Original Article

Selection of the optimal herbal composition of pomegranate concentrated powder from aqueous extracts of *Eucommiae Cortex* and *Achyranthis Radix* to treat osteoarthritis in rats

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Objectives: We investigated whether a mixture of the main component of pomegranate concentrated powder (PCP) with appropriate proportions of *Eucommiae Cortex* (EC) and *Achyranthis Radix* (AR) could act synergistically as an effective treatment for osteoarthritis (OA).

Methods: In order to evaluate the effects of PCP, EC, and AR against OA, knee thicknesses, maximum extension angle of each knee, anti - inflammation effects, the transcript levels of chondrogenic genes mRNA expressions in femur and tibia articular cartilage (AC) with synovial membrane (SM) were analyzed. In addition, the histopathology and immunohistochemistry of the femur and tibia AC or SM were performed.

Results: The surgically-induced OA signs in rats were significantly inhibited by 28 days of continuous treatment of PCP, EC and AR single formulas, and PCP with EC:AR mixed formulas. Especially, PCP with EC:AR 4:1, 2:1 and 1:1 mixed formula treatment constantly showed significantly more favorable inhibitory activities, as compared with those of single formula of PCP, EC and AR treated rats.

Conclusion: PCP and EC:AR 4:1 mixed formula showed similar OA refinement effects through potent anti-inflammatory pathways as compared with those of diclofenac treatment, and showed additional chondrocyte proliferating effects on the both femur and tibia AC.

Key Words : Mixed formulation, Selections of the fittest mixed formula, Dried pomegranate concentrate powders, *Eucommiae Cortex*, *Achyranthis Radix*, Surgically-induced osteoarthritic rats.

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Introduction

Osteoarthritis (OA) is one of the most prevalent articular diseases in elderly adults¹⁾, and clinical syndromes such as joint pain result in functional limitations that reduce quality of life²⁾. OA is a degenerative inflammatory process of articular cartilage (AC) that includes changes in structure and in articulation function^{3,4)}. The extracellular matrix (ECM) of AC is composed mainly of collagen and proteoglycans (PGs), aggrecan, and SOX9^{5,6)}. Tissue destruction and hypocellularity follow once AC components are lost, leading to eventual loss of joint function⁷⁾. In particular, proinflammatory cytokines stimulate cartilage resorption and inhibit synthesis of new matrix components in AC^{8,9)}. Additionally, cytokines increase prostaglandin E₂ (PGE₂) levels and nitric oxide release from chondrocytes^{10,11)}, which have both been implicated in turnover of the cartilage matrix^{12,13)}. However, the etiopathology of OA is not fully understood¹⁴⁾.

Although use of nonsteroidal anti-inflammatory drugs during clinical treatment of OA can be deleterious, they suppress the synthesis of PGs¹⁵⁾. Efforts have been made to develop and identify agents that preserve cartilage^{5,6,16)}. Natural sources and formulations have achieved potent anti-arthritis effects, and pomegranate products have traditionally been used to treat various diseases¹⁷⁾. Pomegranate consists of crude fiber, pectin, sugars, and several tannins¹⁸⁾. Notably, pomegranate seed oil and juice contain abundant flavonoids and anthocyanidins^{19,20)}. Furthermore, accumulating evidence indicates that pomegranate has powerful and favorable protective effects in chondrocytes through potent antioxidant and anti-inflammatory effects²¹⁻²⁴⁾. *Eucommiae Cortex* (EC), which is the dried stem bark of *Eucommia ulmoides* Oliver (Eucommiaceae), has been used as a traditional medicine in Korea for its antihypertensive, anti-inflammatory, antiviral, kidney-invigorating, and hepatoprotective activities, which it achieves without

side effects²⁵⁻²⁷⁾. In particular, as a component of mixed herbal formulations, EC has been reported to have anti-arthritis effects^{28,29)}. *Achyranthis Radix* (AR), the dried roots of *Achyranthes bidentata* Blume, is an important traditional Korean medicine that is used extensively by medical practitioners to treat osteodynia of the lumbar region and knees, spasms, and flaccidity of the limbs³⁰⁾. In particular, the anti-arthritis effects of AR have also been well documented in *in vivo* and *in vitro* studies^{31,32)}.

We hypothesized that a mixed formulation of pomegranate concentrated powder (PCP) and appropriate proportions of EC and AR would enhance its protective effects against OA because, through their diverse ingredients, mixtures of medicinal agents can elevate biological activities that combat many diseases. Thus, this study aimed to select or observe the optimal ranges showing obvious synergistic anti-OA potential of mixtures of PCP, EC, and AR and their effects on surgically induced OA in rats were examined.

Materials and Methods

1. Animals and induction of OA: transection of the anterior cruciate ligament and partial medial meniscectomy

In total, 150 healthy male specific-pathogen-free/VAF outbred-Crl: CD1 rats were used after a 7-day acclimation. The animals were allocated five to a polycarbonate cage in a temperature- (20–25°C) and humidity- (45–55%) controlled room. The light: dark cycle was 12 h: 12 h, and feed (Samyang, Seoul, Korea) and tap water were supplied *ad libitum*. Six days after the OA operation, 10 rats in each group were selected based on body weight and knee thickness deviations. Fifteen groups of 10 rats/group were killed for analyses.

The rats were anesthetized with 2–3% isoflurane in a mixture of 70% N₂O and 28.5% O₂ and were

maintained with 1–1.5% isoflurane in a mixture of 70% N₂O and 28.5% O₂ using a rodent inhalation anesthesia apparatus and a rodent ventilator. The OA treatment group underwent open surgery involving transection of the anterior cruciate ligament and a partial medial meniscectomy via an incision on the medial aspect of the joint capsule, anterior to the medial collateral ligament. The incision was closed in two layers. The joint capsule was sutured independently from peripheral tissues using dissolvable 5-0 Vicryl, and the skin was closed with interrupted silk sutures. This treatment was used to induce OA pathogenesis and is referred to as the operation-induced side. Conversely, the right (non-operated, intact side) knee joint was used for the contralateral treatment. The second group of rats underwent a sham operation in which a similar incision was made in the joint capsule, but the anterior cruciate ligament was not transected and according to our established methods no partial medial meniscectomy was performed³³⁻³⁶.

All laboratory animals were treated according to the national regulations of the usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) prior to animal experiment [Approval No DHU2014-052].

Experimental groups (Fifteen groups, ten rats in each group were finally sacrificed)

- Vehicle control: Sham-operated and distilled water orally administered rats
- OA control: OA-surgery and distilled water orally administered rats
- Diclofenac: OA-surgery and diclofenac sodium 2 mg/kg subcutaneously treated rats
- PCP: OA-surgery and PCP single formula 200 mg/kg orally administered rats
- EC: OA-surgery and EC single formula 200 mg/kg orally administered rats
- AR: OA-surgery and AR single formula 200 mg/kg orally administered rats
- Nine types of PCP with EC:AR mixed formula 200 mg/kg orally administered OA rats
 - 1:1 = PCP 100 mg with EC:AR 1:1 (50:50 mg:mg) mixed formula administered OA rats
 - 1:2 = PCP 100 mg with EC:AR 1:2 (33:67 mg:mg) mixed formula administered OA rats
 - 1:4 = PCP 100 mg with EC:AR 1:4 (20:80 mg:mg) mixed formula administered OA rats
 - 1:6 = PCP 100 mg with EC:AR 1:6 (14:86 mg:mg) mixed formula administered OA rats
 - 1:8 = PCP 100 mg with EC:AR 1:8 (11:89 mg:mg) mixed formula administered OA rats
 - 2:1 = PCP 100 mg with EC:AR 2:1 (67:33 mg:mg) mixed formula administered OA rats
 - 4:1 = PCP 100 mg with EC:AR 4:1 (80:20 mg:mg) mixed formula administered OA rats
 - 6:1 = PCP 100 mg with EC:AR 6:1 (86:14 mg:mg) mixed formula administered OA rats
 - 8:1 = PCP 100 mg with EC:AR 8:1 (89:11 mg:mg) mixed formula administered OA rats

2. Preparations and administration of test substances

PCP (ASYA Meyve Suyu ve Gıda San. A.Ş., Ankara, Turkey) as pink colored powders contains 1.15 mg/g ellagic acid as an active ingredient, EC as deep brown colored powders contains 1.62 mg/g pinoresinol diglucoside, AR as brown colored powders contained 0.25 mg/g ecdysterone, were prepared and supplied by Health Love Ltd. (Anyang, Korea). Briefly, aliquots of the dried EC and AR were boiled at 121°C during 7h, and the resulting liquid was filtered. The filtrate was concentrated, and then spray dried to obtain a dried powder. All test materials were stored at 4°C to protect them from light and moisture. A 200-mg/kg dose was selected as the total dose of a mixed formula consisting of PCP with an EC:AR ratio based on the highest possible clinical dose in humans. The single herbal extracts, such as PCP, EC, and AR, were

administered at 200 mg/kg to compare their effects directly with any synergistic effects produced by the mixed formulas. The nine mixed herbal preparations (200 mg/kg; PCP with EC:AR = 1:1, 1:2, 1:4, 1:6, 1:8, 2:1, 4:1, 6:1, and 8:1) and three single formulas dissolved in distilled water were administered orally by gastric gavage once daily for 28 days beginning at 1 week after OA surgery at a rate of 5 ml/kg. Diclofenac (2 mg) was dissolved in 5 ml sterile saline and subcutaneously injected under the dorsal back skin at a rate of 5 ml/kg (2 mg/kg) once daily for 28 days beginning 7 days after OA surgery, as in our previous study³³⁻³⁶. Equal volumes of vehicle (distilled water) were administered orally to sham and OA control rats. Half quantities of the mixed formulations were prepared as 100 mg PCP in a total of 200 mg in 5 ml distilled water based on our *in vitro* data (not published), and the following amounts of EC and AR were added: 1:1, 1:2, 1:4, 1:6, 1:8, 2:1, 4:1, 6:1, and (mg: mg) 8:1 or 50:50, 33:67, 20:80, 14:86, 11:89, 67:33, 80:20, 86:14, and 89:11, respectively. Each single formula of PCP, EC, and AR was prepared by directly dissolving 200 mg in 5 ml distilled water.

3. Body weight measurements

Changes in body weight were measured at least once weekly beginning 1 day before the OA operation until death (24 h after the last (day 28) administration) using an automatic electronic balance. To reduce any effect of feeding, all experimental animals were fasted overnight for about 18 h before the OA surgery, at the initial administration, and at termination.

4. Knee thickness measurements

The thickness of individual OA-operated hind knees (left side) was measured using an electronic digital caliper (Mytutoyo, Tokyo, Japan) at 6 days after the OA operation, on the day of initial

administration, and at 1, 7, 14, 21, 27, and 28 days after treatment. Additionally, according to our established methods³³⁻³⁶, knee thickness was measured after exposing the joint capsule and after sacrifice to reduce any differences resulting from surrounding tissues.

5. Measurement of maximum extension angle of each knee

The OA-operated knees of all animals were dissected from the coxofemoral to the ankle region, leaving the articular capsule intact. After dissection, the maximum extension angle of each knee was measured according to previous methods³³⁻³⁷, 0° corresponded to the maximum possible extension. All operations and measurements of extension were performed by the same veterinarian to minimize bias.

6. Preparation of the femur and tibia AC with SM tissue homogenates

After measuring the maximum extension angle of each knee, some parts of the femoral and tibial AC with SM tissue were separated individually, and tissue homogenates were prepared using a bead beater (Model TacoTMPre, GeneResearch Biotechnology Corp., Taichung, Taiwan), ultrasonic cell disruptor (Model KS-750, Madell Technology Corp., Ontario, CA, USA), and radioimmunoprecipitation (RIPA) assay buffer (Sigma-Aldrich, St. Louise, MO, USA). The supernatant was separated by centrifugation at 21,000 × g and 4°C to measure PGE₂ levels and 5-LPO, MMP-2, and MMP-9 activities.

7. Detection of the PGE₂ levels on the femoral and tibial AC with SM

The PGE₂ contents in the prepared supernatants of femoral and tibial AC with SM tissue homogenates were separately measured using a commercial PGE₂ assay kit (Cat No. KGE004B; R&D Systems, Minneapolis, MN, USA) with a micro plate reader

(Sunrise; Tecan, Männedorf, Switzerland) as pg/ml levels, according to instructor's protocol, as reported previously¹⁶⁾.

8. Assay of 5-LPO activity

The 5-LPO activities in the prepared supernatants of femoral and tibial AC with SM tissue homogenates were separately measured using a commercial Lipoygenase Inhibitor Screening Assay kit (Cat No. 760700; Cayman Chemical, Ann Arbor, MI, USA) with a micro plate reader as $\mu\text{M}/\text{min}/\text{ml}$ of supernatant according to instructor's protocol, as reported previously¹⁶⁾.

9. MMP inhibitory assays

The MMP-2 and MMP-9 activities in the prepared supernatants of femoral and tibial AC with SM tissue homogenates were separately measured a commercial MMP ELISA kit (Cat No. MBS720217 for MMP-2 and Cat No. MBS722532 for MMP-9; Mybiosource, San Diego, CA, USA) with a micro plate reader at OD 560 nm as ng/ml of supernatant according to instructor's protocol, as reported previously⁶⁾, respectively.

10. Realtime RT-PCR for ECM related chondrogenic gene mRNA expressions

Collagen type II, SOX9 and aggrecan mRNA expressions in the prepared femoral and tibial AC and SM homogenates were detected by realtime reverse transcription-polymerase chain reaction (RT-PCR) analysis according to previously established methods^{38,39)}. Briefly, RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to previously established methods^{6,16)}. The RNA concentrations and quality were determined using a CFX96TM Real-Time System (Bio-Rad, Hercules, CA, USA). To remove contaminating DNA, samples were treated with recombinant DNase I (DNA-free; Ambion, Austin, TX, USA). RNA was

reverse transcribed using the reagent High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. And also briefly, the cDNA strand first was synthesized from the total RNA and then the mixture of the primers and the cDNA products was amplified by PCR, and the conditions of PCR amplification were 58°C for 30 min, 94°C for 2 min, 35 cycles of 94°C for 15 sec, 60°C for 30 sec, 68°C for 1min, and then 72°C for 5 min. The analysis was carried out using an ABI Step One Plus Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and expression levels were calculated relative to those of the vehicle control. The expression of β -actin mRNA was used as a control for tissue integrity in all samples. The sequences of the PCR oligonucleotide primers were as listed in Table 1.

11. Bromodeoxyuridine (BrdU) uptake assay

To assess the effects of the test substances on the proliferation of cells within the rat knee joint, proliferating cells were labeled by an intraperitoneal injection of 50 mg/kg BrdU (Sigma-Aldrich, St. Louise, MO, USA) at a rate of 2 ml/kg, dissolved in saline, and the animals were killed 72 h later, as described previously^{35,40)}. BrdU uptake was determined by immunohistochemistry using an anti-BrdU antibody for the femoral and tibial AC surface and SM tissue, as described previously^{33,34,36)}.

12. Histological process

The knee joints were sampled with the joint capsules preserved, fixed in 10% neutral buffered formalin (NBF), and decalcified in decalcifying solution [24.4% formic acid, and 0.5N sodium hydroxide] for 5 days. Each knee joint was longitudinally trimmed, embedded in paraffin, sectioned (3~4 μm) using a tungsten blade equipped with an automated polycut microtome (Model

Table 1. Oligonucleotides for Realtime RT-PCR Used in This Study

Target	5' - 3'	Sequence	NCBI accession No. (Molecular weights)
Collagen type II	Sense	GAGTGGAAGAGCGGAGACTACTG	NM_012929 (4538 bp)
	Antisense	CTCCATGTTGCAGAAGACTTTCA	
SOX9	Sense	AGAGCGTTGCTCGGAACTGT	NM_080403 (4032 bp)
	Antisense	TCCTGGACCGAAACTGGTAAA	
Aggrecan	Sense	GATGTCCCTGCAATTACCA	NM_022190 (6939 bp)
	Antisense	TCTGTGCAAGTGATTCGAGG	
β -actin	Sense	ATCGTGGGCCCGCCTAGGCA	NM_031144 (1293 bp)
	Antisense	TGGCCTTAGGGTTCAGAGGGG	

NCBI = National Center for Biotechnology Information; RT-PCR = reverse transcription polymerase chain reaction; SOX9 = SRY (sex determining region Y)-box 9

RM2255, Leica, Wetzlar, Germany), and stained with Sirius Red stain for cartilaginous tissues^{33,34,36}. The histological profiles of all samples were interpreted under a light microscope (Model Eclipse 80i, Nikon, Tokyo, Japan) by an assessor blind to the group distribution.

13. Immunohistochemistry

BrdU^{35,40} immunoreactivity was determined using a purified primary antibody with avidin-biotin complex (ABC) reagents and a peroxidase substrate kit (Cat No. PK6200; Vector Labs, Burlingame, CA, USA) on prepared femoral and tibial AC and SM tissues using the apoptotic marker poly(ADP-ribose) polymerase (PARP)⁴¹, proinflammatory cytokine tumor necrosis factor (TNF)- α , and cyclooxygenase (COX)-2^{42,43}. Briefly, endogenous peroxidase activity was blocked by a 30-min incubation with methanol and 0.3% H₂O₂, and the non-specific binding of immunoglobulin was blocked with normal horse serum blocking solution for 1 h in a humid chamber after epitope retrieval using trypsin (Sigma-Aldrich, St. Louise, MO, USA) and 2 N HCl, as described previously³³⁻³⁶, on the prepared unstained sections. Primary antisera were added overnight in a humidity chamber at 4°C and incubated with biotinylated universal secondary antibody and ABC reagents for 1 h at room temperature in the humidity chamber.

Finally, the sections were reacted with a peroxidase substrate kit for 3 min at room temperature.

14. Histomorphometry

The femoral and tibial AC injuries in the knees were evaluated and recorded using the Mankin scoring system and Safranin-O stain to observe the histopathology in more detail. The entire histological evaluation was performed by the same pathologist. Additionally, in accordance with our previously established method³³⁻³⁶, the thickness of the tibial and femoral AC (μ m/cartilage) and the epithelial lining of the SM tissue (μ m/knee joint) and the number of inflammatory cells infiltrating the SM tissue (cells/mm²) were measured in longitudinally trimmed samples using a computer-based automated image analyzer (*iSolution FL ver 9.1*, IMT *i-solution* Inc., Vancouver, Quebec, Canada). The histopathologist was blind to the group distribution during this analysis. Additionally, when cells were occupied by > 20% immunoreactivity, the density of each antibody for BrdU, PARP, TNF- α , and COX-2 was considered positive, and the number of immunoreactive cells was counted separately in each femoral and tibial AC with SM tissue using an automated digital image analyzer as cells/mm². One histological region of femoral and tibial AC with SM tissue in each rat (total 10 fields in each group) was

Table 2. Body Weight Gains in Sham-operated or OA Rats

Periods	Body weights (g) at				Body weight gains (g)	
	OA [A]*	6 days after OA	First treatment [B]*	Sacrifice [C]*	OA recovery [B-A]	Treatment [C-B]
Controls						
Sham	206.00±11.51	264.60±19.48	249.00±18.78	396.80±18.32	43.00±8.83	147.80±17.70
OA	205.60±7.76	252.20±8.59	238.30±9.43	373.50±21.79	32.70±7.26 ^a	135.20±13.73
Diclofenac	204.90±12.14	251.50±20.59 ^b	236.20±20.39 ^b	385.00±52.94	31.30±13.36 ^a	148.80±34.21
Single formula						
PCP	209.80±8.15	255.00±15.57	240.60±14.58	386.00±34.55	30.80±8.84 ^a	145.40±31.37
EC	206.20±8.66	254.40±13.41	241.10±13.16	375.50±42.62	34.90±6.45 ^b	134.40±34.58
AR	205.40±11.30	253.10±16.70	240.60±16.87	381.00±39.11	35.20±7.52 ^b	140.40±23.52
Mixed formula - PCP and EC:AR						
1:1	210.60±8.25	261.90±12.61	246.70±12.05	387.50±37.30	36.10±4.41 ^b	140.80±29.98
1:2	200.70±9.87	246.50±14.14 ^a	231.90±14.08 ^a	371.10±42.66 ^b	31.20±8.56 ^a	139.20±30.48
1:4	206.20±8.36	253.40±13.22	239.10±14.65	392.60±31.59	32.90±7.22 ^a	153.50±19.97 ^c
1:6	204.50±9.28	254.60±12.96	238.70±10.85	381.10±23.39	34.20±4.08 ^b	142.40±19.35
1:8	207.70±9.78	258.10±18.47	243.40±15.61	395.50±48.19	35.70±8.69 ^a	152.10±36.37
2:1	205.30±5.44	253.40±8.57	238.90±7.68	376.10±13.55	33.60±6.40 ^b	137.20±10.99
4:1	207.30±8.41	256.00±8.63	242.00±7.39	379.80±32.77	34.70±4.90 ^b	137.80±26.73
6:1	205.60±10.57	253.50±14.14	237.80±14.20	373.40±32.92	32.20±7.57 ^a	135.60±27.03
8:1	204.20±5.20	252.10±12.85	237.30±12.22	375.30±26.30	33.10±7.92 ^a	138.00±16.38

Values are expressed mean ± SD of 10 rats, g, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder. *All animals were overnight fasted. ^a $p < 0.01$ and ^b $p < 0.05$ as compared with sham control; ^c $p < 0.05$ as compared with OA control.

considered separately for further analysis.

15. Statistical analyses

All values are presented as the mean ± standard deviation of the 10 rats in this experiment. Multiple comparison tests were conducted for the groups receiving different doses. The homogeneity of variance was examined using the Levene test. When the Levene test indicated no significant deviations from the homogeneity of the variance, the data were analyzed by one-way analysis of variance followed by the least-significant difference multi-comparison test to identify the significantly different member of a pair. The non-parametric Kruskal-Wallis H test was performed on data with a heterogeneous variance. When a significant difference was detected by the Kruskal-Wallis H test, the Mann-Whitney U-test with Bonferroni's correction was used to determine the specific pairs that differed. Statistical

analyses were conducted using SPSS for Windows. Differences were considered significant at $p < 0.05$.

Results

1. Effects of PCP, EC, and the AR single formulas and nine PCP and EC:AR mixtures on body weight gain after OA surgery

No significant changes in body weight were observed in the OA control rats during the 28 days of administration compared with sham control rats. However, significant decreases ($p < 0.01$) in body weight were demonstrated in OA control rats compared with the sham control rats during the 7 days of recovery. No changes in body weight were detected during the recovery and administration periods in the single-formula- or the nine PCP and EC:AR mixture-treated rats compared with OA control rats, except for significantly ($p < 0.05$)

increased body weight gain during the 28 days of administration in the PCP and EC:AR 1:4 mixed-formula-treated rats compared with the OA control rats (Table 2).

2. Changes on the knee thicknesses

Knee thickness increased significantly ($p < 0.01$) in OA control rats compared with knee thickness in sham control rats beginning at 6 days after OA was induced. Significant decreases ($p < 0.01$ or $p < 0.05$) in knee thickness were observed in the diclofenac-, AR single formula, PCP, and the EC:AR 1:2, 1:8, 2:1, and 4:1 mixed-formula-treated rats beginning at 1 day after the initial administration compared with values in the OA control rats. At 7 days after the initial administration in the PCP and EC single formula, PCP, and EC:AR 1:1, 1:4, 1:6, and 8:1 mixed formula-treated rats, and at 14 days after initial administration in the PCP and EC:AR 6:1 mixed formula-treated rats knee thickness was decreased compared with the OA control rats. In particular, the PCP and the EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas resulted in significant decreases ($p < 0.01$ or $p < 0.05$) in knee thickness compared with the single formula (Figure 1).

3. Effects on the knee thicknesses after capsule exposure

The thickness of capsule-exposed knees increased significantly ($p < 0.01$) in OA control rats compared with the thickness in sham control rats, whereas knee thickness decreased significantly ($p < 0.01$ or $p < 0.05$) after capsule exposure in all test-substance-treated rats, including the PCP single-formula-treated OA rats, compared with knee thickness in the OA control rats. In particular, the PCP and EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas caused significant decreases ($p < 0.01$ or $p < 0.05$) in capsule-exposed knee thickness compared with each single formula (in that order) (Figure 2).

Fig. 1. Knee thickness changes in sham-operated or OA Rats. PCP and EC:AR 2:1 and 4:1 mixed formula treated rats showed significant ($p < 0.01$ or $p < 0.05$) decreases of knee thicknesses from 7 days after initial administration as compared with each of PCP, EC and AR single formula treated rats (A), from 21 days after initial administration in PCP and EC:AR 1:1 and 1:4 mixed formula treated rats (B), and from 27 days after initial administration in PCP and EC:AR 1:2 mixed formula treated rats as compared with each of PCP, EC and AR single formula treated rats (C), respectively. Values are expressed mean $\pm$ S.D. of 10 rats, mm. Actual compositions of mixed formula – PCP and EC:AR and group identifications were listed in Table 1 and 2; AR = Aqueous extracts of Achyranthis Radix; OA = Osteoarthritis; EC = Aqueous extracts of Eucommiae Cortex; PCP = Pomegranate Concentration Powder. OA 6 means at 6 days after OA operation; 0 means at start of administration, at 7 days after OA operation; 28 means 28 days after initiation of administration, at sacrifice. All animals were overnight fasted before first administration and sacrifice, respectively ($\dagger$).

4. Effects on the knee maximum extension angles

The maximum knee extension angle increased significantly ($p < 0.01$) in OA control rats compared with that in sham control rats, whereas the maximum extension angle of the knee decreased significantly in

all test-substance-treated rats, including the EC single-formula-treated OA rats, compared with that in OA control rats. In particular, the PCP and EC:AR 4:1, 2:1, and 1:1 mixed formulas induced significant decreases ($p < 0.01$) in maximum knee

extension angles compared with each single formula (in that order) (Figure 3).

5. Changes in PGE₂ levels and 5-LPO and MMP activities in femoral and tibial AC

Fig. 2. Knee thickness after capsule exposure in sham-operated or OA Rats. Values are expressed mean $\pm$ S.D. of 10 rats, mm. AR = Aqueous extracts of *Achyranthis Radix*; DC = Diclofenac; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder. ^a $p < 0.01$ as compared with sham control; ^b $p < 0.01$ and ^c $p < 0.05$ as compared with OA control; ^d $p < 0.01$ and ^e $p < 0.05$ as compared with PCP single formula; ^f $p < 0.01$ as compared with EC single formula; ^g $p < 0.01$ as compared with AR single formula.

Fig. 3. Knee maximum extension angles in sham-operated or OA Rats. Values are expressed mean $\pm$ S.D. of 10 rats, degrees ($^{\circ}$). AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder. ^a $p < 0.01$ as compared with sham control; ^b $p < 0.01$ and ^c $p < 0.05$ as compared with OA control; ^d $p < 0.01$ as compared with PCP single formula; ^e $p < 0.01$ and ^f $p < 0.05$ as compared with EC single formula; ^g $p < 0.01$ and ^h $p < 0.05$ as compared with AR single formula.

Table 3. Femoral and Tibial AC with SM PGE₂ Contents in Sham-Operated or OA Rats

Groups	Items	PGE ₂ levels		
		Femoral AC	Tibial AC	SM
Controls				
Sham		36.45±11.08	36.16±10.91	35.99±10.93
OA		104.36±22.82 ^a	106.89±19.53 ^a	172.09±27.41 ^a
Diclofenac		50.56±14.05 ^{bc}	47.16±7.01 ^{bc}	60.79±13.63 ^{ac}
Single formula				
PCP		78.62±7.61 ^{ac}	76.58±10.37 ^{ac}	124.97±22.35 ^{ac}
EC		80.06±10.88 ^{ac}	79.33±11.99 ^{ac}	131.98±16.82 ^{ac}
AR		81.82±8.95 ^{ac}	79.77±10.47 ^{ac}	130.89±20.34 ^{ac}
Mixed formula - PCP and EC:AR				
1:1		61.37±8.26 ^{acegi}	62.49±6.82 ^{acegi}	96.16±13.99 ^{acegi}
1:2		63.65±6.84 ^{acegi}	63.80±10.15 ^{acfgi}	102.46±14.71 ^{acfgi}
1:4		67.19±9.74 ^{acegi}	66.47±6.10 ^{acfhj}	104.30±13.98 ^{acfgi}
1:6		80.84±11.91 ^{ac}	73.10±12.28 ^{ac}	125.13±17.98 ^{ac}
1:8		81.20±11.13 ^{ad}	77.54±13.37 ^{ac}	129.27±18.93 ^{ac}
2:1		60.13±10.93 ^{acegi}	61.30±13.81 ^{acfgi}	87.91±20.71 ^{acegi}
4:1		53.10±11.14 ^{acegi}	52.33±9.16 ^{acegi}	74.87±18.72 ^{acegi}
6:1		70.36±16.31 ^{ac}	71.76±13.35 ^{ac}	119.00±25.14 ^{ac}
8:1		73.21±14.69 ^{ac}	72.44±14.19 ^{ac}	124.62±33.30 ^{ac}

Values are expressed mean ± SD of 10 rats, pg/ml, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; PGE₂ = Prostaglandin E₂. ^a*p* < 0.01 and ^b*p* < 0.05 as compared with sham control; ^c*p* < 0.01 and ^d*p* < 0.05 as compared with OA control; ^e*p* < 0.01 and ^f*p* < 0.05 as compared with PCP single formula; ^g*p* < 0.01 and ^h*p* < 0.05 as compared with EC single formula; ⁱ*p* < 0.01 and ^j*p* < 0.05 as compared with AR single formula.

Table 4. Femoral and Tibial AC with SM 5-LPO Activities in Sham-Operated or OA Rats

Groups	Items	5-LPO activity		
		Femoral AC	Tibial AC	SM
Controls				
Sham		0.10±0.05	0.09±0.05	0.18±0.14
OA		1.23±0.28 ^a	1.22±0.20 ^a	3.56±1.03 ^a
Diclofenac		0.44±0.12 ^{ab}	0.42±0.15 ^{ab}	1.67±0.61 ^{ab}
Single formula				
PCP		0.83±0.12 ^{ab}	0.82±0.10 ^{ab}	2.25±0.37 ^{ab}
EC		0.89±0.16 ^{ab}	0.88±0.09 ^{ab}	2.50±0.46 ^{ac}
AR		0.89±0.18 ^{ab}	0.91±0.14 ^{ab}	2.56±0.36 ^{ac}
Mixed formula - PCP and EC:AR				
1:1		0.62±0.10 ^{abdfh}	0.58±0.07 ^{abdfh}	1.75±0.40 ^{abefh}
1:2		0.63±0.11 ^{abdfh}	0.63±0.12 ^{abdfh}	1.83±0.21 ^{abdfh}
1:4		0.68±0.11 ^{abefi}	0.69±0.13 ^{abefh}	1.93±0.20 ^{abegh}
1:6		0.82±0.14 ^{ab}	0.81±0.15 ^{ab}	2.17±0.51 ^{ab}
1:8		0.83±0.18 ^{ab}	0.84±0.20 ^{ab}	2.28±0.44 ^{ab}
2:1		0.54±0.10 ^{abdfh}	0.53±0.10 ^{abdfh}	1.57±0.28 ^{abdfh}
4:1		0.48±0.09 ^{abdfh}	0.49±0.14 ^{abdfh}	1.43±0.27 ^{abdfh}
6:1		0.78±0.18 ^{ab}	0.85±0.20 ^{ab}	2.15±0.63 ^{ab}
8:1		0.82±0.17 ^{ab}	0.87±0.20 ^{ab}	2.23±0.54 ^{ab}

Values are expressed mean ± SD of 10 rats, μ M/min/ml, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; LPO = Lipoxigenase. ^a*p* < 0.01 as compared with sham control; ^b*p* < 0.01 and ^c*p* < 0.05 as compared with OA control; ^d*p* < 0.01 and ^e*p* < 0.05 as compared with PCP single formula; ^f*p* < 0.01 and ^g*p* < 0.05 as compared with EC single formula; ^h*p* < 0.01 and ⁱ*p* < 0.05 as compared with AR single formula.

with SM tissue

Significant increases ($p < 0.01$) in PGE₂ levels and 5-LPO, MMP-2, and MMP-9 activities were detected in the femoral and tibial AC with SM tissue of OA control rats compared with levels and activities in sham control rats. However, PGE₂ levels and 5-LPO, MMP-2, and MMP-9 activities decreased significantly ($p < 0.01$ or $p < 0.05$) in the femoral and tibial AC with SM tissue of all test-substance-treated rats compared with values in OA control rats. In particular, PCP and EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas induced significant decreases ($p < 0.01$ or $p < 0.05$) in PGE₂ levels and 5-LPO activities (Table 3 and 4), and the PCP and EC:AR 4:1, 2:1, and 1:1 mixed formulas caused significant decreases ($p < 0.01$ or $p < 0.05$) in MMP-2 and MMP-9 activities in femoral and tibial AC with SM tissue compared with each single formula (in that order) (Table 5 and 6).

6. Effects on the ECM-related chondrogenic genes—collagen type II, SOX9, and aggrecan mRNA expression from femoral and tibial AC with SM tissue

Significant decreases ($p < 0.01$) in collagen type II mRNA expression were detected in the femoral and tibial AC with significant increases ($p < 0.01$) in the SM tissue of OA control rats compared with values in sham control rats (Table 7). However, SOX9 and aggrecan mRNA expression levels decreased significantly ($p < 0.01$) in the femoral and tibial AC with SM tissue of OA control rats compared with levels in sham control rats (Table 8 and 9). All test-substance treatments increased collagen type II mRNA expression significantly ($p < 0.01$ or $p < 0.05$) in the femoral and tibial AC and decreased expression in the SM tissue. Rats given all test treatments showed significantly increased ($p < 0.01$) SOX9 and aggrecan mRNA expression levels in the femoral and tibial AC with SM tissue compared with levels in OA control rats. In particular, PCP and the

EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas induced significant increases ($p < 0.01$ or $p < 0.05$) in collagen type II mRNA expression of femoral and tibial AC, but they also induced a decrease in the SM tissue. PCP and the EC:AR 4:1, 2:1, and 1:1 mixed formulas induced significant increases ($p < 0.01$ or $p < 0.05$) in SOX9 and aggrecan mRNA expression levels from femoral and tibial AC with SM tissue compared with each single formula (in that order).

7. Effects on the BrdU-immunoreactivity of femoral and tibial AC with SM tissue

Significant decreases ($p < 0.01$) in the numbers of femoral and tibial AC BrdU-immunopositive cells were detected in OA control rats, whereas the number of SM BrdU-immunolabeled cells increased significantly ($p < 0.01$) in OA control rats compared with numbers in sham control rats. However, the numbers of femoral and tibial AC BrdU-immunoreactive cells increased significantly ($p < 0.01$), whereas the numbers of SM BrdU-immunopositive cells decreased significantly ($p < 0.01$) in all test-substance-treated rats compared with numbers in OA control rats; the only exception was diclofenac-treated rats, in which the numbers of BrdU-immunoreactive cells detected in the femoral and tibial AC were similar to values in OA control rats. In particular, PCP and the EC:AR 4:1, 2:1, 1:1, and 1:2 mixed formulas resulted in significant increases ($p < 0.01$) in the numbers of BrdU-immunolabeled cells in the femoral and tibial AC, but they resulted in decreases in SM tissue compared with each single formula (in that order) (Figure 4, Table 10).

8. Changes in femoral and tibial AC Mankin scores

OA control rats showed a marked increase in surface cartilage damage, decreased numbers of

Table 5. Femoral and Tibial AC with SM MMP-2 Activities in Sham-Operated or OA Rats

Items		MMP-2 activity		
Groups		Femoral AC	Tibial AC	SM
Controls				
Sham		0.74±0.26	0.63±0.22	0.70±0.30
OA		3.14±1.05 ^a	2.74±0.63 ^a	3.80±0.66 ^a
Diclofenac		2.12±0.32 ^{ab}	1.83±0.29 ^{ab}	1.86±0.44 ^{ab}
Single formula				
PCP		2.14±0.27 ^{ab}	1.94±0.23 ^{ab}	2.52±0.44 ^{ab}
EC		2.24±0.25 ^{ab}	2.10±0.23 ^{ab}	2.94±0.58 ^{ab}
AR		2.23±0.30 ^{ac}	2.13±0.21 ^{ab}	3.14±0.42 ^{ab}
Mixed formula - PCP and EC:AR				
1:1		1.67±0.42 ^{abefg}	1.56±0.33 ^{abefg}	1.99±0.25 ^{abefg}
1:2		1.85±0.20 ^{abefg}	1.65±0.15 ^{abdfg}	2.03±0.30 ^{abefg}
1:4		1.85±0.28 ^{abefh}	1.71±0.14 ^{abefg}	2.12±0.19 ^{abef}
1:6		2.08±0.38 ^{ab}	1.97±0.34 ^{ab}	2.74±0.77 ^{ab}
1:8		2.18±0.42 ^{ab}	2.02±0.46 ^{ab}	2.85±0.74 ^{ab}
2:1		1.50±0.35 ^{abdfg}	1.48±0.29 ^{abdfg}	1.79±0.35 ^{abdfg}
4:1		1.41±0.31 ^{abdfg}	1.28±0.17 ^{abdfg}	1.62±0.30 ^{abdfg}
6:1		2.06±0.36 ^{ab}	1.94±0.26 ^{ab}	2.77±0.56 ^{ab}
8:1		2.11±0.36 ^{ab}	1.95±0.39 ^{ab}	2.78±0.63 ^{ab}

Values are expressed mean ± SD of 10 rats, μ g/ml, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; MMP = Matrix metalloproteinase. ^a $p < 0.01$ as compared with sham control; ^b $p < 0.01$ and ^c $p < 0.05$ as compared with OA control; ^d $p < 0.01$ and ^e $p < 0.05$ as compared with PCP single formula; ^f $p < 0.01$ as compared with EC single formula; ^g $p < 0.01$ and ^h $p < 0.05$ as compared with AR single formula.

Table 6. Femoral and Tibial AC with SM MMP-9 Activities in Sham-Operated or OA Rats

Groups	Items	MMP-9 activity		
		Femoral AC	Tibial AC	SM
Controls				
Sham		0.73±0.17	0.60±0.18	0.63±0.23
OA		2.12±0.44 ^a	2.05±0.22 ^a	3.86±0.97 ^a
Diclofenac		1.42±0.26 ^{ab}	1.48±0.22 ^{ab}	2.38±0.55 ^{ab}
Single formula				
PCP		1.66±0.15 ^{ab}	1.51±0.14 ^{ab}	2.73±0.45 ^{ab}
EC		1.68±0.16 ^{ab}	1.60±0.19 ^{ab}	2.81±0.24 ^{ab}
AR		1.70±0.19 ^{ab}	1.67±0.19 ^{ab}	2.82±0.22 ^{ab}
Mixed formula - PCP and EC:AR				
1:1		1.40±0.15 ^{abcef}	1.32±0.13 ^{abdef}	2.19±0.21 ^{abdef}
1:2		1.47±0.13 ^{abdef}	1.34±0.13 ^{abef}	2.28±0.24 ^{abdef}
1:4		1.48±0.12 ^{abdef}	1.37±0.11 ^{abef}	2.31±0.24 ^{abdef}
1:6		1.64±0.19 ^{ab}	1.55±0.20 ^{ab}	2.64±0.31 ^{ab}
1:8		1.66±0.15 ^{ab}	1.56±0.17 ^{ab}	2.72±0.41 ^{ab}
2:1		1.34±0.25 ^{abcef}	1.23±0.13 ^{abcef}	1.95±0.21 ^{abcef}
4:1		1.19±0.44 ^{abcef}	1.03±0.19 ^{abcef}	1.53±0.39 ^{abcef}
6:1		1.60±0.15 ^{ab}	1.51±0.30 ^{ab}	2.58±0.58 ^{ab}
8:1		1.61±0.18 ^{ab}	1.55±0.22 ^{ab}	2.58±0.30 ^{ab}

Values are expressed mean ± SD of 10 rats, μ g/ml, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; MMP = Matrix metalloproteinase. ^a $p < 0.01$ as compared with sham control; ^b $p < 0.01$ as compared with OA control; ^c $p < 0.01$ and ^d $p < 0.05$ as compared with PCP single formula; ^e $p < 0.01$ as compared with EC single formula; ^f $p < 0.01$ as compared with AR single formula.

Table 7. Femoral and Tibial AC with SM Collagen Type II mRNA Expressions in Sham-Operated or OA Rats

Groups	Items	Collagen type II mRNA expressions		
		Femoral AC	Tibial AC	SM
Controls				
Sham		1.05±0.16	1.01±0.12	1.01±0.21
OA		0.34±0.11 ^a	0.31±0.07 ^a	5.41±1.27 ^a
Diclofenac		0.69±0.10 ^{ab}	0.64±0.11 ^{ab}	2.74±0.95 ^{ab}
Single formula				
PCP		0.51±0.10 ^{ab}	0.49±0.04 ^{ab}	3.73±0.35 ^{ab}
EC		0.49±0.10 ^{ab}	0.44±0.07 ^{ab}	4.05±0.50 ^{ab}
AR		0.46±0.06 ^{ac}	0.40±0.06 ^{ab}	4.04±0.47 ^{ab}
Mixed formula - PCP and EC:AR				
1:1		0.62±0.06 ^{abefh}	0.62±0.09 ^{abdfh}	2.79±0.54 ^{abdfh}
1:2		0.61±0.07 ^{abegh}	0.58±0.07 ^{abdfh}	3.04±0.48 ^{abdfg}
1:4		0.61±0.08 ^{abefh}	0.56±0.05 ^{abdfh}	3.12±0.48 ^{abefh}
1:6		0.52±0.10 ^{ab}	0.47±0.11 ^{ab}	3.87±1.00 ^{ac}
1:8		0.49±0.11 ^{ab}	0.46±0.09 ^{ab}	3.96±0.83 ^{ab}
2:1		0.66±0.10 ^{abdfh}	0.66±0.11 ^{abdfh}	2.35±0.61 ^{abdfh}
4:1		0.71±0.11 ^{abdfh}	0.70±0.14 ^{abdfh}	1.98±0.51 ^{abdfh}
6:1		0.52±0.12 ^{ab}	0.49±0.14 ^{ab}	3.88±0.85 ^{ab}
8:1		0.50±0.08 ^{ab}	0.44±0.10 ^{ab}	3.97±0.95 ^{ac}

Values are expressed mean ± SD of 10 rats, Relative to control/ β -actin, AR = Aqueous extracts of *Achyranthis Radix*, OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*, PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; ^a $p < 0.01$ as compared with sham control; ^b $p < 0.01$ and ^c $p < 0.05$ as compared with OA control; ^d $p < 0.01$ and ^e $p < 0.05$ as compared with PCP single formula; ^f $p < 0.01$ and ^g $p < 0.05$ as compared with EC single formula; ^h $p < 0.01$ as compared with AR single formula.

Table 8. Femoral and Tibial AC with SM SOX9 mRNA Expressions in Sham-Operated or OA Rats

Groups	Items	SOX9 mRNA expressions		
		Femoral AC	Tibial AC	SM
Controls				
	Sham	1.01±0.12	1.01±0.17	0.96±0.15
	OA	0.28±0.07 ^a	0.26±0.07 ^a	0.19±0.09 ^a
	Diclofenac	0.57±0.13 ^{ab}	0.55±0.09 ^{ab}	0.45±0.11 ^{ab}
Single formula				
	PCP	0.47±0.06 ^{ab}	0.46±0.08 ^{ab}	0.41±0.06 ^{ab}
	EC	0.43±0.08 ^{ab}	0.42±0.06 ^{ab}	0.39±0.08 ^{ab}
	AR	0.42±0.08 ^{ab}	0.42±0.08 ^{ab}	0.32±0.07 ^{ab}
Mixed formula - PCP and EC:AR				
	1:1	0.59±0.07 ^{abceg}	0.56±0.10 ^{abdeg}	0.51±0.06 ^{abdeg}
	1:2	0.56±0.05 ^{abdeg}	0.55±0.07 ^{abceg}	0.50±0.07 ^{abdeg}
	1:4	0.56±0.09 ^{abdeg}	0.56±0.11 ^{abdeg}	0.48±0.07 ^{abfg}
	1:6	0.47±0.08 ^{ab}	0.48±0.13 ^{ab}	0.39±0.09 ^{ab}
	1:8	0.43±0.05 ^{ab}	0.42±0.10 ^{ab}	0.38±0.10 ^{ab}
	2:1	0.61±0.05 ^{abceg}	0.61±0.11 ^{abceg}	0.56±0.09 ^{abceg}
	4:1	0.69±0.10 ^{abceg}	0.68±0.10 ^{abceg}	0.65±0.11 ^{abceg}
	6:1	0.48±0.09 ^{ab}	0.48±0.11 ^{ab}	0.35±0.10 ^{ab}
	8:1	0.46±0.10 ^{ab}	0.44±0.11 ^{ab}	0.35±0.09 ^{ab}

Values are expressed mean ± SD of 10 rats, Relative to control/ β -actin, AR = Aqueous extracts of *Achyranthis Radix*, OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*, PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; SOX9 = SRY (sex determining region Y)-box 9, ^a $p < 0.01$ as compared with sham control; ^b $p < 0.01$ as compared with OA control; ^c $p < 0.01$ and ^d $p < 0.05$ as compared with PCP single formula; ^e $p < 0.01$ and ^f $p < 0.05$ as compared with EC single formula; ^g $p < 0.01$ as compared with AR single formula.

Table 9. Femoral and Tibial AC with SM Aggrecan mRNA Expressions in Sham-Operated or OA Rats

Groups	Items	Aggrecan mRNA expressions		
		Femoral AC	Tibial AC	SM
Controls				
Sham		1.01±0.13	0.97±0.16	0.98±0.11
OA		0.19±0.06 ^a	0.16±0.06 ^a	0.19±0.04 ^a
Diclofenac		0.54±0.10 ^{ab}	0.43±0.11 ^{ab}	0.45±0.09 ^{ab}
Single formula				
PCP		0.41±0.08 ^{ab}	0.32±0.06 ^{ab}	0.35±0.06 ^{ab}
EC		0.32±0.07 ^{ab}	0.28±0.07 ^{ab}	0.32±0.08 ^{ab}
AR		0.32±0.05 ^{ab}	0.26±0.09 ^{ab}	0.30±0.06 ^{ab}
Mixed formula - PCP and EC:AR				
1:1		0.51±0.06 ^{abdeg}	0.43±0.06 ^{abceg}	0.44±0.05 ^{abdeg}
1:2		0.50±0.07 ^{abdeg}	0.42±0.06 ^{abceg}	0.42±0.06 ^{abdeg}
1:4		0.49±0.07 ^{abdeg}	0.39±0.06 ^{abdeg}	0.41±0.06 ^{abfg}
1:6		0.39±0.13 ^{ab}	0.32±0.08 ^{ab}	0.36±0.09 ^{ab}
1:8		0.35±0.09 ^{ab}	0.28±0.08 ^{ab}	0.35±0.07 ^{ab}
2:1		0.59±0.07 ^{abceg}	0.47±0.07 ^{abceg}	0.48±0.07 ^{abceg}
4:1		0.61±0.10 ^{abceg}	0.52±0.09 ^{abceg}	0.58±0.09 ^{abceg}
6:1		0.37±0.11 ^{ab}	0.30±0.09 ^{ab}	0.35±0.08 ^{ab}
8:1		0.35±0.08 ^{ab}	0.29±0.09 ^{ab}	0.32±0.11 ^{ab}

Values are expressed mean ± SD of 10 rats, Relative to control/ β -actin, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; ^a $p < 0.01$ as compared with sham control; ^b $p < 0.01$ as compared with OA control; ^c $p < 0.01$ and ^d $p < 0.05$ as compared with PCP single formula; ^e $p < 0.01$ and ^f $p < 0.05$ as compared with EC single formula; ^g $p < 0.01$ as compared with AR single formula.

Table 10. Femoral and Tibial AC with SM BrdU-Immunoreactive Cell Numbers in Sham-Operated or OA Rats

Groups	Items	BrdU-immunoreactive cell numbers		
		Femoral AC	Tibial AC	SM
Controls				
Sham		31.70±5.65	25.90±4.53	7.50±2.55
OA		3.90±1.52 ^a	3.70±1.25 ^a	275.90±88.78 ^a
Diclofenac		4.20±1.62 ^a	3.90±0.99 ^a	50.00±14.79 ^{ac}
Single formula				
PCP		15.90±3.38 ^{ac}	15.50±3.89 ^{ac}	107.60±21.75 ^{ac}
EC		11.20±1.81 ^{ac}	11.00±2.83 ^{ac}	151.10±19.14 ^{ac}
AR		10.30±2.54 ^{ac}	10.90±2.23 ^{ac}	135.00±19.67 ^{ac}
Mixed formula - PCP and EC:AR				
1:1		26.50±7.89 ^{cdfig}	27.40±4.79 ^{cdfig}	62.70±11.55 ^{acdfig}
1:2		22.50±2.88 ^{acdfig}	20.50±2.27 ^{acdfig}	76.50±10.60 ^{acdfig}
1:4		17.70±2.98 ^{acfig}	17.10±2.60 ^{acfig}	78.20±14.88 ^{acdfig}
1:6		13.60±5.19 ^{ace}	12.80±3.08 ^{ac}	127.60±35.84 ^{ac}
1:8		13.40±5.40 ^{ace}	12.40±4.35 ^{ac}	134.70±40.95 ^{ac}
2:1		38.80±6.71 ^{bdfg}	33.00±8.03 ^{bdfg}	52.50±10.41 ^{acdfig}
4:1		48.70±6.65 ^{acdfig}	39.00±6.55 ^{acdfig}	44.50±10.54 ^{acdfig}
6:1		13.60±4.27 ^{ac}	14.80±7.67 ^{ac}	119.50±77.76 ^{ac}
8:1		13.20±3.97 ^{ac}	12.30±6.53 ^{ace}	124.50±50.35 ^{ac}

Values are expressed mean ± SD of 10 rats, cells/mm², AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; BrdU = 5-Bromo-2'-Deoxyuridine; ^a $p < 0.01$ and ^b $p < 0.05$ as compared with sham control; ^c $p < 0.01$ as compared with OA control; ^d $p < 0.01$ and ^e $p < 0.05$ as compared with PCP single formula; ^f $p < 0.01$ as compared with EC single formula; ^g $p < 0.01$ as compared with AR single formula.

Fig. 4. Representative immunohistochemical images of the BrdU-immunoreactive cells on the femur and tibia AC with SM, taken from sham-operated or OA Rats. (a) Sham vehicle control (Sham-operated and distilled water orally administered rats); (b) OA control (OA-surgery and distilled water orally administered rats); (c) Diclofenac (OA-surgery and diclofenac sodium 2 mg/kg subcutaneously treated rats); (d) PCP (OA-surgery and PCP single formula 200 mg/kg orally administered rats); (e) EC (OA-surgery and EC single formula 200 mg/kg orally administered rats); (f) AR (OA-surgery and AR single formula 200 mg/kg orally administered rats); (g) PCP 100 mg with EC:AR 1:1 (50:50 mg:mg) mixed formula administered OA rats; (h) PCP 100 mg with EC:AR 1:2 (33:67 mg:mg) mixed formula administered OA rats; (i) PCP 100 mg with EC:AR 1:4 (20:80 mg:mg) mixed formula administered OA rats; (j) PCP 100 mg with EC:AR 1:6 (14:86 mg:mg) mixed formula administered OA rats; (k) PCP 100 mg with EC:AR 1:8 (11:89 mg:mg) mixed formula administered OA rats; (l) PCP 100 mg with EC:AR 2:1 (67:33 mg:mg) mixed formula administered OA rats; (m) PCP 100 mg with EC:AR 4:1 (80:20 mg:mg) mixed formula administered OA rats; (n) PCP 100 mg with EC:AR 6:1 (86:14 mg:mg) mixed formula administered OA rats; (o) PCP 100 mg with EC:AR 8:1 (89:11 mg:mg) mixed formula administered OA rats. AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; BrdU = 5-Bromo-2'-Deoxyuridine; ABC = Avidin-biotin complex. All ABC immunostain. Scale bars = 120 μ m.

Table 11. Femoral AC Mankin Scores in Sham-Operated or OA Rats

Groups	Items	Mankin scores				
		Surface	Hypocellularity	Clone	Stain intensity	Total*
Controls						
Sham		0.80±0.63	0.30±0.48	0.20±0.42	0.40±0.52	1.70±1.25
OA		2.60±0.52 ^a	2.60±0.52 ^a	2.60±0.52 ^a	2.30±0.67 ^a	10.10±0.99 ^a
Diclofenac		1.40±0.70 ^{bc}	1.30±0.48 ^{ac}	0.90±0.57 ^{bc}	1.30±0.48 ^{ac}	4.90±1.20 ^{ac}
Single formula						
PCP		2.30±0.48 ^a	1.60±0.52 ^{ac}	1.80±0.63 ^{ac}	1.80±0.42 ^{ac}	7.50±0.97 ^{ac}
EC		2.20±0.63 ^a	2.00±0.67 ^{ad}	1.90±0.57 ^{ad}	2.00±0.67 ^a	8.10±1.60 ^{ac}
AR		2.40±0.52 ^a	1.90±0.74 ^{ac}	2.10±0.57 ^a	2.10±0.57 ^a	8.50±0.71 ^{ac}
Mixed formula - PCP and EC:AR						
1:1		1.50±0.53 ^{bcehi}	1.80±0.63 ^{ac}	1.10±0.57 ^{acfgi}	1.20±0.42 ^{acfgi}	5.60±0.84 ^{acegi}
1:2		1.80±0.63 ^{acj}	1.10±0.57 ^{acgi}	1.40±0.84 ^{acj}	1.40±0.52 ^{achj}	5.70±1.16 ^{acegi}
1:4		1.50±0.71 ^{bcehi}	1.50±0.53 ^{ac}	1.30±0.48 ^{achi}	1.50±0.53 ^{acj}	5.80±1.23 ^{acegi}
1:6		2.30±0.48 ^a	1.90±0.57 ^{ac}	1.90±0.74 ^{ad}	1.80±0.79 ^a	7.90±0.88 ^{ac}
1:8		2.30±0.67 ^a	1.90±0.57 ^{ac}	2.00±0.67 ^{ad}	1.90±0.57 ^a	8.10±1.79 ^{ac}
2:1		1.50±0.53 ^{bcehi}	1.30±0.48 ^{acgi}	1.50±0.53 ^{acj}	1.10±0.57 ^{befgi}	5.40±1.26 ^{acegi}
4:1		1.60±0.52 ^{acfhi}	1.30±0.67 ^{acgi}	1.10±0.57 ^{acfgi}	1.10±0.57 ^{befgi}	5.10±1.20 ^{acegi}
6:1		2.00±0.67 ^{ad}	1.80±0.63 ^{ac}	1.90±0.57 ^{ad}	1.70±0.82 ^{ad}	7.40±1.78 ^{ac}
8:1		2.10±0.74 ^a	1.60±0.70 ^{ac}	1.80±0.63 ^{ac}	2.00±0.82 ^a	7.50±1.72 ^{ac}

Values are expressed mean ± SD of 10 rats, score (*Max of totalized scores = 12). AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane. ^a*p* < 0.01 and ^b*p* < 0.05 as compared with sham control; ^c*p* < 0.01 and ^d*p* < 0.05 as compared with OA control; ^e*p* < 0.01 and ^f*p* < 0.05 as compared with PCP single formula; ^g*p* < 0.01 and ^h*p* < 0.05 as compared with EC single formula; ⁱ*p* < 0.01 and ^j*p* < 0.05 as compared with AR single formula.

Table 12. Tibial AC Mankin Scores in Sham-Operated or OA Rats

Groups	Items	Mankin scores				
		Surface	Hypocellularity	Clone	Stain intensity	Total*
Controls						
Sham		0.90±0.57	0.40±0.52	0.10±0.32	0.50±0.53	1.90±1.45
OA		2.60±0.52 ^a	2.40±0.52 ^a	2.40±0.70 ^a	2.50±0.53 ^a	9.90±0.99 ^a
Diclofenac		1.70±0.67 ^{ac}	1.20±0.63 ^{ac}	1.20±0.63 ^{ac}	1.10±0.57 ^{bc}	5.20±1.23 ^{ac}
Single formula						
PCP		1.90±0.74 ^{ad}	2.00±0.47 ^a	1.50±0.53 ^{ac}	1.80±0.79 ^{ad}	7.20±1.48 ^{ac}
EC		2.30±0.48 ^a	1.80±0.63 ^{ad}	2.10±0.57 ^a	2.00±0.47 ^a	8.20±1.48 ^{ac}
AR		2.50±0.71 ^a	2.10±0.57 ^a	2.00±0.67 ^a	1.80±0.63 ^{ad}	8.40±1.43 ^{ad}
Mixed formula - PCP and EC:AR						
1:1		1.50±0.53 ^{bcegi}	1.30±0.48 ^{acehi}	1.30±0.48 ^{acgi}	1.90±0.57 ^{cegi}	5.00±0.94 ^{acegi}
1:2		1.70±0.67 ^{achi}	1.10±0.57 ^{acegi}	1.20±0.63 ^{acgi}	1.60±0.70 ^{ac}	5.60±1.26 ^{acegi}
1:4		1.60±0.52 ^{bchi}	1.40±0.52 ^{acfi}	1.30±0.48 ^{acgi}	1.40±0.52 ^{ach}	5.70±0.95 ^{acfgi}
1:6		2.20±0.79 ^a	1.70±0.48 ^{ac}	1.90±0.74 ^a	1.90±0.57 ^{ad}	7.70±1.06 ^{ac}
1:8		2.20±0.63 ^a	1.90±0.57 ^{ad}	2.00±0.67 ^a	1.90±0.74 ^{ad}	8.00±1.63 ^{ac}
2:1		1.60±0.52 ^{bchi}	1.00±0.67 ^{bcegi}	1.10±0.57 ^{acgi}	1.20±0.79 ^{befj}	4.90±1.85 ^{acegi}
4:1		1.40±0.52 ^{cgi}	1.30±0.48 ^{acehi}	0.80±0.63 ^{befgi}	1.00±0.47 ^{cegi}	4.50±1.08 ^{acegi}
6:1		2.20±0.63 ^a	2.20±0.42 ^a	1.90±0.74 ^a	1.30±0.48 ^{ach}	7.60±1.58 ^{ac}
8:1		2.10±0.74 ^a	1.90±0.32 ^{ad}	2.10±0.57 ^{af}	2.10±0.74 ^a	8.20±1.40 ^{ac}

Values are expressed mean ± SD of 10 rats, score (*Max of totalized scores = 12). AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane. ^a*p* < 0.01 and ^b*p* < 0.05 as compared with sham control; ^c*p* < 0.01 and ^d*p* < 0.05 as compared with OA control; ^e*p* < 0.01 and ^f*p* < 0.05 as compared with PCP single formula; ^g*p* < 0.01 and ^h*p* < 0.05 as compared with EC single formula; ⁱ*p* < 0.01 and ^j*p* < 0.05 as compared with AR single formula.

chondrocytes and clones, and decreased Safranin-O stain intensity on femoral and tibial AC. Consequently, significant increases ($p<0.01$) in femoral and tibial AC Mankin scores were observed in OA control rats compared with scores in sham control rats. However, femoral and tibial AC Mankin scores decreased significantly ($p<0.01$ or $p<0.05$) in all test-substance-treated rats compared with values in OA control rats. In particular, PCP and the EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas caused significant decreases ($p<0.01$ or $p<0.05$) in femoral and tibial AC Mankin scores compared with each single formula (in that order) (Figure 5, Table 11 and 12).

9. Changes in the general histopathology of femoral and tibial AC with SM tissue

Femoral and tibial AC thicknesses decreased significantly ($p<0.01$) in OA control rats compared with thicknesses in sham control rats; however, significant increases in femoral ($p<0.01$ or $p<0.05$) and tibial ($p<0.01$) AC thicknesses were observed in all test-substance-treated rats compared with values in OA control rats. In particular, the PCP and EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas induced significant increases in femoral ($p<0.01$) and tibial ($p<0.01$ or $p<0.05$) AC thicknesses compared with each single formula (in that order) (Figure 5, Table 13).

The SM lining epithelium thickness increased significantly ($p<0.01$) in OA control rats compared with values in sham control rats, whereas the SM lining epithelium thickness decreased significantly ($p<0.01$) in all test substance-treated rats, including the PCP and EC:AR 8:1 mixed-formula-treated OA rats, compared with values in OA control rats. In particular, the PCP and EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas caused significant decreases ($p<0.01$) in SM tissue lining epithelium thickness compared with each single formula (in that order) (Figure 5, Table 13).

The number of inflammatory cells infiltrating the SM tissue increased significantly ($p<0.01$) in OA control rats compared with numbers in sham control rats, whereas the number of inflammatory cells infiltrating the SM tissue decreased significantly ($p<0.01$) in all test-substance-treated rats, including the diclofenac-treated OA rats, compared with numbers in OA control rats. In particular, the PCP and EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas caused significant decreases ($p<0.01$ or $p<0.05$) in the numbers of inflammatory cells infiltrating the SM tissue compared with each single formula (in that order) (Figure 5, Table 13).

10. Effects on inhibiting inflammation and apoptosis in femoral and tibial AC with SM tissue

The numbers of PARP, COX-2, and TNF- α -immunopositive cells in femoral and tibial AC with SM tissue increased significantly ($p<0.01$) in OA control rats, whereas the numbers of these cells decreased significantly ($p<0.01$ or $p<0.05$) in all test-substance-treated rats compared with numbers in OA control rats. In particular, the PCP and EC:AR 4:1, 2:1, 1:1, and 1:2 mixed formulas caused significant decreases ($p<0.01$) in the numbers of PARP-immunolabeled cells (Figure 6, Table 14), and the PCP and EC:AR 4:1, 2:1, 1:1, 1:2, and 1:4 mixed formulas induced significant decreases ($p<0.01$ or $p<0.05$) in the numbers of COX2 and TNF- α -immunolabeled cells in the femoral and tibial AC (Figure 7 and 8, Table 15 and 16), as well as in the SM tissue, compared with each single formula (in that order).

Discussion

OA also known as degenerative joint diseases, and is one of chronic inflammatory diseases. The cartilage damages in OA lead to production of PGE₂

Fig. 5. Representative general histological images of the femur and tibia AC with SM, taken from sham-operated or OA Rats, (a) Sham vehicle control (Sham-operated and distilled water orally administered rats); (b) OA control (OA-surgery and distilled water orally administered rats); (c) Diclofenac (OA-surgery and diclofenac sodium 2 mg/kg subcutaneously treated rats); (d) PCP (OA-surgery and PCP single formula 200 mg/kg orally administered rats); (e) EC (OA-surgery and EC single formula 200 mg/kg orally administered rats); (f) AR (OA-surgery and AR single formula 200 mg/kg orally administered rats); (g) PCP 100 mg with EC:AR 1:1 (50:50 mg:mg) mixed formula administered OA rats; (h) PCP 100 mg with EC:AR 1:2 (33:67 mg:mg) mixed formula administered OA rats; (i) PCP 100 mg with EC:AR 1:4 (20:80 mg:mg) mixed formula administered OA rats; (j) PCP 100 mg with EC:AR 1:6 (14:86 mg:mg) mixed formula administered OA rats; (k) PCP 100 mg with EC:AR 1:8 (11:89 mg:mg) mixed formula administered OA rats; (l) PCP 100 mg with EC:AR 2:1 (67:33 mg:mg) mixed formula administered OA rats; (m) PCP 100 mg with EC:AR 4:1 (80:20 mg:mg) mixed formula administered OA rats; (n) PCP 100 mg with EC:AR 6:1 (86:14 mg:mg) mixed formula administered OA rats; (o) PCP 100 mg with EC:AR 8:1 (89:11 mg:mg) mixed formula administered OA rats, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; BrdU = 5-Bromo-2'-Deoxyuridine, Arrows indicated the thicknesses of tibia or femur AC or of SM lining epithelium, All Safranin O stain, Scale bars = 120 μ m.

Fig. 6. Representative immunohistochemical images of the PARP-immunoreactive cells on the femur and tibia AC with SM, taken from sham-operated or OA Rats. (a) Sham vehicle control (Sham-operated and distilled water orally administered rats); (b) OA control (OA-surgery and distilled water orally administered rats); (c) Diclofenac (OA-surgery and diclofenac sodium 2 mg/kg subcutaneously treated rats); (d) PCP (OA-surgery and PCP single formula 200 mg/kg orally administered rats); (e) EC (OA-surgery and EC single formula 200 mg/kg orally administered rats); (f) AR (OA-surgery and AR single formula 200 mg/kg orally administered rats); (g) PCP 100 mg with EC:AR 1:1 (50:50 mg:mg) mixed formula administered OA rats; (h) PCP 100 mg with EC:AR 1:2 (33:67 mg:mg) mixed formula administered OA rats; (i) PCP 100 mg with EC:AR 1:4 (20:80 mg:mg) mixed formula administered OA rats; (j) PCP 100 mg with EC:AR 1:6 (14:86 mg:mg) mixed formula administered OA rats; (k) PCP 100 mg with EC:AR 1:8 (11:89 mg:mg) mixed formula administered OA rats; (l) PCP 100 mg with EC:AR 2:1 (67:33 mg:mg) mixed formula administered OA rats; (m) PCP 100 mg with EC:AR 4:1 (80:20 mg:mg) mixed formula administered OA rats; (n) PCP 100 mg with EC:AR 6:1 (86:14 mg:mg) mixed formula administered OA rats; (o) PCP 100 mg with EC:AR 8:1 (89:11 mg:mg) mixed formula administered OA rats. AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; PARP = Cleaved poly(ADP-ribose) polymerase; ABC = Avidin-biotin complex. All ABC immunostain. Scale bars = 120 μ m.

Fig. 7. Representative immunohistochemical images of the COX-2-immunoreactive cells on the femur and tibia AC with SM, taken from sham-operated or OA Rats. (a) Sham vehicle control (Sham-operated and distilled water orally administered rats); (b) OA control (OA-surgery and distilled water orally administered rats); (c) Diclofenac (OA-surgery and diclofenac sodium 2 mg/kg subcutaneously treated rats); (d) PCP (OA-surgery and PCP single formula 200 mg/kg orally administered rats); (e) EC (OA-surgery and EC single formula 200 mg/kg orally administered rats); (f) AR (OA-surgery and AR single formula 200 mg/kg orally administered rats); (g) PCP 100 mg with EC:AR 1:1 (50:50 mg:mg) mixed formula administered OA rats; (h) PCP 100 mg with EC:AR 1:2 (33:67 mg:mg) mixed formula administered OA rats; (i) PCP 100 mg with EC:AR 1:4 (20:80 mg:mg) mixed formula administered OA rats; (j) PCP 100 mg with EC:AR 1:6 (14:86 mg:mg) mixed formula administered OA rats; (k) PCP 100 mg with EC:AR 1:8 (11:89 mg:mg) mixed formula administered OA rats; (l) PCP 100 mg with EC:AR 2:1 (67:33 mg:mg) mixed formula administered OA rats; (m) PCP 100 mg with EC:AR 4:1 (80:20 mg:mg) mixed formula administered OA rats; (n) PCP 100 mg with EC:AR 6:1 (86:14 mg:mg) mixed formula administered OA rats; (o) PCP 100 mg with EC:AR 8:1 (89:11 mg:mg) mixed formula administered OA rats. AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; COX = Cyclooxygenase; ABC = Avidin-biotin complex, All ABC immunostain, Scale bars = 120 μ m.

Fig. 8. Representative immunohistochemical images of the TNF- α immunoreactive cells on the femur and tibia AC with SM, taken from sham-operated or OA Rats. (a) Sham vehicle control (Sham-operated and distilled water orally administered rats); (b) OA control (OA-surgery and distilled water orally administered rats); (c) Diclofenac (OA-surgery and diclofenac sodium 2 mg/kg subcutaneously treated rats); (d) PCP (OA-surgery and PCP single formula 200 mg/kg orally administered rats); (e) EC (OA-surgery and EC single formula 200 mg/kg orally administered rats); (f) AR (OA-surgery and AR single formula 200 mg/kg orally administered rats); (g) PCP 100 mg with EC:AR 1:1 (50:50 mg:mg) mixed formula administered OA rats; (h) PCP 100 mg with EC:AR 1:2 (33:67 mg:mg) mixed formula administered OA rats; (i) PCP 100 mg with EC:AR 1:4 (20:80 mg:mg) mixed formula administered OA rats; (j) PCP 100 mg with EC:AR 1:6 (14:86 mg:mg) mixed formula administered OA rats; (k) PCP 100 mg with EC:AR 1:8 (11:89 mg:mg) mixed formula administered OA rats; (l) PCP 100 mg with EC:AR 2:1 (67:33 mg:mg) mixed formula administered OA rats; (m) PCP 100 mg with EC:AR 4:1 (80:20 mg:mg) mixed formula administered OA rats; (n) PCP 100 mg with EC:AR 6:1 (86:14 mg:mg) mixed formula administered OA rats; (o) PCP 100 mg with EC:AR 8:1 (89:11 mg:mg) mixed formula administered OA rats. AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; TNF = Tumor necrosis factor; ABC = Avidin-biotin complex. All ABC immunostain. Scale bars = 120 μ m.

Table 13. Femoral and Tibial AC with SM General Histomorphometrical Analysis in Sham-Operated or OA Rats

Items	General histomorphometry			
Groups	Femoral AC thickness (μm)	Tibial AC thickness (μm)	SM epithelial thickness(μm)	SM IF cell numbers (×10 cells/mm ²)
Controls				
Sham	395.97±46.13	413.22±58.94	5.98±2.28	2.06±1.00
OA	184.49±21.39 ^a	142.57±16.15 ^a	36.63±11.55 ^a	166.17±30.00 ^a
Diclofenac	285.01±21.81 ^{ac}	285.71±32.62 ^{ac}	14.14±4.23 ^{ac}	27.86±16.41 ^{ac}
Single formula				
PCP	226.70±16.17 ^{ac}	247.68±17.97 ^{ac}	20.45±1.66 ^{ac}	79.12±14.88 ^{ac}
EC	219.89±16.70 ^{ac}	227.80±35.55 ^{ac}	24.43±5.03 ^{ac}	116.93±21.48 ^{ac}
AR	209.23±15.92 ^{ad}	200.36±37.20 ^{ac}	25.73±2.82 ^{ac}	123.58±23.93 ^{ac}
Mixed formula - PCP and EC:AR				
1:1	321.11±27.44 ^{acegi}	319.45±28.82 ^{acegi}	16.28±2.56 ^{acegi}	48.91±10.60 ^{acegi}
1:2	276.98±18.72 ^{acegi}	295.33±34.93 ^{acegi}	17.21±2.08 ^{acegi}	60.05±13.76 ^{acegi}
1:4	274.97±21.51 ^{acegi}	274.05±29.68 ^{acfgi}	17.65±1.64 ^{acegi}	62.32±11.74 ^{acfgi}
1:6	223.33±31.71 ^{ac}	227.24±46.60 ^{ac}	22.65±3.89 ^{ac}	81.52±21.88 ^{acgi}
1:8	218.38±29.26 ^{ac}	237.05±58.83 ^{ac}	24.46±4.74 ^{acf}	105.97±29.99 ^{acf}
2:1	324.14±30.98 ^{acegi}	350.22±48.95 ^{bcegi}	13.03±2.18 ^{acegi}	39.26±9.30 ^{acegi}
4:1	333.88±25.31 ^{acegi}	373.79±31.66 ^{cegi}	11.98±2.84 ^{acegi}	31.51±8.16 ^{acegi}
6:1	229.59±38.57 ^{ac}	235.80±50.47 ^{ac}	22.61±6.32 ^{ac}	86.35±19.21 ^{acgi}
8:1	218.45±25.97 ^{ac}	223.00±50.33 ^{ac}	23.42±5.09 ^{ac}	88.63±26.11 ^{achj}

Values are expressed mean ± SD of 10 rats, AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; IF = Inflammatory; ^a*p* < 0.01 and ^b*p* < 0.05 as compared with sham control; ^c*p* < 0.01 and ^d*p* < 0.05 as compared with OA control; ^e*p* < 0.01 and ^f*p* < 0.05 as compared with PCP single formula; ^g*p* < 0.01 and ^h*p* < 0.05 as compared with EC single formula; ⁱ*p* < 0.01 and ^j*p* < 0.05 as compared with AR single formula.

Table 14. Femoral and Tibial AC with SM PARP-Immunolabeled Cell Numbers in Sham-Operated or OA Rats

Groups	Items	PARP-immunolabeled cell numbers		
		Femoral AC	Tibial AC	SM
Controls				
Sham		67.80±31.11	64.70±16.57	12.40±11.53
OA		509.60±123.35 ^a	567.70±81.92 ^a	561.00±158.22 ^a
Diclofenac		153.10±28.11 ^{ab}	156.30±53.82 ^{ab}	110.30±28.44 ^{ab}
Single formula				
PCP		205.20±40.92 ^{ab}	235.70±46.80 ^{ab}	214.30±43.73 ^{ab}
EC		293.40±68.78 ^{ab}	255.90±36.56 ^{ab}	281.70±68.85 ^{ab}
AR		266.50±41.44 ^{ab}	318.30±55.25 ^{ab}	345.20±67.85 ^{ab}
Mixed formula - PCP and EC:AR				
1:1		152.90±22.91 ^{abceg}	162.80±54.63 ^{abceg}	143.30±26.93 ^{abceg}
1:2		155.70±30.20 ^{abceg}	158.30±30.85 ^{abceg}	151.80±33.86 ^{abceg}
1:4		204.50±23.02 ^{abceg}	194.40±31.94 ^{abdeg}	189.60±24.10 ^{abeg}
1:6		254.70±79.35 ^{ab}	286.60±96.75 ^{ab}	287.60±59.21 ^{abc}
1:8		288.30±109.10 ^{ab}	296.30±163.33 ^{ab}	289.50±97.76 ^{abd}
2:1		137.90±38.27 ^{abceg}	137.70±45.70 ^{abceg}	110.90±23.97 ^{abceg}
4:1		112.00±22.38 ^{abceg}	119.60±22.53 ^{abceg}	98.10±21.03 ^{abceg}
6:1		216.70±44.44 ^{abfh}	230.90±101.22 ^{abh}	201.50±51.31 ^{abeg}
8:1		297.40±112.71 ^{ab}	266.90±115.36 ^{ab}	257.80±76.87 ^{abh}

Values are expressed mean ± SD of 10 rats, cells/mm². AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; PARP = Cleaved poly(ADP-ribose) polymerase; ^a*p* < 0.01 as compared with sham control; ^b*p* < 0.01 as compared with OA control; ^c*p* < 0.01 and ^d*p* < 0.05 as compared with PCP single formula; ^e*p* < 0.01 and ^f*p* < 0.05 as compared with EC single formula; ^g*p* < 0.01 and ^h*p* < 0.05 as compared with AR single formula.

Table 15. Femoral and Tibial AC with SM COX-2-Immunostained Cell Numbers in Sham-Operated or OA Rats

Groups	Items	COX-2-immunostained cell numbers		
		Femoral AC	Tibial AC	SM
Controls				
Sham		34.40±10.96	54.80±19.79	6.40±3.44
OA		359.70±70.96 ^a	327.10±59.45 ^a	877.40±165.90 ^a
Diclofenac		134.40±46.25 ^{ab}	152.40±47.88 ^{ab}	63.70±25.09 ^{ab}
Single formula				
PCP		237.10±33.72 ^{ab}	234.40±39.30 ^{ab}	112.70±26.59 ^{ab}
EC		278.70±33.66 ^{ab}	256.40±46.86 ^{ab}	236.10±40.46 ^{ab}
AR		303.10±26.11 ^{ac}	257.50±32.15 ^{ab}	258.20±69.60 ^{ab}
Mixed formula - PCP and EC:AR				
1:1		175.50±17.32 ^{abdfh}	158.20±31.84 ^{abdfh}	68.40±13.87 ^{abdfh}
1:2		178.00±20.19 ^{abdfh}	163.20±14.74 ^{abdfh}	79.00±29.97 ^{abefh}
1:4		193.60±18.45 ^{abdfh}	173.40±22.09 ^{abdfh}	81.70±21.93 ^{abefh}
1:6		236.40±48.38 ^{abgh}	222.10±39.32 ^{abi}	123.50±51.64 ^{abgi}
1:8		247.00±42.89 ^{abh}	252.40±46.77 ^{ab}	129.50±47.35 ^{abgi}
2:1		116.60±33.54 ^{abdfh}	144.40±16.82 ^{abdfh}	51.00±15.25 ^{abdfh}
4:1		94.20±19.76 ^{abdfh}	110.50±17.21 ^{abdfh}	31.00±13.04 ^{abdfh}
6:1		253.50±43.61 ^{abi}	221.10±49.04 ^{ab}	104.30±43.73 ^{abfh}
8:1		259.30±70.64 ^{abi}	237.60±85.08 ^{ac}	137.40±50.87 ^{abfh}

Values are expressed mean ± SD of 10 rats, cells/mm². AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; COX = Cyclooxygenase. ^a*p* < 0.01 as compared with sham control; ^b*p* < 0.01 and ^c*p* < 0.05 as compared with OA control; ^d*p* < 0.01 and ^e*p* < 0.05 as compared with PCP single formula; ^f*p* < 0.01 and ^g*p* < 0.05 as compared with EC single formula; ^h*p* < 0.01 and ⁱ*p* < 0.05 as compared with AR single formula.

Table 16. Femoral and Tibial AC with SM TNF- α -Immunopositive Cell Numbers in Sham-Operated or OA Rats

Groups	Items	TNF- α -immunopositive cell numbers		
		Femoral AC	Tibial AC	SM
Controls				
Sham		34.70±19.18	52.10±14.43	4.10±2.28
OA		328.60±56.42 ^a	305.20±38.88 ^a	326.70±80.00 ^a
Diclofenac		148.70±48.90 ^{ac}	121.00±35.00 ^{ac}	35.20±19.66 ^{ac}
Single formula				
PCP		210.80±43.30 ^{ac}	209.70±34.42 ^{ac}	71.30±15.94 ^{ac}
EC		241.30±33.32 ^{ac}	228.70±42.92 ^{ac}	97.50±23.42 ^{ac}
AR		249.70±29.54 ^{ac}	238.00±31.57 ^{ac}	101.30±25.88 ^{ac}
Mixed formula - PCP and EC:AR				
1:1		128.60±32.18 ^{acdfh}	148.80±30.05 ^{acdef}	27.40±7.06 ^{acdfh}
1:2		147.40±26.53 ^{acdfh}	157.90±21.63 ^{acdef}	28.60±9.11 ^{acdfh}
1:4		156.00±30.34 ^{acdfh}	150.60±19.75 ^{acdef}	36.20±9.68 ^{acdfh}
1:6		217.00±32.39 ^{aci}	214.20±38.14 ^{ac}	83.70±21.97 ^{ac}
1:8		220.70±47.50 ^{ac}	215.90±42.92 ^{ac}	89.10±23.00 ^{ac}
2:1		89.60±26.98 ^{acdfh}	105.90±22.94 ^{acdef}	23.10±9.84 ^{acdfh}
4:1		68.30±13.01 ^{acdfh}	81.60±23.48 ^{bcdef}	11.90±4.63 ^{acdfh}
6:1		229.20±44.70 ^{ac}	189.20±32.52 ^{acef}	70.90±17.77 ^{acgh}
8:1		243.30±33.70 ^{ace}	208.10±47.14 ^{acg}	77.20±14.02 ^{acgi}

Values are expressed mean ± SD of 10 rats, cells/mm². AR = Aqueous extracts of *Achyranthis Radix*; OA = Osteoarthritis; EC = Aqueous extracts of *Eucommiae Cortex*; PCP = Pomegranate Concentration Powder; AC = Articular cartilage; SM = Synovial membrane; TNF = Tumor necrosis factor. ^a*p* < 0.01 and ^b*p* < 0.05 as compared with sham control; ^c*p* < 0.01 as compared with OA control; ^d*p* < 0.01 and ^e*p* < 0.05 as compared with PCP single formula; ^f*p* < 0.01 and ^g*p* < 0.05 as compared with EC single formula; ^h*p* < 0.01 and ⁱ*p* < 0.05 as compared with AR single formula.

and leukotriene as one of potent inflammatory mediator by increase of COX-2 and 5-LPO activities coordination with interleukin (IL)-1 and TNF- α – other potent inflammatory mediators. These acute inflammatory processes induced edematous changes on the surround tissues, and the thicknesses of affected joints were marked increased with abnormal bone growth and painful osteophytes, which were frequently encountered in the patients suffering from osteoarthritis^{16,44-46}). In the present study, OA control rats showed significant increases of knee thicknesses, decreases of the femur and tibia AC thicknesses, along significant increases of COX-2- and TNF- α -immunolabeled cell numbers at inspections of histopathology. In addition, OA control rats also showed noticeable and significant increases of the COX-2- and TNF- α -immunopositive cells on the SM, with marked elevations of PGE₂ levels and 5-LPO activities on the femur and tibia AC with SM, respectively. However, these surgically-induced OA related inflammatory signs in OA rats were significantly inhibited by 28 days of continuous treatment of PCP, EC and AR single formulas, and also by all nine types of PCP and EC:AR mixed formulas in this study. Especially, PCP and EC:AR 4:1, 2:1 and 1:1 mixed formula treated OA rats constantly showed significantly more favorable anti-inflammatory activities as compared with those of single formula of PCP, EC and AR treated rats.

Important enzymes involved in deconstruction of chondrocyte and bony tissue, are proteolytic enzymes, MMPs, and they were inhibited by tissue inhibitor of metalloproteinases system at normal conditions⁴⁷). Since MMPs act as protease that degraded the ECM, mainly PGs⁴⁸), they have been regarded as a valuable treatment target in osteoarthritis^{6,49}). As results of the present study, dramatic increases of MMP-2 and MMP-9 activities were observed on the femur and tibia AC with SM of OA control rats, however, these OA related increases of MMP-2 and MMP-9 activities were

significantly inhibited by 28 days of continuous treatment of PCP, EC and AR single formulas, and also by all nine types of PCP and EC:AR mixed formulas in this study. Especially, PCP and EC:AR 4:1, 2:1 and 1:1 mixed formula treated OA rats constantly showed significantly more favorable inhibitory effects against MMP-2 and MMP-9 activities on the femur and tibia AC with SM as compared with those of single formula of PCP, EC and AR treated rats.

Fibrosis occurs during the development of OA due to chronic inflammation, and it subsequently limits joint motion. As stiff joints are a prominent symptom of OA, the maximum extension angle of the joint is used to evaluate joint stiffness, with 0° as maximum extension, i.e., knee function is better with lower values³³⁻³⁷). Noticeable and significant increases of the maximum extension angles in induced knees, detected in OA control rats of this experiment indicated that OA was well induced by proper surgical processes. Marked hypertrophy and hyperplasia of SM tissue have been observed in animals with OA as a result of SM tissue fibrosis-related joint stiffness^{50,51}), and also in OA control rats of present study. However, these OA related increases of the maximum extension angles in induced knees, hypertrophic changes on the SM lining epithelium were significantly inhibited by 28 days of continuous treatment of PCP, EC and AR single formulas, and also by all nine types of PCP and EC:AR mixed formulas in this experiment. Especially, PCP and EC:AR 4:1, 2:1 and 1:1 mixed formula treated OA rats constantly showed significantly more favorable inhibitory effects against knee stiffness and SM proliferations as compared with those of single formula of PCP, EC and AR treated rats.

Our results show that all nine types of the PCP and EC:AR mixed formulas significantly inhibited OA-related increases in femoral and tibial AC Mankin scores and decreased cartilage thickness.

Notably, the PCP- and EC:AR 4:1, 2:1, and 1:1 mixed-formula-treated OA rats had significantly lower femoral and tibial Mankin scores and higher femoral and tibial AC thicknesses than did single-formula PCP-, EC-, and AR-treated rats. The Mankin scoring system is widely used for histopathological evaluations. Higher scores indicate higher levels of OA. It can detect articular cartilage injuries based on cartilage surface damages, chondrocyte numbers, clone formations and stain intensity to Safranin O^{33-37,52}.

Apoptosis occurs through two pathways, an extrinsic pathway involving the interaction of death ligands with their respective cell surface receptors and an intrinsic pathway that is initiated by insults that damage the DNA, such as ultraviolet light and chemotherapeutic agents. Both pathways eventually result in mitochondrial damage with release of cytochrome c and downstream activation of caspases, such as caspase-3. Activation of other downstream caspases results in cleavage of cellular proteins, such as PARP, cytokeratin 18, and other caspases, that lead to the morphologic and biochemical changes of apoptosis^{41,53}. PARP is a nuclear DNA-binding protein that functions in DNA base excision repair⁵⁴. PARP cleavage results in a decreased enzymatic repair function and contributes to the progression of apoptosis, although PARP cleavage is not absolutely necessary for apoptosis to proceed⁵⁵. The inhibition of apoptosis, the immunoreactivities of PARP is considered in a useful strategy for preventing from various organ damages⁵⁶ including OA^{34,57,58}. Therefore, we used PARP as apoptotic marker to observe the protective effects of test substances on the articular cartilages and also on the SM, respectively. In the present study, marked increases of PARP-immunoreactive cells, indicating apoptosis, were demonstrated on the femur and tibia AC with SM of OA control rats, however, these OA related increases of PARP-immunolabeled cells were significantly inhibited by 28 days of continuous

treatment of PCP, EC and AR single formulas, and also by all nine types of PCP and EC:AR mixed formulas in this study. Especially, PCP and EC:AR 4:1, 2:1, 1:1 and 1:2 mixed formula treated OA rats constantly showed significantly more favorable inhibitory effects against OA related increases of apoptosis on the femur and tibia AC with SM as compared with those of single formula of PCP, EC and AR treated rats.

Among the methods for the detection of cell proliferation in histological sections, immunohistochemistry for BrdU are the most preferable ones^{59,60}. BrdU staining was easier to read and seemed to reflect cell proliferations more specifically than other staining⁴⁰, and also have been prevalently used to detect cell proliferation in OA animals³³⁻³⁶. Cells contain BrdU means proliferated or proliferating cells^{33-36,60}. In this experiment, marked decreases of BrdU-immunoreactive cells were demonstrated on the femur and tibia AC of OA control rats, indicating classic OA related chondrocyte proliferation inhibitory histopathological signs. On the contrary, significant increases of BrdU-positive cells were observed on the SM of OA control as compared to those of sham control rats, indicating OA related hyperplasia and fibrotic changes on the SM of the present study. Diclofenac treated rats showed noticeable decreases of BrdU-immunoreactive cells on the SM, may be related its potent anti-inflammatory activity, it did not influenced on the femur and tibia AC BrdU-immunolabeled cell numbers as compared with OA control rats, respectively. However, significant increases of BrdU-immunostained cells on the both femur and tibia AC, indicating chondrocyte proliferations, and decreases in the SM indicating possible anti-inflammatory activities were observed by 28 days of continuous treatment of PCP, EC and AR single formulas, and also by all nine types of PCP and EC:AR mixed formulas in this study. Especially, PCP and EC:AR 4:1, 2:1 and 1:1 mixed formula treated OA rats constantly showed more

favorable increases of BrdU-immunoreactive cells on the femur and tibia AC, and decreases in the SM as compared with those of single formula of PCP, EC and AR treated rats.

The ECM of articular cartilage is composed mainly of collagen and GAGs, PG – aggrecan and SOX9^{5,6,39}. OA is characterized by loss of articular cartilage components, mainly PGs, leading to tissue destruction and hypocellularity, eventually resulting in loss of joint function⁷. It has been believed that degradations of ECM such as collagen, aggrecan and SOX9 from the results of inflammation related chondrocyte deaths and damages are involved in the deterioration of the homeostasis and articular functional limitations in OA⁶¹. SOX9 is a transcription factor essential for chondrogenic differentiation⁶², and aggrecan and collagen is a key component of the ECM of healthy cartilage³⁹. Prevention of these ECM degradations, therefore, has been treated as a valuable anti-OA target for various treatments^{38,39,63}. In the present study, significant decreases of the ECM related chondrogenic mRNA expressions – collagen type II, aggrecan and SOX9 mRNA expressions were demonstrated in the femur and tibia AC OA control as compared with sham control rats. In SMs of OA control rats, significant decreases of aggrecan and SOX9 mRNA expressions were also noticed as compared with sham control rats, but mRNA expressions of collagen type II on the SM were significantly increased in OA control rats as compared with sham control rats, in this experiment. These increases of collagen type II mRNA expressions on the SM, may be results from focal fibrosis and stiffness, a classic signs of OA³³⁻³⁷. However, these OA related decreases of chondrogenic mRNA expressions, and increases collagen type II mRNA expressions on the SM were significantly inhibited by 28 days of continuous treatment of PCP, EC and AR single formulas, and also by all nine types of PCP and EC:AR mixed formulas, respectively. Once again and finally, PCP and

EC:AR 4:1, 2:1 and 1:1 mixed formula treated OA rats constantly showed significantly more favorable inhibitory effects against OA related chondrogenic mRNA expression changes on the femur and tibia AC with SM as compared with those of single formula of PCP, EC and AR treated rats.

Conclusions

Taken together, we demonstrated that an appropriate mixture consisting of PCP and EC:AR synergistically increased the anti-OA effects of each single formula of PCP, EC and AR in rats with surgically induced OA, through synergic anti-inflammatory and chondrogenic activities. The PCP and EC:AR (2:1, 4:1, and 1:1) mixtures enhanced anti-OA effects, suggesting that this mixture may be used as a natural alternative to treat OA.

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