2018

Effect of Fruit Anthocyanin Consumption on Cognition, Blood Pressure and Other Health Parameters in Older Adults

Ezinne Oyidia Igwe
University of Wollongong

UNIVERSITY OF WOLLONGONG AUSTRALIA

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Ezinne Oyidia Igwe

Supervisors:
Professor Karen Charlton
Associate Professor Steven Roodenrys
Dr Yasmine Probst

This thesis is presented as part of the requirement for the conferral of the degree:
Doctor of Philosophy

University of Wollongong
School of Medicine

December 2018

Abstract

Epidemiological evidence has shown that dietary factors, as part of lifestyle behaviours, have significant positive effects on the onset and progression of neurodegenerative disorders including dementia and other chronic diseases. Plant-rich diets especially, have been shown to have significant protective effects on overall health. This is due to the presence of phytochemicals, minerals, vitamins and dietary fibre in these foods. Phytochemicals are classified under carotenoids and polyphenols; which include flavonoids, phenolic acids, and stilbenes/lignans. Anthocyanins are a subclass of flavonoids responsible for the dark red-purple colours in fruits and vegetables. In the last decades, there has been an increased research interest in anthocyanins which is attributed to their antioxidant characteristics - the ability to reduce oxidative stress in living cells. The association between anthocyanin from dietary sources and cognitive function is yet to be fully established. Available evidence suggests 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. In order to determine the clinical effects of anthocyanins, a better understanding is required of the uptake and metabolism of these bioactive compounds found in fruits and vegetables. Intact anthocyanins are believed to reach the large intestine and colon where they undergo intensive metabolism and transformation with observed modulatory effects on gut microbiome. Following further degradation, they are absorbed into the bloodstream, with some of the subsequent metabolites showing potential to be more biologically active than the intact anthocyanins.

The primary aim of this doctoral thesis was to determine the health benefits associated with both acute and longer-term effects of consumption of dietary anthocyanins, delivered in a fruit variant, Queen Garnet plum (QGP), on cognition, blood pressure and other health parameters in younger (acute) and older adults (acute and longer-term). In order to contextualise the findings for dietary guidance to the target population, the anthocyanin intake of the Australian population was assessed using a novel anthocyanin food composition database developed during the course of this research. This thesis addresses the following research questions:

1. What is the current level of evidence on the beneficial health effects of plums that contain considerable amount of anthocyanins?
2. Does consumption of anthocyanin-containing plum juice have an acute effect on various domains of cognitive functioning, and blood pressure, and is there significant difference in the gastro-intestinal metabolism of anthocyanins between young and older adults, assessed using urinary metabolite biomarkers?
3. What is the current level of evidence on the effects of anthocyanins on the gut microbiota in relation to health outcomes?
4. What are the longer-term effects of anthocyanin-containing plum juice consumption on cognition, cardiovascular responses, inflammatory biomarkers (Brain-Derived Neurotrophic Factor (BDNF) and C - reactive protein (CRP)) and gut microbiota in older adults?
5. What is the current dietary anthocyanin intake and sources thereof in the Australian population included in the National Nutrition and Physical Activity Survey (NNPAS) component of the Australian Health Survey (AHS), and is there an association between intake and blood pressure in older adults aged 50+ years?

Five different studies were designed to answer the above research questions. A systematic literature review assessed the current level of evidence on the beneficial health effects of plums known for their high levels of anthocyanins. Results showed that consumption is associated with improved cognitive function, bone health parameters and cardiovascular risk factors. Most of the human trials used the dried version of plums (prunes) rather than fresh fruit thus limiting translation to dietary messages of the positioning of plums in a healthy diet.

A crossover study assessed the acute effects of different dose-timing of Queen Garnet plum juice consumption on cognition, blood pressure, as well as urinary phenolic profile. Queen Garnet plum juice significantly reduced blood pressure but dose-timing did not appear to be a significant factor in the potential acute BP-lowering effect. Native QGP anthocyanins, as well as methylated / glucuronidated metabolites were detected in the urine of participants but there were no significant differences between age groups or dose-timing. No effect was observed on cognition.

A second systematic literature review was carried out to determine the level of current evidence on the effect of anthocyanin treatment/intake on changes in the gut microbiota population, and to compare different techniques used in microbiota determination. With very limited clinical trials done in this area, results showed that anthocyanins induced a significant proliferative effect on *Bifidobacterium spp.*, known for their wide use in probiotics and for the treatment of Irritable Bowel Syndrome (IBS). There was also an observed inhibition of *Clostridium histolyticum*, which has been shown to be pathogenic in humans. Comparison of different techniques used for microbiota determination showed that depth of analysis, with respect to a comprehensive, high-resolution microbiota analysis or analysis of the main microbiota taxa. and budgeted cost are important considerations for future research.

A second clinical trial (8-week randomised controlled crossover study) aimed to assess the longer-term effect of fruit anthocyanins from the Queen Garnet plum juice in comparison to a control (cordial) juice on cognition, cardiovascular responses, gut microbiota, and inflammatory biomarkers in healthy older adults aged 55+ years. Participants consumed 200mL of plum juice (≈ 50mg/L of anthocyanins) or cordial daily for 8 weeks in a cross-over design. Across treatment periods, there was no significant difference on the different domains of cognition measured, blood pressure or anti-inflammatory biomarkers. No intervention effect was found for genera or classes of gut microbes, but there was a trend towards significance in total bacterial count between the control arm and the intervention arm (P= 0.06).

Finally, in response to the lack of a region-specific food anthocyanin database, a first stage (fruits and vegetables) development of anthocyanin food composition database specific to Australian foods was undertaken. Original analytical values for Australian foods were combined with borrowed values from the USDA Database for the Flavonoid content of selected foods and the European Phenol-Explorer database. This novel database was applied to the dietary intake data obtained in a representative national sample of Australians in the Nutrition and Physical Activity component of the 2011-12 Australian Health Survey. Mean intake of anthocyanins in the Australian population was 24.17 ± 0.32 mg/day which is above average in comparison to the world composite database (18.05±21.14 mg/d). Across age-groups, berries were the top food sources: mainly blackberry (5-65%); cherry (2-24 %);

blueberry (2-13%) and raspberry (3-12%). There was a significant inverse association between anthocyanin intake and systolic BP (β= -0.04, $p<0.01$) and diastolic BP (β= -0.01, $p<0.01$), in models that adjusted for covariates (age, gender, BMI, high blood pressure diagnosis, smoking status and physical activity) in adults aged 50+years.

The significance of this body of research can be grouped into three categories including;

- **Knowledge inquiry:** Acute study (to determine metabolism, dose-effect on acute BP) and 8 week Cross over randomised clinical trial of QGP juice.
- **Knowledge synthesis:** Systematic literature reviews.
- **Knowledge translation**: Contextualisation and translation of the clinical trials findings to dietary messages as well as framing of dietary messages in the context of usual dietary practices through assessment of usual intake of anthocyanins in Australians (secondary analysis of national nutrition survey data, using a novel anthocyanin food composition database).

Collectively, this body of research is an original contribution to science. Knowledge gaps in relation to the acute and longer-term health effects of dietary anthocyanins were summarised and addressed. In addition, an Australian anthocyanin food composition database was developed. In a larger context, this body of research further highlights how bioactive components in food (phytonutrients) can impact health outcomes in humans. As projections indicate rapid increases in the prevalence of cognitive impairment and other chronic diseases, dietary interventions offer adjunctive measures to potentially reduce their incidence and progression in the absence of successful conventional treatments.

Acknowledgments

To God who is the author and finisher of my faith…

To Karen, my primary supervisor, who has taught me so much more than research. To be able to work with Professor Karen Charlton has been an enlightening experience. For welcoming me into your space and to be able to partake in your wealth of knowledge, I am eternally grateful. Thank you, Karen, for your patience, encouragement, guidance and above all, friendship which has helped me in innumerable ways. You have taught me resilience and your continuous belief in me will always spur me higher. It has been and will always be an honor.

To my co-supervisors, Associate Professor Steven Roodenrys and Dr. Yasmine Probst, thank you for always challenging my thinking, supporting and encouraging me. In so many ways, your guidance and advice created a welcoming and relaxed environment throughout my PhD. Thank you for all the times you went out of your way to assist me.

As a child, my parents worked hard to teach and instill in me discipline and resilience. Sometimes it was difficult to understand but like most childhoods, you appreciate these lessons more in adulthood. To my parents, Professor S. O Igwe and Ngozi Igwe, who continue to teach me by their own lives that whatever I believe in is achievable. They continue to encourage and pray for me. Every day I take a leaf out of their book and the strength to know that my set goals are reachable.
Speaking of my childhood, I wonder what that story could have been without the soldiers I call siblings, Chie, Ebere, KK (RIP) and Uzunma, my guardians and Barristers (forever have my back) who continue to shower me with love and support despite my shortcomings. I am always encouraged to be better just by the fact that I get to call them family. I love you and continue to live for you all.

To my beautiful daughter, Kamsi, you are a superstar. Being your mother is both fulfilling and a real pleasure. I thank God for your understanding in all my busyness and demands of my PhD, encouraging me even when you could not understand what I had to do. You have been my number 1 cheerleader and I love you.

Ken, for going over and beyond for me, for being my second eye when I over-read my drafts and needed fresh eyes, thank you for loving me, making my problems yours and helping uncloud my judgement when emotions ran high. You are awesome and I truly appreciate your generosity, selflessness, patience and understanding.

In order to produce this body of work, I needed a space to undertake my research and clinical trials which was provided by the University of Wollongong and the Illawarra Health and Medical Research Institute (IHMRI). I am grateful to the clinical research staff at IHMRI for their support during the time I worked in that space and through all the challenges I encountered.

To my friends and colleagues who were a source of encouragement, for the lunch/coffee dates and de-stressing with food (loads!), thank you!

I would also like to thank Professor Victoria Traynor who gave me the opportunity to demonstrate my research skills as a research assistant. Thank you, Vicki, for believing in me and in turn helping me believe in me even more.

Finally, I am grateful for all the participants who gave their time to science (and my PhD!). On behalf of science (and my PhD) I say a big THANK YOU!

Ezinne Oyidia Igwe

Certification

I, Ezinne Oyidia Igwe declare that this thesis submitted in fulfilment of the requirements for the conferral of the degree Doctor of Philosophy, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

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Ezinne Oyidia Igwe
Date

Dedication

This work is dedicated to my father Professor Samuel Okoronkwo Igwe who has afforded me great opportunities to becoming who I am and whose example has spurred me to greater heights.

List of Abbreviations

ABS	Australian Bureau of Statistics
ABPM	Ambulatory blood pressure monitor
AHS	Australia Health Survey
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AUC	Area under curve
AUSNUT	Australian Food and Nutrient Database
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BMD	Bone mineral density
BMI	Body Mass Index
BP	Blood Pressure
BSAP	Bone specific alkaline phosphatase
CBG	Cytosolic β-glucosidase
CI	Confidence Interval
CRF	Cerebral Blood Flow
CRP	C- Reactive Protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DII	Dietary Inflammatory Index
DR	Diet Recall
eNOS	Endothelial Nitric Oxide Synthase
FFQ	Food Frequency Questionnaire
GIT	Gastro-Intestinal Tract
Glut 4	Glucose transporter type 4
H_2O_2	Hydrogen peroxide
HR	Heart rate
IBS	Irritable Bowel Syndrome

IGF-1	Insulin-like growth factor-1
IPE	Immature plum extract
IR	Insulin Resistance
ISU	Iowa State University
LDL	Low-density lipoprotein
LPH	Lactase-Phlorizin Hydrolase
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MAP (kinase)	Mitogen Activated Protein kinase
MAO	Monoamine oxidase
mRNA	Messenger Ribonucleic Acid
MSM	Multiple Source Method
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NO	Nitric oxide
NCI	National Cancer Institute
NHMRC	National Health and Medical Research Council
NNPAS	National Nutrition and Physical Activity Survey
PI3	Phosphoinositide – 3
PPAR	Peroxisome proliferator activated receptor
PTH	Parathyroid hormone
QGP	Queen Garnet plum
QGPJ	Queen Garnet plum juice
RBP4	Retinol-binding Protein 4
ROS	Reactive oxygen species
SE	Standard Error
SGLT1	Sodium-dependent glucose transporter 1
SPADE	Statistical Program to Assess Dietary Exposure
SBP	Systolic blood pressure
TNF-α	Tumor Necrosis Factor Alpha

UOW	University of Wollongong
USDA	United States Department of Agriculture
WFR	Weighted Food Record

Publications constituting this thesis

The chapters of this thesis have been prepared for publication as follows:

Peer reviewed publications

1. Igwe, E.O., and Charlton, K.E., 2016. A systematic review on the health effects of plums (Prunus domestica and Prunus salicina). *Phytotherapy Research*, *30*(5):701-731.

2. Igwe, E.O., Charlton, K.E., Roodenrys, S., Kent, K., Fanning, K. and Netzel, M.E., 2017. Anthocyanin-rich plum juice reduces ambulatory blood pressure but not acute cognitive function in younger and older adults: a pilot crossover dose-timing study. *Nutrition Research*, *47*: 28-43.

3. Igwe, E., Neale, E., Charlton, K.E., Morton, K. and Probst, Y.C., 2017. First stage development of an Australian anthocyanin food composition database for dietary studies–A systematic process and its challenges. *Journal of Food Composition and Analysis*, 64: 33-38.

4. Igwe E.O., Charlton K.E., Probst Y.C., Kent K., and Netzel M.E. 2018. A systematic literature review of the effect of anthocyanins on gut microbiota populations. *Journal of Human Nutrition and Dietetics, 32, 53–62.* https://doi.org/10.1111/jhn.12582

5. Igwe, E.O., Charlton, K.E., Probst, Y.C. (2019) Usual dietary anthocyanin intake, sources and their association with blood pressure in a representative sample of Australian adults. *Journal of Human Nutrition and Dietetics*. 00, 1– 13 https://doi.org/10.1111/jhn.12647.

6. Igwe, E.O., Charlton, K.E., Roodenrys, S., Probst, Y.C., do Rosario, V., Netzel, M.E., Trieu, H. H., Netzel, G., and Phan, A. D. T. 2019. Effect of anthocyanin-containing Queen Garnet plum juice on cognition, blood pressure and gut microbiota in healthy older adults: A

randomised crossover trial. (under review) Submitted to the European Journal of Nutrition. (March 2019).

Conferences abstracts

1. Igwe, E.O, Charlton, K.E., Kent, K., Netzel, M. and Fanning, K., 2016. Acute effect of Queen Garnet plum juice on blood pressure, cognition, and urinary metabolite excretion. In *Nutrition Society of Australia Annual Scientific Meeting*. 29 November - 2 December 2016, Melbourne, Australia. *Journal of Nutrition & Intermediary Metabolism,* 8**,** 82.

2. Igwe, E, and Charlton, K.E., 2016. A systematic approach to the development of an anthocyanin database for Australian foods. The Future of Food and Nutrient Databases: Invention, Innovation, and Inspiration conference proceedings. The 39th National Nutrient Databank Conference, 16-18 May 2016 Alexandria, VA, USA.

3. Igwe, E.O, Charlton, K.E. and Probst, Y.C., 2018. Usual dietary anthocyanin intake, sources and their association with blood pressure in a representative sample of Australian adults. In Nutrition Society of Australia Annual Scientific Meeting proceedings. 27 -30 November 2018, Canberra, Australia.

Prizes and Awards

- HDR student travel grant to present at the 39th National Nutrient Databank Conference in Alexandria, VA, USA, 2016.

- Nutrition Society of Australia student grant to present at the annual conference in Canberra, Australia, 2018.

Media coverage of thesis related research

1. University of Wollongong media release. August 2015. New breed of Australian plum promising in fight against dementia.

2. WIN news Illawarra broadcast, September 2015. Plum juice trial at IHMRI.

3. University of Wollongong Australia Research & Innovation. Issue 1. 2017. Plum target for inflammation: Can fruit improve symptoms of arthritis?

4. WIN news Illawarra broadcast, January 2018, Plum Research – How to improve your memory.

List of funding sources supporting this thesis

- International Postgraduate Scholarship Award (IPTA) funded through the Faculty of Science, Medicine and Health, University of Wollongong (2016-2018).

- University of Wollongong 2013 URC Research Partnerships Grant Scheme ($14,500.00).

- University Global Partnership Network (UGPN) Grants, 2016 (US$ 10,000.00).

- In kind donations:
    - Queen Garnet plum juice: NutraFruit Pty Ltd, Yeerongpilly, QLD, Australia.
    - Gut time lapse kits: uBiome Inc. San Francisco, CA, USA.

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1 Chapter 1

1.1 INTRODUCTION

This chapter provides the background of this thesis from scientific literature. It introduces anthocyanins and describes their beneficial health effects, as well as provides an overview of their metabolism and absorption, and the plausible mechanisms involved. Dietary anthocyanin sources and available methods of anthocyanin-intake measurement in epidemiological studies are described which highlight the gaps in literature and informed the objectives of this thesis. The primary and secondary objectives of this thesis, as well as the conceptual framework for the thesis, are presented in this chapter.

1.1.1 Anthocyanins

Incorporating fruits and vegetables into the usual human diet has been shown to exert protective effects on health. These observed effects have been attributed to the presence of phytochemicals, minerals, vitamins, and dietary fibre in these food groups [1]. Phytochemicals are classified under carotenoids and polyphenols; which include phenolic acids, flavonoids, and stilbenes/lignans. The polyphenols are the largest class of phytochemicals [2]. These bioactive compounds, independently as chemical extracts, and as a group in fruits and vegetables have been associated with reduced premature mortality [3], weight loss [4], and reduced risk of cardiovascular diseases [5] and some cancers [6].

Flavonoids are the most common group of polyphenols and are further divided into six sub-classes namely: flavones, flavonols, flavanones, isoflavones, flavanols, and anthocyanins [7] (Fig. 1-1). In the last decades, there has been increased interest in anthocyanin-based research [8]. This increased research interest has been attributed to the antioxidant characteristics of anthocyanins i.e. the ability to reduce oxidative stress in living cells, which has been shown to prevent certain chronic diseases [8]. Anthocyanins are water soluble plant pigments that are particularly conspicuous in fruits and flower tissues as they are responsible for the diverse range of red, blue and purple colours. High levels of anthocyanins have been observed in some common fruits and vegetables including blueberries, black grapes, raisins, blackberries, plums, purple cabbage, eggplant, purple cauliflower and purple potatoes [9]. In plants, anthocyanins are known to protect the chloroplast from photodegradation by absorbing high energy quanta, while scavenging free radicals and reactive oxygen species (ROS). They are usually present in plants as glycosides (i.e. sugar molecule bound to anthocyanin compounds via a glycosidic bond) or acylglycosides of their respective aglycone anthocyanidins [9]. Anthocyanins are one of the most versatile subgroups of flavonoids. The key characteristic which differentiates anthocyanin glycosides from the rest of the flavonoid group is their ability to form flavylium cations [10]. Flavylium cations are species of a multistate of different molecules reversibly interconverted by external inputs such as pH, light and temperature [11]. In nature, about 17 different anthocyanins have been discovered but only six (cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin) have been shown to be of dietary importance and are ubiquitously distributed in the food supply [10].

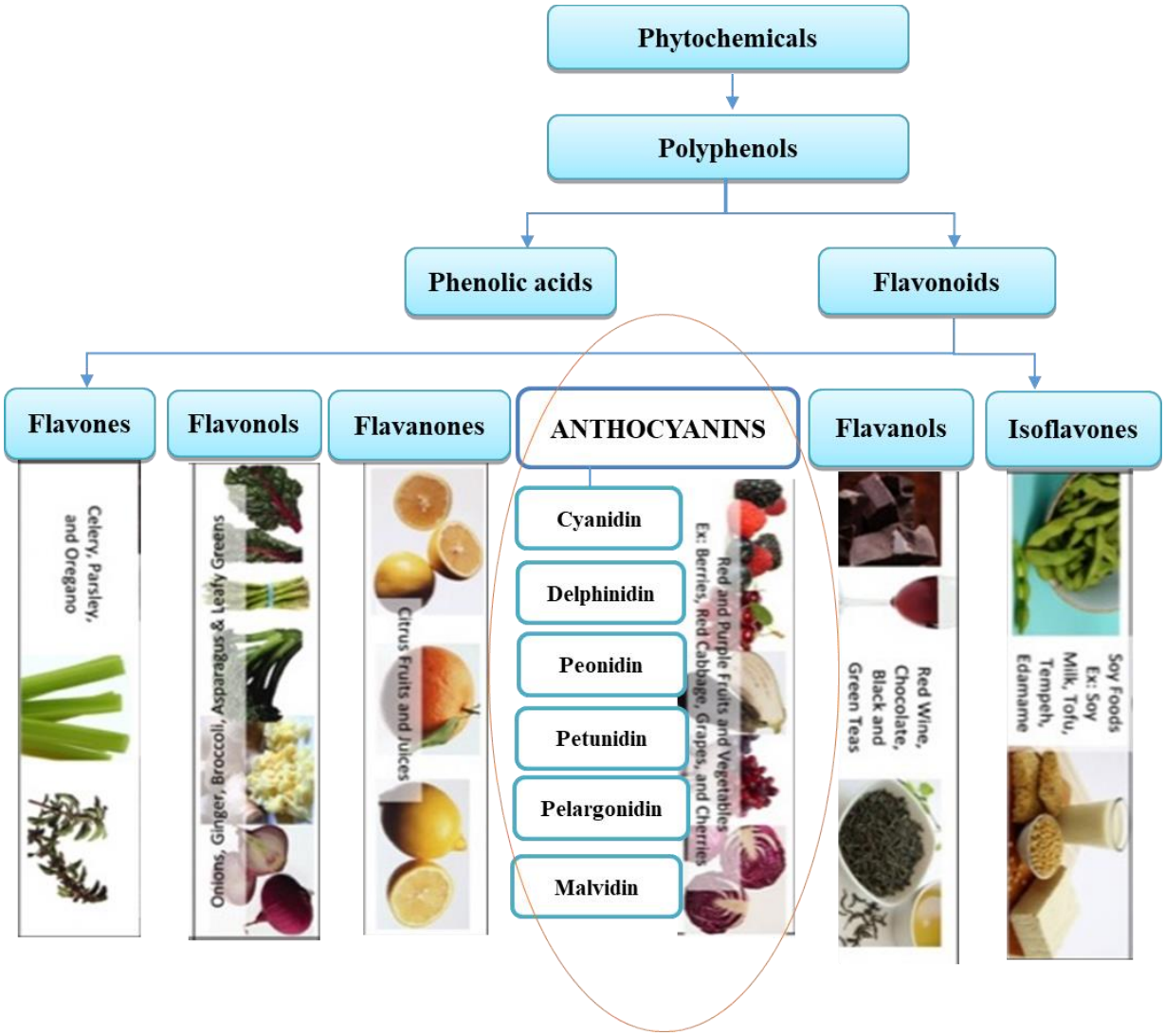


Figure 1-1: Diagram showing the classification of polyphenols and food sources.

Source: [12]

The richest dietary sources of anthocyanins are berries, cherries, apples, red and purple grapes, plums and pomegranates [13, 14] while red wine and certain vegetables (e.g. cabbage, onions and radishes) are also significant sources [15]. Although there is inconclusive evidence regarding the health benefits of anthocyanins, promising findings relate to their anti-inflammatory activity [16], as well as their role in risk factors for CVD [16], weight management [16], alleviation of elevated blood sugar [16] and impaired cognitive functioning [17]. The exact manner in which they exert these protective effects is yet to be fully established as this research area is still emerging [18].

1.1.1.1 Beneficial health effects of anthocyanins

Anthocyanins are regarded as an important component of the human nutrition [19]. Historically, before scientific evidence, they have been used to treat diverse ailments including hypertension, pyrexia, liver disorders, kidney stones and urinary tract infection [8]. Even though research evidence shows their beneficial health effects [20, 21], research in these areas is inconclusive, on-going studies continue to show promising results. Antioxidant activity has been described as one of the most important aspects of anthocyanins in relation to disease prevention [22]. Evidence shows that the antioxidant capacity (molecules able to donate a free electron or hydrogen atoms to reactive free radicals) of a molecule is significantly correlated to its ability to oxidise easily [21]. This ability of anthocyanins to capture free radicals by donation of phenolic hydrogen atoms has been identified as the potential reason for their anti-inflammatory activity [22]. Some studies have suggested that the anthocyanin content in fruits is directly proportional, to some degree, to their corresponding antioxidant activity and in turn related to the protective effects exhibited by the fruits and vegetables against degenerative and chronic diseases [23-25].

1.1.1.2 Absorption and metabolism

Absorption and metabolism are important aspects of understanding the association between anthocyanins and their reported beneficial health effects. Following consumption, the initial absorption of most polyphenols, including anthocyanins, occurs in the small intestine before moving on to the colon where further metabolism and absorption takes place [26]. Evidence from both animal studies and randomised clinical trials (RCTs) have shown that some anthocyanins (delphinidin 3-O-rutinoside, cyanidin 3-O-rutinoside, delphinidin 3-O-glucoside, and cyanidin 3-O-glucoside) are directly absorbed and excreted in their intact glycosylated forms [27], while anthocyanin arabinosides have been observed to be the most resistant to absorption and microbial degradation in the intestines [28]. The primary site of anthocyanin absorption and transformation is the duodenum and jejunum with approximately 10-50% of ingested content being absorbed in these regions of the small intestine, mainly as aglycones (the product when a glycoside is replaced by a hydrogen atom) after

deglycosylation of the original compound [29, 30]. After the glycosides enter the enterocytes in the small intestine, hydrolysis by cytosolic β-glucosidase (CBG) occurs to get rid of glycosidic moieties. In the enterocyte cytosol, polyphenol aglycones are either transferred to the blood stream via passive diffusion or transformed to phase II metabolites via phase II pathways [30]. Enteric and enterohepatic recycling usually leads to isomeric modification before partitioning of anthocyanin metabolites into the urine [31] (Fig. 1-2). The initial absorption of anthocyanins in the intestine is determined, to a large degree, by their hydrophobicity. It has been shown that glycosides that are more hydrophilic than their resultant aglycones cannot readily cross the enterocyte cellular membrane via passive diffusion [16]. Following the initial absorption from the small intestine, the resulting metabolites, and some of the initial intact glycosides travel through the hepatic portal vein to the liver where further metabolism takes place. Anthocyanin interconversion *in vivo* is due to xenobiotic and gut bacterial metabolism involving addition and removal of methyl and hydroxyl groups. Enteric and enterohepatic recycling leads to isomeric modification of anthocyanins before separating some of the resulting metabolites into urine (Fig.1-2)

The exact mechanism involved in anthocyanin absorption and metabolism has not yet been confirmed. Two mechanisms have been described; one of these mechanisms is the cleavage of glycosidic moieties by lactase-phlorizin hydrolase (LPH), a brush border enzyme on the surface of enterocytes. This is known to be very reactive toward flavonoid-O-β-D-glycoside [32]. The second mechanism, first described by Hollman et al. [33], proposes that intact absorption of anthocyanin glycosides could actually occur in the small intestine, using the sodium-dependent glucose transporter 1 (SGLT1). Prior to this theory, it was believed that the typically large and highly polar molecules were not absorbed after oral consumption but underwent hydrolysis to their aglycones by bacterial enzymes in the lower part of the intestine [34]. However, current evidence shows that polyphenol conjugates with sugar moieties that move into the colon are those that are resistant to the action of LPH/CBG [35]. A study on the bioavailability and bioactivity of flavonoids and phenolic acids showed that the absorption and metabolism of different cranberry phenolics occurs at different locations and in different quantities along the gastrointestinal tract (GIT) [36]. An animal study on the metabolism of flavonoids via enteric recycling metabolism observed that the quantity of genistein (a soy isoflavone) and apigenin (a flavone analogue of genistein) that was absorbed differed across various regions of the intestine [37].

For genistein, the highest absorption occurred in the duodenum (44% of perfused amount). The absorption in the duodenum and the colon (35%) was significantly higher than the quantity absorbed in the jejunum (16%) and the terminal ileum (21%). In addition, the metabolism of these two compounds followed a similar trend; apigenin was metabolised faster than genistein, with the highest metabolism of the two compounds occurring in the jejunal microsomes and more readily in the colon, and negligible amount of metabolites observed in the perfusate. The study findings suggested that the rate of metabolism of different flavonoids in the intestinal microsomes could be higher than what is observed in the liver microsomes [37].

Other physio-chemical properties such as molecular size and configuration also regulate the absorption of anthocyanins. Anthocyanins with higher molecular weights tend to have poorer absorption in the body [38]. In food, anthocyanins exist mainly as glycosylated derivatives, the chemical structure of which has been shown to significantly facilitate their absorption through the gut barriers [39]. These glycosylated derivatives are able to survive the acidic environment of the stomach [40] which means that they remain intact for longer. Inter-individual variation is another important factor that determines the rate of absorption, metabolism and excretion of anthocyanins. Inter-individual variation suggests that some individuals are better absorbers than others, possibly due to particular polymorphisms for intestinal enzymes or transporters [41]. To control for this in clinical trials, crossover study designs are usually employed meaning that for each of the trials, participants act as their own control [42].

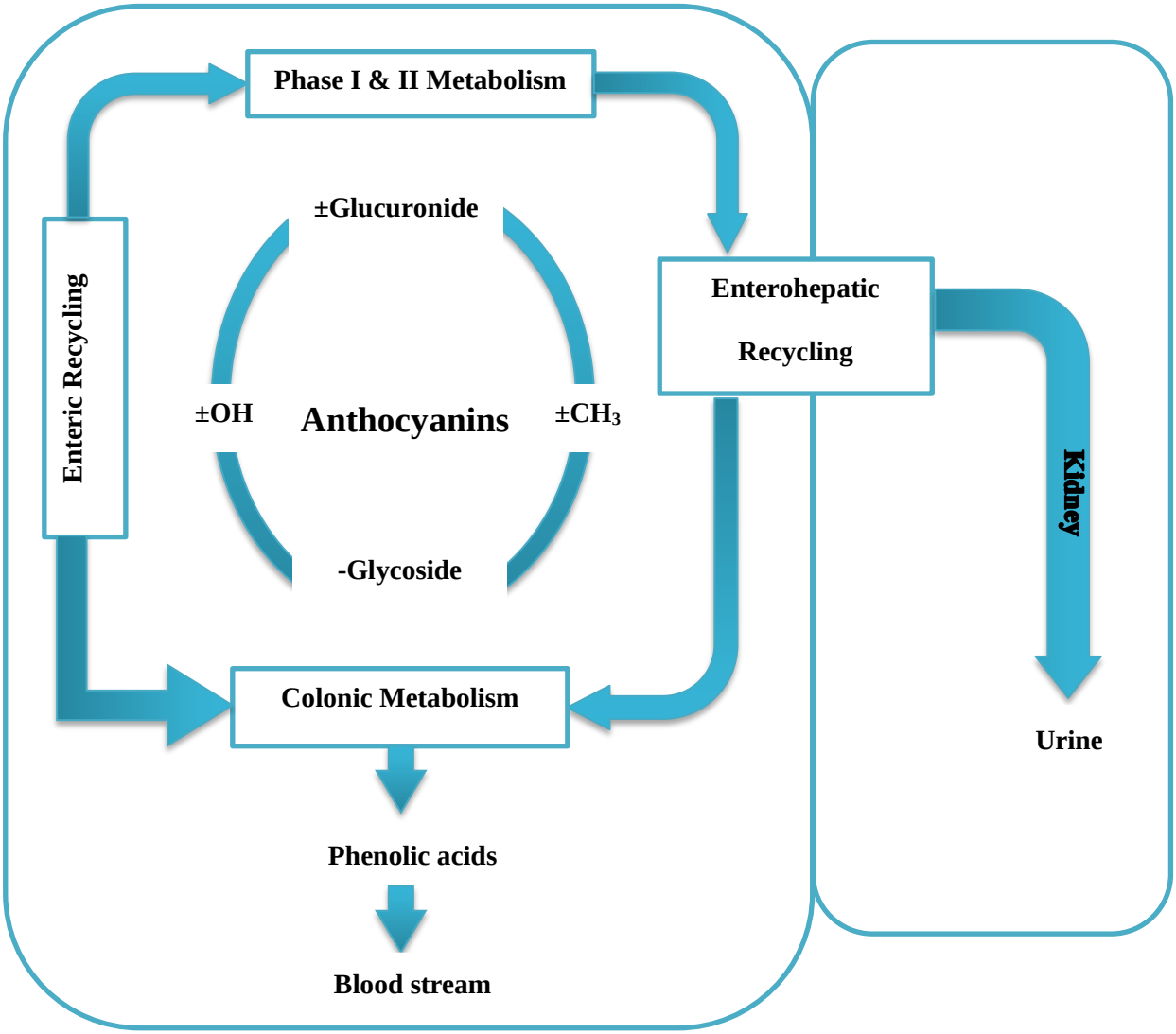


Figure 1-2: Schematic outline of anthocyanin inter-conversion in vivo due to xenobiotic and gut bacterial metabolism involving addition and removal of methyl and hydroxyl groups. Enteric and enterohepatic recycling will lead to isomeric modification before partitioning of anthocyanin metabolites into urine.

Source (Published with permission): [31].

1.1.1.3 Bioavailability

Bioavailability is defined as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action" [43]. For anthocyanins, which are provided from food and ingested orally, bioavailability would be simply defined as the amount or fraction of the ingested quantity that is absorbed.

In addition to the health benefits of dietary anthocyanins, there is also increased interest in their bioavailability. This is due to very low levels observed in the circulatory system following consumption. Studies have shown that the body treats metabolites of anthocyanins and polyphenols in general as xenobiotics (chemical or substance that is foreign to the biological system) by getting rid of them from the body system. This, aside from the issues of their low absorption, may explain the reduced rather than accumulated levels noticed in the circulatory system over time [44].

Bioavailability studies aim to determine which subclasses of dietary anthocyanins are better absorbed and which are associated with the formation of active metabolites [42]. Anthocyanins which are commonly found in some diets are usually the primary dietary phenolic components for people who regularly eat berries and drink red wine [43]. Studies on the bioavailability of anthocyanins have reported low plasma concentrations ranging from 10-50nmol/L with C_{max} mean time of 1.5h (range 0.75-4h) [45] while the C_{max} mean time for anthocyanin metabolites to appear in the urine is 2.5h (range 2-4h) [46]. The exact reason for the low bioavailability, reported as less than 1% of ingested anthocyanins, is not known [45] but low recovery in the plasma and urine has been attributed to dietary anthocyanins undergoing intensive metabolism after ingestion and the subsequent metabolites are the main transport forms *in vivo* [31, 47-49]. Taking into account the different metabolites of anthocyanins, the percentage recovery of consumed anthocyanins found in plasma and urine has been estimated as being 15% and higher [47]. Generally, endogenous factors such as; molecular weight, glycosylation, metabolic conversion and interaction with colonic microbial flora are the main factors that determine anthocyanin bioavailability[50]. Another important factor is the food-matrix. Available evidence shows that dietary fibre (such as hemicellulose), divalent minerals, and viscous and protein-rich meals have the potential to inhibit anthocyanin bioavailability while digestible

carbohydrates, dietary lipids as well as additional antioxidants able to enhance their bioavailability[50]. In the colon, intact anthocyanin compounds are broken down to smaller different metabolites, including phenolic acids and aromatic catabolites [51, 52]. The metabolism and action of anthocyanins in the colon is a bidirectional relationship with the gut microbiota able to enhance the metabolism of anthocyanins in the gut [52] and the anthocyanins able to have a proliferative/inhibitory effect on gut microbiota [53]. Available evidence indicates that colonic metabolites make up a significant portion of the overall metabolic profile following anthocyanin consumption [54, 55]. *In vitro* studies have demonstrated that these colonic metabolites exert more vascular and anti-inflammatory effects in comparison to the metabolites that are formed and absorbed in the small intestine [52]. This highlights the importance of the microbiota–gut-brain axis with evidence showing that the gut microbiota may have a significant association with mental illnesses [56]. Following the metabolism of anthocyanins in the gut, subsequent changes in the gut microbiota composition have been shown to relate with their neuroinflammatory properties [57].

1.1.1.4 Mechanism of Action

There are a number of plausible mechanisms that have been proposed to explain how anthocyanins exert beneficial effects on cognition and other health parameters. Ageing, which is associated with a reduction in the neuronal population and loss of synaptic plasticity leads to reduced cognitive function over time. Spencer (2010) [18], suggested that anthocyanins may exert protective effects on cognition through their ability to act directly (interaction with neuronal signalling and synaptic function in the brain) or indirectly (influence on blood flow and neuroinflammation [58]) on the brain's innate architecture for memory (Fig.1-3). The structural characteristics of *in vivo* metabolites of anthocyanins determine, to some extent, their access to the brain with smaller, lipid-soluble, nonionized compounds able to cross the blood-brain-barrier [59]. Hence it is unclear whether anthocyanins modulate brain plasticity directly or indirectly [58]. Interestingly, substantial evidence continues to support clinically significant beneficial effects of anthocyanins on blood pressure [55, 60, 61]. Blood pressure as part of the vascular system is therefore important for maximum functioning of the brain, as any dysfunction in this process would ultimately affect cognitive function and behaviour [53]. The presence of

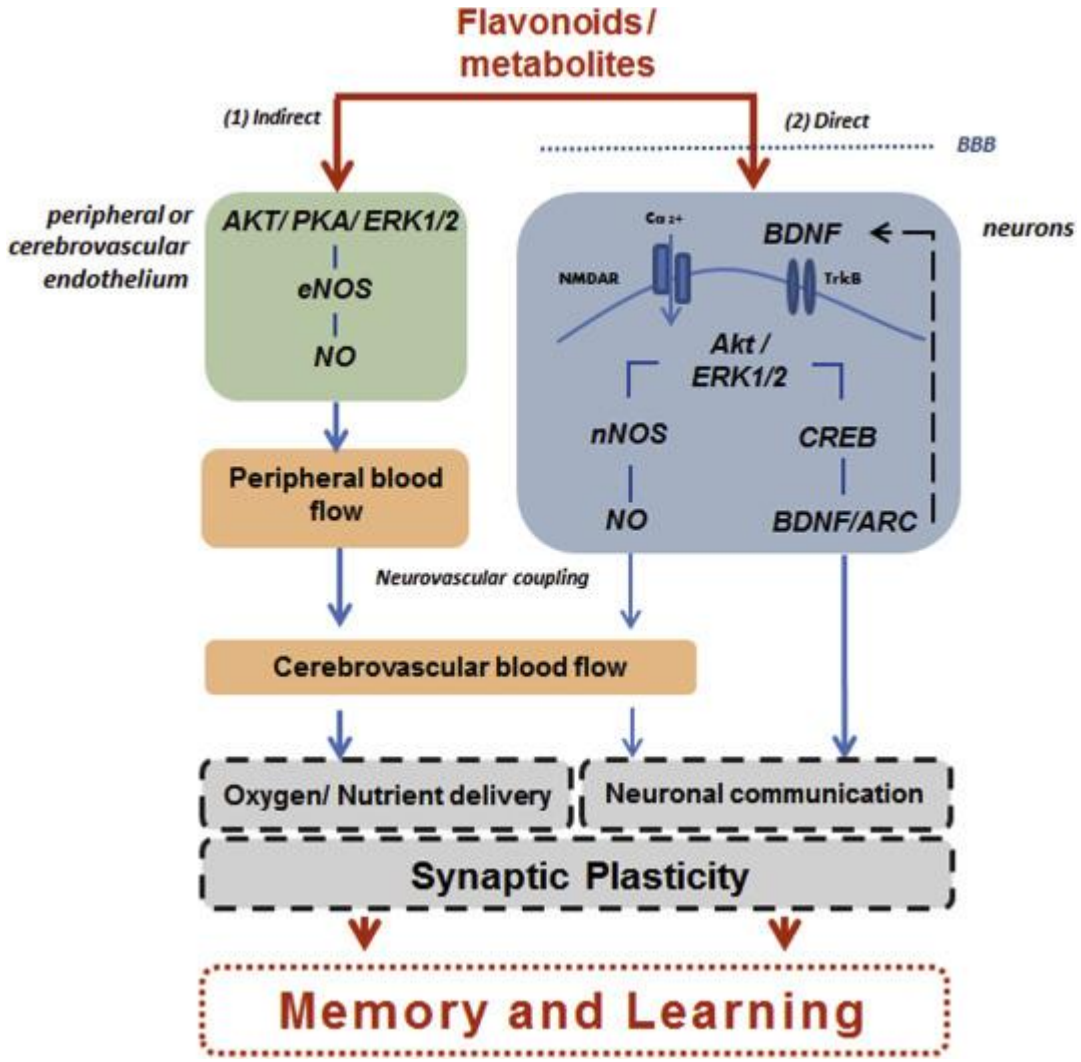
vascular risk factors, as a result of chronic inflammation, impact on brain degeneration, leading to cognitive decline [62]. For example, alterations in mean arterial pressure over time are associated with brain degeneration [62]. Prospective studies report an association between hypertension in mid-life and incidence of dementia at an older age [63] and a higher systolic and diastolic blood pressure (>160mmHg and >95mmHg, respectively) 10-15 years earlier was significantly associated with the onset of Alzheimer's disease [64]. It has been suggested that anthocyanin effects on blood pressure may be facilitated by the actions of absorbed anthocyanin metabolites on arterial nitric oxide (NO) bioavailability by either activating endothelial nitric oxide synthase (eNOS) and/or inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the endothelium [58]. This shows the significance of increase in cerebral blood flow (CRF) in the observed beneficial effects [65]. As the effectiveness of the brain function is dependent on 20% of the body's total oxygen supply and other nutrients supplied via the vascular system [66], the activity of anthocyanins on brain function is highly dependent on the ability of their metabolites to cross the blood brain barrier (BBB) and enter the brain [67]. A number of studies have observed the presence of anthocyanin metabolites in brain tissues following oral administration [68-71] . In addition, it has been suggested that anthocyanins may also exert beneficial effects on cognition and memory by activating MAP (Mitogen Activated Protein) kinases and PI3 kinase pathways in the hippocampus of aged animals [72] as well as modulating mRNA BDNF (Brain-Derived Neurotrophic Factor) levels [73].

Evidence from the last decade has shed more light on the possible mechanism of action of anthocyanins. An updated review on the health benefits of anthocyanins and their molecular mechanisms suggest that some crucial signalling pathways as well as cellular processes, such as cell cycle, apoptosis, autophagy, and biochemical metabolism, are involved in the observed beneficial health effects of anthocyanins [74]. Extensive research on the molecular mechanism of anthocyanins *in vivo* suggests that anthocyanins may have the potential to inhibit a number of signalling pathways that are involved in endothelial function, inflammatory processes as well as tumour proliferation and apoptosis. Delphinidin 3-O-glucoside and cyanidin 3-O-glucoside have been demonstrated to improve endothelial function through the activation of the NO-cGMP (Nitric Oxide Cyclic guanosine monophosphate) signalling pathway in hypercholesteraemic individuals [75]. The NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) pathway regulates inflammatory processes.

The plausible role of anthocyanins in inflammatory processes has also been studied. Anthocyanin-rich extract from wild blueberry reduces the activation of NF-kB in the presence of the pro-inflammatory stimulus IL-1b in human Caco-2 intestinal cells [76]. In addition, anthocyanins induce cell cycle arrest in colon cancer cells by increasing the expression of p21WAF1 and p27KIP1 (cyclin-dependent kinase inhibitors) and decreasing the expression of cyclin A and cyclin B (proteins that function in regulating progression through the cell cycle) [77]. The Wnt signalling pathway is crucial for the regulation of cell proliferation, differentiation, and survival. A primary component of the Wnt pathway is b-catenin (a protein that is involved in the regulation and coordination of cell–cell adhesion and gene transcription). The over activation of b-catenin in the cytosol is related to cancer metastasis, which is normally regulated by dephosphorylated glycogen synthase kinase 3b (GSK3b). Results from an *in vitro* study showed that anthocyanin extract from the Korean wild berry, Meoru, significantly inhibited liver cancer Hep3B cell migration and invasion by reducing the expression of phospho-GSK3b and b-catenin[78].

In addition, anthocyanin metabolites have become the main point of interest as recent evidence shows that anthocyanins are absorbed and transported in the body in the form of their resultant metabolites [74]. Protocatechuic acid (PCA), a major anthocyanin metabolite has been shown to significantly slow the progression of esophageal cancer in rats [79]. While other anthocyanin metabolites, gallic acid, 3-O-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde, showed a significant reduction in cell viability, cause cell cycle arrest and apoptosis in colon cancer Caco-2 cells [80], which emphasizes the potential benefits of anthocyanin metabolites *in vivo*.

With a number of plausible mechanisms that have been described here, there is still need for extensive confirmatory research on the beneficial health effects of anthocyanins and their possible mechanisms of action.



AKT-Protein kinase B (aka Akt); PKA-Protein Kinase A; ERK1/2- Extracellular **signal**-regulated kinase 1/2; eNOS-Endothelial nitric oxide synthase; NO- Nitric oxide; BBB-Blood-brain-barrier; BDNF- Brain-derived neurotrophic factor; ARC- Activity-regulated cytoskeleton; nNOS- Neuronal nitric oxide synthase; CREB- cAMP response element binding (protein); NMDAR- N-methyl-D-aspartate; TrkB- Tropomyosin receptor kinase B

Figure 1-3 Plausible mechanism of anthocyanin effect on memory and learning via direct or indirect routes
Source (Published with permission): [58]

1.1.1.5 Sources of dietary anthocyanins

Anthocyanins are commonly found in a diverse range of red, blue and purple fruits. High levels of anthocyanins have been observed in blueberries and as a result, blueberries have been the major focus of anthocyanin-based research [81]. A number of other fruits also contain similarly high levels of anthocyanins but have not been widely investigated in anthocyanin research. One such fruit is the

plum, a drupe fruit which belongs to the subgenus *prunus* (Family *Rosaceae*). The subgenus can be differentiated from other subgenera (peaches, cherries, etc.) as the shoots have a terminal bud and unclustered single side buds. The flowers combine in groups of one to five on short stems, and the fruit have a crease running down one side and a smooth seed. Between 19 and 40 different species of plum exist. Of these, only two; the hexaploid European plum (*prunus domestica)* and the diploid Japanese plum (*prunus salicina* and hybrids) are of commercial significance across the globe [82].

In the last decade, there has been an increased interest in plum-based research because of the fruit's high levels of polyphenols, and more recently identification of its high anthocyanin content [75]. The major anthocyanins found in the plum are cyanidin (3-rutinoside, 3-glucoside and 3-xyloside) and peonidin (3-rutinoside and 3-glucoside) [83]. The increased interest in plum-based research has also resulted in fruit breeders cultivating different varieties through hybridisation. One of these hybrids is the Australian Queen Garnet plum (QGP), a hybrid of the Japanese plum developed through a breeding program funded by the Queensland Government in Australia. The QGP is known for its exceptionally high anthocyanin levels, reaching up to 277 mg/100 g fruit [84]. Although fruit anthocyanin levels increase progressively during fruit development and ripening, the anthocyanin levels in the QGP is more than two times the total anthocyanin content of regular plums (range of 5 to 173mg/ 100g across harvest years for regular plums [13]) and other common fruits. Preliminary studies using this variant of plum have demonstrated antithrombotic activity in humans [85] and a beneficial effect on metabolic syndrome in rat models, as well as *in vivo* and *in vitro* bioactivity [86]. The proposed research will contribute to the current evidence on the beneficial health effects of plums in general, and for the clinical studies, the Australian QGP will be utilised for its higher levels of anthocyanins, as well as its accessibility to the research team.

1.1.1.6 Anthocyanin food levels and measurement

The ability to measure anthocyanin accurately and specifically is essential in order to investigate the association between anthocyanin intake and health outcomes. Accurate measurement of anthocyanins in food sources, as well as through dietary assessment of individual intake and biomarkers that

indicate absorption and excretion is required. In addition, no consistent biomarkers exist as the 'gold standard' proxy for dietary intake.

For the levels of anthocyanin in major food groups, a number of international databases have been developed including the American USDA Database for the Flavonoid Content of Selected Foods [87] and the European Phenol-Explorer [88]. These databases are freely accessible and usually employed globally to determine individual anthocyanin intake. The problem though is that these databases have been developed overseas and are tailored mainly to foods accessible in those regions of the world. Nutritional content of foods is known to be affected by region, climate and soil conditions [89, 90]. The ability to be able to accurately determine anthocyanin intake across various populations in different regions would involve developing food composition databases tailored to specific regions.
As part of this research, a first stage development of an anthocyanin database for Australian foods was carried out. This, together with borrowed values from the USDA Database for the Flavonoid content of selected foods and the European Phenol-Explorer, was used to measure dietary intake of anthocyanins in the Australian population.

1.1.2 Problem statement

Anthocyanin research has mainly focused on (blue) berries because of their high anthocyanin content with less focus on other anthocyanin-containing (different anthocyanin profile) fruits and vegetables which has limited research conclusions and generalisability to dietary messaging.
Further understanding of the metabolism and mechanism of action of anthocyanins and their metabolites in the body as well as how dose (timing) might affect this process and whether it is age-dependent remains to be addressed.

In addition, the absence of specific food composition databases for anthocyanins, and larger flavonoid subclasses, tailored to countries and regions to measure anthocyanin intake in epidemiological studies continues to limit research evidence and diminishes population estimates.

1.1.3 Hypothesis

Following the problem statement, this research hypothesises that supplementing the diet with plum anthocyanins will have significant positive effects on cognitive function and other health parameters in older adults, while being age- and dose-dependent.

To test this hypothesis the following objectives were developed.

1.1.4 Research Objective

To determine the acute and longer-term effects of consumption of dietary anthocyanins (delivered from plum juice) on cognition and other health parameters in older adults.

1.1.4.1 Specific Objectives:

1) To collate and synthesise the current level of evidence on the beneficial health effects of plum – an anthocyanin-rich fruit.
2) To determine if consumption of anthocyanins from QGP juice has an acute impact on various domains of cognitive functioning, blood pressure, and urinary phenolic profile.
3) To determine the longer-term effects of anthocyanin consumption from QGP juice on cognition, blood pressure, inflammatory biomarkers (BDNF, CRP) and gut microbiota in older adults.
4) To collate and synthesise the current evidence on the effects of anthocyanins on gut microbiota.
5) To determine the anthocyanin intake of older Australian adults from the National Nutrition and Physical Activity component of the Australian Health Survey 2011-13 (a nationally representative sample) in order to obtain current dietary levels of anthocyanin intake and the common food sources thereof.

1.1.5 Conceptual Framework

To test the above hypothesis, acute and longer-term effects of high anthocyanin QGP juice on cognition and other health parameters will be studied in younger and older adults. To achieve this, four studies were developed to answer the above research objectives. These studies were classified under three theoretical research frameworks (Figure 1-4); current evidence synthesis, evidence generation, and evidence translation.

With the dramatic increase in the prevalence of dementia and other CVDs, research continues to find ways to improve the overall wellbeing of the general population especially in older adults. Modifiable risk factors including nutrition have become an area of interest stem the global epidemic of chronic diseases, especially cognitive impairment. Evidence is emerging that anthocyanins, known to be natural antioxidants may exert beneficial effects on memory and cognition, as well as improve metabolic risk factors (blood pressure, insulin resistance etc.).

Two systematic reviews were conducted to synthesise existing knowledge and identify gaps in the literature on the beneficial health effects of plums and the effects of anthocyanins on gut bacteria.

Intervention studies aimed to investigate the effect of acute and chronic consumption of different doses of anthocyanin-rich QGP juice on cognition and blood pressure as main outcome measures, and other health parameters including anthocyanin metabolite excretion, inflammatory biomarkers and gut microbiota as secondary outcomes. A crossover study determined the acute effect of the high anthocyanin QGP on cognition, blood pressure and metabolism (urinary excretion of polyphenolics) in younger and older adults, while an 8-week RCT also assessed these effects, including inflammatory biomarkers and gut microbiota, over the longer term in generally healthy older adults.

The lack of an Australian food composition database related to the anthocyanin content (and flavonoids generally) of local foods hampers interpretation of food-based research. Substitution of values from the U.S. Department of Agriculture (USDA) database and/or the European Phenol-

Explorer database have been used in past studies, but this project further refined research methodology in this area by developing an anthocyanin database tailored to Australian foods (fruits

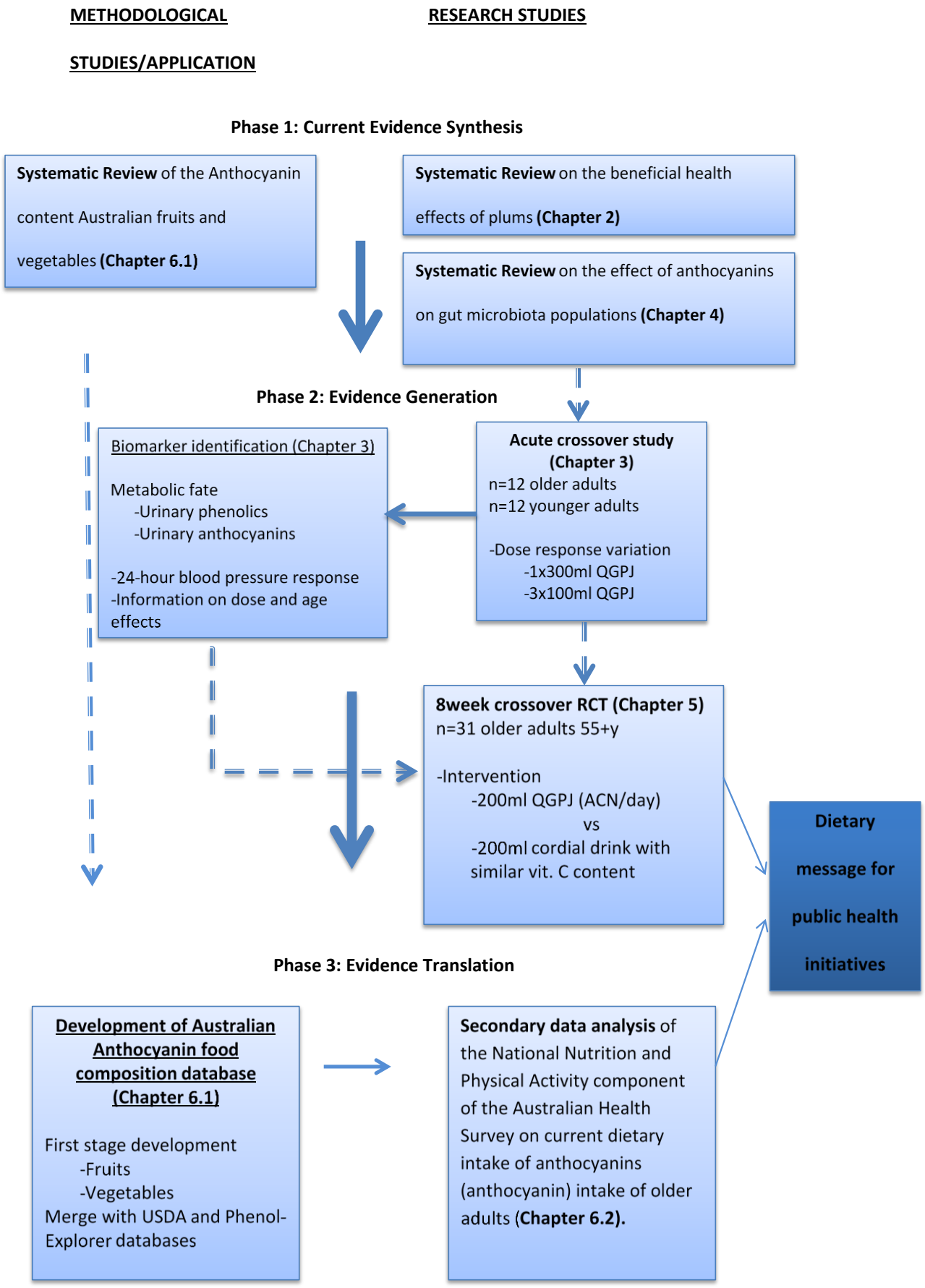


Figure 1-4: Research Conceptual framework

and vegetables as a first step). This developed database was applied to nationally representative nutrition survey data in order to determine anthocyanin intake in the Australian population. This methodological component of the project will contribute to application of clinical trials data to translation of the findings into practical dietary guidance.

1.1.6 Significance and Summary

The role of nutrition as a protective factor in chronic diseases is a promising research area; however, results have been inconclusive. It is important to establish a reliable and robust body of evidence. A review on the health effects of plum will summarise the available evidence to date and identify gaps in the published literature for future plum-based anthocyanin research.

This thesis will make a significant contribution to the area of anthocyanin research. Knowledge synthesis in the systematic reviews will identify gaps for current and future research while the proposed clinical trials would expand the clinical evidence on the health benefits of anthocyanins from a different, less popular anthocyanin source – plum juice. Additionally, differences in anthocyanin metabolism between young and older adults will be investigated in the acute trial in order to inform future clinical trials in older people who may be at an increased risk of cognitive impairment.

The development of an Australian anthocyanin database will facilitate better estimation of the current dietary anthocyanin intake of the Australian population and thereby contribute to dietary methodology. In doing so, it will address some of the gaps in the literature related to the estimation of dietary anthocyanin intake, as is required in studies to determine their beneficial health effects.

In summary, this doctoral thesis addresses significant gaps in the literature, with each study providing novel and significant contributions to the body of evidence in anthocyanin research and advances the scientific knowledge thereof.

2 Chapter 2

2.1 A SYSTEMATIC REVIEW ON THE HEALTH EFFECTS OF PLUMS (*PRUNUS DOMESTICA AND PRUNUS SALICINA)*

Plum as a source of anthocyanin has contributed to the evidence on the beneficial health effects of anthocyanins but the major focus of this bioactive component in food has been blueberries (for their significantly higher anthocyanin content). Little is known about the effectiveness of plum supplementation, as a source of anthocyanin, on health parameters. A systematic review of literature was carried out to summarise the available evidence on the impact of plums (prunus species; *domestica* and *salicina)* on disease-related risk factors and health outcomes. Findings from this review highlighted the low level of evidence for plum-anthocyanin research, with the primary focus on cognitive function in animal models, and bone health parameters in human clinical trials. This review concluded that evidence on the health effect of plums has not been extensively studied and that the available evidence needs confirmation with further well-designed studies.

The majority of this chapter forms the substantive content of a published article: Appendix C

Igwe, E.O. and Charlton, K.E., 2016. A systematic review on the health effects of plums (Prunus domestica and Prunus salicina). *Phytotherapy Research*, *30*(5), pp.701-731.

2.1.1 Introduction

The European plum (*prunus domestica)* is believed to have been discovered about 2000 years ago with its origin somewhere near the Caspian Sea. The fruit was introduced into the U.S. in the 17th century by pilgrims while the Japanese plum has its origin in China but derived its name from the country where it was mostly cultivated and developed; Japan. The Japanese plum (*prunus salicina* and hybrids) was introduced into the U.S in the late 19th century. Today, the main producers of commercially grown plums are the United States, Serbia, China, and Romania. [91]. The nutritional composition of the two species is considered similar (Table 2-1).

Prunes are the dried version of plums and are known for their laxative effect which is commonly attributed to its high fibre content [92]. Earlier studies attributed the laxative effect of prunes to the presence of phenolics (chlorogenic acid) [93] and sorbitol [94] that are in the fruit, together with its high fibre content [95]. In the U.S., prunes are referred to as dried plums. This name change was effected in a bid to promote prunes as a health food instead of being associated with old age [96]. Prunes are produced industrially by drying plums at 85-90^{o}C for 18 hours. This process is believed to have originated thousands of years ago also near the Caspian Sea, the same region where the European plums were discovered. With migration and civilisation, prunes spread throughout Europe. Today, California, USA is the leading producer of prunes (dried plums) worldwide [97].

In classifying whole and natural foods based on their unique nutritional composition, anthocyanin content has become a widely utilised method of classification [98]. The high levels of phenolic compounds including flavonoids, and particularly the subclass of anthocyanins observed in the plum has resulted in a dramatically increased interest in plum based research since the 1990s [99, 100]. A number of health benefits have been associated with the plum fruit and these include improved bone health, cognition and memory, antioxidant anti-inflammatory effects, and easement of constipation. These health promoting properties have been attributed to the plum's antioxidant capacity as a result of the high phenolic content [101-105]. These beneficial health effects have been reported from studies that have used different research designs (*in vitro,* animal studies and clinical studies) and have investigated both plums, and related products and extracts [106].

The aim of this systematic literature review was to determine the level of current evidence on the beneficial health effects of plum and its associated products.

Table 2-1: Major nutritional composition of European (*P. Domestica)* and Japanese (*P. Salicina*) plums (per 100g)

Component	European plum (*p.domestica) and* Japanese plums (*p.salicina*)		
	Fresh plums	Dried prunes	Plum juice[a]
Water/moisture (g)	87.23	30.92	84.02
Energy (kj)	192	1006	243
Carbohydrate (g)	11.42	63.88	15.15
Protein (g)	0.70	2.18	0.51
Fat (g)	0.28	0.38	0.02
Sugars, total			14.22
Glucose (g)	5.07	25.46	23.3[a]
Fructose (g)	3.07	12.45	11.8[a]
Sucrose (g)	1.57	0.15	3.7[a]
Total Dietary fibre (g)	1.4	7.1	0.9
Minerals			
Calcium (mg)	6	43	10
Iron (mg)	0.17	0.93	0.34
Magnesium (mg)	7	41	8
Phosphorus (mg)	16	69	15
Potassium (mg)	157	732	154
Sodium (mg)	0	2	1
Zinc (mg)	0.10	0.44	0.11
Copper (mg)	0.057	0.281	0.054
Manganese (mg)	0.052	0.299	0.033
Fluoride (µg)	2.0	4.0	-
Vitamins			
Ascorbic acid (C) (mg)	9.5	0.6	2.8
Thiamine (B_1) (mg)	0.028	0.051	0.023
Riboflavin (B_2) (mg)	0.026	0.186	0.059
Niacin (B_3) (mg)	0.417	1.882	0.473
Pantothenic acid (B_5) (mg)	0.135	0.422	0.072
Pyridoxine (B_6) (mg)	0.029	0.205	0.027
Total Folate (µg)	5	4	3
Vitamin A, RAE (µg)	17	39	50
Vitamin E (mg)	0.26	0.43	0.18
Vitamin K_1 (µg)	6.4	59.5	4.3
Carotenoids			
Carotene, beta (µg)	190	394	554
Carotene, alpha (µg)	0	57	0
Cryptoxanthin, beta (µg)	35	93	102
Lutein + zeaxanthin (µg)	73	148	49
Phenolic compounds[b]			
Total (mg)	111	184	121[c]
Neochlorogenic acid (mg)	81	131	198.5[a]
Chlorogenic acid (mg)	14.4	44	46.5[a]
Anthocyanins (mg)	7.6	-	0.172[c]
Catechins (mg)	5.4	-	-

Table adapted from [106] and [107], [a][106] (plum juice concentrate/100mL), [b][108], [c] [105], - No available data. Amount reported per 100g weight [109]; data are a mix of Japanese and European plums

2.1.2 Materials and methods

A systematic literature review was conducted according to the guidelines of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement and checklist [110] (Figure 2-1). A number of electronic databases were searched: Scopus, Web of Science, Cochrane library, CINAHL, MedLine, and ScienceDirect up to June 2015 with a combination of search terms, including, plum or prune or *prunus or prunus domestica or prunus salicina* and health effects used as keywords (see appendix B for sample search strategy (Medline)).

Inclusion criteria for journal articles included:

1. Studies carried out *in vitro*, on animal and clinical studies.
2. Studies that utilised the fresh, dried, juice version or extracts of the plum species *prunus domestica* or *prunus salicina.*
3. All studies assessing any health outcome associated with plum consumption/treatment.
4. Studies reported in English. Only studies reported in English were included due to language barrier, reasons of time efficiency and cost of translation not being feasible.

Exclusion criteria for journal articles included:

1. Studies on the quantification of the nutritional composition and antioxidant properties of plums.
2. Studies that utilise a different species of plums e.g. the Japanese apricot also known as Japanese plums in the *prunus mume* specie.
3. Studies assessing properties related to plum cultivation, harvest and the commercial aspects of the plum fruit.

Articles were assessed for peer-reviewed status using Ulrich's Web (available at: http://ulrichsweb.serialssolutions.com.ezproxy.uow.edu.au/). A hand search yielded one additional article which was relevant to this review.

For the clinical trials, all the relevant studies retrieved were classified as either confirmatory or exploratory studies. They were rated for their quality using relevant criteria from The Delphi List, Cochrane Back Review Group and The CONSORT Statement (Appendix A). The strength of evidence of study design was assessed using the Australian NHMRC (National Health and Medical Research Council) hierarchy levels of evidence with rankings from level I - IV. The NHMRC evidence hierarchy has 6 levels according to type of research question with systematic review of level II studies classified as levels I and randomised controlled trials, classified as level II. Studies ranging from a pseudo-randomised controlled trial to a comparative study without concurrent controls are classified as levels III-1 to III-3, and case series with either post-test or pre-test/post-test outcomes classified as level IV [111].

2.1.3 Results

A total of 73 studies were eligible for inclusion in this review (Fig.2-2), most of which were conducted in the last decade. Of these, 18 investigated bone health (2 *in vitro*, 12 animal studies and 4 human clinical trials), and 20 investigated its anti-cancer and anti-inflammatory properties (13 *in vitro* studies, 6 animal studies and 1 human clinical trial). Eleven studies reported on plums' antioxidant properties and their effect on cognition (2 *in vitro* studies, 5 animal studies and 4 human clinical trials) while nine studies investigated the effect of plums on different components of the metabolic syndrome (cholesterol, high blood pressure and anti-thrombosis; 3 animal studies and 6 human clinical trials). For prunes commonly known laxative effect and satiety, 8 clinical studies in humans, including 2 randomised clinical trials were carried out. Five studies examined its anti-allergic, anti-microbial and immune-enhancing properties (2 *in vitro* studies and 3 animal studies) and 2 clinical studies examined its effects on liver function and risk factors for kidney stone formation. Some of the findings reported from the *in vitro* studies such as parameters related to improved bone health and anti-inflammatory properties have also been confirmed in animal and human studies [112-115]. Tables 2-2 to 2-8 summarise the experimental and clinical studies and Appendix A summarises the quality of the clinical studies included in this review.

The quality of the clinical studies included is at best of moderate quality. There were 6 confirmatory studies of moderate quality (1 on bone health, 2 on different components of metabolic syndrome, and 3 on

satiety and laxative effect) and 19 exploratory studies. Evidence on the health effect of plums has not been extensively studied and the available evidence needs further confirmation.

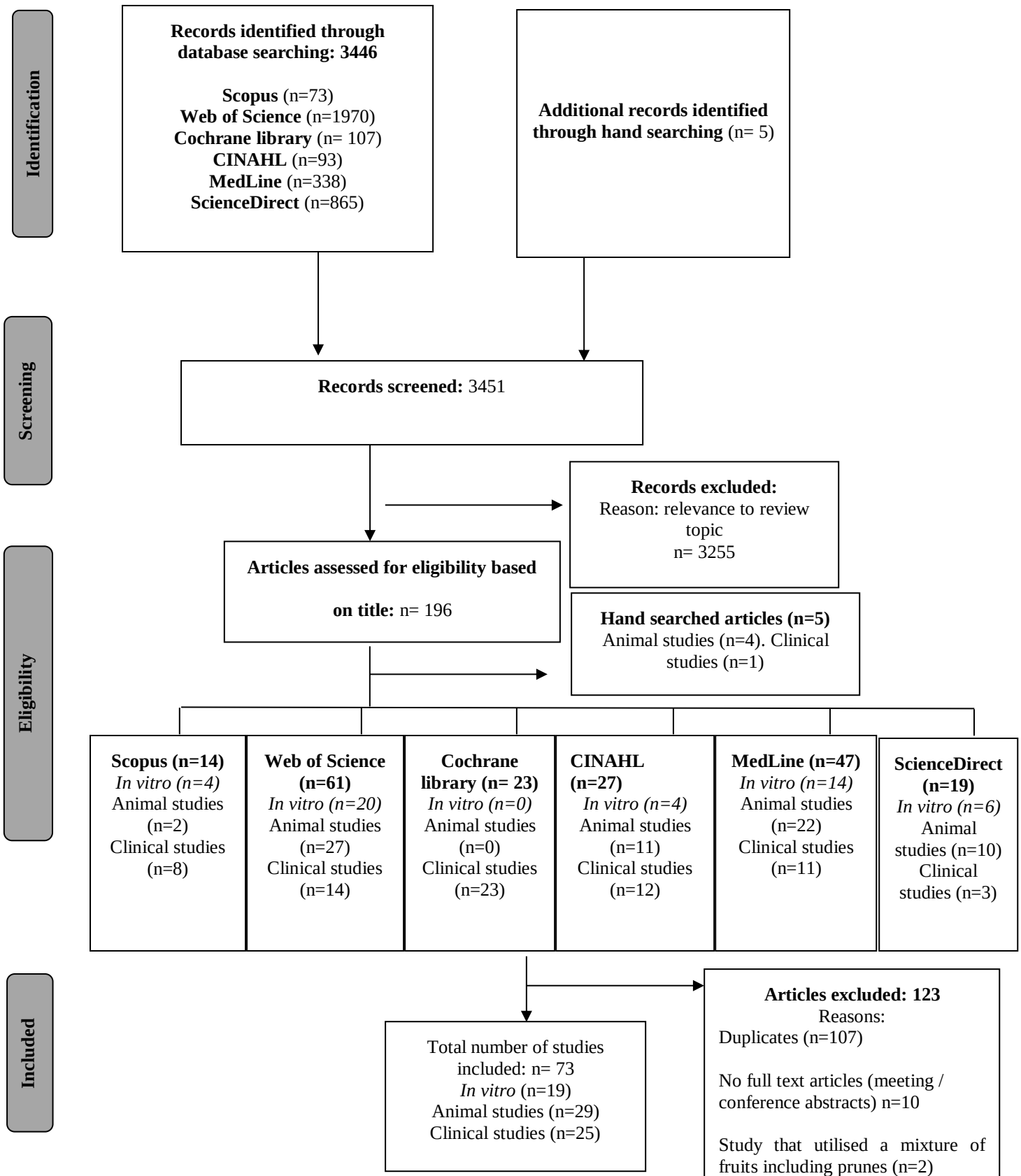


Figure 2-1: Figure 2-2 PRISMA flow diagram for study selection process

2.1.3.1 Bone health

Based on results from 18 studies (2 *in vitro,* 12 animal studies and 4 clinical trials) reported in this review, promising evidence exists on the effect of plum on bone health. This body of evidence has mostly been in agreement and also confirmed in human trials. Bu et al. (2008) [116] in their *in vitro* study involving two groups investigated the effect of dried plum polyphenols on osteoclastogenesis in which one group was stimulated with lipopolysaccharide (LPS) to induce inflammation and the other group stimulated with hydrogen peroxide (H_2O_2) to induce lipid peroxidation. It was observed that the LPS stimulated sample produced NO (nitric oxide) detectable at 8h which further increased at 16hr while the H_2O_2 stimulated cells did not produce NO. This increase in NO associated with LPS was downregulated by different doses (10, 20, 30µg/mL) of plum polyphenol at both 8h and 16h. The authors concluded that dried plum polyphenols directly inhibit osteoclastogenesis which leads to reduced osteoclast activity by downregulation of NFATc1 (Nuclear factor of activated T-cells, cytoplasmic 1) and inflammatory mediators. The results from the above *in vitro* experiment have also been confirmed in animal studies [103, 117-119]. However, one study [119] differed slightly by studying the mechanism of action of dried plum alteration of bone metabolism in comparison to the longer-term effects of dried plum on systemic biochemical markers of bone metabolism and alteration in gene expression [103, 117, 118]. Regulators of osteoblast and osteoclast differentiation and osteoblast activity were studied over a period of 6 weeks. Compared to the anabolic therapy using PTH (parathyroid hormone) that significantly increased systemic and local indicators of bone formation with no effect on systemic marker of bone resorption, dried plum supplementation suppressed bone turnover with no effect on the indices of bone formation at the endocortical surface. In another study by the same research team, dried plum supplementation initially suppressed cancellous bone turnover but demonstrated a biphasic response over time, exerting positive effects on bone mass and bone structure [120]. Plum extract has also been shown to be effective in increasing bone calcium retention by 20% [104].

Rendina et al. (2013) [112] compared dried plum to other dried fruits (apple, apricot, grape and mango) and observed that only the dried plum had an anabolic effect on trabecular bone in the vertebra and prevented bone loss in the tibia. This demonstrates a potentially unique effect of plum that was absent in the other fruits studied. This anabolic effect of dried plum supplementation has also been observed by others in animal models [113]. Prevention of age-associated bone loss was evident because of this anabolic effect,

while bone volume increased, and already lost bone was restored. Investigating further, Monsefi et al. (2013) [114] observed the effect of plum extract on bone parameters in the offspring of pregnant mice as well as in non-pregnant mice. Plum extract was orally administered to the sample population and results showed that in the non-pregnant mice, there was an increase in the femoral and tibial lengths and serum calcium content, while the foetuses and new-borns of the pregnant mice had higher osteogenesis index which was calculated by dividing the ossified length by the total length of each bone.

Ovarian hormone deficiency which is evident in postmenopausal women is a known major risk factor for osteoporosis [121]. For this reason, effect of plum consumption on bone health in human trials has been carried out mostly on postmenopausal women. A dietary supplementation trial compared the effects of consumption of dried plum (100g) with dried apple (75g) for 3 months on markers of bone turnover in postmenopausal women [115]. The difference in the amount of dried plum and dried apple compared was related to comparable quantities of energy, carbohydrates, fat and fibre, obtainable from 100 g of dried plum. Baseline and post-treatment values of serum and urinary biochemical markers of bone status showed that only dried plums significantly increased serum levels of insulin-like growth factor-1 (IGF-1) and bone specific alkaline phosphatase (BSAP) activity.

A similar longer term randomised controlled study compared the effects of dried plum and dried apple on osteopenic postmenopausal women for one year [122]. In addition to similar results in [115], the authors observed that dried plum significantly increased bone mineral density (BMD) of the ulna and spine.
In a slightly different study examining the effects of resistance training and dried plum consumption on strength, body composition, blood markers of bone, and inflammation in breast cancer survivors, results showed that even though breast cancer survivors increased upper and lower body strength, no improvements were observed in their body composition and Bone Mineral Density (BMD)[123].

Table 2-2: Evidence on the effect of plums and its associated products on bone health

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
***In vitro* studies**					
Bu et al., 2008 [116]	USA	Dried plum extract (*P.Domestica)*	RAW 264.7 murine macrophage cells.	NA	Inhibition of osteoclastogenesis under inflammatory and oxidative stress conditions possibly by polyphenol content.
Bu et al., 2009 [124]	USA	Polyphenols extracted from dried plum (*P.Domestica)*	MC3T3-E1 cells pre-treated with dried plum polyphenols (0, 2.5, 5, 10 and 20 μg/mL) and 24 h later stimulated with TNF-α (0 or 1.0 ng/mL).	NA	Improvement of osteoblast activity and function by up-regulating Runx2, Osterix and IGF-I and increasing lysyl oxidase expression, and reduction in osteoclastogenesis signalling.
Animal studies					
Smith et al., 2014a [119]	USA	Dried plum (P. Domestica)	6wk dietary supplementation of dried plum (5%, 15% or 25%) in adult, osteopenic ovariectomized rats.	NA	Restoration of bone Mineral Density by 2 higher doses and bone turnover suppression.
Deyhim et al., 2005 [117]	USA	Dried plum (*P. Domestica)*	Dietary supplementation of dried plum (5%, 15% and 25%) in adult, osteopenic ovariectomized (OVX) rats for 40days.	NA	Bone quality improvement (restoring bone density) with all doses.
Franklin et al., 2006 [103]	USA	Dried plum (*P. Domestica)*	Dietary supplementation of dried plum (5%, 15% and 25%) in orchidectomized rats for 90days.	NA	Prevention of osteopenia in androgen deficient male rats.
Bu et al., 2007 [118]	USA	Dried plum (*P. Domestica)*	Dietary supplementation of dried plum (25%) in osteopenic orchidectomized rats for 90days.	NA	Reversion of bone loss due to orchidectomy.
Smith et al., 2014b [120]	USA	Dried plum (*P. Domestica)*	Dietary supplementation of dried plum (25%) in adult mice for 4 or 12 weeks.	NA	Improvement in bone mass and structure.
Rendina et al., 2013 [112]	USA	Dried plum (*P. Domestica)*	8wk dietary supplementation of dried plum (25%) in adult, osteopenic ovariectomized mice.	NA	Bone loss prevention with anabolic effect.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
Halloran et al., 2010 [113]	USA	Dried plum (*P.Domestica)*	Dietary supplementation of dried plum (15% or 25%) in adult and aged (old) male mice for 6 months.	NA	Restoration of lost bone and increase in bone volume.
Rendina et al., 2012 [125]	USA	Dried plum (*P.Domestica)*	Dietary supplementation of dried plum (5%, 15% or 25%) in ovariectomized adult mice for 4 weeks.	NA	Improvement of bone structure and biomechanical properties and suppression of lymphocyte TNF-α production by higher doses.
Pawlowski et al., 2014 [104]	USA	Dried plum powder extract (*P.Domestica)*	Dietary supplementation of plum extract (9% or 20%) in ovariectomized rats for 6 intervention (10days) and washout (10 days) cycles.	NA	Improvement in bone calcium retention.
Monsefi et al., 2013 [114]	Iran	Plum extract (*P.Domestica)*	Oral administration of plum extract (1.6 g/kg) in distilled water in pregnant mice for 30 days.	NA	Increased osteogenesis index in foetuses of mice treated with plum extract.
Arjmandi et al., 2010 [126]	USA	Dried plum (*P.Domestica)*	180 3-month-old female Sprague-Dawley rats assigned to 15 groups (n=12) and either ovariectomized (14 groups) or sham-operated (Sham, one group) then placed on different dietary treatments including one supplemented with 5% Fructooligosaccharides (FOS) and 7.5% dried plum (DP) for 60 days.	NA	Diets supplemented with 5% FOS and 7.5% DP was most effective in reversing both right femur and fourth lumbar bone mineral density and fourth lumbar calcium loss while significantly decreasing trabecular separation.
Johnson et al., 2011 [127]	USA	Dried plum (*P.Domestica)*	72 3-month-old female Sprague-Dawley rats assigned to 6 groups (n=12/group) and either ovariectomized (5 groups) or sham-operated (Sham, one group) then placed on a semi purified, powdered casein-based diet for 45 days to induce bone loss. Thereafter the groups were placed on different dietary treatments including one supplemented with 5% Fructooligosaccharides (FOS) and 7.5% dried	NA	In combination with soy protein, dried plum and fructooligosaccharides had the most pronounced effect in increasing lumbar bone mineral density.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
			plum (DP) for 60 days.		
Clinical trials					
Hooshmand et al., 2011 [122]	USA	Dried plum (*P.Domestica)*	**Hypothesis**: Dried plums reverses bone loss in osteopenic postmenopausal women. **n**=160 osteopenic postmenopausal women **Study design/Methods**: Randomized controlled trial. Postmenopausal women randomly assigned to treatment groups of dried plum (100g/d) or dried apple (75g) daily for 1 year. Blood and urine samples collected. **Sample size power:** not stated. **Dose:** 100g/day. **Duration:** 1 year.	II Confirmatory	Statistically significant increase in bone mineral density of ulna and spine with decreased serum levels in bone turnover markers (bone-specific alkaline phosphatase and tartrate-resistant acid phosphatase-5b) observed.
Arjmandi et al., 2002 [115]	USA	Dried plum (*P.Domestica)*	**Hypothesis**: Addition of dried plums to the diets of postmenopausal women would positively influence markers of bone turnover. **n**=58 postmenopausal women. **Study design/Methods**: Randomized controlled trial. Postmenopausal women randomly assigned to treatment groups of dried plum (100g/d) or dried apple (75g) daily for 3 months. Blood and urine samples collected. **Sample size power:** not stated **Dose:** 100g/day. **Duration:** 3 months.	II Exploratory	Statistically significant increase in serum levels of insulin-like growth factor-1 (IGF-1) and bone-specific alkaline phosphatase associated with increased rates of bone formation.
Hooshmand et al., 2014 [128]	USA	Dried plum (*P.Domestica)*	**Hypothesis**: Dried plum has an effect on circulating levels of sclerostin and bone metabolism measured in serum levels of RANKL (receptor activator of NF-$_k$B ligand) and OPG (osteopotegerin). **n**=160 women with mild bone loss. **Study design/Methods**: RCT. Subjects randomly assigned to one of 2 groups of dried	II Exploratory	Increase in bone mineral density of the ulna and spine and also the RANKL and OPG levels. A reduction in in serum sclerostin was also observed.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
			plum or dried apple and provided with 500mg Caplus 400 IU (10µg) vitamin D. **Sample size power:** not stated. **Dose:** 100g dried plum per day. **Duration:** 1 year.		
Simonavice et al., 2014 [123]	USA	Dried plum (*P.Domestica)*	**Hypothesis**: 6-month intervention with resistance training (RT) and a combination of resistance training and dried plum (DP) would improve total and regional (lumbar spine, femur, and forearm) BMD. Increase lean body mass, skeletal muscular strength and decrease fat body mass. Additionally, it was hypothesized that the biochemical analyses for both groups would reveal increased levels of bone formation markers, decreased levels of bone resorption markers, and decreased levels of inflammation markers, with the RT+DP group having the most improvements in these areas in breast cancer survivors. **n**=23 female breast cancer survivors. **Study design/Methods**: Case-control. Subjects stratified into 1(RT) or 2(RT+DP) treatment groups. **Sample size power:** not stated **Dose:** 90g dried plum per day **Duration:** 6 months.	II Exploratory	No difference between groups or any group-by-time interaction observed for any of the variables.

*Only clinical trials were ranked for level of evidence

2.1.3.2 Antioxidant and anti-inflammatory activity

The antioxidant property of plums has mostly been attributed to its high phenolic content [129, 130]. Research on this health effect has mostly been carried out with the ripe plum fruit or its products. However, evidence has shown that immature plums may contain higher levels of polyphenols with more significant antimicrobial, antioxidant, and antitumor properties in comparison to mature plums [131]. Studying the antioxidant effect of immature plum extract (IPE) on selected cancer cells *in vitro*, it was observed that even though the IPE was effective in inhibiting growth of the cancer cells (Human hepatocellular carcinoma HepG2 cells, Kato III gastric cancer cells, HeLa human cervical carcinoma cells, U937 leukaemia cells, and MCF 7 hormone-dependent breast cancer cells). This inhibitory effect was not observed in the hormone dependent breast cancer cells and as the fruit ripened, there was a reduction in its inhibitory effect [131]. Another study [102] aimed to identify the phenolic fraction responsible for the potential chemo-preventive and/or chemotherapeutic action in plum. The authors observed that all extract fractions were effective in exerting antioxidant effect on studied cancer cell lines, with the flavonols and procyanidins more effective than the phenolic acids and anthocyanins. In addition, a similar study suggested that the synergistic effect of the total phenolic content of the plum extract significantly increased its antioxidant activity [130].

Investigating this antioxidant effect on human colon cancer cells using prune [132] and plum extract [130], there was no significant reduction in the viable cell number of the human normal colon fibroblast cells while inducing apoptosis of cancer cells. Similar results were also observed with breast cancer cell lines [102]. These results have also been confirmed in animal studies with immature plum extract (IPE) inhibiting the growth of hepatoma HepG2 cells as well as exerting a protective effect against benzo(α)pyrene induced liver toxicity by decreasing serum aminotransferase and hepatic contents of lipid peroxide [133]. In addition, an anti-ulcer effect has also been observed in Wistar albino rats. Following a 7-day feed with 100, 150 or 200mg kg^{-1} of plum extract, peptic ulcer was induced by pyloric ligation. Results from gastric ulcerative index estimation showed that the group pre-treated with plum extract had a significantly reduced gastric volume and a significantly lower ulcerative index [134]. This anti-ulcer effect has also been observed in an acute setting following oral treatments including prune polysaccharides which showed a reduction and inhibition of the gastric lesion area. [135]. Further investigation on the effect of

plum on colon cancer risk factors showed that even though dietary supplementation with dried plum showed no inhibitory effect on aberrant crypt foci formation at the initiation stage of cancer and early progression, it was able to inhibit several risk factors associated with colon carcinogenesis. These include reduction in faecal total and secondary bile acid concentration, decrease in colonic β-glucuronidase and 7α-dehydroxylase activities and increased antioxidant activities [136].

Even though similar results have been observed in *in vitro* and animal studies, results from human trials have not been in agreement. A study that investigated the plasma antioxidant capacity changes after a meal observed that consumption of a meal containing dried plum or dried plum juice did not alter plasma antioxidant capacity (hydrophilic and lipophilic $ORAC_{FL}$) [137]. Contrary to this, another study observed that 9 different fruit juices, including plum juice, exhibited significant antioxidant effects in human plasma within 30 mins of consumption by suppressing reactive oxygen species generation [129]. Similar effects were also observed in young, middle-aged and elderly adults after consumption of 195g of plum twice a day for 5 days [138] and QGP juice in an acute setting [107].

Table 2-3: Evidence on the anti-cancerous and anti-inflammatory properties of plums and its associated products

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
In vitro studies					
Yu et al., 2009 [101]	Korea	Immature Plum extract (IPE) *(P.Salicina)*	Human hepatocellular carcinoma HepG2 cells, Kato III gastric cancer cells, HeLa human cervical carcinoma cells, U937 leukaemia cells, and MCF 7 hormone-dependent breast cancer cells.	NA	Cell growth inhibition by IPE i.e. induction of cancerous cell apoptosis.
Noratto et al., 2009 [102]	USA	Mature red-fleshed plum extract (*P. Domestica)*	MCF-7; the estrogen-positive human breast cancer cell line, MDA-MB-453; the estrogen negative human breast cancer cell line, and MCF-10A; the breast epithelial cells.	NA	Inhibition of breast cancer cell proliferation and significantly reduced toxicity on the normal cells.
Lee et al., 2009 [139]	USA	Plum extract *(P.Salicina)*	Chicken spleen, RP9 tumour cells and HD11 macrophages.	NA	Stimulation of spleen lymphocyte proliferation and NO production by cultured macrophages and inhibition of tumour cell growth.
Fujii et al., 2006 [132]	Japan	Prune extract (*P. Domestica)*	Caco-2 human colon carcinoma cell line, KATO III human stomach carcinoma cell line and CCD-18Co normal human colon fibroblast cell line	NA	Induction of cell apoptosis of cancer cells but not normal cells.
Lea et al., 2008 [130]	USA	Plum extract (*P. Domestica)*	SW1116, HT29, Caco-2 human colon cancer cells and NCM460 human colon cells.	NA	Growth inhibition and induction of differentiation on colon cancer cells.
Hooshmand et al., 2015 [140]	USA	Dried Plum polyphenol extract (*P. Domestica)*	Stimulation of macrophage RAW 264.7 cells with either 1µg mL^{-1} (for measurement of NO production) or 1ng mL^{-1} (for measurement of COX-2 expression) of lipopolysaccharide (LPS) to induce inflammation and treated with different doses of dried plum polyphenols.	NA	Reduction in Nitric oxide and malondialdehyde production with highest dose treatment (1000 µg mL^{-1}). Reduction in LPS-induced expression of COX-2 by the 100 and 1000 µg mL^{-1} dose.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
Kim et al., 2003 [141]	USA	Plum polyphenol extract (*P. Domestica)*	Treatment of two cancer cell lines (HepG2 human liver cancer cells and DLD1 human colon cancer cells) with polyphenol extract of plum.	NA	Antiproliferative activities on both cancer cell lines in a dose dependent manner.
Nishida et al., 2014 [142]	Japan	Plum pectin extract (*P. Domestica)*	Incubation of heparan sulfate in differentiated Caco-2 cells with pectin extracted from plums.	NA	There was an obvious change in the sulphated structures of HS following pectin administration. Also, pectin upregulated human HS 6-O-endosulfatase-2 (HSulf-2) expression and inhibited HSulf-1 expression.
Nishida et al., 2015 [143]	Japan	Plum pectin extract (*P. Domestica)*	Incubation of differentiated Caco-2 cells (cultured in 6-well plates at a cell density of $1.0x10^5$ cells/well), with pectin, extracted from plums.	NA	Pectin-treated differentiated Caco-2 cells promoted growth of IEC-6 cells and also an upregulation of relative mRNA and protein expression levels of Wnt3a protein.
Popov et al., 2014 [144]	Russia	Plum pectic polysaccharide extract (*P. Domestica)*	0.05mL of plum pectic polysaccharide added to peritoneal cell suspension and incubated in a 96-well flat-bottom tissue culture plate in the absence or presence of phorbol-12-myristate-13-acetate at 37^{o}C for 15 mins.	NA	Reduction in the adhesion of peritoneal leukocytes. Inhibition of the production of superoxide anion radicals by reducing xanthine oxidase activity.
Vizzotto et al., 2014 [145]	USA	Plum polyphenol extract (*P. Domestica)*	Oestrogen independent MDA-MB-435, estrogen dependent MCF-7 breast cancer cell lines and one non-cancerous breast line MCF-10A exposed to varying concentrations of plum extracts for 24 hours.	NA	Dose-dependent cytotoxic effect against MDA-MB-435, weak activity against MCF-7 and small or no activity against MCF-10A observed.
Yu et al., 2007 [131]	Korea	Immature, mid-mature and mature plum extract *(P.Salicina)*	6 human cell cancer lines (Hep G2 human hepatocellular carcinoma cells and Kato 111 human gastric carcinoma cells, Hela human cervical carcinoma cells, U937 human leukemia cells, MCF 7 hormone-dependent human breast cancer cells, and MDA-MB-231	NA	Cytotoxic effects observed, and apoptosis observed in MDA-MB-231 cells mediated by the immature plum extract.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
			hormone-independent human breast cancer cells) incubated with varying concentration of plum extract.		
Yu et al., 2009b [146]	Korea	Immature plum extract *(P.Salicina)*	Incubation of PMA-induced HepG2 human hepatocellular carcinoma cells with Immature plum extract.	NA	Antimigrative property in (phorbol 12-myristate 13-acetate) PMA-induced HepG2 cells observed. A strong inhibitory effect on the PMA-induced MMP-9 secretion through suppression of the transcriptional activity of the MMP-9 gene independently of the TIMP gene in HepG2 cells was also observed.
Animal Studies Kim et al., 2008 [133]	Korea	Immature plum extract (IPE) *(P.Salicina)*	Intraperitoneal injection of IPE (2.5 or 5 g/kg bw/day) dissolved in phosphate buffered saline for 5 days in male mice with benzo(α)pyrene induced liver toxicity.	NA	Chemopreventive efficacy by inhibiting the induction of CYP1A1 expression and reducing the activity of glutathione peroxidase, superoxide dismutase and catalase.
Cantu-Jungles et al., 2014 [135]	Brazil	Polysaccharides from prunes (*P. Domestica)*	Inducement of acute gastric ulcer in rats using intragastric administration of ethanol P.A. after 4 different oral treatments including polysaccharides from prunes fraction (3 and 10 mg/kg).	NA	Reduction and inhibition of gastric lesion area by prune polysaccharides fractions.
Noratto et al., 2015 [147]	USA	Plum juice *(P.Salicina)*	Administration of plum juice in drinking water to obese Zucker rats ad libitum for 11 weeks.	NA	Antiadipogenic and anti-inflammatory effects. Reduction in blood glucose, triglycerides and HDL cholesterol levels.
Mishra et al., 2012 [134]	India	Plum extract (*P. Domestica)*	Inducement of peptic ulcer by pyloric ligation in Wistar albino rats after administration of plum extracts (100, 150 or 200mgkg^{-1}) for 7 days.	NA	Antioxidant and anti-ulcerogenic activity.
Yang and Gallaher,	USA	Dried plum (*P. Domestica)*	Dietary supplementation of dried plum (4.75 or 9.5%) in male Wistar rats for 10 days	NA	Inhibition of risk factors associated with colon carcinogenesis (reduction in faecal

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
2005 [136]			followed by administration of 2 doses (1wk apart) of azoxymethane and dietary supplementation for 9 more weeks.		total and secondary bile acids concentration, reduction in colonic β-glucuronidase and 7α-dehydroxylase activities and increased antioxidant activity).
Gallaher and Gallaher, 2009 [148]	USA	Dried plum (*P. Domestica)*	Dietary supplementation of dried plum (4.75 or 9.5%) and cholesterol in apolipoprotein E-deficient mice for 5months.		Development of atherosclerosis Impeded.
Clinical trial					
Kasim-Karakas et al., 2002 [149]	USA	Dried plum (*P. Domestica)*	**Hypothesis**: Prune intake may alter the metabolism of estrogens because prunes are a rich source of both soluble and insoluble fibre and cinnamates and decrease intestinal transit time. **n**=19 healthy premenopausal women **Study design/Methods**: Crossover study. After consuming habitual diets for 3 menstrual cycles (control run-in period), participants replaced dietary simple sugars with prunes for another 3 menstrual cycles (intervention period). **Sample size power:** not stated **Dose:** 100g dried plum per day **Duration:** 6 months.	III-2 Exploratory	Decrease in the independent excretion of 2OHE1 and 16αOHE1 observed but not statistically significant change in the 2OHE1 - 16αOHE1 ratio following prune supplementation.

*Only clinical trials were ranked for level of evidence

2.1.3.3 Cognitive improvement

Cognitive improvement associated with consumption of plum has not been extensively studied in humans. Most of the available evidence is from animal studies. This effect on cognition has mainly been attributed to the antioxidant property of plums as a result its high polyphenolic content. In a study in which four groups of mice were fed a high cholesterol diet, with either 2% or 5% plum powder supplementation, a significant difference was observed in the time taken to complete the Morris water maze task between the group fed just the high cholesterol diet and the control group, as well as both the 5% and 2% plum powder supplementation groups [150]. A similar study reported slightly contradictory results when comparing the effects of 100% plum juice and 2% dried plum powder supplementation to modify age-related deficits in cognitive function in aged rats. It was observed that there was an improvement in cognition with the plum juice, but not with the dried plum powder [105].

Using plum extract to supplement the diets of three groups of mice with different doses (75, 100, 150mg/kg), there was a statistically significant difference in the number of trials to acquisition in the passive avoidance test (evaluates learning and memory) between the control group and the plum extract treated groups. The retention test also showed that the treated groups had increased STLr (step through latency in the retention) in comparison to the control group [151]. These results are in line with similar animal studies [152, 153] that demonstrate a beneficial health effect of plum on cognition.

Examining the effect of chlorogenic acid from plums on anxiety-related behaviours in mice using the light/dark test, the elevated plus maze and the free exploratory test, results showed a decrease in anxiety related behaviours (anxiolytic-like effect) and protection of granulocytes (white blood cells) from oxidative stress. Plum consumption protected against oxidative stress induced by radiation with special attention to spatial learning [154]. A different study observed that plum possesses prophylactic ability against radiation-induced metabolic disorders, and its consumption also improved spatial learning. Exposed mice that had received plum performed better by taking less time to reach the coloured platform in the circular water tank apparatus (proxy for spatial learning and memory) [152]. Similarly, the effect of plum consumption for 2 months on cognitive performance and expression of cerebral neurodegeneration-related

protein in streptozotocin-induced diabetic rats has also been studied. Cognitive performance, assessed using the Morris water maze, showed that the plum supplemented diet had a significant beneficial effect on spatial memory and learning. There was also a significant reduction in expression of cerebral beta-amyloid which is evident in Alzheimer's disease. Significant decreases in hyperglycaemia, insulin resistance and oxidative stress in the sample of rats were also observed [153].

Table 2-4: Evidence on the antioxidant property and effect on cognition of plums and its associated products

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
***In vitro* studies**					
Bouayed et al., 2009 [155]	France	Phenolics from plums (*prunus domestica)*	Polyphenolics extracted from 7 varieties of plum and quantified. Their antiradical activities and protection against oxidative stress evaluated in peripheral blood granulocytes.	NA	Antioxidant activity and protection of blood granulocytes from H_2O_2-induced oxidative stress by preventing granulocytes from intracellular ROS accumulation.
Donovan et al., 1998 [156]	USA	Prune/ prune juice extract (*prunus domestica)*	Human LDL from plasma prepared from blood collected from healthy volunteers.	NA	Inhibition of LDL oxidation.
Animal Studies					
Shukitt-Hale et al., 2009 [105]	USA	Plum juice/dried plum powder (*P. Domestica)*	Two groups of aged rats with either consumption of a mixture of water and plum juice (100%) (Group 1) or dietary supplementation with dried plum powder (2%) (group 2) for 8 weeks	NA	Improved cognitive function assessed by Morris Water Maze.
(Shahidi et al., 2013 [151]	Iran	Plum extract (*P. Domestica)*	Plum extracts (75, 100, 150 mg/kg) administration by oral gavage to male mice once a day for 7 days	NA	Improvement in learning and memory in mice assessed by the passive avoidance test.
Kao-Ting et al., 2013 [153]	Taiwan	Dried plum powder *(P.Salicina)*	Dietary supplementation of dried plum powder (2%) in nicotinamide/streptozotocin-induced diabetic rats for 2 months	NA	Improvement in cognitive performance, antioxidant activity and improvement in insulin sensitivity.
Sharma and Sisodia, 2013 [152]	India	Plum extract (*P. Domestica)*	Administration of optimum dose of plum extract in distilled water to mice for 15 days pre/post whole-body exposure to 10 Gy gamma-radiations	NA	Antioxidant capabilities and improved spatial learning.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
Bouayed et al., 2007 [154]	France	Chlorogenic acid from *prunus domestica*	Administration of chlorogenic acid (20mg/kg) to mice and antioxidant effect on peripheral blood granulocytes.	NA	Decrease in anxiety related behaviours (anxiolytic-like effect) and protection of granulocytes from oxidative stress by chlorogenic acid *in vitro.*
Clinical trials					
Prior et al., 2007 [137]	USA	Dried plum/dried plum juice (*P. Domestica)*	**Hypothesis**: Changes in AOC following consumption of plum juice may be used to assess its potential to alter *in vivo* antioxidant status and provide estimates of dietary antioxidants necessary to prevent postprandial oxidative stress. **n**=6 healthy volunteers. **Study design/Methods**: Randomized cross-over study. Fasting blood sample collected, and participants fed test juices and blood samples collected at 1, 2, and 4 h post juice consumption. **Sample size power:** not stated **Dose:** 315 mL of dried plum juice (DPJ); or dried plums (DP) (131 g blended in 315 mL water **Duration:** 2 weeks (with 2 weeks washout period)	II Exploratory	No effect on plasma hydrophilic (H-) or lipophilic (L-) antioxidant capacity measured as Oxygen Radical Absorbance Capacity ($ORAC_{FL}$).
Ko et al., 2005 [129]	Korea	Plum juice *(P.Salicina)*	**Hypothesis**: Consumption of fruit juices could scavenge ROS generated in human plasma. **n**=10 healthy men. **Study design/Methods**: Cross-over study. Consumption of single dose of 150mL of plum juice. Blood samples collected at 0, 30, 60, 90, and 120 minutes after consumption **Sample size power:** not stated **Dose:** A single dose of 150mL **Duration:** 1 day (with 1-day washout	IV Exploratory	Improved antioxidant activity in human plasma measured by dichlorofluorescein (DCF) fluorescence.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
			period)		
González-Flores et al., 2011 [138]	Spain	Plum *(P.Salicina)*	**Hypothesis**: There is a possible antioxidant effect associated with diets enriched with Japanese plums (*Prunus salicina* Lindl. cv. Crimson Globe) in young, middle-aged and elderly individuals. **n**=18(6 per young, middle aged and older group). **Study design/Methods**: Consumption of plums (390 g/d) divided into two portions: lunch dessert (195g) and dinner dessert (195g) for 5 days. First-void morning urines were collected before treatment (basal values), the immediate day after the last ingestion of plums (assay) and 1 day afterwards (post-assay). **Sample size power:** not stated **Dose:** 2 portions 390g (195g each) daily. **Duration:** 5days.	IV Exploratory	Statistically significant increase in antioxidant capacity and urinary 6-sulfatoxymelatonin (aMT6-s).
Netzel et al., 2012) [107]	Australia	Plum juice *(P.Salicina)*	**Hypothesis**: There is a possible antioxidant effect associated with QGPJ ingestion on the urinary antioxidant capacity and the concentration of malondialdehyde, a biomarker for oxidative stress. **n=**2 healthy male subjects **Study design/Methods**: Crossover study. Consumption of 400 mL of QGPJ or 400 mL of water **Sample size power:** not stated **Dose:** single dose of 400mL. **Duration:** 1 week (1week washout period).	IV Exploratory	Increase in urinary antioxidant capacity and decrease in malondialdehyde excretion (biomarker for oxidative stress).

2.1.3.4 Cardiovascular disease risk factors

Insulin resistance, a major risk factor for metabolic syndrome and selected cancers presents a major public health concern [157]. Studying the beneficial health effects of plums on CVD risk factors, Noratto et al. [147] compared the effect of plum juice with peach juice and a placebo group which received the same amount of sugar in either peach or plum juice in obese Zucker rats. Their results showed that the plum juice group had the lowest weight gain and also that plum polyphenols exerted the highest anti-adipogenic and anti-inflammatory effects in fat tissues. This provides evidence of the reduction of mRNA (messenger Ribonucleic acid) levels of PPAR (peroxisome proliferator activated receptor) associated with plum intake.

Negishi et al. (2007) [158] studied the effect of prune extract on blood pressure elevation in stroke-prone spontaneously hypertensive rats for 5 weeks. They observed that prune extract supplementation in diet suppressed the elevation of systolic blood pressure but not diastolic blood pressure. A study by Gallaher and Gallaher (2009) [148] on apoE-deficient mice, known to be susceptible to rapid development of atherosclerotic lesions when fed cholesterol, investigated the ability of dried plum supplementation to reduce atherosclerosis. The percentage arterial tree atherosclerotic lesion area was significantly lower in groups fed the dried plum supplemented diet (4.75%), either with or without cholesterol, compared to the group fed cholesterol without dried plum supplementation.

In human trials, results have been inconclusive. Chai et al. (2012) [159] studied the effect of daily dried plum consumption in comparison to dried apple on cardiovascular disease risk factors in postmenopausal women over a 1-year period. Results showed that serum total cholesterol levels were significantly lower in the dried apple group in comparison to the dried plum group only at 6 months. There was also a cholesterol-lowering effect for serum total and low-density lipoprotein cholesterol at 12 months, but this was not significant. Neither dried apple nor dried plum had a significant effect on the serum levels of atherogenic cholesterol. Contrary to this observation, Tinker et al. (1991) [92] observed that in adult men with mild hypercholesterolemia, supplementation with prunes significantly lowered plasma low-density lipoprotein cholesterol compared to a grape juice control group. A significantly lower faecal bile acid concentration of lithocholic acid was also reported.

Regarding the effect of prunes on high blood pressure, Ahmed et al. (2010) [160] conducted a study with three groups of pre-hypertensive patients who were randomized to receive on a daily basis, either a single dose of prunes (11.5g), a double dose (23g) or a glass of water (control) for 8 weeks. Participants who received either the single dose or a glass of water on empty stomach in the morning showed significant reduction in both systolic and diastolic BP, while the double dose was associated with only a reduction in systolic blood pressure. The control (water) group also had significant increase in serum HDL that was not seen by prune treated groups. The authors attributed this placebo effect to the common factor between intervention and control- water.

Santhakumar et al.,(2015) [85] observed an inhibition of platelet aggregation induced by adenosine diphosphate, collagen and arachidonic acid from plum juice supplementation with Queen Garnet plum that has higher anthocyanin concentrations than the usual variant.

2.1.3.5 Laxative effect

With a focus on the laxative effect of prunes, Piirainen et al. [161] studied the effect of prunes on individuals with mild gastrointestinal symptoms. This study observed that consumption of prune juice reduced the occurrence of difficulty in defaecation. Similarly, consumption of a daily portion of plum juice before a meal in adults with chronic constipation softened the stool, provided immediate relief and participants showed more preference to prune juice than apple juice [162]. Similar results were also observed with dried plum in patients with mild to moderate constipation by Attaluri et al. [163] in which the effect was attributed to a synergistic effect provided by sorbitol, dietary fibre and polyphenols.

Table 2-5: Evidence on the effect of plums and its associated products on the different components of metabolic syndrome

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed/Conclusion
Animal studies					
Negishi et al., 2007 [158]	Japan	Prune extract (*P. Domestica)*	Dietary supplementation of prune extract (25%) in stroke-prone spontaneously hypertensive (SHRSP) rats for 5 weeks	NA	Suppression of High (systolic) blood pressure.
Kuo et al., 2015 [150]	Taiwan	Plum powder *(P.Salicina)*	Dietary supplementation of plum powder (2% or 5%) in high cholesterol diet in mice for 5 months	NA	Amelioration of some symptoms of neurodegenerative conditions like increased cholesterol and β-amyloid (Aβ) concentration in the brain by both doses.
Lucas et al., 2000 [164]	USA	Dried Plums (*P. Domestica)*	Dietary supplementation (5% or 25% dried plum) with dried plum in 48 ovariectomized (ovx) 90-day old female Sprague-Dawley rats for 45 days.	NA	With elevated serum total cholesterol brought about by ovariectomy, 25% prune diet prevented this increase without affecting HDL cholesterol concentration and also reduction in liver total lipids was observed.
Clinical trials					
(Tinker et al., 1991 [92]	USA	Dried Plums (*P. Domestica)*	**Hypothesis**: **a)** Prunes as a source of fibre can lower plasma cholesterol in men with mild to moderate hypercholesterolemia (5.2-7.5 mmol cholesterol/L). **b)** Faecal bile acid excretion is increased in response to the ingestion of prunes as a source of fibre which may help explain the cholesterol lowering effect of fibre. **n=**41 free living men with mild hypercholesterolemia. **Study design/Methods**: Crossover study. 8 wk period split into two experimental diet periods; each lasting 4 wk. Subjects randomly assigned a diet sequence, starting with either consumption of a grape juice-control supplement (GJ control) or a prune	II Confirmatory	Plasma LDL- cholesterol was statistically significantly reduced after the prune period than the control, faecal bile acid conc. of lithocholic acid was also statistically significantly lower with prune consumption and both faecal wet and dry weights were statistically significantly higher with prune consumption.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed/Conclusion
			supplement. **Sample size power:** not stated **Dose:** 12 prunes ***(***~100 g/d) **Duration:** 8 weeks.		
Chai et al., 2012 [159]	USA	Dried Plums (*P. Domestica)*	**Hypothesis**: Regular intake of apple favourably improves lipid profiles, reduces atherogenic risk ratios, lowers C-reactive protein (CRP) levels, and decreases levels of oxidative stress marker in postmenopausal women **n**=160 postmenopausal women **Study design/Methods**: Case-control study. Subjects randomly assigned to treatment groups of dried apples (75g) or dried plum (100g/d) **Sample size power:** 95% **Dose:** 100g/day **Duration:** 1 year.	II Exploratory	No statistically significant difference between treatment groups in altering serum levels of atherogenic cholesterols observed. For the dried apple group, total cholesterol was statistically significantly reduced at 6mth.
Afaghi et al., 2009 [165]	Iran	Prunes	**Hypothesis**: 8 fruits (Golab apples, Green apples, fresh apricots, prunes, cherries, blueberries, Golden no-seed grapes, and Red sultanas) are low GI and recommendable for diabetics and weight loss. **n**=8 **Study design/Methods**: Crossover study. 8 subjects (healthy, young men aged 20-28, normal weight with body mass index: 20-25 Kg/m2) randomly assigned to one of 8 fruits and blood glucose measured at 0, 15, 30, 45,60, 90 and 120 mins after consumption. **Sample size power:** 95% **Dose:** 143g **Duration:** Not stated - Different occasions after overnight fasting.	II Exploratory	Serving size of prunes was low glycemic load (GL) fruit. Prunes among other tested fruits were low GI and can be recommended for diabetics and weight loss management.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed/Conclusion
Ahmed et al., 2010 [160]	Pakistan	Prunes (*P. Domestica)*	**Hypothesis**: Use of prunes is useful in cardiovascular disorders to bring about changes in blood pressure or prevention of atherosclerosis. **n**=259 pre-hypertensive patients (BP =120/80–139/89 mmHg) **Study design/Methods**: RCT. Patients randomly assigned to 3 groups of A-single dose, B-double dose or C- control group. Blood pressure was recorded fortnightly, and blood samples were taken at 0 and 8 weeks. **Sample size power:** Not stated. **Dose:** group A-11.5gm. Group B-23gm. Control- glass of water. **Duration:** 8 weeks.	II Confirmatory	Reduction of blood pressure (6mmHg drop) by single dose of prunes daily group and the controls with the double dose of prunes showing a reduction in just systolic BP. There was an increase in serum HDL of the control group whereas test groups had significantly reduced serum cholesterol and LDL. The data predicts cardiovascular protective effects of prunes.
(Santhakumar et al., 2015 [85]	Australia	Plum juice *(P.Salicina)*	**Hypothesis**: Anthocyanin-rich Queen Garnet Plum Juice may ameliorate platelet activation related thrombogenesis and maintain haemostatic function by: (1) reducing platelet aggregation and activation through blocking/inhibiting various platelet activation pathways; (2) prolonging clotting time and reducing fibrinogen concentration; and (3) exhibiting favourable effects on lipid profile and inflammation. **n= 21** **Study design/Methods**: Randomised, double blind, placebo Crossover Trial. Healthy volunteers randomly assigned to 3 supplement groups of A-Queen Garnet plum juice, B-prune juice or C- colour matched placebo. Blood samples were collected at least 8h pre-prandial and mid-stream fasting urine samples collected.	II Exploratory	QGPJ supplementation inhibited platelet aggregation induced by adenosine diphosphate (ADP), collagen and arachidonic acid. There was reduction in platelet activation-dependent surface-marker P-selectin expression of activated de-granulated platelets. Increase in activated partial thromboplastin clotting time and reduction in plasma-fibrinogen and malondialdehyde levels, a plasma biomarker of oxidative stress. Prune juice supplementation did not affect blood cell counts, lipid profile, or inflammation markers

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed/Conclusion
			Sample size power: Not stated. **Dose:** 200mL/day of each juice. **Duration:** 28 days with 2 weeks washout period.		
Santhakumar et al., 2015 [166]	Australia	Plum juice *(P.Salicina)*	**Hypothesis**: Anthocyanin-rich QGPJ may impart anti-thrombotic effects via (a) inhibition of platelet aggregation by simultaneously targeting different platelet activation pathways (adenosine diphosphate: ADP-P2Y12/P2Y1; collagen: GPVI/α2β1 and arachidonic acid: cyclooxygenase-1–COX-1), (b) reducing platelet hyper-activation and de-granulation by blocking surface receptors responsible for activation, and (c) favourably altering coagulation parameters and lipid profile. **n=** 13 **Study design/Methods**: Randomised, double blind, placebo Crossover Trial. Healthy volunteers randomly assigned to 2 supplement groups of A-Queen Garnet plum juice or B-a flavoured and coloured formulated cordial placebo. Oxidative stress was induced by constant load exercise bout for 1h at 70% of their VO_{2PEAK} Blood samples were collected at fasting state and at least 8-12h pre-prandial on day 1 and day 29. **Sample size power:** Not stated. **Dose:** 200mL/day of each juice. **Duration:** 28 days with 2 weeks washout period.	II Exploratory	QGPJ supplementation inhibited adenosine diphosphate-induced platelet aggregation both without and under exercise induced oxidative stress, as well as inhibition of arachidonic acid-induced aggregation under oxidative stress. Also, there was reduced platelet activation dependant P-selectin expression both without and under oxidative stress. Favourable effects on coagulation parameters both with and without oxidative stress were also observed for QGPJ.

Table 2-6: Evidence on the satiety and laxative effect of plums and its associated products

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
Clinical trials					
Piirainen et al., 2007 [161]	Finland	Prune juice (prepared from plum juice concentrate, prune puree, water and 7% fructose) (*P. Domestica)*	**Hypothesis:** prune juice alone may have a laxative effect on the bowel function of those adults with certain gastrointestinal symptoms but are otherwise healthy. n=54 volunteers with mild GIT symptoms. Study design/methods: 1-week baseline period, 2-week prune juice (consumption) period followed by 1week follow-up period with daily record of bowel habit Sample size power: not stated. Dose: 125 mL twice a day. Duration: 4 weeks.	IV Confirmatory	Laxative effect with increased flatulence.
(Cheskin et al., 2009 [162]	USA	Plum juice (*P. Domestica)*	**Hypothesis**: Plum juice supplementation diet would induce significant improvements in bowel frequency, and consistency, and possibly decrease appetite compared to baseline as well as placebo and psyllium treatments. **n**=36 adults with chronic constipation symptoms. **Study design/Methods**: Randomized controlled crossover trial. Consumption of a daily portion of plum juice in comparison to psyllium and apple juice in adults with chronic constipation symptoms. **Sample size power:** Not stated. **Dose:** 8 ounces (237mL) per day. **Duration:** 14days.	II Confirmatory	Constipation relief and stool softening evident with consumption of plum juice.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
Attaluri et al., 2011) [163]	USA	Dried plum (*P. Domestica)*	**Hypothesis**: Dried plums are as effective as psyllium in the treatment of adults with chronic constipation. **n**=40 patients with chronic constipation. **Study design/Methods**: Single blind, randomized cross-over study. Consumption of a daily portion of dried plums or psyllium for a treatment period of 3 weeks after which participants continued on their usual remedies for constipation for another 6 weeks. For the duration of the study, subjects maintained daily symptom and stool diaries. **Sample size power:** 80%. **Dose:** 50 g twice a day with meals. **Duration:** 14 weeks (with 1-week washout period between treatments).	II Confirmatory	Effective treatment with dried plum on mild to moderate constipation observed.
Farajian et. al., 2010 [167]	Greece	Prunes (*P. Domestica)*	**Hypothesis**: A preload including dried prunes consumed as a snack before a meal, compared to an isoenergetic bread product preload, would reduce; a) meal time energy intake, b) appetite for dessert offered after lunch and, c) energy intake for the next 24 h. **n**=45 normal weight subjects. **Study design/Methods**: Randomized cross-over study. Fasting participants offered a standardized breakfast followed by a preload of either dried prunes or bread product after 2 hours. 3 hours after the preload, a standardized lunch and desert was provided. Subjects also rated their hunger, thirst, desire to eat, motivation to eat and satiety on 100 mm line visual analogue scales (VAS) just before and right after the preload consumption, every 45 min up till the 180th	II Exploratory	Reduced consumption of dessert with lower energy intake observed. An Increased satiety at all time points between snack and meal was also observed.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
			min. On completion of the test day, detailed record of all foods and beverages intake for the next 24 h was collected. **Sample size power:** not stated. **Dose:** 5 prunes (40g) before meals on each testing day. **Duration:** 2 days (with 1-week washout period).		
Furchner-Evanson et al., 2010 [168]	USA	Dried plum (*P. Domestica)*	**Hypothesis**: Snack choices similar in fat, protein, carbohydrate and sugar contents while differing in fibre content have an effect on satiety, subsequent food intake and plasma glucose, insulin and ghrelin responses. **n**=19 healthy female subjects **Study design/Methods**: Randomized crossover study with at least 1-day washout. Subjects randomly assigned to receive 4 different test foods including dried plums. Blood samples collected at baseline and every 15mins in 1hr then 90 and 120 mins. **Sample size power:** Not stated. **Dose:** served in a 238kcal (1000kj) portion. **Duration:** not stated.	II Exploratory	Satiety index AUC greater for dried plum trial. Consumption of the dried plum elicited lower plasma glucose and insulin AUC and tended to promote a greater plasma ghrelin AOC.
Howarth et al., 2010 [169]	USA	Dried plum (*P. Domestica)*	**Hypothesis**: Snack selection (dried plums vs common carbohydrate-rich low-fat cookies) influences daily energy consumption, nutrient intake and metabolic responses. **n**=26 healthy female subjects **Study design/Methods**: Randomized crossover study with 2 weeks washout. Subjects randomly assigned to receive either dried plums or low-fat cookies for 2 separate 2-week feeding. A 7-day bowel habit questionnaire was completed.	II Exploratory	No change observed with energy intake or weight. In comparison to cookie, dried plum promoted greater intake of fibre, potassium, riboflavin, niacin and calcium. There was an observed reduction in total fat as well as cholesterol intake with dried plum snacks. Dried plum did not alter plasma triglyceride concentration, but softer stool consistency was observed with dried plum consumption.

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
			Sample size power: 80% **Dose:** served in a 100-kcal portion twice a day. **Duration:** 4weeks.		
Lucas et al., 2004) [170]	USA	Dried plum (*P. Domestica)*	**Hypothesis**: Gradual incorporation of 100g of dried plum into the daily diet of healthy postmenopausal women would not cause significant changes in self-reported bowel habits including frequency of defecation, faecal bulk, and stool consistency. **n=58** **Study design/Methods**: RCT. Postmenopausal women randomly assigned to treatment groups of dried plum (100g/d) or dried apple (75g) daily for 3 months. A 7-day bowel habit questionnaire was completed. **Sample size power:** not stated **Dose:** 100g/day. **Duration:** 3 months.	II Exploratory	With both dried plum and apples, no statistically significant differences for any of the parameters used to assess bowel function, showing the absence of any negative side effects associated with prune consumption.
Pasalar et al., 2013 [171]	Iran	Prunes (*P. Domestica)*	**Hypothesis**: Prunes and flixweed (herbal Iranian traditional medicine) are effective in the prevention of constipation among Iranian pilgrims who attended the Hajj ceremony in 2010 in the kingdom of Saudi Arabia for 3 weeks. **Study design/Methods**: RCT. 170 Iranian Hajj pilgrims randomly assigned to two groups of case and control. Case group received measured doses of prunes and flixweed daily before lunch and dinner and control group had their meals with no intervention. **Sample size power:** not stated **Dose:** 40-50g/day with 10-15g of flixweed.	II Exploratory	Using Rome III criteria to define constipation (less than three times of defecation/week, with straining, difficulty in defecation, unproductive urges, feeling of anorectal obstruction, hand manoeuvre to facilitate stool extraction and feeling of incomplete evacuation), a statistically significant difference was observed between the groups with the case group less constipated.

2.1.3.6 Antiallergic and antimicrobial property

On the anti-allergy capability of plum, it was observed that supplementing the animal diet with prune extract following injection of mite allergen for 3 weeks, there was a significant reduction in the number of sneezing events, total and mite allergen-specific immunoglobulin E levels even though there were no identifiable anti-allergic components in the prune extract [172]. Studying the antibacterial property of plums, it was observed that when tested on 5 different gram positive bacteria, ethanol extracts of prunes exhibited an antibacterial property [173]. A similar effect was also observed by a different study [174]

2.1.3.7 Other Effects

Other reported beneficial health effects of plum include its effect on liver function in healthy individuals. In a clinical trial, there was a significant reduction in serum alanine transaminase and serum alkaline phosphatase (clinical biomarkers of liver health) with no changes observed in serum aspartate transaminase and bilirubin [175]. Studying the effect of plum juice on urinary stone risk factors, results showed no significant effect on urinary composition [176]. However, another study using the Australian Queen Garnet plum reported an increase in urinary antioxidant capacity [107]. In normal weight individuals, a preload of prunes in comparison to a bread product before a meal has been observed to result to lower energy intake at later meals, including lunch and the desert (910 kcal ± 233 on prunes day vs 971 kcal ± 249 on bread product day; $p = 0.010$), as well as increased satiety at all time points tested between the snack and meal [167]. Similar results have also been observed in other studies [168, 169].

Table 2-7: Evidence on the anti-allergic, anti-microbial and immune-enhancing property of plums and its associated products

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
***In vitro* Studies**					
Cevallos-Casals et al., 2006 [174]	USA	Plum extract (*P.Salicina*)	Plum extract placed in a well with diluted bacteria inoculum.	NA	Inhibitory effects against *Escherichia coli* 0157:H7 and *Samonella Enteritidis.*
Yaqeen et al., 2013 [173]	Pakistan	Prune extract (*P. Domestica)*	Ethanol extracts of prunes tested against nine bacteria; five Gram positive bacteria *{Staphylococcus aureus, Streptococcuc intermedias. Bacillus cereus. Bacillus pumilus)* and four Gram negative bacteria *{Eschrichia coli, Proteus mirabilis Shigella flexneri. Salmonella typhi* and *Klebsiela pneumoniae).*	NA	Antibacterial activity observed. Several bacteria groups (e.g. *Lactobacillus* and members of *Ruminococcacea*) were found to be more abundant in the plum group. There was also a distinct contrast between the microbiota of control and treatment groups.
Animal studies					
(Karasawa et al., 2012) [172]	Japan	Prune extract (*P.Salicina*)	Dietary supplementation with 25% ovalbumin and 1% prune extract over 6 weeks in rats injected 20L of distilled water containing 20g of mite allergen between the 3rd to 6th week	NA	Reduction in allergic response in comparison to control group
(Lee et al., 2008 [177]	USA	Plum powder (*P.Salicina*)	Dietary supplementation with 0.5% or 1.0% plum in one day old chickens and oral inoculation with 5000 sporulated oocysts of *E. acervulina* at day 12 post-hatch.	NA	There was a reduction in faecal oocyst shedding and chickens fed the plum supplemented diets exhibited greater spleen cell proliferation
Noratto et al., 2014 [178]	USA	Plum Juice (*P. Domestica)*	Administration of plum juice in obese Zucker rats for 11 weeks. Body weight recorded once a week.	NA	Increase in body weight gain, levels of MRNAs for interferon-ϒ and interleukin-15.

*Only clinical trials were ranked for level of evidence

Table 2-8: Evidence on the effect of plum and its associated products on liver function and kidney stone risk factors

Reference	Location	Plum product investigated	Sample/Method	Level of evidence*	Effects observed
Clinical trials					
Ahmed., et al., 2010[175]	Pakistan	Prune juice (*P. Domestica)*	**Hypothesis**: Prune juice does not alter liver function. **n=**107 healthy volunteers **Study design/Methods**: Case-control study. Participants randomly assigned to 3 different groups of A (single dose of 1 pack of prunes = 11.43kg i.e. 3 prunes), B (control; a glass of water) and C (double dose of grp A) consumed daily**.** Blood samples were taken on Week Zero and Week 8 for liver function tests (LFTs) i.e. serum alkaline phosphatase (ALP), bilirubin, aspartate transaminase (AST) and alanine transaminase (ALT). **Sample size power:** not stated **Dose:** 3prunes (~11.43g) or a double dose of 6 prunes (~22.86g) per day. **Duration:** 8 weeks.	II Exploratory	Liver function test showed significant reduction of serum alanine transaminase and serum alkaline phosphatase but no effect on serum aspartate transaminase and bilirubin
(Keßler et al., 2002 [176]	Germany	Plum juice (*P. Domestica)*	**Hypothesis**: Plum-, cranberry- and blackcurrant juice may have an influence on the urinary composition and therefore on multiple risk factors of kidney stone formation. **n=**12 healthy male subjects. **Study design/Methods**: Participants control-fed a standardized diet daily with plum juice. 24h urine sample collected. **Sample size power:** not stated **Dose:** 330mL of plum juice. **Duration:** 5days	II Exploratory	No significant effect on urinary biochemical or physiochemical parameters

*Clinical trials ranked using [111] Levels of Evidence Hierarchy where I is a systematic review (highest rating) and IV is a case series or cross-sectional study (lowest rating) and also classified as exploratory or confirmatory studies.

2.1.4 Discussion

This systematic literature review identified 73 peer-reviewed journal articles on the health effects of plum and its associated products. All identified studies were grouped according to outcome measures but differed considerably based on the plum fraction studied, study design, as well as intervention methods. Despite an increase in plum-based research that has emerged over the past decade, the level of evidence remains low. Of 25 clinical studies in human, nine studies included randomisation to a plum supplementation group but only one of these studies adequately described the method of randomisation and blinding. Nonetheless, results from some of the study outcomes are consistent. Considering bone health as the main study outcome, the polyphenols present in the plum appear to be responsible for the benefits. However, it has been suggested that even though dried plum polyphenols have some bone modulating properties, the synergistic effect of these polyphenols, together with potassium and vitamin K, is required to produce potent effects on bone mass and microarchitecture and to reverse OVX (ovariectomy)-induced bone loss in mature animals [179]. Regardless, compared to other dried fruits (apple, apricot, grape and mango), only the dried plum exhibited an anabolic effect on trabecular bone in the vertebra and prevented bone loss [112].

Some of the findings from the *in vitro* studies have been confirmed in animal studies but remain to be confirmed in human clinical trials. Most of the available human trials used a dried version of plums (prunes) rather than fresh fruit, thus limiting translation to dietary messages of the positioning of fresh plums in a healthy diet. The drying process significantly decreases (more than 90%) anthocyanin and flavanol content of plums [180]. The effect of other processing methods on the antioxidant properties of plums has been studied [181]. Blanching decreased the tannins and antiradical efficiency of the fruits but increased the total polyphenol content, while osmotic dehydration had no effect on the total polyphenol and ferric reducing power. This increase in total polyphenol content was attributed to the thermal disruption of the polyphenol–protein complexes [182]. Fresh plums also have higher free radical scavenging capacity (superoxide and peroxy radicals) and antioxidant activity than dried plums [183]. However, prunes are known to contain higher levels of phenolic compounds than most dried fruits and also possess higher radical scavenging activity, even possibly the highest in dried fruit and vegetable products present in

human diet [184]. Further studies are required to compare the health effects of fresh plum, plum juice and dried plum in human trials.

Extraction methodology is also an important factor in plum based research as different solvents have shown some disparity in extracts. Estimating the antioxidant capacity of the whole plum fruit, results showed that in extracting the bioactive compound in plum, the ethyl acetate and butanol fraction showed the most antioxidant potential in comparison to the hexane and aqueous fraction [185].

Evidence included in this systematic review was gathered from studies that differed in a number of ways including population studied, study design, outcome measures, and methods of randomisation. This limits comparison between studies. Limitations related to different study designs are particularly evident in the animal studies that show inconsistent results. For example, in one of the studies with cognitive outcomes, a significant effect was observed using the Morris water maze task in mice fed a high cholesterol diet that had been supplemented with 2% dried plum [150]. On the contrary, another study [105] showed that plum juice, but not dried plum powder (2% concentration), was effective in alleviating cognitive deficits in aged rats. This may possibly be explained by a difference in the dosage of bioactive compounds provided in the two studies, or the food matrix of this supplement, or both [186] but remains to be elucidated in dose response studies. In addition, even though the nutritional content of study fruits and beverages were described, the anthocyanin contents were lacking. Of all the studies included in this systematic review, only five [85, 137, 138, 166, 187] provided anthocyanin content which ranged from 0-112mg/100mL. The absence of anthocyanin content, an active component, of interventions also limits results comparison across studies.

There have been no reports on the side effects associated with daily consumption of plum and its associated products. Studies have shown that consumption of dried plum over a long period has no significant effect on the levels of insulin and glucose or bowel function [170, 188]. Plums are known to contain considerable levels of oxalates which occur naturally and may increase the risk of kidney stone formation [189]. High levels of oxalate in the body inhibit the absorption of calcium, thereby resulting in precipitation of calcium which can result in stone formation in the kidney and bladder [190, 191]. However, plum juice consumption did not seem to have any significant effect on the risk factors associated with kidney stone

development [176]. In as much as these potential side effects have not been reported with usual plum consumption, it is important for future studies to identify the upper level of safe intake.

With increased interest in plum research which has led to the development of a plum hybrid (QGP), it is important that similar studies be performed with this hybrid, as well as with other hybrids of plum as they become available, to confirm these observed effects and to allow generalisation of plum research findings. Other parts of the plum fruit that are usually discarded or used in animal feed may also provide both nutritive and non-nutritive food components that confer health benefits. For e.g. the plum pomace, a by-product (pulpy residue) obtained during plum juice production has been reported to contain 38-49% dietary fibre and have antioxidant and anti-inflammatory properties that have been demonstrated *in vitro* [192].

2.1.5 Conclusion

In conclusion, this systematic review has identified an emerging body of evidence that demonstrates the beneficial health effects of plum consumption. The largest amount of evidence to date relates to prevention and management of osteoporosis, which shows promising evidence as an adjunctive therapy. However, many of the study designs were of low quality therefore it is important that well designed human trials are conducted to confirm these observed effects. Consideration of the nutritional composition of plums and prunes and the effects of processing on their bioactivity is also important for future research. Elucidation of the mechanism of action of plum polyphenols, identification of potential adverse effects, and the effects of dosage on outcomes is necessary to inform dietary guidelines for chronic disease prevention and management.

3 Chapter 3

3.1 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

This chapter describes a pilot acute cross-over study that was conducted to investigate the impact of plum juice on cardiovascular-related responses, cognitive function, and urinary anthocyanin excretion profiles. A sample of 24 (12 healthy older (65y+) and 12 younger (18-45y) adults) were recruited for this study. Participants received, randomly, either a 1x300mL or 3x100mL QGP juice (QGPJ) 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). Regardless of dose, QGPJ did not impact acute cognitive function. There was a significant reduction in BP responses 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). Dose-timing did not appear to be a significant factor in the potential acute BP-lowering effect. Native QGP anthocyanins, as well as methylated/glucuronidated metabolites were detected in urine with no significant differences between age groups or dose-timing. Results from this study suggests that dose-timing does not seem to be an important factor in a QGPJ intervention.

The majority of this chapter is the substantive content of the published article: (Appendix E)

Igwe, E.O., Charlton, K.E., Roodenrys, S., Kent, K., Fanning, K. and Netzel, M.E., 2017. Anthocyanin-rich plum juice reduces ambulatory blood pressure but not acute cognitive function in younger and older adults: a pilot crossover dose-timing study. *Nutrition Research, 47,* pp.28-43.

Specific role/authorship of all the authors: All authors were involved in designing the study. EI and KK did the data collection. FK and MEN analysed the urine samples for anthocyanin metabolites and were involved in writing the urine section of the manuscript. EI produced the first draft of the manuscript. All Authors contributed to writing and editing the manuscript and approved of the final version of the paper submitted for publication.

3.1.1 Introduction

Research on the beneficial health effects of fruit anthocyanins continue to show promising evidence both *in vitro and in vivo*. However, its effect on cognition in humans has yet to be studied extensively. In Australia, dementia which is preceded by a phase of mild cognitive impairment continues to have a substantial financial burden on individuals as well as the economy [193]. In addition, high BP continues to pose significant health risks. According to the World Health Organization (WHO), the prevalence of high BP in adults aged 18 years and over was around 22% in 2014. This accounted for about 9.4 million deaths or 7% of all deaths [194]. 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 [195]. In global strategies to address neurodegenerative and CVD prevalence, the significant role of modifiable dietary risk factors, including an increased intake of fruits and vegetables is acknowledged [6]. Small (2-5mmHg) but steady decreases in mean BP have been shown to significantly decrease the incidence of cardiovascular events [196]. Given the magnitude and burden of chronic diseases, cost-effective strategies, including dietary intervention, are needed for the prevention and management of chronic diseases. Plant-based foods are integral to a healthy human diet and a plant-rich diet has been found to be associated with prevention of a vast array of diseases [197].

To fully grasp the mechanism of action of dietary anthocyanins *in vivo*, it is important to first understand their absorption and metabolism. Even though anthocyanins have shown favourable health boosting effects in human cell culture, experimental animal and human epidemiological studies, clinical trials are needed to confirm these findings [27, 198, 199].

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 [200, 201] 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 [202-204]. 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 in studies as to the reason each dose was selected, and no consideration generally given to the physiological effects thereof. Even though results from published studies have mostly been in agreement, evidence shows that beyond a point, the bioavailability of anthocyanins decreases with increasing dose [205]. For cyanidin-based anthocyanins, the maximum absorption has been reported to be about 350µmol/L or less, peaking between 1.5-2.5h [206]. This is also believed to differ according to the structure of different anthocyanins found in differing concentrations in foods, the attached sugar moiety and because of wide inter-individual variation in anthocyanin metabolism. All of these factors limit translation of research findings into practical dietary messages [207]. 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.

The aim of this study was to determine the acute impact of differing doses of QGPJ on cognition and other health parameters in young and older adults over a six-hour period. This study also determined and compared the anthocyanin/metabolite profiles of human urine after oral consumption of high anthocyanin QGPJ in healthy young and older adults.

It is hypothesised that the consumption of high anthocyanin QGPJ will have an acute impact by:

1. Improving various domains of cognitive functioning and blood pressure;
2. Demonstrating the bioavailability of QGP anthocyanins as assessed by their urinary excretion over a 24h period;
3. Showing differences in the absorption rate and metabolism of anthocyanins between young and older adults, assessed in urinary biomarkers (24h).

3.1.2 Methods

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 (HE14/343). Written informed consent was obtained from all participants.

Study design

The study was an acute cross-over bioequivalence/non-inferiority study (with at least a two-week washout period between clinic visits) 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 3h) with the aim to determine whether three 100mL of plum juice taken over three hours would have a different pattern of effect on BP in comparison to a single 300ml dose. Results from this pilot study will be used to subsequently inform the methodology as well as sample size calculation for a future crossover randomised clinical trial. This is in line with the assumptions of bioequivalence studies, whereby the future trial will be designed as a crossover study [208-210]. (See Chapter 5).

Sample size

Twenty-four participants were recruited, including 12 younger (18-45y) and 12 older (65+y) adults in order to compare responses between younger and older adult individuals. The sample size was determined according to recommendations for planning a pilot study that investigates bioavailability and bioequivalence of components within food. A sample size of 24 (12 per group), for a pilot study, is recommended based on feasibility, gains in precision about the mean and variance, as well as guidelines for evidence required by regulatory bodies such as the Food and Drug Administration (FDA) [208-210]. For a pilot crossover study, feasibility can be explained as the number of possible period crossover trials (2,3,4 or 6) with balanced Williams square design (defined as a balanced allocation to period trials in a small sample size). [208, 211]. One of the major aims of pilot studies is to provide an estimate of the variance as this could be used in power sample size calculations for a larger follow up study, in this case a larger RCT (Chapter 5). To determine the precision of the variance, the sensitivity of the appropriately powered study to the estimate of the (unknown) variance would be ascertained. Thus, the sample size of a pilot study is required to be adequate to have appropriate degrees of freedom in a sensitivity analysis in

a larger follow-up study. Sim and Lewis (2012) [211] have demonstrated calculations based on this assumption and showed that there were negligible gains in variance precision when the degrees of freedom exceeded 20. Other authors, including Julious (2005) [208] and Van Belle [212] have demonstrated that for sample sizes below n=30, calculations based on confidence interval, assuming a unit variance of s=1, gains in precision for each increase of 1 in the sample size was no longer significant once the sample size reached 12 [208, 212]. Thus, the current study target sample size of 24 (12 per arm) could be considered adequate for the purpose of a crossover pilot study.

A general recommendation for sample size calculation by the ICH is that the number of participants in a clinical trial should always be large enough to provide valid answers to the questions posed [209]. For pilot bioavailability and food investigating studies, the U.S Food and Drug Administration (FDA) guidance on bioequivalence studies states that: "A minimum of 12 evaluable subjects should be included in any bioequivalence study" [210]. Following these recommendations, a sample size of 24 was chosen for this pilot study.

Participants

All participants were recruited from the University of Wollongong (UOW), Australia and the surrounding Wollongong, NSW areas through poster advertising. Potential participants had the opportunity to discuss the study over the phone prior to clinic visits and were screened to determine eligibility. Recruited eligible participants were randomised to a dose-timing allocation and cognitive assessment order by a computer-generated block randomisation by an independent statistician. Participants attended two 6-h clinic visits at the Illawarra Health and Medical Research Institute (IHMRI) at the University of Wollongong, NSW, Australia between June and September 2015.

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 related to flavonoids, and inability to communicate in the English language.

Data collection

On the first study day, a questionnaire was administered to determine participants' socio-demographic characteristics. The validated International Physical Activity Questionnaire (IPAQ) [213] 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).

Dietary instruction and intervention meals

The QGPJ 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. The study beverage was stored at -20^{o}C and thawed (in the refrigerator) 24h prior to use on the days participants came into the study facility [107]. 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. QGPJ was provided with breakfast in random order, as either (i) a single dose of 300mL (369 mg total anthocyanins) [13] 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.

Ambulatory 24h blood pressure and anthropometric measurements

BP 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 [61, 214]. Upon arrival at the testing facility participants were fitted with an ABPM (SpaceLabs Inc., Issaquah, WA, Australia; Model 90207). The ABPM took BP measurements over the next 24h; every 15 minutes while at the testing facility (first 6h) 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 [215]. 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/ (height^2)) was calculated.

Cognitive tasks

Five short cognitive interviewer-administered tests [216, 217] were administered by trained investigators at baseline and 6h on both testing occasions to determine any acute changes in cognition (Table 3-1). 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 at 6h, and in the cross-over arm. Accuracy and response time were recorded for each task. These tasks were chosen based on available evidence from the literature on cognitive tasks and corresponding cognitive domains that appear sensitive to anthocyanin supplementation in acute settings [201].

Table 3-1: Cognitive assessment task description

Instrument	Cognitive Domain	Application	Scoring
Trail making test [218]	Higher executive function	Participants alternate between two simple tasks at once.	The difference in the number of seconds required to complete the task compared to a non-switching version.
The Rey Auditory Verbal Learning test [219]	Verbal learning and memory	Participants learn and recall a list of words over 5 trials.	Each correct word that is identified is associated with a score.
Pattern & letter comparison task [220]	Speed of processing	Participant compares strings of patterns or letters to determine if it is the same or different.	Participants complete as many examples as possible in 30 seconds and scores are tallied.
Reaction time task[221]	General alertness and speed of processing	A left or right arrow-shaped stimulus is displayed on the computer screen and participants will be required to press the corresponding mouse button (left or right) as displayed on the screen.	Scoring will be based on correct and incorrect responses and latency (response speed).
Stroop task [222]	Executive function	Participants say the colour of a word, not what the word says. E.g. for the word, **RED**, they are expected to say "Blue."	Amount of time taken to complete each set of words.

Urine sample collection and preparation

Urine samples were collected at baseline prior to QGPJ consumption, and thereafter collected in sterilised urine containers over the following time periods: 0-2h; 2-6h; 6-12h and 12-24h after QGPJ 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 at the Queensland Allianz for Agriculture and Food Innovation Laboratory, University of Queensland. The methodology was as described by Netzel et. al. (2012) [187]. Acidified urine samples were thawed and maintained for 60 min at room temperature before Solid Phase Extraction (SPE) to obtain the maximal yield of the coloured flavylium cations. The SPE cartridge was activated with 10 mL of methanol and equilibrated with 10 mL of 12 mM aqueous HCl before use. Subsequently, 5 mL of acidified urine was applied to the equilibrated cartridge. The cartridge was then washed

with 10 mL of 12 mM aqueous HCl, and anthocyanins were eluted with 2 mL of 12 mM HCl in methanol. The extracts were filtered (0.2 µm syringe filters; Palls, Cheltenham, VIC, Australia) before UHPLC-PDA-ESI-MS/MS analysis.

3.1.3 Data Analysis

Data was analysed 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 QGPJ 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 BP 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 [223, 224] 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.

3.1.4 Results

3.1.4.1 Subject characteristics

Twenty-four participants (12 younger and 12 older adults) were recruited to participate in the study and sociodemographic characteristics are shown in Table 3-2. Participants were of Caucasian descent (n=21), African descent (n=2) and Asian descent (n=1). All participants attended both visits and there were no withdrawals throughout the study protocol. The average washout period for participants was 20 days (14-26 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." [225]. For the anthocyanin, cyanidin-3-glucoside, which is the main anthocyanin in QGPJ [85, 107], the half-life has been shown to range between 12 and 51h [49].

Table 3-2: Descriptive statistics

Characteristics	Younger adults (n=12) n (%)	Older adults (n=12) n (%)	p-value
Gender			Ns
Male	4 (33.3)	3 (25.0)	
Female	8 (66.7)	9 (75.0)	
Age (Mean & SD)	30.83 (8.04)	77.42 (6.10)	<0.001
BMI (Mean & SD)	22.49 (2.36)	26.36 (3.28)	0.003
Physical Activity			0.97
Low	5 (41.7)	1 (8.3)	
Medium	5(41.7)	10 (83.3)	
High	2 (16.7)	1(8.3)	
Smoking status			Ns
Yes	0(0)	0(0)	
No	11 (91.7)	12 (1000	
Occasionally	0(0)	0(0)	
Rarely	1 (8.3)	0(0)	
Alcohol intake			0.67
Yes	3 (25.0)	5 (41.7)	
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

3.1.4.2 Cognitive tasks

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 (Table 3-3).

Table 3-3: Mean baseline measurements and mean difference of cognitive assessments over 6 hours according to group

Cognitive Task	3 x 100mL QGPJ (0,1,3h)		1 x 300mL QGPJ	
	Baseline Mean (SD)	**6h mean difference**	**Baseline Mean (SD)**	**6h mean difference**
Younger adults (18-45)	**n=12**		**n=12**	
Speed of processing (measured by letter and pattern comparison)[a]	2.39(0.51)	-0.16	2.10(0.48)	0
Stroop effect	18.42(7.63)	0.91	19.42(5.31)	0
Switch cost[b] (measured by task switching)	46.58(13.43)	-0.88	45.46(19.66)	0.84
RAVLT (total)[c]	54.00(9.41)	-1.75	51.50(9.78)	-0.34
RAVLT (20m delay)[c]	11.00 (2.79)	-0.10	11.00(2.87)	-1.78
Reaction time	501.98 (36.28)	12.85	555.31 (87.75)	0.08
Older adults (65+)	**n=12**		**n=12**	
Speed of processing (measured by letter and pattern comparison)[a]	3.33(0.90)	0.33	3.24(0.60)	0.06
Stroop effect	35.58(16.21)	1	47.16(18.98)	3.75
Switch cost[b] (measured by task switching)	73.00(24.62)	8.59	69.21(19.21)	5.16
RAVLT (total)[c]	39.00(8.07)	3.17	38.17(7.73)	4.34
RAVLT (20m delay)	4.56(3.21)	0.17	5.11(3.72)	-2.50
Reaction time	682.54 (70.24)	10.61	781.67 (148.22)	-15.01

[a]Pattern and letter comparison = number of correct answers in 90 secs; [b]Task switching = time taken to complete task; [c]RAVLT – Rey Auditory Verbal learning Test (total) and (20min delay) = number of words recalled

3.1.4.3 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 3-1 to 3-8. Comparison is made between the dosing regimen (single and triple doses) and age groups with an interaction factor (dosing regimen x age-group). Figure 3-1 shows a more

obvious drop in systolic blood pressure of the older adults with the single dose compared to the triple dose (Figure 3-5). This observation was not evident with the younger adults, as shown in Figures 3-1 and 3-5. There was no significant dose-timing effect observed for change in blood pressure following plum juice consumption in the 24h period using the linear mixed model for longitudinal data (Table 3-4).

Area under the curve (AUC) was calculated for the cardiovascular parameters (systolic, diastolic, MAP and HR) for the first 6h (Table 3-5 and Figures 3-9 – 3-12). For both age groups, using an independent sample *t-test*, BP was significantly lower than baseline ($p < 0.05$) at different time points up to 6 h following consumption of the plum juice. The greatest significant BP reduction was observed at 2h for both age groups and was more obvious for systolic BP in the older group with a mean difference of 12.83mm Hg (SD; 16.51, $p=0.001$) from baseline. For the single dose, z-test analysis of the AUC calculations for the younger adult group showed a significant effect of the juice on diastolic BP, MAP and HR (p-values = 0.008, 0.012 and 0.025, respectively). Similarly, a significant effect was seen for the older group: systolic BP, diastolic BP, MAP and HR (p-values = 0.035, 0.028, 0.017 and 0.006 respectively). For the younger age-group on the triple dose, significant effects were observed for diastolic BP and MAP (p-values = 0.008 and 0.013, respectively) with a borderline significance on the HR (p-value = 0.06). In the older group, significant effects of the triple dose were observed for diastolic BP (p-value = 0.00007) and a borderline effect for systolic BP (p-value = 0.063). Plum juice consumption had a significant effect on systolic BP, which was predicted by dose or age group but no interaction term effect (dose x age group) and for MAP, predicted by only age-group. No significant effect was observed on other cardiovascular parameters (Table 3-4).

Table 3-4: Effect of dose-timed plum juice consumption on cardiovascular parameters across age groups

Parameter	Mean ± SE[a]	p-values
SBP		
Intercept	123.7 ± 0.05	<0.0001^
Dose-time[b]		
1 x 300mL	122.3 ± 0.60	0.001^
3 x 100mL	125.2 ± 0.67	
Group[c]		
Younger (n=12)	115.0 ± 0.66	<0.0001^
Older (n=12)	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 (n=12)	72.2 ± 0.53	
Older (n=12)	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	
Group		<0.0001^
Younger (n=12)	86.4 ± 0.54	
Older (n=12)	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	

Parameter	Mean ± SE[a]	p-values
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 (n=12)	72.9 ± 0.54	
Older (n=12)	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; [a]Means and p-values were obtained from linear mixed model; [b]Dose-time represents either a single dose of 300mL or 3 portions of 100mL taken at 0h, 1h and 3h; [c]"Younger" means younger age-group and "older" means older age-group; SBP – Systolic blood pressure; DBP- Diastolic blood pressure; MAP- Mean arterial pressure; HR- Heart rate

Table 3-5: Change in cardiovascular parameters of participants following consumption of different doses of QGP juice

Cardiovascular parameters	**AUC[a] (0-6 h), mmHg**	
	3 x 100mL QGP (0,1,3h)	**1 x 300mL QGP**
Younger adults (18-45)	**n = 12**	**n = 12**
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+)	**n = 12**	**n =12**
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)
[a]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.

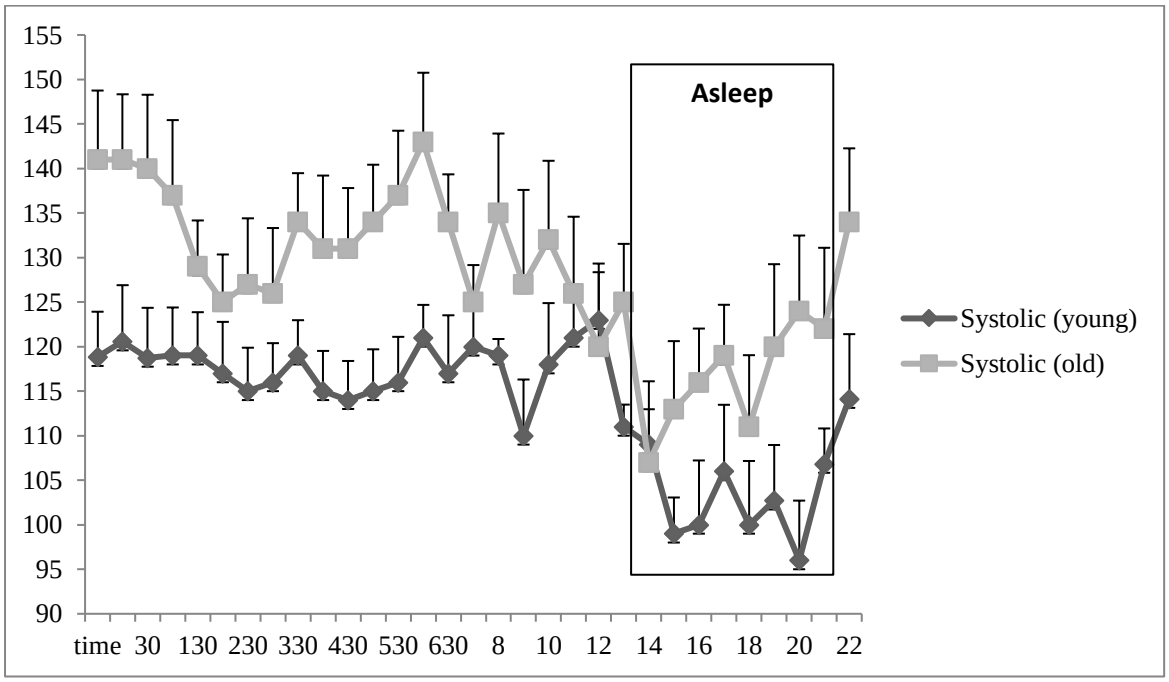


Figure 3-1: Single dose hourly systolic blood pressure of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

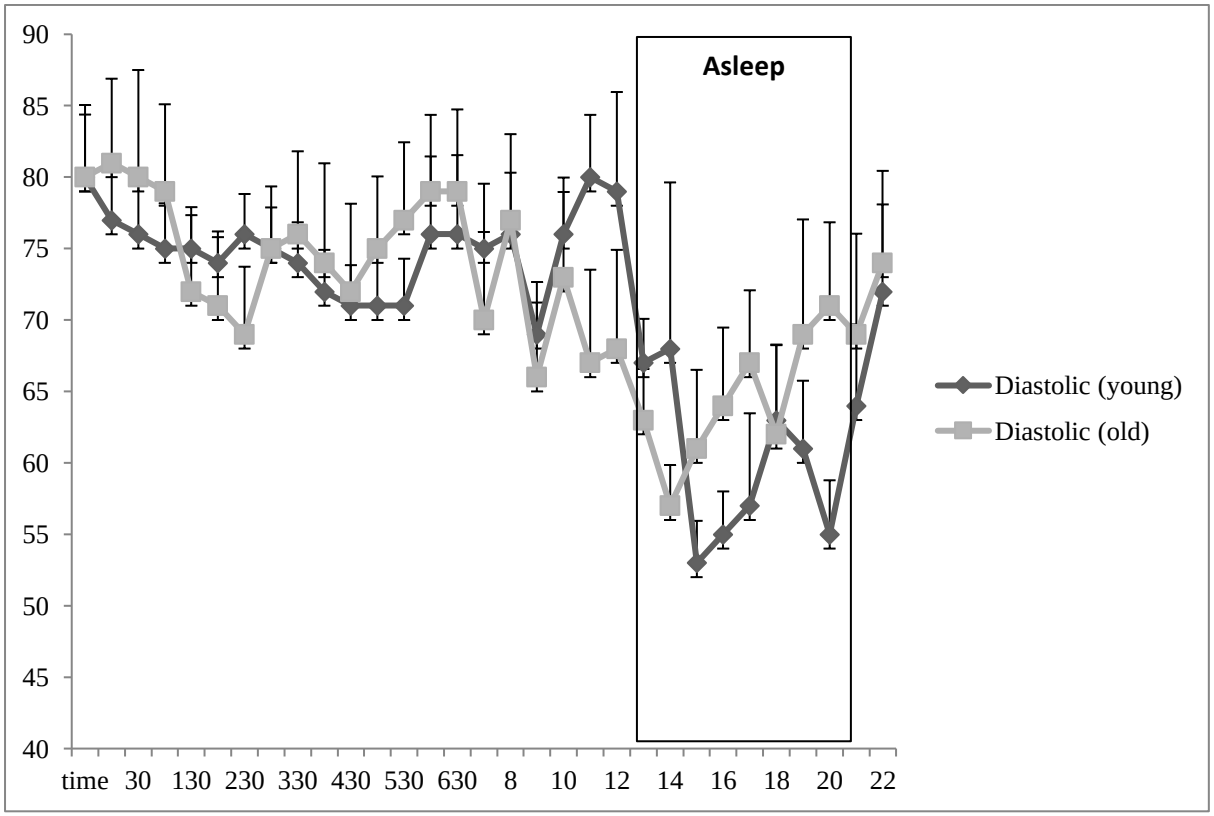


Figure 3-2: Single dose hourly diastolic blood pressure of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

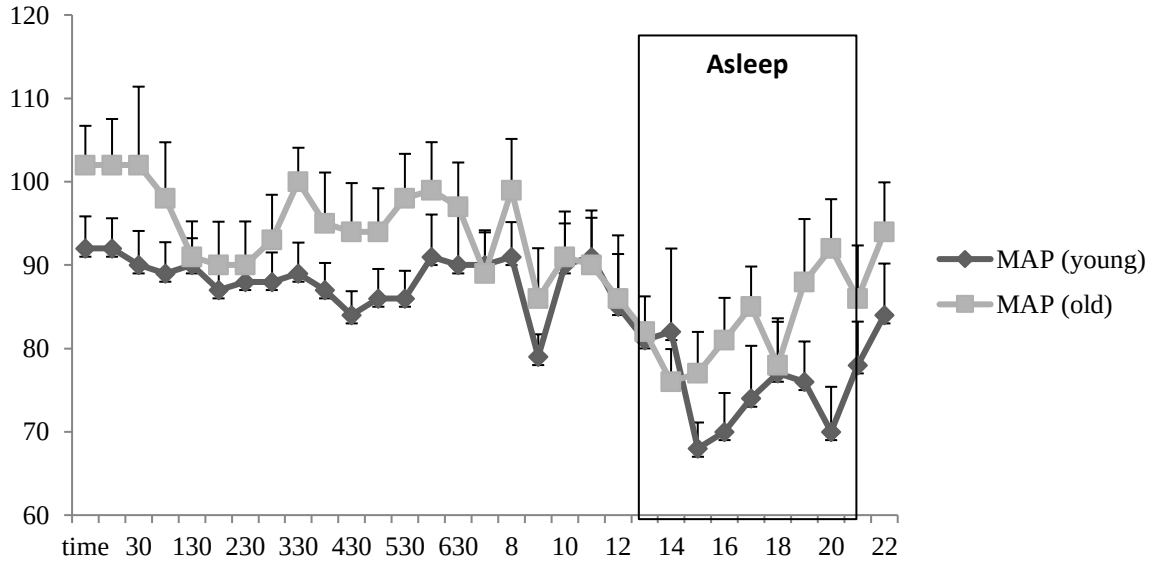


Figure 3-3: Single dose hourly mean arterial blood pressure of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

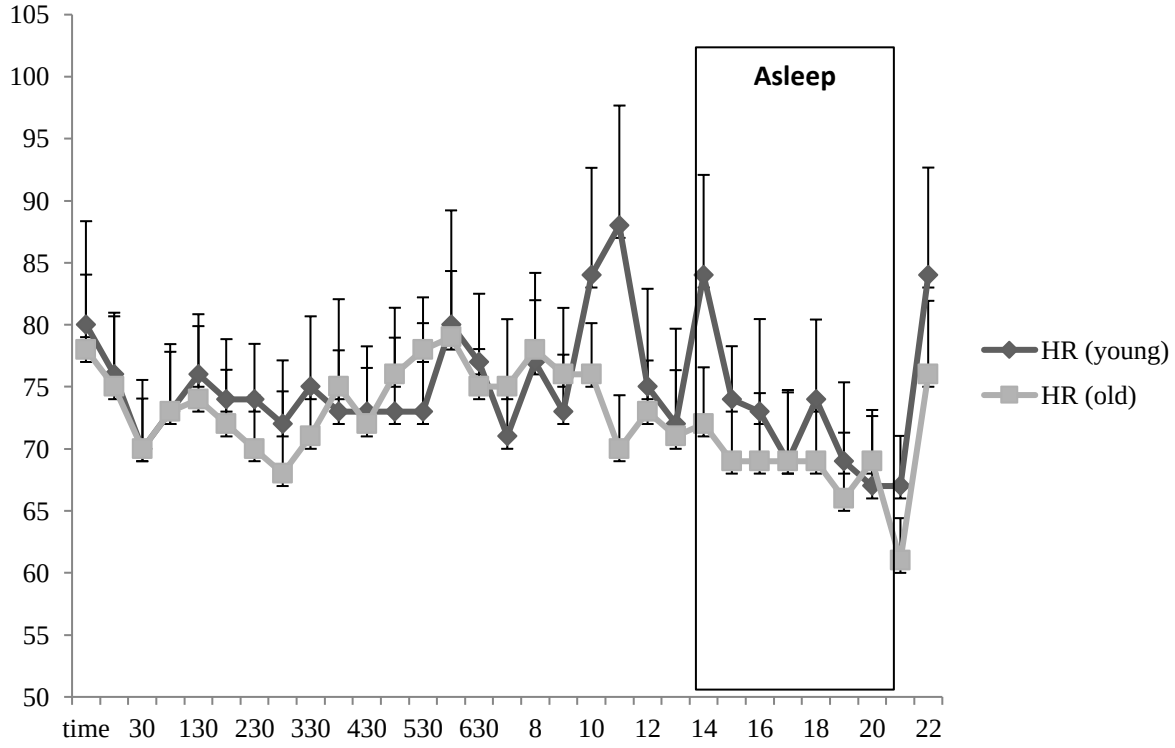


Figure 3-4: Single dose hourly heart rate of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

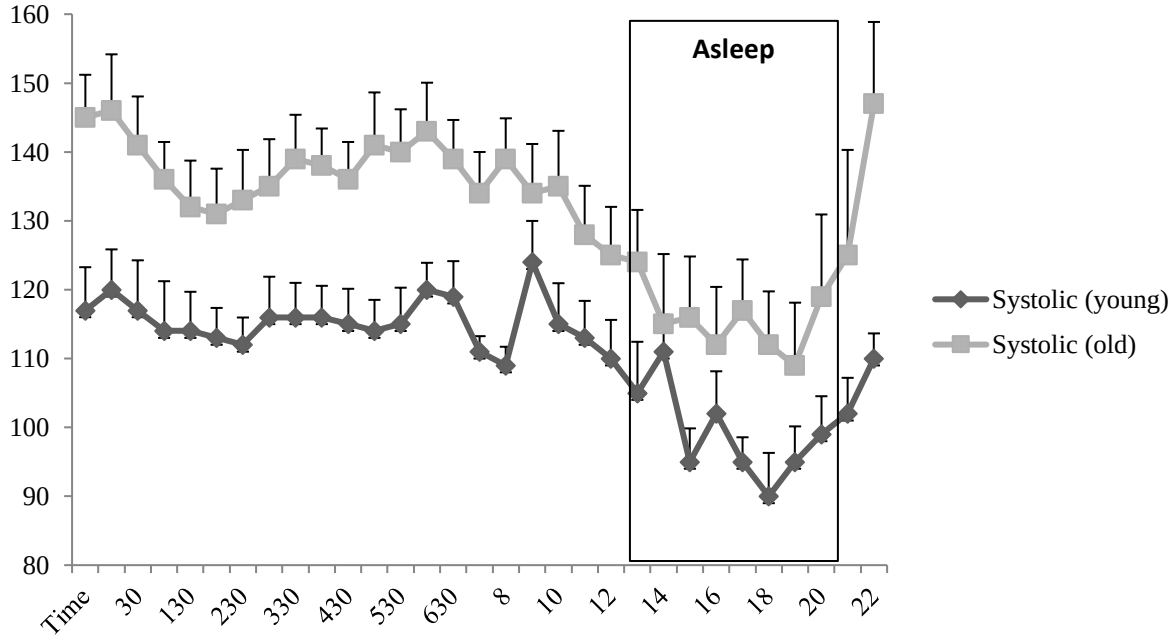


Figure 3-5: Triple dose hourly systolic blood pressure of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

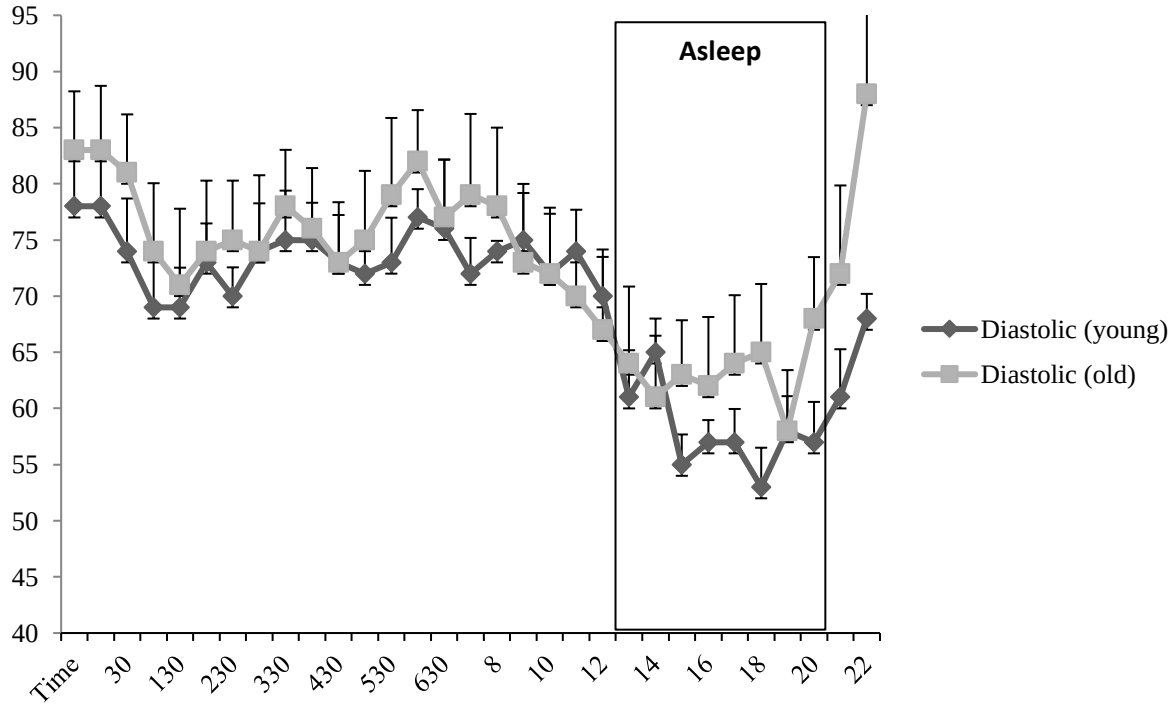


Figure 3-6: Triple dose hourly diastolic blood pressure of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

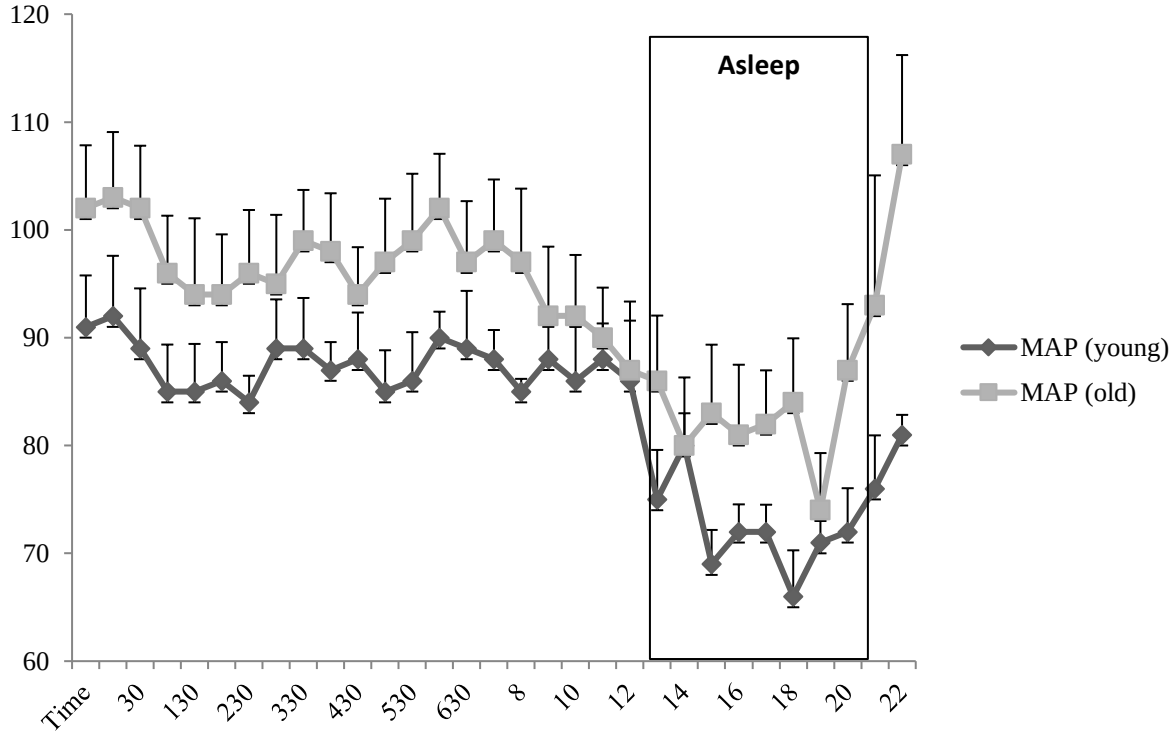


Figure 3-7: Triple dose hourly mean arterial blood pressure of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

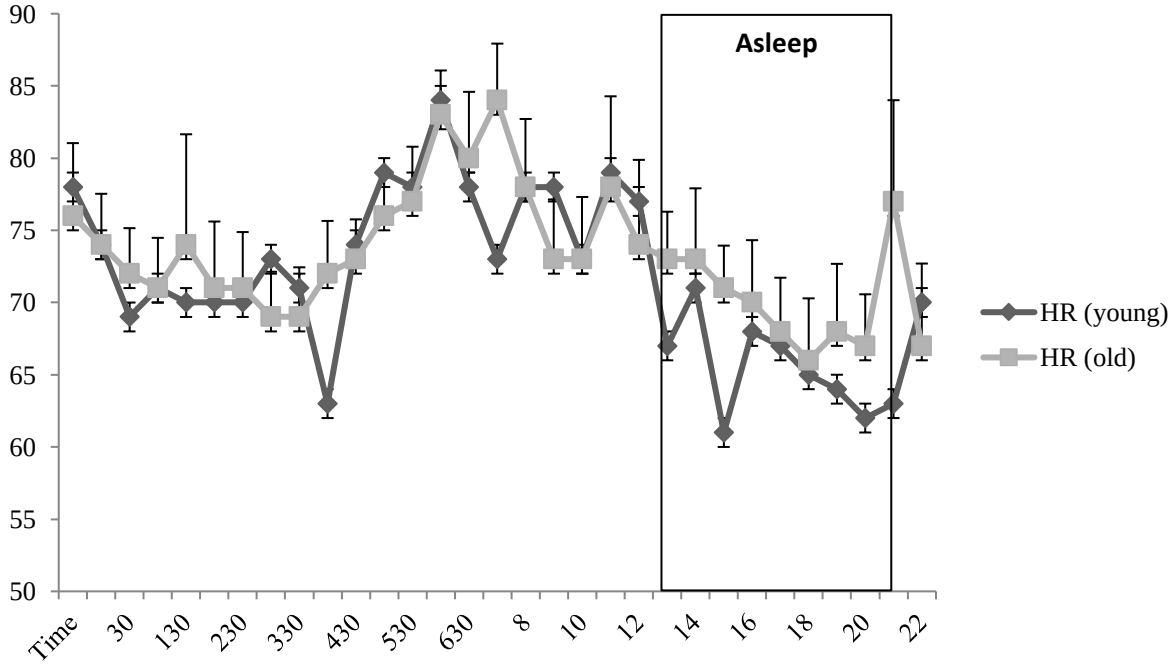


Figure 3-8: Triple dose hourly heart rate of participants by age group over 24 hours after consumption of QGPJ. Values are means n=12 with SE as error bars.

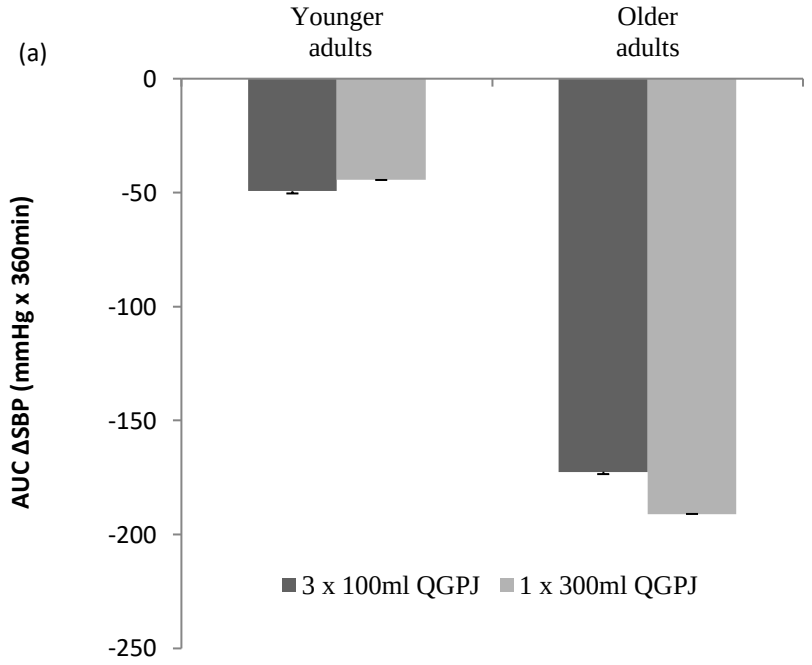


Figure 3-9: Change in systolic blood pressure (0-6h) following consumption of QGP juice.

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

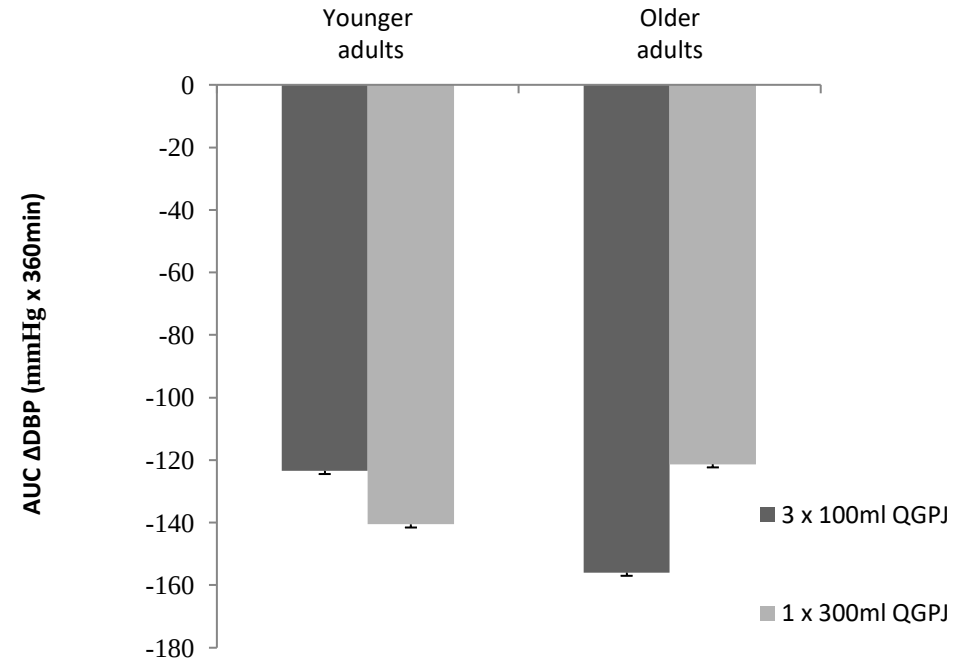


Figure 3-10: Change in diastolic blood pressure (0-6h) following consumption of QGP juice.

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

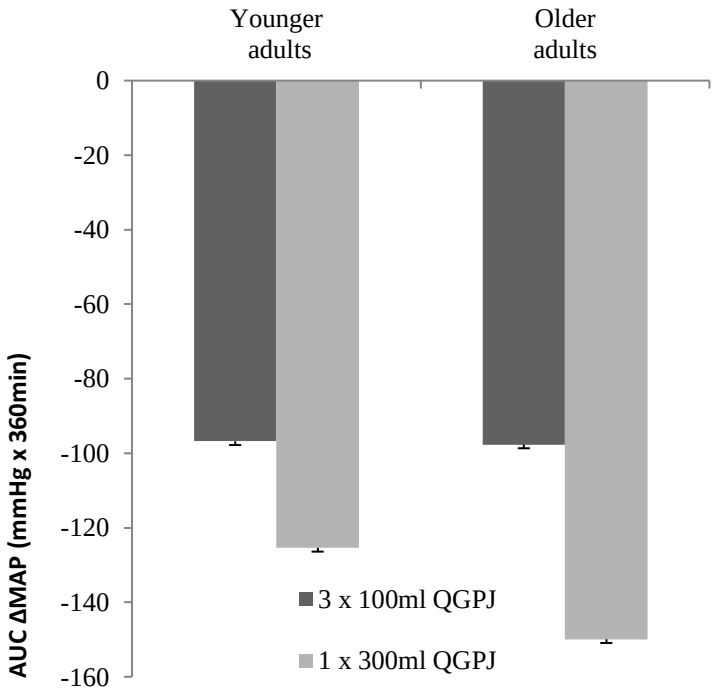


Figure 3-11: Change in MAP (0-6h) following consumption of QGP juice**.**

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

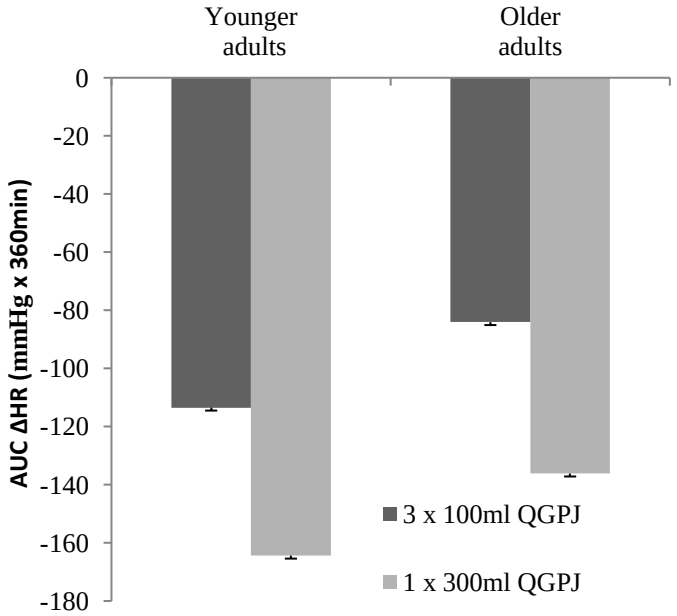


Figure 3-12: Change in heart rate (0-6h) following consumption of QGP juice.

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.

3.1.5 Urinary excretion of anthocyanins and anthocyanin metabolites

The anthocyanin content of the batch of QGP utilised for our study was 123mg/100g [13]. The consumption of QGP juice as a single oral dose of 300mL or in 3 x100mL servings over 3h resulted in the appearance of both intact/non-metabolised 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.

Table 3-6: Urinary excretion of anthocyanins and anthocyanin metabolites in different age groups following the consumption of QGPJ as a single oral dose of 300 mL or as three 100 mL servings

	Dosing (anthocyanins)	**Absolute excretion (µg/24 h)[1]**	**Relative excretion (%)[2]**	**Relative excretion of main metabolites (%)[3]**
Younger age group (n=12)	1x300 mL (369 mg)	811 ± 702	0.22	80
	3x100 mL (123 mg/dose)	759 ± 358	0.21	74
Older age group (n=12)	1x300 mL (369 mg)	871 ± 602	0.24	80
	3x100 mL (123 mg/dose)	693 ± 458	0.19	75

Data are means ± sd; [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 analysed by HPLC-PDA-MS and quantified by an external cyanidin-3-glucoside calibration curve [187].

3.2 Discussion

Following consumption of a single dose of 300mL QGPJ, 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 ($p<0.001$), with a borderline effect on SBP ($p=0.06$) on the triple dose. The acute significant reduction in blood pressure at 2h corresponds with evidence on the maximum absorption and bioavailability of anthocyanins that occurs within 2h post consumption [226]. Anthocyanin concentrations in the body have been observed to reach peak levels between 1-2h and begin to clear from 6h, falling back to baseline levels as they get excreted from the body up to 48h postprandially [47]. The synergistic effect of other nutrients in the QGP cannot be overlooked. There is a possibility that the observed blood pressure lowering effect may have been as a result of this synergistic effect, as well as the presence of potassium in the QGP fruit [13] which is an electrolyte known to lower blood pressure in humans [227]. Despite lack of a significant effect of dose by the different age groups on blood pressure, the greater reduction in blood pressure in response to plum juice consumption in older adults may be explained by their higher baseline blood pressure levels [196]. However, further adequately powered studies are needed to confirm these findings. In the 6h following the QGPJ consumption, no significant effect was observed on the SBP of the younger age group. Similar observation has also been made in a previous study [228] where it was observed that the PacDASH (Pacific Kids Dietary approach to stop hypertension) trial did not affect overall diet quality which was measured by SBP change among other parameters but had a significant effect on DBP by the end of the intervention, by 12.2 mmHg. There is a possibility that the absence of a significant effect on SBP could be associated with age, with interventions having a more pronounced effect among older populations and/or those more prone to age-related vascular stiffening associated with an increased risk of developing CVD.

There was an observed dose-timing and group effect on SBP but not on other blood pressure parameters, however, this was no longer significant after inclusion of an interaction term (age-group x dose-timing). Previously, a similar study observed an acute reduction in blood pressure (SBP, DBP and HR) after consumption of anthocyanin-rich cherry juice, which was found to be dose-timing dependent [214]. The difference between the two studies may be explained by a much higher concentration of anthocyanins in the

QGP juice (123mg/100mL vs 69mg/100mL, respectively), resulting in a physiological threshold that may have been reached in each of the three single 100mL doses.

A possible explanation/mechanism of the observed blood pressure lowering effect of QGPJ could lie in the molecular structure of its main *in vivo* anthocyanin metabolites. Methylation of cyanidin glycosides by catechol-O-methyltransferase (COMT) results in the production of peonidin based metabolites which are the main urinary anthocyanin metabolites after QGPJ ingestion. This reaction also results in a structural modification of the B-ring in the flavonoid skeleton which is structurally analogous to apocynin, an established vasoactive drug [229-231]. Mono-O-methylated anthocyanins/flavonoids can act as inhibitors of NADPH oxidase and as a result can improve vasodilatory processes [231]. Another possible explanation for the blood pressure lowering effect of anthocyanins and/or other polyphenols present in the plum juice is their potential to inhibit the oxidation of Low-Density Lipoproteins (LDLs), a major risk factor for atherosclerosis, through free-radical scavenging and removal of metal ions from catalytic sites via chelation [232]. One study tested this theory and observed that when cells were incubated with OxLDL (100 µg/mL) for 24h, there was an increase in cell death while the additions of mulberry water extracts and mulberry anthocyanin-rich extracts beyond the concentrations of 0.1 and 0.05 mg/mL, respectively, significantly increased the survival of these cell macrophages. In addition, the authors observed that 1 mg/mL of mulberry water extract and 0.1 mg/mL of mulberry anthocyanin-rich extract suppressed the lipid accumulation by approximately 55% and 58%, respectively [233].

Even though anthocyanins have been hypothesized to exert beneficial effects on cognitive functioning, results from our study show that a 300mL serving of QGPJ, regardless of dose-timing or age of participants, had no significant acute effect on various domains of cognitive function. Although previous studies have found no significant acute effect of anthocyanins from fruit sources on cognitive processes [216, 234], the QGPJ used in the present study had a significant higher content of anthocyanins and therefore it was hypothesized that it might induce acute cognitive benefits. In addition, two different cognitive tests that have been shown to be sensitive and target different domains were utilised, namely the Stroop and the Reaction time tasks [217, 235]. Extensive research has been carried out on the long term effect of flavonoid supplementation on cognition [217] with less attention on their acute effects. Recently, there has been an increase in the body of evidence on the acute effects of flavonoids on cognitive processes such as attention, working memory and psychomotor speed in a general population [236]. The precise mechanism by which anthocyanins affect cognition is still not clear but seems to

be dependent on the exposure period. Acute effects on cognition are believed to be as a result of increased cerebrovascular blood flow and possibly monoamine oxidase (MAO) inhibition which has been shown to improve cognitive performance [18, 237]. Following consumption of high anthocyanin fruit/juice, evidence shows that peaks in cerebral blood flow, vasodilation, and anthocyanin metabolite availability is detectable within 2h post consumption [238]. Following blueberry supplementation, plasma anthocyanins and their metabolites were observed to reach peak levels in plasma at 1–2h and 6h [55]. An investigation on the bioavailability of anthocyanins observed an association between colonic 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 from the colon [239, 240]. For this reason, repeat cognitive tasks were administered 6 h post consumption of the plum juice in our study. The absence of a significant effect could be attributed to the timing of cognitive task administration, possibly missing the initial peak action time. Previous research has reported conflicting results regarding the influence of flavonoids on cognition in younger and older people. In one study, anthocyanin-rich blueberry fruit supplementation in younger and older adults resulted in improvements in different acute cognitive domains, whereby a significant improvement in updating ability (constant monitoring and tracking of working memory representations) was reported for younger adults, while improvements in immediate word recognition in older adults were identified [241]. In relation to cocoa flavonoids, consumption of dark chocolate for one week significantly improved endothelial function and reduced BP in younger hypertensive patients, but not in older populations [242]. Overall there is little information that compares responses between younger and older adult populations thus more work comparing these groups is required to elucidate any age-related differences in biological response.

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 in the current study, 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 sources (0.01 – 5.10%) [21, 48, 107]. The bioavailability of anthocyanins has been reported to be low however a recent review indicates that it may be higher than previously reported [243]. Evidence from the review showed that the majority of ingested anthocyanins do reach the large intestine. Here, they are catabolised by the microbiota, producing an array of phenolic components that are absorbed, and some metabolised to phase II conjugates [243]. Furthermore, our finding that methylated and glucuronidated

derivatives of cyanidin-based anthocyanins were the main urinary metabolites is also in agreement with others [187, 244, 245]. 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 [246].

The presence of pelargonidin monoglucuronide, when QGPJ does not contain any (detectable) pelargonidin based anthocyanins, could be explained by the *in vivo* xenobiotic and gut bacterial metabolism of anthocyanins/flavonoids. This includes addition and removal of methyl and hydroxyl groups (pelargonidin is lacking one hydroxyl group compared to cyanidin) [31]. This was also reported in a different study in which significant amounts of pelargonidin based metabolites were detected in the urine of 17 study subjects following consumption of blueberry juice which also did not contain any detectable pelargonidin glycosides [31]. Inter-conversion of anthocyanins due to xenobiotic and bacterial metabolism was suggested by these authors. In the current study, there were no significant differences in the urinary anthocyanin/metabolite excretion profiles either between the age groups or according to the different dosing regimens (1x300 mL dose or 3x100 mL servings).

The main objective with the dose-timing design was to estimate the response according to the dose given. It is worth noting that there were no adverse reactions recorded. Throughout the course of the study, the juice provided was well tolerated and there were no reports of any adverse effects, however the tolerability to the study protocol was not objectively measured. As there is large observed inter-individual variation in the absorption, metabolism and excretion of polyphenols [41], the use of a cross-over study design is appropriate since participants act as their own controls [247].

A notable limitation of our study is the absence of a placebo arm. A placebo (control) arm is essential in dietary intervention studies that investigate the magnitude of effect related to a dietary factor of interest. In the case of anthocyanins, Johnson et. al. (2015)[248] included a placebo control group in their blueberry powder (469mg of anthocyanins/day) study and identified an intervention effect of a 7mmHg and 5mmHg drop in SBP and DBP respectively after 8 weeks ($P<0.05$ and $P<0.01$, respectively). The main purpose of our acute study in which each participant acted as their own control was to identify whether different dosing regimens of a high anthocyanin fruit juice resulted in differences for ether cognitive performance and/or BP. Information related to

the dose-timing administration of an intervention is an important consideration in clinical trial designs in free-living participants. Furthermore, neither intact anthocyanins nor their common metabolites such as glucuronides, sulfates or methylated forms are usually detectable in urine of placebo/control groups, as was demonstrated in a pilot study of QGPJ [107]. 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 may relate to a poor understanding of how dose-timing may affect biological responses.

Another notable limitation is the absence of a blinding 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 at 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 [249, 250]. There is a possibility that a greater BP response would result in more pronounced benefit to 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.

3.3 Conclusion

In conclusion, this study hypothesis was rejected 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 the urine following consumption, but no differences were observed in the absorption rate and metabolism of anthocyanins between young and older adults, as assessed in urinary

biomarkers. 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 to determine effects on cognition in longer-term consumption studies. The greater BP reduction observed in older participants indicates that future studies should focus on this age group where elevated BP is more prevalent, by using a placebo-controlled design. As a first step, an important consideration relates to further understanding the metabolism and subsequent actions of anthocyanins in the colon. This could shed some light on the possible association between anthocyanin intake and observed beneficial health effects. With evidence showing that anthocyanins are further broken down in the colon, it is worth understanding the plausible mechanism and how this could be incorporated into future trials.

4 Chapter 4

4.1 A SYSTEMATIC LITERATURE REVIEW ON THE EFFECT OF ANTHOCYANINS ON GUT MICROBIOTA AND METHODS OF MICROBIOTA DETERMINATION

In order to further the understanding of anthocyanin metabolism and absorption, the role of gut microbiota needs to be examined. The gut microbiota plays a significant role in anthocyanin metabolism. Evidence has shown that in the gut, anthocyanins are metabolised and, in the process, modulate bacterial species and exert bioactive effects through this interaction. A systematic literature review was undertaken to determine the level of current evidence for the association between anthocyanin intake and changes in gut microbiota. Findings from this review showed that anthocyanins induced a significant proliferative effect on *Bifidobacterium spp.,* known for their wide use in probiotics and for the treatment of Irritable Bowel Syndrome (IBS). There was also an observed inhibition of *Clostridium histolyticum,* which have been shown to be pathogenic in humans. Nonetheless, very limited research has been carried out in the area of anthocyanins and gut microbiota and further clinical trials in humans are needed to confirm changes to gut microbes.

The majority of this chapter is the substantive content of the published article: (Appendix F)

Igwe E.O., Charlton K.E., Probst Y.C., Kent K., Netzel M.E. (2018) A systematic literature review of the effect of anthocyanins on gut microbiota populations. J Hum Nutr Diet. https://doi.org/10.1111/jhn.12582

Specific role/authorship of all the authors: EI, KC and YP designed the study. EI and MN contributed to the literature search. EI produced the summary tables and EI and KK produced the first draft of the manuscript. All Authors contributed to writing and editing the manuscript and approved of the final version of the paper submitted for publication

4.1.1 Introduction

The metabolism of anthocyanins has been well reviewed [48]. Briefly, anthocyanins consumed through the diet have shown to be poorly absorbed by the body, with only a small proportion of intact anthocyanins able to pass through the gastrointestinal wall using active transporters like the sodium-dependent glucose transporter 1 (SGLT1) and facilitative glucose transporter 2 (GLUT 2) [251]. Anthocyanins that are absorbed in the GI tract are mainly found as methylated, sulphated or glucuronidated forms but also as intact glycosides in very low concentrations in biological fluids such as blood (plasma) and urine (10 to 2000 nM) [48]. Even though bioavailability of intact anthocyanins in the body is reportedly low, a considerable amount of (food) matrix-bound anthocyanins can reach the large intestine and the colon. Here they undergo further intensive metabolism and degradation and are absorbed into the blood stream, with some of the subsequent metabolites showing potential to be more biologically active than the intact anthocyanins [48, 252]. The recent interest in the gut microbiota and its role in human health has implications for furthering the understanding of the effect of anthocyanins on gut microbiota, and subsequent health benefits [253].

The gut microbiota, which is the microbe population living in the intestine, contain tens of trillions of microorganisms, and is made up of at least 1000 different species of identified bacteria [254]. The majority of the bacteria in the gut are categorised under seven phyla, namely Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria with the Firmicutes and Bacteroidetes phyla making up over 90% of the human gut microbiota [255, 256]. Some of the essential functions of the gut microbiota include vitamin production, regulation of lipid metabolism, short chain fatty acid production as fuel for epithelial cells and regulation of gene expression [257] as well as an important functional role in the gut-brain axis relationship [258]. A heathy gut, which comprises of effective digestion and absorption of food, absence of GI illness, normal and stable intestinal microbiota, and effective immune status, is required in order to sustain a host homeostasis [259]. Irregularities and imbalances in the microbiota at different ages have been linked to different diseases and metabolic conditions across the lifespan, ranging from allergies in young infants to Inflammatory Bowel Disease in young adults [260]. Research on the gut microbiota continues to shed light on the health effects of changes to the gut microbiota modulated by diet.

Different techniques have been described for the determination of gut microbiota. Advancement in the taxonomical composition of the gut microbiota was limited until the 21st century due to bacteriological culture being the only method available to determine its composition. Even to date, only about 30% of the gut microbiota has been cultured [255]. Further understanding of the human gut has been improved by advanced techniques independent of bacteriological culture. Prior to these advancements, culture and biochemical typing were the gold standards for identification of bacteria species but have now been overtaken by methods that are able to produce a more representative information of the overall microbiota [261]. Examples of some of these techniques are sequencing of the 16S rRNA gene or its amplicons, fluorescence *in situ* hybridization (FISH), quantitative polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). With these methods researchers are able to obtain qualitative and quantitative information on the diversity of the gut microbiota, as well as detect changes in the gut microbiota in relation to an intervention or disease [262]. Improved scientific methods of gut microbiota determination have also presented the issue of suitability of the different techniques used, and how the results can be compared to one another.

Alterations of the gut microbiota have been shown to induce and promote or reduce the risk of chronic diseases. This hypothetical mechanism has been applied to the field of obesity and obesity-related non-alcoholic fatty liver disease in relation to both prebiotics / probiotics [263, 264] and anthocyanins [265] as gut modifiers. With this question in mind, given this rapidly advancing area of research in gut health, a systematic literature review has been undertaken to summarise the current evidence on the effect of anthocyanins on the gut microbiota. The included studies are also assessed for the different techniques used in microbiota determination. It is hypothesised that supplementation of the diet with anthocyanin rich foods promotes the proliferation of healthy anaerobic bacterial populations, while inhibiting the pathogenic species, as evidenced using a range of gut microbiota determination techniques.

4.1.2 Methods

A systematic literature review was conducted according to recommendations of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement and checklist [110] (Figure 4-1).

Scientific databases including Scopus, PubMed, ScienceDirect, Web of Science, and MEDLINE were searched up to June 2017. A combination of search terms with truncations included the following keywords: anthocyanins and gut microbiota or colon microbiota (see Appendix B for search strategy). The review was registered on PROSPERO (International prospective register of systematic reviews) as CRD42017073750 [266]. Eligibility criteria for included studies (Table 4-2):

1. carried out either *in vitro*, on animal or conducted in human subjects;
2. quantified the effect of anthocyanin on gut microbial population and described the method used;
3. utilised anthocyanin extracts or anthocyanin-rich foods with anthocyanin (individual compounds or total) content measured and stated;
4. assessed changes in gut microbiota associated to anthocyanin metabolism in the gut;
5. reported in the English language for reasons of time efficiency and cost of translation not being feasible for this review.

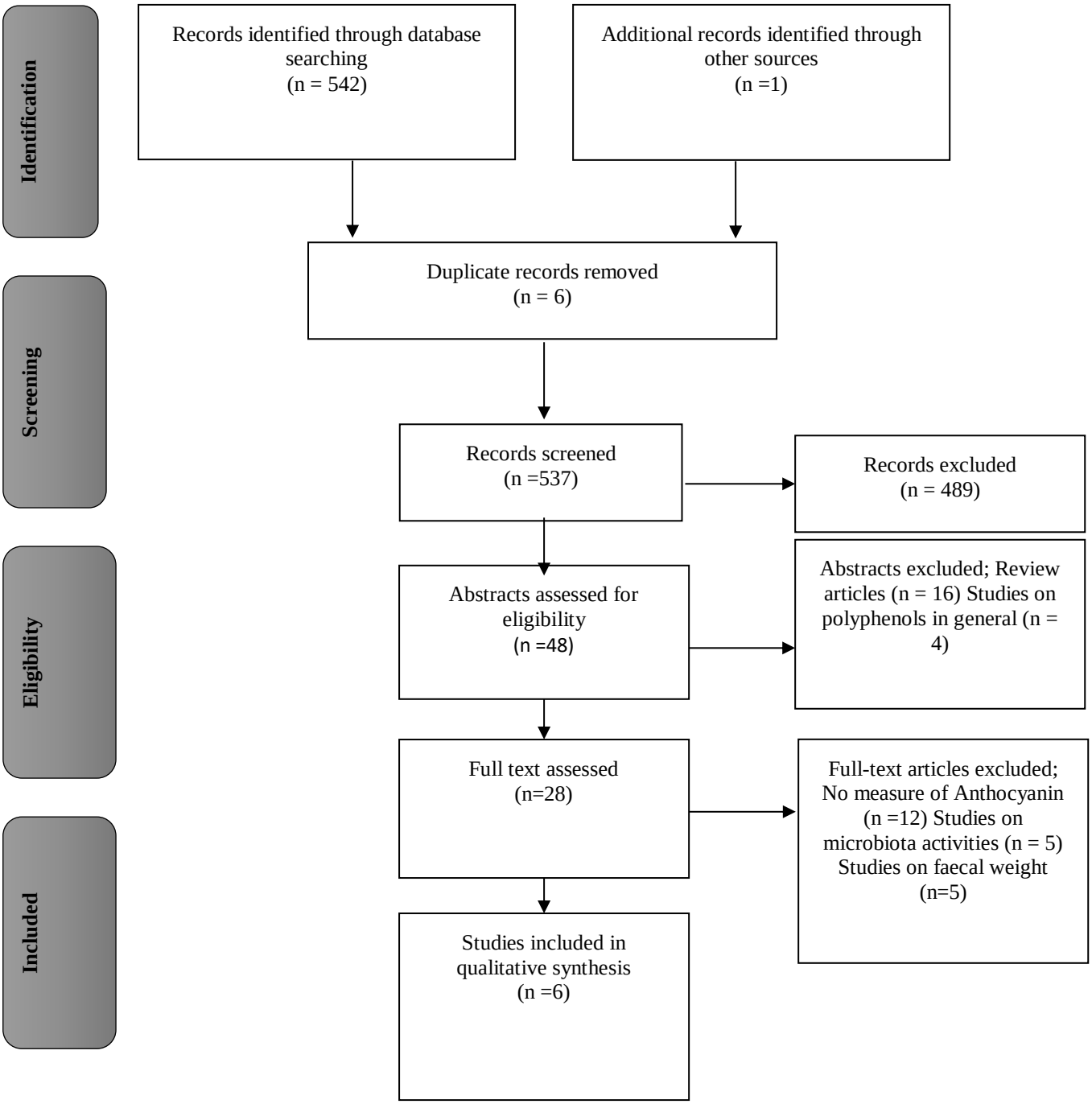
Exclusion criteria

Studies were excluded if:

1. they measured the stability of anthocyanins in the gut without quantifying effects on gut microbial population;
2. there was no measure of anthocyanin content in food/beverage source. One study [267] met this criteria but was included because anthocyanin content was measured indirectly through the use of anthocyanin metabolites in the urine and was the only included clinical trial;
3. they measured total polyphenols or flavonoids in general and not specifically the subclass of anthocyanins.

Review articles were excluded from the search but were considered for hand searching. Hand searching of reference lists was undertaken for all included studies and review articles. All selected abstracts and citations were exported from the scientific databases to the reference management software ENDNOTE X7 (Thompson Reuters, New York, NY, USA). Following extraction and selection of publications according to the above eligibility criteria a tabular summary was developed for this review which included the population, design, intervention, anthocyanin dosage, health outcomes and results.

Figure 4-1 PRISMA flowchart for study selection



4.1.3 Results

The database searches returned 542 articles and one additional study was identified through hand searching of reference lists. Following removal of duplicates and screening of records, 28 full text articles were evaluated and only six studies were eligible and therefore included in the review using the PICOS criteria [268] (Figure 4-1). The included studies are summarised in Table 4-3 and briefly described below.

4.1.3.1 In vitro studies

Three *in vitro* studies were included in this review. Anthocyanins were tested individually or in a mixture, in the case of enocianin, a commercial food colouring from *Vitis Vinifera* grape peel which contains a mixture of anthocyanins including malvidin-3-glucoside (200 mg/L and 20 mg/L respectively), delphinidin-3-glucoside (60 mg/L and 6mg/L respectively), petunidin-3-glucoside (85 mg/L and 8.5 mg/L respectively), peonidin-3-glucoside (traces), and cyanidin-3-glucoside (traces). With high and low concentrations reflecting different levels of intake, anthocyanins showed proliferative effects on beneficial bacterial populations and inhibitory effects on pathogenic species. Incubation of malvidin-3-glucoside with human faecal slurry at 0 (control), 5, 10 and 24 h, showed a significant, ($p<0.05$), increase in total bacteria at 24h. Significant proliferative effects were also observed in the bacteria species with beneficial effects such as *Bifidobacterium spp.* and *Lactobacillus spp.* known for their wide use in probiotics and for the treatment of ulcerative colitis, Irritable Bowel Syndrome (IBS) and constipation [269]. A reduction, although not statistically significant, was observed in the potentially harmful *Clostridium histolyticum* following 24 h incubation. For enocianin, a statistically significant increase ($p<0.05$) was observed in *Lactobacillus spp.* and *Bifidobacterium spp.* following all incubation durations. Bacterial levels were significantly higher than those observed with maldivin-3-glucoside, however, no significant change was observed in *C.coccoides- Eubacterium rectale* group which are known for the critical roles they play in immune homeostasis [270] or the pathogenic *C. histolycum* group [271].
Similar effects have also been observed with purple sweet potato anthocyanin extracts (cyanidins and pelargonidin based anthocyanins) using faecal samples. Following fermentation at 0, 6, 12, and 24 h, a significant increase ($p<0.05$) was observed for the total bacterial count. In comparison to the control medium, a steady increase was observed in the numbers of the *Bifidobacterium spp.* following incubation with purple sweet potato anthocyanins while a significant increase was observed in the bacterial population of

Lactobacillus/Enterococcus spp. On the contrary, the control media had a steady increase in the *Bacteroides-Prevotella* and *Clostridium histolycum* during fermentation, whereas in the media containing anthocyanins, levels were significantly reduced ($p<0.05$).[272]

On the release, metabolism, and effect on gut microbial growth of cyclodextrin encapsulated anthocyanins (cyanidin-3-glucoside, delphinidin-3-glucoside and malvidin-3-glucoside) on faecal slurry, between 0 and 24h, malvidin-3-glucoside showed no significant effect on the population of *Bifidobacterium spp., Clostridium coccoides/Eubacterium rectale* group, *Lactobacillus/Enterococcus spp., C. histolycum* group, *bacteroides spp.,* or *Clostridium* cluster IX. However, a significant increase was observed for the members of the domain *Bacteria (*EUBmix probe). Cyanidin-3-glucoside and delphinidin-3-glucoside on the other hand showed a significant ($p<0.05$) inhibition of the *C. histolycum* group [273].

All three *in vitro* studies utilised the FISH technique for enumeration of bacterial populations.

4.1.3.2 Animal studies

Using six different berries (blackberry, blackcurrant, black raspberry, blueberry, Concord grape, and maqui berry) with structurally diverse anthocyanin profiles, Overall et. al. (2017) [274] supplemented the diets in a mouse model of polygenic obesity. Anthocyanins from these individual berries was normalised to 400µg/g food of total anthocyanins and supplemented with animal chow for each berry category. Six-week old mice were fed LFD (Low-fat diet) or HFD (High-fat diet) for six weeks to initiate the development of obesity in the HFD animals. Thereafter, the HFD animal group were randomised to different berry supplemented diets for a further 12 weeks. Faecal samples were collected from the cages, weighed, and pooled at different time points (weeks 4, 8, 9 and 12) and analysed at the end of the study. Using quantitative real-time PCR, analysis of the bacterial phyla relative abundance in the faecal samples showed that berry supplementation with blackberry and black raspberry did not change the gut microbial population shift that was observed with either LFD or HFD. Supplementation with Concord grape showed a significant increase of the Actinobacterial populations from 2% to 8% in comparison to HFD controls which were similar in kcal/g but without anthocyanin supplementation. Blueberry and blackcurrant supplementation also showed a significant increase in the populations of obligate anaerobes Bacteroidetes from 7% to 10%-12% and Actinobacteria from 2% to 9%-15% which are some of the bacteria species beneficial to humans. Translating the anthocyanin consumption in these animal studies to the

human context , the authors suggested it was equivalent to consuming 2.4 mg/kg/day or 145 mg/day of total anthocyanins for an average adult [275] which could be achieved by daily consumption of 1–2 servings of fresh anthocyanin-rich berries [276].

Similarly, Lacombe et. al. (2013) [277] examined the effect of dietary supplementation of lowbush wild blueberries (LWB) on the colonic microbial population of Sprague Dawley rats. Following control diets or LWB-supplemented diets for six weeks, analysis of the colon contents of the rats using shotgun sequencing showed a significant reduction in the relative abundance of the genera *Lactobacillus* and *Enterococcus* associated with the berry intervention. There was also a significant ($p<0.05$) two-fold increase in the relative abundance of Bifidobacteriaceae and Coriobacteriacaea and the phylum to which they belong, Actinobacteria, in the LWB-supplemented group.

4.1.3.3 Human clinical trials

A randomised crossover clinical trial tested the association between changes in faecal microbiota following wine interventions. Nine male participants were randomized to receive daily 275 mL of alcoholised red wine or dealcoholized red wine or 100mL of gin for 20 days. Participants' usual baseline dietary habits and pattern and lifestyle were maintained, and additional alcoholic beverages were avoided for the duration of the study. At baseline, and following each intervention period, faecal samples were collected from participants. No significant differences in daily energy and dietary intake were observed between baseline and after each intervention. Quantification of the microbial content of faecal samples was done using quantitative real-time PCR. Results showed that in comparison to gin, both red wine and dealcoholized red wine significantly ($p = 0.001$) increased the faecal concentration of *Bifidobacterium, Enterococcus and Eggerthella lenta.* Although the anthocyanin content of the red wine intervention used in this study was not measured prior, results showed that the lowest to the highest changes in Bifidobacteria tertiles was associated with a higher excretion of four phenolic metabolites related to anthocyanin metabolism in participants[267].

Table 4-1: PICOS criteria for inclusion and exclusion of studies

Parameter	Inclusion	Exclusion
Participants	Humans, animals and cell cultures (In vitro)	None
Intervention	Quantified anthocyanins	Other polyphenols or flavonoids
Comparator	Negative controls or different foods/diets/nutrients	None
Outcomes	Changes in gut microbiota	Stability of anthocyanins in the gut
Study design	Randomised and non-randomised experiments	None

Table 4-2: Summary of studies included in the systematic review

Reference	Population/ Sample	*In vitro* / *in vivo*	Design (Intervention/control)	Anthocyanin Dosage mg/100g of food (reported dosage)	Method of microbiota determination	Result
***In vitro* studies**						
Hidalgo, et al (2012) [271]	pH-controlled, stirred, batch-culture fermentation system reflective or mimicking of the distal human large intestine conditions	*In vitro*	Enocianin- commercial food colouring from *Vitis Vinifera* grape peel anthocyanin-rich extract / malvidin-3-glucoside (individual anthocyanin)	34.5 mg (345mg/L)	Fluorescent in situ hybridization (FISH)	Anthocyanins significantly enhanced the growth of *Bifidobacterium spp.* and *Lactobacillus-Enterococcus spp.* This increased proliferation was higher after enocianin treatment. $p<0.005$
Zhang, et al. (2016) [272]	Fresh faecal samples from 8 healthy volunteers (25-30y)	*In vitro*	Purple sweet potato anthocyanins / Fructooligosaccharide (FOS) (prebiotic)	706mg (7.06mg/g) treatment: 1% (w/v)	Fluorescent in situ hybridization (FISH)	Purple sweet potato anthocyanins induced proliferation of *Bifidobacterium* and *Lactobacillus/Enterococcus spp.* and inhibited the growth of *Bacteroides-Prevotella* and *Clostridium histolyticum.* They did not affect the total bacteria count.
Flores, G., et al (2015) [273]	Fresh faecal samples from 3 healthy volunteers	*In vitro*	Individual anthocyanins / a negative control (w/out anthocyanins)	2mg (20mg/L)	Fluorescent in situ hybridization (FISH)	Significant growth of the domain *Bacteria* and slight inhibition of the *Clostridium histolyticum* group
Animal studies						
Overall, et al. (2017) [274]	Seventy-six 6-wk old male mice/ faecal sample (cage collected)	*In vivo (*Animal study)	Berry supplementation / High fat diet (without berry supplementation)	0.04mg (1.14mg/mouse/day)	Quantitative real-time PCR	Significant increase in obligate anaerobic bacterial and Actinobacteria population in the gut (relative abundance).
Lacombe, et al (2013) [277]	Nine males, three-week old Sprague-Dawley/ colon content samples (faeces)	*In vivo (*Animal study).	Blueberry-enriched diet, (AIN93+8% w/w Lowbush Wild Blueberry powder	(24.06 ± 5.2mg/day)	Microbiome shotgun sequencing	Significant increase in the relative abundance of *Actinomycetales*, and several novel genera under the family Bifidobacteriaceae and Coriobacteriaceae and significant reduction in the relative

Reference	Population/ Sample	*In vitro* / *in vivo*	Design (Intervention/control)	Anthocyanin Dosage mg/100g of food (reported dosage)	Method of microbiota determination	Result
			substituting for dextrose) / Control diet (AIN93) without blueberry supplementation for 6 weeks			abundance of *Lactobacillus* and *Enterococcus*.
Human clinical trial						
Boto-Ordóñez, et al (2014) [267]	9 adult men (45-50y)/ fresh faecal samples	*In vivo (*Randomised crossover-controlled trial (3 consecutive periods of 20 days each))	Randomised crossover-controlled trial (3 consecutive periods of 20 days each); Red wine (RW)/ dealcoholized red wine (d-RW) / gin	9.72mg in RW and d-RW/ nd in gin (272mL (26.44mg anthocyanin))	Quantitative real-time PCR	Significant change in bacteria species (*Bifidobacterium, enterococcus, and eggerthella lenta*) concentration following RW and d-RW intervention ($p<0.001$)

RW- Red wine; d-RW-dealcoholized red wine; nd-not detectable

4.1.4 Discussion

Findings from this systematic review support the hypothesis that consumption of foods high in anthocyanins promotes the proliferation of healthy anaerobic bacterial populations, while inhibiting the pathogenic species. Specifically, the presence of anthocyanins significantly increased the microbial population of *Bifidobacterium spp.* and *Lactobacillus-Enterococcus spp.* This observation was consistent in *in vitro,* animal and human studies [267, 271, 272, 277]. These gram-positive bacteria species have been shown to exert beneficial effects in the treatment of diarrhoea and other specific diseases including inflammatory bowel disease (IBD), necrotizing enterocolitis and colorectal cancer [278].

A number of factors including diet, age and antibiotics are important determinants of the gut microbiota profile, and the influence of these factors continue to change throughout the lifetime [279]. The most significant of these factors is diet. In relation to anthocyanin consumption, a diet rich in fruit and vegetables has been shown to significantly alter the gut microbiota. Included studies showed that anthocyanins have proliferative and inhibitory effects on bacteria species. However, some studies have not observed increases in total microbial populations when samples were incubated with anthocyanins. Zhang, et al. (2016) [272] suggested that anthocyanins contained in purple sweet potatoes, including anthocyanin monomers, may enhance both inhibition and proliferation of different bacteria species at similar rates and thereby not affect total overall bacteria count. In addition Flores et al (2015) [273] attributed this to the amount of anthocyanin used in their intervention study (20mg/L of batch culture) which could have been too low to exert any significant effects. Contrary to these observations, with higher concentration (200mg/L) of anthocyanins, Hidalgo et. al. (2012) [271] observed a significant ($p<0.05$) proliferative and inhibitive effect in microbiota population which was more evident in samples that were supplemented with a mixture of anthocyanins showing the synergistic effect of anthocyanin subclasses. Another possibility is that the observed simultaneous proliferative effect on the beneficial bacteria and inhibitory effect on harmful bacteria by anthocyanins could explain the absence of any significant change in total bacterial population as there exists a natural balance between beneficial and harmful bacteria in the gut [280].

Even though observations in *in vitro* models may not be extrapolated to *in vivo* systems, comparison of composition and concentration of anthocyanins across studies which had a significant effect on gut microbiota showed that lower concentrations with a variety of anthocyanins (Malvidin-, delphinidin-, petunidin-, peonidin- and cyanidin-glycosides) [271] had as much effect as higher concentration with only one class of anthocyanins (cyanidins glycosides) [272]. Although anthocyanins were not the highest concentration of flavonoids or polyphenols in wine [267], it is important to note that anthocyanins, not other polyphenols, was associated with the increased level of *Bifidobacteria* in faecal samples. This was due to the observed increase in microbial metabolites in urine presumably derived from anthocyanins. The anthocyanin content of all interventions used in the included studies was determined using High Performance Liquid chromatography (HPLC) coupled to different detectors. HPLC has been identified as the most reliable method of measuring total anthocyanin content as well as individual anthocyanins in foods and biological matrices in comparison to the colorimetric method that measures only total anthocyanins [281].

With these observed beneficial effects, the exact mechanism of action of anthocyanins in the gut remains unclear. Overall et. al., (2017) [274] observed an increased oxygen tension in all gut compartments associated with high-fat diets which was attenuated by supplementing the diet with berries and berry anthocyanins. As a result, they suggested anthocyanins may reduce oxygen tension in the gut lumen and therefore promote the proliferation of oxygen-sensitive bacterial population. However, in the small intestine, there exists high level of oxygen which limits bacterial growth, such that only fast growing, facultative anaerobes with the ability to bind to epithelial/mucus are believed to survive [282]. This highlights the difference in microbiota composition along the lower GI tract and an important consideration in sampling.

On the issue of sampling, all the studies, but one, included in this review utilised faecal samples for the quantification of anthocyanin effects on gut microbiota. In place of faecal samples, rectal mucosal biopsy has been proposed as a better alternative in gut microbiota research in terms of assessment but not participant burden [283]. Durbán et al. (2011) [284] did a comparative study on the bacterial community composition between faecal samples and rectal mucosal biopsies. Samples were collected from an un-prepped healthy population. Comparison of the two samples showed a significant difference in the bacterial

diversity between faecal and rectal mucosal samples from the same participant. Another study compared healthy subjects to IBS subjects, where they observed that there was a reduction in bacterial abundance and diversity in mucosal samples in comparison to stool samples from the same participants [285]. There is a possibility that significant differences exist in the microbial community across the six major subdivisions of the human colon (cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum). However, the degree to which composition and functions differ remain unclear. Studying the microbial diversity of the human colon, Eckburg et. al. (2005) [255] observed significant inter-individual variability and differences between faecal and mucosa community composition. This discrepancy highlights the importance of sampling sites and raises the suggestion that rectal mucosal biopsy samples are more appropriate than faecal samples and should be used instead or together with faecal samples [262]. Regardless, faecal samples are still preferred over rectal mucosa biopsy due to ease of collection and being less invasive.

Another important consideration in gut microbiota research is the methods of determination and quantification. Although bacterial culture, which was the gold standard in the past, is inexpensive, it produces a limited representation of the gut microbiota diversity and results in undervaluation of real changes. Culture independent techniques have become popular and are now the most commonly used techniques [286]. Most of the different culture independent techniques are based on the analysis of the 16rRNA gene. The 16rRNA genes are highly protected across bacterial species. They also have distinctive characteristics that allows for identification of different species. [262]. Studies included in this review employed different techniques including the Fluorescence in situ hybridization (FISH), quantitative polymerase chain reaction (qPCR) and shotgun sequencing in the determination and quantification of the gut microbiota. These techniques have different pros and cons. The qPCR for example, although fast, is unable to identify unknown species and has an amplification bias in that the primer set used in multi-template PCR is required to have a shared sequence across the targets. As often is the case, this shared sequence is absent in the targets. When a primer has one incongruity with some targets, the amplification efficiency is significantly reduced and as a result, a large bias in the amplification will occur [287]. Even though the FISH technique has no PCR bias, it is dependent on probe sequences which mean that it is also unable to identify unknown species. The shotgun sequencing on the other hand, although more adequate than the aforementioned techniques, is expensive and analysis of data involves a complex software [262].

The major limitation of the PCR and FISH methods in comparison to the shotgun sequencing is their inability to identify unknown species. This limitation does not seem to have affected study results as changes in bacteria species were measured with already known species, hence addressing this limitation. The availability of various methods requires deciding the suitability of a given methodology in gut microbiota research. This decision will be dependent on the depth of analysis. So far, the shotgun sequencing techniques produces the most potent data including projections of microbiota function [262]. Although a comparative study of 16S amplicon and shotgun sequencing on water samples found that less than 50% of phyla identified via 16S amplicon sequencing were recovered from shotgun sequencing while also identifying ~27% more families [288].

A notable strength of this systematic review is the side by side comparison of different methods of microbiota determination as well as effects of anthocyanins. At the time of writing this article, to our knowledge, this systematic review is the first to determine the effect of anthocyanins on gut microbiota, while comparing different methods of microbiota determination. Another possible strength is the specific focus on anthocyanins and their effect on gut microbiota. The synergistic effect of compounds in food (nutrients) presents some difficulty in clearly defining causal association in epidemiological research. It is important to note that the presence of procyanidin polymers in anthocyanin containing foods such as berries and some nuts may also contribute to some of the reported microbiome activity [289]. Procyanidin polymers act as microbial substrates similar to anthocyanins; as a result, synergy might explain some of the observed effects. However, there is limited evidence on the absorption and metabolism of procyanidins due to their polymeric nature and high structural complexity which further limit the plausible explanation of their mechanism of action in health [289]. Although nutrients are not consumed in isolation, determining the specific effects of particular nutrients is an important aspect of nutrition research.

As an emerging area of research in the last decade, there are very few *in vitro* and animal studies and even fewer clinical trials that have been carried out. Consequently, there are limitations in the comparison of results and generalisation of conclusions due in part to the diverse sources of anthocyanins, methods of gut microbiota determination and quantification as well as the possibility of synergistic effects of anthocyanins and/with other phytochemicals and nutrients present in the food sources resulting in the observed benefits. Another notable limitation of this review is the different control diets/samples used which restricts the

generalisation of results. Although a limitation, it is important to note that these controlled samples/diets all had in common the absence of anthocyanins demonstrating the modulatory effects of these natural plant pigments.

4.1.5 Conclusion

In conclusion, research on the gut (microbiota) as a metabolic organ is still emerging and characterisation of bacteria species present in the gut is ongoing. Results from this review observed beneficial effects such as significant proliferative effect on *Bifidobacterium spp.*, known for their wide use in probiotics and for the treatment of Irritable Bowel Syndrome and inhibition of *Clostridium histolyticum*, which have been shown to be pathogenic in humans. Research on the possible effect of anthocyanins on gut microbiota population is still in its early stages and conclusions and generalisations cannot be made due to the limited evidence base and varied techniques employed in studies. This is also due to differences in anthocyanin sources, composition, digestive stability, metabolism and biotransformation of anthocyanins in either a food matrix or supplement isolated. This also makes it difficult to understand or elucidate the exact mechanism by which anthocyanins may exert these effects. Therefore, the complete effect and exact mode of action of anthocyanins on gut microbiota needs more research clarification through well-designed human clinical trials. Further research is required to reach a consensus on anthocyanin dose and form, as well as using a uniform approach to control intervention and background diets. In addition, it is imperative to advance the understanding of the direct or indirect beneficial effects of anthocyanins on bacterial growth through research. An important consideration would be to measure concurrently these observed effects on gut microbiota, as well as other health effects, for example vascular function (BP) and cognition to further understand complex *in vivo* processes such as the gut-brain axis relationship. Determination of key techniques to measure [290] and confirm these findings is also vital in this field.

5 CHAPTER 5

5.1 EFFECT OF CONSUMPTION OF FRUIT ANTHOCYANINS ON BLOOD PRESSURE, COGNITION AND GUT MICROBIOTA POPULATION IN OLDER ADULTS OVER 8 WEEKS

This chapter describes an 8-week randomised clinical crossover trial that was conducted to determine the effect and within subject variance on cognition and 24hr ambulatory blood pressure in older adults without cognitive impairment (55+ years) following daily consumption of 200mL QGPJ (approx. 50mg/L anthocyanin/day) or raspberry cordial (control). Secondary outcomes included inflammatory markers (C-reactive protein), Brain derived neurotrophic factor (BDNF), changes in anthropometrics and physical function, and gut microbiota (16S rRNA gene sequencing). Twenty-eight participants (55+ years) were recruited and over 5 clinic visits, data were collected on all outlined parameters. Each study arm lasted for 8 weeks with a 4-week washout period. Order of assignment to intervention arm was randomly allocated. Cognition, BP and urine samples were measured at every visit while blood samples and faecal samples were collected at baseline, end of 1st arm and end of 2nd arm. Across treatment periods, no significant difference was observed on the different domains of cognition measured, blood pressure nor anti-inflammatory biomarkers. No intervention effect was found for genera or classes of gut microbes, but there was a trend towards significance in total bacterial count between the control arm and the intervention arm (P= 0.06). In this group of healthy older adults, anthocyanins provided from QGPJ did not have any significant effects on cognition, blood pressure and other measured parameters but may have an effect on gut microbiota population. Although results from the acute study (Chapter 3) are not comparable to this study based on experimental design (acute vs chronic intervention), it is important to note that the anthocyanin concentration of the QGPJ utilised for this study was considerably lower than that used for the acute study (263mg/100mL vs 50mg/L). This could have been due to seasonal variations, as well as processing and storage conditions.

Research team: Ezinne Oyidia Igwe, Karen Elizabeth Charlton, Steven Roodenrys, Yasmine C. Probst, Vinicius do Rosario, Michael E. Netzel, Hung Hong Trieu, Gabriele Netzel, and Anh Dao Thi Phan

Specific role/authorship of all the authors: The research team were involved in designing the study. EI, VR, KC and SR were involved in data collection. SR advised on the cognitive tasks. KC and YP advised on nutrition monitoring. MN, HT, GN, and AP advised on and analysed the urine samples for anthocyanin metabolites and were involved in writing the urine section of the manuscript. EI produced the first draft of the manuscript. All Authors contributed to writing and editing the manuscript and approved of the final version of the paper submitted for publication.

5.1.1 Introduction

As scientific evidence emerges showing that anthocyanins are more bioavailable than originally perceived [243], the colon has become an emerging area of research as an active site for further metabolism of ingested anthocyanins [253]. Some of the subsequent anthocyanin metabolites are believed to be more biologically active than intact anthocyanins [48, 252] and are possibly responsible for some of the observed beneficial effects on cognition (gut-brain axis) and vascular function (blood pressure). 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 the largest methodological challenge thus far in determining the health effects associated with anthocyanin consumption [207]. Absorption of anthocyanins in the upper gastrointestinal tract is very low, possibly due to their digestive stability and binding interaction within the food-matrix [291]. As a result, a considerable amount of intact anthocyanins can reach the intestinal microbiota which cleave conjugated moieties, resulting in aglycones. These aglycones undergo ring fission to produce low molecular metabolites including phenolic acids and hydroxycinnamates which are then reabsorbed into the bloodstream where they are biologically active [44, 292]. Evidence from *in vitro* studies suggest that bacterial metabolism of anthocyanins in the colon involves the cleavage of glycosidic linkages and the breakdown of the anthocyanidin heterocycle [252, 293].

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 [294], IBD [295], IBS [296] and allergies [297], has increased exponentially in recent years. The gut microbiota profile is also known to possess an important functional role in metabolism, immunity and maintenance of a healthy human gut [298] as well as in regulating the gut-brain axis [258]. The gut-brain axis (GBA) involves a two-way communication between the central and the enteric nervous system that links emotional and cognitive centres of the brain, with peripheral intestinal functions. It has been shown that even though intact anthocyanins possess protective health effects, their further metabolism and absorption in the colon leads to the generation of bioactive metabolites. In turn, these metabolites also regulate the growth of specific beneficial bacteria in the intestinal microbiota. An example is an observed increase in the concentration of faecal *Bifidobacterium* as a result of anthocyanin microbial metabolites following anthocyanin supplementation [267]. Bifidobacteria

are gram-positive, polymorphic rod-shaped bacteria normally found in the gastro-intestinal tract of humans and animals [299]. In healthy breastfed babies these bacteria are the dominant microbial group and their levels remain relatively stable, tending to decrease with advancing age when bacteria belonging to Bacteroidetes and Firmicutes phyla are more dominant in adulthood [300]. 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.

With the gut now considered as an important metabolic site for anthocyanins, as well as other nutrients, there is a better understanding of the potential mechanism of action of anthocyanins and the significant role of the gut microbiota in this process. In acute and longer term studies, anthocyanins have been shown to have significant clinical benefits on different domains of cognition [201] and blood pressure [61, 301]. These effects have been observed in studies using different dietary sources of anthocyanins [201], and in individuals with mild to moderate cognitive impairment [201] and hypertension [248]. However, information in non-cognitively impaired older adults is limited and the current 8-week randomised controlled trial was designed to address this gap.

The design of this trial was informed by the synthesis of current evidence as reported in the systematic literature reviews (Chapters 2 and 4) in this thesis, as well as results from our acute dose-timing trial (Chapter 3). There was limited evidence on the longer-term clinical effects of anthocyanins in healthy older adults and even less clinical trial evidence on anthocyanin effect on gut microbiota. As a result, the aim of this trial was to determine the effect, and within subject variance, on primary measures of cognition and ambulatory blood pressure in healthy older adults following a daily consumption of QGPJ or a control beverage. Changes in the gut microbiota during the trial were also measured, in order to better understand the mechanism of clinical effects. This study hypothesised that consumption of QGPJ for 8 weeks would have significant positive effects on cognitive function and BP, compared to a control cordial.

5.1.2 Methods

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 (UOW) Human Research Ethics Committee, New South Wales, Australia (HE16/278). This study was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617000220369). Written informed consent was obtained from all eligible participants.

Study design

A randomised, crossover clinical trial ran for a period of twenty weeks. Participants were randomly allocated to one of two arms (8 weeks on each with a washout period of 4 weeks). The intervention arm received QGPJ and the control arm received Raspberry cordial, with similar vitamin C content (Table 5-1). Participants were advised to consume 200mL per day (1 bottle) of the provided beverage for the duration of the study. The duration of the trial was based on a previous trial by members of the research team using high anthocyanin cherry juice [61] which detected changes in measured variables as early as 6 weeks.

In comparison to the acute study where a single dose of 300mL was administered, a dose of 200mL was used in this longer term crossover study as consumption was for 8 weeks and 200mL per day was deemed to be a feasible amount that would encourage compliance for the intervention period [61, 302]. A smaller quantity of anthocyanins provided in the reduced quantity of juice will potentially allow translation to habitual dietary intakes and facilitate practical dietary messaging.

Sample size and inclusion criteria

The sample size for this study was calculated using GPower [303]. Our pilot study as well as a similar previous study [61] found effects of anthocyanin supplementation on blood pressure and memory of a moderate size therefore the aim of this study was to detect a moderate effect of 0.3. To obtain a power of 0.90, n = 30 participants are needed, which includes 10% expected dropout. The cross-over design allows each participant to act as their own control, thus improving power of the study.

Based on sample size calculation, participants aged 55 years and above were recruited. Exclusion criteria included individuals with:

1. Uncontrolled hypertension;
2. Type 2 diabetes mellitus;
3. Any unstable physical or mental health condition including dementia that might confound results;
4. Inability to provide informed consent;
5. Inability to communicate in the English language.

Uncontrolled hypertension, Type II diabetes miletus and any unstable physical or mental health condition were not directly assessed at pre-screening interview, but participants were asked about their last regular medical check-up sessions and whether they had been diagnosed with any of the above prior to enrolment.

Table 5-1: Nutrient composition of the two study beverages

	QGPJ (per 100mL)[a]	Raspberry cordial (per 100mL)[c]
Anthocyanins	5[b]	-
Energy (kj)	213	115
Protein (g)	1.1	0
Total fat (g)	0.1	0
Total carbohydrate (g)	6.4	6.5
Sugars (g)	8.7	6.5
Dietary fibre (g)	<0.1	<0.1[a]
Sodium (mg)	4.8	4
Vitamin C (µg)	<0.05	<0.05[a]

[a]Data from Santhakumar et. al. (2015)[85]; [b]Analysed by University of Wollongong and University of Queensland Chemistry laboratories; [c]Nutrient content from label.

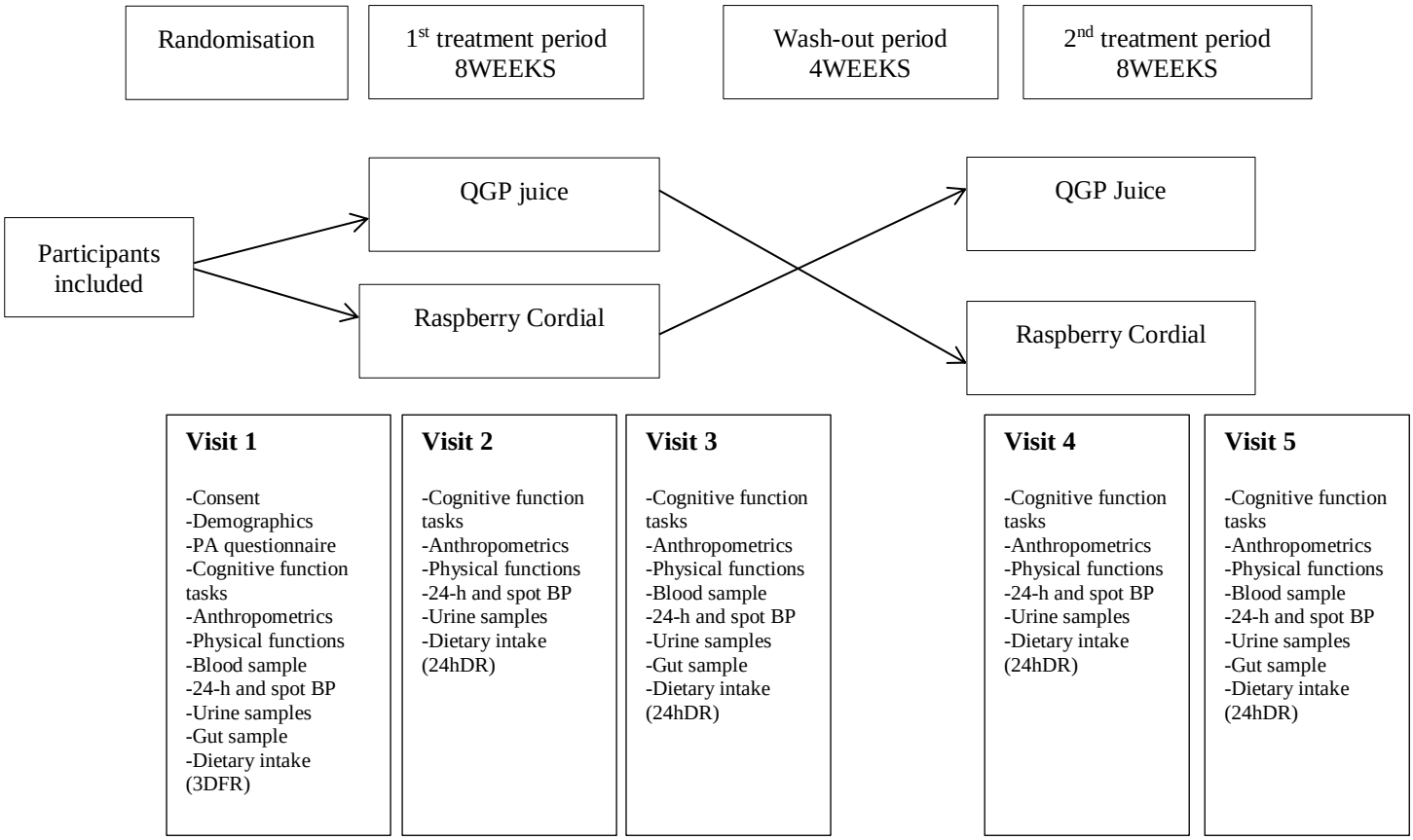
Study Procedure

The QGPJ was donated by Nutrafruit Pty Ltd., while the cordial control beverage (Golden Circle Raspberry cordial) was purchased from a major supermarket chain at a single time-point. The QGPJ was supplied in 20L containers frozen at -20ºC. It was stored frozen in -20ºC freezer for about 8 months prior to the start of the study. At the start of the trial, the plum juice was thawed (in the refrigerator), bottled in

unmarked 200mL plastic containers and frozen again at -20ºC to be delivered to participants. The juice was obtained from two seasonal batches of processing and combined to provide uniform anthocyanin composition for all participants. The juice variant was analysed by two laboratories for anthocyanin content using the colorimetric analysis method (University of Wollongong) and the HPLC method (University of Queensland).

A random allocation schedule prepared by an independent statistician was used to assign participants to either arm. Researchers were blinded to the allocation but due to taste and physical differences, participants were able to determine that there was a difference between the beverages. Participants were required to consume one 200mL bottle of juice daily (approx. 50mg/L) and compliance was recorded from unconsumed returned juice bottles every fortnight. An independent research assistant packed the appropriate beverage in a labelled sealable cooler bag to be delivered to participants' home on a fortnightly basis. No change to regular diet was advised and dietary intake was assessed using estimated 3-day food diaries and 24-h diet recall questionnaires.

Figure 5-1: Study design



Data collection

The primary outcome measure was change from baseline in blood pressure parameters (SBP, DBP, MAP, HR and PP) and cognition measured by 5 different cognitive tasks. Secondary outcome measures included change from baseline in inflammatory biomarkers in blood samples (BDNF and CRP) and gut microbiota. Data collection was performed over 5 visits to the clinical trials research facility: baseline, 4 weeks, 8 weeks which was the end of arm 1 as well as 4 weeks and 8 weeks of arm 2 after crossover. (Figure 5-2). Participants were required to arrive at the research facility after fasting for 10-12 hours for baseline and 8-week data collection visits which involved collection of fasting blood samples. On clinic days, participants were provided with a standardised breakfast (cereal, milk, muffin, and tea/coffee) after blood sample collection and before the administration of the battery of cognitive tasks. For the 4-week visits, participants had their usual breakfast at home prior to attending the testing facility.

At the baseline interview, demographic and lifestyle questionnaires were completed by participants. The International Physical Activity Questionnaire validated by Hagströmer et al. (2006) [213] 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 2 weekdays and 1 weekend day. For baseline and 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.

A battery of five cognitive tests, including The Rey Auditory Verbal Learning test (RAVLT), the verbal fluency task, Digit-span backwards task, the Stroop task, and counting span, was carried out to assess mental functioning of participants as shown in the table 5-1 below. For each cognitive task, accuracy and response time were recorded. Evidence shows that anthocyanins may have protective effects on cognition in both acute and chronic conditions. Different studies have utilised different measures to assess these effects [201]. Some of these protective effects on cognition have been shown to be more sensitive on specific domains of cognition including verbal learning and memory, speed of processing and executive function. As a result, specific cognitive tasks targeting these domains were chosen as study instruments [236].

Enrolment, randomisation and retention of participants are described in Figure 5.2 below. Following dropouts, data for one participant was excluded from ambulatory blood pressure analysis due to significant changes in blood pressure medication and physical activity after completing the first arm of the trial.

Two blood pressure measurements (24h and office) were taken. Twenty-four-hour ambulatory blood pressure was taken using Welch Allyn ABPM (Welch Allyn, NSW, Australia; Model 7100) and office BP measured according to standard protocols [304] using Welch Allyn Spot Vital Signs LXi DXEmed Com. The blood samples collected were spun in a normal centrifuge for 15min at 3000rpm and the serum collected in 2mL sterile tubes and stored at -80^{o}C for batch analysis by an independent laboratory (Cardinal Bioresearch Pty Ltd.) for BDNF (Brain-derived neurotrophic factor) and CRP levels. Serum BDNF was analysed using an R&D Systems Duo ELISA kit. (Intra assay CV 3% and Inter assay CV 4%, Lowest

Detectable Dose 5ng/mL) and serum hsCRP was analysed using a BioBase immunoturbidimetric assay. The assay was run on a BioBase BK400 chemistry analyser. (Intra assay CV 3.5% and Inter assay CV 5%, Lowest Detectable Dose 0.2 ng/mL).

Faecal samples were collected using commercial *uBiome*™ (San Francisco, USA) gut kits. The uBiome gut analysis uses 16S-sequencing on the Illumina Next Generation Sequencing platform that delivers sequence data [262]. 16S is a ribosomal gene present in bacterial DNA that enables 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 PCR primers and is therefore preferable to the other rRNA genes for phylogenetic identification [262]. This method of sequencing is commonly employed in gut microbiota studies creating less heterogeneity in studies for comparison. Trained assessors measured grip strength using a digital Jama handgrip dynamometer (Lafayette Instruments, Indiana USA). Participants were seated with their elbow bent at 90^{o}C. With a neutral wrist position, the dynamometer handle was held at position II, with the dynamometer supported underneath [305] and grip strength recorded for both left and right hands, and hand dominance noted.

Urine analysis was conducted in a similar manner as the pilot study (Chapter 3) using a solid-phase extraction (SPE) cartridge (Sep-Pak C18, Waters Corporation, Milford, MA, USA) according to Felgines et al. (2003) [46] and Netzel et al. (2012) [187]. Briefly, acidified urine samples were thawed and maintained for 60 min at room temperature before SPE extraction to obtain the maximal yield of the coloured flavylium cations. The SPE cartridge was activated with 10 mL of methanol and equilibrated with 10 mL of 12 mM aqueous HCl before use. Subsequently, 5 mL of acidified urine was applied to the equilibrated cartridge. The cartridge was then washed with 10 mL of 12 mM aqueous HCl, and anthocyanins were eluted with 2 mL of 12 mM HCl in methanol. The extracts were filtered (0.2 µm syringe filters; Palls, Cheltenham, VIC, Australia) before UHPLC-PDA-ESI-MS/MS analysis.

Figure 5-2: Enrolment, Randomization, and Retention of Study Participants.

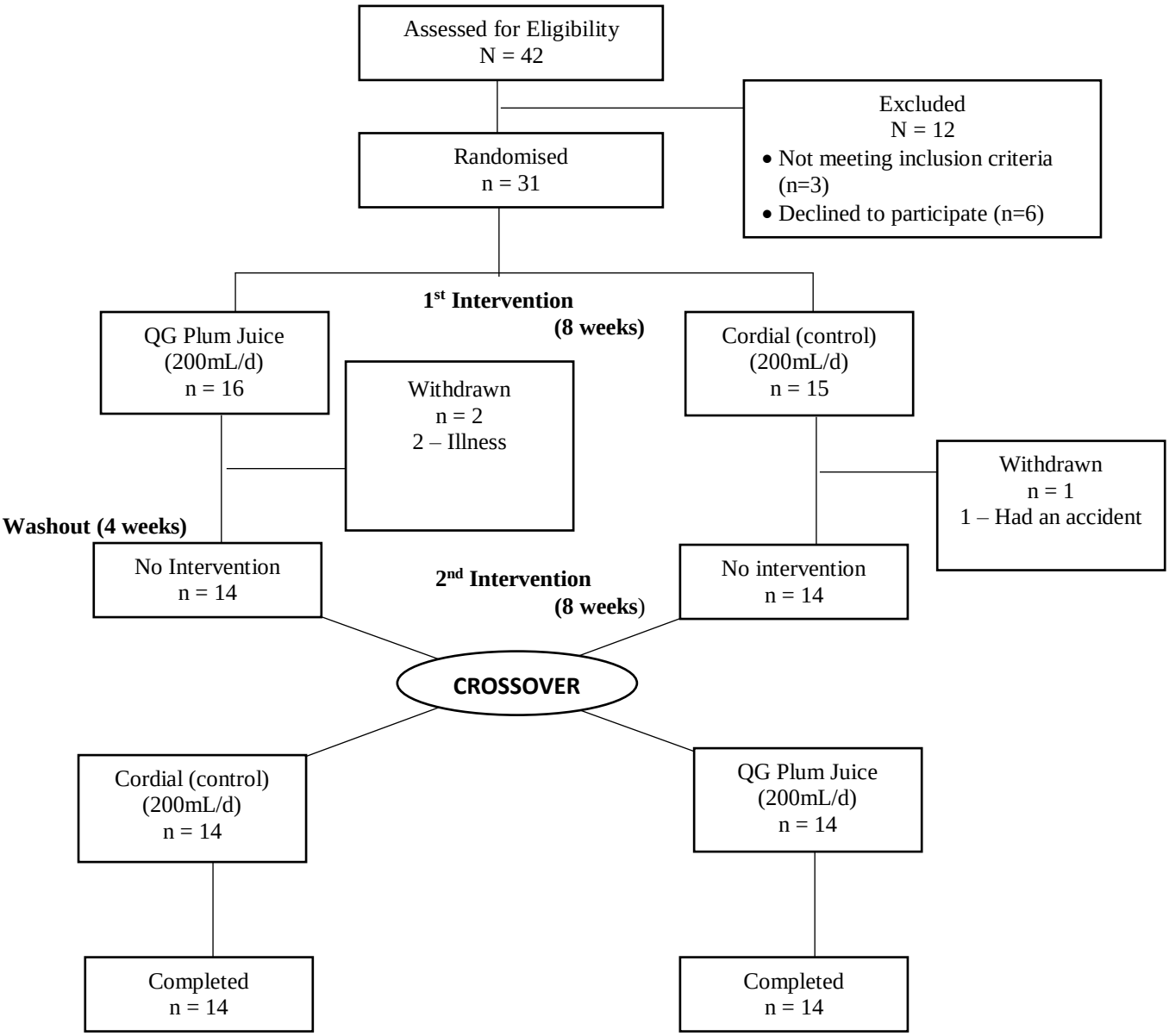


Table 5-2: Cognitive tasks description

Measurements	Application	Scoring
The Rey Auditory Verbal Learning test (RAVLT) [219]	This test measures verbal learning and memory. It requires the participant to learn a list of words over 5 presentation-test trial sequences.	Each correct word that is identified is associated with a score. This score is added, with a higher score indicating greater memory capabilities.
The verbal fluency task [306]	This task is a sensitive measure of executive dysfunction and control processes. The task requires the participant to produce as many words starting with a particular letter or category as possible in one minute.	This task assigns each word that is produced a score of 1. This score is added, where a higher score indicates high executive function.
Digit-span backwards task [307]	This task requires the participant to recall a small set of digits in reverse order and assesses short-term memory storage and executive control processes.	The longer the set of digits that are successfully recalled implies better memory processes.
Stroop task [222]	Participants are provided with a sheet on which the words green, *yellow*, *red*, and *blue* are printed (20 in total). Each word is shown in either congruent or incongruent ink colours (e.g., the word "blue" printed in red). Participants are instructed to read out the actual colour and not the printed word as quickly as possible. This task assesses executive function.	The amount of time taken (seconds) to complete each set of words was recorded.
Counting span [308]	Participants are presented slides with grey and blue shapes (squares and circles) and are asked to count the number of blue circles on each slide. After a certain number of slides (starting with a span size of 2 and going up to 6), participants are asked to remember the number of circles they counted for each card, starting with the first card and going in order.	The responses are typed into free recall boxes.

5.1.2.1 Data analysis

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 mean ± SD. Differences between baseline and 4 and 8 weeks of each study arm were analysed using paired sample t-tests for continuous variables and Chi-square for categorical variables.

Repeated measures ANOVA was used to determine the effect of time, treatment and time*treatment interactions for cognition and blood pressure measurements at 4 and 8 weeks, and markers of inflammation at baseline and 8 weeks. Alpha was set at 0.05 for statistical significance. Eta-squared (η^2) values were also calculated to quantify the difference between the interventions. The Cohen's f effect size estimates were characterised as small (0.10), medium (0.25) and large (0.40) [309]. The statistical analysis controlled for potential cofounders, including age and gender, physical activity and use of blood pressure medication. Treatment order was also included as the between subject factor.

Dietary anthocyanin intake was determined from the dietary assessments using an Australian anthocyanin database [310] available within the dietary analysis FoodWorks software system (Version 8, 2009, Xyris pty ltd, Spring Hill, QLD, Australia) (See Chapter 6.1 for description of database). All participants were included in the final data analysis (i.e. intention-to-treat). Linear discriminant analysis effect size (LEfSe) tool [311] was used to analyse gut microbiota data.

Analysis of urine samples by UHPLC-PDA-ESI-MS/MS

Analysis were carried out on a Shimadzu Nexera X2 UHPLC-PDA-ESI-MS/MS system (Shimadzu, Rydalmere, NSW, Australia). The system consisted of a system controller (CBM-30A), three pumps (LC-30AD), an autosampler (SIL-30AC), column heater (CTO-20AC), photodiode-array (PDA) detector (SPD-M30A) and two degassers (DGU-$20A_{3R}$ and DGU-$20A_{5R}$). Chromatographic separation was carried out on a Waters Acquity UPLC BEH C18 column (100 x 2.1 mm i.d., 1.7 µm) at a column temperature of 60°C and flow rate of 0.25 mL/min. PDA spectrum was scanned from 200-800 nm and anthocyanin compounds were monitored at 520 nm. LC mobile phase A consisted of acetonitrile, deionized water and formic acid (92:7:1, v/v/v) and B of 1% formic acid in acetonitrile. The elution was programmed with 0% A as initial

isocratic hold for 1 min followed by a linear gradient from 0-8% B for 12 min, then increasing to 50% in 5 min followed by a cleaning and equilibration step before the next injection. The Nexera X2 UHPLC system was coupled with a Shimadzu LCMS-8050 triple quadrupole mass spectrometer. The ESI source was operated with nitrogen as nebulizer gas at a flow of 2 L/min and drying gas at a flow of 10 L/min, respectively. Desolvation line (DL) temperature was 250 C and heat block temperature was set at 400 C. Selected ion monitoring (SIM) and product ion monitoring at a collision energy of -20V and full MS scans in positive mode were in the range of m/z 100-1200.

5.1.3 Results

Thirty-one participants (14 males and 17 females) were recruited, 28 (90.3%) of whom completed the trial. Three participants withdrew; two, due to illness and one due to involvement in an accident (see Figure 5-2). The average washout period for participants was 5 weeks (4-7 weeks), a deviation from the original 4-week washout period due to schedule clashes and illnesses. Four weeks was chosen according to recommendations for crossover trials to have a washout period at least five times the half-life of the treatment with the maximum half-life in the study [312]. Even though the half-life of the main anthocyanin (cyanidin-3-glucoside) present in QGPJ [85, 107] has been shown to range between 12 and 51h [49], 4 weeks was chosen in order to allow enough time for dissipation of treatment effects.

Baseline

Baseline characteristics of participants are summarised in Table 5-2 below, with no significant differences in any variables according to order of randomisation.

Table 5-3: Baseline characteristics of the study population

Baseline characteristics	Full group n=31(%)	[a]IC sequence (n=16)	[b]CI sequence (n=15)	p-value
Gender				
Male	14 (45)	6 (37)	8 (53)	ns
Female	17 (55)	10 (63)	7 (47)	
Age (mean ± SD)	70 ± 10	69 ± 10	70 ± 10	ns
BMI (mean ± 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	
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	

Baseline characteristics	Full group n=31(%)	[a]IC sequence (n=16)	[b]CI sequence (n=15)	p-value
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	1.56 ± 1.67	1.44 ± 1.22	1.70 ± 2.11	
BDNF	37.67 ± 10.44	35.44 ± 12.54	40.21 ± 6.99	

[a]IC- intervention- then control order; [b]CI- control then intervention order; RAVLT-Rey auditory verbal learning test; 24-ABPM – 24-h Ambulatory Blood Pressure Monitor; 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

Cognition and physical measurements

There was no observed significant difference from baseline or between groups in cognition or physical tests (Table 5-3). Calculated *p* values were greater than 0.05 (Table 5-4). In addition, calculated eta squared (η^2) for effect sizes for the different cognitive tasks were as follows: RAVLT total (I-V) = 0.024; RAVLT delayed recall =0.012; RAVLT 20m delayed recall = 0.018; Digit Span = 0.019; Letter fluency = 0.00; Category fluency= 0.065; Counting span = 0.00 and; Stroop (secs) = 0.06.

Table 5-4: Crossover analysis of cognitive and physical function variables

	Mean (SD)		p-values of associated ANOVA analysis		
	4-weeks	8-weeks	Treatment effect	Time effect	Treatment* time effect
RAVLT total (I-V)			0.69	0.74	0.44
Baseline	43(12)				
Plum juice	48 (11)	49 (11)			
Cordial	48 (13)	49 (12)			
RAVLT delayed recall			0.49	1.0	0.43
Baseline	8 (4)				
Plum juice	9 (4)	9 (4)			
Cordial	9 (4)	9 (4)			
RAVLT 20m delayed recall			0.41	0.77	0.72
Baseline	8 (4)				
Plum juice	9 (3)	9 (4)			
Cordial	9 (5)	9 (4)			
Digit Span			0.23	0.14	0.49
Baseline	7 (3)				
Plum juice	8 (2)	8 (2)			
Cordial	8 (3)	8 (2)			
Letter fluency					
Baseline	14 (6)		0.73	0.63	0.94
Plum juice	15 (6)	16 (6)			
Cordial	16 (7)	15 (6)			
Category fluency					
Baseline	17 (6)		0.58	0.47	0.20
Plum juice	15 (5)	16 (6)			
Cordial	19 (7)	18 (7)			
Counting span					
Baseline	9 (4)		0.35	0.90	0.96
Plum juice	9 (3)	10 (3)			
Cordial	10 (3)	10 (3)			
Stroop (secs)					
Baseline	12 (6)		0.08	0.72	0.08
Plum juice	11 (5)	10 (5)			
Cordial	10 (6)	9 (5)			
Hand grip strength					
Right					
Baseline	30.1 (10.3)		0.90	0.59	0.88
Plum juice	32.4 (11.0)	31.9 (11.1)			
Cordial	31.3 (10.6)	31.7 (11.0)			
Left					
Baseline	26.8 (10.6)		0.53	0.81	0.30
Plum juice	29.3 (10.7)	29.2 (11.3)			
Cordial	28.6 (11.3)	27.6 (10.1)			
Sit-to-stand					
Baseline	19 (7)		0.34	0.12	0.12
Plum juice	21 (8)	21 (7)			
Cordial	21 (9)	22 (9)			

Ambulatory blood pressure and anti-inflammatory biomarkers

Similar to cognitive outcomes, there was no observed significant effect observed in 24-h ambulatory blood pressure (Table 5-4) or anti-inflammatory biomarkers (Table 5-5).

Table 5-5: Crossover analysis of 24-h Ambulatory blood pressure data

	Mean (SD)		**p-values of associated ANOVA analysis**		
	4-weeks	8-weeks	Treatment effect	Time effect	Treatment by time effect
SBP			0.73	0.97	0.44
Baseline	129 (12)				
Plum juice	125.9(10.0)	127.3(9.5)			
Cordial	124.2 (11.3)	122.5 (7.7)			
DBP			0.41	0.92	0.86
Baseline	80 (8)				
Plum juice	78.5 (7.8)	77.9 (7.8)			
Cordial	76.1 (7.0)	76.1 (7.0)			
MAP			0.75	0.93	0.71
Baseline	102 (9)				
Plum juice	100.1 (7.9)	100.5 (7.6)			
Cordial	98.1 (8.1)	97.4 (6.3)			
Heart rate			0.37	0.06	0.62
Baseline	72 (9)				
Plum juice	70.7 (10.4)	70.9 (8.3)			
Cordial	69.0 (8.8)	70.5 (8.7)			
Pulse pressure			0.77	0.97	0.39
Baseline	49 (9)				
Plum juice	47.6 (8.0)	49.5 (9.7)			
Cordial	48.0 (8.5)	46.4(7.5)			

Table 5-6: Crossover analysis of nerve growth factor / inflammatory biomarkers (BDNF and CRP)

	Mean (SD)	p-values of associated ANOVA analysis	
	8-weeks	Treatment effect	Treatment by baseline
BDNF (ng/mL)		0.70	0.67
Baseline	37.67 (10.44)		
Plum juice	33.61 (12.13)		
Cordial	33.71 (11.52)		
CRP (mg/L)		0.17	0.16
Baseline	1.56 (1.67)		
Plum juice	2.66 (4.02)		
Cordial	2.19 (2.20)		

Gut microbiota

Analysis of gut microbiota data showed that there were no biomarkers associated with QGP juice consumption over eight weeks. There were no significant differences observed for *Bifidobacterium* and *Clostridium* genera. These bacteria genera have previously been shown to be affected by anthocyanin supplementation [53]. Analysis of total bacteria count showed a trend towards significance in the comparison of total bacteria from baseline to week 8 in the control arm. This trend was not observed between baseline and intervention (Figure 5-3).

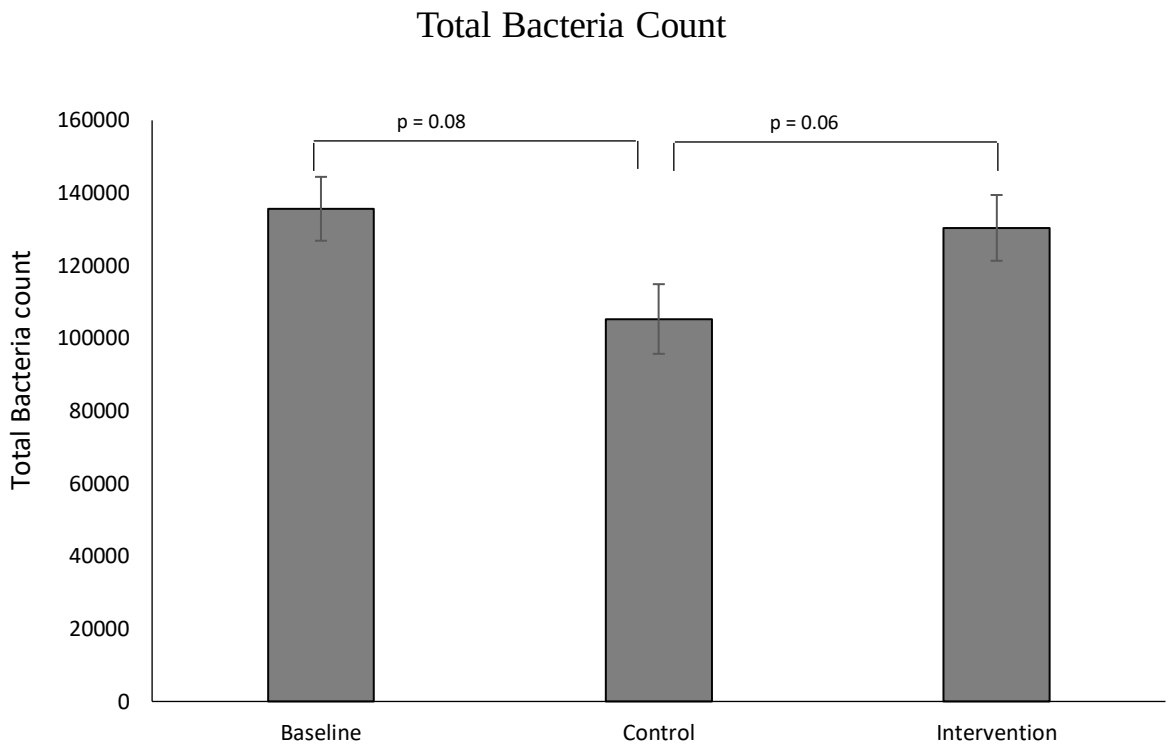


Figure 5-3: Total bacterial count across baseline, and week 8 of intervention and control arms (error bars are SE) (n=31)

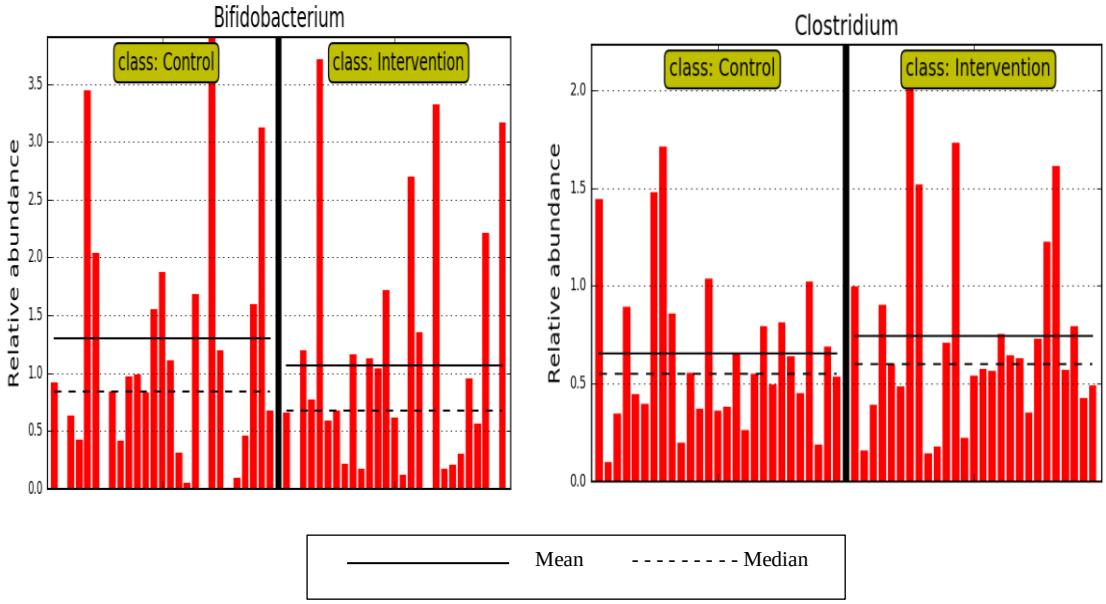


Figure 5-4: Relative abundance of Bifidobacterium and clostridium genera in gut sample across study arms (according to linear discriminant analysis effect size; LefSe).

Urinary analysis

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 QGPJ consumption (Table 5-7). However, further evaluation was not undertaken since the concentrations of these anthocyanins and metabolites were below the limit 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).

Table 5-7: UHPLC-PDA-ESI-MS/MS characterization of QGP anthocyanins and metabolites detected in human urine after the consumption of QGP juice

Compounds Proposed identity	Precursor ions (m/z) [M+H]+	Empirical molecular formula	Fragments
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

Dietary data

Analysis of participants' background diet is presented in Table 5-8 below. The mean intake of dietary anthocyanins was 142.69mg/day with a median of 34.15mg/d (IQR =280.33) and energy intake of 9302kJ/day.

Table 5-8: Dietary data analysis of participants from estimated 3-day food diary data

Nutrient/day	Full group (n=31) mean ± SD (median(IQR))	[a]IC sequence (n=16) mean ± SD (median(IQR))	[b]CI sequence (n=15) mean ± SD (median(IQR))	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	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	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	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

[a]IC- intervention- then control order; [b]CI- control then intervention order; ns- not significant; [+]t-test; [++]Wilcoxon signed-rank test

5.1.4 Discussion

This study hypothesised that consumption of anthocyanin-containing plum juice for 8 weeks would have significant positive effects on cognitive function and BP, compared to a control cordial. However, in a group of healthy older adults, there were no observed effects on blood pressure, cognition or measured inflammatory biomarkers. The significance levels measured using *p* value less than 0.05 and effect size measured using eta squared (η^2) did not show any significant effects of the intervention. Results from this study add to the evidence that in healthy older adults without cognitive decline, supplementation with fruit anthocyanins may provide no beneficial effects [313, 314].

Anthocyanins have been shown to exert protective effects on cognition even in the presence of other polyphenols. Evidence from animal studies continue to show promising results [315] however, clinical trial evidence have not been in agreement. 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). 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 3 months and lower SBP 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) [316]. Other studies using grape or blueberry (87mg of anthocyanins per 100g and 1920mg/100g respectively) observed significant effects on episodic and working memory [317, 318] as well as in a study using encapsulated grape and blueberry extract mix [319] and individuals with mild-moderate cognitive impairment [61]. Similar to the findings of our study, Bensalem et. al. (2018) did not report benefits of anthocyanin supplementation in healthy older adults. However, memory benefits were seen following stratification of participants according to baseline cognitive characteristics, with memory benefits seen in those with poorer function initially [319]. In addition, Different study populations and anthocyanin sources also limits comparison and generalizability between the two studies.

Our study findings are consistent with similar studies on healthy older adults using a crossover study design [320, 321] as well as a parallel study design using elderberries [322]. In one of the studies [321]

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 [323], which may make anthocyanin-consumption easier to control by improving compound stability, the bioactive benefits of extracting and isolating specific flavonoids via encapsulation has been questioned [324], 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: longer duration of intervention (3- 6 months); food source of anthocyanin (usually blueberry anthocyanins); 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 that contain a wide range of anthocyanin sub-classes (i.e. blueberries and grapes), while the fruit vehicle (plum) in the current study mostly contained cyanidins [325]. The synergistic effect of multiple anthocyanin sub-groups found in blueberries could also account for these observed effects. To further demonstrate a synergistic effect of a combination of anthocyanin sub-groups, a study that compared enocianin, (a commercial grape extract food colouring that contains a variety of anthocyanins) with a pure malvidin-3-glucoside extract reported a more significant proliferative effect on gut bacteria by enocianin [271]. The synergistic effect of both nutrients and bioactive non-nutrient components within a food matrix has been highlighted by Jacobs and Tapsell (2007) [326]. 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 [327]. Available evidence suggests that in healthy older individuals, lower levels of anthocyanin supplementation do not seem to have significant effects over the short term ($\leq$ 2 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 blood pressure in healthy people (25-84yr) over 4 weeks [320]. On the other hand, a study of dementia patients (69mg of anthocyanins /100mL of cherry juice) [61] and post myocardial infarction patients [328] reported significantly reduced blood pressure over 12 weeks. Another important consideration for 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 [201, 214, 301] and also in longer-term studies on individuals with mild to moderate cognitive impairment [61, 201]. However, these acute effects in healthy older adults are not generally seen chronically [320] 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 chronic intake of flavonoids do not result in increased levels of metabolites in the tissues compared to single doses, which shows that brain levels are likely reflective of acute intake and may not accumulate in the long term [329]. There is also a possibility that the amount of anthocyanin in the plum juice used in this study could have affected study results. Anthocyanin content of the plum juice used in the acute dose-timing study (Chapter 3) was 123mg/100mL but for this current study, the anthocyanin content of the QGPJ was much lower, approximately 50mg/L. Additionally, the lower concentration of anthocyanins in the QGPJ utilised in this study could explain the lower concentrations of anthocyanin metabolites observed in the urinary analyses, which were below the limits of quantification.

In line with the absence of any significant effects on cognition, no changes in the brain derived neurotrophic growth factor and the inflammatory biomarker, hsCRP were observed following anthocyanin supplementation. BDNF is an important growth factor for synaptic plasticity and learning and memory [330, 331] while C-reactive protein (CRP) is a broad marker for presence of inflammation in the body. 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 [241] which showed stable BDNF plasma levels following blueberry anthocyanins supplementation in adults compared to a reduction associated with consuming a control drink. It is important to note that baseline CRP and BDNF concentrations were within normal ranges (CRP < 3mg/L) and BDNF generally between 8–46 ng/mL [332]) 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 diseases compared to healthy subjects, where no significant effects are observed [322]. 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 [333].

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 (IBS) as well as an inhibitory effect on the pathogenic strain *Clostridium histolyticum* [53]. Our study did not find an intervention effect on these genera of gut bacteria however, but there was an observed reduction in total bacteria in the control arm which was borderline significant. A review on the effects of sweeteners on the gut microbiota showed that some artificial sweeteners reduced bacteria counts *in vivo* in animal models [334]. Although the sugar content of the cordial and QGPJ were comparable, there is a possibility that the consumption of raspberry cordial may affect the gut microbiota population in a different manner. 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 [53] and they may have proliferative and inhibitory effects of a similar magnitude in different species [272]. There is also the possibility that the observed borderline effect on gut microbiota from this study could be attributed to a synergistic effect of other significant bioactive phenolic compounds present in the intervention juice. One of such nutrients is the hydroxycinnamic acids which are present in plums in higher quantities than anthocyanins [180]. Research on hydroxycinnamic acids shows significant biological activities, however, the low bioavailability of hydroxycinnamic acids continues to limit research translation [335]. Evidence from pre-clinical and clinical studies suggest that due to their resistance to substrates in the small intestine namely Lactase-Phlorizin Hydrolase (LPH) and Cytosolic β-glucosidase (CBG), they cannot be absorbed in the small intestine but are transported to the colon where the colon microbiota cleave the conjugating moieties, with resultant beneficial effects on the microbiota [35, 336].

The absence of significant effects on the primary outcome measures could also be associated with background nutrition. Anthocyanin intake in the Australian population has been estimated at between 1.4 - 24.17mg/d (Chapter 6.2). However, the mean intake of dietary anthocyanins in the habitual diet of the current study population was 142mg/d (median (IQR) = 34.15(280.33)). This was higher than intakes reported in other Australian studies [337, 338]. A higher intake of background dietary anthocyanin may hinder any possible additional benefits from anthocyanin supplementation as evidence has shown

significant associations between habitual anthocyanin intake and reduced disease risks in longitudinal studies [231, 339, 340]. Processing and storage times could have also affected anthocyanin stability in the intervention juice and, subsequently, the absence of any significant changes in measured variables. In comparison to the acute dose-timing study (Chapter 3) in which the study beverage was stored for about 2 months (frozen) prior to the start of the trial which lasted for 4 weeks, this study beverage was stored for about 8 months (refrigerated and frozen) while the study duration was 8 months. 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 endeavoured to standardise the provided juice by using two combined batches that had been processed. This necessitated the juice to be kept in storage under controlled conditions for a relatively long time. Evidence shows that processing of fruits to juice, especially in high temperatures significantly degrades anthocyanins [341]. In addition, the thawing and freezing process involved in bottling the juice could have also caused significant degradation of the plum juice anthocyanins [342]. This degradation effect together with the low stability of anthocyanins in general could have significant effects on research findings hence limiting generalisation of research findings [343].

The research team endeavoured to match the study beverages in nutrient content except for anthocyanins. However, there was considerable difference in the energy content between the beverages. There are no indications that this observed difference could have affected study results.
One of the main strengths of this study is the crossover design. Participants acted as their own control which accounted for inter-individual variances and thereby increasing statistical power. In crossover trials, 3 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 [344]. In addition, 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.

There were few dropouts in this study and juice consumption compliance was satisfactory. As with food interventions, blinding of participants was not possible. Other limitations in this study may include the lack of characterization of microbial metabolites of in the plasma and the relatively short duration of

intervention. The major consideration in the design of anthocyanin food-based research is related to preservation of the anthocyanin content in test foods. Losses in anthocyanin content of juice due to the bottling process (thawing and refreezing) and other storage considerations present real challenges.

5.1.5 Conclusion

In conclusion, anthocyanin supplementation provided in a food matrix of plum juice did not have any significant effect on cognition, BP, or nerve growth factor/ inflammatory markers (BDNF and CRP) over 8 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.

6 CHAPTER 6

6.1 DEVELOPMENT OF AN AUSTRALIAN ANTHOCYANIN DATABASE AND SECONDARY DATA ANALYSIS OF CURRENT ANTHOCYANIN INTAKE IN THE AUSTRALIAN POPULATION

To accurately measure nutrient intake in population studies, food composition databases tailored to specific regions need to be developed. As stated in Chapter 1, the available databases are not region-specific which limits measurement of anthocyanin intake in epidemiological research. The aim of this section of Chapter 6 was to describe the first stage development of an Australian anthocyanin food composition database focusing on fruit and vegetables. Development of an anthocyanin food composition database relies on the availability of analytical food data. In the case of Australian fruits and vegetables, there was limited data available for their anthocyanin content using a systematic literature review approach. As a result, imputations (conversion to Australian values using the moisture conversion factor) from other polyphenol datasets was necessary.

The majority of this section of chapter 6 is the substantive content of the published articles: (Appendix D)

Igwe, E., Neale, E., Charlton, K.E., Morton, K. and Probst, Y.C., 2017. First stage development of an Australian anthocyanin food composition database for dietary studies–A systematic process and its challenges. *Journal of Food Composition and Analysis*, *64*, pp.33-38.

Specific role/authorship of all the authors: All authors were involved in the development of the Australian anthocyanin database. All authors were involved in the planning the project. EI and KM conducted the systematic literature reviews for the anthocyanin content of Australian foods. EN, KC and YP advised on methods for data merging (USDA and Phenol-Explorer) and entry into a single database.

All Authors contributed to writing and editing the manuscript and approved of the final version of the paper submitted for publication

Following the first stage development of the Australian anthocyanin database, the database was incorporated into the commercially available FoodWorks software for nutritional analyses (version 9).

6.1.1 Part 1: First stage development of an Australian anthocyanin food composition database for dietary studies – A systematic process

6.1.1.1 Introduction

In order to translate clinical trial findings to dietary messages, it is important that the amount of anthocyanin intake is accurately measured in both epidemiological and experimental studies. This would allow translation of this evidence into dietary messages for overall health improvement. For this purpose, a number of specific and general polyphenol databases, including the USDA Database for the Flavonoid Content of Selected Foods and the European Phenol-Explorer, exist for the measurement of polyphenol (anthocyanin) intake [345, 346]. Comparing these two databases for the estimation of dietary polyphenol intake in Polish adults, significant discrepancies were demonstrated in the amount of flavonoid intake between the USDA and Phenol-Explorer databases (525 mg/day vs 403.5 mg/day, respectively $p<0.001$) [347]. Epidemiological studies commonly utilise either of these databases for the measurement of polyphenol intakes. Such discrepancies between databases may lead to differing conclusions regarding the amount of anthocyanins in the human diet and potential dose responses [338, 347, 348].

Within the USDA and Phenol Explorer databases, analytical data are not country or region specific but are reported as mean values for all available data for a particular food. As a result, there is the possibility that this can lead to inaccurate measurement of polyphenol intakes in specific populations. Evidence has shown that differences in climate, soil conditions and methods of plant harvesting among other factors are major determinants of natural variation in the amounts of nutrients contained in foods [89, 90]. For example, the levels of anthocyanin in fruit may be directly proportional to the amount of sunlight exposure. Anthocyanin levels in grapes have been observed to substantially increase with grape bunch exposure to sunlight in comparison to shading [349]. Average monthly hours of sunshine over the year has been shown to be significantly different in various fruit producing regions of the world [350]. Focusing on this difference in climate and harvesting practices as an example, the amount of micronutrients in apples are found to differ substantially between the USDA [351] and the Australian Food, Supplement and Nutrient Database (AUSNUT) [352] (Table 6-1). For these reasons, it is important that databases tailored to specific regions

of the world are developed to facilitate better measurement of anthocyanin intakes in population level studies.

In utilising the available databases for epidemiological studies, another drawback is the underrepresentation of native fruit and fruit hybrids specific to particular regions. For example, there is limited data for some common Australian native fruit including quandong, riberry and fingerlimes which are known to have high levels of anthocyanins [353].

In summary, the underrepresentation of fruit specific to particular regions in existing databases, as well as the differences in climate and harvesting practices between regions warrants the development of an anthocyanin food composition database specific to different regions.

The aim of this study was, therefore, to describe the first stages of the development of an Australian anthocyanin food composition database using fruit and vegetables as a preliminary step in the systematic development process.

6.1.1.2 Methods

The development of an Australian anthocyanin database involved an expansion of the existing Australian Food and Nutrient database (AUSNUT) 2011-13 database to include anthocyanin content. The AUSNUT 2011-13 database contains over 5,700 foods and beverages reported by Australians and was developed for use in the 2011-13 Australian Health Survey [354].

Table 6-1: Micronutrient comparison between USDA and AUSNUT food composition database values of apple

	AUSNUT* values	USDA+ values
Preformed vitamin A (retinol) (µg)	0	3
Beta-carotene (µg)	10	27
Provitamin A (b-carotene equivalents) (µg)	13	
Vitamin A retinol equivalents (µg)	2	0
Thiamin (mg)	0.022	0.017
Riboflavin (mg)	0.013	0.026
Vitamin C (mg)	4	4.6
Dietary folate equivalents (µg)	13	3

***AUSNUT – Australian Food and Nutrient Database +USDA – U.S Department of Agriculture**
Source: [351, 355].

As a first stage development, the main focus was fruit and vegetables as these are the major food groups with high levels of anthocyanins. The most reliable method to measure anthocyanin content in foods has been identified as High-Performance Liquid Chromatography (HPLC). When evaluating spectrophotometric methods for antioxidant compound measurement, the colorimetric methods were found to be unreliable as reactions could be different for individual compounds within a sub-group (anthocyanins, flavonols or flavan-3-ols) and were not specific to one family [281]. For this reason, only data generated using HPLC methods of analysis were included.

The proposed methodology for estimating the anthocyanin content of Australian foods was adapted from a systematic approach that was developed and used for the estimation of added sugar content in Australian foods via an expansion of the AUSNUT database [356].

Step by step systematic approach to estimating anthocyanins in foods

A step by step process was developed for estimating the anthocyanin content of Australian fruit and vegetables. The first four steps were objective while the rest were considered subjective.

Step 1: Classification of food groups: Food groups were coded into three different categories; plant-based foods, non-plant-based foods and composite foods (made up of plant-based and non-plant-based foods). The difference between the non-plant-based food and the composite food group is that the majority of the former were assigned zero values while in the latter; fewer foods were assigned a zero value (Table 6-2). The grouping system developed for the AUSNUT 2011-13 database classifies foods according to a major (2-digit) based on key ingredients, followed by sub-major (3-digit) and minor food (5-digit) groups. Using this classification system, there are 24 major food groups covering all the reported foods eaten by Australians, as shown in Table 6-2.

Step 2: Assignment of zero values: Zero values were assigned to non-plant-based food and composite food groups with careful attention being paid not to assign 0 values to foods that contained ingredients from the plant-based food groups.

Step 3: Conduct a systematic literature search for analytical data: A systematic literature search of electronic databases was conducted (Scopus, Web of Science, Medline, PubMed central, ProQuest, and Science Direct) using a combination of search terms; "anthocyanin*", "Australia*", "Fruit*", "vege*", and "analy*". Studies were included if they had analysed the anthocyanin content in Australian fruit and vegetables. Additional literature searches using USDA and Phenol-Explorer databases were conducted to identify fruits and vegetables with available anthocyanin analytical values. Searches used scientific and common names to identify all relevant foods in the USDA and Phenol-Explorer databases.

Table 6-2: Major food groups in the Australian Food and Nutrient Database (AUSNUT)

Plant based food groups	**Non-Plant based food groups**	**Composite food groups**
16. Fruit products and dishes	14. Fats and oils	11.Non-alcoholic beverages
22. Seed and nut product and dishes	15. Fish and seafood products	12.Cereals and cereal products
24. Vegetable products and dishes	17. Egg products and dishes	13.Cereal based products and dishes
25. Legume and pulse products and dishes	18. Meat, poultry and game products and dishes	20. Dairy & meat substitutes
	19. Milk products and dishes	21. Soup
	30. Special dietary foods	23. Savoury sauces and condiments
	34. Reptiles, amphibia and insects	26. Snack foods
		27. Sugar products and dishes
		28. Confectionery and cereal/nut/fruit/seed bars
		29. Alcoholic beverages
		31. Miscellaneous
		32. Infant formulae and foods
		33. Dietary supplements

Step 4: Contact local researchers and organisations: Experts in the field of anthocyanin research were identified from the systematic literature search as well as web searches of organisations, groups, and individuals to identify where further sources of analytical anthocyanin data may be located. These sources were contacted to enquire about the existence of any unpublished data for the anthocyanin content of Australian fruits and vegetables.

Step 5: Using borrowed values: Having exhausted steps 1-4, where Australian data was not identified for fruit and vegetables, data will be obtained for similar foods from international databases, including the USDA Database for the Flavonoid Content of Selected Foods and Phenol-Explorer. To assist with the choice of the most appropriate foods from those databases, estimations were made by calculating a conversion factor to apply to the borrowed values in the Australian food anthocyanin database, considering deviation from average moisture content as shown below: [357]

The conversion factor F is calculated using the following formula:

$$F = \frac{100 - actual\ moisture\ content\ (Australian\ food\ composition\ table)}{100 - moisture\ content\ as\ shown\ in\ the\ USDA\ or\ Phenol - Explorer\ food\ comp.table}$$

Moisture content of the foods in the USDA flavonoid database was obtained from the USDA National Nutrient Database for Standard Reference [351] and those of the Phenol-Explorer will be obtained from the Danish Food Composition Databank the data source indicated in the Phenol-Explorer database [358]. Having calculated the conversion factor, the corresponding Australian nutrient value were computed by multiplying specific nutrient value by the conversion factor (F). Following this computation, data from the USDA flavonoid database and Phenol-Explorer were mapped to determine whether to make decisions based on the calculated values from both databases or if further analysis is required. The choice of the international database from which to borrow analytical values was dependent on the similarity of the foods in their macronutrient content and the similarity of the food supply between Australia and the source country including production and processing techniques.

Step 6: Compression of anthocyanin glycosides to main classes:

In the overseas databases, anthocyanin content was listed by specific glycosides. In order to facilitate adequate merging of the anthocyanin values, the anthocyanin glycosides were compressed to the main classes of anthocyanins. For example, cyanidin 3-glucoside was classified under cyanidin.

Step 7: Match values for similar foods: Following compression of anthocyanin glycosides to the main classes, similar species of foods were matched in anthocyanin values. For example, Fuji apple and green apple were matched for values. In the case whereby fresh/raw version of a particular food had a zero value, same value was also assigned to the cooked or processed version of same food.

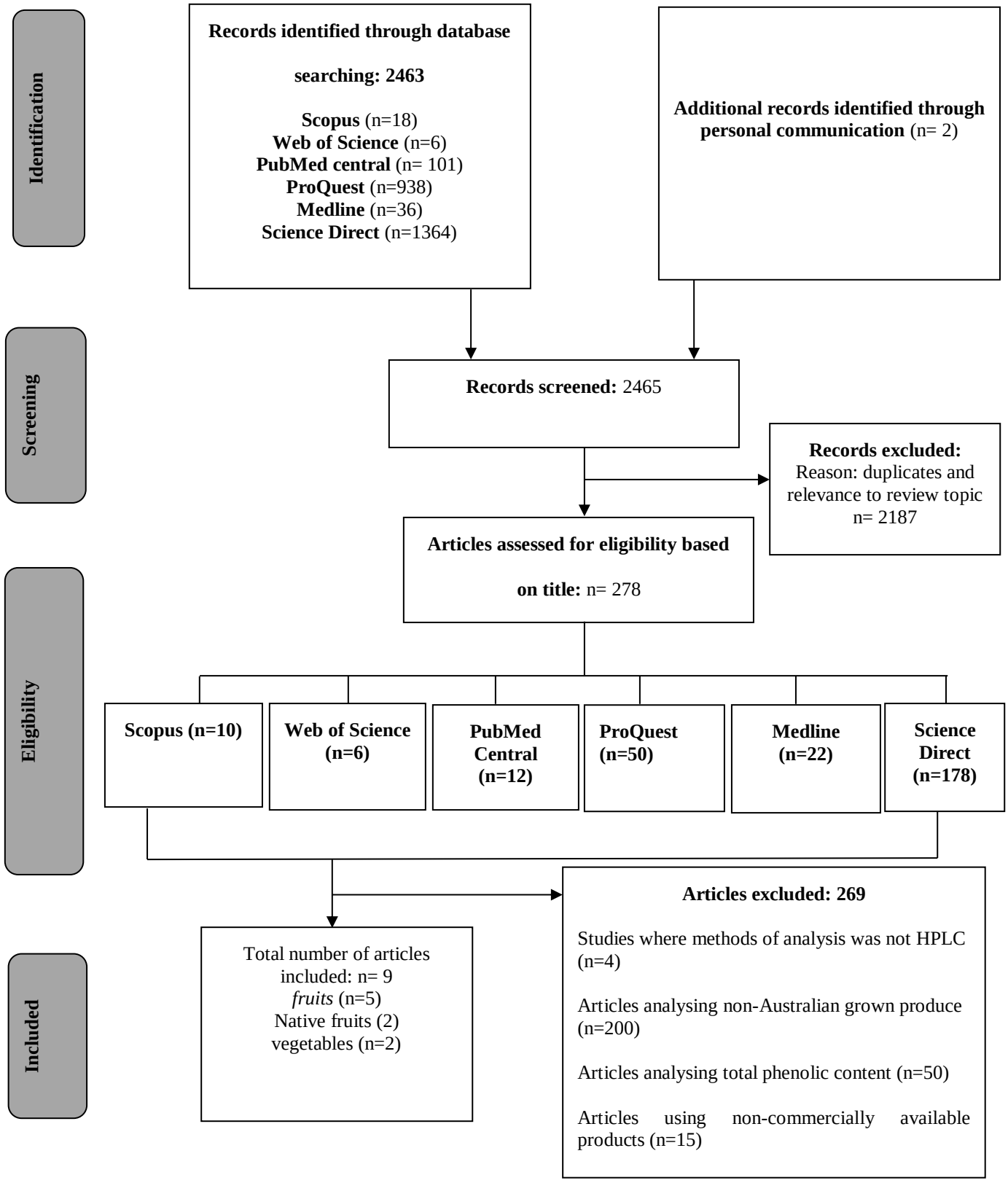


Figure 6-1: PRISMA flow diagram for analytical article selection process

6.1.1.3 Results

Following the four objective steps outlined above, the existing AUSNUT 2011-13 database was expanded to include anthocyanin content. The foods contained in the database are classified under 24 food groups (Table 6-2). Of these, four were grouped as plant-based foods, seven under the non-plant-based food group and 13 under the composite food group. There was a total of 58 individual fruits and 62 vegetables in the database following this method of categorisation.

The systematic literature search (Figure 6-1) for analytical values of Australian fruits and vegetables yielded analytical values for only five Australian fruit (Table 6-3), including berries [359, 360] plums [13, 361], apple [362], grape [363] as well as ten native Australian fruit [360, 364] and two Australian vegetables, namely purple dragon carrots [365] and red cabbage [366]. Unpublished analytical data from local researchers at the Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Australia were obtained for commercially available pomegranate juice, pomegranate and cranberry juice mix, pomegranate and blueberry juice mix, Dr Red products (Purple Shiraz and Purple Carrot Blend; Purple carrot blueberry punch; Blueberry punch), Nature's Goodness (Joint Formula: Cherry juice concentrate with anthocyanin complex) and Sunraysia (100% Prune juice (from concentrate)).

6.1.1.4 Discussion

Using a systematic approach, for the first time, attempts have been made to bring together in a database the analytical data for the anthocyanin content of Australian foods (fruit and vegetables). The first four objective steps were unambiguous as the food groupings based on major ingredients were readily available; hence classification as plant-based or non-plant-based foods was a straightforward process.
Even though there is currently very limited analytical data on the anthocyanin content of Australian foods which was evident in the few studies identified during the systematic literature search, preliminary comparisons of the available data on fruit with the existing polyphenol databases shows a substantial difference in the reported anthocyanin content (Table 6-4). It is expected that more data will become available as this area of research advances, allowing for continuous sourcing of unpublished data.

Expansion of the dataset will enable more precise and consistent estimation of anthocyanin intakes in future studies.

Currently, the major focus has been on raw fruit and vegetables but as this project progresses, a number of considerations will be made in gathering analytical values. Food processing, for example, will be a major consideration as it has been shown to affect the nutritional content of foods with more effect on micronutrients. Blanching for example was observed to decrease the tannins and anti-radical efficiency of plums but increased the total polyphenol content, while osmotic dehydration had no effect on the total polyphenol and ferric reducing power [181]. On drying, it has been observed that some anthocyanin and flavonol content of plums significantly decreased [180]. In line with this observation, the free radical scavenging capacity and antioxidant activity of both fresh and dried fruit samples (plum) have been studied. Results showed that the fresh samples had more oxygen free radical (superoxide and peroxy radicals) scavenging capacity than the dried samples [183].

Some of the strengths of the proposed methodology are its systematic approach and the fact that local researchers were contacted for unpublished data in order to gather additional analytical data. Furthermore, upon completion of the proposed database, there is the possibility of improved study results based on better measurement of anthocyanin consumption. In estimating anthocyanin intake in population studies, a major limitation has been the significant differences in the estimated amount of anthocyanin intake depending on the database used to measure anthocyanin [347]. There is a possibility that the combination of available databases can have a significant effect on accurate measurement of anthocyanin intake. Considering this, a major strength of this proposed methodology will be the combination of analytical data from Australian foods and borrowed data from international databases based on micro and macro nutrient similarity which will provide more accurate estimation [347].

A major limitation of this project was the limited availability of analytical data for Australian foods and thus the need to borrow international data. Some of the published analytical values only reported total anthocyanin content, rather than individual anthocyanins [13, 362, 363, 365, 366]. This could be seen as a limitation as evidence shows that specific anthocyanins vary substantially in their bioavailability [367]. In

addition, even though some foods were assigned a zero value on the assumption that they do not contain any anthocyanins, it will be important to confirm this from analytical data.

Table 6-3: Anthocyanin content of raw Australian fruit, sourced using a systematic literature review approach

Reference	Food name	Food group	Method of analysis[a]	Total anthocyanin (SD) mg/100g fresh weight	Individual anthocyanins reported
Fredericks et al., (2013) [359]	Strawberry	Fruit products and dishes	HPLC	2.02 (0.04)	Yes
Netzel et al., (2006) [360]	Blueberry	Fruit products and dishes	HPLC/ESI-MS-MS	381.75 (6.23)	Yes
Bobrich, A., et al. (2014) [361]	Black diamond plum	Fruit products and dishes	HPLC	89.8mg	Yes
Fanning et al., (2014) [13]	Queen Garnet plum	Fruit products and dishes	HPLC	195	No
Takos et al., (2006) [362]	Apple skin (red)	Fruit products and dishes	HPLC	9mg/100g fruit skin	No
Bidon et al., (2013) [363]	Shiraz grape	Fruit products and dishes	HPLC	159 (5)	No
Zabaras et al., (2013) [366]	Red Cabbage	Vegetables	HPLC/ESI-LC/MS	57.4	No
Singh et al., (2012) [365]	Purple dragon Carrot	Vegetables	HPLC	5 (0.2)	No
Netzel et al., (2006) [360]	Muntries	(Native) fruit products and dishes	HPLC/ESI-MS-MS	25.26 (1.24)	Yes
Netzel et al., (2006) [360]	Tasmanian pepper	(Native) fruit products and dishes	HPLC/ESI-MS-MS	607 (52)	Yes

Reference	Food name	Food group	Method of analysis[a]	Total anthocyanin (SD) mg/100g fresh weight	Individual anthocyanins reported
Netzel et al., (2006) [360]	Molucca raspberry	(Native) fruit products and dishes	HPLC/ESI-MS-MS	73.64 (0.41)	Yes
Netzel et al., (2006) [360]	Davidson's plum	(Native) fruit products and dishes	HPLC/ESI-MS-MS	38.42 (0.83)	Yes
Netzel et al., (2006) [360]	Illawarra plum	(Native) fruit products and dishes	HPLC/ESI-MS-MS	556.82 (20.73)	Yes
Netzel et al., (2006) [360]	Cedar bay cherry	(Native) fruit products and dishes	HPLC/ESI-MS-MS	27.77 (0.54)	Yes
Netzel et al., (2006) [360]	Burdekin plum	(Native) fruit products and dishes	HPLC/ESI-MS-MS	174.55(10.63)	Yes
Netzel et al., (2007) [364]	Riberry	(Native) fruit products and dishes	HPLC-DAD/ESI/MS-MS	72.39 (1.45)	Yes
Netzel et al., (2007) [364]	Bush Cherry	(Native) fruit products and dishes	HPLC-DAD/ESI/MS-MS	105.79(1.24)	Yes
Netzel et al., (2007) [364]	Finger lime	(Native) fruit products and dishes	HPLC-DAD/ESI/MS-MS	11.09 (0.83)	Yes
Unpublished data from source	pomegranate juice	fruit products and dishes	HPLC	13[b]	No
Unpublished data from source	pomegranate cranberry juice	fruit products and dishes	HPLC	12[b]	No
Unpublished data from source	pomegranate blueberry juice	fruit products and dishes	HPLC	12[b]	No

Reference	Food name	Food group	Method of analysis[a]	Total anthocyanin (SD) mg/100g fresh weight	Individual anthocyanins reported
Unpublished data from Source	Dr Red (Purple Shiraz and Purple Carrot Blend)	fruit products and dishes	HPLC	9[b]	No
Unpublished data from source	Dr Red (Purple carrot blueberry punch)	fruit products and dishes	HPLC	40[b]	No
Unpublished data from source	Dr Red (Blueberry punch)	fruit products and dishes	HPLC	610[b]	No
Unpublished data from source	Nature's Goodness (Joint Formula: Cherry juice concentrate with anthocyanin complex)	fruit products and dishes	HPLC	14[b]	No
Unpublished data from source	Sunraysia (100% Prune juice (from concentrate))	fruit products and dishes	HPLC	<<1[b]	No

[a]HPLC- High Performance Liquid Chromatography, HPLC/ESI-MS-MS High-Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry, HPLC/ESI-LC/MS - High-Performance Liquid Chromatography/Electrospray Ionization Liquid Chromatography/Mass Spectrometry, HPLC-DAD/ESI/MS-MS - High-Performance Liquid Chromatography- Diode Array Detection /Electrospray Ionization Tandem Mass Spectrometry

[b] Single item analysis

Table 6-4: Differences in anthocyanin content of similar fruit vegetables from different databases and sources

Fruit/vegetable	**USDA database (mg/100g)**	**Phenol-Explorer database (mg/100g)**	**Australian data[a] (mg/100g)**
Strawberry	2.42	73.01	2.02
Blueberry	163.3	133.99	381.75
Plum	56.05	47.79	89.8
Red cabbage	209.95	- *	57.4

[a]Australian data from systematic literature review [310]; *- not reported

6.1.1.5 Conclusion

In conclusion, this proposed methodology provides insight for the compilation of analytical data for an Australian anthocyanin database for fruit and vegetables. Whilst the literature search produced very limited results, this was to be expected as the database is in the early stages of development and anthocyanins are an emerging area of research. As more analytical data becomes available from organisations and independent researchers, updates will be made to produce a robust database that will facilitate accurate measurement of anthocyanin consumption in Australian population studies and clinical trials.

6.1.2 Part 2: Estimated dietary anthocyanin intake and major food sources in Australia from the National Nutrition and Physical Activity component of the Australian Health Survey and association with blood pressure in older adults

This section of Chapter 6 describes the secondary analysis of the National Nutrition and Physical Activity Survey (NNPAS) component of the Australian Health Survey (AHS). Following the development of the Australian anthocyanin database (Chapter 6.1), a secondary data analysis of the 2011-12 National Nutrition and Physical Activity component of the Australian Health Survey was conducted to estimate the intake of both total anthocyanins and their sub-groups, identify food sources of anthocyanins, as well as determine association between anthocyanin intake and measured BP in older adults aged 50+ years. Results showed that mean anthocyanins intake was 24.17±0.32mg/day in the total population. Across age-groups, berries were the top sources: blackberry (5-65%); cherry (2-24 %); blueberry (2-13%) and raspberry (3-12%). There was also an observed significant inverse association between anthocyanin intake in older adults aged 50+ years and systolic BP (β =-0.04, $p<0.01$) and diastolic BP β= -0.01, $p<0.01$), in models that adjusted for covariates (age, gender, body mass index, high blood pressure (BP) diagnosis, smoking status and physical activity). In comparison to the world composite database, anthocyanin intake in the Australian population was above average (24.17±0.32mg/day vs 18.05±21.14 mg/d). Berries made up the primary sources thereof. Anthocyanin intake in older adults aged 50+y was inversely associated with BP.

The majority of this chapter section is the substantive content of the published articles:

Igwe, E.O., Charlton, K.E., and Probst, Y.C. 2019. Usual dietary anthocyanin intake, sources and their association with blood pressure in a representative sample of Australian adults. *Journal of Human Nutrition and Dietetics* (In press).

6.1.2.1 Introduction

Incorporating fruits and vegetables in the usual diet have been proven to exert protective effects on health. These observed effects have been attributed to the presence of minerals, antioxidant vitamins, phytochemicals and dietary fibre in these foods. These substances, independently and as a group in fruits and vegetables have been associated with reduced mortality [3] weight loss [4] cardiovascular diseases and some cancers [6]. With these observed beneficial health effects, inter-individual variability and optimal intake are still significant limitations in anthocyanin research. Although the source of bioactive compounds, found mainly in fruits and vegetables, cannot be regarded as the primary determining factor responsible for inter-individual variability, evidence has shown that it could have a significant effect on cardiovascular and metabolic responses associated with consumption of these compounds [368, 369]. This could also be attributed to the synergistic effect of other diverse compounds present in the source foods.

Accurate measurement of nutrient intake, in this case, anthocyanins, tailored specifically to individual countries and regions is an important consideration in both epidemiological and experimental studies. There is an observed variation in nutrient content of foods produced across different regions [89, 90] which has led to the development of country/region specific food composition databases. The same is also evident in the micronutrient databases. Currently the American USDA Database for the Flavonoid Content of Selected Foods and the European Phenol-Explorer, exist for the measurement of polyphenol (anthocyanin) intake [345, 346]. As mentioned earlier, these databases have been compared with observed discrepancies [347] which led to the first stage development of an Australian anthocyanin database to determine the amount of anthocyanins consumed by Australians [310] (Chapter 6.1).

Following the development of this database, this study aimed to estimate anthocyanin intake in the Australian population, to describe total and individual anthocyanin intake in the Australian population according to sociodemographic subgroups, and to determine the major sources of total and individual anthocyanins in the diet. Results from this secondary analysis may provide evidence on the possible role of anthocyanins in reducing risks of chronic diseases in Australian older adults.

6.1.2.2 Methods

This study was a secondary data analysis of the 2011–12 National Nutrition and Physical Activity Survey (NNPAS) data from the Australian Bureau of Statistics (ABS) using Basic Confidentialised Unit Record Files (CURF). No ethics approval was required for this study because it was a secondary analysis. However, approval to carry out a secondary analysis with the NNPAS component of the AHS data was obtained by the researchers from the Australian Bureau of Statistics prior to conducting this study.

The National Nutrition and Physical Activity Survey 2011-12

The NNPAS 2011-12 is a component survey of the larger 2011-12 AHS which involved a total of 12,153 persons. It was carried out between 29 May 2011 and 9 June 2012 in approximately 9,500 private abodes selected throughout non-very remote areas of Australia. Face-to-face interview (and by telephone for the second NNPAS interview) was used to collect data on general demographic information (including age, sex, marital status and country of birth) on all individuals while detailed information was collected from one adult and one child aged 2-17 years. This survey covered about 97% of the people living in Australia.

The study design for the 2011-12 NHS and NNPAS was a stratified multistage area sample of private dwellings. To achieve the design objectives, sampling fractions according to state and territories were set as shown in the Table 6-5 below, which also shows the corresponding expected number of completely responding households.

Table 6-5: National Nutrition and Physical Activity Survey, State/territory sample

	NSW[a]	Vic.[b]	QLD[c]	SA[d]	WA[e]	Tas.[f]	NT[g]	ACT[h]	Aust.[i]
Approximate sampling fractions	1/1649	1/1568	1/1131	1/551	1/665	1/208	1/102	1/169	1/901
Expected fully responding households	1 750	1 650	1 580	1 250	1 310	920	740	820	10020

Source: ABS [370] [a]New South Wales; [b]Victoria; [c]Queenland; [d]South Australia; [e]Western Australia; [f]Tasmania; [g]Northern Territory; [h]Australian Capital Territory; [i]Australia

6.1.2.2.1 Data collection

Data for the NNPAS 2011-12 was collected using face-to-face interviewer administered questionnaire. The questionnaire involved multiple approaches to collection. Information collected from the primary questionnaire included:

- Household information - basic demographic data about usual residents of the household (e.g. sex, age, date of birth, birthplace, Indigenous status, marital status) and details of the relationship between individuals in each household.
- Personal Adult Interview - information was collected from the selected adult about demographic, socio-economic and health characteristics (e.g. physical measurements, selected long-term health conditions, and risk factors).
- Personal (or proxy) Child Interview - information was collected on selected demographic and health characteristics, including specific physical activity modules for 2-4 years and 5-17 years. Questions on socio-economic characteristics and smoking were not asked of children aged under 15 years. Physical measurements were taken for children aged 2 years and older (5 years and older for blood pressure measurements).

6.1.2.2.2 Estimated dietary anthocyanin intake and major food sources in Australian adults

Estimation of anthocyanin intake occurred by applying a newly developed Australian anthocyanin database to the dietary records. There is a total of 5740 foods in the Australian Food and Nutrient Database (AUSNUT) 2011-13; of these, anthocyanin values were assigned to 318 individual foods.
The AUSNUT 2011–13 is a food composition database of food, dietary supplement and nutrient intake estimates that was compiled from participants' responses to the 2011–12 NNPAS. For the current analysis,

the NNPAS data was expanded to include anthocyanin content of reported foods and total intakes were calculated based on the amount of food (g) consumed for each day of recall.

Using the two separate days of 24hr dietary recall data, usual anthocyanin intake was calculated using the multiple source method (MSM) [371, 372]. The MSM comprises three steps. First, for each respondent in the study sample, the probability of consumption of the response variable on a randomly selected day was calculated. Secondly, the usual amount of food group intake on reported consumption days was estimated, and finally, the usual overall intakes were calculated by multiplying probability of consumption of the response variable with usual amount of intake on consumption days. Intake values were calculated within the MSM model assuming all participants were habitual consumers, given anthocyanins are primarily found in fruit and vegetables, with age and gender included as covariates. Two variables were produced in the MSM output; usual daily intake of anthocyanins for the total population calculated by the MSM (e.g. measure of habitual intake assuming population are consumers) and the usual intake of anthocyanins in consumers only from the 24-h dietary recall calculated by the MSM. Both variables were used in the statistical analysis (**Figure 6-2)**.

6.1.2.2.3 Statistical Analysis

Statistical analysis was carried out using SAS (release 9.4, 2012; SAS Institute). Daily anthocyanin intake was calculated and expressed as mean and standard error (SE). The total population analysis was based on usual daily total anthocyanin intake for all participants and the consumer population analysis was based on the usual intake of anthocyanins for consumers only. Intake of the major sub-classes of anthocyanins (cyanidins, delphinidins, malvidins, pelargonidin, peonidins and petunidins) was also calculated.

Weighting factors (person weights and replicate weights produced by ABS [370]) were applied to the data in order to generalise results to the total Australian population at the time of the survey and to account for sampling discrepancies.

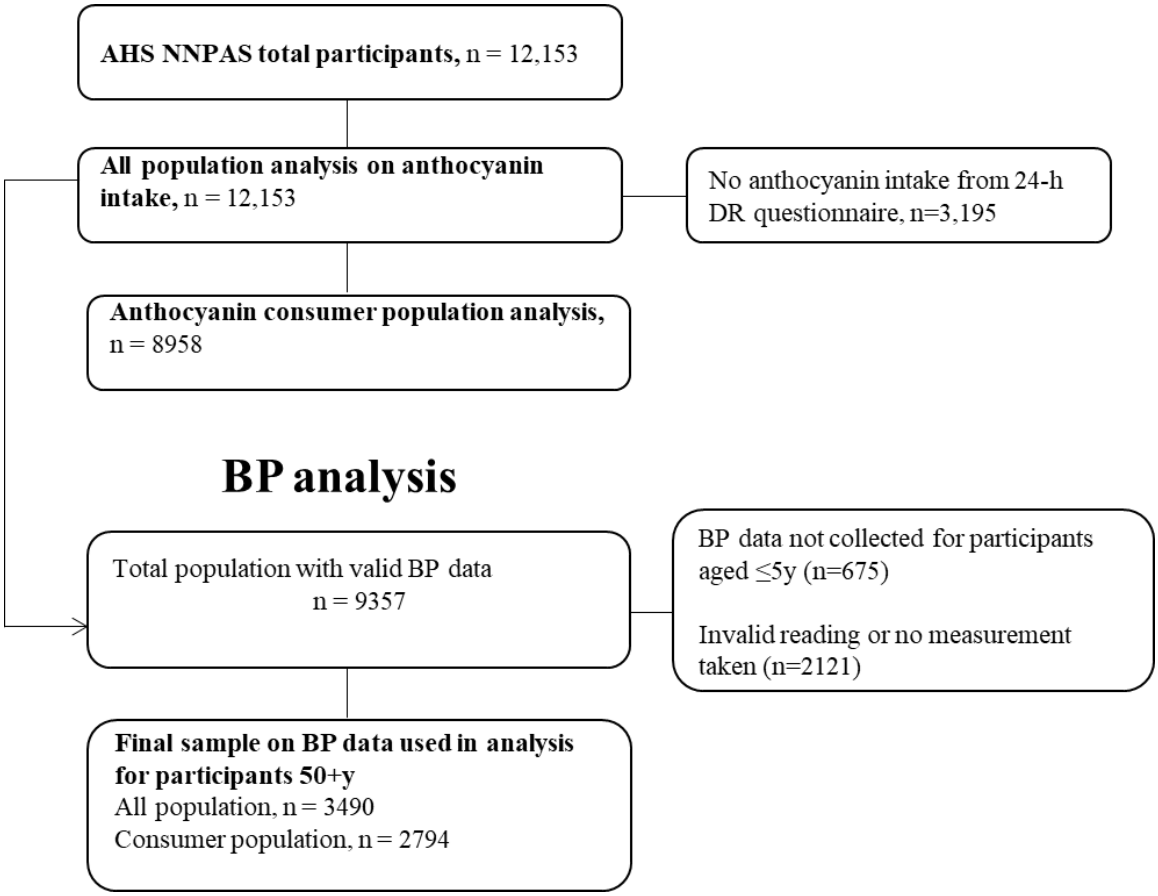


Figure 6-2: Participant flowchart for secondary analysis of the Australian Health Survey, NNPAS component

Mean anthocyanin intake for the total population and across sub-groups (age-groups, sex, Body Mass Index (BMI), level of education, smoking status and level of physical activity) was calculated. Age-groups were categorised according to those used in the National Health and Medical Research Council Nutrient Reference Values (NRVs) for Australia and New Zealand [373]. For the purpose of the analysis, categories of BMI, education, smoking and physical activity were grouped according to the ABS classifications with similar classes grouped together (e.g. underweight Class 3, underweight Class 2 and underweight Class 1 grouped as 'underweight') and individuals aged less than 15y were classified as 'not applicable' to maintain the integrity of the weighting factors applied [374].

Linear regression analysis was used to determine the relationship between anthocyanin intake and blood pressure in the subgroup of adults aged 50+ y (Figure 6-2), while adjusting for age and gender (model 1)

and adjusting for age, gender, BMI, physical activity, smoking status and whether diagnosed with high blood pressure (by a health professional and/or measured BP >=140/90 mmHg) (model 2). Inclusion criteria for this analysis were: i) adults aged 50 years and above and ii) who had a valid blood pressure measurement

Level of significance was set at 0.05 and calculated from t-test for pair-wise comparisons or ANOVA to determine if there are differences in any of the subgroups where appropriate.

6.1.2.3 Results

Dietary anthocyanin intake from the NNPAS was estimated to be 24.17 ± 0.32 mg/d for the total population (n = 12,153) and 37.68mg/d for the consumer population (n = 8,958). Mean intakes for total and subclasses of anthocyanins according to sociodemographic (gender, age-group, level of education) and lifestyle (BMI, smoking status and physical activity) are reported in **Table 6-6**. There were more adults than children (n = 9,341 vs 2,812) with no statistically significant difference in anthocyanin intake. Respondents who had high physical activity levels consumed more anthocyanins compared to those who had a sedentary lifestyle (30.04mg/d vs 20.42mg/d, <0.001). A similar trend was also observed for respondents with a Bachelor's degree compared to those with a diploma or lower (29mg/d vs 24mg/d, t-test p-value <0.001).

The top ten food sources stratified by age are shown in **Table 6-7**. Berries are highly concentrated sources of anthocyanins [310, 375] and were the top contributors to total anthocyanin intake across all age-groups. The top ten food sources were reported as these made up more than 50% of the total anthocyanin intake across all age-groups.

There was a significant inverse association between measured systolic and diastolic blood pressure and anthocyanin intake (**Table 6-8)**. This was evident for both systolic (β = -0.04, F (df) = 7.77 (2), R square = 0.01, p=0.001) and diastolic (β = -0.01, F (df) = 5.72 (2), R square = 0.01, p=0.005) blood pressure (Model 1: adjusted for age and gender). After controlling for further confounders (Model 2: age, gender, BMI, physical activity, high BP diagnosis, smoking status and physical activity), the association remained

significant (β = -0.04, F (df) = 16.8 (6), R square = 0.05, $p<0.01$ for systolic BP; and β = 0.01, F (df) = 5.35 (6), R square = 0.013, $p<0.01$ for diastolic BP).

Table 6-6: Anthocyanin intakes by demographic and lifestyle factors for the Australian population (and consumer population) in 2011–12 NNPAS

Stratification variable	N	Anthocyanins(mg/d)			Cyanidins (mg/d)			Delphinidin (mg/d)			Malvidin (mg/d)			Pelargonidin (mg/d)			Peonidin (mg/d)			Petunidin (mg/d)		
		Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
Total NNPAS population	12153	24.17	0.32		9.33	0.13		3.03	0.07		7.61	0.20		0.12	0.01		1.12	0.02		1.05	0.04	
(consumer population)	**(8958)**	**(37.68)**	**(0.41)**		**(15.22)**	**(0.23)**		**(5.07)**	**(0.12)**		**(14.14)**	**(0.42)**		**(0.21)**	**(0.02)**		**(1.99)**	**(0.05)**		**(1.98)**	**(0.08)**	
Gender				<0.001 **(<0.001)**			<0.001			<0.001			<0.001			<0.001			<0.001			<0.001
Male	5702	23.62	0.41		9.41	0.17		2.83	0.06		7.46	0.29		0.11	0.01		1.08	0.03		1.00	0.04	
	(4030)	**(38.27)**	**(0.63)**		**(16.04)**	**(0.33)**		**(4.89)**	**(0.11)**		**(14.46)**	**(0.67)**		**(0.21)**	**(0.03)**		**(1.98)**	**(0.07)**		**(1.97)**	**(0.09)**	
Female	6451	24.72	0.47		9.27	0.18		3.22	0.13		7.76	0.23		0.13	0.01		1.16	0.04		1.10	0.06	
	(4928)	**(37.14)**	**(0.67)**		**(14.49)**	**(0.29)**		**(5.24)**	**(0.22)**		**(13.85)**	**(0.48)**		**(0.21)**	**(0.02)**		**(2.00)**	**(0.08)**		**(2.00)**	**(0.13)**	
Age group (yrs.)				<0.001 **(<0.001)**			<0.001			<0.001			<0.001			<0.001			<0.001			<0.001
Children (≤18)	2812	22.46	0.58		10.97	0.28		2.51	0.11		4.86	0.33		0.11	0.01		0.89	0.05		0.57	0.07	
	(2071)	35.25	0.90		**(17.96)**	**(0.45)**		**(4.04)**	**(0.18)**		**(9.33)**	**(0.72)**		**(0.20)**	**(0.02)**		**(1.58)**	**(0.09)**		**(1.08)**	**(0.14)**	
2-3	464	21.51	1.40		7.54	0.43		3.45	0.28		6.34	0.89		0.14	0.02		0.99	0.13		0.83	0.16	
	(378)	**(31.11)**	**(1.86)**		**(11.06)**	**(0.83)**		**(5.59)**	**(0.44)**		**(11.18)**	**(1.53)**		**(0.27)**	**(0.04)**		**(1.67)**	**(0.21)**		**(1.49)**	**(0.27)**	
4-8	789	26.06	1.30		12.31	0.75		3.12	0.34		6.51	0.67		0.16	0.02		1.51	0.11		0.83	0.19	
	(633)	**(37.04)**	**(1.78)**		**(18.60)**	**(1.17)**		**(4.59)**	**(0.52)**		**(11.55)**	**(1.25)**		**(0.26)**	**(0.03)**		**(1.87)**	**(0.19)**		**(1.40)**	**(0.31)**	
9-13	787	22.92	0.91		12.02	0.53		2.11	0.12		4.38	0.49		0.09	0.01		0.83	0.06		0.43	0.06	
	(576)	**(36.29)**	**(1.40)**		**(20.01)**	**(0.86)**		**(3.42)**	**(0.21)**		**(8.54)**	**(1.14)**		**(0.16)**	**(0.02)**		**(1.50)**	**(0.11)**		**(0.87)**	**(0.15)**	
14-18	772	18.56	0.87		9.80	0.51		1.94	0.12		3.06	0.44		0.07	0.01		0.64	0.06		0.36	0.07	
	(484)	**(33.69)**	**(1.74)**		**(18.13)**	**(1.09)**		**(3.29)**	**(0.22)**		**(6.41)**	**(1.22)**		**(0.14)**	**(0.02)**		**(1.25)**	**(0.15)**		**(0.72)**	**(0.18)**	
Adults (≥19)	9341	24.66	0.35		8.87	0.13		3.17	0.07		8.39	0.23		0.12	0.01		1.18	0.03		1.19	0.04	
	(6887)	38.38	0.44		**(14.43)**	**(0.23)**		**(5.37)**	**(0.13)**		**(15.52)**	**(0.47)**		**(0.21)**	**(0.02)**		**(2.11)**	**(0.06)**		**(2.24)**	**(0.08)**	
19-30	1592	19.24	0.68		8.48	0.29		2.42	0.13		4.62	0.45		0.16	0.04		0.78	0.05		0.57	0.06	
	(1017)	**(34.24)**	**(1.07)**		**(15.73)**	**(0.60)**		**(4.60)**	**(0.25)**		**(10.08)**	**(1.43)**		**(0.33)**	**(0.09)**		**(1.61)**	**(0.15)**		**(1.29)**	**(0.16)**	
31-50	3565	25.66	0.66		9.58	0.27		3.22	0.14		8.40	0.41		0.11	0.01		1.19	0.05		1.24	0.07	
	(2543)	**(40.55)**	**(0.91)**		**(15.79)**	**(0.45)**		**(5.48)**	**(0.25)**		**(15.84)**	**(0.85)**		**(0.17)**	**(0.01)**		**(2.13)**	**(0.09)**		**(2.35)**	**(0.15)**	
51-70	2907	27.75	0.65		8.88	0.24		3.55	0.13		10.97	0.49		0.11	0.01		1.46	0.06		1.54	0.08	

Stratification variable	N	Anthocyanins(mg/d)			Cyanidins (mg/d)			Delphinidin (mg/d)			Malvidin (mg/d)			Pelargonidin (mg/d)			Peonidin (mg/d)			Petunidin (mg/d)		
		Mean	SE	P	Mean	SE	P	Mean	SE	P	Mean	SE	P	Mean	SE	P	Mean	SE	P	Mean	SE	P
	(2300)	**(40.20)**	**(0.97)**		**(13.39)**	**(0.42)**		**(5.71)**	**(0.27)**		**(18.96)**	**(0.98)**		**(0.18)**	**(0.02)**		**(2.46)**	**(0.11)**		**(2.75)**	**(0.16)**	
71+	1277	24.61	1.15		7.29	0.32		3.64	0.22		9.60	0.61		0.14	0.05		1.27	0.09		1.39	0.15	
	(1027)	**(33.64)**	**(1.37)**		**(10.80)**	**(0.48)**		**(5.37)**	**(0.33)**		**(14.16)**	**(0.99)**		**(0.23)**	**(0.09)**		**(1.91)**	**(0.16)**		**(2.14)**	**(0.24)**	
BMI (kg/m^2)				<0.001 **(<0.001)**			<0.001			<0.001			<0.001			<0.001			<0.001			<0.001
< 25	4876	24.38	0.59		9.91	0.21		3.02	0.14		7.29	0.30		0.14	0.02		1.12	0.04		1.03	0.07	
	(3668)	**(37.76)**	**(0.83)**		**(16.04)**	**(0.39)**		**(4.99)**	**(0.22)**		**(13.73)**	**(0.64)**		**(0.24)**	**(0.03)**		**(1.99)**	**(0.08)**		**(1.96)**	**(0.13)**	
25 to < 30	3044	25.51	0.58		9.21	0.25		3.10	0.09		8.96	0.45		0.12	0.02		1.25	0.05		1.24	0.06	
	(2274)	**(39.10)**	**(0.87)**		**(14.92)**	**(0.41)**		**(5.24)**	**(0.19)**		**(16.70)**	**(0.96)**		**(0.21)**	**(0.04)**		**(2.24)**	**(0.10)**		**(2.36)**	**(0.14)**	
≥ 30	2258	23.23	0.76		8.53	0.30		3.08	0.15		7.36	0.46		0.09	0.02		1.06	0.06		1.00	0.07	
	(1607)	**(37.02)**	**(1.06)**		**(14.09)**	**(0.53)**		**(5.34)**	**(0.28)**		**(13.36)**	**(0.99)**		**(0.17)**	**(0.04)**		**(1.91)**	**(0.12)**		**(1.90)**	**(0.18)**	
Measurement not taken	1975	22.46	0.69		8.96	0.31		2.86	0.13		6.51	0.43		0.09	0.01		0.97	0.05		0.82	0.06	
	(1409)	**(35.68)**	**(1.09)**		**(14.74)**	**(0.56)**		**(4.71)**	**(0.27)**		**(11.67)**	**(0.92)**		**(0.16)**	**(0.01)**		**(1.62)**	**(0.10)**		**(1.47)**	**(0.13)**	
Level of education				<0.001 **(<0.001)**			<0.001			<0.001			<0.001			<0.001			<0.001			<0.001
Not applicable	2180	23.64	0.70		11.36	0.35		2.65	0.14		5.39	0.38		0.12	0.01		0.96	0.06		0.62	0.09	
	(1676)	**(35.87)**	**(1.08)**		**(18.06)**	**(0.61)**		**(4.21)**	**(0.22)**		**(10.10)**	**(0.81)**		**(0.22)**	**(0.02)**		**(1.68)**	**(0.11)**		**(1.16)**	**(0.17)**	
Post-Grad	770	30.09	1.47		10.08	0.57		3.70	0.22		11.46	0.86		0.12	0.02		1.57	0.10		1.71	0.14	
	(629)	**(44.42)**	**(1.92)**		**(15.66)**	**(0.81)**		**(5.88)**	**(0.40)**		**(20.61)**	**(1.68)**		**(0.20)**	**(0.02)**		**(2.69)**	**(0.20)**		**(3.09)**	**(0.28)**	
Bachelors	1615	29.90	1.22		10.84	0.35		3.75	0.27		10.07	0.65		0.17	0.04		1.46	0.09		1.54	0.13	
	(1304)	**(44.07)**	**(1.63)**		**(16.84)**	**(0.54)**		**(6.13)**	**(0.46)**		**(18.23)**	**(1.27)**		**(0.32)**	**(0.08)**		**(2.52)**	**(0.17)**		**(2.86)**	**(0.26)**	
Diploma/TAFE Courses	3252	24.61	0.56		8.74	0.21		3.07	0.12		8.71	0.35		0.12	0.02		1.22	0.04		1.21	0.06	
	(2341)	**(39.10)**	**(0.77)**		**(14.39)**	**(0.38)**		**(5.23)**	**(0.19)**		**(16.56)**	**(0.74)**		**(0.19)**	**(0.03)**		**(2.24)**	**(0.10)**		**(2.35)**	**(0.14)**	
No Non-School Qualification	4190	20.57	0.43		8.08	0.22		2.72	0.07		6.07	0.25		0.10	0.02		0.89	0.03		0.80	0.04	
	(2893)	**(32.99)**	**(0.71)**		**(13.54)**	**(0.44)**		**(4.68)**	**(0.17)**		**(11.02)**	**(0.59)**		**(0.18)**	**(0.04)**		**(1.55)**	**(0.06)**		**(1.48)**	**(0.08)**	
Level not determined	146	29.06	2.40		9.32	1.04		4.43	0.67		10.25	1.60		0.09	0.02		1.35	0.17		1.53	0.27	
	(115)	**(41.95)**	**(3.71)**		**(14.21)**	**(1.69)**		**(7.32)**	**(0.35)**		**(17.42)**	**(3.00)**		**(0.15)**	**(0.04)**		**(2.15)**	**(0.31)**		**(2.70)**	**(0.53)**	
Smoking status				<0.001 **(<0.001)**			<0.001			<0.001			<0.001			<0.01			<0.001			<0.001
Not applicable	2180	23.64	0.70		11.36	0.35		2.65	0.14		5.39	0.38		0.12	0.01		0.96	0.06		0.62	0.09	
	(1676)	**(35.87)**	**(1.08)**		**(18.06)**	**(0.61)**		**(4.21)**	**(0.22)**		**(10.10)**	**(0.81)**		**(0.22)**	**(0.02)**		**(1.68)**	**(0.11)**		**(1.16)**	**(0.17)**	

Stratification variable	N	Anthocyanins(mg/d)			Cyanidins (mg/d)			Delphinidin (mg/d)			Malvidin (mg/d)			Pelargonidin (mg/d)			Peonidin (mg/d)			Petunidin (mg/d)		
		Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
Smoker	1813	18.21	0.68		7.28	0.34		2.20	0.11		5.27	0.33		0.15	0.05		0.78	0.04		0.73	0.05	
	(1073)	**(32.61)**	**(1.10)**		**(13.38)**	**(0.68)**		**(4.14)**	**(0.25)**		**(10.52)**	**(0.87)**		**(0.26)**	**(0.11)**		**(1.43)**	**(0.10)**		**(1.47)**	**(0.13)**	
Ex-smoker	3096	27.68	0.88		9.40	0.28		3.38	0.12		10.44	0.56		0.11	0.01		1.42	0.07		1.52	0.09	
	(2365)	**(41.65)**	**(1.34)**		**(14.81)**	**(0.45)**		**(5.42)**	**(0.21)**		**(18.77)**	**(1.10)**		**(0.17)**	**(0.01)**		**(2.48)**	**(0.13)**		**(2.82)**	**(0.18)**	
Never smoked	5064	24.33	0.45		9.18	0.20		3.24	0.11		7.65	0.27		0.12	0.01		1.12	0.03		1.06	0.05	
	(3844)	**(37.41)**	**(0.70)**		**(14.79)**	**(0.33)**		**(5.46)**	**(0.20)**		**(14.01)**	**(0.58)**		**(0.22)**	**(0.03)**		**(1.97)**	**(0.07**		**(1.96)**	**(0.10)**	
Physical activity				<0.001 **(<0.001)**			<0.001			<0.001			<0.001			ns			<0.001			<0.001
Not applicable	2718	22.64	0.60		10.99	0.28		2.54	0.11		4.98	0.35		0.11	0.01		0.90	0.05		0.57	0.08	
	(2010)	**(35.28)**	**(0.93)**		**(17.91)**	**(0.48)**		**(4.09)**	**(0.19)**		**(9.53)**	**(0.75)**		**(0.20)**	**(0.02)**		**(1.60)**	**(0.10)**		**(1.09)**	**(0.15)**	
High	1328	30.04	1.11		11.34	0.48		3.92	0.35		9.58	0.72		0.11	0.01		1.45	0.09		1.47	0.14	
	(1066)	**(43.83)**	**(1.24)**		**(17.63)**	**(0.67)**		**(6.33)**	**(0.53)**		**(17.28)**	**(1.26)**		**0.20)**	**(0.02)**		**(2.49)**	**(0.17)**		**(2.73)**	**(0.24)**	
Moderate	2574	25.99	0.69		9.17	0.28		3.32	0.10		9.33	0.40		0.13	0.02		1.31	0.05		1.39	0.07	
	(1950)	**(39.50)**	**(0.96)**		**(14.55)**	**(0.43)**		**(5.53)**	**(0.20)**		**(17.07)**	**(0.83)**		**(0.21)**	**(0.04)**		**(2.30)**	**(0.10)**		**(2.62)**	**(0.15)**	
Low	3351	23.57	0.57		8.44	0.20		2.99	0.10		8.12	0.38		0.10	0.01		1.13	0.05		1.08	0.06	
	(2461)	**(37.13)**	**(0.83)**		**(13.92)**	**(0.37)**		**(5.06)**	**(0.19)**		**(15.05)**	**(0.78)**		**(0.19)**	**(0.03)**		**(2.01)**	**(0.09)**		**(2.01)**	**(0.12)**	
Sedentary	2075	20.42	0.73		7.40	0.27		2.68	0.15		6.44	0.45		0.15	0.04		0.90	0.05		0.86	0.07	
	(1398)	**(34.05)**	**(1.27)**		**(12.56)**	**(0.49)**		**(4.74)**	**(0.31)**		**(12.04)**	**(1.04)**		**0.25)**	**(0.08)**		**(1.63)**	**(0.13)**		**(1.62)**	**(0.17)**	
Not stated	107	26.59	4.93		12.55	3.28		3.28	0.67		7.15	1.54		0.21	0.12		0.99	0.17		1.00	0.22	
	(73)	**(39.30)**	**(6.77)**		**(19.03)**	**(5.12)**		**(4.86)**	**(1.15)**		**(11.82)**	**(3.13)**		**(0.37)**	**(0.23)**		**(1.57)**	**(0.32)**		**(1.78)**	**(0.45)**	

p values significant at <0.05 using ANOVA

Table 6-7: Top ten food sources of anthocyanins in the diet of the Australian population, by age-group

	% total anthocyanin intake per age group															
Age (years)	**2-3**		**4-8**		**9-13**		**14-18**		**19-30**		**31-50**		**51-70**		**71+**	
n	**464**		**789[1]**		**787**		**772[2]**		**1592**		**3565**		**2907**		**1277**	
1.	Blackberry, raw	36.6	Blackberry, raw	65.2	Blackberry, raw	37.5	Cherry, raw	23.9	Grape, raw,[3]	15.4	Eggplant,[4]	8.1	Eggplant,[4]	23.1	Eggplant,[4]	26.4
2.	Raspberry, raw	12.8	Blueberry, raw	8.3	Blueberry, raw	12.7	Eggplant,[4]	15.7	Cherry, raw	8.5	Blackberry, raw	7.4	Grape, raw,[3]	9.1	Wine, red,	10.3
3.	Blueberry, raw	10.3	Grape, raw[3]	4.9	Grape, raw[3]	11.2	Grape, raw[3]	14.9	Cranberry, raw	8.2	Cherry, raw	5.7	Blackberry, raw	8.4	Cherry, raw	8.3
4.	Grape, raw[3]	8.6	Cherry, raw	2.6	Cherry, raw	7.3	Raspberry, purchased frozen	8.9	Eggplant,[4]	5.3	Blueberry, raw	5.1	Wine, red	7.8	Blueberry, raw	7.1
5.	Cabbage, red, raw	8.8	Raspberry, purchased frozen	2.4	Eggplant[4]	7.0	Blueberry, raw	8.5	Plum, unpeeled, raw	4.9	Raspberry, raw	4.9	Blueberry, raw	7.1	Blackberry, raw	5.1
6.	Cherry, raw	4.0	Plum, unpeeled, raw	2.4	Plum, unpeeled, raw	4.8	Cabbage, red, raw	5.9	Bean, black,[5]	4.5	Cranberry, raw	4.8	Cherry, raw	6.3	Plum, unpeeled, raw	4.9
7.	Raspberry, purchased frozen	3.4	Blueberry, purchased frozen	2.1	Raspberry, raw	3.2	Plum, unpeeled, raw	5.4	Raspberry, purchased frozen	3.9	Grape, black sultana, raw	4.6	Plum, unpeeled, raw	4.7	Raspberry, purchased frozen	4.7
8.	Plum, unpeeled, raw	3.2	Eggplant[4]	2.0	Raspberry, purchased frozen	2.6	Blueberry, purchased frozen	2.0	Blueberry, raw	3.8	Radish, peeled or unpeeled, raw	4.4	Raspberry, raw	2.9	Grape, Thompson,	3.4
9.	Eggplant[4]	2.7	Raspberry, raw	1.3	Apple, red skin, unpeeled, raw	1.6	Apple, red skin, unpeeled, raw	1.7	Wine, red	3.8	Raspberry, purchased frozen	4.3	Blueberry, purchased frozen	2.6	Radish, raw	3.2
10	Blueberry, purchased frozen	1.4	Apple, pink lady, unpeeled, raw	0.9	Pear, unpeeled, raw, not further defined	1.3	Pear, unpeeled, raw, not further defined	1.5	Raspberry, raw	3.2	Wine, red, sparkling	4.1	Cabbage, red, raw	2.4	Raspberry, raw	2.9
Sum		**91.8**		**92.1**		**89.2**		**88.5**		**61.6**		**53.5**		**74.4**		**76.3**

[1]n=2 excluded due to implausible dietary consumption data, [2]n=1 excluded due to implausible dietary consumption data, [3]Combination of Grape, raw, Grape, red, raw, and Grape, Thompson, seedless; [4]Eggplant, peeled or unpeeled, fresh or frozen, raw; [5]Bean, black, dried, boiled, microwaved or steamed, drained

Table 6-8: Association between anthocyanin intake and change in blood pressure in adults aged 50+yrs

BP parameters	Anthocyanin intake in all population (consumer population) Effect size (regression coefficient and 95% Confidence Interval) per unit (mg) increase					
	Model 1[1] n=4184 (3327)			*Model 2*[2] n=4184 (3327)		
	Regression coefficient	**95% CI**	***p value***	**Regression coefficient**	***95% CI***	***p value***
Systolic BP	-0.04 (-0.03)	-0.06, -0.01 (-0.05, -0.01)	0.001* (0.002)*	-0.04 (-0.03)	-0.06, -0.01 (-0.05, -0.01)	<0.01* (<0.01)*
Gender	-2.50 (-2.51)	-4.28, -0.72 (-4.45, -0.58)	0.01 (0.01)	-2.67 (-2.68)	-4.40, -0.94 (-4.62, -0.74)	0.003* (0.01)*
BMI				0.06 (0.1)	-0.002, 0.12 (-0.004, 0.1)	0.06 (0.07)
High BP diagnosis				-7.92 (-7.38)	-9.80, -6.05 (-9.63, -5.12)	<0.001* (<0.001)*
Smoking status				0.70 (1.06)	-0.06, 1.46 (0.11, 2.01)	0.07 (0.03)*
Physical activity				0.09 (0.15)	-0.61, 0.78 (-0.63, 0.92)	0.80 (0.7)
Diastolic BP	0.01 (0.01)	-0.01, 0.02 (-0.01, 0.02)	0.005* (0.03)	0.01 (0.01)	-0.01, 0.03 (-0.01, 0.02)	<0.01* (0.02)*
Gender	-1.56 (-1.31)	-2.51, -0.62 (-2.40, -0.22)	0.002 (0.02)	-1.52 (-1.30)	-2.49, -0.54 (-2.42, -0.18)	0.002* (0.02)*
BMI				0.06 (0.06)	0.03, 0.09 (0.02, 0.11)	<0.001* (0.01)*
High BP diagnosis				-0.77 (-0.54)	-1.76, 0.22 (-1.64, 0.55)	0.12 (0.33)
Smoking status				-0.24 (-0.19)	-0.64, 0.16 (-0.68, 0.30)	0.24 (0.44)
Physical activity				-0.09 (-0.04)	-0.53, 0.35 (-0.49, 0.42)	0.68 (0.88)

[1] Model 1 covariates = age (included as a class variable) and gender; [2]Model 2 covariates = age (included as a class variable), gender, BMI, hypertension diagnosis, Physical activity and smoking status; * Significant at p<0.05; values in bracket represent analysis for consumer population.

6.1.2.4 Discussion

Using nationally representative dietary survey data, this study reports an observed wide distribution of anthocyanin intake in the Australian population. The estimated mean intake was 24.17 ± 0.32mg/d which is midway between estimates reported for other populations that range between 2.9 and 42.79mg/d (**Table 6-9),** and was above average in comparison to the world composite database [376]. There were variations in anthocyanin intake across subgroups analysed, for example a higher anthocyanin intake in the 51-70y age-group (27.75 ± 0.65 mg/d) compared to the lowest consumption (18.56 ± 0.87 mg/d) in the 14-18y age-group ($p<0.001$). Adults with a post-graduate qualification had higher anthocyanin intakes (30.09 ± 1.47 mg/d) compared to those without school qualifications (20.57 ± 0.43mg/d) ($p<0.001$), while highly active adults (30.04 ± 1.11mg/d) had higher intakes compared to sedentary respondents (20.42 ± 0.73mg/d) ($p<0.001$). These results are consistent with previous reports on the significant association between socio-demographic and lifestyle factors and fruit and vegetable consumption [377-380]. Surprisingly, ex-smokers reportedly consumed significantly higher daily anthocyanins (27.68 ± 0.88 mg/d), more than both smokers (18.21 ± 0.68 mg/d) and non-smokers (24.33 ± 0.45 mg/d). Zamora-Ros et al.(2010) [381] similarly reported that ex-smokers consumed more anthocyanins (11.16mg/d) than non-smokers (10.62mg/d).

Estimation of anthocyanin intake in the Australian population is an important preliminary step in understanding anthocyanin-health relationships. Despite increased research interest on the observed health benefits of anthocyanins provided by food and beverages, the accompanying increased prevalence of consumption of processed foods translates to a reduced consumption of dietary anthocyanins [382]. In the 1970s, average daily dietary anthocyanin intake in the USA. was estimated at 215 mg/d in the summer and 180 mg/d during winter [383]. Current estimates show that dietary anthocyanin intake ranges between 3-43mg/day across countries and tends to be higher in Southern compared to Northern European countries (**Table 6-9).**

Table 6-9: Reported anthocyanin intake (mg/d) in population studies by country

Country (reference)	Sample size	Age/Gender[1]	Dietary assessment	Total anthocyanin intake (mg/d)
Australia (this study)	12,153	≥2yrs	2 x 24h DR (MSM method)	24.17
Australia [337]	10,851	≥2yrs.	24h DR	1.4
Australia [338]	79	≥49yrs	4-day WFR	7.0
China [384]	1393	35-75	FFQ	28[2]
Europe [385]		35-74yrs.	FFQ (GA[2]LEN)	
Denmark	268			7.5
Finland	122			5.9
Sweden	1,085			6.5
UK	139			9.8
Portugal	233			22.1
Belgium	107			10.5
Germany	305			5.5
Netherlands (Amsterdam)	174			8.1
Poland	116			9.2
Europe [386]		35-74yrs.	24h DR	
Greece	2,687			31.82
Spain	3,220			31.58
Italy	3,953	35-74yrs./F (1 out of 5 centres)		42.79
France	4,735			37.42
Germany	4,415	35-74yrs./F		35.09
The Netherlands	3,980			22.56
UK	1,280	35-74yrs./F (1 out of 2 centres)		26.12
Denmark	3,917			28.21
Sweden	6,050			20.96
Norway	1,797	35-74yrs./F		26.56
Finland [387]	1950	42-60yrs./M	4-day food record	6.2
Finland [388]	2007	25-64yrs.	48h diet recall	47
France [389]	4942	45-60yrs.	≥6 24h diet recall	35
Spain [381]	40,683	35-64yrs.	Diet history questionnaire	18.88
United Kingdom [390]	1,997	18-76yrs./F	FFQ	18
USA [391]	8,809	>19yrs.	24h diet recall	3.1
USA [392]	5,420	≥20	24h diet recall	11.48

[1] gender specified when sample size is gender specific; WFR, weighted food record; 24hrDR, 24hr dietary recall; [2]excludes malvidin and petunidin

Consumption of anthocyanin subclasses were also reported for the total and consumer populations.

Cyanidins and malvidins were the most prevalent anthocyanins in both the general and consumer

populations and across subgroups. In the 51-70y age-group, malvidins were the most prevalent anthocyanins. This could be explained by the difference in consumption pattern of the major food contributors of anthocyanins in this age-group being red wine, which has a high content of malvidins. A similar trend was also observed in other Australian population studies [337, 338] with red wine being reported as a major contributor of anthocyanins in older participants. The highest contributing foods (top 10) of dietary anthocyanin in the Australian population were berries, principally blueberries, blackberries, raspberries, and cherries. Berries are known to contain a very high concentration of anthocyanins [393]. Given this, anthocyanin research has generally focused on berries at the expense of other high anthocyanin foods, such as plums and eggplants which also made up some of the top 10 contributors of anthocyanins in the Australian population. Accordingly, it might be worth further exploration to gather epidemiological evidence of the health-related benefits related to these foods.

Results from this study showed a significant association between anthocyanin intake in older adults and lower blood pressure. This result is in agreement with current evidence from clinical and epidemiological studies on the effect of dietary anthocyanins on blood pressure in acute settings and over the longer term [214, 301, 394] . Anthocyanins have also been classified as nutraceuticals in their ability, as part of a food component, to provide health and medical benefits [395]. However, it is unlikely that these health benefits are the independent effects of anthocyanins but rather represent a synergistic effect with other polyphenols found in foods [396]. This emphasises the importance of studying anthocyanin food sources, consistent with this study, rather than isolated anthocyanin extracts. A review by Pascual-Teresa et al. (2010) [397] observed that evidence related to the protective effects of anthocyanins, as part of the diet, against cardiovascular disease risks has been consistent over the years. Dietary patterns high in fruits and vegetables such as blueberries, apples, and leafy greens that are high in natural antioxidants and polyphenols (anthocyanins) have been shown to reduce the risk of high blood pressure [398] and other chronic diseases evident in the Dietary Inflammatory Index (DII) [399]. This evidence has been consistent with similar dietary patterns. Using the Dietary Inflammatory Index, Steck et. al (2014) [400] found that the Mediterranean diet showed anti-inflammatory potential based on the resulting DII scores. In addition, the Nordic diet significantly reduced 24-h ambulatory diastolic blood pressure (DBP) and mean arterial pressure in comparison to a control diet based on mean nutrient intakes in Nordic countries [401]. Other dietary patterns that emphasise high fruit and vegetable intake (the Dietary Approach to Stop

Hypertension), low-carbohydrate, Palaeolithic, high-protein, low-glycaemic index, low-sodium, and low-fat diets) were also found to significantly reduce blood pressure (systolic blood pressure (−8.73 to −2.32 mmHg) and DBP (−4.85 to −1.27 mmHg)) in comparison to control/usual diets [402]. Attributable risk related to the polyphenols present in these diets (i.e. anthocyanins) cannot be disentangled from other health-promoting components of key foods included in these blood pressure lowering dietary patterns.

Previous studies have estimated the dietary flavonoid intake in selected Australian populations using weighed food records [338] and from National Nutrition Survey (1995) data using a single 24-h dietary recall questionnaire [337]. Our results using the latest nationally representative survey data differ significantly from earlier studies with higher values reported in the current study. This is possibly due to differences in the method of dietary assessments, as well as the use of the MSM to calculate usual daily intake from two repeated 24-h dietary recalls. Differences could also be as a result of difference/change in population characteristics. Our analysis indicates that the Australian population consume intermediate quantities of anthocyanins compared to other populations (Table 6-9), but that are similar to estimates from some European countries including Belgium, Norway, Sweden and Denmark [385, 386]. A need for tailored food composition databases for nutritional epidemiological studies cannot be overemphasised. Following the development of the first Australian food composition database in the mid-1980s, comparative analysis showed that using the UK and US databases overestimated specific nutrient contents by up to 60% [403]. As a result, it is imperative that databases be tailored to the specific food supply of the population under study. However, development of nutrient databases is fraught with incomplete coverage of all nutrients hence, borrowing and calculating nutrient values is a known validated method in the absence of analytical values [404].

To our knowledge, this is the first time that dietary anthocyanin intake in the Australian population has been estimated using an Australian-specific anthocyanin database, this being a major methodological strength. Another notable strength is the use of a representative sample of the Australian population, as well as the validated method (MSM) of calculating usual nutrient intake from repeated 24h dietary recalls [371]. Some of the limitations of this study include the use of 24-h diet recall questionnaires in the NNPAS (1-2). The single 24-h diet recall has been deemed insufficient because of the retrospective method of dietary assessment and an inability to describe the typical diet from a single day's intake. In addition, the

recall is dependent on the memory and cooperation of the participant [405]. Four repeated 24-h diet recalls have been recommended as the most appropriate method for large surveys,[406] while two were applied in the NNPAS. This study analysis applied a regression model to these data to better represent usual intake although no adjustments were made to potential misreporting of the data. A further limitation relates to the effects of processing and storage [407] which was not taken into consideration in the borrowed data used from USDA and Phenol-Explorer food composition databases. In addition, the omission of anthocyanin intake from dietary supplements is a notable limitation of this study. It is however unlikely that such supplements are widely used in Australia, and composition of supplements and extracts in the AUSNUT food composition database did not include polyphenols (anthocyanins). Finally, out of the 5,700 foods in the AUSNUT database, about 300 of them, which did not include recipes, were assigned an anthocyanin value. Each of these elements could have led to both over- and underestimation of anthocyanin intake. In addition, for the purpose of this study, total anthocyanins were considered to be the major anthocyanin subgroups (cyanidin, delphinidin, peonidin, petunidin, pelargonidin and malvidin).

The cross-sectional nature of this study limits interpretation of the inverse association found between anthocyanin intake and blood pressure. However, evidence is beginning to emerge from experimental studies that support a protective role of anthocyanins on CVD risk in both young and older adults [408]. For two foods, plum and red cabbage, the Australian analytical data only reported total anthocyanins but not anthocyanin subclasses. Many fruits and vegetables that are high in anthocyanins are seasonal, available only in summer and autumn in Australia, therefore contribution to total intake will depend on the time of year that surveys are conducted. For example, red cabbage is a rich source of anthocyanins but accounted for less than 1% (0.2%) of total anthocyanin intake in the Australian population. This could also be as a result of not many people consuming red cabbage.

Although different methods have been described for estimating usual intake, the MSM method involves similar steps to the National Cancer Institute (NCI) method but uses different modelling procedures and handling of non-consumers [409]. Comparison of the different methods, including those from Iowa State University (ISU), NCI, MSM and Statistical Program to Assess Dietary Exposure (SPADE), showed that with small sample sizes (n = 150), the ISU, MSM and SPADE methods were more reliable and showed

more precise estimates than the NCI method, mainly for the 10th and 90th percentiles. The observed differences between methods became less significant with larger sample sizes (n = 300 and n = 500) [410].

6.1.2.5 Conclusion

In conclusion, this study estimates for the first time, mean daily intake of anthocyanin in the Australian population, according to sociodemographic characteristics, using an Australian-specific anthocyanin food composition database. Given the rapidly emerging evidence base related to the beneficial effects of anthocyanins on cardiovascular risk factors, it is timeous to assess population-level intake of this flavonoid subgroup. Anthocyanin intake in the Australian population was similar to that reported in southern European countries, and higher than in Northern Europe and USA. Identification of major dietary sources of anthocyanins (blackberry, raspberry, blueberry, cherry, red cabbage, eggplant and red wine) allows for focused dietary messaging.

It is worth noting that association does not infer causation and in the general overview of this body of work, results are not comparable across studies (Chapters 3, 5 and 6.2) due to the difference in study and experimental designs: acute crossover trial vs longer-term randomised crossover trial vs secondary analysis of cross-sectional data, respectively. However, results from this secondary analysis showed that there was a significant association between a mean intake of 24.17mg/d of anthocyanins and BP in adults aged 50+ years. In comparison to the acute crossover trial (Chapter 3) and longer-term RCT (Chapter 5), anthocyanin intake in the Australian population was lower than the amount provided in the acute trial intervention (369mg) but higher than the RCT intervention (50mg/L) which further limits comparison of results. Despite this, the current analysis of population-based data on anthocyanin intake provides a minimum benchmark against which to compare anthocyanin interventions in the design of future clinical trials. In addition, the main sources of anthocyanins identified in this nationally representative sample of Australians (berries) are consistent with the anthocyanin sources that have generally been provided in intervention studies. It is unknown whether the research focus to date on berries has influenced consumer behaviour, or if the dietary survey data reflects cultural cuisine or other influences on dietary behaviour. A broader focus

of health-related research that extends to other anthocyanin-containing foods such as plums and other less studied foods with varying anthocyanin profiles may result in changes in fruit preferences in future.

7 CHAPTER 7

7.1 CONCLUSIONS AND RECOMMENDATIONS

7.1.1 Overview of core findings

The body of research presented in this thesis used a mixed method approach to address the central research question. This approach covered domains of: a) current knowledge synthesis (systematic literature reviews), b) knowledge generation (clinical trials) and, c) knowledge translation (epidemiological analysis to identify key dietary sources of anthocyanins in the Australian population for application of clinical trial data to dietary guidance). The central research question investigated was the extent to which consumption of anthocyanins from plums improved health outcomes, specifically the role of anthocyanins derived from the Queen Garnet Plum, on various domains of cognitive function, blood pressure parameters and changes to the gut microbiota. To contextualise these findings, the dietary anthocyanin intake of the Australian population was identified by applying a novel anthocyanin food composition database to national nutrition survey data.

Anthocyanins are ubiquitously distributed in dark red and purple plant foods, with the highest concentration found in berries. As a result, research into the beneficial health effects of anthocyanins to date has focused on this food source [21]. However, the focus on berries in anthocyanin research limits generalisation of findings to broader dietary advice. Anthocyanin-rich foods are classified into three main groups according to their major type of anthocyanin aglycone complexes present in the food: namely, pelargonidin; cyanidin/peonidin; and multiple anthocyanins. Berries have been classified under these three different groups i.e. strawberries (pelargonidin), blueberries (multiple anthocyanins) and raspberries/plums (cyanidin/peonidin) [98] which highlights the need to generate evidence from the different anthocyanin group classifications. With the availability of numerous fruits and vegetables that are rich in anthocyanins but limited in clinical evidence, one of the main objectives of this research was to further investigate the beneficial health effects of anthocyanins from a different anthocyanin-rich source i.e. plum juice.

As a first step, a systematic literature review was undertaken to summarise the evidence on the beneficial health effects of plums (Chapter 2). Results from the review showed that the largest amount of clinical trial evidence relates to the prevention and management of osteoporosis and overall bone health. However, many of the study designs were of low quality and the research conducted with non-healthy participants. Although a number of outcomes have been studied in animal models, there was no confirmatory clinical

evidence from trials in humans, thus limiting the usefulness of the current literature. For example, there was no available evidence from clinical trials on the effects of plum consumption on cognition even though this outcome has been studied using animal models and, likewise, there was only limited evidence related to cardiovascular responses.

To address this gap in the literature on the health effects of plum consumption in humans, a pilot cross-over study was designed to assess the acute impact of differing doses provided by the Queen Garnet plum juice on cognition, blood pressure and bioavailability which was assessed by determination of urinary biomarkers in both younger and older adults (chapter 3). This study was conducted early on in the PhD work in order to further inform the design of a later clinical trial regarding the effect of consuming a previously demonstrated effective physiological dosage of anthocyanins [214] in three smaller quantities rather than as a single dose. Queen Garnet Plum juice was provided to participants, either as a single 300 mL dose (equivalent of 123mg anthocyanins) or as 3x100mL doses consumed over a 3h period. The cross-over study design was used in this study in order to reduce between-subject variability, with each subject acting as their own control, and thus increasing the study power [411]. Results from the pilot crossover study demonstrated a significant reduction in blood pressure for both young and older adults following consumption of QGP juice, with more obvious BP reduction in the older age-group. There was no observed dose-timing effect, nor was there an acute effect on cognitive function.

Following the completion of the acute pilot crossover study, an 8-week randomised crossover trial was planned. To further develop the hypothesis from the pilot trial and better elucidate proposed mechanisms of effect of plum juice anthocyanins, changes in gut microbiota were investigated. Additionally, in order to understand and summarise the current evidence on the effect of anthocyanins on gut microbiota, a second systematic literature review (chapter 4) was undertaken. The gut-brain axis is influenced by the microbiota populations in the gut [412], and this is a rapidly emerging area. The review did not identify any experimental evidence on the effects of anthocyanins derived from plums, specifically on gut microbiota profiles, and there was a general paucity of evidence in totality for the anthocyanin-gut microbiota relationship. With over 1000 species of bacteria in the gut, results from this review highlighted potential bacteria species that might be affected by anthocyanin consumption. There was an observed proliferative effect on *Bifidobacterium spp.*, known for their wide use in probiotics and for the treatment of Irritable

Bowel Syndrome and inhibition of *Clostridium histolyticum*, which has been shown to be pathogenic in humans. The review also informed our analysis of the gut microbiota data obtained in our RCT.

An 8-week randomised crossover clinical trial was undertaken to determine the effect and within patient variation of daily consumption of 200mL of anthocyanin-rich Queen Garnet plum juice or raspberry cordial (control) on cognition and ambulatory blood pressure in healthy older adults (55+ years) (chapter 5). Secondary outcomes included anti-inflammatory markers (C-reactive protein and BDNF), urinary anthocyanin metabolites, changes in anthropometrics and physical abilities and gut microbiota. Results from this study showed that the acute blood pressure effects observed in the pilot study were no longer evident in the longer term, while a lack of effect on cognitive function remained for both studies. There were also no demonstrated effects on anti-inflammatory biomarkers; however, anthocyanins derived from QGP, may affect total population of gut bacteria, but not necessarily impact on specific gut bacteria. The clinical significance of this finding warrants further exploration.

This study has significantly contributed to the body of evidence on the health effects of dietary anthocyanins. For the first time, a well-designed crossover human clinical trial has explored the effects of anthocyanins from plums on outcomes related to the gut-brain axis in otherwise healthy adults. Our results indicate that the effects seen in older adults with dementia are not translatable to healthy ageing individuals. Even though results from the acute trial were not comparable to the longer-term 8-week trial, this research highlighted issues for future considerations in anthocyanin research.

The final study in this thesis comprised development of an Australian anthocyanin food composition database (chapter 6.1). This database development is the first step in achieving appropriate measurement of flavonoid intake, with anthocyanins being the first step, for use in Australian epidemiological studies, as well as dietary clinical trials. Following the development of this database, a secondary data analysis of anthocyanin intake in the Australian population was undertaken. This was done using the National Nutrition and Physical Activity Survey component of the Australian Health Survey (chapter 6.2). Even though this is the first stage in the development of an Australian-specific food composition database for anthocyanin content of local foods, it was adequate to measure anthocyanin intake in the Australian population. Results showed an intermediate anthocyanin intake among Australians, as compared to intakes

in other countries that are reported in the world composite database (24.17 ± 0.32 in the current study vs 18.05 ± 21.14 mg/day). Further progression of the Australian anthocyanin database will include updates in order to produce a robust database which will further facilitate measurement of anthocyanin consumption. In the future, efforts will be made to expand this database to include the full range of other flavonoid classes and subclasses.

7.1.2 Strengths and limitations

The different methods employed in this body of research have their strengths and limitations which are briefly outlined below. These strengths and limitations have been described more comprehensively in each of the thesis chapters.

As research on anthocyanins continues to show beneficial health effects, it is important to note that nutrients are not consumed individually but are consumed within food matrices within a more complex dietary pattern. As such, there is a possibility that any demonstrated health effects may be due to the anthocyanins present in foods or as a result of the synergistic effect of the anthocyanins with other nutrients of non-nutritive components in the same food or present in other foods consumed at the same time as part of a meal. As a result, there needs to be a strong evidence base from confirmatory studies. As a first step, anthocyanin-rich foods rather than anthocyanin standards of individual components should be utilised in clinical trials. For the research included in this thesis, QGPJ was utilised. The QGP was processed to juice using methods that minimise nutrient degradation and utilised 100% of the fruit flesh and skin.

In assessing the positive health effects of anthocyanins, high levels of evidence are needed. Within this framework, the research presented in this thesis has summarised existing evidence but also generated novel findings related to consumption of anthocyanins from plums. The inter-individual variability in the metabolism of anthocyanins have been well documented [41] therefore the cross-over study design was considered best for both the acute and longer term clinical trials (Studies 2 and 4), because participants acted as their own control.

This research highlighted the limitations in precise measurement of flavonoid (anthocyanin) intake in population studies due to a lack of appropriate food composition databases available. Study 5 of this thesis addressed this knowledge gap in the first stage development of an Australian anthocyanin database in order to better estimate anthocyanin intake, and in the long-term flavonoid, intake in Australian population studies.

There are a number of limitations related to this body of research on the effects of anthocyanins from QG plum on cognition, blood pressure, inflammatory biomarkers and the gut microbiota population. The research main focused on anthocyanins and used a specially bred Japanese plum hybrid that was high in anthocyanins as the food delivery vehicle. Extrapolation of the findings to plums in general is limited, therefore it may be worth conducting similar studies with regular plum varieties to confirm these findings. Additionally, current evidence shows that the potential positive health effects of anthocyanins from food may be due to their circulation in blood as phenolic acid metabolites [413]. This body of research was limited in resources to conduct this analysis. Hence the lack of characterisation of these plasma metabolites in the current body of work is a limitation.

Another limitation in the body of anthocyanin research which prevents direct comparison of results across studies is the low stability of anthocyanins and differences in concentrations provided in clinical trials. Anthocyanin stability is affected by a number of factors including; temperature, pH, the structure and concentration of the pigment, light, co-pigments, enzymes, oxygen, metallic ions, sulphur dioxide, and sugar [414]. Processing, and storage have been shown to significantly degrade anthocyanins [342]. The processing of fruits to juice/powder usually involves high temperatures to ensure microbiological stability. In the case of anthocyanins which are heat sensitive, they have been shown to degrade up to 90% when exposed to high temperatures [343]. This is a major limitation in intervention studies in which anthocyanin-rich foods are usually delivered in their juice or power forms [21]. This is not unusual as statistics show that in developed countries, 50% of plum produce are consumed in processed forms [415].This emphasises the need for more efficient ways of processing anthocyanin foods without degrading a significant part of its anthocyanin content [416]. High temperature processing of anthocyanins leads to the production of furfural compounds during the degradation of sugars and ascorbic acid, thereby degrading them in foods [415]. As anthocyanins are responsible for the red, purple and blue pigments in fruits, colour is an

important determinant in product quality hence minimising the pigment losses during processing is an important consideration in the processing and storage of anthocyanin-rich foods [417]. Long-term storage of anthocyanin-rich foods have also been shown to degrade anthocyanin. When plum juice, for example, was stored for a period of 6 months at room temperature, its anthocyanins degraded drastically up to 90%, with lower storage temperature (4ºC) reducing the degradation process (50%) [343]. In the present body of work, there was a considerable difference in the storage times of study beverages used in the acute dose-timing study (2 months frozen) compared to the 8-week RCT (10 months frozen and then refrigerated) as well as duration of the two trials (4 weeks vs 8 months). It is also important to note that the study juice was supplied in batches and as a result stored for the duration of the study. Processing, storage as well as the low stability of anthocyanins has limited results comparison across anthocyanin research, as well as their use in the food industry [416].

Additionally, in assessing the amount of anthocyanin intake in the Australian population and its association with blood pressure, the cross-sectional nature of the data is a major limitation which does not allow for determination of causality.

7.1.3 Future research and recommendations

This research has made a significant contrition to the area of anthocyanin research, specifically in relation to QGP. Nonetheless, there is a need for ongoing research to further contribute to a stronger evidence base. Following on from the findings of this research, a number of key points and recommendations for future research are highlighted below:

- As a first step, this research resulted in the development of an Australian anthocyanin database, and further expansion of using generated analytical values for anthocyanins and flavonoids in general is required. The effect of processing on the flavonoid content of these Australian foods needs to be taken into consideration in order to progress this database development and improve its accuracy;

- Identifying the nutritive and non-nutritive composition of regular plums and prunes in comparison to QG plums and the effects of processing on their bioactivity are also important for future research in order to define the overall health effects of plums/prunes. Further clarification of the mechanisms of action of plum polyphenols, identification of potential adverse effects and the effects of dosage on outcomes is necessary to inform dietary guidelines for chronic disease prevention and management;

- To confirm these observed effects of anthocyanins, it is recommended that further research is conducted using other high anthocyanin foods. This is required in order to identify the synergistic effects of nutrients and food matrices that may be contributing to observed effects. There is also the need for future studies to focus on ways to stabilise or reduce degradation of anthocyanins in foods from processing as a first step. In addition, measuring anthocyanin content for the duration of a trial to monitor anthocyanin deration over time is also an important consideration in order to compare and generalise conclusions across studies.

7.1.4 Conclusion

In conclusion, this body of research has summarised and addressed knowledge gaps in relation to the acute and longer-term health effects of dietary anthocyanins, specifically provided by a high anthocyanin variant of plum (Queen Garnet), as well as developed an Australian anthocyanin food composition database. A total of five studies were undertaken to address the research question on the acute and longer-term effects of consumption of anthocyanin containing plum juice on cognition and other health parameters in older adults.

Each study constituting this thesis contributed novel and original evidence. Current evidence was synthesised and gaps in current literature were identified from the systematic literature reviews. As part of addressing these gaps and generating new evidence, two RCTs were conducted which added to the evidence base of anthocyanin research. Finally, a knowledge translation aspect of this thesis related to

identifying key sources of anthocyanin in the Australian population and amount of intake. Collectively, these studies progress scientific evidence around knowledge gaps on dietary anthocyanins and in a larger context how nutrients as part of food and diet, appropriately modified, can impact health outcomes in humans and influence chronic diseases.

8 List of References

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9 APPENDICES

9.1 Appendix A – Quality rating of included clinical studies (Chapter 2)

Relevant quality rating criteria from The Delphi List, Cochrane Back Review Group and The CONSORT Statement [418-420]

A eligibility criteria specified, **B** randomization appropriate, **C** treatment allocation concealed, **D** similarity at baseline, **E** outcome measures and control intervention explicitly described, **F** co-intervention comparable, **G** outcome measures relevant, **H** adverse events and **I** drop-outs fully described, **J** sample size based on a priori power calculation, **K** point estimates and measures of variability presented for the primary outcome measure, **L** appropriate timing giving a Total Score **(TS)** of 12

	J Nutr **106:** 923-930[122]	*J Wom Health Gend-B* **11:** 61-68 *[115]*	*Br J Nutr* **112:** 55–60[128]	*Appl Physiol Nutr Metab* ***39:*** *730-739[123]*	*Am J Clin Nutr* **76:** 1422-1427.[149]	*J Am Coll Nutr* **26:** 170-181.[137]	*J Med Food* 8**(1):** 41-46[129].	*J Food Nutr Res* **50:** 229-236. [138]
	n = 160 Dried plum (*p. dometica)* 100g/day vs dried apple Parallel 12 months	n = 58 Dried plum (*p. dometica)* 100g/day vs dried apple Parallel 3 months	n = 160 Dried plum (*p. dometica)* 100g/day vs dried apple Parallel 1 year	n = 23 Dried plum (*p. dometica)* 90g/day+ resistance training (RT) vs RT Case-control 6 months	n = 19 Dried plum (*p. dometica)* 100g/day vs habitual dietary simple sugars Cross-over 6 months	n = 6 Dried plum/dried plum juice (*p. dometica)* 131g vs selected fruits Cross-over 2 weeks	n = 10 Plum juice (*p. salicina)* Single dose of 150mL vs selected fruit juices Cross-over 18 days	n = 18 Dried plum (*p. salicina)* 2x195g/day Case series 5 days
A	Postmenopausal women (Osteopenic)	Postmenopausal women	Women with mild bone loss	Female breast cancer survivors	Postmenopausal women (Healthy)	Healthy volunteers	Healthy volunteers	Healthy volunteers
B	Yes	Yes	Yes	Yes	No	Yes	No	Not applicable
C	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	no	No	Not applicable
D	Yes	Yes	Yes	Yes	Yes	Yes	Yes	yes
E	Yes	Yes	Yes	Yes	Yes	Yes	Yes	yes
F	Yes	Yes	Yes	Yes	Don’t know	Yes	Yes	Not applicable
G	Yes	Yes	Yes	Yes	Yes	Yes	Yes	yes
H	Yes	Yes	Yes	Yes	Yes	Yes	Yes	yes
I	Yes	Yes	Yes	Yes	Yes	Probably no	Probably no	Probably no
J	No	No	No	No	No	No	No	no
K	Yes	Yes	Yes	Yes	Yes	Yes	Yes	yes
L	Yes	Yes	Yes	Yes	Yes	Yes	Yes	yes
TS	10	10	10	9	8	9	8	7

	J Food Biochem **36:** 159-170.[107]	*Am J Clin Nutr***53 (5):** 1259-1265 [92]	*J Acad Nutr Diet***112:** 1158-1168 [159].	*Curr Top Nutraceut R* **7:** 157-160 *[165]*	*J Ayub Med Coll Abbottabad* **22(1):** 38-31[160]	*J Funct Foods* **12:** 11-22[85]	*J Funct Foods***14:** 747-757 [166]	*Nutr Res* **27:** 511-513.[161]	*Internet J Nutr Wellness***7:** 1-1 [162]
	n = 2 Plum juice (*p. salicina)* Single dose of 400 mL vs 400mL water Cross-over 1 week	n = 41 Prunes (*p. dometica)* 100g/day vs 360mL grape juice Cross-over 8 weeks	n = 160 Dried plum (*p. dometica)* 100g/day vs dried apple Parallel 12 months	n = 8 Prunes 143g/day vs 8 diff. fruits Cross-over	n = 259 Prunes (*p. dometica)* 11.5g or 23g/day vs water Parallel 8weeks	n = 21 Plum juice (*p. salicina)* 200mL/day vs prune juice/placebo Cross-over 28 days	n = 13 Plum juice (*p. salicina)* 200mL/day vs placebo Cross-over 28 days	n = 54 Prune juice (*p. dometica)* 2 x125mL/day Case-series 4 weeks	n = 36 Plum juice (*p. dometica)* 8 ounces/day vs psyllium and apple juice Cross-over 6 weeks
A	Healthy volunteers	Free living men with mild hypercholesterlemia	Postmenopausal women	Healthy young men	Pre-hypertensive patients	Healthy volunteers	Healthy volunteers	Adults with mild GIT symptoms	Adults with chronic constipation symptoms
B	No	Not stated	Not stated	No	Not stated	Yes	Yes	Not applicable	Yes
C	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	Yes	Yes	Not applicable	no (not feasible)
D	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
E	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
F	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Not applicable	Yes
G	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
H	Probably no	Probably no	Yes	Yes	Yes	No	No	Yes	Yes
I	Yes	Yes	Yes	Yes	Yes	Yes	No	Probably no	Yes
J	No	No	No	No	No	No	No	No	No
K	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
L	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
TS	8	8	9	8	8	9	8	7	10

	Aliment Pharm Ther 33: 822-828[163]	*Eat Behav* 11(3): 201-203[167]	*Appetite*3: 564-569 [168]	*J Am Diet Assoc* 9: 1322-1327[169]	*J Appl Res*4: 37-43 [170]	*Res J Pharm Biol Chem Sci*2: 1195-1204 [171]	*J Pharm Sci* 23: 463-466. [175]	*Eur J Clin Nutr* 56:1020-1023[176]
	n = 40 Dried plum (*p. dometica)* 2 x 50g/day vs psyllium Cross-over 14 weeks	n = 45 Prunes (*p. dometica)* 40 g prune pre-load vs bread product Cross-over Over 1 week	n = 19 Dried plum (*p. dometica)* 238kcal portion vs baked foods & water Cross-over	n = 26 Dried plum (*p. dometica)* 100kcal portion *vs* low fat cookies Cross-over 4 weeks	n = 58 Dried plum (*p. dometica)* 100g/day *vs* Dried apple Parallel 3 months	n = 170 Dried plum and flixweed (*p. dometica)* 40-50g/day Parallel 3 weeks	n = 107 Prunes (*p. dometica)* 3 groups of either single dose, double dose or control Case-control 8 weeks	n = 12 Plum juice (*p. dometica)* 330mL vs selected fruit juices Cross-over 20 days
A	Patients with chronic constipation	Normal weight individuals	Healthy female subjects	Healthy female subjects	Postmenopausal women	Iranian Hajj pilgrims	Healthy volunteers	Healthy male volunteers
B	yes	Yes	Yes	Yes	Yes	Yes	yes	No
C	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	no (not feasible)	No
D	Yes	Yes	Yes	Yes	Yes	Yes	yes	Yes
E	Yes	Yes	Yes	Yes	Yes	Yes	yes	Yes
F	Yes	Yes	Yes	Yes	Yes	No	yes	Yes
G	Yes	Yes	Yes	Yes	Yes	Yes	yes	Yes
H	Yes	Yes	No	No	Yes	Yes	yes	Yes
I	Probably no	Probably no	Yes	Yes	Yes	Yes	yes	Probably no
J	no	No	No	Yes	No	No	no	No
K	yes	Yes	Yes	Yes	Yes	Yes	yes	Yes
L	yes	Yes	Yes	Yes	Yes	Yes	yes	Yes
TS	9	9	8	9	9	8	10	8

9.2 Appendix B - Search Strategy Sample (chapter 2 & chapter 4)

Medline (OVID)

	Searches
1	"plum*1".m_titl.
2	limit 1 to English language
3	Prunes.m_titl.
4	limit 3 to English language
5	"prunus domestica".m_titl.
6	limit 5 to English language
7	"prunus salicina".m_titl.
8	limit 7 to English language
9	2 or 4 or 6 or 8
10	(9 not "plum blossom needle").m_titl.
11	limit 10 to English language
12	(9 not "plum pox").m_titl.
13	limit 12 to English language
14	(12 not "plum curculio").m_titl.
15	limit 14 to English language
16	(15 not "plume").m_titl.
17	limit 16 to English language

a) Scopus search strategy (Chapter 4)

TITLE-ABS-KEY (anthocyan* AND "gut microbiota") AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (DOCTYPE , "cp") OR LIMIT-TO (DOCTYPE , "ip")) AND LIMIT-TO (EXACTKEYWORD , "Human") OR LIMIT-TO (EXACTKEYWORD , "Intestine Flora") OR LIMIT-TO (EXACTKEYWORD , "Humans") OR LIMIT-TO (EXACTKEYWORD , "Polyphenols") OR LIMIT-TO (EXACTKEYWORD , "Metabolism") OR LIMIT-TO (EXACTKEYWORD , "Polyphenol") OR LIMIT-TO (EXACTKEYWORD , "Article") OR LIMIT-TO (EXACTKEYWORD , "Nonhuman") OR LIMIT-TO (EXACTKEYWORD , "Anthocyanins")) AND (LIMIT-TO (LANGUAGE , "English"))

9.3 Appendix C – Published paper: A Systematic Review on the Health Effects of Plums (Prunus domestica and Prunus salicina)

PHYTOTHERAPY RESEARCH
Phytother. Res. **30**: 701–731 (2016)
Published online 16 March 2016 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5581

REVIEW

A Systematic Review on the Health Effects of Plums (*Prunus domestica* and *Prunus salicina*)

Ezinne O. Igwe* and Karen E. Charlton
School of Medicine, University of Wollongong, Northfields Avenue, Wollongong, New South Wales 2522, Australia

In recent times, plums have been described as foods with health-promoting properties. Research on the health effects of plum continue to show promising results on its antiinflammatory, antioxidant and memory-improving characteristics. The increased interest in plum research has been attributed to its high phenolic content, mostly the anthocyanins, which are known to be natural antioxidants.

A systematic review of literature was carried out to summarize the available evidence on the impact of plums (*Prunus* species; *domestica* and *salicina)* on disease risk factors and health outcomes.

A number of databases were searched according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines for relevant studies on plum health effects *in vitro*, animal studies and clinical trials.

A total of 73 relevant peer-reviewed journal articles were included in this review. The level of evidence remains low. Of the 25 human studies, 6 were confirmatory studies of moderate quality, while 19 were exploratory. Plums have been shown to possess antioxidant and antiallergic properties, and consumption is associated with improved cognitive function, bone health parameters and cardiovascular risk factors. Most of the human trials used the dried version of plums rather than fresh fruit, thus limiting translation to dietary messages of the positioning of plums in a healthy diet.

Evidence on the health effect of plums has not been extensively studied, and the available evidence needs further confirmation.

Keywords: plums; *Prunus domestica*; *Prunus salcina*; health effects; systematic review.

9.4 Appendix D – Published paper: First stage development of an Australian anthocyanin food composition database for dietary studies – A systematic process and its challenges

Journal of Food Composition and Analysis 64 (2017) 33–38

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original research article

First stage development of an Australian anthocyanin food composition database for dietary studies – A systematic process and its challenges☆

Ezinne Igwe*, Elizabeth Neale, Karen E. Charlton, Kurt Morton, Yasmine C. Probst

School of Medicine, University of Wollongong, NSW 2522, Australia

ARTICLE INFO

Keywords:
Anthocyanins
Micronutrients
Antioxidants
Food analysis
Australia
Food composition database
Fruits
Vegetables

ABSTRACT

In the last decade, there has been an increased interest in anthocyanin-based research with a growing need to accurately measure anthocyanin intake in population studies. Anthocyanin content in foods is known to vary across regions due to climate, soil content and harvesting practices. To accurately measure nutrient intake in population studies, food composition databases tailored to specific regions need to be developed. The aim of this study was to describe the first stage development of an Australian anthocyanin food composition database focusing on fruit and vegetables. A systematic literature search found analytical data on the anthocyanin content of five fruits and two vegetables (purple dragon carrot and red cabbage) out of the total plant-based food category (58 individual fruits and 62 vegetables). In addition, values were found for ten Australian native fruits, of which 9 are not included in the Australian database. Development of an anthocyanin food composition database relies on the availability of analytical food data. In the case of Australian fruits and vegetables, there are limited data available for anthocyanin content and imputations from other polyphenol datasets will be necessary. Regardless, development of an anthocyanin database tailored specifically for Australian research will facilitate better estimation of intake.

1. Introduction

Anthocyanins are a subclass of flavonoids, a group of polyphenols. They are commonly found in plant-based foods in the human diet and are known to be responsible for the deep rich purple, red, and blue colours in many fruits and vegetables. In nature, about 17 different anthocyanins have been discovered but to date only six (cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin) have been shown to be of dietary importance and are ubiquitously distributed in the food supply (Fernandes et al., 2014). Some common fruit and vegetable sources of anthocyanins include berries, cherries, apples, red and purple grapes, plums and pomegranates (Warner, 2015), as well as red wine and certain vegetables such as red cabbage, red onions and radishes (Zhang, 2015). In the last decade there has been an increased interest in anthocyanin based research (Mahdavi et al., 2014) because of their potential health benefits including improved cognition (Kent et al., 2015a) and vision (Kalt et al., 2014), as well as their protective effects against cardiovascular risk factors (Pojer et al., 2013) and inflammation (Cassidy et al., 2015; Mena et al., 2014). Animal and clinical trials continue to show promising results on these observed beneficial health effects (Wallace et al., 2016; Bhaswant et al., 2015).

As research continues to elucidate beneficial health effects of anthocyanins, it is important that the amount of anthocyanin intake is accurately measured in both epidemiological and experimental studies in order to allow translation of this evidence into dietary messages for overall health improvement. For this purpose, a number of specific and general polyphenol databases, including the American USDA Database for the Flavonoid Content of Selected Foods and the European Phenol-Explorer, exist for the measurement of polyphenol (anthocyanin) intake (Bhagwat et al., 2011; Neveu et al., 2010). Comparing these two databases for the estimation of dietary polyphenol intake in Polish adults, Witkowska et al. (2015) demonstrated significant discrepancies between the amount of flavonoid intake estimated when using the USDA and Phenol-Explorer databases (525 mg/day vs 403.5 mg/day, respectively $p < 0.001$). Epidemiological studies commonly utilise either one of these databases for the measurement of polyphenol intake. Such discrepancies between databases may lead to differing conclusions regarding the amount of anthocyanins in the human diet and potential dose responses (Kent et al., 2015b; Yahya et al., 2015; Witkowska et al., 2015).

Within the USDA and Phenol Explorer databases, analytical data are not country or region specific but are reported as mean values for all

☆ This paper was originally presented at the 39th National Nutrient Databank Conference held May 16–18, 2016 in Alexandria, VA, USA.

* Corresponding author.
E-mail address: ekorie@uow.edu.au (E. Igwe).

http://dx.doi.org/10.1016/j.jfca.2017.04.001
Received 1 August 2016; Received in revised form 11 January 2017; Accepted 3 April 2017
Available online 04 April 2017

Table 1
Micronutrient comparison between USDA and AUSNUT food composition database values of apple.
Source: USDA (2015) and Food Standards Australia New Zealand (2014).

	AUSNUT values[a]	USDA values[b]
Preformed vitamin A (retinol) (μg)	0	3
Beta-carotene (μg)	10	27
Provitamin A (b-carotene equivalents) (μg)	13	
Vitamin A retinol equivalents (μg)	2	0
Thiamin (mg)	0.022	0.017
Riboflavin (mg)	0.013	0.026
Vitamin C (mg)	4	4.6
Dietary folate equivalents (μg)	13	3

[a] AUSNUT – Australian Food and Nutrient Database.
[b] USDA – U.S. Department of Agriculture.

available data for a particular food. As a result, there is the possibility that this can lead to inaccurate measurement of polyphenol intakes in specific populations. Evidence has shown that differences in climate, soil conditions and methods of plant harvesting among other factors are major determinants of natural variation in the amounts of nutrients contained in foods (Karl, 2009; Hornick, 1992). For example, evidence suggests that the levels of anthocyanin in fruit may be directly proportional to the amount of sunlight exposure. A study by Song et al. (2015) showed that anthocyanin levels in grapes were substantially increased with grape bunch exposure to sunlight in comparison to shading. Average monthly hours of sunshine over the year has been shown to be significantly different in various fruit producing regions of the world (World Weather and Climate Information, 2016). Focusing on this difference in climate and harvesting practices, the amount of micronutrients in apples are found to differ substantially between the USDA (USDA, 2015) and the Australian Food, Supplement and Nutrient Database (AUSNUT) (Food Standards Australia and New Zealand, 2014) (Table 1). For these reasons, it is important that databases tailored to specific regions of the world are developed to facilitate better measurement of anthocyanin intakes in population level studies.

In utilising the available databases for epidemiological studies, another drawback is the underrepresentation of native fruit and fruit hybrids specific to particular regions. For example, there is an underrepresentation of some common Australian native fruit including quandong, riberry and fingerlimes which are known to have high levels of anthocyanins (Cherikoff, 2015).

The increased interest in anthocyanin-based research has also resulted in fruit breeders developing different hybrids of fruits and vegetables high in anthocyanin content and antioxidant capacity. One such fruit is a new variety of the Japanese plum (*Prunus salicina* Lindl.), named the Queen Garnet plum (QGP) which was developed within a Queensland Government (Australia) breeding program. (Fanning et al., 2014). It is thus important for the phytochemical content of these novel plant foods to be captured within regionally appropriate food composition databases.

In summary, the underrepresentation of fruit specific to particular regions in existing databases, as well as the differences in climate and harvesting practices between regions warrants the development of an anthocyanin food composition database specific to different regions.

The aim of this study was, therefore, to describe the first stages of the development of an Australian anthocyanin food composition database using fruit and vegetables to pilot the systematic development process.

This paper will also describe the challenges encountered in developing this tailored Australian database.

2. Methods

The development of an Australian anthocyanin database involved an expansion of the existing Australian AUSNUT 2011-13 database to include anthocyanin content. The AUSNUT 2011-13 database contains over 5700 foods and beverages reported by Australians and was developed for use in the 2011–13 Australian Health Survey (Australian Bureau of Statistics, 2013).

As a first stage development, the main focus was fruit and vegetables as these are the major food groups with high levels of anthocyanins. The most reliable method to measure anthocyanin content in foods has been identified as High Performance Liquid Chromatography (HPLC). When evaluating spectrophotometric methods for antioxidant compound measurement, Tabart et al. (2010) found colorimetric methods were unreliable as reactions could be different for individual compounds within a family (anthocyanins, flavonols or flavan-3-ols) and were not specific to one family. For this reason, only data generated using HPLC methods of analysis were included.

The proposed methodology for estimating the anthocyanin content of Australian foods was adapted from a systematic approach used by Louie et al. (2015) for estimation of added sugar content in Australian foods via an expansion of the AUSNUT database, in which analyses showed a strong correlation between two researchers in an inter-researcher repeatability analysis.

2.1. Step by step systematic approach to estimating anthocyanins in foods

A five-staged process was developed for estimating the anthocyanin content of Australian fruit and vegetables. The first four steps were objective while step 5 was considered subjective.

Step 1: Classification of food groups

Food groups were coded into three different categories; plant-based foods, non-plant based foods and composite foods (made up of plant-based and non-plant based foods). The difference between the non-plant based food and the composite food group is that the majority of the former were assigned zero values while in the latter, fewer foods

Table 2
Major food groups in the Australian Food and Nutrient Database (AUSNUT).

Plant based food groups	Non-Plant based food groups	Composite food groups
16. Fruit products and dishes	14. Fats and oils	11. Non-alcoholic beverages
22. Seed and nut product and dishes	15. Fish and seafood products	12. Cereals and cereal products
24. Vegetable products and dishes	17. Egg products and dishes	13. Cereal based products and dishes
25. Legume and pulse products and dishes	18. Meat, poultry and game products and dishes	20. Dairy & meat substitutes
	19. Milk products and dishes	21. Soup
	30. Special dietary foods	23. Savoury sauces and condiments
	34. Reptiles, amphibia and insects	26. Snack foods
		27. Sugar products and dishes
		28. Confectionery and cereal/nut/fruit/seed bars
		29. Alcoholic beverages
		31. Miscellaneous
		32. Infant formulae and foods
		33. Dietary supplements

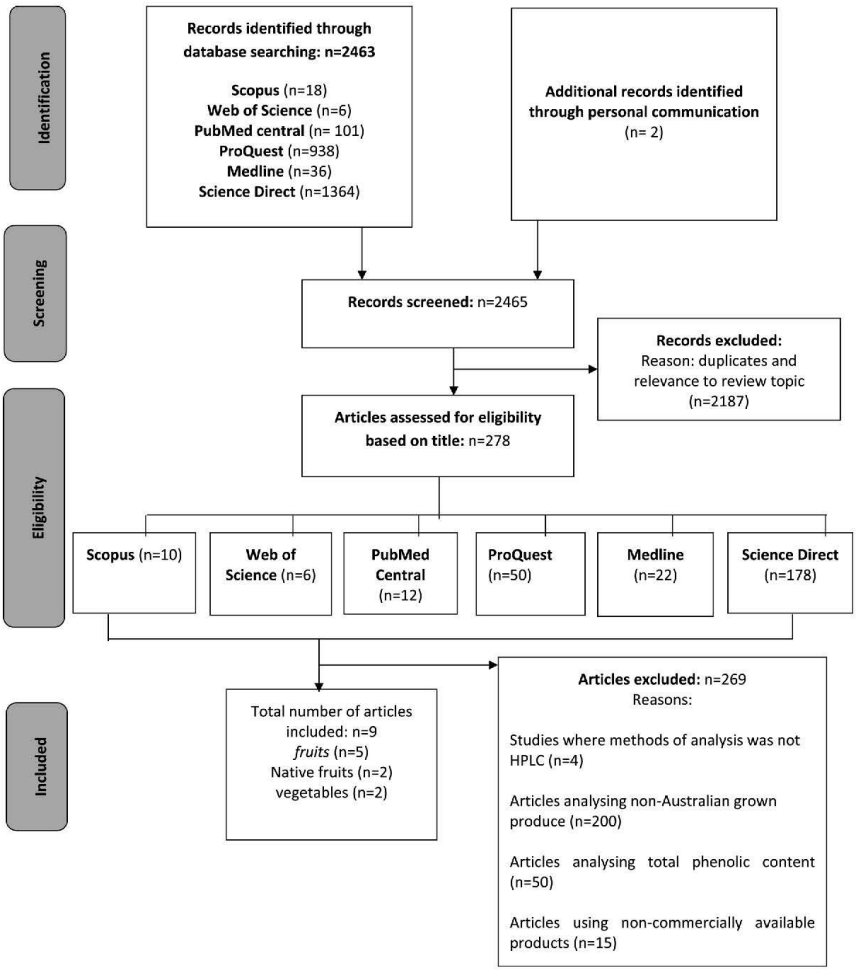

Fig. 1. PRISMA flow diagram for analytical article selection process.

were assigned a zero value (Table 2).

The grouping system developed for the AUSNUT 2011-13 database classifies foods according to a major food group (2-digit) based on key ingredients, followed by sub-major (3-digit) and minor food (5-digit) groups. Using this classification system, there are 24 major food groups covering all the reported foods eaten by Australians, as shown in Table 2.

Step 2: Assignment of zero values

Zero values were assigned to non-plant based food and composite food groups with careful attention being paid not to assign 0 values to foods that contained ingredients from the plant-based food groups.

Step 3: Conduct a systematic literature search for analytical data

A systematic literature search of electronic databases was conducted (Scopus, Web of Science, Medline, PubMed central, ProQuest, and Science Direct) using a combination of search terms: "anthocyanin*", "Australia*", "Fruit*", "vege*", and "analy*". Studies were included if they had analysed the anthocyanin content in Australian fruit and vegetables.

For the vegetable group, additional literature searches using USDA and Phenol-Explorer databases were conducted to identify vegetables with available anthocyanin analytical values. Searches used scientific and common names to identify all relevant foods in the USDA and Phenol-Explorer databases.

Step 4: Contact local researchers and organisations

Experts in the field of anthocyanin research were identified from the systematic literature search as well as web searches of organisations, groups, and individuals to identify where further sources of analytical anthocyanin data may be located. These sources were contacted to enquire about the existence of any unpublished data for the anthocyanin content of Australian fruits and vegetables.

Step 5: Using borrowed values

Having exhausted steps 1–4, where Australian data was not

identified for fruit and vegetables, data will be obtained for similar foods from international databases, including the USDA Database for the Flavonoid Content of Selected Foods and Phenol-Explorer. To assist with the choice of the most appropriate foods from those databases, estimations will be made by calculating a conversion factor to apply to the borrowed values in the Australian food anthocyanin database, considering deviation from average moisture content as shown below (FAO, 1968). The conversion factor F is calculated using the following formula:

$$F = [100 - actual\ MC\ (Australian\ FCT)] \div [100 - MC\ as\ shown\ in\ USDA\ or\ Phenol\ Explorer\ FCT],$$

where MC = moisture content, and FCT = food composition table.

Moisture content of the foods in the USDA flavonoid database will be obtained from the USDA National Nutrient Database for Standard Reference (USDA, 2015) and those of the Phenol-Explorer will be obtained from the Danish Food Composition Databank, the data source indicated in the Phenol-Explorer database (Saxholt et al., 2008). Having calculated the conversion factor, the corresponding Australian nutrient value will be computed by multiplying the specific nutrient value by the conversion factor (F). Following this computation, data from the USDA flavonoid database and Phenol-Explorer will be mapped by the authors (Y. Probst and E. Igwe) to determine whether to make decisions based on the calculated values from both databases or if further analysis is required. The choice of the international database from which to borrow analytical values will be dependent on the similarity of the foods in their macronutrient content and the similarity of the food supply between Australia and the source country including production and processing techniques.

3. Results

Following the four objective steps outlined above, the existing AUSNUT 2011–13 database was expanded to include anthocyanin content. The foods contained in the database were classified under 24 food groups (Table 2). Of these, four were grouped as plant based foods, seven under the non-plant based food group and 13 under the composite food group. There was a total of 58 individual fruits and 62 vegetables in the database following this method of categorization.

The systematic literature search (Fig. 1) for analytical values of Australian fruits and vegetables yielded analytical values for only five Australian fruits (Table 3), including berries (Fredericks et al., 2013; Netzel et al., 2006), plums (Fanning et al., 2014; Bobrich et al., 2014), apples (Takos et al., 2006), grapes (Bindon et al., 2014) as well as ten native Australian fruits (Netzel et al., 2007, 2006) and two Australian vegetables, namely purple dragon carrots (Singh et al., 2012) and red cabbage (Zabaras et al., 2013).

Unpublished analytical data from local researchers at the Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Australia were obtained for commercial pomegranate juice, pomegranate cranberry juice, pomegranate blueberry juice, Dr Red (Dr Purple Shiraz and Purple Carrot Blend), Dr Red (Purple carrot blueberry punch), Dr Red (Blueberry punch), Nature's Goodness (Joint Formula: Cherry juice concentrate with anthocyanin complex) and Sunraysia (100% prune juice (from concentrate)).

4. Discussion

Using a systematic approach for the first time, attempts have been made to bring together in a database the analytical data for the anthocyanin content of Australian foods (fruit and vegetables). The first four objective steps were unambiguous as the food groupings based on major ingredients were readily available; hence classification as plant-based or non-plant based foods was a straightforward process.

Even though there is currently very limited analytical data on the anthocyanin content of Australian foods which was evident in the few studies identified during the systematic literature search, preliminary comparisons of the available data on fruit with the existing polyphenol databases show a substantial difference in the reported anthocyanin content (Table 4). It is expected that more data will become available as this area of research advances, allowing for continuous sourcing of unpublished data. Expansion of the dataset will enable more precise and consistent estimation of anthocyanin intakes in future studies.

To date, the major focus has been on raw fruit and vegetables but as this project progresses, a number of considerations will be made in gathering analytical values. Food processing, for example, will be a major consideration as it has been shown to affect the nutritional content of foods with more effect on micronutrients. Blanching, for example, was observed to decrease the tannins and antiradical efficiency of plums but increased the total polyphenol content, while osmotic dehydration had no effect on the total polyphenol and ferric reducing power (Valero et al., 2012). On drying, Piga et al. (2003) observed that some anthocyanin and flavonol content of plums had significantly decreased. In line with this observation, Najafabad and Jamei (2014) studied the free radical scavenging capacity and antioxidant activity of both fresh and dried fruit samples (plum) and observed that the fresh samples had more oxygen free radical (superoxide and peroxy radicals) scavenging capacity than the dried samples.

Some of the strengths of the proposed methodology are its systematic approach and the fact that local researchers were contacted for unpublished data in order to gather additional analytical data.

Furthermore, upon completion of the proposed database, there is the possibility of improved study results based on better measurement of anthocyanin consumption. In estimating anthocyanin intake in population studies, a major limitation has been the significant differences in the estimated amount of anthocyanin intake depending on the database used to measure anthocyanin (Witkowska et al., 2015). Witkowska et al. (2015) proposed that the combination of available databases can have a significant effect on accurate measurement of anthocyanin intake. Considering this, a major strength of this proposed methodology will be the combination of analytical data from Australian foods and borrowed data from international databases based on micro and macro nutrient similarity which will provide more accurate estimation.

A major limitation of this project was the limited availability of analytical data for Australian foods and thus the need to borrow international data. Some of the published analytical values only reported total anthocyanin content, rather than individual anthocyanins (Singh et al., 2012; Bindon et al., 2014; Takos et al., 2006; Zabaras et al., 2013; Fanning et al., 2014). This could be seen as a limitation as evidence shows that specific anthocyanins vary substantially in their bioavailability (McGhie and Walton, 2007).

5. Conclusion

In conclusion, this proposed methodology provides insight for the compilation of analytical data for an Australian anthocyanin database for fruit and vegetables. Whilst the literature search produced very limited results, this was to be expected as the database is in the early stages of development and anthocyanins are an emerging area of research. As more analytical data becomes available from organisations and independent researchers, updates will be made to produce a robust database that will facilitate accurate measurement of anthocyanin consumption in Australian population studies and clinical trials.

Conflict of interest

The authors have no conflict of interest to declare.

Table 3
Anthocyanin content of raw Australian fruit.

Reference	Food name	Food group	Method of analysis[a]	Total anthocyanin (SD) mg/100 g fresh weight	Individual anthocyanins reported
Fredericks et al. (2013)	Strawberry	Fruit products and dishes	HPLC	2.02 (0.04)	Yes
Netzel et al. (2006)	Blueberry	Fruit products and dishes	HPLC/ESI-MS-MS	381.75 (6.23)	Yes
Bobrich et al. (2014)	Black diamond plum	Fruit products and dishes	HPLC	89.8 mg	Yes
Fanning et al. (2014)	Queen Garnet plum	Fruit products and dishes	HPLC	195	No
Takos et al. (2006)	Apple skin (red)	Fruit products and dishes	HPLC	9 mg/100 g fruit skin	No
Bindon et. al. (2014)	Shiraz grape	Vegetables	HPLC/ESI-LC/MS	159 (5)	No
Zabaras et al. (2013)	Red cabbage	Vegetables	HPLC	57.4	No
Singh et al. (2012)	Purple dragon Carrot	Vegetables	HPLC	5 (0.2)	No
Netzel et al. (2006)	Muntries	(Native) fruit products and dishes	HPLC/ESI-MS-MS	25.26 (1.24)	Yes
Netzel et al. (2006)	Tasmanian pepper	(Native) fruit products and dishes	HPLC/ESI-MS-MS	607 (52)	Yes
Netzel et al. (2006)	Molucca raspberry	(Native) fruit products and dishes	HPLC/ESI-MS-MS	73.64 (0.41)	Yes
Netzel et al. (2006)	Davidson's plum	(Native) fruit products and dishes	HPLC/ESI-MS-MS	38.42 (0.83)	Yes
Netzel et al. (2006)	Illawarra plum	(Native) fruit products and dishes	HPLC/ESI-MS-MS	556.82 (20.73)	Yes
Netzel et al. (2006)	Cedar bay cherry	(Native) fruit products and dishes	HPLC/ESI-MS-MS	27.77 (0.54)	Yes
Netzel et al. (2006)	Burdekin plum	(Native) fruit products and dishes	HPLC/ESI-MS-MS	174.55 (10.63)	Yes
Netzel et al. (2007)	Riberry	(Native) fruit products and dishes	HPLC-DAD/ESI/MS-MS	72.39 (1.45)	Yes
Netzel et al. (2007)	Bush Cherry	(Native) fruit products and dishes	HPLC-DAD/ESI/MS-MS	105.79 (1.24)	Yes
Netzel et al. (2007)	Finger lime	(Native) fruit products and dishes	HPLC-DAD/ESI/MS-MS	11.09 (0.83)	Yes
Unpublished data from source	pomegranate juice	fruit products and dishes	HPLC	13[b]	No
Unpublished data from source	pomegranate cranberry juice	fruit products and dishes	HPLC	12[b]	No
Unpublished data from source	pomegranate blueberry juice	fruit products and dishes	HPLC	12[b]	No
Unpublished data from source	Dr Red (Dr Purple Shiraz and Purple Carrot Blend)	fruit products and dishes	HPLC	9[b]	No
Unpublished data from source	Dr Red (Purple carrot blueberry punch)	fruit products and dishes	HPLC	40[b]	No
Unpublished data from source	Dr Red (Blueberry punch)	fruit products and dishes	HPLC	610[b]	No
Unpublished data from source	Nature's Goodness (Joint Formula: Cherry juice concentrate with anthocyanin complex)	fruit products and dishes	HPLC	14[b]	No
Unpublished data from source	Sunraysia (100% Prune juice (from concentrate))	fruit products and dishes	HPLC	< < 1[b]	No

[a] HPLC – High Performance Liquid Chromatography, HPLC/ESI-MS-MS – High-Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry, HPLC/ESI-LC/MS – High-Performance Liquid Chromatography/Electrospray Ionization Liquid Chromatography/Mass Spectrometry, HPLC-DAD/ESI/MS-MS – High-Performance Liquid Chromatography – Diode Array Detection/Electrospray Ionization Tandem Mass Spectrometry.
[b] Single item analysis.

Table 4
Differences in anthocyanin content of similar fruits and vegetables from different databases and sources.

Fruit/vegetable	USDA database (mg/100 g)	Phenol-Explorer database (mg/100 g)	Australian data (mg/100 g)
Strawberry	**2.24**	**73.01**	**2.02**
Blueberry	**163.3**	**133.99**	**381.75**
Plum	**56.05**	**47.79**	**89.8**
Red cabbage	**209.95**	- [a]	**57.4**

[a] Not reported.

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9.5 Appendix E – Published paper: 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

NUTRITION RESEARCH 47 (2017) 28–43

Available online at www.sciencedirect.com

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Original Research

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

E.O. Igwe[a], K.E. Charlton[a,b,*], S. Roodenrys[a], K. Kent[a,c], K. Fanning[d], M.E. Netzel[e]

[a] School of Medicine, University of Wollongong
[b] Illawarra Health and Medical Research Institute, University of Wollongong
[c] Centre for Rural Health, School of Health Sciences, Faculty of Health, University of Tasmania
[d] Department of Agriculture and Fisheries, Queensland Government
[e] Queensland Alliance for Agriculture and Food Innovation, University of Queensland

ARTICLE INFO

Article history:
Received 28 May 2017
Revised 14 August 2017
Accepted 22 August 2017

Keywords:
Anthocyanins
Blood pressure
Queen Garnet plum juice
Cognition
Acute
Crossover
Human

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 × 300 mL or 3 × 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.

Abbreviations: ABPM, ambulatory blood pressure monitor; ANOVA, analysis of variance; AUC, area under curve; BP, blood pressure; CVD, cardiovascular disease; DBP, diastolic blood pressure; HR, heart rate; LDL, low-density lipoprotein; MAP, mean arterial pressure; MAO, monoamine oxidase; QGP, Queen Garnet plum; QGPJ, Queen Garnet plum juice; SBP, systolic blood pressure; UOW, University of Wollongong.

* Corresponding author.
E-mail address: karenc@uow.edu.au (K.E. Charlton).

http://dx.doi.org/10.1016/j.nutres.2017.08.006
0271-5317/

1. Introduction

Elevated and high blood pressure (BP) is a major public health concern and a significant risk factor for cardiovascular diseases (CVDs). According to the World Health Organization, the prevalence of high BP in adults 18 years and older 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 85 years and older being hypertensive [2]. In global strategies to address noncommunicable diseases including hypertension, the significant role of modifiable dietary risk factors, including an increased intake of fruits and vegetables, is acknowledged [3]. Small (2-5 mm Hg) but steady decreases in mean BP have been shown to significantly decrease the incidence of cardiovascular events [4]. Given the magnitude of hypertension and its contribution toward the burden of CVD, 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 4 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 that a higher consumption of anthocyanin-rich foods is associated with a reduced risk for CVD [8,9]. However, intervention studies do not always support these findings [10]. In the case of BP, plausible mechanisms from experimental studies include their effects on vascular blood flow and flow-mediated dilation [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 BP [13-15]. One of the main pathways linking BP 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 remain 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 has 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, for example, juice, puree, and whole fruit. Consequently, studies administer unfeasibly large doses of anthocyanin-rich foods to elucidate a physiological response, and the selection of dose timings is often unsubstantiated [25-27]. Although splitting a large daily dose of anthocyanin-rich food into 3 or more servings per day may reflect a more feasibly tolerated serve, there are often no justification as to the reason each dose was selected and no consideration given to the physiological effects. Although 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 350 μmol or less. This is also believed to differ according to the structure of different anthocyanins and to the attached sugar moiety and because of wide interindividual 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 to identify any consistent potential health benefits.

The increased interest in anthocyanin-based research has translated into agricultural responses, as the demand for fruits with superior health benefit grows. An example is the Queen Garnet plum (QGP), which is a variety of the popular Japanese plum *Prunus salicina Lindl* that was bred by the Queensland Government to be very high in anthocyanins, providing up to 277 mg/100 g of fruit [30] under "optimal" environmental and harvest conditions. This is more than twice the anthocyanin content of regular plums that ranges from 5 to 173 mg/100 g across harvest years [30]. Previous work from our group has determined the acute effect of anthocyanins provided from a different fruit source (cherries) [15,31]. Learnings from those studies underpin the improved methodologies used in the current study, particularly with regard to more robust assessment of 24-hour BP and measurement of urinary anthocyanin metabolites. Furthermore, the QGP juice (QGPJ) has a completely different anthocyanin and nonanthocyanin polyphenol profile compared with the Australian cherry juice vehicle used previously [15,31], which

may influence its synergistic/antagonist effects on biological activities. Our previous acute trial found that plasma levels of anthocyanin-related metabolites were significantly lower for older adults, but not for younger adults, who consumed cherry juice over 3 smaller servings (3 × 100 mL) compared with consumption of 300 mL at a single time point. This finding warrants further research consideration.

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

1. Have an acute beneficial effect on various domains of cognitive functioning and BP,
2. Be found to be bioavailable through the presence of anthocyanin metabolites excreted in the urine over a 24-hour 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 BP and cognitive function following consumption of 300 mL QGPJ, provided as either a single dose or three 100-mL quantities over 3 hours, in young and older adults. The secondary outcome was to determine the bioavailability of QGP anthocyanins, as assessed by urinary excretion over a 24-hour 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 (UOW) Human Research Ethics Committee, New South Wales, Australia (HE16/278). Written informed consent was obtained from all participants.

2.1. Study design

The study was a pilot crossover bioequivalence/noninferiority study to assess the acute impact of differing dose timings of high-anthocyanin plum juice consumption on acute cognition and BP over 24 hours. This study was considered a bioequivalence/noninferiority design because of the dosage for the 2 crossover arms being the same (a single 300 mL and 3 × 100 mL over 3 hours) with the aim to determine whether three 100 mL of plum juice taken over 3 hours would have a different pattern of effect on BP in comparison to a single 300-mL dose. Results from this pilot study will inform the methodology as well as sample size calculation for a future crossover randomized clinical trial. This is in line with the assumptions of bioequivalence studies whereby the future trial will be designed as a crossover study [32-34].

2.2. Participants

All participants were recruited from the UOW and the surrounding Wollongong areas through poster advertising. Potential participants had the opportunity to discuss the study over the phone prior to clinic visits and were screened to determine eligibility.

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

Recruited eligible participants were randomized to a dose-timing allocation and cognitive assessment order by a computer-generated block randomization by an independent statistician. Participants attended two 6-hour clinic visits at the Illawarra Health and Medical Research Institute at the UOW, New South Wales, Australia, between June and September 2015 with at least a 2-week washout period between clinic visits.

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' sociodemographic characteristics. The International Physical Activity Questionnaire validated by Hagströmer et al [35] was used to determine habitual level of physical activity, and BP measurements were taken using an ambulatory blood pressure monitor (ABPM) (Model 90207; Spacelabs Medical Inc, Issaquah, WA, USA).

2.4.1. Dietary instruction and intervention meals

The QGPJ 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; was processed to juice by research partners at the Department of Agriculture and Fisheries, Queensland Government; and was batch frozen at –20°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 24-hour 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 12-hour fast. A spot urine sample was collected and a battery of cognitive tests was administered by 2 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. QGPJ was provided with breakfast in random order as either (1) a single dose of 300 mL (369 mg total anthocyanins) or (2) 3 × 100-mL servings (123 mg total anthocyanins/serving) of the same plum juice at 0, 1, and 3 hours. A standardized snack (ham and cheese sandwich) was provided at 4 hours, and two (250 mL) bottles of water was provided for the 6-hour duration spent in the study facility to be consumed ad libitum.

2.4.2. Ambulatory 24-hour BP and anthropometric measurements

BP was measured using ABPMs for improved monitoring over 24 hours in comparison to standard digital BP monitors used in similar studies over a 6-hour period [15,31].

Upon arrival at the testing facility, participants were fitted with an ABPM (Model 90207; Spacelabs Medical Inc, Issaquah, WA, USA). The ABPM took BP measurements over the next 24

hours: every 15 minutes while at the testing facility (first 6 hours) and thereafter once per hour while at home. The ABPM uses an oscillometric method for the detection of systolic (SBP) and diastolic blood pressure (DBP) 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, that is, cuff inflation and deflation. After 24 hours, the monitor was removed and collected from participants' homes, and data were downloaded from the monitor for analysis.

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, New South Wales, Australia), respectively, to 2 decimal places, and body mass index (weight/[height^2]) was calculated.

2.4.3. *Cognitive tasks*

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

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

The Trail Making Test [40] required participants to alternate selective responses between 2 types of stimuli in the one task. The difference in the number of seconds required to complete the task was compared to a nonswitching version. This task assesses higher executive function.

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

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

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

The Stroop task [44] provides participants with a sheet on which the words *purple*, *green*, *yellow*, *red*, and *blue* are printed (50 in total). Each word is shown in either congruent or incongruent ink colors (eg, the word "blue" printed in red). Participants are instructed to 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 QGPJ consumption and thereafter were collected in sterilized urine containers over the following time periods: 0-2, 2-6, 6-12, and 12-24 hours after QGPJ consumption. The volume of collected urine samples was measured per container and recorded, and an aliquot of 30 mL of urine sample plus 9 mL of formic acid (100%) was stored in 50-mL tubes, with additional 10 mL of urine for storage. The urine samples were stored at −80°C for batch analysis. Intact (nonmetabolized) 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 high-performance liquid chromatography photodiode array detection mass spectrometry method as described by Netzel et al (2012) [36].

2.5. *Statistical analyses*

Data were analyzed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp, Armonk, NY, USA). Descriptive statistics of participant characteristics were 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 modeling was used to estimate the effect of different timed doses of QGPJ on BP between the 2 age groups while adjusting for correlation due to repeated observations on each participant over 24 hours. Age-group and dose (with interaction term) were entered in the model as fixed effects while controlling for age and sex. Maximum likelihood method of estimation was used with a diagonal covariance structure. As BP measurements were collected over 24 hours, there were a few missing data (less than 1%), and as a result, linear mixed modeling was chosen for analysis because it handles missing data better than the widely used analysis of covariance.

Area under the curve (AUC) was calculated as a summation measure for the first 6 hours of BP measurements, and the baseline observation carried forward 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 6 hours. The period between 0 and 6 hours 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 6 hours postintervention, and 2-way ANOVA was used to determine the difference in cognitive performance at baseline and 6 hours between the 2 age groups and dosing regimens as well as anthocyanin excretion between the 2 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 presented in Table 1. Participants were of

Caucasian descent (n = 21), of African descent (n = 2), and Asian (n = 1). All participants attended both visits, and there were no withdrawals or 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 10× 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 QGPJ [36,46], the half-life has been shown to range between 12 and 51 hours [47].

3.2. *Twenty-four–hour ambulatory BP*

Hourly cardiovascular responses recorded during each of the 24-hour test periods are shown according to plum juice delivery mode in Figs. 1-4. Comparison is made between the dosing regimen (single and triple doses) and age groups with an interaction factor (dosing regimen × age group). Fig. 1a shows a more obvious drop in SBP of the older adults with the single dose compared to the triple dose (Fig. 3a). This observation was not evident with the younger adults, as shown in Figs. 1a and 3a. There was no significant dose-timing effect observed for change in BP following plum juice consumption in the 24-hour period using the linear mixed model for longitudinal data (Table 2).

AUC was calculated for the cardiovascular parameters (systolic, diastolic, mean arterial pressure [MAP], and heart rate [HR]) for the first 6 hours (Figs. 5-6 and Table 3). For both age groups, using a t test, BP was significantly lower than baseline ($P < .05$) at different time points up to 6 hours following consumption of the plum juice. The greatest significant BP reduction was observed at 2 hours for both age groups and was more obvious for SBP in the older group with a mean difference of 12.83 mm Hg (SD; 16.51, $P = .001$) from baseline. For the single dose, z test analysis of the AUC calculations for the younger adult group showed a significant effect of the juice on DBP, MAP, and HR (P values = .008, .012, and .025 respectively). Similarly, a significant effect was seen for the older group: SBP, DBP, MAP, and HR (P values = .035, .028, .017, and .006 respectively). For the younger age group on the triple dose, significant effects were observed for DBP and MAP (P values = .008 and .013, respectively) with a borderline significance on the HR (P value = .06). In the older group, significant effects of the triple dose were observed for DBP (P value = .00007) and a borderline effect for SBP (P value = .063). Plum juice consumption had a significant effect on SBP, which was predicted by dose or age group but no interaction term effect (dose × age group) and for MAP, predicted by only age group. No significant effect of plum juice was observed on other cardiovascular parameters (Table 2).

3.3. *Cognitive tasks*

Using 2-way ANOVA, a significant difference was observed between the 2 age groups ($P < .001$), both at baseline and 6 hours, 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 used for our study was 123 mg/100 g. The consumption of QGPJ as a single oral dose of 300 mL or in 3 × 100-mL servings over 3 hours

Table 1 – Information about the subjects

Characteristics	Younger adults (n = 12) n (%)	Older adults (n = 12) n (%)	P values
Sex			
Male	4 (33.3)	3 (25.0)	NS
Female	8 (66.7)	9 (75.0)	
Age (means ± SD)	31 (8)	77 (6)	<.001
BMI (means ± SD)	22.5 (2.4)	26.4 (3.3)	.003
Physical activity			
Low	5 (41.7)	1 (8.3)	.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)	.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). P values were obtained from χ^2 test for categorical variables.
Abbreviations: BMI, body mass index; NS, not significant.

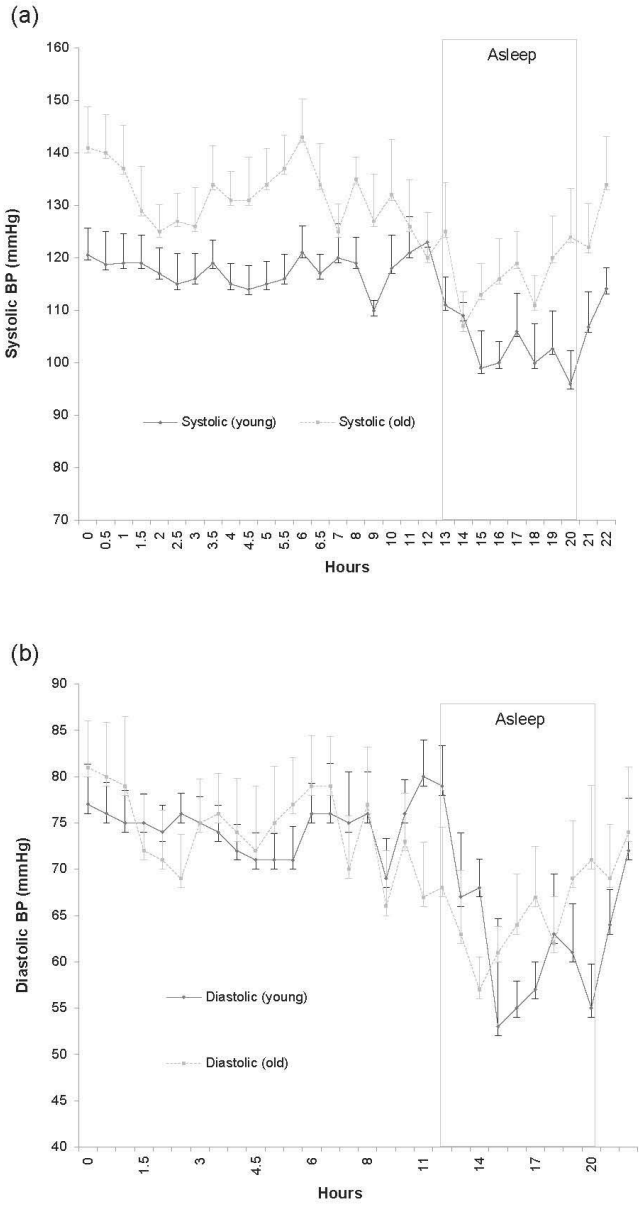

Fig. 1 – a, Single-dose hourly SBP of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). BP indicates blood pressure; QGP, Queen Garnet plum. b, Single-dose hourly DBP of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). BP indicates blood pressure; QGP, Queen Garnet plum.

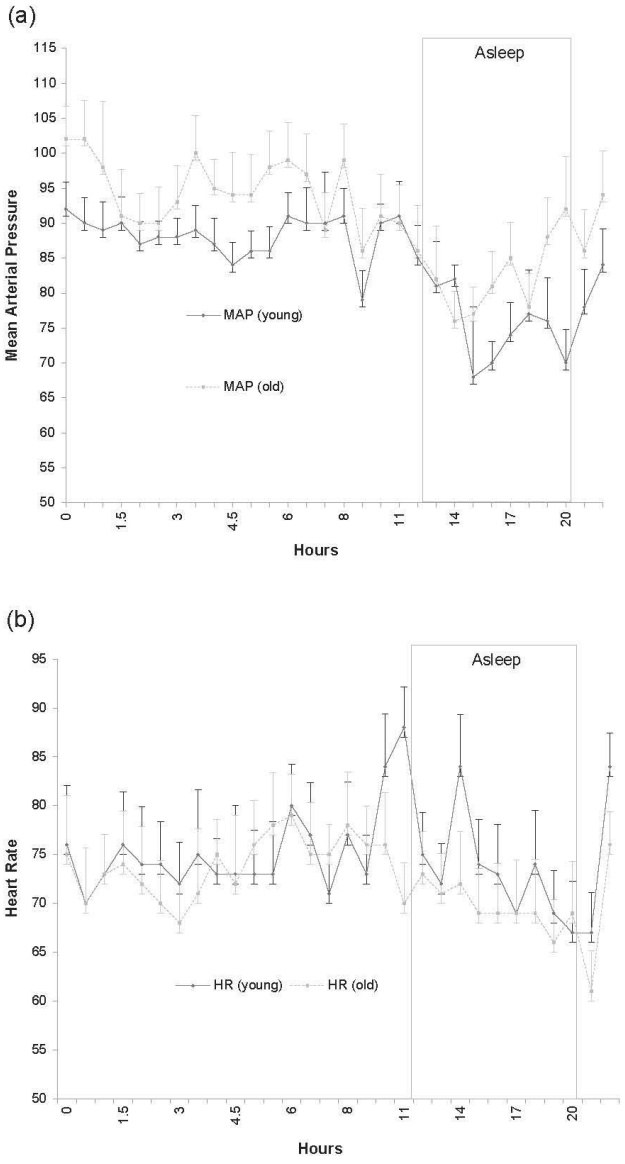

Fig. 2 – a, Single-dose hourly mean arterial BP of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). MAP indicates mean arterial pressure; QGP, Queen Garnet plum. b, Single-dose hourly heart rate of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). HR indicates heart rate; QGP, Queen Garnet plum.

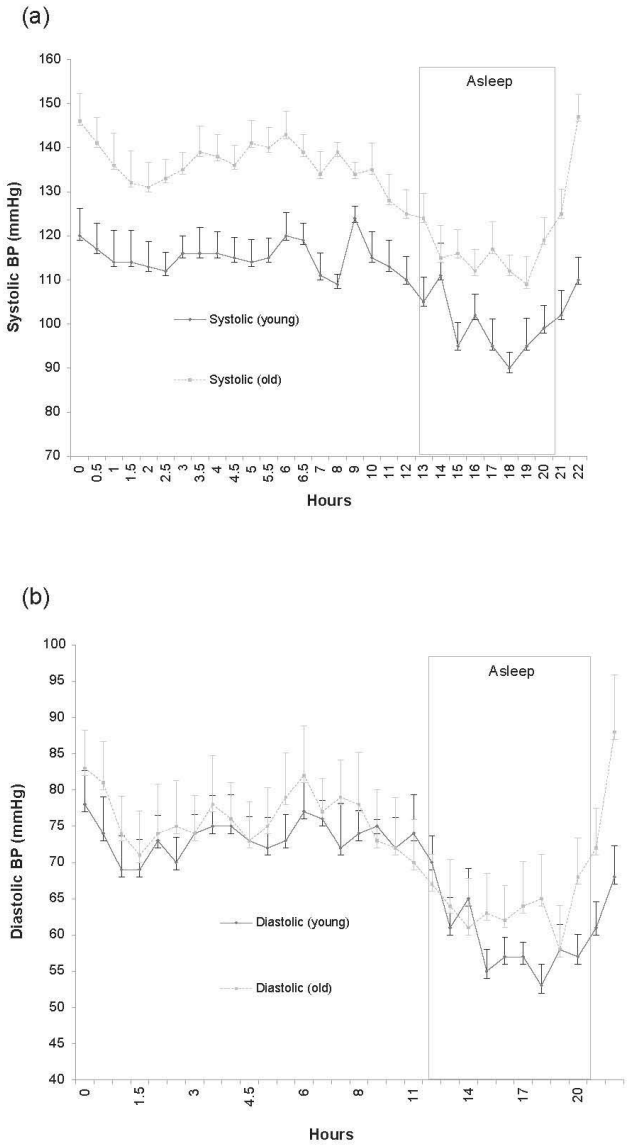

Fig. 3 – a, Triple-dose hourly SBP of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). BP indicates blood pressure; QGP, Queen Garnet plum. b, Triple-dose hourly DBP of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). BP indicates blood pressure; QGP, Queen Garnet plum.

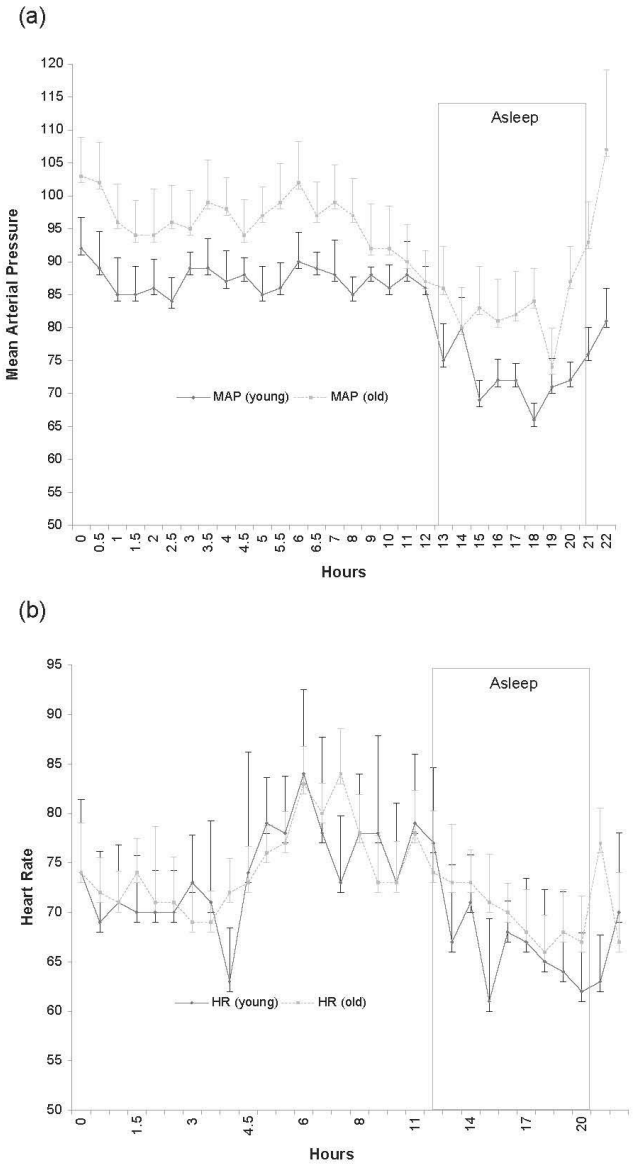

Fig. 4 – a, Triple-dose hourly mean arterial BP of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). MAP indicates mean arterial pressure; QGP, Queen Garnet plum. b, Triple-dose hourly HR of participants over 24 hours after consumption of QGPJ. Values are expressed as mean values ± SE (error bars) (n = 12 per age group). HR indicates heart rate; QGP, Queen Garnet plum.

Table 2 – Effect of dose-timed QGPJ consumption on cardiovascular parameters across age groups

Parameter	Mean ± SE	P values
SBP		
Intercept	123.7 ± 0.05	<.0001[a]
Dose-time		
1 × 300 mL	122.3 ± 0.60	.001[a]
3 × 100 mL	125.2 ± 0.67	
Group		
Younger	115.0 ± 0.66	<.0001[a]
Older	132.8 ± 0.61	
Interaction (group * dose)		.154
Younger * 1 × 300 mL	114.2 ± 0.84	
Younger * 3 × 100 mL	115.8 ± 1.00	
Older * 1 × 300 mL	130.3 ± 0.87	
Older * 3 ×100 mL	134.5 ± 0.87	
DBP		
Intercept	72.9 ± 0.36	<.0001[a]
Dose-time		
1 × 300 mL	72.5 ± 0.49	
3 × 100 mL	73.3 ± 0.54	.25
Group		.11
Younger	72.2 ± 0.53	
Older	73.6 ± 0.49	
Interaction (group * dose)		
Younger * 1 × 300 mL	72.0 ± 0.68	.62
Younger * 3 × 100 mL	72.4 ± 0.81	
Older * 1 300 mL	73.0 ± 0.69	
Older * 3 × 100 mL	74.1 ± 0.70	
MAP		<.0001[a]
Intercept	90.2 ± 0.37	
Dose-time		.077
1 × 300 mL	89.4 ± 0.49	
3 × 100 mL	90.9 ± 0.54	
Group		<.0001[a]
Younger	86.4 ± 0.54	
Older	94.0 ± 0.50	
Interaction (group * dose)		.75
Younger * 1 × 300 mL	85.7 ± 0.68	
Younger * 3 × 100 mL	87.0 ± 0.83	
Older * 1 × 300 mL	93.1 ± 0.70	
Older * 3 × 100 mL	94.9 ± 0.70	
HR		
Intercept	72.6 ± 0.36	<.0001[a]
Dose-time		.69
1 × 300 mL	72.5 ± 0.49	
3 × 100 mL	72.7 ± 0.54	
Group		.76
Younger	72.9 ± 0.54	
Older	72.4 ± 0.49	
Interaction (group * dose)		.91
Younger * 1 × 300 mL	72.8 ± 0.69	
Younger * 3 × 100 mL	73.0 ± 0.82	
Older * 1 × 300 mL	72.2 ± 0.70	
Older * 3 × 100 mL	72.5 ± 0.70	

Values are means ± SE (n = 12 per group).
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 300 mL or 3 portions of 100 mL taken at 0, 1, and 3 hours.
[a] Significant at P < .05.

resulted in the appearance of both intact/nonmetabolized QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) and at least 5 identified anthocyanin metabolites in the volunteers' urine samples (Table 4). The excretion rates and urinary anthocyanin/metabolite profiles were similar (P > .05) between age groups and dosing regimen.

4. Discussion

Following consumption of a single dose of 300-mL dose of plum juice, an acute reduction in SBP (P = .035), DBP (P = .028), MAP (P = .017), and HR (P = .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 2 hours with a mean difference of 12.83 mm Hg from baseline. Significant effects on DBP (P < .001) and MAP (P = .013) were also observed in the younger age group on the single dose and DBP (P < .001) with a borderline effect on SBP (P = .06) on the triple dose. The acute significant reduction in BP at 2 hours is associated with evidence on the absorption and bioavailability of anthocyanins that occur within 2 hours postconsumption [48]. Anthocyanin concentrations in the body have been observed to reach peak levels between 1 and 2 hours and begin to clear from 6 hours, falling back to baseline levels as they get excreted from the body up to 48 hours [49]. The synergistic effect of other nutrients in the QGP cannot be overlooked. There is a possibility that the observed BP-lowering effect may have been as a result of this synergistic effect, as well as the presence of potassium in the QGP fruit [30] which is an electrolyte known to lower BP in humans [50]. Despite lack of a significant effect of dose by the different age groups on BP, the greater reduction in BP in response to plum juice consumption in older adults may be explained by their higher baseline BP levels [4]. However, further adequately powered studies are needed to confirm these findings. In the 6 hours following the plum juice consumption, no significant effect was observed on the SBP of the younger age group. Similar observation has also been made in previous studies. A study by Novotny et al [51] observed that the Pacific Kids Dietary Approach to Stop Hypertension trial did not affect overall diet quality which was measured by SBP change among other parameters but had a significant effect on DBP by the end of the intervention, by 12.2 mm Hg. There is a possibility that the absence of a significant effect on SBP could be associated with age because there is a possibility that interventions may have a more significant effect among older populations and/or those more prone to age-related vascular stiffening with an increased risk of developing CVD.

There was an observed dose timing and group effect on SBP but not on other BP parameters; however, this was no longer significant after inclusion of an interaction term (age group × dose timing). Previously, a similar study observed an acute reduction in BP (SBP, DBP, and HR) after consumption of anthocyanin-rich cherry juice, which was found to be dose-timing dependent [31]. The difference between the 2 studies may be explained by a much higher concentration of anthocyanins in the QGPJ (123 mg/100 mL vs 69 mg/100 mL, respectively), resulting in a physiological threshold to be reached in each of the three 100-mL doses.

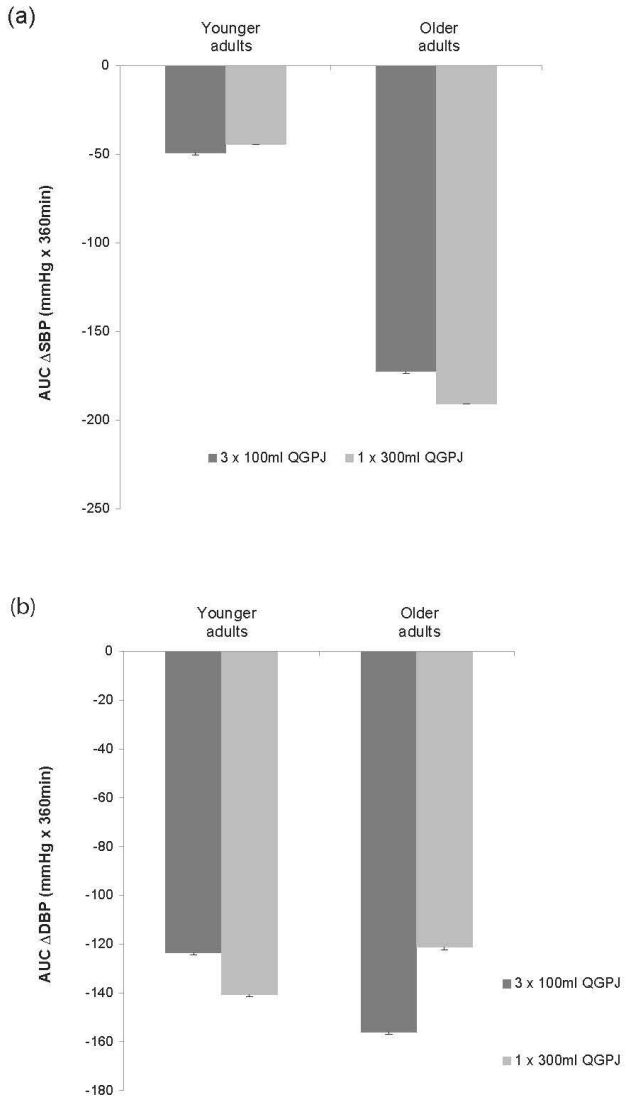

Fig. 5 – a, Change in SBP (0-6 hours) following consumption of QGPJ. Values are expressed as AUC for mean change in SBP from baseline per hour up to 6 hours. Bars represent the sum of AUC for ΔSBP (0-6 hours) ± SE (n = 12 per age group). AUC indicates area under the curve; QGPJ, Queen Garnet plum juice; SBP, systolic blood pressure. b, Change in DBP (0-6 hours) following consumption of QGPJ. Values are expressed as AUC for mean change in DBP from baseline per hour up to 6 hours. Bars represent the sum of AUC for ΔDBP (0-6 hours) ± SE (n = 12 per age group). AUC indicates area under the curve; QGPJ, Queen Garnet plum juice; DBP, diastolic blood pressure.

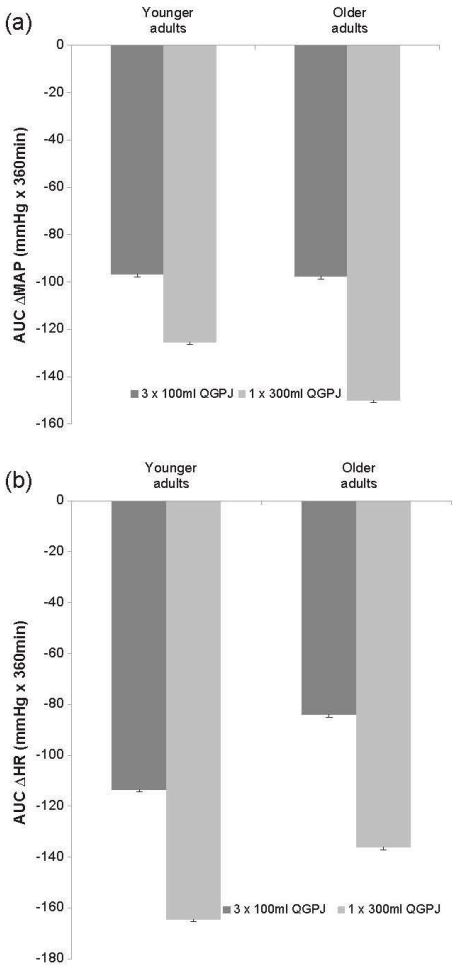

Fig. 6 – a, Change in MAP (0-6 hours) following consumption of QGPJ. Values are expressed as AUC for mean change in MAP from baseline per hour up to 6 hours. Bars represent the sum of AUC for ΔMAP (0-6 hours) ± SE (n = 12 per age group). AUC indicates area under the curve; QGPJ, Queen Garnet plum juice; MAP, mean arterial pressure. b, Change in heart rate (0-6 hours) following consumption of QGPJ. Values are expressed as AUC for mean change in HR from baseline per hour up to 6 hours. Bars represent the sum of AUC for ΔHR (0-6 hours) ± SE (n = 12 per age group). AUC indicates area under the curve; QGPJ, Queen Garnet plum juice; HR, heart rate.

A possible explanation/mechanism of the observed BP-lowering effect of QGPJ could lie in the molecular structure of its main in vivo anthocyanin metabolites. The described methylation of cyanidin glycosides by catechol-O-methyltransferase to peonidin-based metabolites as the main urinary anthocyanin metabolites after QGPJ ingestion results in a structural modification of the B-ring in the flavonoid skeleton which is structurally analogous to apocynin, an established vasoactive drug [8,52,53]. Mono-O-methylated anthocyanins/flavonoids can act as inhibitors of nicotinamide adenine dinucleotide phosphate oxidase and, as a result, can improve vasodilatory processes [8].

Another possible explanation for the BP-lowering effect of anthocyanins and/or other polyphenols present in the plum juice is their potential to inhibit the oxidation of low-density lipoproteins (LDLs), a major risk factor for atherosclerosis, through free-radical scavenging and removal of metal ions from catalytic sites via chelation [54]. The mechanism by which oxidized LDL promotes atherogenesis is believed to be through cytotoxicity, inhibitory effects on macrophage motility, and uptake by the macrophage scavenger receptor resulting in stimulation of cholesterol accumulation and hence foam cell formation, which is critical in early atherosclerosis lesions. To test this theory, Liu et al [55] in their study observed that when cells were incubated with oxidized LDL (100 μg/mL) for 24 hours, there was an increase in cell death, whereas the additions of mulberry water extracts and mulberry anthocyanin-rich extracts beyond the concentrations of 0.1 and 0.05 mg/mL, respectively, significantly increased the survival of these cell macrophages. They also observed that 1 mg/mL of mulberry water extracts and 0.1 mg/mL of mulberry anthocyanin-rich extract suppressed the lipid accumulation by approximately 55% and 58%, respectively.

Although anthocyanins have been hypothesized to promote healthy brain functioning, results from our pilot study show that a 300-mL serving of plum juice, regardless of dose timing or age of participants, has no significant acute effect on various domains of cognitive function. Although previous studies have found no significant acute effect of anthocyanins from fruit source on cognitive processes, the QGPJ used in the present study had a significantly higher content of anthocyanins, and therefore, there was a possibility that it might induce cognitive effects. In addition, 2 different cognitive tests that have been shown to be sensitive and target different domains were used: Stroop and the Reaction Time task [39,56]. Extensive research has been carried out on the long-term effect of flavonoid supplementation on cognition [39] with less attention on their acute effects. Recently, there has been an increase in the body of evidence on the acute effects of flavonoids on cognitive processes such as attention, working memory, and psychomotor speed in a general population [57]. The precise mechanism by which anthocyanins affect cognition is still not clear but seems to be dependent on the exposure period. Acute effects on cognition are believed to be as a result of increased cerebrovascular blood flow and possibly MAO inhibition which has been shown to improve cognitive performance [14,20]. Following consumption of high-anthocyanin fruit/juice, evidence shows that peaks in cerebral blood flow, vasodilation, and anthocyanin metabolite availability is detectable within 2 hours postconsumption [17]. Following blueberry supplementation, plasma anthocyanins and their metabolites were observed to reach peak levels at 1-2 and 6 hours [11]. An investigation on

improvements in different acute cognitive domains, whereby a significant improvement in updating ability was reported for younger adults and improvements in immediate word recognition in older adults were identified [67]. In relation to cocoa flavonoids, consumption of dark chocolate for 1 week significantly improved endothelial function and reduced BP in younger hypertensive patients but not in older populations [68]. Overall, there is little information that compares responses between younger and older adult populations; thus, more work comparing these groups is required to elucidate any age-related differences in biological response.

The main objective with the dose-timing design was to estimate the response according to the dose given to analyze the effect and identify any adverse reactions. Throughout the course of the study, the juice was well tolerated, and there were no reports of any adverse effects; however, the tolerability to the study protocol was not objectively measured. As there is large observed interindividual variation in the absorption, metabolism, and excretion of polyphenols [69], the use of a crossover study design is appropriate because participants act as their own controls [70].

4.1. *Limitations of the study*

A notable limitation of our study is the absence of a placebo arm. While a placebo arm is essential in dietary intervention studies to identify the magnitude of effect related to the dietary factor of interest, in the case of anthocyanins, Johnson et al [71] included a placebo control group in their blueberry powder (469 mg of anthocyanins per day) study and identified a drop of 7 mm Hg and 5 mm Hg after 8 weeks of intervention in both SBP and DBP ($P < .05$ and $P < .01$, respectively) but not in the control group. The main purpose of our acute study in which each participant acted as their own control was to identify whether different dosing regimens of a high-anthocyanin fruit juice resulted in differences in cognitive performance and/or BP. Information related to the dose-timing administration of an intervention is an important consideration in clinical trial designs in free-living participants. Furthermore, neither intact anthocyanins nor their common metabolites such as glucuronides, sulfates, or methylated forms are usually detectable in urine of placebo/control groups as demonstrated in a pilot study with QGPJ 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 long-term 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 hours and 4-6 hours postconsumption 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 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 because there were no differences according to 2 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, whereas 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.

Acknowledgment

Illawarra Health and Medical Research Institute, UOW, Australia. This work was supported by the UOW 2013 University Research Committee research partnerships grant scheme. The authors declare no conflict of interest.

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9.6 Appendix F – Published paper: A systematic literature review of the effect of anthocyanins on gut microbiota populations

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REVIEW

A systematic literature review of the effect of anthocyanins on gut microbiota populations

E. O. Igwe,[1] K. E. Charlton,[1,2] Y. C. Probst,[1,2] K. Kent[3] & M. E. Netzel[4]

[1]School of Medicine, University of Wollongong, Wollongong, NSW, Australia
[2]Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW, Australia
[3]Centre for Rural Health, School of Health Sciences, Faculty of Health, University of Tasmania, Launceston, TAS, Australia
[4]Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Brisbane, QLD, Australia

Keywords
anthocyanins, gut microbiota, health benefits, metabolism, systematic review.

Correspondence
E. O. Igwe, School of Medicine, Faculty of Science, Medicine and Health, University of Wollongong, NSW 2522, Australia.
Tel.: +61 2 42392219
Fax: +61 2 4221 3151
E-mail: ekorie@uow.edu.au

How to cite this article
Igwe E.O., Charlton K.E., Probst Y.C., Kent K., Netzel M.E. (2018) A systematic literature review of the effect of anthocyanins on gut microbiota populations. *J Hum Nutr Diet*.
https://doi.org/10.1111/jhn.12582

Abstract

Background: Evidence has shown that anthocyanins, a subclass of polyphenol, are metabolised in the gut, modulate bacterial species and exert bioactive effects through this interaction.

Methods: A systematic literature review was undertaken to determine the level of current evidence for the association between anthocyanin intake and changes in gut microbiota populations. The studies included were also assessed for the different techniques used in microbiota determination. Following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines, scientific databases, including Scopus, PubMed, ScienceDirect, Web of Science and MEDLINE, were searched up to June 2017. Details on population/sample, study design, intervention/control, dosage and method of microbiota determination were extracted.

Results: Six studies (three *in vitro*, two animal and one human trials) were included in the review, which showed that anthocyanins induced a significant proliferative effect on *Bifidobacterium* spp., known for their wide use in probiotics and for the treatment of irritable bowel syndrome. There was also an observed inhibition of *Clostridium histolyticum*, which was shown to be pathogenic in humans. The depth of analysis is an important consideration for the choice of microbiota determination technique with respect to a comprehensive, high-resolution microbiota analysis or analysis of the main microbiota taxa.

Conclusions: Very limited research has been carried out in the area of anthocyanins and gut microbiota; beneficial effects have generally been observed, and further clinical trials in humans are needed to confirm changes to gut microbes in relation to dietary anthocyanin intake and potential health benefits.

9.7 Appendix E – Published paper: Usual dietary anthocyanin intake, sources and their association with blood pressure in a representative sample of Australian adults

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RESEARCH PAPER

Usual dietary anthocyanin intake, sources and their association with blood pressure in a representative sample of Australian adults

E. O. Igwe,[1] K. E. Charlton[1,2] & Y. C. Probst[1,2]

[1]Faculty of Science, Medicine and Health, School of Medicine, University of Wollongong, Wollongong, NSW, Australia
[2]Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW, Australia

Keywords
anthocyanins, Australia Health Survey, blood pressure, dietary intake, food sources.

Correspondence
E. O. Igwe, Faculty of Science, Medicine and Health, School of Medicine, University of Wollongong, Wollongong, NSW 2522 Australia.
Tel.: +61 2 42392219
E-mail: ekorie@uow.edu.au

How to cite this article
Igwe E.O., Charlton K.E., Probst Y.C. (2019) Usual dietary anthocyanin intake, sources and their association with blood pressure in a representative sample of Australian adults. *J Hum Nutr Diet*. https://doi.org/10.1111/jhn.12647

[Correction added on 14 May after first online publication: There were grammatical and typo errors in the abstract and these have been corrected in this version.]

Abstract

Background: Anthocyanins represent an important subgroup of non-nutritive components of food as evidence continues to build related to their beneficial bioactive effects. Using a recently developed Australian anthocyanin database, the present study aimed to estimate the intake of both total anthocyanins and their subclasses, identify food sources of anthocyanins, and determine associations between anthocyanin intake and measured blood pressure (BP).

Methods: The present study comprised a secondary analysis of the 2011–12 National Nutrition and Physical Activity component of the Australian Health Survey. Anthocyanin intake was estimated using an Australian anthocyanin database. Usual anthocyanin intake, as estimated from 24-h diet recall data, was computed using multiple source methods, whereas food sources were determined by calculating contribution of food groups to total anthocyanin intake. Regression analysis, adjusted for covariates (age, gender, body mass index, high BP diagnosis, smoking status and physical activity) assessed the relationship between anthocyanin intake and BP in adults aged ≥50 years.

Results: Mean anthocyanin intake was 24.17 ± 0.32 mg day^{-1}. Across age groups, berries were the top sources: blackberry (5–65%), cherry (2–24%), blueberry (2–13%) and raspberry (3–12%). There was a significant inverse association between anthocyanin intake and systolic BP ($\beta = -0.04$, $F = 16.8$, d.f. = 6, $r^2 = 0.05$, $P < 0.01$) and diastolic BP ($\beta = 0.01$, $F = 5.35$, d.f. = 6, $R^2 = 0.013$, $P < 0.01$), in models that adjusted for covariates.

Conclusions: In comparison with the world composite database, anthocyanin intake in the Australian population was above average [mean (SD): 24.17 (0.32) mg day^{-1} versus 18.05 (21.14) mg day^{-1}]. Berries were the primary source of anthocyanins. Anthocyanin intake in older adults aged ≥50 years was inversely associated with BP.