

Metabolites of the Ellagitannin Geraniin and Their Antioxidant Activities

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Key words

- ellagitannin
- geraniin
- metabolites
- urolithin
- ORAC
- antioxidant activity

Abstract

▼ Different types of ellagitannins are reported to have various biological activities, such as antioxidant, antiviral, and antitumor activities. However, there are few definitive studies on the absorption and metabolism of ellagitannins. This review compares the absorption and metabolism of ellagitannins, and the antioxidant properties of their metabolites in rats, with those of intact ellagitannins by means of *in vitro* and *in vivo* assays. We isolated 7 urinary and intestinal microbial metabolites in rats after the ingestion of geraniin, which is a typical ellagitannin isolated from *Geranium thunbergii*, an antidiarrheic remedy in Japan. The structures of these metabolites were determined to be dibenzopyran derivatives (1–7), using NMR and mass spectroscopic data. Four major metabo-

lites (1–4) prepared by chemical synthesis were evaluated for their antioxidant activities by using 2,2-diphenyl-1-picrylhydrazyl radical scavenging and oxygen radical absorbance capacity (ORAC) methods. The metabolites exhibited more potent antioxidant activities in the ORAC assay than intact ellagitannins, such as geraniin and corilagin. Furthermore, plasma ORAC scores increased with increases in the plasma concentration of the metabolites after the oral administration of geraniin to rats. These findings suggest that these metabolites may contribute to the health benefits of ellagitannins as antioxidants in the body.

Supporting information available online at <http://www.thieme-connect.de/ejournals/toc/plantamedica>

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Introduction

▼ Tannins are polyphenols that are widespread constituents of vegetables and are classified into 2 groups: hydrolyzable tannins and condensed tannins [1]. Ellagitannins belong to the hydrolyzable tannin group and occur in foods such as raspberries, strawberries, blackberries, pomegranate, almonds, and walnuts [2]. *In vitro* and *in vivo* studies of ellagitannins demonstrate various biological activities, including antioxidant [3], antiviral [4], antimutagenic [5], antimicrobial [6, 7], and antitumor promotion activities [8, 9], suggesting that the consumption of ellagitannins confers health benefits to humans. Nevertheless, the bioavailability of purified ellagitannins after ingestion is not fully understood. Ellagic acid is one of the hydrolysates of ellagitannins; its metabolism has been previously studied by Doyle and Griffiths [10]. The metabolite in urine and feces after oral administration of ellagic acid to rats is characterized as 3,8-dihydroxy-6H-dibenzo[b,d]pyran-

6-one (urolithin A). The absorption and metabolism of punicalagin from pomegranate has recently been reported in rats [11] and humans [12–14]. The metabolism of ellagitannins from several foodstuffs has also been demonstrated in humans [15]. Although the presence of various metabolites in plasma or urine after the ingestion of juice from fruits rich in ellagitannins is suggested, the chemical structures of these metabolites (except for urolithin A) have not been clearly characterized.

Geraniin (8) is the main polyphenolic component in *Geranium thunbergii*, a medicinal plant used to treat diarrhea in Japan. Several biological studies of geraniin show that it possesses antioxidant, antitumor, and antiviral properties. Geraniin is a typical ellagitannin because it is composed entirely of common acyl units such as galloyl, hexahydroxydiphenoyl (HHDP), and dehydrohexahydroxydiphenoyl (DHHDP) groups. We used geraniin as a purified ellagitannin to study the bioavailability of ellagitannins. Seven metabolites,

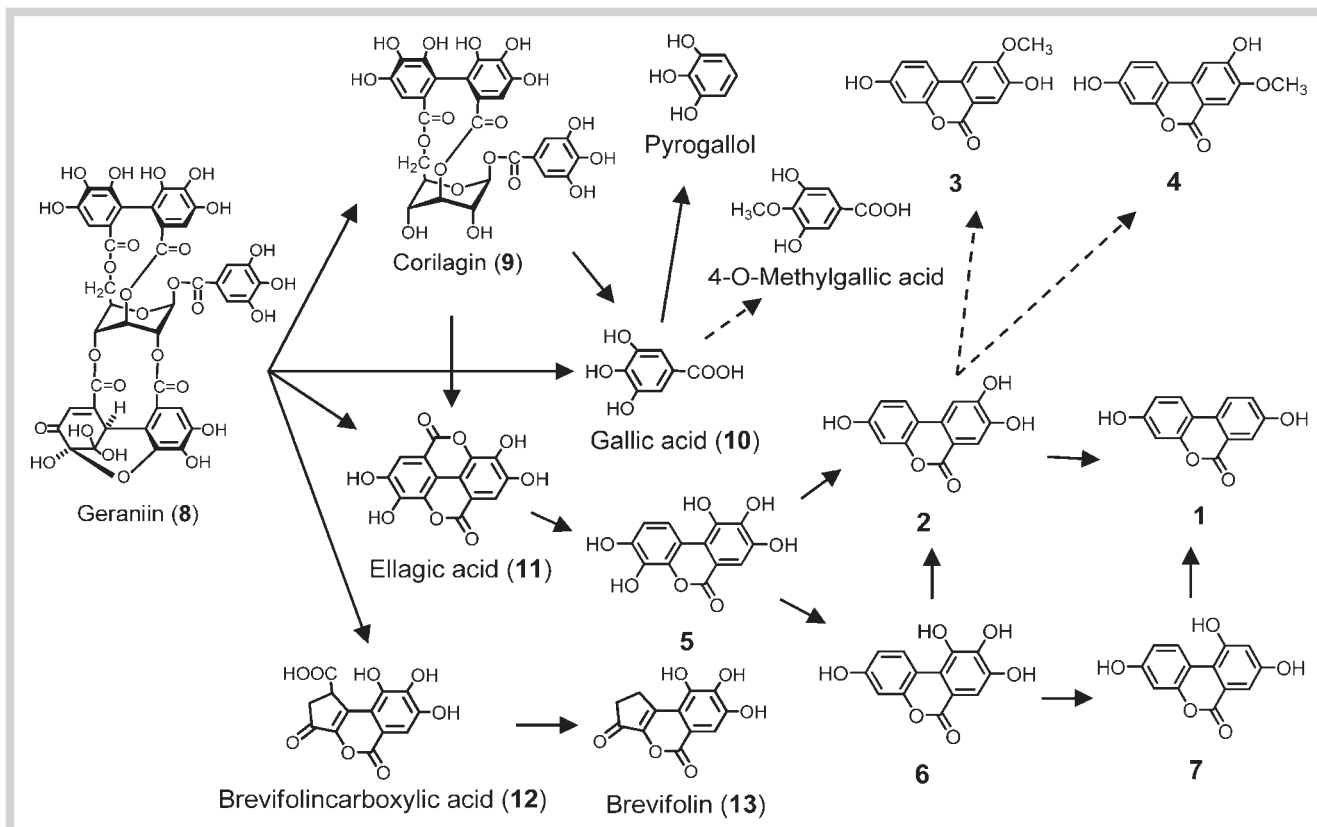


Fig. 1 Proposed metabolic pathway for the formation of metabolites originating from geraniin. Solid and dotted arrows represent possible reactions by intestinal microflora and catechol-O-methyltransferase (COMT), respectively.

including urolithin A, were isolated from rat intestinal microbial suspensions with geraniin and rat urine after oral administration of geraniin. The structures of these metabolites were elucidated using NMR and mass spectral data. We also investigated the absorption of intact ellagitannins and urinary recovery of the metabolites in rats [16]. Furthermore, the antioxidant properties of ellagitannin metabolites were evaluated and compared with those of the intact ellagitannins and related polyphenols by using both *in vitro* and *in vivo* methods. In this review, the metabolic fate of ellagitannins determined through isolation and structural elucidation, as well as the association between antioxidant properties and plasma levels of ellagitannin metabolites are discussed briefly.

Isolation and Structural Elucidation of Ellagitannin Metabolites from Rat Biofluids

The functional constituents of medicinal plants or foods after oral dosing may be primarily affected by intestinal microflora before absorption in the gut. Geraniin (**8**) was anaerobically incubated in a suspension of rat intestinal microflora. After a 96-h incubation, the suspension was filtered and subjected to column chromatography and/or preparative HPLC to give 5 metabolites: **1**, **2**, and **5–7**. Urine samples from rats were collected for 48 h after oral administration of geraniin, incubated with β -glucuronidase and sulfatase, and separated by repeated column chromatography to afford 4 major metabolites: **1–4**.

The isolated metabolites from rat biofluids were identified as follows: 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one (**1**; urolithin A) [17], 3,8,9-trihydroxy-6H-dibenzo[*b,d*]pyran-6-one (**2**) [18], 3,4,8,9,10-pentahydroxy-6H-dibenzo[*b,d*]pyran-6-one (**5**) [19], 3,8,9,10-tetrahydroxy-6H-dibenzo[*b,d*]pyran-6-one (**6**), and 3,8,10-trihydroxy-6H-dibenzo[*b,d*]pyran-6-one (**7**) [20] by comparing spectroscopic data with those reported in the literature (● **Fig. 1**). Both metabolites **3** and **4** were characterized as dibenzopyran derivatives carrying a methoxy unit. The positions of each methoxy group in metabolites **3** and **4** were established using nuclear Overhauser enhancement spectroscopy (NOESY) experiments. The structures of **3** and **4** were determined to be 3,8-dihydroxy-9-methoxy-6H-dibenzo[*b,d*]pyran-6-one and 3,9-dihydroxy-8-methoxy-6H-dibenzo[*b,d*]pyran-6-one [17], respectively (● **Fig. 1**).

Among the ellagitannin metabolites, metabolite **1** has been previously isolated and characterized from rat urine and feces after oral administration of ellagic acid [10] and was also identified as a metabolite in sheep serum and urine after the consumption of *Terminalia oblongata* leaves, which contain abundant amounts of ellagitannins [1]. Metabolite **1** has also been characterized as castoreum pigment I from the scent glands of beavers [21], a constituent of renal calculi in sheep [22], and a plant ingredient of *Shilajit* [23] and *Trapa natans* [24]. Furthermore, metabolite **1** was recently found to be a urinary metabolite in humans after pomegranate juice supplementation [14]. Metabolites **5** and **7** have been identified as phytochemicals from *Tamarix nilotica* [19] and as metabolites from the feces of *Trogopterus xanthipes* [20],

respectively. Compounds 2–7 were unambiguously characterized as ellagitannin metabolites for the first time in our study.

Rat Intestinal Microbial Transformation of Ellagitannins

The time-course profiles of the intestinal microbial transformation of geraniin were investigated using reversed-phase HPLC. The proposed metabolic pathway of geraniin is summarized in **Fig. 1**. Geraniin (**8**) begins to be converted to corilagin (**9**), gallic acid (**10**), ellagic acid (**11**), and brevifolincarboxylic acid (**12**) after 1 h of anaerobic incubation with rat fecal suspension, indicating that geraniin is first hydrolyzed by microflora. Metabolite **5** was produced 6 h after incubation; metabolites **2** and **6** were subsequently detected after a 48-h incubation. Upon further incubation, transformation to metabolites **1** and **7** occurred after 96 h. Similar observations to those of geraniin were made after the re-incubation of both corilagin (**9**) and ellagic acid (**11**) among the intermediate hydrolysates. Furthermore, brevifolincarboxylic acid (**12**) was decarboxylated to brevifolin (**13**) during re-incubation with gut microflora. Geraniin was first subjected to ester hydrolysis with a rat fecal suspension, to give corilagin, and gallic, ellagic, and brevifolincarboxylic acids, indicating that geraniin is similarly hydrolyzed under acidic conditions [25]. Among the hydrolysates, the metabolism of ellagic acid derived from HHDP and DHHDP [26,27] groups was expanded to metabolites **1**, **2**, and **7** through metabolite **5** by the fission of 1 of 2 lactone rings and subsequent dehydroxylation.

Urinary Excretions of Intact Ellagitannins and Their Metabolites in Rats after Oral Dosing of Ellagitannins

The collected urine samples were treated with β -glucuronidase and sulfatase after oral administration of ellagitannins or their related polyphenols to rats. Urinary excretion of metabolites has been demonstrated by reversed-phase HPLC analysis. The identity of each metabolite is based on their individual retention times, which were compared to respective standards. The microbial metabolites, **1** and **2**, and metabolites having a methoxy group, metabolites **3** and **4**, were markedly excreted in urine after geraniin consumption and were identified as major metabolites. The cumulative urinary excretion of major metabolites (**1**–**4**) after oral administration of geraniin is shown in **Fig. 2**. All metabolites started to be detected in rat urine 24 h after geraniin intake; their excretion increased until 72 h. The total excretion of major metabolites in urine over 72 h reached 12.4% of the amount administered at 20 mg/head. Among these, large amounts of metabolite **2** were recovered in urine at a level of 3.56 $\mu\text{mol}/72\text{ h}$ (6.8% of the oral dose of geraniin). The urinary excretion of all metabolites persisted for more than 48 h after geraniin administration. This suggests that ellagitannin metabolites formed by microbial hydrolysis and subsequently reduced in the colon are absorbed and eliminated *via* enterohepatic circulation. These data reinforce the notion that the metabolism of ellagitannins is dependent on intestinal microflora [11, 13].

These metabolites were also detected in urine samples after oral administration of either corilagin (**9**) or ellagic acid (**11**). After oral dosing of brevifolincarboxylic acid (**12**), which is a hydrolysate from geraniin, brevifolin (**13**) due to the decarboxylated derivative of **12** was observed in urine until 12 h. The major metab-

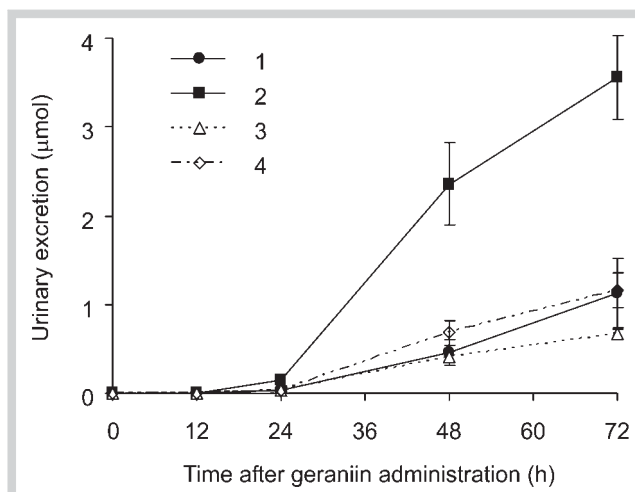


Fig. 2 Cumulative urinary excretion profile of major metabolites in rats after oral administration (50 mg/head) of geraniin. Urine was collected from metabolic cages 0–12, 12–24, 24–48, and 48–72 h after administration. Values are means (SEM) ($n = 4$) represented by vertical bars.

olites were scarcely detected in urine samples that were not treated with β -glucuronidase and sulfatase after geraniin ingestion. However, several other weakly retained peaks were observed on reversed-phase HPLC analysis, suggesting that all metabolites are almost excreted in urine as conjugate forms.

The transformations to both metabolites **3** and **4** (identified as urinary metabolites) were not observed in all rat fecal suspensions with ellagitannins. The methylation of polyphenols with a catechol unit by catechol-*O*-methyltransferase (COMT) is well known among flavonoids and catechins [28]. The production of metabolites **3** and **4** was attributed to the methylation of **2** absorbed from the intestines by COMT in the liver or kidneys. Pyrogallol and 4-*O*-methylgallic acid have been identified as metabolites of gallic acid in both humans [29] and rats [30]. Based on these findings, a metabolic sequence is proposed for the formation of these metabolites that originate from geraniin (**Fig. 1**). We further investigated the presence of intact ellagitannins in rat urine after oral administration by using HPLC-ESI-MS/MS. Corilagin was detected in rat urine after consumption of geraniin, suggesting that it is absorbed and excreted in urine after gut microbial elimination of the DHHDP group in geraniin. In addition, corilagin was clearly observed in rat urine after its intake. The presence of condensed tannins, such as proanthocyanidins, in rat plasma after apple polyphenol consumption is reported [31–33]. Although the absorption of hydrolyzable tannins in both animals and humans is not well understood at present, these findings demonstrate that hydrolyzable tannins having molecular weights > 600 can be absorbed and excreted after oral dosing.

Ellagitannin Metabolites in Rat Serum after Oral Dosing of Geraniin

Rat serum levels of the ellagitannin metabolites after oral administration of geraniin were analyzed by HPLC-ESI-MS/MS. All of the major metabolites found in rat urine were also detected in rat serum. Among the metabolites, serum metabolite **2** contents were highest similarly to the urinary excretion. Serum metabolite

Table 1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and superoxide dismutase (SOD)-like activities of ellagitannins and their metabolites.

Compounds	DPPH IC ₅₀ (μM)	SOD	
		Xanthine-XOD IC ₅₀ (μM)	PMS-NADH IC ₅₀ (μM)
1	> 100	> 100	> 100
2	1.9	> 100	51
3	11	> 100	> 100
4	62	> 100	> 100
Geraniin (8)	0.6	0.8	1.3
Corilagin (9)	0.4	0.8	0.7
Gallic acid (10)	1.9	13	7.8
Ellagic acid (11)	1.7	> 100	21
Pyrogallol	2.1	3.5	4.7

2 and 1 levels reached a maximum after 24 and 48 h, respectively (● Fig. 3). This implies that metabolite 1 is dehydroxylated from 2 by gut microflora, which in turn is absorbed into enterohepatic circulation. Since a trace level of these metabolites was observed in rat serum samples without enzymatic treatment with β-glucuronidase, the ellagitannin metabolites almost existed in rat serum in glucuronide or sulfate forms. Although the metabolites are present in the circulatory system in their conjugated forms, much data exist regarding the biological activity of these conjugates. Quercetin conjugates retain antioxidant activity in plasma [34–36]. Luteolin glucuronide is deconjugated into the aglycone by β-glucuronidase released from neutrophils after the induction of inflammation [37]. Thus, these findings support the idea that these metabolites are absorbed into the circulatory system and are transported in urine after ellagitannin intake, which may contribute to its health benefits.

Association between Ellagitannin Metabolite Plasma Levels and Antioxidant Activities

The major metabolites 1–4 were prepared by condensing resorcinol with bromohydroxybenzoic acid in alkaline solution with a copper catalyst [16]. The resultant products were used to evaluate antioxidant activity. The antioxidant activities of the major metabolites were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effects and 2 superoxide dismutase (SOD)-like activities (● Table 1). Superoxide anions were either generated by a xanthine-XOD enzymatic reaction or by a phenazine methosulfate (PMS)-NADH nonenzymatic reaction. In both of the assays for SOD-like activity, none of the major metabolites exhibited activity except for 2 in the PMS-NADH assay (IC₅₀ = 51 μM). On the other hand, among the tested metabolites, metabolite 2 had a radical scavenging effect with an IC₅₀ of 1.9 μM, which is comparable to that of geraniin and its related polyphenols in the DPPH assay. These data suggest that metabolite 2 possesses radical scavenging activity due to the presence of a catechol unit in the molecule.

The oxygen radical absorbance capacity (ORAC) method is a standard recommended by the United States Department of Agriculture for measuring antioxidant capacity and has the advantage of utilizing a biologically relevant radical source [38, 39]. We measured the antioxidant capacity of ellagitannin metabolites in relation to intact ellagitannins by using the ORAC method (● Fig. 4). The intact ellagitannins and their related polyphenols exhibited

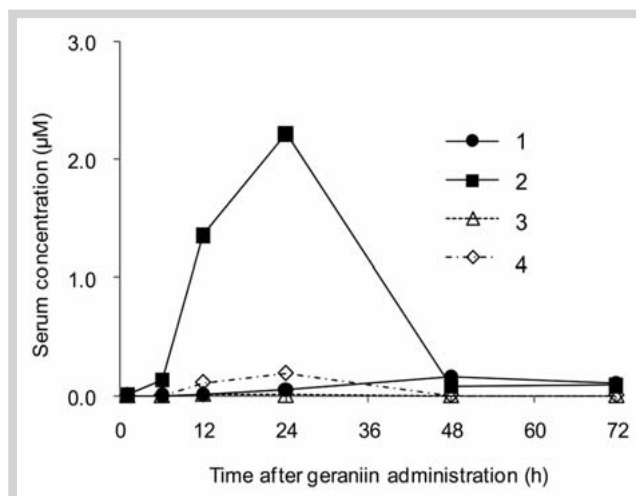


Fig. 3 Serum levels of major metabolites in rats after oral administration (20 mg/head) of geraniin (n = 2–3).

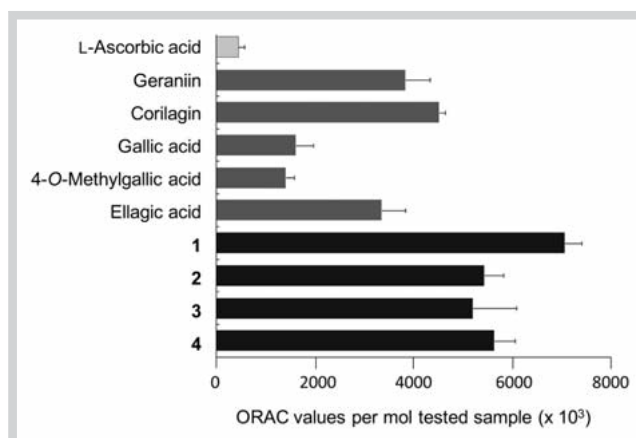


Fig. 4 Oxygen radical absorbance capacity (ORAC) values per mole of intact ellagitannins and their metabolites (n = 3). ** P < 0.01; statistically significant differences between each compound were evaluated by 1-way ANOVA.

strong antioxidant activities compared to ascorbic acid. The ORAC scores of all metabolites indicate potent antioxidant properties compared to those of intact ellagitannins. Furthermore, the ORAC potencies of 1, 3, and 4 without catechol units were significantly higher than those of intact ellagitannins. Metabolite 1 was the most potent among the tested metabolites. This result suggests that the metabolites may function as biological antioxidants after ingestion of ellagitannins.

The antioxidant properties of the ellagitannin metabolites were evaluated using an *in vivo* study. We investigated the association between metabolite plasma levels and plasma ORAC scores after geraniin consumption in rats. The collected rat plasma samples after geraniin oral administration at 5 mg/head were extracted with methanol containing hydrochloric acid. The plasma levels of the metabolites of the samples were analyzed using HPLC-ESI-MS/MS and ORAC scores were subsequently assayed. In this case, metabolite 1 was the main metabolite detected in rat plasma after a low dose of geraniin. However, metabolite 2 was

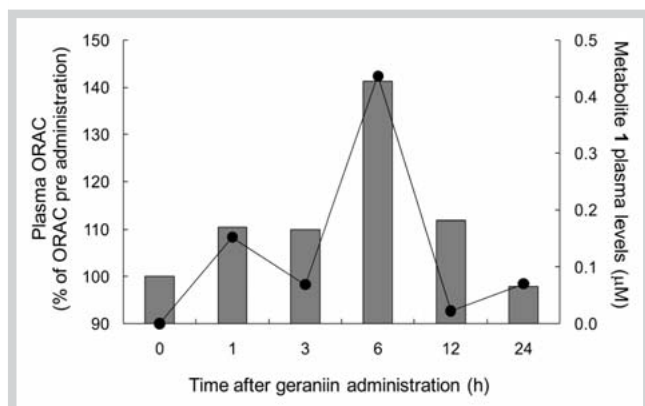


Fig. 5 Plasma levels of **1** and plasma ORAC scores after oral administration (20 mg/head) of geraniin ($n = 3$). Lines represent metabolite **1** plasma levels and bars represent percentages of plasma ORAC scores before geraniin administration.

mainly found in rat serum after a high dose (20 mg/head) of geraniin, as mentioned above. The different main metabolites found in rat blood at low and high doses suggest that the gut microbial conversion of **2** to **1** may be saturated at high doses. The plasma concentration of **1** reached a maximum 6 h after administration. Plasma ORAC scores simultaneously reached their maximum, indicating a strong association between plasma metabolite **1** levels and the plasma ORAC scores (● Fig. 5). This result also indicates that ellagitannin metabolite **1** possesses antioxidant activity *in vivo* as well as *in vitro*. These findings raise the possibility that these metabolites play an important role in biological antioxidants after the oral administration of intact ellagitannins as potent natural antioxidants.

Conclusions

Ellagitannins such as geraniin and punicalagin have a hexahydroxydiphenyl group in their molecules; consequently, ellagic acid, which is readily lactonized from hexahydroxydiphenic acid, is produced upon the hydrolysis of ellagitannins [25]. Metabolic profiles suggest that ellagitannin metabolites are derived from ellagic acid *via* ellagitannin hydrolysis. These metabolites were recently found in the biofluids of rats [10, 11, 40–43], mice [44], and humans [13, 15, 45–47] in *in vivo* studies on the functionalities of foodstuffs rich in ellagitannins, including pomegranates, nuts, and berries. Most of the reported unidentified ellagitannin metabolites may correspond to metabolites **1–7** and/or their conjugate forms. The ORAC method revealed that the antioxidant properties of major metabolites **1–4** are more potent than those of intact ellagitannins. A close relationship between plasma metabolite **1** levels and plasma ORAC scores was observed after the oral administration of geraniin. Urolithin A (**1**) was recently reported to possess several biological activities such as inhibition of prostate cancer [44], anti-inflammatory effects [48], as well as systematic health benefits related to the consumption of ellagitannins. Further study of the biological properties of ellagitannin metabolites, including the bioavailability of ellagitannins, is necessary to clarify the active principles of several biological activities of ellagitannins such as their antioxidant and chemopreventive effects.

Most evaluations of the biological activities of natural products are targeted toward plant components. Our findings raise the possibility that metabolites play important roles in biological antioxidants after oral administration of intact ellagitannins. Studying the bioavailability of natural products is important to clarify the bioactive principles of their biological activities.

Supporting information

Ion chromatograms of corilagin in rat urine samples obtained by HPLC-ESI-MS/MS in the multiple reaction-monitoring mode with negative ionization are available as Supporting Information.

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