Research Article
Chemical Fingerprint and Metabolic Profile Analysis of Tianshu Tablets by Ultra-High Performance Liquid Chromatography/Quadrupole-Time of Flight Mass Spectrometry

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In recent years, the chemical fingerprinting of traditional Chinese medicines and the metabolites in these compounds has been a hot topic. In the present study, the chemical fingerprint of Tianshu tablets (TST) and the metabolic characteristics of compounds in rats after intragastric administration were studied by ultra-high performance liquid chromatography coupled with quadrupole-time of flight mass spectrometry (UPLC/Q-TOFMS). In a preliminary study, 77 chemical components in TST were determined by comparison with retention times, accurate molecular mass, and characteristic fragment ions of the known compounds in the literature and some well-known compounds were analyzed in detail, and the fragmentation pathways for parishins B, gastrodin A, and cnidilide or neocnilide were specifically analyzed. After intragastric administration of TST (4g/kg) to rats, a total of 61 compounds were detected in plasma samples, including 7 prototypes and 54 metabolites. After further analysis, it was found that these metabolites were subjected to glucuronidation, sulfation, methylation, hydroxylation, dehydrogenation, or mixed metabolic processes. Hydroxylation and glucuronidation were finally confirmed as the main metabolic pathways. This is the first research on the chemical fingerprint and metabolites of TST, which lays a foundation for further investigation of TST.

1. Introduction

In recent years, traditional Chinese medicine (TCM) has attracted increasing attention worldwide by virtue of its applications. Da Chuanxiong Formula (DCXF) is a well-known and extensively used traditional Chinese medicine (TCM) decoction for the treatment of migraine caused by blood stasis and wind-heat syndrome. It is composed of two herbs, namely, Chuanxiong (Chuanxiong rhizoma) and Tianma (Gastrodiae rhizoma), with a crude weight ratio of 4:1. Tianshu tablets (TST) are a representative DCXF preparation that is widely used in clinics for treating the blood stasis type of headache and migraine [1–3].

Phytochemical and pharmacological investigations of DCXF have shown that phenols, organic acids, phthalides, and nitrogen-containing compounds are the major active ingredients [4]. At present, several qualitative studies on the main components of DCXF have been performed [5–8]. One study used LC-Q-TOF/MS to identify 17 different components in a 50% ethanol extract of DCXF [5]. In one study, three compounds of Chuanxiong and eight components of Tianma were identified by HPLC-DAD-MSn [6]. Two continuous studies showed that 10 different compounds were detected in rat plasma after intragastric administration of DCXF active components, including 6 compounds from Chuanxiong and 4 compounds from Tianma [7, 8]. These four studies were based on samples of a 50% ethanol extract from a 4:1 mixture of the two herbs or active ingredients from a single crude herb. In one study, 38 components were identified or preliminarily identified from a Tianshu capsule...
by means of HPLC, LC-DAD-MSN, and LC-DAD-ESI IT-TOF/MS analysis, although Tianshu tablets and Tianshu capsules are two different dosage forms [9]. This research is still very important because of its different applicability. This study enriches our understanding of the components of DCXF and studies the metabolites of TST for the first time.

In our study, 77 chemical components of TST were preliminarily determined by a comparison with retention time, accurate molecular mass, and characteristic fragment ions of known compounds in the literature. Furthermore, UPLC/Q-TOF MS was used to analyze the plasma of rats after oral administration of TST. A total of 61 compounds were identified or preliminarily identified, including 7 prototypes and 54 metabolites.

2. Experimental

2.1. Chemicals and Materials. Some reference standards (pyroglutamic acid, 5-(hydroxymethyl)furoic acid, and parishin B) for *Gastrodia rhizoma* were isolated and purified in our laboratory, and other standards (uridine, gastrodin A, and neocnilide) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Purities of the standards were above 98% by HPLC analysis. HPLC-grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (MA, USA). Deionized water was purchased by a Milli-Q Water purification system (Millipore, MA, USA). High-purity nitrogen (99.99%) and helium (99.999%) were purchased from Gas Supplies Center of Peking University Health Science Center (Beijing, China).

*Gastrodia rhizoma* and *Chuanxiong rhizoma* were purchased from Tian Heng pharmacy (Beijing, China). All herbal materials were authenticated by Professor Bei Wu (Nanchang Institute for Food and Drug Control). TianShu tablets were prepared according to Chinese Pharmacopoeia 2015 Edition [1].

2.2. Animals and Drug. Sprague–Dawley rats (male, 12–14 weeks; 200–240 g) were provided by Hunan SJA Laboratory Animal Co., Ltd. Protocols for all animal experiments were approved. Animals were kept in a controlled environment for 3 days and fasted for 12 h before experiments. TST was dissolved in a 9 g/L NaCl solution (NS) (250 mg/ml) and administered by oral gavage at a dose of 1000 mg/kg (equivalent to 4 g of crude drug per kg) body weight.

2.3. Sample Collection and Pretreatment. After oral administration of TST, blood samples were collected at 30, 60, and 120 min (n = 5) in an Eppendorf tube with heparin sodium and then centrifuged (16000 rpm) at 4°C for 10 min. The supernatant was then separated, and all samples were stored at −80°C immediately until analysis. The protocol for sample preparation is described below: 1 mL plasma was mixed with 5 mL methanol, vortexed for 5 min, and centrifuged at 16000 rpm at 4°C for 20 min. The supernatant was then centrifuged at 16000 rpm for 10 min at 4°C. The supernatant was transferred to a vial, and 10 μL was injected for LC-MS analysis. All samples were filtered through a membrane (0.22 μm pore size). At the same time, in order to eliminate the influence of matrix, blank plasma was added to participate in the analysis.

2.4. UPLC/QTOF-MS Conditions. UPLC/QTOF-MS analysis was performed on a Shimadzu LC-30 AD system (Kyoto, Japan) coupled with an AB SCIEX Triple-TOF 5600 mass spectrometer (Foster City, CA, USA). All samples were separated on an Acquity UPLC C18 column (100 mm × 2.1 mm, 1.7 μm, Waters, USA) with a flow rate of 0.3 mL/min at 40°C. The mobile phase consisted of aqueous 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The gradient elution program for TST was set at 0–3.0 min, 5–8% B; 3.0–10.0 min, 8–15% B; 10.0–18 min, 15–20% B; 18–22 min, 20–35% B; 22–37 min, 35–45% B; 37–43 min, 45–95% B; and 43–48 min, 95–95% B. The gradient elution program for the plasma samples was set at 0–2.0 min, 5% B; 2.0–25.0 min, 5–95% B; and 25.0–30.0 min, 95–95%. The equilibration time was 5 min. The conditions for the ion source were as follows: compounds in TST were measured using the total ion chromatograms in negative and positive ion ESI-MS mode in the mass range m/z 50–1250, but plasma samples were analyzed only in positive ion ESI-MS mode. The other operating parameters were optimized as follows: source temperature, 500°C; ion spray voltage, 4500 V; gas 1, 50 psi; gas 2, 50 psi; curtain gas, 45 psi; decluttering potential, 100 V; and collision energy was set to 40 (15) eV.

2.5. Data Process. TST compounds from the extracts and metabolites data were acquired by full scan, which rely on dynamic background subtraction (DBS) and multiple mass defect filtering (MMDF) and includes some compounds with very low concentrations (MDF window was set to ±50 mDa around the mass defects of the templates and over a mass range of ±50 Da around the filter template masses).

Analysis of data on TST compounds in extracts and metabolites was performed using a variety of data mining tools, including extract ion chromatograms (XIC) of PeakView®1.6 (AB SCIEX, CA, USA), MMDF, and NLF&DPLs of Metabolitepilot™1.5 (AB SCIEX, Foster City, CA, USA). All compounds were analyzed after removal of the matrix effects.

3. Results and Discussion

3.1. Optimization of LC/MS Conditions. In order to obtain the best analytical data, our analysis builds upon another recent study [10]. The separation conditions, supplements, and chromatographic columns were optimized at the beginning of the experiment. Firstly, in order to obtain sharp peaks and reduce the pressure on the UPLC column, methanol was used as the mobile phase instead of acetonitrile. At the same time, 0.1% formic acid was added to improve peak shape and ionization of the analytes. The gradient was improved, and it was shown that the
compounds in TST could be separated within 48 minutes, while plasma samples could be separated within 30 minutes (the specific methods can be found in Section 2.4). In addition, in order to obtain the most abundant mass spectrometry information, the collision energy was optimized. The results showed that when the collision energy rose to 40 eV, the main fragments were seen, but when the energy reached 55 eV, the second order fragments were too fragmented to be easily analyzed. Therefore, a collision energy of 40 eV was selected. As for UPLC/Q-TOF MS, mass spectra were recorded in both positive and negative detection modes.

3.2. UPLC/Q-TOF MS Analysis of TST Extracts. To characterize the chemical constituents of TST, a fast, efficient, and reliable UPLC/Q-TOF MS method was established. By virtue of the high resolution and speed of UPLC and the accurate mass measurement of the TOF MS, a total of 77 compounds were identified. The mass spectra of these components were examined in negative ion mode and positive ion mode. The total ion chromatogram (TIC) of TST in positive and negative ion modes are shown in Figures 1 and 2. Details of the identified components are summarized in Table 1.

Through analysis, it was found that the 77 compounds contained 19 organic acids, 9 nitrogen-containing compounds, 11 glucosides, 8 phenols, 24 phthalides, and 6 other compounds. The numbering information of these compounds is shown in Figure 3.

3.2.1. Chemical Fingerprint of TST in Negative Ion Modes. According to the literature, the main components of Tianma are phenols and organic acids [9]. However, there are also glycosides in the components of Tianma [12]. Many characteristic components of Tianma were analyzed and identified in the negative ion mode. Because the structures of organic acids and phenols are relatively simple, the characteristic glycoside compounds X18 and X23 were identified here and the chromatographic and spectral data for compounds X18 and X23 were preliminarily characterized by referring to the literature and reference materials.

Peak X18 gave an [M−H]− ion at m/z 447.1508. Peak X18 produced MS2 base peaks at m/z 269.1028 and 161.0449 corresponding to [M-H-179 Da]−. This suggests that Peak X18 may contain a glucose group and a fructose group. Therefore, we deduced that the molecular structure likely contains sucrose. According to literature reports [12] and reference standards, we identified Peak X18 as gastrodin A. Peak X23 gave a [M-H]− ion at m/z 727.2091 and had characteristic fragment ions at m/z 459.1156, 441.1045, 423.0937, 397.1142, 369.1188, and 217.0496. Based on previous studies [12] and a reference standard, Peak X23 was identified as parishin B. The characteristic fragmentation patterns of gastrodin A and parishin B are described in Figures 4(a) and 4(b).

3.2.2. Chemical Fingerprint of TST in Positive Ion Modes. The analysis of the positive ion mode results showed that the characteristic components of Chuanxiong, including phthalides, were present. Here, compound Y27 was selected for analysis, and the chromatographic and spectral data of this compound were analyzed by comparison with the literature and reference materials. The cleavage pathway of phenyl peptides in Chuanxiong was also analyzed.

Peak Y27 gave a [M+H]+ ion at m/z 195.1378 and fragment ions at m/z 177.1344, 149.1309, and 107.0550. According to previous literature reports [9] and a reference standard, we identified peak Y27 as cnidilide or neocnidilide. The characteristic fragmentation pattern of Y27 is shown in Figure 4(c).

According to our analysis, the main components of Tianma in negative ion mode were organic acids, phenols, and glycosides, with mainly phthalides detected in positive ion mode. The specific pyrolysis fragments were similar to the standards.

3.3. Detection and Identification of the Metabolites of TST in Rat Plasma. In order to identify as many potential pharmacologically active compounds as possible in TST, metabolic profiling of TST in rat plasma was performed. Compounds absorbed in vivo can be further metabolized by a variety of enzymes through oxidation, hydrolyzation, methylation, glucuronidation, and sulfation. Only peaks that were detected in the dosed plasma samples but not in blank samples were considered as probable metabolites. The mass spectra of the metabolites were examined in positive ion mode. These were further analyzed by using Peakview 1.2 to identify expected and unexpected metabolites from different metabolic pathways, and their structures were identified by tandem MS. We selected senkyunolide D or 4,7-dihydroxy-3-butylphthalide and senkyunolide A as examples of the structural identification process. The metabolites of these compounds and others are summarized in Table 2, and their TIC and extract ion chromatogram (EICs) are shown in Figures 5–8.

Metabolite M1, which eluted at 9.75 min, formed a molecular ion of [M+H]+ at m/z 223.0963 corresponding to C12H14O4. M1 was found to have major fragment ions in common with senkyunolide D, so M1 is most likely senkyunolide D or 4,7-dihydroxy-3-butylphthalide. Metabolite M2, which eluted at 10.78 min, formed a molecular ion of [M+H]+ at m/z 253.1071 corresponding to C13H16O5. The characteristic production of m/z 235.0963 and 221.0839 was generated by loss of 18 Da and 18 + 14 Da, which implied loss of a H2O group and methyl group. Other product ions were identical to that of M1. Therefore, M2 may be a metabolite of senkyunolide D after hydroxyl and methyl conjugation. Metabolite M3, which eluted at 9.2 min, formed a molecular ion of [M+H]+ at m/z 303.0535 corresponding to C12H13SO4. Its major fragment ions at m/z 285.0430 and 205.0858 were generated by the loss of 18 Da and 18 + 80 Da, which implied a H2O group and sulfate group. Other product ions were identical to that of M1. Therefore, M3 may be a metabolite of senkyunolide D following sulfation. Metabolite M4, which eluted at 10.6 min, formed a molecular ion of [M+H]+ at m/z 399.1285 corresponding to C18H22O10. Its major fragment ion (m/z 223.0969) was
generated by a loss of 176 Da, which implied a glucuronide group. Other product ions were identical to that of M1. Therefore, M4 might be a metabolite of senkyunolide D after glucuronidation. Metabolites M5 and M6 appear to correspond to M4 plus 2 Da or 16 Da, respectively. The product ions m/z 225.4427 and m/z 227.0584 have both lost 176 Da.
| Peak  | RT  (min) | Formula     | Error  (ppm) | [M–H] Calculated (m/z) Measure | Intensity | Product ions                                                                 | Identification                        | Structure class         | Ref. |
|-------|-----------|-------------|--------------|-------------------------------|-----------|------------------------------------------------------------------------------|---------------------------------------|-------------------------|------|
| X1a   | 0.98      | C₇H₁₂O₆    | −1.2         | 191.0561                      | 191.0599  | 86129                                                                        | Quinic acid                           | Organic acids           | [10] |
| X2    | 1.07      | C₄H₄O₂     | 0.9          | 191.0197                      | 191.0199  | 423157                                                                       | Citric acid                           | Organic acids           | [10] |
| X₃b   | 1.07      | C₇H₁₂N₂O₆  | −1           | 243.0623                      | 243.0620  | 5161                                                                         | Uridine                               | Nitrogen-containing compounds |     |
| X₄b   | 1.08      | C₃H₂NO₃    | 3            | 128.0353                      | 128.0357  | 24352                                                                       | Pyroglutamic acid                     | Organic acids           | [11] |
| X₅b   | 1.36      | C₉H₁₂O₄    | 1.1          | 141.0193                      | 141.0195  | 3248                                                                         | 5-(Hydroxymethyl) furoic acid         | Organic acids           |     |
| X6    | 1.36      | C₁₀H₁₃N₅O₅ | 2.4          | 282.0844                      | 282.0851  | 1153                                                                         | Guanosine                             | Nitrogen-containing compounds |     |
| X7    | 1.63      | C₁₃H₁₆O₇   | −1.1         | 285.0980                      | 285.0977  | 1505                                                                         | Gastrodin                             | Glycosides              | [12] |
| X8    | 2.03      | C₂H₂O₂     | 0.7          | 153.0193                      | 153.0195  | 16114                                                                        | Protocatechuic acid                   | Organic acids           | [13] |
| X₉    | 4.02      | C₂H₂O₃     | 2.5          | 137.0244                      | 137.0248  | 31464                                                                        | 3,4-Dihydroxy benzaldehyde            | Phenols                 | [14] |
| X₁₀   | 4.70      | C₁₉H₂₄O₁₃  | 1.7          | 459.1144                      | 459.1152  | 283440                                                                       | Parishin E or G                       | Glycosides              | [12] |
| X₁₁   | 5.09      | C₁₆H₁₈O₉   | 0.8          | 353.0878                      | 353.0881  | 109164                                                                       | 4-Caffeoylquinic acid                 | Organic acids           | [15] |
| X₁₂   | 5.13      | C₁₇H₂₃N₃O₅S| 0.5          | 412.1184                      | 412.1186  | 11210                                                                        | S-(4-Hydroxybenzyl)-glutathione       | Nitrogen-containing compounds | [12] |
| X₁₃   | 5.24      | C₂₀H₃₃N₃O₁₂S| 2.3         | 574.1712                      | 574.1726  | 7677                                                                         | S-(4-Hydroxybenzyl)-glutathione glucose | Nitrogen-containing compounds | [12] |
| X₁₄   | 5.34      | C₆H₁₀O₄    | −0.2         | 181.0506                      | 181.0506  | 2573                                                                         | 4-(2-Hydroxyethoxy) benzoic acid       | Organic acids           |     |
| X₁₅   | 5.58      | C₂H₂O₂     | 4.3          | 121.0295                      | 121.0302  | 50808                                                                        | 3-P-Hydroxybenzaldehyde               | Phenols                 | [14] |
| X₁₆   | 5.64      | C₁₀H₁₂O₄   | 0.9          | 179.0350                      | 179.0352  | 80360                                                                        | Caffeic acid                          | Organic acids           | [13] |
| X₁₇   | 5.99      | C₁₀H₁₄O₄   | 3            | 167.0350                      | 167.0355  | 275549                                                                       | Vanillic acid                         | Organic acids           | [12] |
| X₁₈b  | 7.31      | C₁₉H₂₆O₁₂  | 0.4          | 447.1508                      | 447.1510  | 14270                                                                        | Gastrodin A                           | Glycosides              | [12] |
| X₁₉   | 7.68      | C₁₇H₁₉N₅O₅ | −1.6         | 372.1313                      | 372.1307  | 2202                                                                         | p-Hydroxybenzyl adenosine             | Nitrogen-containing compounds | [12] |
| X₂₀   | 7.73      | C₁₁H₈O₃    | 0.5          | 151.0401                      | 151.0402  | 7660                                                                         | Vanilline                             | Phenols                 |     |
| X₂₁   | 8.62      | C₁₁H₂₉O₉   | −0.7         | 367.1035                      | 367.1032  | 21071                                                                        | 3-Feruloylquinic acid                 | Organic acids           | [16] |
| X₂₂   | 8.94      | C₉H₁₀O₃    | −0.8         | 165.0557                      | 165.0566  | 2085                                                                         | L-(-)-Phenyllactic acid               | Organic acids           |     |
| Peak | RT (min) | Formula | Error (ppm) | [M+H]+ Calculated | (m/z) Measure | Intensity | Product ions | Identification | Structure class | Ref. |
|------|---------|---------|-------------|------------------|---------------|-----------|-------------|---------------|----------------|-----|
| X23  | 9.11    | C_{32}H_{46}O_{19} | 4.9 | 727.2091 | 727.2151 | 122722 | 459.1156, 441.1045, 423.0937, 397.1142, 369.1186, 217.0496 | Parishin B | Glycosides | [13] |
| X24  | 9.78    | C_{33}H_{42}O_{20} | 4 | 757.2197 | 757.2227 | 659 | 178.0265, 149.0591, 134.0372 | Parishin H or M | Glycosides | [12] |
| X25  | 10.00   | C_{16}H_{16}O_{4} | 0.7 | 193.0506 | 193.0508 | 97733 | 441.0958, 243.0943, 397.1169, 161.0403 | Ferulic acid | Organic acids | [13] |
| X26  | 10.02   | C_{21}H_{22}O_{13} | 0 | 487.1457 | 487.1457 | 4684 | 459.1156, 441.1045, 423.0937, 397.1142 | Parishin O or N | Glycosides | [12] |
| X27  | 10.05   | C_{32}H_{40}O_{19} | 4.9 | 727.2091 | 727.2151 | 122722 | 459.1156, 441.1045, 423.0937, 397.1142, 369.1186, 217.0496 | Parishin C | Glycosides | [12] |
| X28  | 10.71   | C_{14}H_{14}O_{5}S | −2.2 | 261.0591 | 261.0585 | 777 | 205.8269, 167.8694, 137.0057 | 4,4′-Dihydroxybenzyl sulfoxide | Phenols | [14] |
| X29  | 11.74   | C_{14}H_{14}O_{5}S | −2.2 | 223.0612 | 223.0607 | 2431 | 108.0226, 179.0713 | Sinapic acid | Organic acids | [13] |
| X30  | 12.20   | C_{2}H_{6}O_{3} | 2.5 | 137.0244 | 137.0248 | 31464 | 229.0860, 123.0452, 121.0288, 107.0511 | p-Hydroxybenzoic acid | Phenols | [14] |
| X31  | 13.21   | C_{23}H_{20}O_{25} | 4.7 | 995.3038 | 995.3107 | 39727 | 727.2129, 441.1065, 423.0915, 397.1119 | Parishin Glycosides | [12] |
| X32  | 13.56   | C_{20}H_{26}O_{8} | 0.2 | 391.1398 | 391.1399 | 6858 | 229.0860, 123.0452, 121.0288, 123.0452, 107.0511, 93.0357, 71.0265 | Bis-(4-hydroxybenzyl)-ether-mono-β-D-glucopyranoside | Glycosides | [14] |
| X33  | 14.13   | C_{25}H_{22}O_{12} | 2.6 | 515.1195 | 515.1208 | 91406 | 353.0879, 335.0781, 191.0558, 179.0345, 173.0450, 161.0240 | 3,4-Dicaffeoylquinic acid isomer | Organic acids | [15] |
| X34  | 16.48   | C_{25}H_{22}O_{12} | 2.6 | 515.1195 | 515.1208 | 91406 | 353.0879, 335.0781, 191.0558, 179.0345, 173.0450, 161.0240 | 3,4-Dicaffeoylquinic acid isomer | Organic acids | [15] |
| X36  | 17.42   | C_{2}H_{6}O_{3} | −0.8 | 151.0765 | 151.0763 | 1891 | 139.0217 | 4-(Ethoxymethyl)phenol | Phenols | [14] |
| X37  | 23.89   | C_{14}H_{14}O_{2}S | −4 | 245.0642 | 245.0632 | 2498 | 139.0217 | Bis(4-hydroxybenzyl) sulfide | Phenols | [14] |
| X38  | 32.90   | C_{6}H_{8}O_{3} | −1 | 163.0401 | 163.0399 | 4130 | 145.0279, 135.0455, 119.0493, 91.0184, 77.0443 | p-Hydroxycinnamic acid | Organic acids | [15] |
| X39  | 39.73   | C_{4}H_{12}O_{4}S | −0.2 | 277.1445 | 277.1445 | 5380 | 233.1544, 206.8262 | Dibutyl phthalate | Phenols | [14] |
| Y1   | 1.05    | C_{2}H_{2}N_{6} | −1.8 | 136.0618 | 136.0615 | 46698 | nd | Adenine | Nitrogen-containing compounds | Nitrogen-containing compounds | [16] |
| Y2   | 1.08    | C_{10}H_{13}N_{5}O_{4} | 1.5 | 268.1040 | 268.1044 | 174357 | 136.0623, 119.0360, 113.0129 | Adenosine | Nitrogen-containing compounds | Nitrogen-containing compounds | [16] |
| Y3   | 1.09    | C_{6}H_{11}NO_{3} | −1.9 | 182.0812 | 182.0808 | 34675 | nd | Tyrosin | Nitrogen-containing compounds | Nitrogen-containing compounds | [16] |
| Y4   | 8.60    | C_{24}H_{32}N_{4}O_{6}S | −0.5 | 520.1748 | 520.1746 | 7896 | 308.0836, 285.0913, 233.0591, 179.0486, 162.0208, 107.0485 | (2)-g-L-[N-(4-Hydroxy benzyl)] glutamyl-L-[s-(4-hydroxybenzyl)] cysteinylglycine 3-Carboxyethyl-phthalide | Nitrogen-containing compounds | Organic acids | [16] |
| Y5   | 9.77    | C_{10}H_{10}O_{4} | −0.8 | 193.0495 | 193.0494 | 14081 | 178.0257, 150.0323, 133.0281, 122.0361 | Nitrogen-containing compounds | Nitrogen-containing compounds | [16] |
| Peak | RT (min) | Formula  | Error  | [M–H] Calculated | [M–H] Measure | Intensity | Product ions | Identification | Structure class | Ref. |
|------|---------|----------|--------|-------------------|----------------|-----------|--------------|----------------|----------------|------|
| Y6   | 10.55   | C₁₂H₁₈O₅ | −0.1   | 243.1227         | 243.1227       | 13011     | 165.0909, 151.0381, 137.0949 | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxy phthalide Senkyunolide H or I or ligustilidiol or cis-6,7-dihydroxy-ligustilide | Organic acids |      |
| Y7   | 10.55   | C₁₂H₁₆O₄ | −0.5   | 225.1121         | 225.1119       | 21174     | 207.1023, 165.0557, 151.0376, 137.0954 | Senkyunolide R or S | Phthalides [9] |      |
| Y8   | 11.94   | C₁₂H₁₆O₅ | −0.8   | 241.1071         | 241.1069       | 1918      | 150.0677, 107.0497, 71.0498 | Senkyunolide G or K or Z-6,7-epoxyligustilide | Phthalides [9] |      |
| Y9   | 13.27   | C₁₂H₁₆O₃ | 0.1    | 209.1172         | 209.1172       | 288915    | 153.0544, 149.0593, 135.0473, 121.1006, 117.0709 | Senkyunolide J or N or R2 | Phthalides [9] |      |
| Y10  | 13.27   | C₁₂H₁₆O₄ | 0.4    | 227.1278         | 227.1279       | 102387    | 163.1104, 153.0543, 149.0961, 119.0860, 107.0484, 79.054 | Senkyunolide H or I or ligustilidiol or cis-6,7-dihydroxy-ligustilide | Phthalides [9] |      |
| Y11  | 16.56   | C₁₂H₁₆O₃ | −0.6   | 207.1016         | 207.1015       | 1327567   | 189.0917, 165.0550, 146.0732, 133.0653, 119.0841, 105.0693 | Senkyunolide F or chuanxiongol | Phthalides [9] |      |
| Y12  | 16.57   | C₁₂H₁₆O₄ | −0.9   | 225.1121         | 225.1119       | 39845     | 165.0533, 133.0658, 128.0619, 91.0532, 77.0402 | Senkyunolide H or I or ligustilidiol or cis-6,7-dihydroxy-ligustilide | Phthalides [9] |      |
| Y13  | 17.87   | C₁₂H₁₆O₄ | −0.2   | 223.0965         | 223.0966       | 23855     | 177.0921, 149.0591, 121.0308, 103.0523, 77.0401 | Senkyunolide D or 4,7-dihydroxy-3-butylphthalide | Phthalides [9] |      |
| Y14  | 18.08   | C₁₂H₁₆O₃ | −0.1   | 207.1016         | 207.1015       | 425060    | 189.0901, 165.0558, 161.0948, 128.0620, 105.0701, 91.0549, 77.0393 | Senkyunolide F or chuanxiongol | Phthalides [9] |      |
| Y15  | 19.53   | C₁₈H₂₈O₈ | 0.3    | 373.1857         | 373.1858       | 45450     | 211.1335, 193.1228, 147.1172, 105.0702, 79.0577 | Ligusticoside A | Glycosides [17] |      |
| Y16  | 21.97   | C₁₂H₁₂O₂ | −0.5   | 189.0910         | 189.0910       | 151946    | 128.0623, 115.0544, 105.0702, 91.0551, 77.0388 | Butyldienephthalide isomer | Phthalides [9] |      |
| Y17  | 23.67   | C₁₂H₁₄O₄ | 0.3    | 223.0965         | 223.0966       | 43428     | 177.0899, 167.0387, 149.0227, 121.0278, 91.0541, 77.0382, 152.0611, 128.0618, 115.0529, 105.0341, 91.0537, 77.0394 | Butyldienephthalide isomer | Phthalides [9] |      |
| Y18  | 24.29   | C₁₂H₁₂O₂ | 0      | 189.0910         | 189.0910       | 305525    | 207.1020, 189.0907, 161.0364, 133.0640, 119.0840, 91.0533, 187.0745, 168.0574, 144.0573, 131.0493, 115.0541, 103.0552, 91.0533, 77.0401 | Senkyunolide L | Phthalides [18] |      |
| Y19  | 24.50   | C₁₂H₁₄O₂ | −0.9   | 191.1067         | 191.1067       | 340079    | 117.0688, 91.0560, 77.0396 | 3-Butylphthalide or Z-ligustilide or E-ligustilide | Phthalides [9] |      |
| Y20  | 26.27   | C₁₂H₁₅ClO₃ | −0.5   | 243.0783        | 243.0781       | 21244     | 207.0120, 189.0907, 161.0364, 133.0640, 119.0840, 91.0533, 187.0745, 168.0574, 144.0573, 131.0493, 115.0541, 103.0552, 91.0533, 77.0401 | Senkyunolide B or C | Phthalides [9] |      |
| Y21  | 26.63   | C₁₂H₁₂O₃ | 0.6    | 205.0859         | 205.0860       | 90627     | 175.1090, 105.0691 | 2-(1-Oxopentyl)-benzoic | Organic acids |      |
| Y22  | 27.74   | C₁₃H₁₄O₃ | −0.6   | 221.1172         | 221.1171       | 4804      | 115.0541, 103.0552, 91.0533, 77.0401 | Senkyunolide L | Phthalides [18] |      |
| Peak | RT (min) | Formula | Error (ppm) | [M–H] Calculated | (m/z) Measure | Intensity | Product ions | Identification | Structure class | Ref. |
|------|---------|---------|-------------|------------------|----------------|----------|-------------|----------------|----------------|------|
| Y23  | 27.90   | C₁₂H₁₂O₃| 0.6         | 205.0859        | 205.0860       | 90627    | 187.0745, 168.0574, 144.0573, 131.0493, 115.0541, 103.0552, 91.0533, 77.0401 | Senkyunolide B or C | Phthalides [9] |      |
| Y24  | 28.65   | C₁₂H₁₆O₂ | 0.7         | 193.1223        | 193.1225       | 227337  | 153.0704, 145.1007, 135.0440, 117.0695, 91.0548, 77.0391  | Senkyunolide A | Phthalides [9] |      |
| Y25  | 29.49   | C₁₂H₁₄O₂ | 0.3         | 191.1067        | 191.1067       | 348972  | 173.0961, 145.1008, 130.0773, 117.0694, 91.0548, 77.0392  | 3-Butylphthalide or Z-Ligustilide or E-Ligustilide | Phthalides [9] |      |
| Y26  | 32.06   | C₁₂H₁₂O₂ | –0.5        | 189.0910        | 189.0910       | 39396   | 152.0611, 128.0615, 115.0545, 91.0554  | Butylenephthalide isomer | Phthalides [9] |      |
| Y27a | 32.89   | C₁₂H₁₈O₂ | –0.8        | 195.1380        | 195.1378       | 686098  | 173.1344, 149.1309, 79.0550  | Cnidilide or neoCnidilide | Phthalides [9] |      |
| Y28  | 33.04   | C₁₂H₁₄O₂ | 0.3         | 191.1067        | 191.1067       | 637725  | 147.1167, 137.0590, 119.0498, 91.0544, 77.0389, 65.0383 | 3-Butylphthalide or Z-Ligustilide or E-Ligustilide | Phthalides [9] |      |
| Y29  | 33.64   | C₁₂H₁₂O₂ | –0.2        | 189.0910        | 189.0910       | 401466  | 147.1167, 137.0590, 119.0498, 91.0544, 77.0389, 65.0383 | Butylenephthalide isomer | Phthalides [9] |      |
| Y30  | 35.24   | C₁₂H₁₂O₂ | –0.7        | 197.1536        | 197.1535       | 21102   | 177.1344, 149.1309, 79.0550  | 3,7-Dimethyl-3-acetate-1,6-octadiene-3-ol acetate | Others |      |
| Y31  | 36.15   | C₁₂H₁₂O₂ | 0.9         | 317.2475        | 317.2478       | 12477   | 281.2253, 211.1524, 187.1501, 159.1179, 149.1329, 117.0717, 81.0720 | Pregnenolone | Others |      |
| Y32  | 36.37   | C₂₂H₂₆O₅ | 0.1         | 397.2010        | 397.2010       | 71592   | 191.1064, 173.0954, 155.0852, 145.1003, 128.0625, 117.0715, 105.0724, 91.0544, 77.0392 | Chuanxiongdiolide A or B | Others [19] |      |
| Y33  | 38.22   | C₂₂H₂₆O₅ | 0.4         | 397.2010        | 397.2010       | 66000   | 191.1064, 173.0954, 155.0852, 145.1003, 128.0625, 117.0715, 105.0724, 91.0544, 77.0392 | Chuanxiongdiolide A or B | Others [19] |      |
| Y34  | 39.08   | C₂₂H₂₆O₅ | –0.4        | 401.2323        | 401.2321       | 20533   | 279.1401, 261.1264, 211.1524, 187.1501, 159.1179, 149.1329, 117.0717, 81.0720 | Chuanxiongdiolide R2 or chuanxiongdiolide B | Others [20] |      |
| Y35  | 39.48   | C₁₇H₂₄O₄ | 0.8         | 293.1747        | 293.1750       | 22471   | 191.1068, 173.0958, 163.1120, 149.0601, 135.0440, 91.0546, 77.0392 | Senkyunolide M or Q | Phthalides [9] |      |
| Y36  | 41.42   | C₂₄H₃₀O₄ | –0.1        | 383.2217        | 383.2217       | 1160225 | 193.1229, 175.1111347.1159, 137.0587, 119.0871, 93.0687 | Senkyunolide P or 3,8-Dihydro-diligustilide or angelicide or Z,Z′,Z′,Z′-Diligustilide | Phthalides [9] |      |
| Y37  | 41.52   | C₂₄H₃₂O₄ | 0.1         | 385.2373        | 385.2374       | 627373  | 367.2247, 349.2092, 321.2178, 293.1915, 193.1229, 175.1111347.1159, 137.0587, 119.0871, 93.0687 | Chuanxiongdiolide A | Others [20] |      |
| Y38  | 41.88   | C₂₄H₃₂O₄ | 0.1         | 381.2060        | 381.2061       | 4796897 | 191.1070, 173.0955, 163.1126, 149.0596, 135.0437, 91.0551, 79.0549 | Levistolide A or senkyunolide O or tokinolide B or riligustilide | Phthalides [9] |      |

*a“X” in negative ion mode and “Y” in negative-positive mode. bCompared with reference standards.
Figure 3: Information about classification of compounds in TST.

Figure 4: Continued.
Therefore, **M5** might be a metabolite of senkyunolide D after hydrogenation and glucuronidation, while **M6** might be a metabolite of senkyunolide D after hydroxylation and glucuronidation (metabolites of **M1** and extract ion chromatograms (EICs) are shown in Figure 6).

Metabolite **M27**, which eluted at 14.81 min, formed a molecular ion of [M+H]⁺ at m/z 193.1222 corresponding to C₁₂H₁₆O₂. A major fragment ion was shared with senkyunolide A, suggesting that **M27** is prototype of senkyunolide A. Metabolite **M28**, which eluted at 14.81 min, formed a molecular ion of [M+H]⁺ at m/z 223.1328 corresponding to C₁₃H₁₈O₃. Its major fragment ions m/z 205.1224 and 191.1060 were generated by loss of 18 Da and 18 + 14 Da, which implied loss of a H₂O group and methyl group. Other product ions were identical to that of **M27**. Based on the possible metabolic reactions, **M28** might be a metabolite of senkyunolide A after hydroxylation and methylation. Metabolite **M29**, which eluted at 10.35 min, formed a molecular ion of [M+H]⁺ at m/z 372.1475 corresponding to C₁₇H₂₅O₆NS. Its major fragment ions m/z 293.0893 and 191.1074 were generated by loss of 163 Da and 163 + 18 Da, which implied loss of an acetylcysteine group

![Figure 4: MS/MS spectra and the proposed fragmentation pathways.](image-url)

(a) Parishin B in negative ion mode. (b) Gastrodin A in negative ion mode. (c) Cnidilide or neocnidilide in positive ion mode.
| No. | Parent compounds | Metabolic pathways | Formula | tR (min) | [M+H]+ (m/z) | Error (ppm) | Product ions |
|-----|------------------|--------------------|---------|----------|-------------|------------|-------------|
| 1   | 4,7-Dihydroxy-3-butylphthalide, senkyunolide D | Prototype | C_{12}H_{14}O_{4} | 9.75 | 223.0965 | 223.0963 | 177.0899, 167.0387, 149.0226, 121.0278, 91.0541 |
| 2   | 4,7-Dihydroxy-3-butylphthalide, senkyunolide D | Hydroxyl and methyl conjugation | C_{13}H_{16}O_{4} | 10.78 | 253.1071 | 253.1071 | 235.0963, 221.0829, 202.0596, 193.0489, 179.0332, 175.0579, 150.0301, 121.0268, 285.0430, 205.0858, 187.0753, 177.0904, 149.0244, 121.0283, 91.0534 |
| 3   | 4,7-Dihydroxy-3-butylphthalide, senkyunolide D | Sulfate conjugation | C_{12}H_{14}SO_{7} | 9.2 | 303.0533 | 303.0535 | 223.0969, 205.0847, 177.0883, 167.0331, 149.0233, 121.0315 |
| 4   | 4,7-Dihydroxy-3-butylphthalide, senkyunolide D | Glucuronide conjugation | C_{18}H_{22}O_{10} | 10.6 | 399.1286 | 399.1285 | 225.4427, 207.1017, 189.0924, 172.0884, 165.0548, 141.0170, 119.0851, 113.0288 |
| 5   | 4,7-Dihydroxy-3-butylphthalide, senkyunolide D | Hydrogenation and glucuronide conjugation | C_{18}H_{24}O_{10} | 8.67 | 401.1442 | 401.1444 | 227.0584, 221.0824, 165.0929, 137.0955, 123.0434 |
| 6   | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | Prototype | C_{12}H_{18}O_{5} | 7.76 | 243.1227 | 243.1225 | 165.0909, 151.0414, 137.0951, 123.0431, 107.0499, 91.0546, 85.0648 |
| 7   | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | Methyl conjugation | C_{13}H_{20}O_{5} | 10.01 | 257.1384 | 257.1381 | 221.1211, 207.0993, 171.1364 |
| 8   | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | Cystein conjugation | C_{15}H_{23}O_{6}NS | 7.67 | 346.1319 | 346.1319 | 328.1222, 310.1111, 264.1056, 238.0916, 223.0771, 207.1018, 165.0923, 137.0955 |
| 9   | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | Oxidation and cystein conjugation | C_{17}H_{25}O_{8}NS | 5.97 | 404.1374 | 404.1372 | 327.0911, 247.1337, 229.1216, 151.0746 |
| 10  | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | N-Acetyl-L-cysteine conjugation | C_{15}H_{23}O_{7}NS | 9.47 | 362.1268 | 362.1267 | 327.0911, 247.1337, 229.1216, 151.0746 |
| 11  | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | Hydroxyl and glucuronide conjugation | C_{16}H_{22}O_{10} | 5.97 | 404.1374 | 404.1372 | 205.0833, 171.1364 |
| 12  | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | Desat and S-GSH conjugation | C_{22}H_{33}O_{11}N_{35} | 4.39 | 548.1909 | 548.1921 | 473.1636, 419.1465, 205.0860 |
| 13  | 3-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxyphthalide | H_{2}O conjugation | C_{12}H_{20}O_{6} | 4.65 | 261.1333 | 261.1320 | 261.1310 |
| 14  | 3-Carboxyethylphthalide | Prototype | C_{10}H_{10}O_{4} | 7.28 | 193.0495 | 193.0490 | 178.0257, 150.0323, 133.0277, 122.0361, 105.0338, 77.0388 |
| 15  | 3-Carboxyethylphthalide | Methyl conjugation | C_{11}H_{12}O_{4} | 11.52 | 207.0652 | 207.0646 | 147.0441, 131.0502, 103.0546, 91.0533 |
| 16  | 3-Carboxyethylphthalide | Glucuronide conjugation | C_{16}H_{16}O_{10} | 5.51 | 369.0816 | 369.0822 | 193.0493 |
| 17  | 3-Carboxyethylphthalide | Hydroxyl and glucuronide conjugation | C_{16}H_{16}O_{11} | 5.72 | 385.0765 | 385.0758 | 209.0455 |
| 18  | 3-Carboxyethylphthalide | Hydrogenation | C_{10}H_{16}O_{4} | 7.64 | 195.0652 | 195.0651 | 177.0547, 149.0609, 145.0276, 134.0354, 117.0309, 89.0395 |
Table 2: Continued.

| No. | Parent compounds                        | Metabolic pathways | Formula | tR (min) | [M+H]+ (m/z) | Error (ppm) | Product ions |
|-----|----------------------------------------|--------------------|---------|----------|-------------|-------------|--------------|
|     |                                        |                    |         |          | Calculated  | Measure     |              |
|     |                                        |                    |         |          |             |             |              |
| 19  | Cnidilide, neocnilide                  | Prototype          | C12H18O2| 16.14    | 195.1380    | 195.1377    | 1.5          |
|     |                                        |                    |         |          |             |             |              |
|     |                                        | Methyl conjugation  | C13H20O2| 15.63    | 209.1536    | 209.1537    | 0.4          |
|     |                                        |                    |         |          |             |             |              |
| 21  | Cnidilide, neocnilide                  | Acetyl conjugation  | C14H20O3| 12.45    | 237.1485    | 237.1483    | 0.1          |
|     |                                        |                    |         |          |             |             |              |
|     |                                        | Hydroxyl and acetyl conjugation | C14H20O4| 14.26    | 253.1434    | 253.1435    | 0.4          |
|     |                                        |                    |         |          |             |             |              |
| 23  | Cnidilide, neocnilide                  | Oxidation and cystein conjugation | C13H20O4NS| 10.07  | 314.1421    | 314.1424    | 1.1          |
|     |                                        | Hydroxyl and glucuronide conjugation | C18H26O9| 9.62     | 387.1650    | 387.1650    | 0.1          |
|     |                                        |                    |         |          |             |             |              |
| 25  | Cnidilide, neocnilide                  | 2Hydroxyl and glucuronide conjugation | C14H26O4| 8.02     | 403.1599    | 403.1598    | 0.2          |
|     |                                        |                    |         |          |             |             |              |
| 26  | Cnidilide, neocnilide                  | 2Hydroxyl conjugation | C12H18O4| 8.68     | 227.1278    | 227.1279    | 0.7          |
|     |                                        |                    |         |          |             |             |              |
| 27  | Senkyunolide A                         | Prototype          | C12H16O2| 14.81    | 193.1223    | 193.1222    | 0.5          |
|     |                                        |                    |         |          |             |             |              |
| 28  | Senkyunolide A                         | Hydroxyl and methyl conjugation | C13H18O3| 15.85    | 223.1329    | 223.1326    | 1.2          |
|     |                                        |                    |         |          |             |             |              |
| 29  | Senkyunolide A                         | Hydroxyl and acetylcysteine conjugation | C13H25O4NS| 10.35   | 372.1475    | 372.1475    | 0.1          |
|     |                                        |                    |         |          |             |             |              |
| 30  | Senkyunolide A                         | Carboxyl and glucuronide conjugation | C18H22O4| 10.6     | 399.1286    | 399.1285    | 0.3          |
|     |                                        |                    |         |          |             |             |              |
| 31  | Senkyunolide A                         | 2Hydroxyl and glucuronide conjugation | C18H24O4| 8.67     | 401.1442    | 401.1444    | 0.3          |
|     |                                        |                    |         |          |             |             |              |
| 32  | Senkyunolide A                         | H2O conjugation     | C12H14O3| 10.54    | 211.1329    | 211.1327    | 0.9          |
|     |                                        |                    |         |          |             |             |              |
| 33  | Senkyunolide A                         | 2Hydroxyl conjugation | C12H16O4| 9.56     | 225.1121    | 225.1119    | 0.1          |
|     |                                        |                    |         |          |             |             |              |
| 34  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Prototype | C12H16O3| 12.79    | 209.1172    | 209.1173    | 0.3          |

177.1350, 149.1348, 125.0599, 107.0873, 97.0640, 91.0550, 79.0543
193.0211, 167.1088
153.0917, 121.0648, 68.9961
177.0257
235.1340, 193.0856, 157.1012, 135.0816
268.1343, 193.1185
211.1330, 193.1223, 175.1129, 147.1168, 121.0368
227.1287, 209.1174, 191.1065, 171.1373, 163.1123, 153.0549, 145.1025, 141.0186, 135.1164, 121.0995, 191.1057, 163.1115, 153.0554, 145.1001, 135.0444, 105.0705, 91.0541
175.1118, 147.1170, 137.0580, 119.0848, 105.0710, 91.0556, 77.0393
205.1224, 191.1060, 149.0235, 135.0429, 121.0279, 105.0697, 91.0542, 77.0397
330.1375, 284.1322, 267.1048, 239.0756, 209.1169, 191.1074, 162.0210, 153.0540, 130.0492
223.0969, 205.0847, 177.0883, 159.0293, 149.0233, 131.0840, 85.0275
225.1127, 207.1017, 189.0924, 172.0884, 165.0548, 141.0170, 119.0851, 113.0288, 85.0265, 73.0295, 193.1225, 175.1096
147.1156, 129.0700, 105.0693, 93.0692, 79.0548
207.1013, 189.0914, 165.0537, 133.0637, 105.0706, 91.0536, 81.0713
153.0686, 149.0594, 145.0984, 135.0472, 105.0693, 91.0562, 77.0409
| No. | Parent compounds                          | Metabolic pathways            | Formula       | tR  (min) | [M+H]⁺ (m/z) Calculated | Error (ppm) | Product ions                                                                 |
|-----|------------------------------------------|-------------------------------|---------------|-----------|-------------------------|-------------|-----------------------------------------------------------------------------|
| 35  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Methyl conjugation            | C₁₃H₁₄O₄      | 15.85     | 223.1329                | 223.1326    | 191.1060, 173.0946, 149.0355, 135.0429, 105.0697, 91.0542, 79.0551          |
| 36  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Acetyl conjugation            | C₁₄H₁₄O₄      | 15.99     | 251.1278                | 251.1276    | 177.1261, 149.0593, 69.0014, 57.0752                                        |
| 37  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Hydroxyl and acetyl conjugation | C₁₄H₁₄O₅      | 12.27     | 267.1227                | 267.1226    | 249.1137, 193.0479, 189.0582, 135.0435, 119.0846                         |
| 38  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Taurine conjugation           | C₁₃H₁₃O₃NS     | 9.02      | 328.1213                | 328.1214    | 207.1015, 189.0911, 165.0541, 161.0955, 147.0814, 133.0644, 119.0859, 91.0538 |
| 39  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | N-Acetyl-L-cysteine conjugation | C₁₇H₁₃O₃NS     | 11.15     | 370.1319                | 370.1320    | 211.1330, 193.1223, 175.1129, 147.1168, 121.0638, 91.0546, 79.0562         |
| 40  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Hydrogenation and glucuronide conjugation | C₁₈H₂₆O₉      | 9.62      | 387.1650                | 387.1650    | 207.1010, 164.0390, 122.0273, 105.0347, 79.0529                         |
| 41  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Hydroxyl and acetyl conjugation | C₁₇H₁₃O₃NS     | 8.79      | 388.1425                | 388.1422    | 267.1015, 225.1127, 207.1017, 189.0924, 172.0844, 141.0170, 113.0228, 85.0265, 73.0295 |
| 42  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Hydroxyl and glucuronide conjugation | C₁₈H₂₄O₁₀     | 8.67      | 401.1442                | 401.1444    | 227.0584, 221.0824                                                        |
| 43  | Senkyunolide G, senkyunolide K,Z-6,7-epoxyligustilide | Carboxyl and glucuronide conjugation | C₁₈H₂₂O₁₁     | 8.92      | 415.1235                | 415.1238    | 439.1552, 385.1429, 282.1160, 260.1071, 207.0921, 179.0484, 162.0221, 144.0103, 116.0174, 76.0218, 441.1694, 387.1593, 284.1315, 209.1173, 191.1055, 162.0212, 144.0109, 116.0175, 84.0447, 193.1225, 175.1096, 151.0737, 147.1156, 129.0700, 121.0641, 105.0693, 93.0693, 91.0546, 77.0398, 177.0906, 149.0590, 145.0880, 105.0329, 91.0556, 77.0394, 209.1169, 191.1057, 163.1115, 153.0554, 145.1001, 135.0444, 105.0705, 91.0541, 77.0388, 65.0402, 55.0198 |
and H$_2$O group. Other product ions were identical to that of M$_{27}$. Therefore, M$_{29}$ may be a metabolite of senkyunolide A after hydroxyl and acetylcysteine conjugation. Metabolite M$_{30}$, which eluted at 10.60 min, formed a molecular ion of [M+H]$^+$ at m/z 399.1285 corresponding to C$_{18}$H$_{22}$O$_{10}$. Its major fragment ions m/z 223.0969, 205.0847, and 177.0883 were generated by the loss of 176 Da, 176 + 18 Da, and 176 + 18 + 28 Da, which implied loss of a glucuronide group.

Table 2: Continued.

| No. | Parent compounds | Metabolic pathways | Formula | tR (min) | [M+H]$^+$ (m/z) | Error (ppm) | Product ions |
|-----|------------------|--------------------|---------|----------|----------------|------------|--------------|
| 49  | Senkyunolide G, senkyunolide K, Z-6,7-epoxyligustilide | Demethyl and carboxyl conjugation | C$_{12}$H$_{14}$O$_5$ | 9.79 | 239.0914, 239.0915 | 0.4 | 221.0816, 193.0885, 179.0336, 165.0173, 161.0227, 128.0633, 109.0292, 77.0376 |
| 50  | Senkyunolide G, senkyunolide K, Z-6,7-epoxyligustilide | 2hydrogenation and 2hydroxyl conjugation | C$_{12}$H$_{18}$O$_5$ | 7.76 | 243.1227, 243.1225 | -0.7 | 225.1135, 207.1017, 179.1084, 165.0909, 151.0414, 137.0951, 123.0431, 95.0486 |
| 51  | Senkyunolide G, senkyunolide K, Z-6,7-epoxyligustilide | Aromatic hydrocarbon oxidation | C$_{13}$H$_{18}$O$_5$ | 11.38 | 255.1227, 255.1222 | -2.1 | 195.1024, 135.0798, 131.0870 |
| 52  | Senkyunolide J,N,R$_2$ | Prototype | C$_{12}$H$_{18}$O$_4$ | 8.68 | 227.1278, 227.1279 | 0.7 | 163.1115, 153.0554, 145.1001, 107.0705, 91.0541, 79.0544, 65.0402, 55.0198 |
| 53  | Senkyunolide J,N,R$_2$ | Methyl conjugation | C$_{13}$H$_{20}$O$_4$ | 11.42 | 241.1434, 241.1433 | -0.6 | 225.1135, 207.1017, 179.1084, 165.0909, 151.0414, 137.0951, 123.0431, 95.0486 |
| 54  | Senkyunolide J,N,R$_2$ | Hydroxyl and methyl conjugation | C$_{13}$H$_{20}$O$_5$ | 10.01 | 257.1384, 257.1381 | -1 | 221.1211, 207.0993, 165.0913, 161.0984, 137.0951, 123.0434, 93.0699, 79.0549, 67.0538 |
| 55  | Senkyunolide J,N,R$_2$ | Glycine conjugation | C$_{14}$H$_{21}$O$_5$N | 7.49 | 284.1493, 284.1495 | 0.8 | 238.1464, 209.1195, 191.1065, 163.1151, 153.0546, 135.1157, 117.0704, 91.0562, 76.0410, 57.0711 |
| 56  | Senkyunolide J,N,R$_2$ | Cystein conjugation | C$_{15}$H$_{23}$O$_5$NS | 8.48 | 330.1370, 330.1375 | 1.7 | 209.1147, 181.1211, 153.0555, 126.0211, 108.0102, 91.0549 |
| 57  | Senkyunolide J,N,R$_2$ | Taurine conjugation | C$_{15}$H$_{25}$O$_5$NS | 7.56 | 334.1319, 334.1319 | 0 | 328.1222, 310.1111, 264.1056, 238.0916, 207.1018, 195.0853, 165.0923, 137.0955 |
| 58  | Senkyunolide J,N,R$_2$ | Oxidation and cystein conjugation | C$_{15}$H$_{25}$O$_6$NS | 7.67 | 346.1319, 346.1319 | 0 | 207.1010, 164.0390, 107.0491, 122.0273, 105.0347 |
| 59  | Senkyunolide J,N,R$_2$ | N-Acetyl-L-cysteine conjugation | C$_{17}$H$_{25}$O$_5$NS | 8.29 | 388.1425, 388.1422 | -0.6 | 227.1287, 209.1174, 191.1065, 163.1123, 153.0549, 145.1025, 135.1164, 121.0995, 93.0712 |
| 60  | Senkyunolide J,N,R$_2$ | Glucuronide conjugation | C$_{18}$H$_{26}$O$_{10}$ | 8.02 | 403.1599, 403.1598 | -0.2 | 459.1795, 405.1696, 387.1582, 369.1490, 341.1469, 302.1438, 284.1303, 284.1303, 241.0913, 209.1157, 191.1075 |
| 61  | Senkyunolide J,N,R$_2$ | S-GSH conjugation | C$_{22}$H$_{25}$O$_{10}$N$_3$S | 4.96 | 534.2116, 534.2113 | -0.6 |
H$_2$O group, and CO group. Other product ions were identical to that of M$_{27}$. Therefore, M$_{30}$ may be a metabolite of senkyunolide A following carboxylation and glucuronidation. Metabolite M$_{31}$, which eluted at 8.67 min, formed a molecular ion of [M+H]$^+$ at m/z 401.1444 corresponding to C$_{18}$H$_{24}$O$_{10}$. Its major fragment ions m/z
225.1127, 207.1017, and 189.0924 were generated by loss of 176 Da, 176 + 18 Da, and 176 + 18 + 18 Da, which implied loss of a glucuronide group and two H2O groups. Other product ions were identical to that of M27. Therefore, M31 might be a metabolite of senkyunolide A after 2 hydroxylation events and glucuronidation. Metabolite M32, which eluted at 10.54 min, formed a molecular ion of [M+H]+ at m/z 211.1327 corresponding to C12H18O3. Its major fragment ions were m/z 193.1225 and 175.1096. An m/z of 193.1225 (loss of 18 Da) corresponds to senkyunolide...
A, suggesting that M32 might be a metabolite of senkyunolide A after H2O conjugation. Metabolite M33, which eluted at 9.56 min, formed a molecular ion of [M+H]+ at m/z 225.1119 corresponding to C12H16O4. Its major fragment ions m/z 207.1013 and 189.0914 were generated by loss of 18 Da and 18+18 Da, which implied loss of one or two H2O groups. Other product ions were identical to that of M27. Therefore, M33 might be a metabolite of senkyunolide A after 2 hydroxylation events (metabolites of M27 and extracted ion chromatograms (EICs) are shown in Figure 7).

Sixty-one metabolites were identified in rat plasma. Through the analysis of these 61 metabolites, it was found that hydroxylation and glucuronidation were the main metabolic ways following oral administration of TST. From the identified metabolites, it can be speculated that after absorption of TST by human blood, most of the compounds undergo hydroxylation and glucuronidation, which allow TST to play a positive role in the treatment of migraine and blood stasis headaches. This provides a basis for follow-up research on the medical uses of TST. At the same time, from the information obtained on the metabolites, it can be seen that the main metabolites in positive ion mode of TST are concentrated as chuanxiong lactones, but there are no effective metabolites from Tianma. It is possible that Tianma metabolites are mainly present in the negative ion mode of plasma or in feces, urine, and bile, which requires further study.

4. Conclusion

In this study, UPLC/Q-TOF MS was used to comprehensively determine the chemical fingerprint and metabolic profile of TST after intragastric administration. In the analysis of the chemical constituents of TST, 77 compounds were identified, including 39 compounds identified in negative ion mode and 38 compounds identified in positive ion mode. In order to elucidate the mass spectrometric pyrolysis law of the main compounds in TST, gastrodin A, parishin B, and cnidilide or neocnilide were specifically analyzed, and the results were completely consistent with the results in reference standards and the reported literature. And 61 metabolites of TST in rat plasma were detected, which were mainly metabolites of 7 compounds. Two prototypes (senkyunolide D or 4,7-dihydroxy-3-butylyphthalide and senkyunolide A) and their metabolites were analyzed in detail, which showed hydroxylation and glucuronidation were the main metabolic pathways following oral administration. This study expanded our understanding of the chemical constituents of TST, studied its metabolic spectrum for the first time, and clarified its main metabolic pathway in plasma, which will lay the foundation for follow-up studies of the pharmacological mechanism of TST.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest in publication of this study.

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