

Comparative Study of White and Steamed Black *Panax ginseng*, *P. quinquefolium*, and *P. notoginseng* on Cholinesterase Inhibitory and Antioxidative Activity

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This study evaluated the anti-cholinesterases (ChEs) and antioxidant activities of white ginseng (WG) and black ginseng (BG) roots of *Panax ginseng* (PG), *P. quinquefolium* (PQ), and *P. notoginseng* (PN). Ginsenosides Rg₁, Re, Rf, Rb₁, Rc, Rb₂, and Rd were found in white PG, whereas Rf was not found in white PQ and Rf, Rc, and Rb₂ were not detected in white PN. The major ginsenoside content in steamed BG including Rk₃, Rh₄, and 20(S)/(R)-Rg₃ was equivalent to approximately 70% of the total ginsenoside content. The WG and BG inhibited acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in a dose dependent manner. The efficacy of BG roots of PG, PQ, and PN on AChE and BChE inhibition was greater than that of the respective WG roots. The total phenolic contents and 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging activity were increased by heat treatment. Among the three WG and BG, white PG and steamed black PQ have significantly higher contents of phenolic compounds. The best results for the DPPH scavenging activity were obtained with the WG and BG from PG. These results demonstrate that the steamed BG roots of the three studied ginseng species have both high ChEs inhibition capacity and antioxidant activity.

Keywords: *Panax ginseng*, Acetylcholinesterase, Butyrylcholinesterase, Ginseng, Antioxidants, Steamed black ginseng

INTRODUCTION

Panax ginseng (PG) has been used as a general tonic in traditional oriental medicine to increase vitality, health, and longevity, especially in elderly people [1]. Traditionally, white ginseng (WG) has been prepared from raw ginseng through an air-drying process, while red ginseng has been prepared via a steaming process at 98°C to 100°C for 2 to 3 h. It has been reported that steamed ginseng shows enhanced pharmacological effects compared with non-steamed ginseng. Black ginseng (BG), a new ginseng product containing newly discovered ginsenosides (Rg₃, Rg₅, F₄, Rg₆, Rh₂, Rh₃, Rh₄, Rk₃, Rs₃, Rs, etc.) is produced by repeated steaming at high tempera-

tures, upon which the product becomes black in color and exhibits more potent biological activities than white and red ginseng [2].

PG, *P. quinquefolium* (PQ), and *P. notoginseng* (PN) are the three most widely used ginseng herbs [3]. All three species of ginseng are members of the Araliaceae family, and are used primarily as adaptogens. PQ is well known for its antioxidant and free radical scavenging activities [4]. PN has been reported to have beneficial effects on the heart and its constituents ginsenosides are similar to those found in PQ and PG [5]. Several reports have indicated that the medicinal efficacy of ginseng is

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closely linked to its protective properties against scavenging free radicals [6]. Most antioxidant potentials in herbs and spices are due to the redox properties of phenolic compounds, which allow them to act as reducing agents, hydrogen donors, and free radicals quenchers [7]. The oxidative damage that is caused by reactive oxygen species (ROS) has been frequently associated with the pathogenesis of various diseases and health problems such as aging, arthritis, cancer, inflammation, heart disease, and Alzheimer's disease (AD) [8,9].

AD is a brain disorder characterized by progressive and extensive decline of cognitive functions, leading to dementia due to degeneration of the cholinergic neurons in the central nervous system (CNS) [10]. Accordingly, cholinesterases (ChEs) inhibitors, which can effectively increase brain acetylcholine (ACh) levels, alleviate symptoms and delay progress, and are the most prescribed pharmacological agents in the treatment of AD [11]. The use of synthetic ChEs inhibitors has been limited due to their adverse side effects, including gastrointestinal disturbances and bioavailability problems [12]. A great deal of research has meanwhile been carried out on the biological effects of plants traditionally used in infusions or traditional remedies as ChEs inhibitors *in vitro* and also as memory enhancers *in vivo* [13]. Recent studies have noted that AD is associated with inflammatory processes where ROS are liberated [14]. Antioxidants can scavenge ROS and can also attenuate inflammatory pathways. Therefore, their use may be beneficial in the treatment of AD [15].

Although several studies have differentiated the various *Panax* species based on their chemical profiles and individual constituents [16], their anti-ChEs activities have yet to be compared. The present study evaluates the anti-ChEs and antioxidant activities of commercial WG and steamed BG roots of PG, PQ, and PN, as these species have not yet been studied in these respects.

MATERIALS AND METHODS

Ginseng preparation

The roots of PG were purchased from a local ginseng center (Geumsan, Korea). PQ and PN were kindly provided by Daeduk Bio Co. (Daejeon, Korea). To prepare BG, commercial white PG, PQ, and PN roots were respectively subjected to three steps of autoclaving at 121°C for 30 min after being soaked in grape juice followed by drying at 65°C for 18 h, as described by Sun [17]. To prepare the ginseng extracts, all ginsengs were crushed into powder and ultrasonicated three times in 10

volumes of 80% ethanol at 50°C for 1 h, and then filtered and lyophilized.

Analysis ginsenosides

Based on a modified version of the method of Shi *et al.* [18], 1 g of dried ginseng powder was extracted three times with 50 mL of 80% ethanol aqueous solution at 50°C by ultrasonication for 1 h. After filtration, the solvent was removed using an evaporator (Eyela NN; Tokyo Rikakiki, Tokyo, Japan), and the residue was dissolved in 20 mL of distilled water. The solution was transferred to a separatory funnel containing the same volume of ethyl ether. Lipid components in the sample were removed via extraction with ethyl ether three times. The sample was further extracted with 20 mL of water-saturated butanol layer was concentrated *in vacuo*. The samples were then dissolved in 10 mL of 80% methanol and filtered through a 0.45 µm membrane filter. Saponin levels were quantified by a HPLC analysis (SPD 20A; Shimadzu, Kyoto, Japan) using an ACE 5 C₁₈ column (250×0.4 mm, 5 µm) and a UV detector (203 nm). The mobile phase was a gradient of water and acetonitrile. To elute saponin, the acetonitrile concentration was adjusted as follows: 0-30 min, 20%; 30-60 min, 20%-45%; 60-78 min, 45%-75%; 78-80 min, 75%-80%; 80-100 min, 80%-100%. After injecting 10 µL of sample, the mobile-phase flow rate was adjusted to 1 mL/min. As controls, ginsenoside standards (Rg₁, Re, Rf, Rb₁, Rc, Rb₂, Rd, Rk₃, Rh₄, 20(R)-Rg₃, 20(S)-Rg₃) with >98% purity were purchased from Hongjiu Biotech Co., Ltd. (Jilin, China).

In vitro cholinesterases inhibitory activities

ChEs inhibitory activities were measured using the spectrophotometric method developed by Ellman *et al.* [19]. ACh and butyrylcholine were used as substrates to assay the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), respectively. The assay mixture consisted of 340 µL of 0.1 M sodium phosphate buffer (pH 8.0), 40 µL of 2 mM 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), 40 µL of the sample, and 460 µL of either AChE or BChE (0.22 U/mL) prepared in 50 mM Tris buffer (pH 8.0) containing 0.1% BSA. After 10 min, the reactions were initiated with the addition of 10 µL of either 3.75 mM acetylthiocholine iodide (ATCI) or 3.75 mM butyrylthiocholine chloride (BTCC) to the solution. The hydrolysis of ATCI or BTCC was monitored by observing the formation of the yellow 5-thio-2-nitrobenzoate anion at 410 nm for 2 min. This resulted from the reaction of DTNB with thiocholine, which was released by the enzymatic hydrolysis of either ATCI or

BTCC, respectively. Tacrine (9-amino-1, 2, 3, 4-tetrahydroacridine hydrochloride, THA) was used as a positive control. The percent inhibition was calculated from $[A_0 - A_1/A_0] \times 100$, where A_0 is the change in absorbance of the control and A_1 is the change in absorbance in the presence of the test compound. The ChEs inhibitory activities of each sample were expressed in terms of the IC_{50} value (mg/mL required to inhibit the hydrolysis of the substrate; ATCI or BTCC, by 50%).

Total polyphenol contents

Total polyphenol contents were determined according to Folin-Ciocalteu's method modified by Singleton and Rossi [20]. In brief, 0.2 mL of each ginseng extract (10 mg/mL) was mixed with 0.2 mL of Folin-Ciocalteu reagent, 0.4 mL of saturated sodium carbonate, and 3 mL of distilled water in a test tube. The tube was then vortexed and incubated for 1 h. Absorbance was subsequently measured at 725 nm. Quantification of phenol content was performed from the tannic acid calibration curve. The results were expressed as tannic acid equivalents (mg/g extract).

Scavenging activity on 2, 2-diphenyl-1-picryl-hydrazyl radicals

The stable 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity was determined by Blois's method [21]. The samples and references dissolved in ethanol were mixed with DPPH solution (4×10^{-4} M). After 10 min, the amount of remaining DPPH was

measured at 540 nm. Ascorbic acid was used as a positive control. Inhibition of DPPH in percent (%) was calculated according to the following equation: DPPH scavenging activity (%) = $[A_0 - A_1/A_0] \times 100$, where A_0 is the absorbance of the control (containing all reagent except the test sample), and A_1 is the absorbance of the test compound. IC_{50} is the concentration of sample needed to decrease 50% of the DPPH concentration relative to that of control.

Statistical analysis

All data were analyzed using the SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). Ginsenoside content in two groups was analyzed by an independent samples t-test. Differences between groups were analyzed using ANOVA and Duncan's multiple range test. A difference of $p < 0.05$ was regarded as being statistically significant.

RESULTS AND DISCUSSION

Analysis of ginsenosides in the three white ginseng roots

Typical HPLC-UV chromatograms of commercial WG extracts of PG, PQ, and PN are shown in Fig. 1A to 1C, respectively. The contents of the 11 major ginsenosides in the three species of the WG extracts are summarized in Table 1. The total ginsenoside content is 25.89 ± 2.26 mg/g in white *P. ginseng* (WPG), 84.70 ± 2.25 mg/g in white *P. notoginseng* (WPN), and 48.39 ± 2.80

Table 1. Ginsenoside contents in the three white and steamed black ginseng roots

Ginsenoside	White ginseng roots			Steamed black ginseng roots		
	PG	PQ	PN	PG	PQ	PN
Rg ₁	4.78±0.13 ^b	3.13±0.26 ^c	34.61±0.54 ^a	ND	ND	2.61±0.05
Re	2.02±0.30 ^c	16.08±0.47 ^a	11.54±0.40 ^b	ND	ND	1.02±0.01
Rf	0.97±0.05	ND	ND	0.82±0.02	ND	ND
Rb ₁	6.80±1.23 ^c	22.24±1.57 ^b	28.93±1.04 ^a	4.88±0.14 ^a	2.07±0.30 ^c	3.48±0.17 ^b
Rc	6.70±0.14 ^a	3.35±0.29	ND	1.72±0.62 ^b	3.65±0.20 ^a	4.51±1.02 ^a
Rb ₂	3.05±0.30 ^a	0.39±0.04	ND	1.76±0.34 ^b	1.02±0.13 ^c	3.53±0.37 ^a
Rd	1.57±0.11 ^c	4.75±0.16 ^b	9.62±0.27 ^a	0.52±0.04 ^c	0.86±0.01 ^b	3.02±0.19 ^a
Rk ₃	ND	ND	ND	4.28±1.10 ^b	2.11±0.08 ^c	8.33±0.93 ^a
Rh ₄	ND	ND	ND	8.84±0.85 ^b	3.34±0.21 ^c	16.15±1.10 ^a
Rg ₅ (S)	ND	ND	ND	6.73±0.48 ^b	7.22±0.62 ^b	10.64±0.02 ^a
Rg ₅ (R)	ND	ND	ND	5.11±0.62 ^c	7.03±0.5 ^b	8.53±0.03 ^a
Total	25.89±2.26 ^c	48.39±2.79 ^b	84.70±2.25 ^a	36.66±4.21 ^b	27.30±2.05 ^c	61.82±3.89 ^a

Values are expressed as means±SD (mg/g extract, n=3). Different superscripts in the same row indicate significant differences in white ginseng and steamed black ginseng by Duncan's multiple range test. $p < 0.05$ by independent samples t-test.

PG, *Panax ginseng*; PQ, *P. quinquefolium*; PN, *P. notoginseng*; ND, not detected.

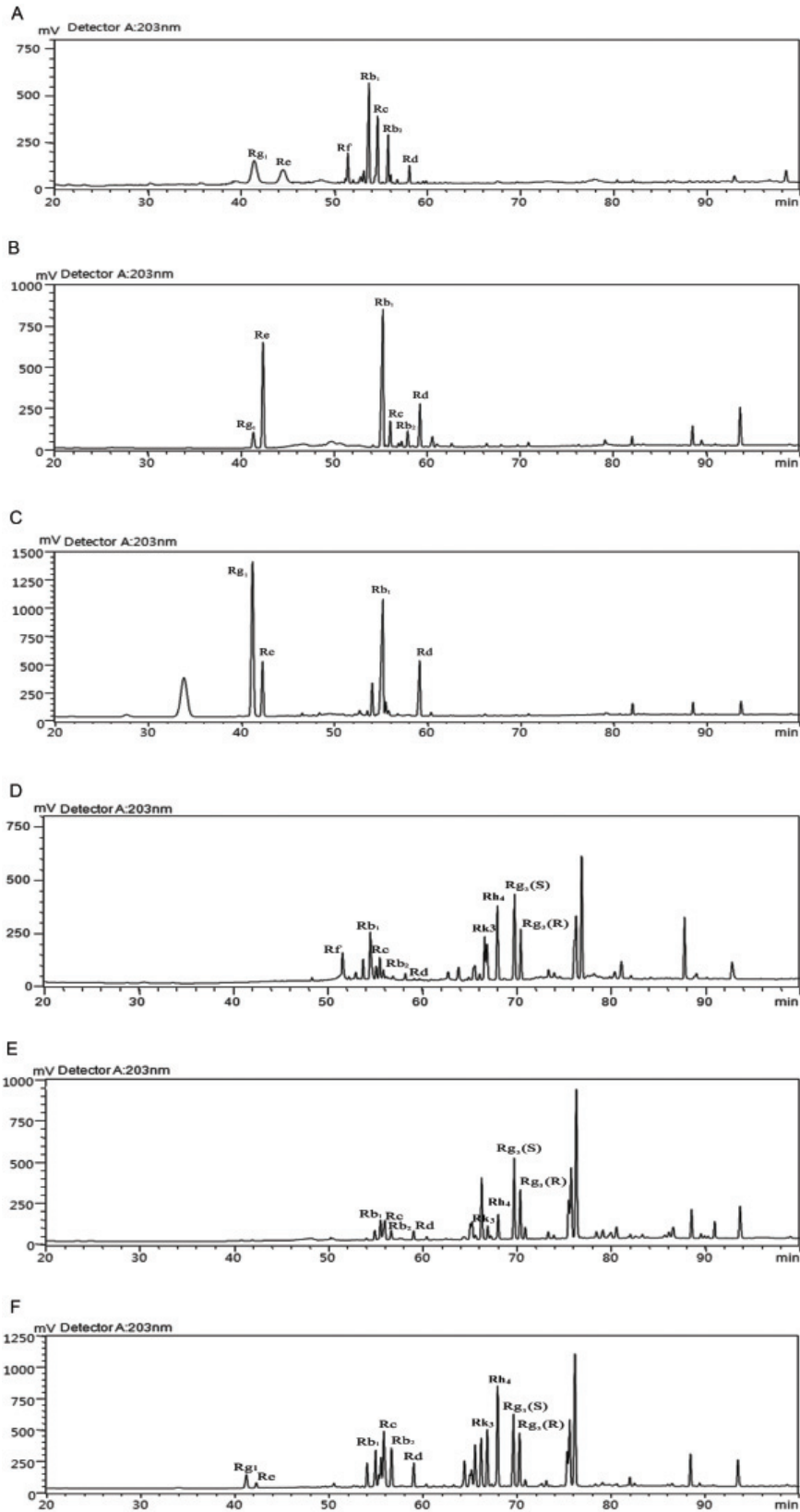


Fig. 1. HPLC-UV chromatograms of ginsenosides in (A) white *Panax ginseng*, (B) white *P. quinquefolium*, (C) white *P. notoginseng*, (D) steamed black *P. ginseng*, (E) steamed black *P. quinquefolium*, (F) steamed black *P. notoginseng*.

mg/g in white *P. quinquefolium* (WPQ). Rg₁, Re, Rf, Rb₁, Rc, Rb₂, and Rd were found in WPG, but Rf was not detected in WPQ, and Rf, Rc and Rb₂ were not observed in WPN. Consistent with the literature, the presence of the ginsenoside Rf in PG with a content in excess of 0.1% (w/w) of the dried root was detected [22], and this is an important parameter for differentiating PG from PN and PQ. Furthermore, the content of the ginsenoside Rg₁ in WPN (34.61±0.54 mg/g) was remarkably higher than that in WPG and WPQ (4.78±0.13 mg/g and 3.13±0.26 mg/g, respectively). WPN had the highest content of ginsenoside Rb₁ (28.93±1.04 mg/g), followed by WPQ (22.24±1.57 mg/g) and WPG (6.80±1.23 mg/g). Furthermore, the ratio of Rb₁ to Rg₁ might be beneficial in differentiating the three ginseng roots. Rb₁/Rg₁ values between 1 and 3 are typically characteristic of PG and PN, while Rb₁/Rg₁ values of 10 or more are indicative of PQ [23].

Analysis of ginsenosides in the three black ginseng roots

Typical HPLC-UV chromatograms of steamed BG extracts of PG, PQ, and PN are shown in Fig. 1D to 1E, respectively, and the contents of the 11 major ginsenosides in the steamed BG extracts are also presented in Table 1. The total ginsenoside content in steamed black *P. ginseng* (BPG) was 36.66±4.21 mg/g, in steamed black *P. quinquefolium* (BPQ), 27.30±2.05 mg/g in BPQ, and 61.82±3.89 mg/g in steamed black *P. notoginseng* (BPN). During the steaming process, the contents of the polar ginsenosides including Rg₁, Re, Rb₁, Rc, Rb₂, and Rd decreased remarkably, while the other ginsenosides increased. The content of the major ginsenosides in the

steamed BG such as RK₃, Rh₄, and 20(S)/(R)-Rg₃ was 24.96±3.05 mg/g in BPG, 19.70±1.41 mg/g BPQ, and 43.65±2.08 mg/g in BPN and was equivalent to approximately 70% of the total ginsenoside content in the steamed BG. Ginsenosides Rh₄ and Rk₃ can be formed by deglycosylation of Rg₆ and F4 at C-6, while ginsenoside Rg₃ can be formed by eliminating the glycosyl residue at C-20 of protopanaxdiol ginsenosides [24]. Recently, many pharmacological studies have shown that the ginsenoside Rg₃ provides a neuroprotective effect against cerebral ischemia [25] and potent anti-tumor activity [26]. Therefore, production of the ginsenoside Rg₃ is expected to be very important and many studies have aimed at converting major ginsenosides to the more active minor ginsenoside Rg₃.

Cholinesterases inhibitory activity

The inhibitory activities on ChEs such as AChE and BChE of ethanol extracts of the three WG and steamed BG using PG, PQ, and PN are shown in Figs. 2 and 3. The results revealed that WG and BG inhibited both AChE and BChE in a dose dependent manner. The BChE inhibitory efficacy of the three WG and BG roots was higher overall than the AChE inhibition. The PG extracts of WG and BG were found to have the highest inhibition of AChE with estimated IC₅₀ values of 5.37±0.20 mg/mL and 2.64±0.19 mg/mL, respectively (Table 2 and Fig. 2). At a concentration of 5 mg/mL, the BChE inhibitory activities of the WG and BG for PG, PQ, and PN reached plateaus at 100%. The WG and BG extracts of PN had the greatest inhibitory effect on BChE with estimated IC₅₀ values of 1.19±0.01 mg/mL and 0.71±0.03 mg/mL,

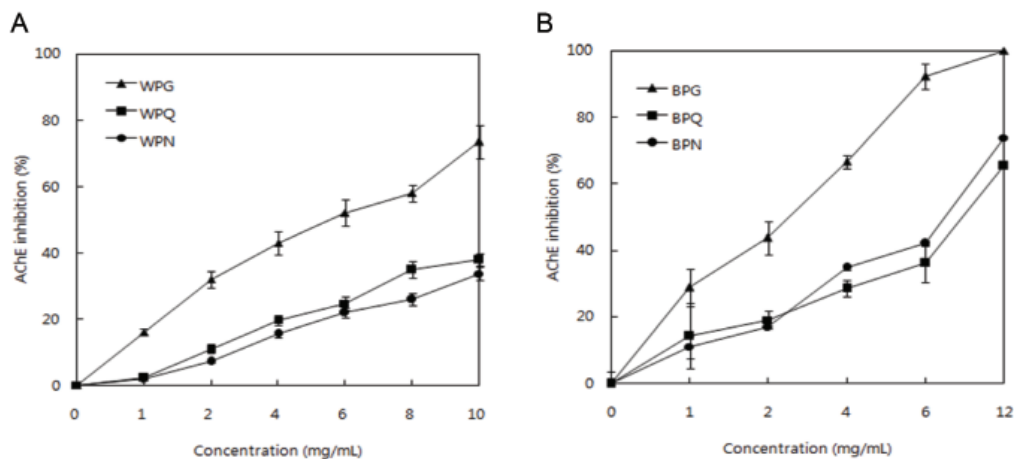


Fig. 2. Effects of the acetylcholinesterase (AChE) inhibition activities according to different concentrations of (A) white *Panax ginseng* (WPG), white *P. quinquefolium* (WPQ), white *P. notoginseng* (WPN), and (B) steamed black *P. ginseng* (BPG), steamed black *P. quinquefolium* (BPQ), and steamed black *P. notoginseng* (BPN). Results are expressed as means±SD ($n=3$).

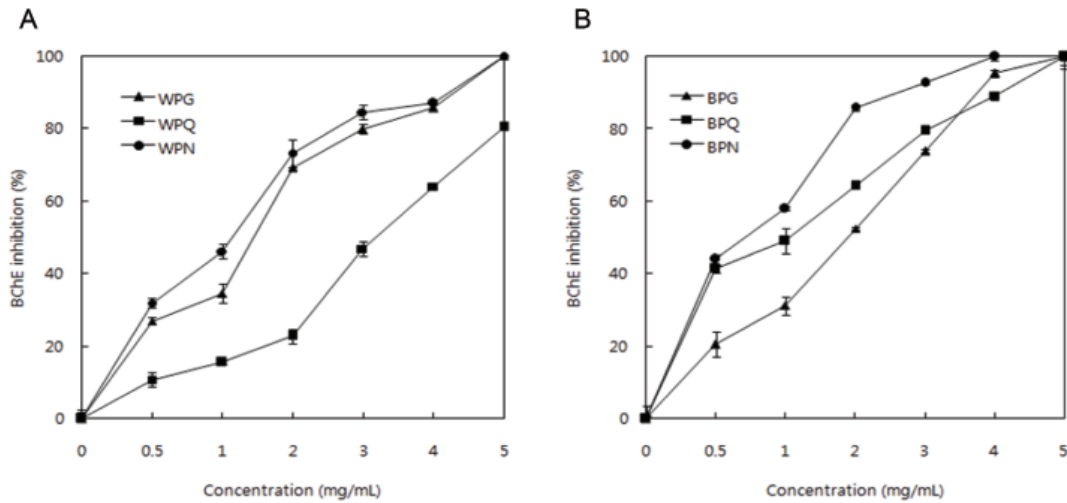


Fig. 3. Effects of the butyrylcholinesterase (BChE) inhibition activities according to the different concentrations of (A) white *Panax ginseng* (WPG), white *P. quinquefolium* (WPQ), white *P. notoginseng* (WPN), and (B) steamed black *P. ginseng* (BPG), steamed black *P. quinquefolium* (BPQ), and steamed black *P. notoginseng* (BPN). Results are expressed as means±SD ($n=3$).

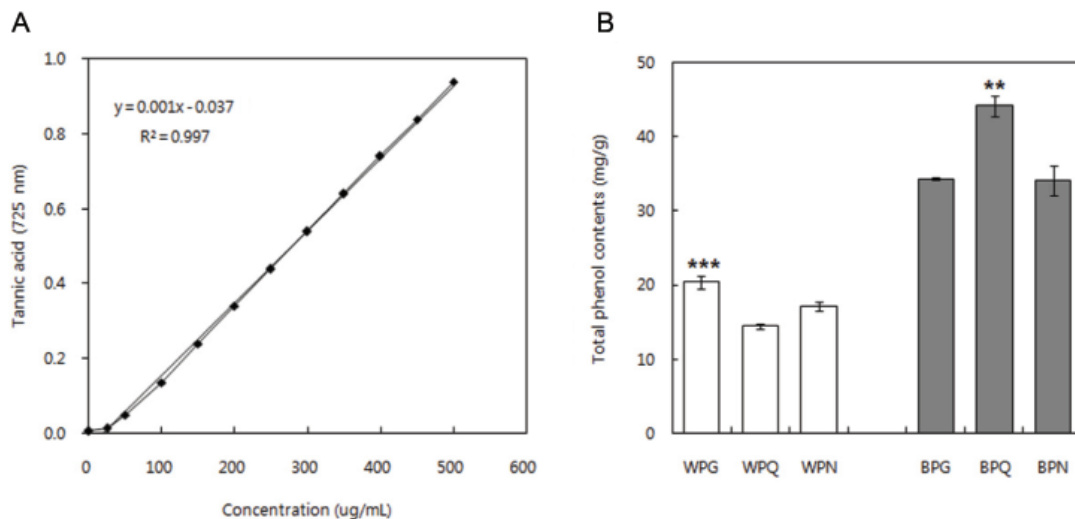


Fig. 4. The (A) standard curve of tannic acid and (B) total phenolic contents of white *Panax ginseng* (WPG), white *P. quinquefolium* (WPQ), white *P. notoginseng* (WPN), and steamed black *P. ginseng* (BPG), steamed black *P. quinquefolium* (BPQ), and steamed black *P. notoginseng* (BPN). Results are expressed as means±SD ($n=3$).

respectively (Table 2 and Fig. 3). The IC_{50} values of THA (positive control) on AChE and BChE were 0.20 ± 0.01 mg/mL and 0.11 ± 0.02 mg/mL, respectively.

In the human brain, both AChE and BChE are found in neurons and glia as well as in neuritic plaques and tangles in patients with AD [27]. AChE co-localizes with β -amyloid and accelerates β -amyloid formation and deposition in AD [28].

The specific BChE inhibitors not only improve cognition, presumably through increased concentration of

ACh, but also reduce levels of amyloid precursor proteins, which are a source of amyloid- β peptides, the main component of plaques in AD [29]. Plant extracts, which have dual anti-ChE activity, may be appropriate for patients in moderate stages of AD [30]. In this screening study on the three ginseng species PG, PQ, and PN, it was found that the extracts of BG for the three ginsengs were effective inhibitors of both AChE and BChE. Therefore, it suggested that the ability of ginseng extracts to prevent or delay memory loss might result from their

Table 2. IC₅₀ values of the three white and steamed black ginseng roots on cholinesterases and antioxidant activities

Ginseng roots	IC ₅₀ (mg/mL)		
	AChE	BChE	DPPH
WPG	5.37±0.20	1.84±0.07	4.30±0.57
WPQ	15.01±0.75	4.63±0.01	11.53±0.68
WPN	13.68±0.50	1.19±0.01	13.76±0.52
BPG	2.64±0.19	1.88±0.06	2.11±0.20
BPQ	8.56±0.80	1.07±0.05	3.53±0.31
BPN	7.50±0.40	0.71±0.03	5.20±0.79
THA	0.20±0.01	0.11±0.02	-
ACA	-	-	0.02±0.00

Values are expressed as means±SD (n=3).

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; DPPH, 2, 2-diphenyl-1-picryl-hydrazyl; WPG, white *Panax ginseng*; WPQ, white *P. quinquefolium*; WPN, white *P. notoginseng*; BPG, steamed black *P. ginseng*; BPQ, steamed black *P. quinquefolium*; BPN, steamed black *P. notoginseng*; THA, tacrine; ACA, ascorbic acid.

cholinergic activity in terms of reduction of the degradation rate of ACh by inhibiting the ChEs activity.

Total phenol contents

Phenolic compounds are widely distributed in plants and are mainly produced to protect plants from stress, ROS, wounds, UV light, disease and herbivores [31]. The total phenol contents of the three WG and BG samples from PG, PQ, and PN were determined by using Folin-Ciocalteu's assay, calculated from the regression equation of the calibration curve ($y=0.002x-0.06$, $r^2=0.998$) in Fig. 4A. As shown in Fig. 4B, PG has a significantly higher content of phenolic compound (20.4±0.90 mg/g),

followed by PN (17.12±0.56 mg/g) and PQ (14.45±0.13 mg/g) in the WG roots. These values in the WG roots increased to 34.3±0.18, 44.15±1.45, and 34.05±2.03 mg/g in the BG roots, respectively. BPQ provided the highest amount of total phenols. More than 10 phenolics in ginsengs, including ferulic, gentisic, cinnamic, syringic, and p-hydrobenzoic acids, have been reported [31]. The increase in the total phenolic contents through heat treatment is thought to be mediated by the increase of free and conjugated phenolic acid contents due to the release of bound phenolic acids linked with glucosides or amine functionalities [32]. In addition, an increase in the Maillard reaction products in ginseng as a result of heat treatment has been well documented and may be a major contributor to enhanced antioxidant activity [33].

2, 2-Diphenyl-1-picryl-hydrazyl radical scavenging activity

DPPH is a stable free radical and has been widely used to assess the ability of compounds or plant extracts to act as free radical scavenging or hydrogen donors [34]. In the DPPH scavenging activity tests of WG and BG roots from the three ginseng species, WPG and BPG scavenged 55.4±1.23% and 86.8±0.85% of the DPPH radicals, respectively, at 5 mg/mL (Fig. 5). However, at the same concentration, the scavenging activity of the WPQ (BPQ) and WPN (BPN) were only 33.02±1.04% (65.20±1.42%) and 29.30±0.73% (53.31±0.57%), respectively. The DPPH scavenging activity of the BG roots increased by 1.5, 1.9, and 1.8 times relative to that of WPG, WPQ, and WPN, respectively. The IC₅₀ values of the three ginseng extracts are shown in Table 2. The

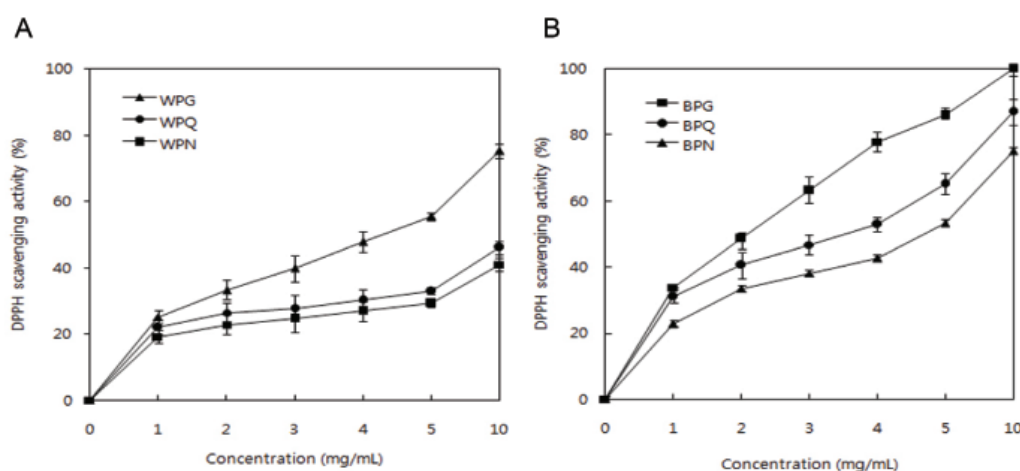


Fig. 5. Free radical 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging activities of (A) white *Panax ginseng* (WPG), white *P. quinquefolium* (WPQ), white *P. notoginseng* (WPN), and (B) steamed black *P. ginseng* (BPG), steamed black *P. quinquefolium* (BPQ), and steamed black *P. notoginseng* (BPN). Results are expressed as Means±SD of triplicate determinations.

best results for the DPPH scavenging activity were obtained with PG, BPG and WPG, with estimated IC₅₀ values of 2.11±0.20 mg/mL and 4.30±0.57 mg/mL, respectively, followed by PQ (BPQ 3.53±0.31 mg/mL, WPQ 11.53±0.68 mg/mL), and PN (BPN 5.20±0.79 mg/mL, WPN 13.76±0.52 mg/mL). The IC₅₀ value of ACA (positive control) was 0.02±0.00 mg/mL. Recently, a method to increase the content of specific ginsenoside such as Rg₃, Rg₅, Rk₃, Rh₄, etc. by steaming WG at higher temperature than employed for RG was developed. This novel heat processed ginseng, BG, showed enhanced free radical scavenging activity and active constituents compared to conventional ginseng [35].

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