

## Article

# Alleviating Effect of a Flower Extract of *Styphnolobium japonicum* L. on Symptoms of Experimentally Induced Osteoarthritis in Rats

Woo Jin Lee <sup>1</sup>, Kyung-Mi Kim <sup>1</sup>, Somin Lee <sup>2</sup>, Seong Yeon Park <sup>2</sup>, Ha-Jin Kim <sup>3</sup>, Jee-Young Imm <sup>3</sup>  
and Jae-Chul Jung <sup>1,\*</sup>

- <sup>1</sup> Life Science Research Institute, NOVAREX Co., Ltd., 80, Osongsaengmyeong 14 ro, Heungdeok-gu, Cheongju-si 28220, Republic of Korea; wj.lee@novarex.co.kr (W.J.L.); kkm3507@novarex.co.kr (K.-M.K.)
- <sup>2</sup> Korea Conformity Laboratories, 8, Gaetbeol-ro 145 beon-gil, Yeonsu-gu, Incheon 21999, Republic of Korea; somin14@kcl.re.kr (S.L.); qkrtd1241@kcl.re.kr (S.Y.P.)
- <sup>3</sup> Department of Foods & Nutrition, Kookmin University, 77 Jeongneung-ro, Seongbuk-gu, Seoul 02707, Republic of Korea; khj0716khj@naver.com (H.-J.K.); jyimm@kookmin.ac.kr (J.-Y.I.)
- \* Correspondence: jchung@novarex.co.kr; Tel.: +82-70-4852-2330

**Abstract:** In the present study, we prepared an ethanol extract from the flowers of *Styphnolobium japonicum* L. (SJFE) and found that it contains rutin as a major constituent as well as quercetin kaempferol and isorhamnetin as minor components. In lipopolysaccharide-stimulated RAW 264.7 macrophages, we observed that SJFE significantly inhibited the production of nitric oxide and the expression of major inflammatory biomarkers such as inducible NO synthase, cyclooxygenase-2, interleukin (IL)-6, and IL-1 $\beta$  significantly. Based on these in vitro results, we investigated the anti-inflammatory properties of SJFE on osteoarthritis (OA) of the left hind knee joints induced by monosodium iodoacetate in rats. SJFE was orally administered to the rats with arthritis for 4 weeks, and the following results were obtained. The rats treated with SJFE exhibited a 24% improvement in the weight-bearing index of their affected legs, as well as reductions of 31.5% and 23.2% in serum levels of cartilage oligomeric matrix protein and C-terminal telopeptide 2, respectively. Additionally, Mankin's score, an indicator used to assess the severity of joint cartilage damage, decreased by 2.75 points compared to the control with no treatment. These findings suggest that SJFE possesses anti-inflammatory properties and can alleviate symptoms of OA, indicating its potential to offer relief to individuals suffering from arthritis.

**Keywords:** *Styphnolobium japonicum*; flavonoids; rutin; HPLC; osteoarthritis; inflammation



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## 1. Introduction

Osteoarthritis (OA) is a common type of degenerative joint disorder that can greatly diminish one's quality of life by hindering mobility [1]. This condition involves various inflammatory reactions and the breakdown of cartilage in joint surfaces. Older individuals, who often have reduced anti-inflammatory capabilities and impaired tissue repair processes, are particularly susceptible to OA [2]. The knee and hip joints, which bear significant weight loads, are frequently affected [3]. Clinically, OA is characterized by noticeable symptoms such as joint swelling, persistent pain, stiffness, deformities, and limited joint mobility [4].

There are two primary approaches for treating OA. One involves surgical intervention, while the other relies on medication. Surgical treatment typically involves artificial joint replacements. However, this option is quite expensive, giving a significant financial burden on patients. Moreover, surgery carries a notable risk of complications, particularly dislocation, which may necessitate further operations [5]. As a result, the alternative option revolves around medication, with non-steroidal anti-inflammatory drugs (NSAIDs) being a common choice [6]. NSAIDs have demonstrated remarkable efficacy in relieving symptoms [7], but they also carry notable adverse effects such as gastrointestinal pain

and bleeding, hepatotoxicity, impaired blood coagulation, and immunosuppression [8,9]. These adverse effects often limit their usage. Consequently, there is a growing demand for medications or functional ingredients with fewer safety concerns, potentially derived from biomaterials like edible herbal plants.

*Styphnolobium japonicum* L. has been commonly used in traditional medicine, particularly in Asia, to treat various disorders, such as appendicitis, hematuria, and intestinal bleeding, among other effects [10]. Recently, we have discovered that the fruit extract of this plant alleviates the symptoms of photo-aging by inhibiting the release of inflammatory mediators and tissue-damaging substances in the skin irradiated with UV [11,12]. Moreover, it has been shown that the flowers of this plant also contain bioactive compounds, such as isoflavonoids, and triterpenes possessing anti-inflammatory properties [13,14], suggesting the flowers may also have an anti-inflammatory property.

Thus, the present study was performed to evaluate the anti-inflammatory properties of the flowers of this plant. For this purpose, we prepared the ethanol extract of the flowers of *S. japonicum* L. (SJFE) and conducted two experiments to assess its anti-inflammatory activities. In the first preliminary in vitro experiment, SJFE was examined for its effects on the production of nitric oxide and the expression of genes of inflammatory mediators in lipopolysaccharide-stimulated RAW 264.7 murine macrophages. In the subsequent animal experiment, SJFE was orally administered to the rats with osteoarthritis in the knee joints induced by monosodium iodoacetate, and its effects were observed on the weight-bearing power of the affected legs and cartilage degradation parameters. We obtained the positive effects in both cellular and animal experiments. These results suggest that the flower extract of this plant has the potential to provide benefits in managing osteoarthritis.

## 2. Materials and Methods

### 2.1. Materials

Lipopolysaccharide (LPS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), the Griess reagent, monosodium iodoacetate, and other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All materials used for RT-PCR analysis, including the high-capacity RNA-to-cDNA kit, Taqman Universal master mix, and Taqman probes, were obtained from Applied Biosystems (Waltham, MA, USA).

### 2.2. Genetic Identification of *S. japonicum* L. Flowers

The flowers of *S. japonicum* L. used in this study were purchased from a traditional herb market (Korea). Thus, the genetic identification of *S. japonicum* L. flowers [Sample No. BM221221-A] was performed by Biomedic Co., Ltd. (Bucheon, Republic of Korea) using a nuclear barcode marker, ITS (specifically the ITS2 region), and chloroplast barcode markers, matK, and trnL. PCR was conducted on the extracted DNA, followed by purification of the amplified products and bidirectional sequencing. Contig assembly was performed on the determined nucleotide sequences, followed by the multiple sequence alignment of the assembled contigs. Contigs from the samples and markers were subjected to a BLASTN search against the NCBI (<https://www.ncbi.nlm.nih.gov/>) nr database. The sequences of amplified ITS, matK and trnL showed 100%, 99.88%, and 100% identity, respectively, to those of the corresponding counterparts in the NCBI database. This conclusively indicates that the flowers originated from *S. japonicum* L.

### 2.3. Preparation of Ethanol Extract of *S. japonicum* L. Flowers (SJFE)

The flowers of *S. japonicum* L. (30 kg) were mixed with 50% ethanol (*v/v*, 300 L) and extracted by reflux circulation (Sungwon E&C, Uljin, Republic of Korea) at 60 °C for 3 h. The extract was filtered through 10–25 µm filters (Sungjin filter, Gwangyang, Republic of Korea), and the filtrate was concentrated using a vacuum concentrator (Sungwon E&C, Uljin, Republic of Korea) and dried. The concentrated filtrate (30.3 kg) was mixed with dextrose (Zhucheng Dongxiao Biotechnology Co., Ltd., Xinxing Town, China) (1:1, *w/w*)

and dried in a spray dryer (Ohkawara Kakohki Co., Ltd., Yokohama, Japan) with an inlet temperature of 188 °C and outlet temperature of 98 °C.

#### 2.4. Identification of Flavonoids in SJFE

Rutin (a quercetin glycoside), quercetin, kaempferol, and isorhamnetin in SJFE were quantified using HPLC. SJFE (20 mg) was dissolved in methanol (10 mL) and sonicated for 1 h (Powersonic 520, HWASIN Technology, Republic of Korea) and then filtered through a 0.45 µm membrane filter (Sartorius, Göttingen, Germany). An aliquot (5 µL) of the filtrate was injected into the column. The authentic compound of rutin (5 µg) was dissolved in methanol (10 mL), and an aliquot (5 µL) of the resulting solution was injected into the column. The conditions of chromatography were as follows: pump, Agilent Tech 1260 Infinity II (Agilent Technology, Santa Clara, CA, USA); column; YMC-Pack Pro, C18, 150 × 4.6 mm, 5 µm, 12 nm (YMC, Kyoto, Japan); mobile phases, 0.2% formic acid in DW (A) and acetonitrile (B); gradient of mobile phases, 0–8 min, (A) 82%, 8–10 min, (A) 75%, 10–20 min, (A) 70%, 20–25 min, (A) 64%, 25–30 min, (A) 60%, 30–31 min, (A) 5%, 31–40 min, (A) 5%; flow rate, 1.0 mL/min; and detection with a diode array detector at 254 nm.

For the analysis of quercetin, kaempferol, and isorhamnetin, the SJFE (300 mg) was dissolved in 10 mL of a hydrolyzing solution (a mixture of 100 mL ethanol, 40 mL DW, and 16 mL HCl) and reacted at 90 °C for 1 h. The reaction was filtered through a 0.45 µm filter. The 3 authentic compounds (quercetin 5.06 µg, kaempferol 4.50 µg, and isorhamnetin 2.39 µg each) were dissolved in 50 µL of methanol. Aliquots (5 µL) of the filtrate or each solution were injected into the column. The conditions of chromatography were the same as those above except for the following: column, YMC-Triart, C18, 250 × 4.6 mm, 5 µm (YMC, Kyoto, Japan); mobile phase; acetonitrile containing 0.1% phosphoric acid (A) and DW containing 0.1% phosphoric acid (B); the gradient of the mobile phase, 0–35 min, (A) 40%; detection with a diode array detector at 370 nm; and temperature, 35 °C.

#### 2.5. RAW 264.7 Murine Macrophage Cell Culture

Before the animal experiments, the anti-inflammatory properties of SJFE were tested preliminarily in the cellular experiment using RAW 264.7 murine macrophages (ATCC, Manassas, VA, USA). RAW 264.7 cells ( $5 \times 10^4$  cells/well) were seeded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Thermo Fisher Scientific Inc. (Waltham, MA, USA) FBS (10%) and penicillin-streptomycin (100 units/mL) and cultured for 24 h in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. The cells were harvested and used for the following experiments.

#### 2.6. Cell Viability

Cell viability was determined using the MTT test, as described previously [12]. The cells ( $5 \times 10^4$  cells/well) were incubated with varying concentrations of SJFE for 3 h, and media were removed and mixed with serum-free DMEM containing MTT dye (0.5 mg/mL). The formed formazan crystals were dissolved in dimethyl sulfoxide (100 µL), and the absorbance of the formazan solution was measured at 540 nm using the microplate reader (Varioskan LUX, Thermo Fisher Scientific, Waltham, MA, USA).

#### 2.7. Nitric oxide Production (NO)

Raw 264.7 cells ( $5 \times 10^4$  cells/well) were seeded for 24 h and incubated for another 24 h in the presence of LPS (1 µg/mL) and SJFE (0, 125, 250, and 500 µg/mL). The supernatant (50 µL) was collected and mixed with the same volumes of the Griess reagent. Absorbance at 546 nm was measured using a microplate reader. The production of NO was calculated from the standard curve obtained from sodium nitrite.

#### 2.8. Inflammatory Gene Expression by Quantitative Real-Time PCR (qPCR)

RAW 264.7 cells ( $2 \times 10^5$  cells/well) were cultured in a 6-well plate and total RNA was extracted using NucleoZOL (Macherey-Nagel, Düren, Germany) according to the

manufacturer's instructions. After RNA was reversely transcribed to cDNA using the high-capacity RNA-to-cDNA kit (Applied Biosystems), qPCR was carried out using a StepOne Plus real-time PCR system (Applied Biosystems) with the Taqman gene expression master mix (Applied Biosystems). For DNA amplification, the preheat step was performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min. The following Taqman probes were used, and the relative expressions of target genes were quantified after normalization to the value of GAPDH using StepOne Plus software v2.0: GAPDH (Mm99999915\_g1), iNOS (Mm00440502\_m1), IL-1 $\beta$  (Mm00434228\_m1), IL-6 (Mm00446190\_m1), and COX-2 (Mm00478374\_m1).

### 2.9. Animal Experiments

Sprague-Dawley rats (6-week-old, female) were purchased from SAMTAKO BIOKOREA Co., Ltd. (Osan, Republic of Korea). The rats were kept at 20–25 °C and 50–55% humidity with a 12 h light/dark cycle. Food and water were provided ad libitum. All animal experiment procedures were approved by the Animal Care and Use Committee at the Korea Conformity Laboratories (Approval No. IA23-00796). After 1 week of the acclimatization period, the following experiments were conducted.

### 2.10. Induction of osteoarthritis (OA) and SJFE Administration

After the cellular experiment, SJFE was tested for its anti-inflammatory properties in an arthritis model in rats.

All rats were anesthetized with isoflurane, hair was removed from both knees, and hairless areas were disinfected. Eight rats were assigned to the sham control group, where saline (50  $\mu$ L) was injected into the joint space of both knees. Forty rats were assigned to the OA group, where 50  $\mu$ L of monosodium iodoacetate (MIA) (60 mg/mL in saline) was injected into the left knee, and saline (50  $\mu$ L) was injected into the right knee. After the procedure, Ketoprofen (2 mg/kg) was administered subcutaneously to relieve any pain. These 40 OA-induced rats were divided into 5 groups (8 rats/group), and each group were orally administered a vehicle (saline), SJFE (50, 100, or 200 mg/kg), or methyl sulfonyl methane (MSM) (150 mg/kg), respectively, once a day for 4 weeks. SJFE and MSM were dissolved in saline, and 10 mL/kg of each of these solutions was given for the final doses to be as above. MSM was used as a positive control, which is known as an anti-inflammatory and anti-arthritic agent [15]. During 4-week experiment, the conditions of the rats were observed every day, and the body weight of all rats was measured once a week for 4 weeks. The following experiments were performed to evaluate the anti-osteoarthritis effect of SJFE.

### 2.11. Weight-Bearing Index

Weight-bearing index was measured at 0, 3, 7, 14, 21, and 28 days using an Incapacitance Meter (Columbus Instruments, Columbus, OH, USA). The rats were placed in an acrylic chamber with their two hind legs extended outside separately. They were positioned on weight sensors, with each leg resting on its respective sensor. The weight loaded on each leg was measured three times for 5 s, and the average value was recorded. Weight-bearing index was calculated by the equation "the weight on the left arthritic leg/[sum of weights on left and right legs]  $\times$  100".

On day 28, the rats were anesthetized with isoflurane. After anesthesia, blood was collected from the posterior aorta, and sera were prepared from the blood samples by centrifugation (3000 rpm, 10 min). Also, the knee joints were collected for histological examination. The sera and knee joints were kept at  $-80$  °C until use.

### 2.12. Serum Analysis

Inflammatory cytokines and cartilage degradation indicators were analyzed from the sera. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , KRC3011, Invitrogen, Waltham, MA, USA), IL-1 $\beta$  (JP27193, Demeditec Diagnostics GmbH, Kiel, Germany), and IL-6 (E-EL-R0015, Elabscience, Houston, TX, USA) were determined using ELISA kits. C-terminal telopeptide

2 (CTX-2, LS-F25032, LS Bio, Lynnwood, WA, USA) was measured using the ELISA kit, and cartilage oligomeric matrix protein (COMP) (SCB197Ra, Cloud-Clone Corp, Katy, TX, USA) was analyzed using the immunoassay kit. All the assays were performed according to the manufacturer's protocols.

### 2.13. Histological Examination of Knee Joints and Evaluation of Arthritic Symptoms

Knee joints were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 mm. Tissue sections were stained with H & E and Safranin O and examined under a light microscope. The microscopic findings were used to rate Makin's modified score [16] with respect to the following 4 categories: the structure of the articular surface, cells, matrix staining, and tidemark integrity.

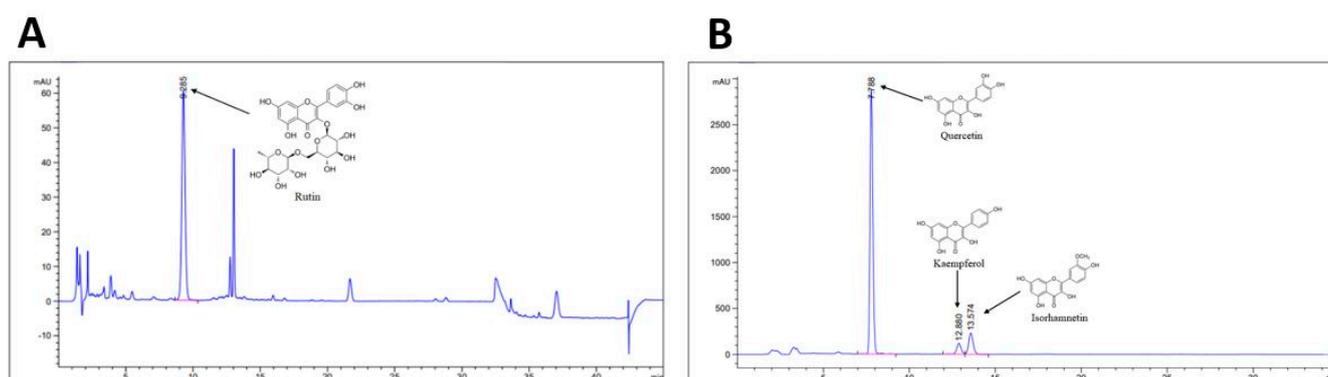
### 2.14. Data Analysis

All statistical analyses were performed using the SPSS 12.0 K program (SPSS, Chicago, IL, USA). Data are presented as the mean  $\pm$  standard deviation for experiments. Differences in means were considered significant at  $p < 0.05$ . The differences between the sham control and negative control were examined using Student's *t*-test. The statistical differences among test groups, except for the positive control, were examined using the standard one-way analysis of variance (ANOVA). The differences between the negative control and positive control were examined using Student's *t*-test. If the equal variance was admitted, Dunnett's test was used, and if the equal variance was not admitted, Dunnett's T3 test was applied.

## 3. Results

### 3.1. Major Antioxidants in SJFE

Antioxidants were identified in SJFE using HPLC. The major antioxidants found in SJFE were quercetin (184.84 mg/g) and rutin (154.03 mg/g), with kaempferol (11.30 mg/g) and isorhamnetin (20.07 mg/g) present as minor components [Figure 1]. It is important to note that rutin was directly measured from SJFE, whereas quercetin was measured from acid-treated SJFE to hydrolyze flavonoid glycosides. This implies that the measured content of quercetin might include both free quercetin and quercetin released from rutin through hydrolysis. It is likely that most of the quercetin exists in the form of rutin, a glycoside of quercetin, and a disaccharide, rutinose.

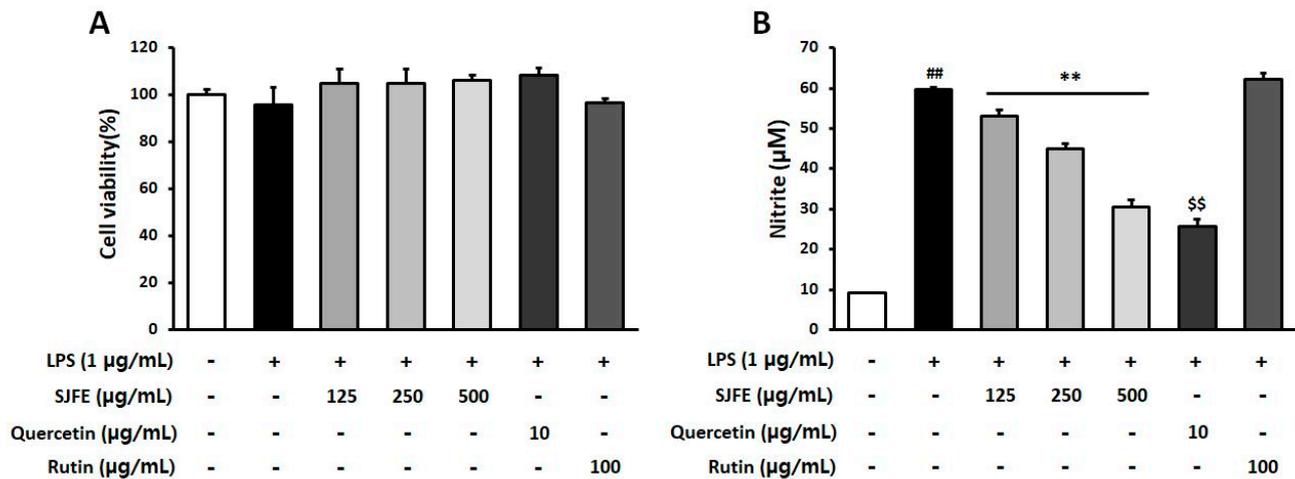


**Figure 1.** HPLC chromatograms of rutin, quercetin, kaempferol, and isorhamnetin identified in SJFE. (A) rutin, (B) quercetin, kaempferol, and isorhamnetin. The conditions of HPLC were described in MATERIALS and METHODS.

### 3.2. Effect of SJFE on Cell Viability and NO Production in LPS-Stimulated RAW 264.7 Cells

SJFE did not show any effect on the cell viability of RAW 264.7 cells up to 500  $\mu\text{g}/\text{mL}$ . Quercetin and rutin also showed no effect [Figure 2A]. However, SJFE effectively suppressed the generation of NO in LPS-stimulated RAW 264.7 cells, and this inhibition was dose-dependent [Figure 2B]. At a concentration of 500  $\mu\text{g}/\text{mL}$ , SJFE caused a 50% reduction

in NO production. Notably, quercetin demonstrated an even more potent inhibition at just 10 µg/mL compared to SJFE. On the other hand, its glycoside form, rutin, did not exhibit any inhibitory effects even at a concentration as high as 100 µg/mL [Figure 2B].



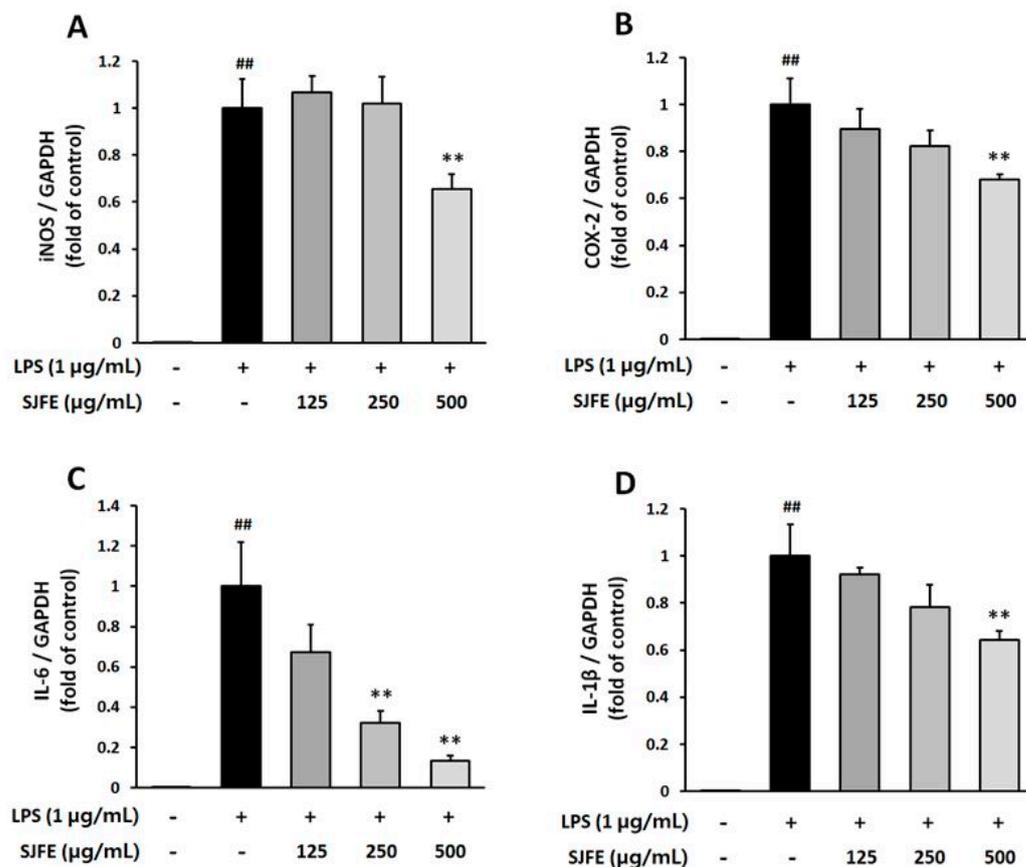
**Figure 2.** Effect of SJFE, quercetin, and rutin on the cell viability of RAW 264.7 cells and NO production in LPS-stimulated RAW 264.7 cells. The cells were incubated with SJFE of varying concentrations, quercetin, or rutin without or with LPS. After incubation, supernatants were used for cell viability using MTT test or NO production with nitrite assay. Data are presented as mean  $\pm$  SD in triplicate experiments. LPS, lipopolysaccharide; SJFE, ethanol extract of *S. japonicum* flowers. <sup>##</sup>,  $p < 0.01$  vs. no addition (student's *t*-test); <sup>\*\*</sup>,  $p < 0.01$  vs. LPS (1 µg/mL) (One-way ANOVA); and <sup>\$\$</sup>,  $p < 0.01$  vs. LPS (1 µg/mL) (student's *t*-test).

### 3.3. Effect of SJFE on Gene Expressions of Proinflammatory Proteins in LPS-Stimulated RAW 264.7 Cells

When exposed to LPS stimulation, RAW 264.7 cells exhibited a significant increase in the expression of genes encoding proinflammatory proteins, such as iNOS, COX-2, IL-6, and IL-1 $\beta$ . However, these gene expressions were significantly suppressed in the presence of SJFE. Specifically, the expressions of iNOS and COX-2 were significantly downregulated by SJFE at 500 and 250 µg/mL, respectively. The expression levels of IL-6 and IL-1 $\beta$  were also reduced by SJFE in a dose-dependent manner [Figure 3].

### 3.4. Effect of SJFE on Weight-Bearing Index (WBI) of OA-Induced Rats

After inducing OA on the left knees of rats, SJFE (0 to 200 mg/kg) was orally administered once a day for 28 days, and changes in body weight and WBI were monitored. The body weights did not show significant differences among the groups [Figure 4A]. On the other hand, WBI, which indicates the distribution of weight between the normal right and affected left legs in the standing position, exhibited a significant difference in 3 days. This suggests that OA was successfully induced in the left knee. However, a significant difference was observed in WBI between the control group and the group with 200 mg/kg of SJFE administration [Figure 4B]. This recovery became particularly significant on day 28 [Figure 4C]. The effect of SJFE was comparable to that of methyl sulfonyl methane (MSM), an anti-arthritic agent used as a positive control (Supplementary Table S1).



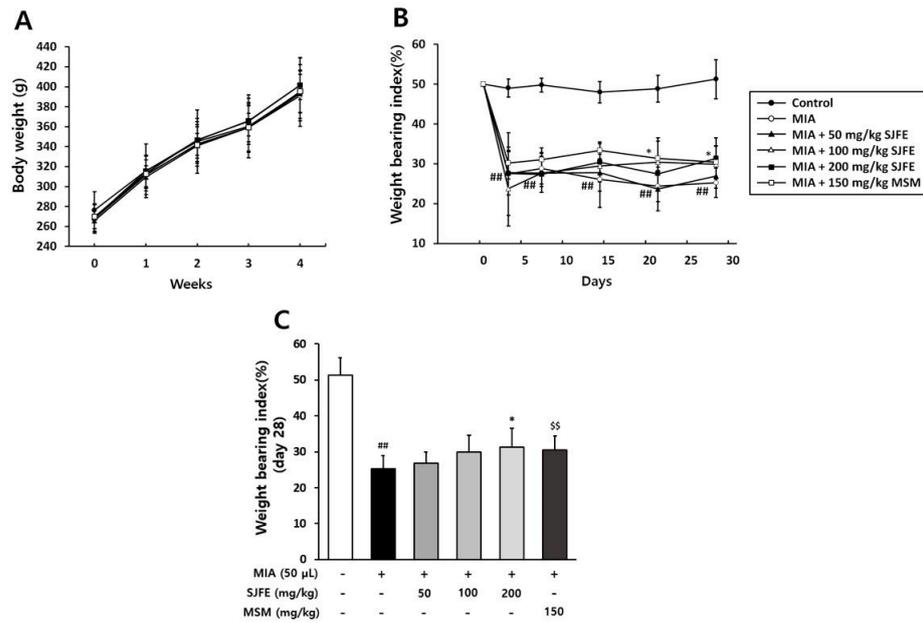
**Figure 3.** Effects of SJFE on the expression of genes for the proteins involved in the inflammatory reaction in LPS-stimulated RAW 264.7 cells. The cells were incubated with SJFE of various concentrations without or with LPS, and cells were harvested and used for real-time RT PCR for the genes of iNOS (A), COX-2 (B), IL-6 (C), and IL-1 $\beta$  (D). Data are presented as the mean  $\pm$  SD in triplicate experiments. ##,  $p < 0.05$  vs. no addition (student's  $t$ -test); \*\*,  $p < 0.05$  vs. LPS (1  $\mu$ g/mL) (One-way ANOVA). iNOS, inducible NO synthase; IL, interleukin; COX-2, cyclooxygenase-2. Other abbreviations are the same as in Figure 2.

### 3.5. Effects of SJFE on Serum Inflammatory Cytokines and Cartilage Damage Marker Proteins in OA-Induced Rats

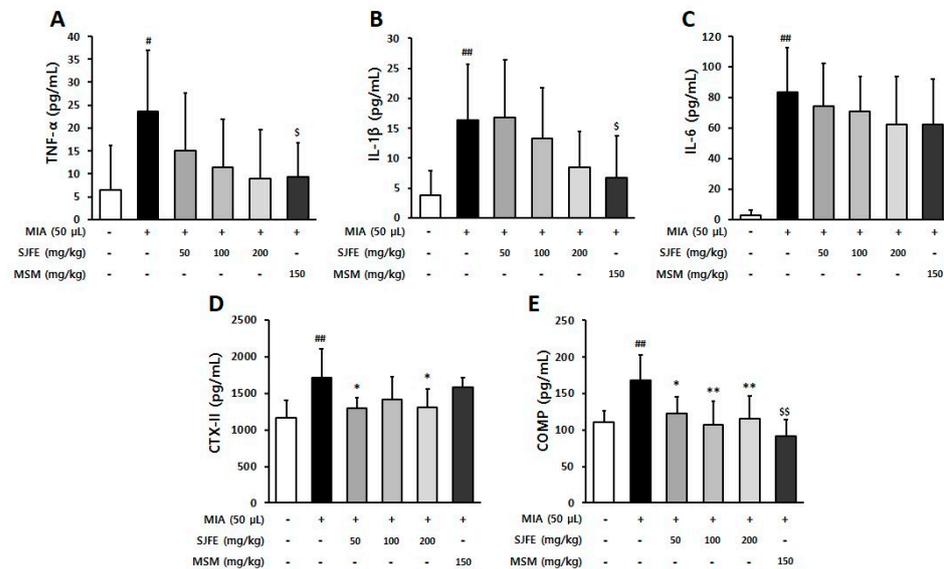
WBI of OA-induced rats increased at the end of the experiment (on day 28) treated with SJFE [Figure 4B], suggesting the alleviation of OA. To support this finding, the levels of serum inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, C-terminal telopeptide 2 (a cartilage-degrading enzyme: CTX-II), and the cartilage oligomeric matrix protein (degraded cartilage products; COMP) were evaluated on the 28th day. The serum levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were significantly increased in OA-induced rats. The increased inflammatory cytokines levels were decreased by the administration of SJFE, but no significant difference was observed [Figure 5A–C]. Even MSM showed no effect. In contrast, the elevated levels of CTX-II and COMP induced by OA were reduced by SJFE (Figure 5D,E). This result indicates that cartilage degradation was significantly inhibited, as was the case with MSM.

### 3.6. Effects of SJFE on Cartilage Damage in OA-Induced Knee Joints of Rats

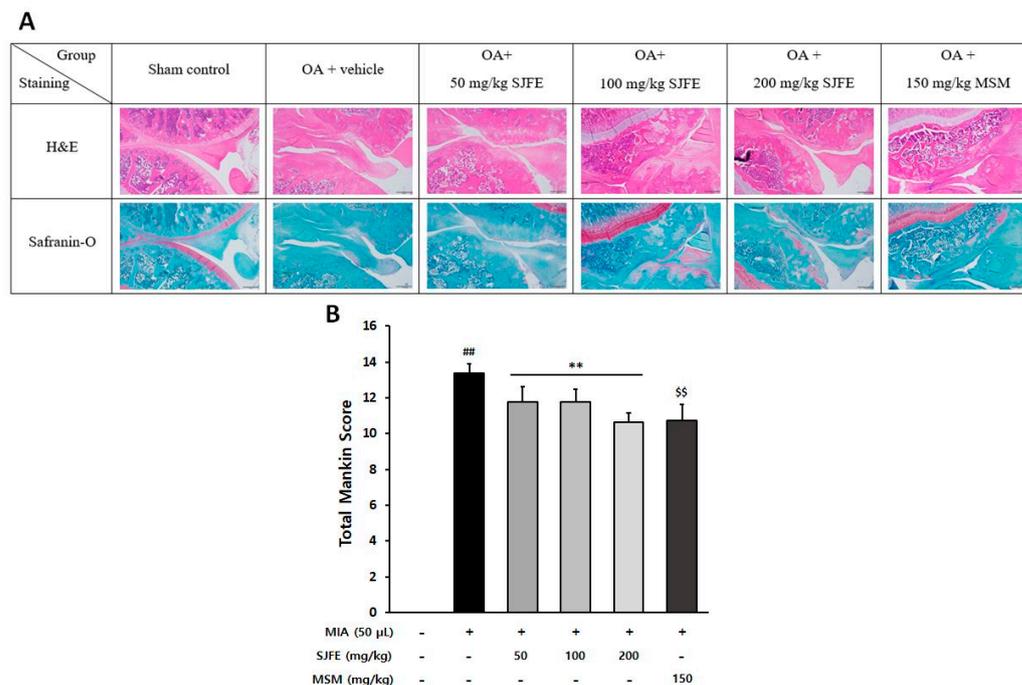
The protective effect of SJFE on cartilage damage was further evaluated by histology of the knee joints stained with H&E and Safranin O [Figure 6A] and modified Mankin's scoring criteria, which is a grading system of cartilage degradation. As shown in Figure 6B, Mankin's score of OA-induced knee joints in the control group was rated as 14, while it was significantly decreased in the groups of both SJFE and MSM.



**Figure 4.** Effects of SJFE on body weight and weight–bearing index (WBI) of osteoarthritis (OA) induced rats. OA was induced by injecting monosodium iodoacetate (MIA) into the left knee joints, and one day after, the rats were treated with the oral administration of SJFE or methyl sulfonyl methane (MSM) once a day for 28 days. Body weight (A), WBI (B), and WBI on day 28 (C) were measured at the time indicated. Data are the mean ± SD for 8 experiments. ##,  $p < 0.01$  vs. sham control (student’s  $t$ -test); \*,  $p < 0.05$  vs. OA-induced rats treated with vehicle (One-way ANOVA); \$\$,  $p < 0.05$  vs. OA-induced rats treated with vehicle (Student’s  $t$ -test). MSM was used as a positive control.



**Figure 5.** Effects of SJFE on the serum levels of inflammatory cytokines and markers of cartilage degradation in OA-induced rats. Experimental conditions were the same as in Figure 4. On day 28, the rats were sacrificed, and inflammatory cytokines and markers of cartilage degradation were measured from the sera. (A), TNF-α; (B), IL-1β; (C), IL-6; (D), COMP; and (E), CTX-II. Data are mean ± SD for 8 experiments. #,  $p < 0.05$ , ##,  $p < 0.01$  vs. the sham control (Student’s  $t$ -test); \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs. OA-induced rats treated with vehicle (One-way ANOVA) and \$,  $p < 0.05$ , \$\$,  $p < 0.01$  vs. OA-induced rats treated with vehicle (student’s  $t$ -test). TNF, tumor necrosis factor; COMP, cysteine-rich protein with proline-rich tandem repeats; CTX-II-cathepsin-L-like cysteine protease. Other abbreviations are the same as in Figure 4.



**Figure 6.** Histology of OA –induced joint tissues in rats and Mankin’s score of arthritic joint tissues. Experimental conditions were the same as in Figure 4. On day 28, rats were sacrificed, and tissue sections of knee joints were prepared and stained with H&E for inflammatory status or Safranin O for cartilage status (A). Using the histological findings of panel A, the severity of arthritis was evaluated by Mankin’s modified scoring (B). Data are presented as mean  $\pm$  SD for 8 experiments. ##,  $p < 0.01$  vs. the sham control (student’s  $t$ -test); \*\*,  $p < 0.01$  vs. OA-induced rats treated with vehicle (One-way ANOVA); and \$\$,  $p < 0.01$  vs. OA-induced rats treated with vehicle (Student’s  $t$ -test). Abbreviations are the same as in Figure 4.

#### 4. Discussion

The present study investigated the anti-inflammatory potential of SJFE in LPS-stimulated RAW 264.7 cells. SJFE was observed to suppress the release of nitric oxide (NO) [Figure 2] and the expression of genes of inflammation-inducing proteins, specifically iNOS, COX-2, IL-6, and IL-1 $\beta$  [Figure 3] in these cells. IL-1 $\beta$  and IL-6 are principal cytokines expressed in chondrocytes and macrophages [17] during arthritic conditions. These cytokines stimulate iNOS and COX-2, leading to the production of NO and prostaglandin E2 [18,19]. Consequently, regulating these cytokines is associated with therapeutic efficacy for arthritic disorders.

Based on the results obtained from the cellular experiments, we proceeded to test the anti-inflammatory properties of SJFE in rats with osteoarthritis (OA) induced by MIA. Our observations revealed that SJFE improved the weight-bearing index of affected knee joints [Figure 4B] and reduced the levels of cartilage-degrading enzymes and degraded products in the serum [Figure 5D, E]. Furthermore, SJFE resulted in a decrease in Mankin’s score, an index used to gauge the severity of cartilage damage [Figure 6B]. These findings support the fact that the flower extract also possesses anti-inflammatory properties capable of alleviating the symptoms of arthritis.

However, it is uncertain what components in SJFE are responsible for its anti-inflammatory properties. This question was raised by the findings where the main component in SJFE was rutin [Figure 1A], but this glycoside (100  $\mu$ g/mL) showed no effect on NO production in LPS-stimulated RAW 264.7 cells, whereas quercetin (10  $\mu$ g/mL) reduced the production by 60%. However, we assumed that the components exerting the anti-inflammatory activities were flavonoids, particularly quercetin, based on the following reasons. First, we were able to measure quercetin as a major component and kaempferol and isorhamnetin as minors when SJFE was acid-hydrolyzed [Figure 1B], meaning that these flavonoids in SJFE

exist as glycosides (possibly inactive forms) in SJFE. [Figure 1B]. Thus, rutin administered orally is hydrolyzed to release quercetin in the body [20]. Second, it has been reported that fruits or leaves of *S. japonicum* contain flavonoids, among which quercetin is the main component [10]. Third, there are the reports demonstrating the anti-inflammatory properties of quercetin on the animal models of inflammatory disorders [21].

In addition, the fruit extract of *S. japonicum* showed a protective effect on prostate inflammation in rats [22]. The flower bud extract of this plant exhibited the suppression of expression of the proinflammatory proteins in experimentally induced contact dermatitis in mice [23]. In these studies, the observed effects were ascribed to the anti-oxidant activity exerted by flavonoids, including quercetin as a principle component. Furthermore, quercetin-rich extracts of *Tabebuia rosea* [24] or *Erica multiflora* [25] reduced the expressions and serum levels of pro-inflammatory genes and proteins in diet-induced obesity in rats. All these reports support the fact that the anti-inflammatory properties of SJFE are exerted by flavonoids like quercetin.

A study demonstrated that the oral administration of quercetin (30 mg/kg) significantly reduced the severity of collagen-induced arthritis in mice and decreased the serum levels of proinflammatory cytokines [21]. In the present study, however, we did not observe significant effects of SJFE on the serum levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 [Figure 5A–C], even at a dosage of 200 mg/kg of SJFE. At this dose of SJFE, the amount of quercetin present was 36 mg, including both free quercetin and that released for rutin. Consequently, the administered free quercetin via SJFE falls considerably below the 30 mg used previously. This may explain the discrepancy between our findings and those of the prior study. Several studies have assessed the antiarthritic effects of different agents by measuring the levels of proinflammatory proteins or expressions in both synovial tissue and fluid [26,27]. We may also observe significant effects if these assays are conducted on joint tissue or fluid.

In summary, our findings support the anti-arthritic effects of SJFE. These effects are evidenced by improvements in the weight-bearing index of knee joints affected by OA, reductions in the levels of cartilage-degrading enzymes and their byproducts in the serum, as well as a decrease in Mankin's score. Therefore, SJFE shows promise in alleviating OA symptoms owing to its anti-inflammatory properties, suggesting potential benefits for individuals suffering from osteoarthritis symptoms.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14104301/s1>.

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