Supplementary materials

1 Materials and Methods

1.1 Materials, chemical and reagents

Rabbiteye blueberry (*Vaccinium ashei* cv. 'Brightwell') fruits were collected from the orchards in Lishui Town, Jiangsu Province (Nanjing, China), and stored at -18 °C in darkness at the Institute of Agro-Product Processing, Jiangsu Academy of Agricultural Sciences (Nanjing, China). A bicinchoninic acid (BCA)-protein-assay kit and phosphate-buffered saline (PBS 10X) were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China). Total antioxidant capacity (T-AOC), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione-peroxidase (GSH-PX) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). A high-purity total RNA extraction kit was purchased from Proteinssci Biotech Co., Ltd. (Shanghai, China). ChamQ Universal SYBR qPCR Master Mix, HiScript III RT Super Mix for qPCR (+gDNA wiper), and 4×gDNA wiper Mix were bought from Vazyme Biotech Co., Ltd. (Nanjing, China). The primers for SOD (Cu, Zn-SOD and Mn-SOD) and GSH-PX (GPX) were ordered from GenScript Biotech Corporation (Nanjing, China). Methanol, formic acid, anhydrous ethanol, and acetic acid were purchased from Shanghai Ningbo Chemical Reagent Co., Ltd. (Shanghai, China), and sodium chloride from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). All the chemicals and reagents were of analytical grade.

1.2 Preparation of blueberry anthocyanin extract (BAE)

The blueberry anthocyanins were extracted according to our previous method (Huang et al., 2018b). At room temperature, the frozen blueberries were thawed and homogenized. A total of 5,000 g of blueberry pulp was soaked in 10 L of methanol containing a 1% HCl solution for 24 h, which was performed in triplicate. The extract was collected and mixed. After centrifugation at 5000*g* for 15 min, the supernatant was evaporated in vacuo at 40 °C to get the crude anthocyanin extract, which was further purified using an AB-8 macroporous resin (Sigma Aldrich, China). Finally, blueberry anthocyanin extract (BAE) powder with a purity of 78.9% was obtained after freeze-drying (Eyela FDU-1200, Tokyo Rikakikai, Tokyo, Japan). About 10 g of BAE powder was recovered and stored at −18 °C until further analysis.

1.3 Animals and experimental design

A total of 66 healthy male C57BL/6J mice (20±2) g from GemPharmatech (Nanjing, China) were kept in standard laboratory conditions ((25±2) °C, 12-h light/dark alternation) with adequate rodent feed and drinking water. All animal experimental procedures were performed in accordance with the guidelines of the Jiangsu Academy of Agricultural Sciences Subcommittee on Research Animal Care and Use Committee. After one week of adaptation, 63 mice were randomly divided into three groups for intragastric administration of 100 μL of BAE aqueous solutions at different concentrations (100, 400, and 800 mg/kg). Each group of mice was anesthetized and sacrificed at different time points (0.1, 0.5, 1, 2, 4, 8, and 12 h). The control group consisted of three mice administrated with the same volume of solvent (100 μL), anesthetized, and sacrificed after 12 h. The blood was collected from eye samples to get plasma. The tissues of the eyeball, intestine, liver, adipose (mesenteric fat), stomach, kidney, lung, spleen, and thymus were quickly removed and weighed (except the intestine and stomach, because they were washed) after execution. Organ index was calculated by organ tissue weight dividing by mouse body weight and expressed as a percentage. The samples were labeled, frozen quickly with liquid nitrogen and then stored in the freezer at -80 °C for further experiments.

1.4 Anthocyanin detection by high-performance liquid chromatography (HPLC) analysis

The blueberry anthocyanins in tissue samples (e.g., liver) were extracted twice using five times the volume (5 V) of the extracting solvent (5% formic acid dissolved in 95% methanol) using an ultrasonic bath (RH7200DB CNC, Kunshan Ultrasonic Instrument Co. Ltd., Suzhou, China) for three minutes. After centrifugation at 4 °C and 10,000 × g for 5 min, the supernatants were collected and mixed. The mixed blueberry anthocyanin extracts in tissues were purified with C18 Sep-Pak cartridges (Kim and Lee, 2002) and collected with 500 μL of the extraction solvent. The effluent was filtered through a 0.22 μm polyvinylidene fluoride membrane before HPLC analysis. The chromatographic analysis was carried out in an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA). An XDB-C18 column (250 mm × 4.6 mm, 5 μm, Agilent Technologies, USA) was used, with an injection volume of 10 μL. The mobile phases were A: 1% phosphoric acid and B: 100% acetonitrile. The elution gradient was as follows: 5% B (from 0 to 2 min), 5% to 10% B (from 2 to 6 min), 10% B (from 6 to 10 min), 10% to 12% B (from 10 to 14 min), 12% to 15% B (from 14 to 20 min), 15% to 18% B (from 20 to 24 min), 18% to 25% B (from 24 to 32 min), 25%

to 30% B (from 32 to 36 min), 30% to 5% B (from 36 to 36.1 min). The column temperature was 25 °C, the detection wavelength was 520 nm, and a flow rate of 0.6 mL/min was used. The anthocyanin content of liver tissues was compared to blueberry extracts' anthocyanin content.

1.5 Antioxidant activity assay

The tissues from the eyeballs, intestine, liver, and adipose (mesenteric fat) were weighted and homogenized by adding normal saline (10 V of 0.9% NaCl solution) and grinding for 1 min in a JY92-IIN ultrasonic cell crusher (Ningbo Xinzhi Biotechnology Co., Ltd., Ningbo, China). The supernatant was collected after centrifugation at 4 °C and 10,000 × g for 5 min. These homogenized tissue supernatants and the blood plasma were analyzed for total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) for antioxidant activity analysis. The assay procedures were performed according to the kit protocol instructions. An Epoch 2 microplate reader was used to measure the absorbance (BioTek Inc., Vermont, USA). The T-AOC results were expressed as Trolox equivalent antioxidant capacity (TEAC), which is defined as mmol Trolox/g protein for tissues and mmol Trolox/L for plasma. SOD activity was expressed in units per gram of tissue protein or liter of plasma. Similarly, the GSH-PX and MDA contents were expressed as molar number per gram of tissue of protein or liter of plasma. The BCA protein assay kit was used to quantify the total protein in the supernatant.

1.6 Total RNA isolation

Mice eyeballs, intestines, livers, and adipose tissues were thawed and homogenized using an ultrasonic cell crusher (JY92-IIN, Ningbo Xinzhi Biotechnology Co., Ltd., Ningbo, China). Because some of the analyzed tissues were not abundant, the mRNA was extracted from mice treated with different concentrations of BAE at two to six of seven-time points. Total RNA was extracted using the procedure described by the high-purity total RNA extraction kit. Quantitative analysis was performed using a NanoDrop ND-2000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). RNA samples with A260/A280 and A260/A230 ratios of 1.8-2.0 were used only for further analysis. The total RNA was dissolved in RNase/DNase-free water and kept at -80 °C for later use.

1.7 Real-time quantitative reverse transcription-polymerase chain reaction analysis

The total RNA (1 μg) of each sample was reversely transcribed into cDNA with 4×gDNA wiper Mix and HiScript III RT SuperMix for qPCR (+gDNA wiper) in a GE4852T gene amplifier

(Hangzhou Bohang Technology Co., Ltd., Hangzhou, China) and the cDNA was stored at -20 °C until used for qRT-PCR expression assay. The cDNA was amplified according to the instructions of the ChamQ Universal SYBR qPCR Master Mixer. The PCR primers used were listed in Table S1, which also included primer sequencing for these genes. Real-time quantitative reverse transcription PCR (qRT-PCR) analysis was carried out in a 96-well plate with an ABI 7300 StepOneTM Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 3 min, 40 cycles of 55 °C for 30 s and 95 °C for 15 s, and a melting curve from 60 to 95 °C to ensure amplification of a single product. The GAPDH primers were amplified as a corresponding control in all samples to represent the housekeeper gene. The cycle threshold (Ct) values were used to calculate the fold differences using the $2^{-\Delta\Delta Ct}$ method, and all relative gene expression data were expressed as a fold increase over the control group.

1.8 Statistical analysis

All experiments were performed in triplicate, and data were presented as the mean ± standard deviation (SD). Figures were obtained by using GraphPad Prism Version 8 (GraphPad Software, Inc., California, USA). A one-way analysis of variance (ANOVA) was used to compare the means of different treatments with the Tukey test. A two-way ANOVA was used to analyze the interactions among different treatments and different samples. Differences with $P < 0.05$ were considered significant.

References

Huang WY, Wu H, Li DJ, et al., 2018b. Protective effects of blueberry anthocyanins against H_2O_2-induced oxidative injury in human retinal pigment epithelial cells. *J Agric Food Chem*, 66(7):1638-1648.
https://doi.org/10.1021/acs.jafc.7b06135

Kim DO, Lee CY, 2002. Extraction and isolation of polyphenolics. *Current Protocols in Food Analytical Chemistry*, 6(1):I.1.2.1-I.1.2.12.
https://doi.org/10.1002/0471142913.fai0102s06

Table S1 Organ indices of mice treated with blueberry anthocyanin extract (BAE) at different concentrations and treatment times (*n*=3)

Treatment		Organ index (%)						
Time (h)	Concentration (mg/kg)	Eyeball	Liver	Kidney	Lung	Spleen	Thymus	Adipose
0.1	100	0.30±0.06^{a}	9.29±0.32^{b}	1.62±0.18^{a,b}	0.83±0.16^{a}	0.42±0.08^{a,b}	0.18±0.03^{a}	0.86±0.17^{a}
	400	0.28±0.05^{a}	8.56±0.25^{b}	1.27±0.17^{b}	0.90±0.18^{a}	0.48±0.09^{a,b}	0.17±0.03^{a}	0.96±0.19^{a}
	800	0.29±0.06^{a}	7.73±0.28^{b,c}	1.43±0.15^{b}	0.82±0.16^{a}	0.66±0.13^{a,b}	0.24±0.05^{a}	0.74±0.12^{a,b}
0.5	100	0.27±0.03^{a}	9.83±0.31^{a,b}	1.53±0.19^{b}	0.78±0.15^{a}	0.47±0.09^{a,b}	0.18±0.04^{a}	0.61±0.12^{a,b}
	400	0.24±0.04^{a}	9.95±0.74^{a}	1.37±0.08^{b}	0.94±0.13^{a}	0.75±0.15^{a,b}	0.29±0.06^{a}	0.99±0.19^{a}
	800	0.26±0.05^{a}	11.21±1.54^{a}	1.70±0.34^{a,b}	0.95±0.19^{a}	0.61±0.12^{a,b}	0.17±0.04^{a}	1.37±0.27^{a}
1	100	0.28±0.06^{a}	9.07±0.41^{b}	2.05±0.13^{a}	0.85±0.17^{a}	0.54±0.08^{a,b}	0.19±0.03^{a}	0.98±0.17^{a}
	400	0.28±0.03^{a}	9.43±0.38^{a,b}	1.92±0.25^{a}	0.82±0.16^{a}	1.32±0.26^{a}	0.22±0.09^{a}	0.74±0.15^{a}
	800	0.23±0.04^{a}	6.54±0.28^{c}	1.41±0.15^{b}	0.68±0.16^{a}	0.36±0.07^{b}	0.17±0.05^{a}	0.65±0.16^{a,b}
2	100	0.29±0.05^{a}	7.46±0.27^{b,c}	1.36±0.13^{b}	0.79±0.13^{a}	0.36±0.05^{b}	0.18±0.03^{a}	1.29±0.25^{a}
	400	0.25±0.04^{a}	8.37±0.45^{b}	1.34±0.16^{b}	0.76±0.17^{a}	0.50±0.10^{a,b}	0.23±0.05^{a}	0.69±0.14^{a,b}
	800	0.27±0.07^{a}	9.51±0.39^{a,b}	1.97±0.08^{a}	0.85±0.15^{a}	0.79±0.16^{a,b}	0.26±0.04^{a}	0.75±0.09^{a,b}
4	100	0.32±0.04^{a}	10.17±0.88^{a}	2.24±0.23^{a}	0.85±0.29^{a}	0.84±0.18^{a}	0.13±0.02^{a}	0.83±0.16^{a}
	400	0.27±0.05^{a}	9.18±0.38^{b}	1.92±0.35^{a}	1.01±0.20^{a}	0.94±0.15^{a}	0.15±0.01^{a}	0.45±0.03^{b}
	800	0.29±0.05^{a}	7.37±0.29^{b,c}	1.48±0.14^{b}	0.77±0.15^{a}	0.56±0.11^{a,b}	0.14±0.02^{a}	0.62±0.13^{a,b}
8	100	0.27±0.03^{a}	7.01±0.27^{c}	1.46±0.15^{b}	0.79±0.45^{a}	1.21±0.24^{a}	0.15±0.03^{a}	0.55±0.11^{b}
	400	0.27±0.05^{a}	8.66±0.35^{b}	1.77±0.14^{a}	0.92±0.18^{a}	0.45±0.09^{b}	0.22±0.04^{a}	0.74±0.16^{a}
	800	0.29±0.06^{a}	8.28±0.33^{b}	1.69±0.25^{a,b}	0.92±0.15^{a}	0.59±0.11^{a,b}	0.17±0.02^{a}	0.49±0.05^{b}
12	100	0.25±0.04^{a}	7.33±0.33^{b,c}	1.65±0.15^{a,b}	0.95±0.19^{a}	0.49±0.08^{b}	0.18±0.03^{a}	1.02±0.21^{a}
	400	0.27±0.05^{a}	7.69±0.29^{b,c}	1.45±0.13^{b}	0.83±0.15^{a}	0.62±0.12^{a,b}	0.16±0.01^{a}	0.47±0.05^{b}
	800	0.32±0.04^{a}	7.29±0.28^{b,c}	1.64±0.17^{a,b}	0.78±0.14^{a}	0.54±0.12^{a,b}	0.20±0.03^{a}	0.75±0.09^{a,b}
0 (Control)		0.25±0.06^{a}	6.61±0.55^{c}	1.75±0.27^{a,b}	0.60±0.15^{a}	0.48±0.10^{a,b}	0.18±0.01^{a}	0.78±0.29^{a,b}

All data are presented as mean±standard deviation (SD), *n*=3; different lowercase letters indicate significant differences among different treatments (P<0.05).

Table S2 In vivo antioxidant activity of the control mice

Sample	T-AOC (mmol/g protein)	SOD (mU/g protein)	GSH-PX (mmol/g protein)	MDA (μmol/g protein)
Plasma	5.29±0.93 mmol/L	6.71±1.94 mU/L	0.48±0.10 mmol/L	6.71±1.94 μmol/L
Eyeball	1.55±0.36	6.12±0.59	1.01±0.11	6.12±0.59
Intestine	1.47±0.98	7.72±2.12	0.16±0.05	7.72±2.12
Liver	2.07±0.84	6.86±1.64	0.89±0.21	6.86±1.64
Adipose	3.51±0.73	8.57±0.79	0.64±0.09	8.57±0.79

Sample	mRNA fold change (normalized to GAPDH)		
	Cu,Zn-SOD	*Mn-SOD*	*GPX*
Eyeball	1.03±0.16	1.04±0.32	1.02±0.27
Intestine	1.14±0.36	1.01±0.21	1.02±0.22
Liver	1.19±0.27	1.10±0.23	1.01±0.13
Adipose	1.08±0.22	1.05±0.17	1.04±0.15

Table S3 Primers used for gene analysis in mice

Target gene	Primer sequences (5′-3′)
Cu,Zn-SOD (copper, zine-superoxide-dismutase)	F: TGGGTTCCACGTCCATCAGT R: ACCGTCCTTTCCAGCAGTCA
Mn-SOD (manganese -superoxide-dismutase)	F: GAACAA TCTCAACGCCACCG R: AGGGCTCAGGTTTGTCCAGAA
GPX (glutathione-peroxidase)	F: CCAGGAGAATGGCAAGAATGA R: AGGAAGGTAAAGAGCGGGTGA
GAPDH (glyceraldehyde 3-phosphate dehydrogenase)	F: ATGACCTTGCCCACAGCCT R: CCTGCACCACCAACTGCTTA

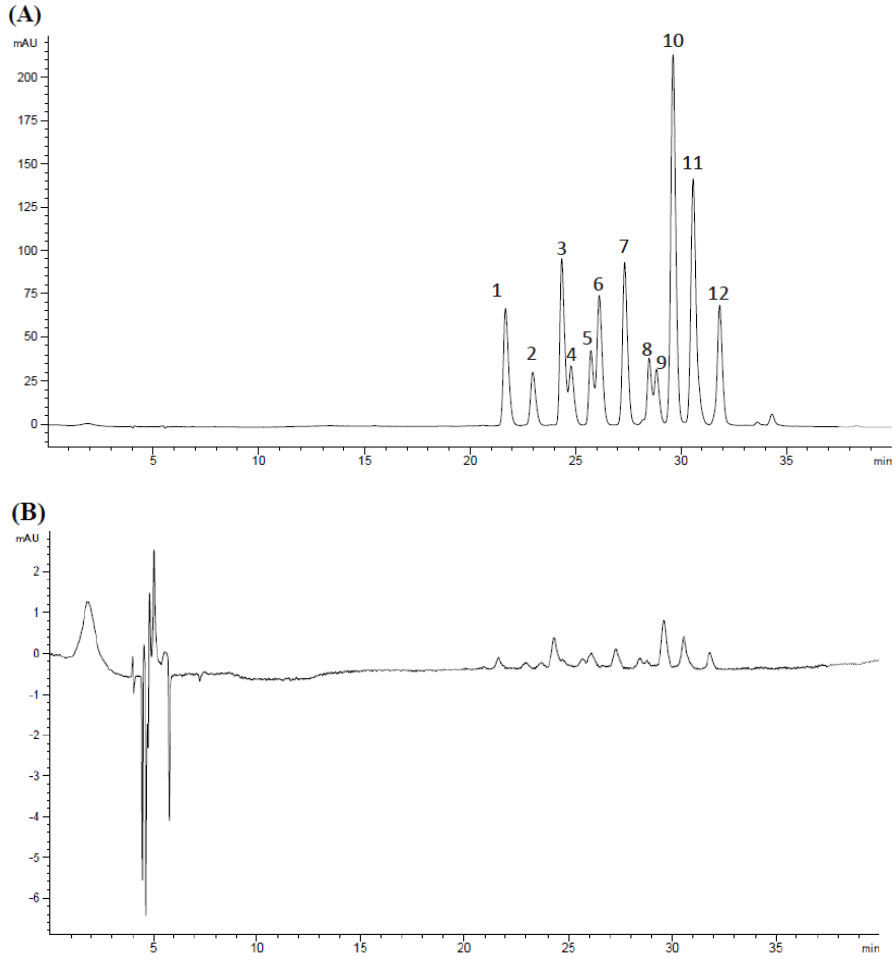

Fig. S1 High performance liquid chromatography (HPLC) chromatogram of blueberry anthocyanin extract (BAE) (A) and blueberry anthocyanins extracted from the mouse liver after treatment with 800 mg/kg BAE for 2 h (B). Peaks: 1. delphindin-3-galactose; 2. delphindin-3-glucose; 3. cyanidin-3-galactose; 4. delphindin-3-arabinose; 5. cyanidin-3-glucose; 6. petunidin-3-galactose; 7. petunidin-3-glucose; 8. petunidin-3-arabinose; 9. peonidin-3-galactose; 10. malvidin-3-galactose; 11. malvidin-3-glucose; 12. malvidin-3-arabinose.