

Prophylactic and Therapeutic Effects of *Acanthopanax senticosus* Harms Extract on Murine Collagen-induced Arthritis

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Evidences are accumulating that extract of *Acanthopanax senticosus* Harms (ASH; syn *Eleutherococcus senticosus* [Rupr. & Maxim.] Maxim), a shrub native to Northeastern Asia, has antiinflammatory effects. In this study, we examined prophylactic and therapeutic effects of ASH extract (ASHE) on rheumatoid arthritis using collagen-induced arthritis (CIA) mouse model. *Acanthopanax senticosus* Harms extract was administered before the onset of arthritis in the prophylaxis model. In the therapeutic model, ASHE was administered after the onset of arthritis with or without anti-TNF- α antibody. The ASHE treatment showed efficacy before onset of CIA but there was no effect after CIA was established. The ASHE treatment delayed the onset and decreased severity of CIA. *In vitro* examinations showed that ASHE is an antioxidant and that ASHE suppresses TNF- α and interleukin-6 production in human peripheral blood mononuclear cells. The combination therapy with ASHE and anti-TNF- α antibody reduced the severity of arthritis compared with anti-TNF- α antibody alone. The present study shows that ASHE has prophylactic effect against CIA and support therapeutic effect of anti-TNF- α antibody. © 2014 The Authors. *Phytotherapy Research* published by John Wiley & Sons Ltd.

Keywords: *Acanthopanax senticosus* Harms; collagen-induced arthritis; reactive oxygen species; inflammatory cytokines; anti-TNF- α antibody.

INTRODUCTION

Acanthopanax senticosus Harms (ASH; syn *Eleutherococcus senticosus* [Rupr. & Maxim.] Maxim), also known as Siberian ginseng or eleuthero, is a shrub that belongs to the family *Araliaceae*. It grows in taiga of China, Korea, Russia, and Hokkaido island of Japan. For thousands of years, the extract of this herb has been used as oriental folk medicine and known to have beneficial effects on psychophysical stress, fatigue, chronic bronchitis, autoimmune diseases, hypertension, ischemic heart disease, gastric ulcer, and allergic responses (Davydov and Krikorian, 2000; Dowling *et al.*, 1996; Fujikawa *et al.*, 1996; Nishibe *et al.*, 1990; Yi *et al.*, 2002). ASH extracts can be obtained in the USA and numerous other countries, and are used as an herbal dietary supplement that exhibits nutritionally fortifying effects.

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder characterized by chronic, symmetric, and erosive synovitis caused by infiltration of activated T-cells and macrophages resulting in hyperproliferation of synovial cells (Feldmann *et al.*, 1996). A number of cytokines, including TNF- α , interleukin 1 β (IL-1 β), and IL-6 are critical pathogenesis of RA; they are produced by synovial cells, macrophages, and T-cells (McInnes and Schett, 2007); and these cytokines are the targets to treat RA (Nam *et al.*, 2010). Osteoclasts are differentiated and stimulated by IL-1 β and TNF- α and are responsible for joint destruction (McInnes and Schett, 2007).

Reactive oxygen species (ROS) are another toxic factor in RA (Hitchon and El-Gabalawy, 2004). ROS are generated by various types of cells in the body, and they are involved in physiological as well as pathological processes (Valko *et al.*, 2007). Cigarette smoking is a risk factor of developing RA (Heliovaara *et al.*, 1993; Silman *et al.*, 1996); it is a well-known source of ROS (Valavanidis *et al.*, 2009). Oxidative damage to hyaluronate (Grootveld *et al.*, 1991), lipoproteins (Dai *et al.*, 2000), and DNA (Hajizadeh *et al.*, 2003) is observed in synovial fluid of patients with RA. Many antioxidants including superoxide dismutase 3, an enzyme that scavenges superoxide, are shown to inhibit arthritis in rodent models (Cuzzocrea *et al.*, 2000; Bandt *et al.*, 2002; Iyama *et al.*, 2001). Although, arthritis is enhanced in superoxide dismutase 3 knockout mice (Ross *et al.*, 2004). Redox signaling is a critical regulator of transcription factors involved in RA (Michiels *et al.*, 2002).

Recently, ASH was demonstrated to inhibit superoxide and hydrogen peroxide production in mouse peritoneal macrophages *in vitro* and *in vivo* (Lin *et al.*, 2008). In this study, we investigated whether or not ASH extract (ASHE) exerts a therapeutic effect in a collagen-induced arthritis (CIA) mouse model and compared it with the effects of vitamin C (ascorbic acid), which is used as a simple, easily available well-known nutritional supplement, and is well-established.

MATERIALS AND METHODS

Reagents. *Acanthopanax senticosus* Harms powder was obtained from Yakuhan Pharmaceutical Co., LTD.,

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(Kitahiroshima, Japan). They are dissolved in distilled water at 100 mg/mL to obtain ASHE solution. Ascorbic acid (AA), cytochrome c, phorbol 12-myristate 13-acetate, lipopolysaccharide (LPS, derived from *Escherichia coli* serotype 026:B26) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine type II collagen (CII) was purchased from Collagen Research Center (Tokyo, Japan). Freund's incomplete adjuvant was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Goat anti-mouse TNF- α antibody was purchased from BioLegend, (San Diego, CA, USA).

Animal and induction of collagen-induced arthritis. The study protocol was approved by the Animal Ethics Committee of Sapporo Medical University (No. 09-089). Male 4–6 weeks old DBA/1J mice were purchased from Charles River Laboratories (Yokohama, Japan). The mice were acclimatized to our facility for at least 7 days before the experiment. They were housed in standard laboratory conditions ($22 \pm 3^\circ\text{C}$; relative humidity, 50–55%; 12 h light/dark cycle) and given free access to food and water.

Collagen-induced arthritis was induced as described, previously (Coutenay *et al.*, 1980). Briefly, the mice were immunized with 100 μg of CII emulsified in Freund's incomplete adjuvant by intradermal injection on day -21. The mice received booster immunization on day 0. After the secondary immunization, severity of arthritis was monitored according to a previously described clinical score (0, normal; 1, erythema and mild swelling confined to the ankle joint and toes; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and severe swelling extended from the ankle to the metatarsal joints; and 4, ankylosing deformity with joint swelling) (Ji *et al.*, 2012). In addition to the clinical score, thickness of hind paw of each mouse was measured using a foot pad thickness gauge (Ozaki Mfg. Co., Ltd., Tokyo, Japan).

Treatment protocol. Treatment protocols are designed as described in Fig. 1. In the Experiment-1, prophylactic

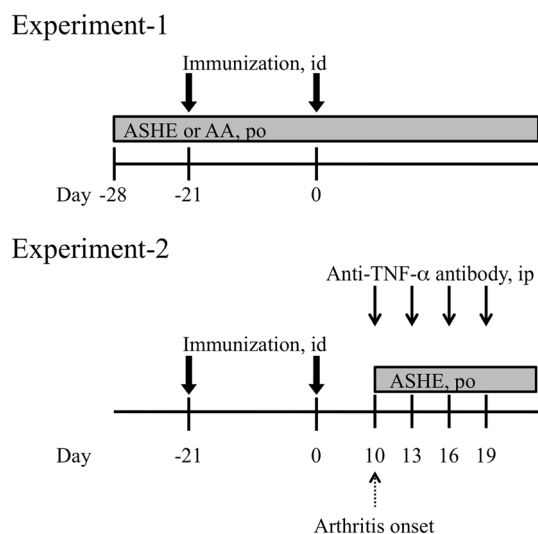


Figure 1. Experimental designs of collagen-induced arthritis in a mouse model. The detail is described in Materials and Methods.

effect of ASHE and AA was compared. A single dose of ASHE (500 $\mu\text{g/g}$ body weight/day) or AA (10 mg/g body weight/day) was administered to each mouse by gastric gavage, everyday from day -28. The doses of ASHE and AA administered to mice were determined with reference to previous studies (Huang *et al.*, 2011a, 2011b; Craven *et al.*, 1997). In the Experiment-2, whether ASHE enhances therapeutic effect of anti-TNF- α antibody was examined. Either 50 μg of anti-TNF- α antibody or its isotype control was administered intraperitoneally to each mouse on day 10, 13, 16, and 19. ASHE or distilled water was administered to each mouse by gastric gavage, everyday from day 10.

Neutrophil and peripheral blood mononuclear cell isolation. Peripheral blood mononuclear cells (PBMCs) and neutrophils were purified from healthy volunteer using Ficoll-Paque (GE Healthcare, Piscataway, NJ), according to the manufacturer's instructions. Briefly, whole blood sample was laid over Ficoll-Paque. After centrifugation, PBMCs were collected from the upper layer. Neutrophils and red blood cells were collected from the sediments. The red blood cells were lysed by hypotonic buffer, and neutrophils were purified. Purity of PBMCs and neutrophils were 96% and 93%, respectively.

Electron spin resonance spectroscopy. Radical scavenging activities of ASHE and AA were examined using electron spin resonance (ESR) spectroscopy, according to a method described (Nakayama *et al.*, 2001). In brief, superoxide and hydroxyl radicals were generated by ultraviolet irradiation (200-W mercury arc RUF-203S, Radical Research, Inc., Tokyo, Japan) of a sodium phosphate buffer (0.1 M, pH 7.4) containing riboflavin (Sigma-Aldrich Biotechnology) or H_2O_2 (Sigma-Aldrich Biotechnology), respectively. The free radicals were trapped by 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline-N-oxide (Radical Research, Inc., Tokyo, Japan), and the ESR signals were recorded using JEOL FE3XG X-band spectrometer (Japan Electron Optics Laboratory, Tokyo, Japan).

Measurement of superoxide production. Superoxide production was determined by the cytochrome c reduction method using 96-well plates, according to a method described (Nagata *et al.*, 1995). Briefly, neutrophils were suspended in PBS at a concentration of 2×10^7 cells/mL, and the reaction was initiated by mixing 200 μL of cell suspension with 1.5 mM cytochrome c and 25 $\mu\text{g/mL}$ phorbol 12-myristate 13-acetate in the presence or absence of ASHE or AA. The absorbance of reduced cytochrome c was recorded at 37°C for 5 min using a spectrophotometer with a wavelength of 550 nm. Superoxide production was expressed in $\text{nmol/min}/1 \times 10^5$ cells.

RNA extraction and reverse transcriptase-polymerase chain reaction. Peripheral blood mononuclear cells were plated in six-well plates at 1×10^5 cells/well and incubated for 1 h with or without 20 $\mu\text{g/mL}$ LPS in the presence or absence of ASHE or AA in serum-free RPMI-1640. Total

RNA was isolated using the RNeasyPlus Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized by TaqMan Reverse Transcription Reagents (Applied Biosystems, Branchburg, NJ). Polymerase chain reaction (PCR) amplification of the TNF- α , IL-6, and glyceraldehyde 3-phosphate dehydrogenase genes was performed using the following primers. For TNF- α mRNA, 5'-TCTCGA ACCCCGAGTGACAA-3' and 5'-GATGAGGGTCC AGGAGAAGT-3' were used as forward and reverse primers, respectively. For IL-6 mRNA, 5'-ATGAACT CCTTCTCCACAAGCGC-3' and 5'-GTCAGGTCGG ACTCCCGAGAAG-3' were used as forward and reverse primers, respectively. For internal control, glyceraldehyde 3-phosphate dehydrogenase was amplified using 5'-GCAGGGGGGAGCCAAAAGGG-3' and 5'-TGCC AGCCCCAGCGTCAAAG-3' were used as forward and reverse primer, respectively. The PCR products were subjected to polyacrylamide gel electrophoresis, and they were visualized and semiquantified using the ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA).

Cytokine detection by enzyme-linked immunosorbent assay. Peripheral blood mononuclear cells were plated in 96-well plates at 1×10^5 cells/well and incubated for 0, 12, or 24 h with or without 20 $\mu\text{g}/\text{mL}$ LPS in the presence or absence of ASHE or AA in serum-free RPMI-1640. Protein levels of TNF- α and IL-6 of the culture supernatants were analyzed using the Human TNF-alpha/TNF-SF1A Quantikine HS enzyme-linked immunosorbent assay (ELISA) and Human IL-6 QuantiGlo ELISA kit (R&D System, Minneapolis, MN, USA), respectively, according to the manufacturer's instructions.

Statistical analyses. Statistical analysis was performed using the Kruskal Wallis tests as appropriate. One-way analysis of variance followed by Dunnett's *post hoc* test was used to compare the differences between three or more groups. The program used for the statistical analysis was the SPSS statistical software package, standard version 22.0 (SPSS, Chicago, IL, USA). *p*-values < 0.05 were considered statistically significant.

RESULTS

Acanthopanax senticosus Harms extract delays onset of collagen-induced arthritis and reduces arthritis severity

We first examined the effects of ASHE and AA on CIA (Fig. 1, Experiment-1). In the control group, signs of arthritis appeared on day 11.3 ± 0.4 and worsened in a time-dependent manner (Tables 1 and 2, Fig. 2). On the other hand, mice treated with ASHE showed signs of arthritis from day 19.6 ± 0.7 (Table 2). ASHE delayed development of CIA from the point of view of its onset and the peak (Tables 1 and 2).

The worst clinical score and maximum hind paw thickness were better in mice treated with ASHE than the control group (Clinical score 11.8 ± 2.1 vs. 5.6 ± 0.8 ,

Table 1. Day of onset and peak severity of collagen-induced arthritis evaluated by hind paw thickness

	Onset	Peak
DW	12.3 ± 1.0	25.4 ± 0.9
AA	11.9 ± 0.8	25.0 ± 1.2
ASHE	25.4 ± 0.9 ($p < 0.01$)	28.3 ± 1.0 ($p < 0.01$)

($n = 8$)

DW, distilled water; AA, ascorbic acid; ASHE, *Acanthopanax senticosus* Harms extract solution.

Statistics analyses were performed between vehicle and administered groups, respectively.

Table 2. Day of onset and peak severity of collagen-induced arthritis evaluated by clinical score

	Onset	Peak
DW	11.3 ± 0.4	26.8 ± 1.3
AA	9.8 ± 0.7	26.0 ± 1.1
ASHE	19.6 ± 0.7 ($p < 0.01$)	28.5 ± 1.1 ($p < 0.01$)

($n = 8$)

DW, distilled water; AA, ascorbic acid; ASHE, *Acanthopanax senticosus* Harms extract solution.

Statistics analyses were performed between vehicle and administered groups, respectively.

$p = 3.81 \times 10^{-4}$; hind paw thickness 2.68 ± 0.09 mm vs. 2.47 ± 0.05 mm, $p = 8.29 \times 10^{-4}$) (Fig. 2). Mice treated with AA had no significant differences compared with the control group in terms of arthritis development and the severity (Fig. 2, Tables 1 and 2).

Acanthopanax senticosus Harms extract and ascorbic acid show antioxidant activities *in vitro*

Next, we examined whether ASHE and AA inhibit ROS production or scavenge free radicals by *cytochrome c* reduction assay and ESR spectroscopy, respectively. ASHE and AA eliminated superoxide (Fig. 3A) and hydroxyl radical (Fig. 3B), dose-dependently. In these concentrations, ASHE and AA inhibited the superoxide production in a dose-dependent manner (Fig. 3C), and the viability of neutrophils was maintained within concentrations (data not shown). These results show that both ASHE and AA have antioxidant activities *in vitro*.

Acanthopanax senticosus Harms extract suppresses production of inflammatory cytokines

The effect of ASHE and AA on TNF- α and IL-6 synthesis was examined by measuring mRNA levels of TNF and IL-6 of human PBMCs after LPS stimulation. Incubation of PBMCs with ASHE resulted in a concentration-dependent suppression of TNF and IL-6 mRNA expression (Fig. 4A and B). Meanwhile, AA showed small effect on TNF and IL-6 mRNA production (Fig. 4A and B).

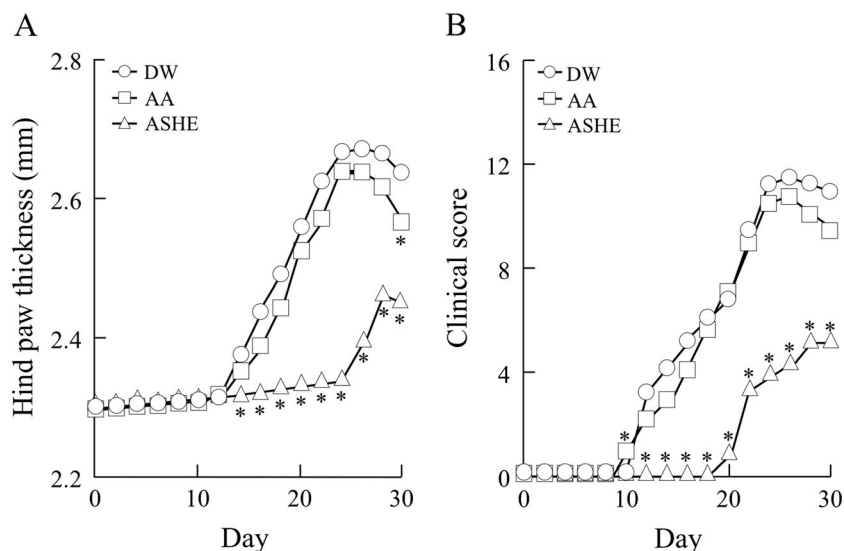


Figure 2. Prophylactic effects of ASHE in collagen-induced arthritis mice. Experiment-1 is carried out as described in Materials and Methods. The figure shows dot plot of hind paw thickness (A) and clinical scores (B). DW, distilled water; AA, ascorbic acid; ASHE, *Acanthopanax senticosus* Harms extract solution. *, statistically significant ($p < 0.05$) compared with the values of DW treated mice. ($n = 8$).

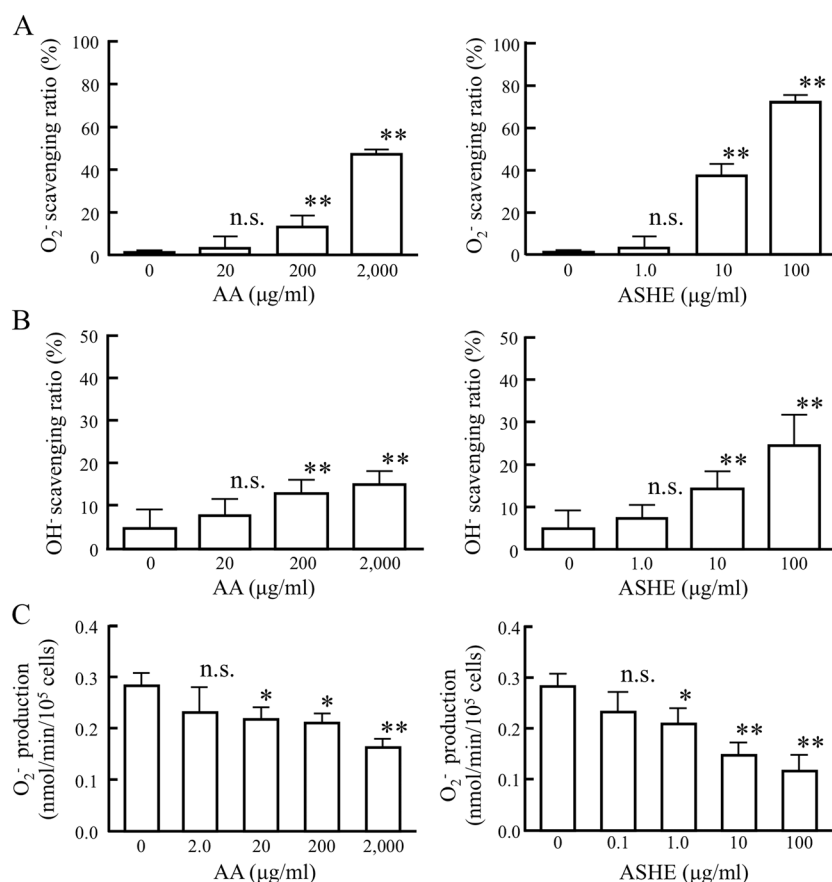


Figure 3. Ascorbic acid (AA) and *Acanthopanax senticosus* Harms extract (ASHE) scavenge free radicals and inhibits the production of superoxide in human neutrophils *in vitro*. Radical scavenging activities of ASHE and AA were measured as described in Materials and Methods. (A) Superoxide scavenging activity were measured by electron spin resonance spectroscopy. (B) Hydroxyl radical scavenging activity was measured by electron spin resonance spectroscopy. (C) Superoxide produced by neutrophils was measured in the presence or absence of AA or ASHE. Error bars denote +2 standard errors of the mean ($n = 9$). *, statistically significant ($p < 0.05$) compared with the values of control. **, statistically significant ($p < 0.01$) compared with the values of control. n.s., statistically not significant compared with the values of control.

Enzyme-linked immunosorbent assay confirmed that production of TNF- α and IL-6 was remarkably reduced with 100 $\mu\text{g/mL}$ ASHE treatment (Table 3). Consistent

to reverse transcription polymerase chain reaction analysis, AA showed smaller effect on TNF- α and IL-6 production, compared with ASHE.

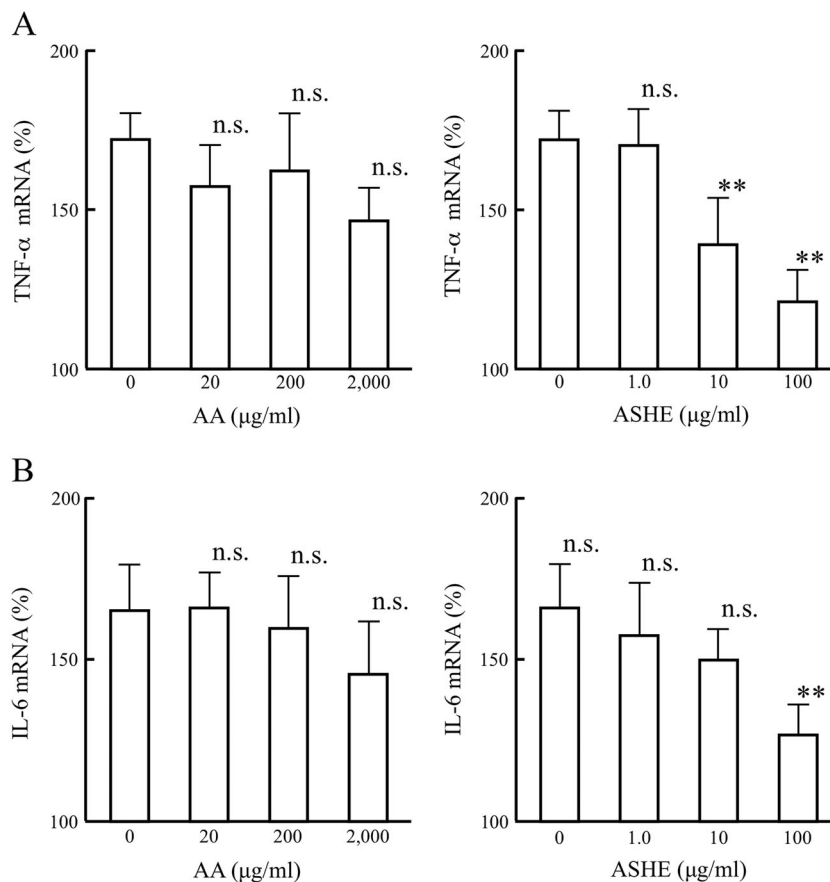


Figure 4. *Acanthopanax senticosus* Harms extract (ASHE) suppresses inflammatory cytokine production in human peripheral blood mononuclear cells. Levels of *TNF-α* (A) and *IL-6* (B) mRNA were quantified by reverse transcriptase-polymerase chain reaction ($n = 9$). Levels of target mRNAs were normalized relative to those of *GAPDH* transcripts. Error bars denote two standard deviations. **, statistically significant ($p < 0.01$) compared with the values of control. n.s., statistically not significant compared with the values of control.

Table 3. Effect of ascorbic acid and *Acanthopanax senticosus* Harms extract on cytokine production in human peripheral blood mononuclear cells *in vitro*

	TNF- α (pg/mL)	IL-6 (pg/mL)
DW	31.5 \pm 6.2	57.0 \pm 12.4
AA		
20 μ g/mL	28.8 \pm 6.2	51.3 \pm 5.6
200 μ g/mL	29.8 \pm 5.1	41.8 \pm 4.0
2,000 μ g/mL	25.2 \pm 6.5	33.0 \pm 6.3
ASHE		
1 μ g/mL	28.6 \pm 5.7	50.0 \pm 6.9
10 μ g/mL	25.1 \pm 4.0	38.7 \pm 8.8
100 μ g/mL	7.3 \pm 1.9 ($P < 0.05$)	12.2 \pm 4.7 ($p < 0.05$)

($n = 6$)

PBMCs, peripheral blood mononuclear cells; DW, distilled water; AA, ascorbic acid; ASHE, *Acanthopanax senticosus* Harms extract solution.

Statistics analyses were performed between vehicle and treatment groups, respectively.

Acanthopanax senticosus Harms extract enhances therapeutic effect of anti-TNF- α antibody

Next, we checked whether ASHE enhances therapeutic efficacy of anti-TNF- α antibody in CIA mice (Fig. 1, animal experiment-2). As shown in Fig. 5, combination of

ASHE and anti-TNF- α antibody significantly reduced arthritis compared with anti-TNF- α antibody alone, as demonstrated by the hind paw thickness (Fig. 5A) and clinical score (Fig. 5B).

DISCUSSION

In the present study, ASHE demonstrated prophylactic and therapeutic effects in CIA mouse model (Figs 2 and 5). Moreover, ASHE showed antioxidant activity and suppressed TNF- α and IL-6 production *in vitro* (Figs 3, and 4). To the best of our knowledge, this is the first study to show beneficial effects of ASHE on arthritis *in vivo*. The results, reduction of ROS and suppression of inflammatory cytokine production, are consistent with the observations reported in previous studies (Lin *et al.*, 2008; Yi *et al.*, 2001; Yokozawa *et al.*, 2003; Chen *et al.*, 2010).

Acanthopanax senticosus Harms extract and AA both had antioxidant activity *in vitro* (Fig. 3); however, only ASHE was beneficial to CIA (Fig. 2). This discrepancy could probably be attributed to: (i) AA was not able to decrease ROS *in vivo* to the level, which is sufficient to prevent arthritis, (ii) in addition to the reduction of ROS, inhibition of cytokine production is required to prevent arthritis, or (iii) there may be a difference between AA and ASH in terms of body clearance. Anti-CII antibody triggers arthritis in CIA (Cho *et al.*, 2007). To gain an insight into the mechanism how ASHE delayed onset of CIA,

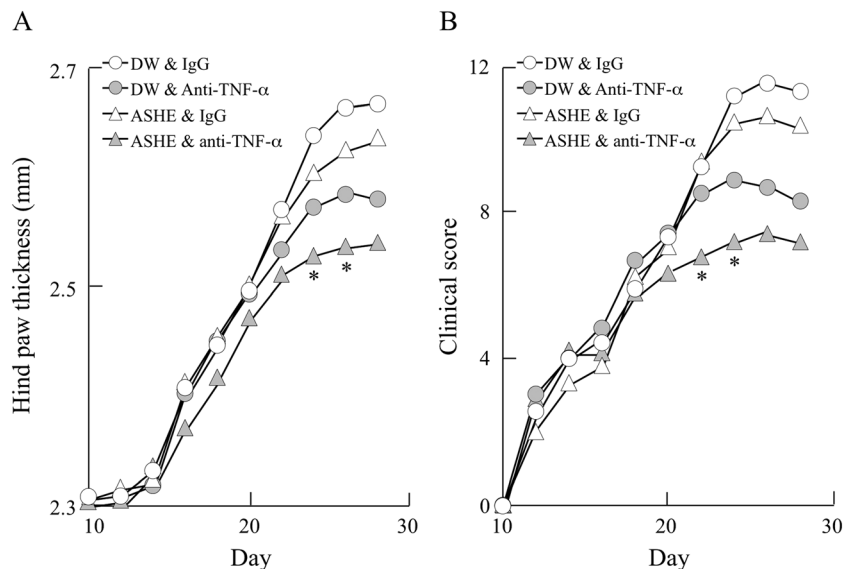


Figure 5. *Acanthopanax senticosus* Harms extract enhances the therapeutic effect of anti-TNF- α antibody. Experiment-2 is carried out as described in Materials and Methods. The figure shows the dot plot of hind paw thickness (A) and clinical scores (B). DW, distilled water; AA, ascorbic acid; ASHE, *Acanthopanax senticosus* Harms extract solution. *, statistically significant ($p < 0.05$) compared with the value of mice treated anti-TNF- α antibody alone. ($n = 6$).

we have monitored titers of antibody against CII in mice treated with vehicle and ASHE. As shown in Figure S1, expression of anti-CII antibody in mice treated with ASHE delayed compared to that of mice treated with vehicle. TNF- α and IL-6 play central role in immune responses including humoral immunity (Hehlgans and Pfeffer, 2005; Kishimoto *et al.*, 1995). This suggests that ASHE, which inhibits inflammatory cytokine production, may therefore also affect the production of anti-CII antibody, thus exerting a prophylactic effect on CIA mice. These observations indicate that inhibition of cytokine production may be the key feature of ASHE to delay CIA in mouse model.

To date, 88 chemical constituents are isolated from ASHE, including volatile compounds, triterpenoid saponins, lignans, coumarins, and flavones (Huang *et al.*, 2011a, 2011b). Among the components, chlorogenic acid, eleutheroside B (syringin), and (+)-syringaresinol-O- β -D-glucoside are able to inhibit free radical and cytokine production (Huang *et al.*, 2011a, 2011b), the compositions of these major compounds in ASHE are shown in Table S1; therefore, these are the candidate components of ASHE that inhibit CIA.

Acanthopanax senticosus Harms extract extract is commercially available in many countries, and several clinical trials are conducted; no obvious adverse effect is reported, when it is administered as single agent (Huang *et al.*, 2011a, 2011b). In this study, we have monitored body weight and performed blood examination every week until day 70 in mice treated with ASHE and found no unfavorable event. ASHE is a safe drug that can be used over a relatively long period.

Acanthopanax senticosus Harms extract had prophylactic effect when it was administered before primary CII immunization in CIA mouse model (Fig. 2). Although, it did not reduce arthritis when it was administered as single

agent after arthritis is established (Fig. 5). From these observations, ASHE should be combined with nonbiological disease-modifying antirheumatic drugs or biological agents targeting inflammatory cytokines to treat active rheumatoid arthritis. Otherwise, it may be administered to patients in remission to prevent reactivation of RA.

According to a study reported by Williams *et al.*, 50 μ g of anti-TNF- α antibody per mouse is a dose that shows minimum therapeutic effect in CIA mouse model (Williams *et al.*, 1992). In the experiment-2, we have chosen this dose of anti-TNF- α antibody to test therapeutic effects of ASHE. It is reasonable to speculate that greater therapeutic response can be obtained in CIA, if dose of anti-TNF- α antibody is increased.

In this study, we investigated the prophylactic and therapeutic effects of the supplements AA and ASHE in CIA model mice. ASHE delayed RA onset and augmented the effect of anti-TNF- α antibody therapy in CIA mouse model. Consequently, ASHE was shown to have potential to lead prophylactic effect or to support therapeutic effect of anti-TNF- α antibody. As ASHE can be commercially purchased as a supplement, it is readily available and immediately accessible to the patients.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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