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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Abstract

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

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

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

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

Keywords: Montmorency, Phenolic Acids, Bioavailability, Cell behaviour

Introduction

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

The health-related benefits have been postulated to arise from the high anthocyanin content of tart cherries; however the biological effectiveness of Montmorency cherries might be due to phytochemical interactions, which accomplish complementary effects. Such synergies occur when combinations of bioactive substances exert effects at target sites that are greater than the sum of individual components [15]. Furthermore, it has been noted that anthocyanins may not be stable during processing or storage [16]. Thus, it is not surprising that Montmorency cherry secondary metabolites could be biologically more active than individual 'whole' components in cherries. The main non-flavonoid polyphenols of dietary significance are the $C_6 - C_1$ phenolic acids; these provide unique taste, flavour, and health-promoting properties and are found in many vegetables and fruits [17]. A human study, feeding blood orange juice suggested that the phenolic acid degradation product, protocatechuic acid (PCA), was a major metabolite of anthocyanins [18]. In addition, a range of phenolic acids, including vanillic acid (VA), syringic acid, caffeic acid and ferulic acid, have been identified within human serum, following the consumption of anthocyanin-rich berries [19,20], but data on the bioavailability of these phenolic acids are very scarce.

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

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

Materials and Methods

Participants

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

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

Study Design

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

Treatments and Dietary Control

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

Montmorency Tart Cherry Analysis

Total Anthocyanins (TACN)

The monomeric anthocyanin pigment content of the MC concentrate and aqueous Montmorency cherry fruit extracts (whole frozen and dried) was determined using the pH-differential method [23]. The MC concentrate 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, respectively. The absorbance was measured spectrophotometrically at 510 and 700 nm (Ultraspec 2000UV/Vis

spectrophotometer, Pharmacia Biotech, Sweden). The absorbance difference A was calculated as A = (A_{510}-A_{700}) pH1.0 – (A_{510}-A_{700}) pH 4.5. The TACN concentration C (mg/L) was expressed as mg cyanidin-3-glucoside equivalents according to the following equation: C=A.MW.DF.1000/(ε.l), where MW was the molar mass for cyanidin-3-glucoside (449.2 g/mol); DF was the dilution factor; 1000 was the conversion from g to mg; ε was the molar extinction coefficient for cyanidin-3-glucoside (26900 L/mol); and l was the path length (1 cm).

Total Phenolic Content (TPC)

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

Trolox Equivalent Antioxidant Capacity (TEAC)

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

Individual Phenolic Analysis

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

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

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

Blood Sampling

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

HPLC Analysis

Under the selected chromatographic conditions, calibration graphs were obtained by preparing standard samples of each compound in triplicate, with increasing concentration of each analyte. From calibration graphs the limit of detection and linearity were calculated (Table 2). The HPLC-DAD was used to identify plasma concentrations of phenolics for the acute phase of the study (pre-supplementation through to 8 h post-supplementation). Plasma samples were extracted using a solid-phase extraction procedure. Briefly, 1 mL of plasma was mixed with 4 mL oxalic acid (10 mM) and 0.1 mL HCl (12.06 M) in 15 mL Falcon tubes and centrifuged at 826 × g for 5 minutes. The supernatant was absorbed on to a primed solid phase extraction cartridge (Waters Sep-Pak C18 plus short cartridge, 360 mg sorbent per cartridge, 55-105 µm), conditioned with MeOH with 0.2% trifluoroacetic acid (TFA) followed by 2 × 5 mL of water. The sample was eluted with 3 mL of MeOH with 0.2% TFA, dried under N_2 at 45°C. Samples were then reconstituted in 400 µl of dilution solvent (0.1% formic acid in water: 2% HCl in MeOH), and filtered through a 0.2µm PTFE filter prior to HPLC analysis. The method's recovery was assessed by analysing separate aqueous solutions of each of the

antioxidants at 1, 25 and 50 μL/mL, as well as blank plasma samples with added antioxidants at the same three concentrations. The recovery for all sample preparations was 99.1–101.4%.

HPLC-DAD analysis of phenolics was carried out using HPLC equipped with a pump, autosampler and UV-Vis detector (UltiMate 3000 HPLC system, Dionex, Camberly, UK). Known volumes of model system solutions (0.1-0.3 mL), were transferred in to an autosampler vial and deionized water was added to afford a final volume of 1.5 mL. Sample aliquots (10 μL) of plasma were injected on a 2.1 cm x 150 mm i.d.; 3 μm particle size reverse-phase column (Phenomenex Luna C18(2) (250 x 2.0 mm, 5 um particle size) that was thermostatically regulated at 30°C. The mobile phase consisted of water with 1% acetic acid (solvent A), and acetonitrile with 1% acetic acid (solvent B). After a 5-minute equilibration with 20% B, the elution programme was as follows: 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-equilibration at the initial conditions for 5 minutes. Detection was performed at the following wavelengths: λ = 260 nm for PCA and VA and λ = 326 nm for CHL. The polyphenolic content of plasma extracts were calculated by interpolation from the calibration graph and expressed as micrograms per millilitre (μg/mL).

LC-MS Analysis

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

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^2 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

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

Results

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

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

Plasma VA (Figure 3) revealed no treatment effect ($F_{1,11}$ = 0.004, p=0.951) or treatment by time interaction effect ($F_{3.456,105}$ = 1.583, p=0.195).However, following supplementation, there was a time effect ($F_{3.456,105}$ = 3.329, p=0.008). VA levels were higher 1 h after consumption of the higher dose (60 mL) when compared to baseline ($p<0.05$). Pairwise comparisons revealed increases in VA from 1 h to 5 h and 8 h post 60 mL MC Juice consumption ($p<0.05$) compared to baseline. However, no significant time effects were observed with the lower dose. For VA, the t_{max} differed depending on the dose administered, occurring at 1 h with the 60 mL dose and 2

h in the 30 mL dose. C_{max} values for VA were not significantly different between the 60 mL (0.29 ± 0.03 μg*h/mL^{-1}) and the 30 mL (0.30 ± 0.01 μg*h/mL^{-1}) dose. Furthermore, AUC_{0-8h} values for VA were not different between the 30 mL and 60 mL doses, 10.7 ± 0.1 μg*h/mL^{-1} and 11.8 ± 0.1 μg*h/mL^{-1}, respectively. The presence of VA was confirmed in plasma by comparison of the experimentally determined monoisotopic molecular weights to literature value, in which all were within ± 1.5 ppm. (Table 3). The current study did not detect CHL in the plasma post MC consumption.

Cell behaviour

The maximum mean concentration of PCA measured in plasma was 2.5μg/mL (range: 0.33 – 6.65 μg/mL) (Figure2) and VA was 0.3μg/mL (range: 0.1-1.32 μg/mL) (Figure 3), these correspond to molar concentrations of 16μM and 2μM, respectively. The concentrations were doubled to 32μM PCA and 4μM VA for the cell experiments to fall within the maximum range of concentrations we observed *in vivo*. Both PCA and VA were applied to VSMC in isolation and showed no significant increase in migration behaviour in comparison to a control. When PCA and VA were combined and added to the culture, there was an increase ($p = 0.038$) in migration of VSMC by 36±12% when compared to the control (Fig 4). Finally, there was no effect of the metabolites on VSMC proliferation (Supplemental Fig. 1).

Discussion

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

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

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

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

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

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

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

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

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

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References

1. Habauzit V, Morand C (2012) Evidence for a protective effect of polyphenols-containing foods on cardiovascular health: an update for clinicians. Ther Adv Chronic Dis 3 (2):87-106. doi:10.1177/2040622311430006

2. Arranz S, Chiva-Blanch G, Valderas-Martinez P, Medina-Remon A, Lamuela-Raventos RM, Estruch R (2012) Wine, Beer, Alcohol and Polyphenols on Cardiovascular Disease and Cancer. Nutrients 4 (7):759-781. doi:10.3390/nu4070759

3. Vinson JA, Su XH, Zubik L, Bose P (2001) Phenol antioxidant quantity and quality in foods: Fruits. J Agric Food Chem 49 (11):5315-5321. doi:10.1021/jf0009293

4. Wang H, Nair MG, Strasburg GM, Chang Y-C, Booren AM, Gray JI, DeWitt DL (1999) Antioxidant and Antiinflammatory Activities of Anthocyanins and Their Aglycon, Cyanidin, from Tart Cherries. J Nat Prod 62 (2):294-296. doi:10.1021/np980501m

5. Seeram NP, Momin RA, Nair MG, Bourquin LD (2001) Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. Phytomedicine 8 (5):362-369. doi:10.1078/0944-7113-00053

6. Kirakosyan A, Seymour EM, Llanes DEU, Kaufman PB, Bolling SF (2009) Chemical profile and antioxidant capacities of tart cherry products. Food Chem 115 (1):20-25. doi:10.1016/j.foodchem.2008.11.042

7. Bell PG, McHugh MP, Stevenson E, Howatson G (2013) The role of cherries in exercise and health. Scand J Med Sci Sport. doi:10.1111/sms.12085

8. Bell PG, Walshe IH, Davison GW, Stevenson E, Howatson G (2014) Montmorency Cherries Reduce the Oxidative Stress and Inflammatory Responses to Repeated Days High-Intensity Stochastic Cycling. Nutrients 6 (2):829-843. doi:10.3390/nu6020829

9. Howatson G, McHugh MP, Hill JA, Brouner J, Jewell AP, Van Someren KA, Shave RE, Howatson SA (2010) Influence of tart cherry juice on indices of recovery following marathon running. Scand J Med Sci Sport 20 (6):843-852. doi:10.1111/j.1600-0838.2009.01005.x

10. Pigeon WR, Carr M, Gorman C, Perlis ML (2010) Effects of a Tart Cherry Juice Beverage on the Sleep of Older Adults with Insomnia: A Pilot Study. J Med Food 13 (3):579-583. doi:doi:10.1089/jmf.2009.0096

11. Howatson G, Bell PG, Tallent J, Middleton B, McHugh MP, Ellis J (2012) Effect of tart cherry juice (Prunus cerasus) on melatonin levels and enhanced sleep quality. Eur J Nutr 51 (8):909-916. doi:10.1007/s00394-011-0263-7

12. Wang H, Nair MG, Strasburg GM, Chang YC, Booren AM, Gray JI, DeWitt DL (1999) Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. J Nat Prod 62 (2):294-296. doi:10.1021/np980501m

13. Bell PG, Gaze DC, Davison GW, George TW, Scotter MJ, Howatson G (2014) Montmorency tart cherry (Prunus cerasus L.) concentrate lowers uric acid, independent of plasma cyanidin-3-O-glucosiderutinoside. J Funct Foods 11:82-90. doi:10.1016/j.jff.2014.09.004

14. Seymour EM, Warber SM, Kirakosyan A, Noon KR, Gillespie B, Uhley VE, Wunder J, Urcuyo DE, Kaufman PB, Bolling SF (2014) Anthocyanin pharmacokinetics and dose-dependent plasma antioxidant pharmacodynamics following whole tart cherry intake in healthy humans. J Funct Foods 11:509-516. doi:10.1016/j.jff.2014.08.007

15. Lila MA, Raskin I (2005) Health-related interactions of phytochemicals. J Food Sci 70 (1):R20-R27

16. Patras A, Brunton NP, O'Donnell C, Tiwari BK (2010) Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. Trends Food Sci Technol 21 (1):3-11. doi:10.1016/j.tifs.2009.07.004

17. Tomas-Barberan F, Espin JC (2001) Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. J Sci Food Agr 81 (9):853-876. doi:10.1002/jsfa.885

18. Vitaglione P, Donnarumma G, Napolitano A, Galvano F, Gallo A, Scalfi L, Fogliano V (2007) Protocatechuic acid is the major human metabolite of cyanidin-glucosides. J Nutr 137 (9):2043-2048

19. de Ferrars RM, Czank C, Zhang Q, Botting NP, Kroon PA, Cassidy A, Kay CD (2014) The pharmacokinetics of anthocyanins and their metabolites in humans. Br J Pharmacol 171 (13):3268-3282. doi:10.1111/bph.12676

20. Nurmi T, Mursu J, Heinonen M, Nurmi A, Hiltunen R, Voutilainen S (2009) Metabolism of Berry Anthocyanins to Phenolic Acids in Humans. J Agric Food Chem 57 (6):2274-2281. doi:10.1021/jf8035116

21. Ross R (1993) The pathogensis of atherosclerosis - a perspective for the 1990s. Nature 362 (6423):801-809. doi:10.1038/362801a0

22. Owens G (1995) Regulation of differentiation of vascular smooth muscle cells. Physiol Rev 75 (3):487-517

23. Buckow R, Kastell A, Terefe NS, Versteeg C (2010) Pressure and Temperature Effects on Degradation Kinetics and Storage Stability of Total Anthocyanins in Blueberry Juice. J Agric Food Chem 58 (18):10076-10084. doi:10.1021/jf1015347

24. Shahidi F HCT (2007) Antioxidant measurement and applications. . ACS Symposium Series 956 American Chemical Society, Washington DC

25. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Lebenson Wiss Technol 28 (1):25-30. doi:10.1016/s0023-6438(95)80008-5

26. Ou B, Bosak KN, Brickner PR, Iezzoni DG, Seymour EM (2012) Processed Tart Cherry Products-Comparative Phytochemical Content, in vitro Antioxidant Capacity and in vitro Anti-inflammatory Activity. J Food Sci 77 (5):H105-H112. doi:10.1111/j.1750-3841.2012.02681.x

27. Wang HB, Nair MG, Iezzoni AF, Strasburg GM, Booren AM, Gray JI (1997) Quantification and characterization of anthocyanins in Balaton tart cherries. J Agric Food Chem 45 (7):2556-2560. doi:10.1021/jf960896k

28. Kamat JP, Ghosh A, Devasagayam TPA (2000) Vanillin as an antioxidant in rat liver mitochondria: Inhibition of protein oxidation and lipid peroxidation induced by photosensitization. Mol Cel. Biochem 209 (1-2):47-53. doi:10.1023/a:1007048313556

29. Chiang LC, Ng LT, Chiang W, Chang MY, Lin CC (2003) Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of plantago species. Planta Medica 69 (7):600-604

30. Fang J (2014) Bioavailability of anthocyanins. Drug Metab Rev 46 (4):508-520. doi:10.3109/03602532.2014.978080

31. Azzini E, Vitaglione P, Intorre F, Napolitano A, Durazzo A, Foddai MS, Fumagalli A, Catasta G, Rossi L, Venneria E, Raguzzini A, Palomba L, Fogliano V, Maiani G (2010) Bioavailability of strawberry antioxidants in human subjects. Brit J Nutr 104 (8):1165-1173. doi:10.1017/s000711451000187x

32. Kirakosyan A, Seymour EM, Wolforth J, McNish R, Kaufman PB, Bolling SF (2015) Tissue bioavailability of anthocyanins from whole tart cherry in healthy rats. Food Chemistry 171:26-31. doi:10.1016/j.foodchem.2014.08.114

33. Kakkar S, Bais S (2014) A review on protocatechuic Acid and its pharmacological potential. ISRN pharmacology 2014:952943-952943. doi:10.1155/2014/952943

34. Stalmach A, Williamson G, Crozier A (2014) Impact of dose on the bioavailability of coffee chlorogenic acids in humans. Food Funct 5 (8):1727-1737. doi:10.1039/c4fo00316k

35. Stalmach A, Mullen W, Barron D, Uchida K, Yokota T, Cavin C, Steiling H, Williamson G, Crozier A (2009) Metabolite Profiling of Hydroxycinnamate Derivatives in Plasma and Urine after the Ingestion of Coffee

by Humans: Identification of Biomarkers of Coffee Consumption. Drug Metab Dispos 37 (8):1749-1758. doi:10.1124/dmd.109.028019

36. Azuma K, Ippoushi K, Nakayama M, Ito H, Higashio H, Terao J (2000) Absorption of chlorogenic acid and caffeic acid in rats after oral administration. J Agric Food Chem 48 (11):5496-5500. doi:10.1021/jf000483q

37. Donangelo AFCM (2006) Phenolic compounds in coffee. Braz J Plant Physiol 18 (1)

38. Farah A, Monteiro M, Donangelo CM, Lafay S (2008) Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans. J Nutr 138 (12):2309-2315. doi:10.3945/jn.108.095554

39. Mountzouris KC, Tsirtsikos P, Kalamara E, Nitsch S, Schatzmayr G, Fegeros K (2007) Evaluation of the efficacy of a Probiotic containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult Sci 86 (2):309-317

40. Louis SF, Zahradka P (2010) Vascular smooth muscle cell motility: From migration to invasion. Exp Clin Cardiol 15 (4):E75-E85

41. Wang H, Liu T-Q, Guan S, Zhu Y-X, Cui Z-F (2008) Protocatechuic acid from Alpinia oxyphylla promotes migration of human adipose tissue-derived stromal cells in vitro. Eur J Pharmacol 599 (1-3):24-31. doi:10.1016/j.ejphar.2008.09.030

42. Lin H, Chen, JH, Chou, FP, Wang, CJ. (2011) Protocatechuic acid inhibits cancer cell metastasis involving the down-regulation of Ras/Akt/NF-κB pathway and MMP-2 production by targeting RhoB activation. Br J Pharmacol 162 (1):237-254

43. Lirdprapamongkol K, Kramb J-P, Suthiphongchai T, Surarit R, Srisomsap C, Dannhardt G, Svasti J (2009) Vanillin Suppresses Metastatic Potential of Human Cancer Cells through PI3K Inhibition and Decreases Angiogenesis in Vivo.J Agric Food Chem 57 (8):3055-3063. doi:10.1021/jf803366f

44. Mudnic I, Budimir D, Modun D, Gunjaca G, Generalic I, Skroza D, Katalinic V, Ljubenkov I, Boban M (2012) Antioxidant and Vasodilatory Effects of Blackberry and Grape Wines. J Med Food 15 (3):315-321. doi:10.1089/jmf.2011.0129

45. Broncel M, Kozirog M, Duchnowicz P, Koter-Michalak M, Sikora J, Chojnowska-Jezierska J (2010) Aronia melanocarpa extract reduces blood pressure, serum endothelin, lipid, and oxidative stress marker levels in patients with metabolic syndrome. Med Sci Monit 16 (1):CR28-CR34

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 ± 0.326 and 1.70 ± 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 ± SEM

Fig 3 VA responses from baseline to 30 mL and 60 mL Montmorency cherry concentrate (MC). Absolute baseline values were 0.158 ± 0.031 and 0.093 ± 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 ± SEM

Fig 4 % Migration of human vascular smooth muscle cells *in vitro* in response to metabolites PCA (32μM) and VA (4μM) 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 ±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*
30 mL MC Concentrate	31.24 ± 0.16	71.37± 0.11	0.30±0.01	0.205±0.24	0.020±0.11	0.253±0.84
60 mL MC Concentrate	62.47 ± 0.31	142.73 ±0.22	0.60±0.03	0.410±0.48	0.040±0.22	0.506±1.68
Frozen cherries	0.03 ± 0.0009	0.005± 0.0004	0.002± 0.0002	-	-	-
Dried cherries	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

Compound	UV/Vis wavelength (nm)	Retention time (min)	LOD (µg/mL)	Range of linearity(µg/mL)
PCA	260	9.263	<0.05	0.5-80
CHL	326	10.140	<0.04	0.4-80
VA	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[1]
PCA	$C_7H_6O_4$	154.034	Negative	154.026611
CHL	$C_{16}H_{18}O_9$	354.102	Negative	354.095093
VA	$C_8H_8O_4$	168.049	Negative	168.042252

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

Fig 1

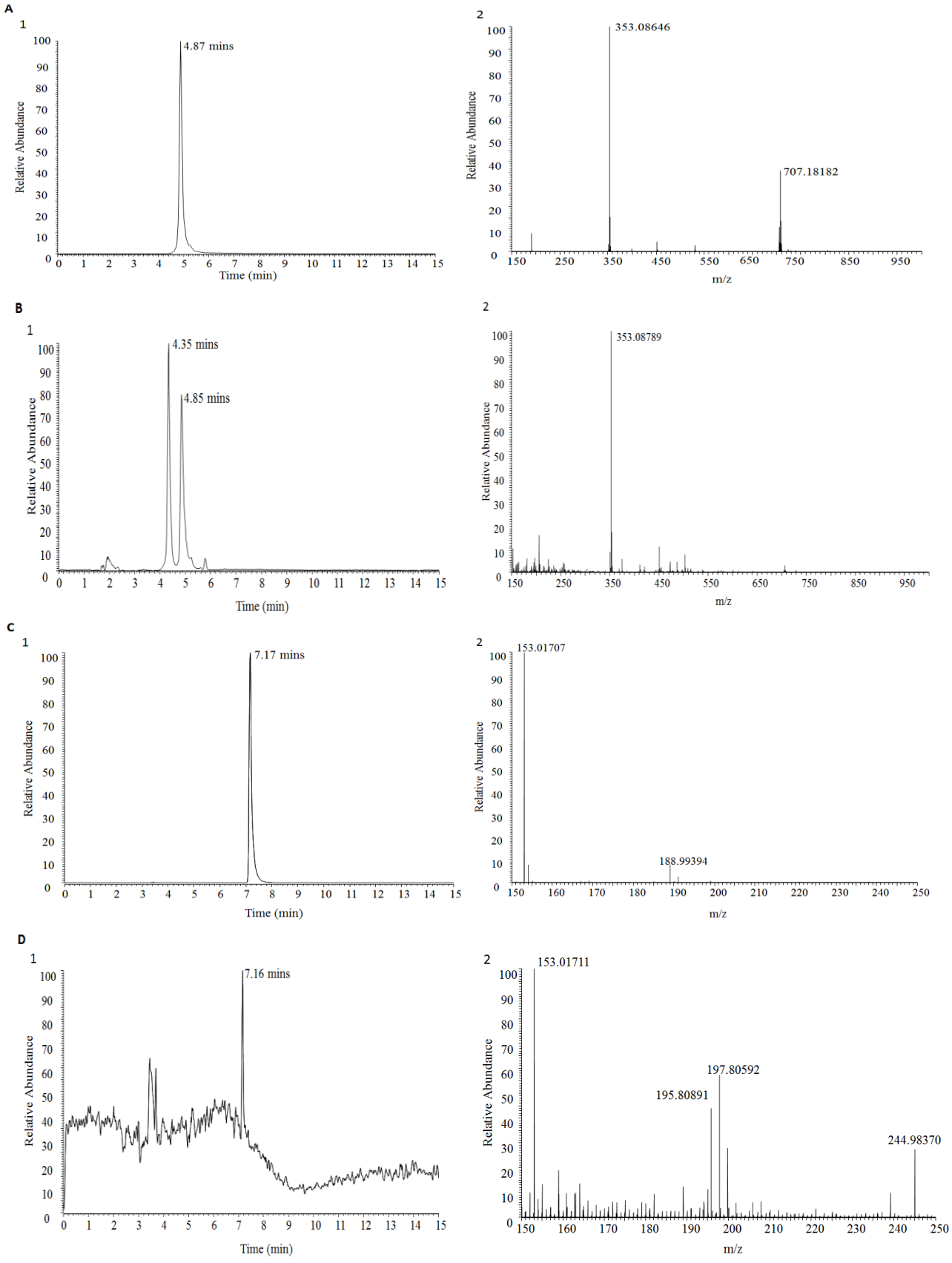

A
1
4.87 mins
Relative Abundance
Time (min)
2
353.08646
707.18182
Relative Abundance
m/z
B
1
4.35 mins
4.85 mins
Relative Abundance
Time (min)
2
353.08789
Relative Abundance
m/z
C
1
7.17 mins
Relative Abundance
Time (min)
2
153.01707
188.99394
Relative Abundance
m/z
D
1
7.16 mins
Relative Abundance
Time (min)
2
153.01711
197.80592
195.80891
244.98370
Relative Abundance
m/z

Fig 2

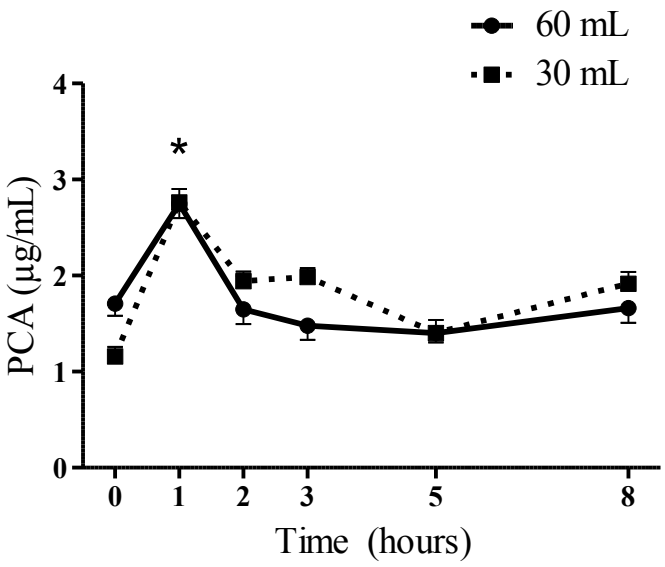


Fig 3

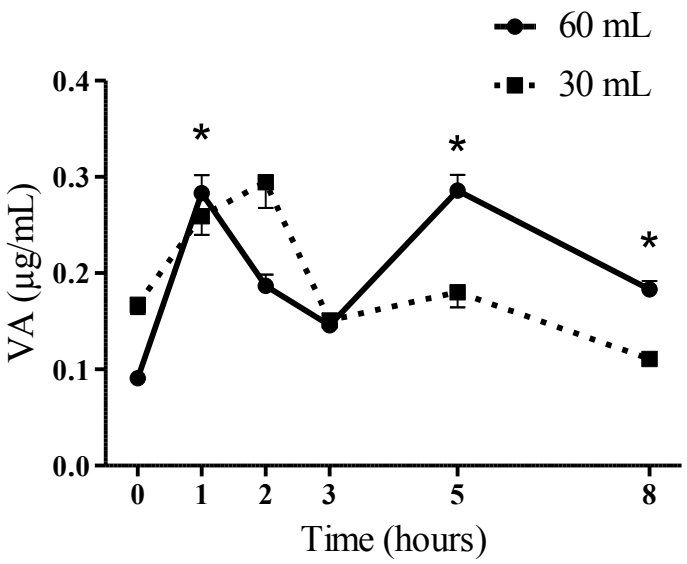
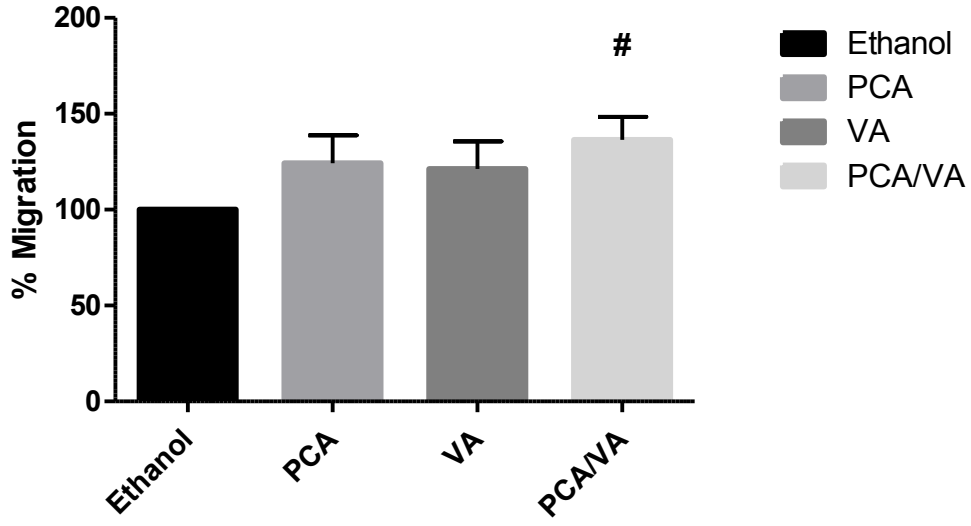


Fig 4


200
150
100
50
0
% Migration
#
Ethanol
PCA
VA
PCA/VA
Ethanol
PCA
VA
PCA/VA

Online Supporting Material

Supplemental Figure 1

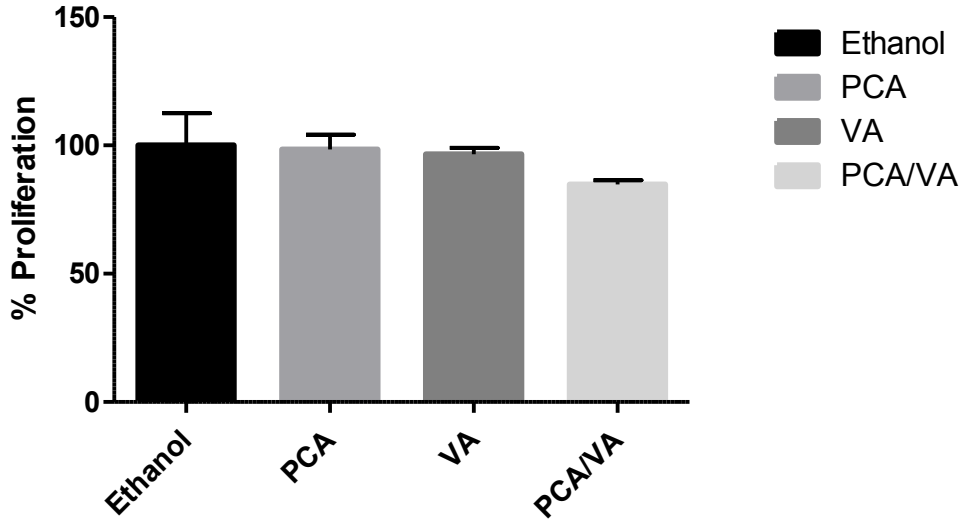


Fig S1 % Proliferation of human vascular smooth muscle cells *in vitro* in response to metabolites PCA (32μM) and VA (4μM) compared to ethanol only control, over 24hours. Combined data from four separate experiments; data presented as mean ±SEM.