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Anti-inflammatory effect of *Rhododendron brachycarpum* D. Don ex G. Don leaves extract on dermatitis

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ABSTRACT

Introduction: *Rhododendron brachycarpum* D. Don ex G. Don (RbGD) leaves have traditionally been used to treat diabetes, rheumatoid arthritis, hypertension, and skin ailments. Although RbGD leaves are used as a medicinal plant, there is no scientific evidence to support skin treatment. Therefore, the purpose of this study was to look into the anti-inflammatory effect of an ethanolic extract of RbGD on skin disease, specifically atopic dermatitis (AD).

Methods: The anti-inflammatory effect of RbGD ethanol leaf extract (RbGDE) on tumor necrosis factor-α (TNF-α)/interferon-γ (IFN-γ)-activated keratinocytes was evaluated using MTT, qPCR, ELISA, and Western blot Procedures. The therapeutic effects of RbGDE were evaluated *in vivo* inflammatory responses by histological observation, quantitative polymerase chain reaction (qPCR), and ELISA using the 1-chloro-2,4-dinitrobenzene (DNCB)/*Dermatophagoides farina* extract (DfE)-induced AD-like skin mouse model.

Results: RbGDE showed the protective effect against irritating and stimulating substances (H_2O_2 and TNF-α/IFN-γ) and inhibited TNF-α/IFN-γ-activated keratinocytes by inhibiting the p38 mitogen-activated protein kinase and nuclear factor-kappa B activation. Furthermore, topical RbGDE treatment reduced the AD features such as thickened skin, erythema, immune cells infiltration (eosinophils and mast cells), and AD-related cytokines (IL-12a, IL-1β, IL-4, and TSLP) in the ear tissues of DNCB/DfE-induced mice. The RbGDE also reduced histamine and immunoglobulins (Igs) levels in the serum, including DfE-specific IgE, total IgE, and IgG2a.

Conclusion: RbGD leaf extract had an anti-inflammatory effect on dermatitis by reducing inflammatory mediators, indicating that it might be used to treat skin disease.

Implication for health policy/practice/research/medical education:
This study provides scientific evidence that *Rhododendron brachycarpum* D. Don ex G. Don leaves extract has anti-inflammatory properties supported by the keratinocytes and an atopic dermatitis model. This extract has the potential to be a viable herbal formulation candidate for skin treatment and management.

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Introduction

Dermatitis is an inflammation of the skin that includes a variety of ailments. Atopic dermatitis (AD) is one of the most common skin diseases, with a significant impact on quality of life and a high global burden in terms of healthcare costs and morbidity. Therefore, the basic management of AD aims to alleviate symptoms and improve the skin's long-term condition by reducing inflammation (1). AD is a chronic skin disease that causes relapses of allergic symptoms. The skin lesions of patients with AD exhibit immunological characteristics, indicating that Th2 signaling plays a role in acute disease, followed by conversion to Th1 signaling in chronic disease (2). Concurrently, abnormal keratinocyte differentiation promotes skin inflammation, which can result in an immune response triggered by harmful factors and the weakening of the epidermal barrier, a phenomenon common in chronic skin diseases (3). Therefore, these

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activated keratinocytes in AD lesions cause inflammatory reactions by producing cytokines and chemokines and exacerbating the underlying immune response (4). Consequently, symptoms of AD skin include irritability, decreased water retention, erythema, dryness, edema, cracking, and itching (5,6). Thus, treatments for AD should control keratinocytes within AD lesions and actively prevent skin moisture loss (7-9). Finally, AD skin is treated with topical or systemic corticosteroids, antihistamines, or calcineurin inhibitors, with topical corticosteroids serving as first-line therapy (10). Long-term use of these drugs, however, results in a variety of side effects (11-13). Hence, AD patients need effective anti-inflammation or anti-itch drugs with few side effects for self-care habits.

Rhododendron brachycarpum D. Don ex G. Don (RbGD) is a plant belonging to the *Ericaceae* family and is a traditional natural medicine called "Manbyeonghco" in Korea (14). *Rhododendron* species have been used as traditional medicine for inflammation, pain, and skin ailments. Over 65 flavonoids have been identified in *Rhododendrons* leaves (15). According to the phytochemical studies, the leaves of this plant contain a high amount of flavonoid and polyphenolic compounds. These compounds were quantified using a preparative high-performance liquid chromatography (HPLC), which revealed that the RbGD leaf extract contained quercetin, hyperin, ursolic acid, and corosolic acid (16,17). Several studies have shown that RbGD leaves have antimicrobial, antioxidant, anti-inflammatory, and anti-diabetic properties. Furthermore, RbGD leaves have been used in traditional medicine to treat diabetes, hypertension, rheumatoid arthritis, and skin diseases (15,18,19). It is known that RbGD extracts contain compounds that reduce skin irritation and increase skin moisture levels (20,21). As such, the leaves of RbGD have skin beneficial effects; however, the use of RbGD for skin ailments has not yet been scientifically confirmed since it is already used folklorically (15). To the best of our knowledge, no studies have been conducted to investigate RbGD's inhibitory action on the inflammatory response associated with AD. Herein, this study aims to provide scientific research data on the anti-inflammatory and therapeutic effects of total ethanol extract of RbGD leaves (RbGDE) on skin inflammation using *in vivo* and *in vitro* experiments.

Materials and Methods

Reagents

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Dexamethasone (Dexa) was used as positive drug control, and dexamethasone-water soluble powder was dissolved in a phosphate-buffered saline (PBS) at pH 7.4 for *in vitro* and *in vivo* experiments. *Dermatophagoides farina* extract (DfE; 1 mg/mL; Prolagen, Seoul, South Korea) powder was a mixed solution with PBS at pH 7.4 plus 0.5% Tween 80. 1-chloro-2,4-dinitrobenzene (DNCB) was dissolved in an acetone/olive oil solution (3:1, v/v) and used at concentrations of 1% or 0.5%. The recombinant human proteins (R&D Systems, Minneapolis, MN), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were dissolved in PBS at pH 7.4 containing 0.1% bovine serum albumin (BSA).

Plant materials and extraction

The leaves of *R. brachycarpum* D. Don ex G. Don (RbGD) were collected from Manbyeonghco cultivated Farm (Gongju, South Korea) in August 2020. The medicinal plant was taxonomically identified and a voucher specimen was deposited with the number KNKB201211080327 at the Korea National Arboretum. Prof. Gil-Saeng Jeong (College of Pharmacy, Chungnam National University, South Korea) received the fresh material right away. The leaves were rinsed with distilled water and thoroughly dried before being ground. RbGD leaf powder (175.4 g) was soaked in 1000 mL of 70% ethanol for 24 hours at room temperature (25 ± 1°C) with stirring. The refluxed extract was filtered, and the filtrate was concentrated using a rotary vacuum concentrator (Heidolph, Schwabach, Germany) to yield 18.2 g of total RbGD leaf extract. The report described the total flavonoid content of the RbGDE (16,17,22,23). The powdery crude RbGD leaf ethanol extract (RbGDE) was qualitatively observed for the flavonoid and polyphenol contents such as quercetin-o-rhamnoside and hyperin by HPLC-MS (Waters, XEVO-TOSmicro) analysis, a method with a well-established phytochemical profile. RbGDE was kept at −70°C until it was used. RbGDE was weighed and dissolved in PBS for use in *in vitro* and *in vivo* experiments.

Cell culture and activation

HaCaT cells (American Type Culture Collection, Manassas, VA), immortalized normal human keratinocytes, were incubated in complete media in 5% carbon dioxide at 37°C. Gibco Dulbecco's modified Eagle medium (Grand Island, NY) containing 10% (v/v) fetal bovine serum (Gibco) and 1× antibiotic-antimycotic solution (Gibco) was served as the completed media. Every two days, the media was changed. For the following experiments, the cells were cultured to 70%–80% confluency. The activated keratinocytes release proinflammatory cytokines and chemokines, which contribute to the pathogenesis of AD (24). Therefore, keratinocytes were treated with TNF-α/IFN-γ at a concentration of 10 ng/mL each to mimic the activated keratinocytes *in vitro*.

Cell viability

The cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Keratinocytes (1×10^4 cells per well)

were seeded into 96-well plates to assess the cytotoxicity or protective effects of RbGDE. For the cytotoxicity assay, keratinocytes were treated with various concentrations of RbGDE (0.1–100 μg/mL) for 24 hours. Keratinocytes were stimulated with TNF-α/IFN-γ for 24 hours after being treated with RbGDE (0.1–100 μg/mL) for 1 hour to perform a protective assay against TNF-α/IFN-γ. Furthermore, keratinocytes were pretreated with RbGDE (0.1–100 μg/mL) for 1 hour before undergoing oxidative damage (400 μM) for 24 hours to investigate the protective effects of hydrogen peroxide (H_2O_2)-induced cell death. After 24 hours of incubation, a solution of MTT (1 mg/mL) was added to each well of the plate, which was then incubated. The formed formazan crystals were checked after 3 hours and dissolved in dimethyl sulfoxide. The absorbance measurement was then performed on a 96-well plate using a VersaMax™ Microplate Reader (Biocompare, Billerica, MA) at 570 nm. Cell viability analysis was expressed as a percentage of cell viability calculated compared to the untreated control.

Quantitative polymerase chain reaction (qPCR)

A StepOnePlus™ Real-Time PCR system (Life Technologies Corporation, Kallang Avenue, Singapore) was used for the qPCR. Table 1 displays the primer sequences. The relative quantification of mRNA was also analyzed using StepOnePlus™ Real-Time PCR software, version 2.3. *In vitro*, keratinocytes (2×10^5 cells/24-well plate) were pretreated with different concentrations of RbGDE (1, 10, or 100 μg/mL) or Dexa (50 μg/mL) for 1 hour. The cells were then stimulated with TNF-α/IFN-γ for 6 hours. *In vivo*, the ears of the mice were homogenized using TissueLyser II (Qiagen, Hilden, Germany). Following that, the RNA was extracted from samples collected *in vitro* and *in vivo* using the RNAiso Plus kit (Takara Bio, Shiga, Japan). The RNA was quantified with a Thermo Fisher NanoDrop 2000 (Wilmington, MA) before being synthesized as complementary DNA (cDNA) with a Thermo Fisher RevertAid RT Reverse Transcription Kit. Each PCR reaction tube held 20 μL of mixture sample, which included 20 μL of mixture sample comprising 200 ng/μL of cDNA, 0.4 μM/μL of primer solution (Forward/Reverse), 2× QGBlue PCR Master Mix (Cellsafe, Yongin, South Korea), and 8 μL of nuclease-free water. For both *in vivo* and *in vitro* samples, quantitative gene expression data were analyzed using the $2^{-\Delta\Delta Cq}$ method and normalized relative to GAPDH.

Detection of released cytokine and chemokine in keratinocytes

In order to assess the effect of RbGDE on inflammatory cytokine and chemokine in activated keratinocytes, 24-well plates were seeded with keratinocytes (2×10^5 cells) and then treated with different concentrations of RbGDE (1, 10, or 100 μg/mL) or Dexa (50 μg/mL) for 1 hour, followed by the addition of TNF-α/IFN-γ. The collected solution was centrifuged for 5 minutes at 2500 g and 4°C after 15 hours, and the clear supernatant was used. The concentrations of the cytokine IL-6 and the chemokine CCL17 were determined immediately after obtaining the supernatant using a specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). The data for calculation and analysis were acquired with a VersaMax™ Microplate Reader using the SoftMax® Pro software version 6.

Protein extraction

Keratinocytes (1×10^6 cells) were seeded in a 6-well plate, and the cells were pretreated with RbGDE (100 μg/mL) or Dexa (50 μg/mL) for 1 hour. The cells were washed twice with ice-cold PBS after being stimulated with TNF-α/IFN-γ for 15 minutes. All buffers contained a protease/phosphatase inhibitor cocktail (Roche, Mannheim, Germany). To extract the total protein, the cells were

Table 1. Primer pair sequences used in quantitative polymerase chain reaction

Primer	Forward (5'→3')	Reverse (5'→3')	Accession numbers
		Human	
CCL17	GTT CGG ACC CCA ACA ACA AG	TGG CTC CAG TTC AGA CAA GG	NM_002987.3
CCL22	AGG ACA GAG CAT GGA TCG CCT ACA	TAA TGG CAG GGA GGT AGG GCT CCT	NM_002990.5
IL-1β	GCT GAT GGC CCT AAA CAG ATG AA	TGA AGC CCT TGC TGT AGT GGT G	NM_000576.3
IL-6	AAA GAG GCA CTG GCA GAA AA	ATC TGA GGT GCC CAT GCT AC	NM_000600.5
GAPDH	CGA CCA CTT TGT CAA GCT CA	AGG GGA GAT TCA GTG TGG TG	NM_001357943.2
		Mouse	
IL-1β	ATA ACC TGC TGG TGT GTG AC	AGG TGC TGA TGT ACC AGT TG	NM_008361.4
IL-4	TCG GCA TTT TGA ACG AGG TC	GAA AAG CCC GAA AGA GTC TC	NM_021283.2
IL-12a	GAT GAC ATG GTG AAG ACG GC	AGG CAC AGG GTC ATC ATC AA	NM_001159424.2
TSLP	CTC CCC TGC ACA CAC CTT AC	TCA GAC CAC CTC ATC ATG GC	NM_021367.2
GAPDH	TGC TCC TCC CTG TTC CAG A	TAC GGC CAA ATC CGT TCA CA	NM_008084.3

IL: Interleukin; CCL: Chemokine (C-C motif) ligand; IFN: Interferon; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TSLP: Thymic stromal lymphopoietin.

scraped and disrupted by sonication for 30 seconds in a lysis buffer (0.5 M Tris [pH 7.5]; 5 M NaCl; 0.5 M EDTA; 10% glycerol; 1% Triton X-100; 0.1 M DTT; and 1 mM Na_3VO_4). The cells were then centrifuged for 20 minutes at 16,000 g and 4°C to obtain the total protein. The cells were scrapped using ice-cold lysis buffer with a pH 7.5 (10 mM HEPES; 2 mM $MgCl_2$; 0.1 mM EDTA; 10 mM KCl; 1 mM DTT; and 0.5 mM PMSF). After then, the samples were centrifuged for 5 minutes at 2500 g and 4°C. Cytoplasmic proteins were found in the supernatant, while cell nuclear proteins were found in the pellet. Thus, we used the clear supernatant as cytoplasmic proteins. Following that, the pellets were washed twice in ice-cold PBS, and then resuspended in ice-cold RIPA buffer (Biosesang, Seongnam, South Korea) for 20 minutes with a vortex on ice. This mixture was then centrifuged for 20 minutes at 16 000 g and 4°C, and the supernatant was collected to obtain nuclear protein.

Western blotting

The Bradford method (Bio-Rad, Hercules, CA) was used to quantify each protein by measuring its absorbance at 590 nm. Samples were run in 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred using a Pall BioTrace™ NT nitrocellulose transfer membrane (Ann Arbor, MI). A blocking buffer was made with 3% BSA in Tris-buffered saline containing 0.05% Tween 20 to reduce the background on the blots. To recognize a specific protein, membranes were incubated with appropriate antibodies: phospho(p)-p38 MAPK$^{Thr180/Tyr182}$ (CST, #9211, 40 kDa, 1:2000), p38 MAPK (CST, #9212, 40 kDa, 1:2000), p-pSTAT1^{Tyr701} (CST, #9167, 84/91 kDa, 1:2000), STAT1 (CST, #9172, 84/91 kDa, 1:2000) in total proteins; IκBα (Santa Cruz, SC1643, 35–41 kDa, 1:1000), β-actin (Invitrogen, MA5-15739, 1:4000) in cytoplasmic proteins; NF-κB p65 (Santa Cruz, SC8008, 65 kDa, 1:1000), Lamin B1 (Santa Cruz, SC374015, 67 kDa, 1:1000) in nuclear proteins. The membranes were analyzed with a chemiluminescent substrate kit (Thermo Fisher) after washing and incubating with secondary antibodies (rabbit, CST, #7074S; mouse, CST, #7076S). The G:BOX Chemi XRQ system (Syngene, Cambridge, UK) detected the target proteins on the membranes.

Animals

BALB/c female mice (age, 4 weeks; weight, 16–18 g) were obtained from DBL Co., Ltd. (Daejeon, South Korea), and all mice were given a week to adjust to our animal facility. All animals (5 animals/cage) were kept at a controlled 22°C ± 2 temperature and 55% ± 5 humidity with a 12 hours light/12 hours dark cycle in a laminar airflow room. The care and treatment of animals were carried out in accordance with the guidelines established by the Public Health Service Policy on the Human Care and Use of Laboratory Animals (25).

Experimental protocol for inducting AD-like skin and sample collection

Following the experimental protocol, DNCB and DfE were spread over both ears of the experimental mice to mimic an AD-like skin. In brief, the mouse ears were sensitized to 1% DNCB (20 µL/ear, twice) for 1 week followed by 0.5% DNCB (20 µL/ear, twice) for 1 week. After 2 weeks, we applied the 0.5% DNCB (20 µL/ear, once) and DfE (20 µL/ear, twice) for 3 more weeks. RbGDE (5, 10, or 20 µg/ear) or Dexa (5 µg/ear) were painted on both ears for 6 consecutive days each week over 2 weeks after 3 weeks of induction. The mice were divided into seven groups: Con (vehicle; PBS), RbGDE (20 µg/ear) only, DNCB/DfE (induced AD) plus PBS, AD plus RbGDE (5, 10, or 20 µg/ear), and AD plus Dexa (5 µg/ear). The change in ear thickness was measured by a thickness dial-gauge (7301-Mitutoyo, Tokyo, Japan). Body-weight changes in DNCB/DfE-induced AD mice were measured using a HKC65050 electronic balance (Cas, Incheon, South Korea) on the last day of each week for 5 weeks.

The mice were put into a euthanasia induction chamber at 35 days and euthanized with carbon dioxide gas flowing into the chamber at a rate of less than 30% of the total volume per minute. We collected blood from mice's inferior vena cava and then centrifuged it for 15 minutes at 400 g to obtain the sera for histamine assays and ELISA. Mice's ears were also collected for RNA extraction and histological examination.

Histological observation

Ear tissues were fixed using 10% formalin for 48 hours at room temperature before histology examination. The ear tissues were then paraffin-embedded according to standard procedures. Within 6 µm, the trimmed paraffin blocks were sectioned. The sectioned ear tissues were stained with hematoxylin and eosin (H&E) and toluidine blue (TB) for the bright-field microscopic observation (Carl Zeiss microscope, Jena, Germany). A stage micrometer 10:100 microscopic lens (Carl Zeiss) with a magnification of 100× was used to measure the epidermal and dermal thickness of H&E-stained ear tissues. Eosinophils infiltration in H&E staining and mast cells in TB staining were counted at three randomly selected sites per ear tissue slide using a high-power field (magnification of 400×).

Histamine assay and ELISA

Histamine levels were measured using *o*-phthaldialdehyde (OPA) spectrofluorimetric methods, as described in previous studies (26,27). In brief, the sera (1:5 dilution) were mixed with 60% perchloric acid and hydrochloric acid (HCl), and centrifuged. The transferred supernatant was then treated with 5 M sodium chloride, 5 N sodium hydroxide, and 1-butanol before centrifuging at 16 000 g for 20 minutes at 4°C. Next, the supernatant was mixed

with HCl and heptane, and centrifuged. The bottom layer was transferred to a 96-well F-Bottom microplate (Greiner bio-one GmbH, Kremsmünster, Austria). Finally, 1% OPA and 3N HCl were added to stop the reaction. To obtain the standard curve, a histamine solution was prepared by dissolving the histamine powder in 0.1 N HCl. The fluorescence intensity was measured using a Perkin Elmer LS50B spectrometer (Norwalk, CT) with excitation (at 355 nm) and emission (at 450 nm) fluorescence filters.

To detect immunoglobulin (Ig) in the sera, the BD Biosciences ELISA kit (Oxford, UK) was used according to the manufacturer's protocol. SoftMax® Pro software version 6 of VersaMax™ Microplate Reader was used to collect ELISA data for DfE-specific IgE, total IgE, and IgG2a. To detect DfE-specific IgE in serum, 10 μg/mL of DfE was coated into Nunc MaxiSorp 96-well plates (Thermo Fisher, Roskilde, Denmark). The optical density of DfE-specific IgE was measured using a VersaMax™ Microplate reader with a 450 nm filter.

Statistical analysis

Statistical analyses were carried out using GraphPad 6 software (San Diego, CA). Following a one-way analysis of variance, the data were examined for treatment effects using Dunnett's multiple comparison test. The results were considered statistically significant if the *P* value was less than 0.05. All results were presented as mean values ± standard error of mean.

Results

Effects of RbGDE on keratinocytes viability

Table 2 shows the viability of keratinocytes that were dependent on RbGDE-dose treatment. RbGDE treatment at various concentrations (0.1–100 μg/mL) had no effect on keratinocytes cytotoxicity up to 100 μg/mL. RbGDE was shown to have a significant protective effect against H_2O_2 toxicity in keratinocytes at a concentration of 10 and 100 μg/mL. The protective effect of RbGDE in TNF-α/IFN-γ-activated keratinocytes was found to be noncytotoxic concentrations of 1–100 μg/mL. As a result, it was decided that the treatment of RbGDE on the activated keratinocytes would use that 1, 10, or 100 μg/mL of RbGDE to measure the expression of inflammation-related cytokines and chemokines, and 100 μg/mL of RbGDE to detect protein molecules.

Effects of RbGDE on inflammatory responses in activated keratinocytes

RbGDE reduced the expression of cytokines (IL-1β and IL-6) and chemokines (CCL17 and CCL22) as a result of qPCR analysis (Figure 1A). Additionally, RbGDE treatment reduced IL-6 and CCL17 secretion in activated keratinocytes as a result of ELISA evaluation (Figure 1B). Western blot analysis was then used to detect the protein molecules involved in the inhibitory signaling pathway to inflammation. RbGDE inhibited the p38 MAPK and p65 NF-κB activity while inhibiting IκBα degradation. On the other hand, RbGDE had no significant effect on STAT1 phosphorylation (Figure 2). These results showed that RbGDE had an anti-inflammatory effect by inhibiting the p38 MAPK and p65 NF-κB activation.

Effects of RbGDE on symptoms of AD-induced mice lesion

To investigate the therapeutic effects of RbGDE, we topically treated RbGDE in both ears of DNCB/DfE-induced AD mice, as shown in the experimental scheme (Figure 3A). In mice, repeated topical application of DNCB/DfE caused AD-like skin inflammation and increased the ear thickness. On the other hand, RbGDE alleviated symptoms of AD such as erythema, edema, keratinization, and mouse ear thickness (Figure 3B and 3C). In addition, we confirmed that the epidermal and dermal thicknesses of mouse ear tissues were significantly reduced through a microscopic examination (Figure 3D). These results showed that RbGDE had a therapeutic effect on AD-like skin.

Effect of RbGDE on inflammatory cells in AD-induced mice lesion

The infiltrated eosinophils of AD skin have been associated with the Th2-mediated immune response in histopathological changes, and histamine derived from mast cells as an inflammatory mediator contributes to itching and inflammation in AD (2,28). Hence, we investigated whether RbGDE could reduce eosinophils

Table 2. Effects of an ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves on keratinocytes viability

MTT assay (%)	Untreated control	RbGDE (μg/mL)				Stimulated only
		0.1	1	10	100	
Cell viability	99.55 ± 2.72	95.36 ± 4.63	96.03 ± 1.67	97.97 ± 3.44	99.41 ± 4.05	-
H_2O_2 treatment	100 ± 1.06	78.68 ± 2.00	83.09 ± 3.40	88.40[b] ± 2.77	93.64[b] ± 0.92	79.90[a] ± 2.26
TNF-α/IFN-γ treatment	100 ± 1.17	83.74 ± 1.36	89.71[c] ± 0.66	89.96[c] ± 1.16	92.51[c] ± 1.29	81.31[a] ± 0.62

RbGDE, Ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; H_2O_2, Hydrogen peroxide; TNF-α, Tumor necrosis factor-α; IFN-γ, Interferon-γ.
Data are presented as the mean ± SEM of five determinations. [a] $P < 0.05$ when compared with the untreated control group, [b] $P < 0.05$ when compared with the H_2O_2-treated group only, and [c] $P < 0.05$ when compared with the TNF-α/IFN-γ-treated group only.

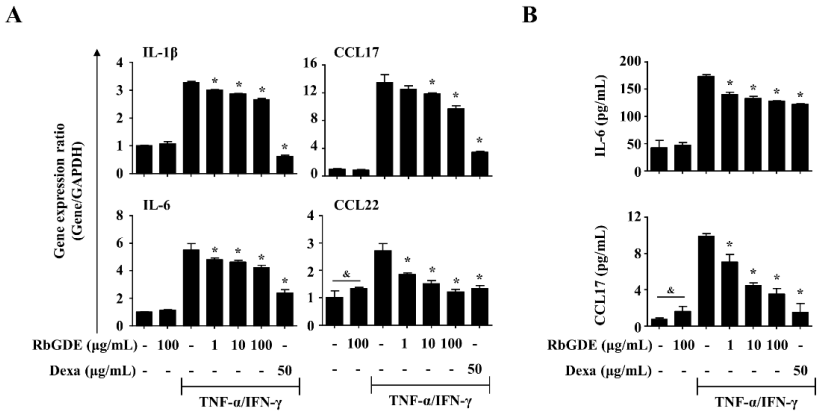

Figure 1. Effects of ethanol extract from *Rhododendron brachycarpum* D. Don ex G. Don leaves on TNF-α/IFN-γ-activated keratinocytes. (A) qPCR was used to measure the expression of IL-1β, IL-6, CCL17, and CCL22. (B) ELISA kits were used to measure the levels of IL-6 and CCL17 released. Data are presented as the mean ± SEM of three determinations. $^{\&}P < 0.05$ compared with untreated control group, and $^{*}P < 0.05$ compared with TNF-α/IFN-γ-activated group only. RbGDE: ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves, Dexa: dexamethasone.

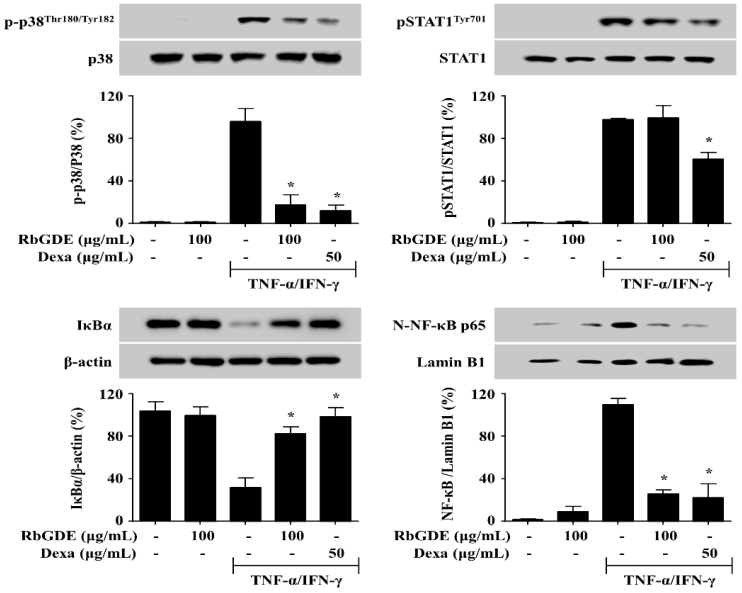

Figure 2. Effects of ethanol extract from *Rhododendron brachycarpum* D. Don ex G. Don leaves on inflammation-related protein activation. Western blot analysis expressed bands for phospho (p)- p38, p38, pSTAT1, STAT1, IκBα, β-actin, nucleus (N)-NF-κB, and lamin B1. The relative protein expression levels (% of TNF-α/IFN-γ-treated group) were normalized to that of p38, STAT1, β-actin, and lamin B1 against each band. The band intensity was quantified using Image J software and displayed as the mean ± SEM of three independent experiments in a bar graph. RbGDE: ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves, Dexa: dexamethasone.

and mast cells in lesions using H&E- and TB-stained ear tissues, respectively. The skin lesions of AD mice were observed to have an increase in infiltrated inflammatory cells such as eosinophils and mast cells in representative photomicrographs of the slides. On the contrary, RbGDE significantly reduced eosinophils (Figure 4A) and mast cells (Figure 4B) infiltration in a dose-dependent manner.

Effects of RbGDE on local and systemic immune response in AD-like mice

The inflammatory mediators were measured using qPCR for local immune responses and ELISA kits for systemic immune response to explain the anti-inflammatory effect of RbGDE. Using qPCR, we first assessed the local immune response to the expression of AD-related inflammatory cytokines in skin lesions. As a result, RbGDE reduced the expression of Th1 (IL-12a), proinflammatory (IL-1β), epithelial cell-derived, and Th2 (IL-4) cytokines (Figure 5A). Following that, RbGDE treatment was found to significantly reduce serum histamine and Ig levels, including DfE-specific IgE, total IgE, and IgG2a (Figure 5B-D). These results showed that RbGDE diminished

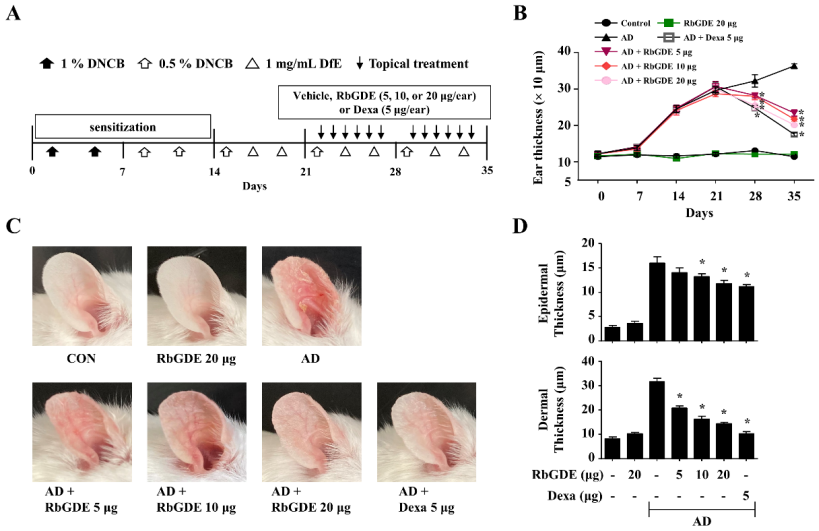

Figure 3. Effects of an ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves on ear tissues from mice with DNCB/DfE-induced AD-like skin. (A) Protocol for inducting AD-like skin lesion with DNCB/DfE and treating RbGDE. (B) Ear thickness over the course of the experiment. (C) A macroscopic view of the ears with AD-like skin lesions. (D) Mouse ear tissue epidermal and dermal thicknesses. The tissues were measured and then represented graphically as bar graphs. Data are presented as the mean ± SEM of five determinations. **P* < 0.05 compared with the DNCB/DfE-treated group only. DNCB: 1-chloro-2,4-dinitrobenzene, DfE: *Dermatophagoides farina* extract, RbGDE: ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves, Dexa: dexamethasone.

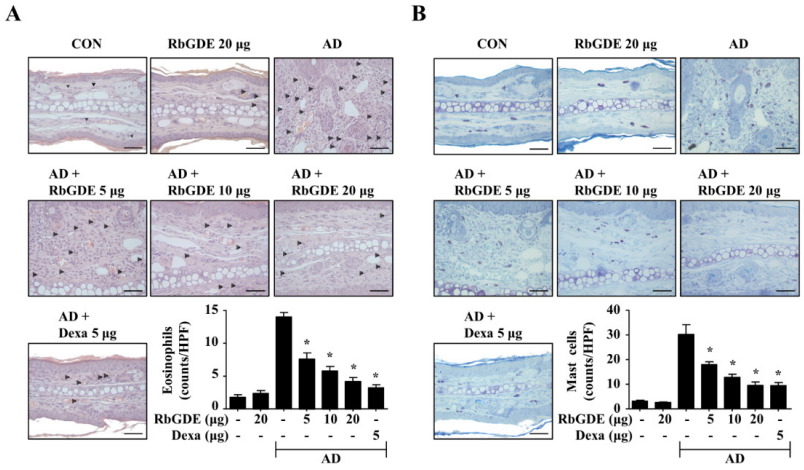

Figure 4. Effects of an ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves on histological findings in AD-like skin lesions. Representative photomicrographs presented that the infiltrated (A) eosinophils (H&E staining; black arrows) and (B) mast cells (toluidine blue staining; deep purple) in AD-like skin tissues were shown. A bar graph was also used to display the number of infiltrating inflammatory cells. Original magnification 400×, Scale bar = 50 μm. Data are presented as the mean ± SEM of five determinations. **P* < 0.05 compared with the DNCB/DfE-treated group only. RbGDE: ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves, Dexa: dexamethasone.

skin inflammation in DNCB/DfE-induced AD mice by suppressing histamine, IgE, and IgG2a levels of sera and AD-related inflammatory cytokines.

Discussion

The current study demonstrated that an ethanol extract of *R. brachycarpum* D. Don ex G. Don leaf (RbGDE) had an anti-inflammatory effect by reducing the overproduction of inflammatory mediators. As illustrated in Figure 6, RbGDE inhibits inflammation in activated keratinocytes via the p38 MAPK/NF-κB pathway and by suppressing AD-related immune responses in an AD mouse model.

RbGDE treatment has been shown to have strong protective activity in human normal cell lines (HEL299 and Chang) as well as human cancer cell lines (A549, AGS, Hep3B, and MCF7) (29). In this regard, we investigated the protective effect of RbGDE against oxidative damage in HaCaT cell lines using hydrogen peroxide production. Additionally, RbGDE treatment inhibited inflammation in TNF-α/IFN-γ-activated keratinocytes. These findings

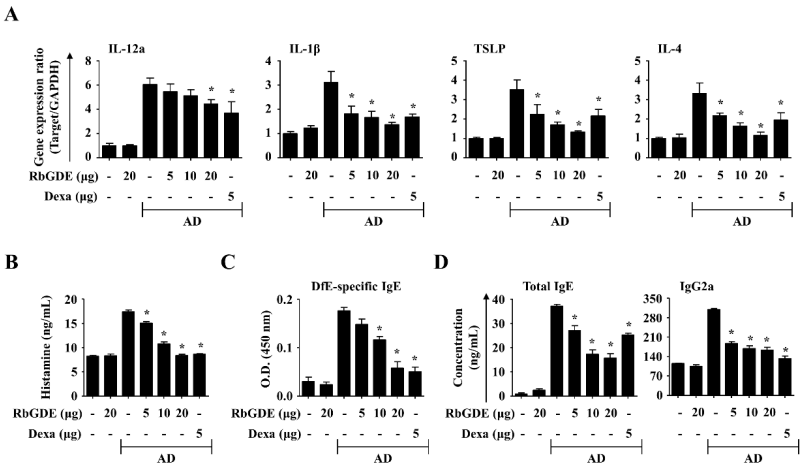

Figure 5. Effects of an ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves on immune response in an AD-like mouse model. (A) Using qPCR, the expression levels of IL-12a, IL-1β, TSLP, and IL-4 cytokines in AD-like lesions were determined. (B) An *o*-phthaldialdehyde spectrofluorimetric assay was used to determine the serum histamine level in an AD-like mouse model. ELISA kits were used to measure the serum levels of (C) DfE-specific IgE and (D) total IgE and IgG2a in mice. Data are presented as the mean ± SEM of five determinations. *$p < 0.05$ compared with the DNCB/DfE-treated group only. DfE: *Dermatophagoides farina* extract, RbGDE: ethanol extract of *Rhododendron brachycarpum* D. Don ex G. Don leaves, Dexa: dexamethasone.

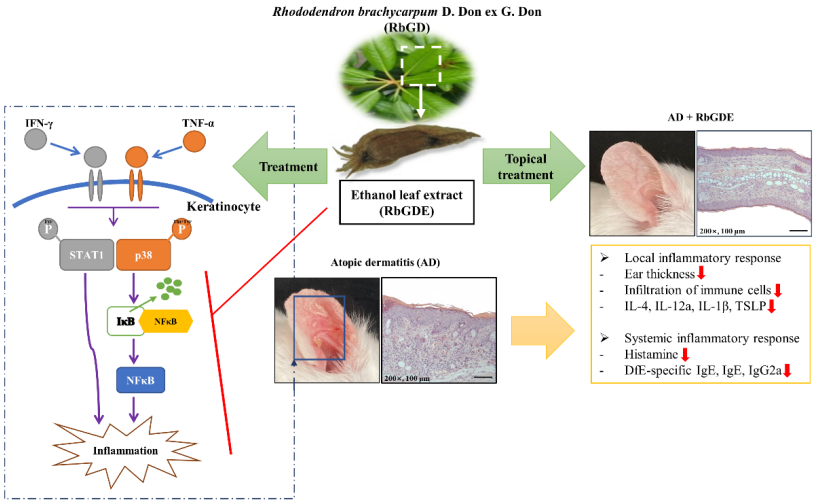

Figure 6. Graphical summary. The leaves of *Rhododendron brachycarpum* D. Don ex G. Don (RbGD) were extracted with an ethanol solvent (referred to as RbGDE) and then applied to activated keratinocytes and DNCB/DfE-induced atopic dermatitis-like skin lesions. RbGDE has been shown *in vitro* and *in vivo* experiments to have an anti-inflammatory effect by inhibiting inflammatory mediators. Thus, RbGD as a topical treatment alleviates dermatitis symptoms.

suggest that RbGDE may have anti-inflammatory properties in human keratinocytes.

Inflammatory cytokines and chemokines produced by TNF-α/IFN-γ-activated keratinocytes are widely regarded as critical mediators of skin inflammation (30). From this, the treatment of RbGDE was shown to reduce the expression of inflammatory cytokines and chemokines, as well as CCL17 and IL-6 secretion in activated keratinocytes. According to research, the transcription factors STAT1 and NF-κB activity are involved in the expression of CCL17 and CCL22, and the activation of the p38 MAPK is an important pathway for the production of IL-1β, IL-6, and TNF-α (31-33). Furthermore, inhibiting the JAK/STAT pathway may help with chronic dermatitis, and p38 MAPK may regulate inflammatory diseases by enhancing the NF-κB signaling (33-35). Based on these findings, we investigated RbGDE's inhibitory effect in TNF-α/IFN-γ-activated keratinocytes via the molecular mechanism involved. The current study's findings indicate that RbGDE may inhibit inflammatory responses by blocking the p38 MAPK and NF-κB activation, but not STAT1. Thus, the treatment of RbGDE provides evidence that it is an anti-inflammatory reaction because it reduces inflammatory mediators in keratinocytes by inhibiting the p38 MAPK/NF-κB signaling pathway.

Dermatitis may cause an allergic reaction when the

skin is irritated or exposed to harmful factors. House dust mites (HDM) are a major allergen source contributing to AD pathogenesis and development. HDM species DfE contributes most to AD pathogenesis and development (36,37). The characteristics of acute AD present edema and thickening of the epidermis, whereas the characteristics of chronic AD show diffuse epidermal hyperplasia caused by infiltrated immune cells, including eosinophils, lymphocytes, and mast cells in histopathological findings (38). Based on these findings, the DNCB/DfE-induced AD-like skin mouse model mimicked AD-like skin lesions. From this, topical RbGDE administration alleviated the typical histopathological changes of AD, such as edema, epidermal hyperplasia, eosinophil infiltration, and mast cell infiltration in AD-like skin lesions. These findings indicate that RbGDE treatment alleviates dermatitis symptoms. Immunologically, Th2 cell activation induces Th2-associated cytokines, such as IL-4, IL-5, and IL-13, allowing B cells to control IgE class switching and detect elevated IgE levels, whereas Th1 cell activation induces the expression of IFN-γ, which induces IgG2a production (39,40). Histamine levels in the serum are higher in patients with AD than in healthy people (13), and histamine also increases the production of various inflammatory cytokines in keratinocytes (41). Keratinocytes eventually produce inflammatory factors that promote self-amplifying loops in chronically inflamed skin (41,42). Thus, these findings suggest that RbGDE attenuated skin inflammation in DNCB/DfE-induced AD mice by reducing serum levels of histamine, IgE (DfE-specific and total), and IgG2a, as well as AD-related inflammatory cytokines. This finding suggests that RbGDE has an anti-inflammatory effect on keratinocyte-involved skin inflammation in AD-like conditions.

Although the leaves of RbGD are known to treat skin diseases and have antioxidative and anti-inflammatory properties, their effects on skin disease have yet to be confirmed (15). Nonetheless, the RbGD is expected to have pharmacological activity due to the presence of flavonoids and polyphenols in RbGD leaf extract. This is most likely because RbGDE has been shown to have antioxidant and anti-inflammatory effects due to flavonoid compounds such as quercetin-o-rhamnoside and hyperin (43,44). Based on these reports, our data suggest that RbGD may be responsible for the anti-inflammatory activity observed with RbGDE. This is supported by the fact that the treatment of RbGDE in this study appeared to improve a therapeutic benefit in the skin lesion. These findings also demonstrate that inflammatory skin has good therapeutic effects on AD. However, additional research is needed to demonstrate the structure-activity of pure bioactive molecules of extracts for herbal applications research. Furthermore, additional research is required to verify pharmacological data and compound interactions, to determine which compounds directly serve beneficial effects or characterize and determine the structure of plant active constituents. The results of this study only relied on the inhibitory effects and symptoms of AD. In addition to this study, it is necessary to investigate whether RbGD can be applied to treat the types of dermatitis such as contact dermatitis, psoriasis, and dyshidrotic dermatitis.

Conclusion

In this study, we showed that an ethanol extract of RbGD leaf inhibits the production of inflammatory mediators in keratinocytes by inhibiting the p38 MAPK/NF-κB signaling pathway. By exerting both lesional and systemic anti-inflammatory effects, topically applied RbGDE effectively alleviated the symptoms and lesions of AD-like skin inflammation. Because of these findings, RbGD leaves could be used as an anti-inflammatory agent to treat dermatitis. Further research is needed to characterize the active phytochemical compounds and elucidate more detailed mechanisms.

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Authors' contributions

JK came up with the idea for the project and designed the experiments. YJK, J-MC, and S-YK contributed resources and participated in plant research. NK and Y-AC helped draw the figures and conduct the statistical analyses. E-NK prepared the extract and conducted the plant literature search. G-SJ and S-HK supervised the research and wrote the paper. The final version of the manuscript was reviewed and approved by all authors.

Conflict of interests

Authors declare no conflict of interests.

Ethical considerations

All mouse experiments were approved by the animal care center at Kyungpook National University (Institutional Animal Care and Use Committee, IRB# 2021-0073; Daegu, South Korea).

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