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Acute supplementation with blackcurrant extracts modulates cognitive functioning and inhibits monoamine oxidase-B in healthy young adults.

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Abbreviations

BCA- Bovine serum albumin
DHPG - Dihydroxyphenylglycine
EDTA - Ethylenediaminetetraacetic acid
H_2O_2 - Hydrogen peroxide
HVA- Homovanillic acid
LH- Lithium heparin
MAO- Monoamine oxidase
MRM- Multiple reaction method
MSEC – Milliseconds
PBS- Phosphate buffered saline solution
PEA- Phenylethylamine
RVIP – Rapid visual information processing
CY-GLU - Cyanidin glucoside
DEL-GLU - Delphinidin glucoside
CY- RUT - Cyanidin rutinoside
DEL- RUT - Delphinidin rutinoside

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Abstract

Background: Berry fruit have been shown to convey a number of benefits in animal models; including improvements in cognitive performance, slowing of cognitive decline and neuroprotection. These findings, along with epidemiological evidence and data showing modulation of factors related to brain function, suggest a potential role for berry polyphenols in improving cognitive performance.

Objective: The current study assessed the effects of two blackcurrant extracts on cognitive outcomes, mood, blood glucose profile and peripheral monoamine levels. Anthocyanin bioavailability was also assessed.

Design: A randomised, double-blind, placebo-controlled, crossover study was conducted in 36 healthy young participants (18-35y). Following a 10 minute baseline assessment, participants consumed sugar, flavour and colour matched drinks containing no polyphenols (control) or 525 +/- 5mg of polyphenols per 60kg body weight from either an anthocyanin enriched powdered blackcurrant extract (Delcyan^{TM}) or cold pressed blackcurrant juice (cultivar Blackadder) in counterbalanced order on separate days. A 70-minute computerised cognitive assessment (COMPASS) designed to be attentionally demanding and mentally fatiguing was then completed following a 60-minute resting absorption period. Blood platelet monoamine oxidase-B (MAO-B), plasma anthocyanin levels, plasma prolactin and plasma monoamines and associated metabolites were also investigated in a subsection of the cohort at 2.5 hours post-consumption.

Results: When compared to control, both blackcurrant extracts improved attention task performance. The juiced extract reduced reaction times during the digit vigilance task, whereas the powdered extract increased accuracy during a rapid visual information processing task. Following the juiced Blackadder extract, platelet MAO-B was inhibited by 96%, dihydroxyphenylglycol (DHPG) was reduced and normetadrenalin was increased in blood plasma, and a rapid decline in blood glucose levels was significantly attenuated, when compared to control.

Conclusion: This is the first illustration of a cognitive benefit of acute blackcurrant supplementation in healthy young humans and the first description of a clinically significant inhibition of MAO-B and MAO-A using a commonly consumed fruit. These data also illustrate that compounds other than anthocyanins are important to observe *in vivo* MAO inhibition and that the degree of processing and cultivar of blackcurrant fruit used substantially alters the neuroendocrinological and cognitive benefits conveyed.

Introduction

Epidemiological evidence suggests a relationship between flavonoid intake and cognitive decline/dementia [1, 2], with a specific benefit indicated for berries (strawberry and blueberry) that was not observed with other individual foods (Devore et al. 2012). Support for this comes from literature demonstrating a slowing or reversal of natural cognitive decline in berry-fed rats. Several different mechanisms of action have been proposed and investigated in an attempt to explain improvements to memory in animal models, including anti-inflammatory and antioxidant responses and improvements to neural signalling (see Spencer 2010 for review). Of particular relevance to the current study, anthocyanins, their aglycones and phenolic acid metabolites have been shown to have monoamine oxidase (MAO) inhibitory effects *in vitro*. As MAO metabolises monoamines, inhibition of this enzyme could reduce oxidative stress associated with this process and lead to increased levels of these neurotransmitters, essential for normal cognitive function. Indeed MAO-B inhibitors are used in the treatment of neurodegenerative symptoms associated with Parkinson's disease. The use of MAO-A inhibitors for several decades in the treatment of mood disorders also suggests that this inhibition, if demonstrated *in vivo*, could lead to enhanced mood [3, 4].

Only three published peer reviewed intervention studies have demonstrated positive effects of berry consumption on human behaviour, impacting verbal memory and spatial memory after supplementation of concord grape juice [5, 6] and blueberry juice [7] in adults with age related memory decline. There is, however, no published evidence pertaining to modulation of cognitive performance in healthy young adults.

One naturally rich source of anthocyanins that has been neglected in the literature is blackcurrant (*Ribes nigrum*). Intact glucosides, galactosides and arbinosides of the berry

100 anthocyanins and their associated metabolites have been found at levels ranging from 0.2 to
101 1.5ng/L in the blood and urine of humans after oral ingestion of flavonoid rich berries such
102 as blackcurrants, blueberries and boysenberries [3, 4]. Bioavailability of these compounds is,
103 therefore, low. As well as anthocyanins, blackcurrant also contains an abundance of other
104 phenolic structures in smaller quantities, which are able to exert physiological changes, such
105 as the rate and pattern of glucose uptake from the small intestine [8-10], and improved
106 vascular function [11] and gut microbiota profile [12], which all have the potential to
107 modulate human behaviour.

108 The aim of the present study was to explore the effects of acute supplementation of
109 two blackcurrant extracts, with matched quantities of polyphenols and sugars but differing
110 phenolic profiles, on attention, subjective mood, peripheral monoamines, prolactin and
111 blood glucose. Anthocyanin bioavailability was also assessed at 2.5 hours post-dose in
112 plasma.

Materials and methods

Participants

Thirty six participants were recruited from Auckland, New Zealand using opportunity sampling and received $120NZ to recompense them for any expense they may have incurred to participate in the trial. Before participants were enrolled in the study they attended a 90 minute screening session. During this session, participants gave their written informed consent to participate in the study and were screened for any contraindications to the study. In brief, all participants reported themselves to be healthy, not pregnant, non-tobacco users. Participants were not using dietary supplements or prescribed, over the counter or recreational drugs (excluding the contraceptive pill), did not have any sensitivities to any of the study treatments and had a body mass index below 35kg/m^2. Participants also completed three repetitions of the study day tasks to ensure they met the required minimum standards (internally set) to participate in the study and to minimise practice effects.

Treatments

Participants received three treatment drinks in an order dictated by random allocation to a counterbalancing (Williams Latin Square) schedule with at least one week washout between visits. Extracts were assessed for the phytochemical constituents using the method described by Schrage *et al* [13]. Anthocyanin stability of the Blackadder juice extract at -20 degrees was confirmed via HPLC. Over the eight week period no significant loss due to storage was observed. Intervention drinks contained either 0mg of polyphenols (control) or 525±5mg of polyphenols per 60kg of bodyweight from an anthocyanin enriched blackcurrant extract, (1.66g of Just the Berries, New Zealand (Delcyan^{TM})) or from 142ml of a

136 cold pressed blackcurrant fruit juice, (Blackadder cultivar, cultivated and processed by Plant
137 and Food Research Ltd, New Zealand (Blackadder juice)). One hundred and forty two
138 millilitres of juice was yielded from approximately 150g of fresh fruit, an amount which
139 could realistically be consumed in one serving. The phytochemical content of each
140 treatment can be seen in table 1. The Blackadder juice was frozen in 50ml aliquots at -20°C
141 until the day of use. The naturally occurring sugars in the Blackadder juice were quantified
142 via HPLC and the same levels were supplemented to the control and Delcyan™ treatments.
143 The total volume of the drink was then made up to 200ml (for a 60kg person) with cold
144 drinking water. All drinks quantities were calculated per kilo of body weight resulting in
145 differing volumes. In each case all drinks contained; 0.78g of glucose, 0.13g of fructose,
146 0.09g of Splenda® sweetener and 3.34µl blackcurrant flavouring (NI #12220, Formula foods
147 NZ) per kilogram of bodyweight. Drinks were coded and prepared fresh from frozen each
148 morning by a third party who had no further part in the running of the study. No member of
149 the investigation team was aware of the coding of the drinks until a blind-data review was
150 completed.

Table 1: Phytochemical constituents of Blackadder juice (mg/100ml of raw juice & mg supplemented per 60kg of body weight) and Delcyan™ extract (mg/g of raw powder & mg supplemented per 60kg of body weight)

Compound	Blackadder juice mg/100ml	Delcyan™ extract mg/g	Blackadder juice mg/60kg	Delcyan™ extract mg/60kg
Caffeoyl quinate	6.3	0.1	9	0.1
Caffeic acid glucoside	1.9	0.2	2.4	0.0
p-Coumaroyl quinate	3.6	0.4	5.4	0.7
Epigallocatechin	8.6	0	12	0
Delphinidin glucoside	24.1	44.6	34.2	73.8
Delphinidin rutinoside	115.9	107.4	164.4	178.2
Cyanidin glucoside	13.6	28.8	19.2	47.4
Cyanidin rutinoside	150.9	149	214.2	247.2
Myricetin rutinoside	15.6	4.5	22.2	7.2
Myricetin glucoside	2.1	0	3	0
Quercetin rutinoside	3.4	1.2	4.8	1.8
Quercetin glucoside	1.9	2.3	2.4	3.6
Quercitin pentoside	1.4	5.5	1.8	9
Myricetin	0.2	0.6	0.0	0.6
Vitamin C	168	0	100.8	0

Cognitive and mood measures

All cognitive measures and mood scales were delivered using the Computerised Mental Performance Assessment System (COMPASS, University of Northumbria), a purpose designed software application for the flexible delivery of randomly generated parallel versions of standard and novel cognitive assessment tasks, which has previously been shown to be sensitive to a range of nutritional interventions [14-16]. For the purpose of behavioural analysis, three tasks were selected with the intention that attention performance and cognitive flexibility could be assessed. Seven repetitions of the digit vigilance task, Stroop task and rapid visual information task were completed in a fashion similar to that of the cognitive demand battery [17] where subsequent repetitions of a ten minute battery are shown to incrementally induce mental fatigue. Mood scales were used at baseline, between each post-dose repetition of digit vigilance, Stroop and rapid visual information tasks and at the end of the cognitive tasks. The logical reasoning task was used at baseline and after the attentionally demanding cognitive battery to assess executive functioning.

Study tasks

Digit vigilance: The digit vigilance task is a measure of sustained attention involving accurate selection of target stimuli. It focuses on alertness and vigilance while placing minimal demands on two other components of attention: selectivity and capacity. A single target digit was randomly selected and constantly displayed to the right of the screen. A series of single digits were presented in the centre of the screen at the rate of 80 per minute. The participant was required to press the response key on the computer keyboard as quickly as possible every time the digit in the series matched the target digit. The task lasted two minutes and there were 30 stimulus-target matches. Task outcomes were accuracy (%), reaction time for correct responses (msec) and number of incorrect responses (false alarms).

Stroop: The Stroop test is a measure of attention, inhibition and cognitive flexibility. Participants were presented with a colour name. The colour name presented was written in a coloured ink which could be the same as the colour name or different. Participants had to respond to the colour of the ink using the peripheral mouse and corresponding colour response buttons. Participants were presented with 60 stimuli. Task measures were accuracy (percent correct) and reaction time (msec).

Rapid visual information processing (RVIP): The RVIP task is a measure of sustained attention and working memory. The participant was required to monitor a continuous series of digits for targets of three consecutive odd or three consecutive even single digits. The single digits were presented at the rate of 100 per minute and the participant responded to the detection of a target string by pressing the response key on the computer keyboard as quickly as possible. The task was continuous and lasted for 5 minutes, with 8 correct target strings being presented in each minute. The task was scored for percentage of target strings

correctly detected, average reaction time for correct detections (msec), and number of incorrect responses (false alarms).

Logical reasoning: The logical reasoning test requires the participant to think logically and analytically and is a measure of cognitive flexibility. A series of statements referring to the relationships between two letters appeared on the screen one at a time (e.g. “a precedes b: ba”). Participants were required to decide if each statement correctly described the order of the 2 letters that followed it by pressing the designated response keys on the computer keyboard. There were 24 stimuli. Mean reaction times were measured in msec, and accuracy of responses were recorded as percentages.

Mood

Bond-Lader visual analogue scales: Bond-Lader visual analogue mood scales [18] which have been shown to be sensitive to a number of nutritional intervention studies were employed [14-16]. The reliability and validity of these visual analogue scales has been demonstrated [19]. The scales comprise a total of sixteen 100mm lines anchored at either end by antonyms (e.g. alert-drowsy, calm-excited) on which participants mark their current subjective position. Scores from the 16 Bond-Lader visual analogue scales were combined as recommended by the authors to form three mood factors: ‘alert’, ‘calm’ and ‘content’ [18].

Visual analogue scales: Following each repetition of the attentional demand battery, participants were asked to subjectively rate how mentally fatigued they felt and how difficult they found the cognitive tasks. The electronic visual analogue scales were anchored “not at all” on the left hand side of the scale and “extremely” on the right, with higher scores representing more mental fatigue/higher difficulty.

Study procedure

Each participant was required to attend a total of three study days which were conducted at least seven days apart to ensure a sufficient wash out between conditions. During the week before, and throughout their participation in the study, participants were asked to abstain from berry consumption. Cognitive testing took place in a laboratory with participants visually and auditorily isolated from each other. On arrival at their first session, participants were randomly allocated to a treatment regime using a Latin square design that counterbalanced the order of treatments across the three active days of the study. On all three study days participants arrived at the lab in the morning (8:30am), after an overnight fast, and firstly gave a 10ml venous blood sample. Heart rate, blood pressure and blood glucose were then measured. Participants then completed one repetition of the ten minute baseline cognitive assessment comprising of the digit vigilance task, the Stroop task, the RVIP task, mood scales and the logical reasoning task. This constituted the baseline measure for that day. Participants were then supplemented with one of the study treatments in the form of a drink, which they were given five minutes to consume. Drinks were served chilled and in a dark brown 300ml plastic bottle with a straw to minimise the possibility of the participant recognising subtle differences in taste, look and mouth-feel between the treatments. After a 60 minute resting absorption period, in which participants read in a waiting area, participants' blood pressure and heart rate were measured again and a second blood glucose reading was taken by finger prick. Participants then completed the post-dose cognitive assessment 65 minutes post consumption of the study treatments, a time when anthocyanins are known to be detectable in plasma after blackcurrant consumption [20]. This paradigm consisted of seven repetitions of the attention tasks (digit vigilance, Stroop, RVIP) and mood scales. This lasted 70 minutes and was followed by the logical reasoning

central executive task. Participants then gave a third blood pressure reading and a third blood glucose reading before providing a second and final venous blood sample. A diagram of the study visit running order can be seen in figure 1.

The study received ethical approval from the New Zealand Regional Northern X Ethics board and was conducted according to the Declaration of Helsinki (1964).

(Place figure 1 here)

Biochemical analysis

Venous blood samples (2x5ml) were collected at baseline and 150 minutes after supplementation of treatments, which coincided with the end of the tasks. Samples were collected in 5ml BD vacutainers© (Becton, Dickinson and company, Plymouth, New Zealand). Both receptacles were treated with anticoagulants, one with lithium heparin (LH) and one with ethylenediaminetetraacetic acid (EDTA).

Whole blood samples treated with LH were immediately centrifuged (4°C, 5000rpm, 10 minutes) (Hitachi Himac preparative ultracentrifuge model CP100MX). Plasma was then extracted and aliquoted into 1ml eppendorf© tubes. Aliquots were spiked with 200µl of 5% trifluorocetic acid for the purpose of measuring plasma anthocyanin content. Plasma samples were stored at -80°C until analysis was performed.

Whole blood samples treated with EDTA were used to isolate blood platelets using the method reported by Snell *et al* [21]. Three and a half millilitres of whole blood were added to 2ml of phosphate buffered saline (PBS) containing 2g of glucose per litre of solution (PBS solution) and gently inverted. The solution was then centrifuged at (22°C, 600g, 3 minutes) and the supernatant placed on ice. The volume of the residual red cell pellet was restored to 7ml with PBS solution, gently inverted to mix and centrifuged again

(22°C, 600g at 22°C, 3 minutes); the supernatant was removed and pooled with the first supernatant fraction. This procedure was performed 5 times. The pooled supernatant fractions were then centrifuged (4°C, 2000g, 10 minutes) and decanted leaving the platelet pellet which was stored at -80ºC until the MAO-B analysis was performed.

Monoamine oxidase-B (MAO-B) activity analysis

A subset of eight participants provided sufficient blood samples for all the study time points to allow for MAO-B analysis.

The isolated platelet pellet was slowly thawed on ice, re-suspended in 1ml of PBS solution, sonicated with a probe sonicator (Microson ultrasonic cell disruptor, model XL2005) for 15 seconds on ice and centrifuged (4°C, 36,000g, 10 minutes) at. Sonication and centrifugation were then repeated after which the supernatant was removed and the pellet consisting of lysed platelets was re-suspended in sodium phosphate buffer. The protein concentration of the lysed platelet solution was determined against a bicinchoninic acid standard curve by using the Pierce BCA protein assay (ThermoFischer Scientific New Zealand Ltd) as per manufacturer's instructions. Each sample was measured in triplicate and the average protein concentration was used. The lysed platelet solution was re-suspended in sodium phosphate buffer to a final concentration of 150µg/ml of protein.

Determination of MAO-B activity was conducted using the Amplex® Red Monoamine Oxidase-B Assay Kit (A12214 Invitrogen), as per manufacturer's instructions. One hundred microlitres of the diluted lysed platelet solution was added to a 96 well plate in triplicate. Two microlitres of the MAO-A inhibitor clorgyline were then added to each well that contained platelet membranes and incubated for 30 minutes at room temperature. During the incubation, 100µl of H_2O_2 standards and the negative control were then added to the

micro-plate in triplicate. After the 30 minute incubation, 100µl of the amplex red working solution were added to each well. The plate was immediately placed into the microplate reader (FLUOstar Omega Plate reader, BMG labtech) and set to incubate at 37°C with an excitation wavelength of 530-560nm and an emission wavelength of 590nm. The micro-plate reader was programmed to take a reading every five minutes for one hour (13 readings in total). The 30 minute reading was used to compare platelet MAO-B activity between treatments.

Glucose

Blood glucose was measured with the use of an Accu-Check (Roche Healthcare, NZ) blood glucose monitor via a finger prick blood sample at baseline, 60 minutes and 150 minutes post supplementation. All 35 participants who completed the study gave all required finger prick blood samples.

Prolactin analysis

Prolactin was measured by diagnostic Medlab, Auckland, New Zealand. Prolactin was analysed in 300µL of blood plasma collected in LH treated vacutainers. Due to technical issues, only 20 sets of blood samples were available for prolactin analysis (8 control, 7 Delcyan™, 5 Blackadder juice). For this reason, prolactin analysis was between subjects.

LCMS analysis

The phytochemical composition of the blackcurrant extracts (Blackadder juice, Delcyan™) and the control sample were determined by liquid chromatography mass spectrometry (LCMS) using a Shimadzu 2020 single-quadrupole mass spectrometer coupled to a Shimadzu 20-Series UFLC system (Auckland, New Zealand) using the method described by Schrage *et al* [13].

306 Levels of anthocyanins and monoamines in plasma at defined time points
307 throughout the study were determined by LCMS using a 5500 QTrap triple
308 quadrupole/linear ion trap (QqLIT) mass spectrometer equipped with a TurboIon-Spray™
309 interface (AB Sciex, Concord, Ontario, Canada) coupled to an Ultimate 3000 UHPLC (Dionex,
310 Sunnyvale, California, USA).

311 **LCMS materials**

312 Formic acid (Riedel-de Haën), ammonium formate and acetic anhydride (Fluka), and Hunig's
313 base were purchased from Sigma Aldrich (Auckland, New Zealand). Optima LC/MS grade
314 acetonitrile (Fisher Scientific) was purchased from ThermoFisher (Auckland, New Zealand).
315 Water was of Milli-Q grade. Analytical standards, dopamine, normetadrenalin, noradrenalin,
316 adrenalin, 3,4-dihydroxyphenylglycol (DHPG), serotonin and homovanillic acid (HVA) were
317 purchased from Sigma-Aldrich, phenylethylamine (PEA) from Acros Organics (Geel,
318 Belgium), cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and
319 delphinidin 3-rutinoside from Polyphenols Laboratories (Sandnes, Norway) and malvidin 3-
320 galactoside chloride from Extrasynthese (Genay Cedex, France). Deuterated acetic
321 anhydride [d6] was purchased from Sigma-Aldrich and deuterated dopamine [d4] from CDN
322 Isotopes (Quebec, Canada). Phree™ Phospholipid removal plates were purchased from
323 Phenomenex (Torrance, CA, USA).

324 **Anthocyanin analysis in plasma**

325 A subset of 17 participants provided sufficient blood samples for all of the study time points
326 for anthocyanin analysis to be conducted.

327 Plasma samples (1ml) were further acidified (1:4 6N HCl:5% formic $acid_{aq}$, 250µl) and
328 then spiked with malvidin galactoside (5ng) as an internal standard. Samples were

centrifuged (4°C, 16100 RCF, 5 minutes) and proteins removed by precipitation via addition of acetone (1:3) to an aqueous aliquot (600µl). The samples were then chilled at -80°C for 30 minutes prior to re-centrifuging (4°C, 16,100 RCF, 5 minutes) and the acetone removed via evaporation. Further clean-up to minimise the presence of phospholipids was achieved via liquid-liquid partition with hexane versus the aqueous sample. A final protein precipitation cleanup of the aqueous aliquot (400µl) with chloroform was performed prior to centrifuging (4°C, 16100 RCF, 5 minutes). Two hundred microlitres of the aqueous phase was transferred to an autosampler vial for immediate analysis by LCMS.

Anthocyanin separation was achieved on a Zorbax SB-C18 Rapid Resolution HD 2.1x100mm ID 1.8 micron column (Agilent Technologies, Santa Clara, CA, USA) maintained at 70°C. Solvents were (A) 5:3:92 acetonitrile/formic acid:water v/v/v and (B) 99.9:0.1 acetonitrile/formic acid v/v and the flow rate was 600µL/min. The initial mobile phase, 100% A was held isocratically for 0.5 minutes, then ramped linearly to 10% B at 5 minutes, followed by another linear ramp to 90% B at 5.1 minutes and held for 1.9 minutes before resetting to the original conditions. Sample injection volume was 20µl. MS data was acquired in the positive mode using a multiple reaction monitoring (MRM) method. The transitions monitored (Q1 and Q3), along with their optimised parameters (declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)) are listed in Table 2.

Table 2: Multiple reaction monitoring transitions used for blackcurrant anthocyanins

Q1	Q3	Name	DP	EP	CE	CXP
465	303	Delphinidin glucoside	70	10	30	10
611	303	Delphinidin rutinoside	130	10	50	30
449	287	Cyanidin glucoside	110	10	35	20
595	287	Cyanidin rutinoside	50	10	55	1
493	331	Malvidin galactoside (IS)	50	10	45	25

Other operating parameters were as follows: dwell time, 100ms; ionspray voltage, 2500V; ion source temperature, 700°C; ion source gas one, 60psi; ion source gas two, 90psi and curtain gas, 40psi.

Quantification was performed using the internal standard ratio method using MultiQuant software v3.0 (AB Sciex).

Monoamine analysis in plasma

Eight monoamines were analysed by LCMS from blood plasma following derivatisation. These were; serotonin, dopamine, phenylethylamine, adrenalin, noradrenalin, normetadrenalin, 3,4-dihydroxyphenylglycol (DHPG) and homovanillic acid (HVA).

Plasma samples were treated to remove proteins and phospholipids and derivatised in two stages to acetylate alcohol and amine functional groups and alkylate free carboxylic acids with Hunig's base prior to LCMS analysis. A double derivatisation was found to be necessary to acetylate the less reactive alkyl hydroxyl groups. The schematic in figure 2 summarises the derivatisation of the different functional groups and the synthesis and use of labelled internal standards for each analyte to facilitate quantitation and to correct for matrix effects during analysis.

(Place figure 2 here)

Briefly, each plasma sample (200µl) was added to an individual well of a Phree™ Phospholipid removal plate already containing cold 600µl acetonitrile, 100µl acetic anhydride and 1ng dopamine-d4 [internal standard (IS)]. The Phree™ plate was centrifuged at 500g for 30 minutes, a further 200µl acetonitrile added to each well, and the plate centrifuged at 500g for a further 10 minutes. The filtrate was transferred to a 2ml micro tube, 100µl acetic anhydride added and heated at 50^{o}C. After 30 minutes 20µl Hunig's base

was added to each sample, vortexed then heated for a further 60 minutes. Samples were then evaporated to near dryness with nitrogen at 40°C. Samples were re-derivatised; 100µl acetonitrile, 100µl acetic anhydride and 10µl Hunig's base heated for 40 minutes at 50°C. Finally, to each sample 1ng of the derivatised labelled internal standard monoamine mixture (d-IS) was added, and the sample made up to 1ml with water and transferred to an autosampler vial ready for analysis.

Monoamine separation was achieved on an Atlantis® T3 150x2.1mm 3 micron column (Waters Corp., Milford, MA, USA), maintained at 40°C. Solvents were (A) MilliQ water +0.03% ammonium formate + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic acid and the flow rate was 0.6ml/min. The initial mobile phase, 98% A, was held for 4 minutes then ramped linearly to 70% A at 11 minutes, 20% A at 14 minutes, and 0% A at 14.5 minutes and held for 5 minutes before resetting to the original conditions. Sample injection volume was 100µl.

MS data was acquired in the positive mode using a scheduled MRM method. In some cases the ammonium adduct was the most abundant ion observed for Q1. The transitions monitored (Q1 and Q3), along with their optimised DP, EP, CE and CXP parameters are listed in Table 3.

Table 3 Multiple reaction monitoring transitions used for monoamines and their isotopically labelled internal standard analogues

Q1	Q3	Time	Name	DP	EP	CE	CXP
164	105	10.6	PEA	30	10	25	10
167	105	10.6	PEA [d3]	30	10	25	10
280	137	11.1	Dopamine	70	6.1	35	15
289	139	11.1	Dopamine [d9]	70	6.1	35	15
284	141	11.1	Dopamine [d4]	70	10	37	15
293	143	11.1	Dopamine [d4] [d9]	70	10	37	15
261	160	11.1	Serotonin	10	5	25	1
267	161	11.1	Serotonin [d6]	10	5	25	1
250	166	11.6	Normetadrenalin	50	9	25	15
256	168	11.5	Normetadrenalin [d9]	50	9	25	15
355	194	11.6	Noradrenalin	10	10	30	1
367	199	11.5	Noradenalin [d12]	10	10	30	1
292	250	12.5	Adrenalin	170	10	20	1
301	257	12.5	Adrenalin [d12]	170	10	20	1

356.	237	13.4	DHPG	90	13	20	20
368	244	13.3	DHPG [d12]	90	13	20	20
308	224	13.9	HVA	110	10	25	20
311	225	13.9	HVA [d3]	110	10	25	20

392

393 Other operating parameters were as follows: ionspray voltage, 2500V; ion source
394 temperature, 700°C; ion source gas one, 40psi; ion source gas two, 50psi and curtain gas,
395 50psi.

396 Quantification was performed using the internal standard ratio method using
397 MultiQuant software v3.0 (AB Sciex).

398 **Statistics**

399 Mood, cognitive scores and the physiological measures were analysed as 'change from
400 baseline' using the SPSS 18 statistics package. Baseline differences were calculated for all
401 measures using a one way (treatment) ANOVA.

402 Two way repeated measures ANOVAs (General linear model) (Treatment [control,
403 Delcyan^{TM}, juice] X completion [1 to 7] for attentional tasks and visual analogue scale
404 outcomes OR Treatment [control, Delcyan^{TM}, juice] X completion [1 to 2] for blood glucose
405 and Bond-Lader) were conducted. Logical reasoning performance, platelet Monoamine
406 Oxidase B activity, plasma monoamines and plasma anthocyanins levels were analysed by
407 one-way (treatment) repeated measures ANOVA. Blood plasma prolactin was analysed
408 using a one way (treatment) between subjects ANOVA. In all instances Mauchly`s test of
409 sphericity was used to assess equality of the variances of the differences between factors.
410 Where sphericity had been violated, Huynh-Feldt corrections for non-sphericity were
411 implemented. Pairwise comparisons were conducted on all treatment-related effects with a
412 p value <0.05 on the initial ANOVA to ascertain any differences between treatments for the
413 whole session or, in the case of interactions, at each task repetition. Partial Bonferroni

corrections were applied to protect for error against multiple comparisons, therefore, the p value was multiplied by the number of treatments being compared to control. All post hoc p values are reported after corrections for multiple comparisons have been applied (x3 for anthocyanins and MAO-B and x2 for all other comparisons).

Results

Prior to analysis of change from baseline data, mean pre-dose scores for all three treatments (control, Delcyan™, Juice) for each outcome were subjected to a one way repeated measures ANOVA. The only significant difference found was between active treatments for homovanillic acid. Data tables for all outcomes can be found in the supplementary materials.

Cognitive performance

A one way ANOVA was conducted on control change from baseline scores for the outcome fatigue to ensure the sustained attention cognitive paradigm was indeed mentally fatiguing. A significant effect of repetition [$F(6,192)=16.44$. $p<0.0001$] confirmed that each subsequent repetition of the tasks caused an increase in rating of mental fatigue.

Digit vigilance

There was a significant treatment × repetition interaction on digit vigilance reaction time [$F(12,384)=1.82$ $p=0.044$] without any effect upon accuracy. Pairwise comparisons revealed an increase in speed of response after supplementation of the juice treatment at repetition 1 ($p=0.028$), 4 ($p=0.011$) and 7 ($p=0.038$). See Figure 3a. There were no effects on any digit vigilance outcomes after supplementation with Delcyan™.

RVIP

There was a significant main effect of treatment on RVIP accuracy [F (2,62)=5.87, p=0.005]. Pairwise comparisons showed an attenuation in the reduction of RVIP accuracy after supplementation of the Delcyan™ extract when compared to control (p=0.011), irrespective of repetition. There were no significant effects on reaction time or false alarms. See Figure 3b. There were no effects on any RVIP outcomes after supplementation with juice.

(Place figure 3 here)

Blood glucose

There was a significant main effect of treatment on blood glucose [F(2,68)=8.89, p<0.001]. Pairwise comparisons showed significantly higher blood glucose levels following supplementation of the juice treatment when compared to control (p=0.002), irrespective of repetition. See figure 4a. There were no significant effects following supplementation of Delcyan™.

Platelet MAO-B

There was a significant effect of treatment on blood platelet MAO-B activity [F (2,16)=15.20 p<0.001]. Pairwise comparisons showed a decrease in platelet MAO-B activity after supplementation with the juice treatment when compared to control (p<0.001). See figure 4b. There were no significant differences between active treatment groups. There was no effect of the Delcyan™ treatment on blood platelet MAO-B.

Monoamines

The repeated measures ANOVA revealed a significant effect of treatment [F (2,32)=12.18 p<0.001] on plasma levels of normetadrenalin. Pairwise comparisons showed levels of

normetadrenalin were significantly higher after supplementation of the juice treatment when compared to the control ($p<0.001$) and Delcyan™ ($p<0.001$). There were no effects of Delcyan™. See figure 4c.

The repeated measures ANOVA revealed a significant main effect of treatment [$F(2,32)=21.30$ $p<0.001$] on plasma levels of DHPG. Pairwise comparisons revealed levels of DHPG were significantly higher after supplementation of the juice treatment when compared to the control ($p<0.001$) and Delcyan™ ($p<0.001$). There were no effects of Delcyan™ versus control. See figure 4d.

(Place figure 4 here)

Blood plasma anthocyanin levels

The repeated measures ANOVA revealed a significant effect of treatment on plasma levels of CY-GLU, CY-RUT, DEL-GLU and DEL-RUT [$F(2,34)=27.5$ ($p<0.001$)], [$F(2,34)=33.7$ ($p<0.001$)], [$F(2,34)=112.51$ $p<0.001$), [$F(1.45,25.25)=96.26$ $p<0.001$) respectively.

Plasma CY-GLU, CY-RUT, DEL-GLU and DEL-RUT levels were significantly higher after supplementation with the Delcyan™ treatment when compared to control ($p<0.001$, $p=0.005$, $p<0.001$, $p<0.001$) and the Blackadder juice treatment ($p<0.001$, $p<0.001$, $p<0.003$, $p<0.001$) respectively.

Plasma CY-GLU, CY-RUT, DEL-GLU and DEL-RUT levels were also significantly higher after supplementation of the Blackadder juice treatment when compared to control ($p<0.001$), ($p<0.001$), ($p=0.003$) and ($p<0.001$) respectively. A graphical representation of anthocyanin levels in blood plasma can be seen in figure 5a.

Following supplementation, the repeated measures ANOVA revealed a significant effect of the treatment on combined levels of CY-GLU, CY-RUT, DEL-GLU and DEL-RUT in

blood plasma (combined anthocyanin level is the total amount of anthocyanins measured in blood plasma after supplementation of the study treatment) after consumption of the study treatments, [F (2,32)=105.34, p<0.0001]. Pairwise comparisons revealed that combined anthocyanin levels were significantly higher after consumption of the Delcyan™ (p<0.001) and juice (p<0.001) treatment when compared to control. Levels were also significantly higher after supplementation of the Delcyan™ extract when compared to the Blackadder juice extract (p<0.001). A graphical representation of combined anthocyanin levels in blood plasma can be seen in figure 5b.

(Place figure 5 here)

Table 3 Means and SD for the amount of measured anthocyanins given to the participants and the amount found in blood plasma (change from baseline) 150 minutes post supplementation of the Delcyan™ and Blackadder juice treatments

Treatment	Anthocyanin	Amount supplemented mg/kilo body weight	Amount supplemented mg/60kg of body weight	Average amount in plasma (nM)
Delcyan™	Cyanidin glucoside	0.8	48	0.4 ± 0.3
	Delphinidin glucoside	1.2	73.8	1.3 ± 0.9
	Cyanidin rutinoside	4.1	247.2	8.6 ± 2.9
	Delphinidin rutinoside	2.9	178.2	11.7 ± 4.5
Blackadder juice	Cyanidin glucoside	0.3	19.2	0.2 ± 0.1
	Delphinidin glucoside	0.5	34.2	0.8 ± 0.6
	Cyanidin rutinoside	3.5	214.2	6.4 ± 2.3
	Delphinidin rutinoside	2.7	164.4	7.8 ± 2.5
Control	Cyanidin glucoside	0	0	0.0 ± 0.1
	Delphinidin glucoside	0	0	0.0 ± 0.01
	Cyanidin rutinoside	0	0	0.0 ± 0.1
	Delphinidin rutinoside	0	0	0.0 ± 0.1

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Discussion and conclusion

The current study has outlined evidence of positive modulation of behaviour following administration of two blackcurrant extracts when compared to control, with no negative effects of either active extract. Improvements in RVIP accuracy were found after supplementation of the Delcyan™ extract and improvements in reaction time on the digit vigilance task were found after supplementation of the Blackadder juice extract. Although there were no other significant effects on behaviour, the Blackadder juice treatment demonstrated a number of physiological effects not present following Delcyan™. These comprised of an inhibition of platelet MAO-B activity (96%) and a significant reduction in plasma normetadrenalin (60%) and increase in DHPG (~35.5%) when measured 2.5 hours after supplementation. The Blackadder blackcurrant juice treatment also showed a significantly sustained (over both time points) increased blood glucose when compared to control at 60 and 150 minutes, despite being sugar matched.

An increase in accuracy was shown during the RVIP task after supplementation with the Delcyan™ treatment, irrespective of task repetition, with no evidence of slowed reaction times. In regards to the juice treatment, there was evidence of an attenuation of the increase of digit vigilance reaction times seen with repeated testing, with no evidence of decreased accuracy. This improvement was seen during repetitions one, four and seven (70, 100 and 140 minutes post-supplementation, respectively). Further evidence for a modulation of behaviour following the blackcurrant extracts comes from non-significant trends observed on the treatment*repetition ANOVAs for Bond-Lader alertness ratings and mental fatigue visual analogue scales showed. These indicated a pattern of attenuation in decreased self-reported alertness and increased ratings of fatigue following supplementation of the Delcyan™ treatment but only reached statistical significance after

518 the final repetition of the 70 minute attentionally demanding cognitive battery. Graphical
519 representations of fatigue and alert ratings can be found in the supplementary materials.
520 There is evidence of direct cellular and molecular interactions of flavonoids on rodent brains
521 [22] and changes in central [23] and peripheral [20] vascular function in humans after
522 consumption of flavonoid-rich fruits. However, definitive mechanisms driving the
523 behavioural effects in the present study are currently unknown, especially after
524 supplementation of the Delcyan^{TM} treatment, which had no significant effect upon any of
525 the physiological outcomes measured.

526 As expected, blood plasma anthocyanins DEL-GLU, DEL-RUT, CY-GLU and CY-RUT
527 were significantly increased 2.5 hours after supplementation of both blackcurrant
528 treatments when compared to control. Measured blood plasma anthocyanins were also
529 greater after supplementation of the Delcyan^{TM} treatment when compared to the juice
530 treatment. When all four measured anthocyanins were combined there was a 30% increase
531 in plasma concentration following Delcyan^{TM} when compared to the juice treatment.
532 However, this extract contained 20% more of the measured anthocyanins than the juice
533 drink. Although there was a significant difference in blood plasma anthocyanins between
534 the two blackcurrant treatments, in line with past published research [20, 24, 25],
535 anthocyanin quantities found in blood plasma were less than one percent of that ingested.
536 It must be noted that vitamins and minerals, other than L-ascorbic were not quantified in
537 any of the study treatments. However, to our knowledge there are no reported cognitive or
538 behavioural effects of acute vitamin or mineral supplementation. Given that the major
539 phenolic constituents of each treatment were anthocyanins and both extracts affected
540 attention based tasks, this may indicate that the effects of blackcurrant upon attention
541 processing are directly related to their anthocyanin content; an acute effect which has

542 previously been indicated in children aged 7-9 years [26]. The specific demands of the two
543 attention tasks are, however, not equal, with a higher demand both in processing, and
544 duration of the RVIP task when compared to the digit vigilance task. The RVIP contains a
545 higher working memory element than the digit vigilance task, potentially indicating changes
546 in working memory processing as well as attention, a cognitive outcome which has
547 previously been shown to be sensitive to flavonoid-rich cocoa [27] and ginkgo biloba [28].
548 However, until replication of the behavioural effects presented in the current study has
549 been achieved, it is difficult to elaborate further at this point.

550 In terms of MAO-B activity, this is the first demonstration of a clinically significant
551 inhibition of platelet MAO-B following blackcurrant supplementation. Central MAO-B
552 inhibitors have been used for several decades for the treatment of depressive disorders and
553 neurodegenerative diseases [29] and have also been shown to improve cognitive processing
554 when given to non-demented Parkinson patients [30]. MAO-B inhibitors also have the
555 potential to attenuate the breakdown of endogenous neurotransmitters, reducing levels of
556 H_2O_2 associated with deamination of dopamine [31]. Although the current study only
557 measured MAO-B inhibition in peripheral tissue, if the inhibition can be shown to be
558 centrally active, the clinical applications of a MAO inhibitor from a commonly consumed
559 fruit could be vast. Potential applications include attenuating cognitive decline associated
560 with natural ageing, as well as in clinical populations, including those suffering from early
561 stage Parkinson's disease, whom are known to respond favourably to MAO inhibitors [30].
562 DHPG, a metabolite largely determined by MAO-A dependent metabolism of noradrenalin
563 [32], which is a marker for reduced MAO-A activity after administration of pharmacological
564 MAO-A inhibitors [33], was also found to be reduced after consumption of the Blackadder
565 blackcurrant juice extract in the current study. This effect was not seen after consumption

566 of the Delcyan^{TM} extract, highlighting that, in addition to MAO-B inhibition, the Blackadder
567 juice treatment possesses MAO-A inhibitory properties. These changes in DHPG did not
568 coincide with an accumulation of adrenalin or noradrenalin in the current study, which is in
569 line with previous research investigating acute supplementation of MAO inhibitors in
570 humans [34]. In addition to decreased levels of DHPG, indicating MAO-A inhibition, we also
571 observed an increase in normetadrenalin, a metabolite of noradrenalin via catechol-O-
572 methyl transferase (COMT). This increase is potentially indicative of increased noradrenalin
573 breakdown through COMT as a result of inhibition of the MAO-A enzyme. A diagram
574 depicting potential inhibition pathways can be found in figure 6. Also related to this MAO
575 inhibitory effect is a non-significant modulation of plasma prolactin observed in the current
576 study where post-dose prolactin was lower after consumption of the Blackadder juice
577 extract when compared to control. Although gamma-aminobutyric acid (GABA), serotonin,
578 adrenalin and noradrenalin are slight rate limiting factors of prolactin secretion, dopamine is
579 the most important hypothalamic prolactin inhibiting factor[35], indicating that general and
580 potentially central dopaminergic tone could have been affected by supplementation of the
581 Blackadder juice drink. Although these prolactin findings are hindered by a small sample size
582 and between subjects design, they illuminate the need for further research.
583
584 ***Place figure 6 here***
585
586 The blackcurrant juice treatment also showed a significant (over both time points)
587 increase in blood glucose when compared to control, despite being sugar matched; an effect
588 not seen with supplementation of the Delcyan^{TM} treatment. Blood glucose was elevated by
589 0.53mmo/L at 60 minutes and 0.23mmol/L at 150 minutes post supplementation of the

juice treatment. Although these results must be interpreted with caution as there were only two post-dose blood glucose measurements, this result shows a clear effect of the juice treatment on blood glucose. Based upon the current findings, the effect on glucose appears to resemble the pattern after supplementation of berry puree where the peak in blood glucose levels following a glucose load when combined with a berry puree is reduced resulting in a higher blood glucose reading one hour after supplementation [36]. The main difference between the study treatments was phenolic acids at 0mg in the Delcyan™ treatment and 61mg in the juice treatment per 60kg of bodyweight, which could provide further evidence of the slowing of glucose transport from the gut via direct inhibition of intestinal epithelial glucose transporters by phenolic acids as described by Manzano and Williamson [8]. A more thorough investigation needs to be completed to ascertain a full post supplementation blood glucose profile.

The findings of the present study demonstrate, for the first time, a positive modulation of behaviour in a young and healthy adult cohort after supplementation of a blackcurrant extract. This is also the first evidence of a clinically significant reduction in MAO activity following ingestion of a commonly consumed fruit. The results suggest that the MAO inhibition found in this study cannot be wholly responsible for the behavioural effects observed as both active conditions positively influenced attention based cognitive tasks, whereas only the juice treatment inhibited MAO-A and MAO-B. The finding of more robust effects on attention following Delcyan™, containing higher levels of anthocyanins, may indicate that these effects are attributable to the anthocyanin content and that any effects on MAO are independent of these. The possibility that a MAO-A and MAO-B inhibiting blackcurrant drink will exert favourable effects on cognitive modulation of clinical and non-clinical populations deserves further investigation. More exploration therefore needs to be

undertaken to ascertain if other cognitive paradigms, especially those which have previously been shown to be sensitive to flavonoid-rich nutritional interventions in rats and humans, specifically memory tasks and paradigms sensitive to changes in levels of dopamine, are modulated after supplementation of a MAO inhibiting blackcurrant juice.

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Conflict of interests

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