**Assessment of the Pharmaceutical Properties of Two *Radix Puerariae* Species through Integrated Metabolomics and Bioactivity Analyses**

**ABSTRACT**

*Pueraria* plants are widely used for both nutritional and medicinal purposes in many Asian countries, with the *Pueraria thomsonii* Benth. (*P. thomsonii*) and *Pueraria lobata* (willd.) Ohwi. (*P. lobata*) species being used interchangeably in these applications. In the present study, the similarities and differences between these two *Pueraria* species were characterized via metabolomics-based ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), desorption electrospray ionization-mass spectrometry imaging (DESI-MSI), ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), and spectrophotometry assays. The antioxidant and protective properties of *P. lobata* when used to treat cardiocytes were assessed through methyl thiazolyl tetrazolium (MTT) and 2,2-diphenyl-2-picrylhydrazide (DPPH) assays, which further emphasized metabolic differences. Through these analyses, the present study not only highlights differences in the potential applications of *P. thomsonii* and *P. lobata,* but also provides a methodological foundation that can be used to assess and quantify isoflavone distributions in specific herbal species of interest.

**Keywords:** *Radix Puerariae*; UPLC-MS; DESI-MSI; Isoflavonoids; Metabolomics; Saccharides

**Abbreviations:** BPI: Base peak intensity; CE: collision energy; CV: cone voltage; DESI-MSI: desorption electrospray ionization-mass spectrometry imaging; DRETM: Dynamic range enhancement; DPPH: 2,2-diphenyl-2-picrylhydrazide; ESI: Electrospray ionization; GC: Gas chromatography; IS: internal standard; LC: Liquid chromatography; MRM: Multiple reaction monitoring; MS: Mass spectrometry; MTT: Methyl thiazolyl tetrazolium; NMR: Nuclear magnetic resonance; *P. lobata*: *Pueraria lobata* (willd.) Ohwi.; PLS-DA: Partial least squares-discriminant analysis; *P. thomsonii*: *Pueraria thomsonii* Benth.; ROI: region of interest; TFA: total flavonoids; UPLC-Q-TOF-MS: Ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry.

**1. Introduction**

*Radix Puerariae* (Kudzu root, Chinese name: Ge Gen), which is the dried root of the *Pueraria lobata* (Willd.) Ohwi plant, has long been used in many Asian countries as a traditional nutritional supplement or herbal medicine. This root is widely planted and harvested in Southern China, and it is reported to exhibit antipyretic, antiemetic, diaphoretic, and antidiarrhoeal properties in addition to being capable of allaying thirst and dispelling heat via the induction of sweating [1, 2]. *Radix Puerariae* contains one of the highest isoflavone levels of known plant species, and is rich in bioactive substances including flavonoids, coumarins, aromatics, and triterpenes [3], all of which lend it robust antioxidative properties [4]. These bioactive compounds have also been linked to the nutritional benefits of this root [5], and to its use for the treatment of hypertension, diabetes, alcoholism cardiovascular disease, and cerebrovascular disease [6, 7]. This root is routinely processed into a number of traditional Chinese medicine (TCM) pharmaceutical preparations including Feng Ningxin tablets, Gegen Qinlian tablets, and Puerarin injections [8].

*P. lobata* and *P. thomsonii* are two members of the *Pueraria* genus, which is a member of the *Fabaceae* pea family. These two herbs are generally regarded as exhibiting similar effects in clinical and industrial contexts, and are typically used interchangeably [9]. According to the 2000 edition of the Chinese Pharmacopeia, the roots of both *P. lobata* and *P. thomsonii* can be used to prepare *Radix Puerariae,* whereas the 2020 revision of the Chinese Pharmacopeia indicates that *Radix Puerariae* specifically refers to the root of *P. lobata,* although the descriptions of the pharmacological activities ofboth *P. lobata* and *P. thomsonii* were still were recorded. The specific pharmacological activities of TCM preparations are tightly linked to the main components found therein. As such, a more thorough investigation of the biochemical composition of *P. lobata* and *P. thomsonii* roots is critical to determine whether they can be interchangeably used in clinical contexts or are better suited to use in specific contexts.

Metabolomics studies are commonly conducted to evaluate the composition of natural herbs and herb-derived products [10, 11]. Rapid advances in analytical techniques in recent years have enabled a wider array of metabolomics strategies to be implemented [12, 13], with ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) being particularly well suited to the analysis of complex plant samples owing to its high speed and excellent resolution. Desorption electrospray ionization-mass spectrometry imaging (DESI-MSI) is another increasingly robust metabolomics approach that enables the detection of metabolites in different tissue samples, enabling researchers to map spatial metabolite distributions in plants [14-16].

The present study was designed to conduct an integrated metabolomics analysis comparing the relative metabolite content and distributions in *P. lobata* and *P. thomsonii* roots through UPLC-Q-TOF-MS, DESI-MSI, and UPLC-MS/MS approaches in an effort to more fully understand the similarities and differences between these two closely related herbs. The biological activities of these herbal preparations were then further tested *in vitro* in cell culture through the use of MTT and DPPH assays with the goal of better differentiating the potential applications of these two herbs.

**2. Materials and Methods**

*2.1. Reagents and herbal samples*

*P. thomsonii* and *P. lobata* reference standards were sourced from six companies: Beijing Rongchengxinde Technology Co., Ltd, Push Bio-Technology, Shanghai Yuanye Bio-Technology Co., Ltd, Chengdu Alfa Biotechnology, Guangzhou Juntang Technology Co., Ltd, and the Institute of Medicinal Plant Development, with detailed information including CAS numbers being compiled in Table S1.

Analytical-grade acetonitrile, methanol, and formic acid were obtained from Fisher Scientific. Ultra-pure water(18.2 MΩ) was prepared with a Milli-Q system (Millipore, MA, USA).

H9c2 cardiac myoblast cells were purchased from iCell Bioscience Inc (Shanghai, China), while 96-well plates were from Nest (Wuxi, China), high-glucose DMEM was from Genom (Hangzhou, China), fetal bovine serum (FBS), was from Biological Industries (Israel), and penicillin/streptomycin were from Haoyang Biological Products Technology Co., Ltd. (Tianjin, China). MTT was obtained from Beyotime Biotechnology (Shanghai, China). Dimethyl sulfoxide (DMSO) was from Solarbio (Beijing, China).

Additional samples of *P. lobata* and *P. thomsonii* were obtained from Jiangxi, China, and were authenticated by professor Xirong He (the Institute of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences, Beijing, China).

*2.2. Standard preparation*

Appropriate standard solutions were prepared using water or methanol as a solvent, and were stored at or below 4℃. *P. lobata* and *P. thomsonii* samples were collected and split into two portions, one of which was crushed and filtered using a sieve (40 mesh) prior to storage in a dryer at room temperature, while the other was stored at −80°C pending subsequent analyses.

For LC-MS analysis, crushed samples (50 mg) were ultrasonically extracted in 1 mL of methanol for 30 min and were centrifuged at 12,000 rpm for 10 min. This extraction step was repeated three times, with the supernatants from these three extracts being pooled together, centrifuged, and injected (1 μL) into the UPLC-Q-TOF-MS system. *P. thomsonii* and *P. lobata* extracts were respectively diluted with methanol 100x and 2000x. Wogonoside at a final concentration of 5 ng/mL was added as an internal standard (IS). Following centrifugation, an aliquot of 1 μL supernatant was injected into UPLC-MS/MS system.

For DESI-MSI analyses, *P. lobata* and *P. thomsonii* sections (60 μm) were prepared at -20°C with a CM1860 cryostat (Leica, IL, USA), mounted on glass microscope slides, and preserved at −80°C prior to analysis.

2.3. *UPLC-Q-TOF-MS conditions*

ACQUITY UPLC and Xevo G2XS Q-TOF instruments (Waters, MA, USA) were used for quantitative analyses of *P. lobata* and *P. thomsonii* metabolite profiles, with an HSS T3 column (Waters ACQUITY; 100 mm×2.1 mm, 1.8 μm) being used to achieve separation. Gradient elution was performed using a mobile phase composed of 0.1% formic acid solution (A) and acetonitrile with 0.1% formic acid (B) as follows: 0-4 min, 0-25% B; 4-15.5 min, 25-80% B; 15.5-16.5 min, 80-100% B; 16.5-18 min, 100% B; 18-18.5 min, 100-0% B; 20.5 min, 0% B. A flow rate of 0.3 ml/min and a column temperature of 40°C were maintained throughout this process.

For the mass spectrometric analyses, an electrospray ionization (ESI) source was operated in both positive and negative ion modes. The desolvation gas was set to 800 L/h at 450℃, and the source temperature was 100°C. Capillary voltage and cone voltage were 2,000 V and 25 V, respectively. LockSprayTM was used for the collection of all mass data to ensure accuracy and reproducibility. To lock the mass in ESI+ and ESI- modes, leucine-enkephalin (100 pg/µL) was used as a reference compound for calibration at a flow rate of 5μL/min, which was detected at m/z 556.2771 and 554.2615 for [M+H]+ and [M-H]- ions, respectively.

*2.4. UPLC-MS/MS conditions*

The UPLC-MS/MS elution conditions used herein were the same as those used for UPLC-Q-TOF-MS. A Xevo TQ-S triple quadrupole mass spectrometer (Wate) equipped with an ESI source in positive ion mode was used with the following settings: desolvation gas = 800 L/h at 400℃, cone gas =150 L/h, capillary voltage = 0.5 kV, sampling cone voltage = 120 V. Maximal responses were achieved through the use of multiple reaction monitoring (MRM) mode, with transitions for each analyte and internal standard (IS), together with their corresponding cone voltage (CV) and collision energy (CE) values, being shown in Table S2. During LC-MS/MS analyses, peak integration and calibration curves generation were conducted using TargetLynx (v 4.1, Waters). Concentrations were calculated using the internal standard method as follows:

Sample concentration = Sample area\* (IS concentration/ IS area)

*2.5. Methodological Validation*

The method developed herein was validated using the bioanalytical method validation guidelines published by the FDA [17]. The limit of quantification (LOQ) and limit of detection (LOD) were defined as the concentration at which the signal-to-noise ratio (S/N) was 10 and 3, respectively. Calibration curves for 10 compounds with different dynamic ranges were established at eight different concentrations, with correlation coefficient (r) values being used to demonstrate linearity. Intra-day and inter-day precision were respectively evaluated through three and six separate standard validation runs. Repeatability was assessed using six replicates of sample solutions, while sample stability was assessed by storing samples at room temperature for 0, 2, 4, 8, and 12 h. In recovery tests, samples were spiked with known quantities of all analytes at a 100% concentration level.

*2.6. DESI-MSI conditions*

Chemical marker distributions in *P. lobata* and *P. thomsonii* were assessed with a Waters Xevo G2XS Q-TOF instrument and a 2D DESI source (Prosolia, IN, USA). 95% Methanol was utilized as a spray solvent at a 5 µL/min flow rate. The DESI sprayer voltage was 4.5 kV, with nitrogen being delivered at 0.45 MPa from an external gas cylinder. Pixel sizes (X and Y) were set at 100 μm, resulting in a spatial resolution of 100 μm. Mass spectra were obtained in positive and negative ion modes in the 50−1200 D range. High-Definition Imaging (v 1.4, Waters) and Masslynx (v 4.1, Waters) were utilized for data acquisition and mass spectrometry image processes. Images were initially normalized via total ion chromatography (TIC), and individual m/z images were then visualized and overlaid. Regions of interest (ROI) were then selected to extract mass spectra from particular regions.

2.7. *Spectrophotometry*

Reference solutions prepared across a range of concentrations were used to generate standard curves, after which sample absorbance was measured following the addition of a chromogenic reagent. A resistant starch analysis was performed using a commercial kit (K-RSTAR, Ziqi Biotechnology, Shanghai, China). For further details regarding the sample processing and measurement methods for different samples, see the Supplementary Materials.

*2.8. Cell culture and viability analyses*

H9c2 cells were cultured in 96-well plates (3×103/well) in high-glucose DMEM containing 0.5% FBS and 1% penicillin-streptomycin in humidified 5% CO2 incubator at 37℃.

Cells in the treatment and positive control groups were treated for 24 h with 10 isoflavones at three appropriate concentrations, with *Pueraria* extracts (in DMSO) at 200 μg/mL, or with quercetin (25 μM). A hypoxia-reoxygenation (H/R) injury model was established via the addition of 100 μL of Na2S2O4 (2.5 mM) for 40 minutes to generate a hypoxic environment, after which cells were cultured in fresh culture media for 5 h prior to being evaluated in an MTT assay.

*2.9. Assessment of DPPH free radical scavenging ability*

Methanol was initially used to prepare 12 mg/mL solutions using the two different *Puerariae* extracts, after which 180μl of DPPH solution (80μg/mL) was added to a 96-well plate. These methanol solutions were then added to appropriate wells and plates were incubated in the dark for 30 min, after which absorbance at 517 nm was measured. Experiments were repeated three times in parallel.

*2.10. Statistical analysis*

Raw Q-TOF data were imported into Progenesis QI (v2.3, Waters, MA, USA) for peak selection, filtering, and alignment. Retention time ranged from 0.5-18.5 min with a tolerance of 0.01 min, masses ranged from 50-1,200 Da with a tolerance of 0.02 Da. A peak intensity threshold of 0 was used during peak integration. Normalized Progenesis QI data outputs were then introduced into SPSS (v26.0, IBM Corp., NY, USA) and Simca (v13.0, Umetrics, Malmö, Sweden) for appropriate multivariate statistical analyses.

3. **Results and Discussion**

*3.1. Sample preparation and the chromatography method development*

Ultrasonic extraction yielded higher spectral intensity and was more convenient than reflux extraction (Fig. S1), and it is also better suited to enhancing isoflavone extraction yields while reducing the amount of solvent utilized and the extraction temperature [18]***.*** Ultrasonic extraction was thus utilized for all analyses in the present study. Three different methanol concentrations (25%, 50%, and 100%) were tested to optimize extraction conditions (Fig. S2), with a 30-minute ultrasonic extraction step using 100% methanol ultimately being found to yield the highest compound levels. This extraction method was therefore used for all subsequent research, and is discussed in detail in our Materials and Methods section.

To optimize peak shapes and resolution, we tested several mobile phase conditions, including 0.1% aqueous formic acid as well as acetonitrile with 0.1% formic acid. Of the two tested column types, the HSS T3 column achieved better separation for all analytes. Under an optimal gradient elution program, we were able to elute the main analytes from the column in 18.5 min while achieving good resolution. Representative BPI ESI-negative and ESI-positive chromatograms for *P. lobata* and *P. thomsonii* extracts are shown in Figure 1. Overlaid QC graphs generated via the reinjection of duplicate QC samples four times in each mode further confirmed the stability and reproducibility of instrument performance in this assay system (Fig. S3).

*3.2. Mass fragmentation*

For the mass spectra generated in positive ion mode, flavonoid C-glycosides and isoflavone C-glycosides exhibited [M+H-18]+ and [M+H-28]+ fragments that were generated by the respective loss of H2O and CO groups. In negative ion mode, most flavonoids and isoflavones followed one of two fragmentation pathways (Fig. 2). Aromatic compounds lost CO2 ([M-H-44]-) or·CH3 ([M-H-15]-) groups, followed by the loss of a CO group ([M-H-28]-). Representative mass spectra and MS/MS spectra for puerarin are shown in Figure S4. In ESI-positive mode, the m/z 417.1198 fragment ion in the MS/MS spectrum matched with the [M+H]+ ion of puerarin with a mass accuracy of 2.9 ppm. It lost a neutral H2O to yield a fragment ion at m/z 399.1082 (with a mass accuracy of 0.5 ppm), while the m/z 381, 363, 335, and 307 ions were attributed to the loss of H2O and CO groups. In negative ion mode, puerarin yielded the m/z 281 and 133 fragment ions, which were characteristic fragments produced via the Retro-Diels-Alder (RDA) reaction (Fig. S5). For other details pertaining to specific fragmentation pathways, see Figures S6-S8.

*3.3. Peak identification and chemical constituent confirmation*

Through comparisons of the empirical molecular formulas and MS/MS data for major detected peaks and reference databases, we were able to match potential chemical markers in *P. lobata* and *P. thomsonii* extracts with specific reference compounds (Table 1). These potential constituent compounds were first identified with the MassLynx software (v 4.1, Waters). Following comparisons of high-resolution mass spectra and MS/MS spectra with previously published spectra for standard solutions and compounds, the structures of these potential chemical markers were clarified. Through this approach, 103 total compounds were identified, including flavonoids, isoflavones, puerosides, aromatics, triterpenes, and coumarins.

*3.4. Spatial metabolite distributions in two Puerariae species as assessed via DESI-MSI*

The spatial distributions of key metabolites of interest in *P. thomsonii* and *P. lobata* extracts were evaluated via DESI-MSI in positive and negative ion modes, with detailed spectra and other information pertaining to m/z ions being shown in Table S3 and Figure S9. There were dramatic differences in the mass spectral profiles for these two samples (Fig. 3). In line with the UPLC-Q-TOF-MS results, the levels of isoflavonoids and flavonoids in *P. lobata* were significantly higher, and these metabolites were primarily concentrated in the pith, xylem, and phloem, whereas in *P. thomsonii s*ome slight cortical accumulation was visible. The xylem and phloem are the primary tissues responsible for transporting water and nutrients from the roots to the upper portions of plants [19], and flavonoids play a key role in regulating root nutrient uptake, growth, and gravireaction [20]. Secondary metabolite distributions in medicinal plants are commonly related to internal structural characteristics, and these DESI-MSI results are consistent with prior studies and provide a basis for periderm removal during herbal medicine preparation [21]. Further research, however, will be required to understand the variations in metabolic pathways in different portions of these roots. The cortical distribution of organic acids in both *Puerariae* speciesmay represent a form of adaptation to conditions of environmental stress [22].

3.5. *Multivariate statistical analyses*

To further evaluate the UPLC-Q-TOF-MS data obtained in both ESI+ and ESI- modes, a supervised partial least squares discrimination analysis (PLS-DA) approach was used to compare *P. lobata* and *P. thomsonii* samples. Such supervised PLS-DA analyses can establish a metabolomic message block based upon known information, after which analyzed can be predicted, identified, and divided into different groups using this known message block. The *R2Y* and *Q2* parameters correspond to the captured variance and predictive accuracy of the model, respectively. When PLS-DA analyses were performed for these two ESI modes (Fig. 4-A, 4-B), a clear distribution of components in *P. lobata* and *P. thomsonii* was evident and model performance was excellent, with respective *R2Y* and *Q2* values of0.935 and 0.673 in positive ion mode and 0.679 and 0.457 in negative ion mode. To further validate this model, permutation tests (n =200) were conducted, demonstrating that the models were reproducible and satisfactory (Fig. 4-C, 4-D).

For DESI-MSI analyses, eight ROIs for each tissue section containing 10 pixels were selected at random and mapped onto the ion images. Raw ion intensity data extracted from ROIs were then analyzed via a PLS-DA approach. The resultant chemical information derived from these DESI-MSI analyses was consistent with UPLC-TOF-MS results (Fig. 4-E, 4-F). The *R2Y* and *Q2* values were consistent with the goodness of fit and the predictive utility of this model, with values of 0.935 and 0.673 in positive ion mode and 0.679 and 0.457 in negative ion mode.

To identify potential chemical markers warranting further study, differentially abundant metabolites were identified using Student’s t-tests. In total, 49 key metabolites were identified, and corresponding p-values were generated. The levels of these 49 metabolites were higher in *P. lobata* samples relative to *P. thomsonii* samples, and there was clear diversity in compound levels. Heat maps were additionally generated to highlight differences in compound levels among samples. While *P. lobata* and *P. thomsonii* share a similar chemical composition, there were nonetheless pronounced differneces in the relative levels of these compounds when comparing samples of these two roots (Fig. 4). When sorted by total content levels, the top 10 differentially abundant compounds were filtered and selected for further quantitative analyses, and these compounds accounted for about half of the weight of the overall identified compounds.

*3.6. Quantitative analyses of the top differentially abundant compounds*

The LOQ, LOD, and standard calibration curves for all analytes exhibited satisfactory linearity (r ≥ 0.9901; Table 2). The precision of this analytical method was assessed based upon relative standard deviation (RSD) values, with intra-day and inter-day RSD values ranging from 4.08% - 8.09% and 3.16% - 4.95%, respectively. Repeatability and stability were within acceptable limits, with respective RSD values below 13.83% and 10.58%. Recovery rates ranged from 83.66–102.17%, and the RSD values were all less than 15.41%. This method was thus considered to exhibit good accuracy.

The method described above was thus next used to analyze the two *Radix Puerariae* species of interest. Variations of the content extracted from these two species were consistent with the variations observed in UPLC-Q-TOF-MS and DESI-MSI analyses. Isoflavones are the primary bioactive pharmaceutical ingredients in Pueraria, and can aid in the treatment of cardiovascular disease. Levels of flavones including genistin, daidzin, and puerarin were 5-7 times higher in *P. lobate* samples, and these compounds have been shown to exhibit a range of health benefits including the prevention or treatment of cancer or cardiovascular disease [23-26].

3.7. *Spectrophotometric analyses of primary metabolites*

Soluble sugar, starch, cellulose, and resistant starch contents in these two Pueraria species were next assessed via a spectrophotometric approach. There were significant differences in the levels of these saccharides between samples (Table 3), with higher levels in samples of *P. thomsonii*, whereas but the total flavonoid (TFA) content was higher in *P. lobata* samples, consistent with the above findings pertaining to isoflavones and flavonoids in the extracts prepared from these roots.

These findings suggest that the levels of primary metabolites in these Pueraria roots were negatively correlated with the levels of secondary metabolites therein. This may be because starch and other primary metabolites accumulate during cultivation, whereas levels of secondary metabolites such as flavonoids remain relatively low in this context. However, a higher starch content may make active ingredient extraction more challenging owing to the impact of gelatinization on such extraction, although Pueraria starches exhibit lower viscosity, a higher gelatinization temperature, and a greater degree of crystallinity relative to cassava starches [27]. In light of these properties, *P. thomsonii* roots may be ideally suited to use in nutritional and health-related contexts. Levels of resistant starch, which were higher in *P. thomsonii*, can impact body weight, energy balance, and increase lipid excretion so as to decrease caloric intake and serum lipid levels [28], potentially making *P. thomsonii* suitable for use in the production of diet foods. *P. thomsonii* samples also contained higher levels of cellulose, which is a valuable additive and can benefit human health by improving the microenvironmental conditions for bacteria within the gastrointestinal tract.

*3.8. Assessment of the impact of Pueraria extracts and chemical markers on H9c2 cell viability*

To more fully understand the biological functions of the major compounds identified in these Pueraria extracts and to attempt to draw correlations between extract chemical composition and bioactivity, the ability of these compounds to protect against H/R-induced H9c2 cell death *in vitro* was assessed in an MTT assay. Survival rates for cells in the model group were significantly lower than for cells in both the control and treatment groups (Fig. 7A-C). This same assay approach was then used to assess the protective effects of the two prepared Pueraria extracts, revealing that H/R exposure significantly impaired H9c2 cell proliferation (p<0.01). Pretreatment with both *P. lobata* extract and quercetin significantly reduced the severity of such H/R-induced injury, with no significant differences between these groups (Fig. 7D). IN contrast, no significant increases in viability were observed when comparing cells pretreated with *P. thomsonii* extract to those in the model group, and *P. thomsonii* extract pretreatment was associated with significantly impaired survival relative to quercetin treatment. Quercetin has previously been shown to prevent cardiomyocyte damage in the context of ischemia/reperfusion injury [29]. Our results suggest that *P. lobata* extracts exhibit protective efficacy against H/R injury in H9c2 cells to a similar to that of quercetin. The enriched isoflavonoid compounds present in *P. lobata* likely account for this improved protective activity in the context of Na2S2O4-induced hypoxia in cardiocytes, and suggest that *P. lobata* may possess the requisite pharmacological activity necessary for its use in the preparation of medicinal compounds.

*3.9. Assessment of the DPPH* *free radical scavenging capacities of different Pueraria extracts*

Both *P. lobata and P. thomsonii* exhibited antioxidant scavenging activity in a DPPH assay in the 0.025-1.0 ng/mL range (Fig. 7), with this activity being positively correlated with the extract concentration. Notably, the scavenging capabilities of *P. lobata* extractswere notably stronger than those of *P. thomsonii* extracts. Isoflavones were the most abundant bioactive compounds in these extracts, and they have previously been shown to act as electron donors in redox reactions wherein they exhibit robust reducing power [30]. Our LC-MS/MS analyses indicated that the isoflavonoid content in *P. lobata* extracts was significantly higher than in *P. thomsonii* extract, consistent with the enhanced antioxidant activity attributable to *P.lobata* in this experiment.

*4.* **Conclusion**

In summary, this study details the successful evaluation of *P. lobata* and *P. thomsonii* metabolite contents through a series of UPLC-Q-TOF-MS, DESI-MSI, LC-MS/MS, and spectrophotometry approaches. TFA and bioactive compounds were found to be abundant in *P. lobata* samples, with levels of the major secondary metabolites puerarin, genistin, and daidzin being 5-7 times higher in *P. lobata* relative to *P. thomsonii.* This was also associated with the superior cardioprotective abilities of *P. lobata* extracts in an H/R injury model and the superior antioxidant activity of these extracts in a DPPH assay. Spatial information also indicated that these compounds were concentrated in the pith, xylem, and phloem of *P.lobata* roots. In contrast, soluble sugars and natural polysaccharides were more abundant in *P. thomsonii*. These data suggest that these two related species are best suited to use in different applications, with *P. lobata* being more suitable for processing into pharmaceutical products, and *P. thomsonii* being of greater value in the production of nutritional supplements or food products. In addition, the methods established in this article represent an accurate, broad, and effective approach to spatial metabolite profiling that may be further used for the evaluation, quantification, and distribution analyses of isoflavones in herbal plants in future research.

**Figure legends:**

Fig. 1. The base peak intensity (BPI) chromatograms for samplescollected in ESI-positive (A, *P. lobata*, B, *P.thomsonii*) and ESI-negative modes (C, *P. lobata*, D, *P. thomsonii*).

Fig. 2. Characteristic fragmentation for isoflavone and flavone compounds in ESI-negative mode.

Fig. 3. DESI-MSI images in positive and negative ion modes demonstrating compound distributions in root samples from the two *Puerariae* species of interest. The m/z ion at 137.0230 corresponds to 4-hydroxybenzoic acid, salicylic acid, 3,4-dihydroxybenzaldehyde, and 2,4-dihydroxybenzaldehyde in negative ion mode; m/z ion 267.0603 corresponds to formononetin, isoformononetin, and isoflavone in negative ion mode; m/z ion 269.0440 corresponds to genistein, 3',4',7-trihydroxyisoflavone, 3'-hydroxyl daidzein, and apigenin in negative ion mode; m/z ion 445.1133 corresponds to 3'-methoxy puerarin, glycitin, 4''-methyloxy genistin, 4'-methoxy puerarin, 3''-methyloxy daidzin, and 5-hydroxyl ononin in positive ion mode; m/z ion 417.1165 corresponds to daidzin and puerarin in positive ion mode; m/z ion 433.1109 corresponds to vitexin, isovitexin, 3'-hydroxy puerarin, genistin, genistein-8-C-glucoside, sophoricoside, and baicalin-7-O-β-D-glucopyranoside in positive ion mode; m/z ion 549.1583 corresponds to mirificin and puerarin-6''-O-xyloside in positive ion mode.

Fig. 4. PLS-DA score plot for *P. thomsonii* (Green) and *P. lobata* (Blue) in ESI-positive mode (A) and ESI-negative mode (B) as established based upon the results of UPLC-Q-TOF analyses, with plots corresponds to 200 PLS-DA model permutations in ESI-positive mode (C) and ESI-negative mode (D). PLS-DA analysis for DESI-MSI results based upon chemical information acquired in ESI-positive mode (E) and ESI-negative mode (F), together with permutation tests (n=200) for each model in ESI-positive mode (G) and ESI-negative mode (H).

Fig. 5. Heatmaps highlighting metabolites that were differentially abundant between *P. thomsonii* (red) and *P. lobata* (green). different *Puerariae* samples are shown on the horizontal axis, while differentially abundant compounds are listed on the vertical axis.

Fig. 6. Levels of the 10 differential components in *P. thomsonii* (Green) and *P. lobata* (Blue). \*\* p<0.01; \*\*\*p<0.001.

Fig. 7. A. Assessment of the ability of formononetin and 3'-methoxy daidzin to protect against H/R-induced cardiac cell death. B. Assessment of the ability of daidzein, genistin, 3'-hydroxy puerarin, and daidzin to protect against H/R-induced cardiac cell death. C. Assessment of the ability of puerarin, mirificin, 3'-methoxy puerarin, and ononin to protect against H/R-induced cardiac cell death. D. Assessment of the effects of *Puerariae* extracts (200 μg/mL) on H/R-induced H9c2 cell viability. Data are normalized to control samples. \*\*\*p<0.001 vs. control; ### p<0.001 vs. model group; &&& p<0.001 vs. quercetin group. E. The DPPH free radical scavenging capacity of *P. thomsonii* and *P. lobate* methanol extracts. Analyses were conducted in triplicate, and data are given as means ± SEM.

**Table legends:**Table 1. Compounds identified in *P. thomsonii* and *P. lobata* methanol extracts via UPLC-Q-TOF-MS.

Table 2. Methodological validation results

Table 3. Standard curve equations and average saccharide and TFA contents in *P. thomsonii* and *P. lobata*. Data are means ± SD.

**Supplementary Materials:**

Table S1. Details regarding the standards used in this study.

Table S2. m/z values with predicted molecular formulas, ion descriptions, error values in ppm, attributions made in negative and positive ion modes as detected via DESI-MSI.

Table S3. Precursor and Product Ions (m/z) for the 10 components of interest with corresponding MS detection conditions.

Figure. S1. Base peak intensity (BPI) chromatograms for *P. lobata* extracts prepared using different methods (A: Ultrasonic extraction, B: reflux extraction) and obtained via UPLC-Q-TOF-MS in ESI-positive mode.

Figure S2. Base peak intensity (BPI) chromatograms for *P. lobata* extracts containing different methanol concentrations (A, 100%, B, 50%, C, 25%) as obtained via UPLC-Q-TOF-MS in ESI-positive mode.

Figure S3. Overlapping BPI chromatograms for all QC samples in positive (A) and negative (B) ion modes.

Figure S4. Representative mass spectrum (A) and MS/MS spectrum (B) for puerarin with a retention time of 4.01 min in ESI-positive mode. (C) The main fragmentation pathways of puerarin in ESI-positive mode.

Figure S5. Representative mass spectrum (A) and MS/MS spectrum (B) for puerarin with a retention time of 4.01 min in ESI-negative mode. (C) The main fragmentation pathways of puerarin in ESI-negative mode.

Figure S6. Representative mass spectrum, MS/MS spectrum, and main fragmentation pathways for Quercetin in ESI-positive mode.

Figure S7. Representative mass spectrum, MS/MS spectrum, and main fragmentation pathways for Quercetin in ESI-negative mode.

Figure S8. Representative mass spectrum, MS/MS spectrum, and main fragmentation pathways for Vanillic acid in ESI-negative mode.

Figure S9. Mass spectra obtained from the section of interest. Ion images of m/z 137.0230 (A), 267.0648 (B), 269.0419 (C), 445.1153 (D), 417.1165 (E), 433.1109 (F), and 549.1583 (G).