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PAPER

The ethyl acetate extract of alfalfa sprout ameliorates disease severity of autoimmune-prone MRL-*lpr/lpr* mice

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Previous study showed that soy isoflavone supplement alleviates disease severity in autoimmune-prone mice. As the ethyl acetate extract of alfalfa sprout (AS) has selective oestrogenic and anti-inflammatory activity, this study evaluated the effects of alfalfa sprout ethyl acetate extract (ASEA) on disease severity of systemic lupus erythematosus, using autoimmune-prone female MRL-*lpr/lpr* mice. In Experiment 1, five groups of 12-week-old female mice were per oral treated with vehicle (control), lyophilized AS (550 mg wt/kg BW), ASEA (ASEA, 25 mg/kg BW), coumestrol (CUM, 0.075 mg/kg BW) and tamoxifen (TAM, 0.375 mg/kg BW) as the positive control. The onset of proteinuria was delayed, and the life span was significantly longer in the ASEA and TAM groups but neither in the AS nor in the CUM groups, compared to the control. To examine the changes in the immunological parameters related to disease process, three more groups of MRL-*lpr/lpr* female mice (control, ASEA and TAM) were fed in a similar manner for 6 weeks in the Experiment 2. Flow cytometric analysis of splenocytes showed a significantly lower percentage of activated T cells in the ASEA and TAM groups. The ex-vivo interferon-γ and interleukin (IL)-4 production from splenocytes and tumour necrosis factor-α and IL-1β production from peritoneal exudate cells were also significantly lower in the ASEA group compared with the control. The ASEA group also had less severe glomerulonephritis. Thus, ASEA attenuated cytokine and inflammatory responses of self-reactive lymphocytes, decreased the disease severity, increased survival and life span of the autoimmune-prone MRL-*lpr/lpr* mice, suggesting a potential of ASEA in the treatment of autoimmune diseases. *Lupus* (2009) **18,** 206–215.

Key words: alfalfa sprout extract; autoimmune; IFN-γ; inflammation; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous disorder with the feature of a multisystem inflammatory disease and the production of a variety of autoantibodies. Although the pathogenic mechanisms are not entirely clear, environmental factors have been suggested to play a role in the aetiology and the exploration of which may help to advance the strategies to treat SLE.[1] The effect of dietary modifications has been extensively studied in lupus animal models such as MRL-*lpr/lpr* and NZB×NZW F1 mice. Restriction of calorie, protein and fat, or addition of n-3 fatty acid or vitamin E supplementation in the oxidised oil diet prolong the life span of lupus mice.[2–4] In addition, Chinese herb such as Ganoderma tsugae, food component such as indole-3-carbinol, a synthetic oestrogen receptor (ER)-binding molecule such as bisphenol A were shown to delay onset of disease symptom or even prolong the life span of lupus mice. These results suggest that dietary change or supplementation may exert immunomodulatory effect on SLE.[5–7]

It is well known that SLE is nine times more prevalent in women than in men,[8,9] and oestrogen enhanced disease progress in animal model of SLE.[10,11] Phytoestrogens are plant-derived compounds that can interact with ER to exert oestrogenic/anti-oestrogen activities.[12,13] In recent years, phytoestrogen supplement, such as soy isoflavones, has become widely used as alternatives to alleviate menopausal syndrome or prevent chronic disease,[14] and thus, its oestrogenic

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or anti-oestrogenic effect on SLE is worth examining. However, phytoestrogens have potential effects on immune modulation through the inhibition of inflammatory responses which may be beneficial for SLE.[15] Our previous study has shown that dietary supplementation of soy isoflavone did not aggravate but alleviate disease severity in MRL-*lpr*/*lpr* mice. We also showed that the soy isoflavone supplement used in that study had higher affinity for ERβ than for ERα, the two isoforms of ERs in human.[16] The ER mediates the function of oestrogen by binding to specific ligands and activating the transcription of specific target gene. Some phytoestrogens with higher affinity for ERβ toward ERα are considered 'natural' selective oestrogen receptor modulators (SERMs) and may play protective roles in inflammatory diseases.[17] Therefore, a transactivation assay that can measure the ligand-dependent transcription of reporter gene through ERα or ERβ has been instrumental in our screening of phytoestrogens from various food materials extracted with methanol, ethyl acetate (EA) or *n*-hexane.[18] Among the plant foods we screened, AS extracted in EA is one of the samples that have the potential for SERM activity.

AS contains high levels of dietary fibre and prevents hypercholesterolemia and atherosclerosis in rats and monkeys.[19,20] It has also been regarded as a functional food to modulate lipid profile because it is enriched with phytoestrogens. In addition, AS extract has the potential to reduce pain from dermatitis in traditional remedy.[21] However, previous reports show that alfalfa seed or sprout provokes SLE-like disease in monkeys and exacerbates disease severity in patients with SLE who ingested alfalfa tablet.[22–24] Later, this causative component has been identified as L-canavanine.[25] However, both basic and clinical studies on alfalfa and the disease process of SLE are quite limited in that even the most recent review articles are based on findings reported more than 10 years ago.[26] It will be interesting to re-evaluate how alfalfa affects SLE in more detail based on more up-to-date knowledge the anti-inflammatory effect.

In this study, we first used MRL-*lpr*/*lpr* mice to evaluate the effects of AS (lyophilized powder), alfalfa sprout ethyl acetate extract (ASEA) and coumestrol (CUM), a known phytoestrogen in AS, on the disease course by following the proteinuria and life span of the lupus mice. Mice treated with tamoxifen (the TAM group), a potent oestrogen antagonist that was shown to alleviate disease severity in lupus mice, served as a positive control.[27] Part of the animal experiment was repeated to examine the phenotypes of splenocytes, cytokine productions from peritoneal exudate cells (PEC) and splenocytes, autoantibody productions and renal pathology of the ASEA-treated mice.

Materials and methods

Lyophilized AS and its extract

Fresh AS (*Medicago sativa L.*) were purchased from Goboul-Grange LTD (Taipei, Taiwan). AS were freeze-dried and finely grounded into the powder. The proximate compositions of this powder were 33.6% carbohydrate, 49.6% protein, 2.6% lipid, 10.5% water and 3.7% ash. AS powders were extracted with EA (1:40, w/v, g/mL) by stirring at room temperature for 2 days. The EA extract (ASEA) was obtained by removing the solvent in a rotary evaporator, with a yield of 43.1 mg/g. The ASEA was stored at −20 °C and was dissolved in sunflower oil (SO) for tube feeding in the mice experiment. In ER transactivation assay, the extract was dissolved in absolute ethanol for cell treatment (final ethanol concentration never exceeded 0.1% in medium).

Experimental animals

Six-week-old-female MRL-*lpr*/*lpr* mice were purchased from the Animal Center of the College of Medicine at the National Taiwan University (Taipei, Taiwan). The mice were kept in stainless steel wire cages in a room with controlled temperature of 23 ± 2 °C and 12-h light–dark cycle. They were fed a non-purified diet (Lab Rodent Chow 5001, Ralston Purina Inc., St Louis, Missouri, USA) for adaptation, and then switched to soy-free and oestrogen-free AIN-76 diet, in which corn oil is substituted by SO. At age 12 weeks, the diet treatment was started.

Diet and supplementation of AS and its extract

In the first experiment, 60 mice were assigned to the following five groups with similar average body weights, urinary protein levels and serum levels of anti–double stranded (ds) DNA immunoglobulin (Ig) G at age 12 weeks. The five groups received the following diet treatments: control group (tube fed with 50 μL SO/day); the AS group (AIN-76 diet containing 0.55% AS powders and tube fed 50 μL SO/day); the ASEA group (tube fed 25 mg/kg BW ASEA in 50 μL SO/day); the CUM group (tube fed 0.075 mg/kg BW CUM in 50 μL SO/day) and the TAM group (tube fed 0.375 mg/kg BW TAM in 50 μL SO/day). Mice were tube fed six days per week. The mice of the AS group averagely received 550 mg/kg BW AS powder per day

from the diet. TAM was used as a positive control, and the dose used was according to the previous study that showed its alleviation of disease course in MRL-*lpr*/*lpr* mice.[27] In the second experiment, the control, ASEA and TAM groups were repeated to further investigate the immune modulation, and each group consisted of 14–18 mice. After 6 weeks' supplementation, mice were sacrificed to determine the renal histology, phenotype of splenocytes and cellular cytokine production.

In both experiments, food intake, body weight and life span of the mice were explicitly recorded. In addition, the urine and serum of the mice were regularly collected for further assay. Animal care and handling conformed to the National Institute of Health's *Guide for the Care and Use of Laboratory Animals*.[28]

Assay of anti-dsDNA antibodies

Mice were retro-orbitally bled and serum samples were analysed for autoantibodies. Serum levels of anti-dsDNA IgG, IgG1 and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated with methylated bovine serum albumin (mBSA, Sigma Chemical Company, St Louis, Missouri, USA). After overnight incubation, the plates were washed and calf thymus dsDNA was coated for overnight at 4 °C. The plates were washed and blocked with gelatin solution for 2 h. After washes, properly diluted serum samples were added in the plates. Two hours later, the plates were washed and peroxidase-conjugated Fcγ fragment-specific antibodies for IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA), biotinylated A85-1 monoclonal antibodies for IgG1 (BD Pharmingen, San Diego, California, USA) or biotinylated R19-15 monoclonal antibodies for IgG2a (BD Pharmingen) were added for 1-h incubation. After washing, the plates for IgG1 and IgG2a assay were added with horseradish peroxidase-conjugated streptavidin (Pierce, Rockford, Illinois, USA) for additionally 30-min incubation. After washing, the plates were incubated with ABTS [2, 20-azino-bis (3-ethylbenzthazoline-6-sulfonic acid); Sigma Chemical Company, St Louis, Missouri, USA]. Absorbance was measured at 405 nm [optimal density (OD)] within 20 min.

Assay of cytokine production from immune cells

In the second experiment, mice were sacrificed at age 18 weeks. Splenocytes and PEC were collected under sterile conditions. The culture medium used in the experiments was phenol-free RPMI-1640 supplemented with 10% charcoal-stripped fetal bovine serum. Splenocytes, at a concentration of 5×10^6 cells/mL/well, were treated without or with 5 mg/L concanavalin (Con) A (Sigma) for 24 h. PEC were seeded at 1.5×10^6 cells/mL/well and treated without or with 10 mg/L lipopolysaccharide (LPS, Sigma) for 48 h. After incubation, cell supernatants were collected for measurement of cytokine production.

The production of cytokines, including interleukin (IL)-2, interferon (IFN)-γ, IL-4, IL-10 (splenocytes), tumour necrosis factor (TNF)-α and IL-6 (PEC), was assayed by using commercial ELISA kit. Briefly, anti-IL-2, IFN-γ, IL-4, IL-6 (BD Pharmingen), TNF-α or IL-10 (R & D, Minneapolis, Minnesota, USA) antibodies were coated to 96-well plate and incubated at 4 °C overnight. After washes, the wells were blocked with 200 μL blocking solution (phosphate buffer saline (PBS) buffer containing 1% bovine serum albumin; Sigma) for 1 h. The plates were washed and added with properly diluted cell supernatant for 2-h incubation. The plates were then washed before adding biotin-conjugated anti-IL-2, IFN-γ, IL-4, IL-6, TNF-α or IL-10 antibodies for 2 h. After washing, horseradish peroxidase-conjugated streptavidin (Pierce) was added and incubated for 30 min. After washing, the plates were incubated with ABTS or tetramethylbenzidine (Clinical Science Products, Mansfield, Massachusetts, USA) and measured for absorbance at 405 nm or 620 nm within 20 min. The data were calculated according to the cytokine standard curve.

Flow cytometry

Phenotypic analysis of splenocytes was performed by flow cytometry. Cells at a concentration of 2×10^6 were suspended in 0.1 mL of PBS (with 0.1 % sodium azide) and incubated at 4 °C for 30 min with predetermined optimal concentration of appropriate fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies (BD Pharmingen, San Diego, California, USA). The cells were washed and resuspended in 0.5 mL of PBS (with 0.1% sodium azide) and subjected to fluorescence-activated cell sorting (FACScan analysis). A total of 10,000 cells were counted, the frequency of each cell surface marker was determined using appropriate software (FACScan, Becton Dickinson, Mountain View, California, USA).

Proteinuria and renal histology

Proteinuria was measured using Bio-Rad Protein Bradford assay to determine protein levels in urine. Briefly, 10 μL of properly diluted urine sample or

BSA at concentrations of 0.2–0.8 g/L as standards were added to 96-well plate. Then 200 μL dye reagent (diluted five fold with deionised water) was added. After mixing for 5 min at room temperature, the absorbance was measured at 595 nm. Proteinuria was defined as a level of urinary protein higher than 1 g/L for two consecutive weeks. For renal histology, kidney samples were fixed in 3% formaldehyde, and 5-μm kidney sections were cut and stained with haematoxylin and eosin. The sections were observed for glomerular nephritis included glomerular enlargement, hypercellularity, mesangial cell proliferation and fibrinoid necrosis.

Assay of oestrogenic activity

Oestrogenic activity was measured by the ER transactivation assay as described in the previous study.[16] Briefly, 1.25×10^5 cells/mL CHO-K1 cells (CCRC 60006) were seeded at 96-well plate and transfected with 0.3 μg of the chimeric receptor construct pBK-CMVGal4-hERα (or β) and pBK-CMV-$(UAS)_4$-tk-alkaline phosphatase (AP) (4:1 or 5:1) in 100 μL serum-free medium OPTI-MEM (Gibco BRL, Gaithersburg, Maryland, USA) containing 1 μL lipofectamine per well. After 5 h, the medium was changed to Ham's F-12 medium containing 10% TCM and 1 nM 17β-estradiol (E2, as the positive control) or the appropriate concentration of ASEA (0.4–50 μg/mL) or CUM (0.01–1 μM). After 48-h incubation, 20 μL (ERα) or 10 μL (ERβ) culture medium was transferred to a new 96-well plate and mixed with 200 μL of SEAP (secreted AP) assay solution. Finally, the absorbance was read at 405 nm for 15 min. Fold activation was calculated by taking the AP activity of vehicle-treated cells as 1. Experiments were repeated four times.

Statistical analysis

Differences in cellular and serological parameters between the treatment and the control groups were analysed by Student's *t*-test, whereas transactivation activity among groups was analysed by one-way ANOVA and Duncan's multiple range test, using the SAS software program (SAS/STAT version 8; SAS Institute, Cary, North Carolina, USA). Data were presented as means ± standard deviation or standard error (SE) for each group. The data of the proteinuria cumulative occurrence and survival were analysed between two different curves using the Cox proportional hazards regression test (STATA version 6.0; Stata Corp., College Station, Texas, USA). Statistical significance was considered at a P value ≤ 0.05.

Results

ASEA delayed the occurrence of proteinuria and increased mice survival

To investigate the effect of AS and its EA extract on SLE, MRL-*lpr*/*lpr* mice were fed AIN-76 diet and supplemented with either lyophilized AS powder, ASEA or CUM beginning at 12 weeks of age. There was no significant difference in body weight, food intake and food efficiency among five groups (data not shown). Figure 1A shows that proteinuria developed early in the control and the AS groups. At age 18 weeks, almost all mice of the control and the AS groups had proteinuria, but only one-third of the ASEA group had proteinuria, less than those of the TAM and CUM groups. The cumulative occurrence of proteinuria in the ASEA group ($P = 0.047$) and the TAM group ($P = 0.049$) were significantly lower than the control group.

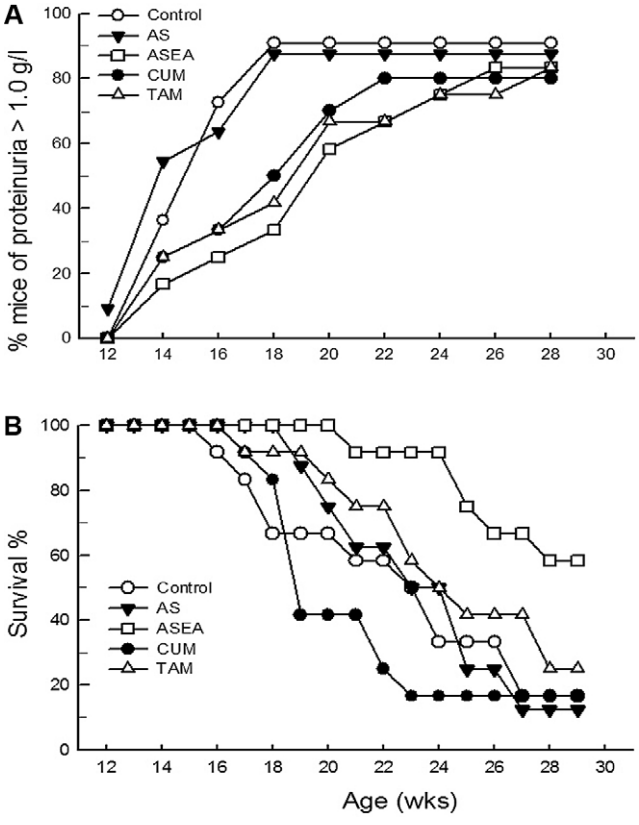


Figure 1 Occurrence of proteinuria (A) and survival curve (B) of MRL-*lpr*/*lpr* mice supplemented without (control) or with alfalfa sprouts (AS), alfalfa sprout ethyl acetate extracts (ASEA), coumestrol (CUM) or tamoxifen (TAM). There were 12 mice, at the age 12 weeks, started on each supplement diet. The data were analysed by COX's proportion hazards regression test. Lower occurrence of proteinuria ($P = 0.047$) and increased survival rate ($P = 0.012$) were in the ASEA group compared to those in the control group.

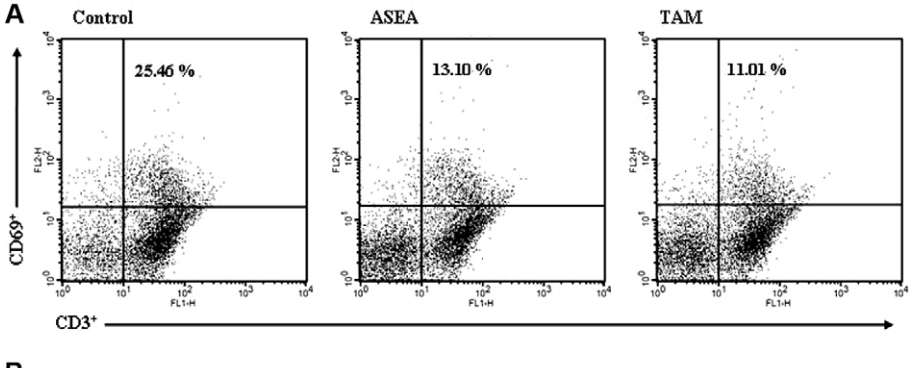
The survival rate of mice in the control group decreased with age and was approximately 50% at the age of 22 weeks (Figure 1B). At this age, 90% of mice in the ASEA group still survived, higher than the TAM and the CUM groups. The survival rate of the ASEA group was higher than that of the control group ($P = 0.012$, Cox proportion hazards regression test). The average life span in the ASEA group (199 ± 14 days) and the TAM group (189 ± 14 days) were significantly longer than the control group (152 ± 10 days) ($P < 0.05$). The life span and the survival rate of the mice in the AS and the CUM groups were not significantly different from that of the control group.

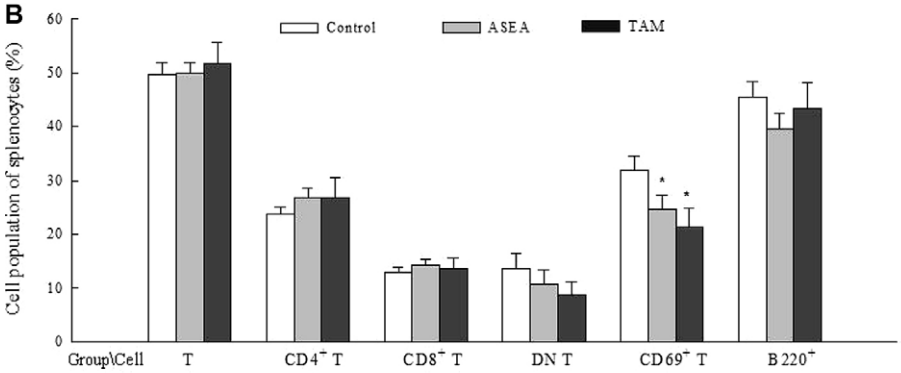
Phenotypic analysis of surface marker expression of splenocytes

To examine the effects of ASEA on immune responses, three more groups of MRL-*lpr*/*lpr* mice were treated in a similar manner to the control, ASEA and TAM groups of the Experiment 1 for 6 weeks. Phenotypic analysis of surface marker expression of splenocytes showed that the expressed level of $CD69^+$ T cells was significantly lower in the ASEA and the TAM groups ($P = 0.04$ and 0.03, respectively, Figure 2). The CD69 molecule is an activated marker for T cells. The percentage of B cells ($B220^+$) tended to be lower in the ASEA group ($P = 0.09$). No significant difference in the distribution of $CD4^+$ T cells, $CD8^+$ T cell or $CD4^-CD8^-$ double negative (DN) T cells was noted among these three groups.

Cytokines production by mitogen-stimulated PEC and splenocytes

Amount of cytokines secreted from LPS-stimulated PEC and Con A-stimulated splenocytes are shown in Table 1. The production of TNF-α was slightly lower





Group\Cell	T	$CD4^+$ T	$CD8^+$ T	DN T	$CD69^+$ T	$B220^+$
Control	49.7 ± 2.18	23.7 ± 1.42	13.0 ± 0.90	13.6 ± 2.91	31.8 ± 2.79	45.6 ± 2.83
ASEA	49.9 ± 1.95	26.9 ± 1.60	14.3 ± 1.03	10.8 ± 2.62	24.7 ± 2.57	39.6 ± 2.75
TAM	51.6 ± 4.00	26.9 ± 3.56	13.7 ± 1.86	8.73 ± 2.53	21.3 ± 3.53	43.3 ± 4.78

Figure 2 Phenotypic analysis of surface marker expression of splenocytes in MRL-*lpr*/*lpr* female mice supplemented without (control), or with alfalfa sprout ethyl acetate extract (ASEA) or tamoxifen (TAM). Splenocytes were collected from mice at age 18 weeks. Each group contains 14–18 mice. (A) Representative examples of two-colour fluorescence-activated cell sorting (FACScan analysis) of $CD69^+$ T populations of splenocytes from these three groups. (B) Cell populations by phenotypic analysis include $CD3^+$ (T), $CD4^+$ T, $CD8^+$ T, $CD4^-CD8^-$ double negative (DN) T, $CD69^+$ T and $B220^+$ cells. The average values were represented means ± SE. The significant difference between the control group and the experimental group was analysed by Student's *t*-test, $^*P < 0.05$.

Table 1 The cytokine production from mitogen-stimulated peritoneal exudate cells and splenocytes of MRL-*lpr/lpr* mice[a]

	PEC (ng/10^6 cells)			*Splenocyte (ng/10^7 cells)*			
Group	*IL-6*	*TNF-α*	*IL-1β*	*IL-2*	*IFN-γ*	*IL-4*	*IL-10*
Control	47.2 ± 5.63	0.14 ± 0.01	0.45 ± 0.04	5.20 ± 0.92	41.2 ± 6.97	0.48 ± 0.07	2.97 ± 0.56
ASEA	39.7 ± 5.04	0.11 ± 0.01*	0.33 ± 0.04**	5.95 ± 0.97	24.3 ± 3.04**	0.31 ± 0.05**	2.04 ± 0.42
TAM	46.9 ± 6.94	0.08 ± 0.01**	0.32 ± 0.05*	6.03 ± 1.21	28.8 ± 4.77	0.39 ± 0.08	1.71 ± 0.37*

[a]Peritoneal exudate cells (PEC) and splenocytes were collected from mice at age 18 weeks. Splenocytes and PEC were stimulated with 5 mg/L Con A and 10 mg/L LPS, respectively. Values are means ± SE. Each group contains 14–18 mice. The significant difference between the control group and the experimental group was analysed by Student's *t*-test.
*$0.05 < P < 0.10$.
**$P < 0.05$.

in the ASEA group ($P = 0.09$) and significantly lower in the TAM group. IL-1β was significantly lower in the ASEA group and slightly decreased in the TAM group ($P = 0.07$). IL-6 level was not significantly different among the groups. The splenocytes of ASEA-supplemented group produced significantly lower IFN-γ and IL-4 while stimulated with 5 mg/L Con A. There was no significant difference in the level of IL-6, IL-2 and IL-10 among these three groups, except a slight decrease in IL-10 production of TAM group ($P = 0.06$).

Effects of ASEA on autoantibodies production and lupus nephritis

The serum levels of autoantibodies were monitored regularly to follow up the disease progress. Serum levels of anti-dsDNA IgG, anti-dsDNA IgM and anti-cardiolipin IgG increased with age but were not statistically different among the groups because of individual variation (data not shown). Further, the level of IgG2a anti-dsDNA antibody tended to be lower in ASEA- and TAM-treated groups but not statistically significant ($P = 0.26$ and 0.22, respectively, Figure 3).

The renal histology shown in Figure 4 indicates that enlarged glomeruli and the proliferation of mesangial cells in the ASEA group were less severe than those in the control group. Less inflammatory cell infiltration in the kidneys of the ASEA and TAM groups was likewise noted.

Estrogenic activity of ASEA and CUM

To determine the oestrogenic activity of ASEA and CUM, ASEA or CUM was added to CHO-K1 cells co-transfected with constructs of either ERα or ERβ ligand binding domain, respectively, as well as a reporter. The results showed that ASEA exhibited a higher oestrogenic activity toward ERβ and a lower ERα oestrogenic activity (Figure 5A). The transactivation of ASEA on ERβ reached comparable levels to 1 nM E2 treatment at a concentration of 10 μg/mL, whereas the activation of ERα was still below half of that of E2 treatment even at a concentration of 50 μg/mL ASEA. However, CUM exhibited a higher oestrogenic activity toward ERα than ERβ (Figure 5B) at the concentrations of 0.1–1 μM. The transactivation of CUM on both ERα and ERβ reached a level comparable to 1 nM E2 treatment at a concentration of 1 μM.

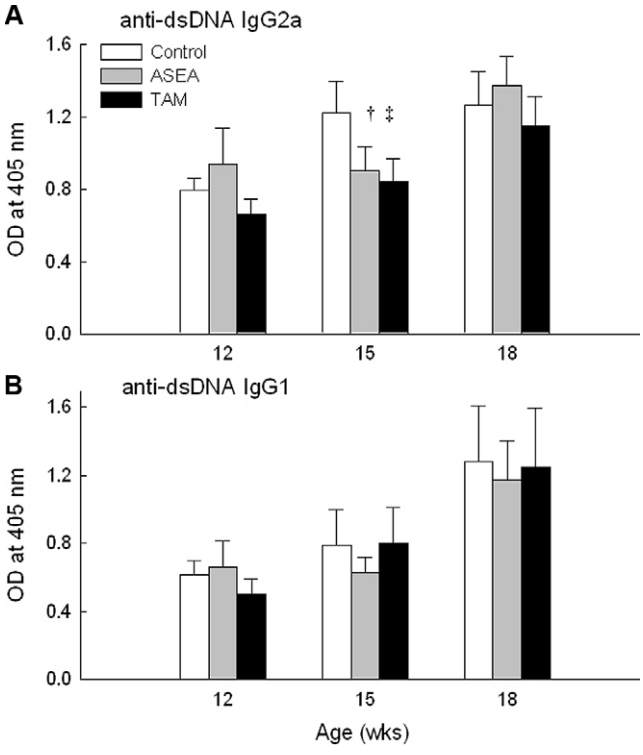


Figure 3 The production of serum anti-dsDNA IgG2a (A) and anti-dsDNA IgG1 (B) of MRL-*lpr/lpr* mice supplemented without (control) or with alfalfa sprout ethyl acetate extract (ASEA) or tamoxifen (TAM). There were 14–18 mice, at the age 12 weeks, started on supplement diet. Bar value is mean ± SE and differs from the control group at the same age by Student's *t*-test. † $P = 0.26$; ‡ $P = 0.22$.

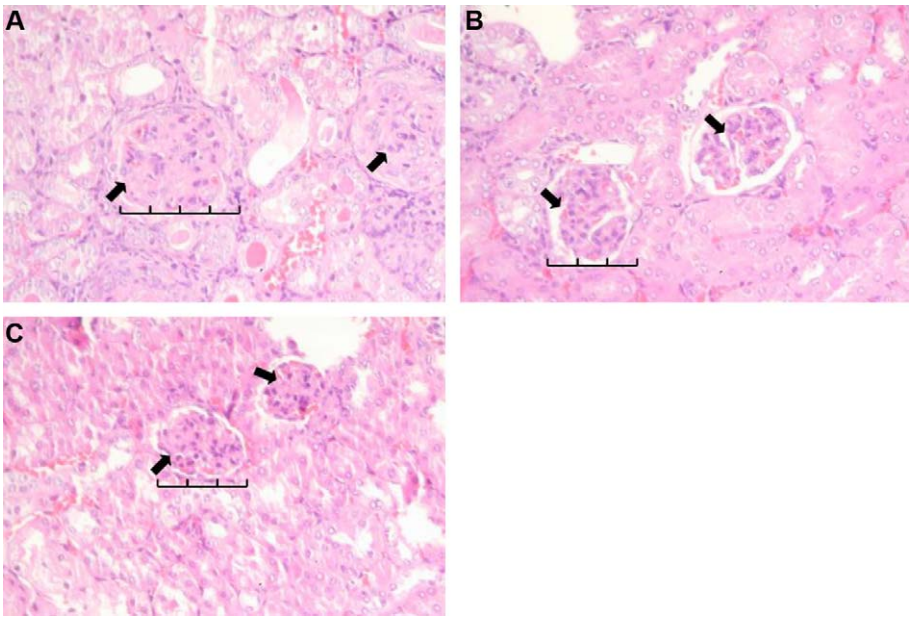


Figure 4 Renal histological change of MRL-*lpr*/*lpr* mice supplemented without (A, control) or with alfalfa sprout ethyl acetate extract (B, ASEA) or tamoxifen (C) for 6 weeks. (A) shows enlarged glomeruli with hypercellularity and mesangial proliferation (arrows). (B) and (C) both show reductions in enlarged glomeruli, and had less mesangial proliferation (more space, arrows) while compared with the control. Sections were stained with haematoxylin and eosin (magnification, 200×).

Discussion

The attempt of dietary manipulation of disease severity in lupus has been extended for decades. Suppression of autoantibodies and inflammatory cytokine productions is suggested to be protective against disease progression. In recent years, ERs are also suggested to play a role in oestrogen-related modulation of lupus.[29] In this study, ASEA, which may exert anti-inflammatory and SERM activity by an in-vitro screening assay developed in our laboratory, was tested in murine lupus. ASEA significantly delayed the onset of proteinuria and prolonged the life span (Figure 1). However, the mice of the AS group had similar proteinuria progression and life span as the control group, suggesting that ingestion of whole AS had no beneficial effect.

Previous studies reported that alfalfa seed or sprout might be an SLE symptom eliciting food in both laboratory animal studies and a few human cases.[22,23] Two inactive patients with SLE were also reported to reactivate serological and clinical symptoms after ingesting 8–15 alfalfa tablets daily.[24] This causative component was considered as a heat-liable amino acid L-canavanine[25] and reported to increase antibody production and higher renal histology scores in autoimmune mice.[30] However, the AS group fed 550 mg/kg BW AS powder (0.55% in diet) in our study did not aggravate disease course. It may be due to the counteraction of L-canavanine and ASEA, or the lower dose in our diet compared to the diet with 40% dried AS or with higher dose of L-canavanine treatment in the previous report.[22,30]

It was suggested that ERβ activation may have an immunosuppressive effect on murine lupus.[29] We also showed that soy isoflavones with higher affinity for ERβ than ERα by transactivation activity alleviated disease severity in MRL-*lpr*/*lpr* in a previous study.[16] In this study, ASEA also exhibited a higher oestrogenic activity toward ERβ than ERα. In contrast, CUM exhibited a higher oestrogenic activity toward ERα than ERβ at the concentrations of 0.1–1 μM (Figure 5). CUM is a well-known phytoestrogen in alfalfa.[31] Our data suggested that phytoestrogenic compounds other than CUM existed in ASEA as CUM did not show a selectivity toward ERβ as ASEA. The difference in ER subtype selectivity between ASEA and CUM seems to be in accordance with the difference in survival rate and life span in Experiment 1. Although in-vivo oestrogenic effect of ASEA needs to be further investigated, results of the present study confirmed our earlier observation that in-vitro screening of plant-derived fraction for both anti-inflammatory and SERM activity could be beneficial for murine lupus.

As previously reported by the other study,[32] CUM neither ameliorated the disease severity nor prolonged life span in murine lupus model in this study.

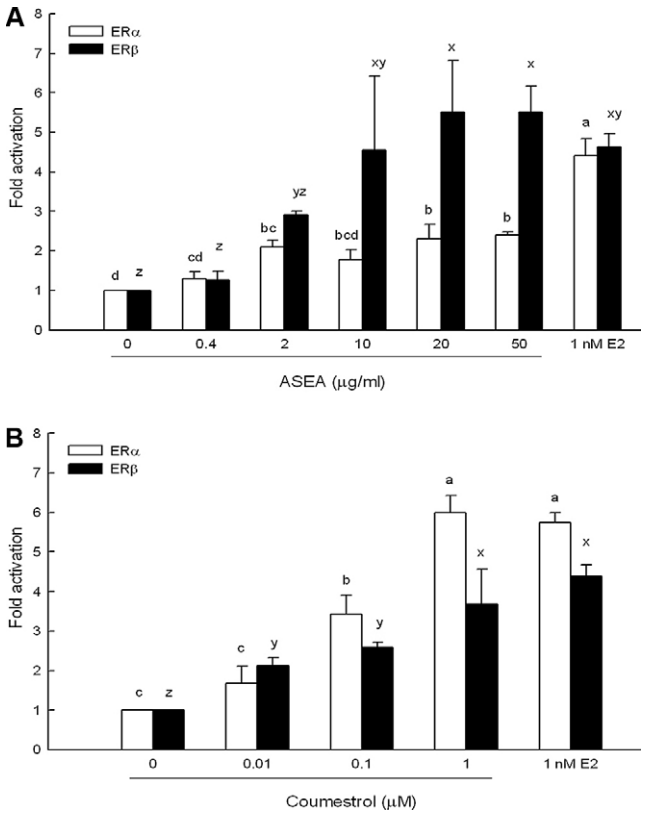


Figure 5 Effects of alfalfa sprout ethyl acetate extract (A) and coumestrol (B) on the transcriptional activation of oestrogen receptors (Gal4-hERα or β). Values are expressed as means ± SE of four separate experiments, and 1 nM E2 (17β-estradiol) is positive control. Values not sharing the same letter are significantly different by Duncan's multiple range test ($P < 0.05$, a–d represents the significant difference for ERα activities, whereas x–z represent for ERβ).

Although CUM supplementation lowered the occurrence of proteinuria at early age, it increased splenocytic B-cell population (CUM, 72.5 ± 7.31% vs control, 45.6 ± 2.83%, $P = 0.053$), serum anti-dsDNA IgM level (CUM, 0.55 ± 0.06 OD vs control, 0.35 ± 0.02 OD, $P < 0.01$) (data not shown) and did not prolong life span while compared to the control. These immunological changes agree with the observations that CUM did not ameliorate lupus onset and was not the main compound that contributed to the beneficial effect of the ASEA.

The isolation and chemical identification of the active compound(s) of ASEA with anti-inflammation and selective ER activation is currently under investigation. It has been reported that alfalfa contains flavonoids such as conjugated apigenin,[33] isoflavones, such as biochanin A, and formononectin,[34] as well as saponins.[35] Although these compounds have been reported to be oestrogenic,[12] they are less selective toward ERβ compared to ASEA observed in this study. Saponins from soybean and Ginseng have also been reported to be oestrogenic.[36,37] The anti-inflammatory effects of these compounds have also been reported.[38,39] Identification of the active compound(s) in ASEA will provide more information for the future application in lupus attenuation.

TAM, a non-steroidal anti-oestrogen compound, has been reported to decrease the percentage of cell number of T cells, activated T cells ($CD69^+$ T cells) and DN T cells ($CD4^-CD8^-$ T cells) of splenocytes and prolong the life span when administrated subcutaneously.[27] Although a decrease in DN T cells was noted in the TAM group of this study but without statistical significance, the percentage of $CD69^+$ T cells was significantly decreased (Figure 2). CD69 is an early T-cell activation marker. The percentages of activated T cells of peripheral blood mononuclear cells were significantly higher for patients with SLE than for healthy control. Activated T cells were reported to significantly correlate to SLE disease activity.[40] Increased activated T cells were also observed in splenocytes from lupus mice compared to normal mice.[41] In this study, ASEA supplementation, such as TAM, significantly decreased the number of activated T cells, indicated that ASEA exerted immunosuppressive effect and thus might alleviate disease severity in murine lupus.

In addition, ASEA significantly suppressed the production of pro-inflammatory cytokines as shown in the ex-vivo IL-1β secretion from PEC and IFN-γ from splenocytes in the present study (Table 1). Serum IL-1β concentrations are increased in patients with SLE.[42] Mice deficient in IL-1β developed lower levels of anti-dsDNA in experimental SLE suggested the role of IL-1β in the immunostimulatory and inflammatory phenomena that mediate the development of SLE.[43] Antagonist of IL-1β has been tested for the alleviation of signs and symptoms of arthritis in SLE, but the results were not conclusive due to side effects.[44] IFN-γ produced by lymph node cells from MRL-*lpr*/*lpr* mice is higher than that from normal mice.[45] The mRNA expression of IFN-γ and IL-10 in severe lupus-like mice increased and thus could be potential targets for immunotherapy.[46] IL-10 secretion were slightly lower in TAM group ($0.05 < P < 0.10$) but not significantly different from the control group. Previous studies showed that dietary manipulation, such as optimal dose of vitamin E, or soy isoflavone supplementation prolonged life span of murine lupus and significantly suppressed splenocytic IFN-γ secretion.[4,16] Our results in the present study with ASEA supplementation (Table 1) agree with our earlier observation that those lupus mice with prolonged life span (Figure 1) by diet treatment

had significantly lower IFN-γ secretion from mitogen-stimulated splenocytes. The suppressive effect of ASEA on IL-1β and IFN-γ secretion implied a decreased risk of interplay of inflammatory and immune mediators.

Ex-vivo IL-4 secretion from Con A-stimulated splenocytes of MRL-*lpr/lpr* mice supplemented with ASEA also significantly decreased compared to the control. Higher serum levels of IL-4 were found in patients with SLE in several reports.[47–49] IL-4 enhanced the production of anti-dsDNA IgG from splenocytes of lupus mice. Administration of monoclonal antibodies against IL-4 before the onset of lupus inhibited the production of anti-dsDNA IgG and prevented the onset of lupus nephritis.[50] Lupus mice spontaneously secrete large quantities of pathogenic IgG1 and IgG2a autoantibodies, this isotype switching is known to be enhanced by IL-4 and IFN-γ, respectively.[51] In the present study, splenocytic IL-4 and IFN-γ secretion in response to Con A were significantly reduced in the ASEA supplemented mice, although the lowering in serum levels of anti-dsDNA IgG1 and IgG2a did not reach statistical difference. The results suggested that the beneficial effect of ASEA might be more attributable to the down-regulation of inflammatory mediators and cytokines instead of autoantibodies. This speculation is also supported by the results of renal histology that also showed less enlarged glomeruli and mesangial proliferation in the ASEA and the TAM groups.

In conclusion, this study showed that autoimmune-prone MRL-*lpr/lpr* mice fed AS EA extract from age 12 weeks had significantly lower activated T-cell population, IL-1β secretion by LPS-stimulated PEC, IFN-γ and IL-4 production by Con A-stimulated splenocytes and prolonged life span. The present study confirmed our earlier observation that plant-derived components with anti-inflammatory and SERM activity may have beneficial effects on disease course of murine lupus.

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