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Metabolomic profiling of the antitussive and expectorant plant *Tussilago farfara* L. by nuclear magnetic resonance spectroscopy and multivariate data analysis

Zhen-Yu Li[a], Hai-Juan Zhi[a,b], Fu-Sheng Zhang[a], Hai-Feng Sun[a,b], Li-Zeng Zhang[a], Jin-Ping Jia[a], Jie Xing[a], Xue-Mei Qin[a,*]

[a] *Modern Research Center for Traditional Chinese Medicine of Shanxi University, No. 92, Wucheng Road, Taiyuan 030006, Shanxi, People's Republic of China*
[b] *College of Chemistry and Chemical Engineering of Shanxi University, No. 92, Wucheng Road, Taiyuan 030006, Shanxi, People's Republic of China*

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ABSTRACT

This study aims to find metabolites responsible for antitussive and expectorant activities of *Tussilago farfara* L. by metabolomic approach. Different parts (roots, flower buds, and leaves) of the title plant were analyzed systematically. The in vivo study revealed that the leaves and flower buds had strong antitussive and expectorant effects. Then ^{1}H NMR spectrometry together with principal component analysis (PCA) and partial least squares discriminant (PLS-DA) analysis were used to investigate the compounds responsible for the bioactivities. PCA was used to find the differential metabolites, while PLS-DA confirmed a strong correlation between the observed effects and the metabolic profiles of the plant. The result revealed that chlorogenic acid, 3,5-dicaffeoylquinic acid, and rutin may be closely related with the antitussive and expectorant activities. The overall results of this study confirm the benefits of using metabolic profiling for screening active principles in medicinal plants.

1. Introduction

The flower bud of *Tussilago farfara* L. (Asteraceae), also called "Farfarae Flos" has been commonly used for the treatment of cough, bronchitis and asthmatic disorders in Traditional Chinese Medicine (TCM). A series of phytochemical studies indicated that the flower bud of *T. farfara* L. contained several kinds of metabolites, including essential oils [1], sesquiterpenes [2–4], triterpenes [5,6], flavonoids [7–9], phenylpropanoids [8–10], chromones [11], and pyrrolizidine alkaloids [12]. Recent pharmacological experiments revealed that the Farfarae Flos also exhibits antioxidant effect, antimicrobial activity, α-glucosidase inhibitory effect, diacylglycerol acyltransferase inhibitory effect, and inhibition of nitric oxide synthesis in lipopolysaccharide (LPS)-activated macrophages [10,13].

Farfarae Flos has been used in many Traditional Chinese Medicine (TCM) prescriptions, such as Juhongwan, Chuanbeixueligao, Kuandongdingchuantang, et al. In Europe, this plant, also known as coltsfoot, has been used as herbal remedies for the same applications, but the leaves are preferred. However, neither the East nor the West uses the roots, which is a commonly used part in other traditional Chinese medicinal plants. The underlying reasons for that the roots could not be used for cough treatment were not clear. Thus, chemical and biological comparison and evaluation of leaves, flower buds, and roots should be conducted to reveal the possible correlation between metabolites and biological effects. However, no systematic study on leaves, flower buds, and roots of *T. farfara* L. has been carried out up to now.

Synergistic effects amongst different compounds contained in a mixture are considered to be key factors for the efficacy of herbal medicinal products. Thus, a technique able to give a complete picture of all of the metabolites present in an extract is essential. Metabolomics, in particular metabolomic fingerprinting, has been widely used as a state of the art technique in medicinal plant research [14]. Of all the analytical methods used, NMR spectroscopy is a key technique due to its non-selectivity, speed, high-throughout and relatively easier sample preparation. NMR is often used in conjunction with multivariate data analysis, such as principal component analysis (PCA), and partial least square discriminant analysis (PLS-DA). In the PLS-DA, an additional Y-matrix can be applied to correlate metabolic profiling with bioactivities. Recently, NMR based metabolomic studies have been successfully used in the bioactive compounds studies of *Galphimia glauca* [15], and *Orthosiphon stamineus* [16]. And flower bud of *T. farfara* L. and its adulteration, rachis, have been compared [17].

In this study, the antitussive and expectorant activities of the hot water extracts from the leaves, flower buds and roots of *T. farfara* L. were compared firstly. Then metabolic profiling using ^{1}H NMR spectroscopy was carried out. Finally, multivariate analysis

* Corresponding author. Tel.: +86 351 7018379; fax: +86 351 7011202.
E-mail addresses: qinxm@sxu.eud.cn, qinxm@sxu.edu.cn (X.-M. Qin).

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techniques were used to find possible correlations between the metabolite profiles and bioactivity, and metabolites highly correlated with the activity were identified.

2. Materials and methods

2.1. Plant materials

The leaves, roots, and flower buds of the plant materials were collected in May 2009, December 2009, and December 2009, respectively, from Yu County, Hebei Province of China, and authenticated by Prof. Xue-Mei Qin of Shanxi University. All the voucher specimens are deposited in the herbarium in the Center of Modern Research for Traditional Chinese Medicine, Shanxi University, China. All samples were freeze-dried and ground into fine powder with pestle and mortar, and then stored at −80 °C until analysis.

2.2. Solvents and chemicals

Analytical grade methanol was from Beijing Chemical Works (Beijing, China). Methanol-d_4 (99.8% D) was obtained from Merck (Darmstadt, Germany). D_2O was purchased from Norell (Landisville, Pennsylvania, USA). Sodium 3-trimethlysilyl [2,2,3,3-d_4] propionate (TSP) was from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), and NaOD was purchased from Armar (Dottingen, Switzerland). Pentoxyverine was obtained from CSPC Ouyi Pharmaceutical Co., Ltd. (Shijiazhuang, China). Ammonium chloride, $NaHCO_3$ and KH_2PO_4 were from Beijing Chemical Works (Beijing, China). Phenol red and ammonium hydroxide were from Tianjin Chemical Works (Tianjin, China).

2.3. Samples for antitussive and expectorant analysis and metabolomic analysis

According to the traditional use, the leaves, flower buds and roots were extracted with water by refluxing for three times (2 h each). The combined solution was filtered and concentrated under reduced pressure to afford the water extract.

2.4. Animals

The animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care [18]. All animals were housed at room temperature (20–25 °C) and constant humidity (40–70%) under a 12 h light–dark cycle in SPF (Specific Pathogen Free) grade laboratory conditions. ICR mice of either sex (19–24 g) were purchased from Beijing Vital Laboratory Animal Technology Company (license number SCKX-2006-0008).

2.5. Antitussive effects against ammonia induced coughing

The antitussive activity was performed as previously described with minor modifications [19]. After 3 days of adaptation, 75 ICR mice were divided into 5 groups ($n = 15$) randomly and orally administered, including control group (distilled water), positive group (pentoxyverine 50 mg/kg), root group (0.9 g/kg), flower bud group (2.8 g/kg) and leaf group (1.7 g/kg). The administration dose of extract was calculated according to the clinical dose of this plant and yield of extract. The administration was carried out at 8:00 a.m. each day for 5 consecutive days and the mice were exposed to a 1000 ml special glass chamber with ammonium hydroxide (1 ml) cotton ball after 1 h of the last administration and the cough incubation period was recorded. After 1 min, the mice were taken out from the chamber and placed in a beaker and the frequency of cough within 2 min was observed and recorded.

2.6. Expectorant test

The procedures were performed as described previously [20]. After 1 week of adaptation, 60 ICR mice were divided into 5 groups (12/group) randomly and orally administered, including control group (distilled water), positive group (ammonium chloride 1 g/kg), root group (0.9 g/kg), flower bud group (2.8 g/kg) and leaf group (1.7 g/kg). All groups were treated with a single dose daily for 5 days and the last dose was given 30 min before intraperitoneal injection of phenol red solution (5% in saline solution, w/v, 0.1 ml/10 g body weight). Mice were sacrificed by cervical dislocation 45 min after application of phenol red. After dissected free from adjacent organs, the trachea was removed from the thyroid cartilage to the main stem bronchi and put into 2 ml normal saline immediately. After ultrasonic for 15 min, 2 ml $NaHCO_3$ solution (5%, w/v) was added to the saline and optical density of the mixture were measured at 558 nm using UV-7501 UV–vis spectrophotometer (Wuxi Keda Instrument Co., Ltd., China).

2.7. NMR measurements

Dried extract (50 mg) was weighed into 2 ml centrifuge tube and added 800 μl mixture (1:1) of CD_3OD and KH_2PO_4 buffer in D_2O (adjusted to pH 6.0 with 1 N NaOD) containing 0.05% TSP and sonicated for 20 min. After centrifuging for 15 min at 13,000 rpm, the supernatant (600 μl) was transferred into a 5 mm NMR tube for NMR analysis.

^{1}H NMR, 2D J-resolved and ^{1}H–^{1}H-correlated spectroscopy (COSY), heteronuclear multiple bonds coherence (HMBC), heteronuclear single quantum coherence (HSQC) experiments were recorded at 25 °C on a Bruker 600-MHz AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. CD_3OD was used for internal lock purposes. Each ^{1}H NMR spectra consisted of 64 scans requiring 5 min acquisition time with the following parameters: 0.18 Hz/point, pulse width (PW) = 30° (12.7 μs), and relaxation delay (RD) = 5.0 s. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline-corrected, and calibrated to TSP at 0.00 ppm. Two-dimensional J-resolved NMR spectra were acquired with 2.0 s relaxation delay using 16 scans and spectral widths of 10,000 Hz in F2 and 78 Hz in F1. J-resolved spectra were tilted by 45° and symmetrized about F1. The COSY spectra were acquired with 2.0 s relaxation delay, 5411 Hz spectra width in both dimensions. The window function for COSY spectra was sine-bell (SSB = 0). The HMBC spectra were obtained with 1.5 s relaxation delay, using 8012 Hz spectral width in F2 and 133,200 Hz in F1. For HSQC spectra, the 1.5 s relaxation delay was used, 6009 Hz spectral width in F2 and 108,000 Hz in F1. All 2D spectra were calibrated at 0.00 ppm to TSP.

2.8. Data analysis

The ^{1}H NMR spectra were processed using MestReNova (version 5.2.5, Mestrelab Research, Santiago de Compostella. Spain). The spectra region between $\delta = 0.50$ and 10.02 ppm was divided ("bucketed") into 228 regions of 0.04 ppm, and the signal intensity in each region was integrated. The spectra were normalized to the integral of the entire processed spectrum in Microsoft Excel before exporting to the software. The regions of δ 4.70–5.02 and δ 3.30–3.38 were excluded from the analysis because of the residual signal of HDO and CHD_2OD, respectively. All spectral data were mean centered and scaled to unit variance, then analyzed by PCA