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# Research Article
# Black Ginseng Extract Prevents Neuroinflammation and Enhances Learning Behavior in Mice

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## Abstract

**Background and Objective:** Ginseng is widely used in the Korean peninsula as a traditional herbal treatment for numerous ailments in the body, like inflammation, tumors and cardiovascular diseases. Therefore the prime objective of the present study was to investigate the effects of ginseng on neuro-inflammation *in vitro* and *in vivo*. **Methodology:** In the present study, two types of ginseng extracts, Red Ginseng (RG) and black ginseng ethanol extract [BG (etoh)] was used and their efficacy in the Morris water maze test for spatial learning behavior and their anti-inflammatory effects in BV2 microglial cells was evaluated. **Results:** Present study showed that both extracts, particularly BG (etoh), improved learning behavior in scopolamine-impaired mice in water maze tests. Moreover, these extracts also inhibited nitric oxide production in BV2 microglial cells, with significant suppression of proinflammatory cytokines, especially iNOS, COX-2 and IL-1β. The protein expression of MAPK and NF-κB pathway factors was also diminished by these extracts, indicating that they not only improve memory impairment, but are also potent anti-inflammatory agents for neuroinflammatory diseases. **Conclusion:** It is concluded that BG (etoh) extract is more effective in preventing neuroinflammation and enhancing learning behavior as compared red ginseng extract.

Key words: Anti-inflammation, cytokines, BV2 cells, black and red ginseng extracts, learning behaviour, ginseng extract

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Neuroinflammation is the root cause of serious and chronic neurological diseases, such as Alzheimer's disease, Parkinson's disease and many others[1]. The underlying cause of any disease in the body can be attributed to the development of inflammation that progresses into other diseases[2]. For centuries, research has been conducted on inflammation and many homeopathic and allopathic treatments have been discovered and produced for the benefit of mankind. The side effects of allopathic medicines, which are essentially chemicals, cannot be ignored, especially if these drugs are taken for long periods of time. However, a suitable alternative to allopathic medicines are herbal drugs, or herbal supplements, that can be used therapeutically or prophylactically without any side effects.

There are numerous studies available on many types of herbal supplements that can alleviate the experimental scopolamine induced amnesia in rodent models[3,4]. Furthermore Nahata *et al.*[5-7] reported the memory enhancement effects of Convolvulus pluricaulis and Evolvulus alsinoides in scopolamine induced memory damage. In addition, Sethiya *et al.*[8] reported the cognition boosting effects of Shankhpushpi (which is the combination of four extracts that are Convolvulus. pluricaulis Choisy. (Convolvulaceae), Evolvulus. alsinoides Linn. (Convolvulaceae), Clitoria. ternatea Linn. (Papilionaceae) and Canscora. decussata Schult. (Gentianaceae). Moreover, in separate studies, the anxiolytic and memory enhancing effects of Canscora decussata Schult are also reported[9,10].

Panax Ginseng has been a widely available and consumed herbal supplement in the Korean Peninsula for centuries. The main reasons for ginseng consumption are longevity and vitality. However, much research has been done on the therapeutic effects of ginseng on minor or major ailments[3-7]. Few studies have compared the effects of different types of ginseng on conditions like inflammation and especially the neuroinflammation harvesting the scopolamine induced memory impairment.

In the current study, the effect of red and black ginseng on scopolamine-induced memory impairment and neuro-inflammation in BV2 cells was evaluated. The underlying concept driving this study is that the processing of ginseng can increase or decrease the ginsenosides present in it. Red Ginseng (RG) is only steamed once and then dried and processed, however, when it is steamed and dried 5 times and then processed, it changes to black ginseng[8]. Therefore, some components in both varieties could be enhanced or deteriorated. Through *in vivo* and *in vitro* experiments we have elucidated that black ginseng ethanol extract [BG (etoh)] has more potential in retaining memory and decreasing neuro-inflammation than RG, thereby implying that some ginsenosides, particularly those related to inflammation, are enhanced by steaming the ginseng 5 times.

## MATERIALS AND METHODS

**Materials:** Dulbecco's Modified Eagle's Medium (DMEM) [Daegu, Korea], fetal bovine serum (FBS) (WelGene Co., Korea), streptomycin and penicillin (Lonza, MD, USA), TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA), oligo dT (Bioneer oligo synthesis, Korea) and iNOS, COX-2, TNF-α, IL-6 and IL-1β primers were obtained from Bioneer, Daejeon, Korea. Lipopolysaccharides (LPS; Escherichia coli 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Specific antibodies against phospho- and/or total forms of ERK, JNK, p38, IKK α/β, IκB, NFκB p65, iNOS, COX-2, β-actin and secondary antibody rabbit HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). All other reagents and chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

**Preparation of ginseng extracts:** The ginseng roots for RG (ginseng roots steamed and dried only once) and BG (ginseng roots steamed and dried 5 times) were obtained from the Daedong Korea Ginseng Cooperation and then boiled with 70% ethanol, cooled, filtered and condensed. The condensed extracts were later subjected to lyophilisation for obtaining their powdered forms.

**Animal experiments:** Male C57BL/6 mice, 6 months old (26-29 g), were purchased from Charles River, Orient Biotechnology, Gyeonggi-do, South Korea. Ten mice were used in each experimental group. The mice were housed in a specific pathogen-free barrier facility at 21±2°C with a relative humidity of 60±10% under a 12 h light and dark cycle. Food and water were provided ad libitum. All animal care and experimental procedures were approved by the Animal care committee of the college of veterinary medicine, Daejeon University (DJUARB 2017-018), Daejeon, South Korea.

**Morris water maze test:** The assessment of learning and memory retention was done using the Morris water maze test as previously described[9].

**Cell culture:** The murine microglial cell line BV2, originating from the American Type culture collection, was cultured in

DMEM supplemented with 8% FBS (WelGene Co, Korea), 100 IU $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin sulfate (Lonza, MD, USA). The incubating conditions were humidified 5% $CO_2$ at 37°C.

**Nitric Oxide (NO) assay:** Nitric oxide was measured using a method based on the Griess reaction assay. Briefly, BV2 cells were seeded in 24-well plates and incubated with or without LPS (0.1 μg $mL^{-1}$) in the absence or presence of ginseng extracts at the indicated concentrations overnight. The cell culture supernatants (100 μL) were then mixed with the Griess reagent (0.2 % naphthyl ethylenediamine dihydrochloride and 2% sulphanilamide in 5% phosphoric acid) in double distilled water at equal volumes and incubated for 10 min at room temperature. The absorbance in each well was then measured at 540 nm in a microplate reader (Versamax, Molecular devices, LLC, CA, USA).

**Cell viability assay:** To determine the cytotoxic effects of the ginseng extracts, a cell viability assay was done according to previous established protocols[11-13]. Briefly, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) reagent that was added to culture medium at a final concentration of 0.1 mg $mL^{-1}$. After a 4 h of incubation at 37°C in 5 % $CO_2$, the resulting violet colored crystals were dissolved in Dimethyl sulfoxide (DMSO) 100 $\mu L^{-1}$ well and absorbance values were measured at 560 nm.

**RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR):** BV2 cells were pretreated with or without ginseng extracts for 30 min and then stimulated with LPS (0.1 μg $mL^{-1}$) for 18 h. Total RNA was extracted using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. The latter procedure was followed according to our previous research[10].

**Western blot analysis:** BV2 cells were treated or left untreated with ginseng extracts in the presence or absence of LPS (0.1 μg $mL^{-1}$). Proteins were then measured using the PROMEASURE assay kit (PRO-PREP, iNtRON Biotechnology). They were then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) (Millipore, Immobilien®-P, Billerica MA, USA). Nonspecific binding on the nitrocellulose filter paper was minimized using a blocking buffer containing 5 % non-fat dry milk and 0.1 % Tween-20 in Tris-Buffered Saline (TBS). The membranes were then incubated with specific primary antibodies overnight at 4°C, followed by a 2 h incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1: 3000 dilution, Cell signaling). Bound antibodies were visualized using enhanced chemiluminescence (Supex) and images were analyzed using ImageJ software. β-actin was used as an internal control.

**Statistical analysis:** Data are presented as Mean±standard deviation. One-way analysis of variance and Dunnett test were used for the statistical evaluation of data. SAS 9.3 (SAS Institute Inc., Cary, NC, USA) was used to perform the analysis. All p values less than 0.001 were considered significant.

## RESULTS AND DISCUSSION

**Supplementation of ginseng extracts enhances scopolamine-induced memory and learning impairment:** The Morris water maze test, which is the most reliable method for evaluating hippocampus-dependent spatial navigation and reference memory,[14] was used in our experiment. Memory impairment was induced by scopolamine, which is a common memory impairing drug for experimental use[15]. Memory enhancement and decreases in inflammation were then checked through supplementation with ginseng extracts and also with a positive control (Galantamine). Hippocampal-dependent memory retention, measured using the Morris water maze, after scopolamine impairment and ginseng extract treatment is represented as latency to find platform graph. Latency refers to the time taken by mice to reach their platform goal. As can be seen from Fig. 1, scopolamine-treated mice showed increased time to reach the platform, indicating that it strongly impaired the targeted region for learning in the mouse brain. However, the ginseng extract-treated mice show reduced latency, especially those treated with BG (etoh).

**Ginseng extracts decreased levels of LPS-induced inflammation in a dose-dependent manner:** Nitric oxide gas (NO) is a free radical gas that is produced when a foreign invader, such as LPS, binds to its toll-like receptor (TLR4) domain[16,17]. When this gas is initially released, it serves as an anti-inflammatory moiety that causes the destruction of invading pathogens. However, if the release of this gas is persistent, it starts destroying the neighboring healthy cells. Therefore, timely control of NO is necessary to reduce the intensity of inflammation. In our experimental setting, we used bacterial LPS-induced NO production[18] in BV2 cells to examine the effects of our ginseng samples on NO inhibition. As shown in Fig. 2(a), both ginseng extracts sharply attenuated LPS-induced NO production when added to culture media

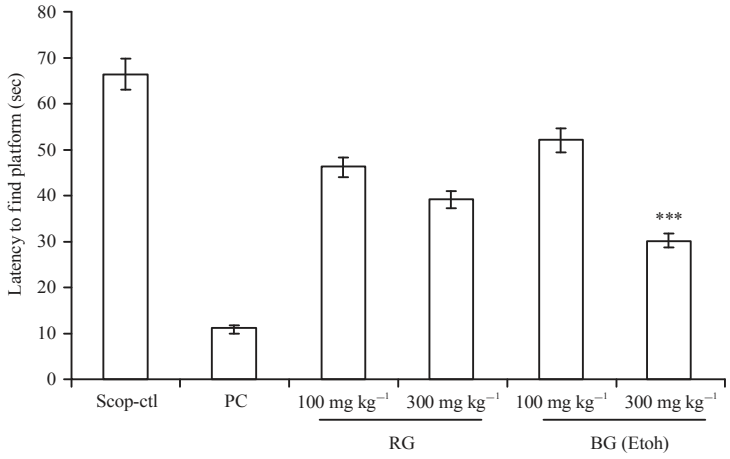


Fig. 1: Latency to reach platform of mice via Morris water maze test. Scopolamine was administered to induce memory damage and then mice were treated with ginseng extracts for 2 weeks. Their memory retention was measured as latency to find the platform in the Morris water maze. BG (etoh) (300 mg $kg^{-1}$) showed maximum memory retention. ***p<0.001, compared to positive control was considered statistically significant. Scop-ctl: Scopolamine control

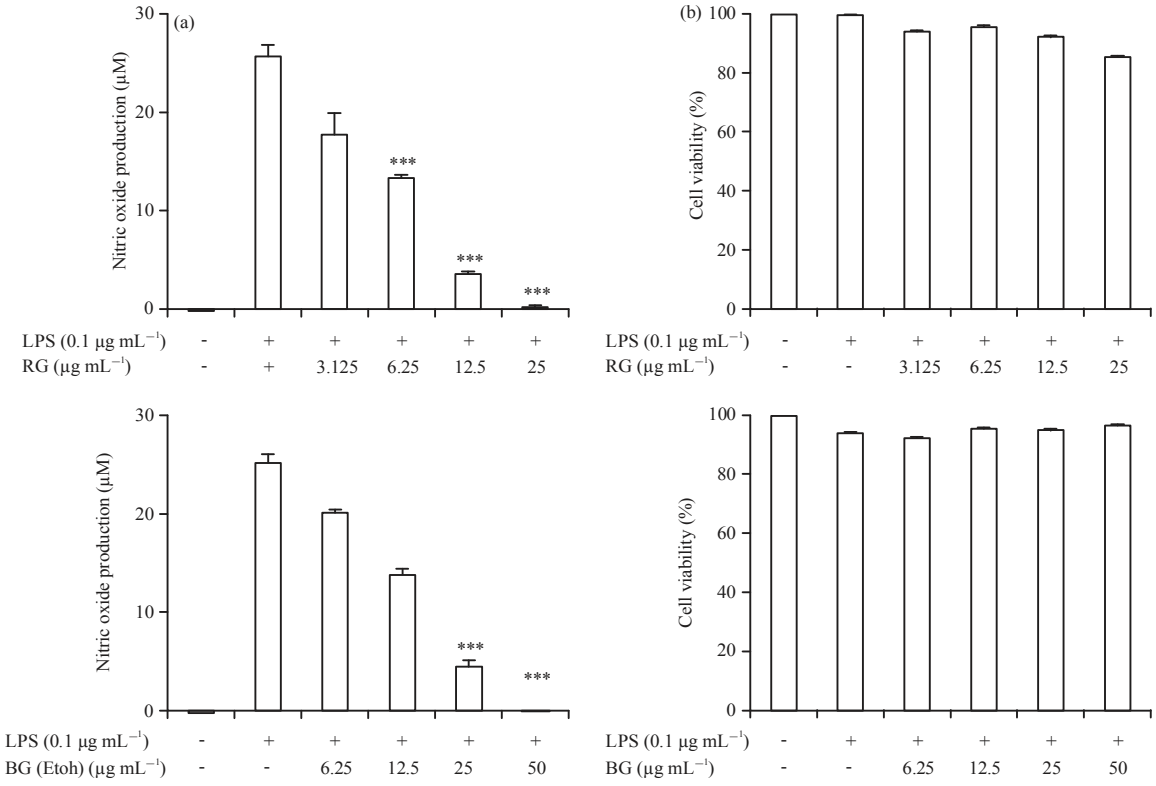


Fig. 2(a-d): Effects of ginseng extracts on nitric oxide production and cell viability. BV2 cells were seeded in 24-well plates overnight, pre-incubated with ginseng extracts for 30 min and then stimulated with LPS (0.1 μg $mL^{-1}$) for 18 h. Next, (a) Cell supernatants were transferred to a 96-well plate and homogenized with equal amounts of Griess reagent and NO production was measured at 540 nm and (b) The effects of ginseng extract on cell viability was determined by an MTT assay and absorbance was measured at 560 nm. Values in the bar graph are Mean±SEM of at least 3 independent experiments. ***p<0.001 compared to LPS-only was considered statistically significant

30 min after the sample injection. Furthermore, they did not show any cytotoxic effects over the concentration range as shown in Fig. 2(b).

**Effects of ginseng extracts on the expression of proinflammatory mediators and cytokines in BV2 cells:** Proinflammatory mediators and cytokines are the basic products that are released after NO production[19]. Previous studies have indicated that the levels of proinflammatory cytokines, especially TNF-α, IL-1β and IL-6, are highly increased in the case of Alzheimer's disease[20]. Moreover, the production of proinflammatory cytokines is a basic biological response towards inflammatory insult in cells. Therefore, we examined whether ginseng extracts can affect proinflammatory mediators and cytokine expression in BV2 cells. As can be seen from Fig. 3 (a-c), ginseng extracts significantly abolished the expression of iNOS, COX-2, IL-1β and IL-6 messenger RNA (mRNA) in a dose-dependent manner with BG (etoh) again showing more potent suppression than the other extract. Moreover, BG (etoh) also potently suppressed the protein expression of iNOS and COX-2 when compared with RG treatment.

**Diminution of NF-κB and MAPK pathways by ginseng extracts:** Inflammatory signaling is the combination of several pathways that activate components or factors in the nucleus responsible for inflammation. There are many direct and indirect pathways for the induction of inflammation, however NF-κB is one of the most extensively characterized transcription factors for inflammatory pathways[11,12]. This pathway is activated when LPS binds to TLR 4[11]. This receptor-ligand association activates a series of factors (i.e., IRAK4, TAK-1, IKK α/β, IκB/α and NF-κB). As shown in Fig. 4(a), both ginseng extracts have suppressed the phosphorylation of every downstream of TLR 4 factors in this pathway. The results show clearly that BG (etoh) exhibited potent inhibition in phosphorylation as compared to RG.

When cells are stressed many inflammatory pathways are activated and the mitogen-activated protein kinase pathway (MAPK) is another very common and well-known inflammatory mediation pathway. It is comprised of upstream and downstream components that work in harmony and continuity to activate inflammation[13,21]. Therefore, we also examined the effects of ginseng extracts on MAPK activation (ERK, JNK and p38). It can be clearly seen from fig. 4(B), that both extracts showed dose-dependent inhibitory effects on the factors (P38, ERK and JNK) involved in the MAPK pathway. Here, again BG (etoh) shows more potent inhibitory phosphorylating effects.

These results indicate that BG (etoh) has more potent memory improvement, retention and inflammatory alleviation properties as compared to RG. Therefore black ginseng extract

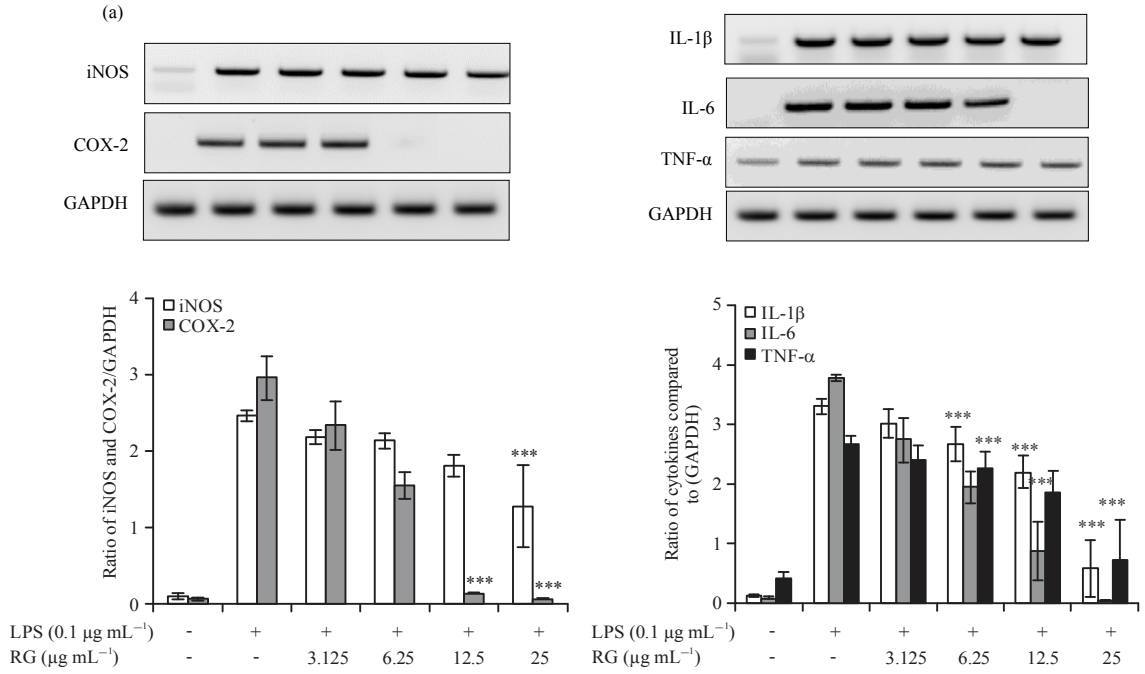


Fig. 3(a-c): Continue

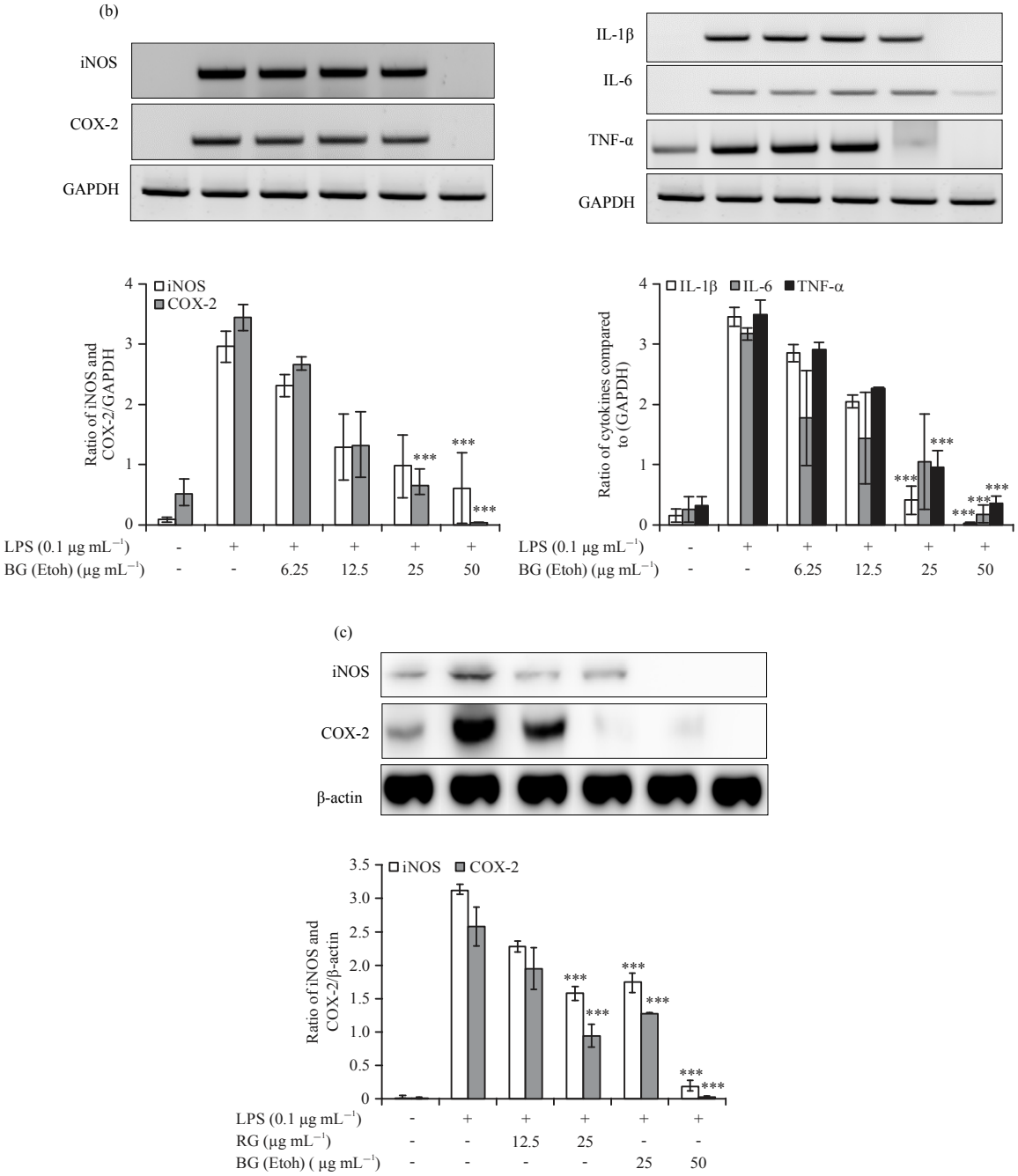


Fig. 3(a-c): Ginseng extracts, particularly BG (etoh), inhibited mRNA and translational expressions of iNOS, COX-2, IL-1β, IL-6 and TNF-α in BV2 cells. BV2 cells were pretreated with ginseng extracts (a) Red ginseng and (b) BG (etoh) for 30 min and then stimulated with LPS (0.1 μg mL$^{-1}$) overnight. Total RNA was extracted by TRIZOL® RNA extraction reagent and mRNA expression of iNOS, COX-2, IL-1β, IL-6 and TNF-α was determined by RT-PCR. GAPDH was employed as a housekeeping gene. Images are representative of 3 independent experiments. Total protein was extracted by Pro-prep lysis reagent. (c) Protein quantitation was estimated by Pro-Measure® and then 35 μg of each protein were run on SDS-PAGE and analyzed by enhanced chemiluminescence (ECL). Western blot images are representative of the 3 independent experiments. Values in the bar graph are the Mean±SEM of the 3 independent experiments. ***p<0.001 compared to LPS-only was considered statistically significant

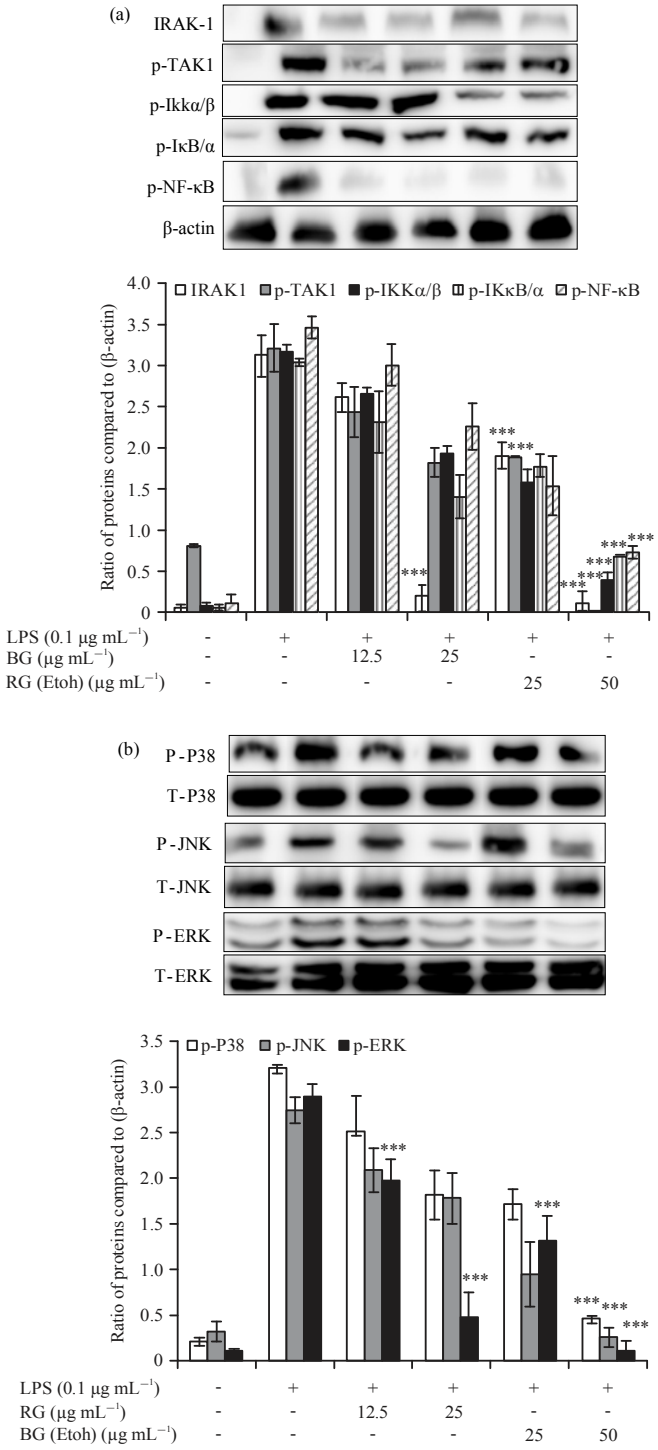


Fig. 4(a-b): Attenuation of NF-κB and MAPK pathways by ginseng extracts. BV2 cells were pretreated with ginseng extracts and then stimulated with or without LPS (0.1 μg mL$^{-1}$) overnight. Nuclear and cytosolic proteins were separated using a NE-PER® extraction kit. Proteins were subjected to SDS-PAGE and then transferred onto PVDF membranes and treated with primary antibodies overnight followed by incubation with secondary antibodies for 1.5 h. Next, membranes were analyzed using an ECL chemiluminescence system. β-actin was used as a loading control. (a) NF-κB pathway (b) MAPK pathway. Values in the bar graph are Mean±SEM of the 3 independent experiments. ***p<0.001 compared to LPS-only was considered statistically significant

can be efficaciously consumed for enhancing and retention of memory as daily food supplement. Although, ginseng is a herbal supplement without any side effect yet some people might have any untoward reaction after consumption of ginseng, thereby posing its limitation in consumption.

## CONCLUSION

Inflammatory damage that results in memory impairment and proinflammatory cytokine production is produced by scopolamine and LPS that have been potently attenuated by both extracts with greater preference to BG (etoh). This study can be used as a reference for future neuro-inflammatory-related anomalies with black ginseng extract consumption as a natural herbal remedy for neuro-inflammation, memory retention and improvement.

## SIGNIFICANCE STATEMENT

This study is first and one of its kinds to present the comparison between the red ginseng and black ginseng's anti-inflammatory activities that has not been elucidated before with regard to neuro-inflammation. This research holds a paramount importance for consumer population in Korean peninsula as well in other countries where ginseng is consumed as routine diet supplement. From research view point, this study is also very unique and opens new dimensions for further elucidation of more beneficial qualities of black ginseng. Moreover this research and further research on black ginseng can lead to the development of black ginseng enriched products for oral consumption primarily for memory retention.

## ACKNOWLEDGMENTS

Evelyn Saba, Seung Hyung Kim and Yuan Yee Lee performed all the experiments and wrote the manuscript. Sung Keun Choi and Byeong Seon Jeon provided the ginseng samples and Man Hee Rhee conceived the idea, supervised all the experiments and helped in the manuscript writing.

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