J Ginseng Res 40 (2016) 437–444

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research article

In vitro and *in vivo* anti-inflammatory activities of Korean Red Ginseng-derived components

Kwang-Soo Baek [1,☆], Young-Su Yi [2,☆], Young-Jin Son [3,☆], Sulgi Yoo [1], Nak Yoon Sung [1], Yong Kim [1], Sungyoul Hong [1], Adithan Aravinthan [4], Jong-Hoon Kim [4,**], Jae Youl Cho [1,*]

[1] Department of Genetic Engineering, Sungkyunkwan University, Suwon, Korea
[2] Department of Pharmaceutical Engineering, Cheongju University, Cheongju, Korea
[3] Department of Pharmacy, Sunchon National University, Suncheon, Korea
[4] Department of Physiology, College of Veterinary Medicine, Chonbuk National University, Iksan, Korea

ARTICLE INFO

Article history:
Received 11 July 2016
Received in Revised form
7 August 2016
Accepted 9 August 2016
Available online 24 August 2016

Keywords:
anti-inflammatory activity
gastritis
Korean Red Ginseng
nonsaponin fraction
saponin fraction

ABSTRACT

Background: Although Korean Red Ginseng (KRG) has been traditionally used for a long time, its anti-inflammatory role and underlying molecular and cellular mechanisms have been poorly understood. In this study, the anti-inflammatory roles of KRG-derived components, namely, water extract (KRG-WE), saponin fraction (KRG-SF), and nonsaponin fraction (KRG-NSF), were investigated.
Methods: To check saponin levels in the test fractions, KRG-WE, KRG-NSF, and KRG-SF were analyzed using high-performance liquid chromatography. The anti-inflammatory roles and underlying cellular and molecular mechanisms of these components were investigated using a macrophage-like cell line (RAW264.7 cells) and an acute gastritis model in mice.
Results: Of the tested fractions, KGR-SF (but not KRG-NSF and KRG-WE) markedly inhibited the viability of RAW264.7 cells, and splenocytes at more than 500 μg/mL significantly suppressed NO production at 100 μg/mL, diminished mRNA expression of inflammatory genes such as inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-α, and interferon-β at 200 μg/mL, and completely blocked phagocytic uptake by RAW264.7 cells. All three fractions suppressed luciferase activity triggered by interferon regulatory factor 3 (IRF3), but not that triggered by activator protein-1 and nuclear factor-kappa B. Phospho-IRF3 and phospho-TBK1 were simultaneously decreased in KRG-SF. Interestingly, all these fractions, when orally administered, clearly ameliorated the symptoms of gastric ulcer in HCl/ethanol-induced gastritis mice.
Conclusion: These results suggest that KRG-WE, KRG-NSF, and KRG-SF might have anti-inflammatory properties, mostly because of the suppression of the IRF3 pathway.

1. Introduction

Inflammation is an innate immune response consisting of a series of complex biological processes to protect the body from infection by pathogens, including bacteria, viruses, and fungi [1,2]. Inflammation is characterized by key symptoms, including recruitment of white blood cells, pain, redness, swelling, heat, tissue damage, and organ dysfunction. During the inflammatory response, different types of immune cells are actively recruited to the inflamed lesions to remove the invading pathogens. Among these immune cells, the macrophage is one of the major effector cells governing inflammatory responses by producing various inflammatory mediators, including nitric oxide (NO), reactive oxygen/nitrogen species (ROS/RNS), prostaglandin E_2 (PGE_2), and different types of proinflammatory cytokines. The latter include tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 [under the control of the activator protein (AP)-1], nuclear factor kappa B (NF-κB), and interferon (IFN) regulatory factor 3 (IRF3) [2–7]. Although inflammation is a host defense mechanism to protect the body from invading pathogens, chronic inflammation, which is a prolonged

* Corresponding author. Department of Genetic Engineering, Sungkyunkwan University, 2066 Seobu-ro, Jangan-gu, Suwon 16419, Korea.
** Corresponding author. Department of Physiology, College of Veterinary Medicine, Chonbuk National University, 79 Gobong-ro, Iksan 54596, Korea.
E-mail addresses: jaecho@skku.edu (J.Y. Cho), jhkim1@chonbuk.ac.kr (J.-H. Kim).
☆ These authors contributed equally to this work.

p1226-8453 e2093-4947/$ – see front matter
http://dx.doi.org/10.1016/j.jgr.2016.08.003

state affecting tissue remodeling for several weeks to years, is regarded as a leading cause in the development of a variety of diseases, such as inflammatory/autoimmune diseases, neurodegenerative diseases, and cancers [8–10].

Korean ginseng (*Panax ginseng*) is a perennial plant that has been traditionally used as an herbal medicine to ameliorate the symptoms of various diseases in eastern Asia. The ginseng root has been used as a common ethnopharmacological remedy to support vitality [11,12]. Because fresh ginseng is easily degraded at room temperature, it needs to be processed to red ginseng by steaming and drying, and accumulating evidence has revealed that red ginseng has higher biological activity and lower side effects compared to fresh or white ginseng [13]. Korean Red Ginseng (KRG) has been known to have various biological activities, including immune enhancement, antioxidant effects, memory enhancement, improvement of menopausal disorder, and induction of metabolic energy [14–17]. However, although KRG has been studied in human health and immunity, the therapeutic potential of each component derived from KRG extract in modulating inflammatory responses and in preventing inflammatory diseases has been poorly understood.

Therefore, in this study, we prepared three fractions of KRG—water extract (KRG-WE), nonsaponin fraction (KRG-NSF), and saponin fraction (KRG-SF)—and investigated their therapeutic potency in inflammatory responses and diseases using lipopolysaccharide (LPS)-stimulated macrophages and an acute inflammatory gastritis mouse model.

2. Materials and methods

2.1. Materials

KRG-WE, KRG-NSF, and KRG-SF were kindly supplied by the Korea Ginseng Corporation (Daejeon, Korea). Male imprinting control region (ICR) mice (6–8 wk old, 17–21 g) were purchased from Orient Bio (Gyeonggi, Korea). RAW264.7 and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium, Roswell Park Memorial Institute 1640 (RPMI 1640) medium, fetal bovine serum (FBS), streptomycin, penicillin, and L-glutamine were purchased from Gibco (Grand Island, NY, USA). LPS, N^{ω}-nitro-L-arginine methyl ester (L-NAME), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyethylenimine (PEI), ranitidine (RNT), and fluorescein isothiocyanate (FITC)-dextran were purchased from Sigma (St. Louis, MO, USA). The primers used for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) were synthesized, and PCR premix was purchased from Bioneer Inc. (Daejeon, Korea). Constructs expressing signaling proteins (FLAG-MyD88 and FLAG-TBK1) and luciferase constructs containing the binding promoters for NF-κB, AP-1, and IFN-β were used as previously reported [18,19]. Antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA, USA). The luciferase assay system was purchased from Promega (Madison, WI, USA).

2.2. Mice

Male ICR mice (6–8 wk old, 17–21 g) were obtained from Orient Bio (Gyeonggi, Korea) and maintained in plastic cages under standard conditions. Water and pelleted food (Samyang, Daejeon, Korea) were supplied *ad libitum*. Studies were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Sungkyunkwan University, Suwon, Korea.

Table 1
Primer sequences used for semiquantitative PCR

Name		Sequence (5′ to 3′)
iNOS	F	CCCTTCCGAAGTTTCTGGCAGCAG
	R	GGCTGTCAGAGCCTCGTGGCTTTGG
COX-2	F	CACTACATCCTGACCCACTT
	R	ATGCTCCTGCTTGAGTATGT
TNF-α	F	TTGACCTCAGCGCTGAGTTG
	R	CCTGTAGCCCACGTCGTAGC
IFN-β	F	CAGGATGAGGACATGAGCACC
	R	CTCTGCAGACTCAAACTCCAC
GAPDH	F	CACTCACGGCAAATTCAACGGCA
	R	GACTCCACGACATACTCAGCAC

COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN-β, interferon beta; iNOS, inducible nitric oxide synthase; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor alpha

2.3. Preparation of splenocytes from mice

Splenocytes from ICR mice were prepared as previously described [20].

2.4. Cell culture

RAW264.7, HEK293 cells and splenocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine, and antibiotics (penicillin and streptomycin) at 37°C in a 5% CO_2 humidified incubator.

2.5. High-performance liquid chromatography analysis

For determination of ginsenosides from KRG-WE, KRG-SF, and KRG-NSF, high-performance liquid chromatography (HPLC) was conducted as described previously [21,22].

2.6. Cell viability assay

The cytotoxic effects of KRG-WE, KRG-SF, and KRG-NSF were determined using the MTT assay, as reported previously [23].

2.7. Phagocytosis assay

RAW264.7 cells treated with KRG-WE, KRG-SF, or KRG-NSF were resuspended in 100 μL phosphate-buffered saline (PBS) containing 1% human AB serum and incubated with FITC-dextran (1 mg/mL) at 37°C for 30 min. The incubations were stopped by adding 2 mL ice-cold PBS containing 1% human serum and 0.02% sodium azide. The cells were then washed three times with cold PBS-azide and analyzed on a FACScan flow cytometer, as reported previously [24].

2.8. NO production assay

RAW264.7 cells were pretreated with either KRG-WE, KRG-NSF, or KRG-SF and incubated with LPS (1 μg/mL) for 24 h. NO production level was determined using Griess reagent as described previously [25].

2.9. mRNA analysis by semiquantitative RT-PCR

RAW264.7 cells pretreated with KRG-WE, KRG-NSF, or KRG-SF for 1 h were incubated with LPS (1 μg/mL) for 6 h. Total RNA was isolated with TRI reagent according to the manufacturer's instructions and stored at −70°C until use. cDNA from 1 μg of total RNA was synthesized using MuLV reverse transcriptase according to the manufacturer's instructions, and semiquantitative RT-PCR

Table 2
HPLC profile of ginseng components from KRG-WE, KRG-NSF, and KRG-SF

	(mg/g)										
	Rg1	Re	Rf	Rh1	Rg2s	Rb1	Rc	Rb2	Rd	Rg3s	Rg3r
KRG-WE	1.03	1.21	1.04	0.96	1.43	5.19	2.02	1.88	0.67	2.10	0.96
KRG-SF	13.33	15.68	13.39	11.54	15.75	64.20	25.98	22.96	8.29	21.69	10.60
KRG-NSF	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

HPLC, high-performance liquid chromatography; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract

reactions were conducted using the primers specific for inducible NO synthase (iNOS), cyclooxygenase (COX)-2, TNF-α, IFN-β, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as previously reported [26]. The primer sequences used in this study are listed in Table 1.

2.10. Luciferase reporter gene assay

HEK293 cells were transfected with β-galactosidase and NF-κB-Luc, AP-1-Luc, IRF3-Luc, MyD88, or TBK1 using PEI. The cells were treated with KRG-WE, KRG-NSF, or KRG-SF for 12 h prior to termination. Luciferase assays were performed using the Luciferase Assay System according to the manufacturer's instructions (Promega, Madison, WI, USA).

2.11. Preparation of whole cell and nuclear lysates and Western blot analysis

RAW264.7 cells were treated with KRG-WE, KRG-NSF, or KRG-SF for the indicated time, and whole cell and nuclear lysates were prepared as described previously [23]. Western blot analysis was performed as previously reported [23] using the indicated antibodies.

2.12. HCl/ethanol-induced gastritis in mice

Fasted ICR mice (7 mice/group) were orally treated with KRG-WE, KRG-NSF, KRG-SF (200 mg/kg), or ranitidine (40 mg/kg) twice daily for 3 d. At 30 min after the final administration, stomach inflammation was induced by oral administration of HCl/ethanol (EtOH), and the degree of inflammation was analyzed according to a published method [27].

2.13. Statistical analysis

All data in this paper are presented as the mean ± standard deviation of an experiment performed with six or three replicates. For statistical comparisons, these results were analyzed using Kruskal–Wallis/Mann–Whitney U tests. A p value < 0.05 was considered statistically significant. All statistical tests were carried

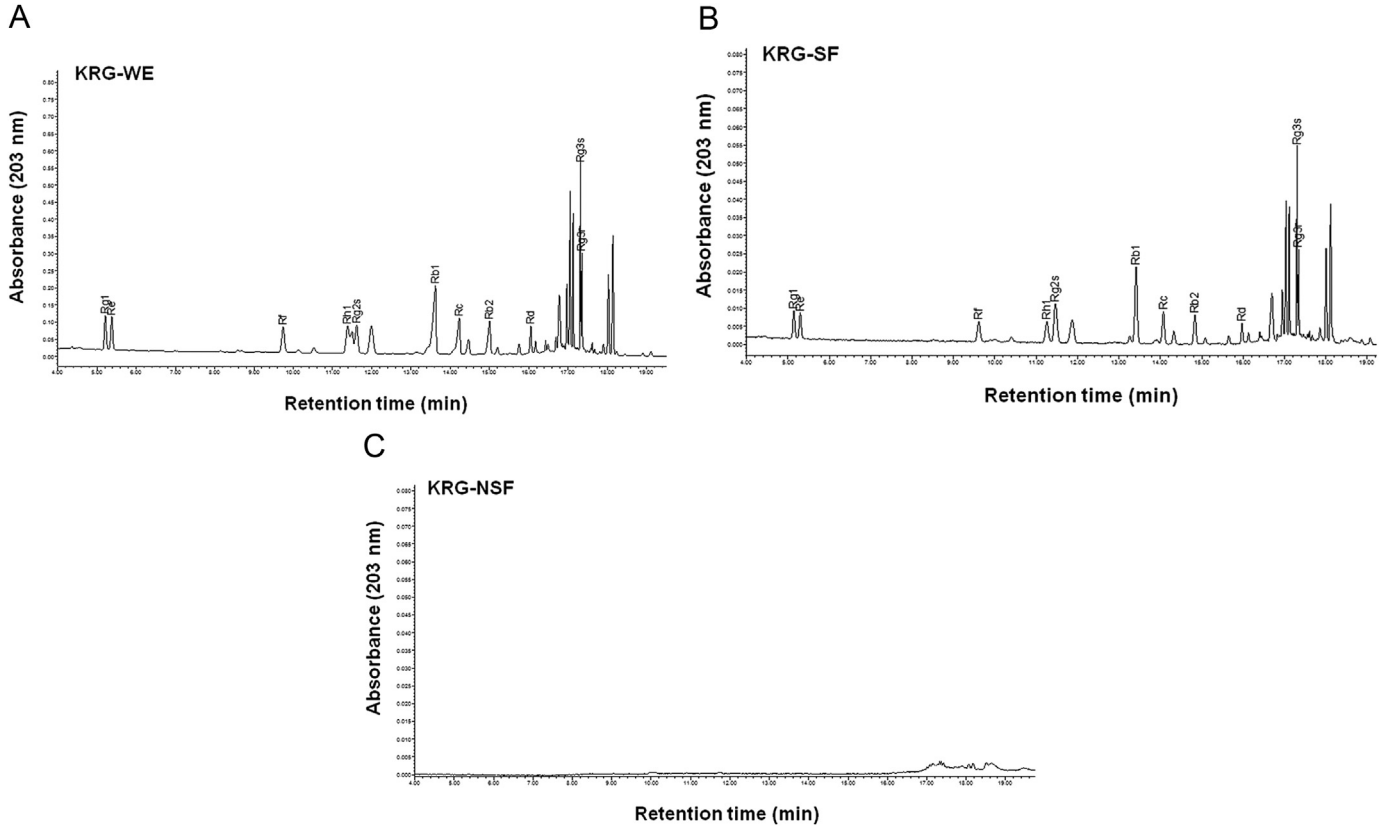

Fig. 1. HPLC profiles. (A) KRG-WE. (B) KRG-SF. (C) KRG-NSF. HPLC, high-performance liquid chromatography; KRG-NSF, Korean Rd Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract.

out using the computer program SPSS (Version 22.0, 2013; IBM Corp., Armonk, NY, USA).

3. Results and discussion

In this study, the regulatory roles of KRG-WE, KRG-NSF, and KRG-SF in the inflammatory response were investigated using macrophages and an inflammatory disease animal model. First, the major active saponin components of KRG-WE, KRG-NSF, and KRG-SF were identified and analyzed by HPLC analysis. A variety of active ginseng components, including ginsenoside (G)-Rg1, G-Re, G-Rf, G-Rh1, G-Rg2s, G-Rb1, G-Rc, G-Rb2, G-Rd, G-Rg3s, and G-Rg3r were identified in both KRG-WE and KRG-SF, in varying amounts (Table 2), whereas none of them was found in KRG-NSF (Figs. 1A–1C). Along with ginseng components, amino acids and sugars were also identified from KRG-WE, KRG-NSF, and KRG-SF in varying amounts (data not shown). These HPLC analysis results indicate that ginsenosides and their metabolites, known as the major active pharmaceutical components of ginseng [28], are contained only in KRG-WE and KRG-SF, but not in KRG-NSF.

To examine the cytotoxicity of KRG-WE, KRG-NSF, and KRG-SF in macrophages prior to exploring their anti-inflammatory activities, RAW264.7 cells were treated with KRG-WE, KRG-NSF, and KRG-SF, and the cell viability was determined. As shown in Fig. 2A, KRG-WE and KRG-NSF decreased the cell viability of RAW264.7 cells by 10–20% with doses up to 800 μg/mL, whereas SF exerted significant cytotoxicity from 400 μg/mL (~80% decrease in viability) to 800 μg/mL (>90% decrease in viability). Similarly, KRG-WE and KRG-NSF did not show cytotoxicity in splenocyte culture, whereas SF significantly reduced splenocyte viability from 250 μg/mL (~40%) up to 1,000 μg/mL (60%) (Fig. 2B). These results suggest that, unlike KRG-NSF, KRG-SF has a severe cytotoxic effect at higher doses, and ginsenosides that are contained only in KRG-SF are regarded as the main contributors for this cytotoxicity. These data highlight the importance of considering the optimal dose of KRG-SF or ginsenosides for their pharmaceutical use.

Inflammatory responses are characterized by the release of inflammatory mediators such as NO and the expression of proinflammatory cytokines such as TNF-α and IFN-β. Therefore, we examined whether KRG-WE, KRG-NSF, and KRG-SF exert anti-inflammatory effects in LPS-treated macrophages. First, NO levels were evaluated in the LPS-stimulated RAW264.7 cells after treating them with KRG-WE, KRG-NSF, or KRG-SF. As shown in Fig. 3A, NO production induced by LPS in RAW264.7 cells was markedly decreased by KRG-SF in a dose-dependent manner (left panel), consistent with the use of L-NAME, an iNOS inhibitor (right panel). The effects of KRG-WE, KRG-NSF, and KRG-SF on the expression of inflammatory genes were further examined in macrophages. The mRNA expression levels of inflammatory genes such as iNOS, COX-2, TNF-α, and IFN-β were determined by semiquantitative RT-PCR in LPS-stimulated RAW264.7 cells after treating them with KRG-WE, KRG-NSF, or KRG-SF. Induced expression of these genes in LPS-stimulated RAW264.7 cells was significantly decreased by KRG-SF, whereas both KRG-WE and KRG-NSF did not exert suppressive effects on the expression of these genes (Fig. 3B). These results strongly indicate that KRG-SF, but not KRG-WE and KRG-NSF, plays an anti-inflammatory role in macrophages through suppressing the release and mRNA expression of inflammatory mediators. The effects of KRG-WE, KRG-NSF, and KRG-SF on the generation of ROS, which are representative inflammatory mediators produced in macrophages, were also examined; however, none of them suppressed ROS generation in LPS-stimulated RAW264.7 cells (data not shown). These results indicate that, although both NO and ROS are inflammatory mediators produced in macrophages, the molecular mechanism by which SF modulates the production of NO and ROS in macrophages is different, and this needs to be further investigated. The phagocytic activity of macrophages is another indicator of macrophage activation, and the effect of KRG-WE, KRG-NSF and KRG-SF on the phagocytic activity of macrophages was examined. As shown in Fig. 3C, only SF markedly suppressed the phagocytic activity of RAW264.7 cells, whereas KRG-WE and KRG-NSF only slightly decreased the phagocytic activity. This suggests that KRG-SF may exhibit its anti-inflammatory effects by suppressing the activation of macrophages.

The molecular mechanism by which SF exerts anti-inflammatory responses was next examined in macrophages. It is well known that inflammatory responses in macrophages are dominantly mediated by the activation of several signal transduction pathways, such as NF-κB, AP-1, and IRF3 signaling pathways [3,4,29–31]. To examine whether KRG-WE, KRG-NSF, and KRG-SF play a role in modulating the transcriptional activities of NF-κB, AP-1 and IRF3, a luciferase reporter gene assay was used using NF-κB-Luc, AP-1-Luc, and IRF3-

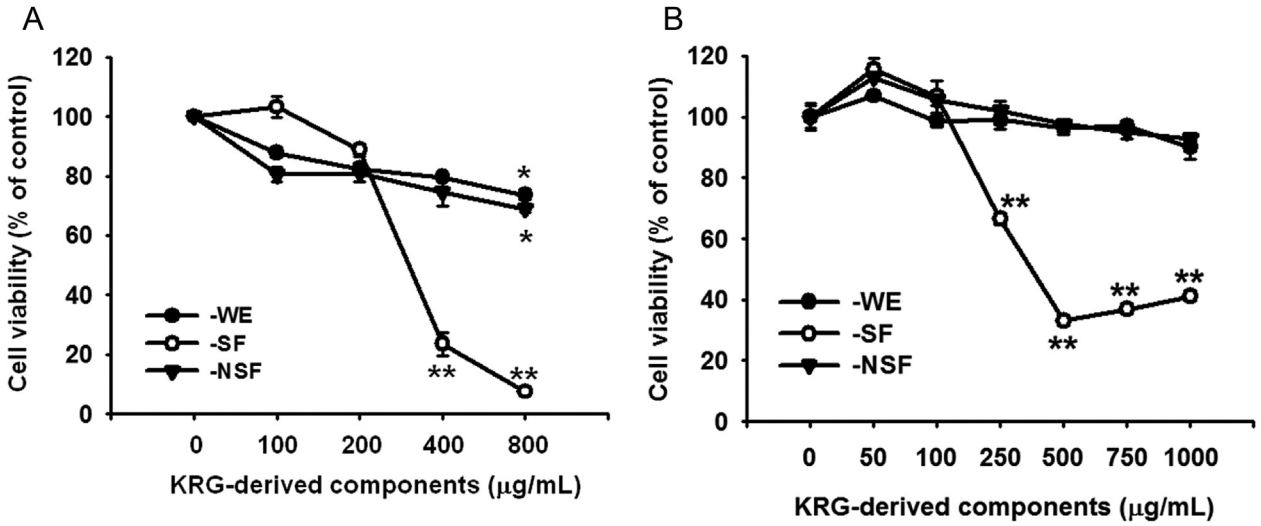

Fig. 2. Effects of KRG-WE, KRG-NSF, and KRG-SF on cell viability. (A) RAW264.7 cells and (B) splenocytes were treated with the indicated concentration of KRG-WE, KRG-NSF, and KRG-SF for 24 h, and the cell viabilities were determined by MTT assay. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to normal controls. KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

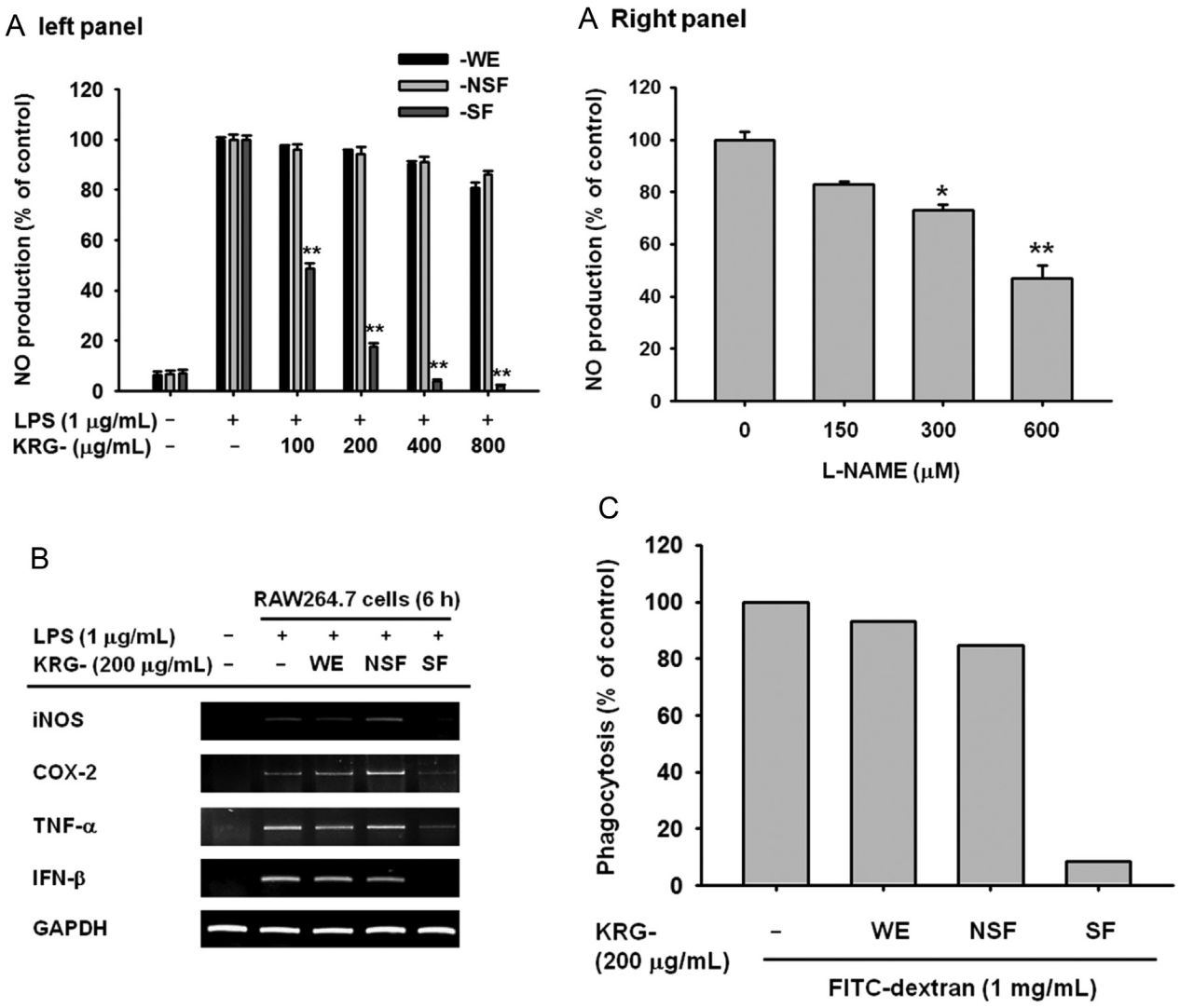

Fig. 3. Effects of KRG-WE, KRG-NSF, and KRG-SF on NO production, mRNA expression, and phagocytic activity. (A) RAW264.7 cells were pretreated with the indicated concentration of KRG-WE, KRG-NSF, KRG-SF, or L-NAME and incubated with LPS (1 μg/mL) for 24 h. NO production was determined by Griess assay using culture supernatants of the cells. (B) RAW264.7 cells were pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) and incubated with LPS (1 μg/mL) for 6 h. mRNA levels of iNOS, COX-2, TNF-α, and IFN-β were determined by semiquantitative PCR. (C) RAW264.7 cells were pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) and incubated with FITC-dextran (1 mg/mL) for 30 min. The uptake levels of FITC-dextran were measured by flow cytometry. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to controls. COX-2, cyclooxygenase-2; FITC, fluorescein isothiocyanate; IFN, interferon; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

Luc constructs in HEK293 cells. KRG-WE, KRG-NSF, and KRG-SF markedly suppressed the luciferase activities of IRF3 induced by TBK1 in HEK293 cells (Fig. 4A), whereas KRG-WE, KRG-NSF, and KRG-SF failed to significantly modulate the luciferase activities of both NF-κB and AP-1 induced by MyD88 (Figs. 4B and 4C). Because KRG-WE, KRG-NSF, and KRG-SF suppressed the luciferase activity of IRF3, we further examined whether they could decrease the nuclear translocation of IRF3 in RAW264.7 cells. As expected, KRG-WE, KRG-NSF, and KRG-SF decreased the nuclear translocation of phospho-IRF3 induced by LPS in RAW264.7 cells (Fig. 4D). Interestingly, and in accordance with the results of the luciferase gene assay, only SF dramatically decreased the nuclear translocation of phospho-IRF3 in RAW264.7 cells (Fig. 4D). To investigate which intracellular signaling molecules are involved in the IRF3 signaling pathways suppressed by KRG-WE, KRG-NSF, and KRG-SF, LPS-stimulated RAW264.7 cells were treated with KRG-WE, KRG-NSF, or KRG-SF, and changes in the activity of TBK1 (located upstream of IRF3) were determined. The phosphorylation of TBK1 induced by LPS was not detectable in the RAW264.7 cells treated with KRG-SF, whereas the phosphorylation levels of TBK1 in the RAW264.7 cells treated with KRG-WE or KRG-NSF were comparable (Fig. 4E). KRG-WE and KRG-NSF suppressed the nuclear translocation of IRF3 (Fig. 4D), but they did not decrease the phosphorylation levels of TBK1 (Fig. 4E), suggesting that the molecular mechanisms of the SF-mediated anti-inflammatory effects differ from those of KRG-WE and KRG-NSF. KRG-SF exerts its anti-inflammatory effects by inhibiting the activity of TBK1 and suppressing the nuclear translocation and transcriptional activity of its downstream molecule, IRF3. In contrast, KRG-WE and KRG-NSF exert their anti-inflammatory effects by suppressing the nuclear translocation and transcriptional activity of IRF3, and by inhibiting molecules upstream of IRF3 (rather than TBK1) in macrophages.

Although *in vitro* and *ex vivo* studies support the anti-inflammatory effects of KRG-WE, KRG-NSF, and KRG-SF, and provide the molecular and cellular mechanisms of their anti-

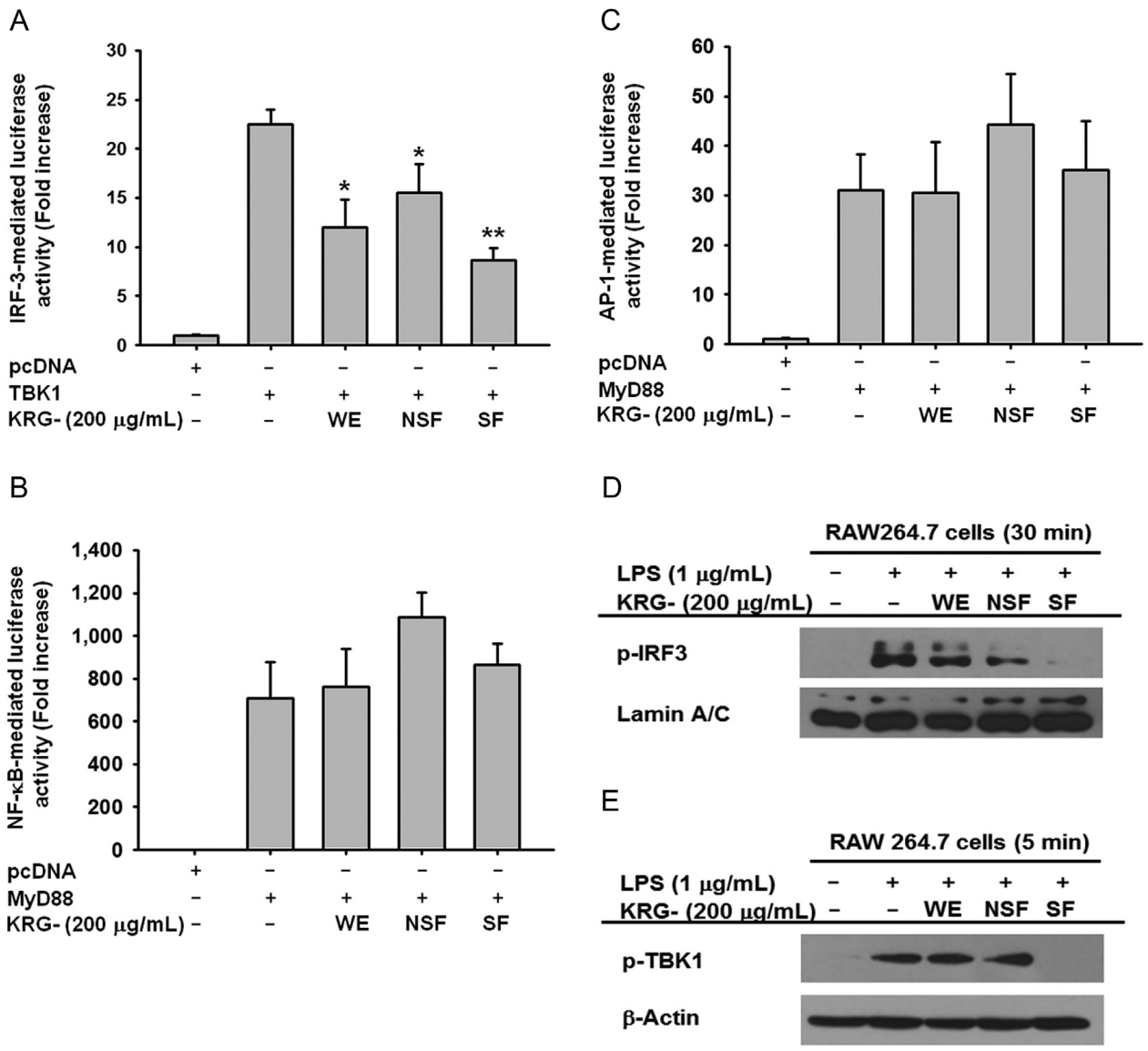

Fig. 4. Effects of KRG-WE, KRG-NSF, and KRG-SF on activation of IRF3, NF-κB, and AP-1 pathways. HEK293 cells transfected with the following constructs for 36 h were treated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) for 12 h. (A) IRF3-Luc and TBK1 expression constructs. (B) NF-κB-Luc and MyD88 expression constructs. (C) AP-1-Luc and MyD88 expression constructs. Luciferase activities were measured using a luminometer. (D) RAW264.7 cells pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) were further incubated with LPS (1 μg/mL) for 30 min, after which the level of phosphorylated IRF3 was determined by Western blot analysis. (E) RAW264.7 cells pretreated with KRG-WE, KRG-NSF, and KRG-SF (200 μg/mL) were further incubated with LPS (1 μg/mL) for 5 min, after which the level of phosphorylated TBK1 was determined by Western blot analysis. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control cells. AP-1, activator protein-1; IRF3, interferon regulatory factor 3; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; NO, nitric oxide; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

inflammatory roles in macrophages, a study regarding their *in vivo* anti-inflammatory roles in animal models of inflammatory diseases is also critical for the development of efficacious and safe drugs to treat inflammatory diseases. The *in vivo* anti-inflammatory effects of KRG-WE, KRG-NSF, and KRG-SF were explored during acute experimental gastritis induced in mice by injecting HCl/EtOH. Because previous studies have revealed that oral administration of ginseng or its metabolites/derivatives is a reasonable route to test their *in vivo* anti-inflammatory effects in animal models [19,32,33], KRG-WE, KRG-NSF, and KRG-SF were orally administered in the HCl/EtOH-induced gastritis mice, and their anti-inflammatory activities were examined according to the time schedule described in Fig. 5A. KRG-WE, KRG-NSF, and KRG-SF ameliorated the symptoms of gastric ulcer (Fig. 5B) and significantly reduced the area of the disease lesions (Fig. 5C) in the HCl/EtOH-induced experimental gastritis mice. These results strongly suggest that oral administration of KRG-WE, KRG-NSF, and KRG-SF could be a potential and effective anti-inflammatory remedy to treat gastritis.

In summary, we found that KRG-WE, KRG-NSF, and KRG-SF exhibited *in vivo* and *in vitro* anti-inflammatory effects by ameliorating the symptoms of gastric ulcer in the gastritis mouse model and by suppressing the IRF3 signaling pathway in macrophages. Interestingly, although KRG-WE, KRG-NSF, and KRG-SF exerted *in vitro* anti-inflammatory effects by suppressing the IRF3 signaling pathway in macrophages, the activity of TBK1 was reduced only by KRG-SF, but not by KRG-WE and KRG-NSF. In addition, although KRG-SF decreased phagocytic activity, NO production, and mRNA expression of inflammatory genes in inflammatory macrophages, KRG-NSF may exert an *in vivo* anti-inflammatory activity by a mechanism other than the modulation of macrophage-mediated inflammatory

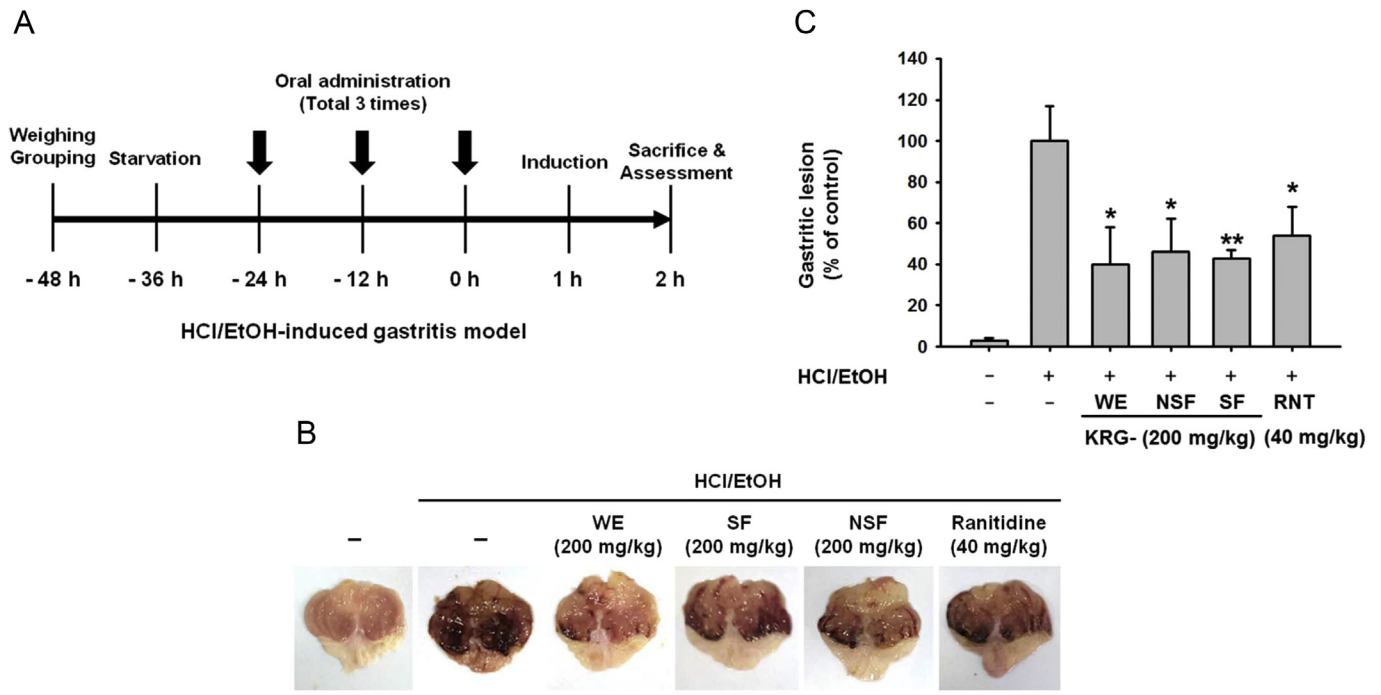

Fig. 5. Effects of KRG-WE, KRG-NSF, and KRG-SF on HCl/EtOH-induced inflammatory symptoms in mouse stomachs. (A) Time schedule for evaluating the anti-inflammatory effects of KRG-WE, KRG-NSF, and KRG-SF in the HCl/EtOH-induced gastritis mouse model. (B) Mice were orally administered KRG-WE, KRG-NSF, KRG-SF (200 mg/kg), or ranitidine (RNT, 40 mg/kg) three times prior to the oral administration of HCl/EtOH. After 1 h, gastric lesions in the stomachs were photographed. (C) Gastric lesions were measured with a pixel counter. The gastric lesions of the control group (inducer alone) are represented by 100%. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with control cells. EtOH, ethanol; KRG-NSF, Korean Red Ginseng nonsaponin fraction; KRG-SF, Korean Red Ginseng saponin fraction; KRG-WE, Korean Red Ginseng water extract.

responses. Exactly how KRG-NSF plays an anti-inflammatory role in macrophages needs to be further investigated. Our data strongly suggest that KRG-WE, KRG-NSF, and KRG-SF have anti-inflammatory activities with different molecular and cellular mechanisms, and could therefore be developed as potential anti-inflammatory remedies to prevent and treat inflammatory diseases.

Conflicts of interest

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

Acknowledgments

This work was supported by a grant (2015–2016) from the Korean Society of Ginseng, funded by the Korean Ginseng Corporation (KGC).

References

[1] Zou J, Guo P, Lv N, Huang D. Lipopolysaccharide-induced tumor necrosis factor-alpha factor enhances inflammation and is associated with cancer (Review). Mol Med Rep 2015;12:6399–404.
[2] Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol 2007;147:227–35.
[3] Yi YS, Son YJ, Ryou C, Sung GH, Kim JH, Cho JY. Functional roles of Syk in macrophage-mediated inflammatory responses. Mediators Inflamm 2014. http://dx.doi.org/10.1155/2014/270302.
[4] 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. http://dx.doi.org/10.1155/2014/352371.
[5] Kim JH, Kim MY, Cho JY. Fisetin suppresses macrophage-mediated inflammatory responses by blockade of Src and Syk. Biomol Ther (Seoul) 2015;23:414–20.
[6] Choi J, Kim H, Kim Y, Jang M, Jeon J, Hwang YI, Shon WJ, Song YW, Kang JS, Lee WJ. The anti-inflammatory effect of GV1001 mediated by the downregulation of ENO1-induced pro-inflammatory cytokine production. Immune Netw 2015;15:291–303.
[7] Anthony Jalin AM, Lee JC, Cho GS, Kim C, Ju C, Pahk K, Song HY, Kim WK. Simvastatin reduces lipopolysaccharides-accelerated cerebral ischemic injury via inhibition of nuclear factor-kappa B activity. Biomol Ther (Seoul) 2015;23:531–8.
[8] Kaur M, Singh M, Silakari O. Inhibitors of switch kinase 'spleen tyrosine kinase' in inflammation and immune-mediated disorders: a review. Eur J Med Chem 2013;67:434–46.
[9] Cha YI, Kim HS. Emerging role of sirtuins on tumorigenesis: possible link between aging and cancer. BMB Rep 2013;46:429–38.
[10] Ham M, Moon A. Inflammatory and microenvironmental factors involved in breast cancer progression. Arch Pharm Res 2013;36:1419–31.
[11] Lu JM, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293–302.
[12] Shin BK, Kwon SW, Park JH. Chemical diversity of ginseng saponins from *Panax ginseng*. J Ginseng Res 2015;39:287–98.
[13] Lee SM, Bae BS, Park HW, Ahn NG, Cho BG, Cho YL, Kwak YS. Characterization of Korean Red Ginseng (*Panax ginseng* Meyer): history, preparation method, and chemical composition. J Ginseng Res 2015;39:384–91.
[14] Zhang D, Yasuda T, Yu Y, Zheng P, Kawabata T, Ma Y, Okada S. Ginseng extract scavenges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. Free Radic Biol Med 1996;20:145–50.
[15] Joo SS, Won TJ, Lee DI. Reciprocal activity of ginsenosides in the production of proinflammatory repertoire, and their potential roles in neuroprotection *in vivo*. Planta Med 2005;71:476–81.
[16] Park HY, Lee SH, Lee KS, Yoon HK, Yoo YC, Lee J, Choi JE, Kim PH, Park SR. Ginsenoside Rg1 and 20(*S*)-Rg3 induce IgA production by mouse B cells. Immune Netw 2015;15:331–6.
[17] Seong MA, Woo JK, Kang JH, Jang YS, Choi S, Lee TH, Jung KH, Kang DK, Hurh BS, Kim DE, et al. Oral administration of fermented wild ginseng ameliorates DSS-induced acute colitis by inhibiting NF-kappaB signaling and protects intestinal epithelial barrier. BMB Rep 2015;48:419–25.
[18] Baek KS, Hong YD, Kim Y, Sung NY, Yang S, Lee KM, Park JY, Park JS, Rho HS, Shin SS, et al. Anti-inflammatory activity of AP-SF, a ginsenoside-enriched fraction, from Korean ginseng. J Ginseng Res 2015;39:155–61.
[19] Yang Y, Lee J, Rhee MH, Yu T, Baek KS, Sung NY, Kim Y, Yoon K, Kim JH, Kwak YS, et al. Molecular mechanism of protopanaxadiol saponin fraction-mediated anti-inflammatory actions. J Ginseng Res 2015;39:61–8.
[20] Endale M, Im EJ, Lee JY, Kim SD, Yayeh T, Song YB, Kwak YS, Kim C, Kim SH, Roh SS, et al. Korean red ginseng saponin fraction rich in ginsenoside-Rb1, Rc and Rb2 attenuates the severity of mouse collagen-induced arthritis. Mediators Inflamm 2014;2014:748964.

[21] Starkenmann C, Luca L, Niclass Y, Praz E, Roguet D. Comparison of volatile constituents of *Persicaria odorata*(Lour.) Sojak (*Polygonum odoratum* Lour.) and *Persicaria hydropiper* L. Spach (*Polygonum hydropiper* L.). J Agric Food Chem 2006;54:3067–71.

[22] Almela L, Sanchez-Munoz B, Fernandez-Lopez JA, Roca MJ, Rabe V. Liquid chromatograpic-mass spectrometric analysis of phenolics and free radical scavenging activity of rosemary extract from different raw material. J Chromatogr A 2006;1120:221–9.

[23] Jeong D, Yi YS, Sung GH, Yang WS, Park JG, Yoon K, Yoon DH, Song C, Lee Y, Rhee MH, et al. Anti-inflammatory activities and mechanisms of *Artemisia asiatica* ethanol extract. J Ethnopharmacol 2014;152:487–96.

[24] Lee YG, Lee WM, Kim JY, Lee JY, Lee IK, Yun BS, Rhee MH, Cho JY. Src kinase-targeted anti-inflammatory activity of davallialactone from *Inonotus xeranticus* in lipopolysaccharide-activated RAW264.7 cells. Br J Pharmacol 2008;154:852–63.

[25] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.

[26] Lee HJ, Hyun EA, Yoon WJ, Kim BH, Rhee MH, Kang HK, Cho JY, Yoo ES. *In vitro* anti-inflammatory and anti-oxidative effects of *Cinnamomum camphora* extracts. J Ethnopharmacol 2006;103:208–16.

[27] Kim SH, Park JG, Sung GH, Yang S, Yang WS, Kim E, Kim JH, Ha VT, Kim HG, Yi YS, et al. Kaempferol, a dietary flavonoid, ameliorates acute inflammatory and nociceptive symptoms in gastritis, pancreatitis, and abdominal pain. Mol Nutr Food Res 2015;59:1400–5.

[28] Hasegawa H. Proof of the mysterious efficacy of ginseng: basic and clinical trials: metabolic activation of ginsenoside: deglycosylation by intestinal bacteria and esterification with fatty acid. J Pharmacol Sci 2004;95:153–7.

[29] Yang WS, Ratan ZA, Kim G, Lee Y, Kim MY, Kim JH, Cho JY. 4-Isopropyl-2,6-bis(1-phenylethyl)aniline 1, an analogue of KTH-13 isolated from *Cordyceps bassiana*, inhibits the NF-kappaB-mediated inflammatory response. Mediators Inflamm 2015. http://dx.doi.org/10.1155/2015/143025.

[30] Hossen MJ, Kim SC, Son YJ, Baek KS, Kim E, Yang WS, Jeong D, Park JG, Kim HG, Chung WJ, et al. AP-1-targeting anti-inflammatory activity of the methanolic extract of *Persicaria chinensis*. Evid Based Complement Alternat Med 2015. http://dx.doi.org/10.1155/2015/608126.

[31] Shen T, Yang WS, Yi YS, Sung GH, Rhee MH, Poo H, Kim MY, Kim KW, Kim JH, Cho JY. AP-1/IRF-3 targeted anti-Inflammatory activity of andrographolide isolated from *Andrographis paniculata*. Evid Based Complement Alternat Med 2013. http://dx.doi.org/10.1155/2013/210736.

[32] Yu T, Rhee MH, Lee J, Kim SH, Yang Y, Kim HG, Kim Y, Kim C, Kwak YS, Kim JH, et al. Ginsenoside Rc from Korean Red Ginseng (*Panax ginseng* C.A. Meyer) attenuates inflammatory symptoms of gastritis, hepatitis and arthritis. Am J Chin Med 2016;44:595–615.

[33] Yang Y, Yang WS, Yu T, Sung GH, Park KW, Yoon K, Son YJ, Hwang H, Kwak YS, Lee CM, et al. ATF-2/CREB/IRF3-targeted anti-inflammatory activity of Korean red ginseng water extract. J Ethnopharmacol 2014;154:218–28.