



Research article

A comparative study on immune-stimulatory and antioxidant activities of various types of ginseng extracts in murine and rodent models

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ABSTRACT

Background: Ginseng (*Panax ginseng*) is a widely used traditional herbal supplement that possesses various health-enhancing efficacies. Various ginseng products are available in market, especially in the Korean peninsula, in the form of drinks, tablets, and capsules. The different ginseng types include the traditional red ginseng extract (RGE), white ginseng, and black red ginseng extract (BRGE). Their fermented and enzyme-treated products are also available. Different treatment regimens alter the bioavailability of certain compounds present in the respective ginseng extracts. Therefore, in this study, we aimed to compare the antioxidant and immune-stimulating activities of RGE, BRGE, and fermented red ginseng extract (FRGE).

Methods: We used an acetaminophen-induced oxidative stress model for investigating the reduction of oxidative stress by RGE, BRGE, and FRGE in Sprague Dawley rats. A cyclophosphamide-induced immunosuppression model was used to evaluate the immune-stimulating activities of these ginseng extracts in BALB/c mice.

Results: Our results showed that most prominently, RGE (in almost all experiments) exhibited excellent antioxidant effects via increasing superoxide dismutase, catalase, and glutathione peroxidase activities in the liver and decreasing serum 8-hydroxy-2'-deoxyguanosine, aspartate aminotransferase, and lactate dehydrogenase levels compared with the groups treated with FRGE and BRGE. Moreover, RGE significantly increased the number of white blood cells, especially T and B lymphocytes, and antibody-forming cells in the spleen and thymus, and it also activated a number of immune cell subtypes.

Conclusion: Taken together, these results indicate that RGE is the best supplement for consumption in everyday life for overall health-enhancing properties.

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1. Introduction

Korean ginseng (*Panax ginseng* Meyer) is the most commonly used herbal supplement throughout the Korean peninsula and in other countries. The marvelous health-enhancing properties of ginseng have been unraveled with the passage of time. The root of ginseng is the treasure of all the biological properties that this plant possesses. Ginseng is extensively used as an adaptogen owing to its antistress and vitalizing properties [1]. In addition, Korean ginseng has been reported to possess a number of healing properties for

various diseases such as inflammation, tumors, cancers, and other pathological ailments [2–6].

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the biological ability of the body to detoxify the reactive components which subsequently leads to cell damage [7]. It is widely associated with pathological conditions such as cancer, Parkinson's disease, Alzheimer's disease, and cardiovascular diseases [8–10]. If the body is unable to rapidly remove ROS, it results in the accumulation of ROS in cells, which in turn damages the whole cell structure and

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ultimately leads to major pathological conditions. Previously, ginseng has been reported for its antioxidative properties [11–16]. In the present study, we compared the antioxidative effects elicited by three types of ginseng extracts, namely, red ginseng extract (RGE), black red ginseng extract (BRGE), and fermented red ginseng extract (FRGE). Immune suppression is a common pathological condition encountered by almost all adults in today's world, and it is caused by high stress levels in studies, work, and emotional conditions. Under immunosuppressed conditions, a person is easily vulnerable to infectious diseases spread through the environment, such as influenza, cough, and cold. Although the effects of ginseng in enhancing the immune system have been reported [17,18], no comparative study has been performed to identify the ginseng extract most suitable for consumption in everyday life for enhancing immunity.

Therefore, for the first time, we performed a comparative study of the antioxidant and immune-stimulating properties of RGE, BRGE, and FRGE. Our results showed that RGE elicited outstanding antioxidant effects and immune-stimulating properties compared with FRGE and BRGE.

2. Materials and methods

2.1. Materials

All commercially available kits for measuring the antioxidant ability of the extracts were purchased from Cayman Chemical (Michigan, USA). Earle's balanced salt solution (EBSS), diethylaminoethyl-dextran, agar, 2-mercaptoethanol, cyclophosphamide (CY), and guinea pig complement serum were purchased from Sigma-Aldrich Co. (St. Louis, Mo., USA). Cell counting kit (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Roswell Park Memorial Institute medium (RPMI) 1640 medium, fetal bovine serum, and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer were procured from Gibco BRL (Grand Island, NY, USA). Ammonium-chloride-potassium lysing buffer was purchased from Lonza (Walkersville, USA). Sheep red blood cells (SRBCs) were purchased from South Pacific Sera Ltd. (Timaru, New Zealand). Splenocyte morphology was measured by flow cytometry (BD FACSCanto II; BD Biosciences, San Diego, CA, USA). Monoclonal antibodies (MoAbs) against CD3e, CD4, CD8, and CD25, and fluorochrome-labeled MoAbs, and isotype control IgGs were purchased from BD Biosciences. The purified anti-mouse CD16/CD32 Fc receptor, peridinin-chlorophyll a-protein-conjugated anti-mouse CD3e (clone: 145-2C11), and R-phycoerythrin (R-PE)-conjugated anti-mouse CD45R/B220 (clone: RA3-6B2) were purchased from BD Pharmingen Co. (San Diego, CA, USA). All other reagents and chemicals used were obtained from Sigma-Aldrich Co.

2.2. Ginseng sample preparation

RGE (Rg1 + Rb1 + Rg3 = 5.5 mg/g), BRGE (Rg1 + Rb1 + Rg3 = 15 mg/g), and FRGE (Rg1 + Rb1 + Rg3 = 7.5 mg/g) were purchased from the local market and then were analyzed for ginsenosides, maltol, arginine-fructose-glucose (AFG), and acidic polysaccharides according to previously reported studies [19–21] by HPLC analysis as shown in Table 1.

2.3. Experimental animals

For determining the antioxidant activity, 6-week-old male Sprague Dawley (SD) rats were purchased from Koatech (Gyeonggi, Korea) and acclimatized for 7 days. During the acclimatization period, the general physical and behavioral symptoms were observed. Only normal animals weighing 200–230 g on the first

Table 1

Amount of ginsenosides and other nonsaponin fractions in RGE, FRGE, and BRGE determined by HPLC

Ginsenosides	Red ginseng extract (RGE) mg/g	Fermented red ginseng extract (FRGE) mg/g	Black red ginseng extract (BRGE) mg/g
Rg1	1.10 ± 0.07	0.47 ± 0.06	1.95 ± 0.06
Re	1.57 ± 0.17	3.39 ± 0.06	5.04 ± 0.11
Rf	0.95 ± 0.01	Not detected	1.21 ± 0.05
Rh1	0.87 ± 0.09	0.12 ± 0.01	0.33 ± 0.15
Rg2(s)	1.83 ± 0.15	1.10 ± 0.04	1.50 ± 0.24
Rb1	4.70 ± 0.17	8.18 ± 0.07	12.02 ± 0.45
Rc	2.03 ± 0.15	2.53 ± 0.05	5.88 ± 0.16
Rb2	1.76 ± 0.15	0.49 ± 0.06	4.94 ± 0.32
Rd	0.67 ± 0.04	2.36 ± 0.12	2.39 ± 0.03
Rg3(s)	2.13 ± 0.05	0.96 ± 0.04	1.34 ± 0.10
Rg3(r)	0.83 ± 0.02	0.53 ± 0.02	0.65 ± 0.04
¹⁾ AFG	44.38 ± 2.13	6.98 ± 0.28	8.01 ± 1.03
Acidic polysaccharide	64.29 ± 1.84	41.24 ± 1.44	49.94 ± 1.23
Maltol	1.16 ± 0.04	0.02 ± 0.00	0.72 ± 0.01

¹⁾ AFG level (elevated amounts) is shown in a box which is responsible for the strong antioxidant and immune-stimulating properties of RGE.

day of administration were selected and used in the experiments. Eight rats per group were used for each experiment. The animal room was maintained at a temperature of 20 ± 2°C, relative humidity of 50 ± 5%, and ventilation rates of 10–12 times/hr. The illumination time was from 7:00 am to 7:00 pm, and illumination was maintained at 150–200 Lux. The rats were provided *ad libitum* access to water and food. The animal feed was procured from Feedlab (Guri, Korea). All the experiments were carried out in accordance with the ethical guidelines of the Animal Experimental Ethics Committee, Kyungpook National University, Daegu, Korea (IACUC-2018-51). For determining the immune-stimulatory activity, 6-week-old male BALB/c mice were purchased from Korea Biological Link (Chungbuk, Korea). The acclimatization period, animal room environment, and monitoring were the same as those selected for SD rats. After acclimatization, mice weighing 22–23 g were selected and used in the experiments. Six mice per group were used for each experiment.

2.4. Ginseng sample administration

To investigate the reduction of oxidative stress by ginseng extracts in SD rats, acetaminophen (APAP) was used to induce oxidative stress in rats (negative control group). The rats were divided into five groups (with each group n = 8 animals) as follows: normal group (fed saline only); negative control group (APAP control); RGE-treated group; BRGE-treated group; and FRGE-treated group. The groups treated with RGE, BRGE, and FRGE were orally fed ginseng extracts daily for 7 days at a dose of 500 mg/kg. For the induction of oxidative stress 2 h after the last administration of ginseng extracts, all groups, except the saline-fed group, were orally administered 800 mg/kg of APAP. Food and water were withdrawn after APAP treatment. The animals were euthanized after 24 h, and blood and organs were collected for later experiments.

For investigating the immune-stimulatory activity of ginseng extracts, CY was used to induce immunosuppression in mice (negative control). Male mice were divided into five groups (with each group n = 6 animals) as follows: normal group (fed saline only); negative control group (CY control); RGE-treated group; BRGE-treated group; and FRGE-treated group. The RGE, BRGE, and FRGE groups were orally fed ginseng extracts daily for 2 weeks at a dose of 500 mg/kg. After 2 weeks of treatment, all groups were intraperitoneally (*i.p.*) injected SRBCs (acting as antigen) for

immune stimulation. On the next day (day 16), 24 h later, all groups, except the saline-fed group, were injected CY (*i.p.*) at a single dose of 50 mg/kg (immunosuppressant drug). Three days later, all animals were euthanized, and blood and organs were collected for later experiments.

2.5. Assessment of serum 8-hydroxy-2'-deoxyguanosine levels

For 8-hydroxy-2'-deoxyguanosine (8-OHdG) measurement and biochemical analysis, blood samples were collected from rats under isoflurane respiratory anesthesia and placed in serum tubes. The 8-OHdG assay kit (Cayman Chemical, Michigan, USA) was used for the measurement of 8-OHdG levels. The content of 8-OHdG in the samples were measured based on the competition of 8-OHdG and Acetylcholinesterase (AChE) enzyme-labeled 8-OHdG for 8-OHdG monoclonal antibody.

2.6. Preparation of liver homogenate

After euthanasia, the liver tissue was immediately extracted and stored in physiological saline on ice until homogenization. The buffer that was used for homogenization of liver tissue consisted of 10 mmol Tris-HCl and 1 mmol ethylenediaminetetraacetic acid (pH 7.4). Whole liver tissues were homogenized in 20 mL of homogenization buffer, and then 10% homogenate was prepared relative to the liver weight. After centrifugation at 1,500 rpm for 30 min at 4°C, the supernatant was separated and stored at -80°C until further analysis.

2.7. Measurement of antioxidant enzyme levels in liver tissue

The activities of all antioxidant enzymes were analyzed using specific enzyme assay kits. Superoxide dismutase activity (Cu/Zn, Mn, and FeSOD) was measured by diluting the samples to 1:1000. Catalase activity was measured by 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald), and the sample was diluted to 1:1000. Glutathione peroxidase (GPx) assay was performed in the presence of glutathione and oxidized glutathione, and GPx activity was evaluated by differences in absorbance at different dihydronicotinamide-adenine dinucleotide phosphate (NADPH) levels (sample dilution of 1:20).

2.8. Evaluation of liver damage by serum biochemical analysis

The blood levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) in rats were measured using a biochemical analyzer (HITACHI 7100, HITACHI Ltd., Tokyo, Japan).

2.9. Measurement of IgM antibody-producing cells in the spleen

To determine the number of IgM antibody-forming cells (AFCs) in the spleen, SRBC was used as an antigen. For injection, SRBC was centrifuged three times with EBSS solution (300 × g, 10 min, 4°C) and adjusted with EBSS solution so that the SRBC concentration would be 5×10^8 cells/mL. This suspension (0.5 mL) was intraperitoneally administered to all experimental animals for induction of immune response. The next day, 50 mg/kg of CY was administered *i.p.* to induce immunosuppression. At this time, no sample was administered to prevent each sample from affecting CY metabolism. On the third day after CY administration, all animals were euthanized with excessive CO₂ gas. Blood was collected from the heart, and the spleen and thymus were excised. The spleen was lightly pulverized with sterile syringes in ice-cold EBSS solution for antibody production and centrifuged (150 × g, 10 min, 4°C). The supernatant was removed, and 3 mL of EBSS solution was added to

prepare a spleen cell suspension (SCS). SCS (200 μL) and 800 μL of EBSS solution were mixed to prepare a fivefold diluted cell suspension. To prepare 0.5% agar solution, 0.5% agar was suspended in EBSS solution, followed by the addition of 3% diethylaminoethyl-dextran to dissolve it completely. Then, this solution was kept at 48°C in a constant-temperature water bath. In a round-bottom tube (12 × 75 mm), 350 μL of agar solution, 100 μL of SCS, 25 μL of SRBC, and 25 μL of guinea pig complement were mixed. The resultant solution (200 μL) was placed in a Petri dish (diameter 100 mm), which was then covered with a cover glass (24 × 50 mm). After agar treatment for around 20 minutes, plaque formation was induced by incubation in a 5% CO₂ incubator at 37°C for 4 h. The number of plaques formed was counted by a 10× magnification microscope (IX-81; Olympus, Tokyo, Japan), and the number of cells in the SCS was counted. Antibody production was calculated as AFCs/10⁶ splenocytes or AFCs/spleen.

2.10. Splenic cell subtype analysis

The spleen was lightly pulverized with sterile syringes in ice-cold EBSS solution for antibody production and centrifuged (150×g, 10 min, 4°C). The supernatant was removed by centrifugation (300×g, 10 min, 4°C). Then, 2 mL of ammonium-chloride-potassium lysing buffer was added to the SCS to hemolyse the red blood cells (RBCs). Then, 5 mL of RPMI 1640 complete medium (10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin, 100 μg streptomycin, and 5×10^{-5} μM 2-mercaptoethanol) was added and left at room temperature for 5 min. The supernatant was removed by centrifugation (300× g, 5 min, 4°C) and washed twice with RPMI 1640 medium again. Fc receptor blockade was performed at 4°C for 15 min with a purified anti-mouse CD16/CD32 Fc receptor (1 μg/tube) in the splenocytes monolayer. To determine the number of T cells, peridinin-chlorophyll a-protein-conjugated anti-mouse CD3e and 1 μg of R-PE-conjugated anti-mouse CD45R/B220 were added to each well to measure the number of B cells. After washing three times with 1 mL of phosphate-buffered saline (PBS), 0.4 mL of PBS was added to each tube, mixed well, and analyzed by flow cytometry.

2.11. Isolation of spleen and thymus and subtyping of immune cells from non-SRBC mice model of immunosuppression by fluorescent antibody cell sorting (FACS)

For investigating the immune-stimulatory activity of ginseng extracts without injecting SRBC, male BALB/c mice were divided into five groups (with each group n = 6 animals) as follows: normal group (fed saline only); negative control group (CY control); RGE-treated group; FRGE-treated; and BRGE-treated groups. The RGE, FRGE, and BRGE groups were orally fed ginseng extracts daily for 2 weeks at a dose of 500 mg/kg. After 2 weeks of treatment, on day 15, 24 h later, after the last sample administration, all groups, except the saline-fed group, were *i.p.* injected with CY at a single dose of 50 mg/kg (immunosuppressant drug). Three days later, all animals were euthanized, and blood and organs were collected for later experiments. For immune cell subtyping by FACS, thymocytes (extracted as given in section 2.10 for splenocytes) were incubated with 10 μL of fluorescein isothiocyanate (FITC)-conjugated anti CD4, PE-Cy 5.5-CD25, and PE-conjugated CD8 and CD4 for 30 min. Later, the acquisition and analysis were performed by multicolor FACS using CellQuest Pro software. The results are expressed as absolute no. of cells in thymus (10⁵).

2.12. Isolation of neutrophils and neutrophil migration assay

The neutrophils were isolated from the whole blood of non-SRBC immune-suppressing mice model. The procedure was

carried out using modified Histopaque gradient technique [22]. Isolation of neutrophils and neutrophil migration assay were performed according to the protocol given by Ahmad et al [23]

2.13. Estimation of Th1/Th2 cytokines using ELISA

For determination of Th1/Th2 cytokines, i.e., interleukin (IL)-4, IL-12 and interferon- γ , 96-well plates were incubated with CD3 monoclonal antibody (mAb) at 0.5 μ g/mL overnight, and the next day, MoAbs-based mouse interleukin enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, MN, USA) were used for determination of their levels according to the manufacturer's instructions.

2.14. Statistical analysis

The data were expressed as mean \pm standard deviation. The groups were analyzed using an analysis of variance followed by

the Dunnett's test for multiple comparisons (SPSS version 20, IBM, NY, USA). A p value less than 0.05 was considered statistically significant. There are some other significant marks shown in the figures, and they are described in their respective figure legends.

3. Results

3.1. Biochemical and liver tissue analysis for antioxidant biomarkers

The blood levels of AST, ALT, and LDH in rats were measured by biochemical methods. There were significant increases in the activity of superoxide dismutase, catalase, and GPx in RGE- and FRGE-treated groups compared with the control group (Figs. 1A–C). Moreover, a significant decrease in 8-OHdG levels was observed in the RGE-treated group compared with the FRGE- and BRGE-treated groups and negative control group (Fig. 1D). AST and LDH levels

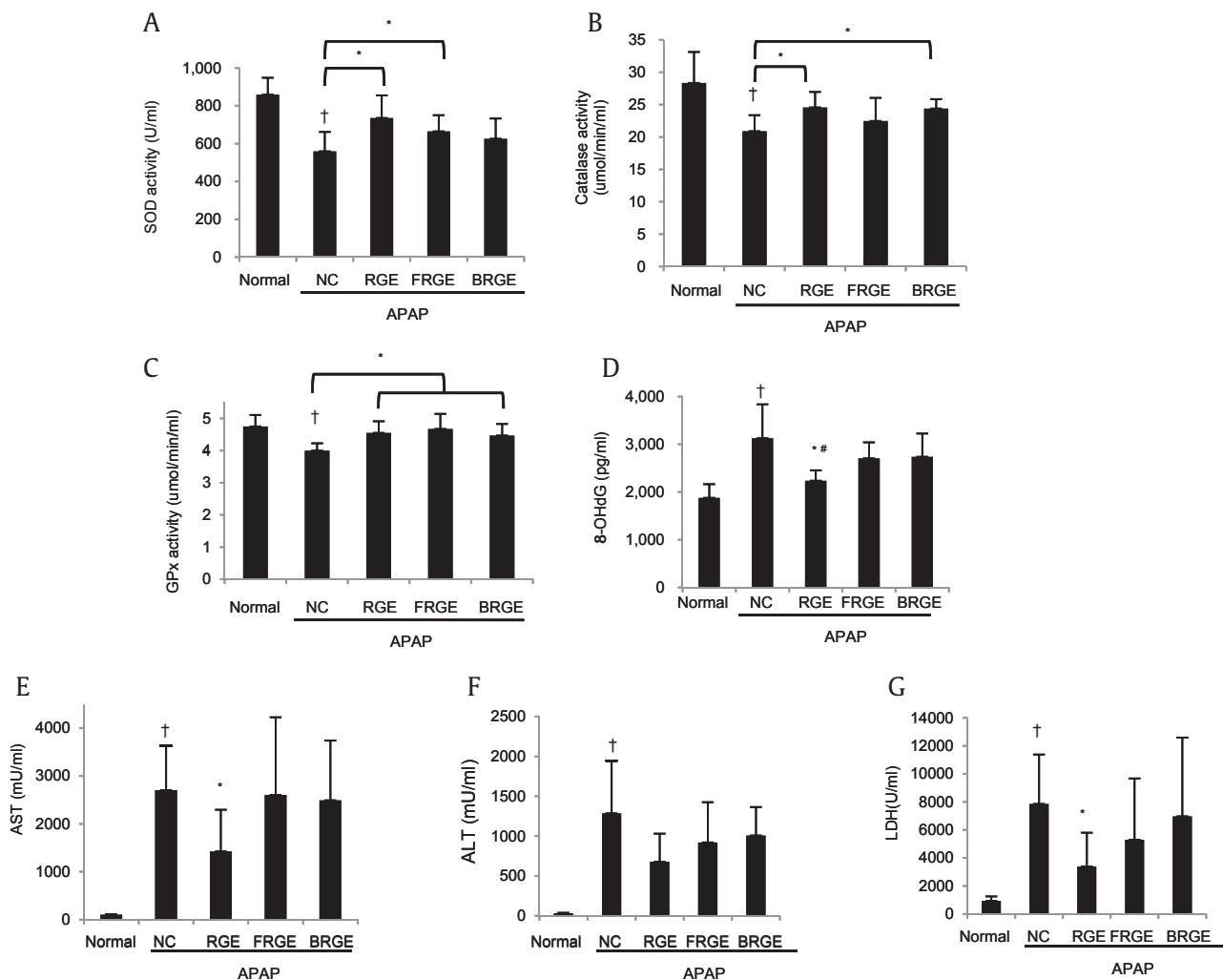


Fig. 1. Effects of RGE, BRGE, and FRGE on oxidative and hepatic pathological parameters. (A) SOD activity in NC group as compared to the normal group, $ip < 0.01$ and SOD activity in RGE- and FRGE-treated groups compared to the control group (NC), $ip < 0.05$. (B) CAT activity as compared to the normal group, $ip < 0.01$ and CAT activity in the RGE- and BRGE-treated groups compared to the control group (NC), $ip < 0.05$. (C) GPx activity as compared to the normal group, $ip < 0.01$ and GPx activity in RGE-, BRGE-, and FRGE-treated groups compared to the control group (NC), $ip < 0.05$. (D) 8-OHdG activity as compared to the normal group, $ip < 0.01$ 8-OHdG levels in the RGE-treated group compared to the control group (NC) (*) and BRGE- and FRGE-treated groups ($^{\#}$), $ip < 0.05$. (E) AST levels in the control group (NC) compared to the normal group, $ip < 0.01$ and AST level in the RGE-treated group compared to the control group (NC), $ip < 0.05$. (F) ALT levels in the control group (NC) compared to the normal group, $ip < 0.01$. (G) LDH levels in the control group (NC) compared to the normal group, $ip < 0.01$ and LDH levels in RGE-treated group compared to the control group (NC), $ip < 0.05$. 8-OHdG, 8-hydroxy-2'-deoxyguanosine; ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; BRGE, black red ginseng extract; CAT, catalase; FRGE, fermented red ginseng extract; GPx, Glutathione peroxidase; LDH, lactate dehydrogenase; RGE, red ginseng extract; SOD, superoxide dismutase.

showed a significant decrease in the RGE-treated group compared with the other groups (Figs. 1E and 1G). No significant difference in ALT levels was observed between the ginseng-treated groups and control group (Fig. 1F).

3.2. Splenic antibody response to SRBCs

Antibody production is known to be the most sensitive indicator of immunity and the most widely used marker for *in vivo* studies [24]. When mixed with rabbit erythrocytes and complement in spleen cells of mice immunized with rabbit erythrocytes was used as antigens, the antibody-producing B cells release immunoglobulin. The released immunoglobulin binds to the hematopoietic RBCs, where the complement acts on the hematopoietic RBCs that produce the antibody [25]. The antibody response of IgM to the T-dependent antigen SRBCs was evaluated as shown in Fig. 2A. CY suppressed the number of AFCs in response to the T-dependent antigen SRBCs when compared with the normal group. When the data were expressed as AFCs $\times 10^3$ per spleen (Fig. 2B), there were 135 ± 42 cells in the normal group, which was significantly reduced to 19 ± 6 , 77 ± 20 , 50 ± 11 , and 48 ± 12 in the CY control, RGE, BRGE, and FRGE groups, respectively. Thereafter, the number of AFCs per million spleen cells was evaluated, and a significant increase was observed in the RGE (398%), BRGE (258%), and FRGE (247%) groups compared with the CY control. Compared with BRGE and FRGE, RGE significantly increased the number of cells by 37% (Fig. 2C).

Table 2
Effects of RGE, BRGE, and FRGE on Hematologic parameters in BALB/c mice

Groups	White blood cells ($\mu\text{L}/10^3$)	Lymphocyte ($\mu\text{L}/10^3$)	Neutrophil ($\mu\text{L}/10^3$)
Normal	4.86 ± 1.49	3.37 ± 0.94	0.27 ± 0.12
CY control	$1.59 \pm 0.39^{**}$	$1.13 \pm 0.28^{**}$	$0.12 \pm 0.04^{**}$
RGE	$2.44 \pm 0.19^{##}$	$1.82 \pm 0.22^{##}$	0.12 ± 0.04
FRGE	$2.15 \pm 0.16^{##}$	$1.56 \pm 0.29^{\#}$	0.12 ± 0.04
BRGE	$2.77 \pm 0.78^{\#}$	$2.06 \pm 0.67^{\#}$	0.13 ± 0.05

BRGE, black red ginseng extract; CY, cyclophosphamide; FRGE, fermented red ginseng extract; RGE, red ginseng extract.

Decrease in white blood cells, lymphocytes, and neutrophils in CY control group compared with the normal group at $^{**}p < 0.01$. Increase in white blood cells and lymphocytes in RGE-, BRGE-, and FRGE-treated groups compared with the CY control at $^{\#}p < 0.05$ and $^{##}p < 0.01$.

3.3. Effects of RGE, FRGE, and BRGE on immune cells subtypes in spleen tissue

As shown in Figs. 2D–E, the numbers of T and B lymphocytes were significantly reduced in the CY control compared with the normal group. In contrast, the numbers of T and B lymphocytes were significantly increased in RGE- and FRGE-treated groups, compared with the CY control group. The number of T and B lymphocytes was significantly increased in the RGE-treated group compared with the FRGE-treated group. It was found that peripheral white blood cell, lymphocyte, and neutrophil counts in CY-treated mice decreased significantly (Table 2).

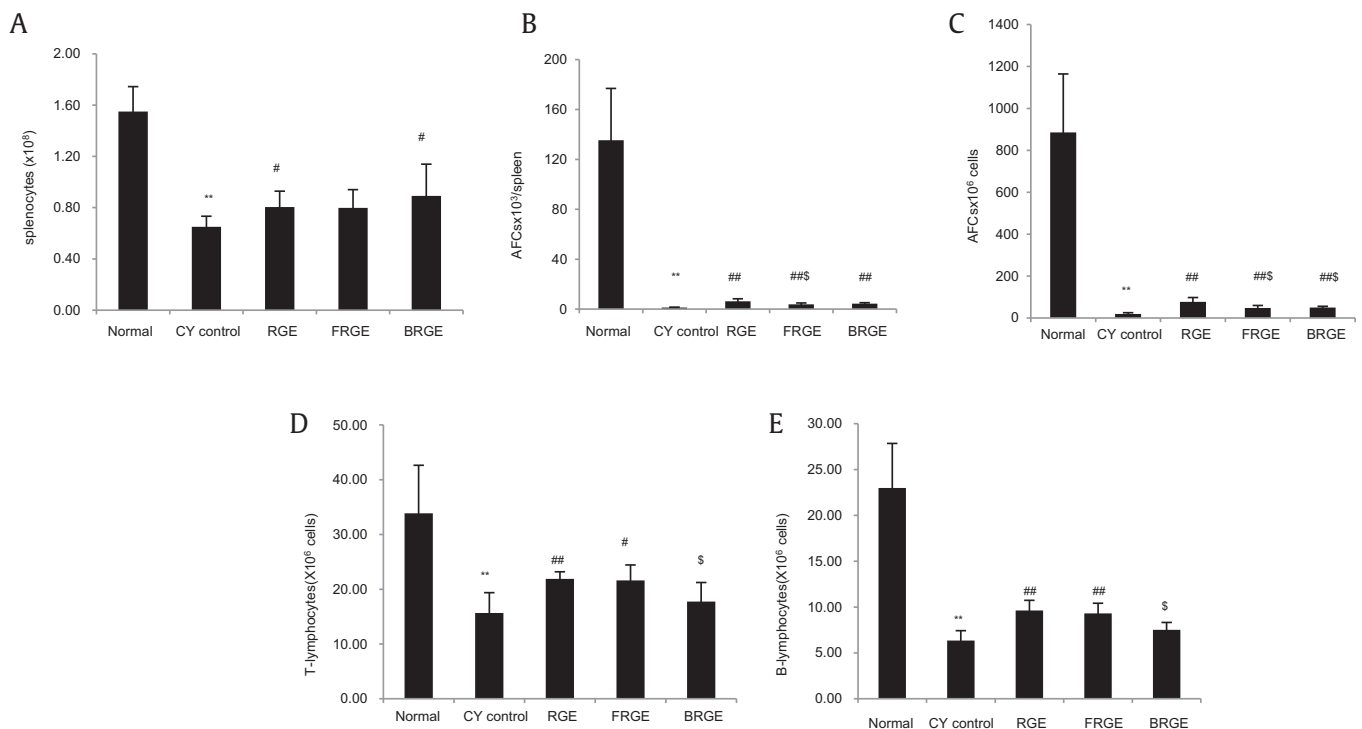


Fig. 2. Effects of RGE, BRGE, and FRGE on antibody-forming cells (AFCs) and splenic subpopulation in BALB/c mice treated with CY. (A) Decrease in splenocytes ($\times 10^8$) from the normal group at $^{**}p < 0.01$. Increase in splenocytes ($\times 10^8$) in the RGE- and BRGE-treated groups compared with the CY control at $^{\#}p < 0.05$. (B) Decrease in AFCs ($\times 10^3$)/spleen from the normal group at $^{**}p < 0.01$, increase in AFCs ($\times 10^3$)/spleen in the RGE-, BRGE-, and FRGE-treated groups compared with the CY control at $^{##}p < 0.01$, and $^{\$}$ significantly less than RGE at $p < 0.05$. (C) Decrease in AFCs ($\times 10^6$)/spleen from the normal group at $^{**}p < 0.01$, increase in AFCs ($\times 10^6$)/spleen in the RGE-, BRGE-, and FRGE-treated groups compared with the CY control at $^{##}p < 0.01$, and $^{\$}$ significantly less than RGE at $p < 0.05$. (D) Decrease in the number of T-lymphocytes compared with the normal group at $^{**}p < 0.01$, whereas increase in the number of T-lymphocytes in RGE- and FRGE-treated groups compared with the CY control at $^{\#}p < 0.05$ and $^{##}p < 0.01$ and $^{\$}$ significantly less than RGE at $p < 0.05$. (E) Decrease in the number of B lymphocytes compared with the normal group at $^{**}p < 0.01$, whereas increase in the number of B lymphocytes in RGE- and FRGE-treated groups compared with the CY control at $^{##}p < 0.01$ and $^{\$}$ significantly less than RGE at $p < 0.05$. BRGE, black red ginseng extract; CY, cyclophosphamide; FRGE, fermented red ginseng extract; RGE, red ginseng extract.

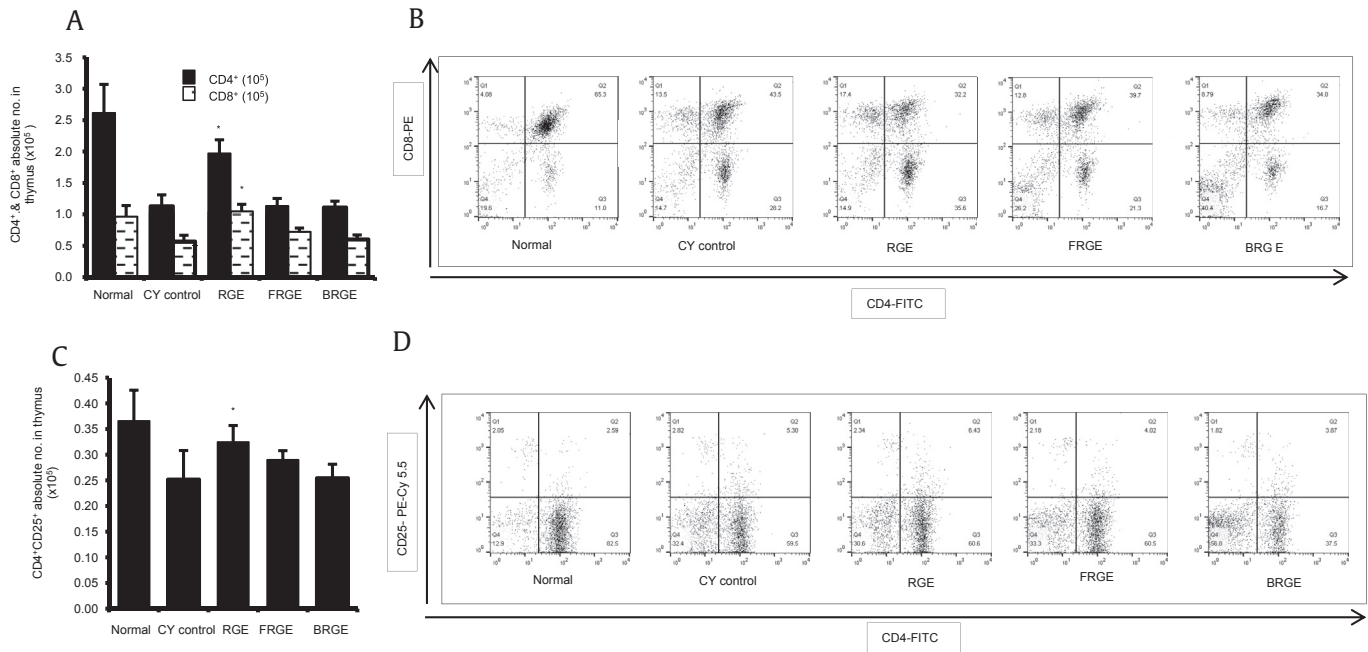


Fig. 3. Effects of RGE, FRGE and BRGE on immune cell subtypes from thymus tissue. (A, B) Absolute number of CD4⁺CD8⁺ cells from thymus tissue for RGE group as compared with CY group at **p* < 0.01 by FACS analysis. (C, D) Absolute number of CD4⁺CD25⁺ cells from thymus tissue for RGE group as compared with CY group at **p* < 0.01 by FACS analysis. Bar graphs are representative of three independent experiments.

BRGE, black red ginseng extract; CY, cyclophosphamide; FACS, fluorescent antibody cell sorting; FRGE, fermented red ginseng extract; RGE, red ginseng extract.

3.4. Effects of RGE, FRGE, and BRGE on immune cells subtypes in thymus tissue

Analysis of the expression of immune cell subtypes (T and B) in thymus tissue was performed by flow cytometry technique. As shown in Figs. 3A–B, CD4⁺/CD8⁺ (CD4 that are the helper T cells and CD8 that are cytotoxic/killer T cells) [26] cells from thymus were found to be significantly increased for the RGE group. Moreover, the amounts of CD4⁺CD25⁺ (markers for regulatory T cells) [27] cells in thymus tissue were also significantly elevated for the RGE group as indicated in Figs. 3C–D.

These results clearly indicate that RGE is potent as immune-boosting extract because it caused the activation of T and B cells subtypes in thymus tissues of mice.

3.5. Effects of RGE, FRGE, and BRGE on neutrophil migration and Th1/Th2-related cytokines

Neutrophil migration assay was performed to investigate the effects of RGE, FRGE, and BRGE on chemotactic activity of neutrophils [28]. As can be seen from Fig. 4A, the relative migration activity was manifolds enhanced for RGE- and FRGE-treated groups, indicating that both these extracts potently activated the neutrophils to combat with CY-induced immune suppression.

Then, to further strengthen our hypothesis that these extracts activate the immune system, we checked the serum IL-12 levels (Th1-related antiinflammatory cytokine) [29] which were elevated for the RGE group, IL-4 (Th2-related antiinflammatory cytokine) [30] which was elevated for all three extracts, and interferon- γ (Th1-related antiinflammatory-type soluble cytokine) [31] which was elevated for both RGE and FRGE groups as can be seen in Figs. 4B–D. These results strongly suggest that these ginseng extracts, especially both RGE and FRGE, are potent immune-stimulating extracts.

4. Discussion

Although extensive industrialization has affected today's lifestyle in a positive manner and made life easy, it has shifted humans toward a sedentary lifestyle with dependence on more synthetic products for consumption, which has led to various health issues, such as cardiovascular disorders, hypertension, and diabetes [32]. Therefore, these days, the orientation of scientific community is shifted toward unraveling the biological properties in natural food sources rather than the commercial ones and to introduce more natural food products than synthetic ones. In our research, we aimed to unravel the antioxidant and immune-stimulatory activities of three different ginseng extracts in rats and mice. Although ginseng is already reported for its antioxidant properties [13,33], our results clearly revealed that RGE possess good antioxidative (Figs 1A–D, E&F) and immune-stimulating (Figs 2A–E, 3A–D and 4A–D) activities among the three extracts. This fact is probably attributed to the processing time of RGE. Since RGE is steamed and dried only once, it contains 4.7–6.3 times more arginine-fructose-glucose (AFG) than FRGE and BRGE. In addition, RGE contains 1.3–1.5 times more polysaccharides than FRGE and BRGE, and it is clearly seen in Table 1. AFG is a specific aminosugar that can be produced by chemical reaction during the process of conversion of fresh ginseng to red ginseng. The AFG content in red ginseng is 1.0–1.5%. The amount of AFG as shown in Table 1 is manifolds higher than the other two extracts, and this is the main reason for its strongest biological activities. The levels of acidic polysaccharide, an immune activator, in red ginseng are 4.5–7.5% [34]. Previous studies have also demonstrated that AFG and polysaccharides derived from RGE exert antioxidant effects against oxidative stress-induced diseases [35–37].

Many previous studies have reported how the processing time of ginseng (one time to many times) can change the composition of single ginseng compounds called "ginsenosides" [13,38–41]. Some studies have reported that excessive heat treatment of ginseng denatures the ginsenosides present in it, whereas some studies

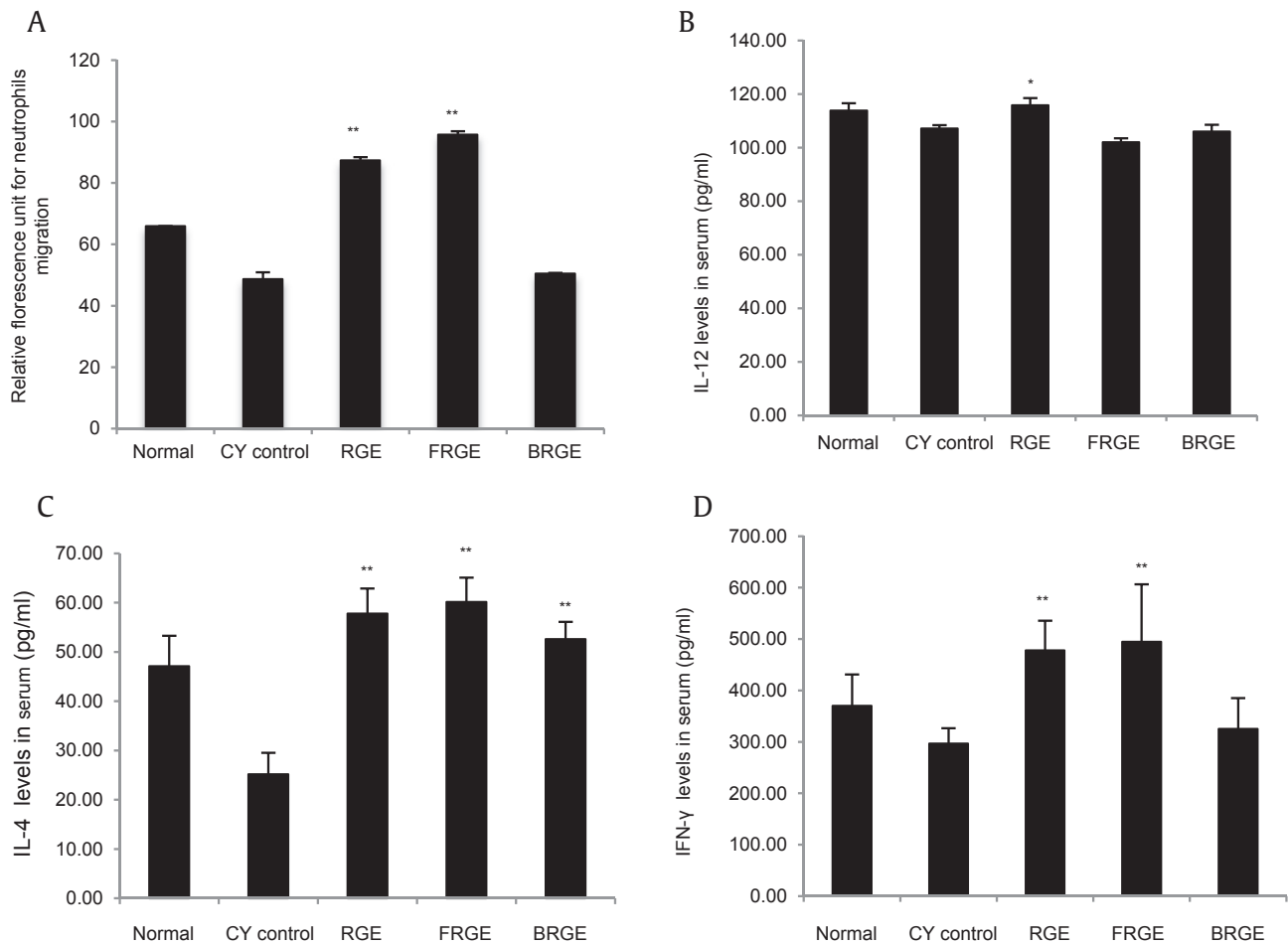


Fig. 4. Effects of RGE, FRGE, and BRGE on neutrophil migration assay and serum levels of anti-inflammatory cytokines. (A) Chemotaxis of neutrophils from RGE-treated and FRGE-treated group as compared to CY-treated group at $^{**}p < 0.05$. (B) Serum levels of IL-12 in RGE group as determined by ELISA when compared to CY-treated group at $^{*}p < 0.01$. (C) Serum levels of IL-4 in RGE, FRGE, and BRGE groups as determined by ELISA when compared to CY-treated group at $^{**}p < 0.05$. (D) Serum levels of IFN- γ in RGE and FRGE groups as determined by ELISA when compared to CY-treated group at $^{**}p < 0.05$. Bar graphs are representative of three independent experiments. BRGE, black red ginseng extract; CY, cyclophosphamide; FRGE, fermented red ginseng extract; IFN, interferon; IL, interleukin; RGE, red ginseng extract.

have reported that extensive processing of ginseng makes some of the important ginsenosides more bioavailable to the body than ginseng boiled and dried once [42–44].

Black ginseng (BG) is obtained from red ginseng roots by nine cycles of steaming at 98°C for 3 h [45,46]. Owing to this extensive steaming process, some ginsenosides in BG, such as Rg3, Rg5, Rg6, Rk3, Rs3, and Rs4, exhibit more potent biological activities than those in RGE [47]. Many studies have reported strong biological activities exhibited by BG [48–51]. In light of these findings, we can conclude that BRGE also has strong antioxidant (Figs. 1B–C) and immune-stimulating (Figs. 2A–C, 4C) activities, but not as strong as RGE.

The process of fermentation generally enhances the effectiveness of bigger pharmacological compounds via degradation into small molecules, which makes it easier for their metabolic absorption. Recent studies on FRGE demonstrated that fermentation of red ginseng by *Lactobacillus fermentum* increased the effectiveness of ginsenosides present in ginseng, making it more bioavailable [52]. In addition, the conversion of some ginsenosides, especially Rb₁, Rb₂, Rc, and Rd, to compound K was reported. Compound K showed enhanced absorption by the intestinal microflora compared with single ginsenosides [53]. Moreover, its other beneficial biological activities such as antioxidant, anticancer,

and antidiabetic properties are already reported [11,54]. Our results have shown that FRGE also exhibited good antioxidant (Figs. 1A–C) and immune-stimulating (Figs. 2A–E, 4A, C and D) activities, but again not as strong as RGE.

In a nutshell, we conclude that RGE possesses strong antioxidant and immune-stimulating activity compared with BRGE and FRGE owing to the processing condition that causes increased amounts of AFG and other acidic polysaccharides present in it. Nevertheless, the strong biological properties posed by both BRGE and FRGE can also never be ignored, and further studies at molecular levels can justify their enhanced efficacies for various pathological disorders.

Conflicts of interest

The authors declare no conflict of interest.

References

- Patel S, Rauf A. Adaptogenic herb ginseng (Panax) as medical food: status quo and future prospects. *Biomedicine & Pharmacotherapy* 2017;85:120–7.
- Dai D, Zhang CF, Williams S, Yuan CS, Wang CZ. Ginseng on cancer: potential role in modulating inflammation-mediated angiogenesis. *Am J Chin Med* 2017;45:13–22.

- [3] Qian J, Kang A, Di LQ, Di YW, Li J, Liu T. Active ingredients and its pharmacokinetic behavior and anti-inflammatory effects of ginseng with different steamed times. *Zhongguo Zhong Yao Za Zhi* 2015;40:3770–4.
- [4] Yuan SM. Potential cardioprotective effects of Ginseng preparations. *Pak J Pharm Sci* 2015;28:963–8.
- [5] Park J. Recent studies on the chemical constituents of Korean ginseng (*Panax ginseng* CA Meyer). *Korean J Ginseng Sci* 1996;20:389–415.
- [6] Rho S, Park J. The effects of ginseng radix preparata extract on anti-thrombotic activity. *J East-west Med* 2008;33:47–61.
- [7] Chandra K, Salman AS, Mohd A, Sweetey R, Ali KN. Protection against FCA induced oxidative stress induced DNA damage as a model of arthritis and in vitro anti-arthritis potential of *costus speciosus* rhizome extract. *Inter J Pharma Phyto Res* 2015;7:383–9.
- [8] Bonomini F, Tengattini S, Fabiano A, Bianchi R, Rezzani R. Atherosclerosis and oxidative stress. *Histol Histopathol* 2008;23:381–90.
- [9] Hwang O. Role of oxidative stress in Parkinson's disease. *Exp Neurobiol* 2013;22:11–7.
- [10] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44–84.
- [11] Kim HJ, Lee SG, Chae IG, Kim MJ, Im NK, Yu MH, Lee EJ, Lee IS. Antioxidant effects of fermented red ginseng extracts in streptozotocin-induced diabetic rats. *J Ginseng Res* 2011;35:129–37.
- [12] Sohn SH, Kim SK, Kim YO, Kim HD, Shin YS, Yang SO, Kim SY, Lee SW. A comparison of antioxidant activity of Korean White and Red Ginsengs on H₂O₂-induced oxidative stress in HepG2 hepatoma cells. *J Ginseng Res* 2013;37:442–50.
- [13] Chung SI, Kang MY, Lee SC. In vitro and in vivo antioxidant activity of aged ginseng (*panax ginseng*). *Prev Nutr Food Sci* 2016;21:24–30.
- [14] Cui X, Sakaguchi T, Ishizuka D, Tsukada K, Hatakeyama K. Orally administered ginseng extract reduces serum total cholesterol and triglycerides that induce fatty liver in 66% hepatectomized rats. *J Int Med Res* 1998;26:181–7.
- [15] Hong SH, Suk KT, Choi SH, Lee JW, Sung HT, Kim CH, Kim EJ, Kim MJ, Han SH, Kim MY, et al. Anti-oxidant and natural killer cell activity of Korean red ginseng (*Panax ginseng*) and urushiol (*Rhus vernicifera* Stokes) on non-alcoholic fatty liver disease of rat. *Food Chem Toxicol* 2013;55:586–91.
- [16] Voces J, Alvarez AI, Vila L, Ferrando A, Cabral de Oliveira C, Prieto JG. Effects of administration of the standardized *Panax ginseng* extract G115 on hepatic antioxidant function after exhaustive exercise. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1999;123:175–84.
- [17] Heo SB, Lim SW, Jhun JY, Cho ML, Chung BH, Yang CW. Immunological benefits by ginseng through reciprocal regulation of Th17 and Treg cells during cyclosporine-induced immunosuppression. *J Ginseng Res* 2016;40:18–27.
- [18] Liu M, Zhang JT. Immunoregulatory effects of ginsenoside Rg1 in aged rats. *Yao Xue Xue Bao* 1995;30:818–23.
- [19] Kang TH, Park HM, Kim YB, Kim H, Kim N, Do JH, Kang C, Cho Y, Kim SY. Effects of red ginseng extract on UVB irradiation-induced skin aging in hairless mice. *J Ethnopharmacol* 2009;123:446–51.
- [20] Kim IW, Sun WS, Yun BS, Kim NR, Min D, Kim SK. Characterizing a full spectrum of physico-chemical properties of (20S)- and (20R)-ginsenoside Rg3 to be proposed as standard reference materials. *J Ginseng Res* 2013;37:124–34.
- [21] Kwak Y-S, Kim E-M. The physicochemical properties of crude polysaccharide fraction isolated from Korean ginseng (*Panax ginseng* CA Meyer). *Korean Journal of Food Science and Technology* 1996;28:389–92.
- [22] Kumar S, Jyoti A, Keshari RS, Singh M, Barthwal MK, Dikshit M. Functional and molecular characterization of NOS isoforms in rat neutrophil precursor cells. *Cytometry* 2010;77:467–77.
- [23] Ahmad W, Jantan I, Kumolosasi E, Bukhari SN. Immunostimulatory effects of the standardized extract of *Tinospora crispa* on innate immune responses in Wistar Kyoto rats. *Drug Des Devel Ther* 2015;9:2961–73.
- [24] Ladics GS. Primary immune response to sheep red blood cells (SRBC) as the conventional T-cell dependent antibody response (TDAR) test. *J Immunotoxicol* 2007;4:149–52.
- [25] Ladics GS. Use of SRBC antibody responses for immunotoxicity testing. *Methods* 2007;41:9–19.
- [26] Koretzky GA. Multiple roles of CD4 and CD8 in T cell activation. *J Immunol* 2010;185:2643–4.
- [27] Pontoux C, Banz A, Papiernik M. Natural CD4 CD25(+) regulatory T cells control the burst of superantigen-induced cytokine production: the role of IL-10. *Int Immunol* 2002;14:233–9.
- [28] Wagner JG, Roth RA. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacol Rev* 2000;52:349–74.
- [29] Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 1993;260:547–9.
- [30] Choi P, Reiser H. IL-4: role in disease and regulation of production. *Clin Exp Immunol* 1998;113:317–9.
- [31] Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol* 2007;96:41–101.
- [32] Hatch T. Impact of industrialization on health. *J Occup Med* 1962;4:569–79.
- [33] Lee JW, Mo EJ, Choi JE, Jo YH, Jang H, Jeong JY, Jin Q, Chung HN, Hwang BY, Lee MK. Effect of Korean Red Ginseng extraction conditions on antioxidant activity, extraction yield, and ginsenoside Rg1 and phenolic content: optimization using response surface methodology. *J Ginseng Res* 2016;40:229–36.
- [34] 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.
- [35] Ansari NA, Moinuddin, Alam K, Ali A. Preferential recognition of Amadori-rich lysine residues by serum antibodies in diabetes mellitus: role of protein glycation in the disease process. *Hum Immunol* 2009;70:417–24.
- [36] Arif B, Ashraf JM, Moinuddin, Ahmad J, Arif Z, Alam K. Structural and immunological characterization of Amadori-rich human serum albumin: role in diabetes mellitus. *Arch Biochem Biophys* 2012;522:17–25.
- [37] Ha KS, Jo SH, Kang BH, Apostolidis E, Lee MS, Jang HD, Kwon YI. In vitro and in vivo antihyperglycemic effect of 2 amadori rearrangement compounds, arginyl-fructose and arginyl-fructosyl-glucose. *J Food Sci* 2011;76:H188–93.
- [38] Kim S. Study on ginsenoside patterns on the steam processing of Korean ginseng and hypoglycemic action on streptozotocin induced diabetic rats of 9 time-steaming ginsengs. PhD Dissertation. Chungnam, Korea: Joongbu University; 2008.
- [39] Ko SK, Lee KH, Hong JK, Kang SA, Sohn UD, Im BO, Han ST, Yang BW, Chung SH, Lee BY. Change of ginsenoside composition in ginseng extract by vinegar process. *Food Science and Biotechnology* 2005;14:509–13.
- [40] Christensen LP. Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. *Adv Food Nutr Res* 2009;55:1–99.
- [41] Shin BK, Kwon SW, Park JH. Chemical diversity of ginseng saponins from *Panax ginseng*. *J Ginseng Res* 2015;39:287–98.
- [42] Jo E-J, Kang S-J, Kim A-J. Effects of steam- and dry-processing temperatures on the benzo (a) pyrene content of black and red ginseng. *The Korean Journal of Food And Nutrition* 2009;22:199–204.
- [43] Kong B-M, Park M-J, Min J-W, Kim H-B, Kim S-H, Kim S-Y, Yang D-C. Physico-chemical characteristics of white, fermented and red ginseng extracts. *Journal of Ginseng Research* 2008;32:238–43.
- [44] Kwon SW, Han SB, Park IH, Kim JM, Park MK, Park JH. Liquid chromatographic determination of less polar ginsenosides in processed ginseng. *Journal of Chromatography* 2001;921:335–9.
- [45] Lee J-H, Shen G-N, Kim E-K, Shin H-J, Myung C-S, Oh H-J, Kim D-H, Roh S-S, Cho W, Seo Y-B. Preparation of black ginseng and its antitumor activity. *Journal of Physiology & Pathology in Korean Medicine* 2006;20.
- [46] Song G-Y, Oh H-J, Roh S-S, Seo Y-B, Park Y-J, Myung C-S. Effect of black ginseng on body weight and lipid profiles in male rats fed normal diets. *Yakhak Hoeji* 2006;50.
- [47] Sun B-S, Gu L-J, Fang Z-M, Wang C-y, Wang Z, Lee M-R, Li Z, Li J-J, Sung C-K. Simultaneous quantification of 19 ginsenosides in black ginseng developed from *Panax ginseng* by HPLC–ELSD. *Journal of Pharmaceutical and Biomedical Analysis* 2009;50:15–22.
- [48] Kim E-K, Lee J-H, Cho S-H, Shen G-N, Jin L-G, Myung C-S, Oh H-J, Kim D-H, Yun J-D, Roh S-S. Preparation of black panax ginseng by new methods and its antitumor activity. *The Korea Journal of Herbology* 2008;23:85–92.
- [49] Kim WY, Kim JM, Han SB, Lee SK, Kim ND, Park MK, Kim CK, Park JH. Steaming of ginseng at high temperature enhances biological activity. *Journal of Natural Products* 2000;63:1702–4.
- [50] Saba E, Jeon BR, Jeong DH, Lee K, Goo YK, Kim SH, Sung CK, Roh SS, Kim SD, Kim HK, et al. Black ginseng extract ameliorates hypercholesterolemia in rats. *J Ginseng Res* 2016;40:160–8.
- [51] Saba E, Jeong DH, Roh SS, Kim SH, Kim SD, Kim HK, Rhee MH. Black ginseng-enriched Chong-Myung-Tang extracts improve spatial learning behavior in rats and elicit anti-inflammatory effects in vitro. *J Ginseng Res* 2017;41:151–8.
- [52] Bae EA, Hyun YJ, Choo MK, Oh JK, Ryu JH, Kim DH. Protective effect of fermented red ginseng on a transient focal ischemic rats. *Arch Pharm Res* 2004;27:1136–40.
- [53] Choi I-D, Ryu J-H, Lee D-E, Lee M-H, Shim J-J, Ahn Y-T, Sim J-H, Huh C-S, Shim W-S, Yim S-V. Enhanced absorption study of ginsenoside compound K (20-O-β-(D-Glucopyranosyl)-20 (S)-protopanaxadiol) after oral administration of fermented red ginseng extract (HYFRG™) in healthy Korean volunteers and rats. Evidence-based Complementary and Alternative Medicine 2016;2016.
- [54] 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.