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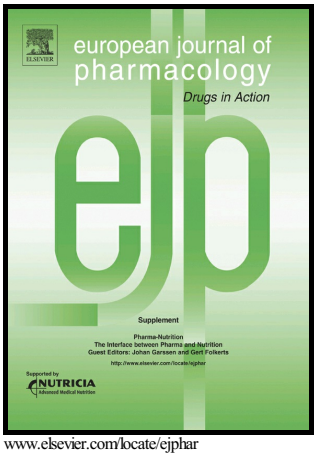

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The surge of flavonoids as novel, fine regulators of cardiovascular Ca_v channels

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ABSTRACT

Ion channels underlie a wide variety of physiological processes that involve rapid changes in cell dynamics, such as cardiac and vascular smooth muscle contraction. Overexpression or dysfunction of these membrane proteins are the basis of many cardiovascular diseases that represent the leading cause of morbidity and mortality for human beings. In the last few years, flavonoids, widely distributed in the plant kingdom, have attracted the interest of many laboratories as an emerging class of fine ion, in particular Ca_v, channels modulators. Pieces of *in vitro* evidence for direct as well as indirect effects exerted by various flavonoids on ion channel currents are now accumulating in the scientific literature. This activity may be responsible, at least in part, for the beneficial and protective effects of dietary flavonoids toward cardiovascular diseases highlighted in several epidemiological studies. Here we examine numerous studies aimed at analysing this feature of flavonoids, focusing on the mechanisms that promote their sometimes controversial activities at cardiovascular Ca_v channels. New methodological approaches, such as molecular modelling and docking to $Ca_v1.2$ channel α_{1c} subunit, used to elucidate flavonoids intrinsic

mechanism of action, are introduced. Moreover, flavonoid-membrane interaction, bioavailability, and antioxidant activity are taken into account and discussed.

Keywords: flavonoid, quercetin, Cav1.2 channel, cardiovascular system, patch-clamp, molecular docking

Chemical compounds studied in this article

Quercetin (PubChem CID: 5280343); Myricetin (PubChem CID: 5281672); Genistein (PubChem CID: 5280961); Daidzein (PubChem CID: 5281708); (S)-(-)-Bay K 8644 (PubChem CID: 6603728).

1. Introduction

Dietary flavonoids comprise a large group of polyphenolic compounds, including approximately 8000 members, predominantly found in fruits, vegetables, chocolate and beverages as tea, coffee and red wine (Geleijnse and Hollman, 2008). These bioactive, non-energetic, non-nutrient secondary metabolites, originating from L-phenylalanine (Marín et al., 2015), possess a variety of physiological functions in plant tissues, such as pigmentation and flavour, growth and reproduction, regulation of metabolic enzymes and protection against different environmental hazards. Their discovery dates 1930s, when the

Hungarian scientists Rusznyak and Szent-Györgi were the first to identify in paprika and lemon peel the so-called “vitamin P” (P for permeability; actually a mixture of the flavonoids hesperidin and eriodictyol glucoside) that reduces capillary permeability (Rusznyak and Szent-Györgyi, 1936).

1.1. The basic structure of flavonoids

The core flavonoid structure consists of two aromatic rings (A and B) bridged by an oxygen containing, heterocyclic benzopyran ring (C) (Fig. 1). According to the degree of hydroxylation, methoxylation, prenylation, glycosylation or even B ring positioning, flavonoids are classified into 6 different main classes: flavanols and catechins (e.g., catechin and epicatechin, mainly found in green tea, cocoa, kola, banana, and pomegranate), flavones (apigenin and luteolin from cereals, parsley, and celery), flavanones [(±)-naringenin and hesperetin from citrus fruits and tomatoes], flavonols (quercetin, myricetin, and kaempferol from onion, kale, spinach, broccoli, caper, and red wine), isoflavones (daidzein and genistein from soy and legumes), and anthocyanidins (malvidin and delphinidin from red wine and berries) (Marín et al., 2015). Flavonoids bearing an open C ring are chalcones. The presence of the double bond in C ring constraints A and C ring to be planar, while its absence renders the non-planar compound chiral at C2 and/or C3 centres. In nature, many flavonoids are found as O- or C-glycosides or exist in polymeric form, dimer being the most common.

Among flavonoids, flavanols are by far the most abundant in fruits and vegetables and, as such, are present in considerable amounts in the human diet. Today, flavonoids content in

food can be verified in web databases developed and continuously updated by national [United States Department of Agriculture (USDA, 2007); the Institut National de la Recherche Agronomique (Phenol-Explorer; www.phenol-explorer.eu) (Neveu et al., 2010)] and international Associations [the European Food Information Resources Network (EuroFir (2009) European Food Information Resource Network Food Composition Databank; http://www.eurofir.net/index.asp?id¼1)]. The Phenol-Explorer, in particular, takes into account also polyphenol retention due to food processing and storage (Rothwell et al., 2015). In recent years, however, extensive efforts were made to develop more reliable dietary biomarkers of food intake (Scalbert et al., 2014; Zamora-Ros et al., 2014) as a more objective method of dietary exposure. In fact, databases present intrinsic limitations inherent to food content variations caused by seasonal effects, local climate, varietal differences, etc.

1.2. Biological activities of flavonoids: focus on the cardiovascular system

Initially investigated for their *in vitro* antioxidant activity [the structural requirements for the antioxidant activity of flavonols, as an example, can be found in Singh et al. (2014)], flavonoids have been at the focus of intense research as they display many other "claimed" interesting biological activities (pro-oxidant, anti-inflammatory, neuroprotective, anti-proliferative, pro-apoptotic, anti-angiogenic, etc.). These features comply with the most recent therapeutic strategies for age- and lifestyle-related diseases, which require the modulation of several therapeutic targets at once to achieve both injury reduction and functional recovery. High consumption of flavonoids (primarily flavanols and flavonols), for

example, has been recently associated with reduced risk of all-cause, cardiovascular disease and cancer mortality in a population of old women (Ivey et al., 2015).

The beneficial effects of flavonoids on the cardiovascular system are by far the most extensively investigated, in both experimental and clinical settings. More than two decades ago, the Zutphen Elderly Study demonstrated the protective effect of flavonoids against mortality from coronary heart disease (Hertog et al., 1993). After the report by the Kromhout's group, a wealth of intriguing but not yet compelling evidence supported this concept (Peterson et al., 2012) without deciphering, however, the biological pathways possibly involved in this effect (Quiñones et al., 2013). Traditionally, their protective activity is attributed to the antioxidant property. However, extensive metabolism impairs flavonoids antioxidant activity (so far never established *in vivo*; Hollman et al., 2011) and markedly reduces their blood concentration to levels much lower than those of other antioxidants.

Cardiovascular diseases are multifactorial: therefore, molecules capable of targeting more than one mechanism involved in their pathophysiology represent excellent opportunities for the development of new, effective drugs. Flavonoids satisfy this requisite (Almeida Rezende et al., 2016), as they present vasodilator effects mainly due to K^+ channels opening and/or Ca_v channels blockade, induce heme-oxygenase-1 and eNOS expression and activity (Chirumbolo, 2012; Shen et al., 2013), improve plasma lipid profiles, attenuate the oxidation of low density lipoproteins, decrease directly or indirectly the expression of inflammatory signalling molecules (e.g., inducible NO synthase, cyclooxygenases, adhesion molecules; Mladěnka et al., 2010), show antithrombotic and anti-platelet activity (Choi et al., 2016), modulate apoptotic processes in vascular endothelium, regulate endothelial cell expression of genes important in cardiovascular health (Nicholson et al., 2010), affect cardiac mitochondrial ion channels, protein kinases or oxidative

phosphorylation (Testai, 2015), and prove more successful than nifedipine in ameliorating high-salt diet-induced hypertension (Olaleye et al., 2013).

Three recent meta-analysis studies support the view that the dietary intake of flavonoids, particularly cocoa and tea flavan-3-ols, significantly decreases the risk of cardiovascular diseases by improving endothelial dysfunction (flow-mediated dilation) and lowering both systolic and diastolic blood pressure (Kay, 2012; Serban et al., 2016; Wang et al., 2014; see also Rodriguez-Mateos et al., 2014). The latter effect, in particular, seems to characterize the beneficial outcome of quercetin intake, as demonstrated by several *in vivo* studies conducted on both hypertensive animals and humans (Larson et al., 2012; Najmanová et al., 2016). Noticeably, the increase in flow-mediated dilation seems to correlate with some phenol metabolites (Rodriguez-Mateos et al., 2016). However, the flavonoids bioactivity does not follow a classical linear but rather a hormetic dose-response relationship and this may have important biological implications. Furthermore, these epidemiological studies still present several limitations: standardised biomarkers of intake were scarce; use of pure compounds was very limited; clinical intervention times were not adequately protracted; and flavonoid blood levels or pharmacokinetics were rarely assessed.

One of the most investigated effects of flavonoids is that on blood vessel mechanics. Essentially, flavonoids are vasodilators that reduce muscle tone through a variety of endothelium-dependent and/or -independent mechanisms (Almeida Rezende et al., 2016), one being their interaction with ion channels.

1.3. Ion channels

Cell types from organs as diverse as brain, heart, and muscles produce fast electrical signals that race along membranes in the form of voltage fluctuations known as action potentials. These signals play a fundamental role in cell-to-cell communication and activate intracellular processes leading, for example, to muscle contraction. Action potentials result from the activity of complex, transmembrane multimeric protein assemblies called ion channels that consist of separate pore-forming and accessory subunits (Ashcroft, 2006). Functionally, ion channels open and close in response to a wide variety of stimuli; this has led to their classification into voltage-gated channels (regulated by changes in membrane potential), and ligand- and sensory-gated channels (responding to changes in various intracellular or extracellular ligands and to mechanical or thermal stimuli, respectively).

Ion channels selectively regulate the flux of ions between the intracellular and the extracellular space, otherwise hampered by the hydrophobic nature of the cell membrane. When ion channels open, ions flow down their electrochemical gradients (Hille, 2001) creating the electrical signals that underpin a vast array of physiological processes. For these reasons, proper ion channel expression, regulation, and function are fundamental for life. Consequently, as ion channels represent a major class of therapeutic targets, it is of primary interest to understand the molecular basis of how they work in order to develop more specific and applicable agents to control their functions (Clare, 2010). Moreover, assessment of new chemical entities on cardiovascular ion channel activity is now an integral component of drug discovery programmes (Safety Pharmacology; Chain et al., 2013) aimed at defining their potential for cardiovascular side effects.

Although ion channels physiological significance and therapeutic relevance were demonstrated in a wide variety of biological systems, they are still underexploited drug targets. This is mostly due to the labour-intensive and low-throughput nature of the patch-clamp technique, the gold standard assay for ion current recordings. The advent of robotic systems, however, will possibly overcome this historical bottleneck in ion channel drug research and discovery (Dunlop et al., 2008), allowing in the near future the development of novel therapeutic agents.

1.4. Aim of the work

Endeavouring the effects of flavonoids on Ca_v channels, central in signalling pathways controlling various biological functions, has increased substantially in the last decade. This paper summarizes the state-of-the-art in this field, by reviewing the continuously growing evidence of flavonoids as cardiovascular Ca_v channel modulators. Data here discussed relate fundamentally to the patch-clamp technique (Neher and Sakmann, 1992), an extremely powerful and exquisitely sensitive means for studying the biophysics and pharmacology of ion channels embedded in a physiological environment to get the best clues of drug-channel interaction (Zahradnikova et al., 2007). Studies carried out on pure compounds (mainly quercetin and genistein) but not on plant extracts (which do not reliably assure a standard content of flavonoids) were reviewed. The physiological significance of the mechanism(s) leading to Ca_v channel function regulation by flavonoids as well as their potential as novel therapeutic agents are discussed, taking into account their membrane interaction, bioavailability and antioxidant activity. New hypotheses for

future investigations are also formulated. Finally, *in silico* molecular docking analysis is also highlighted as a novel approach to understand flavonoid mode of action at target sites.

2. Flavonoids as Ca^{2+} channels modulators

Since the mid-1990s, the number of articles on flavonoids published per year has increased exponentially (almost ten a day in 2015). In spite of the tremendous interest of bio-medical research on the beneficial health effects that flavonoids exert trough the cardiovascular system, only a small fraction of studies deals with their activity on ion channels. Nowadays, there is an increasing body of evidence that flavonoids can directly target cardiovascular ion channels and several findings from this laboratory are in support of this theory.

Preliminary, indirect evidence of ion channel regulation by flavonoids arose largely from pharmacological studies using *in vitro* isolated cardiovascular preparations. Subsequently, several studies combining electrophysiological and molecular biology techniques provided the final evidence for specific flavonoid-channel interaction and insights into the mechanisms of channel regulation. In the following sections, we focussed mainly on $Ca_v1.2$ channels, which play a key role in the maintenance of vascular tone and cardiac activity (Zamponi et al., 2015). To provide a context for understanding how flavonoids regulate Ca^{2+} channels, a brief introduction to ion channel structure, physiology, and pharmacology seems appropriate.

2.1. Ca^{2+} channels of the cardiovascular system: the $Ca_v1.2$ channel

Ca^{2+} channels, present in the membrane of most excitable cells (for their nomenclature see Catterall et al., 2005), represent the major route for Ca^{2+} translocation across the plasma membrane and support several functions, including muscle contraction. Although various families of Ca^{2+} channels are expressed in cardiovascular myocytes, as defined by physiological and pharmacological criteria (Catterall, 2011), Ca_v channels constitute the dominant Ca^{2+} influx route. Other routes, such as non-selective cation channels (Beech et al., 2004), exist but their contribution to total Ca^{2+} influx is generally negligible in contractile cells. The main physiologic functions of Ca_v channels in the cardiovascular system are: (1) action potential generation and pace-making activity (thus regulating heart rate and atrioventricular conduction); (2) generation of systolic Ca^{2+} influx in cardiomyocytes (cardiac inotropism and atrial excitability); (3) generation of Ca^{2+} influx in vascular smooth muscle and endothelial cells (arterial myogenic tone and vascular resistance).

Ca^{2+} channels form hetero-oligomeric complexes that are composed of a pore-forming α_1 subunit. The ten cloned α_1 subunits can be grouped into three families (Catterall et al., 2015): (1) the high-voltage activated dihydropyridine-sensitive (L-type, $Ca_V1.x$; $Ca_v1.2$ in cardiac and vascular myocytes) channels; (2) the high-voltage activated dihydropyridine-insensitive ($Ca_V2.x$) channels and (3) the low-voltage-activated (T-type, $Ca_V3.x$) channels. The α_1 subunit has four homologous repeats (I–IV) bearing six transmembrane domains and a pore-forming region between transmembrane domains S5 and S6. The pore loop contains a unique structural feature, i.e. a pair of glutamate residues, that confers Ca^{2+} selectivity to the channel. The membrane-spanning S4 segment, which contains highly

conserved positive charges, serves as the voltage sensor for activation: it moves outward and rotates under the influence of the electric field, initiating a conformational change that opens the pore. Furthermore, the α_1 subunit presents most of the known sites of channel regulation by second messengers, drugs, and toxins, and confers the functional properties of Ca^{2+}-dependent inactivation, crucial for limiting Ca^{2+} entry during long depolarizations. At least native $Ca_V1.x$ channels comprise co-assemblies of α_1 and regulatory β and $\alpha_2\delta$ subunits that modulate the properties and plasma membrane expression of the channel complex (Bannister et al., 2009). In vascular smooth muscle cells, the α_{1C} subunit represents a splice variant ($\alpha_{1C\text{-}b}$) that differs in four regions from the splice variant ($\alpha_{1C\text{-}a}$) expressed by cardiac myocytes (Liao et al., 2004; Welling et al., 1997).

Free intracellular Ca^{2+} concentration is maintained at 10-100 nM in the cytoplasm, a very low level as compared to the extracellular milieu. $Ca_v1.2$ channel opens in response to membrane depolarization, allowing Ca^{2+} to move down its electrochemical gradient, thus raising intracellular Ca^{2+} concentration. Its activity is essential to coupling of electrical signals in the cell surface to physiological events in the cell. It shows a unitary conductance of 20-30 pS, and is slowly inactivating. Ca^{2+}-dependent inactivation occurs when increasing intracellular Ca^{2+} causes closure of $Ca_v1.2$ channels, even when depolarization is maintained (Morad and Soldatov, 2005); $Ca_v1.2$ channels can also inactivate *via* voltage-dependent inactivation (Lee et al., 1985). Both Ca^{2+}- and voltage-dependent inactivation regulate the amount of Ca^{2+} entry into the cell, thus limiting muscle contraction.

Pharmacological approaches are instrumental to establish the contributions that different Ca_v channels make to various cellular responses. Ca^{2+} current through $Ca_v1.2$ channels is inhibited by three different classes of drugs, namely dihydropyridines (prototype nifedipine;

gating inhibitor), benzothiazepines and phenylalkylamines (diltiazem and verapamil, respectively; channel blockers), which bind to distinct, but allosterically coupled, receptor sites close to the pore and to the proposed activation gate of the channel α_1-subunit (Striessnig et al., 1998; Striessnig et al., 2005; Tang et al., 2016; Tikhonov and Zhorov, 2009) with IC_{50} values ranging from nM to µM concentrations. Some activators also exist, such as FPL64176, (S)-(-)-Bay K 8644 and SZ(+)-(*S*)-202-791. Although cardiac and smooth muscle $Ca_v1.2$ channels exhibit a 95% homology in their amino acid sequences, contribution to total L-type currents, alternative splicing of the α_1 subunit (Liao and Soong, 2010), and resting membrane potential (more depolarized in smooth muscle cells) shape their different sensitivity toward dihydropyridines (Zamponi et al., 2015). For example, dihydropyridine-sensitive splice variants are predominantly expressed in arterial smooth muscles, thus justifying the higher potency of these drugs in vascular smooth muscle as compared to the working myocardium.

$Ca_v1.2$ channel blockers belong to the most widely prescribed drugs worldwide and are used in the treatment of epilepsy, chronic pain, hypertension, angina pectoris, and cardiac arrhythmia (Catterall and Swanson, 2015). Human genetic diseases leading to gain- or loss-of-function have been described for $Ca_v1.2$ channels (for a review see Zamponi et al., 2015).

So far specific blockers for $Ca_v3.x$ channels, which are relatively resistant to $Ca_v1.2$ channel antagonists, do not exist. Only mibefradil is somewhat selective for $Ca_v3.x$ versus $Ca_v1.2$ channels (3- to 5-fold), while the scorpion venom peptide kurtoxin inhibits the activation gating of $Ca_V3.x$ channel.

2.2. The flavonoid scaffold as a template for the design of cardiovascular $Ca_v1.2$ channels modulators

The first evidence of flavonoid effects on $Ca_v1.2$ channels dated back to 1996 (Cataldi et al., 1996), when genistein and daidzein were employed to evaluate channel regulation by protein tyrosine kinase (PTK) in pituitary GH3 cells (Table 1). Since then, quercetin became the most popular and studied among flavonoids, and many papers describing its activity on $Ca_v1.2$ channels have been published in the new millennium. Summanen et al. (2001) were the first to demonstrate that quercetin increases $Ca_v1.2$ current in clonal rat pituitary GH4C1 cells, possibly *via* cAMP-induced activation of PKA. During the ensuing 15-year period, several other groups provided direct electrophysiological evidence, in various cell types, that quercetin is a stimulator of $Ca_v1.2$ channels [e.g., Huang et al. (2009) obtained similar results in smooth muscle cells of the guinea-pig proximal colon and Wu et al. (2003) in GH3 cells]. On the contrary, to our knowledge there is only one work (Hou et al., 2014) demonstrating that $Ca_v1.2$ channel block by quercetin (IC_{50} value of about 10 μM) along with Kv channel stimulation accounts, at least partially, for its spasmolytic effects in rat coronary artery preparations. As both channels play a fundamental role in the physiology of coronary arteries (Berwick et al., 2012), these findings might help to explain quercetin protective effect on heart against ischemia-reperfusion injury (Bartekova et al., 2010; Jin et al., 2012).

In vascular smooth muscle cells, stimulation of $Ca_v1.2$ channels is rather specific, since quercetin does not affect the co-expressed $Ca_v3.1$ channels (Table 2; Saponara et al., 2002), tightly in line with the data reported in INS-1 pancreatic β cell lines (Bardy et al.,

2013), where quercetin likely binds to a site distinct from that of the $Ca_v1.2$ channel stimulator Bay K 8644. Furthermore, quercetin seems to exert a direct effect on the channel protein without involving any of the main, endogenous modulators of the current (namely PKA, PKG, PKC or tyrosine kinase; Mugnai, 2012; Saponara et al., 2011).

Quercetin's stimulation of $Ca_v1.2$ current contradicts its well-known vasodilatory effect (Fusi et al., 2003), as it would be expected to cause muscle contraction. Effectively, following quercetin challenge increases in rat portal vein basal tone (Chiwororo and Ojewole, 2010) and 24 mM KCl-induced contraction of porcine coronary artery (Suri et al., 2010) were reported. However, in rat aorta, the comparison between the electrophysiological and mechanical effects of quercetin points to vasorelaxation as the phenomenon more relevant and hierarchically prevailing over the increase in Ca^{2+} influx that follows $Ca_v1.2$ current stimulation (Fusi et al., 2003). In other words, $Ca_v1.2$ channel stimulation by quercetin determines an influx of extracellular Ca^{2+} that is not sufficient to overcome its myorelaxing activity. This hypothesis is corroborated by the observation that maximal activation of $Ca_v1.2$ channels by Bay K 8644 (i.e. larger influx of extracellular Ca^{2+} as compared to that measured with quercetin alone) indeed prevails over quercetin-induced myorelaxation, thus causing smooth muscle contraction.

Another intriguing aspect of quercetin-induced modulation of $Ca_v1.2$ channel became apparent when the functional interaction between this flavonoid and Bay K 8644 were analysed (Saponara et al., 2008). Pre-incubation of vascular myocytes with concentrations of quercetin *per se* ineffective as $Ca_v1.2$ channel stimulator (100 nM) significantly inhibited the maximal response, and prevented the hyperpolarizing shift of the steady-state inactivation curve as well as the stimulation of tail current intensity evoked by Bay K 8644. These findings led to the conclusion that nutritionally meaningful concentrations of

quercetin (see below section 4) may limit the responsiveness of vascular $Ca_v1.2$ channels to pharmacological stimulations, like that operated by Bay K 8644. To this regard, it is worth mentioning that quercetin, at variance with Bay K 8644 (Fusi et al., 2003), does not displace (+)-[3H]PN-200–110 binding to porcine aortic membranes (Frosini et al., unpublished observation), thus suggesting that quercetin binds a distinct site from that of dihydropyridines. This hypothesis is further supported by the molecular modelling data summarised in section 2.3. It was hypothesized that flavonoid binding causes a negative allosteric modulation, leading to altered plasticity of the channel protein rather than reduction in Bay K 8644 affinity, and tuning down the intrinsic efficacy of the Bay K 8644-receptor complex. The question whether this may have physiological consequences presently has no answer, since endogenous $Ca_v1.2$ channel agonists are so far unknown. However, it is important to underline that Bay K 8644 as well as dihydropyridine Ca^{2+} channel antagonists share the same high-affinity binding site at the α_{1C} subunit pore region of $Ca_v1.2$ channels (IIIS5-IIIS6 and IVS5-IVS6 domains; Yamaguchi et al., 2003). Whether dietary quercetin can modulate the activity of some organic Ca^{2+} channel blockers used in the clinic to treat cardiovascular diseases is currently under investigation at our laboratory.

Quercetin is a polyphenolic molecule endowed with potent radical scavenging and antioxidant properties (Sgaragli et al., 1993). Several reports suggest that vascular ion channels are under control of multiple redox-linked mechanisms (Wolin, 2000), thus raising the hypothesis that significant regional and/or tissue differences in redox-sensitive modulatory properties of cardiovascular $Ca_v1.2$ channels (Campbell et al., 1996). Indeed rutin, the glycosylated analogue of quercetin, is an effective antioxidant as well as a radical scavenger (Rice-Evans et al., 1996); however, it does not affect vascular $Ca_v1.2$ current (Fusi et al., 2003). It therefore appears that quercetin does not modulate $Ca_v1.2$ current in

virtue of its antioxidant activity, once more supporting the view of its direct effect on the channel protein.

Quercetin-induced vasorelaxation displays an endothelium-dependent component, particularly at low concentrations (Fusi et al., 2003). This activity was ascribed to the stimulation of vascular endothelial $Ca_v1.2$ channels, giving rise to the increase of cytoplasmic Ca^{2+} concentration that, in turn, stimulates NO synthase activity (Kubota et al., 2001).

Though quercetin is essentially a vasorelaxing agent, its effects on $Ca_v1.2$ channels are of particular interest, since only few ligands activate these channels. So far, $Ca_v1.2$ channel agonists (such as Bay K 8644) represent valuable probes to prove the role of these channels in cell signalling and investigate channel molecular pharmacology and physiology (Tikhonov and Zhorov, 2009). However, they are not relevant to clinical investigation since they cannot discriminate the four channel isoforms. Furthermore, these agonists activate both brain and cardiovascular $Ca_v1.2$ channels *in vivo*, thus causing toxic effects such as cardiac arrhythmias, hypertension, as well as a severe neurobehavioral dystonic syndrome (Zamponi et al., 2015). Nonetheless, short-term administration of low doses of a $Ca_v1.2$ channel agonist in controlled clinical settings, such as the ischemic insult of brain (Li et al., 2007), could provide some therapeutic benefit. In fact, ischemia-induced inhibition of neuronal $Ca_v1.2$ current, likely mediated by oxidative stress, gives rise to delayed death of hippocampal CA1 pyramidal neurons. Conversely, activation of the same current at a late stage of reperfusion, increases the expression of a group of genes required for neuronal survival. Therefore, if quercetin turns out to activate $Ca_v1.2$ channels also in CA1 pyramidal neurons, it would be of interest to assess its efficacy in reducing brain damage after stroke.

All the electrophysiological features characterising the effect of quercetin on $Ca_v1.2$ channel are also present in myricetin, a flavonoid structurally related to quercetin (Fusi et al., 2005). Myricetin, in fact, acts on $Ca_v1.2$, but not on $Ca_v3.1$ channels. Moreover, it stabilizes the channel in the inactivated state (with an apparent dissociation constant about 5 times lower than that for the resting channel) in a voltage-dependent manner, alters the voltage sensitivity of the inactivation mechanism, and also modifies channel gating kinetics, by slowing the transition from the closed to the open state. Contrary to quercetin, however, $Ca_v1.2$ channel stimulation by myricetin leads to contraction of the vascular musculature, with efficacy similar to that of Bay K 8644 (Fusi et al., 2003). The functional interaction between myricetin and the channel has been defined in its essential lines (Fusi et al., 2005), but does not seem to involve the dihydropyridine receptor. In fact, similarly to quercetin, myricetin has no significant effects on (+)-[^{3}H]PN200-110 binding to porcine aorta membranes, even at concentrations as high as 100 μM. This observation is in line with the molecular modelling data summarised in section 2.3.

Since the specific pharmacological activity of an agent depends on its molecular structure (for a review on the structure-cardiovascular activity relationship of flavonoids, see Mladěnka et al., 2010), a series of 24 flavonoids related to quercetin and myricetin were investigated to define the structural requirements for their effects on $Ca_v1.2$ channels. This study was performed with use of the whole-cell patch-clamp method, in concert with a molecular modelling procedure (Saponara et al., 2011). Both $Ca_v1.2$ current stimulators and inhibitors were found. The key structural requirements for $Ca_v1.2$ current stimulation were the double bond between C2 and C3 and the hydroxylation pattern on the flavonoid scaffold, apigenin expressing the minimal structural requirements. Conversely, the absence of OH groups in B ring played a key role into $Ca_v1.2$ current inhibition. Finally, the

presence of OH groups at 5 and 7 position was important for both stimulators and inhibitors, allowing the interaction with positively charged groups of the $Ca_v1.2$ channel and facilitating the docking of flavonoids. Moreover, the isoflavone genistein behaved as an inhibitor, though possessing OH groups in 5, 7, and 4' position, similarly to the stimulator apigenin. Consequently, the flavonol, but not the isoflavone scaffold, permits the presentation of the compound to the channel in an agonist-like mode. Interestingly, the analysis of the functional interaction between quercetin and either the stimulator myricetin or the inhibitors resokaempferol, chrysin, genistein, and 5,7,2'-trihydroxyflavone, provided evidence of the highest apparent affinity for $Ca_v1.2$ channel of quercetin and led to the hypothesis of a common recognition site for flavonoids. Altogether, these data were the first to point to the vascular $Ca_v1.2$ channel as a novel target for flavonoids structurally related to quercetin. These preliminary *in silico* observations have been recently developed through a novel approach, whose results are summarised in section 2.3.

In our laboratory, two flavonols (kaempferol and galangin), a flavone (chrysin), and an isoflavone (genistein) were recently found to inhibit $Ca_v3.1$ currents in rat tail artery myocytes (Fig. 2). Interestingly, chrysin and galangin antagonised also $Ca_v1.2$ current. Therefore, they might be useful therapeutic agents for the treatment of hypertension, as the classic Ca^{2+} antagonists (e.g., nifedipine) are not capable of blocking $Ca_v3.1$ channels, which play a key role in renal damage associated with this disease (Li and Yang, 2014). Interestingly, a dual $Ca_v1.2$ and $Ca_v3.1$ channel blocker (namely efonidipine) provides broad beneficial effects in the heart, liver, plasma and mitochondria in a mouse model of thalassaemia (Kumfu et al., 2016). Hence, chrysin and galangin may represent an alternative choice for patients unable to follow the current standard therapy based on iron chelators, which are poorly cardio-protective and characterised by serious side-effects.

Alternatively, combined therapy of iron chelators with a dual $Ca_v1.2$ and $Ca_v3.1$ channel blocker may provide additive benefits in preventing cardiac and systemic complications in conditions of iron overload.

Several inhibitors of PTKs have been widely used to dissect modulatory pathways that regulate ion channel functions. PTKs, in fact, specifically phosphorylate tyrosine residues on channel proteins; thus, phosphorylation and de-phosphorylation cycles modify channel activity and hence alter the electrophysiological properties of cell membranes. Among PTK inhibitors, the isoflavone genistein, a major bioactive constituent of soy as well as many other *Leguminosae*, displays marked specificity. In fact, it inhibits PTK by competing for the ATP-binding site (IC_{50} value of 20-111 μM), while it has modest effects on serine/threonine kinases, PKA or PKC (Akiyama et al., 1987). Accordingly, genistein was used in several investigations, essentially to elucidate PTK-mediated modulation of $Ca_v1.2$ as well as other ion channels. Pioneering studies suggest that tyrosine phosphorylation inhibits cardiac (Greiser et al., 2007; Schroder et al. 2004) while stimulating vascular $Ca_v1.2$ channels (Kimura et al., 2000; Liu et al., 1997; Liu and Sperelakis, 1997; Ogata et al., 1997), pointing to indirect, kinase-mediated modulation of the current by genistein. However, a growing body of evidence accumulated over the latest few years, indicates that genistein can modulate $Ca_v1.2$ channel function also *via* PTK-independent mechanisms, including direct interaction with the channel (see Zhao et al., 2008). Hence, genistein should be considered a promiscuous ion channel modulator, and its use as a probe be interpreted with caution. Additionally, to further confound the overall picture, genistein is a phytoestrogen acting at nanomolar concentrations on oestrogen receptors (Escande et al., 2006); these, in turn, can alter ion homeostasis in a variety of tissues and cells, including those of the vascular system (Kelly and Levin, 2001). Finally, evidence of

ion channel modulation by PTKs is also based on the response either to protein tyrosine phosphatase inhibitors, such as the membrane-permeable sodium orthovanadate (Gordon, 1991) or to the structural analogue of genistein, daidzein, which does not inhibit PTK activity. However, orthovanadate is a strong oxidant and, in our experience, it can easily and rapidly oxidize polyphenol derivatives such as quercetin (Fusi et al., unpublished observations) and, reasonably also genistein, thus limiting the power of the conclusions drawn from its use. On the other hand, daidzein is expected to mimic the same effect of genistein where a PTK-independent mechanism is advocated. However, caution is needed, as the structural requirements for genistein activity may be so strict that even the structural analogue daidzein could not fit its role (Teisseyre and Michalak, 2005; Whaley et al., 2006; Zhang and Wang, 2000).

Beyond quercetin and genistein, other flavonoids were investigated for their effects on cardiovascular $Ca_v1.2$ channels. (-)-Epigallocathechin gallate, for example, the most abundant catechin in tea, triggers Ca^{2+} influx into cultured rat aorta smooth muscle cells *via* SKF-96365- and Cd^{2+}-sensitive, Ca^{2+}-permeable, non-selective cation channels, as well as *via* a nifedipine-sensitive channel (Campos-Toimil and Orallo, 2007). The latter effect occurs at resting membrane potential and is enhanced by membrane depolarization. This mechanism may be responsible for the rise of intracellular Ca^{2+} concentration and the ensuing contraction observed in rat aorta preparations under (-)-epigallocathechin gallate challenge. Noticeably, stimulation of $Ca_v1.2$ channel by (-)-epigallocathechin gallate is biphasic, as it shifts time-dependently to an inhibitory effect. This might underpin the endothelium independent, vasorelaxing effect of (-)-epigallocathechin gallate observed in rat aorta (Álvarez et al., 2006) and account for the systolic blood pressure reduction recorded both in spontaneously hypertensive (Potenza et al., 2007) and in normotensive

rats (Sheng et al., 2007) after long-term treatment with (-)-epigallocathechin gallate. On the contrary, another tea catechin, (-)-epicatechin-3-gallate (10 µM) does not affect $Ca_v1.2$ channel current in primary culture of neonatal rat ventricular myocyte (Wu et al., 2013).

A comprehensive functional and electrophysiological analysis of the effects of hesperetin (one of the most common flavonoids in *Citrus*) was performed on rat coronary artery (Liu et al., 2014). Hesperetin relaxes KCl- and U46619-induced contractions, inhibits extracellular Ca^{2+} influx-induced contraction, and reduces intracellular free Ca^{2+} concentration. These effects are due to the blockade of $Ca_v1.2$ channels and to the increase of K_v channel currents, as demonstrated in single, freshly isolated myocytes. Moreover, hesperetin inhibits platelet aggregation (Jin et al., 2007), increases NO production (Liu et al., 2008), suppresses gene expression related to the modulation of vascular tone (Yamamoto et al., 2013b), as well as exerts hypotensive, vasodilatory and anti-inflammatory activities in spontaneously hypertensive rats (Yamamoto et al., 2013a), and inhibits cardiac hERG potassium channel (Scholz et al., 2007) in the same range of concentrations as those achieved in human plasma following oral supplementation (Takumi et al., 2012). Consequently, hesperetin might be beneficial in cardiac ischemic disorders.

The flavonoid kaempferol 3-O-(6″-trans-*p*-coumaroyl)-β-D-glucopyranoside (tiliroside) has shown important biological properties that may concur to reduce the incidence of cardiovascular diseases. These include reduction of low-density lipoprotein oxidation in humans (Schinella et al., 2007), anti-inflammatory, free radical scavenging (Sala et al., 2003) and anti-hyperglycemic activities (Qiao et al., 2011), and amelioration of hyperinsulinemia and hyperlipidemia in obese, diabetic mice (Goto et al., 2012). Using complementary *in vivo* and *in vitro* methods, marked, long-lasting antihypertensive effects

of tiliroside in conscious, DOCA-salt hypertensive rats, as well as the underlying mechanisms thereof have been described (Silva et al., 2013). These are ascribed to the reduction in vascular peripheral resistance, accomplished through the blockade of smooth muscle $Ca_v1.2$ channel. As such, tiliroside represents an interesting model for the development of novel, effective antihypertensive drugs.

Changes in smooth muscle cell membrane potential trigger the activation and define the subsequent inactivation of $Ca_v1.2$ channels. Electrophysiological recordings show that the voltage-dependent activation and inactivation curves show substantial overlap at voltages in close proximity to the resting membrane potential. This reveals the existence of a background Ca^{2+} influx (the so called “window current”) that serves not only myogenic tone maintenance (Zhang et al., 2007) but also tonic contraction regulation of vascular smooth muscle (Fransen et al., 2012). To this regard, it is remarkable that the chalcone, precursor of flavonoids, cardamonin, beyond inhibiting $Ca_v1.2$ and stimulating $K_{Ca}1.1$ channel currents, causes a striking decrease of the window current by leftward shifting both the activation and, more significantly, the inactivation curve (Fusi et al., 2010). Therefore, in *in vivo* conditions, cardamonin might contribute to reduction of steady-state resting vascular tone.

Ca_v channels, and in particular $Ca_v1.2$ channels, are essential for several biological functions and hence key drug targets in the treatment of many disorders, including those of the cardiovascular system. Since compelling evidence points to the cardiovascular $Ca_v1.2$ channel as a novel target for flavonoids, it is not surprising that these natural molecules have surged as protective agents potentially beneficial to human health. Furthermore, flavonoids can be viewed as valuable tools to investigate the novel physiological role of $Ca_v1.2$ channels emerging, for example, in the brain, where they

seem to be involved in Parkinson's disease and other neuropsychiatric disorders (Striessnig et al., 2015). Finally, once proved to be channel isoform-selective, flavonoids could offer novel prospective therapeutic approaches to target $Ca_v\alpha_1$ splice isoforms that are uniquely expressed in a cell- and tissue-specific manner.

Last but not least, Ca_v channels open and close in response to changes in membrane potential, which is finely regulated by K^+ channel activity. Therefore, flavonoid modulation of K^+ channels might concur, though indirectly, to the modulation of Ca_v channel function. Indeed several reports from this as well as other laboratories (e.g. Iozzi et al., 2013; Liu et al., 2014; Saponara et al., 2006 and many others) support this hypothesis that, however, goes beyond the scope of the present review.

2.3. Moving towards the alleged vascular $Ca_v1.2$ channel binding site for flavonoids: an in silico approach

Computational models and simulations are increasingly used to predict and explain the binding of drugs to receptors as well as to complement and, no doubt, expedite the discovery of new leads or drugs from natural products. To further investigate the mechanism of flavonoids stimulation or inhibition of $Ca_v1.2$ currents (see above; Saponara et al., 2011), an *in silico* approach, using a homology 3D model of the $Ca_v1.2$ channel pore domain, has been developed (Saponara et al., 2016). Docking was simulated by means of AutoDock/Vina v.1.1.2 (Trott et al., 2010) using a flexible side chains protocol. Ligand structures were retrieved from the PubChem database in sdf format (Kim et al., 2015) while the pdbqt files for ligands and $Ca_v1.2$ channel α_{1C} subunit were generated using

scripts included in the Autodock/Vina v.1.1.2 tools. The box (size 29Å x 26Å x 18Å) for docking simulation included whole active sites of the protein and residues that might be significant for the interaction with ligands.

The 3D structure of rat $Ca_v1.2$ channel α_{1C} subunit revealed that the binding pocket is located in the region including domain III, segment 5-pore loop-segment 6 and domain IV, pore loop-segment 6 (Senatore et al., 2011) in agreement with a recent crystallographic and functional study of drug binding to the bacterial homotetrameric model Ca_V channel Ca_VAb (Tang et al., 2016). Eighteen flavonoids were selected out of the 24 previously investigated (Saponara et al., 2011) owing to their clear-cut stimulatory or inhibitory activity. The respective, calculated free energies of binding (ΔG_b), summarized in Table 3, were considerably high and comparable to those of the $Ca_v1.2$ channel blockers nifedipine, verapamil, and diltiazem or the agonist (S)-(-)-Bay K 8644, varying within a 1.6 kcal/mol difference window. Values were comparable for both inhibitors and stimulators, which positioned in the same pocket of rat $Ca_v1.2$ channel α_{1C} subunit (Fig. 3A). However, all the inhibitors converged to the same binding site sharing an overlapping pose (Fig. 3B,D), while stimulators converged to another binding site, very close to that associated to the inhibitors (Fig. 3C,E). Nifedipine, verapamil, diltiazem, and (S)-(-)-Bay K 8644, although positioning in the same binding region, involved different sensing residues, as shown in Table 3 and Fig. 3A. This might be due to the different chemical-physical properties of the molecules, flavonoids being characterised by a rigid (see below) and dihydropyridines, benzothiazepines and phenylalkylamines by a flexible molecular structure.

In a subsequent series of simulations, an in-depth analysis of network of interactions between flavonoids and ligand-sensing residues was performed. For each compound, the

best pose with the lowest thermodynamic energy, belonging to the top ranked conformations, underwent additional post-docking analysis. While hydrophobic interactions, hydrogen bonds, and π–π stacking of the compounds are reported in Table 3, overall network of interactions for inhibitors and stimulators is highlighted in Fig. 4. Data show that all inhibitors formed a hydrogen bond with the amino-group of Asn-770 residue while 8 out of 11 (except for 5-hydroxyflavone, chrysin and baicalein) with the hydroxyl group of Tyr-1489 residue (Table 3 and Fig. 4A). As both residues, which belong to the secondary structure of segments IIIS5 and IVS6, respectively, stabilize the channel pore in the closed state (Tikhonov et al., 2009), it is likely that these two hydrogen bonds contribute to the inhibitory activity of the flavonoids. Furthermore, the distance and the angle of these hydrogen bonds (Fig. 4A) are hallmarks of a strong interaction (Jeffrey et al., 1997), allowing flavonoids positioning inside the pocket for a longer time-period, thus favouring the inhibitory effect.

While π–π stacking reasonably involved the flavonoid backbone (specifically A and B rings) and the aromatic rings of Phe-1143 and Phe-1191 residues (Fig. 4B), the hydrophobic interactions took place between the hydrophobic portion of the inhibitor and the hydrophobic-sensing Leu-774, Phe-1143, Met-1188, Phe-1191, and Phe-1495 residues (Fig. 4C). Finally, the oxygen of the carbonylic group in the C ring may impair Ca^{2+} flow through the pore, thus giving rise to current inhibition. The same seems to happen with the nitrile nitrogen of the Ca^{2+} channel blocker devapamil (Cheng et al., 2009).

Interesting pieces of evidence arose from the analysis of the buried surface area (B.S.A.) values of the residues involved in the pore formation. According to the selectivity filter model, permeation of Ca^{2+} through the pore correlates to the degree of metal hydration

that, in turn, is affected by various parameters such as size, rigidity, and solvent accessibility to the pore (Dudev et al., 2014). The latter can be calculated indirectly *via* B.S.A. evaluation. As shown in Fig. 5, posing flavonoid inhibitors into the pocket gave rise to very high B.S.A. values for the pore sensing residues Phe-1191, Leu-774, and Phe-1143. Conversely, B.S.A. values of the same pore residues were equal to 0 when calculated in the presence of the flavonoid stimulators. Therefore, network of interactions with the ligand-sensing residues located inside the binding pocket likely underlie the flavonoid inhibitory activity, promoting the closed state of the channel along with the reduction of solvent accessibility inside the pore.

The analysis of network of interactions between each of the 8 flavonoid stimulators and the ligand-sensing residues of their binding pocket unveiled only polar interactions. The hydroxyl groups of the flavonoids formed hydrogen bonds with those of Ser-1141, Tyr-1178, and Tyr-1489 residues (Fig. 4D), which are part of segment IIIP, IIIS6, and IVS6, respectively. These hydrogen bonds, characterized by such distance and angle values to support a strong interaction (Jeffrey et al., 1997), are known to facilitate the open state of the channel (Tikhonov et al., 2009). In addition, luteloin, myricetin, quercetin, and tamarixetin formed a hydrogen bond between the 3’ hydroxyl group and Thr-1066 residue; this should increase their stability in the binding site, thus promoting the open state of the channel. Finally, sensing-residue interaction analysis has indicated Tyr-1489 residue as a common thread to both inhibitors and stimulators. This residue, in fact, turned out to be crucial for docking of all flavonoids, possibly owing to its peculiar localization at the borderline between the two binding sites.

Though the 18 flavonoids possess very similar structure as well as comparable chemical-physical properties, the computational analysis has identified substantial differences

crucial to confer the molecule either inhibitory or stimulatory activity. Additionally, flavonoids showed variable orientation of the B ring, stimulators being characterized by non-planar geometry (Fig. 6A,B), while inhibitors by a planar one (Fig. 6C,D). This different molecular geometry, caused by the occurrence of intramolecular hydrogen bonds in the inhibitor structure (Hunyadia et al., 2013), is supported by 3D crystal structure of some proteins. In the crystal structure 5AUW (Yokoyama et al., 2015) of the Protein Data Bank, in fact, the stimulator quercetin exhibits non-planar geometry when complexing death-associated protein kinase 1. On the contrary, in the crystal structure 4X2A (Zhang et al,. 2016), the inhibitor baicalein complexes glyoxalase showing planar geometry. These differences in geometry are likely fundamental in determining the flavonoid capability to stimulate or inhibit $Ca_v1.2$ current. The non-planar feature of B ring, in fact, might impede the approach of stimulators deeply inside the pore, whereas planar geometry should facilitate it. Both stimulators and inhibitors, however, may approach their binding site through the same lateral opening of $Ca_v1.2$ channel α_{1C} subunit, as previously hypothesized (Zhorov et al., 2004).

The afore-mentioned analysis and the computational data obtained so far are summarised in Fig. 7 showing the 2D map of sensing residues that interact with flavonoids, either inhibitors or stimulators. The hydroxyl groups positioned in 3', 4' and 5' contributed to the stimulatory activity. Furthermore, those in 4' and 5' increase the polarity of the molecule and steric hindrance of B ring. The hydroxyl group in 3' position is key for the formation of a hydrogen bond with Thr-1066 residue, while those in 5, 6, and 7 position contribute to the formation of hydrogen bonds with Tyr-1178, Ser-1141, and Tyr-1489, respectively. For flavonoid inhibitors, the hydroxyl groups in 4' and 5' position seem to be important for the formation of hydrogen bonds with Tyr-1489 residue, the former being particularly strong

due to the close vicinity of the two groups (Jeffrey et al., 1997). Hydroxyl groups in 3 and 5 position along with the oxygen of the carbonylic group may promote Ca^{2+} coordination (Cheng et al., 2009). Finally, the polar hydroxyl group in 6 position is detrimental as it prevents the hydrophobic bond formation with Leu-774 residue. Conversely, that in 7 position turned out to be important for the formation of a hydrogen bond with the amino group of Asn-770 residue.

The present, novel insights into the molecular interactions of flavonoids with the vascular $Ca_V1.2$ channel contribute to the understanding of the different and sometimes opposite pharmacological activity of such otherwise structurally similar dietary components. Furthermore, these data may be useful to increase flavonoid efficacy or even represent a starting point for the design of novel leads or drugs useful to therapy of human cardiovascular diseases.

3. Membrane interaction of flavonoids

Flavonoids are amphipathic molecules bearing both hydrophilic (hydroxyl groups) and hydrophobic (aromatic rings) structural features. Furthermore, complexation of transition metal cations increases flavonoid lipophilicity, thus favouring their ability to interact with, incorporate and distribute into biological membranes (Selvaraj et al., 2015), particularly with compartments known as lipid rafts (Tarahovsky et al., 2014). This, in turn, may change the lipid bilayer biophysical properties, likely affecting the function of embedded proteins, such as ion channels (Ingolfsson et al., 2014). Although this is an interesting hypothesis, the concentrations of flavonoids and the lipid composition of the membrane

models used to investigate the nature and extent of flavonoid-membrane interactions were so different that diverse and sometimes contrasting results were produced. Furthermore, only a few data have been obtained in *in vivo*-like conditions.

Specific binding sites for flavonoids have not been identified so far. However, these compounds are endowed of extraordinarily diverse activities on the same target protein (see as examples Naylor et al., 2016 and Saponara et al., 2011) that a unifying hypothesis, such as that of membrane perturbation, cannot account for their actions. Finally, the flavonoid scaffold has evolved into chemical entities characterised by several active groups that allow their posing into protein cavities much bigger than the compounds, thus facilitating their binding (Ji et al., 2009).

4. The issue of bioavailability of flavonoids

Though the daily flavonoid consumption is considerable (from 20 mg to 1 g/day, depending on the different flavonoid subclass considered; Bondonno et al., 2015), there is a general consensus that the poor systemic bioavailability of these molecules, along with instability, oxidative degradation, and metabolic transformation, affect their activity, thus limiting their use as drugs or nutraceuticals. This unfavourable characteristic is closely related to the molecular structure (molecular weight, glycosylation and esterification playing a pivotal role), which influences the interaction with food matrices, digestive enzymes, intestinal transporters and blood proteins at various stages of the digestion, absorption and distribution processes (Gonzales et al., 2015). Also environmental, food processing as well as host (species- and gender-dependent differences in metabolism; Dai et al., 2015)

related factors contribute to the limited and highly variable bioavailability of flavonoids (Croft, 2016; Guo and Bruno, 2015). Therefore, a better understanding of the structure-metabolism relationships may identify the crucial structural features that are not subjected to metabolism thus retaining essential biological activities (Jiang and Hu, 2012).

As flavonoids are prone to extensive metabolism (forming O-methylated, glucoronidated and sulfated conjugates), their metabolites, alone or in combination, are likely the principal actors playing pharmacological effects. The mechanisms of action exerted by some flavonoid metabolites in different experimental models of cardiovascular diseases have been recently reviewed by Rodriguez-Mateos et al. (2014). Flavonoid metabolites that appear in the circulatory system at nM concentrations are, among others, O-methylated. To evaluate the *in vitro* effects on vascular smooth muscle ion channels of physiological concentrations of flavonoids, a mixture of quercetin metabolites was assessed at our laboratory on rat tail artery myocyte $Ca_v1.2$ channel current. Individually, kaempferol, isorhamnetin, and tamarixetin caused a negligible decrease of the peak current (Fig. 8A). However, when used in combination, they significantly decreased the current-voltage relationship in a concentration-dependent manner (Fig. 8B), suggesting an additive effect that may have important consequences on blood pressure when occurring *in vivo*. Inhibition seemed to plateau at 1 µM concentration. At higher concentrations, in fact, these flavonoids were weak (tamarixetin) or potent (kaempferol and isorhamnetin) stimulators of the $Ca_v1.2$ channel (see Saponara et al., 2011), thus displaying a hormetic effect. Furthermore, the three metabolites, either separately or in combination, did not affect the steady-state inactivation curve (Fusi et al., unpublished observations), suggesting that they did not stabilise the inactivated state of the channel.

Conjugative metabolism often determines a partial loss of or even hinders flavonoid bioefficacy. Though the literature is not always consistent, some of these metabolites may retain activities either directly or after being converted back to the parent flavonoid. *In vivo*, in fact, flavonoid metabolites can act as temporary reservoirs from which the more active, parent species may be regenerated through the activity of enzymes, such as glucuronidases (Menendez et al. 2011).

While investigation of these aspects continues, several efforts have been devoted to identify novel strategies to prevent and/or bypass metabolism and ameliorate bioavailability of flavonoids. Premise of full exploitation of their health benefit in disease prevention and therapy should be: i) use of absorption enhancers (e.g., ethanol for quercetin; Dragoni et al., 2006); ii) novel delivery systems (e.g. nanodevices; Gormaz et al., 2015; Guo and Bruno, 2015; Leonarduzzi et al., 2010); iii) synthesis of flavonoid pro-drugs (Biasutto and Zoratti, 2014; Hu et al., 2016; Kim et al., 2010); iv) change the site of absorption (Grande et al., 2016; Russo et al., 2012; Thilakarathna and Vasantha Rupasinghe, 2013).

5. The issue of antioxidant activity of flavonoids

Flavonoids have long been acknowledged for their remarkable *in vitro* antioxidant activity. Given the role of oxidation in a number of chronic diseases (in particular heart disease, ischemic stroke, cancer, type II diabetes, and neurodegenerative diseases; Del Rio et al., 2013), it is reasonable to assume that antioxidant activity might explain the link between dietary flavonoids and disease prevention. However, it has been recently argued that *in*

vivo scavenging of radicals is irrelevant with dietary antioxidant defense; conversely, regulation of defence and repair systems along with the maintenance of a nucleophilic tone (i.e. protection against electrophiles, including radicals and oxidants) is consistent with good health (Forman et al., 2014).

Various mechanisms underlie the antioxidant effect of flavonoids: (1) transition metal ions chelation (especially iron and/or copper); (2) radical scavenging activity (Treml and Smejkal, 2016); (3) chain-breaking antioxidant activity; (4) sparing effects on other antioxidants; (5) inhibition of reactive oxygen species-forming enzymes (xanthine oxidase, lipoxygenase, and NADPH oxidase); (6) protection or induction of enzyme and non-enzyme endogenous antioxidants (reduced glutathione (GSH), GSH peroxidase, GSH reductase, GSH S-transferase, superoxide dismutase, and catalase; Akhlaghi and Bandy, 2009); and (7) modulation of an array of mitochondrial processes (De Oliveira et al., 2016). The structural requirements necessary to the radical scavenging activity of flavonoids have been ascribed to the presence of a catechol group in the B ring, a double bond between carbon 2 and 3 of the C ring conjugated with the keto group at position 4, and a hydroxyl group in the position 3 of ring C or in the carbon 5 of the A ring (e.g. quercetin; León-González et al., 2015). However, the overall antioxidant activity is affected by the environment considered (hydrophilic or lipophilic) and the type of radical/oxidative stress inducers employed.

To our knowledge, studies correlating the flavonoid antioxidant activity to the modulation of ion channels have not been published so far. This is somewhat surprising, as considerable pieces of evidence indicate that the function of cardiovascular ion channels in general, and of $Ca_v1.2$ channels in particular, can be altered by changes of cellular redox state (Lee and Griendling, 2008). At our laboratory, flavonoids with very similar antioxidant activity (Rice-

Evans et al., 1996) proved to be either stimulators, inhibitors, or indeed inactive on vascular $Ca_v1.2$ current (Saponara et al., 2011). It therefore appears that the effects of flavonoids on $Ca_v1.2$ current are independent of their antioxidant capability (see also the couple quercetin/rutin, as discussed above).

Finally, one should bear in mind that, at high concentrations, high pH or in the presence of redox-active transition metals, flavonoids can act also as pro-oxidants (León-González et al., 2015). Under these conditions they give rise, directly or through the induction of enzymes such as NADPH oxidase as well as the reduction of transition metal ions involved in redox-cycling, to free radical species. The extent to which this pro-oxidant function may be related to their ion channel modulatory activity deserves further investigation (Margină et al., 2015; Procházková et al., 2011).

6. Conclusion and future perspectives: flavonoids, what else?

This review provides insight into the molecular mechanisms underlying flavonoid-induced modulation of cardiovascular Ca_v channels, which represent effective therapeutic targets for treatment of cardiovascular diseases, the world-wide recognized leading cause of death. Considering the overall decrease of the number of new drugs introduced into the clinical practice (essentially due to the limited success of high-throughput screening of compound libraries), flavonoids can be considered novel, fruitful therapeutic agents, or at least a great source of drug leads providing protection against cardiovascular diseases. However, more efforts are needed to elucidate the structural determinants responsible for their effects on and the binding site (if any) at the channel protein. To develop novel and

specific therapeutic agents, it is necessary to enlarge the number of flavonoids assessed on ion channels beyond quercetin and genistein as well as increase clinical data on their use. Furthermore, the growing awareness of how flavonoids act at molecular and cellular level raises concerns and questions. These include pharmacokinetic issues [are flavonoids tested *in vitro* the same molecules acting at target sites? In other words, can we consider unimportant their metabolism in human body? Is the hormetic effect (Spagnuolo et al., 2012) relevant to their safety?], large scale production issues [can microbial synthesis/synthetic cell factories (Wu et al., 2014) overcome the drawback inherent to the extraction and purification from plants or full chemical synthesis?], and toxicological issues (can the antioxidant quercetin when oxidized to quercetin-quinone arylate protein thiols, thus being toxic?). Solving these concerns is necessary to design reliable flavonoid-derived molecules that are able to counteract cardiovascular diseases.

Finally, further areas of research need to be investigated to answer the following questions.

1. May flavonoids prevent dysfunction of ion channel regulatory proteins? Disorders originating from altered ion channel synthesis, trafficking and targeting were recently defined. Thus, ion channel function is dependent not only on channel biophysical properties but also on channel regulatory proteins (Curran and Mohler, 2015). As previous findings indicate that channel blockers may rescue trafficking-defective channels (Tan et al., 2006), it is mandatory to assess whether flavonoids may be of value in this challenging field.

2. Does metabolism affect flavonoid activity at ion channels? Shall we take into consideration flavonoid metabolites rather than the parent compound? Can selectivity,

bioavailability and tissue distribution of active principles be improved (see as an example Dong et al., 2011)? Addressing the un-metabolised flavonoid to the target either by chemical modification of its structure or by nanoparticle vehiculation is a strategy worth to be pursued to improve its effectiveness.

3. Beyond those centred on the cardiovascular system, might other diseases benefit from the use of flavonoids? One example is cancer, wherein ion channels are associated with various cell functions (Kunzelmann, 2005): some channels, for example, are widely expressed in tumour while barely detectable in normal cells.

In conclusion, the progress occurring in the field of flavonoid pharmacodynamics and pharmacokinetics should drive a new wave of discovery of agents, targeting ion channels, endowed with selectivity, efficacy and safety. These studies are warranted to fully understand whether flavonoids will generate successful, new entries in the medical arsenal or have to be relegated for much longer time to the category of promising drugs. Finally, many ion channels, discovered with extensive gene characterization efforts, have poor or no pharmacology at all, as the ability to define novel pharmacological agents has not been as much prompt (Minor, 2009). Indeed, flavonoids raise the possibility to develop novel pharmacological tools that control channel activity, thus helping investigators to make connections between a particular ion channel gene and its exact biological function.

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References

Akhlaghi, M., Bandy, B., 2009. Mechanisms of flavonoid protection against myocardial ischemia-reperfusion injury. J. Mol. Cell. Cardiol. 46, 309-317.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y., 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. 262, 5592-5595.

Almeida Rezende, BA., Pereira, AC., Cortes, SF., Lemos, VS., 2016. Vascular effects of flavonoids. Curr. Med. Chem. 23, 87-102.

Álvarez, E., Campos-Toimil, M., Justiniano-Basaran, H., Lugnier, C., Orallo, F., 2006. Study of the mechanisms involved in the vasorelaxation induced by (−)-epigallocatechin-3-gallate in rat aorta. Br. J. Pharmacol. 147, 269-280.

Ashcroft, FM., 2006. From molecule to malady. Nature 440, 440-447.

Bannister, JP., Adebiyi, A., Zhao, G., Narayanan, D., Thomas, CM., Feng, JY., Jaggar, JH., 2009. Smooth muscle cell alpha$_2$delta$_1$ subunits are essential for vasoregulation by $Ca_V1.2$ channels. Circ. Res. 105, 948-955. Partial retraction in: 2014. Circ. Res. 115, e10.

Bardy, G., Virsolvy, A., Quignard, JF., Ravier, MA., Bertrand, G., Dalle, S., Cros, G., Magous, R., Richard, S., Oiry, C., 2013. Quercetin induces insulin secretion by direct activation of L-type calcium channels in pancreatic beta cells. Br. J. Pharmacol. 169, 1102-1113.

Barteková, M., Carnická, S., Pancza, D., Ondrejcáková, M., Breier, A., Ravingerová, T., 2010. Acute treatment with polyphenol quercetin improves postischemic recovery of isolated perfused rat hearts after global ischemia. Can. J. Physiol. Pharmacol. 88, 465-471.

Beech, D.J., Muraki, K., Flemming, R., 2004. Non-selective cationic channels of smooth muscle and the mammalian homologues of Drosophila TRP. J. Physiol. 559, 685-706. Erratum in: 2004. J. Physiol., 560, 949.

Berwick, Z.C., Moberly, S.P., Kohr, M.C., Morrical, E.B., Kurian, M.M., Dick, G.M., Tune, J.D., 2012. Contribution of voltage-dependent K^+ and Ca^{2+} channels to coronary pressure-flow autoregulation. Basic Res. Cardiol. 107, 264.

Biasutto, L., Zoratti, M., 2014. Prodrugs of quercetin and resveratrol: a strategy under development. Curr. Drug Metab. 15, 77-95.

Bondonno, C.P., Croft, K.D., Ward, N., Considine, M.J., Hodgson, J.M., 2015. Dietary flavonoids and nitrate: effects on nitric oxide and vascular function. Nutr. Rev. 73, 216-235.

Campbell, D.L., Stamler, J.S., Strauss, H.C., 1996. Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and S-nitrosothiols. J. Gen. Physiol. 108, 277-293.

Campos-Toimil, M., Orallo, F., 2007. Effects of (−)-epigallocatechin-3-gallate in Ca^{2+}-permeable non-selective cation channels and voltage-operated Ca^{2+} channels in vascular smooth muscle cells. Life Sci. 80, 2147-2153.

Cataldi, M., Taglialatela, M., Guerriero, S., Amoroso, S., Lombardi, G., di Renzo, G., Annunziato, L., 1996. Protein-tyrosine kinases activate while protein-tyrosine

phosphatases inhibit L-type calcium channel activity in pituitary GH3 cells. J. Biol. Chem. 271, 9441-9446.

Catterall, W.A., Perez-Reyes, E., Snutch, T.P., Striessnig, J., 2005. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol. Rev. 57, 411-425.

Catterall, W.A., 2011. Voltage-gated calcium channels. Cold Spring Harb. Perspect. Biol. 3, a003947.

Catterall, W.A., Perez-Reyes, E., Snutch, T.P., Striessnig, J., Last modified on 22/01/2015. Voltage-gated calcium channels, introduction. IUPHAR/BPS Guide to PHARMACOLOGY, http://www.guidetopharmacology.org/GRAC/FamilyIntroductionForward?familyId=80 (accessed 23.08.16).

Catterall, W.A., Swanson, T.M., 2015. Structural basis for pharmacology of voltage-gated sodium and calcium channels. Mol. Pharmacol. 88, 141-150.

Chain, A.S., Sturkenboom, M.C., Danhof, M., Della Pasqua, O.E., 2013. Establishing in vitro to clinical correlations in the evaluation of cardiovascular safety pharmacology. Drug Discov. Today Technol. 10, e373-83.

Cheng, R.C.K., Tikhonov, D.B., Zhorov, B.S., 2009. Structural model for phenylalkylamine binding to L-type calcium channels. J. Biol. Chem. 284, 28332-28342.

Chirumbolo, S., 2012. Role of quercetin in vascular physiology. Can. J. Physiol. Pharmacol. 90, 1652-1657.

Chiwororo, W.D., Ojewole, J.A., 2010. Dual effect of quercetin on rat isolated portal vein smooth muscle contractility. Cardiovasc. J. Afr. 21, 132-136.

Choi, J.H., Kim, K.J., Kim, S., 2016. Comparative effect of quercetin and quercetin-3-O-β-d-glucoside on fibrin polymers, blood clots, and in rodent models. J. Biochem. Mol. Toxicol. in press. doi: 10.1002/jbt.21822

Clare, J.J., 2010. Targeting ion channels for drug discovery. Discov. Med. 9, 253-260.

Croft, K.D., 2016. Dietary polyphenols: Antioxidants or not? Arch. Biochem. Biophys. 595, 120-124.

Curran, J., Mohler, P.J., 2015. Alternative paradigms for ion channelopathies: disorders of ion channel membrane trafficking and posttranslational modification. Annu. Rev. Physiol. 77, 505-524.

Dai, P., Luo, F., Wang, Y., Jiang, H., Wang, L., Zhang, G., Zhu, L., Hu, M., Wang, X., Lu, L., Liu, Z., 2015. Species- and gender-dependent differences in the glucuronidation of a flavonoid glucoside and its aglycone determined using expressed UGT enzymes and microsomes. Biopharm. Drug Dispos. 36, 622-635.

de Oliveira, M.R., Nabavi, S.M., Braidy, N., Setzer, W.N., Ahmed, T., Nabavi, S.F., 2016. Quercetin and the mitochondria: a mechanistic view. Biotechnol. Adv. 34, 532-549.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J.P., Tognolini, M., Borges, G., Crozier, A., 2013. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid. Redox Signal. 18, 1818-1892.

Dong, X., Wang, Y., Liu, T., Wu, P., Gao, J., Xu, J., Yang, B., Hu, Y., 2011. Flavonoids as vasorelaxant agents: synthesis, biological evaluation and quantitative structure activities relationship (QSAR) studies. Molecules 16, 8257-8272.

Dragoni, S., Gee, J., Bennett, R., Valoti, M., Sgaragli, S., 2006. Red wine alcohol promotes quercetin absorption and directs its metabolism towards isorhamnetin and tamarixetin in rat intestine in vitro. Br. J. Pharmacol. 147, 765-771.

Dudev, T., Lim, C., 2014. Ion selectivity strategies of sodium channel selectivity filters. Acc. Chem. Res. 47, 3580-3587.

Dunlop, J., Bowlby, M., Peri, R., Vasilyev, D., Arias, R., 2008. High-throughput electrophysiology: an emerging paradigm for ion-channel screening and physiology. Nat. Rev. Drug Discov. 7, 358-368.

Escande, A., Pillon, A., Servant, N., Cravedi, JP., Larrea, F., Muhn, P., Nicolas, J.C., Cavaillès, V., Balaguer, P., 2006. Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. Biochem. Pharmacol. 71, 1459-1469.

EuroFir, 2009. European Food Information Resource Network Food Composition Databank. http://www.eurofir.net/index.asp?id¼1 (accessed 23.08.16).

Forman, H.J., Davies, K.J., Ursini, F., 2014. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. Free Radic. Biol. Med. 66, 24-35.

Fransen, P., Van Hove, C.E., van Langen, J., Schrijvers, D.M., Martinet, W., De Meyer, G.R., Bult, H., 2012. Contribution of transient and sustained calcium influx, and

sensitization to depolarization-induced contractions of the intact mouse aorta. BMC Physiol. 12, 9.

Fusi, F., Saponara, S., Pessina, F., Gorelli, B., Sgaragli, G., 2003. Effects of quercetin and rutin on vascular preparations: a comparison between mechanical and electrophysiological phenomena. Eur. J. Nutr. 42, 10-17.

Fusi, F., Sgaragli, G., Saponara, S., 2005. Mechanism of myricetin stimulation of vascular L-type Ca^{2+} current. J. Pharmacol. Exp. Ther. 313, 790-797.

Fusi, F., Cavalli, M., Mulholland, D., Crouch, N., Coombes, P., Dawson, G., Bova, S., Sgaragli, G., Saponara, S., 2010. Cardamonin is a bifunctional vasodilator that inhibits Ca(v)1.2 current and stimulates K(Ca)1.1 current in rat tail artery myocytes. J. Pharmacol. Exp. Ther. 332, 531-540.

Fusi, F., Durante, M., Spiga, O., Trezza, A., Frosini, M., Floriddia, E., Teodori, E., Dei, S., Saponara, S., 2016. *In vitro* and *in silico* analysis of the vascular effects of asymmetrical *N*,*N*-bis(alkanol)amine aryl esters, novel multidrug resistance-reverting agents. Naunyn Schmiedebergs Arch. Pharmacol. 389, 1033-1043.

Fusi, F., Manetti, F., Durante, M., Sgaragli, G., Saponara, S., 2016. The vasodilator papaverine stimulates L-type Ca^{2+} current in rat tail artery myocytes *via* a PKA-dependent mechanism. Vascul. Pharmacol. 76, 53-61.

Geleijnse, J.M., Hollman, P.Ch., 2008. Flavonoids and cardiovascular health: which compounds, what mechanisms? Am. J. Clin. Nutr. 88, 12-13.

Gonzales, G.B., Smagghe, G., Grootaert, C., Zotti, M., Raes, K., Van Camp, J., 2015. Flavonoid interactions during digestion, absorption, distribution and metabolism: a

sequential structure-activity/property relationship-based approach in the study of bioavailability and bioactivity. Drug Metab. Rev. 47, 175-190.

Gordon, J.A., 1991. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Methods Enzymol. 201, 477-482.

Gormaz, J.G., Quintremil, S., Rodrigo, R., 2015. Cardiovascular disease: a target for the pharmacological effects of quercetin. Curr. Top. Med. Chem. 15, 1735-1742.

Goto, T., Teraminamia, A., Lee, J.Y., Ohyamaa, K., Funakoshia, K., Kim, Y.I., Hirai, S., Uemura, T., Yu, R., Takahashi, N., Kawada, T., 2012. Tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via activation of adiponectin signaling followed by enhancement of fatty acid oxidation in liver and skeletal muscle in obese-diabetic mice. J. Nutr. Biochem. 23, 768-776.

Grande, F., Parisi, O.I., Mordocco, R.A., Rocca, C., Puoci, F., Scrivano, L., Quintieri, A.M., Cantafio, P., Ferla, S., Brancale, A., Saturnino, C., Cerra, M.C., Sinicropi, M.S., Angelone, T., 2016. Quercetin derivatives as novel antihypertensive agents: synthesis and physiological characterization. Eur. J. Pharm. Sci. 82, 161-170.

Greiser, M., Halaszovich, C.R., Frechen, D., Boknik, P., Ravens, U., Dobrev, D., Lückhoff, A., Schotten, U., 2007. Pharmacological evidence for altered src kinase regulation of I(Ca,L) in patients with chronic atrial fibrillation. Naunyn Schmiedebergs Arch. Pharmacol. 375, 383-392.

Guo, Y., Bruno, R.S., 2015. Endogenous and exogenous mediators of quercetin bioavailability. J. Nutr. Biochem. 26, 201-210.

Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B., Kromhout, D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342, 1007-1011.

Hille, B., 2001. Ion channels of excitable membranes, third ed., Sinauer Associates, Inc., Sunderland, MA.

Hollman, P.C.H., Cassidy, A., Comte, B., Heinonen, M., Richelle, M., Richling, E., Serafini, M., Scalbert, A., Sies, H., Vidry, S., 2011. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. J. Nutr. 141, 989S-1009S.

Hou, X., Liu, Y., Niu, L., Cui, L., Zhang, M., 2014. Enhancement of voltage-gated K^+ channels and depression of voltage-gated Ca^{2+} channels are involved in quercetin-induced vasorelaxation in rat coronary artery. Planta Med. 80, 465-472.

Hu, JN., Zou, XG., He, Y., Chen, F., Deng, ZY., 2016. Esterification of quercetin increases its transport across human Caco-2 cells. J. Food Sci. 81, H1825-H1832.

Huang, W.F., Ouyang, S., Li, S.Y., Lin, Y.F., Ouyang, H., Zhang, H., Lu, CJ., 2009. Effect of quercetin on colon contractility and L-type Ca(2+) channels in colon smooth muscle of guinea-pig. Sheng Li Xue Bao 61, 567-576.

Hunyadi, A., Martins, A., Danko, B., Chuang, D.-W., Trouillas, P., Chang, F.-R., Wu, Y.-C., Falkay, G., 2013. Discovery of the first non-planar flavonoid that can strongly inhibit xanthine oxidase: protoapigenone 1′-*O*-propargyl ether. Tetrahedron Lett. 54, 6529-6532.

Ingólfsson, H.I., Thakur, P., Herold, K.F., Hobart, E.A., Ramsey, N.B., Periole, X., de Jong, D.H., Zwama, M., Yilmaz, D., Hall, K., Maretzky, T., Hemmings, H.C. Jr, Blobel, C., Marrink, S.J., Koçer, A., Sack, J.T., Andersen, O.S., 2014. Phytochemicals perturb membranes and promiscuously alter protein function. ACS Chem. Biol. 9, 1788-1798.

Iozzi, D., Schubert, R., Kalenchuk, V.U., Neri, A., Sgaragli, G., Fusi, F., Saponara, S., 2013. Quercetin relaxes rat tail main artery partly via a PKG-mediated stimulation of $K_{Ca}1.1$ channels. Acta Physiol. 208, 329-339.

Ivey, K.L., Hodgson, J.M., Croft, K.D., Lewis, J.R., Prince, R.L., 2015. Flavonoid intake and all-cause mortality. Am. J. Clin. Nutr. 101, 1012-1020.

Jeffrey, G.A., 1997. An introduction to hydrogen bonding. Oxford University Press.

Ji, H.F., Li, X.J., Zhang, H.Y., 2009. Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? EMBO Rep. 10, 194-200.

Jiang, W., Hu, M., 2012. Mutual interactions between flavonoids and enzymatic and transporter elements responsible for flavonoid disposition via phase II metabolic pathways. RSC Adv. 2, 7948-7963.

Jin, Y.R., Han, X.H., Zhang, Y.H., Lee, J.J., Lim, Y., Chung, J.H., Yun, Y.P., 2007. Antiplatelet activity of hesperetin, a bioflavonoid, is mainly mediated by inhibition of PLC-gamma2 phosphorylation and cyclooxygenase-1 activity. Atherosclerosis 194, 144-152.

Jin, J.Y., Park, S.H., Bae, J.H., Cho, H.C., Lim, J.G., Park, W.S., Han, J., Lee, J.H., Song, D.K., 2007. Uncoupling by (-)-epigallocatechin-3-gallate of ATP-sensitive potassium channels from phosphatidylinositol polyphosphates and ATP. Pharmacol. Res. 56, 237-247.

Jin, H.B., Yang, Y.B., Song, Y.L., Zhang, Y.C., Li, Y.R., 2012. Protective roles of quercetin in acute myocardial ischemia and reperfusion injury in rats. Mol. Biol. Rep. 39, 11005-11009.

Kay, C.D., Hooper, L., Kroon, P.A., Rimm, E.B., Cassidy, A., 2012. Relative impact of flavonoid composition, dose and structure on vascular function: a systematic review of randomised controlled trials of flavonoid-rich food products. Mol. Nutr. Food Res. 56, 1605-1616.

Kelly, M.J., Levin, E.R., 2001. Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol. Metab. 12, 152-156.

Kim, S., Thiessen, P.A., Bolton, E.E., Chen, J., Fu, G., Gindulyte, A., Han, L., He, J., He, S., Shoemaker, B.A., Wang, J., Yu, B., Zhang, J., Bryant, S.H., 2016. PubChem substance and compound databases. Nucleic Acids Res. 44, D1202-D1213.

Kim, M.K., Park, K.S., Lee, C., Park, H.R., Choo, H., Chong, Y., 2010. Enhanced stability and intracellular accumulation of quercetin by protection of the chemically or metabolically susceptible hydroxyl groups with a pivaloxymethyl (POM) promoiety. J. Med. Chem. 53, 8597-8607.

Kimura, M., Obara, K., Sasase, T., Ishikawa, T., Tanabe, Y., Nakayama, K., 2000. Specific inhibition of stretch-induced increase in L-type calcium channel currents by herbimycin A in canine basilar arterial myocytes. Br. J. Pharmacol. 130, 923-931.

Krissinel, E., Henrick, K., 2007. Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774-797.

Kubota, Y., Tanaka, N., Umegaki, K., Takenaka, H., Mizuno, H., Nakamura, K., Shinozuka, K., Kunitomo, M., 2001. *Ginkgo biloba* extract-induced relaxation of rat aorta is associated with increase in endothelial intracellular calcium level. Life Sci. 69, 2327-2336.

Kumfu, S., Chattipakorn, S.C., Fucharoen, S., Chattipakorn, N., 2016. Dual T-type and L-type calcium channel blocker exerts beneficial effects in attenuating cardiovascular dysfunction in iron-overloaded thalassaemic mice. Exp. Physiol. 101, 521-539.

Kunzelmann, K., 2005. Ion channels and cancer. J. Membr. Biol. 205, 159-173.

Larson, A.J., Symons, J.D., Jalili, T., 2012. Therapeutic potential of quercetin to decrease blood pressure: review of efficacy and mechanisms. Adv. Nutr. 3, 39-46.

Lee, M.Y., Griendling, K.K., 2008. Redox signaling, vascular function, and hypertension. Antioxid. Redox Signal. 10, 1045-1059.

Lee, K.S., Marban, E., Tsien, R.W., 1985. Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. J. Physiol. 364, 395-411.

Lee, B.H., Pyo, M.K., Lee, J.H., Choi, S.H., Shin, T.J., Lee, S.M., Lim, Y., Han, Y.S., Paik, H.D., Cho, S.G., Yun-Choi, H.S., Rhim, H., Nah, S.Y., 2008. Differential regulations of quercetin and its glycosides on ligand-gated ion channels. Biol. Pharm. Bull. 31, 611-617.

Leonarduzzi, G., Testa, G., Sottero, B., Gamba, P., Poli, G., 2010. Design and development of nanovehicle-based delivery systems for preventive or therapeutic supplementation with flavonoids. Curr. Med. Chem. 17, 74-95.

León-González, A.J., Auger, C., Schini-Kerth, V.B., 2015. Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. Biochem. Pharmacol. 98, 371-380.

Li, X.M., Yang, J.M., Hu, D.H., Hou, F.Q., Zhao, M., Zhu, X.H., Wang, Y., Li, J.G., Hu, P., Chen, L., Qin, L.N., Gao, T.M., 2007. Contribution of downregulation of L-type calcium currents to delayed neuronal death in rat hippocampus after global cerebral ischemia and reperfusion. J. Neurosci. 27, 5249-5259.

Li, X., Yang, M.S., 2014. Effects of T-type calcium channel blockers on renal function and aldosterone in patients with hypertension: a systematic review and meta-analysis. PLoS One 9, e109834.

Liao, P., Soong, T.W., 2010. Understanding alternative splicing of $Ca_v1.2$ calcium channels for a new approach towards individualized medicine. J. Biomed. Res. 24, 181-186.

Liao, P., Yu, D., Lu, S., Tang, Z., Liang, M.C., Zeng, S., Lin, W., Soong, T.W., 2004. Smooth muscle-selective alternatively spliced exon generates functional variation in $Ca_v1.2$ calcium channels. J. Biol. Chem. 279, 50329-50335.

Liu, H., Li, K., Sperelakis, N., 1997. Tyrosine kinase inhibitor, genistein, inhibits macroscopic L-type calcium current in rat portal vein smooth muscle cells. Can. J. Physiol. Pharmacol. 75, 1058-1062.

Liu, H., Sperelakis, N., 1997. Tyrosine kinases modulate the activity of single L-type calcium channels in vascular smooth muscle cells from rat portal vein. Can. J. Physiol. Pharmacol. 75, 1063-1068.

Liu, B., Yang, J., Wen, Q., Li, Y., 2008. Isoliquiritigenin, a flavonoid from licorice, relaxes guinea-pig tracheal smooth muscle in vitro and in vivo: role of cGMP/PKG pathway. Eur. J. Pharmacol. 587, 257-266.

Liu, L., Xu, D.M., Cheng, Y.Y., 2008. Distinct effects of naringenin and hesperetin on nitric oxide production from endothelial cells. J. Agric. Food Chem. 56, 824-829.

Liu, Y., Niu, L., Cui, L., Hou, X., Li, J., Zhang, X., Zhang, M., 2014. Hesperetin inhibits rat coronary constriction by inhibiting Ca^{2+} influx and enhancing voltage-gated K^{+} channel currents of the myocytes. Eur. J. Pharmacol. 735, 193-201.

Margină, D., Ilie, M., Grădinaru, D., Androutsopoulos, V.P., Kouretas, D., Tsatsakis, A.M., 2015. Natural products-friends or foes? Toxicol. Lett. 236, 154-167.

Marín, L., Miguélez, E.M., Villar, C.J., Lombó, F., 2015. Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. Biomed. Res. Int. 2015, 905215.

Menendez, C., Dueñas, M., Galindo, P., González-Manzano, S., Jimenez, R., Moreno, L., Zarzuelo, M.J., Rodríguez-Gómez, I., Duarte, J., Santos-Buelga, C., Perez-Vizcaino, F., 2011. Vascular deconjugation of quercetin glucuronide: the flavonoid paradox revealed? Mol. Nutr. Food Res. 55, 1780-1790.

Mladenka, P., Zatloukalová, L., Filipský, T., Hrdina, R., 2010. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. Free Radic. Biol. Med. 49, 963-975.

Morad, M., Soldatov, N., 2005. Calcium channel inactivation: possible role in signal transduction and Ca^{2+} signaling. Cell Calcium 38, 223-231.

Mugnai, P., Academic year 2011-2012. Studio del meccanismo di azione della quercetina sui canali $Ca_V1.2$ in cellule muscolari lisce vascolari. PhD thesis. Università degli Studi di Siena, Italy.

Mugnai, P., Durante, M., Sgaragli, G., Saponara, S., Paliuri, G., Bova, S., Fusi, F., 2014. L-type Ca^{2+} channel current characteristics are preserved in rat tail artery myocytes after one-day storage. Acta Physiol. 211, 334-345.

Najmanová, I., Pourová, J., Vopršalová, M., Pilařová, V., Semecký, V., Nováková, L., Mladěnka, P., 2016. Flavonoid metabolite 3-(3-hydroxyphenyl)propionic acid formed by human microflora decreases arterial blood pressure in rats. Mol. Nutr. Food Res. 60, 981-991.

Naylor, J., Minard, A., Gaunt, H.J., Amer, M.S., Wilson, L.A., Migliore, M., Cheung, S.Y., Rubaiy, H.N., Blythe, N.M., Musialowski, K.E., Ludlow, M.J., Evans, W.D., Green, B.L., Yang, H., You, Y., Li, J., Fishwick, C.W., Muraki, K., Beech, D.J., Bon, R.S., 2016. Natural and synthetic flavonoid modulation of TRPC5 channels. Br. J. Pharmacol. 173, 562-574.

Neher, E., Sakmann, B., 1992. The patch clamp technique. Sci. Am. 266, 44-51.

Neveu, V., Perez-Jiménez, J., Vos, F., Crespy, V., du Chaffaut, L., Mennen, L., Knox, C., Eisner, R., Cruz, J., Wishart, D., Scalbert, A., 2010. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. Database (Oxford) 2010, bap024.

Nicholson, S.K., Tucker, G.A., Brameld, J.M., 2010. Physiological concentrations of dietary polyphenols regulate vascular endothelial cell expression of genes important in cardiovascular health. Br. J. Nutr. 103, 1398-1403.

Ogata, R., Kitamura, K., Ito, Y., Nakano, H., 1997. Inhibitory effects of genistein on ATP-sensitive K^+ channels in rabbit portal vein smooth muscle. Br. J. Pharmacol. 122, 1395-1404.

Olaleye, M.T., Crown, O.O., Akinmoladun, A.C., Akindahunsi, A.A., 2014. Rutin and quercetin show greater efficacy than nifedipin in ameliorating hemodynamic, redox, and metabolite imbalances in sodium chloride-induced hypertensive rats. Hum. Exp. Toxicol. 33, 602-608.

Peterson, J.J., Dwyer, J.T., Jacques, P.F., McCullough, M.L., 2012. Associations between flavonoids and cardiovascular disease incidence or mortality in European and US populations. Nutr. Rev. 70, 491-508.

Phenol-explorer. www.phenol-explorer.eu (accessed 23.08.16).

Potenza, M.A., Marasciulo, F.L., Tarquinio, M., Tiravanti, E., Colantuono, G., Federici, A., Kim, J.A., Quon, M.J., Montagnani, M., 2007. EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. Am. J. Physiol. Endocrinol. Metab. 292, E1378-E1387.

Procházková, D., Boušová, I., Wilhelmová, N., 2011. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82, 513-523.

Qiao, W., Zhao, C., Qin, N., Zhai, H.Y., Duan, H.Q., 2011. Identification of trans-tiliroside as active principle with anti-hyperglycemic, anti-hyperlipidemic and antioxidant effects from *Potentilla chinesis*. J. Ethnopharmacol. 135, 515-521.

Quiñones, M., Miguel, M., Aleixandre, A., 2013. Beneficial effects of polyphenols on cardiovascular disease. Pharmacol. Res. 68, 125-131.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20, 933-956. Erratum in: 1996. Free Radic. Biol. Med. 21, 417.

Rodriguez-Mateos, A., Vauzour, D., Krueger, C.G., Shanmuganayagam, D., Reed, J., Calani, L., Mena, P., Del Rio, D., Crozier, A., 2014. Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. Arch. Toxicol. 88, 1803-1853.

Rodriguez-Mateos, A., Feliciano, R.P., Boeres, A., Weber, T., Dos Santos, C.N., Ventura, M.R., Heiss, C., 2016. Cranberry (poly)phenol metabolites correlate with improvements in vascular function: A double-blind, randomized, controlled, dose-response, crossover study. Mol. Nutr. Food Res. 60, 2130-2140.

Rothwell, J.A., Medina-Remón, A., Pérez-Jiménez, J., Neveu, V., Knaze, V., Slimani, N., Scalbert, A., 2015. Effects of food processing on polyphenol contents: a systematic analysis using Phenol-Explorer data. Mol. Nutr. Food Res. 59, 160-170.

Russo, M., Spagnuolo, C., Tedesco, I., Bilotto, S., Russo, G.L., 2012. The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem. Pharmacol. 83, 6-15.

Rusznyak, S., Szent-Györgyi, A., 1936. Vitamin P: flavonols as vitamins. Nature 138, 27.

Sala, A., Recio, M.C., Schinella, G.R., Máñez, S., Giner, R.M., Cerdá-Nicolás, M., Ríos, J.L., 2003. Assessment of the anti-inflammatory activity and free radical scavenger activity of tiliroside. Eur. J. Pharmacol. 461, 53-61.

Salentin, S., Schreiber, S., Haupt, V.J., Adasme, M.F., Schroeder, M., 2015. PLIP: fully automated protein-ligand interaction profiler. Nucleic Acids Res. 43, W443-W447.

Saponara, S., Sgaragli, G., Fusi, F., 2002. Quercetin as a novel activator of L-type Ca^{2+} channels in rat tail artery smooth muscle cells. Br. J. Pharmacol. 135, 1819-1827.

Saponara, S., Testai, L., Iozzi, D., Martinotti, E., Martelli, A., Chericoni, S., Sgaragli, G., Fusi, F., Calderone, V., 2006. (+/-)-Naringenin as large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel opener in vascular smooth muscle cells. Br. J. Pharmacol. 149, 1013-1021.

Saponara, S., Sgaragli, G., Fusi, F., 2008. Quercetin antagonism of Bay K 8644 effects on rat tail artery L-type Ca^{2+} channels. Eur. J. Pharmacol. 598, 75-80.

Saponara, S., Carosati, E., Mugnai, P., Sgaragli, G., Fusi, F., 2011. The flavonoid scaffold as a template for the design of modulators of the vascular $Ca_v1.2$ channels. Br. J. Pharmacol. 164, 1684-1697.

Saponara, S., Durante, M., Spiga, O., Mugnai, P., Sgaragli, G., Huong, T.T., Khanh, P.N., Son, N.T., Cuong, N.M., Fusi, F., 2016. Murrayafoline A modulation of rat vascular

myocyte $Ca_v1.2$ channel: functional, electrophysiological and molecular docking analysis. Br. J. Pharmacol. 173, 292-304.

Scalbert, A., Brennan, L., Manach, C., Andres-Lacueva, C., Dragsted, L.O., Draper, J., Rappaport, S.M., van der Hooft, J.J., Wishart, D.S., 2014. The food metabolome: a window over dietary exposure. Am. J. Clin. Nutr. 99, 1286-1308.

Schinella, G.R., Tournier, H.A., Máñez, S., de Buschiazzo, P.M., Del Carmen Recio, M., Ríos, J.L., 2007. Tiliroside and gnaphaliin inhibit human low density lipoprotein oxidation. Fitoterapia 78, 1-6.

Scholz, E.P., Zitron, E., Kiesecker, C., Thomas, D., Kathofer, S., Kreuzer, J., Bauer, A., Katus, H.A., Remppis, A., Karle, C.A., Greten, J., 2007. Orange flavonoid hesperetin modulates cardiac hERG potassium channel *via* binding to amino acid F656. Nutr. Metab. Cardiovasc. Dis. 17, 666-675.

Schröder, F., Klein, G., Frank, T., Bastein, M., Indris, S., Karck, M., Drexler, H., Wollert, K.C., 2004. Src family tyrosine kinases inhibit single L-type Ca^{2+} channel activity in human atrial myocytes. J. Mol. Cell. Cardiol. 37, 735-745.

Selvaraj, S., Krishnaswamy, S., Devashya, V., Sethuraman, S., Krishnan, U.M., 2015. Influence of membrane lipid composition on flavonoid-membrane interactions: Implications on their biological activity. Prog. Lipid Res. 58, 1-13.

Senatore, A., Boone, A., Lam, S., Dawson, T.F., Zhorov, B., Spafford, J.D., 2011. Mapping of dihydropyridine binding residues in a less sensitive invertebrate L-type calcium channel ($L_{Cav}1$). Channels (Austin) 5, 173-187.

Serban, M.C., Sahebkar, A., Zanchetti, A., Mikhailidis, D.P., Howard, G., Antal, D., Andrica, F., Ahmed, A., Aronow, W.S., Muntner, P., Lip, G.Y., Graham, I., Wong, N., Rysz, J., Banach, M., Lipid and Blood Pressure Meta-analysis Collaboration (LBPMC) Group, 2016. Effects of quercetin on blood pressure: a systematic review and meta-analysis of randomized controlled trials. J. Am. Heart Assoc. 5, e002713.

Sgaragli. G.P., Valoti, M., Gorelli, B., Fusi, F., Palmi, M., Mantovani, P., 1993. Calcium antagonist and antiperoxidant properties of some hindered phenols. Br. J. Pharmacol. 110, 369-377.

Sheng, R., Gu, Z.L., Xie, M.L., Zhou, W.X., Guo, C.Y., 2007. EGCG inhibits cardiomyocyte apoptosis in pressure overload-induced cardiac hypertrophy and protects cardiomyocytes from oxidative stress in rats. Acta Pharmacol. Sin. 28, 191-201.

Shen, Y., Ward, N.C., Hodgson, J.M., Puddey, I.B., Wang, Y., Zhang, D., Maghzal, G.J., Stocker, R., Croft, K.D., 2013. Dietary quercetin attenuates oxidant-induced endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice fed a high-fat diet: a critical role for hemeoxygenase-1. Free Rad. Biol. Med. 65, 908-915.

Silva, G.C., Pereira, A.C., Rezende, B.A., da Silva, J.P., Cruz, J.S., de Souza Mde, F., Gomes, R.A., Teles, Y.C., Cortes, S.F., Lemos, V.S., 2013. Mechanism of the antihypertensive and vasorelaxant effects of the flavonoid tiliroside in resistance arteries. Planta Med. 79, 1003-1008.

Singh, M., Kaur, M., Silakari, O., 2014. Flavones: an important scaffold for medicinal chemistry. Eur. J. Med. Chem. 84, 206-239.

Spagnuolo, C., Russo, M., Bilotto, S., Tedesco, I., Laratta, B., Russo, G.L., 2012. Dietary polyphenols in cancer prevention: the example of the flavonoid quercetin in leukemia. Ann. N. Y. Acad. Sci. 1259, 95-103.

Striessnig, J., Ortner, N.J., Pinggera, A., 2015. Pharmacology of L-type calcium channels: novel drugs for old targets? Curr. Mol. Pharmacol. 8, 110-122.

Striessnig, J., Grabner, M., Mitterdorfer, J., Hering, S., Sinnegger, M.J., Glossmann, H., 1998. Structural basis of drug binding to L Ca^{2+} channels. Trends Pharmacol. Sci. 19, 108-115.

Striessnig, J., Hoda, J.C., Wappl, E., Koschak, A., 2005. The molecular basis of calcium antagonist drug action - Recent developments, in: Zamponi, G.W. (Eds.), Voltage-gated calcium channels. Landes Biosciences, Austin, pp. 262-280.

Summanen, J., Vuorela, P., Rauha, J.P., Tammela, P., Marjamäki, K., Pasternack, M., Törnquist, K., Vuorela, H., 2001. Effects of simple aromatic compounds and flavonoids on Ca^{2+} fluxes in rat pituitary GH(4)C(1) cells. Eur. J. Pharmacol. 414, 125-133.

Suri, S., Liu, X.H., Rayment, S., Hughes, D.A., Kroon, P.A., Needs, P.W., Taylor, M.A., Tribolo, S., Wilson, V.G., 2010. Quercetin and its major metabolites selectively modulate cyclic GMP-dependent relaxations and associated tolerance in pig isolated coronary artery. Br. J. Pharmacol. 159, 566-575.

Takumi, H., Nakamura, H., Simizu, T., Harada, R., Kometani, T., Nadamoto, T., Mukai, R., Murota, K., Kawai, Y., Terao, J., 2012. Bioavailability of orally administered water-dispersible hesperetin and its effect on peripheral vasodilatation in human subjects:

implication of endothelial functions of plasma conjugated metabolites. Food Funct. 3, 389-398.

Tan, B.H., Valdivia, C.R., Song, C., Makielski, J.C., 2006. Partial expression defect for the SCN5A missense mutation G1406R depends on splice variant background Q1077 and rescue by mexiletine. Am. J. Physiol. Heart Circ. Physiol. 291, H1822-H1828.

Tang, L., Gamal El-Din, T.M., Swanson, T.M., Pryde, D.C., Scheuer, T., Zheng, N., Catterall, W.A., 2016. Structural basis for inhibition of a voltage-gated Ca^{2+} channel by Ca^{2+} antagonist drugs. Nature 537, 117-121.

Tarahovsky, Y.S., Kim, Y.A., Yagolnik, E.A., Muzafarov, E.N., 2014. Flavonoid-membrane interactions: involvement of flavonoid-metal complexes in raft signaling. Biochim. Biophys. Acta 1838, 1235-1246.

Teisseyre, A., Michalak, K., 2005. Genistein inhibits the activity of $K_v1.3$ potassium channels in human T lymphocytes. J. Membr. Biol. 205, 71-79.

Testai, L., 2015. Flavonoids and mitochondrial pharmacology: A new paradigm for cardioprotection. Life Sci. 135, 68-76.

Thilakarathna, S.H., Rupasinghe, H.P., 2013. Flavonoid bioavailability and attempts for bioavailability enhancement. Nutrients 5, 3367-3387.

Tikhonov, D.B., Zhorov, B.S., 2009. Structural model for dihydropyridine binding to L-type calcium channels. J. Biol. Chem. 284, 19006-19017.

Treml, J., Šmejkal, K., 2016. Flavonoids as potent scavengers of hydroxyl radicals. Compr. Rev. Food Sci. Food Saf. 15, 720-738.

Trott, O., Olso, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J. Comput. Chem. 31, 455-461.

Wang, X., Ouyang, Y.Y., Liu, J., Zhao, G., 2014. Flavonoid intake and risk of CVD: a systematic review and meta-analysis of prospective cohort studies. Br. J. Nutr. 111, 1-11.

Welling, A., Ludwig, A., Zimmer, S., Klugbauer, N., Flockerzi, V., Hofmann, F., 1997. Alternatively spliced IS6 segments of the alpha 1_C gene determine the tissue-specific dihydropyridine sensitivity of cardiac and vascular smooth muscle L-type Ca^{2+} channels. Circ. Res. 81, 526-532.

Whaley, W.L., Rummel, J.D., Kastrapeli, N., 2006. Interactions of genistein and related isoflavones with lipid micelles. Langmuir 22, 7175-7184.

Wolin, M.S., 2000. Interactions of oxidants with vascular signaling systems. Arterioscler. Thromb. Vasc. Biol. 20, 1430-1442.

Wu, S.N., Chiang, H.T., Shen, A.Y., Lo, Y.K., 2003. Differential effects of quercetin, a natural polyphenolic flavonoid, on L-type calcium current in pituitary tumor (GH3) cells and neuronal NG108-15 cells. J. Cell. Physiol. 195, 298-308.

Wu, A.Z., Loh, S.H., Cheng, T.H., Lu, H.H., Lin, C.I., 2013. Antiarrhythmic effects of (-)-epicatechin-3-gallate, a novel sodium channel agonist in cultured neonatal rat ventricular myocytes. Biochem. Pharmacol. 85, 69-80.

Wu, J., Du, G., Zhou, J., Chen, J., 2014. Systems metabolic engineering of microorganisms to achieve large-scale production of flavonoid scaffolds. J. Biotechnol. 188, 72-80.

Yamaguchi, S., Zhorov, B.S., Yoshioka, K., Nagao, T., Ichijo, H., Adachi-Akahane, S., 2003. Key roles of Phe1112 and Ser1115 in the pore-forming IIIS5-S6 linker of L-type Ca^{2+} channel alpha1_C subunit ($Ca_V1.2$) in binding of dihydropyridines and action of Ca^{2+} channel agonists. Mol. Pharmacol. 64, 235-248.

Yamamoto, M., Jokura, H., Hashizume, K., Ominami, H., Shibuya, Y., Suzuki, A., Hase, T., Shimotoyodome, A., 2013a. Hesperidin metabolite hesperetin-7-*O*-glucuronide, but not hesperetin-3'-*O*-glucuronide, exerts hypotensive, vasodilatory, and anti-inflammatory activities. Food Funct. 4, 1346-1351.

Yamamoto, M., Jokura, H., Suzuki, A., Hase, T., Shimotoyodome, A., 2013b. Effects of continuous ingestion of hesperidin and glucosyl hesperidin on vascular gene expression in spontaneously hypertensive rats. J. Nutr. Sci. Vitaminol. (Tokyo) 59, 470-473.

Yokoyama, T., Kosaka, Y., Mizuguchi, M., 2015. Structural insight into the interactions between death-associated protein kinase 1 and natural flavonoids. J. Med. Chem. 58, 7400-7408.

Zahradníková, A., Minarovic, I., Zahradník, I., 2007. Competitive and cooperative effects of Bay K8644 on the L-type calcium channel current inhibition by calcium channel antagonists. J. Pharmacol. Exp. Ther. 322, 638-645.

Zamora-Ros, R., Touillaud, M., Rothwell, JA., Romieu, I., Scalbert, A., 2014. Measuring exposure to the polyphenol metabolome in observational epidemiologic studies: current tools and applications and their limits. Am. J. Clin. Nutr. 100, 11-26.

Zamponi, G.W., Striessnig, J., Koschak, A., Dolphin, A.C., 2015. The physiology, pathology, and pharmacology of voltage-gated calcium channels and their future therapeutic potential. Pharmacol. Rev. 67, 821-870.

Zamponi, G.W., 2015. Calcium channel signaling complexes with receptors and channels. Curr. Mol. Pharmacol. 8, 8-11.

Zhang, Z.H., Wang, Q., 2000. Modulation of a cloned human A-type voltage-gated potassium channel ($hK_v1.4$) by the protein tyrosine kinase inhibitor genistein. Pflugers Arch. 440, 784-792.

Zhang, J., Berra-Romani, R., Sinnegger-Brauns, M.J., Striessnig, J., Blaustein, M.P., Matteson, D.R., 2007. Role of $Ca_v1.2$ L-type Ca^{2+} channels in vascular tone: effects of nifedipine and Mg^{2+}. Am. J. Physiol. Heart Circ. Physiol. 292, H415-H425.

Zhang, H., Zhai, J., Zhang, L., Li, C., Zhao, Y., Chen, Y., Li, Q., Hu, X.P., 2016. *In vitro* inhibition of glyoxalase I by flavonoids: new insights from crystallographic analysis. Curr. Top. Med. Chem. 16, 460-466.

Zhao, Z., Liu, B., Zhang, G., Jia, Z., Jia, Q., Geng, X., Zhang, H., 2008. Molecular basis for genistein-induced inhibition of $K_{ir}2.3$ currents. Pflugers Arch. 456, 413-423.

Zhorov, B.S., Tikhonov, D.B., 2004. Potassium, sodium, calcium and glutamate-gated channels: pore architecture and ligand action. J. Neurochem. 88, 782-799.

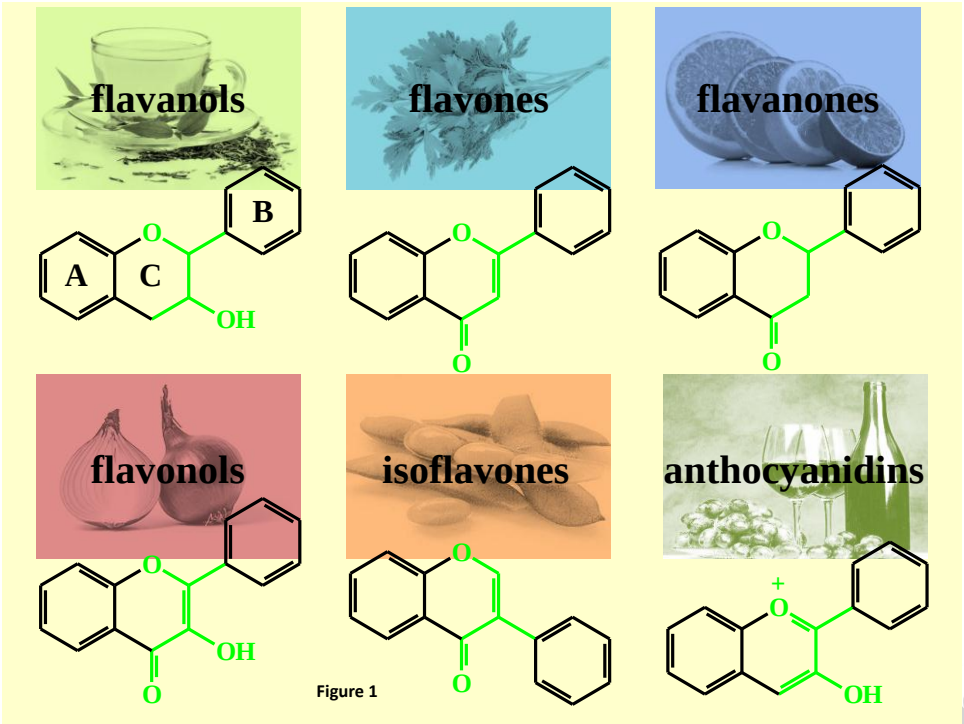


Figure 1

Figure 2

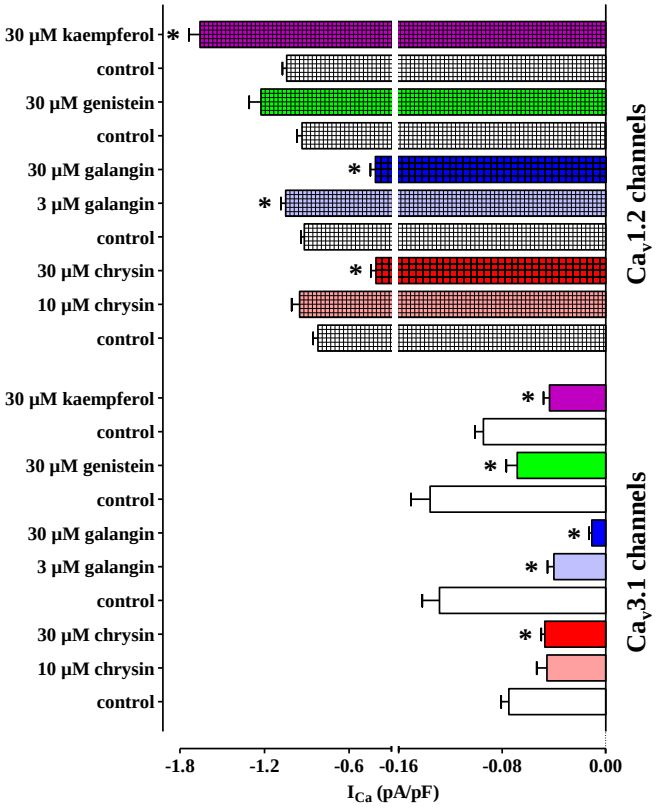
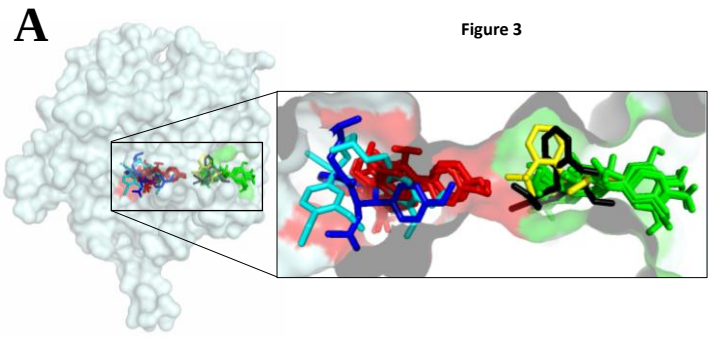


Figure 3



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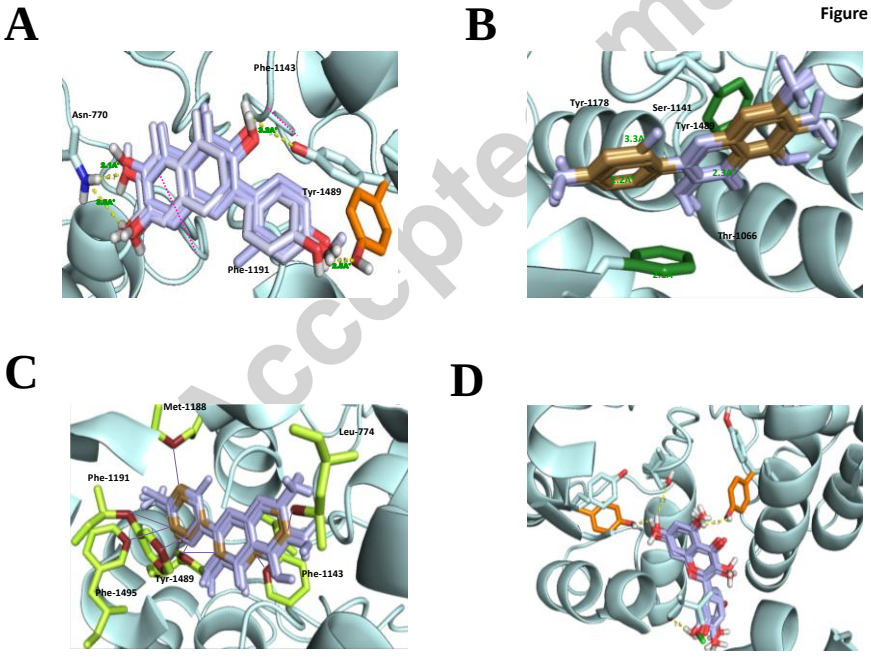

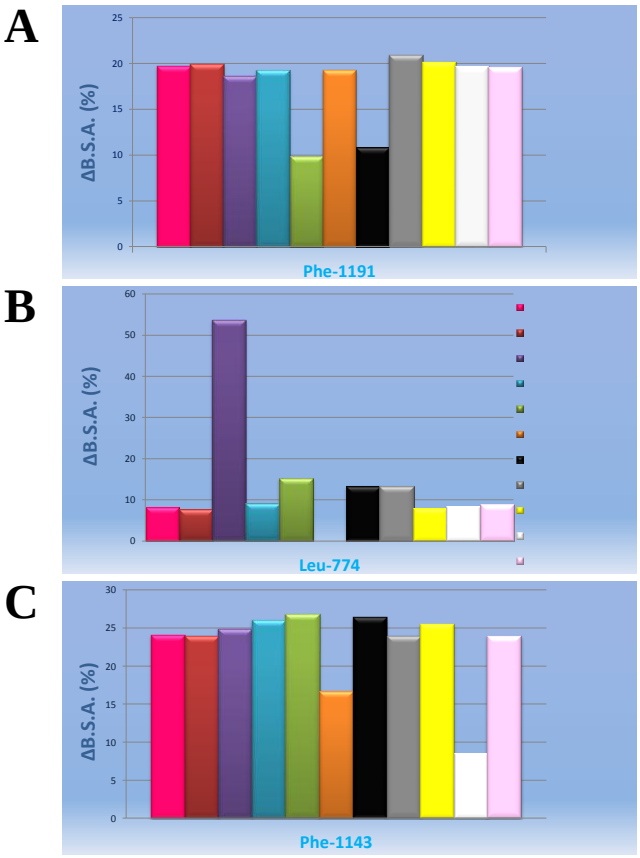

Figure 5

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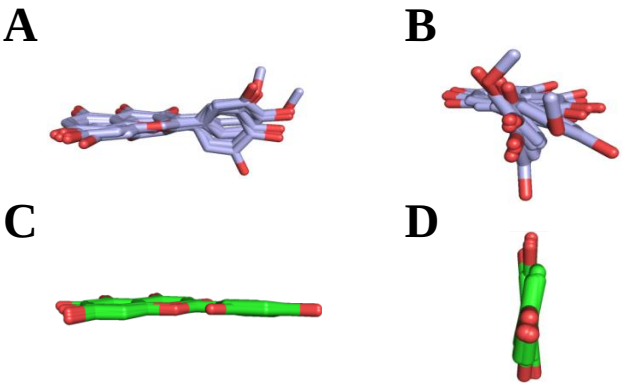
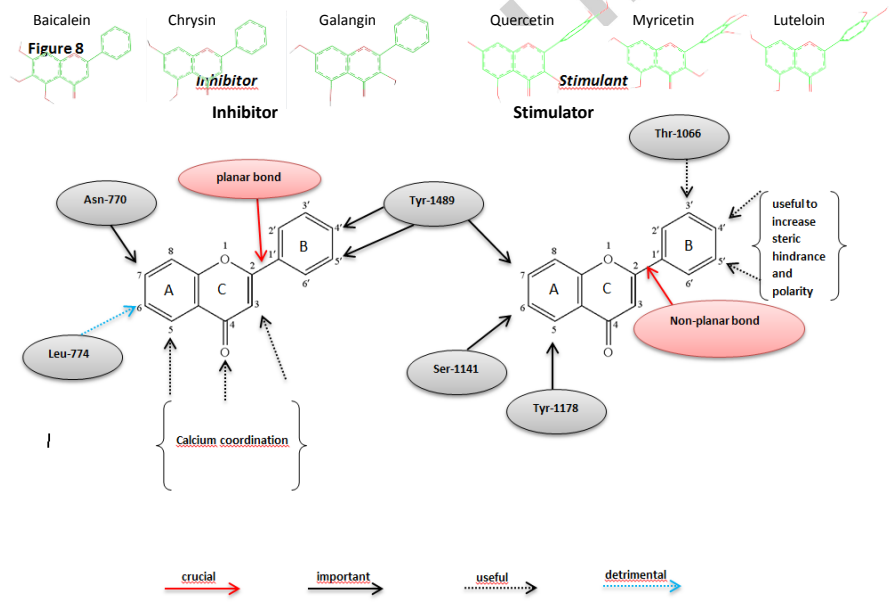


Figure 7



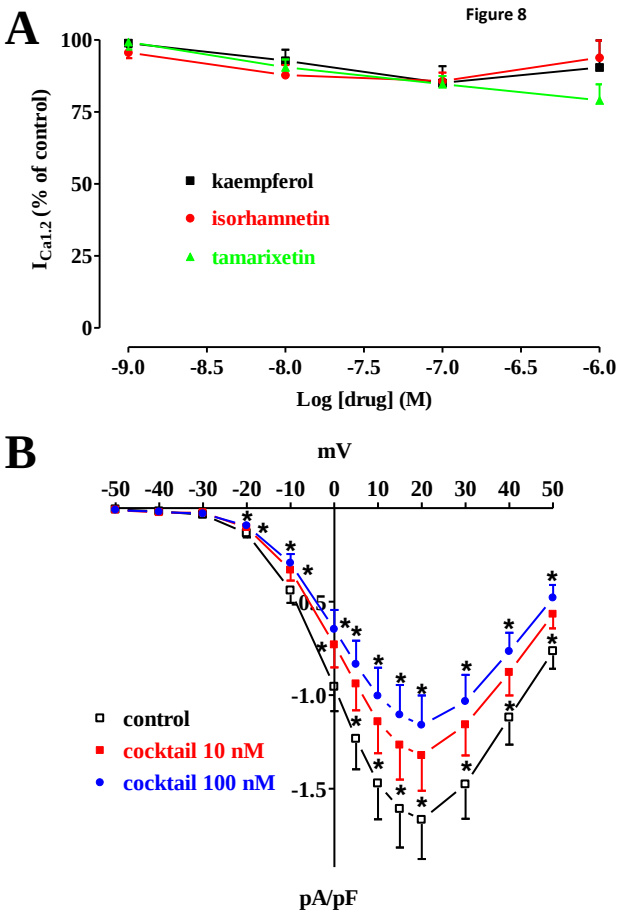


Fig. 1. Flavonoid subclasses, structures and principal food sources. The differences in the skeleton C2-C4 chain are highlighted in green.

Fig. 2. Effects of chrysin, galangin, genistein, or kaempferol on $Ca_v3.1$ and $Ca_v1.2$ currents of rat vascular myocytes. Smooth muscle cells were freshly isolated from the main tail artery (Mugnai et al., 2014). The conventional whole-cell patch-clamp method was employed to voltage-clamp cells, as previously described (Fusi et al., 2016). Recording

electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to obtain a pipette resistance of 2-5 MΩ when filled with internal solution. Data were collected once the current amplitude had been stabilized (usually 7-10 min after the whole-cell configuration had been obtained) by using pClamp 8.2.0.232 (Molecular Devices Corporation, Sunnyvale, CA, USA). Ca^{2+} current, recorded in the presence of 5 mM Ca^{2+} as the charge carrier, was elicited with 250-ms clamp pulses to -40 mV ($Ca_v3.1$, empty columns) and 10 mV ($Ca_v1.2$, cross-hatched columns) from V_h of -90 mV measured in the absence (control) or presence of flavonoids. K^+ currents were blocked with 30 mM tetraethylammonium in the external solution and Cs^+ in the internal solution. Current density is given as pA/pF. Columns are mean ± S.E.M. (n=5-12).

Fig. 3. Docking of flavonoids and classical Ca^{2+} channel modulators at the $Ca_v1.2$ channel α_{1C} subunit. (A) Surface representation of the protein model of the α_{1C} subunit (in light cyan) bearing the binding pocket for flavonoid inhibitors (red) or stimulators (green), as well as classical Ca^{2+} agonist or antagonists [(S)-(-)-Bay K 8644 (yellow), nifedipine (black), diltiazem (blue), and verapamil (cyan sticks)]. (B,C) Model of the $Ca_v1.2$ channel pocket where (B) inhibitors or (C) stimulators preferentially bind. The flavonoid-sensing residues are represented by cyan sticks. (D,E) Poses of (D) inhibitors and (E) stimulators inside the $Ca_v1.2$ channel pocket. Carbon, oxygen, and hydrogen atoms are displayed as green, red, and white sticks, respectively.

Fig. 4. Network of interactions of flavonoid inhibitors and stimulators with the $Ca_v1.2$ channel α_{1C} subunit. (A) Hydrogen bonds (yellow dotted lines) between flavonoid inhibitors

(light blue sticks) and Asn-770 or Tyr-1489 residues (cyan sticks). Hydroxyl groups forming hydrogen bonds are displayed in red (oxygen) and white (hydrogen) sticks. The amino-group of Asn-770 residue is displayed in blue (nitrogen) and white (hydrogen). For ligands with a *p*-hydroxyl group on the B ring, flexible docking simulation revealed orientation change of Tyr-1489 side chain. Green numbers refer to the average distance between donor and acceptor groups engaged in the hydrogen bond. (B) π–π stacking (pink dotted lines) between flavonoid inhibitors backbone and Phe-1143 or Phe-1191 residues. The aromatic rings involved in π–π stacking are shown in sand. Phe-1143 and Phe-1191 residues (cyan sticks) are displayed with a green aromatic ring. (C) Hydrophobic interactions (purple lines) between flavonoid inhibitors and sensing-residues. The flavonoid groups engaged in the hydrophobic bond are displayed in sand, sensing-residues in green, while the groups forming interactions in brown sticks. (D) Hydrogen bonds (yellow dotted lines) between flavonoid stimulators (light blue sticks) and Thr-1066, Ser-1141, Tyr-1178 and Tyr-1489 residues (cyan sticks). Hydroxyl groups forming hydrogen bonds are displayed in red (oxygen) and white (hydrogen) sticks. For all ligands, flexible docking simulation revealed orientation change of Tyr-1178 and Tyr-1489 side chains (orange sticks). Green numbers refer to the average distance between donor and acceptor groups engaged in the hydrogen bond.

Fig. 5. Buried surface area (B.S.A.) of ligand-binding sensing residues. B.S.A. values ($Å^2$) were obtained by means of the internal package of PISA tool (Krissinel et al., 2007) with a probe sphere radius of 1.4 Å (water molecule). ΔB.S.A. values (B.S.A. protein-ligand/B.S.A. protein %) for (A) Phe-1191, (B) Leu-774 and (C) Phe-1143 residues, were calculated in the presence of various flavonoid inhibitors.

Fig. 6. Molecular geometry of flavonoids. The flavonoid backbone is displayed in (A, B) light blue for the stimulators or in (C, D) green sticks for the inhibitors. Oxygen of the hydroxyl groups is colored in red. (A, C) Horizontal view, (B, D) longitudinal view.

Fig. 7. Key elements for flavonoids activity at the $Ca_v1.2$ channel α_{1C} subunit. Outlines of the structure-activity relationship of a flavonoid inhibitor (left) and a flavonoid stimulator (right). Top line: the most representative flavonoid inhibitors (left) and stimulators (right) are depicted. Carbon, oxygen and hydrogen atoms are shown in green, red and grey, respectively. Bottom line: arrows indicate the presence of crucial, important, useful or detrimental hydroxyl groups.

Fig. 8. Effects of quercetin metabolites on $Ca_v1.2$ currents of single rat tail artery myocytes. For a comprehensive method see legend to Fig. 2. (A) Concentration-dependent effect of kaempferol, isorhamnetin, and tamarixetin on conventional whole-cell $Ca_v1.2$ current elicited with 250-ms clamp pulses to 0 mV from a V_h of -50 mV. On the ordinate scale, response is given as percentage of control. Data points are mean ± S.E.M. (n = 4-7). (B) Effect of the combination of the three quercetin metabolites on $Ca_v1.2$ current-voltage relationship, recorded from a V_h of -50 mV, constructed prior to the addition of drug (control) or in the presence of 10 and 100 nM of each drug (cocktail). Data points are mean ± S.E.M. (n = 11). *P<0.05, repeated measures ANOVA and Dunnett post-test.

Table 1 Summary of flavonoids interaction with $Ca_v1.2$ channel.

Flavonoid	Target cell	Activity	References
Apigenin	Rat tail artery myocytes	+	Saponara et al. 2011
Baicalein	Rat tail artery myocytes	-	Saponara et al. 2011
Chrysin	Rat tail artery myocytes	-	Saponara et al. 2011
Daidzein	Rat tail artery myocytes	-	Saponara et al. 2011
	Rabbit portal vein myocytes	0	Liu et al., 1997 Liu and Sperelakis 1997 Ogata et al. 1997
3,4'-Dihydroxyflavone	Rat tail artery myocytes	-	Saponara et al. 2011
(−)-Epigallocatechin-3-gallate	Rat aorta myocytes	+/-	Campos-Toimil and Orallo 2007
Galangin	Rat tail artery myocytes	-	Saponara et al. 2011
Genistein	Pituitary GH3 cells	-	Cataldi et al. 1996
	Human atrial myocytes	+	Greiser et al. 2007

			Schroder et al. 2004
	Rat ventricular myocytes	-	Yokoshiki et al. 1996
			Liu et al., 1997
	Rabbit portal vein myocytes	-	Liu and Sperelakis 1997b
			Ogata et al. 1997
	Canine basilar artery myocytes	-	Kimura et al. 2000
	Rat tail artery myocytes	-	Saponara et al. 2011
Hesperetin	Rat coronary artery myocytes	-	Liu et al. 2014
5-Hydroxyflavone	Rat tail artery myocytes	-	Saponara et al. 2011
Isorhamnetin	Rat tail artery myocytes	+	Saponara et al. 2011
Kaempferol	Rat tail artery myocytes	+	Saponara et al. 2011
Luteloin	Rat tail artery myocytes	+	Saponara et al. 2011
3-Methyl galangin	Rat tail artery myocytes	-	Saponara et al. 2011
Morin	Rat tail artery myocytes	-	Saponara et al. 2011
Myricetin	Rat tail artery myocytes	+	Fusi et al. 2003

			Fusi et al. 2005
			Saponara et al. 2011
(±)-Naringenin	Rat tail artery myocytes	-	Saponara et al. 2011
Naringin	Rat tail artery myocytes	-	Saponara et al. 2011
	Rat pituitary GH4C1 cells	+	Summanen et al. 2001
	Guinea-pig proximal colon myocytes	+	Huang et al. 2009
	Rat pituitary GH3 cells	+	Wu et al. 2003
Quercetin			Fusi et al. 2003
			Mugnai 2012
	Rat tail artery myocytes	+	Saponara et al. 2002
			Saponara et al. 2008
			Saponara et al. 2011
	Vascular endothelial cells	+	Kubota et al. 2001
Resokaempferol	Rat tail artery myocytes	-	Saponara et al. 2011
Rutin	Rat tail artery myocytes	0	Fusi et al. 2003
			Saponara et al. 2011

Scutellarein	Rat tail artery myocytes	-	Saponara et al. 2011
Tamarixetin	Rat tail artery myocytes	0	Saponara et al. 2011
(±)-Taxifolin	Rat tail artery myocytes	0	Saponara et al. 2011
Tiliroside	Rat mesenteric artery myocytes	-	Silva et al. 2013
3,6,4'-Trihydroxyflavone	Rat tail artery myocytes	-	Saponara et al. 2011
5,7,2'-Trihydroxyflavone	Rat tail artery myocytes	-	Saponara et al. 2011

Drug activity on $Ca_v1.2$ channel current: (-) inhibition; (+) stimulation; 0 no effect.

Table 2 Summary of flavonoids interaction with $Ca_v3.1$ channel.

Flavonoid	Target cell	Activity	References
Chrysin	Rat tail artery myocytes	-	This review
Galangin	Rat tail artery myocytes	-	This review
Genistein	Rat tail artery myocytes	-	This review
Kaempferol	Rat tail artery myocytes	-	This review
Myricetin	Rat tail artery myocytes	0	Fusi et al. 2005
Quercetin	Rat tail artery myocytes	0	Saponara et al. 2002
	INS-1 pancreatic β cell	0	Bardy et al. 2013

Drug activity on $Ca_v3.1$ channel current: (-) inhibition; (+) stimulation; 0 no effect.

Table 3 Flavonoids docking at the $Ca_v1.2$ channel α_{1C} subunit.

Ligand	Activity	ΔG_b (kcal/mol)	Hydrophobic interactions	Hydrogen bonds	π–π stacking
Scutellarein	-	-7.3	Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
Morin	-	-7.5	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
5-Hydroxyflavone	-	-7.5	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-	Asn-770	Phe-1143,

			1489, Phe-1495		Phe-1191
3,6,4'-Trihydroxyflavone	-	-7.8	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
(±)-Naringenin	-	-7.5	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
Daidzein	-	-7.8	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
Genistein	-	-7.6	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
Chrysin	-	-7.2	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Asn-770	Phe-1143, Phe-1191
Resokaempferol	-	-7.2	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
Galangin	-	-7.1	Leu-774, Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Tyr-1489, Asn-770	Phe-1143, Phe-1191
Baicalein	-	-6.8	Phe-1143, Met-1188, Phe-1191, Tyr-1489, Phe-1495	Asn-770	Phe-1143, Phe-1191
Nifedipine*	-	-7.9	Phe-1045, Phe-1143, Phe-1191	Tyr-1489	
Verapamil#	-	-6.9	Phe-1143, Ile-1179, and Phe-1191	Tyr-1178, Tyr-1489	Phe-1191
Diltiazem	-	-7.6	Phe-1045, Phe-1143, Phe-1191, Ile-1492		Phe-1143
Myricetin	+	-7.6		Thr-1066, Ser-1141, Tyr-1178, Tyr-1489	
Quercetin	+	-7.4		Thr-1066 Ser-1141, Tyr-1178,	

				Tyr-1489
Isorhamnetin	+	-7.4		Ser-1141, Tyr-1178, Tyr-1489
Luteloin	+	-8.0		Thr-1066, Ser-1141, Tyr-1178, Tyr-1489
Apigenin	+	-8.1		Ser-1141, Tyr-1178, Tyr-1489
Kaempferol	+	-8.4		Ser-1141, Tyr-1178, Tyr-1489
Tamarixetin	+	-7.4		Thr-1066, Ser-1141, Tyr-1178, Tyr-1489
Bay K 8644*	+	-8.0	Leu-1142, Phe-1143, Met-1188, Ile-1486,	Ser-1141, Gln-1069, Tyr-1489

ΔG is the free energy of binding estimated from the top of 20 cluster results and given in kcal/mol. Sensing residues involved in binding interaction were identified according to the best poses of docking solution, calculated by using protein-ligand interaction profiler bioinformatics tool (Salentin et al., 2015). Drug activity on $Ca_v1.2$ channel current: (-) inhibition; (+) stimulation.

* Data are from Saponara et al., 2016. # Data are from Fusi et al., 2016.