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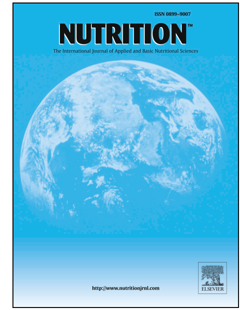

Anthocyanins in chokeberry and purple maize attenuate diet-induced metabolic syndrome in rats

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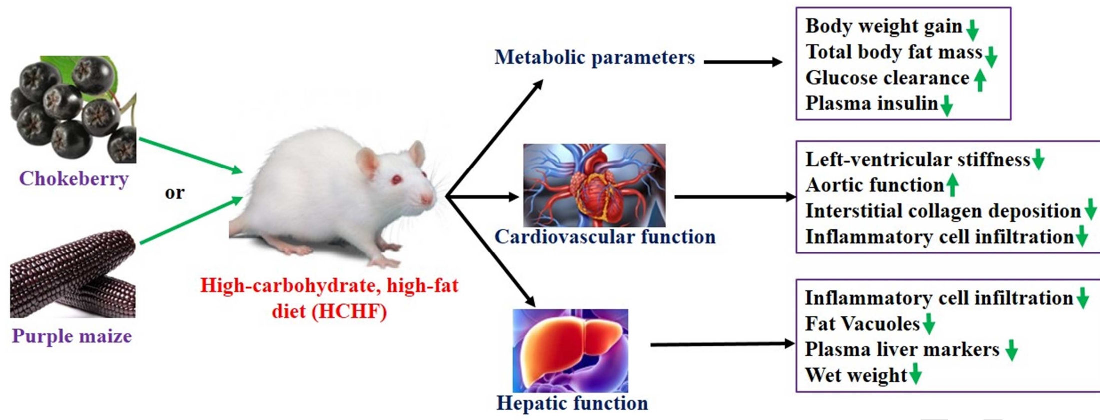

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1 *Nutrition*

2 **Anthocyanins in chokeberry and purple maize attenuate diet-induced metabolic**
3 **syndrome in rats**

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14 **Running Title:** Chokeberry and purple maize in metabolic syndrome
15

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27 **Abstract**

28 *Objective*: Increased consumption of fruits and vegetables as functional foods leads to the
29 reduction of signs of metabolic syndrome. In this study, we have compared and measured
30 cardiovascular, liver and metabolic parameters following chronic administration of the same
31 dose of anthocyanins either from chokeberry (CB) or purple maize (PM) in rats with diet-
32 induced metabolic syndrome. *Research methods and procedures*: Male Wistar rats were fed
33 with cornstarch diet (C) or high-carbohydrate, high-fat diet (H) diet and divided into six
34 groups for 16 week feeding with C, C with CB or PM for last 8 weeks (CCB or CPM), H, H
35 with CB or PM for last 8 weeks (HCB or HPM); CB and PM rats received ~8 mg
36 anthocyanins/kg/day. The rats were monitored for changes in blood pressure, cardiovascular
37 and hepatic structure and function, glucose tolerance and adipose tissue mass. *Results:* HCB
38 and HPM rats showed reduced visceral adiposity index, total body fat mass and systolic
39 blood pressure, improved glucose tolerance, liver and cardiovascular structure and function,
40 decreased plasma triglycerides and total cholesterol compared to H rats. Inflammatory cell
41 infiltration was reduced in heart and liver. *Conclusion:* CB and PM interventions gave similar
42 responses, suggesting that anthocyanins are the bioactive molecules in the attenuation or
43 reversal of metabolic syndrome by prevention of inflammation-induced damage.

44

45 ***Keywords***: anthocyanins; chokeberry; *Aronia melanocarpa*; purple maize; *Zea mays*;
46 metabolic syndrome

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47 **Introduction**

48 Fruits and vegetables rich in antioxidant phytochemicals such as flavonoids and
49 polyphenols are effective in attenuating the signs of metabolic syndrome [1, 2]. An increased
50 intake of fruits and vegetables has been associated with a decrease in cardiovascular diseases
51 [3], and better control of type 2 diabetes [4] and non-alcoholic fatty liver disease [5].
52 Anthocyanins are bioactive flavonoids that give red to purple colours to a wide range of fruits
53 and vegetables including rhubarb, cabbage, berries, cherries and red grapes [6]. Regular
54 consumption of anthocyanins has been credited with reducing risk of chronic diseases such as
55 obesity, non-alcoholic fatty liver, diabetes and cardiovascular diseases [7-9]. It is estimated
56 that ~1000 mg of polyphenols, including up to 215 mg of anthocyanins, are consumed daily
57 by an average adult in the USA [9, 10].

58 This study focuses on black chokeberry and purple maize as two rich dietary sources
59 of anthocyanins similar to purple carrots and Queen Garnet plums [11, 12]. Black chokeberry
60 (*Aronia melanocarpa*) is described as an attractive garden plant, native to eastern North
61 America but now grown widely in northern Europe, primarily Poland, where the sour fruit is
62 eaten raw or processed for incorporation into foods. Black chokeberries are rich in cyanidin
63 anthocyanins, chlorogenic acids and proanthocyanidins, and also contain quercetin flavonols
64 [13]. While these components indicate that black chokeberries may be an effective functional
65 food, more rigorous studies are needed to support popular indications for heart disease,
66 hypertension, hyperlipidaemia and urinary tract infections, as well as actions against bacteria
67 and viruses, and to strengthen memory and digestion [14, 15]. In metabolic syndrome
68 subjects, chokeberry extract decreased blood pressure and plasma lipid concentrations with
69 no change in body weight [16]. Chokeberry attenuated body weight gain and insulin
70 resistance in rats fed with fructose-rich diet [17].

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71 Purple maize has been cultivated in the Andean region, especially Peru and Bolivia, 72 for centuries where it is used as a food and a colourant in a drink believed to improve health 73 [18, 19]. Treatment with purple maize (*Zea mays*) decreased abdominal adiposity [20], 74 improved glucose metabolism [21] and decreased blood pressure in healthy humans [22]. 75 However, there is no clear evidence that intervention with these anthocyanin-containing 76 traditional functional foods will improve the widespread organ dysfunction observed in 77 patients with metabolic syndrome, despite improvements in individual signs.

78 This study has compared the cardiovascular, liver and metabolic responses of two 79 dietary sources of anthocyanins, chokeberry and purple maize, at the same daily anthocyanin 80 dose as in rats fed cyanidin 3-glucoside or Queen Garnet plums [12] using the same high-81 carbohydrate, high-fat diet as a model of human metabolic syndrome [23]. These 82 measurements included systolic blood pressure, echocardiography, vascular reactivity, 83 collagen deposition and stiffness of heart, plasma biochemistry and histology for structural 84 changes on heart and liver. Our results suggest that an adequate intake of foods containing 85 cyanidin-type anthocyanins can normalise the metabolic, cardiovascular and liver changes 86 induced by a high-carbohydrate, high-fat diet by decreasing infiltration of inflammatory cells 87 in the organs.

88 **Materials and methods**

89 *Analysis of chokeberry juice and purple maize flour*

90 Chokeberry juice (CB) was supplied by Fasbay Pty Ltd, Sydney, Australia and purple 91 maize flour (PM) was supplied by Spectrum Ingredients Pte Ltd, Singapore. The anthocyanin 92 contents were determined by HPLC based on the method outlined in the British 93 Pharmacopoeia 2014 (Eur. Pharm 2394) using an Agilent 100 series HPLC system. Briefly, 94 samples were prepared by extraction by 2% v/v HCl in methanol, using sonication for 15

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95 minutes in volumetric flasks, then made up to volume and diluted as required to be within the
96 standard calibration. Analysis was performed using a gradient of mobile phases A (water and
97 formic acid, 91.5:8.5) and B (acetonitrile, methanol, water and formic acid,
98 22.5:22.5:41.5:8.5) over 56 minutes. The gradient ran from 7 to 25% B in 35 minutes, to 65%
99 at 45 minutes followed by 100% B to 50 minutes and return to 7%. The column used was a
100 Phenomenex 250mm C18 5µm column with a flow rate of 1 mL per minute and temperature
101 30°C. Detection and quantification were performed using a diode array detector (DAD) at
102 535nm with cyanidin chloride (PhytoLab, CAS No. 528-58-5, B# 80022 5368) as the
103 calibrating standard. Total anthocyanins were calculated as cyanidin chloride and cyanidin 3-
104 glucoside by mass correction.

105 *Rats and diets*

106 The experimental groups consisted of 72 male Wistar rats (8-9 weeks old; weighing
107 335 ± 3 g) purchased from Animal Resource Centre, Murdoch, WA, Australia and
108 individually housed in a temperature-controlled (20±2°C), 12-hour light/dark cycle
109 environment with *ad libitum* access to water and rat diet at the University of Southern
110 Queensland Animal House. All experimentation was pre-approved by the Animal Ethics
111 Committee of the University of Southern Queensland under the guidelines of the National
112 Health and Medical Research Council of Australia. The rats were randomly divided into six
113 separate groups (n = 12 each) and fed with maize starch (C), maize starch + chokeberry juice
114 (CCB), maize starch + purple maize flour (CPM), high-carbohydrate, high-fat (H), high-
115 carbohydrate, high-fat + chokeberry juice (HCB) and high-carbohydrate, high-fat + purple
116 maize flour (HPM).

117 The preparation and macronutrient composition of basal diets, including the dietary
118 fatty acid profiles, have been described [23-25]. C and H rats received their diets for 16
119 weeks and CCB, CPM, HCB and HPM rats received C or H diets for the first 8 weeks while

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120 both diets were supplemented with chokeberry juice 50 ml/kg or purple maize flour 50 g/kg
121 by replacing equivalent amounts of water for a further 8 weeks. The drinking water in all H
122 diet-fed groups was augmented with 25% fructose for the duration of the study. Body weight
123 and food and water intakes were measured daily and feed efficiency (%) was calculated [24]
124 using the following equation:

$$\text{feed conversion efficiency (\%)} = \frac{\text{increase in body weight (\%)}}{\text{daily energy intake (kJ)}} \times 100$$

125 Increase in body weight (%): body weight difference between day 56 (week 8) and day 112
126 (week 16); daily energy intake: average of daily energy intake from week 8 to week 16.

127 *Oral glucose tolerance test*

128 For the oral glucose tolerance testing, the glucose concentrations in blood collected by
129 tail prick on tail vein of overnight food-deprived rats were measured using Medisense
130 Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, USA). Fructose-supplemented
131 drinking water in H, HCB and HPM rats was replaced with normal water for the overnight
132 food-deprivation period. The rats were given 2 g/kg body weight of glucose as a 40%
133 aqueous solution via oral gavage. Tail vein blood samples were taken at 30, 60, 90 and 120
134 minutes following glucose administration.

135 *Body composition measurements*

136 Dual energy X-ray absorptiometric (DXA) measurements were performed on rats
137 after 16 weeks of feeding, 2 days before rats were euthanased for pathophysiological
138 assessments, using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, USA).
139 DXA scans were analysed using the manufacturer's recommended software for use in
140 laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1, Norland Corp.,

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141 Fort Atkinson, USA) [24]. The precision error of lean mass for replicate measurements, with
142 repositioning, was 3.2%. Visceral adiposity index (%) was calculated [23].

143 *Cardiovascular measurements*

144 Systolic blood pressure was measured under light sedation following intraperitoneal
145 (i.p.) injection of Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst,
146 NSW, Australia) using a MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff
147 connected to a MLT844 Physiological Pressure Transducer using PowerLab data acquisition
148 unit (ADInstruments, Sydney, Australia) [24].

149 Anaesthesia using Zoletil (tiletamine 10 mg/kg and zolazepam 10 mg/kg i.p.) and
150 Ileum xylazil (xylazine 6 mg/kg; Troy Laboratories, Smithfield, NSW, Australia) was used
151 for echocardiographic examination (Hewlett Packard Sonos 5500, 12 MHz transducer)
152 performed at 16 week [23-25], in accordance with the guidelines of the American Society of
153 Echocardiography using the leading-edge method [26].

154 The left ventricular (LV) function of the rats in all groups was assessed using the
155 Langendorff heart preparation [23-25]. Terminal anaesthesia was induced via i.p. injection of
156 pentobarbitone sodium (Lethabarb®, 100 mg/kg). After heparin (200 IU; Sigma-Aldrich
157 Australia, Sydney, Australia) administration through the right femoral vein, blood (~5 mL)
158 was taken from the abdominal aorta. Isovolumetric ventricular function was measured by
159 inserting a latex balloon catheter into the left ventricle of the isolated heart connected to a
160 Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab
161 system (ADInstruments Australia and Pacific Islands, Bella Vista, NSW, Australia). All left
162 ventricular end-diastolic pressure values were measured during pacing of the heart at 250
163 beats per minute using an electrical stimulator. End-diastolic pressures were obtained starting
164 from 0 mmHg up to 30 mmHg.

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165 Thoracic aortic rings (~4 mm in length) were suspended in an organ bath chamber
166 with a resting tension of approximately 10 mN. Cumulative concentration-response
167 (contraction) curves were measured for noradrenaline (Sigma-Aldrich Australia, Sydney,
168 Australia); concentration-response (relaxation) curves were measured for acetylcholine
169 (Sigma-Aldrich Australia, Sydney, Australia) and sodium nitroprusside (Sigma-Aldrich
170 Australia, Sydney, Australia) in the presence of a submaximal (70%) contraction to
171 noradrenaline [25].

172 *Organ weights*

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

177 *Histology*

178 Two rats per group were taken exclusively for histological analysis. Two slides were
179 prepared per tissue specimen and two random, non-overlapping fields per slide were taken to
180 avoid biased analysis. Organs were also collected from rats used for perfusion studies.
181 Immediately after removal, heart and liver tissues were fixed in 10% neutral buffered
182 formalin for 3 days and then dehydrated and embedded in paraffin wax [23-25]. Thin sections
183 (~5 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain
184 for determination of inflammatory cell infiltration with a 20× objective using a Olympus
185 BX51 microscope (Olympus, Melville, NY). Left ventricular sections were stained with
186 picrosirius red to determine collagen distribution. Laser confocal microscopy (Nikon A1R+
187 upright Confocal Microscope) was used to determine collagen distribution in left ventricular
188 sections.

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189 *Plasma biochemistry*

190 Blood was centrifuged at 5,000*g* for 15 minutes within 30 minutes of collection into
191 heparinised tubes. Plasma was separated and transferred to microcentrifuge tubes for storage
192 at -20°C before analysis. Activities of plasma enzymes and analyte concentrations were
193 determined using kits and controls supplied by Olympus using an Olympus analyser (AU
194 400, Tokyo, Japan) [23-25]. Plasma insulin and leptin concentrations (ALPMO, USA) were
195 estimated using commercial ELISA kits according to manufacturer-provided standards and
196 protocols.

197 *Statistical analysis*

198 Data are presented as mean ± SEM. Results were tested for variance using Bartlett's
199 test and variables that were not normally distributed were transformed (using log 10 function)
200 prior to statistical analyses. Data from C, CCB, CPM, H, HCB and HPM groups were tested
201 for effects of diet, treatment and their interactions by two-way ANOVA. When interaction
202 and/or the main effects were significant, means were compared using a Newman-Keuls
203 multiple comparison *post hoc* test. Where transformations did not result in normality or
204 constant variance, a Kruskal-Wallis non-parametric test was performed. A *P*-value of <0.05
205 was considered as statistically significant. All statistical analyses were performed using
206 GraphPad Prism version 6.00 for Windows (San Diego, California, USA).

207 **Results**

208 *Diet and body composition*

209 CB and PM contained similar concentrations of total anthocyanins with cyanidin 3-
210 glucoside as the major anthocyanin (Table 1). The average daily intake of anthocyanins was
211 higher in CCB and CPM rats compared to HCB and HPM rats, as the food intake was higher
212 in CCB and CPM rats (Table 2). Compared to C rats, H rats consumed less food but a similar

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213 amount of water (Table 2). Despite the lower food intake, the mean energy intake, feed
214 efficiency and the increases in body weight were higher in H rats than in C rats (Table 2).
215 Chronic H diet feeding for 16 weeks increased abdominal circumference, body mass index,
216 total body fat mass and the individual abdominal fat pads, and increased the visceral
217 adiposity index (Table 2). No change in total body lean mass was measured (Table 2). The
218 bone mineral content was higher in H rats compared to C rats (Table 2).

Table 1. *Chokeberry juice and purple maize flour analysis*

Variables	Chokeberry juice/ 100 ml	Purple-maize flour/ 100 g
Total anthocyanins (mg)[#$]	240	220
Energy (KJ)[*]	279	1,592
Protein (g)[*]	16.1	7.8
Total fat (g)[*]	0.5	4.2
Total carbohydrates (g)[*]	0.2	76.7

Values are represented as mean of duplicate assays.

[#] Analysed by authors.

[$] Total anthocyanins calculated as cyanidin 3-glucoside.

219 [*] Analysed by a commercial laboratory (Symbio Alliance, Brisbane, QLD, Australia).

220

221 Treatment with either CB or PM for 8 weeks, starting at 8 weeks of the feeding
222 period, did not change food or water intake (Table 2). Compared to controls, CB treatment
223 groups had similar energy intake while PM treatment groups had an increased energy intake.
224 Lower feed conversion efficiency and body weight gain were observed in HCB and HPM rats
225 (Table 2). Both treatments decreased total body fat mass, except CPM rats had higher total
226 body fat mass compared to C rats (Table 2). Abdominal fat (retroperitoneal, epididymal and
227 omental fat pads), body mass index and visceral adiposity index decreased in both HCB and

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228 HPM rats (Table 2). Total lean mass was unchanged in both HCB and HPM rats, and HCB
229 rats had decreased bone mineral content compared to H rats (Table 2).

230 *Plasma biochemistry and glucose handling*

231 Plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids
232 (NEFA) were increased in H rats compared to C rats (Table 2). HCB and HPM rats showed
233 decreased plasma lipid concentrations, compared to H rats. However, HPM rats had increased
234 concentrations of triglycerides and NEFA than HCB rats (Table 2). Plasma leptin
235 concentrations were doubled in H rats compared to C rats. HCB and HPM showed
236 normalised leptin concentrations, consistent with the changes in total fat mass and abdominal
237 fat pads (Table 2).

238 H rats had increased fasting blood glucose concentrations compared to C rats; HCB
239 and HPM rats showed similar concentrations to C rats (Table 2). The plasma glucose
240 response to oral glucose loading was greater in H rats than C rats (Figure 1). At 120 min,
241 HCB and HPM rats along with C rats had lower plasma glucose concentrations compared to
242 H rats (Figure 1). Plasma insulin concentrations almost doubled in H rats compared to C,
243 HCB and HPM rats (Table 2). This change is consistent with glucose tolerance area under the
244 curve (Table 2); similarly, HCB and HPM rats had improved glucose clearance compared to
245 H rats (Table 2).

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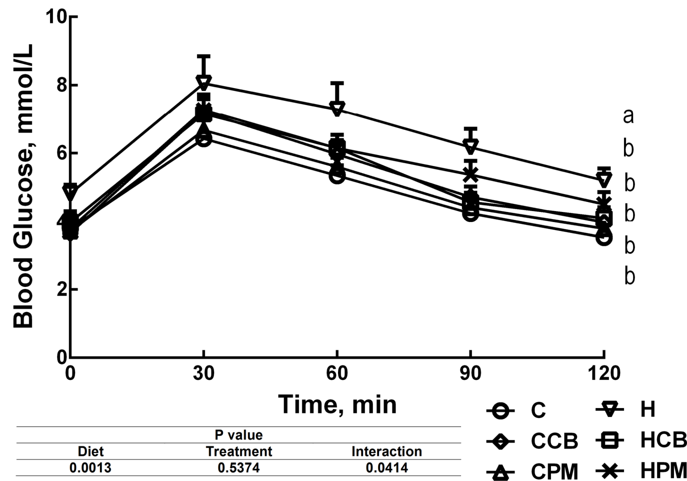

Figure 1. *Effect of CB and PM on oral glucose tolerance in C, CCB, CPM, H, HCB and HPM rats. Data are shown as mean ± SEM. End-point means without a common letter in each data set significantly differ, P<0.05 and n=10/group.*

Cardiovascular structure and function

Compared to C rats, H rats showed eccentric hypertrophy measured as increased left ventricular internal diameter in diastole (LVIDd) without any changes in relative wall thickness or end systolic dimensions (Table 3). H rats showed impaired systolic function with decreased fractional shortening and increased wall stress compared to C rats (Table 3). However, ejection time and ejection fraction were not affected (Table 3). Diastolic and stroke volumes and consequently the cardiac output were increased in H rats compared to C rats. These effects were seen with increased heart rate in H rats compared to C rats (Table 3). These changes in H rats were accompanied by increased LV wet weight and elevated systolic blood pressure (Table 3).

Both CB and PM treatment improved LV function by decreasing LVIDd and normalising developed pressure (Table 3). Ejection time, ejection fraction, fractional shortening and LVIDs were unaffected in both treatment groups (Table 3). Systolic wall stress, cardiac output, diastolic and stroke volumes and heart rate were normalised with CB and PM

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265 treatments. However, systolic volumes were elevated in both HCB and HPM rats with no
266 change in relative wall thickness (Table 3). In the isolated Langendorff heart, LV stiffness
267 was increased while LV dP/dt was decreased in H rats; these changes were normalised in
268 HCB and HPM. These effects were accompanied by decreased LV wet weight and systolic
269 blood pressure in both HCB and HPM rats (Table 3).

270 Histological evaluation of the left ventricle after 16 weeks showed greater infiltration
271 of inflammatory cells into the LV with H diet feeding (Figure 2D), as well as increased
272 interstitial collagen deposition (Figure 2J) compared to C rats (Figure 2A and G). HCB and
273 HPM rats showed normalised inflammatory cell numbers (Figure 2E and F) and markedly
274 reduced ventricular collagen deposition (Figure 2K and L). The reduction in LV fibrosis and
275 inflammation was consistent with the reduced diastolic stiffness in HCB and HPM rats (Table
276 3), while CCB (Figure 2B and H) and CPM (Figure 2C and I) rats showed minimal changes.
277 No other changes were observed and tissue morphology appeared normal.

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Table 2. *Dietary intakes, body composition and anthropometrics, organ wet weights, changes in glucose tolerance test, plasma insulin and plasma biochemistry in C, CCB, CPM, H, HCB and HPM diet-fed rats (n=10 rats/group)*

Variable	C	CCB	CPM	H	HCB	HPM	P values		
							Diet	**Treatment**	**Interaction**
Food intake (g/d)	32.9±1.1^{a}	33.2±0.6^{a}	34.1±0.4^{a}	27.1±0.8^{b}	26.4±0.8^{b}	28.5±0.7^{b}	<0.0001	0.11	0.7
Water intake (ml/d)	27.4±2.1	24.3±1.5	27.2±1.8	26.6±1.4	26.5±1.6	29.7±1.5	0.34	0.2	0.55
Chokeberry juice intake (ml/d)	0.0±0.0	1.7±0.0^{a}	0.0±0.0	0.0±0.0	1.4±0.0^{b}	0.0±0.0	<0.0001	<0.0001	<0.0001
Purple maize powder intake (g/d)	0.0±0.0	0.0±0.0	1.8±0.0^{a}	0.0±0.0	0.0±0.0	1.5±0.0^{b}	<0.0001	<0.0001	<0.0001
Anthocyanins intake (mg/kg/d)	0.0±0.0	9.4±0.0^{a}	9.1±0.0^{a}	0.0±0.0	7.8±0.0^{b}	7.4±0.0^{b}	<0.0001	<0.0001	<0.0001
Energy intake (kJ/d)	369±12^{d}	378±7^{d}	409±5^{c}	580±17^{b}	570±19^{b}	640±17^{a}	<0.0001	0.0005	0.39
Feed conversion efficiency (%)	2.4±0.3^{b}	2.1±0.3^{b}	2.9±0.3^{b}	7.1±0.9^{a}	4.4±1.3^{b}	5.5±0.9^{ab}	<0.0001	0.16	0.25
Body weight gained (8-16 weeks) (%)	8.2±1.4^{b}	7.7±1.4^{b}	8.3±1.3^{b}	21.6±2.6^{a}	9.9±2.8^{b}	10.8±1.6^{b}	0.0004	0.0051	0.0075
Visceral adiposity index (%)	4.9±0.4^{b}	4.4±0.3^{b}	4.8±0.2^{b}	8.9±0.9^{a}	6.1±0.5^{b}	6.0±0.3^{b}	<0.0001	0.0021	0.0137
Abdominal circumference (cm)	19.2±0.1^{c}	18.2±0.2^{d}	19.1±0.1^{cd}	22.3±0.3^{a}	19.7±0.4^{c}	20.7±0.2^{b}	<0.0001	<0.0001	0.0022
Body mass index (kg/m^{2})	5.0±0.2^{d}	4.8±0.1^{d}	4.7±0.1^{d}	7.3±0.2^{a}	5.7±0.1^{c}	6.4±0.2^{b}	<0.0001	<0.0001	0.0002
Bone mineral content (g)	11.3±0.3^{c}	11.4±0.3^{c}	11.5±0.3^{c}	15.8±0.4^{a}	13.5±0.5^{b}	14.8±0.4^{a}	<0.0001	0.0166	0.009
Total body lean mass (g)	309±12	303±12	307±11	311±14	305±11	304±8	0.98	0.87	0.98
Total body fat mass (g)	74.3±7.6^{d}	94.4±8.4^{d}	113.7±8.5^{cd}	224.6±12.6^{a}	144.4±17.2^{c}	177.3±10.4^{b}	<0.0001	0.0203	<0.0001
Tissue wet weight (mg/mm)									
Retroperitoneal adipose tissue	179.7±15.3^{c}	141.9±13.7^{c}	173.0±12.6^{c}	521.7±45.6^{a}	279.2±38.2^{b}	311.8±16.7^{b}	<0.0001	<0.0001	0.0003
Epidydimal adipose tissue	93.4±7.9^{c}	83.3±6.9^{c}	95.4±5.2^{c}	278.5±22.5^{a}	155.4±15.2^{b}	156.8±11.7^{b}	<0.0001	<0.0001	<0.0001
Omental adipose tissue	106.6±8.1^{d}	91.8±8.0^{d}	101.1±5.5^{d}	261.1±21.3^{a}	141.0±12.3^{cd}	193.1±9.9^{b}	<0.0001	<0.0001	0.0002
Liver	214.2±6.7^{c}	188.8±6.2^{c}	204.1±5.5^{c}	319.6±8.9^{a}	250.7±11.1^{b}	268.6±7.0^{b}	<0.0001	<0.0001	0.0111
Glucose metabolism and plasma biochemistry									
Fasting blood glucose (mmol/L)	3.8±0.2	3.7±0.2	3.7±0.3	4.8±0.3	3.9±0.3	3.8±0.3	0.0321	0.17	0.08
OGTT-AUC (mmol/L min)	591±12^{d}	645±9^{c}	613±6^{d}	786±18^{a}	658±7^{c}	694±6^{b}	<0.0001	0.001	<0.0001
Plasma insulin (μmol/L)	1.4±0.3^{b}	1.1±0.2^{b}	1.7±0.3^{b}	4.1±0.5^{a}	2.3±0.4^{b}	2.6±0.6^{b}	<0.0001	0.042	0.07
Plasma leptin (μmol/L)	5.3±0.7^{b}	4.9±0.6^{b}	6.1±0.5^{b}	11.1±0.9^{a}	7.0±1.5^{b}	7.9±1.0^{b}	<0.0001	0.06	0.06
ALP (U/L)	181±12^{c}	214±16^{c}	166±12^{c}	312±18^{a}	252±20^{bc}	265±18^{bc}	<0.0001	0.17	0.0224

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ALT (U/L)	29.6±2.1^{b}	28.1±2.2^{b}	29.7±1.5^{b}	43.2±2.8^{a}	34.6±3.9^{ab}	38.0±3.1^{ab}	<0.0001	0.19	0.4
AST (U/L)	60.4±1.9^{b}	60.9±2.7^{b}	64.0±1.6^{b}	83.5±3.1^{a}	63.1±7.9^{b}	64.6±2.9^{b}	0.0099	0.0383	0.0099
Total cholesterol (mmol/L)	1.5±0.2^{b}	1.5±0.1^{b}	1.6±0.1^{b}	2.2±0.0^{a}	1.6±0.1^{b}	1.6±0.0^{b}	0.0038	0.0166	0.0039
Triglycerides (mmol/L)	0.5±0.0^{c}	0.4±0.0^{c}	0.5±0.0^{c}	1.6±0.2^{a}	0.7±0.1^{c}	1.0±0.1^{b}	<0.0001	<0.0001	0.0005
NEFA (mmol/L)	1.3 ±0.2^{c}	1.2 ±0.1^{c}	1.4 ±0.1^{c}	3.4 ±0.2^{a}	1.8 ±0.4^{c}	2.3 ±0.3^{b}	<0.0001	0.0036	0.0073

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, $P<0.05$.
* In all groups body-weight gained calculated as percentage of body weight increase from 8 weeks to 16 weeks. OGTT-AUC, oral glucose tolerance test-area under the curve; ALP, alkaline phosphatase; ALT, aspartate transaminase; AST, aspartate transaminase; NEFA, non-esterified fatty acids.

Insert Figure 2 here.

Figure 2. *Haematoxylin and eosin staining of the left ventricle (original magnification ×20) showing inflammatory cells (marked as "in") as dark spots outside the myocytes in C (A), CCB (B), CPM (C), H (D), HCB (E) and HPM (F) rats. Picrosirius red staining of left-ventricular interstitial collagen deposition (original magnification ×20) in rats fed the C (G), CCB (H), CPM (I), H (J), HCB (K) and HPM (L) diet. Collagen deposition is marked as "cd" and hypertrophied cardiomyocytes are marked as "hy".*

Table 3. *Changes in cardiovascular structure and function in C, CCB, CPM, H, HCB and HPM diet-fed rats (n=10-8 rats/group)*

							P values		
							Diet	**Treatment**	**Interaction**
Variable	**C**	**CCB**	**CPM**	**H**	**HCB**	**HPM**			
Heart rate (bpm)	277±18^{b}	246±9^{b}	256±15^{b}	335±16^{a}	243±9^{b}	265±11^{b}	0.06	0.0001	0.06
IVSd (mm)	1.9±0.1^{ab}	1.8±0.0^{b}	1.9±0.0^{ab}	2.1±0.1^{a}	1.9±0.1^{ab}	1.9±0.0^{ab}	0.09	0.11	0.38
LVIDd (mm)	6.4±0.2^{b}	6.7±0.3^{b}	7.0±0.1^{b}	7.9±0.3^{a}	7.1±0.2^{b}	7.2±0.2^{b}	0.0005	0.51	0.0139
LVPWd (mm)	1.8±0.1^{b}	1.7±0.0^{b}	1.8±0.0^{b}	2.1±0.0^{a}	1.9±0.1^{b}	1.9±0.0^{b}	0.0001	0.0393	0.23
IVSs (mm)	3.2±0.2^{b}	3.0±0.1^{b}	2.9±0.0^{b}	3.8±0.1^{a}	3.2±0.1^{b}	3.3±0.1^{b}	<0.0001	0.0022	0.42
LVIDs (mm)	3.7±0.2	4.0±0.2	3.7±0.2	4.5±0.2	3.7±0.3	4.3±0.3	0.07	0.58	0.06
LVPWs (mm)	2.9±0.1^{b}	2.6±0.1^{b}	2.7±0.1^{b}	3.4±0.1^{a}	3.2±0.1^{ab}	3.0±0.1^{ab}	<0.0001	0.0099	0.32

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Fractional shortening (%)	50.9±2.1[a]	53.7±0.8[a]	53.4±0.9[a]	47.5±2.1[ab]	58.0±1.2[a]	55.7±1.4[a]	0.39	0.0002	0.0392
Ejection time (ms)	79.6±2.3	93.3±3.4[ab]	86.0±2.7	92.8±2.9[ab]	86.4±3.9	95.0±3.0[ab]	0.0486	0.33	0.0053
Ejection fraction (%)	87.3±1.4	83.3±1.3	84.6±1.6	89.0±2.2	84.1±4.0	83.6±2.9	0.8	0.14	0.85
Diastolic volume (µL)	353±34[b]	365±33[b]	364±22[b]	515±39[a]	386±28[b]	395±37[b]	0.01	0.15	0.07
Systolic volume (µL)	45±10[b]	57±8[a]	56±8[a]	90±10[a]	65±23[a]	63±12[a]	0.0203	0.72	0.11
Stroke volume (µL)	268±18[b]	298±20[b]	307±18[b]	425±29[a]	306±26[b]	322±19[b]	0.0018	0.13	0.002
Cardiac output (mL/min)	92.3±11.2[b]	70.4±13.6[b]	78.6±6.6[b]	144.8±21.7[a]	93.0±7.7[b]	100.3±10.7[b]	0.0038	0.0165	0.41
LV developed pressure (mmHg)	69.6±3.5[a]	71.4±4.1[a]	64.8±5.6[a]	43.7±3.6[b]	63.7±5.7[a]	60.2±3.8[a]	0.001	0.06	0.0444
(+)dP/dt (mmHg/S)	1147±61[a]	1285±53[a]	1195±65[a]	784±66[c]	1089±63[a]	1002±76[ab]	<0.0001	0.0044	0.33
(-)dP/dt (mmHg/S)	-782±51[a]	-804±49[a]	-795±43[a]	-489±57[b]	-700±44[a]	-711±52[a]	0.0002	0.0316	0.08
Diastolic stiffness (k)	22.9±0.8[b]	22.3±0.4[b]	23.1±0.6[b]	28.6±0.6[a]	23.9±0.7[b]	24.2±0.5[b]	<0.0001	0.0002	0.0006
Estimated LV mass, Litwin (g)	0.93±0.06[b]	0.79±0.08[b]	0.88±0.02[b]	1.14±0.05[a]	1.01±0.06[ab]	1.10±0.04[ab]	<0.0001	0.05	0.99
LV+septum wet weight (mg/mm tibial length)	16.1±0.5[c]	15.3±0.5[c]	15.0±0.5[c]	19.5±0.8[a]	16.9±0.8[c]	18.5±0.5[b]	<0.0001	0.0268	0.23
Right ventricle wet weight (mg/mm tibial length)	3.8±0.2	3.8±0.2	4.0±0.2	4.4±0.2	4.3±0.3	5.0±0.4[a]	0.0018	0.18	0.6
Relative wall thickness	0.50±0.03	0.57±0.09	0.53±0.02	0.56±0.03	0.51±0.01	0.48±0.01	0.63	0.69	0.29
Systolic blood pressure (mmHg)	130±2[b]	130±3[b]	132±2[b]	152±2[a]	120±1[b]	134±3[b]	<0.0001	<0.0001	<0.0001
Systolic wall stress (mmHg)	83.0±4.0[b]	79.1±4.3[b]	75.3±11.8[b]	119.6±7.6[a]	76.8±7.5[b]	89.5±9.7[b]	0.0171	0.0129	0.06

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

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297 H diet feeding diminished α_1-adrenoceptor-mediated vascular contraction to
298 noradrenaline (Figure 3A), endothelium-independent relaxation to sodium nitroprusside
299 (Figure 3B) and endothelium-dependent relaxation to acetylcholine (Figure 3C) in isolated
300 thoracic aortic rings compared to C rats. Isolated thoracic aortic rings from HCB and HPM
301 rats showed increased responses to noradrenaline (Figure 3A), sodium nitroprusside (Figure
302 3B) and acetylcholine (Figure. 3C).

303 Insert Figure 3 here.

304 ***Figure 3.*** *Cumulative concentration-response curves for noradrenaline (A), sodium*
305 *nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, CCB, CPM, H, HCB*
306 *and HPM rats. Data are shown as mean ± SEM. End-point means without a common letter in*
307 *each data set significantly differ, P<0.05 and n=10/group.*
308

309 *Liver structure and function*

310 Plasma alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate
311 transaminase (AST) activities were increased in H rats compared to C rats, indicating liver
312 damage (Table 2). HCB and HPM showed lowered but not normalised ALP, ALT and AST
313 activities (Table 2). H rats showed increased inflammatory cell infiltration and lipid
314 deposition as fat vacuoles in the liver (Figure 4D) compared to C rats (Figure 4A). In HCB
315 and HPM rats, macrovesicular steatosis and portal inflammation were decreased compared to
316 H rats (Figure 4E and F). Livers from CCB and CPM rats showed normal tissue architecture
317 (Figure 4B and C).

318 Insert Figure 4 here

319 **Figure 4.** Haematoxylin and eosin staining of hepatocytes (original magnification
320 ×20) showing inflammatory cells (marked as “in”) and hepatocytes with fat vacuoles (marked
321 as “fv”) in C (A), CCB (B), CPM (C), H (D), HCB (E) and HPM (F) rats.

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322 **Discussion**

323 Rats fed with H diet developed abdominal obesity, hypertension with endothelial
324 dysfunction, cardiac fibrosis together with increased left-ventricular stiffness, dyslipidaemia,
325 inflammation in heart and liver, increased plasma liver enzyme concentrations and impaired
326 glucose tolerance [23]. Excess fat deposition in the abdomen increases chronic low-grade
327 inflammation, oxidative stress, dyslipidaemia, non-alcoholic fatty liver disease,
328 cardiovascular diseases, type 2 diabetes and insulin resistance [27-32]. In this study, we
329 showed that intervention with the same dose of anthocyanins as cyanidins from either
330 chokeberry or purple maize attenuated the metabolic, cardiovascular and liver changes in rats
331 fed a H diet. We suggest that these improvements derive from decreased inflammatory cell
332 infiltration into the tissues.

333 Recent studies suggest that modification of the gut microbiome by anthocyanins could
334 be important in mediating the reduced inflammation throughout the body. Anthocyanins are
335 extensively metabolised by gut microbiota to protocatechuic acid [33, 34], one of a group of
336 phenolic acids produced by gut bacteria that may act as potential systemic bioactive
337 compounds to produce the positive responses to anthocyanins [35, 36]. Further, treatment
338 diet-induced obesity in mice with anthocyanins decreased intestinal inflammation and
339 increased gut bacteria especially *Akkermansia* spp. [37]. Increased *Bifidobacteria* in faeces
340 together with increased urinary concentrations of anthocyanin metabolites confirm the
341 important role of anthocyanins as bacterial substrates [38]. Moreover, anthocyanins may act
342 like prebiotics to increase the growth of beneficial gut bacteria [35]. Thus, anthocyanins may
343 improve gut health, possibly decreasing the access of bacterial components into the body, and
344 also by producing metabolites that improve health when absorbed. These gastrointestinal
345 responses may decrease the systemic inflammatory stimulus that increases lipid uptake by

346 adipocytes and so trigger further changes in the body, especially modifying the production
347 and release of adipokines.

348 Abdominal adipose tissue is a dynamic organ producing adipokines that have pro-
349 inflammatory or anti-inflammatory responses. Dysregulated production of these adipokines
350 leads to obesity and also induces low-grade inflammation and insulin resistance [39]. In the
351 current study, plasma insulin concentrations were normalised in rats fed chokeberry and
352 purple maize, consistent with a previous study with the same dose of cyanidin 3-glucoside
353 [12] and in HFD-fed mice treated with purified mulberry anthocyanins [40]. In our diet-
354 induced obese rats, plasma leptin concentrations were increased; further, treatment with
355 either chokeberry or purple maize reduced both plasma leptin concentrations and abdominal
356 fat mass. Similar results were shown with chokeberry extract [17], cyanidin 3-glucoside and
357 Queen Garnet plums [12]. All these interventions contain cyanidin 3-glucoside as the major
358 anthocyanin and this compound is the likely bioactive component. Since leptin is pro-
359 inflammatory, reduced leptin concentrations should reduce inflammation throughout the
360 body, as we have shown in the heart and liver. Thus, we suggest that normalisation of
361 adipokine production may be the key systemic change as fat pads reduce in response to
362 changes by the gut microbiota, thus further reducing inflammation throughout the body and
363 also reducing organ damage. Our results showing fat pad reduction following anthocyanin
364 intervention are consistent with studies with purple maize extract in C57BL/6J mice [20].
365 Further, KK-*Ay* mice treated with anthocyanins showed similar changes together with
366 decreases in mean diameter of the visceral and subcutaneous adipocytes, suggesting that
367 anthocyanin supplementation inhibits lipid accumulation [41].

368 Our study suggests that anthocyanin treatment decreased triglycerides and non-
369 esterified free fatty acids which attenuated the liver steatosis. Cyanidin 3-glucoside showed
370 increased phosphorylated AMP-activated protein kinase (pAMPK) and decreased lipoprotein

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371 lipase activity in skeletal muscle and adipocytes [41]. In addition, purple sweet potato
372 treatment in diet-induced obese mice and HepG2 hepatocytes showed similar
373 phosphorylation of AMPK [42]. AMP-activated protein kinase (AMPK) regulates and
374 monitors cellular energy balance [43] and pAMPK stimulates free fatty acid oxidation via
375 activation of acetyl coenzyme–A carboxylase in skeletal muscle [44] and regulates lipolysis
376 and lipogenesis by converting adipocytes into lipid oxidising cells [45]. Anthocyanins also
377 down-regulated lipid metabolism proteins, sterol regulatory element-binding protein-1c and
378 fatty acid synthase via AMPK inhibitor compound C [42, 46]. Additionally, in C57BL/KsJ
379 db/db mice treated with purple maize extract also increased pAMPK and decreased
380 phosphoenolpyruvate carboxykinase and glucose 6-phosphatase gene expression in liver and
381 glucose transporter 4 expression in skeletal muscle [47]. Therefore, these changes may
382 decrease adipose storage leading to decrease in body weight gain and improved liver function
383 and glucose metabolism.

384 Cardiovascular structure and function was improved by anthocyanins in the CB and
385 PM interventions together with decreased plasma concentrations of non-esterified free fatty
386 acids (NEFA). Increased plasma NEFA inhibited aortic endothelia nitric oxide synthase via
387 oxidative mechanism and caused hypertension [48]. In subjects with metabolic syndrome,
388 supplementation with CB extract and PM extract powder decreased blood pressure and
389 plasma lipids [16, 22]. Similarly, many epidemiological studies suggest that increased dietary
390 intake of anthocyanin-containing strawberries, blueberries and moderate intake of red wine is
391 associated with a reduction in cardiovascular disease [9]. However, there is no direct
392 evidence that anthocyanins are helpful in decreasing cardiovascular disease in humans. In
393 cultured bovine artery endothelial cells, cyanidin 3-glucoside increased the expression of
394 endothelial nitric oxide synthase (eNOS) [49]. Increased expression of eNOS enhanced nitric
395 oxide release to improve endothelial function [50]. Similarly, chokeberry juice treatment

396 showed improved endothelial function in porcine coronary arteries by redox-sensitive
397 activation [51]. Anthocyanin treatment with CB and PM also showed consistent improvement
398 in endothelial function and decreased blood pressure.

399 In this study, we have treated rats either with 3.2 mg/kg BW of CB juice or 3.1 mg/kg
400 BW of PM powder, corresponding to ~46 ml/day CB or ~50 g/day PM to achieve a dose of
401 ~110 mg of anthocyanins in a 70 kg human, based on body surface area comparisons between
402 rats and humans [52]. Similar results to the same dose of anthocyanins either from CB and
403 PM indicate that anthocyanins are the major bioactive compound in CB and PM in reversing
404 or attenuating the signs of metabolic syndrome.

405 **Conclusion**

406 The effects of CB and PM in a diet-induced rat model of human metabolic syndrome
407 are consistent with the reported effects of anthocyanins as they are the only polyphenolic
408 compounds present in sufficient dose in both the treatments. These findings suggest that
409 anthocyanin interventions using chokeberry or purple maize might be beneficial in
410 attenuating obesity and metabolic syndrome in humans. However, further investigations on
411 anthocyanin-containing foods are necessary to understand the mechanism of action,
412 especially to determine the changes in the gut microbiota. Similar responses in CB- and PM-
413 treated rats suggest a clinical trial corresponding to the same dose as in this study is necessary
414 to determine if these positive effects can be translated to humans with metabolic syndrome.

415

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421 **Author contributions**

422 M.B. and L.B. developed the original study aims and analysed and interpreted the data; M.B.
423 and S.R.S conducted the experiments. M.M. provided nutritional advice in the design of the
424 study. P.M. assisted in HPLC techniques. M.B. and L.B. prepared manuscript drafts, with all
425 authors contributing to the final version. L.B. has been the corresponding author throughout
426 the writing process. All authors have read and approved the final manuscript.

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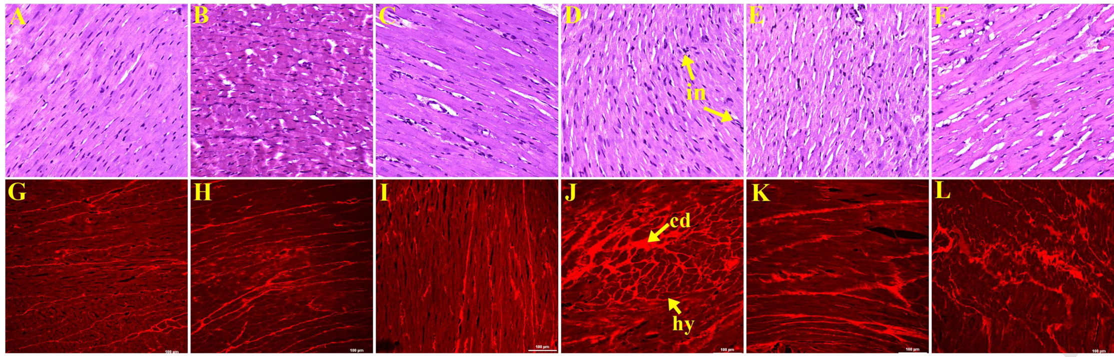

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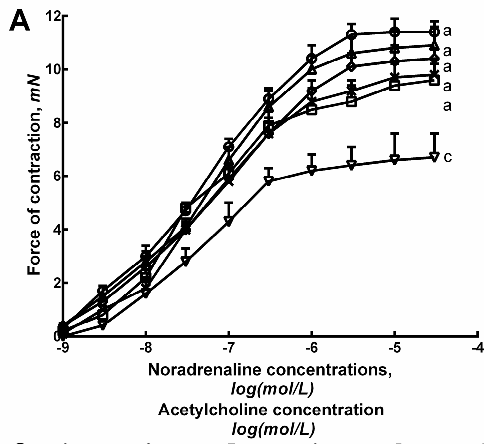

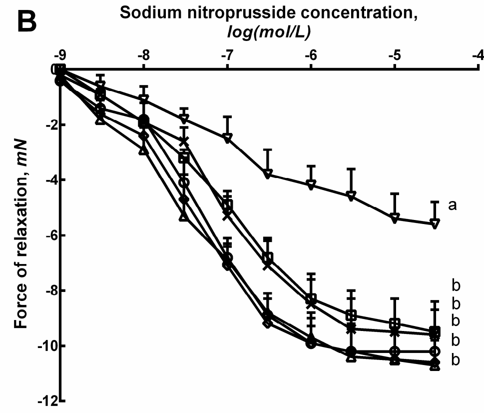

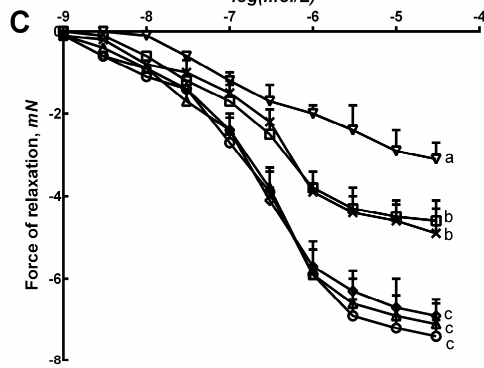

Figure	*P*-Value Diet	Treatment	Interaction
A	< 0.0001	0.1079	0.0043
B	0.0031	0.0233	0.0877
C	0.0002	0.2793	0.0512

C
CCB
CPM
H
HCB
HPM

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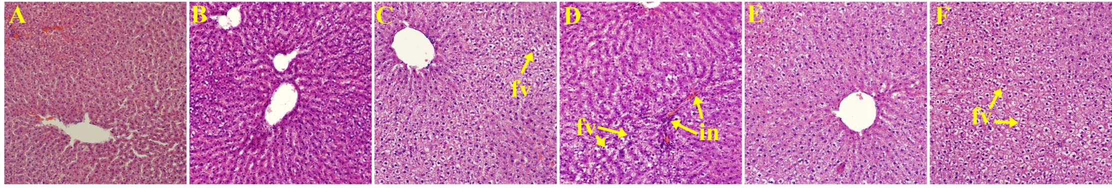

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Highlights

- Chokeberry and purple maize are sources of cyanidin 3-glucoside, an anthocyanin
- Both interventions reverse diet-induced symptoms of metabolic syndrome in rats
- The mechanism is likely to be prevention of infiltration of inflammatory cells

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