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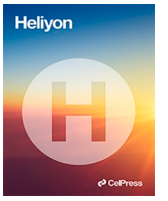

Research article

Metabolomics distinguishes different grades of *Scrophularia ningpoensis* hemsl: Towards a biomarker discovery and quality evaluation

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ABSTRACT

In managing unique complexities associated with Chinese medicinal quality assessment, metabolomics serves as an innovative tool. This study proposes an analytical approach to assess differing qualities of *Scrophularia ningpoensis* (*S. ningpoensis*) Hemsl by identifying potential biomarker metabolites and their activity with the corresponding secondary metabolites. The methodology includes four steps; first, a GC-MS based metabolomics exploration of the *Scrophularia ningpoensis* Hemsl. Second, a multivariate statistical analysis (PCA, PLS-DA, OPLS-DA) for quality assessment and biomarker identification. Third, the application of ROC analysis and pathway analysis based on identified biomarkers. Finally, validation of the associated active ingredients by HPLC. The analysis showed distinct metabolite profiles across varying grades of *S. ningpoensis* Hemsl, establishing a grading dependency relationship. Select biomarkers (gluconic Acid, D-xylulose, sucrose, etc.) demonstrated robust grading performances. Further, the Pentose Phosphate Pathway, deemed as most influential in grading, was tied to the synthesis of key constituents (iridoids, phenylpropanoids). HPLC validation tests affirm a decreasing trend in harpagoside and cinnamic acid levels between first and third-grade samples. In conclusion, this GC-MS based metabolomics combined HPLC method offers a sound approach to assess and distinguish quality variations in *S. ningpoensis* Hemsl samples.

1. Introduction

The quality of Chinese medicine (CM), indisputably linked to its clinical efficiency and safety, underpins the therapeutic basis of disease prevention and management. Affected by the ubiquity and diversity of provenance, CM varies widely in both the variety and concentration of chemical constituents, a response to the plurality of sourcing and cultivation conditions. To optimize the medical potency of CM, numerous analytical strategies have been devised. In the realm of quality assessment, high-performance liquid chromatography (HPLC) fingerprinting has emerged as the favored stratagem. This technique extrapolates on the quality traits of medicinal materials by leveraging a few key chemical entities as detection indices [1–4]. However, the assessment of CM quality and supplementary preparations via HPLC presents considerable challenges, particularly when dealing with low specificity components. Therefore, the demand for more scientifically rigorous and comprehensive analytical methods has become increasingly urgent.

Consequently, a surging demand exists for more sophisticated, wide-ranging analytical modalities [5]. Of note, metabolomics has

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become an eminently utilized platform, offering comprehensive, quantitative scrutiny of small molecules in the totality of a biological system [6]. This would render the metabolomic approach ideal for a holistic examination of metabolites within biological systems [7]. Its employment extends across numerous fields, including disease diagnosis, nutritional study, food sciences, investigation of plant biosynthetic pathways, as well as the examination of plant biotic and abiotic factors [8–12]. Key methodologies in plant metabolomics research, e.g., gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC-MS), and nuclear magnetic resonance spectroscopy (NMR) are prevalently utilized, with GC-MS frequently accorded preference [12]. In the context of the integrated and exhaustive assessment of CM's inner quality, a GC-MS-based metabolomics method may exhibit a high degree of aptness. In particular, its results can be interpreted in conjunction with HPLC results.

S. ningpoensis Hemsl, a known CM derived from the dried roots of *S. ningpoensis* Hemsl, has recently attracted considerable attention [13]. *S. ningpoensis* Hemsl has been used in China for centuries as a traditional medicinal plant for the treatment of many diseases, including inflammation, hypertension, cancer and diabetes. The commodity specification standard of seventy-six kinds of medicinal materials takes traits as the grading index. However, the " standard " formed by the market is more arbitrary and cannot be unified, which leads to confusion about the commodity category, specification, and grade of *S. ningpoensis* Hemsl in the medicinal materials market. Predominantly, the quality evaluation of *S. ningpoensis* Hemsl has been centered on the assessment of its primary components using HPLC methodology [14,15]. A broad variance in both quality and price of SNH across the market, especially between different grades, has been well-established. Traditionally, the quality of the herb is discerned through physical characteristics such as shape, color, cross-section, etc., following standard literature [16]. However, transmuting this discrete and diverse chemical make-up into a quality evaluation based solely on the size and shape of ingredients pose significant challenges [17]. Thus, a comprehensive and practicable model for the quality evaluation of *S. ningpoensis* Hemsl is desirable.

Herein, we explore the possibility of clarifying metabolic differences among grades of *S. ningpoensis* Hemsl using metabolomics method. With the hope that the quality control of different grades of *S. ningpoensis* Hemsl can provide some theoretical reference in their clinical application. After the GC-MS analysis of derivatives derived from the extract of *S. ningpoensis* Hemsl, the data of metabolites were processed with multivariate statistical analysis. PCA, PLS-DA, and OPLS-DA were applied to reveal the metabolic differences among different grades. The pathway analysis and relation between the biomarker metabolites and the biosynthesis of active components were also discussed. Finally, to verify the consistence of results between metabolomics method and HPLC analysis, the primary component of *S. ningpoensis* Hemsl: habaroside and cinnamic acid, were carried out the HPLC analysis.

In this pursuit, we ventured to elucidate metabolic differentiations amongst grades of *S. ningpoensis* Hemsl deploying metabolomic methodology combined HPLC method, with the objective of providing a theoretical framework for quality control of *S. ningpoensis* Hemsl at various grades, thereby benefiting their clinical applications. Following GC-MS analysis of the derivatives harvested from *S. ningpoensis* Hemsl extract, the acquired metabolite data were subjected to multivariate statistical analysis. Techniques such as Principal Component Analysis (PCA), Partial Least Squares-Discriminant Analysis (PLS-DA), and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) were leveraged to disclose metabolic differences among disparate grades [18]. Further, the pathway analysis and proliferation of biomarker metabolites concerning the biosynthesis of active components were examined. Finally, to validate the coherence between the results of this metabolomic approach and conventional HPLC analysis, the main constituent harpagoside and cinnamic acid were analyzed via HPLC.

2. Materials and methods

2.1. Instruments and reagents

Gas chromatography-mass spectrometry (GC-MS-QP 2010, Shimazhu, Japan) and high performance liquid chromatograph (Agilent 1260 diode array detector) were utilized for the analysis. Phosphoric acid of analytical grade, and HPLC grade acetonitrile and methanol were acquired from Merck. Authentic standards of harpagoside (B20480) and cinnamic acid (B21082) with purity more than 98% were obtained from Shanghai Yuanye Biotechnology Co., Ltd. Ultrapure water was procured from Yibao on the market. Methoxyamine hydrochloride, pyridine, and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were used as derivative reagents and obtained from Sigma (USA).

2.2. Medicinal materials

The dry root of *S. ningpoensis* Hemsl was provided by the cultivation base of in Shaoyang City, Hunan Province. This product was identified by Prof. Limin Gong from Hunan University of Chinese Medicine. The *S. ningpoensis* Hemsl batches were divided into three main types based on the "Standard for Commodity Specification of Seventy-six Medicinal Herbs", and these types were classified as first grade, second grade, and third grade according to size, traits, and other characteristics. Under the specification of *S. ningpoensis* Hemsl, the grade is divided according to the number of branches contained per kg, that is, the first grade is ≤ 36 branches per kg, with uniform branches and no vacuoles; the second grade is ≤ 72 branches per kg, without cavitation; the third grade is > 72 per kilogram, the smallest is more than 5g, and there are broken pieces. The batches were stored in the dryer for further use. There were a total of 53 samples, including 17 first-class samples, 18 s-class samples and 18 third-class samples.

2.3. Sample preparation

Each grade of *S. ningpoensis* Hemsl was pulverized into fine powder and sieved through a 100-mesh sieve. Subsequently, 100 mg of

the powdered sample was subjected to extraction using 1400 μL of HPLC-grade methanol. The extraction process involved a sequential combination of vigorous shaking for 20 min and sonication for 1 h. During ultrasound-assisted extraction, the sample tubes were intermittently shaken for 5 s every 3 min using a scroll oscillator, while maintaining a controlled temperature range of 30–55 °C. Following a 10-min stationary phase, 600 μL of the resulting supernatant was collected and subjected to centrifugation (15000 rpm, 5 min). Subsequently, 400 μL of the resulting supernatant was removed, and the solution was then evaporated to dryness under a gentle nitrogen flow. It is worth mentioning that all samples were painstakingly dried under nitrogen to achieve complete dryness, ensuring suitability for the subsequent two-step derivatization process.

2.4. Derivatization process

The dried residue was treated by adding 100 μL of methoxyamine hydrochloride and incubating at 30 °C for 30 min. Subsequently, 100 μL of BSTFA was added and the mixture was incubated at 45 °C for 1 h. Afterward, the prepared mixture was centrifuged at 4 °C for 10 min (15000 rpm), and 150 μL of the resulting supernatant was extracted and transferred to a GC-MS vial for subsequent GC-MS analysis. To ensure the stability and robustness of the analytical system, as well as to evaluate the sample preparation, analytical method, and instrument stability, a system adaptability e *S. ningpoensis* xperiment was conducted. In this experiment, equal proportions of *Scrophularia ningpoensis* Hemsl powders were thoroughly mixed, and the resulting mixture was treated following the aforementioned method. This quality control (QC) sample served as a reference for system calibration and performance assessment.

2.5. GC-MS process

Prior to the formal experiment, preliminary experiments were conducted using quality control (QC) samples to optimize the experimental conditions. The GC-MS parameters obtained from relevant research were employed to optimize the instrument parameters. The instrumental stability was first assessed by testing the QC samples during the formal experiment. Additionally, a QC sample injection was performed every 10 samples within the analytical batch to assess method applicability and instrumental stability. A 1 μL aliquot of the derivatized solution was injected into the GC-MS-QP 2010 Gas Chromatography-Mass Spectrometer (Shimadzu, Japan) operating in split mode, with a split ratio of 10:1. Separation was achieved on a nonpolar DB-5MS quartz capillary (0.25 mm × 30 mm, 0.25 m), utilizing high purity helium as the carrier gas at a flow rate of 1.0 mL/min. Initially, the column temperature was maintained at 80 °C for 2 min, followed by a temperature increase to 170 °C at a rate of 10 °C/min and a subsequent 2 min hold. The temperature was then further increased to 200 °C at a rate of 3 °C/min, and finally to 270 °C at a rate of 9 °C/min, maintaining it for 2 min. The mass spectrometry analysis employed an electron bombardment ion source (EI) with the following settings: ion source temperature of 230 °C, electron energy of 70 eV, inlet temperature of 280 °C, sample analysis time of 41 min, solvent delay time of 4.5 min, and a mass spectrometry scanning range of 35–550 *m/z*.

2.6. Data processing

QC samples and experimental samples of different grades of *S S. ningpoensis* Hemsl were sequentially analyzed on the GC-MS platform. The total ion current spectrum (TIC) of the target compounds obtained from the GC-MS analysis platform was preprocessed using MS-DIAL software, which included deconvolution, peak detection, and peak matching. The data from chromatographic peaks were identified and validated using the professional database NIST 14. Correlated peaks were analyzed and identified based on mass spectrometry and the pyrolysis law of compounds. Variables with more missing values were eliminated according to the 80% rule. Peaks with a similarity greater than 85% were assigned compound names. The corresponding single measurement index, which represents the signal intensity of metabolites, was then listed as the row in a two-dimensional matrix data form, with the samples as the column. The data underwent Pareto scaling (Par) and logarithmic transformation before being imported into the online analysis platform Mateboanalyst (www.metaboanalyst.ca) for pattern discrimination analysis. The analysis involved PCA, PLS-DA, and OPLS-DA [19–21]. Additionally, a random permutation test (n = 100) and 10-fold cross-validation were performed to detect overfitting of the discriminant models. The fold change (FC) of metabolites between first-grade versus second-grade and second-grade versus third-grade samples was calculated, and a heatmap was constructed to observe the trend of metabolites among the three grades. The OPLS-DA models generated using SIMCA 14.1 (Umetrics AB, UMEA, Sweden) software were utilized to screen potential biomarkers based on the projection importance of characteristic variables (VIP) and student's T-test. A VIP value greater than 1.0 indicated significant contribution to the classification, while a p-value less than 0.05 indicated a remarkable difference between the detected groups. Furthermore, the corresponding fold change (FC) was calculated to demonstrate the rate of change of the aforementioned metabolites among the grades. An ROC curve based on potential biomarkers from the three grades of *S. ningpoensis* Hemsl was constructed to assess their contribution to grade classification. Pathway analysis of potential biomarkers was conducted in conjunction with the KEGG database, and the metabolic differences among the grades of *S. ningpoensis* Hemsl were discussed in relation to relevant metabolic pathways. In comparison with targeted metabolomics, the non-targeted metabolomics approach used in this study is a semi-quantitative method that provides relatively lower accuracy in the quantitative analysis of metabolites. However, it still allows for the identification of biomarkers for classification purposes and the establishment of a relatively dependable quality evaluation strategy in this experiment.

2.7. Verification experiment

2.7.1. Preparation of authentic standards solution

The authentic standards of harpagoside and cinnamic acid were precisely weighed, and the mother liquor with mass concentration of 10.0 and 1.0 mg/mL was prepared by adding methanol and stored at 4 °C.

2.7.2. Preparation of samples solution

Approximately 0.5g of *S. ningpoensis* Hemsl powder was accurately weighed and placed in a conical bottle with a plug. The powder was then soaked in 50 mL of 50% methanol for a duration of 1 h, subjected to ultrasonic treatment for 45 min, and precision weighed. Any lost solvent was replenished using 50% methanol, followed by thorough shaking of the solution. Finally, the solution was passed through a 0.45 μm organic filter membrane.

2.7.3. HPLC analysis

HPLC analysis was conducted using the HPLC-DAD method, employing an Elipse XDB-C18 column (250 mm × 4.6 mm, 5 μm) at a temperature of 25 °C. The mobile phase employed was a gradient elution of acetonitrile (A) and 0.05% phosphate water (B), with the following gradient conditions: 0–25 min, 5%~35% A; 25–35 min, 35%~70% A; and 35–40 min, 70%~5% A. The volume flow rate was set at 1.0 mL/min, an injection volume of 20 μL was used, and detection was carried out at a wavelength of 280 nm.

3. Results

3.1. Metabolomics distinguishes different grades of S. ningpoensis hemsl

Metabolomics data processing heavily depends on statistical analysis. In the present study, we have utilized unsupervised PCA as a tool to construct a predictive model to manifest the distinction of various samples among four groups, including the QC group (Fig. 1a). The PCA score plots suggested that these groups differed with the first component explaining 24.4% and the second component justifying 12.7%. The aggregated QC samples (clustered near the central point) indicated the stability of the method we applied. Moreover, the supervised PLS-DA model showed complete separation of all groups, especially in the direction of the first principal component (Fig. 1b). With the direction of the first component from the first to third grade samples, we observed a notable dependence gradient among grades, establishing that the three grade samples could be discriminated. This result suggested that the consistency of quality and grade existed in different grades. The PLS-DA scores plot revealed a clear clustering among the three grades, indicative of the intrinsic differences of the metabolic profiles. For the predictive accuracy of the model, a 10-fold cross-validation (CV) was constructed, and the result demonstrated an accuracy of 0.9167, R^2 equal to 0.9416, and Q^2 equal to 0.8948, signifying good fitness. Moreover, the random permutation test (n = 100) indicated no overfitting occurred in the model.

3.2. Construction of the screening model of potential biomarkers among grades

Supervised OPLS-DA models were utilized to analyze the data, filtering out information with low relevance to the Y value and reducing the complexity of the PLS-DA regression model while improving interpretability without compromising prediction ability

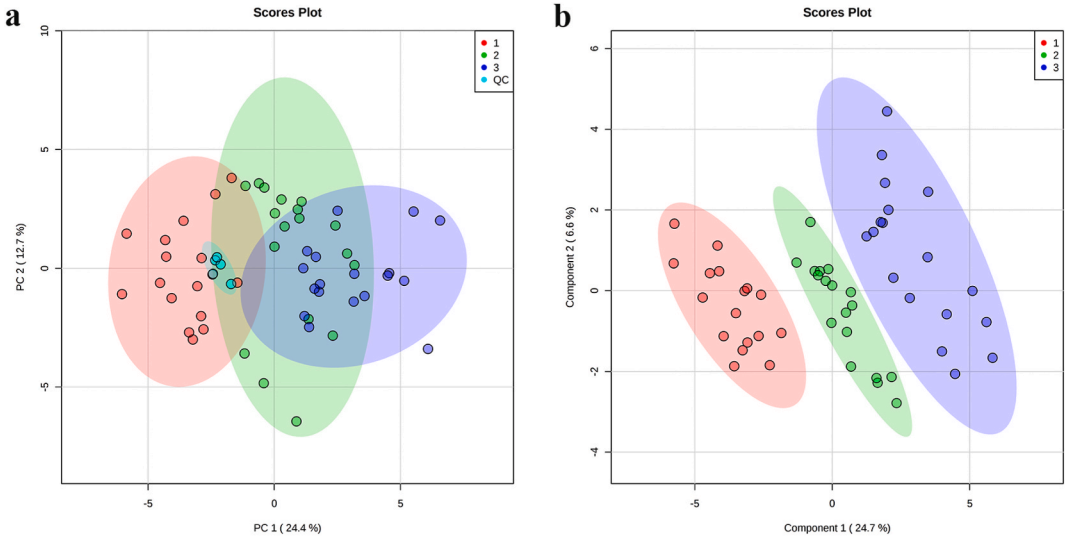

Fig. 1. a. PCA score plot of samples of four groups including QC samples. **b**. PLS-DA score plot of all samples of three grades.

[22]. The OPLS-DA models in this study aimed to discriminate between neighboring grade samples and identify metabolites with differential expression. Discrimination between first-grade and second-grade samples yielded $R^2Y = 0.873$ and $Q^2 = 0.803$, as shown in Fig. 2a. Similarly, discrimination between second-grade and third-grade samples resulted in $R^2Y = 0.854$ and $Q^2 = 0.717$ (Fig. 2b). These results demonstrated the excellent capability of the OPLS-DA model in explaining the differences in metabolite profiling between grades. Additionally, the permutation test (n = 100) shown in Fig. 2c and d indicated no visualized overfitting.

3.3. Discovery and screening of potential biomarkers

The VIP value of OPLS-DA models is universally used to reflect the contribution of variables and to screen potential biomarkers. In this study, OPLS-DA models were performed to present the discrimination between groups, contributing to the discovery of potential biomarkers. The total 44 metabolites were identified, 16 metabolites were screened out as the most significantly changed metabolites between first-grade and second-grade Scrophularia ningpoensis Hemsl with VIP >1.0 and $p < 0.05$ method. While 11 metabolites between second-grade and third-grade Scrophularia ningpoensis Hemsl were confirmed based on this rule. These metabolites were listed in Table 1 and Table 2, respectively. They are carbohydrates, organic acid, inositol, etc. From the corresponding FC used to show change rate of differential metabolites among grades, it can be seen that 1,3-Dihydroxyacetone dimer, L-iditol, sphingosine and other metabolites screened from first-grade versus second-grade Scrophularia ningpoensis Hemsl have a greater contribution to grades separation (Table 1). While D-mannose, gluconic acid and N-acetylgalactosamine in the screened metabolites of second-grade and third-grade Scrophularia ningpoensis Hemsl have greater contribution (Table 2).

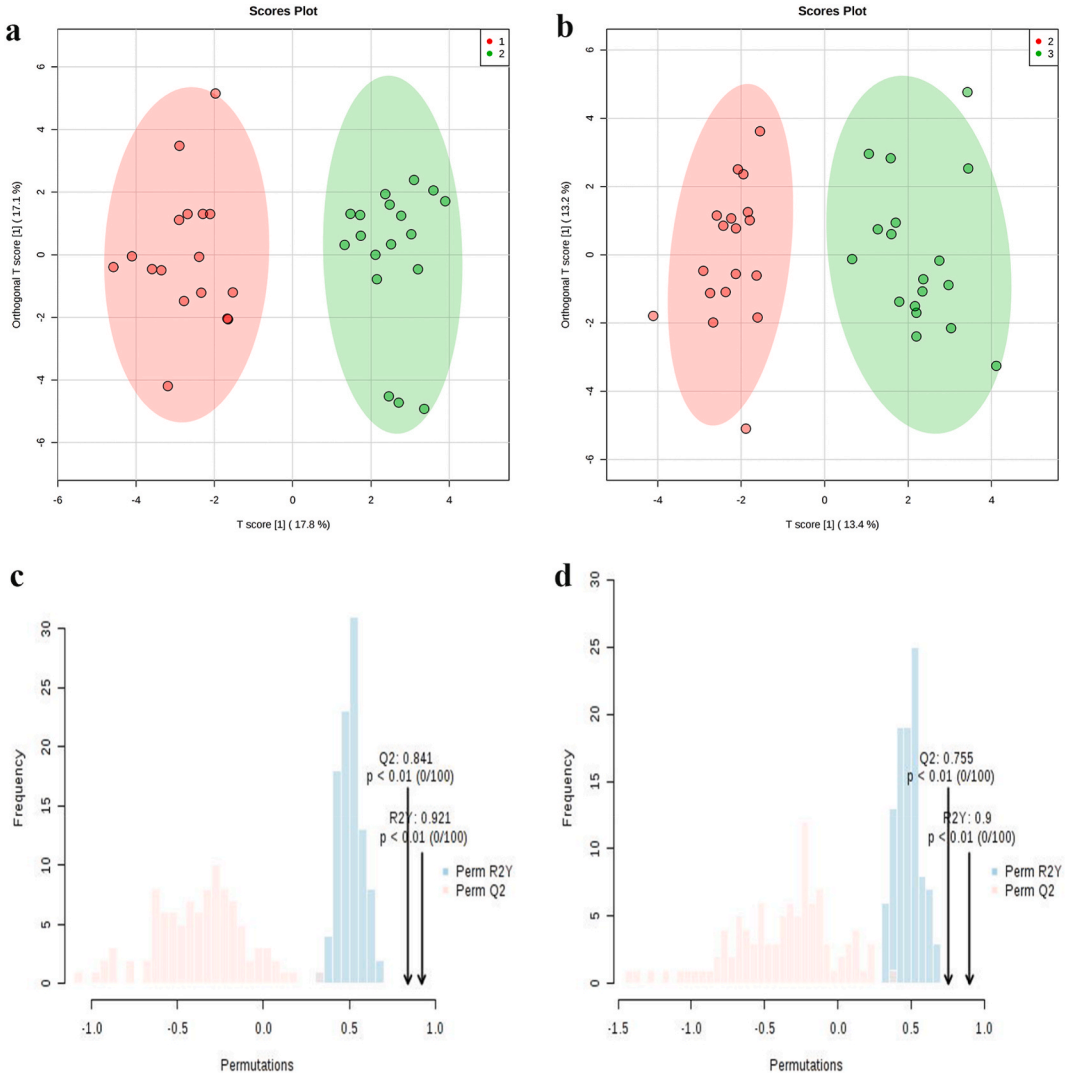

Fig. 2. a. OPLS-DA score plot of the first versus second grades *Scrophularia ningpoensis* Hemsl.**b**. OPLS-DA score plot of the second versus third grades *Scrophularia ningpoensis* Hemsl. **c** and **d**. Permutation tests(100 times) corresponding to the OPLS-DA model **a** and **b**, respectively.

Table 1
Metabolites screened out whose VIP value exceeded 1.0 and *p*-value <0.05 from T-test based on OPLS-DA model of first-grade and second-grade *Scrophularia ningpoensis* Hemsl, and the corresponding FC of these metabolites was calculated and exhibited.

No.	Metabolite name	VIP	p-value	FC
1	1,3-Dihydroxyacetone dimer	2.179	0.000	4.574
2	L-Iditol	1.695	0.001	3.680
3	Sphingosine	1.447	0.012	2.297
4	Gluconic acid	1.302	0.029	0.454
5	D-(−)-Ribose	1.269	0.000	2.159
6	Sucrose	1.218	0.010	0.582
7	Propylene glycol	1.201	0.019	1.708
8	DL-beta-Hydroxybutyric acid	1.191	0.001	1.763
9	1-Aminocyclopropanecarboxylic acid	1.182	0.036	0.507
10	Fructose	1.169	0.000	1.868
11	D-Arabinose	1.145	0.005	1.744
12	Maltotriose	1.135	0.032	1.601
13	D-Mannose	1.130	0.013	0.595
14	D-Xylulose	1.032	0.000	1.551
15	D-Trehalose	1.030	0.006	2.038
16	L-Malic acid	1.002	0.013	1.505

Table 2
Metabolites screened out whose VIP value exceeded 1.0 and *p*-value <0.05 from T-test by OPLS-DA model of second-grade and third-grade *Scrophularia ningpoensis* Hemsl, and the corresponding FC of these metabolites was calculated and exhibited.

No.	Metabolite name	VIP	p-value	FC
1	D-Mannose	1.735	0.000	2.841
2	Gluconic acid	1.660	0.001	3.117
3	N-Acetylgalactosamine	1.620	0.001	2.152
4	Hexose	1.415	0.000	1.738
5	Sucrose	1.397	0.000	0.573
6	D-Galactose	1.367	0.000	1.850
7	Melibiose	1.216	0.002	1.492
8	L-Iditol	1.086	0.037	1.691
9	D-Xylulose	1.084	0.001	1.371
10	Oleic acid	1.029	0.005	1.598
11	N-Acetyl-D-glucosamine	1.016	0.014	1.369

The VIP value of the OPLS-DA models was firstly used to assess variable contribution and screen for potential biomarkers [23]. In this study, OPLS-DA models were employed to discriminate between groups and facilitate the identification of potential biomarkers. A total of 44 metabolites were identified, with 16 metabolites exhibiting significant changes between first-grade and second-grade *S. ningpoensis* Hemsl (VIP >1.0, $p < 0.05$). Similarly, 11 metabolites were confirmed as significantly changed between second-grade and third-grade samples. These metabolites, including carbohydrates, organic acids, and inositol, are listed in Tables 1 and 2 to highlight their differential expression. The corresponding fold change (FC) was utilized to quantify the rate of change for these

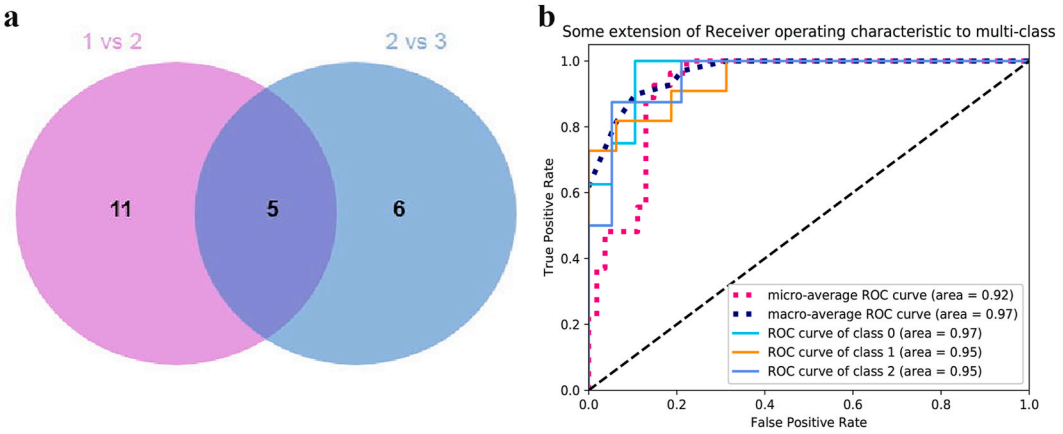

Fig. 3. a. The venn plot drawn based on differential metabolites between different grades of *Scrophularia ningpoensis* Hemsl. **b.** ROC curve of 5 shared potential biomarkers amongst different grades of *Scrophularia ningpoensis* Hemsl.

differential metabolites. It can be revealed the conspicuous impact of certain metabolites in contributing to grade separation among the samples. Specifically, 1,3-Dihydroxyacetone dimer, L-iditol, sphingosine, and accompanying derivatives emerged as prominent contributors to grade differentiation in first-grade and second-grade *S. ningpoensis* Hemsl (Table 1). Contrastingly, the comparison of second-grade and third-grade samples underscored the significance of D-mannose, gluconic acid, and N-acetylgalactosamine attributing to grade segregation within the metabolites assessed (Table 2). Such delineations explicate the diversification of metabolite profiles across different grading distinctions in *S. ningpoensis* Hemsl and underscore potential avenues for further characterization and exploration.

Subsequently, a Venn diagram was employed to visually represent the intersection of biomarkers between the two models under investigation. As demonstrated in Fig. 3a, an amalgamation of the screened biomarkers, including D-mannose, gluconic acid, sucrose, L-iditol, and D-xylulose, was achieved, corroborating these compounds as shared components. To elucidate the contributions of the five shared potential biomarkers in grade classification, receiver operating characteristic (ROC) curves were generated (Fig. 3b). The elevated area under the ROC curve (AUC-ROC) underscores the substantial contribution of these five potential biomarkers to grade classification, substantiating the rationale for selecting them as key metabolites for grading *S. ningpoensis* Hemsl.

In order to further validate the innate disparities among different grades of *S. ningpoensis* Hemsl, a heatmap was constructed, as presented in Fig. 4. The heatmap revealed substantial differences among the three grades concerning the metabolites under study. Notably, L-iditol and D-xylulose exhibited a decreasing trend in concentration and pronounced gradability from the first to the third grade. Conversely, sucrose displayed an opposite pattern, showing an increasing concentration from the first to the third grade. Moreover, the highest concentration of D-mannose and gluconic acid was observed in the second-grade *S. ningpoensis* Hemsl. Subsequent pathway analyses will be implemented to elucidate the intriguing behavior of these metabolites within their respective metabolic pathways.

3.4. Metabolic pathway analysis

In an attempt to navigate the labyrinth of biological functions of differential markers, pathway analyses, typically based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were performed, as presented in Fig. 5. Intriguingly, the exploration, centered around 5 shared potential biomarkers, brought forth the involvement of numerous pathways predominantly including pentose phosphate pathway, pentose and glucuronate interconversions, galactose metabolism, and starch and sucrose metabolism. Notably, the pentose phosphate pathway emerged as the fulcrum of impact.

3.5. Verification test of potential biomarkers

To probe the differences, specifically on cycloartenoid terpenoids and phenylpropanoid terpenoids, across various grades of *S. ningpoensis* Hemsl, we drew on high-performance liquid chromatography (HPLC). The technique allowed for a closer look at the compositions of harpagoside, a type of a cycloartenoid terpenoid, and cinnamic acid, a phenylpropanoid terpenoid, in the studied samples. The results suggest that (Fig. 7) the first-grade sample of *S. ningpoensis* Hemsl contained 0.24 $\pm$ 0.04% of harpagoside and 0.08 $\pm$ 0.01% cinnamic acid. In the second-grade samples, the respective content was 0.21 $\pm$ 0.04% of harpagoside and 0.06 $\pm$ 0.009% cinnamic acid, whereas third-grade samples were found to comprise 0.11 $\pm$ 0.03% harpagoside and 0.03 $\pm$ 0.006% cinnamic acid. The decreasing trend of phenylpropanoid and cycloartenoid terpenoids across different grades aligns with the metabolomics outcomes.

4. Discussion

In this study, *S. ningpoensis* Hemsl's primary metabolites comprise carbohydrates, amino acids, polyols, and organic acids. Among the six essential nutrients (carbohydrates, proteins, lipids, vitamins, minerals, and water), carbohydrates are crucial for sustaining an organism's normal function. Not only do they provide energy, but they also contribute to the synthesis of other vital macromolecules such as nucleic acids, proteins, and fats, through transformations resulting in amino acids, pentose, and others. Moreover, the dominant carbohydrates of *S. ningpoensis* Hemsl include D-xylulose, glucose, fructose, and maltose, which are digestible carbohydrates

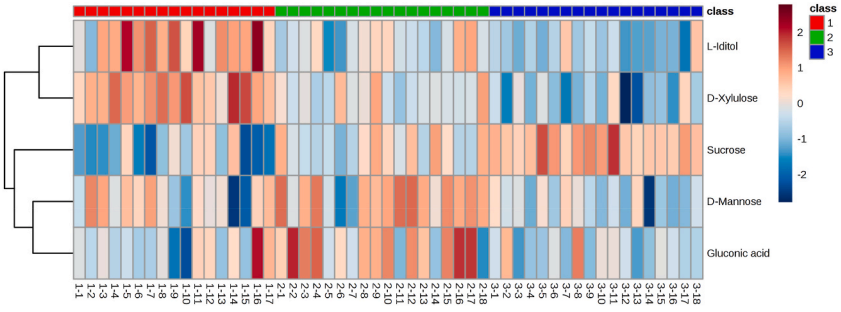

Fig. 4. The heatmap plot of the 5 shared potential biomarkers amongst different grades of *Scrophularia ningpoensis* Hemsl.

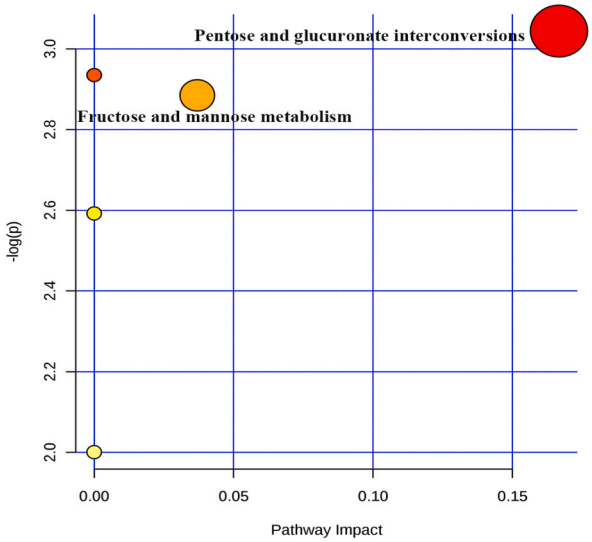

Fig. 5. Pathway analysis concerning 5 shared fifferential metabolites. **b.** Schematic diagram of related metabolic pathways.

crucial for sustaining life processes. These carbohydrates are integral for terpenoid biosynthesis, with plants initially converting CO_2 into glucose or starch, followed by further transformation into terpenoid biosynthetic materials via related pathways [24].

Terpenoids are principally synthesized through two pathways [25]: the classic mevalonic acid (MVA) pathway and the deoxyxylulose-5-phosphate (DXP) or methylerythritol phosphate (MEP) pathway. While both pathways are present in plants, the DXP pathway remains the predominant metabolic route. As illustrated in Fig. 6, glycolysis and the pentose phosphate pathway serve as the primary metabolic pathways for glucose, with the former generating ATP for living processes, and the latter producing NADPH for reductive biosynthesis, pentose phosphate for nucleic acid metabolism, and intermediates for amino acid and fatty acid synthesis [26,27]. Glyceraldehyde-3-phosphate and pyruvate, the products of glycolysis, participate in the DXP pathway of terpenoid synthesis in plants. These distinct raw materials can be converted into a common precursor, isoprenyl diphosphate (IPP), and its isomer, dimethylallyl diphosphate (DMAPP), through a series of disparate biochemical processes [28]. As an increase in glyceraldehyde-3-phosphate is directed towards the DXP pathway, a concomitant reduction in sucrose content channelled into biosynthesis is observed. This phenomenon may be attributed to the utilization of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate as primary precursors for iridoid terpenoid synthesis - the key terpenoids in *S. ningpoensis* Hemsl. Consequently, this results in decreased sucrose allocation towards biosynthetic pathways. Higher levels of sucrose in second and third-grade *S. ningpoensis* Hemsl, compared to first-grade samples, suggest that the corresponding iridoid ether terpenoids may also exhibit lower abundance. Glucose can be converted into pentose forms, such as erythrose-4-phosphate, via the pentose phosphate pathway [29]. Erythrose-4-phosphate is involved in phenylpropanoid synthesis through the shikimic acid pathway [30]. Additionally, glucose can be transformed into D-xylulose via the glucuronic acid pathway [31], and subsequently, D-xylulose-5-phosphate can enter the pentose phosphate pathway, contributing to the synthesis of phenylpropanoid glycosides in *S. ningpoensis* Hemsl [32].

Consequently, elevated levels of D-xylulose in first-grade samples are indicative of corresponding high phenylpropanoid concentrations. An investigation of selected biomarkers revealed that the pentose phosphate pathway exhibited the most substantial impact in discriminating different grades of *S. ningpoensis* Hemsl among the metabolic pathways studied. A more active pentose phosphate pathway results in decreased glucose flux into other metabolic pathways [33]. As first-grade samples display a more vigorous pentose phosphate pathway compared to second-grade samples, the content of gluconic acid and D-mannose, produced through the oxidation and differential isomerization pathway of glucose, is lower in first-grade samples. The presence of gluconic acid and D-mannose is not consistently graded from first to third, with the highest levels found in second-grade samples. This finding may be due to a less active pentose phosphate pathway in second-grade samples, which results in increased glucose oxidation and differential isomerization products.

Furthermore, *S. ningpoensis* Hemsl primarily comprises cyclic enol ether terpenoids and phenylpropanoids. Cyclic enol ether terpenoids include compounds such as harpagide, harpagoside, 8-*O*-feruloyl harpagide, and 6′-*O*-cinnamoyl harpagide, while phenylpropanoids consist of zanthoxoside A (6-*O*-trans-cinnamoyl-1-*O*-α-D-fructosyl-β-D-glucose), ningposide A (3-*O*-acetyl-2-*O*-feruloyl-α-L-rhamnose), among others [34]. There are grade differences in the quality of *S. ningpoensis* Hemsl produced in different producing areas, and different quality grades of *S. ningpoensis* Hemsl have an important impact on clinical efficacy. However, at present, there is no clear and unified method for the quality evaluation of different grades of *S. ningpoensis* Hemsl, and the ' standard ' formed by the market is more arbitrary and can not be unified, resulting in the confusion of the quality, specifications and grades of some medicinal materials in the medicinal market. According to the 2020 edition of the Chinese Pharmacopoeia, harpagide and harpagoside serve as quality control components of *S. ningpoensis* Hemsl herbs [35]. In this study, first-grade samples exhibited higher concentrations of harpagoside and cinnamic acid than other grades, corroborating the metabolomics findings.

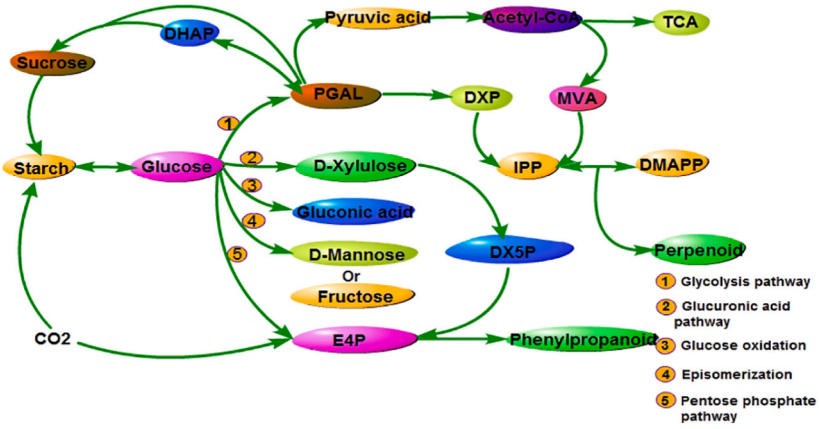

Fig. 6. Schematic diagram of related metabolic pathways.

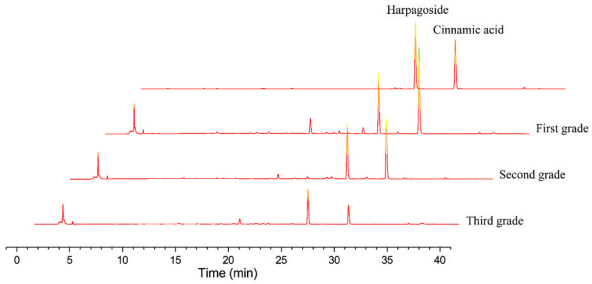

Fig. 7. Schematic diagram related to high performance liquid chromatography (HPLC) method.

5. Conclusion

Distinct variations were observed in metabolite constitution, metabolic functioning, and the activity of relevant mechanisms present within disparate grades of *S. ningpoensis* Hemsl. Utilizing the analytical capabilities of HPLC, we authenticated the levels of iridoids, phenylpropanoids present in a variety of graded. Our findings delineate that the first-grade *S. ningpoensis* Hemsl manifested superior concentrations of harpagoside and cinnamic acid. In terms of quality analysis, The quality of *S. ningpoensis Hemsl* in the first grade is the best in the third grade. Therefore, the GC-MS based metabolomics combined HPLC method, explicated in this investigation, furnished an efficient means of appraising the inherent qualitative disparities among the varied grades of *S. ningpoensis* Hemsl. However, the limitation of this study is that there is no functional evaluation of different grades of *S. ningpoensis* Hemsl and correlation with metabolites and active ingredients.

CRediT authorship contribution statement

Yu-Ai Lu: Writing – original draft. **Shi-Jun Liu:** Supervision. **Shi-Yi Hou:** Validation. **Yu-Ying Ge:** Data curation. **Bo-Hou Xia:** Writing – review & editing. **Ming-Xia Xie:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28458.

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