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Anti-inflammatory effects of phytosphingosine-regulated cytokines and NF-kB and MAPK mechanism

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Abstract

Phytosphingosine (PHS) is a major component of the skin barrier and a multifunctional physiologically active substance. This study aimed to investigate the types of cytokines regulated by PHS, their anti-skin inflammatory effects, and their anti-inflammatory mechanisms. RAW264.7 cells stimulated with Lipopolysaccharides (LPS) were treated with PHS to measure inflammatory factors such as nitric oxide (NO) and prostaglandin E2 (PGE2), and gene expressions of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX2) were confirmed by q-PCR. Cytokines regulated by PHS against LPS-induced inflammation were found through cytokine array, and each factor was reconfirmed through ELISA. Western blot was performed to confirm anti-inflammatory mechanism of Iκbα and MAPK. To confirm anti-skin inflammatory efficacy, HaCaT cells stimulated with TNF-α/IFN-γ were treated with PHS, and TARC, IL-6, and IL-8 were detected by ELISA. PHS suppressed the gene expression of iNOS and COX2, which were increased by LPS, and suppressed NO and PGE2 production. Through cytokine array, it was confirmed that IL-6, IL-10, IL-27 p28/IL-30, IP-10, I-TAC, MCP-5, and TIMP-1 increased by LPS were decreased by PHS. PHS inhibited NF-κB signaling by inhibiting LPS-induced NF-κB nuclear migration and p-Iκbα-mediated Iκbα degradation, and inhibited p38, ERK, and JNK signaling pathways. PHS reduced the production of TARC, IL-6, and IL-8 increased by TNF-α/IFN-γ. These results indicate PHS has anti-inflammatory effects via the suppression of inflammatory factors and pro-inflammatory cytokines through the NF-κB and MAPK pathways. Moreover, these results may explain beneficial effects of PHS in the treatment of skin inflammatory conditions induced by TNF-α/IFN-γ.

Keywords: Anti-atopic dermatitis effect, Anti-inflammatory effect, Cytokine, MAPKs, NF-κB, Phytosphingosine

1. Introduction

Inflammation is a local reaction that occurs when pathogen-caused irritants and damaged cells react, and it is a complex physiological defense response. The excessive inflammatory response then leads to a variety of inflammation types and diseases. Macrophages are one of the cell types that play a significant role in this inflammatory response [1]. Macrophages undergo phagocytosis and have defensive capabilities.

Lipopolysaccharides (LPS) are toxin-causing agents because they increase the production of inflammatory mediators. Activation of signaling pathways by LPS stimulation leads to the induction and secretion of inflammatory factors such as nitric oxide (NO), inducible NO synthase (iNOS), prostaglandin E2 (PGE2), and cyclooxygenase-2 (COX-2) [2]. As well as cytokines (such as TNF-α, IL-1β, and IL-6) cause acute and chronic inflammation by activating macrophages. Controlling the excessive production of cytokines induced by inflammation is crucial for the treatment and prevention of numerous inflammation-related diseases [3].

Overactivation of Nuclear factor-κB (NF-κB) has been demonstrated to be a common cause of inflammation-induced skin diseases [4, 5]. NF-κB regulates the above-mentioned inflammatory responses in macrophages [6]. The mitogen-activated protein kinase (MAPK) pathway mediates LPS-induced activation of KF-κB, leading to the production of pro-inflammatory mediators [7]. There are three subclasses within the mammalian MAPK family: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 mitogen-activated protein kinases (p38) [8-10].

Atopic dermatitis (AD), one of the most common chronic inflammatory skin diseases, is characterized by dry and itchy skin. High concentrations of TARC/CCL17 and MDC/CCL22 are detected in the serum of patients with atopic dermatitis, which is thought to be closely related to this condition. In AD pathogenesis, keratinocytes have similar roles in the immune response to AD by stimulating TNF-α and IFN-γ.

Phytosphingosine (PHS) is a component of the lipids found in the epidermis of the skin [11]. Although it is present in small amounts on human skin, it is a physiologically active ingredient with multiple functions. It is a

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major component of the skin barrier and is known to prevent moisture loss in the skin, and to have sterilization and anti-inflammatory action [12, 13]. Cosmetic formulations of PHS are known to soothe the skin and prevent acne [14, 15]. The PHS used in this study was extracted from *Pichia ciferrii*; it is like sphingosine and is referred to as PHS because it is derived from plants.

However, there has been insufficient research on the anti-inflammatory mechanism and efficacy of PHS in skin inflammation. Therefore, this study aimed to investigate the anti-inflammatory effect and action mechanism of PHS on LPS-stimulated Raw 264.7 macrophages. Additionally, we confirmed the ameliorating effect of PHS on skin inflammation by mimicking the conditions of atopic dermatitis induced by TNF-α/IFN-γ using HaCaT cells, which are human skin keratinocytes typically used in skin immune response studies.

2. Materials and methods

2.1. Experimental materials and equipment

For the Phytosphingosine(PHS) used in the experiment, a CRODA KOREA (Iksan, Korea) product extracted from *Pichia ciferrii* with a purity of at least 95% was used. PHS was utilized via dissolution in DMSO (Sigma, USA). Dexamethasone (Sigma, USA) and indomethacin (Sigma, USA) were used as controls for PHS. LPS (Sigma, USA) was used to induce an inflammatory response in Raw 264.7 cells, and Recombinant Human TNF-α Protein (R&D Systems, USA) and Recombinant Human IFN-γ Protein (R&D Systems, USA) were used to induce atopic stimulation in HaCaT cells. Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), and penicillin-streptomycin were purchased from Gibco. Enzyme-linked immunosorbent assay (ELISA) kits for the measurement of PGE2, IL-6, IL-27 p28/IL-30, I-TAC, MCP-5, and TARC including cytokine array were purchased from R&D System. ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, NF-κB p65, p50, IκBα, phospho-IκBα, β-actin, and α-tubulin were purchased from Cell Signaling Technology, and Lamin B was purchased from Santa Cruz.

2.2. Cell line and cell culture

Murine macrophage RAW264.7 macrophage cell line was purchased from Korean Cell Line Bank, Korea, and spontaneously transformed aneuploid immortal keratinocyte cell line HaCaT cells were purchased from Addex-Bio (San Diego, USA) and used. Both cells were cultured using Dulbecco's Modified Eagle's Medium (Gibco, USA) containing 10% Fetal Bovine Serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). The culture was incubated in an incubator under conditions of 5% CO2, 37°C, and 100% humidity. To prevent overdensity caused by cell proliferation, the cells were subcultured periodically when they reached 80-90% confluence.

2.3. Cell viability evaluation

Using the WST method, cell viability was evaluated. After seeding Raw 264.7 cells at 4 X 10^4 cells/well and HaCaT cells at 1.5 X 10^4 cells/well in a 96-well plate and stabilizing for 24 hours, PHS samples diluted at various concentrations were treated and incubated at 5% CO_2, 37°C for 24 hours. Using a WST assay kit (DoGen, Korea), cell viability was determined. After incubation, the supernatant was removed from each well and replaced with fresh pre-warmed media. 10% of EZ-Cytox was added to the replacement media, which was then incubated in the dark for two hours. Before measuring the absorbance, it was gently shaken for about 1 minute. Absorbance was measured at 450nm using a microplate reader (BioTek, USA).

2.4. Nitric oxide (NO) determination

Raw 264.7 cells were seeded at a density of 4 X 10^4 cells/well in 96-well plates and stabilized for 24 hours. To induce an inflammatory response, PHS was treated with LPS 100ng/ml at various concentrations and incubated at 37°C and 5% CO_2 for 24 hours. A Griess reagent kit (Invitrogen, USA) was used for nitrite quantitation in cultured cells. Equal volumes of N-(1-naphthyl) ethylenediamine and sulfanilic acid were combined to produce Griess reagent. For thirty minutes, the Griess reagent and the cell culture medium interacted. Quantification of nitrite absorbance was accomplished by measuring absorbance at 548 nm with a spectrophotometer (BioTek, USA). The ability to inhibit NO production was expressed as a percentage (%) of the inhibitory activity relative to the control.

2.5. RNA extraction and cDNA synthesis from total RNA

Raw 264. 7 cells and HaCaT cells were seeded at 1 X 10^6 cells/well and 6 X 10^5 cells/well in 6-well plates, respectively. After stabilization for 24 hours, the cells were starved for 24 hours. To induce inflammation, RAW 264.7 cells were treated with LPS 100ng/ml together with samples such as PHS, Dexamethasone, and Indomethcin at each concentration, and incubated under conditions of 5% CO_2 at 37°C. To induce atopic stimulation in HaCaT cells, samples were treated with 2ng/ml of TNF-α and IFN-γ protein and cultured for 24 hours. Following incubation, after the media was removed, PBS was added to wash, and then wash-out was performed. and total RNA was extracted using an RNA purification kit (MACHEREY NAGEL, Germany). RNA was measured with Nanodrop. A cDNA synthesis kit (Bio-Rad, USA) was used to synthesize cDNA. After combining 1μg of total RNA, 1μl of reverse transcriptase, and reaction mix, cDNA was synthesized by priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C, and reverse transcriptase inactivation for 1 minute at 95°C.

2.6. Polymerase chain reaction in real time (RT-PCR)

CFX Opus 96 was used to conduct real-time PCR (Bio-Rad, USA). To measure the expression of the target gene, 1μl of cDNA and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA) were utilized. The relative levels of the target gene were normalized and quantified using GAPDH. The target gene amplification primer sequences for real-time PCR are listed in Table 1. For PCR amplification, pre-denaturation was performed at 95°C for 10 min. The reaction was carried out for 35 cycles under the conditions of denaturation at 95°C for 30 sec. Annealing and extension were performed for 20 seconds at 60°C, and then melt-curve analysis was performed at 65-95°C for 2-5 seconds per step.

2.7. Membrane-based cytokine assay

Raw 264.7 Cells were seeded at a density of 1 X 10^6 cells/well in a six-well plate, stabilized for 24 hours, and

Table 1. Primer Sequences used for real-time PCR.

Gene	Primer	Sequences (5‘-3‘)
Mouse iNOS	Forward	GCCACCAACAATGGCAACAT
	Reverse	CGTACCGGATGAGCTGTGAA
Mouse COX-2	Forward	AGCCCATTGAACCTGGACTG
	Reverse	ACCCAATCAGCGTTTCTCGT
Mouse GAPDH	Forward	GGTCGGTGTGAACGGATTTG
	Reverse	GTGCCGTTGAATTTGCCGT
Human TARC	Forward	CTCCAGGGATGCCATCGTTT
	Reverse	GGTGAGGAGGCTTCAAGACC
Human GAPDH	Forward	TCAAGGCTGAGAACGGGAAG
	Reverse	CGCCCCACTTGATTTTGGAG

then starved for 24 hours. PHS samples were treated with LPS and incubated for 24 hours at 5% CO_2 and 37°C. Following incubation, the media was transferred to a tube and centrifuged to obtain a supernatant for the cytokine assay. The membrane-based immunoassay was purchased and utilized from R&D Systems. The medium and membrane included in the kit were evaluated in accordance with the manufacturer's instructions. Using Image Lab Software (Bio-rad, USA), the density of every spot was analyzed and measured.

2.8. ELISA assay

ELISA (R&D Systems, USA) was used to quantify intracellular Mouse PGE2 and cytokines such as Mouse IL-6, Mouse IL-27 p28/IL-30, Mouse I-TAC, Mouse MCP-5, Human TARC, Human IL-6 and Human IL-8. Raw 264.7 Cells were seeded at a density of 1 X 10^6 cells/well in a six-well plate, stabilized for 24 hours, and then starved for 24 hours. Under conditions of 5% CO_2 and 37°C, cells were treated with PHS samples of various concentrations and LPS 100ng/ml and then incubated for 24 hours. Following incubation, the media was transferred to a tube and centrifuged to obtain a supernatant for the ELISA assay. ELISA Kit (R&D systems, USA) and Reagent Kit (R&D systems, USA) were utilized to measure the concentration of cytokines in the culture medium. Following the manufacturer's instructions, the absorbance at 450 nm was measured after dispensing the cell culture solution into each well of the plate and injecting the supplied standard sample.

2.9. Western blot

Raw 264.7 cells were seeded in 100 mm dishes, stabilized for 24 hours, and then starved. Cells were treated with PHS samples of various concentrations and LPS 100ng/ml and then incubated for 24 hours. After incubation, this was harvested by washing with cold PBS. In order to inhibit Protease and Phosphatase during the protein extraction process, RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma-Aldrich, USA) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, USA) was used. The cells were lysed using ice-cold RIPA buffer. Protein concentration of each sample was determined using a Modified Lowry Protein Assay Kit (Thermo Scientific, USA). Approximately 30 μg of protein supernatant and sample buffer were combined to create a protein mixture for electrophoresis. Each well of TGX gel (Bio-Rad, USA) was loaded with approximately 30 μg of protein mixture and electrophoresed. Using a transfer system (Bio-Rad, USA), the TGX gel protein was transferred to a Western blot nitrocellulose membrane (Bio-Rad, USA) after electrophoresis. Blocker BSA (Thermo Scientific, USA) was diluted with PBS and blocked at room temperature for one hour following transfer. The antibodies such as p38 MAPK, β-actin, Lamin B, α-tubulin, p-SAPK/JNK, COX-2, p-p38 MAPK, p-ERK1/2, SAPK/JNK, ERK1/2, p-IκBα, IκBα, iNOS, p65 and p50 were diluted in Blocker BSA and incubated overnight at 4°C. All antibodies were obtained from CST (Cell Signaling Technology, USA) and utilized. The membrane was washed three times with TBS (Bio-Rad, USA) and Tween-20 (Sigma, USA). The membrane was incubated for one hour after dilution of secondary antibodies conjugated with peroxidase in Clear Milk Blocking Buffer (Thermo Scientific, USA). ECL was used to verify protein expression (Bio-Rad, USA). An imaging system (Bio-Rad, USA) was used to confirm the presence of a protein band.

2.10. Distinction between nucleus and cytoplasm

To measure the amount of NF-κB moving from the cytoplasm to the nucleus, the nucleus and cytoplasm were separated according to the manufacturer's instructions using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA). After measuring the isolated nuclear and cytoplasmic protein concentrations with the Modified Lowry Protein Assay Kit, the expression of p-IκBα, IκBα, NF-κB, α-tubulin, and Lamin B was confirmed by Western blot.

2.11. Statistical analysis

A statistical program, GraphPad Prism 9, was utilized, and analysis of variance for experimental design was evaluated using one-way ANOVA and the independent sample t-test. Each piece of data was evaluated based on the outcomes of three or more repeated experiments, and statistical significance was determined using a *P* value of 0.05.

3. Results

3.1. Cytotoxicity of PHS to Raw264.7 cells and HaCaT cells

The WST-1 assay method was performed to confirm the range of cytotoxicity in Raw 264.7 and HaCaT of PHS. First, cytotoxicity in Raw 264.7 cells, was confirmed through treatment with PHS 2.5, 5, 10, 20, 40, and 80 μg/ml concentration ranges, establishing that PHS concentrations of 5 μg/ml or less are non-toxic (Figure 1A). Next, in order to confirm the cytotoxicity in HaCaT cells, PHS

concentrations in the range of 2.5, 5, 10, 20, 40, and 80 µg/ml were tested (Figure 1B). Results confirmed that PHS concentrations of 2.5 µg/ml or less were non-toxic in HaCaT cells. Therefore, subsequent experiments were conducted in a non-toxic concentration range.

3.2. Inhibition of NO, PGE2 production and iNOS, COX-2 expression in LPS-stimulated RAW264.7 cells

To confirm the effect of PHS on macrophage NO and PGE2 production, Griess assay and ELISA-based PGE2 assay were performed, respectively. After 24 hours of inducing Raw 264.7 cells with LPS-induced inflammatory stimulation, the change in NO and PGE2 production in the cell culture medium was confirmed. The LPS treatment significantly increased NO and PE2 production compared to the untreated control group. It was confirmed that Indomethacin, a nonsteroidal anti-inflammatory drug, inhibited this process. After inducing inflammation, NO and PGE2 production were significantly decreased in a concentration-dependent manner in the experimental group treated with PHS at non-toxic concentrations compared to the LPS-treated group(Figure 2A, B).

Next, gene and protein expression levels were evaluated by qRT-PCR and western blotting to determine whether the NO and PGE2 secretion inhibitory effects of PHS were related to the expression regulation of iNOS and COX, respectively. It was confirmed that the expression levels of iNOS and COX-2 increased compared to the control group when treated with LPS. It was demonstrated that the expression levels of iNOS and COX-2, which were increased by LPS, decreased in proportion to the concentration of PHS(Figure 2C, D). As with COX-2 mRNA expression, LPS increased COX-2 protein, which was confirmed to be inhibited by PHS (Figure 2E, F).

3.3. Inhibition of pro-inflammatory cytokines production in LPS-stimulated RAW264.7 cells

Confirmation of the activity of inflammatory cytokines, one of the factors that increase in response to inflammation, was attempted. A cytokine array was performed to select factors that increase in LPS inflammatory response, among various cytokines, and cytokines whose production is inhibited by PHS at this time (Figure 3A-C). This was confirmed by reacting the cell culture medium of each condition with a membrane coated with various cytokines. The coordinates of the cytokine array on the membrane are shown in Fig. 3A. Fig. 3B shows three membranes that detect multiple analytes in untreated (Control) and treated (LPS 100ng/ml and PHS 5µg/ml) cell culture supernates. The production of IL-6, IL-10, IL-27 p28/IL-30, IP-10, I-TAC, MCP-5, and TIMP-1 increased in response to LPS-induced inflammation and decreased in response to PHS (Figure 3C). An ELISA assay was performed on IL-6,

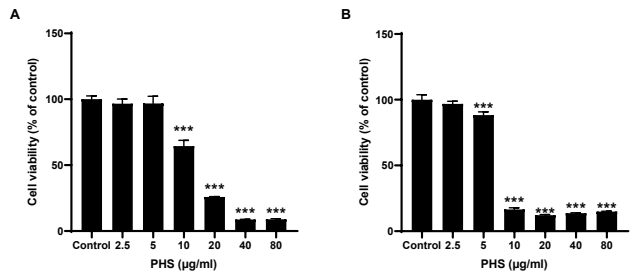

Fig. 1. Cytotoxic effects of Phytosphingosine(PHS) in Raw 264.7 and HaCaT cells. Cell viability was measured with an EZ-cytox reagent. Cells were treated with variable concentrations of PHS (2.5, 5, 10, 20, 40, 80 µg/ml) for 24hr. A: At concentrations below 5 µg/ml, PHS did not significantly affect the cell viability of Raw 264.7 cells after 24 h of treatment. However, at concentrations greater than 5 µg/ml, PHS affected their viability. B: At concentrations below 2.5 µg/ml, PHS did not significantly affect the cell viability of HaCaT cells after 24 h of treatment. However, at concentrations greater than 2.5 µg/ml, PHS affected their viability. The data were expressed as the mean (SD) of three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with control group. PHS, Phytosphingosine.

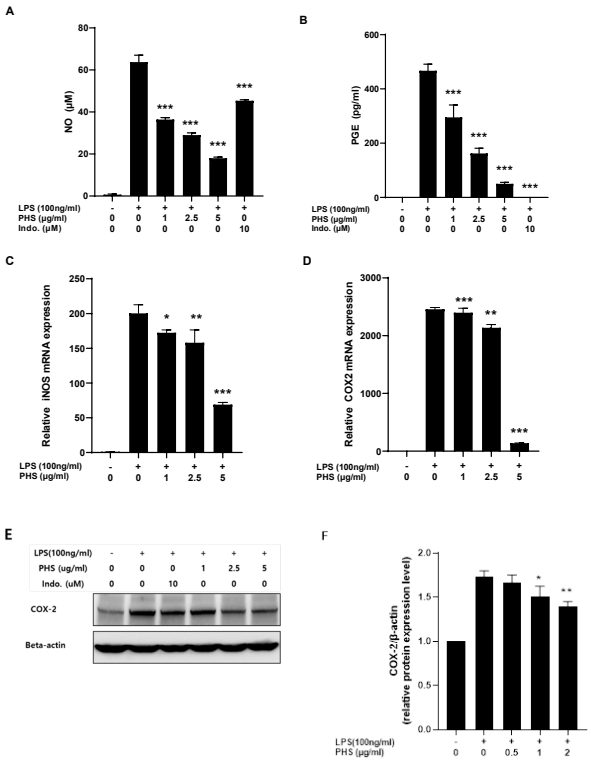

Fig. 2. Inhibitory effect of Phytosphingosine(PHS) on Nitric Oxide (NO) production and Inducible Nitric Oxide Synthase (iNOS) mRNA expression. Raw 264.7 macrophages were treated with 100ng/ml of lipopolysaccharide (LPS), Indomethacin (positive control), and 1, 2.5, 5 µg/ml PHS for 24h. A: Griess assay confirmed the ability of PHS to control NO production. After 24 hours of incubation, the supernatant from the centrifuge was utilized. A microplate reader was used to measure the absorbance of 548nm wavelength. B: After culturing cells for 24 hours, PGE2 of the centrifuged supernatant was measured. The absorbance of 450 nm wavelength was measured using a microplate reader. C: RT-PCR was performed in order to verify that PHS controls iNOS expression. Using iNOS and GAPDH-specific primers, RT-PCR was performed, followed by GAPDH normalization. D: The total RNA was prepared and the mRNA expression of COX-2. RT-PCR analysis was conducted using COX-2 and GAPDH-specific primers, followed by GAPDH normalization. E: COX-2 protein expression was confirmed by western blotting, and lysate of Raw 264.7 macrophages was used. F: Densitometric analysis of COX-2 expression. Anti-actin was used to ensure that the protein loading was equivalent. The data were expressed as the mean (SD) of three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with LPS-stimulated group. PHS, Phytosphingosine. Indo., Indomethacin.

IL-27 p28/IL-30, I-TAC, and MCP-5, among other cytokines (Figure 3D-G). Four factors were increased in the inflammatory response due to LPS stimulation, and it was confirmed that the production was suppressed in a dose-dependent manner according to the concentration of PHS.

3.4. Inhibition of NF-κB activation in LPS-stimulated RAW264.7 cells

To examine the effect of PHS on the activity of NF-κB, a transcriptional regulator that regulates the inflammatory response, and the degradation of p-Iκbα, the nuclei and cytoplasm of cells were isolated, and their protein expression changes were compared (Figure 4).

After 30 minutes of LPS treatment, p-Iκbα expression increased, Iκbα degradation occurred, and nuclear NF-κB p65 and p50 protein expression increased (Figure 4A). However, PHS treatment inhibited LPS-induced phenomena such as Iκbα phosphorylation (Figure 4B)., Iκbα degradation, and translocation of NF-κB p65 and p50 to the nucleus (Figure 4C-F).

These findings confirmed that PHS could inhibit NF-κB signaling, an inflammatory signaling pathway, by inhibiting LPS-induced NF-κB nuclear migration and p-Iκbα-mediated Iκbα degradation.

3.5. Inhibition of MAPKs phosphorylation in LPS-stimulated RAW264.7 cells

MAPK is an activator of the transcription factor NF-κB. It is an important regulator of inflammatory cytokine expression with signaling pathways (ERK, JNK, p38, etc.). To confirm that PHS regulates MAPK activity, the

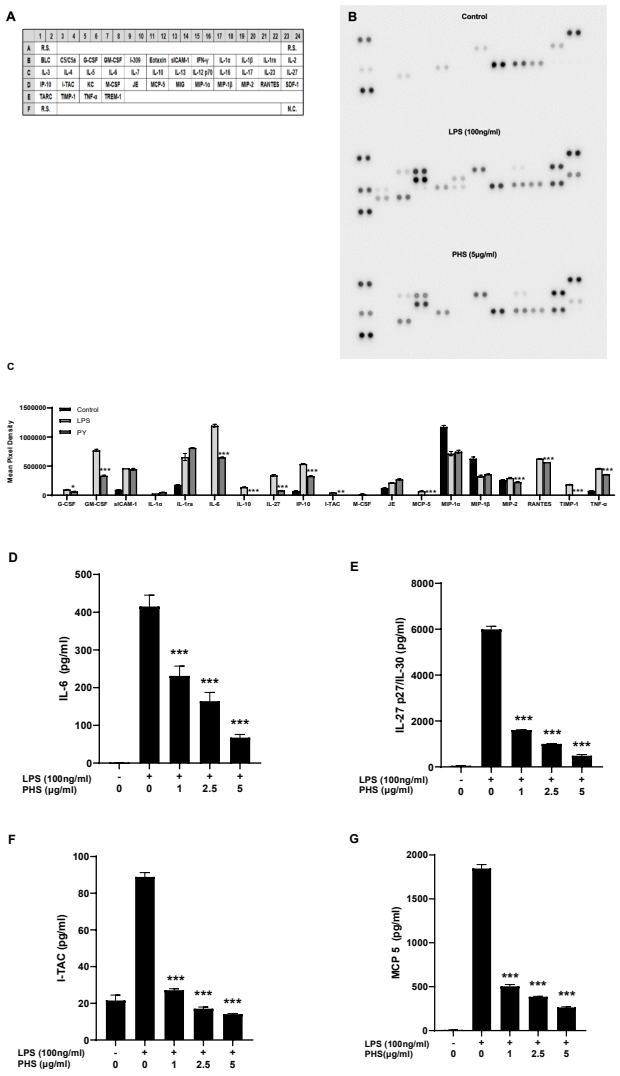

Fig. 3. Changes in the secretion of pro-inflammatory cytokines in Raw 264.7 macrophages by Phytosphingosine(PHS). A: Mouse cytokine array coordinates. B: Analysis of cytokine arrays. Membranes displaying the change in inflammatory cytokines after LPS and PHS treatment for 24 hours. The membrane was subjected to an antigen-antibody reaction with the control group treated with DMSO, 100 ng/ml of LPS, and 5 μg/ml of PHS cultured supernatant, respectively. C: The intensity of a spot was measured densitometrically. Analysis showed significant increase of pro-inflammatory cytokines (IL-6, I-TAC, MCP-5, IL-10, IL-27, IP-10, TIMP-1, TNF-α) when Raw 264.7 cells were treated with 5 μg/ml PHS for 24 h. Four of the pro-inflammatory cytokines were identified by a Sandwich ELISA assay at PHS 1, 2.5, and 5 μg/ml. D: IL-6 production, E: IL-27 production, F: I-TAC production, and G: MCP-5 production. The data were expressed as the mean (SD) of three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with LPS-stimulated group. PHS, Phytosphingosine. Indo., Indomethacin. R.S., Reference Spot. N.C., Negative Control.

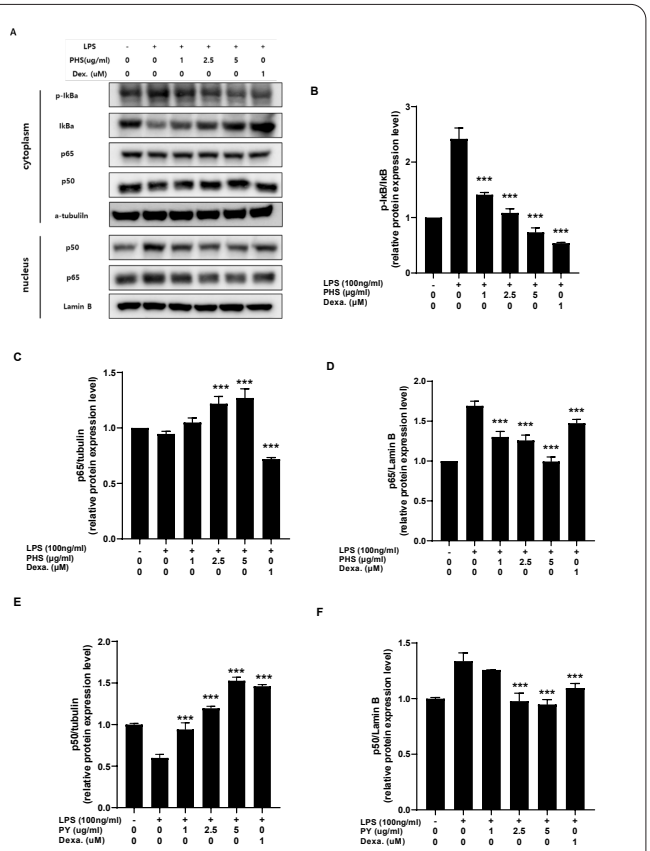

Fig. 4. Phytosphingosine(PHS) inhibits the phosphorylation of Iκbα and the translocation of NF- κB. A: For Western blot analysis of IκBα and NF-κB p65 and p50, various concentrations of PHS and dexamethasone were pre-treated for 2 hours, followed by 30 minutes of LPS treatment. The cells were then harvested and fractionated into the nuclear and cytoplasmic fractions, and the fractions were immunoblotted with anti-p-IκBα, anti-IκBα, anti-p65, anti-p50, anti-tubulin, anti-Lamin B antibodies. Bars represent relative protein quantification of B: p-IκBα/ IκBα, C: p65/tubulin, D: p65/Lamin B, E: p50/tubulin and F: p50/Lamin B. The data were expressed as the mean (SD) of three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA. ***$P<0.001$, compared with LPS-stimulated group. PHS, Phytosphingosine. Dexa., Dexamethasone.

phosphorylation of p38, ERK, and JNK was examined using western blotting (Figure 5A). As a result, it was confirmed that phosphorylation of p38, ERK, and JNK increased during LPS treatment and that treatment with PHS inhibited the activity of p38, ERK, and JNK in a concentration-dependent manner (Figure 5B-D). Consequently, it was determined that the anti-inflammatory effect of PHS was attained by inhibiting the p38, ERK, and JNK signaling pathways.

3.6. Inhibition of TARC, pro-inflammatory cytokines production in TNF-α/IFN-γ-stimulated HaCaT cells

The mRNA expression and extracellular secretion of TARC, a type of chemokine produced during an atopic inflammatory response, were measured using RT-PCR and ELISA. Results confirmed that TNF-α/IFN-γ stimulation increased the expression of TARC mRNA (Figure 6A) and the concentration of TARC in the cell culture medium (Figure 6B). However, it was also confirmed that TARC mRNA expression and extracellular secretion were suppressed in a concentration-dependent manner during PHS treatment. In addition, it was determined that the concentrations of IL-6 and IL-8, typical inflammatory cytokines, increased in the cell culture medium when TNF-α/IFN-γ was added, but were significantly reduced when PHS was added (Figure 6C, D). Consequently, PHS was deemed to effectively inhibit TARC, IL-6, and IL-8 in keratinocytes, making it an effective anti-skin inflammatory agent, such as atopic dermatitis.

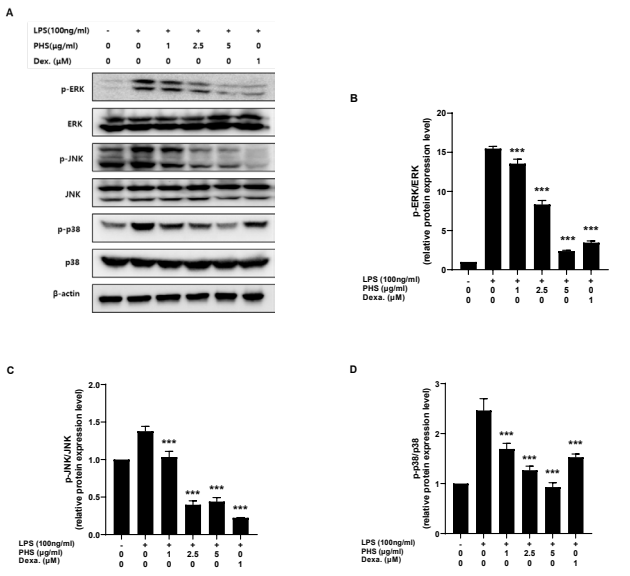

Fig. 5. Western blot results of the mitogen-activated protein kinase (MAPK) pathways in the Raw 264.7 macrophages. A: Representative western blots of total and phosphorylated MAPK family proteins ERK, JNK, and p38. For Western blot analysis of ERK, JNK, and p38 MAPK phosphorylation, pre-treatment with various concentrations of PHS and dexamethasone was performed for 2 hours, followed by LPS for 30 minutes. Immunoblotting with anti-p-ERK, anti-ERK, anti-p-JNK, anti-JNK, anti-p-p38, anti-p38, and anti-β-actin was performed using cell lysate. Semi-quantitative densitometric analysis of B: p-ERK, C: p-JNK, and D: p-p38. The data were expressed as the mean (SD) of three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA. ***$P<0.001$, compared with LPS-stimulated group. PHS, Phytosphingosine. Dexa., Dexamethasone.

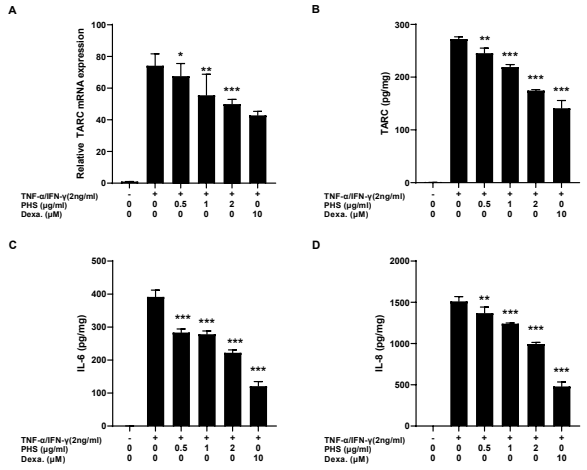

Fig. 6. Effects of Phytosphingosine(PHS) on TARC, IL-6, and IL-8 in TNF-α(2ng/ml)/IFN-γ(2ng/ml)-stimulated HaCaT Cells. A: The total RNA was prepared and the mRNA expression of TARC. Using TARC and GAPDH-specific primers, RT-PCR was performed, followed by GAPDH normalization. Using cell culture supernatant, Sandwich ELISA assay was performed. B: TARC production, C: IL-6 production, D: IL-8 production. The data were expressed as the mean (SD) of three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with TNF-α/IFN-γ-stimulated group. PHS, Phytosphingosine. Dexa., Dexamethason.

4. Discussion

The purpose of this study was to examine the potential of Phytosphingosine (PHS) as a cosmetic material for enhancing anti-inflammation by validating its cytotoxicity, anti-inflammatory effect, and pro-inflammatory cytokine inhibiting effect and mechanism.

Inflammatory responses are induced by macrophages, which migrate other immune cells to the site of inflammation [16-18]. Macrophages are known to play a significant role in the inflammatory response. Raw 264.7 cells are commonly used as an experimental model for validating the efficacy of anti-inflammatory agents and assessing their inhibitory effects on related signaling pathways [19-23]. To confirm the effect of PHS on these macrophages, we confirmed the cytotoxicity of PHS and used LPS to induce an inflammatory response.

To investigate the anti-inflammatory potential of PHS, the concentration range in which no cytotoxicity by PHS was observed was confirmed by cytotoxicity experiments (Figure 1). After inducing inflammation, PHS treatment inhibited NO and PGE2 production in LPS-induced Raw 264.7 cells in a dose-dependent manner, confirming NO and PGE2, which are known early-stage mediators of inflammation (Figure 2). As a result of examining the expression of iNOS and COX-2, which are representative inflammation-related signaling pathway factors of NO and PGE2, PHS was found to decrease iNOS expression in a concentration-dependent manner, as well as COX-2 expression. These findings imply that PHS regulates the PGE2/COX-2 pathway in addition to the NO and iNOS pathways.

Cytokines are involved in all stages of inflammation, and it was determined that LPS-induced cytokines could be inhibited by PHS. Performing a serum cytokine array to investigate the correlation between PHS and inflammatory cytokine secretion confirmed that the expression

of the inflammatory cytokines increased by LPS stimulation, IL-6, IL-27 p28/IL-30, I-TAC, and MCP-5, was suppressed to exhibit an anti-inflammatory effect (Figure 3B). A significant change in relation to the PHS concentration was confirmed for each factor via ELISA (Figure 3D-G). Therefore, it was confirmed that PHS can suppress the cytokines that increase during an inflammatory reaction.

NF-κB is a protein complex that plays a role in the production of these cytokines and is activated by various inflammatory stimuli [24]. In its inactive state, NF-κB is located in the cytoplasm and binds to the inhibitory protein IκBα [25, 26]. NF-κB translocates to the nucleus in an activated state upon phosphorylation, subsequent ubiquitination and dissociation of inhibitory proteins by stimuli such as inflammatory stimuli [27]. The anti-inflammatory biological activity of PHS was confirmed to be associated with inhibition of NF-κB signaling (Figure 4). Iκbα phosphorylation was increased by LPS inflammatory stimulation (Figure 4B), and it was inhibited by PHS. This correlation indicated that the translocalization of NK-κB in the nucleus (Figure 4D), which was increased by LPS, was reduced by PHS.

Various upstream kinases phosphorylate the serine residue (p65 phosphorylation site) of NF-κB/p65 protein [28-30]. MAPK, a Family of serine/threonine kinases, are considered upstream kinases that regulate NF-κB MAPK has also been associated with LPS-induced cytokine production. Here it was determined that PHS inhibited the LPS-induced p38, ERK, and JNK activities, confirming that PHS inhibits phosphorylation (Figure 5). These findings imply that the immune activity of PHS is associated with its primary mechanism of action, which involves the inhibition of MAPK and NF-κB signaling.

Thymus and activation-regulated chemokine (TARC/CCL17) are known to be involved in the production of this chemokine because it contains an NF-κB binding site, according to previous reports [31-33]. TARC is a member of the CC chemokine family and is produced by numerous cells, including skin keratinocytes. TARC induces migration of Th2 lymphocytes to sites of inflammation [34]. In addition, the high concentration of TARC in human blood serum is found in both IgE-mediated and non-IgE-mediated atopic dermatitis, confirming that TARC is a mediator of atopic dermatitis [35]. Therefore, the inhibition of TARC in keratinocytes is anticipated to alleviate atopic dermatitis. In this study, we induced an inflammatory reaction in HaCaT cells, which are human keratinocytes, by TNF-α/IFN-γ treatment, and confirmed changes in TARC mRNA and protein levels (Figure 6A, B). As a result, it was confirmed that TARC protein secretion and mRNA expression were increased by TNF-α/IFN-γ in keratinocytes, which were significantly decreased by PHS concentration-dependent treatment. In addition, the inhibitory effect of the cytokines IL-6 and IL-8 was confirmed (Figure 6C, D).

In conclusion, our findings suppose that PHS has the anti-inflammatory properties that may be mediated at least in part by suppressing the NF-κB and MAPK signaling pathway and inhibiting pro-inflammatory cytokines in macrophages. In addition, inhibition of TARC, a highly useful clinical biomarker for atopic dermatitis, may provide an approach to the treatment of skin inflammation, such as atopic dermatitis. To further develop this study in the future, it is believed that additional research on the effects on other primary immune cells, clinical trials, and the detailed mechanisms that cause atopic dermatitis must be conducted.

5. Conclusion

Phytosphingosine(PHS) is known to prevent moisture loss in the skin, regulate epidermal cell growth, differentiation and apoptosis, and has anti-inflammatory effects. This study aimed to investigate the types of cytokines regulated by PHS, their anti-inflammatory mechanisms, and their anti-atopic effects. It was confirmed that PHS inhibited iNOS/NO and PGE2/COX-2 induced by inflammatory stimulation in a dose-dependent manner. In addition, it was confirmed that PHS inhibited the secretion of cytokines associated with inflammation, and that the NF-kB and MAPK pathways are involved. Inflammation in the skin causes atopic dermatitis, and this study confirmed that the anti-inflammatory effect of PHS could improve this condition. These results showed that PHS has anti-inflammatory effects and can be used as a functional material to help improve atopic dermatitis.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed Consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Mikyung Sung: Research design and perform all laboratory procedures; Sojung Lim: Research design and perform all laboratory procedures; Seungwon Park: Research administration and review; Yongjin Choi: Research administration and review; Sangchul Kim: Research administration and supervision.

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References

1. Locati M, Curtale G, Mantovani A (2020) Diversity, Mechanisms, and Significance of Macrophage Plasticity. Annu Rev Pathol 15: 123-147. doi: 10.1146/annurev-pathmechdis-012418-012718
2. Kim KS, Han CY, Han YT, Bae EJ (2019) Rhodanthpyrone A and B play an anti-inflammatory role by suppressing the nuclear factor-kappaB pathway in macrophages. Korean J Physiol Pharmacol 23 (6): 493-499. doi: 10.4196/kjpp.2019.23.6.493
3. Feghali CA, Wright TM (1997) Cytokines in acute and chronic inflammation. Front Biosci 2: d12-26. doi: 10.2741/a171
4. Kumari S, Van TM, Preukschat D, Schuenke H, Basic M, Bleich A, Klein U, Pasparakis M (2021) NF-κB inhibition in keratinocytes causes RIPK1-mediated necroptosis and skin inflammation.

Life Sci Alliance 4 (6). doi: 10.26508/lsa.202000956
5. Deng Z, Xu S, Peng Q, Sha K, Xiao W, Liu T, Zhang Y, Wang B, Xie H, Chen M, Li J (2021) Aspirin alleviates skin inflammation and angiogenesis in rosacea. Int Immunopharmacol 95: 107558. doi: 10.1016/j.intimp.2021.107558
6. Bae YA, Cheon HG (2016) Activating transcription factor-3 induction is involved in the anti-inflammatory action of berberine in RAW264.7 murine macrophages. Korean J Physiol Pharmacol 20 (4): 415-424. doi: 10.4196/kjpp.2016.20.4.415
7. Fu W, Fang X, Wu L, Hu W, Yang T (2022) Neogambogic acid relieves myocardial injury induced by sepsis via p38 MAPK/NF-kappaB pathway. Korean J Physiol Pharmacol 26 (6): 511-518. doi: 10.4196/kjpp.2022.26.6.511
8. Kim EK, Choi EJ (2015) Compromised MAPK signaling in human diseases: an update. Arch Toxicol 89 (6): 867-882. doi: 10.1007/s00204-015-1472-2
9. Kim EK, Choi EJ (2010) Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802 (4): 396-405. doi: 10.1016/j.bbadis.2009.12.009
10. Hotamisligil GS, Davis RJ (2016) Cell Signaling and Stress Responses. Cold Spring Harb Perspect Biol 8 (10). doi: 10.1101/cshperspect.a006072
11. Kim BH, Lee JM, Jung YG, Kim S, Kim TY (2014) Phytosphingosine derivatives ameliorate skin inflammation by inhibiting NF-κB and JAK/STAT signaling in keratinocytes and mice. J Invest Dermatol 134 (4): 1023-1032. doi: 10.1038/jid.2013.453
12. Choi HK, Cho YH, Lee EO, Kim JW, Park CS (2017) Phytosphingosine enhances moisture level in human skin barrier through stimulation of the filaggrin biosynthesis and degradation leading to NMF formation. Arch Dermatol Res 309 (10): 795-803. doi: 10.1007/s00403-017-1782-8
13. Hasanovic A, Hoeller S, Valenta C (2010) Analysis of skin penetration of phytosphingosine by fluorescence detection and influence of the thermotropic behaviour of DPPC liposomes. Int J Pharm 383 (1-2): 14-17. doi: 10.1016/j.ijpharm.2009.08.038
14. Zhou M, Yang M, Zheng Y, Dong K, Song L, He C, Liu W, Wang Y, Jia Y (2020) Skin surface lipidomics revealed the correlation between lipidomic profile and grade in adolescent acne. J Cosmet Dermatol 19 (12): 3349-3356. doi: 10.1111/jocd.13374
15. Pavicic T, Wollenweber U, Farwick M, Korting HC (2007) Anti-microbial and -inflammatory activity and efficacy of phytosphingosine: an in vitro and in vivo study addressing acne vulgaris. Int J Cosmet Sci 29 (3): 181-190. doi: 10.1111/j.1467-2494.2007.00378.x
16. Viola A, Munari F, Sánchez-Rodríguez R, Scolaro T, Castegna A (2019) The Metabolic Signature of Macrophage Responses. Front Immunol 10: 1462. doi: 10.3389/fimmu.2019.01462
17. Van den Bossche J, O'Neill LA, Menon D (2017) Macrophage Immunometabolism: Where Are We (Going)? Trends Immunol 38 (6): 395-406. doi: 10.1016/j.it.2017.03.001
18. Nelson MC, O'Connell RM (2020) MicroRNAs: At the Interface of Metabolic Pathways and Inflammatory Responses by Macrophages. Front Immunol 11: 1797. doi: 10.3389/fimmu.2020.01797
19. Zhou M, Tang Y, Liao L, Liu M, Deng Y, Zhao X, Li Y (2021) Phillygenin inhibited LPS-induced RAW 264.7 cell inflammation by NF-κB pathway. Eur J Pharmacol 899: 174043. doi: 10.1016/j.ejphar.2021.174043
20. Zeng J, Zhao N, Yang J, Kuang W, Xia X, Chen X, Liu Z, Huang R (2022) Puerarin Induces Molecular Details of Ferroptosis-Associated Anti-Inflammatory on RAW264.7 Macrophages. Metabolites 12 (7). doi: 10.3390/metabo12070653
21. Xie Z, Wang Y, Huang J, Qian N, Shen G, Chen L (2019) Anti-inflammatory activity of polysaccharides from Phellinus linteus by regulating the NF-κB translocation in LPS-stimulated RAW264.7 macrophages. Int J Biol Macromol 129: 61-67. doi: 10.1016/j.ijbiomac.2019.02.023
22. Won S, Ko JH, Jeon H, Park SS, Kim SN (2021) Co-Administration of Gagam-Sipjeondaebo-Tang and Ibuprofen Alleviates the Inflammatory Response in MPTP-Induced Parkinson's Disease Mouse Model and RAW264.7 Macrophages. Pathogens 10 (3). doi: 10.3390/pathogens10030268
23. Giovanni M, Yue J, Zhang L, Xie J, Ong CN, Leong DT (2015) Pro-inflammatory responses of RAW264.7 macrophages when treated with ultralow concentrations of silver, titanium dioxide, and zinc oxide nanoparticles. J Hazard Mater 297: 146-152. doi: 10.1016/j.jhazmat.2015.04.081
24. Thoma A, Lightfoot AP (2018) NF-kB and Inflammatory Cytokine Signalling: Role in Skeletal Muscle Atrophy. Adv Exp Med Biol 1088: 267-279. doi: 10.1007/978-981-13-1435-3_12
25. Magnani M, Crinelli R, Bianchi M, Antonelli A (2000) The ubiquitin-dependent proteolytic system and other potential targets for the modulation of nuclear factor-kB (NF-kB). Curr Drug Targets 1 (4): 387-399. doi: 10.2174/1389450003349056
26. Wang T, Zhang X, Li JJ (2002) The role of NF-kappaB in the regulation of cell stress responses. Int Immunopharmacol 2 (11): 1509-1520. doi: 10.1016/s1567-5769(02)00058-9
27. Yin L, Shi C, Zhang Z, Wang W, Li M (2021) Formosanin C attenuates lipopolysaccharide-induced inflammation through nuclear factor-kappaB inhibition in macrophages. Korean J Physiol Pharmacol 25 (5): 395-401. doi: 10.4196/kjpp.2021.25.5.395
28. Lai JL, Liu YH, Liu C, Qi MP, Liu RN, Zhu XF, Zhou QG, Chen YY, Guo AZ, Hu CM (2017) Indirubin Inhibits LPS-Induced Inflammation via TLR4 Abrogation Mediated by the NF-kB and MAPK Signaling Pathways. Inflammation 40 (1): 1-12. doi: 10.1007/s10753-016-0447-7
29. Dong N, Li X, Xue C, Zhang L, Wang C, Xu X, Shan A (2020) Astragalus polysaccharides alleviates LPS-induced inflammation via the NF-κB/MAPK signaling pathway. J Cell Physiol 235 (7-8): 5525-5540. doi: 10.1002/jcp.29452
30. Ren J, Su D, Li L, Cai H, Zhang M, Zhai J, Li M, Wu X, Hu K (2020) Anti-inflammatory effects of Aureusidin in LPS-stimulated RAW264.7 macrophages via suppressing NF-κB and activating ROS- and MAPKs-dependent Nrf2/HO-1 signaling pathways. Toxicol Appl Pharmacol 387: 114846. doi: 10.1016/j.taap.2019.114846
31. Qi XF, Kim DH, Yoon YS, Li JH, Song SB, Jin D, Huang XZ, Teng YC, Lee KJ (2009) The adenylyl cyclase-cAMP system suppresses TARC/CCL17 and MDC/CCL22 production through p38 MAPK and NF-kappaB in HaCaT keratinocytes. Mol Immunol 46 (10): 1925-1934. doi: 10.1016/j.molimm.2009.03.018
32. Lee H, Lee DH, Oh JH, Chung JH (2021) Skullcapflavone II Suppresses TNF-α/IFN-γ-Induced TARC, MDC, and CTSS Production in HaCaT Cells. Int J Mol Sci 22 (12). doi: 10.3390/ijms22126428
33. Kwon DJ, Bae YS, Ju SM, Goh AR, Youn GS, Choi SY, Park J (2012) Casuarinin suppresses TARC/CCL17 and MDC/CCL22 production via blockade of NF-κB and STAT1 activation in HaCaT cells. Biochem Biophys Res Commun 417 (4): 1254-1259. doi: 10.1016/j.bbrc.2011.12.119
34. Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG (2000) A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol 115 (4): 640-646. doi: 10.1046/j.1523-1747.2000.00115.x
35. Koizumi M, Kuzume K, Ishida Y, Midoro-Horiuti T (2022) Serum thymus and activation-regulated chemokine (TARC) levels correlate with atopic dermatitis disease severity in patients < 6 months. Allergy Asthma Proc 43 (5): 461-467. doi: 10.2500/

aap.2022.43.220034