RESEARCH ARTICLE

In vivo absorption and metabolism of leptosperin and methyl syringate, abundantly present in manuka honey

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Scope: Manuka honey, which shows strong nonperoxide-dependent antibacterial activity, contains unique components, such as methyl syringate 4-*O*-β-D-gentiobioside (leptosperin) and its aglycone, methyl syringate (MSYR). To determine the potential for biological activity evoked by the ingestion of leptosperin and MSYR, we investigated the absorption and metabolism of these components in manuka honey.

Methods and results: The incubation of MSYR with liver microsomes or S9 fractions in vitro resulted in the formation of MSYR-glucuronide (MSYR-GA), MSYR-sulfate (MSYR-S), and syringic acid as metabolites. Then, manuka honey (15 g) was fed to healthy human volunteers. MSYR-GA, MSYR-S, and MSYR were detected in both plasma and urine. Within plasma, their levels were highest within 0.5 h to 1 h post-ingestion, and most metabolites disappeared within 3 h. In conjunction with the disappearances, a significant amount of metabolites along with trace leptosperin was excreted in urine within 4 h. To elucidate the detailed metabolisms of leptosperin and MSYR, each compound was separately administered to mice. In each case, MSYR-GA, MSYR-S, and MSYR were detected in both plasma and urine.

Conclusion: This study shows the major molecular pathway for leptosperin and MSYR metabolism and could facilitate an understanding of biological functions of manuka honey post ingestion.

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1 Introduction

Manuka honey, which originates from the nectar of the manuka flower, *Leptospermum scoparium*, grown in New Zealand, has a strong nonperoxide-dependent antibacterial activity. The main contributor of this activity is known to be methylglyoxal, a highly reactive dicarbonyl compound, which is transformed from dihydroxyacetone present in the nectar of the flower [1]. Since methylglyoxal is also inevitably formed

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Abbreviations: **BMI**, body mass index; **Cr**, creatinine; **GA**, glucuronide; **HPLC**, high performance liquid chromatography; **ICR**, Institute for Cancer Research; **LC-MS/MS**, liquid chromatography tandem-mass spectrometry; **MRM**, multiple-reaction monitoring; **MSYR**, methyl syringate; **PAPS**, 3′-phosphoadenosine-5′-phosphosulfate; **Q3G**, quercetin-3-*O*-β-D-glucoside; **S**, sulfate; **SULT**, sulfotransferase; **SYR**, syringic acid; **UDP**, uridine diphosphate; **UDPGA**, UDP-glucuronide; **UGDH**, UDP-glucose dehydrogenase; **UGT**, UDP-glucuronosyltransferase; **UPLC**, ultra performance liquid chromatography; **UV-Vis**, ultraviolet-visible

as a by-product of glycolysis and/or from nonenzymatic glycation and lipid peroxidation, it may be associated with several pathologies such as diabetes and neurodegenerative diseases [2, 3]; however, it is reported that the intake of methylglyoxal by ingestion of manuka honey is probably safe for consumption by healthy individuals [4]. The ingested methylglyoxal is covalently adducted to digestion enzymes or detoxified by the glyoxalase system [5, 6]. As methylglyoxal disappeared after in vitro simulated digestion of manuka honey, considerable amounts of some aromatic compounds in manuka honey, such as phenyllactic acid, 4-methoxyphenyllactic acid, leptosperin, and methyl syringate (MSYR), remained in the digested sample [7]. Because antibacterial activity of the residue remains, these phenolic compounds may have antibacterial activity directly or synergistically.

Specific components of manuka honey could be used to verify the authenticity of the honey [8–13]. Our group has identified leptosperin and MSYR as inhibitors of an inflammatory enzyme, myeloperoxidase [14] and found that leptosperin is a characteristic compound exclusively found in *Leptospermum* honey [9]. *Leptospermum* honey, including manuka honey, not only exhibits antibacterial activity but also has some biological functions [15]. It has been reported that long-term administration of manuka honey protects middle-aged rats from oxidative damage, probably through the modulation of antioxidant enzyme activities and through direct antioxidative activity by its high phenolic content [16]. However, in general, plant-derived phenolic compounds in food are metabolized as xenobiotics into corresponding conjugates, glucuronides, and sulfates before influx into circulation. This means that plant-derived phenolics that have been evaluated by in vitro experiments have often lost their biological activities because of metabolic structural changes. On the other hand, it has been reported that this conjugation does not always result in reduced biological activity. For example, one of the major metabolites of morphine, morphine-6-glucuronide, is a more potent analgesic than morphine [17]. Furthermore, the permeability of the blood–brain barrier to morphine-6-glucuronide is reduced and delayed compared with that to morphine, resulting in long-term analgesic effects [18]. The pharmacologically active metabolite morphine-6-glucuronide is a well-known example within the pharmaceutical field; however, to date, few studies have investigated the metabolism of leptosperin and MSYR in manuka honey. A study on the processes involved in the metabolism of the specific components may account for the induction of these biological activities by the ingestion of manuka honey.

In this study, we aimed to characterize the metabolic profiles of leptosperin and MSYR. We analyzed ten possible metabolites including intact MSYR and leptosperin in human plasma and/or urine after ingestion of manuka honey. Nine metabolites were detected and four metabolites among these were quantified. Furthermore, the metabolic processes for leptosperin and MSYR have been separately investigated using animals fed with the respective compounds.

2 Materials and methods

2.1 Materials

Leptosperin was chemically synthesized according to the published method [19]. Methyl syringate (methyl 4-hydroxy-3,5-dimethoxybenzoate, MSYR) was purchased from Alfa Aesar, Johnson Matthey Co. (Ward Hill, MA). Manuka honey was obtained from ApBee of Yux Ltd (Richmond, NZ). Quercetin-3-*O*-β-D-glucoside (Q3G) was supplied by Sigma-Aldrich Japan (Tokyo, Japan). Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid, SYR) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Pooled human liver microsomes and S9 fractions obtained from 150 donors of an equal gender mix were purchased from Corning Life Sciences (Woburn, MA, USA).

2.2 Human study design

The following protocol was approved by the Ethical Committee of University of Hyogo, Japan (#093). Three healthy volunteers (one male and two females) participated in this study and provided informed consent. Their ages were 21, 21, and 30 years and had a mean body mass index (BMI) of 20.3 [standard deviation (SD) 0.37] (range 20.0–20.7). Each subject was required to follow a low honey diet for 2 days. All subjects consumed 15 g of manuka honey on a slice of dry toast. The 15 g portion of honey contained 5.8 mg (10.8 μmol) leptosperin and 1.9 mg (8.8 μmol) MSYR, which were quantified as described previously [9]. Peripheral venous blood was collected into a heparinized tube before ingestion (0 h) and at 0.5, 1, and 3 h post-ingestion. Plasma was obtained from the blood samples by centrifugation at 3000 × *g* for 10 min at 4°C, and then stored at −80°C until analysis. Urine was collected before ingestion and at 0–4, 4–8, and 8–24 h periods after post-ingestion of manuka honey. The volume of each sample was recorded and aliquots were then stored at −80°C.

2.3 Animal study design

This study was performed according to the guidelines for the care and use of laboratory animals of the University of Hyogo with approval from the Ethical Committee of the University of Hyogo, Japan (#095). Institute for Cancer Research (ICR) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were housed in a room with regulated environment (temperature, 23 ± 1°C; humidity, 50–60%; light-dark cycle, 12 h/12 h; lights on, 9:00 AM). The mice were provided free access to laboratory feed (CE2; CLEA Japan, Inc., Tokyo, Japan) and tap water for more than 5 days before the initiation of the experiments. For intragastric administration, mice ($n = 3$ for each group) were provided leptosperin or MSYR (1.7 μmol/kg, respectively) dissolved in 300 μL propylene glycol by the use of an oral zonde needle connected to a syringe.

The plasma and urine of mice were collected in the same manner as in the aforementioned human study design.

2.4 Preparation of methyl syringate-glucuronide (MSYR-GA)

MSYR-GA was prepared by incubation of MSYR with resting yeast cells coexpressing uridine diphosphate (UDP)-glucose dehydrogenase (UGDH) and human UDP-glucuronosyltransferases (UGTs) according to the published method with some modifications [20]. Among the 15 strains examined (UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B10, 2B15, and 2B17), yeast cells that expressed UGT1A7 generated MSYR-GA most effectively (Supporting Information Table 1). After the incubation of MSYR with the UGT1A7 expressing yeast cells, the reaction mixture was centrifuged to remove the yeast cells and applied to an open column (2.5 × 30 cm) filled with C18 resins (Cosmosil® 140C18-OPN). The products were eluted with 300 mL water and then concentrated. The concentrates were applied to another open column (2.5 × 15 cm) filled with the same resins. The column was washed with 200 mL water and synthesized MSYR-GA was then eluted with 5% methanol in water. The synthesized MSYR-GA was further purified by a preparative HPLC with Combi-RP (20 ×100 mm, Nomura Chemical Co., Ltd.) using 0.1% acetic acid/acetonitrile (7:3) at a flow rate of 5 mL/min with monitoring at 262 nm and concentrated. The isolate had a specific 262 nm wavelength maximum. The structural identification was performed by LC-MS/MS using an API3000 tandem mass spectrometer (AB Sciex Instruments, Framingham, MA) with ESI mode coupled to a HPLC (Agilent 1100). The isolate showed the 387 $[M–H]^-$ ion as the precursor ion and also produced the 211 $[M–H]^-$ ion as the product ion induced by collision-induced dissociation, indicating that the isolate was MSYR-GA.

2.5 Preparation of MSYR-S

MSYR-S was enzymatically synthesized from sulfotransferases (SULTs) and MSYR using budding yeast, *Saccharomyces cerevisiae*, which expresses human SULT1A3. There are plans to publish a detailed description of the procedure in the near future. Briefly, MSYR was incubated with the yeast in the presence of ammonium sulfate. After the incubation, the reaction mixture was centrifuged and then applied to an open column, as described previously. Products were eluted with 200 mL 5% methanol in water and then concentrated. The concentrates were applied to another open column and then washed as described previously. The synthesized methyl syringate-sulfate (MSYR-S) was eluted with 10% methanol in water and analyzed by HPLC with a Develosil ODS-HG-5 (4.6 ×150 mm, Nomura Chemical Co., Ltd.) using 0.1% acetic acid/acetonitrile at a flow rate of 0.8 mL/min with monitoring at 262 nm, to achieve a purity of 92%. The molecular ion was 291 $[M–H]^-$ and the product ion scan of 291 showed 211 $[M–H]^-$, corresponding to MSYR moiety. Because MSYR-S was relatively unstable during the isolation and concentration steps, it was used without further purification.

2.6 Preparation of MSYR-glucoside

β-Glucosidase (37–40 units/mL, from sweet almond, TOYOBO) was incubated with 0.25 mM leptosperin in 0.1 M acetate buffer (pH 5.0) at 37°C for 15 min. The solution was then ultracentrifuged by Ultrafree 10K (Merck Millipore) at 14 000 ×*g* for 20 min. The filtrate contained unreacted leptosperin, MSYR, and successively formed MSYR-glucoside (methyl syringate 4-*O*-β-D-glucoside). The conversion percentages of leptosperin to MSYR-glucoside, MSYR, and the remaining unreacted leptosperin were calculated as 8.7% ± 0.3%, 1.5% ± 0.1%, and 69.3% ± 0.7%, respectively (n=3). MSYR-glucoside in the filtrate was further purified by conventional HPLC. The profile of UV-Vis scanning showed a similar pattern to leptosperin, with an absorbance maximum at 262 nm and a shoulder absorbance at 300 nm. The structure of MSYR-glucoside was assigned by LC-MS/MS; The compound contained 419 $[M+HCOO^-]^-$ ion and a product ion scan of the 419 generated 211 $[M–H]^-$ ion, corresponding to MSYR.

2.7 Preparation of SYR-gentiobioside, SYR-glucoside, SYR-glucuronide, and SYR-sulfate

Four MSYR-conjugates, namely MSYR-gentiobioside (leptosperin), MSYR-glucoside, MSYR-GA, and MSYR-S, were de-esterified by alkaline treatment to prepare SYR-gentiobioside, SYR-glucoside, SYR-glucuronide (SYR-GA), and SYR-sulfate (SYR-S), respectively, as described previously [8].

2.8 In vitro phase II metabolism of MSYR using human liver microsomes and S9 fractions

For analysis of glucuronide formation, the 90 μL incubation mixture, including pooled human liver microsomes (final concentration 2.0 mg/mL), 100 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, and 100 μM MSYR, were pre-incubated for 2 min on ice. Reactions were initiated by the addition of 10 μL 20 mM UDP-glucuronide (UDPGA) (final concentration 2 mM) to a final reaction volume of 0.1 mL, and incubated at 37°C for 180 min. For the analysis of sulfate formation, the 95 μL incubation mixture, including human liver S9 fractions (final concentration 2.0 mg/mL), 100 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, and 100 μM MSYR, were pre-incubated for 2 min on ice. Reactions were initiated by the addition of

5 μL 5 mM 3′-phosphoadenosine-5′-phosphosulfate (PAPS, final concentration 0.25 mM) to a final reaction volume of 0.1 mL and incubated at 37°C for 180 min. After incubation, each reaction was halted by adding 50 μL ice-cold acetonitrile. The mixtures were then centrifuged (13 000×*g* for 15 min at 4°C) and 5 μL aliquots of the supernatants were injected into the ultra-performance liquid chromatography (UPLC) system with UV-Vis detector (HITACHI LaChrom ULTRA, Hitachi High-Technologies Co. Ltd.). Conditions for UPLC were as follows: Cosmosil® 2.5C18-MS-II column (2.0×100 mm, Nacalai Tesque, Inc.), flow rate of 0.5 mL/min, detection at 262 nm, and temperature at 40°C. The condition of the gradient elution was as follows: water–acetonitrile with 0.1% trifluoroacetic acid, 2% acetonitrile (4 min), 2–40% acetonitrile (4 min), 40–2% acetonitrile (2 min), and 2% acetonitrile (2 min).

2.9 Screening of metabolites in urine

Human urine (5 mL; 0–4 h) was acidified by 6 M HCl and then applied to a solid phase extraction column [Discovery® DSC-18 (3 mL; 500 mg)], which had been pre-conditioned by methanol and 0.1% acetic acid in water. After washing by 2 mL 0.1% acetic acid in water, samples were eluted with 2 mL methanol. The eluate was concentrated to approximately 0.5 mL and then centrifuged to clean up the sample. The sample was separated and scanned by the LC-MS/MS with a precursor ion mode of 211 (MSYR) as described below.

2.10 Analysis of metabolites in plasma

A volume of 20 μL of human plasma or 50 μL of mice plasma was mixed with 300 μL methanol containing 20 pmol Q3G (an internal standard). The mixture was vortexed for 30 s and sonicated for 1 min, and then centrifuged at 20 000 ×*g* for 5 min at 4°C. The supernatant was collected and the pellet was extracted with 300 μL methanol three times. The supernatants were combined and evaporated in vacuo. The extracts were then dissolved in an initial eluent of 100 μL 5% aqueous acetonitrile containing 0.05% formic acid. A volume of 5 μL of the sample was injected into LC-MS/MS and analyzed, as described below.

2.11 Analysis of metabolites in urine

Urine was diluted with distilled water (1:10 or 1:100, V/V). The diluted urine samples of 100 μL each were respectively mixed with 900 μL acetonitrile containing 20 pmol Q3G. The mixture was further treated according to the aforementioned method for plasma. The concentration of urine creatinine (Cr) was measured using an assay kit (Creatinine-test; Wako Pure Chemicals Co.).

Table 1. Multiple-reaction monitoring (MRM) combinations for analysis of metabolites

Compounds	Precursor ion (*m/z*)	Fragment ions (*m/z*)
Leptosperin	581 $[M+HCOO^-]^-$	323, 211
MSYR-glucoside	419 $[M+HCOO^-]^-$	211, 196
MSYR-glucuronide	387 $[M-H]^-$	211, 196
MSYR-sulfate	291 $[M-H]^-$	211, 196
SYR-glucoside	359 $[M-H]^-$	197, 182
SYR-glucuronide	373 $[M-H]^-$	197, 182
SYR-sulfate	277 $[M-H]^-$	197, 182
SYR-gentiobioside	522 $[M-H]^-$	197, 182
MSYR	213 $[M+H]^+$	181, 154
SYR	199 $[M+H]^+$	155, 140
Q3G[a)]	465 $[M+H]^+$	304

a) internal standard
MSYR, methyl syringate; SYR, syringic acid; Q3G, quercetin-3-*O*-β-D-glucoside.

2.12 LC-MS/MS measurement of plasma and urine samples

Leptosperin and metabolites, except MSYR and SYR, were separated by a Chromolith® Performance RP-18e column (2 ×100 mm, Merck Millipore Co.) maintained at 25°C at a flow rate of 0.2 mL/min, using 0.05% formic acid (A) and acetonitrile (B) as solvents. The time program was as follows: 0 min (A95%), 19.2 min (A70%), 21.2 min (A40%), 22.3 min (A95%), and 37.0 min (A95%). For screening of metabolites, LC-MS/MS analysis was performed with the precursor ion mode. The quantitation of metabolites was achieved by multiple-reaction monitoring (MRM) with a negative mode, as shown in Table 1. The MRM for the internal standard Q3G was simultaneously measured with the positive mode.

For MSYR and SYR measurements, samples were analyzed using 0.1% formic acid (A) and methanol (B) as solvents using a Develosil® ODS-SR-5 column (2 × 150 mm, Nomura Chemical Co., Ltd.) maintained at 25°C. The time program was as follows: 0 min (A100%), 20.0 min (A0%), 21.0 min (A100%), and 31.0 min (A100%). MSYR along with the internal standard Q3G were measured using the MRM positive mode.

3 Results

3.1 In vitro phase II metabolism of MSYR using human liver microsomes and S9 fractions

Glucosides are often hydrolyzed in the gut to form aglycones, following which aglycones are conjugated with sulfate or glucuronide as xenobiotic phase II metabolites. Methyl ester in a phytochemical is possibly also hydrolyzed by an esterase enzyme [21]. This indicates that conjugates with MSYR or SYR are possible metabolites of leptosperin/MSYR (Fig. 1). When MSYR was incubated with commercially

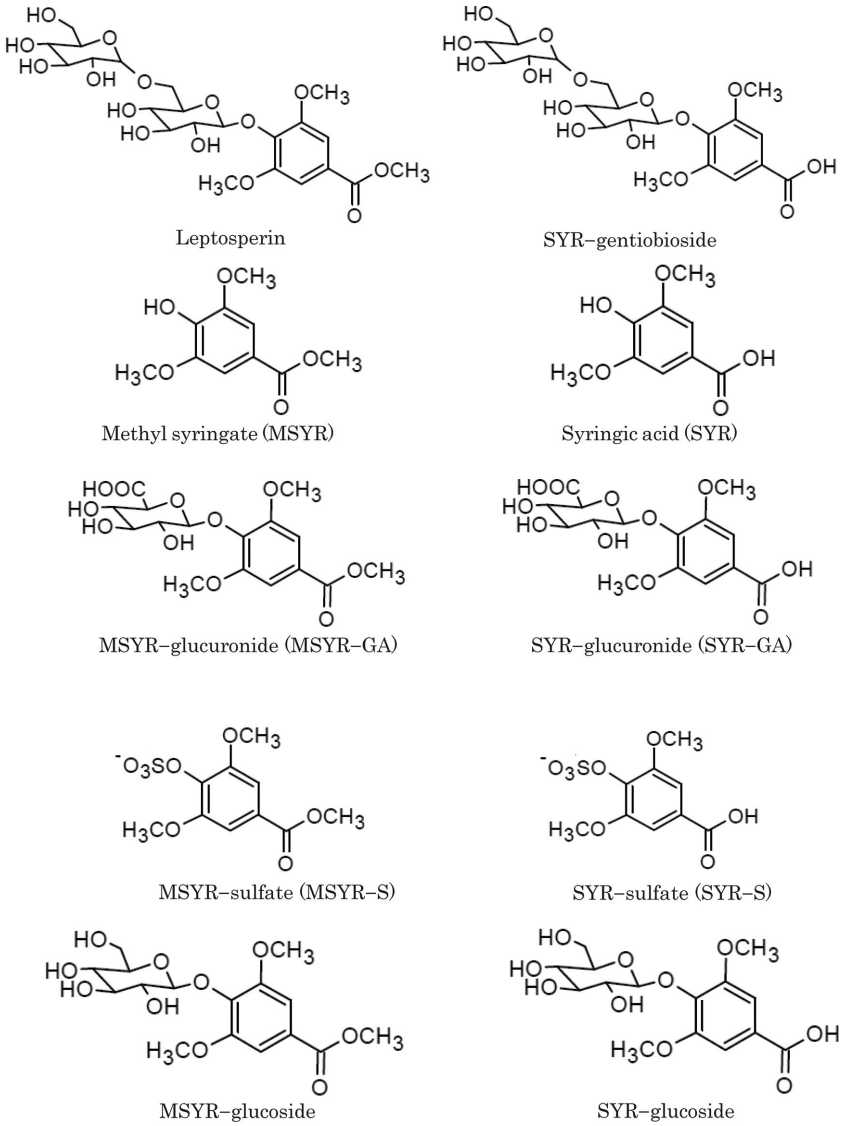

Figure 1. Structures of leptosperin, methyl syringate (MSYR), and their possible metabolites.

available human liver microsomes and UDPGA, MSYR completely disappeared and two peaks were generated (Fig. 2C). The earlier peak at 7.6 min represented SYR and the second peak (7.8 min) represented MSYR-GA. The result indicates that: (i) some UGT isoforms in human liver conjugate MSYR into MSYR-GA; (ii) an esterase cleaves carboxymethylester to generate SYR; and (iii) it is difficult to conjugate SYR into glucuronide. By an incubation of MSYR with human liver S9 fractions, MSYR was lost in 3 h, following which SYR was generated (Fig. 3A and B). MSYR-S (7.5 min) was also formed in the presence of PAPS (Fig. 3C). These results suggest that MSYR was metabolized into the corresponding glucuronide, sulfate, and SYR in the human liver. The experiments were independently conducted three times and a typical chromatogram is shown in Figs. 2 and 3.

3.2 Profiling of metabolites derived from leptosperin and MSYR after ingestion of manuka honey

In vivo metabolisms of leptosperin and MSYR were then analyzed. First, we searched for possible metabolites in urine samples using mass spectrometry with collision-induced dissociation, which cleaves weak bonds such as the *O*-glycosyl bond. A precursor ion scan of 211 (MSYR) showed two major peaks of 387 $[M–H]^-$ and 291 $[M–H]^-$, corresponding to MSYR-GA and MSYR-S, respectively (Fig. 4).

We then established ten sensitive MRM combinations for leptosperin and MSYR, and the possible metabolites, MSYR-GA, MSYR-S, SYR-GA, SYR-S, MSYR-glucoside, SYR-gentiobioside, SYR-glucoside, and SYR, which were analyzed in urine and plasma (Fig. 1). In human urine, leptosperin, MSYR, MSYR-GA, MSYR-S, SYR-GA, SYR-S, and SYR were

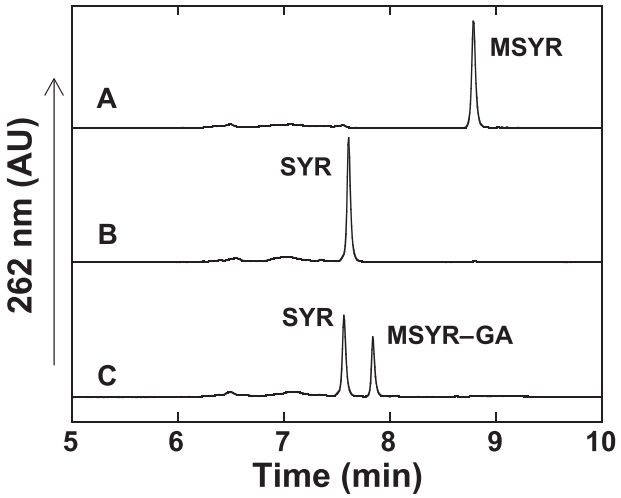

Figure 2. In vitro metabolism of methyl syringate (MSYR) in human liver microsomes. MSYR (100 μM) was incubated with human liver microsomes in the presence or absence of uridine diphosphate-glucuronide (UDPGA) in potassium phosphate buffer. After incubation for 180 min, the supernatant was obtained and injected into an ultra performance liquid chromatography (UPLC). (A) No incubation. (B) Incubation in the absence of UDPGA. (C) Incubation in the presence of UDPGA.

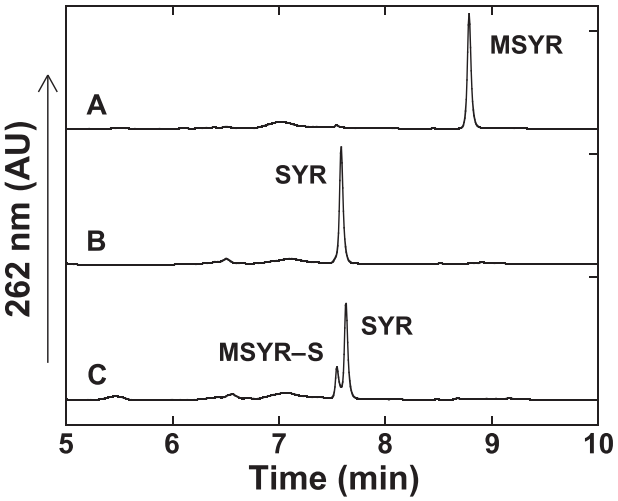

Figure 3. In vitro metabolism of methyl syringate (MSYR) in human liver S9 fractions. MSYR (100 μM) was incubated with human liver S9 fractions in the presence or absence of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) in potassium phosphate buffer. After incubation for 180 min, the supernatant was obtained and injected into an ultra performance liquid chromatography (UPLC). (A) No incubation. (B) Incubation in the absence of PAPS. (C) Incubation in the presence of PAPS.

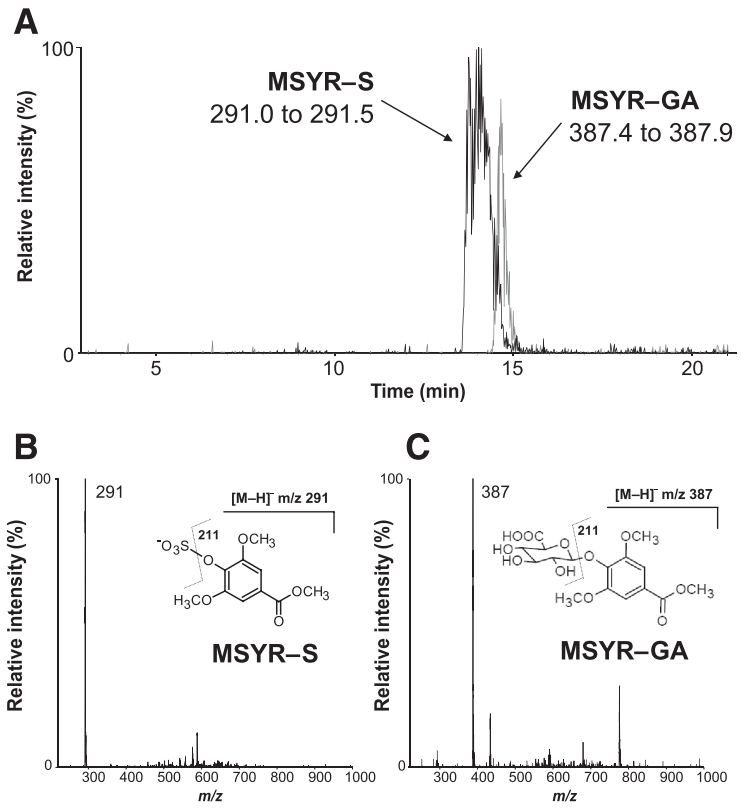

Figure 4. Precursor ion scanning by methyl syringate (MSYR)-derived fragmentation in human urine. After administration of manuka honey to humans, urine was partly concentrated and analyzed by liquid chromatography mass spectrometry (LC/MS) using precursor ion scanning of 211. (A) Extract-ion chromatogram of 291 and 387 from the precursor ion scan of 211. (B) Mass spectrum of 13.6 to 14.3 min. (C) Mass spectrum of 14.6 to 14.7 min. Structures of MSYR-sulfate (S) or MSYR-glucuronide (GA) with their possible fragmentations are shown in insets of B and C.

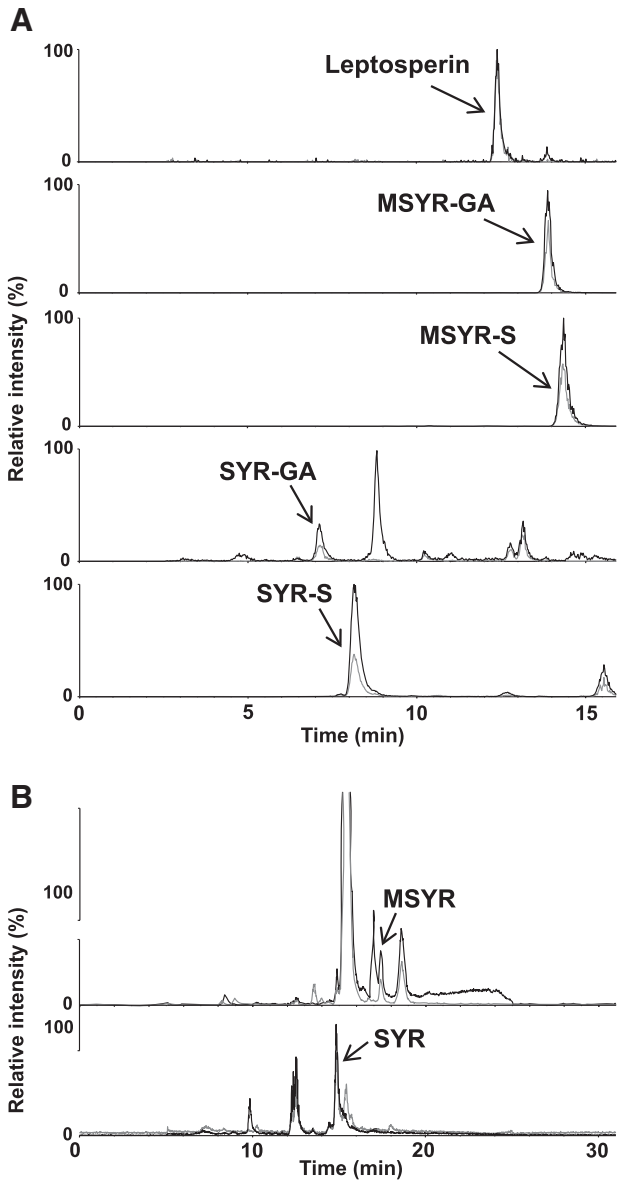

Figure 5. Representative multiple-reaction monitoring (MRM) chromatograms of (A) leptosperin, methyl syringate-glucuronide (MSYR-GA), MSYR-sulfate (S), methyl syringate (SYR)-GA, SYR-S, and (B) MSYR, and SYR in urine from humans 0–4 h post-ingestion of manuka honey.

detected in the 0–4 h sample (Fig. 5). In plasma, MSYR, MSYR-GA, MSYR-S, SYR-GA, SYR-S, and SYR were detected in the 0.5 h sample (Supporting Information Fig. 1). In both plasma and urine samples, the signals of SYR-conjugates were approximately 50 times lower than those of MSYR-conjugates (data not shown). In addition, the elution time of MSYR-S was shifted by the concentration of a sample. Although the elution position of MSYR-S was faster than that of MSYR-GA in the concentrated urine sample (10% of the original volume) (Fig. 4A), the position of MSYR-S was immediately after the elution of MSYR-GA in diluted samples, which were used for routine MRM analyses (Fig. 5A).

3.3 Time-dependent changes in the profile of metabolites after ingestion of manuka honey

Time-dependent changes (0, 0.5, 1, and 3 h) in the concentration of plasma MSYR and their metabolites were investigated (Fig. 6). All three analytes were absent in plasma samples collected prior to ingestion (0 h). The levels of MSYR-GA, MSYR-S, and MSYR were rapidly increased in the plasma and reached peak levels at 0.5 to 1 h post ingestion. Thereafter, the amounts of these compounds decreased continually until 3 h post ingestion. The concentrations of MSYR-GA (approximately 1 μM) at 0.5 and 1 h were highest in analytes and that of MSYR-S was less than half of that of MSYR-GA. Among those, approximately 50% of MSYR remained at 3 h in the plasma sample as compared to 0.5 h. This may suggest that: (i) MSYR is slowly metabolized because of its lipophilicity; and/or (ii) once formed, MSYR-GA and MSYR-S are deconjugated to MSYR by an enzyme.

Leptosperin, MSYR-GA, MSYR-S, and MSYR were also quantified in the human urine samples collected 0, 0–4, 4–8, and 8–24 h post ingestion. The levels of the four compounds increased and reached peak levels at 0–4 h, following which they decreased until 8–24 h in a time-dependent fashion (Fig. 7). The predominant metabolite present in urine was MSYR-GA at a concentration of 3688 ± 781 μmol/mol Cr in 4 h, and thereafter, the amount of MSYR-GA excreted for 4–8 and 8–24 h decreased by 90% (Fig. 7B). A considerable amount of MSYR-S (515 ± 62 μmol/mol Cr) was also detected in 4 h (Fig. 7C). The intact glycoside, leptosperin, was found at 0–4 h (72 ± 14 μmol/mol Cr) and then decreased in 4–8 h. The levels of the data points within the 8–24 h period were below the limit of detection (Fig. 7A). A similar level of MSYR was also observed at 0–4 h (57 ± 26 μmol/mol Cr), and was gradually excreted until 8–24 h post ingestion (Fig. 7D).

3.4 Evaluation of metabolism of leptosperin and MSYR in mice by administration of leptosperin or MSYR

To investigate the bioavailability of leptosperin and MSYR individually, we accomplished an animal study by oral administration of leptosperin or MSYR in mice. The dose of each compound was set to equimolar 1.7 μmol/kg (9.1 μg for MSYR, 23.1 μg for leptosperin per mouse). The amount per mouse was approximately ten-fold higher than that accomplished by human studies in terms of per person normalized by body weight.

The plasma samples from the mice intragastrically administered leptosperin or MSYR at 0.5 h after ingestion were analyzed. As shown in Fig. 8, the concentration of MSYR-GA was much higher in the leptosperin-fed group

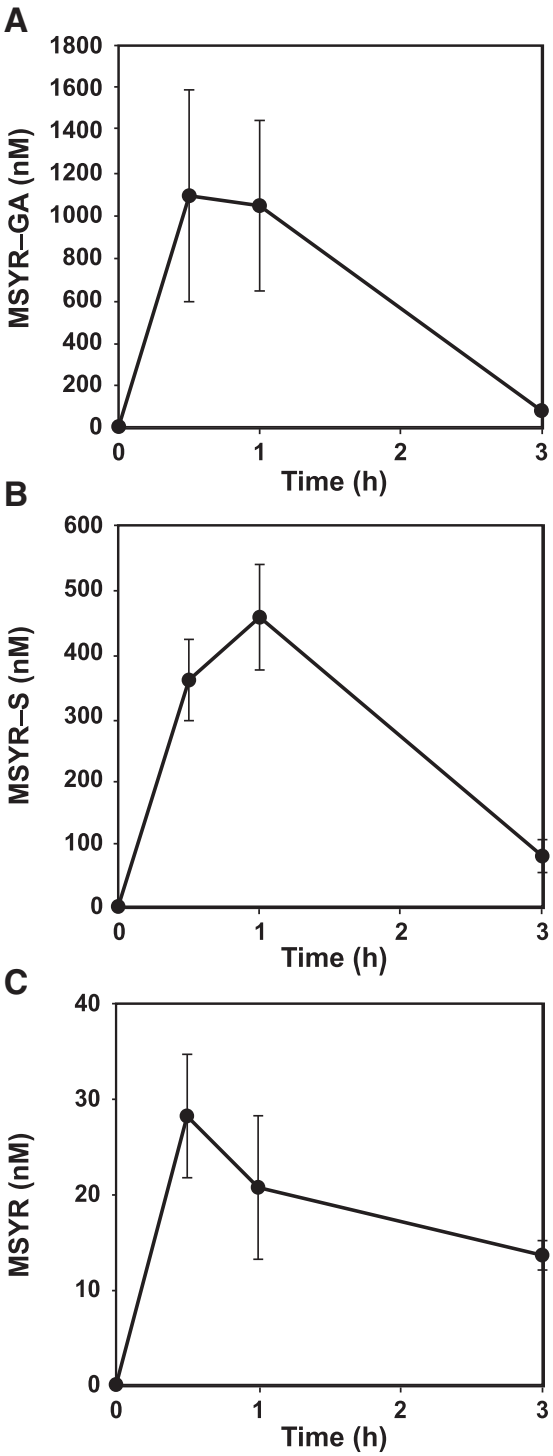

Figure 6. Time–dependent changes in concentrations of leptosperin and methyl syringate (MSYR) derivatives in plasma from humans after ingestion of manuka honey. The concentration of MSYR- glucuronide (GA) (A), MSYR-sulfate (S) (B), and MSYR (C) were measured in human plasma after ingestion of manuka honey (15 g), equivalent to leptosperin 5.8 mg (10.8 μmol) and MSYR 1.9 mg (8.8 μmol). Data represent means ± standard deviation (SD) ($n = 3$).

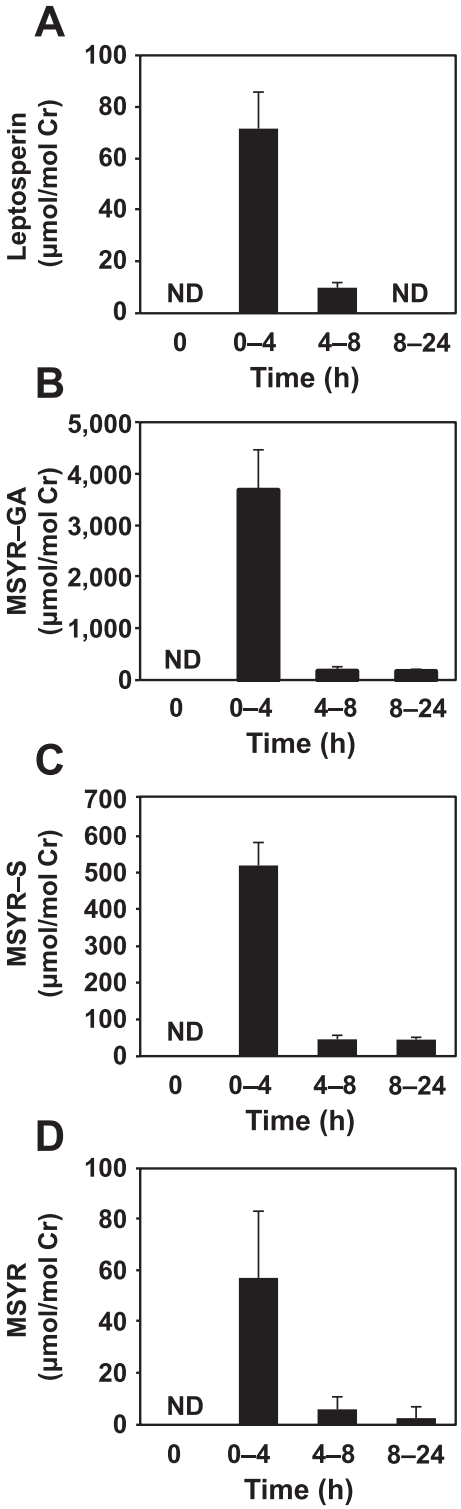

Figure 7. Time-dependent excretion of leptosperin and methyl syringate (MSYR) derivatives in the urine of the human subjects 0–24 h after the consumption of 15 g manuka honey. The results were expressed as μmol/mol creatinine (Cr). A, leptosperin; B, MSYR- glucuronide (GA); C, MSYR-sulfate (S); D, MSYR. Data represent means ± standard deviation (SD) ($n = 3$).

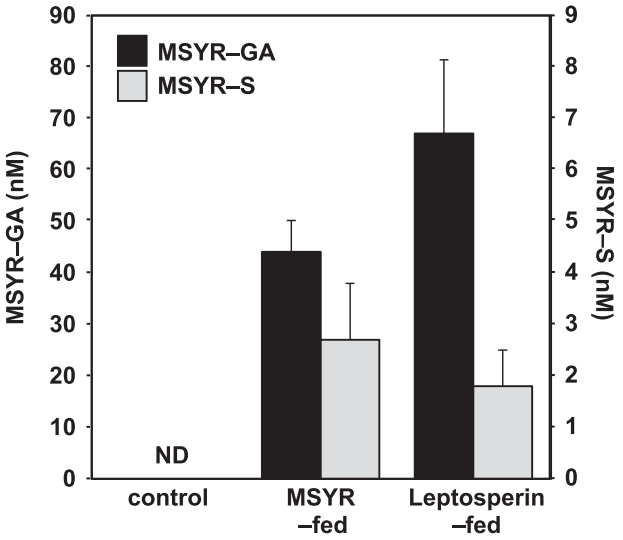

Figure 8. Concentration of methyl syringate-glucuronide (MSYR-GA) and MSYR-sulfate (S) in plasma from mice 0.5 h after oral administration of MSYR or leptosperin. Mice plasma after ingestion of vehicle (control), leptosperin, or MSYR (1.7 μmol/kg, respectively) were collected and analyzed as described in the Materials and methods. Data represent means ± standard deviation (SD) ($n = 3$).

[67 ± 14 nM (leptosperin-fed) versus 44 ± 6.1 nM (MSYR-fed)], whereas the concentration of MSYR-S in the MSYR-fed group was higher than that in leptosperin-fed group [2.7 ± 1.1 nM (MSYR-fed) versus 1.8 ± 0.7 nM (leptosperin-fed)]. The concentration of MSYR-GA in the leptosperin-fed group was 1.5-fold higher compared to that in MSYR-fed group in plasma, whereas the ratio of MSYR-S in the leptosperin-fed group to that in the MSYR-fed group in plasma was 0.67. Meanwhile, leptosperin, MSYR-glucoside, SYR-gentiobioside, MSYR, and SYR were not detected in the mice plasma samples (data not shown).

Leptosperin was not detected in mice urine samples throughout the time course. SYR-GA and SYR-S were only marginally detected in the urine samples collected 0–6 h after the administration of leptosperin and MSYR, respectively (data not shown). MSYR-GA, MSYR-S, and MSYR were quantified in the urine samples collected 0–24 h after the oral administration of each (Fig. 9 and Table 2). These compounds reached maximum levels at 0–1 h after administration. MSYR-GA was the predominant urinary metabolite in the 0–1 h period in both groups (Fig. 9A and B). The concentrations of MSYR-GA were at similar levels between the leptosperin-fed group and the MSYR-fed group. On the other hand, the concentration of MSYR-S in the 0–1 h period was three-fold higher in the MSYR-fed group (33 ± 15 nmol/h/kg) than that in the leptosperin-fed group (9.5 ± 11 nmol/h/kg) (Fig. 9C and D), i.e., the ratio of MSYR-GA in the leptosperin-fed group to that in the MSYR-fed group was 1.25, whereas the MSYR-S ratio of the leptosperin-fed group to the MSYR-fed group in urine (0–1 h period) was 0.29. These results suggest that ingested intact MSYR (as aglycone) is easily transformed to the sulfate conjugate (MSYR-S) compared to digested leptosperin-derived MSYR. As shown in Fig. 9E and F, MSYR was detected at the 0–4 h period, but the levels of the 4–24 h data-points in both groups were below the limit of detection. MSYR-glucoside was detected in urine samples collected 0–6 h after administration of leptosperin, suggesting that the intra-glycoside bond of gentiobioside was also cleaved in vivo (Supporting Information Fig. 2). The detectable amounts of SYR and SYR-glucoside in the mice urine samples (prior ingestion of leptosperin or MSYR) could be explained by the presence of certain amounts of these compounds detected in the mice diet (a commercially available conventional diet) (Supporting Information Fig. 3).

4 Discussion

As shown in Fig. 4, after ingestion of manuka honey, two conjugates, glucuronide and sulfate conjugates of MSYR, were identified in human urine. These conjugates were identified using LC-MS/MS on the basis of their retention times and specific fragmentation patterns compared with their respective standards. Among the specimens obtained from human-ingested manuka honey and from mice that were administered MSYR and leptosperin, MSYR-GA was the most abundant metabolite, followed by MSYR-S (Figs. 6–9). These results suggested that MSYR was preferentially conjugated by glucuronidases rather than sulfotransferases. We have also found that MSYR was effectively conjugated to MSYR-GA by UGT1A7 expressed in yeast cells, followed by the other UGTs (Supporting Information Table 1), indicating that MSYR is a substrate for UGT1A7 and other members of the UGT family in a human liver.

Prior to this study, we conducted a preliminary investigation of the cellular uptake and metabolism of leptosperin and MSYR by human intestinal Caco-2 and by hepatoma HepG2 cell lines, as in vitro models. In both cell lines, although the leptosperin was retained in the medium, a reduction of MSYR supplemented in medium was observed accompanied by the formation of MSYR-GA and MSYR-S (unpublished observation). It has been demonstrated that quercetin derived from ingested rutin, quercetin-3-*O*-rutinoside, was slowly absorbed in comparison with intact quercetin in human, pig, and dog subjects [22–24]. These reports show that rutin is hydrolyzed into quercetin by bacterial α-rhamnosidases and β-glucosidases in the lower parts of the intestinal tract, and that the formed aglycone is then absorbed into the loci. Similarly, gentiobioside in leptosperin might be resistant to an enzyme such as lactose-phlorizin hydrolase expressed in small intestinal cells; therefore, leptosperin was hydrolyzed to MSYR by glucosidases derived from microbes in microflora in the colon. Indeed, the recovery of total excretion of MSYR and MSYR conjugates in 24 h by administration of leptosperin was relatively lower than that by MSYR administration (15% versus 23%) (Table 2).

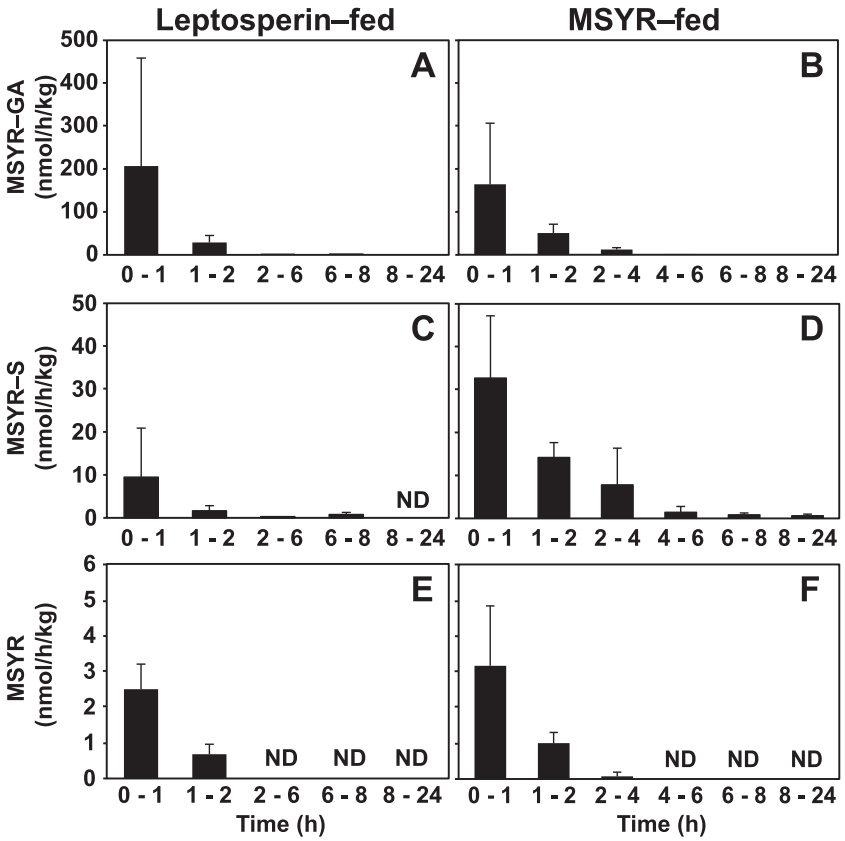

Figure 9. Time-dependent excretion of methyl syringate-glucuronide (MSYR-GA), MSYR-sulfate (S), and MSYR in the urine of the mice subjects 0–24 h after oral administration of leptosperin or MSYR. MSYR-GA (A and B), MSYR-S (C and D), MSYR (E and F). Leptosperin-fed mice urine (A, C, and E). MSYR-fed mice urine (B, D, and F). The abundances of compounds in mice were calculated as nmol/h/kg. Data represent means ± standard deviation (SD) ($n = 3$).

As shown in Fig. 5, the presence of SYR, SYR-GA, and SYR-S has also been confirmed; therefore, an esterase should contribute to the metabolism of MSYR and leptosperin in vivo, similar to the conversion of aspirin to salicylate by human carboxylesterase in vivo [25]. These compounds were not quantified because of their lower contents and a lack of sufficient amounts of standards to construct standard curves. During this study, we found that commercial feed for mice contains SYR and SYR-glucoside. Therefore, it is difficult to distinguish the origin of SYR, SYR-glucoside, and SYR-conjugates between the feed and ingested MSYR/leptosperin; however, we have also detected these SYR compounds from human urine and/or plasma only after ingestion of the honey, suggesting the contribution of a carboxylesterase to the metabolism of MSYR/leptosperin. Because SYR is not a good substrate for human UGT and sulfotransferase (Figs. 2 and 3, and Ikushiro et al., unpublished observation), SYR-conjugates could be formed from

Table 2. Urinary excretion of leptosperin and methyl syringate (MSYR) metabolites in 0–24 h urine of mice following the oral administration of leptosperin or MSYR

	Compounds	(nmol)	S.D.
MSYR-fed mice (1.7 μmol/kg)	MSYR-GA	7.75	4.61
	MSYR-S	1.94	0.56
	MSYR	0.13	0.07
	leptosperin	n.d.	
	Total	9.82 **(22.8%)**	4.27
Leptosperin-fed mice (1.7 μmol/kg)	MSYR-GA	5.94	4.97
	MSYR-S	0.35	0.19
	MSYR	0.07	0.02
	leptosperin	n.d.	
	Total	6.36 **(14.8%)**	5.16

Data represent mean values in nmol ± standard deviation (SD) ($n = 3$). Numbers in bold parentheses indicate excretion as a percentage of leptosperin or MSYR intake. n.d. means not detected (under the detection limit)
GA, glucuronide; S, sulfate.

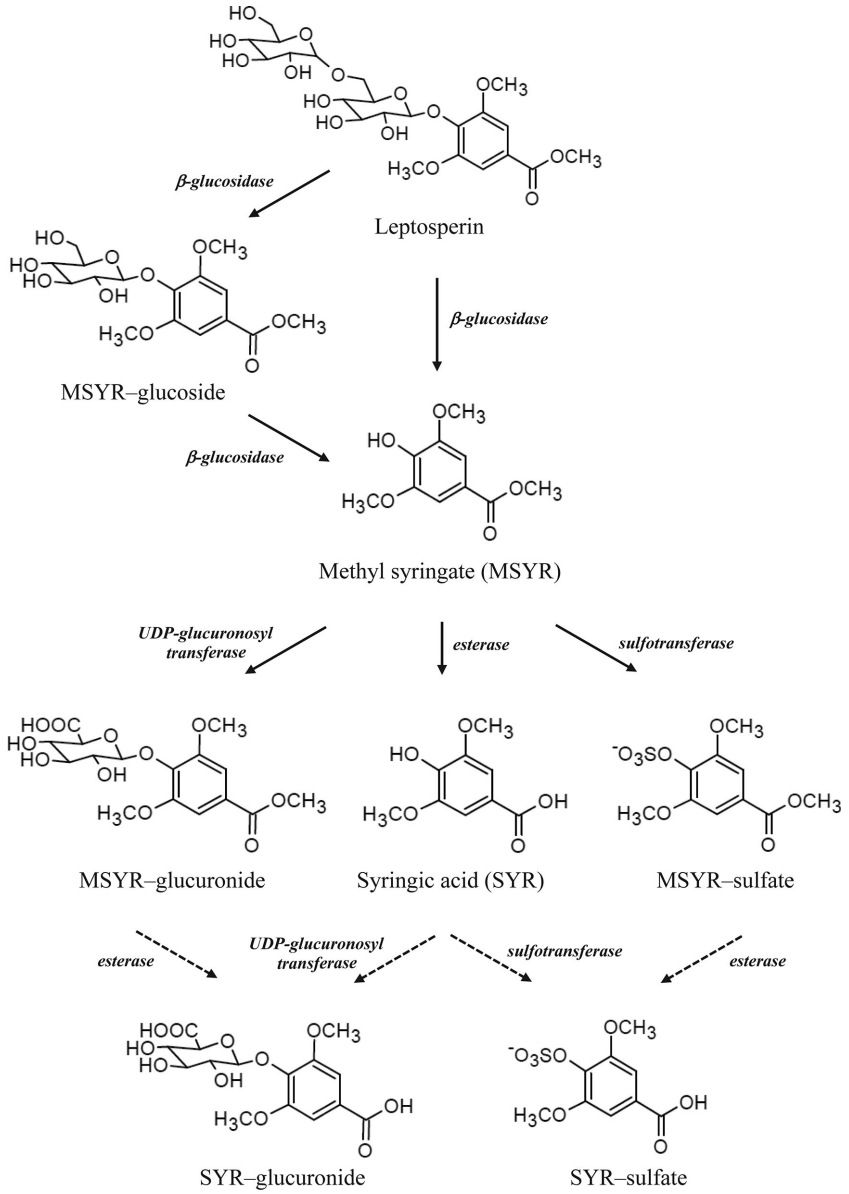

Figure 10. Schematic of the proposed metabolic fate of leptosperin and methyl syringate (MSYR).

respective MSYR-conjugates by an enzymatic action of a carboxylesterase [21]. Nevertheless, when MSYR-GA or MSYR-S was incubated with human liver microsomes/S9 fractions, no peaks of SYR-conjugates were evident in the HPLC chromatogram (data not shown), indicating that at least one carboxylesterase in human liver does not cleave the carboxymethylester in MSYR-GA and MSYR-S. An additional carboxylesterase expressed in other organs, such as intestinal cells or intestinal bacteria, may generate SYR-GA and SYR-S from MSYR-GA and MSYR-S, respectively, via enterohepatic circulation. A possible pathway for in vivo metabolism of leptosperin/MSYR is summarized in Fig. 10.

Leptosperin was detected in human urine but not in mouse urine. In our preliminary study, the metabolite patterns of MSYR were different among the liver homogenates of mice, rats, and humans (unpublished observation). In addition, drug metabolism is different between animals and humans [26]. These facts may account for the differences in leptosperin detection. On the other hand, leptosperin was not detected in human plasma. This result could be due to the lower extraction efficiency of leptosperin from plasma than from urine because blood contains many components, such as proteins (e.g., albumin) and lipids. In any case, the present study showed that at least intact leptosperin also circulates the human body.

In general, natural phenols and polyphenols as types of xenobiotics are metabolized to glucuronide and/or sulfate conjugates after oral administration. Although these phenolics have antioxidant activity and/or biological functions, they often lose their functions by the conjugations in

vivo; however, quercetin-3-*O*-β-D-glucuronide (Q3GA) for example, found in human plasma as a major metabolite of quercetin, has antioxidative and neuroprotective activity in vitro [27,28]. Hence, once conjugated metabolites, such as luteolin glucuronide, Q3GA, and resveratrol sulfates were converted to their respective aglycones in inflammatory cell lines, they then exerted antiinflammatory effects [29–31]. Some reports on MSYR functionality exist; MSYR was identified as a selective agonist of the transient receptor potential channel ankyrin 1 (TRPA1) from extracts of the first leaves of *Kalopanax pictus Nakai* (Araliaceae) [32, 33] and was also isolated from the essential oil of *Betula alba* as an aflatoxin production inhibitor [34]. MSYR was also identified as a superoxide anion radical scavenger in manuka honey [35]. Therefore, even though the concentration of intact MSYR/leptosperin in vivo was low compared to those of the two conjugates of MSYR, MSYR/leptosperin could express some biological functions in vivo.

5 Conclusion

In conclusion, the present study was designed to obtain preliminary/basic information of in vivo metabolism of leptosperin and MSYR which were abundantly found in manuka honey. As a result, the MSYR-glucuronide and sulfates were identified as major metabolites and quantified in plasma and urine after ingestion of leptosperin and MSYR. Further research on the pharmacokinetics, pharmacodynamics, and bioactivity derived from ingested leptosperin and MSYR is required in the near future.

A.I., S.I., and Y. Kato conceived, designed, and performed the experiments and data analysis, with the help of M.T., Y.A., M.J., Y.Y.; Y. Kawai provided technical assistance and contributed to the data collection; N.K. and T.S. supervised this project; T.N. provided technical advice for methodology; H.I. resourced and supervised this project; A.I., S.I., and Y. Kato wrote the paper; Y. Kato planned and supervised all experiments and manuscript preparation. All authors read and approved the final manuscript.

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The authors have declared no conflict of interest.

6 References

[1] Adams, C. J., Manley-Harris, M., Molan, P. C., The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey. *Carbohydr. Res.* 2009, *344*, 1050–1053.

[2] Hansen, F., Pandolfo, P., Galland, F., Torres, F. V. et al., Methylglyoxal can mediate behavioral and neurochemical alterations in rat brain. *Physiol. Behav.* 2016, *164*, 93–101.

[3] Suh, K. S., Choi, E. M., Rhee, S. Y., Kim, Y. S., Methylglyoxal induces oxidative stress and mitochondrial dysfunction in osteoblastic MC3T3-E1 cells. *Free Radic. Res.* 2014, *48*, 206–217.

[4] Wallace, A., Eady, S., Miles, M., Martin, H. et al., Demonstrating the safety of manuka honey UMF 20 + in a human clinical trial with healthy individuals. *Br. J. Nutr.* 2010, *103*, 1023–1028.

[5] Degen, J., Vogel, M., Richter, D., Hellwig, M. et al., Metabolic transit of dietary methylglyoxal. *J. Agric. Food Chem.* 2013, *61*, 10253–10260.

[6] Silva, M., Ferreira, A., Gomes, R., Tomás, A. et al., The glyoxalase pathway in protozoan parasites. *Int. J. Med. Microbiol.* 2012, *302*, 225–229.

[7] Mannina, L., Sobolev, A. P., Coppo, E., Di Lorenzo, A. et al., Antistaphylococcal activity and metabolite profiling of manuka honey (Leptospermum scoparium L.) after in vitro simulated digestion. *Food Funct.* 2016, *7*, 1664–1670.

[8] Kato, Y., Araki, Y., Juri, M., Fujinaka, R. et al., Immunochemical authentication of manuka honey using a monoclonal antibody specific to a glycoside of methyl syringate. *J. Agric. Food Chem.* 2014, *62*, 10672–10678.

[9] Kato, Y., Fujinaka, R., Ishisaka, A., Nitta, Y. et al., Plausible authentication of manuka honey and related products by measuring leptosperin with methyl syringate. *J. Agric. Food Chem.* 2014, *62*, 6400–6407.

[10] Kato, Y., Araki, Y., Juri, M., Ishisaka, A. et al., Competitive immunochromatographic assay for leptosperin as a plausible authentication marker of manuka honey. *Food Chem.* 2016, *194*, 362–365.

[11] Bong, J., Prijic, G., Braggins, T., Schlothauer, R. et al., Leptosperin is a distinct and detectable fluorophore in Leptospermum honeys. *Food Chem.* 2017, *214*, 102–109.

[12] Bong, J., Loomes, K., Schlothauer, R., Stephens, J., Fluorescence markers in some New Zealand honeys. *Food Chem.* 2016, *192*, 1006–1014.

[13] Beitlich, N., Lübken, T., Kaiser, M., Ispiryan, L. et al., Fluorescent pteridine derivatives as new markers for the characterization of monofloral genuine New Zealand manuka (leptospermum scoparium) honey. *J. Agric. Food Chem.* 2016, *64*, 8886–8891.

[14] Kato, Y., Umeda, N., Maeda, A., Matsumoto, D. et al., Identification of a novel glycoside, leptosin, as a chemical marker of manuka honey. *J. Agric. Food Chem.* 2012, *60*, 3418–3423.

[15] Molan, P. C., The evidence and the rationale for the use of honey as a wound dressing. *Wound Practice Res.* 2011, *19*, 204–220.

[16] Jubri, Z., Rahim, N. B., Aan, G. J., Manuka honey protects middle-aged rats from oxidative damage. *Clinics (Sao Paulo)* 2013, *68*, 1446–1454.

[17] Abbott, F. V., Palmour, R. M., Morphine-6-glucuronide: analgesic effects and receptor binding profile in rats. *Life Sci.* 1988, *43*, 1685–1695.

[18] Wu, D., Kang, Y. S., Bickel, U., Pardridge, W. M., Blood-brain barrier permeability to morphine-6-glucuronide is markedly

reduced compared with morphine. *Drug Metab. Dispos.* 1997, *25*, 768–771.

[19] Aitken, H., Johannes, M., Loomes, K., Brimble, M., Synthesis of leptosin, a glycoside isolated from mānuka honey. *Tetrahedron Lett.* 2013, *54*, 6916–6919.

[20] Ikushiro, S., Nishikawa, M., Masuyama, Y., Shouji, T. et al., Biosynthesis of drug glucuronide metabolites in the budding yeast saccharomyces cerevisiae. *Mol. Pharm.* 2016, *13*, 2274–2282.

[21] Laizure, S., Herring, V., Hu, Z., Witbrodt, K. et al., The role of human carboxylesterases in drug metabolism: have we overlooked their importance? *Pharmacother. J. Hum. Pharmacol. Drug Ther.* 2013, *33*, 210–222.

[22] Hollman, P., Bijsman, M., van Gameren, Y., Cnossen, E. et al., The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radic. Res.* 1999, *31*, 569–573.

[23] Reinboth, M., Wolffram, S., Abraham, G., Ungemach, F. R. et al., Oral bioavailability of quercetin from different quercetin glycosides in dogs. *Br. J. Nutr.* 2010, *104*, 198–203.

[24] Cermak, R., Landgraf, S., Wolffram, S., The bioavailability of quercetin in pigs depends on the glycoside moiety and on dietary factors. *J. Nutr.* 2003, *133*, 2802–2807.

[25] Tang, M., Mukundan, M., Yang, J., Charpentier, N. et al., Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterificated in the presence of ethyl alcohol. *J. Pharmacol. Exp. Ther.* 2006, *319*, 1467–1476.

[26] Martignoni, M., Groothuis, G. M., de Kanter, R., Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin. Drug Metab. Toxicol.* 2006, *2*, 875–894.

[27] Moon, J. H., Tsushida, T., Nakahara, K., Terao, J., Identification of quercetin 3-O-beta-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. *Free Radic. Biol. Med.* 2001, *30*, 1274–1285.

[28] Ho, L., Ferruzzi, M., Janle, E., Wang, J. et al., Identification of brain-targeted bioactive dietary quercetin-3-O-glucuronide as a novel intervention for Alzheimer's disease. *FASEB J.* 2013, *27*, 769–781.

[29] Shimoi, K., Saka, N., Nozawa, R., Sato, M. et al., Deglucuronidation of a flavonoid, luteolin monoglucuronide, during inflammation. *Drug Metab. Dispos.* 2001, *29*, 1521–1524.

[30] Kawai, Y., Nishikawa, T., Shiba, Y., Saito, S. et al., Macrophage as a target of quercetin glucuronides in human atherosclerotic arteries: implication in the anti-atherosclerotic mechanism of dietary flavonoids. *J. Biol. Chem.* 2008, *283*, 9424–9434.

[31] Patel, K. R., Andreadi, C., Britton, R. G., Horner-Glister, E. et al., Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence. *Sci. Transl. Med.* 2013, *5*, 205ra133.

[32] Son, H., Kim, M., Park, J.-H., Ishii, S. et al., Methyl syringate, a low-molecular-weight phenolic ester, as an activator of the chemosensory ion channel TRPA1. *Arch. Pharm. Res.* 2012, *35*, 2211–2218.

[33] Kim, M., Son, H., Song, S., Jung, M. et al., The TRPA1 agonist, methyl syringate suppresses food intake and gastric emptying. *Plos One* 2013, *8*, e71603.

[34] Jermnak, U., Yoshinari, T., Sugiyama, Y., Tsuyuki, R. et al., Isolation of methyl syringate as a specific aflatoxin production inhibitor from the essential oil of Betula alba and aflatoxin production inhibitory activities of its related compounds. *Int. J. Food Microbiol.* 2012, *153*, 339–344.

[35] Inoue, K., Murayama, S., Seshimo, F., Takeda, K. et al., Identification of phenolic compound in manuka honey as specific superoxide anion radical scavenger using electron spin resonance (ESR) and liquid chromatography with coulometric array detection. *J. Sci. Food Agric.* 2005, *85*, 872–878.

