Bioactive polyphenols and cardiovascular disease

Bioactive polyphenols and cardiovascular disease: chemical antagonists, pharmacological agents or xenobiotics that drive an adaptive response?

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Abstract

Polyphenols are widely regarded to have a wide range of health-promoting qualities, including in the sphere of cardiovascular disease. Historically, the benefits have been linked to their well-recognised powerful antioxidant activity. However, the concept that the beneficial effects are attributable to direct antioxidant activity *in vivo* does not pay sufficient heed to the fact that polyphenols degrade rapidly, are poorly absorbed and rapidly metabolised, resulting in very low bioavailability.

This review explores alternative mechanisms by which polyphenols, or their metabolites, exert biological activity via mechanisms that can be activated by physiologically relevant concentrations. Evidence is presented to support the action of phenolic derivatives on receptors and signalling pathways to induce adaptive responses that drive changes in endogenous antioxidant, antiplatelet, vasodilatory and anti-inflammatory effects. The implications are that *in vitro* antioxidant measures as predictors of polyphenol protective activity hold little relevance and that closer attention needs to be paid to bioavailable metabolites to understand the mode of action of these diet-derived components.

Key words: polyphenols, cardiovascular disease, antioxidant, cell signalling, bioactivity

Table of links

TARGETS	
Other protein targets[a]	**Enzymes**[d]
TNF-α	AKT serine/threonine kinase 2
GPCRs[b]	COX-1
ET_A	COX-2
ET_B	eNOS
Nuclear hormone receptors[c]	ERK1
ER-α	ERK2
ER-β	GR
	GSK3B
	HO 1
	MAPK
	PI3K
	Src
	PKC
	PKA

LIGANDS	
ADP	IL-6
ATP	IL-10
beta-thromboglobulin	MCP-1
caffeic acid	morin
CD40L	NADPH
cGMP	Nrf2
curcumin	PF4
endothelin-1	quercetin
epigallocatechin-3-gallate	RANTES
gallic acid	TGFβ1
GSH	TXA_2
H_2O_2	VCAM-1
ICAM-1	

This Tables of Links lists key protein targets and ligands in this article that are hyperlinked* to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d]Alexander et al., 2015a,b,c,d).

Abbreviations

A549	adenocarcinomic human alveolar basal epithelial cells
ADP	adenosine diphosphate
Akt 2	AKT serine/threonine kinase 2
ARE	antioxidant responsive element
ATP	adenosine triphosphate
beta-TG	beta-thromboglobulin
CAM	cell adhesion molecule
CD62P	P-selectin
CD63	CD63 molecule
CD40L	CD40 ligand
cGMP	cyclic guanosine monophosphate
CGNs	cerebellar granule neurons
COX-1	cyclooxygenase 1
CRP	C-reactive protein
CSE	cigarette smoke extract
EA.hy926	human endothelial hybrid cell line
ECG	epicatechin-3-gallate
EGCG	epigallocatechin-3-gallate
eNOS	endothelial NOS
ERK1/2	extracellular signal–regulated kinases 1/2
ER -α/β	estrogen receptor -α/β
ET-1	endothelin-1
$ET_{A/B}$ receptors	endothelin A/B receptors
GR	glutathione reductase
GSH	glutathione
GSK3B	glycogen synthase kinase 3 beta
GSL	glutamyl-cysteine ligase
GST	glutathione-S-transferase
H-19-7	neuronal cell line derived from rat's brain/hippocampus
H460	human non-small cell lung carcinoma cell line
HO1	haem oxygenase 1
HUVECs	human umbilical vein endothelial cells
H_2O_2	hydrogen peroxide
$^{\cdot}OH$	hydroxyl radicals
HOCl	hypochlorous acid
ICAM-1	intercellular adhesion molecule 1
IKK	IκB kinase
IκB-α	inhibitor of kappa-B alpha
IL-6, -8, -10	interleukin -6, -8, -10
J774	murine macrophage cell line J774

LDL	low density lipoprotein
LPH	lactase phloridizin hydrolase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MCP-1	monocyte chemoattractant protein-1
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NO	nitric oxide
NQO-1	NADPH: quinone oxidoreductase 1
Nrf2	nuclear factor E2-related factor 2
$O_2^{\cdot-}$	superoxide radical
$ONOO^-$	peroxynitrite
ox-LDL	oxidised low density lipoprotein
PF4	platelet factor 4
PI3K	phosphatidylinositol 3-kinases
PKA	protein kinase A
PKC	protein kinase C
PREDIMED	Prevención con Dieta Mediterránea
RANTES	regulated upon activation normal T-cell expressed and secreted
ROS	reactive oxygen species
Src	SRC proto-oncogene, non-receptor tyrosine kinase
TGFβ1	transforming growth factor beta 1
TNF-α	tumour necrosis factor-α
TRAP	thrombin receptor-activating peptide
TXA_2	thromboxane A_2
VCAM-1	vascular cell adhesion molecule 1

Keywords: polyphenols, antioxidant, signalling, ant-inflammatory, cardiovascular disease

Introduction

Polyphenols have long been recognised to hold health benefits, but their reputation has been boosted recently on account of a number of encouraging clinical studies in a range of disease profiles that appear to confirm efficacy. Increased consumption of fruit and vegetables that are rich in polyphenolic compounds is known to be associated with health benefits related to cardiovascular function. For example, the PREDIMED (Prevención con Dieta Mediterránea) study found that risk of cardiovascular disease (CVD) was reduced by 46% in individuals with a diet rich in polyphenols (Tresserra-Rimbau *et al.*, 2014). Other published reports show that polyphenolics can improve endothelial function (Vita, 2005), inhibit abnormal platelet aggregation (Tangney *et al.*, 2013), reduce inflammation, and improve plasma lipid profiles (Arranz *et al.*, 2012), thereby offering protection to cardiovascular health at a number of levels. However, despite numerous studies conducted in the field, the mechanisms through which these compounds exert cardioprotective actions are not yet fully understood and, as a result, the link between cardiovascular benefits of particular diets and their polyphenolic content is not strictly proven.

For decades, phenolic compounds have been recognised to have powerful free radical scavenging activities, determined by specific structural features, such as the number and position of hydroxyl and catechol units (Castellano, 2012; Fraga *et al.*, 2010). The best known polyphenolic antioxidants are delphinidin, cyanidin, pelargonidin, peonidin, malvidin (anthocyanins), quercetin, keampferol, myricetin, morin, luteolin (flavonols), gallic acid, caffeic acid, syringic acid, protocatechuic acid (phenolic acids), catechin, epicatechin, epicatechin gallate, epigallocatechin gallate (flavanols), ellagic acid, and curcumin (figure 1) (Duthie *et al.*, 2003; Kahkonen *et al.*, 2003; Lianda *et al.*, 2012). Many aspects of cardiovascular disease are associated with oxidative stress – the excessive production of pro-oxidants and/or depression of counteractive endogenous antioxidant systems. Polyphenols are believed to be able to reduce the prevalence of various biomarkers of oxidative stress. It is the association between antioxidant properties of polyphenolic compounds with reduced risk of CVD that has dominated the literature in this arena, although a direct causal link has always been assumed rather than conclusively proven (Acquaviva *et al.*, 2002; Borbalan *et al.*, 2003; Jacob *et al.*, 1996; Lugasi, 2003; Schneider *et al.*, 1996; Wan *et al.*, 2001).

The reason for doubt over the seemingly obvious link between strong antioxidant activity of polyphenols *in vitro* and reduction in oxidative stress *in vivo* is the very poor bioavailability

of native polyphenols on account of extracellular decomposition, poor absorption and rapid metabolism: bioavailable concentrations of polyphenolic compounds are simply too low to mediate direct antioxidant activity *in vivo* (i.e. to act as chemical scavengers of radicals). In addition, a considerable number of studies support the hypothesis that polyphenols can oxidise readily in beverages, tissue culture media and phosphate buffers, with the potential to cause paradoxical adverse effects *in vivo* through pro-oxidative activity (Babich *et al.*, 2008; Prochazkova *et al.*, 2011), thus raising the spectre of counterintuitive toxicity with very high consumption of polyphenols (Martin *et al.*, 2010). However, the pro-oxidant activity of phenolic compounds may also prove to be beneficial at moderate concentrations, since by inducing a mild degree of oxidative stress, they can activate intracellular antioxidant defence mechanisms (Moskaug *et al.*, 2005; Nabavi *et al.*, 2016; Scapagnini *et al.*, 2011). Moreover, it has become clear that the mechanism of action of polyphenols goes way beyond modulation of oxidative stress: their role as mediators in cell-cell signalling, receptor activation, and gene regulation *in vivo* adds interesting dimensions to their complexity and to their scope for preventative and therapeutic applications (Scalbert *et al.*, 2005; Schewe *et al.*, 2008). Thus, polyphenols likely contribute to cardioprotection, but their direct antioxidant effects might only play a very minor role, if any at all. The characteristics that are more likely to determine their *in vivo* efficacy are their stability under physiological conditions, their rate of absorption, metabolism and excretion, their metabolic products, and the pharmacological targets (receptors, enzymes and nuclear factors) of either the original polyphenols or their metabolites, as opposed to their *in vitro* antioxidant potential.

Although increased consumption of polyphenol-rich foods has been associated with reduced risk of many diseases, including cancer, diabetes and neurodegenerative disorders, this review, however, will focus only on the role of polyphenols in cardiovascular health in light of these newly emerging trends.

CVD and oxidative stress

CVD is a global term used for the group of diseases affecting the heart and/or blood vessels and includes coronary artery disease, cerebrovascular disease, peripheral artery disease, congenital heart disease, hypertension, heart failure and stroke (Nicholson *et al.*, 2008). The

incidence rate of CVD has dramatically increased in the past three decades: in 2014, cardiovascular disease, together with cancer, was the biggest cause of death in the UK, causing 28 and 29 % of all deaths in women and men respectively. Coronary heart disease accounted for 45% of all CVD deaths, while 25% of deaths were stroke-related (Jin *et al.*, 2011). Coronary artery disease and ischaemic stroke, as well as peripheral artery disease, are underpinned by a common pathological process - atherosclerosis (Le Brocq *et al.*, 2008). Atherosclerosis is a multi-factorial, progressive disorder of medium-sized and large conduit arteries, which is fuelled by deposition of modified lipids in the vessel wall (Falk, 2006; Megson *et al.*, 2016). Age, smoking, hyperlipidaemia, hypertension and diabetes are the most common risk factors for the disease. Inflammation, oxidative stress and endothelial dysfunction are strongly associated with the atherogenic process (Le Brocq *et al.*, 2008; Loke *et al.*, 2010), while endothelial cells, smooth muscle cells, neutrophils, macrophages and platelets are all potential sources and targets of oxidants (Park *et al.*, 2012). Generation of oxidants occurs during physiological processes, such as cellular respiration and metabolism, and is strictly regulated by antioxidant defence mechanisms in healthy cells (Sies, 1997). However, prolonged exposure to stress (Bouayed *et al.*, 2009), pollution (Lodovici *et al.*, 2011), smoking and excessive drinking (Barreiro *et al.*, 2010; Galicia-Moreno *et al.*, 2014), as well as aging (Finkel *et al.*, 2000), results in an imbalance of oxidative species (also known as reactive oxygen species; ROS) over the endogenous defences - so-called oxidative stress (Khurana *et al.*, 2013; Sies, 1997).

Many functions of the endothelium are affected by ROS (Nicholson *et al.*, 2008). The best-recognised is endothelium-dependent vasorelaxation, which is impaired by a loss of nitric oxide (NO) bioactivity and/or bioavailability (Hopkins, 2013). NO is a powerful vasodilator that also acts to prevent inflammatory cell activation and adhesion (Taniyama *et al.*, 2003). In the presence of superoxide radical ($O_2^{\cdot-}$), endothelium-derived NO rapidly reacts to form peroxynitrite ($ONOO^-$), which acts as a powerful oxidant, and is also harmful to endothelial cells (Curtin *et al.*, 2002). Prolonged exposure of endothelial cells to $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2), $ONOO^-$ and/or oxidised low density lipoprotein (ox-LDL) induces apoptosis (Le Brocq *et al.*, 2008), which leads to cell damage and loss, a key early event in atherogenesis. Atherosclerotic lesions start to develop under an intact but leaky, activated, and dysfunctional endothelium (Falk, 2006). LDL ordinarily diffuses freely across the damaged endothelium in both directions. However, under oxidative stress, LDL undergoes peroxidation to ox-LDL, becoming cytotoxic and pro-inflammatory. Meanwhile, damaged or activated endothelial

cells express adhesion molecules, primarily vascular cell adhesion molecule 1 (VCAM-1), that bind monocytes and T cells prior to transmigration into the vessel wall (Gerhardt *et al.*, 2015). The monocytes become activated and differentiate into macrophages, ultimately becoming engorged with ox-LDL taken up via scavenger receptor-mediated phagocytosis, forming so-called fatty streaks in the vessel wall (Hopkins, 2013; Le Brocq *et al.*, 2008; Martinez-Cayuela, 1995). Lipid engorged macrophages (foam cells) ultimately undergo pro-inflammatory necrotic cell death *in situ*, contributing to the formation of a soft and destabilizing lipid-rich core within ahterosclerotic plaques (Singh *et al.*, 2002). Disease progression can terminate in plaque stabilisation on account of smooth muscle cells secreting a collagen-rich matrix containing fibroblasts and other connective tissue to create a protective cap over the plaque. However, prolonged inflammation can lead to a unstable plaques that are prone to rupture (Singh *et al.*, 2002); ruptured plaques induce a rapid thrombotic response, leading to vessel occlusion and heart attack, ischaemic stroke or peripheral ischaemia, depending on the site of the atherosclerotic lesion (Falk, 2006).

Oxidative stress represents a key feature of the progression of atherosclerosis, influencing both the oxidative modification of LDL and the dysfunction of the endothelium which are central to the aetiology of the disease. However, it is important to recognise that, while oxidative stress is a valid therapeutic target for prevention and treatment strategies, the role of inflammation should not be overlooked.

Polyphenols

Polyphenols are the most widespread class of plant secondary metabolites (Castellano, 2012; Falk, 2006; Tsao, 2010), where they are involved in defence against ultraviolet radiation, cold temperatures or drought, as well as contributing to the colour of leaves, berries and fruits. They also act as anti-feedants and toxins that assist plants in their defence against herbivores, parasites and pathogens (Gould *et al.*, 2009). Approximately 8,000 phenolic structures have been identified so far, several hundred of which are found in edible plants (Perez-Jimenez *et al.*, 2010; Tsao, 2010) (figure 2). Polyphenols are characterised by the presence of several phenolic groups (aromatic rings) (Manach *et al.*, 2004). They are highly diverse and can be divided into several sub-groups, depending on the number of phenol rings that they contain

and of the structural elements that bind these rings to one another (Quideau *et al.*, 2011) (figure 2).

Polyphenols as antioxidants

Historically, polyphenol-induced benefits have been largely attributed to the renowned ability of polyphenols to act as powerful antioxidants. Indeed, under *in vitro* conditions, phenolic compounds can readily donate an electron or H atom from an aromatic hydroxyl group to a free radical, thereby 'neutralizing' it. Direct antioxidant properties of polyphenols depend on the arrangement of functional groups in their core structure (Prochazkova *et al.*, 2011). The antioxidant capabilities of polyphenols are complex: the hydroxylation patterns - such as the 3-hydroxy group in flavanols, or electron deficiency in the case of anthocyanins - as well as the presence of catechol groups are important in the antioxidant activities (Hanif *et al.*, 2008; Kahkonen *et al.*, 2003). It has been reported that the *in vitro* antioxidant activity of several polyphenols is comparable to that of vitamin C and vitamin E (Gardner *et al.*, 1998; Prior *et al.*, 2000). Polyphenols have been reported to scavenge ROS and reactive nitrogen species, including $O_2^{\cdot -}$, H_2O_2, hydroxyl radicals ($^{\cdot}OH$), hypochlorous acid (HOCl) and NO (Tsao, 2010). Moreover, they can act as direct radical scavengers of the peroxidation products of lipids, proteins DNA and RNA (Quideau *et al.*, 2011). Additionally, polyphenols can act as metal ion chelators, thereby reducing the rate of Fenton reactions and formation of highly damaging $^{\cdot}OH$ (Fraga *et al.*, 2010).

Despite their excellent antioxidant activity *in vitro,* the evidence in support of direct antioxidant activity of polyphenols *in vivo* is weak. For example, in the blood, plasma levels of unconjugated polyphenols rarely exceeds 1 μM and, moreover, the products of polyphenol metabolism tend to have lower antioxidant capacity, because the radical-scavenging -OH groups are blocked by methylation, sulfation and glucuronidation (Pollard *et al.*, 2006). In the context of plasma, where highly abundant proteins, thiols, uric acid and vitamin C already constitute a formidable antioxidant barrier, any phenolic contribution is negligible (Turell *et al.*, 2013). Therefore, the concept that the observed increases in antioxidant capacity of plasma after consumption of polyphenol-rich foods is directly attributable to increased polyphenol load is implausible. It is much more likely that other readily absorbed dietary components ingested alongside polyphenols, such as vitamin C, vitamin E (Cao *et al.*, 1998)

or even fructose on account of a recognised interaction with uric acid (Lotito *et al.*, 2004), are responsible for the observed effect of fruit and vegetable rich diets on plasma antioxidant capacity.

Polyphenols as pro-oxidants

Despite the fact that pro-oxidant activities of polyphenols were reported nearly three decades ago (Tulyathan *et al.*, 1989), more attention has been paid to their widely described antioxidant capacities. Spontaneous oxidation of a common phenolic metabolite, gallic acid, leads to generation of a variety of highly reactive species, including $O_2^{\cdot-}$, H_2O_2, quinones and semiquinones (Gil-Longo *et al.*, 2010). Epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG) (Lambert *et al.*, 2010; Severino *et al.*, 2009), quercetin (Lapidot *et al.*, 2002), theaflavin (Babich *et al.*, 2008) and a variety of plant extracts, including apple (Bellion *et al.*, 2009), pomegranate (Weisburg *et al.*, 2010), black and green tea extracts (Severino *et al.*, 2009), as well as red wine (Elias *et al.*, 2009) generate ROS, and H_2O_2 in particular. The extent of pro-oxidant activity of polyphenols is dependent on the polyphenol in question, its concentration and the conditions of the environment (Babich *et al.*, 2008; Bellion *et al.*, 2009). The mechanism by which oxidation takes place remains equivocal, although reduction of iron and copper ions might help to promote Fenton chemistry and pro-oxidant activities of polyphenols (Gil-Longo *et al.*, 2010; Martin *et al.*, 2010).

The relevance of polyphenol oxidation under physiological conditions remains unclear, but the products of polyphenol auto-oxidation have been shown to be toxic in human lung carcinoma cells (H460) (Leung *et al.*, 2007). However, the concentrations used in these studies (50 - 80 μM) were supraphysiological and it is reasonable to assume that physiological levels are non-toxic. Indeed, the potential toxicity of auto-oxidation products of polyphenols is likely the reason for the low absorption in the gut and the rapid conjugation and metabolism of absorbed polyphenols as a means of detoxification.

Polyphenols as xenobiotics (oral bioavailability, metabolism and clearance)

The way polyphenols are handled by the body is characteristic of that for xenobiotics - substances that are not normally found *in vivo* that can become toxic without appropriate

metabolism and excretion (Cardona *et al.*, 2013; Croom, 2012). Thus, regardless of the amount of ingested polyphenol-rich food, the bioavailability of the native polyphenols tends to be maintained in the nM to low μM range (D'Archivio *et al.*, 2007; Mazza, 2007).

Absorption

Polyphenols can be found in wide range of fruit and vegetables. In some plants their concentration can be as high as 750 mg per 100 g of fruit (Bohn, 2014; Manach *et al.*, 2004). Berries, whole-grain cereals, cacao, tea, coffee and red wine are common rich dietary sources of polyphenols. Depending on diet, gender and other socio-economic factors, the total daily intake of polyphenols is around 1g a day (Grosso *et al.*, 2014; Mullie, 2014; Scalbert *et al.*, 2000).

It has been estimated that only 1-10% of total polyphenol intake is found in plasma and urine samples (D'Archivio *et al.*, 2007; Duthie *et al.*, 2003; Nicholson *et al.*, 2008). Bioavailability of polyphenols is first determined by their rate, site and means of absorption. Moreover, direct interaction between polyphenols and other compounds and food components, such as proteins, carbohydrates, fibre, fat and alcohol, can also affect their absorption (D'Archivio *et al.*, 2007). Maximal concentrations are usually reached within 0.5-2 h after ingestion, falling to baseline levels within 8-12 h (Beattie *et al.*, 2005). Just as with drugs, attainment of steady state conditions requires regular, frequent, repeated ingestion (dosing). Most polyphenols (except flavanols) are present in food as glycosides - esters or polymers of very complex structures and high molecular weight, limiting their absorption in native form (Manach *et al.*, 2004). For instance, the molecular weight of proanthocyanidins, oligomers of catechin, epicatechin and their gallic acid esters, ranges from 500 to 20,000 g/mol (Sepúlveda *et al.*, 2011). Most polyphenols remain relatively stable at the low pH experienced in the stomach and resist acid hydrolysis, therefore facilitating their transit to the small intestine intact. Only anthocyanin glycosides are absorbed in both the stomach and small intestine without modification, but the rate of absorption is limited by the type of sugar moiety attached (Hassimotto *et al.*, 2008; Mazza, 2007; Wiczkowski *et al.*, 2010). The remaining intact polyphenols that reach the small intestine undergo hydrolysis by lactase phloridizin hydrolase (LPH) present in the brush-border of the epithelial cells in the small intestine. The released aglycones (deprived of sugar moiety) are then capable of entering the epithelial cells by

passive diffusion on account of their increased lipophilicity (Del Rio *et al.*, 2013). However, only some glycosides are hydrolysed in the small intestine; polyphenols linked to a rhamnose, arabinose and xylose moieties, as well as compounds with more complex structures (e.g. tannins) reach the colon, where they are hydrolyzed by the microflora before absorption can occur (Manach *et al.*, 2004). The colonic microbiota are responsible for extensive breakdown of complex polyphenols, leading to the release of low molecular weight phenolic metabolites (e.g. phenolic acids, urolithins) that only now are available for absorption. However, the rate of absorption in the colon is lower than that in the small intestine. Unabsorbed polyphenols are excreted from the body in faeces (Scalbert *et al.*, 2000).

In addition to the poor absorption of polyphenols, it is important to recognise that some are intrinsically prone to decomposition in aqueous medium. Anthocyanins, for instance, are known to be very unstable in tissue culture medium. Compared to the other anthocyanins, delphinidin has the lowest stability in tissue culture medium; substantial degradation to gallic acid and aldehyde is found as early as 30 min of dissolution (Kay *et al.*, 2009; Woodward *et al.*, 2009). Pelargonidin is the most stable anthocyanin (Kern *et al.*, 2007). Degradation of anthocyanins, as well as the other polyphenols, can be accelerated by light, pH and temperature, as well as by the composition of accompanying substances (enzymes, proteins, other flavonoids) and the redox environment (Fang, 2014; He *et al.*, 2010). For example, the stability of resveratrol is affected by light and alkaline pH (Trela *et al.*, 1996). Similarly, artemetin (another flavonoid), is characterized by low stability at room temperature (Weathers *et al.*, 2012). Moreover, cocoa flavanols ((-)-epicatechin, (+)-catechin) and their dimers are highly unstable in simulated intestinal fluid or alkaline pH (Zhu *et al.*, 2002). Ultimately, the identity of the phenolic compounds that persist in the lumen of the gut is an important consideration in determining the nature and bioactivity of the phenolic derivatives that eventually reach the bloodstream.

Metabolism and excretion

Bioavailable polyphenols can be found in plasma in both their native intact form and the glucuronidated and/or methylated forms; sulfo-conjugates are less common (Matsumoto *et al.*, 2006). Extensive conjugation occurs on first pass through the liver. This metabolic detoxification process is common to many xenobiotics, acting to prevent potential toxic

effects, and is followed by urinary elimination on account of the increased hydrophilicity of the conjugates (D'Archivio *et al.*, 2007). The conjugation mechanisms, together with low stability of many polyphenols under physiological conditions, are implicated in the very low concentrations of aglycones found in the blood, even in individuals on a polyphenol-rich diet. All forms of polyphenols, however, are rapidly excreted from the body. The maximal urine concentration of polyphenols is often attained within 2-4 hours after ingestion (Jin *et al.*, 2011).

Antioxidant therapies in CVD

It is well-recognised that diets rich in fruit and vegetables promote health and attenuate, or delay the onset of CVD (Lopez-Sepulveda *et al.*, 2011). The cardio-protective effects of such dietary interventions have been associated with a wide variety of chemical constituents of fruit and vegetables, many of which are considered to be powerful antioxidants (e.g. vitamins A, C and E) (Beckman *et al.*, 2001; Hozawa *et al.*, 2007; Otero *et al.*, 2005). Given that oxidative stress is a key feature of atherogenesis, antioxidant therapy is a potential option (Nicholson *et al.*, 2008). However, some intervention trials have failed to find a correlation between antioxidant vitamin consumption and reduced CVD (Lonn *et al.*, 2005). Therefore, interest has been directed towards other bioactive compounds found in fruit and vegetables, namely polyphenols (Duthie *et al.*, 2003) that might mediate the benefits independent of the abundant antioxidants. Epidemiological studies have also shown that polyphenols found in berries (Ellingsen *et al.*, 2008; Li *et al.*, 2015), chocolate (Jumar *et al.*, 2016; Larsson *et al.*, 2016), coffee (Grosso *et al.*, 2016), and red wine (Cosmi *et al.*, 2015) are associated with slower CVD progression. However, given their low bioavailability, the assumption that the observed health beneficial effects are driven by their direct antioxidant activity (chemical antagonism) seems very unlikely. Nevertheless, further studies have reported that properties other than antioxidant activity might underpin the benefits of polyphenols in the cardiovascular disease setting. The ability of native polyphenols and/or their metabolites to interact with enzymes, transcription factors (Aggarwal *et al.*, 2006; Kode *et al.*, 2008), and receptors (Chalopin *et al.*, 2010; Grossini *et al.*, 2015) strongly suggest that they might act as signalling molecules and be able to express their beneficial effects at a molecular level (Fraga *et al.*, 2010; Loke *et al.*, 2010).

Polyphenols as pharmacological agents

Collectively, studies have demonstrated that dietary polyphenols are biologically active substances, with therapeutic effects in cells and/or tissues. Phenolic compounds provide a wide spectrum of bioactivities: aside from their broadly described free radical scavenging properties, the existence of both hydrophobic and hydrophilic domains within polyphenols enables them to potentially interact with, and diffuse through biological membranes, and to bind to receptors and enzymes to exert intracellular signalling effects (Bennick, 2002).

Indirect antioxidant activity

One of the hypotheses that has emerged to explain the antioxidant effects imparted by polyphenols is that they act as mild toxins and stimulate a general xenobiotic or/and antioxidant response in the target cells, activating multiple defence genes. Phenolic compounds can activate the nuclear factor E2-related factor 2 (Nrf2)/antioxidant responsive element (ARE) pathway, thereby leading to induction to detoxifying enzymes, such as glutathione-S-transferase (GST), NADPH: quinone oxidoreductase 1 (NQO-1), haem oxygenase 1 (HO1) and glutamyl-cysteine ligase (GSL) (figure 3) (Johnson *et al.*, 2008; Nabavi *et al.*, 2016; Scapagnini *et al.*, 2011). Similar findings have been found for the green tea polyphenol, EGCG: concentrations of 20-100 µM have been shown to induce HO1 expression in rat neurons (H-19-7), possibly via activation of transcription factor Nrf2 (Romeo *et al.*, 2009). Moreover, curcumin (5 - 15 µM; the major component of the spice turmeric) has been reported to reduce hemin-induced oxidative stress in primary cultures of cerebellar granule neurons (CGNs) in rats, as well as to increase intracellular GSH, expression of HO1, glutathione reductase (GR), GST and superoxide dismutase, all of which might be mediated by Nrf2 activation (Gonzalez-Reyes *et al.*, 2013). Artemetin (10 pM and 1µM) was found to protect the endothelial cells against H_2O_2-induced oxidative stress by increasing GSH synthesis (Grossini *et al.*, 2015). Kode *et al.,* reported similar findings in human primary small airway epithelial and human alveolar epithelial (A549) cells. Resveratrol (10 µM) reduced cigarette smoke extract (CSE)-induced ROS production in small airway epithelial and human alveolar epithelial (A549) cells by restoring CSE-depleted GSH levels and upregulating GSL via activation of Nrf2 (Kode *et al.*, 2008).

Activation of antioxidant defence mechanisms might happen on two different levels: firstly, free radicals produced by pro-oxidant anthocyanins might activate protein kinases (e.g phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC)), that subsequently upregulate transcription factor Nrf-2; secondly, the compounds themselves might act as signalling molecules, interacting with protein kinases, thus inducing intracellular signalling cascades (figure 3).

Vasodilation

Anthocyanin glycosides found in elderberry extract can be incorporated into the plasma membrane, and to a lesser extent, the cytosol of endothelial cells (Youdim *et al.*, 2000). Structural similarities of certain anthocyanins to oestrogen (17-β-oestradiol), are responsible for their binding affinity to estrogen receptor α and/or β (ER-α, -β) (figure 4) (Fraga *et al.*, 2010; Grossini *et al.*, 2015; Hidalgo *et al.*, 2012; Zhang *et al.*, 2013). Delphinidin aglycone (17 μM) can induce endothelium-dependent vasodilatation in aorta in mice through activation of ER-α, leading to an increase endothelial NO synthase (eNOS) activity, and increased synthesis of the anti-atherogenic vasodilatory mediator, NO. The PI3K/Akt and Src/ERK1/2 signalling pathways have both been implicated in delphinidin-mediated vasodilatation in endothelial cells (figure 5) (Chalopin *et al.*, 2010; Lopez-Sepulveda *et al.*, 2011; Marino *et al.*, 2006). In support of the relevance of this potential mode of action of polyphenols is the fact that physiologically relevant concentrations can invoke this activity: for example, isoflavones (genistein, daidzein) exhibit oestrogenic activity at ~0.1 μM (Kuiper *et al.*, 1998). However, the aglycone form of delphinidin that has been found to mimic 17-β-oestradiol activity is unlikely to be found in plasma on account of its rapid degradation, which undermines the concept, at least for delphinidin.

Hidalqo *et al.,* (2012) have shown that delphinidin-3-*O*-glucoside (the form, which is more stable than aglycone), pelargonidin-3-*O*-glucoside, gallic acid as well as genistein has higher affinity to ER-β than ER-α (Hidalgo *et al.*, 2012; Kuiper *et al.*, 1998). Moreover, differential tissue distribution of the ER α (uterus, ovary, testis, skin, gut,) and β receptors (fetal ovaries, testes, adrenals, spleen) suggest these compounds may be tissue selective (Brandenberger *et al.*, 1997).

Interestingly, Grossini *et al.,* (2015) demonstrated that artemetin (the main phenolic component of herbs *Artemisia absinthium* and *Achillea millefolium*) at physiologically relevant concentrations (10 pM - 100 µM) increased eNOS-dependent NO production in porcine aortic endothelial cells through involvement of oestrogen receptors and activation of protein kinase A (PKA), ERK1/2, and the Akt pathway. Similar effects were observed for genistein (1 µM) (Grossini *et al.*, 2015). However, the relative instability of artemetin was not taken into consideration in these studies. Weathers *et al.,* (2012) reported that artemetin is poorly extracted from *Artemisia annua* plant and highly unstable in the tea infusion at room temperature (Weathers *et al.*, 2012). There was no information given on its stability under physiologically relevant conditions. The need for utilizing metabolites and degradation products of potentially bioactive polyphenols *in vitro* studies is increasing. Zhang *et al.,* (2013) have shown that *S*-(-)equol (10 nM – 250 nM), a metabolite of isoflavone daidzein, mimics the effects of its parent compound and activate PI3K/Akt pathway through ER-β receptor, increasing Nrf2 expression, an important factor in maintaining vascular redox homeostasis (Zhang *et al.*, 2013). Alternatively, polyphenols can promote vasodilation through inhibition of the release of the vasoconstricting factor, endothelin-1 (ET-1). Lazze *et al.,* (2006) found that delphinidin, and to a lesser extend cyanidin aglycone (50 - 100 µM) inhibit ET-1 synthesis in HUVECs, with a simultaneous increase in eNOS expression (Lazze *et al.*, 2006; Matsumoto *et al.*, 2005). Pre-treatment with delphinidin-3-*O*-rutinoside (10 µM) exerted an inhibitory effect on ET-1 induced contraction in bovine ciliary smooth muscle. Moreover, delphinidin-3-*O*-rutinoside promoted vasodilation via stimulation of ET_B receptors and the cGMP pathway, leading to NO production (Matsumoto *et al.*, 2005). In addition, delphinidin glycosides have been shown to inhibit the activity of other vasoconstrictors, such as angiotensin converting enzyme, but the concentrations required to cause this effect were unlikely to be achieved *in vivo* (IC_{50} ~ 65 µM) (Hidalgo *et al.*, 2012).

Anti-platelet activity

Phenolic compounds exhibited a range of inhibitory effects on platelet activation, related signal transduction pathways, enhancement of NO production, and inhibition of receptors such as thromboxane A_2 (TXA_2) (figure 6).

An *in vitro* study by Yang *et al.* (2012) demonstrated that delphinidin-3-*O*-glucoside inhibited platelet aggregation in platelet-rich plasma and purified platelets from humans and mice by collagen, thrombin, thrombin receptor activating peptide (TRAP), ADP. Substantial effects were observed at supraphysiological concentrations (50 μM), but concentrations as low as 0.5 μM also had a modest but significant effect. Similar concentrations were found to significantly inhibit thrombus formation under high and low shear. However, only 5 and 50 μM delphinidin-3-*O*-glucoside significantly inhibited expression of platelet activation markers, such as P-selectin, CD63 and CD40L ligand in purified platelets (Yang *et al.*, 2012). Furthermore, 5 μM delphinidin was the threshold concentration for inhibition of fibrinogen binding (Yang *et al.*, 2012). Moreover, quercetin (25 – 100 μM) inhibited collagen-induced fibrinogen binding to its receptor. Oh *et al.,* (2012) suggested that quercetin might cause conformational changes to the GPIIb IIIa receptor, therefore reduce the affinity of fibrinogen to the receptor's binding site (Oh *et al.*, 2012). Similar findings have been found for nobiletin (6.25 - 200μM), a flavonoid found in citrus fruit (Vaiyapuri *et al.,* 2015).

In contrast, Garcia-Alonso *et al.,* (2004) reported that delphinidin, petunidin and malvidin glycosides (50 μM) failed to inhibit collagen-induced platelet aggregation in human whole blood samples (Garcia-Alonso *et al.*, 2004). The discrepancy, however, might be a result of some differences in methodology used (e.g different pre-incubation time with the target polyphenol). Another anthocyanin, cyanidin-3-*O*-glucoside (5 and 50 μM), reduced platelet aggregation in healthy and hypercholesterolaemic patients by inhibition of platelet α-, δ-, and γ- granule secretion, as evaluated by P-selectin, RANTES (regulated upon activation normal T-cell expressed and secreted), beta-TG (beta-thromboglobulin), PF4 (platelet factor 4), TGFβ1 (transforming growth factor beta 1), serotonin, ATP, and CD63 release. The mechanism of action proposed by Song *et al.,* (2014) is activation of PI3K/Akt signalling pathway (figure 6) (Song *et al.*, 2014). Meanwhile, resveratrol has been found to inhibit collagen–induced platelet aggregation, to stimulate platelet NO-production, as well as to reduce platelet ROS production, when present at physiologically relevant concentrations (0.1 – 1 μM) (Messina *et al.*, 2015). Simple phenolic acids, such as gallic acid have also been shown to inhibit platelet aggregation and activation via inhibition of the phosphorylation of PKCα/p38 MAPK and Akt/GSK3β (Chang *et al.*, 2012). Similar findings were obtained for hippuric acid, the predominant metabolite of some phenolic acids and polyphenols, which reduced platelet aggregation and P-selectin/CD62P expression at 1 - 2 mM. Hippuric acid, however, is actively excreted from the body with maximal plasma concentrations of ~200 –

300 μM (Santhakumar *et al.*, 2015). Meanwhile, pelargonidin aglycone, but not the glucoside adduct, inhibited thrombin-induced fibrin polymerization and platelet aggregation and elicited anticoagulant effects in mice. Similarly to previous studies, the concentrations used were not attainable physiologically, and were ≥ 10 μM (Ku *et al.*, 2016). Another *in vitro* study by Macakova *et al.,* (2012) reported that differences in chemical structure of several 4-methylcoumarin analogues (absence of hydroxyl group, or altered hydroxyl group at position at C-5, and different substitutions at C-3) determined their anti-platelet function. 5,7-dihydroxy-4-methylcoumarins, especially those with a lipophilic side chain at C-3, had anti-platelet activity that was similar to that of acetylsalicylic acid (aspirin) in arachidonic acid-induced platelet aggregation. Interestingly, only synthetic coumarins, but not the native equivalents (no substitutions on C-5 and C-3) inhibited platelet aggregation. The most effective was synthetic 3-ethoxycarbonylethyl-5,7-dihydroxy-4-methylcoumarin at 10 μM (Macakova *et al.*, 2012). On account of the fact that the effect with this compound was specific to aggregation induced by arachidonic acid, the mechanism of action proposed was inhibition of cyclooxygenase 1 and competitive antagonism at thromboxane A_2 receptors (Macakova *et al.*, 2012).

Polyphenols can, therefore, inhibit platelet aggregation induced by a range of agonists (figure 6). However, the inhibition almost exclusively requires concentrations that are supra-physiological.

Anti-inflammatory mechanisms

Atherosclerosis is an inflammatory disease. Chronic inflammation plays a crucial role in development and progression of CVD. Phenolic compounds exert anti-inflammatory activities by altering the recruitment of inflammatory cells (arresting pro-inflammatory molecules production: tumour necrosis factor α (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP)) and inhibiting the production of adhesion molecules (VCAM-1 and ICAM-1) by the endothelium, thereby impeding cellular migration of monocytes into the subendothelial space (Tangney *et al.*, 2013). Polyphenols are likely to promote their anti-inflammatory properties by modulating transcriptional networks and/or signalling cascades that modulate gene expression, leading to inhibition of inflammatory mediators (figure 7).

There is a significant reduction in secretion of adhesion molecules (MCP-1, ICAM-1, and VCAM-1) when endothelial cells were pre-treated with gallic acid (10 - 100 μM), but concentrations likely attainable *in vivo* (1 μM) were less effective in this regard (Hidalgo *et al.*, 2012). Similarly, the anthocyanins, delphinidin- and cyanidin-3-*O*-glucosides (0.1 – 50 μg/ml; 200 nM – 100 μM) inhibited LPS-induced VCAM-1 expression in porcine iliac artery endothelial cells (Zhu *et al.*, 2013). Moreover, delphinidin aglycone (50 – 200 μM) has been shown to decrease ox-LDL induced expression of adhesion molecules (ICAM-1 and P-selectin) in a human endothelial cell line (EA.hy926), as well as to reduce adhesion of monocytes to endothelial cells by reducing intracellular ROS, p38MAPK expression, IκB-α degradation and NF-κB transcription activity (Chen *et al.*, 2011). Another *in vitro* study showed that quercetin (125 μM) was able to partially supress leptin-induced TNF-α secretion, and significantly inhibit leptin-induced NF-κB expression in HUVECs (Indra *et al.*, 2013). Moreover, epicatechin (1 - 100 μg/ml; approx. 3 – 340 μM), suppressed production of the pro-inflammatory cytokines, IL6, IL-8, with a simultaneous increase in expression of the anti-inflammatory cytokine, IL-10 in whole blood cultures (Al-Hanbali *et al.*, 2009). Additionally, apigenin (30 μM) and kaempferol (30 μM), but not resveratrol (50 μM), supressed expression of LPS-induced interleukine 1 (IL-1). All of these polyphenols, and resveratrol in particular, effectively decreased LPS-induced expression of TNF-α in J774 macrophages (Kowalski *et al.*, 2005).

A wide range of phenolic compounds (daidzein, genistein, kaempferol, pelarginidin, naringenin and isorhamnetin; all at 100 μM) have also been found to significantly reduce LPS-induced NF-κB formation in J774 cells (macrophages) (Hamalainen *et al.*, 2007). Similarly, epicatechin decreased the amount of NF-κB in cytoplasmic fractions (Al-Hanbali *et al.*, 2009).

It has been reported that various flavonoids can suppress expression of others pro-inflammatory mediators (Hou et al., 2005, Lopez-Posadas *et al.,* 2005). Cyclooxygenase 2 (COX-2), an important player in inflammatory responses, has been shown to be inhibited by two anthocyanins, delphinidin and cyanidin (25 – 100 μM), in LPS-activated RAW264 cells (Hou *et al.,* 2005). Other tested compounds such as, pelargonidin, malvidin and peonidin did not show any inhibitory effects (Hou *et al.*, 2005). Similarly, kaempferol (25 and 100 μM) has been found to supress COX-2 and TNF-α gene expression in LPS-treated RAW264.7 cells (Kim *et al.*, 2015). Activation of transcription factor NF-κB plays a crucial role in

inflammation, mainly due to its ability to induce expression of pro-inflammatory genes, thereby regulating the immune response (Tak *et al.*, 2001). Therefore, direct inhibition of NF-κB by polyphenols is recognised to be a fundamental mechanism underpinning their anti-inflammatory activity (figure 7). Banaganapalli *et al.,* (2013) reported that the synthetic resveratrol derivative (propynyl resveratrol; 5 μM -10 μM) suppressed the activity of NF-κB, most likely by interfering with its DNA binding ability possibly via its association with the IκB-α site of NF-κB (Banaganapalli *et al.*, 2013). Interestingly, native resveratrol was not as effective as its synthetic derivative, either on account of lower stability of resveratrol or through modified structure: function relations (Banaganapali *et al.,* 2013). Likewise, curcumin analogues have been found to inhibit NF-κB activation and gene regulation via inhibition of IKK and Akt activation. In the same study, curcumin (50 μM) blocked phosphorylation of IκBα and p65, leading to suppression of events necessary for NF-κB gene expression, mainly degradation of IκB-α and nuclear translocation of p65 (Aggarwal *et al.*, 2006).

Another anti-inflammatory mechanism that can be potentially targeted by polyphenols is the mitogen-activated protein kinase (MAPK) pathway. MAPKs are involved in the production of pro-inflammatory cytokines (IL-6, TNF-α, MCP-1, and iNOS) and downstream signalling events that lead to inflammation and apoptosis (Thalhamer *et al.,* 2008). It has been reported that malvidin (50 μM), an anthocyanin found in red wine, inhibited LPS-induced MAPK signalling in RAW 264.7 macrophages, with simultaneous enhancement of MAPK phosphatase-1 (MKP-1; the protein that down-regulates the activity of all three branches of MAPKs) (Bognar *et al.,* 2013). Similar findings were identified for another anthocyanin, delphinidin: pre-treatment with delphinidin aglycone (100 μM) significantly suppressed phosphorylation of JNK1/2, ERK1/2, and p38 kinases (three branches of MAPKs). Meanwhile, the glycoside, delphinidin-3-sambubioside (50 – 200 μM), only successfully supressed the ERK1/2 phosphorylation with little effect on the phosphorylation of JNK1/2 and p38 in LPS-induced RAW 264.7 (Sogo *et al.,* 2015).

Finding a drug specific for a disease of complex aetiology, like cardiovascular disease, is challenging. However, many natural products are characterised by weak binding affinities for any given target, thereby increasing the likelihood of binding to multiple targets at lower affinity, with a combined effect that is sufficient to drive an overall health benefit (Wang *et al.,* 2016).

Conclusions

That nutritional polyphenols can have cardioprotective activity *in vivo* and are important health-promoting components of our diet is unequivocal. However, the fundamental mechanisms that underpin the protective activity are much less clear, a situation that is not helped by the multitude of *in vitro* data produced using inappropriate concentrations of native polyphenols that have poor bioavailability and are rapidly metabolised to simple phenols, aldehydes and salicylates. In particular, the measurement of direct antioxidant capacity of extracts or even pure compounds *in vitro* as a predictor of *in vivo* antioxidant activity is unfounded, given the low bioavailability and rapid metabolism of the component polyphenols. Instead, there is a far more complex picture emerging of the mechanisms involved in the cardioprotective effects of dietary polyphenols that involves pharmacological activity at receptor, cell signalling and gene expression levels. Crucially, the concentrations required to activate these pathways are often orders of magnitude lower than those necessary for direct antioxidant activity, and many of the products of polyphenol metabolism are as active as the parent compounds.

There is a need to return to first principles in pharmacology to gain a full understanding of the activity of polyphenols in cardioprotection. In particular, not enough attention has been paid to stability, absorption, distribution and metabolism of polyphenols in order to inform the design of experiments to test the mechanism(s) of action *in vitro*. This need extends to *in vivo* pharmacokinetic and pharmacodynamic experiments as a forerunner to mechanistic studies in order to determine the concentrations of polyphenols and/or their metabolites to be tested in cell culture. Critically, acute exposure to a high concentration of a phenolic compound is not an acceptable surrogate for the more realistic chronic exposure to a low concentration that might happen *in vivo*: the mechanisms revealed by each approach are likely to be very different, even if the eventual outcome is the same (e.g. antioxidant protection).

Understanding the pharmacological mechanism by which polyphenols bring about cardiovascular effects is critical not only to inform dietary advice, but also to help design drugs and nutraceuticals with better bioavailability that target the same pathways. Diosmin is an example of a semi-synthetic drug, based on the citrus polyphenol, hesperidin (Tong *et al.,* 2013), that is used widely in Europe and USA, primarily in venous insufficiency (Amato, 1994, Maksimovic *et al.,* 2008). Interestingly, the mode of action of diosmin is through

venous contraction and not through any antioxidant effect. This example serves to highlight that polyphenols have great pharmacological potential, but a universal shift in emphasis away from the direct antioxidant notion for polyphenol activity is the vital first step to a full appreciation of polyphenol activity *in vivo.*

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Conflict of interests

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Figure Legends

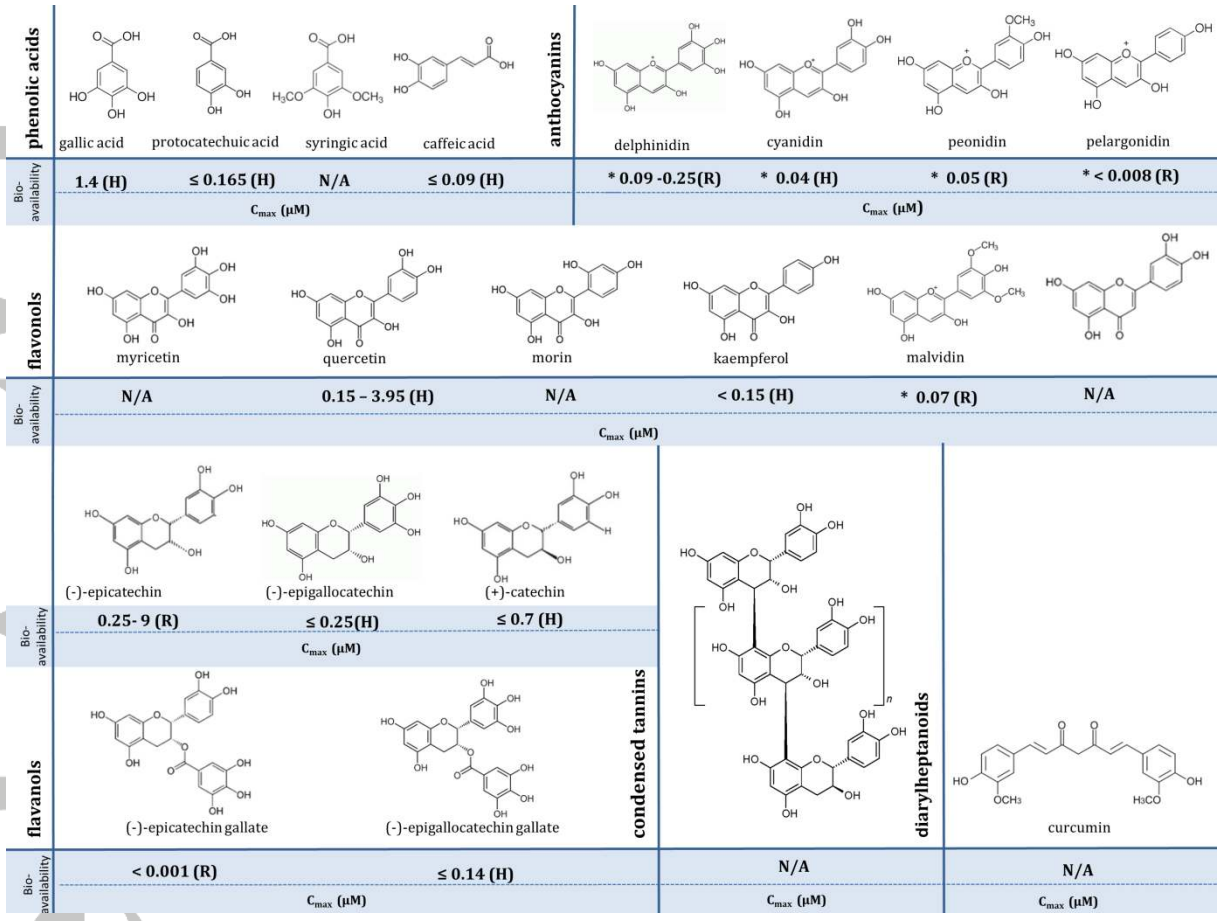

Figure 1. Chemical structures of polyphenols of high antioxidant potential, including their bioavailability in plasma analysed without enzymatic conjugation. Data were obtained from *Phenol-Explorer* data base (www.phenol-explorer.eu). (H) - C_{max} data obtained from human studies; (R) - C_{max} data obtained from animal studies -rats; * presented values correspond to the following glycosides: delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside; N/A- data not available.

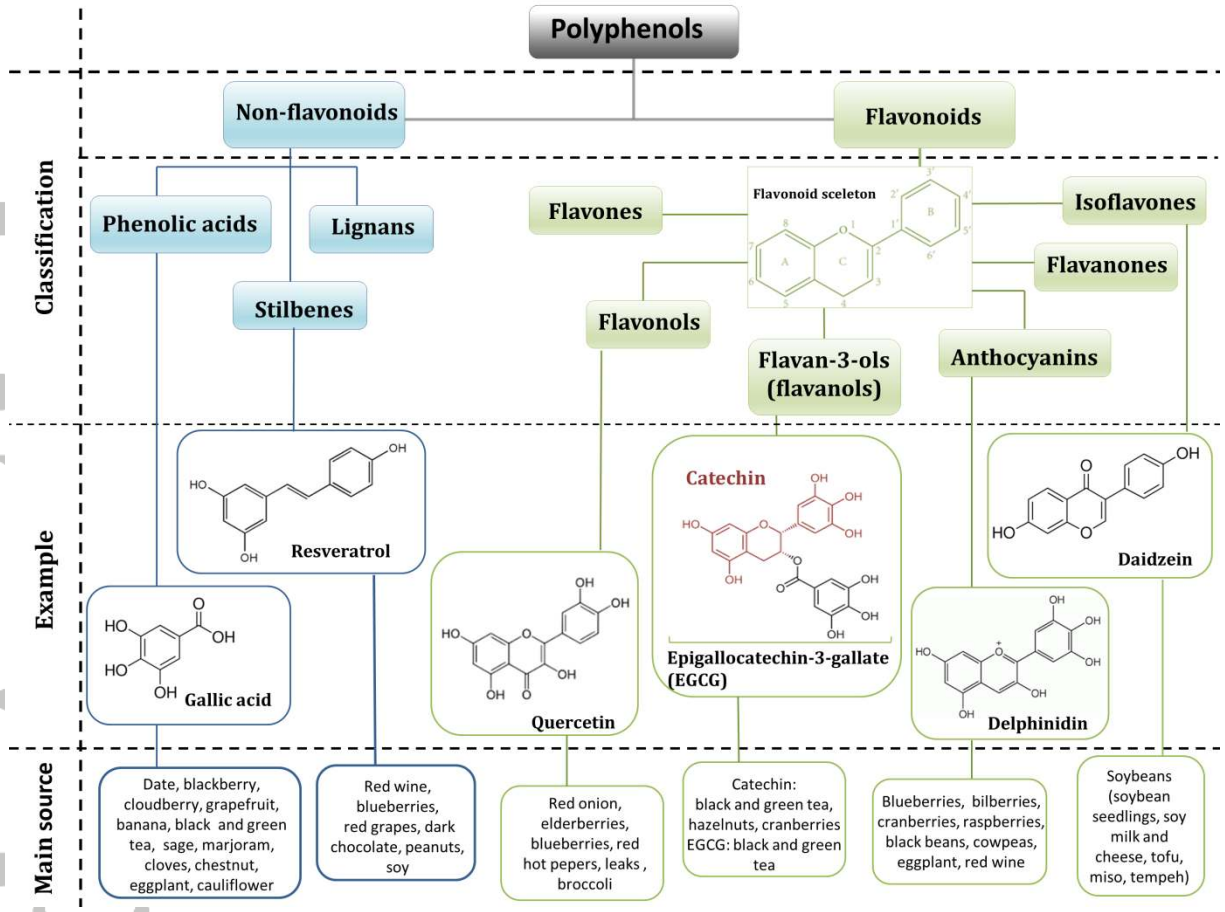

Figure 2. Polyphenol classification. Classes of polyphenols with examples that exhibit possible cardioprotective effects.

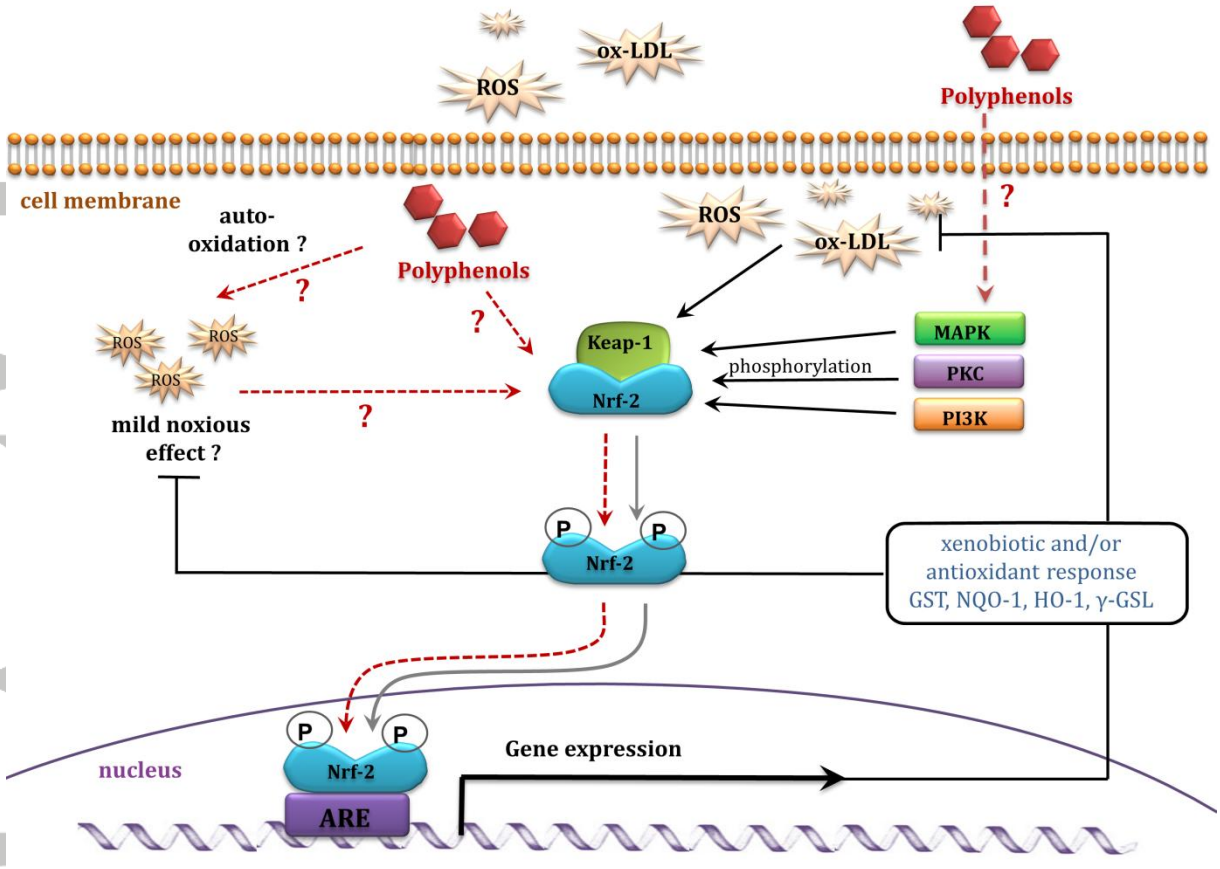

Figure 3. Indirect antioxidant activity of polyphenols mediated via gene expression- a proposed mechanism.

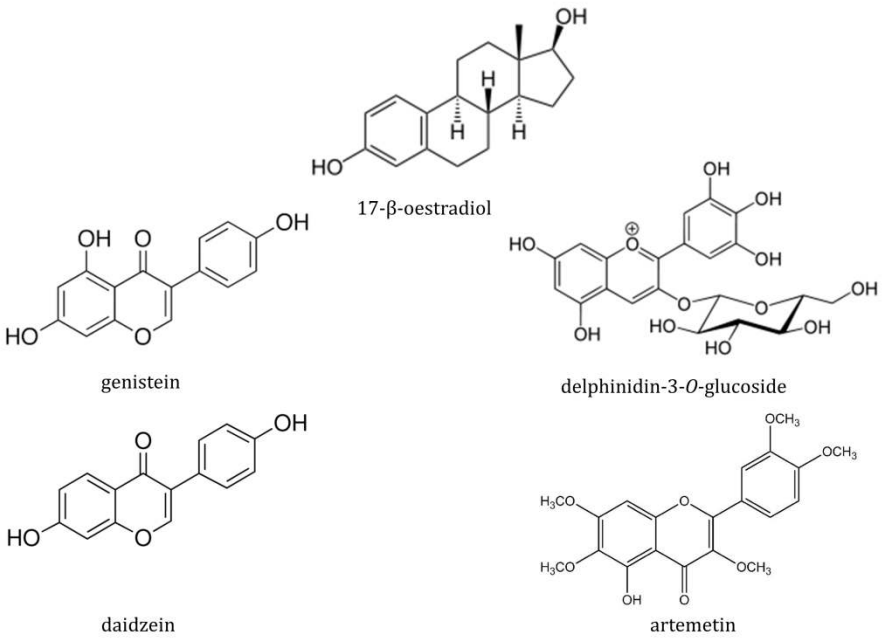

Figure 4. 17-β-oestradiol and phytoestrogens.

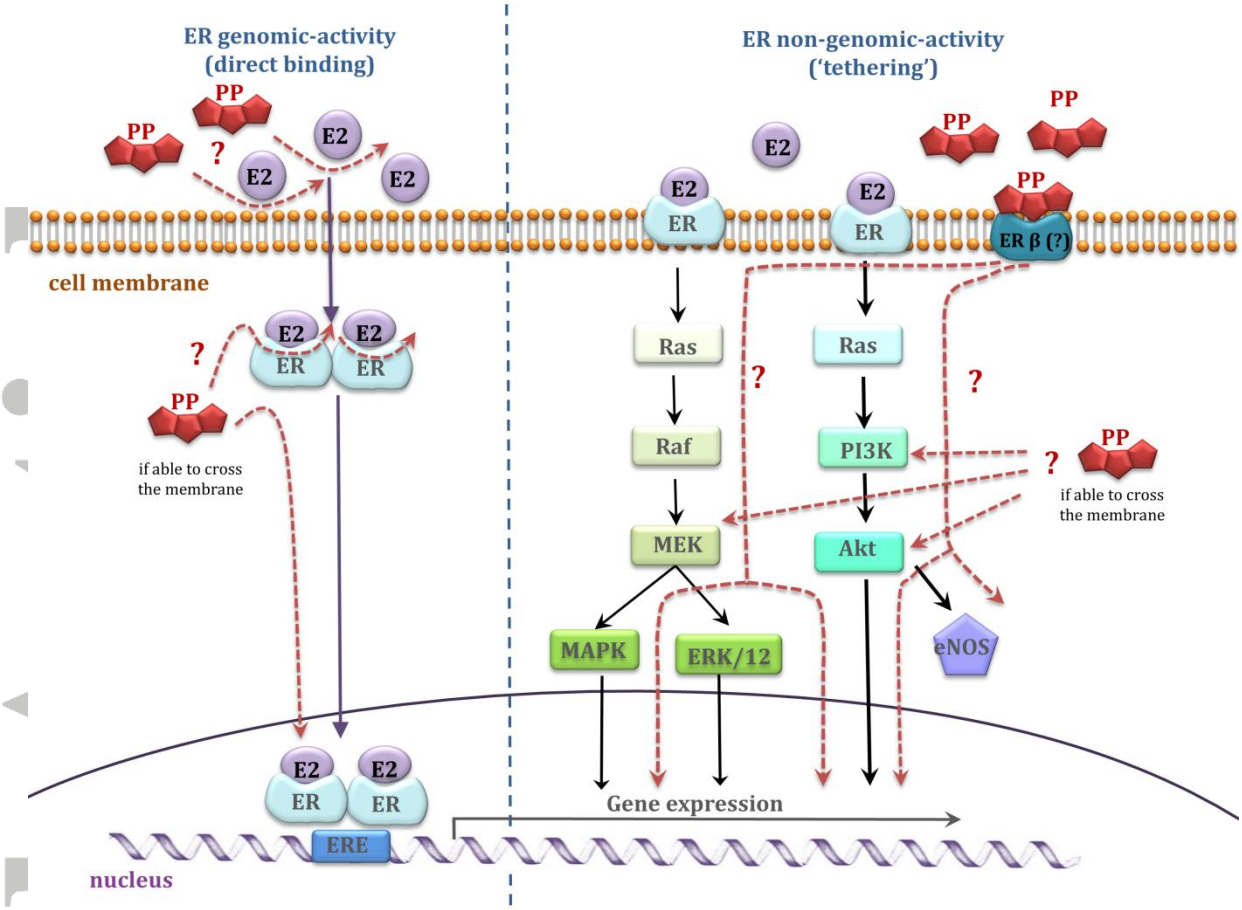

Figure 5. Oestrogen receptor and polyphenols – a proposed mechanism of action, taking into account two main ERs regulatory actions; classical (direct) and tethered pathway.

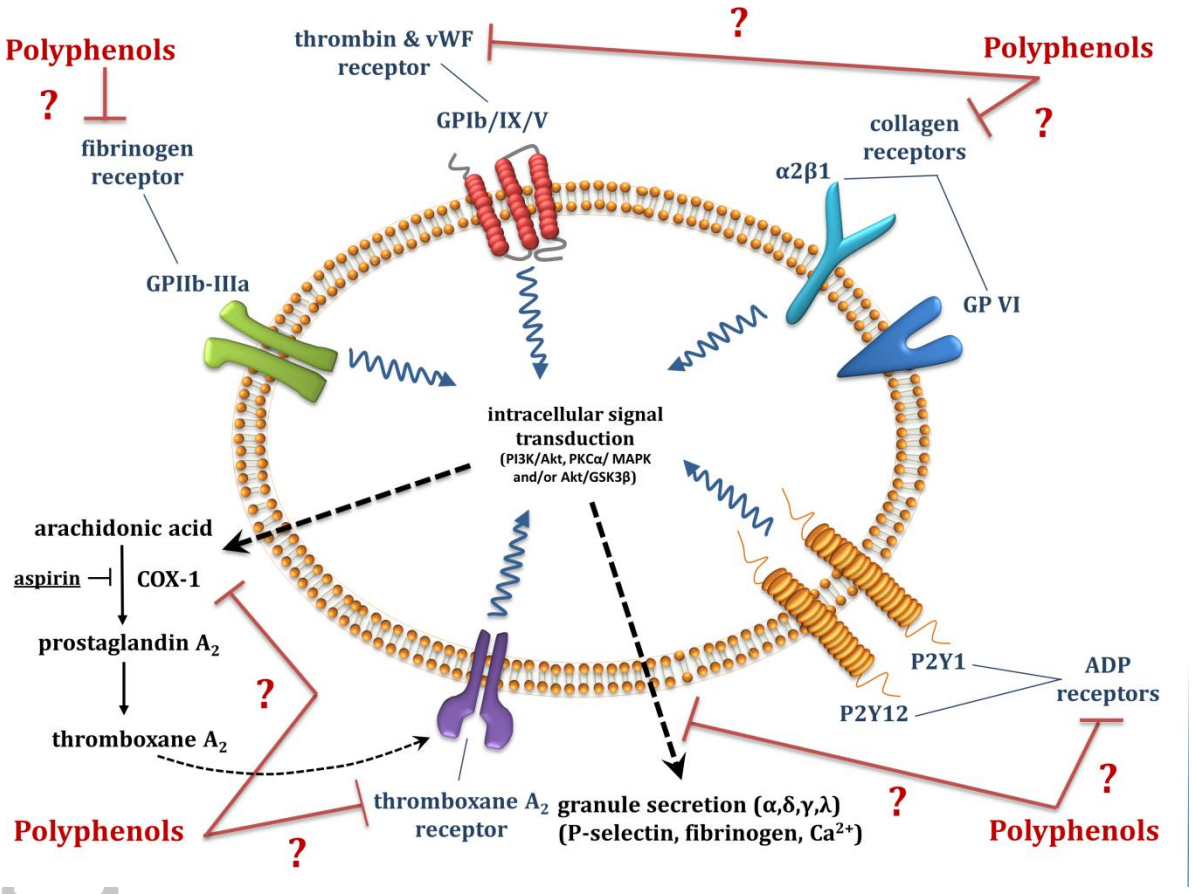

Figure 6. Proposed anti-platelet mechanism of action of polyphenols.

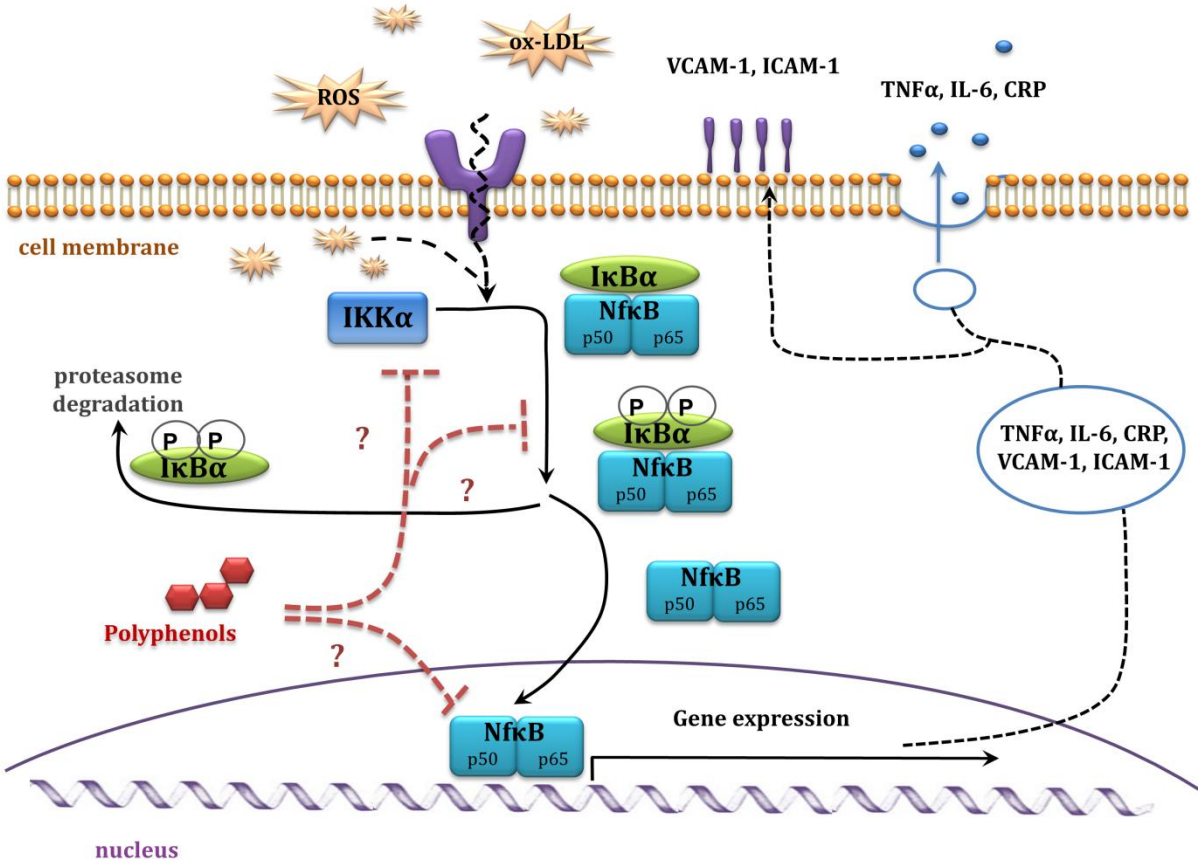

Figure 7. Schematic diagram of NfκB pathways, and proposed anti-inflammatory mechanism of action of polyphenols.