A stepwise strategy integrating metabolomics and pseudotargeted spectrum–effect relationship to elucidate the potential hepatotoxic components in Polygonum multiflorum

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Polygonum multiflorum (PM) Thunb., a typical Chinese herbal medicine with different therapeutic effect in raw and processed forms, has been used worldwide for thousands of years. However, hepatotoxicity caused by PM has raised considerable concern in recent decades. The exploration of toxic components in PM has been a great challenge for a long time. In this study, we developed a stepwise strategy integrating metabolomics and pseudotargeted spectrum–effect relationship to illuminate the potential hepatotoxic components in PM. First, 112 components were tentatively identified using ultraperformance liquid chromatography-quadrupole-time-of-flight-mass spectrometry (UPLC-Q-TOF-MS). Second, based on the theory of toxicity attenuation after processing, we combined the UPLC-Q-TOF-MS method and plant metabolomics to screen out the reduced differential components in PM between raw and processed PM. Third, the proposed pseudotargeted MS of 16 differential components was established and applied to 50 batches of PM for quantitative analysis. Last, three mathematical models, gray relational analysis, orthogonal partial least squares analysis, and back propagation artificial neural network, were established to further identify the key variables affecting hepatotoxicity in PM by combining quantitative spectral information with toxicity to hepatocytes of 50 batches of PM. The results suggested that 16 components may have different degrees of hepatotoxicity, which may lead to hepatotoxicity through synergistic effects. Three components (emodin dianthrones, emodin-8-O-β-D-glucopyranoside, PM 14–17) were screened to have significant hepatotoxicity and could be used as toxicity markers in PM as well as for further studies on the mechanism of toxicity. Above all, the study established an effective strategy to explore the
hepatotoxic material basis in PM but also provides reference information for in-depth investigations on the hepatotoxicity of PM.

KEYWORDS
polygonum multiflorum, hepatotoxicity, pseudotargeted spectrum–effect relationship, plant metabolomics, mathematical model

1 Introduction

*Polygonum multiflorum* (PM) Thunb., known as one of the “Four Great Herbs” in ancient China (PM, Ginseng, Ganoderma lucidum, Cordyceps sinensis), is widely used in many Chinese prescriptions and patent medicines due to its remarkable therapeutic effects. As early as the Song dynasty, the historical Chinese medicine document “Kai Bao Ben Cao” recorded the pharmacological efficacy of PM as “strengthen muscles and bones, benefit the essence, prolong life” (Lei et al., 2015; Teka et al., 2021). With different therapeutic effects, in general, PM can be divided into raw and processed PM in clinical applications. The Chinese pharmacopoeia states that raw PM has the effects of detoxification, eliminating carbuncles, moistening the intestine, and relieving constipation, while the processed product has been used mainly to tonify the liver and kidney, nourish blood, blacken hair, strengthen the body, dissolve turbidity, and lower blood lipid levels (Chinese Pharmacopoeia Commission, 2020). Meanwhile, modern pharmacological research has shown that the main active ingredients of PM are stilbene glycosides, anthraquinones, glycosides, phospholipids, flavonoids and others, which significantly contribute to delaying senescence, preventing cardiovascular diseases, tonifying the kidney and hair, improving intelligence, enhancing immune function, protecting the liver, moistening the intestine, and defecating as well as have antibacterial and anti-inflammatory effects (Lin et al., 2015; Liu et al., 2018).

However, since the 1990s, there has been a rapid increase in reports of liver damage caused by PM, which has attracted attention at home and abroad (But et al., 1996; Park et al., 2001; Han et al., 2019). Thereafter, the drug supervision and administration departments of the United Kingdom, Japan, and China successively issued warnings or regulatory measures for the risk of liver damage from PM and its preparations. In fact, the ancient textbook “Ben Cao Hui Yan” recorded “*Polygonum multiflorum*, taste bitter, astringent, flavor mild, slightly toxic.” Processed PM can significantly relieve the toxicity and change the efficacy of PM, and a relatively complete processing method for PM was used in the Song dynasty. Modern pharmacological studies have also confirmed that processing can greatly reduce the risk of hepatotoxicity of PM. However, the chemical composition of PM is complex and diverse, and PM mainly includes stilbenes, anthraquinones, anthranone, glycosides, phospholipids, flavonoids, and tannins (Lin et al., 2015; Teka et al., 2021). The issue of which components of PM cause hepatotoxicity remains a major subject that needs to be addressed.

In general, the traditional research approach was to first isolate and identify compounds from PM and then to evaluate the compounds for hepatotoxicity *in vivo* or *in vitro*. This process was time-consuming and laborious but also neglected the synergistic toxic effects of the compounds in PM, so the hepatotoxicity of PM could not be comprehensively evaluated. Therefore, it was imperative to develop an effective scientific strategy to efficiently screen out the toxic components of PM.

In recent years, with the development of high-resolution mass spectrometry (MS) and metabolomics techniques, ultraperformance liquid chromatography–quadrupole-time-of-flight-MS (UPLC-Q-TOF-MS) has made it possible to characterize complex components in PM in a short time, and metabolomics combined with chemometrics has enabled the rapid search for differential markers between raw and processed PM (Liu et al., 2016; Shang et al., 2021). Moreover, spectrum–efficiency relationship research has opened a new window for the evaluation of modern traditional Chinese medicine (TCM), which combines the complex chemical information of TCM with pharmacological efficacies and screens the important features related to the efficacy by means of chemometric statistical methods or machine learning (Zhang et al., 2018; Rao et al., 2022). In particular, great progress has been made in the joint analysis of the spectrum–effect relationship based on fingerprinting and pharmacodynamics for illuminating active ingredient markers in complex TCMs. However, the lack of ultraviolet absorption of many compounds and trace components and the lack of standard reference materials pose serious challenges for absolute quantification. Xu’s proposed pseudotargeted metabolomics, establishing a scheduled MRM method for the semiquantification of metabolites, gave us an inspiration of what to do (Luo et al., 2015; Zheng et al., 2020). Compared with previous methods, the established UPLC-coupled scheduled MRM method was a more powerful technique with significant advantages of high sensitivity, wide universality, low matrix effects, and accurate quantification.

In the current study, a stepwise strategy integrating metabolomics and pseudotargeted spectrum–effect relationship was set up to clarify the potential hepatotoxic components in PM (Figure 1). First, the chemical composition of PM was comprehensively characterized.
using UPLC-Q-TOF-MS. Second, based on the theory of detoxification after PM processing, the distinctive differential components between raw and processed PM were screened out using plant metabolomics. Third, the proposed pseudotargeted MRM semiquantitative profiles of the differential marked components were established in different batches of PM. Fourth, the toxicity of various batches of PM to the hepatocytes L02 and HepG2 was investigated. At last, gray relational analysis (GRA), orthogonal partial least squares (OPLS) analysis, and back propagation artificial neural network (BP-ANN) models were established to correlate the peak areas of pseudotargeted spectra with the IC_{50} values of toxicity to further obtain the hepatotoxic components in PM.

2 Materials and methods

2.1 Materials and reagents

Methanol and acetonitrile of LC/MS grade were obtained from Merck (Darmstadt, Germany). High-performance liquid chromatography-grade ethanol and dimethyl sulfoxide (DMSO) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ultra-pure water was prepared using a Milli-Q system (Billerica, MA, United States). Standard products of stilbene glycoside, emodin, etc., were provided by the China National Institute for Food and Drug Control. Physcion-8-O-β-D-glucopyranoside, physcion-1-O-β-D-glucopyranoside, and aloe-emodin-3-hydroxymethyl-β-D-glucopyranoside were purchased from Standard Technology Co., Ltd (Shanghai, China). The purity of all standards was above 98%. Formic acid was acquired from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). A 0.22-µm filter membrane was purchased from Dikema Technology Co., Ltd. (Beijing, China).

The hepatic cell lines HepG2 and L02 were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco’s Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 culture medium (Biological Industries, Israel), fetal bovine serum (FBS; Biosera, France), 1% penicillin-streptomycin (Targetmol, China), and 0.25% trypsin-ethylenediaminetetraacetic acid (Wisent, Canada) were used for cell culture. Staurosporine (STSP) and CCK-8 reagent were obtained from Targetmol (Shanghai, China). A total of 384 cell culture plates were purchased from Jet Bio-Filtration Co., Ltd. (Guangzhou, China). The Victor Nivo multimode plate reader was from PerkinElmer (Shanghai, China).
Sample A: 36 batches of raw and processed PM from different origins or batches. In total, 0.1 g was taken from each batch to make 10 portions of mixed samples as quality control (QC). Sample B: 30 batches of raw PM and 20 batches of processed PM. Samples A and B all met the requirements of the Chinese pharmacopoeia. The samples were stored at the China National Institute for Food and Drug Control (Beijing, China). Detailed sample information can be found in Suplementary Tables S1, S2.

2.2 Sample and standard solution preparation

Sample A (46 samples in total, filtered through a No. 3 sieve): The sample (1.0 g) was weighed precisely and placed in a 50-mL conical flask. Then, 50 mL of 70% ethanol was added, and the mixture was weighed, sonicated for 30 min, cooled, and replenished. The extracted solution was collected for UPLC-Q-TOF-MS analysis.

The standard solution was prepared by weighing 1 mg of standard powder and adding 2 mL of methanol solution to dissolve it for the qualitative test. All standard and sample solutions were filtered through 0.22-μm Millipore filtration before injection.

Sample B (50 samples): 20 g of PM was weighed and extracted with 300 mL of 70% ethanol three times for 30 min each time. Then, the extracted solutions were combined and concentrated under pressure and subsequently freeze-dried to powder. The dry extract powder weighing 40 mg was dissolved in 40 mL of 70% ethanol for UPLC-qqq-MS/MS analysis. Of note, 30 mg of dried extract powder was weighed precisely and prepared as a storage solution of 200 mg/mL. Then, a series of concentrations of working solutions (1000, 400, 160, 64, 25, 10, and 4 μg/mL) were obtained by gradient dilution with culture medium for the in vitro cytotoxicity assay.

2.3 Ulterformance liquid chromatography-quadrupole-time-of-flight-mass spectrometry analysis

2.3.1 Chemical composition characterization

The extract solution of the PM mixed sample in sample A was analyzed using UPLC-Q-TOF-MS. Analysis was performed using an Acquity™ UPLC Class I system equipped with a photodiode array (PDA) detector and Q-TOF SYNAPT G2-Si (Waters, Manchester, United States). Chromatographic conditions: The temperature of the column and autosampler was maintained at 40°C and 6°C. The flow rate was 0.3 mL/min, and the injection volume was 1 μL. The binary mobile phase contained solvent A (0.1% FA in deionized water, v/v) and solvent B (methanol, LC-MS grade). The peptides of the elution gradient were initial 10% B, linear gradient 40% B (22 min), 70% B (33 min), 100% B (44–46 min), 10% B (46.2 min), and holding 10% B to 50 min. The PDA detector used 3D range from 190 to 400 nm. MS conditions: The UPLC-MS system was operated in the negative ion and MS² data acquisition mode. Experimental parameters were set as follows: capillary voltage at −2.5 kV (ESI⁺); source temperature at 115°C; cone voltage at 40 V; ramp trap MS collision energy of 20–50 V; desolvation temperature at 450°C; cone gas flow of 50 L/h; desolvation gas flow of 900 L/h; and scan range of m/z 50–1,500 Da. At the same time, an external reference consisting of 1.0 ng/mL solution of leucine enkephalin was used to produce a reference ion at m/z 554.2615 Da ([M-H]⁻) in negative ion mode for real-time mass correction during acquisition. The obtained mass spectrometric data were analyzed using UNIFI software in combination with a self-built database of PM compounds and reference standards as well as fragment ion matching strategies to fully characterize the components of PM.

2.3.2 Plant metabolomics analysis

Processed sample A (n = 46) was analyzed using UPLC-Q-TOF-MS under the same chromatographic and mass spectrometric conditions as in Section 2.3.1. The acquired data were further deconvolved into a data matrix (Rt-m/z-intensity) by Progenesis QI software (Waters, Milford, MA, United States). After further data preprocessing, chemometric (principal component analysis (PCA), PLS-DA, OPLS-DA) analysis was performed using Simca-P 14.1 software. Combining univariate statistical analysis of P and FC values with multivariate statistical analysis of VIP values further screened out the differential ions between raw and processed PM.

2.4 Ultraperformance liquid chromatography-qqq-MS/MS analysis

2.4.1 Scheduled MRM method development

The scheduled MRM ion pairs were established based on the differential ions and secondary fragment ions of PM from the results of Section 2.3.2. Then, combined with the composition identification results of PM, the MRM ion pairs were further confirmed, and the proposed pseudotarget MRM method was constructed. This method was used to perform semiquantitative analysis in sample B, and the peak area data of the marker components were acquired.

The analysis of samples was performed using a Waters Acquity™ UPLC I-Class system equipped with a Xevo TQ-XS mass spectrometer (Waters, Milford, MA, United States). The chromatographic column and chromatographic separation conditions were the same as the conditions of the previous
UPLC-Q-TOF-MS method. The optimal MS conditions were as follows: capillary voltage at 2.5 kV under negative mode; source temperature at 150°C; desolvation gas temperature at 500°C; desolvation gas flow at 850 L/h; and cone gas flow at 150 L/h. Ion pairs and CV and CE parameters are detailed in Table 1.

The pseudotargeted MRM method was applied for semiquantitative comparison of PM samples (raw PM: S1-S30, processed PM: Z1-Z20).

2.4.2 Method validation
The developed UPLC-MS/MS method was validated with sample Z-1 as an example in terms of specificity, repeatability, precision, linearity, and stability. Specificity was evaluated by comparing samples with the negative control. Repeatability evaluation was carried out by analyzing six replicate samples independently. Precision was investigated by six consecutive injections of the same sample. Linearity was constructed by fitting the peak area of each compound under the injection of 0.5, 1, 1.5, 2, 2.5, and 3 µl of one sample. The same sample was injected at 0, 6, 12, 24, and 30 h to verify the stability. The relative standard deviation (RSD) of the peak area of the characteristic peaks was used to evaluate the results.

2.5 Hepatotoxicity assay in vitro
Two types of hepatocytes, L02 and HepG2, were used to assess the hepatotoxicity of PM extract in vitro. L02 and HepG2 cells were inoculated in 384-well cell plates (density: HepG2 1,000 cells/well; L02 800 cells/well) with 40 µl of cell suspension per well and were incubated overnight at 37°C in a 5% CO2 incubator. HepG2 cells were cultured in DMEM containing 10% FBS and 100 U/mL penicillin and streptomycin, while L02 cells were cultured in RPMI 1640 medium. On the day of the experiment, 10 µl of compound working solution (sample B, PM extracting solution of 0.064, 0.32, 1.6, 8, 40, 200, and 1,000 µg/ml) was added to each well according to the experimental requirements, and this was cultivated at 37 °C for 72 h with 5% CO2 shielded from light. At the end of the incubation, 5 µl of CCK8 reagent was added to the cell plates, and this were incubated for 4 h with 5% CO2 at 37°C. The absorbance at 450 nm was measured, and the inhibition rate was calculated according to the following equation:

\[
\text{Inhibition ratio (\%)} = \frac{OD_{S} - OD_{NC}}{OD_{STSP} - OD_{NC}} \times 100\%
\]

where \(OD_{S}\) stands for the absorbance of the working solution (cell + medium + compound to be tested); \(OD_{NC}\) stands for the absorbance of the negative control (cell + medium + DMSO); and \(OD_{STSP}\) stands for the absorbance of the positive control (cell + medium + 10 µM STSP).

According to the inhibition ratios of the compounds, the IC50 values (the concentration corresponding to 50% of the maximum inhibition response) were calculated from the dose–response curves using GraphPad Prism 9.0. The experiment was performed three times in parallel, and finally, the mean IC50 value was obtained for each sample.

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**Table 1: Optimized ion pairs and CV and CE parameters of 16 compounds.**

| No. | Compounds | Ion pair (m/z) | CV | CE |
|-----|-----------|---------------|----|----|
| X1  | Catechin  | 289.07 > 203.07 | 30 | 29 |
| X2  | Epicatechin | 289.07 > 203.07 | 30 | 29 |
| X3  | Torachrysone-8-O-β-D-glucopyranoside | 407.13 > 245.08 | 30 | 33 |
| X4  | 7-acetyl-3,8-dihydroxy-6-methyl-1-naphthyl-β-D-glucopyranoside | 393.12 > 231.06 | 30 | 33 |
| X5  | Epicatechin-3-O-gallate | 441.08 > 289.07 | 30 | 34 |
| X6  | Emodin-8-O-β-D-glucopyranoside | 431.1 > 269.04 | 30 | 34 |
| X7  | Emodin bianthrenes | 509.12 > 253.75 | 30 | 31 |
| X8  | Emodin-physiocien bianthrenes | 523.14 > 253.83 | 30 | 30 |
| X9  | Physiocien bianthrenes | 537.15 > 254.73 | 30 | 41 |
| X10 | 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-(2-O-monogalloylesters)-glucopyranoside | 557.13 > 243.06 | 30 | 30 |
| X11 | polygonibene E | 581.16 > 243.06 | 30 | 30 |
| X12 | Polygonumnolides Cl-C4 | 671.18 > 416.11 | 30 | 26 |
| X13 | Polygonumnolides A1-A4 | 685.19 > 416.11 | 30 | 26 |
| X14 | PM 14-17 | 757.17 > 458.12 | 30 | 31 |
| X15 | PM 22-25 | 933.24 > 458.12 | 30 | 33 |
| X16 | PM 5 | 919.23 > 458.12 | 30 | 33 |
2.6 Spectrum–effect relationship analysis

2.6.1 Gray relational analysis
GRA is a method to determine the degree of association between factors based on the similarity of the geometry of the change curves in each factor. As a simple and effective method, GRA has been widely used in the evaluation of spectrum–effect relationship in TCM (Wang et al., 2018; Ma et al., 2020). In this study, the peak area of each feature was taken as the comparison series, and the 1/IC_{50} value of the cytotoxicity assessment index was defined as the reference series (all the original data were dimensionless and processed before analysis). The correlation coefficients between the reference series values and each comparison series were calculated, and the average value of the gray correlation coefficient was obtained, which was the gray correlation degree. The influence degree of each characteristic variable on hepatocyte toxicity was evaluated by comparing the gray correlation degrees.

2.6.2 Orthogonal partial least squares analysis
OPLS, a special type of multiple linear regression model, was used to find the relationship between X and Y by considering orthogonal signal correction based on partial least squares regression (Liang et al., 2017; Liao et al., 2020). In this study, an OPLS model was constructed to characterize the correlation between the hepatotoxicity index IC_{50} and the chemical peaks. The peak area of each characteristic ion was used as the independent variable X, and the IC_{50} value was used as the dependent variable Y. In SIMCA 14.0.1 (Umetrics AB, Umea, Sweden), the VIP and regression coefficient were used to find the main characteristic components that were significantly correlated with hepatotoxicity.

2.6.3 Back propagation artificial neural network analysis
The BP-ANN algorithm is a nonlinear mathematical model based on the structure of neural synaptic connections in the brain. The BP neural network is a kind of multilayer feed-forward neural network trained by the error back propagation algorithm and has been one of the most widely used neural network models (Jiang et al., 2018; Shi et al., 2018). The BP neural network can connect the input and output parameters and can continuously modify the weights and biases of each layer through iterative learning to minimize the overall error of the output layer. To screen representative hepatotoxic components from different perspectives, we used MATLAB R2019b (Mathworks, Natick, NJ, United States) to build the BP-ANN model for the association of chromatographic peaks with hepatotoxicity IC_{50}. The BP neural network was established using the characteristic peak area as the input layer neuron, the IC_{50} value as the output layer neuron, the hidden layer of one layer, and the hidden layer node number optimization as 10. Moreover, two parameters were used to evaluate the importance of the variables in the neural network.

MIV was considered to be one of the best indices for evaluating the correlation of variables in the neural network (Xu et al., 2013). The sign of the MIV value represents the direction of the correlation, and the absolute value reflects the importance of the impact. Sensitivity analysis was another important method for evaluating the connection weights in ANN models (Wang et al., 2017; Qiao et al., 2021). The contribution ratios of the characteristic peaks to the cytotoxicity index IC_{50} were calculated by connection weights. The Garson equation was applied to show the relative influence of the independent variables on the dependent variable. The equation was as follows:

$$ P_{wi} = \frac{\sum_{b=1}^{N} \left( \frac{|w_{a}d_{b}|}{\sum_{b=1}^{M} |w_{a}d_{b}|} \right) |e_{b}|}{\sum_{a=1}^{M} \left( \frac{|w_{a}|}{\sum_{a=1}^{N} |w_{a}|} \right) |e_{a}|} $$

where $P$ stands for the percentage influence of input neurons, $w$ indicates the weight between input and hidden neurons, $e$ indicates the weight between hidden and output neurons, $M$ indicates the number of input neurons, $N$ indicates the number of hidden neurons, and $v$ indicates the number of output neurons.

3 Results

3.1 Characterization of chemical components in Polygonum multiflorum

Based on the literature summary and self-built compound library, the main components of PM are stilbenes and anthraquinones. In addition, PM includes flavonoids, lignans, dianthrones, phospholipids, and polysaccharides. Comparing the negative ion response with the positive ion response, the negative ion mode had more peaks and a much stronger response, so negative ion scan was selected for detection (Supplementary Figure S1). Moreover, the peak profiles of PM between raw and processed PM were basically consistent (Supplementary Figure S2), indicating that processing does not change the types of compounds in PM but the relative content of compounds. Considering the differences in the chemical composition of PM from different batches and origins, a mixed sample was chosen for qualitative analysis. The chromatographic column, mobile phase, elution conditions, and MS conditions were further optimized. A total of 112 components were detected and preliminarily identified through self-built database matching, comparison with standard products and the literature, and fragment ion deduction (Table 2). These tentative compounds could be classified into four types according to the structural
| No | Observed RT (min) | Molecular formula | Component name | Observed m/z | Expected m/z | Mass error (ppm) | Fragment |
|----|------------------|------------------|----------------|-------------|-------------|-----------------|----------|
| 1d | 1.03             | C₂₄H₂₄O₄         | Butanedioic acid | 117.0190    | 117.0193    | -3.22          | 71.0138, 59.0137, 55.0187 |
| 2d | 1.05             | C₂₄H₂₄O₄         | 2,3-dihydro-3,5-dihydroxy-6-methyl-4-(H)-pyran-4-one | 143.0350    | 143.0349    | 0.12           | 129.0187, 96.9687, 114.0557, 78.9591 |
| 3d | 1.20             | C₂₄H₂₄O₄         | Gallic acid    | 169.0147    | 169.0142    | 2.44           | 125.0246, 96.9687, 110.0254 |
| 4d | 1.41             | C₁₃H₁₅O₇         | Gallic acid-O-glucoside | 331.0654    | 331.0665    | -3.32          | 169.0107, 125.0221 |
| 5d | 1.53             | C₁₃H₁₅O₇         | Leucine        | 130.0871    | 130.0868    | 2.31           | 88.0363, 85.0303 |
| 6d | 2.25             | C₁₃H₁₅O₇         | Citric acid    | 191.0201    | 191.0197    | 2.05           | 128.0355, 111.0086, 87.0088, 85.0294 |
| 7d | 2.45             | C₁₃H₁₅O₇         | Gallic acid    | 305.0673    | 305.0666    | 2.09           | 213.1246, 241.0027, 125.0245, 96.9604 |
| 8d | 3.16             | C₁₃H₁₅O₇         | Protopaucetchic acid-O-glucoside | 315.0697    | 315.0716    | -6.03          | 153.0177, 195.0297, 111.0094 |
| 9d | 3.82             | C₁₃H₁₅O₇         | 2-vinyl-1H-indole-3-carboxylic acid | 186.0545    | 186.0555    | -5.37          | 289.0716, 559.1279, 451.1047, 407.0772, 125.0243 |
| 10c| 4.07             | C₁₃H₁₅O₇         | Procyanidin B  | 577.1358    | 577.1351    | 1.10           | 259.0612, 255.0660, 125.0242, 407.0769, 368.0900 |
| 11c| 4.22             | C₁₁H₁₈O₃         | Quercetin      | 301.0355    | 301.0354    | 0.56           | 257.0455, 125.0243, 283.0397, 179.0243 |
| 12c| 4.88             | C₁₁H₁₈O₃         | Catechin       | 289.0721    | 289.0717    | 1.02           | 271.0553, 245.0812, 137.0244, 123.0450 |
| 13d| 5.24             | C₂₄H₂₄O₄         | Vanillic acid  | 167.0351    | 167.0344    | 4.19           | 137.0259, 123.0426 |
| 14d| 5.83             | C₂₄H₂₄O₄         | P-hydroxybenzaldehyde | 121.0296    | 121.0295    | 0.99           | 93.0341 |
| 15b| 6.54             | C₁₅H₁₄O₆         | Rumejaposide D | 449.1088    | 449.1089    | -0.15          | 259.0612, 255.0660, 125.0242, 407.0769, 368.0900 |
| 16d| 7.31             | C₁₅H₁₄O₆         | Altechrome A   | 189.0560    | 189.0557    | 1.51           | 147.0448, 124.0157 |
| 17c| 7.92             | C₁₅H₁₄O₆         | Epicatechin    | 289.0718    | 289.0717    | 0.28           | 243.0660, 125.0244 |
| 18e| 8.03             | C₁₅H₁₄O₆         | 3-O-galloyl-procyanidin B2 | 729.1465    | 729.1461    | 0.54           | 499.1267, 589.1452, 247.0619, 243.0660, 125.0244 |
| 19b| 8.58             | C₁₄H₁₀O₉         | 2,3,4,6-tetrahydroxycetophenone-3-O-β-D-glucoside | 345.0832    | 345.0827    | 1.44           | 182.0225, 242.0577, 287.0560, 125.0246, 96.9606 |
| 20a| 8.67             | C₁₉H₂₆O₈         | Isomer-Procyanidin B | 577.1351    | 577.1351    | -0.12          | 439.1056, 289.0715, 345.0818, 182.0225 |
| 21b| 9.12             | C₁₉H₂₆O₈         | Isomer-2,3,5,4′-tetrahydroxystilbene-2,3-dio-β-D-glucopyranoside | 567.1719    | 567.1719    | 0.01           | 405.1186, 387.1069, 241.0503, 281.0445 |
| 22d| 9.61             | C₁₇H₁₄O₅         | 7-hydroxy-3,4-dimethylcoumarin-5-O-β-D-glucopyranoside | 367.1029    | 367.1034    | -1.45          | 243.0665, 225.0554, 109.0293 |
| 23b| 9.61             | C₁₉H₂₆O₈         | Cis-2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucopyranoside | 405.1193    | 405.1191    | 0.52           | 243.0665, 189.0560, 137.0245, 93.0344 |
| 24c| 10.01            | C₁₇H₁₄O₅         | Polygonflavanol A | 693.1821    | 693.1825    | -0.61          | 549.1604, 287.0560, 259.0612, 125.0244, 241.0504 |
| 25a| 10.91            | C₁₅H₁₄O₇         | Emodin anthrone | 255.0660    | 255.0662    | -1.29          | 137.0241, 109.0288, 93.0345 |
| 26a| 10.92            | C₁₅H₁₄O₇         | 1,3-dihydroxy-6,7-dimethoxyxanthone-1-O-β-D-glucopyranoside | 417.1184    | 417.1555    | -1.75          | 259.0609, 255.0659, 109.0288, 137.0242 |
| 27a| 11.08            | C₁₇H₂₆O₈         | 6-methoxyl-2-acetyl-3-methyl-1,4-naphthoquinone-8-O-β-D-glucopyranoside | 421.1137    | 421.1140    | -0.73          | 407.0767, 259.0610, 255.0660, 213.0555 |

(Continued on following page)
| No | RT (min) | Molecular formula | Component name | Observed m/z | Expected m/z | Mass error (ppm) | Fragment |
|----|----------|-------------------|----------------|-------------|-------------|-----------------|----------|
| 28c | 11.11    | C_{14}H_{16}O_{30} | 3,3'-di-O-galloyl-procyanidin B2 | 881.1582 | 881.1571 | 1.24 | 725.1455; 811.1547; 407.0767; 407.0789 |
| 29a | 11.18    | C_{14}H_{16}O_{3} | Carboxyl emodin | 313.0344 | 313.0348 | -0.28 | 243.0465; 243.0466; 169.0107 |
| 30c | 11.65    | C_{22}H_{26}O_{10} | Epicatechin-3-O-gallate | 441.0828 | 441.0827 | 0.16 | 259.0739; 169.0105; 125.0245 |
| 31c | 11.75    | C_{14}H_{16}O_{3} | Kaempferol | 285.0402 | 285.0404 | -1.08 | 193.0142; 125.0245 |
| 32d | 11.89    | C_{34}H_{46}O_{13} | (+)-lyoniresinol-3-O-β-D-glucopyranoside | 581.2239 | 581.2240 | -0.15 | 549.1606; 521.1300; 387.1072; 253.0081 |
| 33b | 12.22    | C_{22}H_{26}O_{10} | 2,3,5,4-tetrahydroxystilbene-2-O-(6-O-acetyl)-β-D-glucopyranoside | 347.1288 | 447.1296 | -1.88 | 259.0608; 227.0713; 185.0608 |
| 34b | 12.70    | C_{22}H_{26}O_{14} | 2,3,5,4-tetrahydroxystilbene-2,3-di-O-β-D-glucopyranoside | 567.1724 | 567.1719 | 0.84 | 405.1179; 269.0455; 243.0664; 225.0553 |
| 35a | 13.16    | C_{14}H_{16}O_{2} | Fallacinol | 299.0558 | 299.0561 | -0.15 | 286.0480; 253.0495; 161.0243; 179.0354 |
| 36b | 13.21    | C_{22}H_{26}O_{11} | β-D-glucoside, 4-[2,3-dihydroxy-6-(hydroxymethyl)-5-(3-hydroxypropyl)-7-methoxy-2-yl]-2-methoxyphenyl | 521.2054 | 521.2023 | 5.95 | 359.1455; 313.1039; 243.0634 |
| 37a | 13.26    | C_{21}H_{26}O_{7} | Isomer-rumexioside D | 449.1090 | 449.1089 | 0.19 | 379.0815; 169.0145; 165.0558; 286.0480 |
| 38b | 13.51    | C_{34}H_{46}O_{12} | Multiflorumiside L/K | 1,217.3710 | 1,217.3718 | 0.08 | 811.2458; 646.1675; 243.0665; 405.1189 |
| 39b | 13.52    | C_{22}H_{26}O_{13} | Trans-2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucopyranoside | 405.1191 | 405.1191 | 0.08 | 243.0665; 225.0554; 109.0293; 215.0173 |
| 40a | 14.08    | C_{22}H_{26}O_{10} | PM 12-13 | 961.2381 | 961.2408 | -2.79 | 693.1812; 503.1164; 555.1137; 393.0699; 839.2375 |
| 41b | 14.47    | C_{14}H_{16}O_{3} | 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-xylloside | 375.1080 | 375.1085 | -0.51 | 243.0665; 225.0553; 109.0291 |
| 42b | 14.61    | C_{22}H_{26}O_{13} | 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-(2-O-mongalloyl ester)-glucopyranoside | 557.1310 | 557.1301 | 0.70 | 243.0666; 241.0504; 313.0567; 405.1189; 125.0243 |
| 43c | 15.54    | C_{22}H_{26}O_{12} | Quercetin-3,5-O-β-D-glucopyranoside | 463.0860 | 463.0882 | -0.43 | 405.1171; 303.0514; 379.0815; 269.0456 |
| 44c | 15.60    | C_{14}H_{16}O_{2} | Dihydroquercetin | 303.0477 | 303.0505 | -9.24 | 153.0177; 125.0221 |
| 45d | 15.84    | C_{14}H_{16}NO_{4} | Trans-5-cafeoyltyramine | 298.1084 | 298.1084 | -0.28 | 169.0836; 227.0710; 135.0450 |
| 46b | 16.45    | C_{22}H_{26}O_{13} | 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-(3-O-mongalloyl ester)-glucopyranoside | 557.1310 | 557.1301 | 1.77 | 243.0664; 313.0567; 405.1180; 407.1097; 125.0244 |
| 47b | 16.59    | C_{22}H_{26}O_{12} | 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-(2'-O-galloyl)-glucopyranoside | 541.1355 | 541.1352 | 0.65 | 243.0664; 313.0567; 105.0145; 405.1180; 467.1097 |
| 48b | 16.82    | C_{14}H_{16}O_{3} | Resveratrol | 227.0716 | 227.0713 | 1.04 | 181.0648; 143.0502; 135.0446; 117.0344 |
| 49b | 17.53    | C_{22}H_{26}O_{12} | β-Glucopyranoside, 3-hydroxy-5-[(1E)-2-4-hydroxyphenyl)ethenyl]phenyl, 2-[3,4,5-trihydroxybenzoate] | 541.1351 | 541.1352 | -0.05 | 485.1242; 313.0564; 169.0145 |
| 50d | 18.00    | C_{14}H_{16}O_{3} | 7-acetyl-3,8-dihydroxy-6-methyl-1-naphthyl-β-D-glucopyranoside | 393.1190 | 393.1191 | -0.24 | 273.0767; 231.0665; 295.0583; 161.0245 |
| 51b | 18.47    | C_{22}H_{26}O_{10} | Polygonibene D | 447.1292 | 447.1296 | -1.06 | 255.0660; 243.0664; 241.0502 |
| 52c | 19.63    | C_{22}H_{26}O_{12} | Tricin-7-O-β-D-glucoside | 491.1191 | 491.1195 | -0.74 | |

(Continued on following page)
| No | Observed RT (min) | Molecular formula | Component name | Observed m/z | Expected m/z | Mass error (ppm) | Fragment |
|----|------------------|-------------------|----------------|--------------|--------------|-----------------|----------|
| 53d | 19.75 | C_{14}H_{19}NO_{4} | N-trans-feruloyltyramine | 312.1240 | 312.1241 | -0.29 | 269.0451; 313.0553; 148.0526; 355.0447; 439.0652 |
| 54a | 19.94 | C_{22}H_{30}O_{10} | Torachryosome-8-O-(6′-O-acetyl)-β-D-glycopyranoside | 449.1447 | 449.1453 | -1.39 | 393.0615; 274.0120; 245.0815; 230.0584; 349.0699 |
| 55b | 20.13 | C_{18}H_{21}N_{2}O_{6} | N-trans-feruloyl-3-methyldopamine | 567.1498 | 567.1503 | -0.88 | 243.0634; 405.1207 |
| 56b | 20.40 | C_{20}H_{22}O_{8} | Polydatin | 389.1237 | 389.1242 | -1.19 | 283.0608; 227.0711 |
| 57d | 20.50 | C_{29}H_{28}O_{12} | Tetrahydroxystilbene-β-(caffeoyl)-glucopyranoside | 567.1498 | 567.1503 | -0.88 | 349.0708; 269.0453; 235.0552; 151.0037 |
| 58b | 20.75 | C_{21}H_{24}O_{8} | Desoxyrhaponticin | 407.1346 | 407.1347 | -0.15 | 389.1003; 241.9957; 405.1207 |
| 59b | 21.11 | C_{19}H_{21}NO_{5} | N-trans-feruloyl-3-methyldopamine | 567.1498 | 567.1503 | -0.88 | 349.0708; 269.0453; 235.0552; 151.0037 |
| 59d | 23.09 | C_{36}H_{36}N_{2}O_{8} | Cannabisin D | 623.2389 | 623.2399 | -1.66 | 389.1026; 245.0814; 225.0555 |
| 60a | 23.73 | C_{22}H_{24}O_{10} | Torachryosome-8-O-β-D-glucopyranoside | 407.1346 | 407.1347 | -0.39 | 245.0820; 230.0587; 215.0352 |
| 61a | 24.05 | C_{14}H_{18}O_{6} | Citreorosein-8-methyl ether | 283.0611 | 283.0612 | -0.51 | 240.0426; 175.0400; 145.0296 |
| 62a | 24.96 | C_{21}H_{24}O_{10} | Quercetin-3-O-rhamnoside | 447.0931 | 447.0933 | -0.45 | 285.0399; 313.0546; 337.0778; 361.0725; 245.0810 |
| 63a | 25.40 | C_{15}H_{10}O_{5} | Isomer-emodin | 269.0458 | 269.0455 | 0.26 | 93.03439; 185.0607 |
| 64a | 25.62 | C_{22}H_{30}O_{10} | Emodin-8-O-β-D-glucopyranoside | 431.0985 | 431.0983 | 0.34 | 269.0459; 225.0559 |
| 65a | 26.09 | C_{45}H_{44}O_{21} | PM 5 | 919.2315 | 919.2302 | 1.35 | 875.2393; 713.1859; 458.1210; 416.1108 |
| 66a | 26.11 | C_{45}H_{44}O_{21} | Isomer-PM 5 | 919.2303 | 919.2302 | 0.07 | 875.2387; 713.1860; 458.1215 |
| 67d | 27.22 | C_{36}H_{36}N_{2}O_{8} | (+)-Grossamid | 623.2395 | 623.2399 | -0.57 | 269.0458; 243.0660; 416.1106 |
| 70a | 27.32 | C_{17}H_{12}O_{7} | Tricin | 329.0660 | 329.0666 | -2.13 | 243.0660; 313.0484; 161.0246; 254.0583 |
| 71a | 27.72 | C_{22}H_{22}O_{10} | Physcion-1-O-β-D-glucopyranoside | 445.1135 | 445.1140 | -1.15 | 283.0611; 240.0426; 145.0295; 387.0501 |
| 72a | 28.11 | C_{15}H_{10}O_{6} | Citreorosein | 285.0410 | 285.0404 | 1.78 | 269.0458; 243.0660; 416.1106 |
| No. | RT (min) | Molecular formula | Component name | Observed m/z | Expected m/z | Mass error (ppm) | Fragment |
|-----|----------|-------------------|----------------|--------------|--------------|------------------|----------|
| 78a | 28.46    | C_{17}H_{14}O_{5} | 1,6-dimethyl ether-emodin | 297.0765 | 297.0768 | -0.06 | 257.0455; 241.0503; 224.0477; 195.0452; 183.0452 |
| 79a | 28.51    | C_{22}H_{22}O_{10} | Physcion-8-O-β-D-glucopyranoside | 445.1138 | 445.1140 | -0.04 | 283.0612; 269.0458; 240.0428 |
| 80a | 28.80    | C_{22}H_{26}O_{10} | Isoemer-torachrysone-8-O-(6''-O-acetyl)-β-D-glucopyranoside | 449.1450 | 449.1453 | -0.66 | 255.0658; 245.0815; 230.0584; 359.0909; 159.0445 |
| 81a | 29.00    | C_{22}H_{24}O_{11} | Citreorosin-O-glucoside | 447.0931 | 447.0933 | -0.50 | 243.0659; 211.1340; 329.2333; 254.0580 |
| 82a | 30.92    | C_{21}H_{22}O_{10} | Isomer-physcion | 283.0611 | 283.0612 | -0.39 | 269.0454; 253.0522 |
| 83a | 31.52    | C_{21}H_{24}O_{13} | PM 26-27 | 889.2553 | 889.2561 | -0.80 | 847.2462; 701.1841; 458.1212; 416.1108; 254.0580 |
| 84a | 33.16    | C_{17}H_{12}O_{6} | 2-Acetyl-emodin | 311.0562 | 311.0561 | 0.15 | 283.0606; 269.0457; 240.0429 |
| 85a | 33.40    | C_{23}H_{14}O_{13} | Polygonumolide E | 685.1922 | 685.1927 | -0.73 | 671.1752; 416.1109; 309.1735; 254.0586 |
| 86a | 34.74    | C_{18}H_{16}O_{4} | Chrysophanol | 253.0498 | 253.05 | -1.19 | 225.0545 |
| 87a | 34.74    | C_{18}H_{16}O_{3} | Emodin | 269.0459 | 269.0455 | 1.32 | 255.0650; 241.0505; 197.0608 |
| 88a | 36.87    | C_{18}H_{22}O_{8} | Trans/cis-emodin dianthrones | 509.1245 | 509.1242 | 0.58 | 254.0582; 225.0545 |
| 89a | 37.13    | C_{18}H_{22}O_{8} | Lunatin | 285.0404 | 285.0404 | -0.26 | 269.0457; 241.0501; 199.1704 |
| 90a | 37.17    | C_{18}H_{22}O_{8} | Physcion | 283.0609 | 283.0612 | -0.96 | 269.0456; 256.0362; 240.0422 |
| 91a | 38.69    | C_{18}H_{22}O_{8} | Trans/cis-emodin-physcion dianthrones | 523.1395 | 523.1398 | -0.68 | 254.0583 |
| 92a | 40.37    | C_{23}H_{16}O_{13} | Polygonumolide E | 537.1541 | 537.1555 | -2.60 | 243.0661; 437.3076; 339.1998 |
| 93d | 41.55    | C_{12}H_{16}O_{5} | Tetradecanoic acid ethyl ester | 255.2333 | 255.2329 | 1.31 | 205.1602; 96.9602 |
| 94d | 42.22    | C_{12}H_{16}O_{5} | Hexadecanoic acid methyl ester | 269.2485 | 269.2486 | -0.47 | 177.9736; 129.9760; 221.0857 |
| 95d | 42.22    | C_{12}H_{16}O_{5} | Hexadecanoic acid ethyl ester | 283.2645 | 283.2642 | 0.75 | 183.0122; 99.0194; 163.1127 |
| 96d | 43.13    | C_{12}H_{16}O_{5} | Ethyl oleate | 309.2796 | 309.2799 | -1.11 | 163.1127; 177.1283; 223.0358; 227.2015 |
| 97d | 43.47    | C_{12}H_{16}O_{5} | Octadecanoic acid ethyl ester | 297.2796 | 297.2799 | -0.89 | 241.0502; 119.0469 |
| 98d | 44.01    | C_{12}H_{16}O_{5} | Octadecanoic acid ethyl ester | 311.2956 | 311.2955 | 0.15 | 229.1596; 163.1130; 130.9451 |
| 99b | 12.37, 13.19 | C_{12}H_{16}O_{5} | (unknown) Dimer of stilbene glycoside | 841.2562 | 841.2561 | 0.12 | 647.1770; 485.1239; 259.0608; 227.0713; 225.0543 |
| 100b | 15.12, 16.18, 17.64, 18.56 | C_{38}H_{42}O_{13} | (Isomer) Multi florumiside A1/B1 | 809.2308 | 809.2298 | 1.15 | 647.1773; 719.1815; 485.1239; 467.1109; 267.0651 |
| 101b | 19.90, 20.67 | C_{38}H_{42}O_{13} | Polygonumolide A/B | 555.1154 | 555.1144 | 1.86 | 393.0615; 274.0120; 245.0815; 230.0584; 349.0699 |
| 102b | 21.48, 22.52 | C_{38}H_{42}O_{13} | Polygonumolide A/B/C | 809.2295 | 809.2298 | -0.44 | 647.1766; 485.1236; 255.0657; 405.1174; 125.0244 |

(Continued on following page)
characteristics, including 43 anthraquinones, 28 stilbene glycosides, 15 flavonoids, and 26 others.

### 3.2 Metabolomics analysis of raw and processed Polygonum multiflorum

The clinical use of PM usually includes both raw and processed PM. Previous studies have shown that the chemical composition of processed PM may change compared with that of raw PM, which may lead to a change in the pharmacological effects. For a fact, various studies have also shown that the toxicity of PM was significantly reduced after processing, which may be due to the significant reduction of toxic ingredients. To date, few studies have been performed to fully clarify the compositional changes in PM after treatment. Here, UPLC-Q-TOF-MS analysis combined with multivariate statistical analysis was used to distinguish between raw and processed PM. The PCA graph shows that the QC samples were closely clustered, indicating that the LC-MS analysis system was credibly reproducible and stable during the testing period. As seen from the PCA plots (Supplementary Figure S3), the raw PM and manufactured PM samples were able to be obviously separated and gathered separately. To further screen out the latent variables for distinguishing between raw and processed PM, OPLS-DA analysis was performed. The R2Y and Q2 of the OPLS-DA model were 0.98 and 0.92, respectively, which indicated excellent fitness and reliability. From the results (Figure 2), it was evident that the raw and processed PM were significantly differentiated under the supervised model. There was no overfitting in the OPLS-DA model by 200-times permutation tests, as shown in Figure 2. Furthermore, with VIP > 1.5, univariate statistical analysis p < 0.5, and fold change < 0.5, 126 differential characteristic ions were screened for significant reduction after preparing PM. Combined with the results of the abovementioned component analysis, 13 potential compounds were identified after excluding the interfering fragments and confirming the molecular ions. The results are shown in Table 3.
3.3 Pseudotargeted spectrum construction of discriminant metabolites

In MRM-based absolute quantification, calibration curves were often drawn for real compounds based on the conversion of the corresponding peak area into the content. However, absolute quantification usually cannot be achieved owing to the limitations of the standards, and the relative content between different groups can be compared by peak area. In consideration of the potential toxic dianthrone components identified in our previous studies and dianthrone aglycon hydrolyzed in acidic gastric juice in vivo, three nuclear parents of dianthrones were summarized (Li et al., 2020; Wang et al., 2021; Yang et al., 2021). Combined with the 13 differential metabolites obtained from the metabolomics analysis, UPLC-qqq-MS/MS semiquantitative profiles were further established. By optimizing the MRM ion pair and CV and CE values, semiquantitative mass spectra of the 16 compounds were constructed. The results are listed below. This method was successfully applied to 30 batches of raw PM and 20 batches of processed PM, and the standardized peak area heatmap is shown in Figure 3.

At last, a methodological investigation on the established scheduled MRM method, including specificity, linearity, precision, repeatability, and stability, was conducted. The

![FIGURE 2](image)

Orthogonal partial least squares analysis-DA score chart and permutation test analysis of Polygonum multiflorum (PM) samples (S: raw PM; Z: processed PM).

| Compounds | Rt-m/z (Da) | VIP  | p-value  | FC-value |
|-----------|-------------|------|----------|----------|
| Catechin  | 4.88_289.0716 | 11.03 | 1.50E−6  | 0.286    |
| Epicatechin| 7.89_290.0786n | 4.08  | 4.60E−4  | 0.397    |
| Torachrysone-8-O-β-D-glucopyranoside | 23.71_408.1413n | 9.99  | 1.00E−6  | 0.225    |
| 7-acetyl-3,8-dihydroxy-6-methyl-1-naphthyl-β-D-glucopyranoside | 17.97_393.1173 | 3.48  | 2.42E−4  | 0.137    |
| Epicatechin-3-O-gallate | 11.59_442.0917n | 7.44  | 9.32E−5  | 0.328    |
| Emodin-8-O-β-D-glucopyranoside | 25.61_431.2031 | 2.47  | 1.75E−4  | 0.441    |
| 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-(2-O-monogalloylesters)-glucopyranoside | 14.58_558.1371n | 13.36 | 5.97E−5  | 0.359    |
| polygonibene E | 22.91_582.1726n | 5.56  | 2.50E−5  | 0.366    |
| Polygonumolides C1-C4 | 32.40_671.1733 | 1.83  | 5.55E−5  | 0.380    |
| Polygonumolides A1-A4 | 34.95_685.1887 | 2.06  | 3.15E−4  | 0.366    |
| PM 14-17 | 30.37_758.1799n | 1.59  | 2.04E−5  | 0.157    |
| PM 22-25 | 27.64_933.2410 | 1.86  | 7.60E−5  | 0.186    |
| PM 5     | 27.10_920.2342n | 1.77  | 1.23E−4  | 0.180    |

Table 3 Detailed information of 13 different compounds between raw and processed *Polygonum multiflorum*. 
FIGURE 3
Heat map of semiquantitative analysis of 16 target compounds between raw and processed Polygonum multiflorum (PM) (S: raw PM, Z: processed PM).

FIGURE 4
Statistical analysis of IC50 values of 50 batches of raw and processed Polygonum multiflorum on two kinds of hepatocytes (p < 0.0001, ****).
735 μl in L02 cells and 281 μl in HepG2 cells. Significantly after processing (p < 0.0001, ****), indicating the basic theories of processing detoxification. In specific, 30 batches of raw PM had an average IC50 value of 250 μg/mL in L02 cells and 281 μg/mL in HepG2 cells. However, 20 batches of processed PM had an average IC50 value of 735 μg/mL in L02 cells and 1,185 μg/mL in HepG2 cells.

### 3.5 Results of spectrum–effect relationship

#### 3.5.1 Gray relational analysis results

The relationship between chromatographic peaks and hepatotoxicity effect was established by the GRA model. The degree of correlation between each chromatographic peak and hepatocyte toxicity is detailed in Table 4. The results showed that the gray relational degree between all 16 chromatographic peaks and the 1/IC50 of L02 cells was between 0.718 and 0.826. The correlation between the 16 peaks and the 1/IC50 of HepG2 cells was between 0.618 and 0.816. These results indicated that the 16 chromatographic peaks were closely correlated with hepatocyte toxicity. In total, dianthrone components X7, X8, X9, X12, X13, X14, X15, and X16; anthraquinone glycoside components X3, X4, and X6; stilbene glycosides X10 and X11; and flavanol compounds X1, X2, and X5 were all associated with hepatotoxicity in hepatocytes, which may synergistically contribute to the hepatotoxicity of PM.

#### 3.5.2 Orthogonal partial least squares analysis results

OPLS analysis was conducted using an orthogonalized multiple linear regression model. In this study, an OPLS model was built to analyze the correlation between the chromatographic peaks of 16 compounds and the IC50 of L02 and HepG2 hepatocytes (Figure 5). For L02 hepatocytes, the constructed model parameters R2X, R2Y, and Q2 were 0.94, 0.82, and 0.67, respectively. The permutation test was performed without overfitting. The results showed that the VIP values of all compounds were greater than 0.7. Combined with the correlation coefficient of less than 0.1, compounds X14, X5, X6, X7, X9, X2, X16, X10, and X11 were screened out. For HepG2 hepatocytes, the model parameters of R2X, R2Y, and Q2 were 0.93, 0.83, and 0.68, respectively, and the model had no overfitting. X5, X14, X10, X16, X7, X9, X4, and X15 were highlighted with correlation coefficients less than −0.1 and VIP values greater than 0.7. For further analysis, the common significant components screened by both models were dianthrone components X7, X9, X14, and X16; anthraquinone glycoside X6; stilbene glycoside X10; and flavanol X5. These components may be of more prominent importance in the multicomponent synergistic hepatotoxicity of PM.

#### 3.5.3 Back propagation artificial neural network results

BP-ANN is a multilayer network that uses an error back propagation algorithm for constant adjustment of weights. In this experiment, a simple 3-layer BP-ANN neural network was modeled with an input layer, one hidden layer, and an output layer.
The fitting degree of the model was evaluated using the mean square error (MSE) and regression R value. In the model, 80% of the random sample data were taken as the training set, and 20% of the sample data were used as the validation set. The results (Figure 6) demonstrated that for L02 cells, the established neural network model, where the R of the training and validation datasets reached 0.9380 and 0.9722, the MSE of the training and validation datasets reached 0.006 and 0.0027, respectively. For HepG2 cells, the R and MSE of the training and validation datasets on the model were 0.9555 and 0.9559, 0.0068 and 0.0125, respectively.

As a result of the sensitivity analysis, the 16 compounds all had relatively average contributions (p value); the L02 cells ranged from 4.07 to 8.50, and the HepG2 cells ranged from 4.08 to 9.43. The specific data are shown in Table 5, and the 16 compounds had a relatively average influence on hepatocyte toxicity. The hepatotoxicity caused by PM may be due to the synergistic result of multiple components. Furthermore, the
average influence value (MIV) of the input variables in the network was used to assess the importance of different variables in the BP-ANN model. Variables negatively correlated with the IC50 values were sieved out. For L02 cells, the screened components were X7, X6, X10, X4, X15, X9, X2, X14, and X16. For HepG2 cells, X7, X4, X11, X13, X6, and X14 were selected. In summary, the common components screened were dianthrone components X7 and X14 and anthraquinone glycosides X4 and X6. These components may be of great significance as potential hepatotoxic components in PM.

For the key characteristic components screened out using the above three models, the intersection of these components included X6, X7, and X14. It was thought that they may play a more significant role in liver injury caused by PM and could be used as toxicity markers of hepatotoxicity. We acknowledge that PM has complex chemical components and that its hepatotoxicity may be the result of the synergistic action of various components. The 16 components screened above all contained a degree of hepatotoxicity. Moreover, there were many potentially hepatotoxic compounds that we had not discovered and identified that need to be further explored and verified.

4 Discussion

As an invaluable treasure of Chinese civilization, Chinese herbal medicine has the characteristics of multiple components, multiple targets, and multiple pathways. Many previous studies have explored the material basis of PM-induced hepatotoxicity through different methods. The results showed that it was not one type of compound that was responsible for hepatotoxicity in PM, which reflected the complexity and holistic nature of TCM. The hepatotoxicity may be a synergistic effect caused by multiple components acting on multiple targets leading to the toxicity result. In this study, MS fingerprints were combined with pharmacological toxicity to target potential hepatotoxic compounds in PM. Sixteen compounds were found to be potentially associated with hepatotoxicity, including dianthrones X7, X8, X9, X12, X13, X14, X15, and X16; anthraquinone glycosides X3, X4, and X6; stilbene glycosides X10 and X11; and flavanols X1, X2, and X5.

It was noteworthy that the dianthrones were the first compounds found by our team to have hepatotoxicity (Yang et al., 2021). The cis- and trans-structures of X7 were shown to
The hepatotoxic components of the stilbene glycosides screened were 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-(2-O-monomaloylesteryl)-glucopyranoside (X10) and polygonibene E (X11). X10 is a stilbene glycoside, and X11 is a stilbene glycoside dimer. At present, few pharmacological studies have been conducted on the above two stilbene glycoside components. However, some studies have reported that the stilbene glycoside component 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucopyranoside could be a risk factor for hepatotoxicity in PM, which indicates that there may be some potential for hepatotoxicity of stilbene glycosides (Meng et al., 2017).

Regarding the flavanol compounds X1, X2, and X5, oxidation and polymerization have been reported to be the main reasons for the reduction of catechins and flavonoids after processing (Xiang et al., 2021). It has been stated that these polyphenols cause different forms of toxicity, including organ toxicity, genotoxicity, mutagenicity, and cytotoxicity (Islam et al., 2021). For instance, studies have shown that catechin (X1) has antitumor effects and can induce tumor cell apoptosis on account of certain cytotoxicity (Miyamoto et al., 2004). In addition, studies have reported that epicatechin has a concentration-dependent inhibitory effect on tumor cell proliferation and promotes cell death through apoptosis (Varela-Castillo et al., 2018). Epicatechin-3-O-gallate (ECG, X5) induced apoptosis through a TGF-beta superfamily protein, NAG-1 (nonsteroidal antiinflammatory drug-activated gene) (Baek et al., 2004). ECG is a strong inducer of NAG-1, and action on HCT-116 cells leads to an increase in the G (1) phase, leading to cleavage of polyribose polymerase, a phenomenon consistent with apoptosis. In addition, ECG has also been shown to be cytotoxic and hepatotoxic in vivo and highly toxic to HSC-2 cancer cells (Babich et al., 2005; Galati et al., 2006).

Other studies have shown that emodin, chrysophanol, and pharmacy anthaquinones in PM could affect bile acid homeostasis and cause hepatotoxicity (Kang et al., 2022). Some studies also concluded that cis-2,3,5,4′-tetrahydroxy-trans-stilbene-2-O-β-D-glucoside (cis-TSG) in PM led to hepatotoxicity through mitochondrial injury (Liu et al., 2022). In addition, cis-TSG was shown to be more closely related to immunological idiosyncratic hepatotoxicity (Meng et al., 2017). Other views also suggested that the synergy between stilbenes and emodin derivatives contributed to hepatotoxicity of PM (Zhang et al., 2020).

In summary, the 16 chemical components all had different degrees of hepatotoxicity and may be responsible for the hepatotoxicity of PM through a synergistic effect. Among these compounds, the three more typical compounds—emodin dianthrones, emodin-8-O-β-D-glucopyranoside, and PM 14–17—showed strong hepatotoxicity in different models. They may be the key hepatotoxic components in PM. However, there were still many limitations in our experiments, such as the toxicity evaluation involving only in vitro cells. In addition, the screened...
hepatotoxic compounds lacked standards, and no further toxicity validation was performed.

5 Conclusion

The complexity and diversity of Chinese medicinal components make the discovery of toxic components in Chinese medicine a challenging task. This study integrated a progressive strategy to explore the hepatotoxic components in PM. First, 112 constituents of PM were characterized using UPLC-Q-TOF-MS. Second, plant metabolomics was used to screen for differential components between raw and processed PM. Third, the pseudotargeted mass spectra of the 16 components of 50 batches of PM were established. Then, the hepatotoxicity of 50 batches of PM was evaluated in two hepatocytes. At last, based on three models, GRA, OPLS, and BP-ANN, a spectrum–effect relationship was established to determine the hepatotoxic components in PM. As a result, 16 components with potential hepatotoxicity were found, among which emodin dianthrones, emodin-8-β-D-glucopyranoside, and PM 14-β-D-glucopyranoside, and PM 14-17 were more significantly prominent. These three markers could be used as hepatotoxic markers in PM as well as for in-depth pharmacological and toxicological studies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

Author contributions

YS, FW, and SM designed and conceived the experiments; YS, XH, PW, HG, and XW carried out the experiments; YS, JY, and YL contributed to writing and supervising the manuscript; FW and SM funded the study. All authors reviewed and approved the final manuscript.

Funding

The work was financially supported by the National Natural Science Foundation of China (Grant No. 81973476 and 81773874) and National Major Scientific and Technological Special Project for “Significant New Drugs Development” (2018ZX09735006).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.935336/full#supplementary-material

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