

Cyanidin-3-Glucoside-Rich Extract from Chinese Bayberry Fruit Protects Pancreatic β Cells and Ameliorates Hyperglycemia in Streptozotocin-Induced Diabetic Mice

Chong-De Sun,^{1,*} Bo Zhang,^{2,*} Jiu-Kai Zhang,¹ Chang-Jie Xu,¹ Yu-Lian Wu,²
Xian Li,¹ and Kun-Song Chen¹

¹Laboratory of Fruit Quality Biology/The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Zijingang Campus, Hangzhou, China.

²Department of Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China.

ABSTRACT Chinese bayberry fruit is a rich source of anthocyanins, especially cyanidin-3-glucoside (C3G). The present study investigated the protective effects of C3G-rich bayberry fruit extract (CRBFE) against pancreatic β cells against oxidative stress-induced injury as well as its hypoglycemic effect in diabetic mice. Bayberry extract from “Biqi” was used for both *in vitro* and *in vivo* testing because of its high C3G content and high antioxidant capacity. Pretreatment of β cells with CRBFE (containing 0.5 $\mu\text{mol/L}$ C3G) prevented cell death, increased cellular viability, and decreased mitochondrial reactive oxygen species production and cell necrosis induced by 800 or 1200 $\mu\text{mol/L}$ H_2O_2 . CRBFE dose-dependently up-regulated *pancreatic duodenal homeobox 1* gene expression, contributing to increased *insulin-like growth factor II* gene transcript levels and insulin protein in INS-1 cells. In addition, administration of CRBFE (150 μg of C3G/10 g of body weight twice per day) significantly reduced blood glucose in streptozotocin-induced diabetic ICR mice and increased the glucose tolerance in an oral glucose tolerance test ($P < .05$). Such results indicated that CRBFE might be useful in prevention and control of diabetes mellitus and diabetes-associated complications.

KEY WORDS: • anthocyanins • antioxidants • diabetes • fruit extract • oxidative stress

INTRODUCTION

ANTHOCYANINS ARE THE MOST ABUNDANT and important water-soluble pigments present in fruit and vegetables. They are glycosides and acylglycoside derivatives of six common anthocyanidins—pelargonidin, cyanidin, malvidin, delphinidin, peonidin, and petunidin—which are classified according to the number and position of hydroxyl groups on the flavan nucleus.¹ These anthocyanins endow fruit and vegetables with diverse colors from blue and red to purple. Recent epidemiological studies showed various bioactivities for these colorful compounds such as antioxidant activity,^{2,3} anti-atherogenic activity,⁴ anticarcinogenesis,⁵ prevention of obesity,⁶ prevention of cardiovascular diseases,⁷ and vision improvement.^{8,9} Therefore, a diet rich in anthocyanins is highly recommended for their potential benefit to human health.

Diabetes is an epidemic disease characterized as the inability to produce or improperly use insulin in the human body. There are two types of diabetes: type 1 (insulin-dependent diabetes) and type 2 (non-insulin-dependent diabetes). The cause of diabetes continues to be a mystery, although both genetics and environmental factors appear to play roles. With the development of economic and lifestyle changes the incidence of diabetes and its multiple complications has increased significantly in the past few decades and caused increasing health problems worldwide. The global prevalence of diabetes is estimated to reach 366 million by 2030, and the regions with the greatest potential increases in diabetes are Asia and Africa.¹⁰ So far, there are more than 40 million people in China who have diabetes. Prevention and control programs are urgent to inhibit the rising incidence of diabetes and its complications.

Recently, several studies showed that a diet rich in anthocyanins may decrease the incidence of diabetes, improve glucose disposal, and inhibit the development of diabetes-associated complications. Five anthocyanins (*i.e.*, malvidin-3-glucoside, delphinidin-3-glucoside, cyanidin-3-glucoside [C3G], petunidin-3-glucoside, and peonidin-3-glucoside) from grape skins showed various inhibitory effects on the diabetic cataract.⁸ C3G-rich purple-colored corn was

*The first two authors contributed equally to this work.

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Address correspondence to: Xian Li, Ph.D., Associate Professor, Laboratory of Fruit Quality Biology/The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Zijingang Campus, Hangzhou 310058, China, E-mail: xianli@zju.edu.cn

demonstrated to prevent obesity and ameliorate hyperglycemia in mice fed with high fat diet.⁶ C3G and delphinidin-3-glucoside showed high-level ability to stimulate insulin secretion from rodent pancreatic β cells in the presence of 4 and 10 mmol/L glucose concentrations.¹¹ Furthermore, C3G from purple corn was proved to ameliorate hyperglycemia and insulin sensitivity in type 2 diabetic mice through down-regulation of retinol binding protein 4 expression.¹² Tart cherry, which is rich in anthocyanins, was shown to reduce blood glucose as well as hyperlipidemia, hyperinsulinemia, and metabolic syndrome in Dahl salt-sensitive rats.¹³

Chinese bayberry (*Myrica rubra* Sieb. & Zucc.), a subtropical fruit native to China, is a fruit with high nutrition and health values. The color of the fruit ranges from white to dark red depending on the cultivar and fruit maturity.¹⁴ The characteristic red color of Chinese bayberry is due to the presence of anthocyanins, especially C3G, which accounts for at least 85% of the total anthocyanins in colored fruit cultivars.³ Therefore, the red Chinese bayberry fruit is a rich source of anthocyanins. High antioxidant capacities were reported for Chinese bayberry fruit under different storage conditions.^{3,15–17} The present study was designed to investigate a possible hypoglycemic effect of the C3G-rich bayberry fruit extract (CRBFE) and its possible mechanisms on the health benefit for prevention of diabetic diseases.

MATERIALS AND METHODS

Chemicals and reagents

Standards of nine anthocyanins (*i.e.*, pelargonin, cyanin, malvin, C3G, delphinidin, cyanidin, pelargonidin, peonidin, and malvidin), dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye and streptozotocin (STZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (high-performance liquid chromatography [HPLC] grade) was obtained from Caledon Laboratories Co. (Georgetown, ON, Canada).

Preparation of CRBFE

Mature Chinese bayberry fruit of four cultivars with different colors—"Biqi" (dark red), "Dongkui" (red), "Fenhong" (pink), and "Shuijing" (white)—were obtained from Yuyao County, Zhejiang Province, China.

Anthocyanins from Chinese bayberry fruit were extracted according to our previous method³ with slight modification. The fruit flesh sample was ground and extracted five times (1:5, wt/vol) with solvent consisting of ethanol and 1.0% formic acid. The extract was filtered and centrifuged at 5000 g for 5 minutes. Five milliliters of upper clear solution was evaporated to dryness at 30°C in a rotary evaporator and dissolved in methanol, H₂O, and formic acid (50:45.5:4.5 by volume). After filtration and centrifugation to remove impurities such as protein and sugar, the solution was evaporated to dryness again, dissolved in 1 mL of double distilled water, and prepared for HPLC analysis. CRBFEs used for experiments with pancreatic β cells and diabetic mice were extracted by the same method from "Biqi" with increased volume.

HPLC analysis of anthocyanins in CRBFE

The composition and concentration of anthocyanins from Chinese bayberry fruit were determined using an HPLC apparatus equipped with a model 2695 pump and a model 2996 diode array detector (Waters Corp., Milford, MA, USA). Separation was achieved on a reverse-phase C₃₀ column (250 mm×4.6 mm i.d.; film thickness, 5 μ m), and the detection wavelength was 520 nm. Chromatography was carried out at 30°C with 4.5% formic acid in water as solvent A and 100% HPLC-grade methanol as solvent B. The gradient elution program (% solvent B) with a flow rate of 1 mL/minute was as follows: 1–5 minutes, 15–35%; 5–25 minutes, 35–50%; 25–40 minutes, 50–80%; 40–42 minutes, 80%; 42–45 minutes, 80–15%; and 45–50 minutes, 15% to finish a cycle. The retention time and peak area were used to identify different anthocyanins and calculate their concentrations, respectively.

Antioxidant capacity of CRBFE

The antioxidant capacity of CRBFE of the four cultivars was determined using the peroxy radical scavenging capacity (PSC) assay as described by Adom and Liu.¹⁸ In brief, for preparations of dichlorofluorescein (DCFH) solution, 900 μ L of 1.0 mmol/L KOH was used to hydrolyze 80 μ L of 2.48 mmol/L DCFH diacetate for 3–5 minutes to remove the diacetate moiety, and then 75 mmol/L sodium phosphate buffer (pH 7.4) was used to dilute to a final volume of 6 mL. A Varioskan[®] fluorescent spectrophotometer (Thermo Electron Corp., Vantaa, Finland) was used to monitor the assay as follows: 100 μ L of standards or fruit extract appropriately diluted in 75 mmol/L sodium phosphate buffer (pH 7.4) was transferred into reaction cells on a 96-well plate, and 100 μ L of DCFH was added. The solution in each cell was mixed in the Varioskan spectrophotometer by shaking at 480 rpm for 20 seconds. The reaction was then initiated by adding 50 μ L of freshly prepared 2,2'-azobis(amidinopropane) (400 mmol/L) in 75 mmol/L sodium phosphate buffer (pH 7.4). For control reaction, 75 mmol/L sodium phosphate buffer (pH 7.4) alone was used. The temperature for reaction was 37°C, and the wavelengths of excitation and emission for monitoring fluorescence were 485 nm and 538 nm, respectively. Data were acquired with Skanlt software version 2.2 (Thermo Electron Corp.). The area under the average fluorescence–reaction time kinetic curve (AUC) values for both control and samples were integrated and used as the basis for calculating antioxidant activity according to the following equation: PSC unit = 1 – (SA/CA), where SA is AUC for the sample or standards and CA is AUC for the control. The median effective concentration was defined as the dose required to cause 50% inhibition (PSC unit = 0.5) for each fruit extract and was used as the basis for comparing different samples. AUCs of samples, which were close to 0.5 PSC units, were compared with a standard curve of Trolox concentrations, and the antioxidant capacity was expressed as millimoles of Trolox equivalents per gram.

Cell culture and reagents

The rat insulinoma cell line INS-1 was purchased from the China Center for Type Culture Collection (Wuhan University, Wuhan, China) and grown in monolayer cultures in regular RPMI 1640 medium supplemented with 10 mmol/L HEPES, 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μ mol/L β -mercaptoethanol, 100 IU/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere (5% CO₂, 95% air). Cells at passages 40–60 were used in the following experiments.

Cell viability assay

The protective effect of CRBFE on cell viability was assessed by using MTT dye. In brief, INS-1 cells were seeded in 96-well plates at a density of 8–10 \times 10³ per well, and 12–18 hours later cells were incubated with CRBFE (containing 0.5 μ mol/L C3G) for 24 hours. After careful washing, cells were further treated with 800 or 1200 μ mol/L H₂O₂, the concentrations of which were based on preliminary experiments. Three hours later, the medium was replaced by fresh medium without H₂O₂, and 20 μ L of MTT (dissolved in phosphate-buffered saline, 5 mg/mL) was added to each well and incubated for 4 hours at 37°C to form formazan crystals. The medium was gently removed, and the crystals were dissolved in 150 μ L of dimethyl sulfoxide. Six wells were used for each treatment. Formazan crystals were quantified at 570 nm using a plate reader (model ELx800, Bio-Tek, Winooski, VT, USA). Cellular viability was expressed as the ratio compared with control cells.

Flow cytometry analysis

To assess the effect of CRBFE on oxidative stress-induced injury of pancreatic β cells, flow cytometry was used to quantitatively evaluate necrosis. In brief, cells were incubated with or without CRBFE (containing 0.5 μ mol/L C3G) for 24 hours, and after the medium was discarded, cells were further stimulated with 800 or 1200 μ mol/L H₂O₂ for another 3 hours. Thereafter, cells (including the detached cells) were harvested, washed twice with prechilled phosphate-buffered saline (pH 7.2), and resuspended in 500 μ L of binding buffer. A volume of 9.8 μ L of propidium iodide was then added to the cell suspension and incubated for 15 minutes in the dark at room temperature. Triplicate wells were used for each treatment. Cell necrosis was determined with a flow cytometry system with CXP software (model FC500, Beckman Coulter, Fullerton, CA, USA).

Mitochondrial reactive oxygen species

The intensity of mitochondrial reactive oxygen species (ROS) was analyzed by fluorescence microscopy and flow cytometry. After various treatments, INS-1 cells were washed with Hanks' balanced salt solution containing Ca²⁺ and Mg²⁺ and then incubated with dimethyl sulfoxide-soluble MitoSOX™ Red Superoxide Indicator (catalog number M36008, Invitrogen, Madison, WI, USA) (5 μ mol/L) for 10 minutes at 37°C, protected from light. The cells

were washed three times, mounted, and observed under fluorescence microscopy. The intensity of fluorescence was measured by flow cytometry.

Real-time quantitative polymerase chain reaction

Total RNA from the cells was isolated with TRIzol® reagent (Invitrogen), and the concentration was detected using a spectrophotometer. Total RNA (2.0 μ g) was reverse-transcribed to cDNA in a reaction mixture in a final volume of 20 μ L using a Fermentas (Vilnius, Lithuania) RNA polymerase chain reaction kit (Moloney murine leukemia virus) according to the manufacturer's directions. Gene expression was quantified using the real-time polymerase chain reaction system (Roche [Basel, Switzerland] Light-cycler 2.0) using SYBR® Green dye (TAKARA Bio, Shiga, Japan). The housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase (gapdh)* served as the internal control. The primers were as follows: *pancreatic duodenal homeobox 1 (pdx-1)*, forward 5'-AAACGCCACACACAAGGAGAA-3', reverse 5'-AGACCTGGCGGTTACATG-3'; *insulin-like growth factor II (Ins2)*, forward 5'-ACCCACAAGTGG CACAAGTGGAG-3', reverse 5'-TTCATTGCAGAGGGG TGGACAGG-3'; and *gapdh*, forward 5'-GGTGGACCTC ATGGCCTACAT-3', reverse 5'-GCCTCTCTTGTCTC TCAGTATCCT-3'. Data analysis was performed using the 2^{- $\Delta\Delta$ CT} method normalized to GAPDH mRNA and expressed relative to the control subjects.

Western blot

Total protein levels were measured (BCA method, Pierce Biotech, Rockford, IL, USA), and 20–40 μ g of protein per lane was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with insulin antibody (1:500 dilution) (Santa Cruz Biotech, Santa Cruz, CA, USA) overnight at 4°C. Equal loading of protein between lanes was confirmed by subsequent actin immunoblots (1:5000 dilution) (Sigma-Aldrich). After incubation with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, immunodetection was performed by enhanced chemiluminescence. The protein density was quantified using Bandscan version 5.0 (Glyko, Hayward, CA, USA). The relative ratios of insulin protein to actin were calculated for both control and CRBFE-treated cells, the former of which was set at 1.

Animals and diets

Male ICR mice (weighing 22–25 g) were used and maintained at 23–25°C and 50–60% humidity in the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The mice were fed with standard laboratory diet of the following composition: 20% protein, 4% fat, 57% carbohydrate, 5% crude fiber, 4% minerals, and 10% water. After 3 days of adaptation, STZ dissolved in citrate buffer (10 mmol/L, pH 4.5) was injected intraperitoneally at a dose of 200 mg/kg of body weight after 6 hours of fasting. Five

days after the injection, the glucose level of serum sampled from the tail vein was determined, and the mice with a serum glucose level higher than 16.7 mmol/L were selected as diabetic mice. The mice were then randomized into four groups (I, II, III, and IV) of seven mice each: Group I, control normal mice; Group II, normal mice treated with CRBFE; Group III, control diabetic mice; and Group IV, diabetic mice treated with CRBFE. Control mice (both Groups I and III) received saline (0.9% NaCl) via oral gavage only, whereas Group II and IV were treated with 0.1 mL of CRBFE (containing 150 μ g of C3G)/10 g of body weight twice per day. Glucose level was monitored for the four groups on the initial day and days 7, 15, and 30 thereafter. All experiments were carried out in accordance with the ethical guidelines of the Animal Experimentation Committee in College of Medicine, Zhejiang University.

Oral glucose tolerance test

Animals were fasted for 6 hours before the oral glucose tolerance test. Mice were given an oral gavage of 0.1 mL of CRBFE (containing 150 μ g of C3G)/10 g of body weight or equivalent saline, and blood glucose was measured 1 hour later as the baseline level. Then, mice in four groups (five mice each) were orally administered glucose (2.5 g/kg). Blood samples were obtained from the tail vein at 0.5-, 1-, and 2-hour intervals, and blood glucose levels were determined using a blood glucose meter (Roche Molecular Diagnostics, Pleasanton, CA, USA).

Statistical analysis

A completely randomized design was used in the present study. All data are mean \pm SD values. Numbers of mice or cell replicates are indicated in individual tables and figures. Data were subjected to analysis of variance using SAS software (release 8.01, SAS Institute, Inc., Cary, NC, USA). Mean separations for anthocyanin content and antioxidant capacity of the four bayberry cultivars were done by Duncan's new multiple range test, and Pearson's correlation coefficient was calculated among them. Student's *t* test was used to compare the effect of CRBFE treatment on the viability and necrosis of β cells treated with different concentrations of H₂O₂ and on the blood glucose level and oral glucose tolerance of diabetic mice. Differences were considered significant at $P < .05$.

RESULTS

HPLC analysis of anthocyanins in Chinese bayberry fruit

Nine anthocyanin standards were readily detected at 520 nm by diode array detection and had fine separations in the HPLC profile (Fig. 1A). The anthocyanin profile of "Biqi," "Dongkui," and "Fenhong" fruit extracts showed several major peaks in the 3–30-minute region of the chromatogram, and four of them were identified as cyanin, malvin, C3G, and cyanidin, respectively, by comparing their

retention times with those of the authentic standards (Fig. 1B–D). Under the same HPLC condition, only the peak for C3G could be detected in "Shuijing" fruit extract (Fig. 1E). C3G was the major anthocyanin (>95% of total area of all peaks) of all four bayberry fruit extracts, although its content differed among cultivars ($P < .05$) (Table 1).

Antioxidant capacity of CRBFE

The PSC assay showed different antioxidant capacities for CRBFE from the four bayberry cultivars ($P < .05$), with the highest antioxidant capacity detected from the dark-red "Biqi" and the lowest from the white "Shuijing" (Fig. 2). Correlation analysis showed that antioxidant capacity was significantly correlated ($r = 0.995$, $P < .0001$) with the C3G content in the four cultivars and was directly related to fruit color (Fig. 3). Therefore, CRBFE from "Biqi" was used for subsequent *in vitro* and *in vivo* tests.

Effect of CRBFE on viability of INS-1 cells under H₂O₂-induced oxidative stress

We investigated whether CRBFE could protect INS-1 cells against oxidative injury. H₂O₂ was added to the cell culture medium to establish the oxidative stress model, and MTT assays were further carried out for quantitatively evaluating cell viability. The 800 or 1200 μ mol/L H₂O₂ treatment induced obvious cell death, where cells appeared to be round and detached (Fig. 4A), and the cell viability was reduced (Fig. 4B). Preincubation of the cells with CRBFE protected INS-1 cells from H₂O₂-induced oxidative injury (Fig. 4A) and increased the cell viability after a 3-hour treatment with 800 or 1200 μ mol/L H₂O₂ ($P < .05$) (Fig. 4B).

Effect of CRBFE on oxidative stress-induced cell necrosis of INS-1 cells

Treatment of INS-1 cells with 800 or 1200 μ mol/L H₂O₂ caused a significant increase in cell necrosis (Fig. 5A). However, pretreatment of INS-1 cells with CRBFE resulted in decreased cell necrosis in both the 800 and 1200 μ mol/L H₂O₂-treated cells ($P < .05$) (Fig. 5B). Also, CRBFE decreased the cell necrosis in β cells without H₂O₂ treatment ($P < .05$).

Effect of CRBFE on mitochondrial ROS production

MitoSOX Red is a new redox-sensitive dye that is targeted to mitochondria and was used in the present study to evaluate mitochondrial ROS production. Our data revealed that H₂O₂ stimulation significantly enhanced mitochondrial ROS production as evidenced by the increased intensity of red fluorescence in H₂O₂-treated INS-1 cells, whereas CRBFE decreased MitoSOX staining intensity (Fig. 6A). Such results were further confirmed by flow cytometry analysis. CRBFE pretreatment reduced the proportion of cells strongly stained by MitoSOX probe, compared with cells treated with H₂O₂ alone (Fig. 6B).

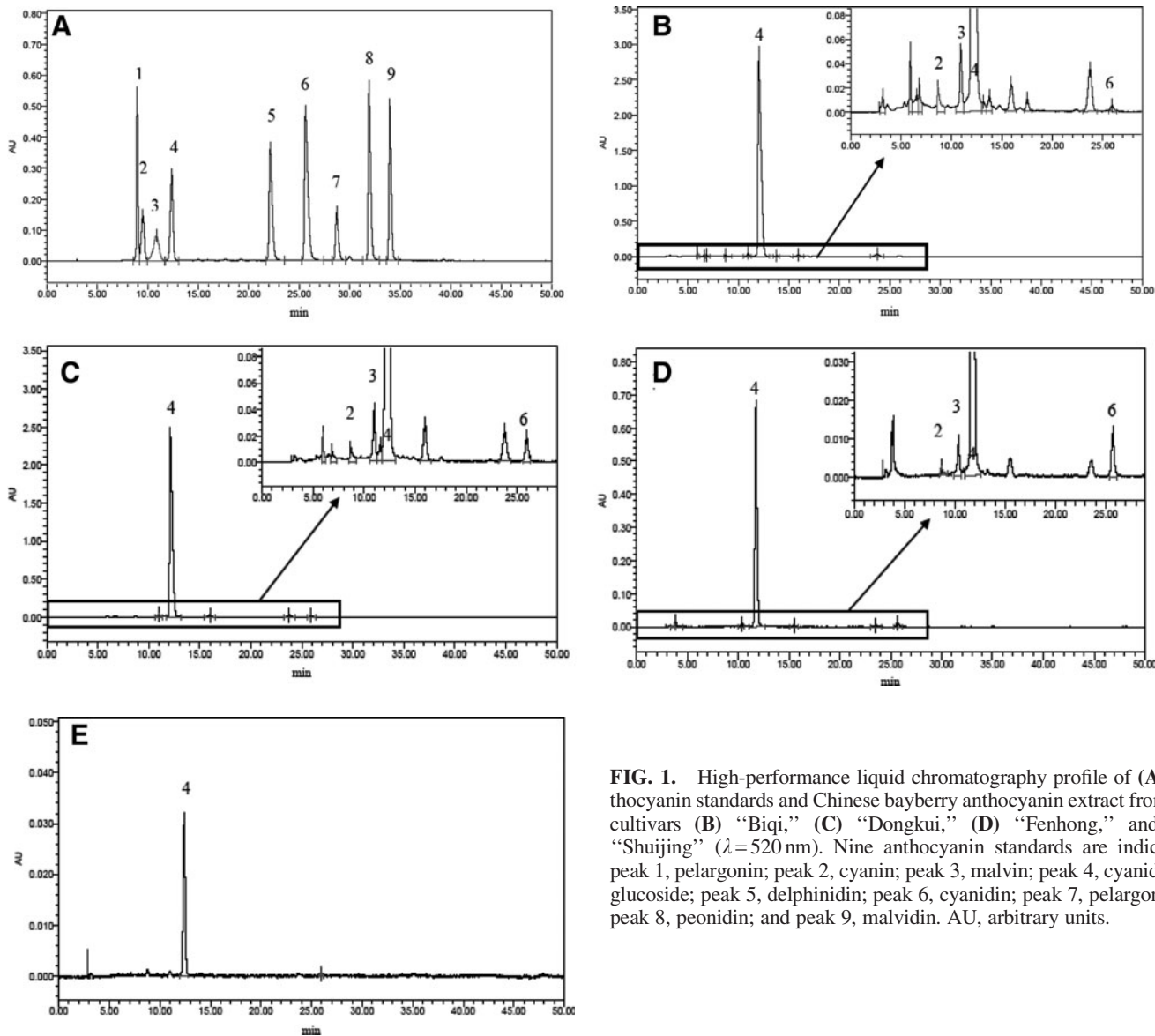


FIG. 1. High-performance liquid chromatography profile of (A) anthocyanin standards and Chinese bayberry anthocyanin extract from the cultivars (B) “Biqi,” (C) “Dongkui,” (D) “Fenhong,” and (E) “Shuijing” ($\lambda=520$ nm). Nine anthocyanin standards are indicated: peak 1, pelargonin; peak 2, cyanin; peak 3, malvin; peak 4, cyanidin-3-glucoside; peak 5, delphinidin; peak 6, cyanidin; peak 7, pelargonidin; peak 8, peonidin; and peak 9, malvidin. AU, arbitrary units.

TABLE 1. CONTENT OF CYANIN, MALVIN, CYANIDIN-3-GLUCOSIDE, AND CYANIDIN IN CHINESE BAYBERRY OF FOUR CULTIVARS

Cultivar	Anthocyanin content ($\mu\text{g/g}$ fresh weight)			
	Cyanin	Malvin	C3G	Cyanidin
“Biqi”	3.4 ± 0.3^a	11.3 ± 1.1^a	467.9 ± 21.3^a	0.4 ± 0.1^c
“Dongkui”	1.7 ± 0.2^b	8.6 ± 0.6^b	344.6 ± 13.3^b	1.4 ± 0.1^a
“Fenhong”	ND	1.9 ± 0.2^c	78.1 ± 5.5^c	0.9 ± 0.1^b
“Shuijing”	ND	ND	3.5 ± 0.3^d	ND

Data are mean \pm SD values ($n=3$).

^{abcd}Means within a column without a common letter differ, $P < .05$.

C3G, cyanidin-3-glucoside; ND, not detected.

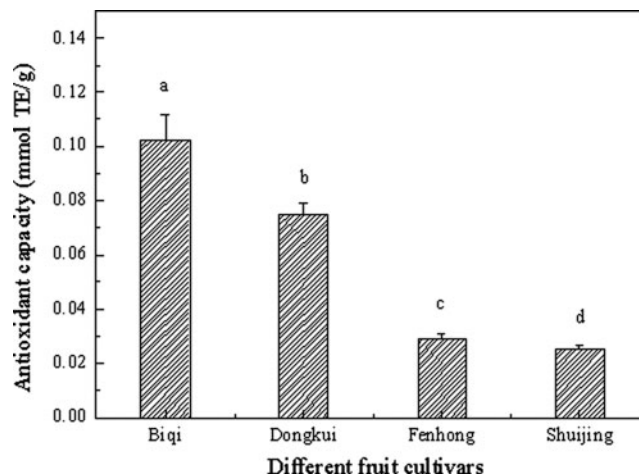


FIG. 2. Antioxidant capacity of C3G-rich extract from four bayberry cultivars. Data are mean \pm SD values ($n=3$). ^{abcd}Means without a common letter differ, $P < .05$. TE, Trolox equivalents.

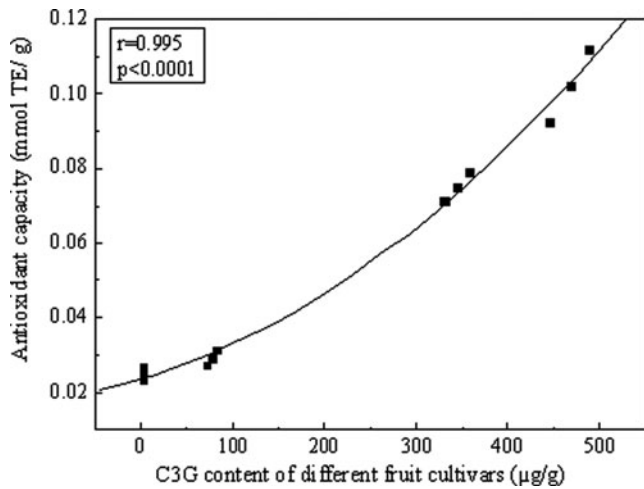


FIG. 3. Correlation analysis of antioxidant capacity and C3G content from four bayberry cultivars.

Effect of CRBFE on *pdx-1* and *Ins2* gene expressions and insulin protein in INS-1 cells

PDX-1 is an important transcription factor for insulin gene transcription, which binds to the A element motif of the insulin genes and contributes to directing their β -cell-specific gene expression. By using different concentrations of

CRBFE, we observed that CRBFE could increase the expression of *pdx-1* gene in INS-1 cells in a dose-dependent manner (Fig. 7A). Also, after 12-hour CRBFE pretreatment of INS-1 cells, increased *Ins2* gene expression ($P < .01$) and increased insulin protein content were observed, compared with those in control cells, as verified by quantitative polymerase chain reaction (Fig. 7B) and western blot (Fig. 7C), respectively.

Effect of CRBFE on body weight, feeding behaviors, and blood glucose levels of mice

Apparent polydipsia and polyphagia symptoms in diabetic mice were observed in our study, and the body weight of mice in all four groups increased slightly during the experiment (30 days). CRBFE administration attenuated the polydipsia and polyphagia symptoms in diabetic mice, although no statistical significance was observed. Overall, the extent of body weight increase was lower in diabetic mice than that of normal control animals ($P < .05$), regardless of whether CRBFE was administered (data not shown). There was no significant difference in body weight between normal Group I and Group II. Also, there was no significant difference in body weight between the two diabetes groups (Group III and Group IV).

Food intake and water consumption of mice in all four groups were investigated for a 24-hour period at the end of

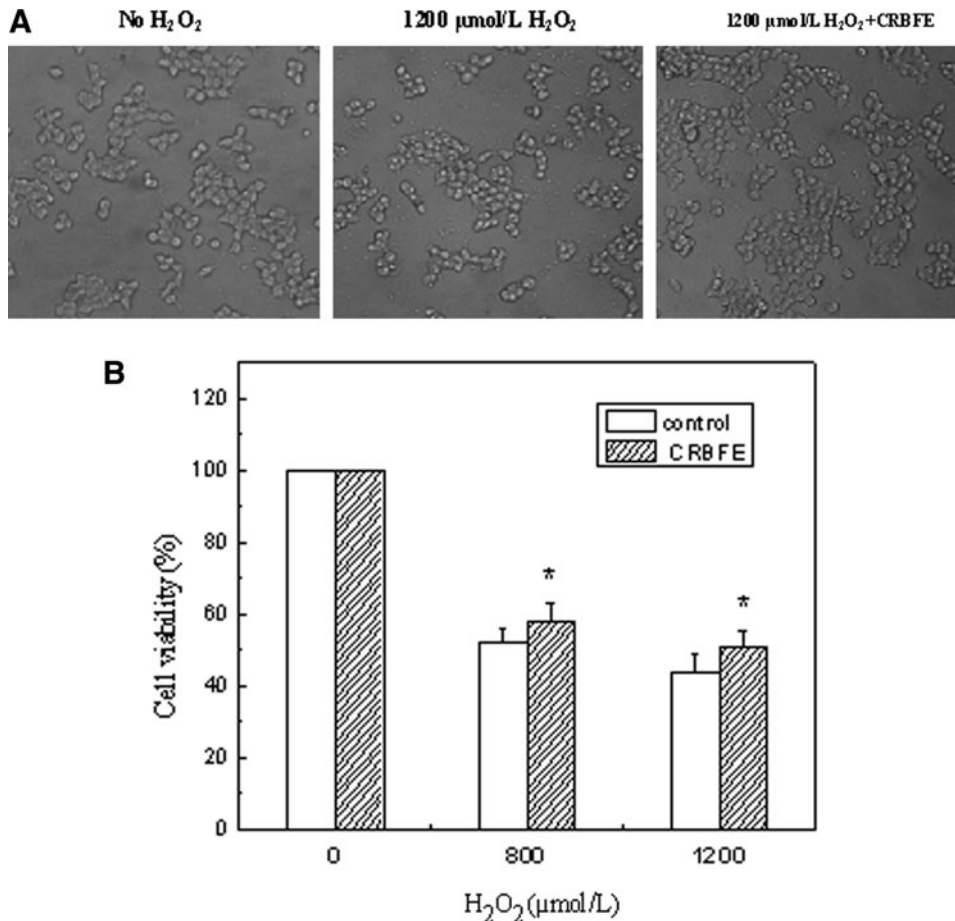


FIG. 4. Effects of C3G-rich bayberry fruit extract (CRBFE) on viability of INS-1 cells under H₂O₂-induced oxidative stress. (A) Representative cell images were obtained with an inverted microscope 3 hours after H₂O₂ treatment. (B) Formazan crystals formed were quantified with a plate reader at 570 nm, and cellular viability was expressed as the ratio of the value for experimental cells versus control cells. Data are mean \pm SD values ($n=6$). *Different from respective control for 800 or 1200 $\mu\text{mol/L}$ H₂O₂ treatment, $P < .05$.

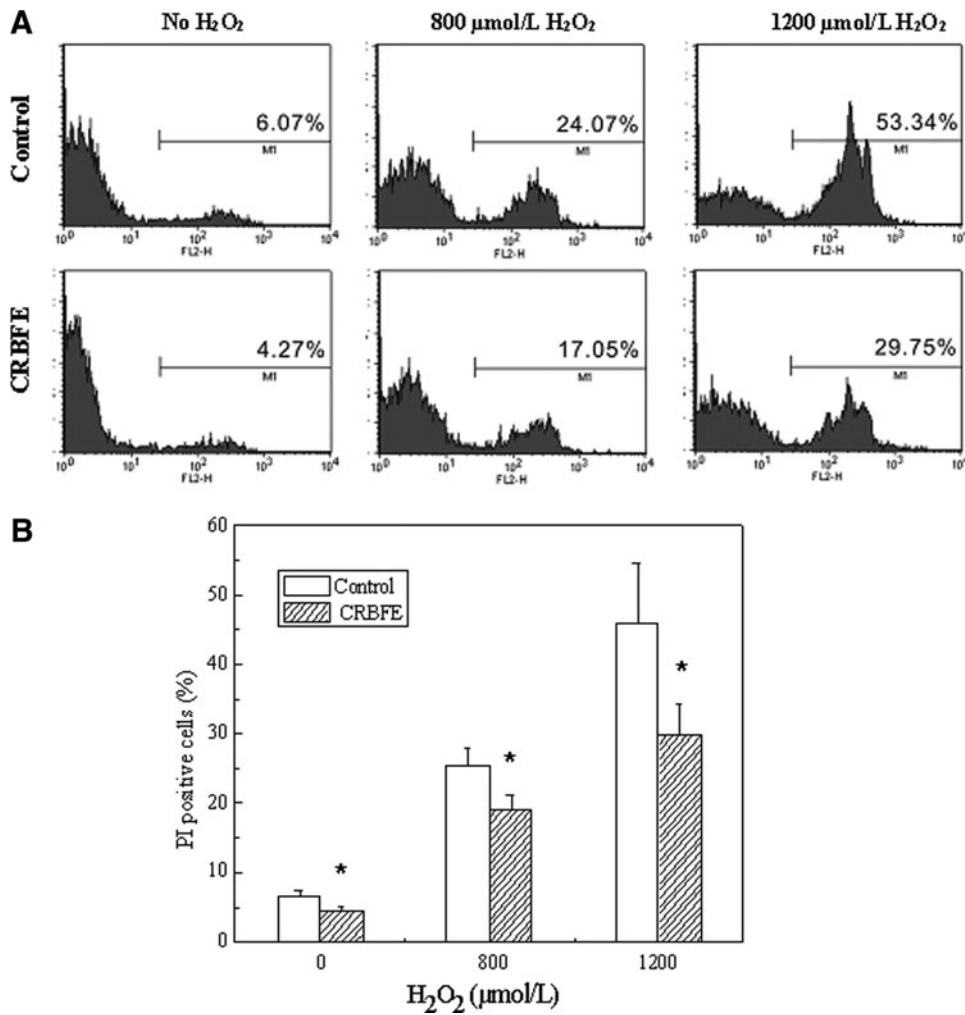


FIG. 5. Effects of CRBFE on oxidative stress-induced cell necrosis of INS-1 cells. **(A)** Flow cytometry quantifying cells with propidium iodide (PI) staining. **(B)** Cell necrosis presented as the percentage of PI-positive cells with the different treatments. Data are mean ± SD values ($n=3$). *Different from respective control for 0, 800, or 1200 μmol/L H₂O₂ treatment, $P < .05$.

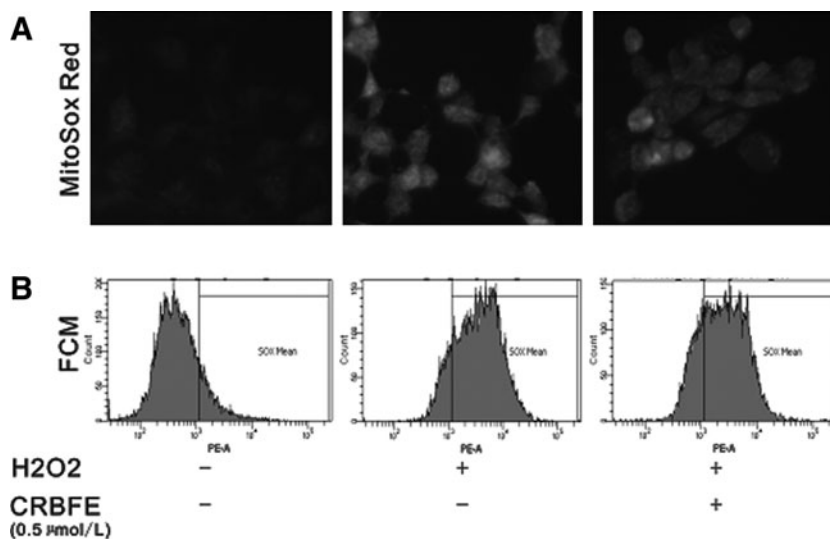


FIG. 6. Effect of CRBFE on mitochondrial reactive oxygen species production detected with the MitoSOX probe. **(A)** Fluorescence microscopy images of INS-1 cells stained with MitoSOX (5 μmol/L): (left) control group, (middle) H₂O₂ group, and (right) H₂O₂+CRBEF group. Changes in the intensity of red fluorescence emitted by MitoSOX in INS-1 cells indicated changes in the mitochondrial reactive oxygen species levels. **(B)** Flow cytometry (FCM) analysis of intracellular MitoSOX Red fluorescence in INS-1 cells.

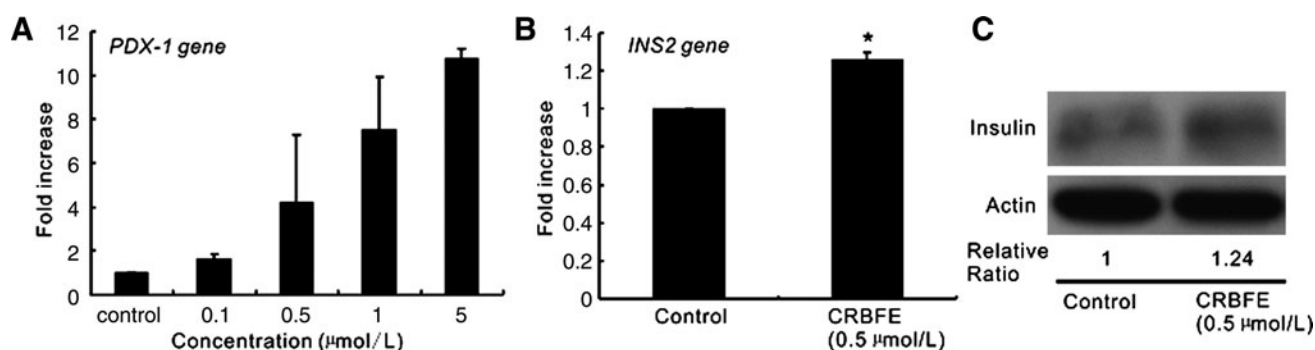


FIG. 7. Effect of CRBFE on *pancreatic duodenal homeobox 1 (pdx-1)* and *insulin-like growth factor II (Ins2)* gene expressions and insulin protein in INS-1 cells. (A) *pdx-1* gene expression assessed by quantitative polymerase chain reaction in INS-1 cells treated with different concentrations of CRBFE. (B) *Ins2* gene expression in INS-1 cells treated with or without CRBFE. (C) Western blot analysis of insulin protein in INS-1 cells treated with or without CRBFE. Protein density was quantified using BandsScan version 5.0 software, and the relative ratios of insulin protein to actin were calculated. In all cases, the control was set at 1.

the experiment. Food intake and water consumption of the two diabetic groups (Group III and Group IV) were higher than those of control mice ($P < .05$) (data not shown). CRBFE administration attenuated the polydipsia and polyphagia symptoms in diabetic mice; however, no statistical significance was observed.

Compared with the initial levels, there was a significant increase in blood glucose levels in STZ-induced diabetic mice within 30 days ($P < .05$) (Table 2). CRBFE from “Biqi” inhibited such increase in diabetic mice, and blood glucose levels decreased 30 days after fruit extract treatment ($P < .05$). Therefore there was a significant difference between the blood glucose levels of the two diabetes groups (Group III and Group IV) on day 30 ($P < .01$). For the normal mice, blood glucose levels were stable during the experimental period, and CRBFE slightly decreased the blood glucose level on day 30 ($P = .053$) (Table 2).

Effect of CRBFE on oral glucose tolerance test

STZ-induced diabetic mice displayed a marked glucose intolerance compared with the normal mice. The blood glucose level remained high at 26.7 ± 5.3 mmol/L at 1 hour

and 15.9 ± 5.3 mmol/L at 2 hours after the oral intake of glucose in diabetic mice, which was an apparent impairment of the glucose tolerance ($P < .05$) (Fig. 8). Administration of CRBFE increased the glucose tolerance in diabetic mice ($P < .05$), and the blood glucose levels were reduced to 16.1 ± 6.0 mmol/L at 1 hour and 8.2 ± 1.8 mmol/L at 2 hours following glucose intake (Fig. 8).

DISCUSSION

Anthocyanins are valuable nutrients because of their various positive effects on human health and marked daily intake.^{1,19} The protective effects of anthocyanins are attributed to their typical chemical structures, which contain

TABLE 2. EFFECTS OF CYANIDIN-3-GLUCOSIDE-RICH BAYBERRY FRUIT EXTRACT ON BLOOD GLUCOSE LEVELS OF DIABETIC AND NORMAL MICE

Group	Blood glucose (mmol/L)			
	Initial	Day 7	Day 15	Day 30
Normal	7.1 ± 1.0	7.0 ± 0.5	6.9 ± 0.3	7.9 ± 0.9
Normal + CRBFE	7.5 ± 0.7	6.9 ± 0.4	6.3 ± 0.7	6.6 ± 0.8
Diabetes	27.7 ± 2.1	29.3 ± 2.9	28.4 ± 2.5	32.6 ± 1.2*
Diabetes + CRBFE	28.1 ± 3.1	29.1 ± 2.2	26.6 ± 2.0	24.3 ± 1.3*†

Data are mean ± SD values ($n = 7$).

*Different compared with the initial level for each treatment, $P < .05$.

†Different compared with the diabetes control group (without CRBFE treatment) on day 30, $P < .0001$.

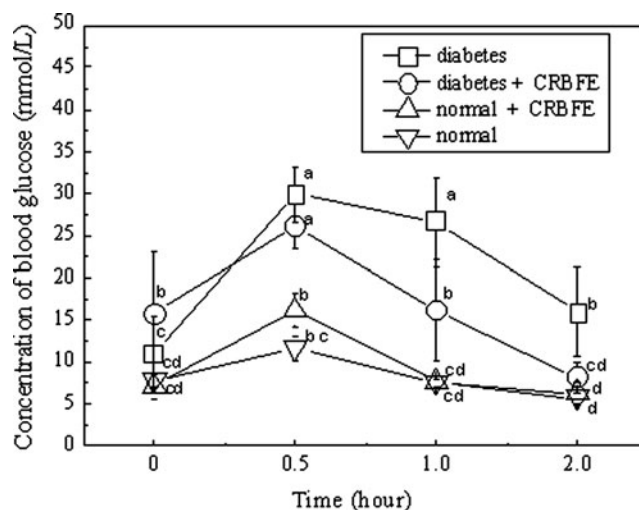


FIG. 8. Effects of CRBFE on the oral glucose tolerance of diabetic and normal mice during an oral glucose tolerance test. The streptozotocin-induced diabetic mice displayed marked glucose intolerance as they maintained a high blood glucose level at 1 hour and 2 hours after the oral intake of glucose (2.5 g/kg). Administration of CRBFE increased the glucose tolerance in diabetic mice. Data are mean ± SD values ($n = 5$). ^{abcd}Means without a common letter differ, $P < .05$.

many hydroxyl groups and various glycosylations and other modifications. The natural electron deficiency of anthocyanins endows these compounds with high reactivity toward ROS, and they are therefore among the most powerful natural antioxidants in dietary plants. Direct interaction of anthocyanins with ROS was demonstrated in different ROS-generating systems by Amorini *et al.*²⁰

C3G alone or together with other cyanidins and their glycosides has been widely investigated for its antioxidant capacities in different experimental conditions.^{2,21} The antioxidant activities of C3G were 3.5 to four times higher than that of Trolox, a vitamin E analog.^{22,23} Also, Sarma *et al.*²⁴ showed higher antioxidant ability for cyanidin-derived anthocyanins than for ascorbic acid due to their ability to chelate metal ions and form an ascorbic acid–metal–anthocyanin complex and thus to prevent oxidation of ascorbic acid. C3G and cyanidin were reported as free radical scavengers, DNA cleavage protectors, and inhibitors of xanthine oxidase.²⁵ C3G from blackberry (*Rubus* species) juice acted as a peroxynitrite scavenger and prevented peroxynitrite-induced endothelial dysfunction and vascular failure in human umbilical vein endothelial cells.²⁶ The cytoprotective effect of C3G against oxidative DNA damage caused by vitamin E deficiency was observed in human clonocytes.²⁷

Oxidative stress is regarded as an important mediator of many cellular dysfunctions. For both type 1 and type 2 diabetes, hyperglycemia associated with diabetes causes oxidative stress due to increased production of endogenous ROS such as O²⁻ and glucose autooxidation.²⁸ These ROS can damage cells by directly oxidizing biological macromolecules such as DNA, proteins, and lipids. On the other hand, ROS can also indirectly attack cells by activating a variety of stress-sensitive intracellular signaling transduction pathways such as mitogen-activated protein kinase.²⁹ In the absence of adequate compensatory response from the endogenous or exogenous antioxidant system against such oxidative stress, numerous stress-related gene products cause further cellular damage in different organs. Eventually, chronic elevations of glucose level may result in dysfunction of pancreatic β cells and development of large-scale diabetes complications.³⁰ Therefore, up-regulation of antioxidant levels and suppression of oxidative stress are important strategies to ameliorate diabetes and its complications.

Recently, the antidiabetic properties of natural products have attracted much attention. The potential therapeutic implications of dietary C3G for prevention and amelioration of diabetic diseases were investigated in C3G-rich dietary food such as colored rice,⁸ purple corn,^{6,12} and tart cherry.¹³ In our study, high antioxidant activity of CRBFE was confirmed by the PSC method. Administration of CRBFE protected pancreatic β cells *in vitro* and attenuated oxidative stress–induced cell necrosis. In addition, we observed that the CRBFE had a hypoglycemic effect in STZ-induced type 1 diabetic mice.

Based on the *in vitro* and *in vivo* data in the present study, we hypothesized that bayberry extract might lower

the blood glucose level through two possible mechanisms. On one hand, CRBFE may protect the pancreatic β cells from oxidative stress, allowing more β cells to survive diabetes-induced oxidative damage. During the onset of STZ-induced diabetes, a significant portion of β cells underwent cellular death (including necrosis, apoptosis, and autophagy), and ROS played a pivotal role.^{31–33} The present results showed that early administration of CRBFE may reduce the cellular ROS level, improve the antioxidant status, and provide a protective microenvironment for pancreatic β cells, thereby facilitating β cell survival under chemical and subsequent hyperglucose-mediated oxidative injury. Such an assumption was consistent with that proposed by Nizamutdinova *et al.*,³⁴ where the rate of apoptotic β cells in diabetic rats was decreased after administration of anthocyanins. On the other hand, the CRBFE may protect the insulin secretion ability of the surviving β cells and therefore prevented the insulin insufficiency that caused the STZ-induced diabetes. Our results showed that CRBFE up-regulated the expression of the insulin transcription factor PDX-1 and increased insulin gene (*Ins2*) expression and insulin protein in β cells. Overall, these effects may lead to enhanced insulin secretion, contributing to the hypoglycemic effect. Studies on *Cornus* fruits extract showed that anthocyanins from this fruit could increase insulin release of INS-1 cells and that C3G was the most effective insulin secretagogue among the nine anthocyanins tested.¹¹ Recently, research showed that administration of black soybean-derived anthocyanins significantly increased serum insulin levels in STZ-induced diabetic rats.³⁴ Also, the insulin-enhancing property of tea polyphenols was demonstrated by Anderson and Polansky.³⁵ The possible role of phenolic compounds other than anthocyanins in CRBFE in protecting β cells remains unknown. Therefore, the mechanisms of the protective effect of CRBFE on ameliorating hyperglycemia in STZ-induced diabetic mice deserve further investigation.

Although plant treatment for diabetes dates back to about 1550 BC as conventional therapy and more than 400 plant treatments for diabetes have been recorded, only a small number of these have been scientifically evaluated for their mechanism.³⁶ In the late 1980s, plant extracts from cerasee,³⁷ xiaoke tea,³⁸ agrimony, alfalfa, coriander, eucalyptus, and juniper³⁹ were all reported to have anti-hyperglycemic effects during the development of diabetes in mice. Recently, various hypoglycemic compounds have been identified, and different possible mechanisms were proposed for different plant extracts that could be preventive and/or curative in diabetic animal models. In the present study, CRBFE showed high antioxidant capacity and a notable protective effect for pancreatic β cells against oxidative stress. In addition, CRBFE significantly reduced the blood glucose level and restored glucose tolerance in STZ-induced diabetic mice. Based on these observations, conclusive studies on the mechanisms and *in vivo* metabolism, absorption, and bioavailability of the CRBFE for preventing and healing of diabetes and its complications are warranted.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interest exists.

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