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Low anthocyanin plum nectar does not impact cognition, blood pressure and gut microbiota in healthy older adults: A randomized crossover trial

Ezinne Oyidia Igwe
University of Wollongong, ekorie@uow.edu.au

Steven J. Roodenrys
University of Wollongong, steven@uow.edu.au

Yasmine Probst
University of Wollongong, yasmine@uow.edu.au

Vinicius do Rosario
University of Wollongong, vadr998@uowmail.edu.au

Michael Netzel

See next page for additional authors

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Abstract

Queen Garnet plum (QGP), known for its high levels of anthocyanins, is a hybrid of the Japanese plum developed in Queensland, Australia. Anthocyanins provide the red, blue and purple pigments in plants with demonstrated beneficial health effects. This study hypothesized that low-dose anthocyanin QGP intake will have a significant positive effect on cognition, blood pressure and gut microbiota in healthy older adults. A randomized crossover trial was conducted to determine the effect and within subject variance on cognition and 24 hr. ambulatory blood pressure in older adults without cognitive impairment following daily consumption of 200 mL low-dose anthocyanin (5 mg/100 g) QGP nectar (intervention) or raspberry cordial (control). Secondary outcomes included inflammatory markers (C-reactive protein), nerve growth factor (BDNF), and gut microbiota (16S rRNA gene sequencing). Twenty-eight participants (55+ years) were recruited. Each randomized treatment arm lasted for eight weeks with a 4-week washout period. Cognition, blood pressure and urine samples were measured at each visit (five total) while blood and fecal samples collected at baseline, eight and 20 weeks. Repeated measures ANOVA was used to analyze the data. Across the treatments, no significant difference was observed for the different domains of cognition, blood pressure or anti-inflammatory biomarkers. No intervention effect was found for genera or class of gut microbes. Low anthocyanin nectar derived from the QGP did not have any significant effects on cognition, blood pressure or gut microbiota in healthy older adults.

Keywords

does, nectar, plum, anthocyanin, low, adults:, older, healthy, microbiota, trial, gut, crossover, pressure, randomized, cognition, impact, blood, not

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Authors

Ezinne Oyidia Igwe, Steven J. Roodenrys, Yasmine Probst, Vinicius do Rosario, Michael Netzel, Hung Hong, Gabrielle Netzel, Anh Phan, and Karen E. Charlton

Low anthocyanin plum nectar does not impact cognition, blood pressure and gut microbiota in healthy older adults: A randomized crossover trial

Ezinne O. Igwe[1,2], Steven Roodenrys[3], Yasmine C. Probst[1,2], Vinicius do Rosario[1,2], Michael E. Netzel[4], Hung T Hong[4], Gabriele Netzel[4], Anh D. T Phan[4], Karen E. Charlton[1,2]

[1]School of Medicine, University of Wollongong, Australia

[2]Illawarra Health and Medical Research Institute, University of Wollongong, Australia

[3]School of Psychology, University of Wollongong, Australia

[4]Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Coopers Plains, QLD 4108, Australia

Corresponding author: Ezinne O. Igwe

Mailing address: School of Medicine, Faculty of Science, Medicine, and Health, University of Wollongong NSW 2522 Australia

Tel: +61 2 42392219

Email: ekorie@uow.edu.au

List of Abbreviation:

BDNF; Brain derived neurotrophic factor;

BP; Blood pressure;

hsCRP; high sensitivity C-reactive protein;

GBA; Gut-brain axis;

QGP; Queen Garnet plum;

QGPJ; Queen Garnet Plum nectar;

RAVLT; The Rey Auditory Verbal Learning test;

Abstract

Queen Garnet plum (QGP), known for its high levels of anthocyanins, is a hybrid of the Japanese plum developed in Queensland, Australia. Anthocyanins provide the red, blue and purple pigments in plants with demonstrated beneficial health effects. This study hypothesized that low-dose anthocyanin QGP intake will have a significant positive effect on cognition, blood pressure and gut microbiota in healthy older adults. A randomized crossover trial was conducted to determine the effect and within subject variance on cognition and 24hr ambulatory blood pressure in older adults without cognitive impairment following daily consumption of 200mL low-dose anthocyanin (5mg/100g) QGP nectar (intervention) or raspberry cordial (control). Secondary outcomes included inflammatory markers (C-reactive protein), nerve growth factor (BDNF), and gut microbiota (16S rRNA gene sequencing). Twenty-eight participants (55+ years) were recruited. Each randomized treatment arm lasted for eight weeks with a 4-week washout period. Cognition, blood pressure and urine samples were measured at each visit (five total) while blood and fecal samples collected at baseline, eight and 20 weeks. Repeated measures ANOVA was used to analyze the data. Across the treatments, no significant difference was observed for the different domains of cognition, blood pressure or anti-inflammatory biomarkers. No intervention effect was found for genera or class of gut microbes. Low anthocyanin nectar derived from the QGP did not have any significant effects on cognition, blood pressure or gut microbiota in healthy older adults.

Keywords: Queen Garnet plum; Anthocyanins; cognition; blood pressure; gut microbiota; fruit nectar

1 Introduction

Plums are part of a group of fruits known to contain phytochemicals known as anthocyanins, a sub-class of flavonoid. There are 19-40 different species of plums that exist of which only two; the hexaploid European plum (*Prunus Domestica)* and diploid Japanese plum (*Prunus Salicina* and hybrids) are of commercial significance globally [1]. Additionally, different hybrids of these species have been developed. One such hybrid of the Japanese plum is the Australian Queen Garnet plum known for its higher levels of anthocyanins (up to 277mg/100g), more than twice the anthocyanin content of regular plums which has been reported to range from 5-173mg/100g [2, 3].

Anthocyanins are red and purple pigments found in some fruits and vegetables. Besides being responsible for the color of the fruits, their beneficial health effects continue to be widely studied for cognition, and blood pressure (BP) [4, 5]. Although these beneficial health effects have been observed in different populations and using a variety of fruit sources, the complex metabolism and subsequent bioavailability of these compounds continue to hinder research efforts. Scientific evidence has emerged showing that anthocyanins are more bioavailable than originally perceived [6]. This observation stems from an increased interest in the colon as more than simply an excretion route, but also as an active site for further metabolism of ingested anthocyanins [7]. Some of the subsequent anthocyanin metabolites are believed to be more biologically active than intact anthocyanins [8, 9] and are possibly responsible for some of the observed beneficial effects on cognition (gut-brain axis) and vascular function (BP). Research on the colonic metabolism of anthocyanins has been faced with limitations, including the wide inter-individual variability in the metabolic fate of anthocyanins. This poses one of the largest methodological challenge thus far in determining the health effects associated with anthocyanin consumption [10].

Absorption of anthocyanins in the upper gastrointestinal tract is very low, possibly due to their digestive stability and binding interaction within the food-matrix [11]. As a result, a considerable amount of intact anthocyanins from fruits can reach the intestinal microbiota which cleave conjugated moieties, resulting in aglycones. These aglycones undergo ring fission to produce low molecular weight metabolites including phenolic acids and hydroxycinnamates which are then reabsorbed into the bloodstream where they are biologically active [12, 13]. The exact metabolite formation is not clear though *in vitro* studies suggest that bacterial metabolism of anthocyanins involves the cleavage of glycosidic linkages and the breakdown of the anthocyanidin heterocycle [9, 14].

The field of research related to the influence of the gut microbiota on a large array of chronic diseases including obesity and type 2 diabetes mellitus [15], inflammatory bowel diseases [16], irritable bowel syndrome [17] and allergies [18], has increased exponentially in recent years. The gut microbiota profile is also known to possess an important functional role in the metabolism, immunity and maintenance of a healthy human gut [19] as well as in the regulation of the gut-brain axis [20]. The GBA involves a two-way communication between the central and the enteric nervous system that links the emotional and cognitive centers of the brain, with the peripheral intestinal functions.

It has been shown that even though intact anthocyanins from fruits possesses protective health effects, their further metabolism and absorption in the colon can lead to considerable concentrations in the colon lumen and also in the central circulation. In turn, these metabolites also regulate the growth of specific bacteria in the intestinal microbiota. An example is an observed increase in the concentration of fecal *Bifidobacterium* as a result of anthocyanin microbial metabolites following anthocyanin supplementation [21]. *Bifidobacteria* are beneficial gram-positive, polymorphic rod-shaped bacteria normally found

in the gastrointestinal tract of humans and animals [22]. In healthy breastfed babies these bacteria are the dominant microbial group and their levels remain relatively stable, tending to decrease with advancing age as the bacteria belonging to Bacteroidetes and Firmicutes phyla are more dominant in adulthood [23]. In recent years, this microbial genus has been studied extensively due to both its important role within the human intestinal microbiota and the widespread use of *Bifidobacterium* strains in probiotic food products.

Evidence from the scientific literature has shown that anthocyanins may exert protective effects on different domains of cognition, including memory and executive processing, either through a direct effect on brain function or indirectly by reducing BP and neuroinflammation [24, 25]. In acute and longer term studies, anthocyanins from fruits including plums have been shown to have significant clinical benefits on different domains of cognition [26] and BP [27, 28] as well as proliferative and inhibitory effects on different species of gut microbiota [29]. These effects have been observed in studies using different study designs, varying dietary sources and doses of anthocyanins, and a range of health conditions including individuals with mild to moderate cognitive impairment and hypertension [5].

Anthocyanin-rich fruits are seasonal and variation in their anthocyanin content can occur due to agricultural factors such as climate, growing conditions, drought and time of harvest [30-32]. During the 2018-19 season, anthocyanin content in fresh QGP fruit ranged from 86 mg/100g (early harvest) to 102mg/100g (mid harvest), 149mg/100g (optimal harvest) and 171 mg/100g (very late harvest) (unpublished data; Department of Agriculture, DAF). In addition, these phytochemicals are highly unstable and susceptible to degradation through processing [33]. This is problematic when seeking to achieve a standardized food

intervention necessary for high quality clinical trials, particularly in studies requiring recruitment over an extended period of time. Unfortunately, many food-based trials do not consider such variability in dosage within interventions, nor do they monitor change in the anthocyanin content over time, which challenges the applicability of study findings to dietary messages. A gap in the literature currently is the investigation of commercial type fruit products that are marketed as being rich in anthocyanin content. As such, the possibility of degradation of the anthocyanins in such anthocyanin-rich foods warrants further research into the potential effect of low anthocyanin foods on different health parameters.

Information in non-cognitively impaired older adults is limited, despite an interest in maintaining cognitive function in old age. Thus, this study hypothesized that the consumption of low anthocyanin QGP nectar for eight weeks by healthy older adults (aged 55+ years) will:

1. Have a significant effect on cognition as measured using validated cognitive tests and 24hr ambulatory blood pressure;
2. Have a significant proliferative and inhibitory effect on gut microbiota genera, *Bifidobacteria* and *Clostridium*, respectively; and
3. have a positive effect on the anti-inflammatory biomarkers, serum hsCRP, and BDNF levels.

From the above hypothesis and expected outcomes, a randomized crossover trial was designed with the aim to determine the effect, and within subject variance, on primary measures of cognition and ambulatory blood pressure in healthy older adults following the daily consumption of processed low anthocyanin QGP nectar or a control beverage with negligible anthocyanin composition. Changes in the gut microbiota during the clinical trial, specifically *Bifidobacteria* and *Clostridium* genera, and selected anti-inflammatory

biomarkers were also measured, in order to better understand the mechanism of the clinical effects.

2 Methods and materials

This study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the University of Wollongong Human Research Ethics Committee, New South Wales, Australia (HE16/278). This study was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617000220369). The research team were trained in accordance with the ICH-GCP guidelines.

2.1 Study design

A randomized, crossover clinical trial was run for a total period of twenty weeks. The cross over design of the trial allowed for baseline measurements to be collected once, prior to the start of the trial treatments, rather than at the beginning of each arm [34] (Figure 1). Participants were randomly allocated to one of two arms (eight weeks on each arm with a washout period of four weeks) see Figure 2. The intervention arm received QGP nectar and the control arm received raspberry cordial beverage, that had a similar vitamin C content but negligible anthocyanin composition. The nutrient composition of the two beverages are presented in Table 1. Participants were advised to consume 200mL per day (one bottle) of the provided beverage for the duration of the study arm. The duration of the trial was based on the findings from previous trial using high anthocyanin cherry nectar [28] which detected changes in the measured variables as early as six weeks.

2.2 Participants

The participant inclusion criteria were generally healthy adults aged 55 years and older. Exclusion criteria included: uncontrolled hypertension; type 2 diabetes mellitus (T2DM); any unstable physical or mental health condition including diagnosed dementia assessed at pre-

screening interview from participants regular medical check-up sessions; an inability to provide informed consent; or an inability to communicate in the English language.

Participants were asked about their last regular medical check-up and whether they had been diagnosed with any of the above conditions prior to enrolment. Participants who were on antibiotic treatment or had any gastrointestinal diseases were also excluded.

All participants of the current study were recruited from mailing lists within the University of Wollongong (UOW) and the surrounding Wollongong region. Potential participants had the opportunity to discuss the study over the telephone prior to the clinic visits and were screened to determine eligibility. All eligible participants were provided with participant information sheets (PIS) and a member of the research team was available to answer any questions that arose. Following provision of the PIS, consenting participants provided written informed consent prior to baseline measurements being taken.

2.3 Intervention

The QGP nectar was donated by Nutrafruit Pty Ltd. (Yeerongpilly, QLD, Australia), while the control cordial beverage (Golden Circle Raspberry cordial) was purchased from a major supermarket chain in Wollongong, NSW, Australia. A random allocation schedule prepared by an independent statistician was used to randomly assign the participants to either arm. Researchers were blinded to the allocation though due to the taste and physical differences, participants were able to tell the difference between the beverages.

An independent research assistant packed the appropriate beverages into a sealable cooler bag to be delivered to participants' home on a fortnightly basis. No change to the regular diet was

advised. Participant compliance was recorded from unconsumed returned bottles every fortnight.

Data collection was performed over five visits: baseline, four weeks, eight weeks which was the end of arm one as well as 16 weeks and 20 weeks which was the end of arm two after crossover (Figures 1 and 2). Participants were required to attend their clinic visits in a fasted state for the baseline, and eight-week data collection time points of each arm which involved collection of fasting blood samples for the anti-inflammatory biomarkers (hsCRP and BDNF) measurements. On clinic days, participants were provided with a standardized breakfast (two wheat biscuits, full cream milk, and English breakfast muffin) after the blood sample collection and before the administration of the battery of cognitive tasks. The standardized breakfast provided 1314kJ energy and 64.8g carbohydrate (source: food label). For the four-week visits of each arm, participants had their usual breakfast at home prior to attending the testing facility.

2.4 Anthocyanin characterization of the intervention beverage

Determination of concentrations of anthocyanins in the intervention nectar was conducted at seven time points during four months of the trial using the validated AOAC pH-differential spectrophotometric method (Spec. AOAC) [35], a spectrophotometric procedure specifically designed to determine the total anthocyanin content in fruit juices and fruit-based products, by a laboratory at the University of Wollongong [36]. This method applies the principle that monomeric anthocyanins reversibly change color with change in pH; colored flavyllium form exists at pH 1.0, while colorless hemi-ketal form predominates at pH 4.5. The difference in absorbance at 520 nm is deemed proportional to the pigment concentration. The anthocyanin

concentration of the nectar was also conducted eight months after the completion of the trial by laboratories of the Queensland DAF using a HPLC-based protocol, as well as the AOAC pH-differential method (Spec.AOAC) described above. The HPLC-chromatograms established that the cyanidin-3-glucoside was the predominant anthocyanin in the QGP nectar, with cyanidin-3-rutinoside the next most abundant, contributing over 95% of the total anthocyanin content. Water soluble anthocyanins were extracted using an acidified water-organic solvent and prepared for separation and determination. Briefly, ≈ 5.0 g of the thawed puree was weighed into a 50 mL centrifuge tube followed by 15 mL of extracting solution (4% formic acid in deionized water: 100% methanol in the ratio 20:80 v/v). This was sonicated for 10 min with occasional shaking at ambient temperature, then centrifuged at 3220g for 5 min. The supernatant was transferred to a 50 mL volumetric flask while the residue was re-submitted for extraction another two times. The three supernatants were combined and made to volume in a 50 mL volumetric flask with extracting solution as above. An aliquot was diluted 50:50 with 2% formic acid in deionized water and filtered through a 13 mm 0.22µm PTFE syringe filter into a vial prior to analysis. All extractions were performed in triplicate. HPLC methods were a modified version of those described by Kammerer et al. (2004) [37]. Samples were analyzed using a Shimadzu HPLC system linked to LabSolutions software (Shimadzu Co., Kyoto, Japan). Separation of the anthocyanins were achieved on a reversed-phase Gemini NX-C_{18} (Phenomenex,Sydney, Australia) 5µm-250 x 4.6 mm with matching guard column, maintained at 30^0C. A calibration equation was calculated from the curve of peak area versus standard cyanidin-3-glucoside concentration and applied to the peak areas of identified anthocyanins; concentrations were calculated after applying the appropriate dilution factor. The concentrations of the predominant anthocyanin, cyanidin-3-glucoside together with all of the other identified anthocyanins are expressed as mg of cyanidin-3-glucoside equivalents/100 g fresh weight.

2.5 Outcome variables

The primary outcome measure was change from baseline in cognitive parameters measured by five different cognitive tasks while the secondary outcome measures included change from baseline in BP parameters (systolic BP, siastolic BP, mean arterial pressure, heart rate and pulse pressure), inflammatory biomarkers (hsCRP) and brain derived neurotrophic factor (BDNF) in the blood samples, as well as the gut microbiota.

A battery of five cognitive tests, including the Rey Auditory Verbal Learning Test (RAVLT) [38], verbal fluency task [39], digit-span backwards task [40], Stroop task [41], and counting span [42], was administered to assess participants' cognition and memory. The RAVLT measured verbal learning and memory, which involved participants learning a list of words over presentation-test and delayed-test trials. Verbal fluency measured executive function, requiring participants to produce as many words as possible that belonged to a category (animals, vegetables or fruits) or began with a specific letter ('A', 'C', 'B', and 'F'). The digit-span backwards task assessed short-term memory storage and executive function by requiring participants to repeat a series of numbers in the reverse order they were given. The Stroop task assessed executive function whereby participants were provided with a sheet on which the words *green*, *yellow*, *red*, and *blue* were printed (20 in total) in either congruent or incongruent ink colors (e.g., the word "blue" printed in red). The aim was to name the actual colors and not the printed words, as quickly as possible. Two versions of the Stroop task (congruent and incongruent ink colors) were applied and analysis was based on the time difference between the two tasks. The counting span task assessed working memory. In this task participants were presented with displays of light and dark blue shapes (squares and circles) and are asked to count the number of dark blue circles on each slide. After a series of displays (between two and six), participants were asked to recall the count for each display in

the series, in the order in which they occurred. Participants were familiarized with the cognitive tests prior to data collection on the first day of each clinic visit, for a period of about 10 minutes.

Demographic and lifestyle questionnaires were completed by participants. The International Physical Activity Questionnaire validated by Hagströmer et al. (2006) [43] was used to determine habitual level of physical activity. For dietary data, participants were required to complete a 24-h dietary recall questionnaire, as well as a 3-day estimated food record all food and beverages consumed over two non-consecutive week-days and one weekend day. For baseline and the follow-up appointments, anthropometric measures including weight, height, waist and hip circumference, as well as physical measure (30-second sit-to-stand, and grip-strength) were collected detailed below.

Two BP measurements (24h and office) were taken. Twenty-four-hour ambulatory BP was taken using Welch Allyn ABPM (Welch Allyn, NSW, Australia; Model 7100) and spot BP measured according to a standard protocols [44] using Welch Allyn Spot Vital Signs LXi DXEmed Com.

The blood samples collected were spun in a centrifuge for 15 minutes at 3000rpm after 30 minutes of collection. The serum was collected in 2mL sterile tubes and stored at -80^{o}C for batch analysis by an independent laboratory (Cardinal Bioresearch Pty Ltd. Queensland, Australia) for BDNF (Brain-derived neurotrophic factor) and hsCRP levels. Serum BDNF was analyzed using an R&D Systems Duo ELISA kit (Intra assay CV 3% and Inter assay CV 4%, Lowest Detectable Dose 5 ng/mL). Serum hsCRP was analyzed using a BioBase immunoturbidimetric assay. The assay was run on a BioBase BK400 chemistry analyzer (BIOBASE, Shandong, China) (Intra assay CV 3.5% and Inter assay CV 5%, Lowest Detectable Dose 0.2 ng/mL). Furthermore, urine samples were screened for anthocyanins and their common conjugated and methylated metabolites by UHPLC-PDA-ESI-MS/MS [45].

Fecal samples were collected using commercial *uBiome*™ (San Francisco, CA, USA) gut kits. Participants were advised to collect their fecal samples following a bowel movement. Samples were collected from toilet paper using the provided instruments. The microbiome analysis by uBiome was done using 16S-sequencing on the Illumina Next Generation Sequencing platform that delivers sequence data [46]. 16S is a ribosomal gene present in bacterial DNA that enables the classification of bacteria at the genus level and in addition filters out genetic material from humans and other organisms. Of the three bacterial rRNA genes (16S, 5S and 23S), the 16S rRNA gene provides the most tractable combination of conserved sites for Polymerase chain reaction (PCR) primers and is, therefore, preferable to the other rRNA genes for phylogenetic identification [46]. This method of sequencing is commonly employed in gut microbiota studies creating less heterogeneity in studies for comparison.

Two trained assessors measured grip strength using a digital Jama handgrip dynamometer (Lafayette Instruments, City, IN, USA). Participants were seated with their elbow bent at 90^{o}. With a neutral wrist position, the dynamometer handle was held at position II, with the dynamometer supported underneath [47] and grip strength recorded for both left and right hands, and hand dominance noted. Height (in meters) and weight (in kilograms) were measured using a stadiometer (Seca, Hamburg, Germany) and an electronic scale (Omron HN286 Digital Personal Body Weight Scale; Omron, Silverwater, NSW, Australia), respectively, to two decimal places, and body mass index (weight/[height^2]) was calculated.

2.6 Statistical Analyses

The sample size for this study was calculated using GPower [48]. Previous work from our group [28] has found a moderate magnitude of effect of anthocyanin supplementation on

memory, however, the aim of the current study was to detect a smaller effect size of 0.3. To obtain a power of 0.90, n = 30 participants were needed, including a 10% expected dropout.

All statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) software (version 22.0, Chicago, IL, USA). Normal distribution of the continuous variables was assessed using the Shapiro-Wilk test, histogram, Q-Q plot and skewness and kurtosis. Continuous data are presented as means ± SD for normally distributed data and median (IQR) for non-normally distributed data. Differences between baseline and four and eight weeks of each study arm were analyzed using paired sample t-tests for continuous variables and chi-square test for categorical variables.

Repeated measures ANCOVA was used to determine the effect of time, treatment and time*treatment interactions for cognition and blood pressure measurements at four and eight weeks of each arm, and the biomarkers of inflammation at baseline and eight weeks of each arm. Alpha was set at 0.05 for statistical significance. The statistical analysis controlled for baseline measurements and randomization order as potential confounders. To avoid a significant reduction in the power of the study, no other covariates were included in the analysis due to the small sample size. However, there were no significant differences in the demographics according to the order of allocation.

Dietary anthocyanin intake was determined from the 24-h recall dietary assessments using an Australian anthocyanin database aligned to the AUSNUT 2011-13 database [27] available within the dietary analysis FoodWorks professional software (Version 8, 2009, Xyris Pty Ltd, Spring Hill, QLD, Australia). All participants were included in the final data analysis (i.e. intention-to-treat).

The linear discriminant analysis effect size (LEfSe) tool [49] was used to analyze gut microbiota data.

3 Results

Thirty-one participants (14 males and 17 females) were recruited between January and March 2018. Twenty-eight (90.3%) participants completed the trial. Three participants withdrew; two due to illness and one due to involvement in an accident (Figures 1 and 2). All participants who completed the study attended all five visits and no adverse reactions were reported throughout the study duration. The mean washout period for participants was six weeks, a deviation from the original four-week washout period due to scheduling clashes and illness.

3.1 Baseline

Baseline characteristics of participants are summarized in Table 2, with no significant differences seen in any variables according to the order of randomization. The mean age of the participants was 70 ± 10 years old with a mean BMI of 26 ± 4 and waist-hip ratio of 0.88 ± 0.09. Forty-five percent of the participants had a post-graduate qualification and 45% reported high physical activity levels. Baseline ambulatory blood pressure was within the normal range with a mean systolic BP of 129 ± 12 mmHg and mean diastolic BP of 80 ± 9 mmHg. Seven participants (23%) were on BP medications that remained stable during the duration of the trial.

3.2 Cognition and physical measurements

There was no observed significant difference from baseline or between arms for the battery of cognitive tests performed with the participants, nor for any of the physical tests (Table 3).

3.3 Ambulatory blood pressure and anti-inflammatory biomarkers (BDNF and hsCRP)

Similar to the cognitive outcomes, there were no observed significant effects observed in the 24-h ambulatory BP (Table 4).

In addition, measured anti-inflammatory biomarkers (BDNF and hsCRP) (Table 5) did not show any significant difference from the baseline measures.

3.4 Gut microbiota

There were no significant differences observed for the *Bifidobacterium* and *Clostridium* genera. These bacteria genera have previously been shown to be affected by anthocyanin supplementation [29]. Further analysis of gut microbiota data showed that there were no biomarkers associated with QGP nectar consumption over the eight-week intervention arm.

3.5 Urine

Cyanidin-3-glucoside and cyanidin-3-rutinoside, the main QGP anthocyanins, as well as cyanidin monoglucuronide, peonidin monoglucuronide and peonidin-3-rutinoside, the most common methylated/conjugated metabolites of cyanidin-based anthocyanins, were tentatively identified in some urine samples after QGP nectar consumption (Table 6). However, further evaluation was not undertaken since the concentrations of these anthocyanins and metabolites were below the limits of quantification; no other conjugated or methylated anthocyanin forms could be detected. Furthermore, no intact anthocyanins or common anthocyanin metabolites could be identified in the control samples (consumption of cordial beverage).

3.6 Dietary data

Analysis of participants' background diet is presented in Table 7. The mean intake of dietary anthocyanins was 143 ± 218 mg/day (median 34.2mg/d (IQR =280)).

During the trial, the anthocyanin concentration of the QGP nectar using the AOAC- pH-differential method ranged from 3.72 to 5.27 mg C3G eq./100g fresh weight which would have provided between 6.74 and 10.54 mg anthocyanins per day. Eight months after completion of the study, mean (SD) anthocyanin concentration of the frozen, stored plum

nectar was 3.7±0.3 (mg C3G eq.)/100g fresh weight and was 6.3±0.5 (mg C3G eq.)/100g fresh weight.

4 Discussion

This study hypothesis that the consumption of QGP nectar for eight weeks would have significant beneficial effects on domains of cognitive function, as well as result in reductions in BP and inflammatory biomarkers (hsCRP, BDNF) and changes in gut microbiota, compared to a control beverage, was not proven in a group of healthy older adults. Results from this study demonstrate that in healthy older adults without cognitive decline, supplementation with commercially available QGP nectar that had undergone heat treatment as part of the industrial processing of fresh plum fruit into nectar, appears to provide no beneficial effects. The QGP has been shown to contain a wide variety of anthocyanins and phenolics [45] and in its fresh state is a rich source thereof. However, preservation of these anthocyanins remains a challenge when the fruit is processed into nectar and stored over time. We have demonstrated that the anthocyanin composition of the intervention nectar was lower than expected in the present study, providing around 9mg per day.

Processing and storage times likely affected the anthocyanin stability in the intervention nectar and, subsequently, may explain the absence of any significant changes in measured variables. Due to the short growing season of plums in Australia, and the extended period of time typically required in clinical trials to recruit sufficient numbers of participants, we endeavored to standardize the provided nectar by using two combined batches that had been processed. This necessitated the nectar to be kept in storage under controlled conditions for over ten months (frozen) while the total study duration was eight months. Evidence shows that the processing of fruits to juice, especially using high temperatures, significantly degrades anthocyanins [33]. In addition, the thawing and freezing process involved in

bottling of the nectar could have also resulted in further degradation of the plum nectar anthocyanins [50]. This degradation effect, together with the low stability of anthocyanins in general, could have significant effects on health outcomes, thereby limiting the generalization of our research findings [51]. However, eight months after completion of this trial, the HPLC analysis of the frozen stored plum nectar showed no further degradation.

Anthocyanins from fruits have been shown to exert a protective effect on cognition even in the presence of other polyphenols. A randomized, double-blind, placebo-controlled study compared the safety and efficacy of encapsulated low dose enhanced wild blueberry extract (WBE111) in healthy older adults (65-80 years). Two doses of wild blueberry powder (500mg and 1000mg) were compared to a formulated wild blueberry extract, Thinkblue™ wild Blueberry Extract (111mg; WBE111). The anthocyanin doses across the three interventions were 1.35, 2.7 and 7mg/dose/day, respectively. The authors observed significantly better episodic memory performance following WBE111 at three months and lower systolic BP following intervention with WBE111 in comparison to placebo, even though one of the doses of wild blueberry powder (1000mg) contained more total polyphenols than the WBE supplementation (70mg/dose/day vs 50mg/dose/day) [52]. Other studies using grape and/or blueberry juice (87mg of anthocyanins per 100g and 1920mg/100g, respectively) observed significant effects on episodic and working memory [53, 54], as well as systolic BP in older healthy adults for six months [55] and individuals with mild-moderate cognitive impairment [28]. However, the study by Bensalem et al. (2019) [55] only found the benefits of anthocyanin supplementation following stratification of the participant data according to baseline cognitive characteristics, with memory benefits only seen in those with poorer function initially. Other studies that have provided higher doses of anthocyanins from fruits than in the current study have similarly not found health benefits in healthy older adults who have no prior cognitive deficit [56, 57].

Our study findings are consistent with similar studies on healthy older adults using a crossover study design [58, 59] as well as a parallel study design using elderberries [60] . In one of the studies [59], 80 mg of anthocyanin extract from bilberry (blueberry) (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*) were consumed in capsule form. While it is possible to encapsulate anthocyanins [61], which may make anthocyanin consumption easier to control by improved compound stability, the bioactive benefits of extracting and isolating the specific flavonoids via encapsulation has been questioned [62], as this process may adversely impact bioavailability.

Comparison across studies that report significant positive effects and those that have not found anthocyanin-related health benefits suggest the following four important differences: a longer duration of intervention (three to six months); food source of anthocyanin (usually blueberry); study design (parallel vs cross over); and cognitive function (individuals with mild cognitive impairment vs no cognitive decline). Positive studies have tended to use fruits such as blueberries and grapes that contain a wider range of anthocyanin sub-classes, rather than a fruit vehicle that contains mostly contains cyanidins such as the plum used in our study [63]. To further demonstrate an effect of a combination of anthocyanin sub-classes, a study that compared enocianin, a grape extract containing a variety of anthocyanins, with a pure malvidin-3-glucoside extract reported a more significant proliferative effect on gut bacteria with enocianin [64]. The synergistic effect of both nutrients and bioactive non-nutrient components within a food matrix has been highlighted by Jacobs and Tapsell (2007) [65, 66]. This is an important consideration in planning and executing nutrition research that is relevant for translation into dietary guidance.

A recent meta-analysis on the effect of anthocyanin-rich foods on cardiometabolic biomarkers found that berries and red grapes/wine significantly reduced BP. These effects were detected in overweight/obese individuals but not in normal weight individuals [55]. Available evidence suggests that in healthy older individuals, lower levels of anthocyanin supplementation do not seem to have significant effects over the short term (≤ two months) on either blood pressure or cognition. A study using anthocyanin-rich blood orange juice (50mg of anthocyanins/500mL) did not observe any effect on BP in healthy people (25-84yrs) over four weeks [58]. On the other hand, studies of dementia patients (69mg of anthocyanins /100ml; 139mg/d) [28] and post myocardial infarction patients (64mg/d) [67] reported significantly reduced BP over 12 weeks. Another important consideration for the study design is the differential effects seen in acute vs longer-term interventions. In acute settings, positive effects have been observed on cognition and BP both in children and adults [26, 68, 69] and also in longer-term studies on individuals with mild to moderate cognitive impairment [26, 28]. However, these acute effects in healthy older adults are not generally seen chronically [58] except in studies where blueberries have been supplemented over a longer term (3-6 months). There is no clear explanation for this observation except for the difference in duration, anthocyanin vehicle, study design and possibly metabolism. Evidence has suggested that a chronic intake of flavonoids does not result in increased levels of metabolites in the tissues compared to single doses; which show that brain levels are likely reflective of acute intake and may not accumulate over the longer term [70]. The lower than expected quantity of anthocyanins present in the plum nectar used in the current study is confirmed by an inability to determine the concentrations of anthocyanin metabolites in the urinary excretion of the participants, which were below the limits of quantification.

In line with the absence of any significant effects on cognition, no changes in either BDNF or the inflammatory biomarker, hsCRP, were observed following anthocyanin supplementation from QGP nectar. BDNF is an important growth factor for synaptic plasticity and learning and memory [71, 72] while hsCRP is a broad marker for inflammatory state. Our hypothesis was that anthocyanins would exert protective effects on memory and cognition through a subsequent increase in BDNF levels, amongst other pathways. In animal models, anthocyanins have been shown to increase BDNF levels [73] but there is little available evidence from human clinical trials. The only available evidence is from an acute trial [74] which showed stable BDNF plasma levels following blueberry anthocyanins supplementation in adults compared to a reduction associated with consuming a control beverage. It is important to note that the baseline CRP and BDNF concentrations were within normal ranges (CRP < 3mg/L and BDNF between 8–46 ng/mL [75]) which may explain the lack of change in these biomarkers. It has been suggested that anthocyanin-rich interventions tend to exert beneficial effects on the cardiovascular system in participants with clinically diagnosed disease compared to healthy subjects, where no significant effects are observed [60]. A review of anthocyanin effects on CRP levels from clinical trials also observed that purified anthocyanins or anthocyanin-rich extract supplementation did not have any significant impact on CRP levels [76].

In terms of effects of anthocyanins on gut microbiota, anthocyanins may have a significant proliferative effect on *Bifidobacterium* spp., known for their wide use in probiotics and for the treatment of irritable bowel syndrome as well as an inhibitory effect on the pathogenic strain *Clostridium* [29]. Our study did not find an intervention effect on these genera of gut bacteria nor on any other microbiota genera. Studies on the effect of anthocyanins on gut microbiota have observed proliferative and inhibitory effects on different bacteria species but

the effect on total bacteria count has not been consistent [29], and they may have proliferative and inhibitory effects of a similar magnitude in different species [77].

The absence of significant effects on the primary outcome measures could also be associated with the background dietary intake. Anthocyanin intakes in the Australian population have been estimated at between 1.4 -24.2mg/d [78]. However, the median intake of dietary anthocyanins in our study population was 34.2 (IQR = 280.3) which is considerably higher than intakes reported in other Australian studies [79, 80]. Significant associations have been demonstrated between habitual anthocyanin intakes and reduced disease risks in epidemiological studies [81-83], therefore a high background dietary anthocyanin intake may have hindered any possible additional benefits from a relatively low dose of anthocyanin supplementation. Additionally, the standardized breakfast provided to participants prior to administration of their cognitive tests at baseline and in both study arms had minimal anthocyanin content but provided 1314kJ and 64.8g of carbohydrate. Research on the effect of breakfast and breakfast composition on cognitive outcomes is inconclusive, however, available evidence shows that there are no observed effects on cognition following a carbohydrate dense breakfast [84].

One of the main strengths of this study was the crossover design. Participants acted as their own control which accounted for inter-individual variances and thereby increasing the statistical power. In crossover trials, three types of baseline measurements have been described: those taken before the start of the first arm, those taken at the end of the first arm and before the start of the second arm, and those taken at the end of the second arm. The first type has been described as a true baseline as there is always the possibility that carry-over effects may be present in the second and third type [34]. Additionally, as comparisons are

within each trial participant, it has been suggested that different baseline measurements are not compulsory. Hence for this study baseline measurements were recorded only once at the start of the trial. It is also of note that there were few dropouts in our study and the nectar consumption compliance was satisfactory (100%).

A limitation of our study was the inability to blind participants due to differences in the taste, texture and appearance of the intervention and control beverages. Other limitations include lack of quantification of the total phenolic content of the study beverage which may shed some light on the lack of effect on gut-brain axis. Additionally, the duration of the intervention may have been too short to elicit effects. A major consideration in the design of anthocyanin food-based research is related to preservation of the anthocyanin composition in test foods. Losses in the anthocyanin composition of the nectar due to the bottling process (repeated freeze-thawing) and other storage considerations presented a real challenge. Another nutrient-related limitation might relate to the difference in energy content between the intervention and control beverages (213kJ vs 115kJ) despite matching for vitamin C content. The nutrient content for the QGP nectar was analyzed by collaborators of the current study and the nutrient content for the control juice was available on the label, unless otherwise stated. The difference in methodology could be seen as a limitation in this study. Closer matching for total nutrient composition, except the relevant bioactives of interest is recommended for future studies. Another recommendation for future studies is quantification of anthocyanin phenolic acid metabolites which was not conducted in the current study.

In conclusion, our research hypothesis was rejected as low-dose anthocyanin supplementation provided in a food matrix of QGP nectar did not have any significant effect on cognition, BP, or nerve growth factor/ inflammatory markers (BDNF and CRP) over eight weeks but may have an effect on the gut microbiota population in healthy older individuals. Future research

may consider stability of anthocyanins while focusing on supplementation with other available fruits with different anthocyanin profiles in longer-term (3-6 months) crossover studies in order to confirm the absence of a chronic effect from fruit anthocyanins at this level of concentration.

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Figure 1

Figure 2

Table 1: Anthocyanin and macronutrient composition of the two study beverages

	QGPJ (per 100mL) [b]	Raspberry cordial (per 100mL) [d]
Anthocyanins (mg C3G[a] eq.)	3.7-5.3[c]	0.4[b]
Energy (kJ)	213	115
Protein (g)	1.1	0
Total fat (g)	0.1	0
Total carbohydrate (g)	8.7	6.5
Sugars (g)	8.7	6.5
Dietary fibre (g)	<0.1	<0.1[b]
Vitamin C (µg)	<0.05	<0.05[b]

[a] Cyanidin-3-glucoside equivalents; [b] Data from Santhakumar et. al. (2015)[85]; [c] Analyzed by University of Wollongong and Department of Agriculture and Fisheries; [d] Nutrient content from label.

Table 2: Baseline characteristics of the study population

Baseline characteristics	Full cohort (n=31)	IC[a] sequence (n=16)	CI[b] sequence (n=15)	p-value
	n (%)	n (%)	n (%)	
Gender				
Male	14 (45)	6 (37)	8 (53)	ns
Female	17 (55)	10 (63)	7 (47)	
Age, years (means ± SD)	70 ± 10	69 ± 10	70 ± 10	ns
BMI, kg/m^2 (means ± SD)	26 ± 4	25 ± 4	27 ± 4	ns
Waist-Hip ratio (mean±SD)	0.88 ± 0.09	0.86 ± 0.09	0.89 ± 0.10	ns
Level of education				ns
Secondary	4 (13)	1 (6)	3 (20)	
TAFE/Diploma	9 (29)	4 (25)	5 (33)	
University degree	4 (13)	4 (25)	0 (0)	
Post-grad	14 (45)	7 (44)	7 (47)	
Marital status				
Married	22 (71)	14 (88)	8 (53)	
Divorced	5 (16)	2 (12)	3 (20)	
Widowed	4 (13)	0 (0)	4 (27)	
Physical activity				
High	14 (45)	7 (44)	7 (47)	
Medium	12 (39)	4 (25)	8 (53)	
Low	5 (16)	5 (31)	0 (0)	
BP medication				
Yes	7 (23)	1 (6)	6 (40)	
No	24 (77)	15 (94)	9 (60)	
24 ABPM (mean ± SD)				ns
SBP	129 ± 12	126 ± 11	132 ±12	
DBP	80 ± 8	79 ± 7	81 ± 9	
MAP	102 ± 9	100 ± 8	104 ± 9	
HR	72 ± 9	72 ± 10	72 ± 8	
PP	49 ± 9	47 ± 10	51 ± 8	
Spot Blood Pressure				ns
SBP	131 ± 19	127 ±20	136 ± 18	
DBP	75 ± 9	74 ± 9	77 ± 9	
PR	74 ± 14	79 ±16	69 ± 9	
Hand grip strength (mean ± SD)				ns
Right	30.1 ± 10.3	29.3 ± 9.6	30.9 ± 11.2	
Left	26.8 ± 10.6	25.1 ± 9.8	28.6 ± 11.5	

Baseline characteristics	Full cohort (n=31) n (%)	IC[a] sequence (n=16) n (%)	CI[b] sequence (n=15) n (%)	p-value
Sit-to-stand (mean ± SD)	19 ± 7	19 ± 8	18 ± 5	ns
Cognitive assessment (mean ± SD)				ns
RAVLT total (I-V)	43 ± 12	44 ± 11	41 ± 14	
RAVLT delayed recall	8 ± 4	8 ± 3	8 ± 4	
RAVLT 20m delayed recall	8 ± 4	9 ± 4	8 ± 5	
Digit Span (mean ± SD)	7 ± 3	7 ± 3	7 ± 3	ns
Letter fluency (mean±SD)	14 ± 6	14 ± 6	14 ± 7	ns
Category fluency (mean±SD)	17 ± 6	18 ± 6	15 ± 6	ns
Counting span (mean±SD)	9 ± 4	8 ± 4	10 ± 3	ns
Stroop (secs) (mean±SD)	12 ± 6	14 ± 7	10 ± 4	ns
Inflammatory biomarkers/ nerve growth factor				ns
hsCRP (mg/L)	1.56 ± 1.67	1.44 ± 1.22	1.70 ± 2.11	
BDNF (ng/mL)	37.7 ± 10.4	35.4 ± 12.5	40.2 ± 6.99	

Data are means ± SD or n (%) (n=31); P values were obtained from $\chi 2$ test for categorical variables; Ns- Not significant
[a]IC- intervention- then control order (n=16); [b]CI- control then intervention order (n=15); RAVLT-Rey auditory verbal learning test; 24-ABPM – 24-h Ambulatory Blood Pressure Monitor; BMI – Body Mass Index; SBP-systolic blood pressure; DBP-diastolic blood pressure; MAP-mean arterial pressure; HR-heart rate; PP-pulse pressure; PR-pulse rate; hsCRP- high sensitivity c- reactive protein; BDNF- Brain-derived neurotrophic factor

Table 3: Crossover analysis of cognitive and physical function variables

	Means ± SD			p-values ANOVA analyses		
	Baseline	4-weeks	8-weeks	Treatment effect	Time effect	Treatment * time effect
RAVLT total (I-V)				0.69	0.74	0.44
	43 ± 12					
Plum nectar		48 ± 11	49 ± 11			
Cordial		48 ± 13	49 ± 12			
RAVLT delayed recall				0.49	1.0	0.43
	8 ± 4					
Plum nectar		9 ± 4	9 ± 4			
Cordial		9 ± 4	9 ± 4			
RAVLT 20m delayed recall				0.41	0.77	0.72
	8 ± 4					
Plum nectar		9 ± 3	9 ± 4			
Cordial		9 ± 5	9 ± 4			
Digit Span				0.23	0.14	0.49
	7 ± 3					
Plum nectar		8 ± 2	8 ± 2			
Cordial		8 ± 3	8 ± 2			
Letter fluency				0.73	0.63	0.94
	14 ± 6					
Plum nectar		15 ± 6	16 ± 6			
Cordial		16 ± 7	15 ± 6			
Category fluency				0.58	0.47	0.20
	17 ± 6					
Plum nectar		15 ± 5	16 ± 6			
Cordial		19 ± 7	18 ± 7			
Counting span				0.35	0.90	0.96
	9 ± 4					
Plum nectar		9 ± 3	10 ± 3			
Cordial		10 ± 3	10 ± 3			
Stroop (secs)				0.08	0.72	0.08
	12 ± 6					
Plum nectar		11 ± 5	10 ± 5			
Cordial		10 ± 6	9 ± 5			
Hand grip strength Right				0.90	0.59	0.88
	30.1 ± 10.3					
Plum nectar		32.4 ± 11.0	31.9 ± 11.1			
Cordial		31.3 ± 10.6	31.7 ± 11.0			
Left				0.53	0.81	0.30
	26.8 ± 10.6					
Plum nectar		29.3 ± 10.7	29.2 ± 11.3			
Cordial		28.6 ± 11.3	27.6 ± 10.1			

	Means ± SD			p-values ANOVA analyses		
	Baseline	4-weeks	8-weeks	Treatment effect	Time effect	Treatment * time effect
Sit-to-stand				0.34	0.12	0.12
	19 ± 7					
Plum nectar		21 ± 8	21 ± 7			
Cordial		21 ± 9	22 ± 9			

Data are means ± SD (full cohort n=31); P values were obtained from repeated measures ANOVA test for categorical.

Table 4: Crossover analysis of 24-h Ambulatory blood pressure data

	Means ± SD			p-values ANOVA analyses		
	Baseline	4-weeks	8-weeks	Treatment effect	Time effect	Treatment * time effect
SBP				0.73	0.97	0.44
	129 ± 12					
Plum nectar		126 ± 10	127 ± 10			
Cordial		124 ± 11	123 ± 8			
DBP				0.41	0.92	0.86
	80 ± 8					
Plum nectar		79 ± 8	78 ± 8			
Cordial		76 ± 7	76 ± 7			
MAP				0.75	0.93	0.71
	102 ± 9					
Plum nectar		100 ± 8	101 ± 8			
Cordial		98 ± 8	97 ± 6			
Heart rate				0.37	0.06	0.62
	72 ± 9					
Plum nectar		71 ± 10	71 ± 8			
Cordial		69 ± 9	71 ± 9			
Pulse pressure				0.77	0.97	0.39
	49 ± 9					
Plum nectar		48 ± 8	50 ± 10			
Cordial		48 ± 9	46± 8			

Data are means ± SD (full cohort n=31); P values were obtained from repeated measures ANOVA test for categorical.

Table 5: Crossover analysis of nerve growth factor / inflammatory biomarkers (BDNF and CRP)

	Measurements		p-values ANOVA analyses	
	Baseline	8-weeks	Treatment effect	Treatment * baseline
BDNF (ng/mL)			0.70	0.67
	37.7 ± 10.4			
Plum nectar		33.6 ± 12.1		
Cordial		33.7 ± 11.5		
CRP (mg/L)			0.17	0.16
	1.56 ± 1.7			
Plum nectar		2.66 ± 4.0		
Cordial		2.19 ± 2.2		

Data are means ± SD (full cohort n=31); P values were obtained from repeated measures ANOVA test for categorical.

Table 6: UHPLC-PDA-ESI-MS/MS characterization of QGP anthocyanins and metabolites detected in human urine after the consumption of QGP nectar.

Compounds	**Precursor ions (m/z)**	**Empirical molecular**	Fragments
Proposed identity	[M+H]+	formula	
Cyanidin-3-glucoside	449.15	$C_{21}H_{21}O_{11}^{+}$	287.05
Cyanidin monoglucuronide	463.10	$C_{21}H_{19}O_{12}^{+}$	287.05
Cyanidin-3-rutinoside	595.15	$C_{27}H_{31}O_{15}^{+}$	449.10, 287.05
Peonidin monoglucuronide	477.10	$C_{22}H_{21}O_{12}^{+}$	301.10
Peonidin-3-rutinoside	609.15	$C_{28}H_{33}O_{15}^{+}$	463.15, 301.10

Table 7: Dietary data analysis of participants

Nutrient/day	Full group (n=31)	[a]IC sequence (n=16)	[b]CI sequence (n=15)	p value[+]
Anthocyanin (mg)	142.69 ± 217.58 (34.15(280.33))	152.42 ± 264.77 (35.05(149.75))	132.97 ± 167.40 (27.35(287.83))	ns[++]
Energy (kJ)	9302.9 ± 3035.89 (9065 (3245))	9716.74 ± 3536.54 (9209 (4310.5))	8889.06 ± 2503.24 (9040.95 (3347.4)	ns
Carbohydrate (g)	209.66 ± 62.71 (193.6 (81.93)	201.37 ± 55.45 (185.05 (62.65)	217.96 ± 70.31 (213.65 (86.93))	ns
Protein (g)	94.59 ± 24.87 (87.4 (50.5))	93.49 ± 33.69 (80.50 (59.45))	95.69 ± 24.35 (92.40 (49.5))	ns
Total Fat (g)	94.92 ± 42.92 (86.9 (59.72))	107.77 ± 51.44 (94.45 (59.8))	82.06 ± 28.70 (78.5 (30.93))	ns
Saturated Fat (g)	30.76 ± 10.55 (29.6 (12.85))	30.96 ± 11.38 (31.4 (17.45))	30.56 ± 10.09 (28.05 (8.95))	ns
Dietary fibre (g)	32.23 ± 12.73 (30.25 (20.55))	34.39 ± 15.16 (33.5 (22.2))	30.08 ±9.86 (28.5 (18.1))	ns
Sodium (mg)	2304.36 ± 815.33 (2118.39 (972.22))	2266.09 ± 752.97 (2095.15 (1013.92))	2342.63 ± 900.30 (2150.07 (968.08))	ns
Potassium (mg)	3746.13 ± 1192.90 (3642.36 (1146.08))	3744.75 ± 1372.60 (3478.31 (1171.54))	3747.51 ± 1035.12 (3807.56 (1396.32))	ns
Magnesium (mg)	404.21 ± 189.00 (379.04 (231.79))	418.47 ± 243.15 (354.89 (344.22))	389.94 ± 120.96 (427.91 (237.05))	ns
Calcium (mg)	1056.97 ± 431.95 (912.71 (471.96))	978.89 ± 432.43 (858.26 (395.33))	1135.05 ± 432.89 (977.80 (456.62))	ns
Iron (mg)	13.83 ± 7.00 (11.82 (7.29))	14.14 ± 6.57 (11.82 (10.00))	13.53 ± 7.64 (11.55 (6.72))	ns
Omega 3 (g)	2.45 ± 1.82 (1.83 (1.75))	3.21 ± 2.25 (2.68 (2.72))	1.69 ± 0.77 (1.41 (1.06))	0.024

Values are means ± SD and (medians (IQR)); IC- intervention- then control order, n=16; CI- control then intervention order, n = 15; ns- not significant; [+]t-test; [++]Wilcoxon signed-rank test

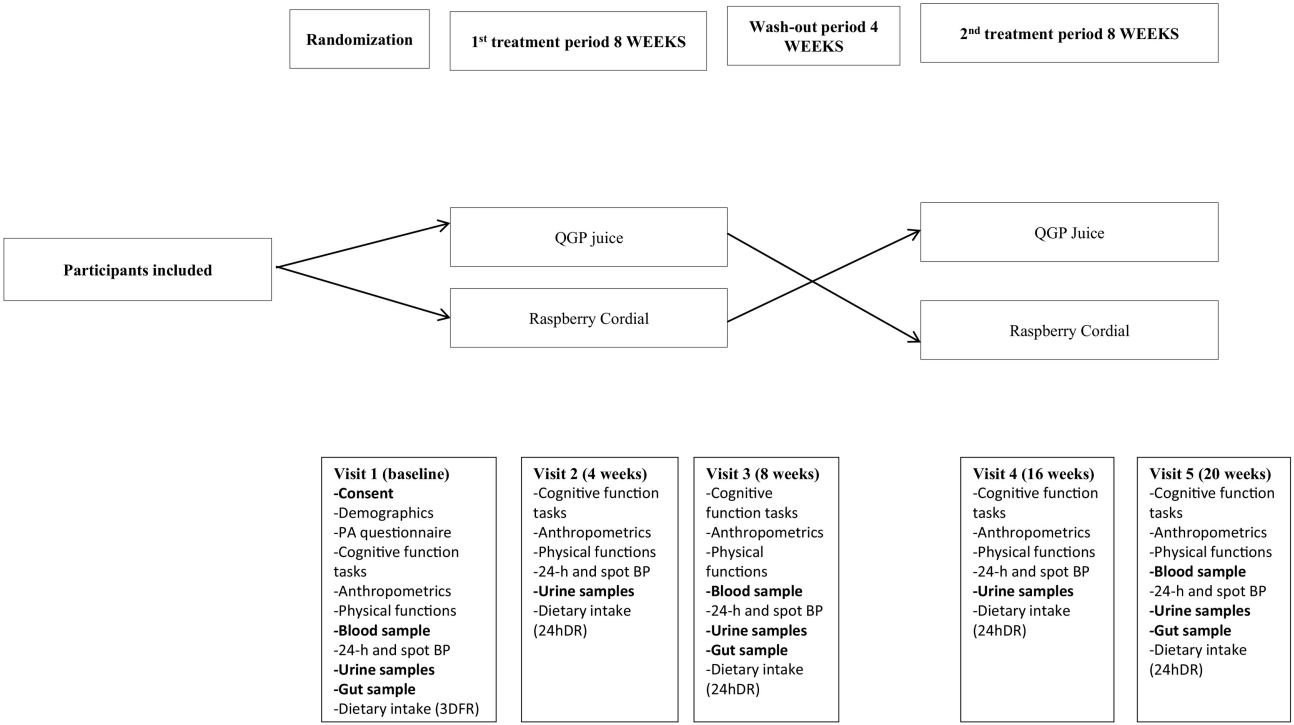
Randomization
1st treatment period 8 WEEKS
Wash-out period 4 WEEKS
2nd treatment period 8 WEEKS
Participants included
QGP juice
Raspberry Cordial
QGP Juice
Raspberry Cordial
Visit 1 (baseline)
-Consent
-Demographics
-PA questionnaire
-Cognitive function tasks
-Anthropometrics
-Physical functions
-Blood sample
-24-h and spot BP
-Urine samples
-Gut sample
-Dietary intake (3DFR)
Visit 2 (4 weeks)
-Cognitive function tasks
-Anthropometrics
-Physical functions
-24-h and spot BP
-Urine samples
-Dietary intake (24hDR)
Visit 3 (8 weeks)
-Cognitive function tasks
-Anthropometrics
-Physical functions
-Blood sample
-24-h and spot BP
-Urine samples
-Gut sample
-Dietary intake (24hDR)
Visit 4 (16 weeks)
-Cognitive function tasks
-Anthropometrics
-Physical functions
-24-h and spot BP
-Urine samples
-Dietary intake (24hDR)
Visit 5 (20 weeks)
-Cognitive function tasks
-Anthropometrics
-Physical functions
-Blood sample
-24-h and spot BP
-Urine samples
-Gut sample
-Dietary intake (24hDR)

Figure 1

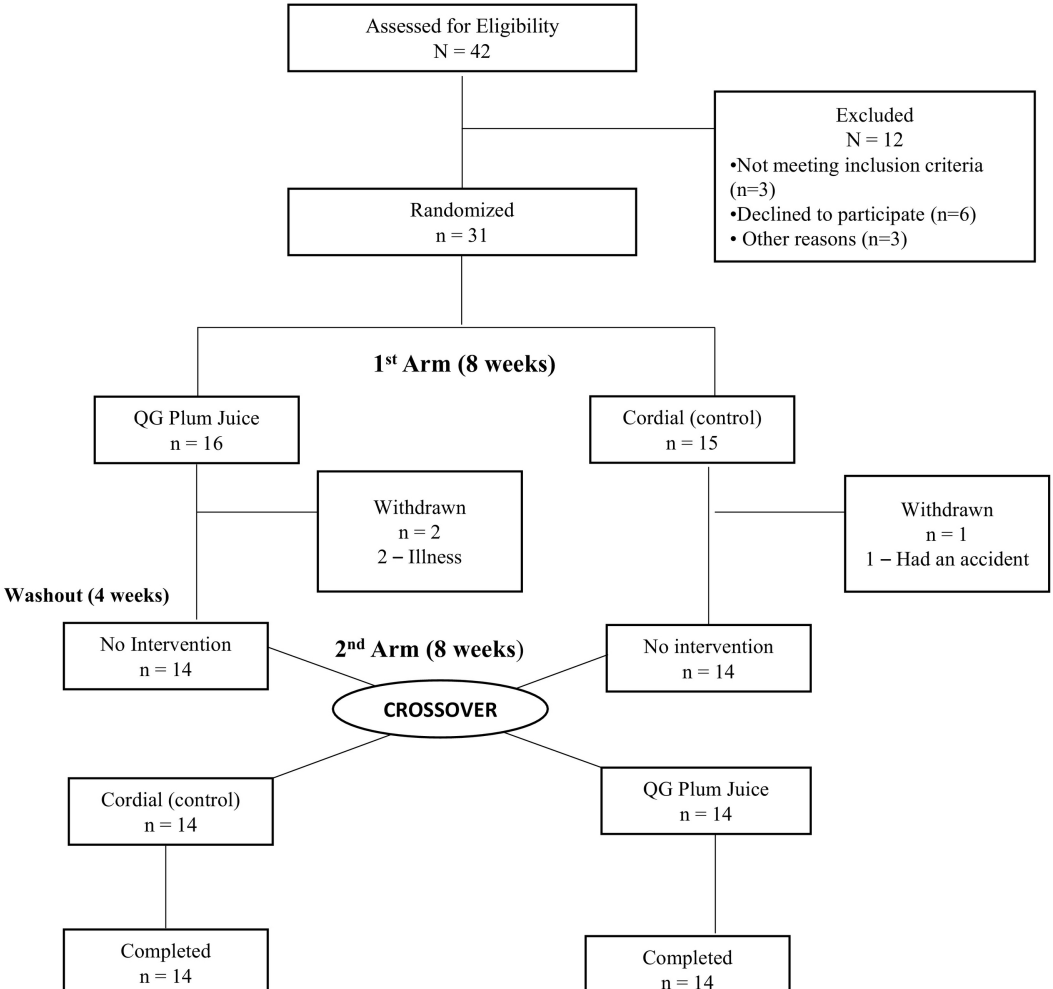
Assessed for Eligibility
N = 42
Excluded
N = 12
•Not meeting inclusion criteria (n=3)
•Declined to participate (n=6)
• Other reasons (n=3)
Randomized
n = 31
1st Arm (8 weeks)
QG Plum Juice
n = 16
Cordial (control)
n = 15
Withdrawn
n = 2
2 – Illness
Withdrawn
n = 1
1 – Had an accident
Washout (4 weeks)
No Intervention
n = 14
2nd Arm (8 weeks)
No intervention
n = 14
CROSSOVER
Cordial (control)
n = 14
QG Plum Juice
n = 14
Completed
n = 14
Completed
n = 14

Figure 2