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Cyanidin 3-glucoside improves diet-induced metabolic syndrome in rats

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ABSTRACT

Increased consumption of dark-coloured fruits and vegetables may mitigate metabolic syndrome. This study has determined the changes in metabolic parameters, and in cardiovascular and liver structure and function, following chronic administration of either cyanidin 3-glucoside (CG) or Queen Garnet plum juice (QG) containing cyanidin glycosides to rats fed either a corn starch (C) or a high-carbohydrate, high-fat (H) diet. Eight to nine-week-old male Wistar rats were randomly divided into six groups for 16-week feeding with C, C with CG or QG, H or H with CG or QG. C or H were supplemented with CG or QG at a dose of ~8 mg/kg/day cyanidin glycosides from week 8 to 16. H rats developed signs of metabolic syndrome including visceral adiposity, impaired glucose tolerance, hypertension, cardiovascular remodelling, increased collagen deposition in left ventricle, non-alcoholic fatty liver disease, increased plasma liver enzymes and increased inflammatory cell infiltration in the heart and liver. Both CG and QG reversed these cardiovascular, liver and metabolic signs. However, no intact anthocyanins or common methylated/conjugated metabolites could be detected in the plasma samples and plasma hippuric acid concentrations were unchanged. Our results suggest CG is the most likely mediator of the responses to QG but that further investigation of the pharmacokinetics of oral CG in rats is required.

1. Introduction

The prevalence of obesity is increasing, now reaching epidemic proportions [1,2]. Obesity is accepted as a chronic, low-grade inflammatory state with increased oxidative stress [3,4]. Controlling inflammation is one mechanism to either reverse or attenuate obesity and associated tissue and organ changes [3]. Eating fruits and vegetables can prevent chronic diseases including cardiovascular disease and possibly prevent body weight gain [5]. Increased consumption of polyphenol-containing fruits and vegetables provides anti-inflammatory responses that could reduce the risk factors for metabolic syndrome, producing cardiac and hepatic protection [6–9]. The most common polyphenols are flavonoids, and many flavonoids have been studied for their role in reducing obesity, probably by antioxidant or anti-inflammatory mechanisms [10,11]. Flavonoids are widespread in nature, including the anthocyanins commonly found in dark-coloured fruits and vegetables including Red Delicious apples, chokeberries, black beans, black plums and wild blueberries [12]; an example is cyanidin 3-glucoside (CG) (Supplementary Fig. 1A). Anthocyanins are produced by plants as secondary metabolites to protect against environmental stress factors and fungal infections [13] and they also promote health in humans [14,15]. Their pharmacokinetics and metabolism have been reported [16,17]. It is estimated that the average daily oral intake is ~1000 mg of polyphenols in adults in the USA [14] and ~65 mg of anthocyanidins in Europe [18]. As vegetables and fruits are rich in polyphenols, they may supply an adequate dietary intake of polyphenols including anthocyanins.

CG has shown responses in experimental models that indicate a potential role in reversing the signs of metabolic syndrome. CG decreased obesity and circulating triglycerides in an *in vivo* study using KK-Ay mice [19]. *In vitro*, CG decreased inflammation in isolated vascular endothelial cells and monocytes [20] and produced

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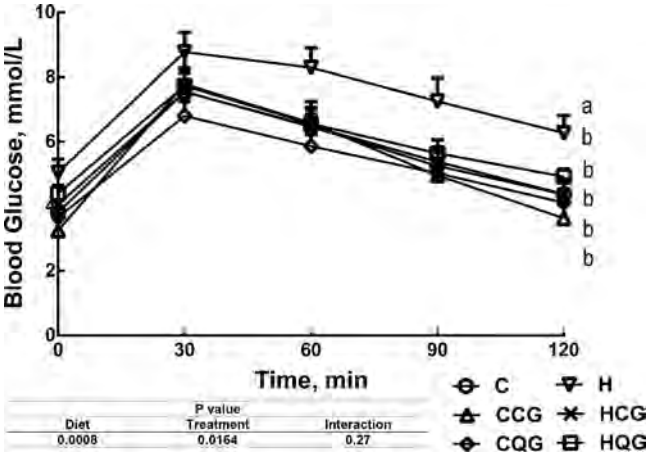

Fig. 1. Effect of cyanidin 3-glucoside (CG) and Queen Garnet plum juice (QG) on oral glucose tolerance in C, CCG, CQG, H, HCG and HQG rats. Data are shown as mean ± SEM. End-point means without a common alphabet in each data set significantly differ, $P < 0.05$ and $n = 10$/group.

an insulin-like effect in human omental adipocytes and 3T3-L1 cells [21].

The Queen Garnet plum is a variety of the Japanese plum (*Prunus salicina* Lindl.) with a high anthocyanin (mainly CG) content up to 272 mg/100 g fresh fruit, being 5–10 fold higher than other plums [22]. Consumption of Queen Garnet plum juice (QG) decreased malondialdehyde concentrations in plasma and urine as a biomarker of oxidative stress [22,23] and reduced platelet activation-related thrombogenesis in healthy volunteers [23]. Other food sources of CG and other cyanidin glycosides also improved signs of metabolic syndrome. As examples, purple corn decreased body fat and hyperglycaemia in high-fat diet-fed mice [24] and Moro orange supplementation decreased the high-fat diet-induced increases in lipid deposition in liver and wet weight in mice as symptoms of non-alcoholic fatty liver [25] and fat accumulation [26]. Similarly, high-fat diet-fed mice given either blueberry or purple corn supplementation reduced central adiposity [27], abdominal fat pads and hyperglycaemia [24]. Additionally, chokeberry juice decreased blood pressure in humans with metabolic syndrome [28]. Purple carrot juice containing anthocyanins improved glucose tolerance, decreased body weight gain, and improved cardiovascular and liver structure and function in rats fed a high-carbohydrate, high-fat diet [29]. However, there is no clear evidence that CG is the active anthocyanin in improving these signs of metabolic syndrome.

Thus, this study has compared CG and QG in rats fed a high-carbohydrate, high-fat diet for attenuation of signs of the human metabolic syndrome. Cardiovascular, hepatic and metabolic parameters were measured for this comparison. Further, plasma samples were screened for intact anthocyanins and their common metabolites (methylated/conjugated forms and hippuric acid) as a measure of anthocyanin absorption and metabolism.

2. Materials and methods

2.1. Cyanidin 3-glucoside and Queen Garnet plum juice

Pure CG was supplied by Biosynth AS, Sandnes, Norway. Fresh Queen Garnet plums were harvested in February 2013 and QG was prepared and analysed for anthocyanins and quercetin glycosides [22,30]. The QG was also analysed for protein, fat, total sugar, dietary fibre and energy content by a commercial laboratory (Symbio Alliance, Brisbane, QLD, Australia). QG was supplied by Nutrafruit Pty Ltd., Toowong, QLD, Australia.

Table 1
Composition of the Queen Garnet plum juice by analysis.

Cyanidin 3-glucoside (mg/100 ml)[c,a]	200
Cyanidin 3-rutinoside (mg/100 ml)[c,a]	30
Quercetin glycosides (mg/100 ml)[c,b]	31
Energy (kJ/100 ml)[d]	374
Protein (g/100 ml)[d]	1.1
Total fat (g/100 ml)[d]	<1
Total sugars (g/100 ml)[d]	15.2
Fibre (g/100 ml)[d]	<1
Sodium (g/100 ml)[d]	0.073

Values are represented as mean of duplicate analysis.
[a] See Supplementary Fig. 1 for chemical structures.
[b] Sum of quercetin 3-glucoside, quercetin 3-rutinoside and quercetin 3-galactoside; calculated as quercetin 3-glucoside equivalents.
[c] Analysed by authors.
[d] Analysed by a commercial laboratory (Symbio Alliance, Brisbane, QLD, Australia).

2.2. Animals and diets

The experimental group consisted of 72 male Wistar rats (8–9 weeks old) purchased from the Animal Resource Centre, Murdoch, WA, Australia and individually housed in a temperature-controlled room (20 ± 2 °C) under 12-h light/dark cycle environment with *ad libitum* access to food and water at the University of Southern Queensland animal house. All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (approval 13REA005), which operates under the guidelines of the National Health and Medical Research Council of Australia. After rats were acclimatised for a week and had reached 336 ± 3 g body weight, they were randomly divided into 6 experimental diet groups ($n = 12$ each) and fed with corn starch (C), C + CG (CCG), C + QG (CQG), high-carbohydrate, high-fat (H), H + CG (HCG) or H + QG (HQG) for 16 weeks. C, CCG and CQG rats were fed with C diet for the first 8 weeks and then with C, C + CG and C + QG diets for the last 8 weeks. H, HCG and HQG rats were fed with H diet for the first 8 weeks and then with H, H + CG and H + QG diets for the last 8 weeks. The composition of C and H diets has been described in detail [31]. H, HCG and HQG rats were also given 25% fructose in drinking water. CG 115 mg/kg food was thoroughly mixed in the diet; 50 ml/kg food QG containing 2.3 mg/ml anthocyanins and 0.31 mg/ml quercetin glycosides replaced an equivalent volume of water. Measurements of body weight and food and water intakes were recorded daily and feed efficiency (%) calculated [32].

Table 2
Dietary intakes, body composition and organ wet weights in C, CCG, CQG, H, HCG and HQG diet-fed rats ($n = 10$ rats/group).

Variable	C	CCG	CQG	H	HCG	HQG	*P* values		
							Diet	Treatment	Interaction
Food intake (g/d)	33.1 ± 1.8^{a}	33.6 ± 0.8^{a}	33.4 ± 0.5^{a}	26.5 ± 0.9^{b}	25.8 ± 0.7^{b}	25.5 ± 0.5^{b}	<0.0001	0.74	0.55
Water intake (ml/d)	30.6 ± 2.1^{a}	30.5 ± 2.3^{a}	29.5 ± 1.3^{a}	25.7 ± 1.1^{b}	23.6 ± 1.0^{b}	22.0 ± 0.7^{b}	<0.0001	0.18	0.57
Plum juice intake (ml/d)	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.0^{b}	<0.0001	<0.0001	<0.0001
Anthocyanins intake (mg/kg/d)	0.0 ± 0.0	9.9 ± 0.0^{a}	9.8 ± 0.0^{b}	0.0 ± 0.0	7.6 ± 0.0^{c}	7.4 ± 0.0^{c}	<0.0001	<0.0001	<0.0001
Quercetin glycosides intake (mg/kg/d)	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.0^{b}	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0^{b}	<0.0001	<0.0001	<0.0001
Energy intake (kJ/d)	370.5 ± 24.1^{b}	375.4 ± 15.1^{b}	375.2 ± 5.0^{b}	557.4 ± 17.1^{a}	553.3 ± 9.3^{a}	531.9 ± 6.2^{a}	<0.0001	0.5	0.33
Feed conversion efficiency (%)	2.1 ± 0.4^{b}	2.2 ± 0.4^{b}	1.3 ± 0.2^{b}	7.9 ± 1.8^{a}	4.1 ± 0.6^{ab}	4.2 ± 0.7^{ab}	<0.0001	0.0293	0.15
Body weight gain (8–16 weeks) (%)*	8.4 ± 1.3^{c}	8.1 ± 1.1^{c}	5.6 ± 0.5^{c}	22.1 ± 3.6^{a}	12.9 ± 1.1^{b}	12.7 ± 0.8^{b}	<0.0001	0.0038	0.1
Visceral adiposity index (%)	4.8 ± 0.4^{b}	4.8 ± 0.2^{b}	4.8 ± 0.3^{b}	9.2 ± 1.1^{a}	6.7 ± 0.5^{b}	6.6 ± 0.4^{b}	<0.0001	0.0485	0.0485
Abdominal circumference (cm)	20.7 ± 0.2^{b}	20.4 ± 0.2^{b}	20.6 ± 0.3^{b}	22.8 ± 0.2^{a}	21.1 ± 0.2^{b}	21.3 ± 0.2^{b}	<0.0001	0.0013	0.0042
Body mass index (kg/m^2)	5.6 ± 0.2^{bc}	5.1 ± 0.1^{c}	4.6 ± 0.1^{c}	6.9 ± 0.3^{a}	5.9 ± 0.2^{b}	5.5 ± 0.1^{bc}	<0.0001	<0.0001	0.31
Bone mineral content (g)	11.3 ± 0.3^{c}	11.5 ± 0.4^{c}	11.4 ± 0.2^{c}	16.9 ± 0.7^{a}	15.5 ± 0.5^{b}	14.4 ± 0.2^{b}	<0.0001	0.0055	0.0029
Total body lean mass (g)	318.4 ± 10.9^{a}	301.6 ± 7.0^{a}	279.0 ± 3.3^{ab}	307.5 ± 19.0^{a}	307.3 ± 6.2^{a}	279.4 ± 8.7^{ab}	0.66	0.0074	0.64
Total body fat mass (g)	72.2 ± 8.0^{c}	86.4 ± 8.5^{c}	94.0 ± 5.2^{c}	216.6 ± 18.1^{a}	162.3 ± 6.1^{b}	159.6 ± 6.4^{b}	<0.0001	0.11	0.0008
Tissue wet weight (mg/mm tibial length)**									
Retroperitoneal adipose tissue	172.1 ± 17.1^{b}	177.3 ± 12.4^{b}	178.1 ± 10.9^{b}	527.7 ± 84.4^{a}	279.6 ± 23.1^{b}	271.8 ± 20.4^{b}	<0.0001	0.0081	0.0057
Epididymal adipose tissue	98.6 ± 8.3^{b}	94.3 ± 7.8^{b}	97.7 ± 7.5^{b}	236.2 ± 31.9^{a}	150.5 ± 10.4^{b}	149.7 ± 9.4^{b}	<0.0001	0.0175	0.0198
Omental adipose tissue	101.8 ± 9.4^{c}	92.2 ± 7.1^{c}	97.2 ± 7.2^{c}	250.4 ± 35.9^{a}	160.4 ± 17.6^{bc}	163.6 ± 10.4^{bc}	<0.0001	0.0255	0.0431
Total abdominal fat	372.5 ± 32.8^{b}	363.7 ± 17.8^{b}	373.0 ± 23.7^{b}	1014.3 ± 151.1^{a}	590.5 ± 43.7^{b}	585.1 ± 36.1^{b}	<0.0001	0.0111	0.0146
Liver	194.2 ± 9.7^{c}	197.5 ± 6.5^{c}	201.9 ± 6.7^{c}	299.2 ± 14.3^{a}	233.7 ± 8.4^{b}	236.7 ± 8.3^{b}	<0.0001	0.0105	0.0014

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, $P < 0.05$.

* In all groups, the body weight gain (8–16 weeks) is calculated relative to body weight at 8 weeks.

** Normalised against tibial length at the time of removal.

2.3. Oral glucose tolerance test

Oral glucose tolerance tests were performed on rats after overnight (12 h) food deprivation; in addition, fructose-supplemented water in H, HCG and HQG groups was replaced with normal water. Basal blood glucose concentrations were measured in blood collected from the tail vein using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA). The rats were given 2 g/kg body weight of glucose as a 40% aqueous solution *via* oral gavage. Tail vein blood samples were taken at 30, 60, 90 and 120 min following glucose administration [31].

2.4. Cardiovascular measurements

Systolic blood pressure was measured at 0, 8 and 16 weeks under light sedation by intraperitoneal injection with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst, NSW, Australia) [31–33]. Measurements were performed using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Bella Vista, NSW, Australia) and an inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) connected to a PowerLab data acquisition unit (ADInstruments).

Echocardiographic examinations using Hewlett Packard Sonos 5500 12 MHz transducer were performed to assess the cardiovascular structure and function at 16 weeks under anaesthesia with intraperitoneal Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg) and Ilium Xylazil (xylazine 6 mg/kg; Troy Laboratories, Smithfield, NSW, Australia), in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method [31–33].

The isolated Langendorff heart preparation was used to assess left ventricular function of the rats in all groups [31–33]. Terminal anaesthesia was induced *via* intraperitoneal injection of Lethabarb (pentobarbitone sodium, 100 mg/kg; Virbac, Peakhurst, NSW, Australia). After heparin (Sigma–Aldrich Australia, Sydney, NSW, Australia) administration (200 IU) into the right femoral vein, blood (~6 ml) from the abdominal aorta was collected into heparinised tubes. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle of the isolated heart connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab system (ADInstruments).

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath chamber filled with Tyrode physiological salt solution bubbled with 95% O_2–5% CO_2 and allowed to stabilise at a resting tension of ~10 mN. Cumulative concentration–response curves (contraction) were obtained for noradrenaline (Sigma–Aldrich Australia) and cumulative concentration–response curves (relaxation) were obtained for acetylcholine (Sigma–Aldrich Australia) and sodium nitroprusside (Sigma–Aldrich Australia) following submaximal (~70%) contraction to noradrenaline [31–33].

2.5. Body composition measurements

Dual-energy X-ray absorptiometric measurements were performed on all rats using a Norland XR36 instrument (Norland Corp., Fort Atkinson, WI, USA) under anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg) and Ilium Xylazil (xylazine 6 mg/kg) *via* intraperitoneal injection at the end of 16 weeks of respective diet feeding, 2 days before the pathophysiological assessments. Scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp.) [31–33]. The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated [31–33].

2.6. Organ weights

The right and left ventricles were separated after perfusion experiments and weighed. Following removal of the heart, the liver, retroperitoneal, epididymal and omental fat pads were collected and blotted dry for weighing. Organ weights were normalised relative to the tibial length at the time of their removal (in mg/mm).

2.7. Histology

Approximately 5–7 min after euthanasia, heart and liver portions from two rats per group were collected and fixed in 10% neutral buffered formalin for 3 days. The samples were then dehydrated and embedded in paraffin wax [31–33]. Thin sections (~5 μm) of left ventricle and liver were cut and stained with haematoxylin and eosin to study infiltration of inflammatory cells and for determining fat vacuoles in liver. Heart sections were also stained with picrosirius red to study collagen distribution in the left ventricle. Laser confocal microscopy (Nikon A1R+ upright Confocal Microscope, Tokyo, Japan) was used to determine the extent of collagen deposition in selected tissue sections.

2.8. Plasma biochemistry and metabolites

Blood was centrifuged at 5000 × *g* for 15 min within 30 min of collection into heparinised tubes. Plasma was transferred to Eppendorf tubes for storage at −20°C before analysis. Plasma concentrations of total cholesterol, triglycerides, non-esterified fatty acids (NEFA), activities of plasma alanine transaminase (ALT), alkaline phosphatase (ALP) and aspartate transaminase (AST) were determined using kits and standards supplied by Olympus (Tokyo, Japan) using an AU 400 Olympus analyser as previously described [31–33]. Plasma insulin and leptin concentrations were estimated using a commercial ELISA kit (ALPCO, Salem, NH, USA) according to manufacturer-provided standards and protocols. Plasma was screened for intact anthocyanins, their common methylated and conjugated metabolites as well as hippuric acid by HPLC [22,23,34].

2.9. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. Data from C, CCG, CQG, H, HCG and HQG groups were tested by two-way anal-

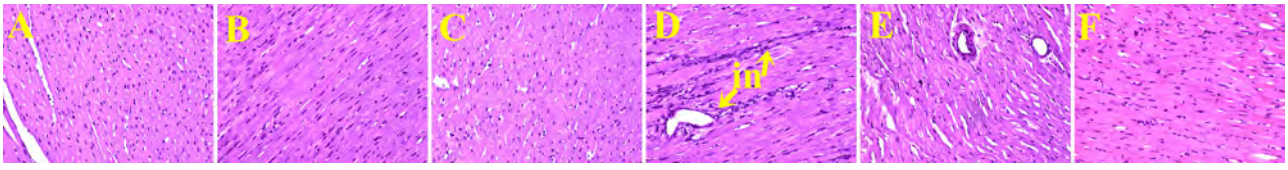

Fig. 2. Haematoxylin and eosin staining of left ventricle (original magnification ×20) showing inflammatory cells (marked as "in") as dark spots outside the myocytes in C (A), CCG (B), CCG (C), H (D), HCG (E) and HCG (F) rats.

Table 3
Changes in glucose tolerance test, plasma hormones, plasma metabolites and plasma biochemistry in C, CCG, CQG, H, HCG and HQG diet-fed rats ($n = 10$ rats/group).

Variable	C	CCG	CQG	H	HCG	HQG	*P* values		
							Diet	Treatment	Interaction
OGTT-AUC (mmol/L· min)	690.5 ± 22.2^{b}	665.7 ± 16.9^{b}	646.1 ± 37.7^{b}	843.1 ± 27.5^{a}	709.1 ± 12.4^{b}	727.4 ± 37.1^{b}	0.0008	0.0164	0.27
Plasma insulin (μmol/L)	1.8 ± 0.5^{b}	1.8 ± 0.7^{b}	1.7 ± 0.2^{b}	3.9 ± 0.3^{a}	2.1 ± 0.4^{b}	1.9 ± 0.3^{b}	0.0019	0.0041	0.0088
Plasma leptin (μmol/L)	7.3 ± 0.7^{b}	7.2 ± 0.9^{b}	7.1 ± 0.9^{b}	11.8 ± 0.9^{a}	8.6 ± 0.8^{b}	8.9 ± 1.0^{b}	0.001	0.09	0.13
Plasma total cholesterol (mmol/L)	1.6 ± 0.1^{b}	1.6 ± 0.1^{b}	1.4 ± 0.1^{b}	2.4 ± 0.1^{a}	1.7 ± 0.0^{b}	1.6 ± 0.1^{b}	<0.0001	<0.0001	0.0049
Plasma triglycerides (mmol/L)	0.4 ± 0.0^{c}	0.4 ± 0.0^{c}	0.4 ± 0.1^{c}	1.9 ± 0.2^{a}	0.9 ± 0.2^{b}	0.6 ± 0.1^{b}	<0.0001	<0.0001	<0.0001
Plasma NEFA (mmol/L)	1.5 ± 0.2^{c}	1.5 ± 0.1^{c}	1.1 ± 0.1^{c}	4.9 ± 0.3^{a}	1.6 ± 0.2^{c}	2.4 ± 0.4^{bc}	<0.0001	<0.0001	0.0005
Plasma hippuric acid (ng/ml)	85 ± 60	86 ± 45	59 ± 82	139 ± 69	91 ± 69	80 ± 49	0.63	0.70	0.69
Plasma ALP (U/L)	123.7 ± 5.2^{c}	116.5 ± 11.6^{c}	123.8 ± 6.5^{c}	315.8 ± 17.2^{a}	192.2 ± 14.5^{b}	212.8 ± 12.0^{b}	<0.0001	<0.0001	<0.0001
Plasma ALT (U/L)	25.9 ± 1.9^{c}	23.0 ± 1.5^{c}	20.4 ± 1.5^{c}	45.9 ± 2.9^{a}	27.0 ± 2.0^{c}	31.9 ± 1.3^{bc}	<0.0001	<0.0001	0.0403
Plasma AST (U/L)	61.1 ± 1.9^{b}	61.8 ± 1.4^{b}	58.2 ± 3.8^{b}	86.6 ± 2.1^{a}	62.0 ± 2.0^{b}	62.4 ± 2.1^{b}	<0.0001	<0.0001	0.0002

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, $P < 0.05$.

ysis of variance. When interaction and/or the main effects were significant, means were compared using Newman–Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal–Wallis non-parametric test was performed. A *P*-value of <0.05 was considered as statistically significant. All statistical analyses were performed using Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Diet intake, body composition and plasma biochemistry

The major flavonoids present in QG were anthocyanins, mainly CG, cyanidin 3-rutinoside (Table 1, Supplemental Fig. 1) and quercetin glycosides (Table 1). The nutritional composition of QG used in this study is shown in Table 1. Food intakes were higher in C, CCG and CQG rats than in H, HCG and HQG rats, respectively (Table 2). Due to these differences, the average daily intake of anthocyanins was higher in CCG and CQG rats than in HCG and HQG rats, respectively (Table 2).

Neither CG nor QG treatment altered food, water or energy intakes (Table 2). H rats had higher feed conversion efficiency, body weight gain, abdominal circumference and body mass index compared to C rats; these parameters were lower in HCG and HQG rats compared to H rats (Table 2). Bone mineral content was higher in H rats than HCG and HQG rats, and all were higher than C, CCG and CQG rats (Table 2). HCG and HQG rats had lower total body fat mass compared to H rats and higher total body fat mass compared to C, CCG and CQG rats. These changes in total body fat are consistent with changes in abdominal fat pads (Table 2). Total body lean mass was unchanged by CG or QG treatment.

3.2. Plasma biochemistry, oral glucose tolerance and plasma metabolites

Plasma concentrations of total cholesterol, triglycerides and NEFA were higher in H rats compared to C or to CG- and QG-treated rats, while HQG rats had higher NEFA concentrations than HCG rats; HCG and HQG rats had higher concentrations of triglycerides than CCG and CQG rats (Table 3). Plasma leptin concentrations were higher in H rats compared to C rats; leptin concentrations were normalised in HCG and HQG rats (Table 3). H rats also had higher fasting blood glucose concentration compared to C rats. CG and QG treatment decreased fasting blood glucose concentrations. The plasma glucose increase after oral glucose loading was greater in H rats than in C rats (Fig. 1). At 120 min, HCG and HQG rats had lower plasma glucose concentrations than H rats (Fig. 1). Plasma insulin concentrations almost doubled in H rats compared to C, and CG and QG-treated rats. This change is consistent with glucose tolerance curves (Table 3).

Neither CG nor cyanidin 3-rutinoside, the main QG anthocyanin, could be detected in the plasma of CG or QG-treated rats. Cyanidin glucuronide, the most common conjugated metabolite of cyanidin-based anthocyanins, was tentatively identified in some plasma samples after QG treatment. However, further evaluation was not undertaken since the concentration of this metabolite was below the limit of quantification. No other conjugated or methylated anthocyanin forms could be detected. Plasma hippuric acid concentrations were unchanged between the groups (Table 3).

3.3. Cardiovascular structure and function

H rats showed increased left ventricular internal diameter in diastole (LVIDd) and left ventricular wet weight as signs of eccentric hypertrophy compared to C rats. This change in LVIDd was observed with no change in relative wall thickness in either of the groups (Table 4). H rats showed impaired systolic function seen as decreased fractional shortening, developed pressure and dP/dt, increased left ventricular diameter in systole (LVIDs), diastolic stiffness and systolic wall stress (Table 4). H rats also showed increased diastolic, systolic and stroke volumes, cardiac output and estimated left ventricular mass compared to C rats, without any change in heart rate (Table 4).

Treatment in HCG and HQG rats with CG and QG decreased left ventricular internal chamber sizes compared to H rats, with increased left ventricular posterior wall thickness in systole in HCG and HQG rats but with constant diastolic thickness. These responses were accompanied by increased fractional shortening with CG and QG (Table 4). Diastolic stiffness, diastolic and systolic volumes, cardiac output, systolic wall stress and wet weight of left ventricle with septum were normalised with CG and QG, while heart rate was decreased with CG and QG and normalised ejection time compared to H rats (Table 4).

Compared to C rats (Figs. 2 A and 3 A), H rats showed increased infiltration of inflammatory cells in the left ventricle (Fig. 2D) and increased interstitial collagen deposition (Fig. 3D). CG and QG suppressed the infiltration of inflammatory cells (Fig. 2B, C, E and F) and reduced collagen deposition (Fig. 3B, C, E and F), while no other changes were observed and tissue morphology appeared normal.

In isolated thoracic aortic rings, H rats showed decreased vascular contraction with noradrenaline (Fig. 4A) and decreased vascular relaxation with sodium nitroprusside and acetylcholine compared to C rats (Fig. 4B and C). CG and QG rats showed improved contraction and relaxation in isolated thoracic aortic rings (Fig. 4A–C).

3.4. Hepatic structure and function

Compared to C rats, H rats had increased liver wet weight with increased plasma activities of ALT, AST and ALP as markers of liver damage. HCG and HQG rats had lower liver wet weight and plasma

Table 4
Changes in cardiovascular structure and function in C, CCG, CQG, H, HCG and HQG diet-fed rats ($n = 10$ rats/group).

Variable	C	CCG	CQG	H	HCG	HQG	*P* values		
							Diet	Treatment	Interaction
Heart rate (bpm)	306.5 ± 15.9[a]	264.3 ± 13.0[b]	253.1 ± 11.0[b]	338.8 ± 18.0[a]	248.9 ± 8.1[b]	269.3 ± 8.0[b]	0.09	0.0001	0.56
IVSd (mm)	1.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.0	2.0 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	0.49	0.0429	0.49
LVIDd (mm)	6.3 ± 0.2[c]	6.9 ± 0.1[b]	7.0 ± 0.2[b]	7.9 ± 0.1[a]	6.9 ± 0.2[b]	7.0 ± 0.1[b]	<0.0001	0.53	<0.0001
LVPWd (mm)	1.6 ± 0.0[b]	1.7 ± 0.0[b]	1.7 ± 0.0[b]	1.9 ± 0.0[a]	1.8 ± 0.1[b]	1.7 ± 0.1[b]	0.0056	0.33	0.0056
IVSs (mm)	2.8 ± 0.2	2.9 ± 0.1	3.0 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	0.27	0.71	0.27
LVIDs (mm)	3.2 ± 0.3[b]	3.5 ± 0.1[b]	3.4 ± 0.1[b]	4.1 ± 0.2[a]	3.7 ± 0.2[b]	3.5 ± 0.1[b]	0.0153	0.31	0.0482
LVPWs (mm)	2.5 ± 0.1	2.9 ± 0.0	2.8 ± 0.1	2.8 ± 0.1	3.0 ± 0.2[ab]	3.0 ± 0.1[ab]	0.0186	0.0186	0.62
Fractional shortening (%)	53.9 ± 2.5[a]	58.4 ± 1.7[a]	59.8 ± 2.3[a]	45.8 ± 2.1[b]	57.3 ± 1.5[a]	57.1 ± 1.3[a]	0.0157	0.0003	0.21
Ejection time (ms)	70.4 ± 2.4[c]	77.6 ± 1.4[bc]	75.3 ± 2.1[bc]	90.6 ± 2.4[a]	83.4 ± 4.1[bc]	84.3 ± 2.1[bc]	<0.0001	0.76	0.0193
Ejection fraction (%)	80.2 ± 1.9	81.6 ± 1.3	82.6 ± 1.9	83.6 ± 1.8	78.6 ± 4.4	82.3 ± 1.8	0.41	0.77	0.33
LV developed pressure (mmHg)	70.2 ± 4.7[a]	70.8 ± 3.7[a]	72.7 ± 5.4[a]	49.4 ± 5.1[b]	67.2 ± 4.1[a]	68.6 ± 3.9[a]	0.0138	0.0302	0.09
(+) dP/dt (mmHg/s)	1096.4 ± 64.8[a]	1046.0 ± 73.5[a]	1077.3 ± 60.4[a]	809.4 ± 74.5[b]	1006.9 ± 59.2[ab]	986.7 ± 60.6[ab]	0.0065	0.23	0.14
(−) dP/dt (mmHg/s)	−714.5 ± 48.4[a]	−756.2 ± 36.8[a]	−734.8 ± 50.6[a]	−506.7 ± 44.8[b]	−669.4 ± 39.5[a]	−686.4 ± 39.4[a]	0.0085	0.0363	0.09
Diastolic stiffness constant (κ)	22.9 ± 0.8[b]	22.5 ± 0.6[b]	23.4 ± 0.8[b]	28.6 ± 0.6[a]	23.8 ± 0.8[b]	24.8 ± 0.9[b]	<0.0001	0.042	0.0093
Diastolic volume (μl)	356.3 ± 23.9[b]	343.5 ± 18.1[b]	356.8 ± 24.0[b]	533.0 ± 48.7[a]	360.2 ± 33.1[b]	360.5 ± 17.7[b]	0.0069	0.0096	0.0093
Systolic volume (μl)	48.4 ± 7.1[b]	45.1 ± 2.7[b]	42.3 ± 3.3[b]	85.8 ± 10.9[a]	70.9 ± 10.0[ab]	47.8 ± 4.7[b]	0.0054	0.0044	0.033
Stroke volume (μl)	307.9 ± 27.3 [ab]	298.4 ± 18.8[ab]	314.5 ± 23.3[ab]	396.7 ± 17.7[a]	289.3 ± 39.9 [ab]	312.7 ± 20.3 [ab]	0.06	0.1	0.05
Cardiac output (ml/min)	88.1 ± 7.5[b]	78.9 ± 6.7[b]	81.0 ± 8.8[b]	127.5 ± 8.7[a]	71.7 ± 10.0[b]	84.7 ± 6.7[b]	0.0115	0.0041	0.0333
Estimated LV mass, Litwin (g)	0.73 ± 0.00[b]	0.84 ± 0.03[b]	0.80 ± 0.03[b]	0.99 ± n0.05[a]	0.82 ± 0.07[b]	0.83 ± 0.03[b]	0.0001	0.18	0.0015
LV+septum wet weight (mg/mm tibial length)	16.1 ± 0.5[b]	14.6 ± 0.4[b]	16.5 ± 0.5[b]	19.5 ± 0.8[a]	16.6 ± 0.5[b]	16.1 ± 0.5[b]	0.0154	0.0154	0.0027
Right ventricle wet weight (mg/mm tibial length)	3.8 ± 0.2[ab]	4.2 ± 0.2[ab]	3.9 ± 0.1[ab]	4.4 ± 0.2[ab]	4.9 ± 0.2[a]	4.0 ± 0.2[ab]	0.06	0.41	0.17
Relative wall thickness	0.52 ± 0.01	0.48 ± 0.01	0.50 ± 0.01	0.50 ± 0.02	0.48 ± 0.03	0.50 ± 0.01	0.46	0.46	0.46
Systolic blood pressure (mmHg)	123.0 ± 3.0[b]	122.7 ± 1.2[b]	125.5 ± 2.5[b]	156.7 ± 4.2[a]	132.3 ± 1.4[b]	128.6 ± 1.6[b]	<0.0001	0.0001	<0.0001
Systolic wall stress (mmHg)	91.3 ± 5.2[b]	81.2 ± 2.5[b]	78.3 ± 4.7[b]	118.0 ± 8.7[a]	86.9 ± 8.6[b]	85.1 ± 3.5[b]	0.007	0.0004	0.1

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, $P < 0.05$.

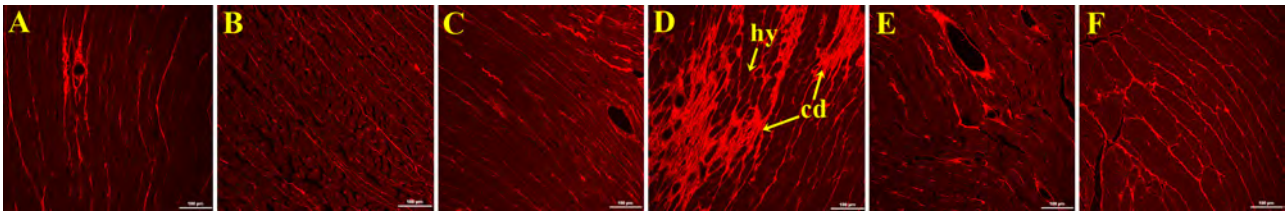

Fig. 3. Picrosirius red staining of left ventricular interstitial collagen deposition (original magnification ×40) in C (A), CCG (B), CQG (C), H (D), HCG (E) and HQG (F) rats. Collagen deposition is marked as "cd" and hypertrophied cardiomyocytes are marked as "hy".

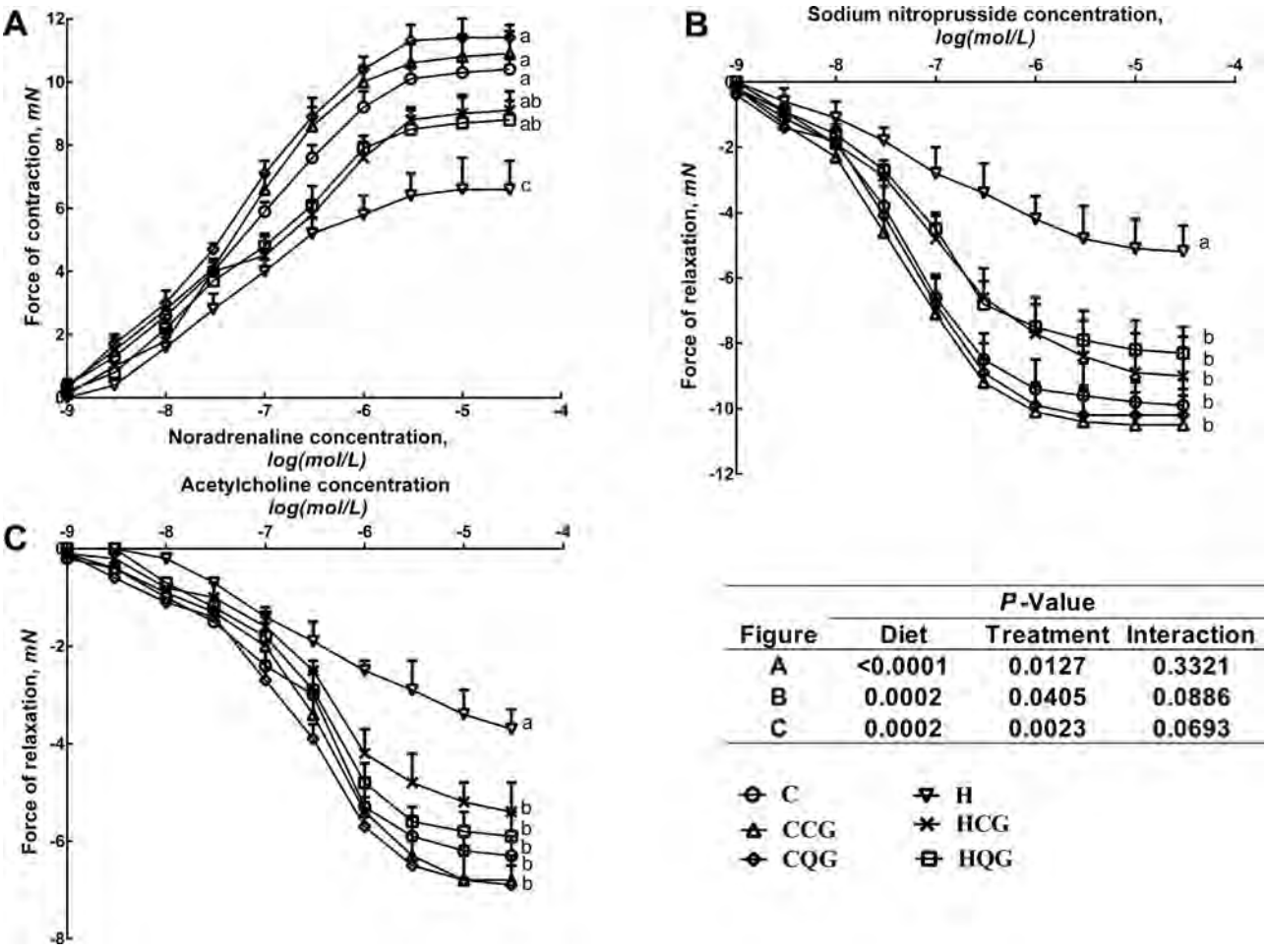

Figure	P-Value		
	Diet	Treatment	Interaction
A	<0.0001	0.0127	0.3321
B	0.0002	0.0405	0.0886
C	0.0002	0.0023	0.0693

Fig. 4. Cumulative concentration–response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, CCG, CQG, H, HCG and HQG rats. Data are shown as mean ± SEM. End-point means without a common alphabet in each data set significantly differ, $P < 0.05$ and $n = 10$/group.

ALT, AST and ALP activities compared to H rats. Liver wet weight and plasma activities of ALT, AST and ALP were unchanged in CCG and CQG rats compared to C rats (Table 3). H rats (Fig. 5D) showed increased hepatic lipid deposition and inflammatory cell infiltration compared to C rats (Fig. 5A) while HCG and HQG rats showed decreased inflammatory cell infiltration (Fig. 5E and F) compared to H rats. CCG and CQG rats showed minimal macrovesicular steatosis and portal inflammation and tissue morphology appeared normal (Fig. 5B and C) as seen in C rats (Fig. 5A).

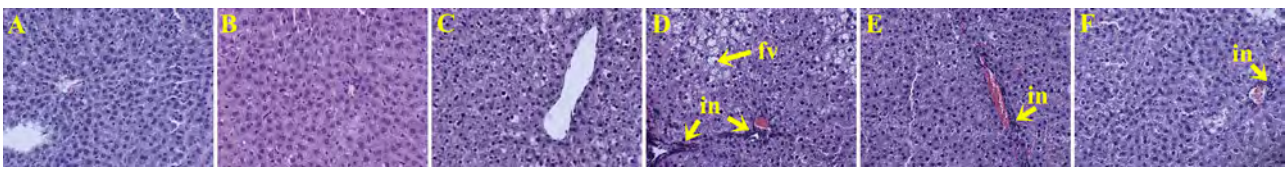

Fig. 5. Haematoxylin and eosin staining of hepatocytes (original magnification ×20) showing inflammatory cells (marked as "in") and hepatocytes with fat vacuoles (marked as "fv") in C (A), CCG (B), CQG (C), H (D), HCG (E) and HQG (F) rats.

4. Discussion

Cyanidin-based anthocyanins, the characteristic polyphenols in QG, are one of the most abundant pigments in nature, being responsible for the dark red colour of many fruits and vegetables [35]. Since human health has been associated with an increased intake of fresh fruits and vegetables, it is important to determine the anti-diabetic, anti-obesity and anti-inflammatory activities of purified cyanidin-based anthocyanins, especially CG, as well as foods containing these compounds in relevant rodent models of human disease. QG contain both anthocyanins and quercetin glycosides, suggesting that either could produce the physiological responses. However, the dose of quercetin glycosides in this study was low at around 1 mg/kg/day, much lower than the quercetin dose of around 50 mg/kg/day or the rutin dose of around 100 mg/kg/day used to reverse signs of metabolic syndrome in the same model [36,37]. This indicates that CG is the major bioactive compound in QG.

The high-carbohydrate, high-fat diet-fed rat mimics most of the signs of metabolic syndrome in humans. This diet increased abdominal fat deposition, plasma lipids, liver enzyme activities, liver weight, infiltration of inflammatory cells in the heart and liver, blood pressure and collagen deposition, and impaired glucose tolerance. In addition, increased left ventricular stiffness and diminished aortic responses were observed when compared to rats fed a low-fat, corn starch-rich diet [31].

Both CG and QG improved cardiovascular and hepatic structure and function and reduced metabolic parameters such as body weight gain, visceral adiposity index and total body fat mass induced by the H diet, consistent with the metabolic responses to purple carrots [29] and purple corn [24], both dietary sources of cyanidin glycosides. CG potentially increased fatty acid oxidation *via* AMP-activated protein kinase (AMPK) signalling [38]. AMPK activation leads to acetyl-CoA carboxylase phosphorylation and inactivation, which stimulates CPT1 expression, thereby increasing fatty acid oxidation, leading to decreased abdominal fat and improved glucose metabolism [38]. Decreases in total body fat mass correlated with decreases in visceral adiposity as CG and QG reduced the weight of all abdominal fat pads. Leptin is a hormone secreted from adipose tissue [39] and the decrease in adipose tissue with CG and QG correlated with decreased plasma leptin concentrations. Similarly, CG and QG supplementation improved plasma lipid profiles by reducing plasma concentrations of triglycerides, total cholesterol and NEFA.

Dysfunction of the left ventricle correlates with metabolic changes, oxidative stress and increased inflammatory cell infiltration, with an increase in left ventricular fibrosis and stiffness [40]. We have also shown that CG and QG improved cardiovascular function. The decrease in left ventricular weight suggests that CG and QG remodelled the structural damage caused by the H diet. It is clear from echocardiographic assessment that CG and QG improved systolic function and left ventricular dimensions, probably by adapting to the reduced wall stress. Additionally, CG and QG rats decreased the ejection time with no change in ejection fraction suggesting that a decreased rate of contraction was required to eject the smaller diastolic volume, correlating with reduced blood pressure and reduced left ventricular internal size during systole and diastole. Decreased blood pressure and diastolic stiffness with CG and QG supplementation also correlated with decreased collagen deposition in left ventricle. Our results also suggested that CG and QG supplementation improved vascular function by improving endothelial responses, perhaps by increasing NO concentrations as shown in similar studies with anthocyanin-containing chokeberry and bilberry extracts [41]. This improved vascular function by CG and QG is consistent with the findings that anthocyanins inhibited inflammatory cytokines such as TNF-α, induced signal transducer and activator of transcription (STAT3) phosphorylation, inducible NO synthase, IL-1β and IL-6 by inhibiting the activation of NF-κB [20,42,43]. Extending these effects could be the reason for reduced inflammatory cell infiltration in left ventricle and liver with supplementation of CG and QG. Both CG and QG supplemented rats showed decreased liver weight, with decreased fat vacuoles and decreased hepatic inflammation. Decreases in liver weight, steatosis and inflammation directly correlated with reduced plasma activity of liver enzymes.

The absence of intact anthocyanins and their common methylated and conjugated metabolites in plasma was similar to the results of previous studies in which these compounds were not detected in plasma of rats and pigs following feeding with anthocyanin-containing foods (blackberries or blueberries) [44–46]. This was presumed to be due to the rapid absorption and metabolism of the anthocyanins. Similar findings of observed biological effects but no detected anthocyanins were also reported with pre- and mildly hypertensive human subjects [47]. In these subjects, blood pressure was lowered with tea containing delphinidin and cyanidin glycosides, but the anthocyanins were not detected in the collected plasma and urine samples, possibly because of insufficient sensitivity of the HPLC method to detect the anthocyanins [47]. Further, hippuric acid, a colon microbial/liver-derived metabolite of dietary polyphenols and anthocyanins, may represent the final product of the *in vivo* biotransformation of these plant compounds. However, it can also be generated by the metabolic degradation and transformation of amino acids and fibre [44]. Similar plasma hippuric acid concentrations between treatments, following 8 weeks of CG or QG diet, is also consistent with the findings of a study in which rats received either a control diet or a blueberry powder-supplemented diet [44]. Plasma hippuric acid concentrations in rats may therefore not be considered as a reliable biomarker to assess anthocyanin absorption and metabolism. However, a relationship between the urinary excretion rate of hippuric acid and the ingested amount of blueberry anthocyanins has been demonstrated [44], suggesting that concentrations of hippuric acid in the urine, in contrast to plasma, may represent a potential biomarker for anthocyanin absorption and metabolism. Due to technical reasons, no urine samples were available for such analysis.

Further, the metabolism of the anthocyanins, especially cyanidin glycosides, by gut microbiota to protocatechuic acid [48,49], one of the hydroxybenzoic acids that reduce blood pressure and improve lipid profiles [50], could be another reason for the improvements in CG and QG rats and could also explain the low or absent anthocyanin concentrations in plasma. Mice fed with a high-fat, high-sucrose diet became obese with increased plasma concentrations of cholesterol and triglycerides; treatment with anthocyanin-containing cranberry juice reversed these features of metabolic syndrome. These effects were associated with decreases in intestinal inflammation and increases in gut bacteria especially *Akkermansia* spp. [51]. In mice, *Akkermansia muciniphila* increased with increased dietary polyphenol intake and attenuated the high-fat diet-induced metabolic syndrome [52]. Increased *Bifidobacteria* in faeces together with increased urinary concentrations of anthocyanin metabolites including syringic acid, *p*-coumaric acid, 4-hydroxybenzoic acid and homovanillic acid confirm the important role of anthocyanins/polyphenols as bacterial substrates [53]. The colonic metabolites of anthocyanins such as phenolic acids produced by gut bacteria may act as potential systemic bioactive compounds to produce the positive responses to anthocyanins [54,55]. Further, anthocyanins may act as prebiotics to increase the growth of beneficial gut bacteria [54]. The current study is limited as we did not analyse the gut microbiota.

5. Conclusion

Both CG and QG showed similar responses in reversing the signs of metabolic syndrome in rats fed a high-carbohydrate, high-fat diet. Reduction of body weight gain with decreased abdominal fat pads and improved lipid profile and glucose metabolism along with improved cardiovascular and hepatic structure and function suggests that both CG and QG can be possible treatments for reversing or attenuating the complications of metabolic syndrome. However, further investigation on CG and QG will be necessary to understand the mechanisms underlying their improvement of the signs of metabolic syndrome. The similar responses observed in CG and QG rats indicate that further investigations with QG are warranted to determine if these positive effects can be translated to obese or overweight humans.

Conflict of interest

The authors declare that there is no conflict of interest in the study.

Author contributions

M.B., S.K.P. and L.B. developed the original study aims. M.B. and L.B. analysed and interpreted the data; M.B. conducted the experiments. M.M. and S.K.P. provided nutritional advice. K.F. and M.N. assisted in Queen Garnet plum juice analysis and plasma analysis for metabolites. M.B. and L.B. prepared manuscript drafts, with all authors contributing to the final version. L.B. has been the corresponding author throughout the writing process. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.10.006.

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