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Full-length article

# Intra-herb pharmacokinetics interaction between quercetin and isorhamentin[1]

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## Key words

drug interaction; pharmacokinetic; flavone; Caco-2 cell; absorption; bioavailability; P-glycoprotein

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## Abstract

**Aim:** Quercetin and isorhamnetin are common constituents of some herb extracts, such as extracts of gingko leaves and total flavones of *Hippophae rhamnoides* L. The intra-herb pharmacokinetics interactions between isorhamnetin and quercetin were investigated in the present study. **Methods:** Human MDR1 cDNA transfected MDCKII cells were used to validate whether isorhamnein interacted with P-gp. Caco-2 transport assays and a randomized, 3-way crossover pharmacokinetics study in rats were used to investigate the pharmacokinetics interactions. HPLC was used to determine cell transport samples. The total plasma concentrations of quercetinand isorhamnetin were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) by treatment with β-glucuronidase and sulfatase. **Results:** The permeability ratio (absorptive permeability/secretive permeability) of isorhamnetin across human MDR1 cDNA transfected MDCKII cells, Caco-2 cells and wild-type MDCKII cells are 0.25±0.02, 0.74±0.05, and 1.41±0.06, respectively. This result proved the role of P-gp in the cell efflux of isorhamnetin. While co-transporting with each other across Caco-2 cells monolayer, the permeability ratio of isorhamnetin and quercetin increased by 4.3 and 2.2 times. After coadministration with each other to rats,the $C_{max}$, $AUC_{0-72\ h}$, and $AUC_{0-\infty}$ of both isorhamnetin and quercetin significantly increased compared with single administration. **Conclusion:** The above results proved intra-herb pharmacokinetics interaction between quercetin and isorhamentin. P-gp might play an important role, whereas other drug efflux pumps, such as multi-drug resistance associate protein 2 and breast cancer resistance protein, might be involved. Accordingly, besides the drug-herb interactions, intra-herb interaction might be brought into view with the wide use of herbal-based remedies.

## Introduction

Many herbs containing flavonoids have been widely used in traditional medicines. Their extracts are increasingly introduced into modern medicines as complementary and alternative medicines, such as Gingko flavones (GF), total flavones of *Hippophae rhamnoides* L (TFH), Soy isoflavones, Pycnogenol (procyanidins extracted from Pinus maritima), grape seeds extracts and Saint John's Wort extracts, *etc*. With the gradually widening use of these herb extracts as food supplements, more and more clinical affairs derived from herb-drug interactions have emerged and are being investigated[1–3]. Most interactions are associated with pharmacokinetics[4].

Quercetin and related bioflavonoids, such as isorhamnetin, were common constituents of some of these extracts, such as GF[5] and TFH[6]. Isorhamnetin, which is also called 3′-*O*-methylquercetin, was a metabolite of quercetin not only in plants, but also in some mammalian species, such as rats[7], pigs[8] and human[9] after oral administration. The *O*-methylation of quercetin in mammals is catalyzed by catechol-*O*-methyltranferase

(COMT)[10]. Both quercetin and isorhamnetin exhibited considerable anti-oxidant activities, such as modification of eicosanoid biosynthesis, protection of low-density lipoprotein from oxidation, prevention of platelet aggregation and promotion of relaxation of cardiovascular smooth muscle[11]. On the other hand, quercetin and related bioflavonoids had been proven to modulate the function and expression of P-glycoprotein[12,13] and CYP3A4[14,15]. Consequently, quercetin and related bioflavonoids affected the pharmacokinetics and pharmacodynamics of some chemical drugs.

Interestingly, similar to the drug-herb interaction, co-occurrence of quercetin and related bioflavonoids in one herb extract would possibly cause potential intra-herb interaction. There was some evidence that not only quercetin but also isorhamnetin interacted with P-glycoprotein[16]. Therefore, we doubted that if there was any pharmacokinetics interaction between quercetin and isorhamnetin, and investigated the interaction by cell transport assays and *in vivo* pharmacokinetics assay.

## Materials and methods

Quercetin (QU, 99%), isorhamnetin (IS, 99%) (Figure 1) and baicalein (internal standard, 99.9%) were purchased from Tauto Biotech (Shanghai, China). β-Glucuronidase (type H-3, from Helix pomatia) and sulfatase (type H-1, from Helix pormatia) were obtained from Sigma-Aldrich (St Louis, MO, USA). The human colon carcinoma cell line (Caco-2) was obtained from American Type Culture Collection (Rockville, MD, USA). MDCK cell clones strain II[17] (MDCKII) and its human MDR1 cDNA transfected strain[18] (MDR1-MDCKII) were gifts from Professor Borst P of the Netherlands Cancer Institute (NKI). Dulbecco's Modified Eagle's medium (DMEM, high glucose, Cat: 61975), Hanks' Balance Salt Solution (HBSS, Cat: 14175), penicillin-streptomycin (Cat: 15070) and Trypsin-EDTA (Cat: 25300) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal calf serum (FCS, Cat: SH30084) was purchased from Hyclone (Logan, UT, USA). Dimethyl sulfoxide (DMSO) was purchased from Millipore Inc (USA). Methanol, glacial acetic acid and formic acid (HPLC grade) were purchased from TEDIA Inc (Fairfield, IA, USA).

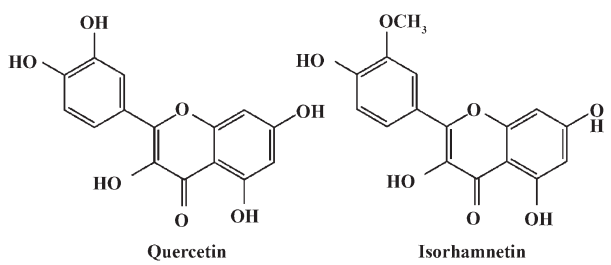


**Figure 1.** Chemical structures of quercetin and isorhamnetin.

**Cell transport assay** Caco-2 cells and MDCKII/MDR1-MDCKII cells were seeded on Transwell polycarbonate membrane filters (0.4 μm pore diameter, 12 mm diameter, Coring Costar Inc. Cambridge, MA, USA) at a density of $5\times10^5$ cells/well and $2\times10^6$ cells/well, respectively. Cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin G and 100 μg/L streptomycin sulfates. For Caco-2 cells, culture medium was replaced 3 times a week for 14 d and daily thereafter until 3 weeks. For MDCKII/MDR1-MDCKII cells, culture medium was replaced every day for 3–4 d. The integrity and confluence of the cells monolayers was determined by measuring the transepithelial electrical resistance (TEER) by and Millicell-ERS voltohmmeter. Inserts were used for transport studies with TEER values >350 $\Omega\cdot cm^2$ for Caco-2 cells, TEER values >400 $\Omega\cdot cm^2$ for MDCKII cells, and TEER values >4000 $\Omega\cdot cm^2$ for MDR1-MDCKIIcells[19].

The inserts were washed twice with warm transport buffer (HBSS containing 1% DMSO [*v/v*] and 1 mmol/L ascorbic acid [to prevent oxidation of quercetin]). The trans-epithelial permeability of QU and IS was measured at a concentration of 50 μmol/L. Transport buffer containing QU or/and IS was added to donor compartment (either the apical [2.0 mL] or basolateral [2.5 mL] side of the inserts), while the receiver compartment contained the corresponding volume of fresh transport buffer. During the incubation at 37 °C for 80 min, samples (100 μL) were collected from both sides at 20, 40, and 80 min and stored at –70 °C until analysis by HPLC. After sampling, 100 μL of fresh transport buffer were added to both transport compartments.

In control experiments, paracellular flux of [$^3$H]inulin

**Table 1.** The drugs administration schemes for the study of the pharmacokinetic interaction between isorhamnetin and quercetin. T1, single oral dose of isorhamnetin (1.0 mg/kg); T2, single oral dose of quercetin (1.0 mg/kg); T3, single oral dose of isorhamnetin (1.0 mg/kg) coadministrated with quercetin (1.0 mg/kg).

| Group | | A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|
| Periods | 1 | T1 | T2 | T3 | T1 | T3 | T2 |
| | 2 | T2 | T3 | T1 | T3 | T2 | T1 |
| | 3 | T3 | T1 | T2 | T2 | T1 | T3 |

(0.15 μCi/mL) through the monolayer was always less than 1.5%, indicating intactness of the monolayer.

**Pharmacokinetics study** This experiment was approved by the Ethics Committee of the West China Center of Medicine, Sichuan University. A latin square study design was introduced into the pharmacokinetic studies. 12 male Wistar rats (200±20 g, Experimental Animals Center, Sichuan University, China) were randomly divided into 6 groups (Group A–F, 2 rats/group). The rats were housed and fed as described in our former published paper[20]. The dose orders of each group of rats were according to Table 1. The wash-out period was 10 d.

In each experimental period, blood samples (0.3–0.4 mL of venous blood) were collected before administration and at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 48, and 72 h postadministration from the tail vein of rats. Blood samples in tubes containing lithium-heparin were centrifuged (1500×*g* for 10 min) within 30 min following collection (EBA21 table centrifuge, Hettich, Germany). Then, due to instability of flavonols at high pH[7], 100 μL of harvested plasma was acidified with 10 μL of 0.5 mol/L acetic acid, which contained 2 mg of vitamin C per mL.

**Samples analysis** An adjusted HPLC method based on the report of Wang *et al*[16] was used to determine the concentrations of samples of transport studies. After adding 100 μL of methanol, the mixture was vortexed for 1 min at 2000 r/min (Vortex Genius 3, IKA, Germany) and centrifuged for 3 min at 5000×*g* (EBA21 table centrifuge, Hettich, Germany). A mobile phase of methanol-acetonitril-0.5 % acetic acid in water (5:23:72, *v/v/v*) was delivered at a rate of 1.0 mL/min using a Shimadzu LC-2010C HPLC system. The Shimadzu VP-ODS column (150×4.6 mm, 5 μm) was maintained at 40 °C, and the wavelength of the UV detector was set at 370 nm.

The ESI-LC-MS/MS method to determine plasma samples was published previously[20]. After being treated with β-glucuronidase and sulfatase, the analytes were extracted with the internal standard (baicalein). The chromatographic separation was carried out on a Diamonsil C18 column with a mobile phase of 2% formic acid-methanol (10:90, *v/v*) at a flow rate of 1.00 mL/min, with a split of 200 μL to mass spectrometer. The mass spectrometer was operated in the positive ion mode with the TurboIonspray heater set at 450 °C (API3000 LC/MS/MS system, Applied Biosystems, Foster City, CA, USA). The samples were analyzed using the transition of m/z 303→153 amu for QU, m/z 317→153 amu for IS and m/z 271→123 amu for baicalein.

**Data analysis** Apparent permeability coefficients ($P_{app}$) across Caco-2 monolayer were calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

In this equation, $Q$ is the amount of analytes in the receiving compartment, in which the sampling amount was added; A is the membrane surface area (4.71 $cm^2$); $C_0$ is the initial concentration in the donor compartment; $dQ/dt$ is the change of analytes in the receiver solution over time. The permeability ratio ($P_{ratio}$) was calculated according to the following equation:

$$P_{ratio} = \frac{P_{app,\,A-B}}{P_{app,\,B-A}}$$

The pharmacokinetics parameters were calculated using DAS 2.0 pharmacokinetics software (Drug and Statistics, Version 2.0) with non-compartment models. All of the data statistics were carried out with SPSS (version 10.0). $AUC_{0-72\,h}$, $AUC_{0-\infty}$ and $C_{max}$ were converted to logarithms before entering the statistics.

## Results

**Transport of IS and QU** No biotransformation between QU and IS was found in Caco-2 cells and MDCKII cells transport systems. Table 2 shows the

**Table 2.** Permeability coefficients of isorhamnetin (50 μmol/L) and quercetin (50 μmol/L) across Caco-2 cells, wild-type MDCKII cells and MDR1-MDCKII cells (n=3).

| $P_{app}\times10^6$ (cm/s) | A–B | B–A | Pratio |
|---|---|---|---|
| Quercetin (transport alone across Caco-2 cells) | 4.97±0.25 | 6.69±0.79 | 0.75±0.05 |
| Quercetin (co-transport with isorhamentin across Caco-2 cells) | 8.00±0.57 | 4.83±0.06 | 1.66±0.13 |
| Isorhamnetin (transport alone across Caco-2 cells) | 2.28±0.09 | 3.09±0.08 | 0.74±0.05 |
| Isorhamnetin (co-transport with quercetin across Caco-2 cells) | 4.61±0.19 | 1.45±0.14 | 3.20±0.34 |
| Isorhamnetin (transport alone across wild-type MDCKII cells) | 2.90±0.06 | 2.06±0.06 | 1.41±0.06 |
| Isorhamnetin (transport alone across MDR1-MDCKII cells) | 1.22±0.11 | 4.96±0.03 | 0.25±0.02 |

permeability coefficients ($P_{app}$) of QU (50 μmol/L)/IS (50 μmol/L) transport alone or together in the Caco-2 cells, wild-type MDCKII cells and MDR1-MDCKII cells. The MDR1-MDCKII cells model was used to validate if IS was the substrate of P-gp. The $P_{ratio}$ of IS in the MDR1-MDCKII cells model was significantly less than the one in the wild-type MDCKII cells model ($P$<0.001, Student's $t$-test). This result proved the role of P-gp in the cell efflux of IS. In Caco-2 cells transport assay, both $P_{app\ (A–B)}$ and $P_{app\ (B–A)}$ of QU were significantly larger than the ones in IS. The secretion (basolateral to apical) rates of transport were higher than absorptive (apical to basolateral) rates of transport ($P$<0.05 for IS, $P$<0.05 for QU, Student's $t$-test). The $P_{ratio}$ of IS and QU was 0.74 and 0.75, respectively, indicating that an efflux mechanism was involved in the transport of either IS or QU.

Although co-transport occurred across Caco-2 cells, the $P_{app\ (A–B)}$ of both IS and QU were increased and the $P_{app\ (B–A)}$ were decreased ($P$<0.001 for IS, $P$<0.05 for QU, Student's $t$-test). Subsequently, the $P_{ratio}$ of co-transport studies increased from 0.74 and 0.75 to 3.20 and 1.66 for IS and QU, respectively. These results indicated that IS and QU could facilitate the absorptive permeation and inhibit the secretion permeation of each other across the Caco-2 cells monolayer. Drug efflux pumps mediated drug-drug interaction might be one of the main reasons[12,15,21].

**Pharmacokinetic interaction of isorhamnetin and quercetin** The plasma concentration of QU and IS versus time curve is illustrated in Figure 2. Corresponding to the previous pharmacokinetics studies in human[22,23], QU and IS showed two or more peaks of maximum plasma concentration in individual rats. The multiple peaks phenomenon was weakened with coadministration. QU was not detected after oral administration of IS alone, whereas IS was detected soon after the oral dose of QU alone. The IS in plasma after the oral dose of IS or QU came from the oral absorption of IS and the biotransformation of QU, respectively. IS in plasma after coadministration of IS and QU was the sum IS absorbed and IS biotransformed from QU. These results confirmed that QU is metabolized to IS, but not vice versa[20]. The main pharmacokinetics parameters of QU and IS were listed in Table 3 and Table 4.

In Table 3, it can be seen that the AUC of QU after the oral dose of QU was significantly different from the ones after coadministration of IS and QU ($P$<0.01 for both $AUC_{0–72\ h}$ and $AUC_{0–\infty}$, Student's $t$-test). After coadministration with IS, the oral bioavailability of QU was significantly enhanced. In Table 4, it can be seen that the AUC of IS prominently increased after coadministration of QU with IS to rats [$P$<0.01 for both $AUC_{0–72\ h}$ and $AUC_{0–\infty}$, Student-Newman-Keuls (SNK) test]. In Table 5, the AUC

**Table 3.** Pharmacokinetics parameters of quercetin after a single oral dose of isorhamentin (1.0 mg/kg), quercetin (1.0 mg/kg) and isorhamnetin (1.0 mg/kg) coadministrated with quercetin (1.0 mg/kg) in male Wistar rats ($n$=12). Data are shown as mean±SD. $^{b}P$<0.05, $^{c}P$<0.01 *vs* Quercetin (Student's $t$-test). $^{\#}P$=0.223 for $C_{max}$ (Student's $t$-test); $^{\dagger}P$=1 for $T_{max}$ (Wilcoxon signed ranks test).

| Dose | Isorhamnetin (1.0 mg/kg) | Quercetin (1.0 mg/kg) | Isorhamnetin (1.0 mg/kg) +quercetin (1.0 mg/kg) |
|---|---|---|---|
| $AUC_{0–72\ h}$ (μg/L·h) | – | 1872.3±426.2 | 2746.8±729.4$^{c}$ |
| $AUC_{0–\infty}$ (μg/L·h) | – | 2042.9±418.3 | 2972.2±818.7$^{c}$ |
| $C_{max}$ (ng/mL)$^{\#}$ | – | 209.5±47.1 | 252.4±85.7$^{b}$ |
| $T_{max}$ (h)$^{\dagger}$ | – | 5.1±1.1 | 5.3±2.2 |

**Table 4.** Pharmacokinetics parameters of isorhamnetin after a single oral dose of isorhamentin (1.0 mg/kg), quercetin (1.0 mg/kg) and isorhamnetin (1.0 mg/kg) coadministrated with quercetin (1.0 mg/kg) in male Wistar rats ($n$=12). Data are shown as mean±SD. $^{c}P$<0.01 (one-way ANOVA).

| Dose | A: isorhamnetin (1.0 mg/kg) | B: quercetin (1.0 mg/kg) | C: isorhamnetin (1.0 mg/kg) + quercetin (1.0 mg/kg) |
|---|---|---|---|
| $AUC_{0–72\ h}$ (μg/L·h) | 2057.4±689.2 SNK test: A↔B ($P$=0.060), A↔C ($P$<0.01), B↔C ($P$<0.01) | 2880.0±930.0 | 6170.0±1136.1$^{c}$ |
| $AUC_{0–\infty}$ (μg/L·h) | 2363.0±870.8 SNK test: A↔B ($P$=0.037), A↔C ($P$<0.01), B↔C ($P$<0.01) | 3524.7±1196.7 | 7459.1±1419.7$^{c}$ |
| $C_{max}$ (ng/mL) | 109.5±28.7 SNK test: A↔B ($P$<0.01), A↔C ($P$<0.01), B↔C ($P$<0.01) | 157.7±29.6 | 235.2±30.1$^{c}$ |
| $T_{max}$ (h) | 8.5±1.9 | 7.3±1.9 | 8.6±2.0 |

**Table 5.** Comparison of the AUC of isorhamnetin between coadministration of isorhamnetin and quercetin, and the sum of single administration ($n$=12). Data are shown as mean±SD. $^{b}P$<0.05 (Student's $t$-test).

| | Isorhamnetin alone (1.0 mg/kg) + quercetin alone (1.0 mg/kg) | Isorhamnetin (1.0 mg/kg) coupled with quercetin (1.0 mg/kg) |
|---|---|---|
| $AUC_{0–72\ h}$ (μg/L·h) | 4937.4±1265.2 | 6170.0±1077.8$^{b}$ |
| $AUC_{0–\infty}$ (μg/L·h) | 5887.7±1534.0 | 7459.1±1346.8$^{b}$ |

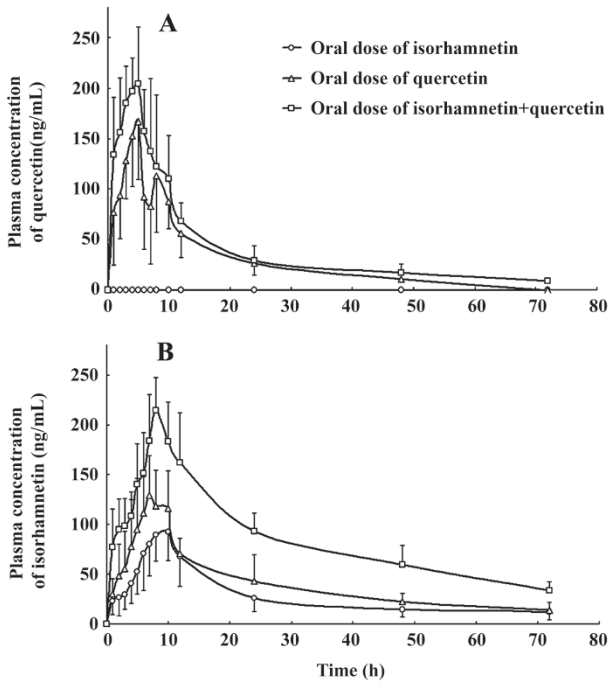


**Figure 2.** Mean plasma concentrations of quercetin (A) and isorhamnetin (B) over time after a single oral isorhamnetin (1.0 mg/kg), quercetin (1.0 mg/kg) and isorhamnetin (1.0 mg/kg) coadministrated with quercetin (1.0 mg/kg) in male Wistar rats ($n$=12).

of IS after coadministration of IS and QU is compared with the sum of AUC of IS after the oral doses of IS alone and QU alone. Significant differences were found in the $AUC_{0-72\,h}$ and $AUC_{0-\infty}$ ($P$<0.05, Student's $t$-test). Assuming the metabolism of QU was not affected by the oral dose of IS, it could be concluded that QU could enhance the oral bioavailability of IS in rats. Therefore, it was concluded that IS and QU could enhance the oral bioavailability of each other. The results of the pharmacokinetics studies were consistent with the results of Caco-2 cells monolayer transport studies. The drug efflux pumps mediated interaction of intestinal epithelia efflux might contribute to the improvements of oral bioavailability.

## Discussion

Drug efflux pumps, such as P-gp, MRP1, MRP2 (multi-drug resistance associate protein 2) and BCRP (breast cancer resistance protein), were expressed not only in Caco-2 cells and intestinal epithelial cells[24–26], but also at the canalicular surface of hepatocytes[27,28]. All might play potential roles in the transport interactions of IS and QU in Caco-2 cells and intestinal epithelial cells. The role of these drug efflux pumps in the ADME of IS and QU might also be taken into consideration while considering the mechanism of the pharmacokinetics interactions.

It had been documented that some naturally-occurring glucosides of QU can be absorbed via sodium dependent glucose transporters (SLGT)[29]. However, the uptake of QU from the intestinal lumen into plasma is limited because of incomplete intestinal absorption, rapid biliary efflux[30,31] or a certain extent of intestinal efflux[20], in which P-gp, MRP2 and BCRP[32,33] were considered to be involved. For most QU glycosides, the transcellular permeation was hindered by their large molecular weight. The intestinal micro flora exhibited an important role in glycosides absorption[34,35]. In the enterocytes and hepatocytes, quercetin and its conjugates were partly transformed to isorhamentin by catechin-*O*-transferase[10]. Both IS and QU undergo extensive conjugations with glucuronide and sulfate after entering intestinal epithelium[20,36]. No CYP metabolism of QU or IS was found. The bile excretion was one of the main routes of elimination, in which MRP2 and BCRP were supposed to be involved[33,36]. Then, considerable reabsorption occurred after bile excretion. Consequently, multiple peaks of maximum plasma drug concentration usually occurred in pharmacokinetics studies. In the present pharmacokinetics study, the attenuation of the multiple peaks phenomenon also hinted that QU and IS might influence the bile excretion of each other.

The present results proved that QU and IS could significantly facilitate the absorptive permeation, inhibit the secretion permeation of each other across the Caco-2 cells monolayer, and enhance the oral bioavailability of each other. Considering the pharmacokinetics of QU and IS, the first possible mechanism of the pharmacokinetics interaction might exist in the methylation of QU. It had been proven that demethylation of IS to QU could not occur in our previous study[20]. Theoretically according to the enzyme kinetics, the increase of metabolic product (IS) could inhibit the methylation of IS, which will result in increase of the plasma level of QU and decrease of IS. In fact, significantly increased systemic exposures of both quercetin and isorhmanetin were found in the present study. Furthermore, COMT is abundant in human tissues and organs and might not be saturated in the tested dose. The metabolic interaction might play a minor role in the present pharmacokinetics interaction between quercetin and isorhamnetin. Therefore, the most possible potential mechanism existed in the intestinal excretion and bile excretion, in which P-pg, MRP2[36] and BCRP[32] might be involved. Because both QU and IS had been identified to interact with P-gp[16,21,37,38]. It could be concluded that

P-gp might contribute to the present pharmacokinetics interaction between QU and IS. But whether MRP2 and BCRP were involved in this intra-herb interaction need further validation.

In the present study, it was proven that IS and QU could enhance the oral absorption of each other in rats. P-gp might play an important role in the absorptive pharmacokinetics interaction between IS and QU, whereas other ABC transporters, such as MRP2 and BCRP, might be involved. Therefore, it could be deduced that the oral dose of mixed flavonols in herb extracts could gain a significant biopharmaceutical advantage compared with the oral dose of single flavonol. Accordingly, besides the drug-herb interactions, inter-herb interaction and intra-herb interaction might be brought into view with the wide use of herbal-based remedies.

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## Author contribution

Ke LAN and Xue-hua JIANG designed research; Ke LAN, Jian-lin HE, Yang TIAN, Fei TAN, and Ling WANG performed research; Li-ming YE contributed new analytical tools and reagents; Ke LAN,Jian-lin HE,and Fei TAN analyzed data; Ke LAN and Xue-hua JIANG wrote the paper.

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