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RESEARCH ARTICLE

In vitro anti-platelet effects of simple plant-derived phenolic compounds are only found at high, non-physiological concentrations

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Scope: Bioactive polyphenols from fruits, vegetables, and beverages have anti-platelet effects and may thus affect the development of cardiovascular disease. We screened the effects of 26 low molecular weight phenolic compounds on two in vitro measures of human platelet function.
Methods and results: After platelets had been incubated with one of 26 low molecular weight phenolic compounds in vitro, collagen-induced human platelet aggregation and in vitro TRAP-induced P-selectin expression (as marker of platelet activation) were assessed. Incubation of platelet-rich plasma from healthy volunteers with 100 μmol/L hippuric acid, pyrogallol, catechol, or resorcinol significantly inhibited collagen-induced platelet aggregation (all $p<0.05$; $n\geq15$). Incubation of whole blood with concentrations of 100 μmol/L salicylic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 4-hydroxyphenylpropionyl glycine, 5-methoxysalicylic acid, and catechol significantly inhibited TRAP-induced surface P-selectin expression (all $p<0.05$; $n=10$). Incubation with lower concentrations of phenolics affected neither platelet aggregation nor activation.
Conclusion: As concentrations of 100 μmol/L are unlikely to be reached in the circulation, it is doubtful whether consumption of dietary phenolics in nutritionally attainable amounts plays a major role in inhibition of platelet activation and aggregation in humans.

Keywords:
Cardiovascular disease / Dietary phenolics / Phenolic acids / Platelet aggregation / P-selectin expression

1 Introduction

Consumption of diets rich in plant-based products protects against the development of cardiovascular disease (CVD) [1–6]. This is not only due to their content in minerals, vitamins, and fibre, but has also been ascribed to phenolic compounds, secondary plant metabolites that are ubiquitous in fruits, vegetables, herbs, spices, teas, and wines. These include both, low molecular weight phenolic acids and related metabolites (e.g. benzoic acid and cinnamic acid derivatives), and more complex polyphenols whose metabolites include benzoic and cinnamic acid derivatives as well as smaller degradation products. Polyphenols and phenolic acids from food are, once consumed, metabolized to glucuronides, sulfates, and methylated metabolites, then partially absorbed from the small intestine into the circulation, and subsequently excreted rapidly [7]. Phenolics that are not absorbed in the small intestine proceed to the colon where they undergo metabolism by microbiota. The

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Abbreviations: ADP, adenosine diphosphate; **APC,** allophycocyanin; **AUC,** area under the curve; **PE,** phycoerythrin; **PRP,** platelet-rich plasma; **TRAP,** thrombin receptor-activating peptide

resulting colonic metabolites are mainly small phenolic acids and their derivatives. Some of them are absorbed into the circulation and excreted with urine [7]. In general, the bioavailability of most phenolic compounds from food sources is considered to be low [8].

Many diet-derived phenolic and polyphenolic compounds have been shown to be potentially beneficial for human health as they exhibit, e.g. antioxidant [9–14], anti-inflammatory [15], and anti-carcinogenic [12, 15] properties. Some dietary phenolic compounds, mainly polyphenols, also have been shown to affect human platelet function in vitro and in vivo [16–20]. Activated blood platelets play a central role in the development of CVD as they are major components of thrombi that occlude arteries [21] and they also contribute to plaque formation within blood vessels in the early stages of atherogenesis [22]. Evidence on the anti-platelet effects of low molecular weight phenolics is, however, scarce and difficult to interpret in a nutritional context. In this study, therefore, we systematically screened the effects of several dietary relevant phenolic acids that are common in plant foods (Tables 1 and 2), and some of their metabolites (Table 3), on collagen-induced in vitro platelet aggregation and thrombin receptor activating peptide (TRAP)-induced P-selectin expression on the platelet surface, a marker for platelet activation.

2 Materials and methods

2.1 Chemicals

Benzoic acid, salicylic acid, 4-hydroxybenzoic acid, protocatechuic acid, gentisic acid, gallic acid, syringic acid, cinnamic acid, caffeic acid, sinapic acid, dihydrocaffeic acid, phloretic acid, 5-methoxysalicylic acid, hippuric acid, pyrogallol, and TRAP were obtained from Sigma-Aldrich Co. Ltd, Dorset, UK. Vanillic acid, *p*-coumaric acid, *m*-coumaric acid, ferulic acid, and catechol were obtained from Fluka Chemika (now Sigma-Aldrich Co. Ltd, Dorset, UK). Resorcinol was obtained from BDH Chemicals (purchased from VWR International Ltd, Lutterworth, UK). 4-Hydroxyhippuric acid, 3-hydroxyhippuric acid, 3-hydroxyphenylacetyl glycine, 3-hydroxyphenylpropionyl glycine, and 4-hydroxyphenylpropionyl glycine were synthesized using a previously described method [23] and kindly provided by Dr. Shika Saha from the Institute of Food Research (IFR), Norwich, UK. All antibodies (i.e. phycoerythrin (PE)-labelled mouse anti-human CD61 and allophyocyanin (APC)-labelled mouse anti-human CD62P) were obtained from BD Biosciences, San Jose, USA. Collagen was obtained from Helena Biosciences Europe (Gateshead, UK). Phenolic compounds were chosen due to their occurrence in plant foods (benzoic and cinnamic acid derivatives) and availability (metabolites). All compounds were solubilized in methanol (analytical grade) as stock dilutions and stored at −20°C until use. All compounds' purities and stability of dilutions in methanol were established by HPLC (data not shown). In all experiments, the final concentration of methanol within the tested compounds and the negative control, PBS, in platelet-rich plasma (PRP) or diluted whole blood was equal to or less than 2%.

2.2 Blood sampling procedure

This study was approved by the North of Scotland Research Ethics Committee (reference number 07/SO8O1/12). Blood samples for light transmission aggregometry were obtained from 26 healthy volunteers (12 males and 14 females) within an age range of 23–58 years. The blood samples for flow cytometry were obtained from 10 healthy volunteers (4 males and 6 females) within an age range of 23–52 years. Each volunteer signed a consent form before donating blood. Volunteers had abstained from anti-inflammatory drugs and food supplements for at least two weeks prior to blood sampling and had normal platelet function. Blood was obtained using siliconized 21 gauge butterfly needles into 10 mL S-Monovette blood collection tubes containing 1 mL 0.106 mol/L trisodium citrate as anticoagulant (Sarstedt Ltd, Beaumont Leys, UK).

2.3 Light transmission aggregometry in platelet-rich plasma

Blood samples were kept at 37°C until aggregometry measurements were completed. They were centrifuged at $200 \times g$ for 10 min within 20 min of sampling in order to obtain PRP. Further centrifugation at $2000 \times g$ for 10 min resulted in platelet-poor plasma, which was used to adjust the platelet number to 300×10^9/L in PRP. Totally, 5 mmol/L stock dilutions of the compounds were diluted 1:5 with PBS (to obtain 1 mmol/L dilutions used for the incubations) on the day of analysis. In all, 450 μL PRP was incubated with 100 μmol/L (a volume of 50 μL of the 1 mmol/L dilution, dilution 1:10) benzoic acid, salicylic acid, 4-hydroxybenzoic acid, protocatechuic acid, gentisic acid, gallic acid, syringic acid, cinnamic acid, caffeic acid, sinapic acid, dihydrocaffeic acid, phloretic acid, 5-methoxysalicylic acid, vanillic acid, *p*-coumaric acid, *m*-coumaric acid, ferulic acid, 4-hydroxyhippuric acid, 3-hydroxyhippuric acid, 3-hydroxyphenylacetyl glycine, 3-hydroxyphenylpropionyl glycine, 4-hydroxyphenylpropionyl glycine, hippuric acid, pyrogallol, catechol, resorcinol, or PBS containing the same amount of methanol as the phenolics (as a negative control), respectively, for 10 min before initiating platelet aggregation by addition of optimal concentrations of collagen (3 or 5 μg/mL, depending on individual response). This incubation time was chosen based on the results obtained in previous studies testing bioactive plant compounds for their effects on platelet function in vitro [24, 25]. Each compound was tested using PRP from at least 15 different volunteers.

Table 1. Structures and sources of phenolic acids found in plant foods (benzoic acid and cinnamic acid derivatives)

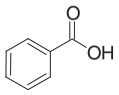
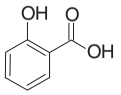
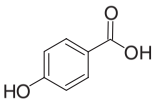
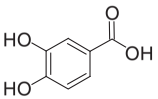
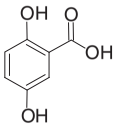
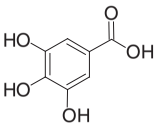
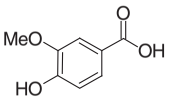
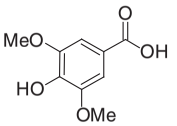
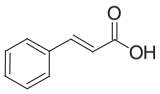
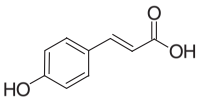
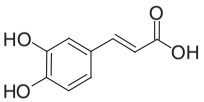
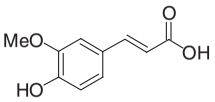
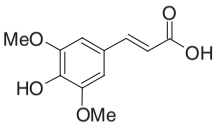
	Benzoic acid derivatives							
	Benzoic acid	Salicylic acid	4-Hydroxybenzoic acid	Protocatechuic acid	Gentisic acid	Gallic acid	Vanillic acid	Syringic acid
Structures								
Sources	Fruits, wine, most berries (especially bilberries and cranberries)	Condiments, fruits, black tea, most berries, cucumbers, tomatoes, dill, wine	Wine, grape juice, beers, tomatoes, lettuce, most berries, herbs, cereals, human urinary metabolite of (−)-epigallocatechin from green tea	Most berries, cloves, cinnamon bark, potatoes, beers, fortified wines (sherry, port etc.), major human metabolite of cyanidin glucosides from citrus juices	Wine, melon, grapes, citrus fruits, pepper, tomatoes, aubergines, cucumber, kiwi fruits, cloves	Most berries, grapes, black and green tea, wine, beers, fortified wines (sherry, port etc.), cloves	Cranberries, tomatoes, wine, potatoes, beers, lettuce, cereals, vanilla beans, human urinary metabolite of chlorogenic acids in coffee	Grape juice, lettuce, cloves, cinnamon bark, potatoes, cereals, beers, fortified wines
References	[42–47]	[42, 48]	[7, 42, 45, 49, 50]	[42, 50–53]	[42]	[42, 49, 50, 54]	[7, 42, 45, 55]	[42]
	Cinnamic acid derivatives							
	Cinnamic acid	*p*-Coumaric acid	Caffeic acid	Ferulic acid	Sinapic acid			
Structures								
Sources	Berries (cranberries, strawberries, raspberries), potatoes	Spinach, sugar beet fibre, cereal brans, wine, fortified wines, ciders, grape juice, orange juice, maple products, most berries, apples, pears, carrots, coffee, plums, cherries, artichokes, aubergines, chicory	Coffee, blueberries, kiwi fruits, plums, cherries, apples, ciders, fortified wines, grape juice, pears, cranberries, cereals, aubergines, artichokes, green mate, chicory	Cereal grains and brans, coffee, citrus juices, sugar beet fibre, grape juice, kiwi fruits, cranberries, blueberries, plums, cherries, apples, pears, spinach, beetroot, maple products, aubergines, ciders, green mate, chicory, artichokes, human urinary metabolite of chlorogenic acids from coffee	Broccoli, kale, other leafy brassicas, citrus juices, kiwi fruits, coffee, tomatoes, blueberries, plums, cherries, apples, maple products, aubergines, ciders, green mate, pears, chicory, artichokes			
References	[45, 56, 57]	[44, 45, 49, 50, 54, 56–59]	[45, 49, 50, 54, 58, 59]	[7, 45, 49, 50, 54, 55, 58, 59]	[49, 50, 59]			

Table 2. Levels of food-based compounds within their food sources (based on data from Phenolexplorer [60])[a)]

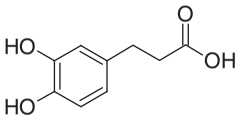
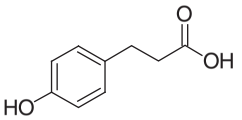
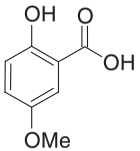
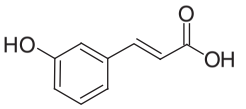
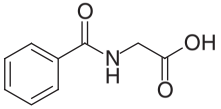
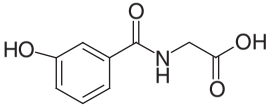
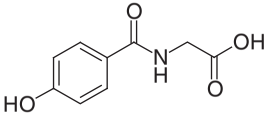
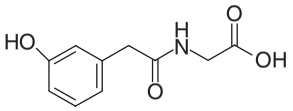
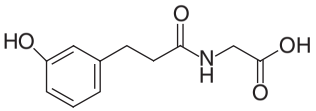
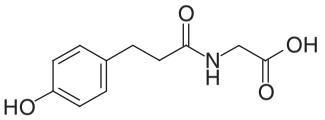
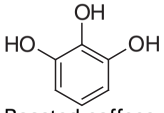
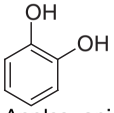
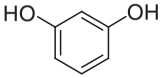
Compound	Food source	Mean content[b)]	Compound	Food source	Mean Content[b)]
Benzoic acid derivatives			**Cinnamic acid derivatives**		
Benzoic acid	American cranberry	48.1	Cinnamic acid	Chinese cinnamon	20.1
	Cocoa powder (commercial)	0.06		Green olives (raw)	14.33
Salicylic acid	Ceylan cinnamon	0.7		Lingonberry	4.12
	Beer (regular)	0.2		Black olives (raw)	0.77
4-Hydroxy-	Green olives (raw)	4.97		Strawberry	0.22
benzoic acid	Black olives (raw)	1.69		American cranberry	0.16
	Beer (regular)	0.96	*p*-Coumaric acid	Cloves	8.49
	Red wine	0.55		Roasted peanuts (dehulled)	6.46
	Oat (whole grain flour)	0.45		Green olives (raw)	5.9
	American cranberry	0.42		Date (dried)	5.77
Protocatechuic acid	Star anise	32.2		Common sage (dried)	4.95
	Green chicory (raw)	21.79		Cloudberry	4.3
	Red chicory (raw)	16.78		Lingonberry	3.93
	Black olives (raw)	6		Date (fresh)	2.89
	Date (dried)	4.94		Peanut (dehulled)	2.53
	Red onion (raw)	2	Caffeic acid	Black chokeberry	141.14
Gentisic acid	White wine	1.82		Spearmint (dried)	25
	Pear (whole)	0.54		Ceylan cinnamon	24.2
	Red wine	0.46		Star anise	20.2
Gallic acid	Chestnut (raw)	479.78		Caraway	16.4
	Cloves	458.19		Nutmeg	16.3
	Green chicory (raw)	25.84		Ginger (dried)	15.5
	Red chicory (raw)	14.56		Lingonberry	6.34
	Common sage (dried)	5.25		Green chicory (raw)	2.61
	Blackberry	4.67	Ferulic acid	Hard wheat (whole grain flour)	72.21
	Black tea infusion	4.63		Dark chocolate	24
	Red wine	3.59		Hard wheat (refined flour)	14.11
	Green tea infusion	0.49		Date (dried)	11.83
Vanillic acid	Sweet basil (dried)	14		Date (fresh)	9.62
	Common sage (dried)	5.85	Sinapic acid	Green olives (raw)	44
	Date (dried)	4.13		Black olives (raw)	10.82
	American cranberry	2.81		Cauliflower (raw)	4.28
	Common sage (fresh)	2.27		Rape seed oil	0.83
	Date (fresh)	1.76		Soybean sprout (raw)	0.28
Syringic acid	Walnut (dehulled)	33.83		Rye (whole grain flour)	0.21
	Black olive (raw)	33.1			
	Common thyme (dried)	11.7			
	Date (dried)	6.06			
	Green olives (raw)	6			
	Date (fresh)	2.45			
Some metabolites are also found in food sources:					
Phloretic acid	Green olives (raw)	6	*m*-Coumaric acid	Black olives (raw)	12.5
	Black olives (raw)	2.8		Green olives (raw)	8
Catechol	Arabica coffee beverage (Filter)	0.54		Beer (regular)	0.02
	Coffee beverage (Filter)	0.41	Pyrogallol	Coffee beverage (Filter)	0.54
	Cocoa powder	0.12		Arabica coffee beverage (Filter)	0.39
	Coffee beverage	0.04		Cocoa powder	0.18
	(Filter, decaffeinated)			Beer (dark)	0.03

a) Examples only.
b) Mean content in (mg/100 g fresh weight) for solid foods and in (mg/100 mL) for beverages.

To establish dose–response relationships for the more potent inhibitors of collagen-induced platelet aggregation, 50 mmol/L stock dilutions of resorcinol, pyrogallol, and catechol, respectively, were prepared in methanol. These stock dilutions were used to incubate PRP from a subset of up to three subjects per compound with 1, 5, 10, 50, 75, 100, 250, 500, or 1000 μmol/L resorcinol, pyrogallol, catechol, or PBS containing the same amount of methanol as the tested compounds (as a negative control), respectively, while keeping the maximum concentration of methanol in each sample to a maximum of 2%. Incubation time was 10 min and thereafter platelet aggregation was initiated by addition of optimal concentrations of collagen (3 or 5 μg/mL, depending on individual response).

The optimal concentration of collagen was determined by measuring collagen-induced platelet aggregation using both concentrations for 5–10 min at the beginning of each experiment for each volunteer. When maximum aggregation levels reached at least 90% and lag time was shorter than 90 s the agonist was considered to be at 'optimal concentration'. Platelet aggregation was assessed using the light transmission method of Born [26] using a Helena

Table 3. Structures and sources of phenolic compounds formed by metabolism in humans

	Dihydrocaffeic acid	**Phloretic acid**	**5-Methoxysalicylic acid**	***m*-Coumaric acid**
Structures				
Sources	Human metabolite of caffeic and ferulic acid from e.g., coffee	Brined black olives, black and green raw olives	Metabolite of gentisic acid	Human urinary metabolite of chlorogenic acid, also found in black and green raw olives, virgin olive oil, beer
References	[7, 34, 61]	[60, 62]	[34]	[60, 61, 63, 64]
	Hippuric acid	**3-Hydroxyhippuric acid**	**4-Hydroxyhippuric acid**	
Structures				
Sources	Human urinary metabolite of benzoic, cinnamic and caffeic acid (glycine conjugate of benzoic acid), red wine/grape juice polyphenols, chlorogenic acids in coffee, and (−)-epigallocatechin from green tea	Human urinary metabolite of chlorogenic acids from coffee and catechin, red wine/grape juice polyphenols, and polyphenols in orange juice (hesperetin glucosides)	Human urinary metabolite of red wine/grape juice polyphenols	
References	[7, 34, 36, 55, 65]	[7, 36, 55, 61, 66]	[36]	
	3-Hydroxyphenylacetyl glycine	**3-Hydroxyphenylpropionyl glycine** (*N*-*m*-Coumaroylglycine)	**4-Hydroxyphenylpropionyl glycine** (*N*-*p*-Coumaroylglycine)	
Structures				
Sources	Glycine conjugate (metabolite) of 3-hydroxyphenylacetic acid, which is a metabolite of rutin (quercetin-3-rutinoside)	Glycine conjugate (metabolite) of *m*-coumaric acid	Glycine conjugate (metabolite) of *p*-coumaric acid	
References	[67]	[68] and Dr. S Saha, Dr. P Kroon, IFR (Norwich, UK): unpublished data	[68] and Dr. S Saha, Dr. P Kroon, IFR (Norwich, UK): unpublished data	
	Pyrogallol	**Catechol**	**Resorcinol**	
Structures				
Sources	Roasted coffees, cocoa powder, human urinary metabolite of red wine/grape juice polyphenols and (−)-epigallocatechin gallate from green tea	Apples, onions, crude sugar beets, roasted coffees, cocoa powder, smoked foods, vanilla beans, human urinary metabolite of (−)-epigallocatechin gallate from green tea	Canihua[a)], broad beans, tobacco leaves	
References	[7, 35, 36, 60]	[7, 31, 35, 60, 69, 70]	[32, 33]	

a) *Chenopodium pallidicaule*, an annual pseudocereal found in semidesert climates at 3600–4400 m altitude (Bolivian Andes).

PACKS-4 aggregometer (Helena Biosciences Europe, Gateshead, UK) over a period of 10 min, with samples randomized over the four different channels.

Aggregation was quantified based on area under the aggregation curve (AUC), maximum aggregation, and lag time of the aggregation curve compared with the control. All tests were carried out within 3 h of blood collection, and we included only those runs where the AUCs for the control were larger than 90% of the AUC for the control in the first run of the day within the same volunteer.

2.4 Assessment of P-selectin expression as a marker of platelet activation by flow cytometry

Blood samples were diluted 1:10 using HEPES-Mg buffer within 20 min of sampling. The diluted blood was incubated with 1, 10, or 100 μmol/L benzoic acid, salicylic acid, 4-hydroxybenzoic acid, protocatechuic acid, gentisic acid, gallic acid, syringic acid, cinnamic acid, caffeic acid, sinapic acid, dihydrocaffeic acid, phloretic acid, 5-methoxysalicylic acid, vanillic acid, *p*-coumaric acid, *m*-coumaric acid, ferulic acid, 4-hydroxyhippuric acid, 3-hydroxyhippuric acid, 3-hydroxyphenylacetyl glycine, 3-hydroxyphenylpropionyl glycine, 4-hydroxyphenylpropionyl glycine, hippuric acid, pyrogallol, catechol, resorcinol, or PBS (as a negative control), respectively, dissolved in methanol (final concentrations of methanol within the diluted blood were 0.02–2%) for 10 min, before platelet activation was initiated by addition of 25 μmol/L TRAP. After a 10-min incubation period at room temperature a combination of PE-labelled CD61 (binds to integrin αIIbβ3 on the surface of platelets and is therefore a general platelet maker) and APC-labelled CD62P (binds to P-selectin expressed on the surface of activated platelets) was added, followed by further incubation at room temperature in the dark. After 20 min, the reaction was stopped by addition of cold PBS. Then the samples were stored at 4°C in the dark, re-suspended just before analysis using cold PBS, and analyzed using a BD FACSArray (BD Biosciences, Oxford, UK) within 2 h. The general platelet marker PE-CD61 as well as side and forward scatter characteristics were used to distinguish platelets from all other cells. Using the gate for integrin αIIbβ3 positive cells (>90%) P-selectin expression was recorded. The results were analyzed as percentage of positive fluorescent platelets binding APC-CD62P in a total of 10 000 events as seen by flow cytometry compared with the control. All antibodies were monoclonal and all samples were run in duplicate. Each compound was tested using bloods from 10 different volunteers.

2.5 Statistical analysis

AUCs, maximum aggregation values and lag times from the platelet aggregation measurements were analyzed by a mixed model using the restricted maximum likelihood (REML) approach. Random effect terms were subject, sample within subject and run. Fixed effect terms were channel, compound and their interaction. Significance was tested by the Wald statistic, with estimated degrees of freedom in the denominator. Post-hoc multiple comparisons were carried out using two-tailed Student's *t*-tests.

Data of the flow cytometric measurements were analyzed using an analysis of variance (ANOVA) approach, using Fisher's protected least significant difference (LSD) test for post-hoc multiple comparisons. All statistical analyses were done with GenStat version 11 (VSN International, UK) and differences were considered to be significant at the level of $p<0.05$.

3 Results

3.1 Subjects

The characteristics of the volunteers are shown in Table 4.

3.2 Platelet aggregation

Of the 26 phenolic compounds tested, four compounds caused a significant decrease in collagen-induced platelet aggregation, measured as AUC, compared with the PBS/methanol control: 100 μmol/L hippuric acid ($-8.9\pm2.2\%$, $p<0.001$), resorcinol ($-30.5\pm2.0\%$, $p<0.001$), pyrogallol ($-8.9\pm2.1\%$, $p<0.001$), and catechol ($-73.2\pm2.0\%$, $p<0.001$) (Fig. 1A and Table 5). They also significantly decreased the percentage of maximum aggregation (all $p<0.001$) (Table 5). Resorcinol and catechol significantly prolonged lag time by 18 s ($25.0\pm2.8\%$) and 33 s ($45.4\pm3.2\%$), respectively, compared with the PBS/methanol control (both $p<0.001$) (Fig. 1B and Table 5). As some of the changes in platelet aggregation were quite substantial we examined if lower concentrations of catechol, resorcinol, and pyrogallol would also decrease collagen-induced platelet aggregation. The dose–response curves of AUCs (Fig. 2A) and lag times (Fig. 2B), depending on the concentration of those compounds and compared with the control, show that concentrations below 50 μmol/L did not significantly change AUCs and that only concentrations of 100 μmol/L or above were able to significantly affect lag times. Platelet viability was clearly not impaired by any of the compounds up to concentrations of 250 μmol/L. Indeed, platelet aggregation in these samples was only incompletely inhibited, and residual aggregating activity remained.

Gallic acid, dihydrocaffeic acid, ferulic acid, and salicylic acid significantly shortened lag time by 4–6 s (6–8%) compared with the PBS/methanol control (all $p<0.05$) (Fig. 1B and Table 5).

This data set was analyzed including and excluding data from four volunteers that smoked and two volunteers

Table 4. Subject characteristics[a),b)]

	Male	Female	Total
Platelet aggregation			
N	12	14	26
Age (range) [years]	38±9 (25–58)	34±11 (23–56)	36±10 (23–58)
Platelet count [$\times 10^9$/L]	237±35	265±54	251±46
Mean platelet volume (fL)	8.55±0.63	8.29±0.68	8.42±0.66
Hematocrit (%)	42.8±3.1	37.7±2.4	40.3±3.8
Smoking	1 (8.3%)	3 (21.4%)	4 (15.4%)
Diabetic (treated by diet only)	1 (8.3%)	1 (7.1%)	2 (7.7%)
P-Selectin expression			
N	4	6	10
Age (range) [years]	34±8 (26–45)	34±11 (23–52)	34±9 (23–52)
Platelet count [$\times 10^9$/L]	252±43	229±52	238±48
Mean platelet volume (fL)	8.78±0.66	8.50±0.66	8.60±0.60
Hematocrit (%)	42.9±2.6	40.3±1.2	41.3±2.2
Hemoglobin (g/L)	145±9	136±4	139±8
Smoking	0 (0%)	0 (0%)	0 (0%)
Diabetic (treated by diet only)	0 (0%)	0 (0%)	0 (0%)

a) Values are mean±SD or absolute numbers (percentage of total).
b) Blood of five of the volunteers (3 males and 2 females) were used for both measurements. Total number of volunteers recruited for the study was 31 (13 males, 18 females).

who had diabetes, and outcomes were identical (data not shown).

3.3 P-selectin expression

Of the 26 phenolic compounds tested, seven caused a statistically significant decrease in TRAP-induced surface P-selectin expression compared with control at a concentration of 100 μmol/L: salicylic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 4-hydroxyphenylpropionyl glycine, 5-methoxysalicylic acid, and catechol (all $p<0.001$) (Fig. 3). Lower concentrations of phenolic compounds (1 and 10 μmol/L) did not significantly affect TRAP-induced surface P-selectin expression (data not shown).

4 Discussion

In this study, we screened 26 low molecular weight phenolic acids and some of their metabolites for their ability to affect collagen-induced platelet aggregation and TRAP-induced platelet activation in vitro. Both platelet activation and aggregation were examined as they represent two separate steps contributing to plaque formation within blood vessels in the early stages of atherogenesis as well as to the formation of thrombi after plaque rupture [22]. Once the vessel wall is wounded, or the plaque ruptured, platelets will come in contact with various extracellular matrix constituents like collagen, leading to adhesion of platelets to the injured vessel wall, which will trigger their activation [21]. The activated platelets then secrete a range of adhesion molecules, such as P-selectin and CD40 ligand, and bind fibrinogen from plasma, which leads to platelet aggregation and thrombus formation. Additionally, platelets synthesize and secrete agonists such as adenosine diphosphate (ADP) and thromboxane A_2, which also induce platelet aggregation and thus amplify and maintain the initial platelet response [27].

Only three studies so far have assessed in vitro anti-platelet effects of some low molecular weight phenolic acids using samples from human volunteers. These studies showed that *p*-coumaric, caffeic, ferulic, and sinapic acid (all hydroxycinnamic acids) inhibit collagen-induced aggregation in washed human platelets by 50% when used in concentrations between 478 and 816 μmol/L [28]. Furthermore, gallic acid inhibited thrombin-induced aggregation in washed human platelets by 10–50% when used in concentrations between 10 and 100 μmol/L [29], and 10 μmol/L dihydrocaffeic acid significantly decreased P-selectin expression in resting platelets as well as showing a trend to decreased P-selectin expression in platelets activated by 4 μmol/L TRAP. In contrast, 10 μmol/L hippuric acid did not affect P-selectin expression [18]. These studies showed mostly anti-platelet effects for the few compounds that were tested, but such potentially beneficial effects were, except for dihydrocaffeic acid, only achieved using high, non-physiological concentrations. Furthermore, extracted phenolic-rich fractions of purple grape juice did not significantly inhibit aggregation or nitric oxide production in human washed platelets, although the fraction rich in cinnamic acids caused a significant decrease of 77% in superoxide release [30].

In our study, we found that only the highest tested concentrations of the very simple phenolics, catechol, and

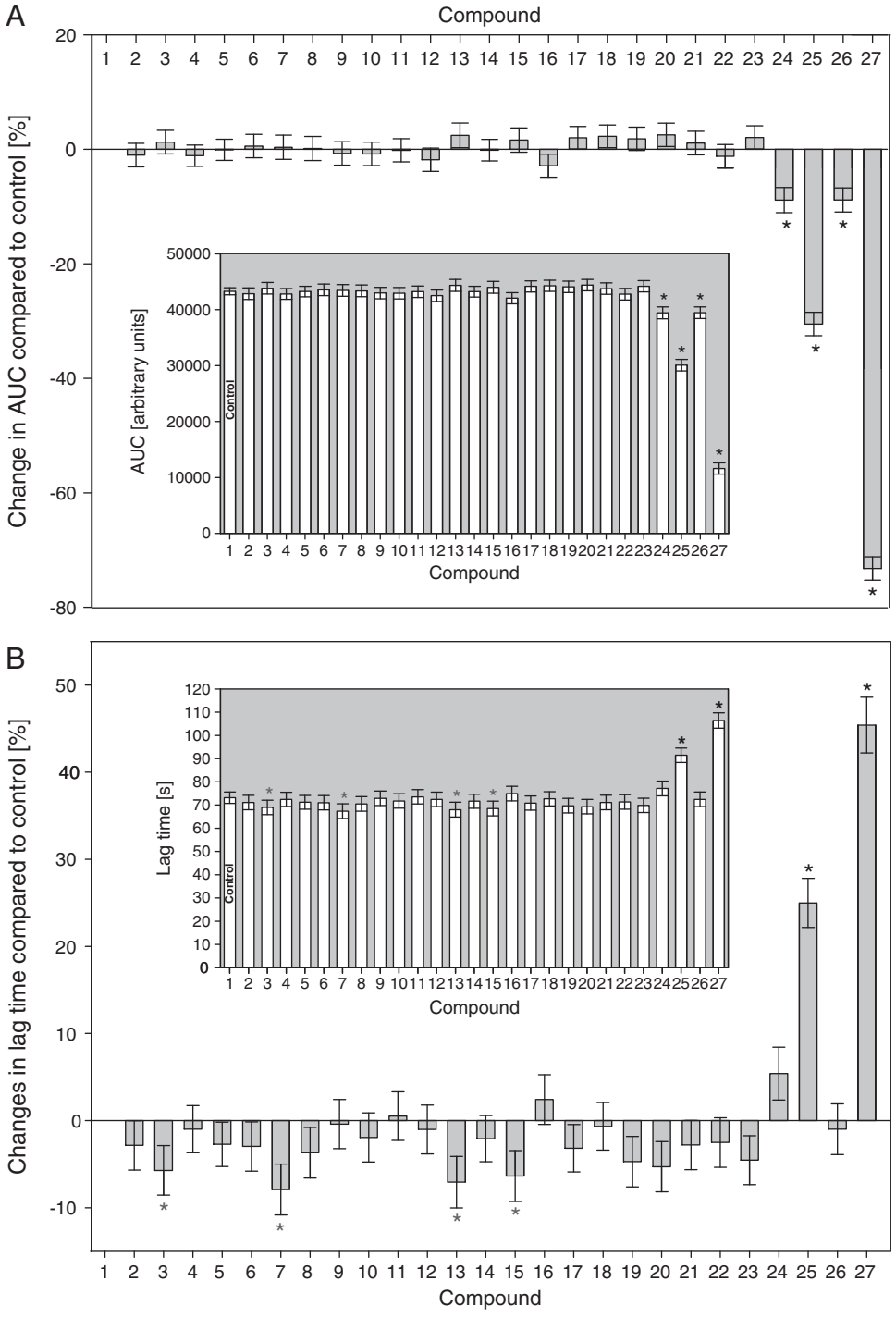


Figure 1. Effects of phenolic compounds on collagen-induced platelet aggregation. Platelet-rich plasma was incubated with 100 μmol/L phenolic compound and collagen-induced platelet aggregation was measured as described in Section 2. Panel A: mean percentage changes in areas under the curve (AUC) compared with PBS/methanol control (mean ± SED, $n \geq 15$ per compound). The inset shows actual AUC values (mean ± SEM, $n \geq 15$ per compound) for all phenolic compounds. Panel B: mean percentage change in lag time compared with PBS/methanol control (mean ± SED, $n \geq 15$ per compound). The inset shows actual lag times (mean ± SEM, $n \geq 15$ per compound) for all phenolic compounds. $^{*}p < 0.05$ versus control. 1, control (PBS+2% methanol); 2, benzoic acid; 3, salicylic acid; 4, 4-hydroxybenzoic acid; 5, protocatechuic acid; 6, gentisic acid; 7, gallic acid; 8, vanillic acid; 9, syringic acid; 10, cinnamic acid; 11, *p*-coumaric acid; 12, caffeic acid; 13, ferulic acid; 14, sinapic acid; 15, dihydrocaffeic acid; 16, phloretic acid; 17, 4-hydroxyhippuric acid; 18, 3-hydroxyhippuric acid; 19, 3-hydroxyphenylacetyl glycine; 20, 3-hydroxyphenylpropionyl glycine; 21, 4-hydroxyphenylpropionyl glycine; 22, 5-methoxysalicylic acid; 23, *m*-coumaric acid; 24, hippuric acid; 25, resorcinol; 26, pyrogallol; 27, catechol.

resorcinol showed a consistent anti-platelet effect by inhibition of collagen-induced platelet aggregation (−73 and −31%, respectively, as measured by the AUC and maximum aggregation), and by an increase in lag time (45 and 25%, respectively). Catechol also significantly decreased TRAP-induced platelet activation by 11% (as measured by P-selectin expression). Catechol occurs naturally in fruits and vegetables such as apples, onions, and crude beet sugar [31] and is a human metabolite of flavan-3-ols from green teas [7], whereas resorcinol is present in canihua and broad beans [32, 33] (Table 3). In addition to catechol and resorcinol, high concentrations of hippuric acid and pyrogallol also showed inhibitory effects on in vitro collagen-induced platelet aggregation. Hippuric acid is a human urinary metabolite of benzoic, cinnamic, and caffeic acid [34], and pyrogallol is present in roasted coffees [35] (Table 3). Both

Table 5. Effects of phenolic compounds on collagen-induced platelet aggregation[a),b)]

Compounds (100 μmol/L)	Δ AUC (±SED)	Δ AUC (%)	Action	*P*	Δ Max (%) (±SED)	Action	*P*	Δ Lag *t* (s) (±SED)	Δ Lag *t* (%)	Action	*P*
25) Resorcinol	−13212 (±884)	−30.5 (±2.0)	↓	<0.001	−28.8 (±2.0)	↓	<0.001	18.3 (±2.1)	25.0 (±2.8)	↑	<0.001
26) Pyrogallol	−3830 (±911)	−8.9 (±2.1)	↓	<0.001	−8.6 (±2.0)	↓	<0.001	−0.7 (±2.1)	−1.0 (±2.9)	↓	NS
27) Catechol	−31695 (±880)	−73.2 (±2.0)	↓	<0.001	−70.3 (±1.9)	↓	<0.001	33.2 (±2.3)	45.4 (±3.2)	↑	<0.001
24) Hippuric acid	−3841 (±947)	−8.9 (±2.2)	↓	<0.001	−8.4 (±2.1)	↓	<0.001	4.0 (±2.2)	5.4 (±3.0)	↑	NS
3) Salicylic acid	544 (±890)	1.3 (±2.1)	↑	NS	1.2 (±2.0)	↑	NS	**−4.2 (±2.1)**	**−5.7 (±2.8)**	↓	**<0.05**
7) Gallic acid	157 (±914)	0.4 (±2.1)	↑	NS	−0.7 (±2.0)	↓	NS	**−5.8 (±2.1)**	**−7.9 (±2.9)**	↓	**<0.01**
13) Ferulic acid	1057 (±934)	2.4 (±2.2)	↑	NS	1.7 (±2.1)	↑	NS	**−5.2 (±2.2)**	**−7.1 (±3.0)**	↓	**<0.05**
15) Dihydro-caffeic acid	704 (±917)	1.6 (±2.1)	↑	NS	1.1 (±2.0)	↑	NS	**−4.7 (±2.1)**	**−6.4 (±2.9)**	↓	**<0.05**

a) Results shown only for compounds that significantly affected platelet aggregation.

b) All values are mean±SEM or mean percentage change±SED, $n \leq 15$ per compound.

↓, decrease; ↑, increase; Δ AUC, mean total change in area under the curve; Δ AUC (%), mean percentage change in area under the curve; Δ Max (%), mean percentage change in maximum aggregation; Δ Lag *t* (s), mean total change in lag time in seconds; Δ Lag *t* (%), mean percentage change in lag time

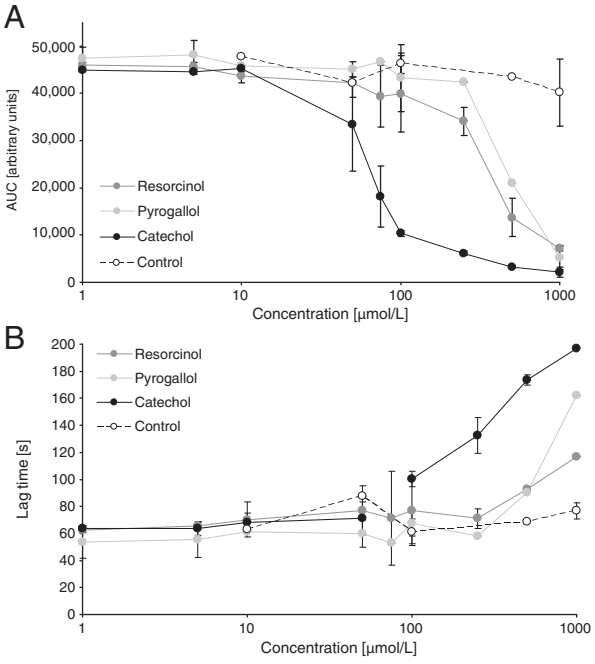


Figure 2. Dose–response curves for resorcinol, pyrogallol, and catechol compared with the control. Platelet-rich plasma was incubated with 1, 5, 10, 50, 75, 100, 250, 500, or 1000 μmol/L resorcinol, pyrogallol, catechol, or PBS/methanol control and collagen-induced platelet aggregation was measured as described in Section 2. (A) mean AUC values±SD ($n \leq 3$ per compound). (B) mean lag times±SD ($n \leq 3$ per compound). Concentrations are shown on a common logarithmic scale.

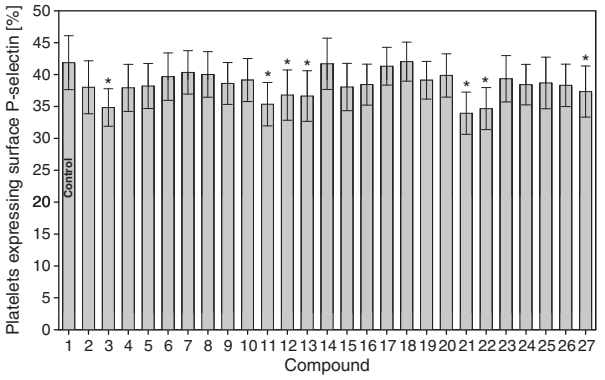


Figure 3. Effects of phenolic compounds on TRAP-induced surface P-selectin expression. Diluted whole blood was incubated with 1, 10, or 100 μmol/L of 27 phenolic compounds and TRAP-induced surface P-selectin expression was measured as described in Section 2. Values represent mean (±SEM) of platelets expressing surface P-selectin as percentage of the total number of platelets ($n = 10$ per compound). $^{*}p < 0.05$ versus control. 1, control (PBS+2% methanol); 2, benzoic acid; 3, salicylic acid; 4, 4-hydroxybenzoic acid; 5, protocatechuic acid; 6, gentisic acid; 7, gallic acid; 8, vanillic acid; 9, syringic acid; 10, cinnamic acid; 11, *p*-coumaric acid; 12, caffeic acid; 13, ferulic acid; 14, sinapic acid; 15,dihydrocaffeic acid; 16, phloretic acid; 17, 4-hydroxyhippuric acid; 18, 3-hydroxyhippuric acid; 19, 3-hydroxyphenylacetyl glycine; 20, 3-hydroxyphenylpropionyl glycine; 21, 4-hydroxyphenylpropionyl glycine; 22, 5-methoxy-salicylic acid; 23, *m*-coumaric acid; 24, hippuric acid; 25, resorcinol; 26, pyrogallol; 27, catechol.

are also found in human urine after consumption of flavan-3-ols from green tea, grape juice, and red wine [7, 36].

Gallic acid, dihydrocaffeic acid, ferulic acid, and salicylic acid slightly but significantly shortened the lag time of collagen-induced platelet aggregation by 6–8% compared with the control without affecting the AUC or maximum aggreation. Ferulic acid and salicylic acid did, however, significantly inhibit TRAP-induced P-selectin expression. Still, the fact that relatively high concentrations of these compounds only had a very small effect on the lag time of collagen-induced platelet aggregation indicates that these findings are unlikely to have a significant physiological impact. Furthermore, salicylic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 4-hydroxyphenylpropionyl glycine, 5-methoxy-salicylic acid, and catechol caused a significant decrease in TRAP-induced surface P-selectin expression at a concentration

of 100 μmol/L, but not at concentrations of 1 or 10 μmol/L. Most of these compounds, apart from catechol, did not significantly affect platelet aggregation, indicating that early reversible mechanisms of platelet activation may have been affected by high concentrations of these phenolic compounds without an irreversible effect on platelet aggregation.

Our data do not allow to distinguish a structure–function relationship, however, they do suggest that structurally related phenolic compounds, such as for example catechol, resorcinol, and pyrogallol, may be acting via different mechanisms. TRAP directly activates platelets through the protease-activated receptors (PAR), initiating platelet secretion reactions including secretion of P-selectin from Weibel–Palade bodies and translocation to the surface [37, 38]. Yet, only a minority of platelets exposed to collagen in suspension express P-selectin on their surface (~11%) [39]. Under these conditions aggregation is induced after a 20–30 s delay, through a pathway primarily mediated by release of the secondary mediators ADP and thromboxane A_2 from platelet dense granules [37, 40]. In the presence of inhibitors of these secondary mediators, strong aggregation by collagen involving activation of G-coupled receptors is replaced by a slow integrin-mediated aggregation involving α2β1 and αIIbβ3 [41]. Mechanisms by which reduction in aggregation induced by collagen can be achieved thus include inhibition of dense granule release (shown by increase in lag time before aggregation – as seen after incubation with 100 μmol/L catechol and resorcinol), and inhibition of the secondary mediators ADP and thromboxane A_2 (shown by reduction in maximum aggregation and AUC – as seen after incubation with 100 μmol/L catechol, resorcinol, pyrogallol, and hippuric acid). Mechanisms of inhibition of P-selectin expression on the surface after exposure to a direct activator such as TRAP are possibly related to inhibition of secretion from Weibel–Palade bodies (observed only after incubation with 100 μmol/L catechol).

Because of the 'proof-of-principle' nature of this screening study, we tested relatively high concentrations of phenolic acids and metabolites for effects on platelet aggregation and activation. Nevertheless, concentrations of low molecular weight phenolics in human plasma are unlikely to be higher than 4 μmol/L [8]. However, lower concentrations ($\leq$10 μmol/L) of phenolic compounds neither affected collagen-induced platelet aggregation (Fig. 2) nor TRAP-induced surface P-selectin expression, indicating that physiologically relevant concentrations of catechol, resorcinol, and pyrogallol that are present in human plasma are unlikely to have anti-platelet effects. Furthermore, most phenolics tested in this study may not be present in human blood in their non-conjugated form as they rapidly undergo metabolism before entering the circulation [7]. However, the phenolics catechol, pyrogallol, and hippuric acid, which showed anti-platelet properties in this study, are found in human urine [7]. Thus, we can assume that these compounds would appear in plasma upon consumption of those or after consumption of certain flavonoids. In addition, the possibility that they might affect platelets indirectly, work synergistically within a mixture of phenolic compounds obtained from the diet, or have other beneficial effects for cardiovascular health cannot be excluded.

In conclusion, only high concentrations (i.e. $\geq$50 μmol/L) of some low molecular weight phenolic compounds show anti-platelet effects in vitro. Our results indicate that inhibition of secretion of Weibel–Palade bodies and secondary mediators may have caused a decreased expression in platelet P-selection and inhibition of aggregation, respectively. It is unlikely, though, that physiological concentrations of low molecular weight phenolic compounds will have significant anti-platelet effect in humans. We cannot exclude, however, that combinations of phenolic acids and their metabolites, as present in certain food products, as well as other phenolic acids that were not tested here, could have more marked beneficial effects on platelet function.

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The authors have declared no conflict of interest.

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