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Effects of Cellular Uptake of Flavonoids against Peroxynitrite-mediated Cell Cytotoxicity

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ABSTRACT

Objective: Flavonoids-derived antioxidant properties have been proposed to be beneficial to various pathologies associated with reactive species including peroxynitrite. It is thus crucial to investigate cellular uptake of flavonoids which are responsible for biological effects in target cells. The objectives of this study were to evaluate the uptake of flavonoids in human colon cancer (HT-29) and chondrosarcoma (SW1353) cells by assessing intracellular phenolic contents and effects against peroxynitrite-mediated SW1353 cytotoxicity.

Methods: The radical scavenging activities of flavonoids (catechin, epicatechin or quercetin) were determined using Thin layer chromatography-1,1-diphenyl-2-picrylhydrazyl (TLC-DPPH) assay. HT-29 and SW1353 cells were treated with the flavonoids and removed after 18-h incubation before the assay. Flavonoid uptake in HT-29 and SW1353 cells was demonstrated by assessing phenolic contents in the cell lysates using the Folin-Ciocalteau method. Additionally, their effects in inhibiting peroxynitrite-mediated SW1353 cell cytotoxicity were studied by using 4 cytotoxicity models: crystal violet-staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction, cellular glutathione (GSH) assay and intracellular oxidant formation.

Results: DPPH assay showed that quercetin exhibited the greatest radical scavenging activity. Phenolic contents detected in both HT-29 and SW1353 cell lines pre-incubated with quercetin were also greater in HT-29 cell lysates. SW1353 cytotoxicity by peroxynitrite was also inhibited by pre-incubation with quercetin. Additionally, phenolic contents and cytoprotective effects were not found in cells pre-incubated with catechin or epicatechin.

Conclusion: There appears to be an uptake of quercetin but neither catechin nor epicatechin in HT-29 and SW1353 cells. Moreover, accumulation of quercetin, the powerful free radical scavenger, in SW1353 cells may contribute to its cytoprotective effect against peroxynitrite-induced cytotoxicity.

Keywords: Antioxidant; cytotoxicity; flavonoids; peroxynitrite; uptake

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Flavonoids, polyphenolic antioxidants abundant in our daily food including tea, fruits and vegetables, are suggested to prevent pathology associated with oxidative stress produced by excess in reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) formation. Epidemiological studies have also reported a correlation between the consumption of diet-derived flavonoids and the prevention of age-related diseases such as cancer, neurodegenerative diseases and cardiovascular diseases[1]. Hence, it is of great interest to develop flavonoids as potential therapeutic antioxidants for clinical use, although cellular uptake of flavonoids related to their effects in target cells should be taken into account and needs clarification.

Peroxynitrite, the product from the diffusion-controlled reaction of nitric oxide ($^{\bullet}NO$) with the superoxide radical ($O_2^{\bullet-}$) ($^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$, $\kappa = (4.3\text{-}19) \times 10^9\ M^{-1}\ s^{-1}$)[2], is capable of oxidising and nitrating a wide range of biomolecules, such as proteins, lipids and DNA, leading to cellular damage. For instance, peroxynitrite-dependent modification of proteins (e.g., tyrosine and cysteine) affect enzymatic function in several cell types. Cell death was also shown to involve excess peroxynitrite generation causing mitochondrial dysfunction and damage[3]. Additionally, increased formation of reactive species responsible for cellular oxidative stress and glutathione depletion could be contributed to cell apoptosis and death[3,4]. Products of reactions between peroxynitrite and biomolecules have also been detected in degenerative disorders such as osteoarthritis[5].

Members of flavonoids shown to provide powerful free radical scavenging properties include quercetin (found in broccoli, onions, apples, grapefruits and red wines), catechin and epicatechin (found in green and black tea) (Fig 1A)[6]. Several *in vitro* and *in vivo* studies have also

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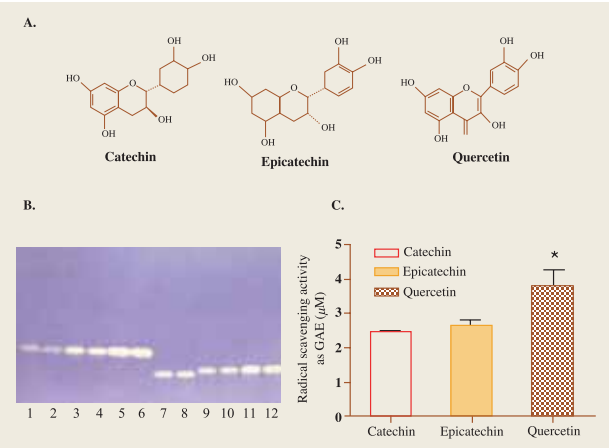


Fig 1. Radical scavenging activity of catechin, epicatechin and quercetin with their chemical structures shown (A) was identified by DPPH assay (B). Lane 1-6 = gallic acid (1.9, 3.75, 7.5 μM in duplicate), lane 7-8 = catechin (3.75 μM), lane 9-10 = epicatechin (3.75 μM) and lane 11-12 = quercetin (3.75 μM). Data were expressed as gallic acid equivalence (C). Statistical significance was evaluated using one-way ANOVA with Tukey's post test for intergroup comparisons. The asterisks stand for the probability of statistical significance, *$p<0.05$.

demonstrated their antioxidant activities capable of detoxifying ROS/RNS (such as superoxide radical, hydrogen peroxide and peroxynitrite), thereby inhibiting cellular damage[7]. Catechin, epicatechin and quercetin have been shown to act as potential peroxynitrite scavengers in previous studies[8]. Since limited data are available regarding cellular uptake of flavonoids and their biological effects in various cells, flavonoid accumulation in HT-29 and SW1353 cells and their effects were investigated in this study by assessing intracellular phenolic contents and their ability to inhibit peroxynitrite-induced SW1353 cell cytotoxicity.

MATERIALS AND METHODS

Materials

Human colon cancer (HT-29) and chondrosarcoma (SW1353) cell lines were from the American Type Culture Collection (ATCC, Rockville, Md, USA). Dulbecco's modified Eagle medium (DMEM) was obtained from Hyclone (Irvine, CA). Chemicals and reagents used were of the highest quality available and purchased from Sigma-Aldrich (Singapore) or Sigma-Aldrich (St.Louis, MO). The assays using microplate readers were done using a Spectra Max Gemini XS or a Spectra Max 190, both from Molecular Devices (Sunnyvale, CA, USA). Reagents used with thin layer chromatography (TLC) analysis were from Merck (Darmstadt, Germany). Chemicals used with DPPH assay were from Sigma-Aldrich (Steinheim, Germany).

Thin layer chromatography (TLC) analysis with DPPH assay

Stock solutions containing 3.75 μM of catechin, epicatechin and quercetin were prepared in 10% DMSO/water (v/v). Gallic acid (1.9-7.5 μM) was used as the standard phenolic compound to identify and compare the free radical scavenging activity of test flavonoids. A drop (1 μl) of each stock solution was loaded on a TLC plate and developed in a solvent system at a distance of 90 mm (TLC; silica gel 60 F254, Merck; mobile phase, chloroform:acetone:formic acid 75:16.5:8.5 (v/v/v). After 5 min air-drying, each plate was sprayed with 0.04% DPPH (1,1-diphenyl-2-picrylhydrazyl) solution to indicate radical scavenging activity yielding the bright yellow bands against the purple background under visible light[9]. Gallic acid equivalence (GAE) used to compare free radical scavenging activity of test flavonoids was calculated from a standard curve relating the chromatographic peak area to concentration using Video Store 2 (version 2.23) (Camag, Muttenz, Switzerland). TLC plate imaging to identify antioxidant activity was performed by TLC combined with video scanning TLC (Camag Video Documentation System connected with Reprostar 3 transilluminator cabinet).

Cell cultures

HT-29 and SW1353 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic antimycotic solution [1% penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml)]. Cells were maintained at 37 °C in humidified air containing 5% CO_2 (PCO_2 = 40 Torr) (a Forma Scientific CO_2 Water Jacketed Incubator) and cultured in physiological concentrations of bicarbonate (22 mM) DMEM equilibrated with 5% CO_2 (1 mM) as a CO_2/bicarbonate buffer to control the pH of the medium (pH 7.4). Cells were processed by trypsin-ethylenediaminetetraacetic acid (EDTA) treatment (10% in sterile phosphate buffer saline, PBS).

Synthesis of peroxynitrite

Synthesis of peroxynitrite was essentially as described[10]. Briefly, an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with KNO_2 (0.6 M) on ice for one second and the reaction quenched with ice cold NaOH (1.2 M). The residual H_2O_2 was removed by mixing with granular MnO_2 pre-washed with NaOH (1.2 M). The stock solution was filtered and then frozen overnight (-20 °C) and the top layer of the solution collected for the experiment. Concentrations of stock peroxynitrite were determined before each experiment at 302 nm using a molar absorption coefficient of 1,670 $cm^{-1}M^{-1}$. Concentrations of 200-250 mM were usually obtained. Once thawed, peroxynitrite solutions were kept on ice for no longer than 30 min before use.

Determination of intracellular phenolic contents

HT-29 and SW1353 cells seeded at a density of 6 x 10^6 per flask on 75-cm^2 culture flasks were incubated with test flavonoids (catechin, epicatechin and quercetin) at final concentrations of 0, 30, 60, 125, 250, 500 μM for 18 h at 37 °C. After washing the cells for 3 times, cell lysis was carried out with 0.1% (v/v) Triton-X 100 and 3-4 cycles of freezing and thawing. Cell lysates were then collected and debris was removed by centrifuging at 10,000 rpm (10,621 ×g) for 10 min. Contents of the flavonoids were determined using the Folin-Ciocalteau (FC) method[11]. 50 μl cell lysate supernatants were mixed with 100 μl Folin-Ciocalteau reagent, 1 ml of H_2O_2, and 500 μl of 15% Na_2CO_3. The absorbance of the mixture was measured at 765 nm after 2 h of incubation at room temperature.

Determination of the effect of flavonoid uptake on SW1353 cytotoxicity induced by peroxynitrite

Various stock concentrations of test flavonoids were freshly prepared in phosphate buffer solution (PBS) (0.25 M K_2HPO_4-KH_2PO_4 buffer, pH 7.4) except for quercetin (dissolved in dimethyl sulfoxide, DMSO, the final concen-

tration of which did not exceed 0.5% (v/v) in culture medium). SW1353 cells were pre-treated with different concentrations of flavonoids (15-125 μM) for 18 h and washed 3 times to remove the flavonoids prior to the addition of peroxynitrite. Cell cytotoxicity was evaluated by different assays. Cells incubated with phosphate buffer only were used as a control with DMSO (0.5% v/v) as a solvent. DMSO alone did not have any effect on the parameters measured. The addition of peroxynitrite did not alter the pH of the reaction mixture; the pH measured after every experiment was 7.4-7.5.

Assay of crystal violet-staining (CVS)

Cell viability was determined using crystal violet-stained intact cell assay as previously described[12]. Cells were seeded in 96-well plates at an initial density of 4×10^4 cells/well. After treatment of SW1353 cells with flavonoids, cells were washed with PBS at least twice before addition of peroxynitrite (1 mM). The attached cells were stained with 0.2% (w/v) crystal violet in 2% ethanol for 15 min at room temperature. Then the plates were washed under running tap water and air-dried. The CV stain was then eluted with 0.5% sodium dodecyl sulfate (SDS) in 50% ethanol and optical density values were read in a Spectra Max190 microplate reader at a wavelength of 610 nm.

Assay of MTT reduction

Cell mitochondrial and metabolic function was determined by measurement of MTT reduction. After removal of flavonoids, the cells were treated with peroxynitrite (250 μM) for 5 min and cells were rinsed with PBS. The cells were incubated with culture medium containing MTT (0.5 mg/ml final concentrations) for 1 h and then washed with PBS. The blue formazan crystals formed were solubilised in 200 μl of DMSO. The absorbance was determined with a microplate reader at 550 nm. MTT conversion was not affected by DMEM and flavonoids were tested.

Measurement of intracellular glutathione (GSH)

GSH contents were assayed as described[13], using the fluorescent reagent *o*-phthalaldehyde (OPA), which reacts specifically with GSH at pH 8. Cells (0.25×10^6 cells/well) in 24-well plate pre-treated with test flavonoids were washed at least twice before addition of peroxynitrite (1 mM). After lysing cells by 6.5% trichloroacetic acid, sample, phosphate/EDTA buffer and OPA (1 mg/ml in methanol) were added. Fluorescence was measured at 350 nm excitation and 420 nm emission using a spectrofluorometer. The GSH levels were calculated by comparing with standard curves using known concentrations of GSH. The results are expressed as nmol GSH/mg protein. The Lowry method was used to determine protein concentrations. The reduction of the folin phenol reagent yielded a blue color read at 750 nm.

Measurement of cellular oxidant formation

2′, 7′-Dichlorofluorescein diacetate (DCF-DA), a stable and non-fluorescent dye, is widely used to measure oxidative stress in cells[14]. Cells pre-treated with flavonoids were washed with PBS and incubated with 5 μM of DCF-DA in DMEM for 1 h in 24-well plates. After washing cells twice with PBS and adding peroxynitrite (1 mM), the DCF fluorescence was then monitored immediately and at 20 min in a Spectra Max Gemini XS spectrofluorometer with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Statistical Analysis

All graphs are plotted with mean ± standard error of the mean (SEM). In all cases the means were calculated from the data taken from at least 6 separate experiments performed on separate days using freshly prepared reagents. Where significance testing was performed, an independent t-test (Student's; 2 populations) and one-way analysis of variance (ANOVA) with Tukey's post test (for intergroup comparisons) were used. The asterisks stand for the probability of statistical significance, *$p \leq 0.05$; **$p \leq 0.01$ and ***$p \leq 0.001$.

RESULTS

Identification of the antioxidant activities of flavonoids

DPPH assay producing yellow bands of catechin, epicatechin and quertecin indicated that they exerted a hydrogen-donating capacity and had radical scavenging activity comparable to that of gallic acid (Fig 1B). In addition, the radical scavenging activity of catechin, epicatechin and quercetin at 3.75 μM was detected to be equivalent to that of 2.46 ± 0.1, 2.64 ± 0.2 and 3.8 ± 0.5 μM of gallic acid, respectively, showing that quercetin significantly yielded the greatest activity ($p<0.05$) (Fig 1C). The radical scavenging activity indicated by GAE thus showed a rank order of quercetin > catechin ≈ epicatechin.

Phenolic contents in colon cancer (HT-29) and chondrosarcoma (SW1353) cells

A HT-29, colonic epithelial cell line, was used in this study as it is an established model system for intestinal drug permeability[15]. Fig 2A-B shows that phenolic contents were detected in a concentration dependent manner in SW1353 and HT-29 cell lysates after pre-treatment with quercetin, whereas their contents were not found in both cells pre-incubated with catechin or epicatechin. Moreover, the phenolic contents observed were greater in HT-29 than SW1353 cells ($p<0.05$).

The effect of flavonoid uptake on the inhibition of peroxynitrite-induced SW1353 cytotoxicity

SW1353 cells are chondrocyte-like cells used as a model to study the cytotoxic effects of peroxynitrite because peroxynitrite has also been suggested to contribute to chondrocyte cell cytotoxicity during the pathogenesis of osteoarthritis[16]. To evaluate whether flavonoids (catechin, epicatechin and quercetin) might exhibit intracellular effects against peroxynitrite-dependent cytotoxicity, cells pre-incubated with flavonoids were washed to remove residual compounds prior to the introduction of peroxynitrite. Catechin and epicatechin (Fig 3A-B) were unable to provide cytoprotective effects as SW1353 cells pre-treated with the flavonoids responded to the addition of peroxynitrite to the same extent compared to cells without pre-treatment with the compounds. However, cells pre-treated with quercetin (15-125 μM) exhibited cytoprotective effects in a concentration-dependent manner as shown by an increase in cell viability (from 51.8% ± 0.8 to 54.8% ± 1.7), MTT reduction (from 32.7% ± 0.7 to 36.3% ± 1.3), cellular GSH (from 49.5% ± 1 to 58.9% ± 1.3) and a decrease in intracellular peroxides (from 3.78 ± 0.05 to 3.34 ± 0.05 RFU).

DISCUSSION

A number of *in vitro* studies have shown that flavonoids

including quercetin, catechin and epicatechin exerted powerful free radical scavenging activities, proposed to be potential therapeutic antioxidants. However, it remains unclear whether they would provide similar activity as seen in both *in vitro* and *in vivo* and studies are needed to show that these agents reach target cells to elicit activities as indicated in *in vitro* experiments. Flavonoids have been extensively discussed whether they have an effect on cells or any other tissues. These molecules need to either interact with cell membranes or be absorbed. It is suggested that flavonoids responsible for protective actions in a number of pathology such as cardiotoxicity and arthritis may be due to their accumulation in the cells to produce the effects[16,17]. In addition, HT-29 cells have been used in established intestinal absorption models to study drug transport properties[15]. Hence, HT-29 and SW1353 cells were used as a model to evaluate cellular uptake of flavonoids.

TLC-DPPH analysis confirmed antioxidant properties of all test flavonoids and quercetin appeared to be the most powerful free radical scavenger among the flavonoids studied here. Cellular uptake of quercetin but neither catechin nor epicatechin was demonstrated, as indicated by increased contents of phenolic compounds in a concentration-dependent manner in the cells pre-incubated with quercetin. Hence, there might be an accumulation of only quercetin in HT-29 and SW1353 cells. Differences in cellular uptake of various flavonoids possibly derived from their lipophilicity. A previous report showed that quercetin possesses the ability to modify membrane-dependent processes and interact with and penetrate the lipid bilayers and can be taken up in cells through passive diffusion, perhaps, due to its higher lipophilicity facilitating its penetration into the cells[18]. Hence, quercetin may be the most lipophilic flavonoid compared to catechin and epicatechin and it therefore has a greater ability to permeate into the cells.

The effects of quercetin accumulated in SW1353 cells were demonstrated by assessing its ability to inhibit peroxynitrite-mediated cytotoxicity after the compounds were removed. Quercetin was shown to inhibit cellular damage, GSH depletion and oxidative stress induced by peroxynitrite. A number of *in vitro* and *in vivo* studies in several cell types (e.g., cardiomyoblasts and hepatocytes) have also demonstrated cytoprotective effects of quercetin against oxidative stress[17,19].

Whereas quercetin accumulation in cells could be responsible for its inhibitory effect against peroxynitrite-mediated SW1353 cell cytotoxicity, intracellular phenolic contents indicating cellular uptake of catechin and epicatechin were not detected, in agreement with the finding showing that pre-treatment of catechin or epicatechin was unable to yield cytoprotective effects against peroxy nitrite.

Cellular accumulation of quercetin was previously demonstrated in breast cancer cells and enterocytes[20]. This study showed for the first time that quercetin could also be absorbed in chondrosarcoma cells, although its accumulation was found in HT-29 cells to a greater extent

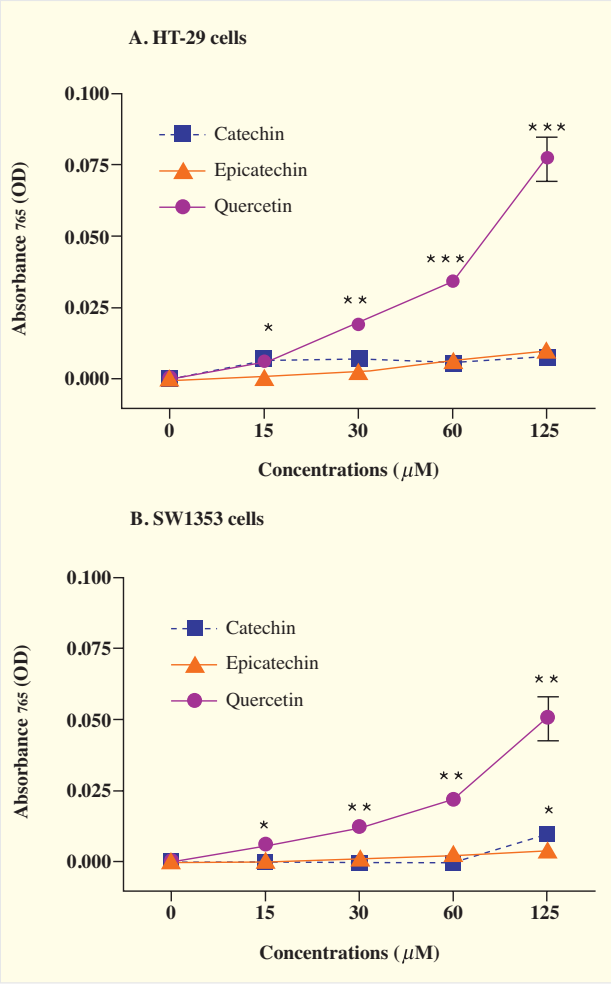


Fig 2. Intracellular phenolic contents of catechin, epicatechin and quercetin in HT-29 (A) and SW1353 (B) cells were assessed using FC method. Phenolic contents were determined spectrophotometrically at 765 nm. Data were expressed as optical density. Statistical significance was evaluated using Student's t-test. The asterisks stand for the probability of statistical significance, *p<0.05, **p<0.01, ***p<0.001, comparing treated cells with untreated cells.

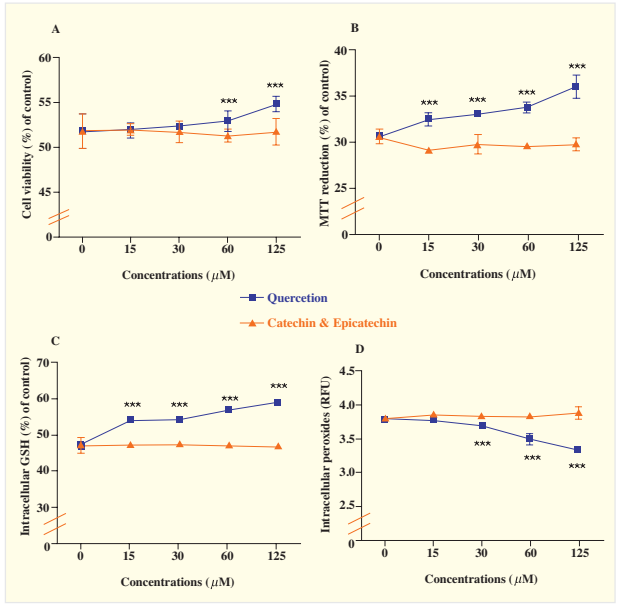


Fig 3. Cytoprotective effects of quercetin but not catechin or epicatechin on peroxynitrite-mediated SW1353 cell cytotoxicity. Cell viability indicated by CV-stained intact cells (A) and MTT reduction (B) and cellular GSH levels (C) were expressed as a percentage of untreated cells (100%). Intracellular peroxides (D) were expressed as RFU. Statistical significance was evaluated using Student's t-test. The asterisks stand for the probability of statistical significance, *p<0.05, **p<0.01, ***p<0.001, comparing treated cells with untreated cells.

than SW1353 cells, probably, because the different cellular transport systems in various cell types may influence the flavonoid uptake, which may be therefore greater in enterocytes than in other cells.

Bioavailability study of flavonoids remains to be resolved. The FC assay used in this study has been widely performed to determine phenolic contents, as it is the colourimetric method based on the reducing capacity of phenolic groups. However, this assay may not have substantial sensitivity and therefore cellular uptake of flavonoids needs further investigation using analytical methods. Thus, our conclusions on undetectable accumulation of catechin and epicatechin in cells should be treated with caution.

In summary, cellular uptake of quercetin but neither catechin nor epicatechin was observed in both cell lines studied, although it appeared to be accumulated to a greater extent in HT-29 cells than in SW1353 cells. Moreover, uptake of quercetin, the powerful free radical scavenger, might be responsible for its cytoprotective effect against peroxynitrite-mediated SW1353 cell toxicity. Further work using analytical high performance liquid chromatography (HPLC) methods is needed for quantitative study of flavonoids in target cells *in vivo*. Additionally, several factors such as cell types, compound structures and methods used should be considered in the study of cellular uptake of flavonoids. Moreover, this finding may be useful in designing a model to investigate whether flavonoids play a pharmacological role in gastrointestinal (GI) diseases because a greater accumulation of flavonoids in enterocytes might be particularly advantageous for preventing diseases in the GI tract.

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