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In vitro bioavailability and cellular bioactivity studies of flavonoids and flavonoid-rich plant extracts: questions, considerations and future perspectives

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In vitro techniques are essential in elucidating biochemical mechanisms and for screening a wide range of possible bioactive candidates. The number of papers published reporting *in vitro* bioavailability and bioactivity of flavonoids and flavonoid-rich plant extracts is numerous and still increasing. However, even with the present knowledge on the bioavailability and metabolism of flavonoids after oral ingestion, certain inaccuracies still persist in the literature, such as the use of plant extracts to study bioactivity towards vascular cells. There is therefore a need to revisit, even question, these approaches in terms of their biological relevance. In this review, the bioavailability of flavonoid glycosides, the use of cell models for intestinal absorption and the use of flavonoid aglycones and flavonoid-rich plant extracts in *in vitro* bioactivity studies will be discussed. Here, we focus on the limitations of current *in vitro* systems and revisit the validity of some *in vitro* approaches, and not on the detailed mechanism of flavonoid absorption and bioactivity. Based on the results in the review, there is an apparent need for stricter guidelines on publishing data on *in vitro* data relating to the bioavailability and bioactivity of flavonoids and flavonoid-rich plant extracts.

Flavonoid: Bioavailability: *in vitro*: Bioactivity: Plant extracts

Flavonoids belong to a large group of secondary plant metabolites called polyphenols. They typically consist of a 15-carbon skeleton consisting of two benzene rings attached via a heterocyclic pyrane ring, labelled as rings A, B and C, in a C_6–C_3–C_6 arrangement. Flavonoids occur either as glycosides, methylated derivatives, bio-conjugates after phase I/II metabolism or aglycones (the basic structure). The position of the B ring may be at the C_2-position in the case of most flavonoids or in the C_3-position in the case of isoflavones. Common hydroxylation points are at positions 5, 7 (A ring), 3′, 4′, 5′ (B ring), 3 and 2 (C ring). Differences also depend on the presence of the $C_2 = C_3$ double bond, and C_4-ketone moiety. Fig. 1 shows the basic structures and common classes of flavonoids[1,2]. They are widely available in plant foods, such as *Brassica* vegetables, onions, fruits and its derivatives like wines and juices.

In nature, flavonoids, except for flavan-3-ols, generally occur as glycosides of, most commonly, single units or polymers or hexose, pentose, rhamnose, arabinose and/or their combinations[3]. These glycosidic moieties are mostly attached to free hydroxyl groups in the A and C rings via a β-glycosidic bond. For instance, flavonols, such as quercetin and kaempferol, are more commonly found as 3-*O*-glycosides (although 7-*O*, 3,7-di-*O*, and B-ring glycosidation also occur in some plants), while 7-*O*-glycosides are more common for flavones, flavanones and isoflavones[4]. Flavonoids may also occur as *C*-glycosides, wherein the glycoside moiety is directly attached to the aglycone backbone via an acid-resistant C–C bond. *C*-glycosylation mostly occurs at the C_6 and

Abbreviations: LPH, lactase phloridzin hydrolase; GAE, gallic acid equivalents; SGLT1, sodium-dependent glucose transporter 1.

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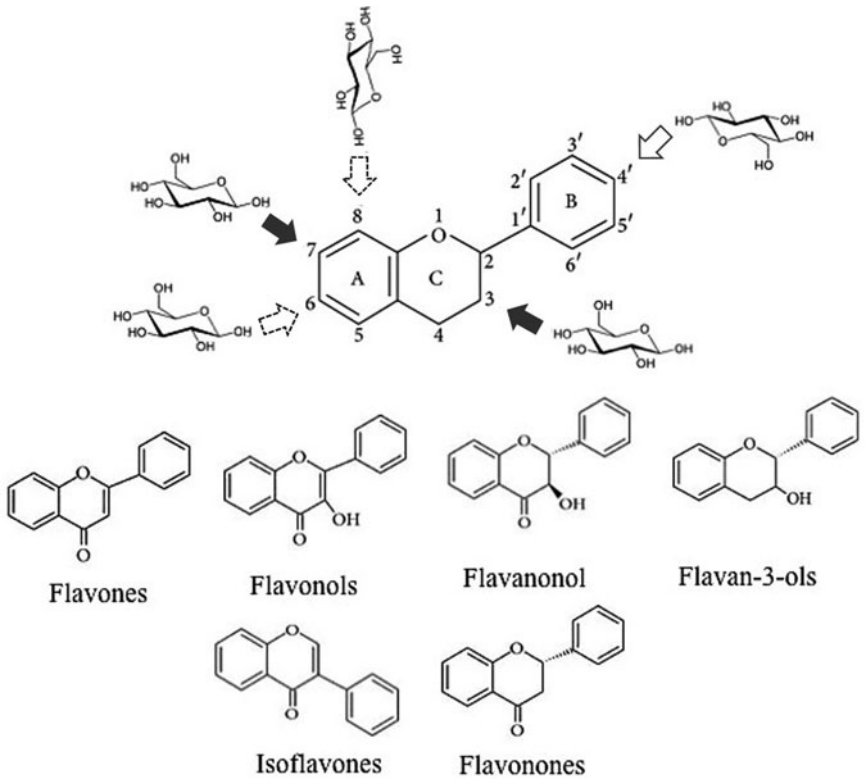

Fig. 1. Basic structure of the common classes of flavonoids and the common points of glycosylation. Common glycosylation points are C_3 and C_7 (black arrows). B ring glycosylation is also observed in some plants (hollow arrow). C-glycosides are least found in plants (arrow with dotted lines).

C_8 positions[3]. Flavonoid-*C*-glycosides however are not as common as their *O*-glycoside counterparts and have received much less attention in the literature[5].

In a survey conducted between 1999 and 2005, adults from fourteen European countries (*n* 30 000) consumed an average of 428 mg flavonoids/d[6], while a study on US adults reported an average consumption of 345 mg/d[7]. For both populations, flavan-3-ols from tea constitute most of the flavonoid intake, followed by flavanones, flavonols and anthocyanins, flavones and isoflavones[6–8]. A survey of the 100 richest sources of polyphenols performed using the online database *Phenol-Explorer*[9] showed that fruit and vegetables, such as berries and artichoke, contain the most polyphenols per serving and are composed of anthocyanins and flavonols[10]. Food in general contains much more flavonoid glycosides compared with their aglycone counterparts[4]. Flavonoid glycosides, especially flavonol and isoflavone glycosides, are also generally stable under cooking conditions[11,12].

Results from epidemiological studies revealed that consumption of flavonoid-rich foods has been associated with a reduced risk of death from CHD[13] and other chronic diseases such as cancer, asthma and diabetes[14]. These results attracted thousands of *in vitro* assays to elucidate the bioactivity of flavonoids, including anti-oxidative[15,16], anti-hypertensive[17], anti-obesity[18,19] anti-viral, hepatoprotective and immune-regulatory activities[20], among many others. This has also sparked the interest of several research groups worldwide to screen for bioactive plants, which could potentially provide these health-promoting effects, based on their flavonoid contents.

Bioavailability of flavonoid glycosides

Although epidemiological studies report health-promoting benefits upon chronic consumption of flavonoid-rich foods, the underlying mechanisms behind these effects are difficult to ascertain because of their poor bioavailability *in vivo*[21] due to factors such as food matrix interactions, food processing, host (human)-related factors (e.g. age, occurrence of certain diseases, lifestyle), and most importantly the bioconversion (microbial, phase I/II metabolism) of flavonoids[22]. Therefore, it is essential to first ensure that flavonoids present in the plant matrices being studied survive the digestion process and are absorbed by the intestine. Once these conditions are met, only then should potential bioactivity towards enzymes, cells and tissues in the body be investigated.

For instance, while earlier studies have demonstrated that quercetin glycosides are found intact in plasma, it is now generally accepted that such compounds are in fact absent from plasma after nutritional doses[23]. Upon reaching the epithelium, flavonoid-*O*-glycosides are hydrolyzed by either lactase phloridzin hydrolase (LPH), cystolic β-glucosidase, or microbial hydrolases

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into aglycones. This deglycosylation step has been found to be a critical step in the absorption and metabolism of flavonoid glycosides from the diet in human subjects[24].

LPH is a brush-border enzyme in small intestinal cells that has a substrate specificity for flavonoid-*O*-β-glucosides[4] to release aglycones, which can then passively diffuse through epithelial cells due to their lipophilicity[25,26]. Dietary flavonoids and isoflavone glycosides are typically hydrolysed by LPH[27]. Alternatively, it has been earlier suggested that cystolic β-glucosidase hydrolyses glycosides intracellularly when they are transported in the intestinal cells by sodium-dependent glucose transporter 1 (SGLT1)[28]. Both LPH and cystolic β-glucosidase are expressed by the Caco-2 cell line and *ex vivo* human small intestinal samples[29]. However, β-glucosidase activity in Caco-2 cells is much lower compared with actual intestinal tissue samples[4,24]. Previously, flavonoid deglycosylation and metabolism has been thought to only occur via microbial metabolism. However, it has been refuted as more and more evidence suggests that flavonoid glycosides can be absorbed in the small intestine, as described earlier. Exemptions to this however are flavonoid rhamnosides, which need to be metabolised by the bacteria due to the inability to metabolise rhamnose[23,30,31].

Once intracellular, phase I and II metabolism converts these aglycones into, commonly, glucuronides, sulphates and methyl-esters, which are then either excreted to the blood or effluxed back to the lumen by ATP-binding cassette transporters. The effluxed metabolites and unabsorbed flavonoids are then passed on to the large intestines, where microbial metabolism occurs that convert them back to aglycones and eventually into smaller phenolic acid metabolites[22]. Blood from the intestines is then directed to the liver, which metabolizes flavonoids that escaped the first-pass metabolism. Therefore, circulating flavonoids are almost exclusively glucuronides > sulphates > methyl-esters (in this order of abundance)[31,32]. These biotransformations along with the role of efflux transporters significantly reduce the bioavailability of flavonoids. In fact, although flavonoid intake reaches >300 mg/d, the concentration of total flavonoid metabolites that reach systemic circulation does not exceed 5 μM after an oral dose challenge[23]. This is very important, as this concentration is mostly insufficient to drive a physiologically relevant bioactivity.

Use of Caco-2 cells as model for intestinal transport of flavonoid glycosides

Human colorectal adenocarcinoma Caco-2 cells have been used in *in vitro* studies as an intestinal model for more than 30 years. The cell line was developed by the Sloan-Kettering Institute for Cancer Research[33]. The use of Caco-2 cells to simulate human intestinal absorption grew as more and more evidence suggested that drug transport in human subjects *in vivo* is highly correlated to the apparent permeability values measured using Caco-2 cells for certain drugs, such as ranitidine HCl, metoprolol tartrate, piroxicam[34], minoxidil[35], naproxen, antipyrine and metoprolol[36]. These drugs however are known to diffuse passively both in Caco-2 cells and human intestinal tissue, thus implying that Caco-2 permeability data are rather correlated to passive diffusion and not for actively transported drugs. Caco-2 cell permeability however was found to be 79- and 27-fold lower for the hydrophilic slowly-passively transported drugs terbutaline and atenolol, respectively. The carrier-mediated transport rates of L-dopa, L-leucine and D-glucose were also much slower in Caco-2 cells than in human jejunum. These data indicate that Caco-2 cells are useful to predict passive intestinal transport of molecules in human intestines, but is not a good model for the transport of hydrophilic and actively transported molecules, such as flavonoid glycosides. This has been attributed to the lack of transporter and enzyme expression in Caco-2 cell lines compared with the real human intestinal epithelium[36]. For instance, the LPH is expressed in Caco-2 cells but its expression is much lower compared with *ex vivo* intestinal samples[24]. Due to this, the metabolism of flavonoid glycosides *in vitro* is usually underestimated. In the previous study, kaempferol glucuronides and sulphates were not found at the basal compartment of a Caco-2 Transwell® set-up upon treatment with a kaempferol glycoside-rich cauliflower leaf extract[37]. *In vivo*, consumption of kaempferol glycoside-rich endive resulted in the appearance of kaempferol glucuronide in plasma[38]. Glucuronides and sulphates of flavonoids were also not observed after transport analysis of Xi-aochaichu-tang (a Chinese herbal remedy) using Caco-2 cells[39]. Conversely, while it is now accepted that quercetin glycosides do not exist in blood, Caco-2 cell transport experiments of quercetin glucosides show basolateral transport of these glycosides[40,41], which could be misinterpreted as being bioavailable. Quercetin metabolites (glucuronides, especially) were not observed when treating Caco-2 cells with quercetin-3-glucoside in contrast to treatment with quercetin aglycones. It therefore appears that pre-deglycosylation during *in vitro* intestinal digestion could enhance the intestinal conversion and metabolic conversion of dietary quercetin glucosides[42].

The mechanism of absorption of flavonoid glycosides in intestinal cells is controversial. Many reports claim that SGLT1 participates in the cellular uptake of quercetin glycosides from the diet. It has been previously shown that flavonoid glycosides can be transported across intestinal cells by the SGLT1 when there is no efficient uptake by passive diffusion[43]. However, contradictory results regarding the involvement of SGLT1 in flavonoid intestinal transport exist and thus require further elucidation. For instance, while it was previously reported that quercetin-3-glucoside is taken up across the intestinal cells by SGLT1 in rat small intestines[44], using SGLT1-expressing *Xenopus laevis* oocytes showed that neither quercetin, luteolin, apigenin, naringenin, pelarginidin, daidzein, genistein, nor any of their glycosylated derivatives are substrates of this transporter[45]. Whether SGLT1 plays a role in flavonoid glycoside uptake or not, it is undoubtedly accepted that deglycosylation exists extracellularly in the intestines and that

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effective deglycosylation is a critical step for flavonoid glycoside absorption. Therefore, the passive diffusion of the liberated aglycone still remains the more efficient route of absorption, indicating that the participation of SGLT1, if present, is less important.

The lack of LPH expression was previously addressed by treating *in vitro* digested shallots and onions with lactase (300 units, 37°C, 20 min) before adding to Caco-2 cells. In this study, quercetin absorption of lactase-treated quercetin glycosides increased 14-fold in Caco-2 cells, indicating that lactase treatment could be a good additional step for *in vitro* intestinal absorption studies[46]. Given the importance of deglycosylation on flavonoid glycoside absorption, the use of Caco-2 cell models for intestinal transport without prior deglycosylation steps should be re-evaluated. Steps for deglycosylation after *in vitro* digestion, i.e. addition of lactase or LPH to the digesta, should be considered prior to Caco-2 transport analysis.

Use of flavonoid aglycones in *in vitro* bioactivity assays

A previous study on the angiotensin-I-converting enzyme inhibitory activity of flavonoids suggested that the IC_{50} (concentration needed to reduce angiotensin-I-converting enzyme activity by 50 %) of flavonoids fall within the range 0·4–9·3 mM[47], which is substantially more than the concentration of flavonoids found in the blood. Moreover, the compounds used in this study were aglycones and not phase I/II metabolites that are normally found in the blood. The use of flavonoid aglycones, without regard to their bioavailability and metabolism has been rampant and still growing rapidly. In 2002, questions on the use of flavonoid aglycones in *in vitro* systems to test bioactivity was raised[31]. In this review, the validity of papers that reported *in vitro* bioactivity of high doses of flavonoids aglycones was questioned. Although it is accurate to say that many studies used aglycones in much higher doses than plasma concentrations, recent evidence on the *in situ* deglucuronidation of flavonoid glucuronides during inflammation suggest that flavonoid aglycones may indeed exist locally in some tissues at concentrations higher than found in the blood[48].

We have also shown previously that cells accumulate flavonoids differently under normal and stressed conditions. Methyl-quercetin was found to accumulate more in valinomycin-stressed cells than in unstressed undifferentiated Caco-2 cells treated with quercetin. More interestingly, quercetin and its methyl-ester derivative were found on the cell membrane of the unstressed cells, whereas they localised intracellularly upon exposure to valinomycin, which caused a recovery in cellular viability and reduction of intracellular reactive oxygen species[49]. These results are interesting as they point to the possibility that local/*in situ* concentrations of flavonoids (in the affected cells) could be much higher than the concentration of the metabolites in the blood. Unfortunately, no comparative study on the local tissue concentration of flavonoids of healthy *v.* unhealthy subjects has been reported. However, a previous study on the effect of quercetin supplementation on the blood pressure of stage I hypertensive men compared with normotensive men reported that quercetin supplementation significantly reduced the blood pressure of hypertensive men that is independent of changes in angiotensin-I-converting enzyme activity, endothelin-I or nitric oxide plasma levels[50]. What is most interesting in the study is that plasma concentrations of normotensive men reached 2·3 (SD 1·8) μM, whereas plasma concentration only reached 0·6 (SD 0·4) μM at 10 h post administration for hypertensive men. It could be possible that the difference in plasma concentrations is caused by local accumulation of quercetin in damaged cells (i.e. vascular cells), which caused the reduction of blood pressure in the hypertensive group.

Given the earlier arguments, it is worth asking whether the dose in *in vitro* bioactivity assays be limited to the concentration of flavonoid metabolites in the blood. If an average person has about 5 litres blood and if indeed deglucuronidation occurs locally, the concentration of flavonoid aglycones at the site of deglucuronidation (i.e. point of inflammation) will be more than the concentration in the blood as a whole. Further, the use of aglycones in *in vitro* assays may in fact be valid for some cases. Unfortunately, it is currently not established whether flavonoids are indeed accumulating selectively in damaged tissues and in what form they are present (aglycones or methyl-conjugates). Thus, studies on the concentration of flavonoids and their corresponding form in localised tissues merits further investigation.

Use of flavonoid-rich plant extracts for bioactivity assays

Even with the growing evidence on the limited (even the absence of) bioavailability of flavonoid glycosides and the present understanding of flavonoid absorption and bioactivity, the number of papers published reporting the *in vitro* bioactivity of flavonoid-rich plant extracts in cellular models is very high and growing.

For instance, a recent study demonstrated that supercritical CO_2 extract of spent hop (*Humulus lupulus* L.), which is dominantly composed of flavanols (procyanidin dimers) and flavonols (quercetin and kaempferol glycosides, including rhamnosides) reduced ADP-induced platelet aggregation, in a concentration-dependent manner when the plant extract was added to both whole blood and platelet-rich plasma of both healthy volunteers and patients with coronary artery disease[51]. In this study however, the plant extracts were directly mixed with the blood samples at a concentration ranging from 1·5–15 μg gallic acid equivalents (GAE)/ml blood. The effect was only observed in coronary artery disease patients when >7 μg GAE/ml was used. Further, human umbilical vein endothelial cells were also exposed to the plant extract to assess cellular viability and anti-platelet activity[51]. However, the data presented would have had more relevance if the bioavailability of the identified compounds in the plant extract was tested. Although an *in vivo* test was performed, the authors did not analyse the concentration and form of the flavonoids that reached the blood. Based on the present

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knowledge on the bioavailability of flavonoid glycosides, it is clear that: (a) the total concentration of phenolic metabolites in blood after a flavonoid-rich diet would not reach 1·5 μg GAE/ml blood and (b) flavonoids in blood after consumption of flavonoid-rich foods do not occur as glucosides and definitely not rhamnosides.

In another study, lotus (*Nelumbo nucifera*) extract, 41·8 mg quercetin equivalents/g (flavonoid profile not reported) was applied to human umbilical vein endothelial cells at a concentration range 10–100 μg GAE/ml culture medium to study its effect against endothelial dysfunction, specifically vascular endothelial growth factor-induced angiogenesis[52]. Also, artichoke extract (1–100 μg GAE/ml) was shown to inhibit inducible nitric oxide synthase expression when directly applied to human coronary artery smooth muscle cells[53]. Lotus and artichoke have however been previously reported to contain mostly quercetin glucoside and glucuronide[54], and luteolin rutinoside and glucoside[55], respectively. Curly kale (*Brassica oleracea* L. convar. acephala var. sabellica) extract was also recently reported to reduce TNF-α induced neutrophil adhesion to human umbilical vein endothelial cells via 24-h pre-incubation of the human umbilical vein endothelial cells with curly kale extract[56]. Curly kale has been previously reported to contain mostly highly glycosylated kaempferol and quercetin[57].

The effect of a flavonoid glycoside-rich extract of a traditional Chinese medicine, semen astragali complanati (*Astragalus complanatus* R.Br.) on the activation of natural killer-92 cells, a model for natural killer cells found in the blood that combats tumours, was investigated by incubating natural killer-92 cells with 12–200 μg (unspecified whether GAE or per dry weight)/ml semen astragali complanati extract for up to 72 h[58]. Apart from problems relating to concentration and the type of flavonoids present in these extracts, the clearance of polyphenols in the blood was also not considered as flavonoid metabolites do not persist in circulation for 72 h when using a single dose[59].

It is surprising that although flavonoid glycoside metabolism and bioavailability has been described in many papers dating decades back, very recent papers still do not consider bioavailability and metabolism of flavonoid glycosides as important considerations when assessing *in vitro* bioactivity. There is therefore a need for stricter acceptance rules or guidelines in reporting *in vitro* bioactivity of flavonoid-rich plant extracts, or any plant extracts, in the literature. This does not only include research data but also papers reviewing the bioactivity of these plant extracts. For instance, a recent review[60] reported the *in vitro* bioactivity of several plant extracts on various cancer stem cells. Comments on neither the identity of the active compound nor the bioavailability of these plant components were however made.

Recently, guidelines and recommendations in reporting requirements for bioactive components, such as flavonoids have been published[61,62]. In both guidelines, the bioavailability of the flavonoids in question should be strictly considered when planning and reporting results from *in vitro* experiments. According to Somoza *et al*[61], studies should address five focus areas when reporting *in vitro* bioactivity of plant-based bioactive components: (1) Identification of the active molecule/s using state-of-art spectrometric and spectroscopic techniques; (2) Quantitation of the active components using validated methods; (3) Demonstration of the bioavailability of the bioactive component using relevant *in vitro* or *in/ex vivo* models; (4) Unequivocal identification and quantitation of metabolites generated in the bioavailability study; (5) Mechanistic study using the relevant compound.

Many papers, both recent and old, usually pass the first two criteria but generally fail to satisfy the third and fourth. Instead, many jump to the mechanistic study of the compounds found in the plant extract; skipping the bioavailability and metabolism criteria. By following these criteria, the relevance of the data published in literature will be preserved. The implementation of this entails a consensus among scientists and publishers to increase the quality standard for publishing data.

Conclusion

In this review, we have shown that current *in vitro* systems and approaches need to be reevaluated. There is clearly a need to improve *in vitro* models for bioavailability, especially for flavonoid glycosides. The analysis of flavonoid accumulation in specific tissues during inflammation or stressed conditions offers a new line of research. Finally, the bioavailability of flavonoids from plant extracts need to be strictly considered when planning *in vitro* experiments and elucidating their bioactivity towards cellular models, such as vascular cells.

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Conflict of Interest

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