Available at www.sciencedirect.com

**ScienceDirect**

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

# Consumption of anthocyanin-rich Queen Garnet plum juice reduces platelet activation related thrombogenesis in healthy volunteers

Abishek Bommannan Santhakumar [a,*], Avinash Reddy Kundur [a], Kent Fanning [b], Michael Netzel [c], Roger Stanley [d], Indu Singh [a]

[a] Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Gold Coast Campus, Queensland 4222, Australia
[b] Crop and Food Science, Department of Agriculture, Fisheries and Forestry, Agri-Science Queensland, Coopers Plains, Queensland 4108, Australia
[c] Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Coopers Plains, Queensland 4108, Australia
[d] Centre for Food Innovation, University of Tasmania, Tasmania 7250, Australia

## ARTICLE INFO

## ABSTRACT

The anti-thrombotic properties of an anthocyanin-rich Queen Garnet plum juice (QGPJ) and anthocyanin-free prune juice (PJ) were studied in this randomised, double-blind, crossover trial. Twenty-one healthy subjects (M = 10, F = 11) consumed QGPJ, PJ or placebo, 200 mL/ day for 28-days followed by a 2-week wash-out period. Only QGPJ supplementation inhibited platelet aggregation induced by ADP (<5%, $P = 0.02$), collagen (<2.7%, $P < 0.001$) and arachidonic acid (<4%, $P < 0.001$); reduced platelet activation-dependent surface-marker P-selectin expression of activated de-granulated platelets (<17.2%, $P = 0.04$); prolonged activated-partial thromboplastin clotting time (>2.1 s, $P = 0.03$); reduced plasma-fibrinogen (<7.5%, $P = 0.02$) and malondialdehyde levels, a plasma biomarker of oxidative stress ($P = 0.016$). PJ supplementation increased plasma hippuric acid content ($P = 0.018$). QGPJ or PJ supplementation did not affect blood cell counts, lipid profile, or inflammation markers. Our findings suggest that QGPJ but not PJ has the potential to significantly attenuate thrombosis by reducing platelet activation/hyper-coagulability and oxidative stress.

© 2014 Published by Elsevier Ltd.

# 1. Introduction

Hyperactivity or hyper-aggregation of platelets is a precursor to a number of pro-thrombotic disease states such as cardiovascular disease (CVD) (Siddiqui, Kumar, & Dikshit, 2013). In the event of endothelial vessel wall damage, circulating platelets attach and aggregate at the surface of the damaged endothelium. They consequently undergo activation in response to stimuli that triggers activation-dependent platelet surface-receptors such as $P2Y_1$ and $P2Y_{12}$ (ADP receptors), thromboxane $A_2$ ($TxA_2$), $\alpha2_A$ epinephrine, PAR-1 and PAR-4 thrombin, GPVI and $\alpha_2\beta_1$ collagen receptors (Coughlin, 2000; Dorsam & Kunapuli, 2004; Kehrel et al., 1998; Rivera, Lozano, Navarro-Nunez, & Vicente, 2009). Current anti-platelet drugs block such receptors to reduce platelet hyper-activation and help reduce risk of thrombosis in vascular conditions. Though anti-platelet drugs such as aspirin and clopidogrel have been mainstay treatments in reducing thrombotic risks, there have been several reports of resistance and sensitivity (Goh, Churilov, Mitchell, Dowling, & Yan, 2013; Petricevic et al., 2013). Combination therapy using aspirin, clopidogrel and warfarin has also been associated with significant bleeding risk (Khurram et al., 2006).

The ability of anthocyanin-rich foods to simultaneously target various platelet activation pathways and potentially attenuate thrombosis is currently receiving significant interest (Alvarez-Suarez et al., 2014; Pojer, Mattivi, Johnson, & Stockley, 2013; Santhakumar, Bulmer, & Singh, 2013). Anthocyanins are a main polyphenol subclass and are some of the most abundant polyphenols in fruits and vegetables with an estimated mean intake in Europe of 65 mg/day (Zamora-Ros et al., 2011). Studies suggesting an ability of anthocyanin-rich foods to attenuate thrombosis include: purple and red grape juice supplements inhibiting platelet aggregation in healthy subjects as well as reducing oxidised LDL and activity of NADPH oxidase in dialysis patients (Castilla et al., 2008; Freedman et al., 2001), red wine preventing the typical postprandial increase in lipid hydroperoxides and cholesterol oxidation products when consumed together with the meal (Natella et al., 2011), and anthocyanins, along with colonic polyphenol metabolites, exhibiting *in vitro* inhibitory properties of platelet activation and aggregation (Rechner & Kroner, 2005).

Queen Garnet is a new anthocyanin-rich Japanese plum (*Prunus salicina*) cultivar that was developed in a Queensland Government breeding program and shown to contain up to 272 mg anthocyanins/100 g fresh weight (Netzel et al., 2012). In a pilot trial with two healthy male subjects, 400 mL Queen Garnet plum juice (QGPJ) containing 1.12 g anthocyanins and 2.66 g total phenolics decreased the urinary excretion of malondialdehyde, a biomarker for lipid peroxidation (McKay et al., 2010), within 24 h (Netzel et al., 2012).

In the present study, the potential of anthocyanin-rich QGPJ was compared with anthocyanin-free commercial prune juice (PJ), in inhibiting platelet hyper-activity mediated by various platelet activation pathways. The hypothesis was that anthocyanin-rich QGPJ 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. Furthermore, the concentrations of hippuric acid, a colon microbial/liver derived metabolite of dietary polyphenols and potential biomarker for total polyphenol intake (DuPont, Bennett, Mellon, & Williamson, 2002), and malondialdehyde were measured using HPLC in urine and plasma of the subjects pre and post treatment to confirm uptake of polyphenols/metabolites and measure their impact on plasma oxidation.

# 2. Materials and methods

## 2.1. Study subjects and experimental design

This trial was approved by the Griffith University Human Research Ethics Committee, Griffith University, Queensland, Australia (GU Protocol No. MSC/02/12/HREC) and registered at the Australian New Zealand Clinical trials registry (ACTRN12612000674831).

Twenty-one healthy volunteers (11 men and 10 women) were recruited from the local community and provided informed, written consent prior to participation in the study. Participants were screened by means of questionnaires to be apparently healthy, non-smokers, with no history of metabolic or cardiovascular diseases, and not consuming daily health/energy supplements, anti-platelet or anti-inflammatory medications during the duration of the study. Volunteers on high antioxidant diets were screened using dietary antioxidant questionnaires and were excluded from the study. Initial screening using baseline full blood counts (FBC), biochemical profile, BMI and blood pressure determined that volunteers were within normal reference ranges (Table 1) as established by the Royal College of Pathologists of Australasia (RCPA) (The Royal College of Pathologists of Australasia, 2004).

A randomised, double blind, placebo-controlled, cross-over trial was performed (Fig. 1). After initial screening

| Table 1 – Baseline parameters of subjects under study. | | |
|---|---|---|
| Parameters | Male (*n* = 10) | Female (*n* = 10) |
| Age (years) | 32.9 ± 11.8 | 34.2 ± 10.8 |
| BMI (kg/m$^2$) | 21.2 ± 2.6 | 19.6 ± 1.5 |
| BP (mm Hg) | 127 ± 9 | 121 ± 8 |
| | 76 ± 6 | 74 ± 7 |
| Haemoglobin (g/L) | 138.3 ± 5.4 | 129.9 ± 9.8 |
| Haematocrit (%) | 40.6 ± 1 | 38.4 ± 2 |
| RBC (×10$^{12}$/L) | 4.9 ± 0.1 | 4.6 ± 0.3 |
| WBC (×10$^9$/L) | 6.0 ± 1.7 | 7.0 ± 2.0 |
| Platelets (×10$^9$/L) | 227 ± 41 | 262 ± 55 |
| MPV (fL) | 8.1 ± 1.0 | 8.0 ± 0.7 |
| TC (mmol/L) | 4.42 ± 0.86 | 4.82 ± 0.86 |
| HDL (mmol/L) | 1.40 ± 0.35 | 1.64 ± 0.42 |
| TG (mmol/L) | 0.85 ± 0.30 | 1.05 ± 0.45 |
| LDL (mmol/L) | 2.63 ± 0.83 | 2.70 ± 0.84 |
| Glucose (mmol/L) | 5.15 ± 0.41 | 4.88 ± 0.37 |

Values are represented as mean ± SD.
Abbreviations: BP, blood pressure; RBC, red blood cell; WBC, white blood cell; MPV, mean platelet volume; TC, total cholesterol HDL, high density lipoprotein; TG, triacylglycerol. Baseline data between men and women were not significantly different (*P* > 0.05).
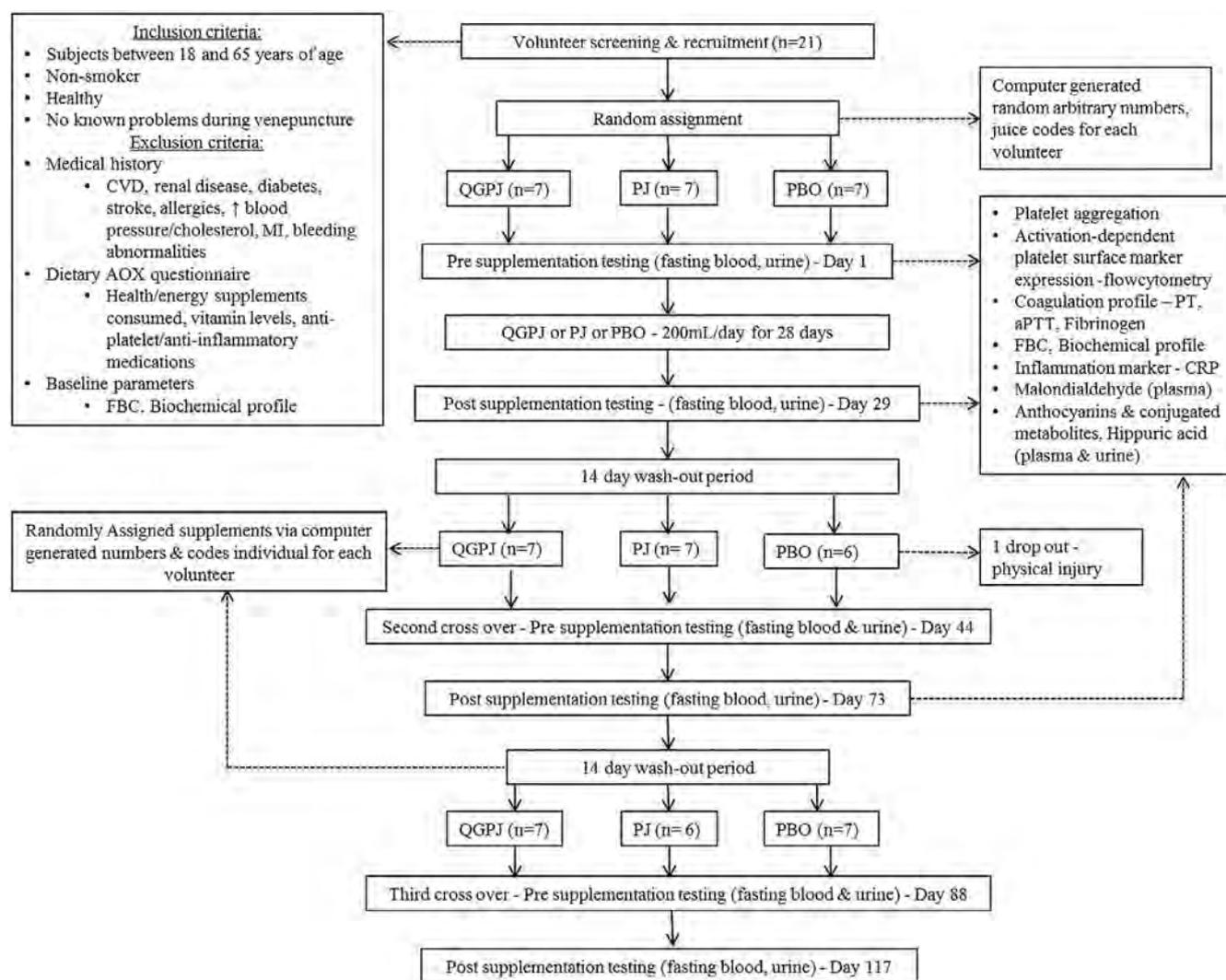
**Fig. 1 – Double-blind, randomised, placebo controlled, dietary intervention trial. Abbreviations: QGPJ, Queen Garnet plum juice; PJ, prune juice; PBO, colour matched placebo drink; PT, prothrombin time; aPTT, activated partial thromboplastin time; FBC, full blood count; CVD, cardiovascular disease; MI, myocardial infarction; AOX, antioxidant; CRP, C-reactive protein.**

volunteers were randomly assigned into three different supplement groups – QGPJ, PJ and colour matched placebo (PBO). The randomisation was made using computer-generated arbitrary number codes for each volunteer and was assigned by a statistician who worked independently to the study investigators. Juice supplementation bottles of 1 L each were identical white plastic bottles labelled with respective volunteer codes and content codes (A, B and C). The labelling of the juice bottles were carried out by a research assistant independent to the clinical investigators of this study, so as to maintain the blinding of the study investigators.

During the study period, volunteers adhered to their usual diet, but consumed 200 mL of either QGPJ, PJ or PBO for 28 days. Six litres of respective juices and a graduated measuring cylinder were provided to each volunteer at the start of each random supplementation bout to facilitate accurate consumption of the juice supplements every day. Compliance with supplement consumption was recorded at the end of the 28 day supplementation period by measuring the quantity of juice remaining in the last bottle. At day 1, baseline fasting blood

and urine samples were collected to evaluate: (1) platelet aggregation, (2) platelet activity – activation-dependent surface marker expression, (3) haemostatic function – coagulation assays, (4) full blood cell counts, (5) biochemical profile, (6) inflammation marker and (7) oxidative stress and polyphenol intake biomarkers. Following 4 weeks of supplementation with any of the three study beverages, the sample collection and assays were repeated on day 29. After a 2-week wash out period, carried out to avoid potential interference from a previous supplementation regime, the supplementation cross-overs were performed, and sample collection and assay procedures were repeated, i.e. second cross-over on day 44 (pre-supplementation testing) and day 73 (post-supplementation testing); third cross-over day 88 (pre-supplementation testing) and day 117 (post-supplementation testing).

### 2.2. Study beverages

QGPJ was prepared as described previously (Netzel et al., 2012), using fruit harvested in February 2012. PJ was commercially

sourced prune juice (100% prune juice from concentrate, Bickford's Australia, Salisbury South, SA, Australia). PBO was prepared by diluting a commercial raspberry flavoured cordial (ingredients: water, sugar, acidity regulator [E330], flavour, natural colour [E163], preservatives [E211, E223] Coles Smart Buy – Raspberry flavoured cordial, Coles, Brisbane, QLD, Australia) 1:4 with water. All study beverages were heated to 72 °C and held for 5 min prior to pack off into identical 1 L, white plastic bottles and stored at 4 °C prior to distribution to participants. The beverages were analysed for anthocyanins, quercetin glycosides, total phenolics (Folin–Ciocalteu method), 5-hydroxymethyl-2-furfural, and oxygen radical absorbance capacity (ORAC assay) as previously described (Bobrich et al., 2014; Netzel et al., 2012). Individual anthocyanin compounds were identified based on their retention time (standards), UV–Vis spectra and mass spectra (Netzel et al., 2012). Energy, protein, fat, total sugar, fibre, vitamin C and several B-vitamins were determined by a commercial laboratory (Symbio Alliance, Brisbane, QLD, Australia).

## 2.3. Dietary intake monitoring

Subjects were required to complete a record of 24 h full food intake once a week during the 4-week supplementation period. Specific guidelines and instructions on how to complete a dietary record were provided to ensure the highest possible accuracy was achieved and dietary pattern was not altered from normal. Subjects were requested to record the type of food eaten and detail its preparation, in addition to the amount and time of consumption. Body weight was measured using a standard scientific scale and height using a stadiometer. These measurements were used to calculate BMI and in turn the estimated dietary requirements. The dietary accounts were analysed using FoodWorks® (Xyris Software Pty Ltd., Kenmore Hillis, QLD, Australia) based on the Australian Food Composition database.

## 2.4. Blood and urine sample collection

All blood samples were collected by a trained phlebotomist at least 8 h pre-prandial from the median cubital vein using a 21-gauge needle. No samples were obtained from traumatic phlebotomy procedures or contained obvious clots. Blood was drawn into ethylenediaminetetraacetic acid (EDTA) (1.8 mg/mL) anticoagulant tubes followed by trisodium citrate (28.12 gm/L) tubes to avoid risk of collecting platelets activated by venepuncture, and used for platelet aggregation, platelet surface marker evaluation and coagulation assays. Blood samples collected into serum separator tubes were used for biochemical analysis. Care was taken to ensure minimal blood sample handling and agitation. All platelet function tests were carried out within 2 h of blood collection.

Mid-stream fasting urine samples were collected into sterile 50 mL urine containers and immediately stored at –80 °C to analyse hippuric acid, anthocyanins and their conjugated metabolites pre and post supplementation.

## 2.5. Full blood examination and biochemical assays

The Coulter® Ac.T™ 5diff CP haematology analyser (Beckman Coulter, Inc., Lane Cove, NSW, Australia) was used to perform a FBC using whole blood collected into EDTA anticoagulant tubes within 10 min of blood collection. Total red blood cell (RBC) count, white blood cell (WBC) count, platelet count, mean platelet volume (MPV), haemoglobin and haematocrit were evaluated at baseline and post intervention for all the three supplementation bouts.

Biochemical parameters such as serum total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triacylglycerols, bilirubin, glucose and C reactive protein (inflammation marker) were evaluated using the Cobas Integra 400® plus biochemistry analyser (Roche diagnostics, Basel, Switzerland).

Hippuric acid (plasma and urine) and plasma malondialdehyde (MDA) were analysed by HPLC coupled with photodiode array and fluorescence detection as previously described (Netzel et al., 2002, 2012). It should be noted that for hippuric acid analysis in plasma, samples were diluted in acetonitrile, vortexed, centrifuged and filtered as detailed by Kubota, Horai, Kushida, and Ishizaki (1988). Furthermore, plasma and urine samples were screened for anthocyanins and their common conjugated metabolites (methylated, glucuronidated and sulphated forms) as outlined by Netzel et al. (2012) and Frank, Netzel, Strass, Bitsch, and Bitsch (2003), respectively.

## 2.6. Platelet aggregation assay

The platelet aggregation assay was carried out using the AggRAM® turbidometric aggregation analyser (Helena Laboratories, Beaumont, TX, USA). Whole blood collected into trisodium citrate anticoagulant tubes were centrifuged at $180 \times g$ for 10 min to obtain platelet rich plasma (PRP). After separating PRP, platelet poor plasma (PPP) was obtained by further centrifugation at high speed ($2000 \times g$ for 10 min). Platelets (count ranging between $200 \times 10^9$/L and $300 \times 10^9$/L) in PRP were stimulated for aggregation by exogenous agonists which were ADP (5 µM), collagen (2 µg/mL), arachidonic acid (200 µg/mL) and the percentage of aggregation was recorded for 6 min. The concentrations used to stimulate optimal platelet aggregation were established by dose response curves performed in our previous in-vitro trials (Santhakumar, Fozzard, Perkins, & Singh, 2013; Santhakumar, Linden, & Singh, 2012). PPP was a clear solution and was used to blank the instrument. All agonists were purchased from Helena Laboratories. Optical channels in the aggregometer were validated each day prior to sample runs.

## 2.7. Activation-dependent platelet surface marker expression

Venous blood collected into trisodium citrate tubes were used to evaluate platelet activation-dependent surface receptor expression. This analysis was performed and interpreted using the BD LSRFortessa cell analyser (BD Biosciences, North Ryde, NSW, Australia) and BD FACSDiva software (version 6.1.3, BD Biosciences, North Ryde, NSW, Australia) respectively. CD42b-peridinin chlorophyll protein (CD42b-PerCp-Cy5.5) conjugated mAb was used to identify the GPIb-IX receptor on the platelet population. Platelet activity was assessed by activation-dependent platelet surface marker expression using PAC-1-fluorescein isothiocyanate-FITC and P-selectin/CD62P-allophycocyanin-APC conjugated mAb. In this assay,

PAC-1-FITC mAb recognises activated conformational changes in the fibrinogen binding receptor GPIIb-IIIa and P-selectin/ CD62P mAb binds to activated, de-granulated platelets. The antibodies and their respective isotype controls were purchased from BD Biosciences (North Ryde, NSW, Australia).

Whole blood, diluted in filtered modified Tyrode's buffer (pH 7.2), was incubated with CD42b, PAC-1 and P-selectin mAb for 15 min in the dark at room temperature. ADP (5 μM) was incubated with the blood–antibody mixture for 10 min in the dark at room temperature to activate the platelets. The suspension was then fixed with 1% paraformaldehyde solution (pH 7.2), incubated in the dark at room temperature for 15 min and analysed in the BD LSRFortessa flowcytometer for antibody expression. The setup and optimal fluorescence compensation of the flowcytometer were validated using BD Cytometer Setup and Tracking beads and BD CompBead compensation particles (BD Biosciences). Ten thousand platelet events were acquired, gated on the basis of light scatter and CD42b mAb expression. Activation dependent-mAb (PAC-1 and P-selectin) expression by activated platelets was articulated as mean fluorescence intensity (MFI).

### 2.8. Coagulation assay

Activated partial thromboplastin time-aPTT, prothrombin time-PT and fibrinogen concentration which are the important factors contributing to the coagulation cascade were evaluated using the C4 coagulation analyser (Helena Laboratories). PPP obtained from tri-sodium citrate tubes was used in the analysis. The assays were performed based on the C4-coagulation analyser operator's manual (Helena Laboratories). Fibrinogen and thromboplastin reagents were purchased from Helena Laboratories and aPTT reagent and controls from Vital diagnostics (Bella Vista, NSW, Australia).

### 2.9. Statistical analysis

A minimum sample size of 17 volunteers in each group was required for 80% power to detect a 5% variation in the laboratory parameters measured, where a 3–5% standard deviation exists in the population, assuming an alpha error of 0.05. Repeated measures analysis of variance (ANOVA) with Newman–Keuls post-test analysis was performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA). Statistical significance was established when $P < 0.05$. Any significant statistical interactions were included in the analysis where applicable. All data are reported as mean ± SD.

### 3. Results

Of the 21 subjects randomly assigned to different supplementation bouts, the analysis excluded 1 volunteer due to the occurrence of an adverse event. There were no significant changes to baseline characteristics between males and females (Table 1). The nutritional composition (including individual anthocyanin compounds, total amount of quercetin derivatives, and antioxidant capacity) of the three study beverages is shown in Table 2. There were no significant changes in blood cell

| Table 2 – Composition of the three study beverages by analysis. | | | |
|---|---|---|---|
| | QGPJ | PJ | PBO |
| Anthocyanin content (mg C3GE/100 mL)[a] | | | |
| Cyanidin-3-glucoside | 76 | ND | 0.1 |
| Cyanidin-3-rutinoside | 25 | ND | 0.1 |
| Other cyanidin based anthocyanins | ND | ND | 0.2 |
| Total | 101 | ND | 0.4 |
| Quercetin derivatives (mg QGE/100 mL)[a] | 43.7 | ND | 0.4 |
| Total phenolic content (mg GAE/100 mL)[a] | 322 | 157 | 7 |
| ORAC (μmol TE/100 mL)[a] | 4207 | 1814 | ND |
| 5-Hydroxymethyl-2-furfural [a,c] | ND | Present | ND |
| Vitamin C (mg/100 mL)[b] | <0.05 | <0.05 | <0.05 |
| Vitamin B1 (thiamin) (μg/100 mL)[b] | 13.5 | 10.8 | ND |
| Vitamin B2 (riboflavin) (μg/100 mL)[b] | 9 | 9 | ND |
| Vitamin B3 (total) (μg/100 mL)[b] | 146 | 77.0 | ND |
| Vitamin B6 (pyridoxine) (μg/100 mL)[b] | 34.7 | 40.0 | ND |
| Vitamin B12 (cyanocobalamin) (μg/100 mL)[b] | <5 | <5 | ND |
| Energy (kJ/100 g)[b] | 213 | 277 | 146 |
| Protein (g/100 mL)[b] | 1.1 | 0.8 | 0.8 |
| Fat (g/100 mL)[b] | 0.1 | 0.1 | 0.1 |
| Total sugar (g/100 mL)[b] | 8.7 | 11.8 | 7.3 |
| Fibre (g/100 mL)[b] | <0.1 | <0.1 | <0.1 |
| pH[b] | 3.28 | 3.80 | 2.70 |

Values are represented as mean of duplicate analysis.
Abbreviations: QGPJ, Queen Garnet plum juice; PJ, plum juice; PBO, placebo drink (diluted raspberry cordial); C3GE, cyanidin-3-glucoside equivalents; GAE, gallic acid equivalents (Folin–Ciocalteu assay); ORAC, oxygen radical absorbance capacity; TE, trolox equivalents; QGE, quercetin glucoside equivalents; ND, non-detectable.
[a] Analysed by the authors.
[b] Analysed by a commercial laboratory (Symbio Alliance, Brisbane, QLD, Australia).
[c] Qualitative analysis only (detectable/non-detectable).

counts, biochemical parameters or inflammation markers, following consumption of the three beverages (Table 3). No statistically significant differences in mean baseline values of parameters tested between each cross-over were recorded thereby eliminating potential carry-over effects.

Intervention with anthocyanin-rich (cyanidin-3-glucoside and cyanidin-3-rutinoside) QGPJ for 4 weeks significantly inhibited platelet aggregation induced by ADP by 5% ($P = 0.02$) (Fig. 2A), collagen by 2.7% ($P < 0.001$) (Fig. 2B) and arachidonic acid by 4% ($P < 0.001$) (Fig. 2C). PJ or PBO supplementation did not have an effect on platelet aggregation (Fig. 2).

QGPJ intervention significantly reduced P-selectin/CD62P activation dependent surface marker expression by 17.2% ($P = 0.04$) (Fig. 3). PJ or PBO supplementation did not affect P-selectin expression (Fig. 3). PAC-1 expression, indicating activation-dependent conformational change of platelets, was not altered post QGPJ, PJ or PBO supplementation.

**Table 3 – Descriptive values of blood cell counts and biochemical parameters in 20 subjects at baseline (pre-treatment) and end of 4 week intervention (post-treatment).**

| Parameters | QGPJ | PJ | PBO |
|---|---|---|---|
| Haemoglobin (g/L) | | | |
| *Baseline* | 136 ± 7 | 136 ± 8 | 137 ± 10 |
| *4 weeks* | 138 ± 9 | 139 ± 9 | 140 ± 8 |
| Haematocrit | | | |
| *Baseline* | 0.39 ± 0.02 | 0.40 ± 0.02 | 0.39 ± 0.02 |
| *4 weeks* | 0.40 ± 0.02 | 0.40 ± 0.02 | 0.40 ± 0.02 |
| RBC ($\times10^{12}$/L) | | | |
| *Baseline* | 4.8 ± 0.2 | 4.8 ± 0.2 | 4.8 ± 0.3 |
| *4 weeks* | 4.9 ± 0.3 | 4.9 ± 0.3 | 4.9 ± 0.3 |
| WBC ($\times10^{9}$/L) | | | |
| *Baseline* | 6.0 ± 1.3 | 6.4 ± 1.5 | 6.2 ± 1.7 |
| *4 weeks* | 6.3 ± 1.6 | 6.2 ± 1.5 | 6.9 ± 1.7 |
| Platelets ($\times10^{9}$/L) | | | |
| *Baseline* | 244 ± 63 | 259 ± 69 | 247 ± 60 |
| *4 weeks* | 249 ± 58 | 260 ± 60 | 254 ± 55 |
| MPV (fL) | | | |
| *Baseline* | 8.1 ± 0.9 | 8.0 ± 0.9 | 8.1 ± 1.0 |
| *4 weeks* | 8.0 ± 0.8 | 8.0 ± 0.8 | 7.8 ± 0.7 |
| TC (mmol/L) | | | |
| *Baseline* | 4.57 ± 0.74 | 4.73 ± 0.82 | 4.80 ± 0.91 |
| *4 weeks* | 4.66 ± 0.62 | 3.91 ± 1.88 | 4.44 ± 1.28 |
| HDL (mmol/L) | | | |
| *Baseline* | 1.45 ± 0.40 | 1.45 ± 0.30 | 1.47 ± 0.36 |
| *4 weeks* | 1.46 ± 0.37 | 1.39 ± 0.33 | 1.41 ± 0.30 |
| TG (mmol/L) | | | |
| *Baseline* | 0.95 ± 0.47 | 1.08 ± 0.63 | 0.93 ± 0.37 |
| *4 weeks* | 0.99 ± 0.48 | 1.13 ± 0.64 | 1.08 ± 0.55 |
| LDL (mmol/L) | | | |
| *Baseline* | 2.67 ± 0.70 | 2.50 ± 1.08 | 2.32 ± 1.41 |
| *4 weeks* | 2.74 ± 0.60 | 2.00 ± 1.79 | 2.54 ± 1.21 |
| Uric acid (μmol/L) | | | |
| *Baseline* | 274 ± 76 | 269 ± 62 | 266 ± 69 |
| *4 weeks* | 278 ± 75 | 265 ± 93 | 283 ± 79 |
| Glucose (mmol/L) | | | |
| *Baseline* | 4.7 ± 0.3 | 4.9 ± 0.3 | 4.9 ± 0.4 |
| *4 weeks* | 4.7 ± 0.3 | 4.6 ± 0.5 | 4.7 ± 0.5 |
| Bilirubin (μmol/L) | | | |
| *Baseline* | 7.9 ± 3.6 | 7.6 ± 2.6 | 7.2 ± 2.2 |
| *4 weeks* | 8.6 ± 3.7 | 6.9 ± 2.1 | 8.4 ± 2.1 |
| HS-CRP (mg/L) | | | |
| *Baseline* | 1.23 ± 1.31 | 1.65 ± 2.81 | 1.33 ± 1.28 |
| *4 weeks* | 1.32 ± 1.31 | 1.04 ± 1.07 | 1.22 ± 1.46 |

Values are represented as mean ± SD.
Abbreviations: RBC, red blood cell; WBC, white blood cell; MPV, mean platelet volume; TC, total cholesterol; HDL, high density lipoprotein; TG, triacylglycerol; HS-CRP, high sensitivity C-reactive protein. QGPJ, PJ or PBO supplementation did not have an effect on blood cell counts, biochemical parameters or inflammation marker ($P > 0.05$).

QGPJ supplementation prolonged aPTT clotting time by 2.1 s ($P = 0.03$) (Fig. 4A) and reduced plasma fibrinogen concentration by 7.5% (pre QGPJ supplementation – 407.1 mg/dL, post QGPJ supplementation – 371.4 mg/dL, $P = 0.02$) (Fig. 4B). QGPJ, PJ or PBO supplementation did not affect PT and no changes to aPTT or fibrinogen concentration were seen post PJ or PBO intervention.

Following QGPJ supplementation there was a significant decrease of 38% ($P = 0.016$) in plasma MDA content (Fig. 5). However, following both PJ and PBO there was no change. Fol-

lowing PJ supplementation, but not QGPJ or PBO, there was a significant increase of 60% ($P = 0.018$) in plasma hippuric acid content (Fig. 6A). However, no statistically significant effects could be observed in urine (Fig. 6B).

In addition to hippuric acid, cyanidin monoglucuronide could be tentatively identified in some of the QGP$_{POST}$ plasma and urine samples. However, since the concentration of this anthocyanin metabolite was below the limit of quantification, a further evaluation was not undertaken.

## 4. Discussion

The aim of this randomised, double-blind placebo controlled, cross-over, human dietary intervention study was to evaluate the effect of an anthocyanin-rich plum juice produced from the novel blood plum variety, Queen Garnet, and an anthocyanin-free commercial prune juice on biomarkers of thrombotic risk. The study demonstrated that only supplementation with 200 mL of anthocyanin-rich (cyanidin-3-glucoside and cyanidin-3-rutinoside) QGPJ for 4 weeks had a significant effect on markers of thrombosis namely: inhibition of platelet aggregation induced by ADP, collagen and arachidonic acid platelet agonists; reduction in P-selectin activation-dependent platelet surface marker expression; prolongation of activated partial thromboplastin clotting time; and reduction of fibrinogen concentration. Only QGPJ also decreased plasma malondialdehyde levels, a biomarker of oxidative stress. These findings suggest that anthocyanin-rich QGPJ has the potential to alleviate platelet activation/aggregation and favourably alter haemostatic function. Queen Garnet plum was chosen for the study due to its high anthocyanin and polyphenolic content, following previous studies which have demonstrated the potential of antioxidants and other polyphenols in reducing biomarkers of thrombosis and risk of vascular diseases (Murphy et al., 2003; Santhakumar et al., 2012, 2013; Singh, Mok, Christensen, Turner, & Hawley, 2008; Singh et al., 2006; Singh, Turner, Sinclair, Li, & Hawley, 2007; Vucinic, Singh, Spargo, Hawley, & Linden, 2010).

The artificial stimulation of platelets by adding exogenous agonists such as ADP (activates P2Y$_1$/P2Y$_{12}$ platelet receptor), collagen (activates GPVI/ $\alpha_2\beta_1$ receptor) and arachidonic acid (activates TxA$_2$) was used in this study to mimic platelet aggregation/activation during vessel wall damage *in vivo*. QGPJ supplementation for 4 weeks effectively and simultaneously targeted the ADP-P2Y$_1$/P2Y$_{12}$, TxA$_2$ and collagen receptors (GPVI/ $\alpha_2\beta_1$) thereby reducing platelet aggregation, which is similar to that seen for an anthocyanin-rich chokeberry extract (10 μg/mL) in a recent *in vitro* trial (Sikora, Markowicz-Piasecka, Broncel, & Mikiciuk-Olasik, 2014). The observed effects of anthocyanin-rich QGPJ on platelet aggregation are also in alignment with the effect of other polyphenol-rich foods such as pomegranate juice (Aviram et al., 2000). We believe that the completely conjugated structure of anthocyanins and the related metabolites play an important role in allowing electron delocalisation hence favouring its antioxidant ability to neutralise hydrogen peroxide in the cyclooxygenase-1 pathway of platelet activation. This assertion is supported by the observed antioxidant effect of QGPJ anthocyanins in reducing arachidonic acid induced platelet aggregation.
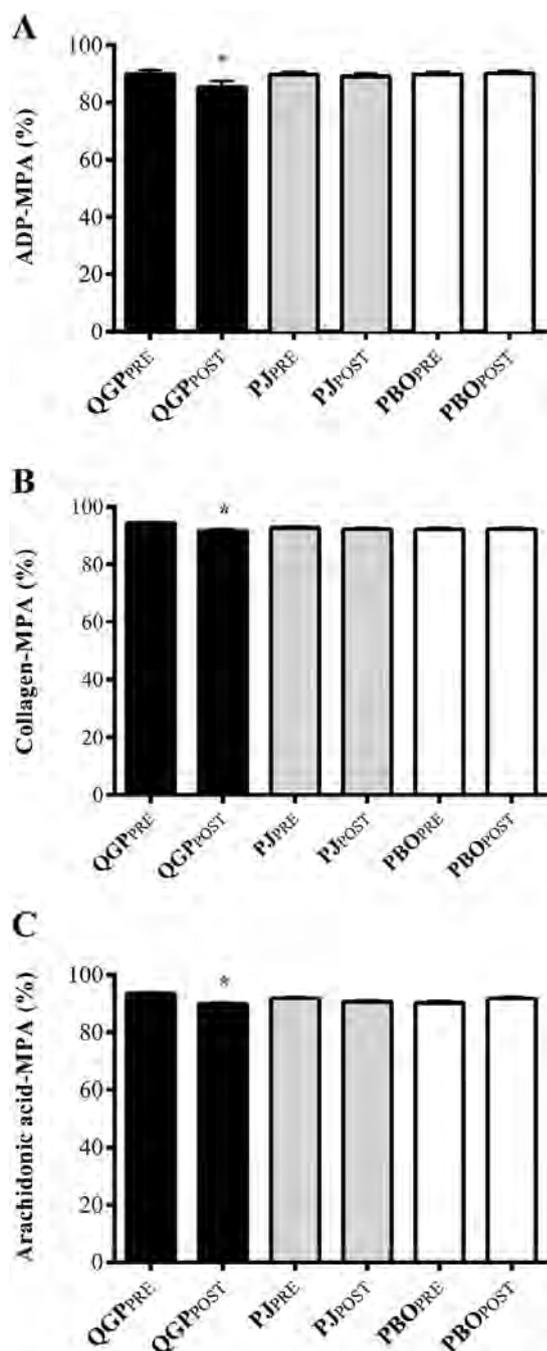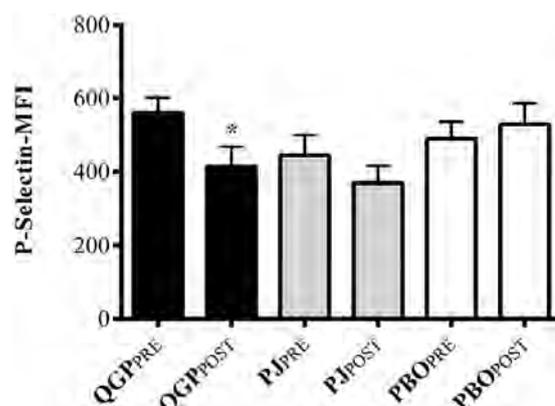
**Fig. 3 – Activation-dependent platelet surface marker P-selectin/CD62P expression before and after QGPJ, PJ and PBO supplementation. Reduction in P-selectin expression on activated platelets was observed (<17.2%, *P = 0.04) post QGPJ supplementation. PJ or PBO supplementation did not alter P-selectin expression. Data are represented as mean ± SD (N = 20). Abbreviations: QGP$_{PRE}$, before Queen Garnet plum juice supplementation; QGP$_{POST}$, after Queen Garnet plum juice supplementation; PJ$_{PRE}$, before prune juice supplementation; PJ$_{POST}$, after prune juice supplementation; PBO$_{PRE}$, before placebo drink supplementation, PBO$_{POST}$, after placebo drink supplementation; MFI, mean fluorescence intensity.**



**Fig. 2 – Effect of 4-week supplementation of QGPJ, PJ and PBO on platelet aggregation induced by platelet stimulants. Inhibition of platelet aggregation stimulated by ADP (A) (<5%, *P = 0.02), collagen (B) (<2.7%, *P < 0.001) and arachidonic acid (C) (<4%, *P < 0.001) post 4-week QGPJ supplementation. PJ or PBO intervention did not alter platelet aggregation stimulated by the agonists. N = 20 in each study group and the data are presented as mean percentage platelet aggregation (MPA%). Abbreviations: QGP$_{PRE}$, before Queen Garnet plum juice supplementation; QGP$_{POST}$, after Queen Garnet plum juice supplementation; PJ$_{PRE}$, before prune juice supplementation; PJ$_{POST}$, after prune juice supplementation; PBO$_{PRE}$, before placebo drink supplementation, PBO$_{POST}$, after placebo drink supplementation.**

The reduction in platelet P-selectin expression, following QGPJ supplementation, signifies the ability of QGPJ to reduce platelet degranulation and inhibit platelet α-granule release consequently assisting in reducing thrombotic risk. Similarly, in subjects with metabolic syndrome, polyphenol-rich extracts of kale and pomegranate have reduced P-selectin and GPIIb-IIIa expression *in vitro* (Konic-Ristic et al., 2013). QGPJ or PJ did not alter PAC-1 expression and consequently had no effect on the initial phase of activation involving the GPIIb-IIIa receptor. Rein and colleagues also observed no changes to PAC-1 expression induced by epinephrine post dietary intervention with de-alcoholised red wine (Rein et al., 2000). Ostertag and colleagues demonstrated the effect of a variety of phenolic compounds in inhibiting activation dependent platelet degranulation (P-selectin expression) induced by thrombin, but at non-physiological concentrations (Ostertag et al., 2011). The observed reduction in P-selectin expression following QGPJ may be due to the desensitisation of activation-dependent platelet surface receptors (Guerrero et al., 2007) interfering with signal transduction or by preventing post activation α-granule release. Hence reduced P-selectin expression post QGPJ supplementation suggests a possible reduction in the risk of platelet activation and subsequent thrombotic episodes, as elevated P-selectin is a marker for increased platelet activation and future CVD risk (Semenov et al., 2000).

The prolongation in aPTT observed after QGPJ supplementation suggests that anthocyanins, quercetin glycosides and/or other potential active metabolites have a favourable effect on the intrinsic or contact activation pathway of coagulation. We believe that the anthocyanins in the juice and/or derived in vivo metabolites might trigger the inhibition of activation
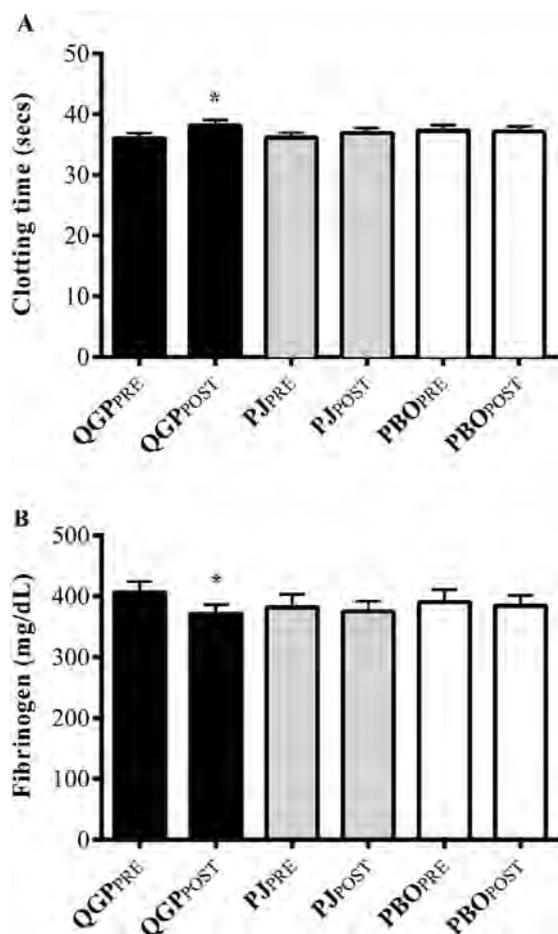
**Fig. 4 – Effect of QGPJ, PJ, and PBO supplementation (4-weeks) on activated partial thromboplastin clotting time and fibrinogen concentration. (A) Prolongation in clotting time (>2.1 s, \*P = 0.03) and (B) reduction in fibrinogen levels (<7.5%, \*P = 0.02) are seen post QGPJ supplementation. PJ and PBO supplementation did not affect haemostatic function. Data are represented as mean ± SD (N = 20). Abbreviations: QGP$_{PRE}$, before Queen Garnet plum juice supplementation; QGP$_{POST}$, after Queen Garnet plum juice supplementation; PJ$_{PRE}$, before prune juice supplementation; PJ$_{POST}$, after prune juice supplementation; PBO$_{PRE}$, before placebo drink supplementation; PBO$_{POST}$, after placebo drink supplementation.**

does not contain anthocyanins or quercetin glycosides (Table 2). The particular commercial prune juice product that was used in this study was reconstituted prune juice concentrate, and the processing (particularly drying and heating) involved to make this product presumably resulted in breakdown and loss of the various polyphenolic compounds which are found in fresh prunes, dried prunes and less processed prune juice (Donovan, Meyer, & Waterhouse, 1998; Pilando & Wrolstad, 1992; Vangorsel, Li, Kerbel, Smits, & Kader, 1992). This was further evidenced by the absence of peaks in the 260 nm and 350 nm absorbance region of the analysis chromatograms where conjugated structures of polyphenols and derivatives should be detected. Furthermore the formation of 5-hydroxymethyl-2-furfural in the prune juice may have been a significant contributor to the antioxidant activity as measured by the total phenolic content and ORAC assays (Donovan et al., 1998; Prior, Wu, & Gu, 2006).

The significantly decreased plasma MDA levels after QGPJ intervention (QGP$_{POST}$ versus QGP$_{PRE}$) are most likely due to QGPJ's anthocyanin and quercetin glycoside content, and their *in vivo* metabolites, as described in a previous pilot trial (Netzel et al., 2012). This result is in line with several other human studies that have reported a significant decrease in plasma/serum MDA after the consumption of anthocyanin-rich foods such as strawberries (–31% after 30 days; Alvarez-Suarez et al., 2014), a cocoa beverage rich in soluble phenolic compounds (–15% after 8 weeks; Sarria et al., 2012) as well as apple and grape juice (–24% after 2 weeks; Yuan et al., 2011). These observations indicate a decreased lipid peroxidation within the plasma compartment. The PJ was less effective in terms of preventing lipid peroxidation *in vivo*, presumably due to the absence of polyphenolics such as anthocyanins and quercetin glycosides as well as their *in vivo* metabolites.
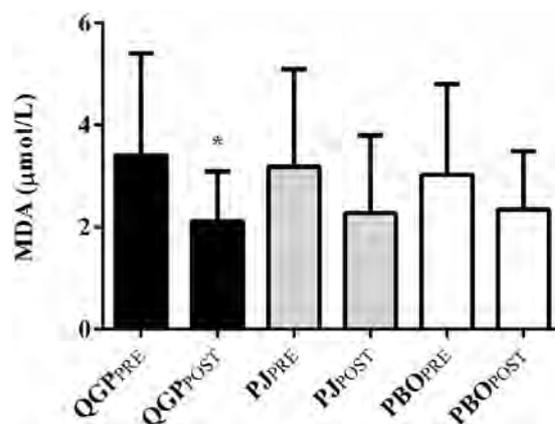
factor XII or the proteins which facilitate the activation of this pathway namely high molecular weight kininogen or prekallikrein. It should be noted that this prolongation in clotting time by 2.1 s is under normal clinical reference ranges and poses no bleeding risk. The observed QGPJ supplementation effect on reducing fibrinogen concentration in plasma indicates alleviation in pro-thrombotic progression, in contrast to high levels seen in conditions such as CVD. Although the exact mechanism of the observed reduction in levels of fibrinogen is unknown it is proposed that anthocyanin-rich QGPJ inhibits overall platelet activation or the amydolytic activity of thrombin (Sikora et al., 2014), consequently blunting fibrin synthesis.

Prune juice did not reduce platelet aggregation, activation or display a favourable activity on the coagulation profile. It



**Fig. 5 – Plasma malondialdehyde (MDA) levels pre and post supplementation. Significant decrease (\*P = 0.016) in plasma MDA levels post QGPJ supplementation. Data are represented as mean ± SD (N = 20). Abbreviations: QGP$_{PRE}$, before Queen Garnet plum juice supplementation; QGP$_{POST}$, after Queen Garnet plum juice supplementation; PJ$_{PRE}$, before prune juice supplementation; PJ$_{POST}$, after prune juice supplementation; PBO$_{PRE}$, before placebo drink supplementation; PBO$_{POST}$, after placebo drink supplementation.**
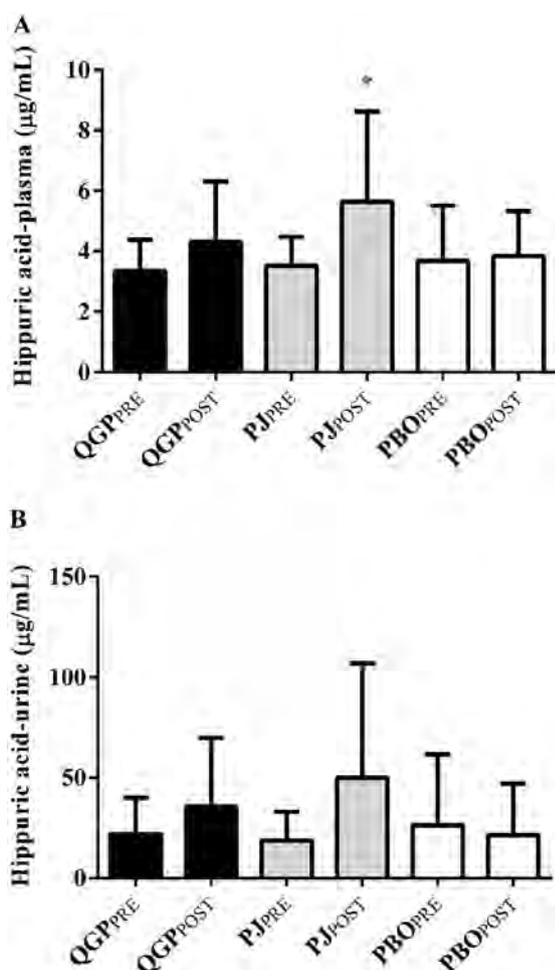
**Fig. 6 – Hippuric acid concentrations in plasma and urine pre and post supplementation. (A) Significant increase (*P = 0.018) in hippuric acid levels in plasma post prune juice supplementation. (B) No effect of QGP, PJ or PBO on urinary hippuric acid concentration. Data are represented as mean ± SD (N = 20). Abbreviations: QGP$_{PRE}$, before Queen Garnet plum juice supplementation; QGP$_{POST}$, after Queen Garnet plum juice supplementation; PJ$_{PRE}$, before prune juice supplementation; PJ$_{POST}$, after prune juice supplementation; PBO$_{PRE}$, before placebo drink supplementation; PBO$_{POST}$, after placebo drink supplementation.**

Increased hippuric acid levels after supplementation with PJ are in accordance with results reported by others: the consumption of prunes (Toromanovic et al., 2008), green and black tea (Henning et al., 2013; Mulder, Rietveld, & van Amelsvoort, 2005), cider (DuPont et al., 2002), dried cranberry juice (Valentova et al., 2007), and lingonberries (Lehtonen et al., 2013) resulted in increased concentrations of hippuric acid, mainly in urine. The significant increase is most likely a direct consequence of the concentrations of typical hippuric acid precursors such as phenolic acids and other simple aromatic acids commonly more abundant in prunes and prune juice than in plum juice (Toromanovic et al., 2008). Prior and colleagues investigated in healthy human subjects the metabolic fate of 5-hydroxymethyl-2-furfural (a typical component in dried plums/prune juice)

following the consumption of prune juice and could identify several urinary glycine conjugates such as N-(5-hydroxymethyl-2-furoyl) glycine. However, hippuric acid was not mentioned as a potential *in vivo* metabolite of 5-hydroxymethyl-2-furfural (Prior et al., 2006). Therefore, our results support the hypothesis to use hippuric acid as a potential biomarker of total polyphenol consumption.

The potential anti-thrombotic mechanism of anthocyanins was investigated by Yang et al. (2012) using delphinidin-3-glucoside which has an identical chemical structure to cyanidin-3-glucoside, except an additional OH-group in 5′-position of the B-ring (Kay, Mazza, & Holub, 2005). The authors concluded that delphinidin-3-glucoside exhibits its anti-thrombotic activity via the inhibition of the expression of P-selectin, CD63 and CD40L, a down-regulation of active integrin αIIbβ3, attenuation of fibrinogen binding, and reduction in phosphorylation of adenosine monophosphate-activated protein kinase. Due to their similar chemical (flavonoid) structure and metabolic fate *in vivo*, cyanidin-3-glucoside and rutinoside, as the major QGPJ anthocyanins, may exhibit the same *in vivo* mode of action as was observed for delphinidin-3-glucoside. Alternatively cyanidin metabolites may be converted to active pelargonidin metabolites via xenobiotic and colonic bacterial action (Kalt, Liu, McDonald, Vinqvist-Tymchuk, & Fillmore, 2014). We also believe that the hydroxylation, methoxylation and the O-diphenyl structure in the B-ring of anthocyanins by virtue of its antioxidant and antiradical activity are instrumental in blocking the P2Y$_1$/P2Y$_{12}$ ADP receptor and blunting the activation of the αIIbβ3 integrin, evident from the reduction in platelet aggregation and P-selectin down-regulation demonstrated in this trial.

Despite rapid uptake of anthocyanins, including from the gastric mucosa, the overall bioavailability of anthocyanins and their derivatives has been considered low due to corresponding rapid elimination from the blood stream mainly in the first 24 h (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014). According to a survey of 97 bioavailability studies (Manach, Williamson, Morand, Scalbert, & Remesy, 2005) the average time taken to reach maximum anthocyanin concentrations in plasma was in the range of 0.75–4 h, between 1 and 6 h in urine and quercetin glycosides were found to be excreted within 6.4 h. Anthocyanins and their derivatives therefore only reach low concentrations in the plasma although enterohepatic cycling may result in more persistent occurrence in the urine beyond 5 days (Kalt et al., 2014). Due to the rapid absorption and elimination of most anthocyanins and other polyphenols post-supplementation, the 2 weeks of wash out used in this study was considered suitable to avoid interference from previous supplementations and confirmed by the different results between the treatment groups despite the cross design of the trial.

Not withstanding the favourable effects of anthocyanin-rich QGPJ on platelet activation, aggregation and coagulation profile, further studies are needed to better understand the mechanism(s) of action. The observed anti-thrombotic activity, which we believe could be due to the anthocyanin content solely from the juice, might also be due to the additional polyphenols and other antioxidants present but not analysed in the QGPJ or their *in vivo* metabolites. In addition, this study demonstrates anti-thrombotic activity exhibited by the acute

ingestion of 200 mL of QGPJ for 4 weeks which is approximately a large glass per day. It is unclear whether the same favourable anti-thrombotic effect would be observed under prolonged administration or with other intake volumes and more dose–response information is needed to make dietary recommendations for consumption to moderate thrombotic risk.

## 5.     Conclusion

This study has demonstrated that consumption of anthocyanin-rich QGPJ resulted in simultaneous targeting of different platelet aggregation pathways, inhibition of activation dependent platelet surface receptor, and amelioration of pro-coagulability, whilst also reducing biomarkers of oxidative stress. The observed anti-platelet properties of anthocyanin-rich QGPJ in contrast to prune juice are promising for reducing thrombotic risk and larger clinical trials evaluating the effect of QGPJ in pro-thrombotic populations are warranted.

## Conflict of interest

The authors of this paper declare no conflict of interest.

## Acknowledgements

REFERENCES

Alvarez-Suarez, J. M., Giampieri, F., Tulipani, S., Casoli, T., Di Stefano, G., Gonzalez-Paramas, A. M., Santos-Buelga, C., Busco, F., Quiles, J. L., Cordero, M. D., Bompadre, S., Mezzetti, B., & Battino, M. (2014). One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular risk, oxidative stress markers and platelet activation in humans. *The Journal of Nutritional Biochemistry*, 25, 289–294.
Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Coleman, R., Hayek, T., Presser, D., & Fuhrman, B. (2000). Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: Studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *The American Journal of Clinical Nutrition*, 71, 1062–1076.
Bobrich, A., Fanning, K. J., Rychlik, M., Russell, D., Topp, B., & Netzel, M. (2014). Phytochemicals in Japanese plums: Impact of maturity and bioaccessibility. *Food Research International*, 65, 20–26.
Castilla, P., Davalos, A., Teruel, J. L., Cerrato, F., Fernandez-Lucas, M., Merino, J. L., Sanchez-Martin, C. C., Ortuno, J., & Lasuncion,

M. A. (2008). Comparative effects of dietary supplementation with red grape juice and vitamin E on production of superoxide by circulating neutrophil NADPH oxidase in hemodialysis patients. *The American Journal of Clinical Nutrition*, 87, 1053–1061.
Coughlin, S. R. (2000). Thrombin signalling and protease-activated receptors. *Nature*, 407, 258–264.
Donovan, J. L., Meyer, A. S., & Waterhouse, A. L. (1998). Phenolic composition and antioxidant activity of prunes and prune juice (Prunus domestica). *Journal of Agricultural and Food Chemistry*, 46, 1247–1252.
Dorsam, R. T., & Kunapuli, S. P. (2004). Central role of the P2Y12 receptor in platelet activation. *The Journal of Clinical Investigation*, 113, 340–345.
DuPont, M. S., Bennett, R. N., Mellon, F. A., & Williamson, G. (2002). Polyphenols from alcoholic apple cider are absorbed, metabolized and excreted by humans. *The Journal of Nutrition*, 132, 172–175.
Fernandes, I., Faria, A., Calhau, C., de Freitas, V., & Mateus, N. (2014). Bioavailability of anthocyanins and derivatives. *Journal of Functional Foods*, 7, 54–66.
Frank, T., Netzel, M., Strass, G., Bitsch, R., & Bitsch, I. (2003). Bioavailability of anthocyanidin-3-glucosides following consumption of red wine and red grape juice. *Canadian Journal of Physiology and Pharmacology*, 81, 423–435.
Freedman, J. E., Parker, C., 3rd, Li, L., Perlman, J. A., Frei, B., Ivanov, V., Deak, L. R., Iafrati, M. D., & Folts, J. D. (2001). Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. *Circulation*, 103, 2792–2798.
Goh, C., Churilov, L., Mitchell, P., Dowling, R., & Yan, B. (2013). Clopidogrel hyper-response and bleeding risk in neurointerventional procedures. *AJNR. American Journal of Neuroradiology*, 34, 721–726.
Guerrero, J. A., Navarro-Nunez, L., Lozano, M. L., Martinez, C., Vicente, V., Gibbins, J. M., & Rivera, J. (2007). Flavonoids inhibit the platelet TxA(2) signalling pathway and antagonize TxA(2) receptors (TP) in platelets and smooth muscle cells. *British Journal of Clinical Pharmacology*, 64, 133–144.
Henning, S. M., Wang, P. W., Abgaryan, N., Vicinanza, R., de Oliveira, D. M., Zhang, Y. J., Lee, R. P., Carpenter, C. L., Aronson, W. J., & Heber, D. (2013). Phenolic acid concentrations in plasma and urine from men consuming green or black tea and potential chemopreventive properties for colon cancer. *Molecular Nutrition & Food Research*, 57, 483–492.
Kalt, W., Liu, Y., McDonald, J. E., Vinqvist-Tymchuk, M. R., & Fillmore, S. A. (2014). Anthocyanin metabolites are abundant and persistent in human urine. *Journal of Agricultural and Food Chemistry*, 62, 3926–3934.
Kay, C. D., Mazza, G. J., & Holub, B. J. (2005). Anthocyanins exist in the circulation primarily as metabolites in adult men. *The Journal of Nutrition*, 135, 2582–2588.
Kehrel, B., Wierwille, S., Clemetson, K. J., Anders, O., Steiner, M., Knight, C. G., Farndale, R. W., Okuma, M., & Barnes, M. J. (1998). Glycoprotein VI is a major collagen receptor for platelet activation: It recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. *Blood*, 91, 491–499.
Khurram, Z., Chou, E., Minutello, R., Bergman, G., Parikh, M., Naidu, S., Wong, S. C., & Hong, M. K. (2006). Combination therapy with aspirin, clopidogrel and warfarin following coronary stenting is associated with a significant risk of bleeding. *The Journal of Invasive Cardiology*, 18, 162–164.
Konic-Ristic, A., Srdic-Rajic, T., Kardum, N., Aleksic-Velickovic, V., Kroon, P. A., Hollands, W. J., Needs, P. W., Boyko, N., Hayran, O., Jorjadze, M., & Glibetic, M. (2013). Effects of bioactive-rich extracts of pomegranate, persimmon, nettle, dill, kale and Sideritis and isolated bioactives on arachidonic acid induced

markers of platelet activation and aggregation. *Journal of the Science of Food and Agriculture, 93*, 3581–3587.

Kubota, K., Horai, Y., Kushida, K., & Ishizaki, T. (1988). Determination of benzoic acid and hippuric acid in human plasma and urine by high-performance liquid chromatography. *Journal of Chromatography, 425*, 67–75.

Lehtonen, H. M., Lindstedt, A., Jarvinen, R., Sinkkonen, J., Graca, G., Viitanen, M., Kallio, H., & Gil, A. M. (2013). H-1 NMR-based metabolic fingerprinting of urine metabolites after consumption of lingonberries (Vaccinium vitis-idaea) with a high-fat meal. *Food Chemistry, 138*, 982–990.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition, 81*, 230S–242S.

McKay, D. L., Chen, C. Y., Yeum, K. J., Matthan, N. R., Lichtenstein, A. H., & Blumberg, J. B. (2010). Chronic and acute effects of walnuts on antioxidant capacity and nutritional status in humans: A randomized, cross-over pilot study. *Nutrition Journal, 9*, 21.

Mulder, T. P., Rietveld, A. G., & van Amelsvoort, J. M. (2005). Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine. *The American Journal of Clinical Nutrition, 81*, 256S–260S.

Murphy, K. J., Chronopoulos, A. K., Singh, I., Francis, M. A., Moriarty, H., Pike, M. J., Turner, A. H., Mann, N. J., & Sinclair, A. J. (2003). Dietary flavanols and procyanidin oligomers from cocoa (Theobroma cacao) inhibit platelet function. *The American Journal of Clinical Nutrition, 77*, 1466–1473.

Natella, F., Macone, A., Ramberti, A., Forte, M., Mattivi, F., Matarese, R. M., & Scaccini, C. (2011). Red wine prevents the postprandial increase in plasma cholesterol oxidation products: A pilot study. *The British Journal of Nutrition, 105*, 1718–1723.

Netzel, M., Fanning, K., Netzel, G., Zabaras, D., Karagianis, G., Treloar, T., Russell, D., & Stanley, R. (2012). Urinary excretion of antioxidants in healthy humans following queen garnet plum juice ingestion: A new plum variety rich in antioxidant compounds. *Journal of Food Biochemistry, 36*, 159–170.

Netzel, M., Strass, G., Kaul, C., Bitsch, I., Dietrich, H., & Bitsch, R. (2002). In vivo antioxidative capacity of a composite berry juice. *Food Research International, 35*, 213–216.

Ostertag, L. M., O'Kennedy, N., Horgan, G. W., Kroon, P. A., Duthie, G. G., & de Roos, B. (2011). In vitro anti-platelet effects of simple plant-derived phenolic compounds are only found at high, non-physiological concentrations. *Molecular Nutrition and Food Research, 55*, 1624–1636.

Petricevic, M., Biocina, B., Konosic, S., Burcar, I., Siric, F., Mihaljevic, M. Z., Ivancan, V., Svetina, L., & Gasparovic, H. (2013). Definition of acetylsalicylic acid resistance using whole blood impedance aggregometry in patients undergoing coronary artery surgery. *Collegium Antropologicum, 37*, 833–839.

Pilando, L. S., & Wrolstad, R. E. (1992). Compositional profiles of fruit juice concentrates and sweeteners. *Food Chemistry, 44*, 19–27.

Pojer, E., Mattivi, F., Johnson, D., & Stockley, C. S. (2013). The case for anthocyanin consumption to promote human health: A review. *Comprehensive Reviews in Food Science and Food Safety, 12*, 483–508.

Prior, R. L., Wu, X. L., & Gu, L. W. (2006). Identification and urinary excretion of metabolites of 5-(hydroxymethyl)-2-furfural in human subjects following consumption of dried plums or dried plum juice. *Journal of Agricultural and Food Chemistry, 54*, 3744–3749.

Rechner, A. R., & Kroner, C. (2005). Anthocyanins and colonic metabolites of dietary polyphenols inhibit platelet function. *Thrombosis Research, 116*, 327–334.

Rein, D., Paglieroni, T. G., Pearson, D. A., Wun, T., Schmitz, H. H., Gosselin, R., & Keen, C. L. (2000). Cocoa and wine polyphenols modulate platelet activation and function. *The Journal of Nutrition, 130*, 2120S–2126S.

Rivera, J., Lozano, M. L., Navarro-Nunez, L., & Vicente, V. (2009). Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica, 94*, 700–711.

Santhakumar, A. B., Bulmer, A. C., & Singh, I. (2013). A review of the mechanisms and effectiveness of dietary polyphenols in reducing oxidative stress and thrombotic risk. *Journal of Human Nutrition and Dietetics, 27*, 1–21.

Santhakumar, A. B., Fozzard, N., Perkins, A. V., & Singh, I. (2013). The synergistic effect of taurine and caffeine on platelet activity and hemostatic function. *Food and Public Health, 3*, 147–153.

Santhakumar, A. B., Linden, M. D., & Singh, I. (2012). Taurine in lower concentration attenuates platelet activity. *Food and Public Health, 2*, 58–64.

Sarria, B., Mateos, R., Sierra-Cinos, J. L., Goya, L., Garcia-Diz, L., & Bravo, L. (2012). Hypotensive, hypoglycaemic and antioxidant effects of consuming a cocoa product in moderately hypercholesterolemic humans. *Food & Function, 3*, 867–874.

Semenov, A. V., Kogan-Ponomarev, M., Ruda, M., Komarov, A. L., Panchenko, E. P., Chazova, I. E., & Mazurov, A. V. (2000). Soluble P-selectin – A marker of platelet activation and vessel wall injury: Increase of soluble P-selectin in plasma of patients with myocardial infarction, massive atherosclerosis and primary pulmonary hypertension. *Terapevticheskii Arkhiv, 72*, 15–20.

Siddiqui, T. I., Kumar, K. S. A., & Dikshit, D. K. (2013). Platelets and atherothrombosis: Causes, targets and treatments for thrombosis. *Current Medicinal Chemistry, 20*, 2779–2797.

Sikora, J., Markowicz-Piasecka, M., Broncel, M., & Mikiciuk-Olasik, E. (2014). Extract of Aronia melanocarpa-modified hemostasis: In vitro studies. *European Journal of Nutrition, 53*, 1493–1502.

Singh, I., Mok, M., Christensen, A. M., Turner, A. H., & Hawley, J. A. (2008). The effects of polyphenols in olive leaves on platelet function. *Nutrition, Metabolism, and Cardiovascular Diseases, 18*, 127–132.

Singh, I., Quinn, H., Mok, M., Southgate, R. J., Turner, A. H., Li, D., Sinclair, A. J., & Hawley, J. A. (2006). The effect of exercise and training status on platelet activation: Do cocoa polyphenols play a role? *Platelets, 17*, 361–367.

Singh, I., Turner, A. H., Sinclair, A. J., Li, D., & Hawley, J. A. (2007). Effects of gamma-tocopherol supplementation on thrombotic risk factors. *Asia Pacific Journal of Clinical Nutrition, 16*, 422–428.

The Royal College of Pathologists of Australasia (2004). *RCPA manual*. Royal College of Pathologists of Australasia.

Toromanovic, J., Kovac-Besovic, E., Sapcanin, A., Tahirovic, I., Rimpapa, Z., Kroyer, G., & Sofic, E. (2008). Urinary hippuric acid after ingestion of edible fruits. *Bosnian Journal of Basic Medical Sciences / Udruženje basičnih mediciniskih znanosti = Association of Basic Medical Sciences, 8*, 38–43.

Valentova, K., Stejskal, D., Bednar, P., Vostalova, J., Cihalik, C., Vecerova, R., Koukalova, D., Kolar, M., Reichenbach, R., Sknouril, L., Ulrichova, J., & Simanek, V. (2007). Biosafety, antioxidant status, and metabolites in urine after consumption of dried cranberry juice in healthy women: A pilot double-blind placebo-controlled trial. *Journal of Agricultural and Food Chemistry, 55*, 3217–3224.

Vangorsel, H., Li, C. Y., Kerbel, E. L., Smits, M., & Kader, A. A. (1992). Compositional characterization of prune juice. *Journal of Agricultural and Food Chemistry, 40*, 784–789.

Vucinic, L., Singh, I., Spargo, F. J., Hawley, J. A., & Linden, M. D. (2010). Gamma tocopherol supplementation prevents exercise induced coagulation and platelet aggregation. *Thrombosis Research, 125*, 196–199.

Yang, Y., Shi, Z., Reheman, A., Jin, J. W., Li, C., Wang, Y., Andrews, M. C., Chen, P., Zhu, G., & Ling, W. (2012). Plant food delphinidin-3-glucoside significantly inhibits platelet activation and thrombosis: Novel protective roles against cardiovascular diseases. *PLoS ONE, 7*, e37323.

Yuan, L., Meng, L., Ma, W., Xiao, Z., Zhu, X., Feng, J. F., Yu, H., & Xiao, R. (2011). Impact of apple and grape juice consumption on the antioxidant status in healthy subjects. *International Journal of Food Sciences and Nutrition, 62*, 844–850.

Zamora-Ros, R., Knaze, V., Lujan-Barroso, L., Slimani, N., Romieu, I., Touillaud, M., Kaaks, R., Teucher, B., Mattiello, A., Grioni, S., Crowe, F., Boeing, H., Forster, J., Quiros, J. R., Molina, E., Huerta, J. M., Engeset, D., Skeie, G., Trichopoulou, A., Dilis, V., Tsiotas, K., Peeters, P. H., Khaw, K. T., Wareham, N., Bueno-de-Mesquita, B., Ocke, M. C., Olsen, A., Tjonneland, A., Tumino, R., Johansson, G., Johansson, I., Ardanaz, E., Sacerdote, C., Sonestedt, E., Ericson, U., Clavel-Chapelon, F., Boutron-Ruault, M. C., Fagherazzi, G., Salvini, S., Amiano, P., Riboli, E., & Gonzalez, C. A. (2011). Estimation of the intake of anthocyanidins and their food sources in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *The British Journal of Nutrition, 106*, 1090–1099.