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Anti-skin inflammatory effects of compounds from *Cudrania tricuspidata* in HaCaT human Keratinocyte

조선대학교 대학원

약 학 과

김 나 연

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꾸지뽕나무 유래 화합물들의 HaCaT 사람 피부각질 형성세포에서 피부염증 억제효과

2020 년 2 월 25 일

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이 논문을 약학 석사학위신청 논문으로 제출함

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IL-6: Interleukin-6

IL-8: Interleukin-8

TNF-α: Tumor necrosis factor-alpha

IFN-γ: Interferon-gamma

RANTES: Regulated upon activation, normal T cell expressed and secreted (CCL5)

TARC: Thymus and Activation-Regulated Chemokine (CCL17)

COX-2: Cyclooxygenase-2

ICAM-1: Intercellular adhesion molecule-1

RP: Reverse phase

C.C.: Column chromatography

HPLC: High performance liquid chromatography

CCK-8: Cell Counting Kit-8

TLC: Thin layer chromatography

PBS: Phosphate-buffered saline

FBS: Fetal bovine serum

DMSO: Dimethyl sulfoxide

DMEM: Dulbecco's Modified Eagle's Medium

CCK8: Cell Counting Kit 8

NF-κB: Nuclear factor kappa B

MAPK: Mitogen-activated protein kinase

ERK: Extracellular signal regulatory kinase

JNK: c-Jun N-terminal kinase

국문 초록

꾸지뽕나무(*Cudrania tricuspidata*) 유래 화합물들의 HaCaT 사람 피부각질 형성세포에서 피부염증 억제효과

김나연
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꾸지뽕나무 뿌리는 전통적으로 한국과 중국에서 사용되었다. 한국에서는 전국 각지에서 자생하고 있으며, 뿌리와 잎에는 항암, 항산화 및 저혈당 효과가 있는 활성물질이 포함되어 있다고 알려져 있다. 꾸지뽕나무 뿌리 껍질은 항경화, 항염증, 항산화, 신경 보호, 간보호 및 세포 독성 활성을 갖는 것으로 보고되어있다. 그러나 꾸지뽕나무를 활용한 피부염증억제에 대한 선행연구는 미미한 상태다. 피부염은 유전적, 환경적, 면역학적 장애의 영향을 받는다. 접촉성 피부염, 건선, 아토피성 피부염 등과 관련된 여러 피부 질환은 각질형성세포와 사이토카인의 상호작용과 밀접한 관련이 있다. 사람각질형성세포인 HaCaT 세포는 여러가지 사이토카인 분비를 통해 국소염증 반응을 일으켜서 피부염증을 일으키는 세포모델로 널리 쓰인다. TNF-α 와 IFN-γ 에 의한 외부 자극은 염증 반응 전사 인자 중 하나인 인 nuclear factor kappaB (NF-κB)를 활성화시켜 cyclooxygenase-2 (COX-2) 와 intercellular adhesion molecule-1 (ICAM-1)를 발현시키고, COX-2 와 ICAM-1 는 다시 사이토카인을 생성시킨다. 이때 생성이 증가된 사이토카인들은 다시 NF-κB 경로를 활성화시켜서 염증 상태를 증폭시킨다. MAPK 는 여러 가지 외부자극에 의해 세포의 성장, 사멸, 분화 등에 관여하며 전사조절인자들은 인산화를 통해 여러 유전자들의 발현을 조절한다. 본 연구에서는 꾸찌뽕나무 유래 추출물 1 종, 분획물 6 종, 단일화합물 16 종의 피부염증조절 효과를 살펴보고자 하였다. 꾸지뽕나무 뿌리유래 70% EtOH 추출물은 HaCaT 세포에서 TNF-α 와 IFN-γ 로 유발된 IL-6, IL-8 생성억제효과를 우수하게 나타났다. 이어서 꾸지뽕나무 유래 70% EtOH 추출물의 하위 분획물 6 개를 제조하였고 피부염증을 조절하는 효과를 살펴보았다. 그 결과 하위분획 6 개 모두 IL-8 억제 효과가 우수했다. 추가적으로 꾸지뽕나무

뿌리유래 화합물 16 개[dihydrokaempferol (1), steppogenin (2), cudraflavanone A (3), cudraxanthone L (4), cudraflavone C (5), cudraflavanone D (6), kuwanon C (7), cudratricusxanthone A (8), macluraxanthone B (9), cudraxanthone M (10), 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (11), cudraxanthone D (12), cudratricusxanthone N (13), cudraflavanone B (14), cudratricusxanthone L (15), and cudratricusxanthone A (16)]를 선행연구로 확보하였고, 이를 이용하여 피부염증에 대한 효과와 작용기전을 살펴보았다. 먼저 HaCaT 세포에서 IL-6 생성억제 효과는 총 11 개의 화합물(1, 2, 4, 5, 6, 7, 9, 11, 14, 15, 16)에서 나타났다. IL-8 억제효과는 총 15 개의 화합물(1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16)에서 나타났다. 이중 가장 효과가 우수한 6 가지 화합물 steppogenin (2), cudraflavone C (5), macluraxanthone B (9), 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone(11), cudraflavanone B (14), cudratricusxanthone L (15)을 선별하여 다음 실험을 진행하였다. 먼저, HaCaT 세포에서 RANTES, TARC 와 같은 케모카인 억제 효과를 살펴보았다. 그 결과 6 개 화합물 모두 고농도 처리 군에서 케모카인 억제 효과가 우수하였다. COX-2 발현 조절실험에서는 cudraflavone C (5), cudratricusxanthone L (15) 2 개의 화합물에서 COX-2 발현 감소효과가 우수하였다. 또한 6 가지 화합물은 모두 ICAM-1 발현을 억제 하였다. 추가로 사이토카인과 케모카인을 조절하는 상위 기전인 NF-κB, MAPK 기전을 살펴보았다. 그 결과 NF-KB 활성 억제에서 IκB-α degradation 억제효과는 cudraflavone C (5), macluraxanthone B (9)화합물이 우수하였고, IκB-α phosphorylation 억제효과는 cudraflavanone B (14), cudratricusxanthone L (15)화합물이 우수하였다. 또한, 핵내 p65 전사억제 효과는 steppogenin (2), cudraflavone C (5), macluraxanthone B (9), cudraflavanone B (14), cudratricusxanthone L (15) 5 가지 화합물에서 우수하게 나타났다. MAPK phosphorylation 조절 효과를 확인한 실험에서는 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (11), cudraflavanone B (14) 화합물이 ERK phosphorylation 을 억제하였고, 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone(11), cudraflavanone B (14)화합물은 p38 phosphorylation 을 억제하였다. 위와 같은 피부염증 억제 효과 및 기전조절 연구결과 가장 우수한 효과를 보인 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (11), cudraflavanone B (14) cudratricusxanthone L (15)가 꾸지뽕나무 추출물과 분획물에 함유된 함량을 평가하고자 HPLC 분석을 하였다. 꾸지뽕나무 추출물과 분획물 안에서의 화합물 11, 14, 15 의 함량을 비교한 결과 추출물에서는 매우 미량 함유된 것으로 나타났으며, 분획물 중 CT-

5 에서 화합물 **14**, **15** 가 함유되어 있었다. 종합적으로 꾸지뽕나무 유래 추출물, 분획물, 그리고 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (**11**), cudraflavanone B (**14**) cudratricusxanthone L (**15**) 화합물은 피부염증을 억제하는 효과가 우수해 향후 피부질환 치료 및 예방 천연소재로 활용 가능할 것으로 사료된다.

Abstract

Anti-skin inflammatory effects of compounds from *Cudrania tricuspidata* in HaCaT human Keratinocyte

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C. tricuspidata roots have traditionally been used in Korea and China. The *C. tricuspidata* blossoms in June, and the fruit ripens from September to October. In Korea, they grow wild all over the country. The roots and leaves of *C. tricuspidata* contain active substances with anti-cancer, anti-oxidant and hypoglycemic effects. The root bark of *C. tricuspidata* has been reported to have anti-sclerotic, anti-inflammatory, anti-oxidant, neuroprotective, hepatoprotective and cytotoxic activities. Dermatitis is affected by genetic, environmental, and immunological disorders while chronic inflammation is characterized by increased epidermal thickness and penetration of macrophages, mast cells, and other inflammatory cells. The stimulation of keratinocytes by TNF-α and IFN-γ is highly dependent on activation and induces the expression and secretion of chemokines, normal T cells (RANTES), IL-8, and thymus and activation regulatory chemicals (TARC). Mitogen-activated protein kinases (MAPKs) are important enzymes in cell signaling, apoptosis, carcinogenesis, and the pathogenesis of different diseases. Nuclear factor-kappa B (NF-κB) translocate to the nucleus, binds to its recognized DNA element, and activates the transcription of the target gene. In the present study, we were investigated the effect of 70% EtOH extract, 6 sub-fractions, and 16 compounds from *C. tricuspidata* skin inflammation. Briefly, 70% EtOH extract decreased IL-6 and IL-8 production. And the sub-fractions from 70% EtOH extract of C. tricuspidata dose-dependently

decreased IL-8 production in TNFα+IFNγ stimulated HaCaT cells. We also used to test on the regulation of skin inflammation by the 16 compounds isolated from the roots of *C. tricuspidata* in our previously study. Among the 16 compounds isolated from *C. tricuspidata*, compounds **1, 2, 4, 5, 6, 7, 9, 11, 14, 15** and **16** reduced IL-6 production, and compounds **1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15,** and **16** compounds decreased IL-8 production. Among the 16 compounds, the six most effective compounds such as steppogenin (**2**), cudraflavone C (**5**), macluraxanthone B (**9**), 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (**11**), cudraflavanone B (**14**), and cudratricusxanthone L (**15**) were selected for the further experiments. RANTES and TARC production were decreased by pretreatment with the all of 6 compounds. Cudraflavone C (**5**) and cudratricusxanthone L (**15**) were found to reduce the expression of COX-2, and all of 6 compounds inhibited ICAM-1 expression. Compounds **2**, **5**, **9**, **14**, and **15** inhibited NF-κβ p65 translocation to nucleus, however compound **11** was not significant. In addition, ERK-1/2 phosphorylation was inhibited by only compound **15** and P38 phosphorylation was inhibited by compounds **11** and **14**. In addition, we were confirmed whether compounds **11, 14** and **15**, which are the most effective among 16 compounds, are included in the extracts and fractions. As a result of HPLC analysis using compounds **11, 14** and **15**, were included in CT-5 fraction. Comprehensively of all results in this study, 70%EtOH extract, sub-fraction, and 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (**11**), cudraflavanone B (**14**), and cudratricusxanthone L (**15**) from *C. tricuspidata* could be further developed as a therapeutic agent that suppresses inflammation in skin cells.

1. Introduction

The *Cudrania tricuspidata* Bureau (Moraceae) is a tree that is found throughout China, Korea, and Japan. Its root has been traditionally used in Korea and China [1]. *C. tricuspidata* is a red apricot that blooms in June and ripens from September to October [2]. Approximately 10 species of *Cudrania* are known. In fact, these species grow wild throughout Korea. The bark of *C. tricuspidata* is grayish brown, its deformed branches have thorns that range in length from 0.5 to 3.5 cm, and its body has hairs. The branches of *C. tricuspidata* are also known to produce blood. Its old bark has a yellowish-gray color and is torn vertically [3] and its leaves are split in three. Further, the edges are flat and egg-shaped, and both shapes are found on a single tree [4]. *C. tricuspidata* can be used throughout the year. In fact, its roots and leaves contain active pharmaceutical substances that exhibit anticancer, antioxidant, and hypoglycemic effects [5]. The *C. tricuspidata* root bark of has also been reported to exhibit anti-sclerotic effects [6], neuroprotective action [7], anti-inflammatory effects [8], mast cell activation [9], cytotoxic activity [10], pancreatic lipase inhibitory action [11], and monoamine oxidase suppressive effects [12]. The physiologically active components in *C. tricuspidata* are mainly polyphenol-based flavonoids. Based on prior research of these components including flavonone[13], flavonol[14], flavonol glycoside[15], flavonone glycoside[16], and xanthone [17], were identified as flavonoids[18] with different structures[19]. Several parts of *C. tricuspidata* have been reported to possess high antioxidant properties; such as di-hydroquercetin 7-o-β-D glucopyranoside, The bark has also been reported to exhibit high antioxidant activity owing to flavonoid compounds, such as taxifolin, orobol, eridictyol, dihydrokaempferol, and steppogenin[20, 21]. Dermatitis is

affected by genetic, environmental, and immunological disorders while chronic inflammation is characterized by increased epidermal thickness and penetration of macrophages, mast cells, and other inflammatory cells [22]. Keratinocytes can secrete various cytokines, and chemokine causing a local inflammatory reaction [23]. Keratinocytes are known to play an important role in inflammation. In fact, many skin diseases are related to inflammation, such as allergic contact dermatitis, psoriasis, and atopic dermatitis. And There are highly related to the function of keratinocytes and cytokines [24]. The stimulation of keratinocytes by tumor necrosis factor α (TNF-α) and interferon α (IFN-γ) is highly dependent on activation and induces the expression and secretion of chemokines, normal T cells (RANTES), IL-8, and thymus and activation regulatory chemicals (TARC), which are regulated accordingly. These factors contribute to the recruitment of inflammatory cells and inflammatory skin penetration [25]. The stimulation of keratinocytes by TNF-α and IFN-γ induced expression of pro-inflammatory cytokines, such as IL-6, IL8, and specific chemokines. These cytokines and chemokines contribute to the penetration of inflammatory cells into inflamed areas in the skin [26]. Mitogen-activated protein kinases (MAPKs) are important enzymes in cell signaling, apoptosis, carcinogenesis, and the pathogenesis of different diseases [27]. MAPK is a serine/threonine kinase that is comprised of the following subfamilies: ERK, p38 kinase, and JNK. ERK is activated by growth factors [28]; this activation can lead to its translocation to the nucleus. G-protein coupled receptor (GPCR) agonists and the stress-activating kinases, p38 and JNK, are activated by cellular stressors, such as UV irradiation, osmotic shock, and oxidative stress. MAPK activation can cause their transformation to the nucleus where they activate transcription factors via phosphorylation, ultimately causing

gene expression changes. Nuclear factor-kappa B (NF-κB) participates in the regulation of different biological processes, such as immune response, cell growth, and apoptosis. There are five well-known mammalian NF-κB/rel proteins: Rel (c-Rel), p65 (RelA), RelB, p50 (NFKB1), and p52 (NFKB2). Further, five or more distinct IkB proteins, including IkBα, IkBβ, and bcl-3, are known. The activation of NF-κβ induces a high rate of IκB phosphorylation and proteolysis by IκB kinase (IKK) [29]. NF-κB translocates to the nucleus, binds to its recognized DNA element, and activates the transcription of the target gene [28]. NF-kB is one of the most important transcription factors that translocate to the nucleus upon activation and cause genetic modifications [30]. The purpose of this study was investigated the effect of 70% EtOH extract, 6 sub-fractions, and 16 compounds from *C. tricuspidata* skin inflammation.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material

The root bark of *C. tricuspidata* was purchased in May 2014 from Daerim Korean crude drug store (Kumsan, Chungnam Province, Korea) and identified by Dr. Kyu-Kwan Jang (Botanical Garden, Wonkwang University). A voucher specimen (No. WP-2014-12) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Iksan, Korea).

2.1.2. Materials for chromatography

To isolate the compounds, pre-coated silica gel F plates (Merck, Art. 5715), RP-18F plates (Merck, Art. 15389), silica gel 60 (40–63 and 63-200 mm, Merck), and LiChroprep RP-18 (40-63 μm, Merck) were used for thin layer chromatography (TLC) and column chromatography (C.C.). Methanol (LiChrosolv, Merck, NJ, USA) was employed as the solvent while YMC-Pack ODS-A (250 × 4.6 mm I. D., S-5 μm, 12 nm, YMC, Kyoto, Japan) was employed as the column for liquid chromatography.

2.1.3. Chemicals and reagents for cell culture

Phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin streptomycin, and DMEM +GlutaMAX^{TM}, which were used for cell culture, were purchased from

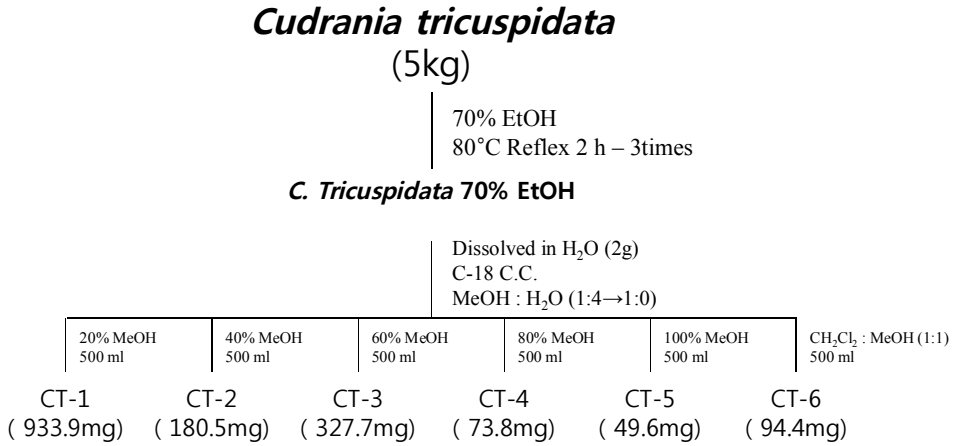
Gibco (Grand Island, NY, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Methods

2.2.1. Extraction and fraction

The roots of the dry *C. tricuspidata* was removed and three rounds of extraction were performed with 70% ethanol (EtOH) at 80 °C for 2 h. Only some of the ethanol was allowed to evaporate. The dried 70% EtOH extract of *C. tricuspidata*(2g) was subjected to reversed-phase (RP) C_{18} open column chromatography (CC, 5 ×9 ×26 cm) and eluted with a stepwise gradient of 20, 40, 60, 80, and 100% (v/v) MtOH in H_2O (500 mL) to yield *C. tricuspidata* fractions 1–6.

A



Scheme 1: Extraction and fraction from the roots of *Cudrania tricuspidata*

2.2.2. Cell culture

HaCaT cells were donated by Hyeonsook Cheong, Chosun University (Kwangju, Korea). The cells (5×10^6/dish cells/dish) were seeded in 100-mm dishes in DMEM +GlutaMAX^{TM} containing streptomycin (100 μg/mL), 10% heat-inactivated fetal bovine serum (FBS), and penicillin G (100 units/mL), and incubated at 37 °C in a humidified atmosphere (5% CO_2 and 95% air).

2.2.3. CCK8 assay

To determine the viability of HaCaT cells, the cells were maintained at 2×10^4 cells/well, treated with the fractions in the absence or presence of glutamate (5 mM), and cultured for 24 h. Subsequently, the Cell Counting Kit-8 (CCK8) (Dojindo Laboratories, Kumamoto, Japan) was employed and a 1h incubation was performed. The absorbance at 450 nm was detected using commercial kits (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions.

2.2.4. Enzyme-linked immunosorbent assay (ELISA)

The cytokines and chemokines in CCL17 (TARC), and CCL5 (RANTES) HaCaT cells were detected by ELISA. The levels of IL-6, IL-8, were measured using commercial kits (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions.

2.2.5. Western blot analysis

The cells were harvested and pelleted by centrifugation at 200 × g for 3 min, washed with PBS, and lysed in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). Protein concentration was determined using a Lowry protein assay kit (Sigma Chemical Co.). Thirty micrograms of protein from each sample was resolved using 7.5% and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Thereafter, the proteins were electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk and sequentially incubated with the primary antibody (Santa Cruz Biotechnology and Cell Signaling Technology) and a horseradish peroxidase-conjugated secondary antibody prior to ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.2.6. Preparation of cytosolic and nuclear fractions

HaCaT cells were homogenized in PER-Mammalian Protein Extraction Buffer (1:20, w:v) (Pierce Biotechnology, Rockford, IL, USA) containing freshly-added protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA) and 1 mM phenylmethylsulfonylfluoride (PMSF). The cytosolic fraction of the cells was prepared via centrifugation at 16,000 × g for 5 min at 4 ℃ while the nuclear and cytoplasmic cell extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents [31].

2.2.7. HPLC analysis

The extract, fractions, and compounds from *C. tricuspidata* were prepared at a concentration of 10 mg/ml. Thereafter, 20 μl of the sample was injected into the HPLC system. The mobile phase was comprised of A: H_2O (0.1% formic acid) and B: methanol (0.1% formic acid). The following gradient elution was employed: 90% A to 0% A over 40 min. The flow rate was maintained at 1 mL/min and wavelengths of 254, 280, and 345 nm were used for detection.

2.2.8. Statistical analysis

Data are expressed as the mean ± SD of three independent experiments. Statistical analysis was conducted using GraphPad Prism software version 5.01 (GraphPad Software Inc., San Diego, CA, USA). The differences between means were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *p <0.05, **p <0.01, and ***p <0.001 indicate a statistical significance.

3. Results and Discussion

3.1. The effects of TNFα+IFNγ-stimulated HaCaT cells.

First, we tested to stimulated with TNFα+IFNγ in normal control HaCaT cells. The normal HaCaT cells showed regular cell morphology. In contrast, the TNFα+IFNγ-stimulated HaCaT cells possessed irregular shapes, large intercellular spaces, and were loose (Figure 1A and B). In addition, we determined the effects of TNFα+IFNγ stimulation on the production of pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, RANTES, and TARC. As shown in Figure 2, TNFα+IFNγ stimulation led to increase on the production of IL-6, IL-8, RANTES and TARC.

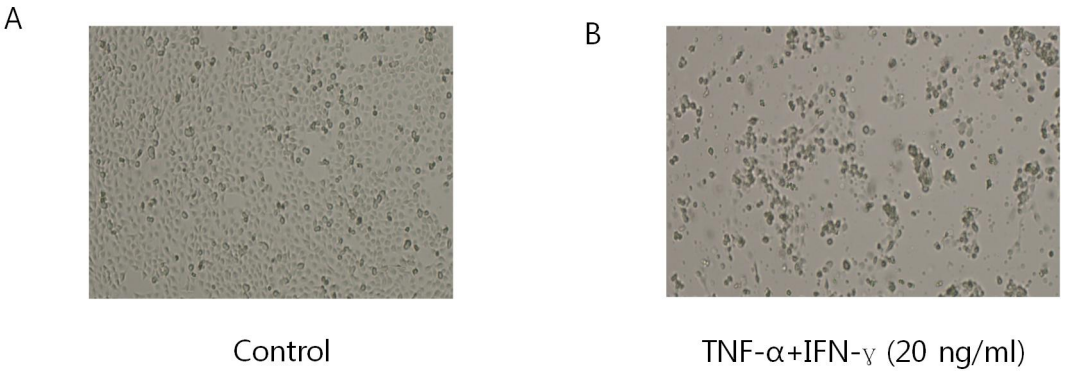


Figure 1. TNFα+IFNγ affects the morphology of HaCaT cells. (A) Normal control HaCaT cells, (B) TNFα+IFNγ (each 20 ng/ml)-induced HaCaT cells (magnification, X100).

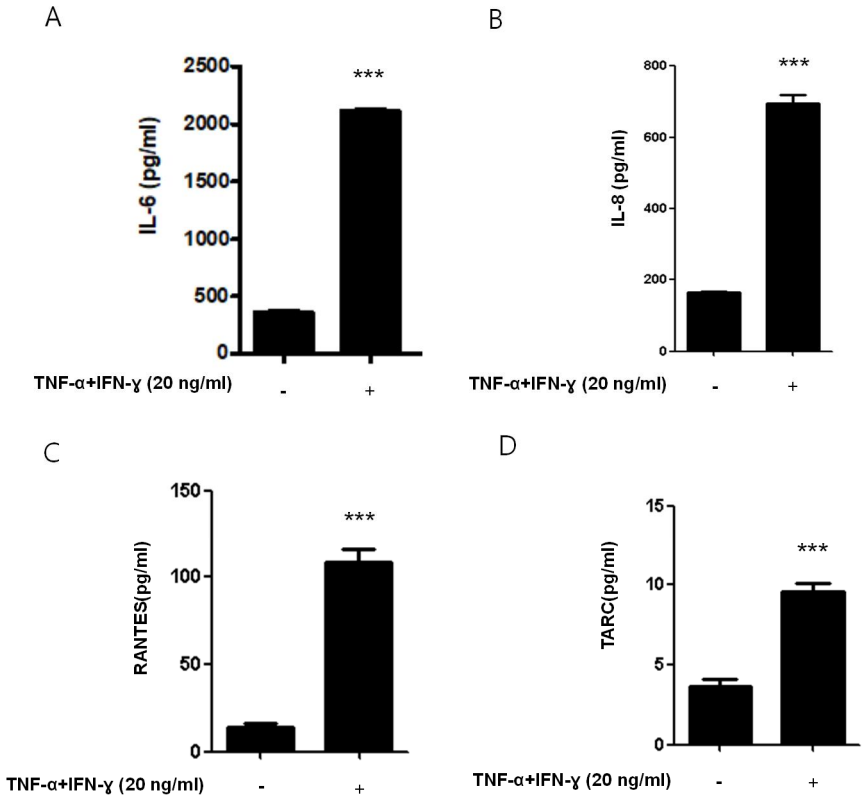


Figure 2. Upregulation of IL-6 (A), IL-8 (B), RANTES (C), and TARC (D), production in HaCaT cells treated with TNFα+IFNγ. The cells were harvested 24 h after TNFα+IFNγ treatment (20 ng/ml) and measured using ELISA. Each value represents the mean ± SD. ***p <0.001 compared to control.

3.2. The cell viability of 70% EtOH extract from *C. tricuspidata*.

The cytotoxicity of the *C. tricuspidata* extract on HaCaT cells was assessed by the Cell Counting Kit-8 (CCK-8) assay. Cell viability was evaluated using the CCK-8 detection kit. It allows sensitive colorimetric assays for the determination of cell viability in cell proliferation and cytotoxicity assays [32]. Based on our findings, cell viability was not significantly affected by 50–200 μg/mL of the 70%EtOH extract from *C. tricuspidat* (Figure 3). As a result, this concentration range was employed in further experiments.

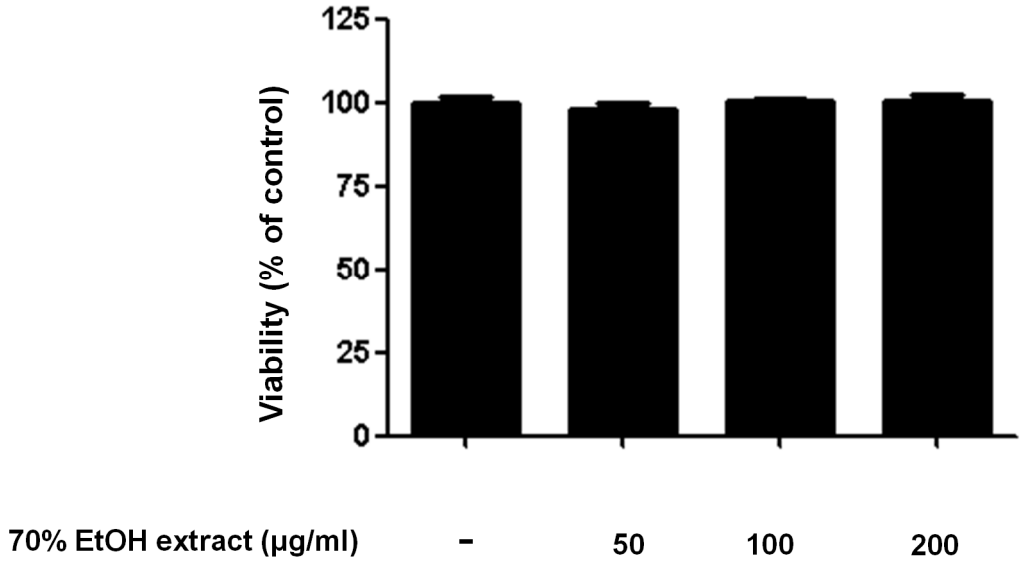


Figure 3. Effects of the 70 %EtOH extract from *C. tricuspidata* on the viability of HaCaT cells. HaCaT cells were incubated with the indicated concentrations for 24 h. The cell viability was determined using the CCK8. The data represent the mean values ± SD of three experiments.

3.3. Effects of *C. tricuspidata* on the production of proinflammatory cytokines in TNFα+IFNγ-stimulated HaCaT cells.

Pro-inflammatory cytokines are known to play an important role in the skin immune response [16]. Accordingly, we sought to determine the effect of the 70% EtOH extract from *C. tricuspidata* on the production of pro-inflammatory cytokines, such as IL-6 and IL-8 in HaCaT cells. Briefly, HaCaT cells were pre-incubated with different concentrations of the 70% EtOH extract for 3 h and subsequently stimulated with TNFα+IFNγ for 24 h. Based on the results of the enzyme immunoassay, 70% EtOH extract decreased IL-6 (A) and IL-8 (B) production in does-dependently manners (Figure 4).

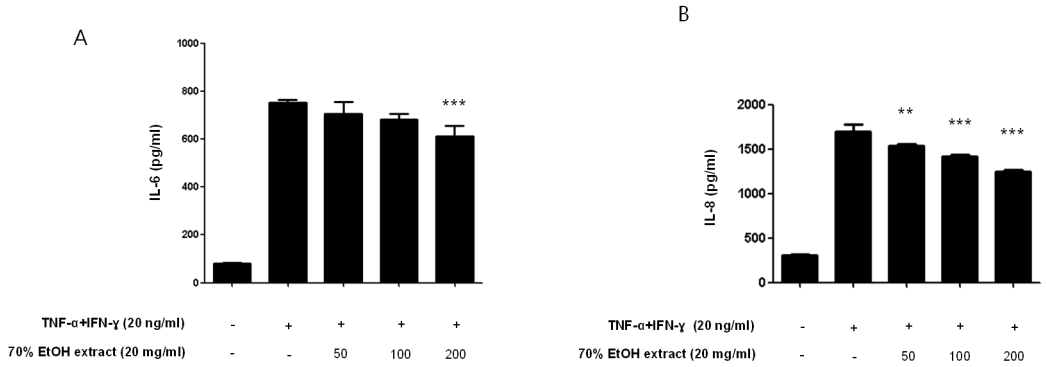


Figure 4. Effects of the 70% EtOH extract from *C. tricuspidata* on IL-6 and IL-8 production in HaCaT cells stimulated with TNFα+IFNγ. Cells were pre-treated with the 70% EtOH extract for 3h and stimulated with TNFα+IFNγ for 24 h. Each value represents the mean ± SD. **p <0.01 and ***p <0.001 as compared with TNFα+IFNγ only.

3.4. The cell viability of sub-fractions from 70% EtOH extract of *C. tricuspidata*.

The cytotoxicity of the sub-fractions 1–6 (CT1-6) from 70% EtOH extract of *C. tricuspidata* were assessed by the CCK8 assay. In this result, the cell viability was not significantly affected by concentrations of 50 μg/mL to 200 μg/mL of the sub-fractions from 70% EtOH extract. These finding suggests that the sub-fractions from 70% EtOH extract of *C. tricuspidata* was not toxic to HaCaT cells at the indicated concentrations (Figure 5). Therefore, the concentration range of 50-200 μg/mL of sub-fractions from 70% EtOH extract of *C. tricuspidata* was employed for further treatments.

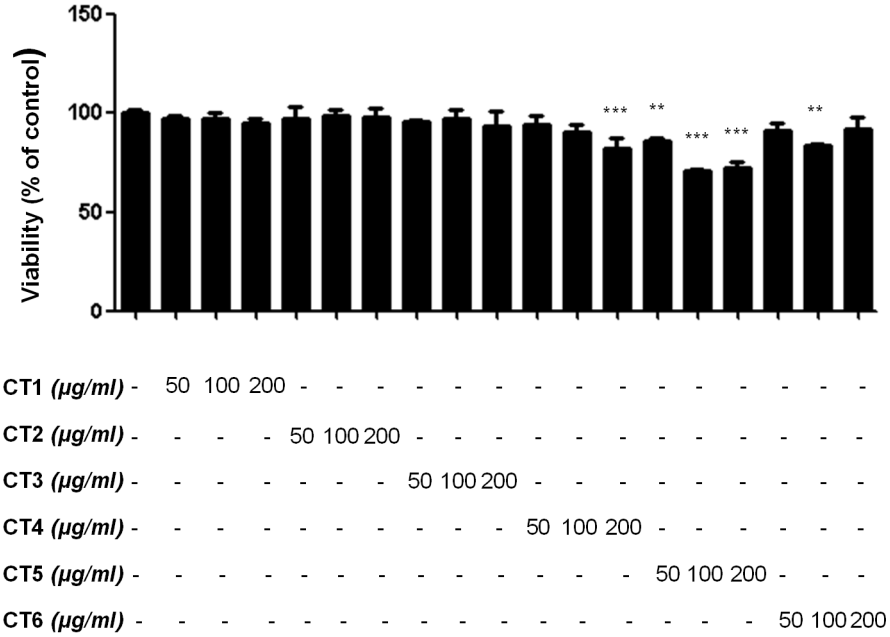


Figure 5. Effects of the sub-fractions 1–6 (CT1-6) from 70% EtOH extract of *C. tricuspidata* on the viability of HaCaT cells. The cells were incubated with the indicated concentrations of fractions CT1–6 for 24 h. The cell viability was determined as described in the Materials and Methods section. The results are presented as the mean ± SD of three experiments. **p. <0.01 and ***p <0.001 compared to compared to control.

3.5. Effects of IL-8 production by the sub-fractions from 70% EtOH extract of *C. tricuspidata* on TNFα+IFNγ induced HaCaT cells.

We tested to determine the effect of the sub-fractions 1–6 (CT1-6) from 70% EtOH extract of *C. tricuspidata* on the production of pro-inflammatory cytokine such as IL-8 in HaCaT cells. HaCaT cells were pre-incubated with different concentrations of sub-fractions from 70% EtOH extract of *C. tricuspidata* for 3 h and then subjected to TNFα+IFNγ stimulation for 24 h (Figure 6). As a result, the sub-fractions from 70% EtOH extract of *C. tricuspidata* dose-dependently decreased IL-8 production in TNFα+IFNγ stimulated HaCaT cells (Figure 6).

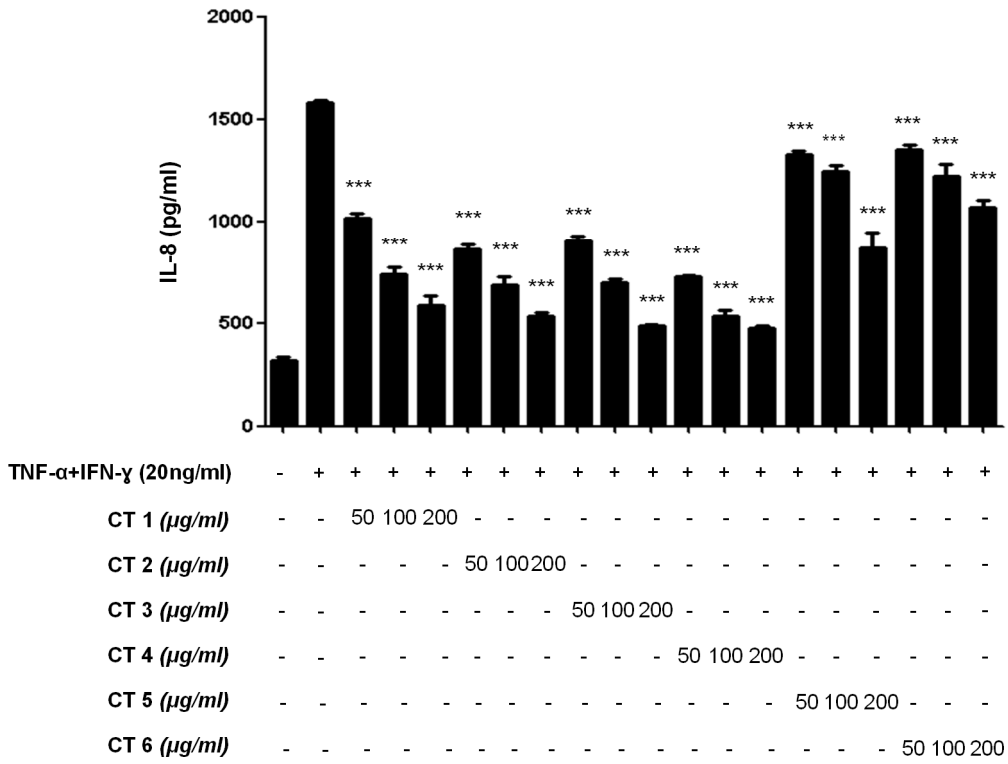
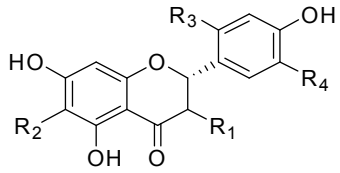


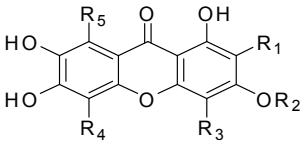
Figure 6. Effect of the sub-fractions 1–6 (CT1-6) from 70% EtOH extract of *C. tricuspidata* on IL-8 production in TNFα+IFNγ-stimulated HaCaT cells. Cells were pretreated with indicated concentrations of fractions CT1–6 and stimulated with TNFα+IFNγ for 24 h. Each value represents the mean ± SD. *** $p < 0.001$ as compared with TNFα+IFNγ only.

3.6. Chemical Structures of 16 compounds isolated from *C. tricuspidata*

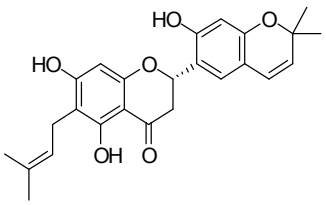
The 16 compounds were isolated from the roots of *C. tricuspidata* in our previously study [31]. The chemical structure of the compounds, dihydrokaempferol (**1**), steppogenin (**2**), cudraflavanone A (**3**), cudraxanthone L (**4**), cudraflavone C (**5**), cudraflavanone D (**6**), kuwanon C (**7**), cudratricusxanthone A (**8**), macluraxanthone B (**9**), cudraxanthone M (**10**), 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (**11**), cudraxanthone D (**12**), cudratricusxanthone N (**13**), cudraflavanone B (**14**), cudratricusxanthone L (**15**), and cudratricusxanthone A (**16**), were previously presented [31].



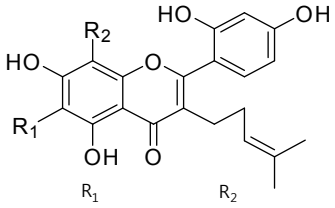
	R_1	R_2	R_3	R_4
1	OH	OH	OH	H
2	H	H	OH	H
6	OH	$CH_2CH=C(CH_3)_2$	OH	$CH_2CH=C(CH_3)_2$
14	H	$CH_2CH=C(CH_3)_2$	OH	H



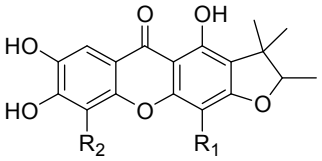
	R_1	R_2	R_3	R_4	R_5
4	$C(CH_3)_2CH=CH_2$	H	H	$CH_2CH=C(CH_3)_2$	H
8	H	H	$C(CH_3)_2CH=CH_2$	H	$CH_2CH=C(CH_3)_2$
9	$C(CH_3)_2CH=CH_2$	H	$CH_2CH=C(CH_3)_2$	H	H
11	$C(CH_3)_2CH=CH_2$	CH_3	H	H	H
15	H	H	$C(CH_3)_2CH=CH_2$	H	H
16	$C(CH_3)_2CH=CH_2$	CH_3	$CH_2CH=C(CH_3)_2$	H	H



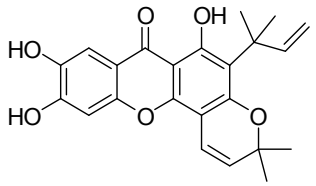
3



	R_1	R_2
5	$CH_2CH=C(CH_3)_2$	H
7	H	$CH_2CH=C(CH_3)_2$



	R_1	R_2
10	$CH_2CH=C(CH_3)_2$	H
12	H	$CH_2CH=C(CH_3)_2$



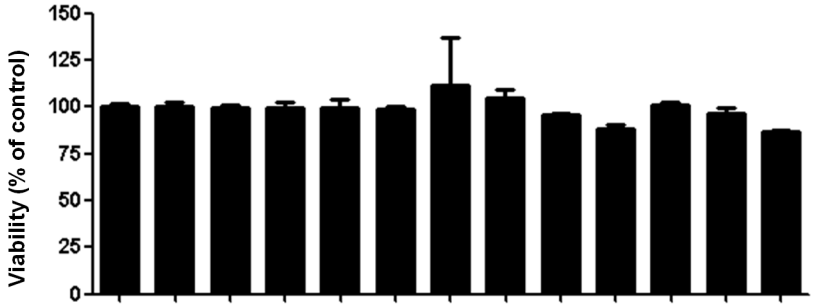
13

Figure 7. Structure of compounds 1-16.

3.7. The cell viability of 16 compounds from *C. tricuspidata* in HaCaT cells.

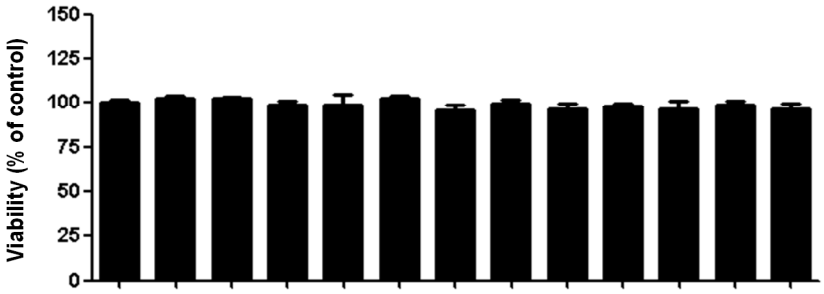
The cytotoxicity of the 16 compounds were evaluated by the CCK8 assay in HaCaT cells. At the indicated concentrations, our results indicated that all of 16 compounds from *C. tricuspidata* did not significantly affect cell viability. These findings suggested that 16 compounds have not cytotoxic effects by the indicated concentrations in HaCaT cells (Figure 8). Therefore, these concentrations were employed in further experiments.

A



Compound 1 (μM)	-	20	40	80	-	-	-	-	-	-	-	-	-
Compound 2 (μM)	-	-	-	-	20	40	80	-	-	-	-	-	-
Compound 3 (μM)	-	-	-	-	-	-	-	10	20	40	-	-	-
Compound 4 (μM)	-	-	-	-	-	-	-	-	-	-	0.5	1	2

B



Compound 5 (μM)	-	1.25	2.5	5	-	-	-	-	-	-	-	-	-
Compound 6 (μM)	-	-	-	-	2.5	5	10	-	-	-	-	-	-
Compound 7 (μM)	-	-	-	-	-	-	-	10	20	40	-	-	-
Compound 8 (μM)	-	-	-	-	-	-	-	-	-	-	1.25	2.5	5

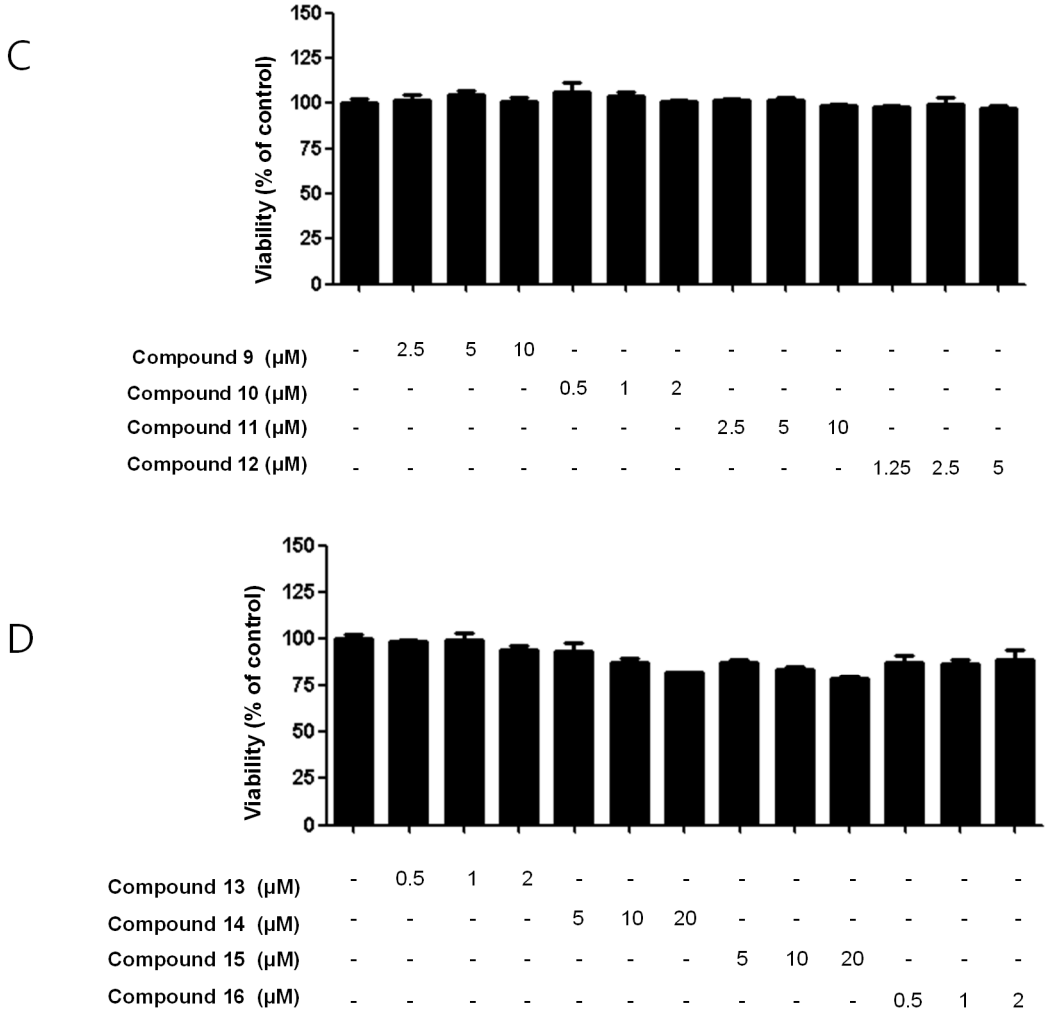
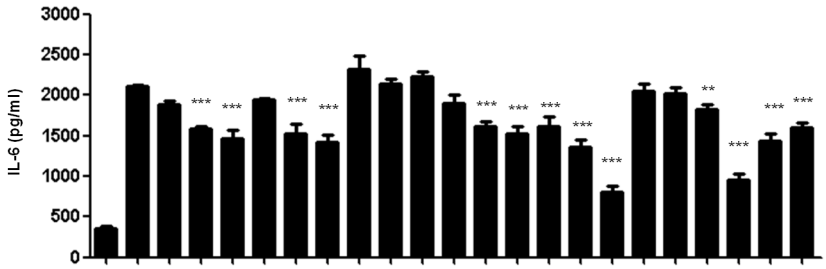


Figure 8. Effects of the 16 compounds from *C. tricuspidata* on the viability of HaCaT cells (A-D). HaCaT cells were incubated with the indicated concentrations for 24 h, and then the viability of cells was determined using the O.D. value.

3.8. Effects of IL-6 production by 16 compounds from *C. tricuspidata* on TNFα+IFNγ induced HaCaT cells.

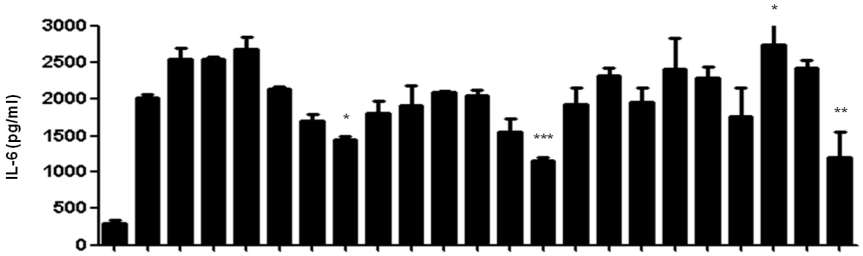
The skin consists of two basic layers the epidermis and the dermis. The epidermis is mainly composed of keratinocytes [33]. As previously mentioned, pro-inflammatory cytokines play an important role in immune response in the keratinocytes [25]. Therefore, we tested to determine the effect of the 16 compounds from *C. tricuspidata* on the IL-6 production in HaCaT cells. Briefly, HaCaT cells were pre-incubated with different concentrations of the 16 compounds for 3 h, and then subsequently stimulated with TNFα+IFNγ for 24 h (Figure 9). Based on the results of enzyme immunoassay, the compounds **1, 2, 4, 5, 6, 7, 9, 11, 14, 15** and **16** from *C. tricuspidata* significantly reduced IL-6 production (Figure 9).

A



TNF-α+IFN-γ (20ng/ml)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Compound 1 (μM)	-	-	20	40	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 2 (μM)	-	-	-	-	-	20	40	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 3 (μM)	-	-	-	-	-	-	-	-	10	20	40	-	-	-	-	-	-	-	-	-	-	-	-
Compound 4 (μM)	-	-	-	-	-	-	-	-	-	-	-	0.5	1	2	-	-	-	-	-	-	-	-	-
Compound 5 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.25	2.5	5	-	-	-	-	-	-
Compound 6 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	5	10	-	-	-
Compound 7 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	20	40

B



TNF-α+IFN-γ (20ng/ml)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Compound 8 (μM)	-	-	1.25	2.5	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 9 (μM)	-	-	-	-	-	2.5	5	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 10 (μM)	-	-	-	-	-	-	-	-	0.5	1	2	-	-	-	-	-	-	-	-	-	-	-	-
Compound 11 (μM)	-	-	-	-	-	-	-	-	-	-	-	2.5	5	10	-	-	-	-	-	-	-	-	-
Compound 12 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.25	2.5	5	-	-	-	-	-	-
Compound 13 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	1	2	-	-	-
Compound 14 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	5	10

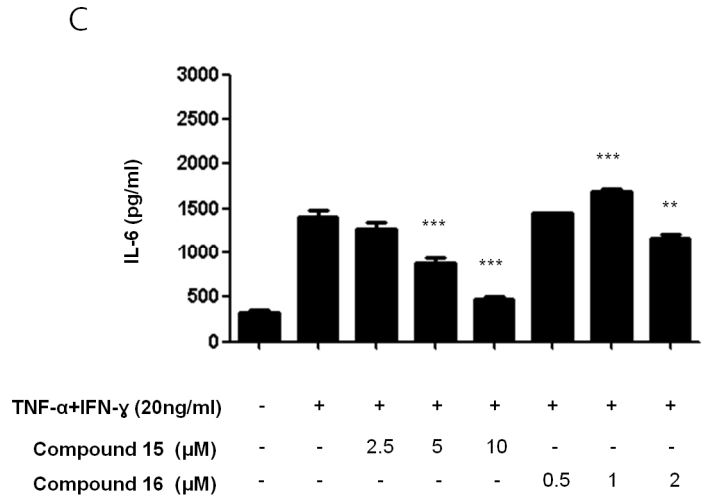
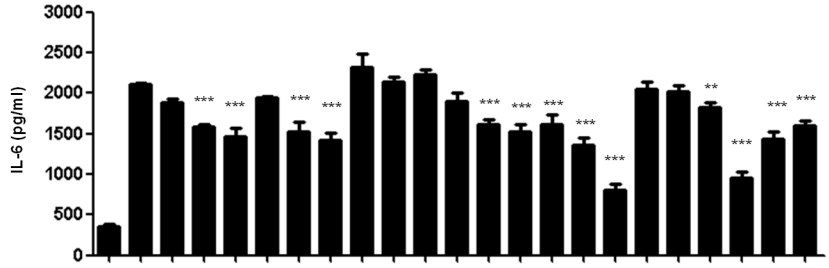


Figure 9. Effects of the 16 compounds from *C. tricuspidata* and TNFα+IFNγ stimulation on IL-6 production in HaCaT cells (A, B, and C). The cells were pre-treated with the indicated concentrations of the 16 compounds from *C. tricuspidata* for 3 h and then stimulated with TNFα+FNγ (20 ng/mL) for 24 h. IL-6 were determined as described in the Materials and Methods section. The results are presented as the mean ± SD of three experiments. *p <0.05, **p <0.01 and ***p <0.001 as compared with TNFα+IFNγ only.

3.9. Effects of IL-8 production by 16 compounds from *C. tricuspidata* on TNFα+IFNγ induced HaCaT cells.

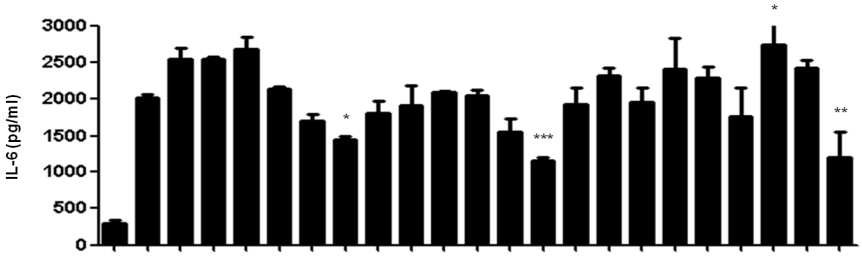
We determined the effect of the 16 compounds from *C. tricuspidata* on the IL-8 production in HaCaT cells. Briefly, HaCaT cells were pre-incubated with different concentrations of the 16 compounds for 3 h. Thereafter, the cells were subjected to TNFα+IFNγ-stimulation for 24 h (Figure 10). Based on the results of enzyme immunoassay, the 15 compounds from *C. tricuspidata* reduced IL-8 production (Figure 10). Further, compounds **1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15,** and **16** compounds from *C. tricuspidata* decreased IL-8 production in the HaCaT cells.

A



TNF-α+IFN-γ (20ng/ml)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Compound 1 (μM)	-	-	20	40	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 2 (μM)	-	-	-	-	-	20	40	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 3 (μM)	-	-	-	-	-	-	-	-	10	20	40	-	-	-	-	-	-	-	-	-	-	-	-
Compound 4 (μM)	-	-	-	-	-	-	-	-	-	-	-	0.5	1	2	-	-	-	-	-	-	-	-	-
Compound 5 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.25	2.5	5	-	-	-	-	-	-
Compound 6 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	5	10	-	-	-
Compound 7 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	20	40

B



TNF-α+IFN-γ (20ng/ml)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Compound 8 (μM)	-	-	1.25	2.5	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 9 (μM)	-	-	-	-	-	2.5	5	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Compound 10 (μM)	-	-	-	-	-	-	-	-	0.5	1	2	-	-	-	-	-	-	-	-	-	-	-	-
Compound 11 (μM)	-	-	-	-	-	-	-	-	-	-	-	2.5	5	10	-	-	-	-	-	-	-	-	-
Compound 12 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.25	2.5	5	-	-	-	-	-	-
Compound 13 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	1	2	-	-	-
Compound 14 (μM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	5	10

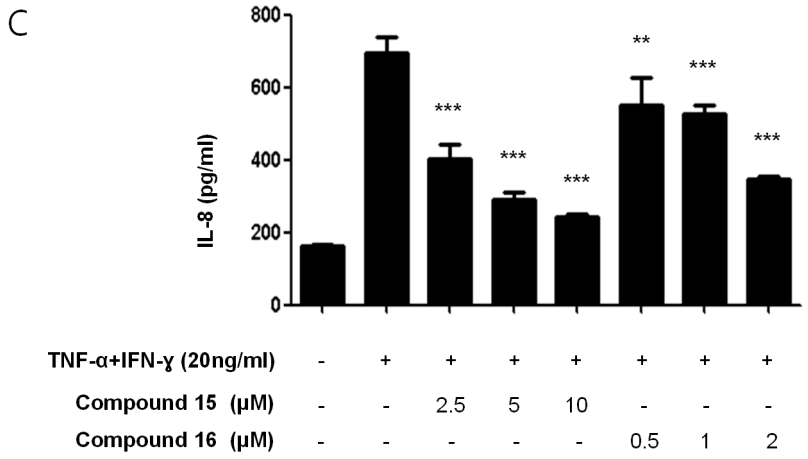


Figure 10. Effects of the 16 compounds from *C. tricuspidata* and TNFα+IFNγ stimulation on IL-8 (A, B, and C) production in HaCaT cells. The cells were pre-treated with the indicated concentrations of the *C. tricuspidata* compounds for 3 h, and then stimulated with TNFα+IFNγ (20 ng/mL) for 24 h. IL-6 was determined as described in the Materials and Methods section. Data are expressed as the mean ± SD values of three experiments. *p <0.05, **p <0.01 and ***p <0.001 as compared with TNFα+IFNγ only.

3.10. Effects of RANTES and TARC production by 6 compounds from *C. tricuspidata* on TNFα+IFNγ induced HaCaT cells.

Among the 16 compounds, the six mostly effective compounds were selected for the further experiments with the production of representative Th2 chemokines, RANTES (CCL5) and TARC (CCL17). The transport of Th2 to the inflamed site via the CC chemokine receptor 4 (CCR4) is known to induce cell migration and invasion [34]. Herein, HaCaT cells were pre-incubated with different concentrations of the 6 compounds from *C. tricuspidata* for 3 h. Thereafter, the cells were induced to TNFα+ IFNγ stimulation for 24 h. The increased production of RANTES was found to be decreased by pretreatment with the all of 6 compounds (Figure 11). Similarly, the TARC production by TNFα+IFNγ (20 ng/mL) was reduced by pretreatment with the all of 6 compounds from *C. tricuspidata* (Figure 12).

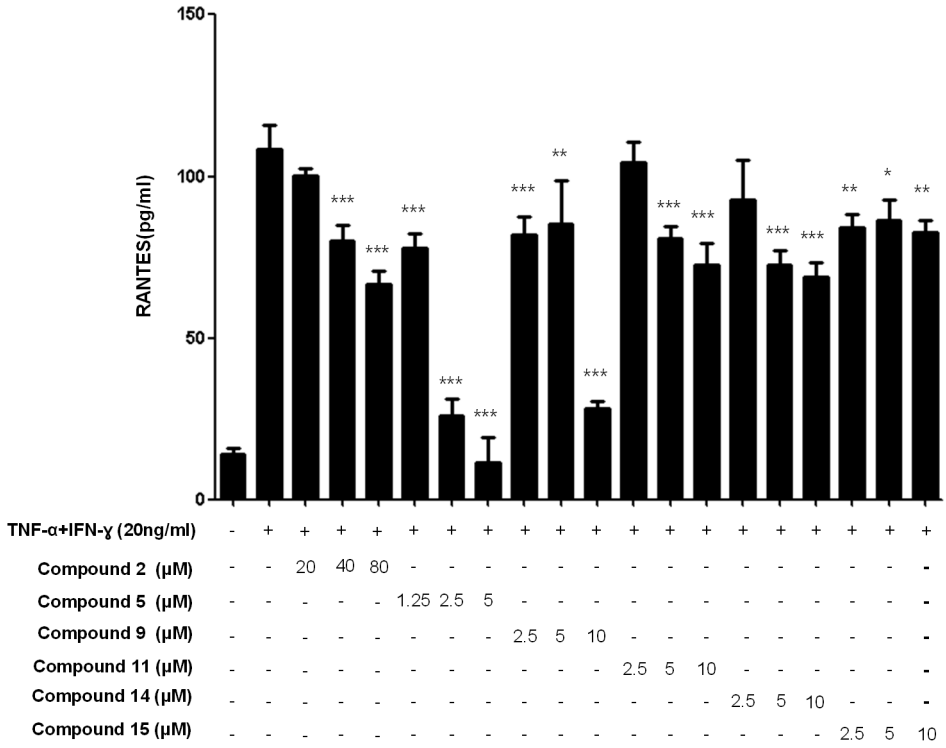


Figure 11. Effects of 6 compounds from *C. tricuspidata* and TNFα+IFNγ stimulation on RANTES production. HaCaT cells were incubated with TNFα+IFNγ (20 ng/ml) in the absence or presence of the indicated concentrations of 6 compounds. After 24 h, chemokine secretions were analyzed by ELISA. Data are presented as mean ± S.D. *p < 0.05, **p < 0.01, and ***p <0.001 as compared with TNFα+IFNγ only.

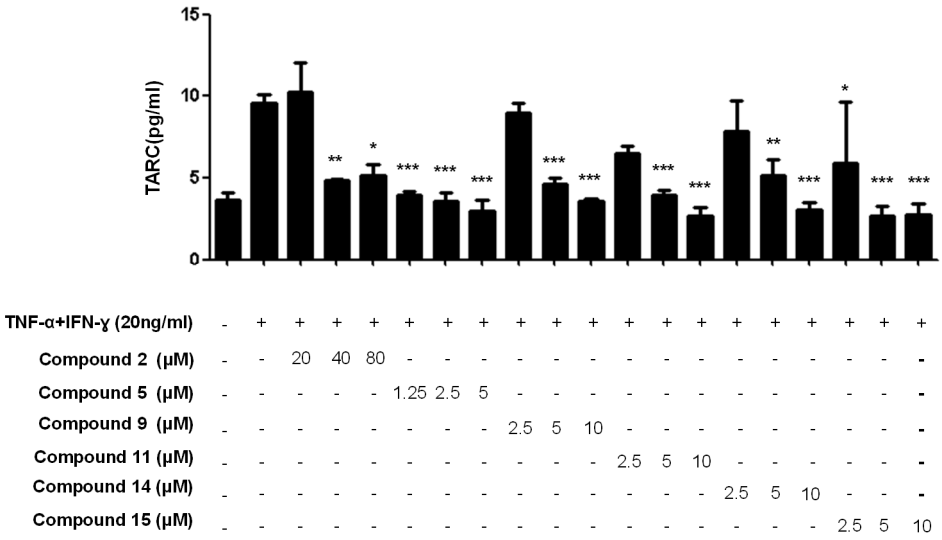
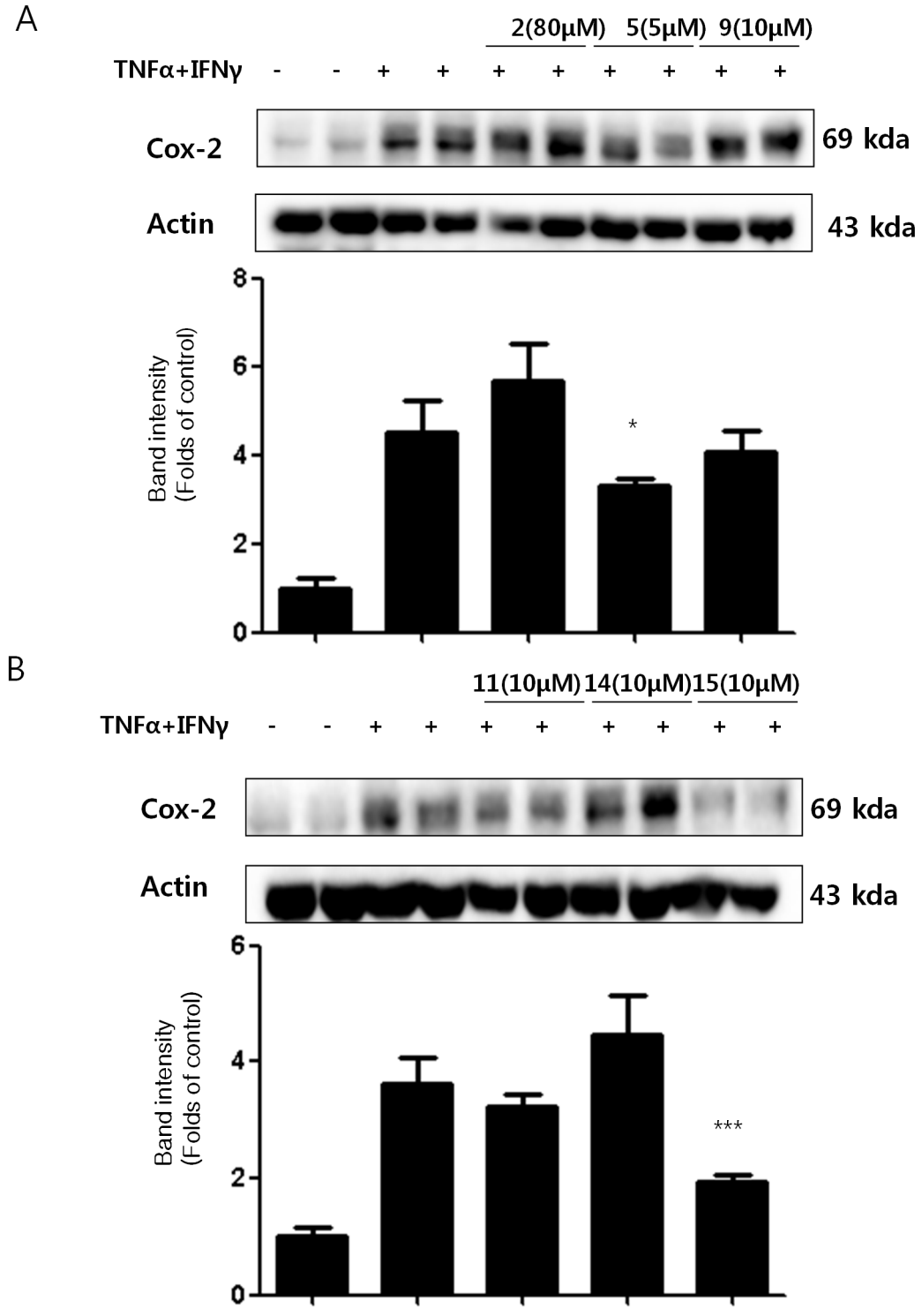


Figure 12. Effects of 6 compounds from *C. tricuspidata* and TNFα+IFNγ stimulation on TARC production. HaCaT cells were incubated with TNFα+IFNγ (20 ng/ml) in the absence or presence of the indicated concentrations of the 6 compounds. After 24 h, chemokine secretions were analyzed by ELISA. Data are presented as mean ± S.D. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared with TNFα+IFNγ only.

3.11. Effects of COX-2 and ICAM-1 protein by 6 compounds from *C. tricuspidata* on TNFα+IFNγ-induced HaCaT cells.

ICAM-1 is up-regulated in response to inflammatory mediators such as the proinflammatory cytokines IL-1β, TNF-α and IFN-γ. The proinflammatory cytokines are well known to induce ICAM-1 on keratinocytes. COX is the enzyme responsible for the conversion of arachidonic acid to prostaglandin H2, the main step in the prostaglandin synthesis pathway. COX-2 expression plays key roles in skin inflammation [35]. The anti-inflammatory effect of the 6 compounds from *C. tricuspidata* on the expression of pro-inflammatory proteins were investigated in TNFα+IFNγ-induced HaCaT cells. Briefly, cells were pre-incubated for 3 h with or without the 6 compounds from *C. tricuspidata* (5-80 μM) and subsequently challenged with TNFα+IFNγ (20 ng/mL) for 24 h. As shown in Figure 13A and B, compounds **5** and **15** were found to reduce the expression of COX-2. Previous studies have reported that compound **5** inhibited the expression of COX-2, however, this study was first reported the inhibition of COX-2 protein by compound **5** in HaCaT cells. In addition, cells were pre-incubated for 3 h with or without the 6 compounds from *C. tricuspidata* (5-80 μM) and subsequently challenged with TNFα+IFNγ (20 ng/mL) for 6 h. The all of 6 compounds significantly inhibited ICAM-1 expression in TNF-α+IFNγ-induced HaCaT cells (Figure 13C and D).



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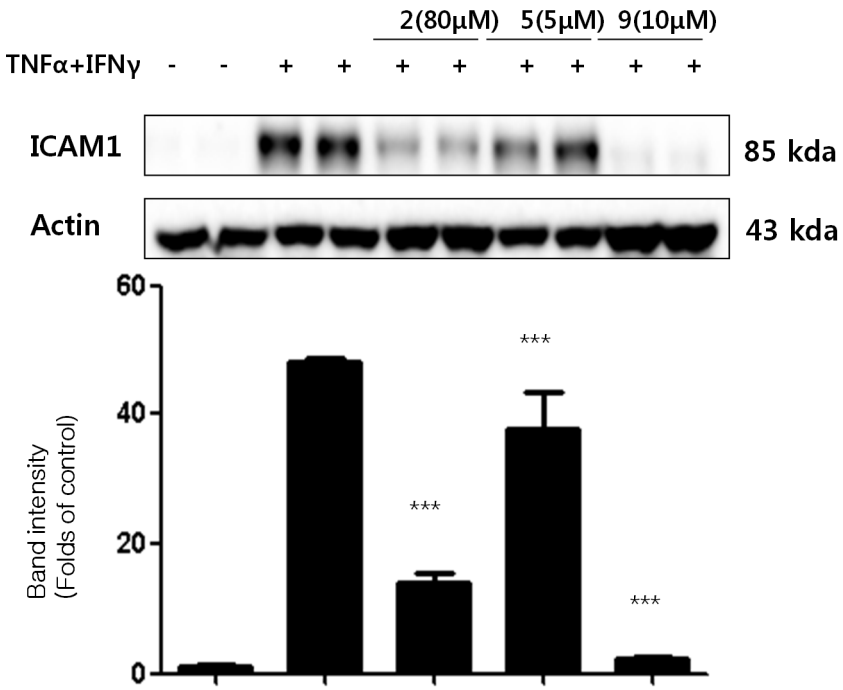

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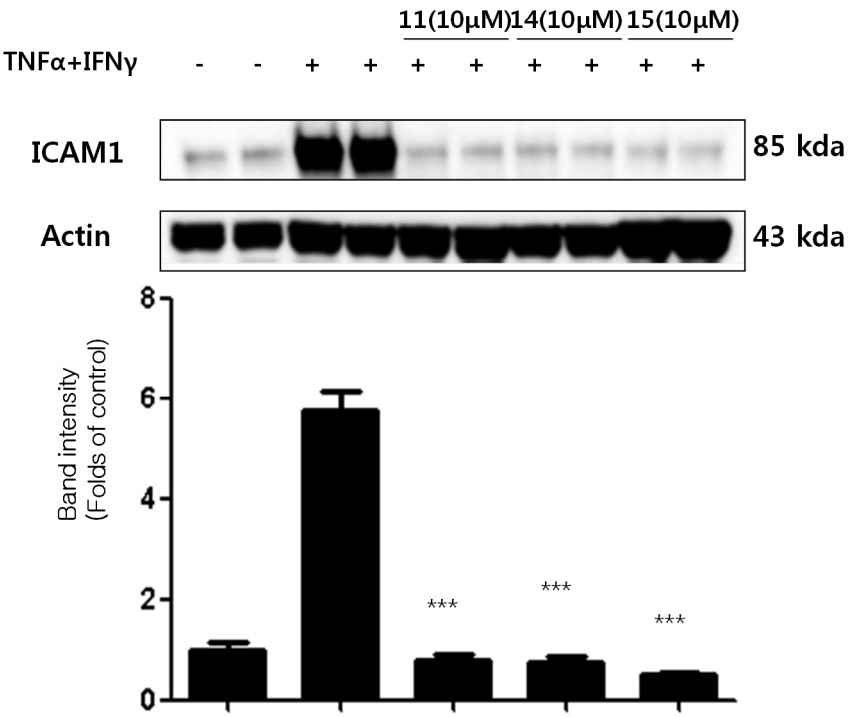
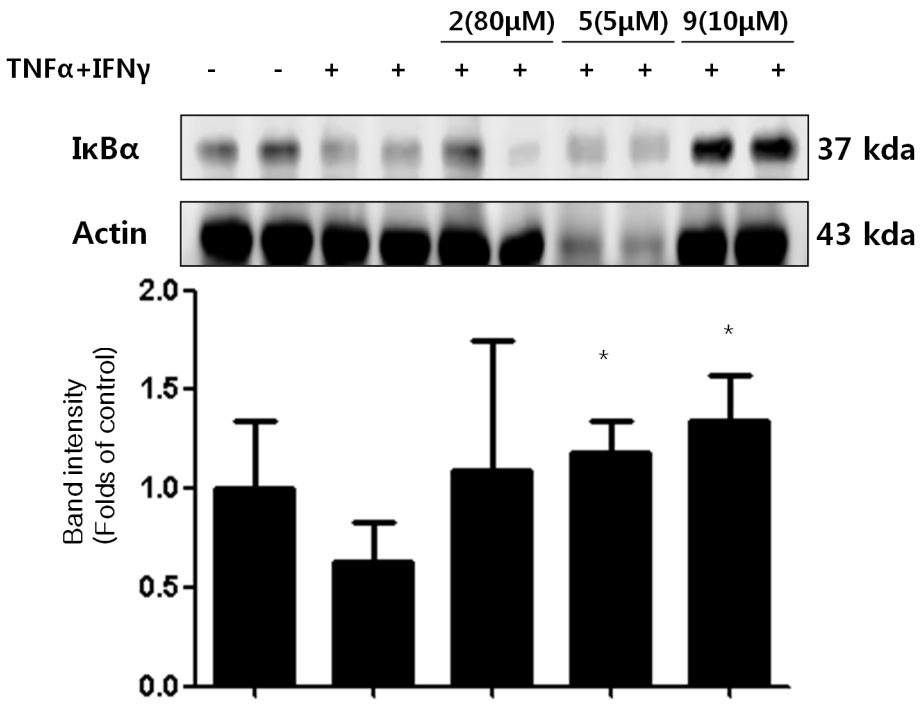


Figure 13. Protein expression of cyclooxygenase-2 (COX-2) (A and B) and intercellular adhesion molecule-1 (ICAM-1) (C and D) on TNFα+IFNγ-induced HaCaT cells. (A and B) The cells were pretreated with the indicated concentrations of 6 compounds from *C. tricuspidata* for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 24 h. (C and D) The cells were pretreated with the indicated concentrations of 6 compounds from *C. tricuspidata* for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 6 h. Western blot analysis was performed as described in the Materials and Methods section. Representative stains from four independent experiments are presented. The immunoblot was quantified using ImageJ software. Band intensity was normalized to that of β-actin. * $p < 0.05$, *** $p < 0.001$ as compared with TNFα+IFNγ only.

3.12. Effects of NF-κB activation by 6 compounds from *C. tricuspidata* on TNFα+IFNγ-induced HaCaT cells

The activation of NF-κβ by TNFα or/and IFNγ induces a high rate of IκB phosphorylation and proteolysis by IκB kinase (IKK) in keratinocytes [29]. To determine the potential mechanisms for the inhibition of TNFα+IFNγ-induced proinflammatory enzymes and mediators by the 6 compounds from *C. tricuspidata*, we investigated the effects of the 6 compounds on the phosphorylation and degradation of cytoplasmic IκBα, and p65 translocation to nucleus. In our results, compounds **5** and **9** inhibited the degradation of IκBα in the cytoplasm. In addition, compounds **5, 14,** and **15** inhibited the phosphorylation of IκBα, however it was not significant (Figure 14C and D). When HaCaT cells were exposed to TNFα+IFNγ, NF-κβ p65 was translocated from the cytoplasm to the nucleus.Among the treatment with 6 compounds, 5 compounds including compounds **2**, **5**, **9**, **14**, and **15**, significantly inhibited NF-κβ p65 translocation to nucleus, however compound 11 was not significant (Figures 14E and F).

A



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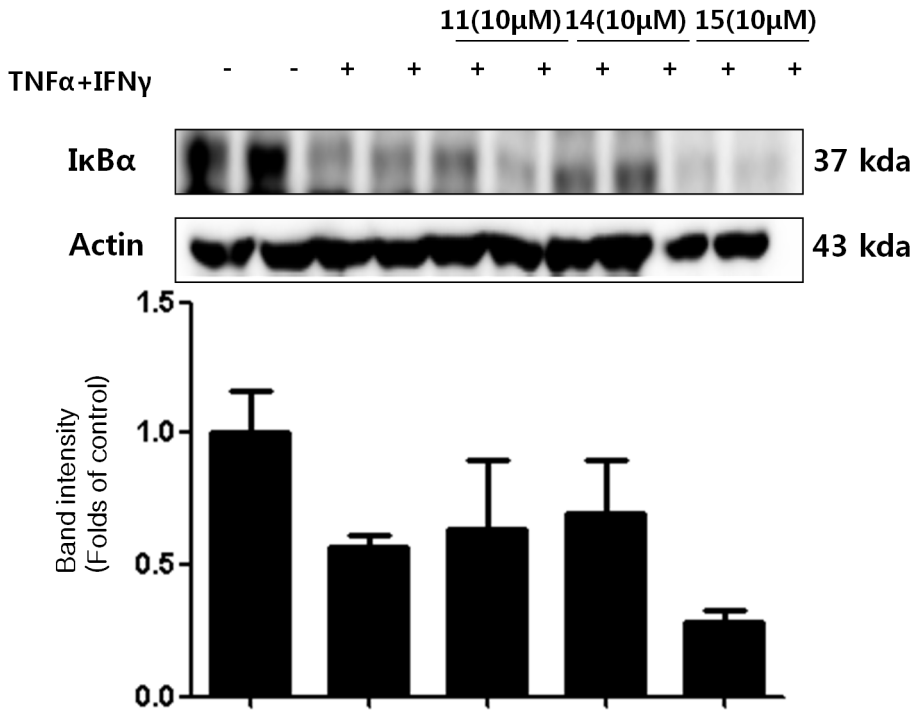

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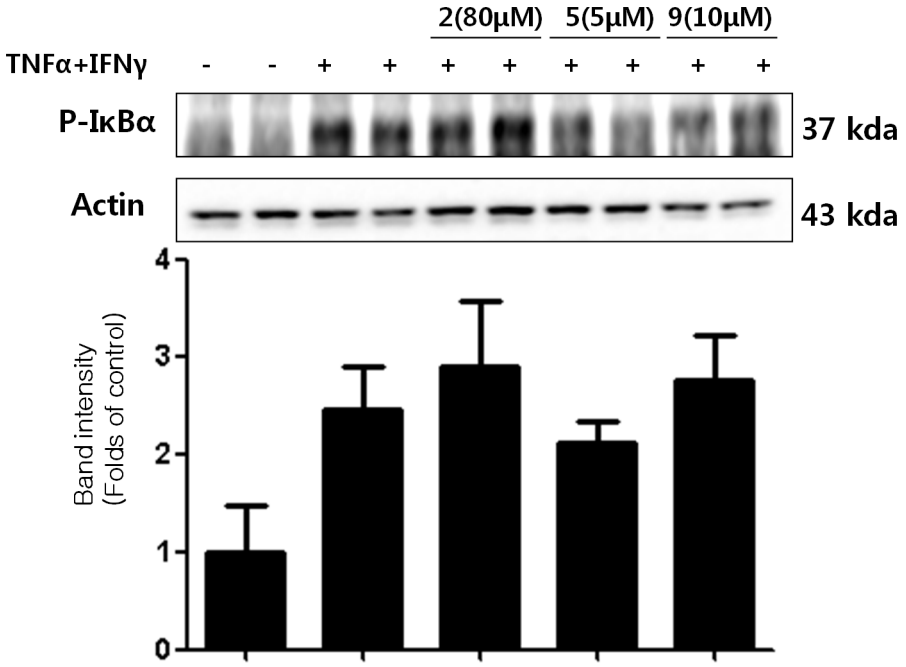

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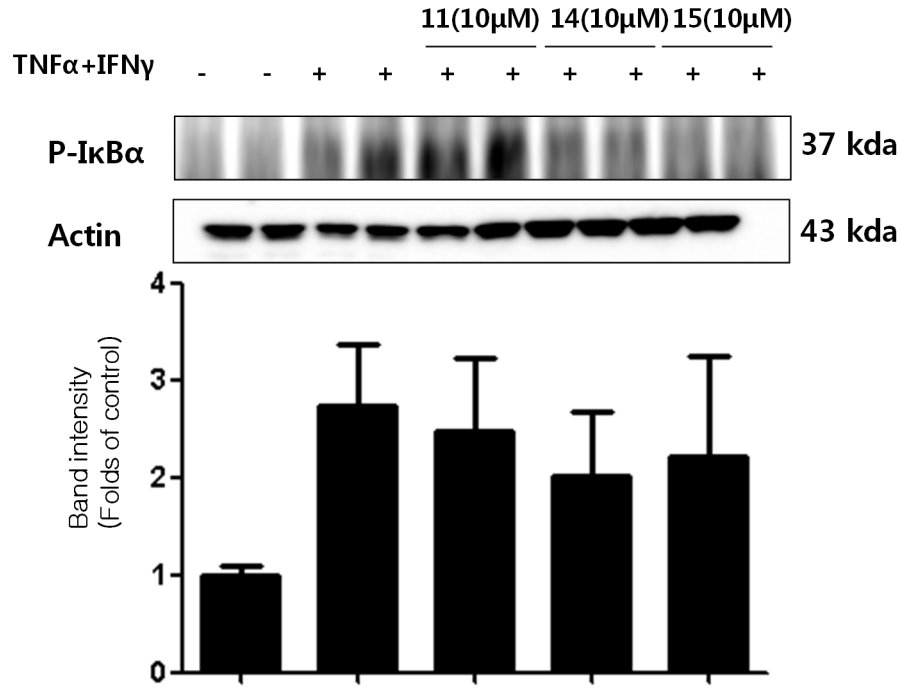

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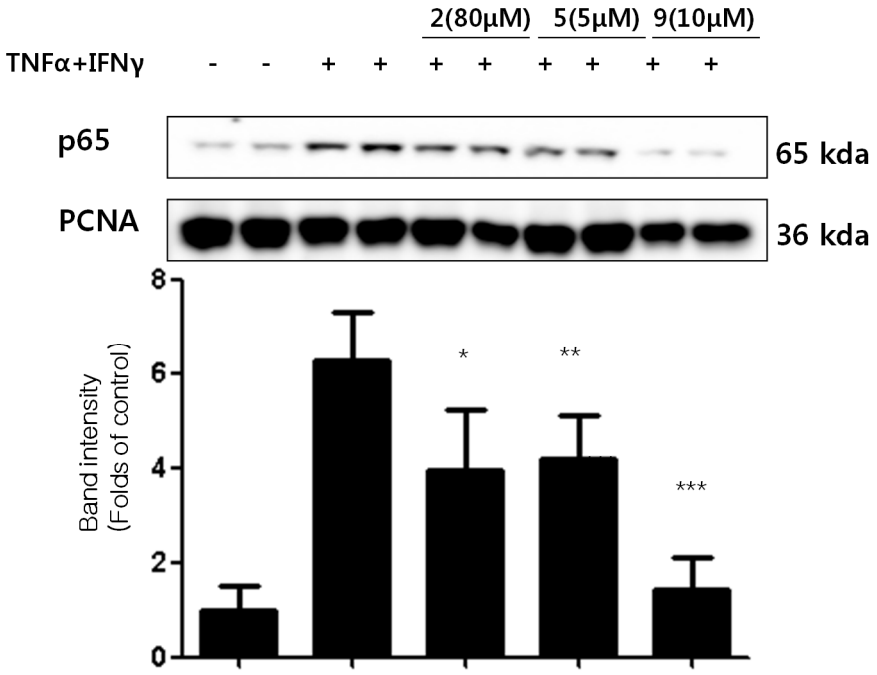

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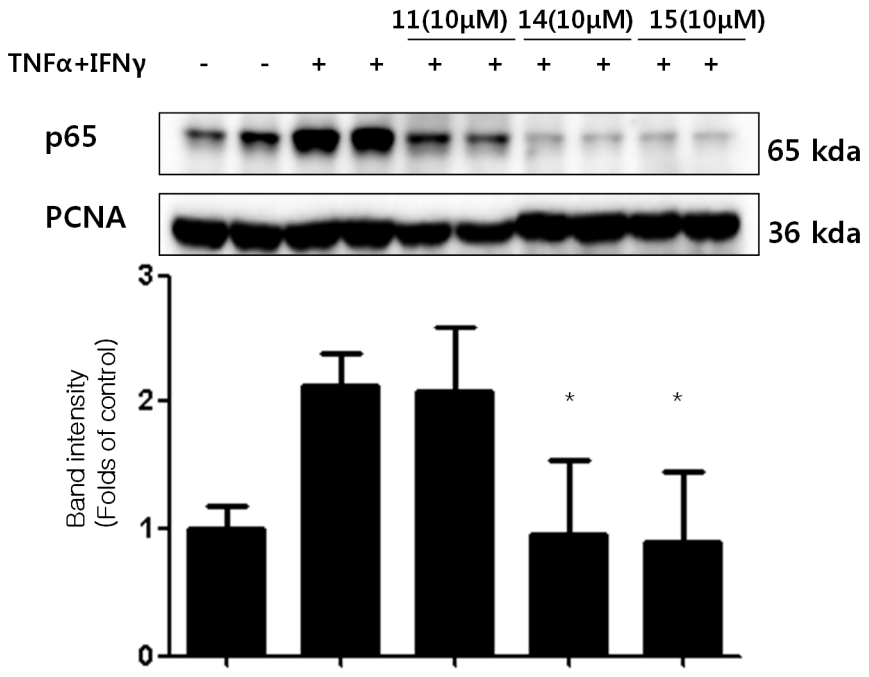
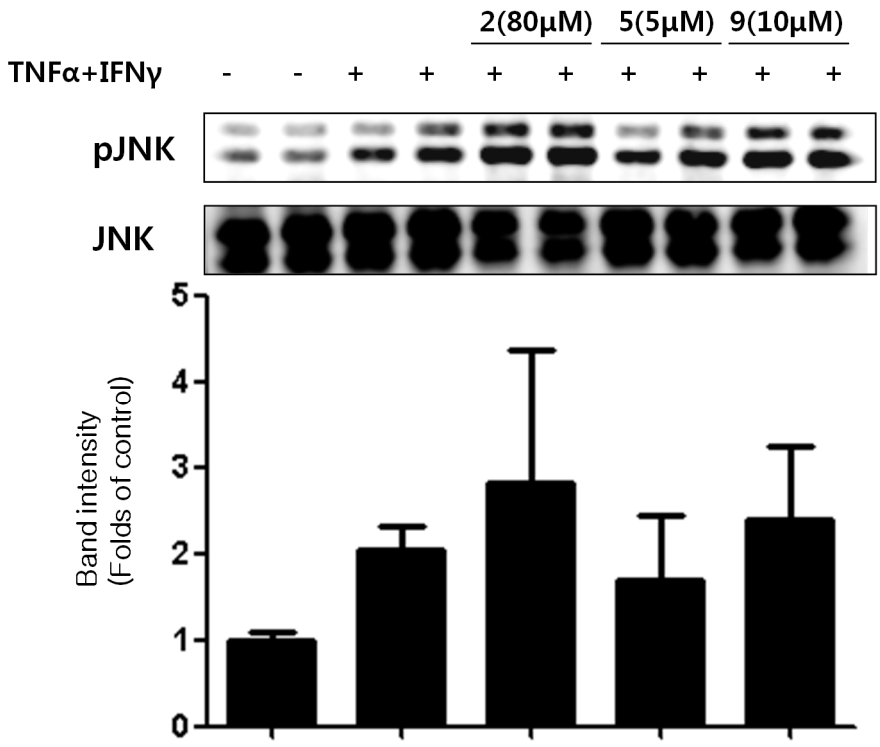


Figure 14. Effects of 6 compounds from *C. tricuspidata* on the degradation of (IκB)-α (A and B), phosphorylation of IκBα (C and D), and NF-κB p65 translocation to nucleus (E and F) in HaCaT cells. Cells were pretreated with the indicated concentrations of 6 compounds from *C. tricuspidata* for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 10 min. IκBα, p-IκBα, and NF-κB p65 were analyzed using Western blotting as described in the Materials and Methods section. Representative blots from four independent experiments are presented. The immunoblots were quantified using ImageJ software. Band intensity was normalized to that of β-actin or anti-proliferating cell nuclear antigen (PCNA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared with TNFα+IFNγ only.

3.13. Effects of MAPK phosphorylation by 6 compounds from *C. tricuspidata* on TNFα+IFNγ-induced HaCaT cells.

To evaluate the anti-inflammatory effects of the 6 compounds from *C. tricuspidata* on the MAPK pathway, western blot analysis was employed to determine the phosphorylation of JNK1/2, ERK1/2, and p38 on TNFα+IFNγ-induced HaCaT cells. Cells were pretreated with 6 compounds from *C. tricuspidata* at the indicated concentrations for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 2 h. Following treatment with TNFα+IFNγ for 2 h, the phosphorylation of JNK-1/2 was not inhibited by all of 6 compounds (Figure 15A and B). Cells were pretreated with 6 compounds at the indicated concentrations for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 15 min. During 15 min treatment with TNFα+IFNγ, ERK-1/2 phosphorylation was inhibited by only compound **15** (Figures 15C and D). Cells were pretreated with 6 compounds at the indicated concentrations for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 6 h. Phosphorylation of p38 was inhibited only by compounds **11** and **14** during 6 h treatment with TNFα+IFNγ. (Figures 15E and F).

A



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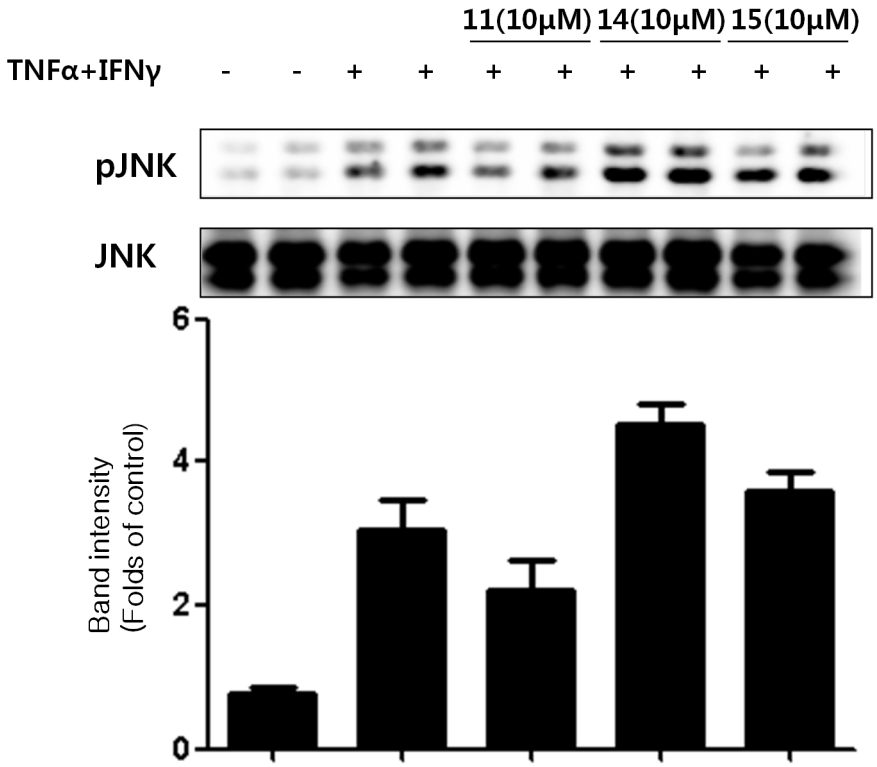

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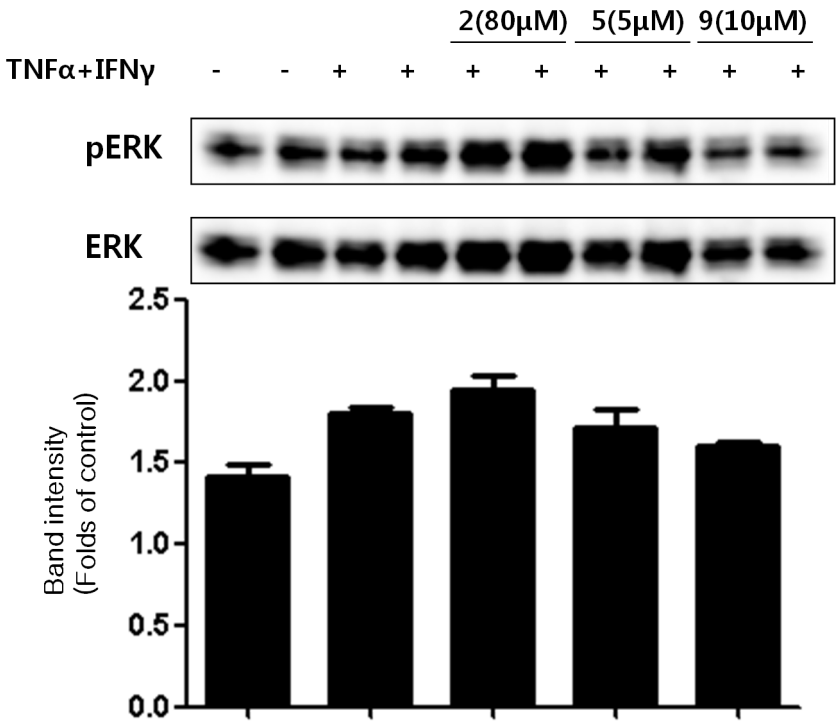

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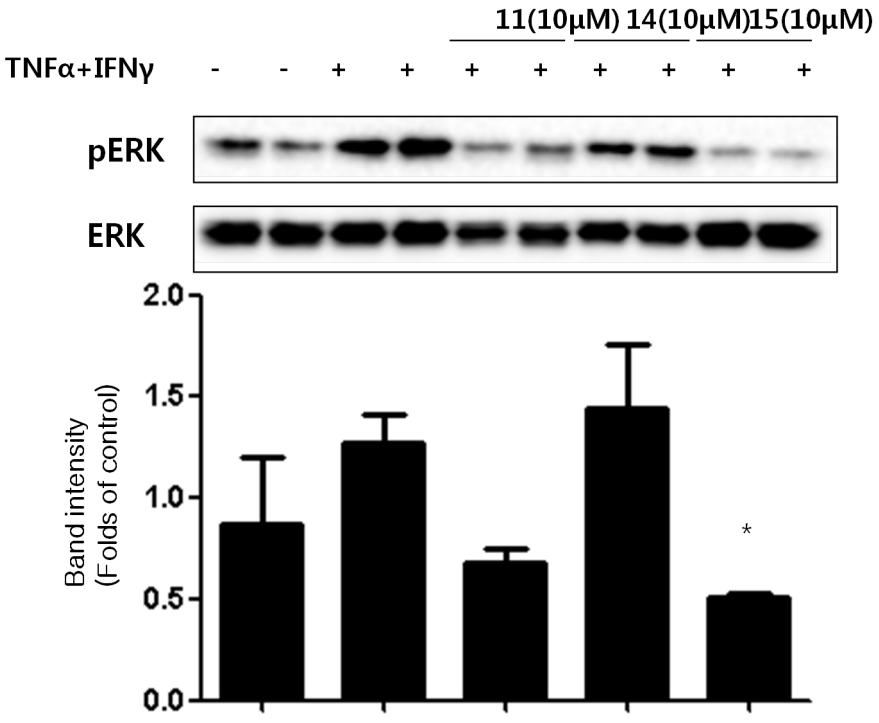

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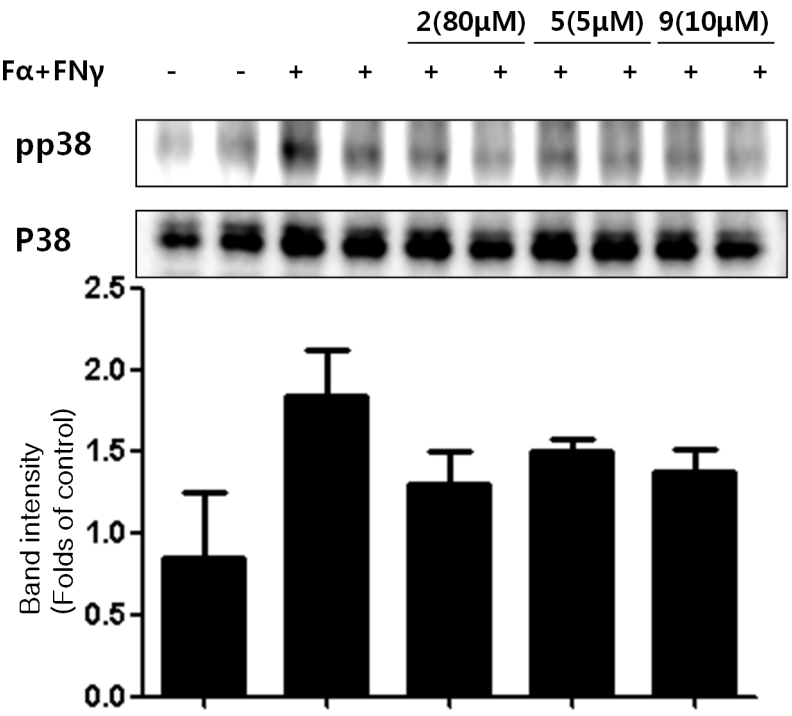

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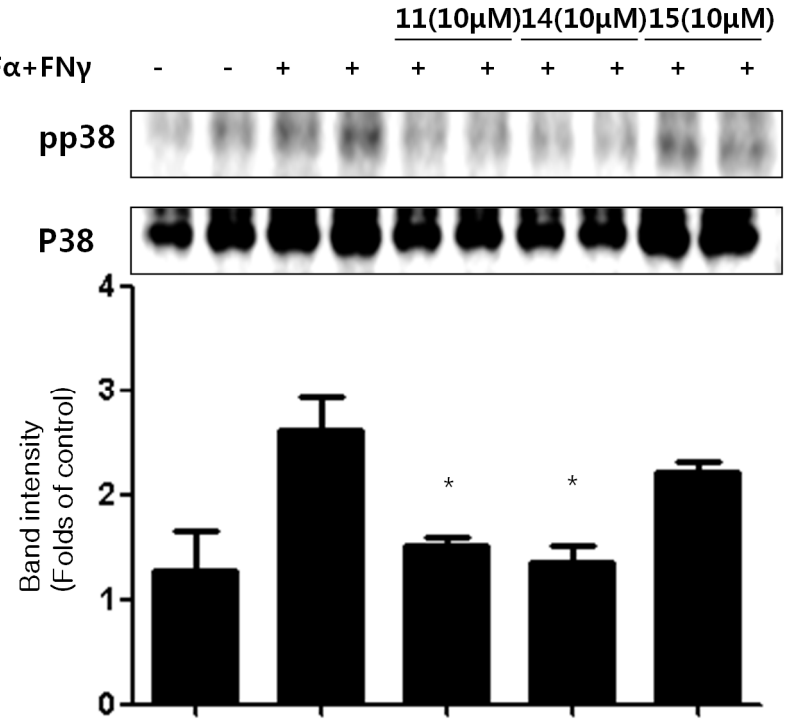

Figure 15. Effect of 6 compounds from *C. tricuspidata* on c-Jun N-terminal kinase (JNK)-1/2 (A and B), extracellular signal regulatory kinase (ERK)-1/2 (C and D), and p38 (E and F) phosphorylation in HaCaT cells. (A and B) Cells were pretreated with 6 compounds at the indicated concentrations for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 2 h. (C and D) Cells were pretreated with 6 compounds at the indicated concentrations for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 15 min. (E and F) Cells were pretreated with 6 compounds at the indicated concentrations for 3 h and stimulated with TNFα+IFNγ (20 ng/mL) for 6 h. The cell extracts were analyzed using western blotting, with antibodies specific for phosphorylated JNK1/2 (p-JNK1/2), pERK1/2, or p-p38. The membrane was stripped and re-irradiated to determine the total abundance of each mitogen-activated protein kinase (MAPK) as a control measure. Representative immunoblot from four independent experiments are presented. The immunoblot was quantified using the ImageJ software. Band intensity was quantified and normalized to the total of each form. *$p < 0.05$ as compared with TNFα+IFNγ only.

3.14. HPLC analysis of *C. tricuspidata* extracts and sub-fractions.

The 70% EtOH extracts and fractions from *C. tricuspidata* were analyzed using HPLC. The results of HPLC analysis using three wavelengths (254, 280, 345 nm) found a lot of peaks (Figure 16). The major components of the *C. tricuspidata* extract were predicted as peaks formed at 12.356 and 14.685 min. Based on the results, it was analyzed that the major components were at most included in CT-2 fraction. In addition, we were confirmed whether compounds **11**, **14** and **15** which are the most effective among 16 compounds, are included in the extracts and fractions. As a result of HPLC analysis using compounds **11**, **14** and **15**, the retention time of peaks were formed at 38.097 min for compound **11**, 34.932 min for compound **14**, and 34.351 min for compound **15** (Figure 16 D). As a result, it was analyzed that compounds **11**, **14** and **15** were included in CT-5 fraction. Further experiment will be needed to analyze the major components in the *C. tricuspidata* extract and sub-fractions.

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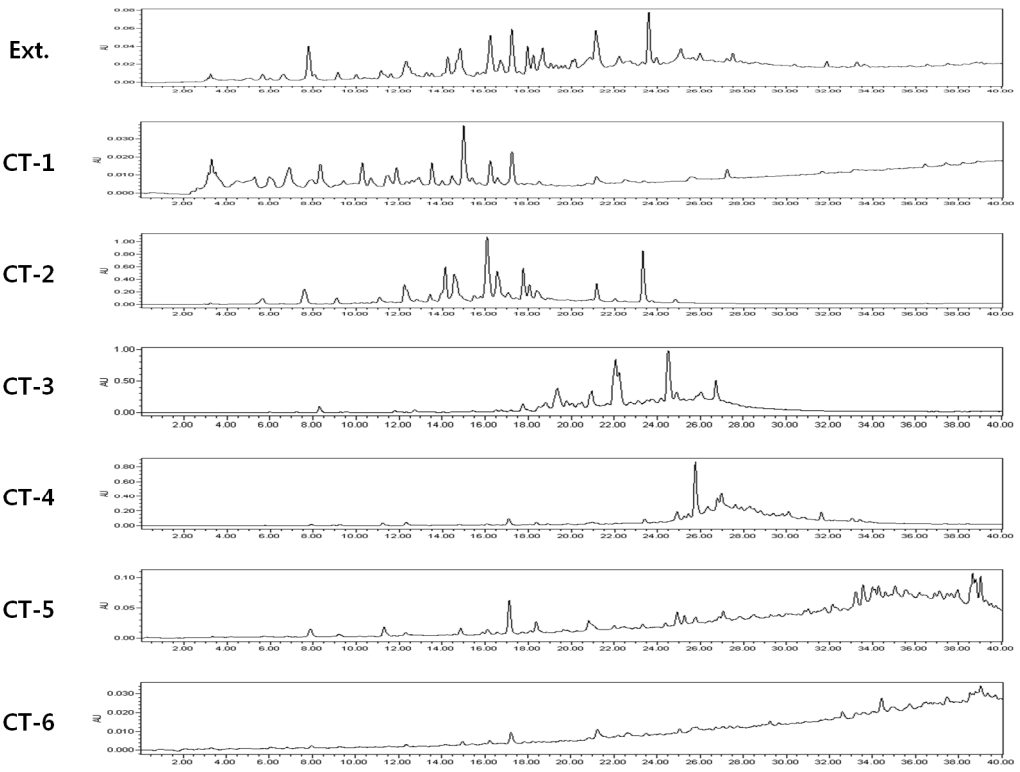

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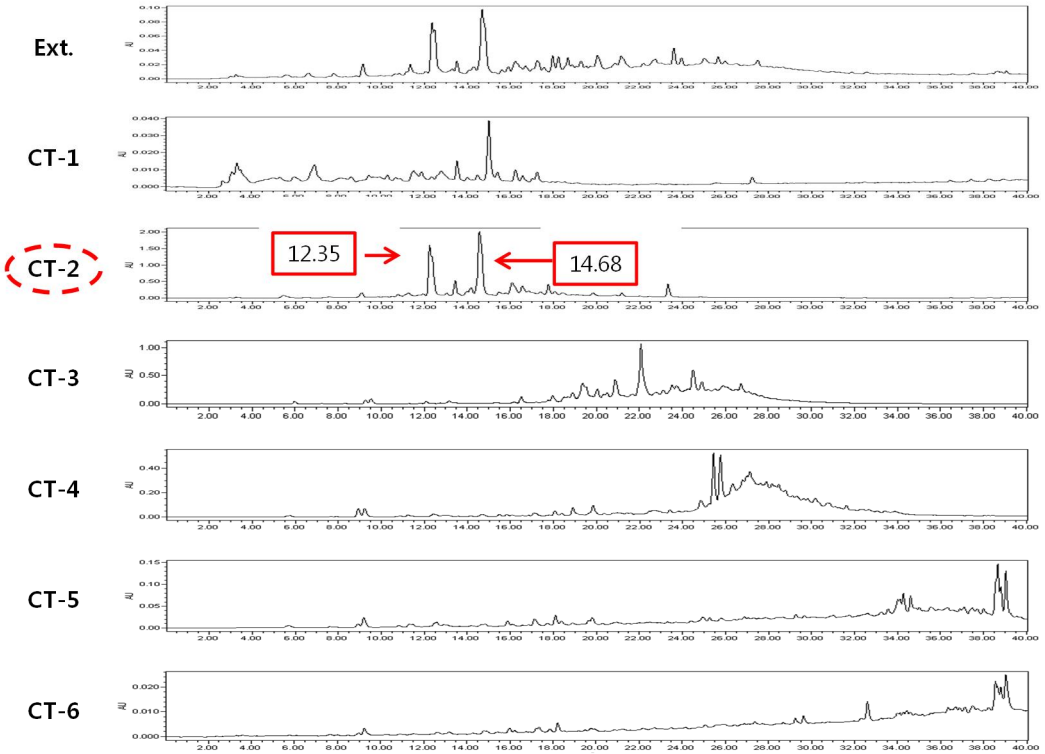

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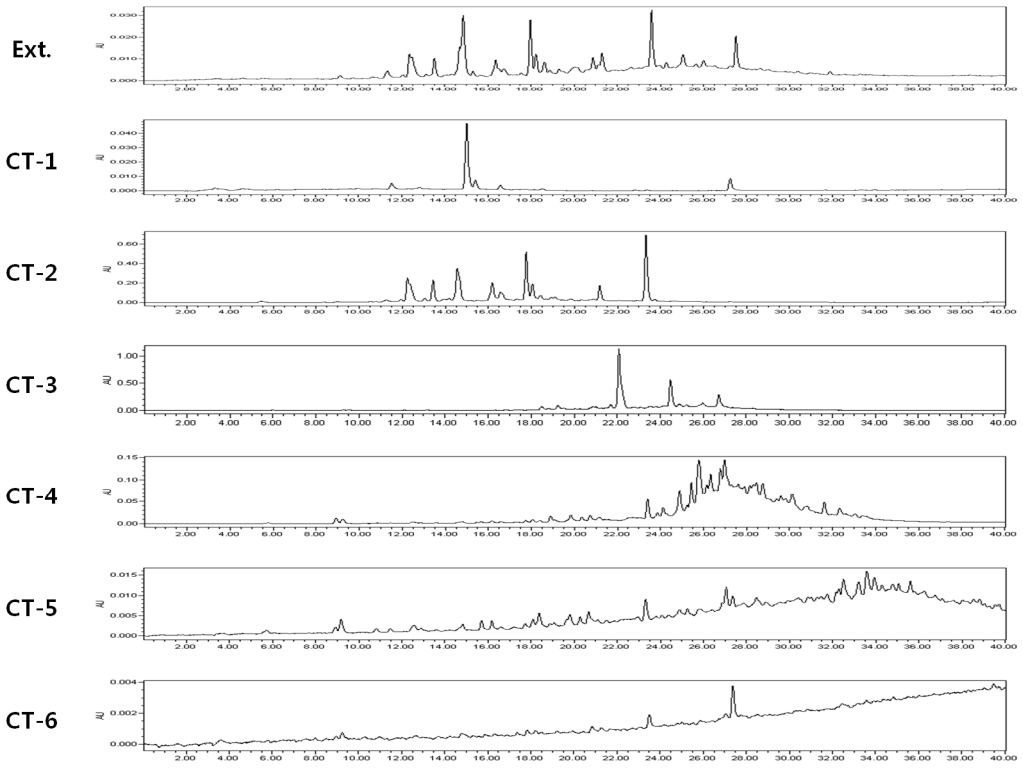

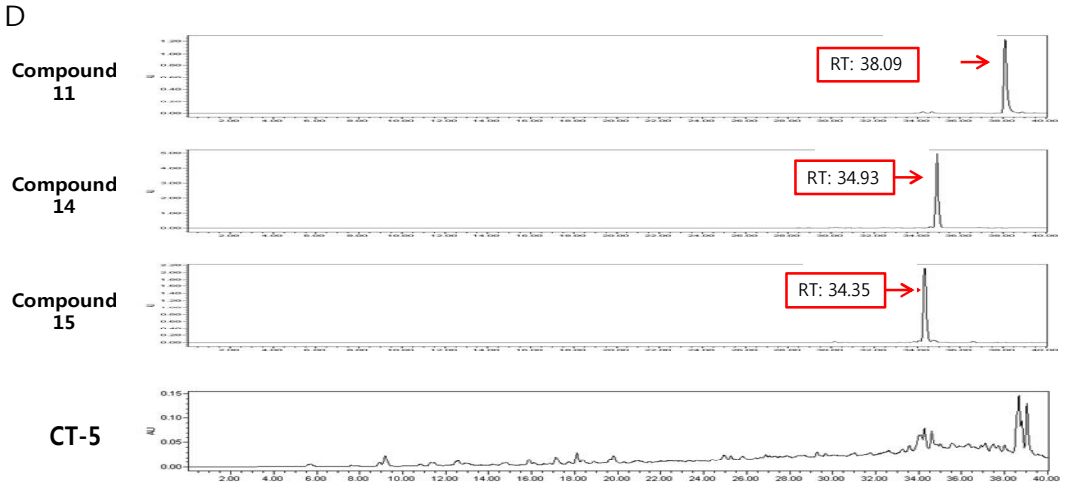

Figure 16. HPLC chromatogram of *C. tricuspidata* extracts and fractions. The wavelengths used in the analysis were detected at 254 nm (A), 280 nm (B) and 345 nm (C). Compounds **11**, **14** and **15** were analyzed at a wavelength of 280 nm (D).

4. Conclusion

In this study, we were investigated the effect of 70% EtOH extract, 6 sub-fractions, and 16 compounds from *C. tricuspidata* skin inflammation. First, 70% EtOH extract decreased IL-6 and IL-8 production in TNFα+IFNγ-induced HaCaT cells. And the sub-fractions from 70% EtOH extract of *C. tricuspidata* dose-dependently decreased IL-8 production in TNFα+IFNγ stimulated HaCaT cells. We also used to test on the regulation of skin inflammation by the 16 compounds isolated from the roots of *C. tricuspidata* in our previously study. Among the 16 compounds, compounds **1, 2, 4, 5, 6, 7, 9, 11, 14, 15** and **16** isolated from *C. tricuspidata* significantly reduced IL-6 production, and compounds **1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15,** and **16** compounds decreased IL-8 production in the HaCaT cells. Among the 16 compounds, the six most effective compounds such as steppogenin (**2**), cudraflavone C (**5**), macluraxanthone B (**9**), 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (**11**), cudraflavanone B (**14**), and cudratricusxanthone L (**15**) were selected for the further experiments. RANTES and TARC production were decreased by pretreatment with the all of 6 compounds. The expression of ICAM-1 and COX-2 proteins plays key roles in skin inflammation [35]. Cudraflavone C (**5**) and cudratricusxanthone L (**15**) were found to reduce the expression of COX-2, and all of 6 compounds significantly inhibited ICAM-1 expression in TNFα+IFNγ-induced HaCaT cells. Among the treatment with 6 compounds, 5 compounds including steppogenin (**2**), cudraflavone C (**5**), macluraxanthone B (**9**), cudraflavanone B (**14**), and cudratricusxanthone L (**15**) significantly inhibited NF-κB p65 translocation to nucleus, however 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (**11**) was not

significant. In addition, the phosphorylation of JNK-1/2 was not inhibited by all of 6 compounds. ERK-1/2 phosphorylation was inhibited by only cudratricusxanthone L (**15**). Phosphorylation of p38 was inhibited only by 1,6,7-trihydroxy-2-(1,1-dimethyl-2-propenyl)-3-methoxyxanthone (**11**) and cudraflavanone B (**14**). In addition, we were confirmed whether compounds **11**, **14** and **15,** which are the most effective among 16 compounds, are included in the extracts and fractions. As a result of HPLC analysis using compounds **11**, **14** and **15**, were included in CT-5 fraction. Comprehensively of all results in this study, 70% EtOH extract, sub-fraction, and compound **11**, **14**, **15** from *C. tricuspidata* could be further developed as a therapeutic agent that suppresses inflammation in skin cells. Further experiment will be need to analyze the major components in the *C. tricuspidata* extract and sub-fractions.

5. References

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