



Research Note

BIOGF1K, a compound K-rich fraction of ginseng, plays an antiinflammatory role by targeting an activator protein-1 signaling pathway in RAW264.7 macrophage-like cells

Eunji Kim^{1,☆}, Young-Su Yi^{2,☆}, Young-Jin Son^{3,☆}, Sang Yun Han¹, Dong Hyun Kim⁴, Gibaeg Nam⁴, Mohammad Amjad Hossain⁵, Jong-Hoon Kim^{5,***}, Junseong Park^{4,**}, Jae Youl Cho^{1,*}

¹ Department of Genetic Engineering, Sungkyunkwan University, Suwon, Republic of Korea

² Department of Pharmaceutical Engineering, Cheongju University, Cheongju, Republic of Korea

³ Department of Pharmacy, Suncheon National University, Suncheon, Republic of Korea

⁴ Department of Engineering Chemistry, Chungbuk National University, Cheongju, Republic of Korea

⁵ College of Veterinary Medicine, Chonbuk National University, Iksan, Republic of Korea



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1. Main text

Inflammation is a host defense mechanism that protects the body from invading pathogens and has five hallmarks: redness, swelling, heat, pain, and loss of function [1–3]. Inflammatory responses are initiated by macrophages recognizing pathogen-associated molecular patterns by pattern recognition receptors expressed on their surfaces and activating inflammatory signaling pathways, such as nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1), and interferon-regulatory factors (IRFs) [4–8]. Toll-like receptors (TLRs) are the main pattern recognition receptors in

macrophages, and TLR4 is a molecular receptor of lipopolysaccharide (LPS), the most powerful agonist derived from gram-negative bacteria able to activate inflammatory responses. LPS binding with TLR4 transduces inflammatory signaling cascades by activating various intracellular signaling kinases in macrophages, resulting in the overexpression of inflammatory genes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and the production of inflammatory mediators, including tumor necrosis factor- α , interleukin-1 β (IL-1 β), IL-6, nitric oxide (NO), and prostaglandin E₂ [4–6,8,9].

Ginsenosides are the main active ingredients found in ginseng and were reported to have many functions, including antiinflammatory, anticancer, antiviral, and antioxidative activities [10–14]. Recently, we prepared a new fraction of Korean ginseng containing a high amount of compound K, named BIOGF1K, and demonstrated its antiinflammatory activity [15]. Despite this study reporting an antiinflammatory role of BIOGF1K, mechanisms by which BIOGF1K plays a protective role in inflammatory responses remains unclear. Therefore, in this study, the antiinflammatory activity of BIOGF1K and the underlying mechanism present during inflammatory responses were investigated using an *in vitro* inflammatory cell model, specifically LPS-stimulated RAW264.7 cells.

RAW264.7 and HEK293 cells (ATCC, Rockville, MD, USA) were cultured in Roswell Park Memorial Institute 1640 medium and Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Gibco), streptomycin, penicillin, and L-glutamine at

* Corresponding author. Department of Genetic Engineering, Sungkyunkwan University, 2066 Seobu-ro, Suwon 16419, Korea.

** Corresponding author. Department of Engineering Chemistry, Chungbuk National University, Seowon-Gu, Cheongju, Chungbuk 28644, Republic of Korea.

*** Corresponding author. College of Veterinary Medicine, Chonbuk National University, 79 Gobong-ro, Iksan 54596, Korea.

E-mail addresses: jhkim1@jbnu.ac.kr (J.-H. Kim), superbody@amorepacific.com (J. Park), jaecho@skku.edu (J.Y. Cho).

☆ These authors contributed equally to this work.

37°C in a 5% carbon dioxide–humidified incubator. To measure NO amount, RAW264.7 cells pretreated with BIOGF1K (0–30 µg/mL) for 30 minutes were treated with LPS (1 µg/mL) for 24 hours, and NO amount in culture media was measured by Griess assay [16]. To determine cell viability, RAW264.7 cells were treated with BIOGF1K (0–30 µg/mL) for 24 hours, and cell viability was measured by a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [17]. To determine mRNA expression levels, RAW264.7 cells pretreated with BIOGF1K (0–30 µg/mL) for 30 minutes were treated with LPS (1 µg/mL) for 6 hours and total RNA was extracted using TRI Reagent® solution (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using 1 µg of total RNA using MuLV reverse transcriptase (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. mRNA expression levels were measured by semiquantitative reverse transcriptase polymerase chain reaction. The nucleic acid sequences of primers are summarized in Table 1.

To determine an AP-1 activity, HEK293 cells were co-transfected with plasmids expressing Flag-MyD88, AP-1-Luc, and β-galactosidase for 24 hours using polyethylenimine (Sigma Chemical Co., St. Louis, MO, USA) and treated with BIOGF1K (0–30 µg/mL) for 24 hours. The cells were lysed by repeating freezing and thawing processes three times, and luciferase activities in the cell lysates were measured, as reported previously in a study by Yi et al [18]. To analyze the activities of signaling molecules during inflammatory responses, nuclear fraction and total cell lysates were prepared, and the active forms of signaling molecules were determined by immunoblotting analysis using specific antibodies recognizing phospho-forms of signaling proteins. RAW264.7 cells pretreated with BIOGF1K (30 µg/mL) for 30 minutes were treated with LPS (1 µg/mL) for the indicated time. HEK293 cells pretreated with BIOGF1K (0–30 µg/mL) for 24 hours were transfected with hemagglutinin (HA)-tumor growth factor-β-activated kinase 1 (TAK1) (0.5 µg/mL) for 48 hours. To prepare total RAW264.7 and HEK293 cell lysates, the cells were washed with phosphate-buffered saline (Gibco) three times and lysed in an ice-cold radioimmunoprecipitation assay lysis buffer (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 0.5% sodium dodecyl sulfate, 150 mM NaCl) by sonication (Thermo Fisher Scientific, Waltham, MA, USA). The lysed cells were centrifuged at 15,000g for 10 minutes at 4°C, and the supernatants were transferred to a fresh tube as total cell lysates. A RAW264.7 cell nuclear fraction was prepared, as previously described in a study by Yi et al [19]. For immunoblotting analysis, total cell lysates or nuclear fraction were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene fluoride membranes. Total and phosphorylated forms of c-Jun, c-Fos, activating transcription factor 2 (ATF2), fos-related antigen 1, c-Jun N-terminal kinase, p38, extracellular signal-regulated kinase (ERK), mitogen-activated protein

kinase (MAPK)/ERK kinase 1/2 (MEK1/2), MAPK kinase 3/6 (MKK3/6), TAK1, HA, lamin A/C, and β-actin were detected by the antibodies specific for the targets and were visualized with an enhanced chemiluminescence solution (AbFrontier, Seoul, Korea). The data are presented as a mean ± standard deviation. Statistical analyses were performed by analysis of variance/Scheffe post hoc test and Kruskal–Wallis/Mann–Whitney U test. A *p* value < 0.05 was regarded statistically significant. All data were analyzed using an SPSS program (SPSS Inc., Chicago, IL, USA).

We first examined the effect of BIOGF1K on NO production, one of the critical inflammatory mediators in macrophage-mediated inflammatory responses. Prednisolone was used as a standard compound because it has been known to downregulate COX-2 and inflammatory cytokines [20]. Like prednisolone, BIOGF1K dose-dependently suppressed NO production in LPS-treated RAW264.7 cells (Fig. 1A) without serious cytotoxicity (Fig. 1B), indicating the antiinflammatory activity of BIOGF1K by the reduction of inflammatory mediators without cytotoxicity at the doses studied herein.

To examine the effect of BIOGF1K on mRNA expression of inflammatory genes, RAW264.7 cells were treated with BIOGF1K and LPS, and mRNA expression of iNOS, COX-2, and tumor necrosis factor-α was measured by semiquantitative and real-time reverse transcriptase polymerase chain reaction. BIOGF1K dose-dependently inhibited mRNA expression of iNOS and COX-2 in LPS-treated RAW264.7 (Fig. 2A). Inflammatory gene expression is governed by inflammatory signaling pathways, such as AP-1, NF-κB, and IRF-3 [4–6,8], and the regulatory activity of BIOGF1K in NF-κB and IRF-3 signaling pathways have been demonstrated in a previous study by Hossen et al [15]. Therefore, the effect of BIOGF1K on an AP-1 signaling pathway was examined by a luciferase assay. HEK293 cells transfected with plasmids expressing AP-1-Luc and Flag-MyD88, an adaptor molecule able to activate AP-1 signaling, were treated with BIOGF1K. BIOGF1K markedly suppressed the AP-1-mediated luciferase activity (Fig. 2B), suggesting that BIOGF1K downregulates mRNA expression of inflammatory genes by suppressing an AP-1 signaling pathway in macrophages.

Mechanisms by which BIOGF1K suppresses an AP-1 pathway in macrophage during inflammatory responses was further investigated by use of immunoblotting analysis. The hallmark of transcription factor activation is their nuclear translocation. Therefore, nuclear translocation of AP-1 transcription factors was examined in macrophages. BIOGF1K inhibited nuclear translocation of c-Jun (at 90 minutes), ATF2 (at 90 minutes), and phospho (p)-fos-related antigen 1 (at 60 minutes and 90 minutes) in LPS-treated RAW264.7 cells (Fig. 3A). The effect of BIOGF1K on the activities of MAPKs in AP-1 signaling was examined. BIOGF1K inhibited the activation of p38 (60 minutes) and ERK (90 minutes) in LPS-treated RAW264.7 cells (Fig. 3B). Phosphorylation of MAPK kinases (MAPKKs), upstream signaling molecules of MAPKs were further investigated and found that BIOGF1K inhibited the activation of MEK1/2 (30 minutes) and MKK3/6 (30 minutes) in LPS-treated RAW264.7 cells (Fig. 3C). This result strongly supports the hypothesis of this study, since MEK1/2 and MKK3/6 are known as upstream MAPKKs of ERK and p38, respectively [21,22]; moreover, BIOGF1K inhibited the activation of MAPKKs earlier (30 minutes) than that of MAPKs (60 minutes) (Fig. 3B, C). These results suggest that BIOGF1K inhibits the AP-1 signaling pathway by inhibiting ERK and p38 MAPKs and MEK1/2 and MKK3/6, the upstream MAPKKs of ERK and p38, in macrophages during inflammatory responses. However, the common upstream molecule of MEK1/2 and MKK3/6, TAK1, was not affected by BIOGF1K. We further confirmed the suppressive effect of BIOGF1K on the activation of these MAPKs and MAPKKs in HEK293 cells by transfecting HA-TAK1, an activator of an AP-1 signaling pathway. As expected, BIOGF1K (30 µg/mL) suppressed the activation of MEK1/2 and ERK (Fig. 3D) and MKK3/6

Table 1
Primer sequences used for semiquantitative RT-PCR in this study

Name		Sequence (5' to 3')
iNOS	F	CCCTCCGAAGTTTCTGGCAGCAG
	R	GGCTGTCAGAGCCTCGTGGCTTTGG
COX-2	F	CACTACATCCTGACCCACTT
	R	ATGCTCCTGCTTGAGTATGT
TNF-α	F	TTGACCTCAGCGCTGAGTTG
	R	CCTGTAGCCACGTCGTAGC
GAPDH	F	CACTCACGCAAATCAACGGCA
	R	GACTCCACGACATACTCAGCAC

COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcriptase polymerase chain reaction; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

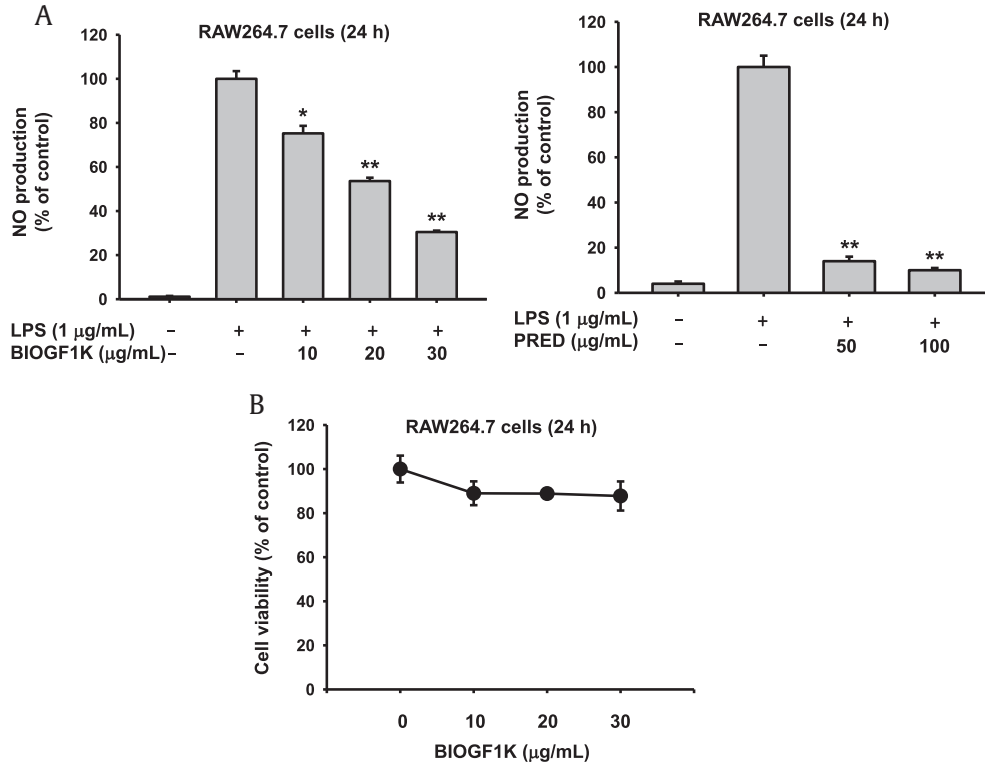


Fig. 1. LPS, lipopolysaccharide; NO, nitric oxide; PRED, prednisolone.

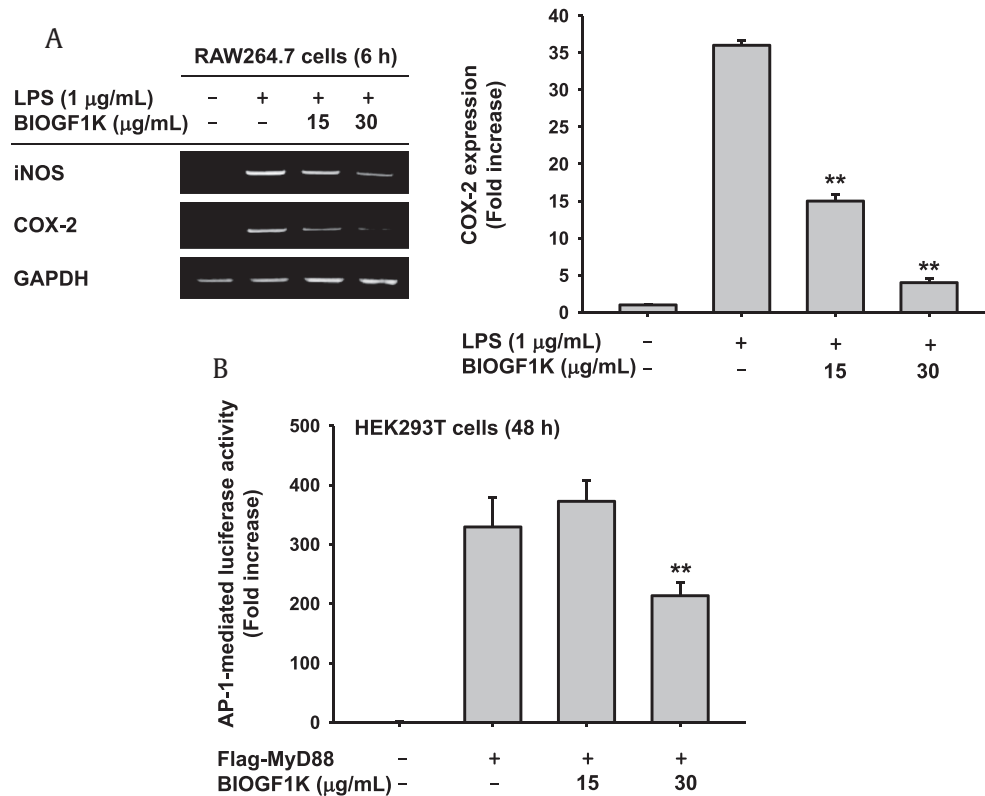


Fig. 2. COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide.

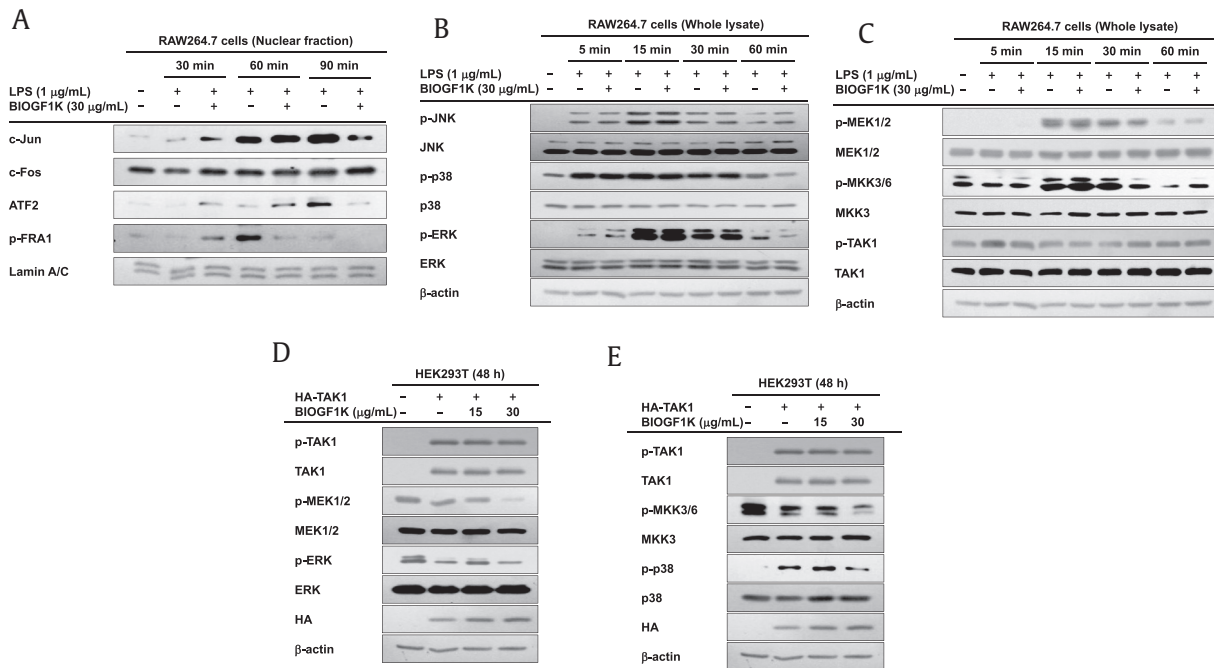


Fig. 3. ATF2, activating transcription factor 2; ERK, extracellular signal-regulated kinase; FRA1, fos-related antigen 1; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MEK1/2, mitogen-activated protein kinase (MAPK)/ERK kinase 1/2; MKK3/6, MAPK kinase 3/6; TAK1, tumor growth factor- β -activated kinase 1.

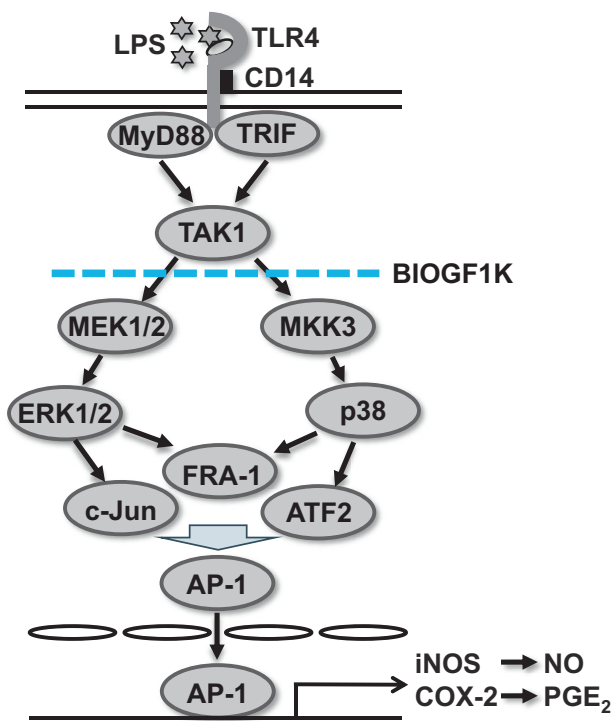


Fig. 4. AP-1, activator protein-1; ATF2, activating transcription factor 2; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; FRA1, fos-related antigen 1; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MEK1/2, mitogen-activated protein kinase (MAPK)/ERK kinase 1/2; MKK3, MAPK kinase 3; NO, nitric oxide; PGE₂, prostaglandin E₂; TAK1, tumor growth factor- β -activated kinase 1; TLR, toll-like receptors; CD14, cluster of differentiation 14; TRIF, Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon beta.

and p38 (Fig. 3E), respectively, in HA-TAK1-transfected HEK293 cells.

In this study, we investigated an antiinflammatory activity of BIOGF1K in macrophage-mediated inflammatory responses. We found that BIOGF1K suppressed the activation of an AP-1 pathway by targeting MAPKs, such as ERK and p38, and MAPKKs, such as MEK1/2 and MKK3/6, as summarized in Fig. 4, thereby suppressing inflammatory gene expression, such as iNOS and COX-2, as well as inflammatory mediator production, such as NO in macrophages during inflammatory response. For further validating inhibitory mechanism of BIOGF1K, whether TAK1 can be directly inhibited by BIOGF1K will be evaluated by employing a kinase assay. Collectively, these results strongly suggest that BIOGF1K, an active ingredient in ginseng, plays a protective role in macrophage-mediated inflammatory responses and provides evidence that BIOGF1K should be further examined as a promising antiinflammatory agent to prevent and treat inflammatory diseases.

Conflicts of interest

The authors declare that there is no conflicts of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jgr.2018.02.001>.

References

- [1] Janeway Jr CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197–216.
- [2] Yi YS. Folate receptor-targeted diagnostics and therapeutics for inflammatory diseases. *Immune Netw* 2016;16:337–43.
- [3] Yi YS. Caspase-11 non-canonical inflammasome: a critical sensor of intracellular lipopolysaccharide in macrophage-mediated inflammatory responses. *Immunology* 2017;152:207–17.
- [4] Yi YS, Son YJ, Ryou C, Sung GH, Kim JH, Cho JY. Functional roles of Syk in macrophage-mediated inflammatory responses. *Mediators Inflamm* 2014;2014:270302.
- [5] Yu T, Yi YS, Yang Y, Oh J, Jeong D, Cho JY. The pivotal role of TBK1 in inflammatory responses mediated by macrophages. *Mediators Inflamm* 2012;2012:979105.
- [6] Byeon SE, Yi YS, Oh J, Yoo BC, Hong S, Cho JY. The role of Src kinase in macrophage-mediated inflammatory responses. *Mediators Inflamm* 2012;2012:512926.
- [7] Italiani P, Boraschi D. New insights into tissue macrophages: From their origin to the development of memory. *Immune Netw* 2015;15:167–76.
- [8] Yang Y, Kim SC, Yu T, Yi YS, Rhee MH, Sung GH, Yoo BC, Cho JY. Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses. *Mediators Inflamm* 2014;2014:352371.
- [9] Kim KH, Kim TS, Lee JG, Park JK, Yang M, Kim JM, Jo EK, Yuk JM. Characterization of proinflammatory responses and innate signaling activation in macrophages infected with *Mycobacterium scrofulaceum*. *Immune Netw* 2014;14:307–20.
- [10] Kim JH, Yi YS, Kim MY, Cho JY. Role of ginsenosides, the main active components of *Panax ginseng*, in inflammatory responses and diseases. *J Ginseng Res* 2017;41:435–43.
- [11] Baek KS, Yi YS, Son YJ, Yoo S, Sung NY, Kim Y, Hong S, Aravinthan A, Kim JH, Cho JY. *In vitro* and *in vivo* anti-inflammatory activities of Korean Red Ginseng-derived components. *J Ginseng Res* 2016;40:437–44.
- [12] Yang J, Yuan D, Xing T, Su H, Zhang S, Wen J, Bai Q, Dang D. Ginsenoside Rh2 inhibiting HCT116 colon cancer cell proliferation through blocking PDZ-binding kinase/T-LAK cell-originated protein kinase. *J Ginseng Res* 2016;40:400–8.
- [13] Lee MH, Lee BH, Jung JY, Cheon DS, Kim KT, Choi C. Antiviral effect of Korean red ginseng extract and ginsenosides on murine norovirus and feline calicivirus as surrogates for human norovirus. *J Ginseng Res* 2011;35:429–35.
- [14] Aravinthan A, Kim JH, Antonisamy P, Kang CW, Choi J, Kim NS. Ginseng total saponin attenuates myocardial injury via anti-oxidative and anti-inflammatory properties. *J Ginseng Res* 2015;39:206–12.
- [15] Hossen MJ, Hong YD, Baek KS, Yoo S, Hong YH, Kim JH, Lee JO, Kim D, Park J, Cho JY. *In vitro* antioxidative and anti-inflammatory effects of the compound K-rich fraction BIOGF1K, prepared from *Panax ginseng*. *J Ginseng Res* 2017;41:43–51.
- [16] Cho JY, Baik KU, Jung JH, Park MH. *In vitro* anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from *Saussurea lappa*. *Eur J Pharmacol* 2000;398:399–407.
- [17] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.
- [18] Yi YS, Cho JY, Kim D. *Cerbera manghas* methanol extract exerts anti-inflammatory activity by targeting c-Jun N-terminal kinase in the AP-1 pathway. *J Ethnopharmacol* 2016;193:387–96.
- [19] Yi YS, Baek KS, Cho JY. L1 cell adhesion molecule induces melanoma cell motility by activation of mitogen-activated protein kinase pathways. *Pharmazie* 2014;69:461–7.
- [20] Ghidini E, Capelli AM, Carnini C, Cenacchi V, Marchini G, Virdis A, Italia A, Facchinetti F. Discovery of a novel isoxazoline derivative of prednisolone endowed with a robust anti-inflammatory profile and suitable for topical pulmonary administration. *Steroids* 2015;95:88–95.
- [21] Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* 2011;75:50–83.
- [22] Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004;68:320–44.