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아토피피부염에서 갈근과 두시 복합추출물의 Endocannabinoid system 조절을 통한 염증조절

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Abstract

Anti-inflammatory Effect of Puerariae Radix and Douchi Complex Extract through Endocannabinoid system Control in Atopic Dermatitis

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Objective

This study aimed to confirm the effect of Puerariae Radix and Douchi complex extracts on skin damage recovery and inflammation relief in atopic dermatitis-induced mice through endocannabinoid system (ECS) control.

Methods

In this study, 4-week-old NC/Nga mice were divided into four groups: control, atopic dermatitis elicited (ADE), palmitoylethanolamide (PEA)-treated after atopic dermatitis elicitation (PEAA), and Puerariae Radix and Douchi complex extract-treated after atopic dermatitis elicitation (PGEA). Ten rats were assigned to each treatment group. After drug administration for 3 weeks following lipid barrier elimination, cannabinoid receptor (CBR) 1, CBR 2, 8-hydroxydeoxyguanosine (8-OHdG), cluster of differentiation (CD) 68, matrix metalloproteinase (MMP)-9, Fc ε receptor, and substance P were observed to confirm the regulation of the ECS, macrophage, and mast cell activities.

Results

The PGEA group showed more positive reactions for CBR1, CBR2, and GPR55 than the ADE and PEAA groups. The number of positive reactions to 8-OHdG, CD68, and MMP-9 was significantly lower in the PGEA group than in the ADE and PEAA groups. Both the Fc ε receptor and substance P showed lower positive reactions in the PGEA group than in the ADE and PEAA groups.

Conclusion

The Puerariae Radix and Douchi complex extract can reduce the inflammation of atopic dermatitis by restoring the structural damage of the skin lipid barrier through ECS activity.

Key words: Puerariae Radix, Douchi, Atopic Dermatitis, Endocannabinoid system (ECS), Inflammation

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Ⅰ. Introduction

The skin protects the internal body and plays a defensive role against external harmful agents such as antigens or pathogens[1,2]. The epidermis consists of keratinocytes, the keratinocyte extracellular matrix, the lipid membrane between keratinocytes, and keratohyalin bodies, which collectively contribute to the protective barrier function[3]. The protein outer membrane of keratinocytes is formed through the binding of proteins such as involucrin, loricrin, and trichohyalin, serving as a physical defense barrier[4,5]. The lipid outer membrane of keratinocytes allows the lipids between the keratinocytes to form multiple layers, contributing to the formation of a complete skin barrier[6].

Atopic Dermatitis is a chronic inflammatory skin disease that arises from a combination of various factors[7]. Over 80% of cases of atopic dermatitis occur in children under the age of 5[8]. Atopic dermatitis often develops in childhood and can progress to other allergic conditions, such as allergic rhinitis or asthma. Therefore, early treatment, management, and avoidance of risk factors are crucial.

In Korean Medicine, it is believed that atopic dermatitis occurs when “taeyeol” (胎熱), or fetal heat, is not properly eliminated[9]. Taeyeol is heat accumulated in the body due to the mother's improper diet or lifestyle, which is then transmitted to the fetus. After birth, this heat emerges on the surface of the body, causing symptoms like erythema, rashes, and itching[10]. Herbs with heat-clearing effects are commonly used in clinical practice for the treatment of atopic dermatitis[11].

The complex extract consists of Puerariae Radix (葛根) and fermented soybean (豆豉, Douchi, fermented *Glycine max* Merr.). Puerariae Radix has effects of promoting sweating and releasing muscles (發汗解肌), dispelling pathogens and relieving rashes (解表透疹), generating body fluids and alleviating thirst (生津止渴), and detoxifying alcohol (解酒毒), which helps to regulate skin inflammatory responses by inhibiting the secretion of Interleukin (IL)-3, IL-4, Tumor Necrosis Factor-alpha (TNF-α), and other cytokines[12]. Fermented soybean (Douchi) has heat-clearing and detoxifying properties, and it is being extensively studied for its effects on inflammatory skin diseases[13].

This study aimed to investigate the regulation of the Endocannabinoid System (ECS) by a complex extract by observing cannabinoid receptors (CBR) 1, CBR2, and G protein-coupled receptor (GPR) 55. Additionally, to examine inflammation regulation, 8-hydroxydeoxyguanosine (8-OXdG), Cluster of Differentiation (CD) 68, and Matrix Metalloproteinase (MMP)-9 were observed. To assess mast cell activation regulation, Fc ε Receptor and substance P were observed. Through this, we report the potential of the complex extract to alleviate inflammation in atopic dermatitis.

Ⅱ. Materials and Methods

A. Materials

1) Animals

The male Nc/Nga mice, 4 weeks old, were obtained from Ja-Bio (South Korea) and adapted for 2 weeks in a germ-free housing system. Mice weighing 15 ± 1 g were selected. The mice were divided into the following groups: Control (Ctrl), atopic dermatitis elicited group (ADE), palmitoylethanolamide (PEA) treated group after atopic dermatitis elicitation (PEAA), complex extract treated group after atopic dermatitis elicitation (PGEA). Ten mice were assigned to each group. Animal experiments were conducted after approval from the Animal Experiment Ethics Committee of 000 University (IACUC No. smecae-22-12-01). The management and use of laboratory animals were carried out following NIH guidelines.

2) Complex extract

A total of 58.5 g of the complex extract, consisting of 26 g of Puerariae Radix and 32.5 g of Douchi (*Glycine max* (L.) Merr.), was added to 1000 mL of distilled water and boiled for 3 hours. The resulting solution was then filtered. The filtrate was concentrated under reduced pressure using a rotary evaporator (Eyela, Tokyo, Japan) to a volume of 50 mL, and then freeze-dried, yielding 8.59

g of extract (yield: 14.7%).

PGEA was orally administered at a dose of 0.143 mg/kg, dissolved in saline solution, after the induction of atopic dermatitis, for a duration of 3 weeks, with 0.2 mL administered each time. PEAA, used as the control drug, was orally administered at a dose of 10 mg/kg, dissolved in saline solution, for 3 weeks after the induction of atopic dermatitis, with 0.1 mL administered each time.

B. Methods

1) Atopic dermatitis

The skin on the dorsal area of the mouse was shaved, and then 1 mL of 5% sodium dodecyl sulfate (SDS: Sigma, USA), a surfactant, was rubbed onto the area 20 times with a cotton swab to remove the lipid lamellae from the stratum corneum. A crude extract of *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) (100 mg, Biostir, Japan) was applied to the area three times a week for three weeks to induce atopic dermatitis.

2) Immunohistochemistry

The skin was fixed via cardiac perfusion with vascular rinse and a 10% neutral buffered formalin (NBF) solution. The dorsal skin obtained was fixed in 10% NBF at room temperature for 24 hours, followed by conventional paraffin embedding, and 5 μm thick continuous sections were prepared.

Changes and mechanisms of the endocannabinoid system (ECS) in the skin were examined using anti-CBR1, anti-CBR2, and anti-GPR55 antibodies. Oxidative stress-induced damage was assessed with anti-8-OXdG, macrophage activity was measured using anti-CD68, edema-induced changes were evaluated with anti-Matrix Metalloproteinase (MMP)-9, mast cell activation was examined with anti-Fc ε Receptor, and scratching behavior was regulated with anti-substance P antibodies. Immunohistochemical staining was performed using these antibodies

First, the skin sections underwent a proteolysis process for 5 minutes using proteinase K (20 μg/mL, Agilent Dako, Santa Clara, CA), and then blocking was performed for 2 hours in a solution containing 1% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 10% normal goat serum (Vector Lab, Burlingame, CA). The sections were incubated for 72 hours at 4°C in a humidified chamber with the primary antibodies: mouse anti-CBR1 (1:100, Santa Cruz Biotec, Dallas, TX), mouse anti-CBR2 (1:50, Santa Cruz Biotec), mouse anti-GPR 55 (1:50, Abcam, USA), mouse anti-8-OXdG (1:100, Santa Cruz Biotec), mouse anti-CD68 (1:100, Santa Cruz Biotec), mouse anti-MMP-9 (1:200, Santa Cruz Biotec), mouse anti-Fc ε Receptor (1:100, Santa Cruz Biotec), and mouse anti-substance P (1:100, Santa Cruz Biotec). The secondary antibody, biotinylated goat anti-mouse IgG (1:100, Abcam), was applied for 24 hours at room temperature, and then an avidin-biotin complex kit (Vector Lab) was used to react for 1 hour at room temperature. The sections were then color developed in a solution of 0.05% 3,3'-diaminobenzidine and 0.01% HCl in 0.05 M tris-HCl buffer (pH 7.4), followed by counterstaining with hematoxylin.

3) Image analysis

The results of immunohistochemistry were quantified (means ± standard error) through image analysis using Image Pro 10 (Media Cybernetics, Rockville, MD). After randomly selecting 10 skin samples from each group, images were captured at a magnification of x200, and image analysis was performed by calculating the positive pixels (intensity 80-100) per 20,000,000 pixels.

4) Statistic

The statistics were analyzed using SPSS software (SPSS 25, SPSS Inc., Chicago, IL). One-way ANOVA was performed to assess significance ($p < 0.05$), and post-hoc analysis was conducted using Tukey HSD.

Ⅲ. Results

A. ECS regulation

The changes in ECS activation in atopic dermatitis were observed immunohistochemically. CBR1 expression increased in all ADE, PEAA, and PGEA compared to the Ctrl group (5,233 ± 252 / 20,000,000 pixels). ADE (24,074 ± 1,889 / 20,000,000 pixels) showed a 360% increase

compared to Ctrl, PEAA (38,159 ± 800 / 20,000,000 pixels) showed a 629% increase, and PGEA (52,768 ± 1,102 / 20,000,000 pixels) showed a 908% increase. The CBR1-positive response in PGEA was significantly increased by 119% compared to ADE and by 38% compared to PEAA (Fig. 1).

After inducing atopic dermatitis, CBR2 showed increased positive responses in ADE, PEAA, and PGEA compared to Ctrl (5,767 ± 241 / 20,000,000 pixels). ADE (24,905 ± 1,475 / 20,000,000 pixels) showed a 332% increase compared to Ctrl, PEAA (34,643 ± 1,060 / 20,000,000 pixels) showed a 501% increase compared to Ctrl, and PGEA (51,604 ± 1,181 / 20,000,000 pixels) showed a 795% increase compared to Ctrl. The positive response of CBR2 in PGEA was significantly increased by 107% compared to ADE and by 49% compared to PEAA (Fig. 1).

GPR55 showed an increased positive response in ADE, PEAA, and PGEA after inducing atopic dermatitis, compared to Ctrl (4,766 ± 101 / 20,000,000 pixels). ADE (23,767 ± 1,104 / 20,000,000 pixels) showed a 399% increase compared to Ctrl, PEAA (35,518 ± 1,427 / 20,000,000 pixels) showed a 645% increase, and PGEA (52,610 ± 1,176 / 20,000,000 pixels) showed a 1,004% increase. The positive response of GPR55 in PGEA was significantly increased by 121% compared to ADE and by 48% compared to PEAA (Fig. 1).

B. Inflammation regulation

Inflammatory changes in atopic dermatitis were observed immunohistochemically. 8-OXdG showed an increased positive reaction in ADE, PEAA, and PGEA compared to the Ctrl (3,611 ± 134 / 20,000,000 pixel). ADE (58,209 ± 1,953 / 20,000,000 pixel) showed a 1,512% increase compared to Ctrl, PEAA (41,570 ± 1,194 / 20,000,000 pixel) showed a 1,051% increase, and PGEA (30,266 ± 1,792 / 20,000,000 pixel) showed a 738% increase. The positive reaction of 8-OXdG in PGEA was significantly less increased by 48% compared to ADE and 27% compared to PEAA (Fig. 2).

CD68 showed an increased positive reaction in ADE, PEAA, and PGEA compared to Ctrl (5,495 ± 322 / 20,000,000 pixel). ADE (170,605 ± 3,006 / 20,000,000 pixel) increased by 3,005% compared to Ctrl, and PEAA (99,694 ± 1,704 / 20,000,000 pixel) increased by 1,714% compared to Ctrl. PGEA (64,795 ± 2,070 / 20,000,000 pixel) increased by 1,079% compared to Ctrl. The CD68 positive reaction in PGEA increased 62% less than in

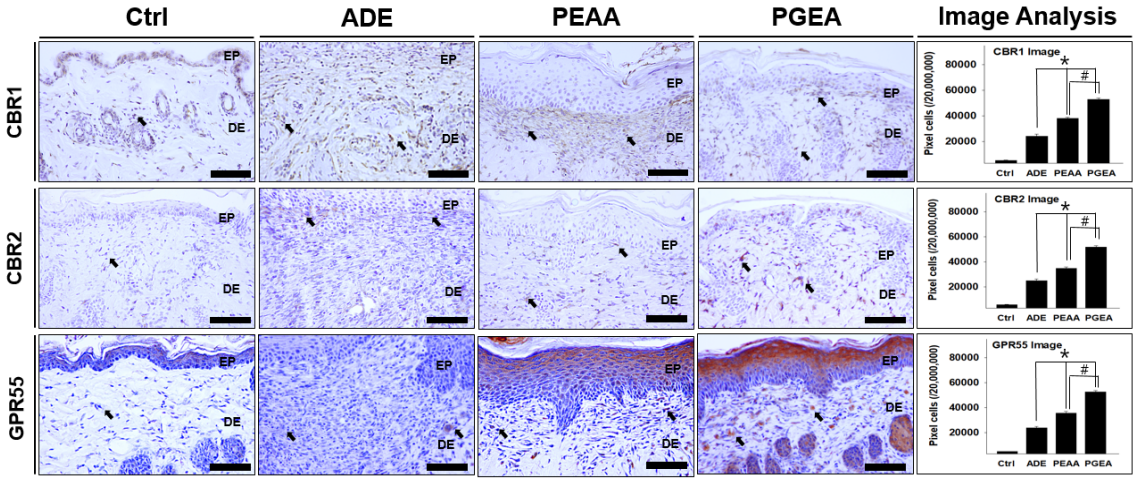

Figure 1. The activation of endocannabinoid system (ECS) in atopic dermatitis by palmitoylethanolamide (PEA) and Puerariae Radix and Douchi complex extract (PGE)

The expression of cannabinoid receptor (CBR) 1, CBR2 and G protein-coupled receptors (GPR) (arrow indicates light brown particle) was significantly increased in PEAA and PGEA as compared with ADE, the data of image analysis showed the same results (immunohistochemistry).
Abbreviations. Ctrl, normal; ADE, atopy dermatitis (AD) elicitate group; PEAA, palmitoylethanolamide (PEA) treated group after AD elicitation; PGEA, Puerariae Radix and Douchi complex extract treated group after AD elicitation; EP, epithelium; DE, Dermis; Bar size, 50㎛; *, $p < 0.05$ compared with ADEG; $^{\#}$, $p < 0.05$ compared with PETG.

ADE and 35% less than in PEAA (Fig. 2).

MMP-9 showed an increase in positive reactions in ADE, PEAA, and PGEA compared to Ctrl (4,850 ± 228 / 20,000,000 pixels). ADE (111,039 ± 2,797 / 20,000,000 pixels) showed a 2,189% increase compared to Ctrl, and PEAA (59,886 ± 2,278 / 20,000,000 pixels) showed a 1,135% increase compared to Ctrl. PGEA (30,675 ± 1,196 / 20,000,000 pixels) showed a 532% increase compared to Ctrl. The MMP-9 positive reaction in PGEA increased significantly less by 72% compared to ADE and by 49% compared to PEAA (Fig. 2).

C. Mast cell activation regulation

The scratching-induced changes were observed immunohistochemically using Fc ε Receptor and substance P antibodies. The positive response for Fc ε Receptor, a marker of mast cell activation, was increased in ADE, PEAA, and PGEA compared to the Ctrl group (5,333 ± 221 / 20,000,000 pixels). ADE (126,879 ± 5,023 / 20,000,000 pixels) showed a 2,279% increase compared to Ctrl, PEAA (85,562 ± 2,351 / 20,000,000 pixels) showed a 1,504% increase compared to Ctrl, and PGEA (50,703 ± 2,608 / 20,000,000 pixels) showed an 851% increase compared to Ctrl. The positive response for Fc ε Receptor in PGEA was significantly less increased by 60% compared to ADE and by 41% compared to PEAA (Fig. 3).

The Substance P-positive response was increased in ADE, PEAA, and PGEA compared to Ctrl (4,325 ± 213 / 20,000,000 pixel). ADE (143,851 ± 4,995 / 20,000,000 pixel) showed a 3,226% increase compared to Ctrl, PEAA (56,549 ± 1,787 / 20,000,000 pixel) showed a 1,207% increase compared to Ctrl, and PGEA (34,076 ± 1,889

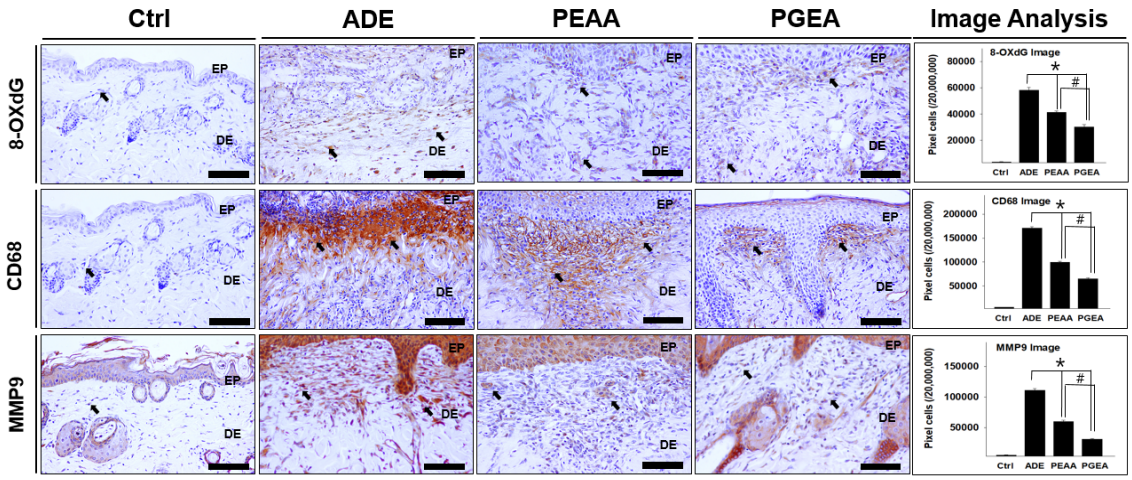

Figure 2. The activation of inflammation in atopic dermatitis by PEA and PGE.

The expression of 8-OHdG, CD68 and MMP-9 (arrow indicates light brown particle) was remarkably decreased in PEAA and PGEA as compared with ADE, the data of these image analysis showed the same results (8-OHdG, CD68 and MMP-9 immunohistochemistry). Abbreviations same as Fig. 1.

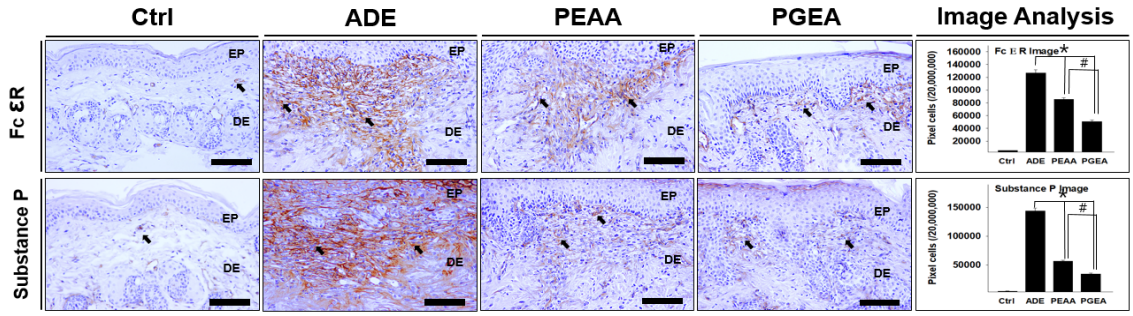

Figure 3. The activation of scratching in atopic dermatitis by PEA and PGE.

The expression of Fc ε Receptor and substance P (arrow indicates light brown particle) was remarkably decreased in PEAA and PGEA as compared with ADE, the data of these image analysis showed the same results (Fc ε Receptor and substance P immunohistochemistry). Abbreviations same as Fig. 1.

/ 20,000,000 pixel) showed a 688% increase compared to Ctrl. The Substance P-positive response in PGEA increased 76% less significantly than in ADE and 40% less significantly than in PEAA (Fig. 3).

Ⅳ. Discussion

The complex extract consists of Puerariae Radix and Douchi. Puerariae Radix is known to regulate the inflammatory response of the skin by inhibiting the secretion of interleukin (IL)-3, IL-4, and tumor necrosis factor-alpha (TNF-α) through its antiperspirant, antiphlogistic, and antidote properties[12]. Douchi is made by boiling and fermenting the mature seeds of soybeans (*Glycine max* Merrill). Douchi has the effects of dispelling pathogens (解表), relieving restlessness (除煩), and promoting the release of trapped heat (宣發鬱熱). One of the isoflavones found in soybeans, genistein, has antioxidant, anti-inflammatory, and anti-apoptotic effects. Several studies have reported its therapeutic effect on inflammatory allergic diseases through its Th2 immune modulation[13-16].

The endocannabinoid system (ECS) is a biological system that regulates memory, cognition, movement, pain, immunity, and more. It consists of cannabinoid receptors (CBRs), the endogenous ligands of CBRs, and enzymes involved in the synthesis and degradation of cannabinoids[17]. The main CBRs are CBR1 and CBR2, and recently, GPR55 has been added as a new CBR[18]. The activation and modulation of the ECS can delay or reduce the progression of diseases or disorders[19]. These changes in the ECS are connected to human tissues such as bones, muscles, the immune system, the gastrointestinal tract, kidneys, skin, central nervous system, and cardiovascular system[20]. When the ECS does not function properly, pathological problems such as skin barrier dysfunction, like atopic dermatitis, may occur[21].

The ECS is involved in various changes that occur in the skin[22]. When CB1, CB2, and CBR55 are activated in the epidermal keratinocytes, they inhibit the proliferation and differentiation of keratinocytes, induce inflammation, and trigger apoptosis[23-25]. Additionally, the activation of CB1 and CB2 receptors reduces inflammation and the infiltration of immune cells[26]. The ECS suppresses symptoms such as itching and regulates inflammatory responses in the skin[27,28]. Due to these effects of the ECS, various studies related to the ECS have been conducted recently, and it is emerging as a new treatment approach[26,29].

In this study, the positive response of PGEA to CBR1 significantly increased by 119% compared to ADE and by 38% compared to PEAA. The positive response of CBR2 was 107% significantly higher in PGEA compared to ADE, and 49% higher in PGEA compared to PEAA. These results suggest that the complex extract increases the production of ECS components such as CBR1, CBR2, and GPR55. This implies the potential for complex extracts to modulate the ECS and alleviate the symptoms of atopic dermatitis.

Patients with atopic dermatitis have an increased secretion of Immunoglobulin E (IgE). The secretion of IgE induces the degranulation of mast cells, which increases the deposition of degranulated cells. Mast cells express Fc ε receptors on their cell surface, and when IgE and antigens bind to these receptors, the mast cells become activated, leading to degranulation. Once degranulated, the activated mast cells secrete inflammatory mediators such as histamine, serotonin, substance P, and matrix metalloproteinase 9 (MMP-9), which trigger inflammatory responses[30]. MMP-9 is synthesized and secreted in response to stimuli such as cytokines and causes changes in cell structures[31]. It also destroys the basement membrane, facilitating the migration of inflammation-related cells[32,33]. CD68 is a protein expressed in macrophages and monocytes, and it is used as an indicator to assess the activation of immune cells and the degree of inflammation in inflammatory and immune diseases. The skin inflammation observed in atopic dermatitis is induced by the proliferation of keratinocytes and the overexpression of inflammatory cytokines, which is associated with CD68 activation[34], 8-OXdG indicates the degree of oxidative stress damage caused by dermatitis. In this study, the positive response for 8-OXdG showed that PGEA significantly increased less compared to ADE and PEAA. The positive responses for CD68 and MMP-9 also showed

that PGEA increased significantly less than ADE and PEAA. These results suggest that the complex extract can inhibit mast cell degranulation through ECS regulation and reduce inflammation by modulating the secretion of inflammatory mediators.

Itching is one of the main symptoms of atopic dermatitis and is the most commonly reported symptom by patients. This itching begins with the degranulation of mast cells triggered by IgE[35]. When IgE and the antigen bind to receptors on the surface of the mast cells, the mast cells are activated. One of the substances involved in this process is the Fc ε Receptor. Activated mast cells release inflammatory mediators that induce an inflammatory response. Substance P, one of the inflammatory mediators, regulates the secretion of cytokines. Additionally, Substance P relaxes blood vessels, increasing permeability, induces histamine secretion, promotes the inflammatory response, and causes itching. Therefore, Substance P is often used as an indirect marker in studies observing changes in inflammation or itching.

In this study, we aimed to confirm that complex extract inhibits the activity of mast cells by regulating the ECS, and observed the positive reaction of Fc ε Receptor. The positive reaction of Fc ε Receptor was significantly reduced by 60% with PGEA compared to ADE, and by 41% with PGEA compared to PEAA. Additionally, to confirm the inhibition of inflammatory mediators released from mast cells, we observed the positive reaction of Substance P. The positive reaction of Substance P was significantly reduced by 76% with PGEA compared to ADE, and by 40% with PGEA compared to PEAA. These results suggest that complex extract can reduce itching by inhibiting mast cell degranulation.

This study showed that the complex extract altered components of the ECS, such as CBR1, CBR2, and GPR55. The complex extract demonstrated the potential to suppress inflammation by affecting changes in 8-OXdG, CD68, and MMP-9. Additionally, changes in Fc ε Receptor and Substance P suggested the possibility of alleviating skin itching. Based on these results, the complex extract is expected to alleviate symptoms of atopic dermatitis through ECS intervention. However, this study was limited to animal experiments and did not clearly elucidate the mechanisms of ECS action. Therefore, we believe that further research is needed in the future.

Ⅴ. Conclusion

This study observed the regulation of the ECS through CB1, CB2, and GPR55, the regulation of inflammation through changes in 8-OHdG, CD68, and MMP-9, and the regulation of mast cell activation through changes in Fc ε receptor and Substance P, resulting in the following findings.

1. The positive response of CBR1 was significantly increased by 119% in PGEA compared to ADE, and by 38% in PGEA compared to PEAA.

2. The positive response of CBR2 was significantly increased by 107% in PGEA compared to ADE, and by 49% in PGEA compared to PEAA.

3. The positive response of GPR55 was significantly increased by 121% in PGEA compared to ADE, and by 48% in PGEA compared to PEAA.

4. The positive response of 8-OXdG was significantly less increased by 48% in PGEA compared to ADE, and by 27% in PGEA compared to PEAA.

5. The positive response of CD68 was significantly less increased by 62% in PGEA compared to ADE, and by 35% in PGEA compared to PEAA.

6. The positive response of MMP-9 was significantly less increased by 72% in PGEA compared to ADE, and by 49% in PGEA compared to PEAA.

7. The positive response of Fc ε receptor was significantly less decreased by 60% in PGEA compared to ADE, and by 41% in PGEA compared to PEAA.

8. The positive response of Substance P was significantly less decreased by 76% in PGEA compared to ADE, and by 40% in PGEA compared to PEAA.

Ⅵ. Acknowledgement

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