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Application of HPLC fingerprint based on acid amide components in Chinese prickly ash (*Zanthoxylum*)

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ABSTRACT

In order to establish a high performance liquid chromatography (HPLC) fingerprint of Chinese prickly ash (*Zanthoxylum*) based on acid amide components, 38 specimen were collected from Western China. Similarity analysis, hierarchical clustering analysis (HCA) and principal component analysis (PCA) were carried out to analyze the obtained fingerprints. Furthermore, uncertainty and reliability values of the established HPLC fingerprints were evaluated. Similarity values ranged between 0.587 and 0.999 for specimen of *Zanthoxylum bungeanum* Maxim. and between 0.991 and 0.999 for specimen of *Zanthoxylum armatum* DC. 17 samples from different habitats were classified by HCA into three groups, i.e. Group I (8 samples), Group II (6 samples) and Group III (3 samples) and were divided into three clusters by PCA. The compound of peak 6 was identified as hydroxyl-*β*-sanshool. The characteristic compounds of peaks 5, 7, 8, 10, 12 and 13 were tentatively identified by LC–MS as other acid amide compounds. As for credibility analysis, the results showed that the values of macroscopic qualitative reliability and macroscopic quantitative reliability of the reference chromatogram fingerprint were more than 0.9772. Statistical analysis indicated that the HPLC fingerprint technology may be applied for quality assessment and classification of *Zanthoxylum*.

1. Introduction

Chinese prickly ash (*Zanthoxylum*) represents a woody plant with a variety of applications and a long cultivation history in China. It is used as one of the traditional condiments in Chinese cooking culture and Sichuan cuisine, offering a unique numb taste. Chinese prickly ash comprises of more than 200 species of *Zanthoxylum* distributed worldwide, with the main varieties *Zanthoxylum bungeanum* Maxim. and *Zanthoxylum armatum* DC. (Li et al., 2016). In recent reports it was demonstrated that *Zanthoxylum* is rich in nutrients (Li et al., 2014), including volatile oils (Wang et al., 2010), acid amide phenol components (Kumar et al., 2014), alkaloids (Wansi et al., 2016), ketones (Wang et al., 2014), etc. Recent reports have shown that acid amide compounds are responsible for the numb taste of Chinese prickly ash. The main components of acid amide compounds comprise of α-sanshool ($C_{16}H_{25}NO$), *β*-sanshool ($C_{16}H_{25}NO$), γ-sanshool ($C_{18}H_{27}NO$), hydroxyl-α-sanshool ($C_{16}H_{25}NO_2$), hydroxyl-*β*-sanshool ($C_{16}H_{25}NO_2$), hydroxyl-γ-sanshool ($C_{18}H_{27}NO_2$) or the isomers of acid amide compounds (Wang et al., 2011; Artaria et al., 2011). The concentration of acid amide compounds in *Zanthoxylum* is of particular interest since it could serve as an indication for the internal quality of prickly ash. The quality of *Zanthoxylum* is considered to be influenced by the growth region, climate and other environmental factors. Therefore, it is critical to establish an effective method to classify the quality of *Zanthoxylum*.

A variety of literature references can be found on the quantitative determination of the chemical components of *Zanthoxylu*m, including volatile oils (Waheed et al., 2011), polyphenols (Nooreen et al., 2017), etc. Moreover, sensory evaluations have been used for the classification of *Zanthoxylum*. Sensory indexes typically include color, smell and impurities. Furthermore, the content of volatile oils represents the only chemical index in forestry industry standards of China, used to evaluate the quality of *Zanthoxylum* (SAC, 2013). Unfortunately, the index lacks an integrity assessment method and national standard for the identification and classification of Chinese prickly ash.

The chromatographic fingerprint method is regarded a comprehensive qualitative and quantitative approach for the purpose of species identification, quality asessment, and ensuring the stability and consistency of herbal drugs (Xie et al., 2006). The chromatographic fingerprint can be obtained by spectroscopic or chromatographic techniques (Deconinck et al., 2013) and the use of chromatographic

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fingerprints is accepted by the World Health Organization (WHO) as a strategy for the identification and quality control of herbal preparations (Sun and Chen, 2012). Accordingly, *Zanthoxylum* may not only be used as a seasoning in food preparation, but may also play a vital role in pharmaceutical industry and medicine (Verma and Khosa, 2010). Therefore, *Zanthoxylum* is now known as a condiment type providing both medical applications and food (Tian et al., 2016). The evaluation of the quality of *Zanthoxylum* using the chromatographic fingerprint technology may provide a useful tool for quality assessment and control.

The high performance liquid chromatography (HPLC) fingerprint technology is widely recognized as an effective method in the quality control of Traditional Chinese Medicine (TCM) (Li et al., 2015). A reference chromatogram fingerprint (RFP) could be established by calculating the mean values of the chromatographic fingerprints of samples. However, there is an obvious disadvantage of RFP often explained by uncertainty and reliability problems (Sun and Wang, 2011). The availability of RFP has a great influence on the assessment credibility of the sample quality but obviously, it is necessary to assess and define the uncertainty and reliability of the chemical fingerprint technology first. Sun and Wang have studied the HPLC fingerprints of a *Shuanghuanglian* capsule and developed an evaluation method to study uncertainty and reliability for traditional Chinese medicine fingerprints. The results showed that the qualitative and quantitative reliability of the *Shuanghuanglian* capsule-RFP were more than 0.981 and 0.938. Importantly, the evaluation method of reliability could accurately reflect the variances in TCM fingerprints and the method of uncertainty and reliability would exactly estimate the degree of reliability of qualitative and quantitative information including both RFP and single sample fingerprint (Sun and Wang, 2011). Before this method was developed, only a few research groups have focused on chromatogram fingerprints of *Z. bungeanum* Maxim. and *Z. armatum* DC. using GC (gas chromatography) or HPLC (Waheed et al., 2011) as analytical tools. Song (Song and Liu, 2012) and Du (Du et al., 2016) established the HPLC-FPs of Chinese prickly ash. Both labs analyzed the similarity of detected samples. In addition, cluster analysis has been used to observe the classification of detected samples. The analytical results were consistent with the distribution of actual origin. Moreover, the findings of both research groups demonstrated that the HPLC-fingerprint could effectively distinguish *Z. bungeanum* Maxim. and *Z. armatum* DC. However, to the best of our knowledge, the uncertainty and reliability of chromatographic fingerprints and quality of the detected samples has not yet been analyzed and evaluated.

In this paper, HPLC was used to establish a fingerprint method in order to determine multi-components by a single reference standard. The method was then adopted to investigate the chemical profiles and active components of *Zanthoxylum*. (Yang et al., 2017). Based on the fingerprint data, the similarity values of 17 *Zanthoxylum* specimen collected from Western China were calculated. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were used to analyze the quality of *Zanthoxylum*. Moreover, LC–MS has been used to confirm the identity of acid amide compounds. Furthermore, uncertainty and reliability assessments were applied to evaluate the credibility of HPLC fingerprints of *Zanthoxylum*.

2. Materials and methods

2.1. Materials and chemicals

Thirty eight seedcase of *Zanthoxylum* specimens were collected from different areas of Western China in November, 2016. The origin of all samples is shown in Table 1. The samples included 11 specimen of *Z. bungeanum* Maxim, 6 specimen of *Z. armatum* DC. and 21 specimen of *Z. bungeanum* Maxim. from Hanyuan district of Sichuan. The standard hydroxyl-*β*-sanshool was purchased from Chengdu Chroma-Biotechnology Co., Ltd. (Chengdu, China, purity ≥98.00%). HPLC grade methanol and acetonitrile were purchased from Chengdu Jere Technology Co., Ltd. (Chengdu, China). Deionized water was prepared using a Milli-Q System (Millipore, USA).

2.2. Chromatographic conditions

HPLC fingerprint analysis was performed on an Agilent 1260 series HPLC-DAD system (Agilent, USA). The chromatographic separation was performed on an Agilent Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm) using acetonitrile (A) and deionized water (B) as mobile phase with a flow rate of 0.8 mL/min. The gradient program was set as follows: 0 min, 35% A; 5–10 min, 35%–40% A; 25–50 min, 45%–65% A; 55–60 min, 90%–35% A. The column temperature was maintained at 35 °C, the detection wavelength was set to 268 nm, the injection volume of each sample as well as standard solution was 2 μL, and the run time was 60 min.

All detected chromatographic peaks were identified and confirmed using a 1260 LC-6120MSD system. MS spectra were recorded in the mass range of m/z = 100–1000, using electrospray ionisation (ESI) as the ionisation source in positive ion-switching mode. The mass spectrometer settings were as follows: capillary voltage 3.8 kV, source temperature 150 °C, drying gas temperature 350 °C, drying gas flow 12.0 L/h and nebulizer pressure 35 psig. Other operating conditions were used as recommended by the instrument manufacturer. All data acquisition and processing steps were performed using ChemStation Edition C.01.05 SP1 [50] software.

2.3. Sample preparation

The seedcase of *Zanthoxylum* were shattered by a universal high-speed smashing machine (FW-100, Beijing Zhongxing Weiye Instrument Co., Ltd., China) and homogenized by a 60-mesh screen. 5.0 000 g of each sample was transferred into separate 250 mL conical flasks (with stoppers) and 50 mL of methanol was added. The conical flasks were placed in an ultrasonic cleaner (KH-50B, Kunshan Ultrasonic Instrument Co., Ltd., China) and subjected to ultrasonication for 30 min at 30 °C. As soon as the sample solutions cooled down to room temperature, methanol was added to supplement the lost weight and the mixtures were shaken well before filtration through a 0.22 μm membrane filter.

The standard hydroxyl-*β*-sanshool was weighed and dissolved in methanol for a final concentration of 2 mg/mL. The solution was filtered through a 0.22 μm membrane filter prior to injection into the HPLC system.

2.4. Method validation

The limits of detection (LODs) for hydroxyl-*β*-sanshool using the chromatographic conditions described above were determined at a signal-to-noise (S/N) ratio of 3. The precision of the method was determined by replicating the HPLC injections of the same solution (Sample 6, *Hanyuan Dahongpao*) five times continuously within the same day. The stability of the sample solutions was assessed by analyzing the same sample solution stored at room temperature for 0, 2, 4, 8, 16 and 24 h. Five individual solutions of the same sample were analyzed to confirm repeatability.

2.5. Data analysis

2.5.1. Identification of the characteristic peaks and similarity analysis

Characteristic peaks were present in all chromatograms of detected samples. The peak retention times (t_R) were the same as the peaks of the reference fingerprints, regarded as characteristic peaks. Identification of the characteristic peaks and similarities of the fingerprint data were performed using the professional software *Similarity Evaluation System for the Chromatographic Fingerprint of Traditional Chinese Pharmacopoeia*