

Anti-inflammatory Effects of KOTMIN13: A Mixed Herbal Medicine in LPS-stimulated RAW 264.7 Cells and Mouse Edema Models

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ABSTRACT

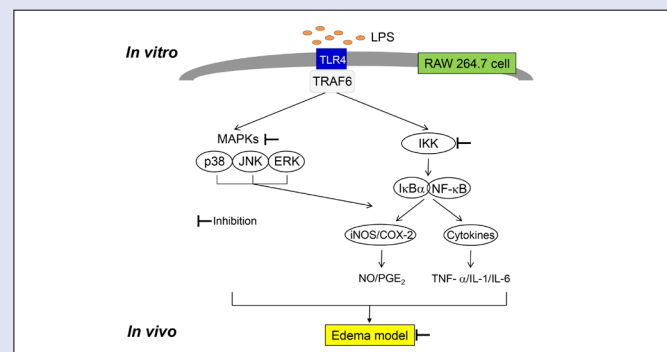
Background: A Korean herbal medicine, KOTMIN13, composed of *Inula japonica* Thunberg, *Trichosanthes kirilowii* Maximowicz var. *japonica* Kitamura, *Peucedanum praeruptorum* Dunn, and *Allium macrostemon* Bge, has been used for anti-allergic and anti-asthmatic treatment in oriental clinics, but its activity has not been investigated. **Materials and Methods:** To evaluate the anti-inflammatory activity of KOTMIN13 for *in vitro* study, LPS-stimulated RAW 264.7 cells were used to induce the production and expression of inflammatory mediators and its mechanisms. 12-*O*-Tetradecanoylphorbol-13 acetate (TPA)-induced ear edema and carrageenan-induced paw edema models were also used to evaluate the effect of KOTMIN13 on acute inflammation *in vivo*. **Results:** KOTMIN13 reduced the release of inflammatory mediators [nitric oxide, prostaglandin E₂, interleukin (IL)-1 β , and IL-6] and the protein expression of inducible nitric oxide synthase and cyclooxygenase-2 in LPS-stimulated RAW 264.7 cells. Mechanism studies showed the attenuation of LPS-induced NF- κ B activation by KOTMIN13 via I κ B α degradation abrogation and a subsequent decrease in nuclear p65 levels. Activation of mitogen-activated protein kinases (ERK, JNK, and p38) was also suppressed. Furthermore, KOTMIN13 ameliorated the development of TPA-induced ear edema and carrageenan-induced paw edema in acute inflammatory edema mouse models. **Conclusion:** Our study demonstrates that KOTMIN13 inhibits inflammatory mediators through the inhibitions of NF- κ B and MAPK activities in LPS-induced RAW 264.7 cells, as well as acute inflammation in edema models, indicating that KOTMIN13 is an effective suppressor for anti-inflammatory activities.

Key words: Inflammatory mediators, Nuclear Factor-Kappa β (NF- κ B), MAP (Mitogen-activated protein), 12-*O*-tetradecanoylphorbol-13 acetate-induced ear edema, carrageenan-induced paw edema

SUMMARY

- KOTMIN13 decrease the production of NO, PGE₂, and proinflammatory cytokine (TNF- α , IL-1 β , IL-6).

- KOTMIN13 Suppressed the degradation of NF- κ B and I κ B α and the phosphorylation of MAP Kinases.
- Topical application of KOTMIN13 reduced mouse ear edema.
- Oral administration of KOTMIN13 decreased carrageenan-induced paw edema.



Abbreviations used: NO: nitric oxide; PGE₂: prostaglandin E₂; iNOS: inducible NO synthase; COX-2: cyclooxygenase-2; TNF- α : tumor necrosis factor- α ; IL: interleukin; NF- κ B: nuclear factor kappaB; MAPK: mitogen-activated protein kinases; ERK: extracellular signal regulated kinase; JNK: c-jun N terminal kinase; TPA: 12-*O*-tetradecanoylphorbol-13-acetate

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INTRODUCTION

Inflammation is a host defense response to external challenge that leads to the release of inflammatory mediators. Among immune cells, macrophages are the major cell type that participates in the inflammatory process. Activated macrophages produce several inflammatory mediators such as nitric oxide (NO) and prostaglandin E₂ (PGE₂), which are synthesized by inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. In addition, they produce cytokines including tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6.^[1]

Expression of these inflammatory mediators is regulated by the activation of downstream signaling pathways such as the nuclear factor-kappa B (NF- κ B) and the mitogen-activated protein kinases (MAPK).^[2,3]

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The NF- κ B signaling pathway is closely related to MAPK activation, which affects the production of inflammatory mediators. MAPK family members, including extracellular signal regulated kinase (ERK), c-jun N terminal kinase (JNK), and p38 MAP kinase, integrate multiple signals from second messengers, resulting in cellular activities, such as gene expression, proliferation, differentiation, and cell survival.^[4] Upon stimulation by LPS, activated MAPK mediates signaling pathways leading to the activation of NF- κ B.^[5,6] Therefore, NF- κ B and MAPK are potential targets for inflammatory diseases.

Phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear and carrageenan-induced paw edema mouse models are widely used to search for new anti-inflammatory agents because these acute inflammatory responses immediately cause the release of several inflammatory mediators such as histamine, serotonin, prostaglandin.^[7-9]

In a search for herbal formulae to treat various inflammatory disorders, KOTMIN13, composed of *Inula japonica* Flowers, *Trichosanthes kirilowii* Semen, *Peucedanum praeruptorum* Radix, and *Allium macrostemon* Bulbs, was evaluated for its anti-inflammatory activity. It is modified from Guaruhaebaekju-tang, which is frequently used for asthma treatment in traditional herbal medicine. Although KOTMIN13 has been used for treatment of anti-inflammatory diseases in a local clinic, there are no investigations of KOTMIN13's inflammatory properties and molecular mechanisms. The present study demonstrates the anti-inflammatory effects of a KOTMIN13 ethanol extract using *in-vitro* assays and *in-vivo* models.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were purchased from Hyclone (Logan, Utah, USA). Lipopolysaccharide (LPS), Griess reagent, SP600125, SB203580, PD98059, and pyrrolidine dithiocarbamate (PDTC, specific inhibitor of NF- κ B) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Antibodies against iNOS and COX-2 were purchased from BD Biosciences (San Jose, California, USA) and Cayman Chemical (Ann Arbor, Michigan, USA), respectively. Anti-Phosphorylated or total antibodies to IKK, I κ B α , NF- κ B p65, JNK, ERK, and p38 were obtained from Cell Signaling (Beverly, Massachusetts, USA). Goat anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Preparation of KOTMIN13

Herbs (*I. japonica* Flowers, *Trichosanthes* Semen, *Anthriscus sylvestris* Radix, and *A. macrostemon* Bulbs) were purchased from Humanherb (Gyeongsan, Korea) and authenticated by Dr. H. Lee, a herbalist, in the Korea Promotion Institute for Traditional Medicine Industry. The herbs were mixed according to the ratio of combination (10:8:8:5), extracted with 30% ethanol at a ratio of 1:10 (w/v) and then refluxed for 24 h at 60°C. The extracted solution was filtered and the solvent evaporated under vacuum at 40°C (Eyela, Tokyo, Japan), before being freeze-dried to obtain a concentrated extract (15.4% yield).

Cell culture and measurement of cell viability

RAW 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and then cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, l-glutamine (2 mM), and 100 μ M MEM non-essential amino acid solution. Cell viability was assessed using the CellTiter 96 Aqueous One kit (Promega; Madison, Wisconsin, USA) as described previously.^[10] Briefly, RAW 264.7 cells (5×10^4 cells) were seeded onto each well of a 96-well plate and incubated at 37°C for overnight. Cells

were incubated with different concentrations of KOTMIN13 for 24 h and 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added to each well and incubated for 2 h. The optical densities were measured at 490 nm using a microplate reader (Tecan System, San Jose, California, USA).

Measurement of nitrite, PGE₂ and cytokine levels

Cells (2×10^5 cells) were pre-incubated with different concentrations of KOTMIN13 for 1 h, followed by LPS treatment for 18 h. The nitrite accumulated in culture medium was measured using Griess reagent and the absorbance was measured at 570 nm using a microplate reader (Tecan System). PGE₂ concentration in the culture supernatant was also measured to determine the inhibitory activity of KOTMIN13 using an enzyme immunoassay kit (EIA; Cayman Chemical, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. The cytokine concentrations of TNF- α , IL-1 β , and IL-6 in the supernatants of cell cultures were quantified using ELISA kits (R and D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions.

RNA isolation and real-time (RT)-PCR

Total RNA was extracted from RAW 264.7 cells using TRI Solution according to the manufacturer's instructions (BSK Bioscience, Gyeongbuk, Korea). Two grams of total RNA was converted into cDNA using OligodT₁₅ and Goscript Reverse transcription system kit (Promega). RT-PCR reaction was carried out on the StepOne Plus (Applied Biosystems, Foster City, California, USA) using HotStart SYBR Green qPCR Master Mix (USB, Cleveland, Ohio, USA). Primer sequences (Bioneer, Daejeon, Korea) were as follows: TNF- α sense GCA GAG AGG TTG ACT TTC and antisense CTA CTC CCA GGT TCT CTT CAA; IL-1 β sense AGT GCA GCT GTC TAA TGG GA and antisense GCC CAT CCT CTG TGA CTC A; IL-6 sense TCA GAA TTG CCA TTG CAC A and antisense GTC GGA GGC TTA ATT ACA CAT G; β -actin sense TGG ACA GTG AGG CCA GGA TAG and antisense TAC TGC CCT GGC TCC TAG CA. Each PCR cycle consisted of the three following steps: 95°C for 2 min, 95°C for 5 s, 60°C for 30 s. The results of RT-PCR were presented as pro-inflammatory cytokine gene (TNF- α , IL-1 β , IL-6) induction fold, and these were calculated using β -actin, which was amplified under the same conditions, as an internal control.

Western blot analysis

Total proteins from RAW 264.7 cells were prepared in RIPA lysis buffer (Pierce, Rockford, Illinois, USA) supplemented with a cocktail of protease inhibitors (Pierce). The nuclear and cytoplasmic extractions were prepared using the NE-PER nuclear protein extract kit (Pierce). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blocked in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) containing 5% non-fat dry milk and blotted with each primary antibody and its corresponding secondary antibody according to the manufacturer's instructions. The antibodies were then visualized using enhanced chemiluminescence solution (Pierce). The intensities of protein were determined by ImageQuant LAS 4000 luminescent image analyzer and ImageQuant TL software system (GE Healthcare, Little Chalfont, UK).

NF- κ B DNA binding activity

To examine the effect of KOTMIN13 on the binding activity of NF- κ B to DNA, nuclear extracts were prepared and NF- κ B activation was assessed using Trans-AM NF- κ B ELISA kit (Active Motif, Carlsbad, California, USA) according to the manufacturer's instructions.

Immunofluorescent staining

RAW 264.7 cells (3 x 10⁵ cells) were seeded in chamber slide (BD Falcon, Glendale, AZ, USA) and pre-treated with KOTMIN13 for 1 h. After stimulation with LPS (200 ng/mL) for 1 h, cells were washed and fixed with 3.7% paraformaldehyde, followed by incubation with 0.2% TritonX-100 (Sigma) for 10 min. Cells was blocked with 5% BSA for 1 h and then incubated with rabbit anti-p65 NF-κB antibody overnight at 4°C. After gently washing with PBS three times, the cells were incubated with Alex Fluor 488-conjugated goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, California, USA) and washed with PBS. Counter staining was performed with DAPI to visualize the cell nuclei for 10 min. NF-κB p65 subunit was observed with a fluorescent light microscope (Nikon, Tokyo, Japan).

Animals

BALB/c (6 weeks old, 16-20 g) mice were obtained from Koatek (Pyeongtaek, Gyeonggi-do, Korea), provided with laboratory feed (Purina, Seoul, Korea) and water ad libitum. Mice were maintained in a specific pathogen-free animal facility. All experimental procedures were approved by the Animal Care Committee of Korea Promotion Institute for Traditional Medicine Industry (Approval No. KOTMIN-2014-09).

Edema models

Ear and paw edema (*n* = 6) were induced according to the previously described procedure.^[11] In brief, the right ear of each mouse was received a topical application of TPA (Sigma) as 1.25 μg/10 μL acetone solution (each side of the ear). KOTMIN13 (0.5, 1, or 2 mg/ear) or Indomethacin (0.25 mg/ear, Sigma) was applied topically immediately after TPA treatment. The thickness of ears was measured before and at 1.5, 3, 4.5, and 6 h after TPA treatment using a micrometer (CD-15APX, Mitutoyo Co., Tokyo, Japan).

For paw edema, KOTMIN13 or Indomethacin (10 mg/kg) were dissolved in PBS and administered orally (50, 100, or 200 mg/kg) 30 min before carrageenan (Sigma) injection. Paw swelling was induced by injection of 30 μL of 1% v/v carrageenan solution into the plantar surface of left hind paw. The paw volume was measured by caliper immediately prior to the carrageenan injections and at 3 min, 0.5, 1, 1.5, 2, 2.5, and 3 h. Paw thickness was determined as the difference between the final and the initial thickness.

Statistical analysis

The data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used to determine the significance differences between the groups, followed by Duncan's multiple range tests using SPSS 19.0 (SPSS, Chicago, Illinois, USA). A probability < 0.05 was considered as significance.

RESULTS

The effects of KOTMIN13 on LPS-stimulated NO/PGE₂ production and iNOS/COX-2 expression

The cytotoxicity of KOTMIN13 in RAW 264.7 cells was examined with different concentration of KOTMIN13 for 24 h and the cell viability was not affected upto 400 μg/mL of KOTMIN13 (data not shown). To evaluate the inhibitory effects of KOTMIN13 on NO and PGE₂ production, the levels of nitrite and PGE₂ in the culture media were analyzed. [Figure 1a and b] shows that LPS stimulation increases the nitrite and PGE₂ production compared with un-stimulated cells. These increases were inhibited by KOTMIN13 treatment in a dose-dependent manner (IC₅₀ value of 17.7 μg/mL for nitrite and 54.3 μg/mL for PGE₂). Next, we measured the protein levels of iNOS and COX-2

by Western blot analysis. The expression was markedly increased with LPS stimulation and KOTMIN13 decreased the expression of iNOS and COX-2 expression in LPS-stimulated cells in a dose-dependent manner [Figure 1c and d].

Effect of KOTMIN13 on pro-inflammatory cytokine production

To investigate the inhibitory effects of KOTMIN13 on pro-inflammatory cytokine production and gene expression, we measured the production and mRNA levels of TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 cells. Figure 2a shows that LPS stimulation significantly increases pro-inflammatory cytokine production in the culture media. However, KOTMIN13 treatment dramatically reduced cytokine production compared with the supernatant of LPS-stimulated cells. We also evaluated the effects of KOTMIN13 on cytokine transcription levels using RT-PCR. The mRNA levels of cytokines were increased in LPS-stimulated cells; however, KOTMIN13 treatment decreased mRNA

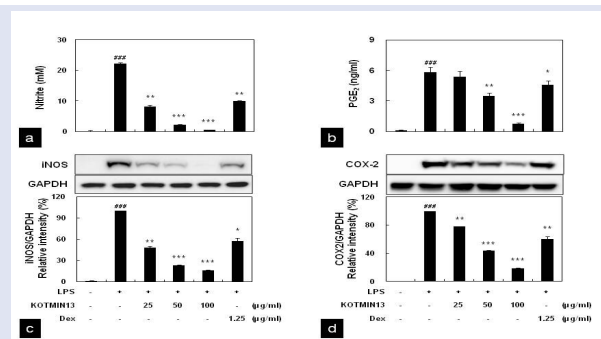


Figure 1

Figure 1: The effects of KOTMIN13 on NO/PGE₂ production and iNOS/COX-2 expression. (a and b) Secreted NO and PGE₂ were measured in the culture media by Griess reagents and an EIA kit, respectively. (c and d) Protein levels of iNOS and COX-2 were evaluated with Western blot analysis. Data represent the means ± SEM of three independent experiments. Significantly different from the LPS-unstimulated value, *###P* < 0.001. Significantly different from the LPS-stimulated value, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. The ratio of intensity between iNOS or COX-2 and GAPDH was calculated. Dex, dexamethasone.

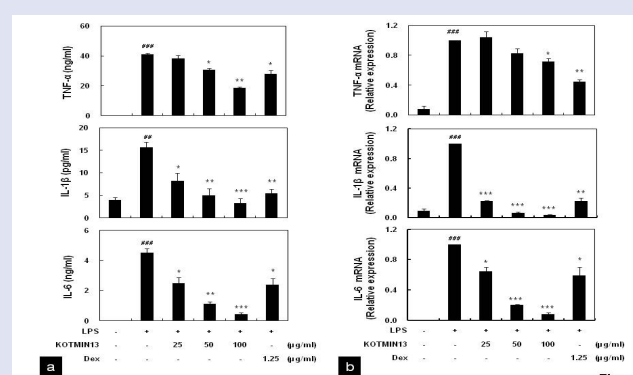


Figure 2

Figure 2: The effects of KOTMIN13 on the production and gene expression of pro-inflammatory cytokines. (a) Secreted TNF-α, IL-1β, and IL-6 protein in the cell culture media were determined using ELISA. (b) mRNA levels of TNF-α, IL-1β, and IL-6 were determined by a real time RT-PCR analysis. Data represent the means ± SEM of three independent experiments. Significantly different from the LPS-unstimulated value, *###P* < 0.001. Significantly different from the LPS-stimulated value, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Dex, dexamethasone.

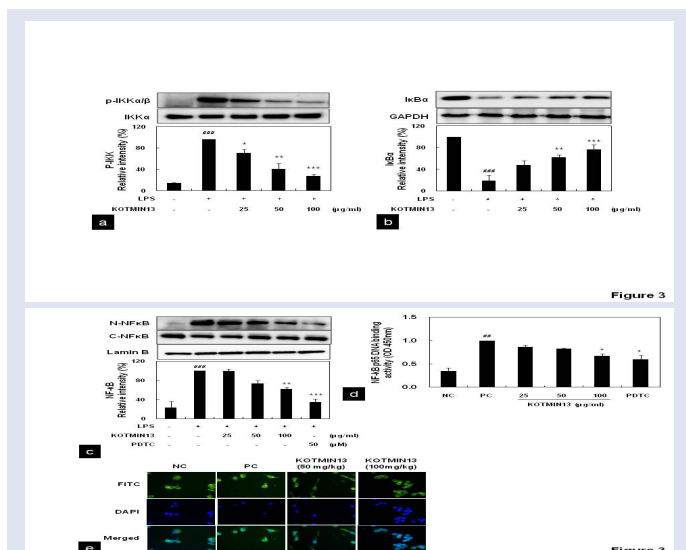


Figure 3: Effect of KOTMIN13 on the activation of NF-κB. (a-c) The levels of NF-κB p65 and IκBα/IKK proteins by Western blot analysis. (d) The binding activity of NF-κB to DNA. (e) Immunofluorescence analysis for detecting the localization of NF-κB p65. Magnification for images was 400x. Data represent the means ± SEM of three independent experiments. Significantly different from the LPS-unstimulated value, [#]*P* < 0.001 and ^{##}*P* < 0.001. Significantly different from the LPS-stimulated value, ^{*}*P* < 0.05, ^{**}*P* < 0.01, and ^{***}*P* < 0.001. NC, negative control (DMSO); PC, positive control (LPS-treatment group).

levels [Figure 2b]. These results suggest that KOTMIN13 can inhibit inflammatory mediators on mRNA and protein levels.

The effects of KOTMIN13 on the activation of IKK/IκBα/NF-κB signal pathways

Since the phosphorylation and degradation of IκBα are regulated by the IKKα/β complex, we first examined the effects of KOTMIN13 on IKK and IκB activation, which are upstream molecules involved in NF-κB signaling pathway. As shown in [Figure 3a], LPS induces IKKα/β phosphorylation and treatment with KOTMIN13 strongly inhibits IKKα/β phosphorylation without affecting total IKK levels. Moreover, KOTMIN13 markedly suppresses LPS-stimulated IκBα degradation [Figure 3b]. Similar inhibition of phosphorylation, as well as nuclear translocation of NF-κB p65 by KOTMIN13 was further confirmed by Western blotting [Figure 3c], TransAM assay [Figure 3d], and immunofluorescence staining [Figure 3e]. These results indicate that KOTMIN13 suppresses the production of inflammatory mediators through the inhibition of IKK/IκB/NF-κB activation.

The effects of KOTMIN13 on MAPK activation

To investigate further the possible mechanisms involved in the anti-inflammatory responses induced by KOTMIN13, we examined whether KOTMIN13 inhibited LPS-stimulated phosphorylation of MAPK by Western blot analysis. [Figure 4] shows that LPS alone significantly elevated the phosphorylation of ERK, JNK, and p38, whereas KOTMIN13 treatment suppressed the LPS-stimulated phosphorylation of all MAPK.

The effects of KOTMIN13 on edema models

Ear edema induced by irritants, such as TPA and croton oil, are applied in models of acute inflammation suitable for topically administered

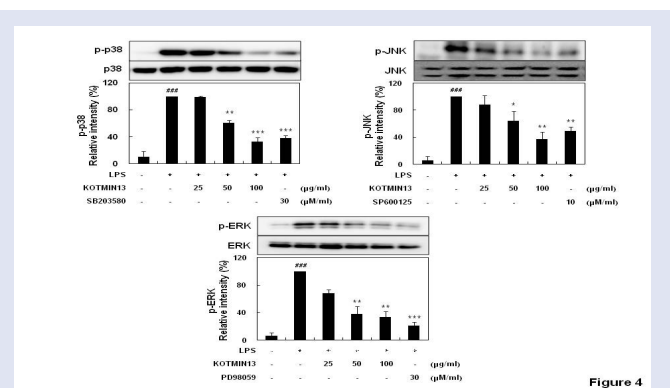


Figure 4: The effects of KOTMIN13 on the MAPK phosphorylation. RAW 264.7 cells were pre-treated with different concentrations of KOTMIN13 for 1 h and then stimulated with LPS (200 ng/mL) for 30 min. Phosphorylation and total protein expression of MAPK were detected by Western blot analysis using specific antibodies. Data represent the means ± SEM of three independent experiments. Significantly different from the LPS-unstimulated value, ^{###}*P* < 0.001. Significantly different from the LPS-stimulated value, ^{*}*P* < 0.05, ^{**}*P* < 0.01, and ^{***}*P* < 0.001.

agent evaluation, that is, substances from plant origins and plant extracts. In this study, a mouse model of ear edema induced with TPA was used to evaluate the anti-inflammatory activity of KOTMIN13. Each side of the right ear was stimulated with TPA (1.25 μg/10 μL) to induce inflammation and KOTMIN13 was applied to examine its effect on acute inflammation. The application of TPA on the ears produced ear swelling within 30 min (data not shown). Swelling was sustained up to 4.5 h and then gradually decreased [Table 1]. KOTMIN13 treatment significantly reduced TPA-induced ear edema at all evaluation time points dose dependently. When compared with indomethacin, a reference drug, KOTMIN13 showed a strong attenuation of ear edema at all doses after TPA application. We also investigated the effect of KOTMIN13 on acute inflammation in mice using a carrageenan-induced paw edema model. As shown in [Table 2], maximum paw edema occurred 1 h after carrageenan exposure and then tissue normalized in 3 h. Oral administration of KOTMIN13 reduced the increase in paw edema dose dependently compared with the TPA-treated control group. Indomethacin (10 mg/kg) inhibited paw edema, similarly to KOTMIN13-treated mice. These results confirm the anti-inflammatory effect of KOTMIN13 on topical acute inflammation *in vivo*.

DISCUSSION

Macrophages activated by LPS produce a variety of inflammatory mediators including NO, PGE₂, and pro-inflammatory cytokines.^[12] Therefore, inhibitors of inflammatory mediators are candidates for anti-inflammatory agents. Our results demonstrate that KOTMIN13 is an effective anti-inflammatory herb that decreases the release of inflammatory mediators. The underlying mechanism is that the production of NO and PGE₂ are downregulated by the decreased expression of iNOS and COX-2, respectively. Furthermore, pro-inflammatory cytokine release is suppressed through KOTMIN13 inhibiting iNOS and COX-2 mRNA expression [Figure 1 and Figure 2]. The transcription factor, NF-κB and MAPK are involved in signal transduction pathways that lead to the regulation of inflammatory mediators.^[13] Activated NF-κB up-regulates a number of genes, including iNOS, COX-2, TNF-α, IL-1β, and IL-6. Phosphorylation of MAPK can also promote the production of pro-inflammatory cytokines.^[14] Therefore, a number of anti-inflammatory drugs that target NF-κB and

Table 1 Effect of KOTMIN13 on TPA-induced ear edema in mice

| Group | Dose (mg/kg) | Ear thickness (mm) (Increase of ear volume) | | | | |
|--------------|--------------|--|--------------------------------------|---------------------------------------|--|--|
| | | 0 | 1.5 h | 3 h | 4.5 h | 6 h |
| Normal | - | 0.24 ± 0.02 (0%) | 0.24 ± 0.02 (0%) | 0.24 ± 0.02 (0%) | 0.24 ± 0.02 (0%) | 0.24 ± 0.02 (0%) |
| Control | - | 0.28 ± 0.03 (0%) | 0.49 ± 0.05 [#] (75.0%) | 0.54 ± 0.05 [#] (92.9%) | 0.75 ± 0.05 [#] (167.9%) | 0.74 ± 0.06 [#] (164.3%) |
| KOTMIN13 | 0.5 | 0.27 ± 0.03 (0%) | 0.38 ± 0.06 [*] (40.7%) | 0.45 ± 0.05 [*] (66.7%) | 0.57 ± 0.07 ^{***} (111.1%) | 0.55 ± 0.05 ^{***} (103.7%) |
| | 1 | 0.28 ± 0.04 (0%) | 0.33 ± 0.03 ^{**} (17.9%) | 0.44 ± 0.08 ^{**} (57.1%) | 0.56 ± 0.03 ^{***} (100%) | 0.54 ± 0.05 ^{***} (92.9%) |
| | 2 | 0.27 ± 0.03 (0%) | 0.31 ± 0.08 ^{**} (14.8%) | 0.39 ± 0.05 ^{***} (44.4%) | 0.52 ± 0.04 ^{***} (92.6%) | 0.51 ± 0.06 ^{***} (88.9%) |
| Indomethacin | 0.25 | 0.27 ± 0.03 (0%) | 0.31 ± 0.08 [*] (14.8%) | 0.44 ± 0.05 ^{**} (57.1%) | 0.49 ± 0.07 ^{**} (81.5%) | 0.47 ± 0.03 ^{***} (74.1) |

Significantly different from the normal value, [#]*p* < 0.05 and ^{**}*p* < 0.01.

Significantly different from the control (TPA-induced) value, ^{*}*p* < 0.05, ^{**}*p* < 0.01, and ^{***}*p* < 0.001.

Table 2 Effect of KOTMIN13 on carrageenan-induced paw edema in mice

| Group | Dose (mg/kg) | Paw thickness (mm) (Increase of paw volume) | | | | | | | |
|--------------|--------------|--|---|---|--|--|---|--|--|
| | | 0 | 3 min | 0.5 h | 1 h | 1.5 h | 2 h | 2.5 h | 3 h |
| Normal | - | 2.38 ± 0.2 (0%) | 2.38 ± 0.2 (0%) | 2.38 ± 0.2 (0%) | 2.37 ± 0.4 (-0.4%) | 2.38 ± 0.3 (0%) | 2.39 ± 0.2 (0.4%) | 2.38 ± 0.3 (0%) | 2.38 ± 0.2 (0%) |
| Control | - | 2.39 ± 0.3 (0%) | 3.38 ± 0.3 [#] (41.4%) | 3.57 ± 0.4 [#] (49.4%) | 3.71 ± 0.4 [#] (55.2%) | 3.49 ± 0.4 [#] (46.0%) | 3.04 ± 0.5 (27.2%) | 2.69 ± 0.6 (12.6%) | 2.55 ± 0.4 (6.7%) |
| KOTMIN13 | 50 | 2.38 ± 0.2 (0%) | 3.40 ± 0.6 (42.9%) | 3.53 ± 0.3 (48.3%) | 3.57 ± 0.4 [*] (50.0%) | 3.31 ± 0.3 ^{**} (39.1%) [*] | 2.94 ± 0.5 (23.5%) | 2.62 ± 0.5 (10.1%) | 2.47 ± 0.4 [*] (3.8%) [*] |
| | 100 | 2.38 ± 0.4 (0%) | 3.38 ± 0.5 [*] (42.0%) [*] | 3.53 ± 0.5 (48.3%) | 3.56 ± 0.6 [*] (49.6%) [*] | 3.27 ± 0.3 [*] (37.4%) ^{**} | 2.79 ± 0.6 ^{**} (17.2%) ^{**} | 2.56 ± 0.4 [*] (7.6%) [*] | 2.47 ± 0.4 [*] (3.8%) [*] |
| | 200 | 2.39 ± 0.2 (0%) | 3.39 ± 0.2 [*] (41.8%) [*] | 3.48 ± 0.6 [*] (45.6%) [*] | 3.52 ± 0.6 ^{**} (47.3%) [*] | 3.15 ± 0.3 ^{***} (31.8%) ^{**} | 2.83 ± 0.3 ^{**} (18.4%) [*] | 2.64 ± 0.6 (7.1%) [*] | 2.41 ± 0.3 ^{**} (0.8%) ^{**} |
| Indomethacin | 10 | 2.38 ± 0.2 (0%) | 3.41 ± 0.3 [*] (43.3%) | 3.52 ± 0.3 [*] (47.9%) [*] | 3.52 ± 0.6 ^{**} (47.9%) [*] | 3.27 ± 0.3 ^{**} (37.4%) ^{**} | 2.86 ± 0.6 [*] (20.2%) [*] | 2.57 ± 0.6 [*] (8.0%) [*] | 2.47 ± 0.4 [*] (3.8%) [*] |

Significantly different from the normal value, [#]*p* < 0.05 and ^{**}*p* < 0.01.

Significantly different from the control (carrageenan-induced) value, ^{*}*p* < 0.05, ^{**}*p* < 0.01, and ^{***}*p* < 0.001.

MAPK control the transcription of COX-2, iNOS, and pro-inflammatory cytokines. In this study, we demonstrate that KOTMIN13 suppressed the phosphorylation of IKK and IκBα degradation as well as NF-κB p65 nuclear translocation in LPS-stimulated RAW 264.7 cells [Figure 3]. Similar inhibition of the nuclear translocation of NF-κB by KOTMIN13 was confirmed further by TransAM assay and immunofluorescence staining [Figure 3]. Additionally, KOTMIN13 markedly suppressed LPS-stimulated MAPK [Figure 4], suggesting that the suppression of MAPK phosphorylation might be involved in inhibition of the LPS-stimulated production of NO, PGE₂, and pro-inflammatory cytokines.

We also used TPA-induced ear and carrageenan-induced paw edema models to verify the anti-inflammatory activity of KOTMIN13 because these are well-established models^[15] for screening the anti-inflammatory drugs *in vivo*. The development of edemas induced by TPA and carrageenan involves the release of inflammatory mediators such as histamine, PG, and cytokines.^[16,17] We observed that the topical application of TPA increased the ear thickness and weight (data not

shown) after exposure to TPA. However, KOTMIN13 treatment right after TPA application clearly reduced ear thickness (50 % decrease at a dose of 200 mg/kg) compared with the TPA-treated control group [Table 1]. The reason that the decreased percentage of ear weight was less than that of thickness is due to leakage, possibly containing plasma, from the punched ear at 6 h. The effect of KOTMIN13 on acute and local inflammation in mice was also evaluated by carrageenan-induced paw edema. Oral administration of KOTMIN13 alleviated paw edema after carrageenan injection compared with the carrageenan-induced control group [Table 2]. The inhibitory effect of KOTMIN13 was comparable to that of indomethacin.

CONCLUSION

In summary, we demonstrate that KOTMIN13 inhibits the production of inflammatory mediators including NO, PGE₂, and pro-inflammatory cytokines in LPS-induced RAW 264.7 cells. These inhibitory effects are mediated through NF-κB and MAPK activity inhibition. In acute inflammatory edema

mouse models, KOTMIN13 ameliorates the development of TPA-induced ear edema and carrageenan-induced paw edemas, indicating KOTMIN13 may have potential as an anti-inflammatory agent.

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Conflicts of interest

There are no conflicts of interest.

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