Transformation of Stilbene Glucosides From Reynoutria multiflora During Processing

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The root of Reynoutria multiflora Thunb. Moldenke (RM, syn.: Polygonum multiflorum Thunb.) has been widely used in TCM clinical practice for centuries. The raw R. multiflora (RRM) should be processed before use, in order to reduce toxicity and increase efficiency. However, the content of trans-2, 3, 5, 4′-tetrahydroxystilbene-2-O-β-D-glucopyranoside (trans-THSG), which is considered to be the main medicinal ingredient, decreases in this process. In order to understand the changes of stilbene glycosides raw R. multiflora (RRM) and processed R. multiflora (PRM), a simple and effective method was developed by ultra high performance liquid chromatography tandem quadrupole/electrostatic field orbitrap high-resolution mass spectrometry (UHPLC-Q-Exactive plus orbitrap MS/MS). The content and quantity of stilbene glycosides have undergone tremendous changes during the process. Seven parent nucleus of stilbene glycosides and 55 substituents, including 5-HMF and a series of derivatives, were identified in PM. 146 stilbene glycosides were detected in RRM, the number of detected compounds increased from 198 to 219 as the processing time increased from 4 to 32 h. Among the detected compounds, 102 stilbene glycosides may be potential new compounds. And the changing trend of the compounds can be summarized in 3 forms: gradually increased, gradually decreased, first increased and then decreased or decreased first. The content of trans-THSG was indeed decreased during processing, as it was converted into a series of derivatives through the esterification reaction with small molecular compounds. The clarification of secondary metabolite group can provide a basis for the follow-up study on the mechanism of pharmacodynamics and toxicity of PM, and for screening of relevant quality markers.

Keywords: Reynoutria multiflora, stilbene glycosides, processed, UHPLC-Q-Exactive plus orbitrap MS/MS, structural and content changes

Abbreviations: DDMP, 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyranone; 5-HMF, 5-hydromethylfurfural; RM, Reynoutria multiflora Thunb; PRM, processed Reynoutria multiflora; RRM, raw Reynoutria multiflora; TCM, Traditional Chinese Medicine; Trans-THSG, tran-2, 3, 5, 4′-tetrahydroxystilbene-2-O-β-D-glucopyranoside; UHPLC-Q-Exactive plus orbitrap MS/MS, ultra high performance liquid chromatography tandem quadrupole/electrostatic field orbitrap high-resolution mass spectrometry.
1 INTRODUCTION

Traditional Chinese medicine processing is a unique pharmaceutical technology derived from the theory of traditional Chinese medicine. It has played a prominent role in the clinical practice of traditional Chinese medicine for thousands of years, ensuring the safety and effectiveness of treatment. After processing with different temperatures, durations, solvents or excipients, the components of traditional Chinese medicine have undergone different changes. Ingredients will be dissolved, decomposed or transformed into new components, resulting in increasing or decreasing of the compounds. All these changes are closely related to the property and efficacy of traditional Chinese medicine. Therefore, it is of great significance to study the changes of chemical components before and after processing of traditional Chinese medicine.

The root of Reynoutria multiflora Thunb. (Polygonum multiflorum Thunb.), well known as He-shou-wu in China, has been widely used in TCM clinical practice for centuries (Li et al., 2017). Lots of research have shown that RRM and its processed products have different pharmacological effects. RRM has the effect of detoxification, carbuncle elimination, relaxing bowel. And PRM shows the effect of tonifying liver and kidney, tonic medicines and hair-blacking (Cheung et al., 2014; Lin et al., 2015; Chinese Pharmacopoeias Commission, 2020). RRM is commonly processed by steaming with black bean or water, which has been officially documented in the Chinese pharmacopoeia. However, the processing time was not specified in the processing specification. Therefore, the processing time of PRM on the market varies greatly, ranging from 2 to 18 h (Lin et al., 2018). But in our previous studies, we have screened out that the best effect of PRM was processing for 24–32 h (Qiu et al., 2008). The quality of PRM is inhomogeneous in the market, the main reason for this phenomenon is that the processing mechanism of PRM is not clear. The increased reports of hepatotoxicity of RRM in recent years (Dong et al., 2014; Lei et al., 2015; Zhang et al., 2019) may also be related to incomplete processing.

Previous research indicated that the main chemical components of RRM were secondary metabolites, including stilbene glycosides, anthraquinone and polyphenols were the most representative (Choi et al., 2007; Lin et al., 2015; Sun et al., 2015). The fragmentation pathways of typical constituents and chemical profiles of RRM have been studied by an on-line UHPLC-ESI-linear ion trap-Orbitrap hybrid mass spectrometry method (Xu et al., 2012; Qiu et al., 2013). The secondary metabolites were quantitatively analyzed by HPLC/IC-MS/MS to study the chemical components before and after processing of R. multiflora, which showed that the content of some chemical substances was changed by processing. In our previous study, the contents of 5-HMF, THSG, emodin and physcion were changed during the processing (Chen et al., 2012). The content of THSG, a compound that possess anti-oxidative, anti-aging, anti-tumor, anti-inflammatory and liver protective activities (Lv et al., 2007; Shao et al., 2012; Lin et al., 2015; Yang et al., 2020), was decreased (Qiu et al., 2006; Fu, 2011). However, there is no research report on the secondary metabolite group produced by stilbene glycosides in the process, and the clarification of secondary metabolite group can provide a basis for the follow-up study on the mechanism of pharmacodynamics and toxicity of PM, and for screening of relevant quality markers.

In this study, a simple and rapid method for the determination of RRM and PRM by UHPLC-Q-Exactive plus orbitrap MS/MS was established, and the qualitative analysis of RRM and PRM were carried out in vitro to obtain a clear chemical map. The fragment ions at m/z 405.1087 and 243.0656 were selected as characteristic fragments, the secondary metabolites in RRM and processed PRM samples prepared with different durations were characterized and identified, then, the changes of stilbene glycosides during processing were further analyzed.

2 MATERIALS AND METHODS

2.1 Materials

RRM and PRMs that had been processed for 4, 8, 12, 18, 24 and 32 h were provided by Shanghai Dehua Traditional Chinese Medicine CO., Ltd., and the corresponding batch numbers were HSW2018051101-S, HSW2018051101-4H, HSW2018051101-8H, HSW2018051101-12H, HSW2018051101-18H, HSW2018051101-24H, and HSW2018051101-32H. The samples were authenticated by Professor Zhihai Huang, and voucher specimens were deposited in the Materials Medica Preparation Lab of the Second Affiliated Hospital of the Guangzhou University of Chinese Medicine.

Trans-2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucopyranoside (THSG), cis-THSG and polydatin were purchased from yuanye Bio-Technology Co., Ltd. Acetonitrile (No. H08J11E115101, P27A11P07214, T15A10F85743, purity ≥98%, Shanghai, China), acetonitrile and methanol (HPLC grade), were supplied by E. Merck (Darmstadt, Germany), formic acid (HPLC grade) was purchased from fisher (United States), ultra-pure water was prepared by a Milli-Q water purification system (Millipore, MA, United States).

2.2 Sample Processing Method

All the samples were prepared using following method: 1 g sample powder was ultrasonicated for 30 min with 25 ml of 70% ethanol, followed by filtration and then evaporated the filtrate. 5 ml of ultrapure water were added to dissolve the residue and then extracted twice with 15 ml of ethyl acetate. The resulting mixture was combined with an ethyl acetate solution and evaporated over a water bath; after that, 1 ml of methanol was added to dissolve the residue and centrifugation (15,000 rpm, 4°C) for 10 min by a 1.5 ml centrifuge tube. Finally, the supernatant of the treatment samples was injected into the UPLC-Q-Exactive plus orbitrap MS/MS system.

2.3 UHPLC-Q-Exactive Plus Orbitrap MS/MS Analysis

2.3.1 Liquid Chromatography

All the samples were analysed using an Ultimate 3000 UPLC system (Dionex, United States) that was controlled with Thermo Xcalibur software (Thermo Fisher Scientific, United States). The samples were separated using a Kinetex UPLC C18 column...
The mobile phase consisted of solvent A (0.1% formic acid) and solvent B (acetonitrile). A gradient elution was applied using the following optimized gradient program: 8-8% B at 0–3 min, 8–28% B at 3–25 min, 28–40% B at 25–26 min, 40–50% B at 26–28 min, 50–70% B at 28–30 min, 70–90% B at 30–32 min, and 90–90% B at 32–35 min. The flow rate was kept at 0.4 ml/min, the sample injection volume was 1 μL, and the column temperature was maintained at 25°C.

2.4 Mass Spectrometry
Mass spectrometry was performed on a Q-Exactive Plus™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, United States) in negative ion mode. The scan mass range was set at m/z 100–1,200. The parameter settings were as follows: a full scan and fragment spectral resolution of 70,000 FWHM and 17, 50 FWHM, respectively; capillary temperature was 350°C; auxiliary gas heater temperature was 350°C; spray voltage was −3.2 KV; sheath gas flow rate was 40 Arb; auxiliary gas flow rate was 15 Arb; and S-lens RF level was set at 50. The acquisition mode of stepped NCE (normalized collision energy) was using with settings of 30, 50, and 70 eV. The accumulated resultant fragment ions were injected into the Orbitrap mass analyzer for single-scan detection.

Considering the possible elemental composition of the RM components, the types and quantities of expected atoms were set as follows: carbon ≤50, hydrogen ≤200, oxygen ≤20, nitrogen ≤3. The accuracy error threshold was fixed at 5 ppm.

3 RESULTS AND DISCUSSION
3.1 Base Peak Chromatograms
The chemical profiles of RRM and processing PRMs were analyzed by UHPLC-Q-Exactive plus orbitrap MS/MS, the representative base peak chromatograms of RRM and processing RM (24 h) are shown in Figure 1. Some differences were observed between the two base peak chromatograms. The stilbene glycosides and their derivatives were distributed from 7 to 22 min. The representative base peak chromatograms of processing of PRM (7–22 min) are shown in Figure 2.

3.2 Fragmentation Pathway of THSG and Derivatives
To identify the derivatives of THSG in the processing RM, the trans-THSG and cis-THSG standard were firstly analyzed by
UPLC-Q-Exactive plus MS/MS under the above-mentioned conditions. Trans-THSG (A3-5, \( t_R = 11.18 \) min) and cis-THSG (A3-2, \( t_R = 7.58 \) min) had a \([\text{M-H}]^-\) ion at \( m/z \) 405.1187 with only a dominant ion at \( m/z \) 243.0654 (C\(_{14}\)H\(_{11}\)O\(_4\)) in MS\(^2\) spectrum. These two ions could be used as a diagnostic ion for identify stilbene glycosides. Compound A3-1, A3-3 and A3-4 (\( t_R = 6.67, 9.75 \) and 9.89 min) also had an \([\text{M-H}]^-\) ion at \( m/z \) 405.1187 (C\(_{20}\)H\(_{21}\)O\(_9\)), and showed a fragment ion at \( m/z \) 243.0654 in their MS\(^2\) spectrum, indicating that they are isomers of THSG. A3-5 was identified as trans-THSG and A3-2 was cis-THSG, and A3-1 should be isomer of cis-THSG, A3-3 and A3-4 should be isomers of trans-THSG. (Figure 3).

3.3 Identification of Tetrahydroxystilbene-O-Hexoside Derivatives

During the processing, Maillard reaction occurred, producing a large number of compounds, including acetone alcohol, 2, 3-butanediol, succinic acid, 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyranone (DDMP), 5-hydroxymethyl furfural (5-HMF) and its derivatives (Liu, 2018). Stilbene glycosides may react with products of Maillard reaction or small molecules, such as gallic acid and catechusic acid, in high temperature and high humidity environment.

Most stilbene glycosides in RM showed common fragmentation pathways and two diagnostic fragment ions at \( m/z \) 405.1192(C\(_{29}\)H\(_{27}\)O\(_9\)) and 243.0654 (C\(_{14}\)H\(_{11}\)O\(_4\)). These were used for rapidly extracting and analyzing unknown stilbene glycosides. According to the structural characteristics of THSG, the linking points of stilbene derivatives with other compounds contain glycosyl hydroxyl moiety and phenyl hydroxyl moiety. According to the cleaved fragments, it can be inferred as follows: 1. if there is a fragment from loss of C\(_6\)H\(_{10}\)O\(_5\) by parent ion, the linking point should be phenyl hydroxyl moiety; 2. The cleavage fragment contains the ion at \( m/z \) 405.1187 of THSG and the ion at \( m/z \) (hexoside + substituent), and there is no fragment to loss of C\(_6\)H\(_{10}\)O\(_5\), so the linking point should be glycosyl hydroxyl moiety; 3. if the fragment is only the ion at \( m/z \) 243.0654, most of the glycosyl hydroxyl moiety may be linked, but there is also a probability that the hexoside of THSG and the substituent on the phenyl hydroxyl moiety will split at the same time, so the linking point cannot be determined in this case. We use tetrahydroxystilbene-O-(substituent)-hexoside to name them.
Compounds A1-1 and A1-2 displayed a [M-H]− ion at m/z 375.1081 (C_{19}H_{19}O_{8}) and the product ion at m/z 243.0654 derived from the loss of a pentose (mostly arabinose). By comparing with literature, Compounds A1-1 and A1-2 were tentatively identified as tetrahydroxystilbene-O-pentose.

Compounds A2-1, A2-2, and A2-3 gave a [M-H]− ion at m/z 389.1242 (C_{20}H_{21}O_{8}) and the product ion at m/z 243.0654 derived from the loss of a deoxyhexose (mostly rhamnose), indicated that it was a THSG derivative. Compounds A2-1, A2-2, and A2-3 were tentatively characterized as tetrahydroxystilbene-O-deoxyhexoside.

Compounds A4-1 and A4-2 displayed a high resolution [M-H]− ion at m/z 423.1295 and gave element composition of C_{20}H_{23}O_{10}. The MS^2 spectra gave identical ions at m/z 261.0764 (C_{14}H_{13}O_{5}) and 243.0654 (C_{14}H_{11}O_{4}), respectively. The loss of C_{6}H_{10}O_{5} (hexoside) and H2O to produce the deprotonated moiety ion at m/z 243.0655, indicated can be identified as stilbene derivatives, but the specific structure is not yet determined.

Compounds A5-1 ~ A5-4 showed the same [M-H]− ion at m/z 433.1136 (C_{20}H_{23}O_{10}) and the MS^2 spectra gave ions at m/z 271.0608 (C_{14}H_{11}O_{5}) and 243.0654 (C_{14}H_{11}O_{4}). Without further information, compounds A5-1 ~ A5-4 were tentatively characterized as tetrahydroxystilbene-O-hexoside-O-formic acid acyl (phenolic hydroxyl moiety).

Compounds A6-1 and A6-2 showed the same [M-H]− ion at m/z 437.1450 (C_{21}H_{25}O_{10}) and the MS^2 spectra gave ions at m/z 275.0922 (C_{15}H_{13}O_{3}) and m/z 243.0655 (C_{14}H_{11}O_{4}), the loss of C_{6}H_{10}O_{5} (hexoside) and CH_{4}O to produce the deprotonated moiety ion at m/z 243.0655, allowed us to infer that they were tetrahydroxystilbene derivative, but the specific structure is not yet determined.

Compounds A7-1 ~ A7-5 gave a [M-H]− ion at m/z 447.1300 (C_{22}H_{23}O_{10}) and loss 204 Da to produce ion at m/z 243.0656 in the MS^2 spectrum, which indicated that the presence of a hexose group and an acetyl. Thus, compounds A7-1 ~ A7-5 were preliminarily characterized as tetrahydroxystilbene-O-(acetyl)-hexoside.

Compounds A8-1 and A8-2 showed the same [M-H]− ion at m/z 449.1086 (C_{21}H_{21}O_{11}) and the MS^2 spectra gave ions at m/z 287.0554 (C_{15}H_{11}O_{6}) and 243.0654 (C_{14}H_{11}O_{4}) form continuous loss of C_{6}H_{10}O_{3} and CO_{2}. Thus, the carbonate acyl substituted THSG was detected and compounds A8-1 and A8-2 were identified as tetrahydroxystilbene-O-hexoside-O-carbonate acyl (phenolic hydroxyl moiety).

Compound A9 displayed a high resolution [M-H]− ion at m/z 457.1116 and gave element composition of C_{23}H_{21}O_{10}, the product ion at m/z 243.0654 originated from the loss of C_{6}H_{10}O_{5} (hexoside + hydroxycyclopropenon). By investigating literature, compound A9 was preliminarily identified as tetrahydroxystilbene-O-(hydroxycyclopropenon)-hexoside. Similarly, compounds A10 and A11 were tentatively identified as tetrahydroxystilbene-O-(acrylic acid acyl)-hexoside and tetrahydroxystilbene-O-(propionyl)-hexoside, since the loss of C_{6}H_{12}O_{5} (hexoside + acrylic acid) and C_{6}H_{14}O_{6} (hexoside + propionic acid) were detected.
Compounds A12-1 and A12-2 showed the same [M-H]− ion at m/z 463.1244 (C_{22}H_{25}O_{11}) and the MS² spectra gave ion at m/z 243.0654 (C_{9}H_{15}O_{7} (C_{10}H_{19}O_{5} and C_{2}H_{2}O_{2}). Thus, the glycolic acid substituted THSG was detected and compounds A12-1 and A12-2 were identified as tetrahydroxystilbene-O-(glycolic acid acyl)-hexoside.

Compounds A13-1, A13-2, and A13-3 showed the same [M-H]− ion at m/z 477.1396 (C_{25}H_{27}O_{12}) and the MS² spectra gave identical ions at m/z 341.1019 (C_{9}H_{15}O_{7}), 297.1125 (C_{18}H_{17}O_{4}) and 243.0655 (C_{14}H_{11}O_{4}). The ion at m/z 477.1396 loss of C_{3}H_{4}O_{2} produce the ion at m/z 405.1184. By comparing literature, compounds A13-1, A13-2, and A13-3 were tentatively identified as tetrahydroxystilbene-O-hexoside-O-valerate (phenolic hydroxyl moiety).

Compounds A14-1 ~ A14-4 showed the same [M-H]− ion at m/z 489.1759 (C_{25}H_{27}O_{12}) and the MS² spectra gave identical ions at m/z 405.1176 (C_{20}H_{23}O_{9}), 327.1222 (C_{19}H_{19}O_{5}) and 243.0656 (C_{14}H_{11}O_{4}). The loss of C_{2}H_{2}O to produce the deprotonated THSG moiety ion at m/z 405.1176. Furthermore, the ion at m/z 327.1222 assigned as loss of C_{2}H_{2}O form the ion at m/z 489.1759. By investigating literatures, compounds A14-1 ~ A14-4 were identified as tetrahydroxystilbene-O-hexoside-O-valerate acyl (phenolic hydroxyl moiety).

Compounds A15 displayed a high resolution [M-H]− ion at m/z 499.1241 and gave element composition of C_{25}H_{25}O_{11}, the product ions at m/z 337.0704 (C_{19}H_{13}O_{5}), 293.0812 (C_{19}H_{11}O_{5}) and 243.0654 (C_{14}H_{11}O_{4}) originated from the consecutive loss of C_{2}H_{10}O_{5} (hexoside), CO_{2} and C_{2}H_{2} (5-hydroxyfuran-2-carbaldehyde). By investigating literature, compound A15 was preliminarily identified as tetrahydroxystilbene-O-hexoside-O-5-hydroxyfuran-2-carbaldehyde (phenolic hydroxyl moiety).

Compounds A16-1 and A16-2 showed the same [M-H]− ion at m/z 501.1393 (C_{23}H_{25}O_{11}) and the MS² spectra gave ions at m/z 339.0858, 321.0756 and 243.0654 form continuous loss of C_{6}H_{10}O_{5} (hexoside), CO_{2} and C_{2}H_{4} (Figure 4). By investigating literature, compounds A16-1 and A16-2 were identified as tetrahydroxystilbene-O-hexoside-O-4-hydroxymethyl-5H-furan-2-one (phenolic hydroxyl moiety).

Compound A17 gave a [M-H]− ion at m/z 503.1553 (C_{23}H_{25}O_{11}) and the product ions at m/z 341.1019 (C_{9}H_{15}O_{7}), 297.1125 (C_{18}H_{17}O_{4}) and 243.0656 (C_{14}H_{11}O_{4}). By comparing literature, compound A17 was tentatively characterized as tetrahydroxystilbene-O-hexoside-O-5-hydroxymethyl-4, 5-dihydrofuranone (phenolic hydroxyl moiety).

Compounds A18-1 ~ A18-5 showed the same [M-H]− ion at m/z 505.1346 (C_{24}H_{25}O_{12}) and in A18-1 and A18-2 MS² spectra, gave ions at m/z 405.1178 and m/z 243.0655, in A18-3 ~ A18-5 MS² spectra, gave ions at m/z 343.0799 and m/z 243.0655. By comparing literature, compounds A18-1 and A18-2 were preliminarily characterized as tetrahydroxystilbene-O-(succinic acid acyl)-hexoside, and compounds A18-3 ~ A18-5 were identified as tetrahydroxystilbene-O-hexoside-O-succinic acid acyl (phenolic hydroxyl moiety).

Compounds A19-1 and A19-2 were eluted at 11.85 and 12.00 min, and the molecular formula was C_{24}H_{27}O_{12} (m/z 507.1500). The MS² spectra gave identical ions at m/z 345.0966 (C_{19}H_{13}O_{5}), m/z 313.0709 (C_{19}H_{13}O_{5}), m/z 285.0763 (C_{19}H_{13}O_{5}), m/z 255.0656 (C_{18}H_{11}O_{4}) and m/z 243.0654 (C_{14}H_{11}O_{4}). By comparing literature, compounds A19-1 and A19-2 were identified as tetrahydroxystilbene-O-hexoside-O-dihydroxybutyrate (phenolic hydroxyl moiety).

Compound A20 gave a [M-H]− ion at m/z 511.1603 (C_{27}H_{25}O_{10}) and the product ions at m/z 349.1068 (C_{19}H_{13}O_{5}) and 243.0655 (C_{14}H_{11}O_{4}) form continuous loss of C_{6}H_{10}O_{5} (hexoside) and C_{2}H_{2}O (salicyloyl). By comparing literature, compound A20 was tentatively characterized as tetrahydroxystilbene-O-hexoside-O-salicyloyl (phenolic hydroxyl moiety).
Compounds A21-1 and A21-2 showed the same [M-H]− ion at m/z 512.1555 (C26H26O16N) and the MS2 spectra gave ions at m/z 405.1175 (C20H13O8) and 243.0655 (C14H11O4). By comparing literature, compounds A21-1 and A21-2 were tentatively identified as tetrahydroxystilbene-O-(aminocatechololy)-hexosides. Similarly, compound A25 was tentatively identified as tetrahydroxystilbene-O-(pyroglutamyl)-hexoside.

Compounds A22-1 ~ A22-4 showed the same [M-H]− ion at m/z 513.1497 (C26H25O12), compounds A22-1, A22-2 and A22-3 loss 162 Da to produce ion at m/z 351.0862, and then loss 108 Da (C6H4O2) to produce ion at m/z 243.0656 in the MS2 spectrum. And in compound A22-4 MS2 spectra, there was a fragment ion at m/z 243.0655, the proposal fragmentation pathway shown in Figure 5. By investigating literatures, compounds A24-1, A24-2, and A22-3 were identified as tetrahydroxystilbene-O-hexoside-O-2, 5-bis-hydroxymethyl furan (phenolic hydroxyl moiety). The MS2 of compound A22-4 showed the same [M-H]− ion at m/z 513.1394, in A26-2, A26-3 and A26-4 MS2 spectra, the fragment ions at m/z 405.1167 (C20H13O8), 357.0967 (C19H12O7), 339.0858 (C18H11O6), 297.0760 (C17H10O5) and 243.0655 (C14H11O4), by investigating literatures, compound A26-1 was identified as tetrahydroxystilbene-O-(glutaryl)-hexoside, A26-2, A26-3 and A26-4 were identified as tetrahydroxystilbene-O-hexoside-O-glutaryl (phenolic hydroxyl moiety).

Compounds A22-1 (tR = 12.84 min), A27-2 (tR = 13.07 min) and A27-3 (tR = 13.82 min) showed the same [M-H]− ion at m/z 521.1294 (C24H25O12) and the MS2 spectra gave identical ions at m/z 405.1177 (C20H13O8), 359.1115 (C19H12O7) and 243.0754 (C14H11O4). The loss of C6H10O5 to produce the deprotonated THSG moiety ion at m/z 405.1177, thus, compounds A27-1, A27-2, and A27-3 identified as tetrahydroxystilbene-O-hexoside-O-malic acid acyl (phenolic hydroxyl moiety).

Compounds A28-1 ~ A28-5 showed the same [M-H]− ion at m/z 525.1398 (C25H25O12), the MS2 of A28-1 ~ A28-3 spectra gave ions at m/z 525.1398, 405.1179, 363.0883, 243.0858 and 137.0228. The product ions at m/z 363.0883 originated from the loss of hexoside (C6H10O5). Thus, the salicylic acid acyl substituted THSG was detected and compounds A28-1 ~ A28-3 identified as tetrahydroxystilbene-O-hexoside-O-salicylic acid acyl (phenolic hydroxyl moiety). The MS2 of A28-4 and A28-5 spectra gave ions 405.1179, 243.0858 and 137.0228, but there were no 363.0833 fragment ion. Thus, compounds A28-4 and A28-5 were identified as tetrahydroxystilbene-O-(salicylic acid acyl)-hexoside.

Compounds A29-1, A29-2 and A29-3 gave a [M-H]+ ion at m/z 527.1190 (C26H23O12) and the MS2 spectra showed identical ions at m/z 365.0652 (C20H13O7) and 243.0659 (C14H11O4). The MS2 spectrum showed losses of C6H10O5 and C6H2O3, respectively, to produce characteristic aglycone ion at m/z 243.0659. By comparing literature, compounds A29-1, A29-2, and A29-3 were tentatively identified as tetrahydroxystilbene-O-
hexoside-O-5-formylfuran-2-carboxylyl (phenolic hydroxyl moiety).

Compounds A30-1 ~ A30-7 showed the same [M-H]\(^-\) ion at m/z 529.1345 (C\(_{26}\)H\(_{25}\)O\(_{12}\)), the MS\(^2\) spectra of A30-1, A30-2 and A30-6 gave ions at m/z 367.0870 (C\(_{20}\)H\(_{15}\)O\(_{7}\)), 323.0914 (C\(_{19}\)H\(_{15}\)O\(_{3}\)) and 243.0651 (C\(_{14}\)H\(_{11}\)O\(_{4}\)). The product ions originated from the consecutive loss of hexoside (C\(_6\)H\(_{10}\)O\(_5\)), CO\(_2\) and C\(_5\)H\(_4\)O. In A30-3 and A30-4 spectra, gave ions at m/z 367.0807 (C\(_{20}\)H\(_{15}\)O\(_{7}\)), 243.0656 (C\(_{14}\)H\(_{11}\)O\(_{4}\)) and 123.0071 (C\(_6\)H\(_3\)O\(_3\)). In A30-5 and A30-7 spectra, gave ions at m/z 405.1176 (C\(_{20}\)H\(_{21}\)O\(_9\)), 243.0656 (C\(_{14}\)H\(_{11}\)O\(_4\)) and 123.0071 (C\(_6\)H\(_3\)O\(_3\)). By investigating literatures, the substituent group of the compound was 5-hydroxymethyl-furfural. And according to the fragmentation fragments, it can be inferred that the binding sites are different (Figure 6). A30-1, A30-2, and A30-6 were tentatively identified as tetrahydroxystilbene (phenolic hydroxyl moiety)-O-5-hydroxymethylfuran-2-carboxylyl-hexosides (hydroxyl moiety), A30-3 and A30-4 were identified as tetrahydroxystilbene (phenolic hydroxyl moiety)-O-5-hydroxymethylfuran-2-carboxylyl-hexoside (carboxyl moiety), A30-5 and A30-7 were identified as tetrahydroxystilbene-O-(5-hydroxymethylfuran-2-carboxylyl)-hexoside.

Compounds A31-1 ~ A31-10 were eluted at 2.58, 2.75, 3.58, 4.66, 5.00 min 5.64, 12.14, 15.02, 15.36, 15.74 min, they both showed an accurate [M-H]\(^-\) ion at m/z 531.1511 (C\(_{26}\)H\(_{29}\)O\(_{12}\)) and 243.0651 (C\(_{14}\)H\(_{11}\)O\(_{4}\)). In their MS\(^2\) spectra, the [M-H]\(^-\) ion showed fragment ions at m/z 405.1180 (C\(_{20}\)H\(_{21}\)O\(_9\)), 369.0966 (C\(_{20}\)H\(_{17}\)O\(_{7}\)), 351.0863 (C\(_{18}\)H\(_{15}\)O\(_{3}\)) and 243.0655 (C\(_{14}\)H\(_{11}\)O\(_{4}\)). All the MS\(^2\) of the compounds have molecular fragment m/z 369.0966, indicated that the compounds are formed by dehydration of substituents and phenolic hydroxyl groups of stilbene glycosides. By investigating literature (Liu, 2018), compounds A31-1 ~ A31-10 were tentatively characterized as tetrahydroxystilbene-O-hexoside-DDMP (phenolic hydroxyl moiety).

Compounds A32-1 ~ A32-5 showed the same [M-H]\(^-\) ion at m/z 533.1658 (C\(_{26}\)H\(_{30}\)O\(_{12}\)) and the MS\(^2\) spectra gave identical ions at m/z 371.1124 (C\(_{20}\)H\(_{18}\)O\(_{7}\)), 327.0863 (C\(_{18}\)H\(_{16}\)O\(_{6}\)) and 243.0657 (C\(_{14}\)H\(_{11}\)O\(_{4}\)). The MS\(^2\) showed losses of C\(_4\)H\(_7\)O\(_3\) and C\(_{6}\)H\(_{11}\)O\(_{5}\) due to substituent and hexose moiety, respectively, to produce characteristic aglycone ion at m/z 243.0657 (C\(_{14}\)H\(_{11}\)O\(_{4}\)). By comparing literature, compounds A32-1 ~ A32-5 were tentatively characterized as tetrahydroxystilbene-O-hexoside-adipic acid acyl (phenolic hydroxyl moiety).

Compound A33-1 and A33-2 gave a [M-H]\(^-\) ion at m/z 537.1609 (C\(_{26}\)H\(_{29}\)O\(_{13}\)) and the product ion at m/z 405.1180 (C\(_{20}\)H\(_{21}\)O\(_9\)) form loss of C\(_{11}\)H\(_{18}\)O\(_{9}\) (C\(_6\)H\(_{10}\)O\(_5\) and C\(_5\)H\(_8\)O\(_4\)). By comparing literature, compound A33-1 and A33-2 were tentatively characterized as tetrahydroxystilbene-O-(arabinoyl)-hexoside.

Compounds A34-1 ~ A34-4 showed a [M-H]\(^-\) ion at m/z 541.1353 (C\(_{27}\)H\(_{29}\)O\(_{12}\)) and the MS\(^2\) spectra of A34-1 and A34-2, showed fragment ions at m/z 405.1175 (C\(_{20}\)H\(_{21}\)O\(_9\)), 297.0610 (C\(_{14}\)H\(_{13}\)O\(_{3}\)), 243.0657 (C\(_{14}\)H\(_{11}\)O\(_{4}\)), 153.0179 (C\(_{7}\)H\(_{5}\)O\(_{3}\)), 89.0459 (C\(_{5}\)H\(_{5}\)O\(_{2}\)) and 57.0225 (C\(_{3}\)H\(_{3}\)O\(_{2}\)).
respectively, indicated that they were THSG derivatives. The loss of C₆H₁₀O₅ to produce the deprotonated THSG moiety ion at m/z 405.1175, allowed us to infer that they were protocatechuic acid substituted THSG products. And the ion at m/z 297.0610 assigned as protocatechuic acid acyl-hexoside moiety, produced protocatechuic acid ion at m/z 153.0179. A34-3 and A34-4 were eluted at 21.70 and 22.07 min, the [M⁺]- ions showed fragment ions at m/z 379.0793 (C₂₃H₁₅O₃) and 243.0657 (C₁₄H₁₁O₄), respectively, originated from the consecutive loss of hexoside (C₆H₁₀O₅) and protocatechuic acid (C₆H₅O₄). There results indicate that protocatechuic acid substituted to the phenolic hydroxyl moiety in compounds A34-3 and A34-4. Therefore, compounds A34-1 and A34-2 were identified as tetrahydroxystilbene-O-hexoside acyl (glycosyl hydroxyl moiety), and A34-3 and A34-4 were identified as tetrahydroxystilbene-O-hexoside-O-protocatechuic acid acyl (phenolic hydroxyl moiety).

Compound A35 gave a [M⁺]- ion at m/z 543.1121 (C₂₆H₂₇O₁₃) and the product ions at m/z 405.1196 (C₂₀H₁₂O₉) and 243.0655 (C₁₄H₁₁O₄) form consecutive loss of C₆H₁₀O₅ and C₆H₁₀O₅. By comparing literatures, compound A35 was tentatively identified as tetrahydroxystilbene-O-(furan-dicarboxylic acid acyl)-hexoside. And compound A36 gave a [M⁺]- ion at m/z 543.1501 (C₂₁H₂₁O₁₀) and the product ion at m/z 381.0963 (C₁₉H₁₃O₇) and 243.0655 (C₁₄H₁₁O₄) form consecutive loss of C₆H₁₂O₄, CO₂ and C₆H₁₀O. By investigating literatures, A36 was tentatively identified as tetrahydroxystilbene-O-hexoside-methoxymethyl-furancarboxylic acid acyl (phenolic hydroxyl moiety).

Compounds A37-1 and A37-2 showed the same [M⁺]- ion at m/z 547.1453 (C₂₆H₂₅O₁₂) and the MS² spectra gave ions at m/z 385.0914 (C₂₅H₁₇O₈) and 243.0655 (C₁₄H₁₁O₄) from consecutive loss of C₆H₁₀O₅ and C₆H₁₀O₅. By comparing literature, compounds A37-1 and A37-2 were tentatively identified as tetrahydroxystilbene-O-hexoside-o xoacidoic acid acyl (phenolic hydroxyl moiety). Similarly, compounds A38-1, A38-2 and A38-3 were tentatively identified as tetrahydroxystilbene-O-hexoside-hydroxydic acid acyl (phenolic hydroxyl moiety), since the ions at m/z 387.1066 (C₂₀H₁₄O₈) and 243.0655 (C₁₄H₁₁O₄) from consecutive loss of C₆H₁₀O₅ and C₆H₁₀O₅.

Compounds A39-1 ~ A39-5 displayed a high resolution [M⁺]- ion at m/z 551.1556 and gave element composition of C₂₉H₂₇O₁₁. In A39-2 ~ A39-5 MS² spectra, the [M⁺]- showed fragment ions at m/z 405.1180 (C₂₉H₂₆O₆), 243.0656 (C₁₄H₁₁O₄), 163.0397 (C₆H₁₅O₃) and 145.0280 (C₆H₁₂O₂). The product ions at m/z 405.1180 and 234.0656 originated from the consecutive loss of p-hydroxyccinnamoyl (C₆H₅O₂) and hexoside (C₆H₁₀O₅). Thus, p-hydroxyccinnamoyl substituted THSG was detected and compounds A39-2 ~ A39-5 were identified as tetrahydroxystilbene-O-(p-hydroxyccinnamoyl)-hexoside. In A39-1 MS² spectra, the [M⁺]- showed fragment ions at 399.1018 (C₂₃H₁₇O₈), 243.0655, 163.0396 and 145.0279, indicated p-hydroxyccinnamoyl was linked to phenolic hydroxyl moiety. Thus, compound A39-1 was identified as tetrahydroxystilbene-O-hexoside-p-hydroxyccinnamoyl (phenolic hydroxyl moiety).

Compounds A40-1 ~ A40-8 showed the same [M⁺]- ion at m/z 557.1295 (C₂₉H₂₅O₁₃) and the MS² spectra gave identical ions at m/z 405.1179 (C₂₀H₁₂O₅), 313.0555 (C₁₉H₁₃O₅), 243.0654 (C₁₄H₁₂O₄) and 169.0127 (C₇H₅O₄). The loss of C₆H₁₀O₅ and C₆H₁₀O₅ to produce the deprotonated resveratrol moiety ion at m/z 243.0654, a galloyl group was present, the ion at 313 ([galloylglucose—H⁺]) appeared as base peak in the MS² spectra. It could further fragment ion m/z 169 ([gallic acid — H⁺]). Therefore, compounds A40-1 ~ A40-8 were identified as tetrahydroxystilbene-O-hexoside-O-galloyl (glycosyl hydroxyl moiety) (Qi et al., 2013).

Compounds 41-1 and 41-2 showed the same [M⁺]- ion at m/z 561.1609 (C₂₇H₁₉O₁₃) and the MS² spectra gave identical ions at m/z 405.1174 (C₂₀H₁₂O₅) and 243.0654 (C₁₄H₁₈O₄). By comparing literatures, compounds 41-1 and 41-2 were identified as tetrahydroxystilbene-O-(galbosine C)-hexoside.

Compounds A42-1 ~ A42-13 gave precursor ion [M⁺]- at m/z 567.1712 (C₂₉H₂₃O₁₄), in A42-2 ~ A42-4 and A42-7 ~ A42-13 MS² spectra, the [M⁺]- ion showed fragment ion at m/z 243.0654 (C₁₄H₁₁O₄), indicated that the consecutive neutral loss of hexoside, they were disaccharide THSGs. By comparing literature, they were tentatively characterized as tetrahydroxystilbene-O-di-hexosides (Figure 7). But in A42-1, A42-5, and A42-6 MS² spectra, the [M⁺]- ion showed fragment ions at m/z 477.1401 (C₂₃H₁₅O₁₁), 447.1278 (C₂₃H₁₅O₁₀), 315.0857 (C₁₇H₁₅O₈), 285.0761 (C₁₆H₁₃O₇), and 243.0655 (C₁₄H₁₁O₄), respectively. The fragment ions 477.1401 [M-H-90 Da, C₃H₆O₃]”, 447.1278 [M-H-120 Da, C₃H₆O₃]” are diagnostic the last two neutral loss fragments suggested that a C-glycoside was connected with the stilbene glycoside. Therefore, A42-1, A42-5 and A42-6 were determined as tetrahydroxystilbene-O-hexoside-C-glycoside.

A42-14 (tR = 18.29 min), A42-15 (tR = 19.50 min) and A42-16 (tR = 21.64 min) showed the molecular formula were C₂₉H₂₆O₁₂ (m/z = 567.1504), which was 162 Da heavier than that of THSG, and different from A42-1 ~ A42-13. In their MS² spectra, the [M⁺]- ion showed fragment ions at m/z 405.1203 (C₂₀H₁₃O₄), 243.0654 (C₁₄H₁₂O₄), 161.0229 (C₉H₆O₃), respectively. Indicated that the consecutive loss of caffeoyl (C₆H₅O₃) and hexoside (C₁₄H₁₀O₄). Thus, the caffeoyl substituted THSG was detected and compound A42-14, A42-15 and A42-16 identified as tetrahydroxystilbene-O-(caffeoyl)-hexoside.

Compounds A43-1 ~ A43-6 showed the same [M⁺]- ion at m/z 573.1251 (C₂₉H₂₅O₁₄) and the MS² spectra gave identical ions at m/z 243.0657 (C₁₄H₁₁O₄), 166.9971 (C₆H₅O₂) and 123.0071 (C₇H₅O₂). By comparing literature, Compounds A43-1 ~ A43-6 were tentatively identified as tetrahydroxystilbene-O-(tetrahydroxybenzoic acid acyl)-hexoside.

Compound A44 gave a [M⁺]- ion at m/z 575.1402 (C₂₉H₂₇O₁₄) and the product ions at m/z 337.0707 (C₁₉H₁₃O₄) and 244.0655 (C₁₄H₁₁O₄). By comparing
literatures, compound A44 was tentatively identified as tetrahydroxystilbene-O- (dioxoheptane-dicarboxylic acid acyl)-hexoside.

Compounds A45-1 ~ A45-5 showed the same [M-H]⁻ ion at m/z 581.1655 (C₃₀H₂₉O₁₂), the MS² spectra gave ions at m/z 405.1177 (C₂₀H₂₁O₉), 337.0921 (C₁₆H₁₂O₆), 243.0655 (C₁₄H₁₁O₄), 193.0493 (C₁₀H₉O₄) and 175.0387 (C₁₀H₇O₃). Its MS² spectrum gave characteristic ions at m/z 337.0921 ([feruoylglucose-H]⁻), m/z 193.0493 ([ferulic acid-H]⁻) and m/z 175.0387, therefore, compounds A45-1 ~ A45-5 were identified as tetrahydroxystilbene-O-hexoside-O-feruloyl (glycosyl hydroxyl moiety).

Compound A46 gave a [M-H]⁻ ion at m/z 591.2075 (C₂₉H₃₅O₁₃) and the product ions at m/z 405.1203 (C₂₀H₂₁O₉), 243.0655 (C₁₄H₁₁O₄) and 185.0806 (C₉H₁₃O₄). By comparing literature, compound A46 was tentatively identified as tetrahydroxystilbene-O- (hydroxynonanedioic acid acyl)-hexoside.

Compound A47 gave a [M-H]⁻ ion at m/z 613.1769 (C₂₇H₃₃O₁₆) and the product ions at m/z 405.1203 (C₂₀H₂₁O₉) and 243.0655 (C₁₄H₁₁O₄). By comparing literatures, compound A47 was tentatively identified as tetrahydroxystilbene-O- (glucoheptanoyl)-hexoside.

Compounds A48-1, A48-2 and A48-3 showed the same [M-H]⁻ ion at m/z 719.1825 (C₃₃H₂₉O₁₈) and the MS² spectra gave identical ions at m/z 557.1287 (C₂₇H₂₅O₁₃), 405.1174 (C₂₅H₂₁O₉), 313.0557 (C₁₃H₁₀O₅), 243.0655 (C₁₄H₁₁O₄) and 169.0126 (C₇H₅O₅), the loss of C₆H₁₀O₅ to produce compound A39 ion at m/z 557.1287, and the fragmentation ions were the same. Therefore, A48-1, A48-2 and A48-3 were identified as tetrahydroxystilbene-O-dihexoside-galloyl (glycosyl hydroxyl moiety).

Compounds A49-1 ~ A49-6 displayed a high resolution [M-H]⁻ ion at m/z 827.2399 and gave element composition of C₄₀H₄₃O₁₉. The MS² spectra gave ions at 405.1165 (C₂₅H₂₁O₅), 259.0607 (C₁₄H₁₁O₂) and 243.0656 (C₁₄H₁₁O₄), forming the ions 405.1165 and 243.0656 indicated that they should be THSG derivatives. By comparing literature, compounds A49-1 ~ A49-6 were tentatively identified as polygonumoside C/D.

Compounds A50-1 ~ A50-8 displayed a high resolution [M-H]⁻ ion at m/z 837.2601 and gave element composition of...
The MS² spectra gave ions at m/z 675.2068 (C₃₆H₃₅O₁₃), 513.1556 (C₃₀H₂₅O₈), 431.1342 (C₂₂H₂₃O₉), 405.1165 (C₂₀H₂₁O₉), 269.0813 (C₁₆H₁₃O₄) and 243.0656 (C₁₄H₁₁O₄). The consecutive neutral loss of hexoside, forming ions at m/z 675.2068 and 513.1556, and forming the ions 405.1165, 243.0656 and 431.1342, 269.0813 indicated that was cleavage into two glycosides. By comparing literature, compounds A₅₀-₁ ~ A₅₀-₈ were identified as polygonumnolide D (Figure 8). Similarly, compound A₅₂ was tentatively characterized as hydroxylation polygonumnolide D, since the [M-H]⁻ ion at m/z 853.2560 (C₄₂H₄₅O₁₉), which was 16 Da (O) higher than that of A₅₀, and the MS² spectra gave ions at m/z 447.1300 (C₂₂H₂₁O₁₀), 405.1175 (C₂₀H₂₁O₉), 285.0765 (C₁₆H₁₃O₅) and 243.0656 (C₁₄H₁₁O₄).

Compounds A₅₁-₁ ~ A₅₁-₅ showed the same [M-H]⁻ ion at m/z 841.2551 (C₄₁H₄₅O₁₉), which was 14 Da (CH₂) higher than that of A₄₉. The MS² spectra gave ions at m/z 405.1182 (C₂₀H₂₁O₉), 273.0764 (C₁₅H₁₃O₅) and 243.0655 (C₁₄H₁₁O₄), and the ion m/z 273.0764 was 14 Da (CH₂) higher than that of A₄₉ ion m/z 259.0607. therefore, compounds A₅₁-₁ ~ A₅₁-₅ were identified as methylation polygonumoside C/D. Similarly, compounds A₅₃-₁ ~ A₅₃-₄ were identified as methylation methylation polygonumoside C/D, since the [M-H]⁻ ions at m/z 857.2502 (C₄₁H₄₅O₂₀), which was 14 Da (CH₂) higher than that of A₅₁, and the MS² spectra gave ions at m/z 405.1172 (C₂₀H₂₁O₉), 289.0709 (C₁₅H₁₃O₆) and 243.0656 (C₁₄H₁₁O₄).

### 3.4 Identification of Trihydroxystilbene-O-Hexoside Derivatives

Compounds B₁-₁, B₁-₂ and B₁-₃ gave a [M-H]⁻ ion at m/z 389.1240 (C₂₀H₂₃O₈) and the product ion at m/z 227.0702 (C₁₄H₁₁O₃). The loss of C₆H₁₀O₅ was confirmed by MS² spectra and indicated a hexose neutral loss. Compared with the control substance, B₁-₂ was identified as polydatin, compounds B₁-₁ and B₁-₃ were identified as isomer polydatin.

Compounds B₂-₁ ~ B₂-₄ showed the same [M-H]⁻ ion at m/z 541.1345 (C₂₇H₂₅O₁₂) and the MS² spectra gave ions at m/z 313.0559 (C₁₃H₁₃O₅), 227.0702 (C₁₄H₁₁O₃), 169.0128 (C₇H₅O₅). Similar with compounds A₄₀, compounds B₂-₁ ~ B₂-₄ were identified as trihydroxystilbene-O-hexoside-O-galloyl (glycosyl hydroxyl moiety).

Compounds B₃-₁ and B₃-₂ showed the same [M-H]⁻ ion at m/z 457.1136 (C₂₉H₂₁O₁₀) and the MS² spectra gave ions at m/z 295.0605 (C₁₇H₁₁O₅) and 227.0702 (C₁₄H₁₁O₃). By comparing literature, compounds B₃-₁ and B₃-₂ were identified as
trihydroxystilbene-O-hexoside-O-acid delticque acyl (phenolic hydroxyl moiety).

Compound B4 gave a [M-H]− ion at m/z 535.1816 \((C_{26}H_{32}O_{12})\) and the product ion at m/z 227.0702 \((C_{11}H_{11}O_{3})\) derived from the loss of \(C_6H_9O_3\) (hexoside) and \(C_6H_6O_4\) (deoxyhexose, mostly rhamnose). By investigating literatures, compound B4 was identified as trihydroxystilbene-(deoxyhexose)-O-hexoside.

Compound B5 displayed a high resolution [M-H]− ion at m/z 359.1132 and gave element composition of \(C_{19}H_{19}O_{7}\), the product ions at m/z 359.1129 \((C_{19}H_{19}O_{7})\) and 227.0701 \((C_{14}H_{11}O_{4})\). The product ion at m/z 227.0701 originated from the loss of pentose (mostly arabinose). Therefore, compound B5 was identified as trihydroxystilbene-O-pentose.

### 3.5 Identification of Pentahydroxystilbene Glycoside Derivatives

Compounds C1-1 ~ C1-7 displayed a high resolution [M-H]− ion at m/z 421.1318, and gave element composition of \(C_{20}H_{21}O_{10}\), which was 14 Da (CH₂) higher than that of THSG, the MS² spectra gave ion at 259.0609 \((C_{14}H_{11}O_{3})\). By comparing literature, compounds C1-1 ~ C1-7 were tentatively identified as pentahydroxystilbene glycosides.

Compound C2 gave a [M-H]− ion at m/z 545.1291 \((C_{26}H_{32}O_{13})\) and the product ions at m/z 421.1128 \((C_{20}H_{21}O_{10})\), 259 \((C_{15}H_{10}O_{3})\) and 123.0070 \((C_{6}H_{6}O_{3})\) derived from the loss of \(C_6H_9O_3\) (5-HMF) and \(C_6H_6O_4\) (hexoside). By investigating literatures, compound C2 was identified as pentahydroxystilbene-(5-HMF)-O-hexoside.

### 3.6 Identification of Tetrahydroxy-Phenanthrene-O-Hexoside Derivatives

Compounds D1-1 and D1-2 gave a [M-H]− ion at m/z 403.1030 \((C_{20}H_{21}O_{10})\) and prominent fragment ion at m/z 241.0497 \((C_{14}H_{13}O_{4})\) in MS² spectrum, which were showed 2 Da less than that of THSG. It can be inferred that they were dehydrogenated product of THSG. By comparing literatures (Qiu et al., 2013), compounds D1-1 and D1-2 were identified as tetrahydroxy-phenanthrene-O-hexoside.

Compounds D2-1 ~ D2-8 showed the same [M-H]− ion at m/z 549.1605 \((C_{26}H_{29}O_{13})\) and the MS² spectra gave ions at m/z 387.1072 \((C_{19}H_{19}O_{8})\), 297.0760 \((C_{17}H_{13}O_{3})\) and 241.0497 \((C_{14}H_{13}O_{4})\). Similarly, compounds A38, compounds D2-1 ~ D2-8 were identified as tetrahydroxy-phenanthrene-O-hexoside-O-p-hydroxycinnamoyl (phenolic hydroxyl moiety).

### 3.7 Identification of Dihydrotetrahydroxystilbene-O-Hexoside Derivatives

Compound E1 gave a [M-H]− ion at m/z 407.1343 \((C_{20}H_{23}O_{8})\) and prominent fragment ion at m/z 245.0811 \((C_{14}H_{13}O_{3})\) in MS² spectrum, which were showed 2 Da higher than that of THSG. It can be inferred that they were dihydrogenated product of THSG. By comparing literatures, compounds E1 was identified as dihydrotetrahydroxystilbene-O-hexoside.

Compounds E2-1 and E2-2 showed the same [M-H]− ion at m/z 527.1552 \((C_{22}H_{27}O_{11})\) and the MS² spectra gave ions at m/z 365.1017 \((C_{17}H_{17}O_{5})\), 335.0918 \((C_{20}H_{20}O_{5})\) and 245.0814 \((C_{9}H_{13}O_{4})\). Similarly, compounds A27, compounds E2-1 and E2-2 were identified as dihydrotetrahydroxystilbene-O-hexoside-saliclyc acid acyl (phenolic hydroxyl moiety).

Compound E3 displayed a high resolution [M-H]− ion at m/z 539.1766, and gave element composition of \(C_{22}H_{24}O_{12}\). The MS² spectra gave ion at 245.0811 \((C_{11}H_{13}O_{3})\) derived from the loss of \(C_6H_9O_3\) (hexoside) and \(C_6H_6O_4\) (pentose, mostly arabinose). Compound E3 was identified as dihydrotetrahydroxystilbene-O-(pentose)-hexoside.

### 3.8 Identification of Pentahydroxy-Phenanthrene-O-Hexoside Derivatives

Compounds F1-1 and F1-2 showed the same [M-H]− ion at m/z 419.0980 \((C_{20}H_{18}O_{10})\) and the MS² spectra gave ion at m/z 257.0542 \((C_{14}H_{13}O_{2})\), which were showed 16 Da higher than that of compounds D1. Therefore, compounds F1-1 and F1-2 were identified as pentahydroxy-phenanthrene-O-hexosides.

### 3.9 Identification of Dihydroxystilbene-O-Hexoside Derivatives

Compound G1 gave a [M-H]− ion at m/z 373.1286 \((C_{14}H_{13}O_{2})\) and prominent fragment ion at m/z 211.0751 \((C_{9}H_{13}O_{3})\) in MS² spectrum, which were showed 32 Da less than that of THSG. It can be inferred that they were dehydroxylation product of THSG. Therefore, compounds G1 was identified as dihydroxystilbene-O-hexoside.

Compounds G2-1 and G2-2 showed the same [M-H]− ion at m/z 525.1396 \((C_{27}H_{25}O_{11})\) and the fragment ions at m/z 525.1392, 313.0558 \((C_{13}H_{13}O_{6})\), 211.0751 \((C_{14}H_{14}O_{2})\), 169.0128 \((C_{9}H_{13}O_{2})\) and 151.0020 \((C_{7}H_{3}O_{3})\) in MS² spectra. Similar with compounds A39 and compounds B2, compounds G2-1 and G2-2 were identified as dihydroxystilbene-O-hexoside-O-galloyl (glycosyl hydroxyl moiety).

### 3.10 Structural Changes of Stilbene Glycosides

#### 3.10.1 Structural Changes of Parent Stilbene Glycosides

Stilbenes are regared to be derived from phenylalanine metabolism in plants (Isvett et al., 2009) (Figure 9). THSG is the highest and most reported compound in RM Studies have shown that resveratrol was the intermediate product of THSG, which was hydroxylated to form terahydroxystilbene, and then glycosylated to form THSG (compounds A3) (Zhao, 2017). Polydatin (compound B1-2) was glycosylated form resveratrol. Pentahydroxystilbene-O-hexoside (compounds C1) was synthesized by rehydroxylation of tetrahydroxystilbene and
FIGURE 9 | The biosynthesis pathway of resveratrol.

FIGURE 10 | The structural changes pathway of stilbene glycosides.
then glycosylation. The phenanthrene moiety was formed by cyclization of 6-H and 2′-H positions of tetrahydroxystilbene, which can stabilize the cyclization of aglycone, and then glycosylated to form tetrahydroxy-phenanthrene-O-hexoside (compounds D1). The tetrahydroxy-phenanthrene was hydroxylated to form pentahydroxy-phenanthrene, then glycosylated to form pentahydroxy-phenanthrene-O-hexoside (compounds F1). Dihydrotetrahydroxystilbene-O-hexoside (compounds E1) was synthesized from tetrahydroxystilbene by double bond opening and then glycosylated. The resveratrol lost a hydroxyl group in the plant, and dihydroxystilbene-O-hexoside (G1) was obtained by the glycosylation of dihydroxystilbene (Figure 10).

3.11 Structural Changes in Stilbene Glycosides Substituents

Compounds containing malonyl, acetyl, caffeoyl and other aromatic acyls are very common in various plants (Barnes et al., 1994; Klejdus et al., 2001; Ye et al., 2007). And galloyl, malonyl, acetyl, caffeoyl, coumaroyl, feruloyl substituent have been reported in raw PM in our previous research (Qiu et al., 2013). In total, fifty-five substituents were found in this study. Except for the above-mentioned substituents, there was also some organic acids existed, such as formic acid, acetic acid, carbonic acid, propionic acid, glycolic acid, lactic acid, valeric acid, succinic acid, dihydroxy butyric acid, salicylic acid, glutaric acid, malic acid, catechic acid and p-hydroxybenzoic acid. Maillard reaction products, mainly includes 2, 3-dihydroxy-3, 5-dihydroxy-6-methyl-4H-pyranone (DDMP, A31) and 5-hydromethylfurfura (5-HMF, A22) and its derivatives from glucose or glycine were also observed as stilbene glycosides substituents (Perez Locas and Yaylayan, 2008; Harishchandra et al., 2011; Liu, 2018). The aldehyde end of 5-HMF is oxidized to carboxylic acid, 5-hydroxymethylfuran-2-carboxyl acid (5-HMF-2-CC, A30) was produced, then methylation and oxidation produced methoxymethyl-furancarboxylic acid (MM-FBA, A36) and furan-dicarboxylic acid (FDA, A35). 2, 5-bis-(hydroxymethyl) furan (2, 5-BHMF, A23) was synthesized by the reduction of 5-HMF. 5-formylfuran-2-carboxyl acid (5-FF-2CC, A29) was formed by oxidation of the hydroxyl end of 5-HMF. Then decarboxylation and reoxidation were carried out to form 5-hydroxymethyl-5H-2-oxidofuran (5HMF-2O, A16), then hydrogenation reaction was reacted to produce 5-hydroxymethyl-4,5-dihydrofuranone (5-HM-4, 5-DF, A17), and the change path of 5-HMF is shown in Figure 11) all substituents information is shown in Supplementary Table S1.

3.12 Content Change Trend of Stilbene Derivatives

The peak areas of all compounds were based on the extracted ion chromatographic peaks. The mean and SD values were calculated and the column diagram of each compound was drawn (Supplementary Table S1). A total of 219 compounds were identified in this study, 73 compounds were not found in RRM, 21 compounds were not found in 4 h PRM, 9 compounds were not found in 8 h PRM and 1 compound
was not found in 12 h PRM. During the process of PM, THSG dehydrated with other small molecules to form new compounds.

The change trend of the highest content compound trans-THSG and its cis-THSG slightly increased before 8 h, then decreased gradually, and was lower than that of RRM at 24 h. This is a model of content change, but the peak time may be 4, 8, 12 or 18 h. The second model, such as compound A3-4 (isomer trans-THSG), gradually increase during the processing time. The third model, such as compound A45-1 (tetrahydroxystilbene-O-hexoside-feruloyl (glycosyl hydroxyl moiety), gradually decreased with processing time.

The results showed that the content and quantity of stilbene glycoside compounds have undergone tremendous changes during the processing process. Although the content of THSG in PRM is indeed lower than that in RRM, a large number of stilbene glycoside derivatives are produced in the processing process, so the total content of stilbene glycoside compounds in the PRM will not be reduced. Conventional understanding, RRM after processing can enhance efficiency and reduce toxicity, and the content of THSG also decreases with the processing time, is THSG toxic? After this experimental study, it can be proved that THSG should not be a toxic component, because its derivatives will metabolize into compounds similar to THSG in vivo, enhancing the efficacy of THSG.

4 CONCLUSION

In the present study, a simple and effective method was developed for characterization of stilbene compounds in the roots of RRM and PRMs by UHPLC-Q-Exactive plus orbitrap MS/MS. Stilbene glycosides were distinguished by diagnostic fragment ions at m/z 405.1087 and 243.0656, accurate mass measurements and fragmentation pathways. Based on the proposed strategy, the metabolic process of 7 stilbene glycosides in plants was identified, and 55 substituent and Maillard reaction process were identified. Finally, 219 stilbene glycosides derivatives were identified, of which 102 compounds may be potential new compounds. The 55 substratants include monosaccharide, disaccharide, organic acid and Maillard reaction products (DDMP, 5-HMF and its derivatives) and so on. The quality and quantity of stilbene glycosides changed during the processing of RM. 73 compounds were not found in RRM, 21 compounds were not found in 4 h PRM, 9 compounds were not found in 8 h PRM and 1 compound was not found in 12 h PRM, and the change trend of the compounds can be summarized into 3 models: gradually increased, gradually decreased, first increased and then decreased. 181 trans-THSG derivative products were obtained through the hydrolysis and dehydration reaction between trans-THSG and small molecules compounds, after this experimental study, it can be proved that THSG should not be a toxic component, because its derivatives will metabolize into compounds similar to THSG in vivo, enhancing the efficacy of THSG.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

XQ and ZH conceived and designed the experiments; JB, WC, and HS preformed the experiments and analyzed the data; JH, WX, and JZ contributed reagents/materials/analysis tools; JB wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.757490/full#supplementary-material

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