Anti-inflammatory and anti-osteoarthritic effect of Mollugo pentaphylla extract

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ABSTRACT
Context: Mollugo pentaphylla L. (Molluginaceae) extract (MPE) has been reported to have anti-inflammatory effect on MSU-induced gouty arthritis in a mouse model.
Objective: This study examined the anti-inflammatory activities of an MPE in vitro and anti-osteoarthritic effects on monosodium iodoacetate (MIA)-induced osteoarthritis (OA) in vivo.
Materials and methods: The dried whole plants of M. pentaphylla were extracted with 70% ethanol under reflux. The anti-inflammatory effect of MPE was evaluated in vitro in lipopolysaccharide (LPS)-treated RAW264.7 cells. The anti-osteoarthritic effect of MPE was investigated in a Sprague–Dawley rat model of MIA-induced OA. Each seven male rats were orally administered MPE (75, 150 or 300 mg/kg) or the positive control drug indomethacin (1 mg/kg) 3 days before MIA injection and once daily for 11 days thereafter. After the treatment with MPE, no evidence of systemic adverse effects was observed in any study group.
Results: MPE exhibited anti-inflammatory activity via inhibition of the production of NO (57.8%), PGE2 (97.1%) and IL-6 (93.2%) in LPS-treated RAW264.7 cells at 200 µg/mL. In addition, MPE suppressed IL-1β (60.9%), TNF-α (37.9%) and IL-6 (40.9%) production and suppressed the synthesis of MMP-2, MMP-9 and COX-2 in the MIA-induced OA rat model.
Conclusions: These results demonstrate that MPE exerts potent anti-inflammatory activities and protects cartilage in an OA rat model. This might be a potential candidate for therapeutic OA treatment.

Introduction
Osteoarthritis (OA) is a degenerative arthropathy that is characterized by a progressive degeneration of articular cartilage, osteophyte formation, and subsequent joint space narrowing (Castaneda et al. 2012). OA primarily affects the articular cartilage and subchondral bone of synovial joints and induces dysregulation of pro-inflammatory and anti-inflammatory pathways, leading to pain during weight-bearing activities, including walking and standing (Krasnokutsky et al. 2008; Glyn-Jones et al. 2015). Inflammation is the most significant cause of the structural changes that occur during the clinical progression of OA (Gong et al. 2012; Musumeci et al. 2012). Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β), IL-6, IL-15, IL-17 and nitric oxide (NO) are produced by chondrocytes in the synovium, and these factors induce extracellular matrix (ECM) degradation and the apoptosis of chondrocytes (Fernandes et al. 2002). These cytokines induce further cytokine expression and activate chondrocytes to synthesize aggrecanases and matrix metalloproteinases (MMPs), which increase cartilage degradation (Thalhamer et al. 2008; Rahmati et al. 2016).

The primary goals of current arthritis therapy are pain control, a reduction in inflammation, and improved joint function (Kapoor et al. 2011; Sokolove and Lepus 2013). Commonly prescribed OA medications, such as corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and hyaluronan preparations, are aimed at reducing stress on joints or improving strength and stopping progression (Barnes and Edwards 2005). These agents may also have serious adverse effects, such as renal toxicity, diarrhea, nausea, vomiting, gastrointestinal toxicity and gastrointestinal bleeding (Le Graverand-Gastineau 2010; Crofford 2013). Therefore, traditional herbal medicinal sources with minor or no side effects have been investigated widely as adjuvant therapeutic agents to control pain and improve joint function (Cameron and Chrubasik 2013; Dhippayom et al. 2015).

Mollugo pentaphylla L. (Molluginaceae) (MP) is found in the tropical regions of China, Malaysia, India, Japan and Taiwan (Zhou et al. 2011). MP is used to treat central nervous system-stimulating, stomachic, antitoxic diuretic, antiperiodic, antitoxitis, anticancer, antiseptic and contusion-alleviating effects. MP contains flavonoids, tannins, and the triterpene alcohol mollugogenol A, which shows considerable antifungal effects (Kim SS et al. 2008; Maiti et al. 2012; Lee et al. 2017). MP decreases the secretion of the pro-inflammatory cytokine IL-8 induced by Propionibacterium acnes in THP-1 cells, suggesting anti-inflammatory effects (Kim SS et al. 2008). MP extract (MPE) exhibited analgesic activity in tail immersion and acetic acid-induced writhing tests (Sahu et al. 2011). Aqueous MPE exhibited anti-inflammatory activity and reduced carrageenan-induced paw edema (Lin et al. 2002). Ethanol MPE significantly suppressed...
paw edema in carrageenan-induced and cotton pellet-induced granuloma models, and these extracts were associated with anti-inflammatory activity in acute and subchronic inflammation models. We have previously reported that MPE suppressed swelling and pain, and exhibited anti-inflammatory activity by inhibiting pro-inflammatory cytokines (IL-1β and TNF-α), NF-κB and NLRP3 inflammasome activation in monosodium urate (MSU)-induced mice (Lee et al. 2017). Although MPE has been reported to have anti-inflammatory effects and ameliorate gouty arthritis in vivo, the effects of MPE in a monosodium iodoacetate (MIA)-induced OA model are still unknown. The MIA-induced OA model is useful and relevant to preclinical or research studies of inflammation and OA (Pitcher et al. 2016). The present study examined the anti-inflammatory effects of MPE in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. The anti-osteoarthritis effect of MPE was measured by the inflammatory response and the suppression of cartilage degradation induced by inflammatory cytokines. We further demonstrated an OA-related pain-relieving effect of MPE on weight-bearing distribution balance in MIA-induced OA rats.

Materials and methods

**MPE preparation**

Whole MP plants were collected in Yangpyung, Kyonggi-do in 2016, and were taxonomically confirmed by Dr. Geung-Joo Lee of Chungnam National University. A voucher specimen (no. KIOM201701018962) was deposited at the Korean Herbarium of Standard Herbal Resources at the Korea Institute of Medicine (KIOM). The dried herb (1 kg) was extracted twice with 70% ethanol (by 3-h reflux), and the extract was concentrated as described previously. The concentrated extract was lyophilized and stored at 4°C. The yield of the dried extract from the crude materials was 13.9% (w/w) (Lee et al. 2017).

**LC-MS analysis**

Liquid chromatography/mass spectrometry (LC/MS) analysis was conducted with an ultra-performance liquid chromatography system (Waters, Milford, MA) with a quaternary solvent manager, flow-through needle autosampler and a column manager. Detection was performed with a photodiode array and mass detectors. Data were processed with Empower software (Waters Co.). An Acquity UPLC BEH column (2.1 x 100 mm; 1.7 μm; Waters, Milford, MA, USA) was used as the stationary phase, and the mobile phase consisting of water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) was used with gradient elution (isocratic elution with 5% B for 5 min, a linear gradient from 5% to 100% B over 60 min, a linear gradient from 100% to 5% B over 5 min, and finally, isocratic elution with 5% B for 5 min). The flow rate was 0.5 mL/min, and quantitative measurements were made at 245 nm. We used a mass spectrometer equipped with an electrospray ionization (ESI) source in positive mode. The instrument parameters were a capillary voltage of 0.8 kV and cone voltage of 15 V. The probe temperature was 600 ± 5°C. MS data were collected in full-scan mode from 300 to 500. The desolvation gas was nitrogen (600 L/h).

**Cell culture**

The murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco Inc., Grand Island, NY, USA) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 5.5% heat-inactivated FBS (Gibco Inc.) at 37°C in a humidified incubator with 5% CO2. The medium was replaced with serum-free DMEM medium. MPE (50, 100 and 200 μg/mL) was dissolved in 100% DMSO, and each concentration was diluted to a final DMSO content of 0.1% when used to treat cells for 2h. LPS (0.5 μg/mL Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was added in the presence or absence of MPE for an additional 24h to stimulate the cells.

**Cytotoxicity assay**

Cells were treated with MPE for 24 h. MTT solution (0.5 mg/mL) was added to each well for 4 h (5% CO2 at 37°C), and 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize purple formazan crystals. The absorbance at 570 nm was read on a microplate reader (SpectraMax M2, Molecular Devices, USA) as a measurement of cell viability.

**Western blot**

Equal amounts of cell extract (30 μg) were resolved on sodium dodecyl sulfate (SDS) gels by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. The antibodies used were anti-cyclooxygenase-2 (COX-2), anti-inducible nitric oxide synthase (iNOS) (Cell Signaling, Danvers, MA, USA), and anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA).

**MIA-induced OA in rats**

Male Sprague–Dawley rats (7 weeks old, 190–210 g body weight) were purchased from Orient Bio (Seongnam, Korea) and housed under controlled conditions with a 12 h light/dark cycle at 22 ± 2°C and 55 ± 15% humidity. All experiments that used animals were approved by the Institutional Animal Care and Use Committee of Daejeon University (Daejeon, Korea DJUARB2016-036). After 1 week of acclimatization, the rats were divided randomly into six groups of 7 animals: (1) control group with saline injection, (2) MIA group with MIA injection, (3–5) MPE-treated group (75, 150 and 300 mg/kg body weight) with MIA injection and (6) indomethacin (IM)-treated group (1 mg/kg body weight) with MIA injection. The doses of MPE and IM used in this study were based on those used in previous studies (Kim SH et al. 2017; Lee et al. 2017). The MIA solution (3 mg/50 μL of 0.9% saline) was directly injected into the intra-articular space of the left knee. MPE and IM were dissolved in 5% carboxymethyl cellulose (CMC)-containing saline immediately before use, and 500 μL of this solution was orally administered 3 days before MIA injection and then once daily for 11 days. The rats were anesthetized using a mixture of ketamine and xylazine and euthanized by isoflurane overdose. Blood samples were centrifuged at 3000 rpm for 10 min at 4°C, and the serum was collected and stored at −70°C until use.

**Measurements of hind paw weight-bearing distribution**

The balance in the weight-bearing capability of the hind paws was disrupted after OA induction. A significant shift in weight from the arthritic site to the contralateral limb, i.e., a weight-
bearing deficit, was considered to be an index of pain, as described previously (Combe et al. 2004). Inflammatory pain was measured via the weight bearing of the paw load using an incapacitance tester (Linton Instrumentation, Norfolk, UK) (McDougall et al. 2006). The weight distribution ratio was calculated using the following equation: [weight on left hind limb/ (weight on left hind limb + weight on right hind limb)] × 100.

**NO and inflammatory cytokine assay**

NO production was analyzed as the accumulation of nitrite in culture supernatants using a Griess Reagent System (Promega, Madison, WI, USA). Levels of IL-1β, IL-6 and TNF-α were measured using ELISA kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Histopathology analysis**

Tissue specimens from the knee joints of rats were removed, fixed in 10% formalin, decalcified using 10% ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, and serially sectioned at 7 μm. Tissue sections were stained with hematoxylin and eosin (H&E) or Safranin-O/Fast Green. Histological changes were examined using light microscopy (Olympus BX51, Olympus, Tokyo, Japan) and photographed (Olympus DP70).

**Real-time PCR analysis**

Total RNA was isolated using TRIzol (Invitrogen, CA, USA), and 0.5 μg of total RNA was reverse transcribed into cDNA and PCR amplified using a one-step RT-PCR kit with SYBR Green (Applied Biosystems, Grand Island, NT, USA). Real-time quantitative PCR was performed using a real-time PCR system (7500, Applied Biosystems). Table 1 shows the primer sequences and probe sequences. cDNA was amplified using a TaqMan® Universal PCR master mixture containing DNA polymerase (Applied Biosystems, Foster, CA, USA) according to the manufacturer’s instructions. The concentration of the target gene was determined using the comparative Ct (threshold cycle number at the cross-point between the amplification plot and the threshold) method (Table 1).

**Statistical analysis**

The results are presented as the means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance with Dunnett’s multiple comparisons test, and a p < 0.05 was considered statistically significant. All analyses were performed using Prism 7.0 (GraphPad Software, San Diego, CA, USA).

**Results**

**HPLC fingerprinting of MPE**

LC/MS revealed that the main component of MPE was arabinopyranosyl apigenin, as expected. The optimized mass transition pair (m/z) was quantified as 535.1437 [M + H]⁺.

Based on the absorption profile and the retention time, the MPE contained 1.34 ± 0.06 mg/g of arabinopyranosyl apigenin (Figure 1).

**Effect of MPE on inflammation in RAW264.7 macrophages**

LPS-induced RAW264.7 cells were treated with MPE to assess the effect of MPE on the inflammatory response. MPE did not affect cell viability and was not toxic to RAW264.7 cells (data not shown). LPS significantly elevated the production of NO, IL-6 and prostaglandin E2 (PGE2) in the cells (Figure 2(A–C)). MPE significantly inhibited LPS-induced NO, IL-6 and PGE2 production at 50, 100 and 200 μg/mL (Figure 2(A–C)).

**Effect of MPE on iNOS and COX-2 protein expression in RAW264.7 macrophages**

The upregulation of pro-inflammatory iNOS and COX-2 in LPS-induced RAW264.7 cells was evaluated using Western blot analysis. Figure 2(D,E) show that MPE dose-dependently decreased the protein expression of iNOS and COX-2 in LPS-stimulated cells.

**Effect of MPE on hind paw weight-bearing distribution**

The weight distribution between the sensitized and contralateral hind limbs was considered to be an index of the joint discomfort in the arthritic knee. We evaluated the hind paw weight bearing using an incapacitance tester for 14 days. The ratio of the hind paw weight distribution between the left and right limbs was used to assess the progression of OA. The weight-bearing distribution of the MIA group decreased rapidly and became significantly different from that of the saline group at 1 day post-MIA injection, and this difference was maintained for at least 11 days. These values were decreased only slightly in the MPE- and IM-treated groups at day 4 compared with that in the MIA group. The MPE- and IM-treated groups exhibited full recovery, and the balance between the hind legs returned to normal. These results demonstrated significant recovery of hind limb weight bearing in the MPE-treated group (Figure 3).

| Gene   | Primer sequence |
|--------|-----------------|
| IL-1β  | Forward 5’-CCCTGCAGCTGGAGAGTGGT-3’ |
|        | Reverse 5’-TGTTGCCTGTTAGGAGGTTG-3’ |
| IL-6   | Forward 5’-TTCTACCCCACTCCTAGT-3’ |
|        | Reverse 5’-ATGAGTTGGAGTCTGCTGTC-3’ |
| COX-2  | Forward 5’-GGTTGCCGCTGTAGT-3’ |
|        | Reverse 5’-GGAATGCGGTTCTGATG-3’ |
| MMP-2  | Forward 5’-CAGGGAATGATGATCGGTCTATT-3’ |
|        | Reverse 5’-ACTCCAGTTAAGCGACTATAC-3’ |
| MMP-9  | Forward 5’-AATCTCTTCTAGACTGGAAGG-3’ |
|        | Reverse 5’-AGCTGATGTATAGATAAGCGA-3’ |
| GAPDH  | Probe Applied Biosystems® Rat GAPDH Endogenous Control (VIC®/MGB Probe, 4352338E) |
Effect of MPE on inflammatory cytokine serum levels

Pro-inflammatory cytokines play important roles in the maintenance of chronic inflammation and tissue damage during OA progression. Therefore, we examined the effects of MPE on the serum levels of IL-1β, IL-6 and TNF-α in the MIA-induced OA model. The serum levels of IL-1β, IL-6 and TNF-α were higher in the MIA group than in the saline group but were suppressed in the MPE- and IM-treated groups compared with the MIA group (Figure 4). These results suggest that MPE may exhibit cartilage-protection
effects in the MIA model via the regulation of inflammatory cytokines.

Expression of cytokine and inflammatory mediator mRNA in knee joint tissues

We also investigated the effects of MPE on the mRNA levels of IL-1β, IL-6, COX-2, MMP-2, and MMP-9 in the knee joint tissues of rats. The expression of cytokine and inflammatory mediator mRNAs increased following MIA injection, but this expression was attenuated in the MPE- and IM-treated groups (Figure 5). These results indicate that MPE suppressed the expression of inflammatory cytokines, mediators, and MMPs in MIA-treated rats.

Effects of MPE on histopathology

The rats were sacrificed, and the knee joints were evaluated histologically to determine the severity of inflammation and cartilage damage using H&E and Safranin-O/Fast Green staining. The MIA group exhibited histological changes indicative of severe arthritis with extensive infiltration of inflammatory cells in the cartilage and synovial membrane, proteoglycan loss, and cartilage structure changes. However, treatment with MPE and IM inhibited the inflammatory cell infiltration, edema, proteoglycan degradation and cartilage structure damage in joints (Figure 6). These histological features demonstrate that MPE attenuated the severity of MIA-induced OA in rats.

Discussion

OA is a common joint disease worldwide, but there is no approved therapy for preventing disease progression. Increasing evidence indicates that inflammation plays a central role in OA joint pathology, which suggests that targeting inflammation in OA may be a suitable therapeutic strategy (Philp et al. 2017). Pro-inflammatory mediators, such as NO, PGE2 and cytokines, are produced by the inflamed synovium and alter the balance of cartilage matrix degradation and repair. These processes exacerbate the clinical symptoms of and joint degradation in OA (Sellam and Berenbaum 2010).

The overproduction of iNOS and COX-2 directly stimulates the high production of PGE2 and NO, respectively, during inflammation (Ahmed et al. 2002; Chabane et al. 2008). iNOS is one of three key enzymes (endothelial NOS, neutral NOS and iNOS) that generate NO, and it is responsible for the overproduction of NO that is often observed during inflammation (Lechner et al. 2005). COX-2 is induced in many cells during the introduction of inflammatory mediators such as LPS and cytokines and produces a significant amount of PGE2, which causes inflammatory reactions (Parente 2001; Chun et al. 2004). The present study examined the anti-inflammatory effects of MPE on the production of inflammatory cytokines and mediators (NO, PGE2, IL-6, COX-2 and iNOS) in LPS-stimulated RAW264.7 cells. Our results demonstrated that MPE significantly inhibited NO, PGE2 and IL-6 production and suppressed the expression of iNOS and COX-2 proteins in LPS-induced RAW264.7 cells. These results suggest that MPE reduces PGE2 and NO levels via suppression of COX-2 and iNOS expression, thus inhibiting inflammation. Joint movement triggers OA pain, and movement of the affected joint is typically reduced, which ultimately affects the ability to perform physical activities (Bhatia et al. 2013). The overproduction of inflammatory cytokines, such as TNF-α, IL-1β and IL-6, plays vital roles in the destruction of the cartilage matrix in OA (Chevalier et al. 2006). IL-1β is synthesized in the joint by chondrocytes, osteoblasts, cells forming the synovial membrane and mononuclear cells that were previously present in the joint or infiltrated its structure during the inflammatory response (de Lange-Brokaar et al. 2012). These same cells in the joint secrete TNF-α, and increased concentrations of IL-1β and
TNF-α are also observed in the synovial fluid, synovial membrane, cartilage and subchondral bone layer (Massicotte et al. 2002; Wojdasiewicz et al. 2014). The production of IL-6 in diseased joint tissues is generally mediated by chondrocytes, osteoblasts and macrophages in response to IL-1β and TNF-α (Guerne et al. 1990). These inflammatory cytokines stimulate a major cascade of events involving the degradation of cartilage matrix and the destruction of articular cartilage, such as the synthesis of MMPs and ECM proteins, which are absent in normal cartilage, and the release of other inflammatory mediators, including COX-2 (Largo et al. 2003; Akhtar et al. 2011; Meszaros et al. 2015; Zeng et al. 2015). We evaluated the therapeutic potential of MPE for pain relief and chondroprotective effects in MIA-induced OA in rats by measuring the weight-bearing distribution, inflammatory cytokines and mediator levels in serum, inflammation-related gene expression in knee joints, and histopathological parameters. We demonstrated an OA-related pain-relieving effect of MPE on the weight-bearing distribution balance in MIA-induced OA rats. Our results demonstrated that MPE significantly protected joints in MIA-induced rats, which suggests that MPE may be useful for the treatment of OA pain. Histological examinations revealed that the MPE-treated group exhibited marked suppression of inflammatory cell infiltration and cartilage degradation in the MIA-treated rat joint. Anti-inflammatory responses were consistently observed in the cartilage and serum after MPE treatment in these rats. These results demonstrated that MPE significantly inhibited IL-1β, TNF-α and IL-6 production and suppressed the synthesis of MMP-2, MMP-9 and COX-2 in MIA-induced rats. These findings suggest that MPE protects joints from cartilage degradation and inflammation, which induces the development and progression of OA. According to the tentative identification of phytochemical, arabinopyranosyl apigenin is the main components of MPE. To the best of our knowledge, arabinopyranosyl apigenin has not been
reported to have an effect on inflammation and cartilage degradation. Therefore, more research is needed to investigate the effects of this compound.

In conclusion, MPE exhibited anti-inflammatory activity via inhibition of NO, PGE2 and IL-6 production in LPS-treated RAW264.7 cells. MPE also attenuated joint pain, inhibited the production of pro-inflammatory cytokines and mediators, and protected cartilage in an OA rat model. Therefore, we hypothesize that MPE exerts beneficial effects on inflammation and shows promise as an anti-OA therapeutic agent.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Disclosure statement
All authors declare that they have no conflicts of interest to disclose.

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