Prev. Nutr. Food Sci. 2025;30(2):141-151
https://doi.org/10.3746/pnf.2025.30.2.141
ISSN 2287-8602

Pyropia yezoensis Extract Attenuates Osteoarthritis Progression *In Vitro* and *In Vivo*

Seul Ah Lee[1], Seul Bi Lee[2], Do Kyung Kim[3], Sook-Young Lee[2], and Chun Sung Kim[1]

[1]Department of Oral Biochemistry and [3]Oral Biology Research Institute, College of Dentistry, Chosun University, Gwangju 61452, Korea
[2]Marine Healthcare Research & Evaluation Center, Chosun University, Jeonnam 59146, Korea

ABSTRACT: Osteoarthritis (OA), a degenerative disease characterized by cartilage degradation and inflammation, occurs due to trauma caused by external stimuli or cartilage aging. *Pyropia yezoensis* is a red alga that belongs to the Porphyra family and is consumed as food in Asia, especially Korea, Japan, and China. *P. yezoensis* contains various bioactive substances, including carotenoids, flavonoids, and vitamins, that exert anti-inflammatory, antioxidant, and anti-photoaging effects. In the present study, the anti-osteoarthritic effects of 30% fermented alcohol extract of *P. yezoensis* (30% FEPY) on interleukin-1 beta (IL-1β)-stimulated chondrocytes and a destabilization of the medial meniscus (DMM)-induced OA rat model were investigated. The results showed that pretreatment with 30% FEPY significantly reduced the IL-1β-induced expression of inflammatory factors (e.g., inducible nitric oxide synthase and cyclooxygenase-2) and cartilage-degrading enzymes [matrix metalloproteinase (MMP) 1, MMP3, MMP13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4, and ADAMTS5], which was analyzed using Griess reaction, enzyme-linked immunosorbent assay, and Western blot analysis. The anti-osteoarthritic effects of 30% FEPY, which were mediated through mitogen-activated protein kinase and nuclear factor kappa-light-chain-enhancer of activated B cell signaling, were analyzed using Western blot analysis. In an *in vivo* study, Safranin O staining and immunohistochemistry analysis revealed that treatment with 30% FEPY significantly increased cartilage degradation and collagen type II protein expression in the DMM group. These findings collectively suggest that 30% FEPY is a promising candidate for alleviating OA progression and developing new therapeutic drugs.

Keywords: chondrocytes, destabilization of the medial meniscus, interleukin-1 beta, osteoarthritis, *Pyropia yezoensis*

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease that develops with age. In knee OA, patients often experience cartilage degradation and knee stiffness, inflammation, and pain (Chen et al., 2017). Traditionally, OA is viewed as non-inflammatory arthritis linked to weight gain or cartilage wear; however, it is currently recognized as an inflammatory joint disease (Robinson et al., 2016; Lee et al., 2023). Inflammation not only initiates OA but also accelerates its pathogenesis and influences pain development (Orlowsky and Kraus, 2015; Lee et al., 2023). Catabolic factors, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs), which are upregulated by inflammation, promote the degradation of chondrocytes in the extracellular matrix (ECM) (Maldonado and Nam, 2013). Specifically, interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are important factors that are implicated in cartilage matrix degradation, chondrocyte death, and local chronic inflammation in the joint tissues of patients with OA (Cai et al., 2015; Jenei-Lanzl et al., 2019). These catabolic factors are expressed by nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) and mitogen-activated protein kinase (MAPK) signaling systems, which are involved in the overall physiological activities of cells, including cell growth, inflammation, and differentiation (Saklatvala, 2007; Cai et al., 2023; Han et al., 2023). Therefore, inhibiting the NF-κB and MAPK signaling pathways is considered a strategy to delay OA progression by reducing the expression of catabolic factors.

Pyropia yezoensis is a red alga that is consumed as food in Asia, especially Korea, Japan, China, and Taiwan (Ha et al., 2020). In East Asia, particularly Korea, *P. yezoensis* is widely cultivated, with an annual global production of

Received 24 December 2024; Revised 22 January 2025; Accepted 25 January 2025; Published online 30 April 2025
Correspondence to Chun Sung Kim, E-mail: cskim2@chosun.ac.kr

more than one million tons (fresh weight) and a market value of more than $1.5 billion annually (Sun et al., 2015; Lee et al., 2018). *P. yezoensis* exerts various physiological effects, including anti-inflammatory, antioxidant, anti-photoaging, and tissue-healing effects (Tao et al., 2008; Ryu et al., 2014; Isaka et al., 2015). It contains 25% to 50% proteins, 20% to 40% polysaccharides, various vitamins, carotenoids, and essential minerals (Noda, 1993; Lee et al., 2018). In particular, porphyra-334 and glycoproteins, which are found in large amounts in *P. yezoensis*, are used as cosmetic or active ingredients to improve liver function and muscle atrophy (Hwang et al., 2008; Ha et al., 2020).

The anti-inflammatory effect of *P. yezoensis* is demonstrated by its ability to suppress the expression of proinflammatory cytokines, treat conditions such as atopy and septic shock, and improve liver fat. However, the effect of *P. yezoensis* on degenerative arthritis remains unclear. Therefore, the present study aimed to analyze the chondroprotective effects of 30% fermented alcohol extract of *P. yezoensis* (30% FEPY) in primary cultured chondrocytes and in an destabilization of the medial meniscus (DMM)-induced experimental model.

MATERIALS AND METHODS

Preparation of 30% FEPY

P. yezoensis was purchased from Wando (Korea). It was washed several times with tap water to remove salt and then dried. Thereafter, the dried sample was extracted with 30% fermented alcohol at 90°C for 4 h and concentrated and spray-dried. Subsequently, the extracted powder was stored at 4°C.

Total sugar, polyphenol, flavonoid, and carotenoid analysis

The contents of bioactive compounds in *P. yezoensis* were analyzed in accordance with the method of Jang et al. (2024) with some modifications. To analyze the total sugar content (TSC), *P. yezoensis* was extracted at 85°C for 25 min. Subsequently, it was filtered, added with 1 mL of 5% phenol solution and 5 mL of 95% sulfuric acid, and allowed to react in the dark for 20 min. The absorbance was measured at 490 nm using an ultraviolet-visible (UV-VIS) spectrophotometer. The TSC was calculated using a standard glucose calibration curve (5 – 500 μg/mL). To analyze the total polyphenol content (TPC), 5 mL of 10% Folin-Ciocalteu reagent was added to 1 mL of *P. yezoensis* extract and reacted for 5 min. Subsequently, the mixture was added with 4 mL of 7.5% Na_2CO_3, and color was developed in the dark for 1 h. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. The TPC was calculated using a standard calibration curve with gallic acid as the standard. To analyze the total flavonoid content (TFC), 1.5 mL of methanol, 0.1 mL of 10% aluminum nitrate [$Al(NO_3)_3 \cdot 9H_2O$], and 0.1 mL of 1 M potassium acetate (CH_3COOK) solution were sequentially added to 0.5 mL of *P. yezoensis* extract. Afterward, the mixture was added with 2.8 mL of distilled water. After thorough stirring and reaction for 40 min at room temperature, the absorbance at 415 nm was measured using a UV-VIS spectrophotometer. The TFC was calculated using a standard calibration curve for quercetin. To analyze the total carotenoid content (TCC), *P. yezoensis* was ultrasonically extracted at 60°C for 1 h using a mixed solvent of hexane : EtOH : acetone : toluene at a ratio of 10:6:7:7. The absorbance was measured at 446 nm using UV-VIS spectrophotometer. The TCC was calculated using a standard calibration curve for lutein.

High-performance liquid chromatography (HPLC) analysis

Chromatographic analyses were performed using a Nexera Series HPLC system. The UV absorbance was monitored at 450 nm. Quantification was performed by integrating peak areas at 450 nm, and separation was conducted using the YMC Carotenoid C30 (particle size, 5 μm; 250× 4.6 mm) with a temperature of 40°C and an injection volume of 10 μL. The mobile phase comprised a 6:4 mixture of acetone : distilled water (A) and a 6:4 mixture of MeOH:acetone (B), with acetone flowing at a rate of 0.5 mL/min. The gradient conditions were as follows: (mobile phase A) 0 – 0.5 min, 40%; 0.5 – 3 min, 70%; 3 – 20 min, 70%; 20 – 25 min, 90%; 25 – 42 min, 90%; 42 – 45 min, 100%; and 45 – 60 min, 100%. *P. yezoensis*, astaxanthin, lutein, and zeaxanthin (Sigma-Aldrich) were used as samples and standards at 10 and 100 ppm concentrations, respectively.

Culture and cell viability assay of primary rat chondrocytes

Primary chondrocytes were isolated from five-day-old postnatal Sprague-Dawley rats. For isolated chondrocytes, the isolated cartilage tissue was washed with phosphate-buffered saline (PBS) containing 2× antibiotics (penicillin and streptomycin) and then enzymatically treated with 0.3% collagenase overnight, in accordance with the method of previous studies with some modifications (Lee et al., 2020; Xia et al., 2023; Jang et al., 2024). After filtering the cells with a cell strainer (0.45 μm), they were plated at a density of 1×10^6 cells/mL. The cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 containing 10% fetal bovine serum and 1% antibiotic at 37°C with 5% CO_2 for three days. The isolated chondrocytes were used directly for experiments without subculture. For cell viability analysis against 30% FEPY, the primary chondrocytes were treated with 0.25 to 1 mg/mL of 30% FEPY, reacted for 24 h, and then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-

trazolium bromide (MTT) assay. The formazan produced was dissolved in dimethyl sulfoxide, and the absorbance was measured at 590 nm using a microplate reader (Epoch Biotek Instruments Inc.). All experiments were performed five times, and all animal procedures were approved by the Institutional Animal Care and Use Committee of Chosun University (CIACUC2022-A0019).

Measurement of nitrite oxide (NO) and prostaglandin E_2 (PGE_2)

The primary chondrocytes were pretreated with 30% FEPY for 1 h and stimulated with IL-1β (10 ng/mL) for 24 h. The culture medium (100 μL) was mixed with 100 μL of Griess reagent [1% (w/v) sulfanilamide and 0.1% (w/v) naphthyl ethylenediamine in 5% (v/v) phosphoric acid]. The absorbance was measured at 540 nm using a microplate reader (Epoch Biotek Instruments Inc.). Nitrite was quantified by using a standard curve for sodium nitrite. The PGE_2 concentration in the culture medium was measured using commercial enzyme-linked immunosorbent assay kits (R&D Systems) in accordance with the manufacturer's protocols. All assays were performed in triplicate.

Western blot analysis

The primary chondrocytes were pretreated with 30% FEPY for 1 h and stimulated with IL-1β (10 ng/mL) for 2 or 24 h. The total proteins were extracted using the PRO-PREP Protein Extraction Solution (iNtRON Biotechnology). Meanwhile, nuclear and cytoplasmic proteins were extracted using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) in accordance with the manufacturer's protocols. The protein concentration in each lysate was quantified using the Bicinchoninic Acid Protein Assay Kit (Pierce). The proteins (10 μg each) were separated by size through 10% to 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were reacted with 5% bovine serum albumin for 20 min and then incubated overnight with specific primary antibodies at 4°C to prevent nonspecific antibody reactions. The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. The protein bands were detected using an enhanced chemiluminescence kit (Millipore) and the MicroChemi 4.2 Imaging System (DNR Bioimaging Systems). The following specific primary antibodies were used: inducible nitric oxide synthase (iNOS; Abcam, 1:2,000), cyclooxygenase-2 [COX-2; Cell Signaling Technology (CST), 1:1,000], MMP1 (Abcam, 1:2,000), MMP3 (CST, 1:1,000), MMP13 (Abcam, 1:1,000), ADAMTS4 (Abcam, 1:1,000), ADAMTS5 (Abcam, 1:1,000), collagen type II (Invitrogen, 1:1,000), aggrecan (Invitrogen, 1:1,000), extracellular signal-regulated kinase (ERK) (CST, 1:2,000), c-Jun N-terminal kinase (JNK) (CST, 1:2,000), P38 (CST, 1:2,000), p-ERK (CST, 1:2,000), p-JNK (CST, 1:2,000), p-P38 (CST, 1:2,000), phosphorylated inhibitor of kappa B α (p-IκB-α) (Invitrogen, 1:1,000), IκB-α (Invitrogen, 1:1,000), and p65 (Invitrogen, 1:1,000).

Alcian blue stain

The primary chondrocytes were plated at a density of 1×10^6 cells/mL in a six-well cell culture plate. After 15 h, they were treated with 30% FEPY and IL-1β (10 ng/mL) at 1-h intervals. After 72 h, the cells were fixed with 95% ethanol for 30 min and stained with 1% Alcian blue in 0.1 N HCL for 18 h, as described in previous studies (You et al., 2017; Lee et al., 2020; Jang et al., 2024). Subsequently, the cells were photographed after removing unstained cells with 1×PBS.

Experimental animals and *ex vivo* and *in vivo* study design

Ex vivo and *in vivo* studies were performed in accordance with the methods of previous studies with slight modifications (You et al., 2017; Lee et al., 2020; Jang et al., 2024). Sprague-Dawley rats (5-days or 6-weeks old) were purchased from Damool Science. For *ex vivo* study, the knees of 5-day-old postnatal rats were removed, and only the skin was removed and cultured in a chondrocyte growth medium at 37°C with 5% CO_2 for 3 days. The culture medium was replaced daily. After 3 days of culture, the cells were pretreated with 30% FEPY for 1 h. Subsequently, they were treated with IL-1β (10 ng/mL) and further reacted for 48 h. After completion of the reaction, the tissues were subjected to histological analysis. For the *in vivo* study, the experimental animals were housed in constant temperature (21°C±1°C) and humidity (55%±5%) conditions with a 12-h light/dark cycle and allowed free access to water and feed. All animal handling procedures were planned following the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Chosun University (CIACUC2022-A0019). The rats (n=24) were randomly divided into the following six groups: group 1 (normal), group 2 (sham, 0.9% normal saline), group 3 (DMM, 0.9% normal saline), and groups 4 to 6 (DMM, receiving 30% FEPY at 50, 100, and 200 mg/kg body weight, respectively). For DMM surgery, the experimental animals were anesthetized with isoflurane, and the medial menisci of both knees were damaged. In the sham group, the medial meniscus was visualized but was not damaged. Starting the day after DMM surgery, 0.9% normal saline or 30% FEPY was orally administered for eight weeks. All experimental animals were sacrificed to obtain knee tissues on the day after the last administration.

Histology analysis and stain

The collected knee tissues were washed with 1×PBS for two days and then fixed with 10% neutral buffered formalin for two days at 4°C. The tissues were decalcified for 10 days in 0.5 M ethylenediaminetetraacetic acid for calcium removal and then dehydrated through a series of ethanol dilutions (70%–100%). For paraffin block fabrication, the samples were infiltrated with xylene for four hours and then embedded in paraffin blocks. Next, the tissues were cut into 10-μm-thick sections, stained with Safranin O/Fast Green, and photographed under a microscope (Leica Camera AG).

Statistical analyses

All data were obtained from at least three independent experiments. The results are presented as the average mean±standard deviation. One-way analysis of variance followed by Tukey's test was used for multiple group comparisons using GraphPad Prism 5.0 (GraphPad Software Inc.). Statistical significance was considered at $P<0.05$.

Ethical approval

The animal study protocols were approved by the Institutional Animal Care and Use Committee of Chosun University (CIACUC2022-A0019 and CIACUC2024-A0018).

RESULTS

Analysis of the phytochemical contents of *P. yezoensis*

The TSC, TPC, TFC, and TCC of *P. yezoensis* are shown in Table 1. The pretreatment method for the analysis of phytochemicals contained in *P. yezoensis* is described in the Materials and Methods section. The analysis results showed that the TSC was 40.68±0.89/100 g of glucose, the TPC was 319.4±0.12/100 g of gallic acid, the TFC was 62.6±0.31/100 g of quercetin, and the TCC was 0.68±0.06 g/100 g of lutein. Carotenoids derived from seaweed are known for their active components, which exhibit various functionalities. Therefore, we analyzed the carotenoid components in *P. yezoensis* using HPLC. The results showed that *P. yezoensis* contains large amounts of organic pigments, including astaxanthin, lutein, and zeaxanthin (Fig. 1).

Table 1. Content of bioactive substances in *Pyropia yezoensis*

Phytochemical (g/100 g dry weight)			
TSC	TPC	TFC	TCC
40.68±0.89	319.4±0.12	62.6±0.31	0.68±0.06

Values are presented as mean±SD.
TSC, total sugar content; TPC, total polyphenol content; TFC, total flavonoid content; TCC, total carotenoid content.

Effects of 30% FEPY on the viability of primary chondrocytes

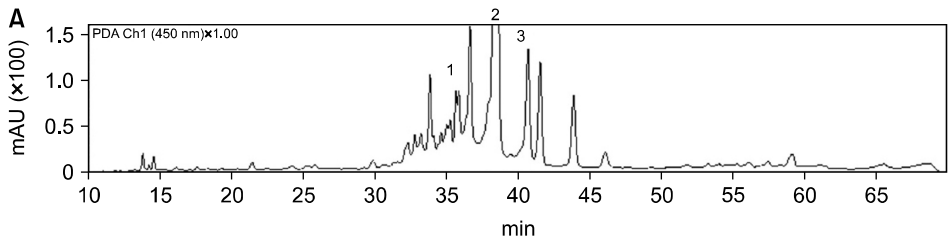
The chondrocytes were treated with 0 to 2 mg/mL of 30% FEPY for 24 h to evaluate its cytotoxicity. No cytotoxicity was observed with treatment of up to 2 mg/mL of 30% FEPY (Fig. 2).

Effects of 30% FEPY on the IL-1β-induced expression of inflammatory factors in primary chondrocytes

Treatment with IL-1β only clearly increased the expression of nitrite (40.95±3.06 μM) and PGE_2 (1,488.24±29.80 pg/mL) compared with the control group (nitrite, 0.90±0.83 μM; PGE_2, 743.65±24.83 pg/mL) as shown in Fig. 3A and 3B. However, pretreatment with 30% FEPY inhibited the induction of nitrite and PGE_2 in a dose-dependent manner. Pretreatment with 1 mg/mL of 30% FEPY resulted in nitrite and PGE_2 inhibition rates of 34% (27.43±1.20 μM) and 31% (1,033.41±62.08 pg/mL), respectively (Fig. 3A and 3B). Moreover, similar to the results of nitrite and PGE_2, pretreatment with 30% FEPY significantly suppressed IL-1β-induced iNOS and COX-2 protein expression (Fig. 3C and 3D).

Effects of 30% FEPY on IL-1β-induced catabolic factor expression in primary chondrocytes

MMPs and ADAMTSs are typical enzymes responsible for degrading the cartilage matrix. Therefore, inhibiting



B

Compound #	R.T. (min)	Predicted M.W. ($g \cdot mol^{-1}$)	Predicted M.F.	Candidate compound
1	35.52	596.841	$C_{40}H_{52}O_4$	Astaxanthin
2	38.42	568.871	$C_{40}H_{56}O_2$	Lutein
3	40.67	568.88	$C_{40}H_{56}O_2$	Zeaxanthin

Fig. 1. Identification of *Pyropia yezoensis*. (A) High-performance liquid chromatography (HPLC) chromatogram of *P. yezoensis*. (B) Accurate mass measurements of the three compounds in *P. yezoensis* using HPLC-electrospray ionization time-of-flight mass spectrometry. R.T., retention time; M.W., molecular weight; M.F., molecular formula.

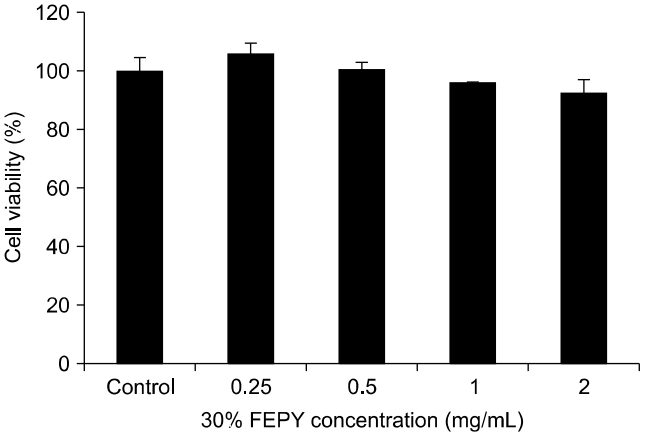


Fig. 2. Effects of 30% fermented alcohol extract of *Pyropia yezoensis* (FEPY) on the viability of chondrocytes. The cells were treated with 30% FEPY (0.25, 0.5, 1.0, and 2.0 mg/mL) for 24 h, and their viability was determined by MTT assay. The cells incubated without 30% FEPY were used as controls and considered 100% viable. Data are represented as the mean±SD of three independent experiments.

their expression is closely associated with the delayed onset of OA (Maldonado and Nam, 2013). In the group treated with IL-1β, the protein expression of MMP1, MMP3, MMP13, ADAMTS4, and ADAMTS5 increased; however, the expression was significantly decreased in the group pretreated with 30% FEPY (Fig. 4).

Effects of 30% FEPY on IL-1β-induced degradation of anabolic factors in primary chondrocytes

Collagen type II and aggrecan are essential components of the ECM and contribute significantly to cartilage structure and function. Matrix-degrading enzymes, including MMPs, cause ECM degradation, which is one of the leading causes of OA (Maldonado and Nam, 2013). IL-1β significantly decreased the protein expression of collagen and aggrecan, but the decreased expression was reversed by pretreatment with 30% FEPY (Fig. 5A and 5B). The *in vitro* effects of 30% FEPY were verified through organ culture. The knee tissues of rats were excised, treated with 30% FEPY and IL-1β (10 ng/mL), and cultured for 48 h. Afterward, they were subjected to histological analysis (Fig. 5C). The articular cartilage was stained using Safranin O and Alcian blue reagents, which stain the cartilage matrix. The results showed that the control group had a dark stain on the cartilage surface, whereas the IL-1β alone group had a fainter stain and color (Fig. 5D and 5E). On the other hand, in the group pretreated with 30% FEPY, the stain was restored to a darker color

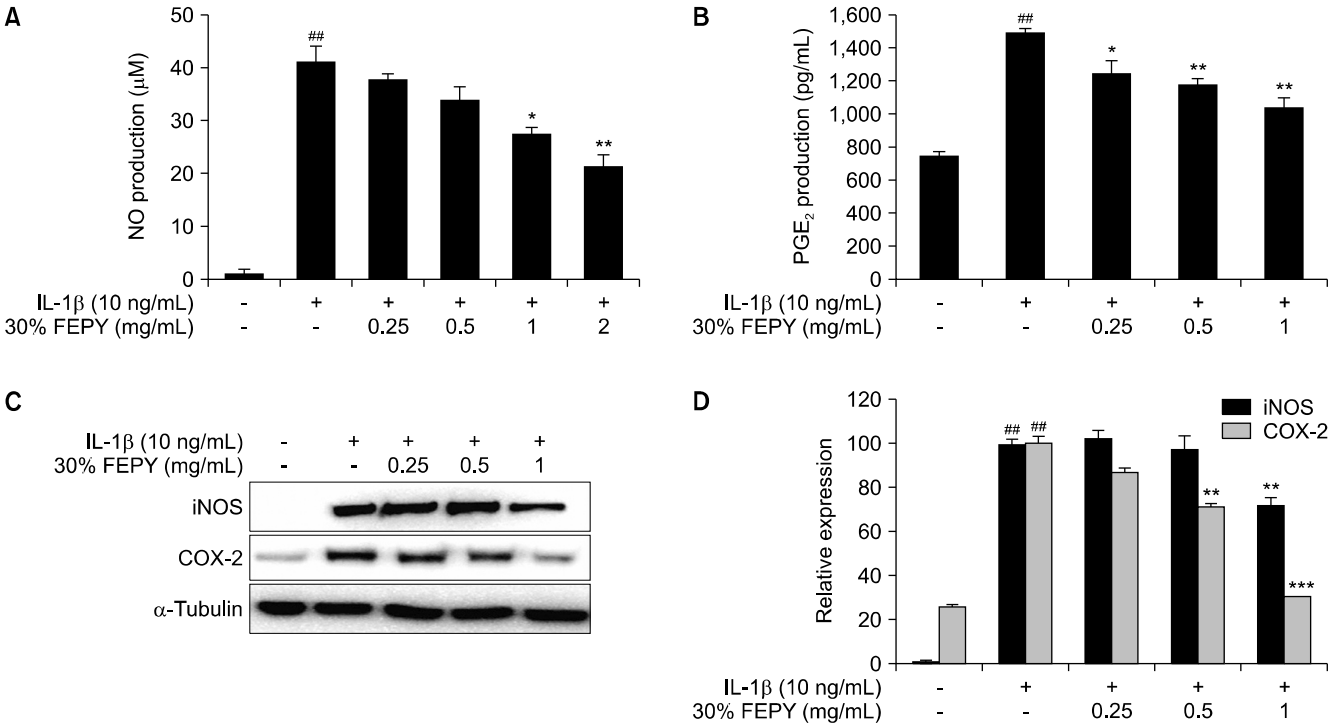


Fig. 3. Effects of 30% FEPY on IL-1β-induced expression of nitrite, PGE_2, iNOS, and COX-2 in primary chondrocytes. The cells were pretreated with 30% FEPY (0.25, 0.5, and 1 mg/mL) for 1 h and treated with IL-1β (10 ng/mL) for 24 h. (A) Nitrite that accumulated in the cultured medium was analyzed by measuring the absorbance at 540 nm after reacting with the Griess reagent. (B) PGE_2 that accumulated in the cultured medium was analyzed using ELISA kits. (C) The expression of iNOS and COX-2 proteins was determined using Western blot analysis. (D) The quantitative data of (C) were analyzed using ImageJ software, with α-tubulin as an internal control. The results are represented as the mean±SD of three independent experiments. $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with IL-1β-treated group. FEPY, fermented alcohol extract of *Pyropia yezoensis*; IL-1β, interleukin-1β; NO, nitrite oxide; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; ELISA, enzyme-linked immunosorbent assay.

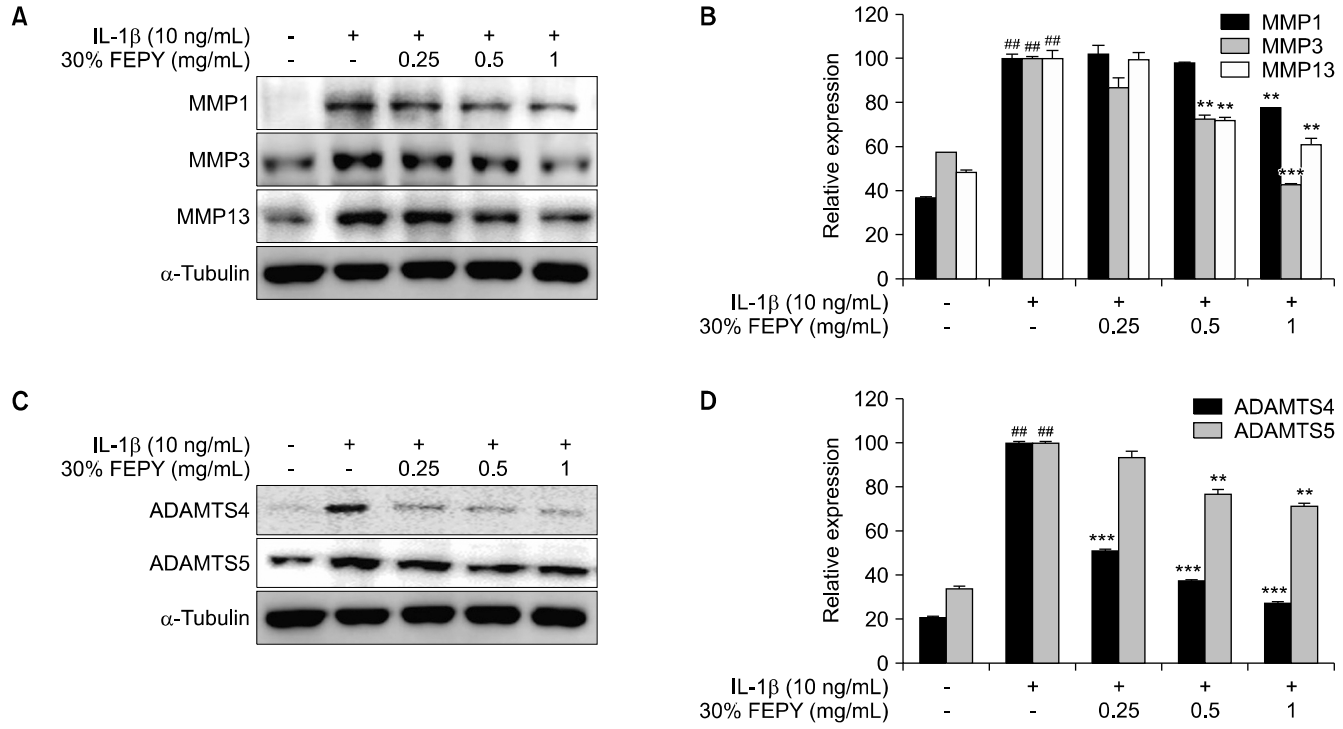


Fig. 4. Effects of 30% FEPY on IL-1β-induced catabolic factors in primary chondrocytes. The cells were pretreated with 30% FEPY (0.25, 0.5, and 1.0 mg/mL) for 1 h and treated with IL-1β (10 ng/mL) for 24 h. (A) The protein levels of MMP1, 3, and 13 were determined using Western blot. (B) The quantitative data of (A) were analyzed using ImageJ software. (C) The protein levels of ADAMTS4 and ADAMTS5 were determined using Western blot analysis. (D) The quantitative data of (C) were analyzed using ImageJ software, with α-tubulin as an internal control. The results are represented as the mean±SD of three independent experiments. $^{\#\#}P<0.01$ compared with the control group; $^{**}P<0.01$ and $^{***}P<0.001$ compared with the IL-1β-treated group. FEPY, fermented alcohol extract of *Pyropia yezoensis*; IL-1β, interleukin-1β; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif.

because of proteoglycan protecting the cartilage matrix (Fig. 5D and 5E).

Effects of 30% FEPY on the IL-1β-induced phosphorylation of MAPKs and NF-κB signaling activation in primary chondrocytes

The phosphorylation of MAPKs (ERK, JNK, and P38) and nuclear translocation of NF-κB p65 were analyzed using Western blot analysis to determine the mechanism underlying the chondroprotective effects of 30% FEPY. IL-1β induced the phosphorylation of ERK, JNK, and P38. However, pretreatment with 30% FEPY significantly inhibited their phosphorylation without affecting the total protein expression levels (Fig. 6A and 6B). In addition, IL-1β induced the phosphorylation and degradation of IκB-α, thereby enhancing the nuclear translocation of the NF-κB subunit p65. Nevertheless, this effect was reversed by pretreatment with 30% FEPY (Fig. 6C and 6D). These findings suggest that the anti-osteoarthritic effects of 30% FEPY may be mediated through MAPKs and NF-κB signaling.

Effects of 30% FEPY administration on DMM-induced OA rat model

To evaluate the anti-osteoarthritic effects of 30% FEPY *in vivo*, an experimental animal OA model was constructed using DMM surgery, which mimics the development of OA in humans. The effects of 30% FEPY on the development of DMM-induced OA were assessed by evaluating collagen type II expression and using Safranin O/Fast Green staining based on the Osteoarthritis Research Society International (OARSI) score (Gerwin et al., 2010). The content of proteoglycans was depleted in the group that underwent DMM surgery, but it was recovered in the group that received daily oral administration of 30% FEPY for eight weeks (Fig. 7A). The OARSI score of the DMM surgery group was 12, whereas the OARSI scores of the groups receiving 30% FEPY at 50, 100, and 200 mg/kg were 7, 6, and 5, respectively (Fig. 7B). Moreover, the oral administration of 30% FEPY significantly suppressed the degradation of collagen type II by DMM. These results suggest that 30% FEPY can alleviate OA development *in vivo*.

DISCUSSION

Cartilage comprises water, chondrocytes, and the ECM. As cartilage functions under avascular, aneural, and anaerobic conditions, chondrocytes are the only cells responsible for cartilage health (Sophia et al., 2009). The ECM, which comprises fibrillar and non-fibrillar collagen and

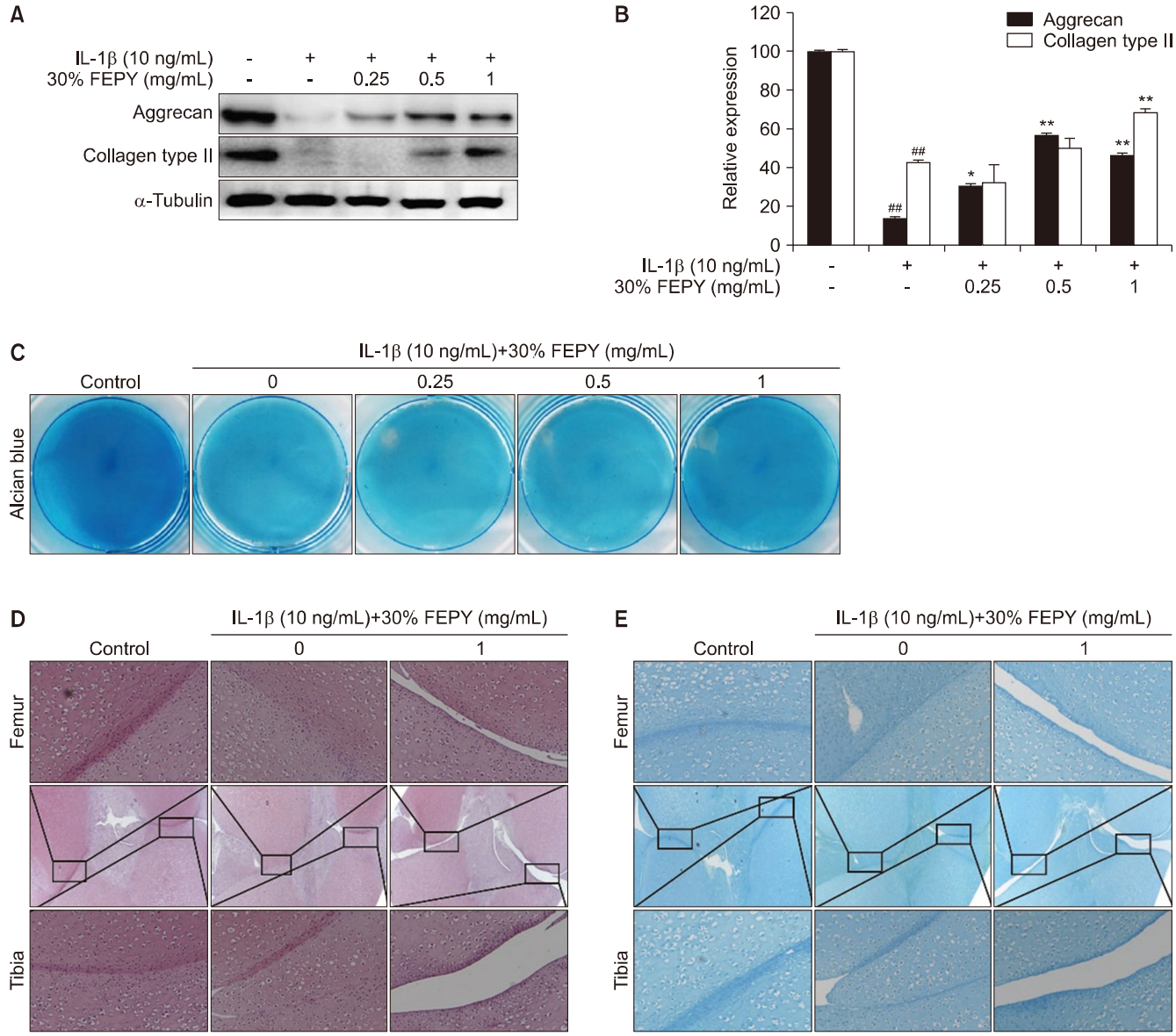


Fig. 5. Effects of 30% FEPY on IL-1β-induced anabolic factors in primary chondrocytes and organ culture. The cells were pretreated with 30% FEPY (0.25, 0.5, and 1.0 mg/mL) for 1 h and treated with IL-1β (10 ng/mL) for 24 h. (A) The protein levels of collagen type II and aggrecan were determined using Western blot analysis, with α-tubulin as an internal control. (B) The quantitative data of (A) were analyzed using ImageJ software. (C) The proteoglycan concentration of chondrocytes was analyzed using Alcian blue staining. Five-day-old postnatal Sprague-Dawley rats were cultured for three days. Then, they were pretreated with 30% FEPY for 1 h and treated with IL-1β (10 ng/mL) for 48 h. (D, E) Histological analysis was performed using Safranin O and Alcian blue staining to analyze the proteoglycan content of articular cartilage. $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$ and $^{**}P<0.01$ compared with the IL-1β-treated group. FEPY, fermented alcohol extract of *Pyropia yezoensis*; IL-1β, interleukin-1β.

negatively charged proteoglycans synthesized from chondrocytes, possesses viscoelastic properties, enabling it to recover from mechanical deformation (Mow et al., 1994; Sanchez-Adams et al., 2014; Al-Maslamani et al., 2022). Therefore, healthy chondrocytes and their dense ECM are essential for maintaining healthy cartilage.

OA is induced by cartilage destruction caused by pro-inflammatory cytokines, including IL-1β and TNF-α (Cai et al., 2015; Jenei-Lanzl et al., 2019). Elevated levels of IL-1β have been reported in the synovial fluid, subchondral bone, and cartilage tissue of patients with OA (Wang et al., 2017; Mai et al., 2023). In the pathogenesis of OA, IL-1β not only triggers the initiation and progression of ECM degradation but also induces the expression of other catabolic factors, including iNOS, NO, COX-2, PGE_2, TNF-α, MMPs, and ADAMTSs (Daheshia and Yao, 2008; He et al., 2020). Among the catabolic factors of the cartilage ECM, the MMP family is a significant factor involved in cartilage destruction as it degrades collagen and proteoglycans, which are the main components of the ECM (Lee et al., 2023). MMP1 and MMP13 have the unique ability to disrupt the triple-helical structure of fibrillar collagen (Visse and Nagase, 2003). MMP3 is upregulated in the early stages of OA and is respon-

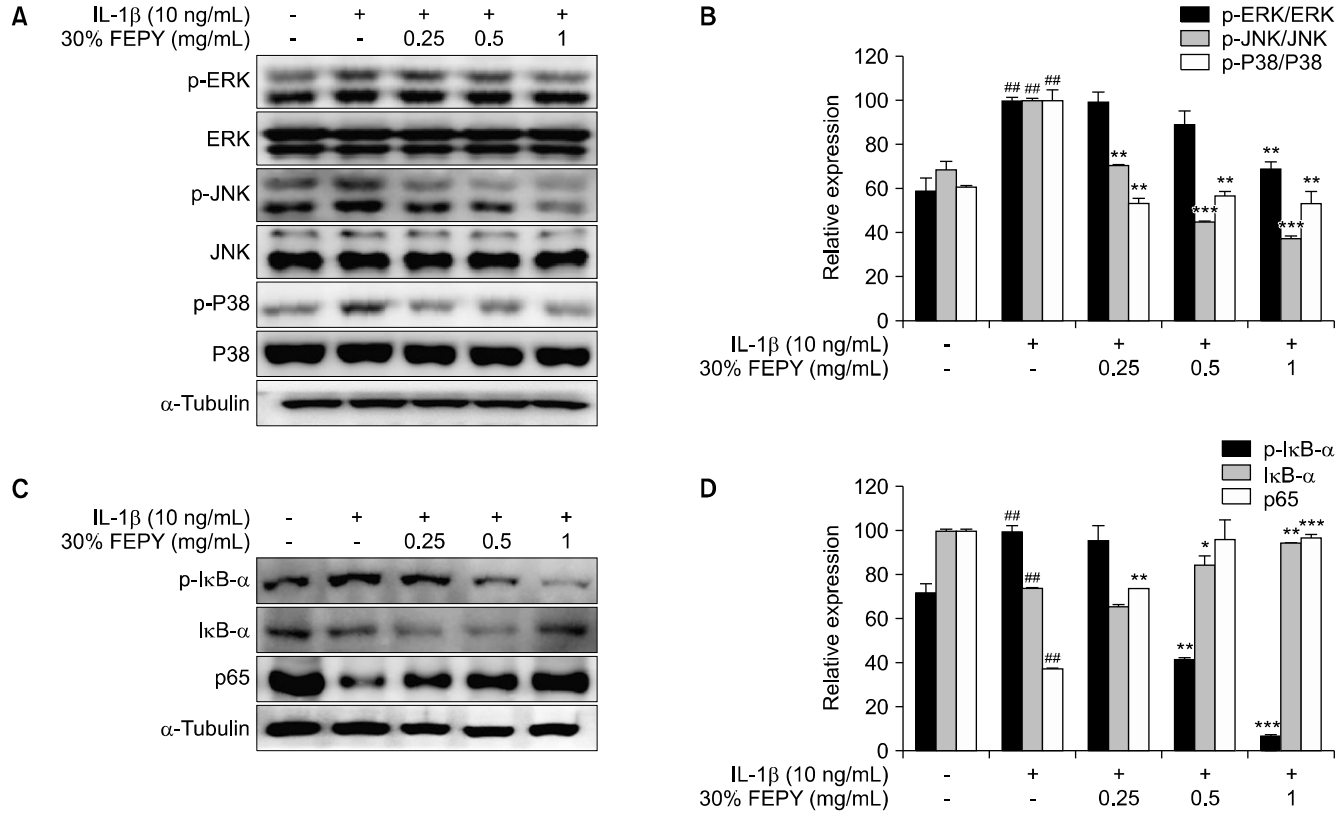


Fig. 6. Effects of 30% FEPY on the IL-1β-induced phosphorylation of MAPKs and activation of NF-κB in primary chondrocytes. The cells were pretreated with 30% FEPY (0.25, 0.5, and 1.0 mg/mL) for 1 h and treated with IL-1β (10 ng/mL) for 1 h. (A) The protein levels of phosphorylated MAPKs (ERK, JNK, and p38) were determined using Western blot analysis. (C) The protein levels of IκB-α and NF-κB p65 were determined using Western blot analysis, with α-tubulin as an internal control. (B, D) The quantitative data of (A) and (C) were analyzed using ImageJ software. $^{\#\#}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with IL-1β-treated group. FEPY, fermented alcohol extract of *Pyropia yezoensis*; IL-1β, interleukin-1β; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cell; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IκB, NF-kappa-B inhibitor alpha.

sible for cleaving collagen type II and aggrecan (Visse and Nagase, 2003; Lee and Kang, 2023). The members of the ADAMTS family, including ADAMTS4 and ADAMTS5, are proteolytic enzymes that accelerate OA by degrading collagen type II and aggrecan (Kapoor et al., 2011; He et al., 2020). Therefore, inhibiting the expression of ECM catabolic factors by suppressing inflammation is one strategy for mediating OA progression. Our results showed that 30% FEPY inhibited the IL-1β-induced elevated expression of iNOS, NO, COX-2, PGE_2, MMP1, MMP3, MMP13, ADAMTS4, and ADAMTS5 and decreased expression of collagen type II and aggrecan. These results are consistent with those of previous studies that have analyzed the ameliorating effects of OA. Bone marrow mesenchymal stem cell-derived exosomes have been reported to promote cartilage repair by inhibiting the IL-1β-induced elevated expression of MMP13 and ADAMTS5 and promoting the synthesis of collagen type II and aggrecan (He et al., 2020). *Schisandra chinensis* extract was reported to exhibit an alleviating effect on OA via inhibiting the expression of MMP3 and COX-2 induced by IL-1β and showed better effects than the active ingredient schisandrol A (Han et al., 2023).

MAPK and NF-κB signaling are essential in various cellular responses, including stress, inflammation, and OA (Gottschalk et al., 2016; Ahmed et al., 2018; Han et al., 2023). IL-1β mainly activates the MAPK and NF-κB signaling pathways and affects MMP, COX-2, and iNOS expression (Daheshia and Yao, 2008; Chow and Chin, 2020). MAPKs, including ERK, JNK, and P38, are activated via phosphorylation to regulate the secretion of inflammatory cytokines, and the MAPK signaling pathway stimulates the activation of the NF-κB signaling pathway. NF-κB signaling is activated through the translocation of the p65 subunit to the nucleus upon degradation of NF-κB-bound IκB protein inhibitor (Chow and Chin, 2020; Park et al., 2024). Our results showed that 30% FEPY inhibited the IL-1β-induced phosphorylation of MAPKs (ERK1/2, JNK, and P38) and translocation of the NF-κB p65 subunit to the nucleus, suggesting that the chondroprotective effects of 30% FEPY are at least partially activated via the MAPK and NF-κB signaling pathway.

In an *in vivo* study, we analyzed the therapeutic potential of 30% FEPY on OA by using a DMM-induced degenerative arthritis animal model. DMM surgery, which induces meniscal damage and subsequent degeneration,

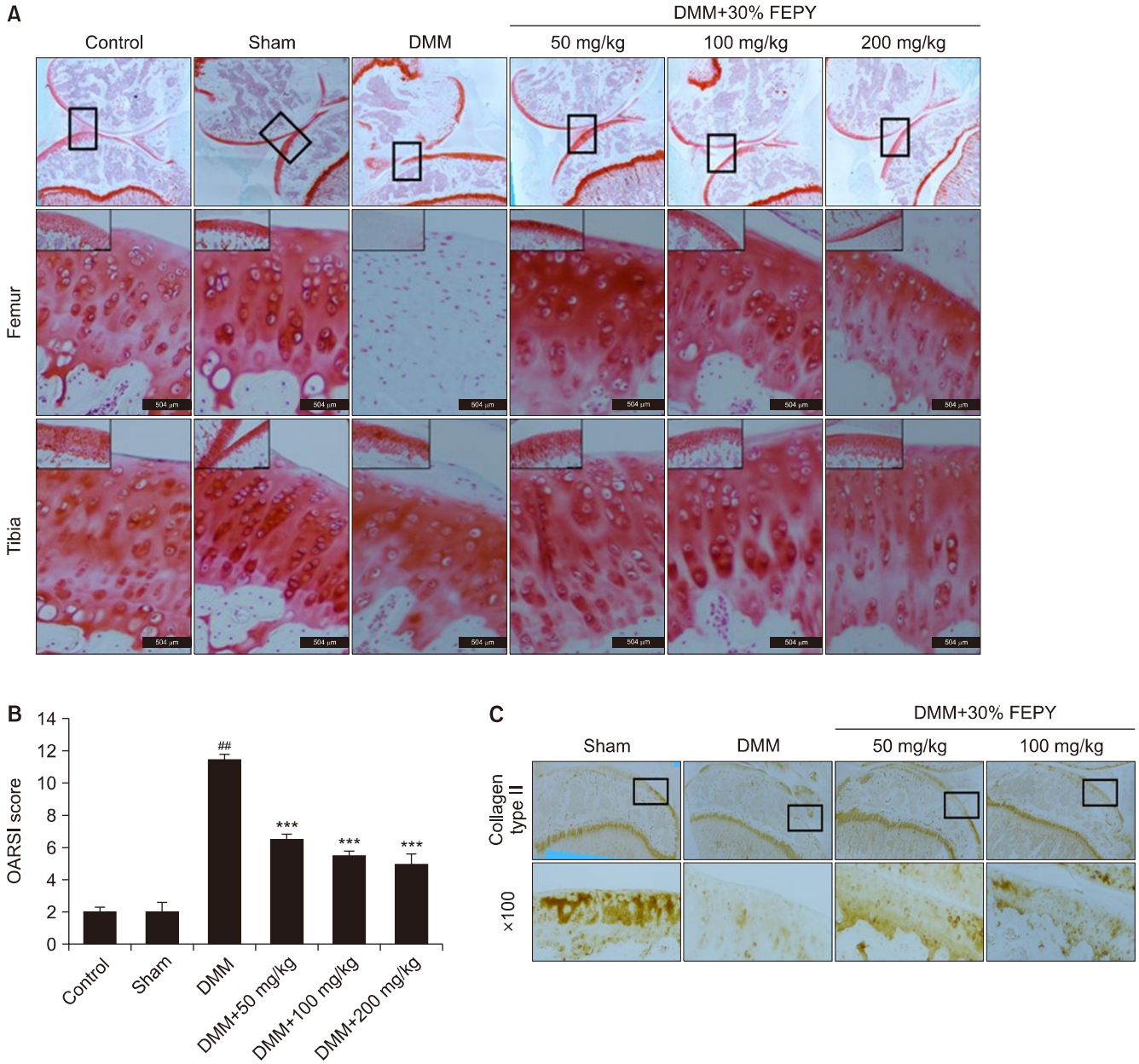


Fig. 7. Protective effects of 30% FEPY against cartilage degradation in DMM-induced rats. (A) Histopathological features of knee joint tissues in DMM-induced rats. (B) OARSI score using blind testing. (C) Immunohistochemical staining of collagen type II protein in cartilage tissue. $^{\#\#}P<0.01$ compared with the control group; $^{***}P<0.001$ compared with the DMM surgery group. FEPY, fermented alcohol extract of *Pyropia yezoensis*; DMM, destabilization of the medial meniscus; OARSI, Osteoarthritis Research Society International.

closely mimics the progression of degenerative arthritis in humans because of aging. It is a surgical technique that is primarily utilized in *in vivo* research for joint improvement (Bove et al., 2006; Gregory et al., 2012). The daily oral administration of 30% FEPY for eight weeks in the DMM surgery group significantly decreased proteoglycan loss. In addition, immunohistochemistry analysis revealed that the anabolic marker, collagen type II, was significantly downregulated in the DMM surgery group. By contrast, the expression of collagen type II was significantly increased in the group pretreated with 30% FEPY. These results suggest that 30% FEPY may help treat OA.

Although this study demonstrated that the excellent chondroprotective effects of 30% FEPY could delay OA progression, it has some limitations. While the total content of the bioactive components of *P. yezoensis* was analyzed, we did not come close to identifying the active ingredients associated with the chondroprotective effects of 30% FEPY. Thus, further studies are needed to identify the active ingredients in order to elucidate the exact mechanism of action of the extract. Another limitation is that only one type of animal model was used to evaluate the efficacy of joint improvement. Although OA induced by DMM surgery closely mimics OA in humans, it cannot represent the development of OA. In most cases, OA

is caused by natural aging. However, the recent increase in leisure exercise has led to an increased incidence of post-traumatic OA among younger generations. Therefore, if the cartilage protection and joint improvement effects of 30% FEPY are proven through efficacy analysis not only in medial meniscotibial ligament (DMM surgery) damage but also in OA induced by anterior cruciate ligament transection or monosodium iodoacetate injection, this extract may become a material worth developing not only as a health functional food but also as a medicine.

In conclusion, pretreatment with 30% FEPY effectively inhibited the IL-1β-induced elevated expression of inflammatory factors (NO, iNOS, PGE_2, and COX-2) and cartilage-degrading enzymes (MMP1, MMP3, MMP13, ADAMTS4, and ADAMTS5). Moreover, 30% FEPY inhibited the degradation of collagen type II and aggrecan induced by IL-1β. In an *in vivo* study, the administration of 30% FEPY to a DMM-induced OA rat model for eight weeks prevented the degradation of collagen type II in the cartilage matrix. These findings suggest that 30% FEPY exerts anti-osteoarthritic effects by modulating MAPK and NF-κB signaling in IL-1β-induced chondrocytes and DMM-induced OA rat model.

FUNDING

This research was supported by the Korea Institute of Marine Science and Technology Promotion (KIMST), funded by the Ministry of Oceans and Fisheries, Republic of Korea (20210656).

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: SAL, CSK. Methodology: SAL, SBL. Validation: DKK. Investigation: SAL, SBL. Data curation: DKK, SYL. Writing the article: SAL. Critical revision of the article: SAL, CSK. Supervision: DKK, SYL. Project administration: CSK. Obtained funding: CSK. All authors have read and agreed to the published version of the manuscript.

REFERENCES

Ahmed AS, Gedin P, Hugo A, Bakalkin G, Kanar A, Hart DA, et al. Activation of NF-κB in Synovium versus cartilage from patients with advanced knee osteoarthritis: A potential contributor to inflammatory aspects of disease progression. J Immunol. 2018. 201:1918-1927. https://doi.org/10.4049/jimmunol.1800486

Al-Maslamani NA, Oldershaw R, Tew S, Curran J, D'Hooghe P, Yamamoto K, et al. Chondrocyte de-differentiation: Biophysical cues to nuclear alterations. Cells. 2022. 11:4011. https://doi.org/10.3390/cells11244011

Bove SE, Laemont KD, Brooker RM, Osborn MN, Sanchez BM, Guzman RE, et al. Surgically induced osteoarthritis in the rat results in the development of both osteoarthritis-like joint pain and secondary hyperalgesia. Osteoarthritis Cartilage. 2006. 14:1041-1048. https://doi.org/10.1016/j.joca.2006.05.001

Cai H, Sun HJ, Wang YH, Zhang Z. Relationships of common polymorphisms in *IL-6*, *IL-1A*, and *IL-1B* genes with susceptibility to osteoarthritis: a meta-analysis. Clin Rheumatol. 2015. 34:1443-1453. https://doi.org/10.1007/s10067-014-2708-x

Cai T, Ye H, Jiang H, Lin C, Lou C, Wang W, et al. Stevioside targets the NF-κB and MAPK pathways for inhibiting inflammation and apoptosis of chondrocytes and ameliorates osteoarthritis *in vivo*. Int Immunopharmacol. 2023. 115:109683. https://doi.org/10.1016/j.intimp.2023.109683

Chen D, Shen J, Zhao W, Wang T, Han L, Hamilton JL, et al. Osteoarthritis: toward a comprehensive understanding of pathological mechanism. Bone Res. 2017. 5:16044. https://doi.org/10.1038/boneres.2016.44

Chow YY, Chin KY. The role of inflammation in the pathogenesis of osteoarthritis. Mediators Inflamm. 2020. 2020:8293921. https://doi.org/10.1155/2020/8293921

Daheshia M, Yao JQ. The interleukin 1β pathway in the pathogenesis of osteoarthritis. J Rheumatol. 2008. 35:2306-2312. https://doi.org/10.3899/jrheum.080346

Gerwin N, Bendele AM, Glasson S, Carlson CS. The OARSI histopathology initiative－recommendations for histological assessments of osteoarthritis in the rat. Osteoarthritis Cartilage. 2010. 18 Suppl 3:S24-S34. https://doi.org/10.1016/j.joca.2010.05.030

Gottschalk RA, Martins AJ, Angermann BR, Dutta B, Ng CE, Uderhardt S, et al. Distinct NF-κB and MAPK activation thresholds uncouple steady-state microbe sensing from anti-pathogen inflammatory responses. Cell Syst. 2016. 2:378-390. https://doi.org/10.1016/j.cels.2016.04.016

Gregory MH, Capito N, Kuroki K, Stoker AM, Cook JL, Sherman SL. A review of translational animal models for knee osteoarthritis. Arthritis. 2012. 2012:764621. https://doi.org/10.1155/2012/764621

Ha Y, Lee WH, Jeong J, Park M, Ko JY, Kwon OW, et al. *Pyropia yezoensis* extract suppresses IFN-gamma- and TNF-alpha-induced proinflammatory chemokine production in HaCaT cells via the down-regulation of NF-κB. Nutrients. 2020. 12:1238. https://doi.org/10.3390/nu12051238

Han SJ, Lee H, Nam J, Pan CH, Jeon J, Yang S. *Schisandra* extract ameliorates arthritis pathogenesis by suppressing the NF-κB and MAPK signalling pathways. J Cell Mol Med. 2023. 27:2071-2081. https://doi.org/10.1111/jcmm.17814

He L, He T, Xing J, Zhou Q, Fan L, Liu C, et al. Bone marrow mesenchymal stem cell-derived exosomes protect cartilage damage and relieve knee osteoarthritis pain in a rat model of osteoarthritis. Stem Cell Res Ther. 2020. 11:276. https://doi.org/10.1186/s13287-020-01781-w

Hwang HJ, Kwon MJ, Kim IH, Nam TJ. Chemoprotective effects of a protein from the red algae *Porphyra yezoensis* on acetaminophen-induced liver injury in rats. Phytother Res. 2008. 22:1149-1153. https://doi.org/10.1002/ptr.2368

Isaka S, Cho K, Nakazono S, Abu R, Ueno M, Kim D, et al. Antioxidant and anti-inflammatory activities of porphyran isolated from discolored nori (*Porphyra yezoensis*). Int J Biol Macromol. 2015. 74:68-75. https://doi.org/10.1016/j.ijbiomac.2014.11.

043
Jang JY, Lee SA, Kim DK, Lee SY, Kim CS. Chondroprotective effect of *Campylaephora hypnaeoides* extract in primary chondrocytes and rat OA model. Int J Mol Sci. 2024. 25:13391. https://doi.org/10.3390/ijms252413391
Jenei-Lanzl Z, Meurer A, Zaucke F. Interleukin-1β signaling in osteoarthritis—chondrocytes in focus. Cell Signal. 2019. 53: 212-223. https://doi.org/10.1016/j.cellsig.2018.10.005
Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol. 2011. 7:33-42. https://doi.org/10.1038/nrrheum.2010.196
Lee MK, Choi JW, Choi YH, Nam TJ. *Pyropia yezoensis* protein prevents dexamethasone-induced myotube atrophy in C2C12 myotubes. Mar Drugs. 2018. 16:497. https://doi.org/10.3390/md16120497
Lee SA, Park BR, Moon SM, Hong JH, Kim DK, Kim CS. Chondroprotective effect of cynaroside in IL-1β-induced primary rat chondrocytes and organ explants via NF-κB and MAPK signaling inhibition. Oxid Med Cell Longev. 2020. 2020:9358080. https://doi.org/10.1155/2020/9358080
Lee SG, Kang H. Protective effects of a mixed medicinal herb extract (NUC1) on collagenase-induced osteoarthritis in rabbits. J Microbiol Biotechnol. 2023. 33:1484-1494. https://doi.org/10.4014/jmb.2303.03044
Lee YM, Kim M, Yuk HJ, Kim SH, Kim DS. *Siraitia grosvenorii* residual extract inhibits inflammation in RAW264.7 macrophages and attenuates osteoarthritis progression in a rat model. Nutrients. 2023. 15:1417. https://doi.org/10.3390/nu15061417
Mai NTT, Hang NT, Hanh DH, Le HY, Hinh NV, Ky ND, et al. Leptin and interleukin-1β levels associated with osteoarthritis in Vietnamese patients: a cross-sectional analysis. Braz J Med Biol Res. 2023. 56:e12746. https://doi.org/10.1590/1414-431X2023e12746
Maldonado M, Nam J. The role of changes in extracellular matrix of cartilage in the presence of inflammation on the pathology of osteoarthritis. Biomed Res Int. 2013. 2013:284873. https://doi.org/10.1155/2013/284873
Mow VC, Bachrach NM, Setton LA, Guilak F. Stress, strain, pressure and flow fields in articular cartilage and chondrocytes. In: Mow VC, Tran-Son-Tay R, Guilak F, Hochmuth RM, editors. Cell Mechanics and Cellular Engineering. Springer New York. 1994. p 345-379.
Noda H. Health benefits and nutritional properties of nori. J Appl Phycol. 1993. 5:255-258. https://doi.org/10.1007/BF00004027
Orlowsky EW, Kraus VB. The role of innate immunity in osteoarthritis: When our first line of defense goes on the offensive. J Rheumatol. 2015. 42:363-371. https://doi.org/10.3899/jrheum.140382
Park DR, Choi BR, Yeo C, Yoon JE, Hong EY, Baek SH, et al. Mume Fructus reduces interleukin-1 beta-induced cartilage degradation via MAPK downregulation in rat articular chondrocytes. PLoS One. 2024. 19:e0302906. https://doi.org/10.1371/journal.pone.0302906
Robinson WH, Lepus CM, Wang Q, Raghu H, Mao R, Lindstrom TM, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. Nat Rev Rheumatol. 2016. 12:580-592. https://doi.org/10.1038/nrrheum.2016.136
Ryu J, Park SJ, Kim IH, Choi YH, Nam TJ. Protective effect of porphyra-334 on UVA-induced photoaging in human skin fibroblasts. Int J Mol Med. 2014. 34:796-803. https://doi.org/10.3892/ijmm.2014.1815
Saklatvala J. Inflammatory signaling in cartilage: MAPK and NF-κB pathways in chondrocytes and the use of inhibitors for research into pathogenesis and therapy of osteoarthritis. Curr Drug Targets. 2007. 8:305-313. https://doi.org/10.2174/138945007779940115
Sanchez-Adams J, Leddy HA, McNulty AL, O'Conor CJ, Guilak F. The mechanobiology of articular cartilage: bearing the burden of osteoarthritis. Curr Rheumatol Rep. 2014. 16:451. https://doi.org/10.1007/s11926-014-0451-6
Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. Sports Health. 2009. 1:461-468. https://doi.org/10.1177/1941738109350438
Sun P, Mao Y, Li G, Cao M, Kong F, Wang L, et al. Comparative transcriptome profiling of *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi in response to temperature stresses. BMC Genomics. 2015. 16:463. https://doi.org/10.1186/s12864-015-1586-1
Tao C, Sugawara T, Maeda S, Wang X, Hirata T. Antioxidative activities of a mycosporine-like amino acid, porphyra-334. Fish Sci. 2008. 74:1166-1172. https://doi.org/10.1111/j.1444-2906.2008.01636.x
Xia T, Zhao R, He S, Wang L, Fu X, Zhao Y, et al. Gardenoside ameliorates inflammation and inhibits ECM degradation in IL-1β-treated rat chondrocytes via suppressing NF-κB signaling pathways. Biochem Biophys Res Commun. 2023. 640: 164-172. https://doi.org/10.1016/j.bbrc.2022.12.016
You JS, Cho IA, Kang KR, Oh JS, Yu SJ, Lee GJ, et al. Coumestrol counteracts interleukin-1β-induced catabolic effects by suppressing inflammation in primary rat chondrocytes. Inflammation. 2017. 40:79-91. https://doi.org/10.1007/s10753-016-0455-7
Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circ Res. 2003. 92:827-839. https://doi.org/10.1161/01.Res.0000070112.80711.3d
Wang SX, Abramson SB, Attur M, Karsdal MA, Preston RA, Lozada CJ, et al. Safety, tolerability, and pharmacodynamics of an anti-interleukin-1α/β dual variable domain immunoglobulin in patients with osteoarthritis of the knee: a randomized phase 1 study. Osteoarthritis Cartilage. 2017. 25:1952-1961. https://doi.org/10.1016/j.joca.2017.09.007