Attenuation of inflammatory responses by (+)-syringaresinol via MAP-Kinase-mediated suppression of NF-κB signaling in vitro and in vivo

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We examined the anti-inflammatory effects of (+)-syringaresinol (SGRS), a lignan isolated from Rubia philippinensis, in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells using enzyme-based immuno assay, Western blotting, and RT-PCR analyses. Additionally, in vivo effects of SGRS in the acute inflammatory state were examined by using the carrageenan-induced hind paw edema assay in experimental mice. As a result, treatment with SGRS (25, 50, and 100 μM) inhibited protein expression of lipopolysaccharide-stimulated inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and nuclear factor kappa B (NF-κB) as well as production of nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and interleukin-6 (IL-6) cytokines in a dose-dependent fashion. Furthermore, carrageenan-induced paw edema assay validated the in vivo anti-edema effect of SGRS. Interestingly, SGRS (30 mg/kg) suppressed carrageenan-induced elevation of iNOS, COX-2, TNF-α, IL-1β, and IL-6 mRNA levels as well as COX-2 and NF-κB protein levels, suggesting SGRS may possess anti-inflammatory activities.

The inflammatory response, a physiological reaction to infection or damage, plays a vital role in the natural defense mechanisms of the body to maintain immune homeostasis1. Inflammation refers to the body's normal protective response to tissue injury caused by physical trauma, toxic chemicals or microbiological agents, and the classical signs of inflammation are skin redness, swelling, pain, heat, and loss of function2. The process of inflammation involves changes in blood flow, destruction of tissues, increased vascular permeability and the synthesis of pro-inflammatory mediators. The injured cells and blood proteins are the sources of inflammatory mediators. The inflammatory process is defined in two critical phases, one as an acute phase, where inflammation occurs a few minutes after tissue damage while failure to acute phase results in the chronic inflammation2. Nevertheless, instability of immune homeostasis as well as a prolonged inflammatory response can result in the development of various chronic diseases such as autoimmune disorders, cancer, and vascular diseases3. Phagocytosis of pathogens via toll-like receptors (TLRs), which distinguish molecular patterns of lipopolysaccharide (LPS), a pathogen-derived material, results in the activation of immune cells (macrophages, neutrophils, and dendritic cells), thereby triggering inflammatory responses4. These immune cells upon activation mediate enhanced production of various pro-inflammatory proteins/enzymes that include cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase.

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(iNOS) along with production of other pro-inflammatory cytokines, such as interleukins (IL-1β and IL-6) and tumor necrosis factor-α (TNF-α). These pro-inflammatory biomarkers are known as important mediators of inflammatory responses. Activation of nuclear factor-kappa B (NF-κB) plays a significant role in the regulation of protein expression levels of iNOS and COX-2, which eventually produce nitric oxide (NO) and prostaglandin E2 (PGE2)6). NF-κB is involved in the trans-activation of a number of genes as an important transcriptional factor, which regulate both immune-inflammatory and acute-inflammatory responses, including the cell survival and tumorigenesis8,9. Furthermore, there is a great deal of involvement of cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-6 in the development of diseases associated with inflammation and inflammatory responses10.

Mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun NH2-terminal kinase (JNK), comprising a group of signaling pathways, play vital roles in the regulation of cell differentiation and growth, and their phosphorylation is known to be a critical component in the production of NO and pro-inflammatory cytokines in activated macrophages8,9. Thus, mounting research has focused on identifying safe candidate materials with a preventive ability to treat inflammatory diseases through their diverse inhibitory action against upstream signaling events involved in the expression profile of inflammatory genes.

Rubia philippinensis is a rambling and a low climbing perennial herb that grows in the Southern part of Vietnam. Local communities have long utilized this medicinal plant to treat ordinary ailments such as wounds, inflammation, and skin infections13. Previous investigations of the species have resulted in the purification of arborinane triterpenoids, which show promising effects on the prevention and treatment of atherosclerosis13. Additionally, rubiarbonone C, a popular chemical entity isolated from R. philippinensis, has been shown to inhibit abnormal proliferation and migration of vascular smooth muscle cells, which plays an important role in the pathophysiology of atherosclerosis. The mechanism by which rubiarbonone C regulates vascular remodeling was further clarified through focal adhesion kinase (FAK), MAPK, and STAT3 Tyr70514. In searching for bioactive components from R. philippinensis, in this study, (+)-syringaresinol was also isolated as a major compound. In addition, Cai et al.15 also reported isolation and characterization of (+)-syringaresinol from Acanthopanax kore-anum along with some other phytoconstituents, including eleutheroside E, tortoside A, and hemlarlensin which were enough cable to inhibit a cytoplasmic protein NFAT playing a significant role in the induction of immune responses.

There is a great increasing demand of natural products as herbal medicines due to their being less toxic, affordable, easily available, and with fewer adversary effects on human. As a part of prior research examining the biological potential of effective phytochemicals and to minimize the side effects of commercial anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs), a lignan, (+)-syringaresinol (SGRS) isolated in this study from R. philippinensis was assessed for its potent anti-inflammatory effects both in vitro and in vivo. In addition, relevant targets involved in the regulation of inflammatory responses were studied to estimate the precise anti-inflammatory mode of action of SGRS. Current research focused on the evaluation of detailed anti-inflammatory mechanism of SGRS in terms of its effect on LPS-stimulated macrophages that influence MAPK signaling pathways. The findings demonstrate that SGRs attenuated the inflammatory response via down-regulation of NF-κB by activating p38 and JNK proteins in RAW 264.7 cells.

Materials and Methods

Plant materials. Root samples of Rubia philippinensis were claimed from the National Park of Lam Dong city, Vietnam. Taxonomic identification of root samples was done at the National Institute of Medicinal Materials, Vietnam by Dr. Phuong Thien Thuong. Specimen samples of R. philippinensis + Acanthopanax kore-anum were deposited under the herbarium identification number VDL20140801 at the National Institute of Medicinal Materials, Hanoi, Vietnam.

Also, the same root sample was deposited under same specimen identification number at the Pharmacy College of Chungnam National University, Daejeon, Korea.

Extraction, isolation, and characterization of (+)-syringaresinol (SGRS). (+)-Syringaresinol was acquired from the root samples of R. philippinensis using various chromatographic techniques. In brief, ethanol-mediated root extract of R. philippinensis (150 g) was suspended in H2O (1.5 L) followed by subsequent partitioning using CH2Cl2 (2 L × 3) to yield CH2Cl2 extract. The CH2Cl2-soluble fraction (50 g) was subjected to silica gel VLC and eluted with n-hexane-EtOAc (20:1, 10:1, 5:1, 3:1, 2:1) and CHCl3-MeOH (8:1) to afford six fractions (D-1 → D-6). Fraction D-6 (10 g) was divided into 11 sub-fractions (D-6-1 → D-6-11) using MPLC with a gradient of MeOH-H2O (10:90 → 100:0, 7 L). (+)-Syringaresinol (t4 42.0 min, 70 mg) was obtained from D-6-3 (360 mg) by eluting HPLC with MeOH-H2O (45:55, 4 mL/min, UV 254 nm).

(+)-Syringaresinol (SGRS): brownish amorphous powder, [α]25D +8 (c 0.1, CHCl3), 1H NMR (300 MHz, methanol-d4): 6.69 (4 H, s, H-2′, H-6′, H-6″, H-6‴), 4.74 (2 H, d, J = 4.0 Hz, H-2, H-6), 4.29 (2 H, dd, J = 6.6, 9.0 Hz, H-4a, H-8a), 3.91 (2 H, dd, J = 3.1, 9.0 Hz, H-4b, H-8b), 3.87 (12 H, s, OCH3-3′, 3″, 5′, 5″), 3.16 (2 H, m, H-1, H-5), 13C NMR (75 MHz, methanol-d4): 55.4 (C-1, C-5), 87.5 (C-2, C-6), 72.7 (C-4, C-8), 133.1 (C-1′, C-1″), 104.4 (C-2′, C-2‴, C-6″, C-6‴), 149.2 (C-3′, C-3″, C-5′, C-5″), 136.1 (C-4′, C-4‴), 56.7 (OCH3-3′, 3″, 5′, 5″).

Cell culture and materials. RAW 264.7 cells were cultured in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 100 units/mL of penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (Grand Island, NY, USA) under a humidified 5% CO2 atmosphere at 37°C. 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), phosphate-buffered saline (PBS, pH 7.4), and dimethylsulfoxide (DMSO) were procured commercially (Sigma Aldrich, St. Louis, MO, USA).
Cell viability and nitric oxide determination. The MTT assay was employed to evaluate the cell viability of SGRS. In brief, a 24-well plate was used to plate the RAW 264.7 cells at the density of $1 \times 10^5$ per well followed by incubation at 37°C for 24 h. For the treatment of the cells, various concentrations (25, 50, and 100 μM) of SGRS were used for 2 h before LPS (1 μg/mL) stimulation, including a vehicle control followed by incubation for an additional 18 h at 37°C. Afterwards, aliquots (100 μL) of the cell-free culture medium were taken for the measurement of NO following the Griess reaction method. For the determination of cell viability, a previously reported method was adopted.

TNF-α, IL-1β, IL-6, and PGE2 assays. RAW 264.7 cells (5 x 10^5 cells/mL) were incubated in media for 16 h. Cells were then pre-treated with various concentrations (25, 50, and 100 μM) of SGRS for 1 h, followed by LPS (1 μg/mL)-mediated stimulation. The cell culture supernatant was collected from the cells after 20 h of LPS-mediated cell stimulation, and an enzyme-linked immunosorbent assay (ELISA) was performed for the quantitative determination of the levels of TNF-α, IL-1β, IL-6, and PGE2.

NF-κB reporter assay. The pNF-κB-luc plasmid (Beyotime Biotechnology, Nantong, China) containing four NF-κB-binding motifs (GGGAATTTCC) and pRL-SV40 (Renilla luciferase driven by SV40) reporter constructs (Promega, Madison, WI, USA) were complexed using GenJetTM Plus DNA In Vitro Transfection Reagent (Signagen, Maryland, USA) in 100 μL of serum-free culture medium. Then, RAW 264.7 cells seeded in six-well plates (Corning, New York, NY, USA) were transfected by adding the mixture. Luciferase activity was assayed after cells were treated with LPS or various concentrations of SGRS using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

Preparation of nuclear extracts. After dishes were washed with ice-cold PBS, cells were scraped and transferred to microtubes. Cells were swollen by adding lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.2% NP-40, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA)] and then incubated for 10 min on ice and centrifuged 15,000 g for 5 min at 4°C. Pellets containing crude nuclei were re-suspended in 50 μL of extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 1 mM dithiothreitol, 420 mM NaCl, 20% glycerol, and protease inhibitor cocktail), incubated for 30 min on a shaker at 4°C, and centrifuged at 16,000 x g for 10 min in order to obtain supernatants (nuclear extracts).

Carrageenan-induced paw edema. Eight-week-old ICR mice were obtained from Central Lab Animals, Inc. (Seoul, Korea) and housed in an air-conditioned animal room at a temperature of 23 ± 1°C, humidity of 55 ± 5%, and 12 h/12 h light/dark cycle with ad libitum access to water and standard laboratory diet. The animals were acclimatized for 1 week and randomly divided into five groups of five mice each. The experiment was conducted in accordance with the guidelines for animal experiments issued by the Kyungpook National University and approved by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU-2017-0035). Mice (N = 25) were randomly divided into four groups (five animals/group): treatment naïve control group (group-1), CA control group (group-2), indomethacin group (group-3), and 50 mg/kg/day SGRS group (group-4). For oral administration, SGRS (dissolved in 40% polyethylene glycol) was administered at the dose of 50 mg/kg/day for 4 consecutive days. For positive control, standard anti-inflammatory drug, indomethacin was employed. For induction of acute phase inflammation, a subcutaneous injection of carrageenan (1%) was administered (60 μL per animal) into the right hind paws of mice after 1 h SGRS or vehicle treatment. A plethysmometer was used for measuring the paw volumes hourly for 4 h after carrageenan injection, after which mice were euthanized. The right hind paw skin was then expunged and immediately frozen in a nitrogen tank for RT-PCR and Western blotting analyses.

mRNA analysis by semi-quantitative RT-PCR. To evaluate mRNA expression levels, RAW 264.7 cells were pre-treated with SGRS (25, 50, and 100 μM) for 30 min before incubation with LPS (1 μg/mL) for 6 h. Total RNA was isolated with TRIzol Reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer’s instructions. Semi-quantitative RT-PCR reactions were conducted as previously reported with minor modifications. In brief, to prepare a cDNA pool from RNAs, total RNA was isolated with TRIzol Reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer’s instructions. Semi-quantitative RT-PCR reactions were conducted as previously reported with minor modifications. To evaluate mRNA expression levels, RAW 264.7 cells were pre-treated with SGRS for 2 h before LPS (1 μg/mL) stimulation, including a vehicle control followed by incubation for an additional 18 h at 37°C. Afterwards, aliquots (100 μL) of the cell-free culture medium were taken for the measurement of NO following the Griess reaction method. For the determination of cell viability, a previously reported method was adopted.

Western blot analysis. Macrophage RAW 264.7 cells were pretreated using above-mentioned concentrations of SGRS or vehicle for 2 h followed by stimulation with LPS (1 μg/mL) for 6 h. Primary and secondary antibodies were obtained commercially (Santa Cruz Biotechnology, Cruz, CA, USA). Ten micrograms of total proteins were separated by SDS-PAGE. Proteins were electro-transferred to nitrocellulose membranes after electrophoresis, blocked with 5% non-fat milk in TBST buffer, and blotted with each primary antibody (1:1000) and with corresponding secondary antibody (1:5000). The antigen-antibody reaction was detected using an ECL solution system (Perkin Elmer). An Image Lab™ Software (version 5.2.1) was used for analyzing the bands. The membrane was stripped using a stripping buffer (Restore™ Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL, USA) for the screening of various biomarkers. Briefly, the membrane was immersed completely in the stripping buffer for 15 min in shaking motion. The stripping buffer was then carefully, but thoroughly washed with TBST twice for 10 min. Then the membrane was again blocked with 5% non-fat milk in TBST buffer, and blotted with β-actin primary antibody (1:1000) and with corresponding secondary antibody (1:5000). The
antigen-antibody reaction was detected using an ECL solution system (Perkin Elmer). An Image Lab™ Software (version 5.2.1) was used for analyzing the bands.

Statistical analysis. Data were presented as the mean ± SD followed by one-way ANOVA analysis. The value (p < 0.05) was considered significant for the differences. For all data analyses, Window’s SPSS Software (Version 10.07 (SPSS, Chicago, IL, USA) was used.

Results

Identification and characterization of (+)-syringaresinol (SGRS). The 1H NMR data of the purified compound, with a specific rotation value \[ [\alpha]_{D}^{25} + 8 \text{ (c 0.1, CHCl}_3]^{18,19}, displayed four symmetric protons at \( \delta_H 6.69 \text{ (4 H, brs, H-2′, H-6′, H-2″, H-6″). In addition, two oxygenated methines at } \delta_H 4.74 \text{ (2 H, d, } J = 4.0, \text{ H-2, H-6), two oxygenated methylene functionalities at } \delta_H 4.29 \text{ (2 H, dd, } J = 6.6, 9.0, \text{ H-4a, H-8a) and } \delta_H 3.91 \text{ (2 H, dd, } J = 3.1, 9.0, \text{ H-4b, H-8b), and two methines at } \delta_H 3.16 \text{ (2 H, m, H-1, H-5), indicating two symmetrical tetrahydrofuran units, were observed in the } 1H \text{ NMR data as well (Fig. S1). The } 13C \text{ NMR spectrum (Fig. S2) showed a total of 18 signals for typical lignan derivatives along with four aromatic methoxy groups (}\delta_C 56.7). Based on the analysis of NMR spectroscopic data and specific rotation values, the compound was identified as (+)-syringaresinol (Fig. 1)\textsuperscript{15}.

Effect of SGRS on production of inflammatory mediators in LPS-induced RAW264.7 cells. Different dosages of SGRS (25, 50, and 100μM) were used to evaluate its inhibitory effects on LPS-induced production of NO and PGE\textsubscript{2} in RAW 264.7 cells. Compared to untreated control cells (column 1 of Fig. 2A), treatment with LPS significantly increased production of NO (column 2 of Fig. 2A). However, treatment with SGRS significantly and dose-dependently reduced NO production (column 3–5 of Fig. 2A). In addition, we examined the effect of SGRS on LPS-induced production of PGE\textsubscript{2} (Fig. 2B). Compared to control cells (column 1 of Fig. 1B), LPS caused an increase in PGE\textsubscript{2} production (column 2 of Fig. 2B), whereas SGRS treatment significantly reduced PGE\textsubscript{2} production (column 3–5 of Fig. 2B) in a concentration-dependent manner. In contrast, SGRS did not affect cell viability, as measured by MTT assay at concentrations that inhibited the LPS-induced inflammatory response (Fig. S3). These results indicate that SGRS inhibits the LPS-induced inflammatory response without affecting cell viability.

Figure 1. Chemical structure of (+)-syringaresinol, a lignan isolated from Rubia philippinensis.
Effect of SGRS on upstream signaling for NF-κB activation in LPS-induced RAW264.7 cells. This study also investigated whether or not SGRS has ability to block activation of the NF-κB pathway because regulation of inflammatory mediators in LPS-stimulated macrophages is transcriptionally implicated with the NF-κB. Phosphorylation of inhibitory kappa B (IκB) and its subsequent degradation by various stimuli is a critical step in NF-κB activation\(^2\), therefore, the effects of SGRS on LPS-induced degradation and phosphorylation of IκBα protein were investigated by immunoblotting analysis. SGRS inhibited LPS-induced phosphorylation of IκB in the cytosol in a dose-dependent manner (Fig. 4A). Free dimer activated subunits of NF-κB (p50/p65) can be translocated from the cytosol to the nucleus upon dissociation of IκB-α from NF-κB. Thus, in order to more specifically evaluate whether or not SGRS can affect the nuclear translocation of NF-κB, Western immunoblotting analysis for NF-κB was conducted using nuclear extracts of LPS-stimulated RAW 264.7 macrophages. Exposure of LPS alone significantly increased the amount of NF-κB in the nucleus (column 2, Fig. 4B). SGRS also inhibited LPS-induced nuclear translocation of NF-κB dose-dependently (column 3–5, Fig. 4B). An NF-κB-driven reporter construct in LPS-stimulated RAW 264.7 cells was employed, since in this system, luciferase reporter activity mediated by NF-κB is greatly induced\(^1\). Consistent with our previous data, NF-κB-driven luciferase activity in LPS-stimulated RAW264.7 cells was significantly reduced by SGRS in a dose-dependent manner (Fig. 4C), indicating that this extract blocked NF-κB activity. These findings indicate the potential role of NF-κB in the possible mode of action of SGRS in suppressing NO, PGE2, and pro-inflammatory cytokines in activated macrophages.

SGRS attenuates MAPK phosphorylation in LPS-stimulated RAW264.7 cells. To confirm whether or not inhibition of NF-κB activation is mediated through MAPK pathways, we examined the effect of SGRS on LPS-stimulated phosphorylation of ERK1/2, JNK, and p38 MAPK in RAW264.7 cells. As depicted in Fig. 4D, LPS markedly induced phosphorylation of ERK1/2, JNK, and p38. Pre-treatment with SGRS significantly inhibited LPS-stimulated phosphorylation of p38 MAPK and JNK, whereas phosphorylation of ERK remained unchanged (data not shown). This result suggests that phosphorylation of p38 and JNK was inhibited by SGRS. However, the degree of inhibition was different for each MAPK, with the maximum inhibitory effect exerted on JNK. This result indicates that signal transduction by p38 and JNK might be effectively blocked by SGRS in activated macrophages.

Inhibitory effects of SGRS on carrageenan-induced mouse hind paw edema. Treatment of mice with carrageenan resulted in significantly increased paw swelling in comparison with the control. However, pre-treatment with indomethacin (10 mg/kg/day, p.o.), a positive control, significantly reduced paw edema formation.
Similarly, treatment with SGRS (50 mg/kg/day, p.o.) significantly reduced paw edema volume (Fig. 5A). In addition, as expected, SGRS treatment significantly mitigated mRNA expression of inflammatory mediators (iNOS and COX-2) and various pro-inflammatory cytokines (TNF-\(\alpha\), IL-1\(\beta\), and IL-6) compared to levels in the CA insult groups (Fig. 5B). Subsequently, protein expression of COX-2 and NF-\(\kappa\)B was also suppressed in the SGRS-treated group compared to levels in the CA insult groups (Fig. 5C). These data suggest that SGRS attenuated carrageenan-induced inflammation in mice, likely via suppression of NF-\(\kappa\)B signaling.

**Discussion**

(\(+\)-Syringaresinol (SGRS) is a naturally occurring lignan found in various plants, including flax seed (\textit{Linum usitatissimum}), sesame seed (\textit{Sesamum indicum}), Brassica vegetables, and grains (rye bran, wheat bran, oat bran, and barley bran)\(^{21-23}\). In this study, SGRS was isolated from the roots of \textit{R. philippinensis} using various chromatographic techniques and characterized based on the spectral data analyses\(^{15}\). SGRS has also been characterized from the roots of \textit{A. koreanum} with an ability to inhibit nuclear factor of activated T-cell protein\(^{15}\). Recently, SGRS has shown a significant potential to act as a neuromodulating agent by suppressing synaptic transmission via presynaptic transmitter release modulation\(^{24}\). Also, a furofuran-like lignan, syringaresinol-4-\(\beta\)-d-glucoside showed a potential efficacy in the treatment of lipid and glucose-based metabolic disorders\(^{25}\). Moreover, the role of SGRS has also been confirmed in the induction of mitochondrial biogenesis via activating PPAR\(\beta\) pathway in muscle cells\(^{26}\). However, the molecular mechanism responsible for the anti-inflammatory action of SGRS has not been evaluated so far. Hence, we evaluated anti-inflammatory effects of SGRS in LPS-induced RAW 264.7 cells as well as in a murine model of carrageenan-induced acute edematous inflammation in mice in order to elucidate the relevant mechanism of action of SGRS.

Inflammation refers as a complex of biological responses to toxic stimuli during the process of host-defense\(^{27}\). Production of NO by NOS after carrageenan administration has significant involvement in the inflammation progression, whereas NO which is produced by iNOS after inflammation is later involved in maintaining the inflammatory responses\(^{28}\). More specifically, high levels of NO generated by inducible NO synthase (iNOS) have been defined as cytotoxic molecules in inflammation and endotoxemia\(^{29}\). Several studies investigated the anti-inflammatory action of lignans from various plant sources on the production of NO/iNOS, PGE\(_2\)/COX-2, TNF-\(\alpha\), and IL-1\(\beta\) in murine macrophages such as RAW 264.7 cells and observed down-regulation of inflammation-associated gene transcription\(^{30-32}\). In the present study, SGRS significantly inhibited LPS-induced production of NO and PGE\(_2\) in RAW 264.7 cells (Fig. 2A,B). Furthermore, SGRS attenuated LPS-induced gene expression as well as translation of iNOS.
and COX-2 (Fig. 2C,D). These findings indicate that NO inhibition and PGE2 production by SGRS might be due to the inhibition of iNOS and COX-2 up-regulation during macrophage activation by LPS.

TNF-α and IL-1β are pro-inflammatory cytokines involved in the pathogenesis of carrageenan-induced inflammation33. Also, IL-6 has been found to be interacted with a variety of target cells and associated with diverse immunological reactions34. In our study, we found that secretion (Fig. 3A–C) and mRNA expression (Fig. 3D) of LPS-stimulated pro-inflammatory cytokines was significantly inhibited by the treatment of SGRS. These findings indicate that inhibition of pro-inflammatory cytokines by SGRS may offer an ideal mean to treat inflammatory disorders.

Expression of iNOS, COX-2, and pro-inflammatory cytokines is regulated at the transcriptional level by NF-κB, which acts as their major transcriptional regulator3. In a resting cell, IκB-α retains NF-κB in the cytoplasm by masking nuclear localization sequences on NF-κB subunits6. Since IκB is dissociated from NF-κB upon phosphorylation, its content in the cytosol reflects the status of NF-κB, i.e. a higher IκB level indicates cytoplasmic localization of NF-κB while a higher p-IκB level indicates the nuclear localization of NF-κB. Many chemo-preventive and anti-inflammatory agents have been shown to reduce inflammatory symptoms by suppressing NF-κB expression. Our results show that treatment with LPS resulted in increased levels of NF-κB and p-IκBα, whereas treatment with SGRS inhibited nuclear translocation of NF-κB (Fig. 4A) and levels of p-IκBα (Fig. 4B). These findings suggest that treatment with SGRS inhibited NF-κB activation by suppressing the p-IκBα level and nuclear translocation of NF-κB in LPS-induced RAW 264.7 cells. Taken together, the current study shows that inhibition of NF-κB activation by SGRS is associated with reduced induction of iNOS, COX-2, TNF-α, IL-1β, and IL-6.

It is well known that extracellular stimuli can activate the members of the MAPK family, such as serine and threonine kinases that have the ability to mediate cell surface signal transduction to the nucleus. The intracellular
signals arising from MAPK cascades invariably lead to the activation of molecules that ultimately cause activation of NF-κB. Hence, inhibition of any or all three MAPKs can be sufficient to block the inflammatory response. A number of lignans with anti-inflammatory properties have been reported to efficiently block LPS-induced phosphorylation of MAPKs. However, the mode of action of lignans depends upon the substitution pattern in the core structure as well as the resultant derivatives that may target various proteins to bring about anti-inflammatory effects. The present study demonstrated that SGRS significantly reduced the phosphorylation and degradation of IκB-α, thereby inhibiting translocation of NF-κB subunits from the cytosol into the nucleus.

Saucerneol F, a new tetrahydrofuran-type sesquilignan isolated from Saururus chinensis, has been shown to directly inhibit IKK activity by oxidizing the critical cysteine residue and further inhibiting IκB-α phosphorylation. A similar mechanism of action may be responsible for SGRS inhibiting the phosphorylation of IκB-α and regulating the transcriptional activity of NF-κB. Further, the present study showed that SGRS prevented phosphorylation of p38 and JNK in response to LPS stimulation; however, inhibition of JNK phosphorylation was dominant compared to inhibition of p38 phosphorylation. It is evident that SGRS mediated inhibition of MAPKs, leading to transcriptional inactivation of NF-κB, which in turn further down-regulated COX-2 and iNOS expression and suppressed cytokine production. Thus, our findings suggest that SGRS can modulate NF-κB directly via IκB modification or indirectly via MAPK inhibition.

Carrageenan as an important phlogistic factor has the ability to induce a variety of inflammatory responses, such as neutrophil-mediated production of free radicals and mediators, neutrophil infiltration, paw edema, and capillary permeability. Mounting amount of research has proven carrageenan-induced hind paw acute edematous inflammation assay as an ideal animal model for evaluating the anti-inflammatory potential of any drug molecule. In our study, treatment with SGRS (50 mg/kg/day) resulted in significant reduction of mice paw edema volumes. In addition, SGRS treatment also mitigated mRNA expression of iNOS, COX-2, TNF-α, IL-1β, and IL-6 in carrageenan-induced mice compared to the control. Subsequently, Western blot analysis revealed that SGRS treatment also suppressed COX-2 as well as NF-κB protein expression in

**Figure 5.** Inhibition of carrageenan (CA)-induced paw edema by (+)-syringaresinol (SGRS). (A) Paw volumes were measured 0–4 h after carrageenan injection as described in materials and methods. Results represent the mean ± S.D. of five animals. *p < 0.01 significant compared with vehicle-treated control; **p < 0.05 and ***p < 0.01 significant compared with LPS alone by one way ANOVA, followed by a post hoc Dunnet test. (B) mRNA expression of iNOS, COX-2, TNF-α, IL-1β, and IL-6 of CA-induced paw skin. (C) protein expression of COX-2, and NF-κB in CA-induced.
carrageenan-induced mice (Fig. 5C). These findings advocate that the inhibitory mechanism of SGRS against LPS-induced NO, PGE2, TNF-α, IL-1β, and IL-6 production in RAW 264.7 cells may represent an important molecular action resulting in the inhibition of carrageenan-induced formation of paw edema. A systematic mechanism of anti-inflammatory effects of SGRS is summarized in Fig. 6.

This study first time reports isolation and characterization of a lignan, (+)-syringaresinol (SGRS), from Rubia philippinensis and demonstrated the anti-inflammatory efficacy of SGRS in LPS-stimulated RAW 264.7 cells in vitro and in a carrageenan-induced hind paw edema assay in experimental mice. Collectively, these results conclusively demonstrate that SGRS is an active ingredient of R. philippinensis that mediates anti-inflammatory effects by down-regulating NF-κB expression through interference with JNK and p38 phosphorylation and by reducing mRNA levels of iNOS, COX-2, TNF-α, IL-1β, and IL-6, thus suggesting its significant therapeutic potential.

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Acknowledgements
This work was supported by a National Research Foundation of Korea (NRF) grant (No. 2017R1A2A205001340 and 2016R1A2B4013374). This study was also supported by the research fund of Chungnam National University.

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V.K.B. and M.B.A. contributed in the study concept and design, acquisition of data, analysis and interpretation of data and drafting of manuscript; K.T.Q., M.K.J., R.M. and S.S. to acquisition of data, analysis and interpretation; Y.S.H., Y.K.H., M.K.N. and S.H.L. in the study supervision, study concept and design and critical revision of the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-27585-w.

Competing Interests: The authors declare no competing interests.

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