Gender discrimination of *Populus tomentosa* barks by HPLC fingerprint combined with multivariate statistics

Cui Wu | Bo Xu | Zhuojun Li | Pingping Song | Zhimao Chao

**Abstract**
A high-performance liquid chromatography (HPLC) fingerprint method with multivariate statistical analyses was applied to discriminate the male and female barks of *Populus tomentosa* for the first time. The samples of 11 male and 13 female barks of mature *P. tomentosa* were collected in Beijing. The chemical fingerprint of methanol extract was established by HPLC method with diode array detector (DAD). The principal component analysis (PCA), hierarchical clustering analysis (HCA), and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to discriminate male and female barks based on the area of common peaks identified in HPLC fingerprints. A clear grouping trend ($R^2_X$, 0.83; $Q^2$, 0.595) among the male and female samples was exhibited by PCA score plot. Two groups were clearly divided into male and female samples by HCA. Both male and female samples were well discriminated with OPLS-DA ($R^2_X$, 0.775; $Q^2$, 0.795). Seven potential chemical markers were screened by variable importance in projection (VIP values >1.0) of OPLS-DA model and four of them were identified as micranthoside, siebolside B, sakuranin, and isosakuranin. The HPLC fingerprint combined with multivariate statistical analyses could be used to discriminate the gender of barks of *P. tomentosa* and revealed the differences in chemical components, which enriched the basic studies on dioecious plant.

**KEYWORDS**
dioecious plant, hierarchical clustering analysis, high-performance liquid chromatography fingerprint, orthogonal partial least squares discriminant analysis, *Populus tomentosa*, principal component analysis

1 | INTRODUCTION

Dioecious plants refer to the seed plants that have male and female flowers grown in different individuals, which is a kind of important category in plant taxonomy (Liu et al., 2012a). Some plants of *Populus* genus of Salicaceae family are dioecious plant with antibacterial, anti-inflammatory, analgesic, antiviral, antioxidant, and cytotoxic activities (Cheng et al., 1994; Marianne et al., 2014; Stéphanie et al., 2011; St-Pierre et al., 2018; Wu et al., 2016). The male and female individuals of dioecious plant are different in physiology (Whitham et al., 2016), biochemistry (Xin et al., 2018), and the content of some active components (Guido et al., 2002; Sun...
et al., 2012). It is of great value to discover the differences in physiology of dioecious plants, just as the differences of androgen and estrogen contents in human body (Cao et al., 2018).

The physicochemical properties of male and female individuals of dioecious plants may differ. For example, the contents of polysaccharides and ticosanthin in male plant were higher than those in female plant, which provide reasonable explanation for that male plant was better (Zhang et al., 2014). So, if there were differences between male and female barks, the pharmacological activities may differ.

The plant of *Populus tomentosa* Carrière (Fam. Salicaceae) is an endemic afforestation tree in north China. Some researches in discrimination of male and female plant based on buds, leaves, and flowers were reported. The phenomena of higher content in volatile compounds of methyl benzoate, benzyl benzoate, and 2-cyclohexen-1-one in female flower buds and higher content of ethyl benzoate in male flower buds can discriminate the mature male and female buds of *P. tomentosa* (Xu et al., 2019a). The polymorphism between male and female trees was tested by using the methods of RAPD and bulked segregate analysis and searched the primer 560 could be used for selecting the gender of *P. tomentosa* (Hou et al., 2009). The isoperoxidase in female plants could be separated into 10–12 bands but male plants only separated in to 4–5 bands by using isoelectric focusing in polyacrylamide gel electrophoresis. In addition, the protein content solvable, peroxidase activity, and the scanning curves of electrophoresis pattern of isoperoxidase between male and female plant of *P. tomentosa* were obviously different (Tian et al., 1993). Transcriptome analysis identified genes significantly differentially expressed between the male and female plant of *P. tomentosa*, including genes related to floral development, phytohormone synthesis and metabolism, and DNA methylation (Song et al., 2013). However, whether there are differences in chemical composition of the barks which are not the sexual organ of *P. tomentosa*, and whether the differences can be used to identify the male and female individual of dioecious plants have not been reported so far. The barks of *P. tomentosa* have the activities of clearing heat, removing dampness, relieving cough, and resolving phlegm (Editorial Board of Chinese Medica, 1999) for the treatment of hepatitis, dysentery, stranguria with turbid urine, cough, and phlegmatic asthma and prevention of infectious hepatitis (Jiangsu New Medical College, 1985; Lv & Tao, 1985). So, it would be valuable and instructive for the clinical use to differentiate male and female barks. In addition, the research on non-sexual organs would enrich the range of dioecious plant.

Previous researchers mostly studied the gender identification on the differences in the mature individuals in morphology (Lu et al., 2018), physiological and biochemical (Song et al., 2014), peroxidase isozyme (Tian et al., 1993), transcriptome, phytohormone, and DNA methylation analysis (Song et al., 2013). It is a complex system to discriminate the gender of dioecious plants. The gender identification based on gene expression products is inaccurate and unreliable, and cannot be primarily applied to the early gender identification of dioecious plants (Yin et al., 2003). The RAPD method has poor stability and reproducibility because the results are highly dependent on certain factors such as the concentrations of the template DNA and Mg ions (Du et al., 2019).

High-performance liquid chromatography (HPLC) is a widely used technique for species differentiation and quality control of herbal medicines. Fingerprinting strategies provide analytical signals related to the composition in a non-selective way such as by collecting a spectrum or a chromatogram (Cuadros-Rodríguez et al., 2016). HPLC fingerprint is a type of quality assessment system with integrated, macroscopic, and “fuzzy” nonlinear characteristics that comprehensively analyzes the complex components of food, plant, and herbal medicines. It emphasizes systematic characterization of the analytical target that contains large amount of information and relates to the classification of samples based on integral chemical background (Wipawee et al., 2008). Identifying the remarkable differences in fingerprints only depend on content determination is hard through it could be an effective strategy to help assess the quality of herbs. It was real a challenge to find remarkable compound in the past, but the wide application of multivariate statistical analysis makes it easy nowadays (Zhang et al., 2015).

The multivariable statistical approach has become more important for analytical chemistry. Such approaches are used to predict a pure component feature, and its concentration profiles from a set of spectra, chromatographs, and any situation where multiple measurements are acquired (Beebe & Kowalski, 2012). Recently, fingerprint methods combined with multivariate analysis have been used to successfully classify and discriminate different complex herbal sources (Liu et al., 2016; Qin et al., 2015; Tang et al., 2015).

In this study, a total of 24 of male and female barks of *P. tomentosa* was collected. A HPLC fingerprint method was established and multivariate statistical analyses including principal component analysis (PCA), hierarchical clustering analysis (HCA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to discriminate the male and female barks of *P. tomentosa*. Then, the potential chemical markers were screened for the discrimination of gender of *P. tomentosa*.

### 2 MATERIALS AND METHODS

The samples of 11 male barks and 13 female barks of mature *P. tomentosa* were collected on February 14, 2020, near the Sanysan Bridge which was located at 39°57′37.31″ north latitude, 116°27′28.53″ east longitude, and an altitude of 44 m (Beijing, China). The sexuality of these samples had already been clearly marked in April 3, 2019, and it could be further distinguished by the sepals on the buds on March 14, 2019. The diameters at the height of 1.5 m of these *P. tomentosa* were 40.2 to 68.2 cm. The male and female barks with the width of 3 cm and the height of 5 cm at the height of 1.5 m of trunk were stripped off. These barks were identified as the mature male and female barks of *Populus tomentosa* Carrière (Fam. Salicaceae) by Prof. Zhimao Chao (Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences) according to the description in Flora of China (Editorial Board of Flora of China, 1984). All of 24 male and
female voucher specimens (MBY-M1-2020 to MBY-M11-2020 and MBY-F1-2020 to MBY-F13-2020) were deposited at the 1,022 room of Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China.

An HPLC analysis for chromatographic fingerprints was performed with an analytical Shimadzu LC-20AT apparatus (Shimadzu Corporation) equipped with CBM-20A system controller, LC-20AT pump, CTO-10ASvp column oven, SPD-M20A UV-vis detector, SIL-20A auto injector, DGU-20A5 degasser, and Shimadzu LC-solution work station. A Liberor EB-620s electronic balance was purchased from Shimadzu Corporation. An HHS electro-thermostatic water bath was purchased from the fifth medical device factory of Shanghai. A KC-02 high-speed disintegrator was purchased from Beijing Kaichuangtonghe Technology Development Co. Ltd. Chromatography grade methanol was purchased from Fisher Scientific (Fair Lawn). Ultrapure water was purchased from Hangzhou Wahaha Group Co., Ltd. Reference standards of micranthoside, siebolside B, sakuranin, and isosakuranin were isolated and purified from male barks of *P. tomentosa* by conventional column chromatography, their structures were identified by ESI-MS, ¹H-NMR, and ¹³C-NMR in comparison with the data from literatures, and related data have been published (Liu et al., 2012b; Xu, et al., 2019b). Their purities were determined to be higher than 95% based on HPLC data have been published (Albu et al., 2015). It revealed the interrelationships between different variables and interpret sample patterns, groupings, similarities, and differences (Xu et al., 2019). $R^2$ and $Q^2$ values were the important parameters of the modeling in PCA. $R^2$ was a measure of how well the model fitted the data. $R^2$ showed how much of the variation in the X variable could be explained by the selected components. $Q^2$ indicated how well the model predicts new data. PCA result was displayed in the form of score plots. The HCA was performed with SIMCA-P version 14.1 software (Umetrics, Malmö, Sweden) based on the PCs scores of samples, and distances between samples were calculated using Ward’s minimum-variance method, with results presented as a dendrogram.

The OPLS-DA extended a regression of the PCA, used the class membership to maximize the variation, introduced an orthogonal signal correction (OSC) filter to separately handle the systematic variation correlated with or uncorrelated with the Y variable, and therefore had better discriminant ability for the samples with larger within-class divergence than PCA (Bylesjoe et al., 2008). The quality of the model was estimated by parameters of $Q^2$, $R^2$X, and $R^2$Y which described how well the model was fitted. The values of $R^2$ and $Q^2$ ranged from 0 to 1, where 1 indicated perfect fitness and predictivity (Huang et al., 2018). In order to identify the components that contribute greatly to the sample classification, the variable importance in projection (VIP) scores were used to screen differential markers. VIP scores were a weighted sum of squares of PLS weights, with scores larger than 1 indicating variables were important in the sample classification (Zheng et al., 2018).

The student's t-test was performed on the SPSS 20.0 software for windows next to confirm the significance of differences in differential markers screened from OPLS-DA between male and female barks. Sample M11 was chosen to identify the chromatographic peaks and analyzed using an Acquity UPLC H-Class from Waters (Milford, MA, America), equipped with a binary solvent delivery pump, an autosampler, a column compartment, and a PDA detector. A reverse-phase Acquity UPLC BEH C₁₈ column (2.1 × 50 mm,
1.7 μm) was used. Flow rate was 0.35 ml/min; injection volume, 1 μL; and column temperature, 35°C. Mobile phases were methanol (A) and water (B) and the elution conditions applied were as follows: 0–3 min, linear gradient 10%-30% A; 3–8 min, linear gradient 30%-35% A; 8–10 min, linear gradient 35%-55% A; and 10–12 min, linear gradient 55%-100% A. UV-visible spectra were recorded at 230 nm.

All MS data acquisitions were performed on a Vion IMS quadrupole time-of-flight (QToF) configuration (Waters) equipped with an electro-spray ionization (ESI) source. The mass spectrometer detector was operated in positive mode, with the capillary voltage at 3.0 kV, 30 V cone voltage, 450 °C desolvation temperature, and 120 °C source temperature. Nitrogen was used as the desolvation and cone gas at flow rates of 1,000 L/hr and 50 L/hr, respectively. Data acquisition was recorded in the mass range m/z 50–1000 with a scan time of 0.2 s. Waters MassLynx V4.1 software was used for data acquisition and processing.

3 | RESULTS

In order to evaluate the feasibility of the HPLC method, the similarities between each of the samples fingerprint and reference fingerprint were calculated to estimate precision, stability, and repeatability. The results of the similarities of precision, stability, and repeatability were found to be higher than 0.998, higher than 0.975, and above 0.998, respectively, indicating that the HPLC measurements were stable and under control.

The 11 male barks and 13 female barks of mature P. tomentosa were analyzed, and their corresponding HPLC chromatographic fingerprints were aligned and matched using the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine. HPLC example chromatograms of F12 and M11 were shown in Figure 1. Fingerprints that represented the characteristic mode were shown in Figure 2. As shown in Figure 2, there were 13 common peaks in the fingerprint of male and female samples. The similarities of every male samples were 0.979–0.999 and of female samples 0.976–0.999 (Tables S1 and S2), which demonstrated that the male and female samples were highly similar.

To evaluate the differences between male and female barks of P. tomentosa, PCA was carried out with the areas of 13 common peaks (Tables S3 and S4). The scores plot obtained by PCA was shown in Figure 3. The first contribution value of two principal components was accounted for 78.3% (R2X) of the total variance (PC1 described 62.4% and PC2 described 15.9% of the sample variability). The predictive ability of the model (Q2) was 0.595, which demonstrated that it was a good model (Q2 ≥ 0.50) (Triba et al., 2015; Zhang et al., 2018; Zheng et al., 2018). There was a clear separation between male and female barks of P. tomentosa. These results indicated that there were differences in some chemical compounds between male and female barks of P. tomentosa. HCA was performed based on two PCs from the above PCA model, and displayed relationships between male and female samples in the form of a dendrogram. As shown in Figure 4, all samples could be clearly divided into two groups, i.e., all of 11 male samples were classified into Group 1 (left) and all of 13 female samples were classified into Group 2 (right) in Figure 4. Some samples distribution in score plot of PCA was close such as sample F4 and sample M9. However, in the dendrograms of HCA, male and female samples were clearly divided into two groups. The results of HCA can further confirm the classification of samples by PCA model.
In the present study, the male and female samples were clearly separated in OPLS-DA score plot (shown in Figure 5a). The $R^2X$ of the OPLS-DA model was approximately 0.775 indicating 77.5% of the variation in the dataset could be modeled by the selected components. The $R^2Y$ of the OPLS-DA model was 0.841 indicating the model was well fitted. The Q$^2$ was 0.795 indicating a very good predictivity. As shown in Figure 5b, the variable importance in projection (VIP) scores of seven peaks (No.3, No.6, No.7, No.8, No.9, No.10, and No.11) of

![HPLC fingerprints of the male and female barks of *P. tomentosa*. F1-F13 are female bark samples; M1-M11 are male bark samples. The X axis refers to retention time (t/min); The Y axis refers to response value (mAU). No. 1–13 refers to the common peaks.](image)

| Peak No. | Assignment       | LC Rt (min) | UV bands (nm) | Formula       | ESI (+) [M + Na]$^+$ (error/mDa) | Adducts & major fragments |
|---------|------------------|-------------|---------------|---------------|----------------------------------|---------------------------|
| 6       | Micranthoside$^a$| 59.41       | 194, 228, 283 | C$_{22}$H$_{24}$O$_{11}$ | 487.1158$^-$5.8 | 487.1158 [M + Na]$^+$; 503.0896 [M + K]$^+$; 465.1338 [M + H]$^+$; 303.0803 [M + H-Glucoside]$^+$; 257.0748; 167.0281 |
| 7       | Siebolside B$^a$ | 66.30       | 195, 229, 283 | C$_{20}$H$_{22}$O$_{9}$ | 429.1108$^-$5.4 | 429.1108 [M + Na]$^+$; 445.0848 [M + K]$^+$; 407.1289 [M + H]$^+$; 245.0753 [M + H-Glucoside]$^+$; 123.0388 |
| 8       | Sakuranin$^a$    | 73.75       | 195, 228, 282 | C$_{22}$H$_{24}$O$_{10}$ | 471.1228$^-$3.9 | 471.1228 [M + Na]$^+$; 487.0968 [M + K]$^+$; 449.1405 [M + H]$^+$; 287.0867 [M + H-Glucoside]$^+$ |
| 9       | Isosakuranin$^a$ | 76.92       | 196, 228, 282 | C$_{22}$H$_{24}$O$_{10}$ | 471.1225$^-$4.2 | 471.1225 [M + Na]$^+$; 487.0965 [M + K]$^+$; 449.1401 [M + H]$^+$; 287.0867 [M + H-Glucoside]$^+$ |
| 10      | Isograndidentatin A$^b$ | 87.30 | 192, 235, 312 | C$_{21}$H$_{28}$O$_{9}$ | 447.1607$^-$2.4 | 447.1607 [M + Na]$^+$; 463.1350 [M + K]$^+$; 425.1783 [M + H]$^+$; 309.0934 [M + H-cyclohexanediol]$^+$; 147.0395 [M + H-cyclohexanediol-Glucoside]$^+$ |
| 11      | NI               | 102.42      | 194, 240, 368 | C$_{22}$H$_{24}$O$_{10}$ | 471.1254$^-$1.3 | 471.1254 [M + Na]$^+$; 487.0994 [M + K]$^+$; 449.1432 [M + H]$^+$; 287.0882 [M + H-Glucoside]$^+$ |
| 3       | NI               | 26.46       | 192, 232, 315 | C$_{21}$H$_{28}$O$_{10}$ | 463.1558$^-$2.2 | 463.1558 [M + Na]$^+$; 433.1661; 281.0491 |

Abbreviation: NI not identified.

$^a$Identified by comparing with reference standards.

$^b$Identified by comparing with literature.
**FIGURE 3** PCA scores plot using areas of common peaks of male and female barks of *P. tomentosa*. Triangles represent female samples, and diamonds represent male samples. The $X$ axis refers to the scores of PC1; The $Y$ axis refers to the scores of PC2. The variances accounted by the first principal component (PC1) and the second principal component (PC2) were 62.4% and 15.9%, respectively.

**FIGURE 4** Dendrograms of the hierarchical cluster analysis (HCA). HCA was performed based on two PCs from the above PCA model, and calculated using Ward's minimum-variance method. F1-F13 refers to female samples and M1-M11 refers to male samples. The $X$ axis refers to sample number. The $Y$ axis refers to distance between different groups.
HPLC fingerprint were higher than 1.0. It was indicated that these seven peaks possessed the most influence on the discrimination between male and female samples. The student’s t-test was performed based on the peak areas of seven compounds screened in OPLS-DA model and the result showed that the differences of these seven peaks between male and female samples were significant ($p < .05$).
The analysis of MS data was showed in Table 1. The MS and MS/MS spectrograms contained the quasi-molecular ion peak and major fragments, respectively, were displayed in Figures S2–S8. The peaks of No.6, No.7, No.8, and No.9 in HPLC fingerprint were identified by comparing the retention time of chromatographic peaks (Figure S1) and the corresponding MS and MS/MS spectrograms of each chromatographic peak with reference standards (Figures S2–S5) obtained from male barks of P. tomentosa. The results showed that the peaks of No.6, No.7, No.8, and No.9 were micranthoside, siebolside B, sakuranin, and isosakuranin (Figures S2–S5). The peak No.10 was identified as isograndidentatin A by comparing the MS and MS/MS data (Figure S6) with literatures (Xu, et al., 2019b; Yang et al., 2013). The peak No.11 was the isomer of sakuranin (No.8) and isosakuranin (No.9) for their same molecular weight and major MS fragments (Figure S7). The molecular formula of peak No.3 was calculated as C_{21}H_{28}O_{10} (Figure S8).

4  | DISCUSSION

In the presented study, an accurate and feasible analytical method based on HPLC fingerprint coupled with multivariate statistical analyses was developed for objective discrimination between male and female barks of mature P. tomentosa for the first time. The informative chemical profiles of 24 barks (11 males and 13 females) of P. tomentosa were obtained and employed in multivariate statistical analyses, including PCA, HCA, and OPLS-DA. Samples of male and female were found to be easily differentiated and seven peaks were screened for the potential markers of the gender. This method provided reference significance for the study of dioecious plants, which discriminated the gender of every individual based on the barks only.

Among the seven compounds screened based on HPLC fingerprint combined with multivariate statistics, four main compounds were identified. The pharmacological activities of sakuranin are allergy-preventive (Yuko et al., 2006), antihyperlipidemic (Weber et al., 2008), anti-inflammatory activities (Zhang et al., 2006), and it can be used as an acetylcholinesterase inhibitor (Remya et al., 2014). Siebolside B and isograndidentatin A have allergy-preventive (Yuko et al., 2006) and antioxidant activity (Si et al., 2009). It is demonstrated that these compounds may be the bioactive components in barks of P. tomentosa. The peak areas of these four compounds in male barks were higher than those in female barks, which might affect the effect of barks of P. tomentosa of different sex. This had important guiding significance for the use and further study of barks of P. tomentosa.

The flowers as the sexual organ for their different chemical components could be used to discriminate the gender. Similarly, the barks, the asexual organ, also can be used to discriminate the gender based on our research, which provide a new idea for researching the dioecious plants.

Some dioecious plants are medicinal in traditional Chinese medicine, such as the barks of mature Eucommia ulmoides Oliv. (Eucommiaceae) and Ilex rotunda Thunb. (Aquifoliaceae). However, there are no clear regulation about using male barks or female barks. The present study demonstrates the differences between male and female barks, which implies the differences in clinical efficacy. The study of the gender discrimination of dioecious plants has referential significance for their medicinal value.

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AUTHORS’ CONTRIBUTIONS

Z.C. designed the experiments. C.W. and B.X. performed the experiments and wrote the original draft preparation. Z.L. analyzed the raw data of HPLC fingerprints. P.S. performed the PCA, HCA, and OPLS-DA analysis. All authors have read and agreed to the published version of the manuscript.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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