

The Evaluation of the Effect of Herbal Extract on Osteoarthritis: *In Vitro* and *In Vivo* Study

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ABSTRACT: In this study, the anti-osteoarthritis effects of *Cynanchum wilfordii*, *Phlomis umbrosa*, and *Angelica gigas* extract (CPAE), observed and confirmed in previously clinical studies were further investigated by *in vitro* and *in vivo* studies. Anabolic biomarkers related to healthy cartilage maintenance, such as aggrecan, type II collagen α -1 (*Col2a1*), sex determining region Y-box-9 (*Sox-9*), and catabolic biomarkers related to osteoarthritis, such as cyclooxygenase-2 (*Cox-2*), matrix metalloproteinase-13 (*Mmp13*), and nuclear factor kappa-light-chain-enhancer of activated B cells (*Nfkb*), were evaluated by quantitative reverse transcriptase polymerase chain reaction and reporter gene assay. *In vitro* study results showed significant changes in both anabolic and catabolic biomarkers. For anabolic factors, significant changes in the level of aggrecan ($P < 0.05$), *Col2a1* ($P < 0.05$), and *Sox-9* ($P < 0.01$) activation were shown after treatment of cartilage cells with CPAE (50 ng/mL) with similar efficacy compared to insulin growth factor, the positive control (100 ng/mL). For catabolic factors, significant changes in the inhibition activity of *Cox-2* ($P < 0.05$), *Mmp13* ($P < 0.01$), and *Nfkb* ($P < 0.05$) were shown for CPAE (50 ng/mL) with similar efficacy compared to Celecoxib, the positive control (10 μ M). In the *in vivo* carrageenan-induced paw edema model study results showed that CPAE-treated groups (100 mg/kg) and Celecoxib-treated groups (60 mg/kg) showed comparably significant efficacy of inhibition by 37.1% and 52.1%, respectively. Furthermore, CPAE (200 mg/kg) showed similar effect to Celecoxib (60 mg/kg) with an inhibition rate of 54.3%. This result confirms that CPAE effectively inhibited the inflammation-induced osteoarthritis symptoms.

Keywords: anti-osteoarthritis, *Cynanchum wilfordii*, *Sox-9*, *Mmp*, edema

INTRODUCTION

Osteoarthritis is a major cause of functional impairment and disability in the elderly (1,2). Epidemiological observations show that after the age of 50, osteoarthritis, particularly of the knee, is more common in women than in men, suggesting that estrogen deficiency may play a role in the onset or progression of this condition (3-5). Estrogen might be involved in the pathogenesis of osteoarthritis, and it has subsequently been described as “arthritis of the menopause” because the rapid development of hand and knee osteoarthritis often coincides with the cessation of menstruation. Furthermore, Nevitt et al. (5) conducted a cross-sectional study on the prevalence of radiological hip osteoarthritis in Caucasian women older than 65 years, comparing those who took hormone replacement therapy (HRT) to those who did not. HRT was associated with a lower relative risk of hip osteoarthritis, and the reduction was particularly marked in moderate to

severe hip osteoarthritis (5-7). Moreover, Hannan et al. (8) evaluated the prevalence of radiological knee osteoarthritis in women (mean age, 73 years) in the Framingham cohort, and the relative risk was lower in women who had been on HRT than those who had not.

Despite all these benefits, HRT has several risks. The results of the Women’s Health Initiative (WHI), a multicenter, randomized, double-blind placebo-controlled study involving 16,608 post-menopausal women, have contributed to changing the current approach to the management of menopausal symptoms (9). The WHI showed a 26%, 29%, and 41% increased risk of breast cancer, heart disease, and stroke, respectively, in participants who took HRT for the relief of menopausal symptoms, compared with those in the placebo group (9). In the “Medical Research Council” (Cancer Research UK) and “Collaborative Group on Epidemiological Studies of Ovarian Cancer” sponsored study of women treated with HRT for less than 5 years, the occurrence of ovarian cancer in-

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creased by 43% compared to the non-HRT treated group. For women treated with HRT for more than 5 years, the occurrence rate increased by 41%, showing high risks of HRT. Moreover, for women who had stopped HRT treatment within the past 5 years, the occurrence rate of ovarian cancer still increased regardless of the treatment period (10). Therefore, the development of alternative therapies to replace HRT is crucial to the long-term health of affected women. CPA extract under the brand name EstroG-100 is a standardized herbal formulation from the root extracts of *Cynanchum wilfordii* (C.W.), *Phlomis umbrosa* (P.U.), and *Angelica gigas* (A.G.). These herbs are non-toxic food materials based on the fact that they have been safely consumed as foods and used in herbal remedies for several hundreds of years in both Korea and China.

EstroG-100 has been proven effective in improving menopausal osteoarthritis in two clinical studies. In a previous human study conducted in Korea, the treatment group administered C.W., P.U., and A.G. extract (CPAE) with vitamins and minerals showed a more significant improvement than the placebo-treated group, in total reported menopausal symptoms including joint pain (11). In another human study conducted in California in the US, EstroG-100 treatment significantly improved the Kupperman index and 10 detailed menopausal symptoms including rheumatic pain (evaluated as joint pain) compared to that of the placebo group (12).

In this present study, *in vitro* biomarkers of cartilage health and osteoarthritis were evaluated to confirm the mechanism of action of CPAE. The activation of anabolic factors such as *aggrecan*, type II collagen α -1 (*Col2a1*), and sex determining region Y-box-9 (*Sox-9*) were evaluated as determinants for the maintenance of healthy cartilage (13,14). Furthermore, the inhibition of catabolic factors such as cyclooxygenase-2 (*Cox-2*), matrix metalloproteinase (*Mmp*), and nuclear factor kappa-light-chain-enhancer of activated B cells (*Nfkb*) were evaluated to determine the inhibition of osteoarthritis (15,16). In addition, there are several animal models for evaluating arthritis related clinical biomarkers, and one of the most popular methods involves the induction of foot, ear, or knee edema after treatment with the test agent, and then measurements are subsequently taken using a plethysmometer (17). In this study, the carrageenan-induced rat paw edema model was used to measure the degree of edema inhibition by CPAE and, thereby, verify its efficacy in treating arthritis (18,19).

MATERIALS AND METHODS

Samples and preparation

The CPAE (EstroG-100) and extracts of C.W., P.U., and

A.G. were provided by Naturalendo Tech Co., Ltd., (Seongnam, Korea). In order to manufacture the CPAE, C.W. was purchased from Jirisan Farming Association (Sancheong, Korea) in July 2015, P.U. was purchased from BaoCheng Chinese Herbal Medicine Plantation Co., Ltd. (Hunan, China) in July 2015, and A. *gigas* was provided by Yeongdong Herb Medicine Farming Association (Jecheon, Korea) in October 2015.

The herbs C.W., P.U., and A.G. were mixed in the ratio of 1:1:1.08 (w/w/w) and extracted with distilled water (1:8, w/v) by boiling under a reflux. After extracting for 8 h, the extract was cooled to room temperature for 30 min and filtered using Whatman no. 4 filter paper. The filtrate was subsequently evaporated using a rotary evaporator (R-215, BÜCHI Labortechnik, Flawil, Switzerland) under reduced pressure until 40° brix and lyophilized in a freeze drier (DW-86L728, Haier, Qingdao, China).

The insulin growth factor (IGF) and Celecoxib were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The test samples used in the *in vitro* study were dissolved in dimethyl sulfoxide and centrifuged before use while those for the *in vivo* study were mixed with water for injection solution before administration.

Primary mouse articular chondrocyte cultures

The chondrocytes were isolated from femoral condyles and tibial plateaus of mice by digesting cartilage tissue with 0.2% collagenase (Sigma-Aldrich Co.). The cells were maintained as a monolayer in Dulbecco's modified Eagle's medium (Gibco-BRL, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (AusGeneX, Oxenford, Australia) and antibiotics (penicillin G and streptomycin). On culture day 3, the cells were treated or co-treated with interleukin (IL)-1 β (5 ng/mL) and graded concentrations of the natural extracts.

Evaluation of anabolic and catabolic factor expression

The total cellular RNA was isolated using the TRI reagent and reverse-transcribed with moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using a polymerase chain reaction (PCR). The quantitative real-time reverse transcription (qRT)-PCR reactions were performed using the ABI plus one system and SYBR Premix Ex TaqTM (TaKaRa Bio, Otsu, Japan). The following conditions and primers were used for the qRT-PCR: *Col2a1* (annealing temperature 60°C), sense 5'-CAC ACT GGT AAG TGG GGC AAG A-3' and antisense 5'-GGA TTG TGT TGT TTC AGG GTT CG-3'; *Sox-9* (annealing temperature 55°C), sense 5'-CAT CAG CAG CAC CGC ACC CA-3' and antisense 5'-CGG GTG ATG GGC GGG TAG GA-3'; *aggrecan* (annealing temperature 60°C), sense 5'-GAA GAC GAC ATC ACC ATC

CAG-3' and antisense 5'-CTG TCT TTG TCA CCC ACA CAT G-3'; *Mmp-13* (annealing temperature 55°C), sense 5'-TGA TGG ACC TTC TGG TCT TCT GG-3' and antisense 5'-CAT CCA CAT GGT TGG GAA GTT CT-3'; *Cox-2* (annealing temperature 62°C), sense 5'-GGT CTG GTG CCT GGT CTG ATG AT-3' and antisense 5'-GTC CTT TCA AGG GAG AAT GGT GC-3'. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was amplified as the control to which the products were normalized, using the following conditions and primers: annealing temperature 50°C, sense 5'-TCA CTG CCA CCC AGA AGA C-3' and antisense 5'-TGT AGG CCA TGA GGT CCA C-3'.

Reporter gene analysis (*Sox-9* and *Nfκb*)

Sox-9 and *Nfκb* activity was determined using a reporter gene assay. Briefly, mouse articular chondrocytes were transfected with a plasmid containing luciferase and three tandem repeats of the serum response element or a control vector. The transfection of the expression vector was performed using the LipofectAMINE Plus (Invitrogen). The transfected cells were cultured in complete medium for 24 h, and then the luciferase activity was determined using an assay kit (Promega, Madison, WI, USA) and subsequently normalized to β-galactosidase activity

Evaluation of anti-inflammation in rat paw edema model

The 5~6-week-old male Sprague-Dawley (SD) rats used in this study were purchased from Samtako (Osan, Korea) and weighed 180~200 g. The animal room environment was maintained at a temperature of 22±3°C, relative humidity of 50±20%, ventilation of 10~15 times/h, and 12-h light/dark cycle, and the animals were provided with food and water. The animals were isolated during the test period and were separated based on test groups. One hour before the carrageenan injection, Celecoxib (60 mg/kg) as positive control was given orally, while the CPAE treatment groups were administered 50, 100, and 200 mg/kg for 4 days, and 1 h following the treatment on day 4, 1% (w/v) carrageenan (100 μL) was injected into the right hind paw of each rat. The volume of the paw edema was measured before, and 1, 3, and 5 h after the carrageenan injection, using a plethysmometer (37140m, Ugo Basile, Comerio, Italy). In addition, images of the paw edema were captured at 3 h to determine the degree of edema inhibition. The rates of edema increase and inflammation inhibition were calculated using the following equations:

$$\text{Rate of increase in edema (\%)} = \frac{A - B}{B} \times 100$$

where, A and B are the paw edema 1, 3, and 5 h after carrageenan administration and at 0 h (right before carrageenan administration), respectively.

$$\text{Rate of inhibition of inflammation (\%)} = \frac{I_c - I_e}{I_c} \times 100$$

where, I_c and I_e are the average edema volume (%) of the normal and treatment groups, respectively.

This study was performed according to the guidelines of the Animal Care and use Review Committee of Jeollanamdo Institute of Natural Resources Research (JINR 1513).

Statistical analysis

The results are expressed as mean±standard error of the mean (SEM), and the Student's *t*-test was used for the statistical analysis and $P < 0.05$ and $P < 0.01$ where considered statistically significant.

RESULTS

Activation of anabolic factors, *Col2a1* and *aggrecan*

Healthy mouse cartilage cells cultured in an *in vitro* system secrete anabolic factors such as *Col2a1* and *aggrecan* that play an important role in cartilage formation. Furthermore, evidence of the regulation of anabolic factor activation of test compound-treated cartilage cells suggests that the test compounds could be used to maintain healthy cartilage. Treatment of the cartilage cells with CPAE and each root extract increased the activation of *Col2a1* as well as that of *aggrecan*, which was significant different following treatment with C.W. ($P < 0.05$) and A.G. ($P < 0.01$) extracts and CPAE ($P < 0.01$) compared to the untreated control group (Fig. 1A). Furthermore, *Col2a1* activation was significantly increased in all groups treated with C.W. ($P < 0.01$), P.U. ($P < 0.05$), and A.G. ($P < 0.01$) extracts, as well as CPAE ($P < 0.01$, Fig. 1B). The results revealed that treatment with CPAE induced a higher activation of the anabolic factors than each of the individual root extracts did, suggesting that the combination of the three roots produced synergistic effects. Furthermore, CPAE at a concentration of 50 ng/mL showed similar activation to that of the control compound, IGF at 100 ng/mL. Therefore, CPAE was confirmed to demonstrate cartilage cell differentiation activity.

Evaluation of *Sox-9* activation

Due to the activation of *Col2a1* induced by CPAE, we further evaluated whether CPAE regulated the activation of *Sox-9*. *Sox-9* is known to be the transcription factor of *Col2a1*, which is responsible for the formation of cartilage. In this study, cartilage cells treated with CPAE exhibited regulation of *Sox-9* activation level in the qRT-PCR analysis (Fig. 2A).

In addition, a promoter analysis was also performed to further confirm the regulation of *Sox-9* activation as well

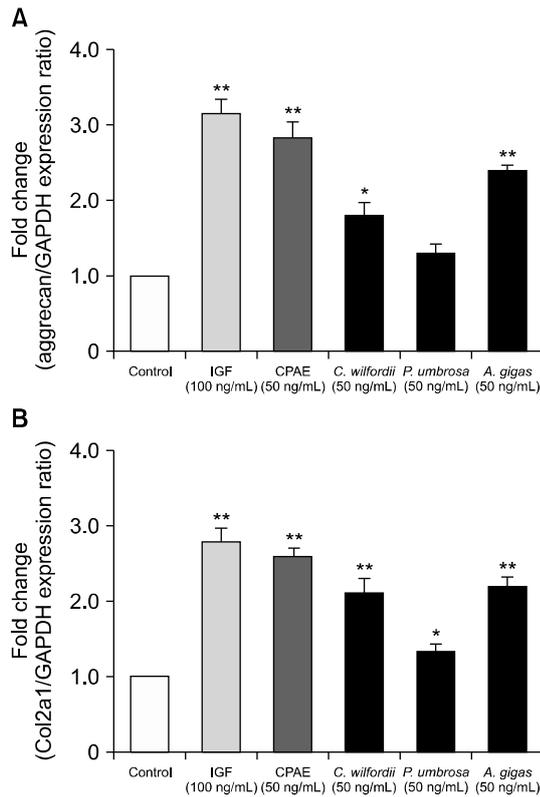


Fig. 1. Effect of *Cynanchum wilfordii*, *Phlomis umbrosa*, and *Angelica gigas* extract (CPAE) on *aggrecan* (A) and *Col2a1* (B) as anabolic factors. Values are mean±standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ compared with control, as analyzed by Student's *t*-test. IGF: insulin growth factor.

as its associated cofactors by CPAE. Treatment with CPAE at different concentrations increased *Sox-9* activation. In addition, the *Sox-9* promoter-containing vector was transfected into the cartilage cell, and the promoter analysis confirmed that different concentrations of CPAE increased *Sox-9* activation, as determined by the luciferase activity measurement (Fig. 2B).

Inhibition of catabolic factor *Cox-2*

The inflammatory cytokine, IL-1 β , activates catabolic factors such as *Cox-2*, which induces osteoarthritis. Therefore, the inhibitory effects of the test compounds on the activation of *Cox-2* were studied in the *in vitro* cell culture system (Fig. 3). The results revealed that CPAE and each of the three root extracts inhibited *Cox-2* activation. In particular, CPAE at a concentration of 50 ng/mL showed a similar inhibitory efficacy to that of the control compound, Celecoxib at 10 μ M. Therefore, CPAE is considered to have potential inhibitory effects against *Cox-2*-induced symptoms of osteoarthritis.

Mmp inhibition

MMP activation is known to cause cartilage destruction. Therefore, the potential inhibitory effect of CPAE on *Mmp-13* was investigated. The IL-1 β -induced activation of

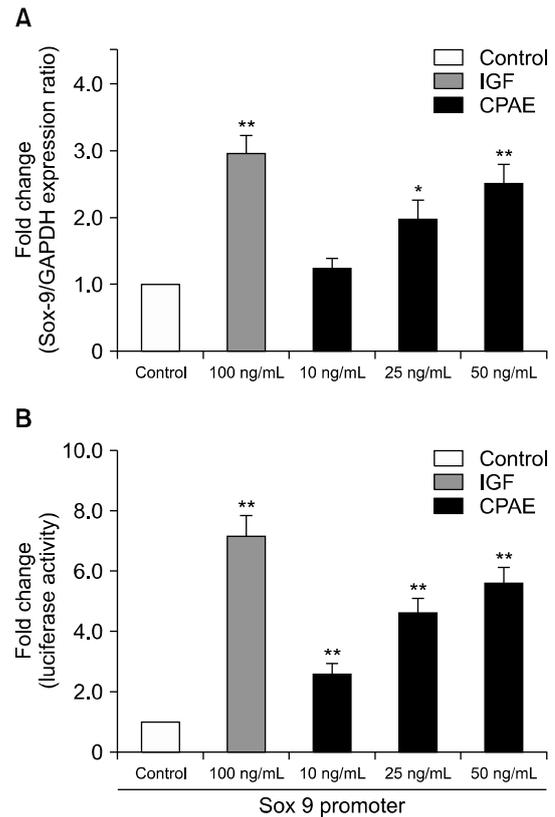


Fig. 2. Effect of *Cynanchum wilfordii*, *Phlomis umbrosa*, and *Angelica gigas* extract (CPAE) on *Sox-9* activation using reverse transcription-polymerase chain reaction (qRT-PCR) (A) and promoter analysis (B). Values are mean±standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ compared with control, as analyzed by Student's *t*-test. IGF: insulin growth factor.

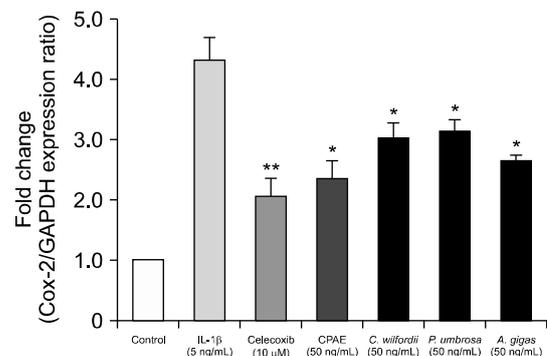


Fig. 3. Inhibitory activity of *Cynanchum wilfordii*, *Phlomis umbrosa*, and *Angelica gigas* extract (CPAE) on catabolic factor *Cox-2*. Values are mean±standard error of the mean, * $P < 0.05$ and ** $P < 0.01$ compared with interleukin-1 β (IL-1 β), as analyzed by Student's *t*-test.

Mmps, which leads to cartilage destruction, was concentration-dependently decreased by CPAE (Fig. 4A). The inhibition of *Mmp-13* activation was statistically significant compared to the control group when treated at concentrations of 25 ng/mL or higher ($P < 0.01$).

Nfκb inhibition

NF- κ B regulates MMP, which causes inflammation and

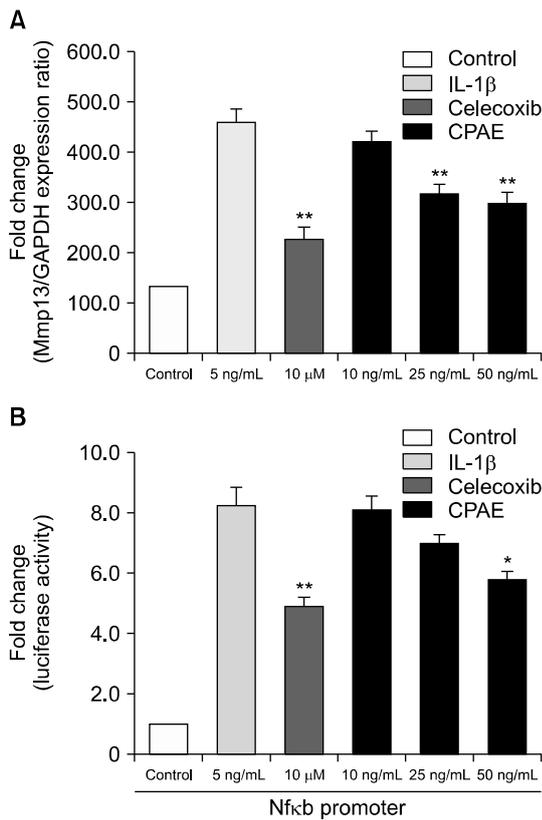


Fig. 4. Inhibitory activity of *Cynanchum wilfordii*, *Phlomis umbrosa*, and *Angelica gigas* extract (CPAE) on *Mmp13* (A) and *Nfkb* (B). Values are mean±standard error of the mean. * $P<0.05$ and ** $P<0.01$ compared with interleukin-1 β (IL-1 β), as analyzed by Student's t -test.

cartilage degeneration. Therefore, the inhibitory effect of CPAE on *Nfkb* activation was evaluated using promoter and other biochemical analyses. The IL-1 β -induced increase in *Nfkb* activity was significantly inhibited by Celecoxib as well as CPAE (50 ng/mL, Fig. 4B, $P<0.05$). In summary, the *Nfkb*-induced activation of *Mmp-13* and *Cox-2*, that leads to osteoarthritis, was reduced by the CPAE-mediated inhibition of *Nfkb* and could potentially inhibit the development of arthritis.

Evaluation of anti-inflammation in carrageenan-induced rat paw edema model

After administrating 50, 100, and 200 mg/kg of CPAE for 4 days followed by the induction of acute inflammation with carrageenan, the hourly change in the level of edema was measured. CPAE significantly reduced the carrageenan-induced paw edema at concentrations above 100 mg/kg compared to Control group, and the inhibition rate was highest at 200 mg/kg of CPAE (Fig. 5A and Table 1). A similar result was obtained with each test compound group at the 3-h time point as shown in the paw edema images (Fig. 5B). In addition, CPAE (200 mg/kg) and Celecoxib (60 mg/kg) significantly inhibited the carrageenan-induced edema by 54.3% and 52.1%, re-

spectively, at 3 h after treatment. Therefore, CPAE at 200 mg/kg showed a similar inhibitory effect as that shown by the positive control (Celecoxib).

DISCUSSION

This study was designed to evaluate the inhibition of arthritis after the administration of CPAE by evaluating its effects on the maintenance of cartilage health and osteoarthritis using a cartilage cell model. Furthermore, a carrageenan-induced rat paw edema model was used to evaluate the improvement of inflammation-induced symptoms of arthritis with treatment of CPAE. The *in vitro* effect of the test extracts on the activation of *Col2a1*, *aggrecan*, and *Sox-9* in cartilage cells was measured to evaluate cartilage health maintenance. The results showed that the related extracellular matrix (ECM), which maintains healthy joints, was continuously activated in the CPAE-treated cartilage cells. In addition, cartilage cells were stimulated with IL-1 β prior to CPAE treatment to confirm its inhibitory effect on osteoarthritis. The activation of *Cox-2*, *Mmp-13*, and *Nfkb* was decreased by CPAE, suggesting that anti-arthritic effects would likely be improved. Consequently, CPAE was shown to induce ECM differentiation as well as inhibit *Cox-2* and *Mmp* activation, which could be beneficial in osteoarthritis treatment. Therefore, the confirmed improvement of arthritis symptoms from the two clinical studies (11,12) could be explained by the mechanisms of action described above.

In the carrageenan-induced rat paw edema model designed to evaluate anti-inflammatory effects, CPAE was confirmed to reduce paw edema. When a foreign substance invades the human body, normal immune responses would produce vasoactive substances, such as histamine, serotonin, bradykinin, prostaglandin, and leukotriene that increase vascular membrane permeability leading to edema, and this inflammation could be the cause for arthritis. This study shows that CPAE has anti-inflammatory effects to inhibit edema, and EstroG-100 could prevent and inhibit arthritis caused by acute inflammation.

In this study, IGF was used as positive control for testing the increase of the anabolic factor, and Celecoxib was used as positive control for testing the inhibition of the expression of catabolic factor and in *in vivo* test. IGF was reported as the major regulating factor of cartilage proteoglycan synthesis in human synovial fluid (20), and Celecoxib that selectively inhibit COX-2 might block inflammation, pain and fever while reducing the side effects (21). The concentration was 100 ng/mL (IGF) and 10 μ M (Celecoxib, 3.81 μ g/mL) in the *in vitro* test, and the dosage was 60 mg/kg in the *in vivo* test.

C.W., the main plant raw material in CPAE, contains

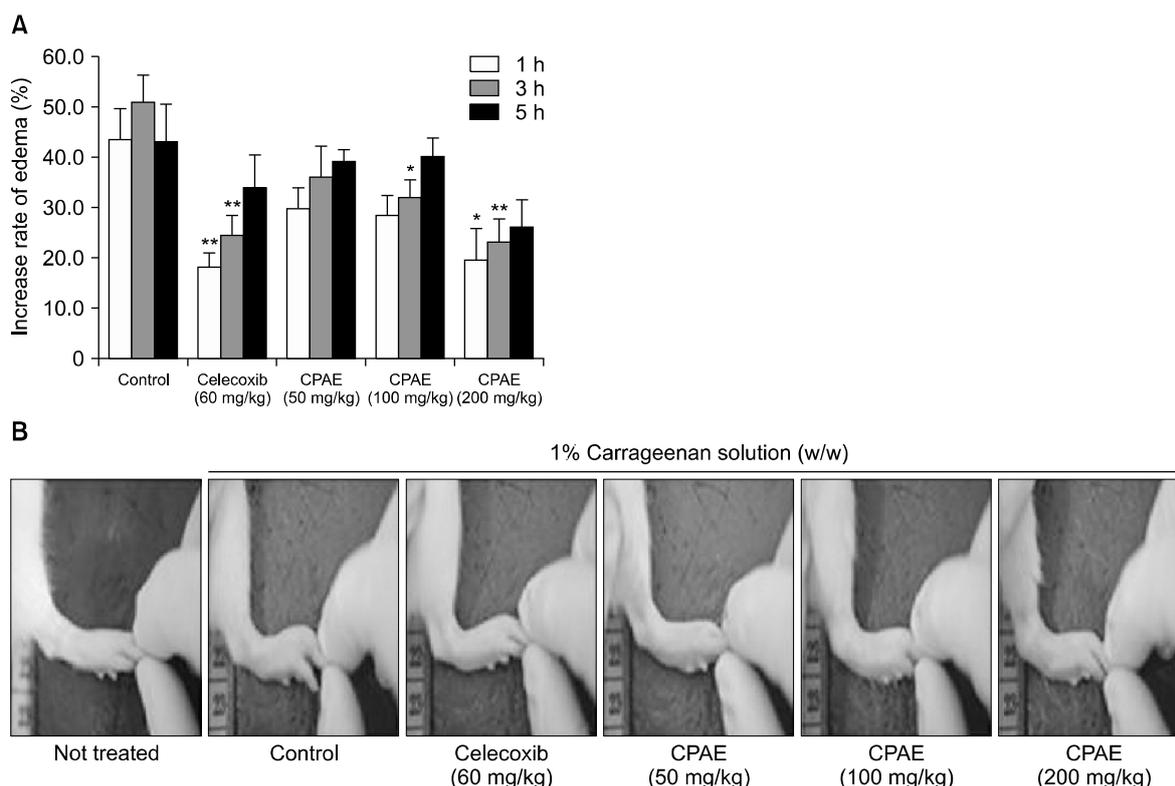


Fig. 5. Paw edema ratio after administration of *Cynanchum wilfordii*, *Phlomis umbrosa*, and *Angelica gigas* extract (CPAE) in carrageenan-induced inflammation in rats (A) and images of hind paw edema 3 h after carrageenan injection in rats (B). Value are mean±standard error of the mean (n=5). * $P<0.05$ and ** $P<0.01$ compared with control.

Table 1. Inhibitory effect of CPAE on carrageenan-induced inflammation in rat hind paws edema

	Time (h)		
	1	3	5
Normal control	43.4±6.2	51.0±5.2	43.2±7.3
Positive control (Celecoxib 60 mg/kg)	18.1±2.8** (58.4)	24.4±4.0** (52.1)	33.8±6.6 (21.7)
CPAE (50 mg/kg)	29.8±4.0 (31.4)	36.0±6.2 (29.4)	39.1±2.2 (9.3)
CPAE (100 mg/kg)	28.6±3.8 (37.0)	32.1±3.4* (37.1)	40.2±3.6 (7.0)
CPAE (200 mg/kg)	19.4±6.4* (55.3)	23.3±4.1** (54.3)	26.0±5.3 (39.7)

Values are mean±standard error (n=5).

* $P<0.05$ and ** $P<0.01$ compared with normal control.

Each value in parentheses indicates percentage inhibition.

CPAE: *Cynanchum wilfordii*, *Phlomis umbrosa*, and *Angelica gigas* extract.

cynandione A, which along with the C.W. root extract significantly decreased the lipopolysaccharide (LPS)-induced nitric oxide (NO) production and the expression of inducible NO synthase concentration-dependently without cytotoxicity (22). C.W. extract has also been reported to downregulate the expression of NF- κ B, and the main chemical constituents are thought to be *p*-hydroxyacetophenone and cynandione A (23). Therefore, the phenolic compounds in C.W. could contribute to its anti-inflam-

matory and anti-osteoarthritic effects.

P.U., another plant component of CPAE, has been reported to have anti-inflammatory effects, and the acetic acid-induced writhing, hot plate, carrageenan-induced paw edema, the xylene-induced ear swelling, and the acetic acid-induced Evans blue leakage and leukocyte infiltration tests were used to investigate the antinociceptive and anti-inflammatory activities of its aqueous extract (24). In addition, these effects are thought to be induced by iridoid glucoside, the main chemical compound in P.U.. Decursin has been confirmed to inhibit the activity of MMP-9 in RAW 264.7 cells. Furthermore, it has been reported to inhibit the induction of NF- κ B, an inflammatory mediator while nodakenin has also been reported to inhibit the NF- κ B pathway (25,26).

The results show that the raw material components of CPAE have synergistic anti-inflammatory effects. Furthermore, the synergistic effects may be mediated by the combined anti-inflammatory effects of the phenolic compounds in C.W., as well as the iridoid and coumarin compounds in P.U. and A.G., respectively.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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