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Chemical Fingerprinting of *Tussilago farfara* by RP-HPLC and Hierarchical Clustering Analysis

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The aim of this study was to establish a method for extraction and chemically fingerprinting of extracts of *Tussilago farfara* and to apply this method to 12 *T. farfara* samples collected from different regions in China. RP-HPLC with gradient elution was performed on rutin. Comparing with the chromatograms, it showed that the samples were divided into two groups. Thirteen characteristic peaks were found in the extracts of all samples. Peak at retention time of 17.45 min was identified as rutin. Hierarchical clustering analysis showed the similar results as the visual comparison. The RP-HPLC method allowed it simple to identify and compare *T. farfara*. This was the first report on hierarchical clustering analysis of *T. farfara*.

Keywords: Chemical fingerprinting, *Tussilago farfara*, Hierarchical clustering analysis, Characteristic peaks.

INTRODUCTION

Tussilago farfara, a member of the compositae family and locally known as "Kuandonghua" in China, is an important traditional Chinese medicine[1]. Many compounds had been isolated from *T. farfara*, such as caffeoylquinic acid, hyperoside, rutin, quercetin and kaempferol[2,3], phenolics, mucopolysaccharides and water-soluble polysaccharides[4,5]. Rutin had been reported to be biologically active to inhibit platelet aggregation and aldose reductase activity[6,7], decrease capillary permeability, improve circulation and present antiinflammatory activity in some animal and in vitro model[8,9]. Its structure was shown in Fig. 1. Recent studies showed rutin could help prevent blood clots, so it could be used to treat patients at risk of heart attacks and strokes[10]. *T. farfara* had been used in many traditional Chinese medicine (TCM) prescriptions, such as Juhongwan, Chuanbeixueligao, *etc.*[11]. Due to the increasing demand of *T. farfara*, adulteration with other similar herbs was frequently encountered in the marketplace. However, to our knowledge, there was still no report about the chemical differences between *T. farfara* and its similar herbs. One common method of quality control on *T. farfara* was to quantify marker compounds[12]. Although the method had been widely used, the chemical markers cannot guarantee it efficacy. The herbs were collected at different times and planted in different regions, causing the difference in the types and quantities of chemical components[13]. It was difficult to evaluate a sample if only depending on some marker compounds. Although HPLC fingerprinting analysis of *T. farfara* had been reported[14], their extraction and analysis methods provided incomplete fingerprints of *T. farfara* extraction.

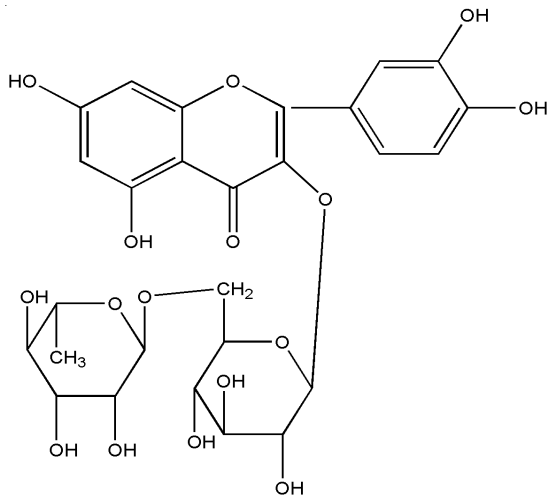

Fig.1. Chemical structure of rutin

In order to establish a new and simple HPLC method for the identification and quality evaluation of *T. farfara*, the chromatograms of extracts of samples from different regions were compared visually and analyzed by hierarchical clustering analysis.

EXPERIMENTAL

Twelve samples were collected from different regions in China. All samples were identified by Professor Quan De Hu in Jilin Agricultural University (Table-1).

TABLE-1
COLLECTION OF *T. farfara* IN DIFFERENT REGIONS

Sample no	Source	Collection time
1	Shaanxi	07.09.20
2	Shaanxi	07.09.20
3	Inner Mongolia	07.09.20
4	Henan	07.09.20
5	Hebei	07.09.20
6	Shanxi	07.09.20
7	Gansu	07.09.20
8	Linjiang, Jilin	07.09.30
9	Linjiang, Jilin	07.10.10
10	Linjiang, Jilin	07.10.20
11	Linjiang, Jilin	07.10.30
12	Linjiang, Jilin	07.11.09

The reference standard rutin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP). HPLC grade acetonitrile and AR grade methanol were purchased from Fisher Scientific (USA). Distilled water was obtained using a Synergy Purification System.

Chromatographic conditions: All HPLC analyses were performed using an Agilent 1100 series HPLC chromatograph with diode-array detector. A Diamonsil™ 5 µm column (25 cm × 4.6 mm i.d.) was used for chromatographic separations. The mobile phase was 0.4 % phosphoric acid solution (A) acetonitrile (B) system. The gradient elution was shown in Table-2. The column temperature was 35 °C and the injection volume was 20 µL. The UV detector was set at 217, 296 and 327 nm.

TABLE-2
TIME-TABLE OF GRADIENT ELUTION FOR HPLC ANALYSIS

Time (min)	CH_3CN (%)	0.4 (%) H_3PO_4 (%)	Flow (mL/min)
0	10	90	1
5	10	90	1
10	20	80	1
32	27	73	1
33	70	30	1
52	90	10	1
55	100	0	1

Sample preparation: 2 g of ground plant material was weighed and extracted with 75 % ethanol, ethyl acetate and *n*-butyl alcohol in an ultrasonic apparatus, each extraction time was set at 20, 30, 40 and 50 min, respectively. The extracts were filtered and evaporated under vacuum and reconstituted with 10 mL methanol. 2 mL was filtered through a 0.45 µm Nylon filter for analysis.

Data analysis: Similarity analysis was performed by similarity evaluation system for Chromatographic Fingerprint of TCM (2004 B version), which was recommended by SFDA of China. The software quantified the similarity indexes among different chromatograms by calculating the correlative coefficient and/or cosine value of vectorial angle[15,16]. In the present study, all of the results were calculated by these two calculated indexes.

The hierarchical clustering analysis (HCA) of samples 1-12 was performed using DPS7.05 software[17]. A method called average linkage between groups was applied and Euclidean distance was selected as the measurement[18].

Method validation: The intra-day and inter-day precision for sample 11 was performed on day one and next four consecutive day. The relative standard deviation (RSD) ranges of the retention time and peak area of the reference peak in the Sample 11 were obtained for intra-day and inter-day analysis. The recovery test was performed using the method of standard addition. Using rutin as a target in the sample 11, the sample11 was spiked with the high, intermediate and low levels of standard solution. The recovery was calculated by comparing the determined amount of those standards with the added amount originally. The stability was assessed by analyzing the same sample solution at 0, 2, 4, 8, 12, 24 h after extraction. The RSD ranges of the retention time and peak area of the reference peak in the extract was also determined.

RESULTS AND DISCUSSION

All the 12 samples were confirmed according to the descriptions in Chinese Pharmacopoeia. The physical appearances of Samples 1-12 were identical to the descriptions of *T. farfara* in Chinese Pharmacopoeia.

The absorption spectrum peaks of most of the components extracted by ethyl acetate or *n*-butyl alcohol extract from *T. farfara* were found to be lower than that by 75 % ethanol extract (Fig. 2). Some high polarity components typically eluting between 0 and 20 min were detected in 75 % ethanol extract, not in the ethyl acetate or *n*-butyl alcohol extract. Hence, to extract and detect as many components as possible, 75 % ethanol was selected as extraction solvent. Fig. 3 showed that the

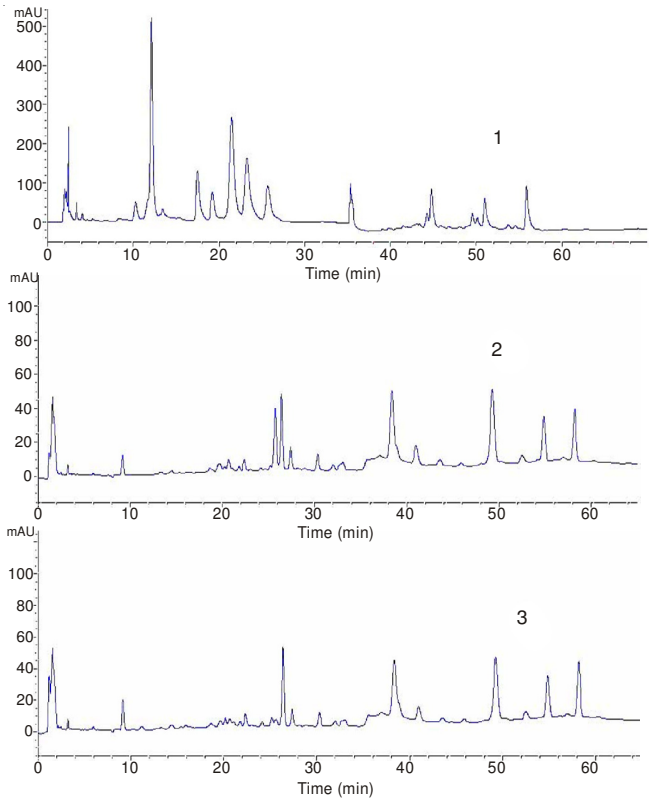

Fig. 2. HPLC chromatogram of different extraction solvent systems. (1: 75 % ethanol, 2: ethyl acetate 3: *n*-butyl alcohol)

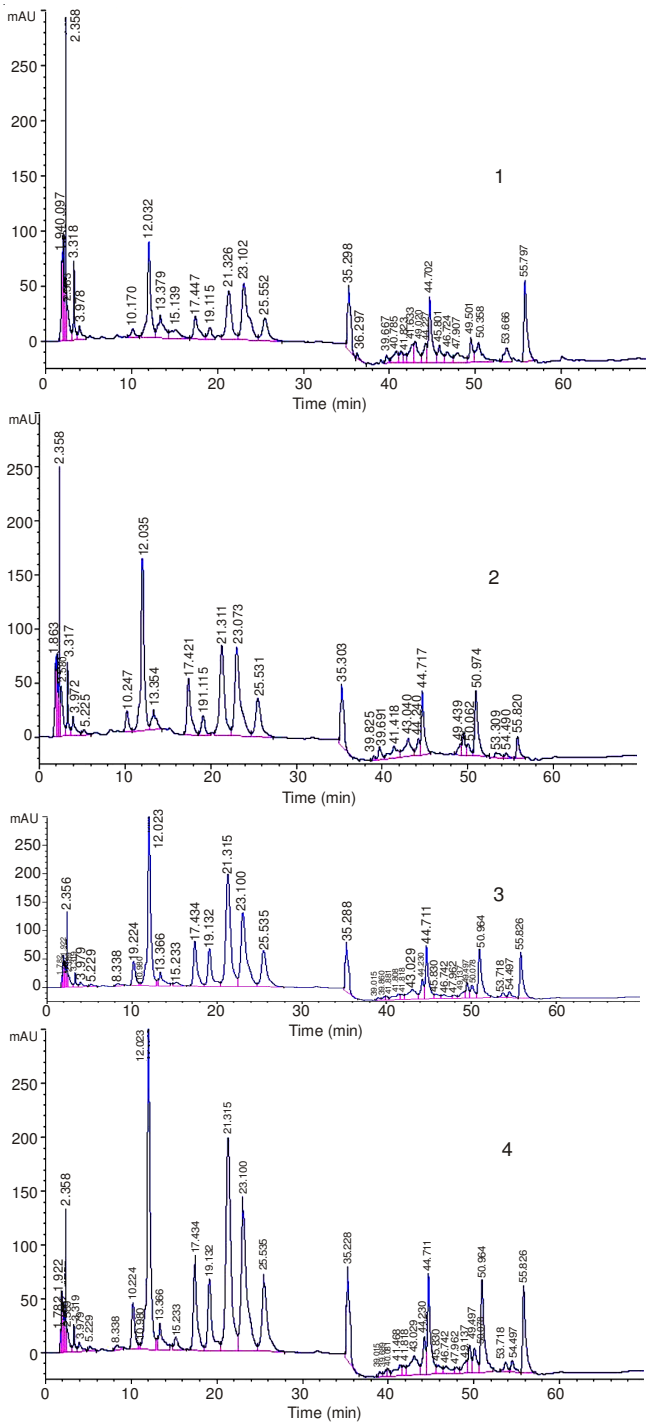

Fig. 3. HPLC chromatogram of different extracting time. (1:20, 2:30, 3:40 and 4:50 min)

number of HPLC absorption spectral peaks in 20 and 30 min extraction was less than those in 40 min and almost unchanged after 40 min. So the extraction time was set at 40 min. DAD full scan (190-600 nm) was used and 3 wavelengths (217, 296 and 327 nm) were specified, because many constituents were detected under these three wavelengths. It was found that there were more constituents of measurable levels at wavelength 217 nm and rutin had maximum absorbance at 217 nm. Hence, the wavelength 217 nm was used for HPLC. Peak of the chemical marker rutin was assigned in the HPLC chromatograms by comparing individual peak retention times. Peak at retention time17.45 min was identified as rutin (Fig. 4).

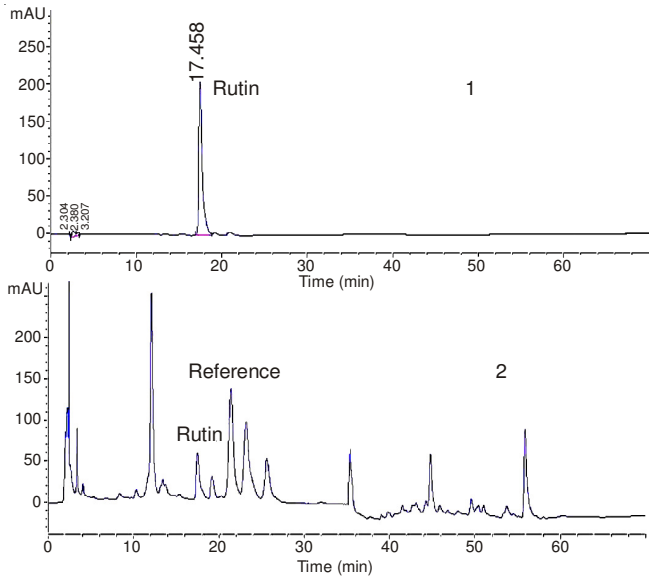

Fig. 4. HPLC chromatograms of 1: rutin reference substance, 2: extract from sample 2

Standardization of fingerprint: The peak corresponding to rutin was found in the HPLC chromatograms of all samples. The chromatographic peaks in different samples with the same relative retention time were defined as the common peaks. The peaks which were too close to the solvent peak (retention time 5 min) were excluded from the list of common peaks. Thirteen peaks were determined to be common peaks and numbered from 1 to 13 (Fig. 5). The peak corresponding to rutin was No. 4 peak in 13 peaks. Each of these 13 peaks contributed >1 % to the total peak area. In Samples 1-12, the area sum of all the common peaks accounted for more than 90 % of the total area of all the observed peaks in the chromatograms. Among the common peaks, peak at retention time of 21.8 min was designated as the reference peak for relative retention time and peak area calculation. Because it was a strong single peak in the middle of the chromatograms of all samples[19]. The points of the relative retention time's superposition is good and there is no points out of the curve and the results of peaks matched well[20]. The relative retention times of the 13 peaks were 10.31, 12.01, 13.51, 17.45, 18.91, 21.8, 22.41, 25.1, 43.9 44.3, 49.3, 51.3 and 55.9 respectively. They could serve as characteristic peaks for identification of *T. farfara*.

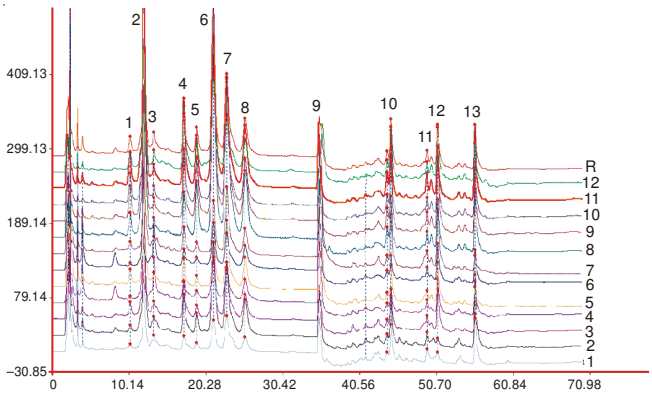

Fig. 5. Overlaid HPLC chromatograms of extracts of Samples 1-12

Hierarchical clustering analysis: Based on the common peaks in 12 samples, 13 common peaks were selected. With respect to the reference peak at retention time 21.8 min, 13 common peaks were selected and the relative retention times of these constituents were calculated. The relative areas of 13 characteristic peaks were calculated based on their ratios to the reference peak. Relative areas of the 13 peaks of samples 1-12 formed a 13 × 12 matrix. Distances among the 12 samples were calculated using the DPS7.05 software. The results of Hierarchical clustering analysis (HCA) were shown in (Fig. 6). It was reasonable that all samples could be divided into two clusters: Samples 1-7 and 12 in Cluster one and Samples 8-11 in Cluster two. HCA provided a quantitative comparison of the samples. In this study, the samples, collected from Shaan' xi, Inner Mongolia, He'nan, He'bei, Shan'xi, Gan'su, showed the similar results. These samples could come originally from the same region. In this study, a concentration of rutin was found to be high in all samples. This suggested that assessing the quality of *T. farfara* using marker compounds was recommended preliminary and not single as evaluation index.

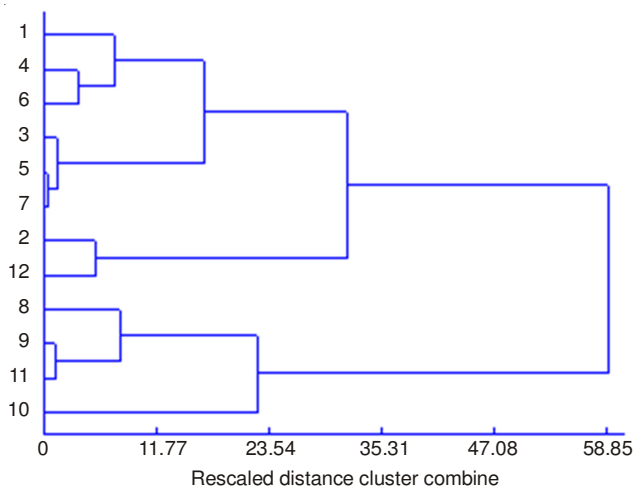

Fig. 6. Cluster analysis of HPLC chromatogram of *T. farfara*

Precision and stability analysis: In order to confirm the repeatability of the extraction technique, HPLC method and HCA, sample 11 was chosen for replicate analysis. The sample was extracted in duplicate and each extract was injected three times. Intra-day analysis was assessed by replicate injections (5 times) of the extract of samples 11. Inter-day analysis was also assessed by replicate injections (3 times) of second extract of sample 11 in 4 consecutive day after the day of extraction. The RSD ranges of the retention time and peak area of the reference peak in sample 11 was 0.23, 1.08 % for intra-day analysis and 0.36, 1.55 % for inter-day analysis. In recovery test, using rutin as a target, the sample11 was spiked with the high, intermediate and low levels of standard solution. Comparing the determined amount of those standards with the amount originally added, the recovery rate was determined to be 94.4 and RSD < 3 %. This suggested that the extraction method, HPLC analysis and HCA method was suitable for differentiating *T. farfara*. For stability analysis, the extract of sample 11 was analyzed by HPLC again 2, 4, 8, 12 and 24 h after extraction. The results indicated that the RSD of the sample 11 was less than 1.614 %, indicating the sample solutions were stable within 24 h.

Conclusion

Based on RP-HPLC, we developed the chromatographic fingerprinting method. After the determination of 13 common peaks and compound marker (rutin), the results showed that the method was reliable and sensitive. Using both common peaks and compound marker ensured the accuracy of the identification of *T. farfara*. Comparing with the current quality standards, the method can provide more information and improve the overall quality of *T. farfara*.

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