

# Kaempferol-3-O- $\beta$ -rutinoside suppresses the inflammatory responses in lipopolysaccharide-stimulated RAW264.7 cells via the NF- $\kappa$ B and MAPK pathways

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**Abstract.** Kaempferol-3-O- $\beta$ -rutinoside is one of the compounds isolated from tartary buckwheat (*Fagopyrum tataricum*), and its biological effects have not been studied yet. The present study examined the anti-inflammatory effects of kaempferol-3-O- $\beta$ -rutinoside and explore its regulatory mechanisms in lipopolysaccharide (LPS)-induced macrophage RAW264.7 cells. Kaempferol-3-O- $\beta$ -rutinoside exhibited no cytotoxic effect in RAW 264.7 macrophage and 293 cell lines up to 300  $\mu$ M. As the concentration of kaempferol-3-O- $\beta$ -rutinoside was increased, the activity of nitric oxide was inhibited in LPS-stimulated RAW264.7 cells. In addition, kaempferol-3-O- $\beta$ -rutinoside treatment down-regulated the expression of inflammation-related cytokines tumor necrosis factor- $\alpha$  and interleukin-6 in LPS-stimulated RAW264.7 cells. Furthermore, kaempferol-3-O- $\beta$ -rutinoside treatment suppressed inflammatory-mediated factors, such as inducible nitric oxide synthase and cyclooxygenase-2. These inflammation-related proteins are known to be regulated by NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) signaling, therefore the effect of kaempferol-3-O- $\beta$ -rutinoside on these pathways was investigated. The results demonstrated that kaempferol-3-O- $\beta$ -rutinoside decreased the expression of inhibitor of  $\kappa$ B (I $\kappa$ B) protein and I $\kappa$ B kinases; as a result, the nuclear translocation and expression of NF- $\kappa$ B was inhibited in LPS-stimulated RAW264.7 cells. Furthermore, kaempferol-3-O- $\beta$ -rutinoside inhibited the phosphorylation of p38, extracellular signal-regulated kinase and stress-activated protein kinase in LPS-stimulated RAW264.7 cells. Thus, the present data demonstrated that kaempferol-3-O- $\beta$ -rutinoside

suppressed inflammation-related gene expression through the NF- $\kappa$ B and MAPK pathways, and suggested that it may be a useful reagent in pharmacological research.

## Introduction

Inflammation is a protective mechanism activated by pathogenic stimuli, which removes harmful factors and repairs damaged tissue through associated signaling pathways (1). Chronic inflammation is associated with multiple diseases, such as asthma, atherosclerosis, rheumatoid arthritis (RA), colitis and inflammatory bowel diseases (IBD) (2). Lipopolysaccharide (LPS) is expressed at the cell wall of gram-negative bacteria and it acts as an endotoxin (3). The LPS stimulus initiates the differentiation of macrophages and promotes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression (4,5). Nitric oxide (NO) is a signaling molecule that has multiple molecular targets and regulates several responses in mammalian cells. Nitric oxide synthases (NOSs) employ L-arginine as a substrate; NOS initiates to oxidize L-arginine to NO and L-citrulline (6). Notably, iNOS is controlled by activated cytokines and induces transcriptional activity in macrophage cells (7). The expression levels of COX-2 have been reported to be increased during inflammation (8). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-6 are regarded as pro-inflammatory cytokines that regulate the inflammatory responses (9,10).

The production of iNOS, COX-2 and pro-inflammatory cytokines are controlled by NF- $\kappa$ B, which is an essential transcription factor in the inflammatory mechanism (11). In the cytoplasm, NF- $\kappa$ B is combined with inhibitor of  $\kappa$ B (I $\kappa$ B) proteins and maintained in an inactive state (12). Following an inflammatory stimulus, I $\kappa$ B is phosphorylated by the I $\kappa$ B kinase (IKK) protein, resulting in the degradation of I $\kappa$ B, and the subsequent release of NF- $\kappa$ B; free NF- $\kappa$ B then translocates to the nucleus and it starts its transcriptional activity (13).

Mitogen-activated protein kinase (MAPK) pathways are activated as a response to numerous signal transductions, including inflammatory signaling (14). MAPK pathways are stimulated by inflammatory cytokines or chemical stress (15).

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LPS stimulation also activates MAPKs; therefore, multiple studies have investigated the involvement of NF- $\kappa$ B and MAPK pathways in inflammatory responses (16-18).

Kaempferol is widely known for its anti-inflammatory activity through the NF- $\kappa$ B pathway and kaempferol-3-O-rutinoside is isolated from tartary buckwheat (*Fagopyrum taticum*) (19,20). The bioactivity or mechanism of kaempferol-3-O- $\beta$ -rutinoside has not been extensively studied; therefore, the present study used kaempferol-3-O- $\beta$ -rutinoside to evaluate its potential anti-inflammatory effect and mechanism in LPS-stimulated RAW 264.7 macrophage cells.

## Materials and methods

**Chemicals and antibodies.** Kaempferol-3-O- $\beta$ -rutinoside (cat. no. 90242), kaempferol (cat. no. K0133) and LPS from *Escherichia coli* (cat. no. L2630) were purchased from Sigma-Aldrich (Merck KGaA). Kaempferol-3-O- $\beta$ -rutinoside was dissolved in DMSO (100 mM), aliquoted and stored at -80°C. Prior to each experiment, kaempferol-3-O- $\beta$ -rutinoside was diluted fresh in warm Dulbecco's modified Eagle's medium (DMEM). After checking that there was no precipitate, RAW264.7 cells were treated.

Monoclonal antibodies directed against GAPDH (cat. no. 2118), iNOS (cat. no. 13120), COX-2 (cat. no. 12282), TNF- $\alpha$  (cat. no. 3707), members of the NF- $\kappa$ B pathway [phosphorylated (p-) IKK $\alpha$ , IKK $\beta$ , p-IKK $\beta$ , IKK $\beta$ , p-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , p-NF- $\kappa$ B and NF- $\kappa$ B; NF- $\kappa$ B sampler kit; cat. no. 9936], p-p38 (Thr180/Tyr 182; cat. no. 9215), p38 (cat. no. 9212), p-p44/42 [also known as extracellular signal-regulated kinase (ERK) 1/2; Thr202/Tyr204; cat. no. 4377], p44/42 (cat. no. 4695), stress-activated protein kinase (SAPK, also known as JNK2; cat. no. 9252) and p-SAPK (Thr183/Tyr185; cat. no. 4671) were purchased from Cell Signaling Technology, Inc. Antibodies against IL-1 $\beta$  (cat. no. sc-12742) and IL-6 (cat. no. sc-57315) were purchased from Santa Cruz Biotechnology, Inc. All antibodies were diluted 1:1,000 with 5% bovine serum albumin.

**Cell culture.** RAW 264.7 and 293 cells were obtained from the American Type Culture Collection. Both cell lines were grown in DMEM (Cellgro; Corning, Inc.) containing 10% heat-inactivated fetal bovine serum (Cellgro; Corning, Inc.), penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml; PAA Laboratories GmbH; GE Healthcare). Cells were maintained in a humidified incubator under 5% CO<sub>2</sub> at 37°C and the medium was refreshed every 2 days. All experiments were performed with healthy cells obtained between passages 3 and 5.

**Cell viability assay.** The cytotoxicity of kaempferol-3-O- $\beta$ -rutinoside was assayed as reported previously (21). Briefly, cells were treated with kaempferol-3-O- $\beta$ -rutinoside for 24 h. The medium was replaced with fresh DMEM, and WST-1 solution (Daeil Lab Services Company, Ltd.) was added for a further 3 h. The absorbance was then measured at 460 nm using a microplate reader (Molecular Devices LLC).

**NO assay.** To measure the inhibitory activity of kaempferol-3-O- $\beta$ -rutinoside on NO production, the protocol from a previous study was followed (21). Briefly, the cells were treated with

kaempferol-3-O- $\beta$ -rutinoside for 2 h, and then LPS (1  $\mu$ g/ml) was added for 18 h. Supernatants were mixed with the same volume of Griess reagent (Sigma-Aldrich; Merck KGaA) and incubated for 15 min. The absorbance of the mixture was measured at 540 nm using a microplate reader (Molecular Devices LLC).

**Western blot analysis.** Western blot analysis was performed according to a previous study (21). Whole-cell protein was lysed with cell lysis buffer and supernatants were obtained and quantified using the Bradford reagent (Biosesang Co., Ltd.). A total of 50  $\mu$ g from each sample was denatured with sample buffer at 95°C for 5 min, separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and blocked with 5% skim milk in PBS with 1% Tween-20 at room temperature (RT) for 1 h. After blocking, the membrane was incubated with specific antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies [anti-rabbit immunoglobulin (Ig) G, cat. no. 7074; anti-mouse IgG, cat. no. 7076; and anti-rat IgG, cat. no. 7077; all 1:20,000; Cell Signaling Technology, Inc.] at RT for 1 h. The membranes were developed on X-ray films using an enhanced chemiluminescence solution<sup>®</sup> (AbFrontier Co., Ltd.).

**Reverse transcription (RT)-PCR.** The mRNA expression levels of the cytokines TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were analyzed by RT-PCR, according to previous studies (22,23). Total RNA was isolated using the RNeasy<sup>®</sup> Plus Mini kit (Qiagen GmbH), and then cDNA was synthesized using AccuPower<sup>®</sup> RT premix (Bioneer Corporation). Each cDNA was amplified by PCR with Prime Tag Premix (Genetbio Co., Ltd.) using specific primers. The specific primers were as follows: TNF- $\alpha$ , 5'-CCCCTCAGCAAACCACCAAGT-3' (forward primer) and 5'-CTTGGGCAGATTGACCTCAGC-3' (reverse primer); IL-6, 5'-GGAGGCTTAATTACACATGTT-3' (forward primer) and 5'-TGATTTCAAGATGAATTG GAT-3' (reverse primer); IL-1 $\beta$ , 5'-AATCTCACAGCAGCA CATCAA-3' (forward primer) and 5'-AGCCCATACTTT AGGAAGACA-3' (reverse primer); and GAPDH, 5'-AAC TTTGGCATTGTGGAAGG-3' (forward primer) and 5'-CAC ATTGGGGGTAGGAACAC-3' (reverse primer). PCR was performed under the following conditions: denaturation (94°C, 1 min), annealing (TNF- $\alpha$ , 59.8°C; IL-1 $\beta$ , 55°C; IL-6, 48°C; and GAPDH, 55°C; 1 min) and extension (72°C, 1 min) for 30 cycles. The amplified PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide. The band intensity was measured using ImageJ 1.48v (National Institutes of Health).

**Immunofluorescence staining of NF- $\kappa$ B protein.** Immunofluorescence staining was performed according to a previous study (21). Cells were stained with DAPI and fixed with 4% formaldehyde at RT for 15 min. After fixation, samples were blocked with 5% rabbit serum (cat. no. sc-2338; Santa Cruz Biotechnology, Inc.) and permeabilized with 0.3% Triton X-100 at RT for 1 h in the dark. Cells were then incubated with a NF- $\kappa$ B p65 antibody (0.1  $\mu$ g/ml; cat. no. 8242; Cell Signaling Technology, Inc.) at RT for 2 h, followed by incubation with Alexa Fluor<sup>®</sup> 488-conjugated secondary antibody (1:2,000; cat. no. 4412; Cell Signaling Technology,

Inc.) at RT for 1 h. Samples were mounted using the Prolong® Gold Anti-fade reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and examined under a Carl Zeiss LSM 700 fluorescence microscope (Carl Zeiss AG). The translocation results were captured and processed with Zen 2011 SP2 software (Carl Zeiss AG).

**Statistical analysis.** All the experiments were performed in triplicate and the results are expressed as the mean  $\pm$  standard error of the mean. Statistical analyzes were performed using a one-way analysis of variance, followed by Dunnett's multiple comparison *post hoc* test for multigroup comparisons. The statistical significance of the results was analyzed by the R 3.5.2 program for Windows (24).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cytotoxicity of kaempferol-3-O- $\beta$ -rutinoside on RAW264.7 macrophage cells.** To determine the cytotoxicity of kaempferol-3-O- $\beta$ -rutinoside on RAW 264.7 and 293 cells, cell viability assays were performed. The viability of the treated cells was compared with untreated cells as a negative control, and with kaempferol-treated cells as a positive control. As Fig. 1A indicates, kaempferol-3-O- $\beta$ -rutinoside and kaempferol in doses up to 300  $\mu$ M did not exhibit any toxic effects in both the RAW264.7 and 293 cell lines. Over 90% of RAW264.7 cells survived at 400  $\mu$ M kaempferol and kaempferol-3-O- $\beta$ -rutinoside, and 86% of 293 cells survived at 400  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside. Approximately 50% of RAW264.7 cells survived at 500  $\mu$ M of kaempferol and kaempferol-3-O- $\beta$ -rutinoside, and 81% of 293 cells survived at 500  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside. Therefore, the results of cell viability assays demonstrated that a concentration up to 400  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside was not toxic to RAW264.7 and 293 cells; thus, concentrations  $< 400$   $\mu$ M were used for further experiments.

**Kaempferol-3-O- $\beta$ -rutinoside suppresses NO production.** To determine whether kaempferol-3-O- $\beta$ -rutinoside has an anti-inflammatory effect, Griess reagent was used to measure NO production, a known inflammatory mediator (21,25). Fig. 1B shows that as the concentration of kaempferol-3-O- $\beta$ -rutinoside was increased, NO production was significantly suppressed in LPS-stimulated RAW264.7 cells. Over 50% of NO production was suppressed at 300  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside treatment. Therefore, concentrations of up to 300  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside were used for further experiments, based on the observations that they did not affect cell viability but inhibited NO production.

**Kaempferol-3-O- $\beta$ -rutinoside downregulates the expression of inflammation-related factors.** Next, the present study investigated whether kaempferol-3-O- $\beta$ -rutinoside inhibited the expressions of the key inflammation-related proteins iNOS and COX-2. When iNOS is upregulated, it causes a high level of NO production, which affects the expression of pro-inflammatory cytokines (7); COX-2 is expressed during inflammation in response to infection or invasion of pathogens (5). Fig. 2

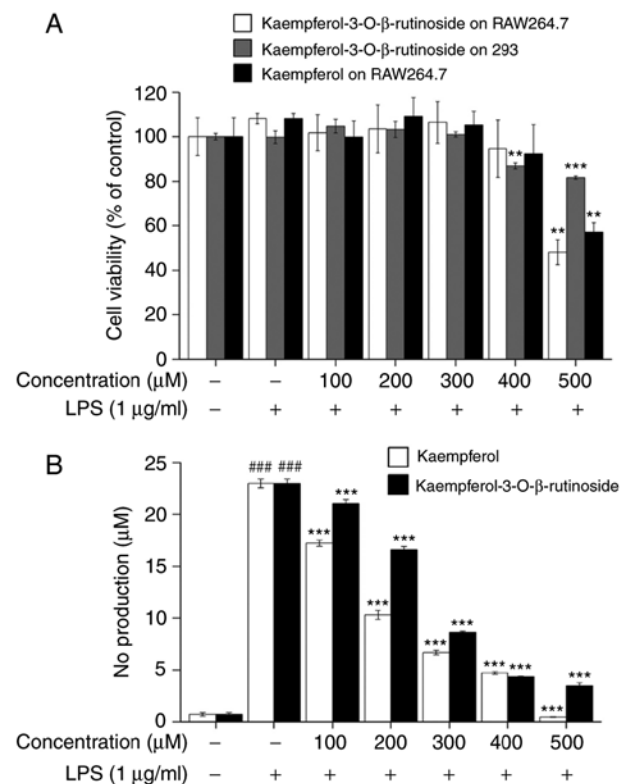


Figure 1. Effect of kaempferol-3-O- $\beta$ -rutinoside on cell viability and NO production. (A) RAW 264.7 macrophage and 293 cells were treated with kaempferol-3-O- $\beta$ -rutinoside (0-500  $\mu$ M) and LPS (1  $\mu$ g/ml) for 24 h. Cell viability was investigated using the WST-1 assay. (B) RAW 264.7 cells were pretreated with the indicated concentrations of kaempferol-3-O- $\beta$ -rutinoside for 2 h and then stimulated by LPS (1  $\mu$ g/ml) for a further 18 h. NO production was measured by Griess reagent. Data are presented as the mean  $\pm$  SEM of three independent experiments. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with the LPS-stimulated group; \*\*\* $P < 0.001$  compared with the untreated control. NO, nitric oxide; LPS, lipopolysaccharide.

shows that following kaempferol-3-O- $\beta$ -rutinoside treatment, the expression levels of iNOS and COX-2 were significantly suppressed in LPS-stimulated RAW264.7 cells, in a dose-dependent manner. Kaempferol-3-O- $\beta$ -rutinoside treatment completely reversed the LPS-induced iNOS and COX-2 expression at 300  $\mu$ M (Fig. 2).

In addition, the expression levels of TNF- $\alpha$ , IL- $\beta$  and IL-6 were analyzed at the mRNA (Fig. 3A) and protein level (Fig. 3B). The results demonstrated that kaempferol-3-O- $\beta$ -rutinoside downregulated the expression levels of the pro-inflammatory cytokines (Fig. 3). Kaempferol-3-O- $\beta$ -rutinoside decreased the TNF- $\alpha$  mRNA expression levels, and it almost completely inhibited its protein expression in LPS-stimulated RAW264.7 cells. In addition, kaempferol-3-O- $\beta$ -rutinoside suppressed IL-6 expression at both mRNA and protein level in LPS-stimulated RAW264.7 cells. However, IL-1 $\beta$  expression was not markedly changed by kaempferol-3-O- $\beta$ -rutinoside in LPS-stimulated RAW264.7 cells. The present data suggested that kaempferol-3-O- $\beta$ -rutinoside had an effect on the expression of inflammation-related factors iNOS, COX-2, TNF- $\alpha$  and IL-6.

**Kaempferol-3-O- $\beta$ -rutinoside suppresses the NF- $\kappa$ B pathway.** NF- $\kappa$ B is a transcription factor and the most significant

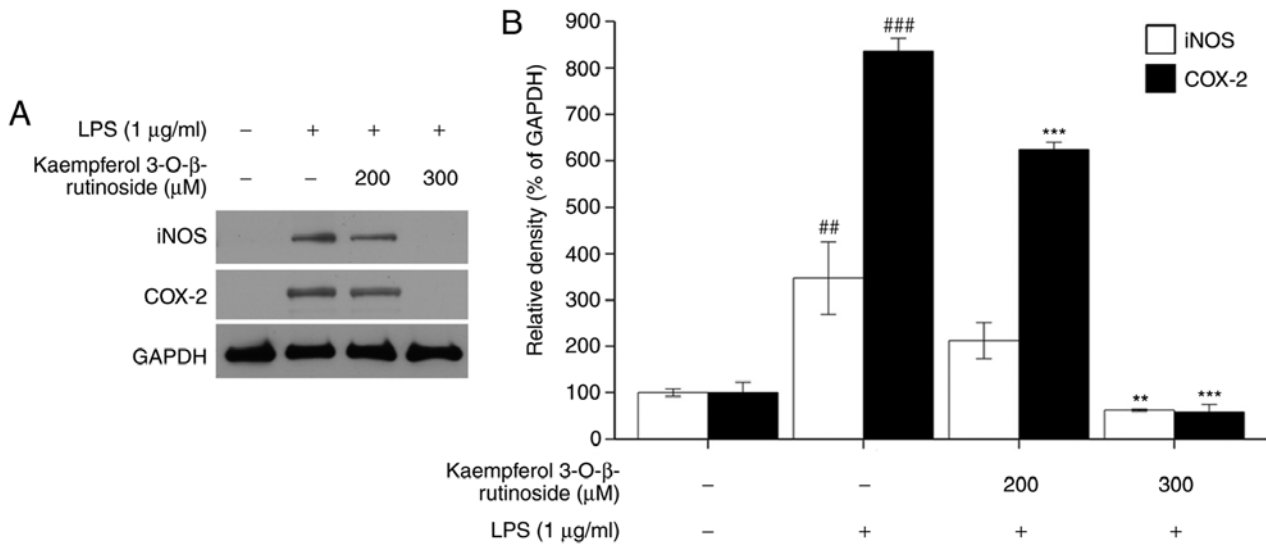


Figure 2. Effects of kaempferol-3-O- $\beta$ -rutinoside on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells. RAW264.7 cells were treated with 200 and 300  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside for 2 h and then stimulated with LPS (1  $\mu$ g/ml) for a further 18 h. iNOS and COX-2 expression levels were examined by western blot analysis. (A) Representative blots and (B) quantification of levels normalized to GAPDH. Data are presented as the mean  $\pm$  SEM of three independent experiments. \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared with the the LPS-stimulated group; ## $P$ <0.01 and ### $P$ <0.001 compared with the untreated control. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide.

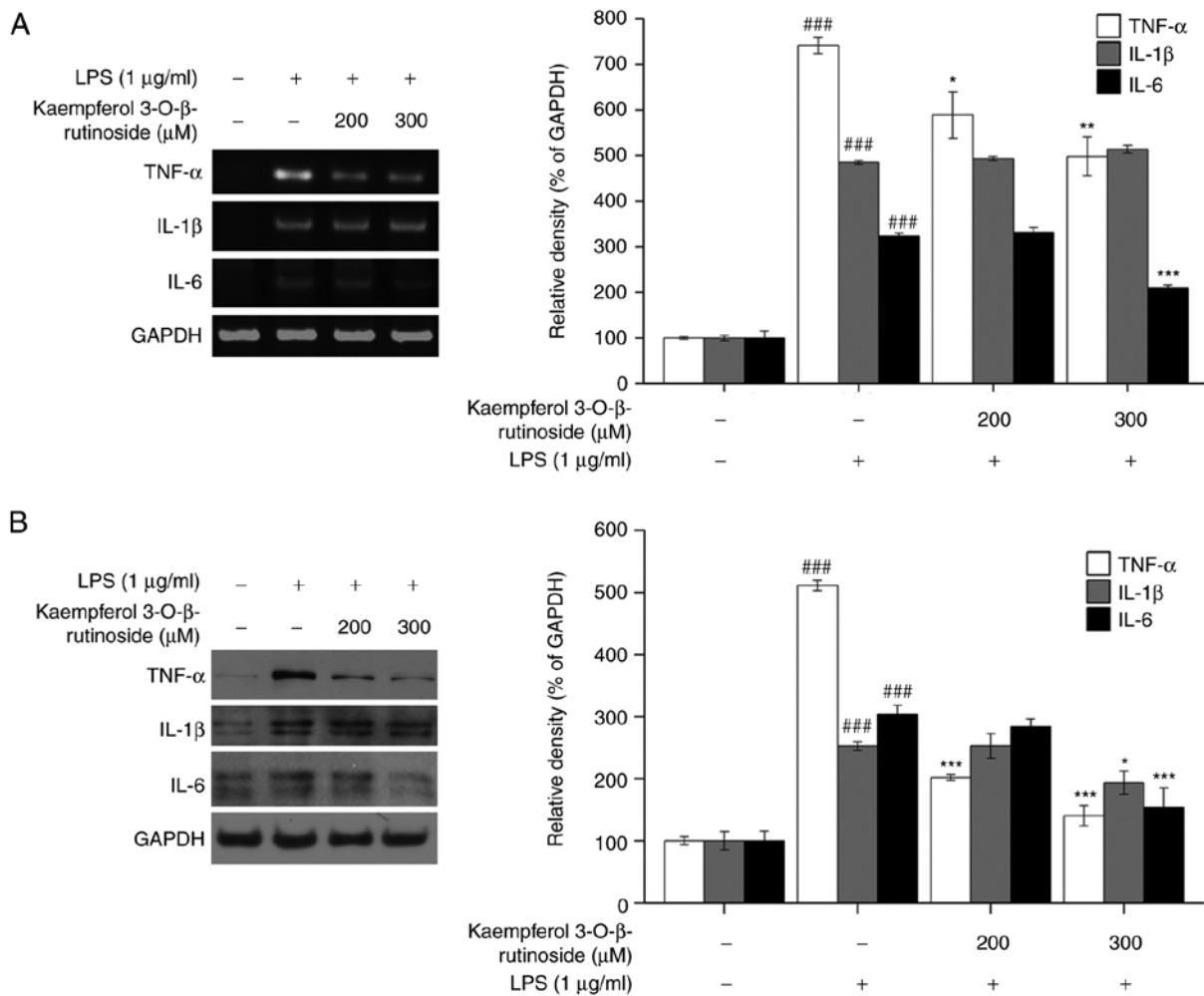


Figure 3. Effects of kaempferol-3-O- $\beta$ -rutinoside on cytokine expression in LPS-stimulated RAW 264.7 cells. RAW264.7 cells were treated with 200 and 300  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside for 2 h and then stimulated with LPS (1  $\mu$ g/ml) for a further 3 h. (A) The mRNA and (B) protein expression levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were detected using reverse transcription-PCR and western blotting, respectively. Data are presented as the mean  $\pm$  SEM of three independent experiments. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared with the LPS group; ### $P$ <0.001 compared with the untreated control. LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

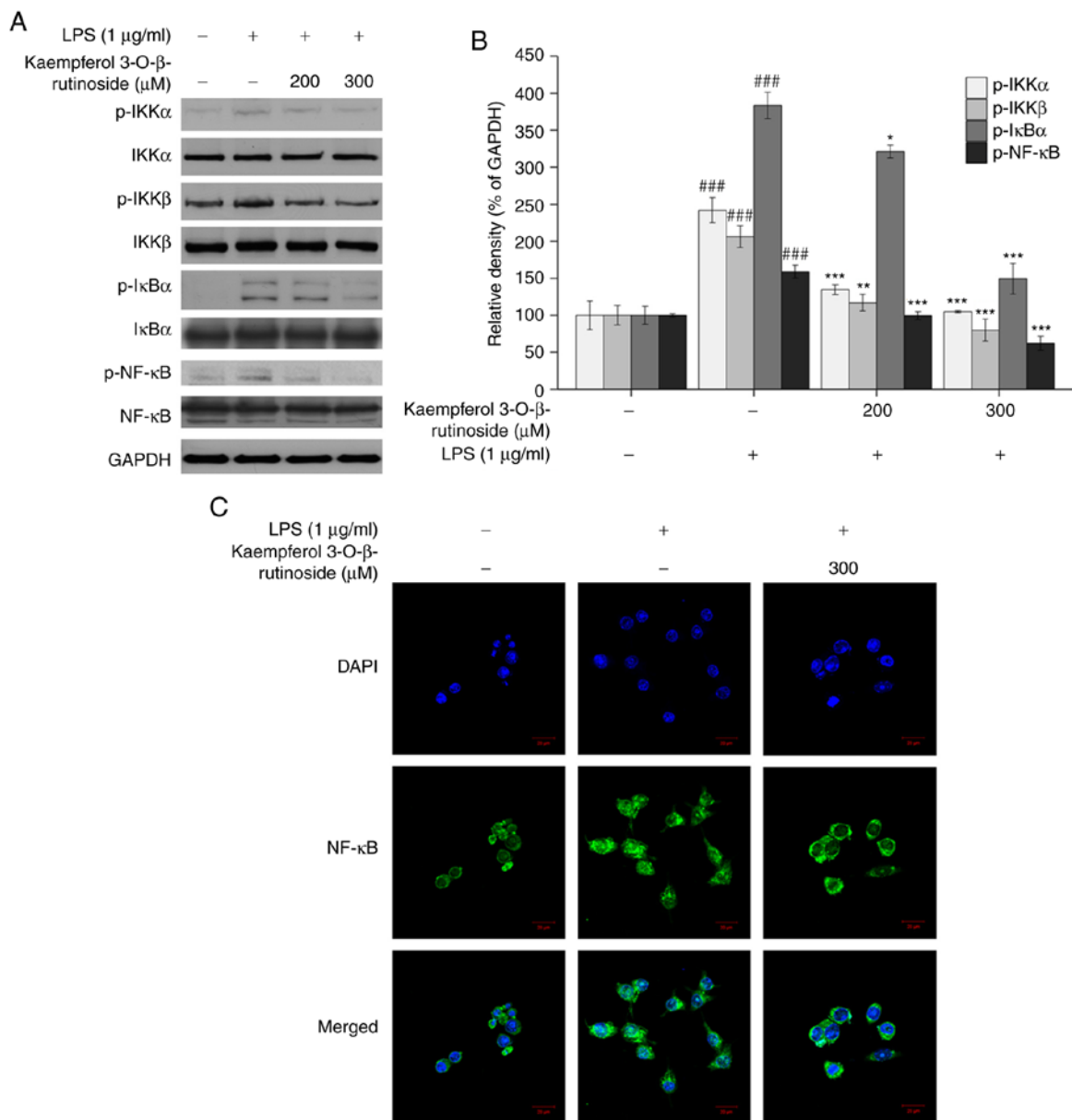


Figure 4. Effects of kaempferol-3-O- $\beta$ -rutinoside on expression of NF- $\kappa$ B pathway proteins in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with kaempferol-3-O- $\beta$ -rutinoside for 2 h and then stimulated with LPS (1  $\mu\text{g/ml}$ ) for a further 2 h (western blot analysis) or 24 h (immunofluorescence). (A) Protein expression levels were measured by western blotting. (B) The density of NF- $\kappa$ B pathway proteins was normalized to GAPDH. (C) The effects of kaempferol-3-O- $\beta$ -rutinoside on NF- $\kappa$ B nuclear translocation were detected by immunofluorescence staining. Nuclei were stained with DAPI (blue) while NF- $\kappa$ B was stained with a specific antibody (green). Magnification,  $\times 400$ ; scale bar, 20  $\mu\text{m}$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with the LPS group; ### $P < 0.001$  compared with the untreated control. LPS, lipopolysaccharide; IKK, inhibitor of  $\kappa$ B kinase; I $\kappa$ B, inhibitor of  $\kappa$ B.

modulator of inflammation-related gene expression, including iNOS, COX-2, TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Therefore, the NF- $\kappa$ B pathway is considered as the main target for investigations of inflammation (12,26). When IKK is activated, it leads to the phosphorylation of I $\kappa$ B. I $\kappa$ B binds to NF- $\kappa$ B under normal conditions; however, phosphorylated I $\kappa$ B is ubiquitinated and degraded, resulting in the activation and nuclear translocation of NF- $\kappa$ B (12). In the present results, as the kaempferol-3-O- $\beta$ -rutinoside concentration was increased, the phosphorylation of NF- $\kappa$ B, I $\kappa$ B $\alpha$ , IKK $\alpha$  and IKK $\beta$  were decreased (Fig. 4A). In addition, the subcellular localization of NF- $\kappa$ B was examined by confocal microscopy. Compared with the LPS group, kaempferol-3-O- $\beta$ -rutinoside treatment inhibited the nuclear translocation of NF- $\kappa$ B (Fig. 4C).

These data demonstrated that kaempferol-3-O- $\beta$ -rutinoside suppressed the phosphorylation of NF- $\kappa$ B by controlling the activities of upstream signaling factors. Furthermore, kaempferol-3-O- $\beta$ -rutinoside treatment reduced NF- $\kappa$ B nuclear translocation, potentially resulting in the iNOS, COX-2 and cytokine downregulation.

*Kaempferol-3-O- $\beta$ -rutinoside inhibits the MAPK pathway.* MAPK pathways are considered as the main pathways of the inflammatory mechanism and are well studied in inflammatory diseases, including RA, pancreatitis, hepatitis, IBD and psoriasis (15). As shown in Fig. 5, kaempferol-3-O- $\beta$ -rutinoside treatment decreased the phosphorylation of p38, ERK, and SAPK in a dose-dependent manner. Among the key MAPK

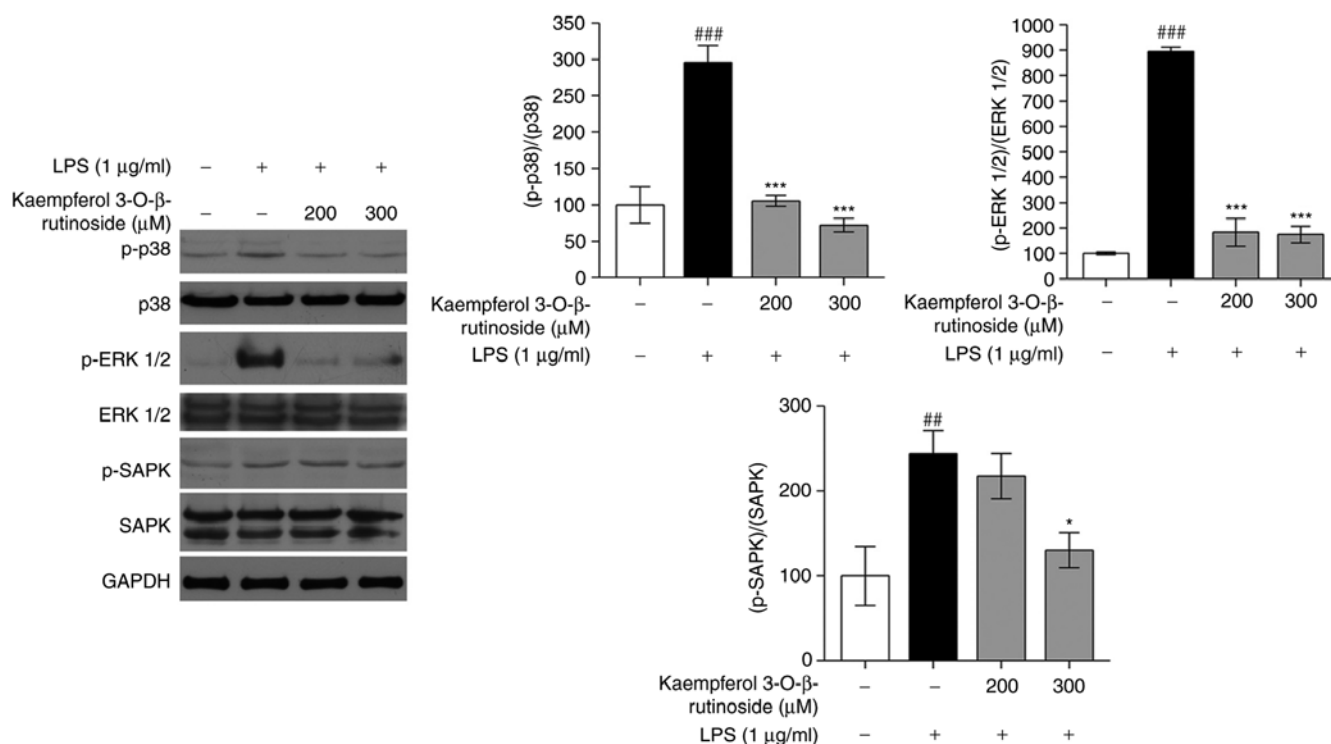


Figure 5. Effects of kaempferol-3-O- $\beta$ -rutinoside on the MAPK pathway in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with 200 and 300  $\mu$ M of kaempferol-3-O- $\beta$ -rutinoside for 2 h and then stimulated with LPS (1  $\mu$ g/ml) for a further 3 h. The protein expression levels of the main effectors of the MAPK pathways were examined by western blot analysis. The densities of phosphorylated p38, ERK1/2 and SAPK were normalized to GAPDH and presented as a ratio to the total protein levels of p38, ERK1/2 and SAPK, respectively. Data are presented as the mean  $\pm$  SEM of three independent experiments. \* $P$ <0.05 and \*\*\* $P$ <0.001 compared with the LPS-stimulated group; ## $P$ <0.01 and ### $P$ <0.001 compared with the untreated control. MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; SAPK, stress-activated protein kinase.

factors, kaempferol-3-O- $\beta$ -rutinoside appeared to inhibit the phosphorylation of ERK1/2 the most (Fig. 5). Hence, kaempferol-3-O- $\beta$ -rutinoside exhibited significant inhibitory activity on MAPK pathway signaling.

## Discussion

Kaempferol-3-O-rutinoside was isolated from tartary buckwheat (*F. tataricum*) (19,20). The photochemistry of tartary buckwheat has been investigated, including quercetin, rutin and kaempferol, and several studies have suggested that flavonoids from tartary buckwheat may be used in preparing novel pharmaceuticals (27,28). Therefore, the present study investigated whether kaempferol-3-O- $\beta$ -rutinoside affects inflammation in LPS-stimulated RAW 264.7 cells.

In the immune response, the overexpression of NO indicates that the body is affected by pathological symptoms, such as endotoxemia, allograft rejection and diabetes (29). It also affects pro-inflammatory responses that cause multiple side effects, including cytotoxicity, vasodilation and edema (7). Therefore, in order to investigate whether kaempferol-3-O- $\beta$ -rutinoside has an anti-inflammatory function, first its effect on NO production was detected. NO is produced by NOSs; iNOS is especially expressed by several stimuli, including LPS or pro-inflammatory cytokines, and then large amount of NO is produced (7). Kaempferol-3-O- $\beta$ -rutinoside inhibited the production of NO in LPS-stimulated RAW 264.7 cells in a dose-dependent manner; the  $IC_{50}$  was  $\sim 260 \pm 2.5$   $\mu$ M (Fig. 1B). The results of the kaempferol-3-O- $\beta$ -rutinoside cytotoxicity

assay revealed that kaempferol-3-O- $\beta$ -rutinoside was cytotoxic at 500  $\mu$ M in macrophage RAW264.7 cells (Fig. 1A). No cytotoxicity effect was observed however at the dose of 300  $\mu$ M. There are several substances which are effective even at high concentration. For example, 5-aminosalicylic acid (5-ASA), known as mesalazine and used to treat IBS safely in pregnancy and breastfeeding, is routinely applied in *in vitro* experiments at 500  $\mu$ M to 20 mM (30-32). Aspirin is also tested at high concentrations, for example, 1- 5 mM (33). The concentration of 5-ASA or aspirin is high in *in vitro* experiments, however, they are both currently used as drugs in humans and are extensively studied. Therefore, the presents study considered the results of the NO and cytotoxicity assays, and doses up to 300  $\mu$ M kaempferol-3-O- $\beta$ -rutinoside were selected for further experiments aiming to investigate its molecular mechanisms.

iNOS and COX-2 are widely known as the main regulatory factors in inflammatory responses; thus, iNOS and COX-2 expression levels have been examined by many studies for the development of anti-inflammatory agents (21,25,34). As shown in Fig. 2, as the concentration of kaempferol-3-O- $\beta$ -rutinoside was increased, iNOS and COX-2 expression was decreased. Pro-inflammatory cytokines are involved in inflammatory reactions, stimulate acute phase inflammation and act as endogenous pyrogens (35). The pro-inflammatory cytokines first activate macrophage cells, then initiate iNOS and COX-2 expression, followed by NO production (7). The reduction of pro-inflammatory cytokine expression is considered an effective way to treat inflammation-related diseases. The present

study examined the pro-inflammatory cytokine mRNA and protein expression levels by RT-PCR and western blotting, respectively. Fig. 3 shows kaempferol-3-O- $\beta$ -rutinoside to be effective in reducing both mRNA and protein expressions of IL-6 and TNF- $\alpha$  in LPS-stimulated RAW 264.7 cells. Kaempferol-3-O- $\beta$ -rutinoside was slightly effective at inhibiting the IL-1 $\beta$  protein expression in LPS-stimulated RAW 264.7 cells. Multiple studies have revealed that IL-6 gene expression is controlled by inflammation triggers and performs important functions in the immune response (10,36). NF- $\kappa$ B is reported to regulate IL-6 gene expression following LPS stimulation in human monocytic cells and human cervical carcinoma cells (10). Therefore, the NF- $\kappa$ B pathway was explored in order to elucidate the mechanism of the anti-inflammatory activity of kaempferol-3-O- $\beta$ -rutinoside.

In the normal state, the NF- $\kappa$ B protein is associated with the I $\kappa$ B $\alpha$  protein in the cytoplasm, thereby maintained in an inactivate state. When the inflammatory response is initiated, IKK phosphorylates I $\kappa$ B $\alpha$ , leading to its degradation. Therefore, the NF- $\kappa$ B protein is free to translocate to the nucleus and to initiate the transcription of inflammation-related genes (13). NF- $\kappa$ B is associated with multiple human inflammation-related disorders, including asthma, RA, IBD and atherosclerosis, and leads to the transcription of pro-inflammatory cytokines, chemokines, iNOS and COX-2 (12). It has been reported that constitutive NF- $\kappa$ B is highly expressed in the inflamed colonic tissue in patients with IBD (37). In addition, NF- $\kappa$ B controls the induction of pro-inflammatory cytokines and chemokines, and promotes the recruitment of monocytes and the progression of atherosclerosis (38). The present results demonstrated that kaempferol-3-O- $\beta$ -rutinoside suppressed the phosphorylation of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ B and reduced the NF- $\kappa$ B translocation to the nucleus.

MAPK pathways are also considered as targets for drug development (15). Both NF- $\kappa$ B and MAPK pathways are associated with various important biological processes, including inflammation, cell survival and apoptosis, in macrophage cells (39). MAPK pathways are involved in regulating inflammatory-related gene transcription, leading to the overproduction of pro-inflammatory cytokines (40,41). LPS is known as one of the potent activators of MAPK pathway proteins (16). Following LPS stimulation, MAPKs are phosphorylated or activated to produce several transcription factors and inflammatory mediators in human monocytic cells (3). Thus, regulating the MAPK pathway is also contemplated as an approach in anti-inflammation treatment. The present data suggested that kaempferol-3-O- $\beta$ -rutinoside significantly downregulated the phosphorylation levels of p38 and ERK, and slightly decreased the phosphorylation of SAPK. These findings indicated that kaempferol-3-O- $\beta$ -rutinoside exerted an anti-inflammatory activity by inhibiting the MAPK pathways.

In conclusion, the present study explored the anti-inflammatory effect of kaempferol-3-O- $\beta$ -rutinoside in LPS-stimulated macrophages via the NF- $\kappa$ B and MAPK pathways. Kaempferol-3-O- $\beta$ -rutinoside substantially reduced NO production at 300  $\mu$ M; this concentration did not show cytotoxicity on RAW264.7 and 293 cells. Kaempferol-3-O- $\beta$ -rutinoside inhibited the expression of inflammation-related factors, such as iNOS, COX-2, TNF- $\alpha$  and IL-6. In addition, kaempferol-3-O- $\beta$ -rutinoside suppressed the phosphorylation

of IKK $\alpha$ , IKK $\beta$ , I $\kappa$ B $\alpha$  and NF- $\kappa$ B, as well as MAPK phosphorylation, including p38, ERK1/2 and SAPK. Therefore, these data demonstrated that kaempferol-3-O- $\beta$ -rutinoside inhibited the inflammation-related gene expression in LPS-induced macrophages via the NF- $\kappa$ B and MAPK pathways. The present study may be used as basic pharmacological information in the field of anti-inflammation reagent development.

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### Availability of data and materials

The analyzed datasets during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

CK and GK conceptualized the experiments. DH and CK collected the data. DH and MK performed the experiments. DH analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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