International Journal of Immunopathology & Pharmacology

Original Research Article

Purpurin suppresses atopic dermatitis via TNF-α/IFN-γ-induced inflammation in HaCaT cells

International Journal of Immunopathology and Pharmacology
Volume 36: 1–12

DOI: 10.1177/03946320221111135
journals.sagepub.com/home/iji
SAGE

Jae-Hoon Oh[1,2], Seung-Ho Kim[1,2], Ok-Kyoung Kwon[1], Jung-Hee Kim[1], Sei-Ryang Oh[1], Sang-Bae Han[2], Ji-Won Park[1] and Kyung-Seop Ahn[1]

Abstract

Objective: We investigated whether purpurin inhibits various pathways of inflammation leading to atopic dermatitis.
Introduction: 1,2,4-Trihydroxyanthraquinone, commonly called purpurin, is an anthraquinone that is a naturally occurring red/yellow dye. Purpurin is a highly antioxidative anthraquinone and previous studies have reported antibacterial, anti-tumor, and anti-oxidation activities in cells and animals. However, the skin inflammatory inhibition activity mechanism study of purpurin has not been elucidated in vitro.
Methods: In this study, we investigated the anti-inflammatory activity of purpurin in HaCaT (human keratinocyte) cell lines stimulated with a mixture of tumor necrosis factor-alpha (TNF-α)/Interferon-gamma (IFN-γ). The inhibitory effect of Purpurin on cytokines (IL-6, IL-8, and IL-1β) and chemokine (TARC, MDC, and RANTES) was confirmed by ELISA and RT-qPCR. We investigated each signaling pathway and the action of inhibitors through western blots.
Results: The expression levels of cytokines and chemokines were dose-dependently suppressed by purpurin treatment in TNF-α/IFN-γ-induced HaCaT cells from ELISA and real-time PCR. Purpurin also inhibited protein kinase B (AKT), mitogen-activated protein kinase (MAPKs), and nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) activation in TNF-α/IFN-γ-stimulated HaCaT cells. Additionally, there was a synergistic effect when purpurin and inhibitor were applied together, and inflammation was dramatically reduced.
Conclusion: Therefore, these results demonstrate that purpurin exhibits anti-inflammatory and anti-atopic dermatitis activity in HaCaT cells.

Keywords

purpurin, inflammatory, HaCaT, atopic dermatitis, cytokine, NF-κB

Date received: 13 April 2022; accepted: 14 June 2022

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by atopic dermatitis lesions and severe itching and is a persistent inflammatory skin disease. It is caused by genetic factors and the environment, involving microbial infections and environmental pollutants.[1–3] Atopic dermatitis develops due to a combination of severe itching, epidermal barrier, genetic predisposition, and

[1]Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju-si, Republic of Korea
[2]College of Pharmacy, Chungbuk National University, Cheongju-si, Republic of Korea

Corresponding author:
Jae-Hoon Oh and Ji-Won Park, Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 30 Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungbuk 28116, Republic of Korea.
Emails: ojh3722@kribb.re.kr; jjiwon87@gmail.com

an imbalanced immune response.[4–6] Patients with AD are caused by an imbalance between T-helper 2 cells (Th2) and T-helper 1 cell (Th1). Activated by antigen penetration, Th2 secretes many cytokines and chemokines. Chemokines are complexes of small cytokines produced by various types of cells. Chemokines are also separated into the CX3C, CXC, CC, and C subfamilies.[7] The main role of chemokines is to adjust inflammatory cell recruitment, involving the migration of T cells, macrophages, eosinophils, and dendritic cells (DCs) at the site of infection and inflammation.[8] Chemokines that lead Th2 cells to the skin include MDC and TARC. Macrophage-derived chemokines (MDCs/CCL22) are also specific ligands for C-C chemokine receptor type 4 (CCR4). Thymus and activation regulatory chemokines (TARC/CCL17) are CC chemokines produced by constitutive expression of monocyte-derived dendritic cells and keratinocytes. Also, regulated on activation (RANTES/CCL5) is a member of a large family of chemokines which are thought to play a regulatory role in inflammatory processes.[9–11]

Tumor necrosis factor (TNF) is an inflammatory cytokine that has many biological correlations in various types of tumor cells, including cell proliferation suppression and cytotoxic effects, differentiation, and proliferation. TNF-α has been reported to be involved in the production of MDCs and TARCs in the nuclear transcription factor (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. In addition, IFN-γ stimulates HaCaT cells and induces the expression of various chemokines such as TARC and MDC and cytokines via the JAK/STAT signaling pathway. Nuclear transcription factor (NF-κB) by TNF-α/IFN-γ is known as an anti-apoptotic gene that plays an important role in cell survival.[12] In addition, phosphorylation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway by IFN-γ is known to be an important anti-inflammatory mechanism.

Purpurin, 1,2,4-trihydroxy anthraquinone, is an alizarin-based anthraquinone with particularly high antioxidant activity.[13–15] Anthraquinone alizarin for purpurin is present as a glycoside in the roots of mother plant, which is the common name for *Rubia tinctorum L.* and *Rubia cordifolia*. This anthraquinone is an ancient natural pigment derived from the mother plant that is used to dye fabrics and colored paints. *Rubia cordifolia* mainly includes purpurin glycosides, while *Rubia tinctorum L.* mainly contains alizarin glycosides.[16] Although not eaten for nutrition, the radiant roots are used as food coloring and have been used in traditional medicine to treat various ailments.[17] Purpurin appears to exhibit antiangiogenic activity, anti-fungal, anti-inflammatory, anti-tumor, anti-oxidation, and anti-bacterial activity. Also purpurin's high anti-inflammatory ability can affect many bioactivities [19]. However, whether purpurin could repress skin cell inflammatory remains unclear.

The purpose of this study was to clarify the anti-inflammatory effect and mechanism of purpurin on TNF-α/IFN-γ-induced inhibition of HaCaT cells. We specifically investigated the effect of purpurin on gene expression and protein production suppression of IL-6, IL-8, IL-1β, TARC, MDC, and RANTES. In addition, the inhibitory properties of purpurin on the major molecular signaling pathways associated with NF-κB and MAPK were confirmed. This study has specific anti-inflammatory effects through the ability of purpurin to inhibit NF-κB and STAT activation and proliferative signaling pathways, and may be a good candidate as an alternative treatment for atopic dermatitis.

Results

Treatment with purpurin inhibits TNF-α/IFN-γ-induced cytokine and chemokine expression in HaCaT cells as determined by qPCR and ELISA

We confirmed the cell viability associated with purpurin via HaCaT cell and MTT assays. It was experimentally confirmed that purpurin did not show toxicity when HaCaT cells were treated with 40 μM purpurin. This is shown in Figure S1. Pro-inflammatory cytokines promote allergic reactions and are responsible for the biological effects of the immune system.[18] Purpurin inhibits the expression of TNF-α/IFN-γ-induced cytokines (IL-1β, IL-8, and IL-6) and chemokines (MDC, TARC, and RANTES) in HaCaT cells. Therefore, we used ELISA and RT-qPCR analysis to investigate the inhibitory effect of purpurin on the production of cytokines and chemokines from TNF-α/IFN-γ-stimulated HaCaT cells. ELISA results showed that treatment with purpurin suppressed the levels of cytokines such as IL-1β, IL-6. and IL-8 compared to those in cells treated with TNF-α/IFN-γ alone (Figure 1). Chemokines are also important mediators of inflammation and the immune response. When keratinocytes are exposed to TNF-α/IFN-γ, chemokine expression increases, and leukocytes penetrate inflammatory lesions of the skin. Purpurin also suppressed the levels of chemokines such as MDC, TARC, and RANTES (Figure 1). Treatment with TNF-α/IFN-γ increased the mRNA expression levels of cytokines (IL-1β, IL-8, and IL-6) and chemokines (MDC, TARC, and RANTES) in HaCaT cells (Figure 2). Purpurin treatment was found to suppress increased expression of IL-1β, IL-8, and IL-6 mRNA. RT-qPCR results confirmed that cytokine and chemokine mRNA levels were significantly increased in TNF-α/IFN-γ-treated cells compared to the untreated group. Similarly, when TNF-α/IFN-γ was added after purpurin was applied to HaCaT cells, the

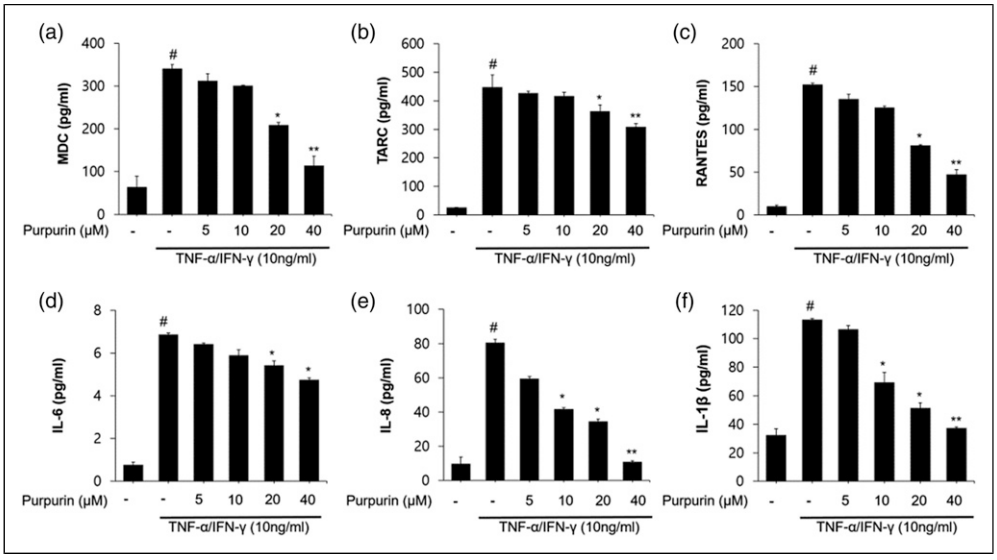

Figure 1. Purpurin affects the expression of cytokines and chemokines in HaCaT cells.
HaCaT keratinogenic cells were pre-treated with purpurin (5, 10, 20, or 40 μM) and stimulated with TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) for 18 h (a) MDC, (b) TARC, (c) RNATES, (d) IL-6, (e) IL-1β, and (f) IL-8 levels were measured using culture supernatants of cells treated with purpurin and TNF-α/IFN-γ for 18 h. The data are displayed as the mean ± standard error of the mean of the two samples. #$p < .01$ vs. the negative control; *$p < .05$, **$p < .01$ vs. TNF-α/IFN-γ stimulated cells.

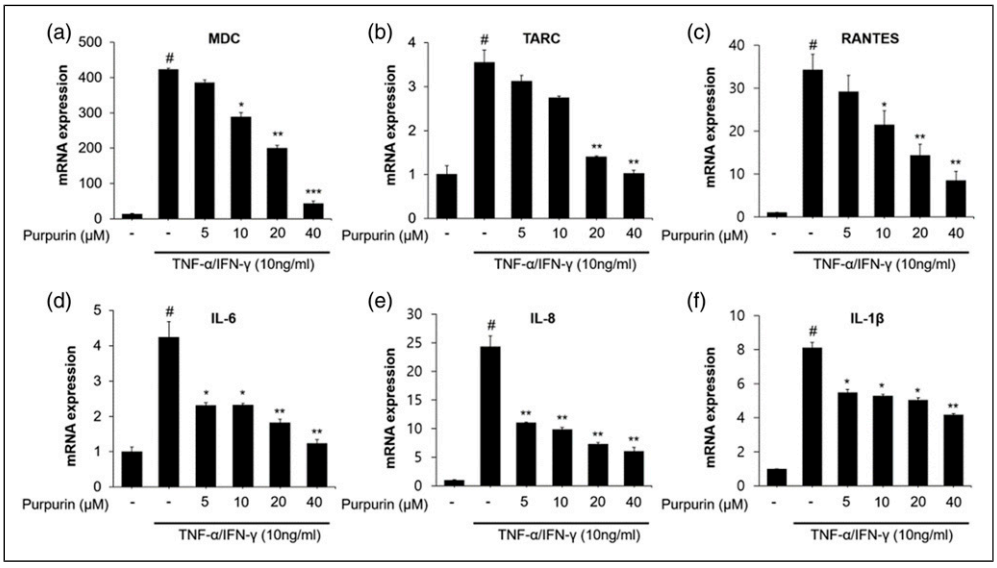

Figure 2. The effect of purpurin on the expression of cytokines and chemokines in HaCaT cells.
HaCaT cells pre-treated with purpurin (5, 10, 20, and 40 μM) were induced with TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) for 24 h (a) MDC, (b) TARC, (c) RNATES, (d) IL-6, (e) IL-1β, and (f) mRNA expression in HaCaT cells. The bar group represents the average of two independent experiments. The data are displayed as the mean ± standard error of the mean of the two samples. #$p < .01$ vs. the negative control; *$p < .05$, **$p < .01$ vs. TNF-α/IFN-γ stimulated cells.

mRNA expression levels of cytokines and chemokines decreased compared to the treatment group with only TNF-α/IFN-γ. (The decrease was found to be concentration-dependent) (Figure 2). As a result, purpurin was shown to suppress the expression of cytokines and chemokines in TNF-α/IFN-γ-induced HaCaT cells via ELISA and qPCR.

Purpurin suppresses the activation of nuclear factor kappa-light-chain-enhancer of activated B cells in HaCaT cells

NF-κB is known to regulate the expression of allergies, inflammation, and immune-related genes by generating

cytokines and chemokines.[19] This proved that purpurin specifically suppresses NF-κB. Purpurin suppresses the phosphorylation of NF-κB p65 in HaCaT cells. The nuclear factor NF-κB signaling pathway is considered to be a circular pre-inflammatory pathway primarily due to the role of NF-κB in the expression of pre-inflammatory genes such as adhesion molecules, chemokines, and cytokines. Therefore, in this study, we analyzed NF-κB p65 phosphorylation in TNF-α/IFN-γ-treated HaCaT cells (Figure 3). Western blotting results showed that phosphorylation of IκBα and NF-κB p65 was increased by TNF-α/IFN-γ treatment, whereas pre-treatment with purpurin reduced IκBα and NF-κB p65 induced by TNF-α/IFN-γ. When IκBα is phosphorylated and degraded in the IκBα complex, NF-κB is translocated from the cytoplasm into the nucleus. It was determined whether NF-κB translocation could be inhibited by treatment with purpurin. Proteins extracted from HaCaT cells were fractionated into the cytoplasm and nucleus, and western blotting was performed. The cytoplasm decreased in the group treated with TNF-α/IFN-γ alone but increased in a concentration-dependent manner when purpurin was administered. Nuclei increased in the group treated with TNF-α/IFN-γ alone but decreased in a concentration-dependent manner when treated with purpurin (Figure 3). From this, it was confirmed that NF-κB inhibits translocation from the cytoplasm to the nucleus.

Treatment with purpurin suppressed the activation of mitogen-activated protein kinases in HaCaT cells

Mitogen-activated protein kinases is a specific protein kinase that regulates multiple cellular processes in response to various external stresses. Therefore, MAPK is activated by TNF-α/IFN-γ stimulation.[20] Purpurin suppresses the activation of mitogen-activated protein kinases (MAPKs) in HaCaT cells. TNF-α/IFN-γ treatment activates the expression of p38, JNK, and ERK. Therefore, the effect of purpurin on p38, JNK, and ERK protein expression in HaCaT cells was confirmed via western blot. Similarly, HaCaT cells pre-treated with TNF-α/IFN-γ and purpurin for 1 h were exposed to TNF-α/IFN-γ for 1 h. We confirmed changes in the phosphorylation of p38, JNK, and ERK, which are MAPK factors known to be important in atopic dermatitis (Figure 4). The results showed that the phosphorylation of p38, JNK, and ERK was increased in HaCaT cells stimulated with TNF-α/IFN-γ for 1 h compared with untreated HaCaT cells. When purpurin was pre-treated in HaCaT cells, phosphorylation of p38, JNK, and ERK was dramatically suppressed compared with the TNF-α/IFN-γ single treatment group (Figure 4). These results indicate that purpurin suppresses the expression of p-p38, p-JNK, and p-ERK after TNF-α/IFN-γ stimulation.

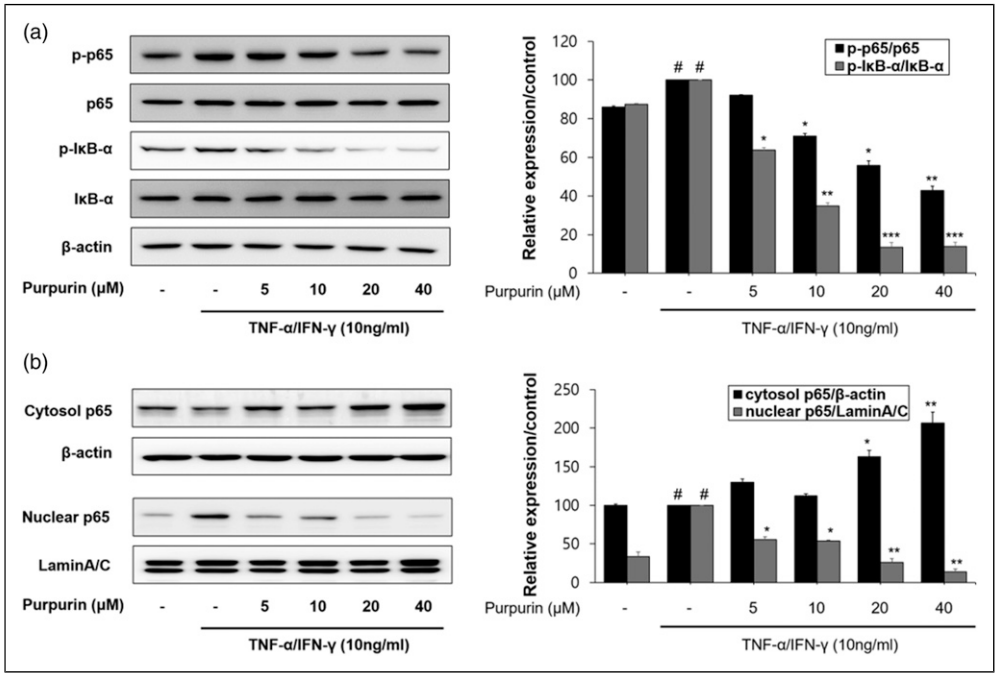

Figure 3. Effect of purpurin on TNF-α/IFN-γ-induced NF-κB activation in HaCaT cells. (a) Phosphorylation of p65 and IκBα was analyzed by western blot. (b) In TNF-α/IFN-γ-induced HaCaT cells, protein was fractionated into the nucleus and cytosol, and translocation was confirmed. It was confirmed that purpurin suppresses the translocation of p65 through western blot. #$p < .01$ vs. negative control group; *$p < .05$, and **$p < .01$ vs. TNF-α/IFN-γ stimulated cells.

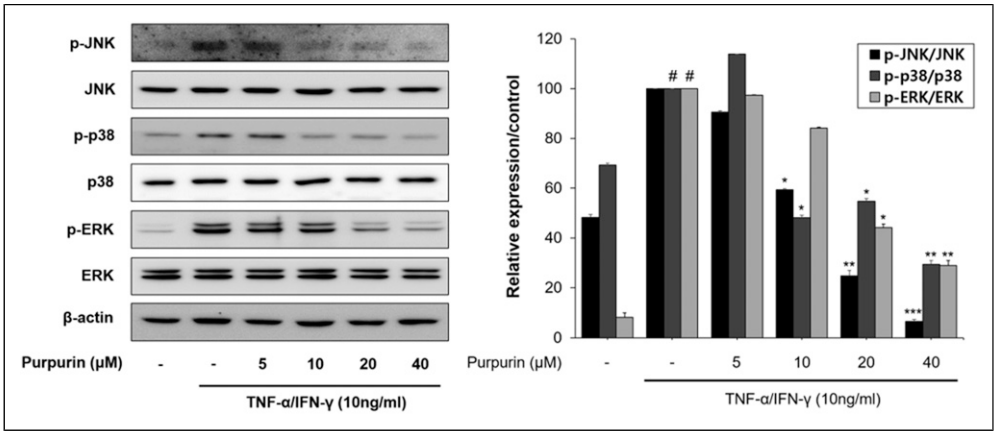

Figure 4. Effect of purpurin treatment on TNF-α/IFN-γ-induced MAPK in HaCaT cells.
Cells were pre-treated with 5, 10, 20, and 40 μM purpurin for 1 h, followed by exposure to TNF-α and IFN-γ (10 ng/mL each) for 1 h. Cell extracts were prepared, and MAPK activation was analyzed by Western blotting using specific antibodies. #$p < .01$ vs. the negative control; *$p < .05$ and **$p < .01$ vs. TNF-α/IFN-γ stimulated cells. #$p < .01$ vs. the negative control; *$p < .05$, **$p < .01$ vs. TNF-α/IFN-γ stimulated cells.

Purpurin suppresses the activation of the AKT, STAT1 and STAT3 signaling pathways in HaCaT cells

The JAK1/STAT pathway is important for atopic dermatitis. Purpurin suppresses the activation of JAK1/STAT in HaCaT cells. Western blots and immunocytochemistry were utilized to investigate whether purpurin suppresses TNF-α/IFN-γ-mediated JAK1/STAT phosphorylation in HaCaT cells. HaCaT cells were pre-treated with purpurin for 1 h, and TNF-α/IFN-γ was applied for 1 h. TNF-α/IFN-γ increased JAK1/STAT phosphorylation after treatment for 1 h. At this time, it was confirmed that the phosphorylation of JAK1/STAT was inhibited in a dose-dependent manner by treatment with purpurin. As a result, purpurin suppressed JAK1/STAT1/STAT3 phosphorylation in HaCaT cells stimulated with TNF-α/IFN-γ (Figure 5). In addition, phosphorylation of p85 and JAK1, which are important factors in JAK1/STAT signaling, was confirmed via western blot. The phosphorylation of JAK1 increased by TNF-α/IFN-γ and was suppressed by purpurin. This was found to suppress purpurin via the upstream factors p85 and JAK1 in JAK1/STAT signaling. Immunocytochemistry experiments were performed to confirm that purpurin suppresses the translocation of STAT1 and STAT3. Immunocytochemistry confirmed that purpurin treatment reduced the translocation of STAT1 and STAT3 in HaCaT cells (Figure 5). These results indicate that purpurin suppresses JAK1/STAT activation in HaCaT cells. When stimulated with TNF-α/IFN-γ in HaCaT cells, PI3K is activated intracellularly. PI3K exists in the form of a complex of p85 and p110. Active PI3K phosphorylates AKT. Phosphorylated AKT produces cytokines and chemokines that translocate to the nucleus and induce inflammation. We confirmed that TNF-α/IFN-γ-stimulated HaCaT cells increased the phosphorylation of p85 and AKT via western blotting. Purpurin suppressed the increased phosphorylation of p85 and AKT. We found that purpurin suppresses inflammation via the AKT signaling pathway.

Purpurin and inhibitors suppress the expression of cytokines and chemokines in TNF-α/IFN-γ-induced HaCaT cells

Purpurin and inhibitors suppress the expression of chemokines and cytokines in TNF-α/IFN-γ-induced HaCaT cells. The translocation of NF-κB was suppressed in purpurin-treated HaCaT cells. Purpurin inhibited p85 and JAK1 in the AKT/STAT signaling pathway. MAPK may be activated by TNF-α/IFN-γ, a key stimulant of the skin inflammatory response of keratinocytes. Purpurin reduced the expression levels of p-p38, p-JNK, and p-ERK in TNF-α/IFN-γ-stimulated HaCaT cells compared to untreated cells (Figure 6). These data indicate that purpurin suppresses the inflammatory response through partial regulation of the NF-κB, AKT, STAT, and MAPK signaling pathways. To confirm the function of NF-κB, STAT1, STAT3, and MAPKs in the development of TNF-α/IFN-γ-induced inflammation, the effects of selective NF-κB, STAT, and MAPK inhibitors on chemokines and cytokines induced by TNF-α/IFN-γ were investigated (Figure 6). ELISA analysis showed that Bay11-7082 (p65 inhibitor) SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), and SB203580 (p38 inhibitor) inhibited MDC, TARC, IL-8, and IL-6 expression. Treatment with purpurin and inhibitors has also been shown to significantly suppress TNF-α/IFN-γ-induced expression of MDC, TARC, IL-8, and IL-6 (Figure 6). These results suggested that purpurin disabled NF-κB

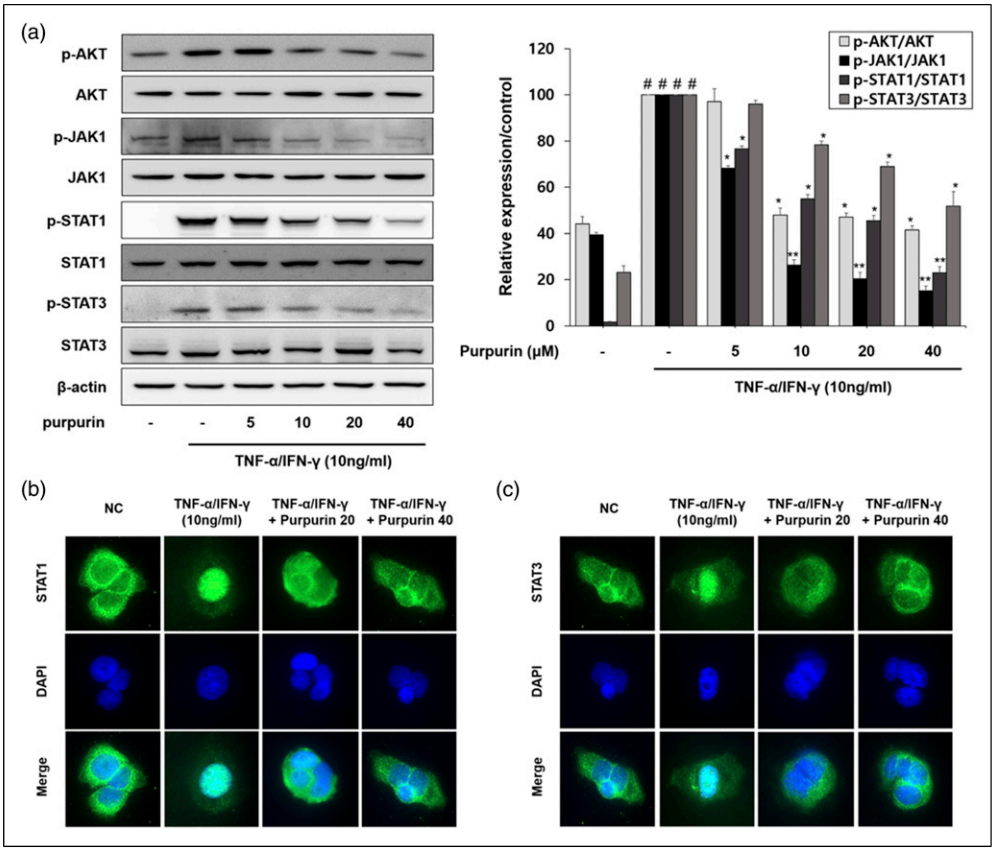

Figure 5. The effect of purpurin on TNF-α/IFN-γ-induced STAT1 activation in HaCaT cells. (a) Phosphorylation of JAK1 and STAT1 was analyzed using Western blotting. (b) Cell localization of STAT1 was determined by immunocytochemistry. Nuclei were visualized using DAPI (blue) and observed at ×400 magnification. (c) Cell localization of STAT3 was determined by immunocytochemistry. Nuclei were visualized using DAPI (blue) and observed at ×400 magnification. The data are displayed as the mean ± standard error of the mean of the two samples. #$p < .01$ vs. negative control group; *$p < .05$, and **$p < .01$ vs. TNF-α/IFN-γ stimulated cells.

p65, p85, JAK, JNK, ERK, and p38 MAPK, thereby suppressing.

Purpurin and each other inhibitors suppress the expression of transcription factor in TNF-α/IFN-γ-induced HaCaT cells

We have shown above that activation of each other inhibitor in HaCaT cells can be targeted through co-treat of purpurin. However, the efficacy of inhibitors may not be activated in transcription factors when co-treated with purpurin in HaCaT cells. Therefore, when purpurin and inhibitor were co-treated, it was confirmed by western blot whether phosphorylation of each target could be inhibited. Each other inhibitor in combination with purpurin had no significant effect on cell survival as compared to drug alone (data not shown). Interestingly, combining purpurin with dual JAK1/JAK2 inhibitor (Ruxolitinib) and p85 inhibitor (Wortmannin) had weak inhibited effect (Figures 7(d) and (f)). Contrastively, combined treatment of ERK (PD98059), p38 (SB203580), JNK (SP600125), and p65 (Bay11-7082) inhibitor further enhanced anti-inflammatory efficacy of purpurin in HaCaT cells (Figure 7(a)–(e)). Collectively, the combination of each other inhibitor with purpurin led to a marked decrease in transcription factor expression as compared to control or treatment with either drug alone (Figure 7).

Discussion

Atopic dermatitis is a common chronic inflammatory skin disease. Many studies have found that AD contributes to skin barrier dysfunction and immunomodulatory disorders and the pathophysiology of atopic dermatitis.[21,22] Various methods have been used to regulate skin inflammation in AD patients and the barrier function of the skin.[23] Traditionally, folk remedies for plants and herbs open up new possibilities for alternative therapies by controlling inflammation and

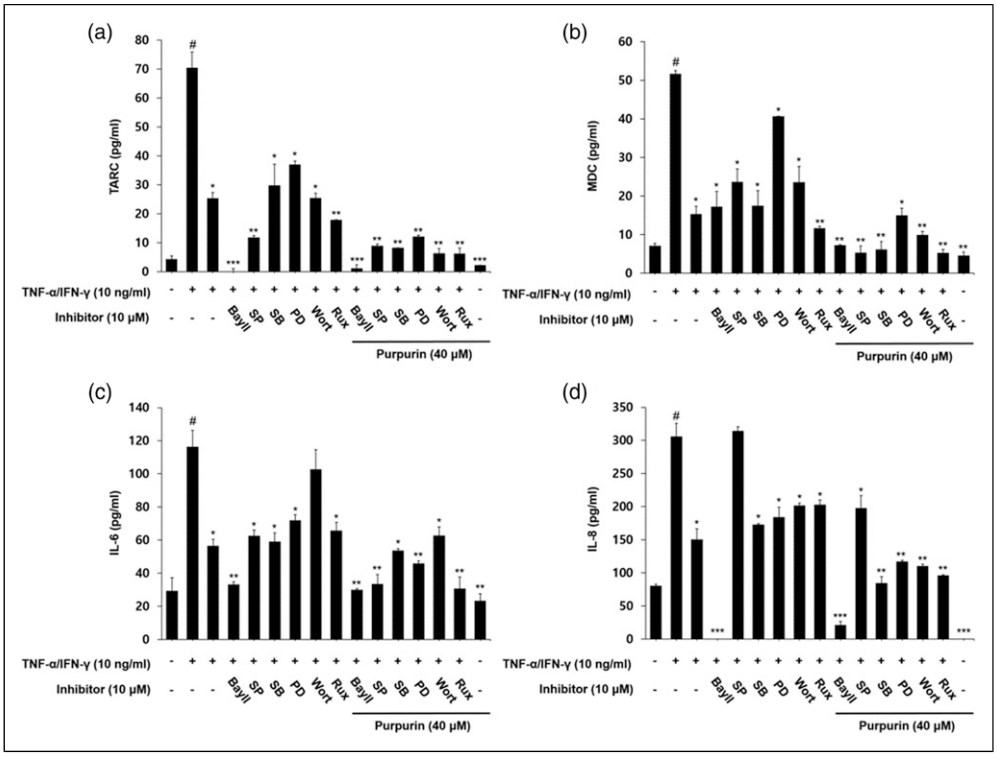

Figure 6. Effects of MAPK, NF-κB, and STAT inhibitors on the expression of pre-inflammatory cytokines and chemokines. HaCaT cells were pre-treated with 40 μM purpurin, 20 μM Bay11-7082, SP600235, SB203580, PD98059 wortmannin, and ruxolitinib for 2 h before culturing with 10 ng/mL TNF-α/IFN-γ. (a) TARC, (b) MDC, (c) IL-6, and (d) IL-8 levels were measured by ELISA. The data are displayed as the mean ± standard error of the mean (n = 2). #p < .01 vs. normal control group, *p < .05, and **p < .01 vs. TNF-α/IFN-γ stimulated cells.

minimizing side effects. Other known medicinal plants and their active compounds have been identified with potential mechanisms of action and are claimed to have anti-inflammatory effects. Purpurin is known to have anti-inflammatory properties. However, the anti-atopic effect of purpurin on TNF-α/IFN-γ-induced HaCaT cells in other embodiments, especially inhibition of NF-κB and STAT activation, is not available. In this study, we investigated the anti-inflammatory and anti-atopic effects of purpurin on HaCaT cells. As a result, it was shown that purpurin suppresses the inflammatory response of keratinocyte cells in a dose-dependent manner. Atopic dermatitis occurs when an externally invading antigen causes an imbalance between Th1 and Th2, and activated Th2 cells migrate to skin tissue to induce many cytokines and chemokines. Therefore, inhibiting the production of cytokines and chemokines in keratinocyte may be advantageous for atopy treatment. We demonstrated that purpurin inhibits cytokines and chemokine in HaCaT cells.

The pathophysiology of Atopic dermatitis acts through signaling pathways and transcriptional regulation in the regulation of T-helper 2 differentiation and includes major cytokines including IL-6, IL-8, andIL-1β. As hormone transmitters, cytokines are responsible for the biological effects of the immune system, such as allergies and immune responses.[18] The cytokines can be further subdivided into Th1 and Th2. Th1 cytokines sustain an autoimmune response and produce a pre-inflammatory response that kills intracellular parasites. IFN-γ is the main Th1 cytokine. Th2 cytokines include IL-6, IL-8, and IL-1β, which produce more anti-inflammatory responses.[24] In this study, we show that purpurin inhibits IL-6, IL-8, and IL-1β gene expression in TNF-α/IFN-γ-activated HaCaT cells. In addition, the chemokines produced by Th2 cells are typically TARC, MDC, and RANTES.[25] In this study, it confirmed that purpurin suppresses chemokines and cytokines increased by TNF-α/IFN-γ treatment at the protein level through ELISA. We also confirmed again via qPCR that purpurin suppresses cytokines and chemokines at the mRNA level. We confirmed that purpurin had a significant effect on AD by demonstrating that it suppressed the final product that induces inflammation.

It is well known that NF-κB plays an important role in the production of pro-inflammatory cytokines such as IL-6, IL-8, and IL-1β, and chemokine including MDC and TARC.[26] TNF-α is known to degrade IκBα, phosphorylate p65, and translocate to the nucleus. Degradation and phosphorylation of IκB occur in response to cell stimulation.

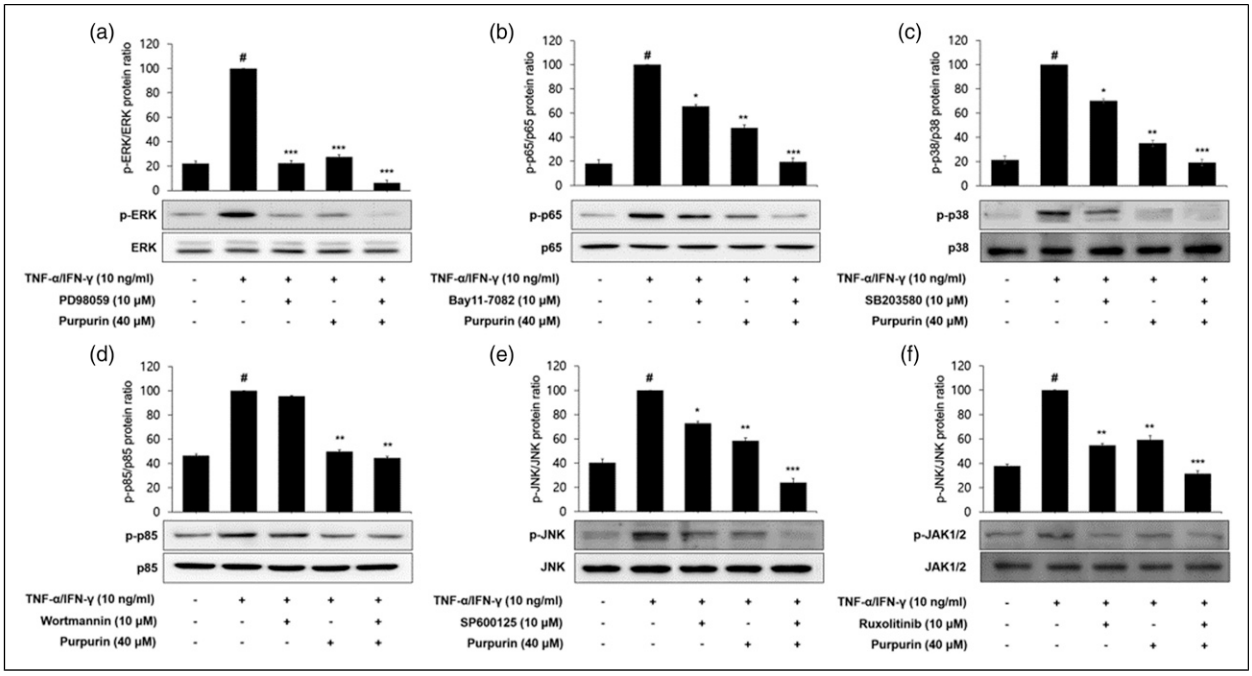

Figure 7. Mechanism of the anti-inflammatory effect of purpurin in TNF-α/IFN-γ-stimulated HaCaT cells.
HaCaT cells were pre-treated with purpurin (40 μM), Bay11-7082 (NF-κB inhibitor), SP600235 (JNK inhibitor), SB203580 (p38 inhibitor), PD98059 (ERK inhibitor), wortmannin (PI3K inhibitor), and ruxolitinib (JAK1/2 inhibitor) for 1 h before culturing with 10 ng/mL TNF-α/IFN-γ. (a) p-ERK, (b) p-p65, (c) p-p38, and (d) p-p85, (e) p-JNK, and (f) p-JAK1/2 levels were measured by western blot.

Active NF-κB releases heterogeneous dimers.[27] As a result, factors such as p65 are phosphorylated and translocate to the nucleus. Since we observed that purpurin reduced the expression of NF-κB downstream pro-inflammatory genes, we can hypothesize that purpurin may interfere with the activation of NF-κB signaling. Therefore, we confirmed through experiments that purpurin reduces NF-κB levels, which shows the anti-allergic effect of purpurin. Also, the protein was separated into the cytosol and nucleus, and the translocation of p65 and IκBα was confirmed via western blot. Purpurin suppressed p65 and IκBα nuclear translocation from the cytosol. These results suggest that purpurin suppresses the mechanism of NF-κB transcriptional activity through its anti-inflammatory effect. We further identified specific inflammatory signaling pathways that purpurin may interfere with. MAPK is a serine/threonine-specific protein kinase that responds to various external stresses. The MAPK signaling pathway adjusts several cellular processes, including gene expression, cell proliferation, cell death, and survival.[28] TNF-α/IFN-γ treatment activates major MAPK factors, such as p38, JNK, and ERK, in HaCaT cells.[29] Inhibition of MAPK is known to reduce intracellular signaling pathway synthesis and inflammatory cytokines.[30] We confirmed that TNF-α/IFN-γ increased the phosphorylation of p38, JNK, and ERK. Additionally, TNF-α/IFN-γ increased the phosphorylation of p65 and IκBα in HaCaT cells. Purpurin suppressed increased MAPK (ps38, JNK, and ERK) and NF-κB p65 and IκBα phosphorylation. This shows that purpurin exerts anti-inflammatory and anti-allergic effects.

We also confirmed the JAK/STAT signaling pathway is induced by IFN-γ. The STAT protein is the main signaling pathway for various factors and cytokines.[31] The STAT family has seven members, of which STAT1 and STAT3 are responsible for the distribution of IFN-γ via JAK1.[32] In particular, IFN-γ signaling is an important vector of inflammation and the immune response. In HaCaT cells, STAT1 and STAT3 are known to mediate the production of chemokines such as TARC and MDC by TNF-α/IFN-γ stimulation.[33] Based on these findings, the JAK/STAT pathway indicates that in human development, it is one of multiple case-expressive cascades that transform signals. Therefore, we considered the JAK/STAT mechanism to be one of the leading targets for AD, and we used purpurin to suppress it. Western blot analysis confirmed that TNF-α/IFN-γ-induced phosphorylation of STAT1, STAT3, and JAK1 was increased, and purpurin suppressed this increase. We also confirmed the translocation of STAT1 and STAT3 via immunocytochemistry. STAT1 and STAT3 were translocated to the nucleus in the TNF-α/IFN-γ-treated group, and it was suppressed when purpurin was administered. The results of this study indicate that purpurin can have an effect on AD based on a decrease in phosphorylated JAK/STAT.

Additionally, we proceeded with the experiment using purpurin and inhibitor together. An inhibitor that prevents activation of the primary target was used in HaCaT cells because a specific inhibitor structure that reduces the level of each gene has not been identified. The NF-κB-specific inhibitor Bay11-7082 was found to suppress NF-κB activation. Inhibitors SP600125, PD98059, and SB203580 were found to suppress specific genes in the MAPK signaling pathway. Wortmannin and ruxolitinib were also used to identify specific structures in the AKT/STAT pathway. Phosphorylation and activation of each gene were more significantly suppressed when the inhibitor and purpurin were treated in parallel. This shows that a synergistic effect appears when the inhibitor and purpurin are applied in parallel rather than when they are applied individually.

In this study, we confirmed that purpurin suppresses the expression of AD-related cytokines and chemokines in TNF-α/IFN-γ-induced HaCaT cells. These effects are considered to be associated with suppression of upstream NF-κB, AKT, JAK/STAT, and MAPK signaling pathway activation. The results of these experiments provide the scientific basis for the use of purpurin in the treatment of AD. We only performed in vitro experiments. It is necessary to confirm how purpurin works in animal models. Proceeding with in vivo experiments will reveal the direct effects of purpurin on atopic dermatitis and will be pathologically identifiable. Additional experiments are currently underway in our laboratory to confirm the in vivo effect of AD.

Materials and methods

Purpurin

Purpurin (1,2,4-trihydroxyanthraquinone; Cat. No. 229148; $C_{14}H_8O_5$, Dye content ≥90%; Table S2) was purchased from Sigma Aldrich (St Louis, MO). The purpurin used in this experiment was the key pigment present in the roots of Indian madder (*Rubia cordifolia*). This pigment produces colors with distinctive heat- and light-resistant properties. In this study, it took 1 year to proceed with each experiment including the sample selection period.

Cell culture

HaCaT human keratinocyte cells were purchased from the American Type Culture Collection (CLS Cell Lines Service). HaCaT cells were grown in DMEM (WELGENE, Cat. No. LM 001-05) medium supplemented with 10% fetal bovine serum (FBS, WELGENE, Cat. No. S 001-07), 100 U/ml penicillin, 100 μg/mL streptomycin, and 25 mM HEPES at 37°C in a 5% CO_2 incubator.

Enzyme-linked immunosorbent (ELISA) assay

HaCaT cells (5×10^4 cells/well) were seeded in a 96-well plate and cultured for 6 h. Then, the cells were pre-treated with purpurin and inhibitors at a specific concentration: NF-kB inhibitor (Bay11-7082; Calbiochem, CAS 16561-29-8), JNK1 inhibitor (SP600125; Calbiochem, CAS 129-56-6), p38 inhibitor (SB203580; Calbiochem, CAS 152121-47-6), ERK inhibitor (PD98059; Calbiochem, CAS 167869-21-8), PI3K inhibitor (Wortmannin; Calbiochem, CAS 19545-26-7), and JAK1/2 inhibitor (Ruxolitinib; MedChemExpress, INCB018424). After 1 h, the cells were treated with TNF-α/IFN-γ (10 ng/mL; TNF-α Cat. No. 300-01A, Peprotech, IFN-γ Cat. No. 300-02, Peprotech, NJ, USA). After a 16 h incubation, the supernatant was harvested. The reduced production of TARC, MDC, RANTES, MCP-1, IL-8 (Cat. No. DY364, DY339, DY278, DY279, DY208, R&D systems), IL-6, and IL-1β (Cat. No. 555220, 557953, BD biosciences) by purpurin was measured. The analysis was performed according to the manufacturers' instructions.

Quantitative reverse transcription polymerase chain reaction

Cells were plated in 12-well plates (1×10^5 cells/per well), pre-treated with purpurin (5, 10, 20, and 40 μM) for 1 h, and subsequently administered TNF-α and IFN-γ maintained for 1 h at 37°C. The extraction of total RNA, synthesis of cDNA, and relative mRNA levels of cytokines and chemokines were determined as described previously. As shown in Table S1, GAPDH was used as the housekeeping gene.

Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis were performed. HaCaT cells were washed with cold phosphate buffered saline (PBS) and then lysed in NP-40 buffer (Cat. No. EBA-1049, ELPIS-Biotech. Inc., Daejeon, Korea) containing protease and phosphatase or NE-PER Nuclear and Cytoplasmic Extraction Reagents (Cat. No, 78833, Thermo Fisher Scientific, Inc., MA, USA). Then, the cells were strongly vortexed on ice for 20 min. Cell lysates were centrifuged at 4°C at 13,000 × g for 15 min, and protein concentrations were measured using the bovine serum albumin (BSA; Thermo, Cat. 23209) method. The sample protein (20 μg) was analyzed with 10% SDS-PADE and electrophoretically transferred to PVDF (polyvinylidene difluoride) membranes. The next PVDF membrane was blocked with 5% defatted milk powder of Tris-buffered saline (TBS, 20 mM Tris, 0.2 M NaCl, pH 7.5). The PVDF membrane was then blocked with 5% defatted milk powder

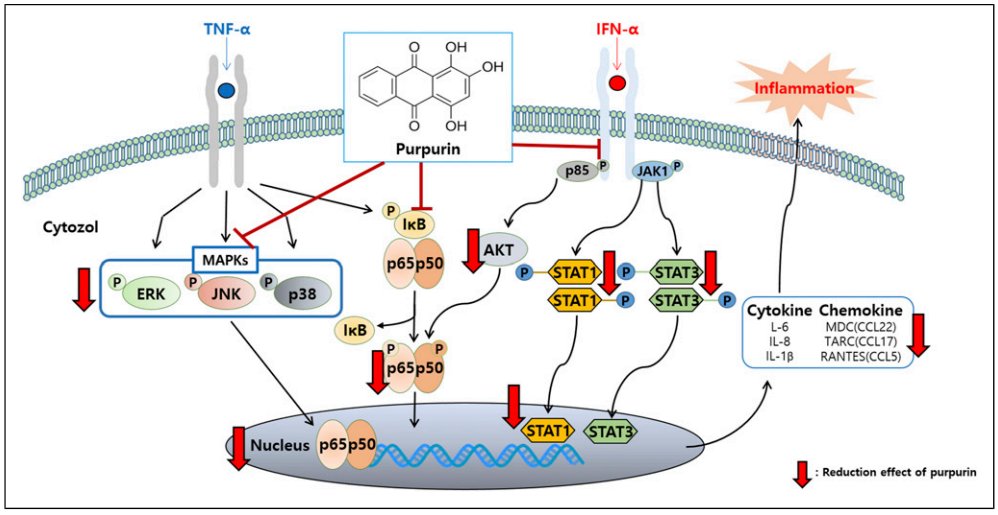

Figure 8. Schematic inhibitory signaling pathway of purpurin on interferon TNF-α/IFN-γ-induced in HaCaT cell. Purpurin reduced the phosphorylation of NF-κB (IκBα, p65), MAPK (p38, JNK, ERK), STAT1, and STAT3. Additionally, purpurin inhibited the nuclear translocation of NF-κB, STAT1, and STAT3. There, purpurin has an anti-inflammatory effect.

dissolved in Tris-buffered saline containing 0.05% Tween-20 (TBS/T) for 1 h at room temperature. The PVDF membrane was incubated overnight at 4°C with the primary antibody. After washing 3 times with TBS/T, the membrane was incubated with HRP (horseradish peroxidase)-conjugated secondary antibody diluted 1:5000 with 5% skim milk of TBS/T at room temperature for 1 h. Membranes were then visualized using SuperSignal West Pico Chemiluminescent Substrate (Cat. No. 32106; Pierce; Thermo Fisher Scientific, Inc.). The primary antibodies used were as follows: anti-NF-κB p65 (cat. no. sc-8242; 1:1000), anti-JNK (cat. no. sc-474; 1:1000), anti-extracellular signal-regulated kinase (ERK; cat. no. sc-154; 1:1000), anti-p38 (cat. no. sc-7149; 1:1000), anti-p-p38 (cat. no. sc-7973; 1:1000), anti-phosphorylated (p-) JAK1 (cat. no. sc-16773; 1:1000), anti-JAK1 (cat. no. sc-376996; 1:1000), anti-p-STAT1 (cat. no. sc-8394; 1:1000), anti-STAT1 (cat. no. sc-464; 1:1000; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-p-ERK 1/2 (cat. no. 9101; cell signaling, USA).

Immunocytochemistry

HaCaT cells were seeded in a NuncTM Lab-TekTM II Chamber SlideTM System (Cat. No. 154534, Nunc, Thermo, MA, USA) at 5×10^4 cells/well and treated with 40 μM purpurin for 1 h. After treatment with 10 ng/mL TNF-α/IFN-γ for 1 h, the cells were fixed in 4% neutral buffered formalin (Cat. No. BBC 0150, BBC Biochemical, WA, USA) for 10 min at room temperature and washed 2 times with cold PBS. HaCaT cells were permeabilized with 0.2% Triton X-100 in cold PBS for 10 min at 4°C, washed 3 times with PBS, and blocked with 3% bovine serum albumin (BSA) in PBS. Next, the cells were incubated with primary antibodies against STAT1 (Cat. #8394, Cell Signaling Technology) and STAT3 (Cat. No. #9145, Cell signaling Technology) at 4°C, overnight. After incubation, HaCaT cells were washed with PBS 3 times, incubated with anti-rabbit Alexa Fluor 488-conjugated secondary antibody at 4°C for 1 h, and washed with PBS 3 times. Then, the cells were incubated with Hoechst 33342 nucleic acid stain (Cat. H3570, Thermo Fisher Scientific, Inc., MA, USA) for 5 min, mounted with coverslips, and visualized with an Axio Observer Z1 microscope (ZEISS, Oberkochen, Germany).

Statistical analysis

For the statistical analysis, the data are expressed as the mean ± SD. Statistical significance among groups was determined by the two-tailed Student's t test. $p < .05$ was considered to indicate a statistically significant difference.

Conclusions

In conclusion, HaCaT cells were treated with TNF-α/IFN-γ to induce cytokines and chemokines. Using qPCR and ELISA, purpurin was found to suppress the final products of this inflammation, cytokines, and chemokines. We also found that the inhibitory effect of purpurin regulates the NF-κB, JAK/STAT, and MAPK signaling pathways (Figure 8). Furthermore, it was confirmed that there was a synergistic effect when purpurin and the inhibitor were applied together. Our findings suggest that purpurin may be a potential treatment for inflammation associated with Atopic dermatitis.

Acknowledgements

The authors thank the National Biodiversity Institute and Tempisque Conservation Area for preparing the plant materials.

Author contributions

JHO analyzed the data and wrote the manuscript. SHK performed in vitro experiments. OKK and JHK prepared the purpurin and analyzed and edited the manuscript. SRO, SBH, JWP, and KSA designed the study and edited the manuscript. All authors have critically revised the article and approved the final version of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The present study was supported by the Bio and Medical Technology Development Program of the National Research Foundation (NRF), funded by the Ministry of Science and ICT (2019M3A9I3091681). This study was funded by the Korean government (MSIT; grant no. NRF-2020R1A2C2101228) and the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Program of the Republic of Korea (grant no. KGM5522211).

ORCID iD

Ji-Won Park https://orcid.org/0000-0003-2899-1937

Supplemental Material

Supplemental material for this article is available online.

References

1. Abramovits W (2005) Atopic dermatitis. *Journal of the American Academy of Dermatology* 53: S86–S93. DOI: 10.1016/j.jaad.2005.04.034.
2. Brandt EB and Sivaprasad U (2011) Th2 cytokines and atopic dermatitis. *Journal of Clinical and Cellular Immunology* 2. DOI: 10.4172/2155-9899.1000110.
3. Portugal-Cohen M, Horev L, Ruffer C, et al. (2012) Non-invasive skin biomarkers quantification of psoriasis and atopic dermatitis: cytokines, antioxidants and psoriatic skin auto-fluorescence. *Biomedicine and Pharmacotherapy* 66: 293–299. DOI: 10.1016/j.biopha.2011.12.009.
4. Hoffjan S and Stemmler S (2007) On the role of the epidermal differentiation complex in ichthyosis vulgaris, atopic dermatitis and psoriasis. *The British Journal of Dermatology* 157: 441–449. DOI: 10.1111/j.1365-2133.2007.07999.x.
5. Nakatsuji T, Chen TH, Two AM, et al. (2016) Staphylococcus aureus exploits epidermal barrier defects in atopic dermatitis to trigger cytokine expression. *Journal of Investigative Dermatology* 136: 2192–2200. DOI: 10.1016/j.jid.2016.05.127.
6. Lee HS, Park JW, Kwon OK, et al. (2019) Anti-inflammatory effects of ethanol extract from the leaves and shoots of Cedrela odorata L. in cytokine-stimulated keratinocytes. *Experimental and Therapeutic Medicine* 18: 833–840. DOI: 10.3892/etm.2019.7639.
7. Zlotnik A and Yoshie O (2000) Chemokines: a new classification system and their role in immunity. *Immunity* 12: 121–127. DOI: 10.1016/s1074-7613(00)80165-x.
8. Kang GJ, Han SC, Yi EJ, et al. (2011) The inhibitory effect of premature citrus unshiu extract on atopic dermatitis in vitro and in vivo. *Toxicological Research* 27: 173–180. DOI: 10.5487/TR.2011.27.3.173.
9. Vestergaard C, Yoneyama H, Murai M, et al. (1999) Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *Journal of Clinical Investigation* 104: 1097–1105. DOI: 10.1172/JCI7613.
10. Horikawa T, Nakayama T, Hikita I, et al. (2002) IFN-gamma-inducible expression of thymus and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in epidermal keratinocytes and their roles in atopic dermatitis. *International Immunology* 14: 767–773. DOI: 10.1093/intimm/dxf044.
11. Sebastiani S, Albanesi C, De PO, et al. (2002) The role of chemokines in allergic contact dermatitis. *Archives of Dermatological Research* 293: 552–559. DOI: 10.1007/s00403-001-0276-9.
12. Pfeffer LM (2011) The role of nuclear factor kappaB in the interferon response. *Journal of Interferon and Cytokine Research* 31: 553–559. DOI: 10.1089/jir.2011.0028.
13. Li Y and Jiang JG (2018) Health functions and structure-activity relationships of natural anthraquinones from plants. *Food and Function* 9: 6063–6080. DOI: 10.1039/c8fo01569d.
14. Zengin G, Degirmenci NS, Alpsoy L, et al. (2016) Evaluation of antioxidant, enzyme inhibition, and cytotoxic activity of three anthraquinones (alizarin, purpurin, and quinizarin). *Human and Experimental Toxicology* 35: 544–553. DOI: 10.1177/0960327115595687.
15. Shen CH, Liu CT, Song XJ, et al. (2018) Evaluation of analgesic and anti-inflammatory activities of Rubia cordifolia L. by spectrum-effect relationships. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* 1090: 73–80. DOI: 10.1016/j.jchromb.2018.05.021.
16. Singh J, Hussain Y, Luqman S, et al. (2020) Purpurin: a natural anthraquinone with multifaceted pharmacological activities. *Phytotherapy Research* 35: 2418–2428. DOI: 10.1002/ptr.6965.
17. Foster M, Hunter D and Samman S (2011) Evaluation of the nutritional and metabolic effects of aloe vera. In: Benzie IFF and Wachtel-Galor S (eds) *Herbal Medicine: Biomolecular and Clinical Aspects*. Boca Raton (FL): CRC.

18. Guenounou M (1998) Cytokines and allergic response. *Annales de Biologie Clinique* 56: 297–304.
19. Liu T, Zhang L, Joo D, et al. (2017) NF-kappaB signaling in inflammation. *Signal Transduction and Targeted Therapy* 2. DOI: 10.1038/sigtrans.2017.23.
20. Seger R and Krebs EG (1995) The MAPK signaling cascade. *The Federation of American Societies of Experimental Biology Journal* 9: 726–735.
21. Brunner PM, Leung DYM and Guttman-Yassky E (2018) Immunologic, microbial, and epithelial interactions in atopic dermatitis. *Annals of Allergy, Asthma and Immunology* 120: 34–41. DOI: 10.1016/j.anai.2017.09.055.
22. Kim BE and Leung DY (2012) Epidermal barrier in atopic dermatitis. *Allergy, Asthma and Immunology Research* 4: 12–16. DOI: 10.4168/aair.2012.4.1.12.
23. Schmitt J, Langan S, Deckert S, et al. (2013) Assessment of clinical signs of atopic dermatitis: a systematic review and recommendation. *Journal of Allergy and Clinical Immunology* 132: 1337–1347. DOI: 10.1016/j.jaci.2013.07.008.
24. Maeda S and Yanagihara Y (2001) Inflammatory cytokines (IL-4, IL-5 and IL-13). *Nihon Rinsho* 59: 1894–1899.
25. Tsuda H, Michimata T, Hayakawa S, et al. (2002) A Th2 chemokine, TARC, produced by trophoblasts and endometrial gland cells, regulates the infiltration of CCR4+ T lymphocytes into human decidua at early pregnancy. *American Journal of Reproductive Immunology* 48: 1–8. DOI: 10.1034/j.1600-0897.2002.01117.x.
26. Vestergaard C, Bang K, Gesser B, et al. (2000) A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. *Journal of Investigative Dermatology* 115: 640–646. DOI: 10.1046/j.1523-1747.2000.00115.x.
27. Giuliani C, Bucci I and Napolitano G (2018) The Role of the transcription factor nuclear factor-kappa B in thyroid autoimmunity and cancer. *Frontiers in Endocrinology* 9: 471. DOI: 10.3389/fendo.2018.00471.
28. Yue J and Lopez JM (2020) Understanding MAPK signaling pathways in apoptosis. *International Journal of Molecular Sciences*: 21. DOI: 10.3390/ijms21072346.
29. Yano C, Saeki H, Komine M, et al. (2015) Mechanism of macrophage-derived chemokine/CCL22 production by HaCaT keratinocytes. *Annals of dermatology* 27: 152–156. DOI: 10.5021/ad.2015.27.2.152.
30. Zhang W and Liu HT (2002) MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research* 12: 9–18. DOI: 10.1038/sj.cr.7290105.
31. Seif F, Khoshmirsafa M, Aazami H, et al. (2017) The role of JAK-STAT signaling pathway and its regulators in the fate of T helper cells. *Cell Communication and Signaling* 15: 23. DOI: 10.1186/s12964-017-0177-y.
32. Darnell JE Jr, Kerr IM and Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264: 1415–1421. DOI: 10.1126/science.8197455.
33. Kwon DJ, Bae YS, Ju SM, et al. (2012) Casuarinin suppresses TARC/CCL17 and MDC/CCL22 production via blockade of NF-kappaB and STAT1 activation in HaCaT cells. *Biochemical and Biophysical Research Communications* 417: 1254–1259. DOI: 10.1016/j.bbrc.2011.12.119.