The potential of anthocyanin-rich Queen Garnet plum juice supplementation in alleviating thrombotic...
The potential of anthocyanin-rich Queen Garnet plum juice supplementation in alleviating thrombotic risk under induced oxidative stress conditions

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ABSTRACT
Increased oxidant production in humans induces a number of thrombotic consequences; including platelet hyperactivity/aggregability, which could be countered through specifically developed functional foods. We sought to determine the antithrombotic properties of anthocyanin-rich Queen Garnet plum juice (QGPJ) supplementation with and without exercise-induced oxidative stress. Thirteen healthy participants were investigated in a randomised, double-blind, placebo-controlled, cross-over trial. Participants consumed 200 mL/day of QGPJ and placebo juice for 28-days, with treatments separated by a two-week wash-out period. Blood samples were collected at baseline and after 1 h of exercise (70% peak-O2 uptake) both before and after oral supplementation and evaluated for platelet function and haemostatic activity. QGPJ supplementation inhibited adenosine diphosphate-induced platelet aggregation both without and under exercise-induced oxidative stress by 10.7% (P < 0.01) and 12.7% (P < 0.001) respectively; arachidonic acid-induced aggregation under oxidative stress by 28.8% (P < 0.05); reduced platelet activation-dependant P-selectin expression by 32.9% (P < 0.01) and 38.7% (P < 0.001) both without and under oxidative stress respectively; and exhibited favourable effects on coagulation parameters both with and without oxidative stress. The anti-thrombotic activity exhibited by anthocyanin-rich QGPJ suggests a potential for cardiovascular disease risk reduction and may be considered as complementary anti-platelet nutritional therapy in pro-thrombotic population.

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Abbreviations: ADP, adenosine diphosphate; ANOVA, analysis of variance; APC, allophycocyanin; aPTT, activated partial thromboplastin time; COX-1, cyclooxygenase-1; CVD, cardiovascular disease; C3GE, cyanidin-3-glucoside equivalents; FBC, full blood count; FITC, fluorescein isothiocyanate; GAE, gallic acid equivalents; HS-CRP, high sensitivity C-reactive protein; MFI, mean fluorescence intensity; MPV, mean platelet volume; ORAC, oxygen radical absorbance capacity; PBO, placebo; PPP, platelet poor plasma; PAC-1, procaspase-1; PRP, platelet rich plasma; PT, prothrombin time; QGE, quercetin glucoside equivalents; QGPJ, Queen Garnet plum juice; RNS, reactive nitrogen species; ROS, reactive oxygen species; TF, tissue factor; TRAP, thrombin receptor-activating peptide; TxA2, thromboxane A2; VO2peak, peak oxygen uptake

Chemical compounds: Cyanidin-3-glucoside (PubChem CID: 441674); Cyanidin-3-rutinoside (PubChem CID: 44256715).

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1. Introduction

Platelet hyperactivity and hyper-aggregability have been associated with a number of thrombotic, cardiovascular and metabolic conditions (Natarajan, Zaman, & Marshall, 2008; Santhakumar, Bulmer, & Singh, 2014). Oxidative stress is a hallmark of many of these disease states, where reactive oxygen (ROS) and nitrogen species (RNS) can cause endothelial injury and trigger the progression of atherosclerosis (Vazzana, Ranalli, Cuccurullo, & Davi, 2012). Injury to the vascular endothelium leads to concurrent stimulation of platelets by agonists resulting in platelet granule release, conformational change and activation of receptors, thereby attracting fibrinogen to form a thrombus (Rivera, Lozano, Navarro-Nunez, & Vicente, 2009). Platelet antagonists, such as aspirin and clopidogrel inhibit platelet aggregation or activation by effectively targeting such platelet activation receptors (Janssen & ten Berg, 2013; Santhakumar et al., 2014). In spite of the anti-thrombotic properties exhibited by such drugs, there is limited benefit in the primary prevention of cardiovascular disease (CVD) in oxidative stress related conditions such as diabetes. Potential reasons for the failure to produce significant therapeutic responses could be hyper-insulinaemia, hyper-glycaemia and increased free radical production related to diabetes (De Berardis et al., 2009). Other instances of resistance and nonresponsiveness to current anti-platelet therapeutics have also been reported (Gasparyan, Watson, & Lip, 2008; Khurram et al., 2006; Petrićević et al., 2013).

Various natural dietary derived polyphenols have been known to mimic the action of current anti-platelet drugs by inhibiting platelet aggregation and platelet activation pathways. A recent study by Alvarez-Suarez et al. (2014) demonstrated anti-thrombotic properties of anthocyanin-rich strawberry supplementation in healthy subjects, backing the results of prior studies on strawberry (Naemura et al., 2006) and mulberry (Yamamoto et al., 2006), where the occurrence of high anthocyanin content cultivars correlated to antithrombotic effects, both in vitro and in vivo. Many other dietary intervention studies have demonstrated a similar effect of anthocyanins in ameliorating platelet hyper-activation (Yang et al., 2011, 2012). Recent reviews have also focussed on the cardio-protective ability of these natural plant polyphenols by reducing the risk of thrombosis and platelet hyper-activity (Fuentes & Palomo, 2013; McEwen, 2014; Norberto et al., 2013; Santhakumar et al., 2014).

Although dietary intervention trials have demonstrated the potential of dietary derived polyphenols in ameliorating platelet activation, it is important to evaluate the mechanistic action of the phenolics by targeting simultaneous platelet-activation pathways in a pro-thrombotic environment.

The aim of this study was to investigate the effect and potential nutritional therapy application of a new variety of anthocyanin-rich Queen Garnet plum juice (QGPJ) supplementation on platelet and haemostatic activity in a healthy population with and without induced oxidative stress. We intend to mimic physiological oxidative stress as seen in conditions such as diabetes (Giacco & Brownlee, 2010) by means of constant-load exercise tests at an intensity equivalent to 70% of peak O2 uptake (VO2peak). We hypothesise that anthocyanin-rich QGPJ may impart anti-thrombotic effects via (a) inhibition of platelet aggregation by simultaneously targeting different platelet activation pathways (adenosine diphosphate: ADP-P2Y1/P2Y12; collagen: GPVI/αIIβ3 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.

2. Materials and methods

2.1. Study participants and experimental design

The study was approved by the Griffith University Human Research Ethics Committee, Griffith University, Queensland, Australia (GU Protocol Number MSC/02/12/HREC) and registered at the Australian New Zealand Clinical Trials Registry (ACTRN12612000674831). Thirteen healthy adults (7 men and 6 women) were recruited from the local community and provided written, informed consent. Participants were healthy, non-smokers, with no history of cardiovascular or metabolic disease, and not taking daily health supplements, and anti-platelet or anti-inflammatory medications during and at least 2 weeks prior to the study. Dietary antioxidant and food frequency questionnaires were administered and used to exclude participants consuming a high antioxidant diet. Baseline full blood counts (FBC), biochemical profile, body mass index (BMI) and blood pressure were within normal reference ranges established by The Royal College of Pathologists of Australasia (2004) for all participants (Table 1). Biochemical profile and FBC was performed using a Cobas Integra 400® plus biochemistry analyser (Roche Diagnostics, Basel, Switzerland) and Coulter® Ac.T™ 5diff CP haematology analyser (Beckman Coulter, Inc., Lake Cove, NSW, Australia), respectively.

A randomised, double-blind, placebo-controlled, crossover study design was utilised. After initial screening, participants were randomly assigned into two different supplement groups – anthocyanin-rich Queen Garnet plum juice (QGPJ) or a flavoured and coloured formulated cordial placebo (PBO) as...
previously described (Santhakumar et al., 2015). Randomisation was performed by a statistician who worked independently to the study investigators, by assigning arbitrary computer-generated numerical codes to each volunteer. Identical, one-litre juice supplementation bottles labelled with respective participant codes and juice codes (A and B) were used. Six litres of respective juice supplements and a graduated measuring cylinder were provided to each volunteer before each random supplementation bout. During the study, participants supplemented their usual diet with 200 mL/day of either QGPJ or PBO for 28 days. Compliance with consumption of juice was recorded by measuring the amount of juice remaining in the last bottle at the end of the supplementation period. Dietary intake was also monitored during the supplementation period. Dietary intake was recorded by measuring the amount of juice remaining in the last bottle at the end of the supplementation period. Dietary intake was also monitored during the supplementation period. Dietary intake was recorded by measuring the amount of juice remaining in the last bottle at the end of the supplementation period.

Fig. 1 – Study design. QGPJ, Queen-Garnet plum juice; PBO, sugar and colour matched placebo; PT, prothrombin time; aPTT, activated partial thromboplastin time; FBC, full blood count; CVD, cardiovascular disease; MI, myocardial infarction; AOX, antioxidant; CRP, C-reactive protein.

2.2. Blood sample collection

Venous blood, 25 mL, was collected by a trained phlebotomist, at least 8–12 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 (for FBC) followed by trisodium citrate (28.12 g/L concentration) tubes (for platelet aggregation, surface marker expression and coagulation assays) to avoid risk of collecting platelets activated by venepuncture. Blood was also collected into serum separator tubes for biochemical
analysis. Care was taken to ensure minimal specimen handling and agitation.

2.3. Induced oxidative stress – exercise tests

Participants were required to refrain from caffeinated beverages, alcohol and strenuous exercise for 24 h before each exercise test to avoid interference of caffeine or alcohol with the exercise output. All exercise tests were performed at the same time in the morning in an environmentally stable laboratory (ambient temperature: -21 °C, relative humidity: 45-55%). Prior to commencing the constant-load exercise tests designed to induce oxidative stress, all participants completed an incremental exercise test to determine peak exercise values on a separate day, followed by a familiarisation trial to determine the power outputs used for the subsequent constant-load exercise tests.

2.3.1. Incremental exercise test

The incremental exercise test was performed on an electronically-braked cycle ergometer (Corival, Lode B.V., Groningen, The Netherlands), and commenced with 3 min of unloaded cycling at a self-selected pedal cadence between 70 and 90 rpm. Thereafter, the power output was increased by 15–25 W·min⁻¹, with the pre-selected increment rate dependant on each participant’s habitual exercise history and gender. Participants were instructed to maintain their preferred cadence for the duration of the test. The incremental exercise test was terminated when participants were unable to consistently maintain a pedal cadence within 5 rpm of their preferred cadence despite strong verbal encouragement. During the test, pulmonary gas exchange was measured breath-by-breath using a calibrated metabolic cart (Ultima CardiO2, MGC Diagnostics, St Paul, MN, USA). Cardiac rhythm was monitored continuously, and heart rate recorded beat-by-beat, with a 12-lead electrocardiogram (ECG) system (X-Scribe, Mortara Instrument Inc., Milwaukee, WI, USA). Gas exchange and heart rate values were averaged over 30 s intervals and peak exercise values recorded as the highest interval observed during the final stages of the incremental exercise test.

2.3.2. Familiarisation trial

The constant-load exercise tests were performed at an intensity equivalent to 70% of VO2peak. Before commencing the first constant-load exercise test, participants returned to the laboratory no earlier than 48 h after the incremental test to perform a familiarisation trial where the constant-load work-rate was determined. During the trial, pulmonary gas exchange and heart rate were measured as described for the incremental exercise test and averaged, in real time, over 60 s intervals. The familiarisation trial commenced with 6 min of exercise at a power output calculated to elicit ~70% of VO2peak, based on published metabolic equations (Gibbons et al., 1997). The power output was then adjusted at 6 min intervals until the O2 uptake was used for all the subsequent constant-load exercise tests.

2.3.3. Constant-load exercise tests

The constant-load exercise tests comprised 60 min of exercise performed at an intensity equivalent to 70% of VO2peak (i.e. at the power output determined during the familiarisation trial). Each test was preceded by 3 min of unloaded cycling and concluded with an active cool down for 6 min. All the constant-load exercise tests were performed on the same cycle ergometer used during the incremental exercise test and familiarisation trial. Cardiac rhythm was monitored via a 5-lead ECG (X-Scribe, Mortara Instrument Inc.) and heart rate recorded every 10 min during the test. Participants were permitted to consume water ad libitum during exercise.

2.4. Juice supplements

The juice supplements, QGPJ and PBO were prepared and processed as described previously (Santhakumar et al., 2015). QGPJ was previously analysed for polyphenol content: cyanidin-3-glucoside - 76 mg cyanidin-3-glucoside equivalents (C3GE)/100 mL, cyanidin-3-rutinoside - 25 mg C3GE/100 mL, quercetin derivatives – 43.7 mg quercetin glucoside equivalents (QGE)/100 mL, and total phenolic content – 322 mg gallic acid equivalents (GAE)/100 mL. The total phenolic content for PBO was previously measured as 7 mg GAE/100 mL (Santhakumar et al., 2015).

2.5. Platelet function and coagulation profile analyses

The platelet aggregation assay was carried out using the AggRAM® turbidometric aggregation analyser (Helena Laboratories, Beaumont, TX, USA) as described previously (Santhakumar et al., 2015). Platelet aggregation response to adenine diphosphate (ADP), collagen and arachidonic acid agonists was evaluated. Optimal agonist concentrations used in platelet aggregation analysis were established using dose response curves performed in our previous preliminary in vitro trials (Santhakumar, Fozzard, Perkins, & Singh, 2013; Santhakumar, Linden, & Singh, 2012). All agonists were purchased from Helena Laboratories.

Activation-dependant platelet surface-marker expression analysis was performed and analysed using the BD LSFRFortessa cell analyser (BD Biosciences, North Ryde, NSW, Australia) and BD FACSDiva software (version 6.1.3, BD Biosciences, North Ryde, NSW, Australia), respectively. Platelet activity was evaluated using activation-dependant monoclonal antibodies, procaspase-1 (PAC-1) conjugated to fluorescein isothiocyanate (FITC) and P-selectin/CD62P conjugated to allophycocyanin (APC) as described previously (Santhakumar et al., 2015). The antibodies and their respective isotype controls were purchased from BD Biosciences (North Ryde, NSW, Australia). Expression of activated platelets bound to PAC-1 and P-selectin was articulated as mean fluorescence intensity (MFI).

The coagulation profile comprising prothrombin clotting time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration were evaluated using the C4 coagulation analyser (Helena Laboratories) based on the C4-coagulation analyser operator’s manual (Helena Laboratories). All reagents and controls were purchased from Helena Laboratories.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA). A
repeated-measures analysis of variance (ANOVA) with Newman–Keuls post-test was carried out. A minimum sample size of 12 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. 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 ± standard deviation (SD).

3. Results

3.1. Exercise induced oxidative stress

The mean \( V_{\text{O2peak}} \) attained during the incremental exercise test was \( 2.22 ± 0.17 \text{ L·min}^{-1} \) or \( 31.9 \text{ mL·kg}^{-1}·\text{min}^{-1} \) (97 ± 5% age-predicted), and peak power output achieved was 207 ± 14 W. The mean power output used during the constant-load exercise tests was 102 ± 9 W and elicited a mean \( V_{\text{O2}} \) of 1.56 L·min\(^{-1}\) (69.9 ± 0.5% \( V_{\text{O2peak}} \)). All participants were able to complete 60 min of exercise during the constant-load exercise tests. The average heart rates attained during constant-load exercise were not significantly different between test conditions (Table 2).

3.2. Ex vivo analysis

Baseline pre exercise blood cell counts, biochemical profile and inflammation marker – high sensitivity C-reactive protein (HS-CRP) remained within normal reference ranges for all volunteers post supplementation with QGPJ and PBO. Exercise-induced increase in haemoglobin, haematocrit, white blood cell (WBC) and red blood cell (RBC) counts were observed post exercise (Table 3). Significant changes to biochemical parameters such as HS-CRP, total cholesterol, high density lipoprotein, low density lipoprotein, triacylglycerol, uric acid and glucose were not observed post QGPJ or PBO supplementation (Table 3). No statistically significant differences in parameters tested between males and females were observed.

Fig. 2 illustrates the change in percentage platelet aggregation after supplementation with QGPJ or PBO, both with and without oxidative stress. ADP-induced platelet aggregation was reduced at baseline (i.e., without exercise-induced oxidative stress).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PREex, before exercise</th>
<th>POSTex, after exercise</th>
<th>PREsupp, before beverage supplementation</th>
<th>POSTsupp, after beverage supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/L)</td>
<td>142 ± 10</td>
<td>142 ± 10</td>
<td>142 ± 10</td>
<td>142 ± 10</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>4.8 ± 0.4</td>
<td>5.0 ± 0.4</td>
<td>5.1 ± 0.4</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>RBC (10(^9)/L)</td>
<td>7.2 ± 1.4</td>
<td>7.3 ± 1.4</td>
<td>7.3 ± 1.4</td>
<td>7.3 ± 1.4</td>
</tr>
<tr>
<td>WBC (10(^9)/L)</td>
<td>8.6 ± 0.6</td>
<td>8.5 ± 0.6</td>
<td>8.5 ± 0.6</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>Platelet (10(^9)/L)</td>
<td>228 ± 16</td>
<td>228 ± 16</td>
<td>228 ± 16</td>
<td>228 ± 16</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>9.1 ± 0.2</td>
<td>9.1 ± 0.2</td>
<td>9.1 ± 0.2</td>
<td>9.1 ± 0.2</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.40 ± 0.40</td>
<td>4.24 ± 0.40</td>
<td>4.13 ± 0.40</td>
<td>4.13 ± 0.40</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.46 ± 0.46</td>
<td>1.47 ± 0.47</td>
<td>1.47 ± 0.47</td>
<td>1.47 ± 0.47</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.48 ± 0.48</td>
<td>2.48 ± 0.48</td>
<td>2.48 ± 0.48</td>
<td>2.48 ± 0.48</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.9 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>Uric acid (µmol/L)</td>
<td>299.2 ± 83.5</td>
<td>269.7 ± 76.1</td>
<td>274.7 ± 80.9</td>
<td>274.7 ± 80.9</td>
</tr>
<tr>
<td>HS-CRP (mg/L)</td>
<td>0.85 ± 0.76</td>
<td>0.83 ± 0.76</td>
<td>0.83 ± 0.76</td>
<td>0.83 ± 0.76</td>
</tr>
</tbody>
</table>

Table 2 - Average heart rate values attained during the constant-load exercise trials.

<table>
<thead>
<tr>
<th></th>
<th>Heart rate (beats·min(^{-1}))</th>
<th>Heart rate (%HR(_{\text{MAX}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBO(_{\text{PRE}})</td>
<td>144 ± 4</td>
<td>81.2 ± 1.7</td>
</tr>
<tr>
<td>PBO(_{\text{POST}})</td>
<td>143 ± 3</td>
<td>80.6 ± 1.2</td>
</tr>
<tr>
<td>QGPJ(_{\text{PRE}})</td>
<td>142 ± 3</td>
<td>80.3 ± 1.3</td>
</tr>
<tr>
<td>QGPJ(_{\text{POST}})</td>
<td>143 ± 3</td>
<td>80.7 ± 1.5</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

PBO\(_{\text{PRE}}\), before placebo supplementation; PBO\(_{\text{POST}}\), after 4 week placebo supplementation; QGPJ\(_{\text{PRE}}\), before Queen Garnet plum juice supplementation; QGPJ\(_{\text{POST}}\), after 4-week Queen Garnet plum juice supplementation.
stress) by 10.7% (P = 0.002), and with exercise-induced oxidative stress by 12.7% (P < 0.001) (Fig. 2A). Arachidonic acid-induced aggregation was inhibited only during oxidative stress by 28.8% (P = 0.030) (Fig. 2B). Collagen-induced platelet aggregation was not inhibited by QGPJ supplementation (data not shown). Placebo juice supplementation did not affect platelet aggregation induced by any of the platelet agonists with or without oxidative stress.

Expression of activation-dependant platelet surface marker, P-selectin, which is secreted in α-granules of activated, de-granulated platelets, was inhibited by 32.9% (P = 0.002) after QGPJ supplementation (Fig. 3). Furthermore, this inhibition of expression was greater (38.7%, P < 0.001) following exercise-induced oxidative stress post supplementation (Fig. 3). PAC-1 surface marker, which binds to activated platelets with a conformational change, was not affected after QGPJ supplementation with or without oxidative stress. P-selectin or PAC-1 expression was unaltered after PBO supplementation.

Prothrombin clotting time (PT), which reflects the extrinsic coagulation pathway, was prolonged by 1.3 s without oxidative stress (P = 0.021) and by 1.5 s under oxidative stress (P = 0.008) conditions after QGPJ supplementation (Fig. 4A). Activated partial thromboplastin clotting time (aPTT), denoting the intrinsic pathway of coagulation, did not change post QGPJ supplementation. PBO supplementation did not alter the intrinsic or extrinsic pathways of coagulation.

Fibrinogen concentration decreased by 92.18 mg/dL (QGP<sub>PRE</sub> = 427.0 mg/dL and QGP<sub>POST</sub> = 334.8 mg/dL) and 78.29 mg/dL (QGP<sub>PRE</sub> = 380.3 mg/dL and QGP<sub>POST</sub> = 302.0 mg/dL) post QGPJ supplementation before (P = 0.023) and after oxidative stress (P = 0.005), respectively, but was unaltered after PBO supplementation (Fig. 4B).
In the past, our group has demonstrated several dietary intervention trials that have emphasised the cardio-protective and anti-platelet properties of polyphenols and antioxidants (Kundur, Bulmer, & Singh, 2014; Murphy et al., 2003; Santhakumar et al., 2012, 2013; Singh et al., 2006; Vucinic, Singh, Spargo, Hawley, & Linden, 2010). Our recent review also elucidates the possible mechanisms of action of various polyphenols in inhibiting platelet activity (Santhakumar et al., 2014). Recently, we have studied the potential of anthocyanin-rich QGPJ supplementation in healthy volunteers in inhibiting platelet aggregation, activation and reducing markers of oxidative stress (Santhakumar et al., 2015). The present study demonstrates the physiological translation of anti-thrombotic properties exhibited by dietary phenolics/antioxidants in an oxidative stress environment. Our approach was to evaluate the potential of anthocyanin-rich QGPJ in reducing pro-thrombotic markers both in the presence and absence of oxidative stress. Sudden exercise performed by sedentary subjects at 70% of their maximum oxygen uptake assisted in inducing an acute physiological oxidative stress which could mimic free radical production in diabetic or obese populations, consequently increasing platelet activity (Singh et al., 2006; Torzi-Ciancarelli, Penco, & Di Massimo, 2002). Evaluating QGPJ’s effect under such pro-thrombotic conditions helps to translate its probable role in reducing thrombotic risk under high oxidant conditions and its capability as a natural complementary anti-platelet therapy for individuals non-responsive or resistant to current anti-platelet drugs.

The effect of QGPJ supplementation on platelet aggregation shows that natural polyphenols, especially anthocyanins in QGPJ, have the potential to simultaneously target different pathways of platelet intracellular signalling and activation. Anti-platelet aggregatory properties of anthocyanins have previously been found by a few in vitro and ex vivo studies (Rechner & Kroner, 2005; Yang et al., 2011), in addition to the role of an anthocyanin in reducing oxidative stress (Yiannakopoulou, 2013), lipid profile (Hursel & Westerterp-Plantenga, 2013), inflammation (Malaguti, Angeloni, & Hrelia, 2013). In contrast, Shanmuganayagam and colleagues have demonstrated an increase in platelet aggregation induced by collagen after in vitro addition of anthocyanins and other phenolic fractions (Shanmuganayagam et al., 2012). The presence of high anthocyanin and antioxidant content in a strawberry cultivar (Naemura et al., 2005, 2006) and in a mulberry cultivar (Yamamoto et al., 2006) was correlated to shear-induced in vitro platelet reactivity, being more countered by thrombolysis activity both in vitro and in vivo.

Various anti-platelet therapeutics act on the P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub> ADP receptor (clopidogrel, ticlopidine and cangrelol) and the COX-1
pathway (aspirin) of platelet activation (Kei, Florentin, Mikhailidis, Elisa, & Liberopoulos, 2011; Rivera et al., 2009). In spite of aspirin’s favourable effects in blocking the formation of TxA2 in the COX-1 pathway, it was observed that this drug does not exert an inhibitory effect on in vitro thrombus formation in high shear stress conditions (Barstad et al., 1996) or in an in vivo model of arterial stenosis (Maalej & Polts, 1996). Several instances of aspirin resistance in diabetic populations (Angiullillo & Suryadevara, 2009; Tasdemir, Toptas, Demir, Esen, & Atmaca, 2014) and inefficacy in reducing oxidative stress and vascular inflammation (Raghavan, Laight, & Cummings, 2014) have also been demonstrated. The potential mechanisms of resistance include increased platelet activity due to decreased endothelial nitric oxide production, increased activity and levels of prothrombotic clotting factors, and hyperglycaemia (Ajjan, Storey, & Grant, 2008). The reduction in arachidonic acid-stimulated platelet aggregation by targeting the COX-1 pathway of activation and inhibition of P.Y., of P.Y., ADP receptors under oxidative stress suggests that QGPJ may confer natural anti-thrombotic effects and prevention of recurrent ischaemic events in diabetic and obese individuals resistant/non-responsive to COX-1 inhibitors or other antiplatelet drugs. The effect on collagen-stimulated platelet aggregation, observed post QGPJ supplementation, was in agreement with an in vitro study by Garcia et al. (2004) where anthocyanins, even at very high concentrations of up to 50 μM, did not inhibit collagen-stimulated platelet aggregation. Although the effect of QGPJ supplementation on platelet aggregation was not dramatic, anthocyanins serve an important complementary therapeutic role to currently used antiplatelet drugs, particularly in alleviating platelet hyperactivity in patients non-responsive to such drugs. It should also be stressed that the inhibitory effect of QGPJ supplementation, on platelet aggregation was in response to strong platelet agonists, and contrasts with in vitro trials which have not exhibited platelet sensitisation to strong exogenous activators (Rechner & Kroner, 2005). We also believe that this irreversible anti-thrombotic activity exhibited by anthocyanins in QGPJ could be due to prolonged elevation in antioxidant response similar to the effect observed after consumption of 90 tart cherries consistently increasing the Trolox equivalent antioxidant capacity over 12 hours (Seymour et al., 2014). This consistent antioxidant potential could directly or indirectly be responsible for irreversible platelet activation surface receptor inhibition thereby reducing the total platelet aggregation response to agonists. The present study is the first to show inhibition of platelet aggregation by anthocyanin-rich natural juice supplements, induced by strong platelet stimulants under physiological oxidative stress conditions. We evaluated the effect of QGPJ supplementation on the surface-marker expression of the fibrinogen binding site, exposed by a conformational change in the GPIIb–IIIa complex and α-granule secretion in ADP-induced activated platelets. It was observed that supplementation with QGPJ did not inhibit the expression of PAC-1 surface markers (recognises platelet activation related conformational change), having no effect on the initial phase of platelet activation involving the GPIIb–IIIa receptor. Rein et al. (2000) also observed a similar effect in epinephrine-induced PAC-1 binding after dietary intervention with de-alcoholised red wine. The role of QGP polyphenols in the inhibition of activation-related platelet degranulation is evident from the reduction in the expression of the α-granule membrane protein, P-selectin. Furthermore, it was observed that this inhibition in expression of ADP-induced P-selectin expression was greater after exercise-induced oxidative stress. This reduced expression of P-selectin is in agreement with an in vitro study performed by Ostertag et al. (2011) which demonstrated inhibition of thrombin receptor-activating peptide (TRAP) induced P-selectin expression by a variety of phenolics, but at non-physiological concentrations. Furthermore, Rechner and Kroner (2005) demonstrated an inhibition of TRAP-induced P-selectin expression of platelets under hydrogen peroxide-induced oxidative stress by a mixture of phenolic compounds. Due to the inhibition in the expression of P-selectin following ADP stimulation, we believe that platelets could have been desensitised either by polyphenols blocking platelet receptors or by interfering with signal transduction. Yang et al. (2012) investigated the effect of delphinidin-3-glucoside, having a structure highly related to cyanidin-3-glucoside, on human and murine models of platelet activation, aggregation and thrombus growth. The authors observed that delphinidin-3-glucoside alleviated platelet activation and aggregation by reducing the phosphorylation of adenosine monophosphate-activated protein kinase. Due to the similar structure to delphinidin-3-glucoside, we believe that cyanidin-3-glucoside and rutinoside may exhibit similar in vivo mode of action. Methoxylation, hydroxylation and the B-ring O-diphenyl structure of anthocyanins could also be an important contributor in blocking the P.Y., ADP platelet activation receptor and the GPIIb–IIIa receptor, evident from the inhibition in platelet aggregation and P-selectin expression seen in this study.

The observed prolongation in prothrombin clotting times suggests a favourable effect of QGPJ supplementation on the extrinsic pathway of coagulation. Note that this increase in clotting time was well within normal reference ranges, suggesting no bleeding risk. We believe that polyphenols in QGPJ trigger the inhibition of the tissue factor (TF) thereby down-regulating the extrinsic pathway. This was reinforced by Carrieri et al. (2013), who demonstrated the ability of grape skin extracts, rich in quercetin and cyanidin, in inhibiting TF synthesis in whole blood as well as isolated mononuclear cells. The observed decrease in plasma fibrinogen concentration after QGPJ supplementation signifies a decrease in pro-thrombotic progression. This finding indicates that natural polyphenols in QGP could have the potency to alleviate high plasma fibrinogen levels seen in conditions such as CVD or in any form of inflammatory condition. Although the exact mechanism of alleviation of fibrinogen levels in plasma is not clear, it is believed that polyphenols rich in procyanidins and anthocyanins could blunt fibrinogen/fibrin synthesis by inhibiting the amyloidotic activity of thrombin (Sikora, Markowicz-Piasecka, Broncel, & Mikicik-Olasik, 2014).

A previous pilot trial has demonstrated that consumption of a single dose of 400 mL QGPJ resulted in the appearance of both native QGPJ anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside) and at least four identified methylated and glucuronidated anthocyanin metabolites (cyanidin monoglucuronide, peonidin monoglucuronide, peonidin-3-glucoside and peonidin-3-rutinoside) in urine being...
approximately 0.5% of the ingested dose (Netzel et al., 2012). Our previous dietary intervention trial also evidenced an increase in hippuric acid, a potential active metabolite of polyphenol intake, concentration in both plasma and urine of 20 healthy volunteers after consumption of 200 mL/day of QGPJ for 28 days (Santhakumar et al., 2015). Due to the rapid absorption, elimination and low bioavailability of anthocyanins and other phenolic compounds (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Williamson & Manach, 2005) in fruit juice supplements, a two-week wash out was considered suitable to avoid interference from previous juice supplementations. This was further confirmed by the baseline results of the tested parameters over the crossovers.

Despite the present study exhibiting a favourable anti-thrombotic effect of acute consumption (200 mL a day for 28 days) of antioxidant-rich QGPJ on platelet function and haemostatic parameters, it is not certain if a similar effect can be observed after prolonged administration or consumption in larger volumes. Although markers of oxidative stress before and after exercise were not measured, we have previously established that prolonged (60 min) constant-load exercise at 70% of VO2peak consistently induces oxidative stress in healthy individuals (Singh et al., 2006; Vucinic et al., 2010). Several other studies in the literature also strongly support the claim that exercise at 70% of VO2peak induces oxidative stress in healthy individuals. Bloomer, Davis, Consitt, and Wideman (2007) have demonstrated that cycling at 70% of VO2peak significantly elevated protein carbonyl concentration, a result of oxidant attack, for at least 60 min post exercise (Bloomer et al., 2007). In another double-blind cross-over trial by Medved et al. (2004) cycling at 71% of VO2peak for 45 min resulted in reduced levels of muscle and blood glutathione compared to pre-exercise samples. Changes in blood parameters such as increase in haematocrit, haemoglobin and WBC count (Table 3) also strongly support our claim that the exercise protocol used in our study induced oxidative stress. A slight increased trend observed in platelet activation marker, P-selectin expression, platelet aggregation induced by ADP, and collagen is also suggestive of pro-oxidant–antioxidant homeostasis disturbances resulting in increased platelet coagulability/activation (Freedman, 2008).

5. Conclusion

This study has demonstrated that anthocyanin-rich QGPJ consumption has the potential to target different platelet activation pathways and alleviate fibrinogen binding to platelet surface receptors, thus reducing further platelet recruitment for aggregation and inhibiting platelet degranulation. Due to its ability to target various pathways of platelet activation under oxidant stress, nutritional supplementation with anthocyanin-rich QGPJ may have the potential to substitute or complement currently used anti-platelet drugs in patient populations resistant or nonresponsive to pharmacological antiplatelet therapy. Further research on the effects of QGPJ supplementation in pro-thrombotic conditions such as diabetic and obese population is also warranted.

Conflict of interest

The authors of this manuscript have no conflicts of interest to declare.

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