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Absorption and Metabolism of Luteolin and Its Glycosides from the Extract of *Chrysanthemum morifolium* Flowers in Rats and Caco-2 Cells

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Supporting Information

ABSTRACT: To elucidate the bioavailability of luteolin and its glycosides in *Chrysanthemum morifolium* flowers, the absorption and metabolism of luteolin from them was investigated in rats and Caco-2 cells using HPLC and LC-MS. After oral administration of *C. morifolium* extract (1.7 g/kg body weight (bw), equivalent to 22.8 and 58.3 μmol/kg bw of luteolin and luteolin-7-*O*-glucoside, respectively) to rats, luteolin and its glycosides were quickly absorbed and luteolin, luteolin monoglucoside, and luteolin monoglucuronide were detected in the plasma. Their levels were highest at 1 h after administration (0.76 ± 0.27 μM). These compounds were also detected in media on the basolateral side from Caco-2 cells treated with the *C. morifolium* extract. These results suggest that luteolin and luteolin monoglucoside are rapidly absorbed after administration of *C. morifolium* flower extract and that luteolin, luteolin monoglucoside, and luteolin monoglucuronide may circulate in humans.

KEYWORDS: *Chrysanthemum morifolium, intestinal absorption, luteolin, luteolin-7-O-β-glucoside*

INTRODUCTION

Chrysanthemum morifolium flowers have been widely and traditionally used in Japan and China as a useful food, healthy beverage, and medicine. For example, dried *Chrysanthemum* flowers are used in common foods such as miso soup and pickles in northern Japan, and *Chrysanthemum* tea has 2000 years of history and is a popular herbal tea in China. The low molecular weight components of *Chrysanthemum* flowers show great diversity, including flavonoids,[1] sesquiterpenes,[2] triterpenes,[3] and unsaturated fatty acids, and some of these compounds contribute to the beneficial functions of *Chrysanthemum* flowers. Above all, the flavonoid luteolin was identified as the representative bioactive compound in these flowers.[4] Luteolin displays beneficial effects, such as anti-inflammation, anticarcinogenesis, and antioxidation.[5,6] Luteolin is a flavone contained in many medicinal herbs and vegetables (e.g., parsley, artichoke, celery, green pepper, perilla leaf) and, together with other flavonoids, is most often found in plant materials in the form of glycosides.

The metabolism of flavonoids has been extensively studied in cultured cells,[7,8] rat intestine,[9,10] and liver cells.[11] In plants and most foods, flavonoids exist as glycosides. Upon ingestion, these glycosides are hydrolyzed to the corresponding aglycone by lactase phlorizin hydrolase (LPH), an enterocyte-specific enzyme localized to the brush border, or bacterial flora in the intestine, followed by diffusion of the aglycone into the cell.[12−15] Meanwhile, several glycosides are taken up into the enterocytes via sodium glucose cotransporter 1 (SGLT1).[16−18] Following cellular accumulation of the aglycone, extensive metabolism occurs within the enterocytes.[19] This metabolism involves the formation of a number of phase II conjugates, most notably sulfates and glucuronides, as well as methylated and mixed conjugates. The conjugation of flavonoids has a major impact on their properties, and the resulting conjugates can be excreted into the digestive tract or passed into the portal vein by specific transporter systems, where they are further metabolized by phase II enzymes in the liver.

Elucidation of the absorption and metabolism of bioactive flavonoids is important to understanding their roles in physiological functions, after the purified compounds or the food including the compounds are administered. There are some studies on the bioavailability of luteolin after administration of *C. morifolium* extract, and they demonstrated that luteolin in the flower extract was absorbed into rat and human bodies.[20−22] However, in these analyses, because they investigated these samples (e.g., plasma, urine) cleaved into aglycon, it remains unknown whether the circulated luteolin exists as a free form or any conjugated form. Our group[20] previously reported that free luteolin and its conjugates were detected in plasma when the purified luteolin and its glucoside were administered to rats and humans. Although our group also showed that luteolin glucoside was absorbed into the intestine in that paper, it remains unclear whether luteolin glucoside exists with or without conjugation.

The purpose of the present study was to investigate the form of luteolin in the body after administration of luteolin and luteolin-7-*O*-glucoside in a *C. morifolium* flower extract for understanding their physical functions in human health. As Lau et al. reported,[24] animal in vivo experiments play a major role in predicting oral in vivo bioavailability in humans. However,

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because different intestinal enzymes exist in animals and humans, animal studies alone may not always be sufficient for predicting compound absorption in humans. There is no report on the absorption of *C. morifolium* in differentiated Caco-2 cells derived from human colorectal carcinoma, a vitro model of absorptive enterocytes. Therefore, we used the differentiated Caco-2 models, with animal in vivo studies, and they might be useful for the aim of predicting the absorption and metabolism in humans. As described above, flavonoids are generally metabolized to their conjugates in the gastrointestinal tract. Because most of these metabolites are not available from commercial sources, their direct analysis is precluded. Thus, in this study on luteolin conjugates in rat plasma and culture media, samples were treated with enzymes having glucuronidase/sulfatase activities to release the parent aglycone for HPLC, and for detailed qualitative analysis, untreated samples were applied to LC-MS.

MATERIALS AND METHODS

Chemicals. Luteolin, luteolin-7-*O*-β-glucoside, and myricetin were purchased from Extrasynthese (Genay, France). MEM nonessential amino acids (NEAA) and penicillin/streptomycin were obtained from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) and β-glucuronidase (type H-5 from *Helix pomatia* and type L-II from limpets) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise specified. HPLC solvents were all of analytical grade. The extraction of the dried *C. morifolium* flowers was performed as briefly described below. *C. morifolium* flowers (200 g) were homogenized in 2.0 L of methanol, incubated at 40 °C for 3 h twice, and evaporated.

Animals. Male Sprague−Dawley rats (7 weeks old; SLC, Hamamatsu, Japan) were housed in an air-conditioned room and given free access to MF commercial food pellets (Oriental Yeast Co., Tokyo, Japan) and tap water for 4 weeks before the experiments. After an overnight fast, the rats (11 weeks old) were intragastically administered the *C. morifolium* flower extract (1.7 g/kg body weight (bw)), equivalent to luteolin (22.8 μmol/kg bw) and luteolin-7-*O*-glucoside (58.3 μmol/kg bw), or propylene glycol as a vehicle control via intragastric intubation. At 0, 0.5, 1, 2, 3, and 6 h after administration, blood was collected from the vein into heparinized glass capillaries and centrifuged to obtain plasma. The rats were maintained and handled according to the Guidelines for the Regulation of Animal Experimentation Committee of the University of Shizuoka.

Cell Culture. Caco-2 human intestinal epithelial cells were purchased from the European Collection of Cell Cultures (Wiltshire, UK). Caco-2 cells were grown in EMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 ng/mL), and NEAA at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. To examine the bioavailability of luteolin and luteolin-7-*O*-glucoside, Caco-2 cells were seeded on cell culture inserts for a 12-well plate (Corning, Tewksbury, MA, USA) at a density of 3.4×10^4 cells/filter and used for experiments on days 15−20. The transepithelial electrical resistance (TEER) value of the monolayers was measured using a Millicell-ERS instrument (Millipore Corp., Bedford, MA, USA) according to the methods of Hidalgo et al.[23,24] and Murota et al.[23,24] After confirmation of differentiation by the TEER value (>600 Ω cm^2), the cells were pre-incubated with transport medium (Hank's balanced salt solution with Ca^{2+}, Mg^{2+}, and glucose; 500 μL in the apical compartment and 1500 μL in the basal compartment of the transwell system) for 30 min at 37 °C. The *C. morifolium* flower extract, luteolin, and luteolin-7-*O*-glucoside dissolved in transport medium were then added to the apical side of the cells at specified total concentrations. After incubation of the cells at 37 °C for the required times, the cell culture media from both compartments (apical and basal) were collected for investigation of the bioavailability.

Sample Preparation for HPLC and LC-MS Analysis. The collected plasma samples and medium samples with or without enzymatic deconjugation were treated as described below for determination of luteolin, luteolin glucoside, and conjugated metabolites using HPLC and LC-MS. Individual samples were divided into two aliquots. To hydrolyze conjugated luteolin metabolites, one aliquot of plasma sample was treated with *H. pomatia* β-glucuronidase type H-5 (200 U), which possesses β-glucuronidase, sulfatase, and glucosidase activities, and then incubated at pH 5.0 for 45 min at 37 °C in 0.5 M acetate buffer. Similarly, one aliquot of medium sample was treated with limpet β-glucuronidase type L-II (400−1400 U), which possesses β-glucuronidase and glucosidase activities in 0.1 M phosphate buffer (pH 5.0), and then incubated at pH 5.0 for 45 min at 37 °C in 0.1 M phosphate buffer. The resulting mixtures were acidified with an equivalent amount of 0.01 M oxalic acid. These conditions and the enzyme mixtures yielded the highest amounts of free luteolin from the plasma of *C. morifolium* flower extract-treated rats and from the medium treated with the *C. morifolium* flower extract, luteolin, and luteolin-7-*O*-glucoside in preliminary analyses. The other portions of the plasma and medium samples were retained without hydrolysis by β-glucuronidase and used for direct quantification of nonconjugated metabolites. These samples were also acidified with 0.01 M oxalic acid. After acidification, the hydrolyzed and unhydrolyzed samples were each mixed with myricetin as an internal standard and applied to a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), which had been pretreated by passage with methanol. The cartridge was washed with 0.01 M oxalic acid, distilled water/methanol/0.5 M oxalic acid (88:10:2), and distilled water, and the methanol eluate was obtained. The eluate was evaporated to dryness, and the residue was dissolved in 20% acetonitrile/0.5% phosphoric acid for HPLC analysis and in methanol for LC-MS, respectively. After filtration (0.45 μm filter unit), the filtrate was applied to HPLC and LC-MS analysis.

HPLC Analysis. The sample was injected into an HPLC-electrochemical detection (ECD) system (model 5600A CA767 CoulArray Detector; ESA, Cambridge, MA, USA) equipped with a CAP CELL PAK C18 UG120 column (S-5 μm, 4.6 mm i.d. × 150 mm; Shiseido, Tokyo, Japan). The separation of the compounds was carried out by gradient elution. For detection of luteolin and luteolin-7-*O*-glucoside using the HPLC-ECD system, two gradient systems were employed. For system 1, solvent A was 20% acetonitrile/0.5% phosphoric acid and solvent B was 40% acetonitrile/0.5% phosphoric acid. The gradient program was as follows: 0−5 min, 0% B; 5−15 min, linear gradient to 100% B; 15−19 min, 100% B hold; 19−22 min, linear gradient to 0% B; flow rate, 0.8 mL/min. For system 2, solvents A and B were the same as in system 1, respectively, and the gradient program was as follows: 0−10 min, 0% B; 10−16 min, linear gradient to 10% B; 16−21 min, 10% B hold; 21−30 min, linear gradient to 25% B; 30−35 min, 25% B hold; 35−40 min, linear gradient to 50% B; 40−43 min, 50% B hold; 43−48 min, linear gradient to 100% B; 48−50 min, 100% B hold; 50−55 min, linear gradient to 0% B; flow rate, 0.8 mL/min. Electrochemical detection was performed with a coulometric electrode at 150 mV (for channel 1) and 200 mV (for channel 2).

LC-MS Analysis. For LC-MS analysis, the samples were injected onto Q Exactive (Thermo Fisher Scientific, Waltham, MA, USA). The mass spectrometer was operated in the negative-ion ESI mode with targeted selected ion monitering (SIM) mode for all LC-MS analytes. The samples were separated by UPLC (Accela; Thermo Fisher Scientific) equipped with an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 mm i.d. × 100 mm; Waters). Gradient elution was performed using a two-solvent system (solvent A, 0.1% formic acid; solvent B, acetonitrile containing 0.1% formic acid). The gradient program was as follows: 0−1 min, 2% B hold; 1−15 min, linear gradient to 98% B; flow rate, 0.3 mL/min.

RESULTS

Time-Dependent Changes in the Plasma Concentrations of Luteolin after Oral Administration of the *Chrysanthemum* Flower Extract. We quantified luteolin and luteolin-7-*O*-glucoside using HPLC-ECD, and representative

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

chromatograms for luteolin, luteolin-7-*O*-glucoside, and myricetin (internal standard) are shown in the Supporting Information (Supplemental Figure 1A). The peaks of luteolin, luteolin-7-*O*-glucoside, and myricetin were observed at 17.767, 11.167, and 14.733 min, respectively, using the system 1 program. On the basis of the analysis using this system, 1 g of *Chrysanthemum* flower extract contained luteolin and luteolin-7-*O*-glucoside at 13.4 and 34.3 μmol, respectively, giving 47.7 μmol of total luteolin in 1 g of the *Chrysanthemum* flower extract. To measure the total content of luteolin derivatives in plasma, we treated plasma samples with *H. pomatia* β-glucuronidase type H-5, which has hydrolysis activity against glucuronides and sulfates, and confirmed that this enzyme altered luteolin-7-*O*-glucoside to luteolin in preliminary analyses (data not shown).

We examined the time-dependent changes (0.5, 1, 2, 3, and 6 h) in the plasma luteolin concentration in rats administered the *Chrysanthemum* flower extract at 1.64 g/kg bw, equivalent to 81 μmol of total luteolin (Figure 1). In this experiment, the levels

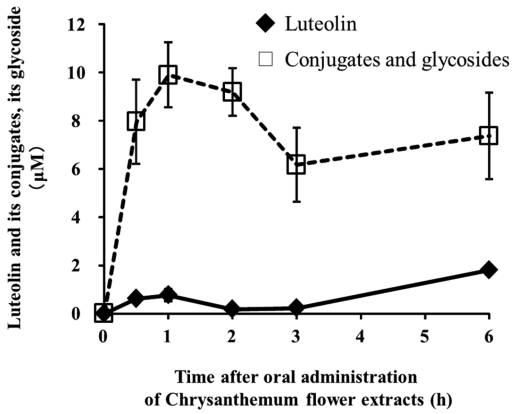

Figure 1. Time-dependent changes in luteolin derivatives in plasma from rats after oral administration of the *Chrysanthemum* flower extract. The concentrations of luteolin and its conjugates were measured in rat plasma after oral administration of the *C. morifolium* flower extract (1.7 g/kg body weight), equivalent to luteolin (22.8 μmol/kg body weight) and luteolin-7-*O*-glucoside (58.3 μmol/kg body weight). Plasma samples were treated with *H. pomatia* β-glucuronidase type H-5, which has hydrolysis activity against glucuronides, sulfates, and glucoside under the present experimental conditions. Thus, the glycosides and conjugates of luteolin were altered to luteolin aglycone. Data represent means ± SE (n = 4).

of free luteolin in plasma increased rapidly at 0.5 h after administration and reached the peak level at 1 h (0.76 ± 0.27 μM). Thereafter, the level of luteolin decreased at 2 h after administration, but increased again at 6 h (1.79 ± 0.17 μM). Meanwhile, the level of conjugated luteolin and luteolin-7-*O*-glucoside in plasma increased rapidly at 0.5 h after administration and reached the peak level at 1 h (9.88 ± 1.35 μM). Thereafter, the level of conjugated luteolin and luteolin-7-*O*-glucoside decreased but also increased again at 6 h after administration (7.35 ± 1.79 μM).

We presumed that the metabolites of luteolin and luteolin-7-*O*-glucoside included luteolin monoglucuronide, luteolin glucoside glucuronide, luteolin diglucuronide, luteolin glucoside sulfate, and luteolin glucuronide sulfate. The presence of these metabolites was confirmed by LC-MS in a qualitative manner. Figure 2A shows the mass chromatogram and mass spectrum of a rat plasma sample obtained 2 h after oral administration of the *Chrysanthemum* flower extract. Compared with the corresponding standard, luteolin-7-*O*-glucoside was identified in the plasma sample at 6.63 min (m/z 449.1033−449.1123). Meanwhile, as shown in Figure 2B, a prominent peak was observed at 6.73 min (m/z 463.0825−463.0917). This peak was possibly one of the presumed conjugates, luteolin monoglucuronide (MW 462.36). The other presumed conjugated metabolites were below the measurable limits.

Changes in Luteolin, Luteolin-7-*O*-glucoside, and Their Conjugated Metabolites in Caco-2 Cells Treated with the *Chrysanthemum* Flower Extract. To investigate the bioavailability in vitro, the *Chrysanthemum* flower extract was applied to the apical side (0.5 mL) of Caco-2 monolayers at a concentration equivalent to 5.0 nmol of total luteolin. As shown in Figure 3A, luteolin-7-*O*-glucoside decreased in the apical solution with incubation time (luteolin, from 0.50 ± 0.24 to 0.09 ± 0.02 nmol/well; luteolin-7-*O*-glucoside, from 4.22 ± 0.65 to 2.62 ± 0.45 nmol/well). Conjugated forms of luteolin in the apical solution appeared at 0.5 h, and their contents tended to increase in a time-dependent manner (from 1.00 ± 0.82 to 1.67 ± 0.65 nmol/well). As shown in Figure 3B, luteolin and luteolin-7-*O*-glucoside increased in the basolateral solution with incubation time (luteolin-7-*O*-glucoside, from 0.006 ± 0.001 to 0.016 ± 0.003 nmol/well; luteolin conjugates, from 0.013 ± 0.010 to 0.052 ± 0.009 nmol/well). Luteolin aglycone in the basolateral solution appeared at 0.5 h (0.016 ± 0.002 nmol/well) and was increased at 2 h (0.056 ± 0.005 nmol/well).

We presumed that the metabolite profile was the same as that observed in the animal experiments. Thereafter, the basolateral solution after 4.0 h of incubation was applied to LC-MS for qualitative analysis. Luteolin-7-*O*-glucoside was identified in the basolateral solution at 6.27 min (m/z 449.1033−449.1123) as shown in Supplemental Figure 2. Meanwhile, as shown in Supplemental Figure 3, prominent peaks were observed at 6.30, 6.78, and 6.97 min (m/z 463.0825−463.0917) and were possibly those of luteolin monoglucuronide (MW 462.36). Luteolin possesses four hydroxyl groups at the 3′-, 4′-, 5-, and 7-positions, and they are available for glucuronidation. Therefore, four types of luteolin monoglucuronides could possibly exist, and these prominent peaks were thought to be three of these monoglucuronides. Because each authentic sample was not commercially available, it was unclear which position of luteolin is a glucuronidation site at the present time. The other presumed conjugated metabolites were below the measurable limits.

Changes in Luteolin, Luteolin-7-*O*-glucoside, and Their Conjugated Metabolites in Caco-2 Cells Treated with Luteolin and Luteolin-7-*O*-glucoside. After luteolin was applied to the apical side of Caco-2 monolayers at the concentration of 10 μM, equivalent to 5.0 nmol of luteolin, both the apical and basolateral solutions were collected. In the apical solution, luteolin aglycone decreased in a time-dependent manner from 3.38 ± 0.22 to 1.40 ± 0.14 nmol/well, and luteolin conjugates tended to increase fom 1.18 ± 0.60 to 1.36 ± 0.34 nmol/well (Figure 4A). In the basolateral solution, luteolin aglycone appeared at 0.5 h (0.95 ± 0.27 nmol/well) and decreased to 0.62 ± 0.10 nmol/well with incubation time (Figure 4B). After application of luteolin-7-*O*-glucoside to the apical side, the detected luteolin-7-*O*-glucoside in the apical solution decreased to 3.66 ± 0.31 nmol/well at 4 h (Figure 5A). Luteolin aglycone tend to increase from 0.08 ± 0.01 to

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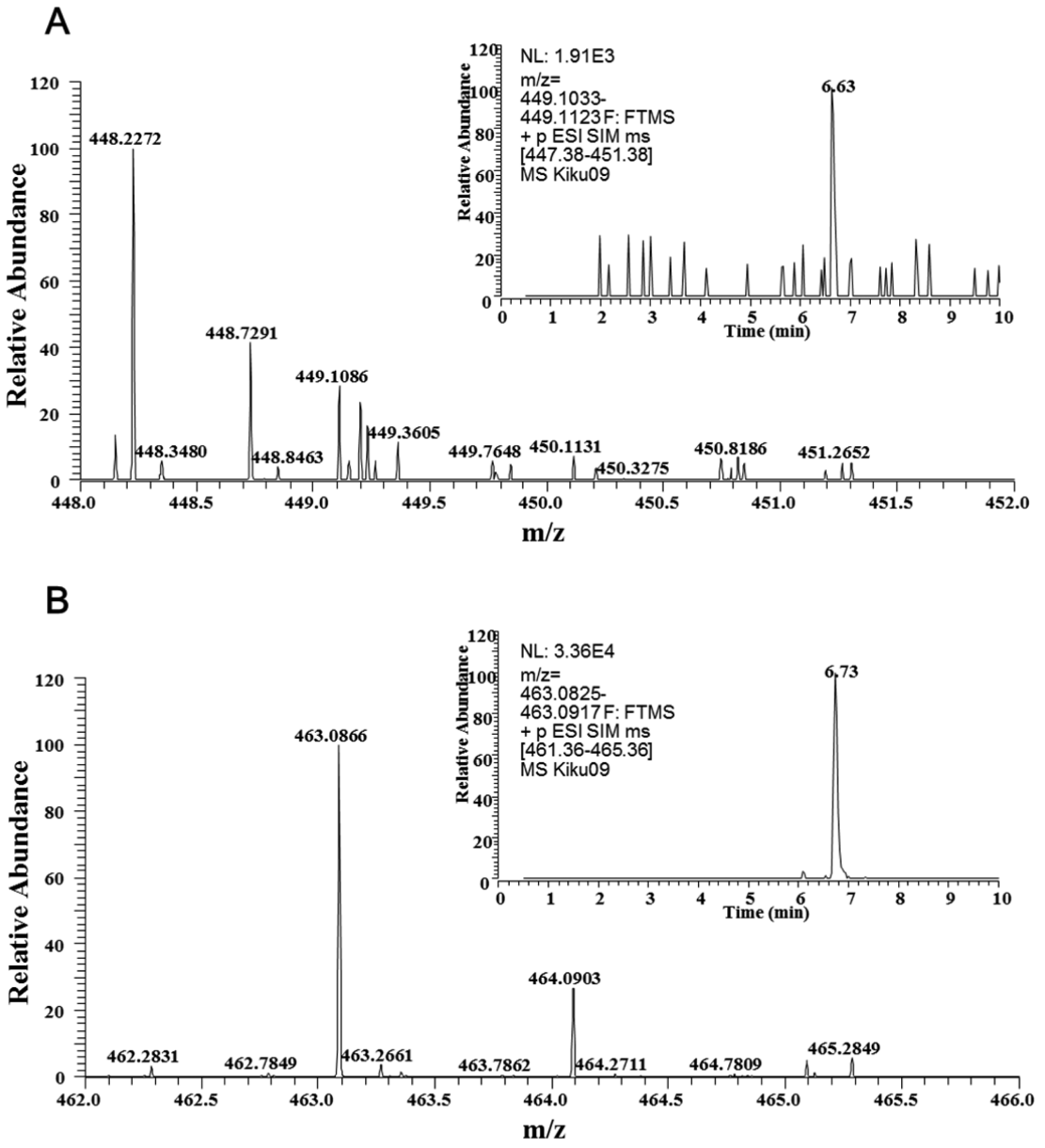

Figure 2. Mass chromatogram and mass spectrum of luteolin-7-*O*-glucoside (A) and luteolin monoglucuronide (B) in plasma from rats 2 h after administration of the *Chrysanthemum* flower extract: (A) mass chromatogram (*m*/*z* 449.1033−449.1123) and mass spectrum (time = 6.63 min); (B) mass chromatogram (*m*/*z* 463.0825−463.0917) and mass spectrum (time = 6.73−6.76 min).

0.12 ± 0.01 nmol/well in a time-dependent manner, and luteolin conjugates were not detected. As shown in Figure 5B, luteolin-7-*O*-glucoside, luteolin, and luteolin conjugates in the basolateral solution tend to increase in a time-dependent manner (luteolin-7-*O*-glucoside, from 0.052 ± 0.014 to 0.125 ± 0.030 nmol/well; luteolin, from 0.006 ± 0.001 to 0.018 ± 0.023 nmol/well; luteolin conjugates, from 0.006 ± 0.014 to 0.018 ± 0.023 nmol/well).

The basolateral solution after 1.0 h of incubation was obtained and applied to LC-MS for qualitative analysis. As shown in Supplemental Figure 4A, luteolin-7-*O*-glucoside was identified in the basolateral solution at 6.09 min (*m*/*z* 449.1033−449.1123). The peak of luteolin monoglucuronide was observed at 6.30 min (*m*/*z* 463.0825−463.0917; Supplemental Figure 4B). The other presumed conjugated metabolites were below the measurable limits.

DISCUSSION

The *Chrysanthemum* flower extract used in the present study contained luteolin and luteolin-7-*O*-*β*-glucoside. The data obtained with rats administered the *Chrysanthemum* flower extract showed that luteolin, luteolin glucuronide, and luteolin-7-*O*-*β*-glucoside were detected in plasma (Figures 1 and 2). Chen et al.[20] reported that luteolin was detected in the deconjugated plasma and urine samples when rats were administered a *Chrysanthemum* flower extract. Li et al.[25] showed that luteolin was detected in human plasma treatment with deconjugating enzyme after administration of a *Chrysanthemum* flower extract. In this study, we find results partly consistent with the knowledge reported previously because luteolin and luteolin glucuronide were detected, and we also newly found that luteolin glucoside can circulate without conjugation.

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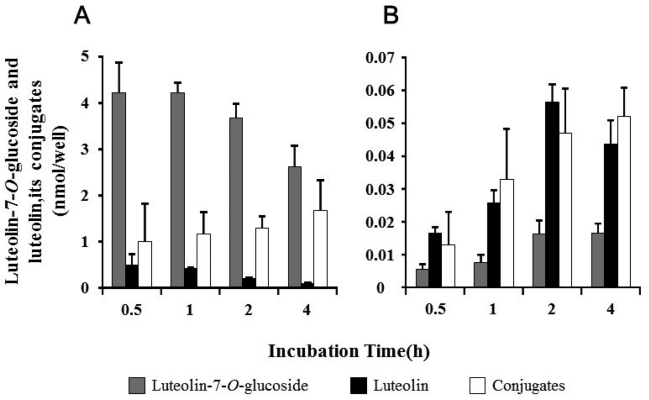

Figure 3. Amounts of luteolin derivatives in culture media from Caco-2 cells treated with the *Chrysanthemum* flower extract. Luteolin-7-*O*-glucoside, luteolin, and its conjugates in the apical solution (A) and basolateral solution (B) of Caco-2 cells treated with the *Chrysanthemum* flower extract, equivalent to luteolin (2.81 μM) and luteolin-7-*O*-glucoside (7.19 μM), are shown. The culture media were treated with limpet β-glucuronidase type L-II, which possesses β-glucuronidase and glucosidase activities in 0.1 M phosphate buffer (pH 5.0), and the conjugates of luteolin were altered to luteolin aglycone. Data represent means ± SD (n = 4−5).

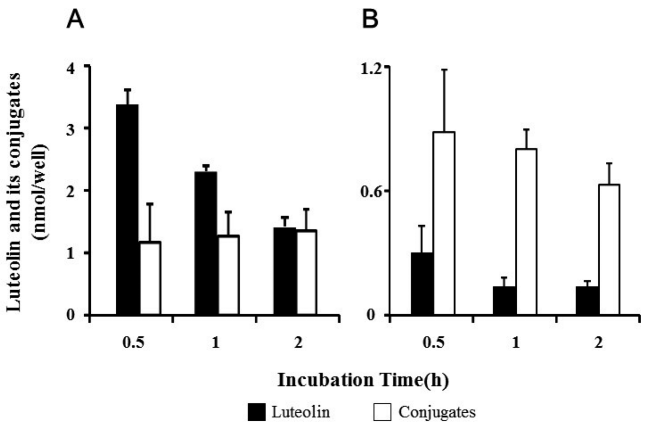

Figure 4. Amounts of luteolin derivatives in culture media from Caco-2 cells treated with luteolin. Luteolin and its conjugates in the apical solution (A) and basolateral solution (B) of Caco-2 cells treated with luteolin (10 μM) are shown. The culture media were treated with β-glucuronidase/sulfatase, and conjugates of luteolin were altered to luteolin aglycone. Data represent means ± SD (n = 5−6).

Glycosylated flavonoids are generally deglycosylated by luminal LPH and/or cleavage within the enterocytes by cytosolic β-glucosidase or bacterial flora in the intestine, followed by diffusion passively across the small intestine brush border.[12−15] According to Shimoi et al.,[26] aglycones of luteolin including the metabolite from the luteolin glycosides were absorbed without any conjugation or converted to glucuronides in the enterocytes of the digestive tract, leading to the appearance of luteolin and glucuronide conjugates in the circulatory system of rats and human. Thus, we showed that luteolin, luteolin glucoside, and luteolin monoglucuronide were detected, and these suggested the possibility that luteolin aglycone and glucoside might be absorbed and circulated with or without conjugation after administration of a *Chrysanthemum* flower extract, partly in agreement with the reported mechanism. Meanwhile, luteolin-7-*O*-glucoside was possibly absorbed also in other mechanisms through SGLT. Yin et al.[27]

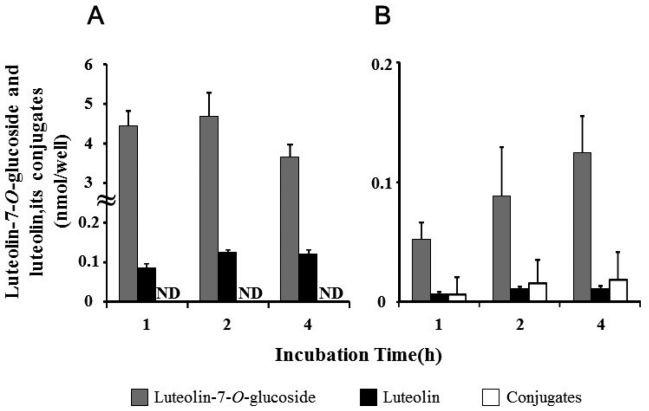

Figure 5. Amounts of luteolin derivatives in culture media from Caco-2 cells treated with luteolin-7-*O*-β-glucoside. Luteolin-7-*O*-glucoside, luteolin, and its conjugates in the apical solution (A) and basolateral solution (B) of Caco-2 cells treated with luteolin-7-*O*-β-glucoside (10 μM) are shown. The culture media were treated with β-glucuronidase/sulfatase, and conjugates of luteolin were altered to luteolin aglycone. Data represent means ± SD (n = 4−5).

investigated the metabolites in rats after administration of an *Ixeris sonchifoliain* (Bge.) extract, containing luteolin glucuronide and luteolin glucoside. They described that the glucuronide and glucoside of luteolin were detected in rat plasma, and a part of the luteolin-7-*O*-β-glucoside, which was not hydrolyzed by LPH and enterobacteria, was possibly absorbed through SGLT1 on the surface of intestinal cells, followed by its appearance in the plasma without conjugation.

As shown in Figure 1, the concentration of luteolin in rat plasma increased to the first and second peak levels at 1 and 6 h (0.76 ± 0.27 and 1.79 ± 0.17 μM), respectively, after dosing with the *Chrysanthemum* flower extract. Conjugated flavonoids are the substrates of efflux transporters (such as MPR2) and can be excreted to the bile and lumen, a phenomenon known as enterohepatic recycling.[20,25,28] This system may entail the reconversion of conjugates that are excreted by efflux transporters with subsequent hydrolysis by intestinal microflora and re-uptake. Otherwise, as described above, flavonoid glycoside is hydrolyzed to its aglycone by LPH in intestinal brush border cells and enterobacteria, and its aglycone is metabolized into conjugates.[12−15] Thus, we further proposed that the conjugates from the glycoside may appear later than those from the aglycone. These results were supported by the case of quercetin glycoside, and they showed that the concentrations of quercetin in rat plasma increased to the first and second peak levels at 30 min and 8 h, respectively, after dosing with αG-rutin, which consists of 4G-α-D-glucopyranosyl rutin and isoquercitrin.[29] However, because the mechanism remains unclear, and to elucidate this phenomena and underlying mechanisms in detail and confirm this speculation, further investigations focusing on kinetic bioavailability are required.

When differentiated Caco-2 cells were treated with the *Chrysanthemum* flower extract, luteolin aglycone, glucoside, and conjugates were detected in basolateral solution (Figure 3) as well as in the plasma from rat after administration of this extract, and luteolin conjugate and glucoside tend to increase in a time-dependent manner in the basolateral solution. Meanwhile, luteolin increased from 0.5 to 2 h there and decreased at 4 h, possibly because luteolin passed from the apical side to the basolateral side through the inside or tight junction of cells, and

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

luteolin was absorbed in cells again. After the differentiated Caco-2 cells were treated with luteolin-7-*O*-glucoside, luteolin glucoside in the apical solution decreased at 4 h, whereas luteolin aglycone tended to increase in a time-dependent manner and conjugates were not detected (Figure 5A). Previously, Chantret et al.[30] and Faria et al.,[31] reported that LPH was expressed in Caco-2 cells. Thus, we suggested the possibility that a part of the luteolin glucoside was hydrolyzed by LPH and that one part of the aglycone was not absorbed and accumulated in the apical solution, whereas the other part was absorbed into cells and secreted into the basolateral solution. Otherwise, Mukinda et al.[32] showed the possibility that luteolin-7-*O*-glucoside could be detected in the basolateral solution because the glucoside was absorbed through SGLT1 or passed through the intracellular tight junctions; this is in agreememt with the case in rat as described above. Therefore, the aglycone appeared in the basolateral solution (Figure 5B) and also because the glycoside from the absorbent through SGLT1 was hydrolyzed by intracellular glucosidase. This aglycone might be converted to conjugates detected in the basolateral solution. After treatment with luteolin in Caco-2 cells, luteolin was simply absorbed and conjugated as reported as the general mechanisms of flavonoid metabolism.[19] These results suggest that luteolin and luteolin glycoside from the *Chrysanthemum* flower extract might be absorbed and metabolized in the intestine.

Taken together, administration of the *Chrysanthemum* flower extract to rats and treatment-differentiated Caco-2 cells led to the detection of luteolin, luteolin glucoside, and luteolin glucuronide in the plasma and basolateral solution, respectively. Thus, when a *Chrysanthemum* flower extract is administered to humans, it is possible that luteolin glucoside is absorbed in the intestine without conjugation and circulated. Luteolin glucoside as well as luteolin have various beneficial effects.[5,6,30] In addition, as our group[31] showed, glucuronidase is released from stimulated neutrophils or certain injured cells, and subsequently deglucuronidates conjugated luteolin derivatives during inflammation. Thus, administration of the *C. morifolium* flower extract might possibly improve human health, and elucidation of the absorption and metabolism in humans should be clarified to assess the efficacy of this extract.

In conclusion, we showed that luteolin and luteolin monoglucoside were quickly absorbed into the plasma or basolateral solution and existed as the aglycone, monoglucoside, and monoglucuronide after administration of the *C. morifolium* flower extract to rats and differentiated Caco-2 cells. As described above, luteolin, luteolin glucoside, and glucuronide can exert biological functions in the human body.[5,6,33,34] The concentrations of these compounds in plasma also showed a secondary increase, meaning that intake of the *C. morifolium* flower extract led to retention of these compounds in the circulatory system for a long time.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental Figure 1. HPLC chromatogram of the flavonoids. L7G, luteolin-7-*O*-glucoside (11.167 min); IS, internal standard (myricetin; 14.733 min); L, luteolin (17.767 min). Supplemental Figure 2. Mass chromatogram and mass spectrum of luteolin-7-*O*-glucoside in the culture medium of Caco-2 cells treated with the chrysanthemum flower extract. The mass chromatogram (m/z 449.1033−449.1123) (inset) and mass spectrum (time = 6.27 min) of luteolin-7-*O*-glucoside in the basolateral solution of Caco-2 cells treated with the chrysanthemum flower extract for 4 h are shown. Supplemental Figure 3. Mass chromatogram and mass spectrum of luteolin monoglucuronide in the culture medium of Caco-2 cells treated with the chrysanthemum flower extract. The mass chromatogram (m/z 463.0825−463.0917) (inset) and mass spectrum at time = 6.30 min (A), time = 6.78 min (B), and time = 6.97 min (C) of luteolin monoglucuronide in the basolateral solution of Caco-2 cells treated with the chrysanthemum flower extract for 4 h are shown. Supplemental Figure 4. Mass chromatogram and mass spectrum of luteolin-7-*O*-glucoside and luteolin monoglucuronide in culture media from Caco-2 cells treated with luteolin-7-*O*-β-glucoside. The mass chromatogram (m/z 449.1033−449.1123) and mass spectrum (time = 6.09 min) of luteolin-7-*O*-glucoside (A) and the mass chromatogram (m/z 463.0825−463.0917) and mass spectrum (time = 6.30 min) of luteolin monoglucuronide (B) in the basolateral solution of Caco-2 cell treated with luteolin-7-*O*-β-glucoside for 1 h are shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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