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Plebeian Sage (*Salvia plebeia* R. Br) Extract Ameliorates Inflammation and Cartilage Degradation in Surgically Induced Osteoarthritis Rats

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Abstract: Osteoarthritis (OA), the most prevalent articular disease with the clinical syndrome of joint pain accompanied by varying degrees of functional limitation, reduces the quality of elderly life. In this study, the effects of Plebeian sage extract (PS) on anti-inflammatory and anti-articular cartilage degradation activities were evaluated in rats with surgically induced OA. PS supplement for 12 weeks significantly decreased Mankin scores, including inflammatory cell numbers, and improved surface cartilage damage and mean femur and tibia articular cartilage (AC) thicknesses in OA rats. PS diminished IL-1β, IL-6, TNF-α, MMP-2, MMP-3, and MMP-9, as well as lipocalin-2 levels in serum or cartilage, which were increased due to OA. The results suggested that PS decreased joint inflammation and loss of articular cartilage by suppressing provocative responses and synovial tissue decimation in the OA model. Thus, PS may be used as a novel potential therapeutic regime for OA in the elderly.

Keywords: *Saliva plebeian*; osteoarthritis; articular cartilage; inflammation; cytokine; MMPs

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1. Introduction

Osteoarthritis (OA) is the most prevalent articular disease in the elderly [1]. The process is characterized by changes in the structure and function of the articulation, mainly due to a degenerative process that takes place in the articular cartilage [2,3]. OA is the most common clinical syndrome of joint pain accompanied by varying degrees of functional limitation that reduces the quality of elderly life [4]. Subchondral bone remodeling and a meniscal damage occur in OA, which is a whole joint disorder, affecting all joint tissues that communicate at the cellular level by releasing and responding to inflammatory mediators. Inflammation and fibrosis present in synovial membrane and in the infrapatellar fat pad (IFP) [5,6]. Synovial inflammation acts as a trigger for several symptoms of OA via the release of soluble factors that, while increasing and perpetuating cartilage damage, are used as biomarkers [7,8]. Inflammation may act as a contributing factor in perpetuating cartilage degradation by promoting destruction and impairing the ability of repair [9].

IL-1β and TNF-α induce other proinflammatory cytokines, such as IL-6, IL-17, and IL-18, and chemokines. Many of these factors synergize with one or another in promoting chondrocyte catabolic responses. The activation of stress- and inflammation-induced signaling, transcriptional, and posttranscriptional events may cause phenotypic shift, apoptosis, and aberrant expression of inflammation-related genes, including catabolic genes [3].

Nuclear factor kappaB (NF-kB) proteins constitute a family of transcription factors that are stimulated by pro-inflammatory cytokines, chemokines, stress-related factors, and extracellular matrix (ECM) degradation products. The activated NF-kB molecules trigger the expression of an array of genes, which induce destruction of the articular joint, leading to OA onset and progression [10]. These include nitric oxide synthase (NOS)-2, cyclooxygenase (COX)-2, and several matrix metalloproteinases (MMPs) [9,10]. Lipocalin-2 induced by pro-inflammatory factors in joint tissues forms covalent complexes with MMP-9 [11], and its circulating levels are elevated in aged individuals [12,13]. Increased levels of Lipocalin-2 have been found in OA SF and OA cartilage [14,15]. It has been reported that Lipocalin-2 contributes to the OA pathologies and other OA risk factors [16].

Loss of articular cartilage components, mainly ECM, which leads to tissue destruction and hypocellularity, and eventually results in loss of joint function, was found in the OA patients [9,10,17]. There is no cure for OA and no effective treatment to stop its progression. Current pharmacologic treatments, such as analgesics and non-steroidal anti-inflammatory drugs, may improve the pain and offer some relief, but they do not affect the progression of the disease. Further, the chronic intake of these drugs may result in severe adverse events [18]. Glucosamine and chondroitin sulfate have been shown to delay OA knee progression in several clinical trials [19,20]. The effectiveness of some products that are considered nutraceuticals has been widely reviewed in the literature [21,22]. The results present that nutrients, vitamins, antioxidants, and other natural components in the normal diet can affect the progression of the disease [23,24]. Many in vitro studies indicate the efficacy of specific nutrients in cartilage metabolism and their involvement in OA [25–27]. *Dipsacus asperoides* and *Mollugo pentaphylla* L. (Molluginaceae) extracts showed potent anti-inflammatory activities and protected cartilage in an OA rat model [28,29]. However, in vivo or rigorous clinical studies that evaluate the efficacy of these compounds in OA models are still missing. The influence of nutrients and diets on the metabolism of cartilage and OA could represent a long-term coadjuvant alternative in the management of OA. The studies show that the diets might be potential candidates for therapeutic OA treatment. Thus, our focus is to find safe and effective nutraceuticals that can control inflammation, cartilage metabolism, and OA progression.

Plebeian sage (*Salvia plebeia* R. Br) is an annual or biennial plant that grows in Korea, China, and India. It is used as a traditional medicine to treat inflammatory diseases, including asthma, hepatitis, and hemorrhoids [30]. Pharmacological investigations have revealed that leaf extract of Plebeian sage (PS) has anti-oxidative [30,31], anti-inflammatory [30,32], anti-asthma [32], and anti-arthritis effects [33]. The active components of Plebeian sage comprise flavonoids [34] and phenolic acid [35], which are known for their antioxidant and anti-inflammatory effects. The homoplantaginin, which is the main flavonoid from Plebeian sage, is known to have inhibitory effects on inflammation by controlling nitric oxide generation [36]. In the previous study, Plebeian sage extracted with 95% v/v EtOH at 70 °C inhibited inflammatory response in a mice model of arthritis [33]. However, 50% v/v EtOH or less concentration of EtOH and 50 °C or lower temperature are preferred as an extracting condition for the clinical trial. Health food producers prefer the condition because of its lower producing price and higher safety compared to the other condition. Thus, we have extracted Plebeian sage with 50% v/v EtOH at room temperature and have evaluated its effect in the osteoarthritis model. It is hypothesized that appropriate treatment of PS inhibits surgically induced OA due to its anti-inflammatory and articular cartilage (AC) preserving effects. The objective of this study is to verify the anti-osteoarthritis effects of PS in rats with surgically induced OA.

2. Materials and Methods

2.1. Plant Material and Its Flavonoids Contents

2.1.1. Sample Preparation

Plebeian sage leaves were obtained from the natural population in a farm in the Paju area of the Gyeonggi-do (South Korea) and were authenticated by the National Institute of Agricultural Sciences. After a multiple-step cleaning process and drying, 300 g of Plebeian sage leaves were extracted twice with 10 times volume of 50% v/v ethanol and distilled water at room temperature for 24 h. The extracts of PS were filtered through No. 6 filter paper (Advantec Co., Tokyo, Japan) and were concentrated by sequential use of a rotary evaporator (EYELA N-1000, Riakikai Co., Ltd., Tokyo, Japan) at 30 °C. Then, they were frozen and lyophilized (PVTFD 10R, Ilsin Lab, Yangju, Korea). The final lyophilized extract (11% yield) was stored at −70 until required for experimental use.

2.1.2. Analyzing Flavonoids Contents in Plebeian Sage and Its Extract

Flavone and flavanone amounts of Plebeian sage leaf and its extract (PS) were measured as major flavonoids compounds. Reference standards of nepetin 7-*O*-glucoside (nepitrin), hispidulin 7-*O*-glucoside (homoplantaninin) and hispidulin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Luteolin 7-*O*-glucoside (cynaroside) and 6-methoxyluteolin (internal standard) were obtained from Extrasynthese (Genay Cedex, France). In addition, methanol, acetonitrile and water (Optima® LC/MS grade) were supplied from Fisher Scientific (Pittsburgh, PA, USA) and formic acid from Junsei Chemical (Tokyo, Japan). By UPLC-DAD-QToF/MS analysis, the flavone and flavanone derivatives of common sage were identified and quantified using an UPLC system equipped with a diode array detector (DAD) (ACQUITY UPLC™ system, Waters Co., Milford, MA, USA) and a QToF/MS (Xevo G2-S QToF, Waters MS Technologies, Manchester, UK). In addition, both main column (CORTECS UPLC T3 C18, 2.1 × 150 mm, 1.6 µm, Waters Co.) and pre-column (CORTECS UPLC T3 VanGuard™, 2.1 × 50 mm, 1.6 µm, Waters Co.) were used to separate flavonoid derivatives. The analysis was conducted at a flow rate of 0.3 mL/min and detection wavelength of 210–400 nm (representative wavelengths of 350 and 280 nm for flavone and flavanone derivatives, respectively). According to mobile phase A (0.5% formic acid in water) and B (0.5% formic acid in acetonitrile), the elution gradient profiles (total 40 min) were detailed as 25% B (20 min), 50% B (25 min), 90% B (30 min), 90% B (32 min), 5% B (34 min) and 5% B (40 min). Mass spectra were simultaneously scanned in the range of 200–1200 m/z in positive ionization mode using an electrospray ionization (+ESI) source, and the parameters used were: capillary voltage 3.5 kV, sampling cone voltage 40 V, source temperature 120 °C, desolvation temperature 500 °C, and desolvation N_2 gas flow 1050 L/h.

2.2. Animal Experiments

2.2.1. Surgically Induced Osteoarthritis Rat Model

OA rats, surgically induced by anterior cruciate ligament transected and partial medial meniscectomy, have been generally used to observe the anti-OA effects of various candidates [22,37]. Forty SPF male Sprague–Dawley (SD) rats (9 weeks old, body weight (BW) 321 ± 36 g) composed of normal and OA model were purchased from the Orient Bio (Sungnam, Korea). Except for the 8 rats as the sham control group (CON), the other 32 rats were used as the OA model. The OA rats confirmed as OA model with data sheets showing increased knee thickness following surgical operation compared to the Sham rats were purchased from the company. Target was left knee joint, including femur and tibia articular cartilages and synovial membranes (SM). Surgery to induce OA was performed in 32 rats [22,37,38]. The normal control group of rats underwent a sham operation in which a similar incision in the joint capsule have been made but anterior cruciate ligament transection and partial medial meniscectomy had not been performed.

2.2.2. Animals Husbandry and Experiment Protocols

After 1 week of an adaptation period following purchase from the company, 10-week-old Sham and OA rats were randomly divided into CON and 4 OA groups (n = 8/group), respectively. Two animals per polycarbonate cage were housed at 22 ± 2 °C and 50–55% humidity in a facility under a 12 h light–dark cycle with free access to a standard pellet diet and water. Experimental doses and duration for this animal study were considered from previous experiments [22,28–30]. The PS was dissolved in distilled water (DW) and fed to rats by 1 mL at 100 (PS1) or 300 mg/kg BW (PS2) every day for 12 weeks. An equal volume of DW used for the melting sample was treated for the sham normal control (CON), negative control (NC), and positive control (PC). PC group was subcutaneously injected with 500 μL of diclofenac sodium salt ($C_{14}H_{10}Cl_2NNaO_2$, Sigma) at 2 mg/kg BW in sterilized saline into the dorsal skin with a 26 G needle [22]. After 12 weeks of treatments, the rats were anesthetized using CO_2 gas. All experimental protocols and procedures were approved by the Small Animal Care and Use Committee of the National Institute of Agricultural Sciences (NAS-201804). Body weight and diet intake were measured every week. Experimental design to evaluate in vivo anti-osteoarthritis effect of PS is shown in Figure 1.

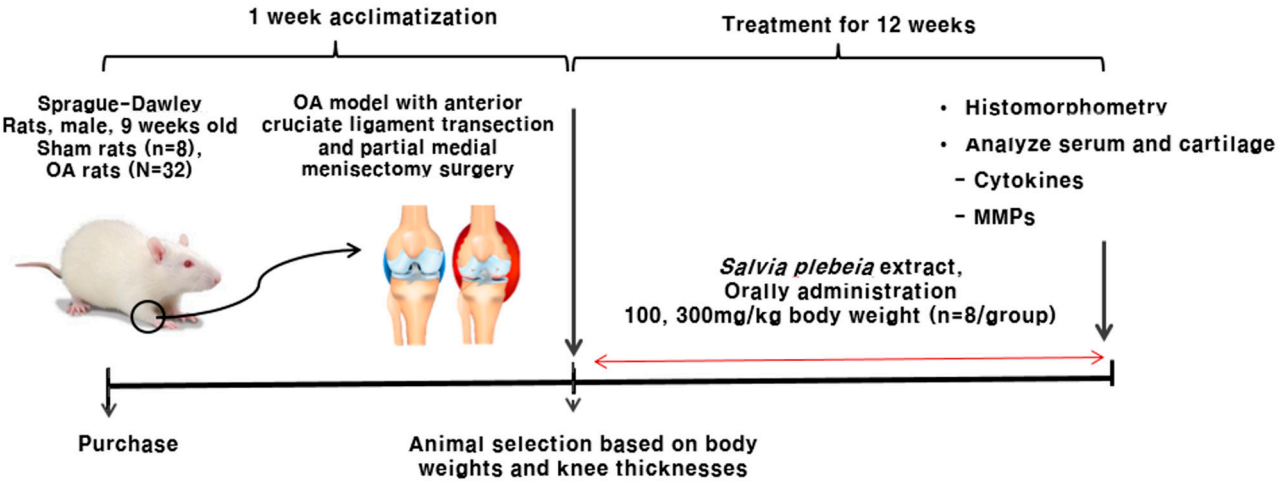


Figure 1. Experimental design for the animal study.

- Group 1: CON (normal control, sham, distilled water (DW)) (n = 8);
- Group 2: NC (negative control, osteoarthritis (OA), DW) (n = 8);
- Group 3: PC (positive control, OA, DW, diclofenac sodium salt 2 mg/kg BW) (n = 8);
- Group 4: PS1 (OA, low dose of PS 100 mg/kg BW) (n = 8);
- Group 5: PS2 (OA, high dose of PS 300 mg/kg BW) (n = 8).

2.2.3. Histological Process

One part in each knee joint, longitudinal section; one synovial cavity, including femur and tibia AC, and SM/IFP histological field in each sectioned knee joint were used (4 or 5 samples/group). All individual knee joint samples were decalcified in decalcifying solution (24.4% formic acid and 0.5 N sodium hydroxide) for 5 days. The mixed decalcifying solution was changed once per day. Each knee joint was longitudinally trimmed on part in each knee joint, including both femur and tibia AC with SM, and then embedded in paraffin using an automated tissue processor (Shandon Citadel 2000, Thermo Scientific, Waltham, MA, USA) and embedding center (Shandon Histostar), and 3–4 μm thick serial two section blocks were prepared using an automated microtome (RM2255, Leica Biosystems, Nussloch, Germany) in each paraffin block. Representative sections were stained with H&E for general histopathology and Safranin O (SO) for AC chondrocytes according to established methods [37,38]. Histological sections were prepared for all samples, and the histological

profiles were interpreted under a light microscope (Model Eclipse 80i, Nikon, Tokyo, Japan) as blinds to group distribution during this analysis.

2.2.4. Histomorphometrical Analysis

The entire histological evaluation was performed by the same pathologist. Mean femur and tibia AC and SM epithelial thicknesses (μm) were measured as histomorphometrical analyses on prepared longitudinally trimmed samples, using a computer-based automated image analyzer (iSolution FL ver 9.1, IMTi-solution Inc., Vancouver, British Columbia, Canada), with mean numbers of inflammatory cells infiltrated on the SM (cells/mm^2) according to previously established methods [22,37]. The histological fields observed in this inspection were selected around the central region of synovial cavity on the knee joint histological specimen. More than five histological fields in each knee joint section were considered to calculate each mean histomorphometrical value. To observe more detailed histopathological changes, the femur and tibia AC injuries found in the knees were evaluated and recorded using the Mankin scoring systems (Table 1) using Safranin O stain. In this system, the higher the score, the higher the level of OA (Semiquantitative scores; Max = 12).

Table 1. Variable scores of the Mankin scoring systems.

Surface Condition	Hypocellularity	Clone Formations	Stain Intensity for Safranin O
0 = normal	0 = normal	0 = normal	0 = normal
1 = irregular	1 = small decrease	1 = occasional duos	1 = small decrease
2 = fibrillation	in color	2 = duos or trios	in color
vacuoles	2 = large decrease in color	3 = multiple	2 = large decrease in color
3 = blisters anderosion	3 = no color	nested cells	3 = no color

2.2.5. Measuring Serum Cytokines, MMPs, and Lipocalin-2 Levels

Approximately 5 mL of whole blood was collected from the vena cava at euthanization, and the serum was separated by centrifugation at 2000 rpm for 15 min. Serum concentrations of TNF-α, IL-1β, IL-6, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, and lipocalin-2 were measured using sandwich enzyme-linked immunosorbent assays (ELISAs). TNF-α (ab46070, Abcam, Cambridge, UK), IL-1β (ab100768, Abcam), IL-6 (ab119548, Abcam), IFN-γ (ab46107, Abcam), MMP-2 (ab213910, Abcam), MMP-3 (LS-F5516, LSBio, Seattle, WA, USA), MMP-7 (LS-F5514, LSBio), MMP-8 (ab100779, Abcam), MMP-9 (LS-F32423, LSBio), MMP-13 (ab221839, Abcam), and lipocalin-2 (ab119602, Abcam) for rats were used in this measurement. The assays were performed according to the manufacturer's instructions, and the absorbance of the contents of each well was measured at 450 nm using a microplate reader (Molecular Devices, Silicon Valley, CA, USA).

2.2.6. Measuring mRNA Expressions of Cytokines and MMPs in the Cartilage of OA Rats

RNA was isolated from the cartilages with synovial membrane of intact or surgically induced OA rats using the RNeasy Mini Plus Kit (74106, Qiagen, Valencia, CA, USA). One-step quantitative polymerase chain reaction (qPCR) was carried out using Quantifast SYBR Green RT-PCR kit (204156, Qiagen) by a one-step cycler. Amplification was performed according to the conditions using the BioRad CFX-96 real-time system (BioRad, Hercules, CA, USA). All results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The primer information (Qiagen) used for qPCR is shown in Table 2.

Table 2. Primer information for gene expression in qPCR experiment.

Gene Symbol	Catalog Number	Detected Transcript(s)	Amplicon Length
GAPDH	QT00199633	NM_017008	149 bp
IL-6	QT00182896	NM_012589	128 bp
IL-18	QT02459506	XM_001070641	105 bp
MMP-2	QT00996254	NM_031054	103 bp
MMP-3	QT00189308	NM_133523	82 bp
MMP-9	QT00178290	NM_031055	149 bp

2.3. Statistical Analysis

All data are expressed as mean ± SEM. One-way ANOVA (one-way analysis of variance) was performed using Statistical Package for the Social Sciences (SPSS ver. 24, IBM Corp, Armonk, NY, USA). NC group was compared to the CON group by Student's *t* test, and a Duncan's multiple-range test was conducted to determine significant differences among the groups. Significant difference was considered at $p < 0.05$.

3. Results

3.1. Characterization of Flavonoids in Plebeian Sage and Its Extract

Plebeian sage leaf contains 77.8 mg of flavonoids, and its extract (PS) contains 365.5 mg flavonoids per each dry matter (g). Six representative compounds consisting of flavone (5) and flavanone (1) derivatives were tentatively identified from the leaf and PS through positive ionized mass fragmentation using UPLC-DAD-QToF/MS analysis (Table 3). The current positive ionized pattern of these compounds provides additional sodium (Na^+, m/z 23) and potassium (K^+, m/z 39) adduct ions with basic protonated molecules $[M+H]^+$ that can help to determine the parent ion. The conjugations with glucose moiety (162 Da) were the 7-OH position of their corresponding aglycones ($[M+H\text{-glucose}]^+$) such as 6-hydroxyluteolin (m/z 303), 5,7,3′,4′-tetrahydroxy-6-methoxyflavanone (m/z 319), luteolin (m/z 287), nepetin (m/z 317) and hispidulin (m/z 301).

Table 3. Characterization of isolated flavone and flavanone derivatives from Plebeian sage leaf and its extract (PS).

Compounds	ESI(+)-QToF/MS Fragmentation of $[M+H]^+$ (m/z)	Contents (mg/g Dry Matters)	
		Leaf	PS
6-hydorxyluteolin 7-*O*-glucoside	487, **465**, 303	13.5 ± 0.4	65.9 ± 4.6
5,7,3′,4′-tetrahydroxy-6-methoxyflavanone 7-*O*-glucoside (naasanone)	503, **481**, 319	9.4 ± 0.2	53.4 ± 1.3
luteolin 7-*O*-glucoside (cynaroside)	471, **449**, 287	11.3 ± 0.4	62.8 ± 1.3
nepetin 7-*O*-glucoside (nepitrin)	501, **479**, 317	23.5 ± 0.6	78.3 ± 1.9
hispidulin 7-*O*-glucoside (homoplantaginin)	485, **463**, 301	19.5 ± 0.4	104.8 ± 2.4
hispidulin	339, 323, **301**	0.6 ± 0.1	1.3 ± 0.1
Total		77.8 ± 1.9	365.5 ± 9.1

All samples analyzed in positive ESI-ionization mode (m/z $[M+H]^+$) of ToF-MS; $[M+Na]^+$ and $[M+K]^+$ adducts are presented. Bold font indicates parent ion ($[M+H]^+$) of flavonoid structures. Each content calculated as means ± SD (n = 3) using internal standard (6-methoxyluteolin).

Among these glycosides (mg/g dry matter of leaf), 6-hydorxyluteolin 7-*O*-glucoside (13.5 g), cymaroside (11.3 g), nepitrin (23.5 g) and homoplantaginin (19.5 g) were found as major constituents. Especially, 5,7,3′,4′-tetrahydroxy-6-methoxyflavanone 7-*O*-glucoside is a new flavanone glycoside (naasanone, 9.4 g). In the PS, most flavonoids increased by more than three times compared to those in the Plebeian sage leaf. PS contains 6-hydorxyluteolin 7-*O*-glucoside (65.9 g), naasanone (53.4 g), cymaroside (62.8 g), nepitrin (78.3 g) and homoplantaginin (104.8 g) as major constituents of flavonoids.

3.2. Animal Experiments

3.2.1. Effects of Treatment with PS on Body Weight and Food Efficiency Ratio

At the beginning of this experiment, there was no significant difference in body weight among all groups of the sham and OA rats. Body weight, body weight gain, and food intake were similar in all experimental groups (Table 4).

Table 4. Effects of PS on the final body weight, body weight gain, and food intake in the surgically induced OA rats.

Groups	Body Weight (g)		Weight Gain (g)	Food Intake (g/rat)
	0 Week	12th Week		
CON	374.6 (6.1 ^{NS})	615.6 (17.7 ^{NS})	241.0 (16.4 ^{NS})	1560.0 (38.5 ^{NS})
NC	371.1 (10.7)	610.8 (10.8)	239.7 (9.8)	1594.7 (44.3)
PC	379.7 (5.4)	613.2 (15.0)	233.6 (15.0)	1534.1 (6.4)
PS1	374.8 (14.8)	622.3 (18.2)	247.5 (14.0)	1566.1 (58.3)
PS2	369.7 (7.2)	620.5 (19.4)	250.9 (12.4)	1544.0 (84.5)

CON: normal sham control; NC: negative control; PC: positive control, which was subcutaneously injected with diclofenac sodium salt at 2 mg/kg BW in sterilized saline into the dorsal skin; PS1: PS at 100 mg/kg BW; PS2: PS at 300 mg/kg BW. The data were presented as the mean ± S.E. (n = 4~5/group). ^{NS} Mean values are not significantly different ($p < 0.05$) among groups by Duncan's multiple range test.

3.2.2. Effects of PS on the Articular Surface Lining Cartilage with SM on the Knee Joint Tissues in OA Rats

The representative general H&E stained histopathological profiles of knee joint tissues are shown in Figure 2. This figure shows that PS affected histopathological profiles of knee joint tissues in rats with surgically induced OA compared to those of the NC group. As shown in the NC group (Figure 2b), significant decreases in the femur and tibia AC thickness and increases on the SM epithelial thicknesses and inflammatory cell numbers infiltrated around the SM as compared to those of the normal CON group (Figure 2a) were found, suggesting classic OA histopathological lesions in the NC group. However, Figure 2d,e showed that PS1 and PS2 significantly increased the femur and tibia AC thickness while they decreased the SM epithelial thicknesses and inflammatory cell numbers in a dose-dependent manner.

Table 5 shows the histomorphometrical results on the articular surface lining cartilage and synovial membrane. Mean femur AC thickness in OA control NC group was reduced to 40.6% as compared with that of the normal CON group, but the value was improved as 209.7%, 220.2%, and 233.9% in PC, PS1, and PS2 groups compared to that of the NC group, respectively. Mean tibia AC thickness in NC was also changed at 43.8% as compared with that of the CON group, but the level increased to 199.2%, 176.5%, and 213.3% in PC, PS1, and PS2 groups compared to that of the NC group, respectively. Mean SM epithelial thicknesses of the knee joints in the NC group increased to 309.9% as compared with that of the CON group, but the value decreased to 52.2%, 49.8%, and 44.6% in the PC, PS1, and PS2 groups, respectively, as compared with that of the NC group. In the NC group, mean numbers of inflammatory cells infiltrated around SM of the knee joints increased by 400.0% as compared with that of the CON group, but the level changed as 48.8%, 35.2%, and 25.9% in the PC, PS1, and PS2 groups, respectively, compared with that of the NC group (Table 5). Thus, PS significantly improved histomorphometrical values of the articular surface lining cartilage and synovial membrane, and the effectiveness was similar or higher than that of the PC group treated with diclofenac sodium salt.

3.2.3. Effects of PS on the Mankin Score of the Knee Joint Tissues

SO-stained histopathology of knee joint tissues in OA rats are shown in Figure 3. In the NC group, significant surface cartilage damages and increased clone formations in both femur and tibia AC were detected. However, PS improved surface cartilage condition and decreased clone formations that affected the histopathological characteristics of the

joints and synovial tissues of the knee joint tissues in the rats with surgically induced OA compared to those of the NC.

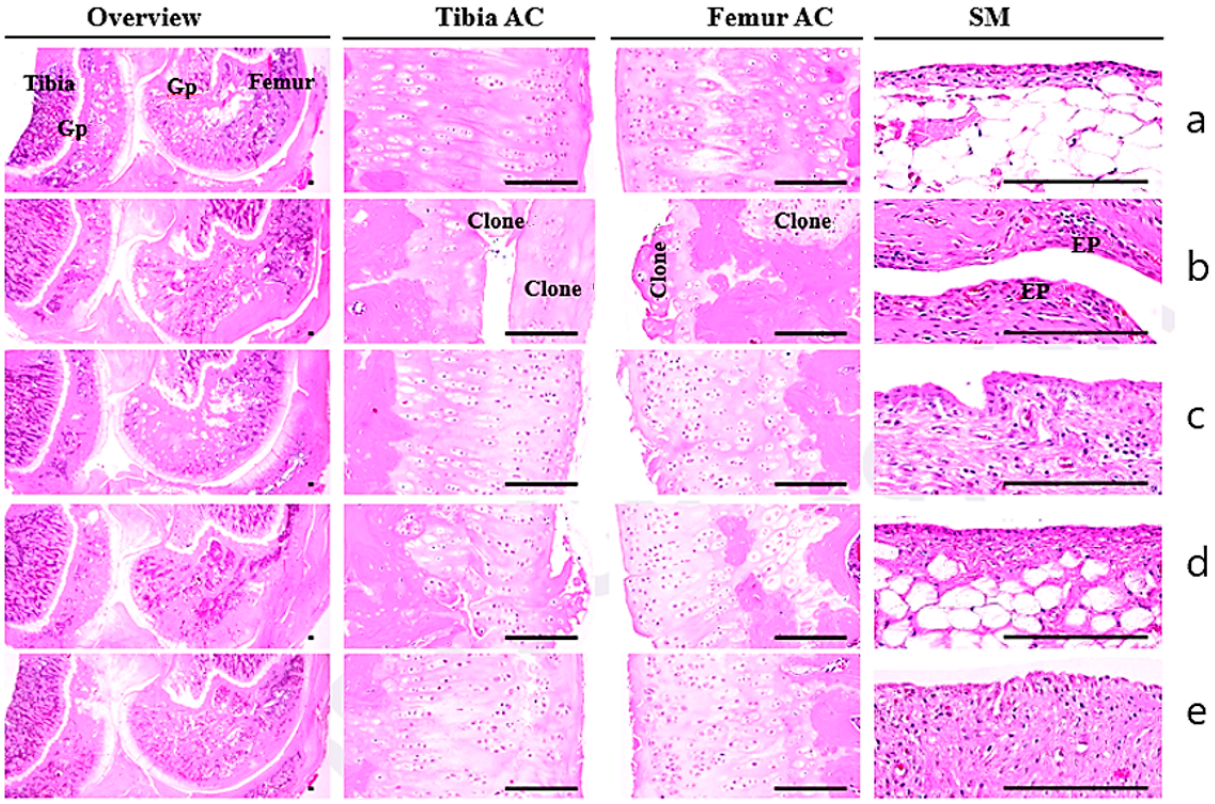


Figure 2. The effects of PS on H&E-stained histopathological profiles of knee joint tissues in rats with surgically induced OA (40×). (**a**) CON: normal sham control; (**b**) NC: negative control; (**c**) PC: positive control, rats treated with diclofenac sodium salt at 2 mg/kg BW in sterilized saline into the dorsal skin; (**d**) PS1: PS at 100 mg/kg BW; (**e**) PS2: PS at 300 mg/kg BW. Gp: growth plate; EP: epithelium; scale bars = 160 μm.

Table 5. The effects of PS on histomorphometrical values of the articular surface lining cartilage and synovial membrane of the knee joint tissues in the OA rats.

Groups	Articular Surface Lining Cartilage			Synovial Membrane
	Femur Thickness (μm)	Tibia Thickness (μm)	Mean Epithelial Thickness (μm)	Mean Inflammatory Cells (Cells/mm^2)
CON	430.0 (39.5 ^{a})	467.2 (49.3 ^{a})	8.1 (0.2 ^{b})	41.5 (31.7 ^{c})
NC	174.4 (25.7 ***,^{b})	204.8 (19.8 ***,^{b})	25.1 (0.6 ***,^{a})	166.0 (15.9 ***,^{a})
PC	365.7 (47.9 ^{a})	408.0 (28.3 ^{a})	13.1 (2.8 ^{b})	81.0 (10.7 ^{b})
PS1	384.0 (57.8 ^{a})	361.5 (33.4 ^{a})	12.5 (2.4 ^{b})	58.5 (3.1 ^{bc})
PS2	408.0 (53.5 ^{a})	437.0 (30.9 ^{a})	11.2 (2.0 ^{b})	43.0 (3.3 ^{c})

CON: normal sham control; NC: negative control; PC: positive control; PS1: PS at 100 mg/kg BW; PS2: PS at 300 mg/kg BW. The data were analyzed by one-way ANOVA using SPSS software and presents as the mean ± S.E. (n = 4~5/group). NC group is significantly different from the CON group at *** $p < 0.001$ by Student's t test. $^{a–c}$ Mean values with different letters are significantly different ($p < 0.05$) among groups by Duncan's multiple range test.

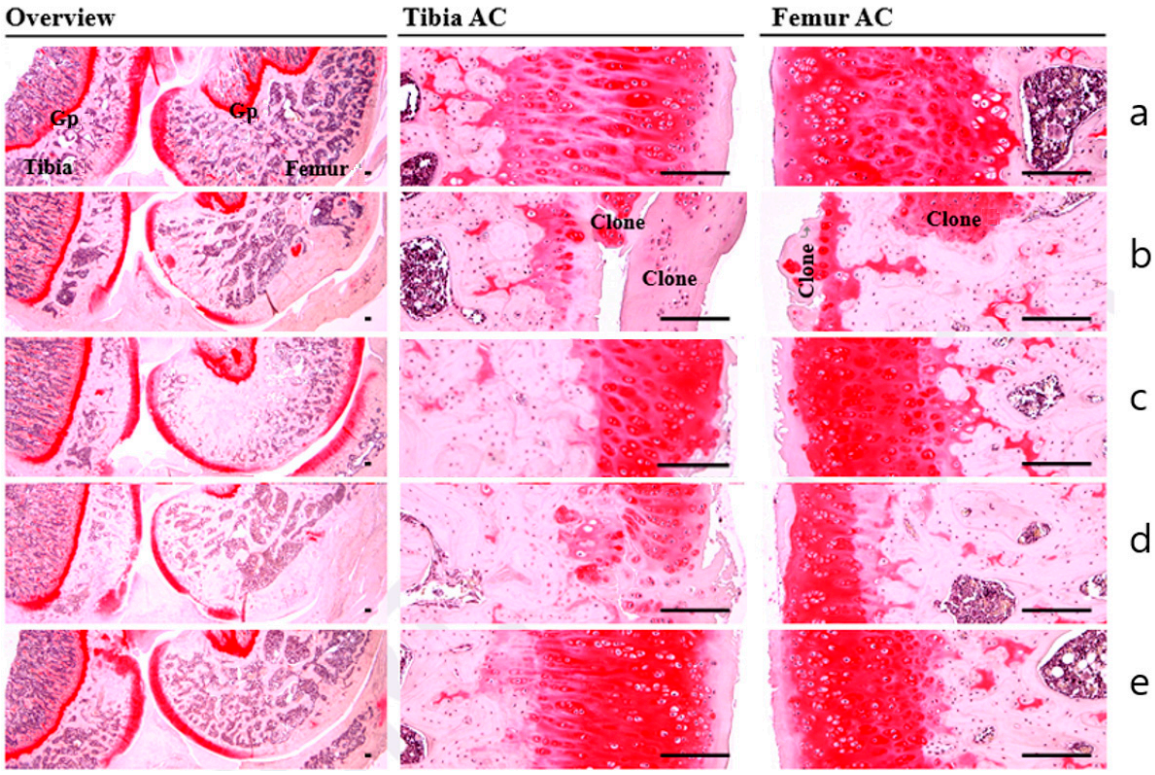


Figure 3. The effects of PS- on SO-stained histopathological characteristics of joints and synovial tissues of knee joint tissues in surgically induced OA rats (40×). (**a**) CON: normal sham control; (**b**) NC: negative control; (**c**) PC: positive control, rats treated with diclofenac sodium salt at 2mg/kg BW in sterilized saline into the dorsal skin; (**d**) PS1: PS at 100 mg/kg BW/day; (**e**) PS2: PS at 300 mg/kg BW/day. Gp; growth plate, EP; epithelium, scale bars = 160 μm.

In Table 6, the OA control (NC) showed marked increases in surface cartilage damages and decreases in chondrocytes, clone formations, and Safranin O stain intensities on both femur and tibia AC. Consequently, significant ($p < 0.01$) increases in the Mankin scores were detected in the NC group as compared with those in the normal CON group. However, the Mankin scores of femoral and tibial AC were lower in PS groups than those of the NC group, in a dose-dependent manner. Totalized Mankin score of the knee joints in OA control NC group was changed by 864.0% as compared with that of the intact control CON group, but they were reduced as 50.9%, 48.6%, and 23.1% in PC, PS1, and PS2 groups, respectively, as compared with that of the NC group.

Table 6. The effects of PS on histomorphometrical data obtained by the Mankin scoring systems on the knee joint tissues of OA rats.

Groups	Surface Condition (Max = 3)	Hypocellularity (Max = 3)	Clones (Max = 3)	Stain Intensity (Max = 3)	Totalized Final Score (Max =12)
CON	0.50 (0.29 ^{c})	0.25 (0.25 ^{cd})	0.00 (0.00 ^{c})	0.50 (0.29 ^{c})	1.25 (0.48 ^{c})
NC	2.80 (0.00 ^{***,a})	2.40 (0.29 ^{***,a})	3.00 (0.00 ^{***,a})	2.60 (0.25 ^{***,a})	10.80 (0.48 ^{***,a})
PC	1.25 (0.25 ^{bc})	1.25 (0.25 ^{b})	1.25 (0.48 ^{b})	1.75 (0.25 ^{ab})	5.50 (0.96 ^{b})
PS1	1.75 (0.48 ^{b})	1.00 (0.41 ^{bc})	1.25 (0.25 ^{b})	1.25 (0.48 ^{bc})	5.25 (1.25 ^{b})
PS2	1.00 (0.00 ^{bc})	0.00 (0.00 ^{d})	0.50 (0.29 ^{c})	1.00 (0.00 ^{bc})	2.50 (0.29 ^{c})

CON: normal sham control; NC: negative control; PC: positive control, which was subcutaneously injected with diclofenac sodium salt at 2 mg/kg BW in sterilized saline into the dorsal skin; PS1: PS at 100 mg/kg BW; PS2: PS at 300 mg/kg BW. The data are presented as the mean ± S.E. (n = 4~5/group). NC group is significantly different from the CON group at *** $p < 0.001$ by Student's t test. $^{a–d}$ Mean values with different letters are significantly different ($p < 0.05$) among groups by Duncan's multiple range test.

3.2.4. Effects of PS on Serum Inflammatory Cytokines Levels

The results indicate that there was a clear increase in the levels of IL-1β, IL-6, and TNF-α in the surgically induced OA, NC group ($p < 0.01$) compared to the CON group (Figure 4). However, daily treatment with PS at a dose of 100 or 300 mg/kg BW significantly decreased the release of IL-1β, IL-6, and TNF-α into the serum ($p < 0.05$ or $p < 0.01$) compared to those of the NC group. PS group showed significantly reduced serum IL-1β level compared to the NC group. Furthermore, the IL-1β level of the PS group was reduced over 34% compared to that of the PC group, without significant differences between the groups. In the PS groups, serum IL-6 levels also significantly decreased compared to the NC group, and the effect was compared to the PC group. The serum IFN-γ levels tend to decrease in the PC and PS groups, although there was no significant difference found (2.4~2.6 pg/mL).

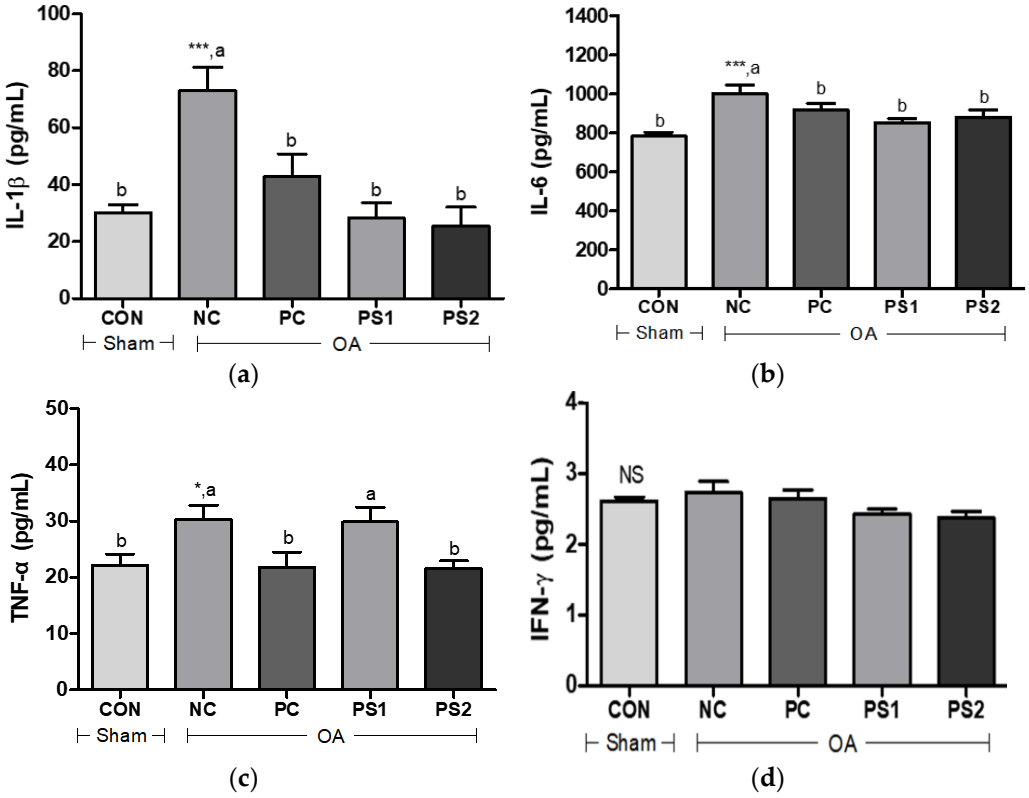


Figure 4. Effects of PS on serum inflammatory cytokine (**a**) IL-1β, (**b**) IL-6, (**c**) TNF-α, and (**d**) IFN-γ levels in OA rats. CON: normal sham control; NC: negative control; PC: positive control; PS1: PS at 100 mg/kg BW; PS2: PS at 300 mg/kg BW. The data are presented as the mean ± SE (n = 4~5/group). NC group was significantly different from the CON group at * $p < 0.05$, *** $p < 0.001$ by Student's t test. ^{NS} Not significantly different among groups. ^{a,b} Mean values with different letters are significantly different ($p < 0.05$) among groups by Duncan's multiple range test.

3.2.5. Effects of PS on Serum MMPs Levels

Serum MMPs (MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13), and lipocalin-2 levels were evaluated in the OA rats and are presented in Figure 5. The serum MMP-2, MMP-7, MMP-8, MMP-9, MMP-13, and lipocalin-2 levels were higher in the OA NC group compared with those in the normal CON group. MMP-2 level decreased in the PS groups, showing a dose-dependent manner, and a significant difference was found in the PS2 group compared to the NC group (Figure 5a). Serum MMP-3 level increased in the OA experimental animals compared to the CON group, and the levels were lower in the PS groups ($p > 0.05$), although there was no significant difference among the experimental animals. Serum MMP-7 and 8 levels were higher in the OA animals compared to those of the normal CON group. However, the values decreased in the PC and PS groups. PS

lowered the serum MMP-7 level in a dose-dependent manner. PS's effect on the MMP-8 was comparable to the diclofenac sodium salt, which was used as a positive control. Serum MMP-9 and 13 values increased in the OA rats compared with the CON group. However, the values significantly decreased in the PC and PS groups compared to the NC group. The lipocalin-2 level, which was increased in the OA rats, was lowered by PS. Moreover, MMP-2, MMP-7, and lipocalin-2 levels more effectively decreased in the groups administered with PS at 300 mg/kg ($p < 0.01$) than in the PC group as a positive control. Generally, PS affected the serum MMPs and lipocalin-2 levels and MMP-2, 7, and lipocalin-2 levels decreased in a dose-dependent manner.

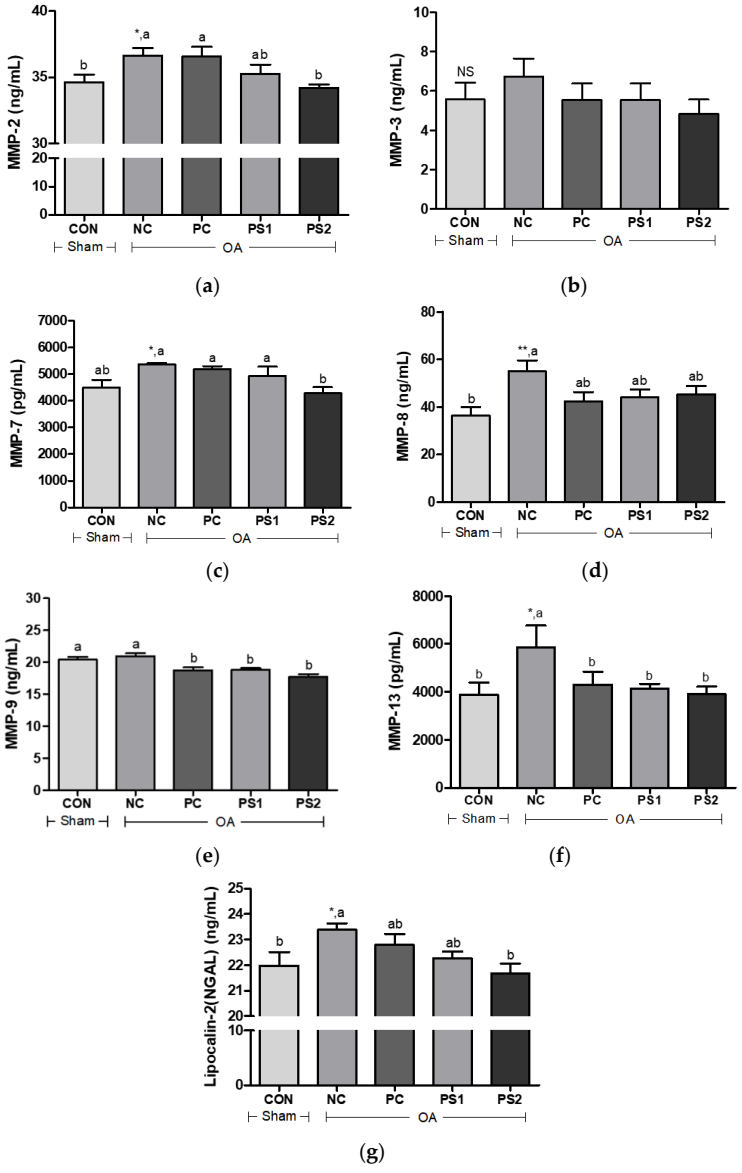


Figure 5. Effects of PS on serum MMPs (**a**) MMP-2, (**b**) MMP-3, (**c**) MMP-7, (**d**) MMP-8, (**e**) MMP-9, (**f**) MMP-13, and (**g**) lipocalin-2 levels in OA rats. CON: normal sham control; NC: negative control; PC: positive control; PS1: PS at 100 mg/kg BW; PS2: PS at 300 mg/kg BW. The data are presented as the mean ± SE (n = 4~5/group). NC group was significantly different from CON group at * $p < 0.05$, ** $p < 0.01$ by Student's t test. ^{NS} Not significantly different among groups. ^{a,b} Mean values with different letters are significantly different ($p < 0.05$) among groups by Duncan's multiple range test.

3.2.6. Effects of PS on mRNA Expressions of Cytokines IL-6 and IL-18 in Cartilage

Figure 6 shows higher levels of IL-6 and IL-18 in the cartilage with synovial membrane of the NC group ($p < 0.01$) compared to the normal CON group. However, daily treatment with PS at a dose of 100 or 300 mg/kg decreased the release of IL-6 and IL-18 expression in the cartilage. The significant difference was found in the IL-6 and IL-18 ($p < 0.01$ or $p < 0.001$) compared to the NC group. The mRNA expression level of cytokine IL-6 was comparable to the diclofenac sodium salt for the PC group. Furthermore, the mRNA expression level of cytokine IL-18 was significantly and effectively reduced in the PS group versus the NC and PC groups.

3.2.7. Effects of PS on MMPs Expression in Cartilage with Synovial Membrane

The expression levels of MMP-2, MMP-3, and MMP-9 in the cartilage with synovial membrane of OA rats are shown in Figure 7. They were significantly higher in the NC group compared with those of the CON group. PC and PS groups decreased the expression levels of MMP-2, MMP-3, and MMP-9 in cartilage. PS2 treatment effectively affected the MMP expression levels of OA rats and showed a similar or higher effect than the diclofenac sodium salt used for the PC group.

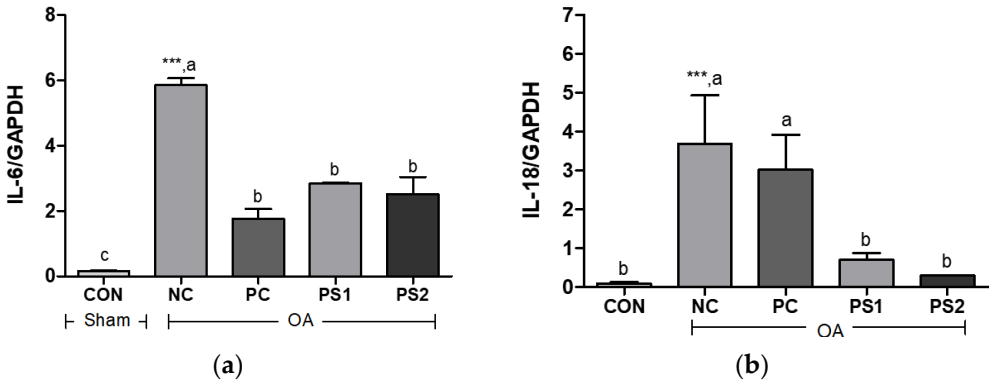


Figure 6. Effects of PS on cytokines expression in the cartilage with synovial membrane of OA rats. (**a**) IL-6 and (**b**) IL-18. Data are expressed as the mean ± SE (n = 4/group). CON: normal sham control; NC: negative control; PC: positive control; PS1: PS at 100 mg/kg BW; PS2: PS at 300 mg/kg BW. NC group was significantly different from the CON group at *** $p < 0.001$ by Student's t test. [a–c] Mean values with different letters are significantly different ($p < 0.05$) among groups by Duncan's multiple range test.

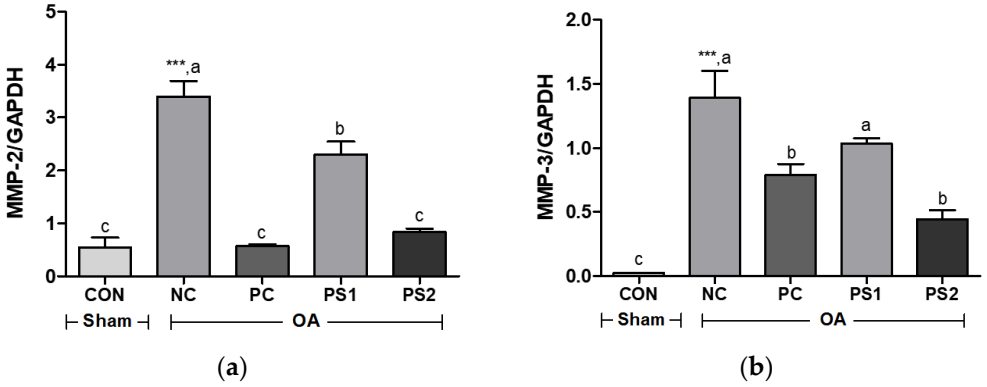
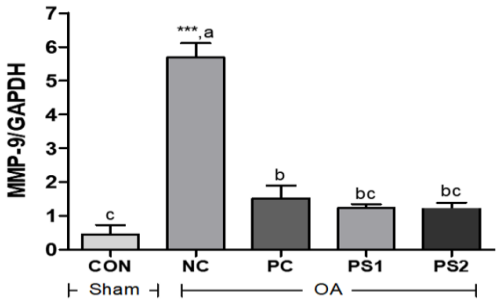


Figure 7. *Cont.*



(**c**)

Figure 7. Effects of PS on cytokines expression in the cartilage with synovial membrane of OA rats. (**a**) MMP-2, (**b**) MMP-3, and (**c**) MMP-9. Data are expressed as the mean ± SE (n = 4/group). CON: normal sham control; NC: negative control; PC: positive control; PS1: PS at 100 mg/kg BW; PS2: PS at 300 mg/kg BW. NC group was significantly different from the CON group at *** $p < 0.001$ by Student's t test. $^{a–c}$ Mean values with different letters are significantly different ($p < 0.05$) among groups by Duncan's multiple range test.

4. Discussion

The primary approach in the clinical treatment of OA involves the use of nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics, and hyaluronan, which allow for symptomatic relief but provide no apparent disease-modifying effects [3]. In some instances, NSAIDs may even have deleterious effects, as they inhibit the synthesis of proteoglycan, which plays a crucial role in maintaining the functions of the cartilage. Therefore, there is a critical need to develop alternative agents that can prevent the destruction of cartilage or stimulate their proper repair [6,8].

OA is the most prevalent articular disease in the elderly [1] and is characterized by loss of articular cartilage components, leading to tissue destruction and hypocellularity, eventually resulting in loss of joint function [19,27]. Alternative agents that can prevent the destruction of cartilage or stimulate its proper repair are needed [6] due to the deleterious effects of NSAIDs [3]. In this study, anti-arthritis effects were evaluated from PS with high anti-oxidative [30] and anti-inflammatory effects [32]. The active components of PS comprise flavonoids and phenolic acid [34,35]. These materials are known to have antioxidant and anti-inflammatory effects, and the inflammation may be controlled by reducing nitric oxide generation [39].

Mankin's 12-point histopathological grading system of cartilage deterioration is based on cartilage surface damage, the number of chondrocytes, and Safranin O staining. In this study, normal cartilage was scored as 0 in the control CON group; the most severe case of cartilage deterioration had a high score of 10.8 in the NC group (Table 5) as shown in the previous report [22]. OA control rats (NC) showed marked increases in surface cartilage damage, decreases in chondrocytes, and clone formations on both femur and tibia AC when compared with those of normal control rats (CON). In addition, NC also showed significant decreases in the mean femur and tibia AC thicknesses, and increases in SM epithelial thicknesses and inflammatory cell numbers infiltrated around the SM/IFP as compared to those of the CON, suggesting classic OA histopathological lesions. Inflammation and fibrosis presented in SM/IFP. However, these histopathological surgically induced histopathological OA lesions were obviously and most significantly inhibited by PC and PS treatments in a dose-dependent manner. PS2 showed more favorable inhibitory effects than those of the commercial reference (PC), and PS1 was comparable to the anti-OA effects of PC (Figure 3, Table 5). In the previous trial using a mixture of dried pomegranate concentrate powder, eucommiae cortex, and achyranthis radix, this improved the decreases in AC thickness and the number of chondrocytes detected in OA rats [22]. OA is the most common clinical syndrome of joint pain accompanied by varying degrees of functional limitation [4]. PS inhibited the inflammatory response in human rheumatoid synovial

fibroblasts and a murine model of arthritis [33]. Thus, effects of PS on joint pain in a rat model of surgically induced OA and comparisons of the mechanisms in different arthritis models should be measured in future studies [40].

OA is a debilitating disease that affects the AC and subchondral bone, and inflammation is closely involved in all steps of OA progression [39]. The inflammatory factors TNF-α, IL-1β, and IL-6 contribute to OA progression [41]. OA-induced rabbits exhibited significant decreases in AC thickness of the femur and tibia. It is also reported that the number of chondrocytes significantly increased synovial IL-1β, IL-6, and TNF-α levels and significantly increased numbers of femoral and tibial subchondral IL-1β and TNF-α-immunolabeled cells [42]. In this trial, treatment of PS favorably inhibited surgically induced OA through anti-inflammatory and AC preserving effects (Figure 3). The inflammatory cytokine (IL-1β IL-6, TNF-α) levels increased in the NC group versus those of the normal control CON group. Conversely, these OA-induced symptoms of inflammation were significantly inhibited by 12 weeks of supplement with PS (100 and 300 mg/kg body weight). PS decreased the release of IL-1β, IL-6, and TNF-α into the serum of OA rats. These findings represent reliable evidence that PS has favorable anti-inflammatory effects on surgically induced OA rats, and these results are consistent with the report showing a strong correlation between low inflammatory levels and anti-osteoarthritis effects in animals supplemented with functional foods [22,25].

MMPs are involved in the degradation of the ECM [42], although their expression can be suppressed by metalloproteinase inhibitors [43]. The MMPs are reliable predictors of cartilage loss during the development of OA [44], and their increase has been reported in the synovium and chondrocytes of surgically induced OA models [45]. Lipocalin-2 is believed to play a significant functional role in OA cartilage [46] as a biomarker for cartilage degradation in arthritic disease [47], although Choi and Chun suggested that its upregulation in osteoarthritic cartilage is not necessary for cartilage destruction in mice [16]. In this study, we found that PS decreased serum MMP-2, MMP-7, MMP-9, MMP-13, and lipocalin-2 levels when administered at a dose of 100 or 300 mg/kg ($p < 0.05$) compared with those of the NC group. After treating the rats with PC, weak effects on the serum MMP-2, -3, -7, -8, and lipocalin-2 levels of OA rats were observed ($p > 0.05$). It is interesting that PS improved the biomarkers such as MMP-2, -7, and lipocalin-2 in a dose-dependent manner and was more effective than diclofenac sodium salt for the PC group. Furthermore, not only Plebeian sage extracted with 95% v/v EtOH at 70 °C [33] but also Plebeian sage extracted with 50% v/v EtOH at room temperature effectively inhibited osteoarthritis. This is a great news to customers and health food producers with its low producing price and higher safety. Thus, the PS used in this study may be used widely for osteoarthritis treatment with lower prices and improved safety.

iNOS is induced by pro-inflammatory cytokines and is involved in high levels of NO production during the inflammatory process [48,49]. NF-κB proteins constitute a family of transcription factors stimulated by pro-inflammatory cytokines, chemokines, stress-related factors, and ECM degradation products. The activated NF-κB molecules trigger the expression of genes that induce destruction of the articular joint [10]. The activation of stress- and inflammation-induced signaling, transcriptional, and posttranscriptional events may cause a phenotypic shift, apoptosis, and aberrant expression of inflammation-related genes, including catabolic genes [25]. These include nitric oxide synthase (NOS)-2, cyclooxygenase (COX)-2, and several matrix metalloproteinases (MMPs) [26,45]. In this study, we evaluated whether PS could ameliorate inflammation and cartilage degradation in surgically induced osteoarthritis rats. Extended evaluation should be performed by measuring joint pain of experimental animals and by studying chondrocytes and synoviocytes to verify its effects on controlling pro-inflammatory cytokines, chemokines, and stress-related factors.

5. Conclusions

The present investigation demonstrates that PS significantly improved surface cartilage damage, Mankin scores, mean femur and tibia AC thicknesses, and inflammatory cell numbers in rats with surgically induced OA. PS significantly decreased serum inflammatory cytokines and MMPs levels, which were elevated due to OA. Thus, it is posited that PS may be used as a novel potential therapeutic regime for various OA and may reduce the risk of cartilage loss and disease progression in the elderly with OA. Further detailed molecular mechanism studies are recommended to verify our findings.

Author Contributions: S.-H.L. and B.-R.C. conceived and designed the study. S.-H.L., E.-B.L., J.-H.C., H.-W.K. and S.-K.K. conducted experiments and analyzed the data. S.-H.L. and E.-B.L. wrote the manuscript. S.-H.L., H.-H.J., H.-W.K. and J.-S.L. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Ethics Committee of the National Institute of Agricultural Sciences (protocol code NAS201804) and was conducted according to the guidelines for animal experiments in the protocol.

Informed Consent Statement: Not applicable.

Data Availability Statement: The study did not report any data.

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Conflicts of Interest: The authors declare no conflict of interest.

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