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Anthocyanin-rich plum juice reduces ambulatory blood pressure but not acute cognitive function in younger and older adults: a pilot crossover dose-timing study

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Abstract

Consumption of anthocyanins from fruit sources may exert protection against hypertension and improve cognition. However, the effect of dose timing in studies is rarely considered. We hypothesized that timed-dose consumption of juice from an anthocyanin-rich Japanese plum variety (Queen Garnet plum, QGP) will have acute and dose-timing effects on cardiovascular responses, cognition, and urinary anthocyanin excretion profiles. Our study objective was to investigate the impact of plum juice on these health parameters. Twelve older (65+ years) and 12 younger (18-45 years) adults participated in an acute crossover study. Participants received, randomly, either 1 x 300 mL or 3 x 100 mL plum juice over 3 hours on 2 different occasions with a 2-week washout period. A battery of cognitive tasks was administered at 0 and 6 hours on each study day. Blood pressure (BP) and urinary anthocyanin/metabolite excretion profiles were measured over 24 hours. Area under the curve for BP was calculated (0-6 hours). A significant reduction in BP and cardiovascular responses was observed in both age groups which was more obvious in the older age group on the single dose for systolic BP, diastolic BP, mean arterial pressure, and heart rate (P values =.035,.028,.017, and.006, respectively). No significant difference was observed between dose-timing regimens for either age group. There was no observed effect on cognition. Native QGP anthocyanins, as well as methylated/glucuronidated metabolites, were detected in urine with no significant differences between age groups or dose timing. High-anthocyanin plum juice significantly reduced BP, but dose timing did not appear to be a significant factor in the potential acute BP-lowering effect of QGP juice.

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Anthocyanin-rich plum juice reduces ambulatory blood pressure but not acute cognitive function in younger and older adults: A pilot cross-over dose-timing study

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List of Abbreviations

ABPM - Ambulatory blood pressure monitor

ANCOVA – Analysis of Covariance

ANOVA – Analysis of Variance

AUC – Area under curve

BMI – Body mass index

BOCF – Baseline observation carried forward

BP – Blood pressure

COMT - Catechol-O-methyltransferase

CVD – Cardiovascular diseases

DAF - Department of Agriculture and Fisheries

DBP – Diastolic blood pressure

FMD - Flow-mediated dilation

HPLC-PDA-MS - High-performance liquid chromatography photodiode array detection mass spectrometry method

HR – Heart rate

IHMRI – Illawarra Health and Medical Research Institute

IPAQ - International Physical Activity Questionnaire

MAP – Mean arterial pressure

MAO - Monoamine oxidase

NADPH – Nicotinamide adenine dinucleotide phosphate

OxLDL – Oxidized low density lipoprotein

PacDASH - Pacific Kids Dietary approach to stop hypertension

QGP- Queen Garnet Plum

QGPJ – Queen Garnet plum juice

RAVLT - Rey Auditory Verbal Learning test

SBP – Systolic blood pressure

UDP – Uridine diphosphate

URC - University Research Committee

UOW – University of Wollongong

WHO – World Health Organization

Abstract

Consumption of anthocyanins from fruit sources may exert protection against hypertension and improve cognition. However, the effect of dose-timing in studies is rarely considered. We hypothesized that timed-dose consumption of juice from an anthocyanin-rich Japanese plum variety (Queen Garnet plum, QGP) will have acute and dose-timing effects on cardiovascular responses, cognition, and urinary anthocyanin excretion profiles. Our study objective was to investigate the impact of plum juice on these health parameters. Twelve (12) older (65y+) and 12 younger (18-45y) adults participated in an acute crossover study. Participants received, randomly, either a 1x300ml or 3x100ml plum juice over 3h on two different occasions with a 2-week washout period. A battery of cognitive tasks was administered at 0h and 6h on each study day. BP and urinary anthocyanin/metabolite excretion profiles were measured over 24h. Area under the Curve for BP was calculated (0-6h). A significant reduction in BP and cardiovascular responses was observed in both age groups which was more obvious in the older age group on the single dose for SBP, DBP, Mean Arterial Pressure and Heart Rate (p-values=0.035, 0.028, 0.017 and 0.006 respectively). No significant difference was observed between dose-timing regimens for either age group. There was no observed effect on cognition. Native QGP anthocyanins, as well as methylated/glucuronidated metabolites were detected in urine with no significant differences between age groups or dose-timing. High anthocyanin plum juice significantly reduced blood pressure but dose-timing did not appear to be a significant factor in the potential acute BP-lowering effect of QGP juice.

Keywords: Anthocyanins, Blood Pressure, Queen Garnet plum juice, Cognition, Acute, Cross-over, human

1. Introduction

Elevated and high blood pressure is a major public health concern and a significant risk factor for cardiovascular diseases. According to the World Health Organization (WHO), the prevalence of high blood pressure in adults aged 18 and over was around 22% in 2014. This accounted for about 9.4 million deaths or 7% of all deaths [1]. In Australia, between 2011 and 2012, almost one-third (31.6%) of all adults were diagnosed with hypertension, which was more prevalent at older ages, with almost 9 in 10 (87.7%) people aged 85 years and older being hypertensive [2]. In global strategies to address non-communicable diseases including hypertension, the significant role of modifiable dietary risk factors, including an increased intake of fruits and vegetables is acknowledged [3]. Small (2-5mmHg) but steady decreases in mean blood pressure have been shown to significantly decrease the incidence of cardiovascular events [4]. Given the magnitude of hypertension, and its contribution towards the burden of cardiovascular disease, cost-effective strategies including dietary intervention are needed for its prevention and management. Plant-based foods are integral to a healthy human diet and a plant-rich diet is associated with the prevention of a vast array of diseases [5]. Bioactive compounds of interest include polyphenols, which are found mainly in plant based foods and have antioxidant properties. More than 8000 different polyphenols have been identified in nature within four different categories (flavonoids, phenolic acids, lignans and stilbenes). Over the past decade, there has been increased research into flavonoids, notably, anthocyanins for their beneficial health effects [6].

Anthocyanins, the largest subclass of flavonoids, comprise a group of water-soluble phytochemicals known to be responsible for the deep rich red to blue-purple colors in fruits

and vegetables [7]. There is some evidence from epidemiological studies that suggests a higher consumption of anthocyanin-rich foods is associated with a reduced risk for cardiovascular disease [8, 9]. However, intervention studies do not always support these findings [10]. In the case of blood pressure, plausible mechanisms from experimental studies include their effects on vascular blood flow and flow-mediated dilation (FMD) [11, 12].

It has been hypothesized that anthocyanins may exert protective effects on cognition, including memory and executive processing, either through a direct effect on brain function or indirectly by reducing blood pressure [13-15]. One of the main pathways linking blood pressure to cognitive degeneration is the decline in vascular reserve capacity which is associated with impaired neurovascular coupling [16]. Despite evidence from epidemiological and intervention studies indicating that anthocyanin intake is linked with improved cognition [15, 17] and a slower cognitive decline [18], the mechanisms by which anthocyanins may exert acute effects on brain function remains unclear and evidence is inconsistent. A crossover study by Caldwell et. al. (2016) [19] found that high anthocyanin cherry juice consumption did not result in any significant acute effects on a battery of cognitive tests in either younger or older adults. Contrary to this, Watson et.al, (2015) [20] observed a cognitive benefit of acute blackcurrant supplementation in healthy younger adults possibly explained by an association between monoamine oxidase (MAO) inhibition and improved attention. There is a possibility that the inhibition of MAO have positive effects on monoaminergic neurotransmission during cognitive performance [21]. This is as a result of monoamine levels, particularly for dopamine, being shown to increase during cognitive tasks (which assess working memory and attention) with a positive correlation with task performance [21]. An acute effect on cognition by fruit anthocyanin supplementation has also been observed in children [17, 22].

Inadequate understanding of the uptake, metabolism distribution and excretion of anthocyanins has limited the design of clinical trials that investigate their effect on health outcomes. The body of evidence on the protective effects of flavonoid-rich foods against CVD is based mainly on epidemiological studies, thus evidence remains inconclusive and acute effects have not been well defined. Systematic reviews of available experimental studies [23, 24] have highlighted an absence of knowledge regarding a 'threshold dose' or appropriate 'dose-timing' required to induce physiological protective effects. This is because the impact of anthocyanin dose has not been studied extensively in humans and different experiments have used varied preparations e.g. juice, puree and whole fruit. Consequently, studies administer unfeasibly large doses of anthocyanin-rich foods in order to elucidate a physiological response, and the selection of dose-timings is often unsubstantiated [25-27]. While splitting a large daily dose of anthocyanin-rich food into three or more servings per day may reflect a more feasibly tolerated serve, there is often no justification as to the reason each dose was selected, and no consideration given to the physiological effects. Even though results have mostly been in agreement, evidence shows that beyond a point, the bioavailability of anthocyanins decreases with increasing dose [28]. For cyanidin-based anthocyanins the maximum absorption has been reported to be about 350µmol or less. This is also believed to differ according to the structure of different anthocyanins, the attached sugar moiety and because of wide inter-individual variation in metabolism which limits translation of research findings into dietary messages [29]. Taking these factors into consideration, there is a need to better understand the acute effects of anthocyanins provided from different foods and beverages, in order to identify any consistent potential health benefits.

141 The increased interest in anthocyanin-based research has translated into agricultural
142 responses, as the demand for fruits with superior health benefit grows. An example is the
143 Queen Garnet plum (QGP), that is a variety of the popular Japanese plum *Prunus salicina*
144 *Lindl* that was bred by the Queensland Government to be very high in anthocyanins,
145 providing up to 277 mg/100 g fruit [30] under "optimal" environmental and harvest
146 conditions. This is more than twice the anthocyanin content of regular plums that ranges
147 from 5 to 173mg/100g across harvest years [30]. Previous work from our group has
148 determined the acute effect of anthocyanins provided from a different fruit source (cherries)
149 [15, 31]. Learnings from those studies underpins the improved methodologies employed in
150 the current study, particularly with regard to more robust assessment of 24 hour BP and
151 measurement of urinary anthocyanin metabolites. Furthermore, the QGP juice has a
152 completely different anthocyanin and non-anthocyanin polyphenol profile compared to the
153 Australian cherry juice vehicle used previously [15, 31] which may influence its
154 synergistic/antagonist effects on biological activities. Our previous acute trial found that
155 plasma levels of anthocyanin-related metabolites were significantly lower for older adults,
156 but not for younger adults, who consumed cherry juice over 3 smaller servings (3 x 100ml),
157 compared to consumption of 300ml at a single time point. This finding warrants further
158 research consideration.

159

160 As a follow-up, this study hypothesized that the consumption of high anthocyanin QGPJ will;

161 1. have an acute beneficial effect on various domains of cognitive functioning and blood
162 pressure,
163 2. be found to be bioavailable through the presence of anthocyanin metabolites excreted
164 in the urine over a 24 h period and,

3. Show differences in the absorption rate and metabolism of anthocyanins between young and older adults.

From the above hypotheses, the primary aim of this study was to determine the dose-timing response on acute ambulatory blood pressure and cognitive function following consumption of 300ml QGP juice, provided as either a single dose or three 100ml quantities over 3h, in young and older adults. The secondary outcome was to determine the bioavailability of QGP anthocyanins, as assessed by urinary excretion over a 24h period, and to assess any significant differences in the anthocyanin/metabolite profiles between young and older adults.

2. Methods and Materials

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Wollongong Human Research Ethics Committee NSW, Australia (HE16/278). Written informed consent was obtained from all participants.

2.1. Study design

The study was a pilot cross-over bioequivalence/non-inferiority study to assess the acute impact differing dose-timings of high-anthocyanin plum juice consumption on acute cognition and blood pressure over 24h. This study was considered a bioequivalence/non-inferiority design due to the dosage for the two crossover arms being the same (a single 300ml and 3x100ml over 3 h) with the aim to determine whether three 100ml of plum juice

188 taken over three hours would have a different pattern of effect on blood pressure in
189 comparison to a single 300ml dose. Results from this pilot study will inform the methodology
190 as well as sample size calculation for a future crossover randomized clinical trial. This is in
191 line with the assumptions of bioequivalence studies whereby the future trial will be designed
192 as a crossover study [32-34].

193

194 *2.2. Participants*

195

196 All participants were recruited from the University of Wollongong (UOW) and the
197 surrounding Wollongong areas through poster advertising. Potential participants had the
198 opportunity to discuss the study over the phone prior to clinic visits and were screened to
199 determine eligibility.

200

201 Twenty-four participants were recruited, including 12 younger (18-45y) and 12 older (65+y)
202 adults. The sample size was determined according to recommendations for planning a pilot
203 study that investigates bioavailability and bioequivalence of components within food [32-34].

204

205 Recruited eligible participants were randomized to a dose-timing allocation and cognitive
206 assessment order by a computer-generated block randomization by an independent
207 statistician. Participants attended two 6-h clinic visits at the Illawarra Health and Medical
208 Research Institute (IHMRI) at the University of Wollongong, NSW, Australia between June
209 and September 2015 with at least a two-week washout period between clinic visits.

210

2.3. Exclusion criteria

Exclusion criteria included self-reported uncontrolled hypertension, any unstable physical or mental health condition, inability to provide informed consent, consumption of specific daily health supplements, and inability to communicate in the English language.

2.4. Data collection

On the first study day, a questionnaire was administered to determine participants' socio-demographic characteristics. The International Physical Activity Questionnaire (IPAQ) validated by Hagströmer, Oja [35] was used to determine habitual level of physical activity and blood pressure measurements were taken using an ambulatory blood pressure monitor (ABPM) (SpaceLabs Inc., Issaquah, WA, Australia; Model 90207).

2.4.1. Dietary instruction and intervention meals

The QGP juice was used as the vehicle to provide a specific and consistent anthocyanin dose to study participants. The plum juice was produced from a single seasonal batch and processed to juice by research partners at the Department of Agriculture and Fisheries (DAF), Queensland Government, and was batch frozen at -20^{o}C until usage [36].

Prior to each study day, participants were advised to avoid consumption of purple/red fruits and vegetables including wine, juices, jams and smoothies in the 24h periods immediately before and after interview day. Verbal compliance to this was received prior to the study. On each study day participants arrived between 08:00 and 09:30 hours at the clinic facility following a 12h fast. A spot urine sample was collected and a battery of cognitive tests administered by two interviewers who had been trained by a senior psychologist (SR). Thereafter, a standardized breakfast (Weet-Bix, milk and sugar) that was low in flavonoids was provided. QGP juice was provided with breakfast in random order, as either (i) a single dose of 300ml (369 mg total anthocyanins) or (ii) 3 x 100ml servings (123mg total anthocyanins/serving) of the same plum juice at 0, 1 and 3h. A standardized snack (ham and cheese sandwich) was provided at 4h and two (250ml) bottles of water provided for the 6h duration spent in the study facility to be consumed ad libitum.

2.4.2. Ambulatory 24h blood pressure and anthropometric measurements

Blood Pressure was measured using Ambulatory Blood Pressure Monitors (ABPM) for improved monitoring over 24 h in comparison to standard digital blood pressure monitors used in similar studies over a 6-hour period [15, 31].

Upon arrival at the testing facility participants were fitted with an ABPM (SpaceLabs Inc., Issaquah, WA, Australia; Model 90207). The ABPM took blood pressure measurements over the next 24 h; every 15 minutes while at the testing facility (first 6 h) and thereafter once per hour whilst at home. The ABPM uses an oscillometric method for the detection of systolic

and diastolic blood pressure and has been shown to be more accurate than casual or in-office BP measurements [37]. Participants were encouraged to go about their usual daily activities, but were advised to stand still and relax their arm whenever the monitor recorded measurements i.e. cuff inflation and deflation. After 24 hours, the monitor was removed and collected from participants' homes and data downloaded from the monitor for analysis.

Height (m) and weight (kg) were measured using a stadiometer (Seca, Hamburg, Germany) and an electronic scale (Omron HN286 Digital Personal Body Weight Scale, (Omron, Australia)) respectively, to two decimal places and BMI (weight/ (height2)) was calculated.

2.4.3. Cognitive tasks

Five short cognitive interviewer-administered tests [38, 39] were administered by trained investigators at baseline and 6h on both testing occasions to determine any acute changes in cognition.

The total duration of the battery of tasks was approximately 30min. To control for cross-over effects, there were 4 versions of the cognitive battery so that each participant had a different version at baseline and 6h and also after cross-over period.

The Trail Making Test [40] required participants to alternate selective responses between two types of stimuli in the one task. The difference in the number of seconds required to complete

277 the task was compared to a non-switching version. This task assesses higher executive
278 function.

279

280 In the Rey Auditory Verbal Learning test (RAVLT) [41], participants learn and recall a list of
281 words over 5 trials and each correct word that is identified is associated with a score. This
282 task assesses verbal learning and memory.

283

284 The Pattern and Letter Comparison task [42] requires participants to compare strings of
285 patterns or letters to determine if it is the same or different. They are required to complete as
286 many examples as possible in 30sec and scores are tallied. This task assesses speed of
287 processing.

288

289 The Reaction Time task [43] involves display of a left or right arrow-shaped stimulus on the
290 computer screen and participants are required to press the corresponding mouse button (left
291 or right). Outcome variables are proportion of correct responses and latency (response speed).
292 This task assesses general alertness and speed of processing.

293

294 The Stroop task [44] provides participants with a sheet on which the words “purple,” “green,”
295 “yellow,” “red,” and “blue” are printed (50 in total). Each word is shown in either congruent
296 or incongruent ink colors (e.g. the word “blue” printed in red). Participants are instructed to
297 read out the actual color and not the printed word as quickly as possible. The amount of time

taken (seconds) to complete each set of words was recorded. This task assesses executive function.

2.4.4. Urine sample collection and preparation

Urine samples were collected at baseline prior QGP juice consumption, and thereafter collected in sterilized urine containers over the following time periods: 0-2h; 2-6h; 6-12h and 12-24h after QGP juice consumption. The volume of collected urine samples were measured per container, recorded and an aliquot of 30ml of urine sample plus 9ml of formic acid (100%) stored in 50ml tubes, with additional 10ml urine for storage. The urine samples were stored at -80^{0}C for batch analysis. Intact (non-metabolized) QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) as well as their main/common conjugated and methylated metabolites such as peonidin-glycosides and –glucuronides were determined by HPLC-PDA-MS as described by Netzel et. al. (2012) [36].

2.5. Statistical Analyses

Data was analyzed using IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp., Armonk, NY, USA). Descriptive statistics of participant characteristics was performed across age groups. Normal distribution of the continuous variables was assessed using the Shapiro Wilk test, histogram, Q-Q plot and skewness and kurtosis.

Linear mixed modelling was used to estimate the effect of different timed doses of QGP juice on BP between the two age groups, while adjusting for correlation due to repeated observations on each participant over 24h. Age-group and dose (with interaction term) were entered in the model as fixed effects while controlling for age and gender. Maximum likelihood method of estimation was used with a diagonal covariance structure. As blood pressure measurements were collected over 24h, there were a few missing data (less than 1%) and as a result linear mixed modelling was chosen for analysis as it handles missing data better than the widely used ANCOVA.

Area Under the Curve (AUC) was calculated as a summation measure for the first 6h of BP measurements and the baseline observation carried forward (BOCF) approach was used for missing data (less than 1%). To determine if the plum juice had a significant effect on BP, a z-test was used to analyze the AUC for the BP. A series of *t*-tests was used to determine whether there was any significant difference between baseline BP and different time points up to 6h. The period between 0 and 6h represents the time that the participants spent at the study facility under standard resting conditions.

One-way analysis of variance (ANOVA) was used to determine differences in performance within each group at baseline and 6h post-intervention and two-way ANOVA was used to determine the difference in cognitive performance at baseline and 6h between the two age groups and dosing regimens as well as anthocyanin excretion between the two age groups.

3. Results

3.1. Subject characteristics

Twenty-four participants (12 young and 12 old adults) were recruited to participate in the study and sociodemographic characteristics are shown in Table 1. Participants were of Caucasian descent (n=21), African descent (n=2) and Asian (n=1). All participants attended both visits and there were no withdrawals, nor adverse events reported throughout the study protocol. The average washout period for participants was 20 days, a deviation from the original 2-week washout period due to schedule clashes and illness. The washout period chosen for our study was 2 weeks. This was informed by the FDA recommendation which states that for bioequivalence studies, "The washout time should be approximately 10x the plasma apparent terminal elimination half-life, to provide for 99.9% of the administered dose to be eliminated from the body." [45]. For the anthocyanin, cyanidin-3-glucoside, which is the main anthocyanin in QGP juice [36, 46], the half-life has been shown to range between 12 and 51h [47].

3.2. 24h ambulatory blood pressure

Hourly cardiovascular responses recorded during each of the 24h test periods are shown, according to plum juice delivery mode in Figures 1-4. Comparison is made between the dosing regimen (single and triple doses) and age groups with an interaction factor (dosing regimen x age-group). Figure 1a shows a more obvious drop in systolic blood pressure of the older adults with the single dose compared to the triple dose (Figure 3a). This observation

366 was not evident with the younger adults, as shown in Figures 1a and 3a. There was no
367 significant dose-timing effect observed for change in blood pressure following plum juice
368 consumption in the 24h period using the linear mixed model for longitudinal data (Table 2).

369

370 Area under the curve (AUC) was calculated for the cardiovascular parameters (systolic,
371 diastolic, MAP and HR) for the first 6h (Figures 5-6 and Table 3). For both age groups, using
372 a *t-test,* BP was significantly lower than baseline ($p < 0.05$) at different time points up to 6 h
373 following consumption of the plum juice. The greatest significant BP reduction was observed
374 at 2h for both age groups and was more obvious for systolic BP in the older group with a
375 mean difference of 12.83mm Hg (SD; 16.51, p=0.001) from baseline. For the single dose, z-
376 test analysis of the AUC calculations for the younger adult group showed a significant effect
377 of the juice on diastolic BP, MAP and HR (p-values = 0.008, 0.012 and 0.025 respectively).
378 Similarly, a significant effect was seen for the older group: systolic BP, diastolic BP, MAP
379 and HR (p-values = 0.035, 0.028, 0.017 and 0.006 respectively). For the younger age-group
380 on the triple dose, significant effects were observed for diastolic BP and MAP (p-values =
381 0.008 and 0.013 respectively) with a borderline significance on the HR (p-value = 0.06). In
382 the older group, significant effects of the triple dose were observed for diastolic BP (p-value
383 = 0.00007) and a borderline effect for systolic BP (p-value = 0.063). Plum juice consumption
384 had a significant effect on systolic BP, which was predicted by dose or age group but no
385 interaction term effect (dose x age group) and for MAP, predicted by only age-group. No
386 significant effect was observed on other cardiovascular parameters (table 2).

387

388 *3.3. Cognitive tasks*

389

Using two-way ANOVA, a significant difference was observed between the two age groups ($p<0.001$), both at baseline and 6h, for performance on cognitive tests. After consumption of the juice, there was no significant difference from baseline values within the groups or by dose-timing.

3.4. Urinary excretion of anthocyanins and anthocyanin metabolites

The anthocyanin content of the batch of QGP utilized for our study was 123mg/100g. The consumption of QGP juice as a single oral dose of 300ml or in threex100ml servings over 3h resulted in the appearance of both intact/non-metabolized QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) and at least five identified anthocyanin metabolites in the volunteers' urine samples (Table 4). The excretion rates and urinary anthocyanin/metabolite profiles were similar ($p>0.05$) between age groups and dosing regimen.

4. Discussion

Following consumption of a single dose of 300ml dose of plum juice, an acute reduction in SBP ($p=0.035$), DBP ($p=0.028$), MAP ($p=0.017$) and HR ($p=0.006$) was observed in the older age group. A similar trend was also observed for the triple dose with the absence of an effect on SBP. This acute effect was more pronounced in the older age group and at 2h with a mean difference of 12.83mm Hg from baseline. Significant effects on DBP ($p<0.001$) and MAP ($p=0.013$) were also observed in the younger age group on the single dose and DBP

413 ($p<0.001$) with a borderline effect on SBP ($p=0.06$) on the triple dose. The acute significant
414 reduction in blood pressure at 2h is associated with evidence on the absorption and
415 bioavailability of anthocyanins that occurs within 2h post consumption [48]. Anthocyanin
416 concentrations in the body have been observed to reach peak levels between 1-2h and begin
417 to clear from 6h falling back to baseline levels as they get excreted from the body up to 48h
418 [49]. The synergistic effect of other nutrients in the Queen Garnet plum cannot be
419 overlooked. There is a possibility that the observed blood pressure lowering effect may have
420 been as a result of this synergistic effect, as well as the presence of potassium in the Queen
421 Garnet plum fruit [30] which is an electrolyte know to lower blood pressure in humans [50].
422 Despite lack of a significant effect of dose by the different age groups on blood pressure, the
423 greater reduction in blood pressure in response to plum juice consumption in older adults may
424 be explained by their higher baseline blood pressure levels [4]. However, further adequately
425 powered studies are needed to confirm these findings. In the 6 h following the plum juice
426 consumption no significant effect was observed on the SBP of the younger age group. Similar
427 observation has also been made in previous studies. A study by Novotny, Nigg [51] observed
428 that the PacDASH (Pacific Kids Dietary approach to stop hypertension) trial did not affect
429 overall diet quality which was measured by SBP change among other parameters but had a
430 significant effect on DBP by the end of the intervention, by 12.2 mmHg. There is a
431 possibility that the absence of a significant effect on SBP could be associated with age
432 because there is a possibility that interventions may have a more significant effect among
433 older populations and/or those more prone to age-related vascular stiffening with an increased
434 risk of developing CVD.

435

436 There was an observed dose-timing and group effect on SBP but not on other blood pressure
437 parameters, however, this was no longer significant after inclusion of an interaction term

438 (age-group x dose-timing). Previously, a similar study observed an acute reduction in blood
439 pressure (SBP, DBP and HR) after consumption of anthocyanin-rich cherry juice, which was
440 found to be dose-timing dependent [31]. The difference between the two studies may be
441 explained by a much higher concentration of anthocyanins in the QGP juice (123mg/100ml
442 vs 69mg/100ml, respectively), resulting in a physiological threshold to be reached in each of
443 the three 100ml doses.

444

445 A possible explanation/mechanism of the observed blood pressure lowering effect of QGP
446 juice could lie in the molecular structure of its main *in vivo* anthocyanin metabolites. The
447 described methylation of cyanidin glycosides by catechol-O-methyltransferase (COMT) to
448 peonidin based metabolites as the main urinary anthocyanin metabolites after QGP juice
449 ingestion results in a structural modification of the B-ring in the flavonoid skeleton which is
450 structurally analogous to apocynin, an established vasoactive drug [8, 52, 53]. Mono-O-
451 methylated anthocyanins/flavonoids can act as inhibitors of NADPH oxidase and as a result
452 can improve vasodilatory processes [8]. Another possible explanation for the blood pressure
453 lowering effect of anthocyanins and/or other polyphenols present in the plum juice is their
454 potential to inhibit the oxidation of Low-Density Lipoproteins (LDLs), a major risk factor for
455 atherosclerosis, through free-radical scavenging and removal of metal ions from catalytic
456 sites via chelation [54]. The mechanism by which OxLDL promotes atherogenesis is believed
457 to be through cytotoxicity, inhibitory effects on macrophage motility, and uptake by the
458 macrophage scavenger receptor resulting in stimulation of cholesterol accumulation and
459 hence foam cell formation, which is critical in early atherosclerosis lesions. To test this
460 theory, Liu, Lee [55] in their study observed that when cells were incubated with OxLDL
461 (100 μg/ml) for 24 h, they was an increase in cell death while the additions of mulberry water
462 extracts and mulberry anthocyanin-rich extracts beyond the concentrations of 0.1 and 0.05

463 mg/ml, respectively, significantly increased the survival of these cell macrophages. They also
464 observed that 1 mg/ml of mulberry water extracts and 0.1 mg/ml of mulberry anthocyanin-
465 rich extract suppressed the lipid accumulation by approximately 55% and 58%, respectively.

466

467 Even though anthocyanins have been hypothesized to promote healthy brain functioning,
468 results from our pilot study show that a 300ml serving of plum juice, regardless of dose-
469 timing or age of participants, has no significant acute effect on various domains of cognitive
470 function. Although previous studies have found no significant acute effect of anthocyanins
471 from fruit source on cognitive processes, the QGP juice used in the present study had a
472 significant higher content of anthocyanins and therefore there was a possibility that it might
473 induce cognitive effects. In addition, two different cognitive tests that have been shown to be
474 sensitive and target different domains were utilized; Stroop and the Reaction time task [39,
475 56]. Extensive research has been carried out on the long term effect of flavonoid
476 supplementation on cognition [39] with less attention on their acute effects. Recently, there
477 has been an increase in the body of evidence on the acute effects of flavonoids on cognitive
478 processes such as attention, working memory and psychomotor speed in a general population
479 [57]. The precise mechanism by which anthocyanins affect cognition is still not clear but
480 seems to be dependent on the exposure period. Acute effects on cognition are believed to be
481 as a result of increased cerebrovascular blood flow and possibly monoamine oxidase (MAO)
482 inhibition which has been shown to improve cognitive performance [14, 20]. Following
483 consumption of high anthocyanin fruit/juice, evidence shows that peaks in cerebral blood
484 flow, vasodilation, and anthocyanin metabolite availability is detectable within 2h post
485 consumption [17]. Following blueberry supplementation plasma anthocyanins and their
486 metabolites were observed to reach peak levels at 1–2h and 6h [11]. An investigation on the
487 bioavailability of anthocyanins observed an association between colon microbiota

metabolism of anthocyanins and a significant increase in the content of generated polyphenols in the brain. There is a possibility that the peak levels observed at 6h is as a result of re-uptake of polyphenols in the colon [58, 59]. For this reason, repeat cognitive tasks were administered 6 h post consumption of the plum juice for our study. The absence of a significant effect could be attributed to the timing of cognitive task administration, missing the initial peak action time. Move cogntion para up here

The urinary recovery of intact anthocyanins and anthocyanin metabolites that had an intact flavonoid skeleton (glucuronides, sulfates and methylated forms) was between 693 and 871 ug/24 h, corresponding to 0.19 – 0.24% of the ingested anthocyanin dose. These ranges are consistent with those reported in human studies for urinary excretion rates of anthocyanins and conjugated/methylated metabolites after consumption of anthocyanin-rich food (0.01 – 5.10%) [7, 36, 60]. The bioavailability of anthocyanins has been reported to be low however a recent review indicates that it may be higher than previously reported [61]. Evidence from this review showed that the majority of ingested anthocyanins reach the large intestine. Here they are catabolized by the microbiota, producing an array of phenolic components that are absorbed, and some metabolized to phase II conjugates [61]. Furthermore, our finding that methylated and glucuronidated derivatives of cyaniding-based anthocyanins were the main urinary metabolites is also in agreement with others [62-64]. The *in vivo* glucuronidation, sulfatation and methylation of anthocyanins by UDP-glucuronosyltransferases, sulfotransferase and COMT in the intestinal epithelial cells, liver and kidney is a common metabolic pathway of dietary anthocyanins and other polyphenolic compounds [65].

511 The presence of pelargonidin monoglucuronide, when QGP juice does not contain any
512 (detectable) pelargonidin based anthocyanins, can be explained by the *in vivo* xenobiotic and
513 gut bacterial metabolism of anthocyanins/flavonoids. This includes addition and removal of
514 methyl and hydroxyl groups (pelargonidin is lacking of one hydroxyl group compared to
515 cyanidin) [66]. Furthermore, Kalt and colleagues reported similar results. After the ingestion
516 of blueberry juice, which did not contain any detectable pelargonidin glycosides, significant
517 amounts of pelargonidin based metabolites could be detected in the urine of 17 study subjects
518 [66]. The described interconversion of anthocyanins due to xenobiotic and bacterial
519 metabolism was suggested by these authors. In the current study there were no significant
520 differences in the urinary anthocyanin/metabolite excretion profiles either between the age
521 groups or according to the different dosing regimens (1x300 ml dose or 3x100 ml servings).

522

523 Previous research has reported conflicting results regarding the influence of flavonoids on
524 cognition in younger and older people. In one study, anthocyanin-rich blueberry
525 supplementation in younger and older adults resulted in improvements in different acute
526 cognitive domains, whereby a significant improvement in updating ability was reported for
527 younger adults and improvements in immediate word recognition in older adults were
528 identified [67]. In relation to cocoa flavonoids, consumption of dark chocolate for one week
529 significantly improved endothelial function and reduced BP in younger hypertensive patients,
530 but not in older populations [68]. Overall there is little information that compares responses
531 between younger and older adult populations thus more work comparing these groups is
532 required to elucidate any age-related differences in biological response.

533

534 The main objective with the dose-timing design was to estimate the response according to the
535 dose given, in order to analyze the effect and identify any adverse reactions. Throughout the
536 course of the study, the juice was well tolerated and there were no reports of any adverse
537 effects, however the tolerability to the study protocol was not objectively measured. As there
538 is large observed inter-individual variation in the absorption, metabolism and excretion of
539 polyphenols [69], the use of a cross-over study design is appropriate since participants act as
540 their own controls [70].

541

542 *4.1. Limitations of study*

543

544 A notable limitation of our study is the absence of a placebo arm. While a placebo arm is
545 essential in dietary intervention studies in order to identify the magnitude of effect related to
546 the dietary factor of interest. In the case of anthocyanins, Johnson et. al. (2015)[71] included
547 a placebo control group in their blueberry powder (469mg of anthocyanins/day) study and
548 identified a drop of 7mmHg and 5mmHg after 8 weeks of intervention in both SBP and DBP
549 ($P<0.05$ and $P<0.01$, respectively) but not in the control group. The main purpose of our
550 acute study in which each participant acted as their own control was to identify whether
551 different dosing regimens of a high anthocyanin fruit juice resulted in differences for ether
552 cognitive performance and/or BP. Information related to the dose-timing administration of an
553 intervention is an important consideration in clinical trial designs in free-living participants.
554 Furthermore, neither intact anthocyanins nor their common metabolites such as glucuronides,
555 sulfates or methylated forms are usually detectable in urine of placebo/control groups as it
556 was demonstrated in a pilot study with QGP juice and water as a control [36]. Food or

beverages used for placebo/control treatments are usually anthocyanin-free or contain only negligible amounts of these pigments.

Previous chronic flavonoid trials have instructed participants to consume an amount of food or beverage over the period of a day, but without specific guidelines on whether this needs to be consumed in totality at a single setting or whether smaller portions can be spread across the day. Nonspecific information on timing of the test food or beverage probably relates to a poor understanding of how dose-timing may affect biological responses.

Another notable limitation is the absence of a double-blinded strategy. This, in addition to cognitive testing time, could have resulted in the absence of a significant effect on cognitive performance after consumption of the plum juice. A consideration for future studies could be to test cognitive effects 2 h and 4-6 h post consumption in order to reflect metabolic processes and thus consolidate available evidence. Another important consideration for future clinical trials may be to screen for individuals with arterial narrowing who may benefit most from blood vessel dilation related to dietary interventions [72, 73]. There is a possibility that a greater BP response would result in more pronounced cognitive functioning, which was not evident in the current study. In addition, it is recommended that blood and fecal samples are included in future human studies in order to allow a more comprehensive analysis of in vivo metabolites, specifically generated by the gut microbiota and thereby elucidate the mode of action of these plant bioactives.

In conclusion, our research hypothesis was not accepted as there were no differences according to two dose-timing regimens of consumption of QGPJ. An acute BP-lowering

effect of anthocyanin-rich plum juice was similarly observed for both dose-timing regimens, while no cognitive effects were observed for either dose, nor were differences in anthocyanin metabolite excretion evident between younger and older adults. Anthocyanin metabolites were bioavailable in urine following consumption but no differences were observed in the absorption rate and metabolism of anthocyanins between young and older adults, assessed in urine. It is important that the mechanism of action is studied further to better understand how anthocyanins exert protective effects on BP and how this reduction effect can be sustained over time, as well as the effects on cognition in longer-term consumption studies. With more significant effects observed in older participants, future studies should focus on this age group where elevated BP is more prevalent by using a placebo-controlled design.

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Conflicts of interest: none

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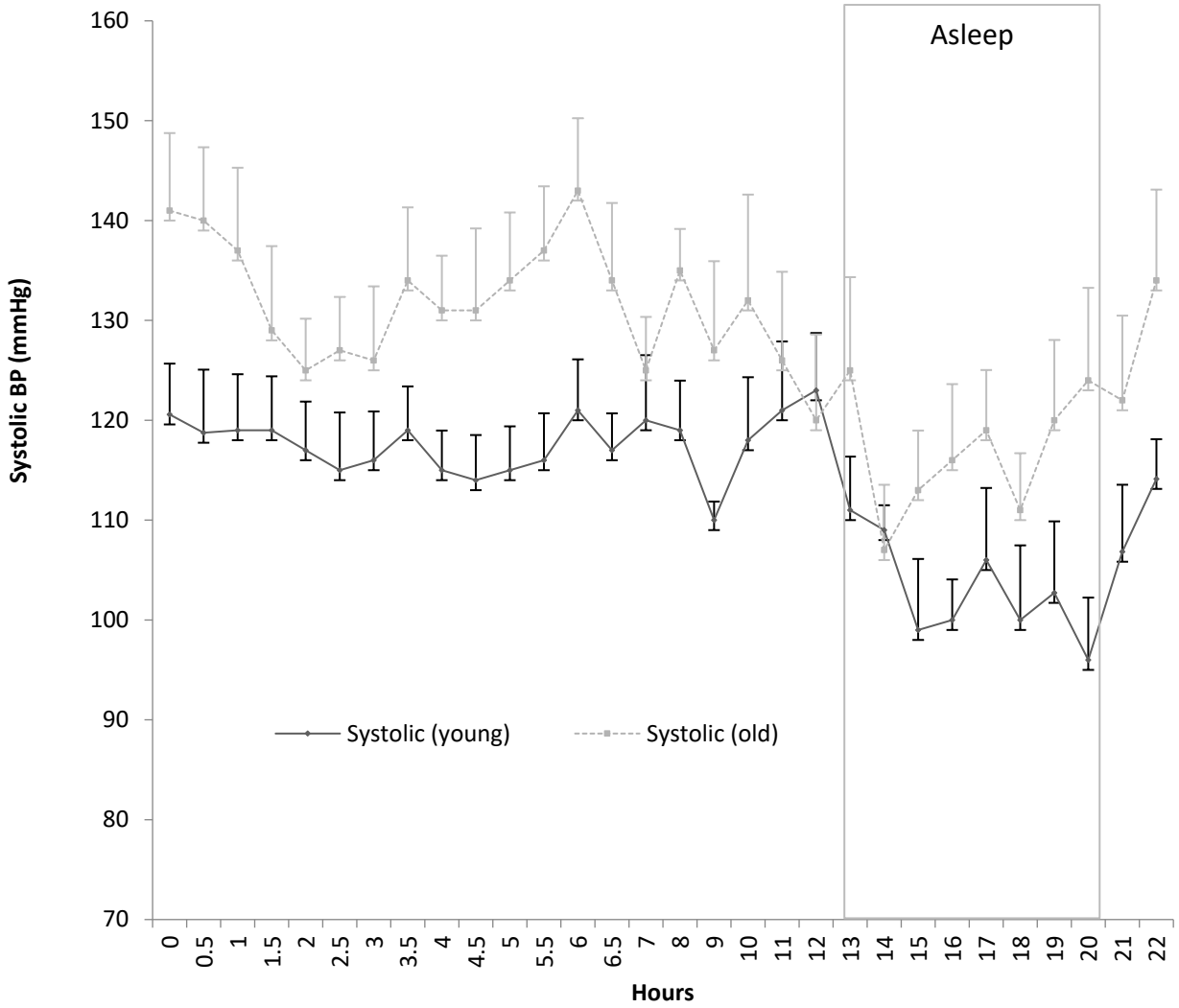
Table 1: Subjects' characteristics

Characteristics	Younger adults (n=12) n (%)	Older adults (n=12) n (%)	p-values
Gender			
Male	4 (33.3)	3 (25.0)	Ns
Female	8 (66.7)	9 (75.0)	
Age (Means ± SD)	31 (8)	77 (6)	<0.001
BMI (Means ± SD)	22.5 (2.4)	26.4 (3.3)	0.003
Physical Activity			
Low	5 (41.7)	1 (8.3)	0.97
Medium	5 (41.7)	10 (83.3)	
High	2 (16.7)	1 (8.3)	
Smoking status			
Yes	0 (0)	0 (0)	Ns
No	11 (91.7)	12 (100)	
Occasionally	0 (0)	0 (0)	
Rarely	1 (8.3)	0 (0)	
Alcohol intake			
Yes	3 (25.0)	5 (41.7)	0.67
No	2 (17.7)	3 (25.0)	
Occasionally	4 (33.3)	2 (16.7)	
Rarely	3 (25.0)	2 (16.7)	

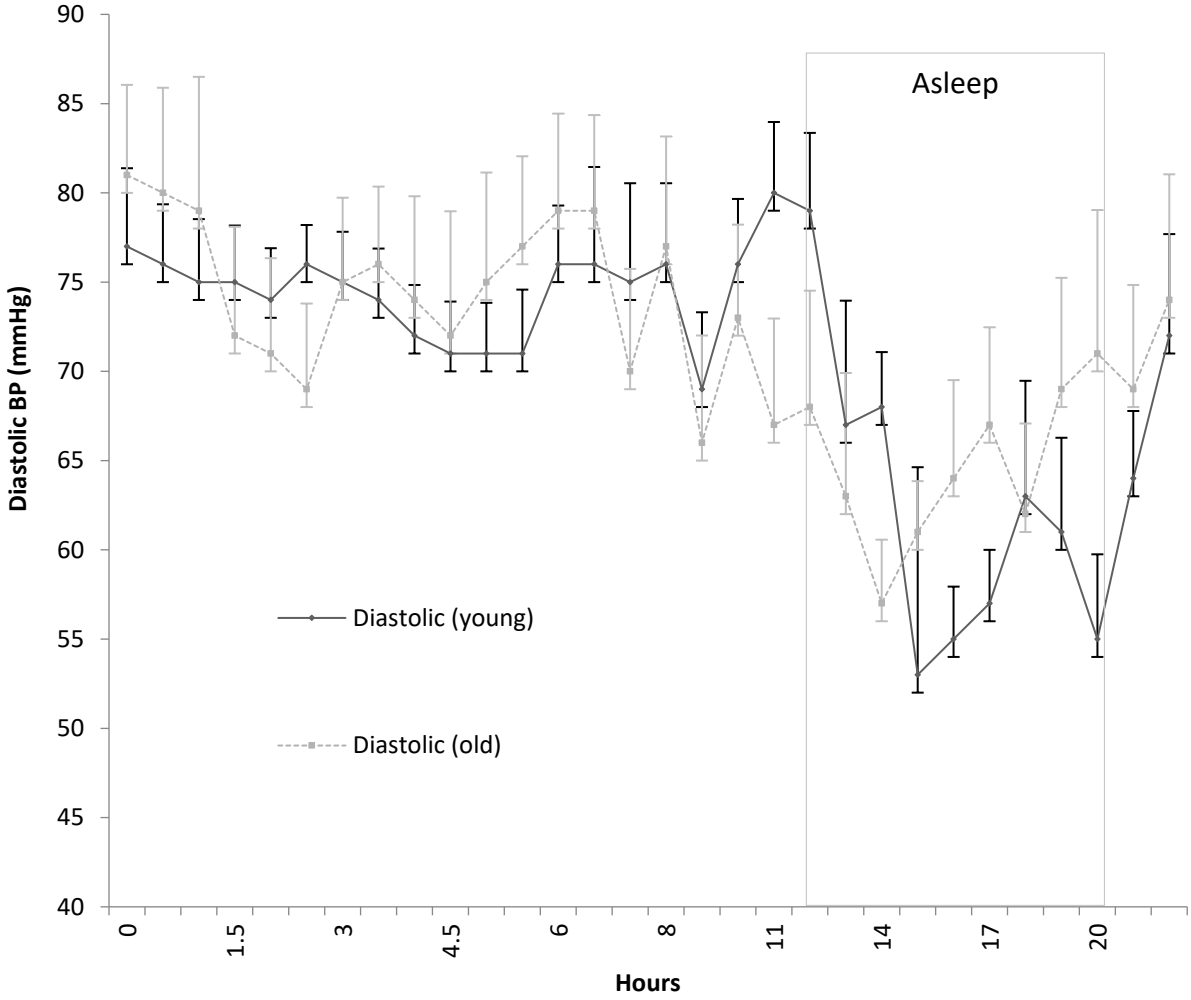
Data are means ± SD or n (%) (n=12)
BMI – Body Mass Index
P values were obtained from $\chi 2$ test for categorical variables.
Ns- Not significant

Fig. 1a: Single dose hourly systolic blood pressure of participants over 24h after consumption of QGP juice



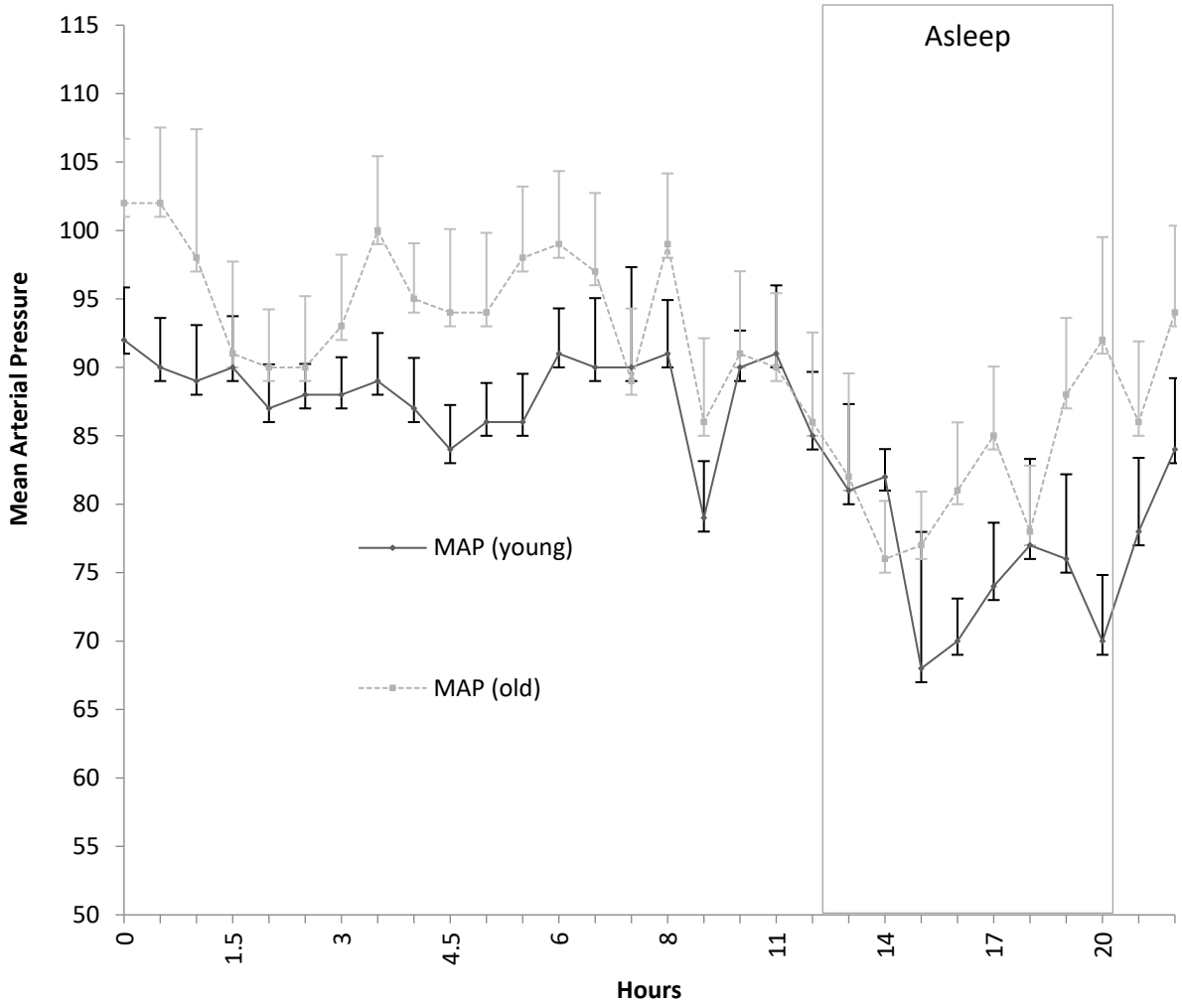
Data are expressed as mean values ± SE (error bars) (n=12 per age group). BP, blood pressure; QGP, Queen Garnet plum.

Fig. 1b: Single dose hourly diastolic blood pressure of participants over 24h after consumption of QGP juice



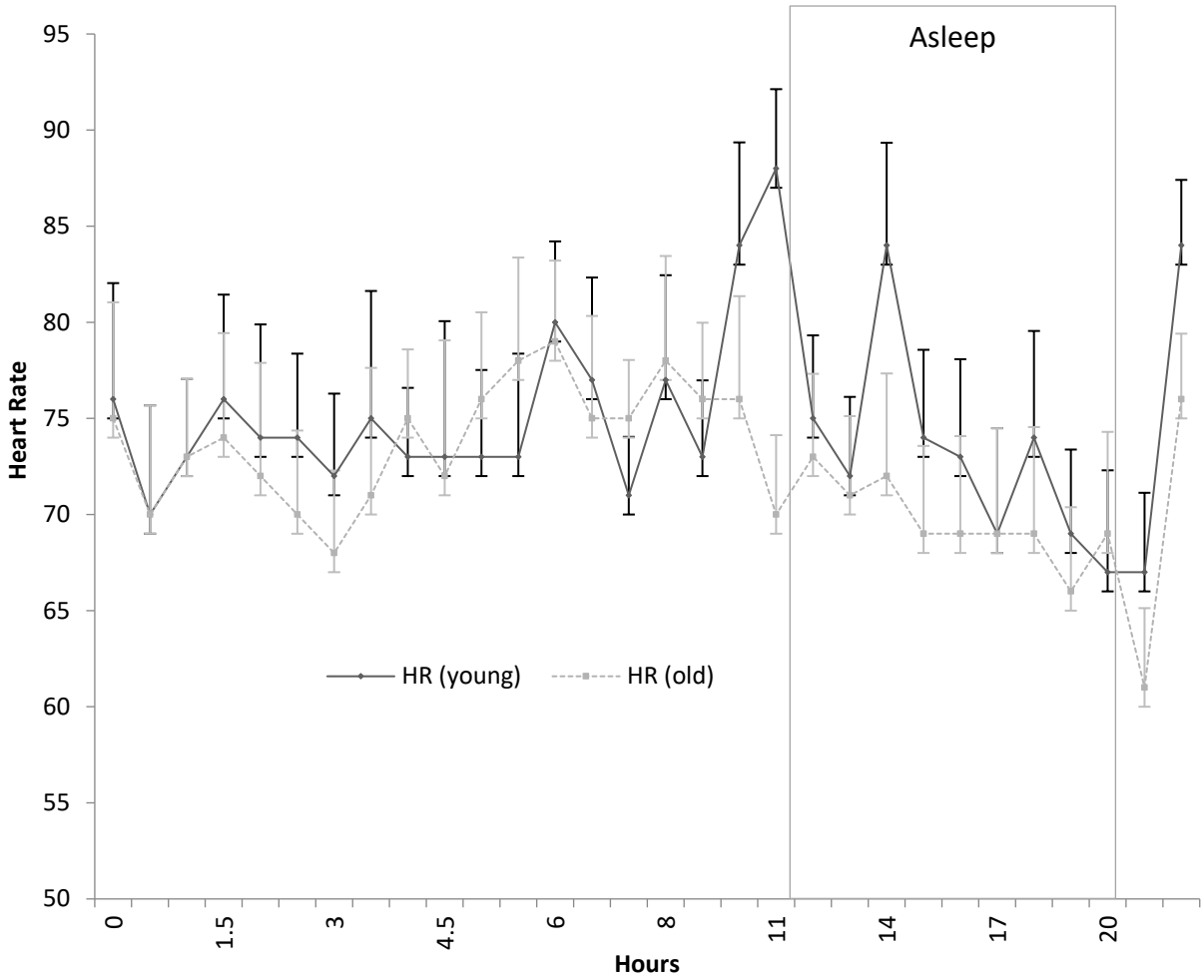
Data are expressed as mean values ± SE (error bars) (n=12 per age group). BP, blood pressure; QGP, Queen Garnet plum.

Fig. 2a: Single dose hourly mean arterial blood pressure of participants over 24h after consumption of QGP juice



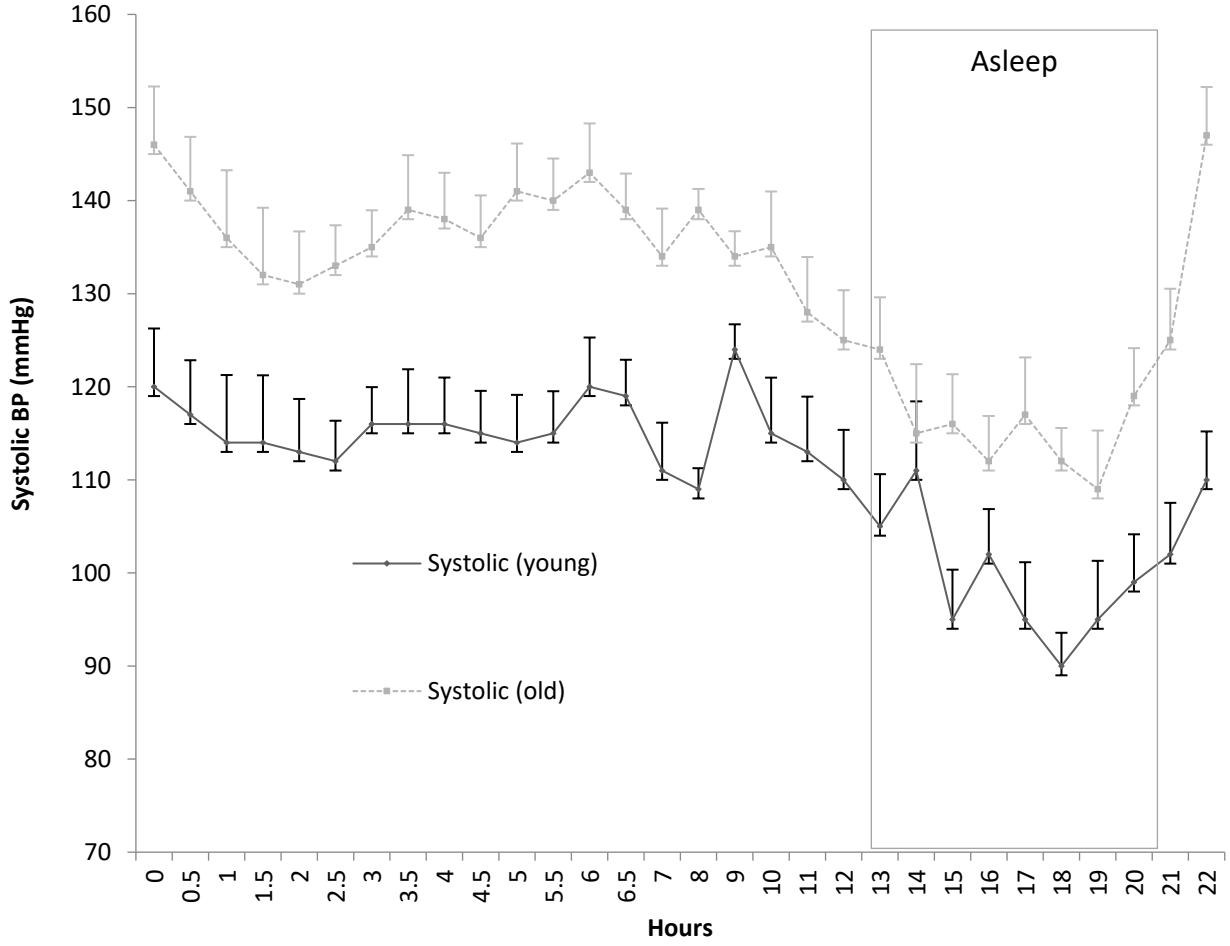
Data are expressed as mean values ± SE (error bars) (n=12 per age group). MAP, mean arterial pressure; QGP, Queen Garnet plum.

Fig. 2b: Single dose hourly heart rate of participants over 24h after consumption of QGP juice



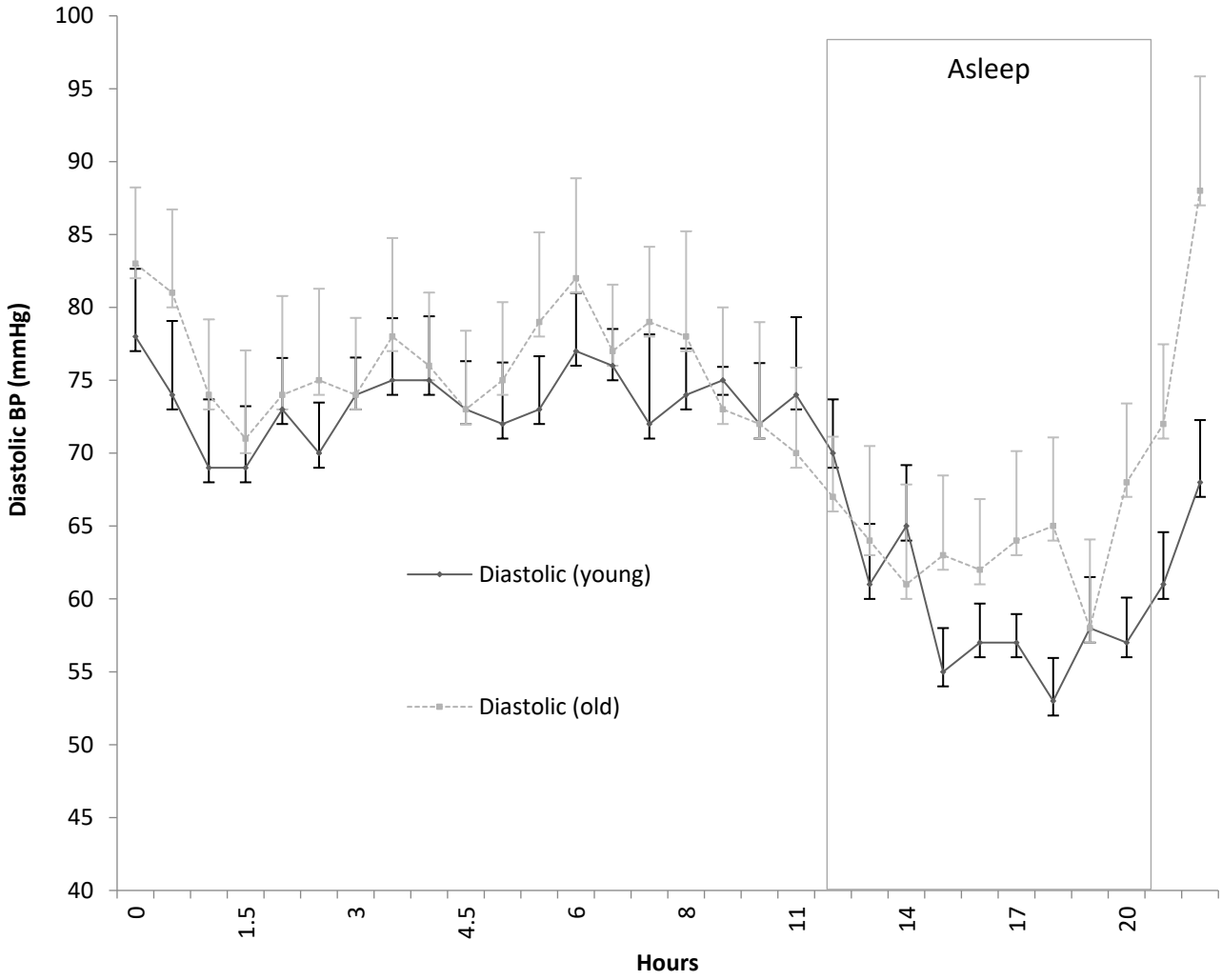
Data are expressed as mean values ± SE (error bars) (n=12 per age group). HR, heart rate; QGP, Queen Garnet plum.

Fig. 3a: Triple dose hourly systolic blood pressure of participants over 24h after consumption of QGP juice



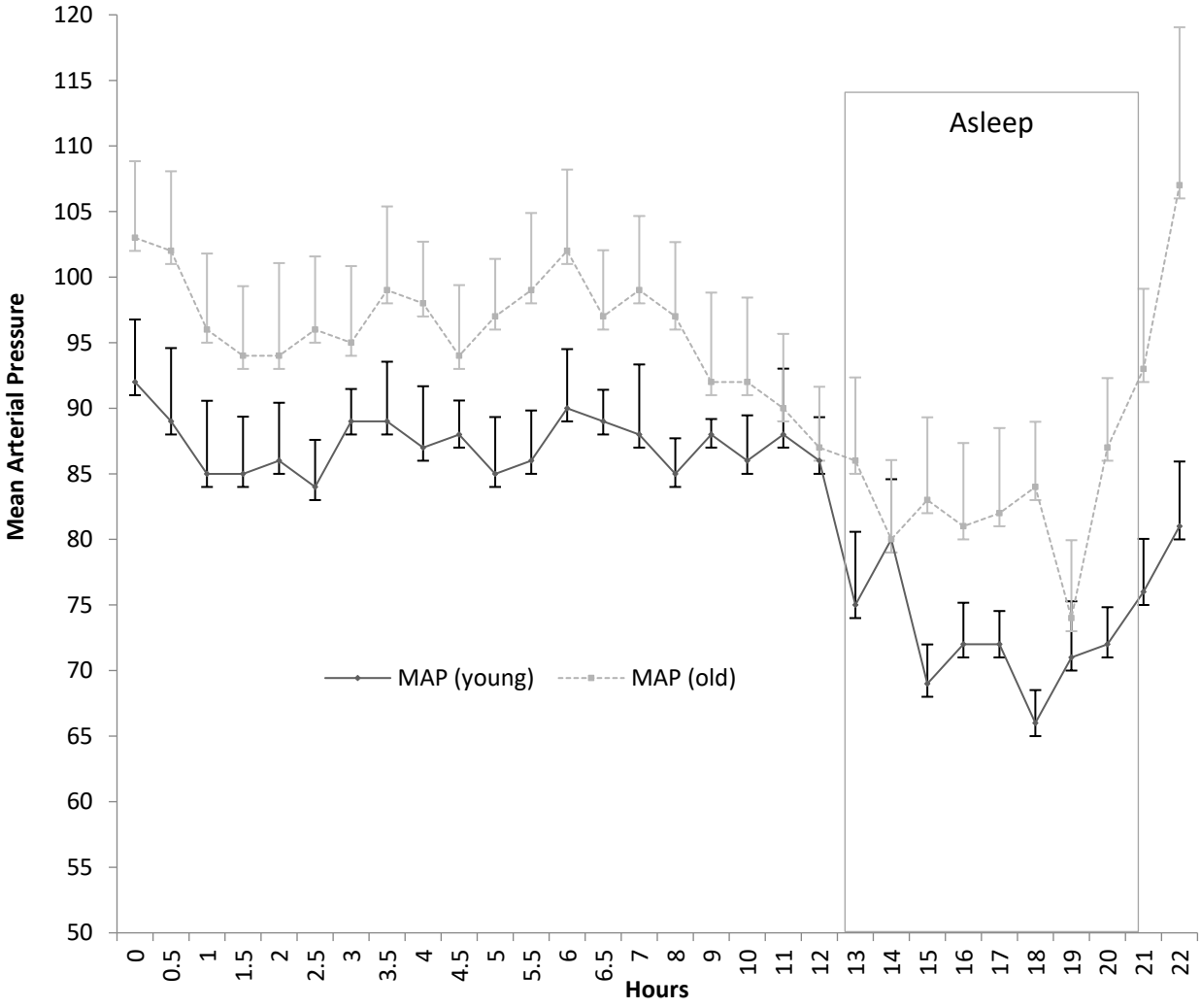
Data are expressed as mean values ± SE (error bars) (n=12 per age group). BP, blood pressure; QGP, Queen Garnet plum.

Fig: 3b: Triple dose hourly diastolic blood pressure of participants over 24h after consumption of QGP juice



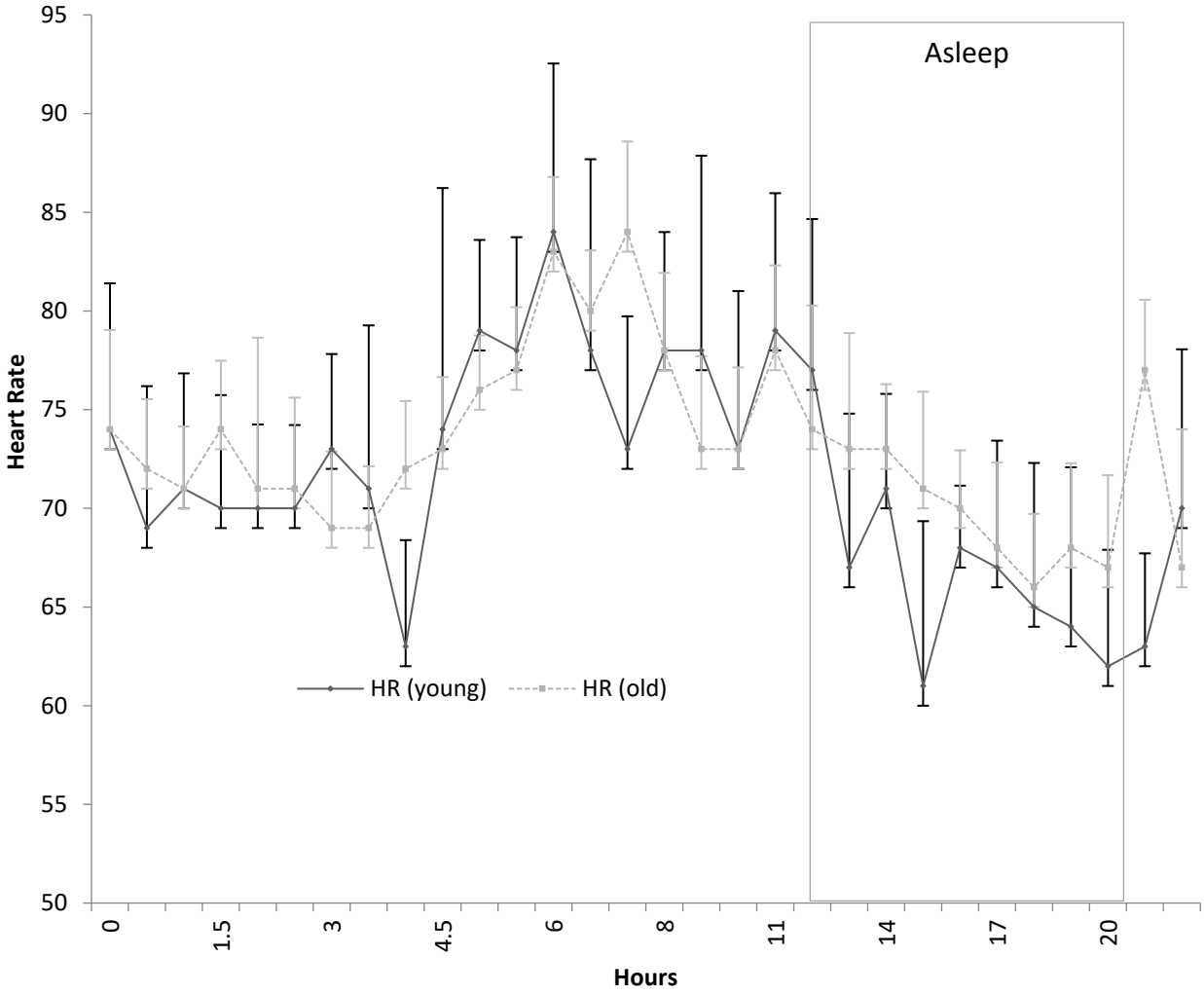
Data are expressed as mean values ± SE (error bars) (n=12 per age group). BP, blood pressure; QGP, Queen Garnet plum.

Fig. 4a: Triple dose hourly mean arterial blood pressure of participants over 24h after consumption of QGP juice



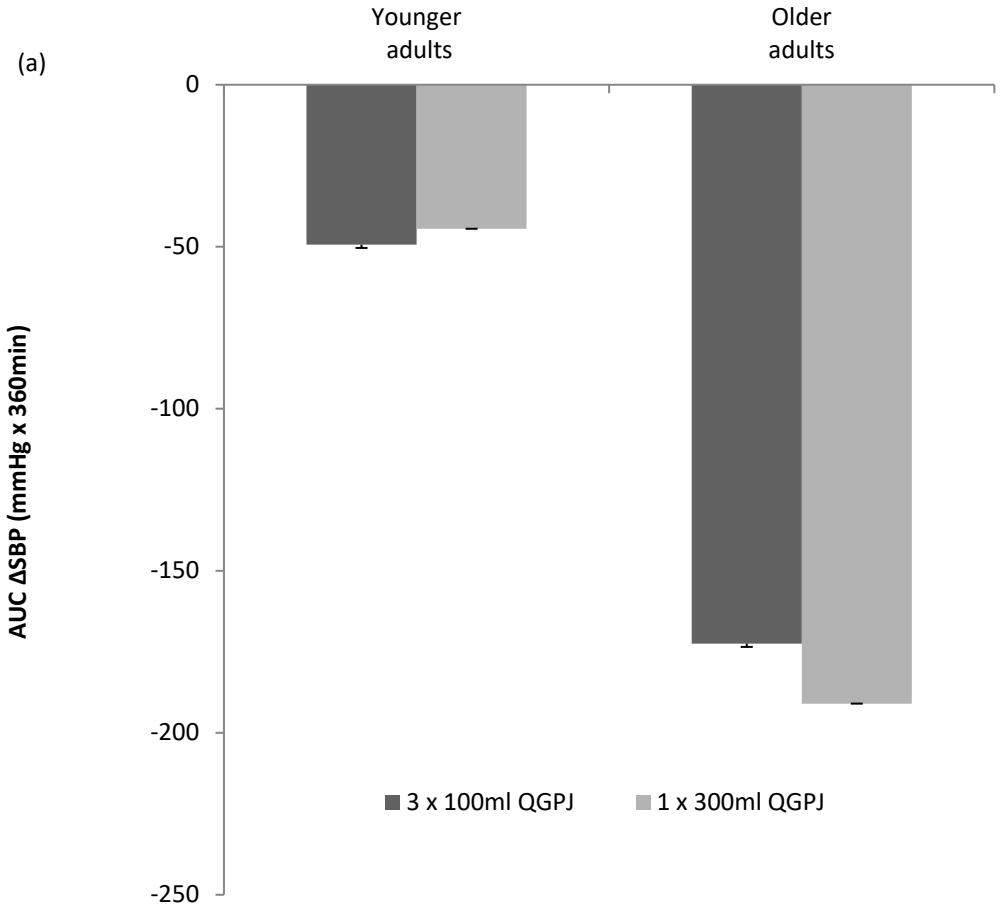
Data are expressed as mean values ± SE (error bars) (n=12 per age group). MAP, mean arterial pressure; QGP, Queen Garnet plum.

Fig. 4b: Triple dose hourly heart rate of participants over 24h after consumption of QGP juice



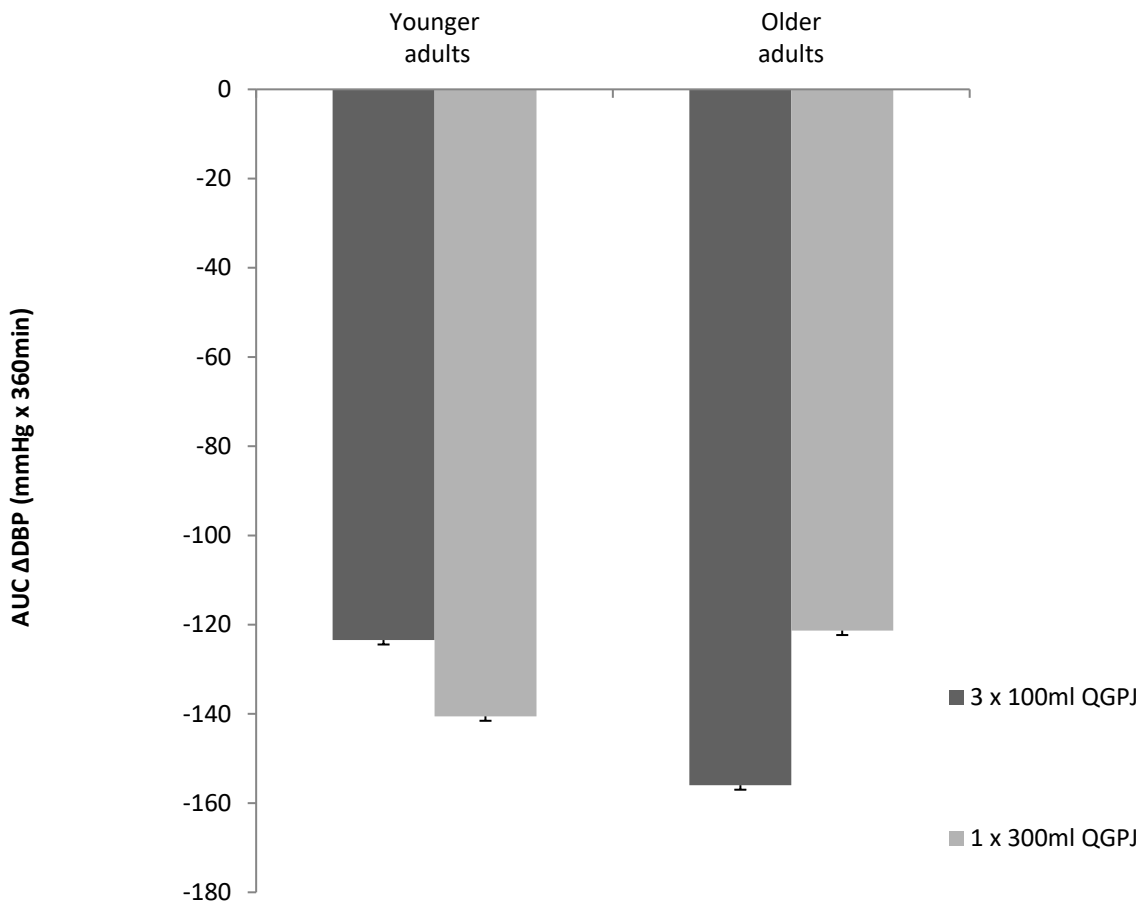
Data are expressed as mean values ± SE (error bars) (n=12 per age group). HR, heart rate; QGP, Queen Garnet plum.

Fig.5a: Change in systolic blood pressure (0-6h) following consumption of QGP juice.



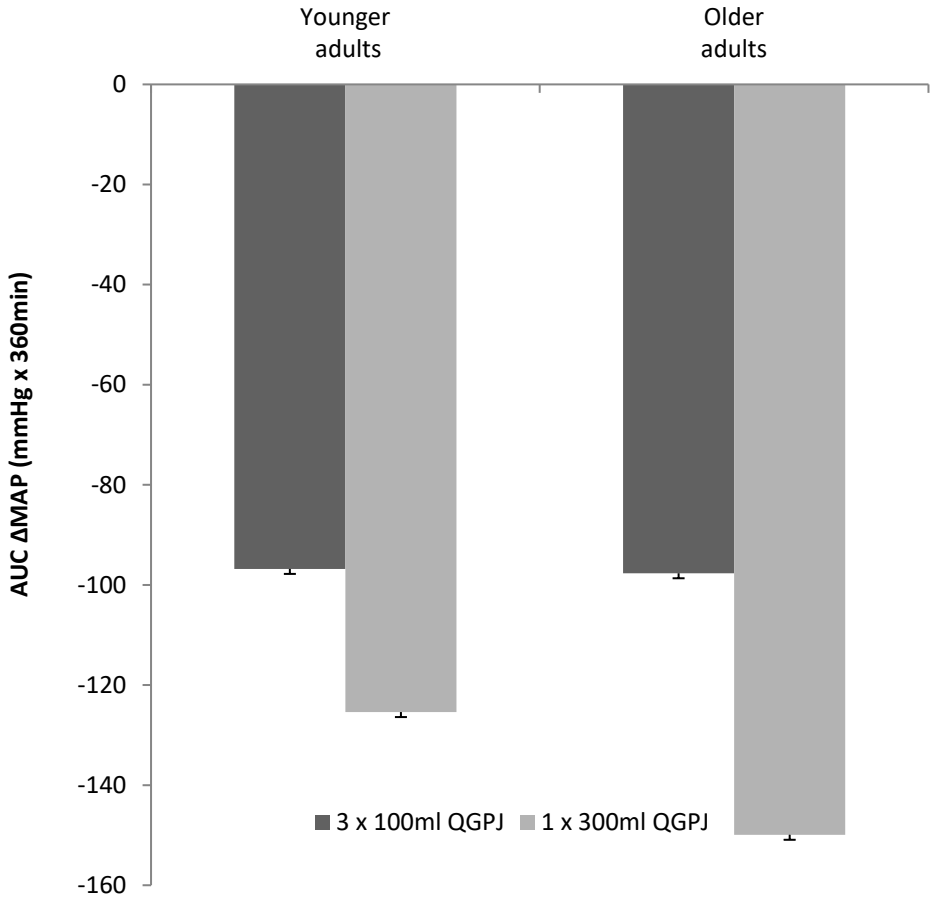
Values are expressed as AUC for mean change in systolic blood pressure from baseline per hour up to 6h. Bars represent the sum of AUC for ΔSBP (0-6h) ± SE (n=12 per age group). AUC, Area Under the curve; QGPJ, Queen Garnet plum juice; SBP, Systolic blood pressure

Fig.5b: Change in diastolic blood pressure (0-6h) following consumption of QGP juice.



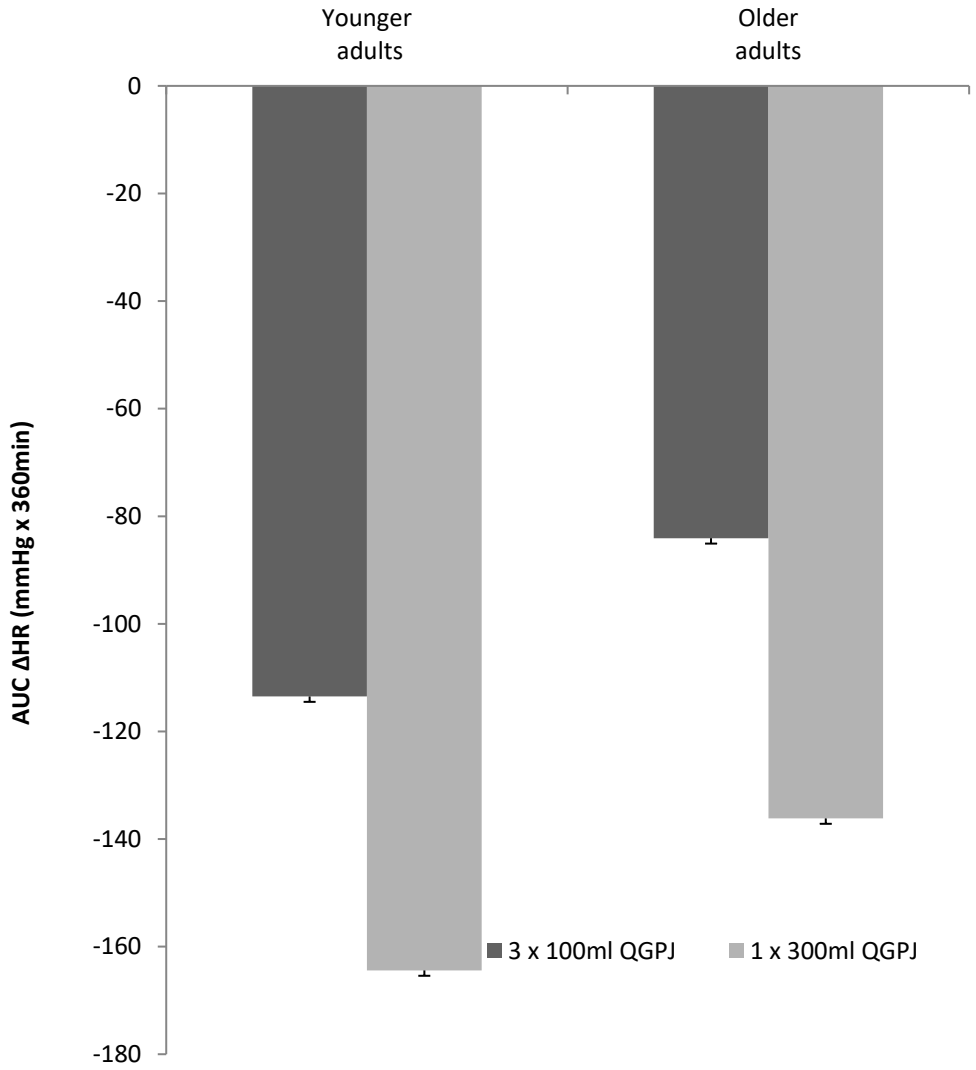
Values are expressed as AUC for mean change in diastolic blood pressure from baseline per hour up to 6h. Bars represent the sum of AUC for ΔDBP (0-6h) ± SE (n=12 per age group). AUC, Area Under the curve; QGPJ, Queen Garnet plum juice; DBP, Diastolic blood pressure

Fig. 6a: Change in MAP (0-6h) following consumption of QGP juice.



Values are expressed as AUC for mean change in MAP from baseline per hour up to 6h. Bars represent the sum of AUC for ΔMAP (0-6h) ± SE (n=12 per age group). AUC, Area Under the curve; QGPJ, Queen Garnet plum juice; MAP, mean arterial pressure

Fig. 6b: Change in heart rate (0-6h) following consumption of QGP juice.



Values are expressed as AUC for mean change in HR from baseline per hour up to 6h. Bars represent the sum of AUC for ΔHR (0-6h) ± SE (n=12 per age group). AUC, Area Under the curve; QGPJ, Queen Garnet plum juice; HR, heart rate.

Table 2: Effect of dose-timed plum juice consumption on cardiovascular parameters across age groups

Parameter	Mean ± SE	p-values
SBP		
Intercept	123.7 ± 0.05	<0.0001^
Dose-time		
1 x 300ml	122.3 ± 0.60	0.001^
3 x 100ml	125.2 ± 0.67	
Group		
Younger	115.0 ± 0.66	<0.0001^
Older	132.8 ± 0.61	
Interaction (group*dose)		0.154
Younger * 1x300ml	114.2 ± 0.84	
Younger * 3x100ml	115.8 ± 1.00	
Older * 1x300ml	130.3 ± 0.87	
Older * 3x100ml	134.5 ± 0.87	
DBP		
Intercept	72.9 ± 0.36	<0.0001^
Dose-time		
1 x 300ml	72.5 ± 0.49	0.25
3 x 100ml	73.3 ±0.54	
Group		0.11
Younger	72.2 ± 0.53	
Older	73.6 ± 0.49	
Interaction (group*dose)		
Younger * 1x300ml	72.0 ± 0.68	0.62
Younger * 3x100ml	72.4 ±0.81	
Older * 1x300ml	73.0 ± 0.69	
Older * 3x100ml	74.1 ± 0.70	
MAP		<0.0001^
Intercept	90.2 ± 0.37	
Dose-time		0.077
1 x 300ml	89.4 ± 0.49	
3 x 100ml	90.9 ±0.54	

Parameter	Mean ± SE	p-values
Group		<0.0001^
Younger	86.4 ± 0.54	
Older	94.0 ± 0.50	
Interaction (group*dose)		0.75
Younger * 1x300ml	85.7 ± 0.68	
Younger * 3x100ml	87.0 ± 0.83	
Older * 1x300ml	93.1 ± 0.70	
Older * 3x100ml	94.9 ± 0.70	
HR		
Intercept	72.6 ± 0.36	<0.0001^
Dose-time		0.69
1 x 300ml	72.5 ± 0.49	
3 x 100ml	72.7 ± 0.54	
Group		0.76
Younger	72.9 ± 0.54	
Older	72.4 ± 0.49	
Interaction (group*dose)		0.91
Younger * 1x300ml	72.8 ± 0.69	
Younger * 3x100ml	73.0 ± 0.82	
Older * 1x300ml	72.2 ±0.70	
Older * 3x100ml	72.5 ± 0.70	

Data are means ± SE (n=12 per group)
^ Significant at p<0.05
Means and p-values were obtained from linear mixed model
"Younger" means younger age-group and "older" means older age-group
Dose-time represents either a single dose of 300ml or 3 portions of 100ml taken at 0h, 1h and 3h

Table 3: Change in cardiovascular parameters of participants following consumption of different doses of QGP juice

Cardiovascular parameters	AUC (0-6 h), mmHg	
	3 x 100ml QGP (0,1,3h)	1 x 300ml QGP
Younger adults (18-45)		
Systolic BP	-49.36 ± 131.26	-44.45 ± 125.29
Diastolic BP	-123.45 ± 122.27*	-140.55 ± 175.93*
Mean Arterial Pressure	-96.80 ± 12.75*	-125.40 ± 136.31*
Heart Rate	-113.50 ± 208.69†	-164.42 ± 253.64*
Older adults (65+)		
Systolic	-172.50 ± 321.86 †	-191.00 ± 313.93*
Diastolic	-156.00 ± 136.28 *	-121.33 ± 190.81*
Mean Arterial Pressure	-97.67 ± 278.55	-149.92 ± 217.91*
Heart Rate	-84.08 ± 195.42	-136.17 ± 171.35*

Data are AUC for mean change in cardiovascular parameters ± SD (n=12 per group)
AUC – area under the curve; BP- blood pressure; QGP – Queen Garnet plum
* Z-test analysis showed statistically significant effect of the juice on the measured parameter.
† Z-test analysis showed borderline significant effect of the juice on the measured parameter.

Table 4: Urinary excretion of anthocyanins and anthocyanin metabolites in different age groups following the consumption of QGP juice as a single oral dose of 300ml or as three 100ml servings

	Dosing (anthocyanins)	Absolute excretion (µg/24 h)[1]	Relative excretion (%)[2]	Relative excretion of main metabolites (%)[3]
Younger age group	1x300 ml (369 mg)	811 ± 702	0.22	80
	3x100 ml (123 mg/dose)	759 ± 358	0.21	74
Older age group	1x300 ml (369 mg)	871 ± 602	0.24	80
	3x100 ml (123 mg/dose)	693 ± 458	0.19	75

Data are means ± SD (n=12 per group); [1]sum of cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-glucoside monoglucuronide, cyanidin monoglucuronide, peonidin-3-glucoside, peonidin monoglucuronide and pelargonidin monoglucuronide; [2]excreted amount vs. ingested dose; [3]excreted amount of peonidin-3-glucoside and peonidin monoglucuronide as the main metabolites vs. absolute excretion; compounds were analyzed by HPLC-PDA-MS and quantified by an external cyanidin-3-glucoside calibration curve [36]