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Short communication

Fingerprint and multi-ingredient quantitative analyses for quality evaluation of hawthorn leaves and Guang hawthorn leaves by UPLC–MS

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ABSTRACT

This paper aimed to evaluate the quality of hawthorn leaves and Guang hawthorn leaves by an UPLC–MS method from two aspects, fingerprint analysis and multi-ingredient quantification. Chromatographic separation was carried out on an UPLC system, the standardized characteristic fingerprints was established by Similarity Evaluation System for chromatographic fingerprinting of traditional Chinese medicine and cluster analysis. Eight components were simultaneously determined by mass spectrometry in multiple reaction-monitoring mode. The method was validated in terms of linearity ($R^2 > 0.9971$), intraday and interday precision (RSD < 2.0%), repeatability (RSD < 2.3%), stability (RSD < 2.5%) and recovery (96.2–103.8%). The developed method was successfully applied to the quality evaluation between hawthorn leaves and Guang hawthorn leaves, and there were differences in the component and the content, hawthorn leaves and Guang hawthorn leaves cannot substitute each other in clinical medication.

Introduction

Chinese medicine is a complex system composed of many ingredients and factors. The China Food and Drug Administration and European Medicines Agency have clearly denoted that appropriate fingerprint chromatograms can be applied to assess the quality consistency of Chinese medicine. Quantitative analysis of effective constituents is also considered as the most direct and important approach for quality control of Chinese medicine avoiding the deficiency of fingerprints in revealing the change of content of each component (Sharma et al., 2016; Wang et al., 2011a,b; Li et al., 2015a,b).

The hawthorn, a member of the Rosaceae family, which belongs to the genus *Crataegus* (Thomas et al., 2009). At present, the extracts from the fruits, leaves and flowers of hawthorn have been confirmed to possess therapeutic effects on the cardiovascular systems including cardiovascular protection, hypotensive activity, hypocholesterolaemic and hypolipidaemic effects (Li et al., 2015a,b; Claudia et al., 2016; Pahlavan et al., 2017; Greenfield, 2018; Gregory and Jeffery, 2018). Hawthorn leaf, known as a commonly used traditional Chinese medicine, there are two major species in China, named as hawthorn leaves and Guang hawthorn leaves. Hawthorn leaves, *Crataegus pinnatifida* var. *major* N.E.Br. or *Crataegus pinnatifida* Bunge, is recorded in the Pharmacopeia of People's Republic of China (2015 edition). Guang hawthorn leaves, *Malus doumeri* (Bois) A. Chev., Rosaceae, or varieties of *M. doumeri*, is included in the 1990 edition of Guangxi Traditional Chinese Medicine Standards. Compared with the hawthorn leaves, Guang hawthorn leaves are grown in the south of China, the leaf is large and strong in fiber. With that in mind, this may result in the difference in chemical composition and content between the two, and further affect the clinical efficacy. These indicate the need for a study to validate an analytical method for quality control of the hawthorn leaves and Guang hawthorn leaves in order to explore whether they can be replaced by each other in therapeutical applications.

In recent years, ultra performance liquid chromatography–mass spectrometry (UPLC–MS) has been widely used in the quality analysis of Chinese medicine because of its fast separation speed, high sensitivity and accurate determination (Liu et al., 2013; Yang et al., 2016a; Yang et al., 2016b; Yang et al., 2013). Multiple reaction-monitoring (MRM) is a tandem MS scan mode unique to triple quadrupole MS instrumentation that is capable of rapid, sensitive and specific quantitation of compounds in highly complex sample matrices (Nabila et al., 2013). Relevant compounds present only in insignificant amounts or accompanied by others with similar structures are difficult to be identified by UPLC alone. MS in the MRM

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mode for identification and quantification is more advantageous for chromatographic fingerprinting of traditional plant remedies.

Several approaches including capillary-zone electrophoresis (Geng et al., 2008), high-performance liquid chromatography (HPLC) (Mudge et al., 2016; Wang et al., 2011a,b) and HPLC–MS (Zhu et al., 2014) for the analysis of hawthorn leaves have been reported. However, there are no investigation concerning the simultaneous determination of epicatechin, chlorogenic acid, vitexin, hyperoside, vitexin-rhamnoside, vitexin-glucoside, rutin and shanyenoside A from hawthorn leaves. The reports about Guang hawthorn leaves are limited to thin layer chromatography analysis and determination of the flavonoid total content (include the technique for this quantification). In addition, the comparison on the chemical composition of hawthorn leaves and Guang hawthorn leaves has not been described.

Therefore, UPLC–MS method was applied to compare the quality of hawthorn leaves and Guang hawthorn leaves from the aspects of fingerprint and multi-ingredient quantitative analysis. This paper would provide a basis for rational use of the crude drugs in clinic by comparison of the type and content of hawthorn leaves and Guang hawthorn leaves.

Materials and methods

Acetonitrile and formic acid (Optima LC/MS, Fisher Scientific). Wahaha pure water (GB17323, Hebei). Other reagents were all of analytical grade. Epicatechin, chlorogenic acid, vitexin, hyperoside, vitexin-rhamnoside, vitexin-glucoside, rutin, Shanyenoside A (purity > 98%, Shanghai Winherb Medical Technology Co. Ltd) were used as external standards.

Hawthorn leaves (*Crataegus pinnatifida* var. *major* N.E. Br. or *Crataegus pinnatifida* Bunge, Rosaceae) were collected in 33 regions, north of China, 116°39′–123°45′ South; 33°44′–41°16′ West. Guang hawthorn leaves (*Malus doumeri* (Bois) A. Chev. or varieties of *M. doumeri* were collected in eight regions, south of China, 111°05′–113°08′ South; 23°39′–25°09′ West. The specimens were identified by Jianxin Dong, from the Hebei Normal University for Nationalities, and preserved in the Institute of Traditional Chinese Medicine of Chengde Medicinal University (herbarium number: 20150921).

Chromatographic separation was conducted on an UPLC system (Waters Co., Milford, MA, USA) consisting of a binary pump, an online degasser, an auto plate sampler, a column oven, and a diode array detector. The chromatographic run with injection volume of 2 μl were carried out on a CORTECS™ UPLC® C_{18} column (100 × 3.0 mm, 1.6 μm; Milford, MA, USA) by maintaining oven temperature 30°. The detection wavelength was set at 320 nm. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid aqueous solution (B) in gradient elution mode, and the flow rate was 0.2 ml/min, the gradient of acetonitrile (A) was changed as follows: 0–4 min, 12%; 4–7 min, 12–17%; 7–14 min, 17%; 14–16 min, 17–25%; 16–25 min, 25–40%; 25–28 min, 40–80%; 28–30 min, 80%.

All 41 batches of sample solutions were injected into UPLC under the above chromatographic conditions. The average chromatogram of the selected batches was regarded as the standardized characteristic fingerprint of hawthorn leaves. Peaks existing in all chromatograms of the samples were assigned as "common peaks" (Tang et al., 2014; Duan et al., 2012). Usually average chromatogram can be affected by the natural deviation of individual plant chemical profiles, this work adopts cluster analysis to eliminate abnormal samples, then the samples with stable chemical composition were selected to re-establish standardized characteristic fingerprint.

Multi-ingredient quantification was carried out on an ABSciex 5500 QTRAP mass spectrometer (Foster City, CA) equipped with an electrospray ionization probe in negative ion MRM mode. This technique needs to be implemented with collision-induced dissociation (CID). The parent ion is detected in the first quadrupole (Q1), the second quadrupole (Q2) is the intermediary stage filled with inert gas where CID of the parent ion occurs, and the resultant ion-fragments are detected in the third quadrupole (Q3). The m/z detected in Q1 and Q3 is set to: epicatechin at 289.1/245.0, chlorogenic acid at 353.1/191.0, vitexin at 431.1/311.0, hyperoside at 463.1/300.1, vitexin-rhamnoside at 577.1/413.1, vitexin-glucoside at 593.2/413.1, rutin at 609.1/300.1 and shanyenoside A at 407.1/245.1. The best response of the analyte is under the following conditions: curtain gas 35, ion spray voltage −4500 V, source temperature 550°, ion source gas (1) 55, ion source gas (2) 60, interface heater on, collision gas medium. The data acquisition were controlled by Analyst software (version 1.6.3, ABSciex). The external standard method was used to determine the content of each component in 41 samples.

For preparation of the sample, the dry leaves were ground into powder and filtered though a 60-mesh sieve. 500 mg of sample powder was ultrasonically extracted with 25 ml 60% methanol for 30 min, and then centrifuged for 10 min at 9345 × *g*, finally filtered through a 0.22 μm micro-porous membrane (Supplementary Table 1).

The stock solution of epicatechin 0.653 μg/ml, chlorogenic acid 0.640 μg/ml, vitexin 0.592 μg/ml, hyperoside 0.572 μg/ml, vitexin-rhamnoside 1.382 μg/ml, vitexin-glucoside 1.232 μg/ml, rutin 0.912 μg/ml, and shanyenoside A 0.72 μg/ml were prepared with methanol individually. Standard solutions were prepared by stepwise dilution with methanol for validation of the method.

The linearity was determined by a series of standard solutions of eight compounds. Each calibration curve was injected in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) are the corresponding concentrations when the signal-to-noise ratio is 3:1 and 10:1 (Supplementary Table 2).

The intraday precision was verified by low, medium, and high concentration mixed standard solutions three times a day. The interday precision, continuous measurements were taken three days, three times a day.

The repeatability test was verified by six copies of the same sample solution in parallel. In the stability test, the same sample solution was tested at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, respectively.

The recovery was determined by accurately adding eight corresponding standard compounds to 6 samples of the same sample

Table 1
Calibration curves, LOD, LOQ of the investigated compounds.

Compounds	Regression equation	R^2	LOD (ng/ml)	LOQ (ng/ml)
Epicatechin	$Y = 17.76X + 148.2$	0.9983	7	23.3
Chlorogenic acid	$Y = 35.71X - 24.84$	0.9973	6	20.0
Vitexin	$Y = 32.45X - 136.3$	0.9986	6	20.0
Hyperoside	$Y = 15.74X + 116.9$	0.9974	8	26.7
Vitexin-rhamnnoside	$Y = 24.46X + 183.5$	0.9986	14	46.7
Vitexin-glucoside	$Y = 21.65X + 54.74$	0.9971	12	40.0
Rutin	$Y = 18.31X + 249.6$	0.9982	9	30.0
Shanyenoside A	$Y = 17.43X + 31.23$	0.9977	7	23.3

LOD, limit of detection; LOQ, limit of quantification.

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