Fingerprint analysis of *Hibiscus mutabilis* L. leaves based on ultra performance liquid chromatography with photodiode array detector combined with similarity analysis and hierarchical clustering analysis methods

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ABSTRACT

Background: A method for chemical fingerprint analysis of *Hibiscus mutabilis* L. leaves was developed based on ultra performance liquid chromatography with photodiode array detector (UPLC-PAD) combined with similarity analysis (SA) and hierarchical clustering analysis (HCA). **Materials and Methods:** 10 batches of *Hibiscus mutabilis* L. leaves samples were collected from different regions of China. UPLC-PAD was employed to collect chemical fingerprints of *Hibiscus mutabilis* L. leaves. **Results:** The relative standard deviations (RSDs) of the relative retention times (RRT) and relative peak areas (RPA) of 10 characteristic peaks (one of them was identified as rutin) in precision, repeatability and stability test were less than 3%, and the method of fingerprint analysis was validated to be suitable for the *Hibiscus mutabilis* L. leaves. **Conclusions:** The chromatographic fingerprints showed abundant diversity of chemical constituents qualitatively in the 10 batches of *Hibiscus mutabilis* L. leaves samples from different locations by similarity analysis on basis of calculating the correlation coefficients between each two fingerprints. Moreover, the HCA method clustered the samples into four classes, and the HCA dendrogram showed the close or distant relations among the 10 samples, which was consistent to the SA result to some extent.

Key Words: Fingerprint, *Hibiscus mutabilis* L. leaves, ultra performance liquid chromatography with photodiode array detector

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INTRODUCTION

Nowadays, traditional Chinese medicine (TCM) has been attracting considerable attention because of its excellent qualities such as low toxicity and less side effects, good medical effects and rare drug tolerance.[1-3] It is well known that medicinal plants collected at different times and from different localities may considerably differ in types and quantities of chemical components, which affect the quality of pharmaceutical products and the standardization of this herbal medicine.[4-6] The quality assessment and control of TCM is an important concern for both the health authorities and the public.[7-9] Most investigations focused on several markers or pharmacologically active constituents to assess the quality and potency of the herbal medicine or preparations, which can not stand for the overall quality of TCM. Therefore, development of reliable, comprehensive quality assessment methods were necessary for TCM.[10] The fingerprint technique, which emphasized on the whole characteristics of samples' compositions and focuses on identifying and assessing the stability of the samples, was accepted by WHO,[11] State Food and Drug Administration (SFDA) of China (2000)[12] and other authorities[13,14] as a strategy for quality assessment of herbal medicines. High Performance Liquid Chromatography (HPLC) played the most important role among all the fingerprint techniques.[15] However, the traditional HPLC fingerprints cannot meet the requirements of high throughput analysis due to the low column efficiency and long analysis time with generally more than 1 hrs.[16-18] In recent years, UPLC has been reported as a viable technique for quantitative and chemical fingerprint analysis of Chinese herbal medicines, prior to HPLC analysis.[1,2,19]

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As a commonly used TCM, *Hibiscus mutabilis* L. leaves have been used in drug treatment as an anodyne, antidote, expectorant and refrigerant.[20,21] With the growing of applications of the *Hibiscus mutabilis* L. leaves, development of a suitable quality control method should be urgently required. Previous studies were performed on pharmacological actions and determining the contents of chemical constituents in the *Hibiscus mutabilis* L. leaves.[21-24] In this paper, a UPLC chemical fingerprinting method combined with similarity analysis (SA) and hierarchical clustering analysis (HCA) was developed for quality control of the *Hibiscus mutabilis* L. leaves from different locations.

MATERIALS AND METHODS

Standards and reagents

Standard rutin (purity ≥ 90.5%, batch NO. 100080-200707) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared using a deionized water treatment system (Thermo Scientific, USA). Analytical grade methanol and ethanol for extraction were purchased from Yongda Chemical Reagent Co. (Tianjin, China). AR grade formic acid and trifluoroacetic acid (Kelong Chemical Reagent Co., Chengdu, China) were used for preparation of mobile phase.

Plant materials

10 batches of *Hibiscus mutabilis* L. leaves samples were collected from different regions of China [Table 1]. The samples were identified based on morphological characteristics by the supervisor Jian-bao Zheng from Zhejiang Chinese medical university Chinese medicine pieces factory. For the chemical fingerprinting analysis, all samples collected were dried at 60°C in an oven, pulverized and passed though an 80-mesh sieve.

Table 1: Geographical origin of 10 batches of *Hibiscus mutabilis* L. leaves samples

Sample no.	Origin	Batch number
S1	Anhui	100316
S2	Zhejiang	100912
S3	Zhejiang	20090217
S4	Jiangsu	101125
S5	Jiangsu	101002
S6	Henan	081201
S7	Jiangxi	20090223
S8	Sichuan	110309
S9	Hunan	20110605
S10	Hubei	20110506

Sample preparation

Sample powder (2.0 g) was accurately weighed and extracted with 30 mL methanol under ultrasonic water bath for 60 min at 25°C with 600 W and 100 Hz of power (Hechuang ultrasonic instruments Co. Ltd, Kunshan, China). After cooling, methanol was added for the lost weight. Then the solution was filtered through a 0.22 μmmicropore membrane filter and 3 μL was injected for UPLC analysis.

Preparation of Standard Solution

Standard stock solution of rutin(985.5 μg/mL) was prepared by dissolving in 20 mL methanol. The concentrations of rutin standard used for calibration were 3.942, 7.884, 19.71, 39.42, 98.55 μg/mL. The standard curve was calibrated using the linear regression equation derived from the peak areas.

UPLC equipment and conditions

Experiments were performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA) equipped with a binary solvent delivery pump, an auto sampler and a PDA detector, and connected to Waters Empower software. The mobile phase consisted of acetonitrile (B2) and water (A1) using a gradient program of 95-92% A1 in 0-1 min, 92-85%A1 in 1-4 min, 85% A1 in 4-4.3 min, 85-92% A1 in 4.3-4.4 min, 92-87% A1 in 4.4-6.3 min, 87-83% A1 in 6.3-8.9 min, 83-80% A1 in 8.9-9 min, 80-74% A1 in 9-12 min, 74% A1 in 12-15 min, 74-60 A1 in 15-16 min, 60% A1 in 16-20 min. The flow rate was 0.4 mL/min. The detection wavelength and column temperature were 340 nm and 30°C, respectively. UV spectra were acquired in the range 190-400 nm (2 nm resolution).

Method validation

Precision, repeatability and stability

To evaluate validation of the method, the precision, repeatability and stability experiments were performed on sample S1. The precision test was determined by replicate injection of the same sample solution for five times. The repeatability test was analyzed by injecting five independently prepared samples. The stability test was determined by seven injections with one sample solution during 12 hours. The relative standard deviations (RSDs) of relative retention times and relative peak areas of each test were calculated.

Data analysis

Similarity analysis was performed by use of the professional software chromatogram analysis and data management system for traditional Chinese medicine which is recommended by National institutes for food and drug control for evaluating the similarity of different chromatograms by calculating the correlation coefficient and the cosine of the vectorial angel. In this paper the correlation coefficients of similarity

among chromatographic profiles of the *Hibiscus mutabilis* L. leaves samples were calculated respectively.10 characteristic peaks in the chromatograms were selected as the common peaks and the peak at retention time 11.467 min was used as a reference. Relative retention time (RRT, the ratio between retention time of characteristic peaks to that of reference peak) and relative peak area (RPA, the ratio between peak area of characteristic peaks to that of reference peak) of each characteristic peak to reference were calculated in the chromatograms.

The hierarchical clustering analysis (HCA) of samples of 10 resources was performed based on the RPA of 10 characteristic chromatographic peaks, using SPSS software (SPSS for Windows 17.0, USA). In this paper, the squared Euclidean distance was chosen as the measure of similarity, and the Ward method was applied for the clustering algorithm.[25]

RESULTS AND DISCUSSION

Optimization of extraction conditions

Factors such as extraction solvent, heating methods and time were investigated to achieve the chromatograms with better repeatability and higher extraction efficiency. The standard rutin was used as one of the references for assessing extraction efficiency. Firstly, ethanol, methanol, water, ethyl acetate and acetone were evaluated as extraction solvents. Poor repeatability was obtained with using ethanol as solvent. Water was also not adopted because the extracts were too sticky to permeate the filter membrane besides its low extraction efficiency. Methanol was found to be better to give higher repeatability and extraction efficiency than ethyl acetate and acetone. Secondly, with comparison of conventional extraction under reflux, higher extraction efficiency was obtained by ultrasonic extraction. Moreover, powdered leaves were extracted with methanol in an ultrasonic water bath for 30, 60 or 90 min and 60 min was thought to be the best for giving high extraction efficiency.

Optimization of UPLC conditions

Conditions such as the mobile phase, flow rate, column temperature and detection wave length were investigated in this paper to get the best resolution for most compositions in *Hibiscus mutabilis* L. leaves.

Mobile phase was thought to be the main effect on the resolution when the column was selected with Waters Acquity BEH C_{18} columns (50 mm × 2.1 mm, 1.7 μm). Among mobile phases investigated such as methanol-water, acetonitrile-water, and acetonitrile-buffer (containing formic acid or trifluoroacetic acid), the acetonitrile (A1)-water (B2) system was the ultimate choice. Gradient elution was essential for the separation. The gradient time, gradient polarity and initial composition of the mobile phase were taken into consideration. The gradient program was finally designed as 95-92% A1 in 0-1 min, 92-85% A1 in 1-4 min, 85% A1 in 4-4.3 min, 85-92% A1 in 4.3-4.4 min, 92-87% A1 in 4.4-6.3 min, 87-83% A1 in 6.3-8.9 min, 83-80% A1 in 8.9-9 min, 80-74% A1 in 9-12 min, 74% A1 in 12-15 min, 74-60 A1 in 15-16 min, 60% A1 in 16-20 min. Column temperature of 25°C and 30°C and flow rate of 0.3, 0.4 and 0.5mL/min was evaluated. When the system pressure, running time and suitability requirements was considered, the flow rate of 0.4 mL/min and 30°C of column temperature can give the best results. Besides, wave length of maximum absorption has been determined by a photodiode array detector and five wave lengths at 256, 267, 313, 340 and 354 nm were selected and compared. Wave length of 340 nm was selected to obtain the lowest baseline noise, a sufficiently large number of detectable peaks and better resolution in the chromatograms.

Under the optimal conditions, almost all the components in the extracts of the *Hibiscus mutabilis* L. leaves were well separated with high peak capacity in 20 min.

Validation of the method

Precision test

The instrument precision was determined by replicate injection of the same sample (sample S1) solution for five times. The results were shown in Table 2. The RSDs of RRT and RPA were not exceeding 0.08 and 2.07% respectively and the similarities of the chromatograms were greater than 0.99, which showed a high accuracy.

Repeatability test

The repeatability test was assessed by analyzing five independently prepared samples (sample S1) using the same method. The results were shown in Table 2. The RSDs of RRT and RPA were below 0.06 and 1.97% respectively and the similarities of the chromatograms were greater than 0.99, which showed a good reproducibility.

Sample stability test

The sample stability test was determined with one sample (sample S1) during 12 hours. During this period, the solution was stored at 4°C. The results were shown in Table 2. The RSDs of RRT and RPA were less than 0.08 and 1.26% respectively and the similarities of the chromatograms were greater than 0.99. The result indicated that the samples remained stable during this period.

UPLC fingerprints development of *Hibiscus mutabilis* L. leaves and identification of common peaks

All the chromatographic fingerprints obtained from 10 batches of *Hibiscus mutabilis* L. leaves from different

Table 2: Results of RRT and RPA of precision test, repeatability test, stability test and ten characteristic peaks on UPLC fingerprint of the *Hibiscus mutabilis* L. leaves from different locations in China

Peak no.	Relative retention time Mean (RSD %)				Relative peak area Mean (RSD %)			
	Rpt	Rrt	Rst	Rcp	Rpt	Rrt	Rst	Rcp
1	0.349 (0.02)	0.349 (0.01)	0.349 (0.02)	0.347 (0.19)	0.183 (1.29)	0.148 (0.34)	0.166 (1.07)	0.141 (11.40)
2	0.388 (0.02)	0.388 (0.01)	0.388 (0.03)	0.387 (0.16)	0.622 (2.07)	0.594 (1.97)	0.588 (1.26)	0.469 (19.18)
3	0.402 (0.02)	0.402 (0.01)	0.402 (0.02)	0.400 (0.12)	0.892 (1.67)	0.842 (1.76)	0.831 (0.47)	0.742 (38.50)
4	0.419 (0.02)	0.420 (0.01)	0.420 (0.03)	0.417 (0.19)	0.099 (0.71)	0.091 (0.70)	0.091 (0.37)	0.125 (8.430)
5	0.548 (0.08)	0.549 (0.06)	0.549 (0.08)	0.539 (0.49)	0.243 (0.74)	0.215 (1.89)	0.242 (0.21)	0.316 (14.24)
6	0.665 (0.04)	0.666 (0.02)	0.666 (0.04)	0.662 (0.20)	0.223 (0.70)	0.207 (1.40)	0.217 (0.36)	0.223 (10.34)
7	0.690 (0.03)	0.691 (0.02)	0.691 (0.04)	0.688 (0.17)	0.088 (0.44)	0.089 (0.75)	0.093 (0.45)	0.105 (1.78)
8	0.712 (0.03)	0.713 (0.02)	0.713 (0.04)	0.710 (0.17)	0.066 (0.63)	0.067 (0.92)	0.070 (0.63)	0.061 (1.27)
9	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)	1.000 (0.00)
10	1.036 (0.00)	1.036 (0.01)	1.036 (0.00)	1.036 (0.02)	0.297 (0.16)	0.293 (0.24)	0.297 (0.10)	0.266 (4.51)

Rpt (n = 5), Rrt (n = 5), Rst (n = 7: determine the same sample solution at 0, 1st, 2nd, 4th, 6th, 8th, and 12th hour after it was prepared, respectively) and Rcp represent the results of relative retention times and relative peak areas of precision test, repeatability test, stability test and ten characteristic peaks on UPLC fingerprint of the *Hibiscus mutabilis* L. leaves from different locations in China, respectively

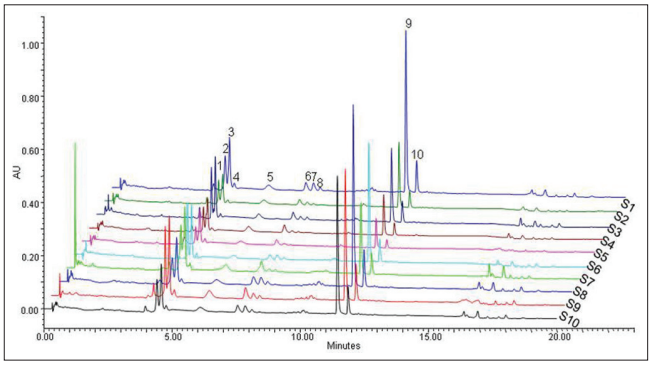

Figure 1: Chromatographic fingerprints obtained from 10 samples from different locations. S1 Anhui, S2 Zhejiang, S3 Zhejiang, S4 Jiangsu, S5 Jiangsu, S6 Henan, S7 Jiangxi, S8 Sichuan, S9 Hunan, S10 Hubei

locations were shown in Figure 1 in different colors. RRT and RPA of main peaks (>1% total peak area) detected were often used to evaluate the quality of fingerprints.[6] In this paper, 10 characteristic peaks with >1% total peak area were selected as the common peaks and labeled from peak 1 to peak 10 which showed in Figure 1. Peak 3 was identified as rutin by using the standards and UPLC-TOF/MS(Waters AcquityUPLC-BrukermicrOTOF-QII).Peak 9 with highest resolution and response was chosen as a reference to calculate the RRT and RPA of all other peaks which were shown in Table 2. From Table 2, the RSDs of RRT of 10 characteristic peaks were not exceeding 0.49% which exhibited the consistency of compositions in *Hibiscus mutabilis* L. leaves. However, RSDs of RPA were relatively higher (the largest was 38.50%), and this result exactly illustrated the differences of contents in sample compositions from different sources.

Similarity analysis

Similarity analysis was reliable for evaluating the chromatographic fingerprint of *Hibiscus mutabilis* L. leaves from various sources based on the correlative coefficients calculation of original data. The correlation coefficients of similarity among the chromatographic profiles of the 10 batches of *Hibiscus mutabilis* L. leaves were calculated by use of the professional software chromatogram analysis and data management system for traditional Chinese medicine. The calculated correlation coefficients (r_{cor}) were listed in Table 3. The results indicated that the samples shared different correlation coefficients of similarity, showing the differences of the internal quality of these samples. The values of r_{cor} are in the range $0 < r_{cor} < 1$. The larger the value of r_{cor} is, the more similar the two samples are. When r_{cor} equals 1, it is identical. It is considered that two samples are worse similar when the correlation coefficient below 0.8.[26,27] The similarity among the samples S2, S6 and S10 were strongly high with the values of $r_{cor} > 0.99$, which were regarded as the most similar ones. Compared to S1, S2, S6 and S10, the S5 sample was less alike ($r_{cor} < 0.80$), while it had higher similarity with S7, S8, S9 ($r_{cor} > 0.93$). The results were accordance with the actual patterns and peak shapes of the chromatograms [Figure 1].

Hierarchical clustering analysis

A hierarchical agglomerative clustering analysis was also performed in order to further assess the resemblance and differences among these *Hibiscus mutabilis* L. leaves samples. The RPA of 10 characteristic chromatographic peaks in 10 batches of *Hibiscus mutabilis* L. leaves from various sources formed a matrix of 10 × 10. The results of HCA were shown in Figure 2. In HCA dendrogram, the shorter distance between two samples indicated their higher similarity and the samples clustered into the same group were the most similar ones. The S2, S4, S6 and S10 with higher similarity ($r_{cor} > 0.95$) were classed into a cluster; moreover, the distance between S5 and S1, S2, S6, S10 was farther with the less similarity ($r_{cor} < 0.80$) among them. In addition, S5 and S9 were nearest, which was in correspondence with their most similarity result

Table 3: Evaluation of the similarities of samples from different locations

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1.0000	0.9395	0.9028	0.9727	0.7979	0.9644	0.9282	0.9143	0.8466	0.9607
S2	0.9395	1.0000	0.8077	0.9472	0.7083	0.9921	0.8500	0.8883	0.7714	0.9956
S3	0.9028	0.8077	1.0000	0.8920	0.8947	0.8499	0.9352	0.9194	0.9175	0.8319
S4	0.9725	0.9471	0.8919	1.0000	0.8451	0.9748	0.9605	0.9585	0.8912	0.9634
S5	0.7976	0.7082	0.8947	0.8451	1.0000	0.7725	0.9356	0.9417	0.9871	0.7364
S6	0.9644	0.9920	0.8500	0.9748	0.7725	1.0000	0.8993	0.9253	0.8280	0.9972
S7	0.9285	0.8501	0.9356	0.9605	0.9356	0.8993	1.0000	0.9708	0.9595	0.8747
S8	0.9143	0.8883	0.9194	0.9587	0.9419	0.9260	0.9708	1.0000	0.9688	0.9042
S9	0.8465	0.7713	0.9175	0.8912	0.9871	0.8280	0.9595	0.9688	1.0000	0.7968
S10	0.9607	0.9956	0.8319	0.9632	0.7360	0.9972	0.8735	0.9030	0.7968	1.0000

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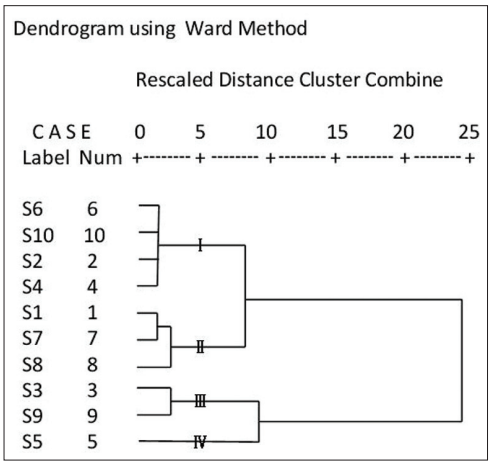

Figure 2: HCA dendrogram for 10 batches of *Hibiscus mutabilis* L. leaves

(r_{cor} = 0.9871) above. To some extent, the cluster results were consistent with SA.We concluded that the clustering results could be caused by the differences of plant origin, the effect of environment, season of collecting, storage conditions, etc. of the samples.In clinical applications,the most similar samples were often used to replace some Chinese traditional medicines which were unavailable.

CONCLUSIONS

A method of UPLC combined with SA and HCA was developed for chemical fingerprint analysis of *Hibiscus mutabilis* L. leaves in this paper. 10 characteristic peaks in the chromatograms were selected as the common peaks for identification of the UPLC fingerprints. Both RSDs of RRT and RPA of 10 peaks were calculated with not more than 3%, which proved that the established method was suitable for fingerprint analysis to control the quality of the *Hibiscus mutabilis* L. leaves. Above all, the similarity analysis result showed abundant diversity of chemical constituents qualitatively in the *Hibiscus mutabilis* L. leaves from different locations based on calculating the correlation coefficients between each two fingerprints. Besides, the HCA method clustered the samples into four classes, which showed the close and/or distant relations among the 10 samples by the dendrogram. The results derived from the two methods were consistent with each other. It had been showed that the method of UPLC with the aid of SA and HCA was able to identify objectively the samples from different locations.

The results also showed that UPLC possessed the advantages of shorter analysis times, higher column efficiency and less solvent consumption for the quality control of the *Hibiscus mutabilis* L. leaves. These superiorities make UPLC an attractive alternative to conventional HPLC technique in TCM fingerprinting analysis. Therefore, the method developed in this study would provide an important reference to establish the quality control method for other related TCM or preparations.

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REFERENCES

1. Wang L, Yuan K, Yu WW.Studies of UPLC fingerprint for the identification of *Magnoliaeofficinalis* cortex processed. Pharmacogn Mag 2010;6:83-8.
2. Kong WJ, Zhao YL, Xiao XH, Jin C, Li ZL.Quantitative and chemical finger print analysis for quality control of Rhizoma Coptidischinensis based on UPLC-PAD combined with chemometrics methods. Phytomedicine 2009;16:950-9.
3. Feng Y, Wu ZH, Zhou XZ, Zhou ZM, Fan WY. Knowledge discovery in traditional Chinese medicine: State of the art and perspectives. ArtifIntell Med 2006;38:219-36.
4. Wang Y, Han T, Zhang XG, Zheng CJ, Rahman K, Qin LP.LC fingerprint and hierarchical clusteranalysis of *Crocus sativus* L. from differentlocations in China. Chromatographia 2009;70:143-9.
5. Caballero-Ortega H, Pereda-Miranda R, Abdullaev Fikrat I. HPLC quantification of major active components from 11 different saffron (*Crocus sativus* L.) sources. Food Chem 2007;100:1126-31.
6. Zhang XG, Han T, Zhang QY, Zhang H, Huang BK, Xu LL, *et al*. Chemical fingerprinting and hierarchical clustering

analysis of Centellaasiatica from different locations in China. Chromatographia 2009;69:51-7.

7. Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, *et al*. Trends in alternative medicine use in the United States, 1990-1997. JAMA 1998;280:1569-75.
8. Xu CJ, Liang YZ, Chau FT. Identification of essential components of *Houttuyniacordata* by gas chromatography/mass spectrometry and the integrated chemometric approach. Talanta 2005;68:108-15.
9. Cardeal ZL, Souza PP, Silva MD, Marriott PJ. Comprehensive two-dimensional gas chromatography for fingerprint pattern recognition in cachaca production. Talanta 2008;74:793-9.
10. Gu M, Ouyang F, Su ZG. Comparison of high-speed counter-current chromatography and high-performance liquid chromatography on fingerprinting of Chinese traditional medicine. J Chromatogr A 2004;1022:139-44.
11. World Health Organization.General guidelines for methodologies on research and evaluation oftraditional medicine. Geneva: WHO; 2000. p.1.
12. State Drug Administration of China. Requirements for studying fingerprint of traditional Chinese medicine injection (Draft). Chin Tradit Patent Med 2000;22:671-5.
13. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. Guidance for industry botanical drug products, 2004.
14. European Medicines Agency. Note for guidance on quality of herbal medicinal products. London: EMA; 2001. p. 6.
15. Zhou X, Zhang YS, Zhao Y, Gong XJ, Zhao C, Chen HG. An LC fingerprint study of Poriacocos (Schw.) wolf. Chromatographia 2009;69:1283-9.
16. Ye J, Zhang X, Dai WX, Yan SK, Huang HQ, Liang X, *et al*. Chemical fingerprinting of Liuweidihuang pill and simultaneous determination of its major bioactive constituents by HPLC coupled with multiple detections of DAD, ELSD and ESI-MS. J Pharm Biomed Anal 2009;49:638-45.
17. Xu LN, Han X, Qi Y, Xu YW, Yin LH, Peng JY, *et al*. Multiple compounds determination and fingerprint analysis of Lidanpaishi tabletand keli by high-performance liquid chromatography. Anal Chim Acta 2009;633:136-48.
18. Xu SJ, Yang L, Tian RT, Wang ZT, Liu ZJ, Xie PS, *et al*. Species differentiation and quality assessment of Radix Paeoniae Rubra (Chi-shao) by means of high-performance liquid chromatographic fingerprint. J Chromatogr A 2009;1216:2163-8.
19. Liu M, Li YG, Chou GX, Cheng XM, Zhang M, Wang ZT. Extraction and ultra-performance liquid chromatography of hydrophilic and lipophilic bioactive components in a Chinese herb Radix Salviae Miltiorrhizae. J Chromatogr A 2007;1157:51-5.
20. Dasuki UA. Hibiscus.In: van Valkenburg JL, Bunyapraphatsara N,editors.Plant resources of South-East Asia No. 12(2): Medicinal and poisonous plants 2. Netherlands: Backhuys Publisher; 2001. p. 297-303.
21. Xie J, Shi LX, Zhu XY, Wang P, Zhao Y, Su WK. Mechanochemical-assisted efficient extraction of rutin from *Hibiscus mutabilis* L. Innov Food Sci Emerg 2011;12:146-52.
22. Meng HN, Luo GP. Determination of rutin in the leaf of *Hibiscus Mutabilis* L. by HPLC.Anhui Med Pharm J 2007;11:705-7.
23. Li H, Song ZJ, Ma RQ. Content determination of rutin in Folium Hibisci Mutabilis. Pharmacy Today 2010;20:34-6.
24. Wu K, Lu LQ. The pharmacological research progress of *Hibiscus mutabilis* L. laeves. Strait Pharm J 2010;22:37-9.
25. Brereton RG.Chemometrics: Data analysis for the laboratory and chemical plant. New York: John Wiley and Sons; 2003. p. 184.
26. Xu CJ, Liang YZ, Chau FT, Heyden Y. Pretreatments of chromatographic fingerprints for quality control of herbal medicines. J Chromatogr A 2006;1134:253-9.
27. Lu HM, LiangYZ, Chen S. Identification and quality assessment of Houttuyniacordata injection using GC-MS fingerprint: A standardization approach. J Ethnopharmacol 2006;105:436-40.

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