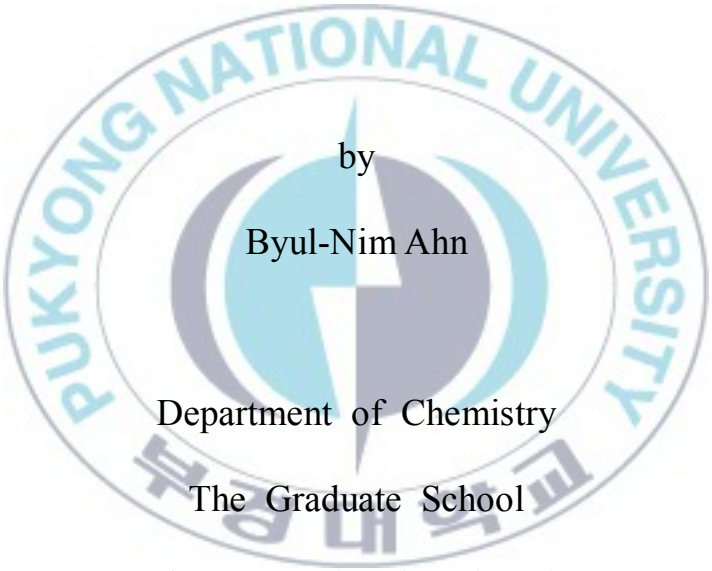
Thesis for the Degree of Master of Science

Effect of Coumarins from *Corydalis heterocarpa* on Photodamage in Human Keratinocyte (HaCaT) cells



by

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February 2011

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HaCaT 각질 세포주에서 *Corydalis heterocarpa* 로부터분리된 coumarin이 광손상에 미치는 효과

Advisor: Prof. Se-Kwon Kim

By

Byul-Nim Ahn

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Science

in Department of Chemistry, The Graduate School,
Pukyong National University

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A dissertation

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Abstract

Solar ultraviolet (UV) radiation, particularly its UVB (315-280 nm) element, has a variety of harmful effects on skin and leads to skin disease. The harmful biological effects of UVB includes, sunburn cell formation, melanoma, photoaging, and skin cancer via metaboliteproduction of ROS. Recently, some studies have been reported on antioxidative activities from isolated salt-tolerant plants such as *corydalis heterocarpa.*

This study focus on investigation of anti-photodamage effects of two coumarin compounds isolated from *corydalis heterocarpa*; columbianetin (A) and libanoridin (B) on UVB induced human keratinocyte (HaCaT) cells. In the results it was observed that compounds **A** and **B** suppressed UVB induced, generation of reactive oxygen species (ROS) and DNA damage. And also the compounds down regulated the UVB induced protein and gene expressions of MMP-2 and MMP-9. Furthermore, formation of apoptotic sunburn cells due to excessive UVB exposure was also alternated by the compounds treatment. The under lying signaling mechanisms of UVB protective effects of compounds A and B was elucidated through ASK1-MAPK-responsive signaliling pathways.

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List of Abbreviations

AP-1	Activatorprotien-1
ASK-1	Apoptosis signal regulating kinase-1
BSA	Bovine serum albumin
DCF	2',7'-dichlorofluorescein
DCFH	2',7'-dichlorodihydroxydrofluorescein
DCFH-DA	2',7'-dichlorofluorescin diacetate
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal regulated kinase
EtBr	Ethidium bromide
FBS	Fetal bovine serum
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
MAPKs	Mitogen activated protein kinases
MMPs	Matrix metalloproteinases
MTT	3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodysil sulphate
TBS-T	Tris buffered saline and tween-20

Introduction

The skin is the largest organ in the body, which includes the accessory structures such as hair and glands. The layer of skin is divided into two parts, epidermis and dermis. Among them, epidermis is the outermost layer of the skin, which is continuously influenced by various harmful environmental factos including ultraviolet irradiation of the sun (Anggakusuma et al., 2010).

Solar UV rays are divided by the wavelength into 3 categories; UVA (400-315 nm), UVB (315 - 280 nm) and UVC (280-100 nm). Most of the UVC rays cannot pass through the ozone layer and it does not reach the earth. Therefore, its role on skin damage is minimal (Zhang et al., 2001). Both UVA and UVB portions reaching the Earth's surface result in significant damage to the human skin (Wang et al., 2005). UVA constitute more than 96% of solar radiation coming to earth's surface. However, with ozone depletion, UVB percentage of the solar radiation has been gradually increasing over the past several decades (Norval et al., 2007). UVA rays penetrate deeply into the dermis and it can cause aging and wrinkling of skin (Matsumura et al., 2004). In contrast, middle-wave UVB ray is almost absorbed by epidermis. As this region of UV has the strongest energy intensity and acts primarily on the epidermal basal cell layer of the skin, inducing harmful biological effects both directly and indirectly, in particular formation of photoproducts, cell cycle arrest, photoageing, inflammation and photocarcinoge-nesis (Zaid et al., 2007; Li et al., 2007; Liu et al, 2007; Kippenberger et al., 2001;

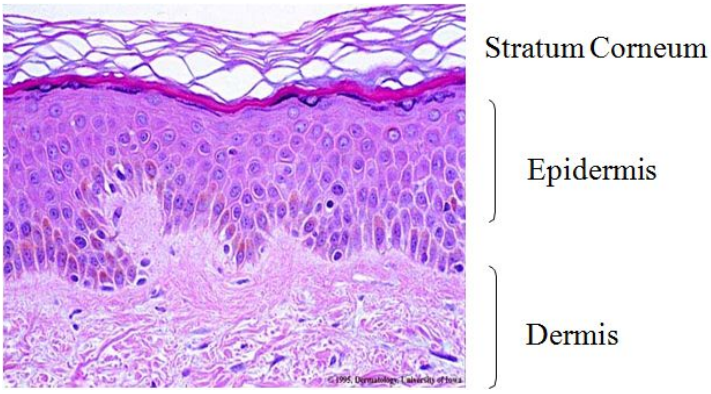

Figure 1. Basic structure of the skin.

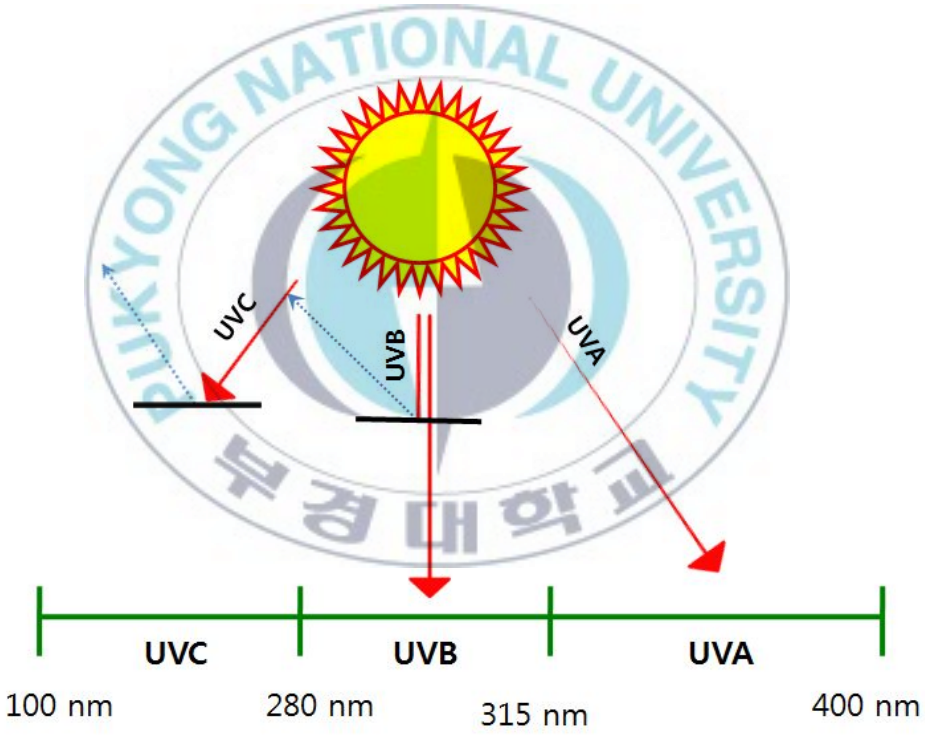

Figure 2. Ultra violet radiation spectrum. Sunlight is composed of a continuous spectrum of electromagnetic radiation that is divided into three main regions of wavelengths.

Afaq et al., 2005). For these important reasons, the destruction of ozone layer is a growing major problem (Svodoba et al., 2009).

Molecular responses of skin to UV exposure are initiated by photochemical generation of reactive oxygen species (ROS). UV produces ROS that can attack cellular components of skin such as lipids, proteins and DNA (Afaq et al., 2007; Park and Lee, 2008).

In addition, ROS induced by UV irradiation is closely related to expression of matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases that play the substantial roles in extracellular matrix (ECM) degradation during pathological processes such as arthritis, inflammation, cardiovascular diseases and cancer (Kong et al., 2010). Among various types of MMPs, MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are capable of degrading epidermal basement membrane including type IV collagen in skin cells (Park et al., 2006). MMPs expressions are regulated by their natural inhibitors, the tissue inhibitors of MMPs (TIMPs) (Steinbrenner et al., 2003). Moreover expression of MMP-9 and MMP-2 is depends on TIMP-1 and TIMP-2 respectively in normal physiological conditions (Corbel et al., 2000).

The critical biological feature of UVB irradiated skin is a form of apoptotic cell death of keratinocytes (Li et al., 2007), which is morphologically characterized by several unique cellular changes, such as cell shrinkage, chromatin condensation, genomic DNA fragmentation and membrane blebbing (Danial and Korsmeyer, 2004). These apoptotic keratinocytes are called sunburn

cells and are regarded as result from a complex molecular balance between survival and apoptotic factors (Laethem et al., 2006). Upon DNA damage by acute UV irradiation, p53 protein and apoptosis-promoting genes such as Bax are activated (Assefa et al., 2005).

A one of the apoptosis signaling mechanism induced by UVB irradiation is mitogen-activated protein kinase (MAPK) signaling pathway (Laethem et al., 2006). The MAPKs are comprised of proline-diredcted Ser/Thr knases including extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p-38 kinase (Anggakusuma et al., 2010). These subfamilies of MAPKs are activated in response to oxidant damage, and influence the cell survival (Afaq et al., 2005).

Apoptosis signal regulating kinase-1 (ASK-1) belonging to MAPK kinase kinase kinase (MAP3K) family that activates the JNK and p38 MAPK pathway in response to various stresses including oxidative stress (Laethem et al., 2006).

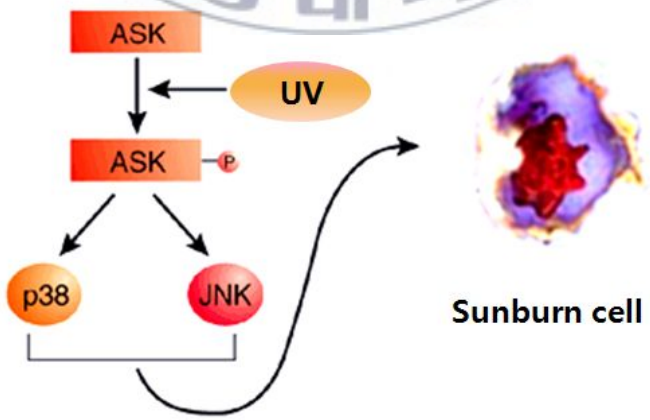

Figure 3. Sunburn cell formation via apoptosis signaling.

When cells are influenced by oxidative stress, Activated ASK-1 leads to the activation of MAPK cascades such as MKK-3/6 and MKK-4/7, which in turn activate p38 MAPK and JNK, respectively (Matsukawa et al., 2004). MAPK subfamilies is associated with the various transcription factors, especially activating protein-1 (AP-1), a complex containing c-Fos and c-Jun which is known to be activated by JNK and p38 MAPK and MMP-9 expression is highly dependent on AP-1 (Jibiki et al., 2003; Overall et al., 2002).

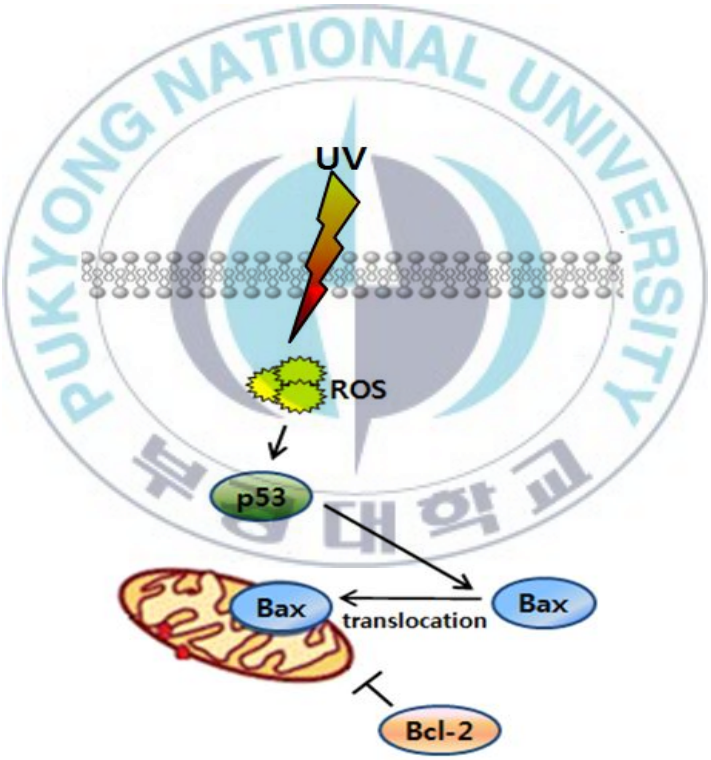

Figure 4. DNA damage in induced UV stimulated cell.

Corydalis heterocarpa (Siebold et Zuccarini) is classified as biennial herb with spikes yellow flowers and is found throughout south Korea (Kim et al., 2010). The salt-tolerant plant mainly inhabit the sandy beaches along the western coast of Korea and has been used as a folk medicine for travailing spasm, several secondary metabolites have been reported from this species (Choi et al., 2007; Kang et al., 2009).

The aim of this study is to investigate the effect of two coumarins, columbianetin **(A)** and libanoridin **(B)**, isolated from *Corydalis heterocarpa* on cellular damage caused by UVB-induced oxidative stress in human keratkinocyte cells.

2. Materials and methods

2.1. Plant materials and isolation of columbianetin and libanoridin

Whole plants of C. *heterocarpa* were collected in Muando, Jeollanamdo, Korea in July, 2003. The collected samples were air-dried, chopped into small pieces, and extracted for 2 days with MeOH (3 L × 2) and CH_2Cl_2 (3 L × 2), respectively. The combined crude extracts (41.1 g) were evaporated under reduced pressure and partitioned to afford the *n*-hexane (7.3 g), 85% aqueous (aq.) MeOH (12.0 g), *n*-BuOH (4.3 g) and water (20.0 g). Compound **A** (658.2 mg) and compound **B** (29.8 mg) were isolated from a portion of the 85% aq. MeOH fraction.

Columbianetin (A) Amorphous white solid, mp. 160–163 ^{o}C; $[\alpha]_{25}^{D}$+ 264^{o} (c 1.1, MeOH); HREI-MS m/z 246.0892 (calcd. for $C_{14}H_{14}O_4$, 246.0892); ^{1}H NMR (300 MHz, CD_3OD) δ: 7.82 (1H, d, *J* = 9.4 Hz, H-4), 7.36 (1H, d, *J* = 8.3 Hz, H-5), 6.76 (1H, d, *J* = 8.3 Hz, H-6), 6.15 (1H, d, *J* = 9.4 Hz, H-3), 4.79 (1H, t, *J* = 9.0 Hz, H-2′), 3.30 (2H, d, *J* = 9.0 Hz, H-1′), 1.30 (3H, s, H-4′/-5′), 1.25 (3H, s, H-4′/-5′); 13C NMR (75 MHz, CD_3OD) d: 165.5 (C-7), 163.0 (C-2), 152.3 (C-9), 146.1 (C-4), 130.2 (C-5), 115.0 (C- 10), 114.2 (C-8), 112.1 (C-3), 107.8 (C-6), 92.5 (C-2′), 72.3 (C-3′), 28.1 (C-1′), 25.4 (C-4′/-5′), 25.3 (C-4′/-5′).

Libanoridin (B) Amorphous white solid, mp. 127–129 ^{o}C; $[\alpha]_{25}^{D}$ + 252^{o} (c 1.0, CHC_{l3}); HREI-MS m/z 288.0998 (calcd. for $C_{16}H_{16}O_5$, 288.0998); ^{1}H NMR (300 MHz, CDCl3) δ: 7.60 (1H, d, *J* = 9.6 Hz, H-4), 7.24 (1H, d, *J* = 8.3 Hz, H-5), 6.72 (1H, d, *J* = 8.3 Hz, H-6), 6.18 (1H, d, *J* = 9.6 Hz, H-3), 5.12 (1H, dd, *J* = 9.6, 7.8 Hz, H-20), 3.35 (1H, dd, *J* = 16.5, 9.6 Hz, H-1′a), 3.26 (1H, dd, *J* = 16.5, 7.8 Hz,

H-1′b), 1.97 (3H, s, H-7′), 1.55 (3H, s, H-4′/-5′), 1.50 (3H, s, H-4′/-5′); 13C NMR (75 MHz, CDC_{l3}) d: 170.0 (C-6′), 163.6 (C-7), 160.8 (C-2), 151.0 (C-9), 143.8 (C-4), 128.7 (C-5), 113.3 (C-10), 112.9 (C-8), 112.1 (C-3), 106.6 (C-6), 88.6 (C-2′), 82.0 (C-3′), 27.6 (C-1′), 22.3 (C-7′), 22.0 (C-4′/-5′), 21.0 (C-4′/-5′).

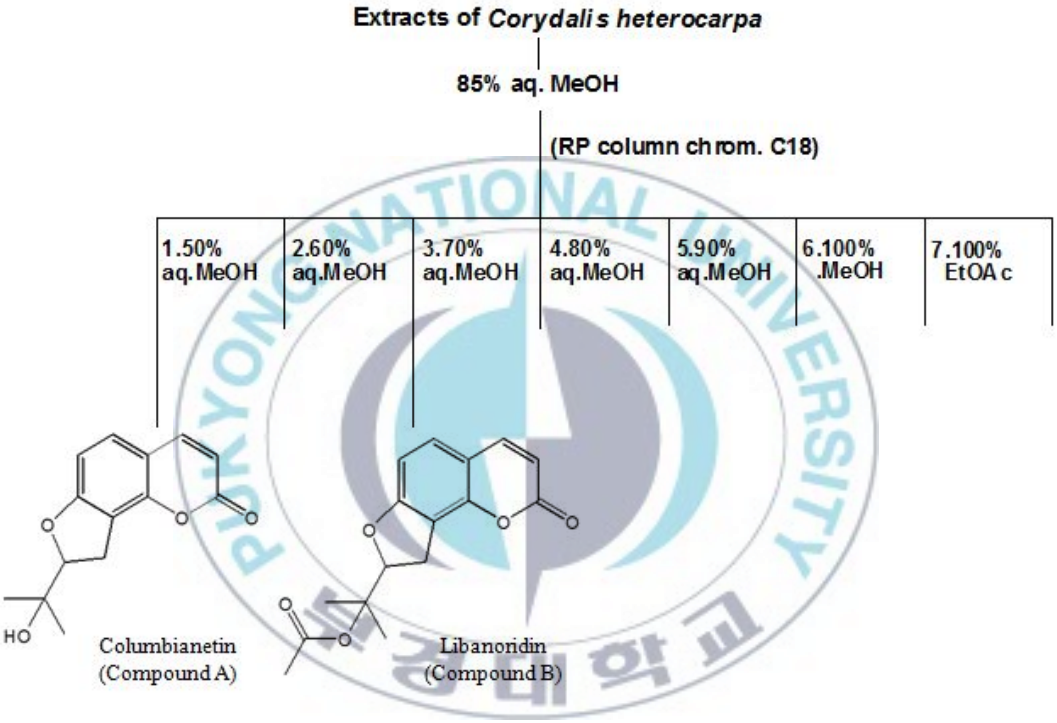

Figure 5. Isolation of the compounds **A** and **B** from *Corydalis heterocarpa.*

2.2. Cell culture

Human keratinocyte (HaCaT) cells were grown in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL, Gaithersbrug, MD USA) containing 10 % fetal bovine serum (FBS), 2 mM glutamine and 100 μg/ml penicillin-streptomycin (Gibco-BRL, Gaithersbrug, MD, USA) at 37°C humidified atmosphere of 5% CO_2. Cells were sub-cultured to about 90-95% confluence by detaching with trypsin-EDTA solution.

2.3. UVB irradiation

In order to determine the optimum level of UVB irradiation intensity, cells at a density of 1 $x10^5$ cells/well were incubated in 24-well plate with DMEM containing 10% FBS, 2 mM glutamine and 100 μg/ml penicillin-streptomycin at 37ºC humidified atmosphere of 5% CO_2. After incubation for 24 h, the cells were exposed to UVB energy at a range of 10-3000 mJ/cm^2 (312 nm UVB light source, Bio-Sun lamp, Vilber Lourmat, Marine, France) in 200 μl of phosphate buffered saline (PBS) in each well. After irradiation, the cells were placed in serum-free DMEM for 6, 12, 24 and 48 h.

2.4. Cell cytotoxicity determination by MTT and LDH assays

2.4.1. MTT assay

The viability levels of HaCaT cells were determined by the ability of mitochondria to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

bromide (MTT) to insoluble formazan product. Briefly, cells were grown in 96-well plates at a density of $1x10^4$ cells/well. After incubation for 24 h, the cells were stimulated by UVB irradiation (40 mJ/cm^2) and incubated for 24 h in the absence or presence of compound A and B at 37 ºC under a humidified atmosphere of 5% CO_2. The supernatant medium was removed and 100 μl of 1 mg/ml MTT reagent was added to each well, followed by incubation for 4 h. After removal of unconverted MTT, the amount of formazan in the cells was determined by adding DMSO (dimethyl sulfoxide) to wells and measuring optical density (OD) at 540 nm using a microplate reader (Tacan Austria GmbH, Salzburg, Austria). Relative cell viability was calculated as the percentage of viability of untreated cells that were considered the control.

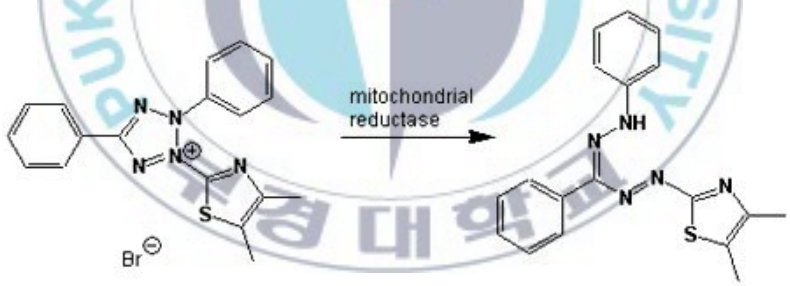

Figure 6. Molecular structure of MTT and its corresponding reaction product.

2.4.2. LDH assay

Cell injury was measured by quantifying the amount of lactate dehydrogenase (LDH)-release using a commercial LDH cytotoxicity detection kit (TaKaRa Biomedicals, Tokyo, Japan). Cells were exposed according to the experiment

design. The conditioned medium of the UVB-exposed cells was collected for LDH measurement. The substrate mix solution was added to aliquots of the supernatant medium at 1:1 ratio and incubated at 37ºC for 30 min under light protected conditions. Then, the absorbance was determined at 490 nm immediately after the addition 1N HCl stop solution (final concentration, 0.2 N) using a microplate reader (Tacan Austria GmbH, Salzburg, Austria). Control was prepared with 0.1% (w/v) Triton X-100, which was defined as 100% LDH release.

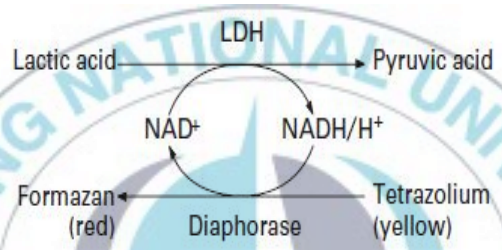

Figure 7. A two-step enzymatic reaction quantifies cell lysis and cell death.

2.5. Intracellular reactive oxygen species (ROS) generation

The levels of intracellular ROS generation were detected using the oxidation sensitive dye 2',7'-dichlorofluorescin diacetate (DCFH-DA). HaCaT cells were grown in a 96-well microplate with fluorescence for 24 h and followed by exposure to UVB (40 mJ/cm^2). The exposed cells were treated with samples for 24 h and then they were loaded with 20 μM of DCFH-DA in PBS and incubated for 30 min in the dark at 37ºC under a humidified atmosphere with 5% CO_2. Finally, cells were washed twice with PBS and the fluorescence of DCF in PBS was detected at an excitation wavelength of 485 nm and an emission wavelength

of 535 nm using a fluorescence microplate reader (Tacan Austria GmbH, Salzburg, Austria).

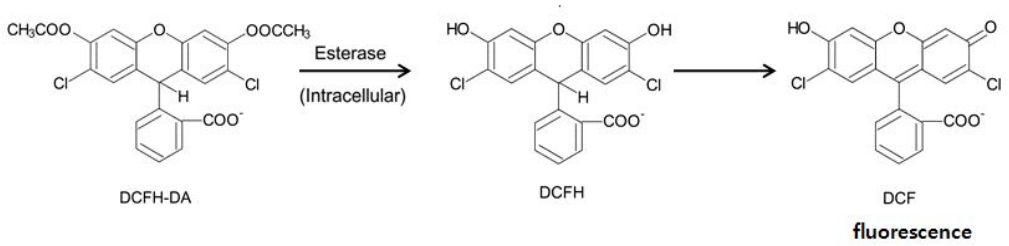

Figure 8. UVB-induced formation of ROS and oxidative damage.

2.6. Genomic DNA extraction and DNA oxidation

Genomic DNA was isolated from HaCaT cells using standard phenol/proteinase K procedure with slight modifications (Sambrook and Russell, 2001). In brief, UVB-stimulated cells in the presence and absence of sample were washed twice with PBS and collected using 1 ml of PBS containing 10 mM EDTA. After centrifugation at 13,400×g for 5 min at 4°C, the deposited cells were resuspended in 410 μl of solution including RNase A (0.5 mg/ml), proteinase K (10 mg/ml), SDS (10%) and NaOAC (0.2 M). After that, This mixture was incubated at 37°C for 30 min and 55°C for 1 h. Following incubation, phenol:chloroform:iso-amylalcohol (25:24:1) was added at 1:1 ratio, after which the mixture was centrifuged at 13,400 ×g for 5 min at 4 °C. Then the upper layer was transferred to new eppendorf tube and 100% ice cold ethanol was added at a 1:1.5 ratio followed by incubation at -20°C for 30 min. After centrifuging at 5,900×g for 5 min at 4°C, the supernatant was carefully removed and the remaining pellet was

dissolved in 20 μl of TE buffer (10 mM Tris-HC1, 1 mM EDTA, pH 8.0). Purity of DNA was determined by ratio of absorbance at 260 and 280 nm using spectrophotometric assay. Aliquot (20 μl) of reaction mixture containing about 1 μg of DNA was separated by 1% agarose gel electrophoresis for 10 min at 100 V. The gels were stained by 1 mg/ml of ethidium bromide (EtBr) for 30 min and photographed under UV illumination using AlphaEase® gel image analysis software (Alpha Innotech, San Leandro, CA, USA).

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated using Trizol reagent (Invitrogen Co., CA, USA). The 2 μg of isolated RNA was reverse transcribed into cDNA using oligo-(dT) primer (Promega, Madison, WI, USA). Primers to specifically amplify the genes of MMP-2, MMP-9, TIMP-1, TIMP-2 and GAPDH were showed in Table 4. Amplification of target cDNA was carried out at 95ºC for 45 sec, 60ºC for 50 sec and 72ºC for 60 sec for 30 cycles. After amplification step, the extension process proceeded consecutively at 72ºC for 5 min. PCR products were separated on 1% agarose gel for 10 min at 100 V by electrophoresis. Gels were stained with 1 mg/ml of EtBr and photographed by UV illumination using AlphaEase® gel image analysis software (Alpha Innotech., San Leandro, CA, USA). Finally, the relative band densities were determined using a LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

Table 1. Chemicals used for reverse transcription reaction

PCR chemical	Amount used	Stock
MMLV reverse transcriptase	0.5 µl	200 U/ µl
dNTP mixture	1 µl	10 mM
DTT	1 µl	100 mM
5X reaction buffer	4 µl	
RNase inhibitor	0.5 µl	80 U/ µl

Table 2. Chemicals used for PCR reaction

PCR chemical	Amount used	Stock
Taq polymerase	0.5 µl	5 U/ µl
dNTP mixture	0.5 µl	10 mM
Forward Primer	0.5 µl	50 pmole/ µl
Reverse Primer	0.5 µl	50pmole/ µl
Template DNA	1 µl	
5X reaction buffer	5 µl	
Nuclease-free water to 25 µl		

Table 3. PCR conditions

Step	Temperature	Time	Number of Cycles
Initial Danaturation	95°C	2 min	1 cycle
Denaturation	95°C	45 sec	30 cycles
Annealing	60°C	50 sec	
Eaxtension	72°C	60 sec	
Final Extension	72°C	5 min	1 cycle
Soak	4°C	Indefinite	1 cycle

Table 4. Gene-Specific primers Used for the RT-PCR

Gene	Direction	Squence
MMP-2	Forward	5'-ATG GCA AGT ACG GCT TCT GT-3'
	Reverse	5'-ATA CTT CTT GTC GCG GTC GT-3'
MMP-9	Forward	5'-CTC GAA CTT TGA CAG CGA CA-3'
	Reverse	5'-GCC ATT CAC GTC GTC CTT AT-3'
TIMP-1	Forward	5'-AAT TCC GAC CTC GTC ATC AG-3'
	Reverse	5'-TGC AGT TTT CCA GCA ATG AG-3'
TIMP-2	Forward	5'-TGA TCC ACA CAC GTT GGT CT-3'
	Reverse	5'-TTT GAG TTG CTT GCA GGA TG-3'
GAPDH	Forward	5'-GAG TCA ACG GAT TTG GTC GT-3'
	Reverse	5'-GAC AAG CTT CCC GTT CTC AG-3'

2.8. Western blot analysis

Whole cells were lysed in RIPA buffer (Sigma-Aldrch Corp., St. Louis, USA) at 4^{o}C for 30 min. After centrifugation, total protein amount of cell lysates were determined using Lowry method (BioRad Laboratories, Hercules, CA). Aliquot of supernatant containing equal amounts of proteins (10 μg) were electrophoresed on 10% or 12% SDS-polyacrylamide gels, transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech., England, UK), blocked with 5% bovine serum albumin in TBS containing 0.1% Tween 20 (TBS-T) for at least 1 h, and hybridized with primary antibodies such as MMP-2, MMP-9, TIMP-1, TIMP-2, ERK, JNK, pERK, pJNK, ASK1, p-ASK1, p38, pp38, c-jun, c-fos, p53, pp53 and Bax (Santa Cruz Biotechnology Inc., CA, USA). All primary monoclonal antibodies were diluted with TBS-T at a 1:1000 ratio. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, and the immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, England, UK) according to the manufacturer's instructions. Western blot bands were visualized using a LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.9. Statistical analysis

Data were expressed as mean ± SD (n=3) and analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS v9.1, SAS Institute Inc., Cary, NC, USA). Significant differences between treatment means were determined using Duncan's multiple range tests at the $p<0.05$ level.

3. Results

3.1. Effect of on viability of UVB-exposed cells

To determine the appropriate energy level of UVB irradiation, the cytotoxicity of cultured keratinocytes was determined by comparing the data obtained for MTT and LDH leakage for 6, 12, 24 and 48 h after UVB irradiation from 10 to 3000 mJ/cm^2. LDH is a stable cytoplasmic enzyme present in many organisms. LDH release from cells into the cultured media is indicative of cellular damage (Bagchi et al., 1995). Exposure of cells to UVB energy significantly reduced the cell viability (Figure 9) and increased LDH release in a dose dependent manner (Figure 10). Exposure of cells to UVB energy at the higher than 40 mJ/cm^2 induced similar cytotoxicity of UVB exposed cells.

Based on these results, effects of compounds **A** and **B** with different concentrations on the viability and injury degree of UVB-exposed HaCaT cells were examined at 40 mJ/cm^2. Exposure of cells to increasing concentrations of compounds substantially enhanced cell viability in a dose-dependent manner, compare with only UVB-exposed cells (Figure 11). LDH release assay revealed that compounds substantially reduced the degree of cell injure by UVB exposure in a dose-dependent manner (Figure 12). The inhibitory effect on cell damage by UVB exposure was more effective in compound **B** treated cells, compared to compound **A**.

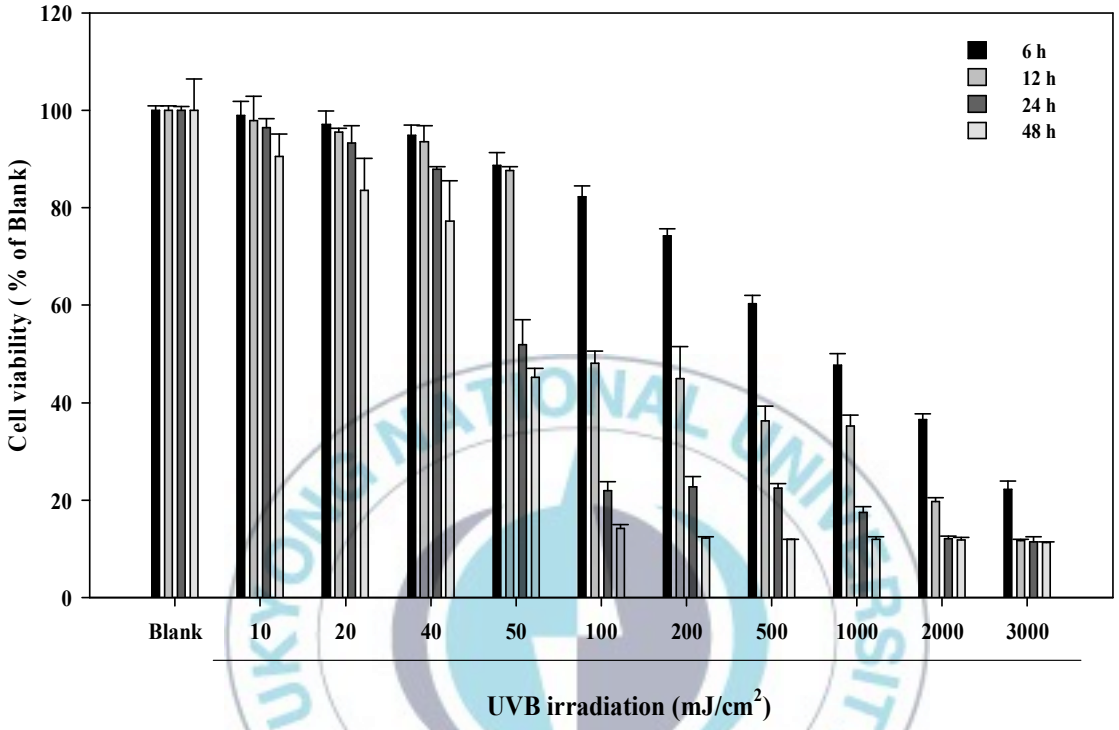

Figure 9. Viability of human keratinocytes exposed to different irradiation intensities of UVB irradiation. Cells were exposed with UVB irradiation and the cytotoxicity level was determined by MTT. Blank: no UVB exposure.

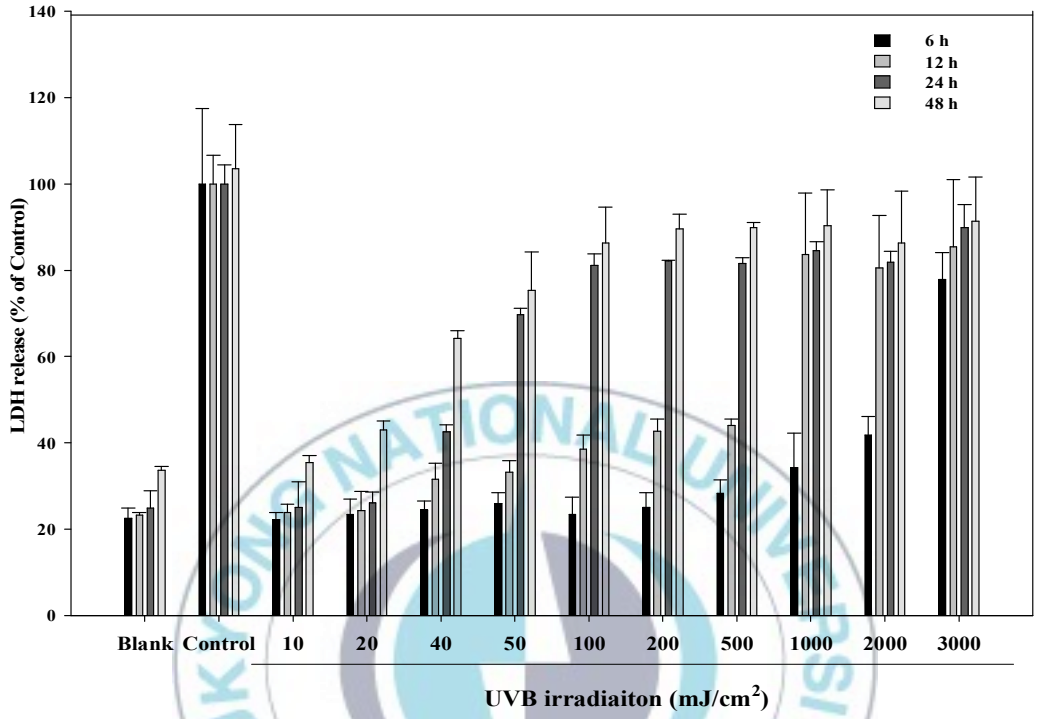

Figure 10. Cytotoxicity of human keratinocytes exposed to different irradiation intensities of UVB irradiation. Cells were exposed with UVB irradiation and the cytotoxicity level was determined by LDH release assays. Blank: no UVB exposure, Control: cells lysed by the addition of Triton X-100.

(A)

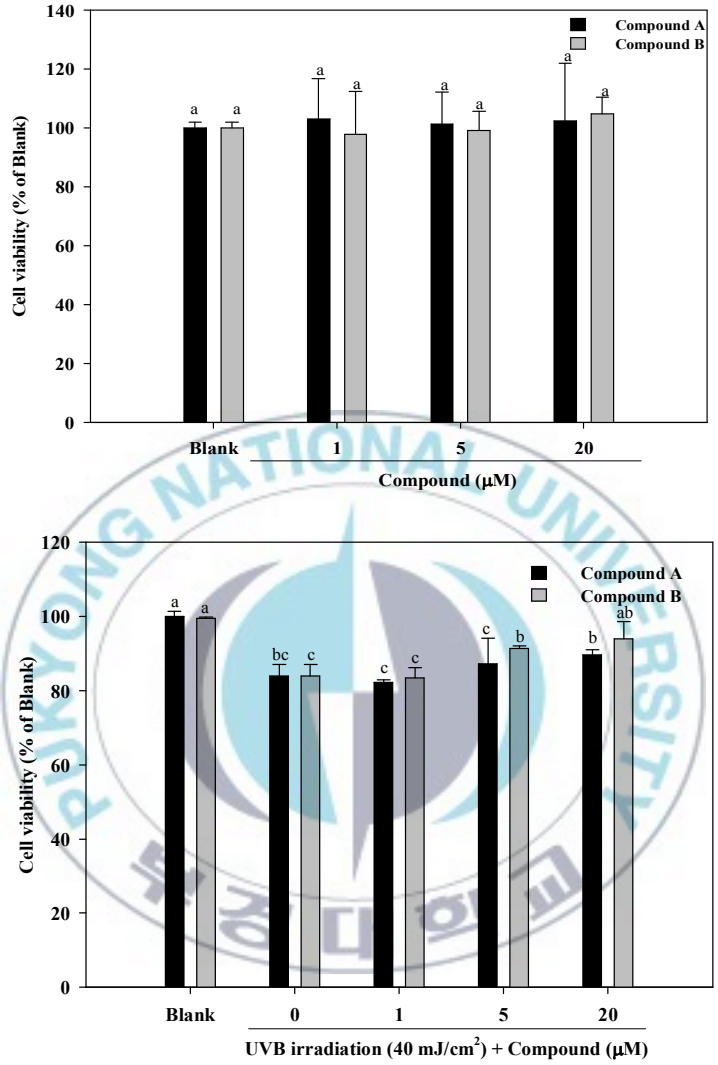

Figure 11. Effect of compounds **A** and **B** with different concentrations on viability of human keratinocytes exposed without **(A)** or with 40 mJ/cm^2 of UVB irradiation **(B)**. Cells were exposed without or with 40 mJ/cm^2 of UVB irradiation and treated with different concentrations of two coumarins. Cell viability was determined by MTT. Blank: no UVB exposure.

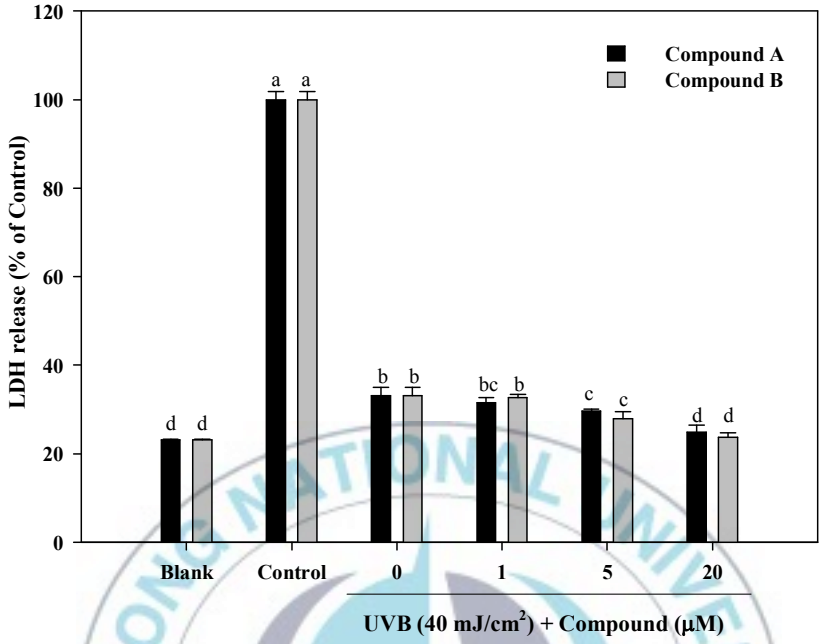

Figure 12. Effect of compounds **A** and **B** with different concentrations on cytotoxicity of UVB induced human keratinocytes exposed to 40 mJ/cm^2 of UVB irradiation. Cells were exposed with 40 mJ/cm^2 of UVB irradiation and treated with different concentrations of two coumarins. Cell viability was determined by LDH. Blank: no UVB exposure, Control: cells lysed by the addition of Triton X-100.

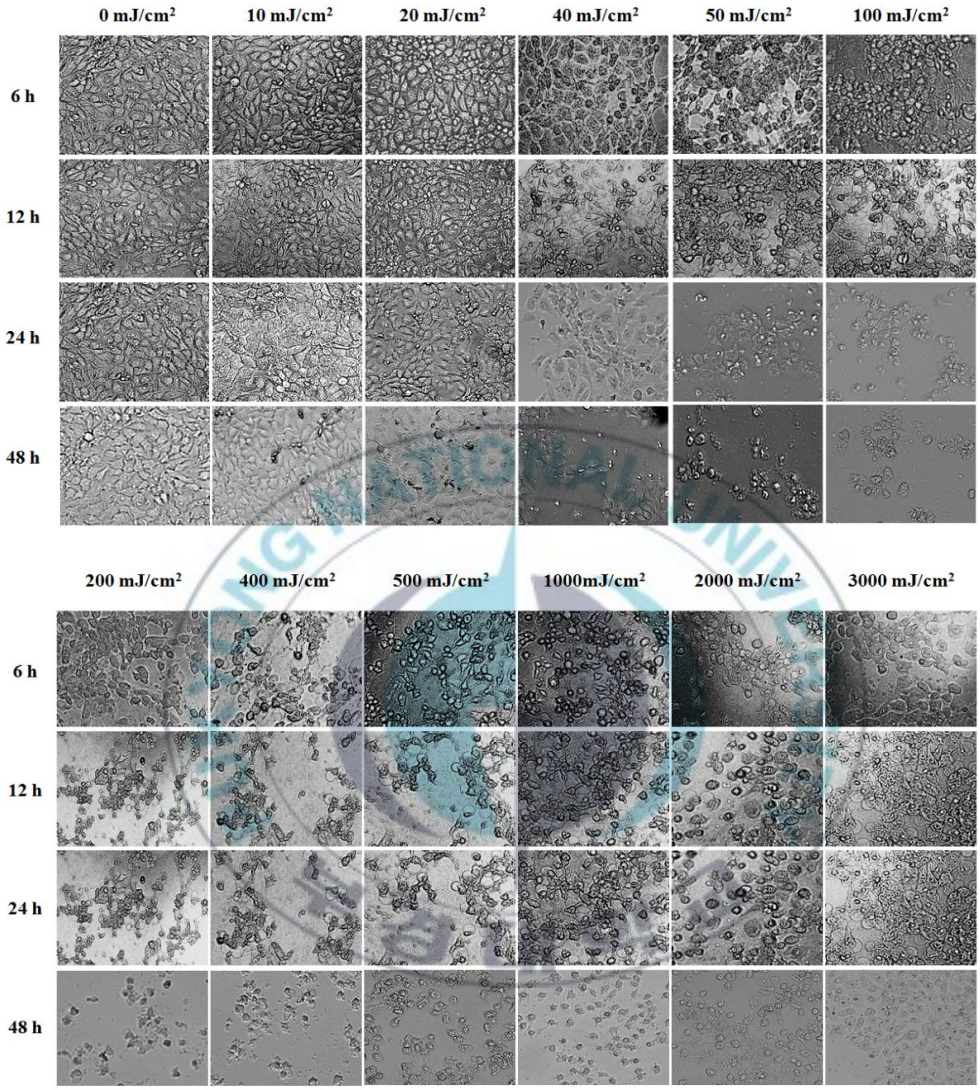

Figure 13. The time course of changes in HaCaT cells after various does of UVB irradiation. After 10-3000 mJ/cm^2 of UVB irradiation, HaCaT cells were incubated in serum-free DMEM and cells cultured for 6, 12, 24 and 48 h following UVB irradiation without fixing were photographed.

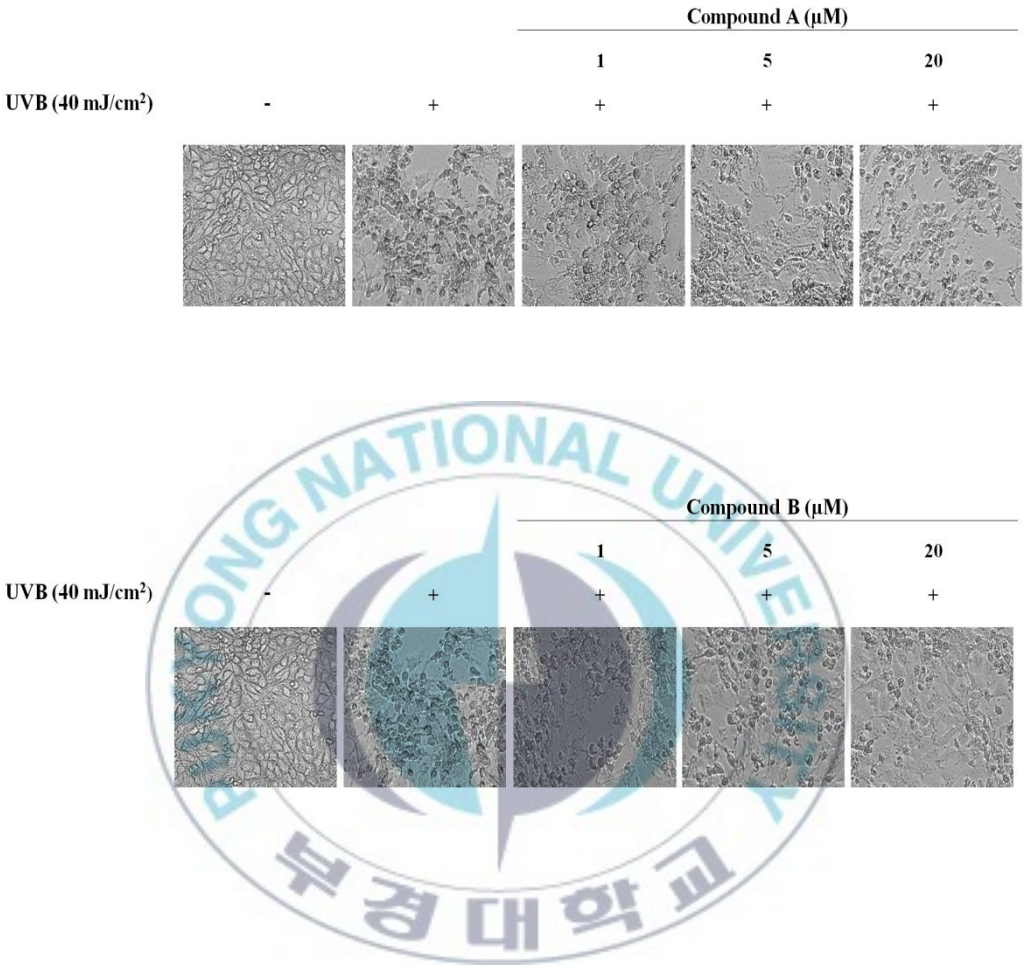

Figure 14. Microscopic image of morphological changes in HaCaT cells in response to UVB irradiation. HaCaT cells examined using microscopes after UVB irradiation (40 mJ/cm^2) and treatment with various concentrations of compounds **A** and **B** for 24 h.

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3.2. Effect of compounds A and B on intracellular ROS generation in UVB exposed cells

To determine the suppressing efficiency of compounds **A** and **B** with different concentrations on intracellular ROS production induced by UVB irradiation, the level of ROS generation in UVB exposed cells was measured using fluorescence dye DCFH-DA (Figure 13). The increments in DCF fluorescence intensity due to UVB exposure were observed. UVB exposure induced higher fluorescence intensity compared with the blank group, which was non-UVB exposed. The presence of compounds in UVB-exposed cells significantly reduced DCF fluorescence intensity in a dose-dependent manner, demonstrating enhanced scavenging activity against intracellular ROS generation. Among two coumarins, compound B effectively suppressed UVB mediated ROS generation.

3.3. Effect of compounds A and B on DNA damage in UVB exposed cells

Inhibitory effect of compounds **A** and **B** with different concentration on DNA oxidative damage was examined using genomic DNA isolated from UVB exposed cells (Figure 14). Although DNA damage was observed in UVB exposed cells, the DNA damage was reduced in the presence of compounds **A** and **B**. These results confirmed that two compounds exerted adequate protective effect on UVB-mediated DNA damage, especially compound **B** effectively protected DNA damage due to UVB exposure.

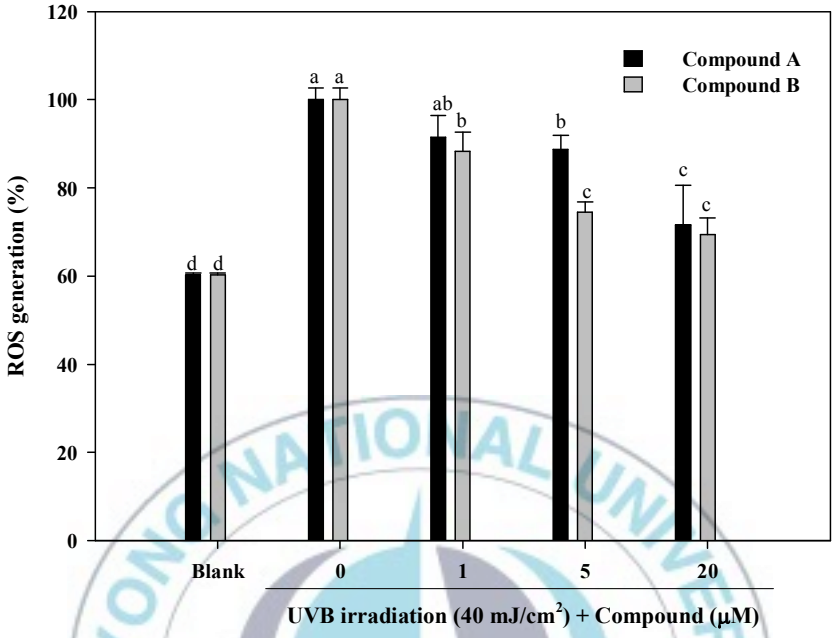


Figure 15. Effect of compounds **A** and **B** with different concentrations on intracellular ROS generation induced UVB irradiation. Cells were exposed with 40 mJ/cm^2 of UVB irradiation were incubated for 24 h and loaded with DCFH-DA. ROS was detected by fluorescence spectrophotometer after DCFH-DA staining. Blank: no UVB exposure.

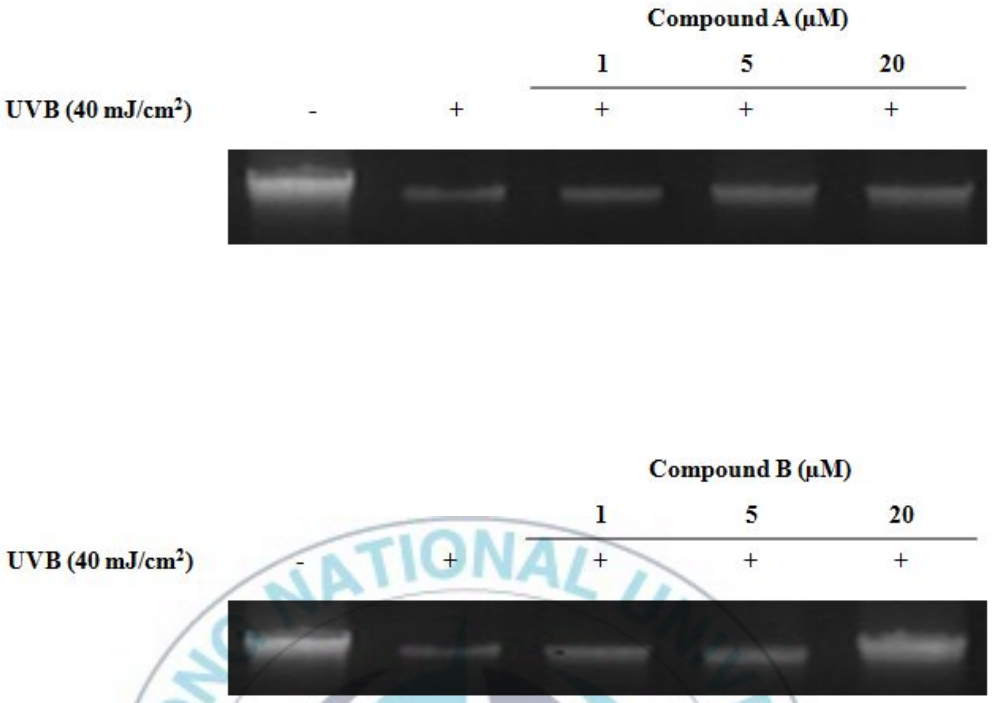


Figure 16. Effect of compounds **A** and **B** of various concentrations on UVB-induced DNA oxidative damage. Cells were exposed with 40 mJ/cm^2 of UVB irradiation and treated with different concentrations of coumarins. DNA was isolated from UVB-stimulated cells in the presence and absence of two compounds and subjected to electrophoresis in 1% agarose.

3.4. Effect of compounds A and B on the regulation of UVB-mediated MMP and TIMP

Effects of compound **A** and **B** on MMP-2, MMP-9 and tissue inhibitor of metalloproteinase TIMP-1 and TIMP-2 expressions in UVB-exposed cells were determined using RT-PCR (Figures 17 and 18). UVB exposed HaCaT cells were treated with various concentrations of compounds **A** and **B** for 24 h. Only UVB alone exposed cells exhibited higher MMP-2 and MMP-9 gene expression than UVB non-irradiated cells. However, the gene expression of UVB-mediated gelatinase A and B gene expression was reduced by compounds **A** and **B** treatment. Especially, MMP-9 gene expression was appreciably decreased by compounds **A** and **B** in a dose-dependent manner and MMP-2 gene expression was decreased by compound **B** in a dose-dependent manner. Furthermore, the expression levels of TIMP-1 gene reduced by UVB irradiation were dose dependently enhanced by treatment with two coumarins, on the other hand, TIMP-2 gene expression were not modulated by UVB exposing, compounds A and B.

Next, the changes in protein expression levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 in presence of two coumarins were examined (Figure 18). Compounds **A** and **B** suppressed UVB-induced increase in MMP-2 and MMP-9 proteins, whether up-modulated TIMP-1 and TIMP-2 protein level. According to these experimental data, among the compounds A and B exhibited the most potent protective effect on UVB-mediated photodamage.

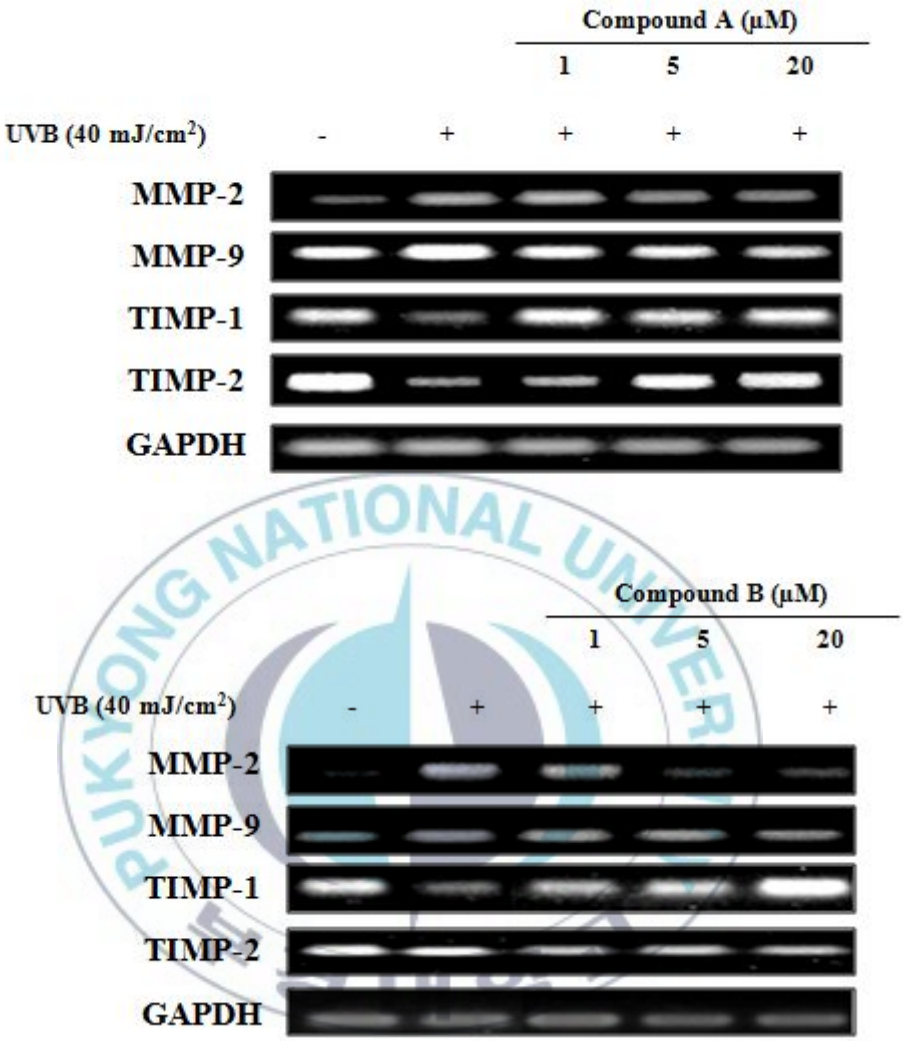


Figure 17. Effect of compounds A and B on mRNA expression levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 in human keratinocytes stimulated with 40 mJ/cm^2 of UVB. Cells were exposed by UVB irradiation and samples were incubated for 24 h. The expression levels of these genes were detected using RT-PCR. GAPDH was used as an internal standard.

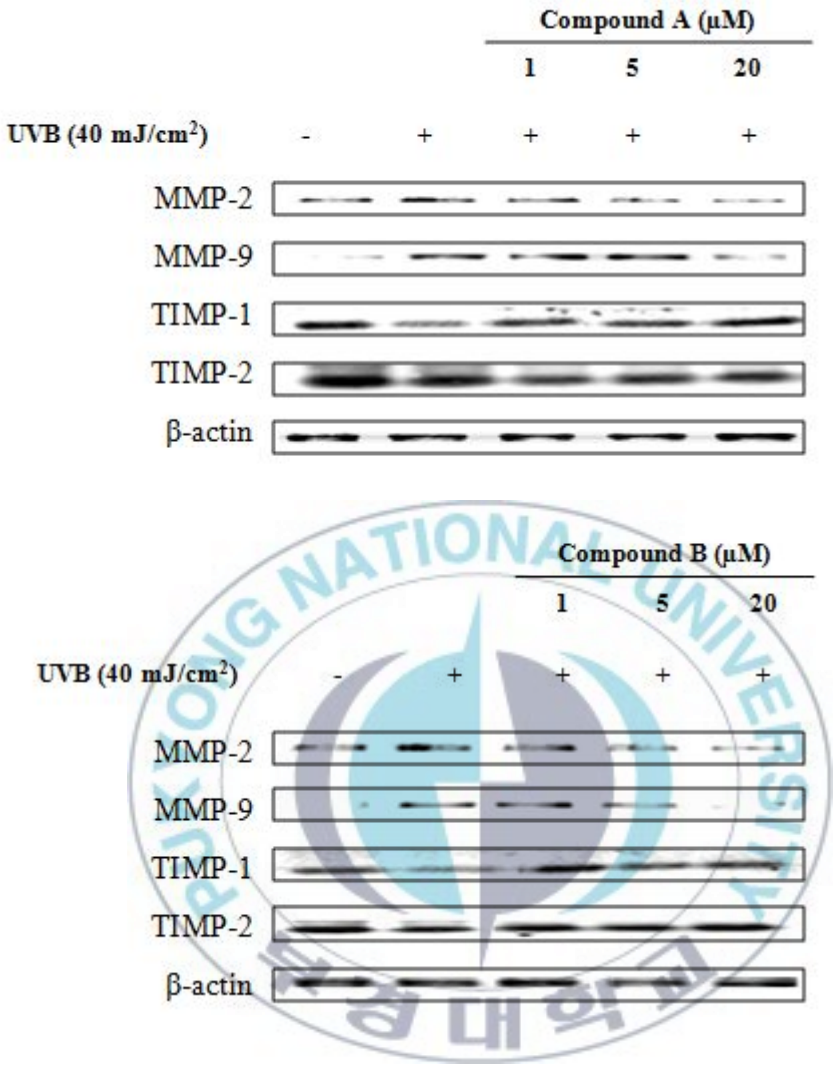


Figure 18. Effect of compounds **A** and **B** on protein expressions of MMP-2, MMP-9, TIMP-1 and TIMP-2 in human keratinocytes stimulated with 40 mJ/cm^2 of UVB. Cells were exposed by UVB irradiation and samples were incubated for 24 h. The protein expression levels were detected using Western blot analysis. β-actin was used as an internal standard.

3.5. Inhibitory effect of compounds A and B on ASK1-p38-MAPK activation

As an attempt to elucidate the signaling cascades responsible for protective effect of compounds **A** and **B** on UVB-exposed cells, ASK1- mitogen-activated protein kinases (MAPK) signaling pathways were studied (Figures 19 and 20). Effects of compound **A** and **B** on the regulations of three major classes of MAPKs, c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK1/2) and p38 MAPK, was investigated in UVB-exposed cells. Although exposure of the cells to UVB irradiation increased the expression of phosphorylated JNK, p38 MAPK and ERK1/2 proteins, the presence of compound **B** effectively decreased regulations. Apoptosis signal regulating kinase-1 (ASK-1) is a critical mediator of acute photodamage, involving oxidative stress. Therefore, we examined the effect of compounds **A** and **B** on the UVB-induced activation of ASK-1, which is required for the sustained activation of JNK and p38 MAPK signaling cascades in response to oxidative stress. The activated nuclear transcription factor, c-fos, due to UVB irradiation was significantly attenuated by compounds A and B treatment (Figure 21). In addition, the phosphorylation of the other transcription factor phosphorylation of p53 and Bax expression were suppressed by treatment with compounds **A** and **B** (Figure 22). Especially, phosporylation of p53expression was decreased by compound **B** in a dose-dependent manner.

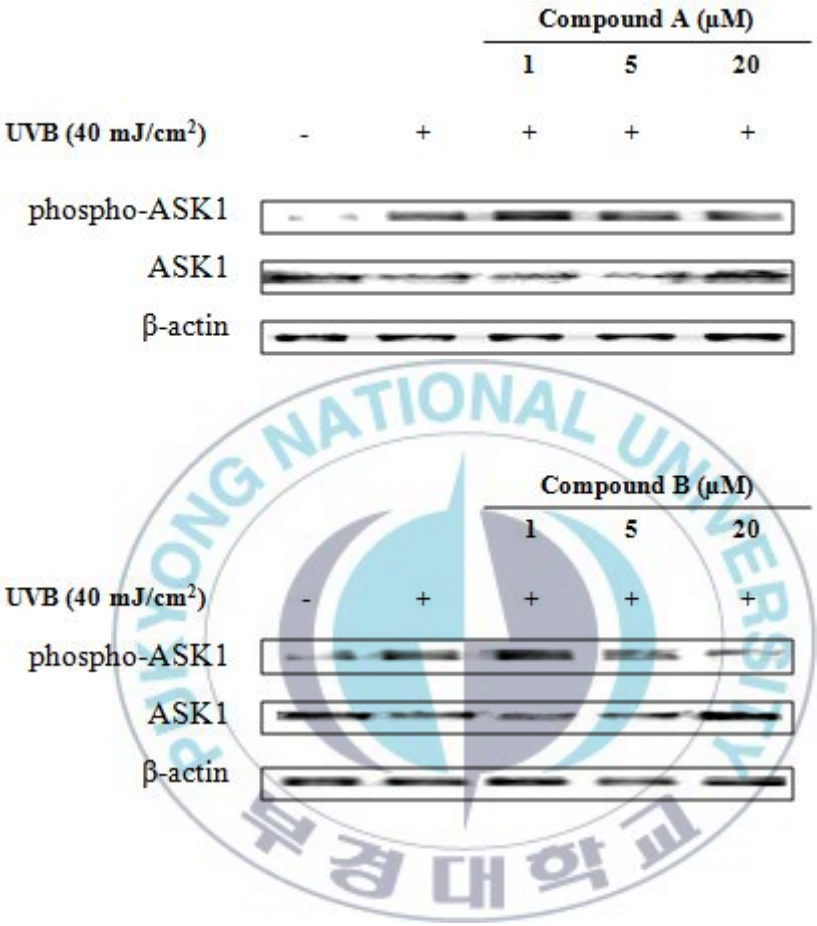


Figure 19. Effect of compounds A and B on phosphorylation of ASK-1 in human keratinocytes stimulated with 40 mJ/cm^2 of UVB. Cells were exposed by UVB irradiation and samples were incubated for 24 h. The protein expression levels were detected using Western blot analysis. β-actin was used as an internal standard.

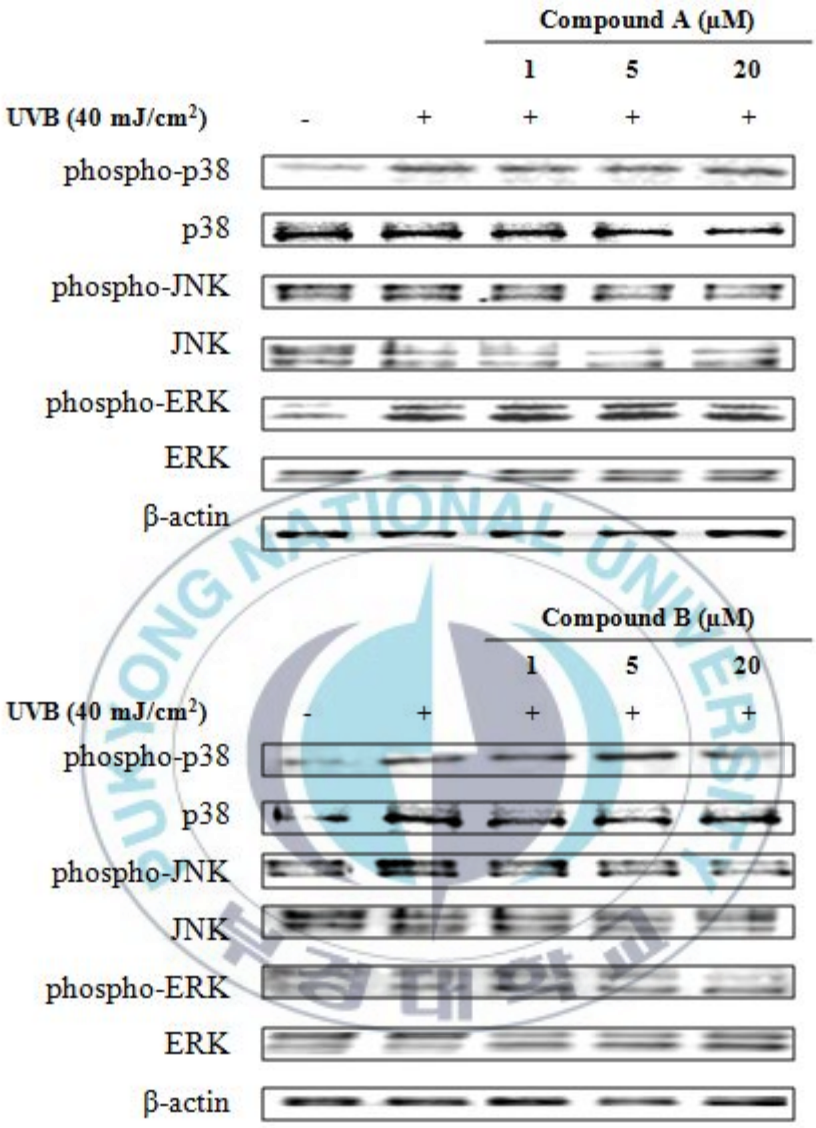


Figure 20. Effect of compounds **A** and **B** on phosphorylation of JNK and ERK in UVB stimulated HaCaT cells. Cells were exposed by UVB irradiation and samples were incubated for 24 h. The protein expression levels were detected using Western blot analysis. β-actin was used as an internal standard.

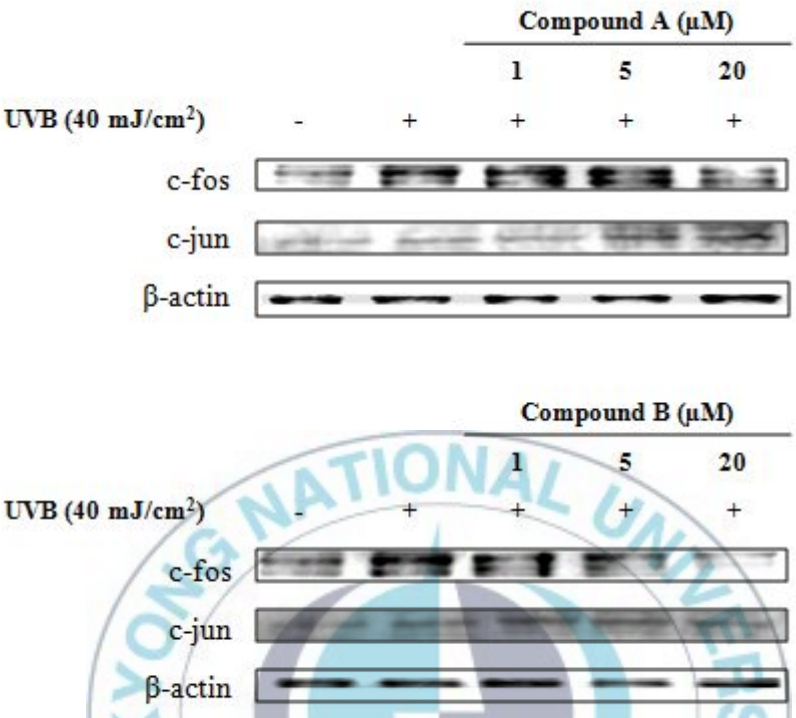


Figure 21. Effect of compounds **A** and **B** on phosphorylation of c-fos and c-jun in human keratinocytes stimulated with 40 mJ/cm^2 of UVB. Cells were exposed by UVB irradiation and samples were incubated for 24 h. The protein expression levels were detected using Western blot analysis. β-actin was used as an internal standard.

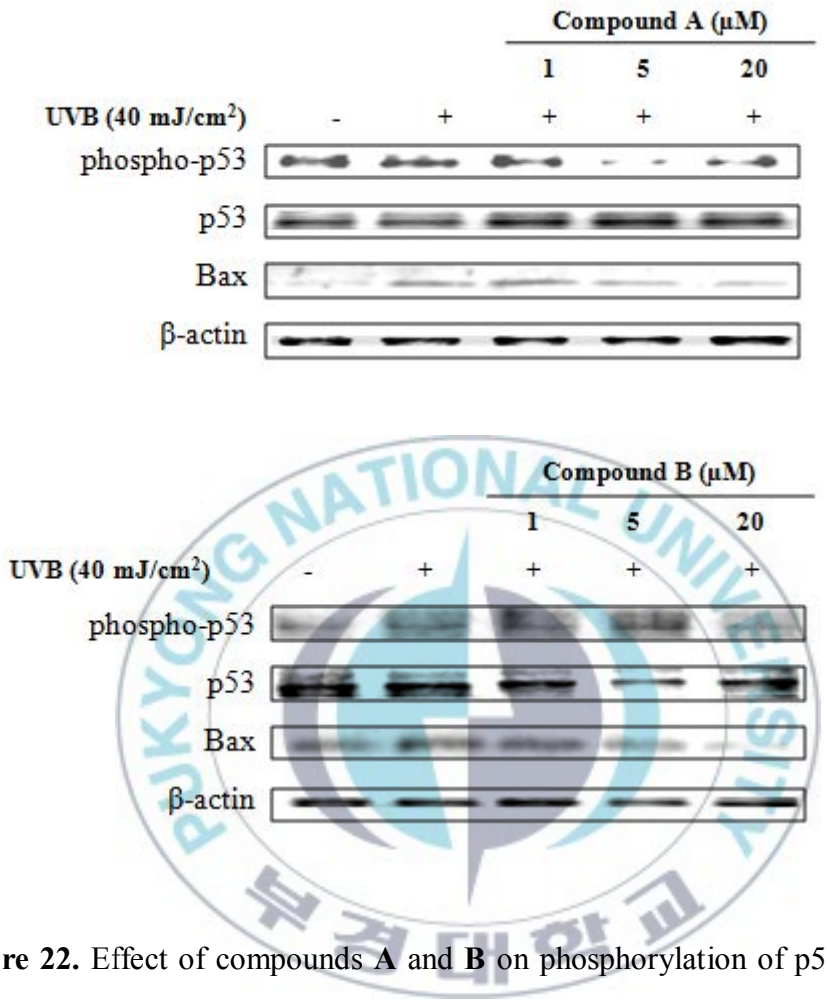


Figure 22. Effect of compounds **A** and **B** on phosphorylation of p53 and Bax in human keratinocytes stimulated with 40 mJ/cm^2 of UVB. Cells were exposed by UVB irradiation and samples were incubated for 24 h. The protein expression levels were detected using Western blot analysis. β-actin was used as an internal standard.

4. Discussion

Photodamaged skin is characterized by increase in MMP expression and decreases in collagen synthesis and abnormal elastic fibers in dermis (Shin and Cherng, 2008). In the present study, inhibitory effect of two coumarins, columbianetin **(A)** and libanoridin **(B)** with different concentration on cellular damage due to UVB-induced oxidative stress was investigated by measuring cell viability, LDH release, ROS generation, and MMPs and TIMPs expression in human keratinocytes. In MTT assay, cell viability was significantly decreased at UVB irradiation energy in a dose dependent manner (Figure 9). LDH assay was performed as another indicator of cell death. Cytosolic enzyme LDH is normally present in culture media or extra cellular fluid upon cell lysis. LDH release in UVB-exposed cells was significantly increased after 24 h at the UVB irradiation energy upto 40 mJ/cm^2 (Figure 10). According to the data obtained, UVB irradiation energy of 40 mJ/cm^2 was identified as the optimum irradiation dose. Therefore, further experiments were carried out at UVB irradiation energy of 40 mJ/cm^2. As shown in Figures 11 and 12, UVB-induced cell toxicity was attenuated by treatment with compounds **A** and **B**. Cell damage by UVB exposure was more effectively reduced in compound **B** (Figure 14).

UVB exposure promotes the intracellular ROS production in skin, which impairs the cellular defense against oxidative stress and causes various cellular changes such as DNA damage and altered intracellular signaling (Sander et al., 2004; Kundu et al., 2009). Therefore, ROS production is believed to act as a key

mediator of cell death due to UVB exposure. Although two coumarins are recognized to have defensive or protective functions against oxidative stress, there are no reports on the protective effect of coumarins against UVB-induced photodamage. In this study, it was found that UVB-induced ROS generation and cell damage via DNA fragmentation assay were suppressed by treatment with compounds **A** and **B**. Among the two types of coumarins, libanoridin (compound **B**) exhibited more inhibitory effect on UVB-stressed keratinocytes. Compound **A** has a hydroxyl group and compound **B** has an ester group. According to the before reports, compounds that have a hydroxyl group would be less reactive when compared to the compounds that have an ester group.

UVB-induced ROS production and ROS-triggered DNA damage are known to play an important role in the UVB initiated signal transduction pathway resulting in MMP induction, which induce connective tissue breakdown (Bae et al., 2008). Two gelatinases, MMP-2 and MMP-9 (type IV collagenase) are UV inducible MMP. Especially, MMP-9 in skin is thought to not only play an important role in the final degradation of fibrillar collagens after initial cleavage by collagenase, but also apoptotic events (Anggakusuma et al., 2010; Onoue et al., 2003).

In addition, this inhibitory effect of two coumarins against UVB irradiation-induced photodamage was confirmed by its capability to block MMP-2 and MMP-9 expression. UV-exposure affects not only the MMP-2 and MMP-9 expression but also tissue inhibitor of metalloproteinase TIMPs (Steinbrenner et

al., 2003). Particularly, TIMP-1 and TIMP-2 are the major endogenous inhibitors as enzymes in regulation of MMP-9 and MMP-2 (Kong et al., 2010). Elevated MMP expression was attenuated and expression level of TIMPs enhanced in presence of coumarins.

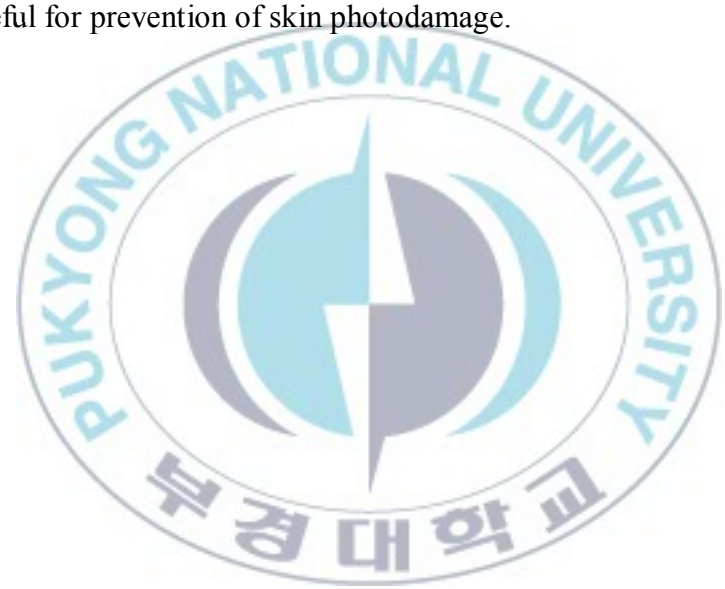
UVB irradiation activated ASK1 mediated signaling pathways such as MAPK. These activated kinases up-regulate expression and functional activation of the ASK-1, and thereby induces the MAPK pathways in response to various stresses including oxidative stress.

In addition, treatment of UVB irradiated cells with two coumarins lead to decreased DNA damage by inhibiting p53 and Bax activation. AP-1 consists of Jun and fos family proteins which bind to a common DNA site, the AP-1 site (Jibiki et al., 2002). The results revealed that the presence of compounds **A** and **B** in UVB-exposed human keratinocytes attenuated regulation/activation of ASK1-JNK and p38 MAPK signaling pathway and c-jun, c-fos and p53.

If the DNA damage caused by UV irradiation is too severe and cannot be repaired, apoptotic pathways are activated to eliminate damaged cells. p53 also plays a leading role in the apoptotic pathways. As a transcativator of transcription, p53 can induce apoptosis by upregulating the expression of apoptosis promoting gene such as Bax, or by downregulating the expression of apoptosis-suppressing genes such as Bcl-2. Upon UVB irradiation of HaCaT cells, the phosphorylated p53 and Bax protein expression were dramatically increased, however, especially compound **B** attenuate both phospho-p53 and Bax expression in a dose-dependent

manner.

In summary, the present study reveals that compound **B** exert the highest protective effect on MMP-2 and MMP-9 production when exposed to UVB irradiation. Down-regulation of MAPK family proteins and transcription factors are responsible for anti-photodamage effect of compound **B** on UVB-stressed HaCaT apoptosis. Therefore, these results suggest that compound **B** may be used as an effective natural source in development of cosmeceutical or pharmaceutical products useful for prevention of skin photodamage.



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Acknowledgement

2년 동안의 대학원 생활은 평생의 자산이 될 소중한 경험이었습니다. 정말 많은 분들의 도움을 받았고, 그 분들의 격려와 애정 어린 질책 속에서 한 걸음 한 걸음 더 나아갈 수 있었습니다. 제 학위논문이 나오기 까지 과학인으로서의 자세와 많은 가르침을 주신 모든 분들께 이렇게 지면으로나마 감사의 말씀을 드리고자 합니다.

먼저 부족한 저에게 기회를 주시고 강의와 연구로 바쁘신 와중에서도 연구할 방향을 인도해 주셨으며 세심한 배려로 돌보아주신 김세권 지도교수님께 감사드리며, 부족한 제 논문을 넓은 안목으로 심사해 주시고 따뜻한 격려와 충고를 아끼지 않으신 심현관교수님과 박선주교수님께 진심으로 감사를 드립니다.

논문이 완성되기 까지의 배움의 여정이 힘들지 않도록 아낌없는 가르침을 주시고 도움을 주신 공창숙박사님께 감사의 말씀을 드리고 실험을 할 수 있도록 sample을 제공해 주신 서영완 교수님께 감사를 드립니다. 또한 함께 하지는 못했지만 많은 가르침을 주신, 강릉대 변희국교수님, 제주대 전유진 교수님 건국대 박표잠교수님, 전남대 제재영교수님, 조선대 정원교교수님, 동의대 김문무교수님께 감사를 드리고 곁에서 지도해 주신 이희봉박사님, 이상훈박사님, 천충길박사님, Dr.Ramjee, 박순선박사님, 윤나영박사님, 조영숙박사님께 감사드리며 실험실에서 동고동락하며 철없는 후배의 투정도 받아주시고 언제나 격려를 아끼지 않으시고 즐거운 생활을 할 수 있도록 많은 도움을 주신 류보미 선배님, 김정애선배님과 외국인 친구들, 김영상후배님께 감사의 말씀을 드리며 연구단의 김희주선생님, 장민정선생님, 박혜미선생님께 감사를 드립니다.

또, 이번에 박사학위를 받으시는 강경화선배님, 엄태길선배님, 석사학위를 받는 Zafer, Uli 에게도 감사와 함께 축하의 말씀을 전합니다.

끝으로 지금까지 곁에서 변함없는 믿음과 사랑을 주시는 어머니, 아버지와 지금 군대에서 나라를 지키고 있는 동생 태환이와 소중한 친구들, 대학원의 선배님, 후배님들 에게도 감사를 드립니다. 이외에 언급하지 못한 많은 분들의 이름을 하나하나 되새기지 못한 것에 대해 죄송하게 생각하며, 이 작은 결실의 기쁨을 나누고 싶습니다.