in Mice

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The aim of this study was to investigate possible analgesic and anti-inflammatory mechanisms of the CR MeOH. Analgesic effect was evaluated in two models including acetic acid-induced writhing response and formalin-induced paw licking. The anti-inflammatory effect was evaluated by λ-carrageenan-induced mouse paw edema and histopathologic analyses. The results showed that CR MeOH (500 mg/kg) decreased writhing response in the acetic acid assay and licking time in the formalin test. CR MeOH (100 and 500 mg/kg) significantly decreased edema paw volume at 4th to 5th hours after λ-carrageenan had been injected. Histopathologically, CR MeOH abated the level of tissue destruction and swelling of the edema paws. These results were indicated that anti-inflammatory mechanism of CR MeOH may be due to declined levels of NO and MDA in the edema paw through increasing the activities of SOD, GPx, and GRd in the liver. Additionally, CR MeOH also decreased IL-1β, IL-6, NFκB, TNF-α, COX-2, and iNOS levels. The contents of two active ingredients, ursolic acid and lupeol, were quantitatively determined. This paper demonstrated possible mechanisms for the analgesic and anti-inflammatory effects of CR MeOH and provided evidence for the classical treatment of Cissus repens in inflammatory diseases.

1. Introduction

Inflammatory reaction, typically characterized by redness, swelling, heat and pain, is one of the most important host defense mechanisms against invading pathogens. However, persistent or overinflammation leads to tissue damage and possibly failure of organs. Proinflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β) are produced in large quantities by activated macrophages/monocytes that stimulate cellular responses via increasing prostaglandins (PGs) and reactive oxygen species (ROS). Additionally, lipid peroxidation (malondialdehyde, MDA) is produced by free radicals attacking the cell membranes. Thus, inflammatory effect results in the accumulation of MDA [1].

Cissus repens Lamk. belongs to the family Vitaceae and is distributed in India to southern China, the Philippines, Malaysia, and Taiwan. Several studies have been performed on the composition of C. repen, and a number of compounds have been identified such as ursolic acid, asiatic acid, lupeol, friedelin, and epifriedelanol [2]. The roots and stems of C. repens are used for snake bites, rheumatic pain, and carbuncles in folk medicine, and the stems are also applied to the treatment of nephritis, long-term coughs, and diarrhoea [3]. However, no research has been investigated on the analgesic and anti-inflammatory mechanisms of C. repens yet.

Many studies have indicated that flavonoids in herbs possess anti-inflammatory activities via scavenging ROS and reducing proinflammatory cytokines (e.g., NF-κB, TNF-α, IL-1β, and IL-6), such as ursolic acid [4–6] and lupeol [7]. These two ingredients have also been isolated from C. repens in previous studies [2, 8]. In this study, not only did we reconfirm the presence of these three compounds
in CRMeOH by establishing its fingerprint chromatogram, but the contents of these two active ingredients were quantitatively determined as well.

In this study, we investigated the analgesic and anti-inflammatory activities of the methanol extract of C. repens (CRMeOH). The analgesic activity was evaluated by acetic acid-induced writhing response and formalin test. Anti-inflammatory activity was determined by using δ-carrageenan-induced mouse paw edema model and histopathologic analysis. In order to evaluate the mechanism of anti-inflammatory effect, we also analyzed the levels of TNF-α, IL-1β, IL-6, NFκB, COX, MDA, and NO in the edema tissues, as well as the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (Grd) in the liver.

2. Materials and Methods

2.1. Chemicals and Drugs. δ-carrageenan, indomethacin, and Griess reagent were purchased from Sigma-Aldrich Chemical Co. (Missouri, USA). Formalin was purchased from Nihon Shiyaku Industry Ltd. (Taipei, Taiwan). Murine IL-1β, IL-6, and TNF-α enzyme-link immunosorbent assay (ELISA) Development Kit was purchased from BioLegend, Inc (California, USA). The SOD, GPx, Grd, COX, TNF-α, and NFκB enzyme-link immunosorbent assay (ELISA) Development Kits were purchased from Cayman Chemical Company (Michigan, USA). Anti-iNOS antibody was purchased from Santa Cruz (rabbit polyclonal to iNOS, sc-650, California, USA). Indomethacin was suspended in 0.5% (w/v) carboxymethylcellulose sodium (CMC) and administered intraperitoneally to animals. LC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). All other reagents used were of analytical grade.

2.2. Plant Material. C. repens was collected from Yunlin Township of Taiwan and was identified by Dr. Yu-Chin Lin, Leader of the School of Sciences and TransWorld University Department of Biotechnology (TWUBIOT). The voucher specimen (Number: TWU-BIOT-PTE-10101) was deposited at TWUBIOT.

2.3. Chromatographic Analysis of CRMeOH. The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10ATvp liquid chromatograph equipped with a DGU-14A degasser, an FCV-10ALvp low-pressure gradient flow control valve, an SIL-10ADvp autoinjactor, an SPD-M10Avp diode array detector, and an SCL-10Avp system controller. Peak areas were calculated using Shimadzu Class-LC10 software (Version 6.12 sp5). The column was a Vercopak Inertsil 7 ODS-3 (5 μm, 4.6 × 150 mm) Vercotech Inc. column. The mobile phase consisted of a mixture of 0.2% phosphoric acid (A) and acetonitrile (B) using a gradient elution. The sample was injected of 10 μL. The following gradient profile was run at 1.0 mL/min over 60 min. Ursolic acid gradient program was set as follows: 0–25 min, 25% B, 25–55 min, 90% B, 55–60 min, 25% B. Peaks were detected at 304 nm with SPD-M10AVP (Shimadzu) detector. Lupeol gradient program was set 0–40 min, solvent A:B = 5:95. Peaks were detected at 205 nm with SPD-M10AVP (Shimadzu) detector. The above conditions were used in both HPLC assay and HPLC fingerprint of CRMeOH.

The contents of ursolic acid and lupeol of CRMeOH were qualified and quantified in the HPLC assay. In qualitative analysis, comparisons were made with the retention time and maximum absorption of the standards. In quantitative analyses, comparisons were made with peak areas under the standard curves.

2.4. Experimental Animals. Male ICR mice (20–25 g) were obtained from BioLASCO Taiwan Co., Ltd. The mice were kept in the animal center of China Medical University at a controlled temperature of 22 ± 1°C, relative humidity 55 ± 5%, and with 12 h light/12 h dark cycles for 1 week before the experiment. Animals were provided with rodent diet and clean water ad libitum. All studies were conducted in accordance with the National Institutes of Health (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 [9]. The experimental protocol was approved by the Committee on Animal Research, China Medical University. This study used cervical dislocation to sacrifice animal.

2.5. Acute Toxicity Study. The acute toxicity test in mice was carried out according to the method of Liao et al. [10]. Male ICR mice were random divided into three groups (10 mice per group). The mice were administered orally with CRMeOH (2.5 g, 5 g, and 10 g/kg). The experimental mice were provided with forage and water ad libitum, and they were kept under regular observation for 14 days for any mortality or behavioral changes. The behavioral changes closely observed for were hyperactivity, tremors, ataxia, convulsions, salivation, diarrhea, lethargy, sleep, and coma.

2.6. Acetic-Acid-Induced Writhing Response. The writhing test in mice was carried out according to the method of Koster et al. [11]. The writhes were induced by intraperitoneal injection of 1.0% acetic acid (v/v, 0.1 ml/10 g body weight). There are three different doses (20, 100, and 500 mg/kg) of CRMeOH administered orally to each groups of mice, 60 min before chemical stimulus. Indomethacin as a positive control was administered 30 min prior to acetic acid injection. The number of muscular contractions was counted over a period of 5 min after acetic acid injection. The data represented the total numbers of writhes observed during 10 min.

2.7. Formalin Test. The formalin test was conducted based on the method of Tjølsen et al. [12]. Twenty microliter of
5% formalin in saline was injected subcutaneously into the right hind paw of each mouse. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured for 5 min after formalin injection (early phase) and 20–30 min after formalin injection (late phase). CRMeOH (20, 100, and 500 mg/kg, p.o.) was administered 60 min before the formalin injection. Indomethacin (10 mg/kg, i.p.) was administered 30 min before formalin injection.

2.8. λ-Carrageenan-Induced Mouse Paw Edema. The anti-inflammatory activity of CRMeOH was determined by the λ-carrageenan-induced paw edema test in the hind paws of mice. The test was conducted according to the method of Vinegar et al. [13]. The basal volume of right hind paw was determined before the administration of any drug. Fifty microliter of 1% λ-carrageenan suspended in saline was injected into the plantar side of right hind paw, and the paw volume was measured at the 1st, 2nd, 3rd, 4th, and 5th hours after the injection using an MK101 CMP plethysmometer (Muromachi Kikai Co., Ltd). The degree of swelling was determined by the delta volume (a-b), where “a” is the volume before the injection and “b” is the volume after the treatment. Indomethacin (10 mg/kg) was administered intraperitoneally 30 min before λ-carrageenan injection. CRMeOH (20, 100, and 500 mg/kg) was orally administered 60 min before λ-carrageenan injection. The control was given an equal volume of saline.

In the secondary experiment, another set of mice were orally administered with 0.5% CMC, indomethacin or CRMeOH 1 h before λ-carrageenan had been injected into their right hind paws. The right hind paws of the animals were taken 4 h later. The paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline four times its volume before homogenization at 4°C. Then, the homogenate was centrifuged at 12,000 rpm for 5 min. The supernatant was obtained and stored at −20°C for upcoming MDA, NO, TNF-α, IL-1β, IL-6, NfKB, and COX analysis. As for the whole liver tissue, similarly, it was rinsed in ice-cold normal saline and immediately placed in cold normal saline of equal volume before homogenization at 4°C. The homogenate was then centrifuged at 12,000 rpm for 5 min. The supernatant was obtained and stored at −20°C for later analysis of antioxidant enzymes (SOD, GPx, and GRd) activities.

2.9. Histological Analysis. For histopathological examination, biopsies of paws were taken 4 h after the induction with carrageenan. Tissue slices were fixed in 10% formalin for 3 days, decalciﬁed overnight, embedded in parafﬁn, and sectioned into 4 μm tissue sections. Tissue sections were stained with hematoxylin and eosin (H&E stain) and examined with a BX60 microscope (Olympus, Melville, NY) for pathological changes. Inflammatory reactions induced by λ-carrageenan, including paw swelling, were examined. The enlarged cavities after CRMeOH (20 mg, 100 mg, and 500 mg/kg) and indomethacin (10 mg/kg) treatments were also examined. Images were captured with a Macroﬁre 599831 camera. The results were identiﬁed in the Animal Disease Diagnostic Center (ADDC), National Chung Hsing University, Taichuang, Taiwan.

2.10. MDA Assay. The production of MDA was induced by λ-carrageenan injection and evaluated by the thiobarbituric acid reacting substance (TBARS) method [14]. In brief, MDA reacted with TBARS at high temperature and formed a red-complex TBARS. The absorbance of TBARS was recorded at 532 nm.

2.11. NO Assay. NO was measured according to the method of Moshage et al. [15]. NO3− was converted into NO2− by nitrate reductase, NO2− subsequently reacted with sulfanilic acid to produce diazonium ion and coupled with N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative (purplish red) which could be recorded at 540 nm.

2.12. TNF-α, IL-1β, IL-6, and NfKB Assay. IL-1β was measured by an enzyme-linked immunosorbent assay [16]. The capture antibody of IL-1β was seeded to each well of a 96-well plate overnight. Next day, a second set of biotinylated antibody was incubated with sample tissues or standard antigens in the plate before streptavidin was ﬁnally added. The color of the reaction converted from purple to yellow and was recorded at 450 nm. IL-6, TNF-α, and NfKB were detected using the same method as IL-1β. Each sample was presented as ng/mg in TNF-α, IL-6, IL-1β, and NfKB concentrations.

2.13. COX-2 Assay. The content of COX-2 was determined by measuring the peroxidase activity of PGHS (prostaglandin endoperoxide H2 synthase) [17]. Peroxidase activity of PGHS was determined by following the oxidation of N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) at 37°C using arachidonate as the substrate. The increase in color was recorded at 590 nm.

2.14. Measurement of Antioxidant Enzymes. SOD was measured according to the method of Vani et al. [18]. Xanthine and xanthine oxidase (XOD) generated superoxide radicals reacted with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye, and the color was recorded at 540 nm. GPx was measured according to the method of Ceballos-Picot et al. by detecting the contents of GR and NADPH [19]. Oxidation of NADPH into NADP+ is accompanied by a decrease in absorbance recorded at 340 nm. GRd was measured according to the method of Ahmad and Holdsworth [20] which detects the decrease of glutathione (GSSG) in the presence of NADPH. NADPH oxidized into NADP+ would result in a decrease in absorbance recorded at 340 nm.

2.15. Western Blotting for iNOS. Freshly isolated paw tissue was homogenized in a lyses buffer. The protein concentration of the tissue homogenate and the cytosolic and microsomal fractions were determined according to the method of Lowry et al. [21]. One hundred μg of protein from paw homogenates or 50 μg of protein from purified microsome...
were loaded per lane on 8% or 12% polyacrylamide gels and electrophoresis was performed. Proteins were then transferred onto nitrocellulose membranes. The membrane was blocked overnight with buffer and then incubated with primary antibodies for 1 h using 1:1000 dilution of rabbit anti-iNOS (100-fold dilution, 2 h at 25°C, Santa Cruz). The membranes were then washed three times in TBST solution containing Tris buffer solution (TBS) with 0.1% Tween-20 for 15 min, incubated with 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG as the second antibody for 1 h. The protein was visualized with an enhanced chemiluminescence Western blotting detection kit (Amersham, Arlington Heights, IL, USA), and exposed to X-ray film for 3 min.

2.16. Statistical Analysis. All data represented the mean ± SEM. Statistical analyses were performed with SPSS software, and were carried out using one-way ANOVA followed by Scheffe’s multiple range test.

3. Results

3.1. Chromatographic Analysis of CRMeOH. The HPLC chromatogram shows that ursolic acid and lupeol with retention times of 28.94 min and 29.18 min, respectively. The maximum absorbance was 304 nm, and the relative amounts were in the order of ursolic acid (69.16 mg/g) and lupeol (8.65 mg/g).

3.2. Acute Toxicity Study. Acute toxicity of CRMeOH was evaluated in mice at the doses of 2.5, 5, and 10 g/kg. After 14 days of oral administration, CRMeOH did not cause any behavioral changes, and no mortality was observed. Therefore, the LD50 value of CRMeOH was concluded to be greater than 10 g/kg in mice, indicating it was practically non acute toxic.

3.3. Acetic-Acid-Induced Writhing Response. Figure 1 shows acetic acid-induced writhing responses in mice which serve as an indication of analgesic activities of CRMeOH. Intraperitoneal injection of acetic acid produced 41.67 ± 5.28 writhes in the solvent control group. The writhing response was significantly reduced by pretreatments with 500 mg/kg CRMeOH (18.83 ± 2.21, P < 0.001) and 10 mg/kg indomethacin (10.17 ± 1.99, P < 0.001).

3.4. Formalin Test. CRMeOH demonstrated a dose-dependent relationship in late phase of formalin-induced pain. In the early phase, CRMeOH (20, 100, and 500 mg/kg) and indomethacin (10 mg/kg) treated groups did not show any significant changes as compared to the control group (Figure 2(a)). In the late phase, subcutaneous injection of formalin-induced licking and biting responses which lasted a duration of 171.0 ± 16.44 s. The time was significantly decreased by pretreatment with 100 and 500 mg/kg CRMeOH (99.29 ± 4.90, P < 0.001 and 76.89 ± 3.47, P < 0.001) and 10 mg/kg indomethacin (85.67 ± 2.74, P < 0.001), as shown in Figure 2(b).

3.5. Effect of CRMeOH on λ-Carrageenan-Induced Mouse Paw Edema. As shown in Figure 3, after the λ-carrageenan injection, the volume of mouse paw increased as edema developed, indicating inflammatory activities. However, indomethacin (10 mg/kg) and CRMeOH (500 mg/kg) obviously decreased paw edema at the 4th and 5th h after the administration. CRMeOH at the dose of 500 mg/kg (29.60 ± 4.40, P < 0.001) showed almost equal amount of inhibition as 10 mg/kg indomethacin (31.67 ± 4.40, P < 0.001).

3.6. Histological Analysis. For histopathological examination, paw biopsies were harvested for 4 h after λ-carrageenan had been injected. Both CRMeOH and indomethacin significantly decreased carrageenan-induced paw oedema (Figure 4). Figure 4(a) shows no inflammation, tissue destruction, and swelling phenomenon in the paws of normal mice. On the other hand, the λ-carrageenan group displayed enlarged cavities in the paw tissue (Figure 4(b)). As for the positive and experimental groups, edematous condition was obviously abated by treatment with indomethacin (10 mg/kg) and CRMeOH (20, 100, and 500 mg/kg), as shown in Figures 4(c), 4(d), 4(e), and 4(f).

3.7. Effect of CRMeOH on MDA Level. In this study, MDA level was used to signify lipid peroxidation. In Table I, MDA level dramatically increased in the λ-carrageenan group (2.62 ± 0.16 nmol/mg); however, pretreatments with 500 mg/kg CRMeOH (1.274 ± 0.065, P < 0.001) and 10 mg/kg indomethacin (1.376 ± 0.096, P < 0.001) showed significant inhibition in the increase MDA level.

3.8. Effect of CRMeOH on NO Level. Pretreatments with 100 and 500 mg/kg CRMeOH (6.918 ± 0.318, P < 0.001 and 6.222 ± 0.501, P < 0.001) and 10 mg/kg indomethacin (6.438 ± 0.321, P < 0.001) showed significant inhibition in
3.9. Effect of CRMeOH on TNF-α, IL-1β, IL-6, and NFκB. TNF-α, IL-1β, IL-6, and NFκB levels in λ-carrageenan-induced edema paws were remarkably raised. CRMeOH (500 mg/kg) and indomethacin (10 mg/kg) significantly reduced the levels of TNF-α ($P < 0.001$). The IL-1β and IL-6 levels increased significantly at the 4th h after Carr injection ($P < 0.01, P < 0.001$). The IL-1β, IL-6, and NFκB levels in serum were significantly decreased by treatment with 500 mg/kg CRMeOH as well as 10 mg/kg indomethacin (Table 1).

3.10. Effect of CRMeOH on COX Level. Table 2 shows that COX-2 level was greatly raised (207.273 ± 9.984 U/mg) in λ-carrageenan-induced edema paw. However, COX-2 levels were decreased by treating with 500 mg/kg CRMeOH (134.936 ± 9.918, $P < 0.001$) as well as 10 mg/kg indomethacin (135.996 ± 7.489, $P < 0.001$). No such result was found in COX-1 activity assay.

3.11. Effect of CRMeOH on the Carr-Induced Mice Paw Edema. Results showed that injection of CRMeOH (500 mg/kg) inhibited iNOS (71.9%) proteins expression in Carr-induced ($P < 0.001$) mouse paw edema at the 4th h (Figures 5(a) and 5(b)). However, 10 mg/kg Indomethacin showed an average down-regulation of iNOS (84.0%, $P < 0.01$, Figure 5(b)).

3.12. Effect of CRMeOH on the Activities of Antioxidant Enzymes. The antioxidant enzyme activities such as SOD, GPx, and GRd at the 4th hour following the intrapaw injection of λ-carrageenan in mice are presented in Table 3. SOD, GPx, and GRd activities in liver tissue were decreased significantly at 4th after λ-carrageenan administration. Treatment with CRMeOH (500 mg/kg) and indomethacin (10 mg/kg) increased the SOD, GPx, and GRd activities significantly ($P < 0.001$).

4. Discussion

Cissus is a genus of about 200 species found in tropical and subtropical regions. In Taiwan, CR is used for the treatment of many diseases, such as epilepsy, stroke, abscess, and diabetes. In addition, it also has anti-inflammatory and antirheumatic activity [22].

Traditional medicines have been popularly used in the treatment of various diseases in recent years. Many medicinal
plants supply analgesic and anti-inflammatory activities to treat acute, chronic, or recurring illnesses. Based on its therapeutic claims in traditional medicine, we investigated possible mechanisms of the analgesic and anti-inflammatory effects of **CR MeOH**.

Analgesic effect of **CR MeOH** was evaluated by two animal models, including acetic acid-induced writhing response and formalin test. Acetic acid indirectly triggers the release of nociceptive endogenous mediators (such as serotonin and prostaglandin) and proinflammatory cytokines (such as TNF-α and IL-1β) to cause painful sensation [23]. This nociceptive effect can be prevented by NSAID drugs and analgesic agents with central actions, such as morphine. In the present study, **CR MeOH** (500 mg/kg) and indomethacin (10 mg/kg) provided antinociceptive effect to relieve abdominal writhes induced by acetic acid in mice.

Formalin test involves a biphasic responses. The first phase (neurogenic nociceptive response) occurs in the first 5 min after the formalin injection. The second phase (inflammatory nociceptive response) occurs between 15 to 30 min after formalin injection. Central acting drugs can inhibit both phases, while peripheral acting drugs, such as NSAID drugs, only inhibit the second phase [24]. The treatments of **CR MeOH** (100 and 500 mg/kg) and indomethacin
Table 1: Effect of CRMeOH and indomethacin on MDA, NO, TNF-α, IL-1β, IL-6, and NFκB level in the edema paws.

| Groups            | MDA (nmol/mg protein) | NO (μM) | TNF-α (pg/mg protein) | IL-1β (ng/mg protein) | IL-6 (ng/mg protein) | NFκB (ng/mg protein) |
|-------------------|------------------------|---------|-----------------------|-----------------------|----------------------|----------------------|
| Carr              | 2.619 ± 0.163          | 9.956 ± 0.437 | 297.600 ± 15.539       | 929.167 ± 60.585      | 1483.900 ± 80.219    | 0.842 ± 0.088        |
| Carr + Indomethacin | 1.376 ± 0.096***       | 6.438 ± 0.321*** | 190.000 ± 11.779***    | 639.500 ± 14.040*      | 1113.500 ± 74.330*     | 0.527 ± 0.032**       |
| Carr + CRMeOH (20 mg/kg) | 2.328 ± 0.149         | 7.699 ± 0.398* | 250.000 ± 16.441       | 922.000 ± 76.033      | 1239.000 ± 70.825     | 0.538 ± 0.039**       |
| Carr + CRMeOH (100 mg/kg) | 2.001 ± 0.187*        | 6.918 ± 0.318*** | 212.000 ± 8.569**      | 740.000 ± 35.407      | 1022.400 ± 89.513**     | 0.516 ± 0.042***      |
| Carr + CRMeOH (500 mg/kg) | 1.274 ± 0.065***      | 6.222 ± 0.501*** | 191.917 ± 9.339***     | 606.000 ± 50.212**     | 978.444 ± 44.539***    | 0.493 ± 0.014***      |

Each value represents the mean ± S.E.M. (n = 8). *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).
CRMeOH and indomethacin (10 mg/kg) were able to diminish the nociceptive response in the second phase induced by the formalin injection. The results indicated that the anti-inflammatory effect of CRMeOH could be due to its anti-inflammatory effect.

λ-Carrageenan-induced paw edema, an in vivo model of inflammation, has also been characterized as a biphasic event [13]. Histamine, bradykinin, and 5-hydroxytryptamine (5-HT) are released in the first phase of edema (0–1 h). In the second phase (1–6 h), TNF-α, IL-1β, COX-2, and PGs are produced more actively, which could in turn worsen the degree of swelling. It is well known that the expression of COX-2 is maximal at the late phase of λ-carrageenan-induced paw edema, which could subsequently increase prostaglandin levels in inflammatory reactions [25]. IL-1β and TNF-α are involved in neutrophil migration in λ-carrageenan-induced inflammation. These mediators are able to recruit leukocytes, such as neutrophils, as reported in several recent experimental models [26, 27]. In this study, CRMeOH and indomethacin (10 mg/kg) showed significant anti-inflammatory effect on λ-carrageenan induced mouse paw edema from the 4th and 5th. Moreover, the levels of TNF-α, IL-1β, and IL-6 were also decreased by treating with CRMeOH and indomethacin (10 mg/kg). Thus, a putative anti-inflammatory mechanism of CRMeOH could be associated with the degree of inhibition on inflammatory mediators, such as TNF-α, IL-1β, and IL-6.

COX-2 selective inhibitor is a form of NSAID that directly targets COX-2, an enzyme responsible for inflammation and pain [28]. Selectivity for COX-2 reduces the risk of peptic ulceration and similar to COX-2, iNOS produces significant amounts of NO and has been identified as playing a central role in inflammatory diseases [29]. Numerous studies have indicated that NO and PGs participate in inflammatory and nociceptive events [30]. Inhibition of NO and PGs production via the inhibition of iNOS and COX-2 expression is beneficial in treating inflammatory diseases [31]. Experimental results indicate that the CRMeOH (500 mg/kg) has an inhibitory effect on iNOS and COX-2 protein expression. The anti-inflammatory mechanism of CRMeOH may be related to the inhibition of PGs and NO synthesis, as described for the anti-inflammatory mechanism of indomethacin in inhibiting the inflammatory process, induced by carrageenan [32].

In histopathological examination of the λ-carrageenan group, paw biopsies displayed obvious enlarged cavities in the connective tissue. However, as shown in Figure 5, the indomethacin and CRMeOH groups significantly improved the edematous condition induced by λ-carrageenan. The intercellular spaces of connective tissues were also obviously decreased.

Neutrophil migration, stimulated by λ-carrageenan, released active substances such as ROS. Nitric oxide, hydrogen peroxide, hydroxyl radicals, and superoxide anions play major roles in terms of producing cellular damage [33, 34]. MDA is a reactive aldehyde caused by toxic stress in cells and form covalent protein adducts which are referred to as advanced lipid peroxidation end products (ALE). Inflammatory effect would result in the accumulation of MDA. Enhancing the level of glutathione and SOD, on the other hand, could reduce MDA production [35]. In this study, SOD, GPx, and GRd activities increased in the liver after treatment with CRMeOH. On the other hand, CRMeOH decreased significantly the MDA level. Therefore, we suspect that the suppression of MDA production was likely associated to the increase in SOD, GPx, and GRd activities.

The fingerprint chromatogram of CRMeOH was established. Its two triterpenoid (ursolic acid and lupeol) contents were quantitatively determined. Two active compounds of

### Table 2: Effect of CRMeOH and indomethacin on COX concentrations in mouse paw edema.

| Groups          | COX-1 (U/mL/mg protein) | COX-2 (U/mL/mg protein) |
|-----------------|-----------------------|-------------------------|
| Carr            | 133.588 ± 5.583       | 207.273 ± 9.984         |
| Carr + Indomethacin | 90.850 ± 2.963***     | 135.669 ± 7.489***      |
| Carr + CRMeOH (20 mg/kg) | 133.188 ± 6.565       | 162.505 ± 12.750        |
| Carr + CRMeOH (100 mg/kg) | 132.383 ± 7.511       | 148.923 ± 14.681*       |
| Carr + CRMeOH (500 mg/kg) | 129.140 ± 5.214       | 134.936 ± 9.918***      |

Each value represents the mean ± S.E.M. (n = 8). *P < 0.05 and ***P < 0.001 as compared with the λ-carrageenan (Carr) group (one-way ANOVA followed by Scheffe’s multiple range test).

### Table 3: Effect of CRMeOH and indomethacin on the liver SOD, GPx, and GRd activities in mice injected with λ-carrageenan.

| Groups          | SOD (U/mg protein) | GPx (U/mg protein) | GRd (U/mg protein) |
|-----------------|--------------------|--------------------|--------------------|
| Carr            | 4.42 ± 0.21        | 0.066 ± 0.005      | 0.217 ± 0.033      |
| Carr + Indomethacin | 6.43 ± 0.27***     | 0.179 ± 0.028*     | 1.215 ± 0.049***   |
| Carr + CRMeOH (20 mg/kg) | 5.31 ± 0.24        | 0.139 ± 0.024      | 0.892 ± 0.098**    |
| Carr + CRMeOH (100 mg/kg) | 5.42 ± 0.23        | 0.230 ± 0.028**    | 1.260 ± 0.095***   |
| Carr + CRMeOH (500 mg/kg) | 6.24 ± 0.17 ***    | 0.255 ± 0.022****  | 1.658 ± 0.035***   |

Each value represents the mean ± S.E.M. (n = 8). *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).
CRMeOH used for alleviating inflammatory pain. Moreover, performed in triplicate.

This study also provides the first evidence for its analgesic and antinociceptive activities [36, 37].

CRMeOH, ursolic acid and lupeol, have been previously demonstrated to possess anti-inflammatory and antinociceptive activities [36, 37].

This study demonstrated that Cissus repens exhibited antinociceptive and anti-inflammatory activities. The anti-inflammatory mechanisms of CRMeOH against λ-carrageenan induced paw edema involved two possible pathways. The first pathway was likely associated to the decrease in MDA and NO levels in the edema paw via increasing the activities of SOD, GPx, and GRd in the liver. The other pathway alleviated inflammation by increasing the levels of inflammatory factors, such as IL-1β, IL-6, TNF-α, NFκB, iNOS, and COX-2 in the edema paw induced by λ-carrageenan. This supported possible mechanisms of CRMeOH used for alleviating inflammatory pain. Moreover, this study also provides the first evidence for its analgesic and inflammation effect in Cissus repens.

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**References**

[1] D. R. Janero, “Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury,” *Free Radical Biology and Medicine*, vol. 9, no. 6, pp. 515–540, 1990.

[2] Y. H. Wand, Z. K. Zhang, H. P. HE et al., “Lignans and Triterpenoids from *Cissus repens*, *Acta Botanica Yunnanica*, vol. 28, no. 4, pp. 437–437, 2006.

[3] Y. H. Wang, Z. K. Zhang, H. P. He et al., “Stilbene C-glucosides from *Cissus repens*, *Journal of Asian Natural Products Research*, vol. 9, no. 7, pp. 631–636, 2007.

[4] S. J. Tsai and M. C. Yin, “Antioxidative and anti-inflammatory protection of oleanolic acid and ursoic acid in PC12 cells,” *Journal of Food Science*, vol. 73, no. 7, pp. 174–178, 2008.

[5] J. Lu, Y. L. Zheng, D. M. Wu, L. Luo, D. X. Sun, and Q. Shan, “Ursolic acid ameliorates cognition deficits and attenuates oxidative damage in the brain of senescent mice induced by d-galactose,” *Biochemical Pharmacology*, vol. 74, no. 7, pp. 1078–1090, 2007.

[6] R. Checker, S. K. Sandur, D. Sharma et al., “Potent anti-inflammatory activity of ursoic acid, a triterpenoid antioxidant, is mediated through suppression of NF-xB, AP-1 and NF-AT,” *PLoS One*, vol. 7, no. 2, Article ID Article number1313, 2012.

[7] V. Saratha and S. P. Subramanian, “Lupeol, a triterpenoid isolated from *Calotropis gigantea* latex ameliorates the primary and secondary complications of FCA induced adjuvant disease in experimental rats,” *Inflammopharmacol*, vol. 20, no. 1, pp. 27–37, 2012.

[8] H. R. Siddique and M. Saleem, “Beneficial health effects of lupeol triterpene: a review of preclinical studies,” *Life sciences*, vol. 88, no. 7–8, pp. 285–293, 2011.

[9] M. Zimmermann, “Ethical guidelines for investigations of experimental pain in conscious animals,” *Pain*, vol. 16, no. 2, pp. 109–110, 1983.

[10] J. W. Liao, Y. C. Chung, J. Y. Yeh et al., “Safety evaluation of water extracts of *Toona sinensis* Roemor leaf,” *Food and Chemical Toxicology*, vol. 45, no. 8, pp. 1393–1399, 2007.

[11] R. Koster, M. Anderson, and E. J. Beer, “Acetic acid for analgesic screening,” *Federation Proceeds*, vol. 18, pp. 412–416, 1959.

[12] A. Tjølsen, O. G. Berge, S. Hunskaar, J. H. Rosland, and K. Hole, “The formalin test: an evaluation of the method,” *Pain*, vol. 51, no. 1, pp. 5–17, 1992.

[13] R. Vinegar, W. Schreiber, and R. Hugo, “Biphasic development of carrageenin edema in rats,” *Journal of Pharmacology and Experimental Therapeutics*, vol. 166, no. 1, pp. 96–103, 1969.

[14] V. L. Tatum, C. Changchit, and C. K. Chow, “Measurement of malondialdehyde by high performance liquid chromatography with fluorescence detection,” *Lipids*, vol. 25, no. 4, pp. 226–229, 1990.

[15] H. Moshage, B. Kok, J. R. Huizenga, and P. L. M. Jansen, “Nitrite and nitrate determinations in plasma: a critical evaluation,” *Clinical Chemistry*, vol. 41, no. 6, pp. 892–896, 1995.

[16] T. Atoğlu, M. Üngör, B. Serpek, S. Halilöglu, H. Atoğlu, and H. Ari, “Interleukin-1β and tumour necrosis factor-α levels in
periapical exudates,” International Endodontic Journal, vol. 35, no. 2, pp. 181–185, 2002.

[17] N. Petrovic and M. Murray, “Using N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD) to assay cyclooxygenase activity in vitro,” Methods in Molecular Biology, vol. 594, pp. 129–140, 2010.

[18] M. Vani, G. P. Reddy, G. R. Reddy, K. Thyagaraju, and P. Reddanna, “Glutathione-S-transferase, superoxide dismutase, xanthine oxidase, catalase, glutathione peroxidase and lipid peroxidation in the liver of exercised rats,” Biochemistry International, vol. 21, no. 1, pp. 17–26, 1990.

[19] I. Ceballos-Picot, J. M. Trivier, A. Nicole, P. M. Sinet, and M. Thevenin, “Age-correlated modifications of copper-zinc superoxide dismutase and glutathione-related enzyme activities in human erythrocytes,” Clinical Chemistry, vol. 38, no. 1, pp. 66–70, 1992.

[20] F. B. Ahmad and D. K. Holdsworth, “Medicinal plants of Sabah, Malaysia, Part II. The Muruts,” International Journal of Pharmcognosy, vol. 32, no. 4, pp. 378–383, 1994.

[21] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, “Protein measurement with the Folin phenol reagent,” The Journal of Biological Chemistry, vol. 193, no. 1, pp. 265–275, 1951.

[22] L. C. L. Araújo, E. C. Marín, and P. Moriguishi, “Estudos preliminares das atividades farmacológicas e toxicológicas do extrato hidroalcoólico de Cissus succacalis Bak er,” in Proceedings of the 14th Simpósio de Plantas Medicinais do Brasil, Florianópolis, Brazil, 1996.

[23] M. N. Manjavachi, N. L. M. Quintão, M. M. Campos et al., “The effects of the selective and non-peptide CXCR2 receptor antagonist SB225002 on acute and long-lasting models of nociception in mice,” European Journal of Pain, vol. 14, no. 1, pp. 23–31, 2010.

[24] A. J. Reeve and A. H. Dickenson, “The roles of spinal adenosine receptors in the control of acute and more persistent noiceptive responses of dorsal horn neurones in the anaesthetized rat,” British Journal of Pharmacology, vol. 116, no. 4, pp. 2221–2228, 1995.

[25] K. Seibert, Y. Zhang, K. Leahy et al., “Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain,” Proceedings of the National Academy of Sciences of the United States of America, vol. 91, no. 25, pp. 12013–12017, 1994.

[26] D. Salvemini, Z. Q. Wang, P. S. Wyatt et al., “Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation,” British Journal of Pharmacology, vol. 118, no. 4, pp. 829–838, 1996.

[27] L. C. Loram, A. Fuller, L. G. Fick, T. Cartmell, S. Poole, and D. Mitchell, “Cytokine profiles during carrageenan-induced inflammatory hyperalgesia in rat muscle and hind paw,” Journal of Pain, vol. 8, no. 2, pp. 127–136, 2007.

[28] J. Chao, T. C. Lu, J. W. Liao et al., “Analgesic and anti-inflammatory activities of ethanol root extract of Mahonia owakensis in mice,” Journal of Ethnopharmacology, vol. 125, no. 2, pp. 297–303, 2009.

[29] Y. Vodovotz, P. K. M. Kim, E. Z. Bagci et al., “Inflammatory modulation of hepatocyte apoptosis by nitric oxide: in vivo, in vitro, and in silico studies,” Current Molecular Medicine, vol. 4, no. 7, pp. 753–762, 2004.

[30] H. Holthausen and J. O. Arndt, “Nitric oxide evokes pain in humans on intracutaneous injection,” Neuroscience Letters, vol. 165, no. 1-2, pp. 71–74, 1994.

[31] C. Bogdan, “Nitric oxide and the immune response,” Nature Immunology, vol. 2, no. 10, pp. 907–916, 2001.