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Bioactive Constituents, Metabolites, and Functions

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Absorption and metabolism of luteolin in rats and humans in relation to *in vitro* anti-inflammatory effects

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ABSTRACT

Luteolin is a flavonoid present in plants in the form of aglycone or glucosides. In this study, luteolin glucosides (*i.e.,* luteolin-7-*O*-β-D-glucoside, luteolin-7-*O*-[2-(β-D-apiosyl)-β-D-glucoside], and luteolin-7-*O*-[2-(β-D-apiosyl)-6-malonyl-β-D-glucoside]) prepared from green pepper leaves as well as luteolin aglycone were orally administered to rats. Regardless of the administered luteolin form, luteolin glucuronides were mainly detected from plasma and organs. Subsequently, luteolin aglycone, the most absorbed form of luteolin in rats, was orally administered to humans. As a result, luteolin-3′-*O*-sulfate was mainly identified from plasma, suggesting that not only luteolin form, but also animal species affect the absorption and metabolism of luteolin. When LPS-treated RAW264.7 cells were treated with luteolin glucuronides and luteolin sulfate (the characteristic metabolites identified from rats and humans, respectively), the different luteolin conjugates were metabolized in different ways, suggesting that such difference in metabolism results in their difference in anti-inflammatory effects.

Keywords

Luteolin aglycone, Luteolin glucuronide, Luteolin sulfate, Metabolism, Anti-inflammatory effects

1. INTRODUCTION

People consume substantial amounts of flavonoids through the intake of plant foods and beverages.[1-4] Intake of flavonoids from such sources is believed to be associated with decreased risk of life-style related diseases such as atherosclerosis, diabetes, and cancer.[5–7] In order to unravel the mechanisms by which dietary flavonoids exhibit their physiological effects, it is important to understand the factors that determine their release from foods, their extent of absorption, and their metabolic fate *in vivo*.[8] Hence, many studies concerning the absorption and metabolism of certain major flavonoids (*e.g.,* catechin and quercetin) have been conducted.[9,10]

Luteolin is a typical flavonoid present in celery, green pepper, and perilla leaf, found in the form of either aglycone or glucosides (*e.g.,* luteolin-7-*O*-β-D-glucoside (L-7G), luteolin-7-*O*-[2-(β-D-apiosyl)-β-D-glucoside] (L-7AG), and luteolin-7-*O*-[2-(β-D-apiosyl)-6-malonyl-β-D-glucoside] (L-7AMG)) (Fig. 1A).[8,11] Intake of luteolin is known to contribute to various beneficial effects such as anti-inflammatory, antioxidative, and anticancer activities.[2,12-14] Nevertheless, only few studies have examined the absorption and metabolic fate of luteolin,[15] which contrasts with the case of catechin and quercetin.[9,10]

In one of such studies, the absorption of luteolin was examined by Lin et al. through the oral administration of luteolin aglycone and L-7G to rats.[16] Whereas luteolin aglycone was directly absorbed, L-7G was mainly hydrolyzed to luteolin aglycone in the gastrointestinal tract, followed by absorption into systemic circulation. Also, Shimoi et al. reported that orally administered luteolin aglycone is mainly metabolized to luteolin glucuronide in rats.[1,8] In a similar way, when L-7G was orally administered to rats, L-7G was first hydrolyzed to luteolin aglycone and then converted

mainly to luteolin glucuronide. Additionally, we recently investigated the position at which glucuronic acid conjugates with luteolin after the oral administration of luteolin aglycone and L-7G to rats, and reported that luteolin-3′-*O*-β-D-glucuronide was the major metabolite found in plasma.[17] Thus, luteolin conjugates, especially luteolin-3′-*O*-β-D-glucuronide, but not luteolin aglycone and luteolin glucosides, are presumably responsible for the physiological effects of luteolin *in vivo*. However, the absorption and metabolism of luteolin glucosides other than L-7G (*e.g.,* L-7AG and L-7AMG) is still unknown, possibly due to the difficulties in preparing such glucosides. In addition, most studies investigating the absorption of luteolin have focused on rodents, and human data are scarcely available.

In this study, we prepared luteolin glucosides (*i.e.,* L-7G, L-7AG, and L-7AMG) from green pepper leaves, and orally administered either these glucosides or luteolin aglycone to rats, in order to investigate the differences with regard to the extent of their absorption and metabolism. We also conducted a human test by which the most easily absorbed form of luteolin in rats (*i.e.,* luteolin aglycone) was orally administered to healthy individuals, and found for the first time that not only the form of luteolin, but also animal species affect the absorption and metabolism of luteolin. To evaluate the relationship of this finding with the physiological effects of luteolin, luteolin metabolites characteristic to rats and humans were each investigated for their effects on inflammation in LPS-treated RAW264.7 cells. The results of this study may help elucidate the relationship between the biological availability and physiological effects of luteolin in animals and humans.

2. MATERIALS AND METHODS

89 **2.1 Chemicals**

90 Luteolin was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).
91 LPS (*Escherichia coli* 0111) was purchased from Sigma (St. Louis, MO, USA).
92 Luteolin-3′-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucuronide, and
93 luteolin-7-*O*-β-D-glucuronide were prepared as reported previously.[18-20] For the
94 preparation of luteolin-3′-*O*-sulfate, 286 mg luteolin was dissolved in 4 mL of
95 anhydrous *N,N*-dimethylformamide, of which was reacted with 318 mg of sulfur
96 trioxide pyridine complex for five hours at room temperature. After addition of 20 mL
97 water, the solution was loaded onto a TOYOPEARL HW-40F column (TOSOH
98 Corporation, Tokyo, Japan). The column was washed with water (200 mL) and eluted
99 with 30% methanol (1 L). The fraction containing luteolin-3′-*O*-sulfate was collected,
100 neutralized with 1.0 M NaOH to pH 6.8, and evaporated to obtain luteolin-3′-*O*-sulfate
101 sodium salt. The purity and chemical structures of luteolin-3′-*O*-β-D-glucuronide,
102 luteolin-4′-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucuronide, and luteolin-3′-*O*-sulfate
103 was confirmed with MS/MS analysis using a micrOTOF-Q II mass spectrometer
104 (Bruker Daltonik, Bremen, Germany) and with ^{1}H and ^{13}C NMR using a
105 JNM-ECX-400P spectrometer (Japan Electronic Co., Ltd., Tokyo, Japan) at 400 MHz
106 with dimethyl sulfoxide-d_6 as the solvent. Other chemicals used were of analytical grade
107 or higher.
108
109 **2.2 Preparation of luteolin glucosides**
110 Green pepper leaves (500 g) were crushed with 1 L of methanol containing 0.5%
111 phosphoric acid. After the addition of 500 mL distilled water, the solution was filtered
112 under reduced pressure. A further 2 L of distilled water was added to the obtained

filtrate. The solution was loaded onto a DIAION^{TM} HP20 column (Mitsubishi Chemical Co., Ltd., Tokyo, Japan), which was then washed with 25% methanol (1 L). L-7AMG was eluted with 2 L of 90% ethanol. The eluate was evaporated, dissolved in methanol, and centrifuged at 1,000×*g* for 5 min at 4°C. The precipitate was collected and freeze-dried to obtain L-7AMG powder (1.9 g).

L-7G was prepared from L-7AMG as follows: L-7AMG (0.5 g) was dissolved in 2.0 M NaOH (20 mL) and hydrolyzed at room temperature for 10 min. Subsequently, 7.5 mL of 6.0 M HCl was added, and L-7AMG was further hydrolyzed at 80°C for 1 h. This solution was loaded onto a TOYOPEARL HW-40C column (TOSOH Corporation), and was washed with 100 mL of 40% methanol. L-7G was fractionated by gradient elution with 70–100% methanol containing 0.1% formic acid (2 L). The fractionated solution was dried to obtain L-7G powder (0.3 g).

For the preparation of L-7AG, L-7AMG (0.5 g) was dissolved in 1.0 M NaOH (20 mL) and hydrolyzed at room temperature for 10 min. After neutralization with 3.3 mL of 6.0 M HCl, this solution was centrifuged at 7,700×*g* for 5 min at 4°C. The precipitates were purified with distilled water and recentrifuged. The obtained precipitate was dehydrated with methanol. After adding an excessive volume of ethyl acetate, the solution was centrifuged twice at 7,700×*g* for 5 min at 4°C. Finally, the collected precipitate was dried to obtain L-7AG powder (0.3 g).

The purity and chemical structures of L-7G, L-7AG, and L-7AMG were confirmed by MS/MS and by ^{1}H and ^{13}C NMR.

2.3 Animal study

Eight-week-old male Sprague-Dawley rats (weight = 208 ± 12 g) were obtained

137 from CLEA Japan, Inc. (Tokyo, Japan). Rats were housed in cages maintained at 23°C
138 with a 12 h light/dark cycle and were given free access to water and commercial rodent
139 chow (CE-2; CLEA Japan, Inc.) for 1 week before the experiments. After 12 h fast, rats
140 (n = 7–8) were administered either luteolin aglycone, L-7G, L-7AG, or L-7AMG (20
141 mg/kg body weight) by oral gavage using 1% sodium cholate as vehicle. After 0, 1, 3, 6,
142 12, and 24 h of administration, blood (about 0.3 mL) was collected from the tail vein
143 using capillary tubes. Blood was centrifuged at 1,000×g for 15 min at 4°C to obtain
144 plasma. In a separate experiment, livers, kidneys, and small intestines (n = 7–8) were
145 excised 6 h after oral administration of either luteolin aglycone, L-7G, L-7AG, or
146 L-7AMG (20 mg/kg body weight). The collected samples were stored at −80°C until
147 analysis. The protocols of the animal study were designed in accordance with the animal
148 experiment guidelines and reviewed by the ethics committee of Tohoku University
149 (approval number: 2016-noudou-006).
150
151 **2.4 Human study**
152 Nine healthy male subjects aged 23–47 years participated in the study and gave
153 written informed consent in accordance with the Declaration of Helsinki. The subjects
154 were refrained from consuming foods containing luteolin (*e.g.,* paprika) for one week
155 prior to the study. Subjects orally consumed 50 mg luteolin aglycone via capsules
156 containing a commercial *Chrysanthemum morifolium* extract (Kiku flower extract-P;
157 Oryza Oil & Fat Chemical Co., Ltd., Aichi, Japan) after fasting overnight. HPLC-UV
158 analysis confirmed that the extract contained about 10% luteolin aglycone and trace
159 levels of apigenin. Luteolin derivatives other than luteolin aglycone were not detected.
160 Blood samples were collected in heparinized tubes before and 1, 3, 6, and 12 h after

administration. Blood was centrifuged at 1,000×*g* for 10 min at 4°C to obtain plasma. The obtained plasma was stored at −80°C until analysis. During the study period, subjects were allowed to drink water ad libitum and to take light meals (Japanese noodles free of luteolin) at 4 and 10 h after administration of luteolin capsules. The protocols were approved by the ethics committee of Tohoku University (approval numbers: 16-A-02 and 17-A-05).

2.5 Extraction of luteolin metabolites from plasma and organs

Luteolin metabolites were extracted from plasma and organs as described previously.[17] Acetonitrile (300 μL) was added to 100 μL of rat/human plasma, and centrifuged at 1,000×*g* for 10 min at 4°C. After collecting the supernatant by decantation, methanol (300 μL) was added to the precipitate. The solution was mixed and centrifuged at 1,000×*g* for 10 min at 4°C. The resultant supernatants were combined, dried, and redissolved in 10% acetonitrile. For the extraction of luteolin metabolites from organs, a 30% (w/v) homogenate solution was prepared by homogenizing each organ (300 mg) in saline containing 1 mM ethylenediaminetetraacetic acid. Luteolin metabolites were extracted from 500 μL of the homogenate in the same way as plasma.

2.6 Cell study

Mouse macrophage RAW264.7 cells were purchased from DS Biomedical Co., Ltd. (Osaka, Japan). RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Dainippon Sumitomo Pharmaceutical, Osaka, Japan), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C under 5%

185 CO_2.

186 RAW264.7 cells (5.0×10^5) were preincubated with 10% FBS/DMEM in 10 cm
187 dishes for 24 h. Luteolin aglycone and conjugates were dissolved in dimethyl sulfoxide
188 (50 mM), and 5 μL of the solution was added to the test medium to achieve a final
189 concentration of 25 μM. Cells were incubated in each test medium for 24 h. After
190 removal of cell medium, the cell surface was washed with PBS several times, after
191 which the cells were collected and homogenized. Intracellular luteolin metabolites were
192 extracted from the cell suspension in the same way as human plasma.

193 For the identification of genes related to the anti-inflammatory effects of luteolin,
194 RAW264.7 cells (8.0×10^5) were treated either with or without luteolin aglycone and
195 conjugates. After incubation for 24 h, LPS was to the medium to achieve a final
196 concentration of 1 μg/mL. Cells were further incubated for 3 h and total RNA was
197 extracted with the use of an RNeasy Mini Kit (Qiagen, Tokyo, Japan). As a control,
198 total RNA was extracted from cells that were not treated with luteolin nor LPS.
199 PrimeScript Master mix (Takara Bio, Otsu, Japan) was used for the synthesis of cDNA
200 from total RNA (500 ng) according to the manufacturer's instructions. PCR
201 amplification was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad
202 Laboratories, New South Wales, Australia) with SYBR Premix Ex TaqII (Takara Bio)
203 and gene-specific primers (Sigma-Aldrich Japan, Tokyo, Japan) for interleukin-6 (*IL-6*),
204 interleukin-1 beta (*IL-1β*), CC chemokine ligand 2 (*Ccl2*), CC chemokine ligand 3
205 (*Ccl3*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). PCR conditions
206 were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 55°C for 30 s.
207

208 **2.7 LC-MS/MS analysis**

LC-MS/MS analysis of luteolin was performed based on our previous method with slight modifications.[17] The extracted samples (10 μL) were injected into a 4000 QTRAP mass spectrometer (SCIEX, Redwood City, CA, USA) equipped with a Prominence HPLC system (Shimadzu, Kyoto, Japan). Samples were analyzed with a C18 column (CAPCELLPAK C18 MGII S3, 4.6×150 mm; Shiseido, Tokyo, Japan) maintained at 40°C with a flow rate of 0.8 mL/min. Gradient elution was performed using a two-solvent system: A, water containing 0.1% trifluoroacetic acid; and B, acetonitrile. The gradient program was as follows: 0–20 min, 90–70% A linear; 20–25 min, 70–50% A linear. The mobile phase was split at the postcolumn, such that the eluate entered the HPLC-MS/MS system at a flow rate of 0.2 mL/min. MS/MS parameters were optimized with luteolin aglycone, luteolin-3′-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucuronide, and luteolin-3′-*O*-sulfate standards under electrospray ionization in the negative ion mode. The MS parameters were as follows: turbo gas temperature, 600°C; spray voltage, −4500 V; nebulizer gas, 40 psi; auxiliary gas, 40 psi; curtain gas, 20 psi; collision gas, 4.0 psi. For the quantification of luteolin aglycone and luteolin glucuronides, the instrument was operated in the multiple-reaction monitoring (MRM) mode and concentrations were calculated based on respective standard curves. Detection limits were in the range of 0.1−0.2 pmol/injection at a S/N ratio of 3, and recovery tests confirmed that recoveries were >70%.[17] The precursor/product ion transitions were the following: luteolin aglycone, *m/z* 284 > 133 (collision energy (CE), −48 V; declustering potential (DP), −100 V); luteolin-3′-*O*-β-D-glucuronide, *m/z* 461 > 285 (CE, −32 V; DP, −95 V); luteolin-4′-*O*-β-D-glucuronide, *m/z* 461 > 285 (CE, −32 V; DP, −95 V); luteolin-7-*O*-β-D-glucuronide, *m/z* 461 > 285 (CE, −32 V; DP, −95 V); luteolin-3′-*O*-sulfate, *m/z* 365 > 285 (CE, −50 V; DP, −90 V). Luteolin of other forms

were qualitatively analyzed using the following MRM transitions either defined in literature[21-23] or predicted MRM ion pairs (CE, −50 V; DP, −90 V): luteolin glucoside, *m/z* 447 > 285; luteolin diglucoside, *m/z* 609 > 285; luteolin diglucuronide, *m/z* 637 > 285; luteolin glucoside glucuronide, *m/z* 632 > 285; luteolin disulfate, *m/z* 444 > 285; luteolin glucoside sulfate, *m/z* 637 > 285; luteolin glucuronide sulfate, *m/z* 541 > 285; methylated luteolin, *m/z* 301 > 285; methylated luteolin glucoside, *m/z* 463 > 285; methylated luteolin glucuronide, *m/z* 477 > 285; methylated luteolin sulfate, *m/z* 381 > 285.

2.8 Statistical Analysis

Data are presented as mean ± standard error (SE). Statistical analysis was performed by one-way ANOVA followed by Tukey's test. A p value of <0.05 was considered to be significant.

3. RESULTS AND DISCUSSION

3.1 Preparation of luteolin glucosides for oral administration studies

Unlike catechin and quercetin, which are flavonoids that have been investigated in detail, studies examining the metabolic fate of luteolin are limited.[15] We previously identified the structures of the major luteolin metabolites (*i.e.,* luteolin glucuronides) found in plasma and organs after oral administration of luteolin aglycone or L-7G to rats,[17] and suggested that luteolin conjugates, especially luteolin-3′-*O*-β-D-glucuronide, are responsible for the biological activity of luteolin *in vivo*. However, the absorption and metabolism of L-7AG and L-7AMG, the primary glucosides detected in certain plants such as pepper fruits,[11] are still unknown. Hence, in this study, the metabolic fate

of luteolin glucosides (*i.e.,* L-7G, L-7AG, and L-7AMG) and luteolin aglycone was investigated by a single oral administration test to clarify how luteolin is absorbed and metabolized.

Since L-7AG and L-7AMG are not commercially available, these luteolin glucosides were prepared for oral administration studies. First, L-7AMG was extracted from green pepper leaves and chromatographically purified with a DIAION^{TM} HP20 column. L-7G was fractionated with a TOYOPEARL HW-40C column after hydrolyzing L-7AMG with NaOH and HCl solutions. L-7AG was produced by hydrolyzing L-7AMG in a NaOH solution and by drying the precipitate. Each luteolin glucoside was pure enough for the use in oral administration tests (HPLC purity > 90%). As a point of reference, although apiosylated flavonoids (*e.g.,* L-7AG and apigenin-7-*O*-apiosyl-glucoside) are known to be present in nature,[11,24] the apiosyl-malonyl type (*i.e.,* L-7AMG) is characteristic to luteolin. It has been suggested that the malonyl group in L-7AMG is easily dissociated in aqueous solutions; thus, it was thought interesting to see whether such property would affect the absorption and metabolism of L-7AMG.

3.2 Absorption and metabolism of luteolin aglycone and glucosides in rats

In the animal study, rats were orally administered 20 mg/kg body weight of either luteolin aglycone or luteolin glucosides (*i.e.,* L-7G, L-7AG, and L-7AMG). This dose was comparable to other studies observing the metabolism of flavonoids, as well as our previous study that investigated the metabolic fate of luteolin.[17] In the present study, HPLC-MS/MS analysis in the MRM mode was utilized to analyze various luteolin metabolites, since the method offers advantages such as selectivity over conventional

techniques (*e.g.,* HPLC-UV).[1] As a result, regardless of the form by which luteolin was administered, luteolin glucuronides (*i.e.,* luteolin-3′-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucuronide, and luteolin-7-*O*-β-D-glucuronide) were mainly detected from plasma and organs (*i.e.,* liver, kidney, and small intestine). Luteolin-3′-*O*-β-D-glucuronide was observed as the largest peak (Fig. 1B and 2). Luteolin diglucuronide, luteolin glucuronide sulfate, and luteolin aglycone were seen at low peak intensities, whereas luteolin glucosides, luteolin diglucosides, luteolin glucoside glucuronide, luteolin sulfate, luteolin disulfate, luteolin glucoside sulfate, luteolin glucuronide sulfate, methylated luteolin, methylated luteolin glucoside, methylated luteolin glucuronide, and methylated luteolin sulfate were either found in only trace levels or not detected, suggesting their low concentrations in plasma and organs. Based on the present results and earlier findings,[1,8,16,17] we can suggest the following absorption and metabolism routes of luteolin. When rats receive luteolin aglycone, a part of luteolin is absorbed from the intestine into the systemic circulation. In case of luteolin glucosides, regardless of sugar chain type, L-7G, L-7AG, and L-7AMG are primarily hydrolyzed to luteolin aglycone in the gastrointestinal tract and then absorbed into the body. Following this absorption, luteolin is mainly converted to luteolin glucuronides, especially luteolin-3′-*O*-β-D-glucuronide. However, further studies are necessary to elucidate whether such hydrolysis of different luteolin glucosides takes place in the gastrointestinal tract.

HPLC-MS/MS analysis of both the plasma and organs demonstrated that the concentration of luteolin glucuronides was larger in the luteolin aglycone administrated group than that of the luteolin glucoside administrated groups (Table 1 and 2). In previous studies, luteolin glucoside was suggested to be hydrolyzed to luteolin aglycone

by the bacterial flora in the intestine, and then converted to luteolin glucuronide by phase II enzymes in the small intestine, liver, and kidney.[8,15] This suggests that luteolin aglycone is easily absorbed compared to luteolin glucoside, because luteolin aglycone does not need to be hydrolyzed, and coincides with the results of the current study in which oral administration of luteolin aglycone resulted in greater amounts of luteolin metabolites in plasma and organs compared to the administration of luteolin glucosides. With regard to the metabolism of luteolin glucosides, the concentrations of luteolin glucuronides were typically higher in order of the L-7G group > L-7AG group > L-7AMG group. Hence, L-7G may be hydrolyzed and absorbed easier than L-7AG or L-7AMG. In a previous study, Chang et al. compared the absorption of quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside and suggested that quercetin-3-*O*-glucoside is rapidly absorbed and transformed into glucuronidated quercetin as compared to quercetin-3-*O*-galactoside.[25] Such results indicate that the absorption of flavonoid glycosides relates to the hydrolysis of the type of sugar chains attached to its aglycone. Furthermore, the withdrawal of sugar chains attached to flavonoids have been suggested to take place as a result of enzymatic reactions. For example, it has been reported that the glucose bound to flavonoids is hydrolyzed by glucosidases that exist in the lower sections of the small intestine or enteric bacteria.[26] These studies suggest that glucose is easily hydrolyzed, and thus L-7G is absorbed easier than L-7AG or L-7AMG. Also, the fact that L-7AG was absorbed easier than L-7AMG can be considered to be due to the presence of a malonyl group in L-7AMG. Since it is known that the malonyl group in L-7AMG is easily dissociated in aqueous solutions, further research will be necessary to identify why the malonyl group seems to be difficult to be dissociated during the metabolism of L-7AMG *in vivo*.

The concentration of luteolin-3′-*O*-β-D-glucuronide in plasma reached its maximum level at 3–6 h after oral administration, and then gradually decreased (Fig. 3). Although the absorbance of luteolin differed according to its different forms, the major metabolite of luteolin (*i.e.,* luteolin-3′-β-D-*O*-glucuronide) remained in rat plasma even after 24 h of administration of both luteolin aglycone and luteolin glucosides (Table 1). Chung et al. reported that the peak concentration of catechin in rat plasma was reached at 0.14–0.18 h after ingestion, and gradually reduced to detection limits at 12 h.[9] Although the study only examined the free forms of catechin, and not its conjugates, the fact that luteolin-3′-*O*-β-D-glucuronide remained to about half of the maximum level even 12 h after administration regardless of administrating luteolin aglycone or glucosides suggests that luteolin remains for a much longer time in rat body compared to other flavonoids. This may be due to enterohepatic recycling, of which previous studies have demonstrated that following initial absorption of flavonoids, their phase II metabolites (*e.g.,* glucuronides) are excreted to the gastrointestinal tract via bile.[27,28] However, further studies are necessary to prove that such mechanism prolongs the bioavailability of luteolin.

3.3 Absorption and metabolism of luteolin aglycone in humans

As mentioned above, it was suggested that luteolin aglycone and glucosides were mainly metabolized to luteolin glucuronide in rats. However, because intestinal enzymatic activities differ between humans and animals,[8] we considered that luteolin is not necessarily converted to only luteolin glucuronide in humans. While human studies related to the metabolism of other flavonoids (*e.g.,* catechin and quercetin) have been conducted,[29-31] knowledge regarding the metabolism of luteolin in humans have been

353 scarce. Therefore, we conducted a human study in which luteolin aglycone, the most
354 absorbed form of luteolin in the animal study, was orally administered. Nine male
355 subjects consumed 50 mg luteolin aglycone, and blood samples were collected over
356 time to investigate time-dependent changes of luteolin in human plasma. As a result,
357 consistent with the oral administration test to rats, luteolin-3′-*O*-β-D-glucuronide and
358 luteolin-4′-*O*-β-D-glucuronide were detected in human plasma (Fig. 4A). However, the
359 maximum peak identified during the LC-MS/MS analysis of human plasma was not
360 luteolin glucuronide but was an unidentified peak. Considering the study by Roubalová
361 et al. in which the major form of quercetin identified in humans was
362 quercetin-3′-*O*-sulfate,[32] and the fact that phenol sulfotransferase, an enzyme that
363 metabolizes flavonoids to flavonoid sulfates, is present in the human intestine,[33] it was
364 speculated that the unidentified peak was luteolin sulfate. Hence, a standard of luteolin
365 sulfates (*i.e.,* luteolin-3′-*O*-sulfate and luteolin-4′-*O*-sulfate) was prepared by reacting
366 luteolin aglycone with sulfur trioxide pyridine complex. Luteolin sulfate in human
367 plasma was analyzed with the use of these standards, and consequently, the unidentified
368 peak corresponded to that of the luteolin-3′-*O*-sulfate standard. Based on these results,
369 it was suggested that the metabolism of luteolin varies according to animal species.
370 Subsequently, time-dependent changes of luteolin-3′-*O*-sulfate concentration in human
371 plasma were further investigated. The plasma concentration of luteolin-3′-*O*-sulfate
372 reached its maximum level (801.9 ± 277.6 nM) at 3 h and was about half of the
373 maximum concentration at 6 h after oral administration of luteolin aglycone. Although
374 the concentration gradually decreased, luteolin-3′-*O*-sufate still remained even after 12
375 h of administration (Fig. 4B). In the study by Mullen et al. where humans consumed
376 261 μmol quercetin (equivalent to 79 mg quercetin aglycone), the concentration of

quercetin-3′-*O*-sulfate in human plasma reached a maximum level (665.0 ± 82.0 nM) at 0.75 h and was half at 1.71 h after administration.[31] Thus, our results suggest that luteolin remains for a relatively long time in the human body compared with other flavonoids. However, compared with the results of the animal study where luteolin glucuronides remained in plasma to about half of the maximum level 12 h after administration (Fig. 3), luteolin sulfate remained in human plasma for a considerably shorter time (Fig. 4B). This may be related to the enterohepatic recycling discussed above, considering that while flavonoid sulfates are mainly excreted only into urine, glucuronides are usually excreted also to bile to undergo enterohepatic recycling.[34-36] Thus, further studies examining luteolin glucuronides and sulfates in urine may provide a more detailed view on the possible mechanism regarding how enterohepatic circulation possibly prolongs the bioavailability of luteolin.

3.4 *In vitro* anti-inflammatory effects of luteolin conjugates in relation to their cellular intake

Based on the above studies, it was elucidated that the metabolism of luteolin is different between rats and humans. As such, a cell study was conducted to examine whether different luteolin metabolites demonstrate different strengths of physiological effects. Recent studies have examined the anti-inflammatory effects of luteolin; Chen et al. reported that luteolin aglycone inhibits the expression of inflammatory genes (*i.e.* *TNF-α*, *IL-6*, *iNOS*, and *COX-2*) in LPS-treated mouse macrophage RAW264.7 cells.[37] In addition, we previously compared the anti-inflammatory properties of luteolin aglycone and glucuronides (*i.e.,* luteolin-3′-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucuronide, and luteoin-7-*O*-β-D-glucuronide) in LPS-treated

RAW264.7 cells.[17] However, the anti-inflammatory properties of luteolin sulfate, which was identified as the major metabolite of luteolin in humans in the current study, has not yet been evaluated. Moreover, studies on how luteolin aglycone and luteolin conjugates are incorporated into cells have been scarce, although it can be assumed that luteolin first has to be incorporated into the cell to exhibit physiological functions (*e.g.,* anti-inflammatory effects). Therefore, the anti-inflammatory effects of luteolin aglycone, luteolin glucuronides (*i.e.,* luteolin metabolites characteristic to rats), and luteolin sulfates (*i.e.,* luteolin metabolites characteristic to humans) were investigated in relation to their cellular intake.

Our previous study along with other reports have demonstrated that luteolin aglycone and luteolin conjugates at a concentration of 25 μM have no effect on cell viability.[17,37] As such, we decided that a concentration of 25 μM was suitable for examining the cellular intake and anti-inflammatory effects of luteolin. The cellular intake of luteolin was investigated by extracting luteolin from RAW264.7 cells treated with either luteolin aglycone, luteolin glucuronides (*i.e.,* luteolin-3′-*O*-β-D-glucuronide and luteolin-4′-*O*-β-D-glucuronide), or luteolin sulfate (*i.e.,* luteolin-3′-*O*-sulfate). Also, to investigate the anti-inflammatory effects of each luteolin form, RAW264.7 cells were first treated with each luteolin form for 24 h, after which LPS (1 μg/mL) was added to the test medium. After further incubating for 3 h, total RNA was extracted, and mRNA expression of *IL-6*, *IL-1β*, *Ccl2*, and *Ccl3* was examined by real-time RT-PCR. These genes related to inflammation were selected based on the results of our previous study, in which it was identified through DNA microarray analysis that luteolin treatment affects their expression.[17]

When RAW264.7 cells were treated with luteolin aglycone, luteolin aglycone was

mainly detected from the cell (Fig. 5). Also, in accordance with our previous study,[17] PCR analysis demonstrated that treatment of cells with luteolin aglycone inhibits the expression of *IL-6*, *IL-1β*, *Ccl2*, and *Ccl3* genes (Fig. 6). With regard to treatment of cells with luteolin conjugates, when cells were treated with luteolin glucuronide (*i.e.,* luteolin-3′-*O*-β-D-glucuronide and luteolin-4′-*O*-β-D-glucuronide), luteolin aglycone was the main metabolite detected from the cell. Shimoi et al. previously reported that β-glucuronidase enzymes convert luteolin glucuronides to luteolin aglycone as a consequence of inflammation *in vitro* in human neutrophils as well as *in vivo* in rats.[38] The predominance of luteolin aglycone after treatment of luteolin glucuronides may be a result of such β-glucuronidase activity; previous studies identifying β-glucuronidase as the major deglucuronidation enzyme that deconjugates quercetin glucuronide in RAW264 cells, further emphasizes this hypothesis.[39,40] The expression levels of inflammatory genes were also suppressed, suggesting that luteolin aglycone is the major form related to the anti-inflammatory effects of luteolin. On the other hand, the intracellular amounts of luteolin metabolites after the treatment of cells with luteolin-3′-*O*-sulfate was very low compared with that of treatment with luteolin glucuronides, suggesting that luteolin-3′-*O*-sulfate is difficult to be incorporated into RAW264.7 cells. Also, metabolites other than luteolin-3′-*O*-sulfate were not detected (Fig. 5), which demonstrates the difference in metabolism between luteolin-3′-*O*-sulfate and luteolin glucuronides. This difference in metabolism was somewhat reflected in the expression levels of inflammatory genes, considering that the inhibitory effects of luteolin-3′-*O*-sulfate was lower than that of luteolin aglycone and luteolin glucuronide (Fig. 6). One exception was the expression level of *Ccl2*, which was increased by treatment with luteolin-3′-*O*-sulfate; the reason for this is unclear and requires further

research. With regard to the physiological effects of phenolic sulfates, Roubalová et al. reported that the bioactivity of quercetin sulfate *in vitro* is weak because the affinity of sulfated quercetin to target molecules is low compared to quercetin aglycone.[32] Similarly, it can be considered that the anti-inflammatory effects of luteolin sulfate was weaker than that of luteolin aglycone and luteolin glucuronide due to the affinity of luteolin sulfate to molecules that control the expression of inflammatory genes being lower.

In conclusion, it was revealed that regardless of administrating luteolin aglycone or glucosides, these molecules are mainly metabolized to luteolin glucuronide (*e.g.,* luteolin-3´-*O*-β-D-glucuronide) in rats. Meanwhile, luteolin-3´-*O*-sulfate was identified as the most abundant luteolin form in human plasma after administration of luteolin aglycone. Although the metabolism of luteolin varied between animal species, it was suggested that luteolin glucuronide and luteolin sulfate, which are the major metabolites of luteolin in rats and humans, remain for a relativity long time in the body. Luteolin glucuronide was mainly converted to luteolin aglycone after being incorporated into the cell and inhibited the expression of inflammatory genes in LPS-treated RAW264.7 cells. Luteolin sulfate also reduced expression levels of inflammatory genes, but this effect was weaker than that of luteolin glucuronide. However, further investigations will be required to fully elucidate the mechanism by which luteolin exhibits its physiological functions *in vitro* and *in vivo*.

Abbreviations Used

CE, collision energy; DP, declustering potential; FBS, fetal bovine serum; L-7AG, luteolin-7-*O*-[2-(β-D-apiosyl)-β-D-glucoside]; L-7AMG,

473 luteolin-7-*O*-[2-(β-D-apiosyl)-6-malonyl-β-D-glucoside]; L-7G,

474 luteolin-7-*O*-β-D-glucoside; MRM, multiple-reaction monitoring

475

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479

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612 **Tables**

613 **Table 1.** Time-dependent changes of luteolin-3'-*O*-β-D-glucuronide in rat plasma

614 following oral administration of luteolin aglycone or glucosides.

Time (h)	Luteolin aglycone administration		L-7G administration		L-7AG administration		L-7AMG administration	
	Concentrations (nM)	Concentrations (μg/L)	Concentrations (nM)	Concentrations (μg/L)	Concentrations (nM)	Concentrations (μg/L)	Concentrations (nM)	Concentrations (μg/L)
0	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*
1	356.5 ± 499.0	164.7 ± 230.5	94.3 ± 91.0	43.6 ± 42.0	25.3 ± 16.6	11.7 ± 7.7	1.2 ± 0.5	0.6 ± 0.2
3	1074.1 ± 909.0	496.3 ± 420.0	301.1 ± 153.8	139.1 ± 195.5	24.3 ± 14.8	11.2 ± 6.8	2.8 ± 1.5	1.3 ± 0.7
6	570.0 ± 519.3	263.1 ± 239.9	153.8 ± 156.4	71.0 ± 72.3	121.2 ± 147.4	56.0 ± 68.1	40.5 ± 72.9	18.7 ± 33.7
12	531.7 ± 742.9	245.6 ± 343.2	133.2 ± 185.0	61.5 ± 85.5	11.0 ± 8.6	5.1 ± 4.0	30.8 ± 27.7	14.2 ± 12.8
24	6.8 ± 6.3	3.1 ± 2.9	4.3 ± 5.3	2.0 ± 2.4	1.3 ± 0.8	0.6 ± 0.4	0.5 ± 0.5	0.2 ± 0.2

Data are mean ± SE (n = 7-8).

L-7G, Luteolin-7-*O*-glucoside; L-7AG, Luteolin-7-*O*-apiosyl-glucoside; L-7AMG, Luteolin-7-*O*-apiosyl-malonyl-glucoside.

*n.d. = not detected

615

616 **Table 2.** The concentration of luteolin glucuronides in rat organs at 6 h after oral
617 administration of luteolin aglycone or glucosides.
618

		Luteolin-3'-*O*-glucuronide		Luteolin-4'-*O*-glucuronide		Luteolin-7-*O*-glucuronide	
		Concentration (pmol/g)	Concentration (ng/g)	Concentration (pmol/g)	Concentration (ng/g)	Concentration (pmol/g)	Concentration (ng/g)
Luteolin aglycone administration	Liver	638.2 ± 344.8	294.8 ± 159.3	277.5 ± 173.1	128.2 ± 80.0	165.1 ± 163.5	76.3 ± 65.4
	Kidney	906.7 ± 344.3	418.9 ± 159.1	184.6 ± 87.9	85.3 ± 40.6	120.2 ± 86.8	55.6 ± 34.7
	Small intestine	4818.5 ± 7008.5	4542.8 ± 3237.9	597.5 ± 440.9	276.0 ± 203.7	3573.4 ± 2814.4	1650.9 ± 1126.0
L-7G administration	Liver	186.7 ± 91.0	86.2 ± 42.1	150.4 ± 109.7	69.5 ± 50.7	23.1 ± 8.8	10.7 ± 4.1
	Kidney	258.7 ± 220.9	119.5 ± 102.1	50.8 ± 38.3	23.5 ± 17.7	32.6 ± 26.2	15.1 ± 12.1
	Small intestine	487.8 ± 443.0	225.4 ± 204.7	92.6 ± 98.8	42.8 ± 45.6	339.5 ± 375.7	142.8 ± 185.4
L-7AG administration	Liver	41.7 ± 58.6	19.3 ± 27.1	16.5 ± 12.7	7.6 ± 5.9	13.6 ± 10.1	6.3 ± 4.7
	Kidney	49.0 ± 38.2	22.6 ± 17.6	12.1 ± 11.0	5.6 ± 5.1	13.1 ± 10.9	6.0 ± 5.0
	Small intestine	211.2 ± 327.6	97.6 ± 151.4	50.5 ± 84.5	23.4 ± 39.1	149.2 ± 246.1	68.9 ± 113.7
L-7AMG administration	Liver	26.5 ± 25.1	12.2 ± 10.1	10.0 ± 6.0	4.6 ± 2.8	6.0 ± 5.1	2.8 ± 2.4
	Kidney	33.5 ± 30.0	15.0 ± 12.0	6.8 ± 5.8	3.1 ± 2.7	6.9 ± 8.0	3.2 ± 3.7
	Small intestine	308.2 ± 496.8	142.4 ± 198.8	39.5 ± 54.2	18.2 ± 25.0	312.4 ± 517.2	144.3 ± 238.9

Data are mean ± SE. (n = 4)

L-7G, Luteolin-7-*O*-glucoside; L-7AG, Luteolin-7-*O*-apiosyl-glucoside; L-7AMG, Luteolin-7-*O*-apiosyl-malonyl-glucoside.

Figure captions

Figure 1. Chemical structures of luteolin aglycone and glucosides (luteolin-7-*O*-β-D-glucoside, luteolin-7-*O*-[2-(β-D-apiosyl)-β-D-glucoside], and luteolin-7-*O*-[2-(β-D-apiosyl)-6-malonyl-β-D-glucoside]) (A) and major luteolin metabolites in rats and humans (B).

Figure 2. MRM chromatograms of luteolin-3′-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucuronide, and luteolin aglycone in rat plasma and organs (liver, kidney, and small intestine) at 6 h after oral administration of luteolin aglycone (20 mg/kg body weight). Similar chromatograms were also obtained by the oral administration of either L-7G, L-7AG, and L-7AMG (20 mg/kg body weight).

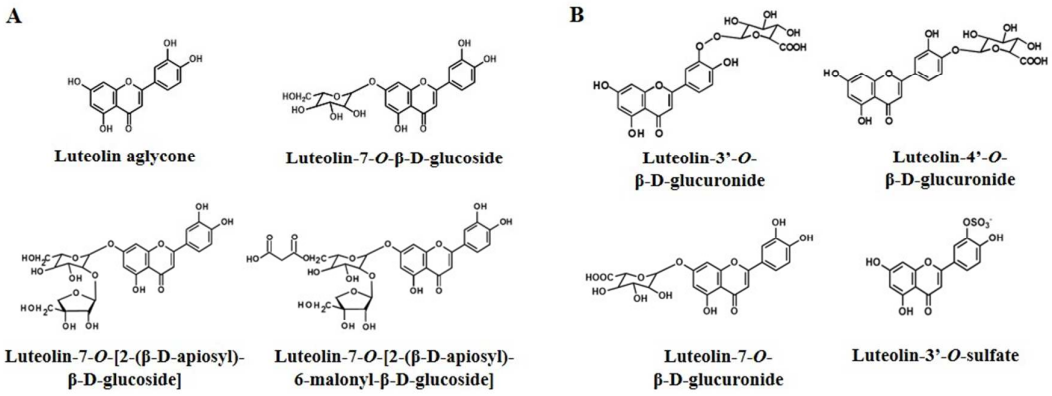
Figure 3. Time-dependent changes of luteolin-3′-*O*-β-D-glucuronide concentrations in rat plasma after oral administration of luteolin aglycone or glucosides (20 mg/kg body weight). Mean ± SE (n = 7–8).

Figure 4. MRM chromatogram of luteolin-3′-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucuronide, luteolin-3′-*O*-sulfate, and luteolin aglycone in human plasma at 3 h after oral administration of luteolin aglycone (50 mg) (A). Time-dependent changes of luteolin-3′-*O*-sulfate in human plasma after consumption of luteolin aglycone (50 mg). Mean ± SE (n = 9) (B).

642 **Figure 5.** MRM chromatograms demonstrating the luteolin metabolites identified from
643 RAW264.7 cells treated with luteolin aglycone, luteolin 3′-*O*-β-D-glucouronide,
644 luteolin-4′-*O*-β-D-glucuronide, and luteolin-3′-*O*-sulfate. RAW264.7 cells (5.0×10^5)
645 were preincubated with 10% FBS/DMEM for 24 h and subsequently treated with 25 μM
646 luteolin aglycone, luteolin-3′-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucuronide, or
647 luteolin-3′-*O*-sulfate for 24 h.
648
649 **Figure 6.** Effect of luteolin aglycone, luteolin-3′-*O*-β-D-glucuronide,
650 luteolin-4′-*O*-β-D-glucuronide, and luteolin-3′-*O*-sulfate on the mRNA expression of
651 genes related to inflammation in LPS-treated RAW264.7 cells. RAW264.7 cells ($8.0 \times$
652 10^5) were pre-incubated with 10% FBS/DMEM in 10 cm dishes for 24h. Cells were
653 treated with 25 μM luteolin aglycone, luteolin-3′-*O*-β-D-glucuronide,
654 luteolin-4′-*O*-β-D-glucuronide, or luteolin-3′-*O*-sulfate for 24 h, and then stimulated
655 with or without LPS (1 μg/mL) for 3 h. mRNA expression of genes was analyzed by
656 real-time RT-PCR. Values are expressed as mean ± SE (n = 3). Means without a
657 common letter differ significantly at $p < 0.05$.
658

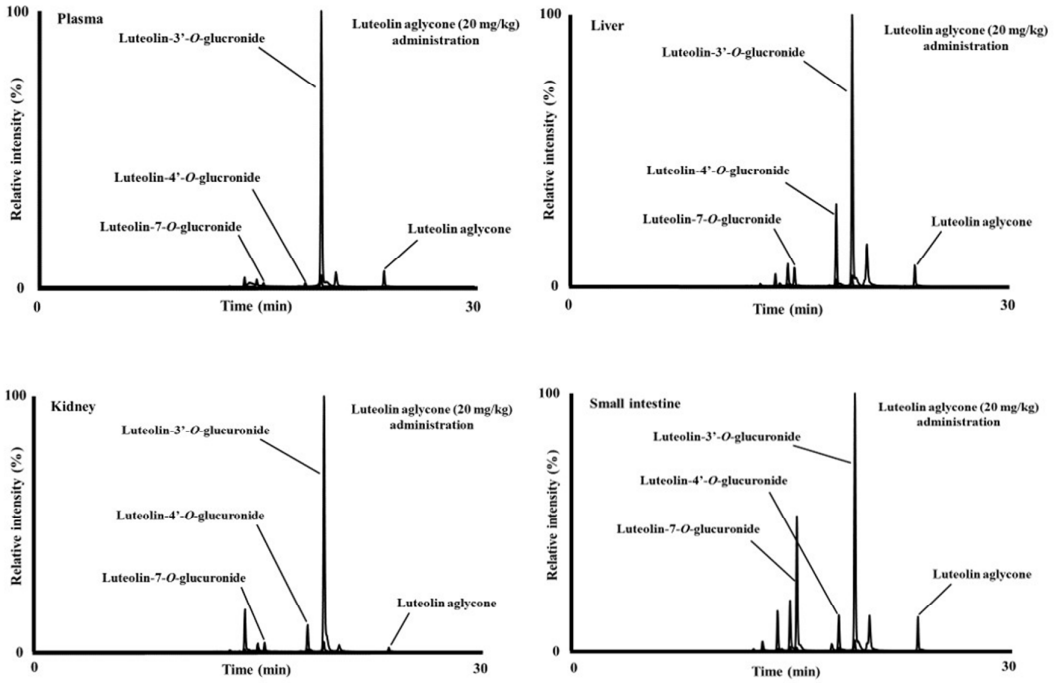
659 **Figure graphics**

660 **Figure 1**



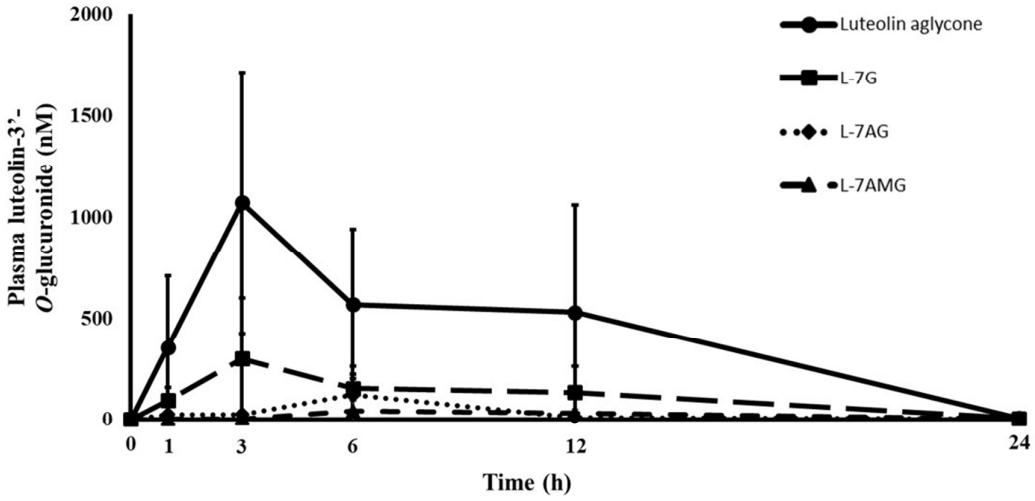
661

662 **Figure 2**



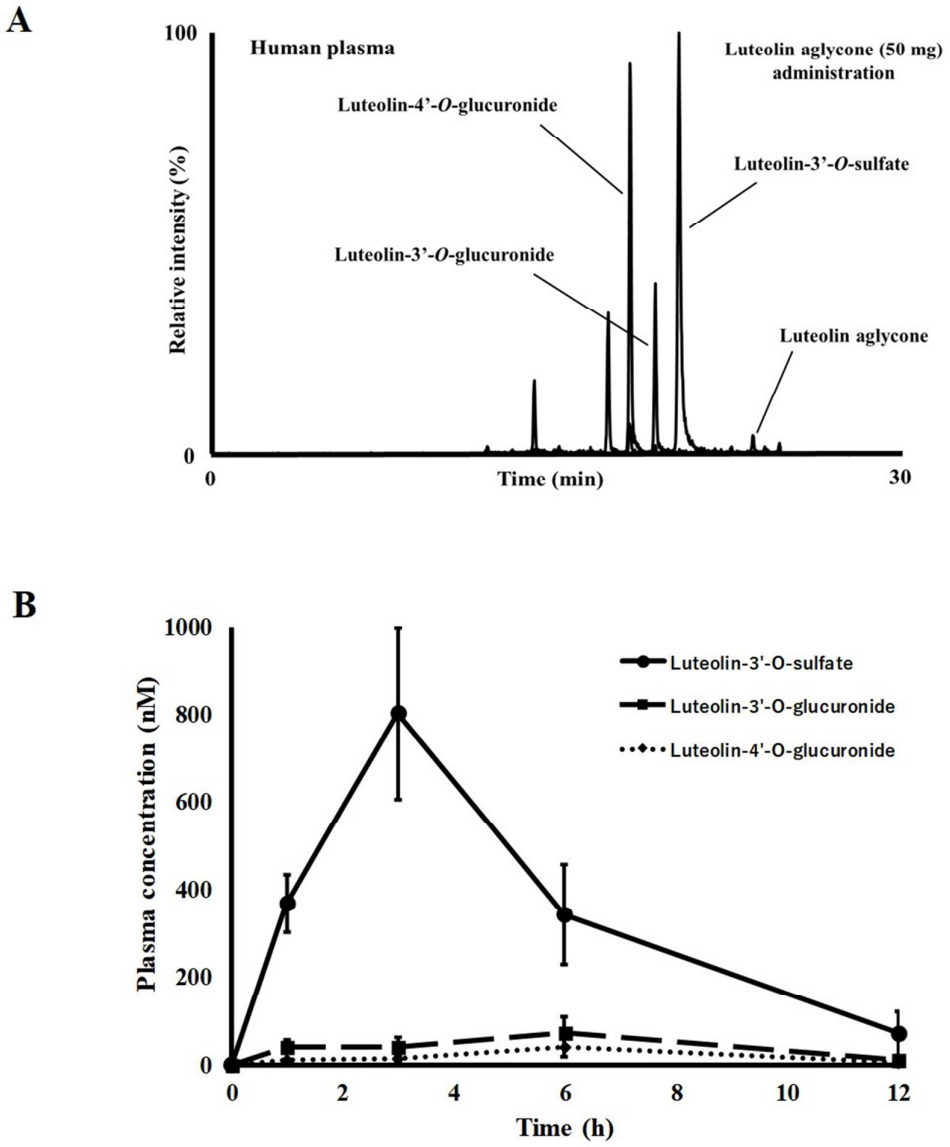
663

664 **Figure 3**



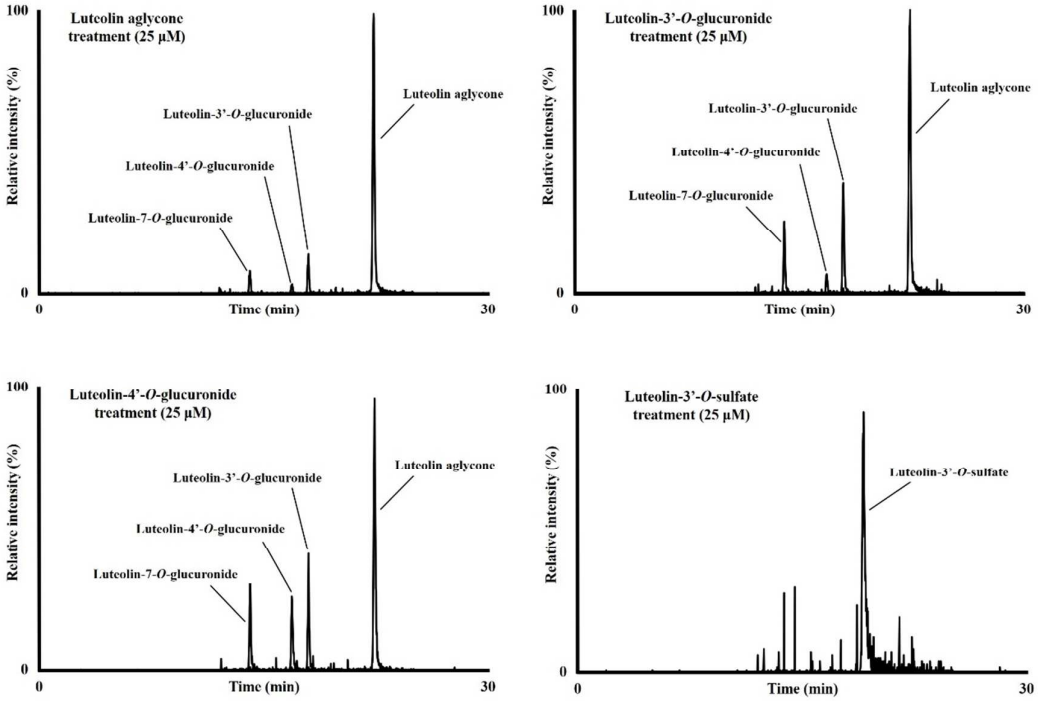
665

666 **Figure 4**



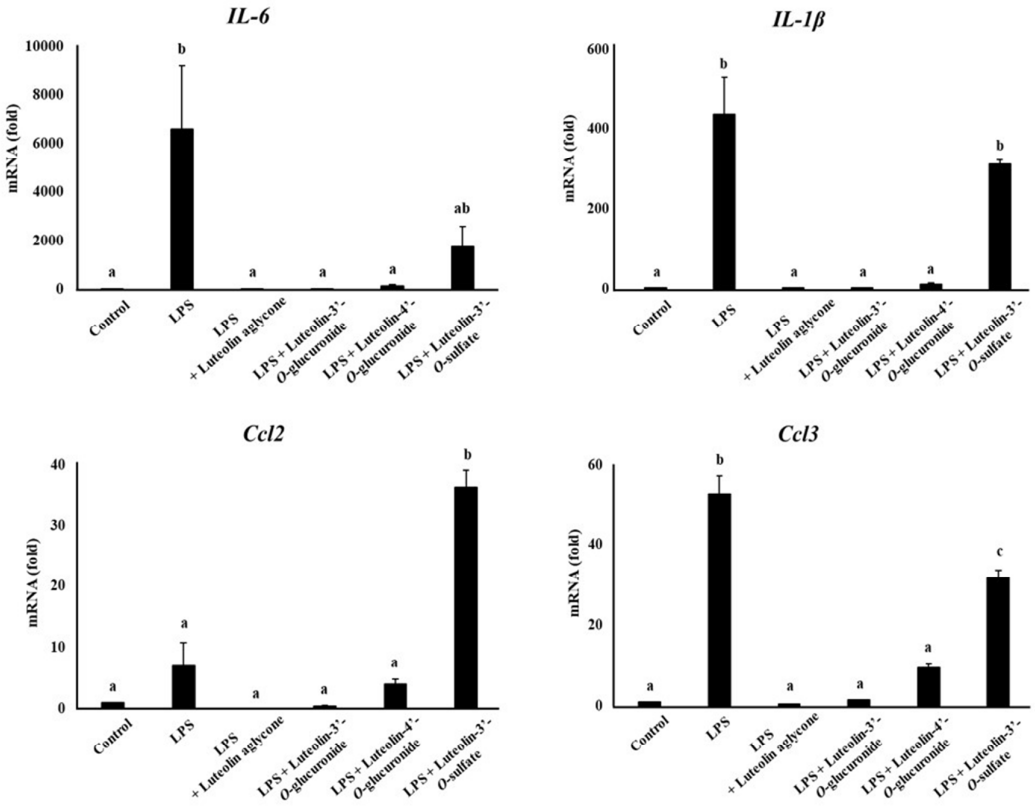
667

668 **Figure 5**



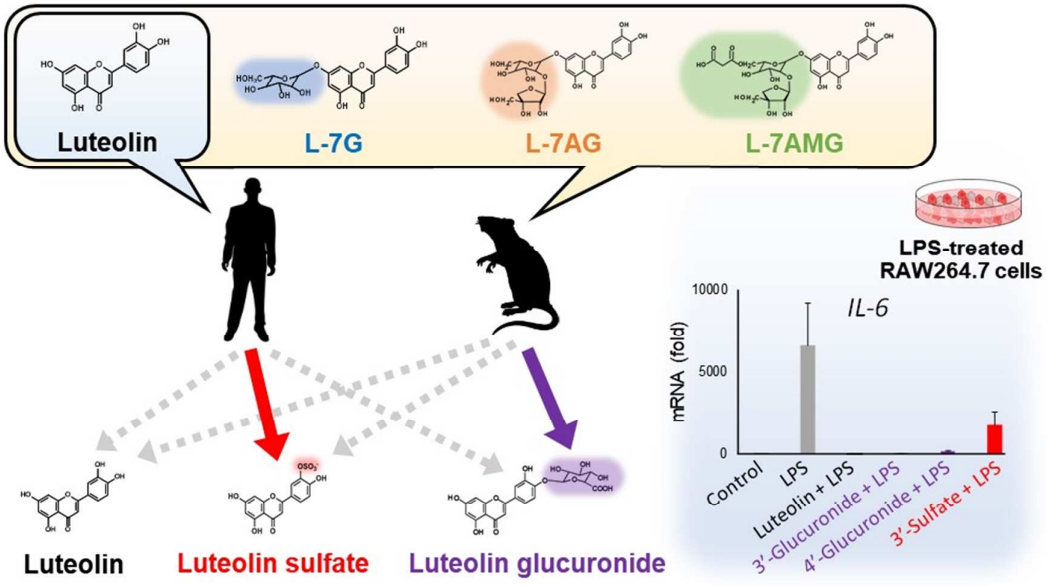
669

670 **Figure 6**



671

672 **Graphic for table of contents**



673