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Citation: Keane, Karen, Bell, Phillip, Lodge, John, Constantinou, Costas, Jenkinson, Sarah, Bass, Rosemary and Howatson, Glyn (2016) Phytochemical uptake following human consumption of Montmorency tart cherry (*L. Prunus Cerasus*) and influence of phenolic acids on vascular smooth muscle cells in vitro. *European Journal of Nutrition*, 55 (4). pp. 1695-1705. ISSN 1436-6207

Published by: Springer

URL: <http://dx.doi.org/10.1007/s00394-015-0988-9> <<http://dx.doi.org/10.1007/s00394-015-0988-9>>

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**Title**

Phytochemical uptake following human consumption of Montmorency tart cherry (*L. Prunus Cerasus*) and influence of phenolic acids on vascular smooth muscle cells *in vitro*.

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## 1 Abstract

2 *Purpose:* To investigate the phytochemical uptake following human consumption of Montmorency tart cherry  
3 (L. Prunus Cerasus) and influence of selected phenolic acids on vascular smooth muscle cells *in vitro*.

4 *Methods:* In a randomized, double blinded, cross-over design, 12 healthy males consumed either 30 or 60 mL of  
5 Montmorency tart cherry concentrate. Following analysis of the juice composition, venous blood samples were  
6 taken before and 1, 2, 3, 5 and 8 h post consumption of the beverage. In addition to examining some aspects of  
7 the concentrate contents, plasma concentrations of protocatechuic (PCA), vanillic (VA) and chlorogenic acid  
8 (CHL) were analysed by reversed-phase high performance liquid chromatography (HPLC) with diode array for  
9 quantitation and mass spectrometry detection (LCMS) for qualitative purposes. Vascular smooth muscle cell  
10 migration and proliferation were also assessed *in vitro*.

11 *Results* Both the 30 mL and 60 mL doses of Montmorency cherry concentrate contained high amounts of total  
12 phenolics ( $71.37 \pm 0.11$ ;  $142.73 \pm 0.22 \text{ mg}\cdot\text{L}^{-1}$ ) and total anthocyanins ( $62.47 \pm 0.31$ ;  $31.24 \pm 0.16 \text{ mg}\cdot\text{L}^{-1}$ ), as  
13 well as large quantities of CHL ( $0.205 \pm 0.24$ ;  $0.410 \pm 0.48 \text{ mg}\cdot\text{L}^{-1}$ ) and VA ( $0.253 \pm 0.84$ ;  $0.506 \pm 1.68$   
14  $\text{mg}\cdot\text{L}^{-1}$ ). HPLC/LCMS identified two dihydroxybenzoic acids (PCA and VA) in plasma following MC  
15 concentrate consumption. Both compounds were most abundant 1-2 h post initial ingestion with traces  
16 detectable at 8 h post ingestion. Cell migration was significantly influenced by the combination of PCA and VA,  
17 but not in isolation. There was no effect of the compounds on cell proliferation.

18 *Conclusions:* These data show new information that phenolic compounds thought to exert vasoactive properties  
19 are bioavailable *in vivo* following MC consumption, and subsequently can influence cell behaviour. These data  
20 may be useful for the design and interpretation of intervention studies investigating the health effects of  
21 Montmorency cherries.

22 **Keywords:** Montmorency, Phenolic Acids, Bioavailability, Cell behaviour

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## 30 Introduction

31 Epidemiologic studies have shown that consumption of food and beverages containing polyphenols are  
32 associated with reduced cardiovascular morbidity and mortality [1,2]. Among the 20 most commonly  
33 consumed fruits, cherries appear to have the fifth highest total phenol content [3]. Tart Montmorency cherries  
34 (*L.Prunus cerasus*) and their processed products are a functional food of growing interest and have been shown  
35 to be high in numerous phytonutrients [4,5]. Data support the presence of several phytonutrients in  
36 Montmorency tart cherries including the flavonoids isorhamnetin, kaempferol, quercetin, catechin, epicatechin,  
37 procyanidins, and anthocyanins [6]. These phytonutrients might be capable of exerting beneficial physiological  
38 effects and could be used as an effective intervention in health maintenance and exercise recovery [7]. It has  
39 been previously shown that tart cherries attenuate circulating inflammatory markers [8,9,4], improve recovery  
40 following exercise [9] and improve sleep quality [10,11]. Despite previous studies in cell culture and animal  
41 models, where cherry extracts have been shown to exert a range of cardio-protective effects [12,5], there has  
42 been only two published studies illustrating the pharmacokinetics of tart cherry phytochemicals and concurrent  
43 evidence of a biologic effect [13] [14].

44 The health-related benefits have been postulated to arise from the high anthocyanin content of tart cherries;  
45 however the biological effectiveness of Montmorency cherries might be due to phytochemical interactions,  
46 which accomplish complementary effects. Such synergies occur when combinations of bioactive substances  
47 exert effects at target sites that are greater than the sum of individual components [15]. Furthermore, it has been  
48 noted that anthocyanins may not be stable during processing or storage [16]. Thus, it is not surprising that  
49 Montmorency cherry secondary metabolites could be biologically more active than individual ‘whole’  
50 components in cherries. The main non-flavonoid polyphenols of dietary significance are the C<sub>6</sub> – C<sub>1</sub> phenolic  
51 acids; these provide unique taste, flavour, and health-promoting properties and are found in many vegetables  
52 and fruits [17]. A human study, feeding blood orange juice suggested that the phenolic acid degradation product,  
53 protocatechuic acid (PCA), was a major metabolite of anthocyanins [18]. In addition, a range of phenolic acids,  
54 including vanillic acid (VA), syringic acid, caffeic acid and ferulic acid, have been identified within human  
55 serum, following the consumption of anthocyanin-rich berries [19,20], but data on the bioavailability of these  
56 phenolic acids are very scarce.

57 Vascular smooth muscle cells (VSMC) are responsible for the provision of vascular tone in normal, healthy  
58 blood vessels and their behaviour is critical in the development of atherosclerotic plaques [21]. The VSMC can  
59 be found in two states – healthy blood vessels are surrounded by differentiated VSMC in a contractile, quiescent  
60 state [22]. Part of the response to vascular injury and the cascade of events that lead to the build-up of an  
61 atherosclerotic plaque cause VSMC to de-differentiate in to a phenotype that proliferates and migrates [22].  
62 These de-differentiated cells migrate through the intima and form part of the fibrous cap on an atherosclerotic  
63 plaque. De-differentiated VSMC in these fibrous caps come in to direct contact with metabolites in the  
64 circulating blood and therefore makes them a biologically relevant cell type to test whether metabolites could  
65 impact on their behaviour.

66 The presence of phytochemical compounds in Montmorency tart cherries that might improve vascular health  
67 have yet to be fully elucidated, and importantly if these compounds can be absorbed and potentially exert a  
68 physiological effect. Furthermore, metabolites from ingestion of cherries that alter the behaviour of VSMC  
69 would be of interest to ascertain further applications of this functional food. Based on previous literature, we  
70 hypothesised that Montmorency tart cherries would contain vasoactive compounds that would be absorbed and  
71 detectable in plasma and that these compounds would modulate VSMC behaviour *in vitro*. Therefore, this  
72 investigation aimed to examine 1) the time-course of selected phenolic compounds following ingestion of a  
73 Montmorency cherry concentrate, and 2) exposure of VSMC to phenolic compounds would influence cell  
74 behaviour *in vitro*.

75

## 76 **Materials and Methods**

### 77 **Participants**

78 Twelve non-smoking males were recruited to take part in the study; the mean  $\pm$  SD age, stature, mass and BMI  
79 was  $26 \pm 3$  years,  $178.5 \pm 7.6$  cm,  $85.2 \pm 11.7$  kg and  $26.7 \pm 3.2$  kg/m<sup>2</sup>, respectively. All participants were in  
80 apparent good health as assessed by a health-screening questionnaire. Exclusion criteria for the study were; food  
81 allergy (as discussed with research team), history of gastrointestinal, renal or cardiovascular disease and current  
82 use of any food supplementations. The study was conducted in accordance with the Helsinki Declaration and

83 ratified by the University's Research Ethics Committee. All enrolled participants provided written informed  
84 consent. This study was registered as a clinical trial with clinicaltrials.gov (NCT01825070).

#### 85 Study Design

86 As a first step in the current study, we analysed three analogues of tart Montmorency cherries (frozen, dried and  
87 concentrated) in order to identify which was superior in terms of the total anthocyanin and phenolic content as  
88 well as total antioxidant capacity and used in subsequent studies. The second part of the study utilised a double  
89 blind, two-phase (separated by at least 10 days), randomised, cross-over, but counterbalanced design in order to  
90 identify the bioavailability of specific phenolic acids and their influence on cell behaviour following the  
91 ingestion of two different doses of Montmorency tart cherry concentrate (MC). Each visit was at the same time  
92 of day and preceded by an overnight fast ( $\geq 10$  h). On arrival to the laboratory, participants provided a baseline  
93 venous blood sample. As previously [13], subsequent blood samples were taken at 1, 2, 3, 4 and 8 h post MC  
94 consumption. No additional food or fluid was provided during the study period except for low-nitrate mineral  
95 water.

#### 96 Treatments and Dietary Control

97 The MC concentrate (CherryActive, Sunbury, UK) was stored at 4°C prior to use. Participants consumed either  
98 30 mL or 60 mL of MC concentrate diluted with 100 mL of water in a double-blind cross over manner.  
99 According to the manufacturer's information, a 30 mL dose of concentrate was equivalent to approximately 90  
100 whole cherries. Participants were instructed to follow a low phenol diet for 48 h prior to each arm of the trial by  
101 avoiding fruits and its equivalents (i.e., juices), vegetables, tea, coffee, alcohol, chocolate, cereals, wholemeal  
102 bread, grains and spices and were asked to refrain from strenuous exercise. Compliance with the dietary  
103 restrictions was examined with a self-completed standardized 2-day dietary record.

#### 104 Montmorency Tart Cherry Analysis

##### 105 *Total Anthocyanins (TACN)*

106 The monomeric anthocyanin pigment content of the MC concentrate and aqueous Montmorency cherry fruit  
107 extracts (whole frozen and dried) was determined using the pH-differential method [23]. The MC concentrate  
108 was diluted 1:20 in 25 M potassium chloride buffer at pH 1.0 and 0.4 M sodium acetate buffer at pH 4.5,  
109 respectively. The absorbance was measured spectrophotometrically at 510 and 700 nm (Ultraspec 2000UV/Vis

110 spectrophotometer, Pharmacia Biotech, Sweden). The absorbance difference A was calculated as  $A = (A_{510}$   
111  $A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5}$ . The TACN concentration C (mg/L) was expressed as mg cyanidin-3-  
112 glucoside equivalents according to the following equation:  $C = A \cdot MW \cdot DF \cdot 1000 / (\epsilon \cdot l)$ , where MW was the molar  
113 mass for cyanidin-3-glucoside (449.2 g/mol); DF was the dilution factor; 1000 was the conversion from g to mg;  
114  $\epsilon$  was the molar extinction coefficient for cyanidin-3-glucoside (26900 L/mol); and l was the path length (1 cm).

#### 115 *Total Phenolic Content (TPC)*

116 Total phenolic content was measured using a modified Folin-Ciocalteu colorimetric method [24]. Samples were  
117 diluted in deionised water (1:10 or 1:100) and 50  $\mu\text{l}$  of the diluted extract, 50  $\mu\text{l}$  of Folin-Ciocaltea reagent  
118 diluted in water (1:25) and 100  $\mu\text{l}$  of 6% (w/v) sodium carbonate were added into corresponding sample wells of  
119 a 96 well plate (Greiner Bio – One, Monroe, USA). Absorbance readings were taken at 725 nm, at 5 minute  
120 intervals, over a 30 minute period at 25°C (BioTek Synergy HT Multi-Mode Microplate Reader, Winooski,  
121 USA). A stock solution of gallic acid (5.8 mM) was prepared in aqueous methanol (80% (v/v) and  
122 quantification was performed on the basis of a standard curve in the range 0-50 mg/mL ( $R^2 = 0.99$ ). The  
123 analysed samples were measured versus a blank sample. All values are expressed as means of gallic acid  
124 equivalents per gram of sample  $\pm$  SE for 6 replications.

#### 125 *Trolox Equivalent Antioxidant Capacity (TEAC)*

126 A modified DPPH assay used for antioxidant activity measurements was adjusted for use in the present study  
127 [25]. The DPPH solution was prepared freshly before analysis, by dissolving the DPPH reagent (2.4 mg) in  
128 80% methanol (100 mL). Then 10  $\mu\text{l}$  of extract, 40  $\mu\text{l}$  of deionized water and 200  $\mu\text{l}$  of DPPH solution were  
129 added into each well of the 96 well plate (CELLSTAR, Greiner Bio-One, Monroe, USA). Absorbance readings  
130 were taken at 515 nm, at 3 minute intervals over a 30 minute period at 37°C, using a Multi Mode Microplate  
131 Reader (BioTek synergy HT, Winooski, USA). A calibration curve using Trolox (0-500  $\mu\text{M}$ ,  $R^2 = 0.99$ ) was  
132 plotted. Final values are expressed as means of Trolox equivalents per milligram of sample  $\pm$  SE for 6  
133 replications.

#### 134 *Individual Phenolic Analysis*

135 The levels of individual phenolics (CHL, PCA and VA) were determined by HPLC and diode array detection  
136 (DAD), using the methods described by Bell and colleagues [13]. These phenolic acids were preferentially

137 selected as they are the most abundant degradation products of cyanidin and peonidin, the two major  
138 anthocyanins detected in the Montmorency whole cherry [6] and concentrate [13]. A 1% solution of MC juice  
139 was prepared in 1:1, 0.1% formic acid: 2% HCl in methanol (MeOH) filtered with a 0.2  $\mu$ m  
140 polytetrafluoroethylene (PTFE) filter and analysed by HPLC-DAD using a Phenomenex Luna C<sub>18</sub>, (250 x 2.0  
141 mm x 5  $\mu$ m). To characterize the major phenolics present, a sample of MC concentrate was analysed by LC-  
142 MS. All of the phenolic acids were identified in the MC concentrate after applying the extractive procedure and  
143 chromatographic method. The MS chromatogram of CHL in MC concentrate is presented in Figure 1: B (1-2).

144 The total anthocyanin, total phenolic and total antioxidant capacities for each analogue of tart Montmorency  
145 cherry are presented in Table 1. Additionally, the individual phenolic acid quantities in the concentrate are also  
146 provided.

#### 147 Blood Sampling

148 Fasting whole blood samples were collected in a 10 mL EDTA vacutainer system (Becton, Dickinson and  
149 Company, Plymouth, New Zealand), inverted to mix the anticoagulant, and immediately centrifuged at 3000  $\times$  g  
150 for 10 minutes at 4°C. Plasma was aspirated and pipetted into ~1 mL aliquots and then immediately stored at -  
151 80°C for later analysis.

#### 152 HPLC Analysis

153 Under the selected chromatographic conditions, calibration graphs were obtained by preparing standard samples  
154 of each compound in triplicate, with increasing concentration of each analyte. From calibration graphs the limit  
155 of detection and linearity were calculated (Table 2). The HPLC-DAD was used to identify plasma  
156 concentrations of phenolics for the acute phase of the study (pre-supplementation through to 8 h post-  
157 supplementation). Plasma samples were extracted using a solid-phase extraction procedure. Briefly, 1 mL of  
158 plasma was mixed with 4 mL oxalic acid (10 mM) and 0.1 mL HCl (12.06 M) in 15 mL Falcon tubes and  
159 centrifuged at 826  $\times$  g for 5 minutes. The supernatant was absorbed on to a primed solid phase extraction  
160 cartridge (Waters Sep-Pak C18 plus short cartridge, 360 mg sorbent per cartridge, 55-105  $\mu$ m), conditioned with  
161 MeOH with 0.2% trifluoroacetic acid (TFA) followed by 2  $\times$  5 mL of water. The sample was eluted with 3 mL  
162 of MeOH with 0.2% TFA, dried under N<sub>2</sub> at 45°C. Samples were then reconstituted in 400  $\mu$ l of dilution solvent  
163 (0.1% formic acid in water: 2% HCl in MeOH), and filtered through a 0.2 $\mu$ m PTFE filter prior to HPLC  
164 analysis. The method's recovery was assessed by analysing separate aqueous solutions of each of the



165 antioxidants at 1, 25 and 50  $\mu\text{L}/\text{mL}$ , as well as blank plasma samples with added antioxidants at the same three  
166 concentrations. The recovery for all sample preparations was 99.1–101.4%.

167 HPLC-DAD analysis of phenolics was carried out using HPLC equipped with a pump, autosampler and UV-Vis  
168 detector (UltiMate 3000 HPLC system, Dionex, Camberly, UK). Known volumes of model system solutions  
169 (0.1-0.3 mL), were transferred in to an autosampler vial and deionized water was added to afford a final volume  
170 of 1.5 mL. Sample aliquots (10  $\mu\text{L}$ ) of plasma were injected on a 2.1 cm x 150 mm i.d.; 3  $\mu\text{m}$  particle size  
171 reverse-phase column (Phenomenex Luna C18(2) (250 x 2.0 mm, 5  $\mu\text{m}$  particle size) that was thermostatically  
172 regulated at 30°C. The mobile phase consisted of water with 1% acetic acid (solvent A), and acetonitrile with  
173 1% acetic acid (solvent B). After a 5-minute equilibration with 20% B, the elution programme was as follows:  
174 0-20 min, 10-100% B, (0.2 mL/min) followed by a washing stage (100% B, 20-28 min, 0.2 mL/min) and re-  
175 equilibration at the initial conditions for 5 minutes. Detection was performed at the following wavelengths:  $\lambda =$   
176 260 nm for PCA and VA and  $\lambda = 326$  nm for CHL. The polyphenolic content of plasma extracts were  
177 calculated by interpolation from the calibration graph and expressed as micrograms per millilitre ( $\mu\text{g}/\text{mL}$ ).

#### 178 LC-MS Analysis

179 A liquid chromatography-mass spectrometry (LC-MS) method, utilising the same chromatographic conditions  
180 as the HPLC-DAD analyses, was used for the identification of individual compounds in the plasma and juice  
181 samples. Briefly, LC-MS analyses were carried out on a Dionex UltiMate 3000 RSLC HPLC System (Dionex,  
182 Camberly, UK) equipped with an UltiMate 3000 RS pump, an UltiMate 3000 RS autosampler and a QExactive  
183 Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Electrospray ionization at  
184 both negative and positive ion modes was performed with a spray voltage of 2.00 kV and capillary temperature  
185 of 280°C. The total ion current (TIC) with a range of 100-1500 m/z and 70000 resolution was measured. Sample  
186 aliquots (2  $\mu\text{L}$ ) were injected on an Phenomenex Luna C18(2) (250 x 2.0 mm, 5  $\mu\text{m}$  particle size) reverse-phase  
187 column thermostatically regulated at 40°C. The mobile phase consisted of water with 1% acetic acid (solvent  
188 A), and acetonitrile with 1% acetic acid (solvent B). The same method as applied for the HPLC analysis was  
189 carried out on the LCMS. The identification of phenolics in the MC concentrate was verified by retention time  
190 and spectral data comparison with the corresponding reference compounds.

191

192

### 193 Vascular Smooth Muscle Cell Culture and Migration

194 Primary human aortic smooth muscle cells (VSMC; Life Technologies, Paisley, UK) were cultured in 231  
195 medium supplemented with smooth muscle cell growth supplement (Life Technologies, Paisley, UK) including  
196 5% foetal bovine serum. The VSMC cultures were maintained in 75 cm<sup>2</sup> tissue culture flasks in a humidified  
197 incubator at 37°C. For all experiments, VSMC were used between passages 3 and 8. Migration of VSMC in  
198 response to metabolites PCA and VA was determined using xCelligence real time cell analyser. PCA and VA  
199 were dissolved in 100% ethanol, to a concentration of 100 mM. VSMCs were serum-starved for 24 h, then  
200 detached from the flask with trypsin. The cells were then incubated with PCA at a molarity concentration of 32  
201 µM and VA at 4 µM or ethanol only (<0.04% (v/v)) control for 1 h at 37°C. These concentrations were based on  
202 the plasma bioavailability concentrations ascertained by the *in vivo* part of this work and fall within the range of  
203 maximum values we observed following consumption of the cherry juice. The VSMC were plated onto an  
204 xCelligence cell invasion and migration (CIM) plate containing serum-free medium in the top and bottom  
205 chambers. The VSMC were added to the top chamber at a density of 8000 cells/chamber. Migration of VSMCs  
206 was determined by measuring impedance, which is created as cells move from the top chamber, through a  
207 microporous membrane to the bottom chamber and attach to a gold electrode on the underside of the top  
208 chamber. Measurements were taken every 15 minutes over a 24 h period. Measurements were then converted to  
209 cell index values, which were used as a relative measure of cell migration.

### 210 Vascular Smooth Muscle Cell Culture and Proliferation

211 Kinetic Proliferation Assay-An xCelligence real time cell analyser was used to monitor cell proliferation in real  
212 time (Acea Biosciences Inc, CA, USA). Primary VSMCs were seeded onto an xCelligence E plate at 6,000  
213 cells/well, with metabolites or ethanol only as control in normal VSMC growth media. E plates consist of a gold  
214 microelectrode and growth of cells is determined by measuring relative electrical impedance across the cell  
215 monolayer. Impedance measurements were taken every 15 minutes for up to 72 h to determine cell  
216 proliferation.

### 217 Statistical Analysis

218 Statistical Analysis was performed using PASW Statistics 21.0 for Windows (SPSS, Inc., Chicago, IL.).  
219 Descriptive statistics are reported as means ± SEM. All dependant variables were analysed by using a treatment  
220 (30 mL v 60 mL) by time (baseline, 1, 2, 3, 5 and 8 h) mixed model analysis of variance (ANOVA). Maulchy's

221 Test of Sphericity was used to check homogeneity of variance for all variables; where necessary any violations  
222 of the assumption were corrected using the Greenhouse-Geisser adjustment. Significant interaction effects were  
223 followed up using LSD *post-hoc* analysis. Further analysis was conducted to identify maximum plasma  
224 concentrations ( $C_{\max}$ ) and times to achieve maximum plasma concentrations ( $t_{\max}$ ), which were directly obtained  
225 from the plasma concentration-time profiles. As a measure of overall plasma bioavailability of individual  
226 phenolic acids, the area under the plasma concentration-time curve ( $AUC_{0-8h}$ ) was estimated by using the linear  
227 trapezoidal rule. For the cellular experiments, significance was judged using Student's t-test. The alpha level  
228 for statistical significance was set at 0.05 *a priori*. Results are reported as means  $\pm$  SEM.

229

## 230 **Results**

231 Protocatechuic (PCA), Vanilic (VA) and Chlorogenic (CHL) Acid

232 Firstly, all participants (n=12) complied with the low-polyphenolic experimental diet according to the food  
233 diaries. The MS chromatogram of PCA in plasma is presented in Figure 1: A (1-2). The PCA (Figure 2)  
234 revealed no significant treatment effect ( $F_{1,11} = 0.59$ ,  $p=0.810$ ) or treatment by time interaction effect ( $F_{5,105} =$   
235  $0.405$ ,  $p=0.845$ ). Following supplementation, there was a significant time effect on PCA plasma levels ( $F_{5,105} =$   
236  $2.956$ ,  $p=0.015$ ). The PCA levels in plasma were significantly higher 1 h following consumption of the low (30  
237 mL) and the high dose (60 mL) of MC when compared to baseline ( $p=0.014$  and  $0.05$ , respectively). For both  
238 the 30 and 60 mL MC dose, the  $t_{\max}$  was 1 h for PCA. The  $C_{\max}$  values for PCA were not different between the  
239 60 mL ( $2.75 \pm 0.13 \mu\text{g}\cdot\text{h}/\text{mL}^{-1}$ ) and the 30 mL ( $2.76 \pm 0.10 \mu\text{g}\cdot\text{h}/\text{mL}^{-1}$ ) dose. Furthermore,  $AUC_{0-8h}$  values for  
240 PCA were not different between the 30 mL and 60 mL doses,  $102.4 \pm 0.9 \mu\text{g}\cdot\text{h}/\text{mL}^{-1}$  and  $106.4 \pm 0.1 \mu\text{g}\cdot\text{h}/\text{mL}^{-1}$ ,  
241 respectively. The presence of PCA was confirmed in plasma by comparison of the experimentally determined  
242 monoisotopic molecular weights to literature value, in which all were within  $\pm 1.5$  ppm (Table 3).

243 Plasma VA (Figure 3) revealed no treatment effect ( $F_{1,11} = 0.004$ ,  $p=0.951$ ) or treatment by time interaction  
244 effect ( $F_{3,456,105} = 1.583$ ,  $p=0.195$ ). However, following supplementation, there was a time effect ( $F_{3,456,105} =$   
245  $3.329$ ,  $p=0.008$ ). VA levels were higher 1 h after consumption of the higher dose (60 mL) when compared to  
246 baseline ( $p<0.05$ ). Pairwise comparisons revealed increases in VA from 1 h to 5 h and 8 h post 60 mL MC Juice  
247 consumption ( $p<0.05$ ) compared to baseline. However, no significant time effects were observed with the lower  
248 dose. For VA, the  $t_{\max}$  differed depending on the dose administered, occurring at 1 h with the 60 mL dose and 2

249 h in the 30 mL dose.  $C_{\max}$  values for VA were not significantly different between the 60 mL ( $0.29 \pm 0.03$   
250  $\mu\text{g}\cdot\text{h}/\text{mL}^{-1}$ ) and the 30 mL ( $0.30 \pm 0.01 \mu\text{g}\cdot\text{h}/\text{mL}^{-1}$ ) dose. Furthermore,  $\text{AUC}_{0-8\text{h}}$  values for VA were not  
251 different between the 30 mL and 60 mL doses,  $10.7 \pm 0.1 \mu\text{g}\cdot\text{h}/\text{mL}^{-1}$  and  $11.8 \pm 0.1 \mu\text{g}\cdot\text{h}/\text{mL}^{-1}$ , respectively.  
252 The presence of VA was confirmed in plasma by comparison of the experimentally determined monoisotopic  
253 molecular weights to literature value, in which all were within  $\pm 1.5$  ppm. (Table 3). The current study did not  
254 detect CHL in the plasma post MC consumption.

#### 255 Cell behaviour

256 The maximum mean concentration of PCA measured in plasma was  $2.5 \mu\text{g}/\text{mL}$  (range:  $0.33 - 6.65 \mu\text{g}/\text{mL}$ )  
257 (Figure 2) and VA was  $0.3 \mu\text{g}/\text{mL}$  (range:  $0.1 - 1.32 \mu\text{g}/\text{mL}$ ) (Figure 3), these correspond to molar concentrations  
258 of  $16 \mu\text{M}$  and  $2 \mu\text{M}$ , respectively. The concentrations were doubled to  $32 \mu\text{M}$  PCA and  $4 \mu\text{M}$  VA for the cell  
259 experiments to fall within the maximum range of concentrations we observed *in vivo*. Both PCA and VA were  
260 applied to VSMC in isolation and showed no significant increase in migration behaviour in comparison to a  
261 control. When PCA and VA were combined and added to the culture, there was an increase ( $p = 0.038$ ) in  
262 migration of VSMC by  $36 \pm 12\%$  when compared to the control (Fig 4). Finally, there was no effect of the  
263 metabolites on VSMC proliferation (Supplemental Fig. 1).

264

#### 265 Discussion

266 Following the identification of which Montmorency cherry analogue (frozen, dried, concentrate) had the  
267 greatest antioxidant activity, total anthocyanin and phenolic content, MC concentrate was used to investigate the  
268 plasma kinetics of selected phenolic acids and their subsequent effect on cell behaviour *in vitro*. This  
269 investigation presents new information on the appearance and time course of phenolic compounds in plasma  
270 following consumption of a lower and higher dose of MC concentrate. The hydroxybenzoic acid content of  
271 edible plants is generally very low, with the exception of certain red fruits, black radish, and onions [25]. As a  
272 result, very little is known about the metabolism and absorption of these compounds. However, this study  
273 showed that both PCA and VA, are most bioavailable in plasma 1-2 h post MC consumption, whilst other  
274 hydroxycinnamic acids (CHL) were not present in the plasma. Furthermore, the combination of PCA and VA  
275 increased cell migration, but had no effect on the proliferation of VSMC.

276 High concentrations of VA *in vivo* can be attributed to the large quantity of anthocyanins in fruit and vegetables  
277 [20]. Ou et al [26] previously established the anthocyanins (in order of decreasing prevalence) in processed tart  
278 cherry products were cyanidin-3-glucosylrutinoside, cyanidin-3-rutinoside, and peonidin-3-rutinoside. These  
279 findings concur with those data from Balaton cherries [27] and Montmorency cherries [6]. VA is the major  
280 degradation product of the parent compound peonidin and this could explain the detection of VA in plasma 1-2  
281 h post MC consumption in the current study. Interestingly, it has been proposed that VA possesses  
282 chemopreventive properties due to its antioxidant activity and its ability to scavenge free radicals [28]. VA has  
283 also been shown to exhibit immunostimulatory effects in enhancing interferon gamma (IFN- $\gamma$ ) secretion and  
284 stimulating proliferation of human peripheral blood mononuclear cells [29].

285 Similar to VA, high concentrations of PCA *in vivo* is likely to be as a direct result of the original anthocyanin  
286 content of both fruit and vegetables [30]. Cyanidin is the main anthocyanin in tart cherry products [6], this was  
287 recently clarified in the MC concentrate (3.346 mg·mL<sup>-1</sup>) where cyanidin accounted for the overwhelming  
288 majority of anthocyanins with far smaller amounts of peonidin and malvidin [13]. Cyanidin-3-glucoside has  
289 been shown to readily degrade to cyanidin, and then further metabolised to PCA. Vitaglione and colleagues  
290 [18] reported that PCA accounts for almost 73% of cyanidin ingested. They [18] concluded that a high  
291 concentration of PCA could explain the short-term increase in plasma antioxidant activity observed after intake  
292 of cyanidin-rich food. PCA has also been shown to remain in biological tissues for longer periods of time than  
293 the parent anthocyanin [31]. A recent addition to the literature [32] examined the tissue bioavailability of cherry  
294 phenolic compounds in rats following three weeks of supplementation. The work showed some tissues  
295 preferentially store these phenolic compounds; but importantly when examined with the data from the current  
296 study the transient increase in compounds seen in plasma might be the first step to increase tissue bioavailability  
297 and hence a potential pathway to the proposed health-enhancing benefits of cherry phenolics. Consequently  
298 more longitudinal supplementation studies should investigate tissue bioavailability in humans to ascertain if  
299 increased tissue concentrations of these compounds is possible in a human model. The detection of high  
300 concentrations of PCA in human plasma 1-2 h post MC ingestion has the potential to exert some physiological  
301 potential; for example, previous research has shown that PCA possess antibacterial, antioxidant, antidiabetic,  
302 anticancer, antiulcer, antiaging, antiviral, anti-inflammatory, anti-atherosclerotic properties [33].

303 Despite there being high quantities of CHL in the MC concentrate, it was not detected in plasma. Other food  
304 and beverage studies administered much higher concentrations of CHL; for example, Stalmach et al [34] gave

305 participants a single serving of a coffee beverage fortified with CHL, the serving consisted of low (412  $\mu\text{mol}$ ),  
306 medium (635  $\mu\text{mol}$ ) and high (795  $\mu\text{mol}$ ) quantities of CHL. Although 412  $\mu\text{mol/L}$  represented the low dose,  
307 this is still far greater than the amounts identified in the MC concentrate in the current study; where 6.8  $\mu\text{g/mL}$   
308 which equates to  $\sim 22.1 \mu\text{mol/L}$ . Unlike the previous work, this study utilized an ecologically valid quantity of  
309 CHL that was found in the MC concentrate that represents a sensible portion to consume, rather than an artificially  
310 derived concentration used previously [34,35]. In support of this, a previous study using comparable  
311 concentrations of CHL also failed to detect its presence *in vivo* (rodent plasma) following CHL ingestion [36].  
312 Given that CHL was present in the MC concentrate, it is quite plausible that CHL was metabolised quickly to  
313 the downstream metabolites caffeic, quinic and ferulic acid. This provides some explanation for the non-  
314 detection in plasma; however, the possibility remains that CHL become degraded during sample treatment  
315 process [37]. A study [38] evaluating the pharmacokinetic profile and bioavailability of CHL in plasma and  
316 urine of 10 healthy participants showed a great deal of inter-individual variation in CHL absorption, metabolism  
317 and kinetics with uptake values ranged from 7.8% to 72.1% amongst participants. Large inter-individual  
318 variations in the plasma concentrations of all compounds in the current study are not unexpected because of the  
319 multifaceted factors such as metabolism and genetic disposition to gut microbial composition [39].

320 The effects of the metabolites PCA and VA on the migration of VSMC *in vitro* were also assessed. Migration  
321 increased when the cells were treated in concert with both metabolites, demonstrating that these metabolites, at a  
322 similar level to that seen in plasma, can alter VSMC function. Migration of de-differentiated VSMC is required  
323 for vessel remodelling which occurs from exercise and vascular injury. The VSMC migration in advanced  
324 atherosclerotic plaques are often considered to be protective as it increases stability, protecting against plaque  
325 rupture and ensuing vascular trauma such as myocardial infarction or stroke [40]. By increasing VSMC  
326 migration, the metabolites may potentially be beneficial for blood vessel remodelling, although this would  
327 require further investigation. Elsewhere there are conflicting reports of the effects of PCA on cell migration; for  
328 example, PCA has been shown to increase the migration of adipose tissue derived stromal cells [41] and to  
329 inhibit the migration of gastric cancer cells [42]; the mechanism of which are thought to involve alterations in  
330 matrix metalloprotease activity. These conflicting reports are likely attributable to the different cell culture  
331 models used in each study. It is interesting to note that the concentration of PCA used in this study was similar  
332 to physiological concentration observed *in vivo*; whereas previous studies required between 15-47 times greater  
333 concentrations to observe an effect on cell behaviour [41,42]. VA is less studied, but has been reported to have

334 a small effect on lung cancer cell migration in comparison to controls, at a concentration 1000 times greater than  
335 used in the current investigation [43]. This is the first study where PCA and VA have been examined in concert  
336 and show that VSMC migration can be influenced at physiologically relevant levels that can be consumed from  
337 MC. Importantly, the work suggests that the examination of phenolic acids (or other phytochemicals) in  
338 isolation may be of limited value, particularly when whole foods and their analogues are far more complex.

339 An acknowledged limitation of the current study is that the analysis was not exhaustive, and so not every  
340 polyphenol was analysed; instead the focus was on the degradation products of two of the main anthocyanidins  
341 reported in the MC juice that could exert a positive effect on vascular function. In addition, we did not  
342 investigate compounds, for instance procyanidins, which appear to have poor bioavailability due to instability,  
343 large molecular weight or are quickly excreted. Conceivably, these compounds might also contribute to any  
344 potential physiological effects exerted by MC and cannot be excluded. Furthermore, enterohepatic metabolism  
345 could predict that the absorption of phytochemicals and their metabolites are not limited to few hours after  
346 intake [14,19]. As a result, the timeframe of the analysis in the current study may be regarded as a potential  
347 limitation.

348 In conclusion, these data provide new information on the presence of phenolic acids in plasma following MC  
349 concentrate consumption in humans. The time course of metabolite absorption peaks at 1-2 hours post-  
350 consumption and this information could inform future *in vivo* work that examines the health-related benefits  
351 associated with Montmorency tart cherries. Lastly, MC concentrate provides a bioavailable source of  
352 phytochemicals that could be helpful in modulating vascular function [44] [45] and influencing cell behaviour.

### 353 **Acknowledgements**

354 The authors would like to thank their participants in this investigation. K.M.K., G.H., J.L. and P.G.B. conceived  
355 and designed the research; K.M.K., P.G.B., S.E.J. conducted the research; K.M.K., G.H., C.L.C., S. E. J., R.B.  
356 analyzed and interpreted the data; K.M.K. and G.H. drafted the paper. G.H. and K.M.K. had primary  
357 responsibility for final content. All authors read, edited and approved the final manuscript. The Cherry  
358 Research Committee of the Cherry Marketing Institute (Lansing, MI, USA), a not for profit organisation,  
359 provided financial support for a PhD studentship to make this work possible. S. E. J. was supported by grant  
360 PG/12/45/29672 from the British Heart Foundation to R. B. All other elements of the study were funded by

361 Northumbria University. The funders had no role in the study design, data collection and analysis, decision to  
362 publish, or preparation of the manuscript. The authors declare no conflict of interest.



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### Figure Captions

**Fig 1 A (1)** LCMS Chromatograms of CHL, extracted ion mode range m/z 352-256 (RT=4.87 min) **(2)** MS output of CHL, @ RT = 4.87 min (the m/z at 707 could be a CHL dimer, formed in the MS) **B (1)** MC Juice, extracted ion, range m/z 352-356. The two distinct peaks in the juice (RT=4.37 and 4.87 min) have similar MS spectra (m/z 353 main ion) which could indicate the presence of another isomer i.e. crypto- or neo- CHL **(2)** MC Juice @ RT = 4.87 min **C (1)** PCA, extracted ion range m/z 150-155 (RT=7.17 min) **(2)** PCA @ RT = 7.17 min **D (1)** Plasma sample (S1-B-Z), extracted ion, range m/z 150-155 (RT=7.17 min) **(2)** Plasma sample (S1-B-Z), @ RT=7.17 min

**Fig 2** PCA responses from baseline to 30 mL and 60 mL Montmorency cherry concentrate (MC). Absolute baseline values were  $1.16 \pm 0.326$  and  $1.70 \pm 0.435$  ug/mL for 30 mL and 60 mL, respectively. \* indicates a significant time effect ( $p < 0.05$ ) (30 mL and 60 mL dose); data presented as mean  $\pm$  SEM

**Fig 3** VA responses from baseline to 30 mL and 60 mL Montmorency cherry concentrate (MC). Absolute baseline values were  $0.158 \pm 0.031$  and  $0.093 \pm 0.024$  ug/mL for 30 mL and 60 mL, respectively. \* indicates a significant time effect ( $p < 0.05$ ) (60 mL dose only); data presented as mean  $\pm$  SEM

**Fig 4** % Migration of human vascular smooth muscle cells *in vitro* in response to metabolites PCA (32 $\mu$ M) and VA (4 $\mu$ M) compared to ethanol only control, over 24hours. Combined data from three separate experiments # indicates a significant difference between condition ( $p < 0.05$ ); data presented as mean  $\pm$  SEM

**Table 1:** Total anthocyanin, phenolics and antioxidant activity in pitted, frozen, whole, dried and concentrated Montmorency tart cherry.

	TACN	TPC	TEAC	Total CHL*	Total PCA*	Total VA*
<b>30 mL MC Concentrate</b>	31.24 ± 0.16	71.37± 0.11	0.30±0.01	0.205±0.24	0.020±0.11	0.253±0.84
<b>60 mL MC Concentrate</b>	62.47 ± 0.31	142.73 ±0.22	0.60±0.03	0.410±0.48	0.040±0.22	0.506±1.68
<b>Frozen cherries</b>	0.03 ± 0.0009	0.005± 0.0004	0.002± 0.0002	-	-	-
<b>Dried cherries</b>	0.008 ± 0.0003	0.006± 0.0005	0.002± 0.0001	-	-	-

*Values are presented as Mean ± SEM, n = 6 per analysis, (\*n=3). TACN, total anthocyanin content, MC = mg cyanidin-3-glucoside /L, Whole Food = mg cyanidin-3-glucoside/100 g; TPC, total phenolic content, MC = mean gallic acid equiv/L, Whole Food = mean gallic acid equiv/g; TEAC, trolox equivalent antioxidant capacity, MC = mean Trolox equiv /L, Whole Food: mean Trolox equiv/g; CHL, chlorogenic acid; PCA, protocatechuic acid; VA, vanillic acid, MC = µg/mL.*

**Table 2:** Retention times (min) and selected UV-Vis wavelengths for quantitation of phenolics by HPLC-UV/Vis

<b>Compound</b>	<b>UV/Vis wavelength (nm)</b>	<b>Retention time (min)</b>	<b>LOD (<math>\mu\text{g/mL}</math>)</b>	<b>Range of linearity(<math>\mu\text{g/mL}</math>)</b>
<b>PCA</b>	260	9.263	<0.05	0.5-80
<b>CHL</b>	326	10.140	<0.04	0.4-80
<b>VA</b>	260	11.326	<0.04	0.5-100

*LOD, limit of detection.*

**Table 3:** LCMS characterization of phenolic peaks.

Polyphenol	Formula	Found	Monoisotopic Mass	
			Ionisation Mode	Literature Value <sup>1</sup>
PCA	C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>	154.034	Negative	154.026611
CHL	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	354.102	Negative	354.095093
VA	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	168.049	Negative	168.042252

<sup>1</sup>Royal Society of Chemistry, (2013), Chemspider chemical database. Found at: <http://www.chemspider.com/>.  
 ND, Not Detected.



Fig 1

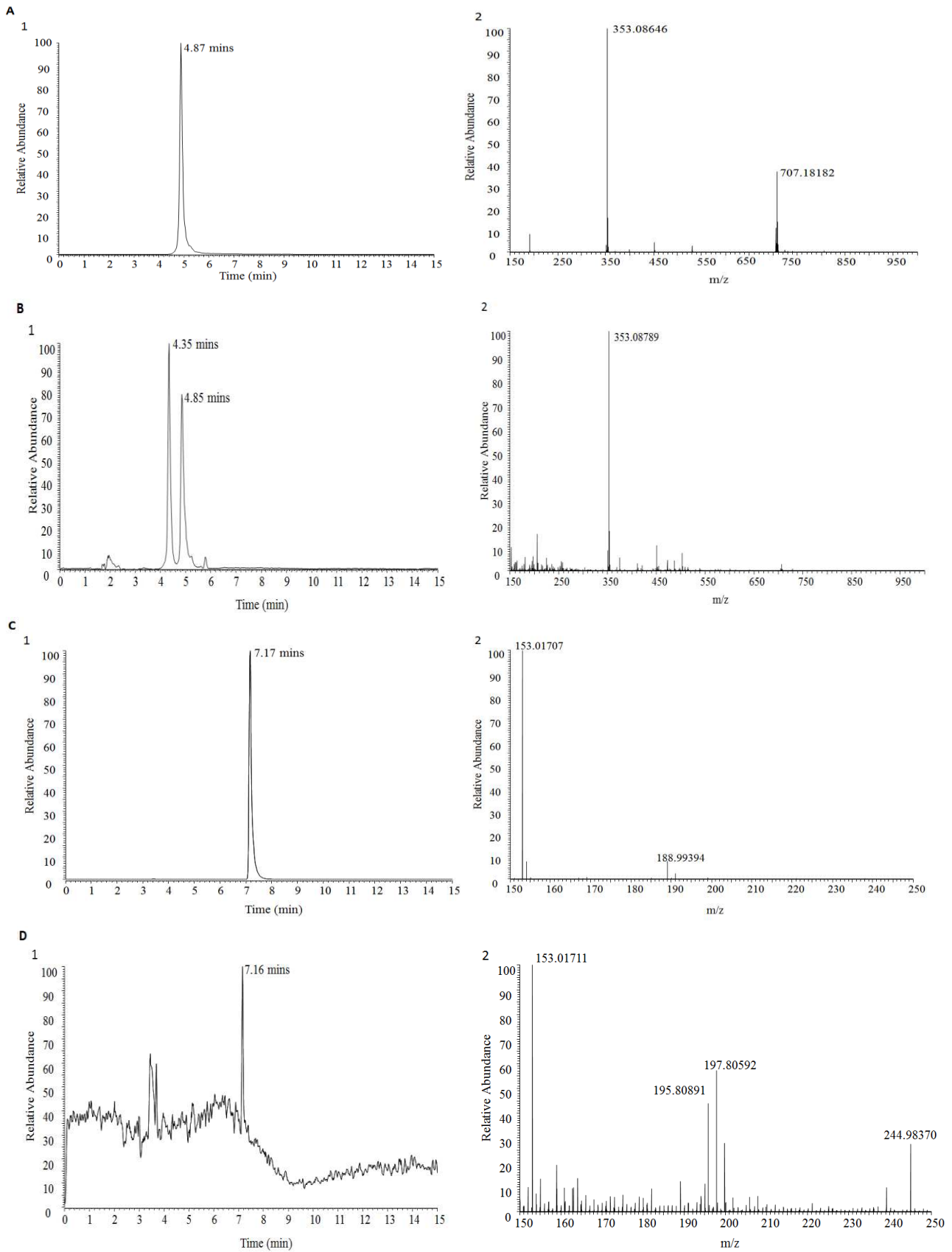


Fig 2

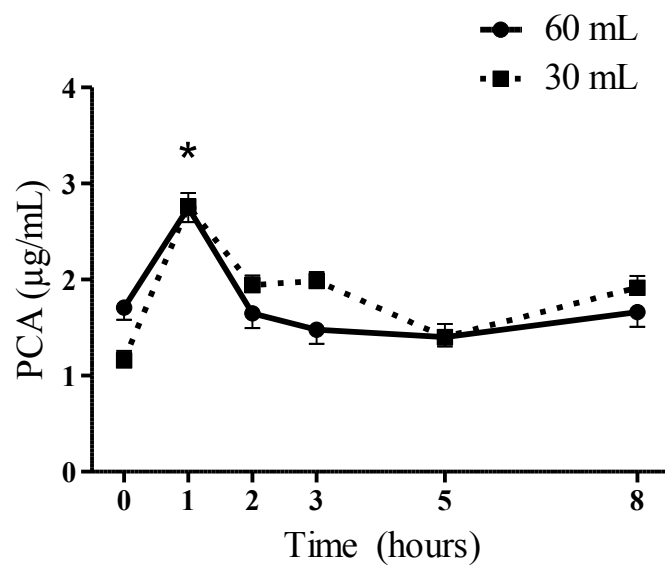


Fig 3

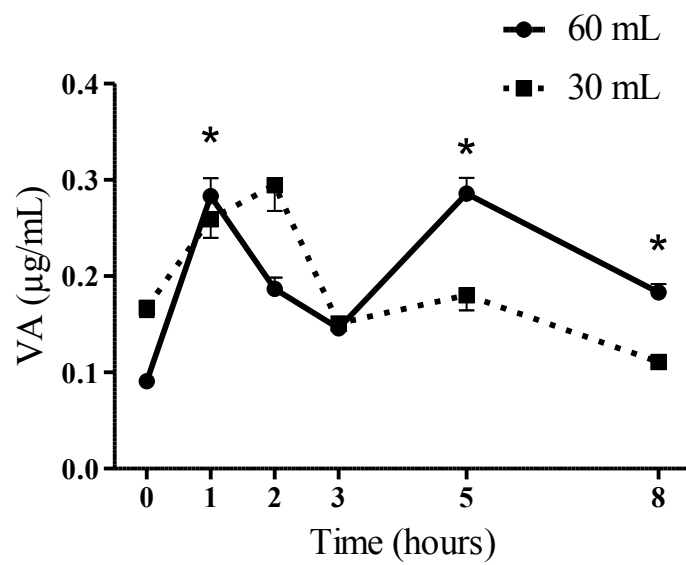
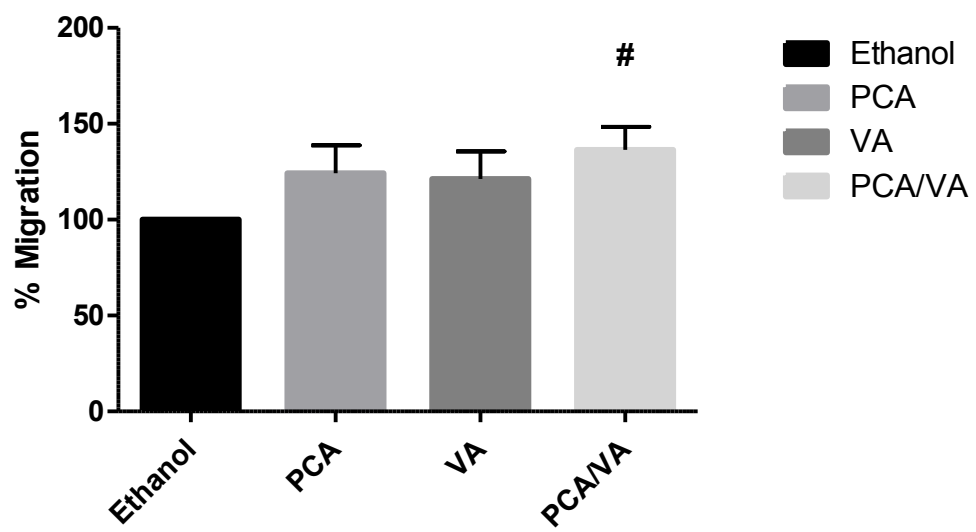
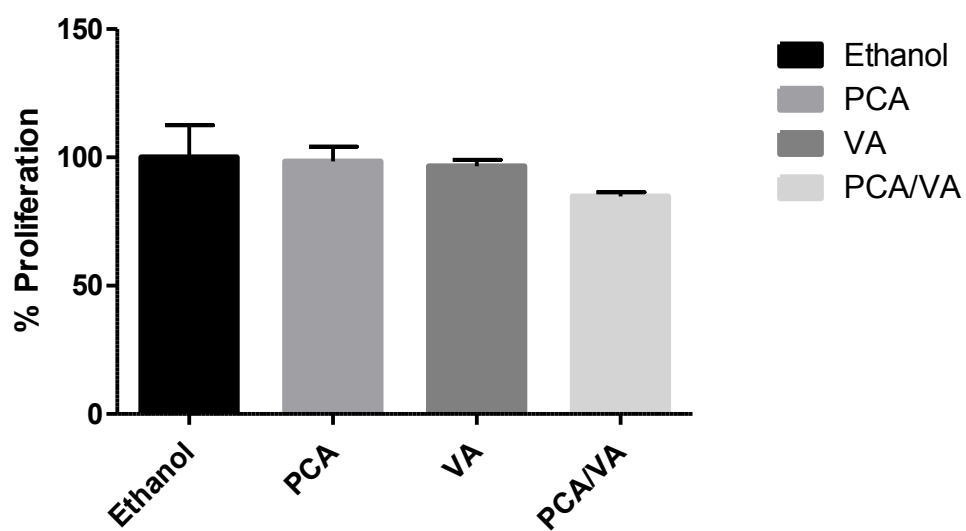


Fig 4



## Online Supporting Material

## Supplemental Figure 1



**Fig S1** % Proliferation of human vascular smooth muscle cells *in vitro* in response to metabolites PCA (32 $\mu$ M) and VA (4 $\mu$ M) compared to ethanol only control, over 24hours. Combined data from four separate experiments; data presented as mean  $\pm$ SEM.