Research Article

Ameliorative Effects of Scopoletin from Crossostephium chinensis against Inflammation Pain and Its Mechanisms in Mice

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Scopoletin exists in nature as an anti-oxidant, hepatoprotective, and anti-inflammatory activities reagent. In this study, we have investigated the analgesic effects of the scopoletin using the models of acetic acid-induced writhing response and the formalin test, the anti-inflammatory effects of scopoletin using model of λ-carrageenan (Carr)-induced paw edema. The treatment of ICR mice with scopoletin inhibited the numbers of writhing response and the formalin-induced pain in the late phase. This study demonstrated that the administration of scopoletin resulted in the reduction of Carr-induced mice edema, and it increased the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) after Carr injection. We also demonstrated scopoletin significantly attenuated the malondialdehyde (MDA) level in the edema paw after Carr injection. Scopoletin decreased the NO, tumor necrosis factor (TNF-α) and prostaglandin E2 (PGE2) levels on serum after Carr injection. Scopoletin decreased Carr-induced inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions in the edema paw. These anti-inflammatory mechanisms of scopoletin might be related to the decrease in the level of MDA via increasing the activities of SOD, CAT, and GPx in the edema paw. Also, scopoletin could affect the production of NO, TNF-α, and PGE2, and therefore affect the anti-inflammatory effects.

1. Introduction

Scopoletin (6-methoxy-7-hydroxycoumarin) was isolated from many herb plants, including Castanea crenata [1], Crossostephium chinensis [2], and so forth, and a traditional Chinese medicine that has been used for the treatment of various rheumatoid diseases for a long history. It has been shown to exert several biological activities, such as anticholinesterasic, antithyroid, antioxidant, anti-hyperglycemic, hypouricemic, antitumoral [3], and anti-inflammatory activities [4]. However, little information is available on the analgesic and anti-inflammatory effects of scopoletin in vivo.

Pain is often associated with inflammation, which resulted from tissue damage, chemical stimuli, or autoimmune processes. These stimuli induce the release of inflammatory mediators (prostaglandins, bradykinin, histamine, growth-factors, and neurogenic factors) [5]. Phlogistic response of mammalian tissues involves a series of complex events and mediators that stimulate the peripheral afferent fibers leading to pain sensation. Involvement of inflammatory events in peripheral sensitization of nociceptors provides scope for anti-inflammatory agents in the therapeutic treatment of the pathologies associated with inflammatory reactions [6]. Subplantar injections of Carr in mice induce a biphasic edema. The first phase peaks at the 3rd hour, and...
the delayed phase peaks at 48 hour after Carr injection. In the early phase, there is a diffuse cellular infiltrate with polymorphonuclear leukocytes (PMNs) whereas the infiltrate of the delayed phase is composed by macrophages, eosinophils, and lymphocytes [7]. Therefore, in this paper, we examined the analgesic effects of scopoletin on nociception induced by acetic acid and formalin. We also evaluated the anti-inflammatory effects of scopoletin on paw edema induced by Carr in mice. And we detected the levels of MDA, NO, TNF-α, and PGE₂ in either paw edema or serum; in addition, the activities of SOD, CAT, and GPx in the paw edema at the 5th hour after Carr injection to investigate the relationship between the anti-inflammatory mechanism of scopoletin and antioxidant enzymes.

2. Methods

2.1. Chemicals. Scopoletin (Figure 1(a)), 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 and PGE₂ were purchased from BioSource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2,
and anti-β-actin antibody (Santa Cruz, USA), and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK) were obtained as indicated. Poly(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA, USA).

2.2. Plant Material. The whole plant of Cossostephium chinensis (L.) Makino (Asteraceae) was collected in July 2007 in Taichung, Taiwan. A voucher specimen (NO CMU 20070880102B1) is deposited at the Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University, Taichung, Taiwan.

2.3. Extraction and Isolation. The dried whole plants of C. chinensis (10 kg) were extracted exhaustively with methanol. The crude methanol syrup was extracted five times with n-hexane. The n-hexane extract (320 g) was chromatographed on silica gel using n-hexane and EtOAc of increasing polarity as eluent and further purified by high-performance liquid chromatography eluting with n-hexane: EtOAc (1:1). Scopoletin was eluted with 10% EtOAc in hexane and recrystallized with EtOH. Scopoletin was obtained 22 mg.

2.3.1. Scopoletin. Colorless needles were obtained from n-hexane-EtOAc, mp was 204-205°C. 1 NMR (acetone-d_6): δ 7.85 (1H, d, J = 9.4), 7.20 (1H, s), 6.80 (1H, s), 6.15 (1H, d, J = 9.4), 3.90 (3H, s); 13 CNMR (50 MHz, acetone-d_6): δ 161.2, 151.9, 151.0, 146.0, 144.6, 113.3, 112.1, 110.0, 103.7, 56.7, EIMS: m/z (relative intensity): 192 (M^+, 100), 177 (70), 164 (28) 149 (59). IR (CHCl_3) ν max 3536, 1719, 1576, 1516, 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio a/b, where a is the volume of the right hind paw after Carr treatment, and b was the volume of the right hind paw before Carr treatment. Indo was used as a positive control [13]. After 5 h, the animals were sacrificed, 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 tissues were taken at the 5th 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°C. Then the homogenate was centrifuged at 12,000  g for 5 min. The supernatant was obtained and stored at −20°C refrigerator for MDA and the antioxidant enzymes (CAT, SOD, and GPx) activity assays. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA).

2.8. MDA Assay. MDA was evaluated by the thiobarbituric acid-reacting substances (TRARS) method [14]. 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.

2.9. Measurement of Nitric Oxide/Nitrite. The production of NO was assessed indirectly by measuring the nitrite levels in plasma determined by a calorimetric method based on the Griess reaction [15]. Plasma samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration
of 15 g/L. After centrifugation at 10,000 × g for 5 min at room temperature, 100 μL supernatant was applied to a microtitrre plate well, followed by 100 μL of Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethenediamine 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.

2.10. Measurement of TNF-α and PGE\textsubscript{2} by an Enzyme-Linked Immunosorbent Assay (ELISA). The levels of TNF-α and PGE\textsubscript{2} were determined using a commercially available ELISA kit (BioSource International Inc., Camarillo, CA) according to the manufacturer’s instruction. TNF-α and PGE\textsubscript{2} were determined from a standard curve [16].

2.11. Antioxidant Enzymes Activity Measurements. The following biochemical parameters were analyzed to check the antioxidant activity of scopoletin in paw edema tissue by the methods given below. Total superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome c reduction [17]. 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 [18]. In brief, the reduction of 10 mM H\textsubscript{2}O\textsubscript{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 [19, 20]. 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.

2.12. Western Blot Analysis of iNOS and COX-2. Total protein was extracted with a RIPA solution (radioimmuno-precipitation assay buffer) at −20°C 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°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, UK). 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.

2.13. 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 5 μm) were deparaffinized with xylene and stained with trichromic Van Gieson. All samples were observed and photographed with BH2 Olympus Microscopy. Histological examination of these tissue slices revealed an excessive inflammatory response with massive infiltration of neutrophils (polymorphonuclear leukocytes (PMNs)) by microscopy. The numbers of neutrophils were counted in each scope (400x) and thereafter obtained their average count from 5 scopes of every tissue slice.

2.14. Statistical Analysis. Data are expressed as mean ± S.E.M. Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe’s multiple range test). Statistical significance is expressed as *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

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

3.2. Formalin Test. Scopoletin (1 mg/kg) significantly (P < 0.05) inhibited formalin-induced pain in the late phase (Figure 1(c)). 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.

3.3. Effects of Scopoletin on λ-Carrageenan-Induced Mice Paw Edema. As shown in Figure 2(a), Carr-induced paw edema. Scopoletin (10 mg/kg) significantly inhibited (P < 0.001) the development of paw edema induced by Carr after 3th and 4th hour of treatment. Indo (10 mg/kg) significantly decreased the Carr-induced paw edema after 3th and 4th hour of treatment (P < 0.001).

3.4. Effects of Scopoletin on MDA Level Measurements. In Figure 2(b), we indicated scopoletin (1 or 5 mg/kg) decreased the MDA level in the edema paw at the 5th hour after Carr injection (P < 0.05 or P < 0.01). And scopoletin (10 mg/kg)
Figure 2: Effects of scopoletin and Indo on hind-paw edema induced by Carr in mice (a), the tissue MDA concentration of foot in mice (b), Carr-induced NO (c), TNF-α (d), and PGE2 (e) concentrations of serum at the 5th hour in mice. Each point represents the average value for eight individual animals. Each value represents as mean ± S.E.M. *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).
3.5. Effects of Scopoletin on NO Level. In Figure 2(c), the NO level increased significantly in the edema serum at the 5th h after Carr injection ($P < 0.001$). Scopoletin (5 or 10 mg/kg) significantly decreased the serum NO level ($(P < 0.01$ or $P < 0.001$). The inhibitory potency was similar to that of Indo (10 mg/kg) at the 5th h after induction.

3.6. Effects of Scopoletin on TNF-α and PGE$_2$ Levels. TNF-α and PGE$_2$ levels increased significantly in serum at the 5th h after Carr injection ($P < 0.001$). However, scopoletin (10 mg/kg) decreased the TNF-α level and PGE$_2$ in serum at the 5th h after Carr injection ($P < 0.001$), as well as 10 mg/kg Indo (Figures 2(d) and 2(e)).

3.7. Effects of Scopoletin on Activities of Antioxidant Enzymes. At the 5th hour following the intrapaw injection of Carr, paw edema tissues were also analyzed for the biochemical parameters such as SOD, CAT, and GPx activities (Table 1). SOD, CAT, and GPx activities in paw edema tissue were decreased significantly by Carr administration. SOD, CAT, and GPx activities were increased significantly after being treated with 10 mg/kg scopoletin and 10 mg/kg Indo ($P < 0.001$) (Table 1).

3.8. Effects of Scopoletin on Carr-Induced iNOS and COX-2 Protein Expressions in Mice Paw Edema. To investigate whether the inhibition of NO production was due to a decreased iNOS and COX-2 protein level, the effect of scopoletin on iNOS and COX-2 proteins expression was studied by Western blot. The results showed that the injection of scopoletin (10 mg/kg) on Carr-induced for 5th h inhibited iNOS and COX-2 proteins expression in mouse paw edema (Figure 3(a)). The intensity of protein bands were analyzed by using Kodak Quantity software in three independent experiments and showed an average of 81.6% and 72.4% downregulation of iNOS and COX-2 protein, respectively, after treatment with scopoletin compared with the Carr-induced alone (Figure 3(b)). In addition, the protein expression showed an average of 61.6% and 57.1% down-regulation of iNOS and COX-2 protein after treatment with Indo at 10.0 mg/kg compared with the Carr-induced alone. The down-regulation of iNOS and COX-2 activity of scopoletin (10 mg/kg) was better than Indo (10.0 mg/kg).

3.9. 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 10 mg/kg, showed a reduction in inflammatory response Carr-induced. 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 4(a)).

decreased the MDA level in the edema paw and serum at the 5th hour after Carr injection ($P < 0.001$).
Figure 4: Histological appearance of the mouse hind footpad after a subcutaneous injection with Carr stained with H&E stain at the 5th hour to reveal hemorrhage, edema, and inflammatory cell infiltration in control mice, Carr-treated mice demonstrating hemorrhage with moderately extravascular red blood cells and a large amount of inflammatory leukocyte mainly neutrophils infiltration in the subdermis interstitial tissue of mice, and mice given Indo (10 mg/kg) before Carr. Scopoletin significantly shows morphological alterations (100x) (a) and the numbers of neutrophils in each scope (400x) compared to subcutaneous injection of Carr only (b). Each point represents the average value for three individual animals. \( *** p < 0.001 \) as compared with the control. \( **** p < 0.001 \) compared with Carr group. Scale bar = 200 μm.

Neutrophil was notified increased with Carr treatment \((P < 0.001)\). Both Indo and scopoletin (10 mg/kg) could significantly decrease the neutrophil numbers as compared to the Carr-treated group \((P < 0.001)\) (Figure 4(b)).

4. Discussion

Scopoletin showed weak inhibitory activities against urease and alpha-chymotrypsin enzymes [21]. Anticancer
potentials of scopoletin from *Gelsemium sempervirens* were demonstrated earlier from *in vitro* and *in vivo* studies [22]. Cytotoxic activity of scopoletin derived from *Artemisia annua* towards cancer cells was reported [23]. Scopoletin was also reported to have anti-inflammatory effects through inhibiting the release of TNF-α, IL-1β, PGE₂, and IL-6 and suppressing the expression of COX-2 in RAW 264.7 macrophages [4]. Therefore, in this study, we focused on the effects of the antinociceptive and anti-inflammatory effects of scopoletin were examined with the objective of elucidating the signaling mechanism through which they exert their effects *in vivo*. Intraperitoneal (i.p.) injection of acetic acid can produce the peritoneal inflammation (acute peritonitis), which causes a response characterized by contraction of the abdominal muscles accompanying an extension of the forelimbs and elongation of the body [24]. The study was based on two primary hypotheses: the repeated presentation of pain stimuli with formalin in a context by a respondent paradigm in a specific context would cause an associative conditioned nociceptive response in mice, and the associative conditioning with nociceptive stimuli would result in a change in pain susceptibility [25].

The acetic writhing test is used to study the peripheral analgesic effects of drugs. Although this test is nonspecific, it is widely used for analgesic screening [26]. In our study, we found that scopoletin exhibited antinociceptive effect in acetic acid-induced writhing response. This effect may be due to inhibit of the synthesis of the arachidonic acid metabolites. Formalin-induced paw pain has been well established as a valid model for analgesic study *in vivo*. It is well known that the formalin test produces a distinct biphasic pain responses (first phase: 0–5 min and second phase: 15–45 min) [27]. Therefore, the test can be used to clarify the possible mechanism of an antinociceptive effect of a proposed analgesic. Centrally acting drugs such as opioids inhibit both phases equally, but peripherally acting drugs such as aspirin and Indo only inhibit the late phase [28]. The inhibitory effect of scopoletin on the nociceptive response in the late phase of the formalin test suggested that the antinociceptive effect of scopoletin could be due to its peripheral action. The findings of this study demonstrated that scopoletin is able to induce analgesic and anti-inflammatory effects in two algesiometric models of tonic (acetic acid writhing or formalin tests) types of pain.

### Table 1: Effects of scopoletin and indomethacin (Indo) on changes in CAT, SOD, and GPx activities were studied in Carr-induced paw edema (5th hr) in mice.

| Groups        | CAT (U/mg protein) | SOD (U/mg protein) | GPx (U/mg protein) |
|---------------|--------------------|--------------------|--------------------|
| Control       | 5.48 ± 0.08        | 4.89 ± 0.08        | 12.87 ± 0.11       |
| Carr          | 3.34 ± 0.12       **| 2.16 ± 0.04      ***| 7.07 ± 0.12       ***|
| Carr + Indo   | 4.96 ± 0.11       **| 4.23 ± 0.06      **| 10.56 ± 0.29      **|
| Carr + scopoletin (1 mg/Kg) | 3.94 ± 0.05      *| 3.35 ± 0.11      *| 8.74 ± 0.09      *|
| Carr + scopoletin (5 mg/Kg) | 4.74 ± 0.06      **| 3.82 ± 0.07      **| 10.19 ± 0.13      **|
| Carr + scopoletin (10 mg/Kg) | 5.13 ± 0.14      ***| 4.61 ± 0.03      ***| 11.64 ± 0.10      ***|

Each point represents the average value for three individual animals. Each value represents as mean ± S.E.M. *P* < 0.001 as compared with the control. *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).

*In vivo* anti-inflammatory activity, Carr-induced edema test, is highly sensitive to nonsteroidal anti-inflammatory drugs and has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs [29]. The Carr-induced edema test is a useful model to determine the anti-inflammatory effects of natural products [30]. According to Figure 2(a), the degree of swelling of the Carr-injected paws was maximal 3th hour after injection, and the mean increase in volume at that time was about 100% in the control group. Statistical analysis revealed that scopoletin inhibited the development of Carr-induced paw edema after 3th hour of treatment, significantly.

The Carr-induced inflammatory response has been linked to neutrophils infiltration and the production of neutrophils-derived free radicals as well as the release of other neutrophils-derived mediators [31]. Some researches demonstrated that inflammatory effect induced by Carr is associated with free radicals. Free radicals, prostaglandin, and NO will be released when administrating with Carr for 1–6 h [32]. MDA production is due to free-radical attack plasma membrane. In a number of pathophysiological conditions associated with inflammation, these reactive oxygen species have been proposed to mediate cell damage via a number of independent mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of antioxidant enzymes [32]. Giving the importance of the oxidative status in the formation of edema, the anti-inflammatory effect exhibited by drug in this model might be related to its antioxidant properties.

Our present results also confirm that Carr-induced paw edema model results in the production of NO and PGE₂. Excess amounts of NO and PGE₂ play a critical role in the aggravation of chronic inflammatory diseases. Scientific papers were observed that while the production of NO and PGE₂ was blocked by the NOS inhibitors in mouse macrophages RAW 264.7 cells, these inhibitory effects were reversed by coinubcation with the precursor of NO synthesis, L-Arginine [7]. Furthermore, inhibition of iNOS activity by nonselective NOS inhibitors attenuated the release of NO from macrophages exerted a stimulatory action on enhancing the prostaglandins production [33]. It is known that Indo acts in this model of inflammation in several ways,
inhibiting prostaglandins synthesis and infiltration of plasma proteins and neutrophils into the inflammatory site [34]. The inhibition of paw edema induced by Carr for 3 hrs coincides with the peak of edema, inhibition of COX-2 [30]. In our study, the levels of NO and PGE2 were decreased significantly by treatment with 5 and 10 mg/kg scopoletin. TNF-α is a mediator of Carr-induced inflammatory incapacitation, and is able to induce the further release of kinins and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting nociceptive response [35]. In this study, we found scopoletin decreased the TNF-α level in serum after Carr injection.

Various bioassays exist in which the anti-inflammatory activity of these products can be evaluated, having demonstrated that scopoletin possesses anti-inflammatory activity in vivo model of inflammation. In this model of acute inflammation, TNF-α, NO, and PGE2 release in mice serum dropped markedly upon pretreatment with scopoletin [29]. There is a significant increase in SOD, CAT, and GPx activities with scopoletin treatment. Furthermore, there is a significant decrease in MDA level with scopoletin treatment. We assume the suppression of MDA production is probably due to the increases of SOD, CAT, and GPx activities. In conclusion, these results suggested that scopoletin possessed analgesic and anti-inflammatory effects. The anti-inflammatory mechanism of scopoletin may be related to iNOS and COX-2 activity [36], and it is associated with the increase in the activities of antioxidant enzymes (SOD, CAT, and GPx). Scopoletin may be used as a pharmacological agent in the prevention or treatment of disease in which free radical formation in a pathogenic factor.

Acknowledgments

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