Lavandulyl Flavonoids from *Sophora flavescens* Suppress Lipopolysaccharide-Induced Activation of Nuclear Factor-κB and Mitogen-Activated Protein Kinases in RAW264.7 Cells

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Oxidized low-density lipoprotein (oxLDL) and reactive oxygen species (ROS) play key roles in the early stage of atherosclerosis. Nitric oxide (NO) and ROS are responsible for regulation of the transcriptional pathways of nuclear Factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK), key regulators of cellular inflammatory and immune responses. Previously, we examined LDL-antioxidant activities of the nine flavonoids isolated from *Sophora flavescens*. Among these, two lavandulyl flavonoids, kurarinone (1) and kuraridin (2) inhibited inducible nitric oxide synthase (iNOS)-dependent NO production and ROS generation, and suppressed remarkably the expression of inflammatory cytokines, CCL2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and iNOS in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Moreover, compounds 1 and 2 attenuated NF-κB activation by inhibition of IκBα proteolysis and p65 nuclear translocation, as well as phosphorylation of extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and p38 MAP kinases.

Key words *Sophora flavescens*; lavandulyl flavonoid; nitric oxide; reactive oxygen species; nuclear factor-κB; mitogen-activated protein kinase

Oxidative stress, which is commonly defined as an imbalance between oxidants and antioxidants at the cellular level, has shown a deep interest constantly as the cause of cardiovascular events and a variety of vascular diseases.[1] One of the crucial steps in oxidative stress augmentation is the overproduction of reactive oxygen species (ROS).[2] ROS encompass a variety of diverse chemical species including hydroxyl radical, nitric oxide (NO), and superoxide anion. In particular, the interaction between the superoxide anion and NO is important because it leads to the formation of the peroxynitrite. The peroxynitrite activates a variety of signaling pathways[3,4] and works deleteriously through its pro-atherosclerotic properties.[1]

In general, NO and ROS are responsible for the regulation of the transcriptional pathways of nuclear factor-κB (NF-κB) that regulates expression of various inflammatory cytokines, chemokines, and adhesion molecules.[5] After activation by inflammatory cytokines and cellular stresses including tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS), NF-κB proteins to translocate to the nucleus and bind their cognate DNA binding sites to regulate the transcription of a large number of genes including antimicrobial peptides, cytokines, chemokines, stress-response proteins, and anti-apoptotic proteins.[6] In addition to NF-κB, mitogen-activated protein kinases (MAPKs) have also been implicated in cytokine production in macrophages.[7] Three MAPK families (extracellular signal-regulated kinase (ERK)1/2, p38 and c-Jun N-terminal kinase (JNK)) are signaling molecules that react to extracellular stimuli (mitogens) and regulate immune responses including proinflammatory cytokine production, mitosis, differentiation, and cell survival/apoptosis.[7,8]

Sophora flavescens has long been traditionally used in asthma, bronchitis, bacterial, fungal infections, and skin disorders, and as an antipyretic, analgesic, anthelmintic, and remedy for stomach ailments.[9] Lavandulylated (C_{10}) or prenylated (C_5) flavanones, lavandulyl chalcones, pterocarpanes, and quinolizidine alkaloids were isolated from *S. flavescens*.[10—14] They possess various biological activities such as anticancer, antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory activities.[15—17] Although the anti-inflammatory activities of flavonoids from *S. flavesens* have been reported, the mechanism of kurarinone (**1**), lavandulyl flavanone and kuraridin (**2**), lavandulyl chalcone on anti-inflammatory activity remains unclear in macrophages.

In this study, we examined the effects of compounds **1** and **2** on LPS-mediated NO production, ROS generation, and expression of inflammatory cytokines, NF-κB activity, and phosphorylation of MAP kinases in murine RAW264.7 macrophages.

MATERIALS AND METHODS

General Experimental Materials and Cell Culture Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Cambrex (Verviers, Belgium). LPS was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2,7′-Dichlorofluorescein diacetate ($DCFH_2$-DA) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Antibody against inducible nitric oxide synthase (iNOS) was obtained from BD Transduction Laboratories (Lexington, KY, U.S.A.). Anti-NF-κB p65, anti-IκBα, and other most antibodies were obtained from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA, U.S.A.). Polyclonal antibodies to MAPK family and phospho-MAPK family were purchased from Cell Signaling Technologies (Beverly, MA, U.S.A.). RAW264.7 cells

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(murine macrophage cell line) were obtained from American Type Culture Collection (Manassas, VA, U.S.A.) and cultured in DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated FBS at 37 °C in a humidified incubator with 5% CO_2/95% air. The cytotoxicity of compounds **1** and **2** was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Measurement of NO and ROS Production RAW264.7 cells (2×10^5 cells/well) in a 96-well plate were treated with or without compounds **1** and **2** for 2 h, followed by incubation with 1 μg/ml of LPS for 18 h. Nitrite accumulation, as an indicator of NO production, was measured in the medium using the Griess reagents. After the removal of media from wells, ROS generation was measured by incubation of the cells with 10 μM $DCFH_2$-DA for 45 min. The fluorescence, corresponding to intracellular ROS, was measured on a Wallac 1420 spectrofluorometer (Perkin-Elmer, Turku, Finland) at 485 nm excitation and 530 nm emission wavelengths.

Western Blot Analysis Total cell extracts (for iNOS), cytoplasmic extracts (for IκBα, ERK, phospho-ERK, JNK, phospho-JNK, p38, and phospho-p38), and nuclear extracts (for NF-κB p65) were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membranes. To investigate the proteolysis of IκBα and phosphorylation of three MAPKs, cells pretreated with or without compounds **1** and **2** for 2 h were stimulated for 0—10 min with 1 μg/ml LPS. Cytosolic extracts were prepared in hypotonic buffer consisting of 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.6), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclear extracts were prepared in hypertonic buffer consisting of 50 mM HEPES (pH 7.9), 400 mM KCl, 0.1 mM EDTA, and 10% glycerol. Each transfer membrane was blocked overnight at 4 °C with a blocking solution [10 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.1% Tween 20, and 5% skim milk] and then incubated with specific antibodies for 1—3 h at room temperature. The blots were washed 3 times with washing buffer (20 mM Tris, 160 mM NaCl, and 0.1% Tween 20), followed by 1 h of incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. The peroxidase bound to the blot was detected using the Immobilon Western HRP detection reagent (Millipore, Billerica, MA, U.S.A.).

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis RAW264.7 cells were treated with or without compounds **1** and **2** for 2 h, followed by incubation with 1 μg/ml LPS for 10 h. The cells were harvested, and total RNA was isolated using RNeasy mini columns (Qiagen, Valencia, CA, U.S.A.) according to the instructions of the manufacturer. A sample (1 μg) of total RNA was used for the synthesis of the first strand cDNA using Omniscript (Qiagen). PCR amplifications were quantified using the SYBRGreen PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) against the expression of genes involved in CCL2 (NM_011333), TNF-α (NM_013693), IL-1β (NM_008361), and iNOS (NM_010927). The following primers were used to amplify CCL2, TNF-α, IL-1β, and iNOS: for CCL2, 5′-TGC TGA CCC CAA GAA GGA AT-3′ (sense), 5′-TGC TTG AGG TGG TTG TGG AA-3′ (anti-sense); for TNF-α, 5′- CTC AGA TCA TCT TCT CAA AAT TCG AGT GAC A-3′ (sense), 5′-CTT CAC AGA GCA ATG ACT CCA AAG T-3′ (anti-sense); for IL-1β, 5′-ATG AGG ACA TGA GCA CCT TC-3′ (sense), 5′-CAT TGA GGT GGA GAG CTT TC-3′ (anti-sense); for iNOS, 5′-GGC AGC CTG TGA GAC CTT TG-3′ (sense), 5′-TGC ATT GGA AGT GAA GCG TTT-3′ (anti-sense). After obtaining real-time fluorescence measurements, cycle threshold values were determined. Standard curves in the linear range (*i.e.* the exponential amplification phase) were used to calculate the quantity of each mRNA. The final data are expressed as the ratio of indicated mRNA to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA.

NF-κB Activity Assay RAW264.7 cells were transfected with a NF-κB luciferase reporter using Lipofectamine Plus reagent (Invitrogen, San Diego, CA, U.S.A.). NF-κB luciferase reporter was kindly provided by Dr. Jongsoon Lee (Joslin Diabetes Center, Boston, MA, U.S.A.). The luciferase assay was performed using dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A.). The results were normalized to the activity of *Renilla* expressed by cotransfected *Rluc* gene under the control of a constitutive promoter. Data are represented as fold induction of NF-κB activity in cells.

Gel Electromobility Shift Assay (EMSA) RAW264.7 cells were treated with or without compounds **1** and **2** and subsequently stimulated with 1 μg/ml LPS for 2 h. The nuclear lysis was performed using a hypertonic buffer. After lysis, the samples were centrifuged at 14000×***g*** for 15 min, and the supernatant was retained for use in the DNA binding assay. EMSA was performed according to the instructions of the manufacturer (Promega) using ^{32}P-labeled double-strand oligonucleotides with consensus recognition sequences for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and Oct-1 (5′-TGTCGAATGCAAATCACTAGAA-3′). DNA–protein complexes were resolved on a 5% native polyacrylamide gel in 0.5×TBE buffer [44.5 mM Tris–HCl (pH 8.0), 44.5 mM boric acid, 1 mM EDTA], at 150 V for 1.5 h, and the results were visualized by autoradiography.

Data Analysis All values are expressed as mean±S.D. Statistical analysis was done using Student's *t*-test. A value of $p<0.05$ was accepted as statistically significant.

RESULTS

Compounds 1 and 2 Inhibit the Production of iNOS-Dependent NO and ROS We demonstrate the effects of lavandulyl flavonone **1** and its chalcone **2** (Fig. 1A) on the production of inflammatory cytokines and mediators in RAW264.7 macrophages. The potential toxicity of compounds **1** and **2** to RAW264.7 cells was assessed by an assay utilizing MTT after 72 h incubation. Compounds **1** and **2** did not display any cellular toxicity against RAW264.7 murine macrophages over 48 h at a concentration to 40 μM (Fig. 1B). To investigate whether compounds **1** and **2** could inhibit ROS generation, cells were incubated with LPS for up to 16 h to increase ROS about 4-fold. In this condition, compounds **1** and **2** significantly reduced LPS-induced ROS production at the indicated concentrations (Fig. 2A). Compound **2** is more potent than compound **1** in the intracellular ROS accumulation. In order to investigate whether compounds **1** and **2**

could regulate the iNOS-derived NO production in RAW264.7 cells, we examined Griess assay and Western blot analysis (Figs. 2B, C). When RAW264.7 cells were treated with 1 μg/ml of LPS only, the levels of NO production and iNOS expression were markedly increased. However, pretreatment with compounds **1** and **2** significantly inhibited LPS-induced production of NO and expression of iNOS protein at the indicated concentrations. In particular, compound **2** showed good inhibition of iNOS-derived NO production.

Compounds 1 and 2 Suppress Pro-inflammatory Cytokines Expression To investigate the effects of compounds **1** and **2** on the LPS-induced expression of CCL2, TNF-α, IL-1β, and iNOS among inflammatory genes in RAW264.7 cells by real time RT-PCR analysis. Cells were pretreated with 40 μM of compounds **1** and **2** for 2 h, and were subsequently stimulated with 1 μg/ml LPS for 12 h. Stimulation of RAW264.7 cells with LPS significantly induced mRNA expression of CCL2, IL-1β, TNF-α, and iNOS (Fig. 2D). However, these mRNA expression levels were remarkably suppressed by the addition of compounds **1** and **2**.

Compounds 1 and 2 Attenuate Phosphorylation of ERK, JNK, and p38 MAPKs To clarify a molecular mechanism of the anti-inflammatory effect of compounds **1** and **2**, we detected the expression of the phosphorylation of ERK1/2, JNK, and p38 by Western blot analysis. LPS caused a rapid and significant increase in the phosphorylation of ERK1/2, JNK, and p38 MAP kinases within 10 min. However, compounds **1** and **2** treatment reduced phosphorylation of three different MAP kinases in a concentration-dependent manner (Fig. 3A).

Compounds 1 and 2 Inhibit LPS-Mediated NF-κB Activation The effects of compounds **1** and **2** on LPS-induced NF-κB activation, we examined Western blot analysis for NF-κB p65 translocation into the nucleus, pNF-κB-Luc plasmid reporter assay, and EMSA analysis. First, NF-κB p65 protein was detectable in the nucleus with LPS treatment for up to 30 min, however, compounds **1** and **2** were able to block NF-κB p65 nuclear translocation (Fig. 3B). In this result, compound **2** is more potent than compound **1**. The effect of compounds **1** and **2** on the IκBα proteolytic pathway was examined by Western blots. Compounds **1** and **2** at 40 μM completely blocked the LPS-induced IκBα degradation (Fig. 3B), thereby inhibiting activation and translocation of NF-κB. In addition, we investigated NF-κB transcriptional activity using a pNF-κB-Luc plasmid reporter. Semi-confluent RAW264.7 cells were transfected with the luciferase reporter plasmid pNF-κB-Luc. Luciferase activity

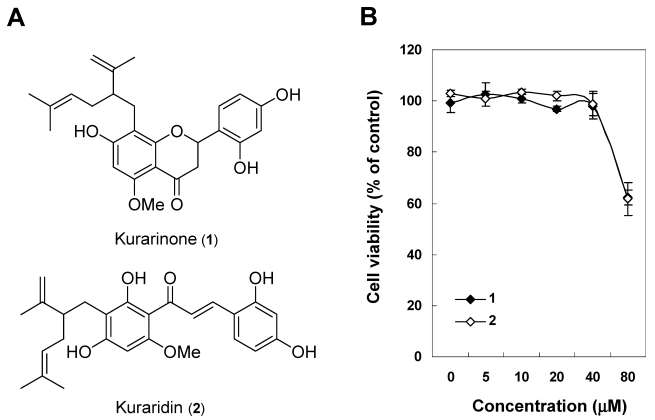

Fig. 1. Structures (A) and Cell Viabilities (B) of Compounds **1** and **2** Isolated from *S. flavescens*

Cell viability was measured by MTT assay. Cells were treated with indicated concentrations of compounds **1** and **2** for 48 h. Data are represented as means±S.D. of two independent experiments.

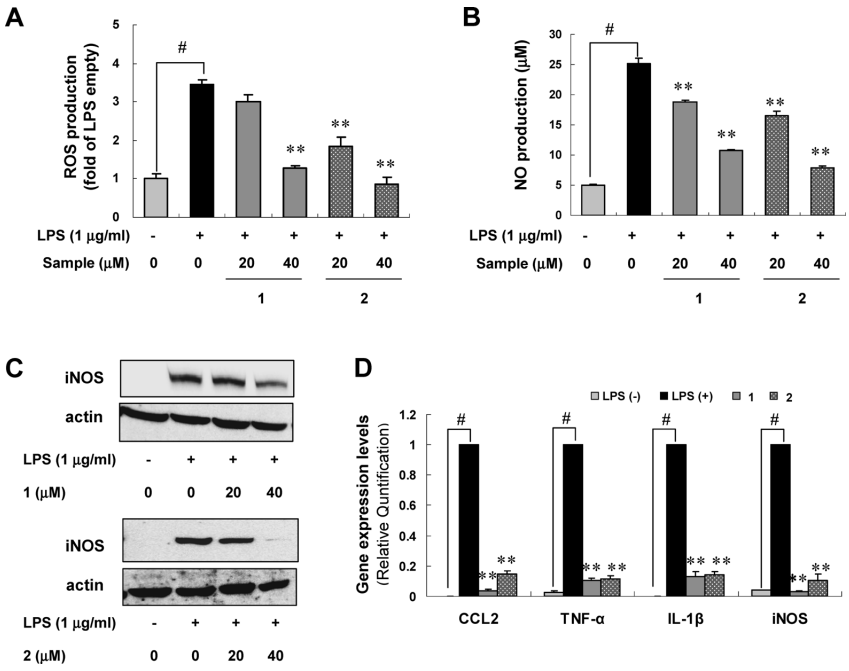

Fig. 2. Effects of Compounds **1** and **2** on the Production of ROS and Inflammatory Mediators in LPS-Stimulated RAW264.7 Cells

(A) The intracellular levels of ROS were determined by fluorescence using a fluorescence microplate reader with excitation/emission set to 485/530 nm. Cells were plated at 1×10^6 cells/ml and pretreated with indicated compounds **1** and **2** for 2 h, followed by incubation with 1 μg/ml LPS for 18 h. (B) The levels of nitrite were measured in the culture medium by Griess reagents. (C) Inducible NOS protein (>30 μg of protein) was determined by Western blot. Actin was used as an internal control. (D) Quantitative real-time PCR analysis of CCL2, TNF-α, IL-1β, and iNOS was examined (control=1). Data reported are mean±S.D. of two independent experiments. $^{\#}p<0.01$ *vs.* media alone-treated group, $^{**}p<0.01$ *vs.* LPS-treated group.

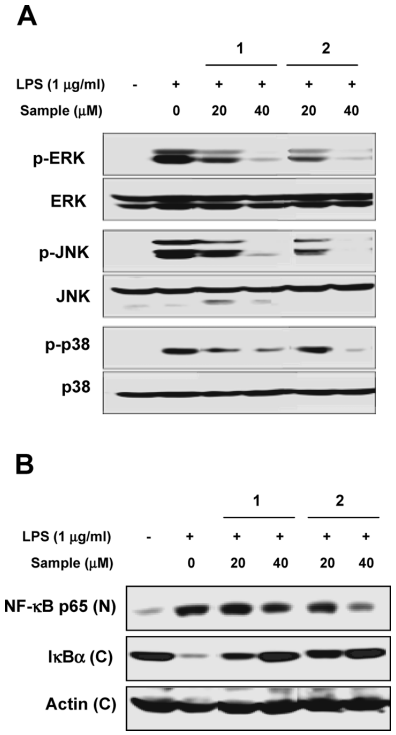

Fig. 3. Effects of Compounds **1** and **2** on the Activation of NF-κB and MAPKs in LPS-Stimulated RAW264.7 Cells

Cells were plated at 1×10^6 cells/ml and pretreated with indicated compounds **1** and **2** for 2 h, followed by incubation with 1 μg/ml LPS for 10 min. (A) Protein extracts (>50 μg) obtained from cells stimulated with LPS for 10 min were assessed by Western blotting, and then protein signals were detected with antibodies against phosphorylated- and total ERK1/2, JNK, and p38 MAPK. (B) Inhibition of NF-κB activation by compounds **1** and **2**. Treatment with compounds **1** and **2** (20, 40 μM) inhibited LPS-induced translocation of NF-κB p65, and degradation of IκBα (>50 μg of protein). N, nuclear extract; C, cytoplasmic extract.

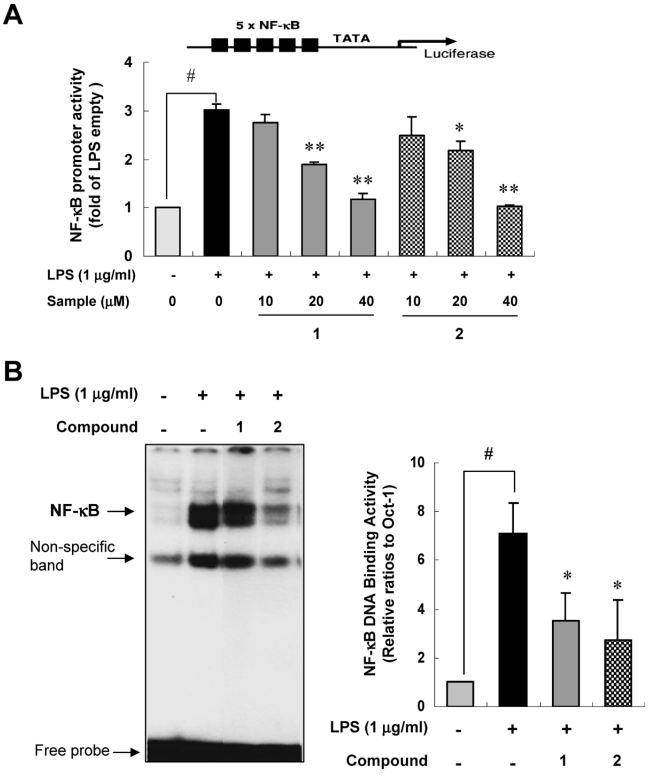

Fig. 4. Effects of Compounds **1** and **2** on the LPS-Induced NF-κB Activation in RAW264.7 Cells

Cells were plated at 1×10^6 cells/ml and pretreated with indicated compounds **1** and **2** for 2 h. (A) Inhibition of NF-κB-mediated transcription of the reporter gene by compounds **1** and **2**. Results were determined by luminometer with a pNF-κB-Luc reporter plasmid, pre-treated with compounds **1** and **2**, and stimulated with 1 μg/ml LPS for 12 h. Data represent mean±S.D. of three independent experiments. (B) DNA-binding activity of NF-κB complex. Cells were pretreated with the indicated concentrations of compounds **1** and **2** for 2 h before incubation with 1 μg/ml LPS for 2 h. EMSA analysis of the nuclear extracts (>5 μg of protein) was conducted using a ^{32}P-labeled NF-κB oligonucleotide probe. Quantification of relative band intensities from three independent experimental results was determined by densitometry. Data represent mean±S.D. of three independent experiments. $^{\#}p<0.01$ *vs.* media-treated group. $^{*}p<0.05$, $^{**}p<0.01$ *vs.* LPS-treated group.

was increased up to 3-fold in cells treated with 1 μg/ml LPS as compared with untreated cells. However, compounds **1** and **2** decreased LPS-induced luciferase activity in a dose-dependent manner (Fig. 4A). The effect of compounds **1** and **2** on the DNA binding of NF-κB transcription factors were evaluated by EMSA. One microgram per milliliter LPS significantly increased the DNA binding activity of NF-κB within 1 h in RAW264.7 cells. These increases were suppressed markedly by compounds **1** and **2** (Fig. 4B). In this study, compound **2** showed stronger inhibitory activities than compound **1** on the NO production, ROS generation, and NF-κB activation in LPS-induced RAW264.7 macrophages. These results suggest that compounds **1** and **2** have anti-atherosclerotic activity due to their ability to decrease intracellular NF-κB signaling, which leads to down-regulation of the expression of inflammation related genes and proteins.

DISCUSSION

Prenylated flavonoids have various biological activities, antiviral[18] and antioxidant activity,[19—21] and inhibitory effects against tyrosinase,[22] cyclooxygenase (COX), and lipoxygenase (LOX).[11] Especially, a variety of naturally occurring lavandulyl (C_{10}) flavonoids from *S. flavescens* such as sophoraflavanone G, kurarinone, and kuraridin exhibit potent inhibitory activity against COX-1 and 5-LOX in RAW264.7 cells,[11] and *in vivo* inflammatory response.[23] The lavandulyl flavonoids exhibited antioxidants potentials in the thiobarbituric acid-reactive substances (TBARS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and peroxynitrite ($ONOO^-$) assays.[19—21]

In the present study, lavandulyl flavonone **1** and its chalcone **2** significantly reduced LPS-induced ROS production. Compound **2** is more potent than compound **1** in the intracellular ROS accumulation. Two compounds have proven to be dual inhibitors, inhibiting not only copper-mediated LDL oxidation[21] but also ROS production from macrophages (Fig. 2A). Moreover, compounds **1** and **2** significantly inhibited iNOS-dependent NO production (Figs. 2B, C). Low NO concentrations play an important physiological role as a defense molecule in the immune system, whereas large amounts of NO produced by iNOS and cytotoxic NO metabolites such as peroxynitrite in macrophages contribute to numerous pathological processes.[24] In atherosclerotic lesions, inflammatory processes upregulate iNOS expression in macrophages, resulting in excessive NO production and vascular damage.[25] Furthermore, excess NO induces oxidation of LDLs within the arterial walls.[26] Therefore, suppression of iNOS expression using drugs might be an important

and attractive therapeutic target for the treatment of numerous pathological processes including inflammation and atherosclerosis.

In inflammatory processes, excessive proinflammatory cytokines or mediators such as CCL2 (MCP-1), TNF-α, IL-1β, IL-6, NO, and prostaglandin E2 (PGE2) play a critical causative role in many human diseases, including rheumatoid arthritis, asthma, and atherosclerosis.[27] In this study, we showed that pretreatment with compounds **1** and **2** remarkably suppressed LPS-induced mRNA expression of CCL2, IL-1β, TNF-α, and iNOS (Fig. 2D). These data indicate that compounds **1** and **2** may possess the potential to prevent inflammation-related diseases *via* modulation of NF-κB or MAPKs target genes.

MAPKs involved in macrophages inflammation play important regulatory roles in the cell growth and differentiation, and they control cellular responses to inflammatory cytokines and stress as well as modulation of NF-κB activity.[28] The compounds **1** and **2** significantly suppress LPS-induced phosphorylation of ERK1/2, JNK, and p38 MAP kinases in a concentration-dependent manner (Fig. 3A). NF-κB is also a critical transcriptional regulator of various genes involved in immune and inflammatory responses. Activation of NF-κB plays a key role in the LPS-induced expression of iNOS, COX-2, and TNF-α.[29] In non-stimulated cells, NF-κB is present in the cytosol as a homo- or heterodimer, and is linked to inhibitor protein, IκB. NF-κB activation requires the phosphorylation and degradation of IκB proteins, followed by nuclear translocation and DNA binding of the NF-κB.[30] Since NF-κB p65 subunit is responsible for the strong transcriptional activity of NF-κB, we conformed by Western blot that the compounds **1** and **2** blocked NF-κB p65 nuclear translocation as well as IκBα degradation in LPS-stimulated RAW264.7 cells (Fig. 3B). In addition, the compounds **1** and **2** decreased LPS-induced luciferase activity in a dose-dependent manner, and inhibited NF-κB activiation by preventing DNA binding (Fig. 4).

For the comparison of biological activities of compounds **1**, lavandulyl flavonone and compound **2**, its chalcone, we searched those biological activities from resent reports. Compounds **2** exhibited more potent antioxidants potentials in DPPH, ABTS, and $ONOO^-$ assay than compound **1**,[20] while compound **1** inhibited more strongly the low-density lipoprotein (LDL)-oxidation than compound **2**.[21] The 5-LOX inhibitory activity of compound **2** was more potent than compound **1**, while the COX-1 inhibitory activity of compounds **1** and **2** was very similar.[11] In this study, compound **2** showed stronger inhibitory activities than compound **1** on the NO production, ROS generation, and NF-κB activation in LPS-induced RAW264.7 macrophages.

In conclusion, two lavandulyl flavonoids, kurarinone (**1**) and kuraridin (**2**) isolated from *S. flavescens* inhibit the production of ROS and NO, and the expression of iNOS in LPS-induced RAW264.7 macrophages. Expression of inflammation-associated genes such as CCL2, TNF-α, and IL-1β are suppressed significantly by compounds **1** and **2**. Moreover, compounds **1** and **2** attenuated NF-κB activation by inhibition of IκBα degradation and p65 nuclear translocation, as well as phosphorylation of MAP kinases in murine RAW264.7 macrophages. These results suggest that the compounds **1** and **2** have anti-atherosclerotic activity due to their ability to decrease intracellular NF-κB signaling, which leads to down-regulation of the expression of inflammation related genes and proteins.

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