The pharmacodynamic profile of 'Blackadder' blackcurrant juice effects upon the monoamine axis in humans: A randomised controlled trial

Running title - Blackcurrants and the monoamine axis in humans

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Abstract

Emerging evidence from human intervention trials indicates health benefits of consuming blackcurrant fruit, including improvements to cognitive performance, modulation of blood flow, regulation of blood glucose and inhibition of enzymes underpinning normal cognitive function. Of particular relevance is our previous demonstration of monoamine oxidase (MAO)-A and B inhibition after the consumption of a New Zealand 'Blackadder' blackcurrant juice in humans.

The current study uses a double-blind, placebo-controlled, randomised cross- over design to assess the pharmacodynamics of the effects on platelet MAO-B inhibition and associated substrates, plasma prolactin levels and blood glucose levels after consumption of a single serve of 'Blackadder' blackcurrant juice standardised to 500mg polyphenols. Eight healthy male (20-–35 years) participants completed the trial. Measurements were obtained at baseline 15, 30, 45, 60, 100, 120, 150, 180, 240 mins and 24 hours post dose.

A fast, absolute and reversible inhibition of blood platelet MAO-B ($P<0.001$) and a significant but delayed reduction in plasma prolactin ($P<0.001$) were observed following the consumption of 'Blackadder' blackcurrant juice when compared to a placebo control. No interpretable changes in substrates of MAO or associated metabolites were seen.

These data provide a clear time course of the reversible inhibition of MAO-B after the single consumption of a of New Zealand 'Blackadder' blackcurrant juice standardised at 500mg of polyphenols and, therefore, provide a therapeutic window on which to base future nutritional interventions.

Introduction

Blackcurrants (*Ribes nigrum*) are a berry fruit high in polyphenols when compared to other similar berries [1], with 3-O-glucosides and the 3-O-rutinosides of anthocyanins delphinidin and cyanidin representing the major phenolic constituents [2]. Other phenolic structures, such as phenolic acids are also present in smaller quantities [3]. Emerging evidence supports health benefits of consuming blackcurrant fruit, including modulation of blood flow [4, 5] and brain wave spectral activity [6]; improvements to cognitive performance [7], and inhibition of monoamine oxidase (MAO) enzymes in humans [7].

Monoamine oxidase enzyme isoforms -A and -B are present in the periphery and the central nervous system and play a major role in the metabolism of both dietary and endogenous monoamines [8]. MAO-A preferentially catalyses the oxidation of serotonin; MAO-B is more active towards β-phenylethylamine and benzylamine; whereas dopamine, adrenaline, noradrenaline tryptamine and tyramine are oxidised by both isoforms [9]. Inhibition of MAO therefore results in an increased concentration of monoamine neurotransmitters and, in the case of MAO-B inhibition, is well documented as a therapeutic treatment for Parkinsonian symptoms [10]. Monoamine inhibition can be reversible or irreversible and can either act non-selectively, affecting both isoforms, or selectively, affecting only one isoform. For example, phenelzine is an irreversible, non-selective MAO inhibitor, which inhibits both MAO-A and B for up to three weeks [11]; in contrast, toloxatone, a reversible inhibitor of MAO-A (RIMA), inhibits MAO-A for only six hours before activity returns to baseline values [12]. This selective inhibition is preferable as the inhibition of both MAO isoforms can in some instances prevent the degradation of dietary amines in the digestive tract and if this continues for prolonged periods tyramine can accumulate to dangerous levels, potentiating a hypertensive crisis. It is therefore important to identify reversible and/or selective MAO inhibitors.

Our previous demonstration of MAO inhibition following blackcurrant consumption, outlined the efficacy of a cold-pressed blackcurrant juice ('Blackadder' cultivar) in inhibiting both MAO isoforms in the periphery. These findings were coupled with a non-significant reduction in plasma prolactin,

potentially indicating modulations in circulating dopamine levels [7]. This inhibition, observed in healthy young adults measured at ~2 hours post consumption, was particularly striking for MAO-B indicating an almost complete inhibition (96 %). It is therefore of great importance to ascertain the pharmacodynamics of this action in order to establish if it is safe, reversible and to determine the optimal timing of dosing. A mild increase in blood glucose was also observed when measured at 1 and 2.5 hours post consumption of 'Blackadder' blackcurrant when compared to control. This increase is contrary to expectation based upon a previous observation of decreased post-prandial peak blood glucose levels following apple juice [13]. It is theorised that attenuation of glucose absorption is due to inhibition of sodium-glucose transport proteins in the gut lumen after exposure to phenolic acids [14]. Therefore, the previously observed higher blood glucose readings may have been due to a slowing of glucose uptake following consumption of 'Blackadder' blackcurrant, rather than an overall increase in blood glucose. It is therefore important that the current study investigates more frequent time points to establish a pattern of glucose modulation after consumption of 'Blackadder' juice.

The current study will assess the pharmacodynamics of peripheral MAO-B inhibition, associated catecholamine and prolactin levels, and venous blood glucose profile following consumption of 'Blackadder' blackcurrant. The trial will utilise the 500 mg/60 kg of body weight dose of cold pressed 'Blackadder' juice (equivalent to ~100 g of fresh fruit) which was shown to have MAO inhibitory effects in our previous report and effects will be measured in a cohort of healthy male adult volunteers.

Materials and methods

Design

The study followed a double-blind, counterbalanced, placebo-controlled, repeated measures design. Participants were randomly allocated to treatment orders as selected through a Williams Latin Square [15].

Participants

Eight healthy male adults participated in the study and provided all samples at all time points. Participant characteristics can be found in Table 1. Women were omitted due to changes in circulating prolactin levels during the menstrual cycle [16]. Power analysis was determined using post dose monoamine-B activity levels after supplementation with a similar blackcurrant juice as described in our previous paper [7]. A two tailed A priori power analysis using G-power [17] was used to indicate the samples size needed to achieve an alpha error probability of 0.01. The power analysis indicated that a sample size of 7 would be needed to achieve a power of 0.99.

TABLE 1 NEAR HERE

Participants were recruited using opportunity sampling. Participants received £70 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 20 minute screening session. During this screening session participants gave their informed consent to participate in the study and were screened for any contraindications to the study with the use of an exclusion questionnaire. Exclusion criteria included: Risk or diagnosis of blood-borne disease, diagnosed history of any psychiatric disorder, aged under 18 or over 35 years, BMI above 30 kg/m^2 or below 17 kg/m^2, diagnosis of diabetes or current use of prescription, over-the-counter or recreational drugs. Recruitment ceased when eight full sets of data were successfully attained.

The study received ethical approval (RE20-10-11208) and was conducted according to the Declaration of Helsinki (1964).

Treatments

The 'Blackadder' juice was assessed for phytochemical profile using the method described by Schrage et al., [18]. Participants received two drinks with at least one week washout between treatments. These drinks contained either 0 mg of polyphenols (control) or ~500 mg/ polyphenols per 60 kg of body weight in the form of a cold-pressed New Zealand blackcurrant juice ('Blackadder' cultivar). Dose ranges of individual phenolic constituents can be found in Table 2 and total dose ranges of phenolic groups can be found in Table 3.

Drinks were matched for sugars and taste. In each case, drinks comprised of 3.44 g of glucose, 4.63 g of fructose, 0.8 g of sucrose, 6 g of Splenda™ and 50 ml of blackcurrant flavouring (Schweppes blackcurrant cordial). The total volume of the drink was made up to 200 ml with water and served chilled in an opaque brown bottle by an independent third party. All quantities discussed are based on a 60 kg person, drink quantities were calculated per kilo of body weight. A breakdown of anthocyanins and other phenolics in the study drinks can be seen in Tables 2 and 3.

TABLE 2 NEAR HERE

TABLE 3 NEAR HERE

Blood collection and storage

Blood was collected from an inlaying cannula in the left median cubital vein (21 Gauge, Becton, Dickinson and Company, UK). Venous blood samples (15 ml) were collected at baseline and 10 further time points post consumption of study treatments. Samples were collected in either BD Vacutainers© (Becton, Dickinson and company) or with 20 µl end-to-end capillary (EKF Diagnostics). Vacutainer 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, 5000 rpm, 10 minutes) (Hitachi Himac preparative ultracentrifuge model CP100MX). Plasma was then extracted and aliquoted into 1.5 ml Eppendorf© tubes and stored at -80 °C until analysis. Whole blood samples treated with EDTA were used to isolate platelet cells prepared for

storage using the methods as described by Snell et al., [19]. Prepared platelet pellets were stored at -80 °C until MAO-B activity analysis was performed.

MAO analysis

Isolated platelet pellets were prepared using methods described by Watson *et al*., [7] and analysed for MAO-B activity using the Amplex® Red Monoamine Oxidase-B Assay Kit (A12214 Invitrogen), as per manufacturer's instructions.

Circulating 3,4-dihydroxyphenylglycol (DHPG) was used as a proxy for MAO-A activity [20]. This method has been used in many pharmacological MAO inhibitor studies [20, 21].

Prolactin analysis

Prolactin analysis was conducted by Diagnostic Medlab, Auckland, New Zealand in 300 µl of LH treated blood plasma.

Catecholamine Analysis

Catecholamines and associated metabolites were analysed in plasma to assess the impact of MAO activity on associated substrates.

Materials

Formic acid (Riedel-de Haën), ammonium formate and acetic anhydride (Fluka), were purchased from Sigma Aldrich (Auckland, New Zealand). Di-sodium tetraborate (BDH) was purchased from Global Science (Auckland, New Zealand). Optima LC/MS grade acetonitrile (Fisher Scientific) was purchased from ThermoFisher (Auckland, New Zealand). Water was of Milli-Q grade. Analytical standards, dopamine, normetadrenaline, noradrenaline, adrenaline, 3,4-dihydroxyphenylglycol (DHPG), serotonin, 3,4-dihydroxyphenylacetic acid (DOPAC), L-3,4-dihydroxyphenyl alanine (DOPA) and homovanillic acid (HMV) were purchased from Sigma-Aldrich and phenylethylamine (PEA) from Acros Organics (Geel, Belgium). Deuterated acetic anhydride [d6] was purchased from Sigma-Aldrich and deuterated dopamine [d4] and DOPA [d3] from CDN Isotopes (Quebec, Canada).

Standard Preparation

Individual stock standards (1000 mg/ml) (PEA, dopamine, serotonin, normetadrenaline, noradrenaline, adrenaline, DHPG, DOPAC, DOPA and HMV) were prepared in 0.1 % formic $acid_{aq}$, and used to create a mixed catecholamine standard of all compounds (10 mg/ml). Two separate labelled internal standards for spiking and recovery were also prepared (IS1) DOPA [d3] 10 mg/ml and (IS2) dopamine [d4] 10 mg/ml. One hundred millilitres of each of these standards was derivatised separately, as described for the samples, to prepare a derivatised mixed standard, and two derivatised internal standards (IS1 and IS2). The derivatised mixed standard was used to prepare calibration standards in the range 0.02 ng/ml to 5 ng/ml.

To facilitate quantitation and to correct for matrix effects during analysis, labelled internal standards for each analyte were prepared (d-IS) by derivatising 100 ml of the mixed catecholamine standard (10 mg/ml), as described for the samples, with the exception that deuterated acetic anhydride [d6] was used in place of unlabelled acetic anhydride.

All calibration standards were spiked with 10 ml of the derivatised internal standards [(IS1) DOPA [d3] 100 ng/ml and (IS2) dopamine [d4] 100 ng/ml; final concentration 1 ng/ml] and 100 µl of the derivatised labelled internal standard catecholamine mixture [(d-IS) 10 ng/ml; final concentration 1 ng/ml] and prepared at a final volume of 1 ml.

Sample Preparation

Plasma samples were treated to remove proteins and derivatised in two stages to acetylate alcohol and amine functional groups prior to LC-MS analysis. A double derivatisation was found to be necessary to acetylate the less reactive alkyl hydroxyl groups. Briefly, each plasma sample (200 µl) was added to a 1.5 ml microtube already containing 600 µl cold acetonitrile, 100 µl acetic anhydride and 10 µl 100 ng/ml DOPA d3 [(IS1); final concentration 1 ng/ml] and mixed well. Samples were chilled at -80 ^{o}C for 15 minutes then centrifuged at 16100 g for 15 min, and the filtrate transferred to a 15 ml screw capped glass culture tube. A further 200 µl acetonitrile was added to each microtube, mixed,

centrifuged at 16100 g for 15 min and the filtrate combined with the original filtrate. To each combined sample filtrate 10 µl 100 ng/mL dopamine d4 [(IS2); final concentration 1 ng/ml] was added and the samples derivatised by the addition of 200 µl 100 mM borate buffer (3.81g in 100 ml of water) and 100 µl acetic anhydride and microwaved at 30 % power for 15 minutes. Samples were then evaporated to just dry with nitrogen at 50 °C. Samples were re-derivatised; 100 µl acetonitrile, and 100 µl acetic anhydride and microwaved at 30 % power for 15 minutes. Finally, to each sample 100 µl of the derivatised labelled internal standard catecholamine mixture [(d-IS) 10 ng/ml; final concentration 1 ng/mL] was added, and the samples made up to 1 ml with water and transferred to an autosampler vial ready for analysis.

LC-MS Analysis

Analysis of catecholamines was performed using an AB Sciex Qtrap 5500 equipped with a Turbo V electrospray source (ESI) (AB Sciex, Foster City, California, USA), coupled to a Dionex UltiMate 3000 HPLC system, which consisted of two UltiMate 3000 RS pumps, an UltiMate 3000 RS autosampler and an UltiMate 3000 RS column compartment (Dionex, Olten, Switzerland) and controlled with Analyst 1.5.2 software. A 150 by 2.1 mm Atlantis® T3 analytical column, (3 µm particle size; Waters Corp., Milford, MA, USA) maintained at 50 °C was used. 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 0.6 ml/min. The initial mobile phase, 100 % A, was ramped linearly to 70 % A at 12 min, 30 % A at 15 min, and 0 % A at 15.5 min and held for 4 min before resetting to the original conditions. Sample injection volume was 50 µl.

The ESI conditions were: gas 1, nitrogen (40 psi); gas 2, nitrogen (50 psi); ion spray voltage, 2500 V; ion source temperature, 700 °C; curtain gas, nitrogen (50 psi). LC/MS data was acquired in the positive mode using the most intense selected reaction monitoring (SRM) transition for each compound. In some cases the ammonium adduct was the most abundant ion observed for Q1. A detailed description

of analyte specific MS parameters is given in table 4 . Quantitation was performed using the internal standard ratio method using MultiQuant software.

Table 4 near here

Glucose and lactate analysis

Twenty microlitres of whole blood were collected from the inlaying cannula in an end-to-end capillary (EKF Diagnostics, Surrey, UK) and immediately transferred into an EKF safe-lock cup prefilled with 1 ml of haemolysis solution. The whole blood in haemolysis solution was then analysed using a Biosen C_line analyser (EKF Diagnostics, Surrey, UK) for glucose (mmol/L) and lactate (mmol/L) within 30 minutes of collection. The manufacturer reports that the Biosen C_line analyser has a coefficient of variance of 1.5 %.

Procedures

Participants were required to attend the laboratory a total of three times. The first was a screening visit to ensure eligibility, the second and third were study visits. On all study day visits, participants arrived in the laboratory at 8 am and confirmed that they had fasted for 12 hours prior and were in good health. An inlaying cannula was then inserted into the participants left median cubital vein by a qualified phlebotomist. Fifteen millilitres of blood were then drawn from the cannula using a 5 ml vacutainer containing EDTA and a 10 ml vacutainer containing LH. Depending upon randomised treatment allocation, the participant then consumed either 'Blackadder' juice or the matched control. Drinks were presented to participants chilled in a sealed opaque bottle and they were given 5 minutes to consume the drink through a straw. A further 15 ml of blood was then removed via the cannula; at; 15, 30, 45 60, 100, 120, 150, 180 and 240 minutes post consumption. Participants were then free to leave. Participants were required to attend a 24 hour follow up visit where 15 ml of blood was obtained via venepuncture. Diet was controlled (nil by mouth other than water) during the 4 hour period after consumption of the treatments. There was no standardisation of food intake across visits during 4-24 hours. Participants were however asked to consume no purple coloured berries. A graphical representation of the study design can be seen in Figure 1.

FIGURE 1 NEAR HERE

Statistical analysis

Data from baseline through to 240 minutes post-consumption were analysed to assess the C_{max} and T_{max} of the biological parameters up to four hours post dose. Twenty-four hour follow-up data was analysed to assess if values had returned to baseline 24 hours post-dose.

For all blood parameters an estimation of the area under the curve (AUC) was calculated using the incremental trapezoidal method. AUC was calculated incrementally from time point; 0 to 15, 15 to 30, 30 to 45, 45 to 60, 60 to 100, 100 to 120, 120 to 150, 150 to 180 and 180 to 240 minutes using "unchanged" raw data (iAUC). Increments were then summed to give an area under the curve from time point zero, to the last observed study day concentration at 240 minutes post consumption (AUC_{0-t}).

Treatment effects were analysed with linear mixed models (LMM) including the terms treatment, assessment, treatment x assessment as fixed effects for AUC. Pairwise comparisons corrected for least squares difference, were conducted on all outcomes with a p value <0.05 from the initial mixed model analysis to ascertain any differences between treatments for the whole session and at specific epochs. Twenty-four hour assessments were analysed on "raw" values using repeated measures LMM including the terms treatment x assessment (baseline and 24h) as fixed effects. All data were tabulated using Microsoft Excel 2013 and analyses were conducted with IBM SPSS Statistics 22.

Results

Analysis of baseline data revealed there were no significant pre-dose differences between treatments on any measures. Area under the curve data can be found in Table 5. Twenty-four hour post-data are presented in Table 6. Outcomes that elicited a significant effect in the initial LMM are outlined below.

Platelet MAO-B activity

Analysis of $AUC_{o\text{-}t}$ data revealed a significant effect of treatment [$F(1,12.43)=67.2$, $p<0.001$]. This was observed to be due to a significant reduction in AUC platelet MAO-B activity after consumption of 'Blackadder' when compared to control. Analysis of iAUC data revealed a significant treatment*increment interaction [$F(8,14.77)=13.00$, $p<0.001$]. Pairwise analysis of the increments revealed significantly lower iAUC at, 15–30 ($p<0.001$), 30–45 ($p=0.002$), 45–60 ($p<0.001$), 60–100 ($p=0.001$), 100–120 ($p<0.001$), 120–150 ($p<0.001$), 150–180 ($p<0.001$) and 180–240 minutes ($p=0.001$) following 'Blackadder' when compared to control. There were no significant effects of treatment at the 24 hour time point. Please see Figure 2a.

Prolactin

Analysis of $AUC_{o\text{-}t}$ data revealed a significant effect of treatment [$F(1,13)=6.24$, $p=0.027$]. This was observed to be due to a significant reduction in prolactin after consumption of 'Blackadder' when compared to control. Analysis of iAUC data revealed a significant treatment*increment interaction [$F(8,24)=3.12$, p0.012]. Pairwise analysis of the increments revealed significantly or trend to significantly lower iAUC at 45-60 ($p=0.083$), 60-100 ($p=0.009$), 100–120 ($p<0.03$), 120–150 ($p=0.012$), 150–180 and ($p=0.05$) following 'Blackadder' when compared to control. There were no significant effects of treatment at the 24 hour time point. Please see Figure 2b.

FIGURE 2 NEAR HERE

TABLE 5 NEAR HERE

TABLE 6 NEAR HERE

Discussion

Results from this randomised, placebo-controlled, double blind, counterbalanced cross-over trial demonstrate a fast and absolute inhibition of blood platelet MAO-B and a significant reduction in plasma prolactin induced by the consumption of the New Zealand 'Blackadder' blackcurrant juice when compared to a placebo control.

The current study illustrates a sustained significant reduction in peripheral platelet MAO-B activity of ~100 % by consuming 'Blackadder' blackcurrant when compared to control. This reduction began within 15 minutes of consumption, during the first post-dose epoch measured, and continued to be significantly reduced through to four hours post dose, that being the last measurement on the day of treatment. Platelet MAO-B activity had returned close to the pre-dose baseline level 24 hours post dose. Due to the extremely rapid inhibition of the platelet MAO-B enzyme, it is not possible to calculate a time to maximal inhibition. It would therefore be useful if future studies were conducted using several doses and shorter initial blood collection epochs. The profile of MAO-B inhibition of 'Blackadder' juice bears a notable similarity, to pharmaceutical reversible MAO-B specific inhibitors such as lazabemide, which have shown a rapid inhibition of MAO-B in platelets of >90 % at 30 minutes post dose, with maximal inhibition subsiding 16 hours post dose and full restoration of enzyme activity returning 48 hours post dose following a 100 mg dose of lazabemide [22]. The active compound or compounds driving the inhibition in the present study are currently not known. Data in the literature outline an inhibition of MAO-B by anthocyanins *in vitro* [23]; however, this study used levels 1000 times higher than quantified in plasma after oral consumption [7]. In addition maximal plasma concentrations of anthocyanins do not occur until one to two hours post oral consumption [24], making it unlikely that they explain the effects seen at 15 minutes in the current study. Coupled with the inability of a blackcurrant anthocyanin-enriched extract (Delcyan^{TM}) to inhibit platelet MAO-B in our previous publication [7], these data suggest that anthocyanins are not likely to be the compound driving MAO inhibition *in vivo*. However, this does not rule out a synergy between anthocyanins and

lower molecular weight phenolic components such as phenolic acids. Further research needs to be conducted to attempt to identify the components responsible for the observed MAO inhibition.

Plasma levels of 3,4-dihydroxyphenylglycol (DHPG) levels were not significantly impacted after the consumption of 'Blackadder' juice. However, the pattern observed highlights that DHPG was reduced by ~30 % when compared to baseline at 100 minutes before rapidly returning to baseline by 3 hours post dose. As a proxy for MAO-A activity [20] these data indicate an inhibition of the MAO-A enzyme in the periphery. AUC analysis showed no significant differences in normetadrenaline up to 4 hours after consumption of `Blackadder' blackcurrant. However, exploration of the data indicates approximately 100 % increase in normetadrenaline when compared to control at 150 minutes, which indicates an increased breakdown of adrenaline via catechol- methyl transferase (COMT) and is in line with our previous observation [7] Analysis of data 24 hours post consumption also shows a significant increase in normetadrenaline, indicating circulating normetadrenaline is still above baseline 24h post dose. There were no other significant changes in measured catecholamines or associated metabolites between treatment groups.

As anticipated, peripheral prolactin was significantly reduced after consumption of 'Blackadder' juice when compared to placebo, confirming our previously reported non-significant reductions. Reductions were seen as early as 30 minutes post consumption, with significantly lower area under the curve increments beginning 45 minutes post dose and continuing until the last measured concentration at 240 minutes post dose. The maximal reduction appeared at the 120 minute epoch with a reduction of 61 %. These findings are consistent with inhibition of prolactin secretion by the central D2 receptor agonist bromocriptine, 12 mg of which reduces peripheral prolactin by ~ 60 % two hours post dose [25]. Since dopamine receptor agonists have been show to inhibit peripheral prolactin secretion [25, 27], these data indicate the possibility of a centrally active inhibition of MAO-B and an increase in central dopamine levels after ingestion of the 'Blackadder' juice in healthy young men. Although it must be noted that there are no reports in the literature of

direct interactions between constituents found in blackcurrants and the suppression of prolactin excretion from the pituitary gland, this mode of action cannot be ruled out.

With regards to blood glucose modulation, post prandial profiles were not statistically different. However, examination of the data showed that consumption of 'Blackadder' juice reduced the post-prandial peak of blood glucose, and delayed the peak by 15 minutes when compared to the control beverage(see figure 2c). Although not significant, a modulation of blood glucose occurs until 100 minutes post consumption of 'Blackadder' juice when compared to the sugar matched control with lower plasma levels until ~35 minutes post dose. Higher blood glucose levels are seen until 100 minutes post dose, after which glucose levels return to a level similar to control and remain that way until the last measured time point at 240 minutes post dose. Blood glucose followed a similar pattern of modulation described by Törrönen *et al.,* [28] and Wilson *et al.,* [29], with reduced levels from the first post-dose measurement at 15 minutes until one hour post dose. In the current study, glucose findings are coupled with a non-significant reduction in post-prandial lactate 15 minutes post consumption of 'Blackadder' blackcurrant when compared to control. As lactate is a by-product of glucose metabolism, this further supports the hypothesis that the pattern of glucose modulation was a result of moderately slowed glucose absorption rather than an increase in metabolism. Although samples in the current study were taken at regular time points via cannulation, it must be noted that the infrequent samples make the assumption that the modulation in blood glucose is linear between each sample. It would be beneficial to use such methods as interstitial continuous glucose monitoring as used by Dye *et al.,* (2010) to allow for a full "real time" profile of the effects of 'Blackadder' blackcurrant upon blood glucose to be monitored.

Monoamine oxidase inhibitors have been highlighted as a tool to attenuate age related decline of behavioural performance and decrease susceptibility to senile depression, Parkinson's disease and Alzheimer's disease" [8]. Data from this current trial provide invaluable pharmacodynamic information pertaining to the impact of 'Blackadder' blackcurrant juice upon platelet MAO-B activity and peripheral prolactin. The findings suggest a reversible non-selective inhibition of the MAO

enzymes by ‘Blackadder’ juice, although a somewhat greater affinity to MAO-B is indicated. These changes in MAO inhibition were evident without a measurable accumulation of monoamines in the periphery. Future research should utilise blackcurrant’s reversible MAO-inhibiting properties and assess its impact upon cognitive functioning in a population at risk of age-related cognitive decline.

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Table 1 Mean participant characteristics

Measure	Average measurement	SD	Range
Age (years)	25.3	4.7	20–35
Height (m)	1.81	0.07	1.7–1.95
Mass (kg)	82.31	4.73	75–89
BMI (kg/m^2)	24.99	2.01	21–27

Table 2 Phytochemical constituents of 'Blackadder' blackcurrant juice (mg/100 ml of raw juice and dosing range supplemented).

Compound	**Quantity (mg/100 ml)**	**Dose range mg**
3-Caffeoylquinic acid	6	5.88- 6.97
Caffeoylhexose	2.5	2.50- 2.97
3-*p*-Coumaroylquinic acid	5.7	5.63- 6.68
Epigallocatechin	4.9	4.88- 5.79
Delphinidin 3-*O*-glucoside	47.7	47.13- 55.92
Delphinidin 3-*O*-rutinoside	184.6	182.38- 216.42
Cyanidin 3-*O*-glucoside	20.6	20.38- 24.18
Cyanidin 3-*O*-rutinoside	206.5	204.00- 242.08
Petunidin 3-*O*-rutinoside	3.5	3.50- 4.15
Pelargonidin 3-*O*-rutinoside	3.7	3.63- 4.30
Peonidin 3-*O*-rutinoside	5.4	5.38- 6.38
Myricetin 3-*O*-rutinoside	15.3	15.13- 17.95
Myricetin 3-*O*-glucoside	2.2	2.13- 2.52
Quercetin 3-*O*-rutinoside	3.6	3.50- 4.15
Quercetin 3-*O*-glucoside	2	2.00- 2.37
Quercetin 3-*O*-pentoside	1.7	1.63- 1.93
Myricetin	2.2	2.13- 2.52
Total anthocyanins	474	465–555
Total polyphenols	636	624–744

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Table 3 Anthocyanins and other phenolic compounds in each of the treatment conditions (mg per kilo of body weight, average dose given (mg) and dose range (mg))

Treatment	Anthocyanins (mg/kg)	Anthocyanin average dose (mg)	Anthocyanin dose range (mg)	Other polyphenols (mg/kg)	Other polyphenols average dose (mg)	Other polyphenols dose range (mg)	Total polyphenols (mg/kg)	Total polyphenols average dose (mg)	Total polyphenols dose range (mg)
Control	0	0	0	0	0	0	0	0	0
'Blackadder'	6.21	511	465–555	2.11	173	158–188	8.33	685	624–744

Table 4: MRM Transitions used for catecholamines and their isotopically labelled internal standard analogues

Q1	Q3	Time	Name	DP	EP	CE	CXP
344	197	8.65	DOPA d3 (IS1)	70	10	35	16
353	201	8.58	DOPA d3 [d9]	70	10	35	16
341	194	8.67	DOPA	70	10	35	16
350	198	8.60	DOPA [d9]	70	10	35	16
164	105	8.98	PEA	30	10	25	10
167	105	8.95	PEA [d3]	30	10	25	10
261	160	9.75	Serotonin	10	5	25	1
267	161	9.70	Serotonin [d6]	10	5	25	1
284	141	9.81	Dopamine [d4] (IS2)	70	10	37	15
293	143	9.74	Dopamine [d4] [d9]	70	10	37	15
280	137	9.85	Dopamine	70	6	35	15
289	139	9.78	Dopamine [d9]	70	6	35	15
242	137	9.89	HMV	50	10	30	16
245	137	9.85	HMV [d3]	50	10	30	16
270	165	9.93	DOPAC	50	10	15	15
276	168	9.87	DOPAC [d6]	50	10	15	15
250	166	10.58	Normetadrenaline	50	9	25	15
256	168	10.50	Normetadrenaline [d9]	50	9	25	15
355	194	10.61	Noradrenalin	10	10	30	1
367	199	10.51	Noradenalin [d12]	10	10	30	1
292	250	12.19	Adrenalin	170	10	20	1
301	257	12.09	Adrenalin [d12]	170	10	20	1
356.	237	14.09	DHPG	90	13	20	20
368	244	14.05	DHPG [d12]	90	13	20	20

Declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP) retention time (time),

parent ion (Q1) and product ion (Q3).

Table 5 Incremental and total AUC data for the control and 'Blackadder' blackcurrant (Ribes nigrum) for blood outcomes MAO-B prolactin, glucose and lactate and linear mixed model outcomes

Outcome	Treatment	N	0 to 15 minutes		15 to 30 minutes		30 to 45 minutes		45 to 60 minutes		60 to 100 minutes		100 to 120 minutes		120 to 150 minutes		150 to 180 minutes		180 to 240 minutes		Total AUC		Effect of treatment	Treatment* increment interaction
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
MAO-B (ng/ml)	Control	7	17305	5835	16850	6095	14774	4600	15052	4066	34649	9936	40411	17103	43400	18830	44437	17936	91500	35631	318379	112471	F=67 p<0.001	F=13 p<0.001*
	'Blackadder'		12758	8394	1375	2390	63	166	0	0	0	0	0	0	1195	2317	2339	2774	8834	9370	26564	19634		
Prolactin (ng/ml)	Control	8	4232	980	3665	793	3391	795	3208	861	6349	1872	6318	1997	5820	2172	5465	2209	11023	4879	49472	14764	F=6.24 p<0.001	F=3.12 p=0.012
	'Blackadder'		4155	927	3523	834	3074	799	2739	744	4514	1127	3733	1014	3557	1174	3753	1399	8286	3928	37333	11362		
Glucose mmol/ml)	Control	8	76.20	14.79	87.06	21.78	73.67	20.82	57.57	15.90	108.13	20.54	113.64	12.48	118.58	9.23	122.21	8.01	249.45	8.52	894.68	450.71	F=0.12 =0.91	F=2.08 p=0.01*
	'Blackadder'		69.39	9.39	77.64	15.16	75.34	16.37	64.04	18.52	112.33	21.81	113.44	12.88	118.95	9.64	123.60	11.03	248.44	15.99	891.71	451.11		
Lactate (ng/ml)	Control	8	13.65	5.16	19.48	8.54	21.83	7.30	20.06	6.17	30.88	10.13	21.88	7.42	20.14	5.92	21.58	5.86	43.84	12.31	213.33	56.17	F=1.55 >0.25	F=1.58 =0.15
	'Blackadder'		14.31	5.30	16.76	5.88	20.80	7.09	22.35	6.15	36.58	7.00	27.84	6.20	23.46	6.76	22.14	6.32	42.21	15.87	226.45	49.07		
Noradrenaline (ng/ml)	Control	8	10.13	2.31	10.55	3.61	10.22	4.75	11.68	6.77	24.56	11.75	23.04	9.03	22.29	8.69	20.88	7.37	39.13	11.43	172.49	54.90	F=0.97 P=0.33	F=0.74.47 P=0.65
	'Blackadder'		7.03	1.54	7.56	2.16	8.15	3.12	9.53	3.68	19.28	7.45	17.99	8.64	19.31	9.28	21.38	6.60	42.02	12.00	152.25	49.77		
Normetadrenaline (ng/ml)	Control	8	6.97	2.60	7.72	4.22	7.39	5.25	8.95	7.48	17.48	14.29	14.04	10.93	14.22	8.98	11.99	4.22	21.64	5.69	110.39	52.62	F=0.01 P=0.92	F=1.03.47 P=0.42
	'Blackadder'		4.99	3.32	6.02	3.85	6.25	3.69	6.99	3.47	14.80	7.96	13.13	7.35	13.32	6.30	14.47	5.41	27.94	8.06	107.92	38.80		
Serotonin (ng/ml)	Control	8	394.78	248.33	319.90	226.52	258.87	161.54	232.74	140.4	978.90	572.89	1481.13	944.93	1254.10	746.35	1071.53	728.45	1322.57	861.70	7314.52	3846.21	F=0.17 P=0.68	F=0.40 P=0.90
	'Blackadder'		438.13	361.61	333.94	239.81	248.67	141.85	215.24	82.75	752.41	417.04	1110.16	884.23	1056.18	722.17	919.20	598.86	1455.84	818.52	6529.76	3678.81		
Dopamine (ng/ml)	Control	8	5.93	6.17	5.51	5.43	4.41	5.03	4.20	4.77	7.74	6.94	6.91	6.40	6.51	5.32	5.30	3.60	9.11	7.05	55.62	45.00	F=1.56 P=0.22	F=0.69 P=0.69
	'Blackadder'		2.60	1.58	2.64	1.38	2.16	0.94	2.53	1.31	5.23	3.03	4.18	2.33	4.47	1.99	5.25	3.23	10.48	6.25	39.54	15.87		
DHPG (ng/ml)	Control	8	18.46	5.01	18.68	6.55	17.99	6.42	20.96	6.54	38.04	13.79	34.11	8.32	37.75	9.90	40.85	12.96	84.81	28.62	310.29	56.80	F=1.93 P=0.17	F=0.33 P=0.94
	'Blackadder'		16.55	4.72	15.91	5.61	14.28	5.93	14.71	7.55	28.61	21.90	27.35	26.17	31.12	20.70	34.98	13.63	73.61	20.14	255.27	111.54		
DOPAC (ng/ml)	Control	8	54.47	21.98	54.23	20.11	46.70	16.57	47.26	15.63	99.98	38.35	94.17	36.94	95.50	31.06	92.04	21.23	176.83	41.90	761.18	213.20	F=0.59 P=0.44	F=0.97 P=0.47
	'Blackadder'		57.97	23.21	52.02	22.85	48.05	21.14	47.68	16.36	84.71	25.72	73.58	30.39	78.54	37.11	88.00	40.72	178.93	68.06	709.47	269.69		
DOPA (ng/ml)	Control	8	22.49	8.99	23.00	8.83	21.87	8.29	20.56	9.21	43.89	16.51	45.89	12.80	51.71	17.84	52.54	22.08	110.57	44.57	391.29	101.25	F=1.43 P=0.24	F=0.71 P0.67
	'Blackadder'		23.94	9.52	20.72	6.69	20.14	7.84	22.18	9.49	44.02	11.13	38.45	8.68	42.45	9.52	45.59	17.13	82.63	36.46	337.12	91.05		
PEA (ng/ml)	Control	8	0.75	0.44	0.73	0.32	0.65	0.35	0.64	0.33	1.36	0.55	1.44	0.76	1.36	0.80	1.18	0.62	2.87	1.24	10.99	4.99	F=0.55 P=0.46	F=1.66 P=0.14
	'Blackadder'		0.68	0.29	0.75	0.34	0.71	0.35	0.86	0.40	1.91	1.00	1.43	0.76	1.38	0.49	1.56	0.53	2.76	0.76	11.89	4.03		
HMV (ng/ml)	Control	8	183.25	52.42	182.55	43.60	184.58	48.48	176.00	51.91	333.74	72.15	296.84	55.03	287.49	47.86	279.83	46.30	544.37	78.47	2461.11	436.17	F=0.21 P=0.64	F=0.65 P=0.72
	'Blackadder'		212.44	87.91	202.63	87.52	183.79	76.57	179.82	50.86	326.59	81.82	281.32	77.11	256.87	95.35	256.36	56.27	483.77	87.04	2367.79	676.80		

Monoamine oxidase-B (MAO-B); Homovanillic acid (HVA); 3,4-Dihydroxyphenylacetic (DOPAC); Dihydroxyphenylalanine (DOPA); phenylethylamine (PEA); 3,5-Dihydroxyphenylglycine (DHPG

Table 6 Mean change from baseline scores, standard deviations and LMM outcomes for all 24 hour parameters

Measure	Treatment	N	Baseline		24h		Treatment* repetition effect
			Mean	SD	Mean	SD	
MAO-B	Control	7	1074.40	376.60	1739.99	681.07	F=1.99 p=0.171
(nmol H_2O_2)	'Blackadder'		1526.02	1095.21	1417.86	542.26	
Prolactin	Control	8	308.32	86.65	332.67	120.66	F=0.72 p=0.79
(mIU/L)	'Blackadder'		296.46	69.68	301.01	122.47	
Glucose	Control	8	4.26	0.47	4.50	0.35	F=0.17 p=0.68
(mmol/L)	'Blackadder'		4.23	0.44	4.34	0.60	
Lactate	Control	8	0.72	0.23	1.01	0.44	F=0.335 p=0.56
(mmol/L)	'Blackadder'		0.93	0.37	0.66	0.30	
HMV	Control	8	12.45	4.44	12.86	7.46	F=0.86 p=0.36
(ng/ml)	'Blackadder'		14.20	5.34	10.85	4.51	
DOPAC	Control	8	3.55	1.51	4.64	4.82	F= 0.85 p=0.36
(ng/ml)	`Blackadder'		3.90	1.61	3.22	0.94	
Noradrenaline	Control	7	0.65	0.14	0.50	0.21	F=6.70 p=0.16
(ng/ml)	'Blackadder'		0.40	0.12	0.54	0.13	
Normetadrenaline	Control	8	0.46	0.14	0.37	0.17	F=5.28 p=0.03*
(ng/ml)	'Blackadder'		0.27	0.17	0.52	0.32	
Serotonin	Control	7	27.24	17.55	17.49	23.60	F=0.14 p=0.71
(ng/ml)	'Blackadder'		30.63	25.17	15.28	13.14	
Dopamine	Control	8	0.41	0.44	0.22	0.17	F=2.97 P=0.96
(ng/ml)	'Blackadder'		0.14	0.10	0.51	0.77	
DOPA	Control	7	1.39	0.55	1.52	0.76	F=1.16 p=0.29
(ng/ml)	'Blackadder'		1.73	0.69	1.31	0.83	
PEA	Control	7	0.05	0.03	0.05	0.03	F=0.65 p=0.42
(ng/ml)	'Blackadder'		0.04	0.03	0.06	0.04	
DHGP	Control	7	1.37	0.31	1.22	0.66	F=2.68 P=0.11
(ng/L)	'Blackadder'		0.95	0.15	1.28	0.34	

Monoamine oxidase-B (MAO-B); Homovanillic acid (HVA); 3,4-Dihydroxyphenylacetic (DOPAC); Dihydroxyphenylalanine (DOPA); phenylethylamine (PEA); 3,5-Dihydroxyphenylglycine (DHPG)

Supplementary table 1. Raw catecholamine data

Outcome	Treatment	Baseline		15 minutes		30 minutes		45 minutes		60 minutes		90 minutes		120 minutes		150 minutes		180 minutes		240 minutes		24h	
		Score	SD	Score	SD	Score	SD	Score	SD	Score	SD	Score	SD	Score	SD	Score	SD	Score	SD	Score	SD	Score	SD
DHPG (ng/ml)	Control	1.37	0.31	1.24	0.50	1.34	0.60	1.30	0.29	1.42	0.63	1.23	0.61	1.41	0.75	1.30	0.52	1.42	0.43	1.32	0.47	1.22	0.66

	'Blackadder'	0.95	0.15	1.11	0.26	1.05	0.41	0.90	0.25	0.86	0.26	0.67	0.30	0.89	0.48	1.07	0.44	1.27	0.46	1.27	0.38	1.28	0.34
DOPAC (ng/ml)	Control	3.55	1.51	3.71	1.46	3.52	1.27	3.09	0.91	3.60	1.50	3.07	1.15	3.21	1.42	3.16	0.80	2.98	0.68	2.92	0.82	4.64	4.82
	'Blackadder'	3.90	1.61	3.83	1.49	3.55	1.42	3.30	1.43	3.06	0.85	2.59	0.90	2.65	0.87	2.92	1.32	2.95	1.41	3.02	0.87	3.22	0.94
DOPA (ng/ml)	Control	1.39	0.55	1.61	0.66	1.46	0.66	1.48	0.82	1.45	0.94	1.48	0.31	1.58	0.69	1.87	0.66	1.63	0.89	2.05	0.71	1.52	0.76
	'Blackadder'	1.73	0.69	1.46	0.64	1.33	0.60	1.52	0.90	1.43	0.65	1.50	0.48	1.22	0.32	1.64	0.44	1.40	0.79	1.35	0.66	1.31	0.83
PEA (ng/ml)	Control	0.05	0.03	0.05	0.03	0.05	0.03	0.04	0.02	0.05	0.02	0.05	0.03	0.05	0.02	0.04	0.03	0.05	0.03	0.05	0.02	0.05	0.03
	'Blackadder'	0.04	0.03	0.05	0.02	0.05	0.03	0.05	0.02	0.06	0.04	0.06	0.03	0.05	0.03	0.05	0.01	0.05	0.02	0.05	0.02	0.06	0.04
HMV acid (ng/ml)	Control	12.45	4.44	11.98	2.85	12.36	3.38	12.85	3.10	12.22	2.93	10.03	2.05	9.76	2.04	9.40	1.68	9.25	1.69	8.89	1.26	12.86	7.46
	Blackadder	14.20	5.34	14.12	6.44	13.93	5.18	12.32	3.99	11.66	3.22	10.12	2.47	9.07	2.86	9.18	2.63	7.91	1.57	8.22	1.70	10.85	4.51
Noradrenaline (ng/ml)	Control	0.46	0.14	0.56	0.40	0.77	0.81	0.54	0.44	0.39	0.14	0.37	0.17	0.46	0.14	0.56	0.40	0.77	0.81	0.54	0.44	0.39	0.14
	'Blackadder'	0.27	0.17	0.47	0.33	0.51	0.34	0.45	0.27	0.47	0.17	0.52	0.32	0.27	0.17	0.47	0.33	0.51	0.34	0.45	0.27	0.47	0.17
Normetadrenaline (ng/ml)	Control	0.46	0.14	0.47	0.24	0.56	0.40	0.48	0.34	0.77	0.81	0.40	0.31	0.54	0.44	0.41	0.23	0.39	0.14	0.33	0.10	0.37	0.17
	'Blackadder'	0.27	0.17	0.40	0.29	0.47	0.33	0.43	0.22	0.51	0.34	0.48	0.29	0.45	0.27	0.49	0.23	0.47	0.17	0.46	0.13	0.52	0.32
Serotonin (ng/ml)	Control	27.24	17.55	25.40	19.22	17.25	13.55	19.73	12.79	13.77	8.39	51.49	33.79	47.25	30.43	36.36	23.54	35.08	27.24	9.01	4.17	17.49	23.60
	'Blackadder'	30.63	25.17	27.78	23.63	19.13	16.93	16.41	8.19	12.28	8.20	37.88	29.80	41.30	29.58	34.28	22.44	27.00	20.49	21.53	16.56	15.28	13.14
Dopamine (ng/ml)	Control	0.41	0.44	0.38	0.40	0.36	0.35	0.27	0.35	0.33	0.32	0.19	0.18	0.27	0.26	0.16	0.11	0.19	0.15	0.11	0.09	0.22	0.17
	'Blackadder'	0.14	0.10	0.21	0.14	0.17	0.07	0.14	0.08	0.20	0.12	0.15	0.11	0.14	0.06	0.17	0.10	0.18	0.15	0.17	0.08	0.51	0.77

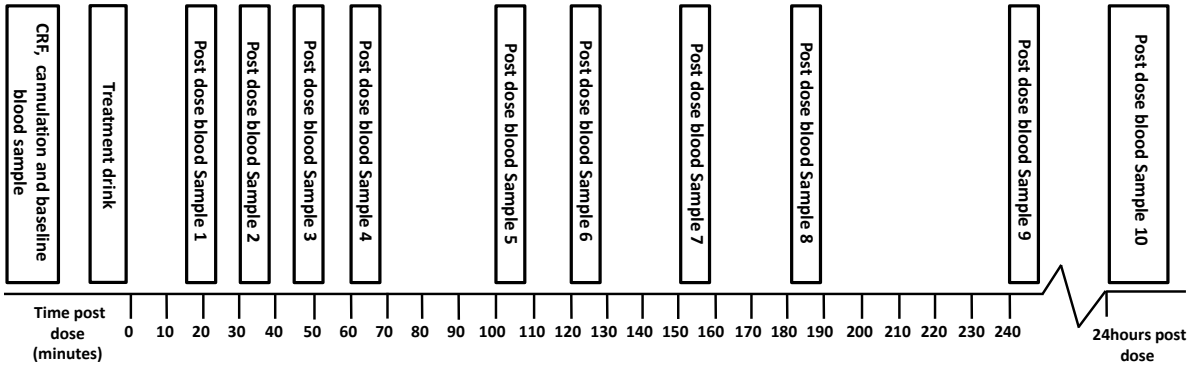

Figure 1 Study day running order. Scale depicts minutes post supplementation of the study intervention. CRF= case report form.

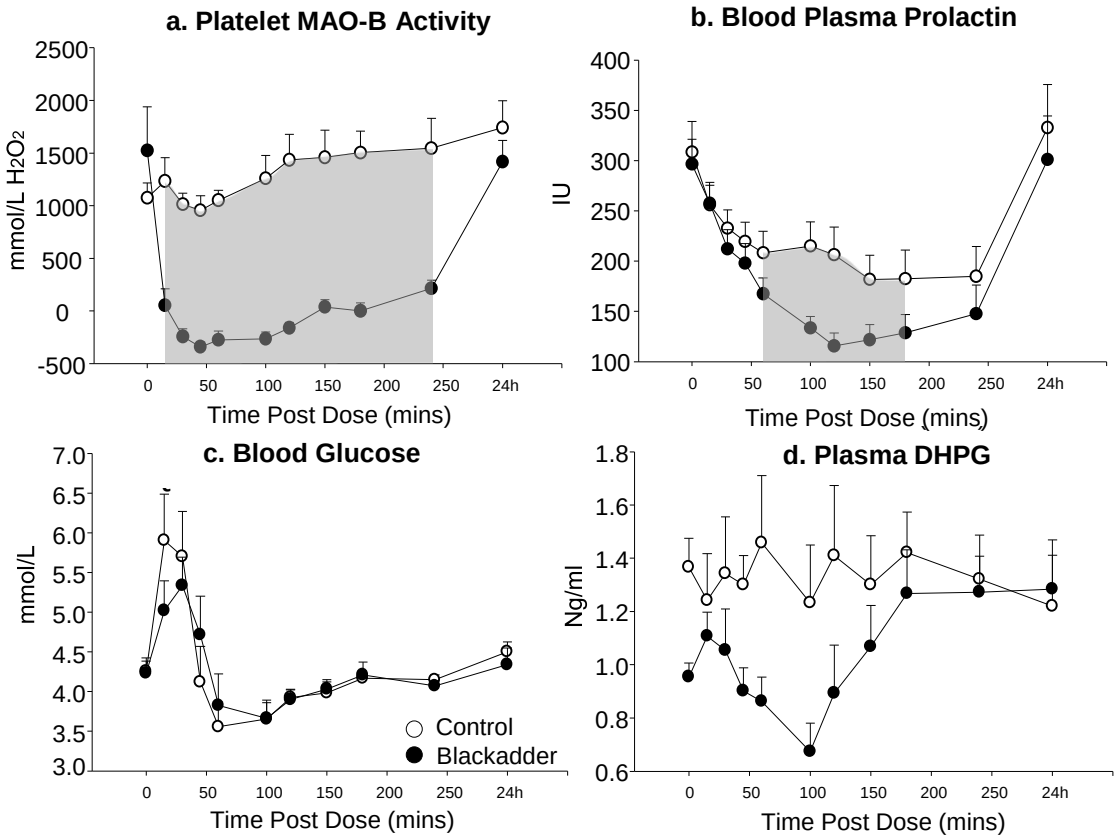

*Figure 2. "Unchanged" raw values for platelet MAO-B activity (a) and blood plasma prolactin levels (b) and blood glucose levels (c) and plasma 3,4-dihydroxyphenylglycol (DHPG) (d) t($p<0.1$). Shaded areas depict increments where $p<0.05$ in the treatment*increment pairwise comparisons. Open circles depict control and closed circles depict 'Blackadder' juice.*