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Metabolic map of osthole and its effect on lipids

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Abstract

1. Osthole, a coumarin compound from plants, is a promising agent for the treatment of metabolic diseases, including hyperglycemia, fatty liver, and cancers. Studies indicate that the peroxisome proliferator-activated receptors (PPAR) α and γ are involved in the pharmacological effects of osthole. The *in vitro* and *in vivo* metabolism of osthole and its biological activity are not completely understood.

2. In this study, ultra-performance chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC–ESI–QTOFMS)-based metabolomics was used to determine the metabolic pathway of osthole and its influence on the levels of endogenous metabolites. Forty-one osthole metabolites, including 23 novel metabolites, were identified and structurally elucidated from its metabolism *in vitro* and *in vivo*. Recombinant cytochrome P450s (CYPs) screening showed that CYP3A4 and CYP3A5 were the primary enzymes contributing to osthole metabolism.

3. More importantly, osthole was able to decrease the levels of lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) in the plasma, which explains in part its modulatory effects on metabolic diseases.

4. This study gives the insights about the metabolic pathways of osthole *in vivo*, including hydroxylation, glucuronidation, and sulfation. Furthermore, the levels of the lipids regulated by osthole indicated its potential effects on adipogenesis. These data contribute to the understanding of the disposition and pharmacological activity of osthole *in vivo*.

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Declaration of interest

The authors report no declarations of interest.

Keywords

Lipid; metabolomics; metabolic disease; osthole; time-of-flight mass spectrometry; ultra-performance liquid chromatography

Introduction

Natural coumarin compounds have been studied extensively since some are known to protect against the metabolic diseases, including hyperlipidemia and hypertension (Katsori & Hadjipavlou-Litina, 2014). Scopoletin, fraxin, and esculin belong to the coumarin family, and show inhibitory effects on hypertension and hyperlipidemia (Ojewole & Adesina, 1983; Tsukamoto et al., 1985; Venugopala et al., 2013). The coumarin osthole is widely distributed in *Cnidium moonieri* (L.) Cussion and *Angelica pubescens*. Accumulating evidence demonstrate that osthole shows protective effects on various metabolic diseases, including hyperglycemia (Liang et al., 2009), alcohol and non-alcohol fatty liver diseases (Qi et al., 2011; Song et al., 2006), and cancers (Jarzab et al., 2014; Wen et al., 2015). Several studies suggested that the effects of osthole on metabolic diseases may be associated with activation of peroxisome proliferator-activated receptors (PPAR) α and γ (Qi et al., 2016; Zhao et al., 2014). The metabolism of osthole has been reported (Lv et al., 2012b; Yuan et al., 2009), however, its metabolic map *in vivo* still remains unclear. The structure-activity analyze of osthole (the structure is provided in Figure 8) was explored, revealing that the 3-methyl-2-butenyl-group at position-8 and the methoxy-group at position-7 in the chemical structure of osthole were essential for the inhibition of concanavalin-induced liver toxicity (Okamoto et al., 2005). Three synthetic osthole derivatives, 7-ethoxy-8-(3-methyl-2-butenyl) coumarin, 8-allyl-7-methoxy-4-methyl-6-nitrocoumarin, and 8-allyl-7-methoxy-3-nitroquinoline, shown higher protective effects on the liver damage than osthole (Okamoto et al., 2007). To date, there are limited studies to determine which cytochrome P450s (CYPs) are responsible for the formation of osthole metabolites.

Extensive studies have demonstrated that ultraperformance chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC–ESI–QTOFMS)-based metabolomics is a powerful tool for the comprehensive profiling of xenobiotic metabolites. It was applied to determine the metabolic behaviors of many clinical drugs, including acetaminophen (Chen et al., 2008a), procainamide (Li et al., 2012), and gefitinib (Liu et al., 2015), yielding new insights on their efficacy and side-effects. The pharmacological activities and the potential action target also could be revealed by metabolomics; for example fenofibrate (Patterson et al., 2009) and tempol (Li et al., 2013b). Using this powerful technology, the metabolic maps of many natural compounds and endogenous compounds have been described, such as arecoline (Giri et al., 2007), noscapine (Fang et al., 2012), and melatonin (Li et al., 2013a).

In this study, a metabolomic approach was used to investigate the metabolic behavior of osthole. The aims of the present study were (i) to elucidate the metabolic pathways of osthole in the mouse; (ii) to identify the drug-metabolizing enzymes involved in osthole metabolism; and (iii) to evaluate the biological activity of osthole based on its regulation of

endogenous metabolites. Forty-one osthole metabolites were identified in this study, including 23 novel metabolites. Osthole undergoes hydroxylation, hydrogenation, demethylation, dehydrogenation, glucuronidation, and sulfation. It was revealed that CYP3A4 and CYP3A5 were the primary enzymes contributing to osthole metabolism. The levels of several lysophosphatidylethanolamines (LPE) and lysophosphatidylcholines (LPC) in the plasma could be decreased by osthole, suggesting its potential benefit effects of osthole on adipogenesis. Overall, this study exemplifies the value of LC–MS-based metabolomics for the simultaneous evaluation of drug metabolism and its effect on host metabolism.

Materials and methods

Chemicals and reagents

Osthole was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). NADPH, chlorpropamide, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse liver microsomes (MLM) and human liver microsomes (HLM) were provided by Bioreclamationivt Inc. (Hicksville, NY, USA). Xenotech, LLC (Kansas City, KS, USA) provided the recombinant CYPs. All other reagents (e.g. acetonitrile and water) were of the highest grade commercially available.

In vitro phase I metabolism of osthole

Microsomal incubations were carried out in phosphate-buffered saline solution (PBS, pH = 7.4), containing 50 μ M osthole, 0.5 mg/ml MLM or 2 pmol/ml of each cDNA-expressed CYPs (Bactosome control, CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C19, 2C8, 2C9, 2D6, 2E1, 3A4, 3A5, and 4A11) in a final volume of 180 μ l. After a 5 min pre-incubation at 37 °C with shaking at 800 rpm, the reaction was initiated by adding 20 μ l of 10 mM NADPH (dissolved in PBS). After 40 min incubation at 37 °C with shaking at 800 rpm, the reaction was stopped by adding 200 μ l of ice-cold acetonitrile. The solution was centrifuged at 15,000 rpm for 10 min following vortexing for 1 min. A 5 μ l aliquot of the supernatant was injected into a UPLC–ESI–QTOFMS system for analysis. Incubations were conducted in duplicate for cDNA-expressed CYPs, and triplicate for MLM and HLM incubation experiments.

In vivo metabolism of osthole

In this study, 6- to 8-week-old male Kunming mice (25–30 g) provided by Shanghai laboratory animal center (SLAC), China were used to investigate osthole metabolism *in vivo*. The mice were maintained under a standard 12 h dark/light cycle with water and chow provided *ad libitum*. The mice were treated with osthole in corn oil at a dose of 40 mg/kg body weight by intragastric (i.g.) and intraperitoneal (i.p.) administration. All the experimental mice were housed separately in metabolic cages for 24 h. Control mice were treated with corn oil alone. Urine and feces were collected for a period of 0–24 h, and blood samples harvested 3 h and 24 h after administration of osthole. The plasma samples were prepared from the blood by centrifugation at 3500 rpm for 5 min. All samples were stored at –80 °C until analysis. Handling was in accordance with study protocols approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Botany, Chinese Academy of Sciences.

Sample preparation

Briefly, plasma samples were prepared through mixing the 10 μ l plasma with 190 μ l 67% aqueous acetonitrile containing 5 μ M chlorpropamide. Urinary samples were prepared by mixing 20 μ l of urine with 180 μ l 50% aqueous acetonitrile containing 5 μ M chlorpropamide. Both the plasma samples and urine samples were separately centrifuged at 15,000 rpm for 20 min. Twenty milligram of feces were mixed with 200 μ l of 50% aqueous acetonitrile containing 5 μ M chlorpropamide. After shaking at room temperature for 20 min, the samples were centrifuged at 15,000 rpm for 20 min to obtain fecal content extract supernatants. Subsequently, 100 μ l of supernatant was transferred to a new Eppendorf vial and diluted with 200 μ l of 50% aqueous acetonitrile. After centrifugation at 15,000 rpm for 20 min, a 5 μ l aliquot of the supernatant was injected into the UPLC–ESI–QTOFMS system.

UPLC–ESI–QTOFMS analysis

The separation of osthole and its metabolites was achieved using a UPLC system containing a 1290 Autosampler and 1290 Quat Pump (Agilent, Santa Clara, CA, USA) equipped with XDB-C18 column (2.1 $\times$ 100mm, 1.8 μ M). Column temperature was maintained at 45 $^{\circ}$ C throughout the run. The flow rate was 0.3 ml/min with a gradient ranging from 5% to 95% acetonitrile containing 0.01% formic acid in 16 min run. The mass signals of ion were collected in both positive and negative mode by the electrospray ionization 6530 QTOFMS (Agilent, Santa Clara, CA, USA). Nitrogen was applied as both drying gas (9 l/min) and the collision gas. The drying gas temperature was set at 350 $^{\circ}$ C and nebulizer pressure was kept at 35 psi. Capillary voltage was set at 3.5 kV.

Multivariate data analysis

Chromatographic and spectral data of samples were acquired by MassHunter Workstation data Acquisition software (Agilent, Santa Clara, CA, USA) and processed by Mass Profinder and Mass Profiler Professional software (Agilent, Santa Clara, CA, USA) to generate a multivariate data matrix. Then, the corresponding data matrix was further analyzed by SIMCA-P+ 13.0 software (Umetrics, Kinnelon, NJ, USA) as described previously (Patterson et al., 2008). Different from unsupervised principal component analysis (PCA), partial least squares-discriminate analysis (PLS-DA), orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was used to identify the major latent variables in the data matrix. Potential metabolites of osthole were identified by analyzing the ions contributing to the separation of the experimental group and control group in the S-plot.

Identification of osthole metabolites and endogenous metabolites

The identification and confirmation of osthole metabolites and endogenous metabolites were performed according the previous approaches (Li et al., 2013b, 2014, 2015). Seven golden rules were applied to calculate the mass error based on the elemental compositions of each metabolite (Kind & Fiehn, 2007). After the putative osthole metabolites in the mouse urine and microsomal incubations were determined by OPLS-DA analysis, tandem MS of selected metabolites was performed in the targeted mode with a default isolation width of 0.01 m/z obtained by ramping collision energies from 5 to 15 V. The chemical structures of osthole metabolites were elucidated by interpretation of their MS/MS fragmentation patterns

compared to that of osthole. The METLIN database was searched to match the changed endogenous metabolites. To confirm metabolites identity, the MS/MS fragmentation patterns of the putative endogenous metabolite were compared with that of an authentic compound.

Data analysis

Statistical analysis was conducted using Prism v. 6 (GraphPad Software, San Diego, CA, USA). *p* Value less than 0.05 was considered significant. The experimental data were presented as mean $\pm$ SD.

Results

Metabolomic profiling of osthole metabolites in mice

UPLC–ESI–QTOFMS coupled with PCA, PLS-DA, and OPLS-DA modeling was used to examine the differences between osthole treatment group and control groups. The osthole treatment and control group could be well separated by these models. To specifically identify the osthole metabolites, the OPLS model was applied to distinguish osthole metabolites from the endogenous metabolites in urine, plasma, and feces (Figure 1A, C; Figure S1A, S1C). Examination of an S-plot of the mouse samples revealed the ions from osthole metabolites. Many ions were elevated in the urine, plasma and feces sample after treatment with osthole as evident in the top right of the S-plot (Figure 1B, D; Figure S1B, D). A comparison of the increased ions in the urine, plasma, or feces samples revealed many ions with similar mass-to-charge (m/z) ratios and retention times. Combined with the trend plots of ions in the control and treatment groups, these ions were detected only in the treatment group and putatively identified as osthole metabolites and their fragments (Figure S2A–D). Overall, 34 osthole metabolites were identified in mouse urine (Table 1), of which metabolites **M1**, **M2**, **M2'**, **M6**, **M17**, **M23**, **M24**, **M26**, and **M28** were main excreted metabolites (Figure 2A, B). In mouse feces, 25 osthole metabolites were identified (**M1**–**M17'**, **M18'**, and **M19**) (Table 1), of which metabolites **M1**, **M2'**, **M3**, **M3'**, **M5**, **M6'**, and **M17** were main excreted metabolites (Figure S3). No phase II metabolites were detected in feces. A total of 8 osthole metabolites (**M3**, **M3'**, **M4**, **M17**, **M23**, **M24**, **M25'**, and **M26**) and unreacted osthole were observed in plasma following the treatment with osthole (Figure 2C, D). Among these metabolites, 23 (**M7**, **M8**, **M10**, **M11**, **M13**–**M17'**, **M19**, **M22**–**M25'**, **M27**–**M30**) were found in the present study and represented as novel metabolites that have never been reported.

Identification and structural elucidation of phase I metabolites of osthole

Initial analysis of mouse samples by OPLS revealed some potential osthole metabolites and their fragments in the S-plot. The chemical compositions and structures of these metabolites were identified based on accurate mass measurements and MS/MS fragmentography. Overall, osthole (**M0**) and 41 metabolites were identified in urine, plasma, and fecal samples. Eighteen metabolites (**M1**–**M6'**, **M9**, **M9'**, **M12**, **M18**, **M18'**, **M20**–**M21'**, and **M26**) were known metabolites, and 23 metabolites (**M7**, **M8**, **M10**, **M11**, **M13**–**M17'**, **M19**, **M22**–**M25'**, and **M27**–**M30**) were novel metabolites. The chemical compositions, retention times of osthole metabolites are listed in Table 1, and the representative MS/MS spectra of phase I and II metabolites are presented in Figures 3 and 4. The fragmentations of osthole

metabolites are present in Table S1 and S2. The major phase I metabolic pathways of osthole were 7-demethylation, dehydrogenation, hydroxylation, hydrogenation, hydration and 3,4-epoxide.

M0 was calculated as $C_{15}H_{16}O_3$ based on the accurate mass measurement m/z 245.1193⁺. The main MS/MS fragmentation ions of osthole were 189, 159, and 131, corresponding to the elimination of the carbon chain, methoxy group, and carbonyl group in Figure 3B.

Neutral losses of 56 (C_4H_8) suggested the existence of 3-methyl-2-butenyl-group in metabolites **M1** and **M5**. Metabolites **M1** and **M5** were calculated as $C_{14}H_{14}O_3$ and $C_{15}H_{16}O_4$ based on the accurate masses of m/z 231.1019⁺ and 261.1140⁺, respectively. Compared with the $[M + H]^+$ ion (m/z 245.1193⁺) of osthole, **M1** was lacked 14 Da (CH_2) as compared with osthole and **M4** was 16 Da (O) higher than osthole. According to the MS/MS fragments shown in Table S1, both metabolites were determined as 7-demethyl and hydroxyl metabolites of osthole.

Neutral losses of 54 (C_4H_6) or 42 (C_3H_6) suggested the existence of a 3-methyl-1,3-dibutenyl group in metabolites **M2**, **M2'**, **M3**, **M3'**, **M6**, **M6'**, **M7**, and **M16**, suggesting that these metabolites were a dehydrogenation product of the carbon chain. Metabolites **M3** and **M3'** were calculated as $C_{15}H_{14}O_3$ based on the accurate masses of m/z 243.1023⁺, and lacked 2 Da (H_2) as compared with osthole, thus indicating that they were dehydrogenation products of osthole. Metabolites **M2** and **M2'** matching the molecular formula $C_{14}H_{12}O_3$ based on the accurate mass m/z 229.0863⁺, were proposed as the 7-demethylation product of **M3** and **M3'** based on the MS/MS fragmentations (Figure 3C, D). Metabolite **M7** was deduced as $C_{14}H_{14}O_4$ based on the accurate mass of m/z 247.0962⁺, and higher by 18 Da (H_2O) than **M2** and **M2'**. The product ion at m/z 193 of **M7** was also higher by 18 Da than the product ion of **M2** and **M2'** at m/z 175 (Table S1), indicating that it was the hydration product of **M2** or **M2'**. Metabolites **M6** and **M6'** were deduced as $C_{15}H_{14}O_4$ based on the accurate mass measurement m/z 259.0969⁺, and higher by 16 Da (O) than **M3** and **M3'**. The product ions at m/z 217 and 189 of **M6** and **M6'** were higher by 16 Da than the product ions of **M3** and **M3'** at m/z 201 and 173 (Table S1), indicating they were the oxidation product of **M3** and **M3'**. Metabolite **M16** was deduced as $C_{14}H_{12}O_4$ based on the accurate mass measurement m/z 245.0840⁺, and was 16 Da (O) higher than **M2** and **M2'**. The product ions at m/z 217 and 175 of **M16** were 16 Da higher than the product ions of **M2** and **M2'** at m/z 201 and 159 (Table S1), indicating it was the oxidation product of **M2** or **M2'**.

Neutral losses of 72 (C_4H_8O or $H_2O + C_4H_6$) or 60 ($H_2O + C_3H_6$) suggested the existence of a hydroxylated 3-methyl-2-butenyl group in metabolites **M4**, **M8**, **M9**, **M9'**, **M10**, **M11**, and **M13**. Metabolite **M4** was calculated as $C_{15}H_{16}O_4$ based on the accurate mass of m/z 261.1131⁺, and higher by 16 Da (O) than osthole, indicating that it was the carbon chain hydroxylation product of osthole based on the MS/MS fragmentations (Table S1). Metabolite **M13** was deduced as $C_{14}H_{16}O_4$ based on the accurate mass measurement m/z 249.1137⁺, indicating that it was the 7-demethylation and hydrogenation product of **M4** based on the MS/MS fragmentations in Table S1. Metabolites **M8**, **M9**, **M9'**, **M10**, and **M11** were deduced as $C_{15}H_{16}O_5$, and higher by 32 Da (O_2) than osthole, indicating they

were the dihydroxylation product of osthole. The other MS/MS fragmental ions of **M8**, **M9**, **M9'**, **M10**, and **M11** were interpreted in Table S1.

Neutral losses of 18 (H_2O) and 46 (CH_2O_2) suggested the existence of carboxyl groups in metabolites **M12** and **M15**, and they were carboxylation products of osthole after a 3,4-epoxide reaction. The other MS/MS fragmental ions of **M12** and **M15** are interpreted in Table S1. Metabolites **M14** and **M14'** were deduced as $\text{C}_{14}\text{H}_{18}\text{O}_4$ based on the accurate mass of m/z 251.1284⁺, and were 2 Da (H_2) higher than **M13**, indicating they were the hydrogenation products on the carbon chain of **M13** based on the MS/MS fragmentations in Table S1. Metabolites **M17** and **M17'** exhibited the molecular ion at m/z 275.0929⁺, which gave a match for $\text{C}_{15}\text{H}_{14}\text{O}_5$. The product ion at m/z 177 of **M17** and **M17'** was the same as the product ion of **M9** and **M9'** (Table S1), indicating that they were further dehydrogenation products of **M9** and **M9'**. Metabolites **M18** and **M18'** were deduced as $\text{C}_{14}\text{H}_{14}\text{O}_4$ based on the accurate mass of m/z 247.0946⁺, and were 16 Da (O) higher than **M1**. The product ion at m/z 175 of **M18** and **M18'** was the same as the product ion found in **M1** (Table S1), indicating that **M18** and **M18'** were the carbon chain hydroxylation products of **M1**. Metabolite **M19** was deduced as $\text{C}_{15}\text{H}_{18}\text{O}_5$ based on the accurate mass of m/z 279.1221⁺, and was 2 Da (H_2) higher than **M11**. The product ion at m/z 261 of **M19** was also 2 Da higher than product ion of **M11** at m/z 259 (Table S1), indicating **M19** was the hydrogenated product of **M11**.

Identification of phase II metabolites of osthole

Metabolites **M20–M30** were identified as conjugated metabolites of osthole (Table 1). All the phase II metabolites showed good responses in the negative ion mode (Table S2), and only partial glucuronic acid conjugate products could be detected in the positive ion mode (Table S1). After elimination of the glucuronic acid group ($\text{C}_6\text{H}_{10}\text{O}_7$) in the negative ion mode, metabolites **M20**, **M21**, **M22**, **M23**, **M24**, and **M25** yielded fragment ions at m/z 229, 259, 257, 275, 273, and 249 that were similar to the masses of **M1**, **M4**, **M6**, **M9**, **M17**, and **M14**, respectively (Table S2). The typical MS/MS fragmentations of **M4** and **M21** are provided in Figure 4B, C. These data suggested that metabolites **M20–M25** were glucuronic acid-conjugated products. Similarly, after elimination of the sulfuric acid group (SO_3) in the negative ion mode, metabolites **M26**, **M27**, **M28**, **M29**, and **M30** gave fragment ions at m/z 229, 259, 245, 275, and 243 that were similar to the masses of **M1**, **M4**, **M7**, **M9**, and **M16**, respectively (Table S2). These data suggested that metabolites **M26–M30** were sulfuric acid-conjugated products.

Comparison of osthole metabolism between oral and intraperitoneal administration

Overall, 34 phase I and II metabolites of osthole were identified in mouse urine after treatment with osthole, and 25 and 8 metabolites of osthole were identified in feces and plasma, respectively (Table 1). The levels of metabolites in mouse urine and plasma are presented in Figure 2. In urine, the excretion of osthole and its metabolites (**M6**, **M8**, **M16**, **M28'**, **M29**, and **M30**) following i.g. administration was more than after i.p. administration ($p < 0.05$) (Figure 2A, B). The levels of osthole and its metabolites (**M4**, **M23**, **M25'**, and **M26**) were higher in plasmas following i.p. administration as compared with i.g. administration ($p < 0.05$) (Figure 2C, D). The higher excretion of unmetabolized osthole in

feces was also detected following i.g. administration as compared to i.p. administration, although no significant difference was found (Figure S3).

Comparison of in vitro metabolism of osthole by human and mouse liver microsomes

The osthole metabolites were analyzed by metabolomics using UPLC–ESI–QTOFMS after osthole was incubated with microsomes. OPLS-DA scores plot of the osthole treatment group and control group indicated two clusters (Figure 5A). The S-plot (Figure 5B) generated from OPLS-DA displayed the differential ions which contributed to the separation of two groups. Combined with the trend plots of ions in the experimental and control groups (Figure 5C–F), the top-ranking ions were putatively identified as osthole metabolites. The potential metabolites of osthole are marked in the S-plot (Figure 5B).

A total of 23 osthole metabolites (**M1–M17'**) were identified in the incubations of osthole with MLM and HML (Table 1), including 11 novel metabolites (**M7, M8, M10, M11, and M13–M17'**). It was noted that osthole could be transformed into metabolites with *cis*- and *trans*-isomers, such as **M3** and **M3'** (Table 1, Figure 3C, D). However, due to the similar MS/MS fragments, the current mass spectrometry technology could not determine the absolute configuration of these *cis*- and *trans*-isomers (Figure 3C, D).

Metabolites **M3, M3', M4, M6, and M6'** were the major metabolites in the MLM incubations, while **M3, M3', M4, M6', M9', and M11** were major metabolites in the HLM incubations (Figure 6A, B). The percentage of hydroxylation metabolite **M4** was decreased and dihydroxylation metabolites **M9'** and **M11** increased in the HLM incubations compared to MLM incubations (Figure 6A, B). Additionally, metabolites **M5, M10, and M13** were not detected in the HLM incubations, but detected in the MLM incubations. The relative abundances of these metabolites in the MLM and HLM incubations are shown in Figure 6A, B.

Screening the CYPs involved in the metabolism of osthole

CYPs are responsible for the transformation of more than 60% of currently marketed drugs (Hu et al., 2015). No study reported which CYPs are involved in the generation of osthole metabolites. In this study, the CYPs were screened by use of different human cDNA-expressed CYPs including the control, CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C19, 2C8, 2C9, 2D6, 2E1, 3A4, 3A5, and 4A11. CYP1A1, 2C8, 2C9, 3A4, 3A5, and 4A11 were identified as the major forms contributing to the metabolism of osthole (metabolic rate >45%) (Figure 6C). Multiple enzymes were responsible for the formation of metabolite **M1, M2, M2', M3, M3', M6, and M6'** (Table 2). CYP1A1, 3A4, and 3A5 were the major enzymes that responsible for the formation of monohydroxylated osthole metabolites (**M4** and **M5**) and dihydroxylated osthole metabolites (**M8, M9, M9', M10** and **M11**) (Table 2). The formation of hydrogenated osthole metabolites (**M14** and **M14'**) was mainly mediated by CYP3A4 and CYP3A5 (Table 2). CYP2D6, 2E1, 3A4, and 3A5 were the primary enzymes involved in the formation of the 3,4-epoxide products **M15** (Table 2).

Evaluation of the biological activity of osthole

Earlier studies reported the protective effects of osthole on various metabolic diseases (Qi et al., 2011; Song et al., 2006). Here, the levels of endogenous metabolites that are affected by osthole treatment were examined. As shown in Figure 1D, two top decreased ions, 478.2909⁺ and 526.2955⁺, were found under the bottom of the third quadrant of the S-plot. Chemical formula calculations showed that two ions were corresponded to C₂₃H₄₄NO₇P and C₂₇H₄₄NO₇P. The MS/MS fragmentation demonstrated that the ions 478.2909⁺ (*R*_t = 9.116) and 526.2955⁺ (*R*_t = 9.083) were [M + H]⁺ ions of LPE18:2 and LPE22:6 (Figure 1E, F). Considering that LPEs and LPCs are closely associated with metabolic syndrome (Garcia-Fontana et al., 2016; Li et al., 2014), the levels of LPEs and LPCs in the plasma were analyzed using targeted metabolomics. LPCs and LPEs have one acyl chain that is linked to either the *sn*-1 or *sn*-2 position of glycerol, which produce the isomer, such as LPC16:0 and LPC16:0' (Okudaira et al., 2014). The levels of three LPEs (LPE18:2, LPE20:4, and LPE22:6) and LPC20:3 were significantly reduced by osthole after 3 h i.g. treatment (*p* < 0.05) (Figure S4). The levels of four LPEs (LPE18:2', LPE20:4, LPE20:4', and LPE22:6) were significantly decreased after 3 h i.p. treatment of osthole (*p* < 0.05) (Figure S4).

After a 24 h treatment by i.g. with osthole, the levels of two LPEs (LPE16:0, and LPE18:2) and five LPCs (LPC16:1, LPC18:1, LPC18:2, LPC20:3, and LPC20:4) were significantly lower than the control group (Figure 7). Similarly, after a 24 h i.p. treatment with osthole, the levels of three LPEs (LPE18:2, LPE20:4, and LPE22:6) and five LPCs (LPC18:1, LPC18:1', LPC18:2, LPC20:3, and LPC20:4) were significantly diminished (*p* < 0.05) (Figure 7). These results indicated that both i.g. and i.p. treatment of osthole could cause the decrease in plasma LPEs and LPCs levels.

Discussion

Using high-resolution UPLC–ESI–QTOFMS combined with OPLS analysis, 41 metabolites of osthole, including 23 novel metabolites, were identified and further structurally elucidated by MS/MS fragmentography. By using this strategy, a series of hydroxylation, hydrogenation, demethylation, dehydrogenation, glucuronidation, and sulfation metabolites of osthole were identified *in vivo* and *in vitro*. Another interesting observation from this study is that two types of lipids LPEs and LPCs were decreased following treatment of osthole. Metabolomic analysis can simultaneously determine the metabolic map of osthole and elucidate its biological activity through its regulation on the endogenous metabolites. The metabolic pathways of osthole are summarized in Figure 8.

Although *in vitro* and *in vivo* metabolism of osthole in mice was previously reported (Lv et al., 2012a,b), a comprehensive profile of osthole metabolism has not been investigated. Results from the current metabolomic analysis provided a detailed view of the potential biotransformation pathway of osthole. The hydroxylation, hydrogenation, demethylation, dehydrogenation, glucuronidation, and sulfation of osthole generated 41 osthole metabolites, of which 23 metabolites (**M7**, **M8**, **M10**, **M11**, **M13–M17'**, **M19**, **M22–M25'**, and **M27–M30**) were novel metabolites. Overall, 34 osthole metabolites from phase I and II metabolism were identified in mice urine of which 7-demethylated-osthole (**M1**, **M2**, and

M2'), dehydrogenated-osthole (**M2**, **M2'**, **M6**, and **M17**), hydroxylated-osthole (**M6** and **M17**), glucuronic acid- and sulfuric acid-conjugates (**M23**, **M24**, **M26**, and **M28**) were the primary metabolites in mouse urine (Figure 2A, B). In mouse feces, metabolites **M1**, **M2'**, **M3**, **M3'**, **M5**, **M6'**, and **M17** were mainly excreted, while no phase II metabolites were found in feces (Figure S3). The major circulating metabolites were **M0**, **M3**, **M3'**, **M17** and the phase II metabolites **M23**, **M24**, **M25'**, and **M26** (Figure 2C, D). It was reported that inhibitors of CYP3A4, 2D6, and 2C9 could inhibit the partial metabolism of osthole in liver microsomal incubations (Hu et al., 2015). However, which CYPs are responsible for the formation of osthole metabolites remains unclear. In this study, using recombinant CYPs indicated that the CYP3A4, 2C9, 1A1, 2C8, 3A5, and 4A11 were the major isoenzymes responsible for the formation of osthole metabolites (Figure 6C).

Coumarin 3,4-epoxide formation is extensive in mice (Born et al., 2003; Lewis et al., 2006). Osthole also had coumarin-structure, so we anticipated it also undergone 3,4-epoxidation. In our studies, we found **M12** and **M15** in osthole metabolites, which were carboxylation product of osthole after 3,4-epoxide reaction (Figure 8). By using CYPs, we found CYP2D6, 2E1, 3A4 and 3A5 were primary enzymes involved in the formation of **M15** (Table 2), which was consistent with previous study who found that CYP2E1 and 3A4 were primary enzymes involved in the formation of 3,4-epoxidation in coumarin (Lewis et al., 2006).

We also found lots of hydroxylation (**M4** and **M5**) and dihydroxylation (**M8–M11**) metabolites of osthole in our studies. By using CYPs, we found that CYP1A1, 3A4 and 3A5 were major enzymes participation in the oxidation reaction (Table 2), which was consistent with previous study who found that CYP3A4 was primary enzyme involved in the formation of 3-hydroxylation metabolites in coumarin (Lewis et al., 2006).

Osthole is metabolized by both phase I and phase II pathways. Phase II metabolism is critical for the drug detoxification, and glucuronidation and sulfation are the most important and widespread conjugation pathways. Previous studies found that the 7-hydroxy group in coumarin is prone to form glucuronic acid and sulfuric acid conjugated products (Duffy & O'Kennedy, 1998), but the reports about phase II conjugates of osthole are limited. In the present study, eight glucuronic acid-conjugated products (**M20–M25'**) and seven sulfuric acid-conjugated products (**M26–M30**) were found of which 11 were novel including **M22–M25'** and **M27–M30**. Their structures were elucidated using MS/MS fragmentation patterns by comparison to the phase I metabolites. Glucuronic acid-conjugated products are produced by UDP-glucuronosyltransferases (UGT) (Duffy & O'Kennedy, 1998), and these products are more water-soluble, less toxic and more readily excreted than the parent compound (de Leon, 2003). Sulfuric acid-conjugated products are formed by sulfotransferases. These products increase the water solubility and their renal excretion, but generally decrease the biological action of parent compound (Duffy & O'Kennedy, 1998). Furthermore, metabolites **M6'**, **M9**, **M9'**, **M10**, **M13**, **M14**, and **M14'** were not found in urine compared with the *in vitro* MLM experiments, probably due to the formation of glucuronic acid and sulfuric acid conjugate products, including **M22**, **M23**, **M25**, **M25'**, **M29**, and **M29'** (Figure 8).

The concentration of almost all osthole metabolites in urine were much higher than that in plasma (Wang et al., 2017), such as metabolite **M6** (Figure 2). This phenomenon was in agree with the report of Wang et al. (2016), who found that the esculin (a coumarin derivative) metabolites in rat urine were much higher than that in plasma. In our studies, metabolite **M6** apparently so dominant in urine yet barely perceptible in plasma (Figure 2). These results might indicate most of the osthole metabolites were potent to excrete from mice (Wang et al., 2017). Beside with liver, these CYPs are also expressed in intestine and kidney. The high abundance of **M6** in urine suggests that the CYPs in the intestine and kidney also involve in the metabolism of osthole.

As we all know, the majority of urine was water (above 95%), and the feces were mainly emerged as solid form (Qiu et al., 2017). Therefore, the metabolites present in mouse urine were more polar than that present in feces. Metabolite **M6** was more polar than **M5**, and we could found the rule from the RT in Table 1 and its conjugated system in Figure 8. Therefore, metabolite **M6** seems the predominant excreted phase I metabolites in mouse urine (Figure 2A); metabolite **M5** seems the predominant excreted phase I metabolites in mouse feces (Figure S3). The differences might come from their polarity, and more **M6** was absorbed through enterohepatic circulation and excreted via kidneys into urine.

Metabolites **M6** and **M1** were the main excreted phase I metabolites in mouse urine. The excretion of metabolites **M6** and **M1** followed the order: **M6** > **M1** (Figure 2A). The excretion of the phase II metabolites of **M6** and **M1** followed the order: **SO₃-M1 (M26)** > **Glu-M1 (M20)** ≈ **Glu-M6 (M22)** > undetected **SO₃-M6** (Figure 2B). Therefore, metabolites **M1** was more liable to be eliminate in the form of phase II metabolites, and more liable to conjugation with sulfuric acid and glucuronic acid than **M6**. This might because the hydroxyl in **M6** could form quinoid conjugated system, and this structure was stable. Furthermore, metabolites **M1** was more liable to conjugation with sulfuric acid (**M26**) than glucuronic acid (**M20**) (Figure 2B).

CYPs was one of the most important enzyme families involved in the metabolism of xenobiotics. CYPs 1–3 were responsible for 70–80% of metabolism of clinically used drugs (Olsen et al., 2015), so we evaluated the contribution of some CYPs 1 to 4 to the metabolism of osthole (Table 2). CYP3A4 functioned as the most important human drug-metabolizing enzyme that cleared over a half of all administered pharmaceuticals, which could catalyze diverse oxidative reactions in addition to traditional hydroxylation reactions. This phenomenon was possible due to a large and malleable action site of CYP3A4, capable of accommodating substrates varying in size and chemical nature, as well as its ability to catalyze diverse chemical reactions such as *O*- and *N*-dealkylation, alkyl carbon and aromatic ring hydroxylation and epoxidation (Olsen et al., 2015). Our studies also found that CYP3A4 and 3A5 had a greater role on the formation of the osthole metabolites than other CYPs (Figure 6C), and they could promote the formation of nearly all the metabolites except **M6** and **M10** (Table 2). In the meanwhile, CYP2D6 had a relatively small role on the formation of the osthole metabolites (Figure 6C), and it could only promote the formation of metabolites **M1**, **M2**, **M3–M5**, **M6'** and **M15** (Table 2).

Previous studies indicated that osthole was able to decrease cholesterol and triglycerides in the liver of stroke-prone spontaneously hypertensive rats, however, it did not affect the levels of total lipids, glucose and apolipoproteins in the serum (Ogawa et al., 2007). In the current study, UPLC–ESI–QTOFMS combined with OPLS analysis revealed that the treatment with osthole significantly decreased the levels of LPE18:2 and LPE22:6 in the serum as early as 3 h after administration. The anomalous high levels of LPEs are related to certain disease, including hyperlipidemia and atherosclerosis (Chen et al., 2008b). Further targeted metabolomic analysis revealed that levels of a series of LPEs and LPCs could be reduced following osthole treatment, including LPE18:2, LPE20:4, LPE22:6, LPC16:1, LPC18:1, LPC18:2, LPC20:3, and LPC20:4. One possible mechanism is that osthole could activate PPAR α and PPAR γ . Earlier studies revealed that osthole decreased the levels of lipids by modulating the expression of lipogenic genes through activation of PPAR α and PPAR γ , including the diacylglycerol acyltransferase, 3-hydroxy-3-methylglutaryl-CoA reductase and cholesterol-7 α -hydroxylase (Qi et al., 2011; Sun et al., 2010). PPAR α plays a pivotal role in lipid metabolism by increasing high-density lipoprotein levels and decreasing serum free fatty acids and triglycerides (Mandard et al., 2004), and PPAR γ has a critical role in glucose metabolism and fatty acid storage by coordinating the expression of genes involved in adipogenesis and lipid metabolism (Tontonoz & Spiegelman, 2008). Although previous studies suggested that osthole might be a dual agonist of PPAR α and PPAR γ (Qi et al., 2016; Zhao et al., 2014), further studies are needed to support this contention. Another potential mechanism is that osthole modulates the levels of the cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Teng et al., 1994; Zhang et al., 2015). cAMP signaling was shown to significantly regulate lipid metabolism, and dysregulation of hepatic cAMP levels plays a critical role in alcohol-induced steatosis (Avila et al., 2016). The exact mechanism by which osthole modulates cellular metabolism and metabolic diseases requires additional experimentation.

Hepatic disease was closely related to lipid metabolism disorder (Beyoglu & Idle, 2013). Therefore, the 3-methyl-2-butenyl-group at position-8 and the methoxy-group at position-7 in the chemical structure of osthole might be essential for its lipid-lowering activity because these group shown protective effects on hepatic diseases (Okamoto et al., 2005).

It was reported that bioavailability was 15.6% at a dose of 20 mg/kg body weight after oral administration of osthole (Yun et al., 2013). In this study, the differences of osthole metabolism between i.g. and i.p. administration were further investigated. Due to the mean peak concentration of osthole at about 3 h after oral administration to the rats (Yun et al., 2013), the levels of osthole and its metabolites in the plasma were determined at 3 h in this study. The results indicate that the phase I and II metabolites of osthole were significantly higher in plasmas at 3 h following i.p. administration as compared to the i.g. administration route (Figure 2C, D). In contrast to the levels of osthole and its metabolites in the plasma, the excretions of osthole metabolites in urine and osthole in feces 24 h following i.g. administration were higher than after i.p. administration (Figure 2A, B; Figure S3). The lower levels of partial LPEs and LPCs in the plasma following i.p. administration were observed in 24 h compared to i.g. administration of osthole, although there were no significant differences between i.p. and i.g. administration (Figure 7). These data suggest that the dose routes will affect the metabolism of osthole and its effect on lipids.

Conclusions

In summary, UPLC–ESI–QTOFMS-based metabolomics was used to determine the metabolic behavior of osthole, including comprehensive defining of the metabolites and endogenous metabolites that were altered after osthole treatment. Drug metabolite profiling of osthole-treated mice indicated that osthole undergoes several metabolic pathways *in vivo*, including the hydroxylation, hydrogenation, demethylation, dehydrogenation, glucuronidation, and sulfation. A total of 41 osthole metabolites were characterized and structurally elucidated, of which 23 were novel metabolites. Recombinant CYPs screening showed that CYP3A4 and CYP3A5 were the primary enzymes contributing to the metabolism of osthole. A comprehensive metabolic map of osthole was generated through use of metabolomics. Furthermore, changes in endogenous metabolites indicated the potential benefit effects of osthole on adipogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Metabolomic analysis of osthole metabolites in mouse urine and plasma. Analysis of control and osthole-treated mice urine by OPLS-DA score plots (A) and S-plot (B). Analysis of control and osthole-treated mouse plasma in the positive mode in OPLS-DA score plots (C) and S-plot (D) (●, control-treated mice in score plots; ▲, osthole-treated mice in score plots). Urine from mice was collected continuously over the 24 h and plasma was collected at 3 h after osthole treatment (40 mg/kg, i.g.). In score plots, the $t[1]$ and $to[1]$ values represent the score of each sample in principal component 1 and 2, respectively. In the S-plots, the X-axis represents the relative abundance of ions and the Y-axis represents the correction of each ion to the model. These S-plots represent the relationship between ions in relation to the first and second components present in the OPLS-DA score plot. Metabolites

are labeled in S-plots. *, M + Na. (E) Tandem MS of LPE18:2. (F) Tandem MS of LPE22:6. MS/MS fragmentation was conducted with collision energy at 10 eV.

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Figure 2.

Comparison of phase I and II metabolites of osthole in mice. (A) Relative abundance of phase I metabolites in urine. (B) Relative abundance of phase II metabolites in urine. (C) Relative abundance of phase I metabolites in plasma. (D) Relative abundance of phase II metabolites in plasma. Urine from mice was collected continuously over 24 h and plasma was collected at 3 h after osthole treatment (40 mg/kg, i.g. and i.p.). The relative quantification was conducted based on the peak areas of ion counts. Statistical analysis was performed using two-tailed Student's t-test and ANOVA ($n = 4$ in each group). * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$.

Figure 3. Identification of phase I metabolites of osthole. (A) Chromatograms of metabolites **M0**, **M3** and **M3'**. (B) Tandem MS and chemical structure of **M0**. (C) Tandem MS and chemical structure of **M3**. (D) Tandem MS of **M3'**. **M3** and **M3'** were the *cis-trans* isoform. MS/MS fragmentation was conducted with collision energy at 5 eV.

Figure 4. Identification of phase II metabolites of osthole. (A) Chromatograms of **M4** and **M21**. (B) Tandem MS and chemical structure of **M4**. (C) Tandem MS and chemical structure of **M21**. The phase II metabolite and its deconjugated metabolite show the similar fragmentation mode. MS/MS fragmentation was conducted with collision energy at 5 eV.

Figure 5.

Metabolomic analysis of MLM incubations with osthole. Separation of metabolites derived from MLM incubated with osthole in the positive mode by OPLS-DA score plots (A) and loading S-plots (B). In the score plots, the $t[1]$ and $t[2]$ values represent the score of each sample in principal component 1 and 2, respectively. In the S-plots, the X -axis represents the relative abundance of ions and the Y -axis represents the correction of each ion to the model. These S-plots represent the relationship between ions in relation to the first and second components present in the OPLS-DA score plot. Metabolites are labeled in S-plots. *, M + Na; **, M + K. (C) Trend plot of **M3** in MLMs with and without osthole. (D) Trend plot of **M3'** in MLMs with and without osthole. (E) Trend plot of **M4** in MLMs with and without osthole. (F) Trend plot of **M6'** in MLMs with and without osthole (●, control-treated MLMs; ▲, osthole-treated MLMs). The absence of NADPH in the microsome system was incubated as control groups in the figure.

Figure 6.

In vitro metabolism of osthole by recombinant CYPs. (A) Relative abundance of metabolites from MLM incubations with osthole. (B) Relative abundance of metabolites from HLM incubations with osthole. The overall abundance of metabolites was set as 100% in each sample. The data are expressed as mean $\pm$ SD ($n = 3$). (C) Metabolic rates of each recombinant CYP for osthole. The data are expressed as mean $\pm$ SD ($n = 2$). The residual amount of unreacted osthole after adding CYPs was collected. We specified the amount of unreacted osthole in control CYPs as the whole. Metabolic rate was calculated by the following formula: metabolic rate (%) = $[1 - (\text{the residual amount of osthole in each CYPs} / \text{the residual amount of osthole in control CYPs})] \times 100\%$.

Figure 7.

Osthole regulates the lipid metabolism at 24 h after treatment. (A) Relative abundance of LPEs in plasma at 24 h after osthole treatment. (B) Relative abundance of LPCs in plasma at 24 h after osthole treatment. LPEs and LPCs have one acyl chain that is linked to either the *sn*-1 or *sn*-2 position of glycerol, which produce the isomer, such as LPC16:0 and LPC16:0'. Statistical analysis was performed using two-tailed student's *t*-test and ANOVA ($n = 4$ in each group). * $P < 0.05$, and ** $P < 0.01$ compared with control mice.

Figure 8.

Metabolic map of osthole. Reaction types: 1: 7-demethylation; 2: dehydrogenation; 3: 3,4-epoxide; 4: hydroxylation; 5: aldehylation; 6: oxidation; 7: hydrogenation; 8: hydration; 9: glucuronidation; 10: sulfation. Metabolites marked with asterisk (*) represent isomers were observed.

Table 1.

Summary of osthole metabolites produced *in vivo* and *in vitro* metabolism.

Metabolite ID	Rt (min)	Observed (m/z)	Mass error (ppm)	Predicted molecular formula	Ion mode	Source
M0	9.3	245.1193	9.0	C ₁₅ H ₁₆ O ₃	Positive	Urine, plasma, feces, MLM, HLM
M1	7.4	231.1019	1.7	C ₁₄ H ₁₄ O ₃	Positive	Urine, feces, MLM, HLM
M2	5.3	229.0863	1.7	C ₁₄ H ₁₂ O ₃	Positive	Urine, feces, MLM, HLM
M2'	5.5	229.0864	2.2	C ₁₄ H ₁₂ O ₃	Positive	Urine, feces, MLM, HLM
M3	6.6	243.1023	2.9	C ₁₅ H ₁₄ O ₃	Positive	Urine, plasma, feces, MLM, HLM
M3'	6.7	243.1023	3.3	C ₁₅ H ₁₄ O ₃	Positive	Urine, plasma, feces, MLM, HLM
M4	7.3	261.1131	3.4	C ₁₅ H ₁₆ O ₄	Positive	Urine, plasma, feces, MLM, HLM
M5	8.7	261.1140	6.9	C ₁₅ H ₁₆ O ₄	Positive	Urine, feces, MLM
M6	6.3	259.0969	1.5	C ₁₅ H ₁₄ O ₄	Positive	Urine, feces, MLM, HLM
M6'	7.4	259.0969	1.5	C ₁₅ H ₁₄ O ₄	Positive	Feces, MLM, HLM
M7 ^a	6.0	247.0962	-1.2	C ₁₄ H ₁₄ O ₄	Positive	Urine, feces, MLM, HLM
M8 ^a	5.2	277.1083	4.7	C ₁₅ H ₁₆ O ₅	Positive	Urine, feces, MLM, HLM
M9	5.6	277.1070	0	C ₁₅ H ₁₆ O ₅	Positive	Feces, MLM, HLM
M9'	5.7	277.1074	1.4	C ₁₅ H ₁₆ O ₅	Positive	Feces, MLM, HLM
M10 ^a	6.0	277.1083	4.7	C ₁₅ H ₁₆ O ₅	Positive	Feces, MLM
M11 ^a	6.9	277.1080	3.6	C ₁₅ H ₁₆ O ₅	Positive	Urine, feces, MLM, HLM
M12	5.6	249.1122	0.0	C ₁₄ H ₁₆ O ₄	Positive	Urine, feces, MLM, HLM
M13 ^a	6.9	249.1137	6.0	C ₁₄ H ₁₆ O ₄	Positive	Feces, MLM
M14 ^a	6.2	251.1284	2.4	C ₁₄ H ₁₈ O ₄	Positive	Feces, MLM, HLM
M14' ^a	6.4	251.1293	5.6	C ₁₄ H ₁₈ O ₄	Positive	Feces, MLM, HLM
M15 ^a	7.8	251.1293	6.0	C ₁₄ H ₁₈ O ₄	Positive	Urine, feces, MLM, HLM
M16 ^a	6.0	245.0820	4.9	C ₁₄ H ₁₂ O ₄	Positive	Urine, feces, MLM, HLM
M17 ^a	6.7	275.0929	5.5	C ₁₅ H ₁₄ O ₅	Positive	Urine, plasma, feces, MLM, HLM
M17' ^a	7.0	275.0924	3.6	C ₁₅ H ₁₄ O ₅	Positive	Urine, feces, MLM, HLM

Metabolite ID	Rt (min)	Observed (m/z)	Mass error (ppm)	Predicted molecular formula	Ion mode	Source
M18	5.3	247.0946	-7.7	C ₁₄ H ₁₄ O ₄	Positive	Urine
M18'	5.6	247.0958	-2.8	C ₁₄ H ₁₄ O ₄	Positive	Urine, feces
M19 ^a	6.5	279.1233	2.1	C ₁₅ H ₁₈ O ₅	Positive	Urine, feces
M20	5.2	405.1222	7.6	C ₂₀ H ₂₂ O ₉	Negative	Urine
M21	5.3	435.1303	2.1	C ₂₁ H ₂₄ O ₁₀	Negative	Urine
M21'	5.4	435.1294	0.5	C ₂₁ H ₂₄ O ₁₀	Negative	Urine
M22 ^a	5.4	433.1103	-8.5	C ₂₁ H ₂₂ O ₁₀	Negative	Urine
M23 ^a	4.9	451.1249	0.4	C ₂₇ H ₃₂ O ₁₇	Negative	Plasma, urine
M24 ^a	5.5	449.1075	-3.1	C ₂₁ H ₂₂ O ₁₁	Negative	Plasma, urine
M25 ^a	5.3	425.1452	-0.2	C ₂₀ H ₂₆ O ₁₀	Negative	Urine
M25' ^a	5.6	425.1438	-3.5	C ₂₀ H ₂₆ O ₁₀	Negative	Plasma, urine
M26	6.2	309.0431	-2.3	C ₁₄ H ₁₄ O ₆ S	Negative	Plasma, urine
M27 ^a	7.1	339.0551	2.1	C ₁₅ H ₁₆ O ₇ S	Negative	Urine
M28 ^a	4.6	325.0395	2.5	C ₁₄ H ₁₄ O ₇ S	Negative	Urine
M28' ^a	4.8	325.0388	0.3	C ₁₄ H ₁₄ O ₇ S	Negative	Urine
M29 ^a	4.6	355.0493	0.0	C ₁₅ H ₁₆ O ₈ S	Negative	Urine
M29' ^a	4.9	355.0497	1.1	C ₁₅ H ₁₆ O ₈ S	Negative	Urine
M30 ^a	5.0	323.0246	4.6	C ₁₄ H ₁₂ O ₇ S	Negative	Urine

^a Novel metabolites identified in this study.

Table 2.

Role of CYPs in the formation of osthole metabolites.

	M1	M2	M2'	M3	M3'	M4	M5	M6	M6'	M8	M9	M9'	M10	M11	M14	M14'	M15	M17	M17'
Control	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-
CYP1A1	9.1	9.9	11.0	3.4	32.6	3.1	63.8	36.9	4.2	3.6	-	-	100	3.3	-	-	-	-	-
CYP1A2	18.0	55.6	11.6	2.7	0.9	0.3	2.6	33.5	24	-	-	-	-	-	-	-	-	6.7	84.2
CYP1B1	0.4	0.8	10	2.6	0.7	0.6	-	-	0.4	-	-	-	-	-	-	-	-	-	-
CYP2A6	1.4	-	-	1	4.4	0.6	7.6	13.1	2.2	-	-	-	-	-	-	-	-	-	-
CYP2B6	41.4	6.1	24.9	18.4	15.8	0.3	4	16.5	7.6	26.6	-	-	-	-	-	-	-	39.5	-
CYP2C19	8	1.6	0.9	6.1	1.7	0.3	10.1	-	1.8	-	-	-	-	-	-	-	-	-	-
CYP2C8	2.5	-	2.2	6.3	1.5	1.4	-	-	1.1	-	-	-	-	-	-	-	-	-	-
CYP2C9	3.4	0.9	5.3	0.5	0.5	0.2	7.2	-	0.6	-	-	-	-	-	-	-	-	-	-
CYP2D6	3.4	2.1	-	19.4	4	1.6	4.7	-	3.8	-	-	-	-	-	-	-	32.1	-	-
CYP2E1	8.6	-	11.4	0.1	0.2	0.2	-	-	0.3	-	-	-	-	-	-	-	17	-	-
CYP3A4	1.5	4.9	-	12.8	10.8	42.2	-	-	22.7	48.1	76.6	64.6	-	62.8	42.5	85.3	16.0	43.5	12.2
CYP3A5	2.3	18.2	14.3	26.7	26.9	48.8	-	-	31.2	21.7	23.4	35.4	-	33.8	57.5	14.7	34.9	10.3	3.6
CYP4A11	-	-	8.4	-	-	0.2	-	-	0.1	-	-	-	-	-	-	-	-	-	-

cDNA-expressed CYPs (control, CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C19, 2C8, 2C9, 2D6, 2E1, 3A4, 3A5, and 4A11) were used to determine the role of individual CYP in osthole metabolism. All samples were analyzed by UPLC-ESI-QTOFMS. The total peak areas of each metabolite of osthole from all the CYPs were set as 100%. All data are expressed as mean ($n = 2$).