Allergy (ISSN: 1398-9995)

Article type: Original Article: Atopic Dermatitis, Urticaria, and Skin Disease

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Title

Piceatannol, a resveratrol analog, attenuates *Dermatophagoides farinae*-induced atopic dermatitis like symptoms in NC/Nga mice

Short title : Piceatannol alleviates AD-like symptoms

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Word count for the abstract : 222

Word count for the text : 2915

Abstract

Background: Piceatannol is a resveratrol metabolite commonly found in red wine, grapes, and passion fruit seeds. Several studies have investigated the immune-modulating effects of piceatannol on processes related to allergic reactions. However, the relationship between piceatannol and atopic dermatitis (AD) has not yet been reported. Therefore, this study sought to investigate the effects of piceatannol in animal and cell line models.

Methods: AD-like symptoms and skin lesions were triggered by repeated topical treatment of *Dermatophagoides farinae* extract (DFE) on the skin of NC/Nga mice. Piceatannol was topically applied five times per week for four weeks. The molecular mechanism of piceatannol was studied in the TNFα/IFNγ-induced HaCaT cell line.

Results: Topical application of piceatannol attenuated DFE-induced AD-like symptoms, as shown by skin thickness, dermatitis score, scratching time, and skin water loss. Histopathological analysis showed that piceatannol suppressed DFE-induced eosinophil and mast cell infiltration into the skin. These results occurred concomitantly with the downregulation of inflammatory markers, including serum TARC, MDC, and IgE. In addition, piceatannol alleviated Th2 cytokines such as IL-4 and IL-13 in the skin tissue. Piceatannol decreased phosphorylation of JAK-STAT protein in the TNFα/IFNγ-induced HaCaT cell line. A molecular docking study showed that piceatannol strongly interacts with JAK1, suggesting a possible piceatannol mode of action.

Conclusions: Piceatannol, a metabolite of resveratrol, has potential therapeutic efficacy in treating AD by targeting JAK1.

Keywords

Atopic dermatitis; JAK1; piceatannol; resveratrol.

List of Abbreviations

AD: atopic dermatitis

DFE: *Dermatophagoides farinae* extract

GYQ : 2-[4-[8-oxidanylidene-2-[(~{E})-(2-oxidanylidenepyridin-3-ylidene)amino]-7~{H}-purin-9-yl]cyclohexyl]ethanenitrile

IL: interleukin

JAK: Janus kinase

MDC: macrophage-derived chemokine

PIC: piceatannol

qRT-PCR: quantitative real-time polymerase chain reaction

RES: resveratrol

SDS: sodium dodecyl sulfate

STAT: signal transducer and activator of transcription

TARC: thymus and activation-regulated chemokine

TEWL: transepidermal water loss

Th2: T helper 2

Introduction

Atopic dermatitis (AD) is a chronic skin disorder characterized by relapsing skin inflammation, disturbance of epidermal barrier function, and IgE-mediated sensitization to allergens.[1] AD has become a significant medical problem worldwide, and its prevalence has increased over the past three decades. AD is related to several other allergic diseases such as asthma, food allergies, and allergic rhinitis.[2, 3] AD is also generally thought to be one of the initial steps in the atopic march, a progression from AD to allergic rhinitis and asthma.[4] AD arises from a complex interaction between the skin as a functional barrier and the immune system.[5] During AD progression, the skin barrier function collapses due to itching and inflammation, resulting in decreased hydration of the stratum corneum and increased transepidermal water loss (TEWL).[6] AD aggravates physical conditions, induces mental stress, and can even lead to suicide.[7] Recently, therapeutic strategies for the treatment of AD have focused on the application of corticosteroids. However, these agents often elicit undesirable side effects, including high blood pressure, nausea, vomiting, kidney problems, headaches, and sensations of tingling and numbness. There are also complications involved when prescribing such treatments to children.[8] Therefore, a considerable unmet need exists for the development of safer and more effective AD treatments.

Primary triggers for AD include allergens such as dust mites, pollen, and pet dander.[9, 10] The pathogenesis of AD involves T-helper cell (Th) type 2 immune responses and the dysregulated production of Th2-derived cytokines such as interleukin 4 (IL-4) and IL-13.[11-13] The influx of Th2 cytokines induces isotype class switching from immunoglobulin M (IgM) to IgE.[14] This leads to Th2 recruitment of eosinophils and mast cells toward inflammatory skin lesions.[15] The expression of CC chemokine receptor (CCR) 4 ligands, such as thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22), leads to the recruitment of Th2 lymphocytes to the inflammatory site, suggesting an important endophenotype of AD.[16-18]

Resveratrol (3,5,4’-trihydroxystilbene) is a natural stilbene found in wine and grapes. It has been suggested as one possible explanation for the “French paradox,” and several research studies have revealed resveratrol’s dramatic effects.[19-21] However, it was reported that the structurally similar component piceatannol (3,5,3’,4’-tetrahydroxystilbene), characterized by the presence of OH groups at the 3’ position in the structure of resveratrol, has been shown to be more efficacious. Piceatannol showed the highest potent scavenging

activity against reactive oxygen species and methyl radicals among the six resveratrol analogs.[22] Furthermore, piceatannol was reported to have the strongest anti-inflammatory activity among the four stilbene derivatives in the macrophage cell line.[23] In addition, piceatannol showed the strongest anti-allergic action, with approximately 90% inhibition among the nine stilbene compounds tested.[24] Therefore, it was hypothesized that piceatannol might be more effective than resveratrol in AD. The AD-like symptoms in NC/Nga mice and the molecular mechanism of the HaCaT cell line were studied to clarify and compare the effects of piceatannol and resveratrol.

Materials and methods

Several experimental details are provided in the Supplementary Information.

Animals

Three-week-old NC/Nga male mice were purchased from SLC Japan. Mice were housed in individually ventilated cages under specific pathogen-free conditions at 22°C with a 12-h light-dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University, Korea (approval number: 150312-4-1).

Induction of AD-like symptoms

After one week of acclimation, the mice were divided into the following five groups (n=8 per group): (1) Non-treated group (Normal), (2) *Dermatophagoides farinae* extract (DFE) + vehicle (ethanol) treated group (negative control), (3) DFE + 500 nmol piceatannol treated group, (4) DFE + 500 nmol resveratrol treated group, and (5) DFE + tacrolimus treated group (positive control). The mice received topical applications of DFE cream twice per week for four weeks from the first week. The NC/Nga mice were anesthetized with 2% isoflurane and dorsally shaved using an electric clipper and shaving cream. To disrupt the skin barrier, 150 mL of 4% sodium dodecyl sulfate was topically applied. After 3 h, 100 mg Biostir cream containing DFE was applied to the shaved dorsal skin twice per week for four weeks to induce AD-like symptoms. Piceatannol and resveratrol were topically applied to the dorsal skin (150 μL each) five times a week for four weeks. Tacrolimus (0.1% Protopic Ointment, 100 mg/mouse/day) was topically applied five times a week for four weeks.

Assessment of dermatitis score and scratching behavior

Skin dermatitis scores were assessed five times in four weeks with a slight modification of the criteria described previously. Scores of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) were assigned to each of the four symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness). The total dermatitis score indication of clinical severity was defined as the sum of all scores (maximum: 12). A video of the scratching behavior was recorded using a digital camera one day before the final experimental day. Scratching behavior was recorded for 20 min, and the total scratching time (s) was analyzed.

TEWL and corneometer units

The TEWL was measured in mouse dorsal skin one day before the experiment's final day under specific conditions of 21–22 °C and 50–60% humidity, using a skin evaporative water recorder (Tewameter TM300). The TEWL was measured after stabilization for approximately 30 s, and eight average data points were counted. Skin hydration was assessed using the Corneometer CM 825. Measurements were conducted five times for each area.

Histological examination

On the last day of the experiment, skin from each mouse was prepared, fixed with 10% neutral-buffered formalin, and embedded in paraffin. Four mm-thick sections were cut and transferred onto slides. Deparaffinized skin sections were stained with hematoxylin and eosin (H&E) and examined at 20× magnification. Deparaffinized skin sections were stained with Congo red (CR) and toluidine blue (TB). The number of eosinophils and mast cells per 1 mm^2 skin was quantified at 100× magnification.

Measurement of serum and skin protein

Blood samples were collected on the final day of the experiment. Serum TARC, MDC, and IgE concentrations were determined using an ELISA kit. Mouse TARC/CCL17, MDC/CCL22, IL-4, and IL-13 protein levels were detected using an ELISA kit.

***In silico* molecular docking**

The piceatannol 3D structures were downloaded from the PubChem database in SDF format and converted to the PDB format using Open Babel version 2.3.2. All ligand structure minimization was performed using UCSF Chimera 1.13.1 before conducting the molecular docking analysis. The protein "Human Janus Kinase 1 (JAK1)" was prepared by retrieving the

three-dimensional crystal structure from the RCSB protein bank (pdb:6HZU). Following receptor and ligand preparation, molecular docking analysis was performed using UCSF Chimera's built-in AutoDock Vina tool to evaluate the hydrogen bond interactions and their binding affinities.

Statistical analysis

Statistical analysis was conducted using the SPSS statistics software. Data are expressed as mean ± standard error (SEM) or mean ± standard deviation (SD). Student's t-test or one-way analysis of variance (ANOVA) followed by Duncan's least significant range (LSR) test was used to determine significance between multiple groups. Differences were considered significant at $p < 0.05$.

Results

Piceatannol has strong preventative, curative effects on DFE-induced atopic dermatitis (AD) in NC/Nga mice.

Piceatannol (3,5,3',4'-tetrahydroxystilbene) is an analog of resveratrol (3,5,4'-trihydroxystilbene) that shares a structural motif with one more hydroxyl group on the B-ring (Fig. 1A). Inflammation and allergic reactions on dorsal skin of NC/Nga mice were induced using DFE cream to test the effect of piceatannol on the AD mouse model. Topical DFE application caused robust atopic dermatitis-like symptoms (Fig. 1B). Piceatannol treatment significantly alleviated the symptoms, such as erythema, scratching marks, and dryness, compared to resveratrol treatment, leading to a decreased dermatitis score (Fig. 1C). DFE induces skin hyperkeratosis and hyperplasia, leading to an increase in the dorsal thickness. The increased dorsal thickness was efficiently alleviated by treatment with piceatannol (Fig. 1D).

Piceatannol attenuates scratching behavior, skin water loss, and increases skin hydration.

Exposure to DFE induces continuous skin barrier disruption and abnormal inflammatory reactions, leading to severe scratching behavior and skin dryness. Treatment with piceatannol decreased the scratching time compared to that in the negative control group (Fig. 2A). Epidermal water loss was also reduced by treatment with piceatannol and increased skin

hydration (Fig. 2B and C). These data showed that piceatannol prevents skin disruption caused by scratching motion and stops the vicious cycle.

Piceatannol suppresses DFE-induced epidermal thickness and eosinophils' infiltration of mast cells into NC/Nga mice skin lesions.

Hematoxylin and eosin staining showed skin hyperkeratosis and hyperplasia, which led to epidermal thickness. Epidermal thickness was increased by DFE treatment, while piceatannol and tacrolimus application significantly decreased skin thickness (Fig. 3A). Topical application of 500 nmol piceatannol dramatically reduced epidermal thickness (46.69%) compared to that of the control group (Fig. 3B). CR staining was used to classify eosinophils in the skin of NC/Nga mice, while TB staining was used to classify mast cells. The number of eosinophils stained by CR per mm^2 in DFE-treated mice skin lesions was significantly decreased by piceatannol (40.49%) and tacrolimus application (Fig. 3C and D). In addition, the number of mast cells stained by TB per mm^2 in skin lesions of DFE-treated mice was significantly decreased in the piceatannol treatment group (59.49%) (Fig. 3E and F).

Piceatannol attenuates DFE-induced increases in TARC, MDC, and IgE levels in NC/Nga mice.

On the last experimental day, blood samples were collected to investigate the effect of piceatannol on DFE induction of TARC, MDC, and IgE in NC/Nga mice. The DFE-treated NC/Nga mice exhibited higher levels of TARC, MDC, and IgE than the non-induced group. Topical application of 500 nmol piceatannol (75.09%, 76.91%, 45.17%) significantly reduced the serum levels of TARC, MDC, and IgE (Fig. 4A, 4B, and 4C).

Piceatannol attenuates DFE-induced increases in mRNA and protein level in NC/Nga mice.

Topical DFE application induces the abnormal expression of chemokines and cytokines, leading to immune cell infiltration and exacerbating inflammatory reactions in the skin. Skin tissues were collected on the last experimental day to investigate the effect of piceatannol on DFE-induced increases in mRNA expression levels. The mRNA expression levels of *Tarc* and *Mdc* were increased in the skin lesions of the DFE-treated group compared to the non-induced group, while application of piceatannol (45.85% and 50.93%) decreased them significantly (Fig. 5A and B). The mRNA expression levels of Th2 cytokines *Il4* and *Il13* were significantly

increased in skin lesions of DFE-treated NC/Nga mice compared to the non-induced group. Topical piceatannol treatment (64.15% and 65.15%) significantly reduced *Il4* and *Il13* mRNA expression levels (Fig. 5C and D). Protein levels were measured to elucidate the effect of piceatannol on the dorsal skin sample. The protein levels of TARC and MDC were increased in skin lesions of the DFE-treated group, while application of piceatannol decreased the values to nearly those of the NC group (Fig. 5E and F). IL-4 and IL-13 protein levels were dramatically increased in skin lesions of DFE-treated NC/Nga mice. Topical treatment with piceatannol significantly reduced the protein levels of IL-4 and IL-13, showing a similar pattern to the TARC and MDC results (Fig. 5G and H).

Piceatannol decreased the TNFα/IFNγ-induced TARC and MDC production levels, and the JAK-STAT protein phosphorylation in the HaCaT cell line.

Chemokines such as TARC and MDC are important mediators produced by co-treatment of TNFα/IFNγ in keratinocytes, as shown by a previous study.[25] After 24-h induction cocktail treatment, cell media was collected to investigate piceatannol's effect on TNFα/IFNγ-induced TARC and MDC production levels. ELISA was used to measure the protein levels. TNFα/IFNγ treatment increased TARC and MDC protein production compared to that of the control group (Fig. 6A and B). However, treatment with 5, 10, and 20 μM piceatannol dramatically decreased TARC levels in a dose-dependent manner (13.18%, 61.01%, 104.89% inhibition, EC_{50} : 9.66 μM) (Fig. 6A). In addition, treatment with 5, 10, and 20 μM piceatannol dramatically decreased MDC levels (58.56%, 87.55%, 90.50% inhibition, EC_{50} : 4.30 μM) (Fig. 6B).

To examine the effects of piceatannol and resveratrol on the JAK-STAT signaling pathway, HaCaT cells were pre-treated for 1 h with piceatannol, resveratrol, or dexamethasone as a positive control. After activation with TNFα/IFNγ cocktail for 1 h, the protein levels of phospho-JAK1, STAT1, STAT3, and total JAK1, STAT-1, and STAT3 were determined. Western blot data showed that 20 μM piceatannol specifically inhibited JAK1, STAT-1, and STAT3 activation by TNFα/IFNγ (Fig. 6C and D). These western blot data showed the possibility of binding between piceatannol and JAK1. To elucidate the possible binding mode, Autodoc vina was used for *in silico* molecular docking. As a result, piceatannol strongly binds to the active site (1nr4) with -8.0 kcal/mol binding energy. It forms three hydrogen bonds with Glu883 (2.91 Å), Glu957 (2.74 Å), Leu959 (3.17 Å, 3.26 Å) and ten hydrophobic interactions

(Asp1021, Gly884, Asn1008, Val889, Ala906, Phe958, Leu1010, Gly962, Leu881, and Gly882), indicating that piceatannol strongly binds to the active site of JAK1 (Fig. 6E)

Discussion

DFE is a common allergen in house dust and has been reported to be a significant inducer of AD. DFE consists of mite bodies and heads, which together trigger strong innate immunity, showing strong haptens that induced robust allergic contact dermatitis in a mouse model. Several studies have reported that repeated application of DFE induced AD-like symptoms in NC/Nga mice that highly resemble the natural process of sensitization in patients with AD. In this model, the animals showed scratching behavior, dryness, elevated blood IgE levels, and significant AD symptoms.[26, 27] Therefore, this animal model was utilized in this study. Keratinocytes account for 90% of the epidermis and outer layer of the skin. They protect against invasion of microbes, fungi, and allergens. TNFα/IFNγ treatment of human keratinocytes (HaCaT) induces a robust immune response resembling the AD skin condition phenomenon. Keratinocytes are the first cells to absorb not only DFE but also the other two chemicals studied. Therefore, this cell model was deemed suitable for use in mechanistic studies.

Recently, our co-operating group developed a low-cost, fast, and extensive production system using an artificial *E. coli* bio-engineering system.[28] Because of this bio-converting enzyme technology, piceatannol might be applicable in the bio-industry to prevent and treat multiple diseases. Previously, our group reported numerous piceatannol studies on the prevention of neuronal cell death and apoptosis, anti-atherosclerotic, anti-adipogenesis, and anti-cancer effects.[29-34] Piceatannol inhibited platelet-derived growth factor (PDGF)-induced migration and proliferation of aortic muscle cells targeting phosphoinositide 3-kinase (PI3K), while resveratrol showed no effects. In addition, piceatannol attenuated 4-hydroxynonenal (HNE)-induced PC12 cell death in a dose-dependent manner (but not resveratrol). In addition, compared to the same concentration of resveratrol (20 μM), piceatannol showed a higher anti-adipogenic effect on human visceral adipose tissue. These studies suggest that the additional 3'-hydroxyl group on resveratrol is critical for biological activity in multiple disease models.

There are pharmacological differences between oral and topical applications in preventing or treating AD using naturally derived phytochemicals. Compared to oral administration, topical treatment requires a relatively low dose with less side effects, and active compounds can

directly interact within the skin disease area.[35] If the chemical is effective in inhibiting allergens, a preventative effect can also be expected. For these reasons, AD could be treated efficiently with almost no common adverse effects, and recently many researchers are finding new active chemical or topical methods to treat AD.

Janus kinase 1 (JAK) is a key regulator of the cytokine signaling pathway. The JAK1/3 pathway is critical for Th2 differentiation in allergic lung response, implicating Jak1 as a potential target of allergic disease.[36] Also, the major Th2 cytokines in AD such as IL4 and IL-13, are controlled by the JAK-STAT signaling pathway. A previous study revealed that JAK1 signaling is critical for regulating itch mechanisms in an AD mouse model.[37] For these reasons, recent studies have focused on JAK1, which become a potent target in the field of dermatology and AD. Upadacitibin (AbbVie) and Abrocitinib (Pfizer), JAK1 inhibitors, are in several ongoing phase 3 clinical studies for AD treatment.[38, 39] Piceatannol is a well-known novel JAK1 inhibitor and has been reported to affect multiple cancers.[40-43] To investigate the direct interaction between piceatannol and JAK1 on a molecular basis, *in silico* molecular docking was performed. GYQ (was used as the ligand of JAK1[44]), which showed a binding energy of -8.9 kcal/mol with one hydrogen bond and 15 hydrophobic interactions. (Supplementary Fig. 1) Interestingly, piceatannol formed ten hydrophobic interactions with JAK1, and nine shared the same interaction with GYQ. Although piceatannol has five fewer hydrophobic interactions than GYQ, the hydrogen interaction may have increased the binding energy to JAK1 (-8.0 kcal/mol). Upon ligand binding to the active site, JAK1 undergoes phosphorylation on tyrosine 1034 and 1035 residues (also known as Y1022/Y1023). As the data showed, piceatannol effectively prevented JAK1 phosphorylation in the HaCaT cell line (Fig. 6C and D), and we assumed that piceatannol competitively blocked ligand binding, resulting in inhibition of auto-phosphorylation of JAK1.

In this study, for the first time, it was found that piceatannol has anti-atopic effects in animal and cell models, which are superior to those of the well-known natural stilbene resveratrol. At a concentration of 500 nm, piceatannol was found to superior to resveratrol in an animal model. To confirm the EC50 value and possible side effects at higher doses, various animal studies are required. Further research is needed to elucidate the possible interactions between piceatannol and DFE. When considering piceatannol for human use, curative and preventative effects could have a significant impact. In the early stage of AD, patients are recommended to use a moisturizer and a low dose of topical immunosuppressors. With strong anti-inflammatory and hydration effects, using piceatannol in topical ointments or cosmetic

components is possible. Taken together, piceatannol is a safe and effective phytochemical for AD.

Acknowledgments

This research was supported by a Seoul Business Agency (SBA) grant funded by the Seoul city (PA140024) and supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the High Value-added Food Technology Development. Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (116030-3). This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2019R1C1C1004387) and Korea National University of Transportation in 2021

Conflicts of interests

The authors declare no conflict of interest.

Author contribution

C.H. Lee and J. Kim designed experiments, analyzed the data, and wrote the paper; C.H. Lee performed experiments; H. Yang, J.H.Y Park, and K.W. Lee reviewed and edited paper. J. Kim and K.W. Lee revised the manuscript and supervised all the processes.

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Figure 1

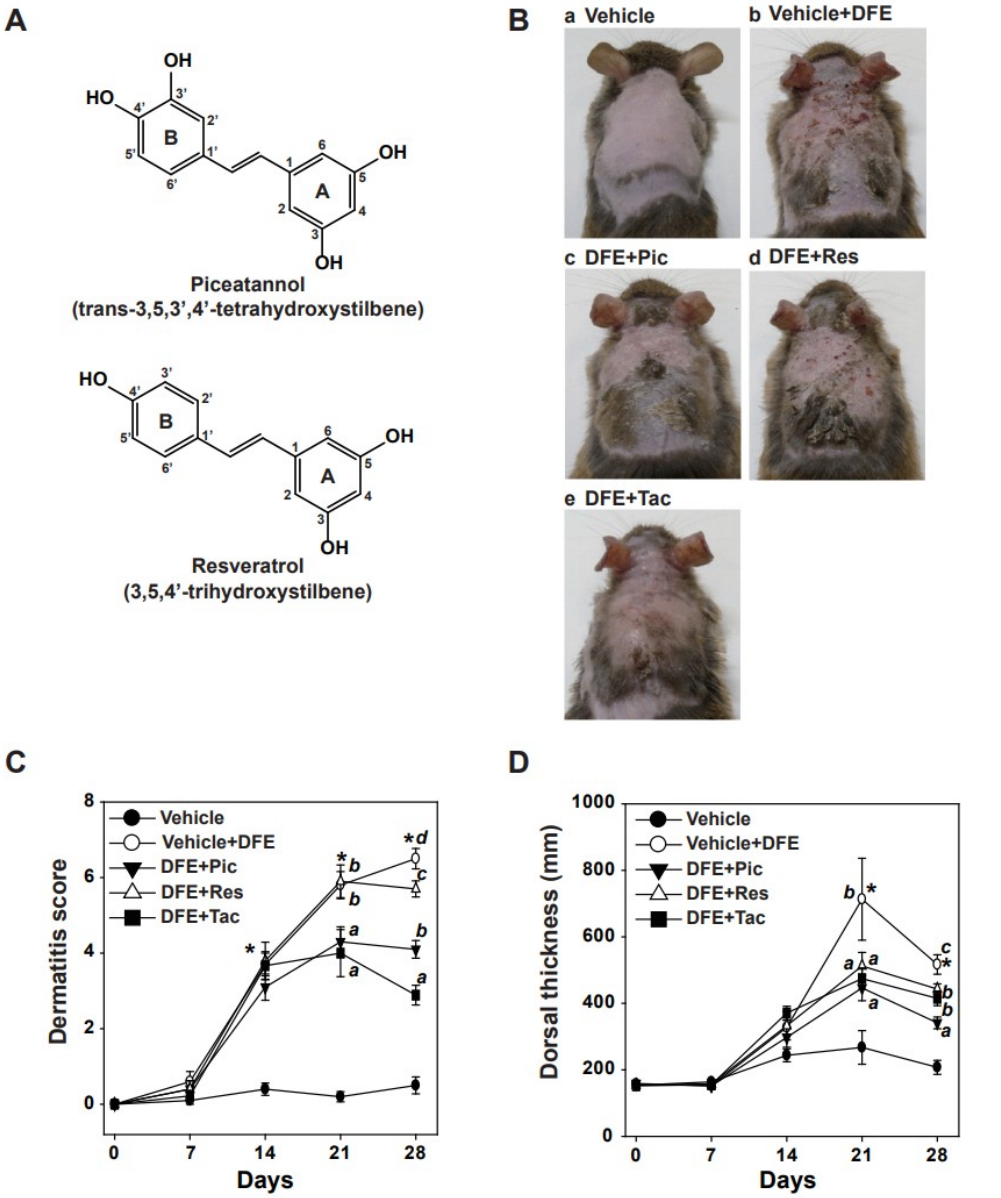

Figure 1.

Effects on DFE-induced AD symptoms in NC/Nga mice. (A) Chemical structure of piceatannol and resveratrol. (B) Picture of a skin lesion induced by DFE on an NC/Nga mice. (C) Dermatitis score during the experiment. (D) Dorsal thickness from day 0 to 28. Data represent the mean values ± SEM (n = 8) * $p < 0.05$ compared with vehicle group.

Figure 2

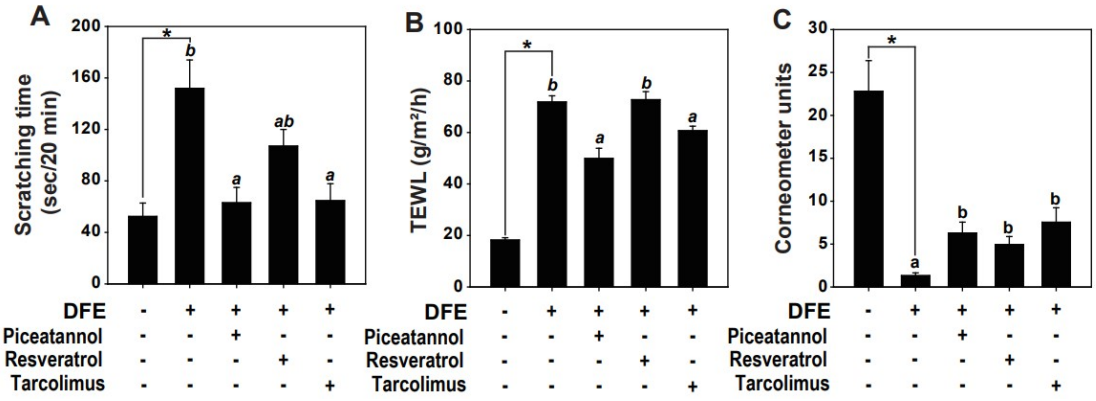

Figure 2.

Scratching time, skin water loss, and skin hydration level in NC/Nga mice. (A) Scratching time was measured by recorded video. (B) TEWL represents water loss through the epidermis. (C) The corneometer unit represents skin hydration. Data represent the mean values ± SEM (n = 7–8) * $p < 0.05$ compared with vehicle group.

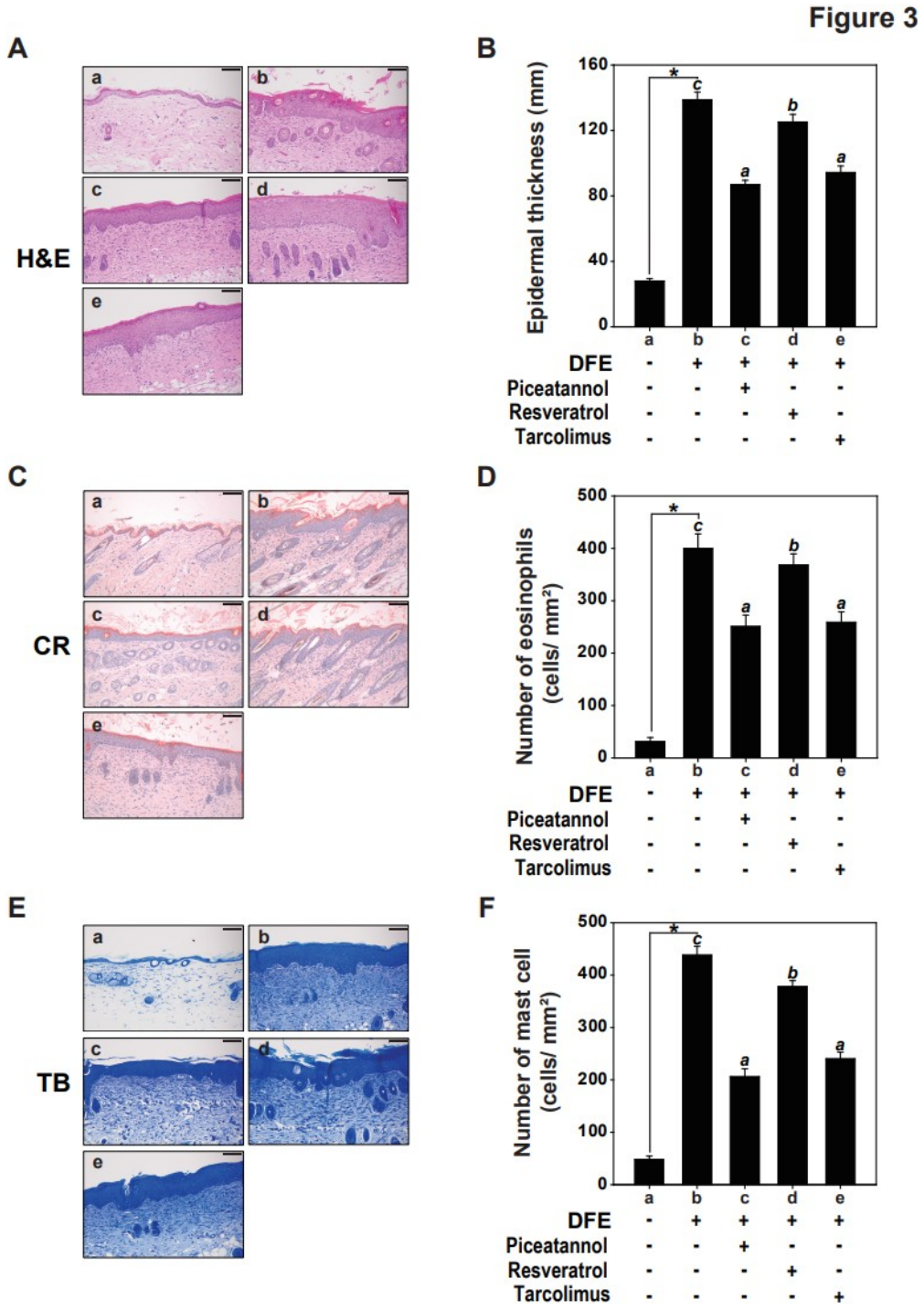

Figure 3.

Epidermal thickness and infiltration of eosinophils, and mast cells into skin lesions in NC/Nga mice. (A) Images of skin stained with hematoxylin and eosin (H&E). (B) Result of epidermal thickness measurement. (C) Image of skin stained with Congo red (CR). (D) The numbers of eosinophils of a skin lesion in 1 mm^2 sections. (E) Image of skin stained with toluidine blue (TB). (F) The numbers of mast cells of skin lesions in 1 mm^2 sections. Skin lesions were evaluated under a microscope at 100× magnification. Scale bar: 100 μm. (a) Untreated control

group; (b) DFE-treated group; (c) DFE plus 500 nmol of piceatannol; (d) DFE plus 500 nmol of resveratrol; (e) DFE plus 0.1% tacrolimus. Data represent the mean values ± SEM (n = 8) * $p < 0.05$ compared with vehicle group.

Figure 4

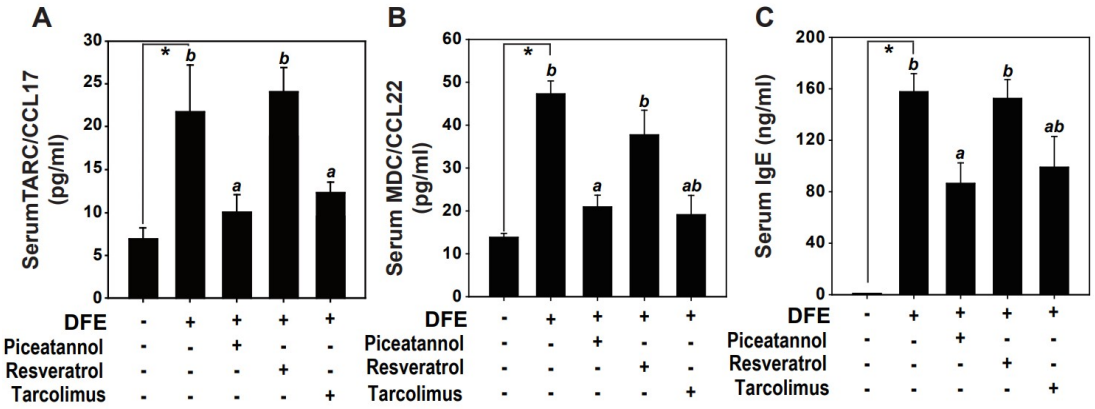

Figure 4.

TARC, MDC, and IgE levels in serum. (A) Serum TARC level. (B) Serum MDC level. (C) Serum IgE. ELISA was used to measure serum TARC, MDC, and IgE levels. Data represent the mean values ± SEM (n = 6–8) * $p < 0.05$ compared with vehicle group.

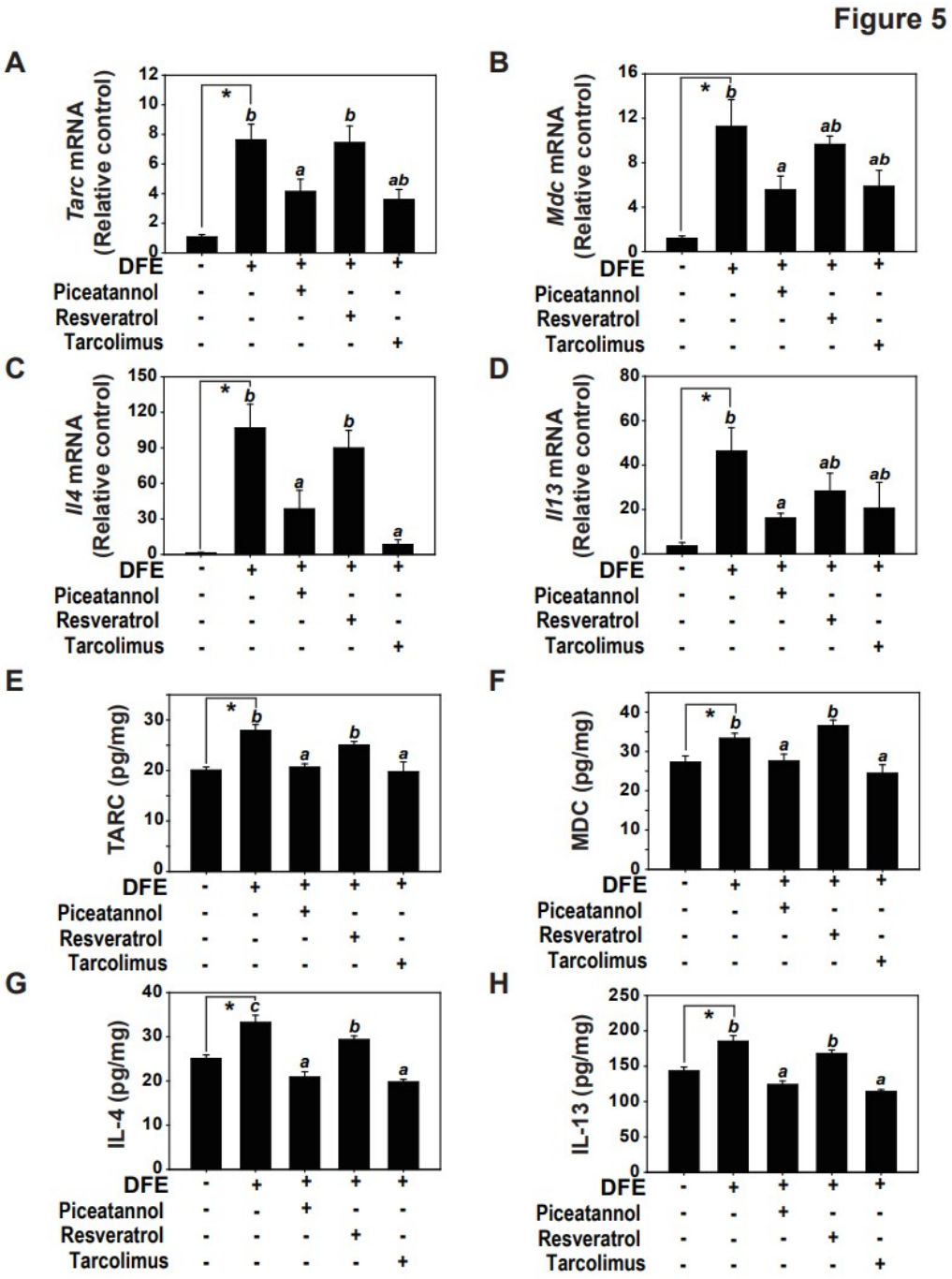

Figure 5.

Chemokine, cytokine mRNA, and protein levels on NC/Nga mice skin. (A~D) Skin *Tarc*, *Mdc*, *Il4*, and *Il13* mRNA levels of NC/Nga mice. *Tarc*, *Mdc*, *Il4*, and *Il13* mRNA levels were measured by real-time quantitative RT-PCR. (E~H) Skin TARC, MDC, IL-4, and IL-13 protein levels of NC/Nga mice. The TARC, MDC, IL-4, and IL-13 levels were measured by

ELISA and normalized by skin total protein concentration. Data represent the mean values ± SEM (n = 6–8) * $p < 0.05$ compared with vehicle group.

Figure 6

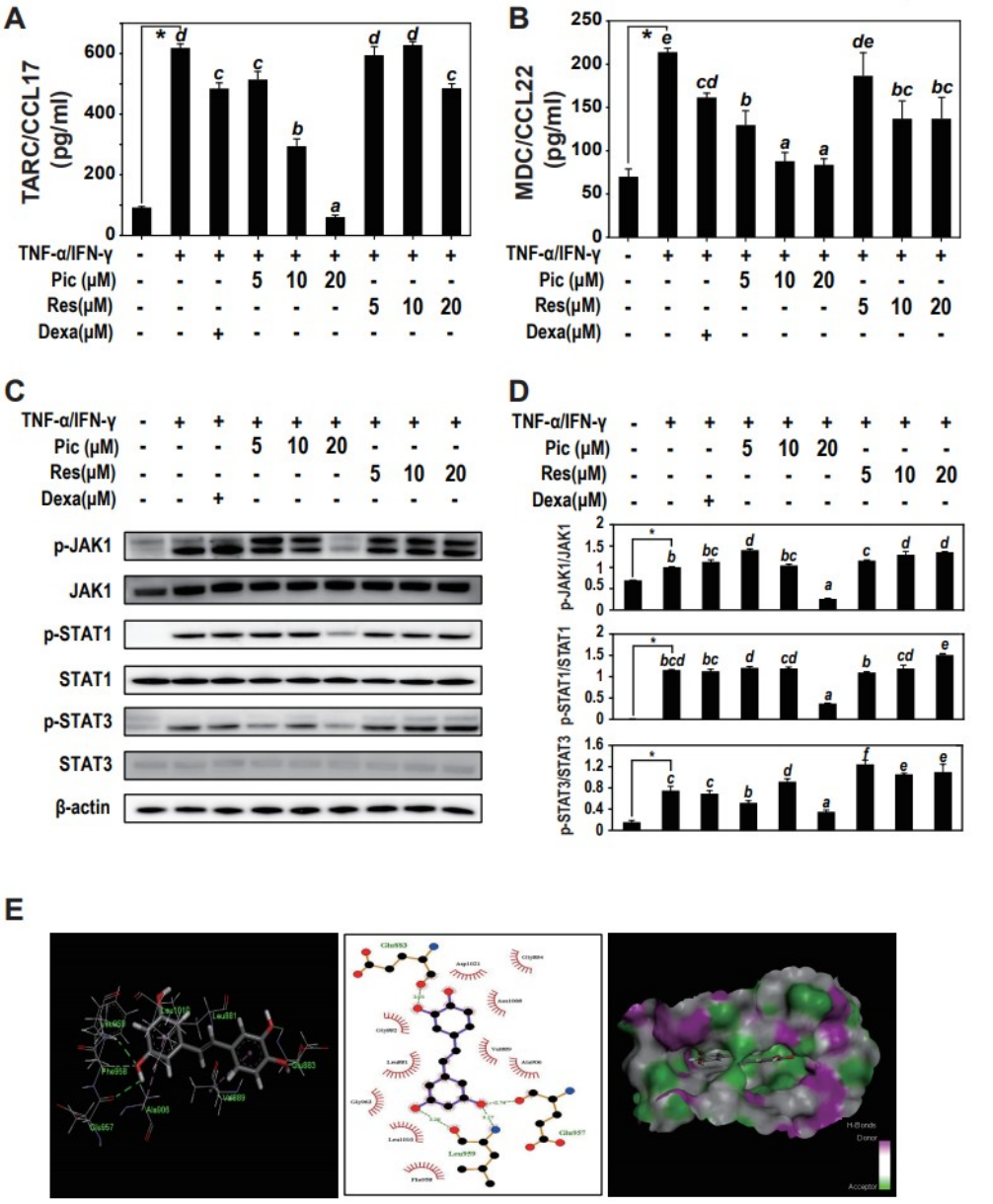

Figure 6.

Chemokine production, JAK-STAT pathway on HaCaT cell line, and JAK1 molecular docking of piceatannol. (A) TARC production level. (B) MDC production level. TARC and MDC levels were measured by ELISA. (C) JAK-STAT western blot data (D) Quantification graph normalized with a specific total protein. (E) Molecular docking image of piceatannol

on JAK1. Data represent the mean values ± SD (n = 3) * $p < 0.05$ compared with non-treated group.