UHPLC-QQQ-MS/MS assay for quantification of dianthrones as potential toxic markers of *Polygonum multiflorum* Thunb: Application to the standardization of TCMs with endogenous toxicity

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Research

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Abstract

Background: The raw and processed roots of *Polygonum multiflorum* Thunb (PM) are commonly used in clinical practice to treat diverse diseases; however, the reports of hepatotoxicity induced by Polygoni Multiflori Radix (PMR) and Polygoni Multiflori Radix Praeparata (PMRP) have emerged worldwide. Thus, it is necessary for researcher to explore the methods to improve its quality standards and further ensure its quality and treatment effect.

Methods: In the present study, an ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QQQ- MS/MS) method has been optimized and validated for the determination of dianthrones in PMR and PMRP, using bianthronyl as the internal standard. Chromatographic separation with a gradient mobile phase (A: acetonitrile and B: water containing 0.1% formic acid (v/v)) at a flow rate of 0.25 mL/min was achieved on a Waters Acquilty UPLC BEH b) C_{18} column (2.1 mm × 50 mm, 1.7 μm). A triple quadrupole mass spectrometer (TQMS) was operated in negative ionization mode with multiple reaction monitoring for the quantitative analysis of six dianthrones. Meanwhile, compounds **5** and **6** were further evaluated for cytotoxicity of HepaRG cells by CCK8 assay.

Results: The UHPLC-QQQ-MS/MS method was first developed to simultaneous determination of six dianthrones in PMR and PMRP, namely polygonumnolides C1**–**C4 (**1–4**), *trans*-emodin dianthrones (**5**), and *cis*-emodin dianthrones (**6**). The contents of **1**~**6** in 90 batches of PMR were in the range of 0.027-19.04, 0.022-13.86, 0.073 -15.53, 0.034 -23.35, 0.38-83.67 and 0.29 -67.00 μg/g, respectively. The contents of **1**~**6** in 86 batches of commercial PMRP were in the range of 0.020-13.03, 0.051-8.94, 0.022-7.23, 0.030 -12.75, 0.098-28.54 and 0.14-27.79 μg/g, respectively. The six dianthrones were almost completely gone after reasonable processing for 24 h. Meanwhile, compounds **5** and **6** showed the inhibitory activity against HepaRG cells with the IC_{50} values of 10.98 and 15.45 μM, respectively. Furthermore, a systematic five-step strategy to realize the standardization of TCMs with endogenous toxicity is proposed for the first time, involving the establishment of determination methods, determination of the toxic markers, the standardization of processing method, the development of limit standards and benefit-risk assessment.

Conclusion: The results of cytotoxicity evaluation of dianthrone indicated that *trans*-emodin dianthrones (**5**) and *cis*-emodin dianthrones (**6**) could be selected as the toxic markers of PMRP. Taking PMR and PMRP for example, we hope this study provided insight into the standardization and internationalization of endogenous toxic TCMs, with the main purpose of improving public health by scientifically using TCMs to treat diverse complex diseases in future.

1. Introduction

Polygonum multiflorum Thunb, including Polygoni Multiflori Radix (PMR) and PMR Praeparata (PMRP), is a commonly used traditional Chinese medicine (TCM) for treating various diseases in China and is also popular in many other countries [1-2]. PMR is commonly used to treat many different conditions, including the efficacy of detoxication, eliminating carbuncle, malaria prevention, and relaxing bowel, while PMRP is well known as tonic medicines for hair-blacking, liver-nourishing, kidney-nourishing, hematopoiesis, and so on [3-5]. However, since the 1990s, a significant number of adverse hepatotoxicity reactions have emerged in China, South Korea, Japan, England, Canada, and other countries [6-8]. The chemical composition of PMR can be significantly altered after processing, and then the hepatotoxicity of PMR can be minimized accordingly. Some studies have shown that the processing could result to a decrease of some compounds, such as 2, 3, 5, 4'- tetrahydroxystilbene-2-O-*β-D*-glucopyranoside (THSG), emodin-8-O-*β-D*-glucoside, catechin, epicatechin, and physcion-8-O-*β-D*- glucopyranoside, whereas these compounds did not disappear [2, 9-10]. These studies demonstrated that there may be no direct link between these compounds and PMR-induced liver injury.

Our previous work on PMR toxicity showed that dianthrones that were first isolated from PMR by our team could be the potential hepatotoxicity, and there are lots of minor dianthrones in PMR [11-17]. Meanwhile, the dianthrones have the characteristics of increasing the content of Fe^{3+} ions and degrading easily when heated [18-19]. These features are very similar to those of hepatotoxic components of PMR [20-21]. However, to the best of our knowledge, there is no report about which types of dianthrones are toxicity markers of PMRP as well as the mechanisms of decreasing the toxicity of TCMs. Therefore, in this study, an effective and sensitive UHPLC-QQQ-MS/MS method is established, and a qualitative analysis of six dianthrones is presented. The excellent selectivity and sensitivity achieved for these target compounds in multi reaction monitoring (MRM) mode allowed satisfactory confirmation and quantitation [22]. In addition, the proposed UHPLC-QQQ-MS/MS method is successfully used for dianthrones determination in PMR and PMRP. To our best knowledge, this work is the most comprehensive study of the content of dianthrones in PMR and PMRP. The results showed that there is a strong correlation between dianthrones and PMR-induced liver damage, and *trans*-emodin dianthrones (**5**) and *cis*-emodin dianthrones (**6**) could be chosen as potential toxicity markers of PMRP. Furthermore Furthermore, a systematic five-step strategy to realize the standardization of TCMs with endogenous toxicity is proposed for the first time, involving the establishment of determination methods, determination of the toxic markers, the standardization of processing method, the development of limit standards and benefit-risk assessment. Taking PMR as an example, it is hoped that these findings will be conducive to improve the standardization and internationalization of endogenous toxic TCMs and provide indispensable evidence for ensuring safe and effective clinical treatment in the future.

In the past several decades, lots of human liver cell lines have been used in vitro screening tests to evaluate hepatotoxic drugs and other compounds. The HepaRG cell line proved to be a suitable human hepatocyte for the assessment of hepatotoxicity in vitro [23]. HepaRG cells were identified from human hepatocellular carcinoma cell lines infected with hepatitis B virus and isolated from non-neoplastic tissue in women with chronic hepatitis C virus infection for the first time [24]. HepaRG cells were derived from a highly proliferating progenitor cells, which differentiated into both biliary and hepatocellular cells in the condition of 2% dimethyl sulfoxide (DMSO) [25]. Compared with HepG2 cells and the others, HepaRG cells which were similar to human primary hepatocytes, were capable of expressing the I phase drug metabolism CYP enzymes, phase drug metabolic enzymes, transporters, and nuclear receptor specificity of liver function [26]. Therefore, in this study HepaRG cells were selected to evaluate the toxicity of the drug to hepatocytes in vitro.

2. Material And Methods

2.1 Reagents and Materials

HPLC-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Merck Inc. (Darmstadt, Germany). Ethanol was of analytical grade and purchased from Shanghai Chemical Reagent (Shanghai, China). Water was purified by a Milli-Q water purification apparatus (Millipore, Billerica, MA, USA). The immortalized hepatic cell line HepaRG was obtained from the Type Culture Collection of the Chinese Academy of Sciences, (Shanghai, China), RPMI 1640 culture medium (Biological Industries, Israel), Fetal Bovine Serum (Biosera, France), penicillin (Targetmol, China), Staurosporine (STSP,Targetmol, China), 0.25 % trypsin-EDTA (wisent, Canada), CCK8 reagent (Targetmol, China), DMSO (Sinopharm, China), Victor Nivo multi-mode board reader (PerkinElmer, China)

P. multiforum samples were authenticated by Associate Professors Ji Zhang and Jian-Bo Yang (Research and Inspection Center of TCM and Ethnomedicine, National Institutes for Food and Drug Control, State Food and Drug Administration) in accordance with the Chinese Pharmacopoeia (edition 2015, volume 1) [3]. A voucher sample of the PMR (No.20191001) was collected from Deqing county, Guangdong Province, China and deposited at the TCM and

Ethnomedicine Research and Inspection Center, National Institutes for Food and Drug Control, State Food and Drug Administration, Beijing, China.

The chemical requirements for Polygonumnolide C4 (**1**), Polygonumnolide C3 (**2**), Polygonumnolide C1 (**3**), Polygonumnolide C2 (**4**), *trans*- emodin dianthrones (**5**), and *cis*-emodin dianthrones (**6**) were isolated and purified. The structures of the six dianthrones (**1**–**6**) were confirmed by UV, MS, ^{1}HNMR and^{13}C NMR analysis, which has been reported in the literature [13-15]. The purity of these compounds was more than 98.0% (determined by HPLC). The internal standard (IS) (Bianthronyl, **IS**) was purchased from Moving Your Chemistry Forward (Shanghai, China). **Fig. 1** shows the six dianthrones structures and one IS. All solvents and samples were filtered through 0.22μm filters before injection into UHPLC.

2.2 Apparatus

The UHPL-MS/MS instrumentation consisted of an Agilent 1200 series UHPLC system equipped with an Agilent 6410B TQMS/MS system (Agilent Technologies, Santa Clara, CA, USA). Chromatographic analyses are performed using an Agilent 1200 series UHPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a quaternary pump, an online degasser, an auto plate-sampler, and a thermostatically controlled column compartment. Chromatographic separation is carried out at 30 °C on an Agilent ZORBAX SB-C_{18} column (2.1 mm × 50 mm, 1.8 μm). The mobile gradient phase is composed of acetonitrile (A) and water containing 0.1% formic acid (v/v) (B) at a flow rate of 0.25 mL/min. The gradient is programmed as follows: 0–8 min, maintaining 37% A; 8–10 min, linear change to 60% A; 10–12 min, linear change to 78% A; 12–20 min, linear change to 90% A; 20–22 min, linear change to 37% A; 22–30 min, maintaining 37% A. The column temperature is maintained at 30 ◦C. The injection volume was 2.0 μL.

All MS experiments are conducted using ESI source in negative ion electrospray mode in a 6410B TQMS (Agilent, USA). The optimal MS conditions are as follows: drying gas temperature 300 °C; drying gas flow rate 10 L/min; nebulizer gas pressure 30 psi; sheath gas temperature 300 °C; sheath gas flow 11 L/min and capillary voltage 4.0 kV. Detection is carried out in MRM mode. All data are processed using MassHunter Workstation software (V.7.0 Quantitative Analysis; Agilent, USA).

2.3 Preparation of Standard Solutions

Standard stock solutions for the six dianthrones, namely, Polygonumnolide C4 (**1**), Polygonumnolide C3 (**2**), Polygonumnolide C1 (**3**), Polygonumnolide C2 (**4**), *trans*-emodin dianthrones (**5**) and *cis*-emodin dianthrones (**6**) are prepared in 70% ethanol, respectively. Accordingly, a standard mixture solution is obtained by precisely mixing the six stock solutions with 70% ethanol so that the concentrations were 0.210 (**1**), 0.214 (**2**), 0.283 (**3**), 0.280 (**4**), 0.318 (**5**) and 0.280 (**6**) μg/mL, respectively. The mixture solution is further diluted to make standard solutions at different concentration ranges. The calibration curve is performed with at least six appropriate concentrations. The bianthronyl (**IS**) is prepared in DMSO/methanol (v/v, 2:1) at a concentration of 100.44 μg/mL. All the standards solutions are stored at 4 °C.

2.4 Sample Preparation

2.4.1 Polygoni Multiflori Radix (PMR)

90 batches of PMR (PMR-01~PMR-90) were collected from different provinces of China, which are all shown in **Table 1**.

2.4.2 Polygoni Multiflori Radix Praeparata (PMRP)

It is well known that PMRP could improve efficacy and reduce the hepatotoxicity of PMR after processing. PMRP could be extracted from PMR using the method of Chinese Pharmacopoeia (2020 edition) [3] and traditional methods [27]. 86 batches of PMR (PMRP-01~PMRP-86) were collected from different provinces of China, which are all shown in **Table 1**.

The water steaming method were as follows: A sample is collected for examination at different points and labeled as PMRP-S_{0h}, S_{2h}, S_{4h}, S_{6h}, S_{8h}, S_{10h}, S_{12h}, S_{16h}, $S_{20h,}$ and S_{24h}, respectively. In addition, ten samples of PMRP-(S_{0h}-S_{24h}) are successfully obtained. Meanwhile, 15 batches of crude PMR (300 g) are infiltrated by distilled water and steamed at 100 °C for 0, 12, and 24 h, respectively. These Processed products are then dried under sunlight. In this way 45 samples of PMRP-(SZ01-0h, 12h, and 24h - SZ15-0h, 12h, and 24h) are successfully obtained.

2.5. Sample analysis

An aliquot of 1.0 g of PMR and PMRP (through No. 3 sieve), accurately weighed, is extracted for 30 min with 50 mL of ethanol-water (7:3, v/v) on an ultrasonic water bath, and the extract is then filtered through a 0.22 μm syringe filter. The successive filtrate is used as the test solution and analyzed with UHPLC-QQQ-MS/MS according to the above procedure.

Weight accurately 1.0 g of the powder (through No. 3 sieve) to a stoppered conical flash, add accurately 50 mL of ethanol-water (7:3, v/v), weigh and ultrasonicate (power, 100 W; frequency, 40 kHz) for 30 min, cool and weigh again, replenish the loss of weigh with ethanol-water (7:3, v/v), mix well. The extract was then filtered through a 0.22 μm syringe filter. The successive filtrate was then used as the test solution. The successive filtrate was used as the test solution and analyzed with UHPLC-QQQ-MS/MS according to the above procedure.

2.6 Effects of the cytotoxicity by dianthrones exposure on HepaRG cells

HepaRG cells were maintained with RPMI1640 medium containing 10% of FBS, 100 U/mL of penicillin and streptomycin at 37 °C with 5% CO2. The effects of dianthrones toxic markers on HepaRG cell viability were determined using a CCK8 assay. According to the experimental operation requirements, the day before detection, the HepaRG cells were inoculated in 384-well cell plates with a density of 1000 cells/well, and 40 μL cell suspension was inoculated in each well. The cell plates were placed in an incubator at 37 °C with 5% CO_2 for overnight incubation. On the day of the experiment, each well was separately added 10 μL compound working solution (0.064, 0.32, 1.6, 8, 40, 200 and 1000 μg/mL respectively), incubate in 37°C, 5% CO2 incubator, and avoid light for 72 hours. After the incubation, 5μL of CCK8 was added into each cell well and incubated for 4 hours. The absorbance at 450nm was measured on NIVO and the inhibition rate was calculated by the following formula:

Inhibition ratio%= (ODs- OD_{NC}) / (OD_{STSP} -OD_{NC})×100%

Where ODs is the absorbance of sample solution (cell + medium + compound to be tested), OD_{NC} is the absorbance of the negative control (cell + medium +DMSO), and OD_{STSP} means the absorbance of the positive control (cell + medium + 10 μM STSP) in the presence tests. According to the inhibition ratio of compounds, the IC50 (the concentration corresponding to 50% of maximum inhibition response) was calculated using Graphpad from the dose-response curves. All the tests were conducted in triplicates, and the mean values were finally obtained.

3. Results

3.1. Optimization of the extraction method

The PMR (No.20191001) are used for optimizing the process of extraction. The optimization of the extraction method is successfully obtained using a three-step approach, described as follows. **Step 1.** *Optimization of extraction solvent system:* The first step in the method of preparation of the sample solution is to select a suitable extraction solvent because of its paramount role to achieve good recovery. Five concentrations of aqueous ethanol, such as H_2O and 30%, 50%, 70%, and 95% ethanol (v/v in water), are systematically compared in virtue of the peak areas of the six dianthrones of PMR. As a result, 70% of ethanol exhibited the highest extraction efficiency among the tested solvents, as shown in **Fig. 2A**. Hence, 70% aqueous ethanol is successfully selected as the best extraction solvent for this study. **Step 2**. *Optimization of solvent volume*: Extractant volume may be another factor that could affect extraction efficiency. This study aimed to obtain the minimum volume of extractant required to achieve the highest extraction efficiency. Four different volumes of 70% ethanol (25, 50, 100 and 150 mL) are systematically studied. From **Fig. 2B**, the peak areas of the six dianthrones could increase with the increase in the volume of 70% ethanol. However, there is no significant difference among the results of four different volumes of 70% ethanol. So, 50 mL of 70% ethanol is eventually selected as the optimized volume to protect the environment. **Step 3**. *Optimization of ultrasonication time:* In this study, an ultrasonic process is used to extract the six dianthrones of PMR. From **Fig. 2C**, there is no significant difference among 15, 30, and 45 min. Accordingly, 30 min is then selected as the best extraction time to save energy.

In conclusion, the optimal sample preparation method is found to be the extraction of 1.0 g sample with 50 mL of 70% methanol in an ultrasonic water bath for 30 min.

3.2. Optimization of UHPLC-QQQ-MS/MS conditions

The chromatographic conditions, especially the composition of the mobile phase, are optimized to achieve the best possible resolution and symmetrically formed peaks of the seven compounds within a suitable run time. In the course of the tests, four mobile phases are examined, i.e., methanol-, acetonitrile-, methanol-water containing 0.1% formic acid (v/v), and acetonitrile-water containing 0.1% formic acid (v/v) in different ratios. The acetonitrile-water containing 0.1% formic acid (v/v) solution mobile phase has the lowest pressure, best baseline stability, and highest ionization efficiency and is eventually selected as the mobile phase. Both the positive and negative ion modes are also tested for MS analysis. The seven compounds showed cleaner mass spectral backgrounds and higher sensitivities in the negative mode than in the positive mode. The parameters of fragmented voltage and collision energy are optimized to obtain the richest relative abundance of patent ions and outputs for the optimization of MRM conditions. In addition, the MRM transitions and parameters of these seven dianthrones compounds are all shown in **Table 2**. Other parameters, such as dry gas flow rate and temperature, nebulizer, and capillary voltage, are set to 10.0 L/min, 300 °C, 15 psig, and 4000 V, respectively. The production mass spectra and proposed fragmentation pathway of **1**–**6** and one IS were also shown in **Fig. 3**. These seven dianthrones (**1–6** and **IS**) could indicate the cleavage of the C10–C10' bond to yield the anthrone free radical in the MS/MS product ion spectra. The MS/MS product ion spectra of **1**–**6** had been reported in the article [12].

3.3. Method validation

3.3.1 Specificity

The peaks of the six dianthrones and the IS presented good separability without interference peaks based on the chromatography and MS conditions mentioned above. The typical MRM chromatograms for a blank test sample and a sample of *P. multiform* are shown in **Figs. 4 A** and **B.** This result showed that the method is highly selective.

3.3.2 Linearity range, limits of detection (LOD) and limits of quantification (LOQ)

The UHPLC-QQQ-MS/MS method developed is further validated in accordance with the guidelines of the Validation of the Quality Standard of TCM (Chinese Pharmacopoeia, 2015, volume 1) [3]. **Table 3** lists the linear calibration curve with R^2, linearity range, LOD, and LOQ. All calibration curves show good linear regression ($r^2 \geq 0.9965$) within the tested ranges; the LOD (*S/N* = 3) and the LOQ (*S/N* = 10) for the six dianthrones are in the ranges of 0.3–0.4 ng/mL and 0.7–1.1 ng/mL, respectively, showing a high sensitivity.

3.3.3 Precision

The precision of the method is evaluated based on intra- and inter-day precision. The intra-day precision is tested with the mixed standard solutions in a day. The standard solutions are examined in triplicate on three consecutive days for the inter-day precision. The corresponding RSD % was calculated. The RSDs for intra-day (n = 6) and inter-day (n = 3) assays are less than 2.73% and 4.63%, respectively (see **Table 4**).

3.3.4 Stability and repeatability

The stability was measured using a sample solution and performed for 0, 2, 4, 8, 12, and 24 h at room temperature. Six independent sample solutions are prepared and analyzed to measure the repeatability. The concentration of each solution is determined by a calibration curve produced on the same day. The RSDs for the stability is less than 3.95% within 24 h. Meanwhile, the RSDs for repeatability are less than 3.30% (see **Table 4**). The results of the stability and repeatability tests show that all analytes are found to be stable within the duration of the whole analysis and the test method is sufficiently effective for conventional analysis.

3.3.5 Recovery

The recovery tests are carried out by adding a known number of mixed standards into a certain amount of six dianthrones. Six replication are performed for the test. The recoveries are calculated using the following equation: Recovery (%) = (total amount detected−amount original)/amount spiked × 100%. **Table 4** also shows that the analytical method developed for the six dianthrones compounds has a good recovery rate from 104.38 to 150.04% and the RSDs are less than 9.70%. Therefore, the UHPLC-QQQ-MS/MS method is precise, accurate, sensitive, and reliable enough for the simultaneous and quantitative determination of the six minor potential hepatotoxic compounds in PMR and PMRP.

3.4 Quantification of 6 dianthrones in different batches of PMR and P-MRP

Comparing UHPLC retention times and *m/z* values of six dianthrones with those of the reference compounds, the identification of the target peaks is successfully developed by UHPLC-QQQ-MS/MS. The contents of each analyte are performed using the respective calibration curves using an IS method. The developed method is successfully applied to analyze the contents of the six dianthrones in PMR and PMRP.

3.4.1 Quantification of 6 dianthrones in 90 batches of PMR

The developed and validated UHPLC-QQQ-MS/MS method is subsequently applied to evaluate six dianthrones in the 90 batches of PMR, and the quantification results are summarized in **Table 5**. The contents of **1**, **2**, **3**, **4**, **5** and **6** were in the range of 0.027-19.04, 0.022-13.86, 0.073 -15.53, 0.034 -23.35, 0.38-83.67 and 0.29 -67.00 μg/g, respectively. The total contents of **1–6** range from 1.39 to 171.45 μg/g. There are distinct differences in the contents of **1–6** in the 90 batches of PMR. It was very interestingly found that the contents of **5** and **6** in the 70% ethanol of PMR are remarkably higher than those of **1–4.** The average content order in the 90 batches of PMR is **5** > **6** > **1** > **4** > **3** > **2**. According to the previous studies [17], dianthrones may be the potential hepatotoxic components in PMR, and **5** and **6** are more toxic than **1**–**4**. Therefore, **5** and **6** could be used as the potential toxicity markers of PMRP.

3.4.2 Quantification of 6 dianthrones in different batches of PMRP (The water steaming method)

The developed and validated UHPLC-QQQ-MS/MS method is subsequently applied to determine six dianthrones in the 10 samples of PMRP (PMRP-S_{0h}, PMRP-S_{2h}, PMRP-S_{4h}, PMRP-S_{6h}, PMRP-S_{8h}, PMRP-S_{10h}, PMRP-S_{12h}, PMRP-S_{16h}, PMRP-S_{20h}, and PMRP-S_{24h}) using the water steaming method, and the quantification results are summarized in **Table 6**. The contents of **1**, **2**, **3**, **4**, **5** and **6** were in the range of 0.18-2.09, 0.58-3.67, 0.26-2.04, 0.71-4.07, 0.25-7.20 and 0.22-6.11 μg/g, respectively. The total contents of **1–6** range from 2.20-20.74 μg/g. The contents of compounds **1–6** in different points with the water steaming method are also shown in **Fig. 5**.

Additionally, the developed and validated UHPLC-QQQ-MS/MS method is subsequently applied to determine six dianthrones in 45 samples of PMRP in 0, 12, and 24h processing, using the water steaming method, and the quantification results are summarized in **Table 7.** The total contents of **1–6** decreased significantly. **1** and **3** could be detected in 5 samples after 12 h processing. **1** could not be detected in 15 samples, and **2** could not be detected in 6 samples after 12 h processing. Meanwhile, **5** and **6** could be detected in all samples after 12 h processing, with the contents of **5** ranging from 0.17 to 0.78 μg/g, and **6** ranging from 0.14 to 0.73 μg/g, repectively. Finally, after 24 hours of processing, the contents of six dianthranones all decreased by more than 80%.

3.4.3 Quantification of 6 dianthrones in 86 batches of commercial PMRP

The developed and validated UHPLC-QQQ-MS/MS method was subsequently applied to determine six dianthrones in the 86 batches of commercial PMRP, and the quantification results are summarized in **Table 8**. The contents of **1**, **2**, **3**, **4**, **5** and **6** were in the range of 0.020-13.03, 0.051-8.94, 0.022-7.23, 0.030 -12.75, 0.098-28.54 and 0.14-27.79 μg/g, respectively. The total contents of **1–6** range from 0.35 to 65.27 μg/g. There were distinct differences in the contents of compounds **1–6** in the 86 batches of commercial PMRP. It was also interestingly found that the contents of **5–6** in the 70% ethanol of PMR were remarkably higher than those of **1–4.** The average content order in the 86 batches of PMP is **5** > **6** > **4** > **2** > **1** > **3**.

According to the literature [9], the best processing technology for PMR process was to steam for 24h in order to eliminate the potential hepatotoxicity of PM. Further analysis was performed by focusing on the 45 samples of PMRP using the water steaming method, since this processing technology is the most commonly used and has been recommended by Chinese Pharmacopoeia. According to the line chart in **Fig.6,** the contents of two potential toxic compounds **5 (Fig.6A)** and **6 (Fig.6B)**were decreased from 17.52 to 0.78μg/g and 13.11 to 0.73μg/g, respectively. The possible limit of total contents of **5** and **6** could be no more than 1.51μg/g in PMRP. If this possible limit is used to evaluation of different PMRP in the market, more than 65% of 86 commercial PMRP will exceed this limit. Therefore, it is noteworthy that there are some problems about the processing technologies of commercial PMRP.

3.4.4 Cytotoxicity evaluation of dianthrone on HepaRG cells

The two potentially toxic compounds 5 and 6 were evaluated for cytotoxicity of HepaRG cells by CCK8 assay. According to the concentration-HepaRG cell inhibition rate curves drawn at different concentrations of the compounds, the IC_{50} values of each compound on the HepaRG cell model were calculated. The IC_{50} values of compounds **5** and **6** were 5.60 μg/ mL and 7.88 μg/ mL, respectively. Those were corresponding to 10.98 μM and 15.45 μM. The results suggested that compounds **5** and **6** had strong hepatocellular toxicity and could be used as the potential toxicity markers.

Discussion

TCMs with endogenous toxicity have a relative narrow treatment window. If they are used improperly in clinic, severe adverse reactions may occur. Attention has been directed towards TCMs with endogenous toxicity because of the negative effects and serious risks they cause to humans. Therefore, it is really essential to develop a standardization system of TCM with endogenous toxicity to guide the clinical use of TCMs. For the first time, in the present study a systematic five-step strategy to realize the standardization of TCMs with endogenous toxicity is proposed, involving the establishment of determination methods, determination of the toxic markers, the standardization of processing method, the development of limit standards and benefit-risk assessment (**Fig.7**).

Firstly, determination methods are expected to be developed to isolate and identify endogenous toxic chemicals in TCMs. The present study innovatively established the UHPLC-QQQ-MS/MS technique to simultaneous detect six dianthrones in PMR and PMRP. The UHPLC-QQQ-MS/MS technique is widely used for applications in chromatography–MS analysis. The method developed could not only provide rapid and improved chromatographic separation and a shorter chromatographic run time but can also provide higher sensitivity and selectivity, which are ultimately helpful for determining the dianthrones in PMR and PMRP.

Secondly, it is conducive to determine the toxic markers and clarify the mechanism of decreasing the toxicity of TCMs. Interestingly, this study showed dianthrones are widely distributed in PMR and demonstrated that dianthrones, especially for *trans*-emodin dianthrones (**5**) and *cis*-emodin dianthrones (**6**), could be selected as the potential toxic markers of PMRP [11, 17]. The possible degradation process of 6 dianthrones (**1**-**6**) in PMRP was also discussed as follows. Free dianthrones (**5** and **6**) may undergo glycosidation and be further converted into combined dianthrones (**1**-**4**). On the other hand, the C_{10}-C_{10}' bond of dianthrones could be easy to be cleaved under the heating conditions. These dianthrones could be converted into anthrones, and then further oxidized into anthracenols. The anthracenols may be further oxidized into anthraquinones, such as emodin and emodin-8-O-glucopyranoside, which may undergo methylation. Finally, the combined anthraquinones could be converted into free anthraquinones originated from the loss of the glucoside unit. Accordingly, postulated degradation process of dianthrones in PMRP was speculated as **Scheme 1**. The contents of dianthrones may be decreased significantly after reasonable processing. Therefore, this study could provide a theoretical basis for exploring the mechanism of decreasing the toxicity of PMRP.

Thirdly, the standardization of processing method is of great significance. Taking *P. multiflorum* preparations (PMP), as an example, this study illustrated the relationship between different solvent extracts and the content of dianthrones in PMR and PMRP for the first time. It is shown that the different extracts using ethanol with different concentrations as an extracting agent could cause a significant influence on the hepatotoxicity of PMR by those reported in the references [28-30]. Therefore, five different concentrations of aqueous ethanol were chosen to evaluate the extraction efficiency of the dianthrones in this study. The results reviewed that 70% ethanol exhibited the highest extraction efficiency among the tested solvents. Interestingly, these results were consistent with our previous research, which had also shown that the toxicity of the extract with 70% ethanol was considered to be higher than that of other extracts such as H_2O extract and 30% ethanol extract [11]. Furthermore, the present study showed that after extraction by the water steaming

method, the total contents of 6 types of dianthones decreased even by more than 80%. Therefore, the extraction method of PMR is quite related to the contents of dianthrones. Besides our study has demonstrated that dianthrones were the potential toxic markers of PMR, representing that the extraction method is of significance for potential toxicity of PMR and PMRP. Base on the results of this study and previous studies, it suggested to pre-treat the PMR by the water steaming method for 24 hours. On the other hand, extracting by 70% ethanol is not encouraged. All in all, in the interest of public health, standardization of pre-treatment method is definitely recommended, in order to minimize the toxicity of TCMs with endogenous toxicity.

Fourthly, considering the public confidence in the safe use of TCMs and TCM preparations, the development of a scientific and practical limit standard for TCMs with endogenous toxicity is urgently needed and beneficial. Taking *P. multiflorum* preparations (PMP), as an example, there are more than 300 Chinese patent medicines (CPMs) containing PMR and PMRP in the Chinese Pharmacopoeia and Drug Standard of Ministry of Public Health of Peoples Republic of China [3, 31]. It has been reported that lots of PMP, such as yangxue shengfa capsules and gastrodia jujube tablets, showed certain hepatotoxicity [31-33]. However, to the best of our knowledge, there is no regulatory standard for PMR or PMP at all. Therefore, it is very necessary to determinate the limit standards for these dianthrones in PMR or PMP to guarantee the medication safety of TMCs in the future. An appropriate method to formulate limit standards is the key. A scientific and practical limit standard should be based on toxicological characteristics of chemicals, the amount of TCMs or TCM preparations ingested by consumers, body weight, and safety factors. The following formula to calculate the maximum theoretical limit is recommended: $L = AW\delta/M$ (1). Where L is the maximum theoretical limit; W is the body weight (70kg); M is the daily ingestion rate of TCMs or TCM preparation(g/day), which could be based on the consumption rate in Pharmacopoeia of the People's Republic of China (PPRC); δ is safety factor, accounting for the contribution of dietary supplements as a component of daily food intake. According to the National Science Foundation (NSF)'s judgment,δcould be 10. A is the acceptable daily intake (ADI),which is defined as the estimated amount of a chemical to which a person can be exposed, on a daily basis over the lifetime, without suffering a detectable deleterious effect. For some endogenous toxic chemicals, such as pyrrolizidine alkaloids, ADI values have been set by organizations involving World Health Organization (WHO) and European Food Safety Authority (EFSA), as references. However, for other endogenous toxic chemicals, such as dianthrones in PMR or PMP, ADI should be determined under the guidance of Good Laboratory Practice (GLP). In the future study, we will take great efforts to determine the crucial parameters ADI, expecially ADI for *trans*-emodin dianthrones (**5**) and *cis*-emodin dianthrones (**6**), based on which the maximum theoretical limit could be acquired. A practical maximum theoretical limit is the basis of a practical limit standard, besides other factors involved in economic development, human cognition, and even history and culture, is recommended to be considered to maintain a balance between public safety and economic progress.

Finally, it is necessary to establish the benefit and risk assessment model of TCMs with endogenous toxicity in order to comprehensively evaluate the benefit and risk of TCMs and to ensure both the safety and effectiveness of TCMs. The evaluation of benefit-risk-ratio is determined by many factors, involving the establishing of the value tree of benefit-risk ratio. The value tree is composed of the characteristics of the disease, clinical efficacy of TCMs, adverse reactions caused by TCMs, ect. On the basis of the severity, duration and incidence of the adverse reactions caused by TCMs with endogenous toxicity, these indexes should be weighted to obtain the estimated benefit-risk ratio. Meanwhile, it is paramount to build a massive mass spectrum database to identify endogenous toxic chemicals, inclding dianthrones, as well as to accumulate a wider range of extensive health risk assessment data on these endogenous toxic chemicals.

Conclusions

In the present study, a rapid, sensitive, precise, and reliable UHPLC-QQQ-MS/MS method was developed to simultaneous determination of six dianthrones in PMR and PMRP for the first time. The results reviewed that trans-

emodin dianthrones (**5**) and cis-emodin dianthrones (**6**) could be considered as the toxic markers of PMRP. Furthermore, taking PMR as an example, a systematic five-step strategy to promote the standardization of TCMs with endogenous toxicity is proposed for the first time, covering the research gap of its field. The systematic strategy is consisted of the following steps, involving the establishment of determination methods, determination of the toxic markers, the standardization of processing method, the development of limit standards and benefit-risk assessment. Taking PMR and PMRP as examples, we hope this study provided insight into the standardization and internationalization of endogenous toxic TCMs, and would be conducive to improve the quality standard of these endogenous toxic TCMs, was well as ensuring safe and effective clinical treatment.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

JBY, FW and SCM designed the study. JBY, YFS and TTZ drafted the manuscript. FW and SCM revised the manuscript. HYG, YL and XWH are responsible for collecting the samples. QW, YW and XLC provided the technical support and advices for the study. HTJ and STW are responsible for pharmacological experiment. All authors read and approved the final manuscript.

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Availability of data and materials

The research data generated from this study is included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table1. Sample collection information in the present study.

Samples	Location	Samples	Location
PMR-01	Bozhou, Anhui Province, China.	PMRP-01	Changsha, Hunan Province, China.
PMR-02	Dingxi, Gansu Province, China.	PMRP-02	Bozhou, Anhui Province, China.
PMR-03	Beijing, China	PMRP-03	Lijiang,Guangxi Zhuang Autonomous Region, China.
PMR-04	Qujing, Yunnan Province	PMRP-04	Anguo, Heibei Province, China.
PMR-05	Bozhou, Anhui Province, China.	PMRP-05	Dunhua, Jilin Province, China.
PMR-06	Xianyang, Shanxi Province, China.	PMRP-06	Tongchuan, Shanxi Province, China.
PMR-07	Bozhou, Anhui Province, China.	PMRP-07	Huanggang, Hubei Province, China.
PMR-08	Taizhou, Jiangsu Province, China.	PMRP-08	Bozhou, Anhui Province, China.
PMR-09	Tianshui, Gansu Province, China.	PMRP-09	Zhangshu, Jiangxi Province, China.
PMR-10	Bozhou, Anhui Province, China.	PMRP-10	Chengduo, Sichuan Province, China.
PMR-11	Bozhou, Anhui Province, China.	PMRP-11	Shijiazhuang, Hebei Province, China.
PMR-12	Zunyi, Guizhou Province, China.	PMRP-12	Beijing, China.
PMR-13	Sichuan Province, China.	PMRP-13	Guigang, Guangxi Zhuang Autonomous Region, China.
PMR-14	Shijiazhuang, Hebei Province, China.	PMRP-14	Shiyan,Hubei Province, China.
PMR-15	Longde, Ningxia Hui Autonomous Region, China.	PMRP-15	Zhangshu, Jiangxi Province, China.
PMR-16	Puyang, Anhui Province, China.	PMRP-16	Quzhou, Zhejiang Province, China.
PMR-17	Yuncheng, Shanxi Province, China.	PMRP-17	Huzhou, Zhejiang Province, China.
PMR-18	Anguo, Heibei Province, China.	PMRP-18	Nanjing, Jiangsu Province, China.
PMR-19	Haikou, Hainan Province, China.	PMRP-19	Kunming, Yunnan Province, China.
PMR-20	Xining, Qinghai Province, China.	PMRP-20	Anguo, Hebei Province, China.
PMR-21	Bozhou, Anhui Province, China.	PMRP-21	Bozhou, Anhui Province, China.
PMR-22	Shangrao, Jiangxi Province, China.	PMRP-22	Beijing, China.
PMR-23	Yulin, Guangxi Zhuang Autonomous Region, China.	PMRP-23	Luoyang, Henan Province, China.
PMR-24	Chengdou, Sichuan Province, China.	PMRP-24	Kunming, Yunnan Province, China.
PMR-25	Shanghai, China.	PMRP-25	Chengduo, Sichuan Province, China.
PMR-26	Anguo, Heibei Province, China.	PMRP-26	Bozhou, Anhui Province, China.
PMR-27	Jining, Shandong Province, China.	PMRP-27	Heze, Shandong Province, China.
PMR-28	Anguo, Heibei Province, China.	PMRP-28	Bozhou, Anhui Province, China.
PMR-29	Linzhi, Tibet Province, China.	PMRP-29	Nantong, Jiangsu Province, China.
PMR-30	Zhongxiang, Hubei Province, China.	PMRP-30	Shanghai, China.
PMR-31	Anguo, Heibei Province, China.	PMRP-31	Zhanjiang, Guangdong Province, China.
PMR-32	Chengdou, Sichuan Province, China.	PMRP-32	Bozhou, Anhui Province, China.
PMR-33	Shanghai, China.	PMRP-33	Hangzhou, Zhejiang Province, China.
PMR-34	Anguo, Heibei Province, China.	PMRP-34	Chongqing, China.
PMR-35	Bozhou, Anhui Province, China.	PMRP-35	Guyuan, Ningxia Hui Autonomous Region, China.
PMR-36	Anguo, Heibei Province, China.	PMRP-36	Bozhou, Anhui Province, China.
PMR-37	Yuncheng, Shanxi Province, China.	PMRP-37	Bozhou, Anhui Province, China.
PMR-38	Yangzhong, Jiangsu Province, China.	PMRP-38	Ningbo, Zhejiang Province, China.
PMR-39	Baoji, Shanxi Province, China.	PMRP-39	Xian, Shanxi Province, China.
PMR-40	Anguo, Heibei Province, China.	PMRP-40	Yulin□Guangxi Zhuang Autonomous Region, China.
PMR-41	Xichang, Jiangxi Province, China.	PMRP-41	Bozhou, Anhui Province, China.
PMR-42	Kunming, Yunnan Province, China.	PMRP-42	Beijing, China.

PMR-43	Shaoxing, Zhejiang Province, China.
PMR-44	Chengdou, Sichuan Province, China.
PMR-45	Chengdou, Sichuan Province, China.
PMR-46	Shangrao, Jiangxi Province, China.
PMR-47	Zhongxiang, Hubei Province, China.
PMR-48	Deqing, Guangdong Province, China.
PMR-49	Deqing, Guangdong Province, China.
PMR-50	Deqing, Guangdong Province, China.
PMR-51	Deqing, Guangdong Province, China.
PMR-52	Deqing, Guangdong Province, China.
PMR-53	Urumqi, Xinjiang Uygur Autonomous Region, China.
PMR-54	Bozhou, Anhui Province, China
PMR-55	Guangxi Zhuang Autonomous Region, China.
PMR-56	Yunnan Province, China.
PMR-57	Dengfeng, Henan Province, China.
PMR-58	Bozhou, Anhui Province, China.
PMR-59	Bozhou, Anhui Province, China.
PMR-60	Bozhou, Anhui Province, China.
PMR-61	Yunnan Province, China.
PMR-62	Puer, Yunan Province, China.
PMR-63	Guizhou Province, China.
PMR-64	Guizhou Province, China.
PMR-65	Henan Province, China.
PMR-66	Bozhou, Anhui Province, China.
PMR-67	Kaili, Guizhou Province, China.
PMR-68	Congjiang, GuizhouProvince, China.
PMR-69	Chengdou, Sichuan Province, China.
PMR-70	Bozhou, Anhui Province, China.
PMR-71	Yuzhou, Henan Province, China.
PMR-72	Henan Province, China.
PMR-73	Sichuan Province, China.
PMR-74	Bozhou, Anhui Province, China.
PMR-75	Hengyang, Hunan Province, China.
PMR-76	Deqing, Guangdong Province, China.
PMR-77	Deqing, Guangdong Province, China.
PMR-78	Henan Province, China.2018Year
PMR-79	Sichuan Province, China.
PMR-80	Deqing, Guangdong Province, China.
PMR-81	Deqing, Guangdong Province, China.
PMR-82	Guizhou Province, China.
PMR-83	Guizhou Province, China.
PMR-84	Sichuan Province, China.
PMR-85	Dengfeng, Henan Province, China.
PMR-86	Sichuan Province, China.
PMR-87	Bozhou, Anhui Province, China.
PMR-88	Lijiang, Yunnna Province, China.
PMR-89	Guangxi Zhuang Autonomous Region, China.

PMRP-43	Tianjin, China.
PMRP-44	Anguo, Hebei Province, China.
PMRP-45	Tianjin, China.
PMRP-46	Bozhou, Anhui Province, China.
PMRP-47	Beijing, China.
PMRP-48	Nanjing, Jiangsu Province, China.
PMRP-49	Anguo, Hebei Province, China.
PMRP-50	Yunfu, Guangdong Province, China.
PMRP-51	Bozhou, Anhui Province, China.
PMRP-52	Anguo, Hebei Province, China.
PMRP-53	Xinyu, Jiangxi Province, China.
PMRP-54	Anguo, Hebei Province, China.
PMRP-55	Qiqihaer, Heilongjiang Province, China.
PMRP-56	Nanjing, Jiangsu Province, China.
PMRP-57	Haerbing, Heilongjiang Province, China.
PMRP-58	Guiyang, Guizhou Province, China.
PMRP-59	Bozhou, Anhui Province, China.
PMRP-60	Yuechang, Guangdong Province, China.
PMRP-61	Chengduo, Sichuan Province, China.
PMRP-62	Bozhou, Anhui Province, China.
PMRP-63	Beijing, China.
PMRP-64	Bozhou, Anhui Province, China.
PMRP-65	Unkonwn, China.
PMRP-66	Unkonwn, China.
PMRP-67	Unkonwn, China.
PMRP-68	Unkonwn, China.
PMRP-69	Chengduo, Sichuan Province, China.
PMRP-70	Bozhou, Anhui Province, China.
PMRP-71	Puer, Yunnan Province, China.
PMRP-72	Xichang,Sichuan Province, China.
PMRP-73	Bozhou, Anhui Province, China.
PMRP-74	Bozhou, Anhui Province, China.
PMRP-75	Chengduo, Sichuan Province, China.
PMRP-76	Bozhou, Anhui Province, China.
PMRP-77	Lijiang, Yunnan Province, China.
PMRP-78	Deqing, Guangdong Province, China.
PMRP-79	Honghe, Yunnan Province, China.
PMRP-80	Henan Province, China.
PMRP-81	Henan Province, China.
PMRP-82	Sichuan Province, China.
PMRP-83	Sichuan Province, China.
PMRP-84	Sichuan Province, China.
PMRP-85	Henan Province, China.
PMRP-86	Henan Province, China.

Table2. Parameters of dianthrones of 6 analytes and 1 internal standard in MRM analysis

No	Compounds	Retention times (RT, min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor voltage (FV)	Collision energy (CE)	Ion mode
1	polygonumnolides C4	6.99	670.7 $[M-H]^-$	415.8	100	20	(-) ESI
2	polygonumnolides C3	7.63	671.0 $[M-H]^-$	415.8	100	20	(-) ESI
3	polygonumnolides C1	11.80	670.9 $[M-H]^-$	415.9	100	20	(-) ESI
4	polygonumnolides C2	13.25	670.9 $[M-H]^-$	416.0	100	20	(-) ESI
5	*trans*-emodin dianthrones	16.51	508.8 $[M-H]^-$	253.8	150	20	(-) ESI
6	*cis*-emodin dianthrones	17.06	508.7 $[M-H]^-$	253.9	150	20	(-) ESI
1S	Bianthronyl	17.37	384.9 $[M-H]^-$	191.8	100	35	(-) ESI

Table 3. Regression equation, LOD and LOQ of the six dianthrones

No	Compounds	Regression equation	R^2	Range (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)
1	polygonumnolides C4	Y = 10.186x +0.0079	0.9980	2.1-126.0	0.4	1.1
2	polygonumnolides C3	Y = 15.446x + 0.0163	0.9990	2.1-128.4	0.4	1.1
3	polygonumnolides C1	Y = 17.122x + 0.0534	0.9985	2.8-169.8	0.3	1.1
4	polygonumnolides C2	Y = 20.117x + 0.0466	0.9983	2.8-168.0	0.3	1.1
5	*trans*-emodindianthrones	Y =30.352x + 0.0800	0.9965	3.2-191.0	0.3	0.7
6	*cis*-emodin dianthrones	Y = 33.308x+ 0.0426	0.9978	2.8-168.2	0.3	0.7

Table 4. Stability, repeatability, precision and recovery of 6 compounds

No	Stability RSD (%) (n=6)	Repeatability RSD (%) (n=6)	Precision		Recovery (n = 6)						
			Intra-day RSD (%) (n = 6)	Inter-day RSD (%) (n = 3)	Sample (g)	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)	Average Recovery (%)	RSD (%)
1	3.41	3.12	2.73	2.85	0.502	1.009	1.092	2.383	125.87	134.46	7.91
					0.502	1.009	1.092	2.654	150.67		
					0.503	1.011	1.092	2.418	128.81		
					0.512	1.029	1.092	2.583	142.33		
					0.501	1.007	1.092	2.494	136.14		
					0.518	1.041	1.092	2.383	122.92		
2	3.55	3.24	1.68	2.79	0.502	0.512	0.535	1.184	125.61	134.05	9.70
					0.502	0.512	0.535	1.318	150.65		
					0.503	0.513	0.535	1.203	128.97		
					0.512	0.522	0.535	1.328	150.65		
					0.501	0.511	0.535	1.183	125.61		
					0.518	0.528	0.535	1.185	122.80		
3	3.64	1.92	2.58	2.12	0.502	0.512	0.594	1.345	140.29	150.04	3.88
					0.502	0.512	0.594	1.430	154.48		
					0.503	0.513	0.594	1.390	147.60		
					0.512	0.522	0.594	1.454	156.79		
					0.501	0.511	0.594	1.398	149.35		
					0.518	0.528	0.594	1.430	151.73		
4	3.47	1.23	2.22	4.05	0.502	0.638	0.672	1.505	129.15	143.88	8.68
					0.502	0.638	0.672	1.674	154.28		
					0.503	0.639	0.672	1.492	126.94		
					0.512	0.650	0.672	1.683	153.70		
					0.501	0.636	0.672	1.630	147.93		
					0.518	0.658	0.672	1.674	151.26		
5	3.95	1.97	2.11	4.63	0.502	2.113	2.131	4.159	96.01	105.53	9.65
					0.502	2.113	2.131	4.457	109.99		
					0.503	2.118	2.131	4.230	99.11		
					0.512	2.156	2.131	4.430	106.71		
					0.501	2.109	2.131	4.201	98.17		
					0.518	2.181	2.131	4.806	123.18		
6	3.73	3.30	1.67	2.90	0.502	1.531	1.512	3.051	100.52	104.38	6.19
					0.502	1.531	1.512	3.092	103.20		
					0.503	1.534	1.512	3.106	103.98		
					0.512	1.562	1.512	3.289	114.24		
					0.501	1.528	1.512	2.974	95.66		
					0.518	1.580	1.512	3.224	108.71		

Table 5. Contents of 6 dianthrones in 90 batches of Polygoni Multiflori Radix (PMR)

Sample No.	Contents of analytes（ug/g, n =2[a]）						Total
	1 (Mean ± SD%)	2 (Mean ± SD%)	3 (Mean ± SD%)	4 (Mean ± SD%)	5 (Mean ± SD%)	6 (Mean ± SD%)	
PMR-01	2.23 ± 0.09	1.75 ± 0.19	1.81 ± 5.81	1.41 ± 0.73	6.94 ± 0.34	4.96 ± 14.32	19.10
PMR-02	0.84 ± 1.48	0.61 ± 3.34	0.69 ± 0.06	0.81 ± 2.58	1.71 ± 2.59	1.18 ± 1.31	5.84
PMR-03	0.60 ± 1.77	0.47 ± 0.98	0.43 ± 0.93	0.62 ± 3.85	1.22 ± 0.20	0.82 ± 4.54	4.16
PMR-04	19.08 ± 10.75	13.86 ± 11.51	15.53 ± 10.02	12.04 ± 13.86	45.33 ± 41.21	37.33 ± 20.60	143.17
PMR-05	0.83 ± 0.32	0.73 ± 0.63	0.67 ± 2.00	0.78 ± 1.07	6.90 ± 15.20	5.22 ± 16.45	15.13
PMR-06	4.62 ± 0.07	3.99 ± 1.43	4.15 ± 1.22	2.30 ± 0.84	10.37 ± 1.79	9.20 ± 0.35	34.63
PMR-07	0.70 ± 1.03	0.49 ± 0.62	0.56 ± 0.93	0.64 ± 1.93	1.38 ± 0.51	0.68 ± 2.72	4.45
PMR-08	1.43 ± 2.01	0.66 ± 0.36	1.06 ± 0.84	1.30 ± 2.94	4.94 ± 9.23	2.89 ± 4.05	12.28
PMR-09	1.87 ± 0.17	1.21 ± 0.21	1.28 ± 0.23	1.24 ± 2.54	1.55 ± 1.51	1.19 ± 3.36	8.34
PMR-10	3.78 ± 0.35	3.33 ± 0.46	3.57 ± 0.08	1.75 ± 1.60	9.07 ± 1.85	7.08 ± 0.77	28.58
PMR-11	1.52 ± 9.04	1.17 ± 3.03	1.35 ± 1.24	1.45 ± 7.84	8.42 ± 59.40	4.85 ± 5.31	18.76
PMR-12	2.24 ± 5.62	1.51 ± 2.21	1.76 ± 1.00	1.72 ± 2.55	6.51 ± 3.46	5.06 ± 4.21	18.80
PMR-13	2.38 ± 2.36	1.41 ± 0.74	1.64 ± 1.09	2.10 ± 2.36	9.36 ± 81.61	5.75 ± 18.09	22.64
PMR-14	2.56 ± 2.36	1.92 ± 3.47	2.04 ± 1.44	1.55 ± 2.20	3.78 ± 18.58	3.03 ± 10.02	14.88
PMR-15	2.52 ± 0.33	3.03 ± 0.74	2.78 ± 1.67	1.50 ± 0.01	10.64 ± 3.47	10.82 ± 2.61	31.29
PMR-16	7.80 ± 2.01	6.18 ± 2.48	6.49 ± 0.69	4.29 ± 0.92	27.53 ± 4.71	25.28 ± 5.37	77.57
PMR-17	0.14 ± 0.50	0.17 ± 0.34	0.16 ± 0.35	0.037 ± 0.16	1.01 ± 10.91	0.46 ± 1.36	1.98
PMR-18	0.25 ± 0.60	0.022 ± 0.00	0.073 ± 0.00	0.74 ± 0.03	1.01 ± 6.87	0.82 ± 1.96	2.92
PMR-19	0.69 ± 0.14	0.52 ± 0.16	0.33 ± 0.63	1.62 ± 2.82	7.31 ± 40.20	5.35 ± 14.37	15.82
PMR-20	5.91 ± 0.12	0.94 ± 0.54	0.69 ± 0.04	3.90 ± 0.17	64.18 ± 8.23	48.48 ± 3.40	124.1
PMR-21	0.20 ± 0.88	2.82 ± 0.33	5.10 ± 5.70	0.50 ± 0.74	4.15 ± 5.15	2.84 ± 0.01	15.61
PMR-22	0.027 ± 0.28	0.48 0.75	0.27 ± 0.92	0.034 ± 0.72	0.47 ± 2.22	0.29 ± 2.25	1.57
PMR-23	0.042 ± 0.10	0.11 ± 0.71	0.10 ± 0.08	0.10 ± 0.43	0.63 ± 3.82	0.41 ± 0.62	1.39
PMR-24	1.87 ± 2.82	0.181 ± 0.33	0.13 ± 0.30	2.04 ± 0.61	2.54 ± 3.68	1.84 ± 0.45	8.60
PMR-25	2.58 ± 0.30	1.091 ± 0.00	1.42 ± 3.62	23.35 ± 19.62	18.06 ± 1.04	13.68 ± 2.07	60.18
PMR-26	0.55 ± 0.02	2.211 0.02	2.63 ± 0.33	1.51 ± 0.12	22.66 ± 7.04	20.44 ± 0.09	50.00
PMR-27	0.42 ± 1.45	0.791 ± 0.10	1.18 ± 0.19	1.54 ± 2.72	8.24 ± 22.57	5.47 ± 18.23	17.64
PMR-28	0.48 ± 0.29	1.22 ± 4.70	0.57 ± 0.19	1.20 ± 0.37	7.93 ± 10.35	6.24 ± 16.38	17.64
PMR-29	0.054 ± 0.03	0.76 ± 1.43	0.54 ± 0.79	0.037 ± 0.39	4.17 ± 2.97	3.31 ± 2.33	8.87
PMR-30	0.27 ± 0.54	0.13 ± 0.53	0.11 ± 0.18	0.15 ± 0.42	1.03 ± 0.08	0.82 ± 1.30	2.51
PMR-31	5.20 ± 1.85	0.19 ± 0.06	0.27 ± 0.02	3.96 ± 0.21	10.74 ± 2.01	8.84 ± 3.84	29.20
PMR-32	1.27 ± 0.35	2.89 ± 3.50	3.22 ± 7.05	0.92 ± 2.08	5.12 ± 5.83	4.66 ± 6.80	18.08
PMR-33	6.83 ± 0.15	0.83 ± 0.97	0.95 ± 1.35	5.82 ± 2.78	51.11 ± 13.81	42.50 ± 3.46	108.04
PMR-34	4.89 ± 1.01	6.02 ± 0.36	6.52 ± 0.38	3.41 ± 2.06	21.71 ± 2.04	15.11 ± 4.99	57.66
PMR-35	7.67 ± 0.42	3.28 ± 0.48	4.34 ± 0.09	5.81 ± 0.60	61.94 ± 0.67	50.02 ± 5.90	133.06
PMR-36	3.38 ± 1.19	6.11 ± 2.08	7.37 ± 0.36	1.73 ± 0.82	16.51 ± 0.62	12.09 ± 0.64	47.19
PMR-37	0.54 ± 0.63	2.47 ± 0.08	3.28 ± 0.33	0.32 ± 2.23	2.12 ± 6.34	1.55 ± 2.14	10.28
PMR-38	4.17 ± 10.69	0.36 ± 1.36	0.48 ± 0.03	2.50 ± 1.15	8.77 ± 5.04	7.51 ± 0.86	23.79
PMR-39	2.01 ± 0.33	2.81 ± 8.05	3.23 ± 4.53	1.13 ± 3.14	5.79 ± 38.26	5.58 ± 11.65	20.55
PMR-40	1.56 ± 0.23	1.37 ± 1.06	1.56 ± 4.16	1.03 ± 0.20	7.07 ± 20.29	5.61 ± 9.30	18.20
PMR-41	6.02 ± 11.72	1.113 ± 0.38	1.32 ± 5.52	3.75 ± 1.79	7.71 ± 6.91	6.26 ± 21.43	26.17
PMR-42	4.68 ± 3.66	3.85 ± 5.45	4.47 ± 2.27	2.86 ± 1.09	6.45 ± 1.67	5.70 ± 23.76	28.01
PMR-43	10.39 ± 0.67	3.16 ± 2.64	3.58 ± 14.24	9.88 ± 0.86	27.34 ± 7.43	20.75 ± 0.12	75.10
PMR-44	1.25 ± 3.60	0.79 ± 3.24	0.66 ± 0.91	1.34 ± 2.87	2.51 ± 2.49	1.87 ± 4.10	8.42
PMR-45	0.92 ± 0.18	0.60 ± 1.06	0.59 ± 0.88	0.64 ± 1.18	4.68 ± 1.77	3.27 ± 17.57	10.7
PMR-46	1.28 ± 0.74	0.83 ± 1.20	0.74 ± 1.03	1.12 ± 0.98	2.35 ± 4.83	1.68 ± 3.90	8.00
PMR-47	2.26 ± 1.93	1.34 ± 3.33	1.35 ± 0.94	1.71 ± 1.61	9.27 ± 16.24	6.60 ± 6.12	22.53

PMR-48	1.45	±	2.29	0.88	±	1.09	0.91	±	0.03	0.72	±	3.24	7.18	±	22.72	6.43	±	31.68	17.57
PMR-49	1.70	±	0.85	0.93	±	0.33	1.02	±	0.67	1.14	±	2.18	6.03	±	0.71	4.94	±	13.15	15.76
PMR-50	0.94	±	0.95	0.53	±	1.70	0.55	±	0.99	0.64	±	0.69	4.48	±	21.93	3.16	±	15.33	10.3
PMR-51	1.22	±	1.04	0.65	±	1.13	0.76	±	1.04	0.80	±	2.97	6.91	±	26.37	6.53	±	28.27	16.87
PMR-52	1.30	±	4.47	0.80	±	1.93	0.74	±	3.43	0.74	±	4.23	3.35	±	18.15	2.60	±	17.49	9.53
PMR-53	7.62	±	0.08	0.69	±	0.19	5.63	±	0.90	7.22	±	0.34	47.24	±	6.47	38.91	±	5.55	107.31
PMR-54	3.01	±	0.17	3.30	±	0.17	2.42	±	1.11	2.85	±	0.93	38.15	±	9.29	33.72	±	1.68	83.45
PMR-55	6.76	±	7.02	4.92	±	7.27	3.93	±	4.57	5.17	±	5.17	83.67	±	3.75	67.00	±	9.16	171.45
PMR-56	0.32	±	0.51	0.22	±	1.18	0.17	±	0.14	0.28	±	1.06	6.78	±	28.19	4.95	±	6.74	12.72
PMR-57	0.61	±	0.50	0.35	±	0.20	0.37	±	0.81	0.42	±	0.51	1.59	±	0.79	1.35	±	0.16	4.69
PMR-58	1.25	±	1.12	0.92	±	0.54	0.81	±	2.83	0.95	±	0.48	9.33	±	11.15	6.29	±	37.35	19.55
PMR-59	1.29	±	0.21	0.90	±	3.74	0.85	±	0.82	0.91	±	1.22	9.48	±	9.83	6.30	±	15.03	19.73
PMR-60	1.46	±	0.75	1.09	±	2.17	0.96	±	1.72	1.03	±	3.02	9.51	±	5.22	6.47	±	4.66	20.52
PMR-61	3.99	±	1.92	2.63	±	2.76	2.30	±	4.61	2.31	±	0.92	12.10	±	15.76	9.72	±	19.35	33.05
PMR-62	1.77	±	4.12	1.01	±	0.31	1.03	±	2.95	1.15	±	1.08	4.55	±	12.20	3.83	±	8.55	13.34
PMR-63	0.82	±	1.08	0.81	±	0.66	0.54	±	1.22	0.80	±	0.91	5.39	±	1.52	4.24	±	5.97	12.6
PMR-64	0.46	±	0.91	0.29	±	0.98	0.27	±	0.13	0.31	±	0.08	1.42	±	1.13	1.40	±	0.53	4.15
PMR-65	1.41	±	0.36	0.94	±	0.51	1.02	±	0.07	1.37	±	0.21	7.01	±	0.84	6.76	±	3.08	18.51
PMR-66	1.16	±	1.65	0.72	±	0.30	0.71	±	0.35	0.74	±	2.46	3.84	±	5.18	3.34	±	9.40	10.51
PMR-67	1.47	±	2.11	0.99	±	0.30	0.91	±	2.47	0.82	±	0.98	7.48	±	22.19	6.91	±	12.61	18.58
PMR-68	1.60	±	0.24	0.97	±	1.97	0.92	±	6.10	1.01	±	2.62	2.57	±	11.58	2.03	±	13.81	9.10
PMR-69	0.77	±	0.25	0.51	±	0.16	0.52	±	0.14	0.78	±	0.34	8.49	±	2.12	8.84	±	0.66	19.91
PMR-70	0.38	±	0.35	0.16	±	0.51	0.18	±	0.64	0.39	±	0.26	3.46	±	9.85	2.66	±	2.92	7.23
PMR-71	1.11	±	5.67	0.71	±	4.43	0.67	±	1.40	0.74	±	5.16	3.50	±	19.82	2.38	±	21.85	9.11
PMR-72	0.96	±	2.83	0.80	±	2.48	0.68	±	0.03	0.72	±	3.11	7.88	±	15.41	5.45	±	6.51	16.49
PMR-73	0.75	±	0.27	0.41	±	0.64	0.37	±	0.99	0.79	±	0.33	3.37	±	7.62	2.49	±	7.62	8.18
PMR-74	2.63	±	0.78	0.97	±	1.51	1.46	±	0.24	2.98	±	0.64	19.43	±	1.13	14.40	±	2.76	41.87
PMR-75	2.34	±	0.29	1.42	±	2.21	0.91	±	1.94	2.22	±	0.21	66.05	±	13.61	41.98	±	3.82	114.92
PMR-76	0.25	±	0.24	0.12	±	1.35	0.15	±	0.11	0.23	±	0.18	1.26	±	2.63	0.83	±	0.57	2.84
PMR-77	0.46	±	0.35	0.39	±	1.12	0.25	±	0.36	0.40	±	1.89	3.92	±	0.17	3.14	±	2.26	8.56
PMR-78	1.14	±	1.65	0.74	±	2.18	0.66	±	0.99	0.70	±	1.99	3.03	±	15.82	2.38	±	17.31	8.65
PMR-79	0.61	±	4.10	0.49	±	4.96	0.34	±	4.95	0.40	±	0.26	5.46	±	11.44	3.59	±	9.55	10.89
PMR-80	0.26	±	0.66	0.15	±	0.62	0.12	±	0.07	0.27		0.10	0.38	±	0.31	0.37	±	0.88	1.55
PMR-81	0.67	±	0.17	0.35	±	0.60	0.45	±	0.13	0.92	±	0.24	8.68	±	3.51	7.25	±	3.26	18.32
PMR-82	4.59	±	0.34	3.78	±	1.03	3.21	±	1.38	4.14	±	0.94	42.84	±	8.21	37.40	±	1.94	95.96
PMR-83	0.18	±	2.23	0.089	±	1.91	0.10	±	1.67	0.14	±	0.07	1.47	±	1.69	0.94	±	0.42	2.919
PMR-84	2.28	±	0.34	1.15	±	1.18	1.35	±	0.89	1.95	±	0.26	11.61	±	11.10	9.29	±	7.78	27.63
PMR-85	0.84	±	0.09	0.47	±	0.14	0.51	±	0.87	0.82	±	0.39	12.74	±	2.17	12.40	±	1.07	27.78
PMR-86	2.06	±	1.19	1.31	±	3.95	1.22	±	3.72	1.33	±	5.86	3.97	±	21.77	3.47	±	18.51	13.36
PMR-87	6.11	±	0.10	5.20	±	1.19	4.61	±	0.93	4.82	±	0.22	52.09	±	15.47	46.51	±	7.87	119.34
PMR-88	0.89	±	6.61	0.53	±	2.83	0.50	±	5.50	0.52	±	1.83	3.23	±	5.44	1.99	±	0.73	7.66
PMR-89	2.13	±	1.71	1.35	±	0.48	1.22	±	5.08	1.28	±	1.00	4.92	±	1.91	3.69	±	4.18	14.59
PMR-90	0.98	±	0.36	0.65	±	2.25	0.59	±	1.27	0.60	±	1.91	4.44	±	17.23	3.93	±	19.42	11.19
Average	2.30			1.53			1.67			1.99			12.23			9.75			29.46

Note: [a] The data are presented as the average of two replicates.

Table6. Contents of 6 dianthrones in 10 samples of Polygoni Multiflori Radix Praeparata (PMRP) with the water steaming method

Sample No.	Contents of analytes（ug/g, n =2[a]）																		
	1 (Mean ± SD%)			2 (Mean ± SD%)			3 (Mean ± SD%)			4 (Mean ± SD%)			5 (Mean ± SD%)			6 (Mean ± SD%)			Total
PMRP -S_{0h}	2.09	±	1.53	1.65	±	2.19	2.04	±	031	1.65	±	4.09	7.20	±	11.48	6.11	±	14.10	20.74
PMRP -S_{2h}	1.46	±	1.16	1.99	±	4.56	1.68	±	2.18	1.87	±	1.66	0.94	±	1.97	0.83	±	1.70	8.77
PMRP -S_{4h}	1.13	±	0.32	2.79	±	0.38	1.66	±	2.35	3.09	±	3.75	0.81	±	1.44	0.71	±	2.17	10.19
PMRP -S_{6h}	1.26	±	2.38	3.67	±	4.92	1.48	±	0.67	4.07	±	0.28	0.88	±	2.86	0.84	±	1.44	12.20
PMRP -S_{8h}	0.61	±	1.18	1.24	±	0.24	0.87	±	0.73	1.67	±	0.21	0.28	±	0.78	0.25	±	1.41	4.92
PMRP -S_{10h}	0.85	±	1.90	1.49	±	0.58	1.13	±	2.05	1.7	±	3.05	0.41	±	0.22	0.36	±	0.66	5.94
PMRP-S_{12h}	0.66	±	0.65	1.27	±	1.36	0.91	±	1.55	1.65	±	1.57	0.36	±	0.78	0.31	±	0.77	5.16
PMRP-S_{16h}	0.52	±	1.98	1.37	±	2.93	0.61	±	1.96	1.52	±	1.79	0.39	±	0.90	0.35	±	1.57	4.76
PMRP-S_{20h}	0.39	±	0.23	0.92	±	0.51	0.49	±	0.075	1.29	±	0.20	0.47	±	0.63	0.42	±	0.37	3.98
PMRP-S_{24h}	0.18	±	0.15	0.58	±	1.20	0.26	±	0.22	0.71	±	0.72	0.25	±	0.16	0.22	±	0.37	2.20

Note: [a] The data are presented as the average of two replicates.

Table 7. Contents of 6 compounds in 45 samples of Polygoni Multiflori Radix Praeparata (PMRP) with the water steaming method

Sample No.	Contents of analytes（ug/g, n =2[a]）																	
	1			2			3			4			5			6		
	0 h	12 h	24h	0 h	12 h	24h	0 h	12 h	24h	0 h	12 h	24h	0 h	12 h	24h	0 h	12 h	24h
PMRP – SZ01	0.37	ND [b]	ND [b]	0.32	0.11	ND [b]	0.37	ND [b]	ND [b]	0.32	0.097	ND [b]	1.05	0.49	0.22	0.68	0.40	0.18
PMRP – SZ02	0.157	ND [b]	ND [b]	0.19	0.12	ND [b]	0.19	ND [b]	ND [b]	0.19	0.10	ND [b]	1.34	0.45	0.17	0.85	0.38	0.14
PMRP – SZ03	1.42	0.36	ND [b]	1.06	0.16	ND [b]	1.18	0.099	ND [b]	0.94	0.16	ND [b]	6.39	0.32	0.20	6.26	0.27	0.16
PMRP – SZ04	0.58	ND [b]	ND [b]	0.45	0.11	ND [b]	0.52	ND [b]	ND [b]	0.50	0.10	ND [b]	1.45	0.29	0.23	1.22	0.25	0.18
PMRP – SZ05	0.72	0.020	ND [b]	0.50	0.16	0.11	0.51	0.089	ND [b]	0.90	0.14	0.085	2.95	0.62	0.29	2.26	0.44	0.24
PMRP – SZ06	0.023	ND [b]	ND [b]	0.10	0.089	0.13	0.089	ND [b]	ND [b]	0.088	0.070	0.10	0.21	0.16	0.68	0.17	0.12	0.54
PMRP – SZ07	0.43	0.04	ND [b]	0.47	1.05	0.12	0.46	0.22	ND [b]	0.49	0.19	0.093	3.73	0.72	0.26	3.41	0.62	0.21
PMRP – SZ08	3.89	0.088	ND [b]	2.67	0.24	0.12	2.91	0.16	ND [b]	2.60	0.25	0.097	10.33	1.69	0.24	8.87	1.53	0.18
PMRP – SZ09	0.92	0.035	ND [b]	0.88	0.15	0.11	0.90	0.11	ND [b]	0.83	0.18	0.088	6.74	0.69	0.21	4.95	0.54	0.17
PMRP – SZ10	1.21	0.060	ND [b]	1.96	0.22	0.11	1.85	0.13	ND [b]	1.87	0.30	0.081	6.99	1.74	0.78	6.11	1.25	0.73
PMRP – SZ11	1.24	0.052	ND [b]	0.98	0.21	0.10	1.12	0.13	ND [b]	1.04	0.25	0.082	8.11	1.19	0.26	5.74	1.01	0.20
PMRP – SZ12	1.99	0.31	ND [b]	1.38	0.43	0.10	1.57	0.31	ND [b]	1.51	0.42	ND [b]	3.46	3.04	0.24	3.16	2.77	0.18
PMRP – SZ13	1.21	0.021	ND [b]	1.00	0.14	ND [b]	1.07	0.099	ND [b]	1.09	0.14	ND [b]	8.01	2.99	0.55	5.75	1.92	0.46
PMRP – SZ14	1.41	0.022	ND [b]	1.17	0.13	ND [b]	1.25	0.10	ND [b]	1.17	0.14	ND [b]	8.12	0.54	0.39	5.88	0.45	0.29
PMRP – SZ15	2.41	ND [b]	ND [b]	1.99	0.14	0.10	2.39	ND [b]	ND [b]	3.67	0.12	0.085	17.52	6.87	0.34	13.11	4.76	0.27

Note: [a] The data are presented as the average of two replicates ; [b] ND, under limits of quantitation.

Table 8. Contents of 6 dianthrones in 86 batches of Polygoni Multiflori Radix Praeparata (PMRP)

Sample No.	Contents of analytes（ug/g, n =2[a]）						
	1 (Mean ± SD%)	2 (Mean ± SD%)	3 (Mean ± SD%)	4 (Mean ± SD%)	5 (Mean ± SD%)	6 (Mean ± SD%)	Total
PMRP-01	0.60 ± 1.32	3.36 ± 1.60	0.76 ± 1.04	2.57 ± 5.40	2.41 ± 6.96	2.16 ± 5.87	11.86
PMRP-02	0.051 ± 0.56	0.070 ± 0.98	0.064 ± 0.61	0.069 ± 0.73	0.41 ± 1.10	0.36 ± 0.74	1.02
PMRP-03	0.25 ± 0.42	0.45 ± 1.68	0.26 ± 1.15	0.60 ± 0.79	4.50 ± 12.09	3.38 ± 5.03	9.44
PMRP-04	0.051 ± 0.04	0.078 ± 0.15	0.071 ± 0.26	0.11 ± 0.59	0.35 ± 0.66	0.36 ± 0.78	1.02
PMRP-05	0.17 ± 0.23	0.23 ± 0.33	0.18 ± 0.11	0.35 ± 0.29	7.05 ± 17.31	6.70 ± 6.99	14.68
PMRP-06	0.081 ± 0.25	0.071 ± 0.09	0.084 ± 0.26	0.082 ± 0.17	5.47 ± 0.75	4.60 ± 17.77	10.39
PMRP-07	0.12 ± 0.14	0.19 ± 0.11	0.13 ± 0.11	0.28 ± 0.04	0.36 ± 0.83	0.37 ± 1.62	1.45
PMRP-08	0.16 ± 0.49	0.36 ± 0.70	0.19 ± 0.11	0.47 ± 0.06	7.21 ± 8.07	5.10 ± 27.48	13.49
PMRP-09	0.16 ± 0.09	0.47 ± 1.30	0.18 ± 0.57	0.42 ± 0.48	6.30 ± 9.12	6.09 ± 18.05	13.62
PMRP-10	0.079 ± 0.09	0.13 ± 0.24	0.094 ± 0.02	0.17 ± 0.99	0.19 ± 0.17	0.24 ± 0.61	0.90
PMRP-11	7.46 ± 2.35	5.73 ± 3.87	4.23 ± 0.28	7.36 ± 4.25	9.47 ± 4.41	8.05 ± 38.33	42.30
PMRP-12	0.10 ± 0.06	0.34 ± 0.26	0.11 ± 0.07	0.27 ± 0.19	1.25 ± 0.88	1.23 ± 2.59	3.30
PMRP-13	0.030 ± 0.00	[b] ND	0.05 ± 0.00	0.031 ± 0.00	0.098 ± 0.12	0.14 ± 0.58	0.35
PMRP-14	0.090 ± 0.22	0.13 ± 0.65	0.11 ± 0.40	0.16 ± 0.30	2.54 ± 1.95	2.27 ± 2.78	5.30
PMRP-15	3.51 ± 4.24	3.08 ± 2.97	2.47 ± 3.51	4.37 ± 9.27	2.68 ± 5.17	2.12 ± 4.77	18.23
PMRP-16	0.062 ± 0.05	0.095 ± 0.15	0.072 ± 0.34	0.085 ± 0.17	4.08 ± 5.99	3.99 ± 3.69	8.38
PMRP-17	0.070 ± 0.35	0.088 ± 0.23	0.081 ± 0.02	0.10 ± 0.34	0.62 ± 2.46	0.62 ± 1.38	1.58
PMRP-18	0.047 ± 0.26	0.082 ± 0.37	0.067 ± 0.26	0.098 ± 0.22	0.24 ± 0.20	0.27 ± 0.31	0.80
PMRP-19	4.42 ± 2.17	4.67 ± 4.59	2.36 ± 5.45	6.42 ± 4.79	8.68 ± 7.05	8.38 ± 28.49	34.93
PMRP-20	0.51 ± 0.27	0.44 ± 0.35	0.67 ± 0.15	0.64 ± 0.10	28.54 ± 7.99	25.95 ± 5.23	56.75
PMRP-21	0.047 ± 0.26	0.051 ± 0.19	0.074 ± 0.55	0.063 ± 0.65	0.52 ± 0.61	0.45 ± 0.39	1.21
PMRP-22	0.22 ± 0.12	0.29 ± 0.00	0.20 ± 0.00	0.40 ± 0.32	9.31 ± 0.00	8.62 ± 0.00	19.04
PMRP-23	0.030 ± 0.08	[b] ND	0.050 ± 0.00	0.030 ± 0.45	0.19 ± 2.99	0.21 ± 2.62	0.51
PMRP-24	0.77 ± 0.99	0.64 ± 0.49	0.46 ± 1.93	0.99 ± 1.74	8.87 ± 10.19	8.22 ± 2.72	19.95
PMRP-25	0.14 ± 0.17	0.28 ± 0.44	0.18 ± 0.62	0.34 ± 0.92	2.81 ± 12.24	2.02 ± 4.31	5.77
PMRP-26	0.10 ± 0.06	0.068 ± 0.04	0.095 ± 0.37	0.098 ± 0.11	0.71 ± 0.52	0.63 ± 1.47	1.70
PMRP-27	0.26 ± 0.59	0.42 ± 0.96	0.27 ± 0.02	0.67 ± 1.75	6.52 ± 5.11	5.13 ± 19.97	13.27
PMRP-28	1.18 ± 0.03	1.75 ± 0.53	1.35 ± 0.05	2.35 ± 0.45	13.77 ± 7.12	10.30 ± 6.31	30.7
PMRP-29	0.063 ± 0.11	0.14 ± 0.39	0.091 ± 0.07	0.16 ± 0.52	0.95 ± 5.51	0.71 ± 2.44	2.11
PMRP-30	0.054 ± 0.29	0.16 ± 0.60	0.098 ± 1.79	0.12 ± 0.08	1.53 ± 3.12	1.19 ± 3.69	3.15
PMRP-31	9.37 ± 8.79	6.32 ± 11.82	5.23 ± 7.88	11.36 ± 14.95	8.23 ± 1.64	7.79 ± 6.42	48.30
PMRP-32	3.48 ± 1.90	2.02 ± 0.60	1.70 ± 1.63	3.40 ± 1.31	6.73 ± 3.92	5.92 ± 22.20	23.25
PMRP-33	0.065 ± 0.41	0.094 ± 0.21	0.084 ± 0.28	0.12 ± 0.19	0.59 ± 5.10	0.51 ± 3.46	1.46
PMRP-34	0.081 ± 0.57	0.10 ± 0.58	0.085 ± 0.27	0.12 ± 0.59	0.89 ± 3.39	0.70 ± 3.10	1.98
PMRP-35	0.16 ± 0.81	0.23 ± 0.18	0.17 ± 0.30	0.41 ± 1.48	0.32 ± 1.99	0.35 ± 1.18	1.64
PMRP-36	0.15 ± 0.25	0.27 ± 0.02	0.17 ± 0.07	0.34 ± 0.03	0.31 ± 0.03	3.52 ± 9.47	4.76
PMRP-	0.11 ± 0.02	0.34 ± 0.14	0.12 ± 0.09	0.35 ± 0.49	0.33 ± 1.51	0.31 ± 1.16	1.56

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PMRP-38	0.056	±	0.01	0.075	±	0.29	0.071	±	0.21	0.089	±	0.01	0.28	±	0.15	0.30	±	1.38	0.87					
PMRP-39	0.21	±	0.64	0.53	±	0.51	0.23	±	0.15	0.59	±	1.60	3.95	±	12.81	2.67	±	2.57	8.18					
PMRP-40	0.057	±	0.08	0.18	±	0.83	0.072	±	0.15	0.12	±	0.36	3.24	±	9.23	2.66	±	3.43	6.33					
PMRP-41	0.025	±	0.29	0.051	±	0.19	0.039	±	0.13	0.063	±	0.31	0.38		0.63	0.27	±	0.86	0.83					
PMRP-42	[b] ND			0.089	±	0.00	[b] ND			0.14	±	0.02	0.36		2.36	0.24	±	0.90	0.83					
PMRP-43	0.13	±	0.028	0.39	±	0.32	0.16	±	1.60	0.62	±	0.53	1.42	±	10.91	1.13	±	14.98	3.85					
PMRP-44	0.025	±	0.08	0.17	±	0.061	0.054	±	0.13	0.23	±	0.11	0.32	±	0.70	0.21	±	0.15	1.01					
PMRP-45	[b] ND			0.089	±	0.043	[b] ND			0.14	±	0.00	0.33	±	0.26	0.24	±	0.71	0.80					
PMRP-46	0.028	±	0.17	0.48	±	0.028	0.14	±	0.37	0.56	±	0.90	3.25	±	16.03	3.29	±	7.35	7.86					
PMRP-47	0.08	±	0.16	0.411	±	0.08	0.073	±	0.44	0.32	±	0.14	0.40		1.05	0.28	±	0.35	1.53					
PMRP-48	0.42	±	0.04	1.63	±	1.42	0.69	±	0.28	2.68	±	0.31	9.86	±	0.14	7.38	±	1.26	22.66					
PMRP-49	0.20	±	0.03	1.21	±	0.04	0.36	±	0.00	1.59	±	0.02	26.99	±	10.44	27.79	±	0.27	58.14					
PMRP-50	7.40	±	2.13	5.61	±	0.37	3.94	±	0.39	10.31	±	1.79	11.01	±	0.97	10.85	±	0.43	49.12					
PMRP-51	0.027	±	0.38	0.13	±	0.01	0.040	±	0.07	0.17	±	0.18	1.90	±	1.42	1.53	±	0.53	3.80					
PMRP-52	13.03	±	18.67	8.94	±	20.21	7.23	±	10.11	12.75	±	16.48	10.82	±	61.54	12.50	±	6.99	65.27					
PMRP-53	0.46	±	0.41	1.06	±	3.01	0.54	±	0.34	1.35	±	3.72	1.26	±	3.35	1.41	±	2.37	6.08					
PMRP-54	0.16	±	0.08	0.54	±	1.02	0.20	±	0.36	0.64	±	0.52	5.29	±	7.85	5.50	±	14.07	12.33					
PMRP-55	0.072	±	0.08	0.36	±	0.60	0.10	±	0.03	0.41	±	0.64	1.81	±	4.11	1.80	±	1.06	4.55					
PMRP-56	8.25	±	4.61	4.64	±	5.20	4.95	±	5.92	8.79	±	10.87	3.80	±	7.98	4.60	±	2.97	35.03					
PMRP-57	0.22	±	0.78	0.58	±	1.31	0.25	±	0.63	0.79	±	1.61	5.18	±	12.17	4.65	±	3.99	11.67					
PMRP-58	0.40	±	0.89	0.92	±	4.89	0.55	±	0.53	1.15	±	0.59	3.24	±	0.48	3.41	±	6.09	9.67					
PMRP-59	0.71	±	0.64	0.87	±	5.14	0.54	±	0.26	0.92	±	1.15	16.79	±	24.06	18.91	±	70.89	38.74					
PMRP-60	5.61	±	0.64	8.19	±	2.47	3.68	±	0.55	10.28	±	0.99	18.71	±	9.53	18.75	±	7.29	65.22					
PMRP-61	0.032	±	0.18	0.17	±	0.24	0.054	±	0.23	0.21	±	0.66	0.29	±	2.00	0.18	±	1.71	0.94					
PMRP-62	0.095	±	0.10	0.16	±	0.75	0.069	±	0.43	0.25	±	0.27	0.34	±	3.00	0.24	±	1.83	1.15					
PMRP-63	0.13	±	0.07	0.57	±	0.69	0.13	±	0.09	0.49	±	0.13	0.56	±	0.41	0.46	±	1.84	2.34					
PMRP-64	[b] ND			0.12	±	0.43	0.032	±	0.10	0.17	±	0.48	0.26	±	0.62	0.15	±	0.60	0.73					
PMRP-65	0.028	±	0.17	0.11	±	0.12	0.044	±	0.04	0.17	±	0.27	0.49	±	0.31	0.40	±	1.86	1.24					
PMRP-66	0.08	±	0.36	0.15	±	0.10	0.040	±	0.24	0.20	±	1.23	0.28	±	0.09	0.19	±	0.92	0.89					
PMRP-67	0.52	±	1.64	0.36	±	0.38	0.31	±	0.04	0.73	±	1.15	0.38	±	0.14	0.31	±	0.58	2.61					
PMRP-68	[b] ND			0.090	±	0.01	0.022	±	0.078	0.14	±	0.080	0.31	±	0.95	0.18	±	0.34	0.74					
PMRP-69	0.028	±	0.16	0.20	±	0.56	0.080	±	0.03	0.27	±	0.42	1.36	±	2.16	1.45	±	4.49	3.41					
PMRP-70	[b] ND			0.089	±	0.01	0.022	±	0.019	0.14	±	0.00	0.48	±	0.49	0.38	±	0.40	1.11					
PMRP-71	1.19	±	4.47	0.85	±	1.93	0.89	±	3.43	0.81	±	4.23	2.32	±	18.15	2.21	±	17.49	8.27					
PMRP-72	0.40	±	1.45	1.21	±	4.70	0.50	±	0.19	1.52	±	2.72	6.52	±	22.57	5.29	±	18.23	15.44					
PMRP-73	0.45	±	0.29	0.78	±	1.43	0.47	±	0.79	1.21	±	0.37	6.23	±	10.35	5.98	±	16.38	15.12					
PMRP-74	0.054	±	0.03	0.19	±	0.53	0.059	±	0.18	0.23	±	0.39	3.41	±	2.97	3.25	±	2.33	7.19					
PMRP-75	0.029	±	0.14	1.25	±	0.29	0.36	±	0.24	1.71	±	0.16	10.67	±	0.60	10.10	±	1.88	24.12					
PMRP-	0.55	±	0.50	0.43	±	0.20	0.45	±	0.81	0.51	±	0.51	1.20	±	0.79	1.14	±	0.16	4.28					

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PMRP-77	1.15	±	1.12	0.97	±	0.54	0.98	±	2.83	1.00	±	0.48	6.13	±	11.15	5.36	±	37.35						15.59
PMRP-78	1.18	±	0.21	0.95	±	3.74	1.03	±	0.82	0.97	±	1.22	6.23	±	9.83	5.37	±	15.03						15.73
PMRP-79	1.34	±	0.75	1.12	±	2.17	1.15	±	1.72	1.08	±	3.02	6.22	±	5.22	5.48	±	4.66						16.39
PMRP-80	1.30	±	0.68	0.91	±	8.23	0.90	±	0.99	1.48	±	1.62	1.41	±	12.83	1.44	±	3.38						7.44
PMRP-81	0.20	±	0.39	0.47	±	1.71	0.22	±	0.93	0.73	±	2.19	1.73	±	1.46	1.44	±	3.51						4.79
PMRP-82	[b] ND			0.090	±	0.01	0.022	±	0.081	0.14	±	0.12	0.38	±	0.60	0.27	±	0.45						0.90
PMRP-83	0.19	±	0.20	3.13	±	1.39	1.84	±	0.42	4.48	±	1.85	19.83	±	2.04	21.69	±	1.97						51.16
PMRP-84	0.849	±	1.01	1.03	±	0.79	0.76	±	1.06	1.73	±	3.45	5.40	±	12.61	5.86	±	22.91						15.63
PMRP-85	0.269	±	1.38	0.51	±	0.96	0.26	±	0.15	0.71	±	1.08	1.38	±	0.96	1.29	±	0.34						4.42
PMRP-86	0.65	±	1.37	6.80	±	1.29	3.53	±	0.35	9.55	±	3.24	15.04	±	3.08	15.83	±	0.01						51.40
Average	0.96			1.08			0.71			1.53			4.47			4.25								12.99

Note: [a] The data are presented as the average of two replicates; [b] ND, under limits of quantitation.

Scheme

Scheme 1 is available in the Supplemental Files section

Figures

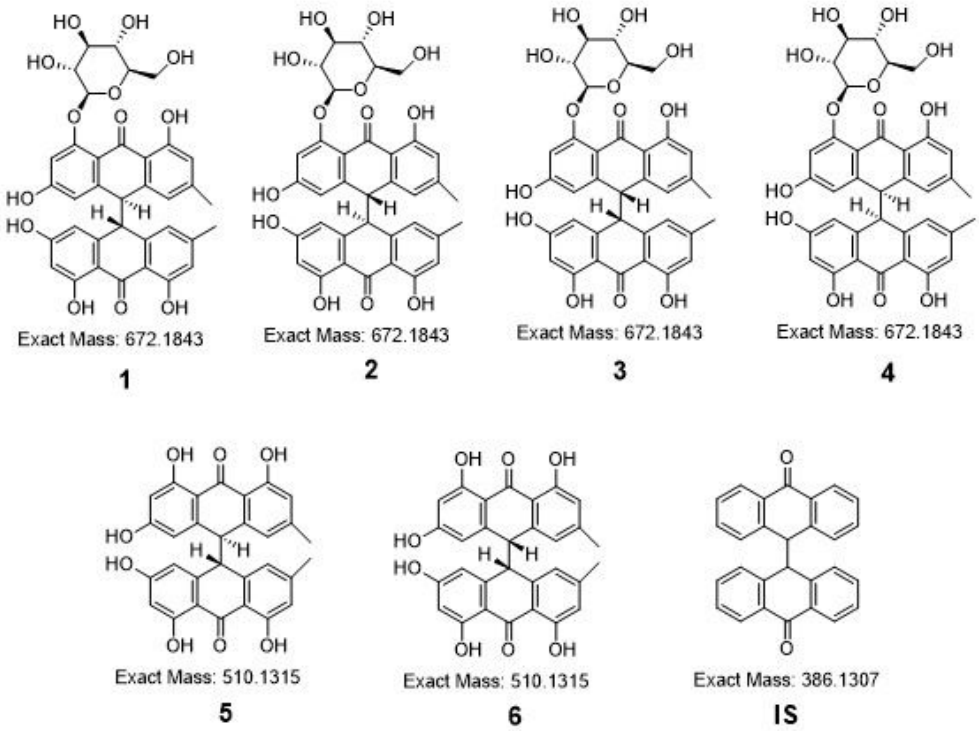

Figure 1

Chemical structure of six dianthrones (1-6) and one internal standard (IS)

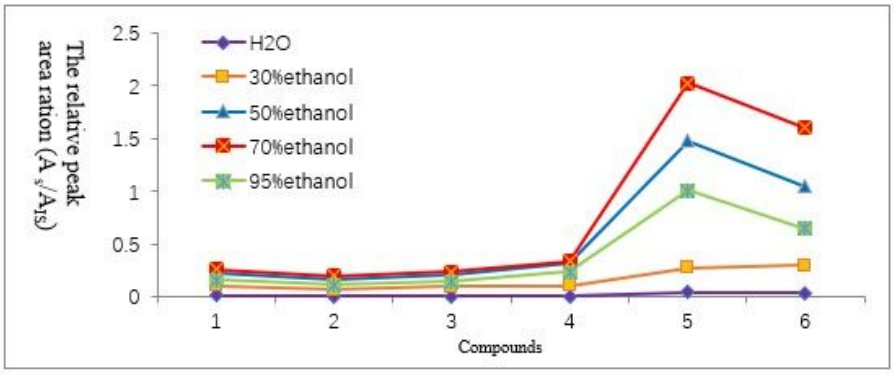

Fig. 2A. Optimization of different extraction solvents

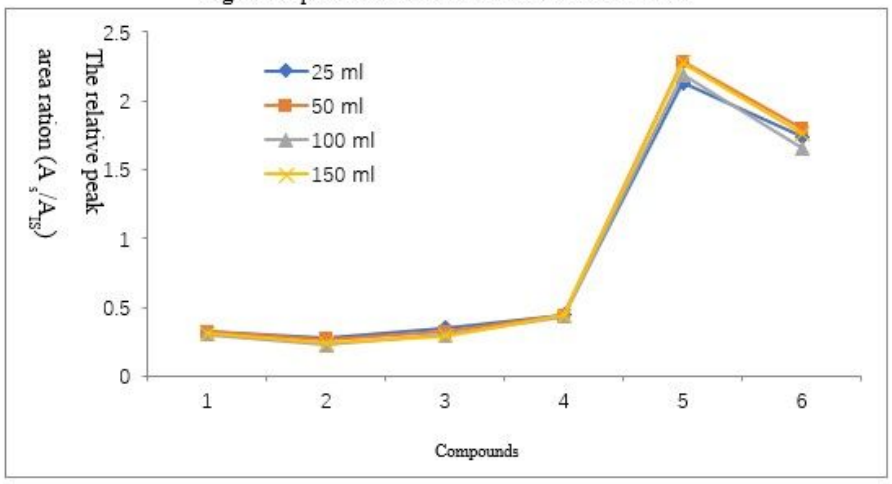

Fig. 2B. Optimization of different solvent volumes

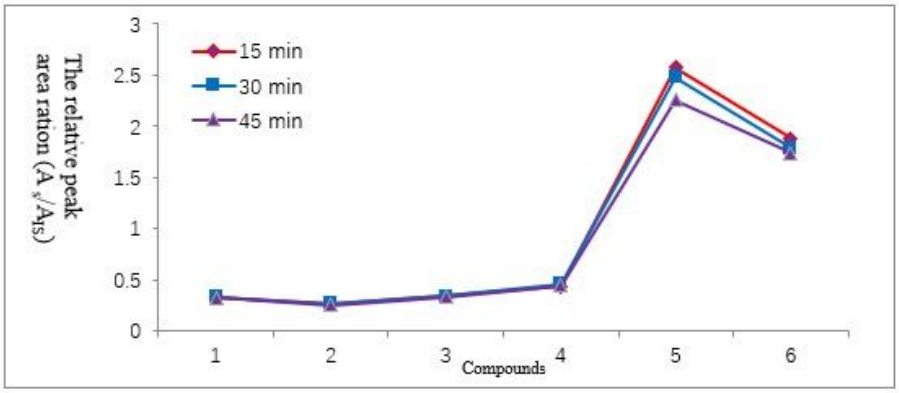

Fig. 2C. Optimization of different ultrasonication times

Figure 2

Optimization of different parameters of the method of sample solution, (A) type of extractant, (B) volume of extractant and (C) ultrasound time

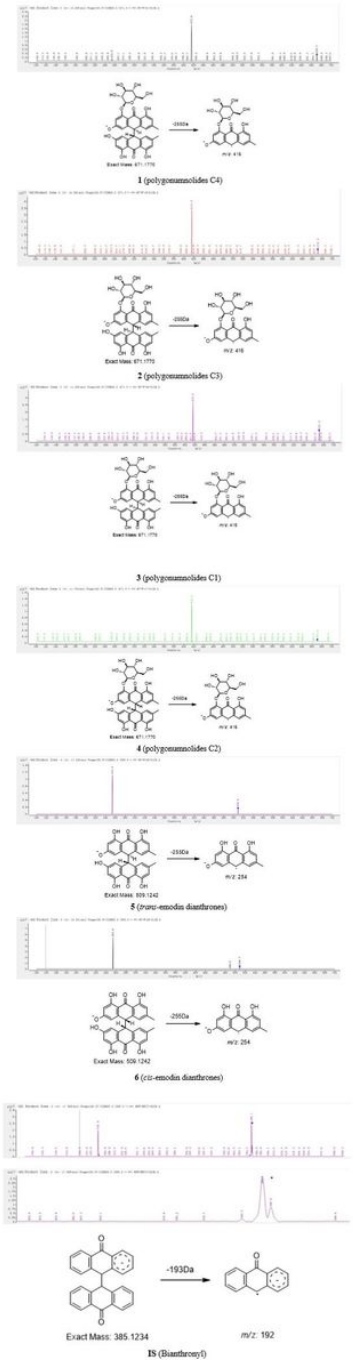

Figure 3

Product ion mass spectra and proposed fragmentation pathway of six dianthrones (1-6) and one internal standard (IS)

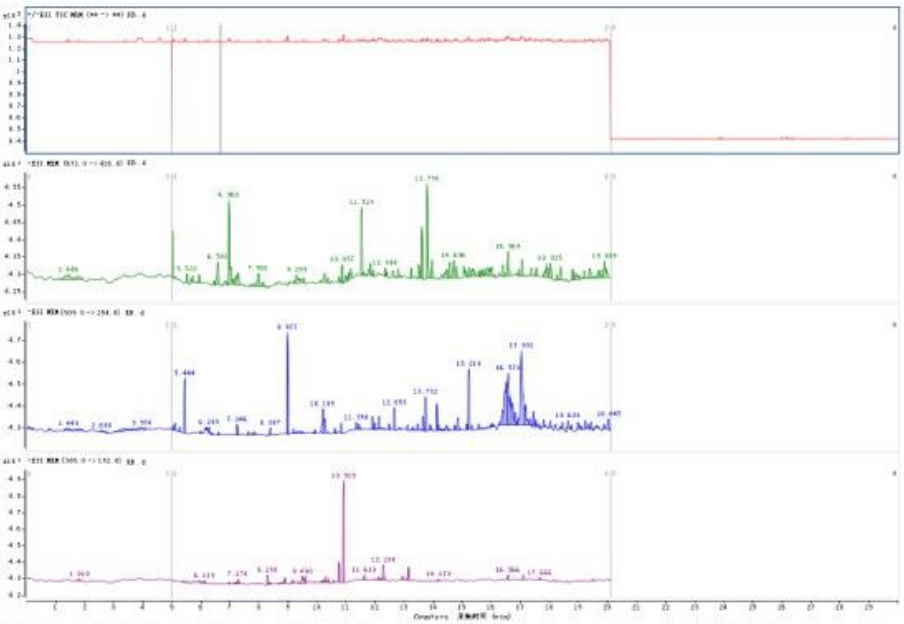

Fig.4A. Typical multiple reaction monitoring (MRM) chromatograms for the blank test sample

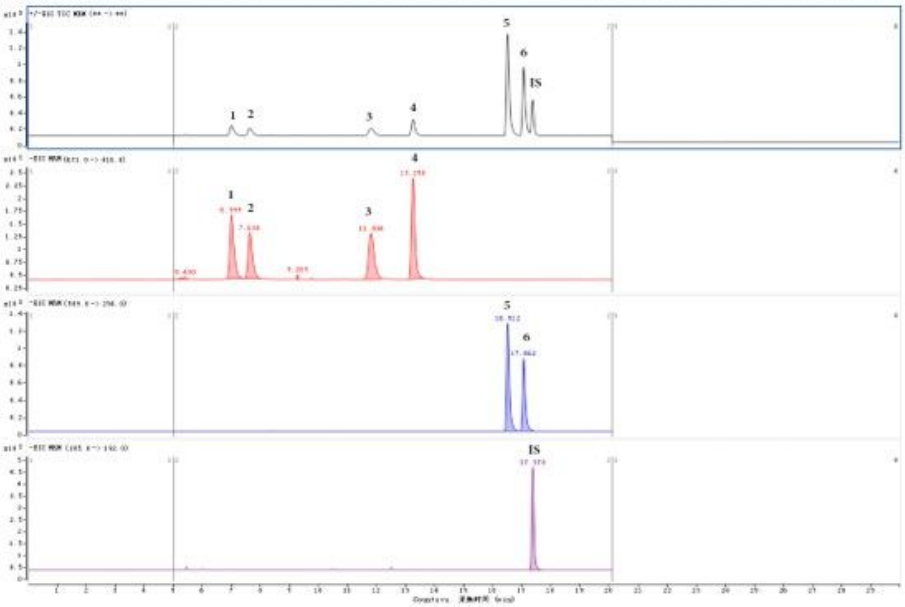

Fig.4B. Typical multiple reaction monitoring (MRM) chromatograms for a sample of *P. multiforum*

Figure 4

Typical multiple reaction monitoring (MRM) chromatograms for a blank test sample and a sample of P. multiforum (1. polygonumnolides C4; 2. polygonumnolides C3; 3.polygonumnolides C1; 4. C polygonumnolides C4; 5. trans-emodin dianthrones; 6. cis-emodin dianthrones; IS. Bianthronyl).

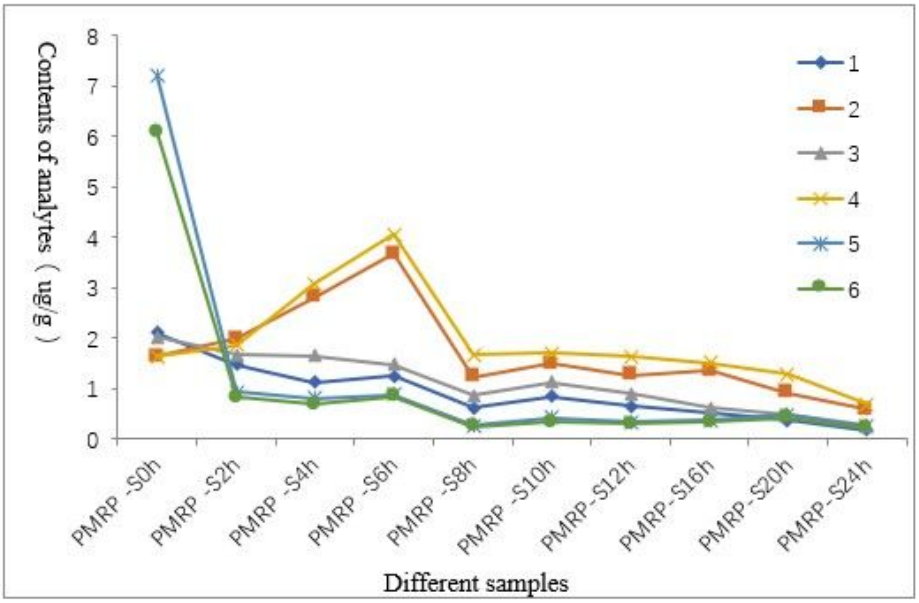

Figure 5

Contents of compounds 1-6 in different points with the water steaming method (PMRP-S0h, S2h, S4h, S6h, S8h, S10h, S12h, S16h, S20h and S24h)

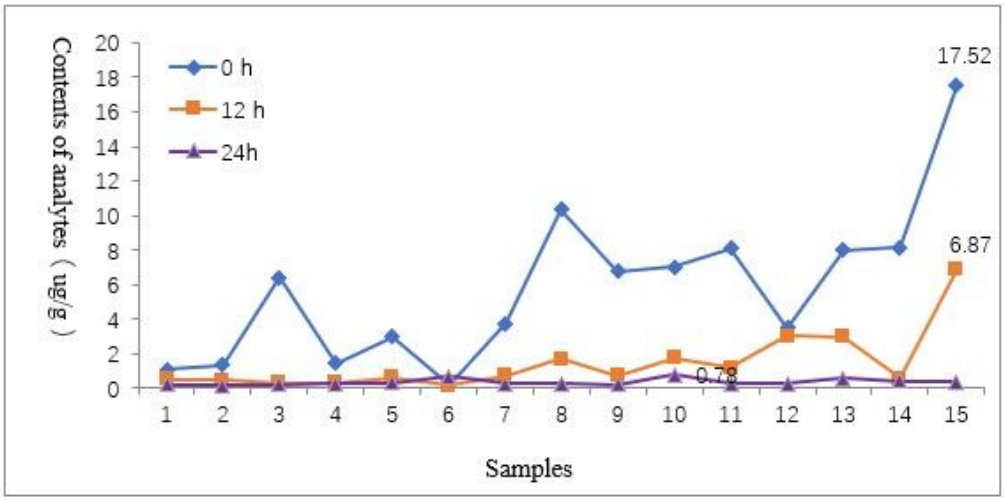

Fig. 6A Contents of **5** in 15 samples

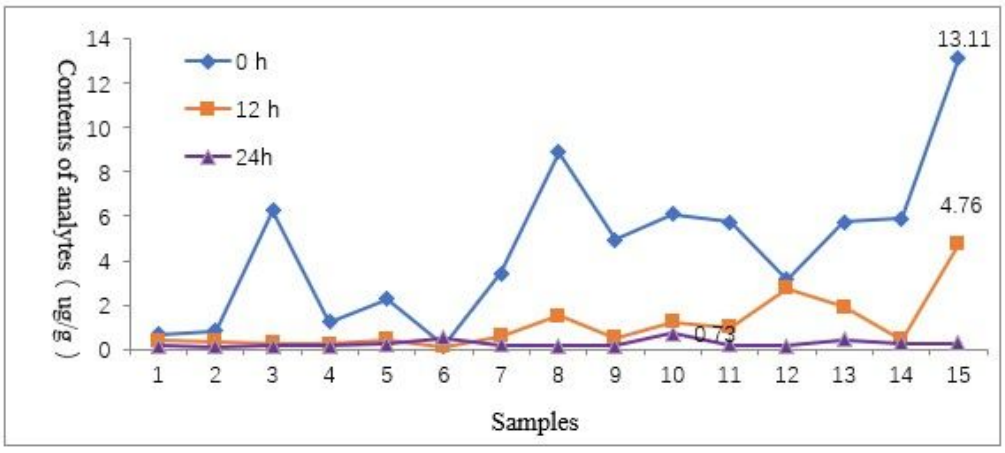

Fig. 6B Contents of **6** in 15 samples

Figure 6

Contents of 5 and 6 in 15 samples of Polygoni Multiflori Radix Praeparata (PMRP) with the water steaming method

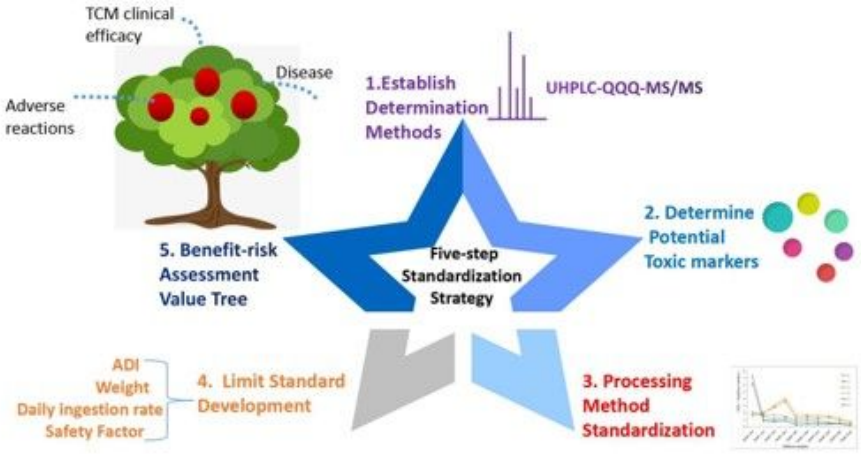

Figure 7

A systematic five-step strategy for standardization of endogenous toxic TCMs

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.jpg