Effects of Polyphenols on Vasomodulatory Factors and Associated Cell Signalling

by

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Abstract

There is increasing evidence that consumption of plant bioactives such as polyphenols reduces cardiovascular disease risk and improves endothelial function. In the Black Sea area, a number of plants are consumed alone and as ingredients in traditional foods, and dill, nettle, kale, *Sideritis*, pomegranate and persimmon were identified as polyphenol-rich traditional food plants. Bioactive-rich extracts of their edible parts were used to treat human umbilical vein endothelial cells (HUVECs), to assess effects on the abundance or activity of signalling molecules related to increased vasodilation, a hallmark of improved endothelial function. Specifically, Akt and eNOS phosphorylation, levels of total eNOS protein and cGMP, secretion of ET-1 and levels of nitrate/nitrite in cell culture media were assayed. Alongside these experiments, HUVECs were treated with quercetin, a flavonol found in a number of the plant extracts, alongside a mix of its human metabolites to assess their effect on a broad range of phosphorylated proteins using an antibody microarray.

Quercetin (50 μM) significantly decreased eNOS phosphorylation ($p < 0.05$), while extracts of pomegranate and persimmon significantly increased levels of phosphorylated (p-) Akt, p-eNOS and cell culture media nitrate/nitrite ($p < 0.05$), and significantly reduced secretion of the vasoconstrictor ET-1 ($p < 0.001$). With regards to pomegranate bioactivity, signalling events upstream of p-Akt were explored, including inhibition of PTEN activity and phosphorylation of receptor tyrosine kinases, though no evidence could be found that either mechanism was involved in this case. Fractionation of the pomegranate extract into its main polyphenol classes revealed that procyanidins were responsible for its bioactivity. These findings suggest that procyanidin-rich foods can improve markers of endothelial function *in vitro*. Further studies on procyanidin bioavailability are required to determine if these effects can also occur *in vivo*.

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Chapter 1

Polyphenols, cardiovascular disease and traditional diets

Chapter 1: Polyphenols, cardiovascular disease and traditional diets

1.1. Cardiovascular disease and endothelial function

Cardiovascular disease (CVD) encompasses a number of conditions that affect the health of the vascular system, including the blood vessels and heart muscle, and is the leading cause of worldwide human mortality (World Health Organisation 2013). Lifestyle factors that have been shown to increase the risk of cardiovascular disease include tobacco use, lack of exercise and poor dietary choices. Higher prevalence is also found amongst those of low income (World Health Organisation 2010). Additional chronic conditions such as type 2 diabetes, hypercholesterolaemia and hypertension can be underlying factors for CVD progression (Sowers et al. 2001). Though disease progress occurs over the course of decades, it often results in severe acute responses, including heart attacks (myocardial infarction) and strokes. The physiological changes that result in these events are often tied to the formation of atherosclerotic plaques (Ross 1999), which in turn are marked by a change in the phenotype of the endothelial cells that form the inner lining of veins and arteries. Such cells are not simply a physical barrier; as an interface to the circulatory system, they activate signalling in response to numerous physio- and pathological stimuli, and play an important role in the control of vascular health. High blood pressure, increased levels of free radicals and oxidised low-density lipoprotein (LDL), infection by *Chlamydia pneumoniae* or damage from shear stress can all contribute to endothelial dysfunction. The outcome of such changes includes elevated levels of oxidative stress and inflammation, high expression of cell adhesion molecules, reduced vasodilatory factors and promotion of vasoconstrictive factors (Mudau et al. 2012; Grassi et al. 2011).

Increased stress at the endothelium increases its permeability which causes build up of lipid-rich molecules, including oxidised lipoproteins, at the vascular wall (Corrado et al. 2010; Libby & Theroux 2005). The rise in numbers of cell adhesion molecules such as I-CAM, V-CAM and P-selectin at the cell surface increases adhesion of white blood cells, including monocytes, to the endothelium. Aided by the adhesion molecules, monocytes migrate to the intima layer between the endothelium and the smooth muscle tissue. Here they differentiate to macrophages, probably through stimulation from interleukins and growth factors such as M-CSF, though current *in vivo* evidence for this is limited (Chang et al. 2012; Shibata & Glass 2009). Proteoglycans in the vascular extracellular matrix contribute to matrix destabilisation, as well as to other pro-atherosclerotic activities, including cell migration, proliferation and lipoprotein retention. Their presence, alongside macrophage scavenger receptors and macrophage-secreted proteoglycans, and proteases promotes the accumulation of lipids in macrophages that leads to the formation of foam cells, a key step in the initiation of atherosclerotic lesions (Ley et al. 2011). Local release of cytokines and growth factors stimulate proliferation and migration of vascular smooth muscle cells (VSMCs), resulting in a fibrous 'cap' separating the lesion from blood flow. Fibrotic material continues to be laid down as the plaque develops, aided by platelet aggregation and thrombus formation, leading to areas of increased thickness and arterial stiffness (Corrado et al. 2010; Ross 1999). Though the plaque may remain stable for a lengthy period, inflammation can lead to thinning of the lesion edges. Episodes of myocardial infarction or stroke occur when the fat-laden lesions rupture and clot vessels with the released material. Assessment of patient CVD risk can be performed by a number of physiological or anatomical measurements, including intima-media thickness, flow-mediated dilation of blood vessels and levels of C-reactive protein or asymmetric dimethylarginine (Davids & Teerlink 2012; Ray et al. 2009).

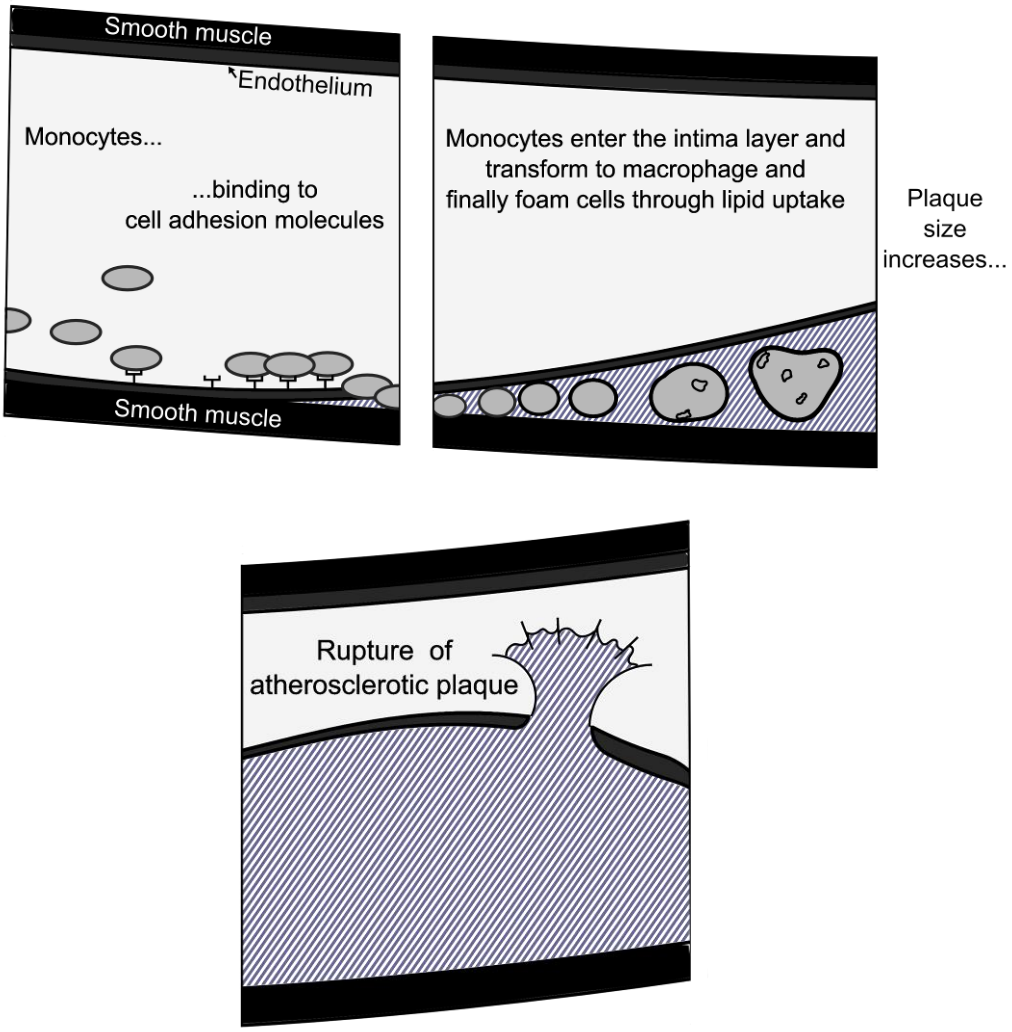

Fig. 1.1: Three stages of atherosclerotic plague formation. **Top l**eft panel:** Factors such as increased inflammation encourage expression of cell adhesion molecules, and binding of white blood cells, such as monocytes. **Top right panel:** Inflammation also increases permeability of the endothelium, allowing monocytes to enter the intima space between endothelial and smooth muscle cells. Oxidised LDL, amongst other lipids, results in the transformation of monocytes to macrophages. Continued lipid uptake produces fat-laden foam cells. **Lower panel:** The atherosclerotic plaque increases in size, while the fibrotic tissue that develops around it can weaken over time. Eventually the plaque may rupture, and the released material can cause clots resulting in myocardial infarction or stroke.

Flow-mediated dilation is particularly useful as it gives a direct measure of one of the key factors resulting in endothelial dysfunction, that of increased constriction of arteries and reduced vasodilatory response. The tone of the vascular smooth muscle tissue is controlled primarily by the balance of a number of vasomodulatory factors, most originating from the endothelium, from where they then diffuse or are transported to the underlying smooth muscle. Relaxation of arterial tissue is induced by increased production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS), prostacyclin (PGI_2) by cyclooxygenase, or by a number of endothelium-derived hyperpolarising factors (EDHFs) which activate K^+ channels in smooth muscle cell membranes. EDHFs include certain small molecules (H_2O_2, carbon monoxide and hydrogen sulphate), epoxyeicosatrienoic acids, K^+ ions and C-natriuretic peptide (Giles et al. 2012; Jin et al. 2011). The decrease in intracellular Ca^{2+} ions in VSMCs that results from K^+ channel activation, as well as NO and PGI_2 signalling, is one of the key changes that reduces vascular tone.

Vasoconstriction factors include prostaglandins, thromboxanes, angiotensin, endothelin and reactive oxygen species (ROS). Similar to a number of EDHFs, prostaglandins and thromboxanes are produced from arachidonic acid by cyclooxygenase. They activate thromboxane receptors on VSMCs to produce direct vasoconstrictive effects, while increased levels of superoxide, produced in endothelial cells by NADPH oxidase, scavenges NO and reduces the efficacy of stimulated NO production (Barton et al. 2012; Toda 2012; Versari et al. 2009). ET-1, the main endothelin peptide expressed in endothelial cells, binds to ETA and ETB receptors in VSMCs to induce vasoconstriction. It is noteworthy that ET_B receptors in endothelial cells stimulate NO production upon ET-1 binding. Evidence from eNOS-blocking studies suggests that there is a link between NO and ET-1 release in endothelial cells, where reduced eNOS activity allows increased ET-1 levels, and that improved NO production inhibits ET-1 production (Bourque et al. 2011). eNOS activity can be increased by the

kinases AMPK, CaMKII, PKA and PKB (Akt), and in turn, these are activated by certain growth factors (such as VEGF), hormones, statins or shear stress (Vanhoutte et al. 2009; Huang 2009; Förstermann & Sessa 2012).

In vitro, human umbilical vein endothelial cells are often used as a model for determining signalling responses to molecules which might be present in the circulatory system. Preparation of cells from the umbilical vein is often carried out with the use of collagenase, facilitating their removal so that they can be collected and cultured for study (Crampton et al. 2007). Vasodilation through NO release in the human umbilical vein is a key method through which, like the arteries of the cardiovascular system, oxygenated blood is carried from the placenta to the foetus, as there is a lack of nervous stimulation to carry out this task (Gude et al. 2004, San Martín and Sobrevia 2006).

1.2. The nitric oxide signalling pathway

As has been mentioned, vasomodulation - the control of arterial and venous blood flow - is likely to play an important role in the development or control of atherosclerosis and CVD, with the enzyme eNOS being key to enabling vascular tissue to relax. Impairment of eNOS function leads to increased vasoconstriction, high blood pressure and oxidative stress in endothelial and smooth muscle tissue. All of these outcomes are associated with endothelial dysfunction (Giles et al. 2012; Atochin & Huang 2010). Indeed, polymorphism of the eNOS gene has been linked to increased incidence of coronary heart disease (Zhang et al. 2012b). Conversely, improvements in vasodilatory response through this mechanism have been shown to attenuate lesion progression and reduce inflammatory responses such as platelet aggregation and expression of adhesion molecules and cytokines (Chatterjee and Catravas 2008, Vanhoutte et al. 2009). CVD risk factors, such as ageing and high cholesterol are also associated with reduced vasodilation

and eNOS expression, as well as increased expression of vasoconstriction factors such as endothelin-1 (Seals et al. 2011; Toda 2012).

Dilation of veins and arteries is controlled at a molecular level by the nitric oxide (NO) signalling pathway (**Fig. 1.2**). NO is a free radical that acts as a signalling molecule in a number of different human tissues (Gao 2010). Formation of NO, as pertains to vasodilation, takes place in endothelial cells, which form the inner lining of veins and arteries (Buerk et al. 2011; Triggle et al. 2012). After production by the enzyme eNOS, it passes to surrounding smooth muscle cells, triggering their relaxation.

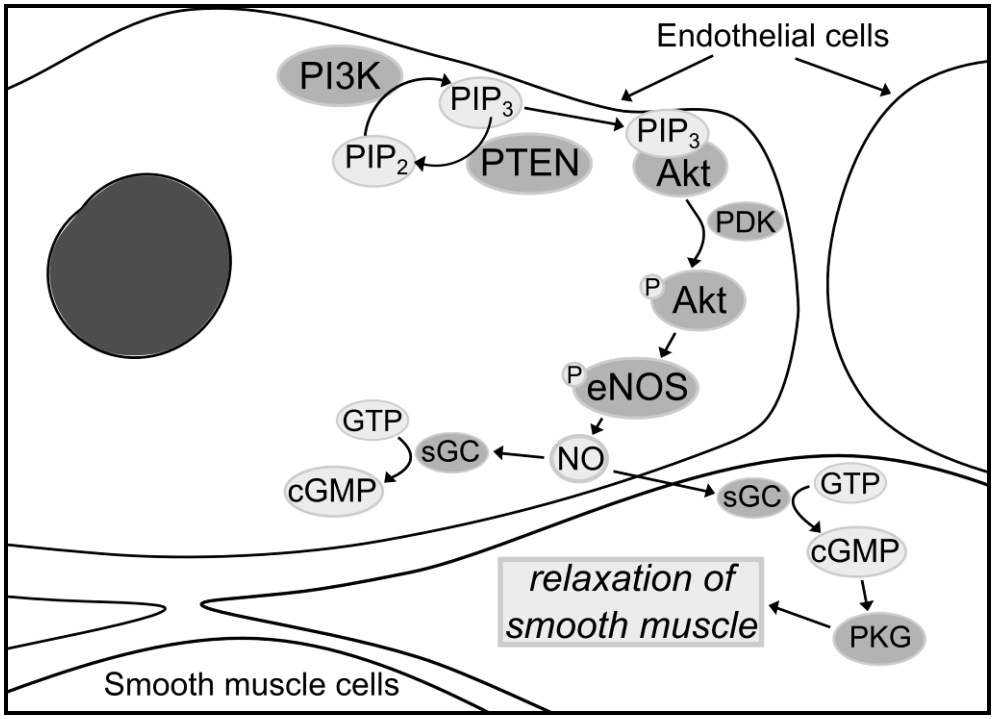

Fig. 1.2: Nitric oxide signalling in endothelial and smooth muscle cells: Increased levels of PIP_3 due to PI3K activity and PTEN inactivity, leads to binding of Akt and the plasma membrane so that it may become active through phosphorylation by PDK. p-Akt then activates eNOS through phosphorylation, allowing production of NO to increase. Diffusion of NO to smooth muscle cells leads to its binding of sGC, which produces cGMP, leading to the activation of PKG and relaxation of the smooth muscle tissue. P - indicates phosphorylation; GTP - guanosine triphosphate; NO - nitric oxide

The regulation of eNOS has been well studied and operates through a number of mechanisms. Acylation (specifically myristoylation and palmitoylation) facilitates eNOS translocation from the cytosol to caveolae in the plasma membrane, where it binds caveolin-1, holding it in an inactive state (Kolluru et al. 2010). Translocation brings it into close proximity with other proteins and cofactors that enable enzyme activity, the first of these being heat shock protein 90 (hsp90) and calmodulin (CaM) which displace caveolin-1. Hsp90 may also act as a scaffolding protein in regulation of signalling events related to nitric oxide signalling, and has been shown to form a complex with eNOS and Akt, an eNOS-activating kinase (Takahashi & Mendelsohn 2003). eNOS protein consists of an N-terminal oxygenase domain and a C-terminal reductase, connected by a calmodulin (CaM)-binding region (Balligand 2002). These contain sequences for binding cofactors, including haem and tetrahydrobiopterin (BH_4) in the oxygenase domain and flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in the reductase domain (Feng 2012). Increased intracellular Ca^{2+} increases CaM's affinity for the CaM-binding region, and enables interdomain electron transfer, facilitating eNOS enzyme activity. One further requirement for active eNOS is that it forms a homodimer, which occurs through to the presence of haem in the oxygenase domain and the linking of two cysteine residues (also in the oxygenase domain) from each monomer by a zinc ion (Förstermann & Sessa 2012). Once activated, eNOS produces nitric oxide according to the formula:

$$\text{L-Arginine} (+ 1.5\ \text{NADPH} + 1.5\ H^+ + 2\ O_2) \rightarrow \text{NO} (+ \text{L-Citrulline} + 1.5\ NADP^+ + 1.5\ H_2O)$$

Electron transfer from the reductase domain to the oxygenase domain, which binds the eNOS substrate L-arginine, facilitates the synthesis of NO from the catalysis of L-arginine to L-citrulline. eNOS is phosphorylated at a number of serine, threonine and tyrosine residues which are key to the increase and decrease of enzyme activity (Fleming 2010). Ser1177 is one of the most well-studied residues involved with increased eNOS enzyme

activity. Its phosphorylation increases the affinity of eNOS for binding CaM and helps facilitate the interdomain electron transfer (Rafikov et al. 2011). Post-translation modifications such as these are not all beneficial to NO production, however. S-nitrosylation of eNOS cysteine residues leads to inhibition of enzyme activity (Dudzinski & Michel 2007) while S-glutathionylation can cause uncoupling of the homodimer subunits. Uncoupled eNOS remains active, but without a partner monomer it acts as an NADPH oxidase, producing superoxide radicals and increasing intracellular oxidative stress (Zweier et al. 2011).

Once NO diffuses to smooth muscle cells it binds to haem in soluble guanylyl cyclase (sGC), greatly increasing cGMP production (Rocha et al. 2009). Besides the vascular smooth muscle, sGC is also found in a number of other different tissues in the human body, including the endothelium, (Potter 2011; Takeuchi et al. 2004). It increases production of cyclic guanosine monophosphate (cGMP), produced from Mg^{2+}-bound GTP (Russwurm & Koesling 2004; Derbyshire & Marletta 2009). cGMP is then targeted to cGMP-regulated ion channels, phosphodiesterases (PDEs) (which catalyse hydrolysis of cGMP to GMP (Bryan et al. 2009)) and cGMP-dependent protein kinases, such as protein kinase G (PKG) (Francis et al. 2010; Hofmann et al. 2000). Binding of cGMP to allosteric sites on PKG cause conformational changes resulting in the release of the enzyme's catalytic domain and interaction with a number of substrates, such as IP_3 receptor-associated cGMP kinase substrate (IRAG). IRAG protein inhibits inositol 1,4,5-trisphosphate (IP_3) receptor activity and the release of intracellular Ca^{2+} stores and increases Ca^{2+}-activated maxi-K^+ channel (BKCa) activity, inhibiting influx of Ca^{2+} through Ca^{2+} channels. This reduces the intracellular Ca^{2+} concentration and the Ca^{2+} sensitivity of the contractile machinery. IRAG also activates MYPT1, which dephosphorylates myosin light chain and reduces myosin ATPase activity, interfering with the interaction between myosin and actin that brings about muscle contraction (Koubassova & Tsaturyan 2011; Schlossmann & Desch 2011). Additionally, increases to

NO levels may be related to decreases in levels of vasoconstriction factors, such as endothelin-1 (ET-1), augmenting the vasodilatory effect (Bourque et al. 2011). ET-1 facilitates increases to vascular tone through its ability to raise the intracellular Ca^{2+} concentration in smooth muscle cells (Bourque et al. 2011; Mazzuca & Khalil 2012)

An important regulator of eNOS activation through phosphorylation of Ser1177 is the kinase Akt (protein kinase B). It has three human isoforms, Akt1, Akt2 and Akt3, with Akt1 being the key isoform in vascular nitric oxide signalling (Matheny & Adamo 2009). Each isoform is composed of an N-terminal pleckstrin homology domain (PH), a kinase domain and a C-terminal hydrophobic domain. Phosphorylation of Thr308 in the kinase domain and Ser473 in the hydrophobic domain are required for full Akt activity. The PH domain is recognised by PIP_3, which is formed from the phosphorylation of PIP_2 by PI3K. PIP_3 holds Akt1 at the plasma membrane after its translocation from the cytoplasm. Phosphorylation is carried out by PDK1 for the Thr308 residue and mTORC2 or DNA-PK (other kinases may also be involved) for Ser473. Dephosphorylation of PIP_3 by phosphatases such as SHIP and PTEN can reverse this process (Bozulic & Hemmings 2009). Akt activity is completely dependent on the phosphorylation of PIP_2 to PIP_3 by PI3K (phosphoinositide 3-kinase) (Hers et al. 2011), as evidenced by inhibited Akt phosphorylation after incubation of cell cultures with the PI3K chemical inhibitor LY294002 (Eisenreich & Rauch 2011). PI3Ks are grouped in three distinct classes, based on catalytic subunit similarity and preference of phosphoinositide substrate, but it is only class 1 PI3Ks which are able to produce PIP_3 (Vadas et al. 2011). Class 1 PI3Ks are divided further, based on their catalytic and regulatory domains. Class 1A PI3Ks have p110 (α, β or δ) as their catalytic domain and p85 (α or β), p55 (α or γ), or p50α as their regulatory subunit, while class 1B PI3Ks are comprised of p110γ and either p84 or p101 (Fougerat et al. 2009).

1.3. Plant polyphenols and other bioactive compounds

One of the key factors behind progression of CVD is poor diet. Conversely, increased consumption of fruit and vegetables is associated with decreased CVD risk (World Health Organisation 1990; He et al. 2007; Pomerleau et al. 2007). There is strong evidence that the polyphenol content, and in particular the flavonoid content, of fruits, vegetables and other plant-derived foods is a major factor behind these health effects. Meta-analyses have shown that flavonoids, particularly those associated with cocoa and soy products, can significantly lower blood pressure and improve the flow-mediated dilation response in patients, though greater consistency between studies, such as in the choice of biomarkers for study outcomes, is required for stronger evidence in the future (Kay et al. 2012; Hooper et al. 2008; Hooper et al. 2012; Ried et al. 2012).

Flavonoids and other polyphenols are plant secondary metabolites, present in various forms throughout the plant kingdom. Their functions in plants encompass numerous roles including pigmentation, bitter or astringent tastes for the dissuasion of herbivores (Landete 2012), antioxidants (Agati et al. 2012) and UV radiation absorption (Pollastri & Tattini 2011; Lattanzio et al. 2008). According to the principles set out by White, Bate-Smith, Swain and Haslam, polyphenols are water-soluble plant phenolics, with mass of 500 to 3000 Da, 5-7 aromatic rings, 12-16 hydroxyl groups, and the ability to precipitate certain proteins, alkaloids and gelatin (Quideau et al. 2011; Haslam 2007). This therefore includes most condensed tannins, hydrolysable tannins and algae-borne phlorotannins. However, much polyphenol research in the last few decades has increasingly focused on smaller phenolic compounds, such as flavonoids, to the extent that the above definition is no longer appropriate. A more contemporary definition is offered by Quideau et al. (2011), which describes polyphenols as:

> "*plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one*

phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression."

Polyphenols are vast in number, encompassing the chemical families mentioned above, with their respective subdivisions, as well as stilbenoids, lignans, phenolic acids and additional derivative compounds (Del Rio et al. 2013). One of the larger families, with over 500 compounds identified so far, hydrolysable tannins consist of esters of gallic acid and sugar molecules, and can be classed as either ellagitannins, where galloyl moieties have bonded through oxidative coupling, or as gallotannins. Degradation of gallotannins releases gallic acid, whereas ellagitannins breakdown can produce numerous derivative compounds, including sugar esters of ellagic acid and gallagic acid (Landete 2011; Larrosa et al. 2010; Quideau 2009). Condensed tannins (also known as proanthocyanidins or non-hydrolysable tannins) represent a large number of oligomeric and polymeric structures, soluble in water up to approximately 10 monomer units (He et al. 2008; Xu et al. 2012b). These monomer units are flavanols, such as epicatechin or catechin, which belong to the extensive flavonoid family of polyphenols. Most flavonoids possess the three-ring flavan backbone structure (**Fig. 1.3)**, coupled with a variety of hydroxyl, methoxy and ketone groups, and are often conjugated with sugars or organic acids in plants.

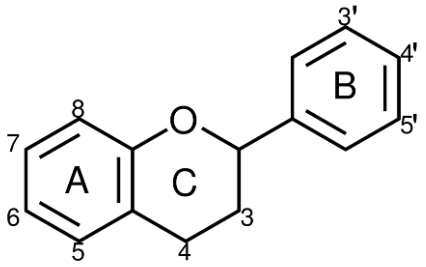

Figure 1.3: Flavan backbone structure, featuring two benzene rings (**A** and **B**), and a dihydropyran ring (**C**).

Other subclasses of flavonoids included flavonols, flavones, isoflavones and anthocyanins. Flavonoids are by far the most commonly expressed polyphenol in plants, with over 5000 plant-derived flavonoids currently described (Keller 2009; Veitch & Grayer 2011).

Stilbenoids are phytoalexins, and are often found in wine due to their presence in grape skins. Of note is resveratrol, which has been the subject of much fervoured study due to its strong effects on cellular mechanisms relating to disease and ageing (Cucciolla et al. 2007; Rivière et al. 2012). Lignans, which are also phytoestrogens, are often present in the seeds of plants and include pinoresinol, lariciresinol and sesamin (Cunha et al. 2012; Peterson et al. 2010). Phenolic acids, while not polyphenols by definition, are often included in polyphenol research, and may also be found as part of the structure of larger polyphenols. Two major classes of phenolic acids, hydroxycinnamic acids (C6-C3 structure) and hydroxybenzoic acids (C6-C1 structure), are commonly found in a variety of plant species (Rice-Evans et al. 1996). Of the numerous phenolics found in nature, over 500 have been identified so far as forming part of human diets, across 400 different foods (Neveu et al. 2010).

Synthesis of polyphenols in plants occurs via enzymes of the shikimate and phenylpropanoid secondary metabolite pathways (Vogt 2010; Knaggs 2003). Dehydrogenation of 3-dehydroshikimate, an intermediary compound of the shikimate pathway, is the primary means for gallic acid formation, a key step in the production of ellagitannins (Haslam & Cai 1994). A sugar, usually glucose, forms an ester with gallic acid to produce β-glucogallin, which accepts galloyl groups from additional β-glucogallin molecules to form 1,2,3,4,6-pentagalloyl glucose (PGG) (Crozier et al. 2009). Oxidative linkage of PGG galloyl groups produces 3,4,5,3',4',5'-hexahydroxydiphenoyl (HHDP) subunits, characteristic of all ellagitannin molecules (Landete 2011; Okuda & Ito 2011). The final structure of these molecules, though, can vary greatly due to a number of factors, including HHDP group position, formation of bonds with additional galloyl

moieties, oligomerisation, further structural changes due to oxidation, and condensation with other molecules (Quideau 2009; Ascacio-Valdés et al. 2011). Breakdown of ellagitannins through hydrolysation releases the HHDP units, which convert to ellagic acid through lactonisation. Ellagic acid, alongside conjugated derivatives, is a common product of ellagitannin degradation. Cleavage of the lactone ring results in the production of urolithins, metabolites found in human plasma after consumption of ellagitannin-rich foods (Larrosa et al. 2010; Landete 2011).

Anthocyanins, like all flavonoids, are derived from the final metabolite of the shikimate pathway, *p*-coumaroyl-CoA, via a number of enzymatically synthesised intermediary compounds. *p*-Coumaroyl-CoA undergoes a condensation reaction, with three malonyl-CoA molecules, to produce naringenin chalcone (**Fig. 1.4**). The C-ring of the flavanone structure is formed, leading to the production of dihydroflavonols, which are converted to leucoanthocyanidins and then anthocyanidins (He et al. 2010; Xie et al. 2011). Anthocya*nins* are formed after the attachment of a sugar group, often at the 3-O or 5-O position (**Fig. 1.5**).

Flavan-3-ols can be synthesised from either leucoanthocyanidins, in which case they have 2*R*,3*S*-stereochemistry (i.e. (+)-catechin), or from anthocyanidins, and so feature 2*R*,3*R*-stereochemistry (i.e. (-)-epicatechin) (He et al. 2008). The exact mechanism for the poly- and oligo-merisation of the flavan-3-ol monomer units into the proanthocyanidin structure is still under investigation. It has been proposed that a laccase-like polyphenol oxidase (PPO) encoded by *TT10* is involved, and that oxidation of flavan-3-ols by PPO assists their formation of proanthocyanidin structures. Leucoanthocyanidin molecules may also be used, after conversion to quinone methide intermediate structures, followed by transportation to plant cells vacuoles, the acidic environment of which aids condensation of proanthocyanidin subunits (Zhao et al. 2010). There does not appear to be a standard order in which flavan-3-ol subunits are arranged in proanthocyanidins, giving rise to a large number of proanthocyanidin structures.

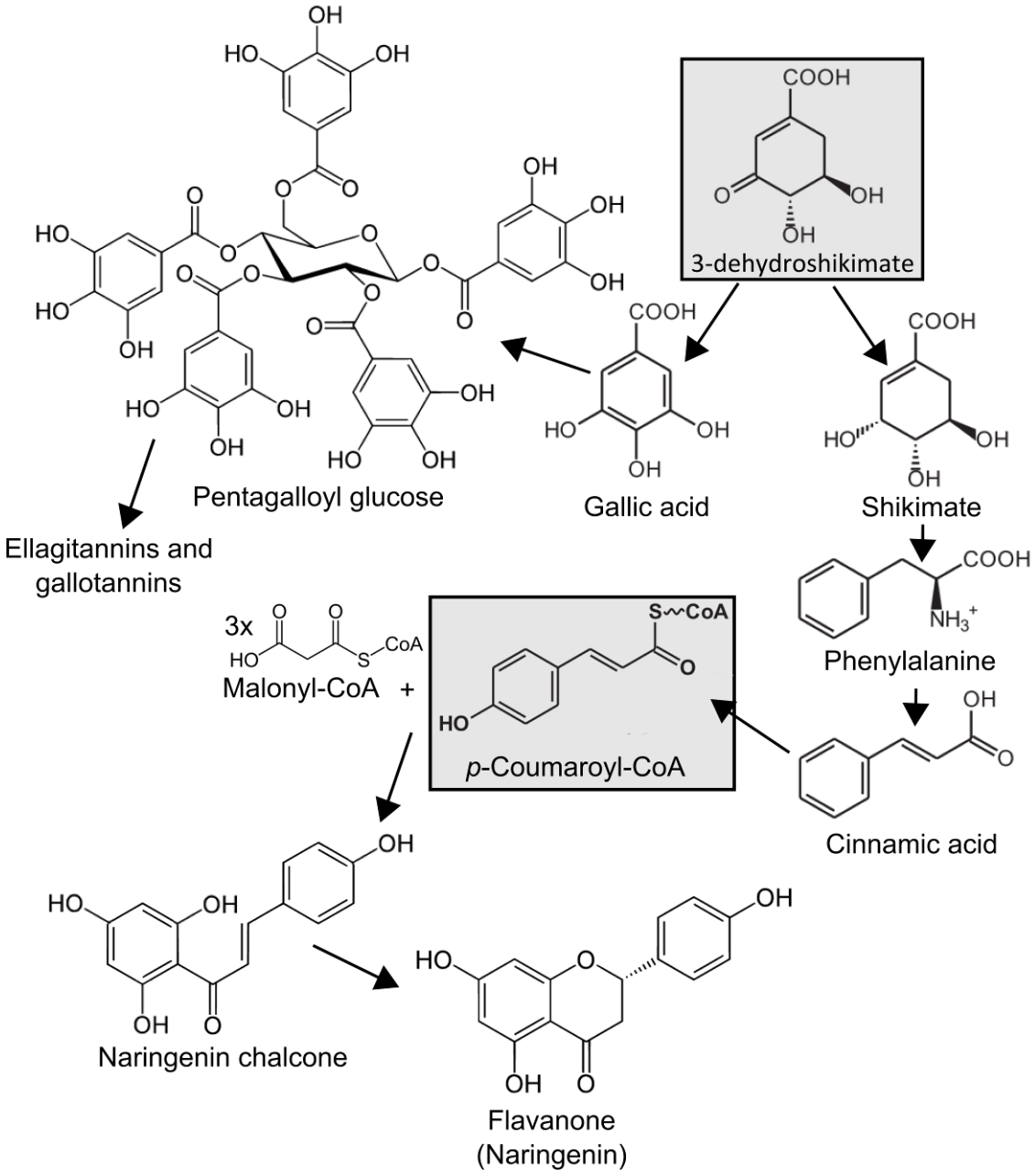

Fig. 1.4: Biosynthesis of ellagitannins and flavonoid precursor compounds from metabolites of the shikimate and phenylpropanoid pathway, through a number of enzymatic reactions. Pathway is continued in **Fig. 1.5**.

Treatment of proanthocyanidins with strong mineral acids results in the release of anthocyanidin molecules, hence their name. Thus, proanthocyanidins capable of releasing cyanidin (those which contain 3'4'-dihydroxyflavan-3-ol subunits, i.e. (+)-catechin) are termed procyanidins (**Fig. 1.6**), while those capable of releasing delphinidin (proanthocyanidins containing 3'4'5'-trihydroxyflavan-3-ol subunits, i.e. (+)-

gallocatechin) are termed prodelphinidins (though it is not unusual for a proanthocyanidin to contain both procyanidin and prodelphinidin monomer units).

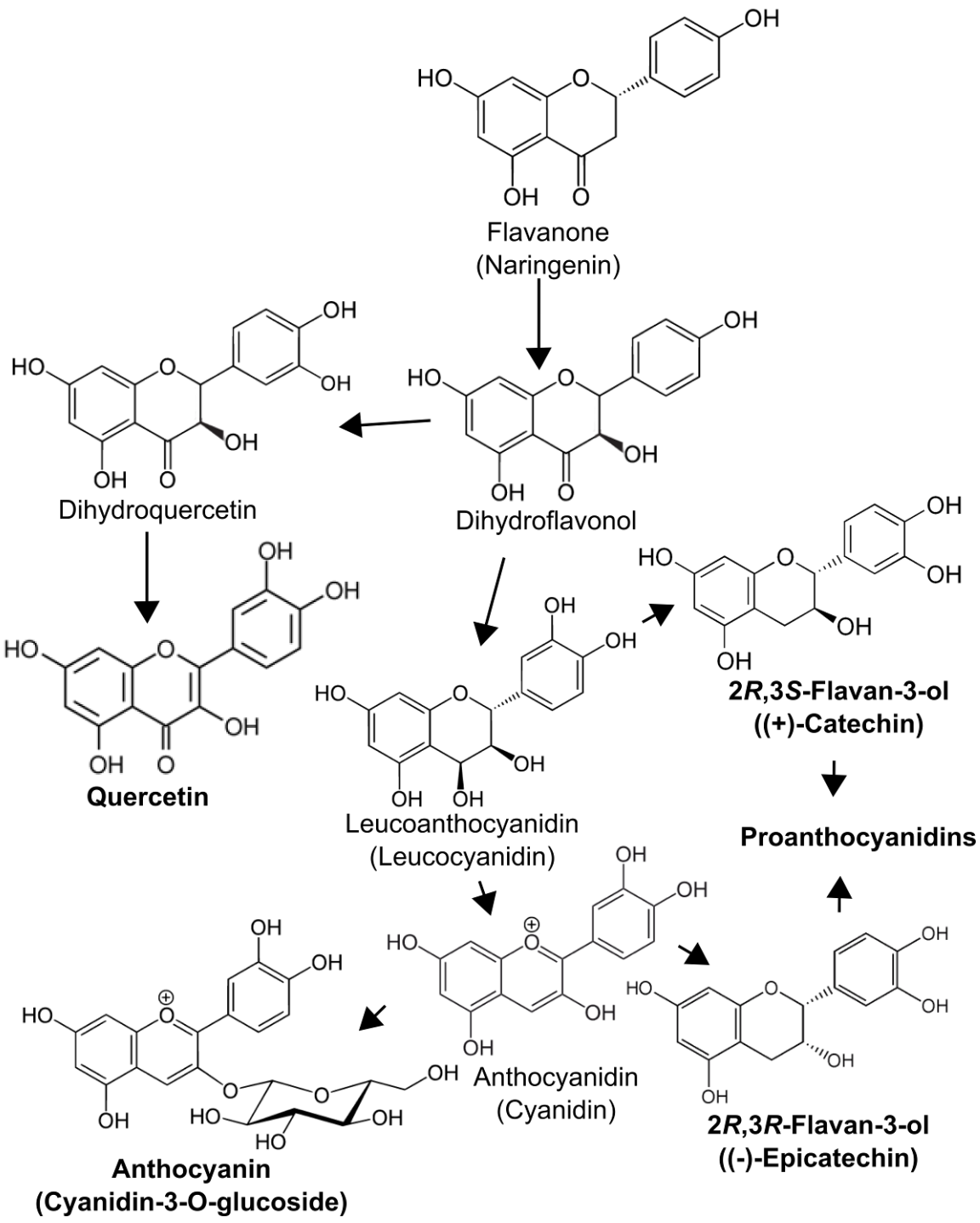

Fig. 1.5: Biosynthesis of flavanols (quercetin), flavan-3-ols ((-)-epicatechin and (+)-catechin), anthocyanins and proanthocyanidins, continued from **Fig. 1.5**. An example of proanthocyanidin structure is demonstrated in **Fig. 1.6** (procyanidin trimer).

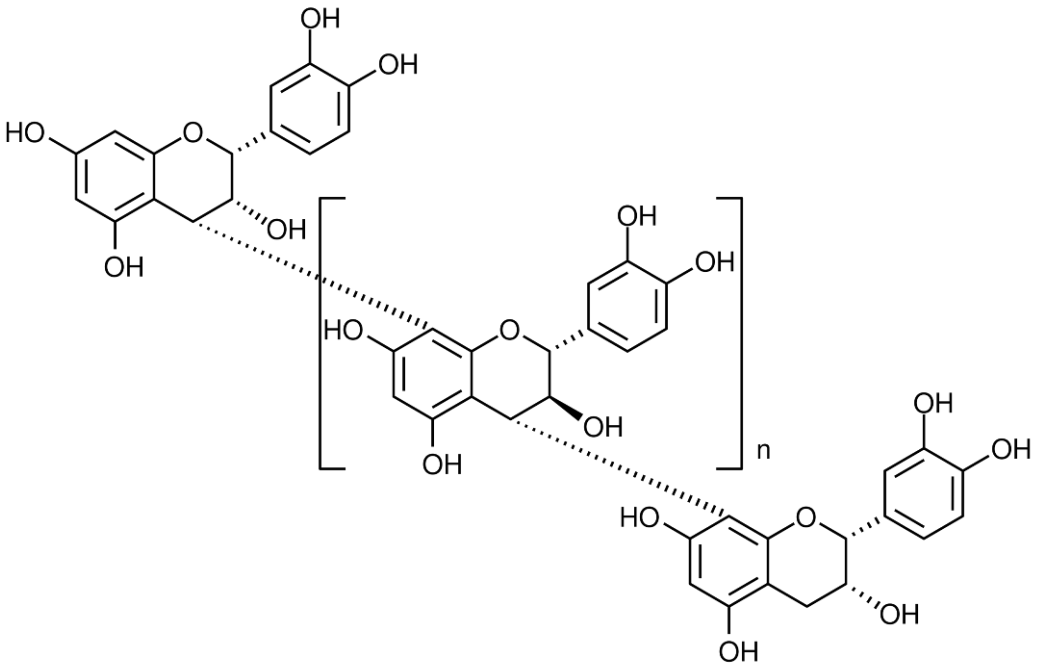

Figure 1.6: Procyanidin trimer with structure (-)-epicatechin-(4α→8)-(+)-catechin-(4α→8)-(-)-epicatechin. Procyanidins can feature multiple (n) extension units.

There are a number of additional bioactive plant secondary metabolites besides polyphenols. Terpenoids have isoprene-based structures, and incorporate a wide range of compounds found across the plant kingdom (Ro 2011). They include numerous classes of phytochemicals, such as phytosterols (triterpenes) and carotenoids (tetraterpenes), of which lycopene may be important for lowering CVD risk (Ried & Fakler 2011; Böhm 2012). Alkaloids are a diverse group of nitrogenous phytochemicals, possessing heterocyclic structure, and have been the basis for numerous pharmaceuticals, such as morphine and codeine, and continue to aid drug-discovery, in areas such as anti-cancer or anti-microbial therapy (Glenn et al. 2013; Hibino & Choshi 2002). In terms of structure variation, glucosinolates are perhaps the most conserved of the phytochemicals mentioned here, though there has still been considerable research into their effects on biological systems. Their structure consists of single thioglucoside and *N*-

hydroxysulphate groups bound by an organic side chain, and are common to plants of the order Brassicales, such as cabbage or mustard (Mithen et al. 2010; Verkerk et al. 2009). Mastication of plants containing glucosinolates releases the enzyme myrosinase from cell vacuoles, which degrades the glucosinolates contained in adjacent 'S-cells' to their bioavailable form, isothiocyanates (ITCs), which can then be absorbed for further metabolism by the human body (Dinkova-Kostova & Kostov 2012; Mithen et al. 2000). The ability to convert glucosinolates to ITCs is also possessed by the gut microflora. One ITC of note is sulforaphane, for which there is now a large body of evidence supporting its beneficial effects on various aspects of human health (Elbarbry & Elrody 2011), particularly in the context of prostate cancer risk (Traka et al. 2008).

1.4. Developments in polyphenol research

Reports of polyphenol use by human populations date back almost three millennia, due to their importance in the process of leather tanning (Quideau et al. 2011), but it is only in the last 200 years that research on individual polyphenols has been performed. Through the 1800s, work was performed to isolate and characterise a number of polyphenols, including catechin (Zwenger 1841) and stilbenoids (Likhtenshtein 2009; Perkin 1868). The preparation of orange bitters by Lebreton (1828) led to the discovery of needle-like crystals, which he called hesperidin (Laszlo 2008), and were revealed in 1876 to be a flavanone glycoside by Hilger and Hoffman (Hendrickson & Kesterson 1954).

The ardent work of Fischer, Freudenberg and Karrer at the turn of the 20th century gave a hint as to the potential vastness of the library of vegetable tannins (Haslam & Cai 1994; Quideau et al. 2011). One of the first investigations into the health benefits of individual polyphenols was performed in the mid-1930s, when Albert Szent-Györgyi recognised that an impure preparation of vitamin C could improve capillary resistance, while isolated vitamin C proved ineffective to his patient. Hesperidin was identified from

the preparation, but despite good results with animal studies, additional human trials were unable to mirror the original effect. The development of paper chromatography by Martin and Synge in 1943 paved the way for a high volume of plant extract analyses, many performed by Bate-Smith and Swain in the following decades (Consden et al. 1944; Haslam 2007). A number of polyphenol classes were first described at this time, including lignans by Haworth in 1948 (Cunha et al. 2012), proanthocyanidins by Masquelier in 1951 (Fine 2000), and ellagitannins by Mayer and Schmidtt in the 1950s and 60s (Lei 2002). In the last few decades, identification of new polyphenols has increased exponentially due to the advances in high and ultra pressure liquid chromatography, gas chromatography, nuclear magnetic resonance and mass spectrometry techniques (Cuyckens & Claeys 2004; Monagas et al. 2010; Stalikas 2010).

The report of the Zutphen Elderly Study by Hertog et al. (1993) was one of the first to show that health benefits associated with high fruit and vegetable intake were related to the presence of flavonoids in the consumed foods. There have been a number of epidemiological studies since which provide evidence that flavonoids confer beneficial effects on CVD mortality and risk factors such as hypertension (Cassidy et al. 2011; Oude Griep et al. 2011; Mink et al. 2007). These studies are also backed by randomised-controlled trials that focus on polyphenol-rich extracts or the compounds themselves, such as cocoa, epicatechin or quercetin, and their effects on markers of endothelial function (Loke et al. 2008a; Monahan et al. 2011; Arranz et al. 2013; Schroeter et al. 2006; Hollands et al. 2013a).

To explain the underlying molecular mechanisms for these effects, there have been a considerable number of *in vitro* and animal studies carried out in the last two decades. A key characteristic of polyphenols is their ability to act as antioxidants, due to their hydroxyl groups (Rice-Evans 1995), allowing them to scavenge radicals or chelate metal ions involved in oxidative reactions (Perron et al. 2008; Ali et al. 2013). As oxidants such as superoxide or hydroxyl radicals contribute to protein damage and

deterioration of cells, one of the initial hypotheses of the mechanism underlying the health benefits of dietary polyphenols was their potent antioxidant activity. However, there has been little evidence to suggest that the concentrations at which polyphenols are present in the human body (nM to low µM range) contribute substantially to the intracellular or extracellular redox environment, especially in comparison to levels of endogenous antioxidants produced by cells (Hollman & Cassidy 2011). Polyphenols may still provide an indirect antioxidant effect, however, by altering the activity of enzymes which reduce oxidants, such as catalase or superoxide dismutase, or by inhibiting pro-oxidant enzymes such as NADPH oxidase or lipoxygenase (Sies 2010). There are a range of additional mechanisms that have been proposed and studied to explain the decrease in CVD risk from consumption of polyphenols. These include anti-inflammatory, anti-thrombotic or anti-atherogenic action, inhibition or promotion of signalling pathways related to the maintenance of vascular health, and changes to the expression of genes related to those pathways (Virgili & Marino 2008; Quiñones et al. 2013; Tangney & Rasmussen 2013). However, linking the *in vitro* or animals experiments which investigate these mechanisms to the human studies that demonstrate the health benefits of polyphenols is generally quite difficult, as compounds are often absorbed in limited quantities, and undergo extensive metabolism.

1.5. Absorption and metabolism of polyphenols

Though the types of phenolic compounds found within species of fruits and vegetables are often consistent between samples, specific concentrations of compounds can still vary due to a number of factors. These include environmental stresses such as temperature and humidity, farming practices, ripeness, length of time between harvest and consumption, transport, storage and method of cooking (D'Archivio et al. 2010). The types of phenolic compounds which are available for absorption in the body can also be affected by certain

nutrients that are consumed alongside or as part of polyphenol-rich foods. For example, association with dietary fibre has been reported to reduce the bioavailability of polyphenols in the small intestine (Palafox-Carlos et al. 2011). On the other hand, increased quantities of dietary fats have been shown to protect cocoa procyanidins during duodenal digestion (Ortega et al. 2009). This was reported as being likely due to increased formation of micellar vesicles, similar to the observed in quercetin micellerisation observed when fat levels in a simulated digestion system were increased (Guo et al. 2013). The same study also showed increases to quercetin plasma concentrations after consumption of a high dose of quercetin (1.1 g) as part of a high-fat meal, compared to a fat-free meal with the same dose. Polyphenols are known for their ability to form complexes with proteins, an interaction which produces the astringent taste of some polyphenol-rich foods and beverages (Ferruzzi et al. 2012; Liang et al. 2013; Ginsburg et al. 2012; Ozdal et al. 2013). Milk has been suggested to inhibit the bioavailability of tea polyphenols, as evidenced by *in vitro* and *in vivo* studies, due to complexes formed between phenolics, milk protein and fat globules (Lorenz et al. 2007; Zhang et al. 2012a). Such complexes were shown to be degraded during simulated digestion, however (van der Burg-Koorevaar et al. 2011), and additional human studies have been unable to show significant effects of co-consumption of milk and tea polyphenols (Roura et al. 2007). There is also *in vitro* evidence that milk may enhance the bioavailability of flavanols (Xie et al. 2012).

There is little evidence that the acidic conditions of the stomach causes breakdown of phenolic compounds, though the digestion of food matrices may improve polyphenol bioavailability (Saura-Calixto et al. 2007; Manach et al. 2004; Scalbert et al. 2002; Larrosa et al. 2010). Once polyphenols reach the small intestine, the increase in pH can aid the release of ellagic acid from ellagitannins, while lactase-phlorizin hydrolase and β-glucosidase activity can hydrolyse and deglycosylate numerous phenolics, and improve their rate of absorption through the intestinal epithelium. Low-molecular weight

phenolics may be absorbed at the small intestine without such modification, though much of the phenolics consumed in the diet will likely pass through to the large intestine, where they can be broken down by colonic microflora. The absorption of polyphenols has been reported so far as particularly low. Systematic review of human studies which account for recovery of phenolics in urine found that recovery of flavonoids varied from 0.2% for anthocyanins, 1-3% for flavonols and flavanols, and 5-7% for flavanones. Recovery of phenolic acids varied from 1-12%, isoflavones from 1-37%, lignans from 13-37%, while resveratrol showed 19% recovery (Pérez-Jiménez et al. 2010). Analytical advances, that allow for improved identification of microbial metabolites in such studies, may help to account for a greater proportion of ingested polyphenols, particularly for compounds which are not readily absorbed in the small intestine, such as anthocyanins (Czank et al. 2013).

Over 160 species of bacteria have been recorded in the human gut, but the particular species and the extent to which they are present varies from person to person, and so the range of microbial metabolites that can be potentially produced varies also (Bolca et al. 2013). It is also likely that the viability of particular species will be affected by certain phenolics in different manners. While some may inhibit bacterial activity, others, such as quercetin-3-O-rutinoside (rutin) may improve microbial attachment and proliferation (Parkar et al. 2008). The particular microbial metabolites varies between polyphenol classes, and includes phenolic acids (proanthocyanidins, anthocyanins and other flavonoids), such as benzoic, phenylacetic, phenylpropionic and phenylvaleric acids, equol (isoflavones), urolithins (ellagic acid) and enterolactone (lignans) (Selma et al. 2009; Williamson & Clifford 2010). The particular compounds produced can also vary between bacterial species. Through β-glucosidase activity, microbes can deglycosylate flavonoid glycosides, for example, hydrolysing rutin to quercetin-3-O-glucoside, and quercetin-3-O-glucoside to quercetin aglycone (Yang et al. 2012; Schneider et al. 1999; Knaup et al. 2007). This activity was shown of the species *Enterococcus casseliflavus* but

it was unable degrade quercetin aglycone further, unlike *Eubacterium ramulus*, which was capable of producing 3,4-dihydroxyphenylacetic acid as a ring-fission metabolite of quercetin (Schneider et al. 1999). Additional microbial phenolic acid metabolites of quercetin glycosides include 3-hydroxyphenylacetic acid, 3-(3-hydroxyphenyl)-propionic acid and 3,4-dihydroxyphenylbenzoic acid (Yang et al. 2013; Rechner et al. 2004; Jaganath et al. 2009). Acetlyation, hydroxylation, dehydroxylation and hydroxymethylation have also been reported of quercetin glycosides by human microflora (Lu et al. 2013). These metabolites can then be absorbed through epithelial cells, in a trans- or para-cellular fashion, or actively via monocarboxylic acid transporter (MCT) (Konishi 2005).

Besides MCT, absorption of phenolics into the epithelial layer of the small intestine is aided by multidrug resistance-associated protein 2 (MRP2) and the sodium-glucose transport protein SGLT1. Compounds which have not already been hydrolysed to remove sugar moieties by lactase-phlorizin hydrolase (LPH) at the surface of enterocytes will be reduced to their aglycone by either intracellular β-glucosidase or LPH, at which point they become substrates for phase II metabolism (Walle 2004; Wu et al. 2011). The enzymes catechol-O-methyltransferase, UDP-glucuronosyltransferase and sulfotransferase in the intestinal epithelium produce a selection of phenolic conjugates, which are then transported via the hepatic portal vein to the liver, where further conjugation/deconjugation occurs. This can lead to a wide range of metabolites present in the blood. For example, phase II metabolites of quercetin (Q) include:
Q-3-glucuronide (Q-3-GlcA), Q-3'-GlcA, Q-diGlcA, isorhamnetin (IR)-3-GlcA, IR-4'-GlcA, Q-3'-sulphate (Q-3'-S), Q-GlcA-S, methyl-Q-GlcA, methyl-Q-diGlcA and Q-glutathione, where Q-GlcA and Q-S are the most common human metabolites (Lee et al. 2012; Mullen et al. 2006).

After passing through the hepatic portal vein, it is likely that further phase II metabolism then occurs in the liver and kidney (Mullen et al. 2006), before plasma

circulation and excretion of conjugates in urine. Nearly all of a radio-labelled quercetin dose of 4 mg/kg (bodyweight) given to rats was found to be excreted after 72 hours, with 69% recovered in the urine, although there was little accumulation of metabolites in plasma or tissues outside the gastrointestinal tract over the three days (Mullen et al. 2008). In this study, much of the quercetin was converted to phenolic acids such as hippuric acid, 3-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid.

Following phase II metabolism, conjugates can also be carried back from the liver to the small intestine in the bile, as demonstrated by reports of *N*-acetylcysteine conjugates of quercetin, likely formed from glutathione conjugates re-entering the gut and undergoing microbial metabolism (Hong & Mitchell 2006). A proportion of metabolites will also enter the circulatory system and be transported in plasma to tissues throughout the body. Quercetin has been observed to accumulate in relatively small quantities in a number of rat tissues, with the highest concentration found in the lungs (Paulke et al. 2012). Deconjugation of metabolites to the aglycone form, however, may take place at the interface with endothelial cells and other target tissues, through enzymes such as β-glucuronidase. It is also possible that polyphenols. including quercetin, may become deconjugated once inside cells (Terao et al. 2011; Perez-Vizcaino et al. 2012). Conjugation has the effect of reducing the hydrophobicity of quercetin, which facilitates transport through lipid bilayers of the plasma membrane. Inflammation of tissues may aid in transport of quercetin conjugates, however, as Q3GlcA was reported to pass through aortic endothelial cells and reach vascular smooth muscle cells under such conditions (Mochizuki et al. 2004). Once in these tissues, further conjugation/deconjugation may occur before efflux back to the circulatory system. Excretion of most phenolic metabolites then occurs 24-48 hours after initial ingestion. While there has been a growing effort in the past decade to include human metabolites of polyphenols in *in vitro* studies alongside the forms found in plants, their synthesis can prove difficult and may not provide the quantities necessary for such experiments.

1.6. Proposed mechanisms underlying polyphenol bioactivity

The means by which polyphenols exert their beneficial effects on the cardiovascular system are still under investigation, though a number of mechanisms have been proposed and shown to be effective *in vitro*. As was previously mentioned, while the physiological concentrations of phenolic compounds are unlikely to provide a strong antioxidant effect, they may still act as antioxidants indirectly by inhibiting ROS-generating enzymes or activating cellular antioxidant responses. Increased expression of antioxidant response enzymes such as thioredoxin or peroxidases by activation of transcription factors such as Nrf-2 can also be trigged by exposure of cells to phenolic compounds such quercetin (López-Alarcón & Denicola 2013). Lipoxygenases, which catalyse the oxidation of fatty acids, can be inhibited by a number of flavonoids, likely through the chelation of iron from its catalytic domain. Production of superoxide by NADPH oxidase can also be inhibited by polyphenols, notably by epicatechin and its methylated/glucuronidated conjugates (Steffen et al. 2007; Steffen et al. 2008). Xanthine oxidase (XO) generates xanthine from hypoxanthine, and uric acid from xanthine, converting water to hydrogen peroxide in the process (Ozyürek et al. 2009). Polyphenols, including quercetin and luteolin (Pauff & Hille 2009), have been shown to inhibit xanthine oxidase activity, and dietary intake of phenolics alongside vitamins has also been shown to reduce uric acid levels in human plasma (Qureshi et al. 2012). A structure-activity study reported that an unsaturated 2,3-bond is important for inhibition of XO by flavonoids, alongside the 4-keto group of flavonols and flavones (Mladenka et al. 2010).

Small changes to a phenolic compound's structure can have a significant effect on its bioactivity. The addition of a single hydroxyl group, i.e. the difference between catechin and gallocatechin, can produce significant differences in the spatial arrangement of the molecule (Du et al. 2012). Methylation of flavonoids can reduce their ability to inhibit ROS-generating enzymes (Loke et al. 2008b), as well as their induction of apoptosis in cancer cells (Landis-Piwowar et al. 2008). Glycosylation of polyphenols can

reduce their suppression of liver cancer cell proliferation (Loa et al. 2009), and a planar structure can improve antioxidant activity (Moalin et al. 2011). Structural changes can also affect the binding affinity of polyphenols for proteins. Increased hydroxylation of the A- and B-rings, the presence of an unsaturated 2,3-bond, a lack of sugar groups and the presence of galloyl groups are all factors which improve polyphenol-plasma protein binding affinity (Xiao & Kai 2012). The binding of plasma and cellular proteins to polyphenols is likely to be important in the context of *in vivo* bioactive effects. For example, flavonoids have been shown to bind to the anti-atherogenic protein PON1 and protect it from inhibition (Atrahimovich et al. 2013). Quercetin has been shown to inhibit a wide array of human kinases at 2 and 30 μM (Boly et al. 2011), and was reported to bind to and inhibit the microbial enzyme D-alanine ligase, with greater potency than apigenin, indicating the importance of quercetin's extra hydroxyl groups for this interaction (Wu et al. 2008). Flavone and flavanone structures were found to be more effective than other polyphenols at inhibiting the activity of the cytochrome P450 enzyme aromatase, in particular flavones and flavanones with hydroxylation at the 3', 4', 5 and 7 positions (Neves et al. 2007). As well as proteins, polyphenols can also interact with the lipid bilayer of the plasma membrane, where they are likely to bind and penetrate the layer and form interlayer bridges, causing the collapse of the interbilayer fluid space and hyperpolarisation of the membrane (Huh et al. 1996).

Additional potential mechanisms for the beneficial vascular effects of polyphenols include prevention of platelet aggregation through inhibition of the thromboxane receptor or synthesis of thromboxane itself (Hubbard et al. 2004; Vita 2005), as well as reducing the expression of cell adhesion molecules, such as ICAM-1 and VCAM-1 (Kim et al. 2011; Song et al. 2011). Evidence from animal studies indicates that polyphenols are also able to cause dilation of vascular tissues, in both endothelium-dependent and endothelium-independent manners, likely involving decreases to intracellular Ca^{2+} concentration and increases to nitric oxide production (Hotta et al. 2006; Villar et al.

2005; Romano & Lograno 2009; Schmitt & Dirsch 2009). It appears that the 4-keto group, as well as hydroxylation of the A-ring of flavonoids are important for this activity, alongside the removal of sugar moieties (Xu et al. 2007). Quercetin, epicatechin-gallate, isoflavones and resveratrol have all been shown to have potent vasodilator effects on vascular tissue.

Signal transduction plays an important role in mediating early responses to circulatory compounds that lead to functional outcomes with important implications for vascular health. While changes to RNA transcription and protein levels are important for mediating downstream functional changes, protein-protein interactions and changes to their structures are a critical part of early signalling events. Introduction of individual polyphenols to cells and tissues may initiate one key signalling event that varies depending on the type of cell, or may start a number of events that cause cascades of various signalling pathways. Crosstalk between signalling pathways downstream of initial signalling events can complicate analyses, especially if sensitivity to additional external factors was the case, and make it difficult to draw conclusions between functional outcomes and upstream signalling. Therefore focusing on early signalling events would theoretically provide a simpler view of the mechanistic effects induced by bioactive compounds.

One common method for proteins to transduce signals is through the attachment of phosphate groups (phosphorylation) to serine, threonine or tyrosine residues. This affects the structural conformation of the protein, changing them into a state where they have enzymatic activity. Often this activity can directly or indirectly lead to phosphorylation of additional proteins (kinase activity), causing a signal cascade (McCubrey et al. 2012). There are many biochemical methods routinely used for measuring levels of phosphorylated (phospho-) proteins, such as ELISA or western blotting, which generally allow assay of a small numbers of targets.

1.7. Consumption of polyphenols in traditional diets

Though there are still limits to the methods by which dietary intake of polyphenols are accessed, mostly by databanks and food diaries (Spencer et al. 2008), consumption is estimated at 1 g/day (Touvier et al. 2011; Ovaskainen et al. 2008). This value can vary, however, as regions across the world have their own particular dietary habits. For example, a national survey of Spanish households found that intake was closer to 3 g/day (Saura-Calixto et al. 2007). This variation in levels of phenolics consumed is also reflected in studies which show that diets of particular regions can provide beneficial effects to cardiovascular health. The so-called 'French paradox' notes that there is a relatively low incidence of CVD amongst populations located along the north Mediterranean coast, in spite of diets that include high levels of saturated fats and red meats, indicative of higher CVD risk. It is suggested this may be in part due to increased consumption of polyphenol-rich foods in these regions, including red wine and olive oil (Shukla et al. 2010; Rasmussen et al. 2005). To date, the focus of many of the studies concerned with the connection between polyphenols and health have been food plants common to the modern diets of Western European and North American countries, including cocoa, coffee, tea, onions and apples (Crozier et al. 2009). Limited attention has been given to less well-known foods of other international regions. Such foods are likely to make use of plant ingredients that, while uncommon in western diets, may prove to be rich sources of polyphenols with potential benefits to human vascular health.

Traditional foods (defined by the European Food Information Resource (EuroFIR) as those that are produced by practices dating to before the Second World War (Costa 2007)) by their nature have the advantage of being both affordable and easy to obtain, and are usually of high cultural importance. There is evidence, however, that such foods can also be perceived as being high value or inappropriate for daily consumption, often being reserved for festive occasions (Almli et al. 2011). Changes to dietary habits, such as inclusion of modern, convenience foods, may threaten the continued use of traditional

foods (Trichopoulou et al. 2006; Milošević et al. 2012). Providing evidence of health benefits, or developing means for improving nutritional value, would help reverse such a trend. Geographical areas where such traditional foods have received little attention with regards to health effects, provide the potential for uncovering new sources of beneficial polyphenols, as well as building evidence for future health claims and providing support for the continued production of traditional foods and sustained crop diversity (Trichopoulou et al. 2007). One such area is that of the Middle Eastern European countries surrounding the Black Sea. This is an area which has had little research into the health outcomes of their traditional and modern diets. It is likely that a number of the plant foods consumed in these countries to provide some health benefit, and by understanding which foods these are, better nutritional advice can be provided for those who suffer health problems such as cardiovascular disease, in these countries.

There is epidemiological evidence from recent studies that high CVD risk, measured using markers which include obesity, C-reactive protein and levels of lipids, is associated with a significant proportion (> 50% in some cases) of the populations of countries surrounding the Black Sea, including Turkey (Onat 2004; Onat et al. 2011), Bulgaria (Dyakova et al. 2008; Kurktschiev et al. 2009), Georgia (Davis et al. 2005; Grim et al. 1999), Romania (Daina et al. 2008; Dorobantu et al. 2010) and Ukraine (Khalangot et al. 2009). There are currently no reports as to whether easily accessible foods, such as those found in traditional diets, are able to counter levels of CVD risk when they are included in the diet. To assess potential health benefits that could be derived from traditional diets in this region, the EU FP7 project 'BaSeFood' was set up to study foods of Romania, Ukraine, Georgia, Turkey, Bulgaria and Russia (D'Antuono et al. 2010). Part of the preliminary work for this project was to compile a list of traditional dishes indigenous to the mentioned countries. This list would act as a basis from which to select foods of plant origin for this project, with an aim to study their polyphenol contents and potential bioactive properties.

1.8. Aims of the project

It was hypothesised that plant foods originating from traditional diets of countries surrounding the Black Sea would contain polyphenols which, in their native or phase-II metabolised form, would be able to influence endothelial cell signalling in a manner associated with improved endothelial function. To test this hypothesis, polyphenols and polyphenol-rich plant extracts would be prepared for incubation with endothelial cell cultures, followed by analysis of specific or broad ranges of signalling molecules. Specifically, this work would involve:

- The systematic selection of plant foods traditionally eaten in countries surrounding the Black Sea, for the preparation of bioactive-rich extracts and representative individual compounds.

- Treatment of endothelial cell cultures with the plant extracts or pure compounds, and preparation of cell lysates for the analysis of signalling molecules, including those related to endothelial function, for the identification of bioactive compounds.

- Determining which compounds were responsible for any observed bioactivity after exposure of cells to plant extracts, and identification of the underlying molecular mechanisms for the polyphenol bioactivity.

Chapter 2

Selection of food plants, and general methods for their biochemical analysis

Chapter 2: Selection of food plants, and general methods for their biochemical analysis

2.1. Selection of traditional food plants

2.1.1. Determining the main ingredients of selected traditional dishes

This PhD project was funded by a Framework Programme 7 (FP7) grant (Sustainable exploitation of bioactive components from the Black Sea Area traditional foods (BaSeFood), grant number: 227118) from the European Union in which the Institute of Food Research was a full beneficiary. The FP7 project 'BaSeFood' was concerned with evaluating the potential benefits of bioactive-rich plants that were used in traditional foods in the little studied Black Sea region countries. At the 2nd BaSeFood Consortium Meeting (October 2009), a shortlist of traditional foods was compiled by the BaSeFood consortium partners (**Table 2.1**), with each of the Black Sea area country representatives selecting a traditional dish in each of the following categories; (i) fruit, (ii) vegetables, (iii) herbs and spices, (iv) oilseeds, (v) cereals and (vi) fermented foods (**Tables 2.2** and **2.3**).

Table 2.1: BaSeFood project partners of countries surrounding the Black Sea

Project Partner	Country
University of Food Technologies	Bulgaria
Elkana Biological Farming Association	Georgia
Academy of Economic Studies	Romania
Moscow State University of Food Production	Russia
Yeditepe University	Turkey
Uzhhorod National University/Odessa National Academy of Food Technologies	Ukraine

Table 2.2: Representative country of project partners and their chosen foods.

Country	Vegetables	Fermented	Herbs & spices
Bulgaria	Tikvenik	Boza	Mursal tea
Georgia	Nettles and walnut sauce	n/a	Wild plum sauce
Romania	Sour nettle soup	Socata	Herbal dish
Russia	Vegetable okroshka	Southern kvass	n/a
Turkey	Kale	Pickled green beans	Black tea
Ukraine	Ukrainian borscht	Sauerkraut	Dill

Table 2.3: Representative country of project partners and their chosen foods.

Country	Fruits	Cereals	Oilseeds
Bulgaria	Fruits of the forest	Wheat bread	Sesame seeds
Georgia	Churchkela	Doli wheat bread	Flax oil
Romania	Plum jam/pomegranate	Mamaliga	n/a
Russia	Watermelon juice	Buckwheat porridge	Mustard oil
Turkey	Persimmon	Corn bread	n/a
Ukraine	Uzvar	Sour rye bread	Sunflower seeds

Information provided by the BaSeFood project partners was used to determine the main ingredients in the dishes listed in **Table 2.2** (work to determine the main ingredients of the dishes presented in **Table 2.3** was carried out by Wendy Hollands, IFR, UK), and where recipe information was not available from the project partners, systematic web searches were performed using the search engine Google (www.google.com) to determine the main ingredients of the dishes. Web searches were performed using either:

(i) the dish's name as given by the project partner, i.e. “vegetable okroshka”

(ii) the name translated into the project partner’s native language, i.e. “овощной окрошка”, or

(iii) single keywords from the whole name using either of the aforementioned methods. i.e. “okroshka” or “окрошка”

Translation was performed using the Google Translate facility. Recipes (date of access, Nov. 2009) were deemed to be of use for determining the dish’s main ingredients when:

- the recipe’s website featured within the top 20 results

- a majority of the ingredients were shared with other recipes in the search results

The main ingredients of each dish, alongside the proportion of the dish that they represent are given in **Tables 2.4** and **2.5**. The sources for the information given in these tables are presented in **Table 2.7**, numbered accordingly between tables. The main ingredients of the dishes listed in **Table 2.3** are presented in **Table 2.6**.

Table 2.4: Main ingredients of the traditional dishes from the '**Vegetables**' category (**Table 2.2**), alongside the approximate percentage of each ingredient in the dish. n/a - ingredient forms the whole of the dish, i.e. close to 100%. nd - no data available. Sources for the presented information are listed in **Table 2.7**.

Dish	Main ingredients	% of dish	Sources
Kale	n/a	n/a	i
Nettles and walnut sauce	Nettles	nd	ii
	Walnuts	nd	
Sour nettle soup	Nettle	80	iii
	Onion	10	
	Bors	n/a	
	Carrots	5	
	Celery Root	5	
	Rice	n/a	
Tikvenik	Pumpkin	92	iv
	Walnuts	7.7	
	Cinnamon	0.3	
	Flour, Sugar, Oil/Butter	n/a	
Ukrainian borscht	Beetroot	25	v
	Cabbage	20	
	Potatoes	25	
	Carrot	10	
	Onion	10	
	White beans	10	
	Parsley root, celery, garlic, tomato paste, kvass, lemon, sugar, sour cream, dill	n/a	
Vegetable okroshka	Potatoes	35	vi
	Cucumber	30	
	Spring Onions	15	
	Carrots	10	
	Turnips	10	
	Radish	10	
	Kvass, dill, parsley, eggs, sour cream	n/a	

Table 2.5: Main ingredients of the traditional dishes from the '**Fermented**' and '**Herbs and spices**' categories (**Table 2.2**), alongside the approximate percentage of each ingredient in the dish. n/a - ingredient forms the whole of the dish, i.e. close to 100%. nd - no data available for the percentage of this ingredient in as part of the whole dish. Sources for the presented information are listed in **Table 2.7**.

Dish	Main Ingredients	% of dish	Sources
Boza	Millet	nd	vii
	Sugar	nd	
Socata	Elderflowers	nd	viii
	Lemons	nd	
	Sugar, yeast	nd	
Southern kvass	Rye bread	nd	ix
	Sugar, yeast	-	
Pickled green beans	Green beans	nd	i
	Onions	nd	
Sauerkraut	Cabbage	nd	x
Mursal tea	Sideritis	n/a	iv
Wild plum sauce	Plum	nd	xi
	Dill	nd	
	Coriander	nd	
Herbal dish	Parsley	40	xii
	Dill	40	
	Onion	19	
	Mint, sweet basil, sage	<1	
Dill	n/a	n/a	n/a
Black tea	n/a	n/a	n/a

Table 2.6: Main ingredients of the traditional dishes from the '**Fruits**', '**Cereals**' and '**Oilseeds**' categories (**Table 2.3**), alongside the approximate percentage of each ingredient in the dish. n/a - ingredient forms the whole of the dish, i.e. close to 100%. nd - no data available for the percentage of this ingredient in as part of the whole dish.

Dish	Main Ingredients
Sunflower hulva	Sunflower seeds, liquorice, cocoa powder, raisins, vanilla, sugar, molasses
Uzvar	Dried apple, pears and prunes, syrup
Sour rye bread	Rye flour, yeast, sugar
Mustard oil	n/a
Water melon juice	n/a
Buckwheat porridge	Buckwheat
Plum jam	Plums
Mamaliga	Water, salt, cornmeal
Flax oil	n/a
Churchkela	Walnut, hazelnut, almonds (skins removed), dried fruits, grape juice and corn flour
Bread baked from Akhaltsikhe Mesfhetion tsiteli doli	Wheat flour, Makhobeli (wild plant) grains
Sesame seeds	n/a
Fruits of the forest	Fruits bearing resemblance to blueberries
Wheat bread	Wheat flour
Persimmon fruit	n/a
Pomegranate	n/a
Fruit of the evergreen cherry laurel	n/a
Corn bread	Corn
Cracked corn soup	Corn, pinto beans, yoghurt
Bulgar pilaf	Bulgar, Chicken broth, onion, tomato, green pepper

Table 2.7: Citations referred to in **Tables 2.4** and **2.5**.

No.	References
i	(Yeditepe University 2009)
ii	(Charlotte's VVeb 2009; World Health Circle of International Cooking 2009)
iii	(Bucharest Academy of Economic Studies 2009; Eculinar 2009; Gastropedia 2009; Retete Mancare 2009)
iv	(University of Food Technologies 2009)
v	(Elena Filatova 2007; Gotovim 2009; Russland Journal 2009a; Say7 2006; The Worldwide Gourmet 2009; Ukraine Orphans 2009)
vi	(Moscow State University of Food Productions 2009; Gastronom 2009; Good-Cook 2009; Gotovim 2009; Povarenok 2009; Russland Journal 2009b; Russian Foods 2009; Wikipedia (RU) 2009)
vii	(Gotvarstvo 2009; Sulekha 2008; Wikipedia (BG) 2009)
viii	(Bucharest Academy of Economic Studies 2009; Europe-Cities 2009; Global Oneness 2009)
ix	(Moscow State University of Food Productions 2009; The Kombucha Journal 2009)
x	(Boyko et al. 2009)
xi	(Elkana - Biological Farming Association 2009)
xii	(Dr Alexandru Stroia, ASE (personal communication), 11/12/2009)

2.1.2. Bioactive constituents of the main plant ingredients

Literature reviews were conducted using the online databases Web of Knowledge and PubMed, to determine main bioactive compounds in the plant ingredients detailed in **Section 2.1.1** (the bioactive content of the food plants listed in **Table 2.6** was researched by Wendy Hollands, IFR, UK). The polyphenol database website Phenol Explorer was also used to determine the phenolic content of food plants (Neveu et al. 2010), along with their total phenolic content (TPC). Reviewed literature was used to estimate the TPC of any foods not present on Phenol Explorer. This information is presented in **Table 2.8**, with sources for the information listed in **Table 2.9**. The main bioactive compounds found in the plant foods listed in **Table 2.6** are presented in **Table 2.10**.

Table 2.8: Plant ingredients from the categories '**Vegetables**', '**Fermented**' and '**Herbs and spices**' (**Table 2.2**) and their main bioactive compounds/classes, with compounds of note given in brackets alongside. The total phenolic content (**TPC**) of each food plant is given as mg/100 g. Sources for the information presented here are listed in **Table 2.9**.

Plant ingredient	TPC	Phenolics and other main bioactives	Sources
Kale	177	Large number glycosides of kaempferol (foremost), quercetin and isorhamnetin Glucosinolates (brassicin) Carotenoids (lutein & β-carotene)	i
Nettles	110	Hydroxycinnamic acids Flavanol glycosides (quercetin) Anthocyanins	ii
Walnuts	1625	Ellagic acid, gallic acid Hydroxybenzoic acids (vanillic and ferulic acid) Hydroxycinnamic acids	iii
Onion	46	Flavanol glycosides (quercetin) Protocatechuic acid	iv
Carrot	58	Flavonoids (quercetin) Phenolics – hydroxybenzoic/cinnamic acids Carotenoids (*β*-carotene)	v
Celery root	59	Flavonoids (apigenin)	iv
Rice	95	Phenolics – hydroxybenzoic/cinnamic acids	iv
Pumpkin	16	Carotenoids (lutein and *β*-carotene) Phenolics – hydroxybenzoic/cinnamic acids	vi
Cinnamon	9700	Catechin, epicatechin, procyanidins (B2 dimer) Cinnamaldehyde, caffeic acid	vii
Beetroot	164	Betalains (betanin), flavonoids (luteolin)	viii
Cabbage	15	Flavonoids (quercetin and kaempferol glycosides) Glucosinolates (sinigrin, glucoberin) Hydroxycinnamic acids (sinapic acid derivatives, 3-*p*-coumaroylquinic acid)	ix
Potatoes	31	Phenolics – hydroxybenzoic/cinnamic acids	x
White beans	138	Hydroxycinnamic acids (ferulic acid), small quantities of flavan-3-ols Glycosides of quercetin and kaempferol	iv
Cucumber	18	Flavones (luteolin), kaempferol, quercetin Lignans (secoisolariciresinol)	iv
Spring Onions	107	Kaempferol and quercetin glycosides Hydroxycinnamic acids (ferulic acid) Flavones (baicalein) and flavanones (hesperidin)	xi

Table 2.8: Plant ingredients from the categories '**Vegetables**', '**Fermented**' and '**Herbs and spices**' (**Table 2.2**) and their main bioactive compounds/classes, with compounds of note given in brackets alongside. The total phenolic content (**TPC**) of each food plant is given as mg/100 g. Sources for the information presented here are listed in **Table 2.9**.

Plant ingredient	TPC	Phenolics and other main bioactives	Sources
Turnips	55	Glucosinolates (gluconapin), Glycosides of kaempferol, quercetin and isorhamnetin	xii
Radish	44	Lignans (pinoresinol) Kaempferol Glucosinolates (glucoerucin and glucoraphenin) Anthocyanins acylated with phenolic acids	xiii
Elderflower	-	Caffeic acid derivatives, Glycosides of kaempferol, quercetin and isorhamnetin	xiv
Lemons	60	Flavanones (eriodictyol, hesperetin)	iv
Rye bread	-	Ferulic acid, lignans	iv
Green beans	304	Catechin, epicatechin, procyanidin dimers Kaempferol and quercetin glycosides	xv
Cabbage (fermented)	15	ITCs (allyl isothiocyanate, sulforaphane)	xvi
Sideritis	480	Flavone glycosides (apigenin, hypolaetin, luteolin) Phenolics – hydroxybenzoic/cinnamic acids Lignans (sesamin) Di- and tri-terpenoids (siderol, squalene)	xvii
Plum	410	Quercetin glycosides (3-O-rutinoside) (Epi)catechin, procyanidin dimers & trimers Peonidin and cyanidin glycosides Caffeic acid	iv
Dill	208	Glycosides of kaempferol, quercetin and isorhamnetin	xviii
Coriander	159	Ferulic, caffeic & gallic acid Quercetin and kaempferol	xix
Parsley	90	Gallic, protocatechuic and caffeic acid Quercetin, kaempferol, Luteolin, myricetin and apigenin	xx
Black tea	105	(Epi)catechin, procyanidins, theaflavins Kaempferol and quercetin glycosides Phenolics – hydroxybenzoic/cinnamic acids	iv

Table 2.9: Citations referred to in **Table 2.8**.

No.	References
i	(Yeditepe University 2009; Neveu et al. 2010; Kopsell et al. 2003; Sarikamis et al. 2008; Schmidt et al. 2010)
ii	(Chrubasik et al. 2007; Pinelli et al. 2008)
iii	(Neveu et al. 2010; Jakopic et al. 2009; Shan et al. 2005; Stampar et al. 2006)
iv	(Neveu et al. 2010)
v	(Neveu et al. 2010; Bub et al. 2000)
vi	(Neveu et al. 2010; Kurz et al. 2008)
vii	(Neveu et al. 2010; Peng et al. 2008)
viii	(Neveu et al. 2010; Kujala et al. 2002; Kujala et al. 2001)
ix	(Neveu et al. 2010; Mattila & Hellström 2007; Sousa et al. 2008; Tolonen et al. 2002)
x	(Neveu et al. 2010; Mattila & Hellström 2007)
xi	(Neveu et al. 2010; Marinova et al. 2005; M. Parvu et al. 2010; Thompson et al. 2005; Aoyama & Yamamoto 2007)
xii	(Neveu et al. 2010; Francisco et al. 2009)
xiii	(Neveu et al. 2010; Malik et al. 2010; Matsufuji et al. 2007)
xiv	(L. Christensen et al. 2008; Kaack & Christensen 2010)
xv	(Neveu et al. 2010)
xv	(Neveu et al. 2010; Hertog et al. 2002; Sarkams et al. 2009)
xvi	(Neveu et al. 2010; Tolonen et al. 2002)
xvii	(Gabrieli et al. 2005; Janeska et al. 2007; Koleva 2007)
xviii	(Neveu et al. 2010; Justesen & Knuthsen 2001; Justesen 2000)
xix	(Neveu et al. 2010; Hadjmohammadi & Sharifi 2009)
xx	(Neveu et al. 2010; Mišan et al. 2011)

Table 2.10: List of plant ingredients from the categories '**Fruits**', '**Cereals**' and '**Oilseeds**' (**Table 2.3**) and their main bioactive compounds/classes, with compounds of note given in brackets alongside.

Plant ingredient	Phenolics and other main bioactives
Sunflower seeds	Phenolics – hydroxybenzoic/cinnamic acids
Liquorice	Flavanols (quercetin)
Cocoa powder	Flavanols - epicatechin, catechin, procyanidins Phenolics - hydroxybenzoic/cinnamic acids
Raisins	Phenolics –hydroxybenzoic/cinnamic acids Flavonols - quercetin-3-O-rutinoside
Dried apple	Flavanols and phenolics (but a reduced quantity due to drying)
Dried pear	Flavanols and phenolics (but a reduced quantity due to drying)
Rye flour	Phenolics – hydroxybenzoic/cinnamic acids (ferulic, sinapic and *p*-coumaric acid) Lignans
Buckwheat	Flavonols (rutin and other quercetin glycosides) Flavanols (catechin, epicatechin, procyanidins)
Flax oil	Lignans, phenolic acids
Almonds	Flavanols – Epicatechin, catechin, epigallocatechin gallate Flavanones – Naringenin Flavonols – Isorhamnetin, Kaempferol, quercetin Phenolics – Hydroxybenzoic acid
Hazelnut	Flavanols – Epicatechin, catechin, epigallocatechin gallate
Grape juice	Flavanols, Flavones (apigenin, luteolin), Flavonols (kaempferol, quercetin, myricetin)
Wheat flour mixed with Makhobeli (wild plant) grains	Flavonoids and phenolic compounds
Pomegranate	Anthocyanins (cyanidin, delphinidin) Ellagitannins (punicalagin) Catechins, procyanidins
Sesame seeds	Lignans
Fruit of the evergreen cherry laurel	Anthocyanins
Persimmon	Flavanols - Catechin, epicatechin, epigallocatechin, Phenolics - chlorogenic acid, caffeic acid, gallic acid

2.1.3. Criteria for the selection of food plants

The following criteria were used to grade and rank the plant ingredients, from which the top six would be chosen for further study.

(i) **In-house expertise for bioactives** - Preference was given to plants which contained bioactives that members of our lab had expertise in. This criteria, which was further divided into the categories of main expertise (A) and additional expertise (B - D) is graded (high to low) as follows:

A. Plant contains flavonoids
B. Plant contains glucosinolates
C. Plant contains non-flavonoid polyphenols
D. Plant contains non-polyphenolic, non-glucosinolate bioactives

(ii) **Proportion in final dish** - Preference was given to plant-ingredients which were present in larger quantities in the final dish. The criteria is graded (high to low) as follows:

A. 50 - 100 % of the final dish
B. 25 - 49 % of the final dish
C. 5 - 24 % of the final dish
D. 0 - 4 % of the final dish

(iii) **Concentration of bioactives in plant-ingredients** - Preference was given to plant-ingredients which contain higher concentrations of bioactives, based on information presented in **Table 2.8**. The criteria is graded (high to low) as follows:

A. > 400 mg bioactives/100 g fresh weight
B. 100 - 400 mg bioactives/100 g fresh weight
C. 40 - 100 mg bioactives/100 g fresh weight
D. 0 - 40 mg bioactives/100 g fresh weight

(iv) **Prevalence of ingredients in Western European diets** - Plant-ingredients which featured less prominently in diets of western Europe were graded higher. This criteria is graded (high to low) as follows:

A. Rare occurrence in Western European diet
B. Occurs in Western European diet to a lesser extent
C. Common in Western European diet

(v) **Prevalence of ingredients in traditional dishes** - Plant-ingredients which appeared in more than one of the dishes listed in **Tables 2.2** and **2.3** were higher preference. This criteria is graded (high to low) as follows:

A. Appearance in more than 2 dishes
B. Appearance in 2 dishes
C. Appearance in 1 dish

2.1.4. Ranking of food plants

Plant ingredients were sorted by criteria grades, with the criteria ranked in the following order (headings for **Table 2.11** given in brackets alongside):

(i) In-house expertise for bioactives (**Expertise)**
(ii) Proportion in final dish (**Propor.)**
(iii) Concentration of bioactives in plant-ingredients (**Conc.**)
(iv) Prevalence of ingredients in Western European diets (**Diet)**
(v) Prevalence of ingredients in traditional dishes (**Dishes)**
(vi) Additional lab expertise of bioactives (**Add. expertise)**

The final selected foods are underlined in **Table 2.11**. While plum was initially selected, a sample of the fruit could not be obtained, so pomegranate was selected instead.

Table 2.11: Plant-ingredients graded by the criteria from **Section 2.1.3**. The highest grading foods which were selected for further study are underlined.

Plant ingredient	Selection criteria					
	Expertise	**Propor.**	**Conc.**	**Diet**	**Dishes**	**Add. expertise**
Sideritis	A	A	A	A	C	C, D
Plum	A	A	A	B	B	C
Kale	A	A	A	B	C	B, C
Nettle	A	A	B	A	B	C
Persimmon	A	A	B	A	C	
Dill	A	A	B	B	A	
Pomegranate	A	A	B	B	C	C
Cherry Laurel	A	A	B	B	C	C
Tea	A	A	B	C	C	C
Green Beans	A	A	B	C	C	
Watermelon	A	A	C	B	C	
Blueberries	A	B	A	C	C	
Beetroot	A	B	B	B	C	D
Parsley	A	B	B	C	B	
Cucumber	A	B	D	C	C	C
Elderflowers	A	C	A	A	C	C
Cocoa Powder	A	C	A	C	C	C
Raisins	A	C	A	C	C	C
Liquorice	A	C	A	C	C	
White beans	A	C	B	A	C	C
Dried Apple	A	C	B	A	C	C
Dried Pear	A	C	B	A	C	C
Almonds	A	C	B	C	C	C
Spring Onions	A	C	B	C	C	C
Grape	A	C	B	C	C	
Hazelnut	A	C	B	C	C	
Onion	A	C	C	C	A	
Turnips	A	C	C	C	C	B
Radish	A	C	C	C	C	B, C
Celery Root	A	C	C	C	C	
Lemons	A	C	C	C	C	
Sage	A	D	B	C	C	C
Mint	A	D	B	C	C	
Celery	A	D	D	C	C	C
Mustard Oil	B	A	A	C	C	

Table 2.11: Plant-ingredients graded by the criteria from **Section 2.1.3**.

Plant-ingredient	Selection Criteria					
	Expertise	**Propor.**	**Conc.**	**Diet**	**Dishes**	**Add. Expertise**
Cabbage	B	A	D	C	B	A, C
Buckwheat	C	A	A	A	C	A
Sesame Seeds	C	A	A	A	C	
Corn	C	A	B	A	B	D
Sunflower Seeds	C	A	B	B	C	
Flax Seed Oil	C	A	C	A	C	
Rye Flour	C	A	C	A	C	
Wheat flour	C	A	C	C	B	A
Potatoes	C	B	D	C	B	
Walnuts	C	C	A	A	B	
Coriander	C	C	B	C	C	A
Cinnamon	C	D	A	C	C	A
Sweet Basil	C	D	B	C	C	
Rice	C	D	C	C	C	
Pumpkin	D	A	D	A	C	C
Carrots	D	C	C	C	B	A, B
Garlic	D	D	C	C	C	

2.1.5. Detailed literature analysis of the bioactive content for selected food plants

Through systematic grading by defined criteria, the food plants dill, nettle, sideritis, kale, pomegranate and persimmon were selected for study in this project. The literature was studied exhaustively to determine all the phenolic compounds that had been identified in each of plants. Only studies which focused on the parts of the plant which would be of focus in this project (i.e. the edible parts) would be used for determining their phenolic content, these being the aerial parts of dill, nettle, kale and sideritis, persimmon fruit and pomegranate arils. The phenolic composition of the six plant foods are given in **Tables 2.12** to **2.21**. The food plants were also analysed by Shikha Saha (IFR, UK), using a high-performance liquid chromatography–diode array detection–mass selective detection system. Compounds similar to those presented in the following tables were detected, and the data from these analyses has been published elsewhere, by Hollands et al. (2013b).

Table 2.12: Phenolic compounds identified in dill by previous studies.

Dill phenolic compounds		Refs.
Quercetin	-3-O-glucuronide	(Justesen 2000)
	-3-O-rutinoside	
	-3-O-galactoside	(Teuber &
	-3-O-glucoside	Herrmann 1978)
Isorhamnetin	-3-O-glucuronide	(Justesen 2000)
	-3-O-rutinoside	
	-3-O-galactoside	(Teuber &
	-3-O-glucoside	Herrmann 1978)
Kaempferol	-3-O-glucuronide	(Justesen 2000)

Table 2.13: Range of concentrations of phenolic compounds found in dill, and the number of samples used to calculate values.

Dill phenolic compounds	mg/100 g fresh weight	Refs. and no. of samples (n)
Quercetin aglycone	48 - 110	(Justesen & Knuthsen 2001)
Kaempferol aglycone	16 - 24	(n=2)
Isorhamnetin aglycone	15 - 72	

Table 2.14: Phenolic compounds identified in either nettle leaf (**L**), stem (**S**) or both, by previous studies, the their range of concentrations found in plant material.

Nettle phenolic compounds	Leaf or stem	mg /100 g fresh weight	Refs.
Quercetin-3-O-glucoside	L & S	8.5 - 60	(Grevsen et al. 2008;
Quercetin-3-O-rutinoside	L & S	34 - 271	Pinelli et al. 2008)
Isorhamnetin 3-O-rutinoside	L & S	5 - 61	
Kaempferol 3-O-rutinoside	L & S	1.7 - 12	
Caffeic acid derivative	L & S	27 - 43	(Pinelli et al. 2008; Chrubasik et al. 2007)
p-Coumaric acid	L & S	3.8 - 11.8	(Pinelli et al. 2008)
5-O-feruloylquinic acid	-	-	(Grevsen et al. 2008)
5-O-caffeoylquinic acid			(Grevsen et al. 2008;
3-O-caffeoylquinic acid	S	6.6 - 15	Pinelli et al. 2008; Chrubasik et al. 2007)
2-O-caffeoylmalic acid	L	139 - 334	(Pinelli et al. 2008; Grevsen et al. 2008)
Chlorogenic acid	L & S	66 - 216	(Pinelli et al. 2008; Chrubasik et al. 2007)
Peonidin-3-O-rutinoside	S	7 - 34	(Pinelli et al. 2008)
Peonidin-3-O-(6″-O-*p*-coumaroyl-glucoside)	S	0 - 3.4	
Rosinidin-3-O-rutinoside	S	11 - 68	
Scopoletin	-	-	(Chrubasik et al. 2007)

Table 2.15: Phenolic compounds identified in persimmon by previous studies, their range of concentrations and the number of cultivars/samples used to calculate values.

Persimmon phenolics compounds	mg/100 g fresh weight	Refs. and no. of cultivars and samples (n)
(-)-Epicatechin	0.5 - 1.49	(Gorinstein et al. 2001) (n=8)
(+)-Catechin	0.4 - 3.3	(Veberic et al. 2010; cultivars=11, n=15)
(+)-Gallocatechin	0.17	(Neveu et al. 2010; Akagi et al. 2009; cultivars=5, n=3; Suzuki et al. 2005; cultivars=5, n=3)
Procyanidin dimer B1	0.13	
Procyanidin dimer B3	0.01	
Procyanidin trimer E→E→C	0.04	
Prodelphinidin dimer B3	0.30	
Quercetin-3-O-rutinoside	Trace	(Veberic et al. 2010; cultivars=11, n=15)
Quercetin-3-O-glucoside		
Quercetin-3-O-galactoside		
Caffeic acid	Trace	
Gallic acid	1.8 - 221	(Veberic et al. 2010; cultivars=11, n=15; Gorinstein et al. 2001; n=8)
Ferulic	9.3 - 11	(Gorinstein et al. 2001; n=8)
Protocatechuic	5.7 - 6.9	
Vanillic acid	0.50	
p-Coumaric acid	56 - 67	
Number of other procyanidin/ prodelphinidin oligomers with E, ECG, EGC and EGCG extender units, and catechin, EGCG and myricetin terminal units.	-	(Li et al. 2010)

Abbreviations: E - epicatechin, C - catechin, ECG - epicatechin-gallate, EGC - epigallocatechin, EGCG - epigallocatechin-gallate.

Table 2.16: Range of concentrations of phenolic compounds found in kale, and the number of cultivars/samples used to calculate values.

Kale phenolic compounds	Cultivar	mg/100 g fresh weight	Refs. and no. of samples (n)
Total phenolics	Reflex	305-478 (GAE)	(Olsen et al. 2009; n=5)
Total flavonols	Reflex	491 - 831 (RE)	
Total hydroxycinnamic acids	Reflex	148 - 250 (RE)	
Kaempferol (as aglycone)	Reflex	52 - 61	
Quercetin (as aglycone)	Reflex	34 - 56	
Kaempferol (as aglycone)	Vates	24 - 35	(Zhang et al. 2003; n=2)
Quercetin (as aglycone)	Vates	7.7 - 24	

Abbreviations: GAE - gallic acid equivalent, RE - rutin equivalent

Table 2.17: Phenolic compounds identified in kale by previous studies.

		Kale phenolic compounds	Refs.
Quercetin	Non-Acetylated	-3-O-(Glc/Gnb/Sph/triGlc) -3-O-Sph,-7-O-(Glc/diGlc) -3-O-Glc,-7-O-Glc -3-O-Glc,-7-O-Glc,-4'-Glc	(Lin & Harnly 2009; Velasco & Francisco 2011; Schmidt et al. 2010; Olsen et al. 2009)
	Acetylated	-3-O-(Fer/Sin/Caff/hFer)-Sph -3-O-(Fer/Sin/Caff/hFer/Coum)-Sph,-7-O-Glc -3-O-Sph,-7-O-(Sin/Fer)-diGlc -3-O-(diSin/Sin-Fer)-triGlc,-7-O-Glc -3-O-Sin-Fer-triGlc,-7-O-diGlc -3-O-Sin-triGlc,-7-O-Sin-diGlc	
Kaempferol	Non-Acetylated	-3-O-(Glc/Gnb/Sph/triGlc) -3-O-Sph,-7-O-(Glc/diGlc) -3-O-(Glc/triGlc),-7-O-Glc -3-O-Glc,-7-O-Glc,-4'-Glc	
	Acetylated	-3-O-(Fer/Sin/Caff/ hFer/Coum)-Soph -3-O-(Fer/Sin/Caff/ hFer/Coum)-Sph,-7-O-Glc -3-O-(Fer/Sin/Caff/ hFer)-Sph,-7-O-diGlc -3-O-Sin-triGlc,-7-O-diGlc -3-O-(diSin/Sin-Fer)-triGlc,-7-O-Glc -3-O-(diSin/Fer-Sin/diFer)-triGlc,7-O-diGlc	
Isorhamnetin	Non-Acetylated	-3-O-(Glc/Gnb/Sph,triGlc) -3-O-Gnb,-7-O-diGlc -3-O-(Sph/Glc/Gnb),-7-O-Glc -3-O-Glc,-7-O-Glc,-4'-Glc	
	Acet-ylated	-3-O-Sin-Sph,-7-O-Glc -3-O-Fer-Sph,-7-O-diGlc -3-O-diSin-triGlc,-7-O-diGlc	
	Hydroxycinnamic Acids	3-(Caff/Coum)/-quinic acid 4-(Caff/Fer)-quinic acid Sinapic acid Sinapylglucoside 1-Sin-,2-(Sin/Fer)-Gnb 1-Sin-,2-(Sin/Fer)-,2'-Sin-Gnb Fer-(diSin/Sin)-triGlc	(Lin and Harnly 2009;Velasco et al. 2011; Olsen et al. 2009)

Abbreviations: Glc - glucoside; Gnb - gentiobioside; Sph - sophoroside; Fer - feruloyl; Sin - sinapoyl; Caff - caffeoyl; hFer - hydroxyferuloyl; Coum - *p*-coumaroyl. There is some discrepancy between glycoside names in the literature (i.e. diglucoside instead of sophoroside or gentiobioside, and triglucoside instead of sophorotrioside (Olsen et al. 2009), so while these glycosides are listed separately, they may in fact be the same compound.

Table 2.18: Phenolic compounds identified in *Sideritis scardica* by previous studies.

	***Sideritis scardica* phenolic compounds**		**Refs.**
Flavones	Isoscutellarein	-7-O-Alo(1→2)Glc -7-O-[6'''-O-Ac]-Alo(1→2)Glc -7-O-[2''',6'''-di-O-Ac]-Alo(1→2)Glc -7-O-[6'''-O-Ac]-Alo (1→2)-[6''-O-Ac]-Glc	(Petreska et al. 2011)
	4'-O-Methylisoscutellarein	-7-O-Alo(1→2)Glc -7-O-[6'''-O-Ac]-Alo(1→2)Glc -7-O-[6'''-O-Ac]-Alo(1→2)-[6''-O-Ac]-Glc -7-O-Alo(1→2)-[6''-O-Ac]-Glc	(Gabrieli et al. 2005; Petreska et al. 2011)
	Hypolaetin	-7-O-Alo(1→2)Glc -7-O-[6'''-O-Ac]-Alo(1→2)Glc -7-O-[2''',6'''-di-O-Ac]-Alo(1→2)Glc	(Petreska et al. 2011)
	3'-O-Methylhypolaetin	-7-O-Alo(1→2)Glc -7-O-[6'''-O-Ac]-Alo(1→2)Glc -7-O-[2''',6'''-di-O-Ac]-Alo(1→2)Glc -7-O-Alo(1→2)-[6''-O-Ac]-Glc -7-O-[6'''-O-Ac]-Alo(1→2)-[6''-O-Ac]-Glc	(Koleva 2007; Koleva et al. 2003)
	Chryseriol		(Janeska et al. 2007)
	4'-O-Methyl-luteolin	-7-O-[6''''-O-Ac]-Alo(1→2)Glc	(Koleva 2007)
	Apigenin	-7-O-Alo(1→2)-[6''-O-Ac]-Glc	
	Phenylethanoids	3''-O-*p*-coumaroyl-6''O-Ac-melittoside Melittoside Echinacoside Forsythoside A Verbascoside Alyssonoside Leucoseptoside A	(Petreska et al. 2011)
	Hydroxycinnamic acids	3-Caffeoylquinic acid 5-Caffeoylquinic acid	(Koleva 2007)

Abbreviations: Alo - allosyl, Glc - glucoside, Ac - acetyl.

Table 2.19: Range of concentrations of phenolic compounds found in *Sideritis scardica*, and the number of samples used to calculate values

***S. scardica* phenolic compounds**	**% dry weight**	**Refs. and no. of samples (n)**
Isoscutellarein aglycone	0.08 - 0.19	(Janeska et al. 2007; n=3)
3'-O-methylhypolaetin aglycone	0.07 - 0.20	
Apigenin aglycone	0.05 - 0.12	
Chryseriol aglycone	Trace	

Table 2.20: Phenolic compounds identified in pomegranate arils by previous studies.

Pomegranate phenolic compounds				Refs.
Anthocyanins	Delphinidin		-3-O-Glc -3,5-O-diGlc	(Alighourchi et al. 2008; Borges et al. 2010; Robert et al. 2010)
	Cyanidin		-3-O-Glc -3,5-O-diGlc	
	Pelargonidin		-3-O-Glc -3,5-O-diGlc	
Anthocyanin-flavan-3-ol Adducts	EGC EC EA	-delphinidin	-3-O-Hex -3,5-O-diHex	(Sentandreu et al. 2010)
	EGC EC EA	-cyanidin	-3-O-Hex -3,5-O-diHex	
	EGC EC EA	-pelargonidin	-3-O-Hex -3,5-O-diHex	
		Flavan-3-ols	Catechin Epicatechin B1 Dimer B3 Dimer Gallocatechin Epigallocatechin	(de Pascual-Teresa et al. 2000)
		Ellagitannins	Punicalagin A Punicalagin B Punicalin A Punicalin B Granatin A Granatin B Ellagic acid 2-O-galloylpunicalagin Ellagic acid-O-hexoside	(Gil et al. 2000; Borges et al. 2010)

Abbreviations: Glc - glucoside, Hex - hexoside, EGC - Epigallocatechin, EC - Epicatechin, EA - Epi-afzelechin

Table 2.21: Range of concentrations of phenolic compounds found in pomegranate, and the number of cultivars/samples used to calculate values.

Pomegranate phenolic compounds		mg/100 ml	Refs. and no. of cultivars and samples (n)
Cyanidin	-3-O-Glc	0.2 - 36	(Alighourchi et al. 2008; cultivars=15, n=3)
	-3,5-O-diGlc	4.4 - 236	
Delphinidin	-3-O-Glc	0.2 - 10	(Mousavinejad et al. 2009; cultivars=8, n=3)
	-3,5-O-diGlc	0.3 - 530	
Pelargonidin	-3-O-Glc	0.1 - 24	
	-3,5-O-diGlc	0.01 - 0.1	
Ellagic acid		0.7 - 16	(Mousavinejad et al. 2009; cultivars=8, n=3)
Total Anthocyanins		30 - 252	(Alighourchi et al. 2008; cultivars=15, n=3)
		81.5 - 776[b]	(Mousavinejad et al. 2009; cultivars=8, n=3)
		16.8 - 133[b]	(Sepulveda et al. 2010; cultivars=8, n=10)
		55[c]	(Robert et al. 2010)
Total Phenolics		420 - 930[a]	(Mousavinejad et al. 2009; cultivars=8, n=3)
		68 - 124[d]	(Sepulveda et al. 2010; cultivars=8, n=10)
		172[d]	(Robert et al. 2010)

[a] Measured by Folin-Ciocalteu assay; [b] Measured by HPLC as cyanidin-3-O-glucose equivalent; [c] Measured by HPLC as malvidin-3-O-glucose equivalent; [d] Measured by HPLC as gallic acid equivalent. Additional details of phenolic concentrations are given in (Borges et al. 2010), but were quantified based on the extract volume (μmol/L) rather than the quantity of plant material, and so were not included.

2.2. General biochemical techniques for analysis of plant bioactivity

The following techniques were used throughout this work, for culture and analysis of endothelial cells. All chemicals were purchased from Sigma-Aldrich unless otherwise specified and water was of MilliQ-grade purity. DMSO was stored under nitrogen to prevent contamination from moisture in the air. All cells and growth media/supplements were purchased from Lonza.

2.2.1. General cell culture techniques

Human umbilical vein endothelial cells (HUVECs) were chosen as the model for *in vitro* experiments in this work. Such cells are commonly used in studies which focus on signalling mechanisms in the endothelium, and as a means for understanding the molecular interactions behind functional outcomes seen in tissue culture studies. While most veins carry deoxygenated blood through the body back to the heart, the umbilical vein differs in that it carries oxygenated blood from the placenta to the foetus' heart. Therefore, there is a similarity between the function of arteries and the umbilical vein. However, the layer of smooth muscle surrounding the umbilical vein endothelium is much thinner that would be normally found around the endothelium of arteries, and so it's ability to contract and dilate is likely to be weaker. Still, such cells are relatively easy to obtain, as well as to characterise through endothelial cell specific proteins, such as von Willebrand factor, PECAM-1 (CD31) and endoglin (CD105), and provide a good model for endothelial cells in a non-diseased state. They also represent a more affordable model for signalling in the endothelium, in comparison to other sources for endothelial cells, such as the aorta, pulmonary artery, or micro-vessels of lung or dermal tissue.

HUVECs were supplied as frozen primaries (Clonetics, Lonza) and cultured at 37 °C to passage 4 (10-11 population doublings) in fully supplemented endothelial growth media (EGM-2) (basal media (EBM-2) plus EGM-2 BulletKit™ supplements) before use

in experiments, which were performed also at 37 °C. Prior to all treatments, cultures were washed twice with warm EBM-2, supplemented with BulletKit™ antibiotics. Post-treatment, cells were washed twice with cold PBS (Gibco, Life Technologies), the remaining liquid was aspirated, and ice-cold cell lysis buffer (1X concentration, Cell Signalling Technologies (CST) #9803) was applied to cells. The lysis buffer contained (at 1X concentration): 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/ml leupeptin. It was supplemented with protease inhibitor (1X concentration, Complete Mini, Roche), and, if target analyte(s) included phosphorylated proteins, with phosphatase inhibitor (1X concentration, PhosStop, Roche). 1 mM phenylmethylsulfonyl fluoride was added to the lysis buffer shortly before use. Plates were held at 4 °C for 2 minutes before harvesting the cells by scraping. Lysates were aspirated to eppendorfs, vortexed briefly, and kept on ice. Eppendorfs were vortexed a further 3 times every 5 minutes, keeping on ice in between vortexes, and centrifuged (4 °C, 10 minutes, 17000 g). Lysates were aliquoted as necessary and stored at -80 °C until further analysis.

2.2.2. BCA assay of total protein

The bicinchoninic acid (BCA) assay provides a fast means for determining protein concentration, in a generally non-specific fashion. Protein structure, the number of peptide bonds, and the proportion of cysteine, tyrosine and tryptophan in the sample can all affect the colorimetric reaction of the BCA assay, but it is generally assumed that the influence of these factors will be minor

A stock of 2 mg/ml bovine serum albumin (BSA) was used to prepare a standard curve from which to measure protein concentrations in the lysates of each cell culture dish/plate well. NaPi buffer (43.85 mM NaH_2PO_4, 6.15 mM Na_2HPO_4, 5 mM EDTA)

was used to dilute lysate samples and the BSA standard, and was used as the 0 mg/ml standard. Lysate aliquots were diluted 1:20 and 25 µl of each standard/sample was pipetted into a 96-well plate in duplicate. 200 µl of a 50:1 mix of bicinchoninic acid and copper (II) sulphate pentahydrate was then added to each well, and the plate was incubated at 37 °C for 30 minutes. The absorbance of each well was read using a Dynex MRX II spectrophotometer set at 550 nm, and the protein concentration of each lysate was calculated.

2.2.3. Western blotting

For semi-quantitative analysis of specific proteins in HUVEC lysate samples, western blotting was chosen as a cost-effective protein assay, were single samples could be sequentially assayed for multiple proteins, or for protein post-translation modification.

30 µg samples of total cell protein were separated on SDS-PAGE gels (Expedeon; 12-well SDS-PAGE gels (8% or 4-12%), RunBlue Rapid SDS buffer, DTT and LDS sample buffer), according to the manufacturer's instructions. Separated proteins were transferred to nitrocellulose membranes (Bio-Rad) using X-Cell II gel tanks (Bio-Rad). Western blot transfer buffer (192 mM glycine, 25 mM Tris base, 0.1% (v/v) SDS) and TBST buffer (20 mM Tris base, 137 mM NaCl, 0.1% (v/v) Tween-20, pH adjusted to 7.6 with HCl) were prepared in house. Blots were washed with TBST (5 mins) after transfer and after the blocking, primary antibody and secondary antibody incubation steps. HRP-conjugated anti-rabbit IgG antibody (CST, #7074), diluted 1:1000 in 5% w/v skimmed milk powder/TBST, was used as the secondary antibody. Blots were incubated with blocking buffer (5% w/v milk powder/TBST) for 60 mins, then overnight at 4 °C with primary antibodies, and finally with secondary antibody at room temperature for 60 mins. Blots were incubated with reagents (2 ml each) from the SuperSignal West Pico chemiluminescence detection kit (Pierce) for 5 mins at room temperature, protected from

light. Blots were imaged using a Bio-Rad FluorS Imager. For staining with additional primary antibodies, blots were incubated with Restore Western Blot Stripping buffer (Pierce) for 40 mins at room temperature, washed for 5 mins with TBST, and then re-blocked and probed as before.

Densitometry analysis of western blots was performed with the software ImageJ (Abràmoff et al. 2004), using the 'Analyze/Gels' menu to produce curves representative of blot bands, and taking the area under the curve as the value represented by each band.

Chapter 3

A broad-targeted approach to determine the effects of quercetin on early endothelial signalling events

Chapter 3: A broad-targeted approach to determine effects of quercetin on early endothelial signalling events

3.1. Abstract

There is a large body of evidence from studies including epidemiological and human intervention trials that quercetin, a flavonol found in the plant foods selected in Chapter 2 and many others world wide, may be beneficial to human health, particularly in the context of the cardiovascular system. The early molecular signalling events that underly these changes to vascular health, however, are not well understood. It would be of interest to study changes to endothelial cell signalling induced by quercetin and its human metabolites, as well as to observe if conjugation of quercetin affects such bioactivity.

Confluent HUVECs were treated with quercetin aglycone (10 μM), a mixture of quercetin human metabolites (total concentration 10 μM) or vehicle for 60 min (n=3, biological replicates). Subsequently, cell lysates were analysed with microarray slides spotted with antibodies representive of 736 different protein phosphorylation sites.

A small number of protein phosphorylation changes (17, quercetin metabolites vs control; 29, quercetin vs control) were significant ($p < 0.05$), but of these, only eNOS phosphorylation at Ser1177 could be confirmed by western blotting. There were no significant results after taking multiple testing into account ($q < 0.05$). It is unlikely, therefore, that the quercetin or a mix of its metabolites had a strong effect on early phosphorylation events in resting endothelial cells, despite the decrease to eNOS phosphorylation. This is the largest reported antibody microarray study to date, the first antibody microarray study to assess HUVEC proteins in phosphorylated and unphosphorylated states, and the first antibody microarray study to assess the intracellular response to an individual polyphenol and its human metabolites.

3.2. Introduction

A number of food plants were selected, as detailed in the previous chapter, for study of their ability to improve markers related to vascular health, in cultured endothelial cells. Of these, dill, nettle and kale have been previously reported to contain relatively high levels of the flavonoid quercetin. Since quercetin is a major dietary plant polyphenol for which there is reasonable evidence of health benefits in humans, it was selected as a relevant bioactive flavonoid for further study.

3.2.1. Quercetin in the diet and its relationship with health

In 1785, Edward Bancroft patented the production of an extract of black oak bark which, being yellow in colour, he called quercitron (from the Latin *quercus citrina*) (Bancroft 1813). In 1854, it was discovered that the extract's main constituent, quercitrin, could be split by treatment with mineral acid to release rhamnose. The aglycone produced from this reaction became known as quercetin (Ott 1873). As flavonols are widespread in the plant kingdom, from bryophytes to higher vascular plants (Iwashina 2000), it is not surprising that quercetin is present in a large number of the food plants consumed in human diets (Larson et al. 2012b). It is found as the aglycone (i.e. without sugar groups attached) (**Fig. 3.1)**, as *O*-methylated derivatives (i.e. isorhamnetin (IR) and tamarixetin) and with glycosidic conjugation, often at the highly reactive 3-hydroxyl group of the flavonol structure (Rice-Evans et al. 1996). In nature, quercetin is almost exclusively found as glycosides. The aglycone quercetin is usually found in a limited number of processed foods such as red wine (Hollman & Arts 2000).

Quercetin has received much interest in diet and health research as not only is it consumed in foods worldwide, but there is mounting evidence for its beneficial health effects in roles such as chemoprevention (Gibellini et al. 2011), improvement to drug

resistance (Chen et al. 2010), improvement in exercise endurance (Kressler et al. 2011) and prevention of cardiovascular disease (Perez-Vizcaino et al. 2006; Russo et al. 2012).

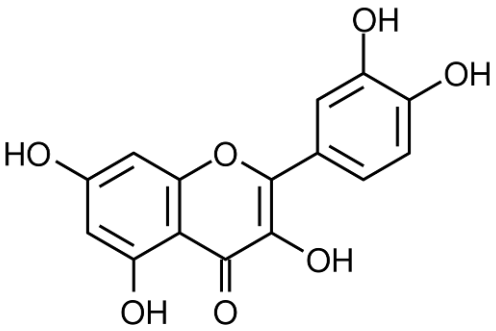

Figure 3.1: Quercetin aglycone.

Concerning the latter, there are a number of reports showing that quercetin can reduce blood pressure, as evidenced by intervention studies where volunteers with hypertension or pre-hypertensive symptoms were given large doses of quercetin (Larson et al. 2012b; Larson et al. 2010). Intervention studies lasting > 8 weeks have found evidence that quercetin can reduce CVD risk factors in cigarette smokers (Lee et al. 2011), and, when coupled with vitamin C, can reduce markers of oxidative stress and inflammation in fit and healthy individuals (Askari et al. 2012). It has also been reported that a dose of 200 mg of quercetin can cause acute increases in plasma *S*-nitrosothiols and nitrites and urinary nitrates in healthy volunteers, which is indicative of increased production of the vasodilator nitric oxide (NO). The same study also reported quercetin-induced decreases in urinary and plasma levels of the vasoconstrictor endothelin-1 (Loke et al. 2008a). Whether or not quercetin-mediated reductions in blood pressure are products of the improvements in NO bioavailability and endothelial function is not yet proven, although there is one report which describes quercetin-mediated decreases in blood pressure that are independent of increased vasodilatory function (Larson et al. 2012a). A study using healthy volunteers also showed decreased platelet aggregation after acute supplementation with quercetin (Hubbard et al. 2006; Hubbard et al. 2004).

In animal models of hypertension, several studies have shown reductions in blood pressure and other factors related to cardiovascular disease (Perez-Vizcaino et al. 2009; Larson et al. 2012b). For instance, hypertensive rats fed daily with a 10 mg/kg dose of quercetin saw a reduction in their blood pressure and augmented effects of vasodilator compounds improvement in isolated aortic rings in an endothelium-dependent manner (Garcia-Saura et al. 2005; Sanchez et al. 2006). Treatment with quercetin also attenuated effects of vasoconstrictor compounds in aortic rings, with a reduced effect if an inhibitor of endothelial nitric oxide synthase (eNOS) was included (Nishida & Satoh 2009; Duarte et al. 1993), or an increased effect if a cofactor of eNOS, such as tetrahydrobiopterin (BH_4), was included (Romero et al. 2009).

Quercetin-induced improvement of hypertension and endothelial function is unlikely to be as a result of a direct antioxidant effect (i.e. radical scavenging *in vivo*) (Loke et al. 2008b; Godycki-Cwirko et al. 2010; Hollman & Cassidy 2011; Askari et al. 2012), although it is possible that changes to antioxidant enzymes, such as glutathione S-transferases (GST) and glutathione peroxidases (GPX), or pro-oxidant enzymes such as NADPH oxidase may be involved. Quercetin treatment was shown to reduce superoxide (O_2^-) produced by NADPH oxidase, as well as expression of its subunit p47phox, to basal levels *in vivo* (Sanchez et al. 2006) and *ex vivo* (Sanchez et al. 2007). Furthermore, quercetin was shown to compete successfully against NO in scavenging O_2^-, allowing levels of the vasodilator to be maintained (Lopez-Lopez et al. 2004).

Further evidence of quercetin's ability to modulate markers of inflammation and endothelial function has been shown through cell culture studies (Chirumbolo 2010; Russo et al. 2012). Additional anti-atherogenic effects observed after quercetin treatment include reduced endothelial cell proliferation and migration (Donnini et al. 2006, Kuhlmann et al. 2005), downregulated components of the apoptotic pathway activated by oxidised-LDL (p38 MAPK, STAT3 and JAK2) (Choi et al. 2007), and upregulation of the apoptosis of intimal-type vascular smooth muscle cell (Perez-Vizcaino et al. 2006).

Furthermore, expression and release of the vasoconstriction factor endothelin-1 has been found to decrease after quercetin treatment, from both HUVECs and activated (aggregating) platelets (Nicholson et al. 2008; Xue-Ying 1996). Quercetin has also been shown to increase levels of cGMP, in a manner involving hyperpolarisation of endothelial cell membrane potential (Kuhlmann et al. 2005).

A significant limitation to much of the *in vitro* work and tissue studies performed with quercetin to date is that studies have focused on the aglycone alone, and have not included any of the metabolites that are found after consumption of quercetin-rich meals, given noted that there is little evidence that quercetin circulates in plasma as the aglycone form. Three of the main phase II metabolites that can be found circulating in plasma are quercetin-3-glucuronide (Q3GlcA), 3'-methyl-quercetin-3-glucuronide (IR3GlcA) and quercetin-3'-sulphate (Q3'S) (Mullen et al. 2006) (**Fig. 3.2**).

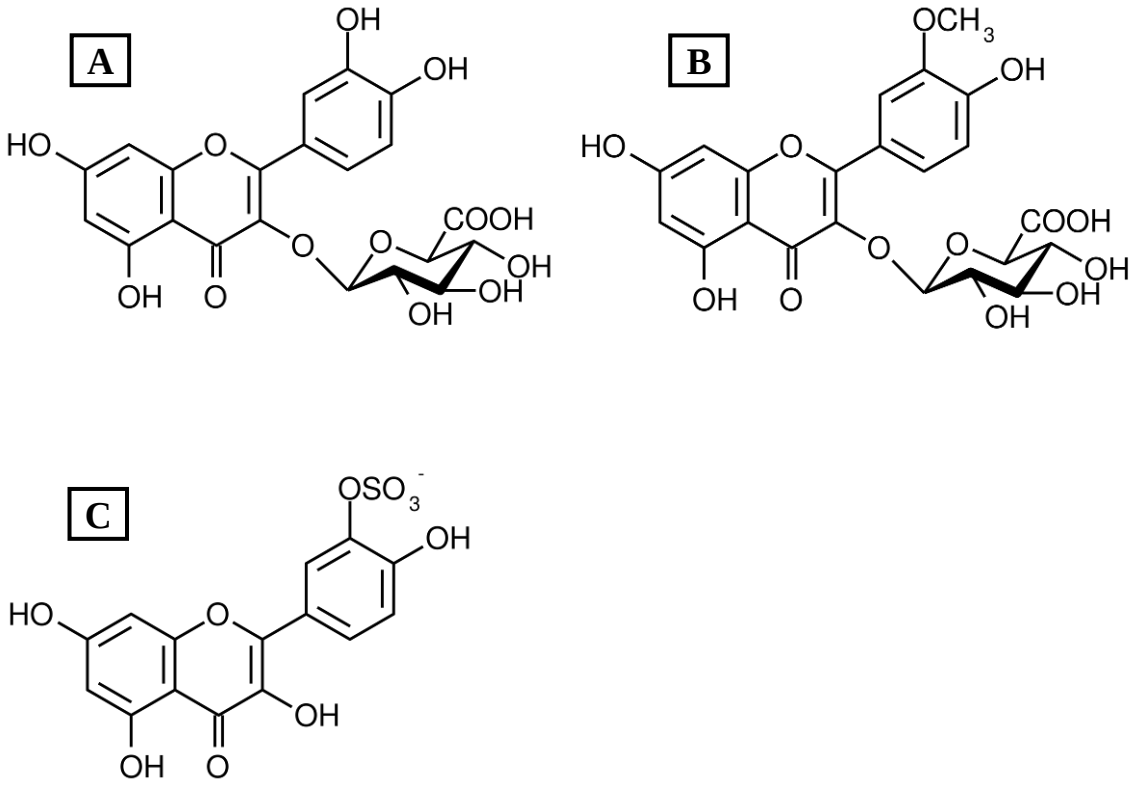

Figure 3.2: Phase II metabolites of quercetin - quercetin-3-glucuronide (**A**), 3'-methyl-quercetin-3-glucuronide (**B**) and quercetin-3'-sulphate (**C**).

Peak concentrations of quercetin metabolites (often measured as the quercetin aglycone, following deconjugation techniques) in plasma can vary widely, ranging from nanomolar quantities to as high as 9.7 μM (Heinz et al. 2010b; Hubbard et al. 2004; Lee et al. 2012).

The bioactivity of quercetin is likely to be affected by phase-2 conjugation. For example, glucuronidation of quercetin reduced its ability to protect dermal fibroblasts from peroxide-induced damage (Spencer et al. 2003) and attenuated pro-oxidant effects of quercetin aglycone, such as nitric oxide scavenging (Lodi et al. 2008). Still, quercetin glucuronides and sulphates (0.3 μM dose) have been shown to reverse apoptosis in hyperglycaemic HUVECs (Chao et al. 2009), inhibit VEGF-induced endothelial functions such as migration and proliferation (> 1 μM dose) (Donnini et al. 2006) and reduce expression of the cell adhesion molecule VCAM-1 (2 μM dose) (Tribolo et al. 2008). Metabolite concentrations from 0.1 to 4 μM attenuated human lymphocyte synthesis of COX-2 mRNA (important for vascular tone) *ex vivo* (though not *in vivo* after consumption of a quercetin rich meal, despite concentrations of the metabolites reaching 4 μM in plasma) (de Pascual-Teresa et al. 2004). 10 μM Q3GlcA was shown to affect the signalling controlling smooth muscle cell migration in rat aortic tissue (Ishizawa et al. 2009), while Q3'S caused increased cGMP levels in porcine coronary arteries, in an endothelium-independent manner, and reduced inflammatory effects of lipopolysaccharide in similar tissues (Suri et al. 2010; Al-Shalmani et al. 2011).

3.2.2. Analysis of vascular cell signalling

The past decade has seen the emergence of technologies that allow for simultaneous analysis of increasingly larger numbers of phosphorylated proteins, utilising mass spectrometry techniques or libraries of binder molecules such as antibodies (Schulze 2010; Kosako & Nagano 2011). The rise in numbers of unique phospho-site specific antibodies has allowed the development of phospho-protein antibody microarrays able to

quantify specific proteins in terms of total levels and phosphorylated levels simultaneously. The ability to target functionally important sites, rather than assess the entire phospho-proteome by mass spectrometry, provides a more convenient means of analysis (Zhang & Pelech 2012; Yang et al. 2011; Borrebaeck & Wingren 2009). "Normal-phase" antibody microarrays involve fixed spots of particular antibodies at known locations on a solid material, such as a glass microscope slide, similar to the format of routine cDNA microarrays, while reverse-phase protein microarrays require the spotting of biological material, followed by antibody incubation (Silvestri et al. 2010). These methods give the potential for tracking large subsets of phospho-proteins equivalent to multiple signalling pathways over the course of one experiment.

The work detailed in this chapter will focus on elucidating changes to early signalling events in endothelial cells after introduction of quercetin, a flavonoid present in a number of the food plants prioritised for study in the previous chapter. Cells will be treated with quercetin aglycone, or a mix of quercetin metabolites alongside a vehicle control. By using antibody microarrays complementary for large a number of proteins (phosphorylated and unphosphorylated), lysates will be analysed to determine what the effects of the quercetin treatments on protein phosphorylation, using a broad-targeted approach to phospho-protein analysis.

3.3. Aims

The overall aim of the research described in this chapter was to identify changes in early signalling events in endothelial cells incubated with quercetin, a flavonoid found in a number of food plants typical of traditional Black Sea area diets. Specifically, the scientific objectives were to:

(i) Measure the effects of quercetin on protein phosphorylation profiles in human vascular endothelial cells.

(ii) Measure the effects of quercetin conjugates on protein phosphorylation profiles in human vascular endothelial cells and determine the impact of human metabolism of quercetin on its bioactivity in this context.

3.4. Methods

3.4.1. Materials

All water used was of MilliQ-grade purity, unless it is stated that deionised water (dH_2O) was used instead. All cells and growth media/supplements were purchased from Lonza. Human umbilical vein endothelial cells (HUVECs) were supplied as frozen primaries (Clonetics, Lonza) and cultured to passage 4 (10-11 population doublings) before use in experiments. Details of the general materials and methods used for the cell culture experiments with HUVECs, including analysis of cell lysates by BCA assay and western blotting, are described in Chapter 2. All purified compounds were stored in single use aliquots at -20 °C. Quercetin, sinigrin, allyl-isothiocyanate (allyl-ITC) (Sigma-Aldrich) and sulforaphane (LKT Laboratories) were stored in DMSO. Quercetin-3'-sulphate (Q3'S), quercetin-3-glucuronide (Q3GlcA) and isorhamnetin-3-glucuronide (IR3GlcA) were prepared by Dr Paul Needs (IFR, UK) according to a published protocol (Needs & Kroon 2006) and stored in water. Tris-buffered saline solution (TBS) was prepared with tris base (20 mM) and NaCl (137 mM), adjusted to pH 7.6 using HCl. Antibodies for eNOS, p-eNOS (Ser1177), VEGFR2, p-VEGFR2 (Tyr951) (Cell Signalling Technologies (CST) #9572, #9570, #2479 and #4991 respectively), caspase-6 and p-caspase-6 (Ser257) (FMB) were used as primary antibodies in western blotting. Antibody microarrays (Phospho Explorer Antibody Microarray, PEX-100) were purchased from Full Moon Biosystems (FMB), who also provided kits containing the following buffers and reagents for processing the microarrays:

- Blocking buffer, produced from 1.8 g dry milk powder dissolved in 60 ml blocking reagent solution
- Labelling reagent, produced from 1 mg biotin reagent dissolved in 100 µl dimethylformamide

- Coupling buffer, produced from 0.36 g dry milk powder dissolved in 12 ml coupling reagent solution
- Wash buffer, produced from a stock solution diluted 1:10 in water
- Coupling chambers, which were 4 x 5 ml rectangular-well plastic dishes, similar to Nunclon #167063
- Detection buffer, containing 0.5 ng/ml Cy3-streptavidin conjugate (GE Healthcare) dissolved in detection reagent
- Cell lysis buffer and eppendorfs containing metal lysis beads to facilitate break up of cell material
- Stop reagent, for halting the reaction of protein biotin-labelling

The antibody microarray slide featured two columns of 8 blocks consisting of 1326 spots in total, each spot printed using the manufacturer's microarray printing buffer. The spots were featured on each microarray in duplicate across two columns, so that the relative location of the spots featured in the left column matched the same relative location in the right column. The microarrays were spotted with 1318 unique antibodies complimentary to phosphorylated and non-phosphorylated versions of 582 phosphorylation sites covering 320 different proteins, plus 154 additional "unpaired" sites (phosphorylated *or* unphosphorylated) covering 126 different proteins. In total, 386 unique proteins were covered by the microarray. The names, targeted phosphorylation residue(s) and Uniprot accession IDs of each protein covered in this microarray are listed in **Appendix I**. Each column also contained two positive control spots, four negative control spots, four blank "spots" (where no materials had been printed) and two spots complimentary for the house-keeping proteins β-actin and GAPDH. The positive controls contained antibodies labelled with cyanine 3 (Cy3) dye, and so demonstrated that fluorescence scanning was working properly, and highlighted the printing boundaries for the microarrays. The negative controls contained bovine serum albumin (BSA) (concentration n/a) in the

microarray printing buffer. While the signal produced by these spots would likely be a close representation of within-spot background signal, there was no evidence to suggest that the signal produced from unspecific binding to BSA would be the same as the signal produced from unspecific binding to the microarray printing buffer, and so they were not used (for calculating within-spot background signal) as such.

3.4.2. Cell culture experiments, BCA assay and western blotting

General materials and methods used for cell culture experiments with HUVECs, unless specified otherwise, are as described in Chapter 2. These include details for the analysis of their lysates by BCA assay and western blotting.

3.4.3. Incubation of cell cultures with quercetin

Confluent monolayers of HUVECs cultured in 10 cm^2 dishes were incubated for 60 minutes with either 10 μM quercetin, a mix of 6 μM Q3'S, 3 μM Q3GlcA and 1 μM IR3GlcA (ratio based on the relative levels of these compounds reported in plasma by Mullen et al. (2006)), or a vehicle control treatment (n=3, biological replicates). Such doses represent concentrations slightly higher than what might be expected *in vivo* after a high-quercetin meal. They are, however, intended to assist in showing changes to protein phosphorylation, in cases where use of physiological doses of quercetin or its metabolites (low μM) would show small, but not measurably significant, changes. Final concentration of DMSO in cell culture media for each treatment was 0.1% v/v. Post-incubation, cells were washed twice with cold 1X PBS (Gibco, Life Technologies). Any remaining buffer was aspirated and 150 μl of FMB lysis buffer was added to each plate. Cells were detached using scrapers and aspirated into eppendorfs, to which metal lysis beads (FMB) were added, filling to approximately half the lysate volume. Eppendorfs were vortexed for 30-60 s and then held on ice for 10 minutes, repeating this for 40-60 minutes,

followed by centrifugation at 13000 x g. Lysates were divided into small aliquots and stored at -80 °C until analysis.

3.4.4. Analysis of cell lysates with antibody microarrays

Cell lysate aliquots containing 330 µg protein were removed from -80 °C storage and brought to room temperature at the bench top. Three aliquots of 100 µg of protein were taken from each and made up to 50 µl total volume with labelling buffer, before addition of 1.5 µl of labelling reagent to each. Eppendorfs were incubated at room temperature for 120 minutes with shaking at 55 rpm. 25 µl of stop reagent was then added to each aliquot, and incubated for a further 30 minutes with shaking on a Luckham R100 Rotatest orbital shaker (used for all subsequent incubations requiring shaking) at 55 rpm.

Alongside this, antibody microarray slides (details of microarray targets given in **Section 3.4.1**) were removed from 4 °C storage and equilibrated to room temperature before removal from foil packaging. Throughout their use, microarray slides were kept antibody-side up. Slides were placed in 30 ml of blocking buffer in a 10 cm^2 dish. Dishes were covered and incubated at room temperature for 45 minutes with shaking at 55 rpm.

After blocking, dishes were filled with TBS. Slides were then transferred to a new dish containing 30 ml TBS, while being kept as horizontal as possible to reduce draining of liquid, which would lead to drying of milk proteins in the blocking buffer to the slide (communication with Full Moon Biosystems). Dishes were covered and incubated at room temperature for 10 minutes with shaking at 55 rpm. Two more 10 minute washes were then performed with fresh TBS each time. Slides were placed in 50 ml polypropylene centrifuge tubes (Corning) containing 45 ml dH_2O. The lid was screwed on, and the tube was shaken vigorously for 10 seconds. Slides were then transferred to fresh dH_2O and shaken for another 10 seconds. This was repeated for a total of 10 washes, and upon completion the slides were kept in fresh dH_2O. The three aliquots of

biotin labelled cell lysate protein were combined and added to 6 ml of coupling buffer, turning gently end over end to mix. The cell protein/coupling buffer mixture was decanted into a coupling chamber well, and a blocked antibody microarray slide was submerged into it. The coupling chamber was then covered and incubated for 120 minutes at room temperature with shaking at 35 rpm.

Coupling chamber wells containing microarray slides were filled with wash buffer before transferring slides, keeping them as horizontal as possible, to a 10 cm^2 dish filled with 30 ml wash buffer. 3 x 10 minute washes with fresh 1X wash buffer were performed, with shaking at 55 rpm, followed by ten washes in polypropylene tubes with 45 ml dH_2O. Slides were then placed in a 10 cm^2 dish containing 30 ml detection buffer. Dishes were covered and wrapped in foil to protect the detection agent from light, and then incubated at room temperature for 45 minutes with shaking at 55 rpm. Slides were washed with wash buffer followed by dH_2O as described above. After the final wash, a flow of compressed nitrogen at 20 psi was used to remove water remaining on the slide.

3.4.5. Analysis of microarray slides

Antibody microarray slides were scanned using a GenePix 4000B microarray scanner (Molecular Devices), connected to a computer running the software GenePix Pro v6.1 (Molecular Devices). The photo-multiplier tube (PMT) gain was adjusted to reduce the number of saturated pixels to < 1% of total image pixels, and this PMT gain setting was used for all the microarrays scanned. Slides were scanned at a resolution of 5 μm/pixel, using a 532 nm excitation laser coupled with a 557.5-592.5 nm emission filter. Images produced from the fluorescence scanning were saved as .tiff files, and microarray slides were then placed into storage at room temperature, protected from light. Slides were also scanned using a 635 nm excitation laser coupled with a 650-690 nm emission filter. Images produced from scanning at 635 nm scans assessed by eye to identify high levels

of signal generated by artifacts such as scratches, so that these could be taken into account when analysing images produced from scanning at 532 nm.

Analysis of the images produced from scanning at 532 nm was carried out with the assistance of Kate Kemsley (IFR, UK), who developed a program script for the Matlab software environment that identified the outlines of the microarray spots in the images produced from the fluorescence scans, and then calculated the median signal of the fluorescence inside these outlines (referred to here as the foreground signal). The median fluorescence intensity was calculated rather than the mean, as the median would be less affected by large outlier values, such as those produced by saturated pixels resulting from scratches or specks of dust. The script also generated a composite of the original image overlaid with the identified outlines, and a score from 0 to 1 of the accuracy of each spot outline (**Fig. 3.3**). The lowest circularity scores were found to represent spots with incorrectly identified outlines, and the median fluorescence of these spots were calculated manually using GenePix Pro. Any additional incorrectly outlined spots were identified by scanning the composite images by eye. The local background fluorescence surrounding each spot was also calculated, by either GenePix Pro (in the case of manually outlined spots) or the Matlab program script.

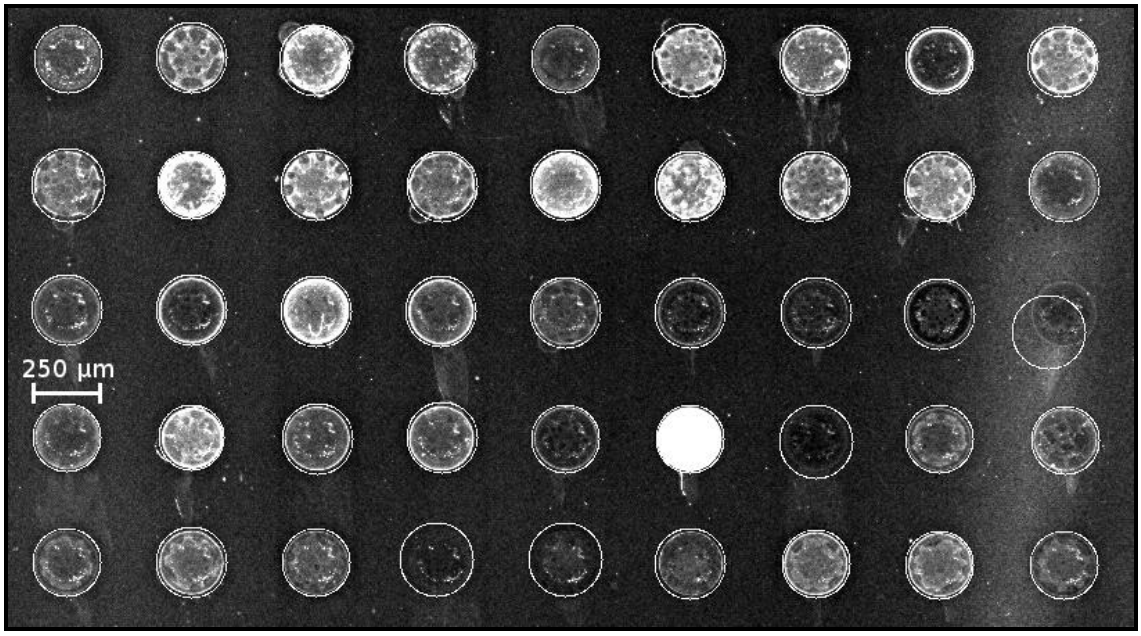

Figure 3.3: Example of fluorescently-scanned microarray, overlaid with circles representing the scored spot boundaries detected by the Matlab script.

Prior to statistical analysis, Microsoft Excel 2007 spreadsheets were used to organise median spot fluorescence intensity (MSFI) values using the following labels:

- *unph* - MFSIs representing an unphosphorylated protein residue.
- *phph* - MSFIs representing a phosphorylated protein residue.
- *nopair* - MSFIs representing a phosphorylated (or unphosphorylated) protein residue, but lacking a 'partner' antibody corresponding to the alternate unphosphorylated (or phosphorylated) protein residue, spotted on the microarray.

Statistical analyses of microarray slide data was carried out using the limma package for the R software environment (Smyth 2005; Smyth 2004; R Development Core Team 2010), in accordance with documentation provided by the package developers. The *phph* to *unph* ratio for corresponding protein residues was calculated, $\log_2$ transformed (to give a Gaussian data distribution), and quantile normalised to adjust for variances due to unspecific binding (Bolstad et al. 2003). An empirical Bayes method, used to shrink standard errors towards a common value, was applied to the data, and p-values were calculated for comparisons of *phph/unph* ratios between each treatment (i.e. quercetin vs

control, quercetin metabolites vs control and quercetin metabolites vs quercetin). Subsequently, p-values were adjusted to account for multiple testing (MT) using the Benjamini-Hochberg method (Benjamini & Hochberg 2009) (p-values adjusted for MT are referred to in this chapter as q-values). To determine significant changes to *nopair* targets, MSFIs of *all* protein targets were first corrected for changes in total protein applied to each microarray, using the average MSFI of the house-keeping protein targets. As above, quantile normalisation and the empirical Bayes method were applied and p- and q-values were calculated for comparisons of *nopair* values between each treatment.

The distribution of foreground and background fluorescence values was also assessed, comparing the spread of values between the left and right hand sides of microarrays, and the spread of values between microarrays processed on the same day.

3.4.6. Bioinformatic analysis of proteins highlighted by the microarray experiment

Bioinformatic analysis of statistically significant changes ($p < 0.05$) to protein phosphorylation was performed using the website DAVID (v6.7) (Huang et al. 2009; Dennis et al. 2003), following instructions given on the website. A list of all the Uniprot accession IDs of the proteins included in the microarray was used as the analysis background list. A separate analysis was performed using this background list of Uniprot IDs as the background *and* experimental list, to highlight the signal transduction pathways represented by the microarray.

3.4.7. Ontological analysis of phosphorylated proteins highlighted by the microarray experiment

Protein ontologies were studied using the websites PhosphoSite (Hornbeck et al. 2004) and Uniprot (The Uniprot Consortium 2012) to determine:

(i) The biological role of the protein ("Protein role")

(ii) The effect of phosphorylation of the particular residue on protein function ("Effect/protein")

(iii) The effect of phosphorylation of the particular residue on cell function ("Effect/cell")

(iv) Upstream proteins that may influence phosphorylation of the particular residue ("Regulated by")

This data was analysed to determine any links between the listed proteins, in the context of associated protein-protein interactions and associated effects on cell function.

3.4.8. Confirmation of changes to selected phospho-proteins by western blotting

Confluent monolayers of HUVECs cultured in 6-well plates were treated with either quercetin, the mix of quercetin metabolites or a vehicle control, in concentrations ranging from 5-50 μM for 60 minutes. Blots were then assayed for changes to the phosphorylation of caspase-6, VEGFR2 or eNOS. For the eNOS assay, cells were pre-treated with epigallocatechin-gallate (EGCG) to stimulate eNOS phosphorylation prior to the above treatments. Etoposide was used as a positive control for the caspase-6 assay and VEGF was used to stimulate VEGFR2 phosphorylation prior to the above treatments. Densitometry values of western blot bands were tested for significance, comparing treatment against the vehicle control, using the Student's *t*-test. Details of the methods used for western blotting are given in **Chapter 2**.

3.5. Results

3.5.1. Pilot antibody microarray experiment

As a pilot experiment, three PEX-100 antibody microarrays were used to analyse lysates from cell cultures treated with quercetin (10 μM), EGCG (10 μM) or a vehicle control (0.1% DMSO), with the slides processed on the same day. All three microarrays suffered from high fluorescence across the slide, affecting both background and foreground values. It was advised to increase the vigour of the water washes after the blocking step, as milk proteins from this step may not have been properly removed in the images from scanned microarray slides (Shannon Zhang [Full Moon Biosystems], personal communication 11 May 2010). This was tested, using two Nuclear/Membrane Receptor antibody microarrays (FMB) to analyse lysates from cell cultures treated with quercetin (10 μM) or a vehicle control. This resulted in images with much lower local background and low variation for inter-microarray target replicates (n=6, no. of unique antibodies = 56). The main study was then undertaken, and the initial problems with high fluorescence were again encountered for the first microarray used. This was resolved after the addition of a TBS wash step after blocking (Shannon Zhang [Full Moon Biosystems], personal communication 11 May 2010). A key factor for the resolution of this issue may be the manner in which the slide was kept horizontal during the transfer between 10 cm^2 dishes (as opposed to the transfer from 10 cm^2 dish to plastic centrifuge tube for the water wash step), though this hypothesis was not fully tested.

3.5.2. Analysis of microarray images

The analysis of the first sample of cell lysate using the antibody microarrays resulted in an image containing very high background signal, adversely affecting the ability to extract meaningful fluorescence values from the spot foreground. As this effect was more prominent on one side of the microarray than the other, the differences between

foreground and background signals of the left and right negative control spots were used to attempt to correct this, according to the following formula, which is explained in detail in **Appendix II**:

$$\mathbf{CSf = Sf – ([(Sb – LNb)/(RNb – LNb)] \times (RNf - LNf)) – LNf + 300}$$

Abbreviations: Corrected Spot foreground (CSf); Spot foreground (Sf); Spot background (Sb); average left negative control spot background (LNb); average right negative control spot background (RNb); average left negative control spot foreground (LNf); average right negative control spot foreground (RNf).

To determine if the data from first microarray was of use, statistical significance testing was performed on the data set by either:

(i) using the data without any correction

(ii) using the data but correcting it as described above, or

(iii) not including the data from the first microarray

Of these three, not including the data from the first microarray resulted in a greater number of significant results ($p < 0.05$) (data not shown) and so this was the method used to produce the final set of results. Significance tests were also performed with and without use of quantile normalisation, do determine if its inclusion increased the number of significant results found ($p < 0.05$), which it did (data not shown). The statistical analysis was also tested by intentionally exaggerating a number of target values to different degrees so that the p- and q-values associated with these targets would be lowered in accordance, which they were (data not shown).

Density histograms of the raw MSFI data revealed a distinct difference between the data distribution of MSFIs from microarrays processed on the same day (treatments were processed so that no two microarrays processed on the same day would represent the same cell culture treatment) (**Fig. 3.4**). This difference was no longer present after

ratios were calculated from the data, as described in **Section 3.4.5** (data not shown). Data distribution of the foreground and background showed a tendency for higher values in the duplicates on left side of the microarrays in both the foreground and background values (**Figs**. **3.5**, only foreground data is shown).

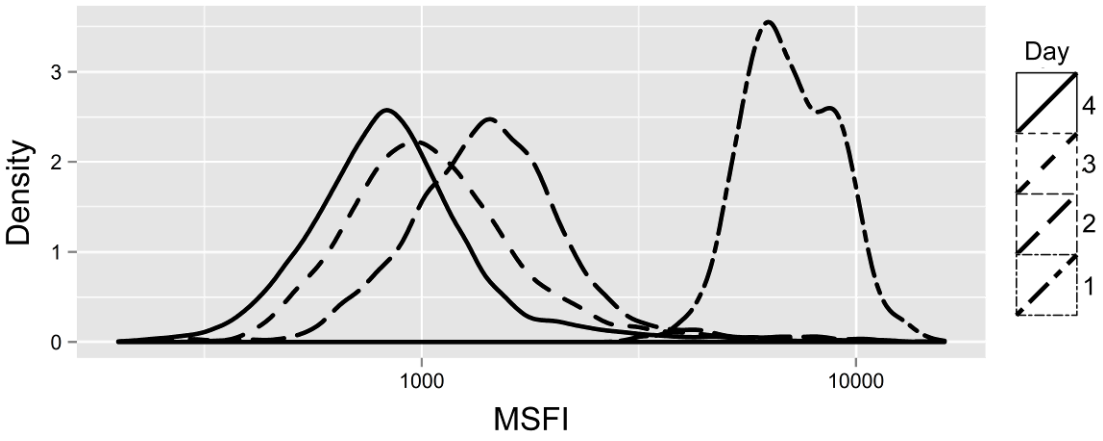

Figure 3.4: Density histogram of MSFI values, from microarrays grouped by the day on which they were performed. n=3 for days 3 and 4, n=2 for day 2 and n=1 for day 1 (the first microarray that was processed).

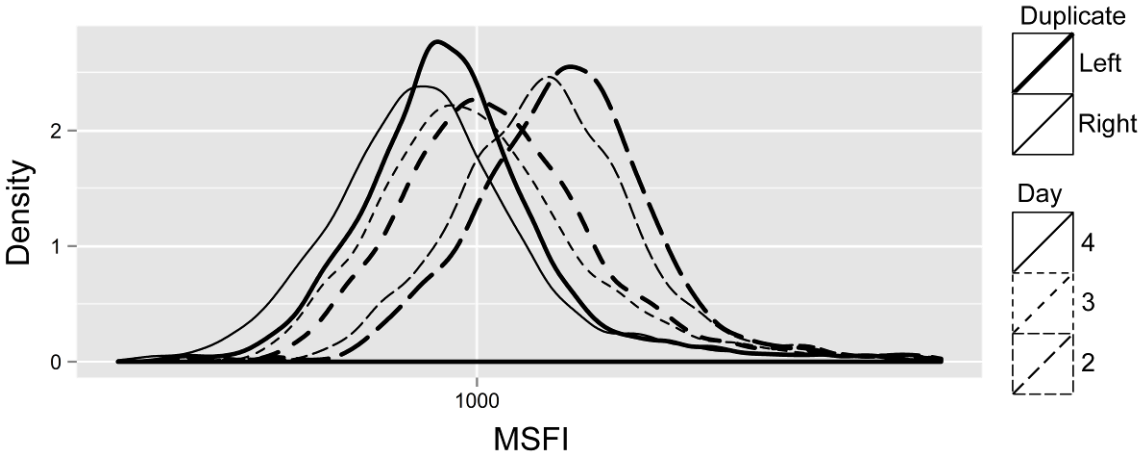

Figure 3.5: Density histogram of MSFI values, from microarrays grouped by the day on which they were performed, and their left or right duplicate values. n=3 for days 3 and 4, n=2 for day 2.

3.5.3. Changes to protein phosphorylation after incubation of HUVECs with quercetin aglycone or quercetin metabolites

Before adjusting for multiple testing, analysis of lysates from HUVECs for significant ($p < 0.05$) changes to protein phosphorylation highlighted 17 protein residues for cells treated with quercetin metabolites (

Table 3.1) and 29 for cells treated with quercetin aglycone (**Table 3.2**) when comparing both treatments to a vehicle control. However, there were no significant changes ($q < 0.05$) after adjusting for multiple testing.

Table 3.1: Quercetin metabolites vs vehicle control. [a] - *nopair* target, represented on the microarray by an anti-phosphoprotein antibody. [b] - *nopair* target, represented on the microarray by an anti-protein (unphosphorylated) antibody.

Phospho-Protein	p-value (unadjusted for MT)	Fold Change
Filamin A (pSer2152)	0.013	0.64
Stathmin 1(pSer37)	0.008	0.74
p90RSK (pThr359/Ser363)	0.011	0.82
Caspase-9 (pSer196)	0.029	0.86
JAK2 (pTyr1007)	0.047	0.87
IkB-α (pSer32/36)	0.044	1.22
PI3K p85-α/γ (pTyr467/199)	0.026	1.28
BLNK (pTyr96)	0.014	1.29
CREB (pSer142)	0.022	1.32
[a]MER/SKY (pTyr749/681)	0.047	1.22
VEGFR2 (pTyr951)	0.031	0.68
eNOS (pSer1177)	0.018	0.77
LCK (pTyr192)	0.039	0.85
CaMK4 (pThr196/200)	0.036	0.87
p130Cas (pTyr165)	0.046	1.17
JAK1 (pTyr1022)	0.019	1.27
GRK1 (pSer21)	0.049	1.28
Casp6 (pSer257)	0.023	1.31
[a]Synuclein-α (pTyr136)	0.022	0.90
[b]Smad2 (pSer255)	0.047	0.81

Table 3.2: Quercetin aglycone vs vehicle control. [a] - *nopair* target, represented on the microarray by an anti-phosphoprotein antibody. [b] - *nopair* target, represented on the microarray by an anti-protein (unphosphorylated) antibody.

Phospho-Protein	p-value (unadjusted for MT)	Fold Change
Ezrin (pTyr353)	0.038	0.60
GluR1 (pSer849)	0.031	0.67
Elk1 (pThr417)	0.016	0.72
Progesterone Receptor (pSer190)	0.028	0.76
p53 (pSer9)	0.044	0.77
ER-α (pSer104)	0.049	0.78
NF-κB-p65 (pSer529)	0.049	0.80
PPAR-r (pSer112)	0.022	0.83
EGFR (pTyr1172)	0.038	0.85
PDGFR-α (pTyr849)	0.047	1.18
Met (pTyr1234)	0.017	1.21
IkB-ε (pSer22)	0.035	1.24
MKK6 (pSer207)	0.046	1.28
BRCA1 (pSer1457)	0.043	1.37
Casp3 (pSer150)	0.019	1.76
[a]Gab2 (pTyr643)	0.010	1.24
[b]Smad2 (Ser255)	0.030	0.76
Synapsin (pSer9)	0.021	0.60
eNOS (pSer1177)	0.005	0.68
STAT1 (pSer727)	0.013	0.72
Smad1 (pSer465)	0.035	0.77
GluR2 (pSer880)	0.028	0.78
Connexin 43 (pSer367)	0.026	0.80
c-Jun (pSer243)	0.022	0.82
eIF2A (pSer51)	0.045	0.84
Chk1 (pSer345)	0.041	0.86
BCL-2 (pThr69)	0.048	1.19
AMPKA1 (pThr174)	0.017	1.22
IKK-β (pTyr188)	0.040	1.28
Casp6 (pSer257)	0.029	1.33
HDAC5 (pSer259)	0.002	1.55
[b]eNOS (Ser1179)	0.035	0.90
[a]VE-Cadherin (pTyr731)	0.013	1.28

3.5.4. Bioinformatic and ontological analysis of phosphorylated proteins highlighted in microarray results

Analysis of the data sets resulting from significance testing (unadjusted for multiple testing) of the microarray data, to determine statistically significant representation of signalling pathways using the website DAVID produced no significant results ($q < 0.05$). The information collected from the websites PhosphoSitePlus and Uniprot are listed in **Tables 3.3** and **3.4**. Associations between proteins as indicated by the ontological study for Q. metabolites vs control are given in **Fig. 3.6**. A number of the phospho-proteins from the quercetin vs vehicle control comparison were identified as being associated with an anti-apoptotic effect (**Fig. 3.7**). Analysis of all the unique Uniprot accession IDs in the list of antibodies featured on the microarray using the website DAVID showed the microarray represented a large number of signal transduction pathways (**Table 3.5**).

Table 3.3: Information regarding the descriptors given in **Section 3.4.7**, detailing the effects of *increased* phosphorylation on proteins from the microarray results (Q. metabolites vs vehicle control).

Protein	Description	Information
Filamin A (pS2152)	Protein role	Cytoskeletal protein, promotes branching of actin filaments, links actin to membrane glycoprotein
p = 0.013	Effect/protein	Protein stabilization
F.C. = 0.64	Effect/cell	Regulates cytoskeletal reorganization
	Regulated by	p90RSK, PAK1, JAK2
CREB (pS142)	Protein role	Induces transcription in response to hormonal stimulation of the cAMP pathway.
p = 0.0383	Effect/protein	Enzyme activity, autophosphorylation, protein degradation, regulates molecular associations
F.C. = 0.85	Effect/cell	Regulates cell growth
	Regulated by	CSFR, EGFR
p90RSK (pT359/S363)	Protein role	Kinase of AGC family, activated in response to hormones, growth factors and neurotransmitters
p = 0.011		
F.C. = 0.82	Regulated by	MKP-7
STMN1 (pS37)	Protein role	Destabilisation of the microtubule filament system by destabilizing microtubules
P = 0.008	Effect/protein	Inhibition, degradation
F.C. = 0.74	Effect/cell	Cell cycle regulation and cytoskeletal reorganization

Table 3.3: Information regarding the descriptors given in **Section 3.4.7**, detailing the effects of *increased* phosphorylation on proteins from the microarray results (Q. metabolites vs vehicle control).

Protein	Description	Information
PI3K p85-α/γ (pY467/199)	Protein role	Regulatory subunit of PI3K. Mediates interactions with a subset of tyrosine-phosphorylated proteins
p = 0.049	Effect/cell	Regulates transcription
F.C. - 0.80	Regulated by	CSFR, EGFR, HS1
eNOS	Protein role	Synthesises nitric oxide
(pS1177)	Effect/protein	Enzyme activity
p = 0.017	Effect/cell	Cytoskeletal reorganization
F.C. = 0.77	Regulated by	Akt1, AMPKA1, PKACa, PKG2
Casp6	Protein role	Activation of caspase cascade, leading to apoptosis
(pS257)	Effect/protein	Inhibition of enzyme activity (Suzuki et al. 2004)
p = 0.0447	Effect/cell	Inhibits apoptosis
F.C. = 0.84	Regulated by	AMPK-related kinase 5
Casp9	Protein role	Activation of caspase cascade, leading to apoptosis
(pS196)	Effect/protein	Inhibition of enzyme activity
p = 0.0288	Regulated by	Akt1
F.C. = 0.86		
$VEGFR_2$	Protein role	Receptor tyrosine kinase, regulates angiogenesis
(pY951)	Effect/protein	Enzyme activity
P = 0.031	Effect/cell	Regulates cell motility
F.C. - 0.68	Regulated by	VEGFR2
LCK (pY192)	Protein role	Tyrosine kinase of the Src family, crucial for antigen-receptor signalling in lymphocytes
p = 0.039		
F.C. = 0.85	Effect/protein	Inhibition, regulates molecular associations
CaMK4	Protein role	Transcriptional activation of CREB protein-mediated signalling pathways
(pT196/200)		
p = 0.036	Effect/protein	Enzyme activity, intracellular translocation, regulates molecular associations
F.C. = 0.87		
	Effect/cell	Regulates transcription
	Regulated by	BMP2, TOB1, TRRAP, BMPR1B
JAK2	Protein role	Tyrosine-kinase involved with cytokine receptor signalling pathways, including IL-3, -5 and GM-CSF
(pY1007)		
p = 0.047	Effect/protein	Regulates molecular association, induce interaction with SOCS1
F.C. = 0.87		
	Regulated by	APS, FGFR1, JAK2, LEPR, SH2-B-beta, SOCS3
p130Cas	Protein role	Docking protein, coordinates tyrosine-kinase-based signalling related to cell adhesion.
(pY165)		
p = 0.028	Regulated by	PXN
F.C. = 0.78		
IkB-alpha	Protein role	Regulatory protein that inhibits NFκB
(pS32/36)	Effect/protein	Enzyme activity, intracellular translocation, autophosphorylation, protein degradation
p = 0.049		
F.C. = 0.78	Effect/cell	Regulates transcription and apoptosis
	Regulated by	PKD2, PLK1, SHIP, IKK-β, IKK-ε, CK2-A1, IKK-α

Table 3.3: Information regarding the descriptors given in **Section 3.4.7**, detailing the effects of *increased* phosphorylation on proteins from the microarray results (Q. metabolites vs vehicle control).

Protein	Description	Information
JAK1 (pY1022)	Protein role	Tyrosine-kinase involved interferon signal transduction pathways
P = 0.026	Effect/protein	Inhibition
F.C. = 0.80	Effect/cell	Regulates cell motility
	Regulated by	Tyk2, JAK3, PTPN2
GRK1 (pS21)	Protein role	Phosphorylates and inactivates rhodopsin
p = 0.022	Effect/protein	Inhibition
F.C. = 0.82	Effect/cell	Regulates transcription
	Regulated by	PKACα
BLNK (pY96)	Protein role	Adaptor protein, regulates B-cell function and development
p = 0.022	Effect/protein	Regulates molecular association
F.C. = 0.83	Effect/cell	Regulates transcription
	Regulated by	Syk

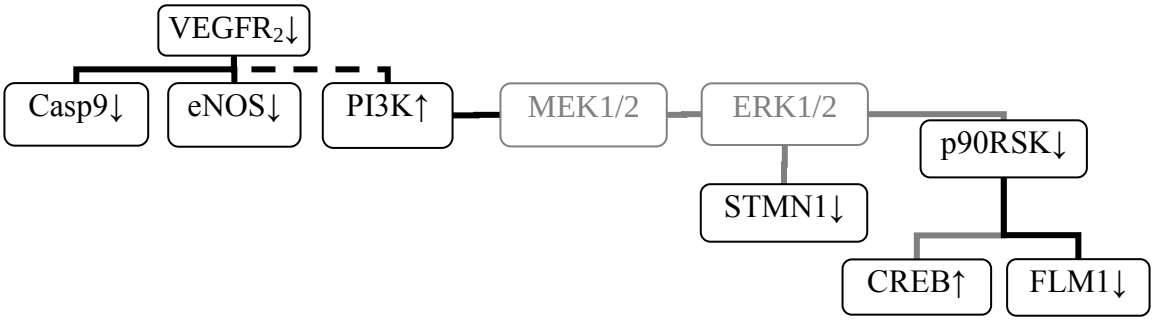

Figure 3.6: Signalling and interactions between phospho-proteins from the microarray results (Q. metabolites vs vehicle control), according to ontological information. Dashed lines indicate interactions between phospho-proteins that are not necessarily supported by the change in phosphorylation recorded by the microarray analysis. Greyed out lines indicated an association between proteins, but not necessarily due to the phosphorylated residue in question. Greyed out proteins indicate that they are not part of the microarray results, but are required to show the signalling pathway.

Table 3.4: Information regarding the descriptors given in **Section 3.4.7**, detailing the effects of *increased* phosphorylation on proteins from the microarray results (quercetin vs vehicle control).

Protein	Description	Information
Casp6	Protein role	Activation of caspase cascade, leading to apoptosis.
(pS257)	Effect/protein	Inhibition of enzyme activity (Suzuki et al. 2004)
p = 0.029	Effect/cell	Inhibits apoptosis
F.C. = 1.33	Regulated by	ARK5 (AMPK-related kinase)
Casp3	Protein role	Activation of caspase cascade, leading to apoptosis.
(pS150)	Effect/protein	Enzymatic inhibition
p = 0.019	Effect/cell	Inhibits apoptosis
F.C. = 1.76	Regulated by	p38α
HDAC5 (pS259) p = 0.002 F.C. = 1.55	Protein role	Transcriptional regulation, cell cycle progression and developmental events
	Effect/protein	Intracellular translocation, regulates molecular association, induces interaction with 14-3-3β
	Effect/cell	Regulates cell differentiation, cell motility, chromatin organization and transcription
	Regulated by	AMPKA1, CaMK4
AMPKA1 (pT174)	Protein role	Regulates cellular energy in response to the balance between AMP/ATP, and intracellular Ca^{2+} levels.
p = 0.017	Effect/protein	Enzyme activity
F.C. = 1.22	Regulated by	ATM, LKB1, p27Kip1, PKCZ, PPP2CA
MKK6	Protein role	Activates p38 MAPK enzyme activity.
(pS207)	Effect/protein	Enzymatic activation
p = 0.046	Effect/cell	Regulates transcription
F.C. = 1.28	Regulated by	ASK1, Cot
STAT1 (pS727) p = 0.0126 F.C. = 0.72	Protein role	Transcription regulation in response to peptide hormone stimulus; NFκB cascade. Regulation of smooth muscle cell proliferation; induction of apoptosis
	Effect/protein	Activation, regulates molecular associations, sumoylation
	Effect/cell	Regulates apoptosis, regulates cell growth, regulates transcription
	Regulated by	MKK6, Raf1
eNOS	Protein role	Synthesises nitric oxide
(pS1177)	Effect/protein	Enzyme activity
p = 0.0046	Effect/cell	Cytoskeletal reorganization
F.C. = 0.68	Regulated by	Akt1, AMPKA1, PKACa, PKG2
p53 (pS9) p = 0.0437 F.C. = 0.77	Protein role	Transcription factor and tumour suppressor, regulates cellular responses to DNA damage
	Effect/protein	Auto-acetylation, enzyme activity (Caporali et al. 2004)
	Effect/cell	Regulates apoptosis, cell cycle and transcription

Table 3.4: Information regarding the descriptors given in **Section 3.4.7**, detailing the effects of *increased* phosphorylation on proteins from the microarray results (quercetin vs vehicle control).

Protein	Description	Information
BCL-2 (pT69) p = 0.0476 F.C. = 1.19	Protein role	Anti-apoptotic protein, inhibits caspase activity
	Effect/protein	Regulates molecular association, likely anti-apoptotic and inducible interaction with Bax (Deng et al. 2006)
	Effect/cell	Regulates apoptosis
	Regulated by	JNK1
IKK-β (pY188) p = 0.0401 F.C. = 1.28	Protein role	Degrades inhibitors of NFκB
	Effect/protein	Activation
	Effect/cell	Regulates transcription
	Regulated by	Src
IkB-ε (pS22) p = 0.035 F.C. = 1.24	Protein role	Inhibits NFκB by complexing with and trapping it in the cytoplasm
	Effect/protein	Ubiquitin-mediated degradation.
	Regulated by	IKK
NFkB-p65 (pS529) p = 0.0489 F.C. = 0.80	Protein role	Subunit of NFκB transcription complex, regulates inflammatory and immune responses.
	Effect/cell	Regulates transcription
	Regulated by	CDK5RAP3, CK2-A1
Ezrin (pY353) (354) p = 0.0377 F.C. = 0.60	Protein role	Regulates cell surface structure adhesion, migration, and organization
	Effect/protein	Activation, regulates molecular association
	Effect/cell	Regulates cell motility, cytoskeletal reorganization, induce interaction with PI3K
	Regulated by	EGFR
Met (pY1234) p = 0.0174 F.C. = 1.21	Protein role	A proto-oncogenic receptor tyrosine kinase.
	Effect/protein	Enzyme activity, autophosphorylation, induce interaction with Grb2
	Effect/cell	Regulates cell growth and motility
	Regulated by	EGFR, Ron
Chk1 (pS345) p = 0.0407 F.C. = 0.86	Protein role	Regulates cell cycle arrest in response to DNA damage
	Effect/protein	Enzymatic activation, protein degradation, regulates molecular associations
	Effect/cell	Apoptosis and cell cycle regulation
	Regulated by	ATM, ATR, Cot, Akt1, EGFR, ER-α, MCM7
EGFR (pY1172) p = 0.0383 F.C. = 0.85	Protein role	Receptor tyrosine kinase, regulates cell growth and differentiation.
	Effect/protein	Enzyme activity, autophosphorylation, protein degradation, regulates molecular association
	Effect/cell	Regulates cell growth
	Regulated by	CSFR
Estrogen Receptor-α (pS104) p = 0.0492 F.C. = 0.78	Protein role	Regulates gene expression and cell proliferation and differentiation
	Effect/protein	Enzyme activity, regulates molecular associations
	Effect/cell	Regulates transcription
	Regulated by	CDK2, ERK2 , GSK3B

Table 3.4: Information regarding the descriptors given in **Section 3.4.7**, detailing the effects of *increased* phosphorylation on proteins from the microarray results (quercetin vs vehicle control).

Protein	Description	Information
Progesterone receptor (pS190) p = 0.028 F.C. = 0.76	Protein role	Regulates gene expression and cell proliferation and differentiation
	Effect/cell	Regulates transcription
	Regulated by	CDK2
c-Jun (pS243) p = 0.022 F.C. = 0.82	Protein role	Component of the transcription factor AP-1
	Effect/protein	Inhibition of enzyme activity, autophosphorylation, regulates molecular associations
	Effect/cell	Regulates transcription
	Regulated by	PPP3CA, CK2-A1, GSK3B
PPAR-γ (pS112) p = 0.022 F.C. = 0.83	Protein role	Regulator of adipogenesis and lipid metabolism, insulin sensitivity, proliferation and inflammation.
	Effect/protein	Protein degradation
	Effect/cell	Regulates differentiation, growth and transcription
	Regulated by	Fyn, PARVB, PPP1CA, ERK2, JNK1
eIF2A (pS51) p = 0.045 F.C. = 0.84	Protein role	Translation initiation factor, active in the early steps of protein synthesis
	Effect/protein	Enzyme activity, regulates molecular associations
	Effect/cell	Regulates apoptosis, cell growth and translation
	Regulated by	GADD34, Nck1, Nck2, PKR, HRI, PERK, PPP1CA
PDGFRα (pY849) p = 0.047 F.C. = 1.18	Protein role	Receptor tyrosine kinase, regulates cell growth, actin reorganization, migration and differentiation
	Effect/protein	Likely enzyme activity, no direct evidence though
Connexin 43 (pS367) p = 0.026 F.C. = 0.80	Protein role	Forms gap junctions with neighbouring cells
	Effect/protein	Regulates cell motility
	Effect/cell	Inhibition of enzyme activity
	Regulated by	PKCE
GluR1 (pS849) p = 0.031 F.C. = 0.67	Protein role	Membrane protein, regulates synaptic transmission
	Effect/protein	Enzyme activity
GluR2 (pS880) p = 0.028 F.C. = 0.78	Protein role	Membrane protein, regulates synaptic transmission
	Effect/protein	Enzyme activity
BRCA1 (pS1457) p = 0.043 F.C. = 1.37	Protein role	Regulation of DNA damage repair, ubiquitination
	Effect/protein	Regulates molecular associations induce interaction with ATM
	Regulated by	ATM, ATR

Table 3.4: Information regarding the descriptors given in **Section 3.4.7**, detailing the effects of *increased* phosphorylation on proteins from the microarray results (quercetin vs vehicle control).

Protein	Description	Information
Synapsin (pS9) p = 0.021 F.C. = 0.60	Protein role	Neuronal protein, associates with synaptic vesicles and binds to the cytoskeleton
	Effect/protein	Inhibits binding to phospholipids and dissociates synapsins from synaptic vesicles
	Regulated by	CaMK1 and PKA
Elk1 (pT417) p = 0.016 F.C. = 0.72	Protein role	Regulates transcription in response to serum and growth factors
	Regulated by	PRP4
Smad1 (pS465) p = 0.035 F.C. = 0.77	Protein role	Regulation of morphogenesis, proliferation, differentiation and apoptosis
	Effect/protein	Enzyme activity, intracellular translocation, regulates molecular associations
	Effect/cell	Regulates transcription

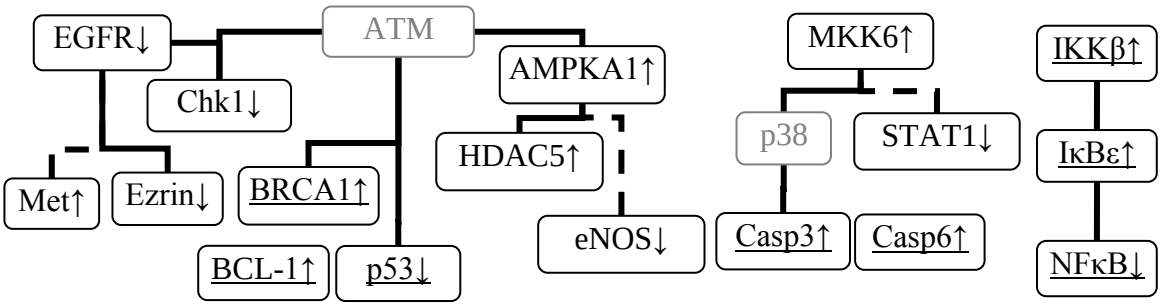

Figure 3.7: Signalling and interactions between phospho-proteins from the microarray results (quercetin vs vehicle control), according to ontological information. Underlined proteins are associated with an anti-apoptotic effect. Dashed lines indicate interactions between phospho-proteins that are not necessarily supported by the change in phosphorylation recorded by the microarray analysis. Greyed out lines indicated an association between proteins, but not necessarily due to the phosphorylated residue in question. Greyed out proteins indicate that they are not part of the microarray results, but are required to show the signalling pathway.

Table 3.5: Signalling pathways represented on the antibody microarray, the number of targets representative of these pathways, and their percentage of the total targets

Signalling pathway	Count	%	Signalling pathway	Count	%
Neurotrophin signalling pathway	65	16.1	Adipocytokine signalling pathway	22	5.5
ErbB signalling pathway	54	13.4	Acute myeloid leukaemia	28	6.9
B cell receptor signalling pathway	36	8.9	Epithelial cell signalling in Helicobacter pylori infection	22	5.5
Prostate cancer	47	11.7	Bladder cancer	17	4.2
MAPK signalling pathway	76	18.9	Wnt signalling pathway	32	7.9
Chronic myeloid leukaemia	42	10.4	Jak-STAT signalling pathway	32	7.9
Focal adhesion	64	15.9	Axon guidance	27	6.7
T cell receptor signalling pathway	47	11.7	NOD-like receptor signalling pathway	18	4.5
Pancreatic cancer	37	9.2	Thyroid cancer	12	3.0
Fc gamma R-mediated phagocytosis	41	10.2	Vascular smooth muscle contraction	23	5.7
Glioma	34	8.4	Melanogenesis	21	5.2
Pathways in cancer	96	23.8	Type II diabetes mellitus	14	3.5
Insulin signalling pathway	46	11.4	Long-term depression	17	4.2
Colorectal cancer	36	8.9	Endocytosis	29	7.2
Fc epsilon RI signalling pathway	33	8.2	Pathogenic Escherichia coli infection	14	3.5
Long-term potentiation	31	7.7	Tight junction	22	5.5
Regulation of actin cytoskeleton	41	10.2	RIG-I-like receptor signalling pathway	15	3.7
Non-small cell lung cancer	27	6.7	Endometrial cancer	24	6.0
Cell cycle	38	9.4	Gap junction	16	4.0
Chemokine signalling pathway	47	11.7	Calcium signalling pathway	24	6.0
Aldosterone-regulated sodium re-absorption	11	2.7	TGF-beta signalling pathway	15	3.7
GnRH signalling pathway	32	7.9	Primary immunodeficiency	9	2.2
Melanoma	26	6.5	Viral myocarditis	13	3.2
VEGF signalling pathway	28	6.9	Prion diseases	8	2.0
Natural killer cell mediated cytotoxicity	37	9.2	Hematopoietic cell lineage	13	3.2
mTOR signalling pathway	23	5.7	Alzheimer's disease	19	4.7
Progesterone-mediated oocyte maturation	30	7.4	Phosphatidylinositol signalling system	11	2.7
Adherens junction	27	6.7	Oocyte meiosis	31	7.7
p53 signalling pathway	25	6.2	Renal cell carcinoma	24	6.0
Apoptosis	28	6.9	Small cell lung cancer	26	6.5
Toll-like receptor signalling pathway	30	7.4	Amyotrophic lateral sclerosis (ALS)	20	5.0
Leukocyte transendothelial migration	32	7.9			

3.5.5. Western blotting of selected protein phosphorylation changes identified using microarray analysis

Three of the proteins identified as being significantly altered by quercetin treatment in the microarray analysis were selected for further analysis using western blotting. The selected protein changes selected were:

(i) increased phosphorylation of caspase-6 (Ser257) by quercetin metabolites and quercetin aglycone

(ii) decreased phosphorylation of VEGFR2 (Tyr951) by quercetin metabolites

(iii) decreased phosphorylation of eNOS (Ser1177) by quercetin metabolites and quercetin aglycone

The increase in caspase-6 phosphorylation and decrease in VEGFR2 phosphorylation identified by the microarray analysis could not be confirmed by western blotting. Bands representing phosphorylated or unphosphorylated caspase-6 (34 kDa) could not be confidently identified in blots of lysates from cells treated with either quercetin, its metabolites or with etoposide (**Fig. 3.8**). Etoposide has been shown previously to increase caspase-6 phosphorylation at Ser257 (as per documentation for Abcam anti-p-caspase-6 (Ser257) antibody [#63440]), though no reports could be found which show such activity in HUVECs or other endothelial cell lines, which may explain the lack of increased phosphorylation observed here. While bands representing phosphorylated VEGFR2 were identified in blots of lysates from cells treated with VEGF, no clear bands could be identified for other treatments, or in blots stained for total VEGFR2 (data not shown).

Blots of lysates from HUVECs treated with EGCG (to stimulate eNOS phosphorylation and allow for more obvious detection of p-eNOS decrease) followed by increasing doses of quercetin metabolites did not result in decreases in eNOS phosphorylation as was putatively identified by the microarray analysis (**Fig. 3.9**). Treatment of HUVECs with EGCG followed by incubation with quercetin aglycone, however, did result in a dose-dependent decrease in eNOS phosphorylation (**Fig. 3.10**),

which was significant at 50 μM ($p < 0.05$) following densitometry analysis (**Fig. 3.11**). Pearson's correlation coefficient was calculated for the dose-dependent decrease in eNOS phosphorylation. The corresponding *t*-statistic showed the decrease to be borderline significant ($p = 0.056$).

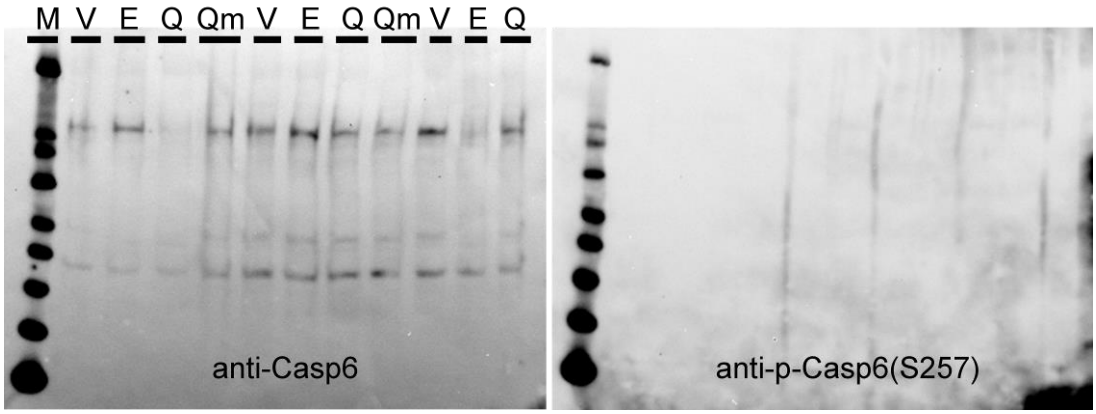

Figure 3.8: Western blots for Casp6 and p-Casp6(Ser257) from lysates of HUVECs treated for 60 minutes with 25 μM etoposide (**E**), 50 μM quercetin (**Q**) and 50 μM quercetin metabolite mix (**Qm**), alongside a vehicle control (**V**). The bands shown on the anti-Casp6 blot could not be confirmed as representative of caspase 6. Molecular weight marker (**M**) bands: **20**, **30**, **40**, **50**, **60**, **80**, **100**, **120** and **220 kDa** (bottom to top). n=3 (culture well replicates) for each treatment, except for the quercetin metabolites treatment (n=2).

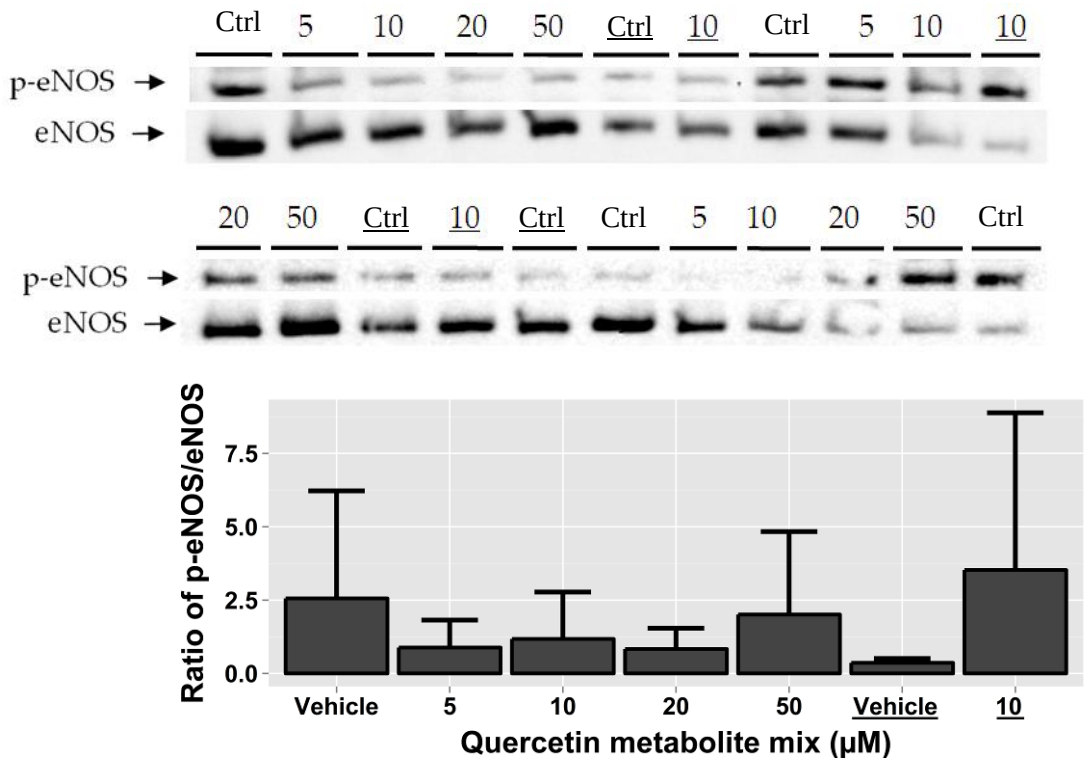

Figure 3.9: Western blots of p-eNOS/eNOS from lysates of HUVECs treated with 50 μM EGCG for 30 minutes prior to varying concentrations (μM) of a mix of quercetin metabolites (in fresh culture media) for 60 minutes alongside a vehicle control (**Ctrl**). Underlined treatments indicate that the assayed lysates were from cells used for the original microarray experiment (no EGCG pre-treatment). n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-eNOS/eNOS)is shown in the lower panel.

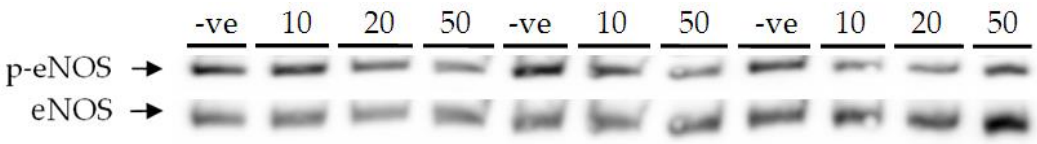

Figure 3.10: Western blots of lysates from HUVECs treated with 50 μM EGCG for 30 minutes prior to varying concentrations (μM) of quercetin (in fresh culture media) for 60 minutes alongside a vehicle control treatment **(-ve**). n=3 (culture well replicates) for each treatment, except for the 20 μM treatment (n=2).

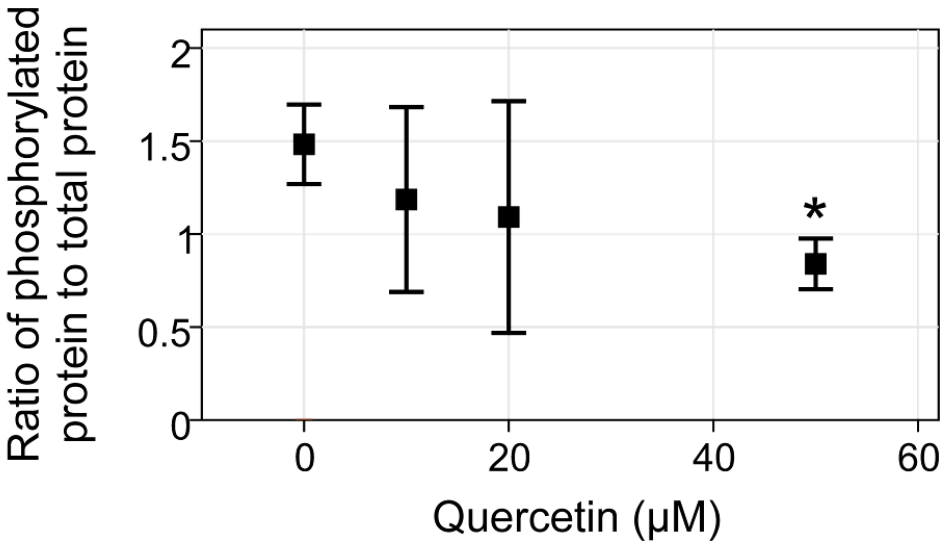

Figure 3.11: Ratio of blot densitometry from western blots shown in **Fig. 3.10**. ***** - $p < 0.05$ vs vehicle control treatment (0 μM).

3.6. Discussion

Analysis of lysates from endothelial cells treated with quercetin, a mix of quercetin metabolites, or a vehicle control, with an antibody microarray complimentary for a large subset of phospho-proteins, provided a small selection of changes that, while unable to stand up to robust statistical testing, provided targets for validation by western blotting. After stimulation of phosphorylation of the enzyme eNOS, treatment with quercetin dose-dependently caused a decrease in phosphorylation ($p = 0.056$), similar to what was observed using the microarray analysis.

3.6.1. Endothelial signalling events in response to quercetin

While a large number of signalling proteins were measured in the endothelial cells, only a small subset of these were shown to be significantly altered by the microarray analysis. There was a consensus amongst a number of the phospho-proteins highlighted by the quercetin treatment towards an anti-apoptotic effect, although this could not be shown as significant by bioinformatic analysis, and so it is unlikely to be a real effect of the quercetin treatment (**Fig. 3.7** and **Table 3.4**). Despite the fact that quercetin can have strong cytotoxic effects at high concentrations, there is evidence that the dose of 10 µM used in this study can improve cell survival in both endothelial and non-endothelial cells (Chao et al. 2009; Spencer et al. 2003). p53, BCL-2 and caspase-3 are three of the phospho-proteins highlighted by the microarray analysis of quercetin-treated cells that were indicative of an anti-apoptotic response. Changes to these proteins have been reported previously in cells treated with quercetin, in terms of changes to phosphorylation or protein expression that are in line with a decrease in apoptotic cells (Choi et al. 2009; Choi et al. 2005; Choi et al. 2003). Such effects would not necessarily be related to the decrease in eNOS phosphorylation (enzyme inactivation), observed as the main effect of quercetin-treated cells, as this decrease is associated with anti-proliferation activity rather

than anti-apoptosis (Mata-Greenwood et al. 2008). The dose of quercetin at which p-eNOS decrease was significant (50μM) was particularly high, however. The involvement of quercetin's cytotoxicity with observed effects/lack of effects should be considered in future experiments, and cell viability assays can be used to take cytotoxic effects into account.

The observation that quercetin can decrease eNOS phosphorylation mirrors previous reports which show decreases to eNOS activity and expression due to quercetin treatment (Jackson & Venema 2006; Wallerath et al. 2005), and while this decrease in phosphorylation was not statistically significant in the microarray analysis for $q < 0.05$, the western blotting results support this as a real effect (**Figs. 3.10** and **3.11**). However, it is also in contradiction to a number of animal studies which show improvement to vasodilation, an effect which would be associated with increased eNOS phosphorylation (at Ser1177) (Sanchez et al. 2006; Nishida & Satoh 2009; Khoo et al. 2010). Indeed, a decrease in Ser1177 phosphorylation of eNOS protein would be associated with increased vascular tone and could be indicative of endothelial dysfunction if such a decrease in phosphorylation was to be maintained for irregular periods. While improvements to vasodilatory function have been seen in human studies which record significantly lower blood pressure after consumption of quercetin (Lee et al. 2011; Egert et al. 2009), this effect has also been reported as being independent of changes to ET-1 and nitrites, markers normally associated with changes to vascular tone (Larson et al. 2012a).

The research reported here details effects in HUVECs after stimulation with only a polyphenol treatment. Bioactive effects due to quercetin are often reported in cells that have been co-treated with additional factors, such as hydrogen peroxide, oxidised low-density lipoproteins, TNF-α or high concentrations of glucose (Chao et al. 2009; Choi et al. 2009; Choi et al. 2003; Mochizuki et al. 2004). Resting HUVECs may be in a state where quercetin or its metabolites cannot influence signalling associated with protein phosphorylation to the extent that is reported in studies where cells are first challenged

with inflammatory factors. It may be that the molecules involved in mediating signals in endothelial cells after exposure to quercetin may first require translocation, or their intracellular levels may be too low, and it is these stresses which can alter location or expression of molecules and allow signalling in response to quercetin to take place.

3.6.2. Study of protein phosphorylation with antibody microarrays

Through the microarray analysis, the phosphorylation of a large subset of endothelial cell proteins was studied. Considering the size of the experiment, it is likely that a number of the proteins complimented by the antibodies present on the microarray have not been previously identified in endothelial cell cultures, specifically those that are of low abundance and are less likely to be identified by mass spectrometry analyses. Therefore, secondary to the main objectives of this work, evidence for the presence of previously unreported proteins in endothelial cells is likely to have been found by this study, which may be of use for future broad-targeted phospho-protein studies in endothelial cell cultures (such evidence would of course be more robust had a cell-free control treatment been analysed by the antibody microarrays alongside the experimental treatments, to measure levels of non-specific binding).

At the time of the microarray analysis described in this chapter, most reported studies using phospho-protein antibody microarrays were focused on a small subset of signalling proteins, usually confined to one specific signalling pathway (He et al. 2009; Zhong et al. 2008; Kang et al. 2010). This highlights the novelty of such a technique involving numbers of target analytes an order of magnitude greater than previous reports. Though not necessarily a symptom of this, there were a number of issues upon initial use of the antibody microarrays.

While there appeared to be an additive effect of background fluorescence on foreground signal in the microarray images which suffered from high background signal,

normal levels (as per manufacturer's examples) of local background did not appear to have significant influence on foreground signal. In a number of cases, the signal surrounding spots would be higher than the foreground spot signal (**Fig. 3.3**, e.g. bottom row, 4th from the left), suggesting that the blocking buffer proteins (if these are indeed the main source of background signal) have lower affinity to the antibodies/spotting buffer than to the glass slide. The manufacturer's recommendation for dealing with normal levels of background fluorescence was to not factor it in to any calculations. A similar case of lower foreground signal compared to background signal was reported in a number of spots of the antibody microarray experiments conducted by Hecker et al. (2012), however they did not ignore the local background signal in their experiments. Their strategy was to increase all negative values to a value of 1, after subtraction of the background signals. While this allows calculations to take into account any influence high background fluorescence might have on foreground signal, information is likely to be lost from spots representing low abundance proteins/phospho-proteins by truncating their values. A third method would be to add a flat value to all spots after background subtraction, to increase lowest value to > 1. While this would not necessarily affect significance testing, there may be a need to take into account the fact that fold-changes for these targets would be skewed.

Analysis of the microarrays was further complicated by an inability of the GenePix software to determine the boundaries of many of the spots in the .tiff image files that were produced from the fluorescence scan of the microarrays. The manufacturer-supplied .gal (genepix array list) file, which stores relative locations of each microarray spot along with each spot's target protein, could not be used for this task due to slight discrepancies between the locations stored in the .gal file and actual locations of spots on the slides. The issue appeared to be with the concentric nature of a number of the spots, and the negative/small differences between spot foreground and local background signals. The Matlab software script used to analyse images made use of a package designed for

detecting objects of a particular shape, and was able to cope much better with both of these factors.

3.6.3. Analysis of antibody microarray data

At the time of analysis, no reports could be found which described statistical significance testing of normal-phase phospho-protein antibody microarray data. The analysis of reverse-phase phospho-protein antibody microarray data by Petricoin et al. (2007) details the use of the Wilcoxon rank test, though such a test is more appropriate for non-parametric data. The use of p-values is mentioned in the report by He et al. (2009), though no description is given as to the method of significance testing used. The limma package for the R software environment was designed around the use of linear models for analysing microarray data. Its use for handling large datasets in experiments with small numbers of microarrays has been well documented (Smyth 2005). The method development involved in this study highlights the of lack of established procedures for performing antibody microarray analysis on a relatively large scale (in terms of analytes), an issue discussed in the report authored by Hecker et al. (2012), where differences between antibody/protein microarrays and cDNA microarrays, such as the distribution of signal and the consistency of total signal across microarrays, were also highlighted.

Similar to other reported studies, Bhola et al. (2011) made no use of significance testing and reported only fold changes, in a study that involved a relatively small set of analytes. Reported fold changes were high enough, however, that changes to phosphorylation are obvious, and do not necessarily warrant thorough significance testing. This is in contrast to the fold changes recorded here for significant ($p < 0.05$) changes to protein phosphorylation (0.60 to 1.76). Such fold changes are also relatively low compared to what might be expected for obvious changes in more-routine biochemical techniques, such as western blotting assays or ELISAs, despite their values for significance.

Regardless of the fact that DAVID was designed for the analysis of data from cDNA microarray, rather than anti-protein antibody microarrays, it is still capable of determining significant analyte relationships in the context of cell signalling, whether or not the analyte is a gene or a protein. Therefore, pathway analysis software that is designed with protein networks in mind, such as Ingenuity Pathway Analysis (http://www.ingenuity.com), or MetaCore (http://portal.genego.com) are likely to have produced similar results. The PhosphoSite and Uniprot websites proved a good source of information on the functions of the protein phosphorylation events highlighted by the microarray study. Though not exhaustive, both sites are under constant development and are likely to continue to be excellent data sources for these types of studies in, and for the development of bioinformatics software in the future. The conclusion from studying these databases, that an anti-apoptotic effect was being represented by the changes in phospho-proteins, was not backed by the statistical significance testing used in this study. It is likely the majority of phospho-proteins highlighted were simply chance occurrences, and that there were no strong effects on protein phosphorylation due to the 60 minute incubations of either quercetin or a mix of its metabolites.

This is the largest reported antibody microarray study to date (1320 unique antibodies per slide), surpassing that of Bodet et al. (2007) (approx. 600 different antibodies). Antibody microarrays capable of assessing > 100 targets at once are produced by a handful of companies, with most taking protein phospho-sites into account (Zhang & Pelech 2012). While microarrays have a number of advantages over techniques such as mass spectrometry analysis, including selectivity, improved reproducibility and relative ease of performance, it is still a relatively new method, and only one out of many techniques that now exist for relative and absolute quantification of large numbers of proteins simultaneously (Taussig et al. 2007; Ray et al. 2010; Rodríguez-Suárez & Whetton 2013). Until such methods become economical enough for routine use, with their capabilities confirmed through published studies, their limitations should not be

underestimated, and compromises in study design will most likely have to be made. The large number of target analytes on the microarrays used meant that their design could only include two replicates per microarray slide, and this may have been costly in obtaining results able to stand up to robust significance testing. Despite this, the decrease in eNOS phosphorylation shows that quercetin may have some effect on early signalling events in resting endothelial cells. By focusing on a small number of markers related to eNOS activity, further details as to the bioactivity of quercetin, or other polyphenols found in the food plants, may be uncovered.

3.7. Conclusion

Quercetin, as a mix of its human metabolites and in its aglycone form, was used to treat endothelial cells to determine early responses of cell signalling related protein phosphorylation. To accomplish this, an antibody microarray, designed to target a large subset of signalling proteins in both their phosphorylated and unphosphorylated forms, was used to analyse cell lysates. Significance testing provided a subset of proteins with altered phosphorylation levels (17 for quercetin metabolites vs control, 29 for quercetin aglycone vs control). Analysis of protein ontologies indicated an anti-apoptotic effect. Using western blotting for validation of the microarray analysis results, quercetin aglycone was shown to dose-dependently decrease eNOS phosphorylation, significant at 50 µM. More detailed analyses of eNOS activity and related signalling molecules may provide further clues as to the bioactivity of quercetin or other bioactive compounds in the food plants selected in Chapter 2.

Chapter 4

Effect of polyphenols on vasodilatory signalling molecules in endothelial cells

Chapter 4: Effect of polyphenols on vasodilatory signalling molecules in endothelial cells

4.1. Abstract

Quercetin, a polyphenol found in numerous foods consumed worldwide, including those selected in **Chapter 2**, was shown to significantly ($p < 0.05$) decrease eNOS phosphorylation in HUVECs, as determined by western blotting and antibody microarray analysis. eNOS is part of the nitric oxide signalling pathway and involved in the regulation of vascular tone, an important factor in the control of endothelial dysfunction. It would be interesting to see if extracts of these and other selected plants could affect eNOS or other molecules related to nitric oxide signalling in endothelial cells.

Food plant extracts were prepared using hot 70% methanol for treatment of HUVECs, alongside a number of pure compound treatments. Quercetin was tested in the aglycone form and as a mix of phase II human metabolites, alongside the kale glucosinolate sinigrin, allyl-ITC, phase II metabolites of sulforaphane, and sulforaphane itself. Phosphorylation (activation) of Akt and eNOS was assayed by western blotting. Changes to cGMP, total eNOS protein and secreted endothelin-1 (ET-1) protein were quantified using ELISA. Levels of nitrates released into cell media were measured using a nitric oxide analyser. Effects of the treatments on cell health were determined using the WST-1 assay, and by making visual observations with a light microscope.

As evidence for their potential to increase endothelial nitric oxide production, the persimmon and pomegranate extracts (and only these two treatments) were able to increase the phosphorylation of both Akt and eNOS in HUVECs, significantly reduce levels of ET-1 in the culture media over 24 hours ($p < 0.001$), and significantly increased levels of nitrates/nitrites ($p < 0.05$), compared to vehicle controls, reported here for the first time.

4.2. Introduction

4.2.1. Potential for polyphenols to affect production of nitric oxide in the endothelium

There is evidence that dietary consumption of polyphenol-rich foods can improve endothelial function and reduce hypertension in humans (Shukla et al. 2010; Manach et al. 2005; Halliwell 2007; Schini-Kerth et al. 2011) based on *in vitro* and *in vivo* evidence, as well as long-term intervention trials (Chong et al. 2010; Oude Griep et al. 2011). The ability of plant-derived polyphenols to regulate nitric oxide release would provide good rational for these effects. Studies with polyphenol-rich foods, such as cocoa and chocolate (Flammer et al. 2012; Ried et al. 2012), wine (Li & Förstermann 2012), tea (Hollman et al. 2010), apple (Jensen 2009), cranberry (Dohadwala et al. 2011) and pomegranate (Lynn et al. 2012), have all shown improvements to markers associated with CVD, such as hypertension. Meta-analyses of intervention trials with foods such as these provide stronger evidence for beneficial effects, as well as highlighting potential method improvements so as to obtain results more comparable to previous studies by others in the field (Kay et al. 2012; Hooper et al. 2008; Peterson et al. 2012). Intervention trials which focus on pure polyphenol compounds, such as epicatechin (Jiménez et al. 2012), quercetin (Perez-Vizcaino et al. 2009), epigallocatechin-gallate (Brown et al. 2009; Widlansky et al. 2007) and chlorogenic acid (Mubarak et al. 2012), as well as subclasses of polyphenols such as lignans (Peterson et al. 2010) or flavonols (Hollman et al. 2010), reinforce results from studies focused on whole foods.

In the context of nitric oxide signalling or endothelium-dependent vasodilation, there are numerous studies showing positive effects in cells or tissues treated with phenolic compounds (Schmitt & Dirsch 2009; Schini-Kerth et al. 2010). The anthocyanin cyanidin-3-O-glucoside (Sorrenti et al. 2007), epicatechin (Ramirez-Sanchez et al. 2011; Brossette et al. 2011), epigallocatechin-gallate (Yamakuchi et al. 2008) resveratrol,

quercetin (Appeldoorn et al. 2009) and thearubigins (Lorenz et al. 2009) have all been shown to increase NO production or phosphorylation of eNOS (Ser1177). Epigallocatechin-gallate has also been reported to decrease secretion of ET-1 (Unger 2010). Many of these studies make use of antibodies complimentary for signalling molecules such as eNOS with and without phosphorylation of enzyme-activity related residues. Other studies measure nitric oxide by way of its oxidation products or using NO-sensitive fluorophores. Cell culture studies are matched by experiments working with whole tissue, measuring the relaxation of endothelium-intact and endothelium-denuded vascular rings. Rings are usually taken from rats, mice and pigs and used as a model for the human vascular system. In such studies, relaxant effects have been reported with, amongst others, epigallocatechin-gallate (Alvarez et al. 2006), procyanidins (Tokoudagba et al. 2010) and grape juice (Anselm et al. 2007). Additional examples of plant extracts reported to increase nitric oxide signalling include treatment of porcine vascular rings with cranberry and lingonberry juices (Auger et al. 2011) and treatment of endothelial cell cultures with black tea (Anter et al. 2005) or grape seed extracts (Liu et al. 2012). Tested compounds may be metabolised *in vivo* to form additional conjugated or deconjugated forms, possibly with altered bioactivity. Such changes in bioactivity after conjugation could provide clues as to which moieties of the original compound are important for influencing cell signalling. For example, it has been reported that polyphenol hydroxyl groups, and their positions, are important for mediating biological effects (Auger et al. 2010).

4.2.2. Plant extract and representative pure compound treatments for endothelial cell culture experiments

The six food plants selected in Chapter 2 (dill, nettle, kale, *Sideritis*, pomegranate and persimmon) may be able to produce similar bioactive effects. By testing polyphenol-rich

plant extracts and representative pure compounds in assays measuring changes to molecules related to nitric oxide signalling, plants can be identified for further study, to examine how and why they are able to affect vasodilatory factors. It is likely that the presence of compounds in the plant extracts at the endothelium *in vivo* will be limited by human absorption and metabolism. Preliminary work to understand the bioactivity of the parent compounds has the potential to provide clues to the bioactivity of the physiologically-relevant metabolites, the measurement of which can be difficult under *in vivo* conditions. Literature evidence for the phenolic content of the six food plants, given in **Tables 2.12 - 2.21** in Chapter 2, demonstrated that quercetin was a common flavonol in a number of the extracts, and was shown to have effects on eNOS phosphorylation in Chapter 3. It was included, along with a mix of its human metabolites, for testing alongside the plant extracts as a representative pure compound. As the extracts were likely to contain other bioactives besides polyphenols that may also be bioactive, the glucosinolate sinigrin was tested, alongside its isothiocyanate (ITC) product, allyl-ITC (found in kale). Phase II metabolites of sulforaphane, and sulforaphane itself, were used in place of sinigrin metabolites, which were not available. Together, these treatments would be used to study the effects of plant bioactives on the phosphorylation (activation) of Akt and eNOS after short incubation periods with HUVEC cultures and, after longer incubations, changes to levels of intracellular cGMP and total eNOS protein, and ET-1 and nitrates/nitrites released into culture media.

4.3. Aim(s)

The aim of this study was to determine if polyphenol-rich extracts of selected plants representative of traditional diets of Black Sea area countries, or representative individual compounds identified from published literature, were able to affect signalling molecules involved with nitric oxide production and the regulation of vascular tone.

4.4. Methods

4.4.1. Materials

All water used was of MilliQ-grade purity, unless it is stated that deionised water (dH_2O) was used instead. The antibodies for Akt (#9272), phosphorylated (p-)Akt (#4060), eNOS (#9572), and p-eNOS (#9570) were purchased from Cell Signalling Technology (CST) and used at their recommended dilutions. All other chemicals were purchased from Sigma-Aldrich and were of analytical-grade purity, unless otherwise specified.

4.4.2. Formulation of quercetin and sulforaphane metabolite mixes

A mix of quercetin metabolites (Q. met.) were prepared based on the relative concentrations of metabolites previously identified in human plasma after consumption of onions (Mullen et al. 2006). Aqueous solutions of quercetin-3'-sulphate, quercetin-3-glucuronide (GlcA) and isorhamnetin-3-GlcA were mixed in a molar ratio of 6:3:1. Likewise, a mix of sulforaphane metabolites (S. met.) were prepared based on the relative concentrations of metabolites previously identified in human plasma after consumption of broccoli (Gasper et al. 2005). Aqueous solutions of sulforaphane-cysteine, sulforaphane-cysteine-glycine, sulforaphane-*N*-acetylcysteine and sulforaphane-glutathione were mixed in a molar ratio of 55:27:9:9. DMSO was also added to formulations before treatment of cells cultures, so that the final concentration of DMSO in culture media was of 0.1% v/v.

4.4.3. Sourcing of plant materials used to produce polyphenol-rich extracts

Plant material for dill, kale, nettle, *Sideritis*, pomegranate and persimmon were sourced from countries surrounding the Black Sea between June and October 2010 and delivered to our lab in November 2010. Aerial parts of *Sideritis scardica* was collected by those

working alongside Dr Maria Glibetic at the Institute of Medical Research (IMR) (Belgrade, Serbia), from five different locations along the Pirin Mountain range near the Macedonian/Bulgarian border. After collection, plants were hung under a wooden roof out of direct sunlight and air-dried for a period of two months. Aerial parts of nettle (*Urtica urens*) and dill (*Anethum graveolens L*) were collected from private gardens and street markets in Uzhhorod, Ukraine by those working alongside Dr Nadiya Boyko at Uzhhorod National University (Uzhhorod, Ukraine). These were then oven-dried at 50-70 °C for a period of 2-3 hours. Persimmon fruit and aerial parts of kale were collected by those working alongside Bike Koçaoglu at Yeditepe University (Istanbul, Turkey). Persimmon was purchased from a supermarket in Istanbul and Kale was collected from a farm in Akcaabat, near Trabzon, Turkey. Both were freeze-dried by Wendy Hollands (IFR, UK). Pomegranate fruit was purchased from a farmers market in Tbilisi by those working alongside Dr Mariam Jorjadze at Elkana (Tbilisi, Georgia) and was not treated with any drying procedure prior to extraction, but was kept refrigerated during transport and storage.

4.4.4. Extraction of polyphenols and glucosinolates from plant material

All polyphenol-rich plant extracts were prepared using hot aqueous methanol, with some minor differences in the method used for each plant. Extracts of dill, nettle and *Sideritis* were prepared at the Institute of Medical Research, Belgrade, Serbia, in the same manner described below.

A kitchen blender was used to prepare a homogenous mix of freshly de-hulled pomegranate arils, or to reduce dried plant material to powder. Arils or the powdered dry plant material were then decanted into polythene bags for weighing prior to extraction. Powdered plant material was decanted into glass containers before addition of hot (65-70 °C) 70% methanol (methanol:water, 70:30, v/v; plant material:70% methanol, 1:10, w/v).

Arils were decanted into measuring cylinders before adding hot methanol (arils:methanol; 30:70, v/v). Methanol/plant material mixtures were maintained at 70 °C for 20 minutes, resuspending material halfway through by swirling. Supernatants were decanted through muslin cloth, held in a porcelain filter/funnel (1 mm pores), and collected in glass containers. Plant material that had accumulated in the muslin cloth was wrapped and pressed to collect remaining liquid, and then back extracted in the original container using fresh solvent under the same conditions used previously. Back extraction was repeated until an obvious difference in the supernatant colour was observed, compared to the colour of the first supernatant produced (kale: 3-5 back extractions, persimmon: 5-7, pomegranate: 2).

Methanolic supernatants of pomegranate, kale and persimmon were decanted into 50 ml plastic tubes and centrifuged in a Heraeus Megafuge 1.0r at 4200 rpm (3000 g) for 10 minutes. They were then filtered through muslin cloth into round-bottom flasks (of size 250 or 500 cm^3) and dried at 40 °C using a rotary evaporator, with further drying of pomegranate and persimmon extracts by vacuum oven (40 °C, 6 x 10^{-2} torr). When flasks remained at a stable weight (measured in grams to 3 decimal places, after equilibration of stoppered flasks to room temperature) the dried extract was removed by scraping and decanted into a new glass container, while keeping the contents of the flask and the container under nitrogen as much as possible. A glass rod was then used to homogenise the extracts.

All extracts were stored at room temperature (or -20 °C in the case of pomegranate) under nitrogen and desiccate in parafilm-sealed containers, in sealed polythene bags. The pomegranate extract was kept at -20 °C, rather than at room temperature, as it was found to adhere to itself and its glass container unless stored frozen.

4.4.5. Measurement of phenolic content by Folin-Ciocalteu assay

Gallic acid (Sigma-Aldrich) was dried in a vacuum oven (40 °C, 48 hours, 6 x 10^{-2} torr pressure) and 31.85 mg were dissolved in 1 ml of absolute ethanol, making up to volume with water in a 25 cm^3 volumetric flask. Dilutions of 100 µg/ml were made with water (in triplicate) and the absorbance of each was measured using a Nanodrop ND-1000, to calculate the extinction coefficient of gallic acid. The extinction coefficient was calculated on two separate days and the average of these was used as the final value (9443.81 M^{-1} cm^{-1}). Dilutions of 500, 400, 300, 250, 200 and 100 µg/ml gallic acid were then prepared to produce a standard curve for the Folin-Ciocalteu assay (Waterhouse 2001). Water containing 0.2% ethanol (equivalent to the 250 µg/ml gallic acid standard) was used as a blank solution, and eppendorfs were prepared containing 0.79 ml water, into which 10 µl of each gallic acid standard, blank or extract (n=3) were added and vortexed. 50 µl of Folin-Ciocalteu reagent was added to each sample, mixing by vortex, and leaving to stand for 0.5-8 minutes. 150 µl of 20% sodium carbonate was added with mixing and samples were maintained at 37 °C for 30 minutes, before being decanted into plastic cuvettes for measurement of their absorbance at 795 nm. Alongside the 37 °C incubation step, the absorbance of the 100, 200, 250 and 300 µg/ml gallic acid standards were measured at 270 nm using a NanoDrop ND-1000. Values obtained were then used to confirm then concentration of the gallic acid solution used to prepare the dilutions, using the extinction coefficient calculated for gallic acid. Total phenolics in each of the extracts were then determined (measured as gallic acid equivalents (GAE)) using the gallic acid standard curve.

4.4.6. Preparation of plant extracts and pure compounds for cell culture studies

Solutions of plant extracts were prepared using DMSO and basal cell culture medium supplemented with BulletKit antibiotics (Lonza). Stocks of pure compounds were

prepared using DMSO, except in the case of the sulforaphane or quercetin metabolites, which were prepared in water. In all cases, treatments were stored in single use aliquots at -20 °C and once applied to cells, the final concentration of DMSO was 0.1% v/v.

4.4.7. Cell culture experiments

The general materials and methods for the culture of HUVECs used in this work, and the analysis of their lysates by BCA assay and western blotting, are described in **Chapter 2**.

4.4.8. Analysis of cell viability by WST-1 assay and observations of cell morphology

Confluent monolayers of HUVECs cultured in 96-well plates were treated with plant extracts, pure compounds or a vehicle control and then washed once with EBM-2 before incubation with EBM-2 containing 10% (v/v) WST-1 reagent (Roche). After 30 minutes, the absorbance of the cell culture media in each well was measured at 450 nm using a microplate reader. The absorbance was read again after a further 30 minutes, and then every 60 minutes after, for a total of 240 minutes. The absorbance values were plotted against time and statistics based on the slopes produced were used to compare treatment effects on WST-1 metabolism. In conjunction with the WST-1 assay, photographs of cells viewed down a light microscope at 40X magnification were recorded, as reduced cell viability is associated with increased detachment of cells from the culture plate surface, a common effect observed in unhealthy endothelial cells (Bombeli et al. 1996).

4.4.9. Treatment of endothelial cells with plant extracts and pure compounds for the study of signalling molecules relevant to vasodilation

Confluent monolayers of HUVECs cultured in 6-well plates were treated with plant extracts, pure compounds or a positive or vehicle control for up to 60 minutes, for analysis of p-Akt and p-eNOS. The flavanol epigallocatechin-gallate (EGCG) had been

previously shown to be a good inducer of Akt and eNOS phosphorylation (Yamakuchi et al. 2008) in HUVECs, and so was used as the positive control for these assays, after confirmation of its bioactivity in-house (data not shown). 24 hour plant extract, pure compound or vehicle control treatments were performed to analyse changes to levels of total eNOS protein, ET-1 secreted into culture media, and nitrate/nitrite found in culture media. To show that eNOS phosphorylation was p-Akt dependent, an inhibitor of PI3K (LY294002) (CST) was used to treat cells for 60 minutes (50 μM dose) prior to and during selected plant extract and pure compound treatments, alongside vehicle controls. For the analysis of levels of cGMP produced by HUVECs, confluent monolayers of cells cultured in 6 cm^2 dishes were treated with 1 mM solutions of 3-isobutyl-1-methylxanthine (IBMX) for 45-60 minutes prior to and during 90 minute plant extract or pure compound treatments, alongside vehicle controls. IBMX inhibits phosphodiesterases, which hydrolyse cGMP to GMP, and treatment of HUVECs with IBMX allows intracellular cGMP to increase to measurable levels. Sodium nitroprusside was used as a positive control, as it had been previously reported to increase cGMP in HUVECs (Kung et al. 2007).

Post-treatment, cell lysates were prepared and the total protein concentration of each sample measured by BCA assay. Analysis of Akt and eNOS phosphorylation was performed by western blotting. This technique provides both a practical and cost-effective analysis, due to the reusability and long-shelf life of the reagents and materials involved. ELISA kits, which were available for both phospho-proteins, would have allowed for analysis of 48 samples (in duplicate) in a single assay, the equivalent to blotting four 12-well gels, and provide quantitative analysis, while western blotting is generally semi-quantitative and quantification of blot bands is often subjective. They are relatively expensive, however, and at the time there were no kits which allowed for assay of phospho-protein and total protein simultaneously within the same microplate wells. Enzyme-linked immunosorbent assays (ELISAs) were used, however, to determine levels

of total eNOS protein and cGMP (ELISA kits purchased from R&D), and ET-1 (Enzo Biosciences), performed according to the manufacturer's instructions, with the addition of extra wash steps (1.5-2 times the number stated in the manufacturer's instructions, based on work by Amy Casgrain (IFR, UK, unpublished)).

4.4.10. Measurement of nitrate levels in spent cell culture media

NO is unstable when left in cell culture media ($t_{½}$ < 4 minutes (Isik 2005)) and oxidises to nitrites and nitrates in the presence of O_2 and superoxide (Chen et al. 1998). Based on a previous report (Boo et al. 2011), cells were incubated for 24 hours before spent media samples were injected in triplicate into a reaction chamber containing a saturated solution of vanadium chloride in hydrochloric acid, heated to 95 °C. This reduced nitrates and nitrites to nitric oxide, the levels of which were then measured using a Sievers 280i Nitric Oxide Analyser that was connected to the reaction chamber. Dilutions (0.1 – 50 µM) of a stock of sodium nitrate were prepared for the production of a standard curve, from which the concentration of nitrates and nitrites in culture media samples could be quantified.

4.5. Results

4.5.1. Yields of extracted material

The weights of material used and obtained for the drying and extraction processes are given in **Table 4.1**. Quantities used/obtained during the drying of the plant material of dill, nettle or *Sideritis* by IMR (Serbia) were not available.

Table 4.1: Quantities of material obtained after drying and extraction of plant material

Plant	Fresh weight (g)	Weight after drying (g)	Yield (%)	Weight of material for extraction (g)	Weight of extract (g)	Yield (%)
Dill	n/a	n/a	n/a	135.0	24.61	18.2
Nettle	n/a	n/a	n/a	132.4	12.96	9.8
Sideritis	n/a	n/a	n/a	93.1	11.5	12.4
Kale	2999.7	312.4	10.4	239.6	69.65	29.1
Pomegranate	1119.9	n/a	n/a	1119.9	139.75	12.5
Persimmon	4745.6	808.5	17.1	336.0	212.28	63.2

n/a - weight of material at the specific stage of preparation not available, or (in the case of pomegranate, as arils were not dried before extraction) not applicable.

4.5.2. Total phenolic content of extracts

Results from the Folin-Ciocalteu assay performed on samples of the plant extracts are given in **Table 4.2**, which were used to determine the mass of extract required for cell culture treatments, based on equivalent doses of phenolics. The highest levels of phenolics were measured in the *Sideritis* extract, while the lowest levels were found in the pomegranate and persimmon extracts.

Table 4.2: Mean concentration of phenolics in each plant extract, ± standard deviation.

Extract	µg GAE/ mg methanolic extract
Dill	58.07 ± 0.11
Kale	42.60 ± 0.06
Nettle	69.44 ± 0.03
Sideritis	156.66 ± 0.01
Pomegranate	9.85 ± 0.20
Persimmon	5.62 ± 0.45

4.5.3. Effects of plant extracts and pure compounds on p-Akt and p-eNOS

Western blotting of lysates from cells treated for 60 minutes with 50 µM of each of the pure compounds, or 100 µg GAE/ml of the dill, nettle, kale or *Sideritis* extracts, showed phosphorylation of Akt (at Ser473) or eNOS (at Ser1177) was not increased by these treatments, in comparison to a vehicle control treatment. The assay was shown to be performing correctly by the increase in phosphorylation seen from cells treated with EGCG from the same experiment (**Figs. 4.1** to **4.4**).

Treatment of cells with 100 µg GAE/ml of the pomegranate and persimmon extracts for 8-60 minutes caused induction of Akt phosphorylation (**Figs. 4.2** and **4.5**), while treatment of cells with 10-100 µg GAE/ml of the pomegranate and persimmon extracts for 60 minutes caused induction of eNOS phosphorylation (**Figs. 4.4** and **4.6**).

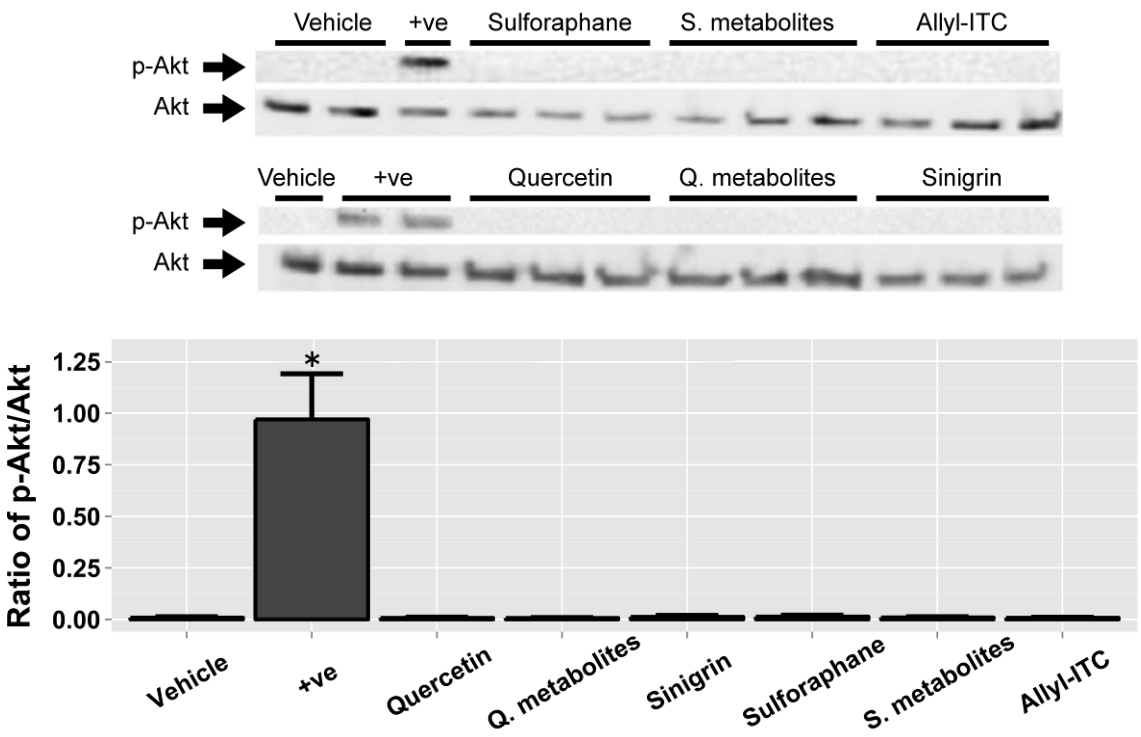

Figure 4.1: Western blots of p-Akt/Akt from lysates of HUVECs treated for 60 minutes with 50 μM pure compounds alongside a vehicle control. 50 μM EGCG was used as the positive (+ve) control. n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

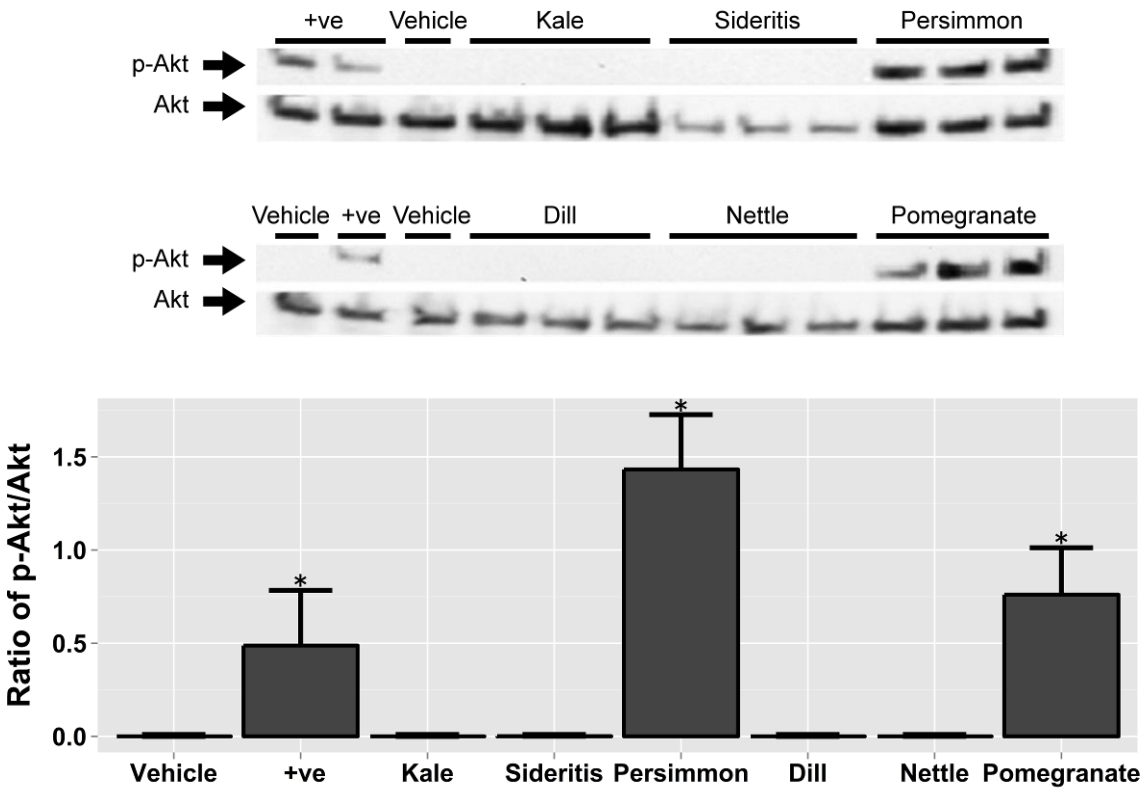

Figure 4.2: Western blots of p-Akt/Akt from lysates of HUVECs treated for 60 minutes with 100 µg GAE/ml of plant extracts alongside a vehicle control. 50 µM EGCG was used as the positive (+ve) control. n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

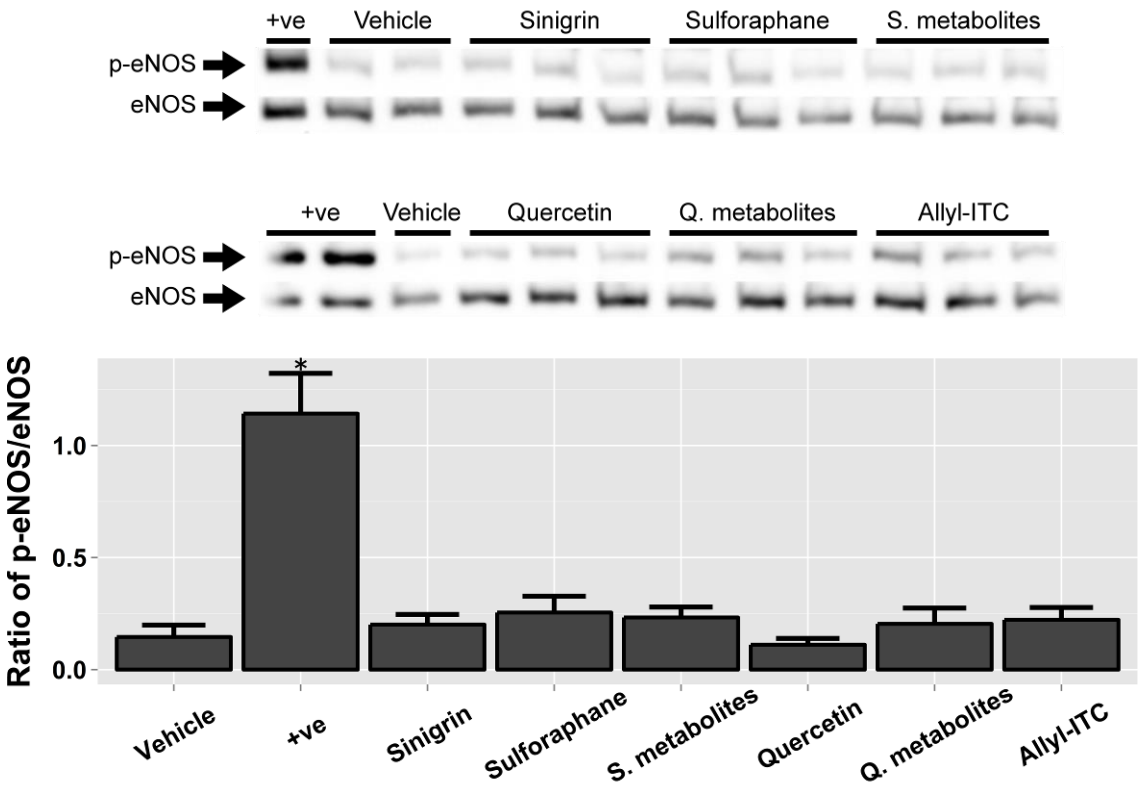

Figure 4.3: Western blots of p-eNOS/eNOS from lysates of HUVECs treated for 60 minutes with 50 μM pure compounds alongside a vehicle control. 50 μM EGCG was used as the positive (+ve) control. n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-eNOS/eNOS) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

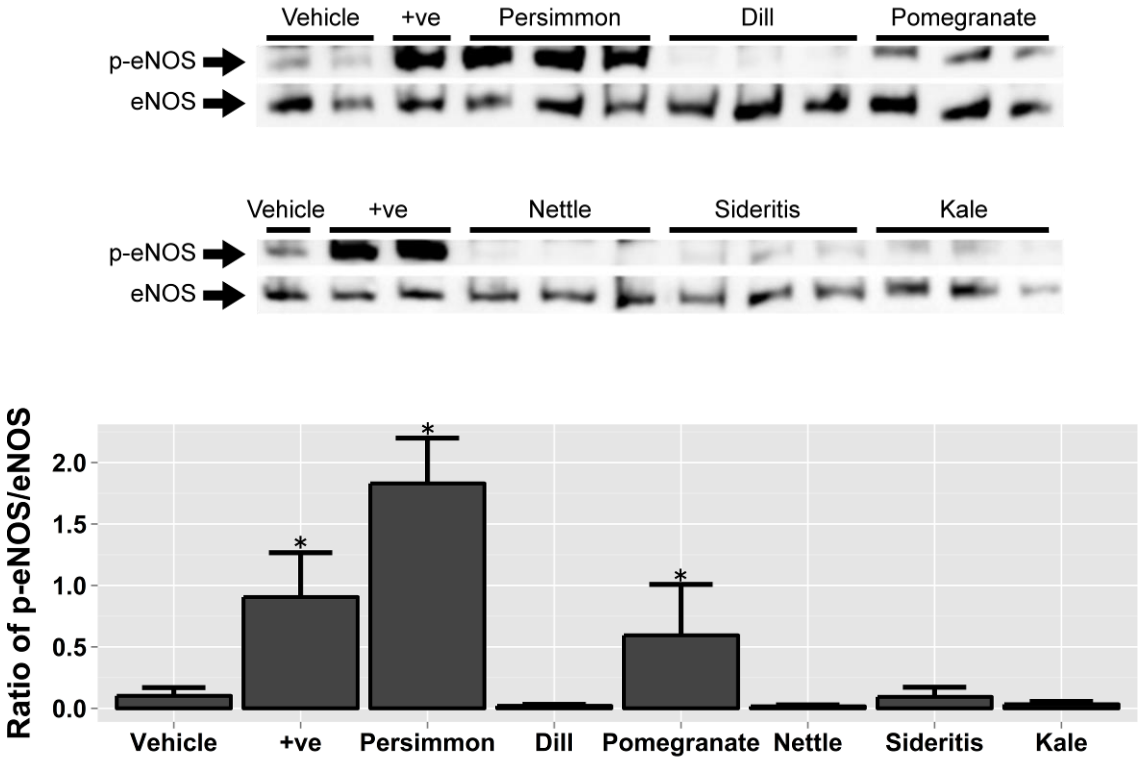

Figure 4.4: Western blots of p-eNOS/eNOS from lysates of HUVECs treated for 60 minutes with 100 μg GAE/ml of plant extracts alongside a vehicle control. 50 μM EGCG was used as the positive (+ve) control. n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-eNOS/eNOS) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

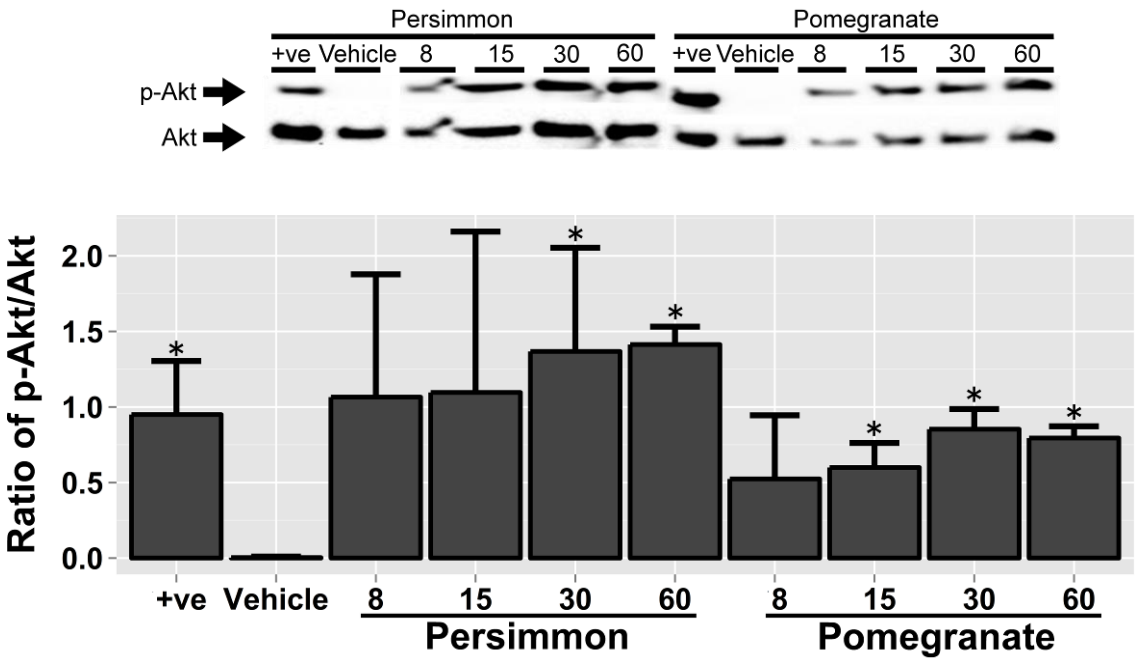

Figure 4.5: Western blots of p-Akt/Akt from lysates of HUVECs treated with 100 µg GAE/ml pomegranate and persimmon extract for different incubation periods (minutes). Blots representative of n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt)is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

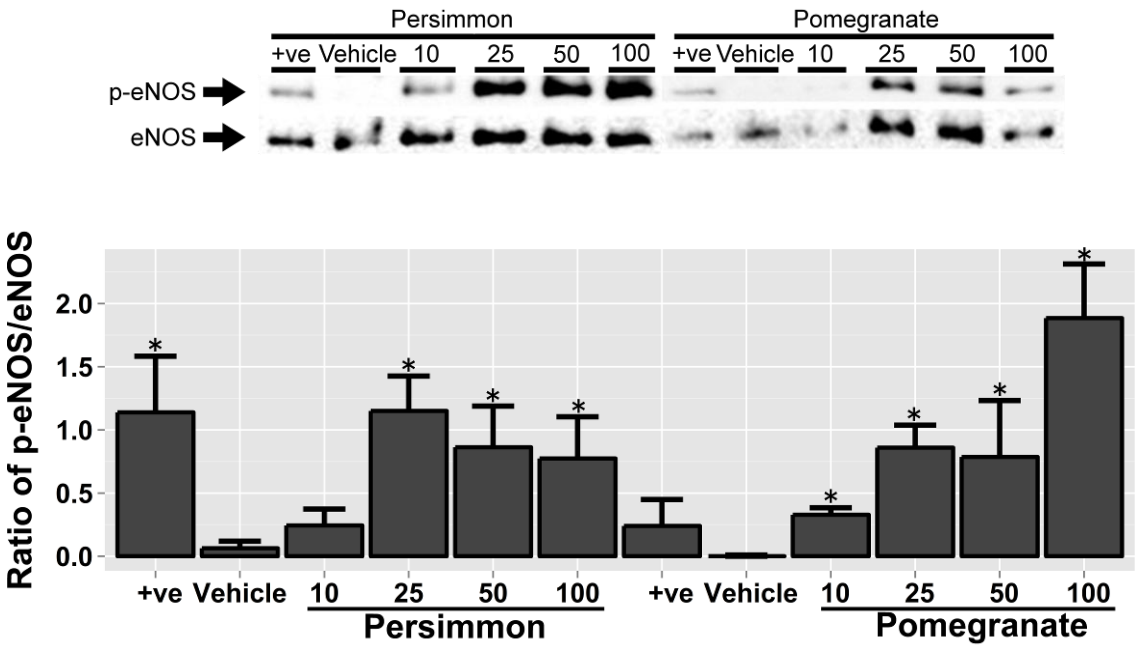

Figure 4.6: Western blots of of p-eNOS/eNOS from lysates of HUVECs treated for 60 minutes with different concentrations (μg GAE/ml) of pomegranate and persimmon extract. Blots representative of n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-eNOS/eNOS) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

4.5.4. Total eNOS protein and secreted ET-1 from endothelial cells treated with plant extracts and pure compounds

Treatment of cell cultures with 5 μM of the pure compound treatments over 24 hours had no effect on levels of total intracellular eNOS protein (**Fig. 4.7**) compared to the vehicle control. Secretion of ET-1 into cell culture media, however, was significantly decreased by quercetin ($p < 0.001$) and by the quercetin metabolites mix, sulforaphane metabolite mix and sulforaphane treatments ($p < 0.05$), compared to the vehicle control treatment (**Fig. 4.8**).

Treatment of cell cultures with pomegranate and persimmon extract at 20 μg GAE/ml for 24 hours resulted in significantly lower levels of ET-1 in the cell culture media ($p < 0.01$) compared to the vehicle control treatment. The pomegranate extract

caused significantly lower levels of intracellular eNOS protein ($p < 0.001$), as did the nettle extract ($p < 0.05$). The other extract treatments were not effective in these assays (**Figs. 4.9** and **4.10**).

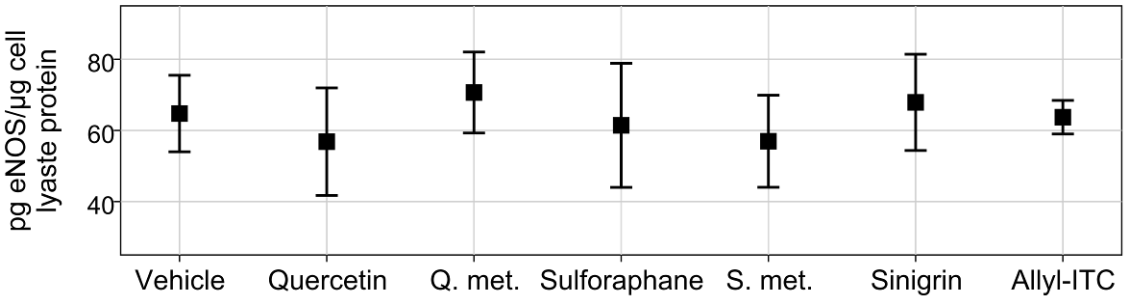

Figure 4.7: Mean levels of total eNOS protein in lysates from HUVECs treated with 5 µM pure compounds for 24 hours, ± standard deviation. n=6 (culture well replicates), over two independent experiments, for each treatment.

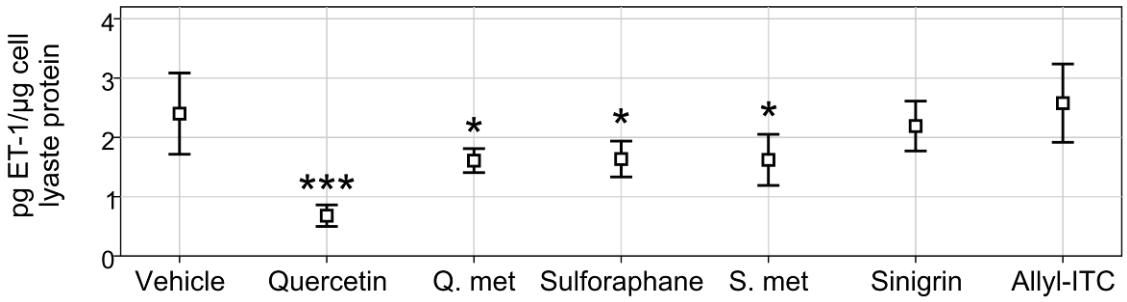

Figure 4.8: Mean levels of ET-1 secreted into culture media by HUVECs treated with 5 µM pure compounds for 24 hours, ± standard deviation. n=6 (culture well replicates), over two independent experiments, for each treatment. *** - $p < 0.001$ vs the vehicle control. * - $p < 0.05$ vs the vehicle control.

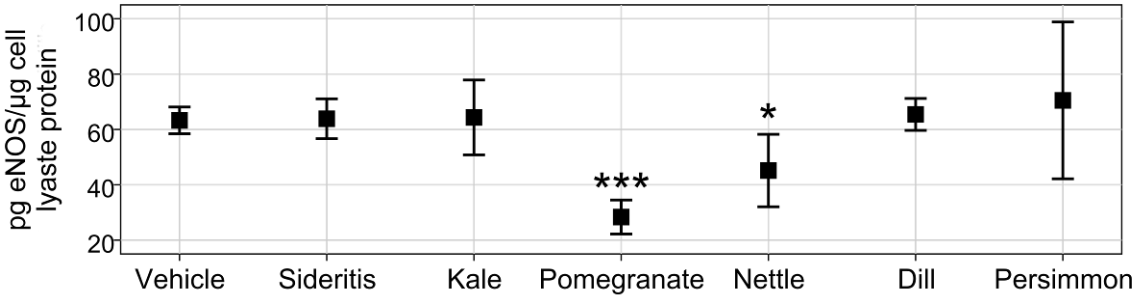

Figure 4.9: Mean levels of total eNOS protein in lysates from HUVECs treated with 20 µg GAE/ml plant extracts for 24 hours, ± standard deviation. n=6 (culture well replicates), over two independent experiments, for each treatment. *** - $p < 0.001$ vs the vehicle control. * - $p < 0.05$ vs the vehicle control.

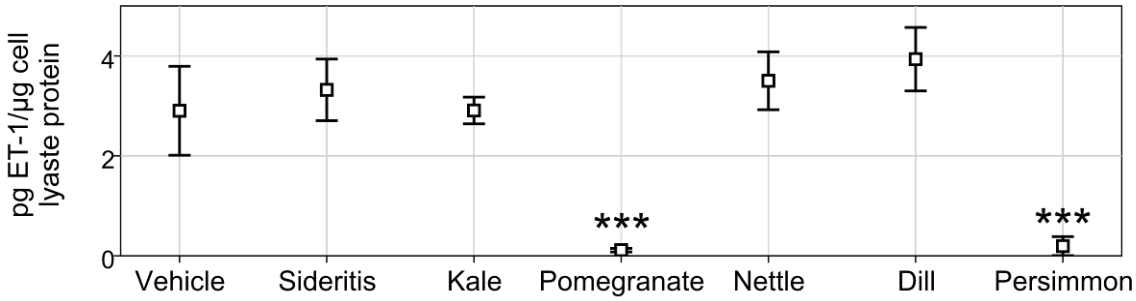

Figure 4.10: Mean levels of ET-1 secreted into culture media by HUVECs treated with 20 µg GAE/ml of plant extracts for 24 hours, ± standard deviation. n=6 (culture well replicates), over two independent experiments, for each treatment. *** - $p < 0.001$ vs the vehicle control.

4.5.5. cGMP production in cells treated with plant extracts and pure compounds

Analysis of the effects of pure compound or plant extract treatment on cGMP production in HUVECs proved inconclusive. A number of data points for the experimental treatments were below the limit of detection and could not be used. Furthermore, a positive control treatment using sodium nitroprusside did not significantly increase

cGMP production. The high degree of variance in results from the cGMP assay (**Figs. 4.11** and **4.12**) may be due to the low quantity of soluble guanylyl cyclase enzyme present in HUVECs.

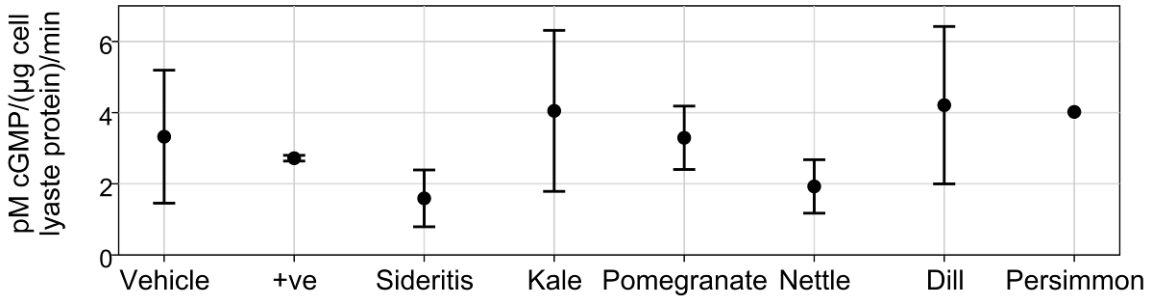

Figure 4.11: Mean levels of cGMP produced in HUVECs after treatment with 100 µg GAE/ml plant extracts, sodium nitroprusside (+ve) or a vehicle control, after inhibition of cGMP-degrading enzymes, ± standard deviation, where replicates allow. n=3 (culture well replicates) for the kale, dill and *Sideritis* treatments, n=2 for the pomegranate, nettle, positive control and vehicle control treatments, and n=1 for the persimmon treatment

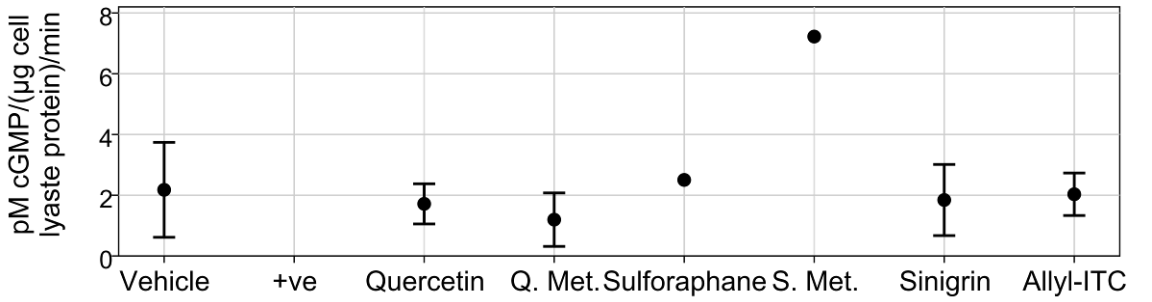

Figure 4.12: Mean levels of cGMP produced in HUVECs after treatment with 50 µM pure compounds, sodium nitroprusside (+ve) or a vehicle control, after inhibition of cGMP-degrading enzymes, ± standard deviation, where replicates allow. n=3 (biological replicates) for the sinigrin treatment, n=2 for the quercetin, quercetin metabolites, allyl-ITC and vehicle control treatments, and n=1 for the sulforaphane and sulforaphane metabolites treatments.

4.5.6. Nitrates/nitrites released into culture media from endothelial cells treated with plant extracts and pure compounds

Treatment of cell cultures with 5 μM of the pure compounds over 24 hours had no effect on levels of nitrates/nitrites released into cell culture media when compared to the vehicle control treatment (**Fig. 4.13**).

The dill, nettle and kale extracts all released very high concentrations of nitrates/nitrites when dissolved in EBM-2 (data not shown). To control for extract nitrate/nitrites, cell-free experiments were also performed with the extracts alongside a vehicle control and a blank media control. This also revealed a basal level of nitrate/nitrite already present in cell culture media (data not shown). Nitrate/nitrite values from the cell-free experiments were subtracted from the cell experiment values. Cell cultures treated with pomegranate and persimmon extract at 20 μg GAE/ml for 24 hours resulted in a significant increase of nitrates/nitrites released into cell culture media ($p < 0.01$) compared to the vehicle control treatment, while no difference was observed in media from cells treated with the *Sideritis* extract (**Fig. 4.14**), or the dill, nettle or kale extracts (**Fig. 4.15**).

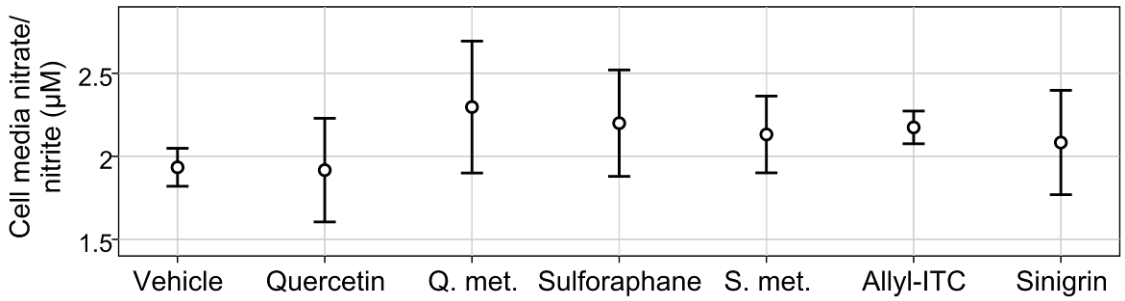

Figure 4.13: Mean levels of nitrate/nitrite in culture media after incubation of HUVECs with pure compounds (5 μM) for 24 hours, ± standard deviation. n=3 (culture well replicates) for each treatment.

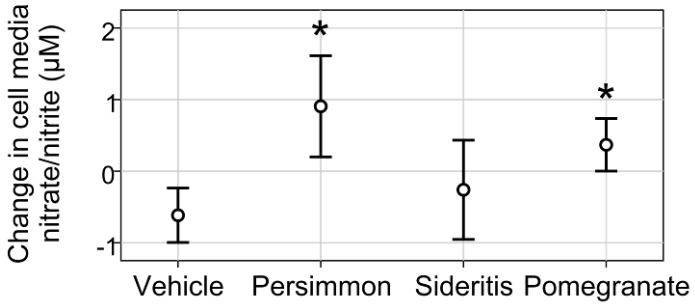

Figure 4.14: Changes to mean levels of nitrate/nitrite in culture media (compared to cell-free experiments) after incubation of HUVECs with *Sideritis*, pomegranate or persimmon extracts (20 μg GAE/ml) for 24 hours, ± standard deviation. n=3 (culture well replicates) for each treatment. * - $p < 0.05$ vs the vehicle control.

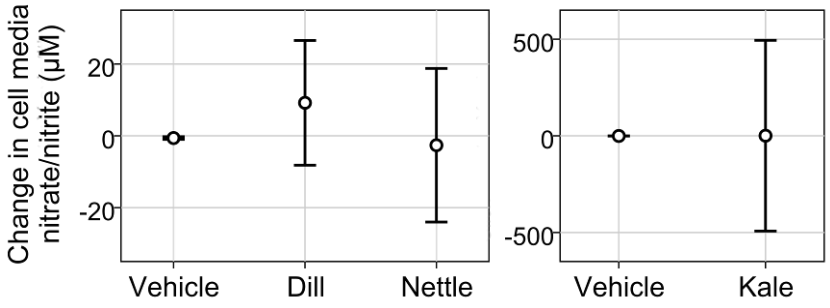

Figure 4.15: Changes to mean levels of nitrate/nitrite in culture media (compared to cell-free experiments) after incubation of HUVECs with dill, nettle or kale (20 μg GAE/ml) for 24 hours, ± standard deviation. n=3 (culture well replicates) for each treatment.

4.5.7. Effect of PI3K inhibition on the increase of Akt and eNOS phosphorylation by extracts of pomegranate and persimmon

Incubation of HUVECs with the PI3K chemical inhibitor LY294002 prior to incubation with the pomegranate or persimmon extract reduced the levels of both p-Akt and p-eNOS, compared to cells treated without the PI3K inhibitor (**Fig. 4.16**).

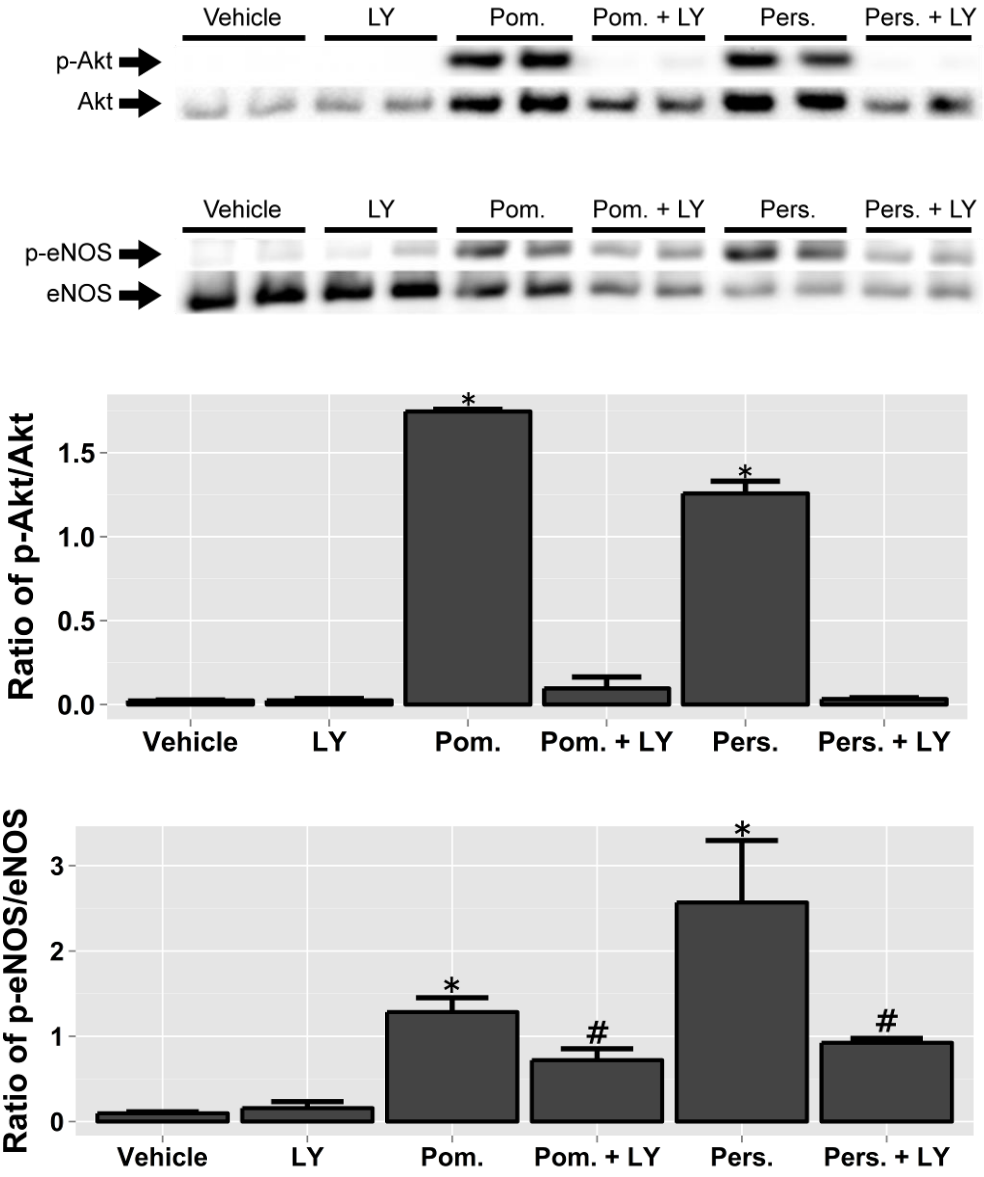

Figure 4.16: Western blots of p-Akt/Akt and p-eNOS/eNOS from lysates of HUVECs treated for 60 minutes with or without the PI3K inhibitor LY294002 (LY), prior to treatment with vehicle control, 50 µg GAE/ml of the pomegranate extract (Pom.) or 50 µg GAE/ml of the persimmon extract (Pers.) for a further 60 minutes. Blots representative of n=2 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt and p-eNOS/eNOS) is shown in the two lower panels. ***** - $p < 0.05$ vs vehicle control treatment. # - $p < 0.05$ vs vehicle control treatment and respective plant extract treatment (i,e. Pom. + LY vs Pom.)

4.5.8. Effects of plant extracts and pure compounds on WST-1 metabolism and cell morphology

The rate of WST-1 metabolism by HUVECs after treatment with plant extracts at a dose of 100 µg GAE/ml for 60 minutes was significantly ($p < 0.05$) reduced in all cases except for kale (**Fig. 4.17**), compared to the vehicle control. The rate was reduced to 85% by dill, to 59% by pomegranate, to 28% by persimmon, to 14% by nettle and to 1% by *Sideritis*. In contrast, 20 µg GAE/ml doses of each extract caused significant ($p < 0.05$) increases in metabolism of WST-1 for dill, kale and *Sideritis* (**Fig. 4.18**). Nettle was still reduced (46% of the vehicle control), as was pomegranate (91%) and persimmon (88%). 24 hours incubations of 10 µg GAE/ml doses of the extracts reduced the rate of WST-1 metabolism to less than 20% in all cases except *Sideritis*, which reduced the rate to 50% (**Fig. 4.19**).

Treatment of cells with 50 µM of sulforaphane and allyl-ITC for 60 minutes had no effect on WST-1 metabolism compared to the vehicle control (**Fig. 4.20**). Similar treatments with quercetin, quercetin metabolites, sinigrin and sulforaphane metabolites all significantly ($p < 0.001$) increased WST-1 metabolism (by 16%, 7%, 6% and 10% respectively). 24 hour incubations of each pure compound treatment at 50 µM reduced the rate of WST-1 metabolism in all cases (data not shown). Cells treated with sinigrin and quercetin metabolite treatments were the least affected in this respect. Following this, cells were treated with the pure compounds at 1, 5 and 10 µM doses (10 µM was the lowest concentration used for the sinigrin and quercetin metabolite treatments due to the relatively weak effects on metabolism of WST-1 mentioned above). Allyl-ITC and sinigrin had no effect at these concentrations compared to the vehicle control (**Fig. 4.21**). Sulforaphane and a mix of its metabolites both caused significant ($p < 0.001$) increases to metabolism of WST-1 (by 14% and 30% respectively, 5 µM dose). 10 µM of quercetin metabolites still reduced metabolism of WST-1 slightly, but significantly ($p < 0.001$), to

93%. Quercetin dose-dependently reduced metabolism of WST-1 to 59% at 10 µM, to 78% and 5 µM and to 97% at 1 µM, compared to the vehicle control.

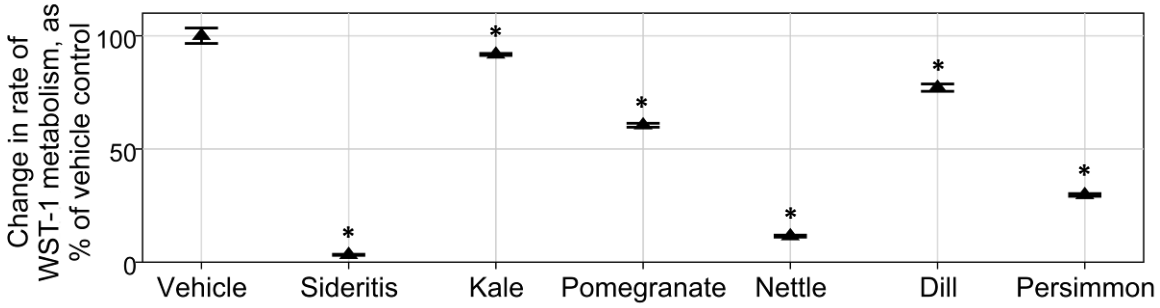

Figure 4.17: Rate, normalised to the vehicle control, at which WST-1 reagent was metabolised by HUVECs, after treatment of cells for 60 minutes with a 100 µg GAE/ml of each extract, ± standard error. n=6 (culture well replicates) for each treatment. ***** - $p < 0.05$ vs vehicle control treatment.

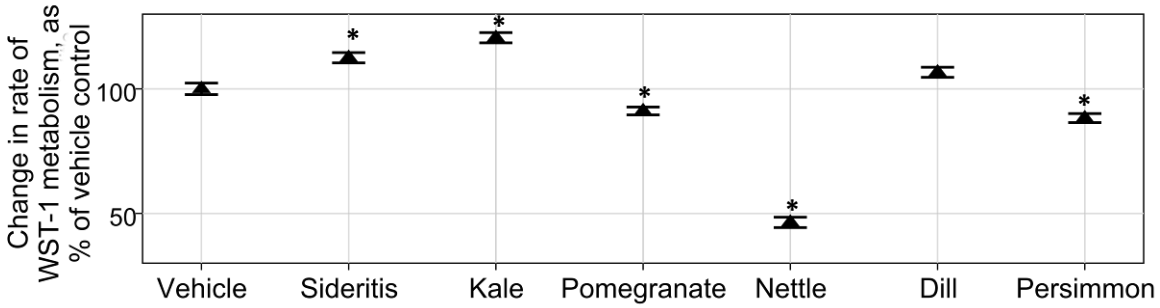

Figure 4.18: Rate, normalised to the vehicle control, at which WST-1 reagent was metabolised by HUVECs, after treatment of cells for 60 minutes with 20 µg GAE/ml of each extract, ± standard error. n=6 (culture well replicates) for each treatment. ***** - $p < 0.05$ vs vehicle control treatment.

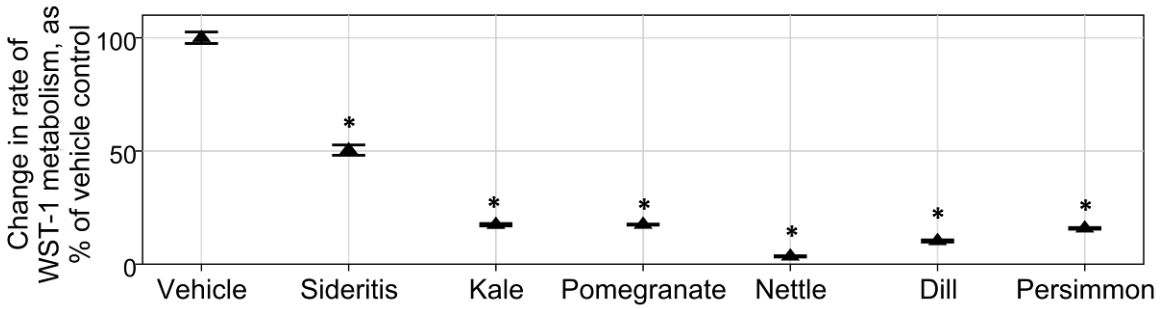

Figure 4.19: Rate, normalised to the vehicle control, at which WST-1 reagent was metabolised by HUVECs, after treatment of cells for 24 hours with 10 µg GAE/ml of each extract, ± standard error. n=6 (culture well replicates) for each treatment. ***** - $p < 0.05$ vs vehicle control treatment.

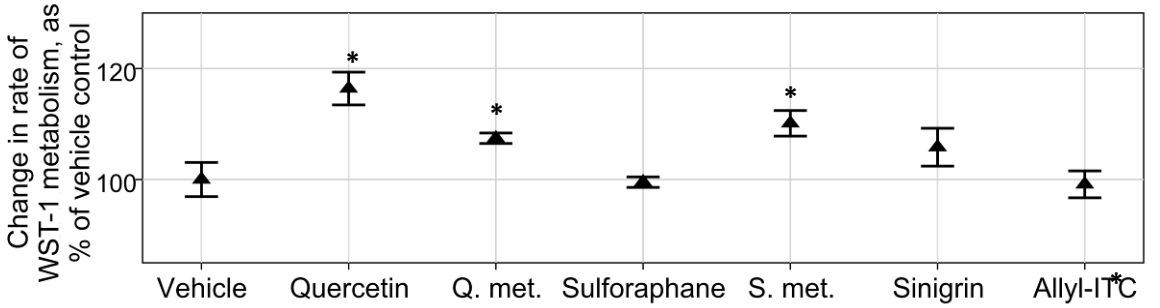

Figure 4.20: Rate, normalised to the vehicle control, at which WST-1 reagent was metabolised by HUVECs, after treatment of cells for 60 minutes with 50 µM of pure compounds, ± standard error. n=6 (culture well replicates) for each treatment. ***** - $p < 0.05$ vs vehicle control treatment.

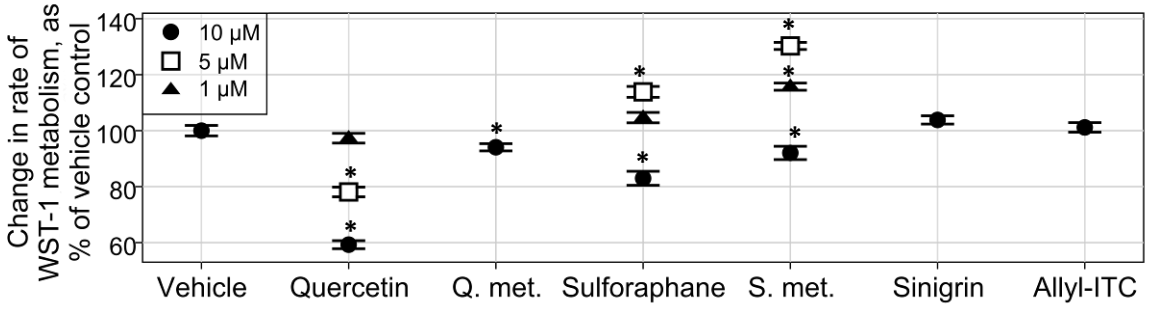

Figure 4.21: Rate, normalised to the vehicle control, at which WST-1 reagent was metabolised by HUVECs, after treatment of cells for 24 hours with indicated concentrations of pure compounds, ± standard error. n=6 (culture well replicates) for each treatment. ***** - $p < 0.05$ vs vehicle control treatment

It was observed that addition of pomegranate extract to cell culture media changed the culture media's pH indicator from pink to yellow. Measurement of the pH of solutions free of cell cultures showed a change of 7.6 to 6.7 after addition of pomegranate extract (at 50 µg GAE/ml). It is likely the extract contained organic acids (such as citric or malic acid (Melgarejo et al. 2000)) from the fruit arils.

Microscope images of cells (40X magnification) after exposure to 20 µg GAE/ml of the plant extract treatments (**Figs. 4.22** to **4.28**), or 5 µM of the pure compound treatments (**Figs. 4.29** to **4.34**), for 24 hours provided little evidence of toxicity. For example, while all treatments resulted in large numbers of detached, floating cells (as demonstrated in **Figs. 4.22** and **4.23**, where photographs were taken before floating cells were washed away), including the vehicle control treatment, there was little evidence of increased detachment of HUVECs from culture plates in comparison to the vehicle control, except in the case of quercetin and the nettle extract, where large cell-free areas were clearly visible, and persimmon, where smaller gaps were visible. The proportion of cells missing due 5 µM quercetin treatment for 24 hours appeared to correspond with the reduction in metabolism of WST-1 for the corresponding quercetin treatment (78%).

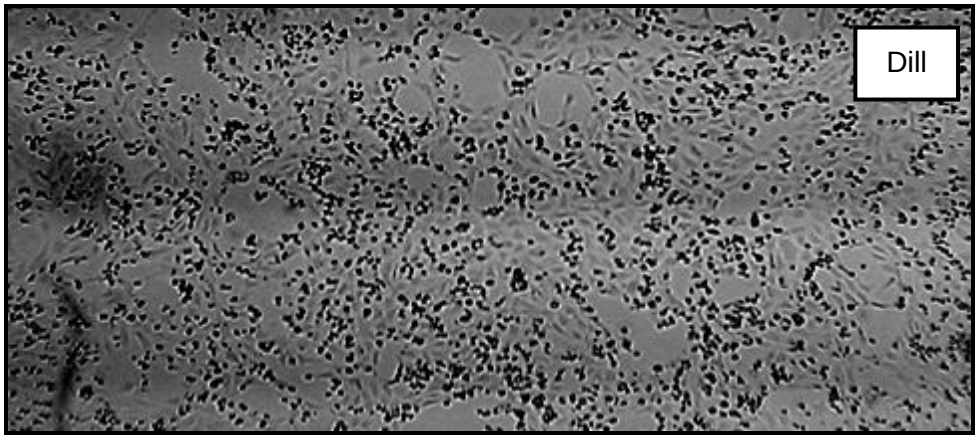

Figure 4.22: Photograph of HUVECs (40X magnification) after incubation with 20 µg GAE/ml of dill extract for 24 hours.

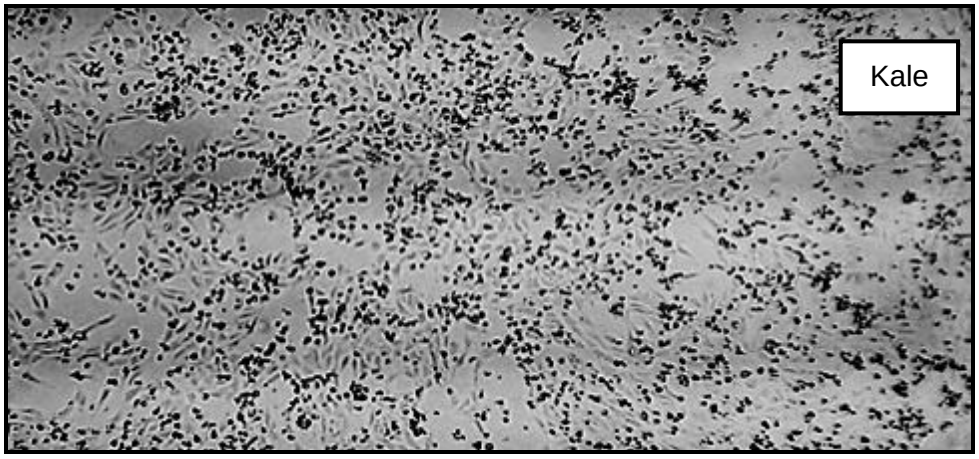

Figure 4.23: Photograph of HUVECs (40X magnification) after incubation with 20 µg GAE/ml of kale extract for 24 hours.

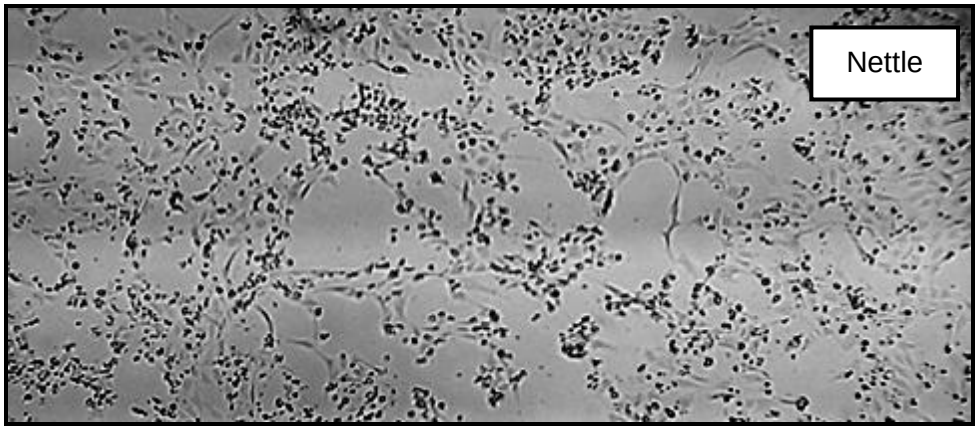

Figure 4.24: Photograph of HUVECs (40X magnification) after incubation with 20 µg GAE/ml of nettle extract for 24 hours.

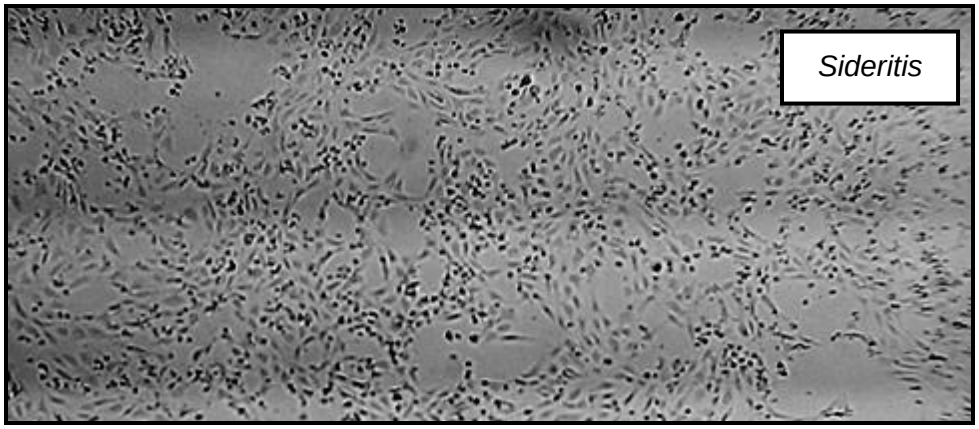

Figure 4.25: Photograph of HUVECs (40X magnification) after incubation with 20 µg GAE/ml of *Sideritis* extract for 24 hours.

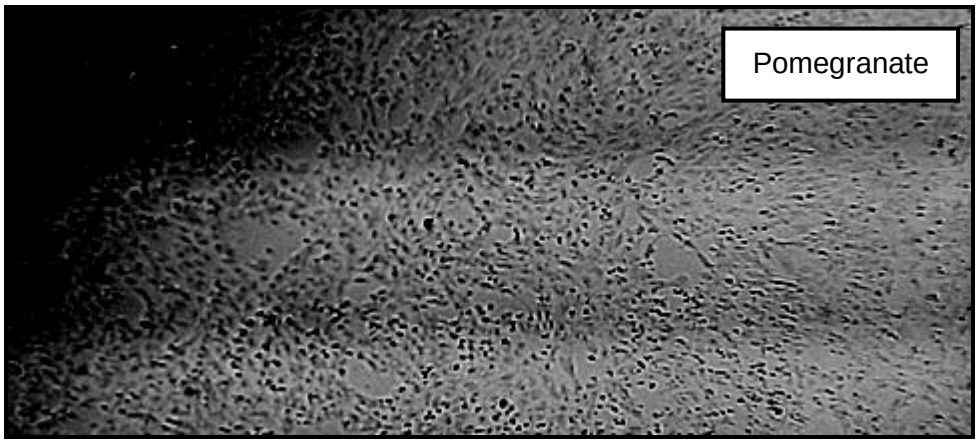

Figure 4.26: Photograph of HUVECs (40X magnification) after incubation with 20 µg GAE/ml of pomegranate extract for 24 hours.

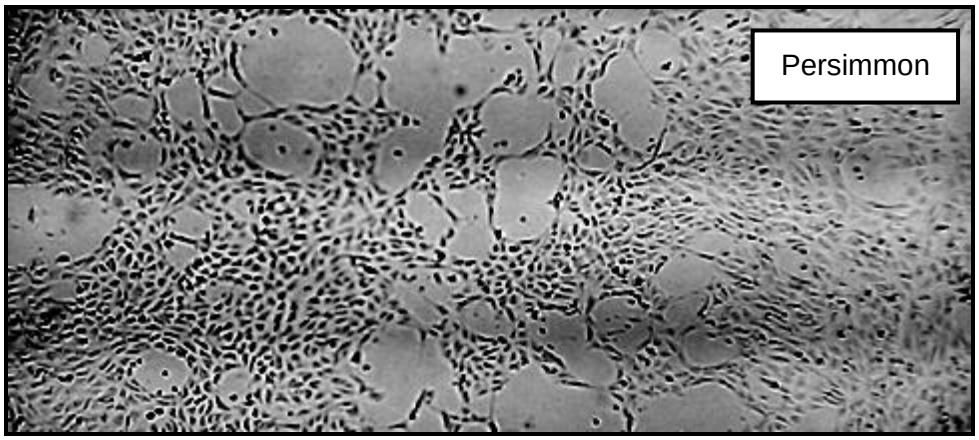

Figure 4.27: Photograph of HUVECs (40X magnification) after incubation with 20 µg GAE/ml of persimmon extract for 24 hours.

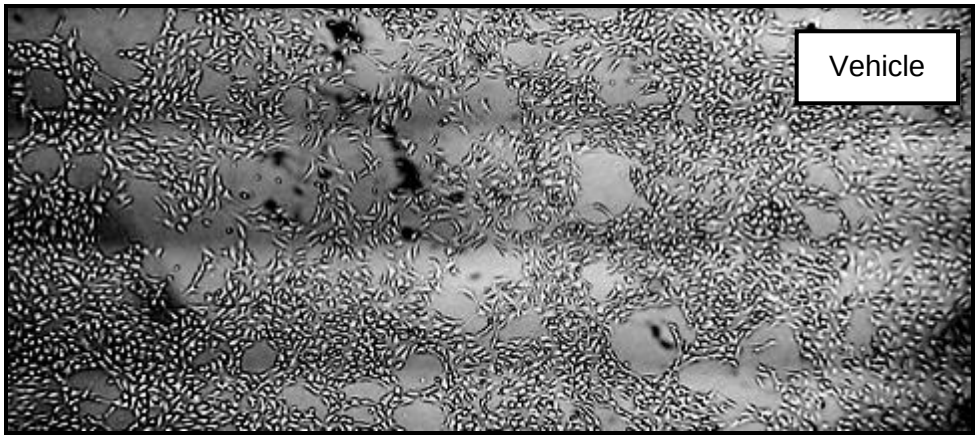

Figure 4.28: Photograph of HUVECs (40X magnification) after incubation with vehicle control (0.1% DMSO) for 24 hours.

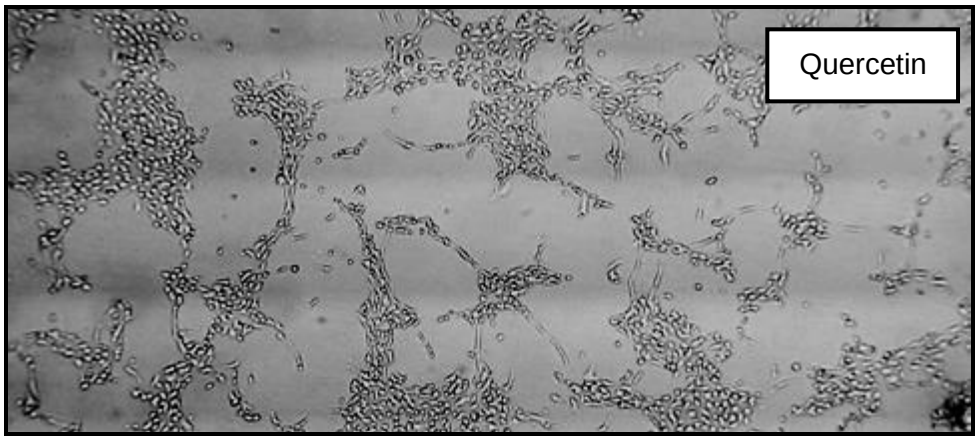

Figure 4.29: Photograph of HUVECs (40X magnification) after incubation with 5 µM quercetin for 24 hours.

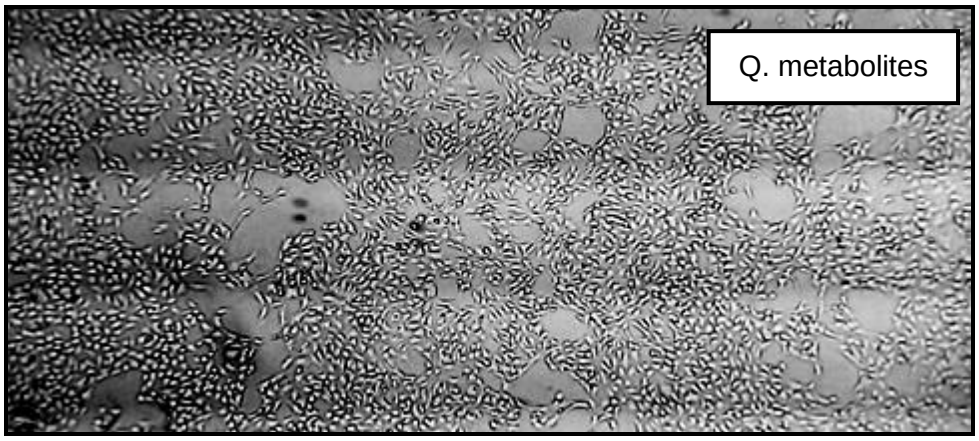

Figure 4.30: Photograph of HUVECs (40X magnification) after incubation with 5 µM quercetin metabolites for 24 hours.

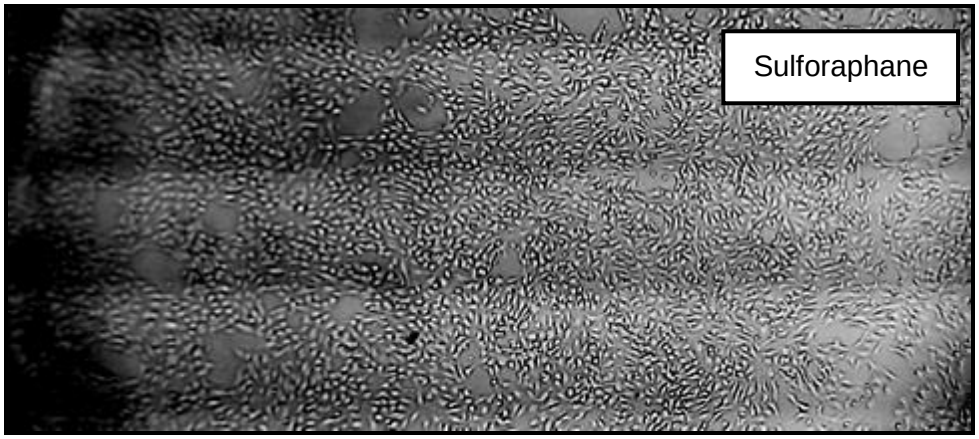

Figure 4.31: Photograph of HUVECs (40X magnification) after incubation with 5 µM sulforaphane for 24 hours.

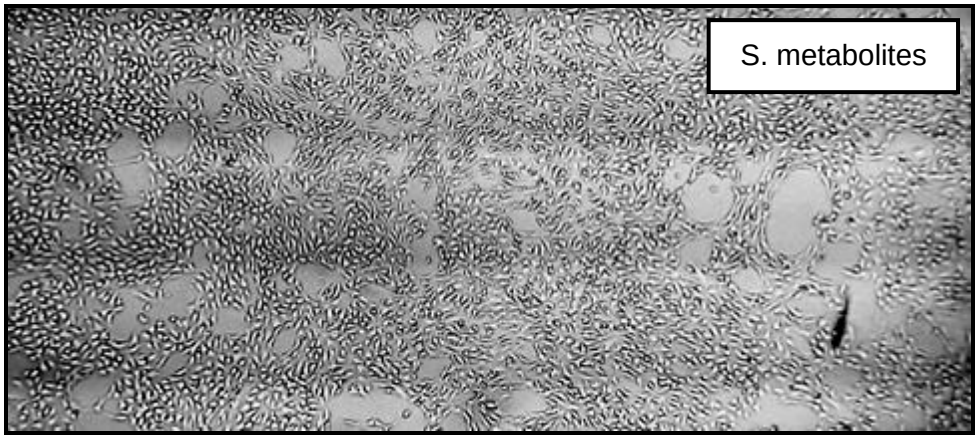

Figure 4.32: Photograph of HUVECs (40X magnification) after incubation with 5 µM sulforaphane metabolites for 24 hours.

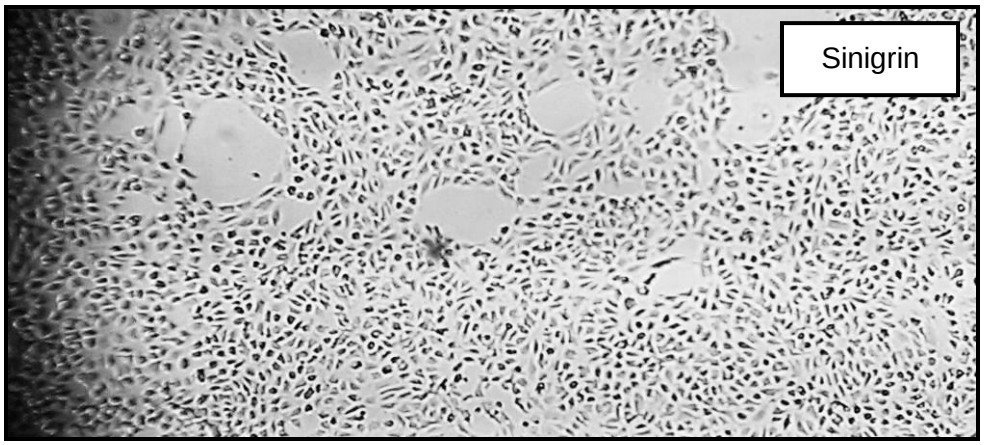

Figure 4.33: Photograph of HUVECs (40X magnification) after incubation with 5 µM sinigrin for 24 hours.

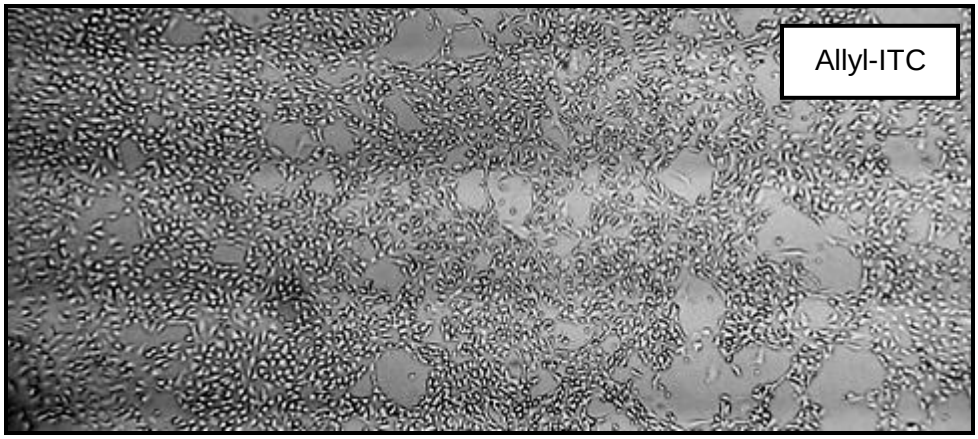

Figure 4.34: Photograph of HUVECs (40X magnification) after incubation with 5 µM allyl-ITC for 24 hours.

4.6. Discussion

Assays measuring changes to signalling molecules involved with endothelial nitric oxide production and the regulation of vascular tone were used to test the affects of extracts prepared from traditional foods characteristic of countries surrounding the Black Sea. Phosphorylation (activation) of Akt and eNOS, and culture media nitrate/nitrite levels were significantly increased, and ET-1 secretion was significantly decreased, by an extract of persimmon and pomegranate, but not by extracts of nettle, *Sideritis*, kale or dill. There were no significant effects in response to treatment with quercetin, quercetin metabolites, sulforaphane, sulforaphane metabolites, sinigrin or allyl-isothiocyanate.

4.6.1. Treatment of endothelial cells with plant extracts and pure compounds for the study of signalling molecules relevant to vasodilation

The effects on Akt and eNOS phosphorylation, total eNOS protein, ET-1 secretion and culture media nitrate/nitrite levels in endothelial cells treated with bioactive-rich extracts of dill, kale, nettle, *Sideritis* and persimmon (edible parts) are reported here for the first time. This is also the first report describing the effects of a persimmon fruit extract on Akt and eNOS phosphorylation (**Figs. 4.2**, **4.5** and **4.6**), the effects of pomegranate extract on phosphorylated and total eNOS protein in endothelial cells cultures (**Fig. 4.9**), and the first to show increased nitrate/nitrite levels, and decreased ET-1 secretion due to pomegranate or persimmon extract (**Figs. 4.10** and **4.14**). The significant, though smaller, decrease in the secretion of ET-1 to cell culture media due to treatment with quercetin or a mix of its metabolites (**Fig. 4.8**) is in agreement with previous reports (Lodi et al. 2012; Zhao et al. 1999), although this appears to be the first report of quercetin and its metabolite lowering ET-1 secretion in resting endothelial cells, and the first to show a significant decrease ($p < 0.05$) in response to sulforaphane or its human metabolites. The stability of ET-1 in biological buffers has been previously demonstrated (Zhang & Luo

1999), and so it is likely that the pomegranate and persimmon extracts either strongly inhibit ET-1 secretion, perhaps as a direct consequence of the increased production of nitric oxide (Bourque et al. 2011), or promote its degradation through another signalling mechanism or by chemical means.

Persimmon and pomegranate have been previously reported to effect markers of endothelial function and CVD. Gorinstein et al. (2011) reported decreased plasma lipid levels and reduced aortic atherosclerotic lesions in rats compared to control treatments, after feeding dried persimmon fruits to rats on a high cholesterol diet. An extract prepared from the leaves of persimmon (but not the fruit) was also able to increase Akt and eNOS phosphorylation in HUVECs, within 15 minutes of exposure to cells (and at a dose approximately 200 times weaker than that used in this study) (Kawakami et al. 2011), and furthermore improved dilation of rat aortic tissue ex vivo in an endothelium- and NO-dependent manner (Yin et al. 2005). de Nigris et al. (2007a; 2007b) reported that a pomegranate extract could increase eNOS expression and slow down the progression of atherosclerosis in atherogenic models of rats and mice, and increase eNOS enzyme activity in human endothelial cells. Ignarro et al. (2006) reported similar effects of pomegranate on eNOS activity in bovine endothelial cells. The concentrations of quercetin used in these experiments matched those which caused a significant decrease to eNOS phosphorylation in Chapter 3 (**Fig. 3.1**). For the work in that chapter, cells were pre-treated with EGCG to stimulate eNOS phosphorylation. No such pre-treatment was performed in this study, however, which may explain why no effect on eNOS phosphorylation after treatment of cells with quercetin was observed.

Despite a lack of effect in the other assays performed, the nettle extract was able to significantly ($p < 0.05$) decrease total eNOS protein in cells over 24 hours (**Fig 4.9**) (but not over 60 minutes (**Fig. 4.4**)). While treatment with pomegranate extract also caused a significant reduction ($p < 0.001$) in total eNOS protein, treatment of cells with the persimmon extract did not. It could not, therefore, be concluded that the decrease in

total eNOS protein over 24 hours was in response to increased nitric oxide production. If increased nitric oxide production does indeed reduce intracellular levels of eNOS protein in the 24 hour period following Akt and eNOS phosphorylation, this could be confirmed by pre-treatment of cells with a chemical inhibitor of Akt activation, such as LY294002.

Nitric oxide binds to soluble guanylyl cyclase, greatly increasing the rate that the enzyme produces cGMP in smooth muscle cells, which ultimately leads to the relaxation of the smooth muscle tissue. There are numerous other pathways dependent on cGMP for activity (Francis et al. 2010), and sGC protein and activity have been reported in most cells and tissues, including endothelial cells (Takeuchi et al. 2004; Boulanger et al. 1990; Potter 2011). Therefore it should have been possible to measure cGMP increase in HUVECs, after inhibition of phosphodiesterases using IBMX, though this was obviously not the case. It is possible that nitric oxide produced by HUVECs was either not reaching sGC in the cells, perhaps due to its diffusion to cell culture media, or the sGC content was too low in the cells to produce measurable levels of cGMP. Where cGMP levels were recorded, they were higher than those obtained by Donnini et al. (2006) (pM cGMP/µg HUVEC lysate protein compared to pM cGMP/mg HUVEC lysate protein). It is likely that the cGMP measurements presented here were false positives, especially considering the inability of the positive control to produce a consistent response, and that a more sensitive assay is required. It has also been reported that vascular smooth muscle cells become desensitised to sGC activation by sodium nitroprusside once they have been passaged five or more time (Boulanger et al. 1990), though such effects have not yet been reported for passaged endothelial cells.

Despite the lack of bioactivity from the dill, nettle, *Sideritis* and kale extracts, and the pure compound treatments, it is still possible that compounds in these food plants could modulate signalling molecules related to vasodilation *in vivo*. Likewise, it is not possible to conclude that the compounds which provide the bioactivity of the pomegranate and persimmon extracts would cause similar effects *in vivo*, due to

metabolism and resulting structural changes to the compounds, and possible limits to their absorption in the gastrointestinal system. The composition of the plant extracts used in the *in vitro* work described here is likely to be quite different from the profile of compounds which would reach the endothelium *in vivo*. after preparation of the plants (including storage and cooking), and consumption in the diet. Polyphenols also tend to be susceptible to oxidation in cell culture media, more so than when they are circulating in plasma. This results in their degradation while incubating with cell cultures, which may attenuate or augment particular bioactive effects. Furthermore, the solubility of compounds can affect they're efficacy *in vitro* compared to their *in vivo* activity. While none of the extracts were observed to produce precipitates when they were first introduced to the cell culture media, observations made under the microscope at the conclusion of cell culture treatments confirmed the presence of particulate matter floating in the media. While this matter would have included dead cell debris, it is also likely that compounds in the plant extracts will have come out of solution over time, possibly due to their degradation. No experiments were performed to determine the solubility over time of the plant extract compounds in the cell culture media used, though it may be more useful to perform such experiments with individual compounds that have been identified as bioactive.

HUVECs were used here as an *in vitro* cell model for endothelial signalling, with the respective *in vivo* situation being the signalling which occurs in the endothelium lining human arteries. While umbilical vein endothelial cells will experience oxygenated blood flow similar to the arteries, the blood pressure in umbilical veins is much lower (6 mmHg at term (Pesola et al. 2001), compared to ~112 mmHg systolic/~64 mmHg diastolic in healthy adults (Spurway et al. 2012)). The smooth muscle tissue surrounding the endothelium of umbilical veins is also much thinner compared to adult human arteries. Therefore it is unlikely that these cells behave exactly as endothelial cells do in adult arteries *in vivo*. This should be taken into consideration when drawing conclusions

on the molecular outcomes in HUVECs that have been used to assay polyphenol bioactivity, even when the polyphenols used are the physiologically relevant human metabolites.

4.6.2. Effect of plant extracts and pure compounds on cell adhesion to culture plate surfaces and metabolism of WST-1

It was clear that some of the treatments (notably the 24 hour incubations of quercetin, nettle and probably persimmon) were cytotoxic at the concentrations used to measure effects on endothelial vasomodulatory factors, and this was taken into account when analysing results from these assays. Both microscope observations and results from the WST-1 assay suggested that these treatments were causing decreases to the number of cells attached to the culture plates, compared to vehicle controls (**Figs. 4.17** - **4.19, 4.21, 4.24, 4.27** and **4.29**). It is interesting to note that the cytotoxicity of quercetin is reduced when quercetin's structure is altered to that of its phase II metabolites. Based on the WST-1 results, 5 µM doses of the pure compounds were used for subsequent 24 hour cell culture experiments. 24 hour cell culture experiments with the plant extracts were performed using a 20 µg GAE/ml dose, as despite large decreases to WST-1 metabolism, microscope images of cells did not suggest that there were significantly detrimental effects to cell cultures due to these treatments.

WST-1 is one of a number of tetrazolium dyes that are used to determine effects on cell viability and metabolism. Upon metabolism by viable cells, WST-1 forms water soluble formazan compounds that are yellow/orange in colour (Assalian & Francoeur 1996). Experiments such as the LDH assay, which measures lactate dehydrogenase released into culture media from burst cells, require the experimental media for proper measurement. As the WST-1 reagent was dissolved in fresh media before application to cells, potential inference in light absorption measurements caused by the pigments of the

plant extracts was avoided. The absorbance of the yellow colour produced can be correlated with the metabolic activity of the cells, though not always with cell viability. For example, it is possible for metabolism of WST-1 to continue, even after cell death (Quent et al. 2010). Metabolism most likely occurs at the cell surface or the plasma membrane via trans-plasma membrane transport, due to the cell-impermeable nature of the formazan compounds produced (Assalian & Francoeur 1996).

It is possible that compounds in the extracts were attaching to the surface of cell cultures, or interfering with cell membrane biology in a manner than allowed cells to remain attached to the culture plate but still reduce metabolism of WST-1 independent of cytotoxic/non-cytotoxic effects. As the plasma membrane is the most probable location for metabolism of WST-1 (Liu et al. 1997; Berridge et al. 2005), interference by compounds here would impede the conversion of WST-1 to formazan dye, and explain reductions seen in the WST-1 assay for cells treated with plant extracts for 24 hours, despite the lack of obvious differences seen between cell cultures viewed down the microscope.

4.6.3. Preparation of plant extracts

So as to give no bias to a particular type of compound when extracting polyphenols and other bioactives from the plant material, the same extraction method was used for each food plant. Aqueous methanol has been previously shown to act as a good solvent for leaching a wide variety of phytochemicals from plant material in a non-selective manner (Tsao 2010; Robards 2003). While care was taken to keep the majority of the extraction method the same for each plant, the nature of each plant's material meant that plant-specific changes to the protocol, such as omittance of the drying steps for the pomegranate arils, were necessary. In this case, as the material was already in a form that could be easily extracted, degradation of bioactive compounds was assumed unlikely due to the short preparation time. During the removal of water from the extract supernatants,

the pomegranate and persimmon extracts required a more intensive evaporation procedure than the kale extract. This was most likely due to the high quantity of sugars extracted from the fruits (reports of > 10 g/100g fresh weight (Glew et al. 2005; Melgarejo et al. 2000), compared to, for example, < 0.1 g/100g fresh weight for kale (Noichinda et al. 2007)), which would make the extracts more hygroscopic. Care was taken to ensure minimal exposure of the pomegranate and persimmon extracts to air, to avoid uptake of additional water into the extracts. The higher moisture/sugar content of these extracts is highlighted by their low TPC values (**Table 4.2**). Sugars such as sucrose and fructose have been previously reported to react with the Folin-Ciocalteu reagent. Such increases, however, are likely to be negligible (Waterhouse 2001; Everette et al. 2010; Lester et al. 2012).

4.7. Conclusion

Of the selected plant extract and pure compound treatments, only the pomegranate and persimmon extracts provided consistent evidence that they could positively affect vasomodulatory factors in a manner consistent with increased nitric oxide production. They increased Akt and eNOS phosphorylation in a PI3K-dependent manner, elevated nitrate and nitrite concentrations in cell culture media and sharply decreased levels of secreted ET-1. It is clear that pomegranate and persimmon both contain compounds able to affect nitric oxide signalling and ET-1 secretion. Further investigation is required, however, to elucidate the effective compounds and the mechanisms involved in the activation of Akt and eNOS by these extracts.

Chapter 5

An investigation of the compounds and mechanisms responsible for pomegranate extract bioactivity

Chapter 5: An investigation of the compounds and mechanisms responsible for pomegranate extract bioactivity

5.1. Abstract

In vitro studies to measure molecules involved in nitric oxide signalling and vasodilation, as detailed in the previous chapter, showed that methanolic extracts of pomegranate and persimmon were able to affect these molecules in a manner associated with increased nitric oxide production. As these extracts are complex mixture of compounds, it is not known which were responsible for the extracts' bioactivity, nor how they were able to increase PI3K/Akt activity. The aim of the research described in this chapter was to identify the compounds responsible and the underlying mechanisms by which the pomegranate extract induced nitric oxide signalling.

Activity-guided fractionation in combination with LC-MS analyses were used to identify the compounds responsible for bioactivity of the extract. The molecular signalling mechanisms underlying the bioactivity were investigated using (1) an antibody microarray spotted with antibodies complementary for a selection of receptor tyrosine kinases (RTK) that lie upstream of the Akt/eNOS pathway and (2) western blotting and enzyme activity assays to determine affects on the phosphatase PTEN (which reverses the effects of PI3K, therefore suppressing Akt activation).

It was shown that procyanidins, of size and concentration similar to those observed in the pomegranate extract, were able to elicit a strong induction of Akt and eNOS phosphorylation. No evidence could be found, however, that this was due to either an increase in the phosphorylation and activation of RTKs upstream of Akt, or a rapid inhibition of PTEN activity. Further research is required to establish the mechanisms responsible for activation of Akt and eNOS by pomegranate polyphenols.

5.2. Introduction

5.2.1. Polyphenol content of pomegranate arils

Pomegranates contain a variety of different phenolic compounds. In terms of those found specifically in the edible arils, most fall into one of three subclasses:

- flavan-3-ols (and oligomers of flavan-3-ols, proanthocyanidins) (de Pascual-Teresa et al. 2000; Sentandreu et al. 2010),
- ellagitannins (Borges et al. 2010; Mertens-Talcott et al. 2006)
- and anthocyanins (Alighourchi et al. 2008; Mousavinejad et al. 2009; Robert et al. 2010; Sepulveda et al. 2010).

A number of phenolic acids, lignans and flavonols may also be present in relatively small quantities, as evidenced by a recent study utilising ultra-HPLC-MS^n analysis (Mena et al. 2012). While these polyphenols are common across most cultivars of pomegranate, the quantities present in each can vary substantially.

Three ellagitannins - punicalagin (**Fig. 5.1**), punicalin and granatin - have been identified in pomegranate aril juice, in both their α- and β- anomeric forms. A galloylated ester of punicalagin has also been described (Borges et al. 2010; Gil et al. 2000), as well as ellagic acid and ellagic acid-O-hexoside.

There are six main anthocyanins which give pomegranate fruit juice its deep red and purple colours, these being 3-O-glucosides and 3,5-di-O-glucosides of cyanidin (**Fig. 5.2**), delphinidin and pelargonidin (Mousavinejad et al. 2009; Alighourchi et al. 2008). Recently, a pentoside and rutinoside of cyanidin were also identified in pomegranate (Fischer et al. 2011), as well as 3-O-glucosides of peonidin, malvidin and petunidin (Borges & Crozier 2012), though no details of the material from which the pomegranate juice was prepared were given. Peonidin has been identified as a component of pomegranate peel (Zhao et al. 2012), and inclusion of peel phenolics in pomegranate juices, depending on the process used, would not be unusual.

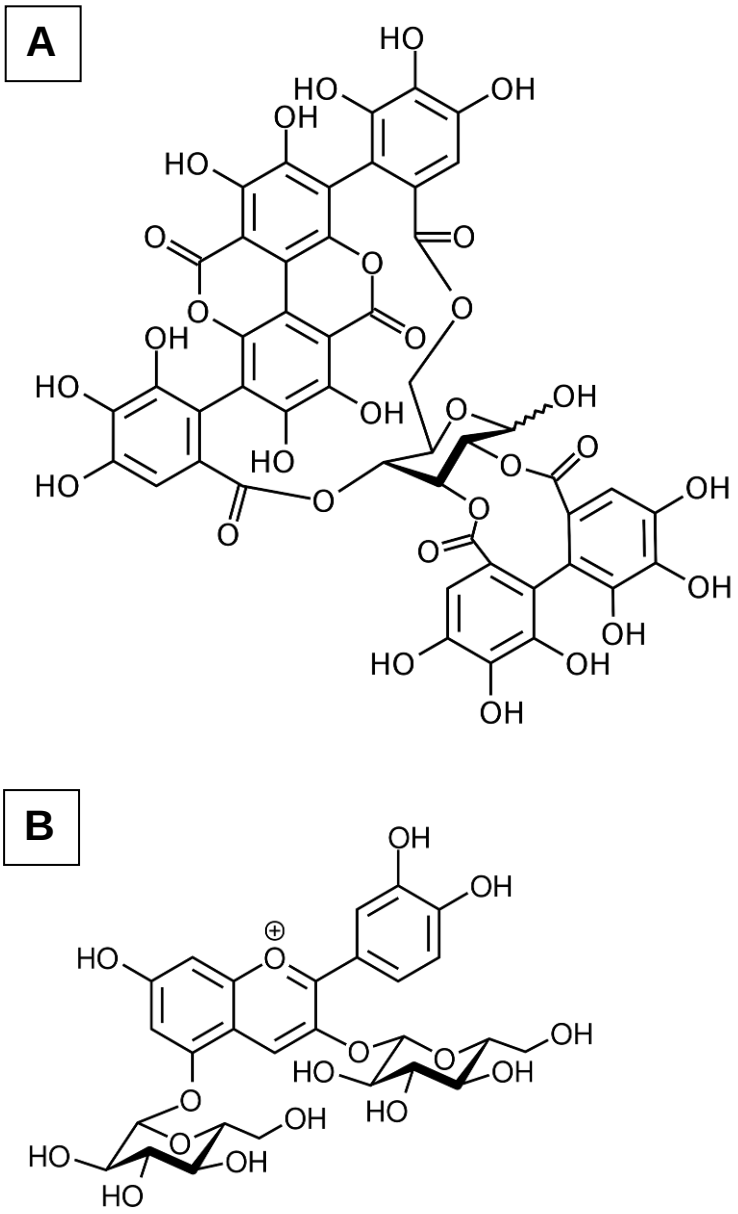

Figure 5.1: (**A**) Punicalagin, an ellagitannin found in pomegranates and (**B**) cyanidin-3,5-di-O-glucoside, an anthocyanin found in pomegranates.

Despite a number of general and specific methods for the identification of proanthocyanidins, the complex nature of their structure can complicate such analyses (Hümmer & Schreier 2008). Though catechin, epicatechin, gallocatechin and epigallocatechin have all been identified in pomegranate, at present what is known of their proanthocyanidin content is limited to procyanidin dimers and trimers (de Pascual-Teresa et al. 2000; Borges et al. 2010). The membranous sacs of pomegranate arils are a

likely source of procyanidins (Dr Francisco A. Tomás-Barberán, personal communication, 24 March 2013), although in preparation of pomegranate juices, particularly in commercial circumstances, proanthocyanidins and other polyphenols from additional parts of the fruit, such as the peel (Saad et al. 2012), are likely to be included in the resulting product (Wang et al. 2010; Türkyılmaz et al. 2013).

5.2.2. Upstream mechanisms for Akt induction

Data presented in **Chapter 4** showed that the increased Akt phosphorylation was PI3K-dependent (**Fig. 4.16**), i.e. dependent on sufficient levels of PIP_3 so that Akt may be present at the plasma membrane to be phosphorylated. Therefore, increased Akt activity is dependent on either increased class 1 PI3K activity or a reduction in the activity of a PIP_3 phosphatase.

Class 1A PI3Ks are activated by either receptor tyrosine kinases (RTKs) or guanine nucleotide-binding (G) protein coupled receptors (GPCRs), whereas class 1B PI3Ks can only be activated by GPCRs (Shiojima & Walsh 2002; Morello et al. 2009). Over 200 GPCRs and their activating ligands have been described in humans. They have a seven-transmembrane structure and couple with G-proteins consisting of α, β and γ subunits upon activation. Gα can activate PI3K indirectly and Gβγ directly (New et al. 2007; Strotmann et al. 2011). Activation of RTKs is accomplished by interaction of growth factors with extracellular domains. RTKs are phosphorylated upon activation and may autophosphorylate further. With assistance from adaptor proteins, RTK pYXXM motifs are able to bind PI3K's regulatory domain, and expose its catalytic domain, so that it may act on phosphoinositides. Their activation leads to the recruitment of proteins such as Lyn, Fyn and Src, which can phosphorylate additional tyrosine residues or act to recruit additional substrate molecules such as PI3K (Engelman et al. 2006). There are 58

RTKs reported in humans, divided into 20 families, with the most extensive families being VEGF, PDGF, ErbB, Eph and FGF (Lemmon & Schlessinger 2010).

While PI3K acts to increase levels of PIP_3, this behaviour is countered by the phosphatases SHIP and PTEN. SHIP has two isoforms, SHIP1 and SHIP2. The former is expressed mostly in haemopoietic cell lineages and in unlikely to be found in endothelial cells (Liu et al. 1998), whereas SHIP2 is more broadly expressed (Erneux et al. 2011). After activation, likely by phosphorylation of tyrosine residues on SHIP, they convert $PI(3,4,5)P_3$ to $PI(3,4)P_2$. PTEN, on the other hand, converts PIP_3 to $PI(4,5)P_2$. PTEN is held in an inactive state in the cytosol through modifications such as phosphorylation at Ser380, Thr382 and Thr383 or acetylation at Lys158 and Lys138 (Singh & Chan 2011). Removal of these modifications allows for its translocation to the plasma membrane, where it performs its phosphatase activity (Das et al. 2003). PTEN is also regulated through ubiquitination in the cytosol and at the plasma membrane, leading to its degradation (Maccario et al. 2010). The removal of PTEN from healthy cells is known to lead to increased levels of Akt phosphorylation (Stambolic et al. 1998). Indeed, PTEN has been well studied in models for cancer where the lack of its expression promotes dysregulation of the cell cycle and activation of Akt (Chetram & Hinton 2012). It would not be unreasonable to assume the basal level of Akt phosphorylation is maintained in resting endothelial cells by the activity of PTEN.

As described above, there are a several ways in which the pomegranate extract used in this work might act on endothelial cells to increase Akt phosphorylation. In this chapter, two of the possible mechanisms that could give rise to increased Akt activity were investigated. The activity of PTEN has been well characterised in HUVECs and other endothelial cell models. Here, a number of assays were performed to measure activity-related changes to PTEN protein. The possibility that exposure of the endothelial cells to the pomegranate extract activates cell surface receptors upstream of PI3K was also investigated. The relatively small number of human RTKs and ease of determining

their activation through their phosphorylation levels made them suitable for study in the context of this project. On top of this, to determine which of the compounds in the pomegranate extract might be responsible for increasing Akt phosphorylation and nitric oxide production in endothelial cells, the extract was separated into fractions representing the main phenolic compounds likely to be found in the extract. These were then tested, alongside individual compounds identified in the extract, to determine their ability to increase Akt phosphorylation.

5.3. Aims

The main objectives of the work described in this chapter were to

(i) determine which chemical class(es) of compounds were responsible for the bioactive effects of the pomegranate extract observed in **Chapter 4** and

(ii) to elucidate receptors or mechanisms upstream of Akt that may be mediating these effects.

5.4. Methods

5.4.1. Materials

C-18 columns were purchased from Biotage (Isolute Flash C18, 25 g). LH-20 sephadex was purchased from GE Healthcare. Malachite green solution, phosphate standard, purified PTEN (supplied as lyophilised stock formulated with various salts and buffer compounds) and PIP_3 (#P-3908) were purchased from Echelon Biosciences. Antibodies for ubiquitin (#3936), p-PTEN (Ser380/Thr382/383) (#9549), PTEN (#9188) and PTEN conjugated to sepharose beads (#4326) were purchased from Cell Signalling Technologies. Antibodies for p-Akt, Akt, p-eNOS and eNOS (Cell Signalling Technologies) were the same as those used in Chapter 4. TBS buffer was prepared with 20 mM tris-base, 137 mM NaCl and 10 mM dithiothreitol (DTT), adjusted to pH 7.6 using HCl. The procyanidin-rich apple extract (Evesse™ OPC, referred to in this chapter as simply OPC) was kindly provided by Coressence Ltd (UK). A sample of isolated apple procyanidin tetramers was prepared from OPC extract by Dr Ana B. Cerezo (IFR, UK). Urolithins were chemically synthesised in-house by Dr Paul Needs. Anthocyanins and procyanidin dimer B1 were purchased from Extrasynthese (France). Punicalagin was purchased as a mixture of its α- and β- anomers from Phytolab (Germany). (-)-Epicatechin and all other chemicals were purchased from Sigma-Aldrich and were of analytical-grade purity, unless otherwise specified. All water used was of MilliQ-grade purity.

5.4.2. Cell culture experiments

General materials and methods for culture of HUVECs, and the analysis of their lysates by BCA assay and western blotting, are described in **Chapter 2**, **Sections 2.2.1 - 2.2.3**. The vehicle control in all cell culture experiments was 0.1% DMSO.

5.4.3. Pomegranate extract fractionation

The fractionation process was based on the method by García-Estévez et al. (2010), which was developed to separate classes of polyphenol compounds from a red wine extract. Pomegranate extract was dissolved in water/2.5% acetic acid to 100 mg/ml, aided by sonication. Aliquots were centrifuged (13000g, 5 minutes) to clarify the solution, which was then loaded onto a polytetrafluoroethylene (PTFE) sample loop by glass syringe, connected to a 6-port injection valve (set in the 'load' position). A 25 g C-18 cartridge was placed in line with a Gilson 306 pump (set for a flow rate of 30 ml/min), a Gilson 805 manometric module and a Gilson 117 UV detector measuring absorbance at 205 and 270 nm. The Gilson pump was also connected to a container of water/2.5% acetic acid (the first elution solvent). The Gilson modules were controlled by a PC running the Gilson Unipoint software, which was also set up to record traces of UV absorbance over time. The cartridges were activated with methanol for approximately 5 minutes before equilibration with water/2.5% acetic acid until UV absorbance stabilised. The pump was stopped and the injection valve set to ‘inject’ to bring the sample loop in line with the cartridge and Gilson system, and then restarted to load the pomegranate extract onto the cartridge. The flow of water/2.5% acetic acid was maintained to elute ellagitannins, sugars and other small polar molecules into a glass container (eluent A), until the UV absorbance was stable for at least 5 minutes. The pump was stopped and the elution solvent changed from acidified water to ethyl acetate. The pump was restarted to elute flavonols, small phenolics, catechins and procyanidins (eluent B) into a new container until the UV absorbance had stabilised once more for at least 5 minutes, and the pump was stopped again. The elution solvent was changed to methanol, and the pump restarted to elute anthocyanins (eluent C) into a new container. Any other compounds still bound to the column would likely be eluted at this point as well.

Eluent A was dispensed into 500 ml round-bottom flasks to approximately 1/3 total volume, and snap-frozen by rotating the flask in an acetone bath chilled with dry-

ice. The ethyl acetate in eluent B and ethyl acetate/methanol in eluent C were first removed by rotary evaporation over a 30 °C water bath, replacing evaporated solvent with water periodically, before snap-freezing. Flasks were placed on dry-ice and flask mouths were covered with muslin cloth secured by parafilm wrapped around flask necks. Flasks were then placed in freeze-driers for 2-5 days to remove water. Fractions were redissolved in acidified water and fractionated again, using the above method, to improve the separation of polyphenol classes. These eluents were freeze-dried and then fractionated a third time before pooling sub-fractions of eluents A, B and C.

Sephadex LH-20 beads, activated in an excess of methanol at room temperature for 2-3 hours, were packed into a glass column connected to an AKTA FPLC system (GE Healthcare) for 24 hours (flow rate of 3 ml/min). After packing, LH-20 was equilibrated with water/2.5% acetic acid for 2 column volumes (1 volume = 35 ml) at a flow rate of 3 ml/min. The dried fraction from eluent A was dissolved in water/2.5% acetic acid and loaded onto a sample loop via syringe and then onto the column. The flow was reduced to 0.1-0.2 ml/min and maintained to elute sugars and other small molecules into a glass container until the UV absorbance (270 nm) had stabilised for approximately 0.5 volumes. The pump was stopped and the solvent changed to methanol. The pump was restarted to elute ellagitannins into a fresh container. Methanol in the second eluent was removed by rotary evaporation, similar to eluents B and C, and then both eluents were snap-frozen and freeze-dried as described above. The resulting C-18 column fractions (PPF (Pomegranate Procyanidin Fraction) from eluent B and PAF (Pomegranate Anthocyanin Fraction) from eluent C) and sephadex column fractions (PSF (Pomegranate Sugar Fraction) from the first elution and PEF (Pomegranate Ellagitannin Fraction) from the second) were prepared for analysis by HPLC and for testing in cell culture experiments. The pellet produced from centrifugation of the pomegranate extract solution at the clarification step was air-dried, weighed, and dissolved in DMSO. It was labelled as PIF (Pomegranate Insoluble Fraction).

Fractions were dissolved in 1-2 ml of water and pipetted into eppendorfs weighed before and after addition of the dissolved fractions. 200 µl aliquots of each (n=2) were pipetted into pre-weighed 2-ml screw-cap eppendorfs, and then frozen on dry-ice, with caps left on loose. The remaining volume of each fraction was then calculated. Once dry, the eppendorfs were weighed again and the mass of aliquoted fraction was calculated. From the total volume of dissolved fraction, the mass of each fraction was calculated. Further aliquots were taken to (i) calculate the total phenolic content (TPC) of each fraction, using the Folin-Ciolcateau method (details in **Chapter 4**, **Section 4.4.5**: *Measurement of phenolic content by Folin-Ciocalteu assay*) and (ii) to analyse their content by HPLC (detailed below), alongside standards of punicalagin for PEF, (-)-epicatechin (EC) and procyanidin tetramers (DP4) for PPF and cyanidin-3-O-Glc (C3Glc), delphinidin-3-O-Glc (D3Glc) and cyanidin-3,5-di-O-Glc (C35diGlc) for PAF. The presence of these compounds, excluding DP4, was confirmed using mass spectrometry analysis, which was carried out by Dr Shikha Saha (IFR, UK), using an Agilent 1100 HPLC-DAD-MSD system, as detailed in Hollands et al. (2013).

5.4.4. HPLC analysis of pomegranate extract and fractions

Before injection onto HPLC columns, samples of crude pomegranate extract and PAF were dissolved in 70% methanol (30% water) acidified with 2.5% acetic acid. PEF and punicalagin were dissolved in water acidified with 2.5% acetic acid, as ellagitannins have been reported to be more stable dissolved in low-pH water as opposed to low-pH methanol (Lei 2002). Stocks of all other samples and standards were prepared in DMSO. A preparation of the vehicle solvent used for each sample was used as the blank standard in each case. For reverse-phase (RP) analysis, samples were injected onto a Luna C18(2) 5 µm column (250 x 4.6 mm; Phenomenex, #00G-4252-E0) connected to an Aglient 1100 HPLC system and eluted at a flow rate of 1 ml/min. The solvents used for elution were

acetonitrile/0.1% trifluoroacetic acid (solvent A) and water/0.1% trifluoroacetic acid (solvent B). A linear solvent gradient was used, varying in terms of solvent A as follows: 0-5 mins: 0%; 15-17 mins: 17%; 22 mins: 25%; 30 mins: 35%; 35 mins: 50%; 40-50 mins: 100%; 55-65 mins: 0%.

For normal-phase (NP) analysis, samples were injected onto a Luna Silica(2) 5 μm column (250 x 4.6 mm; Phenomenex, #00G-4274-E0) connected to an Aglient 1100 HPLC system and eluted at a flow rate of 1 ml/min. The solvents used for elution were dichloromethane (solvent A), methanol (solvent B) and water/50% acetic acid (solvent C). Solvent C formed 4% of the total solvent composition for NP-HPLC analysis; its elution was isocratic. A linear gradient was used for solvents A and B, varying in terms of solvent A as follows: 0 mins: 82%; 30 mins: 67.6%; 45 mins: 56.8% 50 mins: 10%; 55-65 mins: 82%. Where comparison of similar compounds between samples was intended, such samples were injected into the HPLC system within the same run of analyses, in sequence where possible.

To quantify standard compounds in the extract and fractions, increasing concentrations of the respective compounds were analysed alongside extract/fractions so that the concentration of the compounds in the samples could be interpolated from the standard curve produced. Samples of the extract/fractions, spiked with the standard compounds at three increasing concentrations, were also analysed to determine if there were any matrix effects associated with the quantification of the compounds. Analysis of procyanidins and other compounds associated with PPF were performed by NP-HPLC, using fluorescence detection for quantification. All other compounds were analysed by RP-HPLC, with quantification of compounds associated with PEF performed using UV absorbance at 370 nm, and quantification of compounds associated with PAF performed using UV absorbance at 520 nm. The peaks of both punicalagin anomers were used for its quantification.

5.4.5. Akt phosphorylation induced by pomegranate fractions and pure compounds

Confluent monolayers of HUVECs cultured in 6-well plates were treated for 60 minutes with:

(i) doses of pomegranate extract fractions equivalent to 50 μg gallic acid equivalent (GAE)/ml dose of the crude extract

(ii) doses of compounds identified in the extract (**Table 5.5**) equivalent to what would be expected (from HPLC analysis) in the concentrations of extract fractions in (i) or in 50 μg GAE/ml of whole extract

(iii) doses of compounds identified in the extract (at supra-extract-equivalent concentrations) or metabolites of these compounds, including ellagic acid and urolithins A, B and C.

Cell lysates were prepared and the total protein concentration of each sample was determined by BCA assay. Analysis of Akt and eNOS phosphorylation was performed by western blotting.

5.4.6. Inhibition of PI3K and the modulation of vasodilatory signalling molecules by the crude pomegranate extract

Repeats of the total eNOS, ET-1 and media nitrates/nitrites assays from Chapter 4 were performed using cells treated for 8 or 24 hours with either 20 μg GAE/ml of the crude extract or a vehicle control, with or without the PI3K inhibitor LY294002 (Cell Signalling Technologies) (added to cell culture media 60 minutes prior to addition of pomegranate or vehicle treatments). In this case, the media nitrate/nitrite assay was performed without cell-free experiments, as the results from Chapter 4 show that the pomegranate extract by itself does not significantly increase levels of nitrate/nitrite in culture media. Unpaired 2-tailed student *t*-tests were used to determine differences between cell culture treatments, with significance taken as $p < 0.05$.

5.4.7. Phosphorylation of RTKs in endothelial cells treated with pomegranate extract

Confluent monolayers of HUVECs cultured in 6cm^2 dishes were treated with either pomegranate extract (50 μg GAE/ml), punicalagin (20 μM), PF-P (5.5 μg GAE/ml) or vehicle control (0.1% DMSO) (n=2, biological replicates). Cell lysates were prepared and the total protein concentration of each sample measured by BCA. Antibody microarrays (Cell Signalling Technologies, #7949) spotted with antibodies (n=2, technical replicates) (**Fig. 5.2**) corresponding to pan Tyr-phosphorylated RTKs (**Table 5.1**), and related signalling molecules (**Table 5.2**, where antibodies correspond to pan-Tyr phosphorylated proteins unless the phosphorylated residue is specified), were used according to the manufacturer's instructions. After the final wash buffer step, a flow of compressed nitrogen at 20 psi was used to remove remaining buffer from the surface of the slide, which was then stored in a plastic holder, protected from light.

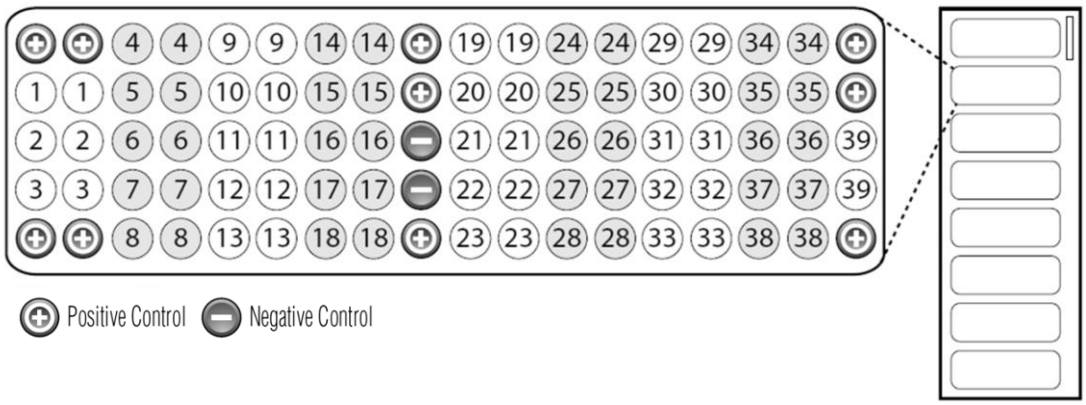

Figure 5.2: Layout of antibodies for phospho-RTKs and related proteins on microarray slide, taken from product data-sheet. Details of targets are given in **Tables 5.1** and **5.2**.

Slides were scanned fluorescently, using a Genepix 4000B microarray scanner, connected to a computer running Genepix software (version 6.1), at a resolution of 5 μm/pixel with a 635 nm excitation laser coupled with a 650-690 nm emission filter. The photo-

multiplier tube (PMT) gain was adjusted so less than 1% of the scanned image's pixels were saturated, Images produced from the slide scanning were saved as .tiff files, and microarray slides were then placed into storage at room temperature, protected from light. Slides were also scanned using an 532 nm excitation laser coupled with a 557.7-592.5 nm emission filter. Images produced from scanning at 532 nm were assessed visually to determine if there had been any signal produced through artifacts such as scratches on the slide, so that these could be taken into account when analysing the images produced by excitation at 635 nm.

Table 5.1: Receptor tyrosine kinases, their RTK family, and corresponding spot number, as featured in the microarray layout of **Fig. 5.2**.

Spot No.	Target	Family	Spot No.	Target	Family
1	EGFR/ErbB1	EGFR	15	PDGFR	PDGFR
2	HER2/ErbB2	EGFR	16	c-Kit/SCFR	PDGFR
3	HER3/ErbB3	EGFR	17	FLT3/Flk2	PDGFR
4	FGFR1	FGFR	18	M-CSFR/CSF-1R	PDGFR
5	FGFR3	FGFR	19	EphA1	EphR
6	FGFR4	FGFR	20	EphA2	EphR
7	InsR	Insulin R	21	EphA3	EphR
8	IGF-IR	Insulin R	22	EphB1	EphR
9	TrkA/NTRK1	NGFR	23	EphB3	EphR
10	TrkB/NTRK2	NGFR	24	EphB4	EphR
11	Met/HGFR	HGFR	25	Tyro-3/Dtk	Axl
12	Ron/MST1R	HGFR	26	Axl	Axl
13	Ret	Ret	27	Tie2/TEK	Tie
14	ALK	LTK	28	VEGFR2/KDR	VEGFR

Table 5.2: RTK-related phospho-proteins, their kinase family, and corresponding spot number, as featured in the microarray layout of **Fig. 5.2**.

Spot No.	Target	Family	Spot No.	Target	Family
29	Akt (Thr308)	Akt	35	Zap-70	Zap-70
30	Akt (Ser473)	Akt	36	Src	Src
31	ERK1/2 (Thr202/Tyr204)	MAPK	37	Lck	Src
32	S6 Ribosomal Protein (Ser235/236)	RSK	38	Stat1 (Tyr701)	Stat
33	c-Abl	Abl	39	Stat3 (Tyr 705)	Stat
34	IRS-1	IRS			

The image produced from scanning at 635 nm was analysed with the software GenePix Pro v6.1, using a manually aligned spot overlay, and the median spot fluorescence intensities (MSFIs) were calculated. Microsoft Excel 2007 spreadsheets were used to organise data-points, and statistical analysis was carried out using the limma package for the R software environment (Smyth 2004; Smyth 2005; R Development Core Team 2010). The data was fitted to a linear model, an empirical Bayes method was applied to the data to reduce statistical errors (Smyth 2004), and p-values were calculated, adjusting for multiple testing (MT) using the Benjamini-Hochberg method, for per-target comparison of MSFIs between each polyphenol/extract treatment and the vehicle treatment.

5.4.8. Changes to PTEN structure and activity in endothelial cells treated with pomegranate extract

5.4.8.1. Phosphorylated and total PTEN

Confluent monolayers of HUVECs cultured in 6-well plates were treated with pomegranate extract at either 50 μg GAE/ml for 60 mins or 20 μg GAE/ml for 8 and 24 hours. Cell lysates were prepared and the total protein concentration of each sample measured by BCA assay. Lysates from HUVECs treated for 60 mins with the pomegranate extract were assayed for changes to phosphorylated PTEN, and lysates from HUVECs treated for 8-24 hours with the extract were assayed for changes for total PTEN protein, both by western blotting.

5.4.8.2. Ubquitinated PTEN

Confluent monolayers of HUVECs cultured in 6cm^2 dishes were treated with 50 μg GAE/ml pomegranate extract or vehicle control for 60 mins. Cell lysates were prepared

and the total protein concentration of each sample was measured by BCA assay. 200 µg of cell lysate protein was diluted in 1X cell lysis buffer (Cell Signalling Technologies) containing 1X protease inhibitor (Roche) to 1 µg/µl. Samples were incubated with anti-PTEN antibody conjugated to sepharose beads, according to the manufacturer's instructions, overnight at 4 °C with gentle agitation. Samples were centrifuged (13000 g for 30 s) and the pellets washed 5 times with fresh cell lysis buffer containing protease inhibitor, with centrifugation between washes.

In preparation for loading onto SDS-PAGE gels, the final wash of cell lysis buffer was aspirated to leave approximately 13 µl of buffer along with the pellet of sepharose beads. 2 µl of a 250 mM stock of DTT and 5 µl of 4X LDS sample buffer (Expedeon) were added and the sample was spun briefly to mix before incubation at 100 °C for 4 minutes. Lysates were assayed for changes to ubiquitinated PTEN by western blotting, using anti-ubiquitin and anti-PTEN antibodies as the primary antibodies. An HRP-linked anti-mouse IgG antibody (CST #7076) was used as the secondary antibody to the anti-ubiquitin antibody. For imaging PTEN protein in this assay, two secondary antibodies were used: a mouse-derived anti-rabbit IgG (conformation specific) antibody (CST #3678) was used first, followed by the HRP-linked anti-mouse antibody was described above. The anti-rabbit IgG (conformation specific) antibody is raised so that it will not detect denatured or reduced rabbit IgG heavy chain, which would be found in western blots from this assay due to the anti-PTEN antibody contained in the immunoprecipitated samples loaded onto the SDS-PAGE gels. Use of the conformation-specific antibody avoids masking of PTEN protein by rabbit IgG heavy chain (both are approximately 50 kDa).

5.4.8.3. Measurement of PTEN phosphatase activity

Confluent monolayers of HUVECs cultured in 6cm^2 dishes were treated with 50 μg GAE/ml pomegranate extract or vehicle control for 30 minutes. Cell lysates were prepared and the total protein concentration of each sample was measured by BCA. 260 μg of cell lysate protein was diluted in cell lysis buffer (CST) to 1 μg/μl. Samples were incubated with sepharose beads conjugated to an anti-PTEN antibody, according to manufacturer's instructions, overnight at 4 °C with gentle agitation. Samples were centrifuged (13000 g for 30 s) and pellets washed 4 times with TBS, centrifuging in between washes. After the final wash, pellets were re-suspended in 27 μl TBS and aspirated into wells of a 96-well plate, alongside blank controls of 30 μl TBS, and substrate only controls of 27 μl TBS plus 3 μl of a 1 mM solution (in TBS) of PIP_3 (PTEN substrate). 3 μl PIP_3 was added to the wells containing HUVEC lysate immunoprecipitate, and the plate was covered and incubated at 37 °C for 120 minutes.

Alongside these samples, microplate wells were prepared that contained 2 μl of a 15 ng/μl solution (in water) of purified PTEN enzyme and 20 μl of procyanidin tetramers diluted in TBS to concentrations of 0, 4, 10, 16, 24, 32 and 40 μM (n=3 for each sample). Samples were incubated immediately at 37 °C for 5 mins, before addition of 3 μl of a 1 mM solution of PIP_3, followed by a further 30 mins incubation at 37 °C. During the substrate incubations, dilutions of a 1 mM phosphate solution were prepared, of concentrations ranging from 4 to 48 μM. Following substrate incubations, aliquots of each standard were pipetted into plate wells (n=2) and 100 μl of malachite green solution was added to each sample, blank or standard. The plate was incubated at room temperature for 20 minutes, protected from light, after which the absorbance of each well at 620 nm was measured using a microplate reader.

Unpaired 2-tailed student *t*-tests were used to determine differences between cell culture treatments, with significance taken as $p < 0.05$. To determine significant correlation between the concentration of procyanidin tetramers and PTEN enzyme

activity, Pearson's correlation coefficient was calculated, along with its corresponding p-value.

5.5. Results

5.5.1. Fractionation of pomegranate extract

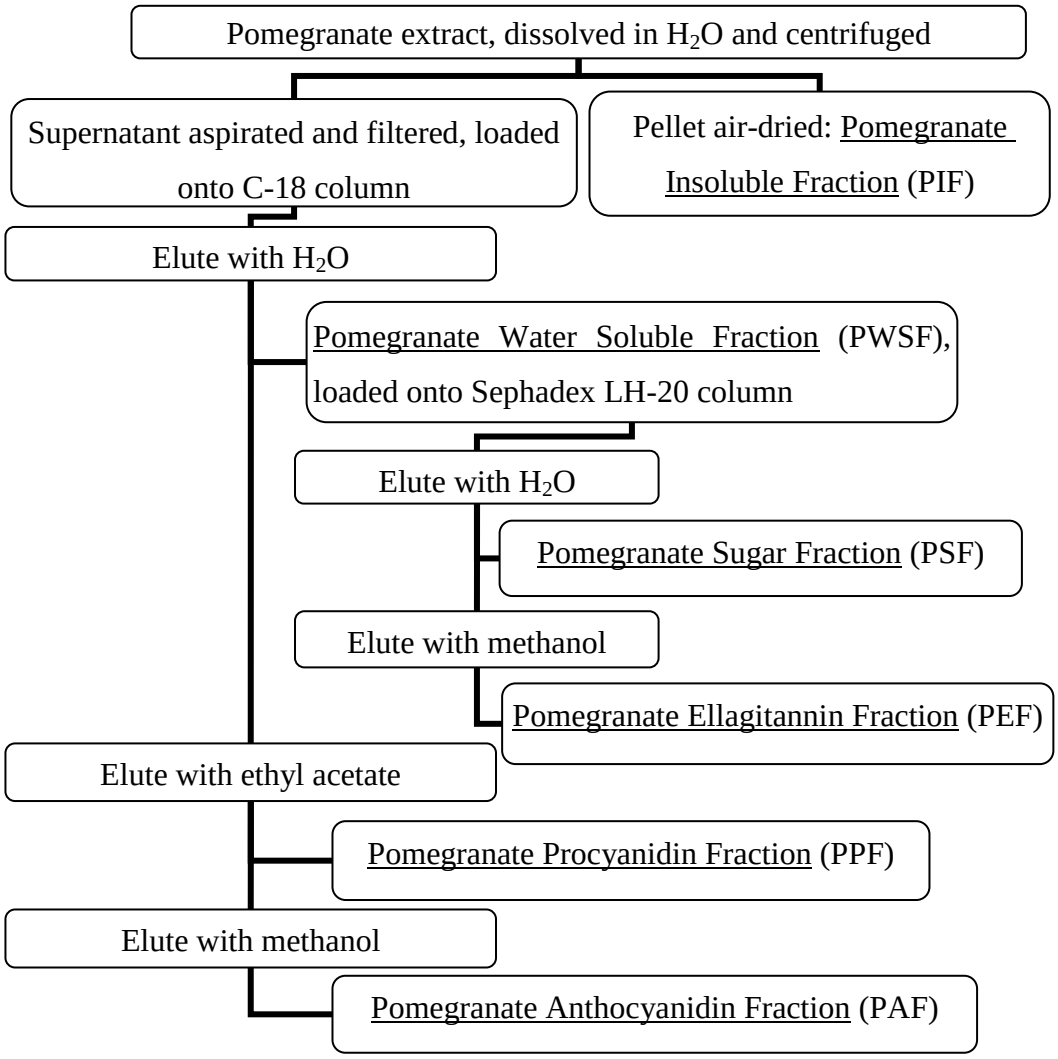

Figure 5.3: Flow chart depicting the steps taken to fractionate the pomegranate extract.

Table 5.3: List of acronyms used for each of the fractions produced from the crude pomegranate extract.

Fraction	Acronym
Pomegranate Ellagitannin Fraction	PEF
Pomegranate Procyanidin Fraction	PPF
Pomegranate Anthocyanin Fraction	PAF
Pomegranate Insoluble Fraction	PIF
Pomegranate Sugar Fraction	PSF
Pomegranate Water Soluble Fraction	PWSF

The steps taken to fractionate the crude pomegranate extract are shown in **Fig. 5.3** with the names and acronyms used for each fraction given in **Table 5.3**. The mass of the final fractions produced and their corresponding TPC values are given in **Table 5.4**. Originally, only use of a C-18 column had been planned for separation of the pomegranate extract. However, the ellagitannin fraction that was produced, despite an ability to increase Akt phosphorylation (data not shown), caused detachment of a large proportion of the endothelial cell cultures from the culture plate surface (data not shown). This was likely due to the decrease in pH, shown by the discolouration of the pH indicator in the culture medium. The inability to evaporate the fraction to dryness due to its hygroscopic nature, likely caused by the sugar content of the extract, in turn would have been causing fruit acids and acetic acid dissolved in the extract solution to be retained, so decreasing the pH of the fraction (PWSF). Sephadex LH-20 material was used to remove this interference, resulting in the high sugar/acid content fraction PSF, in addition to PEF. Centrifugation of the pomegranate extract, dissolved in acidified water, was required to clarify the solution before it was loaded onto the C-18 column. Thus, the pellet produced (PIF) would be expected to contain compounds that, while soluble under the original extraction conditions (70% methanol at 70 ºC), are not soluble in acidified water.

Table 5.4: Weight/yields of fractions prepared from pomegranate extract

Sample	Weight (mg)	Yield (w/w % of crude extract)	TPC (mg GAE/mg)	TPC as % of crude extract TPC)
Crude extract	1482.8 ± 0.10	n/a	14.61 ± 0.30	n/a
PIF	10.74 ± 0.12	0.7	0.01 ± 0.001	0.1
PSF	882.35 ± 1.58	55.4	1.33 ± 0.01	9.1
PEF	2.80 ± 0.21	0.2	0.42 ± 0.02	2.9
PPF	8.47 ± 0.21	0.6	1.61 ± 0.05	11.0
PAF	8.55 ± 0.58	0.6	1.86 ± 0.07	12.7
Fraction total	842.17 ± 1.71	56.8	5.22 ± 0.09	35.8

5.5.2. HPLC analysis of pomegranate fractions and crude extract

Peaks from compounds identified in the crude pomegranate extract by mass spectrometry are labelled in HPLC chromatograms of the crude extract and extract fractions. The fractionation of ellagitannins (the absorbance spectrum of which shows a characteristic peak around 370 nm, as opposed to 520 nm for anthocyanins) into PEF is shown in **Fig. 5.4**, demonstrated by the prominent peaks for punicalagin. Peaks in the chromatogram from PAF are of a magnitude of size smaller than those from PEF, with both fractions injected in similar quantities (10 µg). Absorbance of PPF at 370 nm and 520 nm after similar chromatographic analysis to PAF and PEF did not result in any peaks

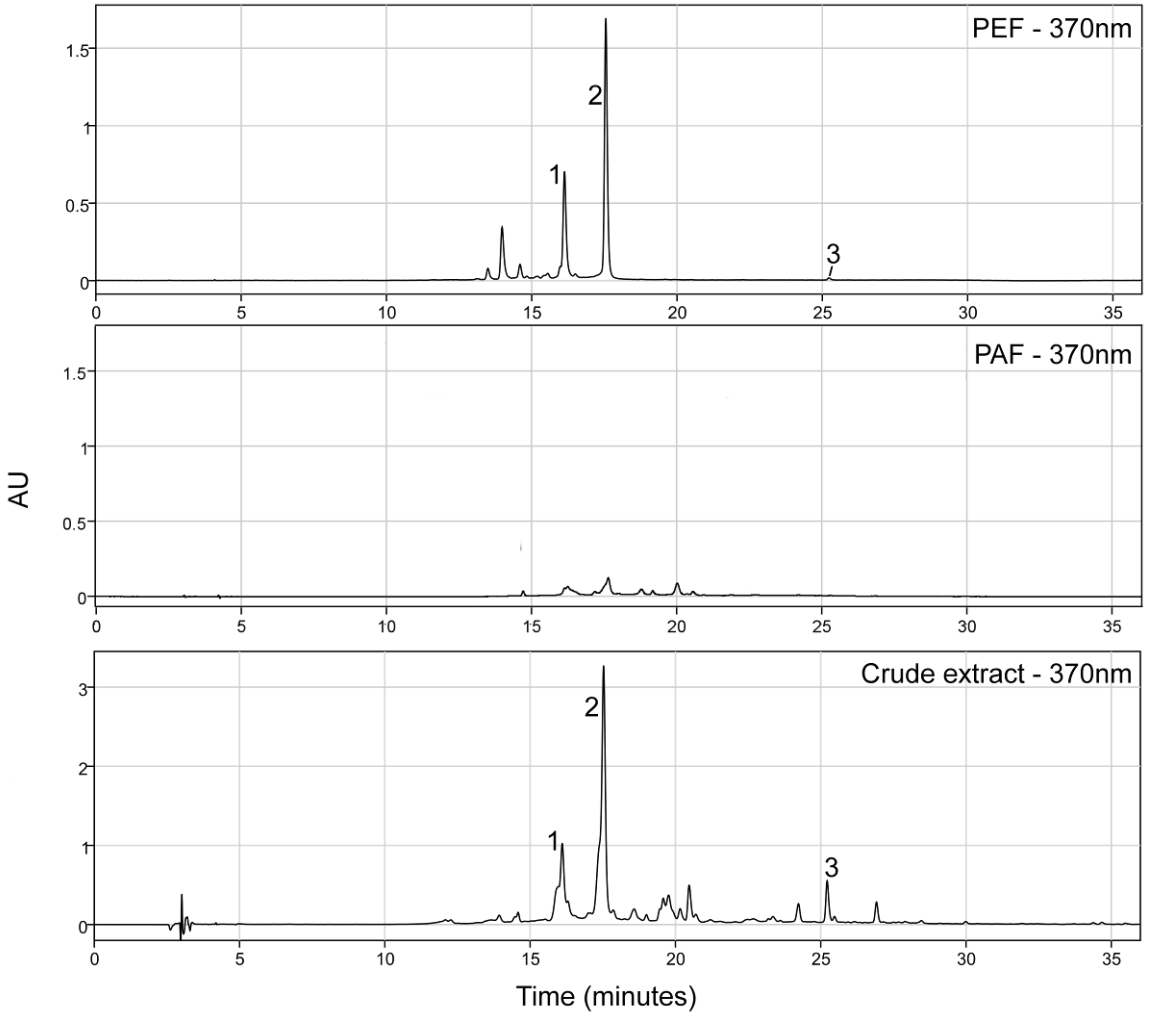

Figure 5.4: Chromatograms of absorbance at 370 nm from RP-HPLC-UV analysis of the pomegranate fractions PEF and PAF, alongside the crude extract. Labelled peaks are (**1**) punicalagin-α, (**2**) punicalagin-β and (**3**) ellagic acid.

discernable from background noise (data not shown). Chromatograms for 520 nm absorbance of PEF showed peaks a magnitude of size smaller than those of PAF (**Fig. 5.5**), again with both fractions injected in similar quantities (10 µg).

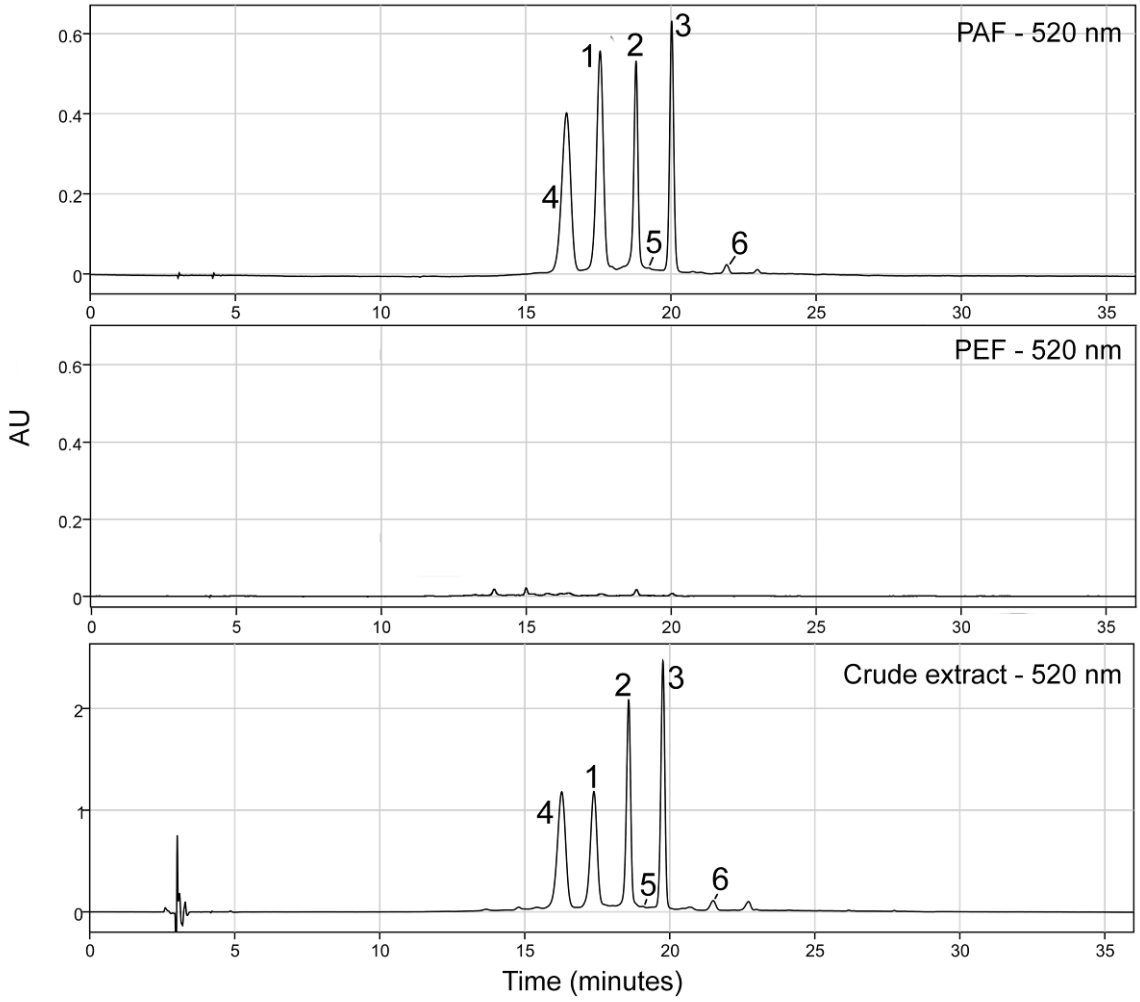

Figure 5.5: Chromatograms of absorbance (measured as absorbance units (AU)) at 520 nm from RP-HPLC-UV analysis of the pomegranate fractions PEF and PAF, alongside the crude extract. Labelled peaks are (**1**) C35diGlc, (**2**) D3Glc, (**3**) C3Glc, (**4**) delphinidin-3,5-di-O-Glc, (**5**) pelargonidin-3,5-di-O-Glc and (**6**) pelargonidin-3-O-Glc.

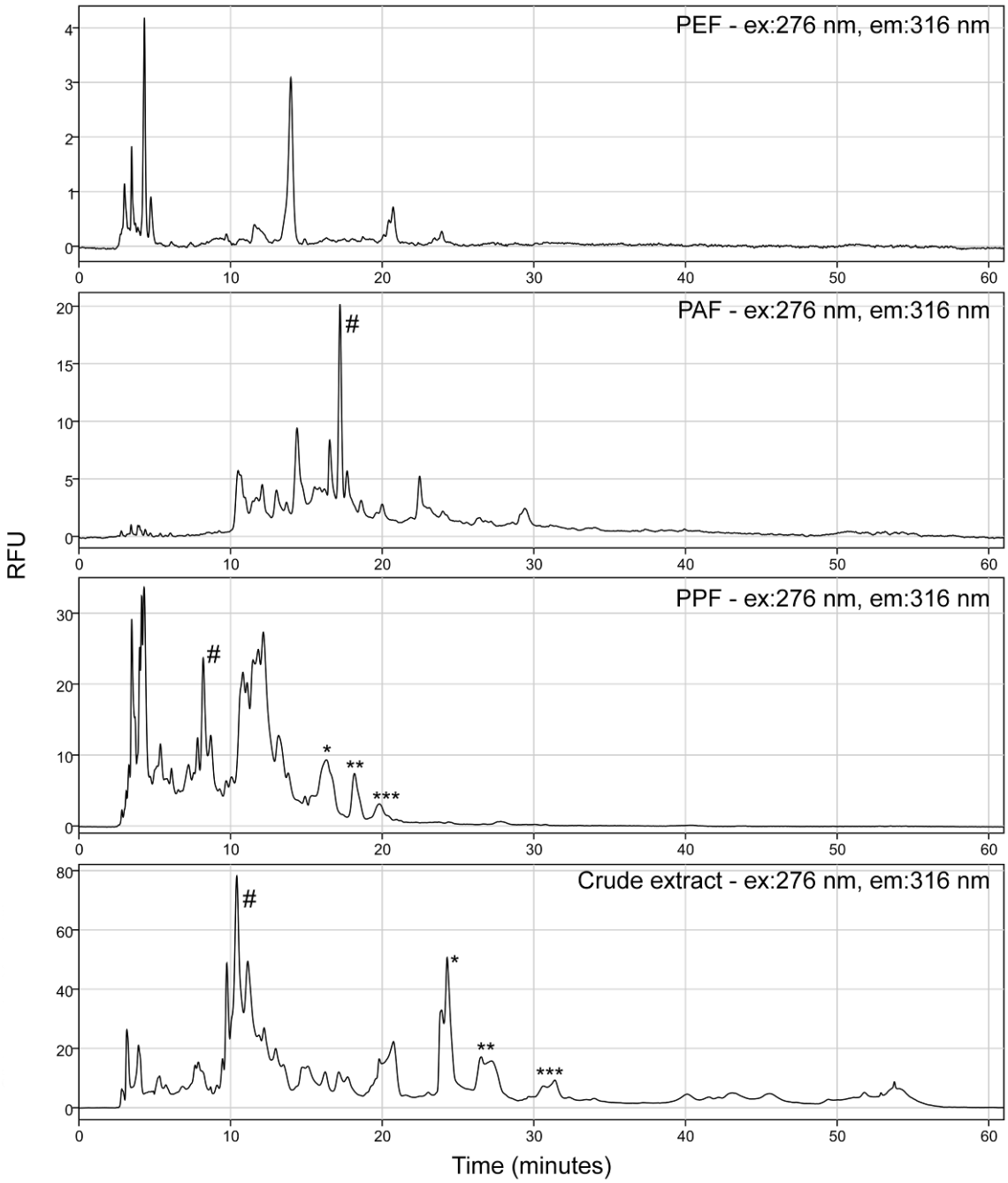

Figure 5.6: Chromatograms of emitted fluorescence (measured as relative fluorescence units (RFU)) from NP-HPLC analysis of the pomegranate fractions PEF, PAF and PPF, alongside the crude extract. Peaks labelled as **#**, *****, ****** and ******* are presumed to each be produced by similar compounds due to the shape of surrounding peaks.

Normal phase HPLC analysis of PEF presented relatively small peaks, with stronger responses from PAF and PPF (**Fig. 5.6**). There did not appear to be consistent retention times between peaks from different samples. This could possibly be due to differences in

their matrices, i.e. the additional compounds present in each besides flavan-3-ols and procyanidins. The crude extract was initially dissolved in acidified water before injection onto the HPLC system. PPF, however, required DMSO for it to solubilise so that it could be analysed by HPLC. While DMSO was also tested as a solvent for the crude extract, there was a large loss of colour (purple to brown) in the extract, even when DMSO was acidified with HCl. This is likely to indicate a change in structure of anthocyanins, and so DMSO was not used as a solvent for the crude extract. It would be interesting to determine whether a DMSO solution of the crude extract would produce NP-HPLC retention times similar to those seen for PPF. Given the current appearance of the PPF and crude extract NP chromatograms, it could be concluded that compounds in PPF have come off the HPLC column quicker than those in the crude extract. Assuming this to be the case, peaks representing similar compounds in each chromatogram have been labelled accordingly.

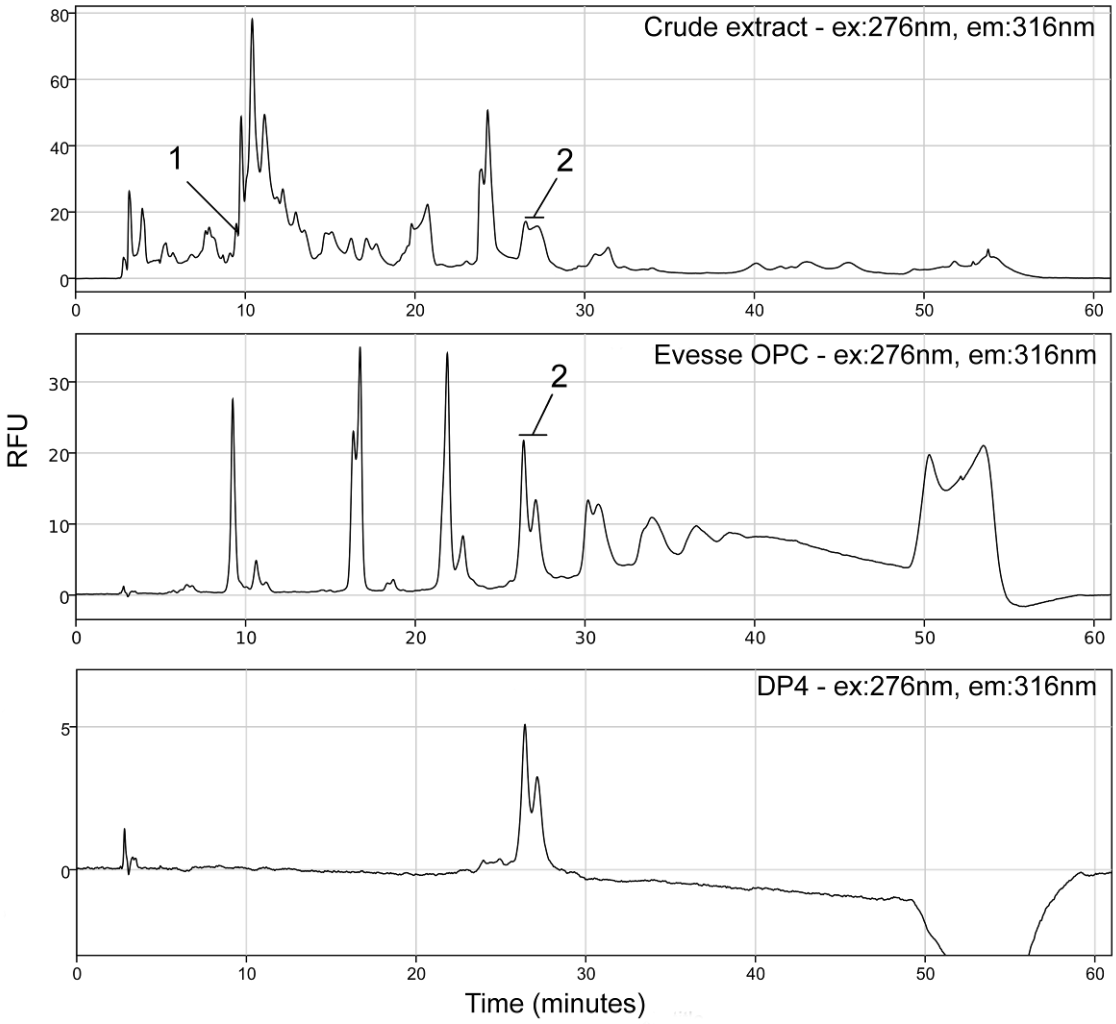

Figure 5.7: Chromatograms of emitted fluorescence (measured as relative fluorescence units (RFU)) from NP-HPLC analysis of the crude pomegranate extract, Evesse™ OPC extract and isolated procyanidin tetramers. Labelled peaks are (**1**) epicatechin and (**2**) procyanidin tetramers.

Procyanidins do not ionise particularly well, and so are difficult to identify by mass spectrometry, unless present in high concentrations (Dr Shikha Saha, personal communication, August 2012). NP-HPLC analysis of the crude extract alongside the procyanidin-rich Evesse™ OPC extract and a purified fraction of procyanidin tetramers (DP4) (**Fig. 5.7**) was conducted to provide evidence for the presence of procyanidins in the extract. The pattern of peak retention produced by the crude extract was similar to that produced by the OPC extract, with one cluster of peaks showing very similar

retention time to the procyanidin tetramers of the other samples. The peak pattern of the OPC extract would also suggest the identification of the peaks in **Fig. 5.6** as (*****) procyanidin trimers, (******) tetramers and (*******) pentamers.

Using a response factor obtained from the peak for the isolated tetramer standard, the concentration of procyanidin tetramers in the extract was calculated as 2.7 μM per 50 μg GAE/ml dose of pomegranate extract. The concentration of other compounds quantified in the extract fractions was also found to be reduced (to varying degrees) compared to the crude extract. The quantification of other polyphenols identified in the extract are shown in **Table 5.5**, where their concentration in quantities of the extract fractions is shown in most cases to be lower than their concentrations in equivalent quantities of the crude extract, indicating the fractionation process caused losses of polyphenols in a number of cases (most prominently for punicalagin).

Table 5.5: Compounds identified in pomegranate extract and fractions, and their equivalent concentrations (**i**) in the crude extract (μM/[mg/ml]), (**ii**) in 50 μg GAE/ml of the crude extract (μM) and (**iii**) in their respective fractions (μM), where the quantity of each fraction is equivalent to 50 μg GAE/ml of the crude extract Column (**iv**) shows the reduction (% w/w) of compounds after fractionation.

Polyphenol	(i)	(ii)	(iii)	(iv)
Punicalagin (α + β)	1.7 ± 0.1	9.3 ± 0.4	0.9 ± 0.01	10
Ellagic acid	0.16 ± 0.02	0.8 ± 0.1	n/a	n/a
C35diGlc	0.21 ± 0.03	1.1 ± 0.1	0.8 ± 0.01	73
D3Glc	0.37 ± 0.05	1.9 ± 0.3	0.5 ± 0.01	26
C3Glc	0.26 ± 0.01	1.3 ± 0.04	0.5 ± 0.04	38
(-)-Epicatechin	0.06 ± 0.01	0.3 ± 0.07	0.4 ± 0.01	-
Procyanidin tetramers	0.53	2.7	n/a	n/a

RP- and NP-HPLC analysis of PIF (**Fig. 5.8**) showed the presence of phenolic compounds. However, considering the concentration the sample of PIF was prepared at for HPLC analysis (100 mg/ml), and the size of peaks produced in contrast to those of the

crude extract (prepared at 50 mg/ml) in **Figs. 5.4**, **5.5** and **5.6**, the concentration of phenolic compounds in this fraction are likely to be very small.

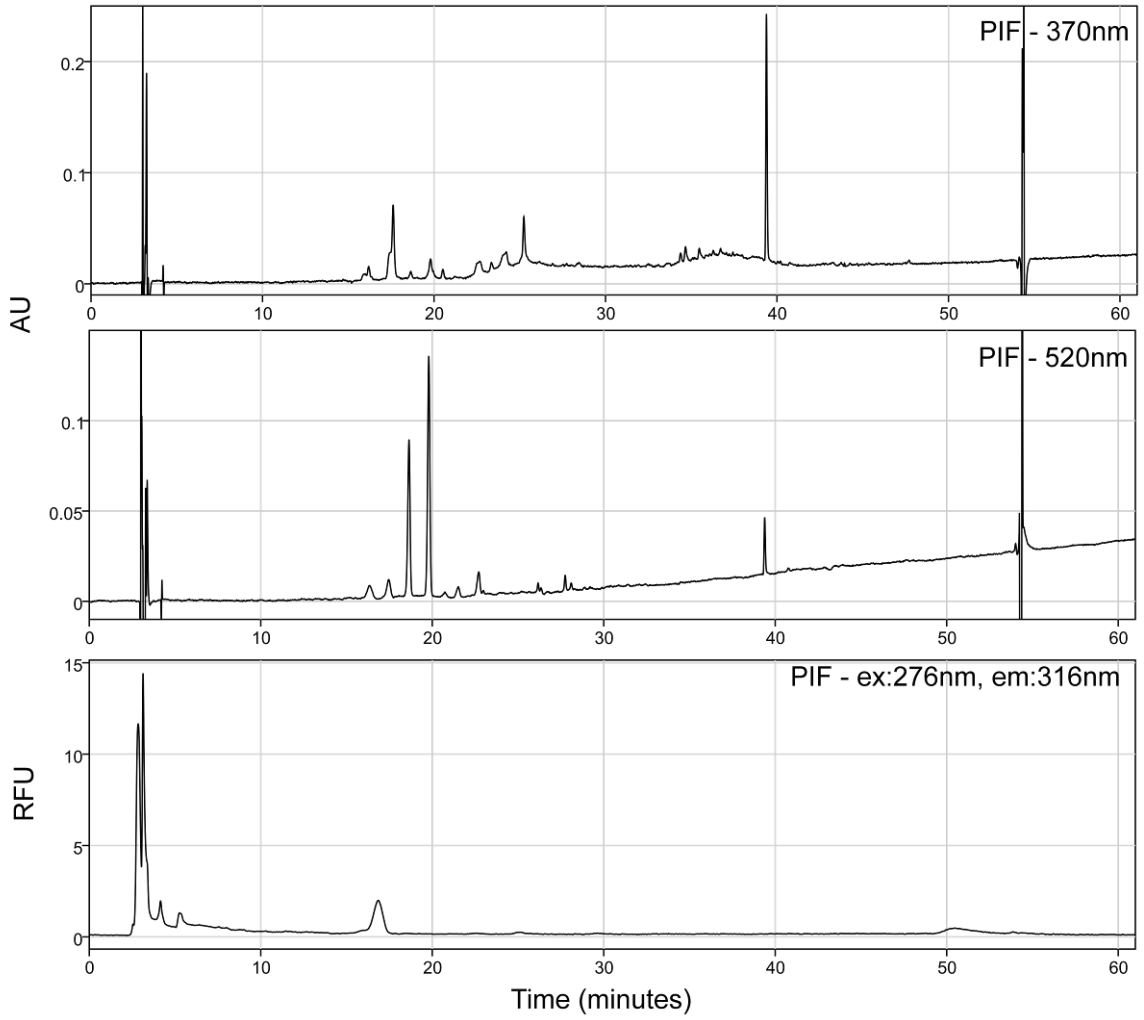

Figure 5.8: Chromatograms of absorbance at 370 and 520 nm from RP-HPLC-UV analysis and emitted fluorescence (from NP-HPLC analysis of the pomegranate fraction PIF. AU - Absorbance Units; RFU - Relative Fluorescence Units.

5.5.3. Increase in p-Akt due to extract fractions or individual compounds

HUVECs were treated with the pomegranate polyphenol fractions at doses equivalent to 50 µg GAE/ml of the whole pomegranate extract. Each fraction was shown to increase Akt phosphorylation in HUVECs, with PPF producing the strongest effect (**Fig. 5.9**).

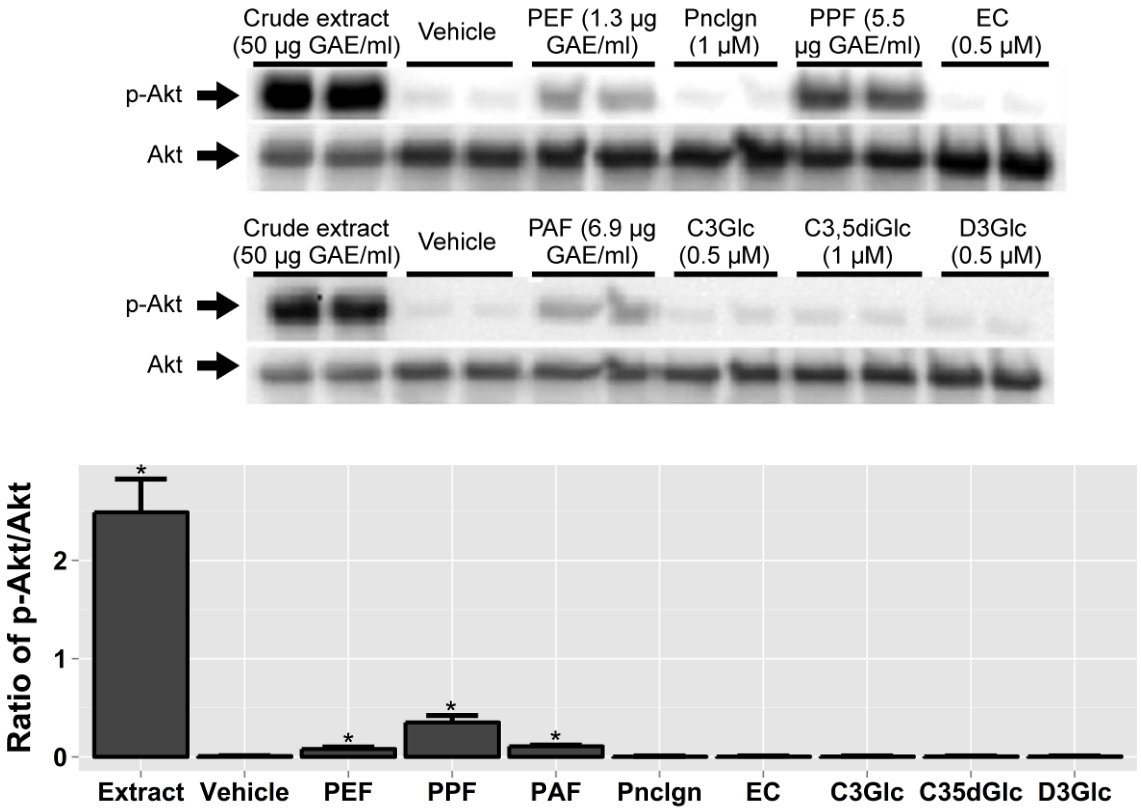

Figure 5.9: Western blots of p-Akt/Akt from lysates of HUVECs treated for 60 minutes with pomegranate fractions PEF, PPF and PAF at doses equivalent to 50 µg GAE/ml of crude pomegranate extract (based on results shown in **Table 5.4**), and cells treated with individual compounds representative of these fractions, at concentrations equivalent to what would be present in the fraction doses used (based on results shown in **Table 5.5**), alongside the crude extract and vehicle controls. Abbreviations: Pnclgn = punicalagin; EC = (-)-epicatechin. n=2 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

Cells were also treated with individual compounds identified in the extract at doses equivalent to concentrations in the fraction and crude extract treatments (**Figs. 5.9 - 5.11**). Of these, only DP4 was able to strongly increase p-Akt (and p-eNOS). Punicalagin and ellagic acid increased Akt phosphorylation, but only at concentrations that were much higher than those present in the crude extract treatments (**Figs. 5.12** and **5.13**). The resulting strong stain for phosphorylated protein in a number of western blots appeared to interfere with subsequent imaging for levels of total protein (resulting in a weak or absent stain), despite the inclusion of extra stripping buffer washes. In these cases, imaging of GAPDH in lysates was also included to demonstrate the relative levels of protein that had been assayed in each sample. Urolithins, human metabolites of punicalagin and ellagic acid were unable to increase p-Akt at similar concentrations. Urolithins A and B were likely in solution at a slightly lower concentration than that stated in **Fig. 5.13**, however, as precipitate was observed after they were diluted into cell culture media.

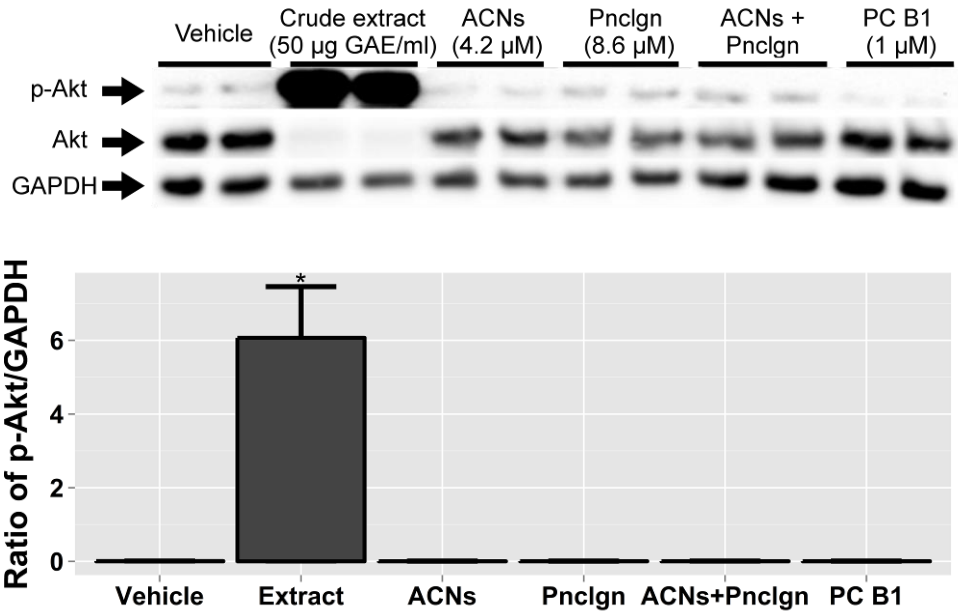

Figure 5.10: Western blots of p-Akt/Akt/GAPDH from lysates of HUVECs treated for 60 minutes with a mix of anthocyanins (ACN = 1.0 µM C3Glc + 1.9 µM D3Glc + 1.3 µM C35dG), punicalagin (Pnclgn), a mix of anthocyanins and punicalagin, or procyanidin B1, alongside the crude extract and a vehicle control. n=2 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/GAPDH) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

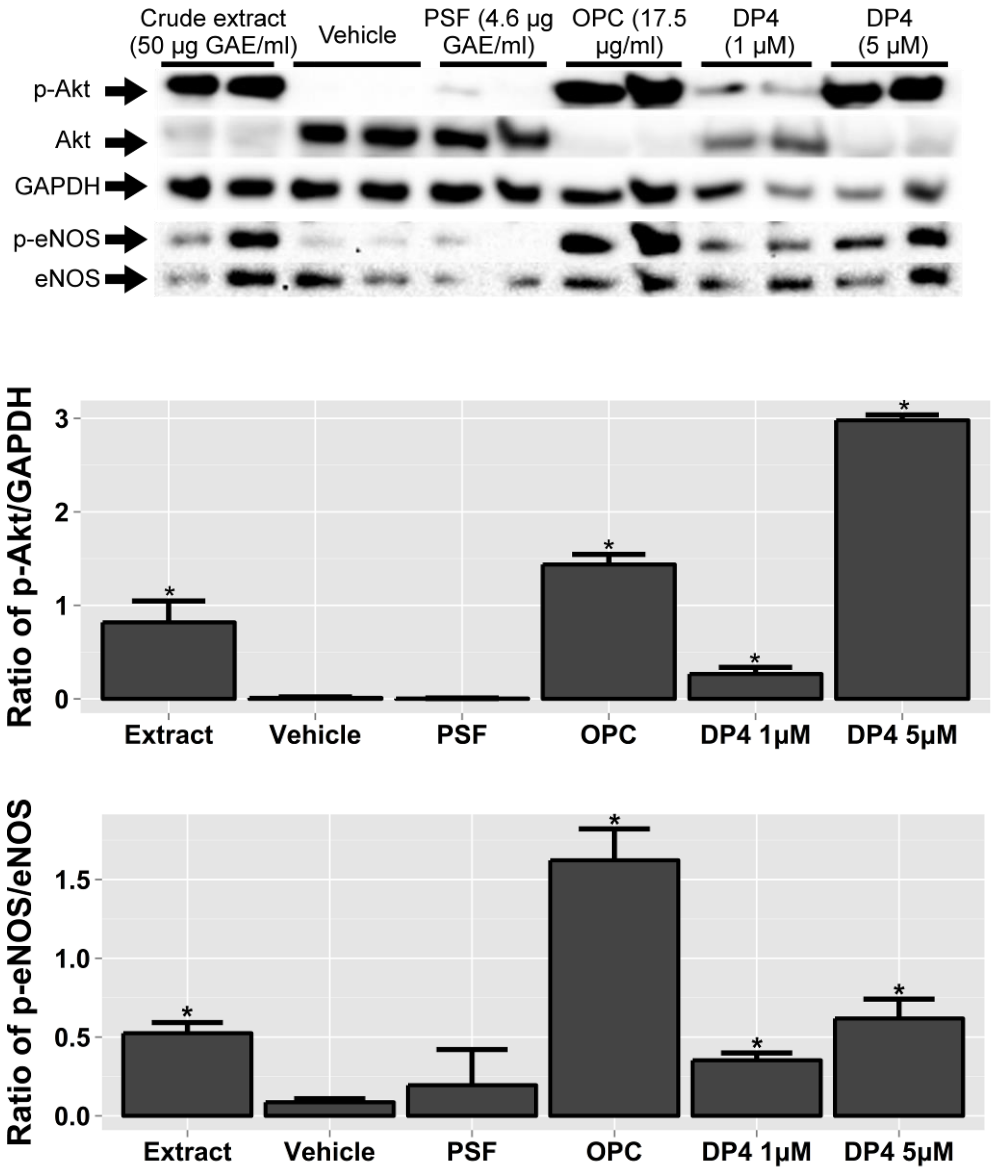

Figure 5.11: Western blots of p-Akt/Akt/p-eNOS/eNOS/GAPDH from lysates of HUVECs treated for 60 minutes with PSF at a dose equivalent to 50 µg GAE/ml crude extract, DP4, and OPC extract (at a concentration containing approximately 2.7 µM procyanidin tetramers), alongside the crude extract and a vehicle control. n=2 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/GAPDH and p-eNOS/eNOS) is shown in the two lower panels. ***** - $p < 0.05$ vs vehicle control treatment.

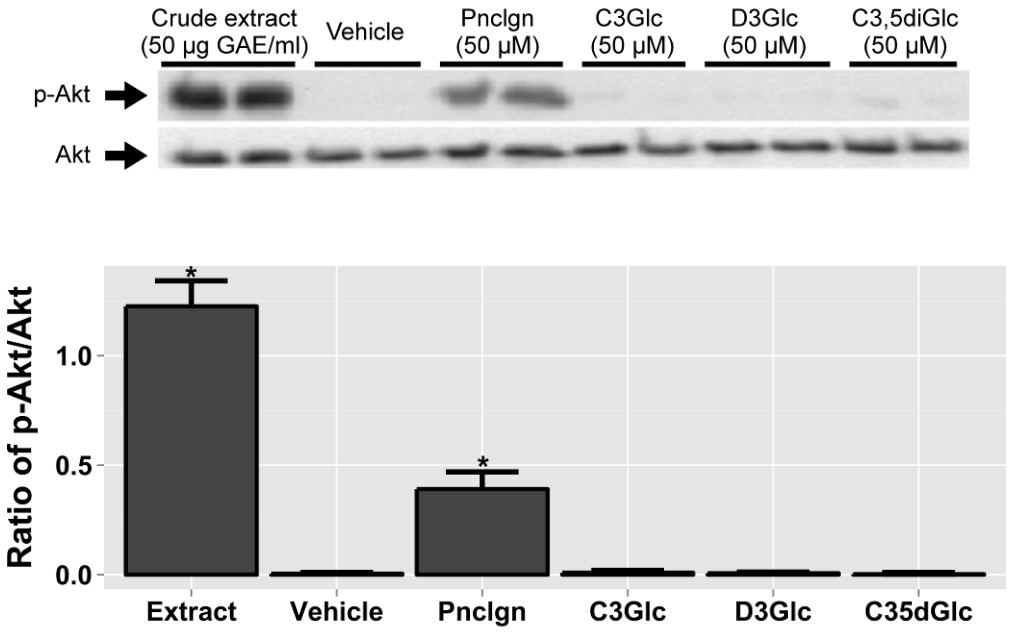

Figure 5.12: Western blots of p-Akt/Akt from lysates of HUVECs treated for 60 minutes with high concentrations of punicalagin (Pnclgn) and anthocyanins found in the crude extract, alongside the extract and a vehicle control. n=2 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

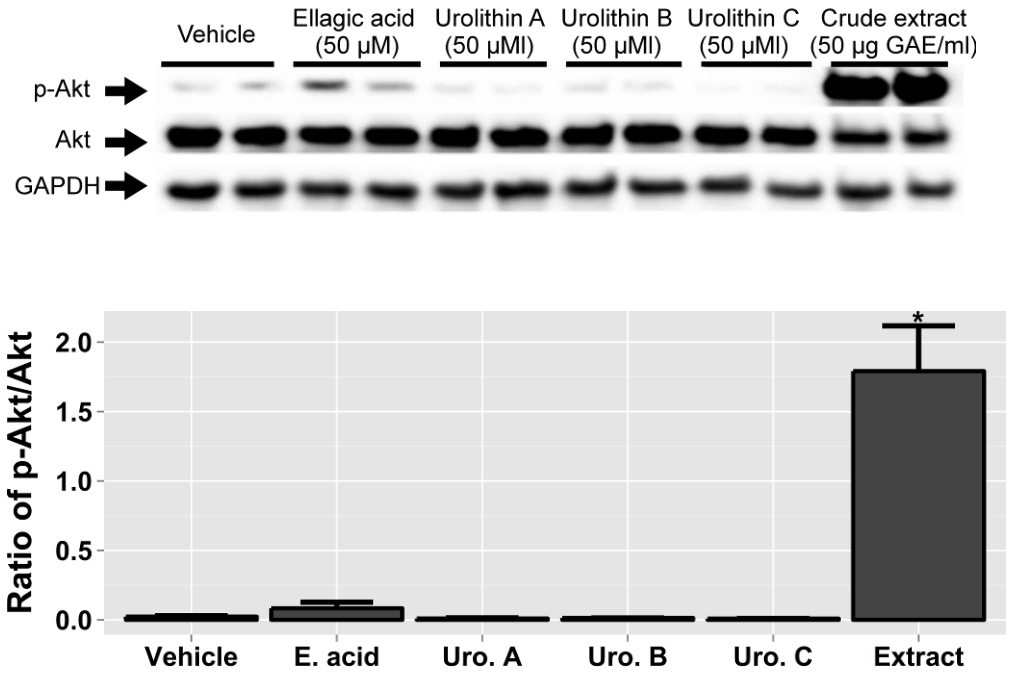

Figure 5.13: Western blots of p-Akt/Akt/GAPDH from lysates of HUVECs treated for 60 minutes with high concentrations of ellagic acid or urolithins, alongside the crude extract and a vehicle control. n=2 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

Treatment of cells with PSF at a concentration equivalent to 50 µg GAE/ml of the crude extract did not cause an increase in Akt phosphorylation (**Fig. 5.11**). HUVECs were not treated with a sample of PIF, due to the precipitation of this fraction when dissolved in cell culture media. However, a sample of pomegranate extract with PIF removed was prepared and used to treat cells. No difference in the levels of phosphorylated Akt were observed between lysates from cells treated with the crude extract and cells treated with the PIF-removed extract (**Fig. 5.14**). It was also shown that adjustment of the culture media pH, down to the same level measured after dilution in media of 50 µg GAE/ml of the crude extract, did not increase basal levels of phosphorylated Akt.

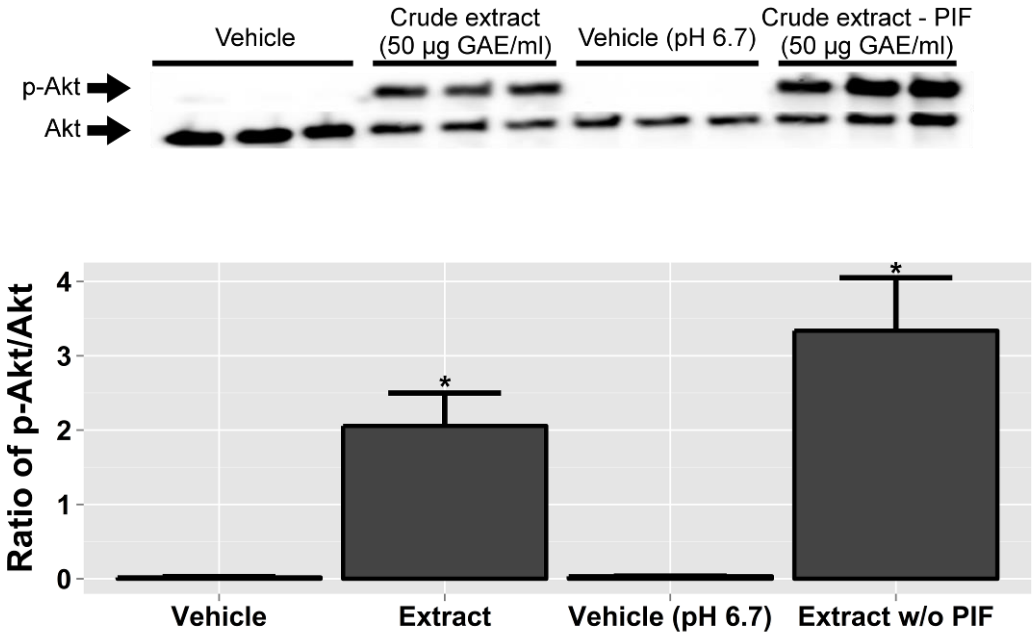

Figure 5.14: Western blots of p-Akt/Akt from lysates of cells treated for 60 minutes with the crude extract after removal of PIF, the vehicle control with cell media pH adjusted using HCl and the whole crude extract, alongside a vehicle control. n=3 (culture well replicates) for each treatment. Ratio of blot densitometry (p-Akt/Akt) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

5.5.4. Role of PI3K in the modulation of additional vasodilatory signalling molecules by the crude pomegranate extract

The crude pomegranate extract was again shown to significantly reduce both total eNOS protein and ET-1 secreted into culture media, and to increase levels of media nitrate/nitrite. Inhibition of PI3K significantly reversed both the increase of media nitrate/nitrite and the decrease in secreted ET-1 (**Figs. 5.15 and 5.16**). This was in spite of the PI3K inhibitor on its own causing a significant reduction in secreted ET-1, possibly due to the increase in the number of cells detached from the culture plate surface that was observed post-treatment, which also appeared to be reversed when cells were treated with the crude extract alongside the inhibitor (data not shown). The PI3K inhibitor

significantly reduced the decrease in eNOS protein by the crude extract even further, an effect also seen in cells treated with the inhibitor on its own (**Fig. 5.18**).

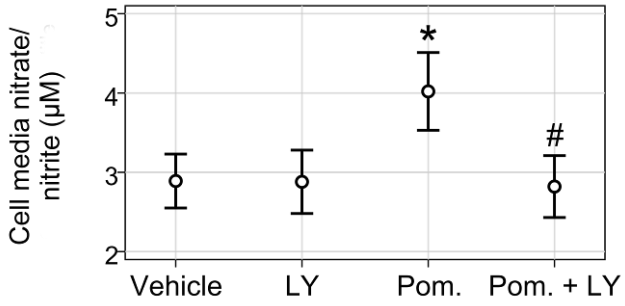

Figure 5.15: Concentration of culture media nitrate/nitrite after treatment of cells for 24 hours with crude pomegranate extract (Pom.) alongside a vehicle control, with or without a PI3K inhibitor (LY). Data representative of mean ± SD. n=3 (culture well replicates) for each treatment. * - $p < 0.05$ vs the vehicle control. # - $p < 0.05$ vs crude extract treated cells.

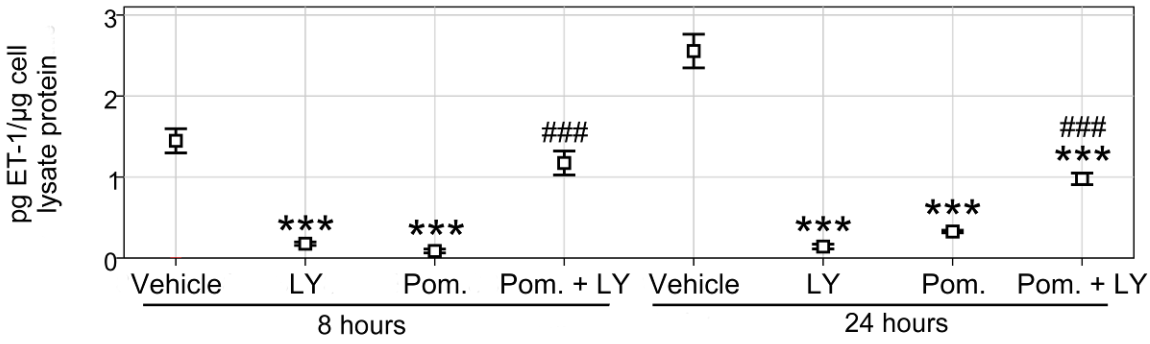

Figure 5.16: Concentration of secreted ET-1 after treatment of cells for indicated times with crude pomegranate extract (Pom.) alongside a vehicle control, with or without a PI3K inhibitor (LY). Data representative of mean ± SD. n=3 (culture well replicates) for each treatment. *** - $p < 0.001$ vs the vehicle control. ### - $p < 0.001$ vs crude extract treated cells.

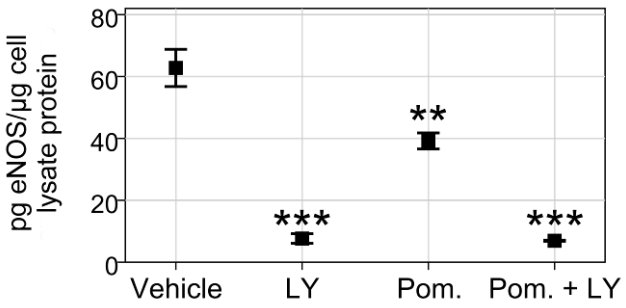

Figure 5.17: Concentration of intracellular eNOS protein after 24 hour treatment of cells with crude pomegranate extract (Pom.) alongside a vehicle control, with or without a PI3K inhibitor (LY). Data representative of mean ± SD. n=3 (culture well replicates) for each treatment. *** - $p < 0.001$ vs the vehicle control. ** - $p < 0.01$ vs vehicle control.

5.5.5. Analysis of RTK phosphorylation in cells treated with pomegranate extract

An antibody microarray slide, spotted with antibodies complimentary for a number of pan-tyrosine-phosphorylated RTKs, was used to analyse cells treated with the pomegranate extract, PPF or punicalagin for 10 minutes. No increase in phosphorylation of any of the multiple RTKs was observed in response to the treatments compared to the vehicle control. Comparison of the pomegranate extract against the vehicle control treatment showed a significant ($p < 0.05$) decrease in the phosphorylation of a number of RTKs featured on the slide, as well as three RTK adaptor proteins, Src, IRS-1 and c-Abl (**Table 5.6**). The only increases to protein phosphorylation in this comparison were to p-Akt (Ser473), p-Akt (Thr308) and p-ERK1/2 (Thr202/Tyr204). There were no significant changes to protein phosphorylation in comparisons of PPF vs. vehicle control or punicalagin vs. vehicle control.

Table 5.6: Kinases with significant differences in their levels of phosphorylation ($p < 0.05$) from cells treated with pomegranate extract, compared to cells treated with a vehicle control. Positive fold change values describe increased phosphorylation, while negative values describe decreased phosphorylation. n = 2 (culture well replicates) for each treatment.

Phospho-protein	Fold Change	p-value (adjusted for MT)
p-ERK1/2 (Thr202/Tyr204)	3.61	7.2E-06
p-Akt (Thr308)	3.50	9.7E-05
p-Akt (Ser473)	5.25	1.3E-04
p-ALK (pan-Tyr)	-1.37	2.8E-03
p-Src (pan-Tyr)	-1.95	4.2E-03
p-EphA3 (pan-Tyr)	-1.24	5.7E-03
p-EphB4 (pan-Tyr)	-1.27	1.4E-02
p-FGFR3 (pan-Tyr)	-1.24	1.5E-02
p-IRS-1 (pan-Tyr)	-1.29	1.5E-02
p-FGFR4 (pan-Tyr)	-1.17	1.7E-02
p-c-Abl (pan-Tyr)	-1.22	2.2E-02
p-FGFR1 (pan-Tyr)	-1.18	2.7E-02
p-TrkA (pan-Tyr)	-1.19	2.7E-02
p-ErbB2 (pan-Tyr)	-1.16	2.8E-02
p-c-Kit (pan-Tyr)	-1.15	3.7E-02
p-Ron (pan-Tyr)	-1.15	3.7E-02
p-EphA1 (pan-Tyr)	-1.13	4.7E-02
p-IGF-IR (pan-Tyr)	-1.13	4.7E-02

5.5.6. Effects of pomegranate extract treatment on endothelial cell PTEN protein and enzyme activity

To determine if PTEN was being prevented from translocating to the plasma membrane and dephosphorylating PIP_3, lysates from cells treated with pomegranate extract were assayed to determine if phosphorylation or ubiquitination of PTEN was increased due to the treatment, and if there were decreases in total intracellular PTEN protein. Western blots showed no change to PTEN phosphorylation (**Fig. 5.18**) or ubiquitination (**Fig. 5.19**) in cells treated with pomegranate extract for 60 mins compared to a vehicle control.

A reduction in total PTEN protein levels was observed following a 24 hour treatment with pomegranate extract (**Fig. 5.20**). To determine if PTEN enzyme activity itself was inhibited by the pomegranate extract, samples of immunoprecipitated PTEN from HUVECs treated with the extract for 30 minutes were incubated with a phosphoinositide substrate for 2 hours, and the concentration of the phosphate released was quantified. No difference was observed between treatments with the pomegranate extract and a vehicle control (**Fig. 5.21**). However, when purified PTEN was incubated for 5 mins with the procyanidin tetramers fraction, its phosphatase activity was significantly reduced ($p = 2.9E-9$) in a dose-dependent manner (0-40 μM) (**Fig. 5.22**).

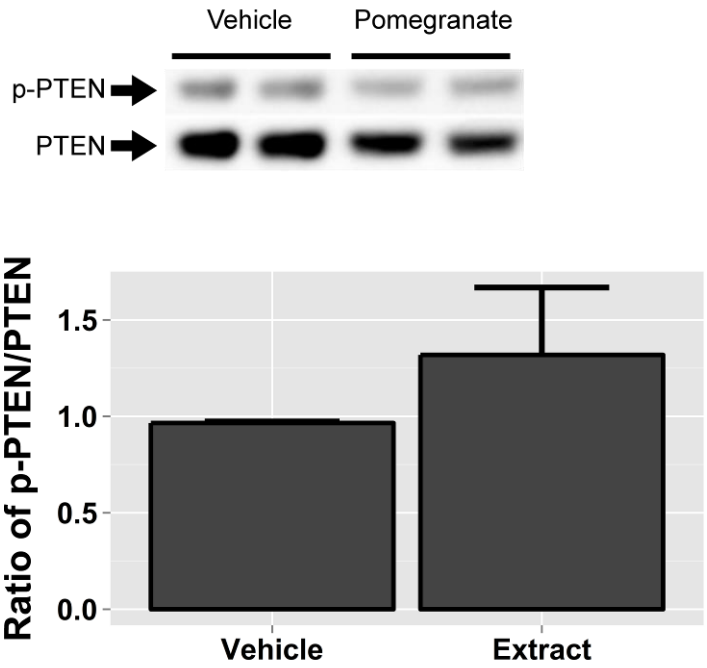

Figure 5.18: Western blots of p-PTEN/PTEN from lysates of HUVECs treated for 60 minutes with 50 μg GAE/ml of the crude pomegranate extract, alongside a vehicle control. n=2 (culture well replicates) for each treatment, representative of two independent experiments. Ratio of blot densitometry (p-PTEN/PTEN) is shown in the lower panel.

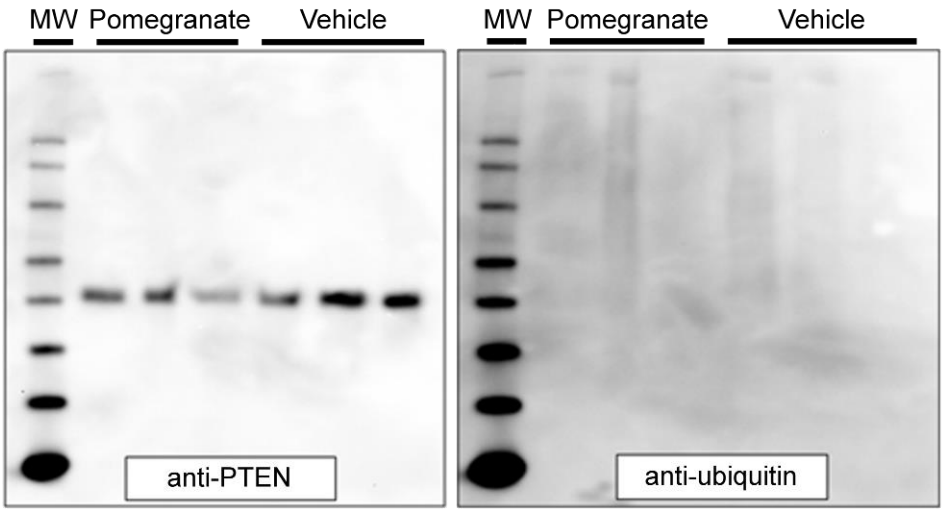

Figure 5.19: Western blots of PTEN/ubiquitin from anti-PTEN immunoprecipitated lysates of HUVECs treated for 60 minutes with 50 μg GAE/ml of the crude pomegranate extract, alongside a vehicle control. Molecular weight marker (**MW**) bands: **20**, **30**, **40**, **50**, **60**, **80**, **100**, **120** and **220 kDa** (bottom to top). n=3 (culture well replicates) for each treatment, representative of two independent experiments.

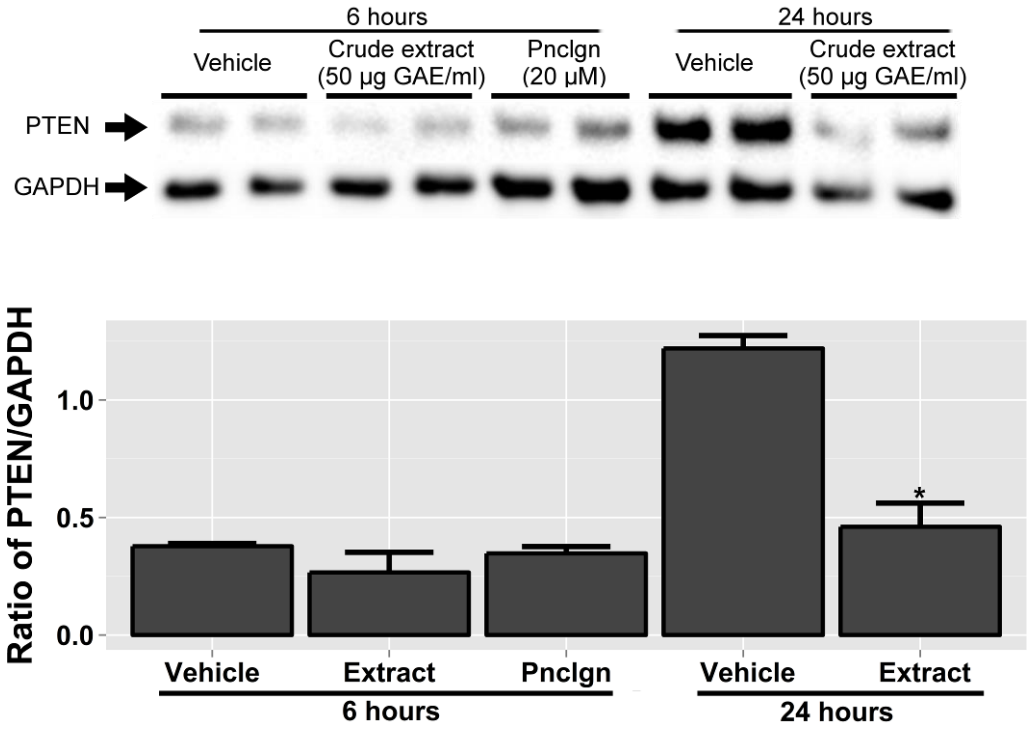

Figure 5.20: Western blots of PTEN/GAPDH from lysates of HUVECs treated for the indicated time periods with the crude pomegranate extract and punicalagin (Pnclgn), alongside a vehicle control. n=2 (culture well replicates) for each treatment, representative of two independent experiments. Ratio of blot densitometry (PTEN/GAPDH) is shown in the lower panel. ***** - $p < 0.05$ vs vehicle control treatment.

Figure 5.21: Enzyme activity of PTEN from cells treated with pomegranate extract (50 µg GAE/ml, 30 mins) is unchanged compared to a vehicle control treatment. Data representative of mean ± SD. n=3 (culture well replicates) for each treatment.

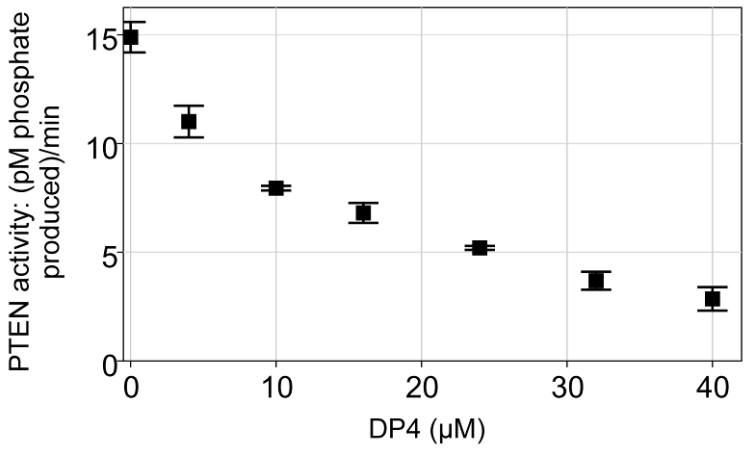

Figure 5.22: Inhibition of PTEN activity (from 30 ng PTEN) by increasing doses of procyanidin tetramers. Data representative of mean ± SD. n=3 (culture well replicates) for each treatment.

5.6. Discussion

Fractionation of the crude pomegranate extract, and subsequent HPLC and *in vitro* analyses, showed the extract's bioactivity to be mostly due to its procyanidin content. The increase to Akt and eNOS activity in endothelial cells was not due to changes to the phosphatase PTEN or receptor tyrosine kinases, leading to the question of what the true nature of the underlying mechanism for this bioactivity was.

5.6.1. Procyanidins as key polyphenols for pomegranate bioactivity

The crude pomegranate extract had been shown to increase Akt phosphorylation in **Chapter 4**. Being a mixture of mostly ellagitannins, anthocyanins, procyanidins and flavan-3-ols, fractions enriched with these compounds were prepared from the extract. The procyanidin/flavan-3-ol fraction (PPF) produced the strongest response with respect to Akt phosphorylation (**Fig. 5.9**). NP-HPLC analysis also suggested the presence of procyanidin tetramers in the crude extract (**Fig. 5.7**), though their presence in PPF could not be confirmed due to apparent drifts in peak retention times, which may require further work to resolve (**Fig. 5.6**). Neither the insoluble fraction. the sugar fraction, nor the decrease in pH caused by the acidity of the extract, were responsible for any considerable part of the extract's bioactivity (**Figs. 5.11** and **5.14**). Though smaller increases to p-Akt were seen from the ellagitannin and anthocyanin fractions, assays conducted using individual compounds showed a strong increase to Akt phosphorylation after treatment of HUVECs with procyanidin tetramers, but not with procyanidin dimer B1, (-)-epicatechin, punicalagin or delphinidin/cyanidin glucosides (**Figs. 5.9 - 5.11**). This is the first case in which a fractionation method has been used to identify the compounds responsible for pomegranate bioactivity.

While procyanidin tetramers in the pomegranate extract appear to play a significant role in increasing Akt phosphorylation in HUVECs, they are unlikely to

account for all of the extract's bioactivity. It has been reported that there is a correlation between the degree of polymerisation (DP) of procyanidins and their bioactivity (Byun et al. 2012; Kimura et al. 2011; Osborne & McNeill 2001; Kondo et al. 2006). This would suggest that if larger procyanidin oligomers, such as pentamers or hexamers, were present, only relatively small quantities of these would be needed to account for the rest of the extract-induced p-Akt response. Running the pomegranate extract on a preparative NP-HPLC method, to isolate the peak presumed to be procyanidin tetramers, would be a sensible means for collecting further evidence of the procyanidins as the key polyphenols responsible for the extracts ability to increase p-Akt.

5.6.2. Fractionation of pomegranate polyphenols

No previous reports were found which described the use of activity-guided fractionation to separate pomegranate into its major polyphenol sub-classes. Though the method used was originally described for the separation of a red wine extract, it was effective for the completion of this study's objectives, with RP- and NP-HPLC showing good separation of the relevant compounds. It appeared that there were short-comings in terms of yields for a number of compounds, comparing the fractions to the whole extract, though the efficiency of the method was not reported in the study in which it was originally described (García-Estévez et al. 2010). As ellagitannins are less stable dissolved in methanol (Lei 2002), its use for the second fractionation with the Sephadex LH-20 material may have been a factor in the loss seen for punicalagin after fractionation. As a further explanation of lost yields, it was observed that during the evaporation of ethyl acetate, small quantities of a cloudy-white precipitate formed around the top of the round-bottom flasks. Attempts were made to redissolve and reclaim this precipitate but they were unsuccessful.

5.6.3. Additional bioactive compounds from the pomegranate extract

Besides procyanidin tetramers, other compounds found in pomegranate arils were able to increase Akt phosphorylation, though the concentration required was too high to conclude that they were responsible for a major part of the observed bioactivity of the crude extract. The increase of p-Akt by punicalagin (**Fig**. **5.12**) and ellagic acid (**Fig. 5.13**) is in concurrence with reports from Chen et al. (2008), where a 50 μM dose of punicalagin and a 25 μM dose of 1-O-galloylpunicalagin increased eNOS (Ser1177) and Akt (Ser473) phosphorylation and Ou et al. (2010) where ellagic acid was similarly shown to increase Akt and eNOS phosphorylation. This was with a smaller dose of 5 μM, though cells were also challenged with oxidised low-density lipoprotein which decreased Akt phosphorylation prior to ellagic acid treatment (the basal level of Akt phosphorylation in this report also appears to be much higher than that observed in the studies performed here). The study by Chen et al. also highlights that other ellagitannins besides punicalagin may be present in the pomegranate extract, and these may be more effective at increasing p-Akt.

Although it was not seen here, (-)-epicatechin has been reported to increase Akt phosphorylation in HUVECs (Ramirez-Sanchez et al. 2012). It has been similarly shown that the flavan-3-ol epigallocatechin-gallate can increase p-Akt as well (Yamakuchi et al. 2008), though this particular flavan-3-ol has not yet been identified in pomegranate. Anthocyanins from black currants, including D3Glc, were also shown to increase Akt phosphorylation (Edirisinghe et al. 2011), again an effect again not seen in this study. There are recent reports of flavan-3-ol-anthocyanin adducts, identified by LC–ESI/MS^n analysis (Sentandreu et al. 2011; Sentandreu et al. 2010). Whether condensation of flavan-3-ols with anthocyanins could affect the potential bioactivity of the individual compounds remains to be investigated.

Pomegranates also contain a number of other secondary metabolites, such as alkaloids, organic acids, fatty acids and a number of volatile organic compounds

(Bonzanini et al. 2009; Wang et al. 2010; Viuda-Martos et al. 2010; Johanningsmeier & Harris 2011; Kumar & Vijayalakshmi 2011; Medjakovic & Jungbauer 2013; Lansky & Newman 2007). No studies could be found, however, that report an increase to Akt or eNOS phosphorylation in endothelial cells due to these compounds.

5.6.4. Evidence of beneficial effects of pomegranate on human health

Many reports have described the potential benefits of pomegranate, for a wide range of aspects of human health. While much has been concluded from *in vitro* and animal studies, such as effects on lipid metablism or gastric ulcer formation, a number of human clinical trials, specially for prostate cancer therapy/prevention, are reportedly now in progress (Colombo et al. 2013; Miguel et al. 2010). In the context of vascular health, a recent human study that measured effects on pulse wave velocity (PWV) and blood pressure after consumption of pomegranate juice for four weeks found that, while PWV was unaffected, systolic and diastolic blood pressure both significantly decreased compared to the control drink, as did mean arterial pressure, though no underlying mechanisms were proposed in this study (Lynn et al. 2012). Such effects are consistent with *in vitro* results showing changes to markers of reduced vascular tone, such as those reported here.

The presence of procyanidins, of sizes large enough to be bioactive towards nitric oxide signalling (i.e. DP4) has still to be confirmed, however, and cannot yet be concluded as the mechanism by which pomegranate exerts health benefits in the cardiovascular system. Procyanidin dimers (i.e. DP2) have been reported in human plasma (Holt et al. 2002), but further development of analytical technologies may be required before the presence or lack of presence of larger procyanidins can be proven.

5.6.5. Underlying mechanisms of pomegranate bioactivity - PTEN protein

Despite the potential for pomegranate arils to provide beneficial effects to human vascular health, little has been reported on the underlying mechanisms in endothelial cells. The effect of pomegranate on eNOS activity has been well reported (Ignarro et al. 2006; de Nigris et al. 2005), but besides this, evidence is limited to an ability to improve levels of antioxidant enzymes in endothelial cells (de Nigris et al. 2007; Nishigaki et al. 2008). The dependence of PI3K for the increase of endothelial nitric oxide production by pomegranate extract, shown in the context of Akt activation in **Chapter 4**, was further underlined in this chapter shown by the reversal to increased media nitrate levels after incubation of cells with a PI3K inhibitor (**Fig. 5.15**). The reversal of decreased ET-1 secretion by the crude extract by this inhibitor also provides strong evidence that the inhibition of ET-1 is a direct result of the increase in nitric oxide production (**Fig. 5.16**).

After studying a number of markers, there was no evidence to suggest that inhibition of PTEN, a phosphatase which acts to limit Akt activity, was behind the PI3K-dependent pomegranate bioactivity. Though there was a reduction in total PTEN protein (**Fig. 5.20**), the relatively long period for this effect to occur would not help to explain the much quicker increase of Akt phosphorylation. The marked reduction in PTEN enzyme activity that was seen after incubation of the protein with procyanidin tetramers (**Fig. 5.22**) is in agreement with previous studies that have reported the capacity for procyanidins to bind to proteins and inhibit enzyme activity (Cho et al. 2009; Huang et al. 2011), however, this observation could not be repeated with an in-cell experiment. This is the first reported use of immunoprecipitation to allow study of the activity of enzymes from endothelial cells treated with polyphenols or polyphenol-rich extracts. It should be noted that while the experiments testing enzyme activity of immunoprecipitated PTEN featured controls for the cell lysis buffer, no controls were used that took account of the immunoprecipitation beads used in the assay. To ensure that the levels of free phosphate measured are certain

to be a result of active PTEN from endothelial cells, this second control should be included in the assay design in any future repeats of this experiment.

While PTEN is a key regulator of Akt activity, SHIP1/2 proteins share similar phosphatase activity. Therefore, study of markers for SHIP2 inhibition (as the presence of SHIP1 in endothelial cells is not well recorded) may be necessary, to rule out the inhibition of phosphatase activity as an underlying mechanism.

5.6.6. Underlying mechanisms of pomegranate bioactivity - RTKs

An investigation into the involvement of endothelial cell surface receptors in the mediation of pomegranate extract bioactivity has not been previously reported. There are previous reports, however, that show the effects of procyanidins on individual RTKs, including reduced expression of ErbB2 (Kenny et al. 2004), the dephosphorylation of which was reported in this study, and the inhibition of VEGFR2 activity (Lu et al. 2010). As the phosphorylation of a number of RTKs was shown to significantly decrease ($p < 0.05$), the extract appears to interfere with either the activating ligands of RTKs or RTK autophosphorylation. It is a key feature of RTKs that their activation is facilitated by increased tyrosine phosphorylation (Lemmon & Schlessinger 2010). If indeed there is a trend of the pomegranate extract decreasing the phosphorylation of RTKs, or even inhibiting their phosphorylation via other signalling molecules, then this suggests that increased PI3K activity through RTK activation is not the key mechanism for pomegranate extract-induced Akt/eNOS phosphorylation. Alongside the decreased phosphorylation of RTKs, the phosphorylation of three RTK cofactors, IRS-1, Src and c-Abl, was also reduced. This phosphorylation decrease may be another indication of interference by the pomegranate extract in the resting activity of RTKs. It is of course still possible that an RTK expressed in human endothelial cells, but not represented by the microarray, is responsible for increasing PI3K activity. Only 12 of the 20 human RTK

families are represented on the microarray slide, though there is evidence that all but one of these are expressed in endothelial cells (Cantarella et al. 2002; Kamiyama et al. 2008; O'Donnell et al. 1999; Song et al. 2012; Suhardja & Hoffman 2003; You & McDonald 2008; Lanckohr et al. 2010). Additionaly, it may be that compounds from the extract can bind to the cell surface and cause RTK activation by interfering with the processes which keep RTKs in a state of low phosphorylation.

As expected, the crude extract increased phosphorylation of Ser473 and Thr308 of Akt. It is surprising that the PPF treatment did not also increase p-Akt in this experiment, but this may be due to the short treatment time, and the likelihood that the relative concentration of bioactive compounds was reduced (through the fractionation process - see **Section 5.5.2)**, in comparison to the crude extract. The crude extract also increased phosphorylation of ERK1/2. These proteins are involved with a number of cellular processes, including proliferation, cell survival and cell adhesion (Roskoski 2012), and have been characterised as a signalling molecule regulated by PI3K activity. Therefore, in this case the increase to its phosphorylation does not appear to provide further information as to the crude extract's underlying signalling mechanisms.

GPCR activation cannot be ruled out as the mechanism for increasing PI3K activity. There are numerous reports detailing the activation of PI3K and Akt through GPCRs, and the stimuli responsible for such activation (New et al. 2007; Oudit & Penninger 2009). Investigation of any dependence of Akt activation on GPCRs could be performed by inhibiting the interaction between G-protein subunits, through the use of, for example, targetted peptides (Chen et al. 1997). As the activity of PI3K, PTEN, Akt and eNOS, in the context of nitric oxide production, takes place at the plasma membrane, it is also possible that exposure of endothelial cells to procyanidins results in changes to the membrane itself, independent of individual signalling receptors, which interfere with signalling processes. For example, the translocation of PTEN to the plasma membrane relies on electrostatic interactions between the membrane and cationic protein residues

(Das et al. 2003). Depolarisation of the membrane to a positive potential could potentially disrupt this interaction, and procyanidins have been reported to cause depolarisation of mitochondrial membranes in breast cancer cells (Ramljak et al. 2005), however, most results in endothelial cells show a hyperpolarisation effect due to procyanidin treatment (Byun et al. 2012; Matsui et al. 2009). It is possible that hyperpolarisation of the membrane may be an underlying mechanism for the activation of Akt and eNOS, but exactly how, and in what manner, remains to be investigated.

5.7. Conclusion

The bioactivity of the pomegranate extract is reported here as being primarily due to its procyanidin content, though further work is necessary to confirm the presence of specific procyanidin oligomers in the pomegranate extract. Phosphorylation, ubiquitination or phosphatase activity of PTEN was not affected by the pomegranate extract treatment, and the activity-related phosphorylation of a number of human RTKs did not increase, prior to the phosphorylation of Akt and eNOS by the extract. Activation of a GPCR or an RTK not covered in this study may explain the observed bioactivity. Alternatively, interference with the stasis of the plasma membrane, possibly through binding of procyanidins to the membrane surface, may be behind the increased production of nitric oxide in endothelial cells by pomegranate procyanidins.

Chapter 6

Concluding remarks and directions for future work

Chapter 6: Concluding remarks and directions for future work

6.1. Identification and analysis of bioactive procyanidins

The studies detailed in this thesis sought to determine the effects of dietary polyphenols on markers of endothelial function, and to understand the underlying mechanisms behind these effects. To accomplish this, a number of biochemical techniques were used to analyse molecular changes in endothelial cell cultures after exposure to phenolic compounds. Western blotting and ELISAs provided simple and practical means for analysis of specific markers, while antibody microarrays allowed analyses of a wide range of targets and consequently provided large sets of data from which to draw conclusions. The flavonoid quercetin was a common phenolic amongst the food plants selected in Chapter 2, being present to varying extents in dill, nettle, kale and persimmon according to previous reports, and confirmed by in-house analysis performed by Shikha Saha (IFR, UK) (Hollands et al. 2013b). However, it was procyanidins, identified after fractionation of the crude pomegranate extract, which had the greatest effect on endothelial cell signalling. As with the crude extract, procyanidins increased both Akt and eNOS phosphorylation in HUVECs, while incubation of cells with pomegranate extract also increased levels of nitrate/nitrite in cell culture media, inhibited ET-1 secretion in a PI3K-dependent manner, and decreased total eNOS protein over 24 hours. As procyanidins and flavan-3-ols constitute a large proportion of persimmon phenolics (Hollands et al. 2013b), it is likely that the persimmon extract's ability to effect markers of endothelial function in a manner similar to the pomegranate extract is also due to the presence of procyanidins or other oligomeric proanthocyanidins (OPACs).

Identification of the particular procyanidins responsible for pomegranate bioactivity remains to be investigated. There are likely to be features of the procyanidin structure that contribute towards its ability to increase endothelial nitric oxide production, and so it would be useful to determine the structure of the bioactive procyanidins so that these features could be better understood. For example, besides the degree of polymerisation (DP), the types of flavanol monomer from which proanthocyanidins are composed may affect their bioactivity. It has been reported in animal studies that gallocatechins and epigallocatechins can be more effective at producing vasorelaxant effects, compared to their catechin and epicatechin counterparts (Huang et al. 1998; Sanae et al. 2002), though no studies could be found which show marked differences between epicatechin and catechin monomers in the context of effects on endothelial function and CVD risk. There are also different manners in which monomer units may be linked, such as A- or B-type OPAC bonds (**Fig. 6.1**), which at the very least may affect their rate of absorption (Appeldoorn et al. 2009). Separation of procyanidins by DP can be achieved using normal-phase (NP) HPLC. Preparation of pomegranate extract fractions by NP-HPLC would allow each set of procyanidin oligomers to be assayed for bioactivity towards p-

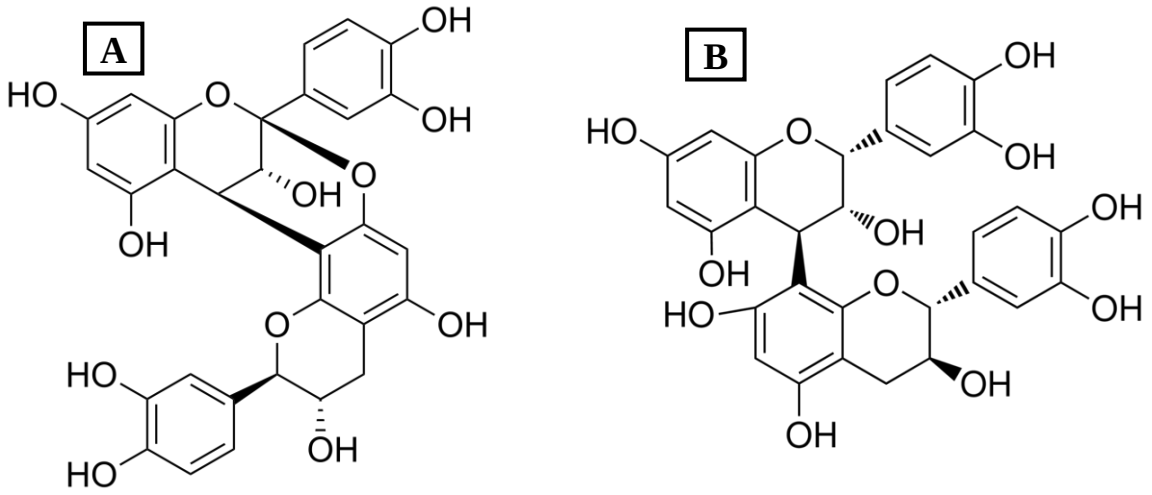

Figure 6.1: Procyanidin dimers demonstrating (**A**) A-type interflavan bonds (C2→O-C5/7, and C4→C6/8) and (**B**) B-type interflavan bonds (C4→C6/8).

Akt in isolation from other procyanidins and pomegranate phenolics. The particular DP of procyanidins that are responsible for the majority of the crude pomegranate extract's bioactivity could be determined by this method. NP-HPLC is often limited by poor reproducibility of peak retention time between runs, however, which could create issues during preparative HPLC runs, as the time points set for collecting each fraction would be based on peak retention times of previous runs. An alternative approach would be to use hydrophilic interaction liquid chromatography (HILIC). As with NP-HPLC, HILIC makes use of a polar stationary phase, often with additional polar functional groups such as diols, but also uses polar solvents such as acetonitrile, similar to reverse phase (RP) HPLC (Buszewski & Noga 2012). Compared to NP-HPLC, however, HILIC analysis provides improved peak resolutions and a high level of reproducibility between chromatography runs. Whereas NP-HPLC is adequate for separation of OPACs by DP alone (Shoji et al. 2006a; Sarnoski et al. 2012; Kelm et al. 2006), and in cases can separate also by A- or B-type interflavan linkage (Gu et al. 2002), recent reports demonstrating HILIC analysis suggest that the technique is powerful enough to not only separate peaks by A- or B-type interflavan linkage (Karonen et al. 2011; Robbins et al. 2009; Wallace & Giusti 2010), but in cases can separate individual OPAC isomers (Furuuchi et al. 2011). It was also demonstrated by Kalili et al. (2012) that chromatography run times can be greatly reduced while retaining peak resolution. Faster HPLC runs alongside improved reproducibility make the repeated collection of fractions highly practical, allowing production of quantities of each fraction to the extent required for incubation with cell cultures. There is potential for further increases to productivity if ultra-pressure liquid chromatography (UPLC) equipment is used, as shown by the comparative study by Ortega et al. (2010). While this study used NP silica columns, the compatibility of HILIC with UPLC equipment has been previously demonstrated (Spagou et al. 2011).

Chromatograms presented in **Chapter 5** (**Fig. 5.8**) demonstrate the presence of compounds in the pomegranate anthocyanin and ellagitannin fractions which have similar retention times to the peaks representative of procyanidins in the pomegranate procyanidin fraction and crude extract. It would be expected, therefore, that fractionation of the crude extract to isolate procyanidins of specific DP would also isolate non-procyanidin compounds in the same fractions. Sephadex LH-20 (Cuevas-Rodríguez et al. 2010; Gu et al. 2002; Wei et al. 2011) and Toyalpearl HW-50 (Xu et al. 2012a; Li et al. 2010; Stringano et al. 2011) have been previously shown to be suitable materials for preparing OPAC rich fractions from crude plant extracts. It was noted in **Chapter 5,** however that fractionation steps can result in losses of certain phenolic compounds and so it would be preferable to keep the number of fractionation steps to a minimum. If the crude extract was to be fractionated by preparative HILIC, without prior purification using LH-20 or HW-50 material, the HILIC fractions could be subsequently separated by RP-HPLC, as has been recently demonstrated. This method has proved to be efficient at separating isomers which share similar NP-HPLC or HILIC retention times (Montero et al. 2013; Kalili & de Villiers 2009; Kalili & de Villiers 2013). The combination of HILIC fractionation with subsequent RP-HPLC analysis would allow identification of individual compounds within the fractions by mass spectrometry, especially if reasonable quantities of each fraction could be collected.

Characterisation of OPACs can be performed by thiolysis (Jerez et al. 2009; Saucier et al. 2001; Xu et al. 2012a) or phloroglucinolysis (Hanlin et al. 2011; Bordiga et al. 2011; Drinkine et al. 2007), where acid-catalysed cleavage of interflavan bonds releases flavanol monomer units. The released flavanols can then be analysed by HPLC and mass spectrometry, so as to identify and quantify the terminal units, the extension units (which become conjugated on release, allowing them to be differentiated from the terminal units) and the mean DP. Electrospray ionisation mass spectrometry (MS-ESI) provides a more certain means of identification for OPACs in concentrated samples.

Besides measurement of the degree of polymerisation, the fragmentation pattern can also provide clues as to the flavanol composition of individual OPAC compounds. (Sendker et al. 2013; Tarascou et al. 2011; Sarnoski et al. 2012). If individual OPACs can be isolated, nuclear magnetic resonance spectroscopy and circular dichroism spectroscopy can be employed for confirming details of the interflavan bonds between monomer units, such as whether linkages are of A- or B-type conformation (Li et al. 2010, Hümmer & Schreier 2008).

HILIC coupled with RP-HPLC-MS-ESI is one of the most powerful means for separation and subsequent identification of OPACs currently available. Such a method may be improved by initial isolation of OPACs using LH-20 or HW-50 columns, and the length of time to perform chromatography would certainly be reduced using a UPLC system, though such an approach may not be currently feasible due to the cost of such equipment. Isolation of groups of OPACs according to their DP from the pomegranate and persimmon extracts, with subsequent preparation of these compounds in reasonable quantities, would allow more detailed analyses of their ability to increase Akt and eNOS phosphorylation. There are currently no commercially available OPACs of DP > 3, and so the production of stocks of individual OPAC oligomers/isomers would be of high value. Concentrated solutions of pomegranate/persimmon OPAC fractions would make identification by MS-ESI more practical, and if successful, may allow conclusions to be drawn on the importance of specific structural features, such as the types of flavanols from which they are composed, or whether galloylation of flavanol units is important for OPAC bioactivity.

6.2. The mechanism by which procyanidins regulate Akt activity

The regulation of PI3K/Akt activity by pomegranate procyandins was shown to be independent of a number of changes to PTEN protein, and of increased activity of a

subset of receptor tyrosine kinases. To date, there are no reports which investigate endothelial signalling upstream of Akt and eNOS phosphorylation, in relation to incubation of cell cultures with pomegranate or persimmon extracts. There are, however, a number of reports that show increased Akt phosphorylation in endothelial cells after incubation with OPACs or flavanols extracted from other sources. Increased phosphorylation of Akt and eNOS has been reported after treatment of endothelial cells with procyanidin-rich fractions of a red wine extract (Auger et al. 2010), and with an OPAC-rich grape seed extract (Edirisinghe et al. 2008). The grape-seed extract also caused relaxation of rabbit aortic rings, but these effects were inhibited after methylation of phenolic hydroxyl groups. The importance of specific flavanol hydroxyl groups for eliciting their bioactivity has been previously demonstrated. The 3" hydroxyl group of epigallocatechin-gallate (i.e. the 3-OH group of the galloyl moiety) was found to be required for its ability to increase Akt and eNOS phosphorylation, whereas methylation of the 4" hydroxyl group caused no reduction of protein phosphorylation (Kurita et al. 2013). As the hydroxyl groups are largely responsible for phenolic antioxidant activity (Rice-Evans et al. 1996), it is probable that OPAC antioxidant activity plays some part in its activation of Akt. The activity of a number of signalling proteins, including AMPK, eNOS and ET receptor B, can be regulated by localised redox changes (Maron & Michel 2012). For example, increased eNOS phosphorylation in endothelial cells has been shown to rely on the availability of non-glutathione oxidant-reducing cysteine thiols inside cells and at the cell surface (Tanaka et al. 2005). The increase to eNOS mRNA in endothelial cells by an OPAC-rich purple grape juice was shown to be partly dependent on intracellular ROS generation, through the incubation of cells with membrane permeable antioxidant enzymes (Alhosin et al. 2013).

As was suggested in Chapter 5, it is possible that the exposure of cells to procyanidins influences the chemistry of the plasma membrane in a manner which activates Akt. OPAC-rich extracts have been reported to cause hyperpolarisation (a

decrease in the membrane electric potential) of endothelial cells and arterial tissue (DalBó et al. 2008; Byun et al. 2012a), and this change to membrane potential has been shown to be reliant on K^+ channels and was induced in less than three minutes after treatment with an epicatechin trimer (procyanidin C1) (Byun et al. 2012b). The increased levels of cell culture media nitrite/nitrate induced by procyanidin C1 treatment was also shown to be partly due to K^+ channel activity. Akt phosphorylation may not necessarily be induced primarily by hyperpolarisation, however. Hyperpolarisation of HUVECs by β_2-adrenoceptor treatment was discovered to be in response to increased nitric oxide production, through increased uptake of the eNOS substrate L-arginine from cell culture media, and so changes to membrane potential may help to sustain eNOS activity, rather than initiate it (Queen et al. 2006). However, increased Akt activity has also been shown to be initiated by depolarisation of endothelial cell plasma membranes (Chatterjee et al. 2012) and endothelial cell mitochondrial membranes (Katakam et al. 2013).

It would be interesting to determine whether the pomegranate or persimmon extract, or their isolated OPACs, are able to hyperpolarise or depolarise the cell culture membrane potential. Measurement of membrane hyper-/depolarisation can be measured using a number of potential sensitive fluorophores, including bis-(1,3-dibutylbarbituric acid) trimethine oxonol, bisoxonol and rhodamine 123, which can be introduced into cells during a pre-incubation period before treatment with phenolic compounds. K^+ channel blocking can be performed by treating cells with iberiotoxin, which could be used to confirm that hyperpolarisation is necessary for Akt activation. C-natriuretic peptide may be used in such experiments as a positive control, as it has been previously shown to hyperpolarise endothelial cell membranes (Simon et al. 2009). The involvement of cell surface cysteine thiols could be investigated using the fluorophore ALM-488, followed by analysis using flow cytometry. It would also be interesting to determine if similar effects to nitric oxide production are seen in these experiments when using inflamed HUVECs, such as through pre-treatment with TNF-α (Sawa et al. 2007). Inflamed

HUVECs are a more commonly reported model, in relation to resting HUVECs, and so such experiments could provide more useful data for comparing to other previously published studies.

It has been shown that *S*-nitrosylation of PTEN leads to increased Akt activity (Numajiri et al. 2011). This particular modification to PTEN protein was not studied as part of Chapter 5, and so it may be useful to determine levels of PTEN *S*-nitrosylation to clarify whether or not PTEN protein is responsible for increasing Akt activity after treatment of cells with pomegranate extract. This can be achieved through reduction of *S*-nitrosothiols to thiols, incubation with a biotin-conjugate followed by subsequent pull-down, and analysis by western blotting. Alternatively, the sub-cellular location of PTEN at various time-points could be tracked using fluorescently tagged recombinant PTEN. As PTEN is required at the plasma membrane to inhibit Akt activity, such an experiment would provide strong evidence of PTEN's involvement in pomegranate extract bioactivity.

6.3. Bioavailability of proanthocyanidins

To date, procyanidins larger than dimers are still to be reported in human plasma, though studies with rats have identified procyanidin trimers in plasma after feeding doses of cocoa or other procyanidin-rich extracts (Serra et al. 2013; Serra et al. 2009; Prasain et al. 2009). Reports of procyanidin dimers and trimers in rat plasma have also shown them to be unaffected by phase II metabolism (Appeldoorn et al. 2009; Serra et al. 2010; Tsang et al. 2007). Human studies have reported the presence of procyanidin dimer B2 after consumption of cocoa, as well as a lack of phase II metabolism of procyanidins (Holt et al. 2002; Kahle et al. 2011). Even though increased size of procyanidins has been shown to correlate inversely with their transport across Caco-2 cell cultures, it is still possible for procyanidins as large as tetramers to pass through these cells (Ou et al. 2012). The study

by Zumdick et al. (2012) reported that the transport of tetramers is comparable to that of procyanidin dimers, indicating the possibility that tetramers could be found in human plasma. It has been hypothesised that procyanidins bind to proteins in plasma, and that this can interfere with their identification after feeding studies (Shoji et al. 2006b). To test this hypothesis, plasma samples from rats fed with apple procyanidins were treated with urea, to reverse the binding of procyanidins to plasma proteins. Following urea treatment, procyanidins up to and including DP5 were then identified. It is likely, though, that the size of OPACs can limit their absorption in the gut. It would be interesting to determine whether the rate of their absorption could be improved. The formulation of liposome vesicles for improved transport of molecules has been demonstrated previously (Parmentier et al. 2010), and as liposome encapsulation has been demonstrated for epigallocatechin-gallate (Gülseren & Corredig 2013), it may be possible to prepare such formulations for OPACs as well. To date, though. there is little evidence to suggest that proanthocyanidins are relatively bioavailable to humans. Any bioactive effects seen from OPACs in *in vitro* experiments cannot yet, therefore, be concluded as likely mechanisms for their *in vivo* health effects, with further human studies still required to conclusively understand the absorption and metabolism of these compounds. In future, technological developments may allow labelling of phenolic compounds such that they can be tracked as native and metabolised compounds *in vivo*. Such advancements would allow the destination of phenolics to be recorded with greater accuracy, and provide a better understanding of the specific metabolites that reach certain tissues.

6.4. Health benefits from consumption of proanthocyanidin-rich foods

Identifying the compounds responsible for the reduction of cardiovascular risk observed in the patients and volunteers of epidemiological and human intervention studies focused on polyphenol-rich diets, as well as determining the underlying molecular mechanisms for their bioactivity, can improve advice for prevention of CVD, and guide drug design

for therapies. Continued investigation of the signalling pathways which are activated or inhibited due to exposure of cells to phenolic compounds provides development of new biomarkers for future human studies. It is important that the physiologically relevant forms of phenolic compounds remain the focus of such studies. While phase II metabolites of quercetin were used to treat cells in **Chapters 3** and **4**, metabolites produced by gut microflora are also present in plasma after consumption of quercetin glycosides. To date, there do not appear to be any reports of studies which investigate the effects of quercetin microbial metabolites on endothelial signalling. Microbial metabolites of quercetin have been recovered in human urine at 22% of the original dose (Jaganath et al. 2006), and so represent a significant proportion of bioavailable quercetin metabolites. There appears to be very few additional studies which quantify levels of quercetin-derived microbial metabolites in human plasma or urine, though validated methods for performing such analyses are now available (Urpi-Sarda et al. 2009; Chen et al. 2012). Future studies could make use of our increasingly greater understanding of the profile of bacterial species which inhabit our gut, and use *in vitro* cultures to determine the microbial metabolites likely to be produced from certain polyphenols *in vivo*, coupled with preparations of microbial metabolite mixes to then be used on human cell cultures, to determine their bioactivity.

Data from appropriate *in vitro* studies in conjunction with human trials can serve as strong supporting evidence in cases of health claims for food products (Aggett et al. 2005). Based on the results of studies described in this thesis, foods rich in procyanidins and other OPACs would be strong candidates for studies focused on markers of endothelial function and reduced CVD risk. Cocoa has been shown to be rich in OPACs (Kalili & de Villiers 2013) and its consumption can effect a number of markers of CVD risk in human studies (Arranz et al. 2013), including improvement of flow-mediated dilation (Monahan et al. 2011) and reduced levels of oxidised low density lipoprotein (Khan et al. 2012). It is of note that cocoa products (and likely other OPAC-rich foods)

are also rich in (-)-epicatechin (Jiménez et al. 2012), which has a higher rate of absorption in the gut than OPACs (Spencer et al. 2001). (-)-Epicatechin has also been shown to increase nitric oxide production, as well as inhibit NADPH oxidase *in vitro* (Ramirez-Sanchez et al. 2012; Steffen et al. 2007) and improve FMD *in vivo* (Schroeter et al. 2006). In future studies that report the bioactivity of an OPAC-rich food or extract, it would be of interest to determine the proportion of activity due to the food's flavanol monomers, in comparison to the activity due to the procyanidin oligomer content.

It is likely that there are food plants besides pomegranate and persimmon, which form part of traditional diets of countries surrounding the Black Sea, that are rich in OPACs. It would be useful to identify such foods, and conduct *in vitro* and human studies which assess markers of endothelial function such as Akt and eNOS phosphorylation, and seek to measure levels of procyanidins or their metabolites in human plasma. Results from these studies could potentially provide good evidence for the ability of the selected foods to lower CVD risk, and would encourage their continued use as part of local and national diets.

Appendix I

List of protein targets featured as pairs of anti-phosphorylated/unphosphorylated antibodies on the PEX-100 antibody microarray:

Uniprot ID	**Protein and phosphorylated residue(s)**
P27348	14-3-3 theta/tau (Phospho-Ser232)
P63104	14-3-3 zeta (Phospho-Ser58)
P63104	14-3-3 zeta/delta (Phospho-Thr232)
Q13541	4E-BP1 (Phospho-Thr36)
Q13541	4E-BP1 (Phospho-Thr45)
Q13541	4E-BP1 (Phospho-Ser65)
Q13541	4E-BP1 (Phospho-Thr70)
O60825	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) (Phospho-Ser483)
P00519	Abl1 (Phospho-Tyr204)
P00519	Abl1 (Phospho-Thr754/735)
Q13085	ACC1 (Phospho-Ser79)
Q13085	ACC1 (Phospho-Ser80)
P68032	ACTIN Pan(α/β/γ) (Phospho-Tyr55/53)
P98177	AFX/FOXO4 (Phospho-Ser197)
P31749	AKT (Phospho-Thr308)
P31749	AKT (Phospho-Tyr326)
P31749	AKT (Phospho-Ser473)
P31749	AKT1 (Phospho-Ser124)
P31749	AKT1 (Phospho-Ser246)

P31749	AKT1 (Phospho-Thr450)
P31749	AKT1 (Phospho-Tyr474)
P31749	AKT1 (Phospho-Thr72)
Q96B36	AKT1S1 (Phospho-Thr246)
P31751	AKT2 (Phospho-Ser474)
Q9UM73	ALK (Phospho-Tyr1507)
Q9UM73	ALK (Phospho-Tyr1604)
Q13131	AMPK1 (Phospho-Thr174)
Q13131	AMPK1/AMPK2 (Phospho-Ser485/491)
Q9Y478	Ampkβ1 (Phospho-Ser182)
P05067	Amyloid β A4 (Phospho-Thr743/668)
P10275	Androgen Receptor (Phospho-Ser213)
P10275	Androgen Receptor (Phospho-Ser650)
P10398	A-RAF (Phospho-Tyr301/302)
P49407	Arrestin-1 (Phospho-Ser412)
Q99683	ASK1 (Phospho-Ser83)
Q99683	ASK1 (Phospho-Ser966)
P18846	ATF-1 (Phospho-Ser63)
P15336	ATF2 (Phospho-Ser112/ 94)
P15336	ATF2 (Phospho-Ser62/ 44)
P15336	ATF2 (Phospho-Thr69/51)
P15336	ATF2 (Phospho-Thr71/53)
P15336	ATF2 (Phospho-Thr73/55)
P18848	ATF4 (Phospho-Ser245)
P05023	ATP1A1/Na+K+ atpase1 (Phospho-Ser23)
P05023	Atpase (Phospho-Ser16)
P53396	ATP-Citrate Lyase (Phospho-Ser454)

Q8WXE1	ATRIP (Phospho-Ser68/72)
Q96GD4	AurB (Phospho-Tyr12)
Q96GD4	AurB (Phospho-Thr232)
O14965	AURORA KINASE (Phospho-Thr288)
Q92934	BAD (Phospho-Ser112)
Q92934	BAD (Phospho-Ser134)
Q92934	BAD (Phospho-Ser136)
Q92934	BAD (Phospho-Ser155)
Q92934	BAD (Phospho-Ser91/128)
P10415	BCL-2 (Phospho-Thr56)
P10415	BCL-2 (Phospho-Thr69)
P10415	BCL-2 (Phospho-Ser70)
Q07817	BCL-XL (Phospho-Thr47)
Q07817	BCL-XL (Phospho-Ser62)
P11274	BCR (Phospho-Tyr177)
P11274	BCR (Phospho-Tyr360)
P55957	BID (Phospho-Ser78)
O43521	BIM (Phospho-Ser69/65)
Q8WV28	BLNK (Phospho-Tyr96)
P15056	B-RAF (Phospho-Ser446)
P15056	B-RAF (Phospho-Thr598)
P15056	B-RAF (Phospho-Ser601)
P38398	BRCA1 (Phospho-Ser1423)
P38398	BRCA1 (Phospho-Ser1457)
P38398	BRCA1 (Phospho-Ser1524)
Q06187	BTK (Phospho-Tyr222)
Q9NYB9	C-Abl (Phospho-Tyr412)

P62158	Calmodulin (Phospho-Thr79/Ser81)
Q9Y2W7	Calsenilin/KCNIP3 (Phospho-Ser63)
Q14012	Camk1-α (Phospho-Thr177)
Q13554	Camk2-β/γ/δ (Phospho-Thr287)
Q16566	Camk4 (Phospho-Thr196/200)
Q9UQM7	CaMKII (Phospho-Thr286)
P29466	CASP1 (Phospho-Ser376)
P42575	CASP2 (Phospho-Ser140)
P55212	CASP6 (Phospho-Ser257)
Q14790	CASP8 (Phospho-Ser347)
P55212	CASP9 (Phospho-Thr125)
P42574	Caspase-3 (Phospho-Ser150)
P55211	Caspase 9 (Phospho-Ser144)
P55211	Caspase 9 (Phospho-Tyr153)
P55211	Caspase 9 (Phospho-Ser196)
P04040	Catalase (Phospho-Tyr385)
P35222	Catenin β (Phospho-Ser33)
P35222	Catenin β (CTNNB) (Phospho-Tyr489)
P35222	Catenin β (Phospho-Ser37)
P35222	Catenin β (Phospho-Thr41/Ser45)
P35222	Catenin β (Phospho-Tyr654)
O60716	Catenin delta-1 (Phospho-Tyr228)
Q03135	Caveolin-1 (Phospho-Tyr14)
P15391	CD19 (Phpspho-Tyr531)
P15941	CD227/mucin 1 (Phospho-Tyr1243)
P20963	CD3Z (Phospho-Tyr142)
P01730	CD4 (Phospho-Ser433)

P06127	CD5 (Phospho-Tyr453)
P06493	CDC2 (Phospho-Tyr15)
P30304	CDC25A (Phospho-Ser124)
P30304	CDC25A (Phospho-Ser75)
P30305	CDC25B (Phospho-Ser323)
P30305	CDC25B (Phospho-Ser353)
P30307	CDC25C (Phospho-Ser216)
P24941	CDK1/CDC2 (Phospho-Thr14)
P24941	CDK2 (Phospho-Thr160)
Q00535	CDK5 (Phospho-Tyr15)
P50613	CDK7 (Phospho-Thr170)
O14757	Chk1 (Phospho-Ser280)
O14757	Chk1 (Phospho-Ser286)
O14757	Chk1 (Phospho-Ser317)
O14757	Chk1 (Phospho-Ser345)
O96017	Chk2 (Phospho-Thr383)
O96017	Chk2 (Phospho-Thr387)
O96017	Chk2 (Phospho-Ser516)
O96017	Chk2 (Phospho-Thr68)
P05412	C-Jun (Phospho-Tyr170)
P05412	C-Jun (Phospho-Thr239)
P05412	C-Jun (Phospho-Ser243)
P05412	C-Jun (Phospho-Ser63)
P05412	C-Jun (Phospho-Ser73)
P05412	C-Jun (Phospho-Thr91)
P05412	C-Jun (Phospho-Thr93)
P48729	CK1-α (Phospho-Thr321)

P67870	CK2-β (Phospho-Ser209)
P10721	C-Kit (Phospho-Tyr721)
O15551	Claudin 3 (Phospho-Tyr219)
O95471	Claudin 7 (Phospho-Tyr210)
P08581	C-met (Phospho-Tyr1003)
P23528	Cofilin (Phospho-Ser3)
P17302	Connexin 43 (Phospho-Ser367)
Q14247	Cortactin (Phospho-Tyr421)
Q14247	Cortactin (Phospho-Tyr466)
P41279	COT (Phospho-Thr290)
Q96A00	CPI17 (Phospho-Thr38)
P47712	C-PLA2 (Phospho-Ser505)
P04049	C-Raf (Phospho-Ser296)
P04049	C-Raf (Phospho-Ser43)
P16220	CREB (Phospho-Thr100)
P16220	CREB (Phospho-Ser121)
P16220	CREB (Phospho-Ser129)
P16220	CREB (Phospho-Ser133)
P16220	CREB (Phospho-Ser142)
P46108	Crkii (Phospho-Tyr221)
P07333	CSFR (Phospho-Tyr561)
P14635	Cyclin B1 (phospho-Ser126)
P14635	Cyclin B1 (phospho-Ser147))
P24385	Cyclin D1 (Phospho-Thr286)
P30281	Cyclin D3 (Phospho-Thr283)
P24864	Cyclin E1 (Phospho-Thr395)
P24864	Cyclin E1 (Phospho-Thr77))

P05783	Cytokeratin 18 (Phospho-Ser52)
P05787	Cytokeratin 8 (Phospho-Ser431)
O75553	DAB1 (Phospho-Tyr220)
O75553	Dab1 (Phospho-Tyr232)
Q9UHF2	DAPP1 (Phospho-Tyr139)
Q9UD71	DARPP-32 (Phospho-Thr34)
Q9UD71	DARPP-32 (Phospho-Thr75)
Q9UER7	DAXX (Phospho-Ser668)
P17844	DDX5/DEAD-box protein 5 (Phospho-Tyr593)
P78527	DNA-PK (Phospho-Thr2638)
P78527	DNA-PK (Phospho-Thr2647)
Q99704	Dok-1 (Phospho-Tyr362)
Q99704	Dok-1 (Phospho-Tyr398)
O60496	Dok-2 (Phospho-Tyr299)
Q05193	DYN1 (Phospho-Ser774)
Q01094	E2F1 (Phospho-Thr433)
P13639	EEF2 (Phospho-Thr56)
O00418	EEF2K (Phospho-Ser366)
P00533	EGFR (Phospho-Tyr1016)
P00533	EGFR (Phospho-Tyr1069)
P00533	EGFR (Phospho-Ser1070)
P00533	EGFR (Phospho-Tyr1092)
P00533	EGFR (Phospho-Tyr1110)
P00533	EGFR (Phospho-Tyr1172)
P00533	EGFR (Phospho-Tyr1197)
P00533	EGFR (Phospho-Thr678)
P00533	EGFR (Phospho-Thr693)

P00533	EGFR (Phospho-Tyr869)
P05198	EIF2a (Phospho-Ser51)
P06730	EIF4e (Phospho-Ser209)
Q04637	EIF4g (Phospho-Ser1108)
P19419	Elk-1 (Phospho-Ser383)
P19419	Elk1 (Phospho-Ser389)
P19419	Elk1 (Phospho-Thr417)
P29474	eNOS (Phospho-Ser1177)
P29474	eNOS (Phospho-Thr495)
P29474	eNOS (Phospho-Ser615)
P11171	EPB41 (Phospho-Tyr418/660)
P29317	EPHA2/3/4 (Phospho-Tyr588/596)
P54762	EPHB1/2 (Phospho-Tyr594/604)
P52799	Ephrin B (Phospho-Tyr330)
P98172	Ephrin-B1 (Phospho-Tyr317)
P19235	Epo-R (Phospho-Tyr368)
Q16659	ERK3 (Phospho-Ser189)
P03372	Estrogen Receptor-a (Phospho-Ser104)
P03372	Estrogen Receptor-a (Phospho-Ser106)
P03372	Estrogen Receptor-a (Phospho-Ser118)
P03372	Estrogen Receptor-a (Phospho-Ser167)
P51813	ETK (Phospho-Tyr40)
P51813	ETK (Phospho-Tyr566)
P15311	Ezrin (Phospho-Tyr353)
P15311	Ezrin (Phospho-Tyr478)
P15311	Ezrin (Phospho-Thr566)
Q13158	FADD (Phospho-Ser194)

Q05397	FAK (Phospho-Tyr397)
Q05397	FAK (Phospho-Tyr407)
Q05397	FAK (Phospho-Tyr576)
Q05397	FAK (Phospho-Tyr861)
Q05397	FAK (Phospho-Ser910)
Q05397	FAK (Phospho-Tyr925)
P16591	FER (Phospho-Tyr402)
P11362	FGFR1 (Phospho-Tyr154)
P11362	FGFR1 (Phospho-Tyr654)
P11362	FGFR1 (Phospho-Tyr766)
P21333	Filamin A (Phospho-Ser2152)
Q12778	FKHR (Phospho-Ser256)
Q12778	FKHR (Phospho-Ser319)
O43524	FKHRL1/FOXO3A (Phospho-Ser253)
P01100	Fos (Phospho-Thr232)
P53539	Fosb (Phospho-Ser27)
Q12778	FOXO1/3/4-PAN (Phospho-Thr24/32)
Q12778	FOXO1A (Phospho-Ser329)
Q13283	G3BP-1 (Phospho-Ser232)
Q13480	GAB1 (Phospho-Tyr627)
Q13480	GAB1 (Phospho-Tyr659)
P18505	GABA-RB (Phospho-Ser434)
P17677	GAP43 (Phospho-Ser41)
P15976	GATA1 (Phospho-Ser142)
P15976	GATA1 (Phospho-Ser310)
P42261	Glur1 (Phospho-Ser849)
P42261	Glur1 (Phospho-Ser863)

P42262	Glur2 (Phospho-Ser880)
Q13322	GRB10/Growth factor receptor-bound protein 10 (Phospho-Tyr67)
Q9UQC2	GRB2 (Phospho-Ser159)
Q15835	GRK1 (Phospho-Ser21)
P25098	GRK2 (Phospho-Ser29)
P49840	GSK3b (Phospho-Ser21)
P49841	GSK3β (Phospho-Ser9)
P49840	GSK3α-β (Phospho-Tyr216/279)
Q9H0H5	GTPase activating protein (Phopspho-Ser387)
P08631	HCK (Phospho-Tyr410)
Q13547	HDAC1 (Phospho-Ser421)
Q92769	HDAC2 (Phospho-Ser394)
O15379	HDAC3 (Phospho-Ser424)
P56524	HDAC4 (Phospho-Ser632)
Q9UQL6	HDAC5 (Phospho-Ser259)
Q9UQL6	HDAC5 (Phospho-Ser498)
Q9UBN7	HDAC6 (Phospho-Ser22)
Q9BY41	HDAC8 (Phospho-Ser39)
P04626	HER2 (Phospho-Tyr1221/Tyr1222)
P04626	HER2 (Phospho-Tyr1248)
P04626	HER2 (Phospho-Tyr877)
P21860	HER3/erbb3 (Phospho-Tyr1222)
P21860	HER3/erbb3 (Phospho-Tyr1289)
Q15303	HER4/erbb4 (Phospho-Tyr1284)
P16104	Histone H2A.X (Phospho-Ser139)
P68431	Histone H3.1 (Phospho-Ser10)
P41235	HNF4α (Phospho-Ser304)

O14964	HRS (Phospho-Tyr334)
Q00613	HSF1 (Phospho-Ser303)
Q05469	HSL (Phospho-Ser552/563)
Q05469	HSL (Phospho-Ser554)
P04792	HSP27 (Phospho-Ser15)
P04792	HSP27 (Phospho-Ser78)
P04792	HSP27 (Phospho-Ser82)
P08238	HSP90β (Phospho-Ser254)
P08238	HSP90β (Phospho-Ser226)
Q16543	Hsp90 co-chaperone Cdc37 (Phospho-Ser13)
P05362	ICAM-1 (Phospho-Tyr512)
P08069	IGF-1R (Phospho-Tyr1161)
P08069	IGF-1R (Phospho-Tyr1165/1166)
P11717	IGF2R (Phospho-Ser2409)
P25963	Ikb-α (Phospho-Ser32/36)
P25963	Ikb-α (Phospho-Tyr42)
Q15653	Ikb-β (Phospho-Thr19)
O00221	Ikb-ε (Phospho-Ser22)
O15111	IKK-α/β (Phospho-Ser180/181)
O15111	IKK-α (Phospho-Thr23)
O14920	IKK-β (Phospho-Tyr188)
O14920	IKK-β (Phospho-Tyr199)
Q9Y6K9	IKK-GAMMA (Phospho-Ser31)
Q9Y6K9	IKK gamma (Phospho-Ser85)
Q13651	IL-10R-A (Phospho-Tyr496)
P78552	IL-13R/CD213a1 (Phospho-Tyr405)
P01589	IL-2RA/CD25 (Phospho-Ser268)

P26951	IL3R (Phospho-Tyr593)
P24394	IL-4R/CD124 (Phospho-Tyr497)
P05556	Integrin β-1 (phospho-Thr788)
P05106	Integrin β-3 (Phospho-Tyr773)
P05106	Integrin β-3 (Phospho-Tyr785)
P15260	Interferon-γ receptor α chain precursor (Phospho-Tyr457)
P06213	IR (Phospho-Tyr1361)
P35568	IRS-1 (Phospho-Ser307)
P35568	IRS-1 (Phospho-Ser312)
P35568	IRS-1 (Phospho-Ser323)
P35568	IRS-1 (Phospho-Ser636)
P35568	IRS-1 (Phospho-Ser639)
P35568	IRS-1 (Phospho-Ser794)
P16144	ITGB4 (Phospho-Tyr1510)
P23458	JAK1 (Phospho-Tyr1022)
P23458	JAK2 (Phospho-Tyr1007)
P23458	JAK2 (Phospho-Tyr221)
P45983	JNK1/2/3 (Phospho-Thr183/Tyr185)
P17275	Junb (Phospho-Ser259)
P17275	Junb (Phospho-Ser79)
P17535	Jund (Phospho-Ser255)
P05783	Keratin 18 (Phospho-Ser33)
P05787	Keratin 8 (Phospho-Ser73)
P10721	KIT (Phospho-Tyr936)
Q8IVT5	KSR (Phospho-Ser392)
P22001	Kv1.3/KCNA3 (Phospho-Tyr135)
P02545	Lamin A (Phospho-Ser22)

P02545	Lamin A/B(lamin A/C) (Phospho-Ser392)
O43561	LAT (Phospho-Tyr171)
O43561	LAT (Phospho-Tyr191)
P06239	LCK (Phospho-Tyr192)
P06239	Lck (Phospho-Tyr393)
P06239	LCK (Phospho-Tyr504)
P06239	LCK (Phospho-Ser59)
P53667	LIMK1 (Phospho-Thr508)
Q15831	LKB1 (Phospho-Thr189)
Q15831	LKB1 (Phospho-Ser428)
P07948	LYN (Phospho-Tyr507)
P49137	MAPKAPK2 (Phospho-Ser272)
P49137	MAPKAPK2 (Phospho-Thr334)
P29966	MARCKS (Phospho-Ser158)
P29966	MARCKS (Phospho-Ser162)
P07333	M-CSF Receptor (Phospho-Tyr809)
Q00987	MDM2 (Phospho-Ser166)
Q02078	MEF2A (Phospho-Thr312)
Q02078	MEF2A (Phospho-Thr319)
Q02078	MEF2A (Phospho-Ser408)
Q06413	MEF2C (Phospho-Ser396)
Q02750	MEK1 (Phospho-Ser217)
Q02750	MEK1 (Phospho-Ser221)
Q02750	MEK1 (Phospho-Thr286)
Q02750	MEK1 (Phospho-Thr291)
Q02750	MEK1 (Phospho-Ser298)
P36507	MEK-2 (Phospho-Thr394)

P35240	Merlin (Phospho-Ser10)
P35240	Merlin (Phospho-Ser518)
P08581	Met (Phospho-Tyr1234)
P08581	Met (Phospho-Tyr1349)
O75030	MITF (Phospho-Ser73)
P46734	MKK3 (Phospho-Ser189
P46734	MKK3/MAP2K3 (Phospho-Thr222)
P52564	MKK6 (Phospho-Ser207)
O14733	MKK7/MAP2K7 (Phospho-Ser271)
P28562	MKP-1 (Phospho-Ser359)
P28562	MKP-1/2 (Phospho-Ser296)
Q9BUB5	Mnk1 (Phospho-Thr385)
O75582	MSK1 (Phospho-Ser360)
O75582	MSK1 (Phospho-Ser376)
O75582	MSK1 (Phospho-Thr581)
Q13043	Mst1/Mst2 (Phospho-Thr183)
P42345	MTOR (Phospho-Thr2446)
P42345	MTOR (Phospho-Ser2448)
P42345	MTOR (Phospho-Ser2481)
P01106	Myc (Phospho-Thr358)
P01106	Myc (Phospho-Ser373)
P01106	Myc (Phospho-Thr58)
P01106	Myc (Phospho-Ser62)
P24844	Myosin regulatory light chain 2 (Phospho-Ser18)
Q12968	NFAT4 (Phospho-Ser165)
Q04206	Nfkb-p100/p52 (Phospho-Ser865)
Q04206	Nfkb-p100/p52 (Phospho-Ser869)

P19838	Nfkb-p105 (Phospho-Ser927)
P19838	Nfkb-p105/p50 (Phospho-Ser337)
P19838	Nfkb-p105/p50 (Phospho-Ser893)
P19838	Nfkb-p105/p50 (Phospho-Ser907)
P19838	Nfkb-p105/p50 (Phospho-Ser932)
Q04206	Nfkb-p65 (Phospho-Thr254)
Q04206	Nfkb-p65 (Phospho-Ser276)
Q04206	Nfkb-p65 (Phospho-Ser311)
Q04206	Nfkb-p65 (Phospho-Thr435)
Q04206	Nfkb-p65 (Phospho-Ser468)
Q04206	Nfkb-p65 (Phospho-Ser529)
Q04206	Nfkb-p65 (Phospho-Ser536)
Q05586	NMDAR1 (Phospho-Ser897)
Q13224	NMDAR2B (Phospho-Tyr1472)
P35372	Opioid Receptor (Phospho-Ser375)
P56945	p130Cas (Phospho-Tyr165)
P56945	p130Cas (Phospho-Tyr410)
P38936	p21Cip1 (Phospho-Thr145)
P46527	p27Kip1 (Phospho-Ser10)
P46527	p27Kip1 (Phospho-Thr187)
Q16539	p38 MAPK (Phospho-Thr180)
Q16539	p38 MAPK (Phospho-Tyr182)
Q16539	p38 MAPK (Phospho-Tyr322)
P27361	p44/42 MAP Kinase (Phospho-Thr202)
P27361	p44/42 MAP Kinase (Phospho-Tyr204)
P04637	p53 (Phospho-Ser15)
P04637	p53 (Phospho-Thr18)

P04637	p53 (Phospho-Ser20)
P04637	p53 (Phospho-Ser315)
P04637	p53 (Phospho-Ser33)
P04637	p53 (Phospho-Ser37)
P04637	p53 (Phospho-Ser378)
P04637	p53 (Phospho-Ser392)
P04637	p53 (Phospho-Ser46)
P04637	p53 (Phospho-Ser6)
P04637	p53 (Phospho-Ser9)
P23443	P70S6K (Phospho-Thr229)
P23443	P70S6K (Phospho-Ser371)
P23443	P70S6K (Phospho-Ser411)
P23443	P70S6K (Phospho-Ser418)
P23443	P70S6k (Phospho-Thr421)
P23443	P70S6K (Phospho-Ser424)
Q9UBS0	P70s6k-β (Phospho-Ser423)
O15350	P73 (Phospho-Tyr99)
Q15418	P90RSK (Phospho-Thr359/Ser363)
Q15418	P90RSK (Phospho-Ser380)
Q15418	P90RSK (Phospho-Thr573)
O60934	P95/NBS1 (Phospho-Ser343)
Q13153	PAK1 (Phospho-Ser204)
Q13153	PAK1 (Phospho-Thr212)
Q13153	PAK1/2 (Phospho-Ser199)
Q13153	PAK1/2/3 (Phospho-Ser141)
Q13153	PAK1/2/3 (Phospho-Thr423/402/421)
Q13177	PAK2 (Phospho-Ser192)

O75914	PAK3 (Phospho-Ser154)
P49023	Paxillin (Phospho-Tyr118)
P49023	Paxillin (Phospho-Tyr31)
P16234	PDGF R α (Phospho-Tyr849)
P09619	PDGF R β (Phospho-Tyr1021)
P09619	PDGF R β (Phospho-Tyr740)
P09619	PDGF R β (Phospho-Tyr751)
O15530	PDK1 (Phospho-Ser241)
Q15121	PEA-15 (Phospho-Ser116)
P16284	PECAM-1 (Phospho-Tyr713)
P27986	PI3-kinase p85-subunit alpha/gamma (Phospho-Tyr467/Tyr199)
P06803	Pim-1 (Phospho-Tyr309)
P17612	PKA CAT (Phospho-Thr197)
P17252	PKC α (Phospho-Tyr657)
P17252	PKC α/β II (Phospho-Thr638)
P05771	PKC β/PKCB (Phospho-Ser661)
Q05655	PKC δ (Phospho-Thr505)
Q05655	PKC δ (Phospho-Ser645)
Q02156	PKC ε (Phospho-Ser729)
P17252	PKC pan activation site (Phospho)
Q04759	PKC theta (Phospho-Thr538)
Q04759	PKC theta (Phospho-Ser676)
Q05513	PKC zeta (Phospho-Thr410)
Q05513	PKC zeta (Phospho-Thr560)
Q15139	PKD1/PKC mu (Phospho-Ser205)
Q15139	PKD1/PKC mu (Phospho-Tyr463)
Q15139	PKD1/pkcmu (Phospho-Ser910)

Q9BZL6	PKD2 (Phospho-Ser876)
P19525	PKR (Phospho-Thr446)
P19525	PKR (Phospho-Thr451)
Q01970	PLC-β (Phospho-Ser1105)
Q01970	PLC-β (Phospho-Ser537)
P19174	PLCG1 (Phospho-Tyr771)
P19174	PLCG1 (Phospho-Tyr783)
P16885	PLCG2 (Phospho-Tyr1217)
P16885	PLCG2 (Phospho-Tyr753)
Q13393	PLD1 (Phospho-Tyr561)
P62136	PP1alpha (Phospho-Thr320)
P67775	PP2A-α (Phospho-Tyr307)
Q15648	PPAR-β (Phospho-Thr1457)
P37231	PPAR-r (Phospho-Ser112)
P06401	Progesterone Receptor (Phospho-Ser190)
P60484	PTEN (Phospho-Ser370)
P60484	PTEN (Phospho-Ser380)
P60484	PTEN (Phospho-Ser380/Thr382/Thr383)
Q14289	Pyk2 (Phospho-Tyr402)
Q14289	Pyk2 (Phospho-Tyr580)
Q14289	Pyk2 (Phospho-Tyr881)
P63000	Rac1/cdc42 (Phospho-Ser71)
P43351	RAD52 (Phospho-Tyr104)
P04049	Raf1 (Phospho-Ser259)
P04049	Raf1 (Phospho-Ser338)
P04049	Raf1 (Phospho-Tyr341)
P04049	Raf1(Phospho-Ser621)

Q13972	Ras-GRF1 (Phospho-Ser916)
P06400	Rb (Phospho-Ser608)
Q13972	Rb (Phospho-Ser780)
P06400	Rb (Phospho-Ser795)
P06400	Rb (Phospho-Ser807)
P06400	Rb (Phospho-Ser811)
Q04864	Rel (Phospho-Ser503)
Q01201	Relb (Phospho-Ser552)
P07949	Ret (Phospho-Tyr905)
Q8TDA3	Rho/Rac guanine nucleotide exchange factor 2 (Phospho-Ser885)
Q15418	RSK1/2/3/4 (Phospho-Ser221/227/218/232)
Q92736	Ryr2 (Phospho-Ser2808)
P62753	S6 Ribosomal Protein (Phospho-Ser235)
P45983	SAPK/JNK (Phospho-Thr183)
P45983	SAPK/JNK (Phospho-Tyr185)
P45985	SEK1/MKK4 (Phospho-Thr261)
P45985	SEK1/MKK4 (Phospho-Ser80)
P45985	SEK1/MKK4/JNKK1 (Phospho-Ser257)
P29353	Shc (Phospho-Tyr349)
P29353	Shc (Phospho-Tyr427)
Q06124	SHP-2 (Phospho-Tyr542)
Q06124	SHP-2 (Phospho-Tyr580)
Q13094	SLP-76 (Phospho-Tyr128)
Q15797	Smad1 (Phospho-Ser187)
Q15797	Smad1 (Phospho-Ser465)
Q15796	Smad2 (Phospho-Thr220)
Q15796	Smad2 (Phospho-Ser250)

Q15796	Smad2 (Phospho-Ser467)
P84022	Smad2/3 (Phospho-Thr8)
P84022	Smad3 (Phospho-Thr179)
P84022	Smad3 (Phospho-Ser204)
P84022	Smad3 (Phospho-Ser213)
P84022	Smad3 (Phospho-Ser425)
Q14683	SMC1 (Phospho-Ser957)
P08047	SP1 (Phospho-Thr739)
P12931	Src (Phospho-Tyr418)
P12931	Src (Phospho-Tyr529)
P12931	Src (Phospho-Ser75)
P36956	SREBP-1 (Phospho-Ser439)
P11831	SRF (Phospho-Ser77)
P11831	SRF (Phospho-Ser99)
O75886	STAM2 (Phospho-Tyr192)
P42224	STAT1 (Phospho-Tyr701)
P42224	STAT1 (Phospho-Ser727)
P52630	STAT2 (Phospho-Tyr690)
P40763	STAT3 (Phospho-Tyr705)
P40763	STAT3 (Phospho-Ser727)
Q14765	STAT4 (Phospho-Tyr693)
P42229	STAT5A (Phospho-Tyr694)
P42229	STAT5A (Phospho-Ser780)
P51692	STAT5B (Phospho-Ser731)
P42226	STAT6 (Phospho-Tyr641)
P42226	STAT6 (Phospho-Thr645)
P16949	Stathmin 1(Phospho-Ser15)

P16949	Stathmin 1(Phospho-Ser24)
P16949	Stathmin 1(Phospho-Ser37)
O15392	Survivin (Phospho-Thr117)
P43405	SYK (Phospho-Tyr348)
P43405	SYK (Phospho-Tyr525)
P17600	SYN1-Synapsin1 (Phospho-Ser62)
P17600	Synapsin (Phospho-Ser9)
P21579	Synaptotagmin (Phospho-Thr202)
P21579	Synaptotagmin (Phospho-Ser309)
P37840	Synuclein alpha (Phospho-Tyr125)
P37840	Synuclein alpha (Phospho-Tyr133)
O43318	TAK1 (Phospho-Thr184)
P10636	Tau (Phospho-Thr181)
P10636	Tau (Phospho-Thr205)
P10636	Tau (Phospho-Thr212)
P10636	Tau (Phospho-Ser214)
P10636	Tau (Phospho-Thr231)
P10636	Tau (Phospho-Ser235)
P10636	Tau (Phospho-Ser262)
P10636	Tau (Phospho-Ser356)
P10636	Tau (Phospho-Ser396)
P10636	Tau (Phospho-Ser404)
P10636	Tau (Phospho-Ser422)
Q9NYV6	TIF-IA (Phospho-Ser649)
P11388	TOP2A/DNA topoisomerase II (Phospho-Ser1106)
Q16620	Trk B (Phospho-Tyr515)
P49815	Tuberin/TSC2 (Phospho-Thr1462)

P49815	Tuberin/TSC2 (Phospho-Ser939)
P29597	TYK2 (Phospho-Tyr1054)
P07101	Tyrosine Hydroxylase (Phospho-Ser19)
P07101	Tyrosine Hydroxylase (Phospho-Ser31)
P07101	Tyrosine Hydroxylase (Phospho-Ser40)
P07101	Tyrosine Hydroxylase(TH) (Phospho-Ser8)
P50552	VASP (Phospho-Ser157)
P50552	VASP (Phospho-Ser238)
P15498	VAV1 (Phospho-Tyr174)
P52735	VAV2 (Phospho-Tyr142)
P17948	VEGFR1 (Phospho-Tyr1333)
P35968	VEGFR2 (Phospho-Tyr1054)
P35968	VEGFR2 (Phospho-Tyr1059)
P35968	VEGFR2 (Phospho-Tyr1175)
P35968	VEGFR2 (Phospho-Tyr1214)
P35968	VEGFR2 (Phospho-Tyr951)
P18206	Vinculin (Phospho-Tyr821)
P42768	WASP (Phospho-Tyr290)
Q92558	WAVE1 (Phospho-Tyr125)
P98170	XIAP (Phospho-Ser87)
P43403	Zap-70 (Phospho-Tyr292)
P43403	Zap-70 (Phospho-Tyr319)
P43403	Zap-70 (Phospho-Tyr493)

List of protein targets featured as either an anti-phosphorylated or an anti-unphosphorylated antibody on the PEX-100 antibody microarray:

Uniprot ID	**Protein and phosphorylated/unphosphorylated residue(s)**
P63104	14-3-3 zeta/β (Ab-184/186)
P00519	Abl1 (Phospho-Tyr412)
Q07912	ACK1 (Phospho-Tyr284)
P35611	ADD1 (Ab-726)
P31749	AKT1 (Ab-129)
P31749	AKT1 (Ab-308)
P31749	AKT1/2/3 (Ab-315)
Q13315	ATM (Ab-1981)
O14965	Aura (Ab-342)
Q9UQB9	Aurb/C (Ab-202/175)
P30530	AXL (Phospho-Tyr691)
Q07812	BAX (Ab-167)
P10415	BCL-2 (Phospho-Ser87)
P41182	BCL-6 (Ab-333)
Q8WV28	BLNK (Phospho-Tyr84)
Q13882	Breast tumor kinase (Phospho-Tyr447)
Q06187	BTK (Phospho-Tyr550)
P00519	C-Abl (Phospho-Tyr245)
Q9UQM7	Camk2 (Phospho-Thr305)
P22681	CBL (Phospho-Tyr700)
P22681	CBL (Phospho-Tyr774)
P20273	CD22/BL-CAM (Phospho-Tyr807)
P10747	CD28 (Phospho-Tyr218)

P31994	CD32 (fcgammariib) (Ab-292)
P08575	CD45 (Phospho-Ser1007)
P30304	CDC25A (Ab-178)
P30307	CDC25C (Phospho-Thr48)
O14757	Chk1 (Phospho-Ser296)
O14757	Chk1 (Phospho-Ser301)
P48729	CK1-A/A2 (Phospho-Tyr294)
P56747	Claudin 6 (Phospho-Tyr219)
P13726	Coagulation Factor III (Phospho-Ser290)
P46109	Crkl (Phospho-Tyr207)
P61073	CXCR4 (Phospho-Ser339)
P24863	Cyclin C (Phospho-Ser275)
P24385	Cyclin D1 (Ab-90)
P30279	Cyclin D2 (Ab-280)
O96020	Cyclin E2 (Ab-392)
P78527	DNA-PK (Ab-2056)
P00533	EGFR (Ab-998)
P23588	EIF4b (Phospho-Ser422)
P29474	eNOS (Ab-1179)
P98172	Ephrin B1/B2/B3 (Phospho-Tyr324)
Q8TD08	ERK8 (Phospho-Thr175/Tyr177)
P25445	FAS (Ab-291)
P36888	FLT3 (Ab-599)
P36888	FLT3 (Phospho-Tyr842)
P36888	FLT3 (Phospho-Tyr969)
P01100	Fos (Ab-374)
P01100	Fos (Phospho-Ser362)

Q12778	FOXO1A/3A (Phospho-Ser322/325)
Q8WU20	FRS2 (Phospho-Tyr436)
P06241	Fyn (Phospho-Tyr530)
Q9UQC2	Gab2 (Ab-623)
Q9UQC2	Gab2 (Phospho-Tyr643)
Q9NRY4	GRF-1 (Phospho-Tyr1105)
P25098	GRK2 (Phospho-Ser685)
P04626	HER2 (Ab-1112)
P04626	HER2/erbb2 (Ab-686)
Q9UPZ9	ICK (Phospho-Tyr159)
P17936	IGFBP-3 (Ab-183)
P25963	Ikb-α (Phospho-Tyr305)
Q15653	Ikb-β (Phospho-Ser23)
O15111	IKK-α/β (Ab-176)
P16871	IL7R/CD127 (Phospho-Tyr449)
P05556	Integrin β-1 (Ab-789)
P17181	Interferon-α/β receptor α chain (Ab-466)
P06213	IR (Phospho-Tyr1355)
P35568	IRS-1 (Phospho-Ser1101)
P35568	IRS-1 (Phospho-Ser612)
P10721	KIT (Phospho-Tyr703)
Q14721	Kv2.1/Kcnb1 (Phospho-Tyr128)
O43561	LAT (Ab-161)
P53667	LIMK1/2 (Ab-508/505)
Q15831	LKB1 (Ab-334)
Q13233	MAP3K1/MEKK1 (Phospho-Thr1381)
O43318	MAP3K7/TAK1 (Ab-187)

O43318	MAP3K7/TAK1 (Ab-439)
P41279	MAP3K8/COT (Ab-400)
P49137	MAPKAPK-2 (Phospho-Thr222)
O15151	MDM4 (Phospho-Ser367)
Q14814	MEF2D (Phospho-Ser444)
Q12866	MER/SKY (Phospho-Tyr749/Tyr681)
Q09YK0	Met (Phospho-Tyr1356)
O14733	MKK7/MAP2K7 (Phospho-Thr275)
O75582	MSK1 (Phospho-Ser212)
O75676	MSK2 (Phospho-Thr568)
O14974	MYPT1 (Phospho-Thr696)
O14974	MYPT1 (Phospho-Thr-853)
Q99640	MYT1 (Ab-83)
Q14934	NFAT3 (Ab-168/170)
Q14934	NFAT3 (Ab-676)
Q00653	Nfkb-p100 (Phospho-Ser872)
Q04206	Nfkb-p65 (Ab-281)
Q04206	Nfkb-p65 (Ab-505)
Q12879	NMDA NR2A/B (Phospho-Tyr1246/1252)
Q09472	P300 (Ab-89)
P04637	P53 (Ab-376)
P04637	P53 (Ab-387)
P04637	P53 (Phospho-Ser366)
P04637	P53 (Phospho-Thr81)
Q9H3D4	P63(Phospho-Ser455)
P23443	P70S6K (Ab-427)
P23443	P70S6K (Phospho-Thr389)

Q13177	PAK2 (Ab-197)
Q13177	PAK2 (Phospho-Ser20)
O96013	PAK4/PAK5/PAK6 (Ab-474)
P09619	PDGF R β (Ab-1009)
Q15121	PEA-15 (Phospho-Ser104)
P27986	PI3-kinase p85-alpha (Phospho-Tyr607)
Q9Y2I7	PIP5K (Phospho-Ser307)
P31323	PKA-R2B (Phospho-Ser113)
Q05655	PKC delta (Phospho-Tyr52)
Q05655	PKC delta (Phospho-Tyr313)
Q05655	PKC delta/PKCD (Phospho-Tyr64)
Q15139	PKD1/2/3/PKC mu (Ab-744/748)
P19174	PLCG1 (Phospho-Tyr1253)
O14939	PLD2 (Phospho-Tyr169)
P53350	PLK1 (Ab-210)
P18433	PTPRA (Phospho-Tyr798)
Q14289	Pyk2 (Phospho-Tyr579)
Q06609	RAD51 (Ab-309)
Q06609	RAD51 (Phospho-Tyr315)
P04049	Raf1 (Ab-289)
Q13905	Rapgef1 (Phospho-Tyr504)
P06400	Rb (Phospho-Thr821)
Q08999	Rb-like-2 (RBL2) (Ab-952)
O15492	RGS16 (Phospho-Tyr168)
P61586	Rhoa (Ab-188)
P29350	SHP-1 (Phospho-Tyr536)
Q15796	Smad2 (ab-245)

Q15796	Smad2 (Ab-255)
P84022	Smad3 (Phospho-Ser208)
P12931	Src (Phospho-Tyr216)
P42229	STAT5A (Phospho-Ser725)
P43405	SYK (Phospho-Tyr323)
P37840	Synuclein alpha (Phospho-Tyr136)
P78347	TFII-I (Phospho-Tyr248)
P36897	TGFBR1 (Ab-165)
P37173	TGFBR2 (Ab-250)
Q02763	TIE2 (Phospho-Tyr1108)
Q9Y4F6	TLK1 (Ab-764)
P04629	Trk A (Ab-496)
P04629	Trk A (Phospho-Tyr680/681)
P04629	Trk A (Phospho-Tyr701)
P04629	Trk A (Phospho-Tyr791)
Q16620	Trk B (Phospho-Tyr705)
P49815	Tuberin (Ab-981)
P15498	VAV1 (Ab-160)
P33151	VE-Cadherin (Phospho-Tyr731)
P30291	WEE1 (Ab-53)
P30291	WEE1 (Phospho-Ser642)
Q96KM3	WWOX (Phospho-Tyr33)
P43403	Zap-70 (Phospho-Tyr315)

Appendix II

To correct for the high variance in spot values on the first microarray slide processed for the main microarray study of **Chapter 3**, estimation of unspecific binding was performed using the values of the negative control spots. This calculation (equation given at the bottom of page) was performed for each of the protein targets on the first microarray:

Firstly, the difference between the background of protein target's spot (**Sb**) and the mean background of the left negative control spots (**LNb**) was calculated, alongside the difference between the mean background of the right negative control spots (**RNb**) and the mean background of the left negative control spots (**LNb**). The ratio between these two differences was then calculated [(**Sb – LNb**)/(**RNb – LNb)**]. This ratio was then used to multiply the difference between the mean foreground of the right negative control spots and the mean foreground of the left negative control spots (**RNf – LNf**). This calculation, therefore, uses the differences in background fluorescence of the aforementioned spots to the estimate the proportion of the fluorescence at the location of the protein target in question that is due to unspecific binding. The value of this unspecific binding is then subtracted from the protein target's spot fluorescence (**Sf**). The mean foreground of the left negative control spots (**LNf**) is also subtracted, and a value of 300 is added to ensure that all values calculated by this method remain over 0. The equation used is shown here:

$$\mathbf{CSf = Sf – ([(Sb – LNb)/(RNb – LNb)] \times (RNf - LNf)) – LNf + 300}$$

Abbreviations: Corrected Spot foreground (CSf); Spot foreground (Sf); Spot background (Sb); average left negative control spot background (LNb); average right negative control spot background (RNb); average left negative control spot foreground (LNf); average right negative control spot foreground (RNf).

Abbreviations

Ac	Acetyl
ACN	Anthocyanin
Alo	Allosyl
AMPK	5' adenosine monophosphate-activated protein kinase
BH4	Tetrahydrobiopterin
BKCa	Large conductance Ca(2+)- and voltage-activated potassium channel
BSA	Bovine serum albumin
C35diGlc	Cyanidin-3,5-diglucoside
C3Glc	Cyanidin-3-glucoside
CaMKII	Ca2+/calmodulin-dependent protein kinase II
cGMP	Cyclic guanosine monophosphate
Coum	Coumaryl
CST	Cell Signalling Technologies
Cy3	Cyanine 3
D3Glc	Delphinidin-3-glucoside
dH2O	Deionised water
DMSO	Dimethyl sulfoxide
DP4	Degrees of polymerisation 4 (procyanidin)
DTT	Dithiothreitol
E. acid	Ellagic acid
EA	Epiafzelechin
EBM-2	Endothelial basal media
EC	Epicatechin
ECG	Epicatechin-gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin-gallate
EGM-2	Endothelial growth media

EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
EU	European Union
EuroFIR	European Food Information Resource Network
Fer	Feruloyl
FMB	Full Moon Biosystems
FMD	Flow Mediated Dilation
FP7	Framework Programme 7
FPLC	Fast protein liquid chromatography
GAE	Gallic acid equivalent
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glc	Glucose
GlcA	Glucuronic acid
Gnb	Gentiobioside
GPCR	G-protein coupled receptor
GPX	Glutathione peroxidase
GST	Glutathione S-transferase
Hex	Hexose
hFer	Hydroxyferuloyl
HHDP	3,4,5,3',4',5'-hexahydroxydiphenoyl
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High pressure liquid chromatograpy
HPLC-DAD-MSD	High pressure liquid chromatograpy-diode array detection-mass spectrometry detection
HPLC-MSn	High pressure liquid chromatograpy-mass spectrometry in series
HRP	Horse-radish peroxidase
HUVEC	Human umbilical vein endothelial cells
I-CAM	Intracellular adhesion molecule

IFR	Institute of Food Research (UK)
IMR	Institute of Medical Research (Serbia)
IP3	Inositol trisphosphate
IR	Isorhamnetin
ITC	Isothiocyanate
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoprotein
LDS	Lithium dodecyl sulfate
LPH	Lactase-phlorizin hydrolase
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MCT	Monocarboxylate transporters
MRP2	Multidrug resistance-associated protein 2
MSFI	Median spot fluorescence intensity
MT	Multiple testing
MW	Molecular weight
MYPT1	Myosin phosphatase target subunit 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NaPi	Sodium phosphate
NO	Nitric oxide
nopair	"MSFIs representing a phosphorylated (or unphosphorylated) protein residue, but lacking a 'partner' antibody corresponding to the alternate unphosphorylated (or phosphorylated) protein residue, spotted on the microarray"
NP-HPLC)	Normal phase HPLC
OPAC	Oligomeric proanthocyanidin
OPC	Evesse™ procyanidin-rich apple extract
PAF	Pomegranate anthocyanin fraction
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline solution

PC	Procyanidin
PEF	Pomegranate ellagitannin fraction
PEX-100	Phospho Explorer Antibody Microarray
PGG	1,2,3,4,6-pentagalloyl glucose
phph	MSFIs representing a phosphorylated protein residue
PIF	Pomegranate insoluble fraction
PIP2	Phosphatidylinositol-bisphosphate
PIP3	Phosphatidylinositol-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKG	Protein kinase G
Pnclgn	Punicalagin
PPF	Pomegranate procyanidin fraction
p-X (where X is a protein, i.e. p-Akt)	phosphorylated version of X (where is a protein)
PSF	Pomegranate sugar fraction
PTEN	Phosphatase and tensin homolog
PTFE	Polytetrafluoroethylene
PWSF	Pomegranate water soluble fraction
PWV	Pulse wave velocity
RE	Rutin equivalent
RNA	Ribonucleic acid
RP-HPLC	Reverse phase HPLC
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
Ser	Serine
sGC	Soluble guanylyl cyclase
SGLT1	Sodium-glucose transport protein 1
SHIP	Src-homology 2-containing inositol 5' phosphatase
Sin	Sinapoyl

Sph	Sophoroside
TBS	Tris-buffered saline solution
TBST	Tris-buffered saline solution plus Tween-20
Thr	Threonine
TNF-α	Tumor necrosis factor alpha
TPC	Total phenolic content
Tris	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
UDP	Uridine diphosphate
unph	MFSIs representing an unphosphorylated protein residue
UPLC	Ultra pressure liquid chromatography
UV	Ultraviolet
V-CAM	Vascular cell adhesion molecule
VEGFR2	Vascular endothelial growth factor receptor 2
VSMC	Vascular smooth muscle cells
WST-1	Water soluble Tetrazolium salt 1
XO	Xanthine oxidase

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