

Original Research Article

Anti-inflammatory and human chondrocyte protective effect of *Drynariae rhizoma* extract

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Abstract

Purpose: To evaluate the anti-inflammatory properties of *Drynariae rhizome* (Polypodiaceae) extract (DRE) on lipopolysaccharide RAW264.7 macrophage cells and its protective effects on interleukin (IL)-1 β -stimulated SW1353 chondrocyte cells.

Methods: The anti-inflammatory effect of DRE (12.5, 25, 50, and 100 μ g/mL) were measured by 5-lipoxygenase (LO) and cyclooxygenase (COX)-2 enzyme inhibitory activities, nitric oxide (NO) production in LPS-stimulated RAW264.7 cells using Griess reagent assay. Prostaglandins (PGE₂) and leukotriene (LTB₄) production were measured using enzyme-linked immunosorbent assay (ELISA). To investigate the protective effect on chondrocytes, the level of matrix metalloproteinases (MMP)-3, -9, and tissue inhibitor of matrix metalloproteinase (TIMP)-1 was determined using zymography assay and ELISA kit in SW1353 cells stimulated with IL-1 β .

Results: *Drynariae rhizome* (Polypodiaceae) extract (DRE) had a significant ($p < 0.05$) inhibitory effect on 5-LO and COX-2 enzymes in a concentration-dependent manner compared to untreated group. In RAW264.7 cells, levels of NO, PGE₂ and LTB₄ of DRE group were significantly ($p < 0.05$) decreased in study group (treated with DRE) at 12.5 μ g/mL compared to the group treated with LPS (100 ng/mL) alone. Additionally, in SW1353 human chondrocytes stimulated with IL-1 β , MMP-3 and MMP-9 were significantly ($p < 0.05$) suppressed in DRE-treated group compared to IL-1 β group alone, with no significant impact on TIMP-1 production.

Conclusion: *Drynariae rhizome* (Polypodiaceae) extract (DRE) possesses anti-inflammatory and cartilage protection effects. It therefore has the potential to be developed as an effective natural pharmaceutical agent for inflammation or osteoarthritis.

Keywords: *Drynariae rhizoma*, Interleukin-1 beta (IL-1 β), SW1353, Matrix metalloproteinases (MMPs), Prostaglandin E₂ (PGE₂)

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INTRODUCTION

Osteoarthritis is the most common joint disease related to joint trauma and aging, characterized by erosion of cartilage and bone formation. It

progresses due to an imbalance of metabolic pathways [1], and changes in signaling pathways due to excessive production of oxidative stress such as reactive oxygen species (ROS) and this is believed to be the leading cause [2].

Interleukin-1 beta (IL-1 β) significantly increases the expression of matrix metalloproteinases (MMPs), which play a central role in cartilage degradation in osteoarthritis [3]. It has been reported that MMPs induce osteoarthritis by releasing inflammatory mediators such as prostaglandin E₂ (PGE₂), nitric oxide (NO), and cyclooxygenase-2 (COX-2) [4]. This inflammatory response is promoted by oxidative stress generated in the body, which initiates inflammatory response by increasing gene expression of specific cells that cause cell death as well as degenerative diseases. When inflammation is expressed due to harmful stimuli, infection or trauma, arachidonic acid (AA) is released from cell membrane phospholipids, and then various inflammatory response mediators such as leukotriene, thromboxane, and prostaglandin are produced through the action of lipoxygenase (LO) or cyclooxygenase (COX) [5].

Herbal medicines have long been used as folk remedies in Korea and the East due to their low toxicity and side effects [6]. It has various physiological activities such as pain relief, anti-inflammation, antiseptic, antipyretic, detoxification, and astringency, and is used for treatment and prevention [7]. *Drynariae rhizoma* is the dried root and stem of *Davallia mariesii* T. Moore, a perennial plant belonging to the family *Polypodiaceae*, and the name means to attach broken bones. *Drynariae rhizoma* has long lateral roots and is shaped like an awl or an earthworm. Each root has projections like ears attached and is covered with hairs. In oriental medicine, it has been reported to be effective for inflammation, hypertriglyceridemia, arteriosclerosis, osteoporosis, bone fracture, bone resorption, and gynecological diseases [8]. In addition, it has been reported in the literature that *Drynariae rhizoma* is used for the prevention and treatment of hyperlipidemia, promotion of calcium absorption from bones, and increasing blood calcium concentration in fractures and osteoporosis [9].

A recent study on *Drynariae rhizoma* extract showed that it inhibits bone resorption by cathepsin K in mouse osteoclasts, promotes bone cell function for osteoclast activity, and inhibits collagen degradation and bone resorption [10]. Therefore, this study was aimed at investigating the inhibitory effect of *Drynariae rhizoma* ethanol extract (DRE) on inflammatory mediators in RAW264.7 macrophage cells, effect on cartilage degrading enzymes (MMPs, which play a central role in the pathogenesis of osteoarthritis) as well as interleukin-1beta (IL-1 β) stimulated SW1353 chondrocytes.

EXPERIMENTAL

Preparation of DRE

Drynariae rhizoma used in this experiment was purchased from Gyeongdong Market (Seoul, Korea). It was taxonomically identified by a plant biotechnologist, Dr. Jong Bo Kim (College of Biotechnology, Konkuk University Global Campus, Chungju, Republic of Korea). A voucher specimen was deposited at the College of Health Science, Dankook University, Korea. *Drynariae rhizoma* was extracted after grinding to an appropriate size. A total of 100 g of the ground sample was extracted with 1 L of 80 % ethanol twice for 3 h using an ultrasonic extractor (JAC-4020, KODO Technical Research Co., Ltd., Hwaseong, Korea) at 40 °C and thereafter filtered (Whatman No. 2, Maidstone, England). The filtered ethanol extract was concentrated under reduced pressure (N-1000S-WD, Eyela Co., Tokyo, Japan), and the obtained ethanol extract was dissolved in 100 mL of 80 % ethanol, left at 4 °C for 24 h, and then the supernatant was concentrated under reduced pressure and thereafter frozen. The *Drynariae rhizoma* ethanol extract (DRE) was then dried (FDU-1100, Eyela Co., Tokyo, Japan).

Evaluation of 5-LO inhibitory activity

The inhibitory activity of 5-lipoxygenase (5-LO) was measured using an assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) [13]. To 10 μ L of the sample (DRE; 12.5, 25, 50 and 100 μ g/mL), 90 μ L of 5-LO (220 units/mL) and 10 μ L of 1 mM arachidonic acid were added at room temperature (RT) for 5 mins. Then, 100 μ L of chromogen was added and reacted at RT for 5 mins. Absorbance was measured at 490 nm using ELISA auto reader (Biochrom Ltd., Holliston, MA, USA). Nordihydroguaiaretic acid (5 μ g/mL) (NDGA, Sigma Co., St. Louis, Mo. USA) was used as the positive control group, and the inhibition rate (I) was calculated using Eq 1.

$$I (\%) = (C-S)/C \dots\dots\dots (1)$$

where S = absorbance of sample and C = absorbance of control

Determination of COX-2 inhibitory activity

COX-2 inhibitory activity was measured using a COX inhibitor screening assay kit (Cayman Chemical Co.) [13]. To 20 μ L of the sample (DRE; 12.5, 25, 50 and 100 μ g/mL), 10 μ L of hematin, 5 μ L of the enzyme, and 950 μ L of buffer were added and reacted at 37 °C.

Thereafter, 10 μL of arachidonic acid was added and reacted at 37 °C for 2 mins to activate COX-2. Also, 100 μL of chromogen was added and the mixture was reacted at 25 °C for 90 mins, and the absorbance was read at 420 nm using an ELISA autoreader (Biochrom Ltd.). Indomethacin (1 $\mu\text{g}/\text{mL}$, Sigma Co.), one of the non-steroidal anti-inflammatory drugs, was used as the positive control group, and inhibition rate was calculated using Eq 1.

Cell culture

Murine macrophage cell line RAW264.7 cells used in this experiment were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea), and the human chondrosarcoma cell line (SW1353 cell) was purchased from the American Type Culture Collection (ATCC, USA). RAW264.7 cells and SW1353 cells were sub-cultured once every 2 - 3 days in a 37 °C incubator in the presence of 5 % CO_2 using Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (Gibco-BRL, Rockville, MD, USA), 100 $\mu\text{g}/\text{mL}$ penicillin (Gibco-BRL), and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco-BRL).

Cell viability

RAW264.7 and SW1353 cells were cultured in a 96-well plate at 1×10^5 cells/mL for 24 h, then treated with DRE 12.5, 25, 50, and 100 $\mu\text{g}/\text{mL}$ for 1 h. The RAW264.7 cells were then treated with lipopolysaccharide (LPS, 100 ng/mL, Sigma Co.) while the SW1353 cells were treated with IL-1 β (10 ng/mL, Sigma Co.) for 24 h. Cell viability was analyzed with 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) at a wavelength of 540 nm [11].

Measurement of NO production

Production of NO was measured by Griess reaction assay. After dispensing RAW264.7 cells into a 96-well plate at a concentration of 1×10^5 cells/mL using DMEM medium, the DRE (12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$) and new medium containing LPS (100 ng/mL, Sigma Co.) were simultaneously treated for 24 h and cultured.

After mixing 100 μL of the cell culture supernatant with 100 μL of Griess reagent (1 % sulfanilamide, 0.1 % naphthyl ethylenediamine in 2.5 % phosphoric acid) in a 96-well plate, it was left to stand for 10 mins, and absorbance was read at 540 nm using an ELISA autoreader (Biochrom Ltd.). A standard curve was prepared with sodium nitrate (Sigma Co.) to calculate the amount of NO present [12].

Assessment of LTB₄ and PGE₂ production

The amount of Leukotriene B₄ (LTB₄) and prostaglandins E₂ (PGE₂) produced in the culture medium was measured using an ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA). RAW264.7 cells were dispensed into a 96-well plate at a concentration of 1×10^5 cells/mL using DMEM medium, and then treated with new medium containing DRE (12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$) and LPS (100 ng/mL) at the same time and cultured for 24 h. Thereafter the cell culture supernatant was taken and LTB₄ and PGE₂ were measured [10].

Gelatin zymography

Matrix metalloproteinases-2 (MMP-2) and -9 (MMP-9) activities were investigated by treating SW1353 cells cultured in DMEM with DRE (0, 12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$) and IL-1 β (10 ng/mL). Cell culture medium containing 50 $\mu\text{g}/\text{mL}$ of total protein was electrophoresed using 10 % polyacrylamide gels under non-reducing conditions containing gelatin (1.5 mg/mL).

Gelatin decomposed bands appear as transparent bands on a blue background and the intensity of the band appears in proportion to the activity of MMPs, and this was observed using the LAS 3000 Rimage analyzer (Fujifilm Life Science, Tokyo, Japan) [13].

Evaluation of MMP-3, -9, and TIMP-1 production

The SW1353 cells were dispensed into a 96-well plate at a concentration of 1×10^5 cells/mL using DMEM high glucose medium and treated for 1 h with different DRE concentrations (0, 12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$) and thereafter left for 24 h. After that, IL-1 β (10 ng/mL) was introduced into a 96-well plate for another 24 h. The cell culture supernatant was taken and the amount of MMP-3, -9, and TIMP-1 produced was quantified using ELISA kit (R&D Systems, Inc. USA) according to the manufacturer's instructions [14].

Statistical analysis

All experiments were performed in triplicate and expressed as mean \pm standard deviation (SD). Statistical analysis was performed using SAS software version 6.12 (SAS Institute, Cary, NC, USA). Duncan's multiple range test was carried out to determine significant differences among the groups and $p < 0.05$ was considered significant.

RESULTS

Inhibitory activity of 5-LO and COX-2

Enzyme inhibitory activity was measured using NDGA and indomethacin as positive controls for 5-LO and COX-2, respectively. Results revealed that DRE significantly inhibited ($p < 0.05$) 5-LO in a concentration-dependent manner with 20.51, 32.87, 78.09, and 85.78 % at 12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$ respectively (Figure 1 A). Inhibitory activity of DRE on COX-2 also showed 32.07, 45.85, 52.51 and 56.64 % inhibition at 12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$ respectively. Indomethacin (positive control) exhibited excellent COX-2 inhibitory activity (82.98 %) at 1 $\mu\text{g}/\text{mL}$ (Figure 1 B).

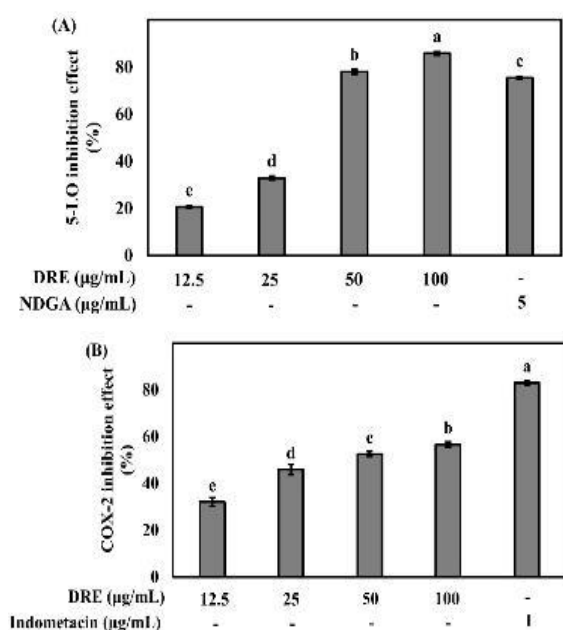


Figure 1: (A) 5-LO and (B) COX-2 inhibitory effects of DRE. NDGA and indomethacin were used as positive control. $abcde P < 0.05$ vs. control

Cytotoxicity and NO, LTB₄, and PGE₂ production inhibitory effect

Cell viability was over 90 % at all concentrations of DRE using the MTT assay (Figure 2). Furthermore, in the group treated with only LPS, NO was approximately 30.88 μM , producing about 7 times more NO than in untreated group, while in the study group (treated with DRE), NO production was significantly ($p < 0.05$) inhibited (Figure 3 A). Also, DRE at 12.5 $\mu\text{g}/\text{mL}$ significantly reduced ($p < 0.05$) LTB₄ and PGE₂ compared to lone LPS treatment group (Figure 3 B and C).

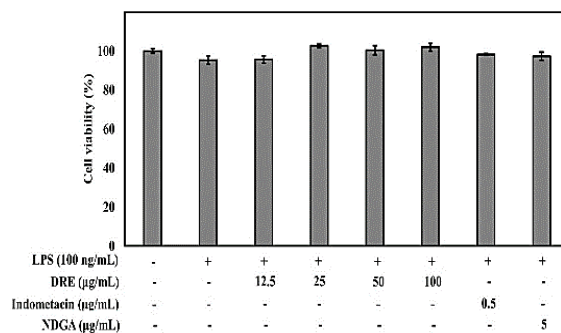


Figure 2: Effect of DRE on RAW264.7 cell viability in the presence and absence of LPS (100 ng/mL). RAW264.7 cells treated with DRE at 12.5, 25, 50, and 100 $\mu\text{g}/\text{mL}$, and LPS at 100 ng/mL. $P < 0.05$ vs. indomethacin and NDGA control

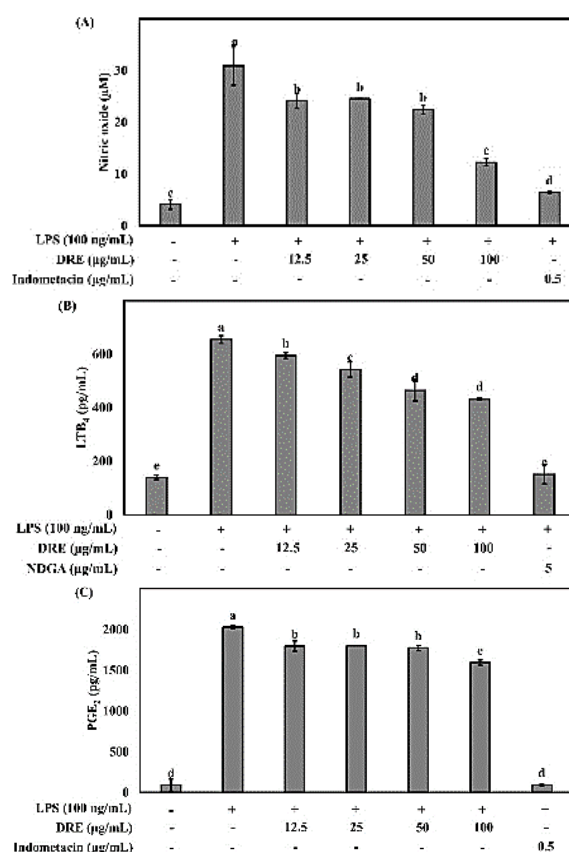


Figure 3: Inhibitory effect of DRE on NO, LTB₄, and PGE₂ production (A) NO in the culture supernatant (B) LTB₄ and (C) PGE₂ in the culture supernatant. $abcde P < 0.05$ vs. control.

Effect of DRE on MMPs and TIMP regulation

There was no cytotoxicity on SW1353 cells up to the highest concentration of 100 $\mu\text{g}/\text{mL}$ in the LPS and DRE-treated group compared to untreated group (Figure 4). Zymography analysis of MMP-9 revealed its activity significantly increased ($p < 0.05$) in IL-1 β (10 ng/mL) treated group, but DRE significantly decreased ($p < 0.05$) MMP-9 band (Figure 5). In the case of

changes in the amount of MMP-3 production, the IL-1 β treatment group alone increased MMP-3 production by about 5 times compared to control group (untreated group). However, on treatment with different concentrations of DRE, production of MMP-3 following IL-1 β treatment was significantly ($p < 0.05$) reduced in all treatment groups (Figure 6 A). Production of MMP-3 significantly increased ($p < 0.05$) approximately 6.2 times in IL-1 β treated group compared to control group. However, when the samples were used, production of MMP-3 significantly reduced ($p < 0.05$) (Figure 6 B).

Result of the measurement of change in level of the tissue inhibitor of metalloproteases (TIMP), showed that no significant change was produced on treatment with DRE compared to the MMPs (Figure 6 C).

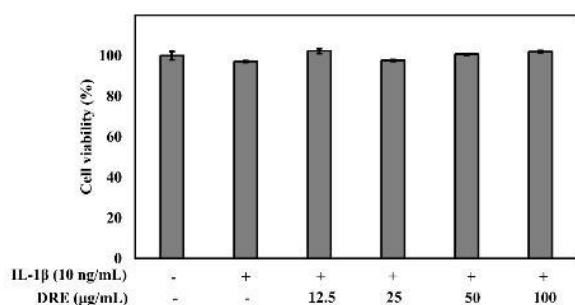


Figure 4: Effect of DRE on SW1353 cell viability in the presence and absence of IL-1 β (10 ng/mL). SW1353 cells treated with DRE at 12.5, 25, 50, and 100 μ g/mL without IL-1 β (10 ng/mL). $P < 0.05$ vs. control

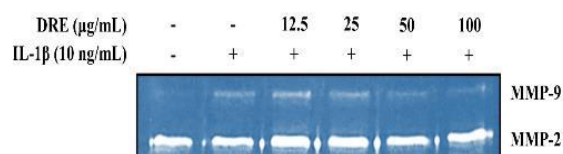


Figure 5: Effect of DRE on MMP-9 enzyme activity observed by gelatin zymography

DISCUSSION

The inflammatory response is an internal defense mechanism that restores function against external invasion or tissue damage, and many studies are being conducted to find and remove mediators involved in the inflammatory response in various diseases, including rheumatoid arthritis [15]. Therefore, this study aimed to investigate the production and expression of inflammatory mediators involved in osteoarthritis by using RAW264.7 cells stimulated by LPS, and SW1353 chondrocytes stimulated by IL-1 β .

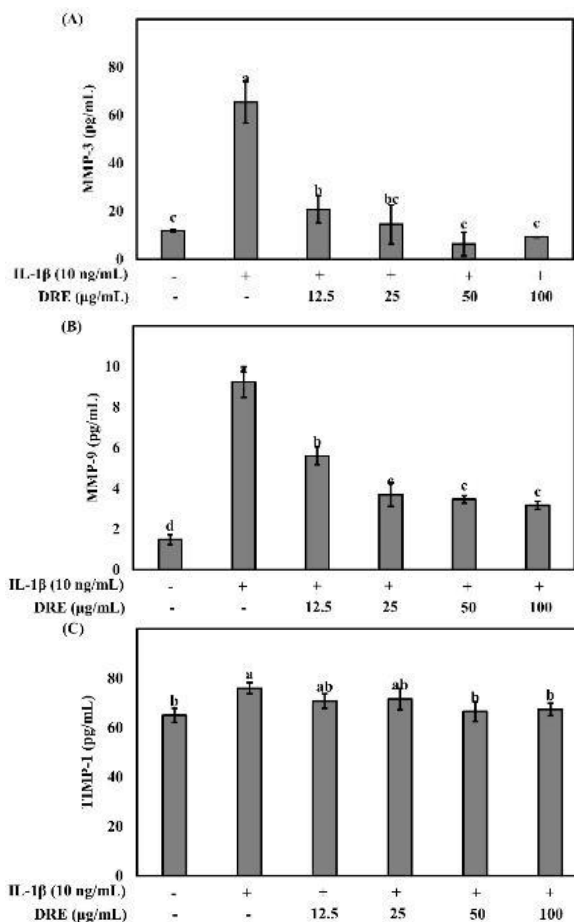


Figure 6: Effect of DRE on MMP-3, -9, and TIMP-1 regulation in SW1353 cells treated with DRE at 12.5, 25, 50, and 100 μ g/mL with IL-1 β at 10 ng/mL. (A) MMP-3, (B) -9, and (C) TIMP-1 in the culture supernatant. $abcdP < 0.05$ vs. control

The inhibitory activity of DRE on 5-LO and COX-2 enzymes (enzymes involved in the synthesis of inflammatory cytokines LTB $_4$ and PGE $_2$ respectively), revealed that DRE has the effect of simultaneously inhibiting both 5-LO and COX-2 enzymes. Monocytes or macrophages are activated by a small amount of LPS and this results in the release of cytokines, arachidonic acid metabolites, ROS, and NO [16].

Nitric oxide (NO) mediates important physiological functions such as vascular homeostasis and apoptosis induction under normal physiological conditions, but to a large extent kills normal cells and induces inflammation, acting as a substance that causes acute or chronic inflammatory diseases [17]. Therefore, effective regulation of NO secretion is a known treatment method for acute or chronic inflammatory diseases, and research on this is still ongoing. Therefore, DRE inhibitory action on NO production was investigated as an anti-inflammatory pathway, and the results revealed

that DRE inhibited NO production in a concentration-dependent manner. In addition, when inflammation occurs in the human body, PGE₂, (an important mediator in the pathogenesis of inflammation) is rapidly produced by COX-2 of macrophages [18]. Nitric oxide (NO) plays a large role in the early stage of leukocyte migration to inflammatory sites, while PGE₂ mainly acts in the later stage of fever and pain [19], and forms osteophytes to cause abnormal bone growth and pain, and these symptoms are common in osteoarthritis [20].

Furthermore, LTB₄ affect bone metabolism and its formation is increased in inflammatory bone loss diseases such as osteoarthritis, rheumatoid arthritis, and periodontitis [21]. In this study, DRE showed a significant decrease in PGE₂ and LTB₄ production at all concentrations. MMPs are matrix proteolytic enzymes that compose cartilage, such as proteoglycan, collagen, gelatin, and aggrecan, and are induced by inflammatory cytokines such as IL-1 and TNF- α . There are several types of MMPs, but among them, MMP-1 (collagenase) and MMP-3 (stromelysin) have the major action on inflammation, and TIMP has an inhibitory action on MMPs. Matrix metalloproteinases (MMPs) and their inhibitor, TIMP, are zinc-dependent enzymes that degrade extracellular matrix components such as collagen and proteopolysaccharide and play an important role in the pathogenesis of osteoarthritis [22].

A recent report, using arthritic cartilage extracts, suggests that both MMP and TIMP concentrations increased in arthritis compared to normal cells, but the increase in concentration was much higher for MMP compared to TIMP. An over-production of MMPs is thought to be related to cartilage degeneration [23]. Results of this study confirmed that DRE had no significant effect on TIMP-1 production compared to suppressing MMP-3 and -9 production in SW1353 human chondrocytes stimulated by IL-1 β , thus having a cartilage-protective effect. These results suggest the possibility of DRE as a new natural pharmaceutical lead effective in suppressing inflammation or osteoarthritis.

CONCLUSION

Drynariae rhizome (Polypodiaceae) extract (DRE) inhibits the production of inflammatory mediators induced by LPS and significantly inhibits the production of cartilage-degrading enzymes, MMPs, in chondrocytes induced by IL-1 β . Therefore, DRE is a potential source for agents with inhibitory activity on inflammation and cartilage degradation.

DECLARATIONS

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Funding

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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