Tropical Journal of Pharmaceutical Research July 2018; 17 (7): 1317-1324
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i7.13

Original Research Article

Purification, compositional analysis and antioxidant properties of polysaccharides from black ginseng

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Sent for review: 9 April 2018 *Revised accepted: 26 June 2018*

Abstract

Purpose: *To extract, purify black ginseng polysaccharides, and study their compositional analysis and antioxidant properties.*
Methods: *Crude polysaccharides from black ginseng were prepared by hot water extraction and subjected to chromatographic purification on Sephadex G-75 and DEAE-cellulose and Sephadex G-75 columns to yield 4 polysaccharide components: BGP-60, BGP-65, BGP-70 and BGP-80. The BGPs were characterized by chemical analysis, gel permeation chromatography (GPC), gas chromatography-mass spectrometry (GC-MS), and Fourier transform-infrared spectroscopy (FT-IR). Finally, the in vitro antioxidant activities of the BGPs were determined through their capacities to scavenge superoxide anion, as well as 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals.*
Results: *The four fractions designated BGP-60, BGP-65, BGP-70 and BGP-80 were polysaccharides with glucose as the main component. They were acidic in nature, with estimated molecular weights (MWs) of 28.6, 26.7, 11.4 and 3.05 kDa, respectively. Fractions BGP-60, BGP-65 and BGP-80 had α-type glycosidic linkage, whereas BGP-70 had β-type glycosidic linkage. Compared with vitamin C (vit C), it was found that BGP-60, BGP-65, BGP-70 and BGP-80 had strong potential antioxidant activities; BGP-60 exhibited a stronger antioxidant activity than BGP-65, BGP-70 or BGP-80 against DPPH and superoxide anion radicals, while BGP-65 had the highest antioxidant activity against hydroxyl radical.*
Conclusion: *These results suggest that BGPs may be beneficial in the development and manufacture as potential therapeutic agents and functional foods.*

Keywords: *Black ginseng, Polysaccharides, Purification, Antioxidant activity, Functional foods*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

The dried roots and rhizome of *Panax ginseng* were important components of a famous traditional Chinese medicine for thousands of years. Black ginseng is a processed product from fresh ginseng, which is made by repeated steaming and drying [1]. Research on black ginseng is famous in South Korea, but rare in Europe and America. Most of the research are focused on technology, while a few addressed the chemical constituents. In contrast, studies on its polysaccharide content are rare [2]. At present, South Korea is leading the world in the processing and research on black ginseng. Black

ginseng has appreciable biological activities such as anti-inflammatory, anti-tumor, and immune boosting capacities [3,4]. Some rare ginsenosides with strong anti-cancer effects have been isolated from black ginseng [5]. At present, there are no uniform quality standards for black ginseng polysaccharides. These carbohydrates promote thymus and spleen indices, and they also have immunity-enhancing potential [6]. They activate lymphocyte proliferation or maturation [7], and stimulate cytokine secretion [8]. Their biological activities are closely related to their physicochemical properties and structures [9-11]. The black ginseng used in this experiment was prepared in the laboratory by non -traditional technology.

The present study involved a preliminary characterization, and determination of antioxidant activities of black ginseng polysaccharides (BGPs).

EXPERIMENTAL

Materials and chemicals

DPPH radicals, DEAE-cellulose and standard monosaccharides (L-rhamnose, L-arabinose, D-glucose, D-galactose, D-mannose and D-xylose) were products of Sigma (UK). Dextrans of various MWs were Pharmacia products. Other solvents and chemicals used were all of analytical grade.

Extraction and purification of black ginseng polysaccharides

Powder of black ginseng was extracted successively with ethanol in a reflux unit, and then extracted thrice using 8 volumes of water for 12 h. The aqueous extracts were combined and filtered. Then using a rotary evaporator, the volume of the pooled extract was reduced by nine-tenth. It was subsequently subjected to precipitation for 12 h at 4 ^{o}C using 80 % ethanol, and clarified by centrifugation for 15 min at 4000 g. The resultant residue was suspended in water and de-proteinated as described earlier [12]. The aqueous fraction was passed through a 0.22-um molecular membrane and freeze-dried to yield the crude BGP which was re-constituted to 50 % solution with ethanol, and kept refrigerated at 4 ^{o}C for 12 h to allow for polysaccharide precipitation. Following centrifugation for 15 min at 4000 g, the supernatant was subjected to successive BGP fractionation using increasing gradients of ethanol i.e. 50 - 60 %, 60 – 65 %, 65 - 70 %, 70 - 75 %, and 75 - 80 %), which resulted in five precipitation fractions. These fractions were subjected to chromatographic purification using Sephadex G-75 and DEAE-cellulose columns, to yield 4 purified fractions coded BGP-60, BGP-65, BGP-70 and BGP-80.

Assays for sulfate, total sugar, uronic acid and protein levels

The phenol-sulfuric acid method was used to determine the sugar contents of BGP-60, BGP-65, BGP-70 and BGP-80, with glucose serving as standard [13], while protein levels were assayed with Bio-Rad Protein Assay [14]. Uronic acid was assayed using sulfuric acid-carbazole method [15], while the barium chloride-gelatin method was employed for the assay for sulfate levels [16]. Bovine serum albumin (BSA), D-glucuronic acid, and potassium sulfate were used as standards in the protein, uronic acid and sulfate assays, respectively.

Estimation of average molecular weights (MWs) of BGPs

The average MWs of the various BGPs were determined by GPC using a Waters 515 HPLC equipment (Waters Co. Ltd., USA) bearing an ultra-hydrogel column (PL aquagel-OH MIXED; ultra-hydrogel column: length×aperture: 300×7.5 mm ID), with 0.1 M $NaNO_3$ as mobile phase. Using pure water as mobile phase, each sample was analyzed at flow rate, column temperature and injection volume values of 0.6 mL/min, 40 ^{o}C, and 20 µL, respectively, and the MWs were extrapolated from dextran-T standard calibration curves prepared under the same operating conditions. The average MWs were calculated with empower software.

Determination of monosaccharide contents of BGPs

The monosaccharide contents of the BGPs were determined by GS-MS (QP2010, Shimadzu, Japan) as described previously [17]. Sample (2 mg) was subjected to hydrolysis with trifluoroacetate (2 M) for 1½ h. The solution was placed in a rotary evaporator and 2 mL of methanol was added until it was dried up. This was done two times, and the resultant residue was taken up in water (double-distilled) and subjected to borohydride reduction for 8 h. The sodium borohydride was neutralized by addition of acetic acid. Then the mixture was placed in the rotary evaporator and dried by sequential addition of three 3-mL batches of methanol (each 3 mL added was allowed to dry before the next addition), followed by oven-drying at 110 ^{o}C. Acetylation was done by adding 1 mL of acetic anhydride at 100 ^{o}C for 1 h, and the mixture was cooled by addition of 3 mL toluene. Thereafter, the product was vacuum-concentrated to dryness

(this process was repeated 5 times to remove unreacted acetic anhydride). The product was dissolved in 3 mL chloroform, and fractionated in a separating funnel by addition of 10 mL distilled water, followed by thorough shaking. The upper aqueous layer was discarded (this operation was carried out 4 times), and the $CHCl_3$ layer was dried with anhydrous sodium sulfate, and subjected to GC-MS analysis, with He as carrier gas at a flow rate of 1.0 ml/min. The detector and injection temperatures were 250 ^oC. The temperature of the column was increased at 3 ^oC intervals from 120 to 250 ^oC, and maintained at 250 ^oC for 5 min.

UV–VIS spectral analysis

Aqueous solutions of BGP-60, BGP-65, BGP-70 and BGP-80 were separately scanned in the wavelength range of 200- 400 in a Shanghai UV–VIS spectrophotometer.

Fourier-transform infrared spectra (FT-IR) analysis

The samples (BGP-60, BGP-65, BGP-70 and BGP-80) were ground with KBr powder and pelleted prior to use for FTIR analysis (frequency range: 4000 – 400 cm^{-1}) in a Perkin Elmer Fourier transform IR spectrophotometer [18].

Evaluation of antioxidant activity

DPPH scavenging assay

The DPPH scavenging capacities of BGP-60, BGP-65, BGP-70 and BGP-80 were determined by the method of Yang *et al* [19]. 1 mL each of different concentrations of polysaccharide solution was incubated with 0.1 mM ethanolic solution of DPPH (2mL) at 25 ^oC for 20 min, and the absorbance was read at 517 nm. Assays done with distilled water in place of sample served as blank, while vitamin C served as positive control. The absorbance values were used to calculate the DPPH scavenging capacity (D) as in Eq 1.

$$D\ (\%) = \{1-(A_1-A_2)/A_0\}100 \quad \ldots\ldots\ldots\ (1)$$

where A_0 is control absorbance, A_1 is absorbance of sample and DPPH, and A_2 is the absorbance of the sample blank. The IC_{50} values of the samples were extrapolated from the standard curve.

$OH^{\cdot}$ scavenging capacity

The OH scavenging capacities of BGP-60, BGP-65, BGP-70 and BGP-80 were determined as outlined by Chen *et al*. [20]. A range of concentrations of each BGP (I mL each) was incubated for 30 min at 37 ^oC with 2 ml of 9 mM $FeSO_4$, 0.1 mL of 20 mM H_2O_2 , 1 mL of deionized H_2O and 1.5 mL of 9 mM solution of salicylic acid in ethanol. Vitamin C was used as standard. At the end of reaction, absorbance was read at 510 nm in a spectrophotometer, and the OH scavenging ability (Y) obtained using Eq 2.

$$Y\ (\%) = \{1 - (A_1-A_2)/A_0)\} \times 100 \quad \ldots\ldots\ldots\ (1)$$

where A_0 is control absorbance, A_1 is sample absorbance (with $OH^{\cdot}$), and A_2 is sample blank absorbance. The corresponding IC_{50} values were extrapolated from a standard curve.

SO_2^{-} scavenging capacity

This was assayed by a slight modification of the procedure described by Han *et al* [21]. A range of concentrations of each BGP (0.5 mL each) was incubated at 25 ^oC with 45 mM pyrogallate (0.1 mL), 0.05 M Tris buffer, pH 7.8, and 0.5 mL of deionized H_2O for 20 min. The reaction mixture consisted of 3 mL 0.05 M Tris-HCL buffer, pH 7.8, and 0.1 mL pyrogallate (45 mM), 0.5 ml of deionized H_2O) for 20 min. Then, 0.1 mL of 45 mM pyrogallate was added, followed by further incubation for 4 min, after which absorbance was read at 420 nm. Ascorbic acid was used as control. The SO_2^{-} scavenging capacity (Q) was obtained for each BGP using Eq 3.

$$Q\ (\%) = \{1-\%A_1-A_2)/A_0\} \times 100 \quad \ldots\ldots\ (3)$$

where A_0 is control absorbance value, A_1 is sample absorbance (with SO_2^{-}), and A_2 is the sample blank absorbance. The corresponding IC_{50} values were extrapolated from a standard calibration curve.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences between BGPs were analyzed statistically with one-way analysis of variance (ANOVA) and Duncan's least significant test. All analyses were carried out using SPSS version 13. Values of $p < 0.05$ were taken as indicative of statistical significance.

RESULTS

Purified BGPs

Black ginseng polysaccharide was obtained in an overall yield of 17.63 % through water extraction, ethanol precipitation, filtration through 0.22 µm molecular membrane, and lyophilization.

Following chromatographic purification with Sephadex G-75 and DEAE-cellulose, four fractions were obtained: BGP-60, BGP-65, BGP-70 and BGP-80. The recoveries of BGP-60, BGP-65, BGP-70 and BGP-80 based on the amount of crude BGPs used were 9.78, 8.65, 14.62 and 4.21 %, respectively. The four fractions yielded single, narrow and symmetrical peaks when eluted in GPC, indicating their homogeneity (Figure 1). The GPC analysis indicated that the average MWs of BGP-60, BGP-65, BGP-70 and BGP-80 were approximately 28.6, 26.7, 11.4 and 3.05 kDa, respectively. Results from UV-VIS spectrum in the wavelength range of 200 - 400 nm showed no absorption from 260 to 280 nm, suggesting the absence of DNA, RNA and proteins.

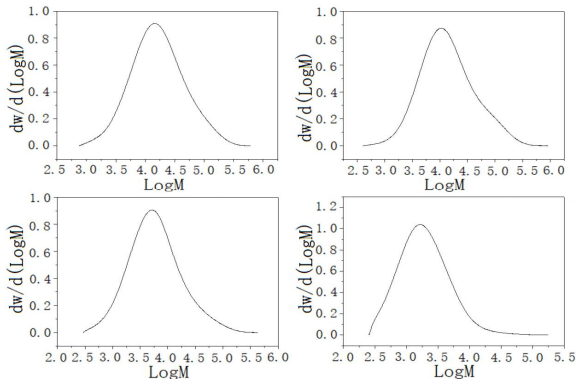

Figure 1: Molecular weights of BGP-60, BGP-65, BGP-70 and BGP-80, as obtained using GPC

Compositions of the BGPs

The levels of carbohydrate, protein, uronic acid and sulfate in BGP-60, BGP-65, BGP-70 and BGP-80 are shown in Table 1. The carbohydrate contents of BGP-60, BGP-65, BGP-70 and BGP-80 were 79, 54, 78 and 17 %, respectively. None of the four polysaccharide fractions contained proteins. The contents of uronic acid in BGP-60, BGP-65, BGP-70 and BGP-80 were 4, 23, 18 and 63 %, respectively, while sulfate contents were 17, 23, 4 and 20 %, respectively. These results indicate differences in chemical components of the BGPs.

Monosaccharide composition of BGPs

The monosaccharide composition of BGP-60, BGP-65, BGP-70 and BGP-80 are presented in Table 2 and Figure 2. BGP-60 contained galactose and glucose in an approximate molar ratio of 22:78, with glucose as the main monosaccharide, while BGP-65, BGP-70 and BGP-80 contained mainly arabinose, galactose and glucose, with glucose also as the predominant monosaccharide.

FT-IR spectra of BGPS

The FT-IR spectra of BGP-60, BGP-65, BGP-70 and BGP-80 are presented in Figure 3. Fraction BGP-60 had specific absorptions at 3433, 2927, 1635, 1026, 1111, 1153 and 853 cm^{-1}, while BGP-65 had specific absorptions at 3384, 2933, 1636, 1043, 1078, 1151 and 854 cm^{-1}. Fraction BGP-70 had specific absorptions at 3420, 2927, 1635, 1044, 1078, 1151 and 892 cm^{-1}, while BGP-80 had specific absorptions at 3395, 2928, 1633, 1041, 1078, 1149 and 860 cm^{-1}.

Antioxidant properties of BGPs

DPPH radical scavenging capacity

The DPPH scavenging capacities of BGP-60, BGP-65, BGP-70, BGP-80 were lower than that of vitamin C (Figure 4). The scavenging activities of BGP-60, BGP-65, BGP-70 and BGP-80 at 8 mg/mL reached 96.33, 81.47, 91.07 and 92.38 %, respectively, with IC_{50} values of BGP-60, BGP-65, BGP-70 and BGP-80 for 1.80, 3.26, 1.93 and 2.36 mg/mL, respectively.

Table 1: Carbohydrate, protein, uronic acid and sulfate levels in BGP-60, BGP-65, BGP-70, and BGP-80

Sample	Protein (%)	Carbohydrate (%)	Uronic acid (%)	Sulfuric radical (%)
BGP-60	0	79	4	17
BGP-65	0	54	23	23
BGP-70	0	78	18	4
BGP-80	0	17	63	20

Table 2: Monosaccharide compositions of the four BGPs

Sample	Sugar content (%)						
	Rhamnose	*Fucose*	*Arabinose*	*Xylose*	*Mannose*	*Glucose*	*Galactose*
BGP-60	-	-	-	-	-	77.67	22.33
BGP-65	-	-	3.72	-	-	59.23	37.05
BGP-70	-	-	5.18	-	-	51.43	43.39
BGP-80	-	-	6.66	-	-	51.61	41.73

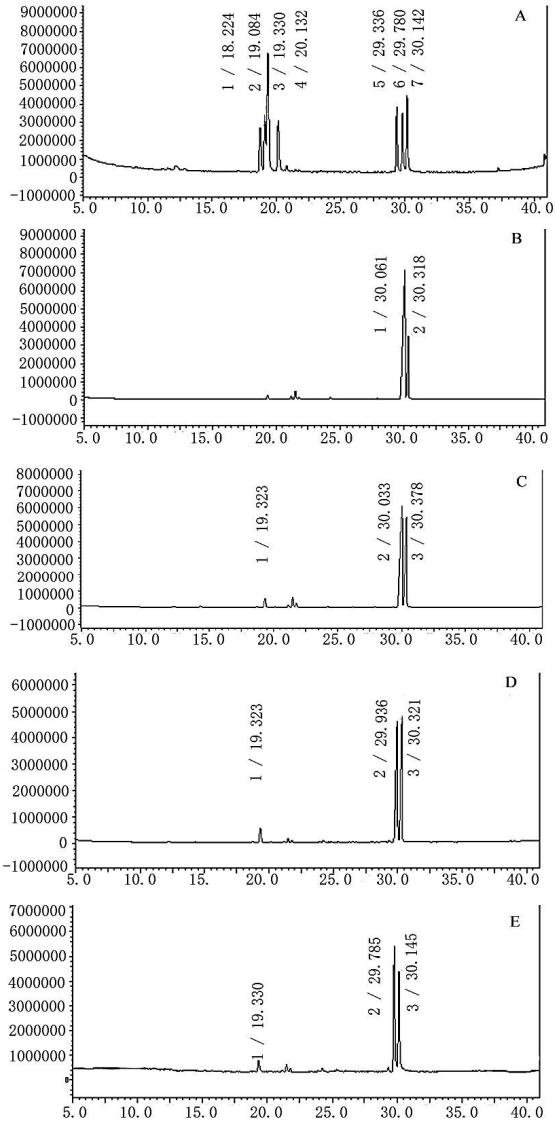

Figure 2: GC-MS chromatograms of monosaccharide standards (A), BGP-60 (B), BGP-65 (C), BGP-70 (D) and BGP-80 (E)

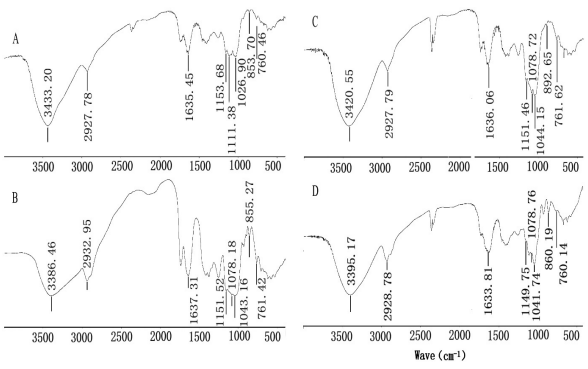

Figure 3: FTIR spectra of BGP-60 (A), BGP-65 (B), BGP-70 (C) and BGP-80 (D)

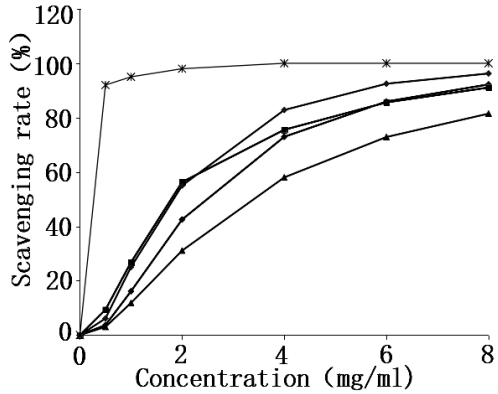

Figure 4: Scavenging effect of BGP-60, BGP-65, BGP-70 and BGP-80 on DPPH radicals ●*: BGP-80,* ■*: BGP-70,* ▲*: BGP-65,* ◆*: BGP-60,* ∗*: Vitamin C*

OH• scavenging capacity

The OH• scavenging capacities of BGP-60, BGP-65, BGP-70, BGP-80 and vitamin C are shown in Figure 5. The OH• scavenging capacities produced by BGP-60, BGP-65, BGP-70 and BGP-80 (87.30, 83.28, 87.71 and 90.89 %), respectively were concentration-dependent below 0.5 mg/mL, although they were lower when compared with vitamin C. The IC_{50} values of BGP-60, BGP-65, BGP-70 and BGP-80 were 2.28, 1.90, 2.05 1.91 mg/mL, respectively, and the OH• scavenging activity was in the order: BGP-60 < BGP-70 < BGP-80 < BGP-65.

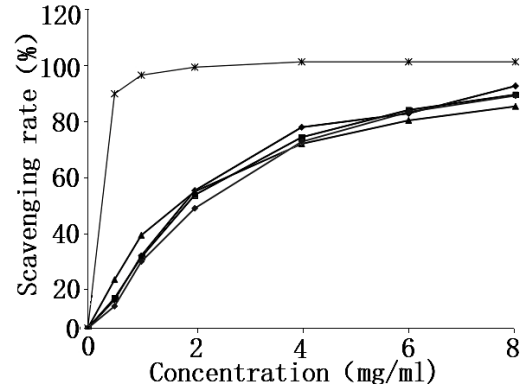

Figure 5: Scavenging effect of BGP-60, BGP-65, BGP-70 and BGP-80 on hydroxyl radical. ●*: BGP-80,* ■*: BGP-70,* ▲*: BGP-65,* ◆*: BGP-60,* ∗*: Vc*

O_2^- scavenging capacity

Figure 6 shows that the O_2^- scavenging capacities of BGP-60, BGP-65, BGP-70, and BGP-80 were concentration-dependent below 0.5mg/ml, although they showed lower scavenging abilities when compared to vitamin C. The potential scavenging activities of BGP-60, BGP-65, BGP-70 and BGP-80 were 83.07, 78.37, 77.12 and 82.97 % (8 mg/mL),

respectively; and their IC_{50} values were 1.95, 2.88, 2.61 2.47 mg/mL, respectively.

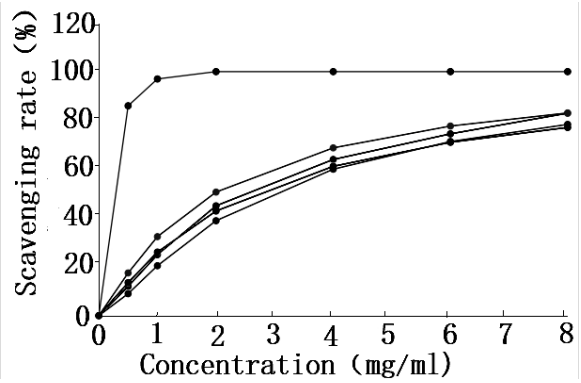

Figure 6: $O_2^{\bullet-}$ scavenging effects of BGP-60, BGP-65, BGP-70 and BGP-80. ●*: BGP-80,* ■*: BGP-70,* ▲*: BGP-65,* ◆*: BGP-60,* ∗*: Vitamin C*

DISCUSSION

Black ginseng is a processed product from fresh ginseng. Polysaccharides from black ginseng possess hypoglycemic, anti-tumor, antithrombotic and antioxidant properties. In order to avoid the destruction of the biological activities of these polysaccharides, cold extraction method was used in this study. Free radicals are associated with many diseases. Hydroxyl, DPPH and superoxide radical scavenging activities are widely used for the quantitative determination of antioxidant capacities of biological samples and foods.

The present study reports the isolation and purification of pharmacologically-active polysaccharides from black ginseng. Four major polysaccharide fractions coded BGP-60, BGP-65, BGP-70 and BGP-80 were identified. The four polysaccharide fractions showed different degrees of antioxidant activities. The biological effects of polysaccharides are associated with their structural characteristics. Results from GPC analysis indicated wide variations in molecular weights of the BGPs, with values of 28.6, 26.7, 11.4 and 3.05 kDa for BGP-60, BGP-65, BGP-70 and BGP-80, respectively. Monosaccharide compositional analysis showed that BGP-60 consisted of glucose and galactose, while BGP-65, BGP-70 and BGP-80 were contained mainly galactose, arabinose and glucose.

In the infrared spectra analysis, stretches in OH groups occur within 3600 and 3200 cm^{-1} (BGP-60, BPG-65, BPG-70, and BPG-80 had bands at 3433, 3384, 3420, and 3395 cm^{-1}, respectively); while C–H stretching occurs at 2923 cm^{-1} (BGP-60, BPG-65, BPG-70, and BPG-80 had bands at 2927, 2933, 2927, and 2928 cm^{-1}, respectively). The 1635 cm^{-1} is ascribed to bound water (BGP-60, BPG-65, BPG-70, and BPG-80 had bands at 1636, 1635, and 1633 cm^{-1}, respectively) [22].

Each GBP produced a band within the zone 1200–1000 cm^{-1}, which is usually attributed to the overlap between ring vibrations and vibrations due to (C–O–C) glycosidic bonds and side groups (C–OH) [23]. Therefore the absorptions at 1026, 1111 and 1153 cm^{-1} (for BGP-60); 1043, 1078 and 1151 cm^{-1} (for BGP-65); 1044, 1078 and 1151 cm^{-1} (for BGP-70); and 1041, 1078 and 1149 cm^{-1} (BGP-80) indicate a pyranose sugar configuration [24]. The absorptions at 853 cm^{-1} (BGP-60), 854 cm^{-1} (BGP-65) and 860 cm^{-1} (BGP-80) are characteristic absorption peak of α-dominating configuration in pyranose sugars [25]. A significant feature peak at 892 cm^{-1} was seen in BGP-70, revealing the β-configuration of the sugar units [26]. These results indicate that BGP-70 possessed β-configuration of pyranose ring structure, while BGP-60, BGP-65 and BGP-80 consisted of α-dominating configurations in pyranose forms.

At room temperature, DPPH is a stable free radical and it is commonly employed in assessing antioxidant properties of crude extracts and purified fractions. In this study, the ability of the BGPs to scavenge DPPH radicals generally increased in the order: BGP-65 < BGP-80 < BGP-70 < BGP-60. Iron-chelating compounds which participate in Fenton reaction exert $OH^{\bullet}$ scavenging capacities [27]. The neutralization of $OH^{\bullet}$ is extremely important for antioxidant defenses in cells. The $OH^{\bullet}$ scavenging capacity contributed to the observed antioxidant effects of the BGPs, which increased in the order of BGP-60 < BGP-70 < BGP-80 < BGP-65.

On decomposition, $O_2^{\bullet-}$ generates $O^{\bullet}$ and $OH^{\bullet}$ which are more powerful ROS; these damage lipids, proteins and DNA through deleterious oxidation [28]. Thus, the neutralization of $O_2^{\bullet-}$ is a critical aspect of all antioxidant investigations. The $O_2^{\bullet-}$ scavenging capacities of the BGPs were in the order: BGP-65 < BGP-70 < BGP-80 < BGP-60, and contributed substantially to the overall antioxidant effects of the BGPs.

CONCLUSION

These results demonstrate that BGPs are potential natural antioxidants that may be used as functional foods. The antioxidant capacities of BGP-60 with respect to DPPH and $O_2^{\bullet-}$ scavenging are superior to those of BGP-65,

BGP-70 or BGP-80; while BGP-65 has the strongest antioxidant activity against $OH^{\bullet}$.

DECLARATIONS

Acknowledgement

This work was financed by Research Fund of University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (no. UNPYSCT-2017172), Natural Science Foundation of Heilongjiang Province (no. H2016090), Research project of Heilongjiang Provincial Health and Family Planning Commission (no.2017-367), both in China.

Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Jia-Wei Liu conceived and designed the study, Li-Hong, Tao Lei, Zhao-Li Zhang, Qi-Chao Liang, Feng-Guo Zhai, Yi-Yan Wu, Xiu-Ping Zhang, Jia-Qi Liu collected and analyzed the data, and Li-Hong Gong wrote the manuscript.

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