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ORIGINAL ARTICLE

Metabolomics Analysis of Region-Specific Polygoni Multiflori Radix Metabolite Profiles

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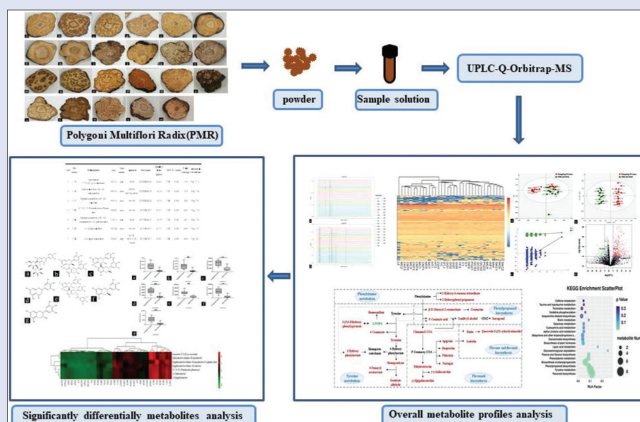
ABSTRACT

Background: Polygoni Multiflori Radix (PMR) is a widely distributed herb that has been used for centuries in the treatment of a range of systemic diseases among practitioners of traditional Chinese medicine. **Objectives:** The present comparative metabolite analysis study was conducted in an effort to understand the relationship between the geographical origin of PMR samples and their medicinal properties. **Materials and Methods:** As a metabolomics analysis of 35 PMR samples collected from Guangdong and other provinces in China was conducted via ultra-high performance liquid chromatography-quadrupole-electrostatic field orbitrap high-resolution mass spectrometry. **Results:** Differential metabolite profiles in these PMR samples were evaluated through multivariate statistical analyses. In total, this approach led to the identification of 778 differential metabolites [value of group contribution (VIP) >1, $P < 0.01$, and fold-change (FC) >2 or <0.5)] that were primarily associated with glycosaminoglycan degradation, lipoic acid metabolism, flavonoid biosynthesis, tyrosine metabolism, flavone and flavonol biosynthesis, phenylpropanoid biosynthesis, and phenylalanine metabolism. Of these metabolites, catechins accounted for seven significantly altered metabolites (VIP ≥2, $P < 0.01$, and FC >2 or <0.5) when comparing PMR samples from Guangdong with those from other regions. This suggests that PMR samples contain metabolite profiles characteristic of their provenance. **Conclusion:** As such, metabolomic profiling may be effective means of differentiating between PMR samples from different geographic regions within China, thus providing a sound theoretical basis for the reliable differentiation among and selection of pharmacologically optimal PMR samples.

Key words: Geographical origin, metabonomics, Polygoni Multiflori Radix, provenance, ultra-high-performance liquid chromatography-quadrupole-electrostatic field orbitrap high-resolution mass spectrometry

SUMMARY

- In this study, using PMR as an example, a novel strategy exploring the quality markers of Chinese material medica for the reliable differentiation among and selection of pharmacologically optimal PMR samples has developed. Firstly, untargeted metabolomics data of 35 batches of PMR from Guangdong and other regions in China was obtained by UPLC-Q/Orbitrap-MS. Seven hundred and seventy-eight differentially abundant metabolites were successfully screened by UPLC-Q/Orbitra-MS and multivariate statistical analysis, and the types, expression levels, and relationships of these differential metabolites involved in the five enriched metabolic pathways were demonstrated on the pathway map. Secondly, seven potential marks were further screened out, and their profiles characteristic, structure, and relative content were studied in detail. Finally, the scientificity of these seven compounds as the chemical index to assess the authenticity of PMR was successfully verified by performing bidirectional clustering of the seven significantly different metabolites and PMR samples from different sources. Thus, using a metabolomics-based approach to evaluate PMR provenance offers value as a means of studying the intrinsic mechanisms governing PMR authenticity, providing a firm theoretical foundation for additional studies of PMR composition and quality.



Abbreviations used: PMR: Polygoni Multiflori Radix; TCM: traditional Chinese medicine; ESI: electrospray ion source; PCA: principal component analysis; OPLS-DA: orthogonal partial least-squares discrimination analysis; UPLC: ultra performance liquid chromatography.

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INTRODUCTION

Polygoni Multiflori Radix (PMR) is a medicinal herb derived from the dried tuberous roots of the Polygonaceae perennial herb *Polygonum multiflorum* Thunb that is cultivated in China, Japan, Korea, and other Asian countries.[1] PMR is commonly used as a medicine and exhibits laxative, antimalarial, and detoxifying properties that have led to its use for the treatment of chronic malaria, constipation, scrofula, and

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rubella pruritus.[2,3] Processed PMR samples have been found to exhibit immunomodulatory, hepatoprotective, and renoprotective properties and can stimulate hematopoiesis.[4,5] PMR is thus commonly utilized in both culinary and medicinal contexts and is a resource in over 240 traditional Chinese medicinal formulations.[6]

PMR plants are widely distributed and are primarily grown in the Guangdong, Guangxi, Sichuan, and Guizhou provinces of China.[7] Increased urbanization has led to a growing demand for PMR that necessitates its artificial cultivation. However, genetic and environmental factors are known to impact the properties of PMR grown in different regions.[8] These factors can alter metabolite levels in prepared PMR samples, potentially resulting in inconsistent quality and clinical efficacy that may constrain the commercialization of this important medicinal herb.

"Genuine medicinal material" is a Traditional Chinese medicine (TCM) term referring to a medicinal plant that is grown in an appropriate environment that is associated with optimal clinical efficacy and quality.[9] High-quality TCM materials are thus referred to as originating from geo-authentic producing areas, with Deqing County in Guangdong Province being the traditional geo-authentic producing area for PMR. However, cultivated and wild PMR can also be found in other Provinces including Guangxi, Sichuan, and Guizhou. Whether PMR grown in these regions exhibits any intrinsic differences in pharmacologic activity or quality, however, remains to be determined. As such, it is essential that researchers fully assess the composition and consistency of PMR cultivated in geo-authentic and non-authentic producing areas.

Metabolomics studies enable researchers to quantitatively analyze metabolite components in particular tissues, organs, or organisms to understand the relationship between these compounds and particular phenotypes of interest.[10-12] Plant metabolomics studies are of particular value in the context of TCM, as they enable researchers to assess sample origin, classification, or quality based upon chemometric parameters.[13] Indeed, such metabolomics approaches have been leveraged to compare the relationship between TCM material composition and different plant sources,[8,9] varieties,[14] medicinal parts,[15] processing methods,[2,16] growth periods, and environmental conditions.[17,18] Owing to their comprehensive nature, such metabolomics studies are superior to single-target analyses and are ideal for the assessment of the quality of TCM formulations and materials.[13]

In the present study, we analyzed samples of PMR from Guangdong and compared them with samples from other provenances via a comprehensive ultra-high performance liquid chromatography-quadrupole-electrostatic field orbitrap high-resolution mass spectrometry (UPLC-Q-Orbitrap/MS)-based metabolomics profiling approach to highlight origin-related differences in PMR composition and consistency. All these data provide a scientific framework for analyzing the origin and quality of different PMR samples.

MATERIALS AND METHODS

Plant material

Fresh PMR samples were collected from different regions in China and were positively identified by Associate Professor Jizhu Liu of Guangdong Pharmaceutical University. After collection and identification, samples were rinsed with water, cut into 1 cm-thick slice, dried, and stored. For details regarding sample collection see Table 1.

The Guangdong sample group included samples CNT1-5, MC1-7, WFC, HT1-2, MM, STJ, and SKC1-2, the Guangxi sample group included samples GG1, GG2, GG7, GG9, and LZ, the Guizhou sample group included SKS and GY, the Sichuan sample group included PQZ1-2, QCS1-3, and ZWY, the Jiangsu sample group included YY, the Shanxi sample group included YC, and the Jiangxi sample group included YFX.

Chemicals and reagents

Methanol, formic acid, acetonitrile, and pure water (≥99.0% pure; chromatographic grade) were obtained from CNW Technologies GmbH (Germany). L-2-chlorophenyl alanine was obtained from HC Biotech Co., Ltd. (Shanghai, China). LysoPC17:0 were from Avanti (USA).

Instrumentation

A Nexera UPLC (Shimadzu, Japan) and a QE High-resolution mass spectrometer (Thermo Fisher Scientific, USA) instrument were used for this study. An ACQUITY UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) (Waters) was used for chromatographic separation. In addition, an LNG-T98 Freeze concentration centrifugal dryer (Taicang Huamei Biochemical Instrument Factory, China), a TGL-16 MS High speed refrigerating centrifuge (Shanghai Lu Xiangyi Centrifuge Instrument Co., Ltd., China), a JXFSTPRP-24/32 Automatic sample rapid grinding machine (Shanghai Jingxin Industrial development CO., LTD., China), and an SB-5200DT Ultrasonic cleaner (Ningbo SCIENTZ Biotechnology CO., LTD., China) were used for this experimental procedure.

Sample preparation

PMR samples were ground into powder, after which 30 mg of powdered samples were combined with 20 μL of internal standard (IS; 0.3 mg/mL L-2-chlorophenyl alanine and 0.01 mg/mL Lyso PC17:0, prepared using methanol), and 1 mL of methanol–water (7:3, V/V). Samples were then vortexed for 30 sec, after which two small steel balls were added to the sample tube, which was cooled for 2 min at −20°C. Samples were then processed using a grinding machine (60 Hz, 2 min), after which steel balls were removed and ultrasonic extraction was performed for 30 min. The resultant solution was then transferred to −20°C for 20 min, after which it was spun for 10 min at 13,000 rpm at 4°C. Next, a 300 μL volume of supernatant was removed, dried, dissolved in 400 μL of methanol–water (1:4, V/V), vortexed for 30 sec, and ultrasonicated for 2 min. Samples were then spun for an additional 10 min at 13,000 rpm at 4°C, after which 150 μL supernatant volumes were collected via syringe, filtered through a 0.22 μm organic pinhole filter, and used for UPLC-Q-Orbitrap-MS analysis.

UPLC-Q-Orbitrap-MS analysis

A UPLC-Q-Orbitrap-MS system was employed for PMR metabolic analyses. Briefly, chromatographic separation was achieved by injecting 10 μL sample aliquots into an ACQUITY UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) for separation, with a fixed column temperature of 45°C. Separation was achieved using a mobile phase composed of water containing 0.1% formic acid (A) and methanol (B). The flow rate was fixed at 0.35 mL/min, and the following gradient elution settings were used: at 0.01 min 95% A; at 1.5 min 95% A; at 3 min 70% A; at 7 min 40% A; at 9 min 10% A; at 11 min 0% A; at 12 min 0% A; at 12.1 min 95% A; and at 15 min 95% A.

For MS analyses, an electrospray ion source (ESI) was operated in both negative and positive ion scanning modes, with respective positive and negative spray voltage values of 3.5 kV and 3.1 kV. HCD energy set to ladder mode (20, 40, 60ev), the HCD MS/MS scanning resolution was set to 17,500. The full-mass scan was conducted with a resolution of 70,000 and an m/z range of 50–1,500.

Data analysis

Data were imported into Progenesis QI for baseline filtering, peak identification, integration, retention time correction, peak

Table 1: Sample information

Name	Geographical origin	Provenance	Type
CNT1	Chunniuting Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
CNT2	Chunniuting Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
CNT3	Chunniuting Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
CNT4	Chunniuting Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
CNT5	Chunniuting Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
MC1	Mo Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
MC2	Mo Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
MC3	Mo Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
MC4	Mo Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
MC5	Mo Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
MC6	Mo Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
MC7	Mo Village, Deqing County, Guangdong Province	Deqing, Guangdong	Cultivated
WFC	Wufu Village, Deqing County, Guangdong Province	Deqing, Guangdong	Wild
HT1	Hetai Town, Gaoyao District, Guangdong Province	Guangdong Province	Wild
HT2	Hetai Town, Gaoyao District, Guangdong Province	Guangdong Province	Wild
MM	Maoming City, Guangdong Province	Guangdong Province	Wild
STJ	Shantangjiao Village, Xinxing County, Guangdong Province	Guangdong Province	Cultivated
GG1	Guigang City, Guangxi Province	Guangxi Province	Cultivated
GG2	Guigang City, Guangxi Province	Guangxi Province	Cultivated
GG7	Guigang City, Guangxi Province	Guangxi Province	Cultivated
GG9	Guigang City, Guangxi Province	Guangxi Province	Cultivated
SKC1	Shankou Village, Shibing County, Guizhou Province	Deqing, Guangdong	Cultivated
SKC2	Shankou Village, Shibing County, Guizhou Province	Deqing, Guangdong	Cultivated
SKS	Sankeshu Town, Shibing County, Guizhou Province	Guizhou Province	Wild
GY	Guiyang City, Guizhou province	Guizhou Province	Wild
PQZ1	Pingquan Town, Jianyang City, Sichuan Province	Sichuan Province	Wild
PQZ2	Pingquan Town, Jianyang City, Sichuan Province	Sichuan Province	Wild
QCS1	Qingcheng Mountain, Dujiangyan City, Sichuan Province	Sichuan Province	Wild
QCS2	Qingcheng Mountain, Dujiangyan City, Sichuan Province	Sichuan Province	Wild
QCS3	Qingcheng Mountain, Dujiangyan City, Sichuan Province	Sichuan Province	Wild
ZWY	Medicinal Botanical Garden of Chongqing city	Sichuan Province	Wild
YY	Nanjing University of Chinese Medicine, Jiangsu Province	Jiangsu Province	Cultivated
YC	Yuncheng city, Shanxi Province	Shanxi Province	Wild
YFX	Yifeng County, Jiangxi Province	Jiangxi Province	Wild
LZ	Guangdong Pharmaceutical University, Guangdong Province	Guangxi Province	Cultivated

alignment, and normalization, leading to the generation of a final sample metabolite matrix. This data matrix was then imported to the SIMCA 14.1 software to conduct principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA). OPLS-DA mode was assessed by the intercepts of R2Y and Q2 in a permutation test to avoid over-fitting. The permutation tests were performed multiple times ($n = 200$) to generate different random Q2 values that were used to further test the model validity. Metabolite differences were screened based upon the value of group contribution (VIP), significance values ($P < 0.01$), and fold-change (FC) values in univariate analyses. Heat maps and volcano plots were generated using R (http://www.r-project.org/) to visualize differences among samples. Mass and MS/MS fragmentation data were used to identify metabolites in combination with database and literature searches. Mbrole2.0 was used for differential metabolite and metabolite-related pathway analyses, while the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was employed to fully explore metabolite-related pathways and biological roles.

RESULTS

Comparative assessment of PMR sample cross sections

When transverse cross sections of PMR plant tissues collected from different sites of origin were compared, clear differences in root color and structure were observed among these samples [Figure 1].

PMR samples were next analyzed in both negative- and positive-ion modes, and the resultant base peak chromatogram (BPC) profiles revealed that there were clear differences in certain BPC peaks when comparing different sample groups [Figure 2].

Total metabolite hierarchical clustering analysis

After processing using Progenesis QI, we extracted 10309 effective components that were subsequently hierarchically clustered based upon relative levels in analyzed samples [Figure 3]. PMR samples in these analyses clustered into two groups, with LZ samples exhibited metabolite profiles that were significantly different from those of other samples. Samples clustered on the other branch of this analysis were also primarily separated into two categories, with samples from Guangdong (including samples CNT1-5, MC1-3, MC5, MC7, and HT1-2) largely clustering together separately from other samples. Metabolites in the Guangdong STJ, the Guangxi GG, Shanxi YC, and the Sichuan ZWY samples were also similar and clustered together in a small group, while the SKS and GY samples from Guizhou were clustered with the PQZ and QCS samples from Sichuan. In addition, metabolites from the transplanted sample SKC1-2 (transplanted from Deqing, Guangdong province to Guizhou Province) and Guangdong samples MM, MC6, WFC, and MC4 were similar to one another in cluster analyses. Together, these clustering results revealed that PMR samples of a given provenance or cultivation site were generally similar to one another with respect to their metabolite profiles.

Multivariate analyses of overall metabolite profiles

PCA

PCA was initially conducted to visualize the relationships between different PMR samples and to detect outliers [Figure 4a]. The R^2X values for this analysis were 0.53 (>0.5), consistent with good model fitting accuracy. All samples other than sample LZ were within the 95% confidence interval (Hotelling's T-squared ellipse), indicating that sample LZ was an outlier. A distinct trend of group clustering was observed in the PCA score plot. There were differentially abundant metabolites between the Guangdong Province group and the other Province groups. This thus suggested that there may be a relationship between PMR provenance and sample metabolite composition.

OPLS-DA

To identify metabolites that were differentially abundant between different PMR sample groups, an OPLS-DA model was used to classify all samples other than sample LZ, which was identified as an outlier in PCA evaluations. As OPLS-DA is a supervised approach to data analysis, it can be more reliably used to identify differences among samples relative to PCA.[19]

OPLS-DA plots revealed a clear separation between PMR samples of different provenance [Figure 4b], with respective R^2Y and Q^2 values of 0.978 and 0.777 confirming the ability of this model to explain differences between sample groups. The permutation test was also conducted to assess model validity, yielding respective R^2 and Q^2 values

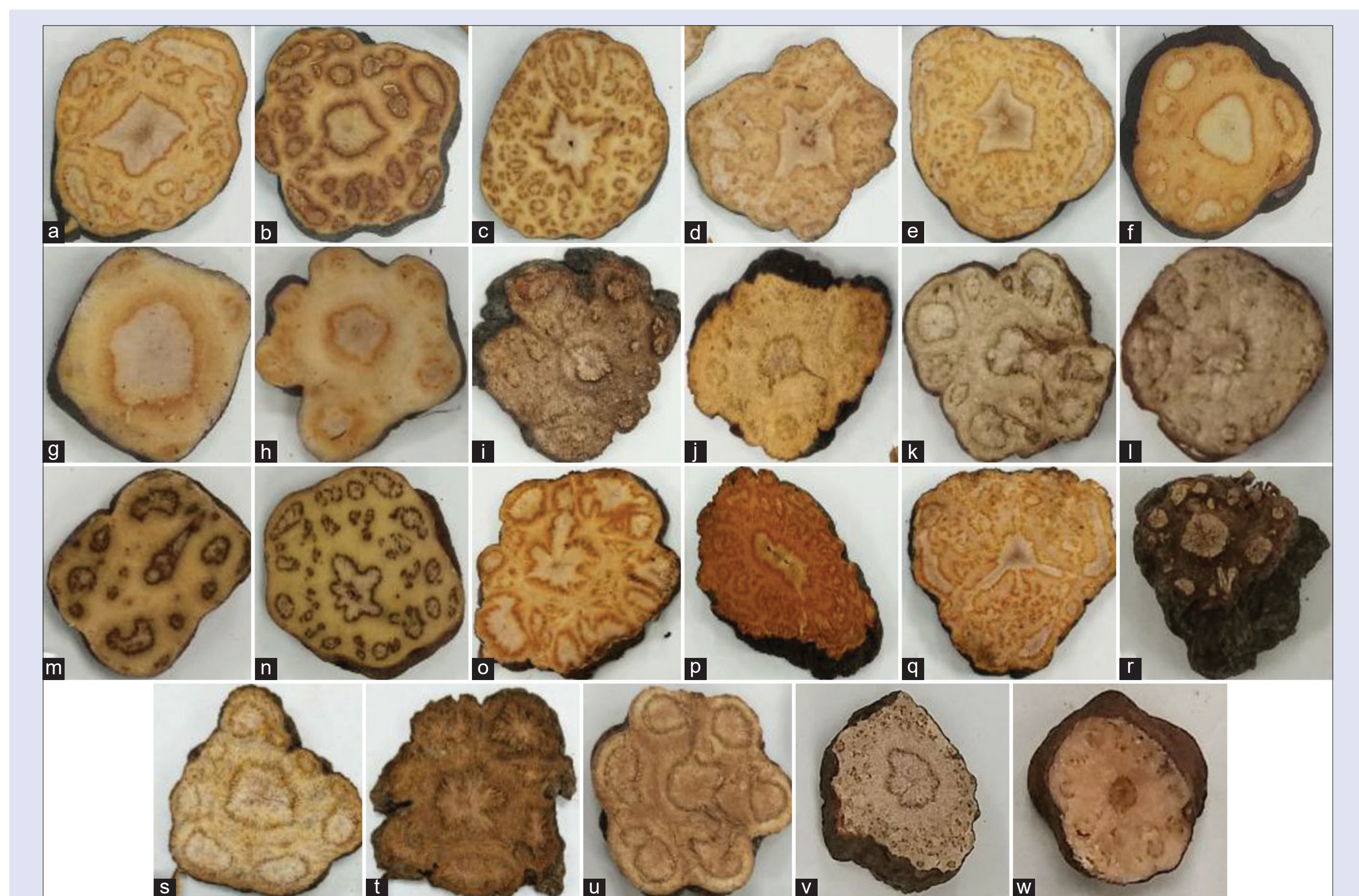

Figure 1: Transverse section of PMR samples from CNT (a and c), MC (d and f), HT (g and h), SKS (i), GY (j), SKC (k and l), GG (m and n), QCS (o and q), PQZ (r and s), YY (t), YC (u), YFX (v), and LZ (w). Assessment of PMR base peak chromatogram profiles

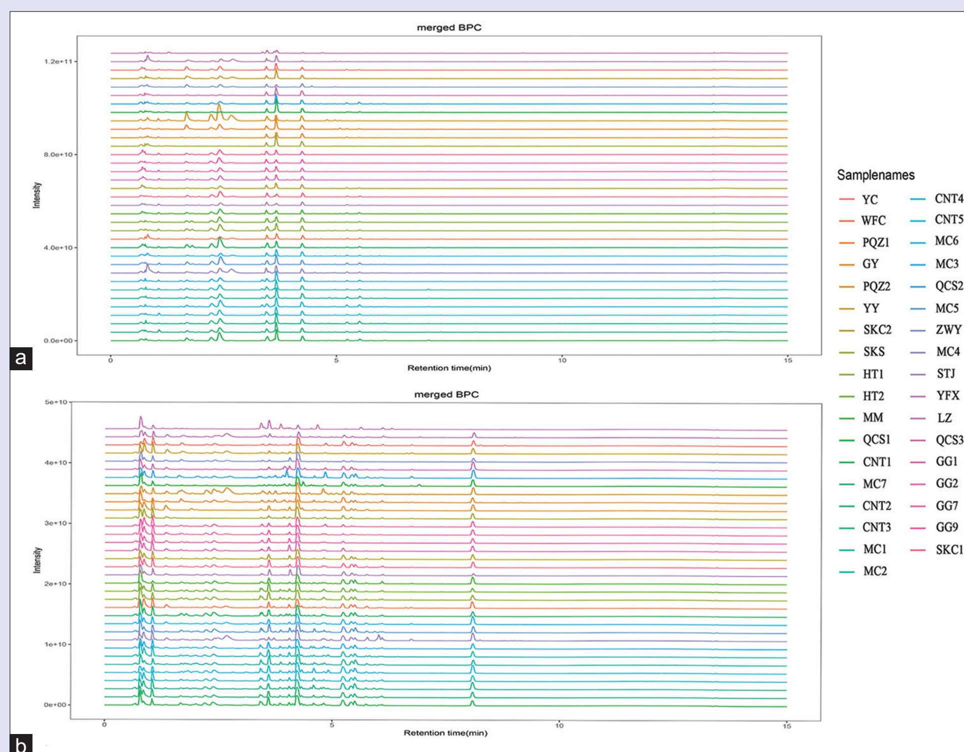

Figure 2: PMR base peak chromatogram profiles. a. positive-ion modes, b. negative-ion modes

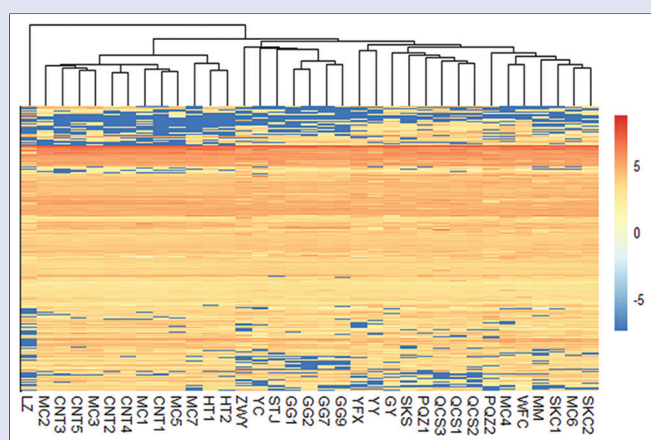

Figure 3: Total metabolite hierarchical clustering analysis

of 0.875 and −0.257 after 200 permutations [Figure 4c]. As the R^2 value was close to 1 and the Q^2 value was negative, the model was considered to be reliable and not subject to over-fitting.

Differential metabolite analysis

Differentially abundant metabolite identification

We next constructed volcano plots to identify metabolites that were differentially abundant between PMR sample groups [Figure 4d]. As per the results of the OPLS-DA analysis, differential metabolites were selected as those chemical species with a VIP >1, a *P* value < 0.01, and an FC >2 or <0.5. Individual data points in this plot correspond to specific metabolites, with *X* and *Y* axes in turn corresponding to FC values and Student's t-test-derived *P* values when comparing sample groups. The color of the data points corresponds to the ratio of the average expression of metabolites in the two groups of samples (FC). Red points indicate upregulation, with 654 upregulated differential metabolites (FC >2), while green points indicate downregulation, with a total of 124 downregulated differential metabolites (FC <0.5). Black points correspond to metabolites that were not significantly differentially abundant. In total, we screened 778 differential metabolites between the Guangdong and the non-Guangdong sample group.

Pathway analyses of differentially abundant metabolites

To systematically assess differences in the metabolite profiles of PMR samples from Guangdong Province and other provinces, we next conducted KEGG pathway mapping for the 778 metabolites that were differentially abundant when comparing these samples. For pathway analysis purposes, the model play *Arabidopsis thaliana* was utilized. Of these metabolites, 164 were matched to annotated pathways within the Mbrole 2.0 and KEGG databases.

The top 20 most enriched pathway terms were shown in Figure 5. KEGG enrichment analyses revealed that these differentially abundant metabolites were primarily associated with glycosaminoglycan degradation, lipoic acid metabolism, flavonoid biosynthesis, tyrosine metabolism, phenylpropanoid biosynthesis, phenylalanine metabolism, and flavone and flavonol biosynthesis.

Pathway maps incorporating details pertaining to known metabolite interactions, reactions, and relationships were additionally constructed to fully elucidate the metabolic networks that differ between PMR samples from different sites of origin [Figure 6].

The pathway map shows the types, expression levels, and relationships of differential metabolites involved in the five enriched metabolic pathways. We observed that the different metabolites in these

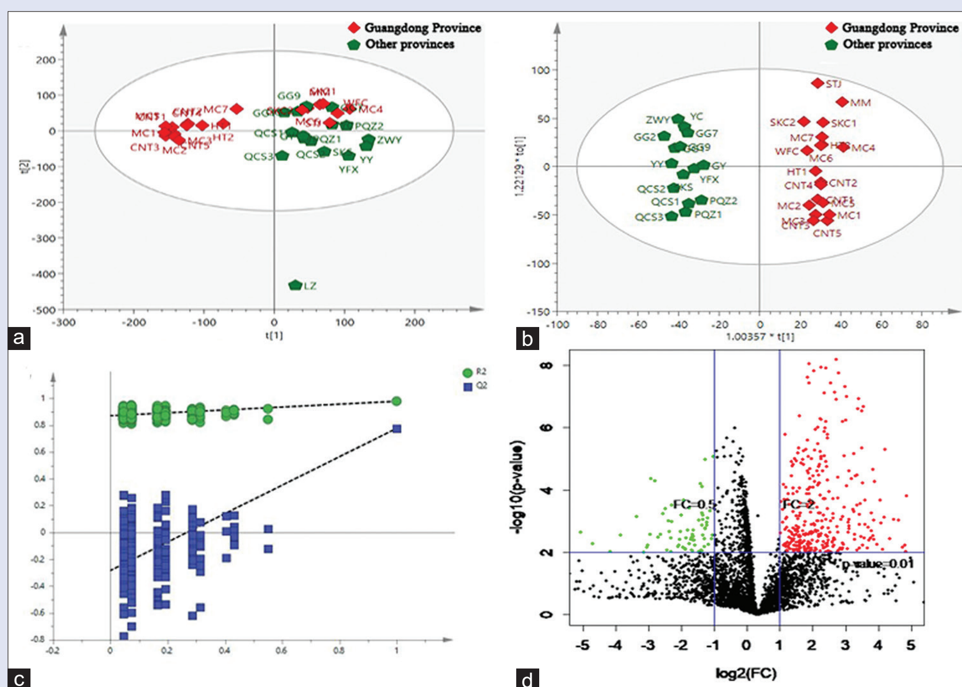

Figure 4: Score plots of PCAmodels (a), orthogonal partial least-squares discriminant analysis model (b), validation of OPLS-DA model with the permutation test (c), volcano figure (d), based on the data of PMR samples from UPLC-Q-Orbitrap/MS profiling data

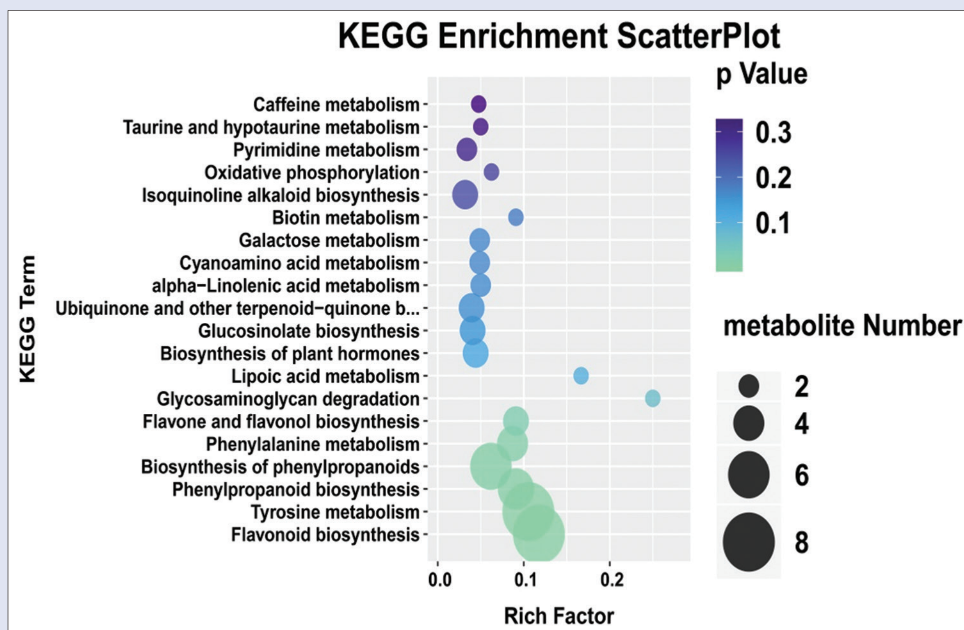

Figure 5: KEGG enrichment analysis of differential metabolites (the most enriched top 20 pathway terms). The sizes of circles were proportional to the number of metabolites; the statistical significance was represented in the color gradient, and the abscissa represented the ratio of the number of differential metabolites in the corresponding pathway to the total number of identified metabolites and the ordinate showed the impact of the pathway

metabolic pathways were largely upregulated and that the majority of them were flavonoids and tyrosine metabolites. The results of this analysis suggested that PMR provenance had a significant impact on metabolic activity and metabolite profiles in harvested plant tissues.

Increase (red font) and decrease (green font) in metabolite levels as observed in two provenances PMR are indicated.

In-depth analysis of significantly differentially abundant metabolites

Some studies have shown that the major active components of PMR are stilbene, anthraquinones, flavonoids, and catechins,[20] the result is the same as our study. To identify key metabolite profiles characteristic of PMR samples from Guangdong and other regions, we further screened

out seven metabolites with a VIP ≥2 [Table 2, Figure 7]. These seven metabolites were primarily flavonoids and polyketides. Catechins are flavonoid components. Catechin is mainly found in natural plants such as tea and croton[21,22] and has the same structure as the catechin metabolites in the present study. Plants containing catechins have antioxidant effects, hypoglycemic and hypolipidemic activities, and anti-tumor effects, and so does PMR.[23]

The seven differential metabolites we screened included Isoorientin-2´´-O-(E)-p-coumarate, Gallocatechin-(4α→8)-epicatechin, Epigallo catechin-(4β→8)-catechin, Epigallocatechin-(4β→8)-epicatechin-3-O -gallate ester, 5,7,3´,4´,5´-Pentahydroxyflavanone, (+)-Gallocatechin, and (−)-Epigallocatechin. We then compared the mean relative peak area values for these seven metabolites between samples from Guangdong Province and samples from other areas [Figure 8], revealing that these metabolites were all present at significantly higher levels in the former set of samples relative to the latter set.

Lastly, we performed bidirectional clustering of the seven significantly different metabolites and PMR samples from different sources [Figure 9]. The results revealed that seven significantly different metabolites facilitated the clustering of PMR from different provenances into two categories. The first category was composed of samples from the traditional geo-authentic producing areas (Guangdong Province), while the second category was composed of samples from regions other than Guangdong province. This indicates that the seven identified significantly differentially abundant metabolites can be used as markers to distinguish PMR from geo-authentic and non-authentic producing areas. In addition, we found that the relative levels of these seven significantly differentially abundant metabolites in samples from the geo-authentic

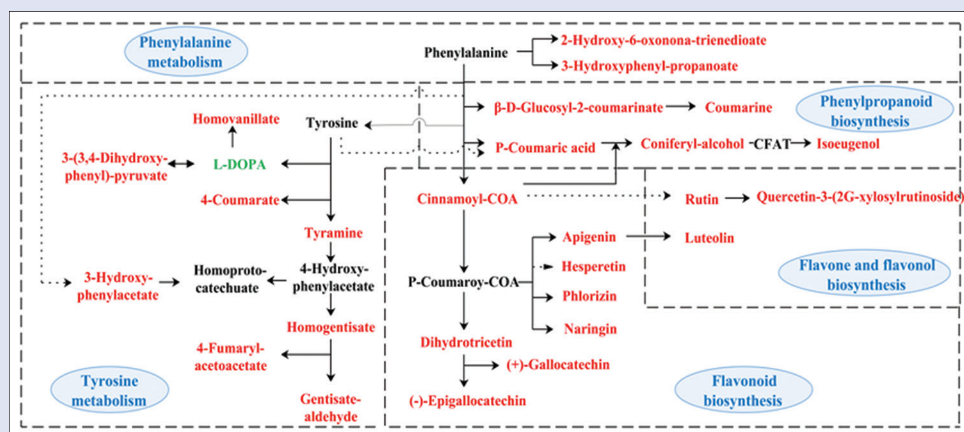

Figure 6: Integrated metabolic pathways related to PMR from different provenances

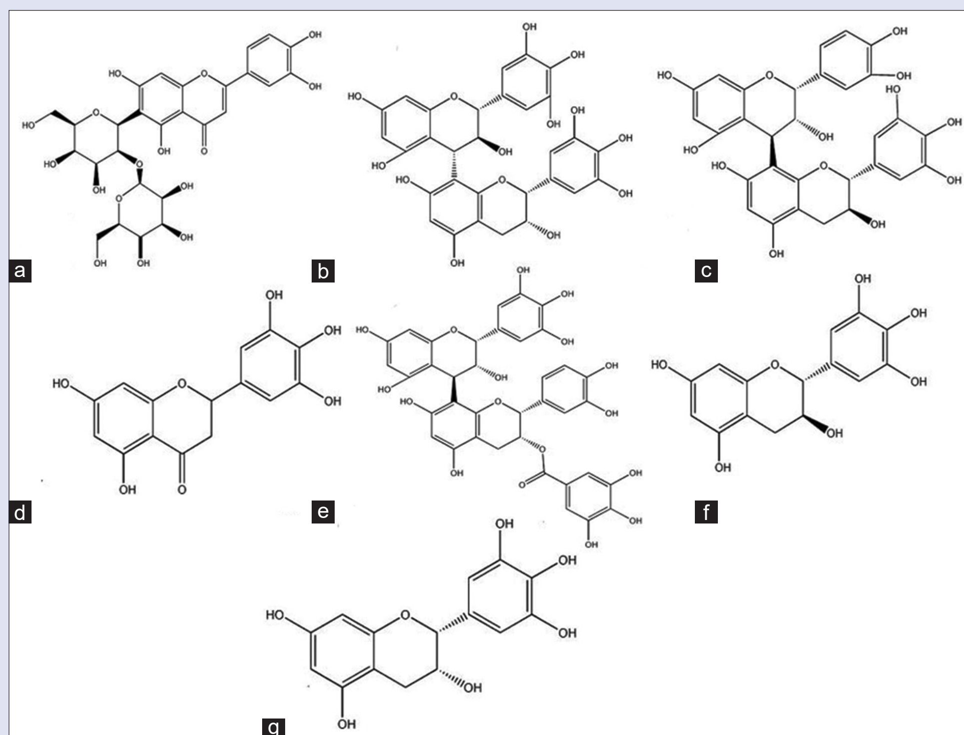

Figure 7: (a) Isoorientin-2´´-O-(e)-p-coumarate, (b) Gallocatechin-(4α→8) -epicatechin, (c) Epigallocatechin-(4β→8)-catechin, (d) 5,7,3´,4´,5´-Pentahydroxyflavanone, (e) Epigallocatechin-(4β-8)-epicatechin-3-O-gallate ester, (f) (+)-Gallocatechin, (g) (–)-Epigallocatechin.

Figure 8: The compounds were the seven significant differentially expressed metabolites between samples from Guangdong Province and samples from other areas and the changes in their relative contents were shown by the boxplots. (a) Isoorientin-2´´-O-(e)-p-coumarate, (b) Gallocatechin-(4*α*→8) -epicatechin, (c) Epigallocatechin-(4*β*→8)-catechin, (d) 5,7,3´,4´,5´-Pentahydroxyflavanone, (e) Epigallocatechin-(4*β*®8)-epicatechin-3-O-gallate ester, (f) (+)-Gallocatechin, (g) (–)-Epigallocatechin. Data are means ± standard deviation (SD) of samples from the same provenance group. *P < 0.05 and **P < 0.01 vs other provenances group, respectively

Figure 9: The hierarchical cluster diagram of seven significantly differential metabolites. Rows: samples; Columns: significantly differential metabolites

producing areas were significantly higher than those in samples from other production regions, indicating that they can be used as a chemical index to assess the authenticity of PMR.

DISCUSSION

"Deqing Shouwu" is a desirable medicinal quality that has been validated by thousands of years of medicinal practice, and as such, Deqing County in Guangdong Province is regarded as the home of PMR.[24] This geo-authentic producing area has a higher temperature, greater annual precipitation, a more suitable ecological environment, and a larger area suitable for PMR growth than other regions.[25] The study on the difference in chemical composition of PMR in the geo-authentic and non-authentic producing areas is of great significance to reveal the formation mechanism of the authenticity of PMR from the perspective of secondary metabolites.

LZ is considered to be a variant of PMR and is also known as *Cynanchum auriculatum*.[26] Its chemical and pharmacological components are quite different from those of PMR.[27] Using PCA clustering and

Table 2: Selected seven significantly differential metabolites profiles characteristic

Rt(min)	Metabolites	*m/z*	Ion mode	Adducts	Formula	Quality error (ppm)	VIP	*P*	FC	chemical structure
1.75	Isoorientin-2′′-O-(E)-p-coumarate	593.13	neg	M−H	$C_{30}H_{26}O_{13}$	0.85	2.09	0	7.53	Figure 7a
1.76	Gallocatechin-(4α→8)-epicatechin	595.14	pos	M+H, M+Na, M+K	$C_{30}H_{26}O_{13}$	−0.44	2.07	0	7.54	Figure 7b
3.71	Epigallocatechin-(4β→8)-catechin	595.14	pos	M+H	$C_{30}H_{26}O_{13}$	−0.53	2.06	0	4.09	Figure 7c
2.02	5,7,3′,4′,5′-Pentahydroxyflavanone	305.07	pos	M+H	$C_{15}H_{12}O_7$	0.37	2.05	0	6.59	Figure 7d
3.96	Epigallocatechin-(4β→8)-epicatechin-3-O-gallate ester	305.07	neg	M−H	$C_{37}H_{30}O_{17}$	−0.55	2.04	0	4.81	Figure 7e
2.02	(+)-Gallocatechin	745.14	pos	M+H	$C_{15}H_{14}O_7$	0.22	2.02	0	6.4	Figure 7f
2.00	(−)-Epigallocatechin	305.07	neg	M-H, M+FA-H, 2M-H	$C_{15}H_{14}O_7$	0.82	2.00	0	6.56	Figure 7g

Rt=retention time; VIP=the contribution rate of different substances to the OPLS-DA model; *P* value=a significance value for t-test; FC=Fold-change

sample cross-sectional analyses, we confirmed that LZ samples were significantly different from other analyzed samples. In our clustering results based upon sample origin and total metabolite levels [Figure 3], Deqing provenance or samples grown in Guangdong Province clustered with one another. Samples with different growth environments but the same provenance clustered together in some cases. For example, SKC transplanted from Deqing and grown in Guizhou clustered together with Deqing MC samples in this study. However, samples from similar geographic sources also exhibited certain differences. For example, MC4 and MC6 were not grouped with other Deqing MC samples. This may suggest that the growth environment of these two samples was quite different from that of other MC samples. These samples were also grown for different numbers of years, with MC4 and MC6 having been grown for 1 year and other MC samples having been grown for 4 years. Wild products (Deqing WFC, Guangdong Gaoyao HT, Guangdong Maoming MM) clustered together with Deqing cultivated products (MC4, MC6), indicating that there was little difference in metabolite composition between wild products and cultivated products in Guangdong province. In summary, we found that the composition of PMR was jointly determined by genes and the growth environment. When the provenance and the environment were the same, the samples were still able to cluster together even when the growth years were different. Ecological geographical environment, germplasm resources, and cultivation/processing strategies may be the reasons for the differences in the chemical composition of the medicinal materials, and also the factors related to the formation of genuine herbs.[28-32] Ensuring a similar ecological environment to that in the geo-authentic producing area is an effective way to ensure the quality of these medicinal materials.

PMR is a widely utilized and valuable TCM. In prior analyses, the primary bioactive components of PMR were found to include stilbenes, anthraquinones, flavonoids, and polysaccharides that exhibit a range of anti-tumor, anti-microbial, antioxidant, and hepatoprotective activities.[33,34] Among them, stilbene glycosides, free anthraquinones, and combined anthraquinones are the primary medicinal components of PMR, and they are also important indicators that can be used to test the quality of PMR and related medicinal products. Stilbene glycosides are closely related to the beneficial effects of PMR on the liver and kidney, while anthraquinones are utilized to treat malaria, for detoxification, and to improve intestinal health.[20,35-38] Catechins have antibacterial, antioxidant, anti-tumor, and cardioprotective functions.[39,40] The origin of PMR has an important influence on the formation of stilbene glycosides and anthraquinones.[30] We found that catechins were the primary metabolites that were differentially abundant between PMR samples that were and were not from Guangdong Province, with the levels of these metabolites being significantly higher in samples from the Guangdong region. In addition, the Guangdong sample group included the SKC sample that had been cultivated off-site, a sample transplanted from Deqing County in Guangdong Province to Guizhou Province. According to our clustering and PCA results, these SKC samples were metabolically similar to the samples of PMR that had been planted in Deqing. This suggests that environmental and genetic factors may influence catechin levels in PMR plants. These findings also raise the possibility that the superior quality and medicinal efficacy of PMR from Deqing in Guangdong Province may be linked not only to stilbenes and anthraquinones, but also to a number of flavonoids including catechins that are differentially abundant in these samples.

To improve efficacy and reduce toxicity, PMR is usually processed before clinical use[2] and is most commonly processed via a black bean juice steaming method.[41,42] The chemical composition of PMR will be altered by processing, as combined anthraquinone will be hydrolyzed, free anthraquinone levels will increase, while the content of stilbene

glycoside will gradually decrease.[2,6] Another study found that levels of catechins in PMR may be reduced after heating for 16 h.[6] Further work is needed to understand differences in the chemical composition of PMR from different geographical origins after processing.

CONCLUSION

PMR is an important TCM. In this study, we conducted a UPLC-Q-Orbitrap/MS-based metabolomics profiling of 35 different PMR samples from Guangdong and other regions in China. Cluster analyses revealed that samples of Deqing provenance and samples were grown in Guangdong Province clustered with one another. When comparing samples from Guangdong to other samples, we identified 778 differentially abundant metabolites, highlighting the important metabolic pathways associated with these differentially abundant metabolites, leading to the identification of seven markers that can distinguish between PMR samples from geo-authentic and non-authentic producing areas. This suggests that the metabolic composition of PMR samples differs based on their provenance and that these metabolite profiles can consequently be used to distinguish between PMR isolates from different regions. Using a metabolomics-based approach to evaluate PMR provenance offers value as a means of studying the intrinsic mechanisms governing PMR authenticity, providing a firm theoretical foundation for additional studies of PMR composition and quality.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Lee MS, Hxiao HJ. Rapid and sensitive authentication of *Polygonum multiflorum* (He-Shou-Wu) of Chinese medicinal crop using specific isothermal nucleic acid amplification. Ind Crops Prod 2019;129:281-9.
2. Huang J, Zhang JP, Bai JQ, Wei MJ, Zhang J, Huang ZH, *et al*. Chemical profiles and metabolite study of raw and processed Polygoni Multiflori Radix in rats by UPLC-LTQ-Orbitrap MSn spectrometry. Chin J Nat Med 2018;16:375-400.
3. Luo DQ, Jia P, Zhao SS, Zhao Y, Liu HJ, Wei F, *et al*. Identification and differentiation of *Polygonum multiflorum* radix and *Polygoni multiflori* Radix preaparata through the quantitative analysis of multicomponents by the single-marker method. J Anal Methods Chem 2019;2019:7430717.
4. Gong X, Liu M, Gong L, Li Y, Peng C. Study on hepatotoxicity of different dosages of Polygoni Multiflori Radix praeparata in rats by metabolomics based on UPLC-Q-TOF-MS. J Pharm Biomed Anal 2019;175:112760.
5. Zhang Z, Yang L, Huang X, Gao Y. A Serum metabolomic study on rats induced by Polygoni Multiflori Radix and Polygoni Multiflori Radix preparata by pattern recognition and pathways analysis. Biol Pharm Bull 2020;43:306-18.
6. Lin L, Ni B, Lin H, Zhang M, Li X, Yin X, *et al*. Traditional usages, botany, phytochemistry, pharmacology and toxicology of *Polygonum multiflorum* Thunb.: A review. J Ethnopharmacol 2015;159:158-83.
7. Li H, Cao S, Wang X, Zuo Q, Chen P, Liu Y, *et al*. Quality evaluation of heshouwu, a taoist medicine in wudang. Exp Ther Med 2016;12:2317-23.
8. Feng Y, Fu TX, Zhang L, Wang C, Zhang D. Research on differential metabolites in distinction of rice (Oryza sativa L.) origin based on GC-MS. J Chem 2019;2019:1-7.
9. Lv X, Li Y, Tang C, Zhang Y, Zhang J, Fan G. Integration of HPLC-based fingerprint and quantitative analyses for differentiating botanical species and geographical growing origins of Rhizoma coptidis. Pharm Biol 2016;54:3264-71.
10. Saito K, Matsuda F. Metabolomics for functional genomics, systems biology, and biotechnology. Annu Rev Plant Biol 2010;61:463-89.
11. Sumner LW, Lei Z, Nikolau BJ, Saito K. Modern plant metabolomics: advanced natural product gene discoveries, improved technologies, and future prospects. Nat Prod Rep 2015;32:212-29.
12. Weitzel KM. Bond-dissociation energies of cations--pushing the limits to quantum state resolution. Mass Spectrom Rev 2011;30:221-35.
13. Ning Z, Lu C, Zhang Y, Zhao S, Liu B, Xu X, *et al*. Application of plant metabonomics in quality assessment for large-scale production of traditional Chinese medicine. Planta Med 2013;79:897-908.
14. Lee EJ, Shaykhutdinov R, Weljie AM, Vogel HJ, Facchini PJ, Park SU, *et al*. Quality assessment of ginseng by (1) H NMR metabolite fingerprinting and profiling analysis. J Agric Food Chem 2009;57:7513-22.
15. Gao H, Xu D, Zhang H, Qian J, Yang Q. Transcriptomics and metabolomics analyses reveal the differential accumulation of phenylpropanoids between Cinnamomum cassia Presl and Cinnamomum cassia Presl var. Macrophyllum Chu. Ind Crops Prod 2020;148:112282.
16. Zhang Z, Yang L, Huang X, Gao Y. Metabolomics profiling of Polygoni Multiflori Radix and polygoni multiflori radix preparata extracts using UPLC-Q/TOF-MS. Chin Med 2019;14:46.
17. Clancy MV, Zytynska SE, Moritz F, Witting M, Schmitt-Kopplin P, Weisser WW, *et al*. Metabotype variation in a field population of tansy plants influences aphid host selection. Plant Cell Environ 2018;41:2791-805.
18. Wei B, Hou K, Zhang H, Wang X, Wu W. Integrating transcriptomics and metabolomics to studies key metabolism, pathways and candidate genes associated with drought-tolerance in Carthamus tinctorius L. under drought stress. Ind Crops Prod 2020;151:112465.
19. He S, Liu X, Wang Y, Xie J, Gao H, Li X, *et al*. Metabolomics analysis based on UHPLC-Q-TOF-MS/MS reveals effects of genistein on reducing mycotoxin citrinin production by Monascus aurantiacus Li AS3.4384. LWT Food Sci Technol 2020;130:109613.
20. Qiu X, Zhang J, Huang Z, Zhu D, Xu W. Profiling of phenolic constituents in *Polygonum multiflorum* Thunb. by combination of ultra-high-pressure liquid chromatography with linear ion trap-Orbitrap mass spectrometry. J Chromatogr A 2013;1292:121-31.
21. Yang C, Hu Z, Lu M, Li P, Tan J, Chen M, *et al*. Application of metabolomics profiling in the analysis of metabolites and taste quality in different subtypes of white tea. Food Res Int 2018;106:909-19.
22. Nonaka G, Miwa N, Nishioka I. Tannins and related compounds. Part 2. Stilbene glycoside gallates and proanthocyanidins from *Polygonum multiflorum*. Phytochemistry 1982;21:429.
23. Higdon JV, Frei B. Tea catechins and polyphenols: Health effects, metabolism, and antioxidant functions. Crit Rev Food Sci Nutr 2003;43:89-143.
24. Wang GY, Shang J, Wu Y, Ding G, Xiao W. Rapid characterization of the major chemical constituents from Polygoni Multiflori Caulis by liquid chromatography tandem mass spectrometry and comparative analysis with Polygoni Multiflori Radix. J Sep Sci 2017;40:2107-16.
25. Li M, Yu Y, Zhang XB, Guo LP. Comparative study on distribution of Polygoni Multiflori Radix based on different methods. Zhongguo Zhong Yao Za Zhi 2019;44:4082-9. Chinese.
26. Moon BC, Choo BK, Cheon MS, Yoon T, Ji Y, Kim BB, *et al*. Rapid molecular authentication of three medicinal plant species, *Cynanchum wilfordii*, *Cynanchum auriculatum*, and *Polygonum multiflorum* (Fallopia multiflorum), by the development of RAPD-derived SCAR markers and multiplex-PCR. Plant Biotechnol Rep 2010;4:1-7.
27. Xie HQ, Chu SS, Zha LP, Cheng ME, Jiang L, Ren DD, *et al*. Determination of the species status of Fallopia multiflora, Fallopia multiflora var. angulata and Fallopia multiflora var. ciliinervis based on morphology, molecular phylogeny, and chemical analysis. J Pharm Biomed Anal 2019;166:406-20.
28. Li X, Wang S, Guo L, Huang L. Effect of cadmium in the soil on growth, secondary metabolites and metal uptake in Salvia miltiorrhiza. Toxicol Environ Chem 2013;95:1525-38.
29. Ncube B, Finnie JF, Van Staden J. Quality from the field: The impact of environmental factors as quality determinants in medicinal plants. S Afr J Bot 2012;82:11-20.
30. Tang JF, Li WX, Zhang F, Li YH, Cao YJ, Zhao Y, *et al*. Discrimination of Radix Polygoni Multiflori from different geographical areas by UPLC-QTOF/MS combined with chemometrics. Chin Med 2017;12:34.
31. Wei Y, Liu M, Liu J, Li H. Influence factors on the hepatotoxicity of polygoni multiflori radix. Evid Based Complement Alternat Med 2019;2019:5482896.
32. Zhao Q, Song Z, Fang X, Pan Y, Guo L, Liu T, *et al*. Effect of genotype and environment on Salvia miltiorrhiza Roots using LC/MS-based metabolomics. Molecules 2016;21:414.
33. Lin L, Ni B, Lin H, Zhang M, Yan L, Qu C, *et al*. Simultaneous determination of 14 constituents of Radix polygoni multiflori from different geographical areas by liquid chromatography-tandem mass spectrometry. Biomed Chromatogr 2015;29:1048-55.
34. Zhu ZW, Li J, Gao XM, Amponsem E, Kang LY, Hu LM, *et al*. Simultaneous determination of stilbenes, phenolic acids, flavonoids and anthraquinones in Radix polygoni multiflori by

LC-MS/MS. J Pharm Biomed Anal 2012;62:162-6.

35. Kim HK, Choi YH, Choi JS, Choi SU, Kim YS, Lee KR, *et al*. A new stilbene glucoside gallate from the roots of *Polygonum multiflorum*. Arch Pharm Res 2008;31:1225-9.
36. Lin L, Hao Z, Zhang S, Shi L, Lu B, Xu H, *et al*. Study on the protection of water extracts of Polygoni Multiflori Radix and Polygoni Multiflori Radix Praeparata against NAFLD and its mechanism. J Ethnopharmacol 2020;252:112577.
37. Lv LS, Gu XH, Tang J, Ho CT. Antioxidant activity of stilbene glycoside from *Polygonum multiflorum* Thunb. *in vivo*. Food Chem 2007;104:1678-81.
38. Rui W, Xia W, Zhao W, Li B, Li J, Feng Y, *et al*. Differential constituents in roots, stems and leaves of Polygonum *multiflorum* Thunb. screened by UPLC/ESI-Q-TOF-MS and multivariate statistical analysis. J Chromatogr Sci 2020;58:136-43.
39. Liu M, Li X, Liu Q, Xie S, Zhu F, Chen X. Preparative isolation and purification of 12 main antioxidants from the roots of Polygonum *multiflorum* Thunb. using high-speed countercurrent chromatography and preparative HPLC guided by 1,1'-diphenyl-2-picrylhydrazyl-HPLC. J Sep Sci 2020;43:1415-22.
40. Singh BN, Shankar S, Srivastava RK. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem Pharmacol 2011;82:1807-21.
41. Ho TT, Murthy HN, Dalawai D, Bhat MA, Paek KY, Park SY. Attributes of *Polygonum multiflorum* to transfigure red biotechnology. Appl Microbiol Biotechnol 2019;103:3317-26.
42. Liu Z, Chao Z, Liu Y, Song Z, Lu A. Maillard reaction involved in the steaming process of the root of *Polygonum multiflorum*. Planta Med 2009;75:84-8.