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Metabolomics Based on UPLC-QTOF/MS Applied for the Discrimination of Cynanchum wilfordii and Cynanchum auriculatum

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Abstract

Recently, it has been a big issue to distinguish the dried roots of Cynanchum wilfordii and Cynanchum auriculatum in Korean herbal medicine market. Although C. wilfordii and C. auriculatum have similar morphology, the types and quantities of metabolites may differ depending on the species. Thus, in this study, UPLC-QTOF/MS based metabolomics was applied to discriminate the roots of C. wilfordii and C. auriculatum. In the optimal LC/MS conditions, 64 known metabolites were analyzed in the two species. PCA and PLS-DA of metabolic profile data was able to differentiate between C. wilfordii and C. auriculatum. Furthermore, OPLS-DA and S-plot were applied to find the potential biomarkers for the discrimination of C. wilfordii and C. auriculatum. Finally, 4 known and 10 unknown metabolites were determined as the biomarkers, and their repeatability and reliability were also validated. This indicated the metabolite profiling is a robust approach to find discriminating biomarkers of C. wilfordii and C. auriculatum.

Keywords: Cynanchum wilfordii; Cynanchum auriculatum; Metabolomics; UPLC-QTOF/MS; PCA; PLS-DA; OPLS-DA

Abbreviations

UPLC-QTOF/MS: Ultra-Performance Liquid Chromatography Coupled with Quadrupole Time-of-Flight/Mass Spectrometry; PCA: Principal Component Analysis; PLS-DA: Partial Least Square-Discriminant Analysis; OPLS-DA: Orthogonal Partial Least Square-Discriminant Analysis

Introduction

The roots of Cynanchum wilfordii and Cynanchum auriculatum have been widely used as traditional herbal medicines in Eastern Asia. C. wilfordii is an ingredient for tonic herbal drugs, and it shows pharmaceutical benefits against tumors, antioxidants, vascular diseases, and diabetes mellitus [1-4]. In China, C. auriculatum has been used as a tonic agent having the activities of anti-tumor, gastroprotective, antidepressant, and anti-aging [5-7]. However, in Korea, the human consumption of C. auriculatum is still not approved due to its safety concerns. Thus, only the C. wilfordii is registered in Korean Herbal Pharmacopoeia [8].

C. wilfordii and C. auriculatum belong to the Asclepiadaceae family and appear morphologically similar. In order to discriminate them, it is needed to find the presence of sap and the leaf shapes: C. auriculatum has a blade ovate leaf comparing to C. wilfordii [9]. However, in the herbal medicine market, they have been handled as cut and dried roots. Due to their similar morphology, it is limited to distinguish the roots of C. wilfordii and C. auriculatum. Recently in Korea, it has been a critical issue to misuse these two roots in the herbal market and food industry [10]. Thus, it is required to establish a robust tool for the discrimination and quality control of them.

In order to discriminate C. wilfordii and C. auriculatum, several analytical methods have been previously reported. HPLC–UV method was used to analyze eight marker compounds including conduritol F to discriminate the two species [11]. Phytochemical study was also performed to discover effective chemical markers for their identification [12]. Furthermore, multiplex polymerase chain reaction with specific primers was tried to perform the molecular authentication of similar medicinal plant species [13,14].

Recently, metabolomics based on liquid chromatography (LC) coupled with mass spectrometry (MS) have been used to assess the contents of plant metabolites [15,16]. Plants produce various metabolites in response to developmental, environmental, and stress-induced physiological changes [17-19]. In particular, metabolite profiling by LC/MS is an emerging tool to phenotype and evaluate the quality of plants [20-23]. In this study, we applied the ultra-performance liquid chromatography (UPLC) coupled with quadrupole time-of-flight (QTOF)/MS to analyze various metabolites in the roots of C. wilfordii and C. auriculatum. Their metabolic profile data was subjected to several multivariate analysis including principal component analysis (PCA), projection to latent structure discriminant analysis (PLS-DA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA) to observe the dissimilarities of metabolites between C. wilfordii and C. auriculatum. Finally, significant metabolites were selected as the biomarkers to differentiate two species.

Materials and Methods

Herbal medicine samples and reagents

Dried one-year old Cynanchum wilfordii (CW) and Cynanchum auriculatum (CA) roots were purchased from Yeongju, Gyeongbuk Province, South Korea in 2014. Voucher specimen (NIHHS2014-3) was deposited at the herbarium of the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea. HPLC-grade acetonitrile, methanol, and water were obtained from Merck (Darmstadt, Germany). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Sample preparation

Each root sample was dried at 35°C in a forced-air-convection-drying oven for three days after washing, and then weighed. The roots were ground (<0.5 mm) using a mixer (Hanil, Seoul, Korea) and thoroughly mixed, after which the subsamples were homogenized further using a Retsch MM400 mixer mill (Retsch GmbH, Haan, Germany) for the analyses. Fine powder was weighed (50 mg), suspended in 10 mL of 70% (v/v) methanol, and ultrasonically extracted for 1 h at 50°C. The extract was filtered and evaporated in a vacuum, and the residue was dissolved in 70% methanol. The solution was filtered through a syringe filter (0.22 μm) and injected directly into the UPLC system.

UPLC-QTOF/MS analysis

UPLC was performed using a Waters ACQUITY H-Class UPLC (Waters Corp.). Chromatographic separations were performed on an ACQUITY BEH C18 column (2.1 mm × 100 mm, 1.7 μm). The column oven was maintained at 40°C and the mobile phases consisted of solvent A [0.1% formic acid (v/v) in water] and solvent B [0.1% formic acid (v/v) in acetonitrile]. The gradient elution program was as follows: 0–2 min, B 10–40%; 2–3.5 min, B 40–45%; 3.5–4.2 min, B 45–55%; 4.2–4.8 min, 55–65%; 4.8–5.2 min, 65–70%; 5.2–10 min, B 70–100%; 10–11 min, B 100–10%; 11–13 min, B 10%. The flow rate was 450 μL/min and 2 μL aliquot of each sample was injected onto the column.

Next, MS analysis was performed using a Waters Xevo G2-S QTOF/MS (Waters Corp.) using an electrospray ionization (ESI) operated in the positive and negative ion mode. The mass spectrometers performed alternative high- and low-energy scans, known as the MS² acquisition mode. The operating parameters were as follows: cone voltage, 40 V; capillary voltage, 3.0 kV; source temperature, 120°C; desolvation temperature, 300°C; cone gas flow, 30 L/h; and desolvation gas flow, 600 L/h. The scan mass range was from 100 and 2,000 m/z. The QTOF/MS data was collected in centroid mode, using the lock spray to ensure accuracy and reproducibility. A concentration of 200 pg/mL leucine enkephalin was used as lock mass (m/z 556.2766 (ESI+), m/z 554.2620 (ESI-)). The lock spray frequency was set at 10 s, and the lock mass data were averaged over 10 scans for correction.

Data processing and multivariate analysis

All MS² data were collected and processed within UNIFI 1.7.1 and 1.8 Beta (Waters Corp., Milford, USA). Data within UNIFI 1.7.1 and 1.8 Beta is passed through the apex peak detection and alignment processing algorithms. This enables related ion components to be grouped together and analyzed as a single entity. The intensity of each ion was normalized with respect to the total ion count to generate a data matrix that consisted of the retention time, m/z value, and the normalized peak area. Charged species, salt adducts, and fragments are all automatically aligned and grouped. The three-dimensional data including peak number (RT-m/z pair), sample name, and normalized peak areas were exported to the EZinfo software 3.0.3 (UMETRICS) for multivariate analysis such as unsupervised PCA and the supervised PLS-DA and OPLS-DA. The data were mean-centered and Pareto-scaled prior to PCA, PLS-DA, and OPLS-DA.

Results and Discussion

UPLC-QTOF/MS analysis of various metabolites in the roots of CW and CA

For the effective profiling of various metabolites in the roots of CW and CA, we established a metabolomics platform based on UPLC-QTOF/MS that enables the fast and sensitive analysis with high mass accuracy. Using the optimal UPLC, metabolites extracted using 70% methanol was separated well in 12 min. Both positive and negative ion modes were estimated for the ESI of molecules. As a result, the positive mode showed poor efficiency of ionization (data not shown), and various metabolites of the two roots were successfully analyzed in the negative mode. Thus, only negative mode analysis was performed in this study. Figure 1 represented the base peak intensity (BPI) chromatograms of various metabolites in the two roots.

Next, we constructed the in-house library to identify the metabolites of CW and CA, analyzed by UPLC-QTOF/MS. In practical, it is limited to assign a number of mass spectrums without the database. Previous studies have already reported the presence of various metabolites in CW and CA [6,12,24-26]. Thus, we added the molecular formula of reported compounds into the in-house library. The m/z of ions from the raw data of CW and CA was automatically matched to the library compounds. As a result, 69 metabolites were determined with retention time (RT) and mass accuracy (ppm) (Table 1).

PCA and PLS-DA analysis

To visualize the general clustering trends between CW and CA, we applied the multiple pattern recognition methods such as PCA and PLS-DA [27]. First, the constructed method was applied for the metabolite profiling of the extracts obtained from three sample groups including 12 CWs, 12 CAs, and 5 tests (CA:CW mixtures=5:5, 6:4, 7:3, 8:2, 9:1 (w/w)). And then, the processed data was analyzed by PCA and PLS-DA in order to classify the metabolic phenotypes of samples and identify the differentiating metabolites. Unsupervised PCA reduces the dimensionality of complex datasets and provides an overview of all observations, such as groupings, trends, and outliers [28,29]. In the PCA analysis, the score plot of each sample was shown in Figure 2A. Each point represents an individual sample, and the scatter of samples indicates the similarities or differences of metabolic compositions. Samples having similar metabolite contents are clustered together, whereas those having different metabolites are dispersed. In the PCA score plot, two groups of 12 CWs and 12 CAs were separated well. Furthermore, 5 test samples having different ratio of CW and CA (50%, 60%, 70%, 80%, and 90% CA) were scattered between two groups of CW and CA. However, 5 test samples and CA group were not distinguished clearly. Thus, we also performed the supervised PLS-DA to classify the samples. As a result, the PLS-DA score plot (for the first five components, the goodness of fit of the model (R²Y)=98%) reflected a clear separation trend among three groups of 12 CWs, 12 CAs, and 5 tests (Figure 2B). Of 5 test samples, the point of 50% CA was positioned almost in the middle of both CW and CA groups. Besides, when the percentage of CA was increased from 50 to 90%, each point was closer to CA groups. This indicated that the metabolic profiles are applicable to not only discriminate CW and CA, but also evaluate the contents ratio of two species.

OPLS-DA analysis

Next, OPLS-DA analysis was performed to find the potential biomarkers for the discrimination of CW and CA. The OPLS-DA score plot showed the clear separation between these two groups (Figure 3A). The scores t [1] (x-axis) and to [1] (y-axis) are the two most important variables in summarizing and separating the data. The separation of CW and CA groups was visible in t [1], and the [1] score values showed the variation within each group. Furthermore, S-plot that shows the covariance p [1] and correlation p (corr) [1] between variables and...
Figure 1: Representative UPLC/QTOF MS-BPI chromatograms of the roots of CW (A) and CA (B) in the negative ion mode.

Figure 2: The score plots of PCA (A) and PLS-DA (B) of CA, CW, and Test samples, indicated by boxes, dots, and triangles, respectively. (a) 50% CA; (b) 60% CA; (c) 70% CA; (d) 80% CA; and (e) 90% CA.

Figure 3: The score plots of OPLS-DA (A) and S-plot (B) of CW and CA samples.
OPLS-DA model was applied to select the critical variables contributing to differentiate CW and CA (Figure 3B). The points in the S-plot represent the m/z-RT pairs of molecules. The upper right quadrant of the S-plot shows the components elevated in CW, while the lower left quadrant shows the components elevated in CA. When the point is positioned in the farther along the x-axis and y-axis, the metabolite has the greater contribution and higher reliability to the variance between two groups. In the S-plot, several metabolites were selected as the biomarker candidates. And then, we checked the intensities of each metabolite in whole data to monitor their abundantly difference between CW and CA. As a result, 14 metabolites were selected as the biomarkers. The bar graph represented their altered abundances in CW and CA (Figure 4A). In the analysis of 12 CWs and 12 CAs, 7 and 4 metabolites were almost uniquely detected in CW and CA, respectively. In addition, 3 metabolites were detected in both sides, and showed different abundances. This indicated that the 14 metabolites can be useful biomarkers to discriminate CW and CA.

**Identification and validation of selected biomarkers**

Based on the in-house library, we tried to identify the selected 14 biomarkers. As a result, 4 ions with m/z-RT (1201.6001-4.99, 1435.7114-5.443, 1131.5733-5.78, and 1111.6055-6.13) were identified as Cynauricoside E, Wilfoside C1GG (Cynauricoside C), wilfoside K1N, and wilfoside C1N, respectively. The molecular formula of other 10 ions were also determined by the calculating program, and their RT, mass accuracy, and adducts were listed in Table 2. Next, we performed the validation of 14 biomarkers to find if these biomarkers are repeatable and reliable to discriminate CW and CA. For this purpose, the biomarkers were analyzed in the additional samples of 6 CWs and 6
| No. | RT (min) | Compound names | Molecular formula | Expected neutral mass (Da) | Observed neutral mass (Da) | QTOF/MS (ESI) m/z | Mass accuracy (ppm) | Adducts |
|-----|----------|----------------|------------------|--------------------------|--------------------------|----------------|-------------------|---------|
| 1   | 0.48     | 20-O-vanillyl-kidjoranin | C_{35}H_{54}O_{10} | 634.3719 | 679.3701 | 0.36 | +HCOO |
| 2   | 0.48     | Geniposide | C_{49}H_{78}O_{19} | 969.5072 | 913.5056 | 0.22 | -H |
| 3   | 0.48     | Qingyangshengenin | C_{50}H_{80}O_{20} | 969.5072 | 913.5056 | 0.22 | -H |
| 4   | 0.53     | Conduitol F | C_{65}H_{96}O_{23} | 1246.6346 | 1246.6328 | 0.34 | -H |
| 5   | 0.68     | Sarcosic acid | C_{51}H_{74}O_{16} | 913.5056 | 987.5038 | 0.05 | +HCOO |
| 6   | 0.84     | Cynanoneside B | C_{62}H_{100}O_{22} | 1246.6346 | 1246.6328 | 0.34 | +HCOO |
| 7   | 1.76     | p-hydroxyacetophenone | C_{62}H_{94}O_{23} | 1289.6329 | 1233.6219 | 0.17 | +HCOO |
| 8   | 1.82     | Acetovanillone | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 9   | 2.03     | Resacetophenone | C_{62}H_{94}O_{22} | 1332.7231 | 1276.7121 | 0.17 | +HCOO |
| 10  | 2.14     | Cynandione A | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 11  | 2.14     | Scoptolin | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 12  | 2.6      | Penupogenin | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 13  | 2.81     | Cynanchone A | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 14  | 2.83     | Cynauricoside H | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 15  | 3.39     | Cynauricoside F | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 16  | 4.68     | Kidjoranin | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 17  | 4.87     | Caudatin | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 18  | 4.87     | Cynauricoside E | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 19  | 4.87     | Cynauricoside F | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 20  | 4.87     | Wilfoside K 1GG | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 21  | 4.93     | Otophyllide L | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |
| 22  | 4.94     | Caudatin 3-O-β-D-digitoxypyranoide | C_{62}H_{94}O_{22} | 1290.6329 | 1234.6219 | 0.17 | +HCOO |

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CA, and the bar graph of 14 biomarkers validation was represented in Figure 4B. As a result, the additional analysis of 14 biomarkers showed the almost similar results with previous analysis. Although the fold change of Biomarker 12, 13, and 14 was altered, CA showed the higher abundance of them.

Conclusion

In this study, metabolomics approach based on UPLC-QTOF/MS provided the effective discrimination of CW and CA. By the optimal LC/MS conditions, the fast and sensitive analysis of 64 metabolites in CW and CA were successfully performed. In the PCA and PLS-DA, not only two groups of CW and CA but also their mixture samples having different ratio were differentiated well. Next, OPLS-DA and S-plot were also applied for the selection of biomarkers to discriminate CW and CA. Based on the quantification of 14 selected molecules, we determined 7 CW unique, 4 CA unique, and 3 both sides biomarkers. Our results indicated the UPLC-QTOF/MS based metabolite profiling is promising to differentiate CW and CA that have morphological similarity. Hence, this work is of great importance to prevent the misuse of CW and CA especially in the Korean herbal market.

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Table 1: The in-house library of various metabolites in CW and CA.

| No. | Biomarkers          | RT (min) | Molecular formula | Expected neutral mass (Da) | Observed neutral mass (Da) | QTOF/MS (ESI) m/z | Mass accuracy (ppm) | Adducts |
|-----|---------------------|----------|-------------------|---------------------------|---------------------------|------------------|---------------------|---------|
| 1   | CW unique marker 1  | Wilfoside C 1GG | 5.43 C_{48}H_{70}O_{17} | 504.3087 | 504.3078 | 549.306 | -1.72 | +HCOO |
| 2   | CW unique marker 2  | Unknown  | 6.23 C_{48}H_{70}O_{17} | 58.99 | 58.99 | 58.99 | 0.00 | -H |
| 3   | CW unique marker 3  | Unknown  | 6.47 C_{48}H_{70}O_{17} | 62.99 | 62.99 | 62.99 | 0.00 | -H |
| 4   | CW unique marker 4  | Unknown  | 6.89 C_{48}H_{70}O_{17} | 66.99 | 66.99 | 66.99 | 0.00 | -H |
| 5   | CW unique marker 5  | Unknown  | 7.31 C_{48}H_{70}O_{17} | 70.99 | 70.99 | 70.99 | 0.00 | -H |
| 6   | CW unique marker 6  | Unknown  | 6.905 C_{48}H_{70}O_{17} | 74.99 | 74.99 | 74.99 | 0.00 | -H |
| 7   | CW unique marker 7  | Unknown  | 7.44 C_{48}H_{70}O_{17} | 78.99 | 78.99 | 78.99 | 0.00 | -H |
| 8   | CA unique marker 1  | Unknown  | 6.26 C_{48}H_{70}O_{17} | 82.99 | 82.99 | 82.99 | 0.00 | -H |
| 9   | CA unique marker 2  | Unknown  | 6.39 C_{48}H_{70}O_{17} | 86.99 | 86.99 | 86.99 | 0.00 | -H |
| 10  | CA unique marker 3  | Cynaroside E | 4.99 C_{48}H_{70}O_{17} | 90.99 | 90.99 | 90.99 | 0.00 | -H |
| 11  | CA unique marker 4  | Unknown  | 6.45 C_{48}H_{70}O_{17} | 94.99 | 94.99 | 94.99 | 0.00 | -H |
| 12  | BS marker 1         | Unknown  | 6.6 C_{48}H_{70}O_{17} | 98.99 | 98.99 | 98.99 | 0.00 | -H |
| 13  | BS marker 2         | Wilfoside K1N | 5.78 C_{48}H_{70}O_{17} | 102.99 | 102.99 | 102.99 | 0.00 | -H |
| 14  | BS marker 3         | Wilfoside C1N | 6.14 C_{48}H_{70}O_{17} | 106.99 | 106.99 | 106.99 | 0.00 | -H |

Table 2: The list of biomarkers to discriminate CW and CA (*Both sides).
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