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Anti-inflammatory effect of taxifolin in TNF-α/IL-17A/IFN-γ induced HaCaT human keratinocytes

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Abstract

Taxifolin, a bioactive flavonoid, has been attracting attention as a beneficial and valuable phytochemical due to its antioxidant, anticancer, and anti-inflammatory properties. Recently, an improvement effect of taxifolin against psoriasis has been reported in an animal experimental model. However, its exact mechanism of action at molecular and cellular levels is not known. Thus, the purpose of this study was to verify the anti-inflammatory effect of taxifolin on psoriasis at cellular/molecular level using HaCaT human keratinocytes. First, a CCK-8 assay was performed to evaluate cytotoxicity of taxifolin. Results revealed that taxifolin was a relatively safe material, showing no cytotoxicity at concentrations up to 300 μg/mL. In TNF-α-induced HaCaT cells, taxifolin significantly inhibited mRNA expression levels of pro-inflammatory cytokines (IL-1α, IL-1-β, and IL-6) and chemokines (CXCL8 and CCL20). The ability of taxifolin to regulation expression of inflammatory cytokine genes was associated with phosphorylation of IκB/STAT3 protein. In addition, taxifolin inhibited expression levels of IL-1α/β, IL-6, CXCL8, and CCL20 by inhibiting IκB/STAT3 protein phosphorylation upon stimulation of TNF-α, IL-17A, and IFN-γ. These results show that taxifolin has the potential to be developed as a treatment for psoriasis and skin inflammation.

Keywords Anti-inflammation, HaCaT, STAT3, Psoriasis, Taxifolin

Introduction

Psoriasis is a chronic skin disease related to the typical human immune system. In patients with psoriasis, erythema occurs mainly on the skin or joints. Hyperproliferation of the epidermis caused by abnormal differentiation of the stratum corneum can stimulate deterioration of the skin barrier structure and an excessive inflammatory reaction [1–10]. The cause of psoriasis has not been clearly identified yet, although Th17 cells are known to be closely related to its pathogenesis. Normally, Antigen presenting cell (APC) dendritic cells activated by a foreign antigen can promote the differentiation of naive CD4+T cells, thereby promoting the differentiation of T helper cells. A large amount of differentiated Th17 cells have been found in the psoriatic skin tissue, strongly suggesting an association between Th17 and psoriasis disease development [11]. Th17 cells can produce IL-17A and IL-22 cytokines. IL-17A can induce excessive proliferation of keratinocytes in epidermis and secretion of chemokines such as chemokine CXC motif ligand 1/-3/-5/-8 and chemokine CC motif ligand 20 (CCL20). Moreover, it can trigger neutrophil infiltration into the lesion and amplify the inflammatory response through STAT3 dependent CCL20 chemokine expression [12–15]. Th1 cells are also involved in the mechanism of psoriasis [16]. TNF-α secreted from Th1 cells can stimulate skin keratinocytes to secrete pro-inflammatory cytokines (IL-1, IL-6, IL-23) [16]. Since overexpressed

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TNF-α is a central cause inducing keratinocyte proliferation, inhibitors of TNF-α are used to treat psoriasis in medical field [16]. As IFN-γ is also known to be overexpressed cytokine in psoriatic lesions, studies targeting IFN-γ overexpression are being actively conducted. Therefore, TNF-α, IL-17A, and IFN-γ can be viewed as central cytokines inducing psoriasis, a chronic skin inflammatory disease [17].

Bioactive compounds such as polyphenols and flavonoids derived from plants are known to be beneficial agents for human health. Specially, flavonoids have a wide range of cellular activities such as antioxidant, anti-inflammatory, and anticancer effects. Vegetables, fruits, and other plant foods containing high levels of flavonoids can decrease the risk of chronic inflammatory disorders and cancer, indicating that phytochemicals from natural products can be important resources for managing these diseases [18]. Taxifolin (dihydroquercetin) is commonly isolated in many plants including onions [18], milk thistle (*Silybum marianum*) [19], and oriental raisin tree (*Hovenia dulcis*) [20]. Taxifolin has been used as one component of dietary supplements and functional foods [21]. Various pharmacological effects of taxifolin have been reported, including its antioxidant, anticancer, hepatoprotective, antimicrobial, and anti-inflammatory activities [22, 23]. Recently, the anti-inflammatory activity of taxifolin in both RAW264.7 mouse macrophages and Imiquimod (IMQ)-induced mouse animal experiments has been reported [24–26]. However, taxifolin are not well-characterized in the anti-inflammatory activity for skin disease. Thus, this study was focusing on the determination about the anti-inflammatory effect of taxifolin on psoriasis.

Material and methods

Chemicals and reagents

Taxifolin, dimethylsulfoxide (DMSO), and TNF-α were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-17A and IFN-γ were purchased from R&D systems (Minneapolis, MN, USA). Brazilin used as a positive control was purchased from Chem Faces (Wuhan, Hubei, China).

Cell culture

Human keratinocyte (HaCaT) was purchased from CLS (Cell Lines Service GmbH, Eppelheim, Baden-Wurttemberg, Germany) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Gyeongbuk, Korea) supplemented with 1% penicillin-Streptomysin (Gibco, Grand Island, NY, USA), 10% fetal bovine serum (Welgene, Gyeongbuk, Korea) and incubated at 37 °C in a cell incubator with 5% CO_2.

Cell cytotoxicity

CCK-8 (Cell Counting Kit-8, DoGen bio, Seoul, Korea) assay was performed to observe the cytotoxicity of taxifolin. HaCaT cells were aliquoted into 24-well plates at density of 7.0×10^4 cells/well and stabilized for 24 h. Taxifolin was diluted to different concentrations and used to treat the HaCaT cells for 24 h. The supernatant of the culture medium was suctioned, a mixture of phenol red free-DMEM and EZ-Cytox prepared at a 10:1 ratio was added to each well and then reacted for 30 min. In living cells, the reaction solution will change color from colorless to yellow. Cell viability was determined by measuring absorbance at 450 nm with an ELISA reader (BioTek, Highland Park, USA). To observe taxifolin-induced cytotoxicity in TNF-α alone or TNF-α, IL-17A, and IFN-γ stimulated HaCaT cells, CCK-8 assay was performed in the same way.

RNA extraction and RT-PCR

To extract total RNA, cultured cells were lysed using Trizol (Ambion, Carlsbad CA, USA). Then 200 μL of chloroform was added and vortexed, followed by reaction at room temperature for 5 min. The supernatant was obtained after centrifuging at 14,000 rpm for 15 min at 4 °C. After mixing the supernatant and 2-propanol at a 1:1 ratio, layers were separated by centrifuging at 14,000 rpm for 15 min at 4 °C. Reverse transcription of RNA into cDNA was performed at 42 °C for 20 min, 99 °C for 5 min, and 4 °C for 5 min) using a Revertra ace -α- kit (Toyobo, Osaka, Japan). RT-PCR was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Using a Taqman probe (Applied Biosystems, Foster City, CA, USA) for each gene, PCR was performed with 45 cycles of 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. Probes used in this experiment are listed in Table 1.

Western blotting

To extract total proteins, RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) was used. After

Table 1 List of gene name and assay ID number in RT-PCR

Symbol	Gene name	Assay ID
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02786624_g1
CXCL8	C–X–C motif chemokine ligand 8	Hs00174103_m1
CCL20	C–C motif chemokine ligand 20	Hs01011368_m1
IL-1α	Interleukin 1 alpha	Hs00174092_m1
IL-1β	Interleukin 1 beta	Hs01555410_m1
IL-6	Interleukin 6	Hs00174131_m1

quantification of total protein, each sample was loaded onto a 10% polyacrylamide gel, separated by protein sizes through electrophoresis at 160 V for 1 h, and transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Waltham, MA, USA) for 1 h at room temperature. After blocking with 10% (w/v) skim milk, PVDF membrane was washed with 1 × TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and incubated with a primary antibody (1:1000) at 4 °C for 12 h. After washing with 1 × TBST 3 times for 10 min each, the membrane was incubated with a secondary antibody (1:10000) diluted in 4% skim milk for one hour. After 3 times of washing with 1 × TBST, western ECL substrates (Biorad, CA, USA) were dispensed onto the PVDF membrane. To visualize protein expression, an image processing device (Microchemi-DNR, Neve Wamin, Israel) was used. Protein quantification was performed using Image J software (N.I.H., Bethesda, MD, USA). Antibodies used in this experiment are listed in Table 2.

Statistical processing

All experiments data were analyzed with Student's t-test using average value and standard deviation of three measurements. Statistical significance was considered if *p*-value was less than 0.05 in Student's t-test.

Results

Cytotoxicity and regulation of psoriatic skin inflammation by taxifolin

Taxifolin, a polyphenol compound, might exhibit similar activity to quercetin because of its structure similarity (Fig. 1A). First, to determine the cytotoxicity of taxifolin to skin cells, a CCK-8 assay was performed using HaCaT human keratinocytes. Cell viability was observed after treatment with taxifolin at different concentrations (from 5 to 500 μg/mL) in FBS-free DMEM medium for 24 h, respectively. Taxifolin at concentrations up to 300 μg/mL showed no cytotoxicity to HaCaT cells (Fig. 1B), confirming that it was a relatively safe material. Inhibitory effect of taxifolin on hyperproliferation of epidermis was then determined using TNF-α treated HaCaT cells in FBS-free DMEM medium for 72 h as TNF-α could promote cell proliferation. Taxifolin at 20 μg/mL suppressed cell proliferation like brazilin, the positive control. It was observed about 1.5-fold over-proliferation of keratinocytes in the TNF-α-only group compared to the untreated group. However, taxifolin significantly inhibited the

Table 2 List of protein name and antibody used in western blot analysis

Symbol	Gene name	Assay ID
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	MA5-15738[a]
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	4812[b]
p-IκBα	Phospho-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2859[b]
ERK1/2	Extracellular signal-regulated kinase	4695[b]
p-ERK1/2	Phospho-extracellular signal-regulated kinase	9101[b]
p38	p38 mitogen-activated protein kinase	9212[b]
p-p38	phospho-p38 mitogen-activated protein kinase	9211[b]
STAT3	Signal transducer and activator of transcription 3	4904[b]
p-STAT3	Phospho-Signal transducer and activator of transcription 3	9145[b]
Goat anti-Rabbit IgG (H + L) Secondar Antibody, HRP		31460[a]
Goat anti-Mouse IgG (H + L) Secondar Antibody, HRP		31430[a]

a: Invitrogen™

b: Cell signaling Technology™

(See figure on next page.)

Fig. 1 Taxifolin suppressed hyperproliferation of epidermis and expression levels of pro-inflammatory cytokines (IL-1α, IL-1β, and IL-6) and chemokines CXCL8 and CCL20) in TNF-α treated HaCaT cells. **A** Structure of taxifolin, **B** Cytotoxicity of taxifolin to HaCaT cells. Taxifolin did not show any cytotoxicity at concentrations up to 300 μg/mL. **C** TNF-α-induced epidermal hyper-proliferation was suppressed by taxifolin in a concentration-dependent manner. Here, the non-cytotoxic concentration of taxifolin was set to be 20 ug/ml and brazilin was used as positive control. **D** IL-1α mRNA expression levels in TNF-α induced HaCaT cells were verified by real-time RT-PCR. Taxifolin downregulated IL-1α mRNA expression in a concentration-dependent manner. Taxifolin inhibited **E** IL-1β **F** IL-6 gene expression levels in TNF-α signal pathway. Taxifolin also inhibited expression levels of chemokines such as **G** CXCL8 and **H** CCL20 in TNF-α treated HaCaT cells. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ versus control; *, $p < 0.05$, **, $p < 0.01$ versus TNF-α treated HaCaT cells

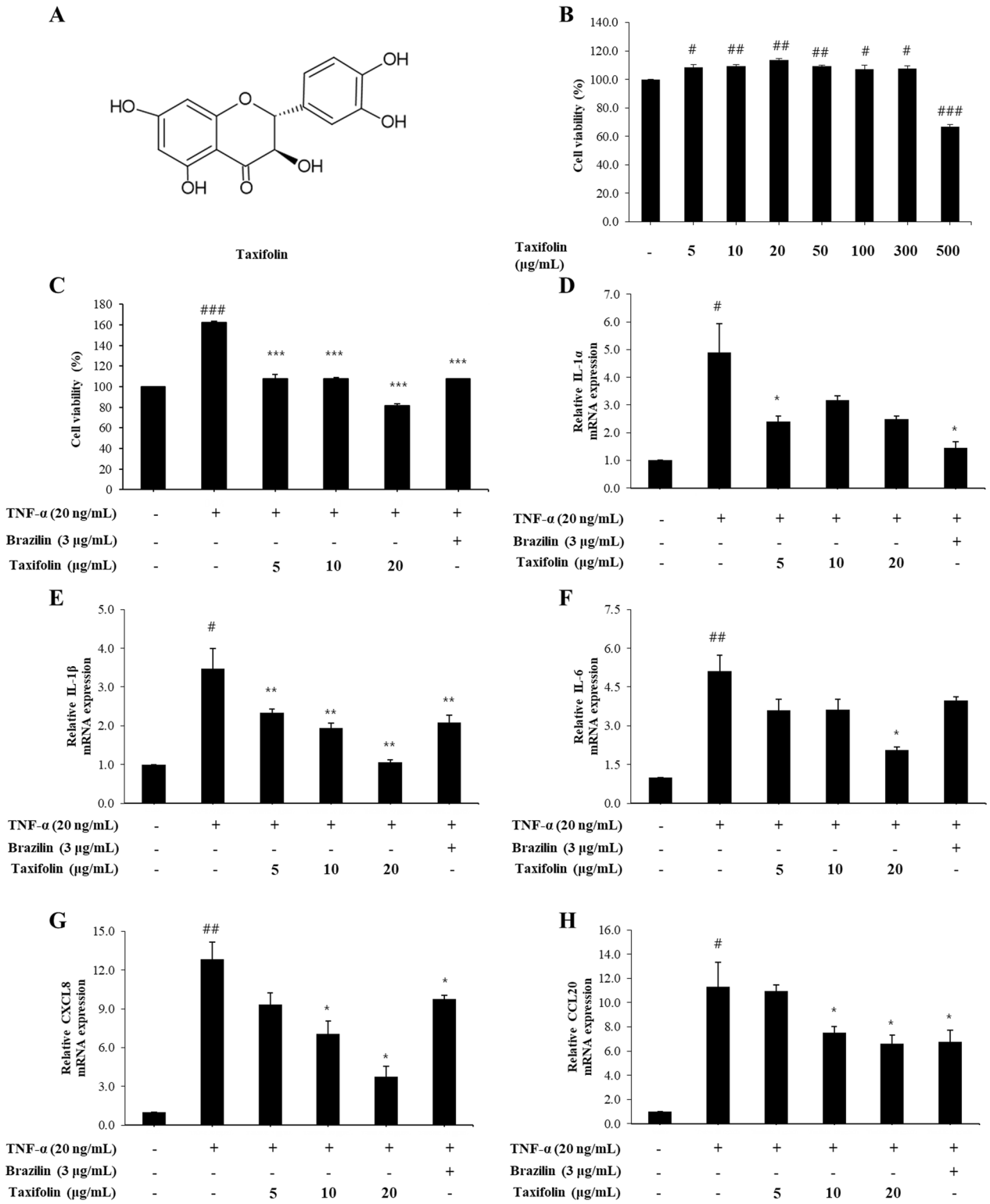

Fig. 1 (See legend on previous page.)

hyper-proliferation induced by TNF-α (Fig. 1C). These results confirmed that the epidermal hyper-proliferation phenomenon characteristic of psoriasis was significantly regulated by taxifolin. Expression levels of inflammatory cytokine and chemokine in TNF-α-induced HaCaT cells after treatment with taxifolin at safe concentrations of 5, 10, and 20 μg/mL were then examined. Brazilin (3 μg/mL) was used as a positive control. IL-1α mRNA expression was induced in TNF-α only treated group by 4.89 ± 1.05 folds compared to that in the untreated group. Its expression was inhibited by 36.35 ± 13.53%, 48.28 ± 13.04%, and 72.22 ± 2.62% in groups treated with taxifolin at 5, 10, and 20 μg/mL, respectively, showing a concentration-dependent inhibition (Fig. 1D). The expression of IL-1β, another member of the IL-1 family, was increased 3.47 ± 0.52 times in TNF-α treatment group. However, it was significantly inhibited by taxifolin (Fig. 1E). In addition, IL-6 expression was increased by 5.11 ± 0.64 times in TNF-α only treated group. It was inhibited by 59.09 ± 6.88% by taxifolin at a concentration of 20 μg/mL (Fig. 1F). These results confirmed that taxifolin significantly reduced mRNA expression levels of inflammatory cytokines IL-1α/1β and IL-6. CXCL8 and CCL20 chemokines are found at high concentrations in psoriatic lesions. CXCL8 and CCL20 mRNA levels were increased by 12.85 ± 1.33 and 11.33 ± 2.03 times, respectively, in TNF-α alone treated group compared to those in the untreated group. CXCL8 mRNA expression in the group treated with taxifolin at 20 μg/mL was inhibited by 70.50 ± 7.99% compared to that in the TNF-α alone treated group (Fig. 1G). In addition, CCL20 expression in the group treated with taxifolin at 20 μg/mL was inhibited by 41.04 ± 4.60% (Fig. 1H). These results confirmed that taxifolin not only suppressed epidermal hyperproliferation in the TNF-α-induced psoriasis environment, but also suppressed mRNA expression levels of inflammatory cytokines and chemokines.

Inhibitory effect of taxifolin on IκB/STAT3 protein phosphorylation in TNF-α treated HaCaT cells

NF-κB or STAT3 pathway is closely related to the pathogenesis of skin inflammation. Phosphorylation of IκB protein in TNF-α-induced HaCaT cells was increased by about 2.9 ± 0.29 times compared to that in the untreated control, whereas treatment with taxifolin at concentrations of 10 and 20 μg/mL inhibited phosphorylation of IκB protein by 71.41 ± 1.68% and 73.30 ± 3.26%, respectively. These results confirmed that taxifolin was involved in the regulation of IκB phosphorylation in the NF-κB pathway (Fig. 2A, B). In addition, TNF-α can induce secretion of IL-6, IL-23, and CCL20 through STAT3 transcription factor activation, which in turn can accelerate chronic inflammatory responses. Thus, we examined phosphorylation and expression changes of STAT3 protein in the presence of taxifolin. STAT3 protein level was increased by 7.04 ± 0.77 times in TNF-α alone treated group compared to that in the untreated group. When cells were treated with taxifolin at concentrations of 5, 10, and 20 μg/mL, STAT3 protein expression levels were significantly decreased by 26.25 ± 3.36, 75.23 ± 1.62, and 87.08 ± 1.35%, respectively (Fig. 2C, D).

Anti-inflammatory effect of taxifolin in TNF-α/IL-17A/IFN-γ-induced psoriasis model

Psoriasis is related with IL-17A, IFN-γ and TNF-α. Among these three cytokines, psoriasis is most strongly associated with IL-17A. Here, to set an in vitro psoriasis model, HaCaT cells were simultaneously treated with all three cytokines. In HaCaT cells stimulated with all three cytokines (TNF-α, IL-17A, and IFN-γ), cell viability was increased about 116.5 ± 0.74% compared to that of the untreated group, suggesting epidermal hyper-proliferation. When HaCaT cells were co-treated with taxifolin and all three cytokines (TNF-α + IL-17A + IFN-γ), cell cytotoxicity was not observed when taxifolin was used at concentration up to 300 μg/mL, showed a pattern similar to the result of TNF-α alone treatment (Fig. 3A). In HaCaT cells treated with all three cytokines (TNF-α + IL-17A + IFN-γ) and taxifolin at a concentration up to 20 μg/mL, mRNA expression levels of IL-1α, IL-1β, and IL-6 were significantly decreased compared to all three cytokines (TNF-α + IL-17A + IFN-γ) treated group (Fig. 3B, C, D). In addition, CXCL8 and CCL20 mRNA were inhibited by taxifolin as concentration dependent manner (Fig. 3E, F). Therefore, taxifolin could effectively regulated mRNA expression levels of inflammatory cytokines and chemokines in an in vitro psoriasis model.

Taxifolin also regulated IκB/STAT3 protein phosphorylation under the same condition. Phosphorylation level of IκB protein was increased 5.69 ± 0.46 folds in TNF-α + IL-17A + IFN-γ treated HaCaT cells. Although no change was observed in the group treated with taxifolin at a concentration lower than 20 μg/mL, phosphorylation level of IκB protein was significantly decreased by 64.81 ± 6.03% in the group treated with taxifolin at 20 μg/mL (Fig. 4A. B). STAT3 phosphorylation was also increased by 5.86 ± 0.07 times in HaCaT cells treated with all three cytokines (TNF-α + IL-17A + IFN-γ) compared to that in the untreated group. STAT3 phosphorylation was significantly decreased by 54.35 ± 1.90% by taxifolin (Fig. 4C, D). These results indicate that taxifolin can significantly inhibit phosphorylation of IκB and STAT3 and expression levels of cytokines and chemokines in the psoriasis model treated with all three cytokines (TNF-α + IL-17A + IFN-γ).

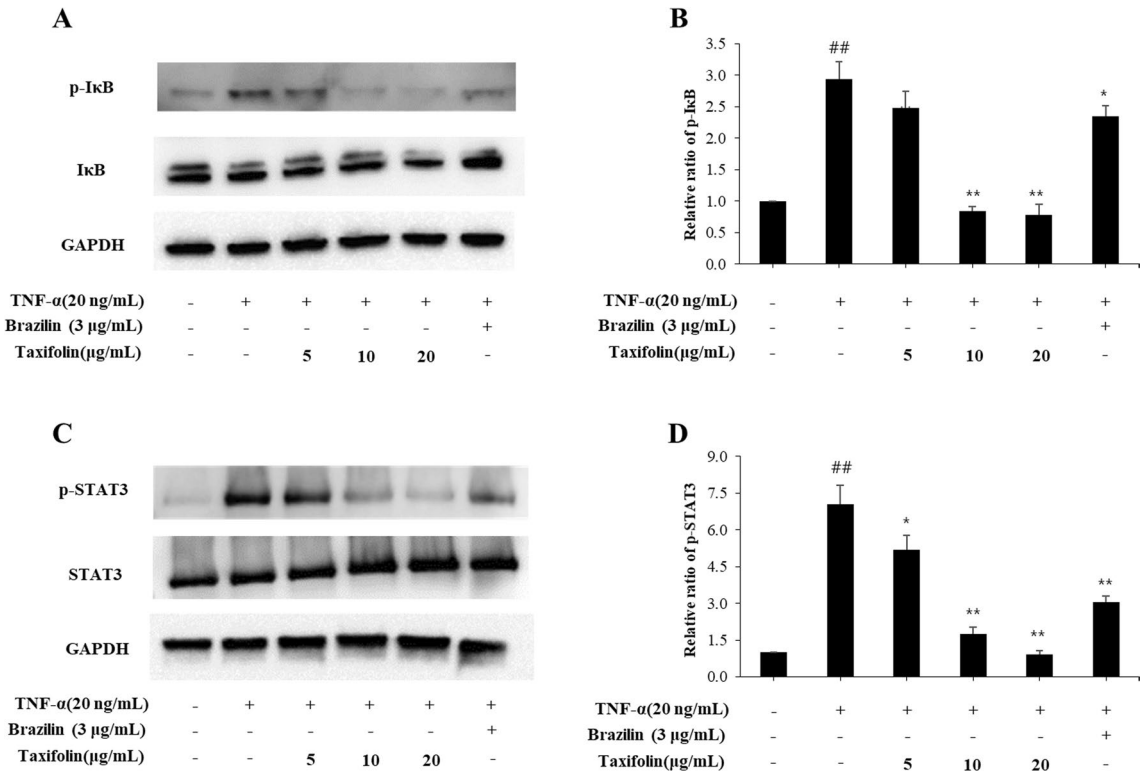

Fig. 2 Taxifolin regulates IκB and STAT3 protein activation in TNF-α signal pathway. **A** IκB phosphorylation in the presence of TNF-α was inhibited by taxifolin. Although similar expression levels of IκB protein were observed under various conditions, IκB phosphorylation was significantly inhibited by taxifolin, strongly suggesting an anti-inflammatory activity of taxifolin through regulation of IκB phosphorylation. **B** p-IκB/IκB level was quantified with ImageJ program. **C** Taxifolin inhibited STAT3 phosphorylation as a concentration-dependent manner. GAPDH was used as a loading control. **D** Quantification of p-STAT3/STAT3 level with ImageJ program. ##, $p < 0.01$ versus untreated control; *, $p < 0.05$ and **, $p < 0.01$ versus TNF-α treated group

Discussion

Psoriasis is an intractable skin disease, showing overgrowth of epidermal keratinocytes due to an abnormality of the skin's immune system and weakened skin barrier. It is accompanied by silvery white scales and severe inflammation. The pathogenesis of psoriasis is directly related to IL-17A secreted by Th17 cells differentiated from CD4 + T cells. On the skin of psoriasis patients, increased expression levels of cytokines (such as TNF-α, IL-6, IL-17A) and chemokines (such as CXCL8 and CCL20) have been observed in the epidermal differentiation process. Currently, vitamin D derivatives, steroids, UVB phototherapy, and TNF-α inhibitors are used as treatments for psoriasis. However, no cure with risk of recurrence is their biggest problem. Thus, developing natural product-based materials with safety guaranteed due to reports of side effects caused by chemicals such as steroids is very important at this time.

The anti-inflammatory activity of taxifolin has already been confirmed in the RAW264.7 mouse macrophage model [24, 25]. Recently, the psoriasis alleviating effect of taxifolin was reported in an IMQ-induced psoriasis-like model [26]. However, the exact mechanism of action by taxifolin at the cellular/molecular level is unknown. Therefore, in this study, we tried to identify the mechanism of action of taxifolin using an in vitro psoriasis-like human keratinocyte model. First, keratinocytes were activated with TNF-α known as an epidermal hyperproliferative factor of psoriasis using HaCaT cells, an immortalized keratinocyte line. Inhibition of hyperproliferation by taxifolin and changes in proinflammatory cytokine/chemokine expression were then examined. As a result, taxifolin was confirmed as a relatively safe compound among natural product-based compounds as it showed no cytotoxicity at concentrations up to 300 ug/mL. Taxifolin also significantly inhibited mRNA expression levels of TNF-α-stimulated IL-1α, IL-1β, and IL-6. It also significantly regulated the expression of chemokines CXCL8 and CCL20. To investigate the role of taxifolin in TNF-α signaling mechanism, NF-κB/STAT3 protein expression in keratinocytes was observed. As a result, taxifolin significantly decreased IκB and STAT3 protein phosphorylation. This suggests that taxifolin is involved in upstream regulation of IκB/STAT3 protein in TNF-α signaling. This indicates that taxifolin could

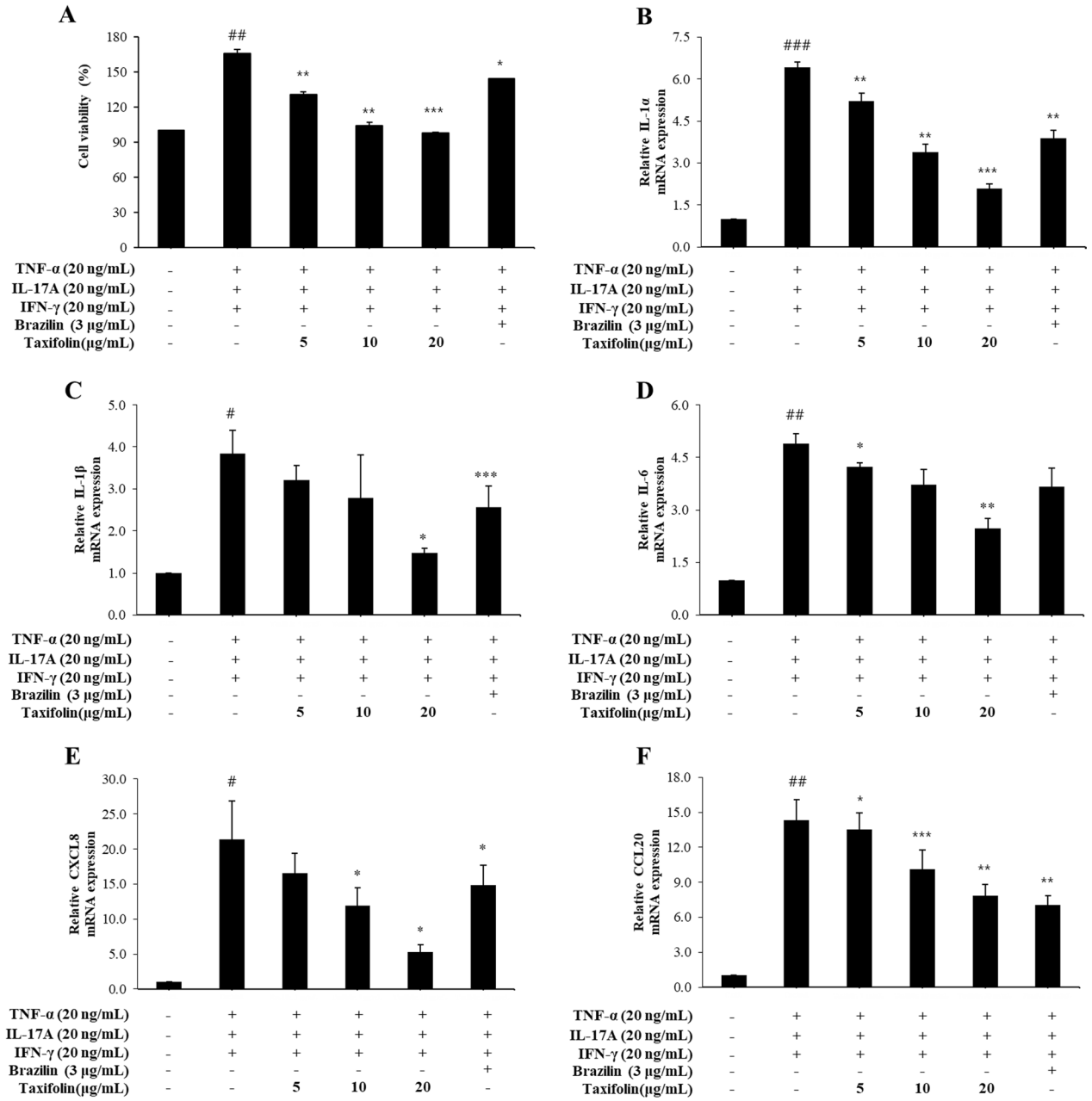

Fig. 3 Taxifolin suppresses psoriasis-related cytokines and chemokines in HaCaT cells treated with TNF-α + IL-17A + IFN-γ. **A** Taxifolin at concentrations up to 300 μg/mL is not cytotoxic to TNF-α + IL-17A + IFN-γ treated HaCaT cells. **B** IL-1α, **C** IL-1β, **D** IL-6, **E** CXCL, (F) CCL20 mRNA expression levels determined by real-time RT-PCR. Brazilin was used as positive control. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ versus control; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus TNF-α + IL-17A + IFN-γ treated HaCaT cells

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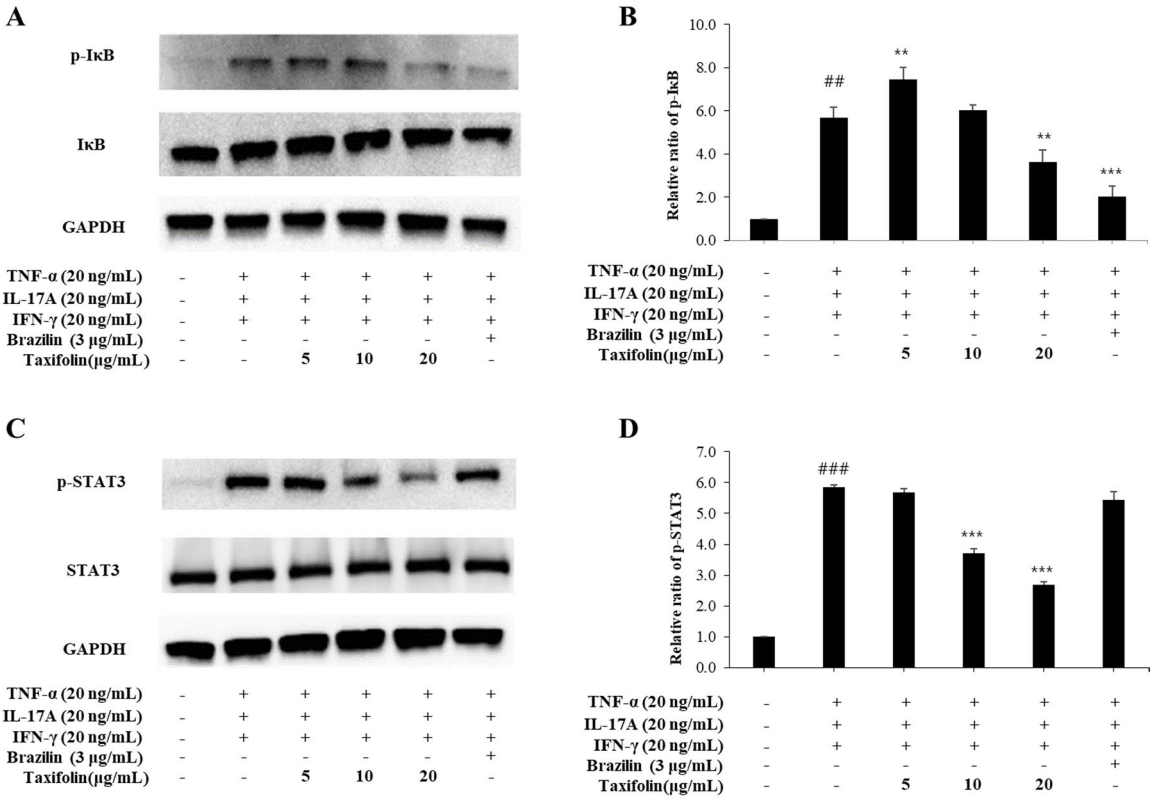
Fig. 4 Taxifolin down-regulates phosphorylation of IκB/STAT3 in TNF-α + IL-17A + IFN-γ treated HaCaT cells. **A** IκB and phospholated IκB protein levels were determined by western blot analysis. GAPDH was used as a loading control. **B** Quantification of p-IκB/IκB levels using ImageJ program. **C** STAT3/pSTAT3 protein level validated by western blot analysis. **D** Quantification of p-STAT3/STAT3 levels using Image J program. **E** Taxifolin suppress inflammatory cytokine and chemokine expression by inhibiting IκB/STAT3 protein phosphorylation in a human keratinocyte psoriasis-like model. ##$p < 0.05$ and ###$p < 0.001$ versus untreated control, **$p < 0.01$ and ***$p < 0.001$ versus the group treated with TNF-α + IL-17A + IFN-γ



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regulate gene expression of pro-inflammatory cytokines and chemokines and alleviate inflammatory responses. In this study, we applied an in vitro cell model closest to the psoriatic environment, that is, HaCaT cells treated with TNF-α + IL-17A + IFN-γ or under TNF-α alone treatment condition. It was confirmed that taxifolin significantly decreased the expression of IL-1α/β and IL-6. It also strongly inhibited the expression of CXCL8 and CCL20 chemokines. In addition, taxifolin showed inhibitory regulation on the expression of inflammatory cytokines and chemokines throughout the regulating IκB/STAT3 protein phosphorylation. In addition, taxifolin is expected to be involved in the regulation of epidermal differentiation by regulating the expression of keratin 5 and keratin 16/17 known to be psoriasis marker proteins [27]. Therefore, we understand that the psoriasis-regulating function of taxifolin is related to the reduction of inflammatory cytokine and chemokine expression by inhibiting IκB/STAT3 protein phosphorylation in keratinocytes (Fig. 4E). Results of this study confirmed that taxifolin could be used as an active ingredient for psoriasis regulation, with the potential to be developed as a novel psoriasis treatment.

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Author contributions
JE Park led in conducting experiments and interpreted the law data in the psoriasis skin model and in vitro experiments. HJ Kwon performed the CCK-8 assay and RT-PCR, and HJ Lee suggested experiment idea in material selection and wrote the manuscript. HS Hwang designed experiments, analyzed and review all data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Competing interests
The authors declare that they have no competing interests.

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