Food & Function

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

rsc.li/food-function

View Article Online
DOI: 10.1039/C6FO01404F

The colonic metabolites dihydrocaffeic acid and dihydroferulic acid are more effective inhibitors of *in vitro* platelet activation than their phenolic precursors

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DOI: 10.1039/C6FO01404F

Abstract

Cardiovascular diseases (CVD) are the major cause of morbidity and mortality worldwide. The consumption of healthy diets rich in polyphenols has been inversely associated with the development of CVD. This study evaluated the effects of green coffee bean (GCBE) and yerba mate (YMPE) phenolic extracts, the main phenolic and methylxanthines constituents (5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, caffeine, and theobromine), and their main metabolites (caffeic acid, ferulic acid, dihydrocaffeic acid -DHCA- and dihydroferulic acid -DHFA-) on platelet activation *in vitro*. Upon incubation with different doses (0.01 – 100 µg/mL or µM) of each compound, adenosine 5'-diphosphate-induced P-selectin expression and fibrinogen binding were determined using whole blood flow cytometry. Platelet P-selectin expression was significantly decreased by YMPE and all phenolic and methylxanthines constituents at physiological concentrations, compared with control, whereas fibrinogen binding on platelets was significantly increased. The colonic metabolites (DHCA and DHFA) had stronger inhibitory effects on P-selectin expression than their phenolic precursors, suggesting an increase in the efficacy to modulate platelet activation with the metabolism of the phenolic compounds.

Running title: Colonic metabolites of yerba mate inhibit *in vitro* platelet activation

Keywords: Green coffee; yerba mate; phenolic compounds; methylxanthines; metabolites; platelet activation.

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Introduction

Cardiovascular disease (CVD) is a main cause of mortality worldwide. The development of atherosclerosis and subsequent thrombus formation are believed to be the underlying reason of CVD.[1] Consumption of certain dietary compounds lowers the risk of CVD.[2,3,4] Indeed, consumption of various plant extract infusions was associated with cardioprotective effects in animal models and in humans.[5,6,7] One such compound is yerba mate, a popular infusion originating from South America prepared from the dried leaves of *Ilex paraguariensis*. Due to its perceived hypocholesterolemic, anti-oxidant and anti-obesity activity, consumption of yerba mate is now spreading around the world.[8] In addition, various studies have shown that moderate intake of coffee may have cardioprotective effects,[9,10,11] questioning the negative effects on vascular function traditionally associated with coffee consumption.

Yerba mate and coffee are a rich source of different bioactive compounds, especially cinnamoylquinic acids and methylxanthines (Figure 1). Cinnamoylquinic acids, collectively known as chlorogenic acids (CGA),[12] are a family of esters formed between quinic acid and one or more *trans*-cinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic or dimethoxycinnamic acid). Caffeoylquinic and dicaffeoylquinic acids isomers (Figure 1a) represent 80-90% of total CGA in green coffee and yerba mate.[13,14] Methylxanthines are natural purine alkaloids including caffeine, theophylline and theobromine (Figure 1b), with caffeine being the most abundant in both beverages.[15,16,17]

Physiological effects of dietary compounds are potentially limited by the bioavailability and biotransformation of their bioactive components in the organism. CGA are absorbed and metabolized in the stomach, small and large intestine (Figure 2), and their bioavailability depends on the ingested dose.[18] Plasma concentrations of 5-caffeoylquinic acid (5-CQA), the main CGA in green coffee and yerba mate, are low at 6 - 30 nM after consumption of roasted coffee,[18,19] and 5.9 μM after consumption of green coffee.[20] In addition, plasma levels of 3,5-dicaffeoylquinic acid (3,5-DCQA) have been reported to be as high as 2.5 μM after the intake of a green coffee extract,[20] although most studies suggested the hydrolysis of 3,5-DCQA to monoacylquinic acid as the main biotransformation pathway.[18,19] Caffeic (CA) and ferulic (FA) acids are the main early metabolites,

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with plasma levels from 0.08 to 1.1 and from 0.14 to 0.8 μM, respectively, after intake of roasted and green coffee.[20,21] Nevertheless, the main metabolites from CGA are dihydrocaffeic (DHCA) and dihydroferulic (DHFA) acids, produced by the microflora in the large intestine. These metabolites are found in plasma at levels up to 0.7 and 1 μM, respectively, 5 - 10 h after intake of 400 mL of coffee containing approximately 600 mg CGA.[19] On the contrary, caffeine is quickly and completely absorbed in the small intestine and transported to the liver where it is metabolized into the dimethylxanthines paraxanthine, theobromine and theophylline, which are further metabolized to monomethylxanthines.[22] Previous studies have shown that plasma levels of caffeine can increase to 2 - 12 μM, and theobromine can increase to 0.5 - 16 μM, after intake of 9.9 - 70 mg of caffeine and 0.2 - 84 mg of theobromine, respectively, which are present in 3.5 g of coffee and 15 g of cocoa.[23,24]

Roasting of green coffee beans causes significant degradation and/or transformation of polyphenols, affecting the physical and chemical properties of roasted coffee beans,[14,25,26] and causing a loss of its antioxidant [27] and anti-inflammatory capacity in animal models.[28] Moreover, the consumption of green coffee has been associated with a lower risk of cancer and CVD [29] and may therefore be a healthier alternative to roasted coffee.

One possible mechanism by which these dietary compounds may lower CVD risk is by modulation of platelet function.[30] Activated platelets are involved in the formation of blood clots to stop bleeding and heal wounds.[31] However, excessive platelet activation has been associated with both the physical blocking of blood vessels as well as the development of chronic inflammation. Therefore, platelet activation has been proposed as an independent risk factor of CVD.[32] Platelet function can be beneficially modulated by different dietary compounds, such as those found in garlic, onions, kiwi, olive oil, chocolate and mushrooms.[33,34,35,36,37] In this study we assessed the effects of green coffee bean (GCBE) and yerba mate (YMPE) phenolic extracts, the main methylxanthines and phenolic constituents, caffeine, theobromine, 5-CQA, 3,5-DCQA, including their main metabolites (CA, FA, DHCA and DHFA), on activation of human platelets *in vitro*.

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2. Material and methods

Reagents

Green coffee (*Coffea arabica* L. from Colombia) and yerba mate (*Ilex paraguariensis* L.) were purchased in a local supermarket in Madrid (Spain). 3,5-DCQA was acquired from PhytoLab (Vestenbergsgreuth, Germany). Caffeine was obtained from Fluka (Madrid, Spain). 5-CQA, CA, FA, theobromine, DHCA, DHFA were obtained from Sigma-Aldrich (Madrid, Spain). Phycoerythrin (PE)-conjugated mouse anti-human CD61 (CD61-PE), allophycocyanin (APC)-conjugated mouse anti-human P-selectin (CD62-APC), PE-conjugated mouse IgG1κ, APC-conjugated mouse IgG1κ, AF488-conjugated mouse IgG1κ, sodium chloride (NaCl) and FACS Flow sheath fluid were acquired from BD Biosciences (Oxford, UK). AF488-conjugated fibrinogen from human plasma was obtained from Fisher Scientific (Loughborough, UK). Adenosine 5`-diphosphate (ADP), phosphate-buffered saline (PBS), dimethylsulfoxide (DMSO), potassium chloride (KCl), magnesium sulfate heptahydrate ($MgSO_4$-$7H_2O$), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), phorbol 12-myristate 13-acetate (PMA) and quercetin were acquired from Sigma-Aldrich (Dorset, UK). All other chemicals were of analytical grade.

Green coffee bean (GCBE) and yerba mate (YMPE) phenolic extracts and pure compound preparation

Soluble phenolic compounds were prepared extracted in triplicate according to Bravo and Calixto[38] and characterized as previously described.[39] Briefly, 1 g of green coffee beans, previously ground and sieved to 0.5 μm particle size, and dried leaves of yerba mate, were washed once with 2 N hydrochloric acid in aqueous methanol (50:50, v/v, 1 h at room temperature, constant shaking) followed by acetone:water (70:30, v/v, 1 h at room temperature, constant shaking). After each extraction step, the samples were centrifuged (10 min, 3000 *g*) and the supernatants combined. The organic solvents were evaporated under reduced pressure with a rotavapor and the phenolic extracts were lyophilized.

The main phenolic and methylxanthine compounds of both extracts were analyzed as previously described[40,39] using a Superspher 100 RP18 column (4.6 x 250 mm, 4 μm; Agilent

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Technologies) and an Agilent 1200 series liquid chromatographic system equipped with an auto-sampler, quaternary pump, diode-array detector and quadrupole mass spectrometer (Agilent Technologies, Germany). Chromatographic analysis showed that the main constituents of both extracts were mono- and dicaffeoylquinic acids (80% and 8% of the total phenolic content of GCBE and 65% and 18% for YMPE, respectively). YMPE also contained up to 10% of feruloylquinic acids and other cinnamoylquinic acids, as well as over 9% of flavonol-glycosides. Caffeine was the major methylxanthine in both extracts (up to 95% of total alkaloids and less than 10% of total compounds).[40,39]

GCBE and YMPE powder and the pure standards were dissolved in 100% DMSO at 100 mg/mL and 100 mM, respectively, and diluted with PBS to prepare working dilutions for incubation with whole blood at 0.01, 0.1, 1, 10, 20, 50 and 100 µg/mL and µM (final concentration in 0.1% DMSO in blood).

Blood sample collection

Blood sampling for testing the *in vitro* effects of dietary compounds on platelet function was approved by the Rowett Human Studies Ethical Review Panel, Aberdeen, Scotland, United Kingdom and the experiments were carried out in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice. Eligible volunteers were 20 - 70 years old, healthy, non-smokers, not suffering from chronic pathologies, had abstained from any medication or dietary supplements that affect platelet function and had not given blood for transfusion purposes within the previous month. We included volunteers who had normal platelet function as assessed for previously executed *in vitro* platelet function experiments.

Venous blood samples were taken from a group of 15 healthy men and women after an informed consent was obtained as described previously.[41] Volunteers were allowed to continue their habitual diet but were asked to fast for at least 10 h before the blood sampling including to abstain tea and coffee consumption. Multiple blood donations were separated by at least four weeks to allow recovery of platelet counts. Blood samples were collected using a siliconized 21 gauge butterfly needle and closed s-monovette blood collection tubes containing 1 mL trisodium

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citrate (0.106 mol/L) as anticoagulant (Sarstedt, Beaumont Leys, UK) with the pressure of the tourniquet released before blood was drawn. The first 5 ml of blood were discarded.

Assessment of platelet activation markers by flow cytometry

P-selectin expression and fibrinogen binding was assessed with flow cytometry in diluted whole blood after pre-incubation with compounds on a 96-well plate as described previously with modifications. [42] Briefly, blood was diluted 1:10 in HEPES-Mg buffer and platelets were allowed to rest for 10 min before incubation with the compounds (final concentration 0.01, 0.1, 1, 10, 20, 50 and 100 µM for pure standards and µg/mL for GCBE and YMPE) for 10 min in duplicate. Platelet activation was subsequently initiated with 10 µM ADP (final concentration) for 10 min followed by incubation for 20 min with PE-CD61, APC-CD62 and AF488-fibrinogen. All incubations were done at room temperature. The reaction was stopped by addition of cold (4°) PBS and the samples were measured using a BD FACS Calibur (BD Biosciences, Oxford, UK) and an Automated Microsampling System (Cytek Development Inc., Ely, UK) within 6 h of sampling. 0.1% DMSO/PBS, 1 µM PMA and 10 and 25 µM quercetin in 0.1% DMSO/PBS final concentration were used as control, positive control marker in the flow cytometry and positive control of compounds, respectively. The layout of the 96-well plate was as follows: each compound was analyzed in columns with an individual control at the top of each set (with five sets per plate) whereas the duplicate of each compound/concentration combination was measured in rows. The order of the sets in which the compounds were measured but not the concentrations were randomized between plates. Acquisition of cells were stopped when 10000 cells were detected in the platelet gate or after 75 seconds whichever came first. In case of a time out, data were accepted as valid when more than 8000 platelet events per sample were detected.

Statistical analysis

GenStat version 13 (VSN International, UK) was employed for the statistical analysis of data. A mixed model was fitted using residual maximum likelihood (REML) without adjustment for missing values. Significance of treatment effects was tested by the Wald statistic, with estimated degrees of freedom in the denominator after treatment with compounds adjusted for control. Fixed effect

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terms were: plate, volunteer, set, compound and concentration. Volunteer, plate and set were defined as random effect terms. Data are presented as percentage of change of adjusted mean ± SED compared with control. The percentage of change was calculated as follows ((adjusted mean compound – adjusted mean control)/adjusted mean control)*100. The adjusted means and SED of adjusted means were calculated as part of the REML analysis. Results were considered significant when difference of adjusted mean with control was higher than 2 x standard error of difference (SED).

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Results

Effect on P-selectin expression

Incubation with 10, 20 and 50 µg/mL YMPE significantly decreased ADP-induced P-selectin expression by 10.5%, 8.5% and 6.0%, respectively compared with a 0.1% DMSO/PBS control. Contrary, incubation with 100 µg/mL YMPE and 50 and 100 µg/mL GCBE significantly increased ADP-induced P-selectin expression (Figure 3a and Table 1 from supporting information). Incubation with the mayor compounds present in yerba mate and green coffee beans (Figure 3a and Table 1 from supporting information) showed a high capacity to decrease ADP-induced P-selectin expression. Incubation with 1, 10 and 20 µM 5-CQA reduced ADP-induced P-selectin expression by 7.5%, 7.6% and 8.3%, respectively; 10 and 20 µM 3,5-DCQA decreased ADP-induced P-selectin expression by 8.0% and 6.7%, respectively; 20, 50 and 100 µM caffeine decreased ADP-induced P-selectin expression by 5.8%, 8.0% and 8.0%, respectively; and 1 to 100 µM theobromine decreased ADP-induced P-selectin expression by 6.4%, 11.3%, 12.9%, 8.9% and 8.2%, respectively. However incubation with 0.01 µM 5-CQA and 0.01 µM caffeine significantly increased ADP-induced platelet P-selectin expression by 7.2% and 6.3%, respectively compared with control. Finally, ADP-induced P-selectin expression, compared with 0.1% DMSO/PBS control, was significantly lower after incubation with the main metabolites from yerba mate and green coffee at different concentrations: 20 µM CA by 8.6%, 10 to 100 µM FA by 5.3%, 7.4%, 8.4% and 9.9%, 1 to 100 µM DHCA by 7.7%, 10.6%, 13.5%, 10.7% and 7.7%, and 10 and 20 µM DHFA by 6.3% and 10.6%, respectively. Contrary incubation with 0.01 µM FA significantly increased ADP-induced P-selectin by 6.3%.

Effect on fibrinogen binding

Fibrinogen binding was generally increased after incubation with compounds, compared with control (Figure 3b and Table 2 from supporting information). Incubation with YMPE, GCBE, 5-CQA and CA across all concentrations significantly increased ADP-induced fibrinogen binding from 3.4% after 10 µM 3,5-DCQA and 10 µM DHCA to 17.0% after 100 µg/ml GCBE.

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Discussion

In this study, we have shown that the crude phenolic extracts of green coffee beans and yerba mate, as well as their main compounds and metabolites, were effective in modulating agonist-induced platelet activation markers *in vitro*. Platelets play an essential role maintaining hemostasis upon vascular damage, recognizing exposed connective tissue components from endothelial cells, such as collagen or von Willebrand factor. P-selectin is one of the first molecules released from α–granules in platelets, so its expression on the surface is commonly used as an early marker for platelet activation.[31,43]

The intake of green coffee and yerba mate has been associated with a lower risk of CVD due to its anti-hypertensive effect and capacity to reduce blood viscosity.[44,45,46] Moreover, the results of this study demonstrated, for the first time, a potentially beneficial effect of YMPE at physiological concentrations on the modulation of platelet activation by reducing ADP-induced P-selectin expression. The anti-platelet effects of these compounds are similar to those shown for other bioactive plants, specifically fruits and vegetables with a high phenolic content, such as strawberry or grape. Such compounds inhibit ADP- and arachidonic acid-induced platelet aggregation at concentrations of 100 - 1000 μg/mL, and thrombin receptor activating peptide (TRAP)- and thrombin-induced platelet activation and aggregation at 1.2 - 50 μg/mL, respectively.[47,48,49] However, whilst these studies showed modulation of platelet function for a large range of concentrations, i.e. 1 to 1000 μg/mL, we found that ADP-induced P-selectin expression was increased rather than reduced when platelets were incubated with high concentrations of GCBE and YMPE, i.e. 50 and 100 μg/mL. Our results suggest that the beneficial effects of YMPE are only present at lower concentrations, which may be observed in the blood stream, and that higher, pro-oxidant concentrations of these compounds may possibly have detrimental effects on platelet function.

Traditionally, methylxanthines have been associated with negative effects on health due to their stimulatory properties on the central nervous system. However, recently many studies have focused on understanding some of the molecular mechanisms of methylxanthines, associating their moderate consumption with neuroprotective, hypoglycemic, anti-inflammatory or

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cardiovascular protector effects. [50,51,52] In this study we show, for the first time, an important role of methylxanthines on modulation of platelet function. Previous studies have evaluated the effect of 300 - 600 mg orally administered caffeine on human platelet function.[53,54] These studies showed that only when caffeine was administered after clopidogrel treatment (a typical treatment as part of coronary stenting), enhanced platelet inhibition could be observed, whilst caffeine itself had no significant effect on ADP- and collagen-induced platelet aggregation or activation in caffeine-treated subjects compared with a placebo group. However, caffeine significantly decreased *in vitro* ADP-induced P-selectin expression at equivalent plasma concentrations, yet, the effect may be small. In contrast, physiologically relevant theobromide concentrations (i.e. 10 and 20 μM), which are found in plasma after intake of a methylxanthines-rich cocoa, [24] reduced ADP-induced P-selectin expression.

On other hand, polyphenols have been also associated with a number of beneficial effects on health, such as anti-carcinogenic, anti-inflammatory, antioxidant, and also anti-platelet activity.[55,56] However, these beneficial effects could be limited by their bioavailability which is lower than methylxanthines, with phenolic plasma concentrations lower than 10 μM, depending of the degree of roasting and the consumed dose of coffee.[18,20] The results of this study showed a significant effect of 5-CQA on the modulation of platelet activation at 1 - 20 μM, close to concentrations found in plasma after intake of green coffee extract (6 μM).[20] On the contrary, much higher doses of 3,5-DCQA, the other main polyphenol in green coffee and yerba mate, are needed (10 - 20 μM) in order to significantly decrease ADP-induced P-selectin expression, much higher than the maximal plasma concentration (2.5 μM) reported for this compound.[20] This may be due to the higher molecular weight of 3,5-DCQA, making it more difficult to pass the platelet membrane. With respect to the metabolites of 5-CQA and 3,5-DCQA, only DHCA showed the ability to significantly modulate platelet activation at 1 μM, the maximal plasma concentration found after coffee intake.[18]

We found that the colonic metabolites DHCA and DHFA significantly decreased P-selectin expression in ADP-stimulated platelets at 10 μM, with others showing a trend to decreased P-selectin expression on platelets activated by TRAP after incubation with DHCA.[57] On the contrary, concentrations of at least 100 μM were necessary to inhibit the expression of TRAP- and ADP-induced P-selectin by the early metabolites CA and FA, and 5-CQA, respectively,[42,58] while the

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results of this study showed a significant decrease of ADP-induced P-selectin expression at noticeably lower concentrations (20, 10 and 1 μM for CA, FA and 5-CQA, respectively), indicating that the colonic metabolites may have an even stronger effect on platelet function than their phenolic precursors. Early metabolites from CGA appear at 1 - 2 h after coffee intake, and have a short life time of approximately 30 min,[21,59] whereas DHCA and DHFA appear in the circulation 5 - 10 h after consumption and their life time is between 0.7 and 2.1 h.[19] A longer circulation time of colonic metabolites, together their higher effect on platelet function, suggest that these colonic metabolites may be the main contributors to the beneficial effect of CGA-rich foods on human platelet function. The differences in efficacy could relate to their molecular structure. Indeed, our previous data have suggested that the very simple phenolic structures are more likely to show anti-platelet effects.[42] On the contrary, the early metabolite CA originating in the small intestine showed less effect than its 5-CQA precursor, while the microbial metabolite DHCA was more effective, suggesting that the reduction of some cinnamic acids could be favoring their anti-platelet effect. Therefore, this study confirms the capacity of CGA and overall their metabolites, at physiological concentrations, to modulate P-selectin expression on platelets. This effect is probably due to its cinnamic acid molecules, which have been associated with a reduction in the expression of P-selectin in previous studies, whilst quinic acid have not shown any of these effects.[60,61] However, the underlying mechanisms of the anti-platelet effects of polyphenols are not clear.

There are only few publications about the bioavailability of yerba mate compounds. The daily intake of yerba mate is approximately 100 g infused in 2 liter, being traditionally consumed in single mouthfuls throughout the day and not per coup.[8] We recently showed that a single serving could be prepared from 5 g yerba mate in 250 mL water, containing approximately 400 mg CGA (5 mM) from which up 800 nM would be reported in plasma after their intake.[62] In comparison, a single serving of espresso coffee provides between 24 - 422 mg of CGA and 51 - 322 of caffeine, corresponding to approximately 6 - 12 mM CGA and 8 - 16 mM caffeine depending on the type of roast and the volume consumed.[63] In the studies about the bioavailability of coffee phytochemicals, plasma levels up 1.5 μM CGA and 13 μM methylxanthines have been reported after the intake of coffee beverages providing 2 - 4 mM CGA and 1 mM methylxanthines,[19,21,23] with higher plasma concentrations of CGA (up to 10 μM) reported after the intake of roasted coffee.[64]

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Based on previous results,[40,39] the highest dose tested of phenolic extracts was 100 µg/mL in the present study, corresponded to approximately 200 µM CGA and 50 µM methylxanthines; however, the doses tested for phenolic extracts ≤ 1 µg/mL would be within the physiological range for CGA (equivalent to approximately ≤ 2 µM CGA), while all tested concentrations would be physiological for methylxanthines. In comparison, methylxanthines as well as polyphenols and their metabolites have a similar ability to modulate platelet activation by reducing the expression of ADP-induced P-selectin as quercetin at the same range of concentrations (Table 1 from supporting information).

Unexpectedly, the binding of fibrinogen onto platelets was significantly increased by the majority of compounds, compared with control. However, only the effect of the highest concentrations of phenolic extracts (20 - 100 µg/mL GCBE and 100 µg/mL YMPE) induced a >10% change versus control. In contrast, incubation with quercetin significantly decreased the binding of fibrinogen in ADP-stimulated platelets (Table 2 from supporting information). The activation of the platelet integrin glycoprotein IIb/IIIa (αIIbβ3) receptor is one of the last steps in the process of full platelet aggregation. The signaling cascade that initiates platelet activation allows the conformational change of this receptor. The transformation from a quiescent to an activated conformation of the receptor permits the interaction with soluble fibrinogen, which plays an important role in maintaining the stability of a thrombus.[31,65] However, the activation of integrin αIIbβ3 receptor may involve other receptors or molecules not directly related to platelet activation, such as the cMpl receptor, tyrosine kinases or GTPasa Rap1b.[66] Thus, polyphenols and methylxanthines from green coffee and yerba mate and to lesser extent their main metabolites, may be able to indirectly increase the ADP-induced binding of fibrinogen to integrin αIIbβ3 via such receptors or molecules. Furthermore, platelets also express the integrin αVβ3 which shares several ligands with αIIbβ3, including fibrinogen and von Willebrand Factor.[67] Based on the results, we cannot exclude the possibility that the tested compounds interfered with the binding of these other ligands and thus increased to probability for fibrinogen binding. Overall, it should be noted that the percentage increase of fibrinogen binding was lower compared with the percentage decrease in P-selectin expression.

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One of the proposed mechanisms by which polyphenols affect platelet function is that they increase levels of cyclic adenosine monophosphate (cAMP), which inhibits P-selectin expression on platelets through activation of protein kinase A.[68,69] Previous studies have demonstrated that polyphenols, such as caffeic acid, quercetin or epigallocatechin-3-gallate, act through this mechanism.[70,71,72] Methylxanthines also have been associated with an increase of cAMP levels due to up-regulation of platelet Gs protein-coupled adenosine 2A receptor,[53,54] which mediates the production of cAMP by adenylyl cyclase in platelets.[73] Moreover it has recently been demonstrated that chlorogenic acid presents an active site to the adenosine 2A receptor, favoring the increase of cAMP levels and therefore an inhibition of platelet activation.[58] Therefore, the decrease in ADP-induced P-selectin expression by the tested polyphenols and methylxanthines in our study could be associated with an increase of cAMP levels in platelets mediated by adenosine 2A receptor.

In conclusion, this study has shown, for the first time, the capacity of green coffee beans and yerba mate phenolic extracts to decrease ADP-induced P-selectin expression probably due to their phenolic and methylxanthine content, leading to a possible protective effect against CVD. Additionally, this cardio-protective effect could be strengthened *in vivo* by the action of the own colonic metabolites from the intake of both beverages. These results demonstrate that these compounds and metabolites have beneficial effects on human platelet function *in vitro* at physiological concentrations suggesting that continued exposition to physiological levels of CA, FA, DHCA or DHFA through moderate consumption of green coffee, yerba mate or CGA-rich foods may have beneficial health effects *in vivo*.

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Conflicts of interest: none

Acknowledgements

The authors thank all the volunteers for participating in this study. The personnel of the Human Nutrition Unit at the Rowett Institute of Nutrition and Health are acknowledged for their excellent and expert contributions during the blood sampling.

Funding:

This work was funded by the Spanish Ministry of Economy and Competitivity (projects AGL2010-18269 and AGL 2015-69986-R). G.B. is a FPI fellow (BES-2011-047476) granted with a bursary for short stays from MINECO (EEBB-I-14-08802). The Rowett Institute of Nutrition and Health receives funding from the Scottish Government Rural and Environment Science and Analytical Services (RESAS). The funding bodies had no involvement in the design and execution of the study.

Abbreviations*:* 5-CQA, 5-caffeoylquinic acid; 3,5-DCQA, 3,5-dicaffeoylquinic acid; ADP, adenosine 5`-diphosphate; APC, allophycocyanin; CA, caffeic acid; cAMP, cyclic adenosine monophosphate; CGA, chlorogenic acids; cGMP, cyclic guanosine monophosphate; CVD, cardiovascular disease; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; DMSO, dimethylsulfoxide; FA, ferulic acid; GCBE, green coffee bean phenolic extract; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PBS, phosphate-buffered saline; PE, phycoerythrin; PMA, phorbol 12-myristate 13-acetate; SED, standard error of difference; TRAP, thrombin receptor activating peptide; YMPE, yerba mate phenolic extract.

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DOI: 10.1039/C6FO01404F

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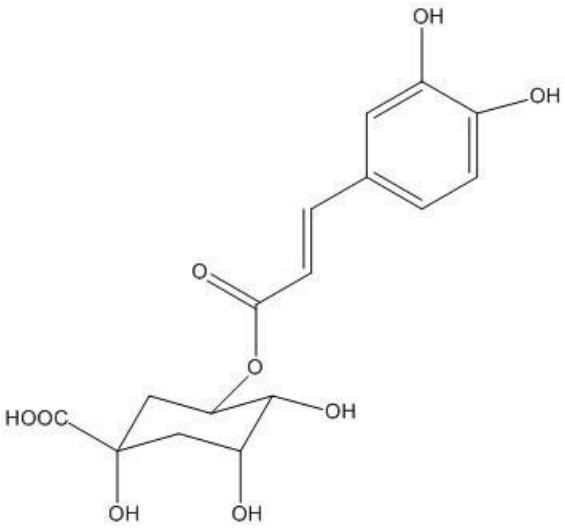
Figure Captions

Figure 1. Structures of the main phenolic compound chlorogenic acids (a) and methylxanthines (b) found in green coffee and yerba mate.

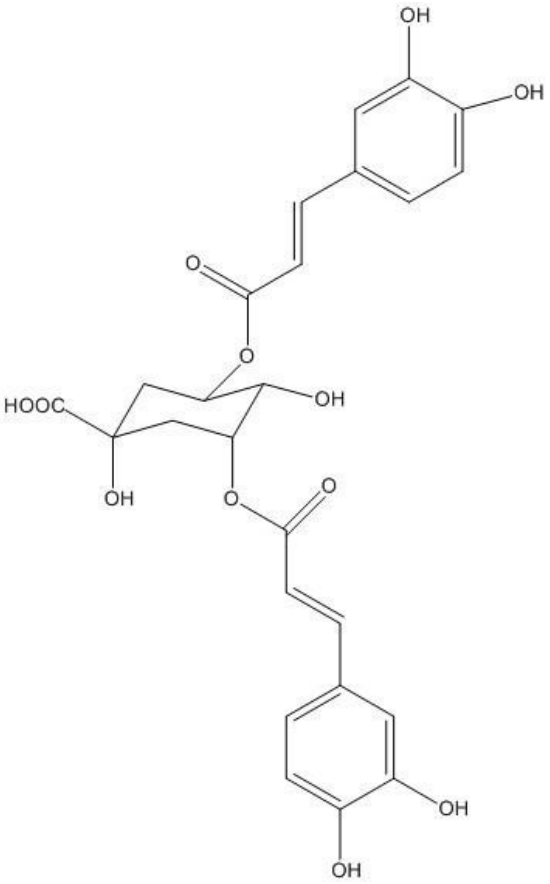
Figure 2. Schematic overview of chlorogenic acids metabolism in digestive tract after the intake of roasted and green coffee according to previously published studies.[18-21] 5-CQA, 5-caffeoylquinic acid; 3,5-DCQA, 3,5-dicaffeoylquinic acid; CA, caffeic acid; COMT, catechol-*O*-methyltransferase; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; EST, esterase; FA, ferulic acid; RA, reductase.

Figure 3. Effects of green coffee, yerba mate and their main compounds on platelet activation markers. Diluted whole blood was incubated with 0.01 to 100 µg/mL of green coffee bean (GCBE) and yerba mate phenolic extracts (YMPE), 0.01 to 100 µM of caffeine (CAF) and theobromine (TB), 5-caffeoylquinic acid (5-CQA), 3,5-dicaffeoylquinic acid (3,5-DCQA), as well as caffeic (CA), ferulic (FA), dihydrocaffeic (DHCA) and dihydroferulic acids (DHFA). ADP-induced P-selectin expression (a) and fibrinogen binding (b) were measured as described in 2.4. Results are shown as percentage change of adjusted means compared with control (0.1% DMSO/PBS) ± SED ($n \geq 9$). * $p < 0.05$.

a. Chlorogenic acids

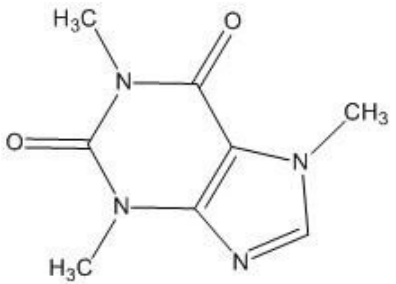


5-Caffeoylquinic acid (5-CQA)

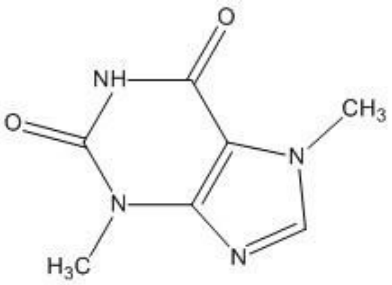


3,5-Dicaffeoylquinic acid (3,5-DCQA)

b. Methylxanthines



Caffeine (CAF)



Theobromine (TB)

Figure 1

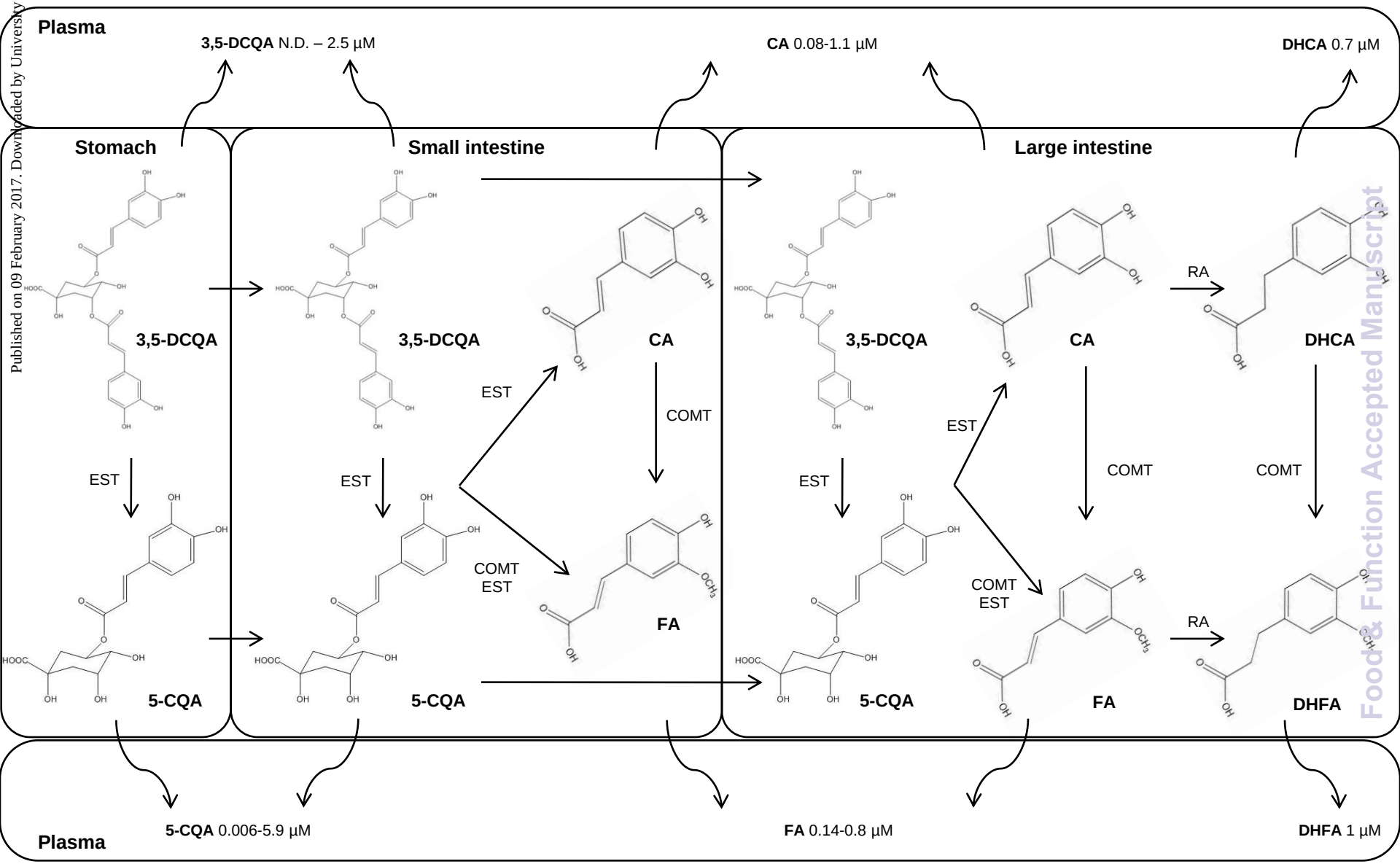


Figure 2

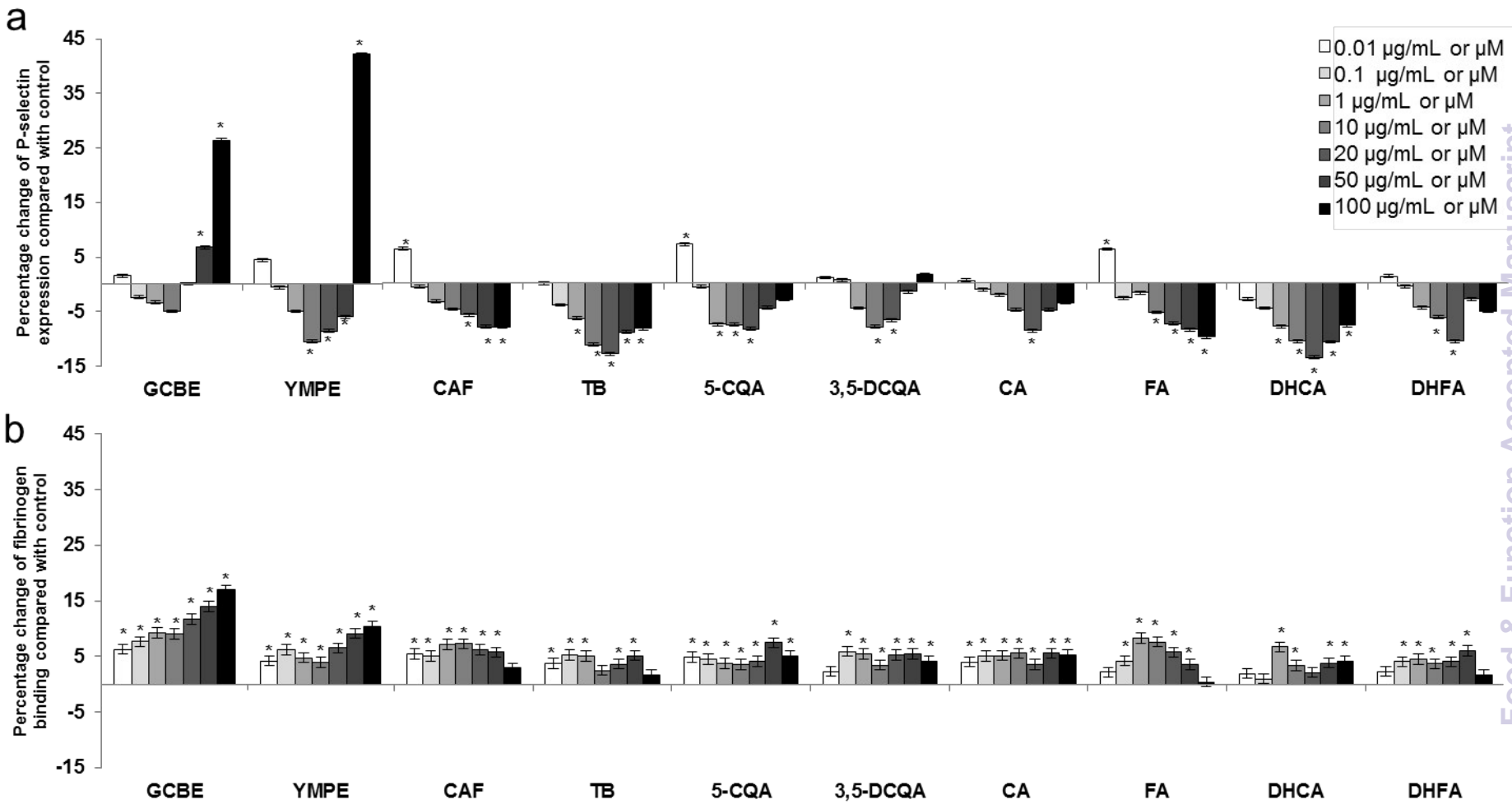


Figure 3