

Anti-allergic effect of *Aster yomena* on ovalbumin-sensitized mouse and RHL-2H3 cells via Th1/Th2 cytokine balance

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ARTICLE INFO

Keywords:

Aster yomena

Allergy

Th1 cytokines

Th2 cytokines

ABSTRACT

Recently, the incidence of allergies is increasing worldwide. Immunoglobulin E (IgE)-mediated hypersensitivity is central to the pathogenesis of asthma, and other allergic diseases. *Aster yomena* (AY) have been valued for their medical properties including anti-inflammation, anti-pyresis and analgesia properties. In this study, we investigated the anti-allergic effects of AY and isoquercitrin, a functional compound of AY, using a mouse allergy model and cells. In mice with ovalbumin (OVA)-induced allergic rhinitis, oral administration of AY or isoquercitrin reduced secretion of IgE and histamine in the plasma. AY or isoquercitrin regulated both Th1 and Th2 cytokines levels in the plasma. In addition, AY and isoquercitrin showed dose-dependent inhibition of β -hexosaminidase and reduction in mRNA levels of Th2 cytokines in RBL-2H3 cells. Thus, our results demonstrate that AY or isoquercitrin suppresses cytokine production of the allergy response pathway, resulting in prevention or alleviation of allergy symptoms.

1. Introduction

Allergies such as asthma, atopic dermatitis, and allergic rhinitis are caused by immunoglobulin E (IgE)-mediated immediate hypersensitivity reactions. Approximately 10–20% of the world population is affected by allergies, with the number of allergy patients increasing annually (Oh, Kim, & Jeong, 2011; Shin et al., 2010). Allergy is caused by allergic mediators such as inflammatory cytokines, and histamine (Ren, Deng, Xiao, Wang, & Tao, 2013). They have been recognized as key players of inflammatory reactions because the mediators interact with each other to trigger acute inflammatory reactions upon exposure to allergens (Lee & Arriola, 1999). At the onset of the allergic reaction, mast cells combine with IgE receptors on the basophil surface, which trigger the release of inflammatory cytokines and histamine (Pawankar, Bunnag, Khaltayev, & Bousquet, 2012). These steps increase the recruitment of inflammatory cells and eosinophil migration to affected tissue (Shinmei et al., 2009).

Multiple medications are available for treating allergy, such as anti-histamines, calcium influx blocker, phosphodiesterase inhibitor, cAMP stimulator, thromboxane inhibitor, leukotriene antagonist, and Th2 cytokine inhibitor. However, these drugs can only be used for palliative care of hypersensitivity symptoms and not for curing the condition (Howarth et al., 2005). Therefore, alternative treatments with improved drug safety are required. Recently, natural medicines are being used for treatment of various allergic diseases to maintain the natural

balance of immune factors. Hence, several studies aimed at finding appropriate treatment for allergy have focused on the use of traditional medicines, which have fewer side effects than synthetic drugs (Bernstein et al., 2002; Resnick, Bielory, & Bielory, 2008; Sheehan & Atherton, 1992).

Aster yomena (AY) is a traditional herbal medicine used for the treatment of runny nose, sneezing, and congestion (Jung, Lim, Park, & Bae, 2005; Kim, Jin, Jin, Ko, & Woo, 2014). And we have previously reported that *Aster yomena* extract decreases pulmonary inflammation of asthma, in the ovalbumin (OVA)-induced asthma model (Sim et al., 2014). Nevertheless, the exact molecular mechanism of AY action and functional components remain unknown.

According to Kim et al. (2014), asteryomenin, esculetin, 4-O- β -D-glucopyranoside-3-hydroxy methyl benzoate, caffeic acid, isoquercitrin, isorhamnetin-3-O-glucoside, and apigenin have been identified as the major compounds in AY extracts. To identify the major compounds of AY, we analyzed the phytochemicals of them. In our results, we also confirmed that they contain isoquercitrin, caffeic acid, apigenin and esculetin, etc. The esculetin and apigenin were present in few amounts and were difficult to quantify. In the case of caffeic acid and isoquercitrin, 0.31 ± 0.017 mg/g and 0.47 ± 0.016 mg/g were detected in AY extract, respectively (supplementary Table 1). Several studies reported that isoquercitrin and caffeic acid have significant anti-inflammation and anti-allergy effects (Hossen et al., 2006; Kempuraj et al., 2005; Makino et al., 2013; Park et al., 2004, 2008; Zhao, Shin,

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Satsu, Totsuka, & Shimizu, 2008). There are many differences in the compounds of natural materials at different harvesting times. In this study, the variation of isoquercitrin was lower than that of caffeic acid. And the anti-allergic effect was higher in AY extract when the content of isoquercitrin was high in preliminary experiments (data not shown). Therefore, isoquercitrin was considered as a major component. Therefore, we set up positive control to isoquercitrin which might be the major compounds of AY extract.

Hence, in this study, we investigated the anti-allergic effect of AY and isoquercitrin which is AY's putative major functional compound in an OVA-induced murine model of type I allergy and the anti-allergic mechanism of action in vitro using RBL-2H3 mast cells.

2. Material and methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin (PS) were obtained from GIBCO/Life Technologies Inc. (Rockville, MD, USA). Compound 48/80, isoquercitrin, and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of extracts

AY was obtained from the Gurye-gun Agricultural Center, Gurye, Jellanam-do, Korea. Voucher specimens (SCHAY150722) were deposited in the Department of Life Science and Biotechnology herbarium, Soonchunhyang University, Korea. AY were cleaned, dried and weighed, and then extracted twice with 50% ethanol at 80 °C for 4 h. Subsequently, the extracts were filtered and evaporated. And then the concentrated extracts were sterilized at 95 °C for 1 h and spray dried. Before, preparation for further analysis, the dried extracts were stored at –20 °C.

2.3. Animals

The 6-week-old female BALB/c mice were purchased from Orient Bio (Seong-Nam, Korea). All animal experiments were approved according to the guidelines of the Institutional Animal Care and Use Committee of the National Academy of Agricultural Science (NAAS-201411), and all procedures were conducted in accordance with the Animal Experiments Guidelines of the National Academy of Agricultural Science. The mice were housed under controlled temperature (23 ± 3 °C) with a relative humidity of 40–60% and 12 h light/dark cycles. Food and water were provided ad libitum.

2.4. Sensitization and challenge

The BALB/c mice were divided into four groups (5 mice in each group): (1) control group; no sensitization (2) OVA sensitized (OVA) group; sensitization with OVA and aluminum hydroxide (Alum; Sigma) (3) AY extracts group; sensitization with OVA and Alum and AY extracts by oral administration (p.o.) and (4) Iso group; sensitization with OVA, Alum and isoquercitrin by p.o. Briefly, after the mice were acclimated for 1 week, they were OVA-sensitized by intraperitoneal injection of 50 µg OVA (chicken egg albumin; Sigma) in 200 µL (PBS) containing 2 mg Alum on day 7, and 21. Sensitization was followed by daily intranasal challenges for 3 days with OVA five days after the second injection. The mice were sacrificed one day after the last challenge on day 28. The mice were treated with AY extracts (1 g/kg bodyweight) (Sim et al., 2014) or Iso (15 mg/kg bodyweight) by p.o. on from days 7 to 28. The mice in the control and OVA groups were administered saline by p.o. (Fig. 1). At the end of 4 weeks, all mice fasted overnight and were euthanized with CO₂. Blood samples were collected from abdominal aorta with heparinized syringes and centrifuged at 3000g for 15 min.

Plasma samples were stored at –70 °C until further analysis. The spleens were aseptically removed and placed in RPMI 1640 and splenocytes were isolated to observe the Th1/Th2 cell ratio.

2.5. Measurement of total plasma IgE and histamine in plasma

Total IgE was quantified using an enzyme-linked immunosorbent assay (ELISA) (Abcam, Billerica, MA, USA). Histamine concentration in plasma was determined using a histamine EIA kit (Spi-bio, Montigny-le Bretonneux, France) according to the manufacturer's instructions.

2.6. Measurement of cytokines in plasma

To determine the effect of AY on allergic responses in the OVA-induced mouse model, the levels of IL-4, IL-5, IL-13, IFN-γ, TNF-α and IL-12 were measured using ELISA kit (Abcam) according to the manufacturer's protocols.

2.7. Cell culture

The RBL-2H3 (rat basophilic leukemia cells) and EL-4 (mouse T cell lymphoma) cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). RBL-2H3 and EL-4 cells were cultured in DMEM supplemented with 10% FBS and 1% PS in an incubator with 5% CO₂ at 37 °C.

2.8. Cell viability

RBL-2H3 cells seeded in 24-well plates were treated with AY or isoquercitrin. The AY extracts was dissolved in DMSO and diluted in PBS to obtain final concentrations of 25, 50, and 100 µg/mL. Cells were treated with the extracts for 24 h, and cell viability was measured using the Cell Titer-Glo assay (Promega, Madison, WI, USA). Viability was expressed as the percentage of live cells in each well.

2.9. Degranulation in RBL-2H3 mast cells

RBL-2H3 cells were harvested by trypsinization, transferred to 24-well plates, and incubated overnight with AY extracts or isoquercitrin for compound 48/80 stimulation. Experiments on intact cells were performed in PIPES-buffered medium. Cultures were incubated for 10 min with 10 µg/mL compound 48/80. The secretion of granules containing various allergic mediators was determined by measuring the release of the granule marker, β-hexosaminidase, using a colorimetric assay in which the release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-d-glucosaminide is measured. Values are expressed as the percentage of intracellular β-hexosaminidase that was released into the medium.

2.10. Real-time reverse transcription–polymerase chain reaction (RT–PCR) analyses

Real-time RT-PCR using a real-time thermal cycler Qiagen rotorgene Q (Qiagen, Valencia, CA, USA) was performed according to the manufacturer's instructions. The cells were treated with the AY extracts, isoquercitrin and compound 48/80. Thereafter, cDNA was synthesized from the total RNA isolated from the cells. The real-time PCR reaction was performed using 2 × SYBR Green mix (Qiagen). All results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The following rat primer sequences were used: GAPDH, 5'-GAG CCA AAA GGG TCA TCA TC-3' (forward), 5'-TAA GCA GTT GGT GGT GCA GG-3' (reverse); IL-4, 5'-GGA GAG ACT ATC AAG ATA GT-3' (forward), 5'-ATG GTC AGT AGA CTT TTA CA-3' (reverse); IL-5, 5'-AGC ACA GAA AGC ATG ATC CG-3' (forward), 5'-GTT TGC TAC GAC GTG GGC TA-3' (reverse); IL-13, 5'-CGA TGA TGC ACT TGC AGA AA-3' (forward), 5'-TGG AAA TTG GGG TAG GAA GG-3' (reverse); MCP-1, 5'-

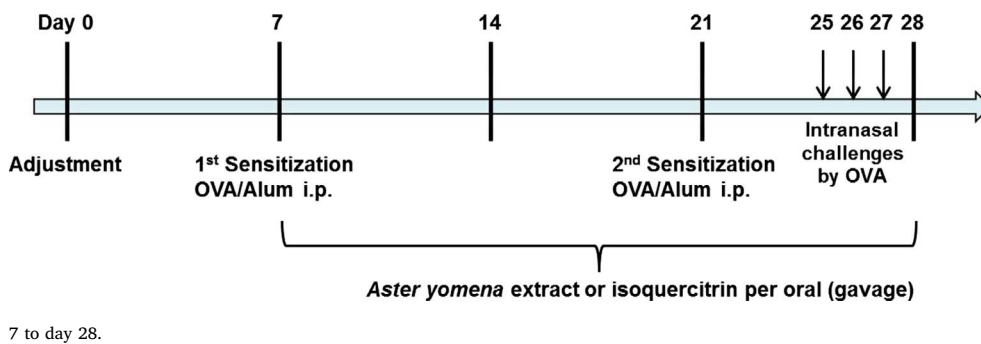


Fig. 1. Experimental design of OVA-induced allergy in a mouse model. Female BALB/c mice (6 weeks old) were divided into control group, OVA sensitized group, *Aster yomena* extracts group and isoquercitrin group. To induce an allergic response, mice were immunized twice with 50 μ g OVA adsorbed in 2 mg/mL Alum by intraperitoneal injection on days 7 and 21. Sensitization was followed by daily intranasal challenges with OVA for 3 days, five days after the second injection. The *Aster yomena* extracts (1 g/kg body weight) or isoquercitrin (15 mg/kg body weight) were orally administered daily from day

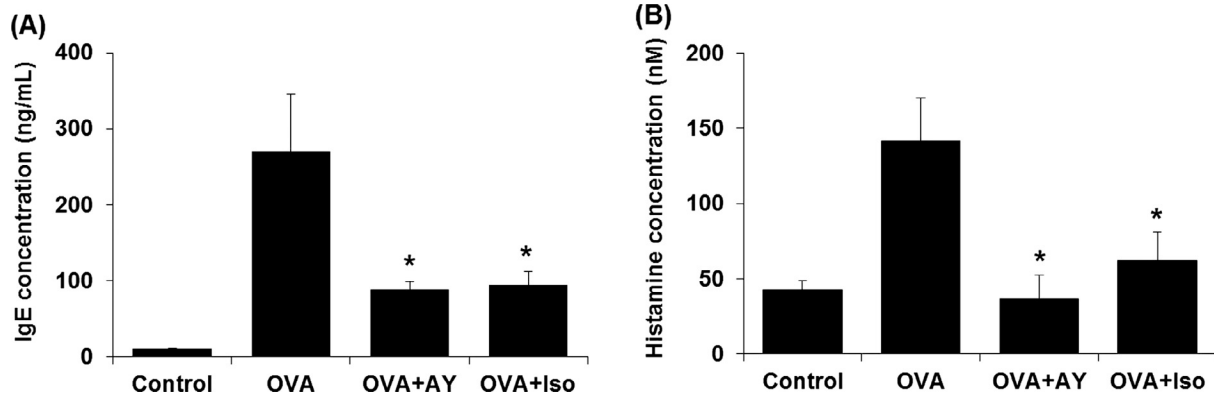


Fig. 2. Effect of *Aster yomena* and isoquercitrin on IgE and histamine levels in plasma of OVA-sensitized mice. (A) Plasma IgE levels. (B) Histamine levels in plasma. Total IgE and histamine were measured using ELISA. Columns and error bars represent the mean $\pm$ standard error. * $p < 0.05$, significantly different from the OVA group. OVA = ovalbumin sensitization.

GGA CAG AAT ATC AAC CAA CAA GTG ATA-3' (forward), 5'-GTG TGC CGT CTT TCA TTA CAC AG-3' (reverse).

2.11. Western blot analysis

EL-4 cells were seeded in 6-well plates and treated with AY (100 μ g/mL) or isoquercitrin (20 μ g/mL) at 37 $^{\circ}$ C for 1 h, and the cells were then stimulated with OVA (200 μ g/mL) for another 24 h. The cell were harvested and resuspended in lysis buffer on ice for 40 min to obtain total protein. Protein concentration was estimated using a bicinchoninic (BCA) protein assay kit (Gendepot, Barker, TX, USA) and loading buffer was added to the sample. The sample was then heated at 100 $^{\circ}$ C for 5 min. Equal amounts of protein were separated by 4–20% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene (PVDF) membranes. After blocking with 5% skim milk for 1 h, the PVDF membranes were incubated overnight at 4 $^{\circ}$ C with the primary antibodies against GATA3, T-bet, p-STAT1, p-STAT6, STAT1, STAT6, c-Maf and IRF1. Anti-rabbit horseradish-linked IgG was used as the secondary antibody. Signals were detected using electrochemiluminescence (ECL, Thermo, Rockford, IL, USA).

2.12. Flow cytometric analysis of Th1 and Th2 cells

A single cell suspension was prepared by disrupting the spleen passed through cell strainer (BD Biosciences, Durham, NC, USA). And the red blood cells were lysed with Tris-NH₄Cl. And the isolated cells were equally divided into two tubes. Then cells were labeled with anti-mouse CD3 (fluorescein isothiocyanate; FITC) and CD4 (phycoerythrin; PE) antibody (BD Biosciences) at 4 $^{\circ}$ C for 30 min. Following surface staining, cells were fixed and permeabilized in perm/wash buffer for 20 min at 4 $^{\circ}$ C in the dark, then incubated with anti-IL-4 (Allophycocyanin; APC) and anti-IFN- γ (APC) antibody (BD Biosciences) in each tube for 30 min at 4 $^{\circ}$ C in the dark. After fixing with 1% paraformaldehyde, the stained cells were detected by flow

cytometry (BD Biosciences).

2.13. Statistical analysis

Statistical analyses were performed using the SPSS v12.0 software (SPSS, Chicago, IL, USA). Data are represented as the mean $\pm$ standard error of mean (SEM) from three independent experiments, unless stated otherwise. Statistical analyses included the Student's *t*-test and $p < 0.05$ was considered significant.

3. Results

3.1. Effect of AY extracts on total IgE levels in plasma

The total plasma IgE level was 270.6 ± 75.5 ng/mL in the OVA group, which was significantly higher than that in the control group (10.9 ± 0.47 ng/mL). The plasma IgE level in the Iso group (95.50 ± 16.9 ng/mL) and AY group (89.04 ± 10.5 ng/mL) decreased significantly compared to that of the OVA group (Fig. 2A). Moreover, the histamine released into the plasma of OVA-sensitized mice was reduced by the oral administration of AY extracts and isoquercitrin (Fig. 2B).

3.2. Effect of AY extracts on plasma cytokine levels

It is well known that Th2 cytokines, including IL-4, IL-5, IL-6, IL-10, and IL-13 play important roles in establishing type I allergies. Th2 cytokines are required during the production of antigen-specific IgG or IgE, and Th1 cytokines, including IL-12, IFN- γ , and TNF- α levels are relatively suppressed and vice versa (Lehman & Blaiss, 2006).

To examine whether AY extracts and isoquercitrin can suppress plasma Th2 cytokine levels in OVA-sensitized mice, the production of IL-4, one of the major Th2 cytokines, was determined using ELISA. As shown in Fig. 3A, there was a significant increase in IL-4 production by

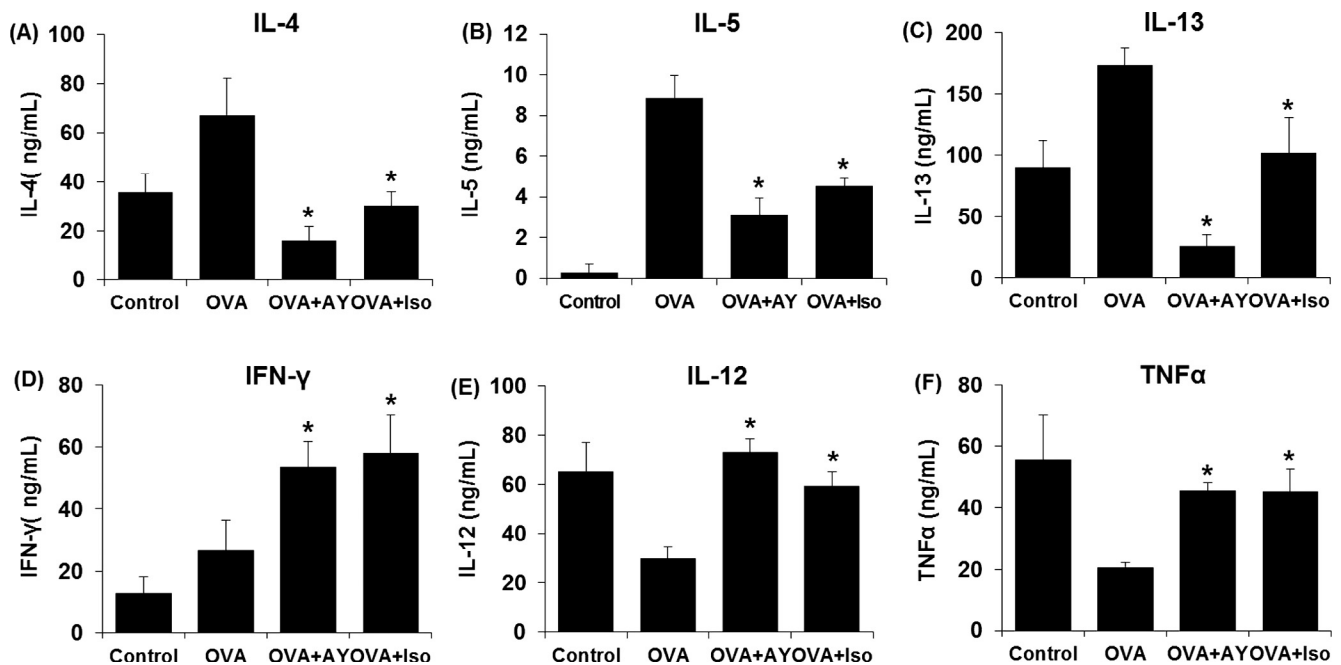


Fig. 3. Immunomodulatory effects of *Aster yomena* extracts on cytokine production in plasma of OVA-sensitized mice. Cytokines produced from plasma were detected using ELISA. (A) IL-4, (B) IL-5, and (C) IL-13 levels were measured (Th2 cytokines). (D) IFN-γ, (E) IL-12, and (F) TNF-α levels were measured (Th1 cytokines). Columns and error bars represent the mean $\pm$ standard error. * $p < 0.05$, significantly different from the OVA group. OVA = ovalbumin sensitization.

OVA stimulation. AY and isoquercitrin significantly inhibited the production of IL-4. In addition, the production of other Th2 cytokines such as IL-5 and IL-13 were also clearly inhibited by the AY extracts and isoquercitrin (Fig. 3B & C).

Among Th1 cytokines, IFN-γ production did not change significantly in the OVA-group; however, IFN-γ production was significantly increased in the AY and Iso groups (Fig. 3D). AY and isoquercitrin tended to increase the TNF-α and IL-12 levels as well (Fig. 3E & F).

Th1 and Th2 cells play an important role in allergy induction. An allergic reaction occurs when either Th1 cell activity was reduced or Th2 cell activity was enhanced (Deo, Mistry, Kakade, & Niphadkar, 2010). Consequently, the percentages of CD4⁺ IL-4⁺ T (Th2) cells in the OVA-treated group were higher than those in the control group; however, the percentages of CD4⁺ IFN-γ⁺ T (Th1) cells decreased significantly. Compared to the OVA-treated group, the AY extracts reduced the number of Th2 cells and increased the number of Th1 cells. Furthermore, the Th1/Th2 ratio increased significantly in the AY group (Fig. 4).

3.3. Cytotoxic effect of AY extracts on RBL-2H3 cells

The cytotoxicity of various concentrations of the AY extracts or isoquercitrin on RBL-2H3 cells was determined by measuring the intracellular ATP level. AY in the range of 25, 50 and 100 μg/mL did not cause significant cytotoxicity after 24 h treatment compared to the control (Fig. 5).

3.4. Effect of AY extracts on degranulation stimulated by compound 48/80 in RBL-2H3

The release of mediators by degranulation is one of the key phenomena observed during allergic inflammation (Theoharides et al., 2012). We investigated the effects of AY extracts and isoquercitrin on compound 48/80-induced degranulation in RBL-2H3 cells. AY reduced β-hexosaminidase release in a dose-dependent manner (14.5% and 37.9.1% inhibition at 25 and 100 μg/mL, respectively) (Fig. 6). Similarly, isoquercitrin inhibited β-hexosaminidase release (26.8% inhibition at 20 μg/mL). This indicates that AY is capable of inhibiting the

degranulation of mast cells.

3.5. Effect of AY extracts on the mRNA levels of pro-inflammatory cytokines

The inhibitory effects of AY extracts and isoquercitrin on pro-inflammatory cytokine production were assessed using quantitative PCR. Activation of RBL-2H3 cells by an IgE-antigen complex improved the mRNA levels of interleukins (IL-4, -5, and -13), and monocyte chemoattractant protein-1 (MCP-1); pre-treatment with AY extracts and isoquercitrin before stimulation decreased their levels in activated RBL-2H3 cells (Fig. 7).

3.6. Effect of AY extracts on expression of transcription factors regulating Th1/Th2

To investigate the mechanism by which AY extracts improves the Th1/Th2 cytokine imbalance, the levels of various transcription factors were explored in mouse EL-4 cells using western blot. The levels of p-STAT6, GATA3, and c-Maf were elevated in OVA-sensitized cells than in the control; however, the expression of p-STAT1, T-bet, and IRF1 were reduced. AY extracts and isoquercitrin inhibited the increase in expression of p-STAT6, GATA3, and c-Maf and increased the expression of p-STAT1, T-bet, and IRF1 compared to those in the OVA-sensitized cells (Fig. 8).

4. Discussion

In this study, we investigated whether AY and its putative functional compound isoquercitrin exert an anti-allergic effect in the widely used OVA-sensitized mice. In an in vivo study, we confirmed that administration of AY extracts and isoquercitrin diet significantly reduced production of IgE and histamine release. To elucidate the molecular basis for these observations, we investigated the effect of AY and isoquercitrin on cultured cells. We predicted that the mechanism of AY action involves modulation of Th1/Th2 cytokine levels. Allergy and inflammation are associated with enhanced production of Th2 cytokines (IL-4, IL-5, and IL-13) and decreased production of Th1 cytokines

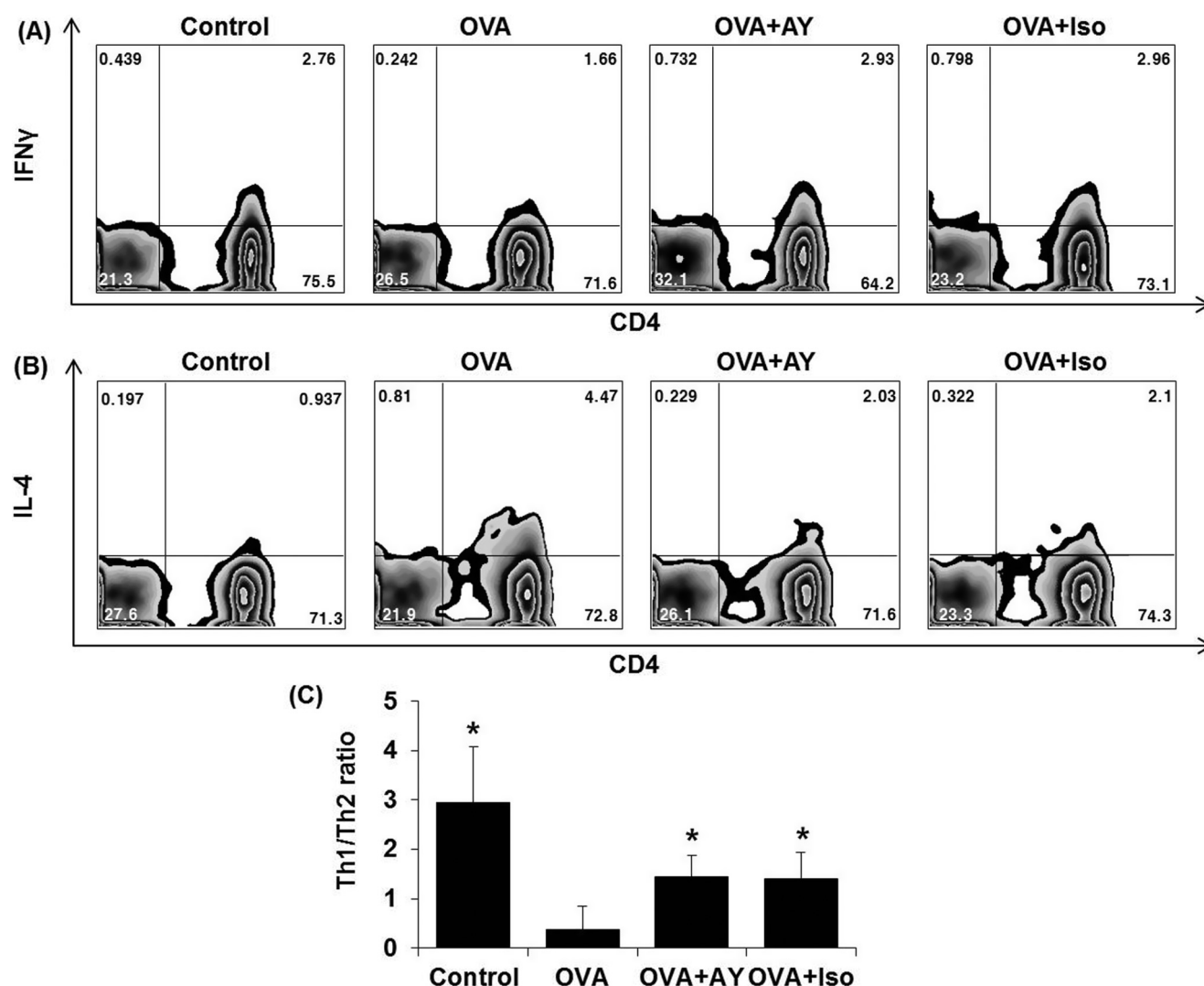


Fig. 4. Effect of *Aster yomena* on percentages of Th1 and Th2 cells among CD4⁺ T cells in spleens of OVA-sensitized mice. (A) Flow cytometric analysis of splenocytes stained with Anti-CD3 (FITC), Anti-CD4 (PE) and anti-IFN γ (APC). (B) Flow cytometric analysis of splenocytes stained with Anti-CD3 (FITC), Anti-CD4 (PE) and anti-IL-4 (APC). (C) The ratio of CD4⁺ IFN γ ⁺ (Th1) cell and CD4⁺ IL-4⁺ (Th2) cell. Columns and error bars represent the mean $\pm$ standard error. * p < 0.05, significantly different from the OVA group. OVA = ovalbumin sensitization.

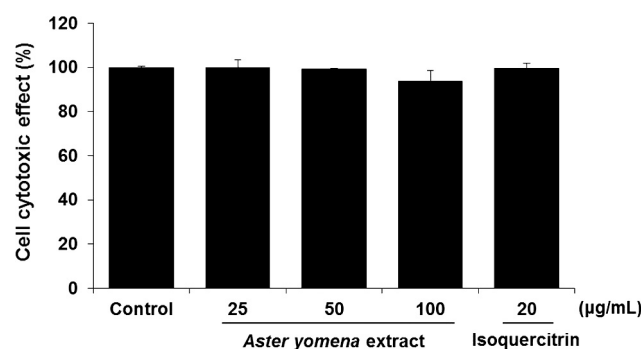


Fig. 5. Cytotoxic effects of *Aster yomena* extracts in RBL-2H3 cells. Cytotoxic effect was quantified by measuring intracellular levels of ATP. Columns and error bars represent the mean $\pm$ standard error.

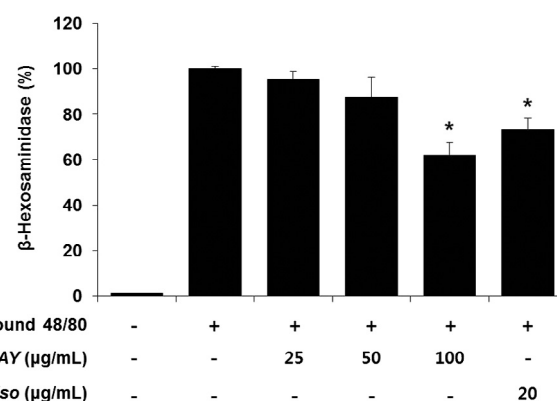


Fig. 6. The inhibitory effect of *Aster yomena* extracts on release of β -hexosaminidase from RBL-2H3 cells. Columns and error bars represent the mean $\pm$ standard error. * p < 0.05, significantly different from the Compound 48/80-control group.

(IFN- γ , TNF- α , and IL-12) (Quraishi, Davies, & Craig, 2004). T cells activated in the presence of IL-12 differentiate into Th1 cells, predominantly secrete IFN- γ and TNF α and promote delayed-type hypersensitivity responses (Trinchieri, 1995). In contrast, Th1 cells that are activated in the presence of IL-4 differentiate into Th2 cells, produce mainly IL-4 and IL-5, and promote humoral and allergic responses

(Nelms, Keegan, Zamorano, Ryan, & Paul, 1999; Romagnani, Annunziato, Piccinini, Maggi, & Romagnani, 2000). Thus, many scientists are currently attempting to regulate the activity of Th1 cells in case of allergy and Th2 in case of inflammation. Mijatovic, Kruys, Caput,

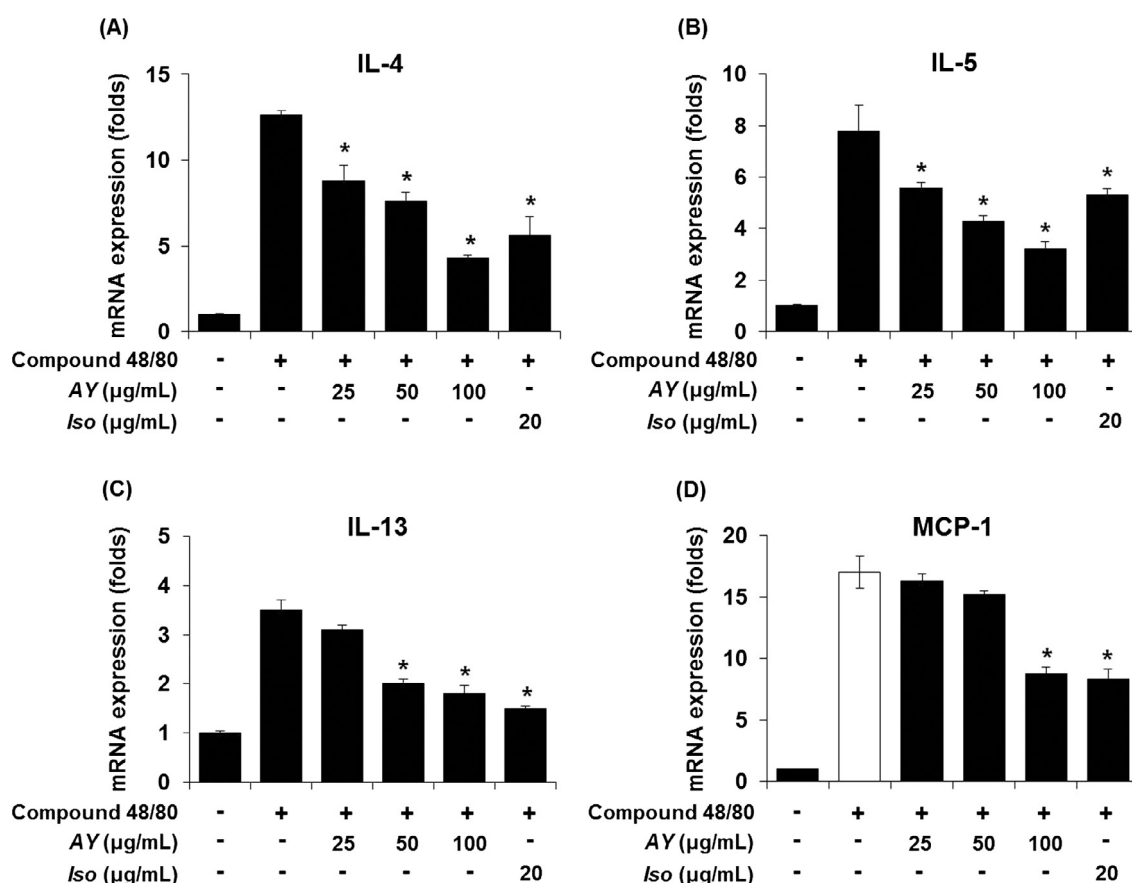


Fig. 7. Immunomodulatory effects of *Aster yomena* extracts on cytokine production in RBL-2H3 cells. Cytokines were detected by RT-PCR. (A) IL-4, (B) IL-5, (C) IL-13 and (D) MCP-1 levels were measured. Columns and error bars represent the mean $\pm$ standard error. * $p < 0.05$, significantly different from the compound 48/80-control group.

Defrance, and Huez (1997) reported that the production of TNF- α can be markedly inhibited by IL-4 and IL-13. In addition, according to Levings and Schrader (1999), the regulation of TNF- α production is suppressed by IL-4, IL-10, or IL-13. As is the case for TNF- α , the production of IL-12 is suppressed by IL-4, IL-10, or IL-13. IL-4 didn't inhibit the production of TNF- α and IL-12 from STAT6 null macrophages that were stimulated by LPS and IFN- γ . These data show that STAT6 is contributed for the stimulated by LPS, and IL-4 also inhibit the IFN- γ -mediated enhancement of IL-12 and TNF- α production. Our data show that AY significantly suppresses IL-4, IL-5, and IL-13 cytokine levels as well as p-STAT6, whereas IFN- γ , TNF- α , and IL-12 cytokine levels was significantly increased by AY. Taken together, AY inhibits the IL-4 production by STAT 6 regulation may lead to increase of IFN- γ , TNF- α ,

and IL-12 productions for treatment of allergic rhinitis. And isoquercitrin also suppressed Th2 cytokine such as IL-4 and increased Th1 cytokine in mouse and cultured cells as with AY extract results. Isoquercitrin is a glycoside of quercetin and is likely to play a role similar to quercetin. Previous studies have shown that quercetin increases Th1 cytokines and inhibits Th2 cytokines (Nair et al., 2002). And also, our results are in agreement with results of previous studies, isoquercitrin decrease the Th2 cytokines (Li, Zhang, Liu, Liu, & Dong, 2016).

Various transcription factors control the production of Th1 and Th2 cytokines in allergic reactions. In particular, T-bet is a Th1 transcription factor that controls the expression of the hallmark Th1 cytokine, IFN- γ (Mullen et al., 2001). Furthermore, GATA3 induces Th2 cytokine production, which depends on STAT6 activation, while suppressing Th1

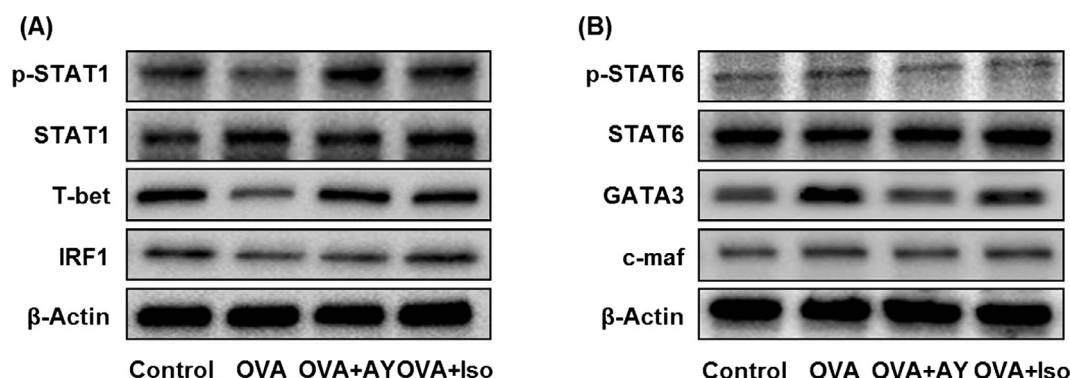


Fig. 8. Effects of *Aster yomena* on protein expression of p-STAT6, GATA3, c-Maf, p-STAT1, T-bet and IRF1 in OVA-stimulated EL-4 cells. EL-4 cells treated with AY extracts (100 μg/mL) or isoquercitrin (20 μg/mL) for 1 h and then cultured with OVA for another 24 h. The total cell proteins were extracted to detect (A) the expression of Th1 regulating transcriptional factor p-STAT1, T-bet and IRF1; (B) Th2 regulating transcriptional factor p-STAT6, GATA3 and c-Maf via western blot.

differentiation (Zhu, Yamane, Cote-Sierra, Guo, & Paul, 2006). Th2 cells secrete IL-4, which induces IgE antibody production by B cells and contributes to the development of allergic responses (Ray & Cohn, 1999). Here, we measured the expression of GATA3 and T-bet and the levels of IFN- γ and IL-4 in OVA-sensitized EL-4 cells to investigate the regulatory effect of AY on T cell differentiation. Our findings showed that AY extracts directly improved the imbalance in the ratio of Th1/Th2 cytokines by inhibiting the production of IL-4. The reduction in IL-4 levels was either due to the decreased expression of the Th2 transcription factors GATA3, p-STAT6, and c-Maf or the increase in expression of Th1 transcription factors T-bet, p-STAT1, and IRF1.

Some researchers reported that isoquercitrin, caffeic acid and apigenin possess anti-allergy activity and correlation with some signaling pathways to influence the cellular processes involved in allergy, asthma and inflammation (Liu, Zhang, Guo, & Dong, 2014; Rogerio et al., 2007; Zhou et al., 2014). Previous studies showed significant physiological effects of orally administered isoquercitrin in mice using doses ranging from approximately 10 to 30 mg/day/kg (Kempuraj et al., 2005; Makino et al., 2013; Park et al., 2008). Anti-allergy effects of isoquercitrin confirmed through inhibition of the passive cutaneous anaphylaxis reaction, intracellular calcium ion levels and protein kinase C phosphorylation in mast cells. Reports show that caffeic acid, another component of AY, suppressed lipopolysaccharide and concanavalin A-stimulated spleen cell proliferation (Park et al., 2004). Caffeic acid inhibits the synthesis of proinflammatory cytokines including IL-8 in TNF- α -induced Caco-2 cell (Zhao, et al., 2008); in addition, caffeic acid significantly suppresses delay type allergy (Hossen et al., 2006). Apigenin significantly inhibited T cell activation because of the down-regulation of mitogen-activated protein kinases. Additionally, apigenin inhibited T cell activation induced by the activation of T cell receptors by anti-CD3/CD28 antibodies and protein kinase C stimulation by PMA and ionomycin. These results clearly suggest that onset of T cell activation is one of the important molecular targets of apigenin (Kang, Ecklund, Liu, & Datta, 2009; Xu, Zhang, Bertucci, Pope, & Datta, 2008).

In present study, we confirmed that there are various functional compounds existing in AY extracts including phenolic acid and flavonoid such as isoquercitrin, caffeic acid, apigenin, esculetin, etc. And we found that the anti-allergy effect of AY extracts was higher than that of isoquercitrin. It seems to be synergistic effect of a small amount of compound such as caffeic acid, apigenin, etc.

According to Saini et al. (1999), cross-linking of Fc ϵ RI induced by the complex formation of IgE with an antigenic protein is an essential event in the IgE-mediated allergic reaction, so that inhibition of the binding between IgE and Fc ϵ RI, has been reported to be a powerful tool for suppressing allergic reactions. However, it is difficult to speculate that flavonoid compounds can directly regulate the intracellular signaling, because many literatures demonstrate that flavonoid compounds might be not physiologically incorporated into the cells. Therefore, it is acceptable to speculate that the flavonoid compounds such as quercetin and its aglycon, isoquercitrin may interact with the antigen-antibody-coupled Fc ϵ RI complexes on the cell surface of RBL-2H3 cells. For examples, the disturbance of antigen binding to IgE, aggregate formation and conformational change of receptor complexes may occur on the cell surface in the presence of flavonoid compounds. However, further study is needed to clarify the anti-allergy effect and precise mechanism such as the inactivation of intracellular signaling of the various compounds isolated from the AY extract. Nonetheless, our results using AY extracts and isoquercitrin can partially explain the anti-allergic mechanism of AY.

5. Conclusions

We proposed that AY extracts can be used for prevention of IgE-mediated allergic disorders and that AY extracts is a potential immunosuppressive agent that contributes to alleviate cytokine-mediated immune responses.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgments

This study was supported by “Research Program for Agricultural Science & Technology Development (Project No. PJ01082601)”, National Academy of Agricultural Science, Rural Development Administration, Korea.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jff.2018.02.026>.

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Supplementary method

Phytochemical characterization of *Aster yomena* extracts

The phytochemical of 50% ethanol extract of AY was identified by the high performance liquid chromatography (HPLC). HPLC was performed on an Agilent 1260 Infinity. Detecting wavelength was set at 360 nm. The separations were obtained with a reversed phase column (Thermo synchronis C18, 4.6 × 250 mm, 5µm) eluted at a flowing rate of 0.7 mL/min. The mobile phase consisted of (A) 0.3% formic acid in H₂O and (B) acetonitrile under gradient conditions. The gradient conditions were following profile: 0–5 min, 80% A, 20% B; 5–30 min, 40% A, 60% B; 30–32 min, 10% A, 90% B; 32–33 min, 80% A, 20% B; 33–40 min, 80% A, 20% B. Phytochemicals such as isoquercitrin, caffeic acid were quantified using the calibration curves obtained from commercial standards.

Supplementary table 1.

Compound	Isoquercitrin	Caffeic acid	Apigenin	Esculetin
Quantification (mg/g)	0.4711 ± 0.0158	0.3097 ± 0.0173	LDL ¹⁾	LDL

¹⁾ LDL: lower than detection limit