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Characterization of Black Ginseng Extract with Acetyl- and Butyrylcholinesterase Inhibitory and Antioxidant Activities

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Black ginseng and white ginseng were extracted with 80% ethanol and evaluated for relative ginsenoside composition, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities, and antioxidant properties. As analyzed by HPLC, black ginseng contained a greater proportion of ginsenoside Rg_3 compared to white ginseng. White ginseng was characterized by undetectable ginsenoside Rg_3 but it contained more total ginsenosides than black ginseng. Black ginseng extract exhibited higher ($p<0.05$) free radical-scavenging activity, as well as higher antioxidant activities than white ginseng against 2,2-diphenyl-1-picryl-hydrazyl, superoxide dismutase, and xanthine oxidase, despite the fact that the total saponin content was higher in white ginseng than black ginseng. In addition, the black ginseng extract displayed greater AChE and BChE inhibitory activities. These results suggest that black ginseng has stronger effects on anti-oxidation and AChE and BChE inhibition than white ginseng.

Keywords: Black ginseng, Acetylcholinesterase, Butyrylcholinesterase, Antioxidation, Acetylcholine

INTRODUCTION

Ginseng, one of the most popular herbs in the world, is considered a tonic or adaptogen that enhances physical performance, promotes vitality, and increases resistance to stress and aging [1]. In Asia, a variety of commercial ginseng products are sold, including white, red, and black ginseng. White ginseng is produced as a result of dehydration by the sun, whereas red ginseng is produced by steaming at 95–100°C for 2–3 h. Black ginseng is produced from white ginseng through nine cycles of steam treatment, at which point it becomes black in color [2].

The non-saponin components of Korean red ginseng exhibit the scavenging effects on free radicals that are related to the aging of cells. Moreover, phenolic compounds and maltol of ginseng have been reported to play an important role in inhibiting aging. Maltol is a substance produced by the pyrolysis of hexose when steamed at high temperatures during the manufacturing process of red ginseng [3]. Besides maltol, red ginseng contains antioxidant components, including salicylic acid, ferulic acid [4], gentisic acid [5], caffeic acid, and vanillic acid. Such phenolic compounds show antioxidant effects in tissues of animals with extreme oxidative injury. A comparison of the phenolic compounds found in Korean ginseng and American ginseng has indicated that certain polyphenol components exist only in Korean ginseng [6].

Acetylcholine (ACh) is a key neurotransmitter in

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learning and memory and its activity is terminated by its hydrolysis into acetate and choline by acetylcholinesterase (AChE) [7]. Two forms of cholinesterase coexist ubiquitously throughout the body, AChE and butyrylcholinesterase (BChE), and although highly homologous (>65%), they are products of different genes on chromosomes 7 and 3, respectively, in humans [8].

In this study, we investigated black ginseng for possible AChE and BChE inhibitory activities, which may explain the effect it has on mental performance. Moreover, the objectives of this study were to compare the antioxidant properties and contents of potential antioxidants between black and white ginseng extracts.

MATERIALS AND METHODS

Preparation of ginseng extracts

White ginseng was made from 4-year-old ginseng purchased from a local ginseng center (Geumsan, Korea). Black ginseng was manufactured via nine cycles of repeated steaming of white ginseng at 98°C for 3 h and drying at 60°C for 18 h. To prepare ginseng extracts, ginseng was crushed into a powder and ultrasonicated three times in 10 volumes of 80% ethanol at 50°C for 1 h, then filtered and lyophilized.

Sample preparation for HPLC analysis

Based on the method of Shi *et al.* [9] with modification, 1-g dried ginseng powder was extracted three times with 50 mL of 80% ethanol aqueous solution at 50°C by ultrasonication (60 Hz, heat power 600 W; Powersonic 510, Hwashin, Korea) for 60 min. After filtration using filter paper (Advantec, Dublin, CA, USA), the solvent was removed using an evaporator (EYELA N-N series; Rikakikai Co., 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 by extracting with ethyl ether three times. The sample was further extracted with 20 mL of water-saturated butanol and concentrated *in vacuo*. The samples were then dissolved in 10 mL of 80% methanol and filtered through a 0.45-μm membrane filter.

Saponin analysis

Saponin levels were quantified by HPLC analysis (SPD 20A; Shimadzu, Kyoto, Japan) using an ACE 5 C_{18} column (250±0.4 mm, 5 μm) and 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% [9]. After injecting 10 μL of sample, the mobile-phase flow rate was adjusted to 1 mL/min. As controls, ginsenoside standards (Rg_1, Re, Rh_1, Rb_1, Rc, Rb_2, Rd, 20(R)-Rg_3, and 20(S)-Rg_3) of >98% purity were purchased from Hongjiu Biotech Co. (Jilin, China).

In vitro cholinesterase inhibitory activity assay

The inhibitory activities against cholinesterases (ChEs) were measured using the spectrophotometric method developed by Ellman *et al.* [10]. ACh and butyrylcholine were used as the substrates to assay the inhibition of AChE and 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% bovine serum albumin). After 10 min, the reactions were initiated with the addition of 10 μL of either 3.75 mM acetylthiocholine iodide (ATCI) or 40 μL of 3.75 mM butyrylthiocholine chloride (BTCC) to the solution. The hydrolysis of ATCI or BTCC was monitored by following the formation of the yellow 5-thio-2-nitrobenzoate anion at 410 nm for 2 min, which resulted from the reaction of DTNB with thiocholine that was released by the enzymatic hydrolysis of either ATCI or BTCC, respectively. The percent inhibition was calculated from $[(A_0–A_1)/A_0]'100$, 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 ChE inhibitory activity of each sample was expressed in terms of the IC_{50} value (mg/mL or nM/mL required to inhibit the hydrolysis of the substrate, ATCI or BTCC, by 50%)

Determination of total polyphenol contents

The method of Folin–Denis was used for measuring total polyphenol contents [11]. Samples of 0.2 mL of each extract (10 mg/mL) and 0.2 mL of Folin–Ciocalteu reagent was mixed with 1.8 mL of distilled water. After 3 min of incubation, 0.4 mL of sodium carbonate saturated solution and 1.4 mL of distilled water was added and the mixture was incubated for 1 h. The absorbance at 725 nm was measured and converted to phenolic contents according to a calibration curve of tannic acid. The concentration of total phenolic compounds in the extracts was calculated using the following linear equation based on the calibration curve (r=0.998):y=0.002x–0.06,

where *y* is the absorbance and *x* is the total phenolic contents in micrograms tannic acid equivalent per milliliter extract.

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

The free radical scavenging activity of ethanol extracts and their solvents were measured by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method proposed by Blois [12]. Briefly, a 0.4 mM solution of DPPH was prepared in ethanol and 0.8 mL of this solution was added to 0.2 mL of sample at different concentrations. After 10 min, the absorbance was measured at 540 nm and compared to the concentrations of ascorbic acid as a positive control. The SC_{50} (scavenging activity) is the concentration of sample needed to reduce the DPPH concentration by 50% compared to that of the control. The DPPH radical-scavenging activity was calculated according to the equation: DPPH scavenging activity (%)=$[(A_0–A_1)/A_0]\times100$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the test compound.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured by the inhibition of pyrogallol autoxidation at 420 nm for 2 min using a kinetic assay according to Marklund and Marklund [13]. The enzyme activity was expressed relative to that of the control. The assay mixture consisted of 1.8 mL of 50 mM Tris-HCl buffer (pH 8.5) containing 10 mM EDTA plus 0.1 mL of the sample. The measurement started with the addition of 0.1 mL of 7.2 mM pyrogallol to the solution. The SOD-like activity was calculated according to the equation: SOD-like activity (%)=$[(A_0–A_1)/A_0]\times100$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the test compound.

Xanthine oxidase inhibition

Xanthine oxidase (XO) inhibition activity was measured by the inhibition of XO at 295 nm for 3 min according to the method of Stirpe and Corte [14]. The assay mixture consisted of 250 μL of 50 mM potassium phosphate buffer (pH 7.5), 300 μL of 0.15 mM xanthine solution (prepared in buffer with a minimum 1 N NaOH), and 150 μL of the sample. The measurement started with the addition of 300 μL of XO (0.2 U/mL) in cold buffer. The inhibitory activity was represented as IC_{50}, which is the concentration of sample required to reduce the XO concentration by 50% compared to that of the control. The XO inhibition activity was calculated according to the following equation: XO inhibition activity (%)=$[(A_0–A_1)/A_0]\times100$, 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.

Statistical analysis

All data were analyzed using the SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using ANOVA and Duncan's multiple range test. A p-value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Saponin analysis

The 20(S)-ginsenoside Rg_3 might provide neuroprotection against cerebral ischemia-induced injury in the rat brain through reducing lipid peroxides, scavenging free radicals, and improving energy metabolism [15]. 20(S)-ginsenoside Rg_3 and Rg_5/Rk_1 significantly reversed the memory dysfunction induced by ethanol or scopolamine (SCOP) and their neuroprotective actions against excitotoxicity may have been attributable to their memory-enhancing effects [16]. In this study, we found that the black ginseng extract (BGE) contained 20(S)- and 20(R)-ginsenoside Rg_3, whereas white ginseng extract (WGE) did not. Thus, BGE has potential neuroprotective and memory improvement properties. Also, note that during the heating process used to process black ginseng, protopanaxadiol-type saponins including Rb_1, Rg_1, Rc, and Rd are converted to ginsenoside Rg_3 by the hydrolysis of a sugar residue at C-20 or isomerization of a hydroxyl group at C-20 [17,18].

The amounts of nine ginsenosides analyzed by HPLC and the chromatograms of the ginseng extracts used in this study are presented in Table 1 and Fig. 1. The total amount of ginsenosides was 54.45±5.08 mg/g in WGE and 14.17±4.33 mg/g in BGE. WGE had a higher total ginsenoside content, but the ginsenoside Rg_3 was not detected in WGE; 7.77±1.95 mg/g of the ginsenoside Rg_3 was found in BGE. Ginsenoside Rg_3 comprised 55% of the total ginsenoside content in black ginseng, and 25% of the ginsenoside Rg_3 in the BGE was 20(S)-ginsenoside Rg_3. The typical difference between the saponin contents of these two ginsengs was the amount of ginsenoside Rg_3 present.

In vitro cholinesterase inhibition activity

To investigate mechanisms of the memory-enhancing

Table 1. Ginsenoside contents in ginseng extracts

Ginsenoside (mg/g)	White ginseng extract	Black ginseng extract
Rg_1	7.81±4.83	Not detected
Re	9.30±0.88	Not detected
Rh_1	0.74±0.31	0.67±0.15
Rb_1	14.14±1.35	2.96±1.60
Rc	12.62±3.02	1.61±0.71
Rb_2	6.97±1.48	0.63±0.21
Rd	2.88±1.37	0.53±0.44
Rg_3(R)	Not detected	5.80±1.42
Rg_3(S)	Not detected	1.97±0.53
Total	54.45±5.08	14.17±4.33

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

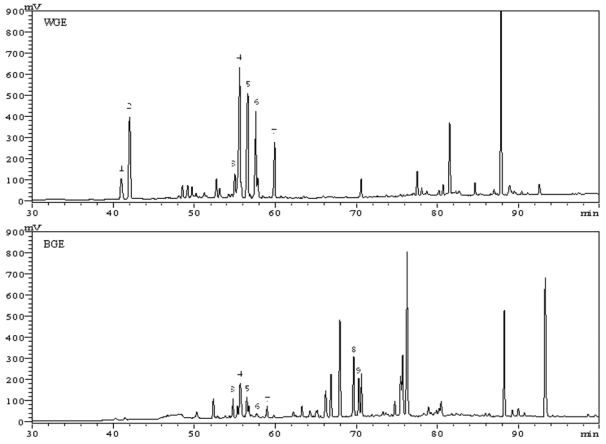

Fig. 1. HPLC-UV chromatograms of ginsenosides in ginseng extracts. Peaks: 1, Rg_1; 2, Re; 3, Rh_1; 4, Rb_1; 5, Rc; 6, Rb_2; 7, Rd; 8, Rg_3(R); 9, Rg_3(S). WGE, white ginseng extract; BGE, black ginseng extract.

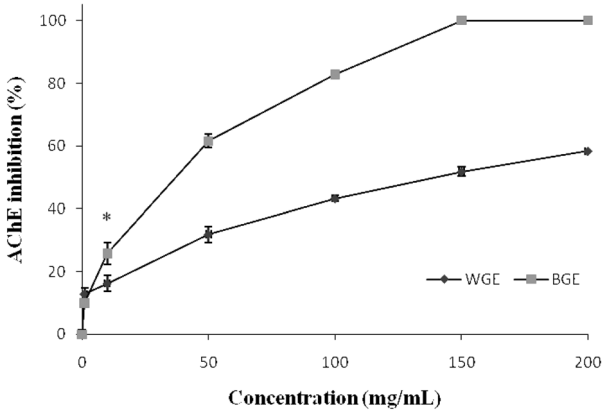

Fig. 2. Inhibitory effects of various concentrations of ginseng extracts on acetylcholinesterase (AChE) activity. Results are expressed as means±SD from triplicate determinations. WGE, white ginseng extract; BGE, black ginseng extract. *p<0.05 vs. WGE.

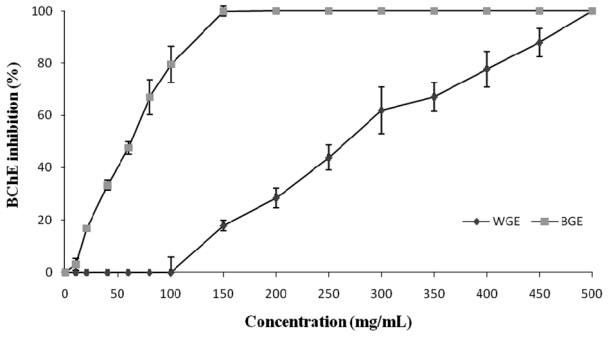

Fig. 3. Inhibitory effects of various concentrations of ginseng extracts on butyrylcholinesterase (BChE) activity. All values are expressed as means±SD from triplicate determinations. WGE, white ginseng extract; BGE, black ginseng extract.

effects of ginseng extract, we measured the activity of cholinergic marker enzymes including AChE (Fig. 2) and BChE (Fig. 3). The rate of the AChE-mediated hydrolysis of acetylthiocholine was determined by measuring the rate of production of free sulfur groups produced as acetylthiocholine was hydrolyzed to thiocholine [19]. On learning and memory deficits associated with SCOP treatment model, the increased AChE activity produced by SCOP was significantly inhibited by WGE and BGE (p<0.001) [17].

The ethanol extracts of black ginseng and white ginseng exhibited detectable ChE inhibitory activities against both AChE and BChE, with IC_{50} values of 48.08±7.49 mg/mL and 66.32±3.00 mg/mL, and 146.94±4.66 mg/mL and 281.75±5.29 mg/mL, respectively. The IC_{50} for AChE inhibition by BGE (48.08±7.49 mg/mL) was 32.7% of that by WGE (Table 2). The IC_{50} for BChE inhibition by BGE was 23.5% of that by WGE. BGE showed more potent ChE inhibitory activity than WGE, probably due to the compositional differences between their active components. Ginseng extracts, especially BGE, inhibited ChE activity, which can lead to greater binding of ACh to the postsynaptic ACh receptor.

In vitro antioxidant activity assay

The anti-oxidative and free radical scavenging effects of ginseng and some of its selected ingredients have been investigated extensively. In the current study, both samples tested exhibited marked antioxidant activities that were attributable to the total phenolic contents (p<0.05). Typical antioxidant activities of BGE and WGE are shown in Table 2, indicating that the BGE displayed stronger antioxidant activity. A typical difference in the characteristics between these two ginseng extracts is the higher ginsenoside Rb_1/Rb_2 ratio in the black gin-

Table 2. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory and antioxidant activities

	Ascorbic acid (mg/mL)	Tacrine (nM/mL)	Ginseng extracts	
			White ginseng extract (mg/mL)	Black ginseng extract (mg/mL)
AChE (IC_{50})	-	824.70±135.21	146.94±4.66	48.08±7.49
BChE (IC_{50})	-	394.45±137.39	281.75±5.29	66.32±3.00
Polyphenol content	-	-	20.40±0.90	34.12±0.18
DPPH (SC_{50})	0.11±0.14	-	4.12±0.80	2.28±1.33
Superoxide dismutase (IC_{50})	0.77±0.11	-	88.99±5.80	42.23±4.16
Xanthine oxidase (IC_{50})	0.48±0.38	-	11.20±0.99	2.41±0.17

Values are expressed as the means±SD (*n*=3).
DPPH, 2,2-diphenyl-1-picryl-hydrazyl.

seng (4.85±2.61) compared to that of the white ginseng (2.09±0.43) [20].

We analyzed the compositional differences in active compounds between these two kinds of ginseng. The total phenolic contents of extracts from WGE and BGE are summarized in Fig. 4. BGE, which exhibited stronger antioxidant activities than WGE, showed a higher concentration of polyphenols. BGE had a total phenolic content of 31.12 mg/g, whereas that of WGE was 20.40 mg/g (p<0.05). The phenolic compounds of ginseng have been reported to play an important role in the inhibition of aging [21,22].

The SC_{50} values for the DPPH radical-scavenging activities of the ginseng extracts at various concentrations are presented in Fig. 5; ascorbic acid was used as a positive control. Compared to ascorbic acid (0.11±0.14 mg/mL), BGE (2.28±1.33 mg/mL) exhibited higher radical-scavenging activities than WGE (4.12±0.80 mg/mL). Non-saponin components of Korean ginseng exhibit free radical-scavenging effects that are related to the aging of cells [23].

From studies on the autoxidation of pyrogallol, the superoxide anion radical in the reactions was investigated with the aid of SOD. SOD activity according to various concentrations of ginseng extracts is depicted in Fig. 6. In the case of BGE, the SOD activity reached 100% effect at 100 mg/mL; however, with WGE, that activity was observed at 200 mg/mL. From 10 to 100 mg/mL, BGE showed a stronger effect (average 48%) than WGE, and BGE showed significantly higher inhibition activity (p<0.05) between 50 and 100 mg/mL. As a positive control, ascorbic acid was also examined for SOD-like activities. The IC_{50} values for the SOD-like activities in ascorbic acid, BGE, and WGE were 0.77±0.11, 42.23±4.16, and 88.99±5.80 mg/mL, respectively.

XO, which induces gout through the formation of uric acid, also causes oxidative damage to tissues in the living body through generation of the superoxide anion

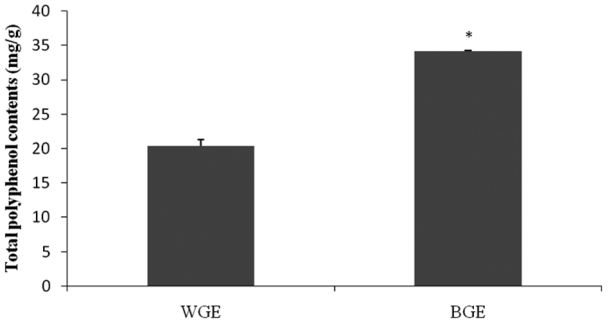

Fig. 4. Contents of polyphenol compounds in extracts from *Panax ginseng* C.A. Meyer. Results are expressed as means±SD from triplicate determinations. WGE, white ginseng extract; BGE, black ginseng extract. *p<0.05 vs. WGE.

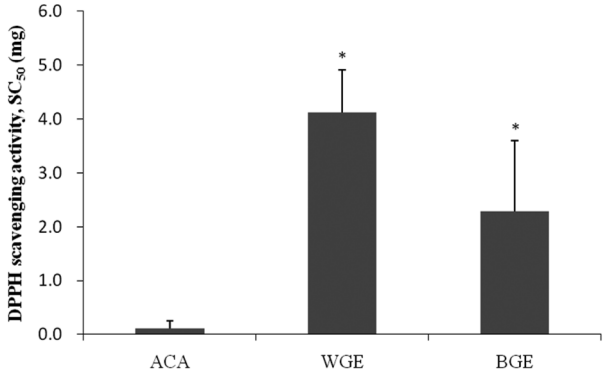

Fig. 5. SC_{50} values for 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activities according to the concentration of various ginseng extracts. ACA, ascorbic acid; WGE, white ginseng extract; BGE, black ginseng extract. *p<0.05 vs. ACA.

radical [24]. The IC_{50} values for the XO inhibition activities of ginseng extracts of various concentrations are presented in Fig. 7. As a positive control, ascorbic acid was also examined for XO inhibition activities. Compared to ascorbic acid (0.68 mg/g), BGE (2.41 mg/g) showed significantly higher inhibition activity (p<0.05) than WGE (11.20 mg/g).

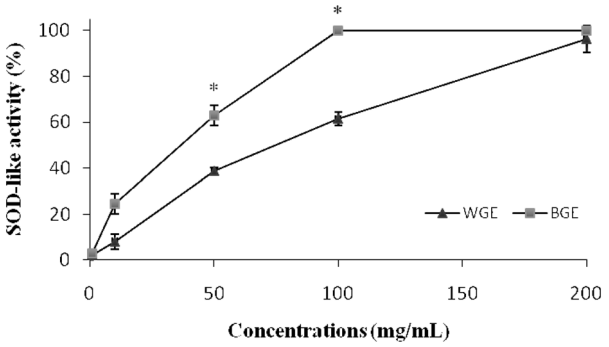

Fig. 6. Superoxide dismutase (SOD) activity according to various concentrations of ginseng extracts. Results are expressed as means±SD from triplicate determinations. WGE, white ginseng extract; BGE, black ginseng extract. *p<0.05 vs. WGE.

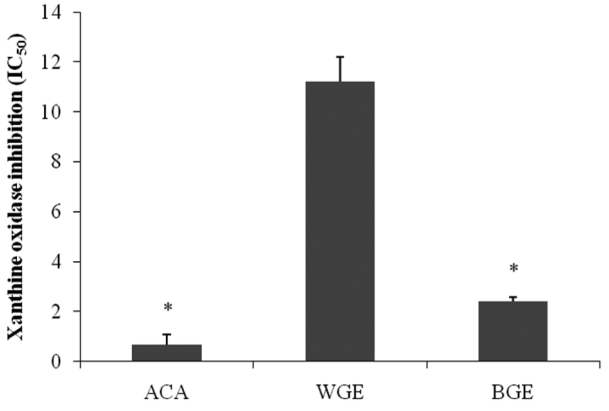

Fig. 7. IC_{50} values for the inhibitory effects of different concentrations of various ginseng extracts on xanthine oxidase activity. Results are expressed as means±SD from triplicate determinations. ACA, ascorbic acid; WGE, white ginseng extract; BGE, black ginseng extract. *p<0.05 vs. WGE.

In conclusion, the total amount of ginsenosides was higher in WGE than in BGE, but the ginsenoside Rg_3 was detected in BGE and not in WGE. The antioxidant activity of BGE was greater than that of WGE, through all of the assays, suggesting that the relationship between the total saponin content and antioxidant activity is not important. Nonetheless, ginsenoside Rg_3 and non-saponin components may be related to the antioxidant and ChE inhibitory activities [25,26].

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