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Edible prodelphinidins that improve postprandial hyperglycemia were discovered in Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) fruits

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ABSTRACT

Plant polyphenols are potential substitutes for clinical α -glucosidase inhibitors. Our previous studies indicated that prodelphinidins from Chinese bayberry leaves (BLPs) have stronger α -glucosidase inhibitory activity than other common polyphenols, but they have no safe history of consumption. There is a reasonable prospect that prodelphinidins from Chinese bayberry fruits (BFPs) can improve postprandial hyperglycemia, though known active components are only myricetin and cyanidin-3-*O*-glucoside. Hence, the aim of this study was to analyze structure and hypoglycemic effect of BFPs of “Biqi” (BQPs) and “Dongkui” (DKPs) cultivars, and compare their difference with BLPs. The contents of BQPs and DKPs were (221.73 ± 0.60) and (136.82 ± 4.33) mg epicatechin (EC) equivalents/g dry weight, respectively. The most subunits were epigallocatechin gallate along with a small amount of epigallocatechin, epicatechin gallate and EC connected by B-type linkages and a small portion of A-type linkages with mean polymerization degree of 4.25 and 4.08, respectively. Importantly, BQPs and DKPs were α -glucosidase inhibitors with half inhibitory concentration of 11.91 and 9.47 μ g/mL respectively, which were significantly stronger than BLPs. DKPs could also improve postprandial hyperglycemia of normal mice and high fat diet-induced type II diabetic mice. Therefore, edible prodelphinidins, which have stronger hypoglycemic effect than BLPs, were first found in Chinese bayberry fruits.

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1. Introduction

Postprandial blood glucose is the main indicator for predicting or diagnosing type II diabetes, and its abnormality will lead to

insulin resistance, lipid metabolism disorder and oxidative stress^[1]. Inhibition of α -glucosidase represents an effective strategy to improve postprandial hyperglycemia, which can delay carbohydrate absorption, but have little impact on protein and lipid metabolism^[2]. Clinical α -glucosidase inhibitors, including acarbose, voglibose and miglitol are marred by unpleasant side effects and poor compliance^[3]. Hence, there is an urgent need to develop new substitutes for clinical α -glucosidase inhibitors.

Screening potential α -glucosidase inhibitors from natural products is a hotspot recently. Numerous studies have shown that plant polyphenols possess α -glucosidase inhibitory activity, stronger than acarbose^[4-6]. Our previous study indicated that compared with other polyphenols, prodelphinidins from Chinese bayberry (*Myrica rubra*

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Sieb. et Zucc.) leaves (BLPs) have stronger α -glucosidase inhibitory activity, due to their poly-hydroxy B ring and galloyl C ring. BLPs exhibited inhibitory activity of 517.01 mmol acarbose equivalents/g for α -glucosidase, stronger than common polyphenols from *Lepisanthes alata* leaves (0.30 mmol acarbose equivalents/g), grape seeds (117.46 mmol acarbose equivalents/g) and so on^[7]. Acid-catalyzed transformation of BLPs generated dimeric prodelphinidin gallate, and its half inhibitory concentration (IC_{50}) against α -glucosidase is one thousandth of acarbose^[8].

However, Chinese bayberry leaves have no edible history, and their safety is difficult to guarantee. It was known that Chinese bayberry fruits can regulate blood glucose, and reported active components are mainly myricetin and cyanidin-3-*O*-glucoside^[9-10]. Based on our earlier work, we reasoned that prodelphinidins from Chinese bayberry fruits (BFPs) may be excellent components for regulating blood glucose. But there are few studies to our knowledge has reported the improvement of postprandial hyperglycemia of BFPs. In addition, the structure of BFPs remains unclear, and its difference from BLPs may lead to stronger hypoglycemic activity. If the hypothesis is true, prodelphinidins with improved regulation of postprandial hyperglycemia will be first discovered in edible food.

Therefore, the objective of this study was to isolate and purify BFPs of “Biqi” and “Dongkui” cultivars. Then analyze their structural characteristics, as well as difference from BLPs. In addition, their structure-activity mechanism of regulating hyperglycemia was investigated. Our results provided unambiguous structural information of BFPs, and presented a new evidence for hypoglycemic effect of Chinese bayberry fruits, which lay the foundation for dietotherapy of diabetic mellitus.

2. Materials and methods

2.1 Chemicals and reagents

Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) fruits of “Biqi” and “Dongkui” cultivars were obtained from Xianju County, Zhejiang Province, China. Acetic acid, formic acid, acetonitrile of high performance liquid chromatography (HPLC) grade, α -glucosidase from *Saccharomyces cerevisiae*, sucrose were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, USA). *p*-Nitrophenyl α -*D*-glucoside (pNPG) was purchased from Haohong Biomedical Technology Co., Ltd. (Shanghai, China). Acarbose was purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). HPD500 macroporous adsorption resin and Sephadex LH-20 were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Other chemicals of analytical grade were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Extraction and purification of BFPs

BFPs of “Biqi” and “Dongkui” cultivars were extracted according to previous studies with minor modification^[11]. Briefly, seeded Chinese bayberries fruits were juiced and extracted with 70% aqueous acetone containing 0.1 g/100 mL ascorbic acid at room temperature for 12 h. The above procedure was repeated twice. The extracts were pooled and concentrated under reduced pressure at 50 °C to remove acetone. The extracts were filtered and then purified using HPD500

macroporous adsorption resin. The column was equilibrated with water and then the concentrated extracts were applied to it. The column was then rinsed with 3 column volumes of water to remove carbohydrate and protein. The proanthocyanidins were then eluted with 3 column volumes of 80% ethanol. The eluents were concentrated under reduced pressure at 50 °C to remove ethanol, and then lyophilized to obtain crude proanthocyanidins. The crude proanthocyanidins were further purified by Sephadex LH-20. The column was equilibrated with 90% methanol and the crude proanthocyanidins powders dissolved in this mobile phase were loaded onto it. Next, the column was rinsed with 5 column volumes of 90% methanol to remove low-molecule-weight flavonoids, and proanthocyanidins were then eluted with 3 column volumes of 70% acetone. The eluents were concentrated under reduced pressure at 50 °C to remove organic reagents, and then lyophilized to a dry powder, which was stored at -80 °C to further analysis. BFPs of “Biqi” and “Dongkui” cultivars purified by Sephadex LH-20 were termed as BQPs and DKPs, respectively.

2.3 Determination of proanthocyanidins content

Proanthocyanidins content in Chinese bayberry fruits was determined with vanillin assay as reported previously with slight modifications^[12]. The 1 mL of sample solution was mixed with 2.5 mL of 1 g/100 mL vanillin in methanol and 2.5 mL of 20% (V/V) H_2SO_4 in methanol. The mixture was mixed and incubated at 30 °C for 15 min to carry out vanillin reaction. Methanol was served as blank control. The absorbance was measured at 500 nm against a blank control. Proanthocyanidins content was expressed as mg epicatechin (EC) equivalents per gram of dry weight (DW).

2.4 Determination of ultraviolet (UV)-visible spectra

UV-visible spectra of BQPs and DKPs in methanol were recorded in the range of 200–800 nm using a UV-visible spectrometer (UV-2600, Shimadzu Co., Kyoto, Japan). The UV-visible spectra of methanol were subtracted as the background.

2.5 Determination of Fourier transform infrared (FTIR) spectra

FTIR spectra of BQPs and DKPs were detected using FTIR spectrophotometer (AVATAR370, Thermo Nicolet Co., Waltham, USA) in the range of 4 000–400 cm^{-1} . BQPs or DKPs were ground with KBr in a ratio of 1:20 and pressed into pellets for FTIR determination in the range of 4 000–400 cm^{-1} .

2.6 Acid-catalyzed depolymerization of BQPs and DKPs in the presence of phloroglucinol

Constituent units and mean degree of polymerization (mDP) of BQPs and DKPs were determined as described by previous studies with minor modifications^[13]. The 5 mg of BQPs or DKPs were reacted in 0.1 mol/L methanolic HCl containing 50 g/L phloroglucinol and 10 g/L ascorbic acid at 60 °C for 1 h. The reaction was terminated by adding 40 mmol/L aqueous sodium acetate with volume ratio of 5. Phloroglucinol adducts were analyzed by HPLC system (Waters Co.,

Milford, USA) equipped with triple TOF 5600+ system (AB Sciex Co., Framingham, USA). Chromatographic separation was carried on a Eclipse XDB-C₁₈ column (4.6 mm × 250 mm, 5 μm, Agilent Technologies Co., Ltd., Palo Alto, USA) using a binary mobile phase consisting of water containing 0.1% formic acid (A) and methanol (B). The flow rate was 1 mL/min and elution profile was as followed: 0–10 min, 5% B linear; 10–30 min, 5%–20% B linear; 30–55 min, 20%–40% B linear; 55–60 min, 40%–90% B linear; 60–63 min, 90%–5% B linear; 63–70 min, 5% B linear. The injection volume was 10 μL and the column temperature was set up to 35 °C. Eluting peaks were monitored at 280 nm. Mass spectrometry (MS) ionisation at negative mode was operated in a negative ion mode under the following conditions: desolvation temperature was 550 °C, capillary voltage was 3.5 kV and nebulizer pressure was 44 psi. The mass spectra were recorded in the *m/z* range of 100–2 000.

2.7 HPLC-quadrupole time-of-flight (QTOF)-MS/MS analysis

HPLC-QTOF-MS/MS of BQPs and DKPs were analyzed by HPLC system (Waters Co., Milford, USA) equipped with triple TOF 5600+ system (AB Sciex Co., Framingham, USA). Chromatographic separations of BQPs and DKPs were performed on a Luna hydrophilic interaction liquid chromatography (HILIC) column (250 mm × 4.6 mm, 5 μm, Phenomenex Technologies Co., Ltd., Torrance, USA). The elution utilized a binary gradient with mobile phases containing 0.5 g/100 mL acetic acid-acetonitrile (A) and water (B): 0–30 min, 10%–27% B linear; 30–40 min, 27%–80% B linear; 40–42 min, 80%–10% B linear; and 42–50 min, 10% B linear. The flow rate was 1 mL/min and eluting peaks were monitored at 280 nm. The 10 μL of sample solutions filtered with a 0.22 μm syringe filter were injected and separated at 30 °C. MS ionisation at negative mode was operated in a negative ion mode under the following conditions: desolvation temperature was 550 °C, capillary voltage was 3.5 kV and nebulizer pressure was 44 psi. The mass spectra were recorded in the *m/z* range of 100–2 000.

2.8 ¹³C nuclear magnetic resonance (NMR) analysis

The 30 mg of BQPs or DKPs were deuterium-exchanged through dissolving in 0.5 mL D₂O (99.8% D) and lyophilized for three times. ¹³C NMR spectra of BQPs and DKPs were recorded on a Avance III 600 MHz spectrometer (Bruker Group, Fällanden, Switzerland) at 25 °C.

2.9 Inhibition of α-glucosidase activity in vitro

Inhibition assay for α-glucosidase activity by BQPs and DKPs *in vitro* were carried out according to the method of Kim et al.^[14] with slight modifications. Sample solutions at various concentrations (0.04 to 1 mg/mL in 0.2 mol/L PBS, pH 6.9) were premixed with α-glucosidase (14.5 units/mg solid in 0.2 mol/L PBS, pH 6.9), and pNPG (1 mmol/L in 0.2 mol/L PBS, pH 6.9) as a substrate was added to start the reaction. The reaction was carried out at 37 °C for 30 min and terminated by addition of 0.2 mol/L Na₂CO₃. α-Glucosidase activity was determined by measuring absorbance at 405 nm after cooling to room temperature. Acarbose and PBS (0.2 mol/L, pH 6.9) were used

as positive and negative control, respectively. The inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{B - B_{\text{blank}}}{A - A_{\text{blank}}}\right) \times 100 \quad (1)$$

Where, *A* and *B* represent the absorbance before and after adding the inhibitor, and *A*_{blank} and *B*_{blank} represent the absorbance of the corresponding control.

2.10 Kinetics of inhibition against α-glucosidase activity

Inhibition modes of BQPs and DKPs against α-glucosidase were determined with rising concentrations of pNPG (0.5 to 3.0 mmol/L in 0.2 mol/L PBS, pH 6.9) as a substrate in absence and presence of sample solutions at various concentrations (0.08, 0.1, and 0.12 mg/mL in 0.2 mol/L PBS, pH 6.9). The inhibition modes of BQPs and DKPs against α-glucosidase were analyzed by Lineweaver-Burk plot (1/reaction velocity vs. 1/substrate concentration).

2.11 Determination of fluorescence spectra

Fluorescence spectra were measured using fluorescence spectrophotometer (Cary Eclipse, Varian Co., Ltd., Palo Alto, USA) as described by previous studies^[15]. α-Glucosidase (2 units/mg solid in 0.2 mol/L PBS, pH 6.9) was premixed with sample solutions at various concentrations (0 to 220 μg/mL in 0.2 mol/L PBS, pH 6.9) and then incubated at 298.15, 304.15, and 310.15 K for 5 min before analysis. The fluorescence spectroscopy of PBS was subtracted as background. The excitation wavelength was set at 280 nm and the emission wavelength was set in a range of 300–400 nm with a 5-nm slit width. The mechanism of fluorescence quenching was confirmed by Stern-Volmer equation and its variant form:

$$\frac{F_0}{F} = 1 + K_{sv} [Q] \quad (2)$$

$$\lg \left(\frac{F_0 - F}{F} \right) = \lg K_a + n \lg [Q] \quad (3)$$

Where, *F* and *F*₀ are fluorescence intensity before and after adding the inhibitor, *K*_{sv} is fluorescence quenching constant (L/g), *n* is the number of binding sites, [*Q*] is the concentration of the inhibitor (mg/mL), and *K*_a is binding constant (L/g).

Thermodynamic parameters were calculated from the following equation:

$$\lg K_a = -\frac{\Delta H}{2.303RT} + \frac{\Delta S}{2.303R} \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

Where, Δ*H* is enthalpy change (kJ/g), Δ*S* is entropy change (J/(g·K)), *R* is gas constant (8.314 J/(mol·K)), *T* is incubated temperature (K), and Δ*G* is Gibbs free energy change (kJ/g).

2.12 Determination of circular dichroism (CD) spectra

CD spectra were measured using J-1500-150ST CD spectropolarimeter (Jasco Group, Tokyo, Japan). The 2.5 mg/mL of α-glucosidase dissolved in 0.1 mol/L PBS, pH 6.9) was premixed

with sample solutions at various concentrations (0 to 220 $\mu\text{g/mL}$ in 0.2 mol/L PBS, pH 6.9) and then incubated at 298.15 K for 5 min before analysis. The CD spectra of sample solutions were subtracted as background. The contents of secondary structure were calculated by an online SELCON3 program (<http://dicroweb.cryst.bbk.ac.uk/html/home.shtml>).

2.13 Oral sucrose tolerance test in normal mice

Six-week-old male ICR mice from Zhejiang Academy of Medical Sciences (Hangzhou, China) were used and housed in a 12-h light-dark cycle, a temperature of $(21 \pm 2)^\circ\text{C}$ and a relative humidity of $(50 \pm 10)\%$. They were maintained on a standard rodent chow diet and water ad libitum for one-week acclimatization. After depriving of food for 16 h with free access to water, mice were assigned to four groups based on fasting blood glucose: in group 1, mice were administrated with water; in group 2, mice were administrated with 200 mg/kg of acarbose; in group 3, mice were administrated with 200 mg/kg of DKPs; in group 4, mice were administrated with 400 mg/kg of DKPs. After 15 min, mice in all groups were administrated with 2 g/kg of sucrose. Blood glucose levels were measured at 0, 15, 30, 60, 90 and 120 min after administration of sucrose using an Accu-Check Performa portable kit (Roche Diagnostics Korea Co., Ltd., Seoul, Korea). Animal care policies and protocols used in the experiments were approved by Institutional Animal Care and Use Committee (IACUC), Laboratory Animal Center, Zhejiang University (ZJCLA) (Approval No. ZJCLA-IACUC-20020130).

2.14 Oral glucose tolerance test in high fat diet-induced type II diabetic mice

Four-week-old male C57BL/6J mice from Zhejiang Academy of Medical Sciences (Hangzhou, China) were maintained on a 12 h light-12 h dark cycle with a temperature of $(21 \pm 2)^\circ\text{C}$ and a relative humidity of $(50 \pm 10)\%$. They had a standard rodent chow diet and water ad libitum. After one-week of acclimatization, mice were fed with a high-fat diet (60% calories from fat; Xietong Pharmaceutical Bioengineering Co., Ltd., Nanjing, China). After 12 weeks, mice with a fasting blood glucose concentration ranging from 11.0 to 20.0 mmol/L were considered to have type II diabetes and were randomly assigned to two groups (6 mice in each group): model group, mice were administrated with water; treatment group, mice were administrated with 400 mg/kg of DKPs. Mice were administrated daily for 4 weeks. Mice fasted for 12-h were administrated with 2 g/kg of glucose. Blood glucose levels were measured at 0, 15, 30, 60, 90 and 120 min after administration of glucose using an Accu-Check Performa portable kit (Roche Diagnostics Korea Co., Ltd., Seoul, Korea). Animal care policies and protocols used in the experiments were approved by IACUC, ZJCLA (Approval No. ZJCLA-IACUC-20020130).

2.15 Statistical analysis

All experiments in this study were performed in triplicate and data obtained were analyzed by GraphPad Prism for Windows (version 7.0). The statistical significance ($P < 0.05$) comparing data was evaluated

by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison.

3. Results and discussion

3.1 Analysis of extraction and purification of BFPs

The crude proanthocyanidins extracted from Chinese bayberry fruits of "Biqi" and "Dongkui" cultivars were purified by HPD500 macroporous adsorption resin and the yields were 1.83% and 1.43%. In order to remove low-molecule-weight flavonoids, they were further purified by Sephadex LH-20, which lead to yields of 0.55% and 0.43%. The UV-visible spectra of BQPs and DKPs were shown in Fig. S1A. The general shapes of the spectra were similar and maximum absorption peak appeared at about 280 nm, which indicated that the extracts were all proanthocyanidins with the same structure. The results of vanillin- H_2SO_4 assay showed that the content of BFPs of "Biqi" and "Dongkui" cultivars were (221.73 ± 0.60) and (136.82 ± 4.33) mg EC equivalents/g DW, respectively. Hence, Chinese bayberry fruits are good resource of proanthocyanidins.

3.2 Analysis of FTIR spectra

As shown in Fig. 1A, BQPs and DKPs were basically the same in FTIR spectra, showing that they have similar functional groups. The wide absorption band at approximately 3388 cm^{-1} was attributed to the stretching vibrations of O-H in phenols^[16]. The presence of band at 1710 cm^{-1} representing C=O stretching indicated BQPs and DKPs contained galloyl groups and the strong band at 1610 cm^{-1} was assigned to ortho O-H stretching of gallic acid^[17]. The absorption bands at 1532 and 1445 cm^{-1} corresponded to aromatic C=C stretching and presented for galocatechin-based compounds^[18]. The absorption bands observed at 1340 and 1040 cm^{-1} originated from C-O asymmetric stretching^[19]. The presence of pyran C rings was confirmed by the C=O aliphatic stretching band at 1220 cm^{-1} ^[20]. The apparent band at 1110 cm^{-1} was assigned to C-H deformation of phenylene rings^[21]. Moreover, two bands at about 830 and 800 cm^{-1} demonstrated the epicatechin subunits of BQPs and DKPs^[18].

3.3 Analysis of cleavage products from acid-catalyzed depolymerization of BQPs and DKPs

Under acid conditions, interflavonoid bonds of proanthocyanidins are cleaved to release terminal subunits as monomeric flavan-3-ols and extension subunits that can trap nucleophilic reagents, such as phloroglucinol used here^[22]. Analyzing phloroglucinol adducts by HPLC-QTOF-MS/MS can identify subunit composition and mDP of proanthocyanidins. Heterocyclic ring fission (HRF), retro-Diels-Alder (RDA) fragmentation, quinone methide (QM) fragmentation and benzofuran formation (BFF) have been identified as characteristic fragmentation routes of flavan-3-ols and their phloroglucinol adducts^[23].

As shown in Fig. 1B and Table 1, four monomeric flavan-3-ols and their corresponding phloroglucinol adducts from BQPs following acid-catalyzed cleavage were identified. Peaks at 3.23 and 6.48 min assigned as ascorbic acid and phloroglucinol produced the molecular ions $[\text{M}-\text{H}]^-$ at m/z 175.03 and 125.03, respectively.

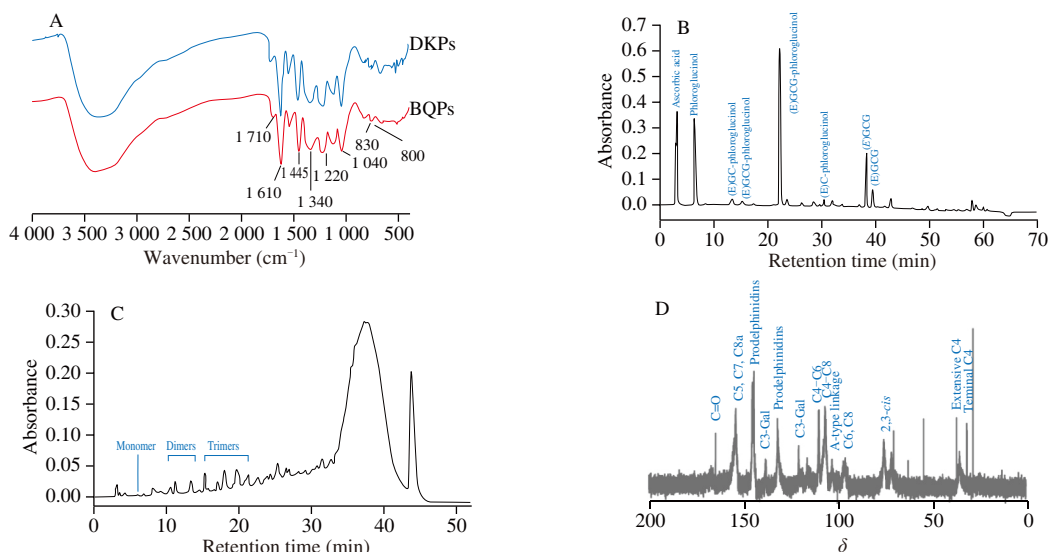


Fig. 1 Structural characterization of BQPs and DKPs. (A) FTIR spectra of BQPs and DKPs; (B) HPLC spectra of BQPs reacting with phloroglucinol using C_{18} column; (C) HPLC spectra of BQPs using HILIC column; (D) ^{13}C NMR spectra of BQPs.

Table 1

Flavan-3-ol monomers of BQPs and their corresponding phloroglucinol adducts analysed by HPLC-QTOF-MS/MS.

Retention time (min)	Molecular formula	$[M-H]^-$ (m/z) (observed)	$[M-H]^-$ (m/z) (calculated)	Fragments (m/z)	Remark
3.23	$C_6H_8O_6$	175.03	175.13	88.02	Ascorbic acid
6.48	$C_6H_6O_3$	125.03	125.11	65.01	Phloroglucinol
13.51	$C_{21}H_{18}O_{10}$	429.08	429.37	305.06, 263.04, 177.02, 125.02	(E)GC-phloroglucinol
15.38	$C_{28}H_{22}O_{14}$	581.09	581.47	455.08, 285.04, 261.05, 177.01, 143.06	(E)GCG-phloroglucinol
22.17	$C_{28}H_{22}O_{14}$	581.09	581.47	287.05, 243.07, 177.06, 161.02, 125.02, 57.04	(E)GCG-phloroglucinol
24.51	$C_{15}H_{14}O_7$	305.07	305.27	220.85, 184.88, 169.01, 124.03	(E)GC
25.94	$C_{15}H_{14}O_6$	289.04	289.28	218.06, 163.00, 125.04, 57.05	(E)C
29.49	$C_{21}H_{18}O_{10}$	429.08	429.37	285.04, 261.04, 217.05, 175.04, 125.02, 83.02	(E)GC-phloroglucinol
30.42	$C_{21}H_{18}O_9$	413.09	413.37	395.08, 245.05, 167.03, 125.02	(E)C-phloroglucinol
31.89	$C_{28}H_{22}O_{13}$	565.10	565.47	413.09, 287.06, 269.05, 217.05, 169.01, 161.02, 125.02	(E)CG-phloroglucinol
37.14	$C_{21}H_{18}O_9$	413.09	413.37	283.03, 261.04, 161.02, 125.03	(E)C-phloroglucinol
38.32	$C_{22}H_{18}O_{11}$	457.08	457.38	304.07, 257.05, 243.03, 199.04, 171.04, 125.02, 79.02	(E)GCG
39.39	$C_{22}H_{18}O_{11}$	457.08	457.38	305.07, 169.01, 125.02	(E)GCG
47.02	$C_{22}H_{18}O_{10}$	441.08	441.38	331.06, 289.07, 245.09, 175.08, 169.01, 125.02	(E)CG

Note: (E)GC: (epi)gallocatechin; (E)GCG: (epi)gallocatechin gallate; (E)C: (epi)catechin; (E)CG: (epi)catechin gallate.

Peak at 22.17 min with the highest response value was identified as (E)GCG phloroglucinol adducts generating the molecular ions $[M-H]^-$ at m/z 581.09 and fragment ions at m/z 287.05 by QM. Peak at 15.38 min was identified as (E)GCG phloroglucinol adducts with similar molecular ions and fragment ions. Peak at 13.51 min was assigned as (E)GC phloroglucinol adducts producing the molecular ions $[M-H]^-$ at m/z 429.08. It generated fragment ions at m/z 263.04 by QM, and m/z 177.02 and 125.02 by HRF. Peak at 29.49 min was also assigned as (E)GC phloroglucinol adducts with similar molecular ions and fragment ions. Peak at 31.89 min detected as (E)CG phloroglucinol adducts produced the molecular ions $[M-H]^-$ at m/z 565.10. It produced fragment ions at m/z 413.09 by RDA, m/z 287.06 by QM, and m/z 169.01 and 125.02 corresponding to deprotonated gallic acid and decarboxylated gallic acid moiety, respectively. Peak at 30.42 min detected as (E)C phloroglucinol adducts produced the molecular ions $[M-H]^-$ at m/z 413.09 and fragment ions at m/z 395.08 by the loss of water molecule, m/z 245.05 and 167.03 by RDA, and m/z 125.05 corresponding to decarboxylated gallic acid moiety. Peak at 37.14 min was also

assigned as (E)C phloroglucinol adducts with similar molecular ions and fragment ions. Peaks at 24.51, 25.94, 38.32, 39.39, and 47.02 min were identified as (E)GC, (E)C, (E)GCG, (E)GCG, and (E)CG, respectively on the basis of above analysis. Hence, the most extension and terminal units of BQPs were (E)GCG along with a small portion of (E)C, (E)CG and (E)GC. The similar results of cleavage products from acid-catalyzed depolymerization of DKPs analyzed by HPLC-QTOF-MS/MS were shown in Fig. S1B. What is more, the mDP of BQPs and DKPs were 4.25 and 4.08, respectively. This is significantly different from BLPs, which were composed of EGCG and EGC as subunits with mDP of 6.30^[8].

3.4 Analysis of BQPs and DKPs by HPLC-QTOF-MS/MS

Oligomeric BQPs and DKPs can be separated according to degree of polymerization (DP) by HILIC column. Based on retention time and MS/MS results, 8 compounds with DP ranging from 1 to 3 were identified in BQPs (Fig. 1C and Table 2). Peak at 6.06 min detected as (E)GCG produced the

molecular ions $[M-H]^-$ at m/z 457.08 and fragment ions at m/z 303.04 by QM, and m/z 167.03 and 140.05 by RDA. Peak at 11.42 min was assigned as (E)GC-(E)GCG producing the molecular ions $[M-H]^-$ at m/z 761.14. It generated fragment ions at m/z 289.07 by QM, and m/z 169.01 and 125.03 by HRF. Peak at 13.44 min was identified as (E)GCG dimer generating the molecular ions $[M-H]^-$ at m/z 913.15 and fragment ions at m/z 423.08 by RDA, m/z 305.07 by QM and m/z 287.06 by QM. Peak at 15.35 min was assigned as (E)GCG-(E)GC-(E)C producing the molecular ions $[M-H]^-$ at m/z 1049.20. It generated fragment ions at m/z 921.18 by HRF, and m/z 607.12 by RDA. Peak at 18.05 min detected as (E)GC-(E)GCG-(E)GC produced the molecular ions $[M-H]^-$ at m/z 1065.19. It produced fragment ions at m/z 647.12 and 405.08 by RDA. Peaks at 21.31 and 22.75 min producing the molecular ions $[M-H]^-$ at 1369.21 and 1353.25 were identified as (E)GCG trimer and (E)GCG-(E)CG-(E)GCG, respectively. Therefore, the constituent units of BQPs were (E)GCG, (E)GC, (E)CG and (E)C, which was consistent with the results of acid-catalyzed depolymerization. Due to the diversity of constituent units and the existence of isomers, BQPs with DP over 3 were difficult to separate. The similar structure of DKPs were analyzed by HPLC-QTOF-MS/MS and shown in Fig. S1C.

3.5 Analysis of BQPs and DKPs by NMR

^{13}C NMR spectrum used to in further characterize BQPs was shown in Fig. 1D. Resonances between δ 153 to 156 was assigned to C5, C7 and C8a in A-ring of the flavan-3-ol units, while the resonance at δ 96.7 represented unsubstituted C6 and C8 of A-ring^[24]. The signals at δ 106 and 110 were attributed to C4–C8 and C4–C6 interflavanoid linkages^[25]. Furthermore, the proportion of C4–C8 linkages in BQPs was relatively high according to the intensity of their bands. The presence of prodelphinidin units and procyanidin units was confirmed by the resonance near δ 145. The strong signal at δ 131 also confirmed the existence of large amount of prodelphinidin units. The resonance at δ 167 corresponded to carbonyl carbon, which also had chemical shift at δ 120 and 138^[26]. These results indicated that BQPs contained 3-*O*-gallate. The stereochemical configurations of 2,3-*cis* and 2,3-*trans* can be determinate by the resonances at δ 76 and 82, respectively^[27]. Obviously, 2,3-*cis* configuration in BQPs was more abundant and 2,3-*trans* configuration was rare. Extensive and terminal C4 exhibited resonances at δ 36 and 30, respectively^[28]. A small portion of A-type linkages with resonances between δ 102 to 104 was also detected

in BQPs^[29]. But, subunits in BLPs were only connected by B-type linkages^[30]. Hence, the most extensive and terminal units of BQPs were EGCG and contained a small amount of EGC, ECG and EC, which were consistent with above results of FTIR and HPLC-QTOF-MS/MS. ^{13}C NMR spectrum of DKPs was similar with that of BQPs and shown in Fig. S1D.

3.6 Inhibitory effect of BQPs and DKPs against α -glucosidase in vitro

Control of postprandial hyperglycemia is crucial to prevent and treat diabetic mellitus and its complications. α -Glucosidase located in the brush border of the small intestinal mucosa are closely related to carbohydrate absorption, and inhibiting their activity is a significant measure to improve postprandial hyperglycemia^[31]. As shown in Fig. 2A, BQPs and DKPs significantly inhibited α -glucosidase with concentration ranging from 0.04 to 1 mg/mL in a concentration-dependent manner, and the maximum inhibition rates reached 86.88% and 86.74%, respectively. What is more, IC_{50} of BQPs (11.91 $\mu\text{g/mL}$) and DKPs (9.47 $\mu\text{g/mL}$) were much lower than that of acarbose (3.20 mg/mL). Surprisingly, IC_{50} of BLPs (37.00 $\mu\text{g/mL}$) we previously reported was higher than that of BQPs and DKPs, which predicted the potential of Chinese bayberry fruits in dietotherapy of diabetic mellitus. Lineweaver-Burk plots showed that BQPs and DKPs are non-competitive inhibitors of α -glucosidase, which means that BQPs and DKPs could combine with α -glucosidase and its substrate into stable ternary complex (Figs. 2B and S2A)^[32]. Hence, BQPs and DKPs were potential α -glucosidase inhibitors.

3.7 Inhibition mechanism of BQPs and DKPs against α -glucosidase in vitro

3.7.1 Fluorescence spectra

It is well known that lysine, tryptophan and phenylalanine residues in α -glucosidase can be used to reflect the change of spatial conformation^[33]. As shown in Fig. 3C, at excitation wavelength of 280 nm, the maximum emission wavelength of α -glucosidase was near 337 nm. Its fluorescent strength weakened as the concentration of BQPs and DKPs increasing, and the largest fluorescence emission wavelength red shift occurred. This was probably due to the interaction between BQPs or DKPs and α -glucosidase, resulting in the quenching of intrinsic fluorescence and the change of spatial

Table 2
Chromatographic separation and analysis of BQPs by HILIC-QTOF-MS/MS.

Retention time (min)	Molecular formula	$[M-H]^-$ (m/z) (observed)	$[M-H]^-$ (m/z) (calculated)	Fagmentions (m/z)	Remark
6.06	$\text{C}_{22}\text{H}_{18}\text{O}_{11}$	457.08	457.38	303.04, 167.03, 140.05	(E)GCG
11.42	$\text{C}_{37}\text{H}_{30}\text{O}_{18}$	761.14	761.63	729.16, 441.09, 407.08, 289.07, 243.03, 169.01, 151.04, 125.03	(E)GC-(E)GCG
13.44	$\text{C}_{44}\text{H}_{34}\text{O}_{22}$	913.15	913.74	423.08, 305.07, 287.06, 193.02, 177.02, 169.01, 137.03, 125.03	(E)GCG dimer
15.35	$\text{C}_{52}\text{H}_{42}\text{O}_{24}$	1049.20	1049.89	921.18, 877.17, 769.16, 725.12, 607.12, 557.11, 523.09, 485.11, 463.07, 323.06, 285.04, 257.04, 177.02, 169.01, 125.03	(E)GCG-(E)GC-(E)C
18.05	$\text{C}_{52}\text{H}_{42}\text{O}_{25}$	1065.19	1065.89	799.14, 647.12, 465.08, 405.08, 299.03, 246.02, 177.02, 169.02, 163.00, 125.05	(E)GC-(E)GCG-(E)GC
21.31	$\text{C}_{66}\text{H}_{50}\text{O}_{33}$	1369.21	1370.10	683.12, 607.11, 571.09, 423.08, 391.05, 319.05, 285.04, 169.02, 125.03	(E)GCG trimer
22.75	$\text{C}_{56}\text{H}_{38}\text{O}_{39}$	1353.25	1353.11	761.15, 752.14, 676.13, 591.12, 423.08, 319.05, 285.04, 243.03, 169.02, 125.03	(E)GCG-(E)CG-(E)GCG

Note: (E)GC, (epi)gallocatechin; (E)GCG, (epi)gallocatechin gallate; (E)C, (epi)catechin; (E)CG, (epi)catechin gallate.

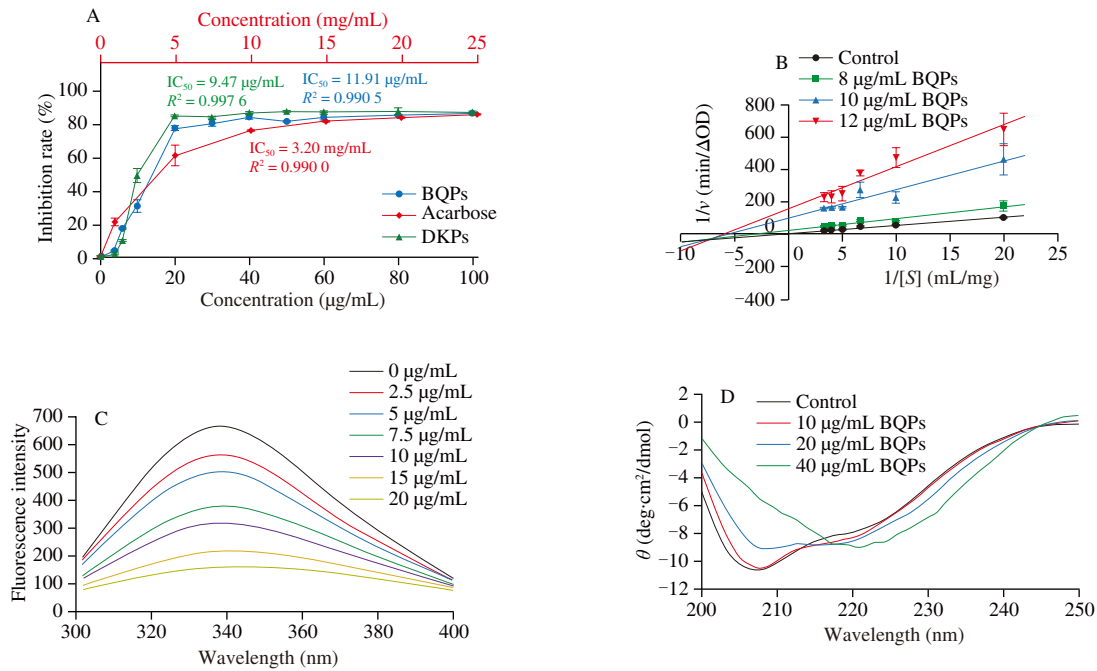


Fig. 2 Inhibition of BQPs against α -glucosidase activity *in vitro*. (A) α -Glucosidase inhibition curve of BQPs; (B) Lineweaver-Burk plots of the inhibition kinetics against α -glucosidase by BQPs; (C) Fluorescence spectra of α -glucosidase with different concentrations of BQPs; (D) CD spectra of α -glucosidase with different concentrations of BQPs. Values are expressed as the mean $\pm$ standard deviation (SD) of three independent experiments.

conformation of α -glucosidase. Similar results of DKPs were shown in Figs. S2B-C. The Stern-Volmer equation was employed to determinate the mechanism of fluorescence quenching including dynamic quenching and static quenching^[34]. As observed in Fig. S3, the slope of Stern-Volmer equation representing K_{SV} gradually decreased with increased temperature, indicating BQPs and DKPs were static quenchers of α -glucosidase.

3.7.2 Fluorescence quenching parameters and thermodynamic parameters

Considering the static quenching effects of BQPs and DKPs on intrinsic fluorescence of α -glucosidase, Stern-Volmer equation can convert to Scatchard equation^[35]. As shown in Table 3, K_a values of BQPs and DKPs were relatively large, indicating their strong affinity with α -glucosidase. However, K_a decreased as the temperature increased, so high temperature was not conducive to the formation of BQPs or DKPs and α -glucosidase complex. n values of BQPs and DKPs were all close to 1, indicating there was only one binding site between BQPs or DKPs and α -glucosidase.

The thermodynamic parameters were calculated to confirm interaction types between BQPs or DKPs and α -glucosidase. ΔG

values of BQPs and DKPs were negative, suggesting that they bound with α -glucosidase spontaneously^[36]. ΔH values of BQPs and DKPs were negative, while ΔS values were positive, indicating that main interaction types between them were hydrogen bonds, hydrophobic interaction and van der Waals forces^[37].

3.7.3 CD spectra

CD spectra was applied to analyze the effects of BQPs and DKPs on the secondary structure of α -glucosidase (Figs. 2D and S2C). α -Glucosidase had characteristic negative absorption peaks at 208 and 222 nm, but addition of BQPs and DKPs could reduce the intensity of absorption peaks, suggesting the change of secondary structure of α -glucosidase. As shown in Table S1, with the concentration of BQPs and DKPs increased from 0 to 40 $\mu\text{g/mL}$, α -helix content of α -glucosidase decreased from 31.0% to 25.2% and 3.8%, respectively, and β -sheet content increased from 1.9% to 6.1% and 15.2%, respectively. Besides, the contents of random coil increased in varying degrees in presence of BQPs and DKPs. Hence, BQPs and DKPs increased the false folding probability of α -glucosidase, making their structure loose and activity decreased.

Table 3

Effects of BQPs and DKPs on the fluorescence quenching parameters and thermodynamic parameters of α -glucosidase.

Prodelphinidins	T (K)	K_{SV} (L/g)	K_a (10^3 L/g)	n	ΔH (kJ/g)	ΔG (kJ/g)	ΔS (J/(g·K))
BQPs	298.15	211.90	5.53	1.83		-21.35	
	304.15	186.10	4.60	1.80	-0.26	-21.78	71.61
	310.15	174.90	3.53	1.77		-22.21	
	298.15	177.60	1.73	1.57		-18.50	
DKPs	304.15	176.00	1.35	1.54	-0.35	-18.87	62.04
	310.15	162.30	1.35	1.57		-19.24	

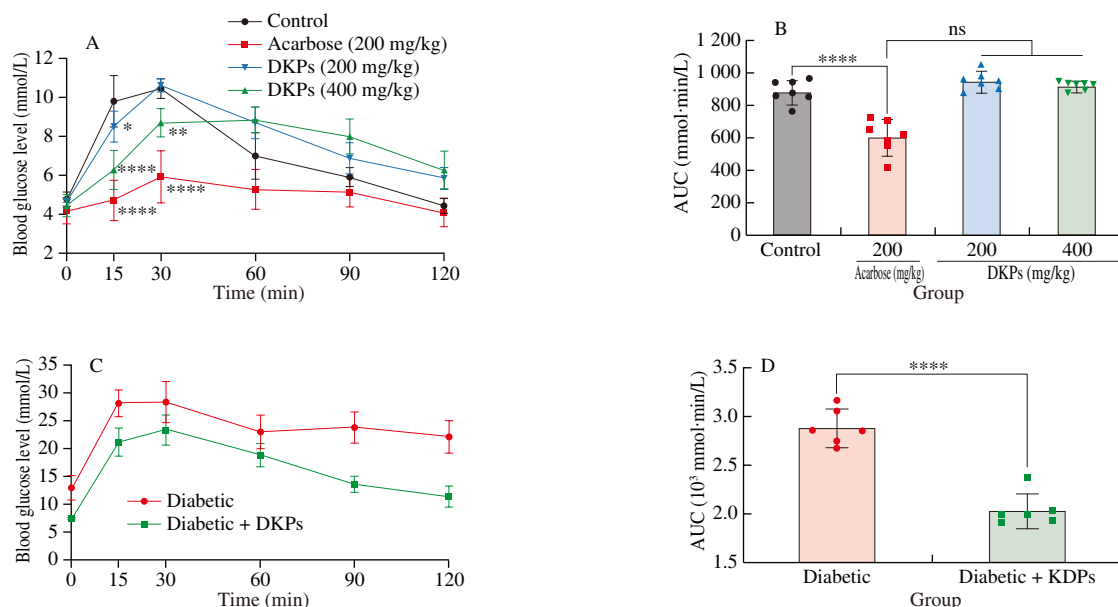


Fig. 3 Effects of DKPs on blood glucose levels in normal ICR mice and high fat diet-induced type II diabetic mice. (A) Blood glucose levels of normal ICR mice after oral administration of acarbose, DKPs and sucrose; (B) Incremental AUC_{0-120 min} of normal ICR mice after administration of acarbose, DKPs, and sucrose; (C) Blood glucose levels of high-fat diet-induced type II diabetic mice after oral administration of DKPs and glucose; (D) Incremental AUC_{0-120 min} of type II diabetic mice after oral administration of DKPs and glucose. Asterisks indicate statistically significant differences from the control group (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; ns, not significant).

3.8 Improve postprandial hyperglycemia in normal ICR mice

To determinate the effects of BQPs with the lowest IC₅₀ on postprandial hyperglycemia *in vivo*, oral sucrose tolerance test was carried out. As shown in Figs. 3A and B, blood glucose concentrations of normal ICR mice increased by 50.81% 15 min after administration of sucrose, while mice administrated DKPs of 200 or 400 mg/kg only increased by 46.31% and 29.16%. DKPs of 400 mg/kg also suppressed blood glucose levels significantly 30 min after administration of sucrose. After that, the blood glucose level tended to be stable and returned to normal at 120 min. The difference between areas under the curve (AUC) after oral administration of DKPs with different doses was not statistically significant, compared with that of control group. The value of DKPs was similar with that of BLPs^[8]. It was noteworthy that acarbose with same dose in regulation of postprandial hyperglycemia was better than that of DKPs, but a large amount of carbohydrates is difficult to be broken down and absorbed in the small intestine, resulting in abdominal distension, diarrhea and other side effects. Fortunately, DKPs may delay but do not hinder the absorption of carbohydrates in small intestine, so it can inhibit the sharp rise of postprandial blood glucose without side effects. However, BFPs have no such effect of delaying digestion we previously reported.

3.9 Improve glucose tolerance in high fat diet-induced type II diabetic mice

In order to explore the therapeutic effect of DKPs on type II diabetes, oral glucose tolerance test in DKPs treated type II diabetic mice was performed. As shown in Figs. 3C and D, administration of DKPs (400 mg/kg) suppressed hyperglycemia in high fat diet-induced type II diabetic mice after glucose loading, and the AUC of DKPs

administrated group reduced significantly by 29.37% compared to that of control group ($P < 0.0001$). Hence, DKPs improved obviously glucose tolerance in high fat diet-induced type II diabetic mice and can be considered as a potential drug for the treatment of type II diabetes.

4. Conclusions

Edible prodelphinidins, which have stronger improvement of postprandial hyperglycemia than BLPs, were first found in Chinese bayberry fruits. Structure and hypoglycemic effect of BFPs and that of difference from BLPs were investigated in this study. Collectively, the content of BPFs of “Biqi” and “Dongkui” cultivars were (221.73 ± 0.60) and (136.82 ± 4.33) mg EC/g DW, respectively. The most extensive and terminal units of them were EGCG along with a small amount of EGC, ECG and EC, which were connected by B-type linkages and a small portion of A-type linkages. The structure of BFPs was obviously different from that of BLPs. Interestingly, BQPs and DKPs were uncompetitive inhibitors of α -glucosidase with IC₅₀ of 11.91 and 9.47 μ g/mL respectively, which were significantly stronger than that of BLPs. They combined with α -glucosidase by hydrogen bonds, hydrophobic interaction and van der Waals forces, leading to loose structure and low activity of α -glucosidase. Oral sucrose tolerance test in normal mice showed that DKPs improved postprandial hyperglycemia in a dose-dependent manner without side effects. Oral glucose tolerance test in high-fat diet-induced type II diabetic mice showed that DKPs were able to be a potential drug for the treatment of type II diabetes. Therefore, BQPs and DKPs are potential substitutes for clinical α -glucosidase inhibitors.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://doi.org/10.26599/FSHW.2024.9250065>.

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