Anti-inflammatory effect of quercetin and galangin in LPS-stimulated RAW264.7 macrophages and DNCB-induced atopic dermatitis animal models

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Received February 15, 2017; Accepted November 16, 2017

DOI: 10.3892/ijmm.2017.3296

Abstract. Flavonols are compounds that have been shown to possess potent anti-inflammatory effects in cellular and animal models of inflammation. In the present study, the anti-inflammatory effects and mechanisms of two natural flavonols, quercetin and galangin, in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages were investigated. It was identified that quercetin and galangin markedly reduced the production of nitric oxide (NO), inducible NO synthase and interleukin-6, and the nuclear translocation of nuclear factor-κB (NF-κB). In addition, LPS-induced activation of extracellular signal-regulated kinase 1/2 (Erk1/2) and c-Jun N-terminal kinase (JNK) was suppressed by quercetin and galangin. Taken together, these data implied that NF-κB, Erk1/2 and JNK may be potential molecular targets of quercetin and galangin in an LPS-induced inflammatory response. Subsequently, the effects of oral administration of quercetin or galangin, either alone or in combination, in a 2,4-dinitrochlorobenzene-induced atopic dermatitis (AD) mouse model were investigated. As a result, measurements of ear thickness and the levels of serum immunoglobulin E, and histological analysis revealed that the two flavonols led to a decrease in inflammation, whereas, in combination, they were even more effective. These results suggested that quercetin and galangin may be promising therapeutic agents for AD. Additionally, their combination may be a novel therapeutic strategy for the prevention of AD.

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Key words: hydroxy group, flavonol, anti-inflammation, nuclear factor-κB, mitogen-activated protein kinase

Introduction

The flavonoids comprise a large group of unique compounds that are widely distributed throughout the plant Kingdom. Flavonoids are widely consumed in foodstuffs, including fruits, vegetables and tea (1). Flavonoids can be divided into various classes on the basis of their molecular structure. Flavonols are a subgroup of dietary flavonoids, consisting predominantly of myricetin, quercetin, kaempferol and galangin, contai ning 3, 2, 1, and 0 hydroxy groups on the B-ring structure, respectively (2). Flavonols are the strongest antioxidants among flavonoids. 3,3',4',5,7-Pentahydroxyflavone (quercetin) (Fig. 1A), with two hydroxy groups on the B-ring, located in herbs, fruits and vegetables, has long been used as a component in certain traditional Chinese medicines as an anti-inflammatory and anticancer agent (3,4). 3,5,7-Trihy droxyflavone (galangin) (Fig. 1A), with no hydroxy group on the B-ring, exists in high concentrations in propolis and *Alpinia officinarum*, a plant that has been used as a spice and as a herbal medicine for various ailments in Asia (5).

Inflammation is a natural host defense response to invading pathogens and tissue injury involving phagocytic cells, such as macrophages, mast cells, dendritic cells and innate lymphocytes, ultimately leading to the restoration of normal cell structure and function (6). Nevertheless, the instability of immune homeostasis and a prolonged inflammatory response may result in the development of various chronic diseases, such as cancer and inflammation (7,8).

Atopic dermatitis (AD) is an inflammatory skin disease associated with severe pruritic, long-term swelling and

redness of the skin. It is characterized by the infiltration of inflammatory cells, such as eosinophils, mast cells and macrophages, into lesioned skin (9). The causes of AD have yet to be completely elucidated, but complex inflammatory immune dysregulation and the response to allergens are considered to be involved (10).

Macrophages, which are a differentiated tissue cell type originating as blood monocytes, serve a critical role in the initiation and propagation of inflammatory responses by releasing proinflammatory mediators, including nitric oxide (NO), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and prostaglandins by inducible cyclo-oxygenase (COX-2) (11).

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria, and one of the most potent initiators of inflammation. LPS activates monocytes and macrophages to produce proinflammatory cytokines (12). The mitogen-activated protein kinase (MAPK) family consists of extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) (13). MAPKs are serine-threonine kinases that mediate intracellular signaling associated with various cellular activities, including cell proliferation, differentiation, cell death and inflammation (14).

Nuclear factor-κB (NF-κB) regulates the transcription of numerous genes involved in immunity, inflammation and protection from programmed cell death (apoptosis). The activation of NF-κB is mediated by various upstream protein kinases, including MAPKs (15). p50/p65 NF-κB is bound to inhibitory inhibitor of κB (IκB) proteins in the cytoplasm (16). The cytoplasmic NF-κB/IκB complex is activated by phosphorylation; in the case of IκB-α, this modification occurs at serines 32 and 36 by the IκB kinase (IKK) complex (17). A free p50/p65 NF-κB complex translocates from the cytosol to the nucleus, and ultimately binds to the promoter region of target genes encoding various proinflammatory factors (18).

Several pharmacological studies have shown that quercetin and galangin exhibit anti-inflammatory efficacy *in vitro* and *in vivo* (19-21). However, those studies had limitations, and it is unknown whether quercetin or galangin exhibits higher activity. Therefore, the aim of the present study was to analyze the efficacy of flavonols as anti-inflammatory compounds in RAW264.7 macrophages by evaluating the generation of NO, prostaglandin E_2 (PGE_2), inducible NO synthase (iNOS), COX-2, TNF-α, and IL-6, and also to investigate the activation of NF-κB and MAPK signaling. Subsequently, the anti-inflammatory effects of quercetin, galangin, and co-administration of the two flavonols were measured by assessing ear thickness, immunoglobulin E (IgE) production, inflammation and mast cell infiltration in 2,4-dinitrochlorobenzene (DNCB)-induced AD models.

Materials and methods

Chemicals, drugs and antibodies. Quercetin and galangin (purity >95%) were both purchased from Merck KGaA (Darmstadt, Germany), dissolved in dimethyl sulfoxide (DMSO), and stored at -20˚C. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS) were all purchased from Welgene (Gyeongsan, Korea). LPS, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were purchased from Merck KGaA. The nitrate/nitrite colorimetric assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA); the mouse TNF ELISA and mouse IL-6 ELISA kits were both purchased from BD Biosciences (San Jose, CA, USA). β-actin (#4967), iNOS (#2982), COX-2 (#4842), p-NF-κB/p65 (#3033), IκB-α (#9242), ERK1/2 (#9102), phospho-ERK1/2 (#4376), p38 MAPK (#9212), phospho-p38 MAPK (#9211), JNK (#9252), phospho-JNK (#4668) and anti-rabbit horseradish peroxidase (HRP; #7074) antibodies, as well as Alexa Fluor 594-conjugated anti-rabbit immunoglobulin G (IgG) (#8889), were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture and stimulation. The RAW264.7 macrophage line was obtained from the Korean Cell Line Bank (Seoul, Korea), and maintained in DMEM supplemented with 5% FBS/1% penicillin-streptomycin at 37˚C in a 5% CO_2-humidified air environment. The cells were incubated for 24 h in medium supplemented with 10% FBS. Subsequently, the cells were pre-incubated with or without the indicated concentrations of quercetin and galangin for 2 h in serum-free media, prior to the addition of LPS (1 μg/ml).

Cell viability assay. RAW264.7 cells were seeded in a 96-well plate at a density of $1x10^5$/ml and a volume of 200 μl/well. After incubation for 24 h at 37˚C, the cells were treated with quercetin and galangin at the indicated concentrations for 24 h, followed by the addition of 5 mg/ml MTT solution to each well, and the plates were further incubated for 2 h at 37˚C. The supernatant was removed, and 200 μl DMSO was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbance at a wavelength of 595 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The percentage of viable cells compared with that in untreated control cells was estimated.

Measurement of NO. RAW264.7 cells were seeded ($1x10^5$/ml) and cultured in 96-well plates. After incubation for 24 h at 37˚C, the cells were treated with quercetin and galangin at the indicated concentrations for 2 h in serum-free medium, prior to the addition of LPS (1 μg/ml). After 24 h, the supernatants were measured for NO production using the nitrate/nitrite assay kit (Cayman Chemical Company). NO was measured as the accumulation of nitrite and nitrate reductase, which were determined spectrophotometrically using Griess reagent at an optical density of 540 nm.

Determination of TNF-α and IL-6 production. Production of the proinflammatory cytokines, TNF-α and IL-6, in the culture medium was determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences), according to the manufacturer's protocol.

Western blot analysis. The cells were preincubated with or without the indicated concentrations of quercetin and galangin for 2 h prior to exposure to LPS (1 μg/ml), and the cells were subsequently harvested at various time points. The cells were washed with phosphate-buffered saline (PBS) and treated with trypsin-EDTA. The cell pellets were obtained by centrifugation at 260 x g for 5 min at 4˚C, lysed in

lysis buffer (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and were centrifuged at 27,237 x g for 5 min at 4˚C to obtain whole-cell lysates. The protein concentrations were determined by the Bradford protein assay (Bio-Rad Laboratories, Inc.). The proteins were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Transferred membranes were blocked with Tris-buffered saline (TBS) containing 5% non-fat dried milk and 0.1% Tween®-20 at 4˚C for 2 h. After blocking, the membranes were incubated with primary antibodies (1:1,000) overnight at 4˚C with gentle shaking. After incubation with the primary antibodies, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibodies (1:1,000) for 2 h at room temperature with gentle shaking. After washing the membranes three times for 10 min in TBS containing 0.1% Tween®-20, bands were detected using enhanced chemiluminescence western blotting detection reagents (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. β-actin was used as a loading control. Band density was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence assay. RAW264.7 cells were cultured directly on glass coverslips in 6-well plates at a density of $1x10^5$/ml for 24 h to detect NF-κB/p65 localization by fluorescence microscopy. After stimulation with LPS for 15 min in the presence or absence of quercetin and galangin, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 100% methanol for 10 min at 20˚C. After several washings with PBS, the cells were blocked in PBS containing 3% BSA (Cell Signaling Technology, Inc.). The cells were subsequently incubated with the primary antibody, diluted 1:200, at 4˚C overnight. The cells were then washed with PBS, and Alexa Fluor 594-conjugated anti-rabbit IgG, diluted 1:200, was applied for 1 h to the cells. This process was performed in the dark at room temperature. After washing with PBS, the nuclei were stained with DAPI, and fluorescence was visualized using a fluorescence microscope (BX41; Olympus, Tokyo, Japan) at a magnification of x400.

Animals. BALB/c female mice (4 weeks old) were purchased from the animal production company of Orient Bio, Inc. (Gyeonggi-do, Korea) and maintained at 23±5˚C at 40±10% relative humidity with artificial lighting from 8:00 a.m. to 8:00 p.m. in facilities approved by the Companion and Laboratory Animal Science Department of Kongju National University (Chungnam, Korea). The animals were housed in cages and allowed access to sterilized water and commercial rodent chow (Biopia, Seoul, Korea) *ad libitum*. All the animal experiments were performed with the approval of the Institutional Animal Care and Use Committee, following the guidelines of Kongju National University. All experiments with mice were performed in accordance with the national guidelines, and approved by Kongju National University (approval no. KNU_2015-10; Yesan, Korea).

Induction of AD. AD was induced in BALB/c mice according to a published method, but with minor modifications (22). The schematic experimental procedure is described in Fig. 6A. Briefly, BALB/c mice were divided into seven groups (n=3/group). Group I (normal control) and group II (DNCB) were provided with vehicle orally, while group III (50 mg/kg quercetin), group IV (100 mg/kg quercetin), group V (50 mg/kg galangin), group VI (100 mg/kg galangin), and group VII (50 mg/kg quercetin + 50 mg/kg galangin) received quercetin, galangin or their combination, respectively. For the induction of AD, the surfaces of both ears of the mice were stripped with surgical tape. After stripping, 1% DNCB dissolved in acetone/olive oil solution (acetone:olive oil, 3:1) was painted on each ear. On day 0, the mice were challenged again by applying 0.3% DNCB to the ears every other day for up to 36 days. From day 7 until the completion of the experiment, the mice were treated with quercetin, galangin and quercetin + galangin via gavage every other day. The mice were sacrificed on day 37. A vernier caliper (Mitutoyo, Kawasaki, Japan) was used to measure ear thickness 24 h after the application of DNCB. On day 37, blood samples were collected from abdominal aorta, and the plasma was stored at -70˚C until further analysis. After blood collection, the ears were excised and subjected to histopathological analysis.

Measurement of immunoglobulin levels. Blood samples were obtained from each treatment group 37 days following AD induction. Total IgE levels in serum were measured using an ELISA kit, following the manufacturer's protocol.

Histological observation. The ears were immediately fixed in 10% formaldehyde, and embedded in paraffin. Blocks were then cut into 5-μm-thick slices. For the measurement of thickening of the epidermis, hematoxylin and eosin (H&E) staining was performed. For the measurement of mast cells, the skin sections were stained with toluidine blue.

Statistical analysis. The results are expressed as the mean ± standard deviation (SD). Differences between the mean values for the individual groups were assessed by one-way analysis of variance (ANOVA), with Dunnett's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of quercetin and galangin on the viability of RAW264.7 macrophages. The cytotoxic effects of quercetin and galangin were evaluated after treatment with various concentrations of quercetin and galangin for 24 h using an MTT assay. This analysis revealed that quercetin and galangin did not affect the viability of RAW264.7 macrophages at concentrations of 6.25, 12.5 and 25 μM (Fig. 1B). Hence, these concentrations of quercetin and galangin were considered suitable for further assays.

Effects of quercetin and galangin on LPS-induced NO production. To investigate the effects of quercetin and galangin on NO production in macrophages, RAW264.7 cells were pretreated with or without quercetin and galangin (0, 6.25, 12.5 and 25 μM) for 2 h prior to stimulation with 1 μg/ml LPS for 24 h. Consequently, LPS alone markedly induced NO production compared with the untreated control. However,

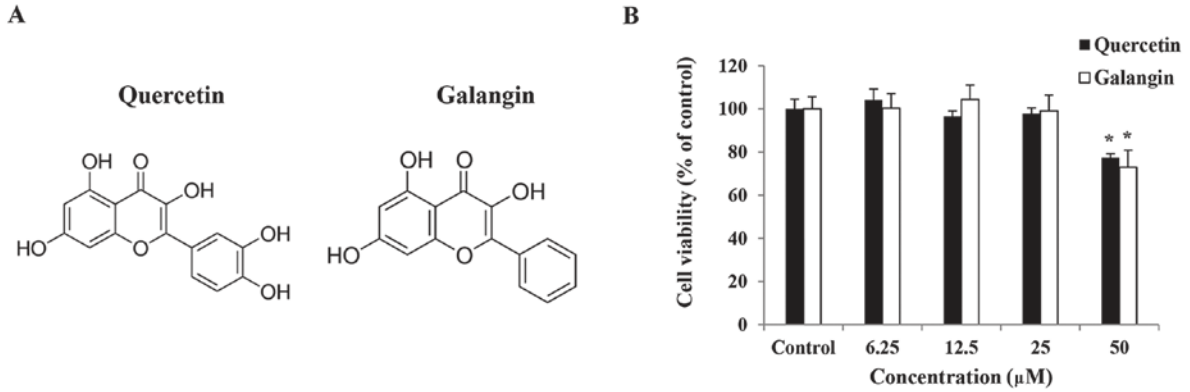

Figure 1. Effects of quercetin and galangin on cell viability in RAW264.7 cells. (A) Chemical structures of quercetin and galangin examined in the present study. (B) RAW264.7 cells ($1x10^5$ cells/ml) were treated with 6.25, 12.5, 25 and 50 μM quercetin and galangin for 24 h, and cell viability was determined by the MTT assay. Each bar represents the mean ± standard deviation (SD) calculated from three independent experiments. Significance was determined by Dunnett's t-test. *P<0.05 vs. untreated controls.

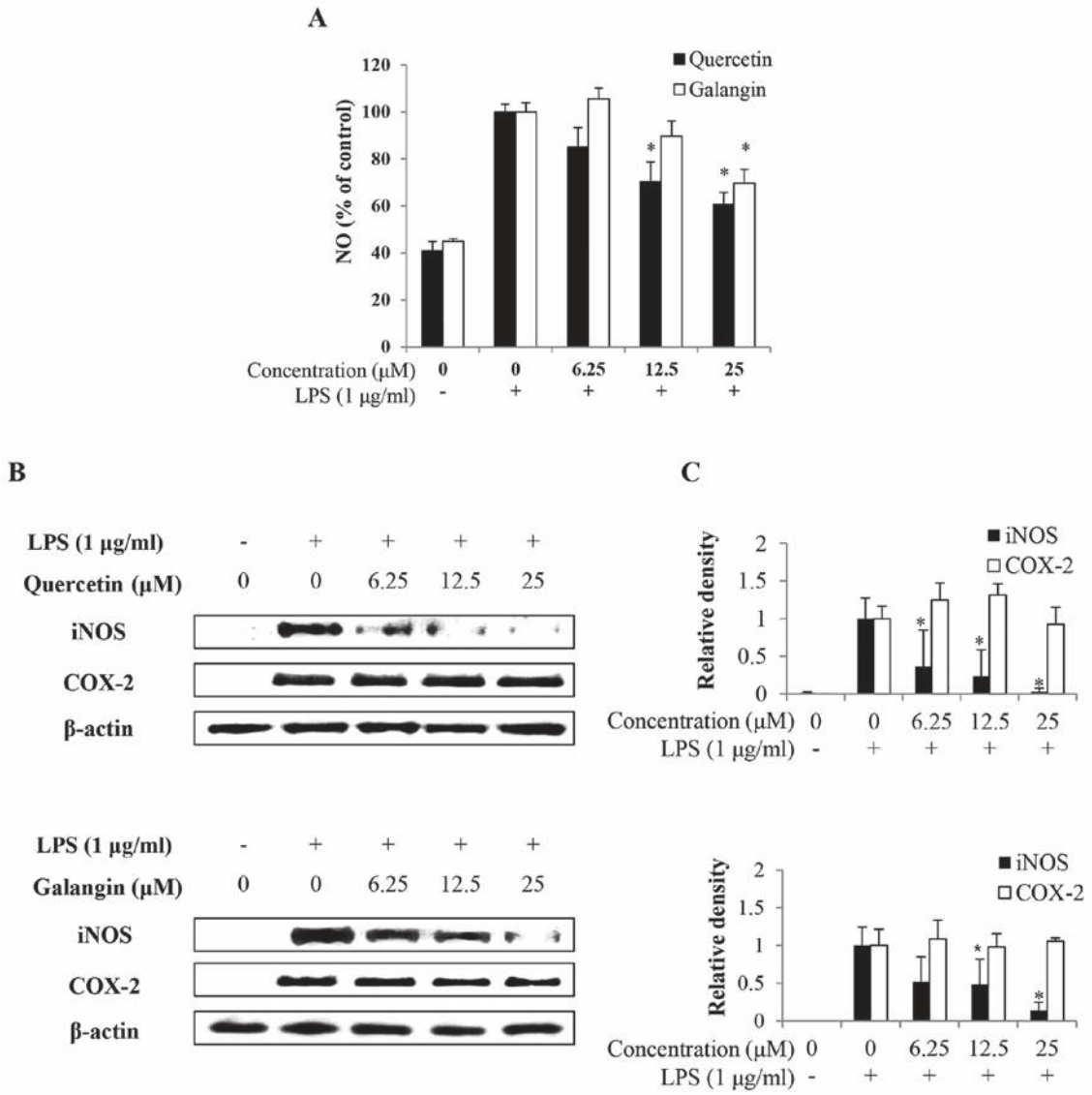

Figure 2. Effects of quercetin and galangin on the LPS-induced iNOS and COX-2 protein levels and nitrite production in RAW264.7 cells. (A) RAW264.7 cells were pre-incubated with 6.25, 12.5 and 25 μM quercetin or galangin for 2 h, and then treated with 1 μg/ml LPS for an additional 24 h. Dimethylsulfoxide was used as a vehicle. NO was measured according to the Griess reaction. (B) The cells were sampled and lysed following treatment for 24 h, and the protein levels of iNOS and COX-2 were determined by western blot analysis. Blots were also probed with the β-actin antibody to confirm equal sample loading. (C) Data analysis was performed using ImageJ software by measuring the integrated band densities following background subtraction. Each bar represents the mean ± standard deviation calculated from three independent experiments. Significance was determined by Dunnett's t-test. *P<0.05 relative to the LPS-treated group. LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclo-oxygenase-2.

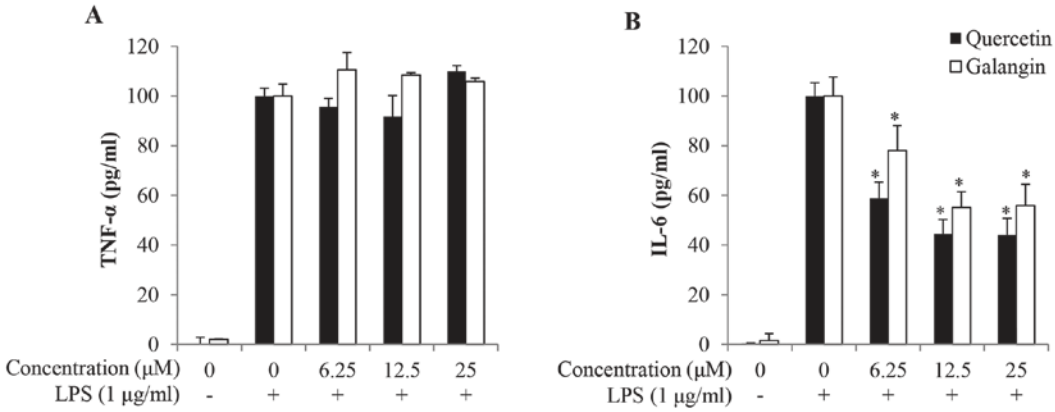

Figure 3. Effects of quercetin and galangin on LPS-induced proinflammatory cytokine production in RAW264.7 cells. RAW264.7 cells were treated with LPS (1 μg/ml) in the absence or presence of quercetin and galangin at the indicated concentration for 24 h. (A) TNF-α and (B) IL-6 in the cultured supernatant were measured by ELISA. Each bar represents the mean ± standard deviation calculated from three independent experiments. The significance was determined by Dunnett's t-test. *P<0.05 vs. the LPS-treated group. LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

pretreatment with 12.5-25 μM quercetin and 25 μM galangin significantly reduced NO production in LPS-stimulated RAW264.7 cells in a dose-dependent manner (Fig. 2A).

Effects of quercetin and galangin on LPS-induced iNOS and COX-2 protein. To confirm whether the effects of quercetin and galangin involved modulation of the expression of iNOS and COX-2, which are associated with NO and PGE_2 production, the expression levels of iNOS and COX-2 were examined by western blot analysis. After treatment with 1 μg/ml LPS, the expression levels of iNOS and COX-2 were significantly increased, whereas pretreatment with 6.25-25 μM quercetin or with 12.5-25 μM galangin markedly decreased iNOS expression, but had no effect on the expression of COX-2 (Fig. 2B and C). These results indicated that the reduced expression of iNOS at the protein level contributed to the inhibitory effect of quercetin and galangin on LPS-induced NO production.

Effects of quercetin and galangin on LPS-induced proinflammatory cytokine production. To investigate the anti-inflammatory effects of quercetin and galangin, the production of TNF-α and IL-6 in the culture supernatants of RAW264.7 cells was quantified. As shown by ELISA, the expression levels of both TNF-α and IL-6 were relatively low in untreated control cells, but were markedly increased upon exposure to LPS alone. Pretreatment with quercetin and galangin significantly reduced IL-6 production in LPS-stimulated RAW264.7 cells in a dose-dependent manner from 6.25 to 25 μM quercetin and galangin. However, quercetin and galangin had no effect on the production of TNF-α following 24 h of incubation (Fig. 3A and B).

Effects of quercetin and galangin on the activation and nuclear translocation of NF-κB in LPS-stimulated RAW264.7 macrophages. Subsequently, whether quercetin and galangin suppressed the degradation of IκB-α, and translocation of NF-κB into the nucleus, was investigated. As shown in Fig. 4A, LPS stimulation induced the degradation of IκB-α and activation of NF-κB in RAW264.7 cells. However, quercetin and galangin pretreatment significantly attenuated IκB-α degradation and NF-κB activation. Immunofluorescence staining of NF-κB revealed that NF-κB was exclusively distributed in the cytoplasmic compartment prior to LPS stimulation. Treatment with LPS resulted in the enrichment of NF-κB in the nucleus. The nuclear translocation of NF-κB was markedly attenuated by quercetin and galangin treatment (Fig. 4B). These results indicate the potential role of NF-κB in the suppression of inflammatory mediator production by quercetin and galangin.

Effects of quercetin and galangin on LPS-induced MAPKs phosphorylation. Whether quercetin and galangin suppressed MAPK signaling pathways in LPS-stimulated RAW264.7 cells was subsequently evaluated. It was identified that the phosphorylation of p38 by LPS stimulation in RAW264.7 cells was not affected by quercetin or galangin. However, phosphorylation of Erk1/2 and JNK was markedly reduced by quercetin and galangin treatment in a concentration-dependent manner, without a change in total protein expression (Fig. 5). These results suggest that suppression of phosphorylation of Erk1/2 and JNK may be involved in the inhibitory effect of quercetin and galangin on LPS-stimulated NF-κB activation in RAW264.7 cells.

Effect of orally administered quercetin, galangin and their combination on DNCB-induced AD-like skin lesions in BALB/c mice. Treatment of mice with DNCB (see the scheme in Fig. 7A) resulted in severe discernible inflammation, with a significant increase in ear thickness compared with the normal group. The oral administration of quercetin and galangin in AD mice led to a noticeable reduction in ear thickness and AD symptoms, which were significant on day 21 and thereafter. Additionally, the data revealed that the combination of quercetin and galangin was more effective in suppressing ear thickness compared with each flavonol alone (Fig. 6B). Subsequently, the morphological changes in the lymph nodes of AD mice were measured. The lymph nodes from mice in the DNCB-only group were very swollen, whereas those from quercetin and galangin mice were smaller and weighed less (Fig. 6C). The IgE levels in AD mice were also measured. As expected, AD mice receiving quercetin and galangin produced significantly less IgE than did DNCB-only mice.

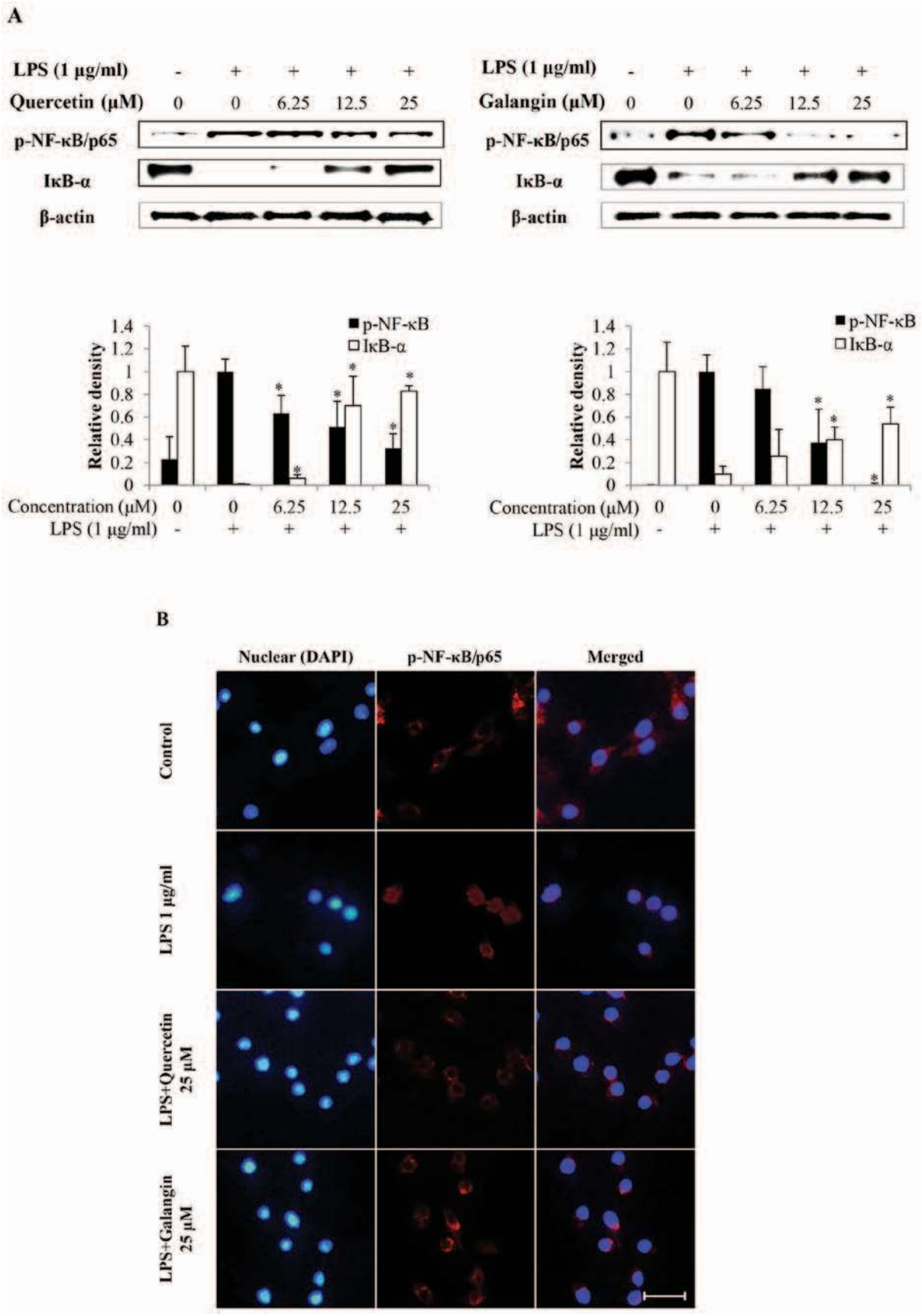

Figure 4. Effects of quercetin and galangin on degradation of IκB-α and phosphorylation of NF-κB/p65 protein in LPS-induced RAW264.7 cells. RAW264.7 cells were pre-incubated with the indicated concentrations of quercetin and galangin for 2 h, and then were treated with 1 μg/ml LPS for an additional 15 min. (A) NF-κB phosphorylation and IκB-α degradation were assessed by western blot analysis. Each bar represents the mean ± standard deviation calculated from three independent experiments. Significance was determined by Dunnett's t-test. *P<0.05 vs. the LPS-treated group or the control group, respectively. (B) Localization of NF-κB/p65 was visualized with a fluorescence microscope after immunofluorescence staining with anti-NF-κB/p65 antibody and Alexa Fluor 594-conjugated anti-rabbit IgG (red). Nuclei of the corresponding cells were visualized with DAPI (blue), and the cells were visualized using a fluorescence microscope (x400). Scale bar, 10 μm. IκB-α, inhibitor κB-α; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide.

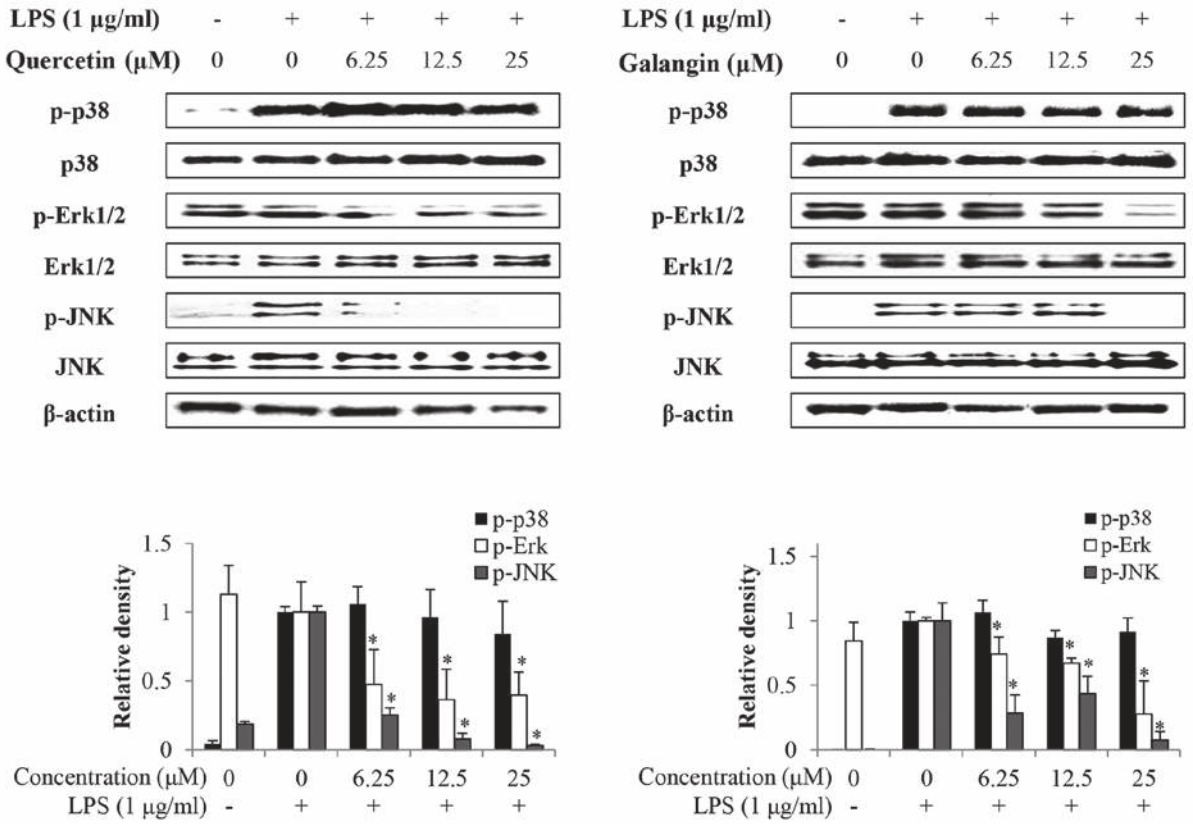

Figure 5. Effects of quercetin and galangin on theMAPK signaling pathway in LPS-induced RAW264.7 cells. RAW264.7 cells were pre-incubated with the indicated concentrations of quercetin and galangin for 2 h, and were then treated with 1 μg/ml LPS for an additional 15 min. MAPKs were assessed by western blot analysis from whole-cell lysates. Each bar represents the mean ± standard deviation calculated from three independent experiments. Significance was determined by Dunnett's t-test. *P<0.05 vs. the LPS-treated group. MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide.

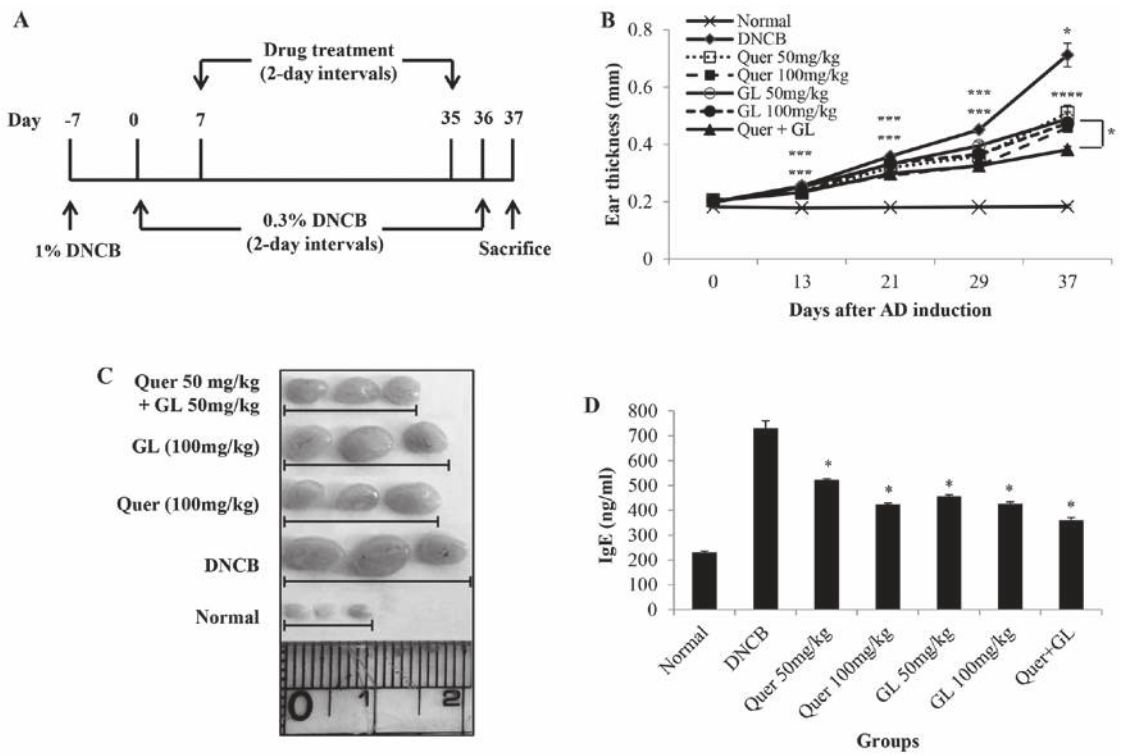

Figure 6. Effects of orally administered Quer, GL and their combination on DNCB-induced AD in BALB/c mice. (A) Schematic diagram showing the induction of AD and treatment of Quer and GL. (B) Ear thickness during the course of AD. (C) The lymph nodes were photographed to record morphological changes. (D) Levels of serum IgE were measured by ELISA. Blood samples were collected on day 37 post-induction. Data are expressed as the mean ± standard error of the mean, and significance was determined by Dunnett's t-test. *P<0.05 vs. the AD group. Quer, quercetin; GL, galangin; AD, atopic dermatitis; DCNB, 2,4-dinitrochlorobenzene; IPgE, immunoglobulin E.

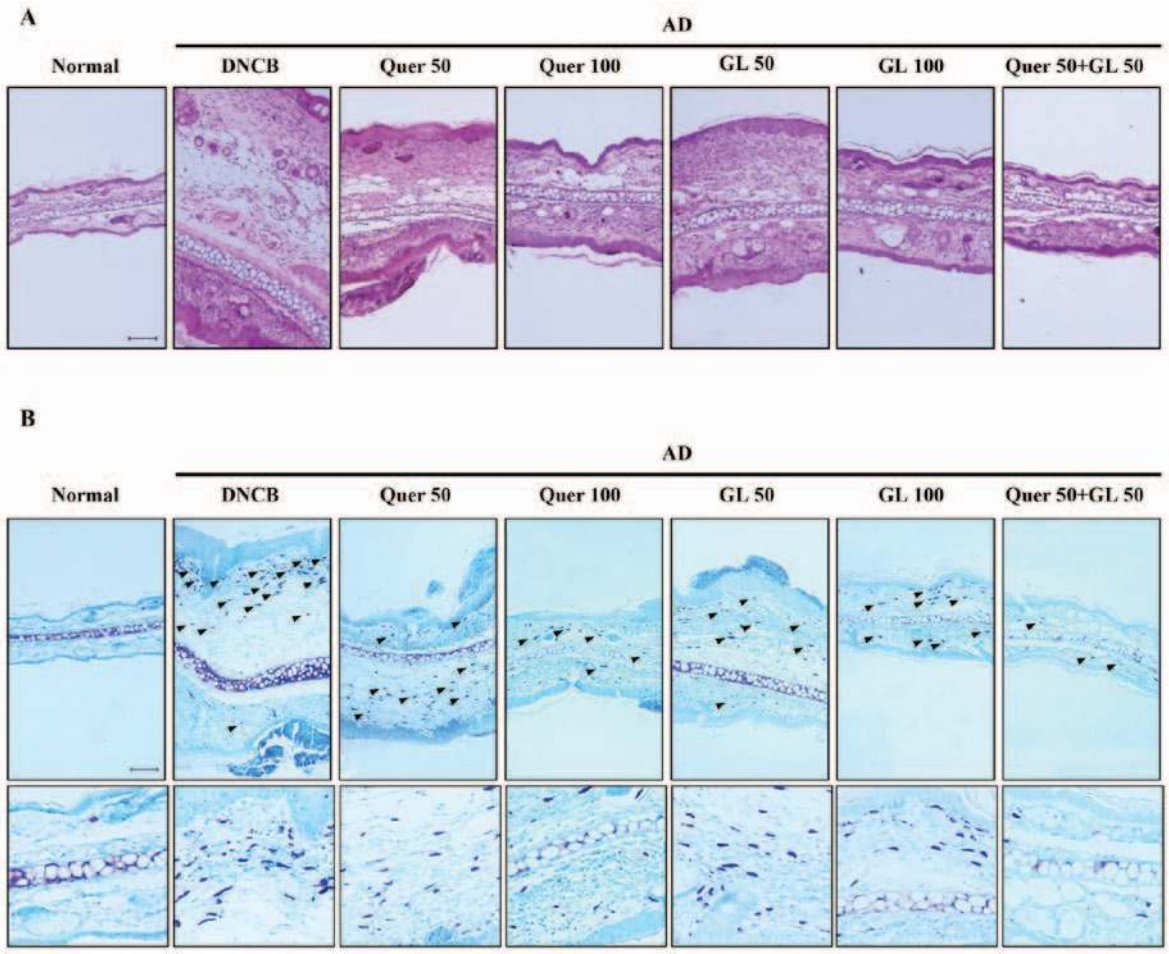

Figure 7. Effects of orally administered Quer (at concentrations of 50 or 100 mg/kg), GL (also at concentrations of 50 or 100 mg/kg), and their combination (both compounds at 50 mg/kg) on tissue inflammation and infiltration of immune cell in AD mice. (A) Hematoxylin and eosin (red) and (B) toluidine blue (blue) stainingwas performed, and the cells were examined under a light microscope (magnification, x200). The arrows indicate mast cells. AD, atopic dermatitis; Quer, quercetin; GL, galangin; AD, atopic dermatitis; DCNB, 2,4-dinitrochlorobenzene.

Furthermore, the combination of quercetin and galangin was more effective in reducing IgE levels compared with each flavonol alone (Fig. 6D). These data indicated that quercetin and galangin ameliorated AD symptoms in mice, thereby suggesting that quercetin and galangin may be effective modulators of immune responses in AD.

Effect of quercetin and galangin on DNCB-induced immune cell infiltration in BALB/c mice. Following induction of AD in mice, the ears were excised, and the thickness of ear tissues and the number of mast cells in the skin lesions were examined. First, H&E staining revealed extensive epidermal and dermal changes in the ears of AD mice, whereas these changes were attenuated in AD mice that were administered quercetin and galangin. The epidermal and dermal tissues in AD mice were significantly thinner following the administration of quercetin and galangin (Fig. 7A). Secondly, toluidine blue staining of ear tissue sections revealed mast cell infiltration on AD mice. This was abrogated by administration of quercetin and galangin (Fig. 7B). Taken together, these results suggested that quercetin and galangin reduce inflammation and mast cell infiltration in the skin, thereby attenuating AD.

Discussion

Flavonoids, also known as natural substances, possess various biological activities, including antioxidant, anti-inflammatory and anticancer properties (23). However, analyses of the anti-inflammatory properties of quercetin and galangin are limited to *in vitro* studies, and whether quercetin or galangin exhibits higher activity has yet to be elucidated. Thus, in the present study, the anti-inflammatory effects of quercetin and galangin in LPS-stimulated RAW264.7 cells *in vitro*, and the DNCB-induced AD model *in vivo*, were evaluated. Furthermore, whether the number of hydroxy groups on the B-ring of two flavonols would influence their anti-inflammatory effects was also investigated. The difference between the chemical structures of the two flavonols is associated with the number of hydroxy groups on the B-ring. This is considered to be involved in the biological activities of flavonols (24). Therefore, the present study aimed to compare the anti-inflammatory effects of the two flavonols.

Macrophages are crucial for the host's defense against infections and in inflammatory processes through the release of molecules, including NO, TNF-α and IL-6. The overproduction of these mediators has been implicated in several

inflammatory diseases and cancer (25). Thus, the murine macrophage RAW264.7 cell line was used in the present study.

In the MTT assay results, quercetin and galangin exhibited no cytotoxicity towards RAW264.7 macrophage cells up to concentrations of 25 μM, and therefore concentrations above this were not considered for further *in vitro* experiments (Fig. 1B).

NO is produced from L-arginine by three NOS enzymes: Endothelial NOS (eNOS), neuronal NOS (nNOS), and iNOS. Low physiological levels of NO are produced by constitutively expressed eNOS and nNOS, whereas iNOS is responsible for prolonged production of larger amounts of NO (26). iNOS is induced by bacterial products and inflammatory cytokines in macrophages and several other cells (27). In the present study, it has been demonstrated that quercetin and galangin markedly attenuated the LPS-induced expression of NO from RAW264.7 cells (Fig. 2A). Furthermore, these results indicated that quercetin, which has a higher number of hydroxy groups, was more effective compared with galangin.

Another important enzyme, COX-2, is an inducible enzyme that catalyzes the conversion of arachidonic acid into prostaglandin. Numerous studies have suggested that increased levels of prostaglandin and COX activity promote inflammatory pain (28). Thus, modulation of iNOS and COX-2 expression is considered to be a putative strategy for alleviating inflammatory disorders. The present study showed that quercetin and galangin inhibited the production of NO through downregulation of iNOS expression in LPS-stimulated RAW264.7 cells. However, quercetin and galangin had no effect on the production of COX-2 (Fig. 2B). In a previous study, the expression of iNOS and COX-2 by drug treatment yielded different results, i.e. that iNOS expression was decreased, but COX-2 expression was not affected (29). The differences noted in these results may be due to the degree of dependence of iNOS and COX-2 promoters on the various transcription factors.

TNF-α serves a major role in the cascade of proinflammatory cytokines, and subsequent inflammatory processes (30). IL-6 production is rapidly increased in acute inflammatory responses associated with infection, injury, trauma, and other stresses. As such, a dysregulated, high-level production of IL-6 may induce an undesirable inflammatory state (31). In the present study, IL-6 production was also inhibited by quercetin and galangin (Fig. 3B), but no effect was observed with respect to TNF-α production (Fig. 3A). These results provide the possibility that potentially separate mechanisms may exist in the production of TNF-α and IL-6 in LPS-stimulated macrophages. Therefore, the effects of quercetin and galangin on the expression of the NF-κB signaling pathway associated with IL-6 were examined in the following experiments.

NF-κB is a transcription factor that promotes the expression of inflammatory cytokines such as IL-6, and its downstream targets, including iNOS. Under normal conditions, NF-κB is present in an inactivated form in complex with IκB in the cytoplasm (32). However, LPS stimulation leads to the phosphorylation and degradation of IκB. As a result, NF-κB is released from the inhibition mediated by IκB, and activated NF-κB is subsequently translocated into the nucleus (33). The present study indicated that quercetin and galangin inhibited the nuclear translocation of NF-κB/p65 by suppressing degradation of the IκB-α protein levels (Fig. 4A). Subsequently, nuclear translocation of NF-κB/p65 was observed under a fluorescence microscope. Quercetin (25 μM) and galangin (25 μM) markedly inhibited the LPS-induced nuclear translocation of NF-κB/p65 (Fig. 4B). These data suggested that the inhibitory effect of quercetin and galangin on the production of various inflammatory factors, including NO, iNOS and IL-6, occurs through blocking the activation and nuclear translocation of NF-κB/p65.

A previous study has reported that MAPKs (p38, Erk1/2 and JNK) are associated with TLR4-mediated signaling events that lead to activation of the transcription factors, NF-κB and activator protein-1 (AP-1), in LPS-stimulated macrophages (34). The effects of quercetin and galangin on MAPK phosphorylation in RAW264.7 cells were also investigated. In the present study, it was shown that quercetin and galangin inhibited the LPS-induced phosphorylation of Erk1/2 and JNK, but had no effect on the phosphorylation of p38 (Fig. 5), suggesting that the Erk1/2 and JNK pathway may be involved in the quercetin- and galangin-mediated inhibition of LPS-induced inflammation. Taken together, these results suggested that quercetin and galangin may suppress the LPS-induced expression of iNOS and IL-6 by inhibiting phosphorylation of Erk1/2 and JNK in RAW264.7 cells.

In accordance with the *in vitro* results, quercetin and galangin significantly inhibited the LPS-induced inflammatory response. A noteworthy feature is that quercetin, which has two hydroxy groups on the B-ring, exhibited markedly more pronounced anti-inflammatory effects compared with galangin, which possesses no hydroxy groups on the B-ring. According to a previous study, the presence of hydroxy groups on the B-ring was shown to increase the pro-apoptotic activity of the flavonoids (35). Also, the antioxidant effect of various flavonoids was increased, depending on the number of hydroxy groups on the B-ring (36). Taken together, the number of hydroxy groups on the B-ring in quercetin and galangin is considered to exert an influence on the inflammatory response of LPS-induced RAW264.7 macrophages.

The number of published studies on the bioactive effects of various flavonoids has been continually increasing, although the majority of these studies has been focused on the efficacy of a single compound. However, synergistic effects have been reported for several flavonoids (37-39). Based on these results, the anti-inflammatory effects of quercetin, galangin and their combination in the DNCB-induced AD-like skin lesions of BALB/c mouse models were investigated.

DNCB is a representative irritant that induces contact dermatitis. Numerous studies have indicated the importance of the method used to deliver a compound to target tissues and cells in studying diseases in animal models. Topical or oral administration is widely used in AD models. Topical administration has relatively rapid effects on the administration site, although it is difficult to control the dosage. Oral administration is more convenient for AD animal studies, and it is much easier to control the dosage compared with topical administration (40). Since the symptoms of AD are systemic, rather than being limited to the skin, oral administration was selected for the present study.

Skin inflammatory diseases, such as contact dermatitis and AD, have features in common, including the infiltration

of immune cells, epidermal hyperplasia, elevated serum IgE levels and increased inflammatory cytokines (41). To investigate the anti-inflammatory effect, ear thickness of the mice was measured. It was revealed that the increase in DNCB-induced ear thickness was markedly suppressed by quercetin, galangin and their combination compared with the DNCB control in the following order: Quercetin + galangin > quercetin 100 > galangin 100 > galangin 50 > quercetin 50 > DNCB-control (see the Materials and methods section for a fuller description of these groups). Among them, the most effective group was the flavonol combination group (Fig. 6B).

Lymph nodes exert a critical role in the cell-mediated immune response. Therefore, morphological changes in AD-induced mice were investigated. The lymph nodes from mice in the DNCB-only group were significantly larger compared with the quercetin- and galangin-treatment groups (Fig. 6C).

IgE is an important therapeutic target for AD, as it is a major activator of mast cells, which release histamine. IgE expression causes acute- and chronic-phase skin inflammation (42). Consequently, the levels of serum IgE in DNCB-induced AD mouse models were also investigated. As a result, the quercetin, galangin and flavonol combination groups exhibited significantly reduced levels of IgE compared with the control group. Among them, the most effective group was the flavonol combination group (Fig. 6D).

Histological examination of the skin lesions stained by H&E and toluidine blue revealed thickening of the epidermis and dermis due to leukocyte infiltration, hyperplasia, dermis ulcers, and infiltration of immunocytes on the skin, including T-cells, mast cells and eosinophils (43). Mast cells are key effector cells in IgE-mediated allergic disorders, and are activated by cross-linking of the high-affinity IgE receptor. In addition, it has been reported that numerous mast cells may be identified in AD skin lesions (44). In the quercetin, galangin and flavonol combination groups, the thicknesses of the epidermis and dermis were reduced, and the expression of mast cells was significantly decreased, compared with the DNCB-only group (Fig. 7A and B). These experimental results indicated that the anti-inflammatory effect of quercetin combined with galangin is more potent compared with that of either flavonol alone.

For the *in vitro* experiments, marked anti-inflammatory effects were revealed, depending on the number of hydroxy groups on the B-ring, whereas the *in vivo* experiments showed no significant effects according to the difference in the number of hydroxy groups on the B-ring. However, these findings cannot be completely correlated, due to the differences that result from performing experiments *in vitro* and *in vivo*. Thus, it may be necessary to perform further experiments to gain an improved understanding of the differences between these findings.

In conclusion, the present study has demonstrated that pretreatment with quercetin and galangin ameliorated the LPS-induced inflammatory response in RAW264.7 macrophages through the inhibition of NF-κB, Erk1/2 and JNK signaling. Furthermore, the presence of hydroxy groups in flavonols has been confirmed to lead to significant anti-inflammatory effects *in vitro*. Additionally, to the best of our knowledge for the first time, it has been reported that the combination of quercetin and galangin works much more effectively in DNCB-induced AD-like skin lesions compared with either compound alone. Compared with the *in vitro* results, no significant differences attributable to the hydroxy groups were identified for quercetin and galangin treatment in the *in vivo* experiments. However, quercetin and galangin both exerted anti-inflammatory effects on the DNCB-induced AD animal model. Therefore, it may be hypothesized that quercetin and galangin treatment may be further developed as a medicine for AD once further experimental work has helped to delineate the molecular mechanisms of how quercetin and galangin act.

Acknowledgements

This study was supported by a research grant of the Kongju National University in 2016.

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