A Thesis

For the Degree of Master of Science in Medicine

Galangin (3,5,7-trihydroxyflavone) attenuates apoptosis induced by ultraviolet B radiation in human keratinocytes

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인간 각질형성세포에서 자외선 B 조사로 유도되는 아포토시스에 대한 Galangin (3,5,7-trihydroxyflavone)의 경감작용

지도교수 현 진 원

마두마 헤와게 수사라 루완 쿠마라

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마두마 헤와게 수사라 루완 쿠마라의 의학 석사학위 논문을 인준함

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Abstract

Background: Reactive oxygen species (ROS) is the root cause for most of the skin damages caused by ultraviolet B (UVB) radiation including skin cancer and photoaging of the dermis and epidermis. Phytochemicals are known to act as antioxidants against UVB-induced oxidative stress. Galangin (3,5,7-trihydroxyflavone) is a flavonol abundantly found in plants such as *Alpinia officinarum* and *Helichrysum aureonitens* which has been proven for antiviral and anticancer properties. This study was focused to investigate the cytoprotective effects of the flavonol galangin against UVB-induced oxidative damage and subsequent induction of apoptosis in human keratinocytes.

Methods: Human skin keratinocytes (HaCaT) were cultured in RPMI 1640 containing 10% FBS. Cell viability, ability of galangin to scavenge ROS were analyzed. Protective nature of galangin against UVB induced cellular macromolecule damage was evaluated via detecting DNA strand breaks, lipid peroxidation and protein carbonylation. Formation of apoptotic bodies, mitochondrial membrane depolarization and expression of pro-apoptotic as well as anti-apoptotic proteins were assessed to determine the degree of apoptosis.

Results: Galangin efficiently scavenged free radicals and reduced UVB-induced damage to cellular macromolecules, such as DNA, lipids, and proteins. Furthermore, galangin rescued cells undergoing apoptosis induced by UVB radiation via recovering mitochondrial polarization and down-regulating pro-apoptotic proteins and up-regulating anti-apoptotic proteins.

Conclusion: These findings proved that galangin shields human keratinocytes against UVB radiation-induced oxidative stress and apoptosis via its ROS scavenging effects.

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1. Introduction

Solar radiation can be classified into three main types based on the wavelength which are ultraviolet (UV), visible light, and infrared (Lyons and O'Brien, 2002). Of these, UV radiation is the most responsible for photoaging and skin cancer. UV radiation is subcategorized into UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm). The ozone layer reflects UVC radiation, allowing only UVA and UVB to reach the earth's surface. UVB radiation is particularly absorbed by human skin and causes erythema, burns, immune suppression, and skin cancer (Park *et al*., 2013). Though UVA contributes for the majority of UV radiation that reaches the earth's surface and can get across the skin deeper than UVB, it is less carcinogenic and results aging and wrinkling of the skin (Yoshikawa *et al*., 1990; Donawho *et al*., 1996; Matsumura and Ananthaswamy, 2004).

UVB directly or indirectly damages skin cells via formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone(6-4) photoproducts or via generation of reactive oxygen species (ROS), such as hydroxyl radicals (•OH), superoxide anions ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Cunningham *et al*., 1985; Hattori *et al*., 1996; Meeran *et al*., 2008). UVB-exposure generates ROS by activating specific small molecules such as riboflavin, tryptophan, and porphyrin in the cells (Ikehata and Ono, 2011). The antioxidant defense system in cells equilibrates ROS production; however, when the ROS levels are raised, this antioxidant defense system is overwhelmed, resulting in oxidative stress. Uncontrolled release of ROS causes single- and double-strand DNA breaks and DNA-protein cross-linking (Caldwell *et al*., 2007). ROS also attack important cellular structural and functional molecules, such as proteins and lipids, causing the malfunction of cellular activities, finally leading to apoptosis, a process of programmed cell death (Tsoyi *et al*., 2008; Dhumrongvaraporn and Chanvorachote, 2013).

With the frequent occurrence of skin cancers and other damaging effects of UVB exposure, the protection of skin from UVB-induced oxidative damage has become a main consideration in the pharmaceutical industry. Phytochemicals are well-known for their defensive effects against oxidative stress in the skin (Sumiyoshi and Kimura, 2009). Galangin (3,5,7-trihydroxyflavone, IUPAC name: 3,5,7-

trihydroxy-2-phenylchromen-4-one, Fig. 1) is a type of flavonoid that is commonly found in *Alpinia officinarum* and *Helichrysum aureonitens* (Afolayan and Meyer, 1997; Ciolino and Yeh, 1999). Galangin has antibacterial (Cushnie and Lamb, 2005; 2006) and antiviral (Afolayan *et al*., 1997) properties and represses breast tumor cell growth (So *et al*., 1996; Diffey, 2004). However, the cytoprotective effects of galangin against UVB-induced oxidative damage in human keratinocytes have not been studied yet. Therefore, the objective of this study was to investigate the protective effects of galangin against UVB-induced oxidative stress in human keratinocytes.

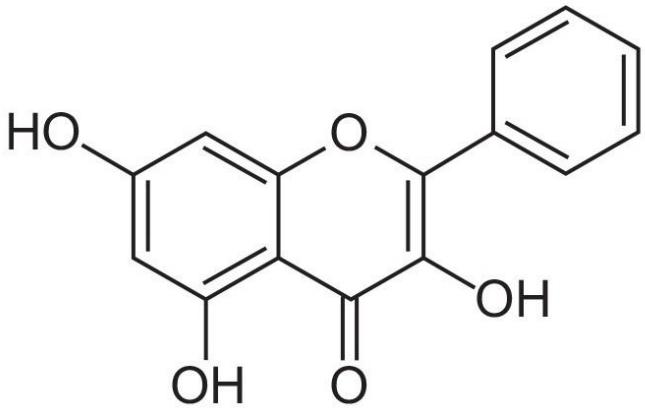

Figure 1. Chemical structure of galangin (3,5,7-trihydroxyflavone, IUPAC Name: 3,5,7-trihydroxy-2-phenylchromen-4-one).

2. Materials and Methods

2-1. Reagents

3,5,7-trihydroxyflavone (Galangin) was bought from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5-dimethyl-1-pyrroline n-oxide (DMPO), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), N-acetyl cysteine (NAC), Hoechst 33342 and primary antibody for actin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) was purchased from Invitrogen (Carlsbad, CA, USA). Primary antibodies for Bax and Bcl-2 were bought from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Primary antibodies for caspase-3 and caspase-9 were bought from Cell Signaling Technology (Danvers, MA, USA). All other chemicals and reagents were of analytical grade.

2-2. Cell culture and UVB exposure

HaCaT human keratinocyte cells were purchased from Amore Pacific Company (Yongin, Korea) and maintained in a humified atmosphere conditions of 5% CO_2 incubator at 37^{o}C. RPMI 1640 culture medium with 10% heat-inactivated fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 units/ml) was used to culture cells. Cells were exposed to UVB radiation at a dose of 30 mJ/cm^2. The CL-1000M UV Crosslinker (UVP, Upland, CA, USA) was used as the UVB source and delivered a UVB energy spectrum of 280-320 nm.

2-3. Cell viability assay

The influence of galangin on cell viability was examined using the MTT assay. Cells were seeded in a 96-well plate at a density of 1×10^5 cells/ml. After 24 h, galangin was added to a final concentration of 20, 40, 80, or 100 μM and cells were incubated for 24 h. MTT stock solution (50 μl, 2 mg/ml) was added to

each well to yield a final reaction volume of 200 μl. The supernatant was aspirated 4 h later and formazan crystals were dissolved in 150 μl of dimethyl sulfoxide (DMSO). The absorbance at 540 nm was read using a scanning multi-well spectrophotometer (Carmichael *et al*., 1987).

2-4. Detection of DPPH radicals

The ability of galangin to scavenge DPPH radicals was assessed. Various concentrations of galangin (20, 40, 80, or 100 μM) or 1mM of NAC were added into a 96-well plate. DPPH dissolved in ethanol (0.1 mM) was added to each well to yield a total volume of 200 μl. After shaking for 3 h, unreacted DPPH was detected by measuring the absorbance at 520 nm using a spectrophotometer.

2-5. Detection of hydroxyl radicals

Hydroxyl radicals generated by the Fenton reaction (H_2O_2 + $FeSO_4$) were reacted with DMPO. The resultant DMPO/•OH adducts were detected using an electron spin resonance (ESR) spectrometer (Li *et al*., 2004). The ESR spectrum was recorded 2.5 min after a phosphate buffer solution (pH 7.4) was mixed with 0.02 ml each of 0.3 M DMPO, 10 mM $FeSO_4$, 10 mM H_2O_2, and 40 μM of galangin. The ESR spectrometer parameters were set as follows: central magnetic field, 336.8 mT; power, 1.00 mW; frequency, 9.4380 GHz; modulation width, 0.2 mT; amplitude, 600; sweep width, 10 mT; sweep time, 0.5 min; time constant, 0.03 sec; and temperature, 25°C.

2-6. Detection of intracellular ROS

DCF-DA fluorescence was detected to measure intracellular ROS generated by H_2O_2 or UVB (Rosenkranz *et al*., 1992). Cells were seeded at a density of 1.5×10^5 cells/ml and incubated at 37°C for 24 h. Galangin (40 μM) or NAC (1 mM) was added to each well. After 1 h, cells were treated with H_2O_2 (1 mM) or exposed to UVB. After 30 min, H_2O_2-treated cells were treated with DCF-DA (25 μM) and incubated for another 20 min. UVB-treated cells were incubated for 24 h, treated with DCF-DA (50 μM), and incubated for a further 30 min. Fluorescence of 2',7'-dichlorofluorescein (DCF) was detected and

quantified using a PerkinElmer LS-5B spectrofluorometer (PerkinElmer, Waltham, MA, USA). Intracellular ROS scavenging effect of galangin (%) = ((absorbance of control cells - absorbance of galangin - or NAC-treated cells)/absorbance of control cells) × 100. Only H_2O_2 or UVB-treated cells were considered as controls.

2-7. Single cell gel electrophoresis (Comet assay)

DNA damage caused by oxidative stress was detected by the comet assay (Singh, 2000; Rajagopalan *et al*., 2003). Cells were seeded at a density of 1×10^5 cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm^2). The cell suspension was collected and mixed with 120 μl of 0.7% low melting agarose (LMA) at 37°C. The mixture was spread on a fully frosted microscopic slide pre-coated with 200 μl of 1% normal melting agarose. After this had solidified, a further 170 μl of LMA was applied to the slide. Slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 90 min at 4°C. Slides were then immersed in an unwinding buffer (300 mM NaOH and 10 mM Na-EDTA, pH 13) for 30 min at 4°C. Slides were subjected to electrophoresis in unwinding buffer solution with an electrical field of 300 mA and 25 V for 20 min at room temperature. The slides were washed three times with neutralizing buffer (0.4 M Tris, pH 7.5) for 10 min each time and then washed with 70% ethanol for 5 min. Slides were stained with 70 μl of ethidium bromide and observed under a fluorescence microscope using an image analyzer (Kinetic Imaging, Komet 5.5, UK). The tail length and the percentage of fluorescence in comet tails were recorded for 50 cells per slide.

2-8. 8-Isoprostane assay

Cells were seeded at a density of 1×10^5 cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm^2), and incubated at 37°C for another 24 h. Lipid peroxidation was assayed by colorimetric determination of the amount of 8-isoprostane secreted into the culture medium by HaCaT keratinocytes (Beauchamp *et al*., 2002). A commercial enzyme immunoassay

(Cayman Chemical, Ann Arbor, MI, USA) kit was used according to the manufacturer's instructions.

2-9. Protein carbonylation assay

Cells were seeded at a density of 1×10^5 cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm^2), and incubated at 37°C for another 24 h. The extent of protein carbonyl formation was determined using an OxiSelect™ Protein Carbonyl ELISA Kit from Cell Biolabs (San Diego, CA, USA) according to the manufacturer's instructions.

2-10. Nuclear staining with Hoechst 33342

Cells were seeded at a density of 1×10^5 cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm^2), and incubated at 37°C for another 24 h. The DNA-specific fluorescent dye Hoechst 33342 was added to each well and cells were incubated for 10 min at 37°C. Stained cells were visualized under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera. The degree of nuclear condensation was evaluated and apoptotic cells were counted. The ratio between apoptotic bodies and total number of cells were determined within randomly selected 0.3 mm^2 area of each well.

2-11. Analysis of mitochondrial membrane potential ($\Delta\psi_m$)

Cells were seeded in chamber slides (Nalge Nunc International) at a density of 1×10^5 cells/ml and incubated for 24 h at 37°C. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm^2), and incubated at 37°C for another 24 h. JC-1 (1 μM of final concentration) was added to each well and cells were incubated for 30 min at 37°C. Stained cells were washed with phosphate-buffered saline (PBS), coverslips were mounted onto microscopic slides in mounting medium (DAKO, Carpinteria, CA, USA), and slides were examined using a confocal microscope. Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) (Cossarizza *et al*., 1993). In addition, mitochondrial membrane potential was analyzed by flow cytometry (Becton Dickinson,

Mountain View, CA, USA). Cells were harvested, washed, suspended in PBS containing JC-1 (1 μM of final concentration), incubated for 30 min at 37°C, and analyzed using a flow cytometer (Troiano *et al*., 2007).

2-12. Western blot analysis

Harvested cells were lysed by incubation on ice for 30 min in 150 μl of lysis buffer (iNtRON Biotechnology, Republic of Korea). The resultant cell lysates were centrifuged at 13,000 rpm for 5 min. Supernatants were collected and protein concentrations were determined. Aliquots were boiled for 5 min and electrophoresed on 12% SDS-polyacrylamide gels. Protein blots of the gels were transferred onto nitrocellulose membranes. The membranes were incubated with the appropriate primary antibodies (1:1,000) followed by horseradish peroxidase conjugated anti-IgG secondary antibodies (1:5,000) (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

2-13. Statistical analysis

All measurements were performed in triplicate and all values are expressed as means ± standard error. The results were subjected to an analysis of variance using Tukey's test to analyze differences between means. In each case, a p-value of <0.05 was considered statistically significant.

3. Results

3-1. Galangin attenuates UVB-induced ROS generation

The MTT assay elucidated that galangin was not toxic to HaCaT cells at any concentration used. Following the treatment with each of the concentrations of galangin tested, cell viability was above than 96% of that of control cells (Fig. 2A). Galangin exhibited significant ability of DPPH radical-scavenging at the concentrations of 20-100 μM. A well-known antioxidant NAC (1 mM) was used as the positive control (Fig. 2B). Therefore, considering both cell viability and DPPH radical scavenging data, 40 μM was selected as the optimal concentration of galangin for further experiments. To assess the ability of galangin (40 μM) to scavenge hydroxyl radicals, ESR spectrometry was performed. In the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$), DMPO/•OH adducts generated a signal of 2,915 in control cells and this signal was reduced to 1,803 in the presence of galangin (Fig. 2C). Next, the intracellular ROS scavenging activity of galangin was assessed using the DCF-DA fluorescent probe. Galangin scavenged 27% of ROS (versus 52% for NAC) in H_2O_2-treated cells and 27% of ROS (versus 22% for NAC) in UVB-treated cells (Fig. 2D). Taken together, these results show that galangin efficiently scavenges ROS.

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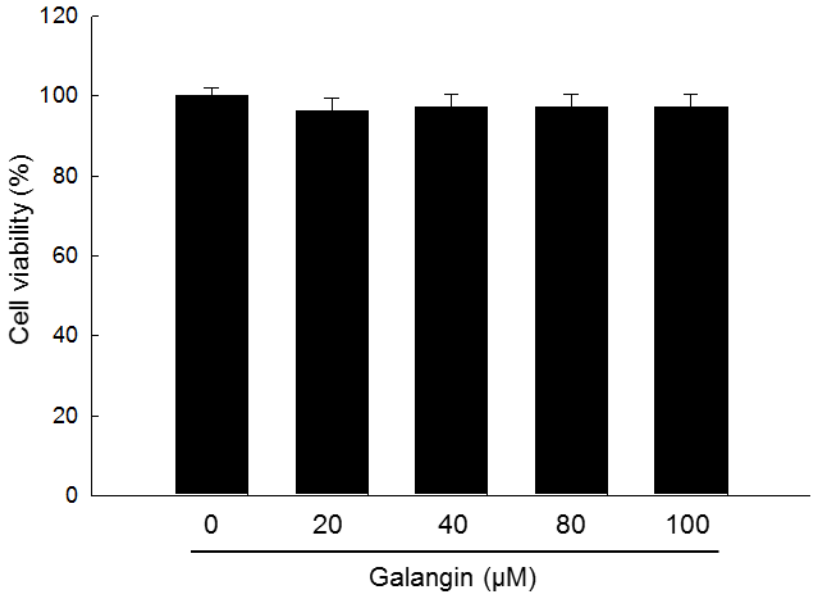

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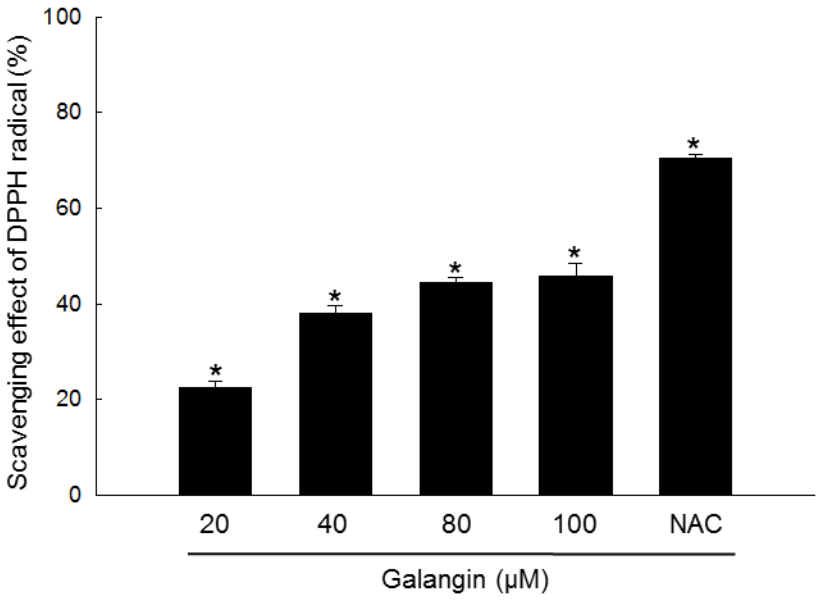

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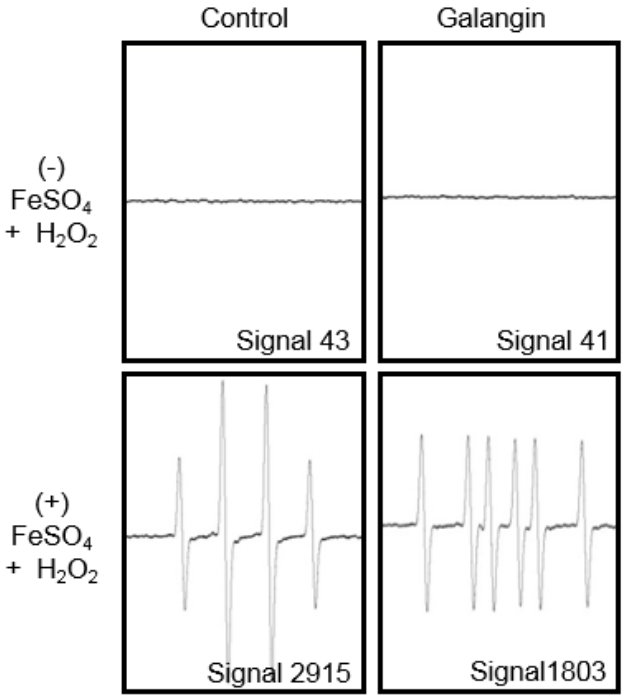

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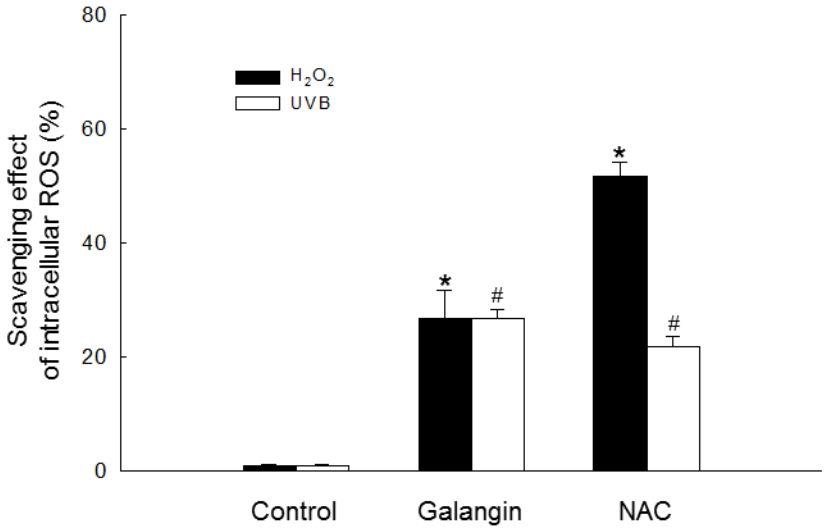

Figure 2. Galangin scavenges reactive oxygen species (ROS). (A) Human skin keratinocytes (HaCaT cells) were treated with various concentrations of galangin for 24 hours and cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (B) Degrees of the 1-diphenyl-2-picrylhydrazyl (DPPH) radical were measured spectrophotometrically at 520 nm. N-acetyl cysteine (NAC, 1 mM) was used as the positive control. *Significantly different from the control group ($p < 0.05$). (C) Hydrodroxyl radical-scavenging ability was evaluated using the Fenton reaction system. *Significantly different from the control group ($p < 0.05$). (D) The ability of galangin to clean intracellular ROS generated by H_2O_2 or UVB was determined using a spectrofluorometer followed by DCF-DA staining. $^{*,\#}$Significantly different from cells treated with H_2O_2 or UVB alone ($p < 0.05$).

3-2. Galangin significantly attenuates UVB-induced damage of cellular macromolecules

I next investigated whether galangin can protect cellular macromolecules, such as DNA, lipids, and proteins, from UVB-induced oxidative damage. UVB-induced DNA damage and the cytoprotective effects of galangin were first studied using the comet assay. Representative microscopy images indicating the length of comet tails and the percentage of fluorescence in the tails are shown in Fig. 3A. UVB treatment increased the comet tail length and increased the percentage of fluorescence in the tail to 52%, indicating an increased level of DNA damage in keratinocytes, while galangin pretreatment significantly reduced this value to 34%. UVB-induced cellular membrane damage was measured by detecting lipid peroxidation via colorimetric determination of the level of 8-isoprostane secreted into the culture medium by HaCaT cells. The amount of 8-isoprostane was 219 pg/ml in the culture medium of UVB-exposed cells and 32 pg/ml in that of cells pretreated with galangin prior to UVB exposure (Fig. 3B). Finally, protein carbonylation was measured to assess the degree of protein damage. Oxidative stress usually modifies the amino acid side chains of proteins into carbonyl derivatives, which can be used to quantify protein damage caused by UVB-induced oxidative stress (Dalle-Donne, 2006). Protein carbonylation was markedly higher in UVB-treated cells than in control cells, whereas this increase was significantly reduced in cells pretreated with galangin prior to UVB exposure (Fig. 3C). These findings confirm that galangin can protect cellular macromolecules from UVB-induced oxidative damage.

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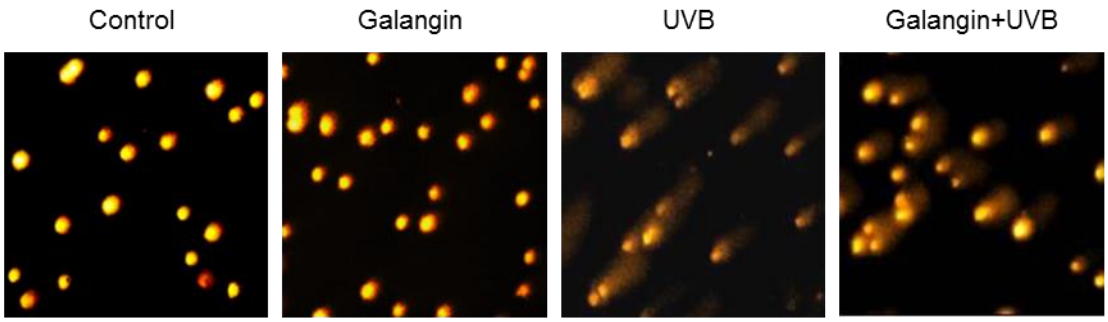

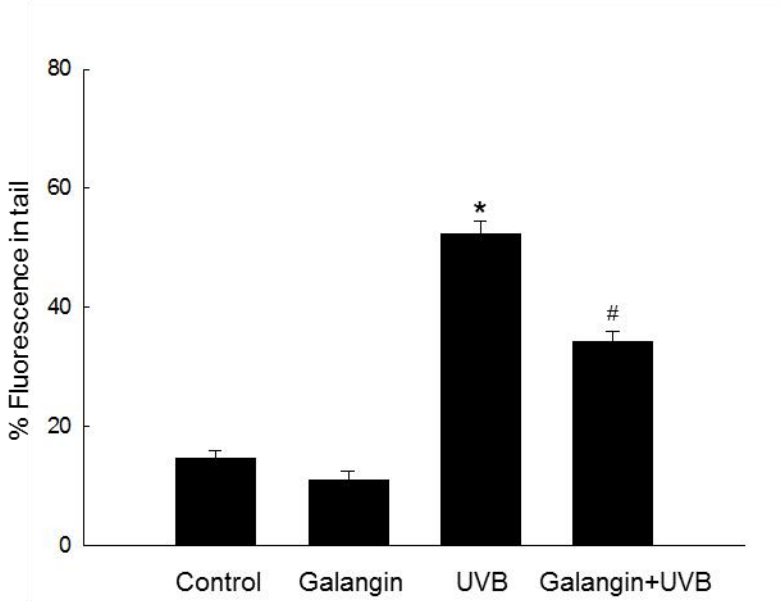

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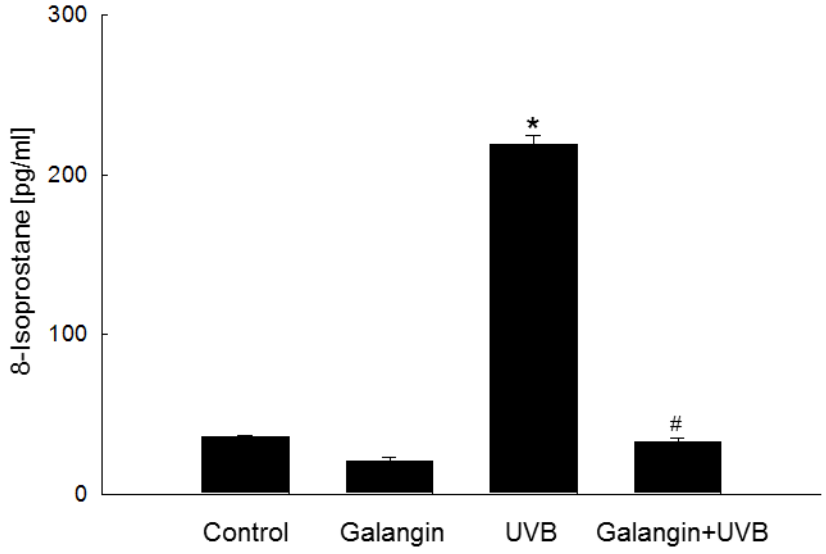

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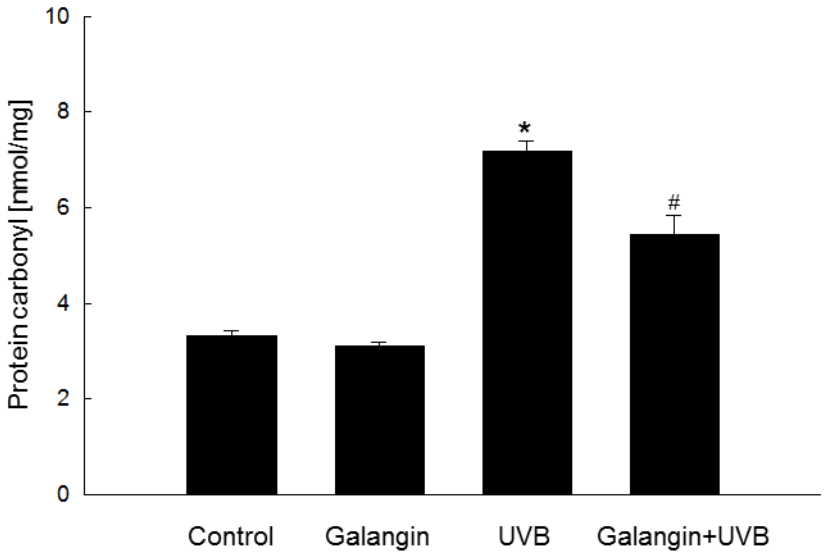

Figure 3. Galangin reduces UVB-induced cellular macromolecule damage. Cells were pretreated with 40 μM of galangin and exposed to UVB (30 mJ/cm^2) 1 hour later. After 24 hours, (A) the comet assay was accompanied to assess the DNA damage. Representative images and the percentage of cellular fluorescence within comet tails are given. (B) Lipid peroxidation was measured by assessing the concentration of 8-isoprostane in the conditioned medium. (C) Protein oxidation was assessed by measuring the amount of carbonyls formed. *Significantly different from control cells ($p < 0.05$); $^{\#}$significantly different from UVB only-exposed cells ($p<0.05$).

3-3. Galangin reduces UVB-induced apoptosis

The viability of HaCaT cells were assessed using the MTT assay (Fig. 4A). UVB exposure reduced the viability of keratinocytes to 60% in comparison to that of control cells. However, pretreatment with galangin increased cell viability to 76%, while pretreatment with the well-known antioxidant NAC increased cell viability to 88%. Next, cells were stained with Hoechst 33342 staining dye to visualize nuclear condensation and the formation of apoptotic bodies, which are characteristic of apoptosis. Normal nuclei were observed in control and galangin-treated cells, whereas notable nuclear condensation was found in UVB-exposed cells (Fig. 4B). The nuclear condensation was significantly lowered in galangin- and NAC-pretreated cells than in UVB only-exposed cells. Permeability of mitochondrial membrane plays a crucial role in the mitochondria-mediated apoptosis pathway. To elucidate the mechanism underlying how galangin protects keratinocytes against UVB-induced apoptosis, changes in the mitochondrial membrane potential were assessed. UVB treatment strongly increased the level of green fluorescence caused by JC-1 monomers, indicating mitochondrial depolarization (Fig. 4C). However, galangin treatment prior to UVB exposure significantly reduced the intensity of green fluorescence, showing that galangin attenuated UVB-induced mitochondrial depolarization. To confirm these data, flow cytometry analysis of JC-1 was accompanied. As expected, the green fluorescence peak was increased in UVB-treated cells. However, galangin pretreatment markedly deducted the peak of this fluorescence (Fig. 4D). Caspase-9, an initiator caspase, is cleaved to activate followed by the releasing cytochrome c from the depolarized mitochondria into the cytosol, cleaved caspase-9 propagates further activation of downstream apoptotic proteins which causes for cleavage of procaspase-3 to activate caspase-3 which execute the apoptotic process (Adrain and Martin, 2001). To assess the caspase-9 and -3 expression levels, a western blot analysis was performed. UVB exposure resulted up-regulation of cleaved caspase-9 and -3 in HaCaT cells, interestingly galangin pretreatment significantly down-regulated the levels of cleaved caspase-9 and -3. The vulnerability of cells towards apoptosis is ultimately determined by the pro-apoptotic and anti-apoptotic proteins in the B-cell leukaemia/lymphoma-2 (Bcl-2) family (Basu and Haldar, 1998). Bcl-2 itself is considered as an anti-

apoptosis protein while Bcl-2-associated x protein (Bax) is known as a pro-apoptotic protein in the Bcl-2 protein family (Wei *et al*., 2001) Therefore levels of Bcl-2 and Bax proteins were examined after galangin treatment (Fig. 4E). Galangin treatment prior to UVB exposure resulted up-regulation of Bcl-2 proteins whereas down-regulation of Bax proteins. These results indicate that galangin inhibits apoptotic process via establishing mitochondrial membrane polarity and regulating apoptotic proteins.

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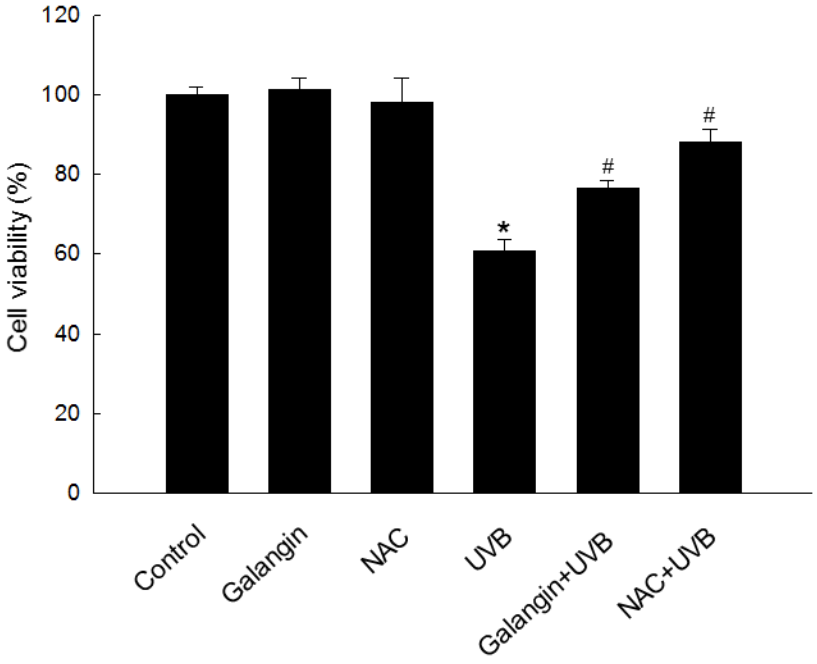

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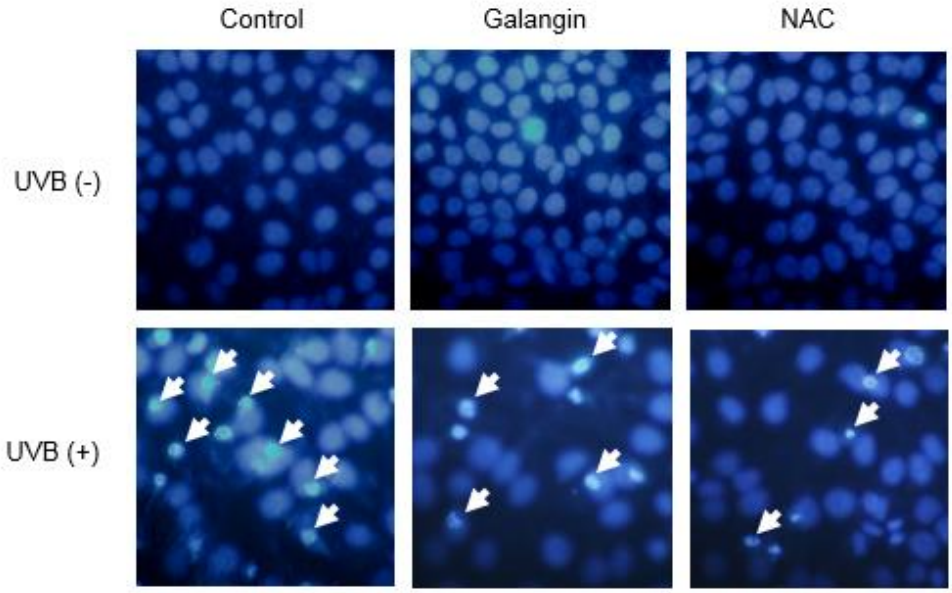

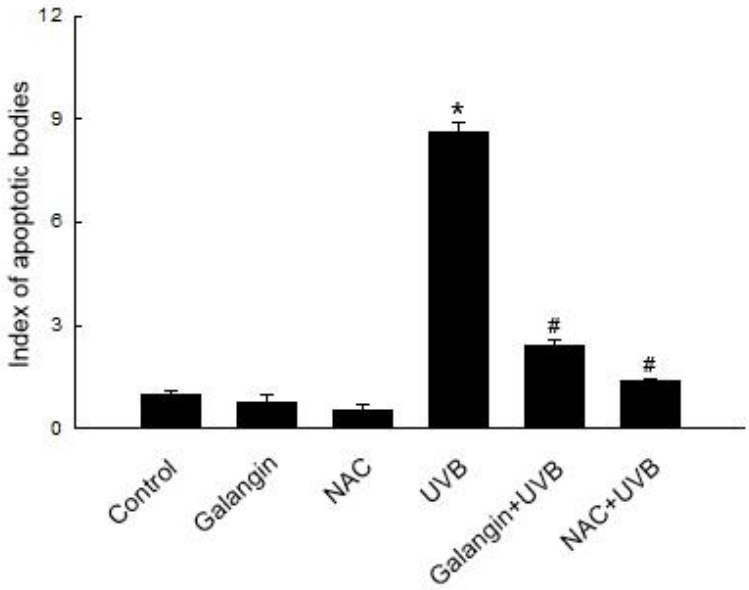

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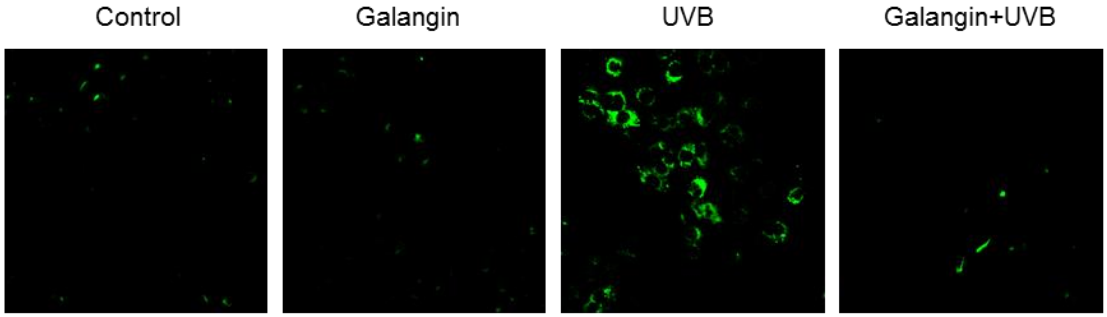

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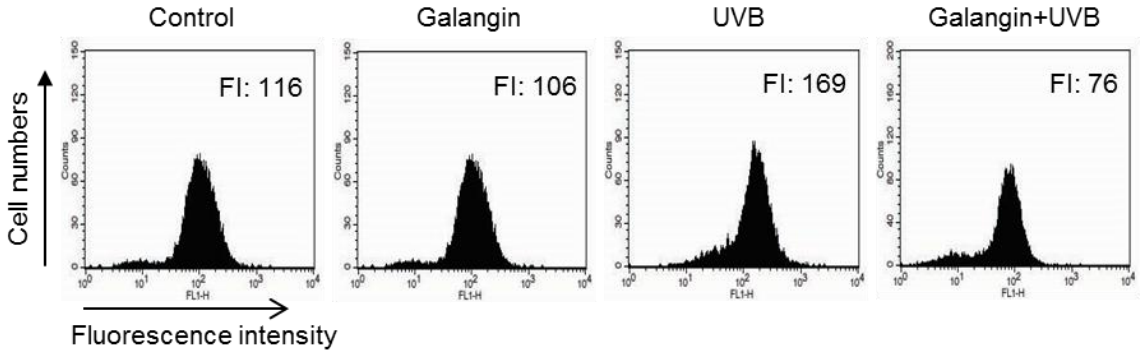

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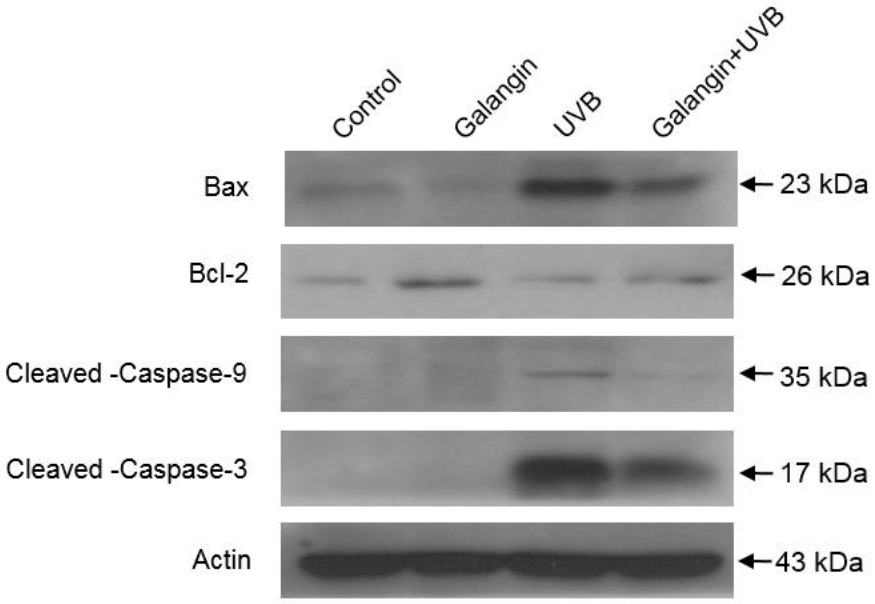

Figure 4. Galangin elevates the viability of UVB-irradiated cells and attenuates apoptosis. HaCaT cells were pretreated with 40 μM of galangin and exposed to UVB (30 mJ/cm^2) 1 hour later. (A) After 24 hours, the MTT assay was committed to assess cell death. *Significantly different from control cells ($p < 0.05$). $^{\#}$Significantly different from UVB only-exposed cells ($p < 0.05$). (B) Cells were stained with Hoechst 33342 staining dye and observed by a fluorescence microscopy. Apoptotic bodies were quantified (arrows). JC-1 staining was performed to examine the mitochondrial membrane potential via (C) confocal microscopy and (D) flow cytometry. (E) Western blot analysis was accompanied to detect the expression levels of cleaved caspase- 9, -3, Bcl-2 and Bax proteins.

4. Discussion

Chronic solar UVB exposure, also known as photoaging, is the most well understood skin aging mechanism (Nichols and Katiyar, 2010; Svobodova and Vostalova, 2010). Notwithstanding the skin possesses a complex enzymatic and non-enzymatic defensive mechanism to protect it from adverse effects, prolonged UVB exposure can overwhelm this system. Solar UVB radiation has become one of the most common carcinogens in the environment and excessive UVB exposure leads to skin cancer. Meanwhile, due to the depletion of the stratospheric ozone layer and climate change, the earth welcome elevated levels of UV radiation (De Gruijl *et al*., 2003; Diffey, 2004). UVB damages DNA directly via the formation of pyrimidine dimers and indirectly via ROS generation (Ichihashi *et al*., 2003). DNA is the prime information molecule in the cell; therefore, nuclear DNA must exist intact for the whole life time of the cell. DNA damage can critically affect cellular functions. ROS attack not only nucleic acids but also proteins and lipids, thereby interrupting cellular metabolism. In this regard, prevention of the adverse effects caused by exposure to harmful UV radiation has become a popular theme in the cosmetic and pharmaceutical industries. Phytochemicals are well-known for their abilities to protect the skin against harmful UV radiation. Galangin is a flavone whose antioxidant ability depends on the donation of hydrogen atoms to free radicals (Sim *et al*., 2007). In this study, galangin scavenged DPPH radicals and hydroxyl radicals (Fig. 2B, C). DCF-DA staining showed that intracellular ROS generated via H_2O_2 and UVB (Fig. 2D) were removed by galangin. Flavonoid is known to possess antioxidant activity by donating hydrogen to radicals, depending on the substitution pattern of hydroxyl groups. Hydroxylated position is related with stability of the resulting phenoxyl radical by hydrogen donation or electron delocalization. The presence of 3-OH and 5-OH and C2-C3 double bond conjugated with the 4-oxo function increase antioxidant activity (Rice-Evans *et al*., 1996; Jung *et al*., 2005). In general, O-dihydroxy groups known as catechol structure in B ring increases radical scavenging activity, however it is reported that absence of catechol structure in the B ring like galangin is not always related with low antioxidant activity by compensating lack of catechol structure through combination of C2-C3 double bond with the 3-OH and 4-keto groups (Amic *et al*., 2003),

demonstrating antioxidant effect as shown in the results of my study.

UVB-induced ROS cause oxidative damage to DNA, lipids, and proteins in keratinocytes. Formation of CPDs and 6-4 photoproducts are considered as the main types of lesions caused by UVB radiation in DNA, and these adducts causes DNA strand breaks and induction of mutations (You *et al*., 2000). ROS especially hydroxyl radicals abstract allylic hydrogen forming carbon centered lipid radicals which will rapidly react with oxygen to form lipid peroxy radicals, interestingly these peroxy radicals are capable of abstract hydrogens from another lipid molecules generating lipid radicals initiating chain reactions (Ayala *et al*., 2014). ROS attack proteins and cause reversible and/or irreversible modifications, such as protein carbonylation, formation of adducts with lipid peroxidation products and protein protein cross-linking (Perluigi *et al*., 2010). Formation of protein carbonyls could be either by oxidative cleavage of proteins or by direct oxidation of lysine, arginine, proline, and threonine residues (Sander *et al*., 2002). The comet assay showed that galangin significantly suppressed DNA strand breaks induced by UVB (Fig. 3A). Detection of 8-isoprostane released by cells into the extracellular environment is an accurate means of assaying lipid peroxidation. Galangin-pretreated cells showed notably lower levels of 8-isoprostane in the culture medium than UVB alone-treated cells (Fig.3B), suggesting that galangin attenuated UVB-induced lipid peroxidation in skin cells. Proteins are oxidized by ROS and formation of protein carbonyls is a hallmark of oxidative stress. These results illustrated that the level of carbonyls was significantly attenuated in cells pretreated with galangin prior to UVB exposure (Fig. 3C).

At elevated concentration of ROS overwhelms the antioxidant defense system and interrupts cellular metabolism. In response to DNA damage, cells can undergo apoptosis (Barzilai and Yamamoto, 2004). Recent studies show that UVB induced cell death is basically governed through apoptotic pathway (Ji *et al*., 2012). In addition to DNA damage and cell cycle arrest by UVB induced oxidative stress, following the membrane damage via lipid peroxidation and protein carbonylation, ROS weaken the inner cellular structures especially mitochondrial membranes which causes to release cytochrome c into the cytosol and activates apoptotic proteins (Kulms *et al*., 2002). Galangin pretreatment significantly increased the viability of UVB-exposed cells (Fig. 4A). Hoechst 33342 staining revealed that galangin pretreatment effectively

reduced the formation of apoptotic bodies and DNA condensation induced by UVB exposure (Fig. 4B). Furthermore, I investigated the impact of galangin pretreatment on mitochondria-mediated apoptosis via JC-1 staining. Confocal microscopy (Fig. 4C) and flow cytometry (Fig. 4D) analyses confirmed that galangin restored mitochondrial membrane polarization. These data showed that galangin reduced apoptosis of UVB-radiated cells by establishing mitochondrial polarization. Pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 regulate the release of cytochrome c into the cytosol (Atan *et al*., 1999). Caspases are synthesized in their inactive forms in the cells and activated in respond to apoptotic signals (Thornberry and Lazebnik, 1998). Released cytochrome c into the cytosol binds to apoptotic protease activating factor 1, which then activates caspase-9 by cleaving procaspase-9 (Bossy-Wetzel *et al*., 1998). Cleaved caspase-9 activates caspase-3 and which then triggers downstream caspase cascades to execute apoptosis (Soengas *et al*., 1999). Results of the study elucidated that galangin suppressed the expression of Bax, cleaved caspase-9 and -3 while increased the expression of Bcl-2 protein level (Fig. 4E).

Concluding the results of this study it is justice to confirm that galangin own antioxidant properties and it intervenes the intrinsic pathway of apoptosis through down regulating apoptotic proteins and thereby galangin shields the HaCaT cells from UVB-induced oxidative damage and ultimately from the apoptosis

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6. Abstract in Korean

배경: 활성 산소종 (ROS)은 자외선 B (UVB) 조사에 의한 피부 손상의 근본적인 원인이며 피부암 또는 표피와 진피의 광 노화가 이에 포함된다. 파이토 케미컬은 UVB로 유도되는 산화적 스트레스에 대하여 항산화제 작용을 하는 것으로 알려져 있다. Galangin은 일종의 플라보놀로서 *Alpinia officinarum, Helichrysum aureonitens*과 같은 식물에 풍부하게 함유되어 있으며 항바이러스와 항암특성을 나타낸다는 것이 이미 증명되었다. 이 연구는 인간의 각질형성세포에서 UVB로 유도되는 아포토시스에 대한 플라보놀 galangin의 세포 보호 효과를 조사하기 위해 초점을 맞추었다.

방법: 인간 피부 각질형성세포(HaCaT)는 10% 소 태아 혈청이 함유된 RPMI1640 배양액으로 배양하였다. 세포 생존율, galangin의 ROS 소거능을 분석하였고 Galangin의 보호 특성은 UVB로 유도되는 세포 내 거대분자의 손상 즉 DNA의 사슬 붕괴, 지질 과산화작용 및 단백질의 카보닐화의 측정을 통해 평가하였다. 아포토틱 소체의 형성, 미토콘드리아 막 탈분극 및 아포토시스의 촉진 또는 억제 단백질의 발현을 통해 아포토시스의 정도를 측정하였다.

결과: Galangin은 효율적으로 프리 라디칼을 소거하고 UVB로 유도되는 DNA, 지질, 단백질과 같은 세포 내 거대분자의 손상을 감소시켰다. 또한, galangin은 미토콘드리아 분극화의 복구, 아포토틱 촉진 단백질들의 하향조절과 아포토틱 억제 단백질들의 상향조절을 통하여 UVB 조사로 유도된 아포토시스를 보호하였다.

결론: 이러한 발견은 galangin이 ROS 소거작용을 통하여 UVB 조사로 유도되는 산화적 스트레스와 아포토시스에 대하여 인간 각질형성세포를 보호한다는 것을 증명하였다.

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