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Protective effects of galangin against UVB irradiation-induced photo-aging in CCD-986sk human skin fibroblasts

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Abstract

Photo-aging is caused by cumulative oxidative stress from ultraviolet B irradiation with up-regulating intracellular reactive oxygen species, 4-hydroxynonenal, and matrix metalloproteinases. MMPs are the enzyme that degrades collagens so that impair the function of the dermis. Galangin was identified by ^{1}H-NMR and ^{13}C-NMR spectroscopy and is a natural flavonol that recently known to have many pharmacological effects such as anti-viral, anti-inflammatory, anti-atopic dermatitis and anti-oxidative activities. In this study, the protective effect of galangin on UVB-induced photo-aging in human skin fibroblasts (CCD-986sk) was conducted by Western blot analysis and enzyme-linked immunosorbent assay. Activator protein 1 and nuclear factor-kappa B are the main transcription factors from activated mitogen-activated protein kinases that up-regulates MMPs. Galangin showed down-regulation of intracellular ROS, 4-HNE, and MMPs through inhibition of phosphorylation of the MAPK pathway and showed a protective effect against skin fibroblasts under oxidative stress caused by UVB irradiation. This lead to up-regulation of fibroblast growth factor 2 and type 1 pro-collagen. These findings suggest that galangin can be developed as a potential agent for functional food and cosmetics of UVB-induced skin photo-aging.

Keywords: Anti-wrinkle, CCD-986sk, Galangin, Photo-aging

Introduction

Skin aging can be caused by two types of aging factors: intrinsic aging factors and extrinsic aging factors. Intrinsic aging is a natural process of skin aging that depends on the passage of time perse. Over time, the physical function gets impaired and the production of reactive oxygen species (ROS) is increased, leading to skin aging [1]. Extrinsic aging is caused by several environmental factors such as smoking [2], pollution factors and ultraviolet (UV) irradiation. Consistently exposed UV is a major extrinsic aging factor that causes photo-aging and exhibits symptoms such as deep wrinkles, lack of moisture, reduced elasticity and skin disorders [3, 4]. As more frequent exposure of strong intensity of UV cause aged appearance and damage to the connective tissues such as elastin and collagen that are produced by fibroblasts in the dermis [5, 6].

Ultraviolet-B (UVB) irradiation is known to be responsible for the biological damage on the skin more than UVA, and cause skin photo-aging by up-regulation of two transcription factors, activator protein-1 (AP-1) and nuclear factor-kappa B (NF-kB), by producing ROS in human skin fibroblasts [4, 7]. UVB-induced ROS activate mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), p38 kinase and c-Jun N-terminal kinase (JNK). These enzymes induce the generation of transcription factor AP-1, a heterodimer consisting of c-Jun and c-Fos. Phosphorylation of MAPKs results in activation of AP-1, causing gene expression of MMP-1, MMP-3, MMP-9 and inhibition of type 1 procollagen expression [8, 9].

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NF-kB, a heterodimer composed of p65 and p50, usually in the binding state with inhibitor-kappa B (IkB) and is presented in the cytoplasm of human skin fibroblasts. UVB irradiation activates IkB kinase (IKK) to phosphorylate IkB and separate IkB and NF-kB [10, 11]. The freed-up transcription factor NF-kB thereby migrates from the cytoplasm to the nucleus [12] and expresses the MMP-1, MMP-3 genes. Therefore, transcription factor AP-1 and NF-kB up-regulates collagen breakdown by MMPs such as MMP-1, MMP-3, and MMP-9 [13].

Galangin is a natural flavonol that has pharmacological effects such as anti-viral [14], anti-inflammatory [15], anti-atopic dermatitis [16], anti-oxidative activity [17] and used as materials for skin care cosmetics [18]. However, it is not known whether galangin is effective to prevent photo-aging in human skin fibroblasts. The structure of galangin was identified by ^{1}H-NMR and ^{13}C-NMR spectroscopy and this study were carried out to examine the effect of galangin on preventing photo-aging through MAPK signaling pathways in UVB irradiated human skin fibroblasts. Therefore, CCD-986sk human skin fibroblasts were used.

Materials and methods

Chemicals

CCD-986sk human skin fibroblasts were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone Laboratories, Inc. (Logan, UT, USA), while fetal bovine serum (FBS), penicillin-streptomycin were obtained from Gibco (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) obtained from Sigma Chemical Co. (St. Louis, MO, USA) and enzyme-linked immunosorbent assay (ELISA) was performed using ROS-Glo™ H_2O_2 assays (Promega, Madison, WI, USA) and pro-Collagen type 1 C-peptide protein Elisa kit (Takara, Shiga, Japan) and human FGF basic Quantikine ELISA kit (R&D Systems., Minneapolis, MN, USA). All the antibodies used were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Identification of the purified bioactive compound

The ^{1}H-NMR (CDCl3, 600 MHZ) spectrum showed a profile of 6.234 (1H, d, J=2.1 Hz, H-6), 6.485 (1H, d, J=2.1 Hz, H-8), 7.505–7.561 (3H, m, H-3′, H-4′,5′), 8.156 (2H, dd, J=1.5 and 8.0 Hz, H-2′,6′), and the ^{13}C-NMR (CDCl3, 62.9 MHz) spectrum showed a profile of 176.18 (C-4), 164.14 (C-7), 160.49 (C-5), 156.40 (C-9), 145.64 (C-2), 137.02 (C-3), 130.92 (C-1), 129.89 (C-4), 128.48 (C-2′, C-6′), 127.51 (C-3′, C-5′), 103.16 (C-10), 98.22 (C-6), 93.53 (C-8). The purified compound, a yellow-green powder, was spread out in TLC which the color was developed into yellow color when heated with 10% H_2SO_4 sprayed. In the ^{1}H-NMR spectrum, two protons were identified to be coupling in doublet at 8.156 ppm (2H, dd, *J*=1.7,9.7 Hz, H-2′,6′). 6.485 ppm (1H, d, *J*=2.1 Hz, H-8) and 6.234 ppm (1H, d, *J*=2.1 Hz, H-6) was found to be coupling in *J*=2.1 Hz. 13 Carbon signals were detected in the ^{13}C-NMR spectrum but an overlapping peak of 128.48 ppm (C-2′-,6′) and 127.51 ppm (C-3′,5′) was detected which totals 15 carbons. With these results, purified compound from *Alpinia officinarum* Hance was identified as galangin.

Cell culture

Cells were plated in 75 cm^2 culture flasks and grown in DMEM supplemented with 10% FBS and 5% penicillin-streptomycin in a 5% CO_2 incubator at 37 °C. For the treatment, cells were cultured in fresh medium for 24 h. After overnight incubation, the cells were washed with phosphate buffered saline (PBS; Gibco, Grand Island, NY, USA) and pretreated by UVB irradiation. After UVB irradiation, PBS was removed, and the cells were incubated in serum-free medium with galangin-treated for further 48 h more.

Elastase and collagenase inhibition assay

The elastase inhibitory activity was evaluated by the method of [19] with minor modifications. Simply, 1.0 U/mL porcine pancreatic elastase and the sample or distilled water were added to the mixed solution of 0.2 M Tris-HCl buffer (pH 8.0) and 0.8 mM N-succinyl-(Ala)$_3$-ρ-nitroanilide, then the absorbance was measured at 410 nm.

The collagenase inhibition activity was proceeded by the method of [20] with minor modifications. Briefly, 0.2 mg/mL collagenase and sample or distilled water were added to the mixed solution of 0.1 M Tris-HCl buffer (pH 7.5) and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (0.3 mg/mL), then the absorbance was measured at 320 nm. The inhibition rate of both elastase and collagenase was calculated as the following formula: Inhibition (%) = 1 − (absorbance of sample/absorbance of control) × 100.

Cell viability

The toxicity of the galangin in skin fibroblasts was measured by using a MTT colorimetric assay. Briefly, 5×10^3 cells were seeded in 48-well plate and incubated for 24 h. Skin fibroblasts were subsequently exposed to UVB (20 mJ/cm^2) then incubated in the presence of galangin at a concentration of 5, 10, 20, 25, 50 µg/mL for 48 h in serum-free medium. After incubation, cell medium was replaced with MTT reagent and incubated for additional 4 h. Then, all the medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve insoluble formazan

crystals. Cell viability was then measured at an absorbance of 540 nm with a micro-plate reader.

Measurement of ROS

Skin fibroblasts were seeded in 96-well plates and after 24 h incubation, the culture medium was replaced and pretreated with UVB (20 mJ/cm^2) for 1 min. After UVB irradiation, they were treated with 5, 10, 25 μg/mL of galangin and incubated for 48 h in serum-free medium. The production of ROS was quantified using hydrogen peroxide assay kit (ROS-Glo™ H_2O_2, Promega).

Enzyme-linked immunosorbent assay (ELISA)

Skin fibroblasts were seeded in plates and cultured for 24 h. They were pretreated with UVB and 5, 10, 25 μg/mL of galangin then incubated for 48 h in serum-free medium. The production of type 1 procollagen was quantified according to manufacturer's introductions (Takara, Shiga, Japan) so do fibroblast growth factor 2 (FGF-2) (R&D Systems Inc., Minneapolis, MN, USA). Then detected by using ELISA plate reader.

Western blot analysis

Skin fibroblasts were seeded in 6-well plates and grown for 24 h, followed by galangin treatment for 48 h. Skin fibroblasts were then lysed in radio immune precipitation assay (RIPA) lysis buffer (Pierce, Rockford, IL, USA) with protease inhibitor cocktails (100X). To obtain a supernatant, the lysate was centrifuged at 12,000 rpm for 20 min at 4 °C and protein concentration was determined with a bicinochoninic acid (BCA) protein assay kit (Thermo scientific, USA). 20 μg of each protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were immediately placed in blocking buffer (5% skim milk with TBST) and blocked for 1 h. Subsequently, PVDF membranes were incubated at 4 °C with primary antibodies against IkB, phosphor-IkB, nucleus NF-kB, cytoplasm NF-kB, c-Fos, c-Jun, MMP-1, MMP-3, MMP-9, p-38, phosphor-p-38, ERK, phospho-ERK, 4-HNE, β-actin (1:1000 dilution) for 24 h. To detect primary antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000 dilution) were incubated for 2 h. Signals were detected using the Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) under a LAS 4000 image analyzer (Fuji Film Life Science, Tokyo, Japan).

Statistical analysis

All the results in this study were obtained from the average of triplicate experiment data, and the mean values and standard deviations were analyzed using one-way ANOVA in SPSS 23 program (Statistical Package for Social Science, Chicago, IL, USA). Analysis of variance Duncan's multiple range test and one-way ANOVA were used to compare the significance of differences between the samples at $P < 0.05$ level. Differences among the means were determined by a *t*-test, and values of $P < 0.05$ and $P < 0.01$ were considered statistically significant.

Results and discussion

Elastase and collagenase inhibitory activities of galangin

The elastase inhibitory effect of galangin was conducted in the concentration range of 50–200 μg/mL and was compared with ursolic acid. At 50–100 μg/mL, inhibition rates between the two concentrations were similar, all lower than that of ursolic acid. However, at 150–200 μg/mL, the inhibition rates of both concentrations exceeded the inhibition rate of ursolic acid, reaching 78–85% (Fig. 1a). For the collagenase inhibitory effect, epigallocatechin gallate (EGCG) was used as a positive control.

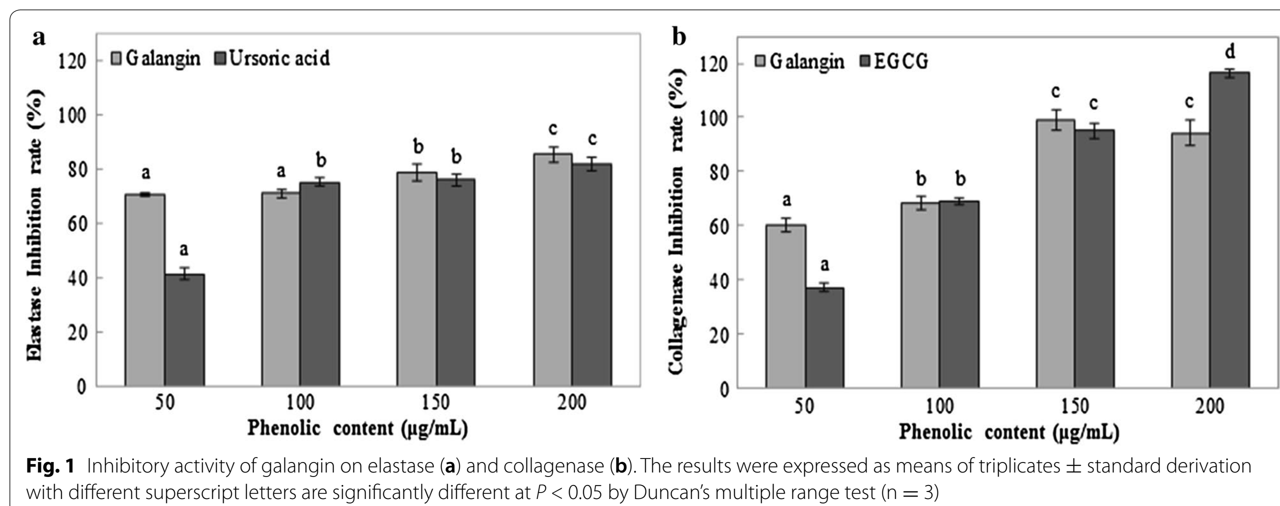


Fig. 1 Inhibitory activity of galangin on elastase (**a**) and collagenase (**b**). The results were expressed as means of triplicates ± standard derivation with different superscript letters are significantly different at $P < 0.05$ by Duncan's multiple range test (n = 3)

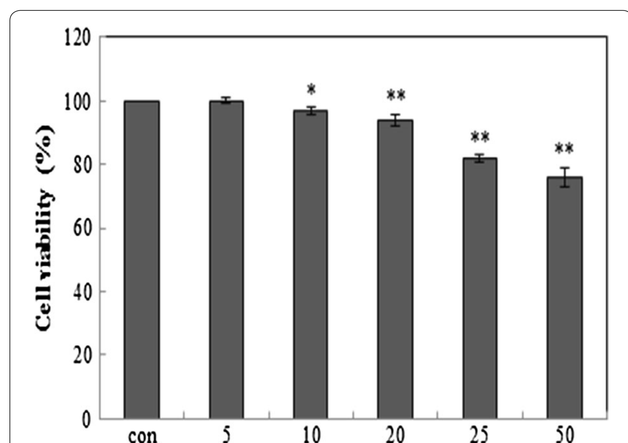


Fig. 2 Cell viability of CCD-986sk human skin fibroblasts with galangin for 72 h. The results were expressed as means of triplicates ± standard derivation. *$P < 0.05$ and **$P < 0.01$ compared with control

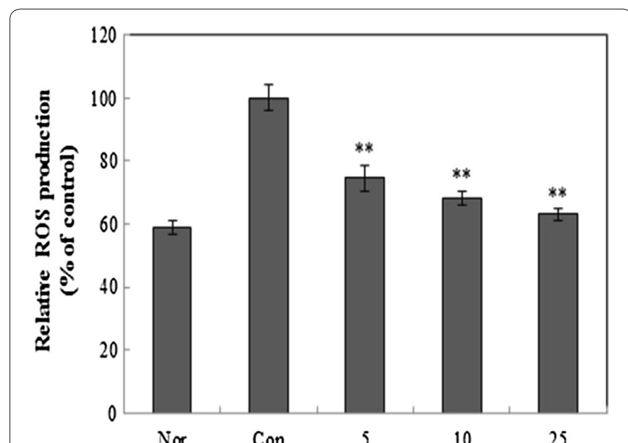


Fig. 3 ROS of CCD-986sk human skin fibroblasts after pre-treatment by UVB (20 mJ/cm^2), then treatment with a various concentration of a galangin for 72 h. The results were expressed as means of triplicates ± standard derivation. *$P < 0.05$ and **$P < 0.01$ compared with control

At 150–200 μg/mL, galangin inhibition rates was higher than 90% (Fig. 1b).

Cell viability

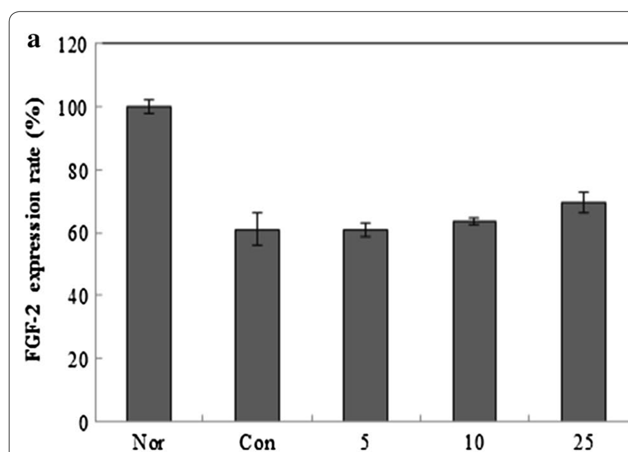
MTT assay was performed to determine the viability of 5, 10, 20, 25, and 50 μg/mL galangin-treated cells, and the concentration range of galangin to be used in the remaining experiments was set. The results showed that galangin concentration did not affect cell viability when the concentration was lower than 25 μg/mL, so the concentration of 5, 10 and 25 μg/mL galangin was used for subsequent experiments (Fig. 2).

Measurement of ROS

This experiment was conducted to evaluate the effect of reducing the generation of UVB-induced intracellular ROS by galangin. Galangin-treated cells inhibited UVB-induced intracellular ROS levels up to 74.5, 68.2, 63.2% at 5, 10, 25 μg/mL respectively, compared to that of the UVB irradiated control (Fig. 3).

Measurement of FGF-2 and type 1 procollagen

The effect of galangin on a synthesis of FGF-2 and type 1 procollagen was assessed by ELISA. UVB irradiation on skin fibroblasts reduced FGF-2 and type 1 procollagen by 60.9% and 70.9%, respectively. However, after treatment with galangin at a concentration of 5, 10 and 25 μg/mL, the expression of FGF-2 gradually increased to 60.8, 63.6 and 69.8%, respectively, and type 1 procollagen increased to 75.7, 77 and 79.1%. These results suggest that skin fibroblasts irradiated with UVB can be restored by treating galangin, although the expression of FGF-2 and type 1 procollagen is reduced (Fig. 4).



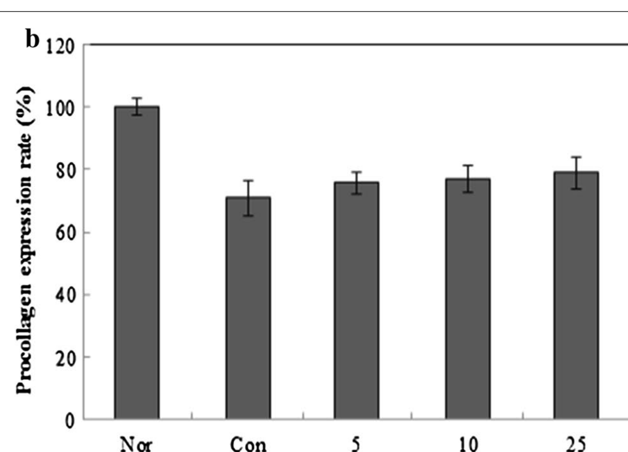


Fig. 4 Effect of galangin on FGF-2 (**a**) and type 1 procollagen (**b**) expression. CCD-986sk human skin fibroblasts were pre-treated by UVB (20 mJ/cm^2), then treated with various concentration of galangin for 72 h. The results were expressed as means of triplicates ± standard derivation. *$P < 0.05$ and **$P < 0.01$ compared with control

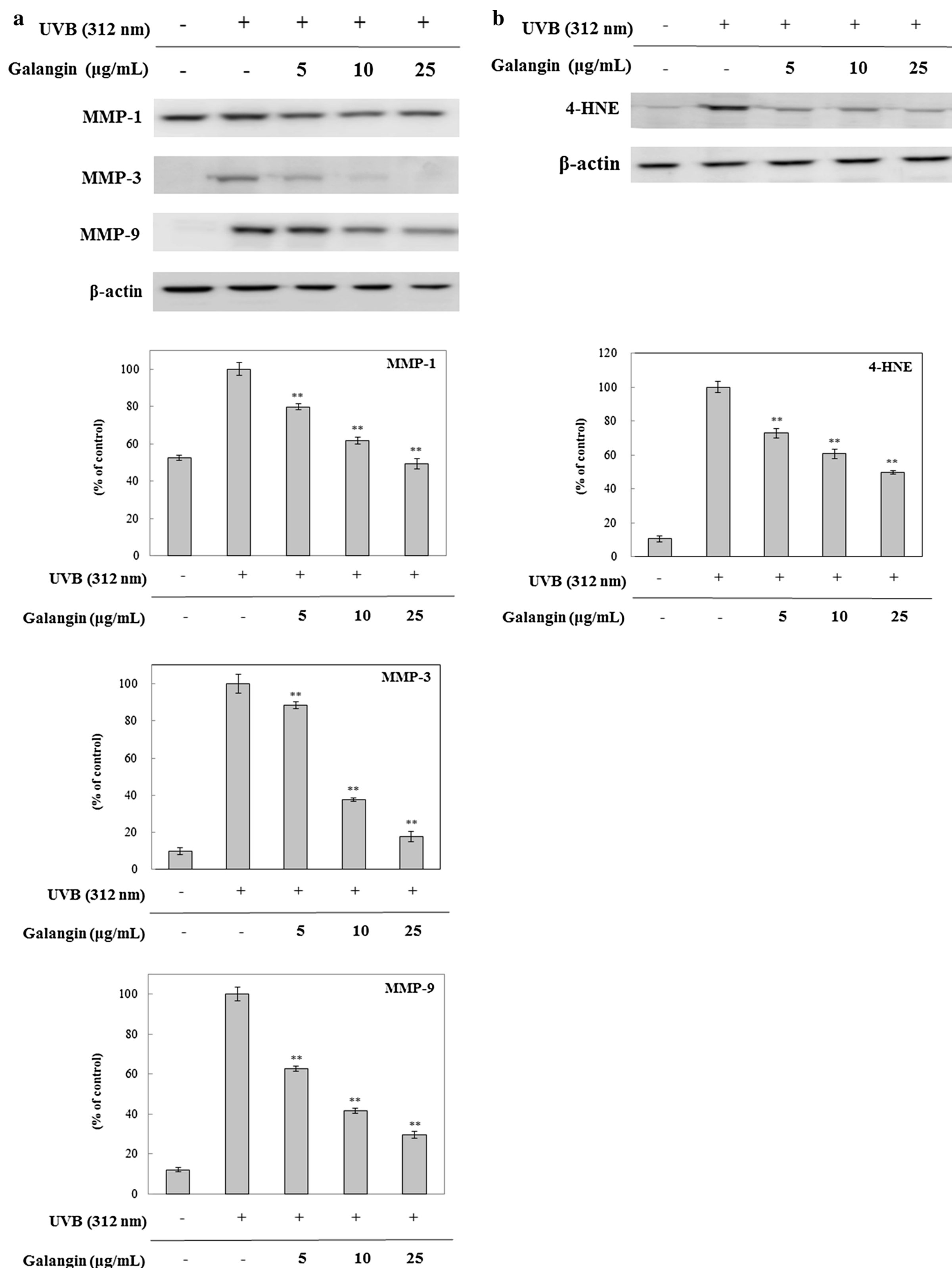


Fig. 5 Effect of galangin on modulation of MMPs (**a**) and 4-HNE (**b**) expression. CCD-986sk human skin fibroblasts were pre-treated by UVB (20 mJ/cm^2), then treated with various concentration of galangin for 72 h. The results were expressed as means of triplicates ± standard derivation. *P < 0.05 and **P < 0.01 compared with control

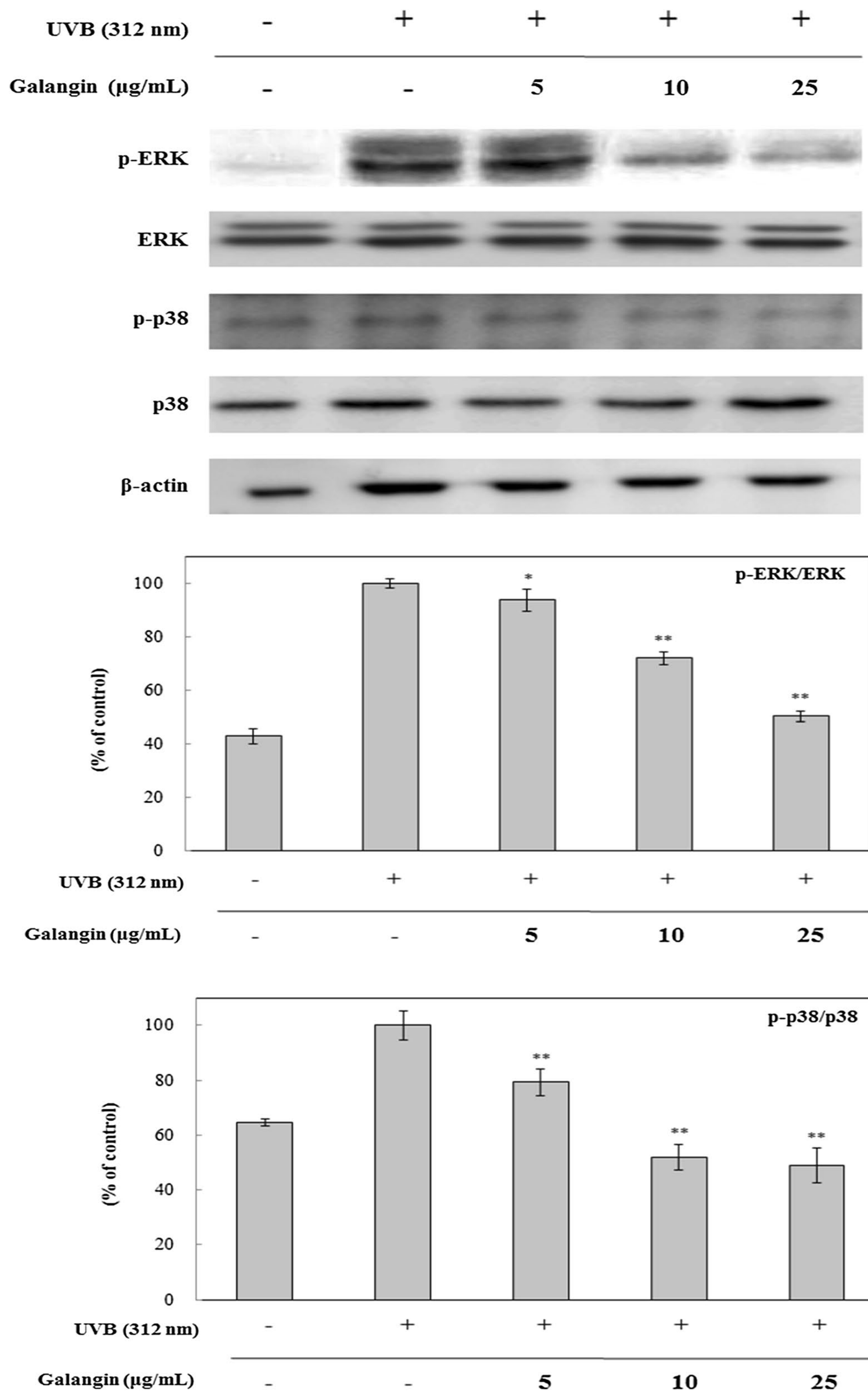


Fig. 6 Effect of galangin on MAPK signaling. CCD-986sk human skin fibroblasts were pre-treated by UVB (20 mJ/cm^2), then treated with various concentration of galangin for 72 h. The results were expressed as means of triplicates ± standard derivation. *$P < 0.05$ and **$P < 0.01$ compared with control

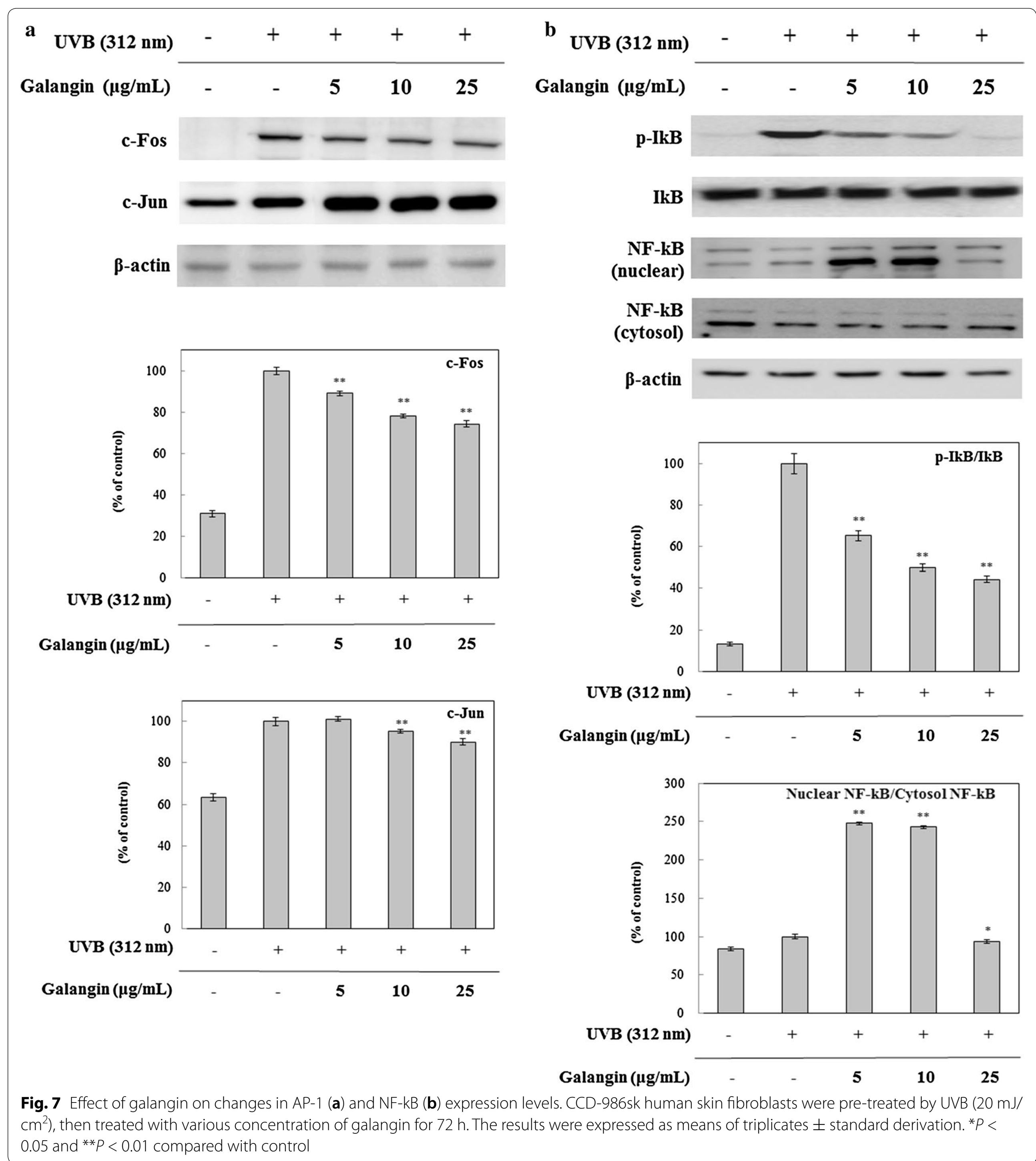


Fig. 7 Effect of galangin on changes in AP-1 (**a**) and NF-kB (**b**) expression levels. CCD-986sk human skin fibroblasts were pre-treated by UVB (20 mJ/cm^2), then treated with various concentration of galangin for 72 h. The results were expressed as means of triplicates ± standard derivation. *$P < 0.05$ and **$P < 0.01$ compared with control

Effect of galangin on MMPs and 4-HNE expression

UVB irradiation causes ROS in the skin fibroblasts, which produces lipid peroxidation products such as 4-HNE and activates transcription factors such as AP-1 and NF-kB [21]. Activated AP-1 and nuclear NF-kB upregulate the expression of MMP-1, MMP-3, and MMP-9 that degrades collagen of intracellular fibroblasts or inhibit the production of type 1 procollagen [8]. As shown in Fig. 5, UVB induced the expression of MMPs and after treatment with 5, 10, 25 μg/mL galangin, the expression of MMPs and 4-HNE was decreased in a dose-dependent manner.

Effect of galangin on MAPK signaling pathway

Activation of the MAPK signaling pathway by sustained exposure of UVB can damage the skin dermis and produce collagen-degrading enzyme, MMPs [22]. To investigate the inhibition effect of galangin on phosphorylation of MAPK proteins due to UVB irradiation, the expression level of phosphorylated ERK and p38 were measured by western blot. The results show that phosphorylation of MAPKs (ERK, p38) activated by UVB irradiation is inhibited by treatment with galangin in a dose-dependent manner (Fig. 6). When the MAPK signaling pathway is activated by UVB stimulation, the MAPK protein phosphorylates the heterodimers c-Jun and c-Fos of transcription factor AP-1 and NF-kB to upregulate MMPs [23]. First, expression levels of c-Jun and c-Fos were measured by Western blotting and showed galangin reducing the activation of c-Jun and c-Fos (Fig. 7a). Next, gene expression of related genes IkB and NF-kB were also examined by Western blotting (Fig. 7b). The results showed that galangin inhibited the phosphorylation of IkB in a dose-dependent manner, inhibiting NF-kB from migrating to the nucleus at a concentration of 25 μg/mL.

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Authors' contributions
YJK and EHL analyzed the data and wrote the paper. DHK, BOK and IKK provided technical assistance to YJK and helped with the data collection. EBC edited the manuscript. HYJ and YJC supervised the work. EHL revised and edited the manuscript and supervised the work. All authors read and approved the final manuscript.

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

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