Physiologically relevant screening of polyphenol-rich commercial preparations for bioactivity in vascular endothelial cells and application to healthy volunteers: a viable workflow and a cautionary tale

H.S. Jones[1]*, M. Papageorgiou[2], A. Gordon[2], Ehtesham[2], Z. Javed[2], L.K. Wells[2], S. Greetham[2], B.Doyle[1], N. Hayes[1], A. Rigby[2], S.L. Atkin[3], F.L. Courts[2], T. Sathyapalan[2]

[1] Department of Chemistry and Biochemistry, University of Hull, Hull, HU6 7RX

[2] Hull York Medical School, University of Hull, Hull, HU6 7RX

[3] Royal college of surgeons in Ireland, Bahrain

*corresponding author

huw.jones@hull.ac.uk

+44 1482 463320

Declarations of interest: NONE

ABSTRACT

This study describes the screening of 13 commercially-available plant extracts for pharmacological activity modulating vascular function using an endothelial cell model. A French maritime pine bark extract (FMPBE) was found to have the greatest effect upon nitric oxide availability in control (181% ± 36% of untreated cells) and dysfunctional cells (132% ± 8% of untreated control cells). In healthy volunteers, the FMPBE increased plasma nitrite concentrations 8 h post-consumption compared to baseline (baseline corrected median 1.71 ± 0.38 (25% IQR) and 4.76 (75% IQR) μM, $p<0.05$). This was followed by a placebo-controlled, healthy volunteer study, which showed no effects on plasma nitrite. It was confirmed that different batches of extract had been used in the healthy volunteer studies, and this second batch lacked bioactivity, assessed using the in vitro model. No difference in plasma catechin levels was seen at 8 h following supplementation between the studies (252 ± 194 nM versus 50 ±64 nM, $p>0.05$), however HPLC-UV fingerprinting showed that the new batch had a 5-15% in major constituents (including procyanidins A2, B1 and B2) compared to the original batch. This research describes a robust mechanism for screening bioactive extracts for vascular effects. It also highlights batch variability as a significant limitation when using complex extracts for pharmacological activity, and suggests the use of in vitro systems as a tool to identify this problem in future studies.

Keywords: Nitric oxide; vascular function; plant extracts; batch variability.

1. INTRODUCTION

Plant extracts are a popular potential source of therapeutic interventions for chronic diseases such as hypertension, due to a range of factors including accessibility, low toxicity/high tolerance, and user perceptions regarding benefits and ethics of use (Sauer & Plauth 2017). Of those plant extracts shown to have beneficial effects on blood flow and blood pressure in humans, many are rich in polyphenols (e.g. quercetin, epicatechin), which have been shown to exert positive effects on blood flow and blood pressure in human volunteers (Schroeter et al 2006, Edwards et al 2007). Thus, there is interest in identifying either isolated polyphenols or extracts rich in certain polyphenols for use as supplements with health benefits. The major limiting factors for studies using plant extracts are i) their poor/inadequate characterisation, ii) the limited bioavailability of constituents, iii) the properties and nature of the active component, which may be a metabolic derivative of another component in the extract before ingestion and, iv) limited knowledge of the mechanisms that explain any reported bio-active effects (Manach et al., 2005). It is also anecdotally described that an additional limitation of the use of crude plant extracts is the substantial batch-to-batch variability which forms a significant barrier to the use of crude extracts for therapeutic benefit. Despite the evidence of potential batch variation in extract composition, there is little published evidence on this type of variability in the bioactivity of commercially sourced and partially characterised extracts.

Therefore, in the present study, we employed a comprehensive experimental approach to assess the bioactivity of partially-characterised, commercially sourced plant extracts (i.e. some of the polyphenolic content of these extracts have been

identified). We initially screened polyphenol-rich commercial preparations for bioactivity (modulation of nitic oxide availability and Angiotensin Converting Enzyme (ACE) activity) in vascular endothelial cells under both healthy and dysfunctional conditions at physiologically-relevant concentrations. Commercially available extracts were purposefully used in this study, as they are at least partially characterised (i.e., several polyphenolic species are listed on the certificate of analysis and extracts are reported to have amounts of these species within defined ranges) in order to address potential issues with extract characterisation. Based on the results of this in vitro screening, a French maritime pine bark extract was selected for assessment in healthy volunteers. In order to assess the bioactivity of this extract in vivo, we undertook , two trials in healthy volunteers; the first study aimed to provide initial validation of the findings of the in vitro screening results (does the selected extract demonstrate bioactivity in vivo as well as in vitro?). The second study aimed to confirm these findings in a more robust manner, using a placebo-controlled, cross-over design.

2. MATERIALS AND METHODS

Materials

All materials, unless otherwise stated, were purchased from Sigma-Aldrich (Poole, UK). The following commercially available polyphenol-rich preparations (termed NP – native preparations) were used in this study, and kindly provided by Boots Pharmaceuticals: NP1 – Amlamax Indian gooseberry (*Emblica officinalis*) extract 15% (Arjuna Natural Extracts LTD, India, lot# AET-301/1207/RD-11); NP2 –Black currant 25% anthrocyanins 82001 *Ribes nigrum* (Frutarom, Belgum, lot # PB0306) ; NP3 – Vineatrol 30 grapevine shoot (*Vitis vinifera*) extract (Breko GMBH, Bremmen,

Germany, lot# R283-12); NP4 – Naturex cocoa 45% PE (Gee Lawson, London, UK, Lot #V163/016/A12) ; NP5 – Naturex grape skin extract (Gee Lawson, London, UK, Lot # A101/060/A12); NP6 – Naturex green tea extract (Gee Lawson, London, UK, Lot # A30/026/A12) ; NP7 – Oligopin French maritime pine bark extract (DRT, France); NP8 – Mirtoselect bilberry extract 35% (Indena SAS, France, Lot # 30392/M1) ; NP9 – Vinitrox apple and grape polyphenol extract (Nexira, France, lot# 1203297) ; NP10 - OliOla olive extract (Nexira, France, lot#1112092); NP11 – Green tea extract (Slater and Frith, Norwich, UK, Lot# PBH43782); NP12 – Worldway soy isoflavones (WorldWay Inc., China, Lot# PBH43594) ; NP13 – Fruitflow lycopene-free tomato concentrate (DSM Nutritional Products, Basel, Switzerland, powder format, lot# CH2012.01C).

Human umbilical vein endothelial cell (HUVEC) culture

HUVEC were cultured and treated as described by Jones et al 2016. Briefly, HUVEC were purchased from Promocell (Heidlberg, Germany) and cultured in endothelial cell growth media (Promocell, Heidlberg, Germany) supplemented with 20% fetal bovine serum (FBS) and grown up to passage 6 for use in experiments. Cells were seeded at a density of 14700 cells/cm^2 and cultured to a density of 80% confluency before incubation in serum deprived M199 media (0.5% v/v FBS) 24 h before experiments. In order to generate the healthy and dysfunction models, the cell media was changed to medium-199 supplemented with 0.5% FBS containing either water (solvent control) or 100 nM angiotensin II for 8 h. This treatment has been shown to reduce nitric oxide bioavailability in HUVEC cultures, providing a useful model system for assessing the pharmacological effects of polyphenols *in vitro* (Jones et al 2016).

Standardisation of polyphenol-rich preparations using the Folin-Ciocalteu assay

All preparations were dissolved in dimethyl-formamide (DMF) at a concentration of 50 mg/ml, using vigorous vortex mixing and centrifugation to remove any precipitate and debris with the supernatant retained (16000 x g, 5 min, room temperature). These preparations were further diluted 100-fold in 18.2 MΩ water prior to use in the assay. The diluted NP samples (15 µl) were added to 18.2 MΩ water (170 µl), Folin-Ciocalteu reagent (12 µl) and sodium carbonate (200 g/L, 30 µl) in a 96-well plate and incubated at room temperature for 1 hour in the dark. Water (18.2 MΩ, 73 µl) was added to each well and the absorbance at 765 nm was measured using a BMGlabtech Omega plate reader. Quantification of phenolic content was done using a standard curve of epicatechin ranging from 0-200 µM, with the solvent concentration matched to that of the NP samples. All samples were assessed in triplicate, with mean concentrations (relative to epicatechin) calculated.

MTS assay

HUVEC were seeded in 96-well plates as described above, and incubated with 10 µM of each preparation for 8 h. The cells were then assayed for viability using the MTS assay kit (Promega, Hampshire, UK) as instructed by the manufacturer.

Nitric oxide bioavailability assay

Nitric oxide bioavailability was assessed in HUVEC cultures as previously described by Jones et al (2016) using the fluorescent probe DAF-2DA (Enzo life-sciences,

Exeter, UK). Briefly, HUVEC were exposed to the polyphenol-rich extracts at a standardised concentration of 1 µM (DMF final concentration of 0.1% v/v) for 8 h prior to assay. Cells were then washed in HBSS (containing calcium and magnesium) and incubated with 2 µM DAF-2DA, with fluorescence measured every minute for 30 minutes at λ_{ex} = 485 nm and λ_{em} = 520 nm (Tecan infinite X200 plate reader, 10 flashes per point, 4 x 4 grid per well, manual gain = 100). The linear rate of fluorescence for each well was calculated and expressed as a percentage of solvent only control cells.

Initial assessment of bioactivity of French maritime pine bark extract in human volunteers for validation of in vitro screening results.

The first healthy volunteer study aimed to assess potential bioactivity of the French maritime pine bark extract (Oligopin®). According to the manufacturer certificate of analysis, this extract has a high content of low molecular weight procyanidins and <1% content in tannins. Thirteen healthy volunteers (7 males and 6 females) were screened after identification from the local research database of healthy volunteers (Academic Diabetes, Endocrinology and Metabolism Unit, Hull University Teaching Hospitals NHS Trust). Inclusion and exclusion criteria are listed in table 1. One individual was excluded from participation during screening due to difficult venous access. Thus, a total of 12 subjects were entered into the clinical study. All the study procedures were approved by National Research Ethics Services Committee, Yorkshire and the Humber (14/YH/0084). Written informed consent was obtained from all participants prior to their commencement in the study.

Participants attended an initial preliminary visit to establish that the inclusion criteria were met and a single study visit following an overnight fast. Participant age, gender,

blood pressure, height, weight and body mass index (BMI) from this preliminary visit are presented in table 2. Participants were instructed by a registered dietitian on how to follow a polyphenol low diet for one week before starting the trial and for the duration of the trial. In addition, participants avoided alcohol for 24 hours before attending the laboratory. Body weight, height, blood pressure, heart rate, temperature, hip and waist circumference were measured. Baseline blood samples were collected prior to consumption of the French maritime pine bark extract (Oligopin®, 1.1.g contained in 4 capsules) with ab libitum water. Blood samples were collected at 0 and 30 minutes and 1, 2, 4 and 8 hours post-ingestion and analysed for plasma nitrate and nitrite concentrations and for plasma catechin and epicatechin concentrations (see below).

A placebo-controlled assessment of the bioactivity of French maritime pine bark extract in healthy volunteers.

Twenty-four healthy volunteers (11 males and 13 females, age: 36 ±14 years; BMI: 26.2 ± 3.1 kg/m^2) were screened initially after identification from the local research database of healthy volunteers (Academic Diabetes, Endocrinology and Metabolism Unit, Hull University Teaching Hospitals NHS Trust). Three subjects dropped out from the study before (n=1) or after (n=2) entering the intervention phase without giving any reasons and therefore, replaced by three healthy volunteers from the healthy volunteers dataset. In total, twenty-four subjects (11 males and 13 females) entered the study. This study was approved by the National Research Ethics Services Committee, Yorkshire and Humber (14/YH/0084) and written informed consent was obtained. The inclusion/exclusion criteria are listed in table 1.

Participants attended five study visits (Visits 1-5). During Visit 1, participants were screened against inclusion and exclusion criteria by medical history and clinical

examination, routine blood tests (i.e., full blood count, liver function tests, biochemical profile, clotting screen and a pregnancy test, if applicable) and anthropometric measurements (see table 3). Similar to the procedure followed in the initial healthy volunteer study, participants were instructed to follow a low-polyphenol diet throughout the study and avoid alcohol for 24 hours before attending the laboratory for the main experimental visits (visit 2-5). An independent person not involved in the study oversaw participant randomisation using a computer generated randomisation list, and they un-blinded the samples at the end of the study. The visit 2 and 4 were the intervention visits when participants consumed the French maritime pine bark extract (Oligopin®, 1.1.g contained in 4 capsules) or placebo (methylcellulose-filled capsules, 1.1 g contained in 4 capsules) as a first intervention in a randomised order. These visits were followed by visit 3 and 5 the following mornings (24h following visit 2 and visit 4, respectively). Figure 1 shows a flow diagram schematic of this volunteer trial.

Blood samples were collected at baseline following an overnight fast and at 2, 4, 8 and 24h (visit 3 & 5 - following an overnight fast) post-ingestion of French maritime pine bark and placebo, and were analysed for nitrate and nitrite concentrations. A washout period of at least 7 days was used between each intervention, as it was expected that any bioactive constituents would have been excreted within this time-frame, and cross-contamination between the placebo and French maritime pine bark extract would be minimised.

Nitrate and Nitrite quantification in human plasma

For the first healthy volunteer study, nitrate and nitrite levels in human plasma were measured using the Cayman Chemicals colourmetric Nitrate/Nitrite assay kit (Cambridge Biosciences, Cambridgeshire, UK), as directed by the manufacturer

after filtration of plasma samples using 10 kDa molecular weight cut off filters (Millipore). It was noted during the analysis of plasma nitrite levels in the first volunteer study that the colourmetric assay kit lacked the necessary sensitivity nitrite detection in our hands. Thus, in the second healthy volunteer study, the Cayman Chemicals Fluorometric Nitrate/Nitrite assay kit (Cambridge Biosciences, Cambridgeshire, UK) was used as directed by the manufacturer, after filtration as described above. Absorbance and fluorescence measures were undertaken using a BMGlabtech omega series multimodal plate reader at the wavelengths recommended by the assay kit manufacturer.

Human plasma catechin and epicatechin measurements

Commercial preparations of French Maritime pine bark are rich in polyphenols and more specifically, procyanidins, with the main constituents being catechin and epicatechin. Other active ingredients include polyphenolic monomers, phenocarbonic acids and their glycosides (Rohdewald, 2002). Plasma concentrations of catechin and epicatechin were quantified using a LC-MS approach, using diadzein as an internal standard. Plasma samples (200 µl) were incubated with a deconjugation mix (60 µl sulfatase, 5.1µl β-glucuronidase and 1 µM final concentration of diadzein as an internal standard) for 2 hours at 37 °C. These samples were then deproteinated by addition of 120 µl of acidified DMF (100 µl of DMF plus 20 µl of formic acid) and incubation at room temperature for 10 minutes with regular mixing by vortex. The precipitated protein was removed by centrifugation (16000 x g, 10 minutes) and the supernatant was retained. The supernatant was loaded into a HPLC insert vial and analysed as detailed below.

Chromatographic separation was achieved using a Shimadzu LC20-AD quaternary pump, SIL-20A HT autosampler and CTO-10A column oven connected via a FCV-20AH$_2$ switching valve to a LC2020 single quadropole mass spectrometer. An Agilent Eclipse-XDB-C$_{18}$ column (5 µm pore size, 4.6 x 150 mm, Agilent Technologies, Cheshire, UK) was used for separating the analytes as detailed below, with solvent A comprising of 0.5% v/v formic acid in water, and solvent B comprising 0.5% v/v formic acid in methanol. The column was maintained at 40 °C. The method began with a gradient of solvent B from 35% to 45% over 5 minutes, followed by an increase in solvent B from 45% to 80% over 5 minutes. Solvent B was then maintained at 80% for 5 minutes before returning to 35% over 30 seconds. The initial starting conditions were re-equillabrated over 4.5 minutes. Under these conditions catechin eluted at RT = 3.7 min, epicatechin eluted at RT = 4.7 min, and diadzein at RT = 12.6 minutes. Both catechin and epicatechin were detected at a *m\z* = 289, diadzein at *m\z* = 253. Standard curves of catechin and epicatechin were prepared in plasma from fasted individuals, shown not to contain these analytes, for the quantification of the samples collected in this study. A representative chromatogram and standard curve for each analyte are shown in Figure 2. This method showed quantification of both catechin and epicatechin at a concentration of 100 nM in plasma samples.

HPLC-UV fingerprinting analysis of French maritime pine bark extracts

The two batches of Oligopin French maritime pine bark extract were assessed for differences in composition using a HPLC-UV method, with absorbance measured at 280 nm for each extract. Both extracts were standardised to a concentration of 5 mg/ml of extract in 10% v/v/ dimethyl-formamide (DMF). A solvent control (10% v/v

DMF) was also assessed at both wavelengths using the method described below. Standards of procyanidin A2, B1, B2 and C1 (Cambridge Biosciences, Cambridgshire, UK) were also run at a concentration of 0.1 mM in 10% DMF.

Separation was achieved using a Shimadzu LC20 HPLC system (autosampler, quaternary pump, column oven, and diode array detector) at a flow rate of 1 ml/min and an injection volume of 15 µl. An Agilent Eclipse-XDB-C_{18} column (5 µm pore size, 4.6 x 150 mm, Agilent Technologies, Cheshire, UK) was used for separating the analytes as detailed below, with solvent A comprising of 0.1% v/v formic acid in water, and solvent B comprising 0.1% v/v formic acid in methanol. The column was maintained at 40 °C. The method began with a plateau of solvent B at 2% for 12 minutes, followed by an increase in solvent B from 2% to 25% over 18 minutes. Solvent B was then increased over 2 minutes to 38%, and maintained at this composition for 28 minutes before increasing to 80% over 2 minutes. These conditions were maintained for 6 minutes before returning to the initial starting conditions.

Statistical analysis

Normality of distribution of the data was tested using the Shapiro-Wilk test. For parametric data, a t-test (with or without Welch correction) or ANOVA with post-hoc testing was used as appropriate. If the data was not normally distributed, ANOVA on ranks with comparisons versus control or baseline samples was done using an appropriate post-hoc test. The Sigmaplot v.12 and Graphpad Prism v. 8 software were used to do these statistical tests.

For the second healthy volunteer study a two-way ANOVA with repeated measures was used to determine the effects of treatment and interaction effects (intervention x time) for blood pressure, nitrite and nitrate concentrations. Non-normally distributed data were log-transformed prior to this analysis. Significant main or interaction effects were followed by Bonferroni's post-hoc analysis. Statistical significance was set at $p \leq 0.05$ and SPSS v.24.0 software was used to perform this analysis.

3. RESULTS

In vitro screening of polyphenol-rich plant extracts – MTS assay

In order to test the extracts for any toxic effects in cultured cells, the extracts were incubated for 8 hours with cells at a concentration of 10 µM prior to assessment of cell viability using the MTS assay. There were no significant decreases in assay response observed for any of the tested extracts, suggesting that the extracts are not toxic at this concentration (Figure 3).

In vitro screening of polyphenol-rich plant extracts – nitric oxide bioavailability

All extracts were screened at a standardised concentration of 1 µM, with an 8 hour incubation period, in cells that had either been treated with solvent control (Figure 4A) or angiotensin II (Figure 4B, n=2-4 independent experiments per extract, 6 wells per treatment, per experiment). At this concentration, with the exception of NP4 and NP6, all tested extracts returned nitric oxide bioavailability to near control levels in the angiotensin II-treated cells, whereas only NP1 (121 ± 20%, n=3 independent experiments), NP5 (121 ± 6%, n=2 independent experiments), NP7 (118 ± 5%, n=2

independent experiments) and NP10 (121 ± 8%, n=2 independent experiments) appeared to improve nitric oxide availability under control conditions. Thus NP1, NP5 and NP7 were taken forward for more detailed dose-response assessment under both control (Figure 4C and dysfunctional conditions (Figure4D). The extract NP10 was discounted at this stage due to a significant lack of solubility in aqueous media. Through this dose-response assessment (ranging from 1 nM to 1 µM), the French maritime pine bark extract (NP7) was observed to be the only preparation to positively affect nitric oxide availability in both healthy and dysfunctional conditions (Figure 4C and 4D).

In vivo assessment of the effects of the French maritime pine bark extract in human volunteers

During this study, no adverse effects of supplementation were reported or observed. Potential vascular bioactivity was assessed using the measurement of plasma nitrate and nitrite concentrations. Although no differences were observed between baseline, 4 h, and 8 h post ingestion for plasma nitrate (mean ± SD, baseline = 31.35 ±11.99 µM, 4 h = 25.32 ±8.47 µM, 8 h = 24.22 ± 9.21 µM, $p>0.05$, one-way ANOVA), there was a significant increase in plasma nitrite at 8 h compared to baseline (Table 3: median concentration at 4h relative to baseline = -0.96 ± range of -10.25 to 0.00 µM, median concentration at 8h relative to baseline = 1.71 ± range of -0.19 to 4.79 µM, n=11, Kruskal-Wallis test $p<0.001$, Dunn's post-hoc test $p<0.05$ for 8 h versus baseline). This finding prompted a second clinical study to further investigate the vascular bioactivity of French maritime pine bark extract in healthy volunteers. In the placebo-controlled, randomised cross-over design study no effect of French maritime pine bark extract on plasma nitrite was observed at any time point (placebo (mean ±

SD): 4 hours = 0.04 ± 0.09 µM, 8 hours post = 0.06 ± 0.09 µM; Oligopin: 4 hours = 0.04 ± 0.18 µM, 8 hours post = -0.05 ± 0.17 µM; all values corrected for baseline concentration, raw data in Table 4)). After the completion of these healthy volunteer studies it became apparent that two different batches of French maritime pine bark had been supplied (one used in the initial in vitro and first healthy volunteer assessments, and the second in the placebo-controlled study). This observation led us to hypothesise that there was a either a compositional difference between the two batches of extract that underlay the differences in apparent bioactivity, or that the in vitro screening model used in this study did not reliably predict in vivo bioactivity.

In vitro comparison of French maritime pine bark extracts – nitric oxide bioavailability

We first assessed the validity of the in vitro screening assay by comparing both extracts for effects on nitric oxide availability using the in vitro endothelial cell culture model, under control conditions using a dose-response of extract ranging from 1 nM to 1 µM. It was found that there was no effect of the second batch (Figure 5, n=2 independent experiments, 6 wells per treatment per experiment). This lack of previously observed increase in nitric oxide availability suggested that the in vitro model reflected in vivo bioactivity, and that a likely explanation for the observed batch variation was due to compositional differences of the French maritime pine bark extract.

Assessment of French maritime pine bark extract catechin and epicatechin concentrations in the extracts used in the healthy volunteer studies

Based on composition information from the manufacturer, catechin and epicatechin were expected to be the major constituents observed in volunteer plasma samples,

however only catechin was detected by the LC-MS method used in all samples (epicatechin was only detected in a single sample in the placebo-controlled study). In the first study, plasma catechin was detected at baseline, with no obvious C_{max}, half-life or elimination of catechin detected, despite a trend of an increase in plasma catechin concentration at 8 h (Figure 6). In the second study (placebo-controlled), an increase in plasma catechin levels was detected at 8 hours post ingestion of Oligopin® compared to baseline (mean increase of 54 nM ± 64 nM, n=24). This was a smaller increase than that observed within the first study (mean increase of 252 nM ±194 nM, n=6), however this difference between the two studies was not statistically significant (p=0.083, unpaired t-test with Welch correction). To further assess the two batches for compositional differences, HPLC-UV fingerprinting of each batch was undertaken (Figure 7). It is clear from the 280 nm UV traces that compositionally there are no obvious differences between the batches, however there is a noticeable difference in the height of each peak, with greater signal in the original batch compared to the second batch, suggesting that the second batch contains a lower amount of constituents compared to the original batch. The composition of both batches (based upon comparison with the procyanidin A2, B1, B2 and C1 standards) are detailed in table 6, and the representative peak areas showed a 5 to 15% reduction of each major constituent in the new batch compared with the original batch. Taken together, the fingerprinting and pharmacokinetic data suggest that the second batch has some compositional deficiency that underlies its lack of bioactivity, compared with the original batch.

4. DISCUSSION

In this work, we present the results of the application of a screening workflow from a primary cell culture model to human volunteers. Specifically, we initially screened 12 commercially available, polyphenol-rich extracts for potential pharmacological effects using a cell culture model of the vascular endothelium, which has been previously shown to predict pharmacological activity in vivo (Jones et al 2016). Through this screening, we identified a potentially bioactive extract, French maritime pine bark extract (Oligopin), which was further assessed in two separate trials in healthy volunteers. In these studies, we came across a major challenge of working with crude plant extracts, namely batch-to-batch variability. The two different batches of the extract used in the two studies had a similarly characterised composition (based on manufacturer certificates of analysis and HPLC-UV fingerprint analysis) and resulted in comparable plasma circulating levels of catechin and epicatechin (two possible candidate bioactive structures, and major constituents of the extract); however, they yielded different responses in healthy volunteers. Additionally, when compared using the in vitro screening tool, the two extract batches showed different pharmacological properties. As such, our work confirms anecdotally reported batch-to-batch variability in plant extracts and provides evidence that suggests such variability is due to composition differences and has significant pharmacological consequences. Additionally, this also highlights a major challenge that will need to be overcome for the production of a viable and efficacious plant extract, and will likely involve substantial standardisation of plant strain, growth conditions, and manufacturing processes.

There are several key challenges identified within the in vitro research of the pharmacology of dietary polyphenols and polyphenol-rich preparations. These include (i) the inherent limitation related to how well the in vitro model mimics the in vivo environment, (ii) the consideration of the bioavailability of the test compound(s) (i.e. are the test compound exposure concentration and duration reflective of the in vivo ADME (absorption, distribution, metabolism, excretion) processes), (iii) the lack of reflection of oxidative, conjugative and bacterial metabolism of test compounds, and (iv) the relevance of testing isolated single chemicals rather than considering of food matrix effects (i.e. diet derived chemicals are part of a complex mixture of numerous polyphenols and other species) (Alvia-Galvez et al 2018). The in vitro screening model used in this study addresses several, but not all, of these issues. In particular, the model system uses well-characterised primary vascular endothelial cells (HUVEC) and endothelial dysfunction is induced using a physiologically relevant stimulus, angiotensin II (for a detailed characterisation of this approach please see Jones et al 2016). In brief, the application of this model system to explore the bioactivity of quercetin, demonstrated that the HUVEC model showed similar healthy/dysfunctional responses to those reported in human patient trials (Jones et al 2016), indicating that this in vitro system reflects in vivo responses at least to some extent. The in vitro screening process also aimed to mimic in vivo ADME characteristics, exposure times reflecting the pharmacokinetics of major constituents (as identified from manufacturer certificates of analysis), along with the application of physiologically-relevant concentrations of test compounds (low micromolar to nanomolar range). In order to standardise, directly compare and rank the concentrations of the tested extracts, we chose to quantify total phenolic content (using the Folin Colciateu assay, relative to epicatechin) rather than focus on a

single polyphenolic species. It is also likely that our approach better reflected the complex nature of the extract. Conversely the focus on a single phenolic species may have resulted in some skewing of the concentrations of the constituents of the extract. The complex nature of the plant extracts used in this study also provided a degree of realism in terms of the food matrix effect issues as highlighted by Avilia-Galvez et al (2018). Despite the advantages, our study is is limited by the lack of integration of human and bacterial metabolic processes. As highlighted by Avila-Galvez et al (2018) and previously by Jones et al (2016), there is currently a lack of availability of conjugated metabolites of polyphenols, and also the platforms to synthesise them. Thus this limitation could not be overcome in this study.

The top “hit” from our in vitro screen was a French maritime pine bark extract (Oligopin). French maritime pine bark extracts have previously been reported to modulate vascular and cardiovascular function in a range of disease models and systems, including humans (Liu et al., 2004a; Hosseini et al., 2001, Araghi-Niknam et al., 2000; Wang et al., 1999, Devarahj et al., 2002, Ohkita et al 2011), although not all studies have reported bioactive effects (Drieling et al., 2010). It should also be noted that these studies utilised different commercial preparations French maritime pine bark extracts (e.g., Oligopin, Pycnogenol, Flavagenol) in variable dosages over different study durations and have assessed several endpoints, in a range of different populations (healthy vs. patients with existing comorbidities). Thus it is challenging to make direct comparisons between our trials and other published studies. We used a relatively large (1.1 g) single acute bolus of French maritime pine bark extract in two separate healthy volunteer experiments. In the initial human study we showed that French maritime pine bark extract was active, whereas bioactivity was absent in our second more comprehensive study in healthy

volunteers. After the completion of both trials, it became apparent that different batches of extract were used. The in vitro screening model confirmed that there were differences in bioactivity between the two batches of the extract. This observation of batch-to-batch variability may well contribute to the inconsistent reports of bioactivity of French maritime pine bark extract in humans, and, represents a significant challenge of using complex extracts for therapeutic benefits. To address this issue, we propose pre-screening of different batches of an extract using an appropriate in vitro system that has or can be shown to reflect in vivo biology to an appropriate degree.

In summary, this research describes the use of an in vitro primary cell culture model of endothelial cell function to identify potentially bioactive plant extracts. The most effective “hit” from this screening phase showed initial promise in the modulation of nitric oxide metabolites, however this observation was not repeatable due to the use of a different batch of extract for the repeat experiment. Thus, this research highlights a significant limitation of using complex plant extracts for pharmacological effects. It also suggests a potential route to identify this issue in future studies, the use of a robust in vitro model system that can quickly identify bioactivity in vitro.

Acknowledgements

This work was co-funded by Innovate UK and Boots Pharmaceuticals (TSB 101173), and HEIF5 funding. The authors would like to acknowledge the help and contribution of the support staff at the Academic Diabetes, Endocrinology and

Metabolism Unit, Hull University Teaching Hospitals NHS Trust. The authors would also like to thank Mr Dean Moore (Department of Chemistry and Biochemistry, University of Hull) for his help and technical support with the HPLC-UV fingerprinting experiments.

References

Araghi-Niknam, M., Hosseini, S., Larson, D., Rohdwald, P., Watson, R.R. (2000). Pine bark extract reduces platelet aggregation. Integrative medicine, 2, 2-3, 73-77.

Avila-Galvez, M.A., Gonzalez-Sarrias, A., Espin, J.C. (2018). In vitro research on dietary polyphenols and health: a call of caution and a guide on how to proceed. Journal of agricultural and food chemistry, 66, 30, 7857-7858.

Devaraj, S., Vega-Lopez, S., Kaul, N., Schonlau, F., Rohdewald, P., Jialal, I.(2002). Supplementation with pine bark extract rich in polyphenols increases plasma antioxidant capacity and alters the plasma lipoprotein profile. Lipids, 37, 10, 931-934.

Drieling RL, Gardner CD, Ma J, Ahn DK, Stafford RS. No beneficial effects of pine bark extract on cardiovascular disease risk factors. Arch Intern Med. 2010;170(17):1541-7

Edwards, R. L., Lyon T, Litwin, S. E., Rabovsky, A., Symons, J. D., Jalili, T. (2007). Quercetin reduces blood pressure in hypertensive subjects. Journal of Nutrition, 137, 11, 2405-2411.

Hosseini, S., Lee, J., Sepulveda, R.T., Rohdewald, P., Watson, R.(2001). A randomised, double-blind, placebo-controlled, prospective, 16 week crossover study to determine the role of Pycnogenol in modifying blood pressure in mildly hypertensive patients. Nutrition research, 21, 9, 1251-1260.

Jones, H.S., Gordon, A., Magwenzi, S., Naseem, K., Atkin, S.L., Courts, F.L. (2016). The dietary flavonoid quercetin ameliorates angiotensin II-induced redox imbalance in a human umbilical vein endothelial cell model of endothelial dysfunction via ablation of p47phox expression. Molecular nutrition and food research, 60, 4, 787-797.

Liu, X., Wei, J., Tan, F., Zhou, S., Wurthwein, G., Rohdewald, P, (2004) a. Pycnogenol, French maritime pine bark extract, improves endothelial function in hypertensive patients. Life Science, 74, 7, 855-862.

Manach, C., Andrzej, M., Scalbert, A. (2005). Polyphenols and prevention of cardiovascular diseases. Current opinion in Lipidology, 16, 1, 77-84.

Ohkita, M., Kiso, Y., Matsumura, Y. (2011). Pharmacology in health foods: improvement of vascular endothelial function by French maritime pine bark extract (Flavangenol). Journal of Pharmacological Sciences, 115, 4, 461-465.

Rohdewald, P. (2002). A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. International Journal of clinical pharmacology and therapeutics, 40, 4, 158-168.

Sauer, S., and Plauth, A. (2017). Health-beneficial nutriceuticals – myth or reality? Applied microbiology and biotechnology, 101, 951-961.

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C.L., Hollenberg, N.K., Sies, H., Kwik-Uribe, C., Schmitz, H.H., Kelm, M. (2006). (-)-Epicatechin mediates beneficial effects flavanol-rich cocoa on vascular function in humans. PNAS, 103, 4, 1024-1029.

Wang, S., Tan, D., Zhao, Y., Gao, G., Gao, X., Hu, L. (1999). The effect of Pycnogenol on the microcirculation, platelet function and ischemic myocardium in patients with coronary artery diseases. Eur Bull Drug Res.

552

553 Table 1: Inclusion and exclusion criteria for both the initial human trial and the placebo-
554 controlled volunteer trial.

Inclusion criteria	Exclusion criteria
Male or Female subjects between the age of 18-65 who can speak and understand English	Patients not wishing to allow disclosure to their GPs.
	Concomitant medication including herbal medicines and food supplements
No concomitant medication including herbal medicines and food supplements	Concomitant disease processes
	History of drug/alcohol abuse or Alcohol intake within 24 hours of dosing visit (visits 2-4)
No concomitant disease processes	Body Mass Index <21 and > 29kg/m^2
	Systolic blood pressure >150 mm Hg and or a diastolic pressure>90 mm Hg
Body Mass Index 21- 29 kg/m^2	Unable to tolerate polyphenol products or adhere to low polyphenol diet
Systolic blood pressure ≤150 mm Hg and diastolic pressure <90 mm Hg	Vegetarian
Subjects who have given informed consent	Subjects not willing or able to fast until 12 noon (a total of 14 hours).
	Pregnant females or planning to conceive in the next 3 months
	Participation in any other study currently or in the last three months

555

556

557

558

559

560

Table 2: Subject Characteristics Screening visit 1 for the initial human volunteer study

Volunteer number	**Sex**	**Age** (y)	**Height** (m)	**Weight** (kg)	**BMI**	**SBP**	**DBP**	**HR**
P001	M	40	1.80	78.7	24.3	107	59	59
P002	F	18	1.62	73.0	27.8	127	87	92
P003	M	19	1.72	82.0	27.7	118	69	62
P004	F	43	1.65	72.0	26.4	107	67	73
P005	M	30	1.87	99.0	28.3	124	88	84
P006	F	36	1.58	58.0	23.2	113	69	82
P007	F	25	1.78	90.2	28.5	140	86	95
P008	M	21	1.80	75.3	23.2	130	76	55
P009	M	31	1.78	81.1	25.6	133	81	90
P010	F	24	1.64	58.9	21.9	107	73	58
P011	F	35	1.60	56.0	21.9	96	70	68
P012	M	33	1.86	98.0	28.3	121	72	69
P013	M	32	1.83	93.7	28.0	99	68	90

Abbreviations (Units): Age (years); BMI, body mass index ($kg/m^{2)}$; SBP, systolic blood pressure in (mmHg); DBP, diastolic blood pressure (mmHg); Weight (Kg); Height (meters); HR, heart rate (per minute).

Table 3 Screening Characteristics for the healthy volunteer, placebo-controlled trial.

Volunteer number	Sex	Age (y)	Height (m)	Weight (kg)	BMI	SBP	DBP	HR
PS001	F	53	1.62	62.4	23.8	134	83	86
PS002	F	19	1.62	74.8	28.5	135	85	95
PS003	M	20	1.72	83.6	28.3	121	73	70
PS004	M	30	1.69	82	28.7	114	69	77
PS005	F	30	1.71	61.6	21.1	106	76	60
PS006	M	18	1.88	86.2	24.4	110	59	60
PS007	M	38	1.88	92	26.0	110	68	58
PS008	M	46	1.79	88.9	27.7	130	81	69
PS009	F	47	1.65	78.2	28.7	138	84	78
PS010	M	56	1.78	90	28.4	116	86	71
PS011	F	41	1.7	72	24.9	136	82	78
PS012	F	55	1.55	58.2	24.2	130	73	58
PS013	M	41	1.83	81	24.2	120	73	57
PS014	F	36	1.62	59.6	22.7	122	80	69
PS015	F	46	1.6	62.1	24.3	130	80	54
PS016	F	21	1.74	87.5	28.9	118	76	73
PS017	M	38	1.73	73.8	24.7	102	62	68
PS018	F	39	1.7	72.4	25.1	122	70	50
PS019	M	22	1.81	75.4	23.0	124	64	68
PS020	M	30	1.75	87.9	28.7	116	72	80
PS021	M	20	1.81	81.2	24.8	128	67	64
PS022	F	19	1.7	61.6	21.3	122	81	66
PS023	F	24	1.77	66	21.1	110	73	80
PS024	F	29	1.64	58	21.6	94	65	60
PS025	F	62	1.57	65	26.4	138	86	90
PS026	F	50	1.55	48.9	20.4	110	65	69
PS027	F	27	1.59	72.6	28.7	136	82	92

Abbreviations (Units): Age (years); BMI, body mass index ($kg/m^{2)}$; SBP, systolic blood pressure in (mmHg); DBP, diastolic blood pressure (mmHg); Weight (Kg); Height (meters); HR, heart rate (per minute).

Table 4: Plasma nitrite concentrations for healthy volunteers given the French maritime pine bark extract (Study 1). Values shown are concentrations corrected for baseline measures in µM (n=11).

Volunteer number	**4 h post ingestion**	**8 h post ingestion**
001	0.00	1.10
002	0.00	2.56
003	-10.25	4.76
005	0.00	7.32
006	-4.03	1.46
007	0.00	4.03
008	0.00	4.79
009	-0.96	-0.19
010	-1.33	0.38
011	-3.86	1.71
012	-2.47	0.38
Median	**-0.96**	**1.71**
25% IQR	**-3.86**	**0.379**
75% IQR	**0.00**	**4.76**

615

616

617 Table 5: Plasma nitrite concentrations for healthy volunteers given the French maritime pine bark
618 extract (Study 2). Values shown are concentrations corrected for baseline measures in µM (n=24).

619

	Placebo		French maritime pine bark extract	
Volunteer number	**4 h post ingestion**	**8 h post ingestion**	**4 h post ingestion**	**8 h post ingestion**
PS001	-0.11	-0.10	0.07	0.07
PS002	0.07	0.07	0.01	0.01
PS003	0.11	0.08	-0.18	-0.23
PS004	0.03	0.08	-0.08	-0.24
PS005	0.04	0.05	0.02	-0.02
PS006	0.08	0.10	0.06	0.14
PS007	-0.04	0.00	-0.05	0.05
PS008	-0.05	-0.05	0.23	0.08
PS009	0.11	0.15	0.12	0.08
PS010	0.00	-0.01	-0.06	-0.02
PS011	0.00	0.00	0.08	0.18
PS012	0.07	0.12	-0.24	-0.23
PS013	0.09	0.10	0.00	-0.03
PS014	0.11	0.14	-0.60	-0.55
PS015	0.08	0.11	0.20	0.04
PS016	0.14	0.10	-0.35	-0.37
PS017	0.06	0.11	0.15	0.13
PS018	0.03	0.08	-0.15	-0.15
PS019	0.06	0.06	-0.01	0.04
PS020	0.02	0.17	-0.08	-0.13
PS021	-0.12	-0.12	-0.08	-0.10
PS023	0.25	0.26	-0.11	-0.05
PS025	-0.12	-0.11	0.09	0.08
PS027	0.04	-0.01	0.03	0.01
Mean	**0.04**	**0.06**	**-0.04**	**-0.05**
SD	**0.09**	**0.09**	**0.18**	**0.17**

620

621

622

623

624

625

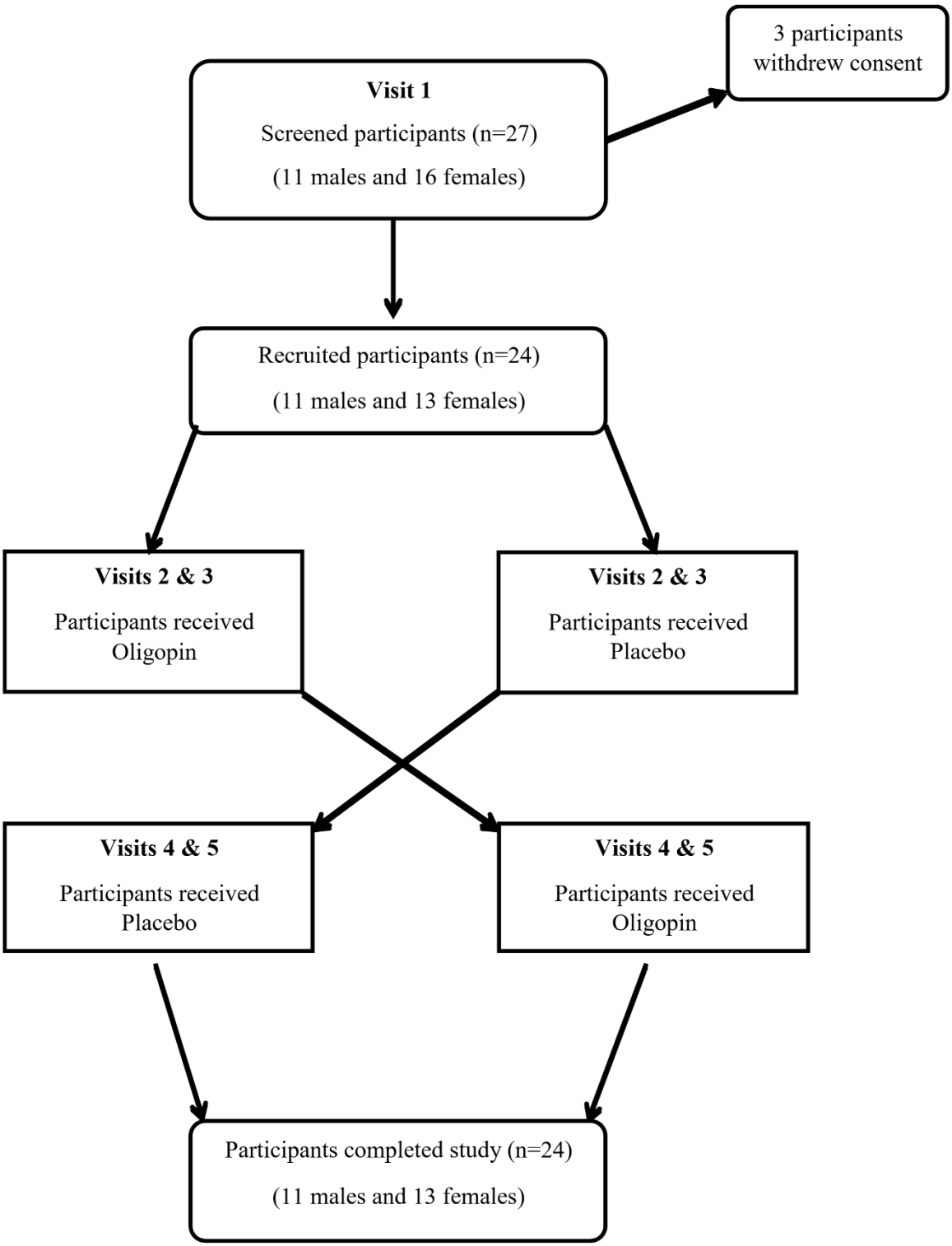
626

627

628

Table 6: Major peak identifications based upon comparisons with procyanidin standards (A2, B1, B2 and C1) for the two different batches of Oligopin French maritime pine bark extract. A comparison of the peak areas for these peaks is also prevented to illustrate the reduced levels of major constituents in the new batch compared to the original screened batch is also shown.

		Peak area
Retention time (min)	**Peak identity**	**% difference between batches (new:original)**
18.03	Unknown	13.25
18.30	Unknown	5.92
18.63	B1	9.83
19.36	Unknown	14.77
20.23	B2	8.34
22.98	A2	9.89

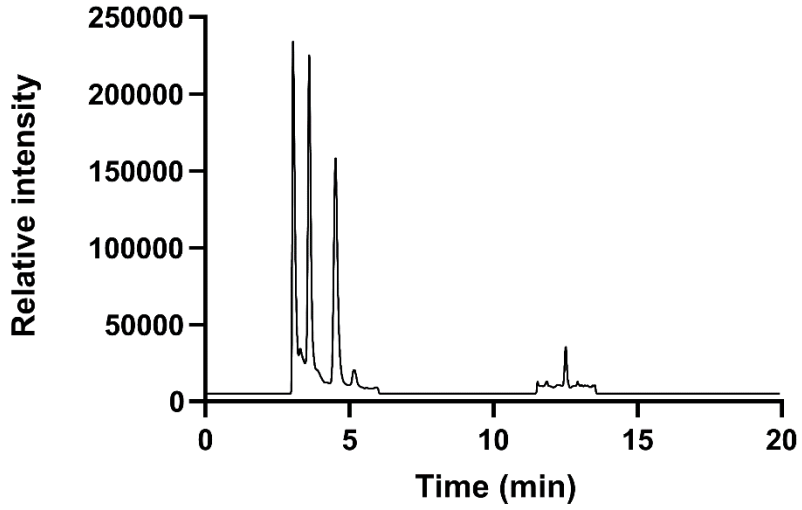


639

640 Figure 1: Flow diagram showing the design of the placebo-controlled, randomised,

641 cross-over trial.

642



643

644 Figure 2: Representative chromatogram of catechin and epicatechin using the

645 optimised LC-MS method. Catechin (retention time = 3.7 min), epicatechin (retention

646 time = 4.7 min), and diadzein (retention time = 12.6 min) are shown.

647

648

649

650

651

652

653

654

655

656

657

658

659

660

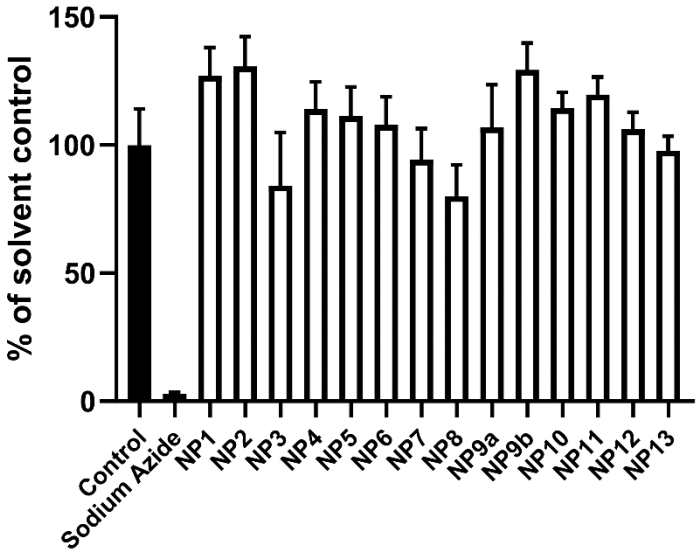
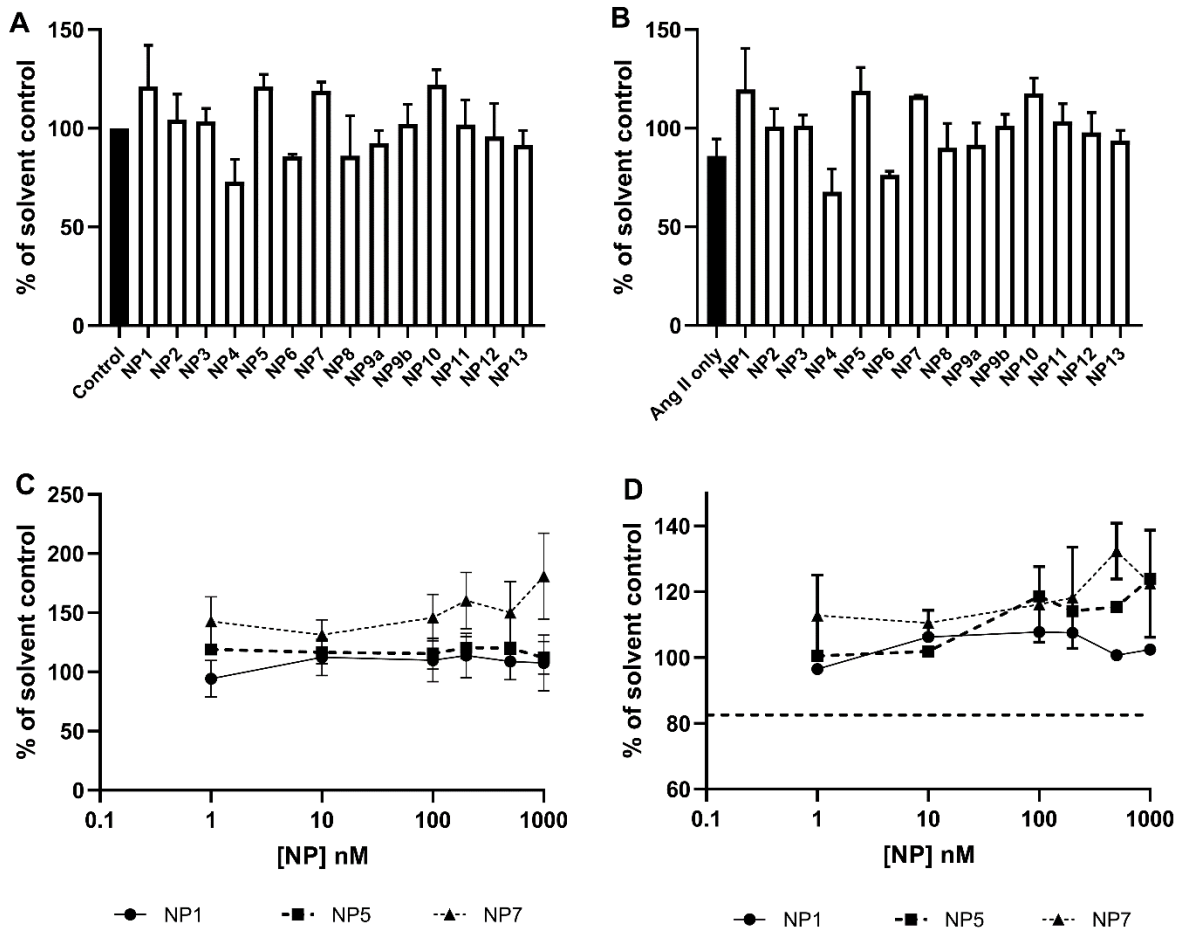


Figure 3: Assessment of cell viability after incubation with 10 µM of each plant extract for 8 hours. Data is presented as mean ± standard deviation of n=3 experiments.



677

678 Figure 4: In vitro screening of polyphenol-rich plant extracts using a HUVEC culture
679 system. (A) The effect of the different plant extracts (NP1-13) on HUVEC cultures
680 under control conditions. Black bars indicate cultures without NP incubation (1 µM, 8
681 h), with nitric oxide availability expressed as a percentage of control cultures. (B)
682 The effect of the different plant extracts (NP1-13) on HUVEC cultures under
683 Angiotensin II treated conditions (100 nM, 8 h). Black bars indicate cultures without
684 NP incubation (1 µM, 8 h), with nitric oxide availability expressed as a percentage of
685 angiotensin II treatment only cultures. (C) Dose-response curves of NP1, NP5 and
686 NP7 under control and angiotensin II treated (D) conditions. Nitric oxide availability
687 is expressed as percentage of control cells. The dotted lines in the angiotensin II
688 plots indicates the percentage of solvent control activity for cells without NP
689 treatment.

690

691

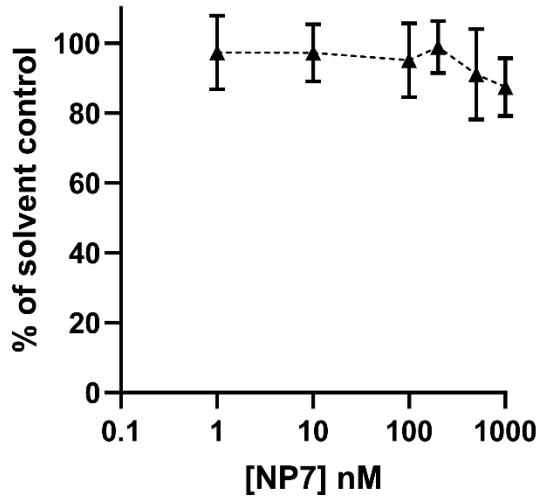


Figure 5: In vitro assessment of the second batch of French maritime pine bark extract for its effect on nitric oxide availability in control HUVEC cultures. Graph shows mean ± SD for n=2 independent experiments (6 wells per experiment). In contrast to figure 3D, no effect of the extract is observed.

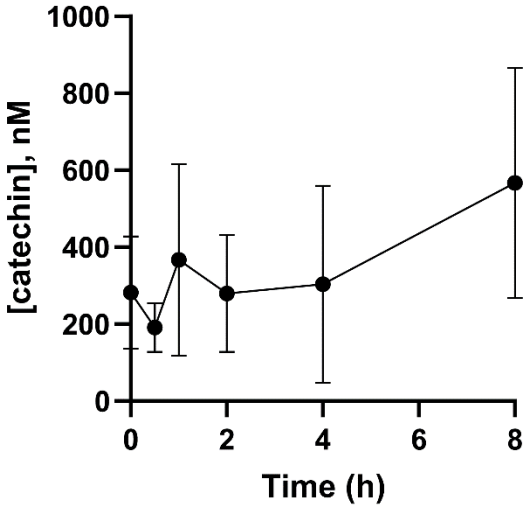
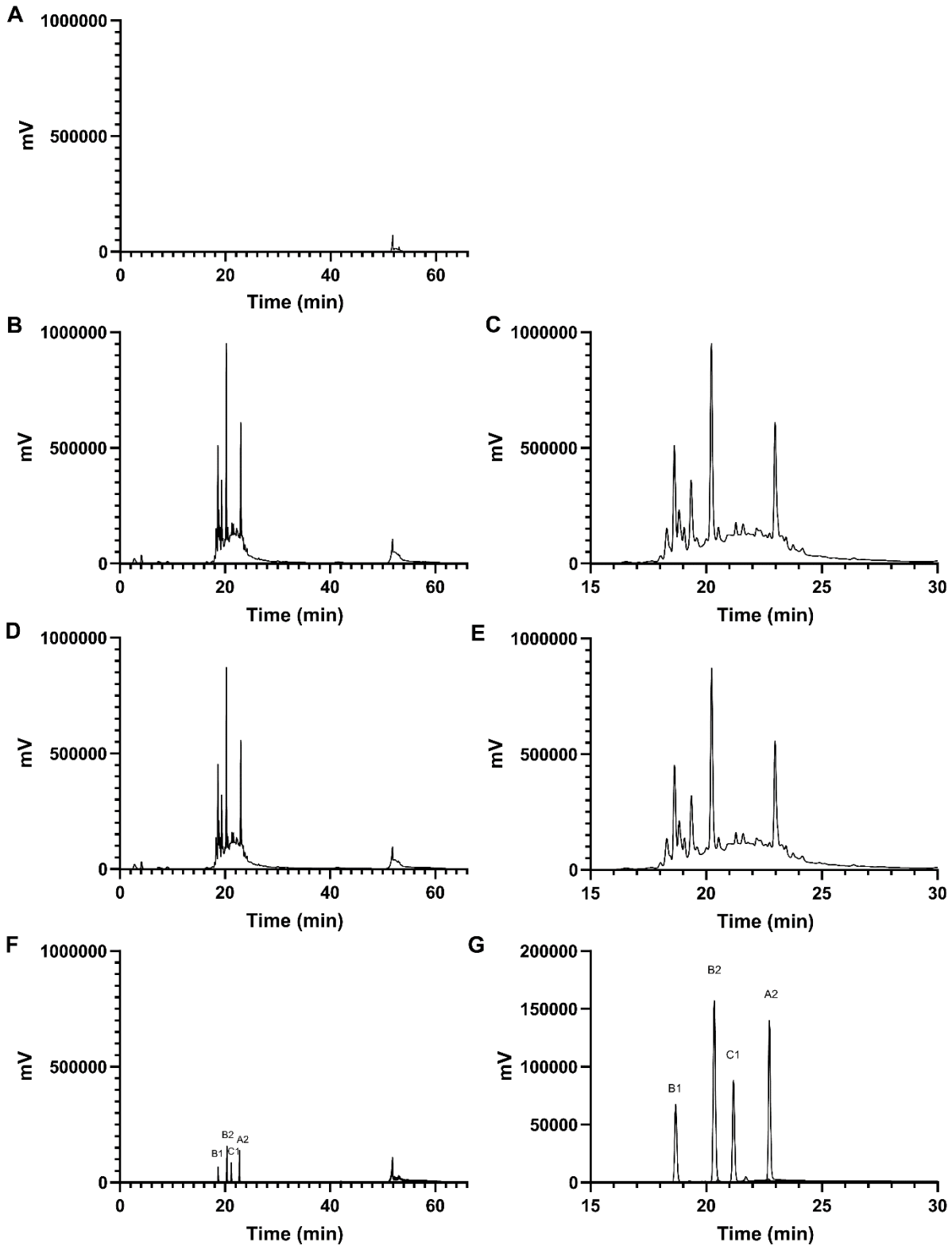


Figure 6. Plasma concentrations (nM) of catechin for n=5-6 volunteers from the first healthy volunteer study. Graph shows mean ± SD for sampling times up to 8 hours post ingestion.



728

729 Figure 7: HPLC fingerprint assessment of the two batches of French maritime pine

730 bark extract, and comparison with procyanidin standards (A1, B1, B2 and C1), with

absorbance measured at 280 nm. (A) Solvent control (10% DMF). (B) The original batch of Oligopin used in the in vitro screening process and first healthy volunteer study. (C) The fingerprint shown in panel B, focussed upon the time range of 15 to 30 minutes. (D) The second (new) batch of Oligopin used in the placebo-controlled healthy volunteer study. (E) The fingerprint shown in panel D, focussed upon the time range 15 to 30 minutes. (F) Representative chromatogram of the separation of procyanidin standards using the fingerprinting method. (G) The same fingerprint of standards as shown in panel F, focussed upon the time range 15 to 30 minutes. The identities of the various standards used are indicated on the chromatograms in panels F and G.