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Original Article

Metabolic profiling investigation of *Fritillaria thunbergii* Miq. by gas chromatography–mass spectrometry

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ABSTRACT

Thunberg fritillary bulb (the dry bulbs of *Fritillaria thunbergii* Miq.), a traditional Chinese Medicine, is widely applied as an expectorant and antitussive. In this investigation, the primary metabolites of bulbs, flowers, leaves, and stems of *F. thunbergii* were analyzed by gas chromatography–mass spectrometry. Principal component analysis, partial least squares-discriminate analysis, orthogonal projection to latent structures-discriminate analysis, and heat map analysis showed that there were dissimilar metabolites, and a negative correlation between amino acids and saccharides in different analytes. Furthermore, carbodiimide, tryptophan, glucose-6-phosphate, xylose, 2-piperidinecarboxylic acid, monoamidomalonic acid, phenylalanine, and histidine were found to play an important role in the plant metabolism net of *F. thunbergii*.

1. Introduction

Fritillaria thunbergii Miq. is a perennial herbaceous plant, mainly distributed in Zhejiang, Jiangsu, and Anhui provinces in China [1]. In Chinese medical clinical practice, Thunberg fritillary bulb (Zhebeimu), the dry bulb of *F. thunbergii*, is often utilized in the treatment of cough caused by wind-heat and phlegm-heat in Traditional Chinese Medicine, and bronchitis, inflammation, hypertension, gastric ulcer, diarrhea, and bacterial infection [2]. Additionally, Zhebeimu is nowadays extensively used to treat drug resistant leukemia [3].

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Comparing to bulbs of *F. thunbergii*, phytochemical researches have shown that the flowers, stems, and leaves also contain various kinds of chemical constituents including flavonoids, essential oils, saponins, and alkaloids [4,5], which are potential medicinal resources.

Gas chromatography–mass spectrometry (GC-MS) was utilized for biomarkers screening in primary metabolites as a metabolomic technique [6] for its reliable and versatile characters comparing with nuclear magnetic resonance and liquid chromatography–MS [7]. In this study, metabolomic multivariable analysis of GC-MS data, covering principal component analysis (PCA), partial least squares-discriminate analysis (PLS-DA), orthogonal projection to latent structures-discriminate analysis (OPLS-DA), heat map analysis, and total metabolites correlation analysis were applied to reveal the metabolic regularity of *F. thunbergii*. Metabolic distinctions between the different parts of the plant were identified. This work will contribute to the metabolic composition and comprehensive usage of *F. thunbergii*.

2. Methods

2.1. Samples and reagents

The bulbs (FT0001–0006), flowers (FT 0007–0012), stems (FT 0013–0018), and leaves (FT0019–0024) of *F. thunbergii* were collected in March 2015 from Yinzhou District, Ningbo City, Zhejiang Province of China, the place of origin of *F. thunbergii*. The harvested samples were identified by Professor Jian-Wei Chen at Nanjing University of Chinese Medicine, Nanjing, China. The standard substances (ribitol) were supplied by Sigma. Solvents and reagents (methyl alcohol, chloroform analytical or chemical grade) were supplied by Aladdin Industrial Corporation (Shanghai, China). Pyridine solution was ordered from TCI, Japan. Calibration solutions (C8-C20, C21-C40) were ordered from Fluka Chemika (Switzerland).

2.2. Sample preparation

Experimental procedure of extract preparation [2]: samples (bulbs, leaves, stems, and flowers of *F. thunbergii*) were accurately weighted (100 mg for each sample); then they were rapidly frozen and grinded in liquid nitrogen. Samples were transferred to 10 mL centrifuge tubes, and 1.4 mL of 100% methanol (precooled at −20°C) was added into each of them, with vortexing for 30 seconds. Next in the process, 60 mL of ribitol (0.2 mg/mL) as interior label was added, then vortexed for 30 seconds. They were sonicated for 15 minutes, and centrifuged for 15 minutes at 4000 rpm. Next, chloroform (750 μL) was added and 1400 μL of dH_2O was added, with vortexing. Samples were centrifuged for 15 minutes. Supernatants were transferred to glass vials. Extracts were dried with a stream of nitrogen gas in a vacuum container. Pyridine solutions (60 μL) were added, vortexed for 30 seconds, and deposited for 16 hours. Bis(-trimethylsilyl)trifluoroacetamide reagent (TCI) 60 μL was added and deposited in normal atmospheric temperature for 60 minutes [8].

2.3. GC-MS method

Agilent 7890A/5975C GC-MS (Agilent, J&W Scientific, Folsom, CA, USA). Gas chromatographic conditions were as follows: HP-5MS apillary column (5% phenyl methyl silox: 30 m × 250 μm, 0.25 μm; Agilent J&W Scientific). Split sampling was with injection volume 1 μL and split ratio 20:1. The injection, ion source, and interface temperatures were set at 280°C, 250°C, and 150°C, respectively. The oven temperature raising procedure was set to 80°C for 5 minutes, and then increased by 20°C/min, then to 300°C for 6 minutes. The total run time was set at a 22 minutes measurement period. The carrier gas was helium (1.0 mL/min). Mass spectrum conditions: electrospray ionization source, electron energy 70 eV; mass data collected in a full-scan mode (m/z 35–780).

2.4. Data preprocessing

Raw data of GC-MS from Agilent MSD ChemStation was transformed into CDF format files (Net CDF) by Xcalibur (Thermo Fisher Scientific Inc., Waltham, MA, USA). Peaks were automatically detected and aligned by XCMS software (www.bioconductor.org/) [9].

Besides the default parameters, the parameters of XCMS were adjusted as follows: xcmsSet [fwhm = 3, snthresh = 3, mzdiff = 0.5, step = 0.1, steps = 2, max = 300), retcor method = "obiwarp", plottype = c ("deviation")], bandwidth (bw) = 2, minfrac = 0.3.

A series of results including data tables, RT-m/z pair, observations, and variables were listed in a *.t file, and then they were normalized in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) ahead of multivariate analyses.

2.5. Statistical analysis

Data was statistically analyzed by SIMCA-P 11.0 (Umetrics AB, Umea, Sweden) in Suzhou BioNovoGene Company, with variates mean-centered of PCA, PLS-DA, and OPLS-DA. Firstly, outliers were dislodged according to the cluster analysis results by means of PCA which is an unsupervised method. Secondly, PLS-DA, as a supervised method, was utilized to analyze the sample data, preventing from over-fitting with permutation test. Then, OPLS-DA was utilized to filter out the noise and increase the discrimination. Lastly, variant metabolites were found in Student *t* test ($p < 0.05$) and variable importance plot (VIP) values (it is significant if VIP > 1) of the first principal component. R 3.0.3 (www.r-project.org) was utilized for the *t* test.

Further qualitation of metabolome was conducted on the basis of Metabolome Alkane Retention Index on the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/). Derivatization of metabolites can produce a series of trimethylsilyl substances. One typical trimethylsilyl substance was chosen for the analysis .After normalization, raw data was processed with an internal standard method (ribitol) [10].

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