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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.

with known component content (Supplementary Table 3). The average recovery was estimated according to the following formula:

$$\text{recovery}(\%) = \left[\frac{\text{amount found} - \text{original amount}}{\text{amount spiked}}\right] \times 100\%.$$

Results and discussion

Different conditions including extraction method (ultrasonic, reflux, and soxhlet extraction), extraction solvent (ethanol and methanol), solvent ratio (50, 60, 70% methanol) and extraction time (20, 30, and 40 min) were optimized in order to seek the highest efficient extraction procedure with the highest yields of the above eight compounds. Finally, 60% methanol and ultrasonic extraction of 30 min were selected as the optimum conditions. Different types of columns were tested to reach an optimum separation on the Cortecs™ UPLC® C_{18} column, which can give the widest usable pH range (pH 1–12), superior low pH stability, and ultra-low column bleed for high sensitivity MS applications. Acetonitrile and 0.1% formic acid aqueous solution were determined as the most appropriate eluent with gradient elution due to the satisfactory resolution and acceptable peak parameters.

The linearity results for eight compounds are shown in the Table 1, and the data show a good linear correlation ($R^2 > 0.9971$). The LOD and LOQ values range from 6 to 14 ng/ml and 20 to 46.7 ng/ml, respectively.

In the intraday precision test, the RSD values of the peak areas of the eight components ranged from 0.2 to 1.7%. Interday precision assay obtained similar results with the range of 0.3–1.9%.

The repeatability test and the stability test, both exhibited RSD values of each component content were less than 2.5%. For the recovery results, recoveries were between 96.2 and 103.8% with RSD values ranged from 0.89 to 2.7%.

Under the same chromatographic conditions, the standardized characteristic fingerprints showed ten common peaks (tR = 2.59–12.2 min), with coefficient of similarity values (R^2) ranging between 0.808 and 0.977, were found in hawthorn leaves (Fig. 1A); while, twenty common peaks (tR = 2.59–28.6 min) with similarity values ranging from 0.937 to 0.977 were detected in Guang hawthorn leaves (Fig. 1B). Five common peaks were unambiguously identified as chlorogenic acid, vitexin, hyperoside, vitexin-rhamnoside and vitexin-glucoside. Before 12.3 min, the difference between the two fingerprints was not obvious. Starting from 16.1 min, the number of common peak in Guang hawthorn leaves reached 6, but none in the hawthorn leaves. The tR values of the common peaks are different, which indicate that there are differences in the characteristic components of the hawthorn leaves and Guang hawthorn leaves.

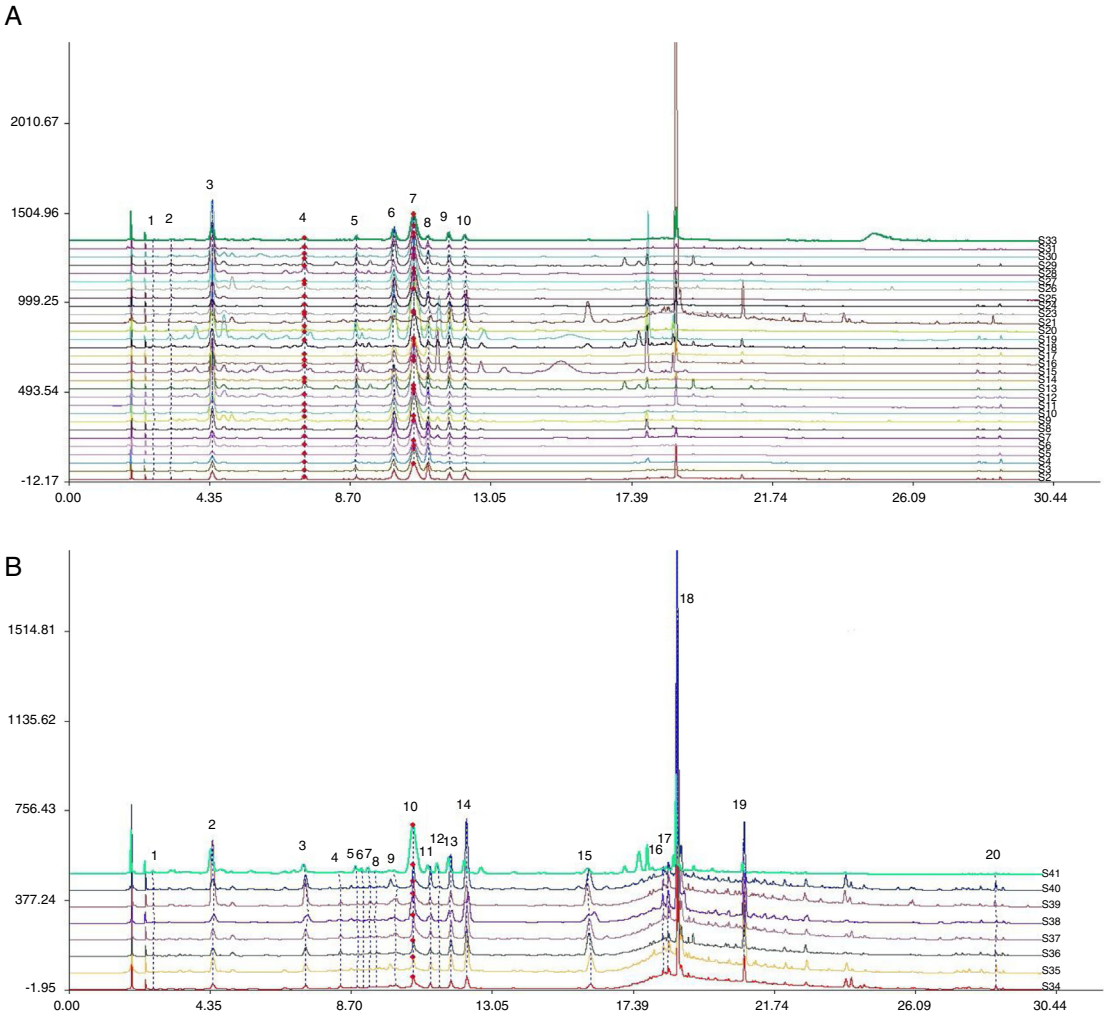

Fig. 1. The standardized characteristic fingerprints of hawthorn leaves (A) and Guang hawthorn leaves (B).
Note: common peaks, marked by 1–20 (chlorogenic acid-A3/B2, vitexin-glucoside-A6/B9, vitexin-rhamnoside-A7/B10, vitexin-A8/B11, hyperoside-A9/B13).

Table 2
The contents of the determination based on LC–MS methods of hawthorn leaves and Guang hawthorn leaves.

Origin(No.)	Epicatechin (mg/g)	Chlorogenic acid (mg/g)	Vitexin (mg/g)	Hyperoside (mg/g)	Vitexin-rhamnoside (mg/g)	Vitexin-glucoside (mg/g)	Rutin (mg/g)	Shanyenoside A (mg/g)
1	0.15	1.68	1.50	0.46	4.59	0.99	0.49	0.44
2	0.17	1.37	1.68	0.47	3.50	0.97	0.40	0.27
3	0.28	3.35	0.96	0.68	4.63	1.54	0.62	0.54
4	0.05	1.15	5.45	0.29	2.97	0.84	0.12	0.35
5	0.03	0.88	1.14	0.11	1.61	0.60	–	0.06
6	0.07	0.54	1.74	0.41	2.48	1.72	0.32	0.30
7	0.02	0.39	0.58	0.10	1.13	0.37	–	0.03
8	0.03	0.65	0.71	0.21	1.82	0.48	0.07	0.08
9	0.18	1.70	0.07	0.35	1.73	0.47	0.25	0.18
10	0.08	2.91	0.34	1.31	3.81	0.66	0.18	0.16
11	0.82	2.91	0.24	1.31	3.81	0.66	0.17	0.16
12	0.33	4.37	0.18	0.40	4.82	1.14	0.91	0.12
13	0.17	5.58	0.97	0.69	2.87	1.45	1.22	0.03
14	0.12	2.00	0.72	0.46	2.71	0.86	0.36	0.20
15	0.45	3.97	0.28	0.60	3.84	0.30	1.13	0.04
16	0.13	1.93	0.65	0.61	3.41	0.89	0.34	0.23
17	0.08	1.38	1.39	0.38	2.64	0.63	0.21	0.07
18	0.88	1.16	0.41	0.71	4.37	0.21	1.23	0.10
19	0.94	1.55	0.13	0.80	6.36	1.86	4.41	0.01
20	0.20	2.05	0.47	0.96	3.95	3.95	0.98	0.12
21	–	1.30	0.28	2.23	2.35	0.75	0.41	–
22	0.29	0.62	0.43	0.29	2.72	0.97	2.15	–
23	0.14	4.59	0.38	0.19	4.02	1.67	0.35	0.32
24	–	3.73	2.41	0.64	1.73	0.87	0.07	0.07
25	0.03	1.17	3.21	0.40	2.79	0.69	0.02	0.11
26	0.85	2.33	0.13	0.76	0.16	0.03	1.02	0.32
27	0.58	1.06	0.63	1.53	0.05	0.01	0.22	0.19
28	0.71	2.84	0.44	1.63	0.15	0.01	0.97	0.06
29	0.61	1.31	0.26	1.65	0.08	0.03	–	–
30	0.47	0.79	0.46	2.25	0.03	0.02	1.05	0.02
31	0.70	3.91	0.12	1.27	0.07	0.03	0.81	0.36
32	0.55	4.95	0.09	2.93	0.26	0.01	0.76	0.28
33	0.11	2.55	0.19	0.54	1.85	1.07	0.36	0.16
34	–	1.23	6.49	1.41	0.49	0.23	–	0.01
35	–	1.99	0.26	0.71	0.39	0.17	1.15	0.03
36	0.04	0.71	0.57	0.24	0.33	0.04	0.67	0.03
37	–	3.67	0.41	1.19	0.97	0.88	0.80	0.02
38	–	3.36	0.04	0.59	0.90	0.80	1.89	0.02
39	–	4.28	0.99	0.29	0.47	0.50	1.28	0.01
40	–	3.00	0.05	0.97	0.75	0.92	3.02	0.02
41	0.02	2.01	0.03	0.38	0.35	1.13	0.80	–

According to the data collected by UPLC-MS, eight compounds epicatechin, chlorogenic acid, vitexin, hyperoside, vitexin-rhamnoside, vitexin-glucoside, rutin and shanyenoside A were identified accurately, that enriched the research indexes compared to UPLC fingerprinting studies. The quantitative results of the eight components in the 41 batches were found in Table 2. The hawthorn leaves and Guang hawthorn leaves, what they all have in common was that the content of vitexin-rhamnoside, vitexin-glucoside and chlorogenic acid were high in all components. But from the overall analysis, the differences were more pronounced. First of all, for vitexin-rhamnoside, the content in hawthorn leaves is much higher than that in Guang hawthorn leaves. Then for epicatechin and shanyenoside A, the content in both of hawthorn leaves and Guang hawthorn leaves were low-level, sometimes even was undetectable in Guang hawthorn leaves. Finally for vitexin, the range of content (0.03–6.49 mg/g) in Guang hawthorn leaves fluctuated greatly, but it was more stable in hawthorn leaves. In brief, hawthorn leaves matrices were more homogeneous and with a higher content of components than Guang hawthorn leaves.

Conclusion

For the quality evaluation of hawthorn leaves and Guang hawthorn leaves, an accurate and reliable UPLC–MS method was applied. From the two aspects, fingerprint analysis and multi-ingredient quantification, the differences in the type and content of components between the hawthorn leaves and Guang hawthorn leaves were evident. Thus, the leaves of these two crude drugs cannot be replaced by each other and should be carefully screened in clinical practices.

Authors' contributions

LYD, NSZ and RYL contributed in collecting plant samples and conducting the laboratory work. XBX and YMY performed the experiments and analyzed the data. FHP designed the study and contributed to the critical reading of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.03.005.

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