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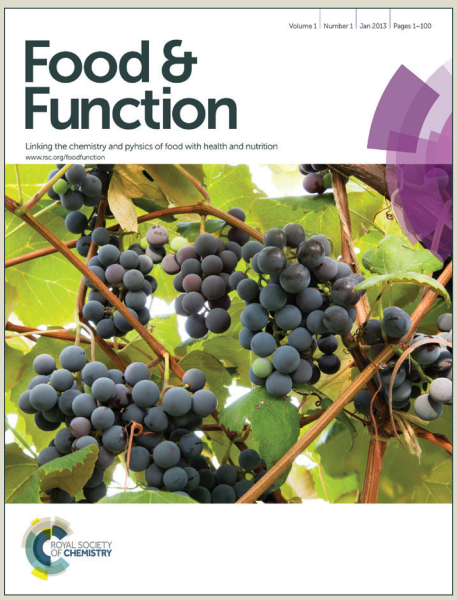

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The *ex vivo* antiplatelet activation potential of fruit phenolic metabolite hippuric acid

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Abstract

Polyphenol-rich fruit and vegetable intake has been associated with reduction in platelet hyperactivity, a significant contributor to thrombus formation. This study was undertaken to investigate the possible role of hippuric acid, a predominant metabolite of plant cyclic polyols, phenolic acids and polyphenols, in reduction of platelet activation-related thrombogenesis. Fasting blood samples were collected from 13 healthy subjects to analyse the effect of varying concentrations of hippuric acid (100 μM, 200 μM, 500 μM, 1 mM and 2 mM) on activation-dependant platelet surface-marker expression. Procaspase activating compound-1 (PAC-1) and P-selectin/CD62P monoclonal antibodies were used to evaluate platelet activation-related conformational changes and α-granule release respectively using flow cytometry. Platelets were stimulated *ex vivo* via the P_2Y_1/P_2Y_{12} – adenosine diphosphate (ADP) pathway of platelet activation. Hippuric acid at a concentration of 1 mM and 2 mM significantly reduced P-selectin/CD62P expression (p=0.03 and p<0.001 respectively) induced by ADP. Hippuric acid at 2 mM concentration also inhibited PAC-1 activation-dependant antibody expression (p=0.03). High *ex vivo* concentrations of hippuric acid can therefore significantly reduce P-selectin and PAC-1 expression thus reducing platelet activation and clotting potential. However, although up to 11 mM of hippuric acid can be excreted in the urine per day following consumption of fruit, hippuric acid is actively excreted with a recorded C_{max} for hippuric acid in human plasma at 250-300 μM. This is lower than the blood concentration of 1-2 mM shown to be bioactive in this research. The contribution of hippuric acid to the protective effects of fruit and vegetable intake against vascular disorders by the pathways measured is therefore low but could be synergistic with lowered doses of antiplatelet drugs and help reduce risk of thrombosis in current antiplatelet drug sensitive populations.

Keywords: Platelet activation, polyphenol, hippuric acid, thrombosis, P-selectin

INTRODUCTION

According to a recent World Health Organisation report, 17.5 million people died from cardiovascular disease in 2012, representing 31% of all global deaths [1]. Amongst several risk factors including smoking, hypertension, genetic association and obesity, poor nutrition has also been one of the important contributors. Several studies have reported the benefits of a healthy diet of fruit and vegetables in reducing the risk of vascular disorders [2-5]. Interest in plant derived antioxidants and polyphenols such as anthocyanins and quercetin continues to grow as a result of their reported antithrombotic and cardiovascular benefits [6-13]. Analysis of the pharmacokinetics and bioavailability of plant derived compounds after polyphenol/anthocyanin rich food intake has shown that the concentrations of anthocyanins and structurally related metabolites only reach micro-molar concentrations *in vivo* (less than 5 μM) [14]. However hippuric acid is a major metabolite post polyphenol consumption that is excreted in urine in up to millimolar concentrations *in vivo* [15, 16]. Consumption of prunes [16], black and green tea solids [17], cranberry juice [18], red wine, grape juice [19], and fruits/vegetables in general [15] have been shown to increase urinary hippuric acid levels. According to a dietary intervention study by Toromanovic and colleagues [16], urinary excretion of levels of hippuric acid reached up to 11 mM per 24 hours after consumption of 300 g of edible fruits [16]. The increased levels are believed to be due to plant derived simple phenolic compounds and the microbial degradation of anthocyanins, chlorogenic acids and quinic acid in the colon and their catabolism to benzoid type compounds [20-22]. In spite of hippuric acid being a consistent biomarker/metabolite that can be excreted via the urine in grams per day, there are no well-documented cardiovascular benefits associated with it.

Several *in vitro* and *in vivo* trials have demonstrated the prospective bioactivity of natural dietary phenolics rich in antioxidants, such as anthocyanins [6, 23, 24] and quercetin derivatives [25] in reducing thrombotic risk by alleviating platelet hyperactivity/aggregation.

Hippuric acid, however, occurs in very high concentrations *in vivo* versus very low levels of most other phenolics [26]. Similar to the action of current anti-platelet drugs such as aspirin and clopidogrel, polyphenols have been demonstrated to block platelet activation pathways (cyclooxygenase-1 – COX-1 and P_2Y_1/P_2Y_{12}) consequently reducing the risk of thrombosis [9, 27, 28]. We hypothesize that, active metabolites occurring *in vivo* at high concentration such as hippuric acid, could also be partially responsible for the claimed anti-platelet properties of plant derived phenolic acids and polyphenols.

This *ex vivo* trial aimed to evaluate the effect of hippuric acid, a potential active metabolite, in targeting the P_2Y_1/P_2Y_{12} pathway of platelet activation. We have analysed the effect of various concentrations of the metabolite on activation-dependant platelet surface receptor P_2Y_1/P_2Y_{12} induced by adenosine diphosphate (ADP), a physiological platelet activator, by evaluating the expression of platelet activation-related conformation change and degranulation.

MATERIALS AND METHODS

Subject recruitment and sample collection

The study protocol was approved by the Griffith University Human Research ethics committee (GU Protocol no. MSC/13/11/HREC) and was performed in compliance with the relevant laws and institutional guidelines. Thirteen healthy subjects were recruited from the general population by means of advertisements placed around Griffith University, Gold Coast campus. Subjects were healthy, non-smokers with no history of metabolic or cardiovascular diseases and were not on any anti-inflammatory, anti-platelet medications or health supplements at least 7 days prior to blood collection. Informed consent was obtained from all

volunteers prior to commencement of the study. Fasting whole blood samples were collected from the median cubital vein by a trained phlebotomist at the School of Medical Science, Griffith University. Care was taken to ensure minimal sample handling or agitation to prevent platelet activation. No samples were obtained from traumatic phlebotomy procedures or contained obvious clots. Blood was drawn into tri-potassium EDTA (1.8 mg/mL) anticoagulant tubes (used for full blood examination - FBE) before collection into tri-sodium citrate (28.1 g/mL) tubes (used for platelet surface-marker expression) to avoid risk of collecting venepuncture activated platelets. Baseline FBE and biochemical profile was carried out for initial subject screening and recruitment using a Coulter® Ac.T™ 5diff CP haematology analyser (Beckman Coulter, Inc., Lane Cove, NSW, Australia) and Cobas Integra 400® plus biochemistry analyser (Roche diagnostics, Basel, Switzerland) respectively.

Hippuric acid

Hippuric acid used in the analysis was purchased from Sigma-Aldrich, Australia. Working solutions of 100 μM, 200 μM, 500 μM, 1 mM and 2 mM were prepared from a stock solution of 20 mM concentration diluted in 1x concentration of phosphate buffered saline (PBS). Aliquots of different concentrations of hippuric acid were stored at 4ºC for a maximum of 2 weeks.

Activation dependant platelet surface-marker expression

Venous blood collected into tri-sodium citrate tubes were used to evaluate platelet surface marker 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. Platelet activation dependant monoclonal antibodies (mAb) were used to evaluate the expression of positive activated platelets induced by ADP agonist. CD42b-peridinin chlorophyll protein

(CD42b-PerCp-Cy5.5) conjugated mAb was used to identify the GPIb-IX receptors, the most abundant receptor on the platelet population. Platelet activity was assessed by activation-dependant platelet surface marker expression using PAC-1-fluorescein isothiocyanate (PAC-1-FITC recognises activated conformational changes in the fibrinogen binding receptor GPIIb-IIIa) and P-selectin/CD62P- (expressed in activated de-granulated platelets) conjugated mAb. The antibodies and their respective isotype controls were purchased form BD Biosciences (BD Biosciences, North Ryde, NSW, Australia).

Whole blood was incubated with respective concentrations of hippuric acid and control (PBS) for 15 min at room temperature (25ºC). After incubation, the samples were then diluted in filtered modified tyrode's buffer (pH 7.2) and incubated with CD42b, PAC-1, P-selectin mAb's for 15 mins 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 stimulate the platelets via the P_2Y_{12}/P_2Y_1 platelet activation pathway. 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 flowcytometer for antibody expression. The setup and optimal fluorescence compensation of the flowcytometer was validated using BD Cytometer Setup and Tracking beads and BD CompBead compensation particles (BD Biosciences, North Ryde, NSW, Australia). Ten thousand platelet events were acquired, gated on the basis of light scatter and CD42b MAb expression. Activation dependant-MAb (PAC-1 and P-selectin) expression by activated platelets was articulated as mean fluorescence intensity (MFI).

Statistical analysis

A two-way ANOVA following Tukey's post comparison test was performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, California, USA). A minimum sample size of 12 subjects in each group is required for 80% power to detect a 5% variation in the laboratory parameters measured, where a 3-5% standard deviation exists in the

149 population, assuming an alpha error of 0.05. All the data is expressed as mean ± standard
150 deviation. Differences between the groups were considered to be significant when $p<0.05$.
151 Any significant statistical interactions were included in the analysis where applicable.
152

153 **RESULTS**

154 The baseline full blood cell counts, inflammation marker and biochemical profile of the
155 subjects under study were within normal reference ranges (Table 1) as established by the
156 Royal College of Pathologists Australasia [29].

157 It was observed that whole blood *ex vivo* treatment with 1 mM and 2 mM
158 concentrations of hippuric acid reduced ADP-induced P-selectin/CD62P expression by 7.5%
159 ($p=0.03$) and 14% ($p<0.001$) respectively (Figure 1). Lower concentrations of hippuric acid
160 (100 µM, 200 µM or 500 µM) did not have a statistically significant effect on P-selectin
161 platelet surface marker expression but demonstrated a trend in inhibition of the activation-
162 dependant monoclonal antibody expression (Figure 1).

163 Hippuric acid at a concentration of 2 mM inhibited the initial platelet activation phase,
164 by 11% ($p=0.03$), involving its conformational change targeting the GPIIb-IIIa receptor,
165 evident from PAC-1 activation-dependant monoclonal antibody expression (Figure 2). Lower
166 concentrations of hippuric acid did not affect PAC-1 expression.
167

168 **DISCUSSION**

169 In this trial we have demonstrated the potential of hippuric acid in alleviating platelet
170 activation stimulated by an exogenous agonist, ADP, which is responsible for thrombus
171 formation and platelet adhesion *in vivo*. This potent platelet stimulant was used to activate

resting platelets *ex vivo* and target the P_2Y_1/P_2Y_{12} receptor of platelet activation. Similar to the action of the anti-platelet drug clopidogrel, i) hippuric acid at 1 mM and 2 mM concentrations reduces P-selectin/CD62P mAb expression indicating its ability to inhibit platelet degranulation consequently platelet activation ii) 2 mM concentration of hippuric acid reduces PAC-1 mAb expression therefore suppressing the initial phase of activation – GPIIb-IIIa platelet receptor-related conformational change induced by ADP platelet agonist. However we did not observe inhibition of platelet activation by lower concentrations of hippuric acid in relation to the expression of activation-dependant conformational change or degranulation markers.

In platelet related thrombogenesis, platelets adhere to damaged endothelium; undergo a conformational change followed by activation and degranulation [30]. This activation results in the binding of fibrinogen to platelet surface receptors consequently leading to thrombus formation [30]. Current antiplatelet drugs target different receptors on the surface of platelets or their activation pathways in order to reduce platelet hyper-activation or hyper-aggregability. For example, aspirin, the most commonly used antiplatelet drug and salicylate derived from fruit and vegetables in the diet [31], inhibit platelet activity by targeting the cyclooxygenase-1 (COX-1) enzyme hence blocking the production of thromboxane A_2 (TxA_2), a pro-thrombotic agent [32]. Furthermore, clopidogrel binds irreversibly to the P_2Y_1/P_2Y_{12}-ADP platelet surface-receptor consequently reducing ADP-induced platelet activation [33]. In the current *ex vivo* trial it was observed that 1 mM and 2 mM concentrations of hippuric acid efficiently target the P_2Y_1/P_2Y_{12} platelet surface-receptor induced by ADP. In an *in vitro* study performed by Ostertag and colleagues, 100 µM hippuric acid did not inhibit collagen induced P-selectin expression but reduced platelet aggregation [26]. Furthermore, Rechner et al. demonstrated that lower concentrations of hippuric acid (10 µM) did not exhibit an inhibitory effect on thrombin receptor activating peptide induced P-selectin expression in an *in vitro* trial

evaluating the effect of anthocyanins and colonic metabolites of polyphenols on platelet function [23]. The action of hippuric acid on platelet activation inhibition could also potentially be due to the internalization and metabolism by enzymes in platelets capable of modifying molecules by the addition of a methyl, sulphate or a glucorinide group. These functional groups, conjugated to polyphenols or their metabolites within the platelets, might impact the regulation of activation [34]. Our results are in agreement with the above *in vitro* trials, where low concentrations of hippuric acid did not effectively block platelet activation dependant receptors. Studies had not previously been extended into the higher concentrations of 1-2 mM. Consumption of a 300 g portion of blueberries, cranberries and prunes resulted in excretion of 11 mM hippuric in 24 hours and other fruits such as raspberry, melon and blackberry resulting in excretions of 5-6 mM hippuric in 24 hours peaking at 10-15 hours [16]. Children and adolescents eating fruit and vegetable averaged 2 mM hippuric acid per day in urine [15]. Since this millimolar excretion of hippuric acid occurs via the portal circulation it is believed that high concentrations could also be detected in plasma post polyphenol rich fruit or vegetable consumption. Hippuric acid, following a bolus dose of benzoic acid, has a recorded maximum concentration (C_{max}) in human plasma of 250-300 μM with elimination of hippuric acid occurring at a first-order rate or in a non -saturable manner with the maximum urinary excretion rates of hippuric acid independent of the dose size [35]. Serum hippuric acid concentrations were in the range 1.1 – 6.1 mM when 5g (28 mM) hippuric acid was bolus dosed for antibacterial effects [36]. While the *in vivo* levels of hippuric acid in plasma that can be reached following fruit consumption have not been tested they are not likely to reach the recorded C_{max} of 250-300 μM but could be higher than the 100 μM hippuric acid shown by Ostertag and colleagues to reduce platelet aggregation *in vitro* [26]. Although several dietary intervention and *in vivo* studies have shown the mechanism of inhibition of platelet function and anti-clotting activity by polyphenols, the prospective of active metabolites such hippuric

222 acid being a possible reason for the observed anti-thrombotic effect has not been
223 demonstrated. We have shown the potential of the metabolite in reducing platelet activation
224 related conformational change and inhibition of α-granule release although at a level at least
225 three times higher than what is currently known to occur *in vivo* as a result of fruit and
226 vegetable consumption.

227 Microbial degradation of polyphenols especially anthocyanins and quinic acid or
228 chlorogenic acid in the colon, followed by hepatic conjugation with glycine, is believed to be
229 responsible for the observed increased levels of hippuric acid [20, 21]. It has also been suggested
230 that majority of the hippuric acid seen in urine is due to synthesis from quinic acid present in
231 antioxidant rich fruits [21]. Ronald W. Pero recently proposed that quinic acid and its end
232 catabolite, hippuric acid may themselves not be efficacious but may lead to the production of
233 increased levels of nicotinamide and tryptophan as antioxidants through the shikimate
234 pathway [21]. The present study shows the anti-thrombotic effect of acute *in vitro* addition of
235 varying concentrations of hippuric acid to whole blood directly. It is unclear whether a
236 similar effect of active metabolites can be seen after prolonged consumption of large
237 quantities of polyphenol rich food.

238 In addition to increased levels of hippuric acid post consumption of a polyphenol rich
239 diet, it is also a metabolite of acetylsalicyclic acid (aspirin). The other major metabolites of
240 aspirin include salicyluric acid and gentisuric acid which have very similar structural moieties
241 as hippuric acid [37]. Acetylsalicylic acid is converted to salicylic acid and conjugates with
242 benzoic acid in the liver to result in the synthesis of hippuric acid. This pharmacokinetics of
243 acetylsalicylic acid might partly explain the reason behind the anti-thrombotic activity of its
244 potential metabolite, hippuric acid. Aspirin alleviates platelet activation by blocking the
245 COX-1 pathway resulting in reduced arachidonic acid production hence preventing
246 thromboxane synthesis consequently thrombus formation. Further mechanistic studies

247 evaluating the potential of hippuric acid in reducing arachidonic acid induced platelet
248 activation by blocking the COX-1 pathway of platelet activation is warranted.

249 It has also been demonstrated that thiol derivatives of antiplatelet drugs such as
250 clopidogrel and ticlopidine bind in a covalent manner to the P_2Y_1/P_2Y_{12} ADP receptor and
251 block the activation of platelets [38]. Hippuric acid at concentrations attained *in vivo* post
252 polyphenols rich food consumption could potentially help block the ADP receptor of
253 activation thereby mimicking the action of anti-platelet drugs. We hence believe that
254 consumption of polyphenols rich foods could be synergistic with lowered doses of
255 antiplatelet drugs and help platelet activation inhibition and thus reduce risk of thrombosis in
256 current antiplatelet drug sensitive populations.

257

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261

262 **CONFLICT OF INTEREST AND FUNDING DISCLOSURE**

263 The authors of this manuscript have no conflicts of interest to declare. This work was not
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265

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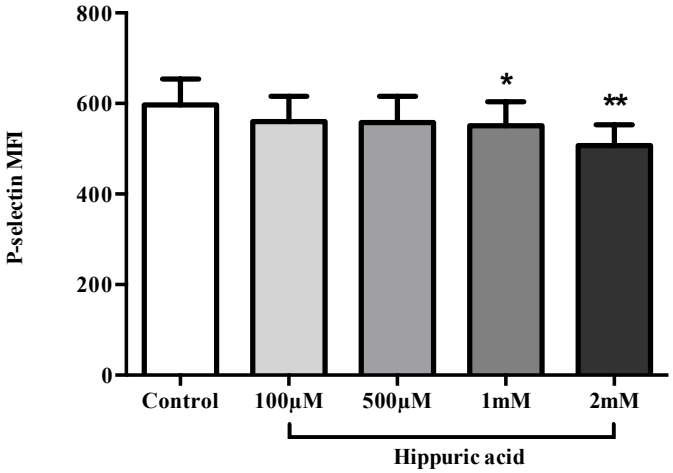

Figure 1: The effect of varying concentrations of hippuric acid on P-selectin/CD62P surface marker expression. Hippuric acid at 1 mM and 2 mM concentration inhibits P-selectin activation dependant monoclonal antibody expression consequently platelet de-granulation/α-granule release (↓45.9 ± 50.3, p=0.03 and ↓89.6 ± 74.6, p<0.001 respectively). N=13 and the data is represented as mean fluorescence intensity (MFI) versus hippuric acid concentrations. *signifies statistical significance p<0.05. **signifies greater statistical significance p<0.001. Data expressed as Mean ± SD.

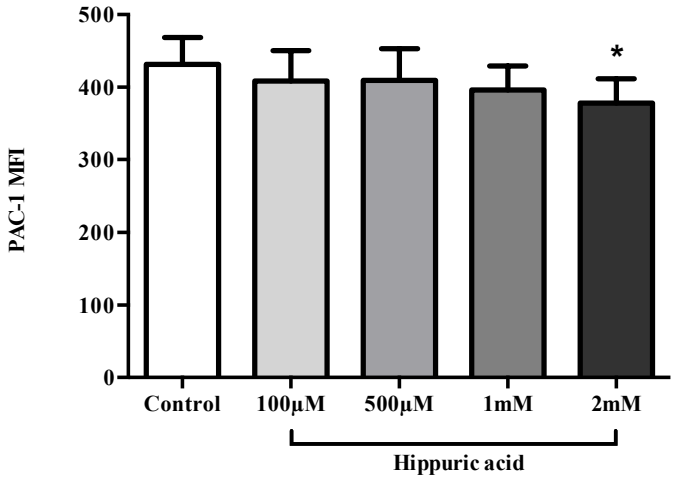

Figure 2: The effect of different concentrations of hippuric acid on PAC-1 activation-dependant surface marker expression. Hippuric acid at 2 mM concentration reduces activation-dependant conformational changes in platelets (↓53.0 ± 81.2, p=0.03). N=13 and the data is represented as mean fluorescence intensity (MFI) versus hippuric acid concentrations. *signifies statistical significance p<0.05. Data expressed as Mean ± SD.

373 **Table 1:** Baseline parameters of subjects under study

Parameters	Mean± SD	Reference ranges
n (M/F)	13	NA
Age (y)	30 ± 3	NA
Body mass (kg)	73 ± 8	NA
BMI (kg/m^2)	23.6 ± 0.7	20 – 25
Haemoglobin (g/L)	140 ± 10	120 – 180
Haematocrit (%)	42 ± 2	40 – 54
RBC (×10^{12}/L)	4.9 ± 0.4	3.8 – 6.5
WBC (×10^9/L)	6.2 ± 1.2	4.0 – 11.0
Platelet (×10^9/L)	217 ± 13	150 – 400
MPV (fL)	9.0 ± 0.9	7.5 – 11.5
Total cholesterol (mmol/L)	4.40 ± 0.81	3.10 – 6.50
HDL (mmol/L)	1.52 ± 0.32	> 1.0
Triglycerides (mmol/L)	0.85 ± 0.35	0.20 – 1.90
LDL (mmol/L)	2.49 ± 0.72	< 6.00
Glucose (mmol/L)	4.87 ± 0.62	3.0 – 6.0
Uric acid (μmol/L)	274.7 ± 75.5	200 – 430
HS-CRP (mg/L)	0.85 ± 0.76	0.0 – 6.0

374

375 BMI, body mass index; RBC, red blood cell; WBC, white blood cell; MPV, mean platelet
376 volume; HDL, high density lipoprotein; LDL, low density lipoprotein, HS-CRP, high
377 sensitivity C-reactive protein, NA, Not applicable.

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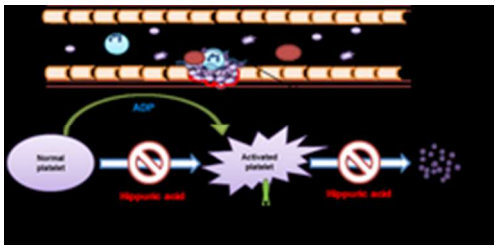

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