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Absorption and Excretion of Luteolin and Apigenin in Rats after Oral Administration of *Chrysanthemum morifolium* Extract

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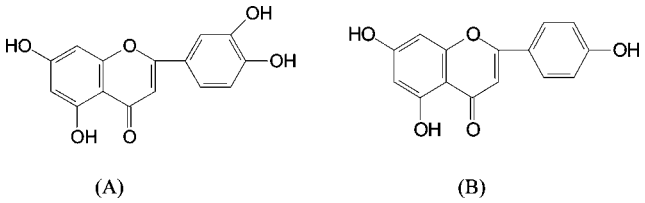
Chrysanthemum morifolium extract (CME) has the protective effect on cardiovascular diseases. Luteolin and apigenin are two major bioactive components in vivo when CME is orally administrated to experimental animal. The present paper shows the study of the absorption and excretion of luteolin and apigenin in rats after a single oral dose of CME (200 mg/kg). The levels of luteolin and apigenin in plasma, urine, feces, and bile were measured by HPLC after deconjugation with hydrochloric acid or β-glucuronidase/sulfatase. The results showed that the plasma concentrations of luteolin and apigenin reached the highest peak level at 1.1 and 3.9 h after dosing, respectively. The area under the concentration−time curves (AUC) for luteolin and apigenin were 23.03 and 237.6 μg h mL^{-1}, respectively. The total recovery of the dose was 37.9% (6.6% in urine; 31.3% in feces) for luteolin and 45.2% (16.6% in urine; 28.6% in feces) for apigenin. The cumulative luteolin and apigenin excreted in the bile was 2.05% and 6.34% of the dose, respectively. All of the results suggest apigenin may be absorbed more efficiently than luteolin in CME in rats, and both luteolin and apigenin have a slow elimination phase, with a quick absorption, so a possible accumulation of the two flavonoids in the body can be hypothesized.

KEYWORDS: ***Chrysanthemum morifolium* extract (CME); luteolin; apigenin; absorption; excretion**

INTRODUCTION

The flower of *Chrysanthemum morifolium* Ramat. (CM) has been used as Chinese traditional healthy food and medicine for hundreds of years. It is used for drink (just like tea) more usual than for medicine, especially in the summer. The flower of CM contains flavonoids, amino acids, vitamins, and some trace elements. In addition to well-known antioxidant values (*1*, *2*), it has various biological features such as cardiovascular protection effects (*3*), protection against terminal tumors (*4*), and anti-inflammatory features (*5*). The health benefits of CM as preventive nutrition from cardiovascular diseases and cancer have received considerable attention.

Dietary intake of flavonoids, and their absorption, metabolism, and bioavailability have been reviewed (*3*, *6*): flavonoids in a free form or a glycosylated form are absorbed from the intestinal tract and are metabolized to glucuronide or sulfate conjugates. These metabolites circulate in the blood and are excreted into bile and urine. The main flavonoids in CM were luteolin-7-O-β-D-glucoside and apigenin-7-O-β-D-glucoside (*7*), which would be bio-transformed into their aglycones, luteolin and apigenin (**Figure 1**) in the intestine by microorganism and hydrolase in epithelial cell (*8*, *9*) after an oral administration of the extract of CM to animals. The previous studies of our laboratory have proved that luteolin and apigenin are the main bioactive components of *Chrysanthemum morifolium* extract (CME) in vivo. The potent biological effects of luteolin and apigenin have been described as anticarcinogenic, antimutagenic in vitro and in vivo (*10*, *11*). Luteolin has the vasodilation effect on rat thoracic aorta (*12*), and apigenin can suppress skin tumor promotion and also has the effect of vasodilation (*13*).



(A) (B)

Figure 1. Chemical structures of luteolin (**A**) and apigenin (**B**).

Even though the biological and health properties of CM, luteolin, and apigenin have been extensively studied, very limited information is available on the absorption and excretion of luteolin and apigenin in human or in experimental models, especially when they were in the extract of herb. It is possible that the concomitant components in the extract will influence the process of the other compounds in the extract in vivo, so the limited information about absorption and excretion of luteolin and apigenin could not elucidate those of in CME. However, the absorption and excretion of luteolin and apigenin in CME will play an important part in the total fate of CME in vivo and provide a foundation to clinic research. Therefore, in

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the present study, we investigated the absorption and excretion of luteolin and apigenin in rats after oral administration of CME, to provide primary elements concerning the kinetic parameters of both compounds. The elimination of contents was measured in urine and feces. Also, a bile excretion work was carried out to check whether, like other flavonoids, luteolin and apigenin metabolism can occur via an enterohepatic pathway.

MATERIALS AND METHODS

Chemicals. Luteolin was purchased from J&K-ACIoS (purity >99%). Apigenin (lot 064K0653) was purchased from Sigma Co. (St. Louis, MO; purity >99%). Methanol, HPLC-grade, used for HPLC analysis, was purchased from Merk Co. Ltd. CME was provided by the Institute of Medicine, Zhejiang University, China, containing 7.60% and 5.19% of luteolin and apigenin determined by HPLC after hudrolyzed with hydrochloric acid. β-Glucuronidase (type IX-A; lot 093K8600) and sulfatase (type H-1; lot 075K3801) were purchased from Sigma Co. (St. Louis, MO). All other chemicals were of analytical grade.

Animals and Administration. Male SD rats weighing 200−250 g were obtained from the Experimental Animal Center of the Zhejiang Academy of Medical Sciences. They were housed in cages at a temperature between 20 and 23 °C, with food and water ad libitum. All animals were cared for in accordance with the principles and guide for the care and use of laboratory animals of the National Institutes of Health. Animals were fasted for 12 h before the start of experimentation and 2 h after drug administration.

Rats were orally administrated with CME (0.5% CMC-Na aqueous solution) at the dosage of 200 mg/kg according to the dosage of pharmacodynamic study.

Sample Collection. *Plasma.* Blood samples were collected from the tail vein of rats in 0.5 mL tubes containing heparin at 0, 0.17, 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48, and 72 h after dosing. Plasma was quickly obtained by centrifugation (4000 r/min, 10 min).

Urine and Feces. The urine and feces samples were collected before and in intervals of 0−2, 2−5, 5−8, 8−12, 12−24, 24−36, 36−48, and 48−72 h after administration, using metabolic cages. Urine was collected in glass flasks containing ascorbic acid (0.1% concentration) as preservative. Residual urine adhering to the metabolic cages and the surface of feces were carefully washed with a small volume of saline, the former was then pooled with the collected urine, and the latter to avoid the inference of urine. The final volume of urine was measured.

Bile. Rats were lightly anaesthetized with ether. The bile duct was cannulated with polyethylene tubing. After being resuscitated, rats were orally administered 200 mg/kg CME. Next, bile was continuously collected for 36 h in nine portions (0−1, 1−2, 2−4, 4−6, 6−8, 8−10, 10−12, 12−24, 24−36 h). During the experimental procedures, the body temperature of the rat was maintained at 38 ± 0.5 °C with a heating lamp to prevent hypothermic alteration of the bile flow. Bile volume was recorded. The samples were frozen and stored at −20 °C until analysis.

Sample Preparation. *Plasma.* Samples were analyzed according to the method as previously described (*14*) with a little modification. In short, plasma samples were hydrolyzed by acid, and then were extracted by ethyl acetate. After the extraction was evaporated to dryness, the residue was reconstituted in mobile phase and analyzed by HPLC.

Urine and Bile. Samples were analyzed according to a method as previously described (*15*). Sample was hydrolyzed to release luteolin and apigenin from glucuronic acid and sulfate conjugates with enzyme. Briefly, the samples (200 μL of urine, 100 μL of bile) were adjusted with 0.58 mol/L acetic acid (1/10, v/v) and incubated with 5 μL of β-glucuronidase and sulfatase−sodium acetate buffer solution (pH 5.0) (200 and 10 U/mL, respectively) for 2.0 h at 37 °C with continuous shaking. After hydrolysis, the samples were diluted with mobile phase to 1.00 mL and then centrifuged at 13 000 r/min for 10 min; the supernatant was analyzed by HPLC.

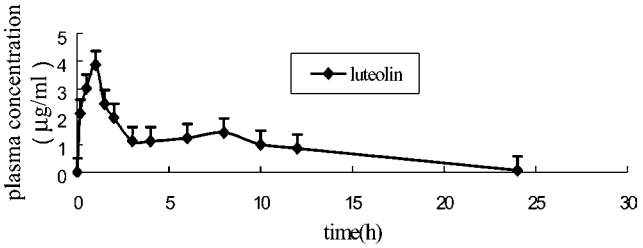

Figure 2. Plasma concentrations–time curve of luteolin in male rats after a single oral dose of CME 200 mg/kg body wt. Data were expressed as mean ± SD (n = 5).

Feces. Samples were dried in vacuo for 2.0 h, and the dried weight was measured before pulverization with a mortar and pestle. 50 mg of pulverized feces was homogenized in 5 mL of methanol with a vortex and sonicated for 15 min. After centrifugation of the homogenate, 200 μL of supernatant was diluted with mobile phase to 600 μL, and centrifugated again before being analyzed by HPLC.

HPLC Analysis. HPLC analysis to measure concentrations of luteolin and apigenin was carried out using an Agilent 1100 HPLC system, equipped with a G1314A isocratic pump, a thermostatted column compartment, and a variable-wavelength UV detector (VWD) set at 350 nm linked to Agilent Chemstation software. The analysis of treated samples was performed on Agilent SB-C_{18} (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol and 0.2% phosphoric acid aqueous solution (52:48, v/v) with the flow rate of 1.0 mL/min. The injection volume was 50 μL. The column temperature was maintained at 30 °C.

Method Validation. The calibration curves were prepared by spiking blank plasma, urine, feces, and bile with standard luteolin and apigenin at a series of concentration levels in triplicate prior to sample analysis. To ensure reproducibility and recovery of the method, a selected blank plasma, urine, feces, or bile sample, respectively, was repeatedly spiked and worked up.

Statistical Analysis. Noncompartmental pharmacokinetic analysis of plasma data was conducted using WinNonlin 4.1 (Pharsight). The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were taken directly from the data. The elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2/Lz$, where Lz is the elimination rate constant. The concentrations of luteolin and apigenin in urine, bile, and feces, which were calculated from the respective standard curve, were multiplied by the respective urinary, biliary volume, and fecal dried weight to obtain the amount excreted during a given sampling time. All data are expressed as mean ± SD.

RESULTS

Methodology. The concentration of luteolin in plasma, urine, feces, and bile was linear over the range of 0.0460−8.09, 0.0195−1.560, 0.2992−31.92, and 0.1745−6.986 μg/mL, respectively. The concentration of apigenin in plasma, urine, feces, and bile was linear over the range of 0.145−25.7, 0.03488−2.790, 0.1800−19.20, and 0.3693−11.08 μg/mL, respectively. The recoveries for luteolin and apigenin at low, medium, and high concentrations were all higher than 80.0%, and the intra- and inter-day variations (RSD %) at low, medium, and high concentrations for both compounds were below 13.5% in all sample types.

Plasma Level and Pharmacokinetic Parameters. The profiles of total concentrations of luteolin and apigenin in rat plasma with time after oral administration of CME (200 mg/kg) are shown in **Figures 2** and **3**. Luteolin and apigenin reached the highest peak level at 1.1 and 3.9 h after dosing, respectively. The concentration of luteolin was lower than apigenin in rat plasma at all time points. The other pharmacokinetic parameters

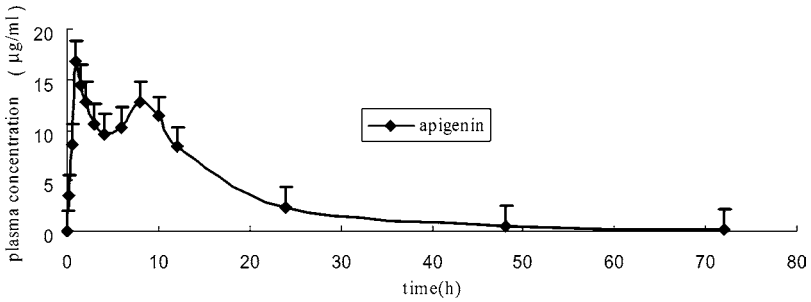

Figure 3. Plasma concentrations–time curve of apigenin in male rats after a single oral dose of CME 200 mg/kg body wt. Data were expressed as mean ± SD ($n = 5$).

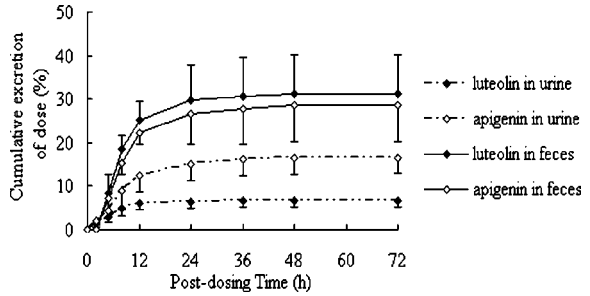

Figure 4. Urinary and fecal excretion–time profile of luteolin and apigenin after oral administration of CME (200 mg/kg) to rats. Data were expressed as mean ± SD ($n = 5$).

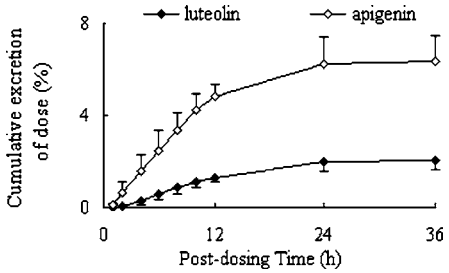

Figure 5. Biliary excretion–time profile of luteolin and apigenin after oral administration of CME (200 mg/kg) to rats. Data were expressed as mean ± SD ($n = 5$).

showed the area under the concentration−time curves (AUC) was 23.03 and 237.6 μg h mL^{-1} for luteolin and apigenin, respectively, the apparent distribution volume (V_d/F) was 65.12 L kg^{-1} for luteolin and 13.78 L kg^{-1} for apigenin, the renal clearance (CL/F) was 8.473 and 0.8569 L/kg h, and the elimination half-life ($t_{1/2}$) of luteolin and apigenin was 2.7 and 3.4 h, respectively.

Excretion. *Urinary and Fecal Excretion.* **Figure 4** shows the cumulative urinary and fecal excretion as a percentage of the dose of luteolin and apigenin in rats all time points. The rate of the total urinary excretion was 6.6% and 16.6% in rats during 72 h after oral administration, respectively; meanwhile, the rate of accumulative fecal excretion of luteolin and apigenin during the same time was 31.3% and 28.6%; thus, the total recovery of luteolin and apigenin in urine and feces was 37.9% and 45.2%. The peak excretion rates appeared both at about 8 h, then, the concentration of them in urine and feces decreased slowly. Luteolin and apigenin were excreted completely in 72 h after ingestion of CME. The amounts of luteolin and apigenin in feces were more than those in urine.

Biliary Excretion. The cumulative luteolin and apigenin excreted into the bile over 36 h after oral administration of CME are depicted in **Figure 5**. The cumulative luteolin and apigenin excreted in the bile were 2.05% and 6.34% of the dose, respectively. Also, the rate of excretion was similar during 3−10 h, approximately at 6 and 24 h appeared the peak excretion, and the excretion reached the highest amount at 24 h. It was indicated a slow and low excretion of luteolin and apigenin from the bile.

DISCUSSION

According to the reference, flavonoids may be metabolized to phase II metabolites, glucuronide or sulfate conjugates (*16*), and the retention time of the conjugates was shorter than luteolin and apigenin themselves in HPLC analysis. For determination of total luteolin and apigenin in plasma, urine, feces, and bile, the sample should be pretreated to disconnect the luteolin and apigenin from conjugates if they were formed phase II metabolites. So, before quantitive analysis of the sample, the forms of phase II metabolites of luteolin and apigenin were studied. The concentrations of luteolin and apigenin of samples hydrolyzed by hydrochloric acid and a mixture of β-glucuronidase and sulfatase were compared to those of samples untreated. The result showed the concentrations of luteolin and apigenin in urine and bile hydrolyzed by acid and mixture of β-glucuronidase and sulfatase were much higher than those in untreated samples, and the concentration in urine and bile hydrolyzed by acid and hydrolyzed by a mixture of β-glucuronidase and sulfatase had no significant difference. However, in feces, the situation was very different. The amounts of luteolin and apigenin in feces untreated, enzymic hydrolysis, and acid hydrolysis were similar. It is indicated that the main forms of luteolin and apigenin were glucuronoconjugates and sulfoconjugates in rat urine and bile, while in rat feces, the main forms were aglycones. This result is partly consistent with previous researches (*9*, *17*, *18*). Therefore, the samples of urine and bile should be pretreated with enzymic hydrolysis before HPLC analysis.

To our knowledge, little information has concerned the pharmacokinetics, metabolism, and excretion of CME or bioactive components of CME, luteolin and apigenin, in rats. Much research on flavonoids was around quercetin (*19*−*23*), which is not similar to luteolin and apigenin in absorption and excretion. The plasma levels of the present study suggested a relatively rapid absorption and a slow elimination phase of luteolin and apigenin. There were two peaks in plasma concentration−time curves, which indicates that luteolin and apigenin are reabsorbed by enterohepatic cycling or from the distal parts of the small intestine or the colon. In addition, our results prove the biliary excretion's existence of luteolin and apigenin. Although the amounts of biliary excretions were not

large, this existence shows the conjugates of luteolin and apigenin undergo enterohepatic cycling, resulting in deconjugation and reabsorption of them. Actually, many kinds of flavonoids have enterohepatic circulation, and the plasma concentration would increase again after the first absorption (*24*). The study published in 1995 using GC/MS showed the profiling of luteolin and its metabolites in rat urine and bile, and the bile levels indicated quick absorption and rapid transformation, followed by a slow elimination phase of luteolin (*25*).

Luteolin and apigenin have some differences in absorption and excretion. The plasma concentration of apigenin was higher than that of luteolin (**Figures 3** and **4**), and the area under the plasma concentration−time curve (AUC) for apigenin was approximately 10-fold higher than that of luteolin, whereas the content of luteolin (7.60%) in CME was higher than that of apigenin (5.19%). Moreover, we have demonstrated that luteolin could not be bio-transformed to apigenin in vivo and in vitro in our other experiment. We also applied the everted gut sac from rat to study the absorption of apigenin and luteolin in hydrolyzed CME, and the result showed that the absorption rate constants (K_a) and apparent permeability coefficients (P_{app}) were 0.0185 min^{-1}, 3.941 $\times$ 10^{-5} cm/min for luteolin and 0.0253 min^{-1}, 10.15 $\times$ 10^{-5} cm/min for apigenin; therefore, we deduce that apigenin may be absorbed more efficiently. Otherwise, even though some papers reported that apigenin could be bio-transformed to luteolin in hepeatic microsome and primary cultured rat hepatocytes (*16*, *17*, *26*), the study in our laboratory showed luteolin was not detectable in plasma of rat after oral administration with apigenin, which indicates that apigenin cannot be bio-transformed to luteolin of rat in vivo.

The urinary excretions of luteolin and apigenin were only 6.6% and 16.6%. It is suggested that renal excretions of them are both low. Also, the total recoveries of luteolin and apigenin in urine and feces were only 37.9% and 45.2%, respectively. So much of luteolin and apigenin may be metabolized to simple compounds (*27*), or probably there would be an accumulation of them in vivo, which should be studied in further research.

In conclusion, the results of the present study show a relatively rapid absorption and slow elimination of luteolin and apigenin in rats after oral administration of CME. After 3 days, all of the luteolin and apigenin seem to be excreted from the body. This study is useful in completing the total research on pharmacokinetics of CME.

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