Quality evaluation of *Houttuynia cordata* Thunb. by high performance liquid chromatography with photodiode-array detection (HPLC-DAD)

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Abstract: A new, validated method, developed for the simultaneous determination of 16 phenolics (chlorogenic acid, scopoletin, vitexin, rutin, afzelin, isoquercitrin, narirutin, kaempferitrin, quercitrin, quercetin, kaempferol, chrysosplenol D, vitexicarpin, 5-hydroxy-3,3',4',7-tetramethoxy flavonoids, 5-hydroxy-3,4',6,7-tetramethoxy flavonoids and kaempferol-3,7,4'-trimethyl ether) in *Houttuynia cordata* Thunb. was successfully applied to 35 batches of samples collected from different regions or at different times and their total antioxidant activities (TAAs) were investigated. The aim was to develop a quality control method to simultaneously determine the major active components in *H. cordata*. The HPLC-DAD method was performed using a reverse-phase C_{18} column with a gradient elution system (acetonitrile-methanol-water) and simultaneous detection at 345 nm. Linear behaviors of method for all the analytes were observed with linear regression relationship (r^2>0.999) at the concentration ranges investigated. The recoveries of the 16 phenolics ranged from 98.93% to 101.26%. The samples analyzed were differentiated and classified based on the contents of the 16 characteristic compounds and the TAA using hierarchical clustering analysis (HCA) and principal component analysis (PCA). The results analyzed showed that similar chemical profiles and TAAs were divided into the same group. There was some evidence that active compounds, although they varied significantly, may possess uniform anti-oxidant activities and have potentially synergistic effects.

Keywords: Hierarchical clustering analysis (HCA), *Houttuynia cordata* Thunb., phenolics, principal component analysis (PCA), quality evaluation

INTRODUCTION

Houttuynia cordata Thunb., as a potentially medical and edible functional food (Wu *et al.*, 2005a; Wu *et al.*, 2005b), is a traditional Chinese medicine (TCM) that is officially listed in the Chinese Pharmacopoeia (CP) (2010 edition) (Pharmacopoeia, 2010). In some Asian countries (e.g. Thailand, Korea, India and Vietnam), While the mature *H. cordata*, which are commonly used as a traditional medical herb (Xu *et al.*, 2011), possess a variety of pharmacological activties (e.g., anti-oxidant, antibacterial, immunomodulatory effects, anti-leukemic, anti-platelet aggregation, anti-inflammatory, anti-tumor and antimicrobial (Chang *et al.*, 2001; Jong *et al.*, 1993; Nishiya *et al.*, 1988; Proebstle *et al.*, 1994). Recently, *H. cordata* showed significant anti-SARS activity (Lau *et al.*, 2008). The flavonoids and chlorogenic acid, which are two of the most common components in *H. cordata*, possess anti-oxidant, free radical scavenging, antipyretic, antibiotic, anti-neoplastic and anti-mutagenic capacities (Chen *et al.*, 2003; Choi *et al.*, 2002). It is usually believed that these components all contribute to the therapeutic effects of *H. cordata*.

Because of the complexity of the components, it is often a difficult process to establish quality control standards for TCMs. The quality evaluation of *H. cordata* was only based on morphological characteristics in the CP (2010 edition). Previous research related to *H. cordata* has isolated a number of compounds of various structural types. Recently, the antioxidants identified in aqueous extracts of *H. cordata* using high performance liquid chromatography–mass spectrometry (HPLC-MS) (Nuengchamnong *et al.*, 2009) were reported. Eight bioactive components (including flavonoids and alkaloids) of *H. cordata* and related Saururaceae medicinal plants were simultaneously analyzed (Meng *et al.*, 2009). The quality evaluation of HPLC-MS fingerprinting in *H. cordata* had been established previously (Meng *et al.*, 2005; Meng *et al.*, 2006), which was based on a fingerprinting correlation coefficient developed according to similarity of components and their contents.

The clinical effects of *H. cordata* are closely related to its quality. Phenolics (e.g. flavonoids and chlorogenic acid, etc.) varied remarkably in *H. cordata* plants with different provenances, with different biological characteristics and the geographic region where the plant grows (Wu *et al.*, 2009). However, more and more evidence is now available that shows that the quality evaluation of the fingerprinting characteristic is not mediated by the clinical effects of *H. cordata* for the potential synergistic

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effects among the bioactive compounds. Although the phenolics varied remarkably, anti-oxidant activity may be relatively uniform for potential synergistic effects among the phenolics. It is therefore essential to establish a method to evaluate the relationships between the phenolics in *H. cordata*. In this regard, a simple and comprehensive method for evaluating the quality of *H. cordata* is urgently needed.

The aims of this study were to develop a quality control method to simultaneously determine the major active components in *H. cordata* using HPLC. The 16 markers (Chlorogenic acid, scopoletin, vitexin, rutin, afzelin, isoquercitrin, narirutin, kaempferitrin, quercitrin, quercetin, kaempferol, chrysosplenol D, vitexicarpin, 5-hydroxy-3,3',4',7-tetramethoxy flavonoids, 5-hydroxy-3,4',6,7-tetramethoxy flavonoids and kaempferol-3,7,4'-trimethyl ether) contents of 35 *H. cordata* batches were simultaneously determined and their antioxidant activities evaluated by DPPH assay. The samples were differentiated and classified according to their active marker content and the total antioxidant activity (TAA) by both hierarchical clustering analysis (HCA) and principal component analysis (PCA). This may provide important information for the selection or evaluation of candidate cultivars of *H. cordata* from a pharmacological perspective.

MATERIALS AND METHODS

Chemicals and reagents

Sixteen markers (chlorogenic acid, scopoletin, vitexin, rutin, afzelin, isoquercitrin, narirutin, kaempferitrin, quercitrin, quercetin, kaempferol, chrysosplenol D, vitexicarpin, 5-hydroxy-3,3',4',7-tetramethoxy flavonoids, 5-hydroxy-3,4',6,7-tetramethoxy flavonoids and kaempferol-3,7,4'-trimethyl ether) (fig. 1) were purchased from Sigma (USA). Acetonitrile (HPLC) and methanol (HPLC) were purchased from MERCK, Inc. (Germany). DPPH was purchased from Sigma-Aldrich Chemie (Steinheim, Germany) and formic acid was purchased from TianJin Chemical Reagents Development Center (TianJin, China). Ultrapure water (18.2 M) was prepared using a Sartorius Arium 611UF water purification system (Sartorius, Germany). Other reagents were analytical grade.

Plant materials

35 samples of *H. cordata* (table 1), which were collected from different regions of Guizhou Province in China and authenticated by Professor Chen Deyuan of Guiyang Chinese Medical College, were air dried at room temperature. Voucher specimens were stored in sealed bottles at the Key Laboratory for Information System of Mountainous Area and Protection of Ecological Environment of Guizhou Province, Guizhou Normal University, until they were required.

Standard solution

Preparation of a stock solution is that 16 markers weighed accurately were dissolved in methanol in a 10mL volumetric flask. Preparation of working solutions is that the stock solutions were further diluted with the appropriate methanol. The solutions prepared were stored in the dark at 4°C.

Sample solution

Samples that had been pulverized using a homogenizer were accurately weighed into 100 mL triangular flasks and then extracted three times at 40°C (30 min each) by sonication with 30 mL methanol. The extracts were centrifuged using a centrifuge (Model 80-2, Jinda, Jiangsu) for 8 min at 4000 r/min and then combined and concentrated to about 15 mL at 40-50°C using rotary evaporators (R-210, BUCHI, Switzerland). The concentrated extracts were diluted to 25mL with methanol, and then filtered through a 0.45 μm membrane filter.

HPLC conditions

A HPLC system LC-20AT series (Shimadzu, Japan) including a diode array detector, two pumps, a thermostated column compartment, an online vacuum degasser and Chem Station software was performed for chromatographic analysis. All chromatographic separations were performed on a reversed-phase Shim-pack CLC-ODS (6.0 mm × 150 mm, I.D., 5 μm; No.61626630). A linear gradient elution using eluent A (acetonitrile: methanol=11: 5 (v/v)) and eluent B (0.1% formic acid (m/v)) was carried out for the separations. The elution program optimized was conducted as follows: 0-5 min, linear gradient 5% A; 5-8 min, linear gradient 5-16% A; 8-30 min, linear gradient 16-24% A; 30-47 min, linear gradient 24-32% A; 47-68 min, linear gradient 32-64% A; 68-75 min, linear gradient 64% A; 75-78 min, linear gradient 64-100% A; 78-88 min, linear gradient 100% A; 88-89 min, linear gradient 100-5% A and 89-95 min, linear gradient 5%. The flow rate program was conducted as follows: 0-5 min, 1.4mL/min; 5-10 min, 1.4-0.6 mL/min; 10-47 min, 0.6-0.8 mL/min; 47-50 min, 0.6-1.4 mL/min and 50-95 min, 1.4mL/min. The set detection wavelength was 345 nm, the volume of injection was 20μL, and the column temperature maintained was 40°C.

DPPH assay

The DPPH assay was performed the standard method (Brand-Williams *et al.*, 1995) and slightly modified. The reaction mixture is that a sample solution of *H. cordata* (0.3mL) and 0.1mM DPPH (9.7mL) was mixed in methanol. The reaction mixtures were incubated in the dark for 30min. The absorbances (A) of the reaction mixtures were measured on a Cary 100 (Warian, USA) at 515nm by methanol as a blank. The total antioxidant activity (TAA) was obtained and calculated by the following equation: TAA (%) =100× [(A control-A sample)/A control], where A control and A sample is the

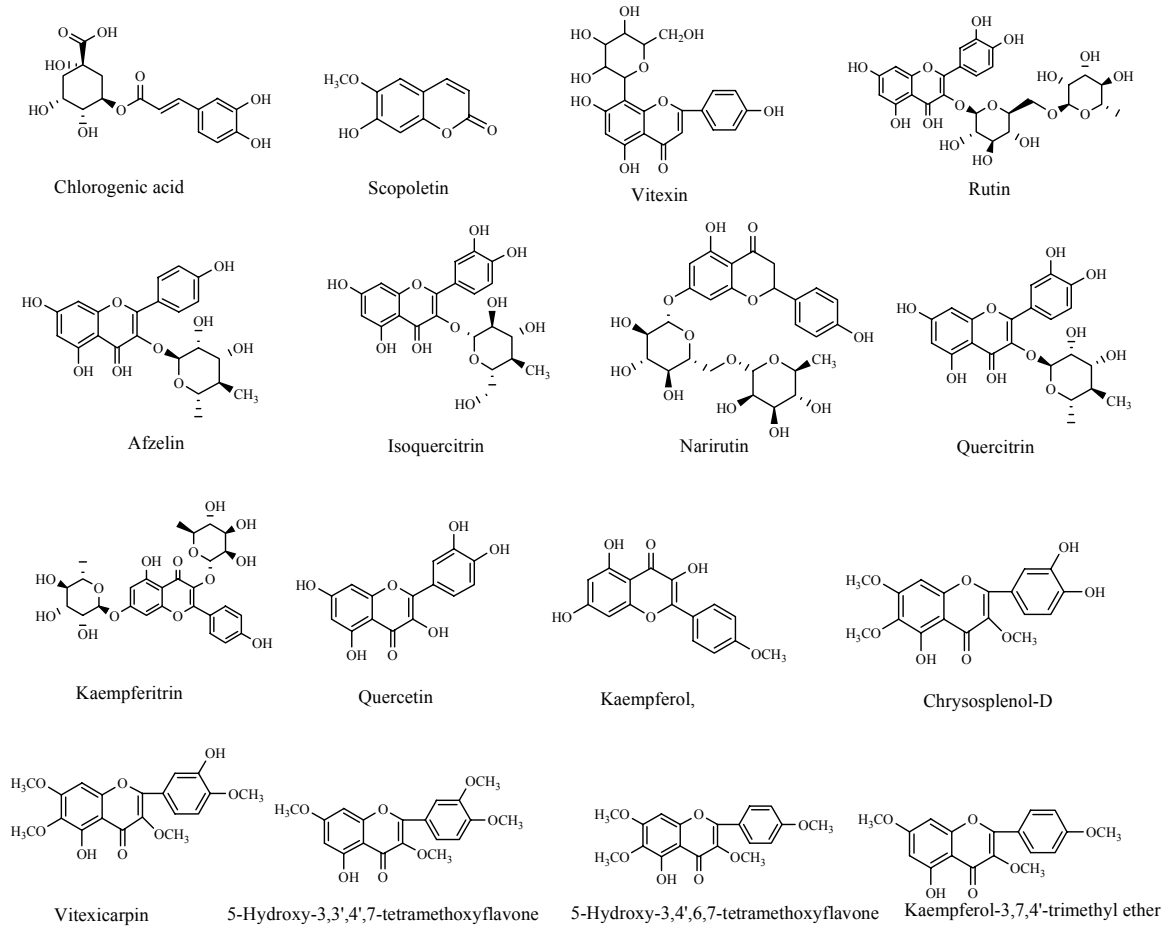

Fig. 1: Chemical structures of the sixteen markers

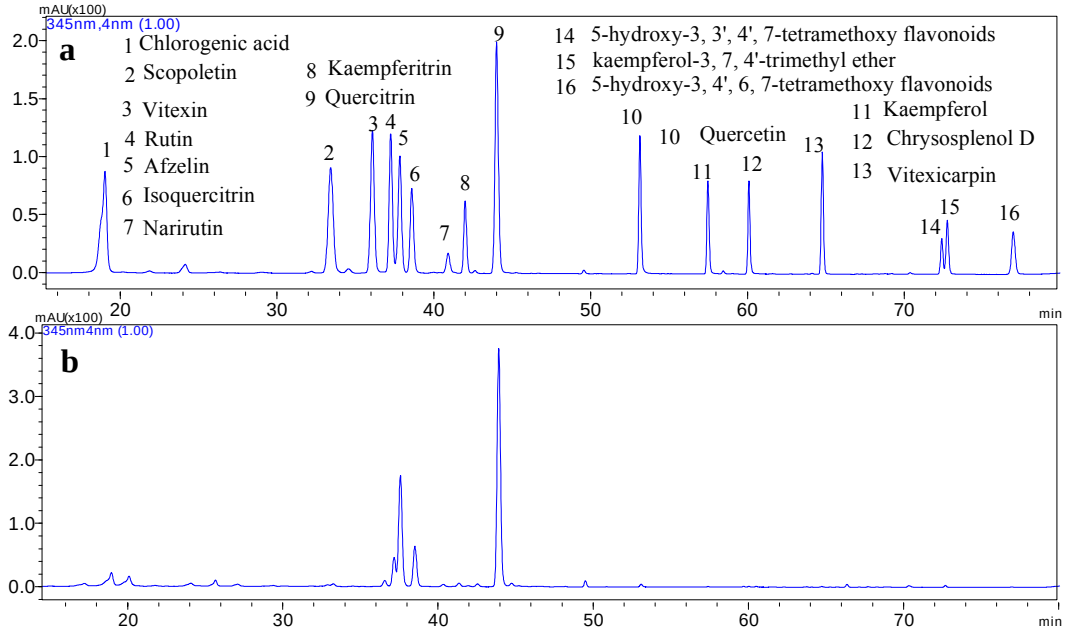

Fig. 2: Representative HPLC-DAD chromatographic profiles of mixed standard solution containing the 16 markers (**a**) and the extract of *H. cordata* batch (samples no. 35) (**b**) at 345 nm.

Table 1: Collection information of the samples and their total antioxidant activity (% TAA) by DPPH assay

No.	Voucher specimen	Sources (From Guizhou province, in China)	Altitud (m)	Acquisition time	TAAs (%) ± S.D.s
1	JCS001	Gaoan in Congjiang county (N, 25°43'49.48", E, 109°13'22.31")	159	2010.7.16	88.9±0.10
2	JCS002	Shibantian in Chishui county (N, 28°31'16.27", E, 105°44'20.67")	248	2010.7.13	89.9±0.11
3	JCS003	Fengxiang in Yanhe county (N, 28°34'49.83", E, 108°30'04.96")	302	2010.8.9	86.3±0.10
4	JCS004	Miaoer in Yanhe county (N, 28°31'05.07", E, 108°29'16.83")	331	2010.8.9	72.6±0.10
5	JCS005	Guakou in Jiangkou county (N, 27°43'34.42", E, 108°54'21.53")	333	2010.8.8	72.9±0.12
6	JCS006	Changping in Fanjing mountain (N, 27°53'48.73", E, 108°54'56.64")	358	2010.8.2	87.5±0.18
7	JCS007	Banpotian in Tongren county (N, 27°44'36.10", E, 109°17'17.80")	382	2010.8.11	91.2±0.12
8	JCS008	Zhenjiang in Jiangkou county (N, 27°43'37.16" E, 108°49'56.17")	394	2010.8.8	90.2±0.21
9	JCS009	Guandong in Liling county (N, 26°03'51.20", E, 108°54'18.91")	405	2010.7.16	89.9±0.17
10	JCS010	Tucheng in Daozhen county (N, 28°52'54.26", E, 107°40'51.13")	443	2010.7. 7	93.2±0.14
11	JCS011	Xinmin in Luodian county (N, 25°25'08.97", E, 106°47'02.83")	454	2010.9.1	88.8±0.32
12	JCS012	Changkan in Dejiang county (N, 28°19'00.28", E, 108°06'50.31")	470	2010.8.6	90.7±0.18
13	JCS013	Sandaoyan in Yinjiang county (N, 27°29'13.33", E, 108°13'24.17")	497	2010.8.5	86.9±0.22
14	JCS014	Qingqi in Zhenyuan county (N, 27°07'23.86", E, 108°44'57.46"	507	2010.7.20	62.3±0.14
15	JCS015	Majinggang in Wuchuan county (N, 28°33'21.10", E, 107°55'00.66")	509	2010.7.7	92.9±1.14
16	JCS016	Gancun in Zhengan county (N, 28°32'52.03", E, 107°29'39.17")	533	2010.7.6	91.4±0.36
17	JCS017	Dagao in Jianhe county (N, 26°43'24.41", E, 108°26'02.71")	584	2010.7.20	88.7±0.17
18	JCS018	Tiansheng in Zhengan county (N, 28°31'44.04", E, 107°27'44.42")	634	2010.7.6	87.2±0.06
19	JCS019	Guatang in Shiqian county (N, 27°36'05.07", E, 108°16'12.97")	634	2010.8.7	86.2±0.14
20	JCS020	Xiangyang in Dejiang county (N, 28°13'51.51", E, 108°07'35.02")	660	2010.8.5	87.6±0.10
21	JCS021	Gaoyang in Liping county (N, 26°02'26.67", E, 109°06'34.43")	660	2010.7.16	73.9±0.71
22	JCS022	Baiyuan in Fanjing mountain (N, 27°59'25.28", E, 108°32'25.82")	726	2010.8.2	92.5±0.20
23	JCS023	Luotang in Liuzhi county (N, 26°04'27.35", E, 105°10'19.38")	769	2010.8.20	88.7±0.51
24	JCS024	Qingchi in Jinsha county (N, 27°42'59.65", E, 105°56'17.53")	778	2010.7.13	91.3±0.77
25	JCS025	Caijiadi in Dushan county (N, 25°50'18.10", E, 105°33'31.72")	993	2010.7.10	87.0±0.90
26	JCS026	Dongfeng in Guiyang (N, 26°38'39.68", E, 106°49'02.42")	1004	2010.8.23	55.0±0.31
27	JCS027	Changtian in Huishui county (N, 26°16'16.43", E, 106°40'41.34")	1060	2010.9.1	92.6±0.42
28	JCS028	Dazai in Xinren county (N, 25°22'14.00", E, 105°18'49.52")	1282	2010.7.19	90.1±0.86
29	JCS029	Shaoshan in Zhengfeng county (N, 25°22'14.88", E, 105°38'05.94")	1328	2010.8.17	71.7±0.63
30	JCS030	Fanjing mountain (N, 27°50'33.63", E, 108°41'20.28")	1411	2010.8.2	64.4±0.21
31	JCS031	Guantian in Qinglong county (N, 25°50'38.38", E, 105°13'40.38")	1479	2010.8.17	89.1±0.26
32	JCS032	Duimen in Nayong county (N, 26°44'32.07", E, 105°24'39.03")	1537	2010.8.4	88.8±0.44
33	JCS033	Hongyan in Bijie (N, 27°20'22.20", E, 105°20'50.07")	1658	2010.8.11	89.9±0.08
34	JCS034	Fanjing mountain (N, 27°55'17.43", E, 108°39'35.83")	2393	2010.8.2	88.3±0.11
35	JCS035	Caohai in Weining county (N, 26°50'12.60", E,104°05'52.49")	2555	2010.8.10	52.6±0.16

Notes: The activity data obtained are the average of three analyses ± standard deviations (S.D.s)

absorbance of the control and the tested sample after 30 min, respectively.

Calculations and statistical analyses

Each sample was carried out in triplicate. The data obtained and calculated by the Excel (2003) were reported as a mean (n=3). The analysis of variance were followed by S.D.s and R.S.D.s.. HCA and PCA were undertaken using SPSS 13.0 (SPSS Inc., USA).

RESULTS

Optimization of the extraction condition

The extraction efficiency was evaluated using methanol, ethanol and acetonitrile, respectively. Methanol produced fewer interfering peaks and obtained the highest values for the contents of 16 compounds. Orthogonal array design (OAD) based on a four-factor-three-level, including the following components: number of times the

sample was subjected to sonication (one, two, and three times), volume of methanol (20, 30 and 40mL) and duration of extraction (10, 20, and 30min), was developed so that the extraction could be optimized. The results show that the optimized extraction condition was suitable and appropriate for the analysis.

compounds investigated were determined and compared using different analytical chromatographic columns (Shim-pack CLC-ODS, Diamonsil C18 or CAPCELL PAK C18) with methanol-0.1% formic acid, acetonitrile-0.1% formic acid and acetonitrile-methanol-0.1% formic acid at different programs of gradient elution, respective. The results showed that the markers investigated could efficiently been separated by the Shim-pack CLC-ODS column with a gradient elution using mixed system of acetonitrile-methanol-0.1% formic acid (fig. 2). After analyzing the UV spectra for the 16 compounds recorded by DAD, 345 nm was selected for monitoring the 16 compounds.

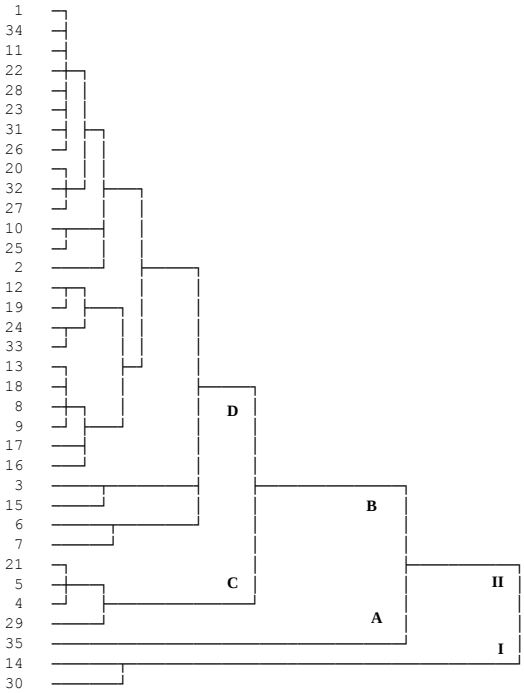

Fig. 3: Dendrogram of HCA for the 35 tested *H. cordata* batches.

HPLC method Validation

Calibration curves, Limits of detection (LOD) and quantitation (LOQ) Standard solutions of different concentration levels were prepared by diluting the stock solution of the 16 markers and the appropriate concentration ranges needed to create the calibration curves. The respective calibration curves were plotted by linear regression to the mean peak areas versus concentrations. LOD and LOQ under the optimal chromatographic condition were tested at signal-to-noise ratios (S/N) of 3 and 10, respectively. The data of LOD and LOQ are summarized in table 2.

Precision, repeatability and stability The precision was examined, using the mixed standards solution of appropriate concentration level and the sample solution under the optimal extraction conditions, the inter-day and intra-day variation. Repeatability was tested using different working solutions prepared independently from sample no. 35 and one of them was determined every 4 h over a 20 h period in order to calculate the stability of the sample solution. The results obtained are expressed in R.S.D.s, which are shown in table 3.

Recovery Recovery test was undertaken by adding known amounts of the 16 markers to *H. cordata* sample no. 35 at three different levels (80%, 100% and 120%, respectively). The resultant samples extracted and processed with the proposed methods were analyzed by the HPLC method developed. The results are given in table 4.

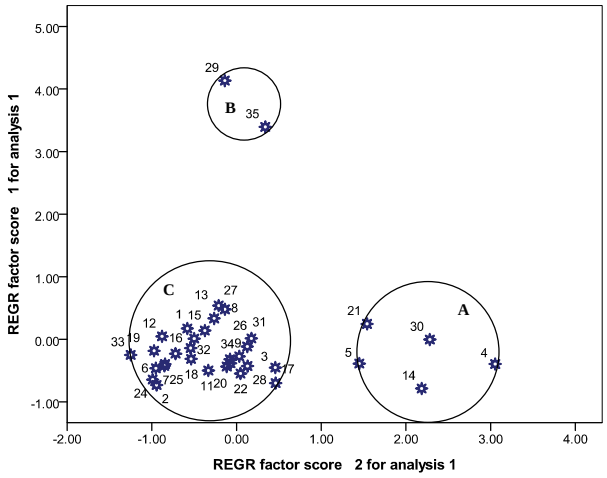

Fig. 4: The scatter plot obtained by PCA of the 35 *H. cordata* batches.

Robustness Method robustness test was evaluated using Shim-pack CLC-ODS (6.0 mm × 150 mm, I.D., 5 μm) and CAPCELL PAK C18 (150 mm × 4.6 mm, I.D., 5 μm). The same working solution of *H. cordata* sample no.35 was separately tested and the percent contents of the 16 compounds were calculated. The mean percent contents of the 16 compounds (chlorogenic acid, scopoletin, vitexin, rutin, afzelin, isoquercitrin, narirutin, kaempferitrin, quercitrin, quercetin, kaempferol, chrysosplenol D, vitexicarpin, 5-hydroxy-3,3',4',7-tetramethoxy flavonoids, 5-hydroxy-3,4',6,7-tetramethoxy flavonoids and kaempferol-3,7,4'-trimethyl ether) were 0.161, 0.013, 0.073, 0.049, 0.116, 0.025, 0.030, 0.016, 0.550, 0.018 0.317, 0.018, 0.017, 0.019, 0.027 and 0.011%, respectively, for the Shim-pack CLC-ODS column and 0.160, 0.014, 0.073, 0.048, 0.117, 0.025,

Table 2: Regression equation, regression relationship (r^2), Linear range, limits of detection (LOD) and quantitation (LOQ) of the sixteen markers

No.	Markers	Calibration curves [a]			LOD (ng)	LOQ (ng)
		Linear regression equation	r^2	Linear range (μg)		
1	Chlorogenic acid	Y = 40828X-23026	0.9991	0.0401-1.003	0.275	0.916
2	Scopoletin	Y = 120214X-41728	0.9991	0.0120-0.300	0.088	0.292
3	Vitexin	Y = 121488X-51466	0.9996	0.0128-0.320	0.119	0.395
4	Rutin	Y = 50329X-31137	0.9991	0.0258-0.644	0.090	0.301
5	Afzelin	Y = 46720X-46272	0.9998	0.0250-0.625	0.172	0.575
6	Isoquercitrin	Y = 66196X-51166	0.9996	0.0130-0.325	0.135	0.451
7	Narirutin	Y = 8028.8X-13330	0.9995	0.0275-0.686	0.142	0.474
8	Kaempferitrin	Y = 49297X-16833	0.9994	0.0121-0.303	0.269	0.896
9	Quercitrin	Y = 92666X-140888	0.9997	0.0248-0.619	0.051	0.169
10	Quercetin	Y = 76009X-39600	0.9997	0.0126-0.316	4.208	14.028
11	Kaempferol	Y = 49723X-34557	0.9991	0.0122-0.305	0.111	0.370
12	Chrysosplenol D	Y = 45312X-29331	0.9993	0.0122-0.305	0.076	0.253
13	Vitexicarpin	Y = 49135X-23556	0.9991	0.0150-0.375	0.217	0.725
14	5-Hydroxy-3,3',4',7-tetramethoxy flavonoids	Y = 13397X-15695	0.9995	0.0190-0.474	0.124	0.413
15	5-Hydroxy-3,4',6,7-tetramethoxy flavonoids	Y = 33817X-22774	0.9991	0.0123-0.308	0.114	0.380
16	Kaempferol-3,7,4'-trimethyl ether	Y = 22670X-11449	0.9995	0.0195-0.488	0.257	0.855

0.031, 0.016, 0.552, 0.017 0.316, 0.019, 0.017, 0.020, 0.026 and 0.011%, respectively, for the CAPCELL PAK C_{18} column. A *t*-test (P>0.05) showed that there were no significant differences between the results from the two columns, indicating that the proposed HPLC method was enough for evaluating results with performance.

Sample analysis

The newly validated HPLC-DAD method was applied to analyze the 16 markers in the *H. cordata* batches, coded 1-35. The results showed that the contents of the 16 markers in the 35 *H. cordata* batches were chlorogenic acid (0.01-0.701%), scopoletin (0.001-0.016%), vitexin (0.002-0.073%), rutin (0.003-0.170%), afzelin (0.005-0.839%), isoquercitrin (0.001-0.119%), narirutin (0.002-0.034%), kaempferitrin (0.001-0.019%), quercitrin (0.002-0.550%), quercetin (0.001-0.018%), kaempferol (0.001-0.317%), chrysosplenol D (0.001-0.018%), vitexicarpin (0.001-0.017%), 5-hydroxy-3,3',4',7-tetramethoxy flavonoids (0.002-0.119%), 5-hydroxy-3,4', 6,7-tetramethoxy flavonoids (0.001-0.077%) and kaempferol-3,7,4'-trimethyl ether (0.002-0.142%), respectively. The contents of the markers varied significantly in the 35 *H. cordata* batches.

Antioxidant activity analysis

The antioxidant activities of the 35 *H. cordata* batches were analyzed by DPPH assay. The screening results are listed in table 1 and show that the TAAs of batch nos. 4, 5, 14, 21, 29, 30 and 35 were 52.6-73.9% and the others were 86.2-92.5%.

DISCUSSION

HCA of the samples

The contents of the 16 markers and the TAA were defined as 17 characteristics in the analysis so that the *H. cordata* samples could be analyzed, differentiated and classified (fig. 3), which revealed the relationships among the *H. cordata* samples. The 35 samples of *H. cordata* were divided into two main clusters. Sample nos. 14 and 30 were in cluster I and the other samples were in cluster II, which was subdivided into two subgroups. Sample no. 35 was in subgroup A, and the others were in subgroup B, which was further subdivided into another two subgroups. Sample nos. 4, 5, 21 and 29 were in subgroup C and the others were in subgroup D. The results obtained indicated that tested samples which had similar chemical profiles and TAAs were divided into the same group.

PCA of the samples

The contents of the 16 markers and the TAA were analyzed as variables, which were then translated mathematically into two main comprehensive factors in order to analyze the samples. The 35 *H. cordata* batches were further analyzed and classified using PCA. The scatter plot is presented in fig. 4, where each *H. cordata* batch was represented as a marker. It is noticeable that the 35 *H. cordata* batches were clearly clustered into three domains. Sample nos. 4, 5, 14, 21 and 30 were in domain A, nos. 29 and 35 were in domain B and the others were in domain C. The results were similar to those obtained using HCA.

Table 3: Intra- and Inter-day variability, repeatability and stability for the assay of the sixteen markers

No.	Markers	Precision (n = 6)			
		Intra-day			
		Mean (%)[a]	R.S.D.s (%)	Average peak area[b]	R.S.D.s (%)
1	Chlorogenic acid	0.0161	1.25	1998963.5	1.05
2	Scopoletin	0.0013	2.43	615998.7	2.13
3	Vitexin	0.0073	2.59	1767481.5	2.49
4	Rutin	0.0049	2.77	1494550.0	2.67
5	Afzelin	0.0116	2.45	1340618.8	0.92
6	Isoquercitrin	0.0025	1.79	921319.3	2.23
7	Narirutin	0.0030	2.67	237446.7	1.46
8	Kaempferitrin	0.0016	2.15	43516.3	2.51
9	Quercitrin	0.0550	2.88	2511508.7	1.98
10	Quercetin	0.0018	2.38	1041720.2	1.80
11	Kaempferol	0.0317	2.92	640144.5	2.02
12	Chrysosplenol D	0.0018	2.94	585709.2	1.14
13	Vitexicarpin	0.0017	2.54	806094.3	1.19
14	5-Hydroxy-3,3',4',7-tetramethoxy flavonoids	0.0019	2.94	271253.5	1.63
15	5-Hydroxy-3,4', 6,7-tetramethoxy flavonoids	0.0027	2.49	93862.0	2.92
16	Kaempferol-3,7,4'-trimethyl ether	0.0011	2.69	87092.7	2.18

Precision (n = 6)				Repeatability		Stability	
Inter-day				Mean (%)	R.S.D.s (%)	Mean (%)	R.S.D.s (%)
Mean (%)	R.S.D.s (%)	Average peak area	R.S.D.s (%)				
0.0160	3.43	2015630.2	3.03	0.0159	2.31	0.0157	1.05
0.0012	2.05	617665.3	2.95	0.0014	1.23	0.0012	0.43
0.0070	2.99	1767481.5	2.59	0.0075	1.54	0.0075	1.89
0.0047	1.41	1497883.3	2.46	0.0050	2.07	0.0048	1.77
0.0115	2.70	1338952.2	2.71	0.0117	2.13	0.0112	2.12
0.0024	1.99	917986.0	1.19	0.0026	1.25	0.0027	0.59
0.0031	2.38	235946.7	1.45	0.0032	1.64	0.0032	1.67
0.0015	2.29	44016.3	2.27	0.0017	2.75	0.0017	3.18
0.0551	3.07	2544842.0	3.47	0.0558	2.89	0.0552	0.88
0.0019	2.10	1058386.8	3.12	0.0017	1.55	0.0016	2.30
0.0314	1.93	641811.2	2.93	0.0320	0.92	0.0318	2.02
0.0017	3.19	587375.8	3.32	0.0016	1.94	0.0017	2.01
0.0018	2.04	806094.3	2.54	0.0015	2.00	0.0016	3.33
0.0016	2.41	272086.8	3.42	0.0017	1.04	0.0020	2.27
0.0025	2.52	93695.3	2.72	0.0028	1.47	0.0026	3.40
0.0012	3.39	87142.7	2.60	0.0010	2.80	0.0011	2.95

Sample solution. [b]Standard mixture solution.

CONCLUSION

In this study, chlorogenic acid, scopoletin, vitexin, rutin, afzelin, isoquercitrin, narirutin, kaempferitrin, quercitrin, quercetin, kaempferol, chrysosplenol D, vitexicarpin, 5-hydroxy-3,3',4',7-tetramethoxy flavonoids, 5-hydroxy-3,4',6,7-tetramethoxy flavonoids and kaempferol-3,7,4'-trimethyl ether in *H. cordata* were simultaneously analyzed using a HPLC-DAD method developed by this study. It is the first reported that these 16 markers have been determined simultaneously with acceptable performances for linearity, repeatability, precision, accuracy and robustness for 90 min. Furthermore, the method developed was successfully used to test 35 *H. cordata* batches. HCA and PCA were performed in order to classify and differentiate the 35 *H. cordata* batches, based on the contents of the 16 markers and the TAA. There is some evidence that although the activity of the compounds varied significantly, their activities may possess uniform anti-oxidant activities and potentially synergistic effects. The blending quality evaluation has been shown to be able to save and guide rational herb resources use in medicinal and herbal production.

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Table 4: Recovery of the sixteen markers in *H. cordata*

No.	Markers	Samples	Contents			Recovery (%)	Mean Recovery (%)±R.S.D.s
			$M_{Original}$ (mg)	M_{Added} (mg)	M_{Found} (mg)		
1	Chlorogenic acid	S1[a]	0.331	0.265	0.593	99.12	99.91±1.52
		S2[b]	0.325	0.322	0.652	101.64	
		S3[c]	0.322	0.386	0.704	98.96	
2	Scopoletin	S1	0.027	0.022	0.049	99.46	100.33±1.78
		S2	0.027	0.027	0.054	101.15	
		S3	0.026	0.032	0.058	100.37	
3	Vitexin	S1	0.149	0.119	0.269	101.21	101.23±2.34
		S2	0.146	0.145	0.294	101.98	
		S3	0.145	0.174	0.320	100.52	
4	Rutin	S1	0.100	0.080	0.178	98.25	99.27±1.82
		S2	0.098	0.098	0.196	99.18	
		S3	0.097	0.117	0.214	100.38	
5	Afzelin	S1	0.238	0.190	0.427	99.69	100.27±1.50
		S2	0.234	0.229	0.461	99.12	
		S3	0.231	0.278	0.515	102.01	
6	Isoquercitrin	S1	0.051	0.041	0.093	100.91	100.51±1.33
		S2	0.051	0.050	0.101	101.11	
		S3	0.050	0.060	0.110	99.50	
7	Narirutin	S1	0.062	0.050	0.112	98.56	99.53±2.36
		S2	0.061	0.061	0.123	100.71	
		S3	0.061	0.073	0.133	99.31	
8	Kaempferitrin	S1	0.032	0.026	0.058	100.62	99.55±1.24
		S2	0.032	0.032	0.063	98.94	
		S3	0.031	0.038	0.069	99.10	
9	Quercitrin	S1	1.127	0.902	2.033	100.4	99.51±1.42
		S2	1.108	1.109	2.209	99.25	
		S3	1.097	1.317	2.399	98.88	
10	Quercetin	S1	0.037	0.030	0.067	100.12	101.26±1.11
		S2	0.037	0.037	0.074	101.56	
		S3	0.036	0.043	0.081	102.10	
11	Kaempferol	S1	0.650	0.520	1.159	98.10	98.93±2.76
		S2	0.638	0.630	1.263	99.21	
		S3	0.632	0.759	1.387	99.47	
12	Chrysosplenol D	S1	0.036	0.029	0.065	101.65	100.76±1.61
		S2	0.035	0.035	0.071	102.21	
		S3	0.035	0.042	0.076	98.43	
13	Vitexicarpin	S1	0.036	0.029	0.064	99.19	100.29±1.89
		S2	0.035	0.035	0.070	100.26	
		S3	0.035	0.042	0.077	101.42	
14	5-Hydroxy-3,3',4',7-tetramethoxy flavonoids	S1	0.039	0.032	0.071	100.54	99.41±0.98
		S2	0.039	0.039	0.078	99.56	
		S3	0.038	0.046	0.084	98.12	
15	5-Hydroxy-3,4',6,7-tetramethoxy flavonoids	S1	0.054	0.044	0.098	99.42	100.38±2.02
		S2	0.053	0.053	0.107	100.48	
		S3	0.053	0.064	0.117	101.23	
16	Kaempferol-3,7,4'-trimethyl ether	S1	0.023	0.019	0.042	102.54	100.88±2.14
		S2	0.023	0.023	0.046	100.21	
		S3	0.023	0.027	0.050	99.89	

% Recovery = $((M_{found}-M_{original})/M_{added}) \times 100$. The results obtained showed that the proposed method was accurate for the determination of the 16 markers.

[a]The samples added 80% of the known amounts. [b] The samples added 100% of the known amounts. [c] The samples added 120% of the known amounts.

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