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New Phytoformula (CAS) Containing the Roots of *Cyathula officinalis*, *Achyranthes japonica* and *Sophora subprostrata* Inhibits Collagen-induced Arthritis in Mice

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Abstract – The combined new phytoformula (CAS), a mixture (5 : 3 : 2, w/w/w) of the ethanol extracts of the roots of *C. officinalis* and *A. japonica*, and the *n*-butanol fraction of the *S. subprostrata* strongly inhibited arthritic severity score as well as IL-6 production in serum of collagen-induced arthritic mice. Histological observation also indicated that the CAS-treated group showed less breakdown of joint cartilage of the collagen-treated mice. In contrast, *C. officinalis* alone or a combination of *A. japonica* and *S. subprostrata* did not show significant inhibitory action on the same animal model. Thus, it is thought that CAS possesses a synergistic inhibitory action on arthritic condition. All these results strongly suggest that CAS may be a potential anti-arthritic agent.
Keywords – Arthritis, *Cyathula officinalis*, *Achyranthes japonica*, *Sophora subprostrata*, Inflammation

Introduction

The roots of *Cyathula officinalis*, *Achyranthes japonica* and *Sophora subprostrata* are frequently used plant materials in traditional medicine to treat inflammatory conditions. For instance, the roots of *C. officinalis* have been used for the restoration of normal menstrual cycle, as well as being used as anti-inflammatory agents and diuretics.[1] Previously, we have found that the ethanol extract of the roots of *C. officinalis* and its constituent, atractylenolide I, inhibited matrix metalloproteinase-13 (MMP-13) expression in IL-1β-treated SW1353 chondrocytes, suggesting a favorable effect on joint inflammation and cartilage degradation,[2] since MMP-13 is mainly responsible for degrading cartilage collagen materials under arthritic condition.[3,4] The roots of *A. japonica* and a related species, *A. bidentata*, have also been frequently used for the treatment of inflammatory disorders, especially for alleviating arthritic conditions in Korea.[5] Some analgesic and anti-inflammatory activities of *A. bidentata* were described,[6] and an herbal combination having *A. bidentata* and *Atractylodes japonica* was prepared and its anti-arthritic activity was demonstrated previously.[7] In addition, *S. subprostrata* is an anti-inflammatory agent in Chinese medicine.[8] In particular, we have previously reported that the methanol extract of the roots of this plant material inhibited an animal model of rheumatoid arthritis, adjuvant-induced arthritis in rats.[9]

Based on the potential of these plant materials, a new phytoformula (CAS) having the ethanol extract of the roots of *C. officinalis* and *A. japonica*, and the *n*-butanol fraction of the roots of *S. subprostrata* (5 : 3 : 2, w/w/w) was prepared, and its anti-arthritic activity was evaluated in the present investigation for an expectation of the improved and synergistic action towards the effects of arthritis.

Experimental

Chemicals – Prednisolone was obtained from Upjohn Co. (Kalamazoo, MI). Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA).

Animals – Male DBA mice (4 - 6 weeks, specific pathogen-free) were obtained from SLC (Shizuoka, Japan). Animals were fed with laboratory chow and water was freely available. They were acclimatized in an animal facility under conditions of 20 - 22 ºC, 40 - 60% relative humidity and 12 h/12 h light-dark cycle for at least 7 days. *In vivo* study was carried out in accordance with procedures adhering to the regulation of Korean Food and Drug Administration controlling experiments in live animals. And the experiment was approved by the local

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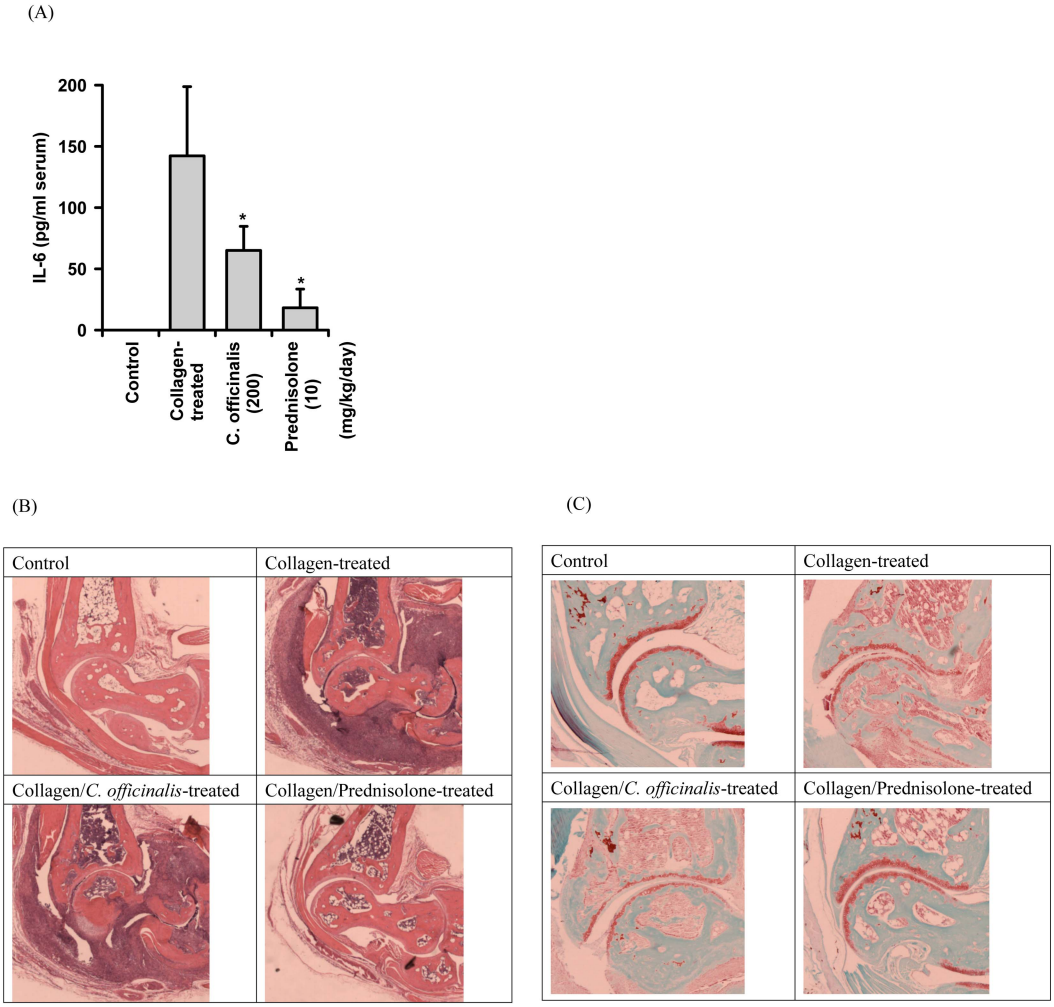

Fig. 1. Effects of the ethanol extract of *C. officinalis* on CIA in mice. (A) Effects on serum IL-6 concentration ($n = 3$), (B) Photomicrograph of the joints by H&E staining ($n = 3$), (C) Photomicrograph of the joints by Safranin O staining. Note: No apparent change in joint inflammation (B) and cartilage degradation (C) was observed between collagen-treated control and *C. officinalis*-treated groups. *: $P < 0.05$, **: $P < 0.01$, Significantly different from the collagen-treated control group.

ethical committee (KNU) for the experimentation on animals (KIACUC-11-0007).

Plant materials and preparation of the extract – The roots of *Cyathula officinalis* Kuan (Amaranthaceae), *Achyranthes japonica* (Miq.) Nakai (Amaranthaceae) and *Sophora subprostrata* (Leguminosae) were purchased from the Kyungdong herbal market (Seoul, Korea) and they were authenticized by one of the authors (Prof. Y. S. Kwon). Voucher specimens were deposited in College of Pharmacy (KNU).

These plant materials (1 kg, each) were extracted with 100% ethanol twice for 3 days at room temperature and the extracts were dried under vacuo to afford each ethanol extract of *C. officinalis*, *A. japonica* and *S. subprostrata* (20.3 g, 148.2 g and 45.8 g, respectively). The ethanol extract (40.0 g) of the roots of *S. subprostrata* was dispersed in water and fractionated with *n*-hexane followed by *n*-butanol. The *n*-butanol fraction was dried under vacuo, affording the butanol fraction (12.4 g). New phytoformula (CAS) is a mixture (5 : 3 : 2, w/w/w) of the ethanol extracts of *C. officinalis* and *A. japonica*, and the butanol fraction of *S. subprostrata* and used throughout this study.

Collagen-induced arthritis (CIA) in mice – Bovine type II collagen (2 mg/ml) dissolved in 0.05 M acetic acid (Chondrex, Inc., Redmond, WA, USA) was emulsified with an equal volume of complete Freund's adjuvant (Chondrex, Inc.). For an induction of CIA, this emulsion was injected intradermally to the base of the tail of DBA mice (120 μl/mouse). Booster injection was carried out (150 μl/mouse) after 3 weeks of initial immunization.[10] Starting from the day of the booster injection, test

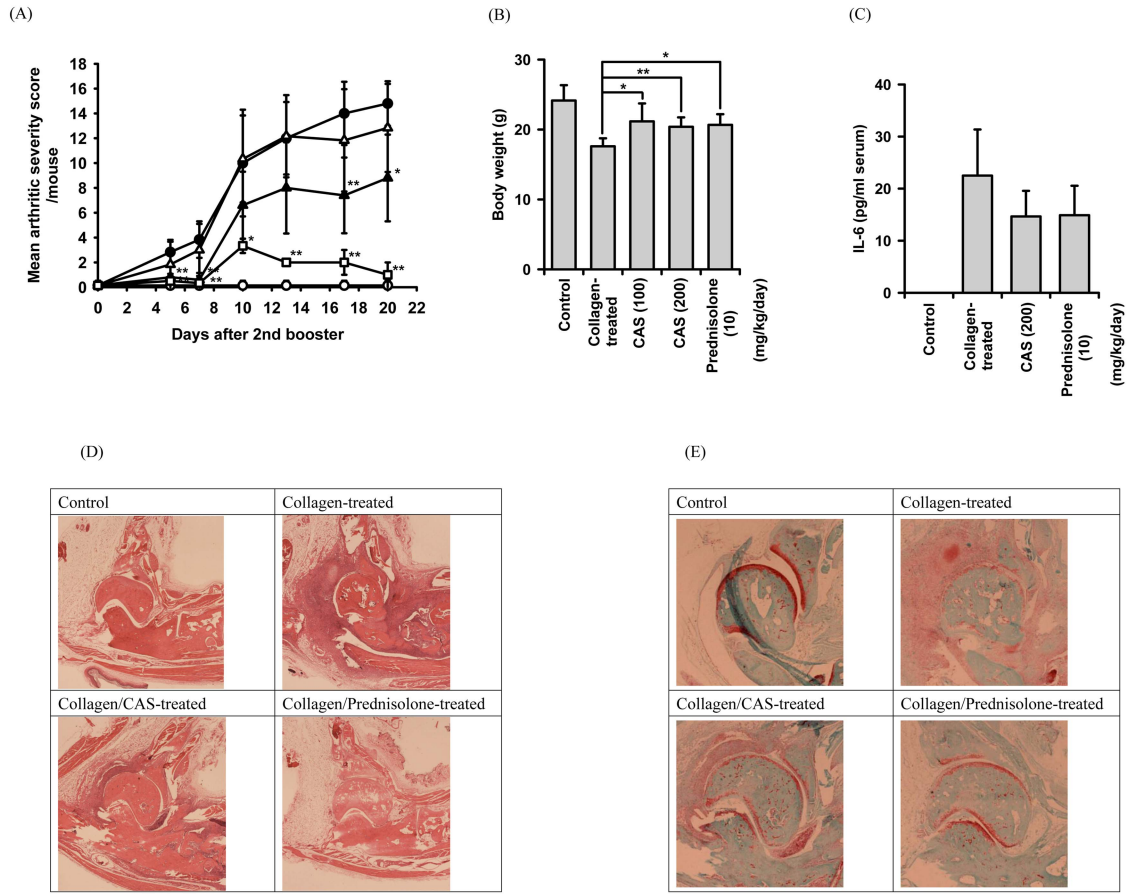

Fig. 2. Effects of the new phytoformula (CAS) on CIA in mice. (A) Effects on the arthritic severity score ($n = 6$), Control (○), collagen-treated (●), collagen/CAS-treated (△), collagen/CAS-treated (▲), prednisolone-treated (□), (B) Effects on the body weight increase ($n = 6$), (C) Effects on the serum IL-6 concentration ($n = 5$), (D) Photomicrograph of the joints by H&E staining, ×100 (e) Photomicrograph of the joints by Safranin O staining, x100. *: $P < 0.05$, **: $P < 0.01$, Significantly different from the collagen-treated control group.

compounds dissolved in 0.1% CMC were administered orally for 3 weeks at the indicated doses. The control group only received sterile saline instead of the emulsion intradermally and 0.1% CMC orally. Assessment of arthritic severity was evaluated by visual inspection. All four legs of the mice were evaluated and scored from 0 to 5 grades according to the severity (0: normal to 4: severe inflammation on whole limb. 5: deformed joint). The score of all four legs were combined and expressed as mean severity score per mouse. For histology, mice were sacrificed and knee joints were removed. After routine fixation and decalcification, tissue sections were stained with hematoxylin and eosin (H&E) or safranin O. For determination of serum levels of IL-1 and IL-6, blood was withdrawn and the cytokine concentrations were measured using ELISA (Cayman Chem., Ann Arber, MI, USA) according to the recommended procedure of the manufacturer.

Statistical analysis – Experimental values were represented as arithmetic mean ± SD. Statistical analysis was evaluated using one-way ANOVA followed by Dunnett's analysis. P values less than 0.05 were regarded to be significantly different.

Results

The anti-arthritic activity of the ethanol extract of *C. officinalis* alone was examined in the CIA model. As shown in Fig. 1A, an immunization with type II collagen provoked a drastic increase of serum IL-6 level, but no significant change of the IL-1α level in collagen-treated mice was observed (data not shown). Under this condition, oral administration of the ethanol extract of *C. officinalis* (200 mg/kg/day) alone significantly reduced the serum IL-6 concentration. However, the arthritic severity score was not significantly reduced in the *C. officinalis*-treated group (data not shown), and the clear improvement of cartilage inflammation and degradation was not seen in the *C. officinalis*-treated group by

histological observation (Fig. 1B and 1C). On the other hand, the reference drug, prednisolone (10 mg/kg/day) almost completely reduced IL-6 concentration, and histology clearly demonstrated the inhibitory action against joint inflammation.

Furthermore, the anti-arthritic activity of CAS was examined. When CAS (100 and 200 mg/kg/day) was orally administered, a high dose-treatment group (200 mg/kg/day) strongly inhibited the arthritic severity score, and significantly reversed body weight loss induced by collagen treatment (Fig. 2A and 2B). In addition, CAS strongly reduced the IL-6 level in serum (Fig. 2C). Moreover, histological observation showed much improvement of joint inflammation and cartilage degradation (Fig. 2D and 2E). Prednisolone (10 mg/kg/day) potently inhibited the arthritic severity score, joint inflammation and cartilage degradation, as expected. In another experiment, the ethanol extract of *A. japonica* and the butanol fraction of *S. subprostrata* (3 : 2, w/w, 100 mg/kg/day) were administered in the same CIA model. However, no reduction of the arthritic severity score and no improvement by histological observation were found (data not shown).

All these results clearly demonstrated a synergistic enhancement of anti-arthritic activity and cartilage protection of CAS compared to that upon treatment of *C. officinalis* alone or the combined treatment of *A. japonica* and *S. subprostrata*.

Discussion

The present investigation clearly demonstrated that a new phytoformula (CAS), having the roots of *C. officinalis* as major component, possesses anti-arthritic activity with some cartilage protective action.

Particular pathological conditions, such as rheumatoid arthritis and osteoarthritis, cause cartilage matrix materials to be rapidly hydrolyzed, resulting in cartilage degradation. Matrix metalloproteinases (MMPs) and aggrecanases are largely responsible for this process, and particularly, MMP-13 is a key collagenase that acts in synovial joint space. Thus, it is reasonably considered that MMP-13 down-regulator(s) may have some potential to block cartilage degradation and to alleviate the symptoms of arthritis. In this respect, medicinal plants may provide a good source of potential agents. Based on this concept, many plant extracts were examined in the screening procedure in our laboratory, and the roots of *C. officinalis* were determined to act as a down-regulator of MMP-13 expression.[2] However, the pharmacological activity of *C. officinalis* alone is not strong enough to be developed as new anti-arthritic drug, as has been determined through the present investigation. It has some inhibitory activity on CIA, but it did not improve the arthritic severity score. On the other hand, CAS showed strong inhibitory action against all parameters examined, including CIA and several associated biomarkers. Compared to the pharmacological potency of each plant material, it is clear that CAS possesses an improved and synergistic activity on arthritic condition.

Many constituents were previously isolated from the roots of *C. officinalis*. They include cyasterone derivatives,[11] and oleanolic acid derivatives.[12] We also isolated several components, and one of the isolated constituents, atractylenolide I, was shown to inhibit MMP-13 induction in chondrocytes, and to inhibit λ-carrageenan-induced paw edema in mice.[2] It is worth to mention that one previously reported herbal combination showing anti-arthritic activity contains *A. bidentata* and *Atractylodes japonica*.[7] The latter herbal material possesses atractylenolide I as one of the major constituents.[13] Therefore, it is suggested that atractylenolide I might contribute to the anti-arthritic activity in this combination, and in our preparation, CAS.

As mentioned above, *A. japonica* and a related species, *A. bidentata,* have been widely prescribed to treat arthritic conditions in Korea.[5] *A. japonica* may possess some anti-arthritic activity, since one publication of the combined use of *A. bidentata* and *Atractylodes japonica* showed anti-arthritic activity.[7] In our experiment, an addition of *A. japonica* and *S. subprostrata* to the extract of *C. officinalis* certainly showed strong arthritic inhibitory action in an animal model, although a combined preparation of *A. japonica* and *S. subprostrata* only did not exert significant inhibitory activity.

S. subprostrata is an anti-inflammatory agent in Chinese medicine.[8] In particular, this plant material was demonstrated to inhibit an animal model of adjuvant-induced arthritis in rats. Moreover, the *n*-butanol fraction of the roots of *S. subprostrata* showed stronger inhibitory activity on the same animal model.[9] Based on these previous observations, CAS is formulated to contain the *n*-butanol fraction of the roots of *S. subprostrata* instead of the ethanol extract. Although the detailed action mechanisms are not elucidated in this study, the three plant materials *C. officinalis*, *A. japonica* and *S. subprostrata,* together provide improved and synergistic action on an animal model of arthritis. CAS may be safely used in human arthritic conditions.

As a conclusion, the present investigation indicates that the combined new phytoformula (CAS), a mixture (5 : 3 : 2,

w/w/w) of the ethanol extracts of the roots of *C. officinalis* and *A. japonica*, and the *n*-butanol fraction of the *S. subprostrata*, may have potential for the treatment of arthritic conditions.

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