



Effects of pelargonidin-3-O-glucoside and its metabolites on lipopolysaccharide-stimulated cytokine production by THP-1 monocytes and macrophages

Article

Accepted Version

Creative Commons: Attribution-Noncommercial-No Derivative Works 4.0

Amini, A. M., Spencer, J. P. E. and Yaqoob, P. (2018) Effects of pelargonidin-3-O-glucoside and its metabolites on lipopolysaccharide-stimulated cytokine production by THP-1 monocytes and macrophages. *Cytokine*, 103. pp. 29-33. ISSN 1096-0023 doi: <https://doi.org/10.1016/j.cyto.2017.12.031>
Available at <http://centaur.reading.ac.uk/75012/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1016/j.cyto.2017.12.031>

Publisher: Elsevier

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

1 **Effects of pelargonidin-3-O-glucoside and its metabolites on lipopolysaccharide-**
2 **stimulated cytokine production by THP-1 monocytes and macrophages**

3

4 Anna M. **Amini**^a, Jeremy PE. **Spencer**^a, Parveen **Yaqoob**^a

5

6 ^aDepartment of Food and Nutritional Sciences, University of Reading, Whiteknights PO Box
7 226, Reading RG6 6AP, UK

8

9 **Corresponding author:** Parveen Yaqoob, Telephone: +44 (0) 118 378 8720, E-mail:
10 p.yaqoob@reading.ac.uk

11

12 **Anna M. Amini's E-mail:** amini.anna@gmail.com

13 **Jeremy PE. Spencer's E-mail:** j.p.e.spencer@reading.ac.uk

14

15 **Abbreviations:** 4-HBA, 4-hydroxybenzoic acid; CVD, cardiovascular disease; FCS, fetal
16 calf serum; IL, interleukin; LPS, lipopolysaccharide; PCA, protocatechuic acid; Pg-3-glc,
17 pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; PMA, phorbol-12-myristate-13-
18 acetate; TNF- α , tumor necrosis factor- α ;

19 **Abstract**

20 Epidemiological evidence suggests cardioprotective effects of anthocyanin consumption. This
21 study examined the predominant strawberry anthocyanin, pelargonidin-3-O-glucoside (Pg-3-
22 glc), and three of its plasma metabolites (protocatechuic acid [PCA], 4-hydroxybenzoic acid,
23 and phloroglucinaldehyde [PGA]) for effects on the production of selected cytokines by
24 lipopolysaccharide-stimulated THP-1 monocytes and macrophages. Concentrations of tumor
25 necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, IL-8 and IL-10 were determined using a
26 cytometric bead array kit. PCA at 0.31, 1.25 and 20 μ M and PGA at 5 and 20 μ M decreased
27 the concentration of IL-6 in the monocyte cultures, but there were no effects on TNF- α , IL-1 β ,
28 IL-8 and IL-10 and there were no effects of the other compounds. In the macrophage cultures,
29 PGA at 20 μ M decreased the concentrations of IL-6 and IL-10, but there was no effect on
30 TNF- α , IL-1 β and IL-8 and there were no effects of the other compounds. In conclusion,
31 while the effects of PGA were only observed at the higher, supraphysiological concentration
32 and are thus considered of limited physiological relevance overall, the anti-inflammatory
33 properties of PCA were observed at both the lower, physiologically relevant, and the higher
34 concentrations; however, effects were modest and limited to IL-6 and monocytes. These
35 preliminary data suggest potential for physiologically attainable PCA concentrations to
36 modulate IL-6 production by monocytes.

37

38 **Keywords:** Anthocyanin, Cytokine, Inflammation, Pelargonidin-3-O-glucoside, Strawberry

39 **2.1 Introduction**

40 Epidemiological evidence links anthocyanin consumption with lower risk of cardiovascular
41 disease (CVD) [1] and CVD risk factors [2]. The mechanisms are not yet fully elucidated, but
42 evidence suggests modulation of vascular function, platelet aggregation and inflammation [3-
43 9]. TNF- α , IL-1 β , IL-6, IL-8 and IL-10 are inflammatory cytokines that play a critical role in
44 atherosclerosis, the underlying cause of most CVDs [10].

45 Strawberries are particularly rich in anthocyanins, predominantly pelargonidin-3-O-glucoside
46 (Pg-3-glc). Glucuronidated pelargonidin has been reported as the predominant metabolite in
47 three pharmacokinetic studies [11-13], but there is ambiguity regarding the position of
48 glucuronidation and glucuronidated pelargonidin compounds are currently commercially
49 unavailable and hence cannot be tested in cellular models. 4-hydroxybenzoic acid (4-HBA)
50 and protocatechuic acid (PCA) have also been reported in plasma following strawberry
51 consumption in low micromolar concentrations (0.1-2 μ M) [13-15]. In addition, it is likely
52 that phloroglucinaldehyde (PGA) might appear in plasma following strawberry consumption,
53 as it is a A-ring degradant, reported in plasma upon anthocyanin consumption in low to high
54 nanomolar concentrations (20-600 nM) [16, 17]. However, most studies exploring the effect
55 of anthocyanins to modulate cytokine secretion used unmetabolized parent anthocyanins,
56 often at supraphysiologically high doses [3, 4]. In addition, although macrophages are an
57 important source of inflammatory cytokines [18], there are no studies exploring the potential
58 modulation of cytokine production by anthocyanins or their metabolites by human-derived
59 macrophages, or studies comparing the effects in human-derived monocytes versus
60 macrophages. Thus, the aim of this study was to examine the parent anthocyanin Pg-3-glc and
61 three physiologically relevant plasma metabolites for effects on the production of selected

- 62 pro- and anti-inflammatory cytokines (tumor necrosis factor- α [TNF- α], interleukin [IL]-1 β ,
63 IL-6, IL-8 and IL-10) in lipopolysaccharide (LPS)-induced THP-1 cells.

64 **2.2 Materials and methods**

65 **2.2.1 Chemicals and reagents**

66 Pg-3-glc was purchased from Extrasynthese (Genay, France). PCA (3,4-dihydroxybenzoic
67 acid), 4-HBA, PGA (2,4,6-trihydroxybenzaldehyde), LPS from *Escherichia coli*, phorbol 12-
68 myristate 13-acetate (PMA), methanol and formic acid were purchased from Sigma-Aldrich
69 (Dorset, United Kingdom). RPMI 1640 culture medium, fetal calf serum (FCS), penicillin
70 and streptomycin were purchased from Lonza (Basel, Switzerland). The cytometric bead
71 array kit to analyze cytokine concentrations was purchased from BD Biosciences (Oxford,
72 United Kingdom).

73 **2.2.2 Preparation and culture of THP-1 cells**

74 THP-1 cells (human monocytic leukemia, ECACC 88081201) were cultured in RPMI 1640
75 culture medium supplemented with 100 UI/mL streptomycin, 100 µg/mL penicillin and 10%
76 (v/v) FCS at 37 °C in a humidified atmosphere of 5% CO₂ and kept at a density of 2-
77 9 x 10⁵ cells/mL. For the experiments, cells were seeded in 24 well plates at a density of
78 1 x 10⁶ cells/mL. For differentiation into macrophages, cells were exposed to PMA at a final
79 concentration of 0.1 µM for 72 h [19]. Polyphenols were added to provide final
80 concentrations of 0.08, 0.31, 1.25, 5 and 20 µM. Polyphenols were added in 20 µL of 10.98%
81 methanol and 0.22% formic acid to produce a final concentration of 0.22% methanol and
82 0.004% formic acid in culture. The final culture volume was 1 mL. LPS (20 µL; 1 µg/mL
83 final concentration) was added to stimulate cytokine production.

84 After 24 h incubation at 37 °C in a humidified atmosphere of 5% CO₂, plates were
85 centrifuged at 260 x g for 5 min and culture supernatants were collected and stored in aliquots
86 at -20 °C until analysis.

87 **2.2.3 Measurement of cytokine concentrations**

88 Concentrations of TNF- α , IL-1 β , IL-6, IL-8 and IL-10 in the culture supernatants were
89 measured using a cytometric bead array kit from BD Biosciences (Oxford, United Kingdom)
90 according to the manufacturer's instructions. Data were acquired on a BD FACS Canto™ II
91 flow cytometer and analyzed using the BD FCAP Array v3 software. Limits of detection of
92 the cytokine assays were 0.13 pg/mL (IL-10), 1.2 pg/mL (TNF- α and IL-8), 1.6 pg/mL (IL-6)
93 and 2.3 pg/mL (IL-1 β).

94 **2.2.4 Cytotoxicity**

95 To determine whether test compounds had any cytotoxic effects, cell viability of THP-1
96 monocytes and macrophages was assessed using Trypan blue staining.

97 **2.2.5 Statistical analyses**

98 Results are expressed as percentage of cytokine production versus control (no polyphenols)
99 and shown as means with their standard deviations (SD). One-way ANOVA was performed
100 to determine whether test compounds affected the cytokine production, followed by Dunnett
101 as post hoc analysis versus control group. Statistical analysis was performed using SPSS 21
102 (IBM Corporation, New York, USA) and a lowered $P < 0.01$ was considered significant to
103 account for multiple comparisons.

104 **2.3 Results**

105 **2.3.1 Effects of Pg-3-glc, PCA, 4-HBA and PGA on viability of THP-1** 106 **cells**

107 There were no cytotoxic effects of the studied compounds on THP-1 monocytes or
108 macrophages at any of the tested doses (data not shown).

109 **2.3.2 Effects of Pg-3-glc, PCA, 4-HBA and PGA on cytokine** 110 **production by THP-1 monocytes**

111 Stimulation with LPS increased IL-1 β production 135-fold, TNF- α production 105-fold, IL-6
112 production 470-fold, IL-8 production 640-fold and IL-10 production 5-fold. PCA
113 significantly reduced IL-6 production at 0.31, 1.25 and 20 μ M compared to the control
114 cultures (all $P < 0.01$, Table 1). PGA also significantly inhibited IL-6 production and while the
115 effects were only significant at 5 and 20 μ M ($P < 0.01$ and $P < 0.001$ respectively, Table 1),
116 they were slightly more potent (production was lowered by 25-35% compared to 20% for
117 PCA) and appeared to be concentration-dependent. There was no significant effect of any
118 tested compound, at concentrations up to 20 μ M, on the production of IL-1 β , TNF- α , IL-8 or
119 IL-10 by THP-1 monocytes (Table 1).

120 **2.3.3 Effects of Pg-3-glc, PCA, 4-HBA and PGA on cytokine** 121 **production by THP-1 macrophages**

122 Stimulation with LPS increased IL-1 β production 10-fold, TNF- α production 45-fold, IL-6
123 production 220-fold, IL-8 production 80-fold and IL-10 production 5-fold. PGA was the only
124 compound that induced significant changes in cytokine production. PGA at the highest dose

125 tested (20 μ M) significantly lowered IL-6 production ($P<0.001$, Table 2), similar to the
126 monocyte results and with a similar magnitude of effect. Furthermore, it also significantly
127 decreased IL-10 production (20 μ M; $P<0.01$; Table 2), an effect not observed in THP-1
128 monocytes. There was no significant effect of any of the tested compounds, at concentrations
129 up to 20 μ M, on the production of IL-1 β , TNF- α or IL-8 by THP-1 macrophages (Table 2).

130
131

Table 1 Effect of Pg-3-glc, PCA, 4-HBA and PGA on IL-1 β , TNF- α , IL-6, IL-8 and IL-10 production by THP-1 monocytes

	Cytokine production (% of control)				
	IL-1 β	TNF- α	IL-6	IL-8	IL-10
Pg-3-glc					
0 μ M	100	100	100	100	100
0.08 μ M	91.8 \pm 18.9	114.2 \pm 20.5	85.6 \pm 17.5	108.6 \pm 15.6	113.4 \pm 12.4
0.31 μ M	92.7 \pm 10.7	110.8 \pm 20.3	78.2 \pm 13.2	102.3 \pm 19.6	104.0 \pm 8.9
1.25 μ M	93.2 \pm 8.4	112.9 \pm 20.1	78.4 \pm 11.8	96.1 \pm 13.0	102.9 \pm 10.4
5.00 μ M	92.3 \pm 9.6	106.4 \pm 19.4	84.5 \pm 10.4	101.9 \pm 12.1	102.5 \pm 8.0
20.00 μ M	96.4 \pm 13.9	101.1 \pm 20.0	88.5 \pm 18.6	103.9 \pm 19.4	98.0 \pm 4.9
PCA					
0 μ M	100	100	100	100	100
0.08 μ M	94.5 \pm 7.8	103.5 \pm 18.9	87.3 \pm 7.0	110.3 \pm 13.0	107.0 \pm 6.9
0.31 μ M	97.7 \pm 7.5	93.4 \pm 15.3	77.8 \pm 9.7 **	98.2 \pm 7.1	104.6 \pm 5.9
1.25 μ M	104.9 \pm 5.2	92.9 \pm 10.7	77.2 \pm 10.2 **	106.0 \pm 9.0	105.4 \pm 7.0
5.00 μ M	95.2 \pm 12.8	85.7 \pm 7.9	88.3 \pm 15.9	100.5 \pm 14.9	99.0 \pm 9.5
20.00 μ M	89.3 \pm 19.3	85.9 \pm 11.0	79.8 \pm 14.1 **	102.4 \pm 10.1	93.6 \pm 7.6
4-HBA					
0 μ M	100	100	100	100	100
0.08 μ M	83.4 \pm 7.6	104.2 \pm 21.2	99.1 \pm 14.5	94.0 \pm 18.2	99.9 \pm 8.6
0.31 μ M	97.7 \pm 10.4	109.3 \pm 22.5	90.9 \pm 5.1	91.3 \pm 15.7	104.8 \pm 8.9
1.25 μ M	97.3 \pm 15.3	110.4 \pm 26.4	91.9 \pm 3.6	90.9 \pm 16.5	101.8 \pm 6.9
5.00 μ M	100.2 \pm 8.6	107.2 \pm 19.5	85.1 \pm 21.6	93.5 \pm 17.8	103.7 \pm 8.0
20.00 μ M	105.7 \pm 13.0	114.2 \pm 16.5	93.4 \pm 25.0	90.2 \pm 22.2	102.3 \pm 7.1
PGA					
0 μ M	100	100	100	100	100
0.08 μ M	97.2 \pm 11.6	111.4 \pm 21.3	90.0 \pm 16.2	103.2 \pm 13.7	110.6 \pm 13.1
0.31 μ M	102.1 \pm 7.4	107.5 \pm 21.4	91.9 \pm 17.2	96.8 \pm 21.1	104.6 \pm 11.0
1.25 μ M	106.5 \pm 11.8	112.9 \pm 18.9	79.7 \pm 10.6	97.0 \pm 13.8	101.2 \pm 9.0
5.00 μ M	110.4 \pm 12.3	117.0 \pm 22.1	73.6 \pm 6.9 **	95.2 \pm 20.6	97.1 \pm 9.2
20.00 μ M	106.2 \pm 12.6	111.6 \pm 21.7	65.9 \pm 17.6 ***	94.6 \pm 19.3	95.6 \pm 6.1

132 THP-1 monocytes (1×10^6 cells/mL) were treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at
 133 concentrations of 0-20 μ M, prior to lipopolysaccharide stimulation (1 μ g/mL) and incubated for 24 h at 37 $^{\circ}$ C.
 134 Cytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are
 135 expressed as percentage of cytokine concentration vs. control (no polyphenols). Data are represented as the
 136 mean \pm SD of six independent experiments. Data were analyzed by one-way ANOVA and Dunnett post hoc
 137 analysis, where applicable, and a lowered $P < 0.01$ was considered significant to account for multiple
 138 comparisons. Statistically significant differences are denoted as ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control.
 139 4-HBA, 4-hydroxybenzoic acid; IL, interleukin; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-
 140 glucoside; PGA, phloroglucinaldehyde; TNF- α , tumor necrosis factor- α .

141
142

Table 2 Effect of Pg-3-glc, PCA, 4-HBA and PGA on IL-1 β , TNF- α , IL-6, IL-8 and IL-10 production by THP-1 macrophages

	Cytokine production (% of control)				
	IL-1 β	TNF- α	IL-6	IL-8	IL-10
Pg-3-glc					
0 μ M	100	100	100	100	100
0.08 μ M	113.2 \pm 18.8	117.3 \pm 31.0	113.5 \pm 24.7	111.4 \pm 12.8	100.9 \pm 17.4
0.31 μ M	111.6 \pm 15.1	115.8 \pm 22.2	108.0 \pm 21.6	106.5 \pm 12.2	87.3 \pm 15.1
1.25 μ M	108.9 \pm 12.3	112.4 \pm 16.0	101.4 \pm 24.1	105.8 \pm 16.9	88.0 \pm 16.8
5.00 μ M	107.8 \pm 16.7	110.5 \pm 24.5	101.6 \pm 20.1	103.4 \pm 16.1	80.1 \pm 13.5
20.00 μ M	99.2 \pm 14.7	106.0 \pm 18.1	107.7 \pm 19.8	102.9 \pm 11.8	84.3 \pm 16.0
PCA					
0 μ M	100	100	100	100	100
0.08 μ M	102.7 \pm 9.3	117.2 \pm 23.2	116.2 \pm 25.2	106.5 \pm 16.1	100.8 \pm 12.0
0.31 μ M	103.8 \pm 10.0	101.0 \pm 18.6	111.7 \pm 22.9	104.4 \pm 14.1	85.0 \pm 24.9
1.25 μ M	100.8 \pm 6.8	96.5 \pm 26.0	101.8 \pm 23.6	101.7 \pm 12.5	84.1 \pm 22.2
5.00 μ M	100.9 \pm 10.8	101.7 \pm 17.5	107.2 \pm 23.9	98.7 \pm 12.0	83.2 \pm 19.6
20.00 μ M	99.1 \pm 9.9	100.6 \pm 22.9	88.6 \pm 19.4	103.7 \pm 12.0	77.8 \pm 27.2
4-HBA					
0 μ M	100	100	100	100	100
0.08 μ M	108.7 \pm 17.9	115.3 \pm 28.7	107.9 \pm 21.4	111.3 \pm 13.6	106.2 \pm 13.5
0.31 μ M	107.4 \pm 17.1	112.2 \pm 27.7	104.2 \pm 17.0	103.4 \pm 16.6	93.1 \pm 15.0
1.25 μ M	110.2 \pm 18.2	109.6 \pm 29.3	101.1 \pm 14.4	102.6 \pm 19.5	90.8 \pm 19.0
5.00 μ M	109.9 \pm 16.8	97.8 \pm 27.3	97.4 \pm 27.4	103.0 \pm 15.2	88.5 \pm 16.5
20.00 μ M	112.2 \pm 18.4	109.7 \pm 27.9	94.7 \pm 25.8	95.2 \pm 16.1	94.8 \pm 11.6
PGA					
0 μ M	100	100	100	100	100
0.08 μ M	106.6 \pm 7.4	113.9 \pm 21.7	109.5 \pm 17.6	110.9 \pm 16.4	110.3 \pm 21.2
0.31 μ M	111.4 \pm 11.9	107.6 \pm 18.0	95.7 \pm 18.6	104.8 \pm 11.9	77.6 \pm 15.3
1.25 μ M	112.3 \pm 8.7	100.5 \pm 18.7	79.7 \pm 16.0	103.1 \pm 21.2	72.1 \pm 16.0
5.00 μ M	110.9 \pm 16.3	98.5 \pm 21.4	71.8 \pm 19.1	106.3 \pm 12.3	70.9 \pm 19.1
20.00 μ M	113.7 \pm 17.9	110.5 \pm 23.7	58.8 \pm 25.6 ***	115.6 \pm 23.2	65.4 \pm 26.2 **

143
144
145
146
147
148
149
150
151
152

THP-1 monocytes (1×10^6 cells/mL) were converted to macrophages through exposure to 0.1 μ M phorbol-12-myristate-13-acetate for 72 h. THP-1 macrophages were treated with Pg-3-glc, PCA, 4-HBA, PGA or vehicle control at concentrations of 0-20 μ M, prior to lipopolysaccharide stimulation (1 μ g/mL) and incubated for 24 h at 37 $^{\circ}$ C. Cytokine concentrations in the culture supernatants were measured using a cytometric bead array kit. Results are expressed as percentage of cytokine concentration vs. control (no polyphenols). Data are represented as the mean \pm SD of six independent experiments. Data were analyzed by one-way ANOVA and Dunnett post hoc analysis, where applicable, and a lowered $P < 0.01$ was considered significant to account for multiple comparisons. Statistically significant differences are denoted as ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control. 4-HBA, 4-hydroxybenzoic acid; IL, interleukin; PCA, protocatechuic acid; Pg-3-glc, pelargonidin-3-O-glucoside; PGA, phloroglucinaldehyde; TNF- α , tumor necrosis factor- α .

153 **2.4 Discussion**

154 This study aimed to compare the effects of Pg-3-glc and its plasma metabolites PCA, 4-HBA
155 and PGA on cytokine secretion by LPS-stimulated THP-1 cells. There were modest anti-
156 inflammatory effects of some of the tested compounds in THP-1 monocytes, with PCA and
157 PGA inhibiting IL-6 production, but there were no effects on TNF- α , IL-1 β , IL-8 and IL-10
158 and there were no effects of the other compounds. The effects in macrophages were slightly
159 different. Whilst PGA inhibited IL-6 production by THP-1 derived macrophages, it also
160 inhibited IL-10 production, which was not observed in THP-1 monocytes.

161 IL-6 is generally classified as a pro-inflammatory cytokine and the inhibition of LPS-
162 stimulated IL-6 secretion by PCA and PGA in the monocyte cultures and PGA in the
163 macrophage cultures is a novel observation. Importantly, the inhibitory effect of PCA was
164 observed at physiologically attainable [14, 15, 17] low micromolar concentrations. Taken
165 together with previous investigations, the current results contribute to the suggestion that
166 PCA acts on multiple cell types involved in IL-6 secretion during atherosclerosis, including
167 endothelial [7] and dendritic cells [4], although the latter experiment applied a
168 supraphysiological PCA dose of 25 μ M. It is important to note in this context that the
169 presence of PCA in plasma has also been reported following ingestion of other anthocyanins
170 [42, 53, 54], the flavonol quercetin [55] and it is also naturally present in several other dietary
171 sources, such as chicory, olives, raspberries, dates and onions [2, 56, 57], but it is currently
172 not known what proportion in plasma comes from these sources and what proportion
173 indirectly as a metabolite. Whilst the bioactivity of PGA on IL-6 was a novel observation, the
174 effect was only significant at the higher 5 and 20 μ M concentrations. As levels in circulation
175 remain below micromolar concentration (even after anthocyanin consumption), this finding is
176 likely of limited physiological relevance [16, 17].

177 IL-10 is generally classified as an anti-inflammatory cytokine. PGA decreased the production
178 of IL-10 in THP-1 macrophages, suggesting a pro-inflammatory tendency and this seems to
179 contrast its IL-6-reducing (i.e. anti-inflammatory) effect. However, the IL-10 decreasing
180 effect was observed only at high concentrations that are unlikely to be achieved *in vivo*. To
181 our knowledge, no other studies have investigated the effect of PGA on IL-10 production by
182 monocytes or macrophages. There is evidence to suggest that the inhibition of IL-6 and IL-10
183 production could be linked in a way that inhibition of IL-6 production could indirectly inhibit
184 IL-10 production [20]. There were no effects of any of the other tested compounds on IL-10
185 levels, which is in line with experiments on the effect of PCA in human monocyte-derived
186 dendritic cells [4] and Pg-3-glc, PCA and 4-HBA in THP-1 monocytes [6]. Beneficial effects
187 on IL-10 production were observed in human leukocytes with tea-derived polyphenols [21],
188 suggesting differential bioactivity of different polyphenol classes, although effects were only
189 observed at the higher, supraphysiological polyphenol doses (10 and 20 μM).

190 4-HBA had no effect on the secretion of any of the cytokines. Consistent with this, 4-HBA at
191 0.1-10 μM was previously reported to have no effect on TNF- α secretion by LPS-stimulated
192 THP-1 monocytes. Interestingly, however, this study is indicative of additive/synergistic
193 effects between polyphenols as an inhibitory effect on TNF- α was reported when 4-HBA was
194 coincubated with PCA or PCA plus vanillic acid, none of which was bioactive in isolation [6].
195 Furthermore, the reported IL-1 β -reducing effect of 4-HBA is in contrast to the present study,
196 despite both studies employing similar concentrations of 4-HBA, the same cell line and
197 similar culture conditions, but the current experiment conducted no preincubation with test
198 compounds. In peripheral blood mononuclear cells, 1 μM 4-HBA had no effect on IL-1 β or
199 IL-6 levels in accordance with the present data, but a modest 10% increase in TNF- α levels
200 was observed, which could be related to the differences in cell type and culture conditions.

201 The discrepant findings regarding the effect of 4-HBA on IL-1 β and TNF- α merit further
202 investigation.

203 In this study only PCA and PGA modulated cytokine secretion, whose effects were targeted
204 towards IL-6 and IL-10, while having no impact on the other cytokines. In contrast, 4-HBA
205 and Pg-3-glc did not affect any of the cytokines tested. These results suggest that at least two
206 hydroxyl groups (either *ortho* or *para* to each other) might be required for bioactivity. In line
207 with this suggestion, potent radical scavenging activity of polyphenols was previously linked
208 to the presence of an *ortho*-dihydroxy group [22] and was furthermore correlated with the
209 number of hydroxyl groups on the B-ring [23]. In order to identify chemical structures or
210 properties required for anti-inflammatory effects, screening studies with a larger number of
211 related compounds are required.

212 **2.5 Conclusion**

213 In conclusion, the data suggest that PCA may possess anti-inflammatory properties through
214 modulation of IL-6 production, which could contribute to protective effects in inflammatory
215 diseases. Importantly, this action was observed at physiologically attainable concentrations,
216 although the effect was modest and limited to monocytes. The IL-6 and IL-10-reducing
217 effects of PGA were only observed at the higher, supraphysiological concentration and are
218 thus considered of limited physiological relevance. Pg-3-glc and 4-HBA had no effect on any
219 of the tested cytokines. Future studies should focus on screening a larger number of related
220 compounds in order to identify chemical structures or properties required for anti-
221 inflammatory effects and underlying mechanisms.

222 **2.6 Acknowledgments**

223 The authors declare no conflicts of interest related to this experiment.

224

225 **Funding:** This work was supported by a BBSRC CASE studentship with GlaxoSmithKline
226 as industrial sponsor. The sponsors had no role in the interpretation of the study. The study
227 design was approved by GlaxoSmithKline. Apart from that, the sponsors had no role in the
228 collection, analysis and interpretation of the data, in preparation of the manuscript and in the
229 decision to publish.

230 2.7 References

- 231 [1] Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH, Rimm EB. High anthocyanin intake is associated
232 with a reduced risk of myocardial infarction in young and middle-aged women. *Circulation*. 2013;127:188-96.
- 233 [2] Jennings A, Welch AA, Fairweather-Tait SJ, Kay C, Minihane A-M, Chowienczyk P, et al. Higher
234 anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women. *Am J Clin*
235 *Nutr*. 2012;96:781-8.
- 236 [3] Noratto GD, Angel-Morales G, Talcott ST, Mertens-Talcott SU. Polyphenolics from acai (*Euterpe oleracea*
237 *Mart.*) and red muscadine grape (*Vitis rotundifolia*) protect human umbilical vascular endothelial cells (HUVEC)
238 from glucose- and lipopolysaccharide (LPS)-induced inflammation and target MicroRNA-126. *J Agric Food*
239 *Chem*. 2011;59:7999-8012.
- 240 [4] Del Corno M, Varano B, Scazzocchio B, Filesi C, Masella R, Gessani S. Protocatechuic acid inhibits
241 human dendritic cell functional activation: Role of PPAR γ up-modulation. *Immunobiology*. 2014;219:416-24.
- 242 [5] Min SW, Ryu SN, Kim DH. Anti-inflammatory effects of black rice, cyanidin-3-O-beta-D-glycoside, and its
243 metabolites, cyanidin and protocatechuic acid. *Int Immunopharmacol*. 2010;10:959-66.
- 244 [6] di Gesso JL, Kerr JS, Zhang QZ, Raheem S, Yalamanchili SK, O'Hagan D, et al. Flavonoid metabolites
245 reduce tumor necrosis factor- α secretion to a greater extent than their precursor compounds in human THP-1
246 monocytes. *Mol Nutr Food Res*. 2015;59:1143-54.
- 247 [7] Amin HP, Czank C, Raheem S, Zhang QZ, Botting NP, Cassidy A, et al. Anthocyanins and their
248 physiologically relevant metabolites alter the expression of IL-6 and VCAM-1 in CD40L and oxidized LDL
249 challenged vascular endothelial cells. *Mol Nutr Food Res*. 2015;59:1095-106.
- 250 [8] Rodriguez-Mateos A, Heiss C, Borges G, Crozier A. Berry (poly)phenols and cardiovascular health. *J Agric*
251 *Food Chem*. 2014;62:3842-51.
- 252 [9] Erlund I, Koli R, Alftan G, Marniemi J, Puukka P, Mustonen P, et al. Favorable effects of berry
253 consumption on platelet function, blood pressure, and HDL cholesterol. *Am J Clin Nutr*. 2008;87:323-31.
- 254 [10] Ait-Oufella H, Taleb S, Mallat Z, Tedgui A. Recent advances on the role of cytokines in atherosclerosis.
255 *Arterioscler Thromb Vasc Biol*. 2011;31:969-79.
- 256 [11] Mullen W, Edwards CA, Serafini M, Crozier A. Bioavailability of pelargonidin-3-O-glucoside and its
257 metabolites in humans following the ingestion of strawberries with and without cream. *J Agric Food Chem*.
258 2008;56:713-9.
- 259 [12] Sandhu AK, Huang YC, Xiao D, Park E, Edirisinghe I, Burton-Freeman B. Pharmacokinetic
260 characterization and bioavailability of strawberry anthocyanins relative to meal intake. *J Agric Food Chem*.
261 2016;64:4891-9.
- 262 [13] Banaszewski K, Park E, Edirisinghe I, Cappozzo J, Burton-Freeman BM. A pilot study to investigate
263 bioavailability of strawberry anthocyanins and characterize postprandial plasma polyphenols absorption patterns
264 by Q-TOFLC/MS in humans. *J Berry Res*. 2013;3:113-26.
- 265 [14] Russell WR, Scobbie L, Labat A, Duthie GG. Selective bio-availability of phenolic acids from Scottish
266 strawberries. *Mol Nutr Food Res*. 2009;53:S85-S91.
- 267 [15] Azzini E, Vitaglione P, Intorre F, Napolitano A, Durazzo A, Foddai MS, et al. Bioavailability of strawberry
268 antioxidants in human subjects. *Br J Nutr*. 2010;104:1165-73.
- 269 [16] de Ferrars RM, Cassidy A, Curtis P, Kay CD. Phenolic metabolites of anthocyanins following a dietary
270 intervention study in post-menopausal women. *Mol Nutr Food Res*. 2014;58:490-502.

- 271 [17] de Ferrars RM, Czank C, Zhang Q, Botting NP, Kroon PA, Cassidy A, et al. The pharmacokinetics of
272 anthocyanins and their metabolites in humans. *Br J Pharmacol*. 2014;171:3268-82.
- 273 [18] Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev*.
274 2006;86:515-81.
- 275 [19] Kavanagh IC, Symes CE, Renaudin P, Nova E, Mesa MD, Boukouvalas G, et al. Degree of oxidation of
276 low density lipoprotein affects expression of CD36 and PPAR gamma, but not cytokine production, by human
277 monocyte-macrophages. *Atherosclerosis*. 2003;168:271-82.
- 278 [20] Steensberg A, Fischer CP, Keller C, Moller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and
279 cortisol in humans. *Am J Endocrinol Metab*. 2003;285:E433-E7.
- 280 [21] Crouvezier S, Powell B, Keir D, Yaqoob P. The effects of phenolic components of tea on the production of
281 pro- and anti-inflammatory cytokines by human leukocytes in vitro. *Cytokine*. 2001;13:280-6.
- 282 [22] Yokozawa T, Chen CP, Dong E, Tanaka T, Nonaka GI, Nishioka I. Study on the inhibitory effect of tannins
283 and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem Pharmacol*. 1998;56:213-22.
- 284 [23] Yi L, Chen CY, Jin X, Mi MT, Yu B, Chang H, et al. Structural requirements of anthocyanins in relation to
285 inhibition of endothelial injury induced by oxidized low-density lipoprotein and correlation with radical
286 scavenging activity. *FEBS Lett*. 2010;584:583-90.
- 287