Standardized Ethanolic Extracts of *Boswellia serrata* Ameliorate Symptoms of Osteoarthritis by Direct Effects on Chondrocytes

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Abstract We investigated whether standardized ethanolic extracts of Boswellia (FJH-BS) could alleviate the symptoms of osteoarthritis, including pain, inflammation, and degradation of articular cartilage. Sprague-Dawley rats with monosodium iodoacetate (MIA)-induced osteoarthritis received supplementation of FJH-BS at 80 and 125 mg/kg body weight. We found that FJH-BS supplementation reduced histological and architectural changes and pain levels in rats with MIA-induced osteoarthritis. In addition, FJH-BS supplementation suppressed mRNA expression of matrix metalloproteinases (MMPs) and pro-inflammatory mediators, including cyclooxygenase-2, prostaglandin E2, and pro-inflammatory cytokines. Furthermore, FJH-BS treatment directly suppressed cell death, inflammation, and expression of MMPs in H₂O₂- or LPS-treated primary chondrocytes. Our results suggest that supplementation with standardized FJH-BS may prevent osteoarthritis progression by directly influencing chondrocytes.

Keywords: Boswellia, osteoarthritis, chondrocytes


1. Introduction

Joint pain because of cartilage degradation and synovial inflammation is a typical symptom of osteoarthritis, a degenerative joint disease. Osteoarthritis is a whole joint disease that involves not only cartilage but also the menisci, subchondral bone, infrapatellar fat pad, and synovial membrane. The exact etiology of osteoarthritis is not yet clear, but it typically occurs later in life, usually after age 50, although it may occur earlier in the case of joint injury [1,2,3]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are common pharmacological interventions used to relieve the associated pain and inflammation. The NSAIDs have the ability to inactivity of cyclooxygenase (COX), which impairs the production of prostaglandins, important mediators of the inflammatory response. However, NSAIDs cause gastrointestinal adverse effects, such as stomach pain, ulcers, and heartburn [4,5]. Therefore, herbal medicines are often used to treat osteoarthritis because of the low risk of side effects and toxicity and increased demand for functional foods [6,7].

Articular cartilage degeneration in osteoarthritis has been linked to abnormal mechanical stresses that are known to cause chondrocyte apoptosis and metabolic derangement [8]. Although mechanical stimulation is essential for the maintenance of homeostasis in the articular cartilage, changes in matrix loading can induce the production of various pro-inflammatory mediators from chondrocytes, which cause cartilage matrix degradation and osteoarthritis development [9]. Evidence from several studies indicates that synoviocytes, chondrocytes, and cells from other joint tissues can produce and/or respond to a number of pro-inflammatory mediators, including COX-2, prostaglandin E2 (PGE2), pro-inflammatory cytokines, and chemokines [10,11]. These pro-inflammatory mediators are capable of activating matrix metalloproteinases (MMPs) and other catabolic genes and cleaving collagen type II in the regions of matrix depletion in articular cartilages of osteoarthritis patients [12,13]. Subsequently, the release of these components is recognized by integrins and other cell surface receptors, triggering inflammatory responses and sustaining cartilage destruction [14].

*Boswellia serrata*, belonging to the family Burseraceae, is a plant used in traditional Ayurvedic medicine in India.
The main component of *B. serrata* is boswellic acid, which is effective in treating asthma and various chronic inflammatory diseases [15,16]. Alluri *et al.* demonstrated that gum resin extracts of *B. serrata* had the ability to relieve pain and protect cartilage in monosodium iodoacetate (MIA)-induced osteoarthritis in rats [17]. However, the exact mechanism underlying these effects is unclear. Here, we investigated the protective effect of the standardized ethanolic extract of *B. serrata* (FJH-BS) in rats with MIA-induced osteoarthritis and H$_2$O$_2$- or LPS-treated primary chondrocytes to identify the underlying mechanisms involved in anti-inflammatory effects and inhibition of cell damage via direct effects in chondrocytes.

2. Materials and Methods

2.1. Preparation of the Extract

We obtained the standardized ethanolic extract of *B. serrata* from Frombio (Suwon-si, Gyeonggi-do, Korea). The compounds from *B. serrata* (30 kg) were extracted with 452 L of 95% ethanol at 50 ± 5 °C for 3 h in a reflux apparatus. The extract was concentrated, filtered, and dried at 90 °C, resulting in a total FJH-BS yield of 34.8%. Thereafter, we analyzed sum of 11-keto-β-boswellic acid (KBA) and acetyl CH$_3$CO acetyl-11-keto-β-boswellic acid (AKBA) levels of FJH-BS using high-performance liquid chromatography. The sum of KBA and AKBA was 59.73 mg/g in FJH-BS (Figure 1) and stored in an air-tight container at −20 °C until use.

2.2. Animals and Induction of Osteoarthritis

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Chonnam National University approved the protocol (no. CNU IACUC-YB-R-2020-115, approval date: 15/01/2021) for animal study.

Sprague-Dawley rats (Six-week-old males) were obtained from SaeRon Bio (Uiwang, Korea) and were housed in cages with automatically controlled environment (temperature: 22 ± 2°C; humidity: approximately 50%; lighting: 12-h light:dark cycle). Rats fed an AIN 93G diet for an acclimation period of 7 days. All the rats were randomly divided into eight groups of eight mice each as follows: Normal Control (AIN93G diet), Control (AIN93G diet + MIA injected group), MSM (positive control, AIN93G diet supplemented with MSM 150 mg/kg body weight (b.w.) + MIA injected group), FJH-BS Low (AIN93G diet supplemented with FJH-BS 80 mg/kg b.w. + MIA injected group), and FJH-BS High (AIN93G diet supplemented with FJH-BS 125 mg/kg b.w. + MIA injected group). Two weeks after each diet supplement, the rats were anesthetized with isoflurane and were administered MIA (50 μL of 60 mg/mL) (Sigma–Aldrich) through a single injection into the right knee joint. The normal group was administered 0.9% saline through injections. The rats were sacrificed by cervical dislocation 3 weeks after MIA injection.

2.3. Treadmill

Three weeks after injecting MIA, we used a rat-specific treadmill (Jeollanamdo Institute of Natural Resources Research, Korea) to measure rear pressure, rear propel, and running speed.

2.4. Micro-CT Image Scan

Micro-CT imaging of the formalin-fixed articular cartilage from rats was used to measure the roughness of the bone surface. Micro-CT image scanning was conducted using the Skyscan 1172 ® X-ray μCT scanning system (Bruker, Belgium). After standardized reconstruction of the scanned images, the data for each sample were obtained using the micro-CT software to orient each sample in the same manner.

Figure 1. High-performance liquid chromatography analysis of 11-keto-β-boswellic acid (KBA) and acetyl CH$_3$CO acetyl-11-keto-β-boswellic acid (AKBA) levels in FJH-BS
2.5. Histologic Hematoxylin and Eosin (H&E) Staining

Knee joints were collected from rats, fixed in 10% neutral buffered formalin, and decalcified. The samples were embedded in paraffin, sliced into 7-μm-thick sections, and stained with H&E for histological evaluation.

2.6. Measurement of CRP and COMP Levels in the Serum

Serum samples were obtained from rats, and CRP and COMP levels were determined using the Rat COMP ELISA and Rat CRP ELISA Kits (MyBioSource, San Diego, CA, USA).

2.7. Primary Culture of Chondrocytes

Sprague-Dawley rats (160-180 g, 5 weeks, male) were purchased from SaeRon Bio (Uiwang, Korea) and were sacrificed by cervical dislocation. Cartilages were isolated and incubated overnight on a shaker at 100 rpm in Hank's balanced salt solution (Hyclone Laboratories, Logan, Utah, USA) containing 2 mg/mL collagenase (Sigma-Aldrich Co, St. Louis, MO, USA). The chondrocytes seeded in 75T flasks in Dulbecco’s minimal essential medium (Hyclone Laboratories) with 10% fetal bovine serum (Hyclone Laboratories), 100 mg/L penicillin-streptomycin (Hyclone Laboratories), and 2 mmol/L glutamine (Hyclone Laboratories) at 37°C in a humid atmosphere and 5% CO₂.

2.8. Cell Viability

Diverse concentrations of FJH-BS were added to the H₂O₂- or LPS-treated primary chondrocytes (1×10⁴ cells/well in 96-well plate). To evaluate the effect of FJH-BS on viability of the chondrocytes, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used in the chondrocytes. 20 μL of MTT solution (5 mg/mL in PBS as stock solution) was added into chondrocytes for 3 h at 37°C. In order to measure the absorbance, supernatants were extracted through suction and 200 μL of DMSO was added to each well. The plates were read at 560 nm.

2.9. 5-lipoxygenase Activity in Chondrocytes

The chondrocytes (5×10⁶ cells/well in 6 well plate) were cultured with FJH-BS and 50 μg/mL of LPS for 24 h. 5-lipoxygenase activity was determined using a Rat 5-lipoxygenase ELISA Kit (MyBioSource).

2.10. mRNA Expression in Chondrocytes and Rat Cartilage

mRNAs from the chondrocytes and rat articular cartilage were extracted using the RNaseasy Mini Kit (QIAGEN, Maryland, USA). The iScript™ cDNA Synthesis Kits (BIORAD, Hercules, CA, USA) were used for cDNA synthesis. RT-PCR was conducted using the SYBR Green PCR Master Mix (iQ SYBR Green Supermix, BIORAD, Hercules, CA, USA). All PCRs for cDNA amplification included an initial denaturation step (95°C for 30 s), followed by annealing (56°C for 30 s) and extension (72°C for 45 s) for 40 cycles. The primer pairs are listed in Table 1. Data analysis was conducted using the 7500 System SDS software version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

2.11. Statistical Analysis

All results are presented as mean ± standard deviation (SD). To evaluate the differences among the groups, the results were statistically evaluated using one-way ANOVA and Duncan’s multiple range test. We used with SPSS statistical procedures for Windows (SPSS PASW Statistic 23.0, SPSS Inc. Chicago, IL, USA). Differences were considered statistically significant at p < 0.05.

Table 1. Primer set sequence used for Real-Time PCR.

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<th>Reverse Sequence (5’-3’)</th>
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<td>CAG CAA CTG AGG GCC TCT CT</td>
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<td>CTG ACC TGT CTC GAT CAT GTT GCA</td>
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<tr>
<td>Aggrecan</td>
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<td>GGA TAG GGC TGG GTC ACA CTT</td>
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3. Results

3.1. Statistical -BS Suppressed Pain Levels in Rats with MIA-induced Osteoarthritis

We measured rear left pressure, rear right pressure, rear left propel, rear right propel, and running speed of rats with MIA-induced osteoarthritis using the treadmill to investigate the effect of FJH-BS on pain levels. The levels of rear left pressure, rear right pressure, rear left propel, rear right propel, and running speed were significantly lower in the rats with MIA-induced osteoarthritis (control) than in rats without osteoarthritis (normal control). However, dietary supplementation with methylsulfonylmethane (MSM) or FJH-BS significantly increased rear left pressure, rear right pressure, rear left propel, rear right propel, and running speed as compared to those of the control group (p < 0.05) (Figure 2).

3.2. FJH-BS Suppressed Architectural and Mineralization Parameters Changes in Rats with MIA-induced Osteoarthritis

The morphological change, apparent bone mineral density (BMD), material BMD, trabecular separation, trabecular thickness, trabecular number, and bone volume/total tissue volume of rats with MIA-induced osteoarthritis were measured using Micro-computerized tomographic (Micro-CT). The joints from rats with MIA-induced osteoarthritis showed an irregular articular cartilage surface and significantly lower levels of BMD, trabecular thickness, trabecular number, and bone volume/total tissue volume than the joints from rats without osteoarthritis. The groups where diets were supplemented with MSM or FJH-BS showed significantly suppressed changes in these architectural and mineralization parameters (p < 0.05) (Figure 3).

Figure 2. The effect of FJH-BS on rear left propel (A), rear right propel (B), rear left pressure (C), rear right pressure (D), and running speed (E) of rats with MIA-induced osteoarthritis on using the treadmill. Normal Control: AIN93G diet; Control: AIN93G diet + MIA injected group; MSM (positive control): AIN93G diet + MIA injected group supplemented with MSM 150 mg/kg b.w.; FJH-BS Low: AIN93G + MIA injected group supplemented with FJH-BS 80 mg/kg b.w.; FJH-BS High: AIN93G + MIA injected group supplemented with FJH-BS 125 mg/kg b.w. Values are presented as mean ± SD (n=8). Different letters (a > b > c > d) indicate a significant difference with p < 0.05, as determined using the Duncan’s multiple range test.
Figure 3. The effect of FJH-BS on morphological change (A), apparent BMD (B), material BMD (C), trabecular separation (D), trabecular thickness (E), trabecular number (F), and bone volume/total tissue volume (G) of rats with MIA-induced osteoarthritis using micro-CT. Normal Control: AIN93G diet; Control: AIN93G diet + MIA injected group; MSM (positive control): AIN93G diet + MIA injected group supplemented with MSM 150 mg/kg b.w.; FJH-BS Low: AIN93G + MIA injected group supplemented with FJH-BS 80 mg/kg b.w.; and FJH-BS High: AIN93G + MIA injected group supplemented with FJH-BS 125 mg/kg b.w. Values are presented as mean ± SD (n=8). Different letters (a > b > c > d) indicate a significant difference with p < 0.05, as determined using the Duncan’s multiple range test.
3.3. FJH-BS Suppressed Histological Changes in Rats with MIA-induced Osteoarthritis

To study the effect of FJH-BS on rats with MIA-induced osteoarthritis, we observed histological changes in the articular joints using H&E staining and measured Mankin’s scores. Rats with MIA-induced osteoarthritis showed an irregular articular cartilage surface and cartilage matrix degradation and increased Mankin’s scores. However, dietary supplementation with MSM or FJH-BS suppressed histological changes and decreased Mankin’s scores in rats with MIA-induced osteoarthritis (p < 0.05) (Figure 4).

3.4. FJH-BS Suppressed the mRNA Expression Changes in Articular Cartilage from Rats with MIA-induced Osteoarthritis

We investigated the mRNA expression of the inflammatory, anabolic, and catabolic factors in the articular cartilage to investigate the effect of FJH-BS in rats with MIA-induced osteoarthritis. The inflammatory factors (IL-1β, IL-6, and TNF-α) and catabolic factors (MMP-3, MMP-7, and MMP-13) were significantly decreased in groups where diets were supplemented with MSM or FJH-BS as compared to those in the group with MIA-induced osteoarthritis (p < 0.05) (Figure 5).

3.5. FJH-BS Decreased Serum C-reactive Protein (CRP) and Cartilage Oligomeric Matrix Protein (COMP) Levels in Rats with MIA-induced Osteoarthritis

We measured the serum levels of the inflammatory biomarker CRP and osteoarthritis biomarker COMP in rats with MIA-induced osteoarthritis. The CRP and COMP levels were significantly higher in the groups of rats with MIA-induced osteoarthritis than in the groups of rats without osteoarthritis. The groups where the diets were supplemented with MSM or FJH-BS showed a significant decrease in the levels of CRP and COMP as compared to those in the group with MIA-induced osteoarthritis (p < 0.05) (Figure 6).
Figure 5. The effect of FJH-BS on the mRNA expression of IL-1β (A), IL-6 (B), TNF-α (C), aggrecan (D), collagen type I (E), collagen type II (F), TIMP-1 (G), TIMP-2 (H), TIMP-3 (I), MMP-3 (J), and MMP-13 (L) in the articular cartilage from rats with MIA-induced osteoarthritis using RT-PCR. Normal Control: AIN93G diet; Control: AIN93G diet + MIA injected group; MSM (positive control): AIN93G diet + MIA injected group supplemented with MSM 150 mg/kg b.w.; FJH-BS Low: AIN93G + MIA injected group supplemented with FJH-BS 80 mg/kg b.w.; and FJH-BS High: AIN93G + MIA injected group supplemented with FJH-BS 125 mg/kg b.w. Values are presented as mean + SD (n=8). Different letters (a > b > c > d > e) indicate a significant difference with p < 0.05, as determined using the Duncan’s multiple range test.
3.6. FJH-BS Ameliorated H$_2$O$_2$-induced Damage and mRNA Expression Changes of Primary Chondrocytes

The death of chondrocytes was induced by treating them with 200 μM H$_2$O$_2$. However, FJH-BS pre-treatment inhibited H$_2$O$_2$-induced chondrocyte death in a dose-dependent manner (p < 0.05) (Figure 7).

Moreover, the expression levels of the anabolic factors aggrecan, collagen type I, collagen type II, TIMP-1, TIMP-2, and TIMP-3 mRNAs were significantly increased in the groups with MSM or FJH-BS treatments as compared to the group with H$_2$O$_2$-induced damage of primary chondrocytes. The expression levels of catabolic factors MMP-3, MMP-7, and MMP-13 mRNAs were significantly decreased in the groups with MSM or FJH-BS treatments as compared to the group with H$_2$O$_2$-induced damage of primary chondrocytes (p < 0.05) (Figure 8).

3.7. FJH-BS Suppressed Inflammation Development in LPS-treated Primary Chondrocytes

We measured mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), PGE2, and COX-2 and levels of 5-lipoxygenase in LPS-treated primary chondrocytes. All inflammatory factors were significantly increased in LPS-treated primary chondrocytes; however, FJH-BS treatment suppressed mRNA expression of IL-1β, IL-6, TNF-α PGE2, and COX-2 and levels of 5-lipoxygenase in LPS-treated primary chondrocytes (p <.05) (Figure 9).
Figure 8. The effect of FJH-BS on H2O2-induced changes in the mRNA expression of aggrecan (A), collagen type I (B), collagen type II (C), TIMP-1 (D), TIMP-2 (E), TIMP-3 (F), MMP-3 (G), MMP-7 (H), and MMP-13 (I) in primary chondrocytes. Normal Control: no treatment; Control: H2O2 200 μM treatment; MSM 10: H2O2 200 μM and MSM 10 μg/mL treatment; MSM 20: H2O2 200 μM and MSM 200 μg/mL treatment; FJH-BS 1: H2O2 200 μM and FJH-BS 1 μg/mL treatment; FJH-BS 5: H2O2 200 μM and FJH-BS 5 μg/mL treatment; FJH-BS 10: H2O2 200 μM and FJH-BS 10 μg/mL treatment; and FJH-BS 20: H2O2 200 μM and FJH-BS 20 μg/mL treatment. Values are presented as mean ± SD (n=4). Different letters (a > b > c > d > e) indicate a significant difference with p < 0.05, as determined using the Duncan’s multiple range test.
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4. Discussion

Recently, the use of natural products as complementary and alternative medicine has gained attention in osteoarthritis research because of reduced side effects and toxicity [6]. In the present study, we investigated the effect of FJH-BS in rats with MIA-induced osteoarthritis and H2O2- or LPS-treated primary chondrocytes to assess the effect of novel alternative medicines in osteoarthritis treatment. MIA inhibits glyceraldehyde-3-phosphate dehydrogenase activity. Thus, an intra-articular injection of MIA induces the development of osteoarthritis caused by chondrocyte death in articular cartilage [18]. Herein, we found that an intra-articular injection of MIA induces matrix degradation and inflammation in articular cartilages from rats, and H2O2 or LPS caused cell death, increased proteinases expression, and inflammation in primary chondrocytes.

The pathophysiology of osteoarthritis involves several catabolic mediators that cause progressive damage of articular cartilage by inflammatory substances, including MMPs [19,20]. Collagen is the major protein in the extracellular matrix and connective tissue, accounting for 30% of total body protein. Aggrecan, a large aggregate proteoglycan, is an important component of the extracellular matrix and is essential for the normal functioning of joints. The quality of the extracellular matrix is critical for maintaining the functional properties of the cartilage [19,21]. As MMPs with the collagenolytic ability (collagenases) are rate-limiting during collagen degradation, collagen degradation is almost exclusively mediated by MMPs [22]. MMPs have been divided into several categories: MMP-3 (stromelysins), which degrade non-collagen matrix proteins (proteoglycans); MMP-7 (matrilysin), which degrade casein, type I, II, IV, and V gelatin, fibronectin, and proteoglycans; and MMP-13 (collagenases), which degrade the interstitial collagens (types I, II, and III). All MMPs are inhibited by TIMPs and greatly increased by pro-inflammatory mediators during osteoarthritis progression [23]. We showed that MMPs and pro-inflammatory mediators decreased and TIMPs increased after supplementation with FJH-BS in rats with MIA-induced osteoarthritis. These results indicated that FJH-BS effectively suppressed inflammation and degradation of articular cartilage in osteoarthritis.

Lo et al. demonstrated that apoptosis was induced by exposure to H2O2 in primary human chondrocytes through caspase activation, in a time and dose dependent manner [24]. Chondrocytes, the unique cellular component of articular cartilage, secrete extracellular matrix to maintain the cartilage under normal conditions. In the chondrocyte metabolism, several signaling factors control articular cartilage development, growth, maintenance, and repair by regulating the expression of MMPs [25]. It is well known that the osteoarthritis is associated with increased

Figure 9. The effect of FJH-BS on LPS-treated inflammatory factors, IL-1β (A), IL-6 (B), TNF-α (C), 5-lipoxygenase (D), PGE2 (E), and COX-2 (F) in primary chondrocytes. Normal Control: no treatment; Control: LPS 50 μg/mL treatment; MSM 10: LPS 50 μg/mL and MSM 10 μg/mL treatment; MSM 20: LPS 50 μg/mL and MSM 200 μg/mL treatment; FJH-BS 1: LPS 50 μg/mL and FJH-BS 1 μg/mL treatment; FJH-BS 5: LPS 50 μg/mL and FJH-BS 5 μg/mL treatment; FJH-BS 10: LPS 50 μg/mL and FJH-BS 10 μg/mL treatment; and FJH-BS 20: LPS 50 μg/mL and FJH-BS 20 μg/mL treatment. Values are presented as mean ± SD (n=4). Different letters (a > b > c > d > e) indicate a significant difference with p < 0.05, as determined using the Duncan’s multiple range test.

Values are presented as mean ± SD (n=4). Different letters (a > b > c > d > e) indicate a significant difference with p < 0.05, as determined using the Duncan’s multiple range test.
inflammatory mediators that interfere with the catabolic and the anabolic process of articular cartilage [23], thus we investigated whether FJH-BS have a protective effect in H2O2- or LPS-treated primary chondrocytes. We found that treatment with FJH-BS suppressed cell death, MMP expression, extracellular matrix degradation in H2O2-treated primary chondrocytes. In addition, treatment with FJH-BS suppressed inflammation in LPS-treated primary chondrocytes. These results suggest that the effect of dietary supplementation with FJH-BS in rats with osteoarthritis may have been induced by direct effects on chondrocytes in articular cartilage.

Yu et al. [26] and Kimmakatkar et al. [27] reported that administration of Boswellia and its extract induced a decrease in knee pain and an increase in knee flexion and walking distance. In the systemic review and meta-analysis by Yu et al. [26], they showed that Boswellia and its extract may relieve the pain and improve the function of joint, compared with the control group and suggested a duration and dose of at least 100-250 mg of Boswellia for 4 weeks. This suggestion is similar to our experimental period of 5 weeks in the present animal study. We suggest that FJH-BS may reduce the clinical symptoms of osteoarthritis via inhibition of the inflammatory response. Although there are various causes of osteoarthritis, we used an animal model which was induced forcibly osteoarthritis using MIA. Therefore, we need to further confirm the efficacy of that Boswellia in various osteoarthritis models, such as aging and female. Moreover, although our data provided clues for further studies on the mechanisms of FJH-BS, additional studies on active ingredients of Boswellia and the underlying molecular mechanisms are needed to establish the effectiveness of the osteoarthritis treatment.

5. Conclusions

We demonstrated that a standardized FJH-BS ameliorated osteoarthritis symptoms, including inflammation and degradation of articular cartilage in rats with MIA-induced osteoarthritis. Furthermore, FJH-BS treatment directly suppressed cell death, inflammation, and MMP expression in primary chondrocytes under H2O2- or LPS-induced stress. Based on these findings, we suggest that supplementation with standardized FJH-BS may be useful for preventing osteoarthritis progression because of the direct effects on chondrocytes.

Conflicts of Interest

The authors declare no conflicts of interest.

References


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