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만성 피부 질환으로 발생하는 스트레스 개선을 위한 호박, 작약, 타트체리 복합물의 효능 연구

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Study on the Efficacy of Paeonia Japonica, Cucurbita Moschata and Prunus Cerasus Complex Extract for Alleviating Stress Associated with Chronic Skin Conditions

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요 약 : 현대사회에서 스트레스와 긴장감은 피할 수 없는 요인이다. 다양한 피부질환은 스트레스를 일으키는 중요한 요인으로 언급되고 있다. 피부질환을 가진 환자들은 수면상태가 원활하지 않아 전반적으로 수면 효율이 낮다. 또한 피부질환으로 인해 심리적 스트레스 수치가 높아지고, 이와 같은 과정은 반복적으로 발생하고 있다. 피부질환과 스트레스는 상호적으로 연관되어 있으며, psychodematology에 대한 연구가 증가하고 있다. 이에 본 연구에서는 피부질환을 저하 시킬 수 있는 호박, 작약, 타트체리 복합물을 활용하여 피부 각질 형성 세포에서 스트레스로 인한 만성 피부질환을 개선할 수 있는 소재를 개발하고 효능을 입증하고자 하였다. HaCaT 각질형성세포에 복합 추출물은 12.5, 25, 50, 100 μg/mL 농도 의존적으로 TNF-α, IL-1β, IL-6, MDC, TARC 발현량이 저해되었으며 특히 IL-1β의 경우, 100 μg/mL의 농도에서 40% 이상 저해하는 우수한 효능을 확인하였다. 또한 AQP-3, HA, filaggrin의 생성량 농도 의존적으로 유의미한 증가를 보이며 TNF-α/IFN-γ로 증가된 p-ERK, p-JNK, p-p38의 단백질 발현은 복합 추출물의 처리로 유의하게 감소시 키는 것으로 나타났다. 이를 통하여 해당 복합 추출물은 피부질환을 치료

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및 예방할 수 있는 소재로서 활용가치가 있는 것으로 판단되며, 이는 피부질환과 스트레스 간의 상호 관계의 악영향을 낮춰 줄 것으로 판단된다.

주제어 : 피부 장벽, 스트레스, 면역, 항염증, 각질 세포

Abstract : In modern society, where tension and stress are ubiquitous, individuals often experience psychological imbalances. These stressors not only affect mental well-being but also manifest physically, through the skin. Consequently, a new term psychodermatology combining psychiatry and dermatology, has emerged, garnerning attention and research focus. In this study, we aimed to develop materials improving chronic skin conditions caused by stress by utilizing a compound of *Cucurbita moschata*, *Paeonia japonica*, and *Prunus cerasus* known to alleviate skin disorders. We sought to develop and validate the efficacy of materials alleviating chronic skin conditions induced by stress in keratinocytes..Therefore, in this study we analyzed the effects of a complex extract using *Cucurbita moschata*, *Paeonia japonica*, and *Prunus cerasus* on HaCaT keratinocyte cells to understand how it influences them. The complex extract on HaCaT keratinocyte cells showed a concentration-dependent decrease in the expression levels of TNF-α, IL-1β, IL-6, MDC, and TARC at concentrations of 12.5, 25, 50 and 100 μg/mL. Particularly noteworthy was the efficacy observed in inhibiting IL-1β, with a reduction of over 40% at a concentration of 100 μg/mL. Additionally, the production levels of AQP-3, HA, and filaggrin exhibited a significant concentration-dependent increase. The protein expression of p-ERK, p-JNK, and p-p38, which were elevated by TNF-α/IFN-γ, was significantly decreased with the treatment of the complex extract. These findings suggest that the compound extract may be utilized as a material for treating and preventing skin conditions, potentially mitigating the adverse effects of the mutual relationship between skin disorders and stress.

Keywords : Skin barrier, Stress, Immune, Anti-inflammatory, Keratinocyte cells

1. Introduction

Modern society may enjoy progress and a prosperous lifestyle, but it also grapples with a mental health imbalance caused by tension and stress [1]. Excessive stress, accompanying mental disorders such as depression, sleep disorders, chronic fatigue, and mood disorders, significantly diminishes the quality of life [2]. Conditions like depression and skin issues may have a high correlation. The skin, being rich in nerves, blood vessels, and smooth muscles, is influenced by the autonomic nervous system, making it one of the most sensitive organs to stress. Recent discoveries indicate that nerves, including Langerhans cells and the neuropeptide calcitonin gene-related peptide (CGRP), which plays a crucial role in the skin's immune response, are in contact with the skin. This suggests direct involvement of the nervous system in regulating the immune function of the skin [3].

The prevalence of chronic inflammatory skin diseases is increasing in developing countries, and many researchers are conducting studies on mental health and its negative impact related to stress, depression, sleep disorders, etc [4].

According to one of the studies, patients with common skin disorders have been found to have a higher prevalence of depression, anxiety disorders, and suicidal ideation [5]. Similarly, it is known that patients with skin disorders often experience disrupted sleep

patterns, leading to overall decreased sleep efficiency, and high levels of psychological stress, forming a vicious cycle. Stress and skin disorders are mutually associated [6, 7].

The main cells involved in the formation of the epidermal barrier, keratinocytes, play an essential role in recognizing external substances or potential threats through pattern recognition receptors, cytokines, chemokines, etc., in innate immune responses. Interferon-γ (IFN-γ) is a cytokine that regulates various cellular activities, including antiviral immunity, cell death, and cell cycle degradation, inducing the expression of chemokine [8].

Chemokines, which play a key role in immune and inflammatory responses, are small proteins that regulate the controlled migration of white blood cells [8]. Thymus and activation-regulated chemokine (TARC, CCL17) and macrophage-derived chemokine (MDC, CCL22), both belonging to the CCR4+ chemokine family, play a crucial role in the migration of inflammatory cells, particularly contributing to the migration of CCR4+ Th2 cells [9]. During the acute exacerbation phase of atopic dermatitis, CCR4+ Th2 cells infiltrate the skin, and the expression of TARC (CCL17) and MDC (CCL22) is known to increase. After steroid treatment, their expression tends to decrease [9].

Cytokines are primarily secreted by monocytes or macrophages and lymphocytes, but they can also be secreted by brain cells such as neurons, endothelial cells, astrocytes, and microglia. They are categorized into interleukins, chemokines, tumor necrosis factors, and interferons. In pathological conditions like acute or chronic inflammation or tissue damage, immune dysfunction is activated, leading to increased activity of immune cells and elevated levels of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α. This immune response also regulates the production of the autonomic nervous system. Thus, the immune system, through the secretion of cytokines from immune cells, can influence the central nervous system, impacting the behavior and emotions of the brain [10].

Tumor necrosis factor (TNF) is known to promote immune responses, inflammation, insulin resistance, and induce the expression of chemokines as a cytokine. TNF also facilitates the infiltration of monocytes/T-cells from the bloodstream into inflammatory areas in the skin [9]. Additionally, in experimental research utilizing Yigan Powder, commonly used for conditions like insomnia and night sweats, cases have been reported where the expression of inflammatory cytokines such as TNF-α and IL-4 was inhibited, leading to an improvement in symptoms of atopic dermatitis [10].

Paeonia japonica, belonging to the Paeoniaceae family, has been traditionally used as a therapeutic remedy for abdominal pain, pain relief, hypertension, and inflammation. According to recent research, peony has been reported to have anti-inflammatory, antioxidant, and immunomodulatory effects [11, 12].

Cucurbita moschata contains carotenoids, which act as antioxidants similar to neutralizers in the body, and is rich in beta-carotene, effective for inflammation in the skin and lungs [13]. Among them, *Cucurbita moschata* seeds contain a significant amount of fat and protein, with higher content compared to other parts [14, 15]. Pumpkin seeds are reported to have effects as antioxidants, anti-inflammatory agents, and antidepressants, improving chronic stress-induced depression in mice [15, 16].

Prunus cerasus is a type of cherry native to the region spanning Western Europe to Turkey, known for its strong sour taste. It has been used for medicinal and plant purposes for Approximately 100 years. *Prunus cerasus* is known for its efficacy in reducing chronic inflammation, and it contains anthocyanin, an antioxidant that contributes to its anti-aging effects [17]. The melatonin contained in tart cherry is involved in regulating sleep and circadian rhythms, inducing sleep through specific enzyme activity at night [18].

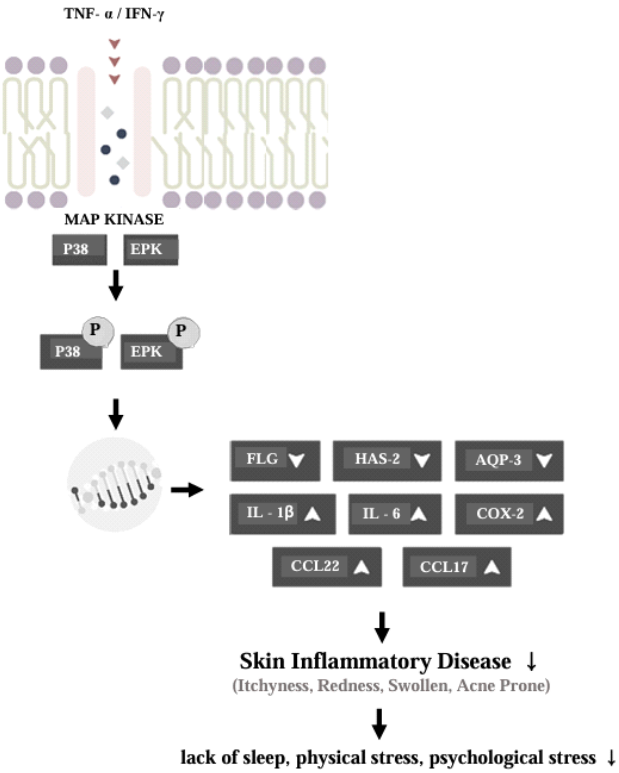


Fig. 1. The mechanism of the relationship between skin inflammation of cytokines ans Stress.

Therefore, in this study, we aim to develop and demonstrate the efficacy of materials that can simultaneously treat skin inflammatory conditions and sleep disorders using three substances. These materials are intended to improve chronic skin conditions caused by stress in skin keratinocytes.

2. Material and Methods

2.1. Preparation of the Extract

The *Paeonia japonica* root (Hello Green), *Cucurbita moschata* seeds (Nuts Brother), and *Prunus cerasus* fruit (Nature Dream) used in this experiment were directly purchased and used after washing. The sample was prepared and processed in a ratio of 1:2:1, consisting of 1 part *Cucurbita moschata* seeds, 2 parts *Paeonia japonica* root, and 1 part *Prunus cerasus* friut. It was extracted with a 70% ethanol solvent at room temperature for 24 hours with agitation. Each extract was filtered through Whatman No. 1 filter paper (Whatman Inc., Piscataway, NJ, USA) and then freeze-dried at −85℃ using a freeze dryer(FD8518, Ilshinbiobase Co., Dongducheon, Korea) (Fig. 1). N.ex is a complex extract composed of three ingredients: *Paeonia lactiflora* root, *Cucurbita moschata* seeds, and *Prunus cerasus* fruit. The final yield of N.ex was determined to be 14.5%.

2.2. Reagents

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and 0.4% trypan blue stain used in cell culture were purchased from Gibco BRL Co (Grand Island, USA). The haemacytometer (Marienfeld, Germany) and 3-[4,5-dimethylthiazol]-2-yl]-

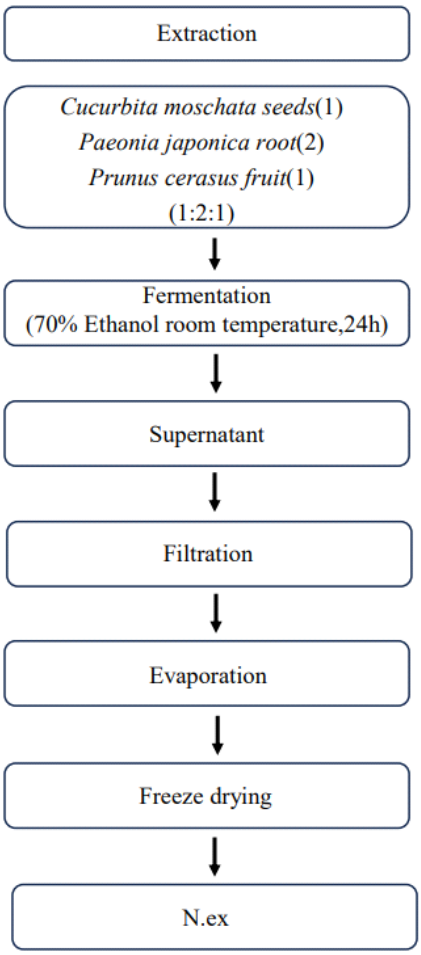


Fig. 2. Flow chart of manufacturing precess to produce the extract from dried N.ex. (*Paeonia lactiflora* root, *Cucurbita moschata* seeds, and *Prunus cerasus* fruit)

2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α, IL-1β, IL-6 kits were purchased from Thermo Fisher Science, MDC, TARC kits from LSbio, Aquaporin-3 enzyme-linked immunosorbent assay (ELISA) from Elabscience, and Hyaluronic acid (HA) kit and Filaggrin Kit from LSbio. Primary antibodies such as p-ERK(#4370), ERK(#4695), pp-38 (#4511), p38(#9212), p-JNK(#9251), JNK (#9522), iNOS(#95423) and COX-2(#12282) protein expression verification were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). For RT-PCR as an experimental material for confirming mRNA expression, SYBR green master mix was purchased from Promega (Madison, WI, USA). Oligonucleotide primers for IL-6, TNF-α, TARC, MDC, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained through Bioneer (Daejeon, Republic of Korea) (Table 1).

2.3. Cell culture

The human keratinocyte cell line HaCaT was obtained and used from Keimyung University. Cells were cultured using DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/mL). The cells were adapted and cultured in a 37℃, 5% CO_2 incubator.

2.4. Cell toxicity measurement by MTT assay

HaCaT cells were seeded at a density of 1×10^4 cells/well in a 96-well plate, and samples were prepared at concentrations of 12.5, 25, 50, 100 and 200 μg/mL, with 0.02 mL added to each well. The cells were incubated for 24 hours in a 37℃, 5% CO_2 incubator. Afterward, an MTT solution, prepared at a concentration of 5 mg/mL, was added at 0.02 mL per well, and the cells were cultured for an additional 3 hours. The culture medium was then removed, and 0.15 mL of DMSO was added to each well. After reacting at room temperature for 15 minutes, the absorbance was measured at 540 nm using an ELISA reader. Cell toxicity was expressed as the percentage decrease in absorbance compared to the non-addition group.

Table 1. Primer sequences for real-tome PCR

Gene symbol	Accession Number	Primer sequence	Product Size(bP)
AQP3	NM_004925	F: 5′ -CACCTCCATGGGCTTCAACT-3′ R: 5′ -TGCCCATTCGCATCTACTCC-3′	278
HAS2	NM_005328	F: 5′ -GAGCACCAAGGTTCTGCTTC-3′ R: 5′ -CTCTCCATACGGCGAGAGTC-3′	154
TNF-α	NM_00594	F: 5′ -TGA GCC ATC GTG CCA ATG-3′ R: 5′ -AGC CCG TCT TCA TCC A-3′	79
FLG	NM_002016	F: 5′ -CAAATCCTGAAGAATCCAGATGAC-3′ R: 5′ -TGCTTGAGCCAACTTGAATACC-3′	126
IL-1β	NM_000576	F: 5′ -ATGCACCTGTACGATCACTG-3′ R: 5′ -ACAAAGGACATGGAGAACACC-3′	142
CCL22	NM_009137	F: 5′ -TGGTGCCAAGGAAGAC-3′ R: 5′ -GAAGAACTCCTTCACTACGCGC-3′	82
CCL17	NM_002987	F: 5′ -GTGCTGCCTGGAGATCTTCA-3′ R: 5′ -TGGCATCCCTGGGACACT-3′	89
GAPDH	NM_002046	F: 5′ -ACATCGCTCAGACACCATG 3′ R: 5′ -TGTAGTTGAGGTCAATGAAGGG 3′	143

2.5. Enzyme-Linked immunosorbent assay analysis

The Enzyme-linked immunosorbent assay (ELISA) was utilized to measure TNF-α, IL-1β, IL-6, CCL22, CCL17, AQP-3, HA, and Filaggrin. After treating HaCaT cells with 10 ng/mL concentration of TNF-α/IFN-γ, the N.ex was treated at concentrations of 25, 50, 100 μg/mL. The supernatant was collected 24 hours after treatment. The collected supernatant was then used to perform ELISA assays according to the manufacturer's manual.

2.6. Quantitative real-time PCR analysis

After treating, base HaCaT cells with a concentration of 10 ng/mL of TNF-α/IFN-γ, N.ex at concentrations of 25, 50, 100 μg/mL was applied. The cells were cultured for 24 hours, washed with cold PBS, and total mRNA was extracted using Quiazol lysis reagent (Qiagen, Crawley, U.K.) RNA extracted from the cells was then used to synthesize cDNA using the Goscrip™ Reverse Transcriptase system (Promega, Madison, WI) following the manual. The synthesized cDNA was used for real-time PCR with Goscrip® qPCR Master Mix (Promega, Madison, WI) and primers. The oligonucleotide primers used in the PCR were designed based on known nucleotide sequences, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal positive control for each reaction. The primers used in the experiment are listed in Table. 1 and were purchased from Bioneer (Korea). PCR reaction conditions for AQP-3, HAS2, FLG, TNF-α, IL-1β, CCL22, and CCL17 mRNA were denaturation (95℃, 30 seconds), annealing (60℃, 60 seconds), and extension (72℃, 60 seconds).

2.7. Western blot analysis

After treating HaCaT cells with TNF-α /IFN-γ at a concentration of 10 ng/mL and then with N.ex at concentrations of 25, 50

and 100 μg/mL, the supernatant was removed 24 hours later. The harvested cells were washed with cold PBS and then lysed using RIPA buffer (Thermo, USA) to extract proteins. The extracted proteins were quantified using the BCA protein kit. To visualize the proteins, 20 μg of protein was separated using 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hercules, CA, USA) and transferred to an Immobilon®-P PVDF Membrane (Merck KGaA, Darmstadt, Germany). Primary antibodies against iNOS, COX-2, and β-actin were used, followed by secondary antibodies, HRP-conjugated mouse antibody, and HRP-conjugated rabbit antibody. After completing the antibody reactions, the Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA, Darmstadt, Germany) was used for detection, and the results were measured using LAS-4000 (Fujifilm Life Science, Tokyo, Japan).

2.8. Statistical processing

The results were statistically analyzed using SPSS 12.0 Significance testing was performed using analysis of variance (ANOVA) and *t*-tests, with a statistical significance level set at $p<0.05$.

3. Results and Discussion

3.1. The impact of N.ex on cell survival rate in HaCaT keratinocyte cells.

HaCaT keratinocyte cells were treated with N.ex at concentrations of 12.5, 25, 50, 100 and 200 μg/mL for 24 hours and the cell survival rate was measured through MTT analysis. The results showed that there was no significant cytotoxicity at concentrations of 12.5, 25, 50 and 100 μg/mL. However, at a concentration of 200 μg/mL, the cell survival rate of HaCaT keratinocyte cells decreased by more than 20%. Therefore, the concentration of 200 μg/mL, which exhibited lower cell survival, was excluded. The three concentrations of 25, 50 and 100 μg/mL, which showed no toxicity and were expected to have effects were used in the study (Fig. 2).

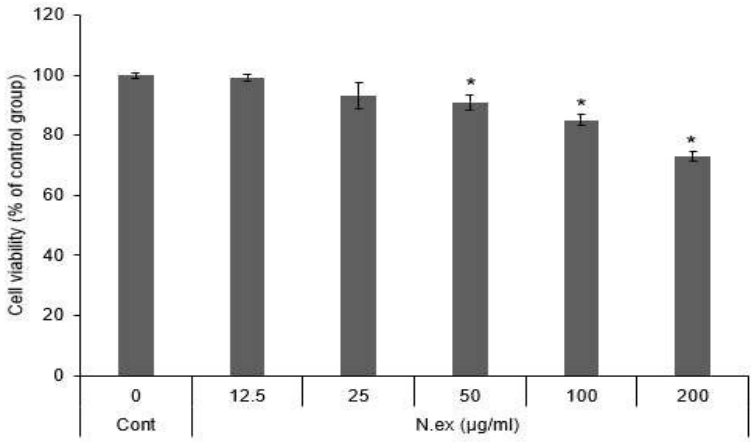


Fig. 3. Effects of N.ex on cell viability in HaCaT keratinocytes. HaCaT cells were treated with different concentrations of N.ex for 24 h and cell viability was measured from MTT reagent. The values shown represent mean ± SEM of three times assays.
* indicates significant difference ($p<0.05$) from control group.

3.2. The effects of N.ex on the expression of cytokines and Th2 chemokines in TNF-α and IFN-γ stimulated HaCaT keratinocytes

Keratinocytes, known to induce inflammatory responses through the secretion of various cytokines in response to external stimuli, are particularly influenced by stimulation with TNF-α and IFN-γ. This stimulation leads to abnormal secretion of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 from keratinocytes, inducing the infiltration of T cells and white blood cells into the inflamed skin area. Prolonged exposure to these conditions is associated with skin conditions such as skin aging, atopic dermatitis, and psoriasis [19]. The expression levels of TNF-α, IL-1β, and IL-6, which act as mediators in the inflammatory response, were measured. When treating TNF-α/IFN-γ-induced HaCaT cells with the compound extract at concentrations of 25, 50 and 100 μg/mL, a concentration-dependent inhibition of TNF-α, IL-1β, IL-6, MDC and TARC expression was observed. Particularly, IL-1β showed excellent efficacy, inhibiting more than 40% at a concentration of 100 μg/mL (Fig. 3A). The mRNA expression of TNF-α, IL-1β, IL-6, MDC, and TARC also decreased in a dose-dependent manner, as shown in (Fig. 3B).

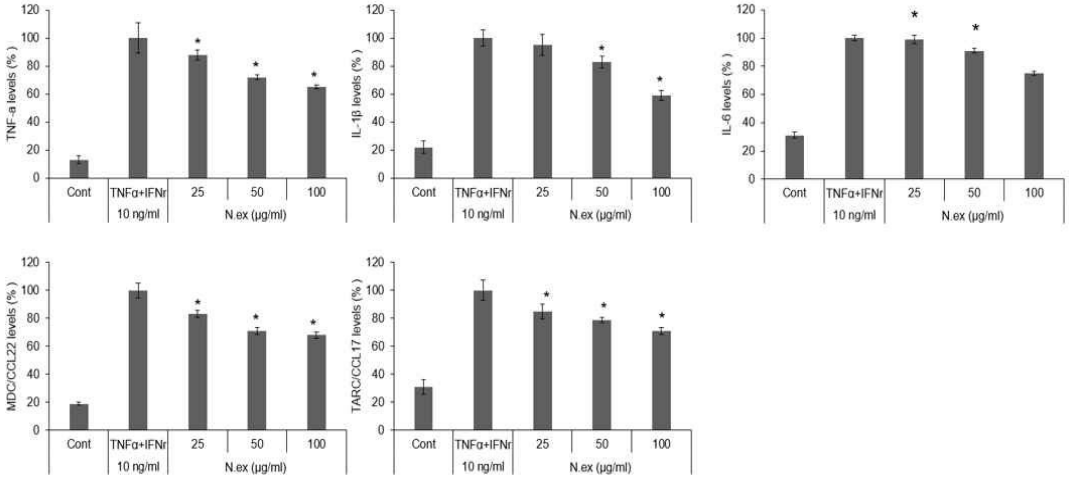
3.3. The effect of N.ex on moisturizing and skin barrier factor expression in TNF-α /IFN-γ stimulated HaCaT keratinocytes

Hyaluronic acid and Aquaporin are high-molecular-weight compounds known to not only prevent moisture evaporation from the epidermis to maintain skin elasticity but also exhibit various functions such as antioxidant, anti-inflammatory, antimicrobial, and anti-edema effects [18, 20]. Mutations in filaggrin can lead to damage to the skin barrier, making it more susceptible to immunoglobulin E reduction through the damaged skin barrier, leading to increased skin inflammation reactions. The reduction of filaggrin can also result in decreased moisture content in the stratum corneum. The study measured the effects of N.ex on the production of AQP-3, HA, and filaggrin in TNF-α/IFN-γ-stimulated HaCaT cells. The results showed a concentration-dependent significant increase in the production of AQP-3, HA, and filaggrin when treated with N.ex at concentrations of 25, 50, and 100 μg/mL (Fig. 4).

3.4. Effects of N.ex on the Phosphorylation of MAPKs in TNF-α /IFN-γ -stimulated HaCaT Keratinocytes

The phosphorylation reactions of MAPKs play a central role in cell proliferation, differentiation, and apoptosis. This signaling cascade involves the transfer of phosphate groups from upstream kinases to downstream kinases, regulating various cellular functions, including gene expression and protein production [21]. In this context, understanding the activation of specific genes and the subsequent production of proteins within cells is crucial. p-JNK (Jun N-terminal Kinase), JNK, p-ERK (Extra-signal Response Kinase), ERK, p38 kinase, p-p38, p-38, β-actin are key molecules involved in cell survival and inflammation. Therefore, they were selected as markers, and experiments were conducted. To investigate the effects of N.ex on the phosphorylation of MAPKs in TNF-α/IFN-γ -stimulated HaCaT cells, protein extraction was performed, and western blotting analysis was conducted for p-ERK, ERK, p-JNK, JNK, p-p38, p-38, β-actin. The results showed a significant decrease in the protein expression of p-ERK, p-JNK, and p-p38, which were increased by TNF-α/IFN-γ, upon treatment with N.ex (Fig. 5).

(A)



(B)

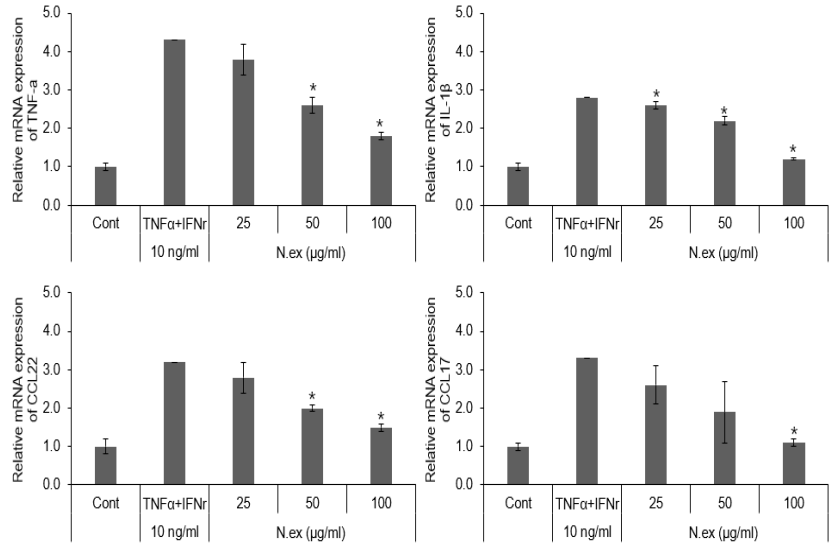


Fig. 4. Expressions of cytokines and chemokines evaluated by protein level(A) and mRNA level(B) in TNF-α/IFN-γ induced. HaCaT cells after N.ex HaCaT cells were treated with different concentrations of N.ex (25, 50 and 100 μg/mL) after stimulation with 10 ng/mL TNF-α/IFN-γ. After 24h, cytokine expression was determined in the cell supernatant according to the kit manual. Each value represents mean ± SD for the three individual experiments. n=3, *p<0.05 compared with the TNF-α/IFN-γ group.

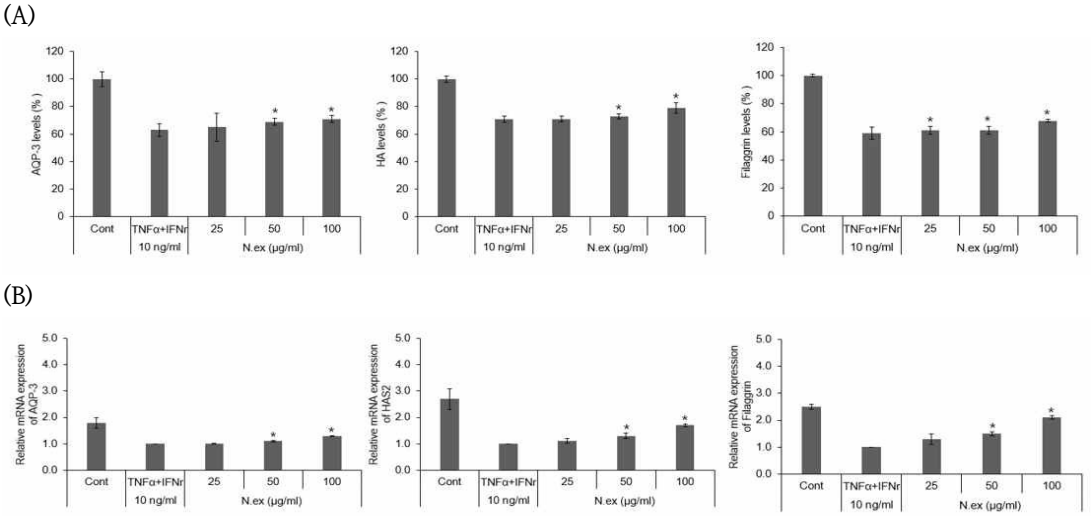


Fig. 5. Expressions of AQP-3, HA, Filaggrin evaluated by protein level(A) and mRNA level(B) in TNF-α/IFN-γ induced. HaCaT cells after N.ex. HaCaT cells were treated with different concentrations of N.ex (25, 50 and 100 μg/mL) after timulation with 10 ng/mL TNF-α /IFN-γ. After 24h, cells were harvested and relative mRNA levels were determined. Each value represents mean ± SD for the three individual experiments. n=3, *p<0.05 compared with the TNF-α/IFN-γ group.

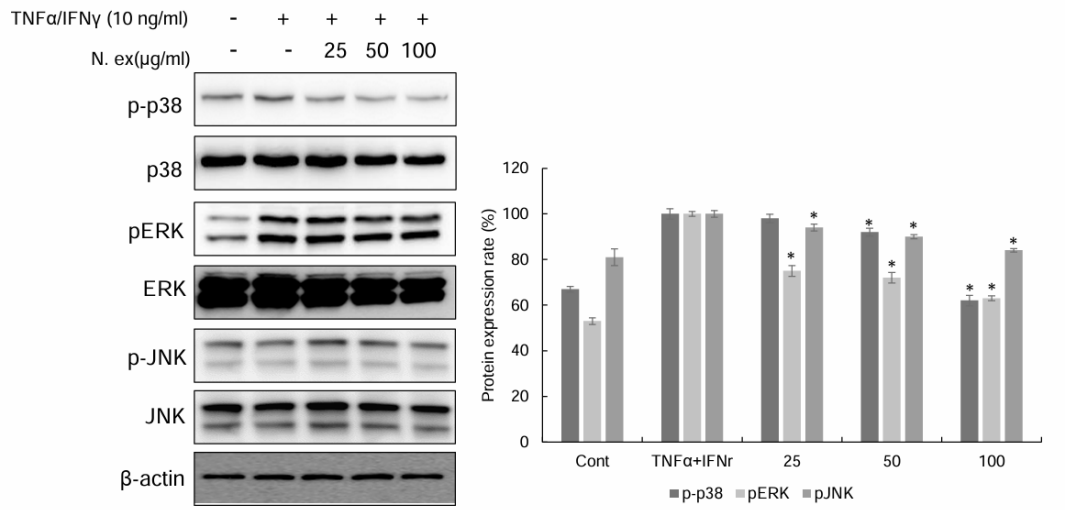


Fig. 6. Effects of N.ex on TNF-α,/IFN-γ stimulated phosphorylation of MAPKs in HaCaT keratinocytes. HaCaT cells were treated with different concentrations of N.ex (25, 50 and 100 μg/mL) after stimulation with 10 ng/mL TNF-α/IFN-γ. After 30 min, cells were harvested and relative protein levels were determined. Histogram shows the densitometry of phosphorylated-p38, extracellular signal-regulated kinase (ERK), -c-Jun N-terminal kinase (JNK) proteins normalized to GAPDH. Each value represents mean ± SD for the three individual experiments. n=3, *p<0.05 compared with the TNF-α/IFN-γ group.

4. Conclusion

Cytokines play important roles in immunity, infectious diseases, hematopoiesis, tissue repair, and the development and growth of cells. They are categorized into types such as interleukins (IL), chemokines, tumor necrosis factors (TNF), and interferons (IFN-) [10, 21]. In this study, it was observed that the combination extract N.ex exhibits anti-inflammatory effects by inhibiting the expression of inflammatory cytokines IL-1β, IL-6, TNF-α, as well as inflammatory enzymes iNOS and COX-2. The inhibitory effects were confirmed to occur through the suppression of inflammatory signaling molecules, especially the activity of JNK and ERK, components of MAPKs, leading to the transcriptional regulation inhibition of NF-κB [22]. Confirming the inhibition of these cytokines shed light on the impact of N.ex, a compound comprising *Paeonia lactiflora*, *Cucurbita moschata*, and *Prunus cerasus*, on HaCaT keratinocytes. the cells responsible for the formation of the epidermis.

Therefore, it has been confirmed that N.ex can suppress inflammatory skin conditions, and it is speculated that alleviating skin conditions can reduce both mental and physical stress. According to one study, there appears to be a high correlation between psychological anxiety about work and the perceived psychological anxiety related to physical factors, indicating a strong interrelationship between these two factors in relation to experiences of acne and daily stressors [23].

Thus, in contemporary society, acne can arise from both psychological and physical stress, and considering the interaction between skin conditions and mental health, this study suggests that N.ex, identified and developed through this research, holds potential as a material capable of alleviating skin conditions that may arise from stress in the future.

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