

Effects of *Hovenia dulcis* Thunb. extract and methyl vanillate on atopic dermatitis-like skin lesions and TNF- α /IFN- γ -induced chemokines production in HaCaT cells

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Keywords

Hovenia dulcis; atopic dermatitis; immunoglobulin E; immunoglobulin G2a; Th1/Th2-related mediators; NC/Nga mice

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Abstract

Objectives Here, we hypothesized that *Hovenia dulcis* branch extract (HDB) and its active constituents ameliorates 2,4-dinitrochlorobenzene-induced atopic dermatitis (AD)-like skin lesions by modulating the T helper Th1/Th2 balance in NC/Nga mice and TNF- α - and IFN- γ -induced production of thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) in HaCaT cells.

Methods HaCaT cells were stimulated by TNF- α /IFN- γ in the presence of HDB and its constituents. TARC and MDC were measured by ELISA and RT-PCR. For the in-vivo study, oral feeding of HDB was performed for 5 weeks with 2,4-dinitrochlorobenzene (DNCB) treatment every other day. The efficacy of HDB on parameters of DNCB-induced AD was evaluated morphologically, physiologically and immunologically.

Key findings In-vitro studies showed that HDB and its constituents suppressed TNF- α /IFN- γ -induced production of TARC and MDC in HaCaT cells by inhibiting MAPK signalling. In-vivo studies showed that HDB regulated immunoglobulin (Ig) E and immunoglobulin G2a (IgG2a) levels in serum and the expression of mRNA for Th1- and Th2-related mediators in skin lesions. Histopathological analyses revealed reduced epidermal thickness and reduced infiltration of skin lesions by inflammatory cells.

Conclusion These results suggest that HDB inhibits AD-like skin diseases by regulating Th1 and Th2 responses in NC/Nga mice and in HaCaT cells.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease, the prevalence of which is increasing in industrialized nations. It generally occurs during infancy and childhood but can also occur in adults.^[1] AD is characterized by chronic eczematous and pruritic skin lesions, which are caused by infiltration of inflammatory cells such as mast cells, eosinophils, monocytes/macrophages and T lymphocytes.^[2,3]

The development of AD is influenced by several factors, including immunological abnormalities.^[4] The developmental trigger in most patients is elevated levels of serum immunoglobulin E (IgE) and circulating eosinophils due to

increased production of interleukin (IL)-4, IL-5 and IL-13 by Th2 cells.^[5,6] Th1/Th2 immune response underlies the known pathogenic mechanism of AD; thus, AD is a biphasic inflammatory skin disease induced by the Th1/Th2 immune response.^[7]

Keratinocytes, which accounts for the majority of epidermis, play a key role in the pathogenesis of inflammatory skin disorders such as AD.^[8] The pathogenesis of AD is known to be greatly affected with production of chemokines such as thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22).^[9] TARC is expressed in the thymus and is abundant in macrophages, endothelial cells, bronchial epithelial cells, keratinocytes, dendritic cells and peripheral

blood mononuclear cells^[10–12] and binds to the chemokine receptor CCR4,^[13] which is expressed at high levels by Th2 cells. Neutralizing MDC with specific antibodies reduces airway hyper-reactivity and inhibits interstitial lung inflammation in a model of ovalbumin-induced lung allergic inflammation.^[14] MDC is a potent chemoattractant for Th2 monocytes and lymphocytes.^[15] For these reasons, TARC and MDC are considered to be important mediators in the Th2-dominant skin diseases such as AD.

Mitogen-activated protein kinase (MAPK) signalling pathways are an important target for anti-allergy mechanisms. Extracellular signal-regulated kinase (ERK) activity is induced by growth factors via a Ras-dependent signalling pathway.^[16] Another kinase, c-Jun N-terminal kinase (JNK), is activated by inflammation-related cytokines and by certain environmental conditions. JNK activation increases during costimulation of T lymphocytes.^[17] Crucially, activation of ERK and JNK is biochemically and functionally separate.

In East Asia, *Hovenia dulcis* Thunb. (Rhamnaceae), which is well-known as 'oriental raisin tree', is commonly used both as a pharmaceutical supplement and as a nutraceutical. The roots, seeds, bark, leaves and peduncles of *H. dulcis* are used in traditional Japanese, Chinese and Korean medicine to quench thirst, strengthen the stomach, alleviate alcoholic intoxication, promote digestion and provide antifebrile therapy.^[18] According to recent studies, extracts of the seeds and fruits of *H. dulcis* have an effect on ethanol-induced muscle relaxation, attenuate acute liver toxicity and show antilipid peroxidation and antitumour activity.^[19,20] A previous study shows that *H. dulcis* extract has inhibitory effects on mast cell-mediated allergic reactions and passive cutaneous anaphylaxis in mice,^[21] anti-inflammatory actions in lipopolysaccharide-stimulated inflammatory responses^[22]; however, its effect on AD has not been investigated. Therefore, this study examined whether *H. dulcis* branch extract (HDB) alleviates AD in 2,4-dinitrochlorobenzene (DNCB)-induced AD model.

Materials and Methods

Chemical reagents

Foetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) were obtained from Hyclone (South Logan, UT, USA). DNCB, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185), SAPK/JNK (Thr183/Tyr185), phospho-p44/42 MAPK (ERK1/2;

Thr202/Tyr204), ERK1/2 and β-actin were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA).

Plant material and preparation of chemical constituents

The branches of *H. dulcis* were purchased in traditional herbal market (Kyungdong oriental market, Seoul, Korea), and their identification was verified by M. Kim (co-author). The voucher specimen was deposited at the Korea Institute of Science and Technology Gangneung Institute, Korea. We isolated seven compounds from HDB, as previously described.^[21]

Cell culture and cell viability assay

HaCaT cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS and antibiotics. Cells were grown at 37 °C in 100 mm dishes in a humidified atmosphere containing 5% CO₂. Cell viability was assayed using the MTT colorimetric assay. Briefly, HaCaT cells were plated in 96-well plates (4 × 10³ cells/ml) and grown for 24 h. After treatment with various concentrations of HDB or its active constituents for 24 h with or without of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (each at 20 ng/ml), cells were washed, treated with MTT (1 mg/ml) and incubated for 4 h. Cells were then washed, and the insoluble formazan products were dissolved in DMSO. Absorbance at 550 nm was measured in a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

Enzyme-linked immunosorbent assay

IgE and immunoglobulin G2a (IgG2a) levels in serum samples and the production of TARC and MDC by HaCaT cells were measured in enzyme-linked immunosorbent assays (ELISAs) (Abcam, Cambridge, UK), according to the manufacturer's instructions. Immunoglobulin and chemokine concentrations were calculated using a linear regression equation derived from standard absorbance values.

Animal experiments

Specific pathogen-free male NC/Nga mice (4 weeks old; Central Laboratory, Seoul, Korea) were bred in a controlled environment (22 ± 2 °C, 55% ± 15% relative humidity, 12-h light/dark cycle). After a one-week acclimation period, the mice were fed a standard diet (Cargill Agri Purina, Seongnam, Korea) and water. After complete removal of the dorsal hairs, 30 mice were randomized into a blinded, placebo-controlled trial where they were topically treated with

200 µl of 1% DNCB solution (dissolved in a 3 : 1 mixture of acetone and olive oil) five times per day. Five days after sensitization, the skin was challenged with 200 µl of 0.2% DNCB solution three times per week for 35 days. AD induction was verified by macroscopic observation (e.g. skin eruptions and erythema). After 5 weeks, the thirty mice were randomized into five experimental groups ($n = 6$): vehicle group (vehicle without DNCB treatment); DNCB group (vehicle plus DNCB treatment); HDB50 group (oral administration of HDB 50 mg/kg per day plus DNCB treatment); HDB200 group 4 (oral administration of HDB 200 mg/kg per day plus DNCB treatment); and DEX group (oral administration of dexamethasone 2.5 mg/kg per day plus DNCB treatment). M.K randomized the mice using a random number generation software (<http://www.graphpad.com/quickcalcs/randomize1.cfm>). S.J.L., who was blinded to which the mice had been randomized, performed all clinical and histological analysis. All mice (except those in vehicle group) were topically treated with 200 µl of 0.2% DNCB three times per week for 5 weeks (15 challenges in total). The HDB50, HDB200 or DEX groups were fed on a daily basis for 5 weeks using oral feeding needles along with receiving topical DNCB. At the end of the oral administration period, blood was collected via cardiac puncture. After the experiments, the skin lesions were removed and stored at -80°C . The experimental protocol was approved by the Animal Use and Care Committee of the Korea Institute of Science and Technology (2015-051), Seoul, Korea.

Histopathological analyses

Skin tissues were fixed in 10% formalin. After fixation, specimens were embedded in paraffin and sectioned (4 µm thick). To measure the number of mast cells and epidermal thickness, sections were stained with haematoxylin–eosin (H&E) and toluidine blue. The epidermal and dermal images were observed using Inverted DIC Fluorescence Microscope fitted with twin CCD cameras (Nikon, Tokyo, Japan; magnification, $\times 200$). The epidermal thickness of each H&E-stained section was measured, and the number of mast cells in each toluidine blue-stained section was counted, in more than ten fields per each sample using ProgRes CapturePro software (Jenoptik, Altenstadt, Germany). Data are expressed as the mean and standard deviation (SD).

Semi-quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from HaCaT cells and skin tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol, and the concentration was measured by spectrophotometry at 260 nm. cDNA was synthesized with total RNA, oligo(dT) and

reverse transcriptase (Elpis-Biotech, Daejeon, Korea). The oligonucleotide primers used are shown in Table 1. Primers were denatured at 94°C for five min before PCR amplification. Amplification was then performed as follows: 32 cycles of 94°C for 30 s, followed by annealing for 1 min and extension at 72°C for 1 min and a final five-min extension at 72°C . β -actin was used as a loading control and for normalization of data between samples. The PCR products were analysed by agarose gel and visualized by ethidium bromide staining. Densitometric analysis was used for measuring reverse transcriptase-polymerase chain reaction (RT-PCR) amplicons.

Western blot analysis

The protein concentration of each cell lysate was measured using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal volumes (18 µl per lane) from each sample were loaded on sodium dodecyl sulphate-polyacrylamide gels. After separation, the proteins were transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) and initially blocked with 3% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline containing 0.1% Tween-20 for 1 h, followed by an overnight incubation with primary antibodies at 4°C . After three washes in Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) for 2 h.

Statistical analysis

Data are presented as the mean $\pm$ SD. Statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Group differences were evaluated by one-way analysis of variance, followed by the Duncan test. A P value < 0.05 was considered to indicate statistical significant.

Results

Hovenia dulcis branch reduces expression of chemokine ligands in HaCaT cells stimulated with TNF- α and IFN- γ

An MTT assay was used to measure the cytotoxic effects of HDB on HaCaT. Cells were treated with culture medium containing different concentrations of HDB (0–20 µg/ml). HDB was cytotoxic at concentrations above 10 µg/ml (Figure 1a). Thus, HDB was used at 1–10 µg/ml in future experiments. We also measured the cytotoxic effects of HDB in HaCaT cells in the presence of TNF- α and IFN- γ . Again, the results showed that HDB was not cytotoxic below 10 µg/ml (Figure 1b). We then examined the effect of HDB on TARC and MDC levels in HaCaT cells induced by TNF- α

Table 1 Primers used for RT-PCR

Gene	Primer	Sequence	Product length (bp)
Human TNF- α	Forward	5'-AGC CGC ATC GCC GTC TCC TA-3'	163
	Reverse	5'-CAG CGC TGA GTC GGT CAC CC-3'	
Human IL-6	Forward	5'-TCG AGC CCA CCG GGA ACG AAA-3'	403
	Reverse	5'-AGG CAA CTG GA C CGA AGG CG-3'	
Human CCL17	Forward	5'-TGA CTG TAC CCG GAC TGG AT-3'	233
	Reverse	5'-TGG CTC CAG TTC AGA CAA GG-3'	
Human CCL22	Forward	5'-AGA AGC CTG TGC CAA CTC TC-3'	161
	Reverse	5'-GGG AAT CGC TGA TGG GAA CA-3'	
Human b-actin	Forward	5'-TCC ACC AAA TTC GCT GAC GA-3'	434
	Reverse	5'-ATG CTG AAC TCA GCT GGG AC-3'	
Mouse IFN- γ	Forward	5'-CGG CAC AGT CAT TGA AAG CC-3'	268
	Reverse	5'-TGC ATC CTT TTT CGC CTT GC-3'	
Mouse IL-1 β	Forward	5'-TCG GCC AAG ACA GGT CGC TC-3'	231
	Reverse	5'-CCT CAG TGC GGG CTA TGA CC-3'	
Mouse IL-4	Forward	5'-CCA TAT CCA CGG ATG CGA CA-3'	254
	Reverse	5'-AAG CCC GAA AGA GTC TCT GC-3'	
Mouse IL-5	Forward	5'-GCT GAA GGC CAG CGC TGA AGA-3'	160
	Reverse	5'-ACA GAG CTC GGT GAG CGG ACA-3'	
Mouse IL-10	Forward	5'-CCA GTC GGC CAG AGC CAC AT-3'	208
	Reverse	5'-GGC CAT GCT TCT CTG CCT GGG-3'	
Mouse IL-12	Forward	5'-TCT CTG GAC CTG CCA GGT GT-3'	542
	Reverse	5'-CCT GTT GAT GGT CAC GAC GCG-3'	
Mouse CCL5	Forward	5'-AGG AAC CGC CAA GTG TGT GTG CC-3'	67
	Reverse	5'-AGT GGC ATC CCC AAG CTG GCT A-3'	
Mouse CCL11	Forward	5'-ATG CAC CCT GAA AGC CAT AGT-3'	97
	Reverse	5'-CAG GTG CTT TGT GGC ATC CT-3'	
Mouse CCL17	Forward	5'-CCG AGA GTG CTC CCT GGA TTA-3'	123
	Reverse	5'-AGC TTG CCC TGG ACA GTC AGA-3'	
Mouse T-bet	Forward	5'-CAG CCT GGG GAC GCC CTA CT-3'	280
	Reverse	5'-CAG GTG GTT GCT GAG CGC GA-3'	
Mouse GATA-3	Forward	5'-CGG GTC GGC CAG GCA AGA TG-3'	386
	Reverse	5'-TAA GGG GCC GGT TCT GCC CA-3'	
Mouse Foxp3	Forward	5'-ATC CAG CCT GCC TCT GAC AAG AAC C-3'	401
	Reverse	5'-GGG TTG TCC AGT GGA CGC ACT TGG AGC-3'	
Mouse β -actin	Forward	5'-TAA CCA ACT GGG ACG ATA TG-3'	203
	Reverse	5'-ATA CAG GGA CAG CAC AGC CT-3'	

and IFN- γ . TARC and MDC specifically regulate the infiltration of Th2 cells to sites of inflammation and both are thought to be key mediators of AD.^[22] HaCaT cells were pretreated with various concentration of HDB (1, 5 or 10 μ g/ml) for 1 h and then treated with TNF- α and IFN- γ for 24 h. TARC and MDC levels in the culture medium were then measured in ELISAs (Figure 1c and d). The results showed that HDB decreased the production of TARC and MDC by HaCaT cells stimulated with TNF- α and IFN- γ .

Hovenia dulcis branch decreases expression of inflammatory cytokines by HaCaT cells stimulated with TNF- α and IFN- γ

We next examined whether HDB affects the expression of inflammatory cytokines and the MAPK pathway, both of

which are important mediators of AD^[22] because phosphorylation of MAPKs induces cytokine production.^[23] HDB treatment of TNF- α - and IFN- γ -stimulated HaCaT cells reduced their expression of mRNAs encoding inflammatory genes and inhibited the phosphorylation of ERK, JNK and p38 (Figure 2a and b). Thus, HDB prevented the release of AD mediators by inhibiting MAPK signalling pathways.

Several compounds derived from *Hovenia dulcis* branch reduce chemokine ligand expression, and MV suppresses the MAPK pathway, in HaCaT cells stimulated with TNF- α and IFN- γ

Next, we measured TARC and MDC levels in HaCaT cells induced by TNF- α and IFN- γ to confirm the effects of

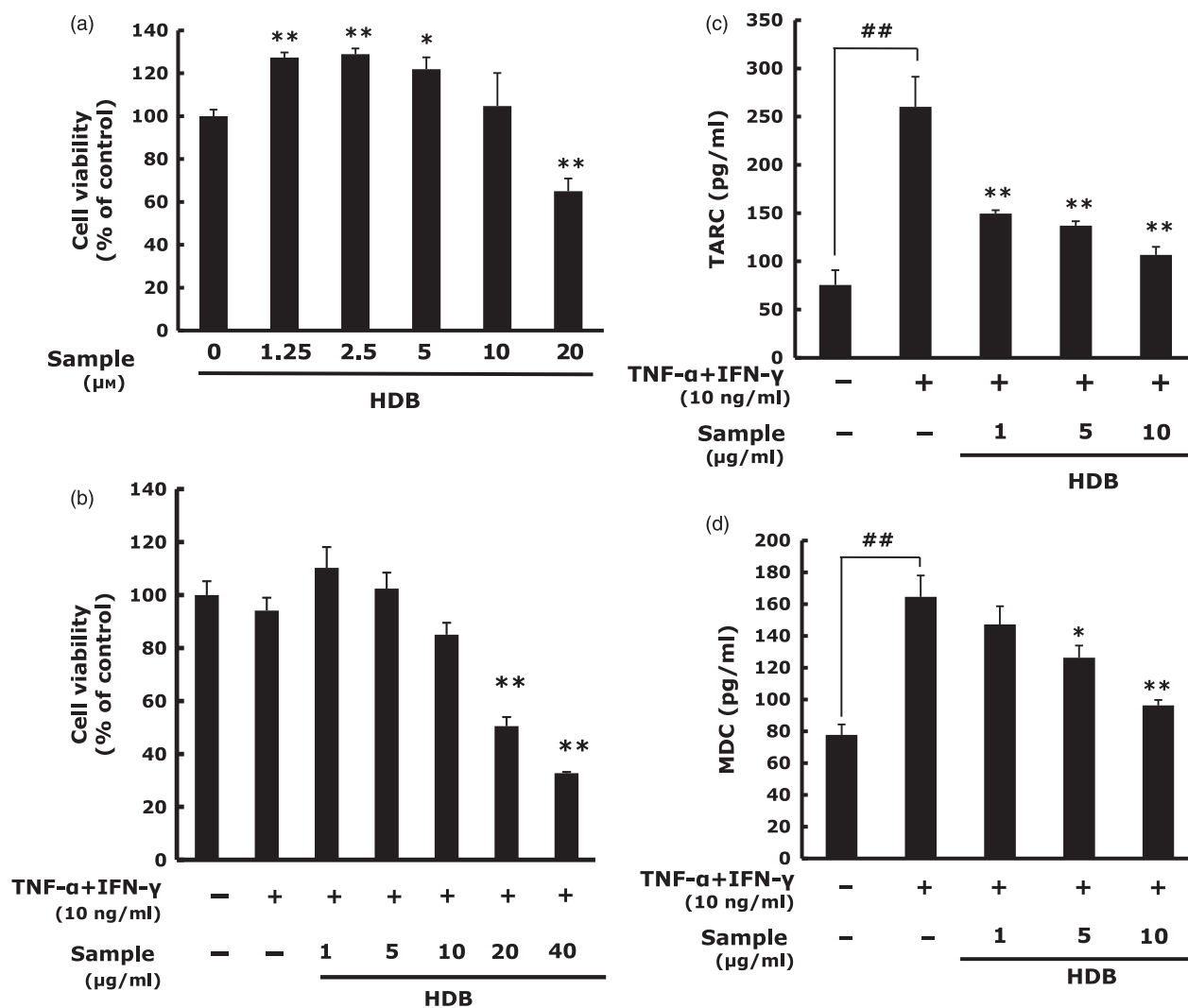


Figure 1 Effects of the branches of *Hovenia dulcis* extract (HDB) with TNF- α and IFN- γ on production of thymus and activation-regulated chemo-kine (TARC) and macrophage-derived chemokine (MDC) in HaCaT cells. (a and b) Cell viability of various concentration of HDB (1–20 μ M) with or without TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) in HaCaT cells. The cell viability was measured by MTT assay. HaCaT cells were pretreated with HDB (1–20 μ g/ml) for 1 h and then stimulated with or without TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 24 h. (c) TARC and (d) MDC production in the culture medium were determined by Enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean $\pm$ SD of triplicate experiments. (## P < 0.01, ** P < 0.01 and * P < 0.05).

HDB-derived compounds (Figure 3a and b). Ferulic acid (FA), vanillic acid (VA), methyl vanillate (MV), taxifolin, 3,5-dihydrokaempferol (DHK) inhibited production of both TARC and MDC. However, MV had a stronger inhibitory effect than the other compounds. Thus, we examined whether MV regulates expression of genes encoding AD mediators or members of the MAPK pathway. First, we showed that MV was not cytotoxic up to a concentration of 20 μ M (Figure 3c and d). HaCaT cells were pretreated with MV for 1 h and stimulated with TNF- α and IFN- γ for 24 h before measurement of expression of AD mediator and MAPK pathway member proteins (Figure 3e and f). The

results showed that MV reduced the expression of AD mediators (TNF- α , IL-6, TARC and MDC) (Figure 3e) and ERK, JNK and p38 (Figure 3f). These results suggest that MV is a possible therapeutic compound for AD.

***Hovenia dulcis* branch has an inhibitory effect on the atopic dermatitis-like skin lesions in NC/Nga mice**

An NC/Nga mouse model of AD was generated by twice weekly application of DNCB solution to the dorsal skin. DNCB initially caused skin dryness, followed by

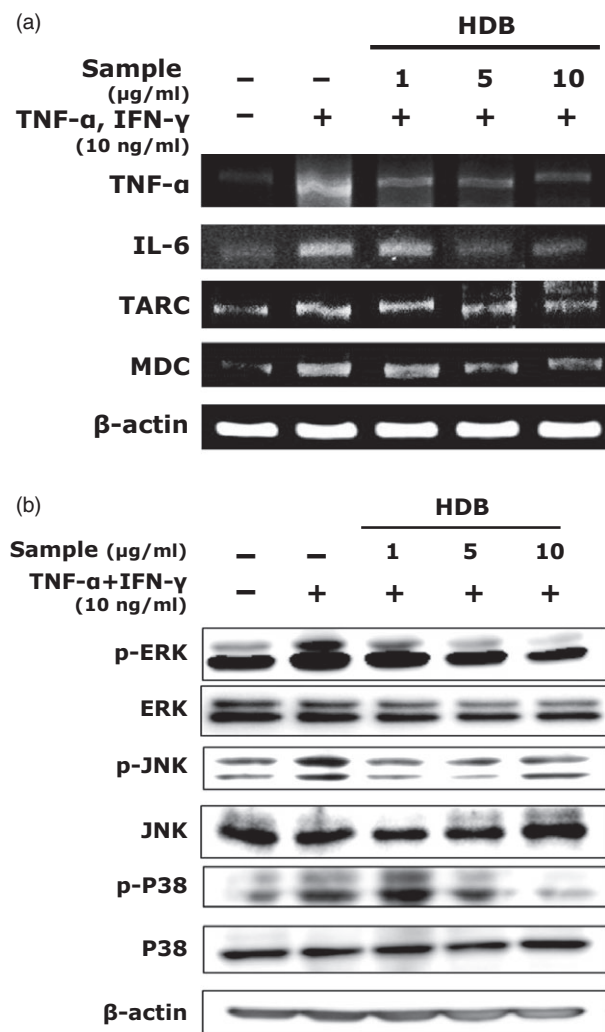


Figure 2 Decrease in inflammatory cytokine expression and mitogen-activated protein kinase (MAPK) pathway in HaCaT cells treated with *Hovenia dulcis* branch (HDB). (a and b) HaCaT cells were pretreated with HDB (1–10 μg/ml) for 1 h and then stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 24 h. mRNA levels were determined with RT-PCR. (c and d) HaCaT cells were pretreated with HDB (1–10 μg/ml) for 1 h and then stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 30 min. Phosphorylation of ERK, JNK and p38 was determined by Western blot analysis. β-actin was used as internal control. Results are expressed as a representative data of triplicate experiments.

haemorrhage, oedema, erythema, itching and thickening. DNCB solution continued to be applied twice a week while mice were orally administered HDB (50 mg/kg per day or 200 mg/kg per day) or DEX (2.5 mg/kg per day) for 42 days. HDB treatment inhibited the noticeable hyperkeratosis, haemorrhage and itching compared to DNCB group (Figure 4a). Furthermore, HDB attenuated epidermal thickening and infiltration of inflammatory cells,

particularly mast cells by the findings of H&E and toluidine blue staining of the dorsal skin sections (Figure 4b and c). Taken together, these results suggest that HDB reduces mast cell degranulation and skin hypertrophy in AD. The effects of oral administration of HDB200 were comparable with those of DEX.

Hovenia dulcis branch regulates the production of serum immunoglobulin E and immunoglobulin G2a

Immunoglobulin E and IgG2a play a pivotal role in AD progression.^[24–27] Thus, we investigated the effects of HDB on IgE and IgG2a levels in serum samples from NC/Nga mice. IgE levels increased, and IgG2a levels decreased, in the DNCB-treated group when compared with those in the vehicle-alone group. However, oral administration of HDB reduced IgE levels and augmented IgG2a levels when compared with those in the DNCB-treated group (Figure 5a and b).

Hovenia dulcis branch reduces the expression of mRNA for Th1- and Th2-associated transcription factors, chemokines and cytokines in skin lesions

Finally, we examined whether HDB affected the expression of mRNA encoding cytokines, chemokines and important markers of AD in the skin of NC/Nga mice.^[5,7,28] We found that HDB significantly inhibited the expression of cytokines and chemokines associated with Th1 and Th2 immune responses (Figure 6a and b). In addition, HDB reduced the expression of IL-1β in skin lesions of NC/Nga mice (Figure 6c). Taken together, the results showed that oral administration of HDB inhibits expression of mRNA encoding Th1, Th2 and inflammation-related cytokines and chemokines in the skin lesions of NC/Nga mice.

T-bet, GATA-3 and Foxp3 are Th1/Th2/T regulatory (Treg) cell-associated transcription factors.^[29–31] Thus, we measured the effects of HDB on expression of T-bet, GATA-3 and Foxp3 mRNA in skin lesions (Figure 6d) and confirmed the results using RT-PCR. HDB200 attenuated expression of T-bet and GATA-3. These results suggest that HDB treatment inhibits the expression of inflammatory genes in AD skin lesions.

Discussion

Hovenia dulcis, an important medicinal plant in the *Hovenia* species, has been used as herbal medicines in China, Japan and Korea. Previous reports have revealed that *H. dulcis* have antidiabetic, antioxidative, antifatigue, hepatoprotective, neuroprotective and

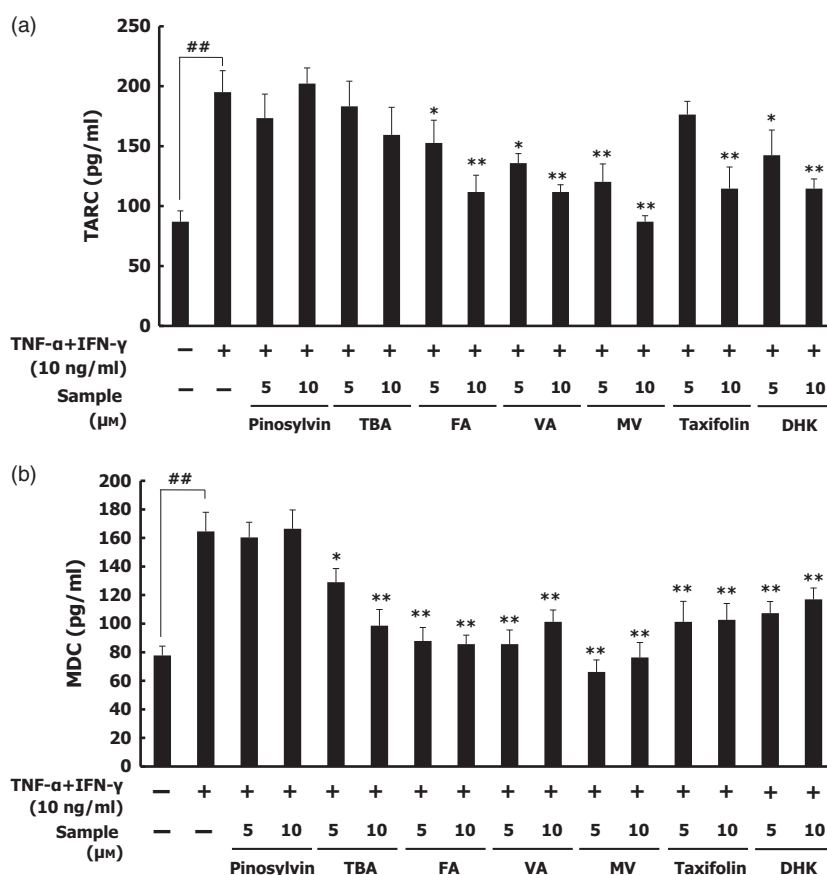


Figure 3 Among several compounds from *Hovenia dulcis* branch (HDB), effects of MV from HDB with TNF- α and IFN- γ on production of thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), expression of inflammatory cytokines and chemokines, and mitogen-activated protein kinase (MAPK) pathway in HaCaT cells. HaCaT cells were pretreated with seven compounds from HDB (5 and 10 μ M) for 1 h and then stimulated with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 24 h. (a) TARC and (b) MDC production in the culture medium were determined by ELISA. (c and d) Cell viability of MV (5–40 μ M) with or without TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) in HaCaT cells. The cell viability was measured by MTT assay. HaCaT cells were pretreated with MV (5–40 μ M) for 1 h and then stimulated with or without TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 24 h. (e and f) HaCaT cells were pretreated with MV (1–10 μ M) for 1 h and then stimulated with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 24 h. mRNA levels were determined with RT-PCR. (g and h) HaCaT cells were pretreated with MV (1–10 μ M) for 1 h and then stimulated with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 30 min. Phosphorylation of ERK, JNK and p38 was determined by Western blot analysis. TBA, 2,3,4-trihydroxybenzoic acid; FA, ferulic acid; VA, vanillic acid; MV, methyl vanillate; DHK, 3,5-dihydrokaempferol. β -actin was used as internal control. Results are expressed as mean $\pm$ SD of triplicate experiments. (## P < 0.01, ** P < 0.01, * P < 0.05).

immunostimulatory activity.^[18–20] It is reported that *H. dulcis* extract inhibits the allergy-mediated activity of IgE-triggered mast cells and reduces passive cutaneous anaphylaxis in mice.^[21] An allergic reaction is a hypersensitivity disorder mediated via immunological mechanisms; thus, an allergic response can lead to tissue damage, AD, eczema, anaphylactic shock and asthma.^[32,33] Therefore, we hypothesized that HDB might also inhibit the mechanisms underlying AD.

Allergens induce the production of antigen-specific IgE antibodies, which bind to high-affinity IgE receptors on the surface of mast cells or basophils, resulting in extreme inflammatory responses.^[32,34,35] In addition, keratinocytes

play an important role in the pathogenesis of AD.^[36] When keratinocytes are stimulated with IFN- γ and TNF- α , they increase their expression of pro-inflammatory chemokines, cytokines and adhesion molecules.^[37]

Keratinocytes which are stimulated with TNF- α and IFN- γ treatment induce the production of inflammatory chemokines and cytokines that mediate infiltration of inflammatory cells in the epidermis.^[37] It has reported that TNF- α and IFN- γ synergistically induce generation of TARC and MDC in HaCaT cells and in primary human keratinocytes.^[9] Furthermore, patients with AD showed the increased production levels of TARC and MDC in keratinocytes in the skin lesion.^[38] Thus, we used TNF- α and

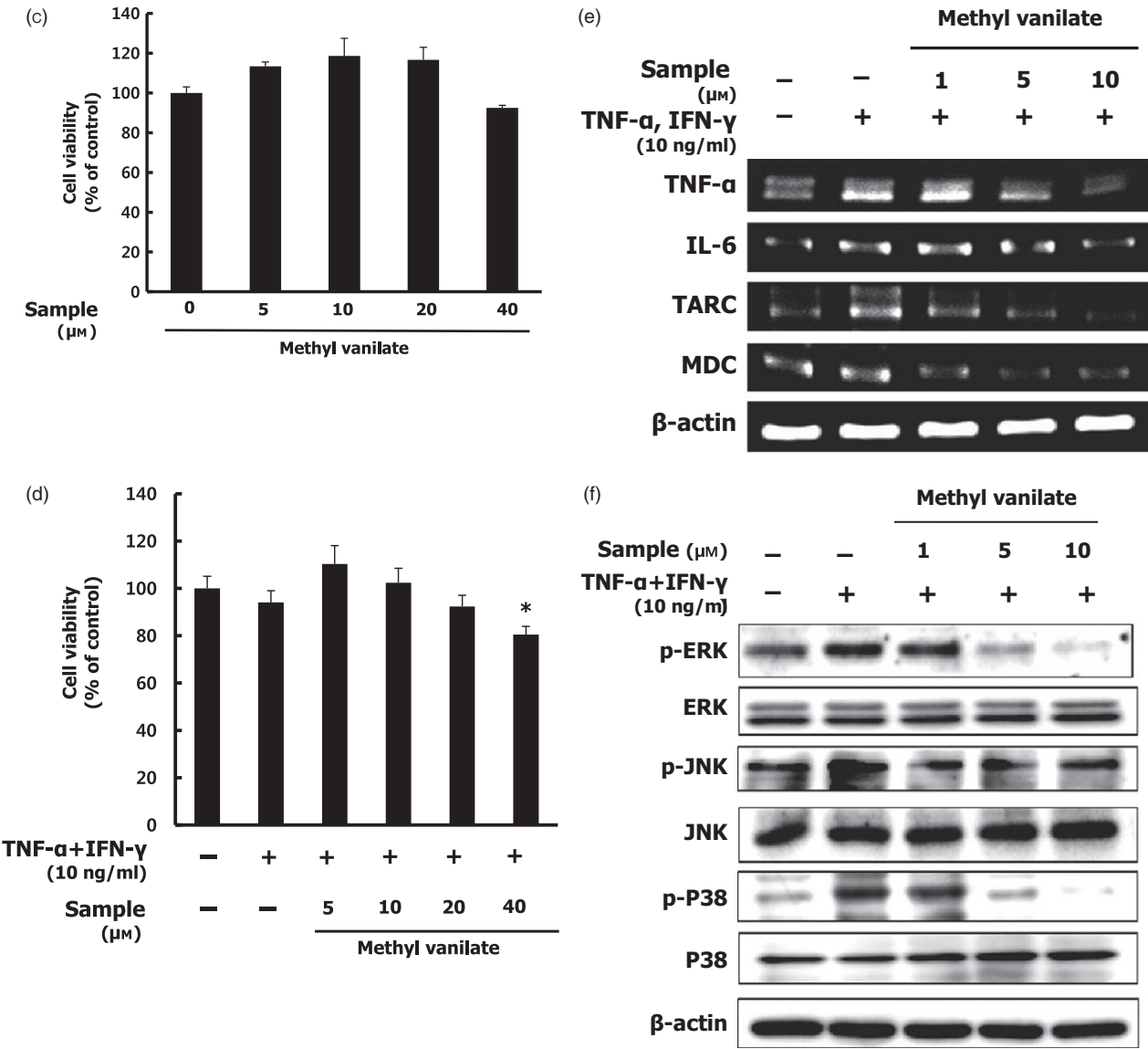


Figure 3 Continued

IFN- γ to stimulate HaCaT before measurement of MDC and TARC.

Macrophage-derived chemokine and TARC are members of the CC chemokine subfamily and are released by diverse cell types.^[9] Both are present in the skin lesions of NC/Nga AD model mice and human patients with AD.^[39,40] We found that HDB inhibited the production of MDC and TARC in HaCaT cells stimulated with TNF- α and IFN- γ (Figure 1). Previous studies show that the keratinocytes of AD sufferers produce cytokines and chemokines that promote development of inflammatory skin diseases; the secretion of pro-inflammatory cytokines is triggered by activation of the MAPK signalling

pathway.^[41] Indeed, the intracellular MAPK signalling pathway is activated in keratinocytes stimulated by TNF- α and IFN- γ .^[42] Pro-inflammatory cytokines, including IL-1 and IL-6, then induce keratinocyte migration and proliferation.^[36] Here, we showed that HDB inhibited TNF- α - and IFN- γ -induced expression of mRNA for pro-inflammatory cytokines and chemokines and inhibited phosphorylation of ERK, JNK and p38 (Figure 2a and b). Previously, we demonstrated that HDB inhibits MAPK activation in IgE-stimulated mast cells.^[21] Thus, it seems that the inhibitory effect of HDB on chemokine production results at least partly from the suppression of MAPK signalling.

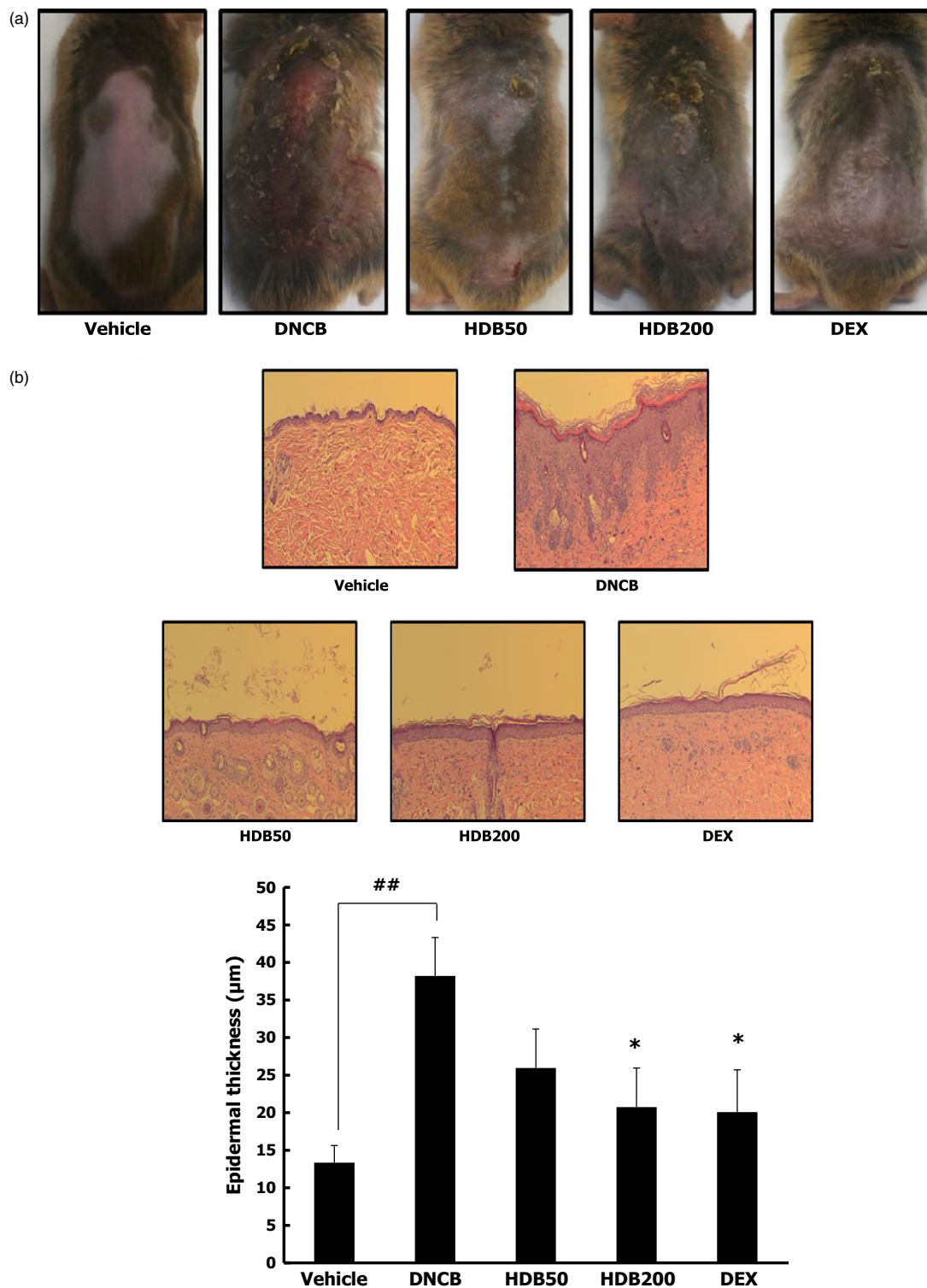


Figure 4 Effects of *Hovenia dulcis* branch (HDB) on atopic dermatitis (AD)-like skin lesions treated topically in NC/Nga mice. (a) Clinical features of AD-like skin lesions after 2,4-dinitrochlorobenzene (DNCB) application in NC/Nga mice. (b and c) Histopathological analysis in skin lesions. Skin lesions were excised, fixed in 10% formalin, embedded in paraffin and sectioned. The sections were stained with haematoxylin and eosin (H&E) and toluidine blue. Epidermal thickness was observed, and mast cells were counted with a microscope at a magnification of $\times 200$. Results are expressed as mean $\pm$ SD ($n = 6$) ($^{##}P < 0.01$, $^{**}P < 0.01$ and $^{*}P < 0.05$).

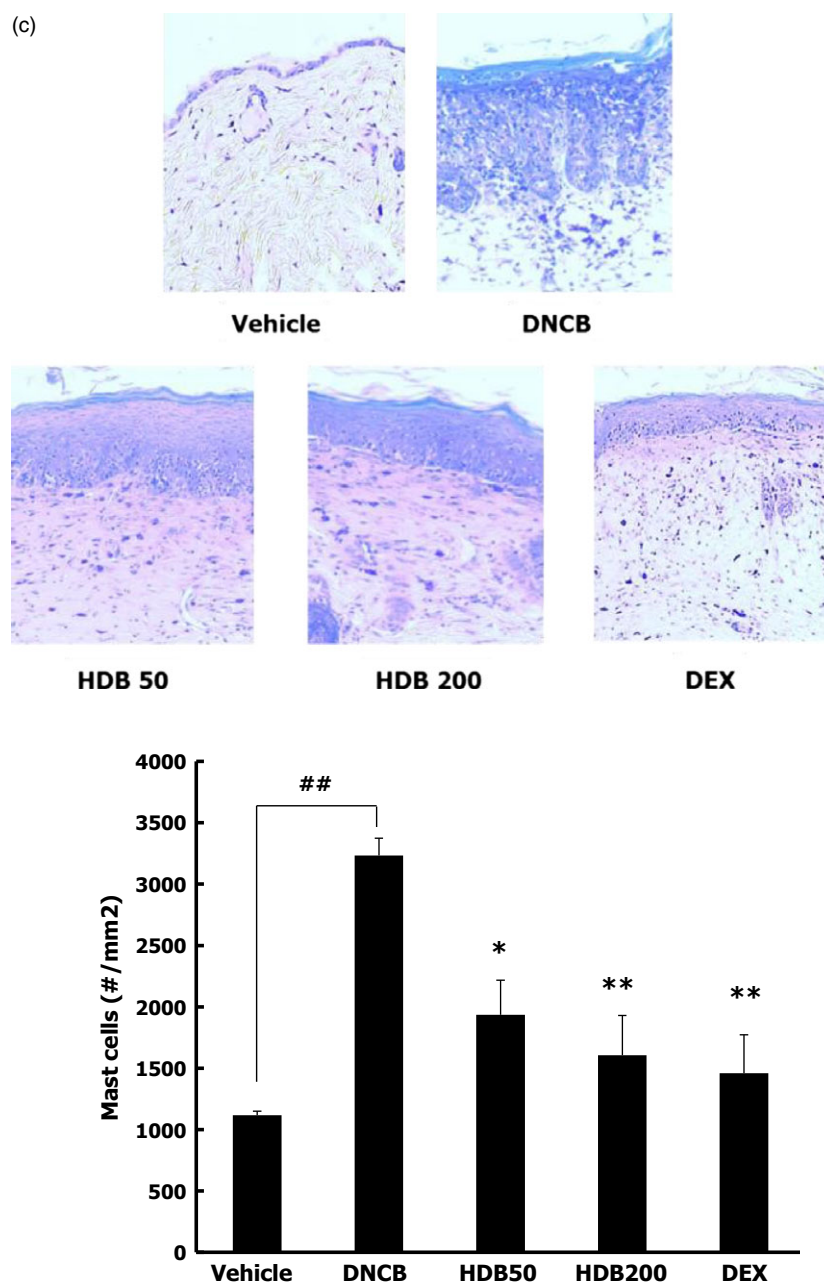


Figure 4 Continued

To date, more than 10 bioactive compounds have been isolated from HDB, including flavonoids ((+)-afzelechin, (+)-aromadendrin (–)-catechin, DHK), organic acids (FA, VA, MV, 2,3,4-trihydrobenzoic acid (TBA)) and stilbenoid (pinosylvin).^[43] The seven HDB-derived compounds were identified in a previous study by Li *et al.* (pinosylvin, TBA, FA, VA, MV, taxifolin and DHK).^[44] Screening for anti-atopic activity identified MV as a strong inhibitor of AD-

like phenomena in HaCaT cells (Figure 3a and b). MV is contained in many plants, including the HDB and *Zanthoxylum scandens*, and has been reported to have antioxidant and antiplatelet activity.^[45–47] Here, we showed that MV decreased expression of inflammatory genes, including cytokines and chemokines, at both the mRNA and protein levels (Figure 3e and 3f). Curiously, high-performance liquid chromatography revealed that MV is only a minor

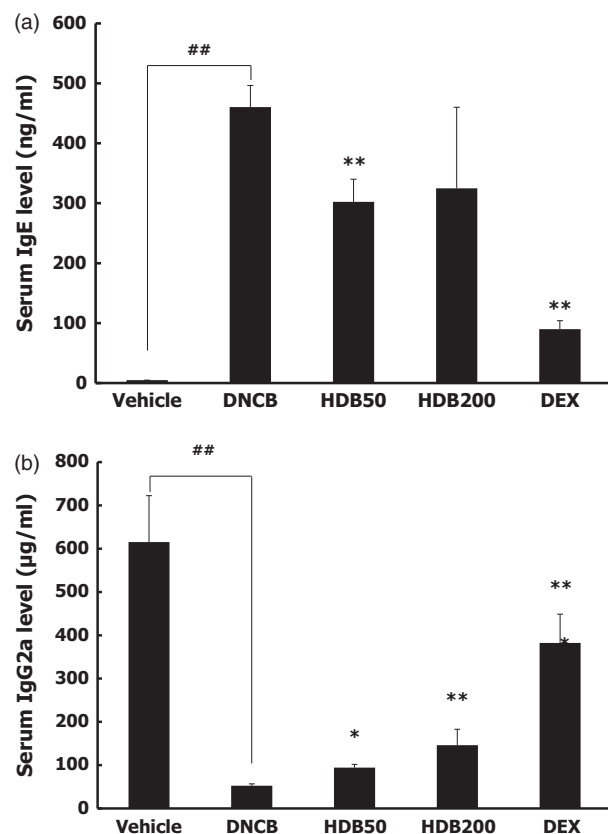


Figure 5 Effects of *Hovenia dulcis* branch (HDB) on serum levels of total immunoglobulin E (IgE) and immunoglobulin G2a (IgG2a) in NC/Nga mice. The concentration of (a) IgE (b) IgG2a in collected serum on day 42 was determined by Enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean $\pm$ SD ($n = 5$) (## $P < 0.01$, ** $P < 0.01$ and * $P < 0.05$).

component of HDB (data not shown). Thus, it may be that MV acts synergistically with another compound(s) in HDB. A detailed analysis of the anti-AD properties of the compounds in HDB is needed.

We used NC/Nga mice as an in-vivo model of AD and examined the effects of HDB on skin lesions. The inbred NC/Nga strain was established in 1957 and is the most well-known animal model of AD.^[48] NC/Nga mice develop AD under conventional conditions and show the same symptoms (erythema, oedema, itchiness/scratching, dry skin and haemorrhage) as human patients with AD. Here, we used DNCB to induce AD. DNCB is an important environmental allergen and generates lesions similar to those in AD sufferers.^[48]

The inhibitory effects of HDB on AD in NC/Nga mice were confirmed by pathological analyses (Figure 4a). HDB reduced AD-like skin symptoms (hyperkeratosis, haemorrhage and erythema) (Figure 4a and b); however, analysis of serum samples did not reveal dose-

dependent effects on IgE levels. Indeed, IgE levels in mice treated with 200 mg/kg per day were higher than those in mice treated with 50 mg/kg per day (Figure 5a). Conversely, serum IgG2a levels in the HDB50 group were lower than those in the HDB200 group (Figure 5b). Although the effects were not dose dependent, the data show that HDB reduced IgE levels and increased IgG2a levels in the serum of DNCB-induced mice. Thus, HDB ameliorates the symptoms of AD by regulating IgE and IgG2a levels.

The production of Th2 cytokines, such as IL-4 and IL-5, is affected to development of AD. GATA-3, which is a transcription factor of Th2 cells, transcriptionally regulates the production of cytokines. Because of GATA-3, IgE is affected by IL-4.^[49] On the other hand, the expression of T-bet, which is a transcription factor of Th1 cells, is correlated with the expression of IFN- γ in Th1 cells.^[50] Also, IL-12 is up-regulated by IFN- γ , which is an important cytokine in the inhibition of IgE.^[51] Furthermore, interactions between chemokines and chemokine receptors cause the recruitment of inflammatory cells. CCL are also essential in the development of AD.^[52] Oral administration of HDB induced a significant decrease in Th1-mediated molecules (IL-12p35, IFN- γ and T-bet) and Th2-mediated molecules (IL-4, IL-5, CCLs, CCRs and GATA-3) in skin lesions (Figure 6a, b and d). It was previously reported that HDB could inhibit inflammatory mediators such as TNF- α , IL-4, COX-2, NFKB1 and two in mast cells and passive cutaneous anaphylaxis.^[21] The report is consistent with ours, which shows that treatment with HDB ameliorated DNCB-induced AD-like skin inflammation in NC/Nga mice. Therefore, these results indicate that HDB has an anti-allergic effect and may regulate Th1- and Th2-associated immune responses by inhibiting the production of Th1/Th2 molecules.

The production of pro- or anti-inflammatory cytokines, especially IL-1 β and IL-10 in epidermal cells, is the important events in initiation of AD.^[53] IL-1 β produced at the AD initiation triggers the secretion of various chemokines that induce the infiltration and recruitment of inflammatory cells in the skin lesion. IL-10 is a key mediator in the prevention of allergic inflammation. IL-10 plays an important role in the modulation of immune reactions in AD-like skin lesions.^[54] Recently, Tregs as pivotal regulators of immune responses have been reported as essential cells in the prevention and treatment of allergic disorders^[55] and any studies have shown that Foxp3, which regulates AD, is highly expressed in Tregs.^[56] In the present study, the mRNA expression of IL-1 β in skin lesion was reduced in the HDB group, but IL-10 and Foxp3 were not increased by HDB (Figure 6c and d).

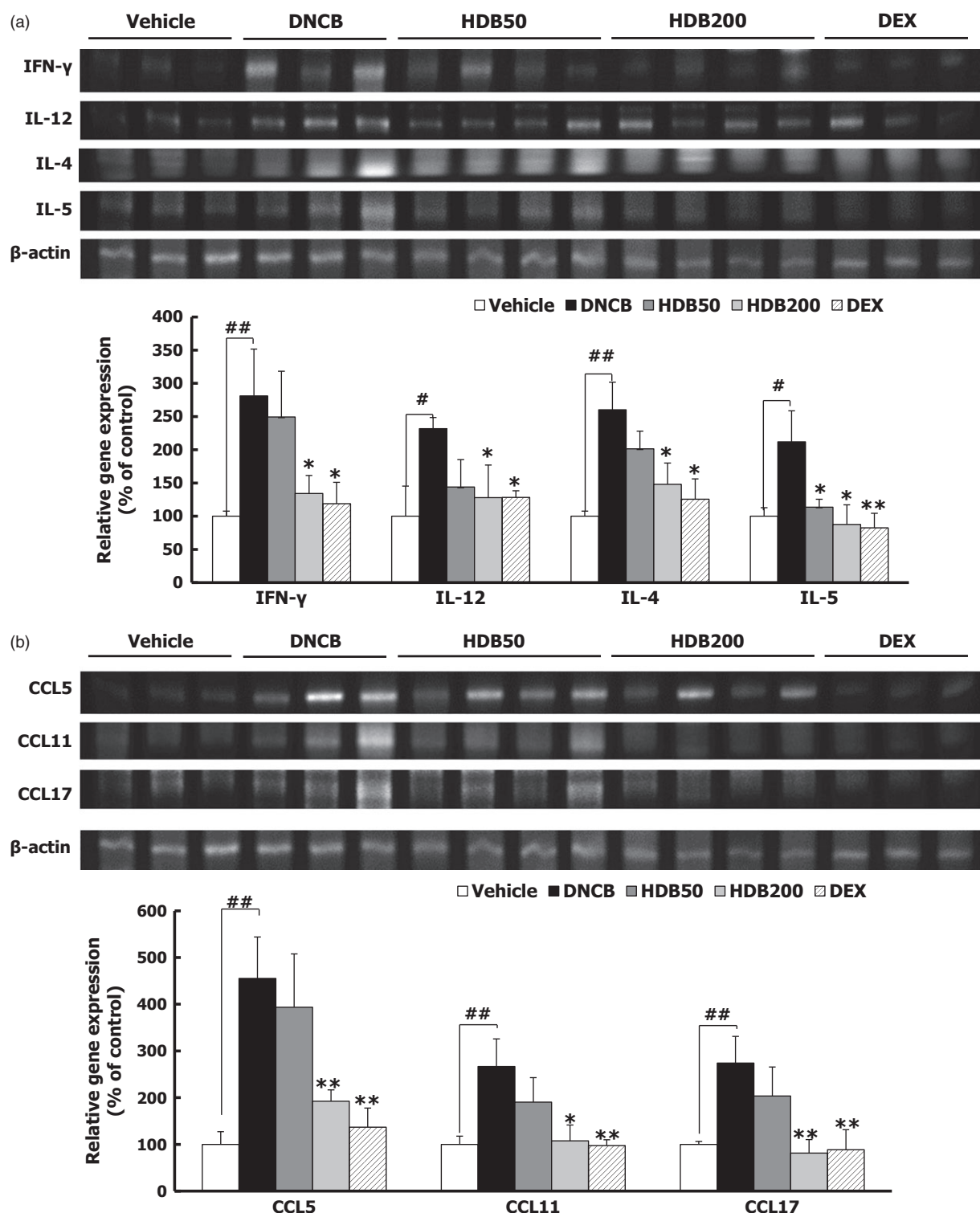


Figure 6 Decrease in Th1/Th2-associated cytokines, chemokines and transcription factors mRNA expression in skin by *Hovenia dulcis* branch (HDB). mRNA expression of (a) Th1 and Th2-related cytokines (IFN- γ , IL-12p35, IL-4 and IL-5) (b) chemokines (CCL5, CCL11 and CCL17) (c) inflammatory cytokines (IL-1 and IL-10) and (d) Th1/Th2/Tregs-related transcription factors (T-bet, GATA-3 and Foxp3) were determined by RT-PCR. Total RNA was isolated from the skin lesion of NC/Nga mice fed with HDB and semi qRT-PCR was performed. β -actin was used as internal control. Results are expressed as mean $\pm$ SD ($n = 5$) (## $P < 0.01$, ** $P < 0.01$ and * $P < 0.05$).

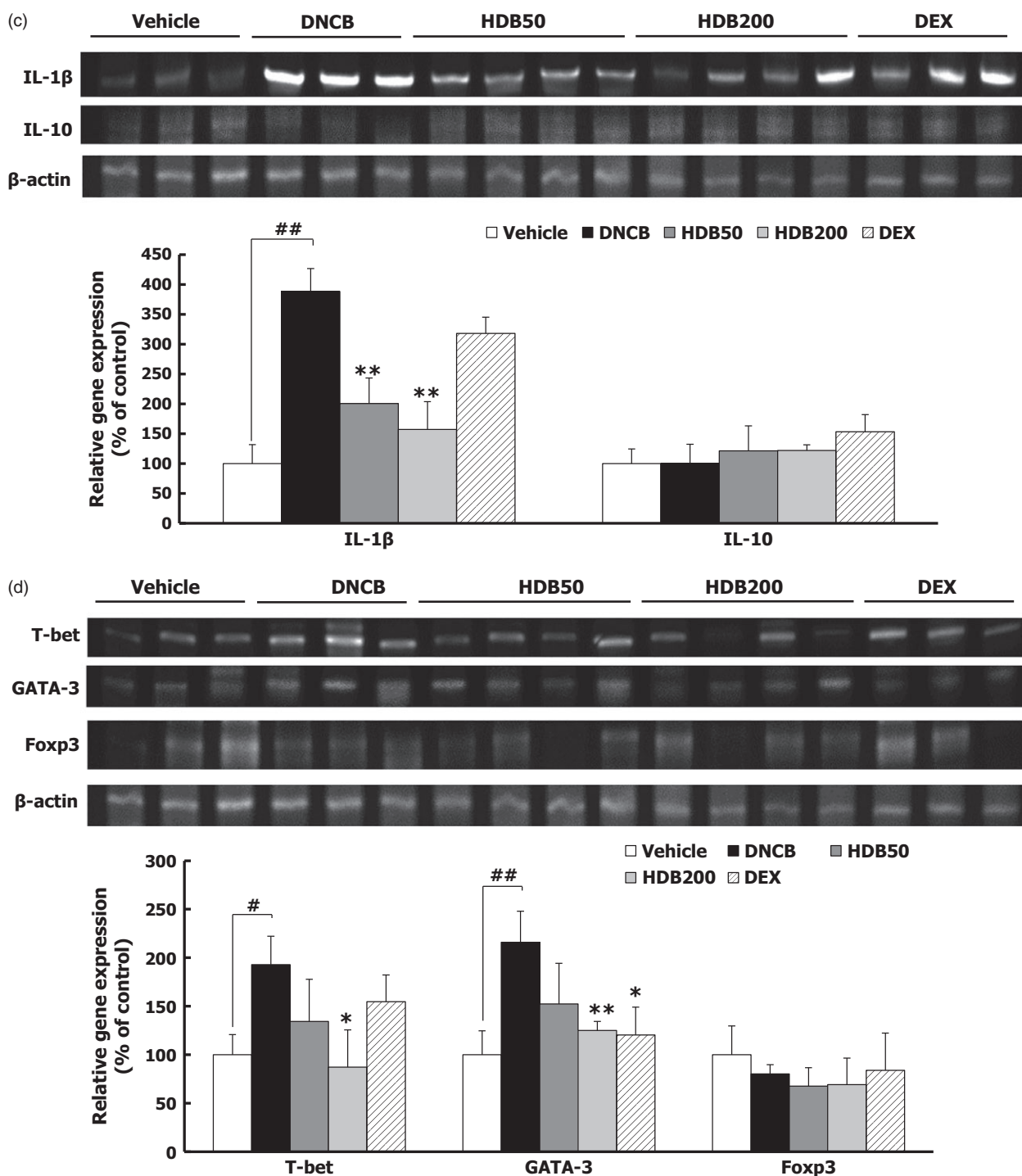


Figure 6 Continued

Conclusion

In summary, the results presented herein suggest that oral administration of HDB regulates Th1 and Th2 responses

and inhibits inflammatory cell infiltration, thereby ameliorating the symptoms of AD. Also, we provide evidence that HDB (or the active compounds contained within it) inhibits TARC and MDC production and MAPK

activation in TNF- α - and IFN- γ -stimulated HaCaT cells. Taken together, these results suggest that HDB may be an effective candidate for the prevention and treatment of AD. We think that further studies are required to investigate the therapeutic potential and the underlying molecular mechanisms of HDB for the AD treatment.

Declarations

Conflict of interest

The Authors declare that there is no conflict of interests.

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Author Contributions

M. Kim and S.J. Lim contributed equally to this work and M. Kim, S.J. Lim and C.H. Nho designed the studies. M. Kim, S.J. Lim, A. Randy and E.J. Nam performed the experiments. M. Kim, S.J. Lim and C.H. Nho analysed the data. M. Kim, S.J. Lim and C.H. Nho wrote the manuscript.

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