Metabolites characterization of chamaechromone in vivo and in vitro by using ultra-performance liquid chromatography/Xevo G2 quadrupole time-of-flight tandem mass spectrometry

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A B S T R A C T

Ethnopharmacological relevance: Stellera chamaejasme L. (Thymelaeaceae) was a toxic perennial herb and widely used as pesticide and dermatological agents in China. Chamaechromone was a major component in the dried roots of Stellera chamaejasme with anti-HBV and insecticidal activity. Analysis of metabolic profile in vivo and in vitro plays a pivotal role to unravel how TCM works. And the metabolites of chamaechromone might influence the effects and toxicity of Stellera chamaejasme. Moreover, the metabolic routes of chamaechromone provide an important basis for toxicological safety evaluation. Until now, little is known about the metabolism of chamaechromone. The current study was designed to characterize the whole metabolic pathways of chamaechromone in vitro and in vivo.

Materials and methods: Twenty-four rats were randomly divided into four groups, including two oral administration groups (100 mg kg\textsuperscript{-1}), one intravenous injection group (5 mg kg\textsuperscript{-1}), and one control group. The metabolites in rat urine and feces and bile were identified by UPLC/Q-TOF MS analysis and β-glucuronidase hydrolysis. Moreover, the possible metabolic mechanism was further confirmed by Phase I and Phase II metabolism and catechol-O-methyltransferase methylation in rat liver S9 fraction and degradation in rat intestinal bacteria.

Results: A total of 24 metabolites from chamaechromone were detected and identified in vivo and in vitro, 20 of which were novel. And the major metabolic processes were hydroxylation, methylation, glucurononation, acetylation, dehydroxylation and degradation.

Conclusions: The present study revealed the whole metabolic pathways of chamaechromone in rat through both in vivo and in vitro experiments for the first time. And chamaechromone could undergo extensive phase I and phase II metabolism in rat. These findings would provide an important basis for the further study and clinical application of chamaechromone. In addition, the results of this work have showed the feasibility of the UPLC/Q-TOF-MS approach for rapid and reliable characterization of metabolites.

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1. Introduction

Stellera chamaejasme L. (Thymelaeaceae) is a toxic perennial herb widespread in northern and southwestern China and Nepal.

Abbreviations: UPLC, ultra-performance liquid chromatography; Q-TOF MS, quadrupole time-of-flight mass spectrometer; TCM, traditional Chinese medicine; MeOH, methanol; HPLC, high-performance liquid chromatography

This herb has been shown to possess both toxic and therapeutic effects. Its root was known as Langdu and embodied in the Pharmacopoeia of The People’s Republic China as a toxic traditional Chinese medicine (Chinese Pharmacopoeia Commission, 2010). It was used as a pesticide and remedy for stubborn skin ulcers (Zhao et al., 2013) with antivirus (Asada et al., 2011; Yang and Chen, 2008), antitumor (Wang et al., 2010), antibacterial (Shi et al., 2004), immunomodulatory activities (Xu et al., 2001) and insecticidal activity (Tang and Hou, 2011; Wang et al., 2002). Chamaechromone, a biflavone constituent, was a major active component in dried roots of Stellera chamaejasme (Su et al., 2003). Many pharmacologic actions of chamaechromone might well contribute to the utilization of Stellera chamaejasme. There...
have been reported that chamaechromone possessed anti-HBV effect against HBsAg secretion (Yang and Chen, 2008) and insecticidal activity (Tang and Hou, 2011).

Analysis of metabolic profile in vivo and in vitro plays a pivotal role in the development of TCM and its modernization (Zhang et al., 2010a). From the metabolic methods standpoint, possible in vivo methods include administering the drug to a test subject, resulting in metabolite identification from such a complex biological system (Lou et al., 2010). In vitro methods, rat liver S9 fraction is one of the most commonly used and played a significant role in the research of drug metabolism (Bi et al., 2008). In addition, rat intestinal microflora system was also necessary for the metabolism in vitro owing to various toxicological sequelae by intestinal microflora (Xue et al., 2011). From the instrumentation standpoint, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS) has a pronounced capability for superior selectivity, sensitivity and high-resolution analysis with mass accuracy for metabolite identification (Lu et al., 2012).

Until now, only pharmacological activity and methods for identifying and quantifying chamaechromone in raw herbs have been reported (Su et al., 2003; Yang and Chen, 2008; Zhao et al., 2008), little is known about its metabolism. Our previous research has developed and validated a highly sensitive HPLC/MS bioanalytical method to estimate chamaechromone in rat plasma and applied it to investigate the pharmacokinetics and absolute bioavailability of chamaechromone in rats (Lou et al., 2011). The result showed that the concentration–time curves of chamaechromone in rat plasma after oral administration showed significant double-peak phenomenon. Absolute bioavailability (F) is calculated based on the AUICα→∞ obtained after oral and i.v. administration. And its absolute bioavailability is low with a value being 8.9%. To further understand the mechanism of the effects and toxicity of chamaechromone, studies on metabolism need to be performed.

In this paper, metabolites of chamaechromone in rat were detected with UPLC coupled with Xevo G2 Q-TOF MS. The metabolites were reliably characterized by accurate fragment ions spectra and their different fragmentation pathways. In addition, the formations of the major metabolites were further investigated in vitro using rat liver S9 fraction and rat intestinal microflora systems. In total, 24 metabolites were detected in vivo and in vitro, and 20 (M1–M7, M9–M10, M13–M16, M18–M24) are reported here for the first time.

2. Experimental

2.1. Materials and methods

Chamaechromone was isolated and purified from the dried roots of Stellera chamaejasme in our laboratory, and the structure was confirmed by MS, 13C- and 1H NMR as described in the literature (Niwa et al., 1984). Trisodium isotiocarboxylic acid, isotiocarboxylic dehydrogenase, β-NADP and its reduced form (β-NADPH), UDP-glucuronic acid (UDPGA), and β-glucuronidase were purchased from Sigma-Aldrich (St. Louis, MO, USA), S-Adenosyl-L-methionine p-toluene sulfonate salt was obtained from Aladdin Reagent (Shanghai, China) (purity > 80%). Calcium chloride, epsom salt, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium bicarbonate, and sodium chloride were supplied by Sinopharm Chemical Reagent (Beijing, China). Tryptone, yeast extract and cysteine hydrochlorate were obtained from BBI (Canada). Solutol-HS15 was obtained Sigma Chemical Co. (St. Louis, MO). Phenylpropionic acid and phenylpropenol were obtained from Zhejiang Institute for Food and Drug Control (Hangzhou, China). HPLC grade acetonitrile, methanol and formic acid were purchased from TEDIA Inc. (Fairfield, USA). Ultra-pure water (18.2 MΩ) was obtained fromman ELGA-purelab Ultra system (HighWycombe, UK). All other chemicals were from standard commercial sources and were of the highest quality.

Male Sprague-Dawley rats (200–220 g) were obtained from the Animal Center of Zhejiang Academy of Medical Sciences (Hangzhou, China). Animals were bred in a breeding room with temperature at 25 °C, humidity of 50 ± 10%, and a 12 h dark–light cycle. They had free access to water and rodent chow all the time. All the experiment animals were housed under the above conditions for one week for acclimation, and were fasted overnight before the experiments.

2.2. Metabolites in vivo

Twenty-four rats were divided into four groups at random. The oral and injection doses of chamaechromone in rats were confirmed by the quality standard of Stellera chamaejasme (Zhuo et al., 2008), the dose of the extract in rat (Zhang et al., 2003, 2004), and its absolute bioavailability (Lou et al., 2011). The rats were fasted for the first 2 h with free access to water after dosing. Two groups were administered 100 mg kg–1 chamaechromone by oral administration after an overnight fasting period. After administration, the urine and feces samples were collected using a metabolic cage from one group and bile samples were collected via the bile duct catheter immediately from another group for over 24 h. And the other two groups were intravenous injection of 5 mg kg–1 via the tail vein or left untreated as a control group. The blank and the drug-containing urine and feces samples were collected during 0–24 h, respectively. The powder of chamaechromone was dissolved in Solutol-HS15 and then diluted with water to get 8.5 mg mL–1 solution for oral administration. And the drug for intravenous administration was formulated by dissolving chamaechromone with a Solutol-HS15 – physiologic saline (1:3, v-v) and mixed well. Rats were euthanized with CO2 after completion of the studies. All samples were stored at –80 °C until analysis. All experimental procedures were approved by the Ethics Committee of Zhejiang University.

2.2.1. Urine, fecal and bile samples preparation for UPLC/Xevo G2 Q-TOF MS analysis

Urine sample (1 mL) was extracted with ethyl acetate (1 mL) by vortex-mixing for 5 min after filtration. The supernatant was transferred into another test tube and evaporated to dryness with vacuum at room temperature. Finally, the residue was reconstituted in 100 μL incipient mobile phase by vortex-mixing for 4 min and centrifuged at 13,000 rpm for 20 min to await analysis. The fecal samples were twice extracted ultrasonically with MeOH (3 mL·g–1) for 30 min. The combined MeOH extracts were concentrated to nearly 1.0 mL in vacuum and centrifuged at 13,000 rpm for 20 min and the supernatant stored at –80 °C until analysis. The bile sample was prepared similarly as urine sample except that the extraction solution was MeOH (1:1, v-v).

2.3. Metabolites in vitro

2.3.1. Preparation of rat liver S9 fraction

The liver S9 fraction was prepared, as described in the literature (Bi et al., 2008). Male rats were sacrificed by cervical dislocation after fasting for 12 h, and livers were perfused with ice-cold normal saline and homogenized in four volumes of ice-cold phosphate buffer. The homogenate was centrifuged (9000 g, 10 min) at 4 °C to remove cell debris, mitochondria and nuclei. The resultant supernatant (S9 fraction) was stored at –80 °C until use. Protein concentration was determined by the modified
method (Yu et al., 2010), using bovine serum albumin as the standard.

2.3.2. Phase I metabolism of chamaechromone by rat liver S9 fraction

The phase I metabolism of chamaechromone in rat liver S9 fraction was conducted using the method described by Zhang et al. (2010b), except that rat liver microsome was replaced by rat liver S9. In brief, 100 μL of mixture containing 0.1 M Tris–HCl (pH 7.4), 15 mM MgCl₂, the NADPH – generating system, rat liver S9 fraction (1.0 mg mL⁻¹), and 100 μM chamaechromone was preincubated at 37 °C for 3 min. And the reaction was initiated by adding NADP⁺ and NADPH. The reaction was performed at 37 °C and stopped after 30 min by the addition of 300 μL of ice-cold methanol. After centrifugation (13,000 rpm, 10 min), the supernatant was analyzed by UPLC/Xevo G2 Q-TOF MS. Control incubations with inactive liver S9 fraction or without cofactors were performed in parallel.

2.3.3. Phase II metabolism of chamaechromone by rat liver S9 fraction

Glucuronidation was investigated by incubating rat liver S9 fraction protein (1.0 mg mL⁻¹) at 37 °C for 60 min in a medium (100 μL) containing 0.1 M K₂HPO₄ buffer, 112.5 μg mL⁻¹ Triton X – 100, 10 mM MgCl₂, 50 mM Tris–HCl (pH 7.4), and 100 μM chamaechromone. Then 4 μL of UDPGA solution was added to reaction mixture. The reaction was terminated by addition of 300 μL of ice-cold methanol. After centrifugation, the supernatant was analyzed by UPLC/Xevo G2 Q-TOF MS. Control incubations with inactive S9 fraction or without cofactors were performed in parallel.

2.3.4. Bile sample β-glucuronidase Hydrolysis

β-Glucuronidase assay was performed as follows: 1000 units of β-glucuronidase in KH₂PO₄ buffer (0.1 M, pH 5.0) were added to the bile samples which were then incubated for 37 °C for 2 h. And the control part without β-glucuronidase was treated in parallel. After treatment, 300 μL of ice-cold methanol was added to both parts. After centrifugation, the supernatant was for analysis.

2.3.5. Methylation of chamaechromone in rat liver S9 fraction with or without catechol-O-methyltransferase inhibitor

The reaction mixture containing rat liver S9 fraction protein (1.0 mg mL⁻¹), 0.1 M Tris–HCl (pH 7.4), 15 mM MgCl₂, 12 mM DL–isocitrate trisodium, 0.08 unit of isocitrate dehydrogenase, 1.0 mM EGTA, and 100 μM chamaechromone, with or without 30 nM entacapone (catechol-O-methyltransferase (COMT) inhibitor) (Chen et al., 2011) in 150 μL of phosphate buffer (5 mM, pH 7.8), was preincubated for 3 min at 37 °C, and then 2 μL of NADP⁺/NADPH solution was added to each reaction mixture. After incubation at 37 °C for 30 min, 1.5 μL of 25 mM S-adenosylmethionine dissolved in phosphate buffer (5 mM, pH 7.8) was added to initiate the reaction. Reactions were terminated by adding 150 μL of ice-cold methanol after incubation for 90 min at 37 °C. The mixture was centrifuged (13,000 rpm, 10 min), and the supernatant was analyzed by the UPLC/Xevo G2 Q-TOF MS.

2.3.6. Preparation of intestinal bacteria in vitro

All anaerobic incubations were conducted in an anaerobic jar (BioMérieux, Marcy l’Etoile, France) containing two BioMérieux GENbox Anaer small bags (bioMérieux Marcy l’Etoile, France). And the atmosphere of the jar was made anaerobic by using two packages of GENbox anaer. Next, the jar was removed to incubator (Thermo, USA) and the temperature was set at 37 °C. Fresh stools (0.5 g) were suspended in 20 volumes of sterile physiological saline in a cooled tube and centrifuged at 2000 rpm for 10 min. The supernatant was added with 18 volumes of anaerobic medium, cultivated overnight in anaerobic incubator. The anaerobic medium was modified PY medium contained 20 mg mL⁻¹ tryptone, 10 mg mL⁻¹ yeast extract, 0.5 mg mL⁻¹ cysteine hydrochloride, 40 mL⁻¹ VPI salt solution, pH 7.2. The VPI salt solution was consisted of 0.2 mg mL⁻¹ calcium chloride, 0.2 mg mL⁻¹ epsom salt, 1 mg mL⁻¹ dipotassium hydrogen phosphate, 1 mg mL⁻¹ potassium dihydrogen phosphate, 10 mg mL⁻¹ sodium bicarbonate, 2 mg mL⁻¹ sodium chloride. All procedures were performed at 4 °C.

2.3.7. Incubation of chamaechromone in rat intestinal bacteria

Chamaechromone (final concentration 100 μM) were added to the overnight cultivated intestinal bacteria cultivated in the anaerobic jar. Incubation in the absence of intestinal bacteria was used as negative control. The incubation mixture was incubated for 0, 1, 2, 4, 8 and 24 h, collected and frozen immediately to stop the reaction. The sample was centrifuged (13,000 rpm, 10 min), and the supernatant were analyzed by the UPLC/Xevo G2 Q-TOF MS.

2.4. Instrumentation and conditions

The Xevo G2 Q-TOF mass spectrometer (Waters, Manchester, UK) was connected to the ACQUITY UPLC system (Waters, Milford, MA, USA) via an electrospray ionization (ESI) interface. Chromatographic separation of metabolites of chamaechromone was performed on the ACQUITY UPLC with a conditioned autosampler at 4 °C, using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters, Milford, MA, USA). The column temperature was maintained at 40 °C. The mobile phase consisting of water with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) was pumped at a flow rate of 0.4 mL min⁻¹. The gradient elution program was as follows: 0–0.94 min, 2% B; 0.94–9.37 min, 2–70% B; 9.37–10.00 min, 70–100% B; 10.00–11.87 min, 100% B; 12.5–15.00 min, 2% B for equilibration of the column. The injection volume was 5 μL.

The Xevo G2 Q-TOF mass spectrometer was used in positive ESI mode for data acquisition using UPLC/MS², which allows both precursor and product ion data to be acquired in one injection. Typical source conditions for maximum intensity of precursor ions were as follows: capillary voltage, 3.0 kV; sample cone, 30 V; extraction cone, 4.0 V; source temperature, 120 °C; desolvation temperature 450 °C; cone gas flow rate 50 L h⁻¹; desolvation gas (N₂) flow rate 800 L h⁻¹. All analyses were performed using the lockspray, which ensured accuracy and reproducibility. Leucine–enkephalin (5 ng mL⁻¹) was used as the lockmass generating a reference ion in positive mode at m/z 556.2771 and introduced by a lockspray at 10 μL min⁻¹ for accurate mass acquisition. Data acquisition was achieved using MS², which has two separate scan functions that are programmed with independent collision energies. Function 1 (low energy): 100–1000 mass-scan range; 0.3 s scan time; 0.014 s inter-scan time; 6 eV collision energy; Function 2 (high energy): 100–1000 mass-scan range; 0.3 s scan time; 0.014 s inter-scan time; collision energy ramp of 20–35 eV. Acquiring data in this manner provided for the collection of information of intact precursor ions as well as fragment ions.

2.5. Data processing

Data processing was performed using a Metabolynx XS program (Waters Corp., Milford, MA, USA), which relies on an expected metabolites list of potential biotransformation reactions (Yan et al., 2010). This software automates the detection and identification of metabolites by comparing the sample with the control to
eliminate endogenous interfering ions from the complex matrices. Comparison of fragment ion spectra between the parent compound and metabolites further aided in the identification of metabolite structures and site(s) of modifications in the parent molecule (Deng et al., 2011).

The key parameters were carefully modulated as follows: the maximum tolerance of the mass defect filter (MDF) was set at 5 mDa. In the window of the expected metabolites list, the parent compound alone was added, and the mass window was set to 0.01 mD. The unexpected metabolite chromatograms were created over the full acquisition mass range in the mass window of 10 amu. The peak detection was accomplished using the ApexTrack algorithm, and the threshold of the peak area was set to 0.5 unit.

3. Result

3.1. UPLC/Xevo G2 Q-TOF MS analysis of chamaechromone

The chromatographic and MS fragmentation behaviors of the parent drug were investigated by UPLC/Xevo G2 Q-TOF MS. The protonated chamaechromone (M0) at m/z 543 was eluted at a retention time of 6.30 min. For a better understanding of product ions of the metabolites, the fragmentation pattern of M0 was examined (Fig. 1A). Product ions at m/z 389.1025, 219.0293, 163.0395, 199.0795, and 191.0344 (100% abundance) were observed. The fragment ion at m/z 389 was generated by the cleavage of the C-C bond, followed by the loss of C10H6O4 (190 Da) to form the ion at m/z 199. And the fragment ion at m/z 163 came from the further loss of C14H10O3 (226 Da) from the fragment ion at 389. The formation of the ion at m/z 219 was the result of the loss C19H16O5 (324 Da) from the protonated molecular ion. This fragment ion could further loss CO (28 Da) to give the ion at m/z 191. According to the results obtained, the fragmentation pathways of M0 were proposed as shown in Fig. 1B. And the structure of chamaechromone was divided into parts A, B, and C (Fig. 1B). These fragment ions were used as references to aid interpretation of fragment ions of the metabolites, as well as to examine the high resolution and mass accuracy of the instrument.

3.2. Metabolic profile of chamaechromone

As shown in Fig. 2, a total of 24 metabolites of chamaechromone were detected in vivo and in vitro, and 20 metabolites (M1–M7, M9 and M10, M13–16, M18–M24) were new metabolites. Table 1 lists the detailed information of these metabolites, including the retention times, proposed elemental compositions, and the characteristic fragment ions. The structures of metabolites were characterized by mass spectral fragmentation patterns or confirmed by available reference or corresponding standards. On the basis of the metabolites identified, hydroxylation, methylation, glucuronation, acetylation, dehydroxylation and degradation were the main metabolic pathways of chamaechromone in rats. The possible pathways for the formation of all of the metabolites are shown in Figs. 3 and 4.

3.3. Identification of metabolites in vivo

Among them, 10 metabolites (M1–M10) were discovered in fecal sample after intravenous administration, and 2 metabolites

![Fig. 1. Mass spectrum of chamaechromone obtained on Q-TOF mass spectrometry at high collision energy (A) and tentative structures of the most informative fragment ions for chamaechromone (B).](image)
were detected in urine sample (M11 and M12). After oral administration, 8 metabolites were obtained in fecal sample (M5, M7–M10, and M13–M15), 3 metabolites were detected in urine sample (M11, M16 and M17) and 2 glucuronide conjugations were found in bile sample (M18 and M19).

3.3.1. Metabolites M1 and M2

M1 and M2 were eluted at retention times of 6.34 and 6.48 min, respectively. Both metabolites showed a protonated molecular ion at \( m/z \) 573, which was 30 Da higher than that of \( m/z \) 543, indicating that they were hydroxylation and methylation metabolites. The fragment ion of M1 at \( m/z \) 543 was formed by the loss of CH2O (30 Da) from the precursor ion (\( m/z \) 573), which further confirmed the above conclusion. The fragment ion at \( m/z \) 229 lost a CH2O to form the ion at \( m/z \) 199. In addition, M1 showed the same fragment ion at \( m/z \) 219 as M0, revealing that the part B was intact. The fragment ions of M2 were the same as those of M1, suggesting that the metabolites M1 and M2 were isomers of each other and the hydroxylation and methylation must occur on the part C. Moreover, the results of metabolism in rat liver S9 fraction suggested that the methylation was mediated by COMT. On the basis of the structure, the substrate of COMT must has a catechol ring (Yan et al., 2010). Accordingly, the site of hydroxylation was
Table 1  

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Description</th>
<th>Retention time (min)</th>
<th>Formula</th>
<th>Measured mass [M+H]+</th>
<th>Calculated mass [M+H]+</th>
<th>Fragment ions</th>
<th>After oral administration</th>
<th>After IV administration</th>
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<tr>
<td>M0</td>
<td>Parent</td>
<td>6.30</td>
<td>C_{30}H_{22}O_{10}</td>
<td>543.1292</td>
<td>543.1291</td>
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<td>No</td>
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<tr>
<td>M1</td>
<td>Hydroxylation + methylation</td>
<td>6.34</td>
<td>C_{31}H_{23}O_{11}</td>
<td>573.1381</td>
<td>573.1397</td>
<td>543, 229, 219, 199</td>
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<td>No</td>
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<tr>
<td>M2</td>
<td>Hydroxylation + methylation</td>
<td>6.48</td>
<td>C_{31}H_{23}O_{11}</td>
<td>573.1361</td>
<td>573.1397</td>
<td>543, 229, 219, 199</td>
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<td>C_{30}H_{22}O_{11}</td>
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<td>559.1240</td>
<td>215, 219, 199</td>
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<td>559.1240</td>
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<td>541.1499</td>
<td>199, 219, 191, 163</td>
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<td>Degradation</td>
<td>5.36</td>
<td>C_{20}H_{18}O_{2}</td>
<td>435.094</td>
<td>435.088</td>
<td>219, 199</td>
<td>Yes</td>
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<td>Degradation</td>
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<td>C_{20}H_{18}O_{2}</td>
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<td>C_{31}H_{24}O_{10}</td>
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<td>557.1448</td>
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<td>163, 101, 219, 209</td>
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<td>Degradation</td>
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<td>C_{17}H_{12}O_{2}</td>
<td>135.0811</td>
<td>135.0810</td>
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<td>719.1612</td>
<td>543, 521, 345, 219, 199</td>
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<td>C_{31}H_{24}O_{10}</td>
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<td>543, 521, 219, 199</td>
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<tr>
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<td>371, 191, 163, 153</td>
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occurred on position C-22 or C-28 of the benzyl in part C. On the basis of the above analysis, M1 and M2 were identified as 22/28-hydroxy, 23/29-methoxyl chamaechromone, or 22/28-methoxyl chamaechromone, respectively.

3.3.2. Metabolites M3–M6
Metabolites M3–M6 were eluted at retention times of 5.93, 5.96, 6.08 and 6.18 min, respectively. All metabolites showed a protonated molecular ion at m/z 559, which was 16 Da higher than that of m/z 543, suggesting the addition of one oxygen atom. The major fragment ion of M3 was m/z 215, which was 16 Da higher than the fragment ion at m/z 199 of the parent compound, implying the modification was occurred in the part C. Then the fragment ion at m/z 215 lost an oxygen atom (16 Da) to form the fragment ion at m/z 199. Moreover, the fragment ion at m/z 219 indicated the part B was intact. The metabolite M4 had the same fragment ions at m/z 219, 215 and 199 as M3, suggesting that M4 was another hydroxylation of M0 and the two metabolites were isomers of each other. Based on these observations, M3 and M4 were identified as the ortho- (o-) or meta- (m-) position of the hydroxy group left could not be characterized. M5 and M6 had the same fragment ions at m/z 199 and 219 as M0, implying M5 and M6 were the hydroxylation metabolites of M0. However, the exact site of hydroxylation could not be characterized.

3.3.3. Metabolite M7
The metabolite M7 showed an UPLC profile with a retention time at 6.51 min. And the protonated molecular ion at m/z 557 was 14 Da higher than M0 indicating the introduction of methyl group into the parent molecule. The major fragment ions of M7 were m/z 543 and 199. The fragment ion at m/z 543 due to 14 Da lost, illustrating that the methylation occurred. And the remaining fragment m/z 199 indicated that part C was intact. In view of the analysis, M7 was identified as the methylation of M0, and the methylation occurred on part A or part B.

3.3.4. Metabolites M8 and M9
Metabolite M8 had a retention time of 6.46 min. And it had a protonated molecular weight of 527, which was 16 Da lower than that of protonated M0. The elemental composition of C_{30}H_{22}O_{9}...
indicated an oxygen atom had been lost from the parent drug. M8 was consequently proposed as a dehydroxylation metabolite. The fragment at \( m/z \) 199 suggested that the site of dehydroxylation was not on part C. M8 was proposed to be dehydroxylation metabolite of M0.

Metabolite M9 had a retention time of 6.93 min, and exhibited a protonated molecule at \( m/z \) 541. And it had the elemental composition of \( \text{C}_{31}\text{H}_{24}\text{O}_{9} \), suggesting the addition of \( \text{CH}_2 \) and loss of one oxygen atom from M0. M9 was consequently proposed as a dehydroxylation and methylation of chamaechromone. The characteristic fragment ion at \( m/z \) 199 was the same as that of the parent drug, indicating that part C was intact. And the fragment ions at \( m/z \) 219, 191 and 163 originated from modified parts A and B. M9 was proposed to be dehydroxylation and methylation metabolite of M0 and the modifications occurred on part A or B.

3.3.6. Metabolite M11

Metabolite M11 had a retention time of 6.34 min and exhibited the protonated ion at \( m/z \) 165. The elemental composition of \( \text{C}_{9}\text{H}_8\text{O}_3 \) suggested that it was a degradation metabolite of M0. The high energy mass spectrum of M10 showed the fragment ion at \( m/z \) 219 which was formed by the protonated molecular ion at \( m/z \) 435 lost a \( \text{C}_{13}\text{H}_{12}\text{O}_2 \) (part C) and O radical. In addition, the major fragment ion at \( m/z \) 199 was identical to the fragment ion of M0 and lophiraic acid (Murakami et al., 1991). Comparing the elemental composition of M10 with that of lophiraic acid indicated that an oxygen atom had been added to the lophiraic acid. According to the results obtained, M10 was tentatively identified as 5-hydroxyl lophiraic acid.

![Proposed metabolic pathways of M0 in vitro.](image)
3.3.7. Metabolite M12

Metabolite M12 was eluted at a retention time of 6.62 min and showed the protonated ion at \( m/z \) 151, which was 14 Da lower compared with the protonated molecule of M11 (\( m/z \) 165). Fragment ions of M12 were \( m/z \) 135 and 107, which were 14 Da lower than the fragment ions at \( m/z \) 149 and 121 of M11, respectively. Considering the elemental composition of M12, this metabolite was the addition of two hydration atoms (2H) and loss of one oxygen atom (O) from M11. In accordance with the data on the retention time and characteristic fragment ions of the commercially available corresponding standards, M12 was identified as phenylpropionic acid.

3.3.8. Metabolite M13

Metabolites M13 was eluted at 6.54 min, and had a precursor ion at \( m/z \) 557, 14 Da higher than M0 (\( m/z \) 543). M13 had the same protonated molecular ion and fragment ions (\( m/z \) 543 and 199) as M7, indicating that the metabolites M13 and M7 were isomers of each other. In view of the analysis, M13 was identified as the methylation of M0.

3.3.9. Metabolites M14 and M15

Metabolites M14 and M15 were eluted at the retention times of 6.57 and 7.88 min, respectively. Both metabolites displayed the same protonated molecular ion at \( m/z \) 569, which was 26 Da (C\( _2 \)H\( _2 \)) higher than that of the protonated M0 at \( m/z \) 543. This suggested the addition of acetyl group (CO\( \text{CH}_3 \)) and loss of one oxygen atom (O) from M0. The high energy mass spectrum of the protonated molecular obtained from M14 provided the major fragment ion at \( m/z \) 555, which were 14 Da (CH\( _2 \)) lower than protonated molecular ion at \( m/z \) 569. This fragment ion was further lost the C\( _7 \)H\( _6 \)O\( _2 \) (part C) to form the fragment ion at \( m/z \) 357. The fragment ion at \( m/z \) 369 was acquired by the loss of C\( _7 \)H\( _7 \)O\( _2 \) (part C) from the fragment ion at \( m/z \) 569. In addition, the fragment ions at \( m/z \) 219, 191 were generated by the loss of C\( \text{H}_3 \)\( \text{O}_2 \) (150 Da) and C\( \text{H}_2 \)\( \text{O}_4 \) (178 Da) from the fragment ion at \( m/z \) 369, respectively. Based on these observations, the sites of acetylation and dehydroxylation were not on part C. This conclusion was further confirmed by the characteristic fragment at \( m/z \) 199.

The metabolite M15 had the same fragment ions as M14 at \( m/z \) 219, 191, and 191. Moreover, the formation of the fragment ion at \( m/z \) 371 of M15 was due to the loss of part C (C\( _7 \)H\( _7 \)O\( _2 \)) from the precursor ion at \( m/z \) 569. Therefore, the metabolites M14 and M15 were isomers of each other. And they were identified as acetylation and dehydroxylation metabolites and the modifications occurred in part A or part B.

3.3.10. Metabolite M16

The metabolite M16 was detected with the retention time at 5.86 min and exhibited the protonated ion at \( m/z \) 345, indicating the molecular formula was C\( _{17} \)H\( _{12} \)O\( _8 \). The high energy mass spectrum of M16 showed fragment ions at \( m/z \) 191, 219, 163 and 209. The fragment ion at \( m/z \) 191 of M16 was formed by the loss of C\( \text{H}_3 \)\( \text{O}_4 \) (154 Da) from the precursor ion, followed by the loss of CO (28 Da) to form the ion at \( m/z \) 163. The fragment ion at \( m/z \) 219 was generated by the loss of C\( _{6} \)H\( _{6} \)O\( _3 \) (126 Da) from the precursor ion at \( m/z \) 345. Moreover, the fragment ion at \( m/z \) 209 was formed by the loss of C\( _{6} \)H\( _{6} \)O\( _3 \) (136 Da) from the precursor ion at \( m/z \) 345.

The major fragment ions were the same as that of the parent drug, whereas the fragment ion at \( m/z \) 199 was absent, indicating the loss of the part C. Accordingly, M16 was thus identified as the degradation metabolite of M0 resulting from the removal of the part C and named as debiphenyl chamaechromone.

3.3.11. Metabolite M17

Metabolite M17 was eluted at 8.39 min. And it showed a precursor ion at \( m/z \) 135, which was 16 Da lower compared with the protonated molecule of M12 (\( m/z \) 151), revealing that the metabolite was the reduced metabolite of M12. Considering that the difference in elemental composition between M17 and M12 was an oxygen atom, we propose that M17 underwent deoxidation from M12. The fragment ion at \( m/z \) 119 of M17 was due to the loss of O from the precursor ion at \( m/z \) 135. And the fragment ion at \( m/z \) 105 was generated by the loss of CH\( _2 \)O (30 Da) from the ion at \( m/z \) 135. Accordingly, M17 was identified as phenylpropenol by comparison their mass and mass fragmentation patterns to reference standards.

3.3.12. Metabolites M18 and M19

Metabolites M18 and M19 were eluted at retention times of 5.64 and 5.94 min, respectively. Both metabolites showed a protonated molecular ion at \( m/z \) 719. And the elemental composition of this metabolite was C\( _{30} \)H\( _{24} \)O\( _{16} \), corresponding to the monogluconuride conjugate of M0. The fragment ion at \( m/z \) 543 was due to a neutral loss of 176 Da. The major fragment ion at \( m/z \) 521 was formed by the loss of C\( _{17} \)H\( _{14} \)O\( _3 \) (part C) from the protonated molecular ion, followed by the loss of glucuronic acid (176 Da) to generate the fragment ion at \( m/z \) 345, and then lost C\( _{6} \)H\( _{6} \)O\( _3 \) (126 Da) to form fragment ion at \( m/z \) 219. In addition, the major fragment ion at \( m/z \) 199 was identical to the fragment ion of M0, which also revealed that the site of glucuronidation was not on part C.

M19 had the same fragment ions as M18, and both of them were monogluconuride conjugates of M0 indicating that the two metabolites were isomers.

3.4. Identification of metabolites in vitro

3.4.1. Metabolism of chamaechromone in rat liver S9 fraction

Compared with the control sample, 4 oxidative metabolites (M3–M6) were obtained in Phase I metabolism of chamaechromone. And 2 oxidative and methylated metabolites (M1 and M2) were detected in methylation of chamaechromone by rat liver S9 fraction. In addition, 4 monogluconuride conjugates of M0 (M18–M21) were detected in Phase II metabolism of chamaechromone.

3.4.1.1. Metabolites M20 and M21

Another two metabolites were detected in rat liver S9 fraction. The two chromatographic peaks (M20 and M21) that eluted at the retention times of 5.61 and 5.82 min, respectively, and both of them exhibited the same protonated ion at \( m/z \) 719. The characteristic neutral losses of 176 Da were found in the high energy mass spectrum of both M20 and M21, which indicated the presence of glucuronide residues on the structures. Their different retention times on the UPLC system indicated different positions of glucuronation. M20 and M21 had the same fragment ions as M18 and M19, indicating that they were isomers of each other. The observed formation of M18–M21 indicated that an isomerization took place in the conjugation reaction.

3.4.2. Metabolism of chamaechromone by intestinal microflora

By a comprehensive analysis of the peaks from the incubated sample, 12 metabolites were detected in rat intestinal microflora incubate compared with blank sample. And three acetylation and dehydroxylation metabolites (M22–M24) were detected in vitro, which were not observed in vivo. The results indicated that methylation (M7 and M13), dehydroxylation (M8), dehydroxylation and methylation (M9), acetylation and dehydroxylation (M14,
M15, and M22–M24) and degradation (M16 and M17) were the major metabolic pathways of M0 by intestinal microflora in vitro.

3.4.2.1. Metabolites M22, M23, and M24. At retention times of 7.54, 7.71 and 8.31 min, M22, M23 and M24 were detected with the protonated molecule at m/z 569, implying that they were acetylation and dehydroxylation metabolites of M0. The major fragment ions of M22 and M23 were the same as M14 at m/z 369, 357, 219, 199, and 191, illustrating that the three metabolites were isomers, and the acetylation and dehydroxylation maybe occur at the similar positions.

The metabolite M24 had the same fragment ions at m/z 371, 191 and 163 as M15, suggesting that the two metabolites were isomers of each other and with modifications at the similar positions.

4. Discussion

Previous studies have shown the biflavone had a poor absorption through the gut barrier and a limited metabolism by the intestinal microflora as compared to its monomeric flavone. Studies with rats fed biflavone had reported the bioavailability is low and characterized by a low urinary recovery (Baba et al., 2002; Tsang et al., 2005; Shoji et al., 2006). And although methylated form had been observed (Shoji et al., 2006; Rezppa et al., 2012), there are no reports of glucuronidated or sulfated metabolites in vivo. However, glucuronidation and methylation of procyanidins B2 and B2G2 occurred but were minor processes in vitro (Shrestha et al., 2012). The methylation of parent drug was catalyzed by the enzyme catechol-O-methyltransferase (COMT) (Weinert et al., 2012). There is also evidence for the flavonoids, especially flavone dimers and/or other related higher molecular weight oligomers were transformed into various aromatic acid by intestinal microflora (Xue et al., 2011; Stoupi et al., 2010; Mattheis et al., 2012; Takagaki and Nanjo, 2010).

In this study, chamaechromone may undergo extensive modification before initial absorption or after biliary excretion before reabsorption, further metabolism, possible degradation, and ultimate excretion in rat.

The metabolites and unchanged chamaechromone were excreted more through feces than urine. Hydroxylation and methylation were the main metabolic pathways of chamaechromone in liver after intravenous administration (M1 and M2). A possible mechanism for the formation of M1 and M2 could involve hydroxylation of the M0 by cytochrome P450 to form the catechol ring intermediate, the substrate of catechol-O-methyltransferase (COMT) (Chen et al., 2011), followed by COMT to produce the major metabolites (M1 and M2). This hypothesis was verified by in vitro experiments using rat liver S9. After mediated by COMT, two metabolites (M1 and M2) were also obtained in rat liver S9 incubate. Furthermore, entacapone, an inhibitor of COMT, was incubated with rat liver S9, and the result showed that entacapone almost completely inhibited the formation of metabolite M1 and M2. Together with the mass spectral data, the hydroxylation sites of M1 and M2 were proposed to be on C-22 or C-28 in part C. In addition, methylation occurred in the part A or B was a minor metabolic pathway in rat feces.

Glucuronide conjugates, because of their large molecular weight and relative hydrophilicity, underwent biliary excretion in the rat and enterohepatic recirculation. Indeed, two glucuronide conjugates (M18 and M19) were found in rat bile samples. After incubation with rat liver S9 fraction in the presence of UDPGA, 4 glucuronide conjugates (M18–M21) were detected. M18 and M19 were obtained in both in vivo and in vitro. The metabolites were verified by enzymatic hydrolysis. After incubation, the peak intensity of metabolites M18–M21 decreased obviously and, meanwhile, the appearance of M0 (m/z 543) with the retention time at 6.30 min was detected. Other metabolic processes lead to the production of acetylated, dehydroxylated forms of chamaechromone. Moreover, the parent form was detected in rat feces while no chamaechromone was observed in urine samples within 24 h.

Modifications of chamaechromone also occurred in the colon where the resident microflora degrade them to smaller phenolic acids. Using UPLC/Xevo G2 Q-TOF as an analytical tool, we observed several degradation metabolites of M0 in rat (M11, M16 and M17) after oral administration. The in vitro anaerobic metabolism of chamaechromone by fecal suspensions resulted in their partial degradation metabolites (M16 and M17). Several previous researches showed that isoflavones had a higher susceptibility to cleave the C-ring and give the biphenyl-ketone derivatives through the microbial transformation (Hur et al., 2002). Similarly, M16 was biodegraded to M17 through the intermediates of the C-ring reduced metabolites by intestinal microflora. Moreover, methylation, dehydroxylation and acetylation reactions which represented phase II drug biotransformations were also recognized in vitro incubation.

5. Conclusion

It is important to characterize the metabolic profile of the constituent of traditional Chinese medicine. But this process is often challenging and time-consuming. The present study explored a rapid, sensitivity and selectivity method for identifying the metabolites in a single sample run by a Metabolynxs program using UPLC/Xevo G2 Q-TOF. And the present study revealed chamaechromone could undergo extensive phase I and phase II metabolism. The possible metabolic mechanism was verified by in vitro experiments. In conclusion, this study has increased our knowledge of the whole metabolic pathways of chamaechromone in rat, and these findings would provide an important basis for further study and clinical application.

Acknowledgments

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References


