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 praeuptorum 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)-1β, 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-κB activation by KOTMIN13 via IκBα 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-κB 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-KappaB (NF-κB), 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-1β, IL-6).

• KOTMIN13 Suppressed the degradation of NF-κB and IκBα and the phosphorylation of MAP Kinases.

• Topical application of KOTMIN13 reduced mouse ear edema.

• Oral administration of KOTMIN13 decreased carrageenan-induced paw edema.

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)-1β, 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-κB) and the mitogen-activated protein kinases (MAPK). [2,3]

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The NF-kB 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. Upon stimulation by LPS, activated MAPK mediates signaling pathways leading to the activation of NF-kB. Therefore, NF-kB 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.

In a search for herbal formulae to treat various inflammatory disorders, KOTMIN13, composed of *Inula japonica* Flowers, *Trichosanthes kirilowii* Semen, *Paeceudanum praeruptorum* Radix, and *Allium macrostemum* Bulbs, was evaluated for its anti-inflammatory activity. It is modified from Guaruhaebaebaekju-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 pyrrolidin dithiocarbonate (PDTC, specific inhibitor of NF-kB) 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 IκK, IκBa, NF-κB 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. macrostemum* Bulbs) were purchased from Humanherb (Gyeongbuk, 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 (Eylau, 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 μg/ml streptomycin, l-glutamine (2 mM), and 100 μM MEM non-essential amino acid solution. Cell viability was assessed using the CellTiter 96 Aqueous One kit (Promega; Madison, Wisconsin, USA) as described previously. Briefly, RAW 264.7 cells (5 x 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 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-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 x 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 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-1β, 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 OligoDT and Gsorscript 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 AAC TTC and antisense CTA CTC CCA GGT TCT CCT CAA; IL-1β sense AGT GCA GCA GCT GTC TAA TGG GA and antisense GCC CAT CTG TTA GCA GAA and antisense GCC CAT CTG TTA GCA GAA and antisense GCC CAT CTG TTA GCA GAA and antisense GCC CAT CTG TTA GCA GAA and antisense GCC CAT CTG TTA GCA GAA and antisense GCC CAT CTG TTA GCA GAA. Each PCR cycle consisted of the 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-1β, 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 onto 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-κB DNA binding activity**

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

RAW 264.7 cells (3 x 10^5 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 were blocked with 5% BSA for 1 h and then incubated with rabbit anti-p65 NF-κB 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, Gyeonggido, 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. 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/ PGE_2 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 up to 400 μg/mL of KOTMIN13 (data not shown). To evaluate the inhibitory effects of KOTMIN13 on NO and PGE_2 production, the levels of nitrite and PGE_2 in the culture media were analyzed. [Figure 1a and b] shows that LPS stimulation increases the nitrite and PGE_2 production compared with un-stimulated cells. These increases were inhibited by KOTMIN13 treatment in a dose-dependent manner (IC_50 value of 17.7 μg/mL for nitrite and 54.3 μg/mL for PGE_2.). 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...
The effects of KOTMIN13 on the activation of IKK/IκB/NF-κB signal pathways

Since the phosphorylation and degradation of IκBα are regulated by the IKKa/β complex, we first examined the effects of KOTMIN13 on IKK and IκB activation, which are upstream molecules involved in NF-kB signaling pathway. As shown in Figure 3a, LPS induces IKKa/β phosphorylation and treatment with KOTMIN13 strongly inhibits IKKa/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-kB 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-kB 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 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 is downregulated by the deceased 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-kB and MAPK are involved in signal transduction pathways that lead to the regulation of inflammatory mediators.13 Activated NF-kB 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-kB and
Table 1 Effect of KOTMIN13 on TPA-induced ear edema in mice

| Group     | Dose (mg/kg) | Ear thickness (mm) | Ear (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%)                         |
| Control   | -           | 0.28 ± 0.03         | (0%)                         | 0.49 ± 0.05*                 | (92.9%)                      | 0.54 ± 0.05*                 | (167.9%)                     |
| KOTMIN13  | 0.5         | 0.27 ± 0.03         | (0%)                         | 0.38 ± 0.06                  | (46.1%)                      | 0.45 ± 0.05                  | (66.7%)                      |
|           | 1           | 0.28 ± 0.04         | (0%)                         | 0.33 ± 0.03**                | (57.1%)                      | 0.44 ± 0.08                  | (100%)                       |
|           | 2           | 0.27 ± 0.03         | (0%)                         | 0.31 ± 0.08**                | (44.4%)                      | 0.39 ± 0.05**                | (103.7%)                     |
| Indomethacin | 0.25       | 0.27 ± 0.03         | (0%)                         | 0.31 ± 0.08                  | (14.8%)                      | 0.44 ± 0.05                  | (103.7%)                     |

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) | Paw (Increase of paw volume) |
|-----------|--------------|--------------------|------------------------------|
|           | 0           | 1 h                | 1.5 h                        | 2 h                          | 2.5 h                        | 3 h                          |
| Normal    | -           | 2.38 ± 0.2         | (0%)                         | 2.37 ± 0.4                   | (-0.4%)                      | 2.38 ± 0.3                   | (0%)                         |
| Control   | -           | 2.39 ± 0.3         | (0%)                         | 3.57 ± 0.4**                 | (55.2%)                      | 3.49 ± 0.4**                 | (27.2%)                      |
| KOTMIN13  | 0.5         | 2.38 ± 0.2         | (0%)                         | 3.53 ± 0.3                   | (50.0%)                      | 3.31 ± 0.3**                 | (23.5%)                      |
|           | 1           | 2.38 ± 0.4         | (0%)                         | 3.53 ± 0.5                   | (42.9%)                      | 3.56 ± 0.6                   | (23.5%)                      |
|           | 2           | 2.38 ± 0.4         | (0%)                         | 3.53 ± 0.5                   | (42.0%)                      | 3.56 ± 0.6                   | (27.2%)                      |
| Indomethacin | 0.25       | 2.39 ± 0.2         | (0%)                         | 3.48 ± 0.6                   | (49.6%)                      | 3.27 ± 0.3                   | (7.6%)                       |

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-kB 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 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. 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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