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Evaluation of hepatic clearance and drug-drug interactions of luteolin and apigenin by using primary cultured rat hepatocytes

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The hepatic clearance and drug-drug interactions of luteolin and apigenin were studied by using primary cultured rat hepatocytes. Luteolin and apigenin experienced extensive first-pass metabolism. The elimination percent of luteolin and apigenin was found to be 91.9% and 86.7% after 120 min of incubation. The predicted % liver blood flow was 82.3% and 85.4% for luteolin and apigenin, respectively. Total glucuronidated/sulfated conjugates of luteolin/apigenin were determined by an enzyme hydrolysis method. Compared with the elimination of pure luteolin and apigenin, the elimination of luteolin and apigenin was much lower in hydrolyzed *Flos Chrysanthemi* extract (FCE) containing comparable amounts of luteolin and apigenin. The effect of a series of flavonoids, including flavonols, flavones, isoflavone, flavanone, flavanonols and catechins, on the elimination of luteolin and apigenin was studied. At least four key determinants in the chemical structures of flavonoids are necessary for exerting the inhibitory effects on the conjugation: 1) catechol structure (3',4'-dihydroxylation) in the B-ring; 2) B-ring is attached to the C-2 position on the C-ring; 3) the C2–3 double bond in conjunction with the C4 carbonyl group on the C-ring; 4) no glycoside present. Investigation of clearance and interaction among flavonoids could help us better understand their bioavailability and offer insight into the approaches to be taken to minimize competitive effects, and to design appropriate bioavailability studies in humans.

1. Introduction

Flavonoids are a large group of poly-phenolic compounds commonly found in plant-derived beverages from fruits, vegetables such as tea and red wine. More than 5000 different flavonoids have been isolated from various plants. Due to growing evidence of their various health benefits, there has been increasing interest in research of flavonoids from dietary substances (Yao et al. 2004; Ross and Kasum 2002).

Luteolin and apigenin are typical flavones that co-exist in many plant medicines (Yang et al. 2008). They have anticancer (Wang et al. 2009) protective effects on the cardiovascular (Xu et al. 2007), hepatic (Zheng et al. 2005) and neurological systems (Rezai-Zadeh 2008). The main naturally occurring luteolin and apigenin are luteolin-7-*O*-β-D-glycoside and apigenin-7-*O*-β-D-glycoside, respectively, which can be deconjugated to their aglycones during passage through the intestine, and then conjugated to their phase II metabolites in intestine and liver (Lu et al. 2010; Chen et al. 2007; Li et al. 2005).

Previous pharmacokinetic studies of luteolin and apigenin demonstrated their extensive oral first-pass metabolism as their glucuronidated and sulfated conjugates appeared rapidly in systemic circulation (Chen et al. 2007; Li et al. 2005). Conjugate metabolism in intestine and liver implies an important role in causing low oral bioavailability of flavonoids (Zhang et al. 2007). Antioxidative activity is associated in part with the number of hydroxyl groups and their structural relationship in the molecule, it is very likely that during metabolic conjugation, at least some of this activity is lost (Piskula and Terao 1998). Luteolin and apigenin are usually ingested in the form of plant extracts which contain various other flavonoids. Therefore, drug-drug interactions can occur, affecting their bioavailability (Williams et al. 2004).

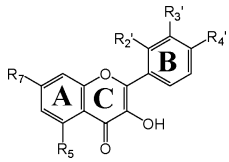
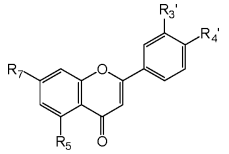
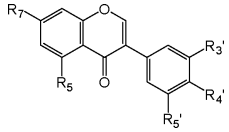
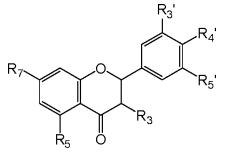
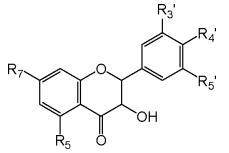
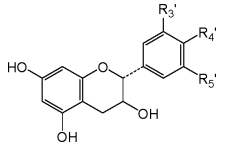
Accordingly, the objectives of the current experiments were to study: 1) hepatocytes clearance of luteolin and apigenin 2) elimination of luteolin and apigenin in a typical flavonoids-rich medication-*Flos Chrysanthemi* extract (FCE); 3) elimination of luteolin and apigenin in the presence of a series of flavonoid subgroups (Table 1). The structural features of flavonoids necessary for exerting an inhibitory effect on the clearance of luteolin and apigenin were evaluated.

2. Investigations and results

2.1. Extensive first-pass metabolism of luteolin and apigenin

To assess the role of phase II metabolism in the elimination of luteolin and apigenin, 10 μM of pure luteolin or apigenin was incubated with hepatocytes for the designed time (0–120 min) and hydrolyzed with β-glucuronidase (100 U)/sulfatase (10 U) as described above. The profiles of total elimination or phase II elimination of luteolin and apigenin vs incubation time are shown in Fig. 1. After 120 min incubation of 10 μM of

Table 1: Variation in arrangement of hydroxyl substitutions among flavonoids

Flavonoids subgroups	Flavonoids structure	Flavoniods	Substitutions						
			C3	C5	C6	C7	C8	C3'	C4'
Flavonols		quercetin	OH	OH	H	OH	H	OH	OH
		kaempferol	OH	OH	H	OH	H	H	OH
		rutin	O-rha-glu	OH	H	OH	H	OH	OH
		quercitrin	O-rha	OH	H	OH	H	OH	OH
		hyperin	O-gal	OH	H	OH	H	OH	OH
Flavones		apigenin	H	OH	H	OH	H	H	OH
		luteolin	H	OH	H	OH	H	OH	OH
		acacetin	H	OH	H	OH	H	H	OCH3
		baicalein	H	OH	OH	OH	H	H	H
		wogonin	H	OH	H	OH	OCH_3	H	H
		baicalin	H	OH	OH	O-glu	H	H	H
		scutellarin	H	OH	OH	O-glu	H	H	OH
		apiin	H	OH	H	O-apio-glu	H	H	OH
Isoflavone		ipriflavone	H	H	H	isoproxy	H	H	H
		daidzein	H	H	H	OH	H	H	OH
		puerarin	H	H	H	OH	glu-py	H	OH
Flavanone		hesperidin	**H**	OH	**H**	O-rha-glu	**H**	OH	OCH_3
		naringin	H	OH	H	O-rha-glu-	H	H	OH
Flavanonols		astilbin	O-rha	OH	H	OH	H	OH	OH
Catechins		catechin	OH	OH	H	OH	H	OH	OH
		epicatechin	OH	OH	H	OH	H	OH	OH

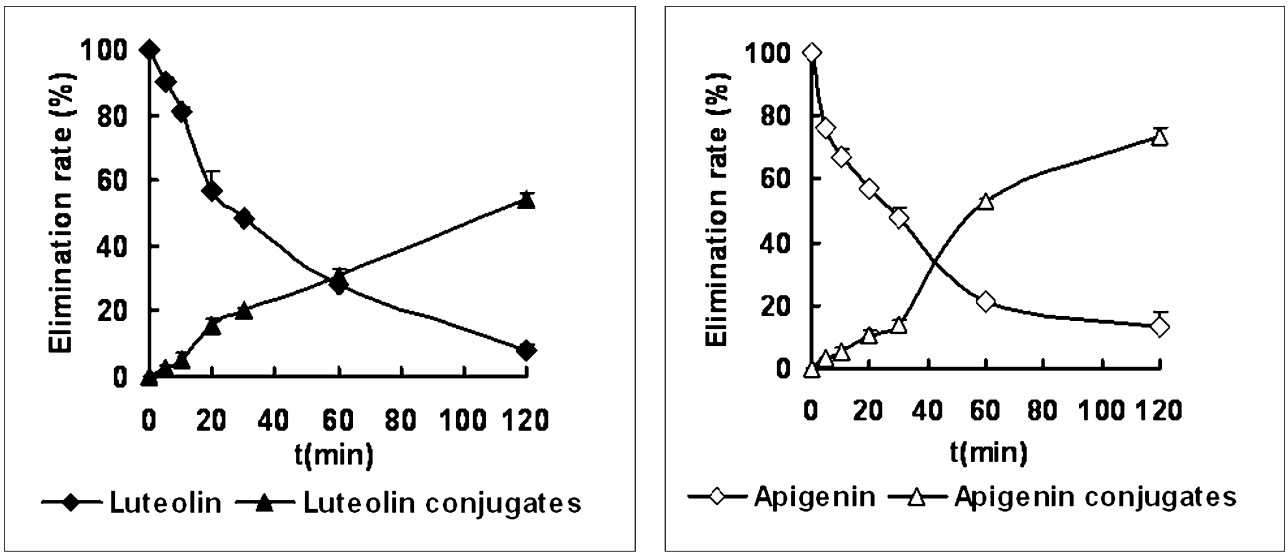
rha-glu (rhamnoglucoside); rha (rhamnoside); gal (galactoside); glu (glucuronide); apio-glu (apiosylglucoside); glu-py (glucopyranoside)

individual luteolin or apigenin with hepatocytes, the elimination percents of luteolin and apigenin were 91.9% and 86.7%, respectively. *In vitro* intrinsic clearance (Cl'int) of luteolin and apigenin was 255.6 and 321.6 ml/min/kg, and corresponding *in vivo* intrinsic clearance (Cl'int) was 45.3 and 47.0 ml/min/kg, respectively, and the predicted % liver blood flow was 82.3% and 85.4%, respectively. The total glucuronidation/sulfation elimination percents of luteolin and apigenin were found to be 54.6% and 73.6% of initial concentrations, respectively, which suggested that they were metabolized mainly as conjugated derivatives in rat hepatocytes.

2.2. Elimination of luteolin and apigenin in hydrolyzed FCE

FCE contains luteolin-7-*O*-β-D-glucoside and apigenin-7-*O*-β-D-glucoside which are transformed into luteolin and apigenin in the intestine following oral ingestion (Chen et al. 2007; Li et al. 2005). In order to determine whether the ingredients affecting the pharmacokinetic behavior of luteolin and apigenin originated from FCE, elimination comparison between pure luteolin/apigenin and hydrolyzed FCE mixture containing approximately the same dose of luteolin and apigenin were conducted in hepatocytes (Fig. 2). The half-lifes of pure luteolin and apigenin were found to be 33 and 27 min, respectively, while the half life of luteolin and apigenin in hydrolyzed FCE increased to 56 and 57 min, respectively. The results demonstrated that the clearance of luteolin and apigenin in hydrolyzed FCE was much lower than that of pure compounds.

To show whether this decreased clearance in hydrolyzed FCE was affected by co-existing of luteolin and apigenin, the elimination of luteolin and apigenin in a mixture of pure luteolin (10 μM) and apigenin (10 μM) and in the hydrolyzed FCE containing equivalent luteolin and apigenin were compared. In the mixture, inhibition (%) were $24.3 \pm 4.3\%$ and $33.5 \pm 1.2\%$ for luteolin and apigenin, respectively, while inhibition (%) for luteolin and apigenin were $34.7 \pm 6.7\%$ ($n = 3$) and $33.2 \pm 7.8\%$



(A) (B)

Fig. 1: Time-dependent elimination of luteolin (A) and apigenin (B) and formation of their conjugates by primary cultured hepatocytes (0.4 million cells/well) from male rats after 120 min incubation. Elimination rates of luteolin and apigenin as well as the formation rates of their conjugates were expressed as percents of originally administered amounts (defined as 100%). Data are expressed as mean ± S.D. ($n = 3$).

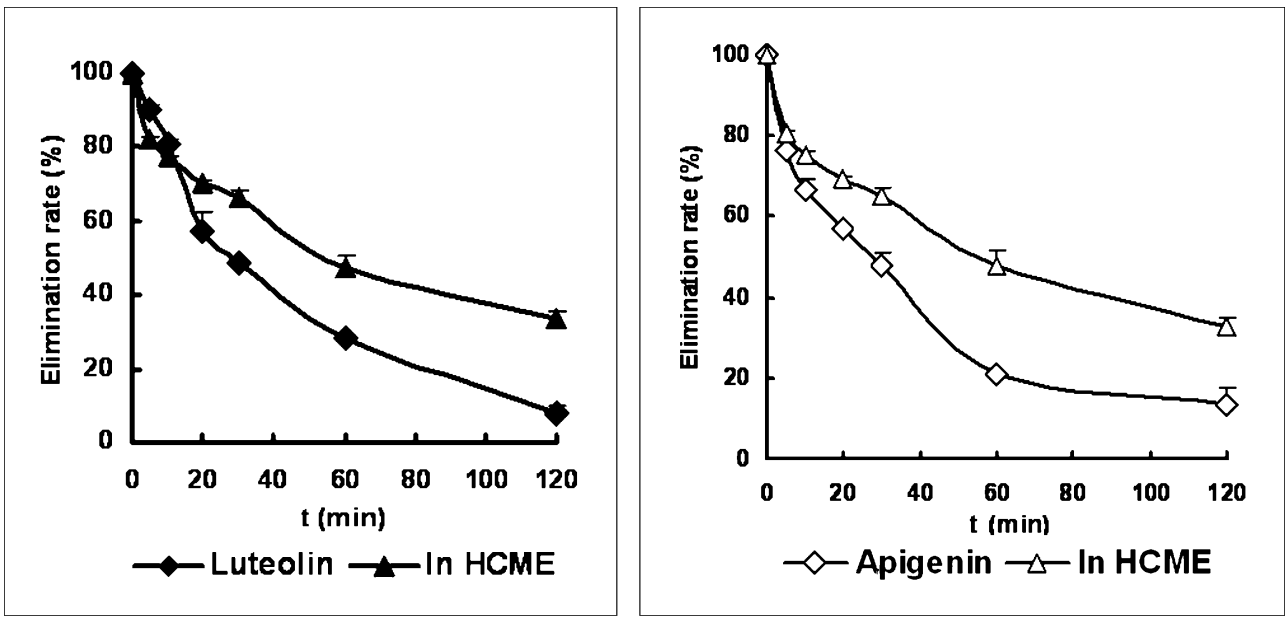
($n = 3$), respectively in hydrolyzed FCE. These results indicate that the interaction between luteolin and apigenin accounts for their decreased clearance in hydrolyzed FCE. In contract, other components had little effect.

2.3. Inhibitory effects of other flavonoids on the elimination of luteolin and apigenin

The effect of a series of flavonoids, including flavonols, flavones, isoflavone, flavanone, flavanonols, catechins, on the elimination of luteolin and apigenin was studied (Fig. 3). There was a large variation among flavonoids in inhibiting the clearance of luteolin and apigenin. The inhibitory activity of flavonoids (100 μM) on the elimination of luteolin and apigenin was calculated. The flavonol aglycones, such as quercetin, kaempferol showed the strong inhibition of the clearance of luteolin and apigenin Flavone aglycones such as luteolin, apigenin and acacetin, had almost equal potency to flavonols. Flavone aglycones baicalein and wogonin had relatively weak inhibitory potency. Isoflavone aglycone and catechins had little inhibitory activity. None of the glucosides had inhibitory effects on the elimination of luteolin and apigenin. Among six effective flavonoids, most of the IC_{50}s were less than 30 μM, indicating high inhibitory effects (Table 2).

3. Discussion

A primary hepatocytes culture system is a useful tool for predicting *in vivo* clearance and drug-drug interactions. There were good correlations between *in vitro* data in hepatocytes and *in vivo* data (Naritomi et al. 2003). It is well known that metabolic stability is an important property of drug candidates since that will



(A) (B)

Fig. 2: The elimination of luteolin (A) and apigenin (B) as pure compounds or in hydrolyzed FCE by primary cultured hepatocytes (0.4 million cells/well) from male rats after 120 min incubation. The initial concentration of luteolin and apigenin, in the pure samples, and in the hydrolyzed samples was 10 μM. Elimination rates of luteolin and apigenin were expressed as percents of originally administered amounts (defined as 100%). Data are expressed as mean ± S.D. ($n = 3$).

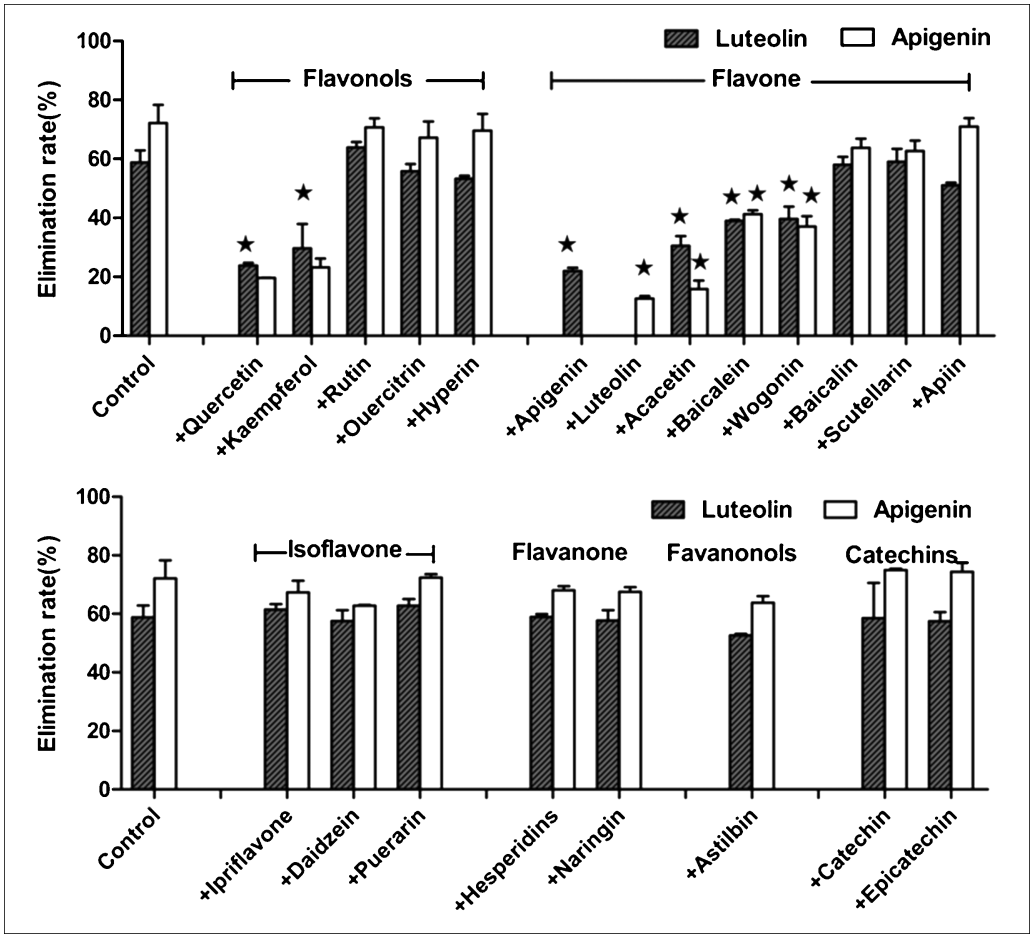


Fig. 3: Effect of other flavonoids on the elimination of luteolin or apigenin in primary cultured hepatocytes (0.4 million cells/well) from male rats after 20 min incubation. The initial concentrations of luteolin and apigenin were 10 μM. The concentration of other flavonoids as inhibitors was 100 μM. Elimination rates of luteolin and apigenin were expressed as percents of originally administered amounts (defined as 100%). Data are expressed as mean ± S.D. ($n = 3$).

Table 2: The IC_{50}s of flavonoids on the elimination of luteolin and apigenin in primary cultured rat hepatocytes

	Compounds	IC_{50}(μmol/l)	
		Luteolin	Apigenin
Flavonols	Quercetin	29.7 ± 8.2	28.2 ± 4.2
	Kaempferol	15.7 ± 8.3	24.1 ± 8.7
Flavones	Luteolin	–	17.3 ± 0.9
	Apigenin	38.2 ± 3.2	–
	Acacetin	24.3 ± 7.8	14.2 ± 0.6
	Baicalein	61.1 ± 7.9	58.9 ± 10.9
	Wogonin	58.8 ± 8.7	67.5 ± 12.5

Data are expressed as mean ± S.D. ($n = 3$).

affect pharmacokinetic parameters such as clearance, half-life and oral bioavailability. Luteolin and apigenin were found to be extensively metabolized in hepatocytes which is in line with previous results (Gradolatto et al. 2004). The present results confirmed that phase II metabolism plays a major role in the clearance of luteolin and apigenin.

The elimination process of flavonoids in hepatocytes includes oxidation metabolism, phase II metabolism (including glucuronidation,sulfation and methylation) (Gradolatto et al. 2004) as well as cellular uptake (Mukai et al. 2009). In the present study, the total levels of glucuronidation/sulfation of luteolin and apigenin reached 54.6% and 73.6% of the initial values, respectively. Flavonoids are polyphenols and, therefore, are considered to be metabolized mainly as conjugates with glucuronic acid or sulfuric acid. In our previous *in vivo* study, luteolin and apigenin in plasma, urine and bile were found mainly in conjugate form. The concentrations of luteolin and apigenin in samples hydrolyzed by acid or mixture of glucuronidase/sulfatase were reported to be much higher than those in untreated samples (Chen et al. 2007; Li et al. 2005), which supports the results of current article.

Previous work has shown that the phase I metabolism of apigenin (hepatic rat microsomes) mainly led to the formation of luteolin *in vitro* (Gradolatto et al. 2004), whereas trace or little luteolin were recovered in *ex vivo* and *in vivo* experiments (Gradolatto et al. 2004; Wan et al. 2007). In the current experiment, only 2.4 ± 0.1% ($n = 3$) of the phase I derivatives of apigenin in hepatocytes were found. Phase II conjugation of flavonoids in hepatocytes is more efficient than P450-mediated oxidation, and is clearly the dominant metabolic pathway (Gradolatto et al. 2004).

Flavonoids are all excellent substrates for glucuronidase and sulfatase. Since components of herbal flavonoids must be eliminated from the body by various metabolic enzymes, there is a potential for interaction among flavonoids. Flavonoids have been found to have inhibitory effects on phase II conjugative reactions including glucuronidation, sulfation, acetylation and *O*-methyltranslation in cultured hepatocytes (Morimitsu et al. 2004; Kadowaki et al. 2005; Mizoyama et al. 2004).

The effect of different flavonoids on the elimination of luteolin and apigenin in the cultured cells varied depending on the specific flavonoid used, which was similar to previous reports (Morimitsu et al. 2004; Kadowaki et al. 2005; Mizoyama et al. 2004). Flavone aglycones such as luteolin, apigenin and

acacetin, in which the C3 hydroxyl on the C-ring is unsubstituted, had almost equal potency to flavonols, suggesting that the C3 hydroxyl on the C ring was not a very important contributor to the observed inhibitory effects. The inhibitory efficiency of active flavonol and flavone was greater with increasing numbers of hydroxyl substitutions in the B-ring. Isoflavone, in which the B-ring is attached to the C-3 position on the C-ring, and catechins in which the C2–3 double bond and carboxyl group at the C4 position on the C-ring are absent, had no such inhibitory activity despite having a number of hydroxyl substitutions. Glycosylation also affects the inhibitory activity of flavonoids. Glycosylation not only masks hydroxyl groups, but also makes flavanoids less planar. In addition, glycosylation decreases the hydrophobicity of flavonoids. All of above conditions could be expected to impact binding to a protein, according to Xiao et al. (2009).

These results suggest that at least four key determinants in the chemical structures of flavonoids are necessary for exerting the inhibitory effects on the conjugation: 1) catechol structure (3′,4′-dihydroxylation) in the B-ring; 2) B-ring is attached to the C-2 position on the C-ring;.3) the C2–3 double bond in conjunction with the C4 carbonyl group on the C-ring; 4) no glycoside present.

Base on the present results, it can be concluded that both phase I and phase II metabolism contribute the hepatic first-pass metabolism of luteolin and apigenin. Phase II metabolism is the major pathway for their elimination which may be hindered by flavonoids, the extent of which depends on the specific flavonoid present. The identification of the clearance and interaction among flavonoids could improve our understanding of their bioavailability, and permit design of appropriate studies in humans.

4. Experimental

4.1. Chemicals and reagents

FCE was provided by the Institute of Medicine, Zhejiang University, China, containing 7.60% and 5.19% of luteolin and apigenin determined by HPLC after hydrolysis with hydrochloric acid (Lu et al. 2010; Chen et al. 2007; Li et al. 2005). Luteolin was purchased from J&K Chemical Co. Ltd. (Beijing, China). Apigenin, collagenase (type IV), MTT, β-glucuronidase (type IX-A) and sulfatase (type H-1) were purchased from Sigma Co. Ltd. (St. Louis, MO, USA). Other flavonoids were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

4.2. Animals

Male Sprague-Dawley rats weighing 180–200 g were obtained from the Experimental Animal Center of the Zhejiang Academy of Medical Sciences. All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. The rats were housed in cages at a temperature between 20–23 °C and exposed to 12:12 h light-dark cycle, with food and water available *ad libitum*. Animals were fasted for 12 h before experiment.

4.3. Preparation of solutions

Hydrolyzed FCE was prepared by an acid hydrolysis process ($HC1{:}H_2O{:}CH_3OH = 2{:}1{:}3$, at 90 °C for 45 min), and then vacuum concentrated. Hydrolyzed FCE, luteolin, apigenin as well as other flavonoids were dissolved in dimethyl sulfoxide (DMSO), and then diluted with DMSO to create a series of stock solutions. Working solutions were prepared by diluting their stock solutions with the standard Hanks' balanced salt (HBSS) to contain a final concentration of DMSO of 0.4%.

4.4. Isolation of hepatocytes

Hepatocytes were isolated based on a modified two-step collagenase digestion method of Seglen (1976). The isolated cells were resuspended in RPMI 1640 medium supplemented with fetal calf serum (10%), insulin (0.1 μM), dexamethasone (0.1 μM), penicillin (100 U/ml)/streptomycin (100 μg/ml), and then seeded in 12 well plates (Costar Corning Inc, NY, USA) at a density of 0.4×10^6 cells/well. Seeded cells were allowed to attach for 4 h in an incubator in a humidified atmosphere of 5% CO_2 at 37 °C.

4.5. Elimination of luteolin and apigenin *(Kamath et al. 2008)*

Following 4 h cultivation of hepatocytes as described above, culture medium was removed, and the cell monolayers were rinsed twice with HBSS. Cells were exposed to 1.0 ml working solutions of luteolin, apigenin or hydrolyzed FCE at 37 °C. After incubation for 20 min, 0.2 ml medium was taken from each well ($n = 3$). For time-course experiments, 0.2 ml medium was taken from each well, and three wells were used at each time point. Samples were mixed with 0.2 ml 50 mM ice cold glacial acetic acid prepared in methanol to terminate the enzyme reaction, and the mixture was centrifuged at 13000 rpm for 10 min. The supernatant (50 μl) was subjected to HPLC analysis.

Elimination of luteolin and apigenin is expressed as the percentage of remaining parent compound. It is calculated from the peak area ratio of remaining compound after different times of incubation compared to the same ratio at the time zero incubation. The half-life ($t_{1/2}$) is calculated using the following equation: $t_{1/2} = -\ln(2)/k$, where k is the slope of the ln% remaining versus time regression.

In vitro intrinsic clearance (Cl′int), *in vivo* intrinsic clearance (Cl′int) and predicted % liver blood were calculated using the following equation:

$$\text{In vitro } Cl'_{int}(ml/\min/g) = \frac{0.693}{T_{1/2}(\min)} \times \frac{\text{incubation volume (ml)}}{\text{cell per incubation}} \times \frac{1.2 \times 10^8 cells}{\text{gm liver}}$$

$$\text{In vivo } Cl'_{int}(ml/\min/kg) = \text{In vitro } Cl'_{int} \times \frac{40 \text{ gm liver}}{\text{kg body wt}}$$

$$\text{Predicted in vivo Cl′int (ml/min/kg)} = \frac{Q \times \text{in vivo} Cl'_{int}}{Q + \text{in vivo} Cl'_{int}}$$

$$\%\text{ liver blood flow} = \frac{\text{Predicted in vivo Cl′int}}{Q} \times 100\%$$

where the liver blood flow (Q) of rat is 55 ml/min/kg.

4.6. Enzyme hydrolysis

In our primary tests, phase II conjugates of luteolin and apigenin were found in the incubation system. For determination of the amount of conjugates formed, luteolin or apigenin was incubated with hepatocytes for the designed time, and then the incubation solution was further treated with β-glucuronidase (100 U)/sulfatase (10 U) at pH 5.0 and incubated at 37 °C for 30 min with shaking to degrade the conjugates. Then, samples were processed and analyzed as described previously. The formation percent of phase II metabolism was calculated using the equation:

$$\text{Formation } (\%) = \frac{\Delta C_{\text{Enzyme hydrolysis}}}{C} \times 100\%$$

where $\Delta C_{\text{Enzyme hydrolysis}}$ means the concentration difference with or without β-glucuronidase/sulfatase treatment, and C is the initial concentration before incubation.

4.7. Inhibitory effect of other flavonoids on the elimination of luteolin and apigenin

Flavonoids were studied for their ability to inhibit the elimination of luteolin and apigenin in cultured hepatocytes. Cell monolayers were pre-incubated with inhibitors (100 μM) (Morimitsu et al. 2004; Kadowaki et al. 2005; Mizoyama et al. 2004) for 10 min, then luteolin or apigenin at a final concentration of 10 μM was added to incubation medium. The cells were incubated for another 20 min as described above. Elimination of luteolin and apigenin in the presence of other flavonoids were expressed as percents of originally administered amounts (defined as 100%). The IC_{50}s were determined by measuring the elimination of luteolin/apigenin under inhibitor concentrations of 5.0 to 100 μM, and Graphit software was used to calculate the IC_{50}.

4.8. HPLC analysis

Chromatographic separation and quantitative determination was carried out as described previously (Chen et al. 2007; Li et al. 2005). Briefly, separation of luteolin and apigenin was performed on a 5 μm Agilent ZOR BAX SB-C_{18} column (250 x 4.6 mm, 5 μm, USA). The mobile phase was methanol: 0.2% H_3PO_4 (52: 48, v/v) at a flow rate of 1 ml/min. The UV detector (VWD)

was set at 350 nm. The assays for analysis of luteolin and apigenin in the medium were validated according to the guidance of the FDA for industry bioanalytical method validation. The concentrations of luteolin and apigenin were linear over the range of 1–20 μM. The recoveries of both compounds at low, medium, and high concentrations were in the range of 97.3–103.8%, and the intra- and inter-day precision of assays (expressed as RSD %) were below 4.3%.

4.9. Statistical analysis

All experiments were carried out in triplicate. The concentrations of luteolin and apigenin in the incubation medium were calculated from the standard curves. All data were expressed as mean ± standard deviation (S.D.).

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