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Quality consistency evaluation of *Isatidis Folium* combined with equal weight quantified ratio fingerprint method and determination of antioxidant activity

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ABSTRACT

Isatidis Folium has been known as a valuable traditional Chinese medicine for thousands of years. Little attention, however, has been paid to its quality control. The aim of the present study was to establish a novel strategy to monitor and assess the quality consistency of *Isatidis Folium*. First, 20 samples were separated and identified simultaneously by high-performance liquid chromatography in an effective, quick and sensitive way. Then, Single-wavelength fingerprint was fused into multi-wavelength fingerprints to show fingerprints' information thoroughly. The similarity analyses of fingerprints were performed by equal weight quantified ration fingerprint method in terms of qualitative and quantitative aspects. The evaluation result showed that 20 batches of samples were classified into different grades. In addition, the relationship between fingerprints and antioxidant activity were investigated by partial least-squares model, which offered significant medicinal efficacy information for quality control. This comprehensive strategy provided a valuable reference for *Isatidis Folium* to ameliorate their quality control.

1. Introduction

Traditional Chinese medicine (TCM) has received an increasing acceptance for over a millennium for the prevention, treatment and diagnoses of various diseases due to exact clinical efficacy and complete theoretical system [1–3]. However, because of the complexity of TCM which includes multi-target, multi-level, and multi-ingredient that usually display synergistic effects, the quality control (QC) of TCM has become more difficult than chemical drugs [4–7]. Moreover, the chemical content of herbs varies greatly due to the difference in cultivation areas, climatic conditions, storage, harvest time and pretreatment [8, 9]. Obviously, it is insufficient for controlling the quality of TCM by identification or content determination of one or several markers. Therefore, the fingerprint technique, especially chromatographic fingerprint, has been recognized by World Health Organization (WHO), US Food and Drug Administration (FDA), China Food and Drug Administration (CFDA) and European Medicines Agency (EMA) [10–13] as a convenient and efficient technology of quality inspection, which includes thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [7, 12, 14, 15]. Among them, HPLC is a popular method used in the QC of TCM due to its high separation efficiency and satisfactory detection sensitivity [16, 17]. In the previous study, samples were mostly analyzed by HPLC in a single wavelength, which is unable to show the maximum absorption of different chemical components. And so, this study was aimed to fuse single wavelength fingerprint into multi-wavelength fingerprints, which could enhance the discernibility of fingerprints, monitor the difference of response values of different components, and compensate for the shortcomings of single-wavelength fingerprint [18, 19]. In the fingerprint assessments, equal weight quantified ration fingerprint method (EWQRFM) has been applied to evaluate the similarity of samples in terms of qualitative and quantitative aspects based on ratio qualitative ($\boldsymbol{S}_r$), ratio quantitative similarity ($\boldsymbol{P}_r$) and α.

Isatidis Folium, derived from the dry leaves of *Isatia indigotica* Fort., is applied for the treatment of various diseases such as measles, acute infectious hepatitis, acute gastroenteritis, acute pneumonia, influenza, dysentery and epidemic encephalitis B [20–22]. Most published reports

Abbreviations: AD, adenosine; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EWQRFM, equal weight quantified ratio fingerprint method; PCA, principal component analysis; P_r, equal weight quantitative similarity; PLS, partial least square; QC, quality control; RFP, reference fingerprint profiles; RPA, relative peak area; RRT, relative retention time; RSA, radical scavenging ability; RT, rutin; S_r, equal weight qualitative similarity; TCM, traditional Chinese medicine; UR, uridine

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have suggested that *Isatidis Folium* also improve anti-inflammatory and strengthen immunity [23]. In addition, the chemical compositions of *Isatidis Folium* have a great variety that includes alkaloids [24], organic acids, glycosides and flavonoids [20]. Actually, *Isatidis Folium* has been documented into the first section of the 2015's edition of Chinese Pharmacopoeia. However, only microscopic, TLC and assay of one makers by HPLC methods are prescribed for quality evaluation. Moreover, few reports have focused on the quality control of *Isatidis Folium*. Therefore, it is necessary for us to study the quality safety and efficacy of *Isatidis Folium*.

Reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2\cdot^-$) and hydroxyl radical (·OH) are harmful to the human body [25]. However, antioxidants can clear free radicals to protect living system from chronic diseases. Some studies also have shown that measles is associated with the generation of free radicals [26–29]. The important information encourages us to investigate the antioxidant ability of *Isatidis Folium*. On the one hand off-line DPPH radical scavenging assay was performed to research the antioxidant activities of *Isatidis Folium*; on the other hand, PLS model was constructed to explore the relationship between chemical constituents and chromatographic fingerprints.

In the present study, we established the multi-wavelength fusion fingerprints by HPLC-DAD and evaluated the quality consistency of 20 batches of *Isatidis Folium* samples through EWQRFM. An off-line DPPH radical scavenging assay was proposed for determining the antioxidant activities and PLS model was applied to explore the fingerprint-efficacy relationship in a simple way. It has been demonstrated that the present study provides a scientific way for the QC of *Isatidis Folium*.

2. Materials and methods

2.1. Chemicals and reagents

Ten batches of *Isatidis Folium* samples (labeled S1–S10) were provided by the manufacturer, and the remaining ten batches (labeled S11–S20) were purchased from pharmacies (Liaoning, China). Standard substances including UR, AD and RT were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPP, Beijing, China). The purity of standards was over 98%. Their molecular structures were showed in Fig. 1.

DPPH was obtained from Sigma-Aldrich (Steinheim, Germany). Methanol, ethanol and acetonitrile were HPLC-grade, they were obtained from Shandong Yuwang Industry (Shandong, China). Phosphoric acid (HPLC grade) were purchased from Tianjin Kermel Chemical Reagent (Tianjin, China). Deionized water was used for all the experiments.

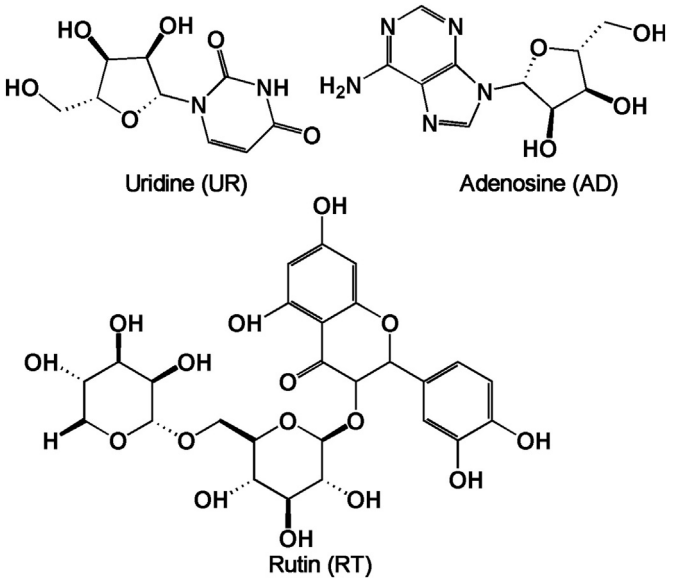

Fig. 1. The molecular structures of the three marker compounds.

2.2. Apparatus and analytical conditions

HPLC analysis was carried out on an Agilent 1100 HPLC system (Agilent Technology, USA), equipped with a G1315-DAD, a G1313A autosampler, a G1311A low pressure mix quaternary pump, a G1379A online degasser, and a HPLC system organizer. The gained data was performed on Agilent ChemStation.

To gain more chromatographic information, we optimized the analytical conditions. The chromatographic separation was performed on a COSMOSIL C_{18} column (4.6 × 250 mm, 5 μm). The mobile phase was made up of water-phosphoric acid (A, 1000:1, *v/v*) and acetonitrile-phosphoric acid (B, 1000:1, *v/v*). The gradient elution program of the mobile phase was as follows: 100% (A) in 0–3 min; 100–95% (A) in 3–9 min; 95–88% (A) in 9–22 min; 88–84% (A) in 22–30 min; 84–82% (A) in 30–45 min; 82–70% (A) in 45–60 min; 70–40% (A) in 60–80 min; 40% (A) in 80–120 min. The injection volume, and flow rate were carried out at 5 μL, 1.0 mL/min, respectively. The column temperature was maintained at 35 °C, the effluent was detected by DAD and wavelengths were set at 203 nm, 254 nm, 265 nm, 289 nm, 320 nm.

2.3. Preparation of samples and standard solutions

2.3.1. Sample solutions

Isatidis Folium samples, using a grinder, were ground into powder. Each powder sample was weighted accurately into a round-bottomed flask. 15 mL of ethanol (75%, *v/v*) was added into flask to extract the chemical substances by refluxing for 2 h, then the supernatant was percolated and remaining was extracted with 10 mL of ethanol (75%, *v/v*) for half an hour. The combined extracts were mixed and diluted with ethanol (75%, *v/v*) in a 25 mL as sample stock solution.

2.3.2. Standard solutions

Each standard substance (UR, AD and RT) was weighted and dissolved in absolute methanol as standard stock solutions. A mixed standard solution was prepared in methanol by mixing the individual standard stock solutions. Then mixed standard solution was diluted in a range of suitable concentration for the calibration curves.

All the sample and standard solutions were stored in a refrigerator at 4 °C and brought to room temperature before use, they were filtered through 0.45 μm Millipore filters (Beijing Sunrise T&D Company, China) before HPLC analysis.

2.4. The off-line DPPH antioxidant activity assay

DPPH which is a radical-containing compound is usually used to quantify the antioxidant activity of ingredients or extracts of TCM by spectrophotometry. It has a characteristic absorption at 517 nm [30, 31]. When a free radical scavenger is present, the DPPH radical receives electrons or hydrogen atom to form a stable DPPH-H compound, which result in its methanol solution to change from deep purple to yellow. The degree of discoloration is comparable to that of antioxidants. Rapid quantitative analysis can be performed with a spectrophotometer. The smaller absorbance values, the stronger the antioxidant activity of samples.

A stock solution (64 μg/mL) of DPPH was prepared with methanol before the experiments and protected from light. A series of proper concentration sample solution were mixed with 2 mL DPPH solution and placed 40 min from light. Radical scavenging ability (RSA) was expressed by the Eq. (1):

$$RSA = (A_{blank} - A_{sample})/A_{blank} \times 100\% \quad (1)$$