Research Article

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Comparative biotransformation of luteolin and apigenin from the flower extract and the stem-and-leaf extract of *Dendranthema morifolium* Ramat. Tzvel. in rats

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Abstract

BACKGROUND: The flower of *Dendranthema morifolium* Ramat Tzvel has been widely used as a nutritional health supplement worldwide. However, most of the studies have focused on the flower and the rest of the plant was neglected. Our hypothesis is that similar flavonoids may be present at different parts of *D. morifolium*, and the flavonoids may undergo a similar biotransformation pathway within the body. To investigate this hypothesis, an *in vivo* pharmacokinetic experimental model was developed to explore the comparative biotransformation of luteolin and apigenin after administration of *D. morifolium* extracts (10 g kg^{-1}, p.o.) in freely moving rats. Because luteolin and apigenin mainly underwent phase II metabolism, the metabolic enzymes of β-glucuronidase/sulfatase or β-glucuronidase were used to hydrolyze the plasma sample, depending on the biotransformation pathway involved.

RESULTS: The results revealed that luteolin and apigenin mainly went through glucuronide and sulfate conjugations, respectively, in both the extract of flowers and the stem-and-leaf group. In addition, the area under the concentration curve (AUC_{last}) of luteolin glucuronides and sulfates in the group administered the stem-and-leaf extract was approximately 4.6 times higher than that of the flower extract group. The dominant products of biotransformation for apigenin were sulfates.

CONCLUSION: These findings support our hypothesis that not only the flower parts of *D. morifolium*, but also the stem-and-leaf parts contain rich flavones, including glycosides and aglycone, and they undergo similar biotransformation pathways.

Keywords: luteolin; apigenin; *Dendranthema morifolium*; comparative pharmacokinetics; biotransformation

INTRODUCTION

The flower of *Dendranthema morifolium* Ramat. Tzvel. (*Chrysanthemum morifolium* Ramat.), has been used as a popular beverage as a result of its desirable taste and aroma. In addition, chrysanthemum flowers have been widely used as medicinal herbs and common health supplemental products in many Asian and European countries. The extract of *D. morifolium* has been reported to possess several potential biological activities, such as antioxidation[1] and anti-inflammation properties,[2,3] modulation of the intestinal microbiota community,[4] neuroprotective effects[5] and antitumor activities.[6] Previous studies have indicated that chrysanthemum flowers contain diverse constituents, such as flavonoids,[7] polyphenols,[8] lignans,[9] phenolic acids[10] and polysaccharides.[4]

Flavonoids can be subdivided into subgroups such as flavones, flavonols, flavanones, flavanonols, flavanols, anthocyanins and chalcones, etc. These flavonoids not only control the color, aroma and growth of the plants, but also play various important roles in biological activities such as the antioxidant activities of phase I and II metabolism of luteolin and apigenin.[11] The basic skeleton of flavonoids is the A-B-C tricyclic ring (C6-C3-C6). The double bonds between positions C-2 and C-3 in the C ring donate hydrogen or electrons to stabilize free radicals, and the oxo group at position C-4 can bind metal ions, contributing to its antioxidant activity.[12] In addition, some other natural products have been reported to affect lipid metabolism as a result of changing the gut microflora,[13] intestinal morphology[14] and nutritional dietary supplements.[15,16]

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Flavonoids in *D. morifolium* have been found to possess antitumor activities, such as inhibiting the growth and inducing the apoptosis of gastric,[6] breast and colon[17] cancer cells. By surveying the *in vivo* biotransformation of the extract of *D. morifolium*, luteolin (3′,4′,5,7-tetrahydroxyflavone) and apigenin (4′,5,7-trihydroxyflavone) (Fig. 1) were found to be the main active flavonoids *in vivo* after oral administration of flower extract of *D. morifolium*.[18,19] Luteolin has been reported to have biological functions with respect to antihypertension, antihyperlipidemia,[20] antihyperglycemia[21] and antihistamine[22] effects, and well as in reducing the generation of ROS in body after radiation exposure.[23] Apigenin has been found to have anti-anxiety and mild sedative effects in animal experiments,[24] and the mechanism of action is similar to that of benzodiazepines on GABA receptors.[25] Inflammation could be inhibited by apigenin through the inhibition of cyclooxygenase-2, lipopolysaccharide-induced inducible nitric oxide synthase, inflammatory factors and AP-1 expression.[26]

Luteolin and apigenin are usually presented in the form of aglycones, glycosides or glucuronic acid in the flower part, such as luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside.[27] After oral administration, luteolin-7-*O*-glucoside can be easily hydrolyzed to luteolin in the small intestine and then absorbed via the intestinal mucosa,[28] and luteolin is mainly found as luteolin-3′-*O*-β-D-glucuronide in human plasma.[29] In addition to the small intestine, the liver also plays an important role in the metabolism of luteolin, which goes through phase II metabolism and is catalyzed by uridine diphosphoglucuronosyltransferases (UGTs) and catechol-*O*-methyltransferases (COMTs); the glucuronidation and methylation processes of luteolin can compensate for each other.[30,31] Apigenin mainly goes through phase I biotransformation, followed by glucuronidation and sulfation reactions by phase II enzymes in rats. *In vitro* studies have shown that apigenin can be transformed to luteolin by phase I metabolism and conjugated phase II derivatives such as glucuronic acid and sulfoconjugates.[32,33]

Recently, the issues of conserving and improving the availability of good herbal medicines in a sustainable way and plant part substitution for medical uses have received increasing attention.[34,35] The major reasons for this attention involve the desire to achieve sustainability and to prevent the depletion of natural resources; thus, the discovery and evaluation of the use of renewable/alternative parts, such as leaves and stems, to replace the originally used parts are becoming increasingly important.[36] In addition, byproducts produced during manufacturing in the food/herbal industries can be useful for further applications. For example, the phenolic compounds in olive mill wastewater can be used as active ingredients for functional foods.[37] Because most of the studies have focused on flowers, the remaining parts of *D. morifolium* have often been neglected. Only a few reports have mentioned that the leaf parts also contain flavonoids that were different from the common major flavonoids of the flower parts.[8,38,39] A previous report demonstrated that supplementation with a leaf ethanol extract of chrysanthemum (*D. morifolium*) and luteolin significantly decreased weight gain and epididymal white adipose tissue weight, suggesting that the leaf ethanol extract of chrysanthemum provides a significant effect for the prevention of obesity and metabolic disease.[40] As a result of the limited information of the chemical components in the leaf parts of *D. morifolium*, we aimed to analyze the contents of luteolin, apigenin and their glucosides in the flower and stem-and-leaf extracts of *D. morifolium*.

Luteolin-7-*O*-glucuronide and glycoside derivatives of apigenin were previously isolated from the stem-and-leaf extract of *D. morifolium* in our laboratory. Thus, we hypothesized that the stem-and-leaf extract of *D. morifolium* could also be a resource for flavonoid supplements, such as luteolin and apigenin. The present study aimed to develop an analytical system to determine the contents of luteolin and apigenin in the flower and stem-and-leaf extracts of *D. morifolium*. This method was then utilized to compare the pharmacokinetics and biotransformation of luteolin and apigenin after oral administration of the extracts of the flower and stem-and-leaf parts of *D. morifolium*. To investigate the biotransformation of the flowers and stem-and-leaf extracts of *D. morifolium*, lyophilized powders of the flower and stem-and-leaf parts (10 g kg^{-1}, p.o.) were separately administered to rats ($n = 6$) by gavage. The *in vivo* biotransformation and pharmacokinetics of luteolin and apigenin from the flower and stem-and-leaf extracts of *D. morifolium* were assessed to investigate whether the stem-and-leaf extract can be used as a potential source for the supplementation of luteolin and apigenin.

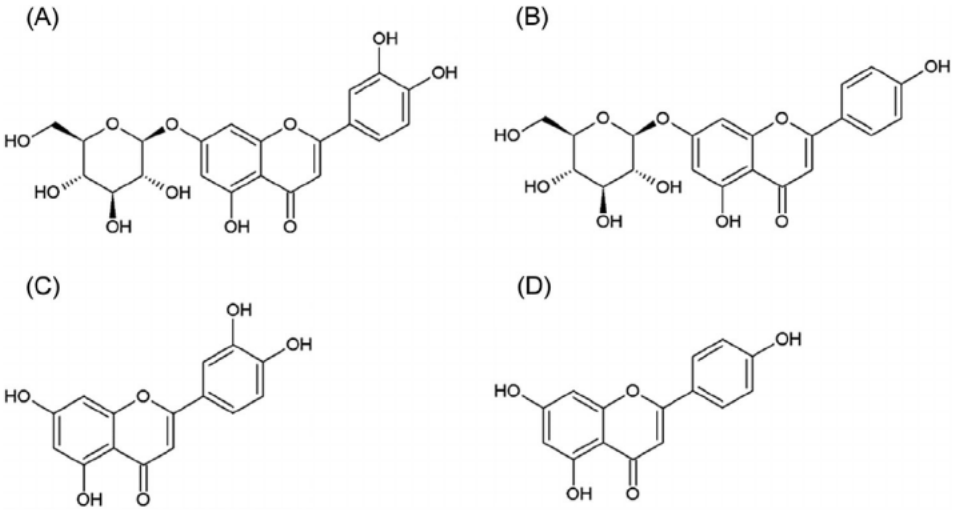

Figure 1. The chemical structures of (a) luteolin-7-*O*-glucoside, (b) apigenin-7-*O*-glucoside, (c) luteolin and (d) apigenin.

MATERIALS AND METHODS

Chemicals and reagents

Luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside and luteolin standards were isolated from the flower of *D. morifolium* by our laboratory, and their structures were confirmed using nuclear magnetic resonance and mass spectral analysis (Fig. 1). Apigenin, ornidazole (internal standard for plant extract component analysis), clozapine (internal standard for plasma sample analysis), *β*-glucuronidase (type B-1 from bovine liver), sulfatase (type H-1 from *Helix pomatia*), heparin and acetic acid were obtained from Sigma-Aldrich Chemicals (St Louis, MO, USA). Pentobarbital was acquired from SCI Pharmtech, Inc. (Taoyuan, Taiwan). Sodium acetate anhydrous and ortho-phosphoric acid were purchased from Merck (Darmstadt, Germany). Potassium phosphate monobasic was obtained from Riedel-de-Haën (Seelze, Germany). Polyethylene glycol 400 was purchased from Tokyo Chemical Industry (Tokyo, Japan). Triple-deionized water for aqueous solutions was from Millipore (Bedford, MA, USA). Acetonitrile was purchased from Spectrum (New Brunswick, NJ, USA). Methanol was purchased from Macron (Hamilton, PA, USA). The solvents used in the experiments were all liquid chromatography grade.

High-performance liquid chromatography (HPLC)

The chromatographic system (Shimadzu, Kyoto, Japan) for sample analysis included an HPLC pump (LC-20AT), an autosampler (SIL—20C), a photodiode array detector (SPD-M20A) and a degasser. The analytes were separated by an Alltima C8 column (250 × 4.6 mm, 5 μm; Alltech, Deerfield, IL, USA). The mobile phase consisted of solvent A (10 mmol L^{-1} potassium phosphate monobasic in water, adjusted to pH 2.5 by ortho-phosphoric acid) and solvent B (100% methanol). Gradient elution used for the analysis of the components of the extracts linearly ran from 35% to 45% B for 0–10 min, 45% to 50% B for 10–20 min, 50% to 60% B for 20–21 min and 60% to 70% B for 21–36 min, with rebalance for 15 min. Isocratic elution with the mobile phase consisted of solvent A: B = 50:50 (v/v) and solvent A: B = 40:60 (v/v) used for the analysis of plasma samples incubated with *β*-glucuronidase/sulfatase and *β*-glucuronidase, respectively. The flow rate was 1.0 mL min^{-1} and the injection volume was 10 μL. The optimal detection UV wavelength was set at 340 nm.

Method validation

The analytic method in this experiment was validated according to the Bioanalytical Method Validation Guidance for Industry by the US Food and Drug Administration (Docket Number: FDA-2013-D-1020). The calibration curve was generated by plotting the peak area ratios of the analyte to the internal standard against the nominal concentrations. Regression analysis was used to evaluate linearity, and the coefficient of determination (r^2) had to be greater than 0.995. The limit of detection (LOD) was defined as a signal-to-noise ratio (S/N) of greater than 3, and the lowest limit of quantification (LLOQ) was defined as an S/N of 10. The mean values for the accuracy and the precision of calibration samples must be within ±15%, except at the LLOQ, which must be within ±20%. Six replicates of calibration samples were analyzed on the same day (intraday) and on six continuous, different days (interday) to evaluate the accuracy and precision. The accuracy was presented as bias representing the proximity of mean results (observed concentration, C_{obs}) from this analytical method to the true concentrations (nominal concentration, C_{nom}): Bias (%) = $(C_{obs} - C_{nom})/C_{nom}$. The precision was presented as the percentage of the ratio of the SD to the observed concentration and was expressed as the percentage relative SD (RSD %): RSD (%) = $[SD/C_{obs}] \times 100$. The recovery was evaluated by the ratio of the peak area of the pre-extraction spiked matrix sample to that of the neat sample prepared with acetonitrile instead of plasma. Stability was established by placing the validation samples under different conditions: autosampler (12 h at 4 °C), short term (6 h at room temperature), long term (7 days at −20 °C) and three freeze–thaw cycles (freeze at −20 °C for 24 h and then thaw at room temperature for 12 h repeated for three cycles) and comparing their before and after concentrations.

Plant materials

The fresh stem-and-leaf parts of *D. morifolium* were obtained from farmers in Miaoli county, Taiwan, and the dried flowers of *D. morifolium* were purchased from Tongluo Farmer's Association (Miaoli County, Taiwan). Fresh stem-and-leaf parts were dried at 45 °C overnight. Both the stem-and-leaf and flower parts of *D. morifolium* were ground into powders before extraction. A herbal powder sample (500 g) was extracted with 5 L of 50% ethanol by reflux for 30 min for two extraction times. Then, the extract solutions were combined and the alcohol in the solution was removed under vacuum evaporation. After lyophilization, the freeze-dried extracts of flower and stem-and-leaf parts were obtained and used for the subsequent experiments. The yields of the 50% ethanol extracts from the flower and stem-and-leaf parts were 25.32% and 27.83%, respectively.

Preparation and analysis of plant extract samples

The standard stock solutions of luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin, as well as the internal standard (ornidazole) solution, were all prepared to concentrations of 1 mg mL^{-1} with methanol. The standard working solutions of luteolin-7-*O*-glucoside, luteolin and apigenin were diluted from the standard stock solutions to concentrations of 1, 5, 10, 50 and 100 μg mL^{-1}, and the standard working solution of apigenin-7-*O*-glucoside was diluted from the standard stock solution to concentrations of 1, 5, 10, 50, 100 and 500 μg mL^{-1} with methanol. The internal standard working solution was diluted from the internal standard stock solution to 200 μg mL^{-1} with methanol. All solutions were sealed with parafilm and stored at −20 °C until use. The calibration samples were prepared at final concentrations of 0.1, 0.5, 1, 5 and 10 μg mL^{-1} (for luteolin-7-*O*-glucoside, luteolin and apigenin) and 0.1, 0.5, 1, 5, 10 and 50 μg mL^{-1} (for apigenin-7-*O*-glucoside). The flower and the stem-and-leaf extracts were filtered through 0.22-μm membrane filters (Millex-GV; Millipore) and 10 μL was injected into the HPLC for further analysis. To identify the existence of analytes in the extract, the individual peak was confirmed by the retention time, UV spectrum, and addition of the spiked authentic standard to reconfirm the peak in the chromatograms.

Animal experiments

The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) by the Institute Animal Experimentation Committee of National Yang-Ming University, Taipei, Taiwan (IACUC approval no: 1080328). All animal experiments followed the National Yang-Ming University guidelines and procedures. Adult male specific pathogen-free Sprague–Dawley rats (250 ± 50 g) were obtained from the Laboratory Animal Center of the National Yang-Ming University. The animals were given free access to water and food (Laboratory Rodent Diet

5001; PMI Feeds, Richmond, IN, USA) and were maintained under a 12:12 h light/dark photocycle.

To conduct pharmacokinetic studies under the usual physiological status, a model of conscious and freely moving rats was applied to exclude the stress caused by restraint or anesthesia. The rats were anesthetized with pentobarbital (50 mg kg^{-1}, i.p.) during the surgery. A polyethylene tube (PE-50) was implanted in the right jugular vein for blood sampling. The tube crossed the subcutaneous tissue and was fixed at its exit at the dorsal neck region. To avoid blood coagulation, the polyethylene tube was rinsed with heparinized saline (20 units mL^{-1}). The rats were placed in experimental cages for 1 day until recovery from anesthesia after surgery and were fasted for 12 h but given *ad libitum* access to water before oral administration.

Drug administration

The flower and stem-and-leaf extracts of *D. morifolium* were dissolved in water, and a dose of 10 g kg^{-1} was orally administered to the rats ($n = 6$) by gavage individually. An aliquot of blood (200 μL) was collected from the jugular vein at baseline, 5, 15, 30, 60, 120, 180, 240, 360, 480, 600, 720, 960 and 1440 min after dosing and was stored in a plastic centrifuge vial rinsed with heparin. Blood samples were centrifuged at 16 000 × *g* for 10 min at 4 °C to separate plasma. The plasma was stored at −20 °C before sample preparation and analysis.

Preparation of rat plasma samples

Enzymatic hydrolysis reactions using *β*-glucuronidase/sulfatase or *β*-glucuronidase were performed to obtain aglycone, such as luteolin and apigenin. An aliquot of plasma blank (50 μL) or sample with *β*-glucuronidase/sulfatase (50 μL; sulfatase 100 units mL^{-1} in acetate buffer, pH 5.0, containing *β*-glucuronidase 3000 units mL^{-1}) or *β*-glucuronidase (3000 units mL^{-1} in acetate buffer, pH 5.0) was incubated in a 37 °C water bath for 1 h. The hydrolyzed plasma (50 μL) sample was fortified with the internal standard solution (10 μL) and acetonitrile (90 μL) to terminate the hydrolysis. The mixture of sample was vortexed for 5 min and centrifuged at 10 000 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.22-μm membrane filter (Millex-GV; Millipore) before HPLC analysis.

Preparation of calibration standards for rat plasma analysis

The standard stock solution containing luteolin and apigenin (1.0 mg mL^{-1}) and the internal standard solution containing clozapine (1.0 mg mL^{-1}) were both prepared in acetonitrile. The standard working solutions containing both luteolin and apigenin were diluted from the standard stock solutions to concentrations of 5, 10, 20, 100, 200 and 500 μg mL^{-1} with methanol. The internal standard working solution was diluted from the internal standard stock solution to 200 μg mL^{-1} with acetonitrile. All the solutions were sealed with parafilm and stored at −20 °C until use. Blank plasma (40 μL), standard working solutions (10 μL), acetate buffer (50 μL), internal standard working solution (10 μL) and acetonitrile (90 μL) were used to prepare the various concentrations of 0.25, 0.5, 1, 5, 10 and 25 μg mL^{-1} for the calibration curve for the analytes. Validation samples at low, medium and high concentrations (0.25, 5 and 25 μg mL^{-1}) were prepared in the same manner.

Statistical analysis

WinNonlin Standard Edition, version 5.3 (Scientific Consulting, Apex, NC, USA) was used for calculating the pharmacokinetic parameters, including the maximum plasma concentration (C_{max}), time to reach the maximum concentrations (T_{max}), half-life ($t_{1/2}$), area under the concentration−time curve (AUC) and oral clearance (CL). The results were estimated by a noncompartmental analysis. Statistical data are expressed as the mean ± SD. The comparison between two groups was determined by Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Optimization the conditions of HPLC coupled to a photodiode array detector

The organic solvents of methanol and acetonitrile were used to optimize the peak shape of the analytes, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin, and using methanol provided a sharper peak shape than that of acetonitrile. Although a phenyl-hexyl column (150 mm × 4.6 mm i.d.; particle size 5 μm) coupled with acetonitrile as the mobile phase was used in a previous study,[18] the differences in conditions may result from the different properties of the C8 and phenyl-hexyl columns. In addition, the peak tailing was improved by using KH_2PO_4 solution adjusted to pH 2.5 as the water phase, which was consistent with a previous study using sodium dihydrogen phosphate solution.[18] The internal standards of ornidazole and clozapine were selected in the present study as a result of the lack of interference during the chromatogram monitored at UV 340 nm. The retention times of the internal standard (ornidazole), luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin were 7.9, 16.3, 20.6, 28.9 and 31.7 min, respectively (Fig. 2). The UV spectra of the analytes recorded by the photodiode array detector are presented in Fig. 3.

Analysis of flavonoids in the plant extracts

The analysis of flavonoids in the plant extracts was validated according to the Bioanalytical Method Validation Guidance for Industry by the US Food and Drug Administration (Docket Number: FDA-2013-D-1020). Luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin were analyzed in the plant extracts. The calibration curves resulted in good linearity ranging from 0.1 to 50 μg mL^{-1} for extract component analysis. The coefficient of determination (r^2) values were all > 0.995. The LLOQ values for luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin were all 0.1 μg mL^{-1}, and the LOD values were all 0.05 μg mL^{-1} in the standard solution.

The accuracy (bias %) and precision (RSD %) of luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin in standard solution (50% MeOH) are shown in Table 1. The intra- and interday accuracy and precision values of the four standard solutions at the LLOQ were within ±15.76%, and the others were all within ±9.33%, which demonstrated that this method has good reproducibility (Table 1).

To identify the analytes in the chromatogram, the peak retention time (Fig. 2), UV spectra (Fig. 3) and spiking with authentic standards were used to reconfirm the analytes of luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin in the chromatogram (Fig. 2). The contents of luteolin, apigenin, luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside in the flower extract and the stem-and-leaf extract are summarized in Table 2. The contents of luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside in the flower parts were 3973 ± 104 μg g^{-1} and 31 780 ± 83.9 μg g^{-1}, respectively. These results correlated with their aglycones, and the contents of luteolin and apigenin were

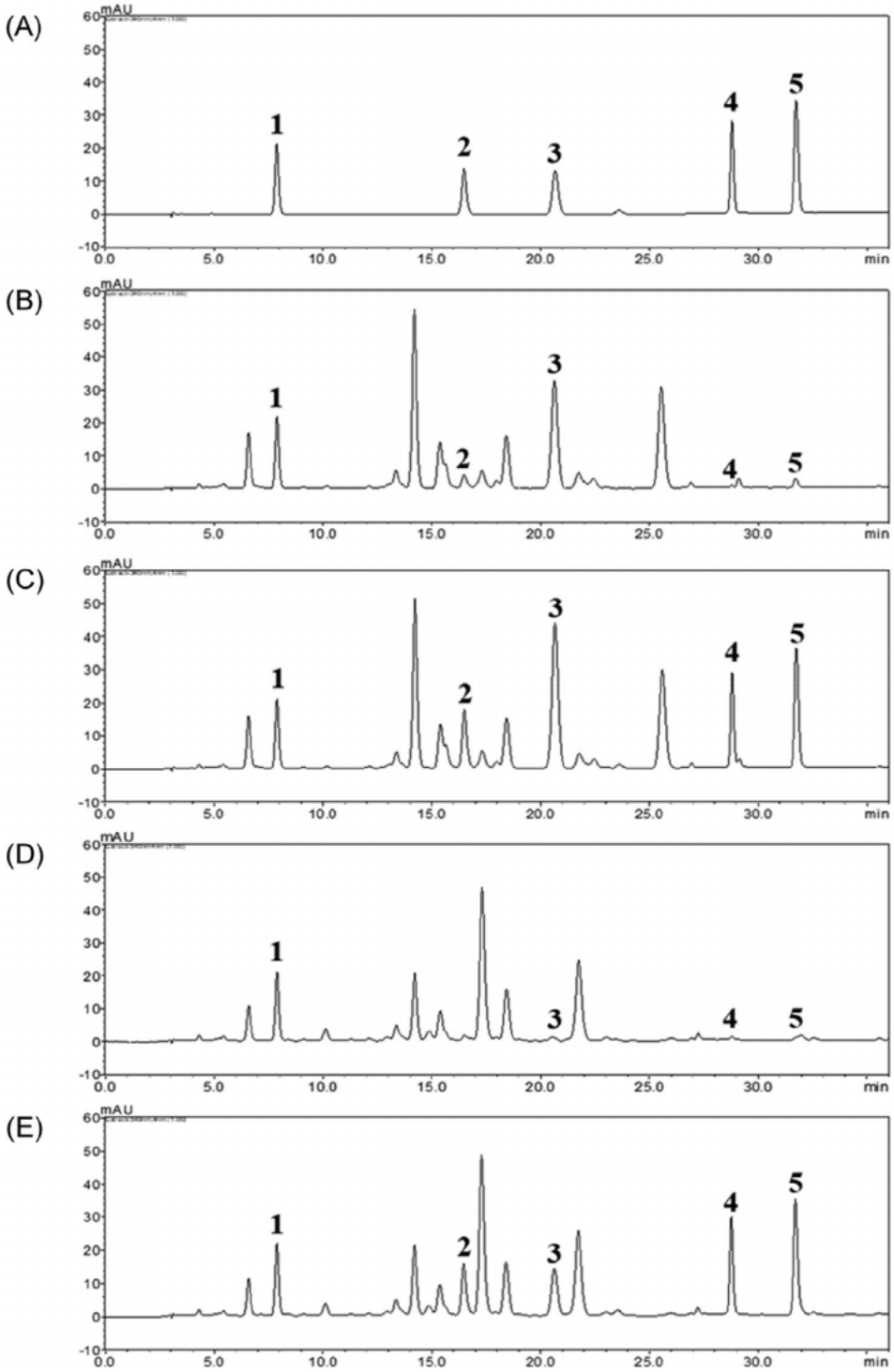

Figure 2. HPLC chromatograms of (a) analyte standards of luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin each at a concentration of 10 μg mL^{-1} containing internal standard (IS); (b) flower extract 0.8 mg mL^{-1} spiked with internal standard (IS); (c) flower extract 0.8 mg mL^{-1} spiked with standards 10 μg mL^{-1} and IS; (d) stem-and-leaf extract 0.8 mg mL^{-1} spiked with IS; and (e) stem-and-leaf extract 0.8 mg mL^{-1} spiked with standards 10 μg mL^{-1} and IS. Peak 1: ornidazole (IS) 20 μg mL^{-1}, 2: luteolin-7-*O*-glucoside, 3: apigenin-7-*O*-glucoside, 4: luteolin, 5: apigenin.

261.2 ± 2.74 μg g^{-1} and 931.6 ± 8.19 μg g^{-1}, respectively. Compared with the content ratios of glycoside and aglycone, the ratios ($C_{glycoside}/C_{aglycone}$) of luteolin and apigenin in the flower extract were 15.2 and 34.1, respectively, which suggested that glycoside was the major form present in the flower parts (Table 2). Among the four flavonoids, three (luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside and apigenin) had higher amounts in the flower extract than in the stem-and-leaf extract, with luteolin being the exception. The content of luteolin in the stem-and-leaf extract was higher than that in the flower extract. However, luteolin-

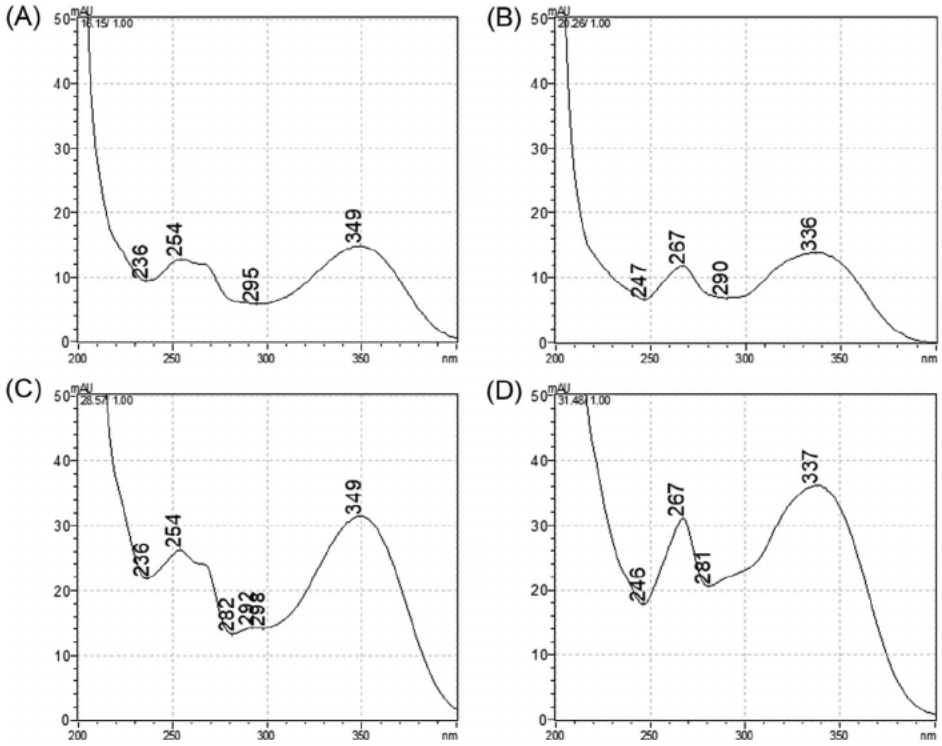

Figure 3. UV spectra of (a) luteolin-7-*O*-glucoside, (b) apigenin-7-*O*-glucoside, (c) luteolin and (d) apigenin.

Table 1. Accuracy (bias %) and precision (RSD %) of luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin and apigenin standards

	Intraday			Interday		
C_{nom} (μg mL^{-1})	C_{obs} (μg mL^{-1})	Accuracy (%)	Precision (%)	C_{obs} (μg mL^{-1})	Accuracy (%)	Precision (%)
Luteolin-7-*O*-glucoside						
0.1	0.09 ± 0.01	−12.14%	9.17%	0.09 ± 0.01	−8.06%	14.26%
1	0.92 ± 0.02	−7.88%	1.73%	0.95 ± 0.07	−5.17%	7.90%
10	9.97 ± 0.02	−0.30%	0.23%	10.08 ± 0.94	0.76%	9.33%
Apigenin-7-*O*-glucoside						
0.1	0.1 ± 0.01	3.34%	8.63%	0.1 ± 0.01	2.19%	11.92%
1	1.01 ± 0.02	1.22%	1.56%	1.01 ± 0.05	0.56%	4.59%
10	9.87 ± 0.01	1.33%	0.14%	10.07 ± 0.33	0.65%	3.31%
50	50.02 ± 0.09	0.03%	0.18%	49.75 ± 0.14	−0.5%	0.28%
Luteolin						
0.1	0.09 ± 0.01	−11.72%	8.11%	0.09 ± 0.01	−7.97%	5.53%
1	0.96 ± 0.02	−4.06%	1.61%	0.97 ± 0.02	−3.47%	2.53%
10	9.98 ± 0.03	−0.17%	0.27%	10.18 ± 0.21	1.81%	2.02%
Apigenin						
0.1	0.08 ± 0.00	−15.76%	2.71%	0.08 ± 0.00	−16.38%	2.85%
1	0.97 ± 0.01	−3.18%	1.46%	0.97 ± 0.02	−2.79%	2.51%
10	9.98 ± 0.01	−0.19%	0.15%	10.08 ± 0.18	0.82%	1.77%

Data are expressed as the mean ± SD (n = 6). C_{nom}, nominal concentration; C_{obs}, observed concentration.

7-*O*-glucoside was not detected in the stem-and-leaf extract, which may be a result of the content being lower than the detection limit of the current analytical system.

Method validation for the analytes in rat plasma

The analytical method for the analytes in rat plasma was also validated in accordance with the Bioanalytical Method Validation Guidance for Industry by USFDA.[18] Luteolin and apigenin were analyzed in plasma. The r^2 values of the calibration curves ranging from 0.25 to 25 μg mL^{-1} for plasma sample analysis were all > 0.995. The LLOQs for luteolin and apigenin were both 0.25 μg mL^{-1} and the LODs were both 0.1 μg mL^{-1} in plasma.

The accuracy (bias %) and precision (RSD %) of luteolin and apigenin in rat plasma are shown in Table 3. The intraday accuracy of

Table 2. Contents of the 4 flavonoids in flower and stem-and-leaf extracts of *D. morifolium*

	Flower extract	Stem-and-leaf extract
Luteolin-7-*O*-glucoside	3973 ± 104 μg g^{-1}	ND[a]
Apigenin-7-*O*-glucoside	31 780 ± 83.9 μg g^{-1}	990.8 ± 168 μg g^{-1}
Luteolin	261.2 ± 2.74 μg g^{-1}	467.6 ± 9.53 μg g^{-1}
Apigenin	931.6 ± 8.19 μg g^{-1}	453.2 ± 11.6 μg g^{-1}

[a] Not detected. Data are presented as the mean ± SD ($n = 4$).

luteolin ranged from 0.74% to −10.04% and apigenin from −0.46% to −16.27%, and the intraday precision ranges were 1.50–5.86% and 1.67–5.79%, respectively. The interday accuracy of luteolin ranged from 0.32% to 14.14%, and that of apigenin ranged from 0.58% to 7.00%, and the interday precision ranges were 1.91–5.86% and 1.26–9.76%, respectively. All the accuracy and precision values were within ±15%, except at the LLOQ, which was within ±20%. The results indicated that the method was repeatable on the same day and on different days (Table 3).

The recoveries (%) of low-, medium- and high-quality control (QC) concentrations of luteolin and apigenin are presented in Table 4 and were obtained by dividing the peak area of the QC concentrations prepared from the standard solutions by the peak area of the blank plasma spiked with the QC concentrations. The extraction rates demonstrated the good efficiency of this method (Table 4).

Stability (%) analysis was conducted to test the unprepared and prepared flavone-spiked validation samples under various conditions, and the results are shown in Table 5. The stability of the prepared QC samples in the autosampler (12 h at 4 °C) ranged from 98.17% to 111.3%. The stability values of the unprepared QC samples assessed in the short term (6 h at room temperature), long term (7 days at −20 °C) and three freeze–thaw cycles (freeze at −20 °C for 24 h and then thaw at room temperature for 12 h, repeated for three cycles) ranged from 92.1% to 100.8%, 85.04% to 97.71%, and 83.29% to 97.9, respectively. Both luteolin and apigenin in plasma showed good stability under the four conditions (Table 5).

Optimization of the enzymatic hydrolysis reactions

To optimize the efficiency of enzyme activity, a pretest was conducted to examine the unit activities of β-glucuronidase/sulfatase and β-glucuronidase used for hydrolysis reactions. An aliquot of 50 μL of β-glucuronidase/sulfatase was added to 50 μL of plasma sample and incubated in a 37 °C water bath for 1 h. Different concentrations of β-glucuronidase/sulfatase were used to optimize the reaction such as 1000, 3000, 6000 or 15 000 units mL^{-1} were used for β-glucuronidase and 33.33, 100, 200 or 500 units mL^{-1} were used for sulfatase. The results suggested that the 3000 units mL^{-1} of β-glucuronidase and 100 units mL^{-1} of sulfatase provided the best reaction for the enzymatic hydrolysis. The plasma sample was collected at 450 min after stem-and-leaf extract administration (10 g kg^{-1}, p.o.), which was a result of the time reach C_{max}

To optimize the dose of botanic extracts for oral administration in rat, a pilot study was performed. According to the analytical results from the extracts of *D. morifolium*, the flavone contents of luteolin and apigenin were 261.2 ± 2.74 and 931.6 ± 8.19 μg g^{-1}, respectively (Table 2). However, after oral administration of the botanic extract (10 g kg^{-1}, p.o.), the peaks of luteolin and apigenin were all undetectable by the analytical system, which may be a result of the low bioavailability or fast biotransformation of the flavones. Thus, the β-glucuronidase/sulfatase and β-glucuronidase were used for enzymatic hydrolysis reactions. Based on the pilot study, the dose of botanic extract (10 g kg^{-1}, p.o.) was selected for pharmacokinetic investigation, as well as to explore the potential metabolic mechanism of luteolin and apigenin in rat.

Typical chromatograms are provided for luteolin, apigenin and internal standard in rat plasma treated with β-glucuronidase/sulfatase or β-glucuronidase (Fig. 4) after administration of the flower

Table 3. Accuracy (bias %) and precision (RSD %) of luteolin and apigenin in rat plasma

	intraday			interday		
C_{nom} (μg mL^{-1})	C_{obs} (μg mL^{-1})	Accuracy (%)	Precision (%)	C_{obs} (μg mL^{-1})	Accuracy (%)	Precision (%)
Luteolin						
0.25	0.22 ± 0.01	−10.04%	5.86%	0.29 ± 0.02	14.14%	5.86%
0.5	0.51 ± 0.02	2.85%	3.2%	0.53 ± 0.01	6.06%	1.91%
1	1.04 ± 0.03	3.74%	2.54%	1 ± 0.05	0.32%	5.36%
5	5.11 ± 0.12	2.1%	2.31%	4.66 ± 0.12	−6.78%	2.59%
10	10.07 ± 0.15	0.74%	1.5%	9.22 ± 0.2	−7.85%	2.18%
25	25.25 ± 0.85	0.98%	3.37%	23.11 ± 0.82	−7.56%	3.53%
Apigenin						
0.25	0.21 ± 0.01	−16.27%	5.79%	0.26 ± 0.03	5.56%	9.76%
0.5	0.49 ± 0.01	−2.34%	2.85%	0.53 ± 0.02	7.00%	4.48%
1	1.01 ± 0.03	0.72%	2.76%	1.05 ± 0.07	4.51%	6.74%
5	4.98 ± 0.08	−0.46%	1.67%	5.03 ± 0.14	0.58%	2.70%
10	9.86 ± 0.21	−1.4%	2.16%	9.92 ± 0.12	−0.84%	1.26%
25	24.44 ± 0.70	−2.24%	2.88%	24.48 ± 0.51	−2.10%	2.07%

Data are expressed as the mean ± SD ($n = 6$). C_{nom}, nominal concentration; C_{obs}, observed concentration.

Table 4. Recovery of luteolin and apigenin in rat plasma

Nominal concentration (μg mL^{-1})	Peak area		Recovery (%)
	Neat sample	Pre-extraction spiked matrix sample	
Luteolin			
0.25	7104 ± 323	7063 ± 164	99.48 ± 2.27
5	162 500 ± 4729	154 500 ± 437	95.15 ± 3.01
25	790 900 ± 27 108	741 200 ± 11 721	93.81 ± 4.33
Apigenin			
0.25	11 480 ± 163	10 710 ± 735	93.23 ± 5.42
5	261 800 ± 6654	256 776 ± 2529	98.13 ± 2.27
25	1 260 000 ± 29 730	1 191 498 ± 15 560	94.58 ± 3.29

Data are expressed as the mean ± SD ($n = 3$). Recovery (%) = (Peak area of the pre-extraction spiked matrix sample)/(Peak area of the neat sample).

Table 5. Stability of luteolin and apigenin in rat plasma

Nominal concentration (μg mL^{-1})	Autosampler stability (%)	Short-term stability (%)	Long-term stability (%)	Freeze–thaw stability (%)
Luteolin				
0.25	98.17 ± 5.86	100.8 ± 1.94	97.71 ± 1.39	97.90 ± 0.84
5	100.1 ± 0.33	94.66 ± 0.42	94.60 ± 3.91	89.15 ± 1.70
25	98.41 ± 0.48	92.10 ± 3.76	85.04 ± 3.63	83.29 ± 2.79
Apigenin				
0.25	111.3 ± 4.59	95.96 ± 1.03	96.19 ± 1.28	94.92 ± 2.89
5	100.9 ± 0.70	94.17 ± 1.03	94.32 ± 2.46	94.03 ± 1.83
25	98.98 ± 2.28	92.97 ± 0.40	86.23 ± 2.32	89.73 ± 2.42

Data are expressed as the mean ± SD ($n = 3$). Stability (%) = (C_{after}/C_{before}) × 100. Autosampler stability: the samples were kept in a 4 °C autosampler for 12 h. Short-term stability: the samples were kept at room temperature for 6 h. Long-term stability: the samples were kept at −20 °C for 1 week. Freeze–thaw stability: the samples were frozen at −20 °C for 24 h and then thawed at room temperature for 12 h, repeated for three cycles.

and stem-and-leaf extracts (10 g kg^{-1}, p.o.). The results showed that the apigenin levels in both the flower and stem-and-leaf extract administration groups were higher than the luteolin levels when treated with β-glucuronidase/sulfatase. Then again, with the same processes, the concentration of luteolin was higher than that of apigenin when treated with β-glucuronidase. These results suggested that the predominant phase II metabolic pathways of luteolin and apigenin were glucuronidation and sulfation conjugation, respectively.

Hydrolysis of glucuronide and sulfate conjugation for luteolin and apigenin

To investigate the glucuronide or sulfate conjugations for luteolin and apigenin, the β-glucuronidase/sulfatase or β-glucuronidase enzymes were used to treat the plasma sample after oral administration of flower and stem-and-leaf extracts. The data demonstrated that, for the plasma sample collected at 720 min after flower extract administration (10 g kg^{-1}, p.o.), only a small amount of luteolin could be detected, although apigenin was undetectable. When the same plasma sample was incubated with β-glucuronidase/sulfatase via the same processes, the luteolin and apigenin concentrations were 0.75 and 9.37 μg mL^{-1}, respectively. For the plasma sample collected at 720 min after stem-and-leaf extract administration (10 g kg^{-1}, p.o.), both luteolin and apigenin were undetectable. When the same plasma sample was treated with β-glucuronidase/sulfatase, the luteolin and apigenin concentrations were 3.67 and 5.62 μg mL^{-1}, respectively. These results demonstrated that most luteolin and apigenin existed as glucuronic and sulfate conjugates in the rat plasma. These phenomena revealed that reversible phase II metabolic pathway had taken place for luteolin and apigenin.

Glucuronidation of luteolin and apigenin

To identify the catalyze enzyme, only β-glucuronidase was used instead of mixed β-glucuronidase/sulfatase in the above study. The results demonstrated that, for the plasma sample collected at 720 min after administration of flower extract (10 g kg^{-1}, p.o.) and stem-and-leaf extract (10 g kg^{-1}, p.o.), only a small amount of luteolin could be detected, and apigenin was undetectable. When plasma samples were incubated with β-glucuronidase under the same conditions, the luteolin concentrations in the groups administered flower and stem-and-leaf extracts were 0.63 and 2.85 μg mL^{-1}, respectively. These results suggested that luteolin primarily underwent glucuronidation rather than apigenin. The mean plasma concentration-time profiles of rat plasma incubated with β-glucuronidase/sulfatase and β-glucuronidase are shown in Fig. 5. The pharmacokinetics of luteolin and apigenin after the administration of flower or stem-and-leaf extract (10 g kg^{-1}, p.o.) are shown in Table 6.

Luteolin and apigenin, distributed in different parts of *D. morifolium*, mostly present as forms of glucoside or glucuronic acid conjugation, which can be hydrolyzed to aglycones in rat cultured hepatocytes[41] and in rabbit, rat and human fecal flora.[42] Previous studies have shown that both luteolin and apigenin

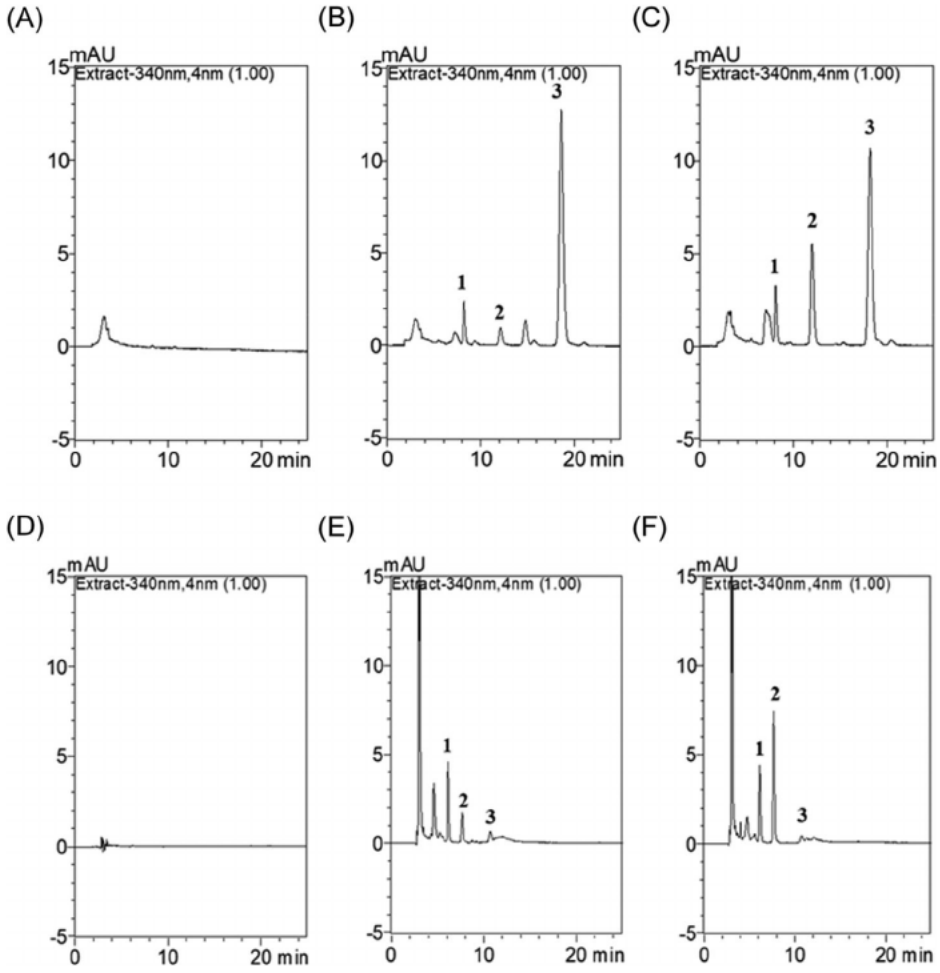

Figure 4. HPLC chromatograms of (a) blank plasma sample incubated with β-glucuronidase/sulfatase; (b) luteolin (0.75 μg mL^{-1}) and apigenin (9.37 μg mL^{-1}) in plasma sample collected at 720 min after flower extract of *D. morifolium* (10 g kg^{-1}, p.o.) administration; (c) luteolin (3.67 μg mL^{-1}) and apigenin (5.62 μg mL^{-1}) in plasma sample collected at 720 min after stem-and-leaf extract of *D. morifolium* (10 g kg^{-1}, p.o.) administration incubated with β-glucuronidase/sulfatase; (d) blank plasma sample incubated with β-glucuronidase; (e) luteolin (0.63 μg mL^{-1}) in plasma sample collected at 720 min after flower extract of *D. morifolium* (10 g kg^{-1}, p.o.) administration; and (f) luteolin (2.85 μg mL^{-1}) in plasma sample collected at 720 min after stem-and-leaf extract of *D. morifolium* (10 g kg^{-1}, p.o.) administration incubated with β-glucuronidase. Peak 1: internal standard, clozapine, 2: luteolin, 3: apigenin.

undergo phase II metabolism by UGTs, sulfotransferases and COMTs in the intestine and liver, resulting in very little detectable luteolin and apigenin after oral administration of the extracts. Therefore, β-glucuronidase and sulfatase were used to determine the amounts of luteolin and apigenin conjugates in plasma and to compare the oral bioavailability of flavonoids between the flower and stem-and-leaf extracts of *D. morifolium*. An *ex vivo* study demonstrated that, during apigenin perfusion using an isolated rat liver, none of the phase I metabolites could be recovered. By contrast, the glucuronic conjugations and sulfonic conjugations of apigenin were recovered and identified.[32] This phenomenon is consistent with our finding that only a small amount of aglycone can be detected, and the conjugated metabolites were major forms present within the body after the ingestion of the herbal extracts.

Pharmacokinetics of luteolin and apigenin treated with β-glucuronidase/sulfatase and β-glucuronidase

The pharmacokinetic results demonstrated that the AUC values of luteolin in plasma after administration of flower extract (10 g kg^{-1}, p.o.) showed no significant difference between enzymatic hydrolysis by β-glucuronidase/sulfatase and β-glucuronidase. These results again confirm that the major enzyme responsible for luteolin metabolism was β-glucuronidase (Table 6). Other pharmacokinetic parameters, such as the C_{max}, T_{max} and $t_{1/2}$, were not significantly different in the hydrolyzed results of the two enzymes (Table 6). For apigenin, the AUC values of hydrolysis by β-glucuronidase/sulfatase were approximately 45 times higher than those of β-glucuronidase hydrolysis alone and the C_{max} of obtained by the β-glucuronidase/sulfatase treatment was approximately 19.6 times higher than that obtained by the β-glucuronidase alone treatment. Although there were no significant differences between their T_{max} and $t_{1/2}$ values, suggesting that apigenin primarily went through the metabolic pathway of sulfate conjugation (Table 6).

In the stem-and-leaf extract group, the AUC_{0-t} and C_{max} values of luteolin after hydrolysis by β-glucuronidase/sulfatase and β-glucuronidase were statistically significant. When the plasma sample was treated by the hydrolysis of β-glucuronidase/sulfatase, the AUC_{last} of apigenin was 6223 ± 1000 min μg mL^{-1},

although the pharmacokinetic parameters were not applicable after β-glucuronidase hydrolysis. This result confirms that sulfate conjugations favor the metabolic pathway for apigenin (Table 6).

β-Glucuronidase/sulfatase and β-glucuronidase were individually used in our experiment to clarify the metabolic pathways for luteolin and apigenin. The results demonstrated that, regardless of whether the flower extract or stem-and-leaf extract was administered, luteolin mainly existed as glucuronide conjugated, and apigenin mainly appeared as sulfate conjugated in plasma. These results are consistent with a previous study showing that luteolin mainly undergoes UGT metabolism between UGTs and COMT metabolism.[30] Integrated with the results of this experiment, luteolin mainly undergoes UGT metabolism *in vivo*.

To evaluate the predominate phase II metabolic mechanism of luteolin and apigenin, a previous report demonstrated that the conjugated metabolites of amentoflavone (AMF) treated with β-glucuronidase/sulfatase in plasma in various routes of administration to rats, including oral gavage and intravenous and intraperitoneal injection, had conjugation ratios of AMF (%) = 100 × (AUC_{0-t} of AMF conjugates)/(AUC_{0-t} of free AMF + AUC_{0-t} of AMF conjugates) that were approximately 3-, 3- and 10-fold higher than that of free AMF, respectively.[43] These results suggested that the phase II metabolite was the predominant metabolic pathway, although not sufficient to distinguish between glucuronidation or sulfation. It was also revealed that the metabolites were altered by the different routes of administration, which may be a result of the composition of the enzymes in various organs and tissues.[39] Another study also provides evidence that glucuronidation in the intestinal mucosa is the first conjugation step for bioflavonoids.[44] These results are all supported by our previous study, which demonstrated that fisetin went through the metabolic pathways of glucuronidation and

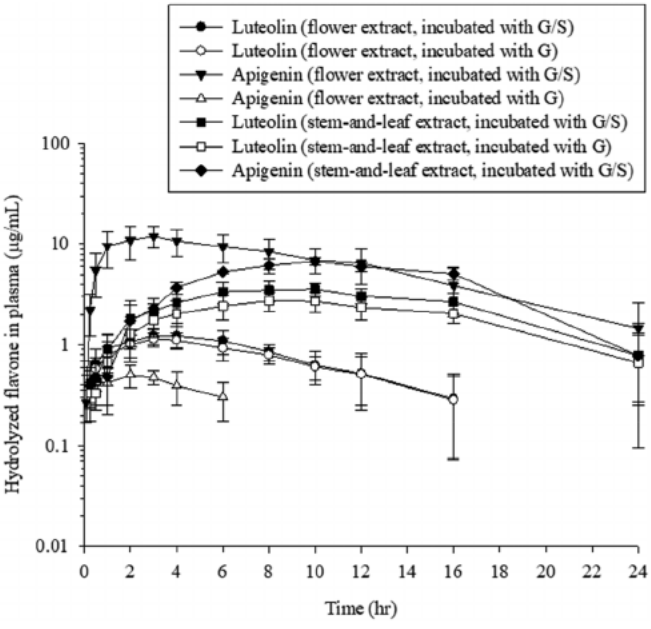

Figure 5. Mean plasma concentration-time profile in rat plasma incubated with β-glucuronidase/sulfatase (G/S) or β-glucuronidase (G) of luteolin (●○) and apigenin (▲△) after administration of flower extract of *D. morifolium* (10 g kg^{-1}, p.o.), and luteolin (■□) and apigenin (◆) after administration of stem-and-leaf extract of *D. morifolium* (10 g kg^{-1}, p.o., n = 6).

Table 6. Pharmacokinetic parameters of luteolin and apigenin after administration of the flower and stem-and-leaf extracts of *D. morifolium* (10 g kg^{-1}, p.o.) in rat plasma

	Flower extract				Stem-and-leaf extract			
	Luteolin		Apigenin		Luteolin		Apigenin	
Parameter	Glucuronide / sulfatase	Glucuronide	Glucuronide / sulfatase	Glucuronide	Glucuronide /sulfatase	Glucuronide	Glucuronide/ sulfatase	Glucuronide
AUC_{last} (min μg mL^{-1})	742.1 ± 118	668.3 ± 124	8820 ± 1830[a]	192.8 ± 95.7[b]	3415 ± 492[c]	2543 ± 660[d]	6223 ± 1000[e,f]	ND[†]
AUC_{INF} (min μg mL^{-1})	992.6 ± 243	944.9 ± 222	9867 ± 2050[a]	679.2 ± 377[b]	4109 ± 627[c]	3662 ± 638	8062 ± 2240[e]	ND
C_{max} (μg mL^{-1})	1.31 ± 0.25	1.19 ± 0.20	12.52 ± 2.76[a]	0.64 ± 0.17[b]	3.97 ± 0.53[c]	2.94 ± 0.55[d]	7.18 ± 1.13[f]	ND
T_{max} (min)	220 ± 73	200 ± 31	160 ± 31	118 ± 86	440 ± 145[c]	500 ± 118	540 ± 126[f]	ND
$t_{1/2}$ (min)	412 ± 322	457.4 ± 113	361.4 ± 213	899 ± 580	407.9 ± 158	606.1 ± 204	494 ± 221	ND

[†] Not detected. Data are presented as the mean ± SD ($n = 6$); AUC_{last} is the area under the curve from time 0 to the last measurable concentration; AUC_{INF} is the area under the curve from time 0 extrapolated to infinite time; C_{max} is the maximum plasma concentration; T_{max} is the time to reach the maximum plasma concentration. $t_{1/2}$ represents half-life. [a]$P < 0.05$, statistically significant. Comparison of luteolin and apigenin from β-glucuronidase/sulfatase incubation in the flower extract group by Student's *t*-test. [b]$P < 0.05$, statistically significant. Comparison of apigenin from β-glucuronidase/sulfatase and β-glucuronidase incubation in the flower extract group by Student's *t*-test. [c]$P < 0.05$, statistically significant. Comparison of luteolin from β-glucuronidase/sulfatase incubation in the flower extract group and β-glucuronidase/sulfatase incubation in the stem-and-leaf extract group by Student's *t*-test. [d]$P < 0.05$, statistically significant. Comparison of luteolin from β-glucuronidase/sulfatase and β-glucuronidase incubation in the stem-and-leaf extract group by Student's *t*-test. [e]$P < 0.05$, statistically significant. Comparison of luteolin and apigenin from β-glucuronidase/sulfatase incubation in the stem-and-leaf extract group by Student's *t*-test. [f]$P < 0.05$, statistically significant. Comparison of apigenin from β-glucuronidase/sulfatase incubation in the flower extract group and β-glucuronidase/sulfatase incubation in the stem-and-leaf extract group by Student's *t*-test.

sulfation. The biotransformation ratio of fisetin into glucuronides and sulfates was defined as k (%) = $AUC_{conjugates}/AUC_{parent}$. The AUC values of fisetin and its glucuronide and sulfate conjugations in plasma were 275.9, 1719 and 6429 min μg mL^{-1}, respectively, with a relative ratio of 1:6:21.[45] The phase II biotransformation of apigenin is consistent with fisetin, which mainly goes through sulfate conjugation.

The absorption of luteolin and apigenin in the stem-and-leaf group were slower than the flower group reflected by the T_{max} values (Table 6). This result may be a result of the difference of conjugated glycoside group with luteolin and apigenin between different plant part extracts because we can see their compositions of flavonoids were not the same in Table 2. The above pharmacokinetic results also demonstrated that the amount of luteolin absorbed from the stem-and-leaf extract was approximately 4.6 times higher than that from the flower extract even though the dose administered was the same. However, the amount of apigenin in the flower extract was about 1.4 times higher than that from the stem-and-leaf extract. Perhaps the stems and leaves of *D. morifolium* could be used as a better source of nutritional supplement.

CONCLUSIONS

In conclusion, the present study determined the contents of luteolin, apigenin, luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside in the flower extract and the stem-and-leaf extract. The glycoside and aglycone content ratios ($C_{glycoside}/C_{aglycone}$) of luteolin and apigenin in the flower extract were 15.3 and 34.2, respectively, which indicated that the glucosides of luteolin and apigenin are more abundant than the aglycone and the glycosides in the flower parts. Based on the biotransformation study, the phase II conjugated reactions for luteolin and apigenin were predominated by glucuronide and sulfate conjugation, respectively, which was represented in both the flower and stem-and-leaf extracts. The luteolin glucuronide products in the group administered the stem-and-leaf extract were approximately 5-fold higher than those in the group administered the flower extract. These results also provide the constructively important information that the stem-and-leaf parts of *D. morifolium* may be used as alternative parts of chrysanthemum flower regarding the health-promoting effects contributed by the flavonoid derivatives.

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AUTHOR CONTRIBUTIONS

Y-HC performed the study, analyzed the data and prepared the manuscript. Y-TW prepared the manuscript. L-CL and T-HT designed the experiments, edited the paper and secured funding.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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