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Research Article

Availability of blueberry phenolics for microbial metabolism in the colon and the potential inflammatory implications

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Blueberries are a rich source of phenylpropanoid-derived phytochemicals, widely studied for their potential health benefits. Of particular interest for colonic health are the lower molecular weight phenolic acids and their derivatives, as these are the predominant phenolic compounds detected in the colon. Blueberries contained a wide variety of phenolic acids, the majority of which (3371.14 ± 422.30 mg/kg compared to 205.06 ± 45.34 mg/kg for the free phenolic acids) were attached to other plant cell-wall components and therefore, likely to become available in the colon. Cytokine-induced stimulation of the inflammatory pathways in colon cells was four-fold up-regulated in the presence of the free phenolic acid fraction. Incubation of the bound phenolic acids with human faecal slurries resulted in qualitative and quantitative differences in the phenolic compounds recovered. The metabolites obtained by incubation with faecal slurries from one volunteer significantly decreased (1.67 ± 0.69 ng/cm^3) prostanoid production, whereas an increase (10.78 ± 5.54 ng/cm^3) was obtained with faecal slurries from another volunteer. These results suggest that any potential protective effect of blueberry phenolics as anti-inflammatory agents in the colon is a likely result of microbial metabolism. Studies addressing a wide-range of well-characterised human volunteers will be required before such health claims can be fully established.

Keywords: Colon cancer / Inflammation / Nutrition / Phytochemicals / Prostanoids

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1 Introduction

Many diseased states including cancer and cardiovascular disease [1, 2] and some of the deficits of age-related neuronal function [3] are associated with an oxidative and inflammatory stress imbalance. Consumption of plant-based foods, rich in antioxidant and anti-inflammatory compounds is considered to be a potentially protective factor and regular intake of fruits and vegetables is known to markedly decrease the risk of developing certain diseases such as colon cancer [4, 5]. The human diet consists of a large variety of fruits and vegetables and contains an extremely complex mixture of phytochemicals. Studies on the beneficial effects of single plant foods are limited. However, in the Western diet many berries (including blackberries, strawberries, cranberries, raspberries and blueberries) contained the highest amounts of redox-active compounds compared to other fruits and vegetables [6]. In particular, blueberries have been shown to reverse age-related neuronal and behavioural dysfunction [7, 8]. These conditions are associated with the oxidative/inflammatory stress balance [8] and it is likely that the compounds in blueberries could also be chemoprotective in colon cancer development. The phenolic compounds from blueberries have also been shown to inhibit colon cancer cell proliferation and induce apoptosis [9]. However, *in vitro* studies invariably do not account for the bioavailability and metabolism of the parent compounds by the colonic microbiota. The human colon harbours more than 500 different bacterial species that posses a wide range of metabolic activities [10–12]. Previous studies have shown that certain strains of bacteria can specifically metabolise dietary constituents including certain flavanoids and the dietary lignan secoisolariciresinol [13–15]. This study will focus on phenolic acids (Fig. 1), as it tends to be these lower molecular-weight compounds that are detected in the colon [16]. The aim of the study is to analyse both the freely available and bound phe-

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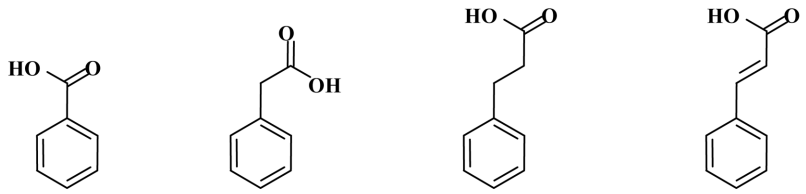

benzoic acid **phenylacetic acid** **phenylpropionic acid** **cinnamic acid**

Figure 1. Structures of the unsubstituted phenolic acids.

nolic acids in blueberries and to assess the availability of blueberry phenolics for metabolism by the colonic microbiota and the potential inflammatory implications.

2 Materials and methods

2.1 General reagents

The 3-hydroxyphenylpropionic, 4-hydroxyphenylpropionic, 3,4-dihydroxyphenylpropionic and 3-methoxy-4-hydroxyphenylpropionic acids were synthesised as reported previously [17]. The remaining compounds and general laboratory reagents were purchased from (Sigma/Aldrich, Gillingham, England).

2.2 Extraction and analysis of phenolics acids from blueberries

Blueberries (*Vaccinium ashei*) rabbit-eye variety from Spain were washed, weighed and stored at −80°C. They were then lyophilised (Heto Lab Equipment, SICC, type CD 2.5, Allerød, Denmark) and moisture loss was recorded. They were freeze-milled (Spex 6700, Edison, USA) and the powder stored in a desiccator prior to extraction. Samples (2 g dry weight, $n = 3$) were suspended in water (100 cm^3), in which the pH was reduced to 2 with HCl (6 mol/dm^3), extracted into ethyl acetate (EtOAc, 50 cm^3) and the layers were separated by centrifugation (15 min, 5000 rpm). The extraction was repeated three times and the EtOAc extracts were combined. The organic layer was left to stand over sodium sulphate (anhydrous) for 1 h and filtered. The combined organic layers were then evaporated to dryness under reduced pressure at a temperature not exceeding 40°C and stored in a desiccator prior to analysis by HPLC. The pH of the aqueous fraction was increased to 7 with sodium hydroxide (4 mol/dm^3) and freeze dried. The samples were then redissolved in NaOH (1 mol/dm^3, 100 cm^3) and were mixed thoroughly and stirred at room temperature for 4 h under nitrogen. The pH was reduced to 2 with HCl (6 mol/dm^3) and the samples extracted into EtOAc (50 $cm^3 \times 3$) and processed as described above. The pH of the aqueous fraction was then increased to 7 with NaOH (4 mol/dm^3) and freeze dried. The samples were then redissolved in HCl (2 mol/dm^3, 100 cm^3) and incubated at 95°C for 30 min, cooled and extracted with EtOAc (50 $cm^3 \times 3$) and processed as described above. The extracts were then redissolved in methanol and filtered through a 0.2 μm PVDF membrane. Separation of the phenolic compounds was by HPLC using AcCN and TFA (0.05% v/v, pH 2.3) and employing gradient elution which involved 11 – 14% AcCN (35 min), 14 – 50% AcCN (5 min), 50% AcCN (10 min) and 50 – 11% AcCN (5 min). Detection was at 215 and 280 nm and the metabolites were quantified by internal standardisation and use of response factors calculated from pure compounds.

2.3 Incubation of blueberry phenolics with human faecal microbiota

Freeze-dried blueberries (2 g, $n = 3$) were extracted with ethyl acetate as described above. The aqueous fractions were combined, the pH increased to 7 with sodium hydroxide (4 mol/dm^3) and freeze dried. Samples (500 mg, $n = 3$) of the freeze-dried blueberry extract were incubated (72 h, 37°C, pH 6.8 ± 0.2, under CO_2) with faecal slurries (0.2% v/v in YCFA medium, 10 cm^3) from two human volunteers consuming an unrestricted Western-style diet and who had not received antibiotics within at least 3 months of sampling. The pH was reduced to 2 with HCl (2 mol/dm^3) and extracted into ethyl acetate (5 cm^3), separating the layers by centrifugation (3000 rpm, 5 min). This process was repeated three times and the organic layers combined, filtered through $MgSO_4$ and evaporated to dryness under reduced pressure at a temperature of less than 40°C. The extracts were then redissolved in methanol (0.2 cm^3) and filtered through a 0.2 μm PVDF membrane. Separation of the phenolic compounds was by HPLC employing two gradient elution methods using AcCN and TFA (0.05% v/v, pH 2.3). Method 1: 11 – 14% AcCN (35 min), 14 – 50% AcCN (5 min), 50% AcCN (10 min) and 50 – 11% AcCN (5 min). Method 2: 11 – 40% AcCN (40 min), 40 – 50% AcCN (10 min) and 50 – 11% AcCN (5 min). Detection was at 215 and 280 nm and the metabolites were quantified by internal standardisation and use of response factors calculated from pure compounds.

2.4 Effect of blueberry phenolics and metabolites on prostanoid production

Inflammatory properties were compared by measuring the ability of the parent compounds and their metabolites to inhibit prostanoid production in a cell system in which the inflammatory pathways were up-regulated following a cytokine-induced insult as reported previously [18]. In brief, fibroblast (CCD-Co18) cells (ATCC, Middlesex, UK) at P5 (PDL 27) were seeded at a density 1×10^5 well^{-1} in a 24-well cell culture plate and treated with IL-1β. At the end of the 2-h stimulation period, aliquots were removed and screened with a prostaglandin screening EIA (Cayman Chemicals, Ann Arbor, MI) designed to measure a wide range of prostanoids. Samples were appropriately diluted and immediately assayed according to the manufacturers' instructions. The solvents were removed from the extracts and they were reconstituted in methanol and appropriately diluted in cell culture media. All extracts and appropriate controls were added 30 min prior to stimulation and were representative of 125 mg of blueberry (dry weight). This corresponded to mean individual concentrations of phenolic acids of 0.048 ± 0.117 mmol/dm^3, which is potentially achievable in the human colon [16].

3 Results

3.1 Phenolic acids in blueberries

Blueberries were solvent extracted to remove the free phenolic acids and the bulk of the other free phenolic components. The free phenolic acids were analysed and found to contain syringic, vanillic, salicylic, ferulic, gallic and protocatechuic acid (Table 1). One portion of the remaining aqueous fraction containing the phenolic acids bound to other cell components was reserved for microbial metabolism studies and the bound phenolic acids were released by alkali and acid hydrolyses and analysed. The majority of the phenolic acids were in this fraction (3371.16 ± 422.30 mg/kg dry weight) compared to the free phenolic acid fraction (205.04 ± 45.34 mg/kg dry weight). This fraction contained caffeic, syringic, protocatechuic, *p*-hydroxybenzoic, vanillic, ferulic, *p*-coumaric, gentisic, sinapic, *m*-coumaric and salicylic acids (Table 1).

3.2 Metabolism of blueberry phenolics

The fraction containing the ethyl acetate insoluble phenolic acids bound to other cell components represents the fraction of phenolic acids most likely to reach the colon. To assess the potential of these phenolic acids to be released and metabolised by the gut microbiota, this fraction was incubated with faecal slurries from two human volunteers consuming an unrestricted Western-style diet. From the same quantity of extracted blueberries, the free phenolic compounds released and metabolised by volunteer 2 was almost two-fold higher than that of volunteer 1 (839.08 ± 208.98 mg/kg compared to 433.52 ± 153.08 mg/kg). Only very small quantities of the parent phenolic acids were detected after metabolism with faecal inoculants from either volunteer (Table 2), which is in keeping with previous results which indicated that the cinnamic acids are readily hydrogenated, demethoxylated and selectively dehydroxylated following release by the gut microflora. The compounds and metabolites detected were mostly derivatives of the benzoic, phenylacetic and phenylpropionic acids (Table 2). Significant differences between volunteers were observed for 2-hydroxybenzoic (salicylic), 4-hydroxybenzoic (*p*-hydroxybenzoic), 3-methoxy-4-hydroxybenzoic (vanillic), 3,5-dimethoxy-4-hydroxyben-

Table 1. Free, alkali-labile and acid-labile phenolic acids from blueberries

Compound; t_R	Blueberry (mg/kg)		
	Free	Alkali labile	Acid labile
3,4,5-Trihydroxybenzoic acid; gallic acid; 4.46	27.06 ± 0.00	93.66 ± 50.42	49.14 ± 6.38
3,4-Dihydroxybenzoic acid; protocatechuic acid; 6.72	11.98 ± 2.70	74.66 ± 5.68	207.54 ± 59.68
4-Hydroxybenzoic acid; *p*-hydroxybenzoic acid; 10.40	n.d.	18.24 ± 3.00	180.72 ± 22.34
2,5-Dihydroxybenzoic acid; gentisic acid; 11.58	n.d.	3.98 ± 1.76	52.18 ± 8.10
3,4-Dihydroxycinnamic acid; caffeic acid; 12.85	n.d.	1047.04 ± 88.38	93.50 ± 16.86
3-Methoxy-4-hydroxybenzoic acid; vanillic acid; 13.45	40.24 ± 16.22	77.10 ± 20.16	89.90 ± 15.58
3,5-Dimethoxy-4-hydroxy benzoic acid; syringic acid; 15.37	60.60 ± 8.28	806.12 ± 47.40	287.94 ± 42.44
4-Hydroxycinnamic acid; *p*-coumaric acid; 22.21	n.d.	68.18 ± 2.62	13.80 ± 0.94
3-Hydroxycinnamic acid; *m*-coumaric acid; 27.35	n.d	34.32 ± 2.38	n.d.
3-Methoxy-4-hydroxycinnamic acid; ferulic acid; 29.23	29.64 ± 4.52	93.88 ± 6.18	24.60 ± 3.66
3,5-Dimethoxy-4-hydroxycinnamic acid; sinapic acid; 31.71	n.d.	40.14 ± 7.46	4.60 ± 4.90
2-Hydroxybenzoic acid; salicylic acid; 33.71	35.52 ± 13.62	7.42 ± 2.20	2.50 ± 2.32

Values are specified on a dry weight basis for blueberries that contained 82.5% moisture and are given as mean ± SD ($n = 3$). n.d., not detected. Retention times (t_R) are given in minutes.

Table 2. Phenolic compounds recovered from treatment of bound blueberry phenolic acids with faecal inoculants from two human volunteers consuming a Western-style diet

Compound	Blueberry (mg/kg)			*p*
	Bound phenolics	Metabolised volunteer 1	Metabolised volunteer 2	
2-Hydroxybenzoic acid	9.92 ± 4.52	6.06 ± 0.64	18.40 ± 2.58	0.011
4-Hydroxybenzoic acid	n.d.	49.94 ± 11.38	92.16 ± 15.54	0.022
2,5-Dihydroxybenzoic acid	56.16 ± 9.86	62.44 ± 12.52	50.06 ± 7.12	0.228
3,4,5-Trihydroxybenzoic acid	142.80 ± 56.80	15.82 ± 2.60	18.70 ± 2.88	0.269
3-Methoxybenzoic acid	n.d.	2.14 ± 3.70	15.5 ± 9.06	0.110
3,4-Dimethoxybenzoic acid	n.d.	n.d.	0.32 ± 0.56	0.423
3-Methoxy-4-hydroxybenzoic acid	167.00 ± 35.74	17.76 ± 4.06	80.74 ± 12.28	0.008
3,5-Dimethoxy-4-hydroxybenzoic acid	1094.06 ± 89.84	n.d.	2.94 ± 0.34	0.004
Phenylacetic acid	n.d.	24.46 ± 6.12	28.12 ± 8.00	0.564
3-Hydroxyphenylacetic acid	n.d.	24.60 ± 4.00	43.48 ± 3.72	0.131
4-Hydroxyphenylacetic acid	n.d.	26.22 ± 2.12	33.96 ± 9.62	0.297
3,4-Dihydroxyphenylacetic acid	n.d.	24.24 ± 1.92	58.46 ± 16.68	0.069
3-Methoxy-4-hydroxyphenylacetic acid	n.d.	n.d.	74.20 ± 11.18	0.007
Cinnamic acid	n.d.	11.30 ± 6.86	3.10 ± 5.38	0.183
3-Hydroxycinnamic acid	34.32 ± 2.38	2.04 ± 3.52	0.98 ± 1.08	0.660
4-Hydroxycinnamic acid	81.98 ± 3.56	n.d.	n.d.	
3,4-Dihydroxycinnamic acid	1140.54 ± 105.24	18.92 ± 5.52	77.48 ± 25.50	0.052
4-Methoxycinnamic acid	n.d.	11.14 ± 10.42	n.d.	0.205
3,4-Dimethoxycinnamic acid	n.d.	45.80 ± 15.64	59.34 ± 20.84	0.423
3-Methoxy-4-hydroxycinnamic acid	118.48 ± 9.84	n.d.	0.50 ± 0.88	0.423
3,5-Dimethoxy-4-hydroxycinnamic acid	44.74 ± 12.36	n.d.	n.d.	
Phenylpropionic acid	n.d.	17.00 ± 0.54	22.64 ± 4.26	0.147
3-Hydroxyphenylpropionic acid	n.d.	16.42 ± 4.84	9.00 ± 7.84	0.249
4-Hydroxyphenylpropionic acid	n.d.	n.d.	47.86 ± 5.18	0.004
3,4-Dihydroxyphenylpropionic acid	n.d.	57.22 ± 56.76	94.90 ± 26.20	0.378
3-Methoxy-4-hydroxyphenylpropionic acid	n.d.	n.d.	6.22 ± 2.08	0.036

Values are specified on a dry weight basis for blueberries that contained 82.5% moisture and are given as mean ± SD ($n = 3$). n.d., not detected. Significant differences between the products obtained from metabolism of the bound phenolic fraction by faecal slurries from volunteers 1 and 2 were determined by the *t*-test (ρ).

zoic (syringic), 3-methoxy-4-hydroxyphenylacetic, 4-hydroxy phenylpropionic and 3-methoxy-4-hydroxyphenyl propionic acid.

3.3 Inflammatory effect of blueberry phenolics and their metabolites

Comparison of the anti-inflammatory properties was made by measuring the ability of the blueberry extracts and their metabolites to modulate prostanoid production in a cell system in which the inflammatory pathways were up-regulated following a cytokine-induced insult [18]. Following IL-1β-treatment of these cells both the free phenolic acids and the bound phenolic acids (released by hydrolysis) increased the amount of total prostanoids produced from 3.17 ± 1.36 to 12.40 ± 2.19 and 7.79 ± 4.46 ng/cm^3, respectively (Fig. 2). Compared with the control, bound phenolic compounds which were metabolised by faecal slurries obtained from volunteer 1 significantly decreased the amount of prostanoids produced to 1.67 ± 0.69 ng/cm^3 (Fig. 2). Conversely, the bound phenolic compounds which were metabolised by faecal slurries from volunteer 2 significantly increased prostanoid production to 10.78 ± 5.54 ng/cm^3 (Fig. 2).

4 Discussion

Blueberries are a rich source of a variety of phenolic acids, most of which are attached to other plant cell components and have the potential to reach the colon before they are released. In a cell model in which the inflammatory pathways have been up-regulated, the parent free phenolic acids significantly increased the amount of prostanoids produced. This is not surprising as this fraction contained a large portion of hydroxylated phenolic acids, previously found to increase prostanoid production in normal colon cells treated with IL-1β [18]. However, despite the potential for the free phenolic acid fraction to be pro-inflammatory, it may be that these compounds are absorbed in the small intestine where they are conjugated and/or rapidly excreted in the urine. The fraction containing phenolic acids released by acid and alkaline hydrolysis also increased the amount of prostanoids produced, but this did not attain significance. The phenolic acids attached to other cell wall components have the potential to be released and extensively metabolised by the human gut microbiota. These components of the faecal matrix are an important factor to consider, as they have been shown to influence several cellular variables

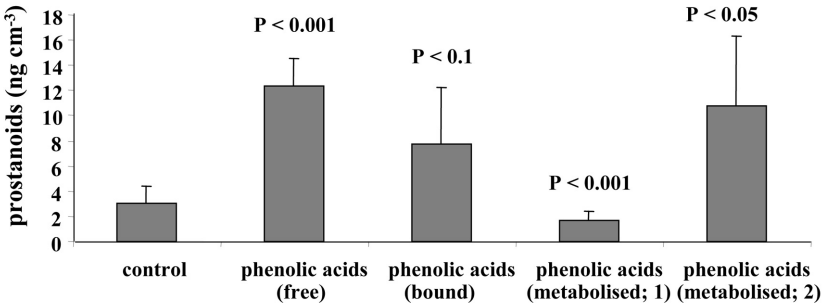

Figure 2. Effect of the parent blueberry extracts and their metabolites on prostanoid production. The phenolic acid extracts included the free phenolic acids and bound phenolic acids released by alkali/acid hydrolysis and the bound phenolic acids metabolised by faecal inoculants from two human volunteers consuming a Western-style diet. Human colon fibroblast cells at passage 5; population doubling 27 (P5) were treated with the individual phenolic extracts 30 min prior to cytokine stimulation. Prostanoid concentration was measured after 2 h stimulation with IL-1β (10 ng/cm^3). Values are given as mean ± SD ($n = 6$). Significant differences between the IL-1β-stimulated cells and IL-1β-stimulated cells in the presence of extracts were determined by the *t*-test (ρ).

including proliferation, apoptosis, cell signalling pathways and markers of tumorigenesis [19–21]. Microbial metabolism of the bound phenolic acid fraction, which occurred by incubation with mixed faecal microbiota from two volunteers, resulted in significant differences in both the amount and variety of phenolic compounds available (Table 2). There were also marked differences in the effect of these phenolic extracts on prostanoid production with both a significant decrease and increase in prostanoids production being observed for incubation with samples obtained from each of the volunteers (Fig. 2). These results suggest that any potential protective effect of blueberry phenolics as anti-inflammatory agents in the colon is likely to occur as a result of microbial metabolism and that this is likely to depend on the composition of each individual's colonic microbiota. Before such health claims can be fully established for dietary phenolics from plant-based foods, studies addressing a wide range of human volunteers, well characterised with respect to the relative abundance of bacterial groups and both demographic and nutritional status will be required.

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