Comparative analysis of the metabolites in Pinellia ternata from two producing regions using ultra-high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry

1 Introduction

Pinellia ternata (Thunb.) Breit. is a perennial herb belonging to the Araceae family, which has great medicinal value in the form of Pinelliae rhizome (PR) [1]. P. ternata, also referred to as Banxia in China, was first documented in the “Shen Nong’s Herbal Classic” nearly 2,000 years ago. The plant matures in mid-summer and has eight species spread across the world. Several of these species are endemic to China and are distributed mainly in Sichuan, Hubei, Henan, and other provinces [2].

PR is described as pungent and bitter in taste and reportedly affects the liver, spleen, stomach, and kidney meridians. The main functions of PR include drying dampness and resolving phlegm, lowering the reversal and terminating vomiting, and dissipating swelling and stagnation [3]. In addition, PR is applied externally to reduce swelling and relieve pain [4]. Pharmacological studies have demonstrated that PR exhibits anti-fungal, anti-inflammatory, anti-oxidant, anti-coughing, sedative-hypnotic, and anti-vomiting effects. PR also inhibits the proliferation of cancer cells, exerts anti-epileptic effects, and exhibits insecticidal activity [5–11]. Modern pharmacological studies have revealed that PR contains a wide variety of chemical components, including alkaloids, flavonoids, organic acids, amino acids, sterols, sugars, and certain inflammatory components [12–16]. The study of the chemical constituents and the pharmacological functions of PR revealed that it contains 25 alkaloids, 20 organic acids, 28 amino acids, 12 flavonoids, 6 steroids and their glycosides, and 65 volatile oils [17].

Anthropological activities and environmental pollution have destroyed the habitat of wild P. ternata to a large extent, because of which the population of wild P. ternata has decreased, and its germplasm resources...
have been reduced [18]. Therefore, it is imperative to pay further attention to wild \textit{P. ternata} and protect its existing germplasm resources. Jing banxia is an authentic medicinal PR distributed in Jingmen, Hubei Province, China. According to the “Drug Production Debate,” “the quality of PR from Jingzhou, Hubei Province, is very good” in terms of excellent processing, appearance, and medicinal value [19]. Xi banxia is produced in Xi County, Henan Province, and its earliest records are from the “Xixian Chronicle,” which was documented in the fourth year of Jiaqing in the Qing Dynasty. It is stated that just 9 g of Xi banxia could be used in place of PR 15 g of PR. Xi banxia is an authentic medicinal material from Henan, Xi banxia is a treasure among similar kinds of medicinal materials because of its large-sized, white and pink roots and good curative effects [20]. The quality analysis of the multi-components of \textit{P. ternata} is the basis for the quality evaluation of \textit{P. ternata} herbs. The existing reports have focused mainly on the analysis of certain chemical components of \textit{P. ternata}, while little attention has been paid to the metabolomics of wild \textit{P. ternata} from different regions.

Widely targeted metabolomics, which is also referred to as next-generation metabolomics, has been applied widely to mine novel secondary metabolites, plant metabolism and the associated effective metabolic pathways and regulatory mechanisms, gene function elucidation, medicine development, etc., and accurate qualitative and quantitative results, high throughput, high sensitivity, and wide coverage have been achieved [21–24]. Although being an authentic medicinal material from Henan, Xi banxia has not been studied so far for its chemical composition, and no database of its chemical composition is available currently.

In the present study, the metabolites of Jing banxia and Xi banxia were isolated and identified using the ultra-high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC–ESI-MS/MS) analysis. To provide theoretical and data support for the chemical composition analysis, pharmacological mechanism analysis, and further application of Xi banxia, the metabolic differences between the \textit{P. ternata} plants from two different producing regions were investigated based on the involved metabolic pathways.

2 Materials and methods

2.1 Plant materials

The authentic medicinal materials, Jing banxia (Pin.t) and Xi banxia (XPin.t), used in the present study, were collected from the wild \textit{P. ternata} in Jingzhou, Hubei Province and Xi County, Xinyang, Henan Province, respectively. The collected samples were identified by Professor Huiyuan Ya (the School of Food and Drug, Luoyang Normal University, Luoyang, Henan Province, China). The process of sample collection involved collecting a minimum of five only fresh samples of PR, which were then randomly sampled.

2.2 Sample extraction process

Fresh Pin.t and XPin.t samples were vacuum freeze-dried in a lyophilizer (Scientz-100F) and then ground to powder using a grinder (MM 400, Retsch) at 30 Hz for 1.5 min. Afterward, 100 mg of the sample powder was dissolved in 1.2 mL of 70% methanol extraction solution, followed by vortexing once every 30 min, each time for 30 s, and six times in total. Subsequently, the sample was placed overnight in a refrigerator at 4°C. The next day, the samples were centrifuged at 12,000 rpm for 10 min, and the resulting supernatant was collected, filtered through a microporous membrane with a pore size of 0.22 µm, and then stored in a sample vial until to be used for UPLC–MS/MS analysis. Three biological replicates were analyzed independently from each group. The samples were analyzed under the following HPLC conditions: chromatographic column, TSKgel ODS-80Ts C18 (5 µm, Tosoh Co. Ltd, Japan); solvent system, water (0.04% acetic acid):acetonitrile (0.04% acetic acid); gradient program, 100:0 V/V at 0.0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, and 95:5 V/V at 15.0 min; flow rate, 0.8 mL/min; temperature, 40°C; sample size: 2 µL. The effluent was connected alternatively to electrospray ionization (ESI)-triple quadrupole-linear ion trap (Q-TRAP) MS.

2.3 Chromatographic mass spectrometry acquisition conditions

The construction of the metabolome database was commissioned by Wuhan Metware Biotechnology Co. Ltd. The data acquisition instrument system included the UPLC equipment (SHIMADZU Nexera X2, https://www.shimadzu.com.cn/) and the Tandem mass spectrometry (MS/MS) equipment (Applied Biosystems 4500 QTRAP, http://www.appliedbiosystems.com.cn/).

The liquid conditions mainly included the following: column: Agilent SB-C18 1.8 µm, 2.1 mm × 100 mm; mobile
phase: phase A – ultrapure water (containing 0.1% formic acid) and phase B – acetonitrile (containing 0.1% formic acid); elution gradient: 0.00 min – the initial proportion of phase B = 5%, the proportion of phase B increased linearly to 95% within 9.00 min and was maintained at 95% for 1 min, 10.00–11.10 min – the proportion of phase B decreased to 5% and the proportion of phase B decreased to 5%; equilibration, 14 min; flow rate, 0.35 mL/min; column temperature, 40°C; and injection volume, 4 µL.

The mass spectrometry conditions mainly included the following: the LIT and triple quadrupole (QQQ) scans were acquired using a triple Q-TRAP mass spectrometer named the AB4500 Q TRAP UPLC–MS/MS system equipped with an ESI Turbo; the ion spray interface could be controlled using the Analyst 1.6.3 software (AB Sciex) to run the positive and negative ion modes. The ESI source operating parameters were as follows: ion source, turbo spray; source temperature, 550°C; ion spray voltage (IS), 5,500 V (positive-ion mode)/-4,500 V (negative-ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) pressures were set to 50, 60, and 25.0 psi, respectively, and the collision-induced ionization parameter was set to ‘high’. Instrument tuning and mass calibration were performed using 10 µmol/L of polypropylene glycol solution in the QQQ mode and 100 µmol/L of polypropylene glycol solution in the LIT mode, respectively. The QQQ scans were performed in the MRM mode, and the collision gas (nitrogen) level was set to ‘medium’. The DP and CE were further optimized, through which the DP and CE of the individual MRM transitions were completed. A specific set of MRM transitions in each epoch were monitored based on the metabolites eluted in each epoch.

Metabolite quantification was performed using the MRM mode of QQQ mass spectrometry. In the MRM mode, the quadrupole filtered the precursor ions of the target substance and excluded the ions corresponding to the other molecular weights to eliminate interference. After obtaining the metabolite mass spectrometric data, the integration of the peak area was performed using MultiQuant version 3.0.2 (AB SCIEX, Concord, Ontario, Canada). Finally, the chromatographic peak area was utilized to determine the relative metabolite contents.

### 2.5 Statistical analysis

The present study mainly involved multivariate statistical analysis. All normalized data were subjected to the principal component analysis (PCA) and the orthogonal partial least squares discriminant analysis (orthogonal partial least squares) using the Umetrics SIMCA 14.1 software. The orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using R software (www.r-project.org) for the hierarchical clustering analysis of metabolites in the samples. To identify the differentially accumulated metabolites (DAM), fold change (FC) ≥2 or FC ≤0.5, and variable importance (VIP) ≥1 in the items were used as the screening criteria. The data were log transformed (log2) and mean centered prior to OPLS-DA. The VIP values and the corresponding plots were extracted from the OPLS-DA results using the R package MetaboAnalystR (https://github.com/xia-lab/MetaboAnalystR). To prevent overfitting, a permutation test (200 permutations) was performed.

The identified metabolites were annotated using the KEGG Compound database (http://www.kegg.jp/kegg/compound/), and the annotated metabolites were then mapped to the KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html). The pathways to which the significantly regulated metabolites were mapped were subjected to the metabolite sets enrichment analysis, and their significance was determined based on the p-values obtained in the hypergeometric test.

### 3 Results and analysis

#### 3.1 Metabolic profiling of Pin.t and XPin.t using UPLC–MS/MS

To investigate the chemical compositions of Pin.t and XPin.t, the primary and secondary metabolites were
identified through a UPLC–MS/MS analysis. The identified metabolites were then analyzed quantitatively using the software Analyst under the MRM mode (Figure 1a and b). As depicted in Figure 1c, 573 metabolites under 12 different categories were detected, including 114 flavonoids, 97 amino acids and their derivatives, 72 lipids, 63 organic acids, 51 phenolic acids, 49 kinds of alkaloids, 48 nucleotides and their derivatives, 17 lignans and coumarins, 12 terpenes, 8 tannins, 1 quinone, and 41 other metabolites. Flavonoids (19.9%), amino acids and their derivatives (16.93%), lipids (12.57%), and organic acids (10.99%) were the four main metabolites identified (Table 1 and Table S1). The flavonoids could be further categorized into eight classes, among which flavonoids (41.23%), flavonols (14.91%), flavonoid carbon glycosides (14.04%), and dihydro-flavonoids (9.65%) were the main flavonoid metabolites (Figure 1d).

3.2 Multivariate statistical analysis

Multivariate statistics were used for further assessing the identified metabolites in Pin.t and XPin.t. According to the results of the hierarchical clustering analysis, the Pin.t and XPin.t samples could be distinctly categorized into two groups, with the metabolites exhibiting different accumulation patterns between these two groups (Figure 2a). To further analyze the degree of variability in the intergroup and intragroup sample data, the metabolite profiles of six samples were subjected to PCA (Figure 2b), and according to the results, the extracted two principal components PC1 and PC2 could explain 45.86% and 17.83% of the total variation, respectively. In the PCA plot, the three biological replicates of Pin.t were concentrated on the left side of the plot, while the three biological replicates of XPin.t were distributed on the right side of the plot (Figure 2b). The clustering and correlation analysis categorized the six samples into two distinct groups, with significantly and distinctly different metabolic profiles between Pin.t and XPin.t.

3.3 Differential metabolites between Pin.t and XPin.t.

The differential metabolites between Pin.t and XPin.t were screened based on a combination of FC and VIP values obtained from the OPLS-DA model. VIP ≥1.0 and

Figure 1: Qualitative and semi-quantitative analysis of the metabolites in Pin.t and XPin.t. The multi-peak mass spectral chromatogram of the metabolites acquired in the negative-ion mode (a) and positive-ion mode (b). (c) The types and proportions of the identified metabolites in Pin.t and XPin.t. (d) The types and proportions of flavonoids.
FC ≥ 2 or ≤ 0.5 were used as the screening criteria. A total of 155 differential metabolites were detected between Pin.t and XPin.t, among which 68 putative metabolites were upregulated while 87 metabolites were downregulated in XPin.t compared to Pin.t (Figure 3a and b; Table S2). The differential metabolites were categorized into 11 classes, among which the main differential metabolites were categorized into three classes, namely, flavonoids (20.64%), lipids (18.06%), and alkaloids (11.61%) (Figure 3c).

Table 2 lists the differential primary and secondary metabolites identified between Pin.t and XPin.t. Sixty-five differential metabolites were identified as the primary metabolites, including 28 lipids, 17 amino acids and their derivatives, 12 organic acids, and 8 nucleotides along with their derivatives. In comparison to Pin.t, the differential primary metabolites that were upregulated in XPin.t included 13 lipids, 8 amino acids and their derivatives, 4 phenolic acids, and 3 nucleotides along with their derivatives. On the other hand, 90 differential metabolites were identified as secondary metabolites, including 32 flavonoids, 18 alkaloids, 17 phenolic acids, 8 lignans and coumarins, 4 tannins, 4 terpenoids, and 4 other kinds of compounds. A few secondary metabolites were upregulated in XPin.t, including 20 flavonoids, 6 alkaloids, 6 phenolic acids, 2 lignans and coumarins, 2 tannins, 1 terpenoid, and 3 other compounds.

Furthermore, the KEGG database was employed to map the metabolites in Pin.t and XPin.t to various
Figure 2: Analysis of the identified metabolites. (a) Cluster analysis of the identified metabolites in Pin.t and XPin.t. (b) PCA of Pin.t and XPin.t.

Figure 3: DAM between Pin.t and XPin.t. (a) A volcano plot of the differential metabolites. Log$_{2}$FC ≥1 or ≤−1 and VIP ≥1 were used as the screening criteria. (b) Heatmap of the differential metabolites. (c) The types and proportions of the differential metabolites. (d) An overview of the KEGG pathway analysis of the differential metabolites.
metabolic pathways. A total of 235 metabolites, including 52 differential metabolites, could be mapped to 44 metabolic pathways. The most enriched KEGG pathways were flavonoid biosynthesis, arginine and proline metabolism, tryptophan metabolism, pyrimidine metabolism, and cysteine and methionine metabolism (Figure 3d).

3.4 Analysis of the key differential metabolites

The dynamic distribution maps were plotted to highlight the differences in the metabolite content, and the top 10 metabolites with FC change are presented in Figure 4.
Moreover, the top 10 metabolites that were significantly upregulated in XPin.t included eight flavonoids, one amino acid and its derivatives, and one other metabolite. The eight flavonoids included kaempferol-3-O-glucoside-7-O-rhamnoside, luteolin-7-O-neohesperidoside (Lonicerin), pinostrobin, alpinetin, luteolin-5,7-di-O-rutinoside, naringenin-7-O-neohesperidoside (Naringin), naringenin-7-O-rutinoside (Narirutin), and luteolin-7-O-rutinoside. The top 10 metabolites in XPin.t that were differentially downregulated were resveratrol, N-feruloylagmatine, naringenin.

**Figure 5:** The heatmap depicting the main differentially accumulated secondary metabolites between Pin.t and XPin.t.
Table 3: Overview of the main differentially accumulated secondary metabolites between Pin.t and XPIn.t

<table>
<thead>
<tr>
<th>Primary classification</th>
<th>Secondary classification</th>
<th>Pin.t vs XPIn.t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Down</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Flavonoid</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Dihydroflavone</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Flavonols</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Flavonoid carbonoside</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Isolavones</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Dihydroflavonol</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chalcones</td>
<td>0</td>
</tr>
<tr>
<td>Phenolic acids</td>
<td>Phenolic acids</td>
<td>6</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Alkaloids</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Phenolamine</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Plumerane</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Steroid alkaloids</td>
<td>1</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Triterpene saponin</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sesquiterpenoids</td>
<td>1</td>
</tr>
</tbody>
</table>

chalcone, butin, alpha-epoxydihydroartemisinic acid, mucic acid-1,4-lactone-3,5-di-O-gallate, acetylsakosaponin E, phloretin, phloretin-2′-O-glucoside (Phlorizin), and dihydrocarcone-4′-O-glucoside.

The main secondary metabolites in Pin.t and XPIn.t were analyzed further, with a focus on flavonoids, phenolic acids, alkaloids, and terpenes. As visible in Figure 5 and Table 3, most of the flavonoids were present in higher concentrations in XPIn.t compared to Pin.t. Conversely, most of the alkaloids, phenolic acids, and terpenes had lower concentrations in XPIn.t compared to Pin.t.

4 Discussion

PR is the most widely used medicinal herb, and its tubers are used for treating a variety of diseases. As PR has extensive pharmacological activities, several studies have investigated its chemical constituents. Li et al. studied the chemical constituents of Pinellia ternata and identified 186 compounds [17]. The report by the above authors serves as a reference for research on the chemical constituents of Pinellia ternata. The present study also investigated the primary and secondary metabolites in Pin.t and XPIn.t, and a total of 573 metabolites were identified under 12 subclasses, including flavonoids, phenolic acids, alkaloids, nucleotides, and their derivatives mainly (Table 1 and Table S1). Over 400 chemical compounds were identified in PR initially, including betaine, kaempferol, luteolin, coruscin, kaempferol, naringin, etc. The results of the present study contributed to advancing the knowledge of the chemical constituents of PR (Table S1).

The metabolites in medicinal plants are crucial for the physiological activities of these plants and are closely associated with the defense of the plant against pests, diseases, and environmental stress. Therefore, these metabolites have important pharmacological activities [25]. Differential metabolites reflect the differences between different plant varieties, and PR from different origins differ greatly in terms of their contents of flavonoids, alkaloids, etc. [18]. Peng et al. studied 16 different wild provenances of Pinellia ternata from Hunan Province and reported that the contents of total organic acids, alkaloids, and guanosine presented great significant differences among the different provenances and that the total organic acids and alkaloids exhibited no evident pattern of geographical and spatial variation [26]. Wang analyzed 15 samples of Pinellia ternata from 7 different provinces and reported significant differences in organic acids, alkaloids, polysaccharides, and other metabolites in the Pinellia ternata samples from different regions [27]. Jingzhou and Xixian are the two main production regions of PR, and the PR samples from these regions have been analyzed for the first time in the present study. The results revealed differences in flavonoids, alkaloids, phenolic acids, amino acids, and their derivatives, and organic acids between the samples from the two regions, which was consistent with the findings of previous studies (Figure 3c).

The alkaloids are among the important and effective medicinal components of Pinellia ternata, which exhibit anti-tussive, anti-emetic, anti-tumor, and other effects [28]. Zeng et al. studied the relationship between the main components of Pinellia ternata and the antitussive and expectorant effects of these components. The authors reported that the total alkaloids in Pinellia ternata also exhibited the greatest correlation with the antitussive and expectorant effects [29]. The alkaloids in Pinellia ternata were also observed to inhibit the spontaneous contraction of the isolated guinea pig ileum, which could then inhibit the 5-HT3 receptor and the NK1 receptor [7]. Chen et al. reported that a certain dosage of the total alkaloids in Pinellia ternata could evidently inhibit the proliferation of human hepatoma cells in vitro [30]. In the present study, the relative expression levels of alkaloids were observed to be relatively higher in Pin.t, with 12 alkaloids exhibiting higher expression in Pin.t compared to XPIn.t. Flavonoids exhibit pharmacological effects, such as anti-tumor, anti-oxidant, anti-inflammatory, and circulation improvement effects [31–33]. For instance, kaempferol attenuates the IL-32-induced differentiation of monocytes into macrophage-like
cells. In addition, kaempferol reportedly inhibited the IL-32-induced activation of p38 and nuclear factor-kB in a dose-dependent manner in THP-1 cells [34]. Luteolin-7-O-neohesperidin (loniceratin) was reported to improve neurological function in rats [35]. Alpinin may reduce inflammation in rats with coronary heart disease via the inhibition of the MEK/ERK signal pathway, thereby improving vascular endothelial function, blood lipids, and cardiac function [36]. In the present study, 20 flavonoids exhibited higher concentrations in XPin.t compared to Pin.t. Eight among the top 10 upregulated metabolites in XPin.t compared to Pin.t were flavonoids, including kaempferol-3-O-glucoside-7-O-thannoside, luteolin-7-O-neohesperidoside (Lonicerin), pinostrobin, alpinetin, etc. (Figure 4). Therefore, the authors of the present study believe that these differences in the chemical composition could be due to the different pharmacological activities of Jing banxia and Xi banxia.

5 Conclusions

The UPLC–ESI-MS/MS-based metabolomics analysis was adopted to identify the chemical constituents of two authentic PR Jing Banxia and Xi banxia. The results revealed a total of 573 metabolites under 12 sub-categories. Among the identified metabolites, 155 metabolites exhibited significant differences between Jing Banxia and Xi banxia. Most of the DAMs were concentrated under three categories of compounds, namely, flavonoids (20.64%), lipids (18.06%), and alkaloids (11.61%). The relative contents of 68 metabolites in Xi banxia were upregulated compared to Jing Banxia. On the other hand, the relative contents of 87 metabolites in Jing Banxia were upregulated compared to Xi banxia. Flavonoids and alkaloids were identified as the main putative bioactive components. While most of the alkaloids exhibited higher relative concentrations in Jing banxia, flavonoids had relatively higher expression levels in Xi banxia. The results of the present study improved the understanding of the chemical components and the medicinal value of Jing Banxia and Xi banxia and would serve as a theoretical reference when using PR for medicinal and other purposes.

Funding information: This work was supported by Henan Provincial Science and Technology Research Project (Grant number: 212102110183).

Author contributions: S.L. – formal analysis, writing acquisition, investigation, validation, writing – original draft, writing – review & editing. Y.C. – data curation, formal analysis, writing – original draft, writing – review & editing. X.L. – formal analysis, writing – original draft. C.Z. – formal analysis. H.Y. – conceptualization, investigation, project administration, resources, supervision, writing – original draft, writing – review & editing.

Conflict of interest: The authors declare no competing financial interest.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: All data generated or analyzed during this study are included in this published article and its supplementary information files.

References


