

Communication

Anti-Inflammatory Effect of *Cudrania tricuspidata* Extract and *Stewartia koreana* Extract Mixture in a Collagen-Induced Arthritis Mouse Model

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Abstract: *Cudrania tricuspidata* extracts (CTE) and *Stewartia koreana* extracts (SKE) are viable drugs for managing inflammation. We investigated the nitric oxide levels of CTE and a mixture of CTE/SKE (CTE mix) against lipopolysaccharide-induced RAW264.7 cells. In addition, we administered the CTE and CTE mix to mice with collagen-induced arthritis to confirm an anti-inflammatory effect against rheumatoid arthritis. We analyzed arthritis symptoms by oral administration of CTE mix using a CIA-induced animal model and analyzed the inhibitory activity of NO production with in vitro experiments. Both the CTE and CTE mix decreased nitric oxide levels, and a 2:1 ratio of CTE mix was most effective in vivo among the varying ratios of CTE mix tested. The spleen size increased by about 2.1 times, and the lymph node size decreased by about 2.5 times relative compared to the vehicle group. In blood biochemical analyses, tumor necrosis factor- α levels decreased by about three times, interleukin-1 β and interleukin-6 levels were reduced by about eight times and three times, and PRG4 expression levels were increased by about 2.5 times relative to the vehicle group. We suggest that the CTE mix was superior to CTE alone and has potential as an anti-inflammatory treatment for patients with rheumatoid arthritis.

Keywords: *Stewartia koreana* extract; *Cudrania tricuspidata* extract; anti-inflammation; rheumatoid arthritis



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1. Introduction

Rheumatoid arthritis is a type of inflammatory arthritis associated with progressive disability, systemic complications, and early death by affecting diarthrodial joints like those in the hands and knees [1,2]. Rheumatoid arthritis triggers the production of sub-inflammatory mediators such as proteolytic enzymes and various chemokines by secreting large amounts of inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 from the synovial membrane surrounding each joint together with macrophages [3–8]. As a result, the inflammatory response and joint damage are amplified. Selective cyclooxygenase-2 antagonists; nonsteroidal anti-inflammatory drugs; and anti-rheumatic drugs such as methotrexate, sulfasalazine, and auranofin have been adopted at the early onset of rheumatoid arthritis to arrest disease progression [9–11]. Meanwhile, TNF- α antagonists and IL-1 and IL-6 receptor antagonists can also prevent novel anti-rheumatic rheumatoid arthritis [12–17]. Proteoglycan-4 (PRG4) is a mucous glycoprotein secreted from synoviocytes and is known to exhibit strong anti-inflammatory activity in binding with TLR2 and TLR4 in activated cells isolated from patients with

rheumatoid arthritis and osteoarthritis [18]. In summary, the inhibition of inflammatory cytokine is the most important factor in the early progression of rheumatoid arthritis [10,19]. In Asian countries, a variety of herbs and plants have been used for the treatment of inflammatory diseases and wounds [20–22]. In addition, natural products from plants commonly contain phenolic flavonoids, which may have antioxidant and anti-inflammatory functions in vitro and in vivo [23–25]. One of these products, *Cudrania tricuspidata* extract (CTE), is used as a folk remedy in the treatment of various diseases such as eczema, bruises, mumps, and acute arthritis [26]. According to a recent study, CTE has anti-inflammatory, antioxidant, and antibacterial activities due to its unique components such as xanthone, flavonoids, and hydroxybenzyl flavonoids [20,27,28]. Meanwhile, *Stewartia koreana* extract (SKE) has been adopted as a folk remedy for acute gastroenteritis, quadriplegia, and various pains [29,30]. It includes a variety of compounds such as flavonoids, proanthocyanin, and glucosides, which can help to improve bone cells against IL-1 and IL-6, enhance osteoclast differentiation, and inhibit bone resumption [8,31,32]. These two natural products have already displayed anti-rheumatic arthritis potential independently, and we also expect that a similar effect would be attained by the synergic effect of those complexes. In this study, we assessed various ratios of the CTE/SKE complex and demonstrate that these extracts exhibit strong activity in certain proportions.

2. Materials and Methods

Materials and extraction; The CTE and SKE samples used in the experiment were collected and air-dried in Sinan-gun, Jeollanam-do, South Korea, then boiled in the ratio of 1:50 (*w/v*) at 90 °C to 100 °C for four hours. Subsequently, these extracts were cooled down at 50 °C with water, then filtered to remove impurities. The filtered extracts were concentrated at 50 °C to 60 °C with a solid content of more than 10%, then sterilized, stirred, and spray-dried for one hour at 95 °C with the addition of a dose of dextrin (50:50, *w/w*).

Cell culture; RAW264.7 mouse macrophage cells were purchased from the Korean Cell Line Bank (Seoul, Korea), then cultured in a growth medium. The RAW264.7 were cultured in Dulbecco's modified Eagle medium (Gibco Laboratories, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories) and 1% antibiotic antimycotic solution (Gibco Laboratories) at 37 °C in an incubator at 95% humidity and a 5% mixture of air and CO₂.

Cell viability assay; Mouse macrophage RAW264.7 cells for in vitro experiments were dispensed into 3×10^5 cells/well in a 96-well plate and incubated for 24 h in a 5% CO₂ incubator at 37 °C. Subsequently, the cells were treated with different concentrations (0, 100, 200, 500, 1000, and 2000 µg/mL) of a mixture of CTE and SKE in various ratios. After incubation for 24 h, 10 µL of the EZ cytox (DoGenBio, Seoul, South Korea) was added to each well and further incubated for 30 min at 37 °C and 5% CO₂. The index of the cell viability was determined by measuring the formazan production with an enzyme-linked immunosorbent assay (ELISA) reader (Thermo Fisher Scientific, Waltham, MA, USA) at an absorbance of 450 nm.

Inhibition of nitric oxide (NO) production (Griess assay); mouse macrophage RAW264.7 cells for in vitro experiments were dispensed into 3×10^5 cells/well in a 96-well plate and incubated for 24 h in a 5% CO₂ incubator at 37 °C. Subsequently, the cells were treated with different concentrations (0, 100, 200, 500, 1000, and 2000 µg/mL) of a mixture of CTE and SKE in various ratios. Simultaneously, 1 µg/mL of lipopolysaccharides (LPS) (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 24 h. Then, after adding and reacting the same amount of Griess reagent (Sigma-Aldrich) equal to 100 µL of the culture solution, the absorbance was measured at 540 nm with an ELISA reader. The NO concentration in the culture medium was determined using a standard curve for each concentration of sodium nitrite.

Animals and maintenance; previously, an antibody against collagen was found in the serum of rheumatoid arthritis patients, and it was conceived that type 2 collagen present in the joint could act as an autoantigen. As a representative animal model of

rheumatoid arthritis, collagen-induced arthritis (CIA) DBA/1J mice were introduced. The total experimental group included the following six mouse groups containing six DBA/1J mice each: a “control” group without CIA or treatment, a “vehicle” group with CIA but no treatment, a CTE-alone treatment group, a CTE/SKE 2:1 treatment group, a CTE/SKE treatment 1:1 group, and a CTE/SKE treatment 1:2 group, respectively. All mice were in cages and allowed to acclimatize for one week before use. The temperature of the breeding room was maintained at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, the humidity was maintained at $5\% \pm 10\%$, and a 12-h light/dark cycle was set. To observe the arthritis-prevention effect, oral administration was performed every two days after the date of the induction of arthritis. CTE/SKE mixtures in the ratios of 2:1, 1:1, and 1:2 were prepared using 300 mg/kg, 400 mg/kg, and 300 mg/kg as appropriate concentrations. Physiological saline was used as the drug administered in the negative control group and positive control group. All experimental groups were orally administered 0.1 mL at regular time intervals every two days, with water and solid feed supplied freely. The animal experiments were approved by the Institutional Animal Care and Use Committee at Kyung Hee University (KHGASP-20-369).

Development of the CIA model; To establish the CIA mice, seven-week-old male DBA/1J mice were purchased (Orient Bio Inc., Seongnam, South Korea), and, following a one-week adaptation period, CIA was induced. Bovine type 2 collagen (Chondrex, Redmond, WA, USA) at a concentration of 2 mg/mL of *M. tuberculosis* mycobacteria sold in liquid form was mixed with complete Freund’s adjuvant (Chondrex) at the same concentration at a ratio of 1:1. Then, 0.1 mL of the mixed solution (100 µg of type 2 collagen) was slowly injected intradermally, avoiding the blood vessel 2 cm below the base of each mouse’s tail. In this context, when the concentration or dose of the mixed solution was increased or if injected further downward, the induced arthritis becomes more severe; thus, the dosage and administration site were maintained consistently. Boosting was performed two weeks after the first injection (Day 14), and the same amount of collagen was injected by mixing with incomplete Freund’s adjuvant (Chondrex) instead of complete Freund’s adjuvant again using the tail as the injection site. To establish a similar incidence rate of arthritis between subjects and to obtain more severe arthritis, 20 µg of LPS was additionally injected intraperitoneally two weeks after the second injection (Day 28).

End of experiment and sample analysis; the bodyweight of each mouse was measured at two-day intervals from the day CIA induction was started (Day 0), while arthritis-related indicators were measured and visually observed at two-day intervals from the second injection time point (Day 14). At the end of the experiment, the point at which the arthritis evaluation index of the vehicle group without oral administration of the sample naturally decreased was set as the endpoint, while the index began to decrease from 44 to 48 days after the start of CIA induction. The experiment was terminated on Days 46 to 50 after CIA induction. After the end of the experiment, all mice were sacrificed, and blood samples, spleen, lymph nodes, and paws were collected and stored for use in analysis experiments. Specifically, the size and weight of the spleen and lymph nodes, which are the organs majorly involved in the immune response, were assessed, while the blood cells were removed from blood samples, and plasma was separated using a centrifuge at $4\text{ }^{\circ}\text{C}$. Using the separated plasma, the nature of both proteoglycan synthesis and inflammatory cytokine production was analyzed by ELISA.

Statistical processing; The result values are expressed as mean \pm standard error of the mean values. Statistical comparative analysis was performed when the statistically significant *p*-value was 0.05 or less after an unpaired *t*-test verification process using the GraphPad Prism 6 software program (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Cell Viability Assay

Prior to analyzing the activity of the sample in RAW264.7 cells, which are mouse macrophages, the cytotoxicity of the sample itself was confirmed. Samples were diluted by concentration (0–2000 µg/mL) in a serum-free medium, treated with cells for 24 h, and

analyzed. As a result, for the SKE and the CTE/SKE ratios of 2:1 and 1:1, more than 80% cell viability at the concentration treated with 500 $\mu\text{g}/\text{mL}$ was attained. Meanwhile, the CTE and 1:2 CTE/SKE mixture showed more than 80% cell viability at the concentration treated with 1000 $\mu\text{g}/\text{mL}$ (Figure 1).

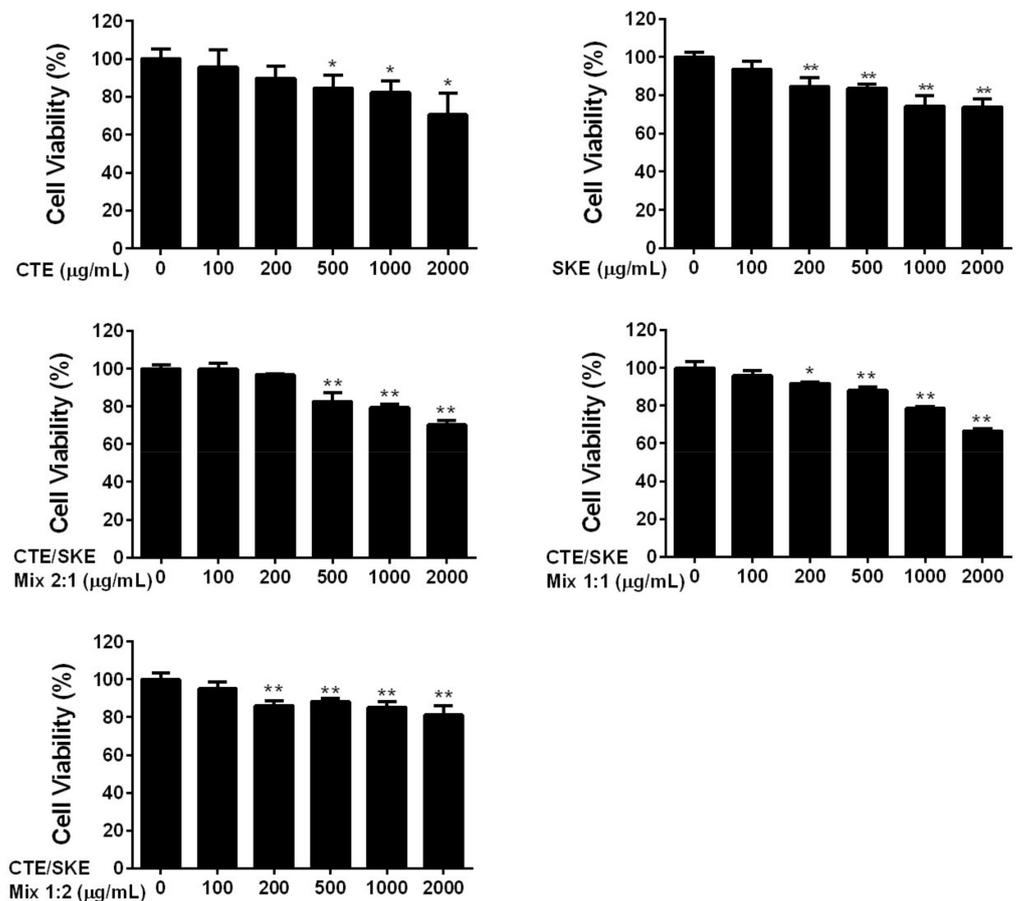


Figure 1. Cytotoxic effect of CTE and CTE mix in RAW264.7 cells. Cell-viability analysis results of CTE only; SKE only; and these two extracts mixed at ratios of 1:1, 1:2, and 2:1. The cells were treated with different concentrations (0, 100, 200, 500, 1000, and 2000 $\mu\text{g}/\text{mL}$) of a mixture of CTE and SKE in various ratios. After incubation for 24 h, an ELISA reader at an absorbance of 450 nm. (Control vs. * $p < 0.05$, ** $p < 0.01$).

3.2. CTE and CTE Mix Inhibited the Production of NO

To analyze the effect on the production of nitric oxide (NO), each sample was diluted by concentration (0–2000 $\mu\text{g}/\text{mL}$) in a serum-free medium containing 1 $\mu\text{g}/\text{mL}$ of LPS and added to cells for 24 h. As a result of analyzing the culture medium by Griess assay after treatment, it was confirmed that LPS-induced NO production was inhibited depending on the concentration. The IC_{50} values (50% inhibition rate) for the respective concentrations were 531.6 ± 4.31 $\mu\text{g}/\text{mL}$, 198.9 ± 5.72 $\mu\text{g}/\text{mL}$, 212.4 ± 5.86 $\mu\text{g}/\text{mL}$, 208.2 ± 5.92 $\mu\text{g}/\text{mL}$, and 204.1 ± 6.812 $\mu\text{g}/\text{mL}$ (Figure 2).

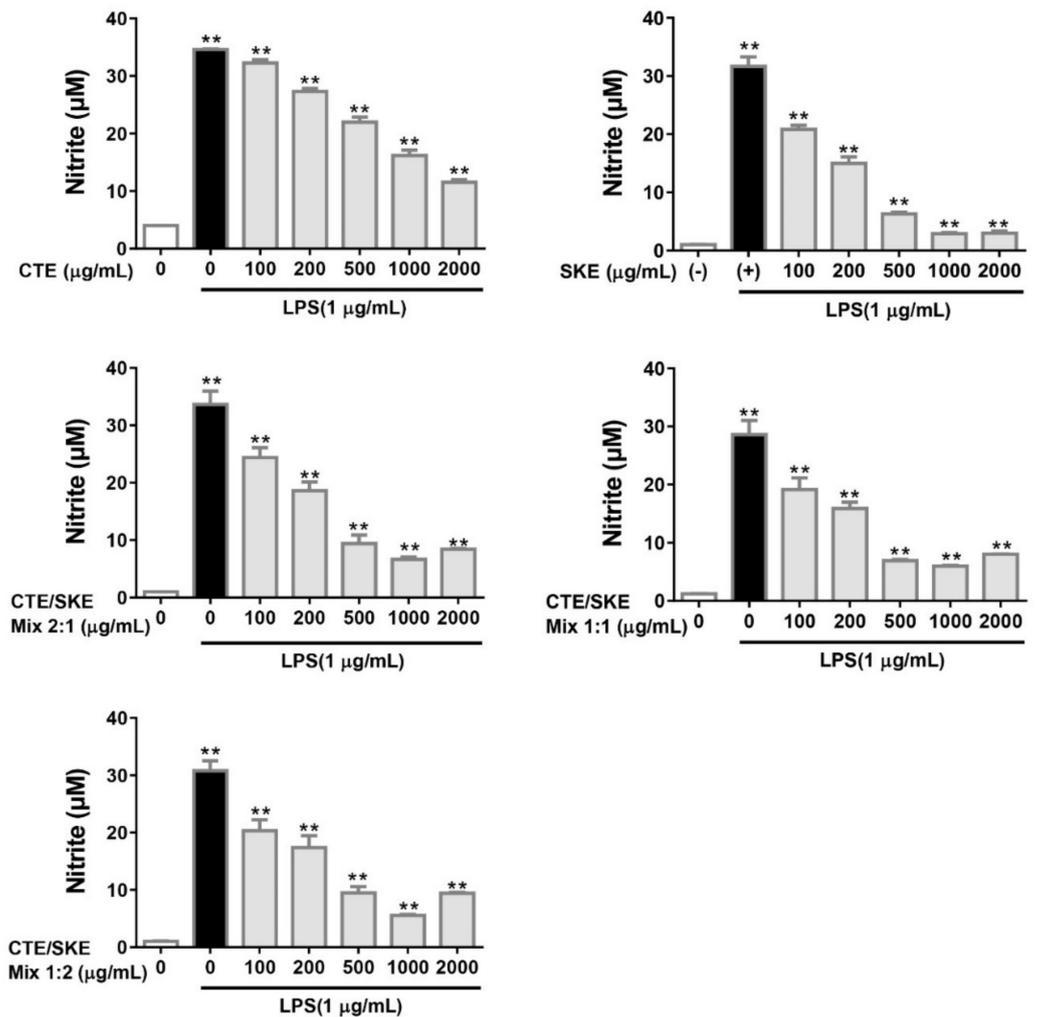


Figure 2. Effect of CTE and CTE mix on Nitric Oxide (NO) production in LPS induced RAW264.7 cells. Inhibition of NO production results of CTE only; SKE only; and these two extracts mixed at ratios of 1:1, 1:2, and 2:1. The cells were treated with different concentrations (0, 100, 200, 500, 1000, and 2000 $\mu\text{g/mL}$) of a mixture of CTE and SKE in various ratios. Simultaneously, 1 $\mu\text{g/mL}$ of LPS was added and incubated for 24 h. The ELISA reader at an absorbance of 540 nm. (Control vs. ** $p < 0.01$).

3.3. CTE and CTE Mix Attenuated the Arthritis Symptom

Weight changes with arthritis progression. The vehicle group with induced CIA relative to the control group showed significant weight loss due to insufficient food intake resulting from decreased joint function and mobility following an inflammatory response. Meanwhile, the weights of the CTE group and the CTE/SKE 2:1, 1:1, and 1:2 groups were initially decreased due to CIA induction but then gradually increased (Figure 3B).

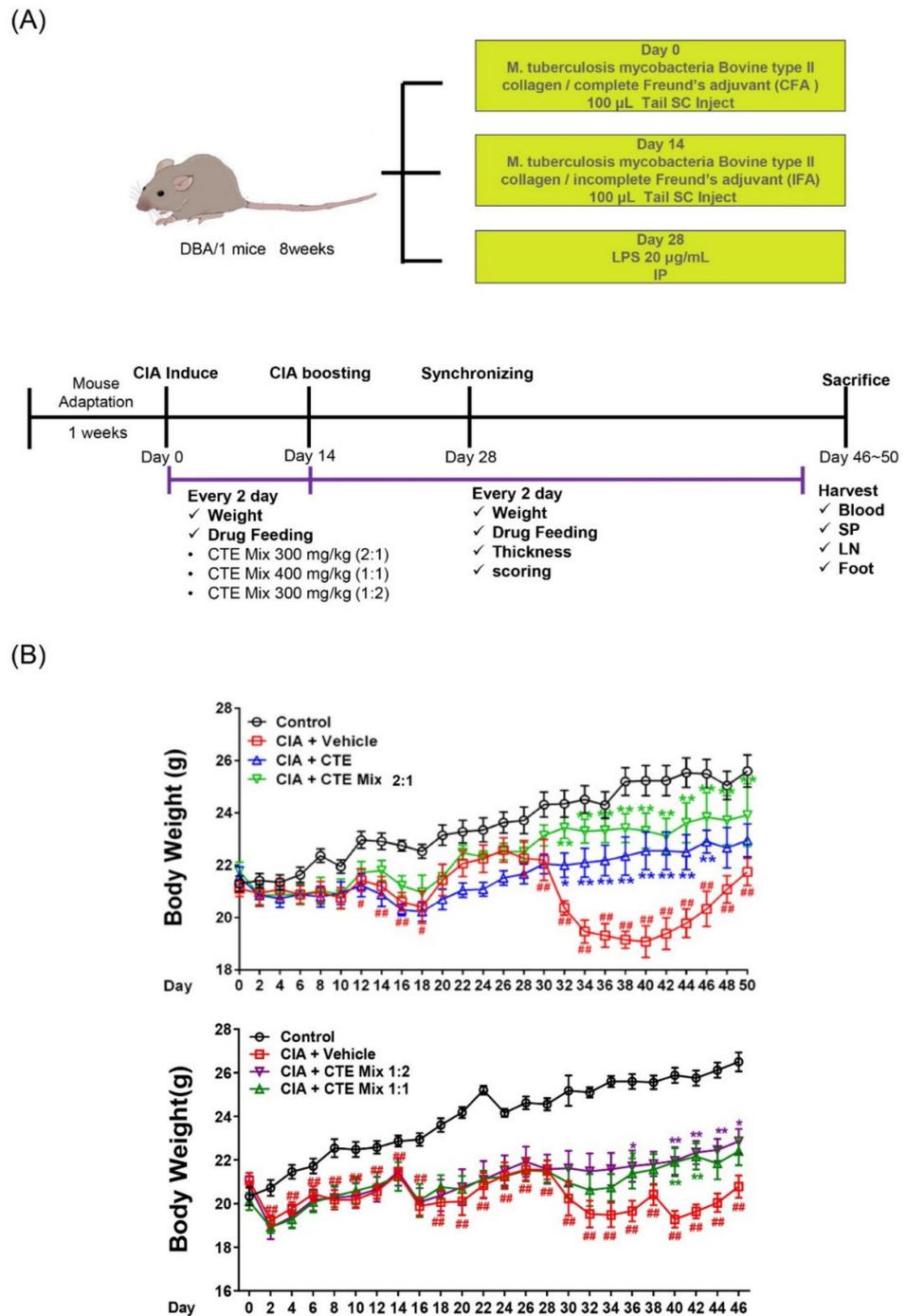


Figure 3. Cytotoxic effect of CTE and CTE mix in CIA-induced animal model. (A) In vivo research scheme. (B) The control group and vehicle group were administered orally physiological saline. In the CTE group, only CTE was administered orally, while in the CTE/SKE combination groups, these extracts were mixed at the respective concentrations of 2:1, 1:2, and 1:1 and administered orally. The weight change due to the progression of arthritis was then confirmed (control vs. $## p < 0.01$, vehicle vs. $* p < 0.05$, $** p < 0.01$; $n = 5$).

As the arthritis progressed in the CIA mice, edema was measured, and the vehicle group was found to have significantly increased edema relative to the control group. Meanwhile, the CTE group and CTE/SKE 2:1, 1:1, and 1:2 groups experienced a decrease in edema as compared with the vehicle group (Figure 4).

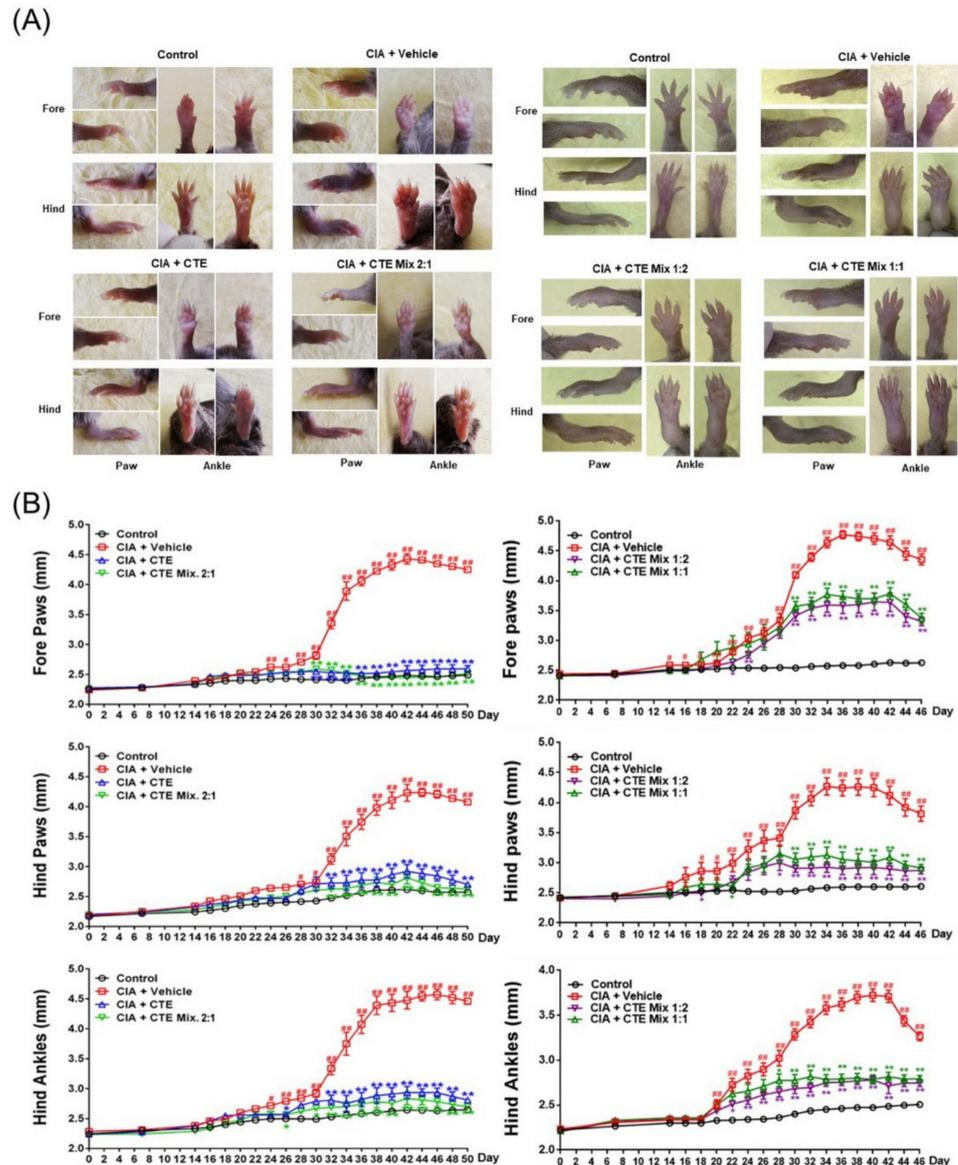


Figure 4. Effect of CTE and CTE mix on arthritis symptoms. (A) The control group and vehicle group were orally administered physiological saline. In the CTE group, only CTE was administered orally, while in the CTE/SKE combination groups, these extracts were mixed at the respective concentrations of 2:1, 1:2, and 1:1 and administered orally. Joint swelling was then confirmed (control vs. $p < 0.01$; vehicle vs. $* p < 0.05$, $** p < 0.01$; $n = 5$). (B) The control group and vehicle group were orally administered physiological saline. In the CTE group, only CTE was administered orally, while in the CTE/SKE combination groups, these extracts were mixed at the respective concentrations of 2:1, 1:2, and 1:1 and administered orally. Joint swelling due to the progression of arthritis was then confirmed (control vs. $## p < 0.01$; vehicle vs. $* p < 0.05$, $** p < 0.01$; $n = 5$).

3.4. Measurement of Spleen and Lymph Node Changes Following Arthritis

As shown in Figure 5, the size and weight of the spleen and lymph nodes in the vehicle group were increased significantly relative to the control group, while, in the CTE group and CTE/SKE 2:1, 1:1, and 1:2 groups, the spleen and lymph node were decreased in size as compared with the vehicle group, with the weight of the spleen reduced by 1.5, 2.1, 1.3, and 1.4 times (Figure 5A) and the weight of lymph nodes reduced by 2, 2.5, 3, and 3.5 times (Figure 5B).

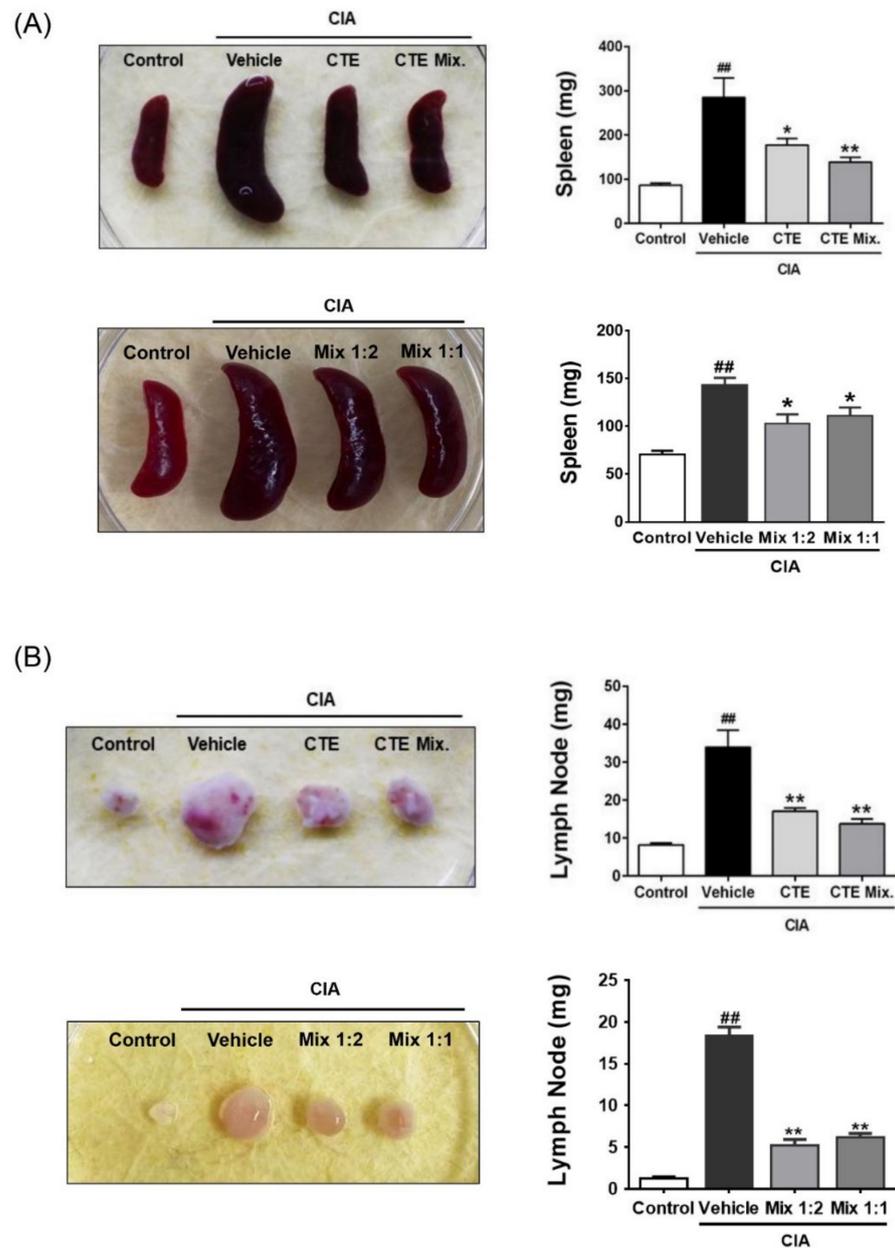


Figure 5. Effect of CTE and CTE mix immune responses in CIA-induced animal model. (A) The control group and vehicle group were orally administered physiological saline. In the CTE group, only CTE was administered orally, while in the CTE/SKE combination groups, these extracts were mixed at the respective concentrations of 2:1, 1:2, and 1:1 and administered orally. A comparative analysis of spleen size and weight according to group was then performed (control vs. ^{##} $p < 0.01$; vehicle vs. ^{*} $p < 0.05$, ^{**} $p < 0.01$; $n = 5$). (B) The control group and vehicle group were orally administered physiological saline. In the CTE group, only CTE was administered orally, while in the CTE/SKE combination groups, these extracts were mixed at the respective concentrations of 2:1, 1:2, and 1:1 and administered orally. A comparative analysis of lymph node size and weight by according to group was then performed (control vs. ^{##} $p < 0.01$; vehicle vs. ^{*} $p < 0.05$, ^{**} $p < 0.01$; $n = 5$).

After completion of the CIA experiment, isolated plasma was used to ascertain the level of inflammatory cytokine production by ELISA. The expression levels of IL-6, TNF- α , and IL-1 β among the inflammatory cytokines increased by CIA induction were significantly increased in the vehicle group relative to the control group. Meanwhile, in the CTE group and the CTE/SKE 2:1, 1:1, and 1:2, the expression level of IL-6 was decreased by about

2.1, 3, 1.3, and 1.4 times, while TNF- α expression levels were decreased by about 2, 3, 1.5, and 2.1 times and IL-1 β expression levels were decreased by about 4, 8, 1.3, and 1.4 times. In addition, the degree of PRG4 synthesis was significantly reduced in the vehicle group relative to the control group, while, in the CTE group and the CTE/SKE 2:1, CTE/SKE 1:1, and CTE/SKE 1:2 groups, it was increased by about 1.9, 2.5, 1.9, and 2.9 times relative to the vehicle group (Figure 6).

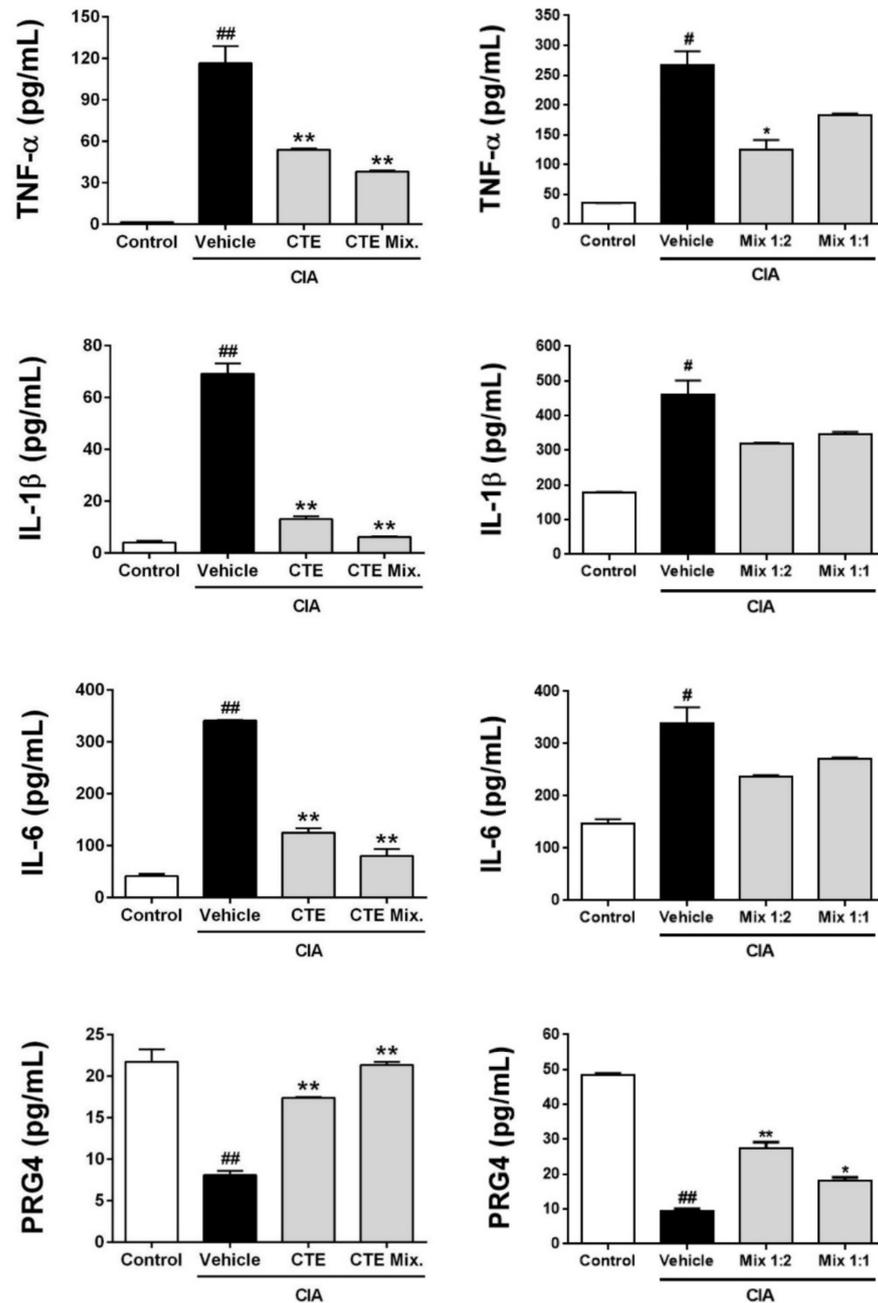


Figure 6. Effect of CTE and CTE mix on pro-inflammatory cytokines in CIA-induced animal model. The control group and vehicle group were orally administered physiological saline. In the CTE group, only CTE was administered orally, while in the CTE/SKE combination groups, these extracts were mixed at the respective concentrations of 2:1, 1:2, and 1:1 and administered orally. Inflammatory cytokine production and proteoglycan synthesis production by specimen administration were confirmed through ELISA (control vs. ## $p < 0.01$; vehicle vs. * $p < 0.05$, ** $p < 0.01$; $n = 5$).

4. Discussion

NO is essentially a free radical involved in the immune system and inflammation. Furthermore, NO is commonly expressed with and binds with thiol-contained proteins in rheumatoid arthritis patients [33]. In recent studies, SKE has been investigated for possible anti-inflammatory properties, and it is believed that SKE decreases NO levels due to components such as spinasterol-Glc contained within SKE [34,35]. In addition, xanthone and flavonoids included in CTE have been used to have anti-inflammatory and antioxidant effects for a long time [36,37]. Previously our study has shown that SKE strongly inhibits (Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL)-induced MAPKs phosphorylation, activation of NFATc1, and expression of c-FOS in Bone Marrow Macrophage (BMM). Moreover, we confirmed that SKE (5 µg/kg of body weight, intraperitoneally injection) increased bone density loss by LPS in the LPS-induced bone loss animal model [23]. Ethyl acetate *Cudrania* extracts are known to inhibit the proliferation of rheumatoid synovial fibroblast and the production of MMPs, COX-2, and PGE2 by induced IL-1β. This extract strongly inhibited the differentiation of osteoclasts via downregulation of MAPKs/c-FOS/NFATc1 signaling pathways [31,32]. As such, we designed this study to assess the synergy of different components thought to be effective in improving inflammatory diseases such as rheumatoid arthritis. We designed an in vitro model of LPS-induced inflammation in RAW264.7 mouse macrophages, where it was confirmed that it had a significant inhibitory effect on NO levels. Based on the in vitro results, more diverse inflammatory cytokines such as TNF-α, IL-6, and IL-1β were considered in an in vivo model. Among the various cytokines expressed in the synovial membrane, TNF-α and IL-1β, which are produced by macrophages and are known as upper cytokines that induce lower inflammatory mediators such as IL-6, various chemokines, and proteases amplify inflammation and cause damage to joints by promoting the proliferation of synovial cells [12–17]. TNF-α and IL-1β cytokines can be observed at high concentrations in the synovial fluid of rheumatoid arthritis patients and promote the production and secretion of metalloproteinase, prostaglandin, and NO in various cells while inhibiting the production of matrix components, thereby reducing inflammation and damage in joints [7–9]. In addition to stimulating the secretion of inflammatory mediators from synovial cells, it triggers systemic effects such as fever, muscle wasting, and loss of appetite. The expression levels of IL-6, TNF-α, and IL-1β among inflammatory cytokines induced by LPS were significantly decreased with the administration of the sample [10–13]. Among the study samples, it was found that the CTE/SKE 2:1 ratio displayed the best anti-rheumatoid arthritis effects both in vitro and in vivo. In the mice treated with this ratio of CTE/SKE, the spleen increased by about 2.1 times, and the lymph nodes decreased by about 2.5 times relative to the vehicle group. During ELISA, TNF-α, IL-1 β, and IL-6 levels were decreased by about three, eight, and three times, respectively, while PRG4 expression was increased by about 2.5 times. These values quantify the greatest effect among all samples. In addition, PRG4, bound with LPS receptors including TLR2 and TLR4, increases the expression of both receptors and is considered a key factor in regulating chronic inflammatory inhibitory activity in joints by operating as an antagonist during the TLR signaling process. In this study, we couldn't reveal the cause of the difference in activity according to the CTE/SKE mixing ratio. However, the CTE/SKE 2:1 ratio mixture has decreased the expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in a CIA-induced animal model. We suggest that the combination of these extracts has potential as a therapeutic agent for rheumatoid arthritis. However, additional studies focusing on the identification of clear chemical constituents to better understand the effect at this specific ratio are required.

Author Contributions: C.S.N., G.J., and T.H.L. designed the study; H.K. and I.K. performed all the experiments; K.K., E.H.L., and T.H.L. analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal experiments were approved by the Institutional Animal Care and Use Committee at Kyung Hee University (KHGASP-20-369).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this article.

Conflicts of Interest: The authors declare no conflict of interest.

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