Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Metabolic Profiling Analysis of Three Processed Rhizomes of *Curcuma wenyujin Y.H. Chen et C. Ling* by Ultra-performance Liquid Chromatography/Time-of-Flight Mass Spectrometry

Min Hao, De Ji, Lin Li, Lianlin Su, Ji Zhang, Qiaohan Wang, Wei Gu, Chengxi Jiang[1,2], Tulin Lu, Chunqin Mao

School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, [1]School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, [2]Life Sciences Institute, Wenzhou University, Wenzhou, China

Submitted: 17-07-2018 Revised: 05-09-2018 Published: 23-01-2019

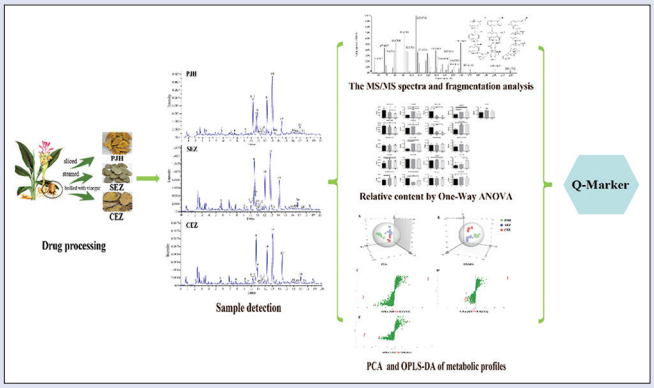
ABSTRACT

Background: In China, rhizome of *Curcuma wenyujin* (CW) is used to improve blood stasis syndrome-related diseases for many years. Nonsteamed, steamed, and boiled with vinegar rhizomes of CW can be used as three different traditional Chinese medicine s, named as Pian-Jiang-Huang (PJH), Sheng-E-Zhu (SEZ), and Cu-E-Zhu (CEZ), respectively. After processing, the therapeutic effects have changed. **Objective:** In order to illustrate the effective substance of the three kinds of rhizome of CW, an ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q/TOF-MS) method coupled with statistical approach was developed. **Materials and Methods:** Fresh rhizomes of CW were processed into PJH, SEZ, and CEZ according to Chinese pharmacopoeia 2015. UPLC-Q/TOF-MS coupled with chemometric analysis was applied to compare the differences in chemical profiles of the three kinds of rhizome of CW. **Results:** In this study, 21 compositions in the rhizome of CW were identified. The one-way ANOVA of relative intensities of the 21 compositions was processed by SPSS software version 20.0. The column superposed graph of the relative intensities of 21 components shows that the total relative intensities of the 21 components in SEZ are the topmost and that of PJH is about the same as CEZ, but the proportion of each component is quite differ from each sample. Principal component analysis and orthogonal partial least squares discrimination analysis were processed by Simca-p14.1 software. Finally, five ions including curcumenone, curcumol, curzerenone, furanodiene, and germacrone were discovered to be the Q-Makers of the three kinds of rhizome of CW. **Conclusion:** This study provides a reliable research basis for the clinical application of the three kinds of rhizome of CW.

Key words: Boiled with vinegar, multivariate statistical analysis, Q-Marker, rhizome of *Curcuma wenyujin,* steamed, ultra-performance liquid chromatography/time-of-flight mass spectrometry

SUMMARY

- The relative content of the chemical components varied greatly among the three kinds of rhizome of *Curcuma wenyujin*
- Five ions including curcumenone, curcumol, curzerenone, furanodiene, and germacrone were discovered to be the Q-Makers of the three kinds of rhizome of *C. wenyujin*
- Chemometric analysis provided an accurate and strong proof to identify these three kinds of rhizome of *C. wenyujin*.



Abbreviations used: PJH: Pian-Jiang-Huang; SEZ: Sheng-Er-Zhu; CEZ: Cu-Er-Zhu; CW: *Curcuma wenyujin*; PCA: Principal component analysis; OPLS-DA: Orthogonal partial least squares discrimination analysis; RT: Retention time; TCM: Traditional Chinese medicine; TIC: Total ion chromatogram; QC: Quality control; RSD: Relative standard deviation.

Correspondence:

Dr. Tulin Lu,
Nanjing University of Chinese Medicine, No. 138, Xianlin Road,
Qixia District, Nanjing, Jiangsu, PR China.
E-mail: lutuling2005@126.com
Prof. Chunqin Mao,
Nanjing University of Chinese Medicine,
No. 138, Xianlin Road, Qixia District, Nanjing,
Jiangsu, PR China.
E-mail: mcq63@163.com
DOI: 10.4103/pm.pm_399_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Before the arrival of Western medicine into China, ancient Chinese people relied on traditional Chinese medicine (TCM) and its processed products to treat various human diseases and achieved remarkable effects. *Curcuma wenyujin* (CW) is mainly planted in Wenzhou, Zhejiang province, in China, which has been widely used in clinic because of its various pharmacological activities. Nonsteamed, steamed, and boiled with vinegar rhizome of CW have been used as three different herbal medicines named as Pian-Jiang-Huang (PJH), Sheng-Er-Zhu (SEZ), and Cu-Er-Zhu (CEZ), respectively.[1] All of them have been used to treat blood stasis, inflammation, pain, and tumor, but each has its own characteristics in clinic.[2] The anti-inflammatory effect of PJH is even more prominent. SEZ is good at treating blood stasis, whereas CEZ can

Cite this article as: Hao M, Ji D, Li L, Su L, Zhang J, Wang Q, *et al.* Metabolic profiling analysis of three processed rhizomes of *Curcuma wenyujin Y.H. Chen et C. Ling* by ultra-performance liquid chromatography/time-of-flight mass spectrometry. Phcog Mag 2019;15:164-71.

enhance hepatoprotective effect. The same medical plant parts have different effects after different processing methods. The material basis must have changed significantly. Therefore, in order to more effectively and safely use TCM and its processed products, an in-depth analysis of the chemical composition of TCM is very necessary by more sensitive and accurate analytical methods.[3]

Nontargeted metabolomics is a potent tool for the systematic biomarker discovery among different biological classes and thus is very attractive in the authentication of TCM, especially those with similar chemical compositions. Typically, a nontargeted metabolomics approach is composed of global metabolite profiling and biomarker discovery. Among the approaches used for metabolite profiling, ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC-Q/TOF-MS) is the most powerful,[4] and it has been widely accepted to be the predominant tool for qualitative analysis of herbal medicines in complex samples as it provides favorable specificity and sensitivity.[5-7] Programmed data analysis in metabolomics is achievable by the software platforms, such as XCMS (www.bioconductor .org, Seattle, Washington, USA), Progenesis QI (Waters Corporation, Milford, Connecticut, USA), SIEVE (ThermoFisher Corporation, Waltham, Massachusetts, USA), and SIMCA-P (Umetrics Corporation, Malmo, Sweden). Nontargeted metabolomics has been employed to differentiate TCMs derived from different congeneric species,[8] different parts of an herb,[9] different production regions,[10] even different ages,[11] etc.

Modern pharmacological studies have shown that the rhizome of CW possesses wide activities, such as antitumor,[12,13] anti-inflammatory,[2] and antiviral activities.[14] The rhizome of CW is rich in volatile components consisting of monoterpenes and sesquiterpenes. It was reported that eucalyptol, camphor, isoborneol, borneol, b-elemene, curzerene, germacrone, curdione, neocurdione, and curcumenone were the dominant and bioactive ingredients.[15-17] In addition, curcuminoids in the rhizome of CW including curcumin, demethoxycurcumin, and bisdemethoxycurcumin have also demonstrated various pharmacological activities such as anti-inflammation, antimicrobial, anti-oxidation, antiparasitic infection, antimutagenic effect, and anticancer.[18] Previous phytochemical researches were limited to analyze the chemical compositions of SEZ and CEZ by gas chromatography–mass spectrometry method. However, several sesquiterpenoids in the rhizome of CW are heat-sensitive components. For instance, furanodiene degrades to curzerene through a 3,3-sigmatropic reaction[19] and (4S, 5S)-germacrone-4, 5-epoxide cyclizes via a transannular reaction under heating condition.[20] Moreover, little information was available about the difference of chemical constituents among three of them. Therefore, in this study, a systematic, comprehensive, and untargeted UPLC-Q/TOF-MS metabolomics approach along with one-way ANOVA analyses was designed to assess and characterize the differentiations of three kinds of processing materials of rhizome of CW.

MATERIALS AND METHODS

Sample collection and processing

In this research, 15 batches of fresh rhizome of CW were collected from Rui-an, Zhejiang, China, in December, 2016. The samples were identified as rhizome of CW by Professor Lu Tulin from Nanjing University of Chinese Medicine. Each batch of samples was divided into three parts on an average, and then processed into PJH, SEZ, and CEZ according to Chinese pharmacopoeia 2015, respectively. Specific processing methods were as follows: (1) PJH: One-third of the fresh rhizome was sliced in 3-mm thickness directly and oven dried (No. P1–P15); (2) SEZ: One-third of the fresh rhizome was steamed 1.5 h, then sliced in 3 mm thickness, and oven dried (No. S1–S15); and (3) CEZ: The last part of the fresh rhizome was boiled with vinegar until the water was finished (per 100 kg samples with vinegar 20 kg, percentile of vinegar is 20% approximately) and oven dried (No. C1–C15). All samples were oven dried by forced air at 50°C about 36 h until moisture content was ≤14% according to Chinese pharmacopoeia 2015. Before this study, all the samples underwent a strict quality evaluation, including water, ash, and total volatile oil, according to the Chinese pharmacopoeia 2015 edition Part 1.[1]

Chemicals and reagents

Reference substances

Curdione (Lot: K19D5C1), germacrone (Lot: P10S6F3197), curcumol (Lot: RM0331FB14), curcumin (R25M6S1), and demethoxycurcumin (Lot: Y14J7S9060) were purchased from Yuanye Biotechnology Co. Ltd., Shanghai, China. The purities of the compounds were all ≥98%.

Liquid chromatography-mass spectrometry (LC-MS)-grade acetonitrile, LC-MS-grade methyl alcohol, high-performance liquid chromatography-grade methanoic acid, (Merck Co. Inc., Germany), and ultra-pure grade water were obtained from a Milli-Q system (Millipore, Bedford, MA, USA). The other solvents were of analytical grade. SPE columns (CNWBOND, LC-C_{18}, 500 mg/3 ml) were purchased from ANPLE scientific Instrument (Shanghai, China).

Sample preparation

Different rhizomes of CW samples were powdered to pass through 65-mesh sieve. The 0.5 g sample powders were weighed precisely and those powders were extracted ultrasonically (200 W, 40 kHz) with 70% ethanol (v/v, 20 ml) for 45 min at room temperature. The extracted solution was then filtered through a filter paper. After that, 4 ml of the filter liquor was loaded onto a C18 RP SPE column and the gradient elution was conducted in the following sequence: 1 mL of 20% ethanol in water (20:80, v/v), 1 mL of 40% ethanol in water (40:60, v/v), 1 mL of 60% ethanol in water (60:40, v/v), 1 mL of 80% ethanol in water (80:20, v/v), and 1 mL of ethanol. After that, all of the eluents were collected and fully mixed. Then, the mixed liquor was centrifuged at 12,000 for 5 min, and the supernatant was taken to test.

Standard solution preparation

Standard stock solutions were separately prepared by dissolving the accurately weighed five reference substances (curdione, germacrone, curcumol, curcumin, and demethoxycurcumin) with methanol. A mixed reference substance solution was obtained by mixing germacrone, curcumol, curcumin, demethoxycurcumin, and bisdemethoxycurcumin stock solutions above to obtain a final concentration of 5 μg/ml for each compound approximately. In addition, curdione and curzerene were diluted to 5 μg/ml (these two are isomers).

Apparatus and chromatographic conditions

Shimadzu UPLC (Japan) coupled with LC-30AD Binary liquid pump, SIL-30SD autosampler, DGU-20A5R On-Line Solvent Degasser, CTO-30A column oven, AB SCIEX Triple TOF 5600+ system coupled with ESI source. Data acquisition software: Analyst TF 1.6 software (AB Sciex Corporation, Redwood, California, USA); data processing software system: Peakview1.2 software (AB Sciex Corporation, Redwood, California, USA), Simca-P14.1 software (Umetrics Corporation, Malmo, Sweden) and Markerview1.2.1 software (AB Sciex Corporation, Redwood, California, USA); SPE column, CNC ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd); BP121S electronic analytical balance (Mettler Toledo).

Chromatographic condition

Agilent C18 reversed phase column (2.1 mm × 100 mm, 1.8 μm), mobile phase A (0.1% formic acid aqueous solution)-B (acetonitrile), gradient elution program: 0–1 min, 5%~25%B; 1–3 min, 25%~30%B; 3–13 min, 30%~55%B; 13–15 min, 55%~70%B; 15–25 min, 70%~100%B; 25–28 min, 100%~5%B; flow rate: 0.3 ml/min; column temperature: 35°C; Injection volume: 1 μl.

Mass spectrometer condition

ESI source, data collection in positive ion mode. The source parameters are set as follows: Ion spray voltage floating: +4500/−4500; declustering potential: +60/−60 V; source temperature: 550°C; the atomizing gas is N_2, Curtain gas: 35 psi; Gas 1 (nebulizer gas): 55 psi; Gas 2 (heater gas): 55 psi; Collision energy: +35/−35eV; Using tandem mass spectrometry (MS/MS) secondary mass spectrometry mode: The MS spectrometer ion scanning range is m/z 100–2000. The MS/MS spectrometer ion scanning range is m/z 50–1000; dynamic background subtraction is turned on.

Validation of ultra-performance liquid chromatography/time-of-flight mass spectrometry method

The method was validated by a quality control (QC) sample. The QC sample solution was obtained by mixing equal volumes of all sample extracts to provide an average sample which contains all the chemical components at an average content. Before sample test, the QC sample needs to be analyzed six times to examine the precision of the system. After every five tested samples, the QC sample needs to be tested one time to monitor the stability of the analysis condition. The QC sample also needs to be tested at 2, 4, 6, 8, 12, and 24 h to inspect the stability of the sample. To validate the repeatability, six repeat QC samples were extracted and analyzed. The metabolites of demethoxycurcumin (retention time [Rt] 11.23, m/z 339.1230), curcumin (Rt 11.57, m/z 369.1339), curdione (Rt 13.03, m/z 237.1848), curcumol (Rt 16.55, m/z 237.1850), and germacrone (Rt 17.02, m/z 219.1745) which were identified by standard substances were selected for the method validation in the positive ion mode. The relative standard deviation of ion strength and Rt of the selected metabolites were calculated to value the reproducibility and stability of the method. The results of the method validation are shown in Table 1.

Data processing and multivariate statistical analysis

The MarkerView1.2.1 software (AB Sciex, USA) was used to perform peak detection and alignment of the raw UPLC-Q/TOF-MS data; the parameters were set as follows: minimum spectral peak width: 25 ppm; minimum RT peak width: 6 scans; noise threshold: 100; mass tolerance within 10 ppm; Rt tolerance within 0.5 min; and use area integrated from raw data. After normalized to the total ion intensity per chromatogram, the original data were translated into three-dimensional data matrices including peak name (Rt −m/z value), sample ID, and normalized peak areas. Then, the three-dimensional data matrices were imported into the Simca-P14.1 software (Umetrics AB, Sweden) for multivariate chemometrics analysis, including principal component analysis (PCA) and orthogonal partial least squares discrimination analysis (OPLS-DA). The predictive capability of model was evaluated by the R^2X (cum) and Q^2X (cum) values in its score plot. The R^2X (cum) indicates the explanatory capacity of the variables, whereas the Q^2X (cum) value represents the predictive capability of the model. The method indicates good fitness when the R^2X (cum) and Q^2 (cum) values were both close to 1.0. A scatter plot (S-plot) of OPLS-DA was conducted to find the potential chemical biomarkers among the three kinds of samples.

RESULTS

Overall analysis of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu

First, from the perspective of color, the transection of PJH is yellowish-brown. After process, the color of the transection of SEZ and CEZ deepens in varying degrees [Figure 1a]. Second, by comparing of the typical total ion chromatogram (TIC) in both positive and negative ion modes, it was found that the components were more sensitive in positive ion mode than that in negative ion mode. TICs of PJH, SEZ, and CEZ in positive ion mode are shown in Figure 1c-e. It can be seen from the TIC that there is a great difference among the three, especially in 3–4 min, 7 min, and 9–11 min. TIC of PJH has two peaks between 3 and 4 min, but TIC of SEZ and CEZ has three peaks, what's more, the middle peak of CEZ between 3 and 4 min is lower than that of SEZ. TIC of PJH has a lower peak compared with SEZ and CEZ at 7 min. In addition, there are some changes in these peaks appearing within 9–11 min and the peak in 10 min is reduced obviously after processing. As a whole, the TIC of PJH is more different from SEZ and CEZ. Last, the average values of total ionic strength of three kinds of samples which were extracted by PeakView1.2 software were analyzed by one-way ANOVA by SPSS Statistics Software version 20.0 (IBM, Chicago, IL, USA). The result shows that the total ionic strength of PJH was significantly different from that of SEZ and CEZ and there was no statistical significance between SEZ and CEZ [Figure 1b]. The statistical differences of the total ionic strength of the three samples reflect the quality differences partly. In addition to the overall analysis of the three samples, the internal differences among them need further multivariate statistical analysis.

Identification of chemical compositions in Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu

A chemical composition database in PJH, SEZ, and CEZ was established by searching PubMed, ChemSpider, SciFinder Mass Bank, and Chinese National Knowledge Infrastructure. This database includes Rt, molecular formula, accurate molecular weights, indicated molecular weights, tolerance (ppm), prominent MS2 fragmentation, compound name, and compound types. Analyst TF 1.6 software (AB Sciex, USA) is used to collect the raw data of PJH, SEZ, and CEZ. Then, these raw data would be analyzed by Peakview 1.2 software through the information of element compound and fragmentations of the different peaks matched

Table 1: Method validation of precision, repeatability, and stability of five identified components

Compounds	Precision (*n*=6)		Repeatability (*n*=6)		Stability (*n*=6)	
	Rt (RSD %)	Relative intensity (RSD %)	Rt (RSD %)	Relative intensity (RSD %)	Rt (RSD %)	Relative intensity (RSD %)
Demethoxycurcumin	1.74	3.29	0.98	2.44	0.57	2.62
Curcumin	3.36	4.90	2.13	3.76	1.06	3.75
Curdione	2.81	5.47	1.70	4.81	1.59	3.66
Curcumol	2.93	3.04	1.39	3.43	3.28	4.31
Germacrone	1.26	2.83	2.68	5.06	2.67	4.99

Rt: Retention time; RSD: Relative standard deviation

with the information in the established chemical composition database. Finally, the compounds could be identified when their purity scores were all above 80%, ions in different samples illustrated both the same m/z value (tolerance of 10 ppm) and the same Rt (tolerance of 0.2 min).

In this study, 21 chemical compositions were identified in positive ion mode [Figure 2]. Five of them including demethoxycurcumin, curcumin, curdione, curcumol, and germacrone were identified by comparison with reference substance and literature.[21,22] The last 16 chemical compounds were identified in positive ion mode by comparing the accurate mass, Rt, mass spectrometric fragmentation characteristic ions, and matching empirical molecular formula with the database of chemical components that has been established. The details of these 21 components are presented in Table 2. The MS/MS spectra and fragmentation of germacrone and curcumin are shown in Figures 3 and 4. Germacrone produced $[M + H]^+$ ions at m/z 219.1732, 201.1635, 177.1280, 163.1116, 159.1165, 145.1010,

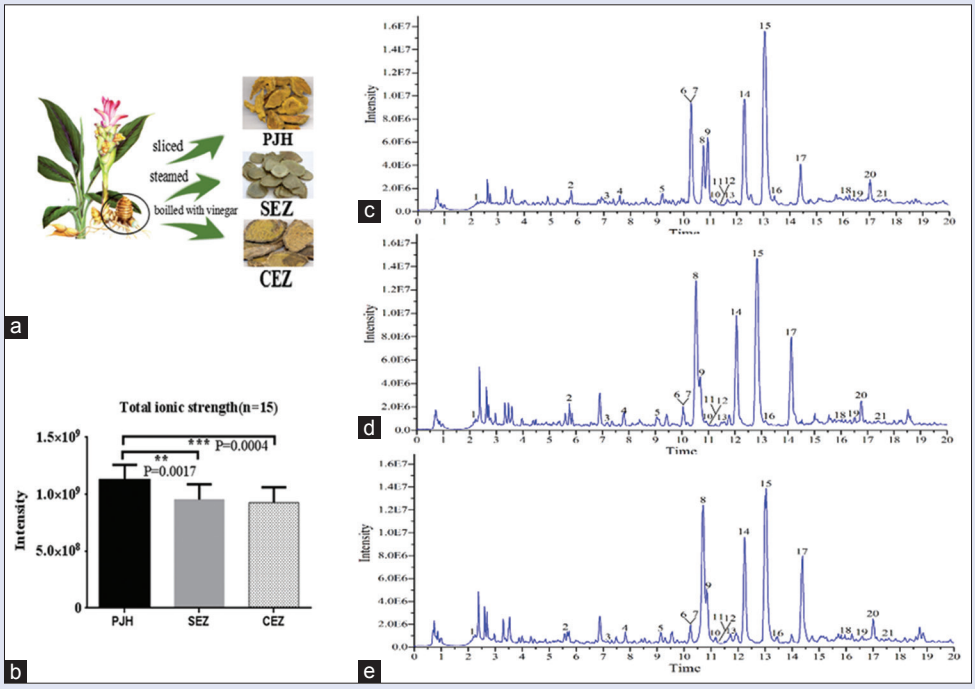


Figure 1: (a) The processing of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu. (b) Total ionic strength of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu. Data represent the mean values ± standard deviation (n = 15). $^{**}P < 0.05$, $^{***}P < 0.001$. (c-e) The typical total ion chromatograms of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu in positive ion mode

Table 2: Identification of 21 chemical compounds by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry

Rt (min)	Formula	M (Da)	$[M+H]^+$ (m/z)				Metabolite class
			Indicated	ppm	Prominent MS^2 fragmentation	Identification	
2.16	$C_{15}H_{20}O_5$	280.1310	281.1389	1.8	281, 245, 227, 203, 156, 107, 91	Zedoalactone B	Guaiane type
5.74	$C_{15}H_{22}O_3$	250.1569	251.1644	1.1	233, 215, 191, 173, 165, 159, 145	Aerugidiol	Guaiane type
7.06	$C_{15}H_{20}O_4$	264.1362	265.1434	2.5	247, 229, 121, 105, 91	Zedoarofuran	Eudesmane type
7.51	$C_{15}H_{18}O_3$	246.1256	247.1333	1.5	247, 229, 189, 183, 128, 105	Zederone	Germacrane type
9.16	$C_{15}H_{24}O_3$	252.1725	253.1800	0.6	253, 235, 189, 175, 147, 133, 107	Zedoarondiol	Guaiane-type
10.25	$C_{15}H_{20}O$	216.1514	217.1588	0.3	217, 199, 169, 157, 128, 131, 55	Furanodiene	Germacrane-type
10.25	$C_{15}H_{22}O_2$	234.1620	235.1693	0.3	235, 217, 189, 135	Curcumenol	Guaiane-type
10.71	$C_{15}H_{22}O_2$	234.1620	235.1693	0.5	235, 177, 161, 105, 91	Curcumenone	Carane type
11.15	$C_{15}H_{16}O$	212.1201	213.1274	−0.2	213, 197, 155, 128, 69	Pyrocurzerenone	Laserane type
11.17	$C_{15}H_{18}O_2$	230.1307	231.1381	−0.2	231, 213, 185, 157, 142, 128	Curzerenone	Elemane type
11.20	$C_{21}H_{22}O_6$	370.1416	371.1489	0.5	371, 177, 137, 117	Dihydrocurcumin	Curcumins type
11.23	$C_{20}H_{18}O_5$	338.3539	339.1230	1.0	339, 255, 223, 177, 147, 119	Demethoxycurcumin	Curcumins type
11.57	$C_{21}H_{20}O_6$	368.1260	369.1339	1.0	369, 285, 253, 177, 145, 117	Curcumin	Curcumins type
12.25	$C_{15}H_{24}O_2$	236.1776	237.1851	0.9	237, 219, 135, 107, 93, 81	Neocurdione	Germacrane type
13.03	$C_{15}H_{24}O_2$	236.1776	237.1848	−0.5	219, 135, 107, 93, 79, 67	Curdione	Germacrane type
13.47	$C_{15}H_{16}O_2$	228.1150	229.1223	2.1	229, 213, 201	Curzeone	Cadinane type
14.37	$C_{15}H_{20}O_2$	232.1463	233.1536	0.8	233, 175, 147, 105, 119, 91	Furanogermenone	Germacrane type
16.38	$C_{15}H_{20}O_3$	248.1410	249.1488	1.0	249, 203, 163, 143, 105, 91, 69	Curcumenolactones A	Carane type
16.55	$C_{15}H_{24}O_2$	236.1776	237.1850	0.5	237, 163, 135, 95, 69	Curcumol	Guaiane-type
17.02	$C_{15}H_{22}O$	218.1671	219.1745	0.5	219, 159, 129, 105, 81	Germacrone	Germacrane-type
17.48	$C_{15}H_{24}O$	220.3505	221.1900	0.2	221, 203, 151, 123, 109, 95, 81	Bisacurol	Bisabolane type

*Means this component is identified by compared with standard substance. Rt: Retention time

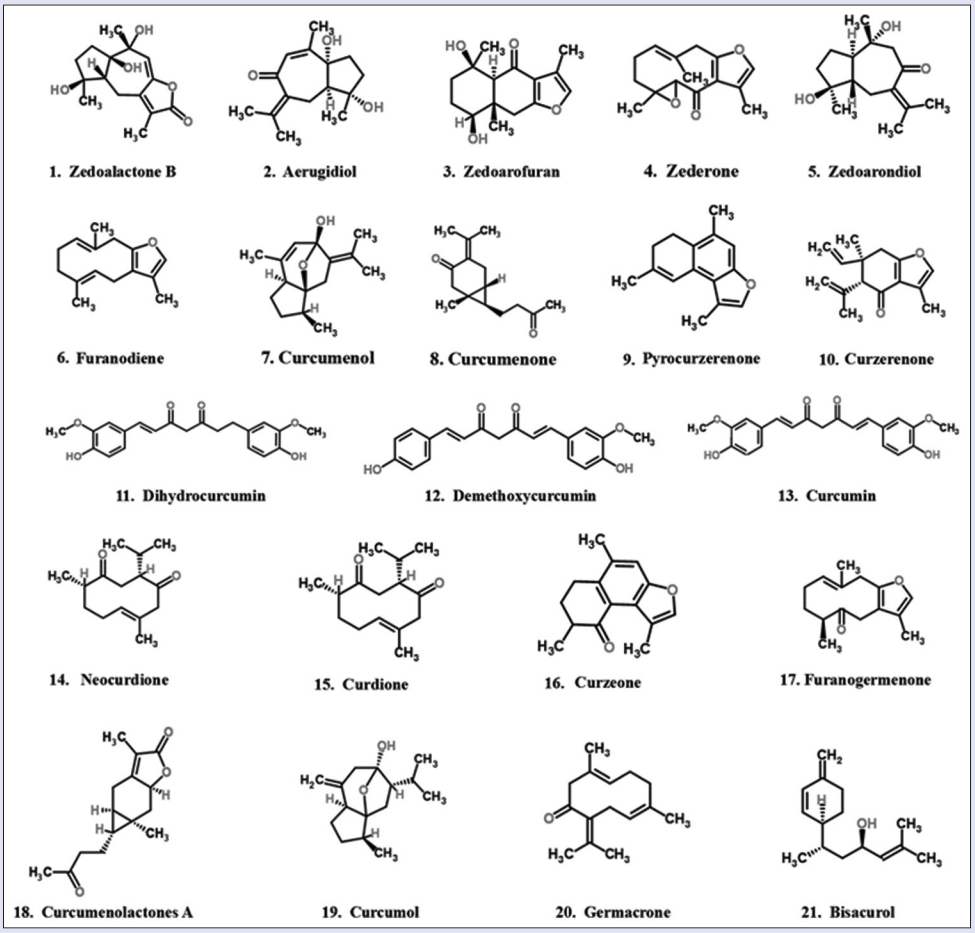


Figure 2: The structure of 21 compounds

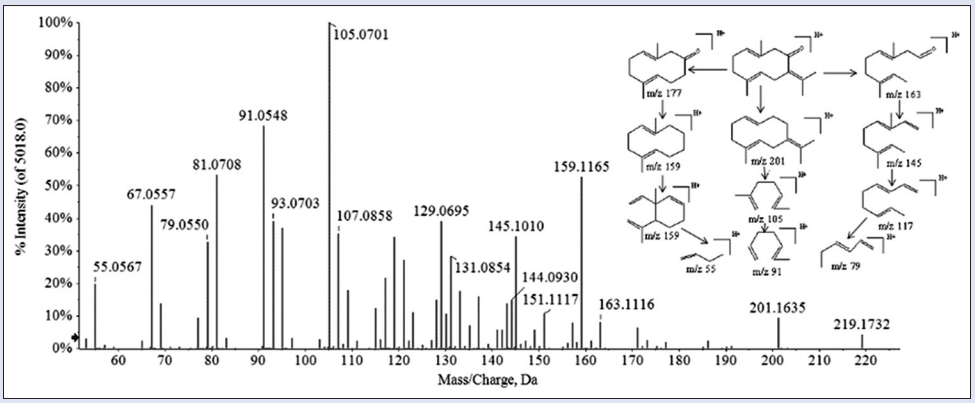


Figure 3: The tandem mass spectrometry and fragmentation of germacrone in positive ion mode

105.0701, and 91.0548, respectively. The characteristic loss of 18 Da, 42 Da and 56 Da represent H_2O, C_3H_6, and C_4H_8, respectively. Curcumin produced $[M + H]^+$ ions at m/z 369.1296, 285.1116, 253.0859 177.0541, 145.0283, and 117.0334, respectively. The characteristic loss of 84Da, and 177Da represent $-CH_3$+COH = CHCO, and $C_{10}H_9O_3$, respectively.[23] The 21 compounds can be found in all the three kinds of samples, but the relative intensities are large differences and the relative intensities of some ingredients are increased after processing, this is probably due to constituent transformation in the sample processing.[24] The relative intensities of 21 components identified was statistically analyzed by one-way ANOVA using SPSS 20.0 statistical software (n = 15). The results show that the relative intensities of four components including zedoalactone B, zedoarofuran, curcumenol, and curdione are gradually declining in SEZ and CEZ. The relative intensity of curcumenolactones A in SEZ is lower than that of PJH, but it is higher in CEZ. We speculate that this maybe related with acidic condition. As a whole, the relative intensities of 11 components in SEZ and CEZ are higher than those of PJH. Among the 11 compounds, the relative intensities of two components including zederone and curzeone in CEZ are

higher than those of SEZ; the relative intensities of aerugidiol, furanodiene, and curcumin in CEZ are lower than those of SEZ; the relative intensities of curcumenone, curzerenone, demethoxycurcumin, furanogermenone, germacrone, and bisacurol have no significant change from SEZ to CEZ; the relative intensities of four components including pyrocurzerenone, dihydrocurcumin, neocurdione, and curcumol have no significant change in all the three kinds of samples [Figure 5]. In addition, the column superposed graph of the relative intensities of 21 components of three kinds of samples (n = 15) is shown in Figure 5; it shows that the total relative intensities of 21 components in SEZ are the topmost and that of PJH is about the same as that of CEZ, but the proportion of each component is quite different from PJH, SEZ, and CEZ.

Principal component analysis of metabolic profiles of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu

In order to evaluate the overall quality change between the three kinds of samples, PCA was performed based on metabolic profiles which have been translated into three dimensional data matrices above. The PCA 3D score-plot is shown in Figure 6a. The R^2X (cum) and Q^2X (cum) of the PCA model are 0.81 and 0.69, respectively, which indicates that the PCA model has good fitness. The result of PCA shows that 45 batches of samples can be clearly divided into three groups. The quality markers (Q-Makers) lead to the difference between the three kinds of samples, which needs further OPLS-DA model to search.

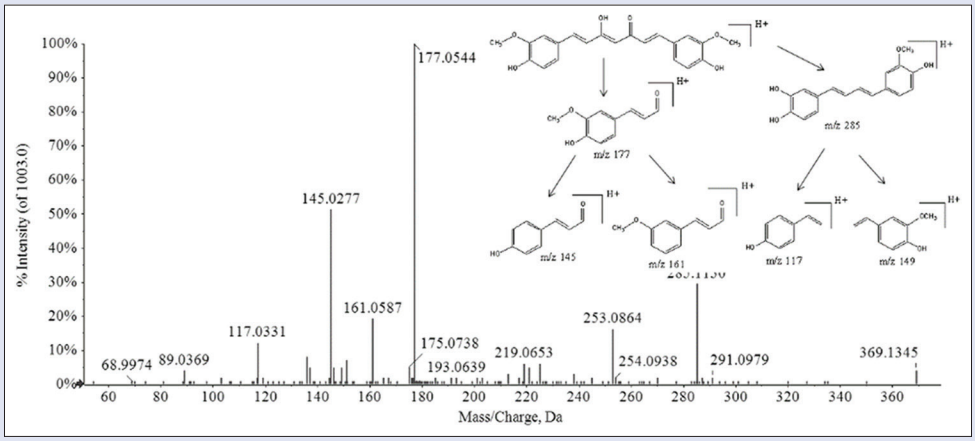


Figure 4: The tandem mass spectrometry and fragmentation of curcumin in positive ion mode

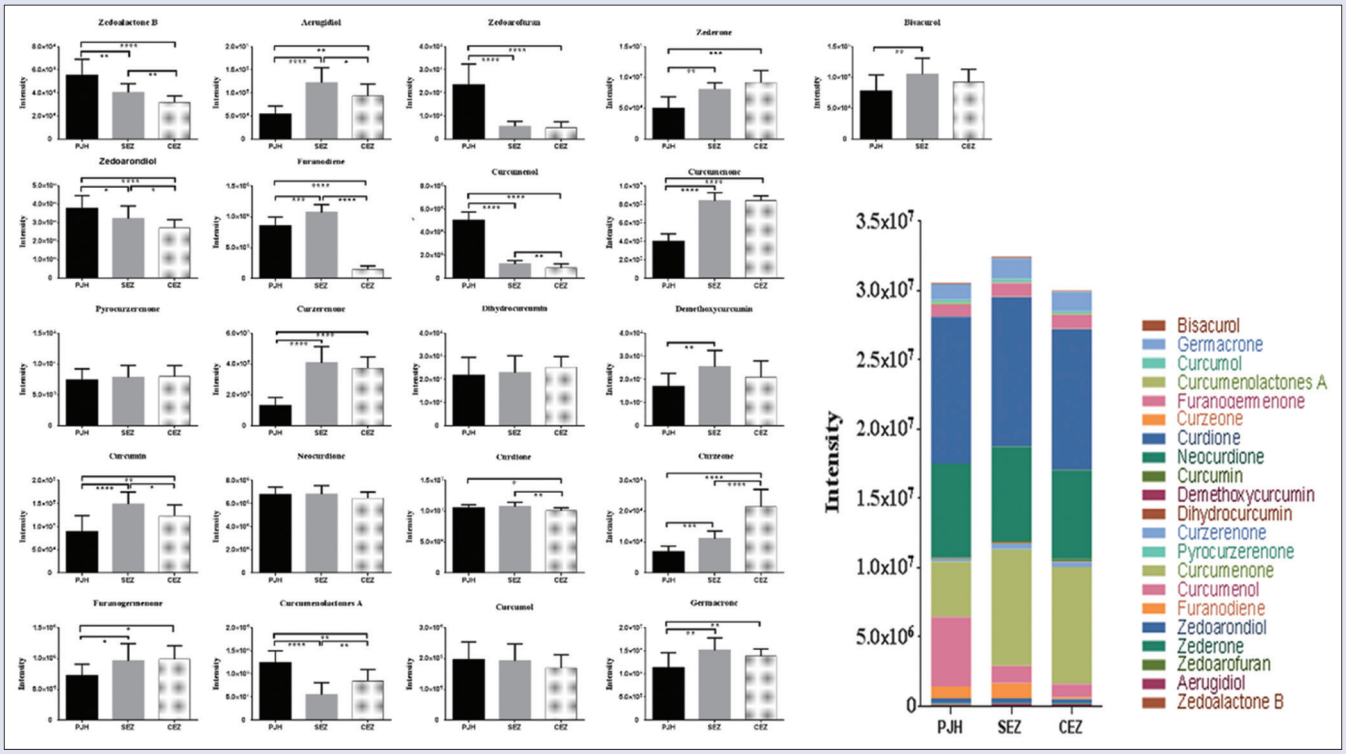


Figure 5: Left: Relative intensities of the 21 components in Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu. Data represent the mean values ± standard deviation (n = 15). *, **, ***, and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively. Right: The column superposed graph of the relative intensities of the 21 components of three kinds of samples

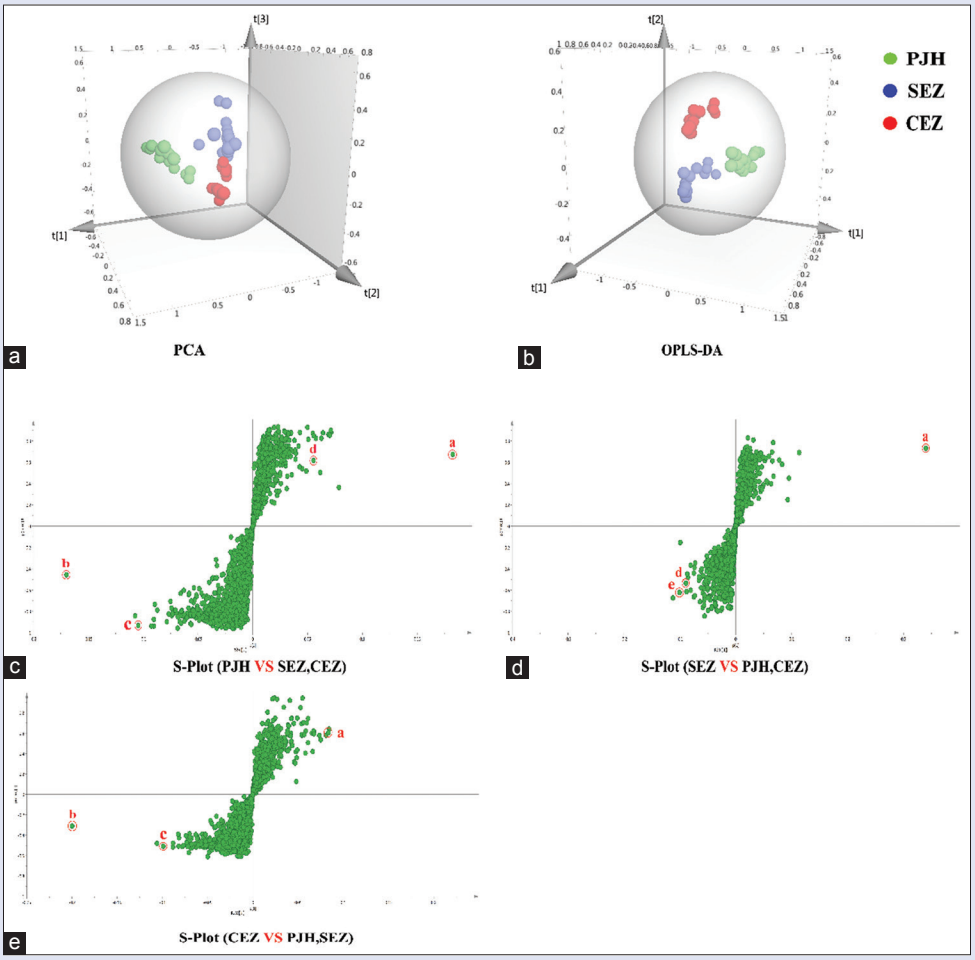


Figure 6: (a) Principal component analysis score plot of metabolic profiles of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu. (b) Orthogonal partial least squares discrimination analysis score plot of metabolic profiles of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu. (c-e) S-plots of orthogonal partial least squares discrimination analysis constructed based on a comparison metabolic profiles of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu. The dot in red circle: (a) Curcumenone (b) curcumol (c) curzerenone (d) furanodiene (e) germacrone

Q-markers of Pian-Jiang-Huang, Sheng-Er-Zhu, and Cu-Er-Zhu by orthogonal partial least squares discrimination analysis

The R^2X (cum) and Q^2X (cum) of the OPLS-DA model are 0.80 and 0.97, respectively, which indicates that the OPLS-DA model has good fitness. An S-plot of the OPLS-DA was structured to confirm the identities of the Q-markers, as well as to identify the characteristic chemicals of each samples [Figure 6b]. In this study, we took PJH as an example. The 15 batches of PJH samples were marked as one group, whereas the remaining 30 batches of SEZ and CEZ samples were defined as the second group, which formed the basis of the OPLS-DA model. The result of the S-plot is shown in Figure 6c. In this S-plot, every point represents an ion m/z–Rt pair. The X axis represents the contribution of the variables, whereas the Y axis represents the confidence of the variables. The farther the data point from the center, the greater contribution of this point to the separation of the two groups. Therefore, the points at either ends of the S-shaped curve represent potential Q-markers with the highest contribution.[25] The result turns out that four ions (a, b, c, and d) at the end of the S-shaped curve were considered to be Q-Makers capable of discriminating PJH from SEZ and CEZ [Figure 6c]. Three ions (a, d, and e) were found to be Q-markers which made the greatest contribution to distinguish SEZ from PJH and CEZ [Figure 6d]. Three ions (a, b, and c) were found to be Q-markers for the CEZ derived from PJH and SEZ [Figure 6e].

DISCUSSION

Optimization of extraction method and chromagraphic separation

The extraction methods (ultrasonic and reflux extraction) were performed by the single-factor test with different extraction solvents (50%, 70%, 90%, and 100% ethanol and 50%, 70%, 90%, and 100% methanol) and different extraction time (30, 45, and 60 min). Finally, the samples were ultrasonically extracted in 70% methanol for 45 min because of its best extraction rate. In order to obtain higher separation efficiency and sufficient chromatographic resolution, chromatographic conditions such as mobile phase and column temperature were optimized. At last, the chromatographic conditions were optimized as a mixture of acetonitrile and 0.1% (v/v) aqueous formic acid solution at a temperature of 30°C.

Optimization of mass spectrometric detection

Positive and negative ion MS modes were both estimated to analyze fragments as comprehensively as possible, and mass spectrometric detection condition was investigated, including the collision energy, ion spray voltage, turbospray temperature, and declustering potential.

The repeatability and sample stability evaluation results show that the sample can be used for analysis by UPLC-Q/TOF-MS within 12 h after preparation, which indicates the stability and reliability of the method.

Possible chemical conversion in the sample processing

A previous study indicated that the contents of eucalyptol and borneol camphor in SEZ were less than those in PJH for the low-boiling point monoterpenoids in SEZ were distilled off during the high temperature steaming process.[3] In addition, we have already mentioned that several sesquiterpenoids including furanodiene and (4S, 5S)-germacrone-4, 5-epoxide in the rhizomes of CW are heat-sensitive components. Therefore, we speculate that the clinical efficacy differences among different processing products of rhizome of CW are closely related with chemical conversion. In a word, composition changes during processing remain to be explored in-depth.

CONCLUSION

An UPLC-Q/TOF-MS method coupled with multivariate statistical analysis has been successfully applied to analyze the changes of chemical components among PJH, SEZ, and CEZ. In this study, 21 chemical compositions in the rhizome of CW were identified by standard substance, mode of target compounds searching and literature. The relative intensities of 21 compositions vary greatly after processing. An OPLS-DA S-plot has been constructed to identify curcumenone (a), curcumol (b), curzerenone (c), furanodiene (d), and germacrone (e) as unique chemical markers of PJH, SEZ, and CEZ.

Changes in the chemical components of herbs could be the main reason for their change in pharmacological effects. In this article, a study on different components in PJH, SEZ, and CEZ could provide a scientific basis for the basic research of pharmacodynamics, while the relationship between efficacy and components remains to be verified.

Acknowledgement

This research was financially supported by the National Natural Science Foundation of China (81673598, 81473349, 81503249), the National traditional Chinese medicine industry special project (2015468002-2), National Standardization Program for Chinese Medicine (ZYBZH-Y-SC-40), The Opening Project of Zhejiang Provincial Top Key Discipline of Pharmaceutical Sciences (201701), Research and Innovation program for graduate students in Jiangsu Province, China (KYCX18_1607), and Graduate Research and Innovation Project of Jiangsu province (KYCX18_1607).

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. The Pharmacopoeia Committee of China. Pharmacopoeia of the People's Republic of China. Vol. 1. Beijing: Chemical Industry Press; 2015.
2. Zhou J, Qu F, Zhang HJ, Zhuge XH, Cheng LZ. Comparison of anti-inflammatory and anti-nociceptive activities of *Curcuma wenyujin* Y.H. Chen et C. Ling and *Scutellaria baicalensis* Georgi. Afr J Tradit Complement Altern Med 2010;7:339-49.
3. An YW, Hu G, Yin GP, Zhu JJ, Zhang QW, Wang ZM, *et al.* Quantitative analysis and discrimination of steamed and non-steamed rhizomes of *Curcuma wenyujin* by GC-MS and HPLC. J Chromatogr Sci 2014;52:961-70.
4. Gika HG, Theodoridis GA, Plumb RS, Wilson ID. Current practice of liquid chromatography-mass spectrometry in metabolomics and metabonomics. J Pharm Biomed Anal 2014;87:12-25.
5. Liu K, Song Y, Liu Y, Peng M, Li H, Li X, *et al.* An integrated strategy using UPLC-QTOF-MSE and UPLC-QTOF-MRM (enhanced target) for pharmacokinetics study of wine processed *Schisandra chinensis* fructus in rats. J Pharm Biomed Anal 2017;139:165-78.
6. Su D, Li W, Xu Q, Liu Y, Song Y, Feng Y, *et al.* New metabolites of acteoside identified by ultra-performance liquid chromatography/quadrupole-time-of-flight MS (E) in rat plasma, urine, and feces. Fitoterapia 2016;112:45-55.
7. Song Y, Su D, Lu T, Mao C, Ji D, Liu Y, *et al.* Differential pharmacokinetics and the brain distribution of morphine and ephedrine constitutional isomers in rats after oral administration with keke capsule using rapid-resolution LC-MS/MS. J Sep Sci 2014;37:352-9.
8. Sun H, Wang H, Zhang A, Yan G, Han Y, Li Y, *et al.* Chemical discrimination of cortex *Phellodendri amurensis* and cortex *Phellodendri chinensis* by multivariate analysis approach. Pharmacogn Mag 2016;12:41-9.
9. Lv M, Chen J, Gao Y, Sun J, Zhang Q, Zhang M, *et al.* Metabolomics based on liquid chromatography with mass spectrometry reveals the chemical difference in the stems and roots derived from *Ephedra sinica*. J Sep Sci 2015;38:3331-6.
10. Li L, Zhao C, Chang Y, Lu X, Zhang J, Zhao Y, *et al.* Metabolomics study of cured tobacco using liquid chromatography with mass spectrometry: Method development and its application in investigating the chemical differences of tobacco from three growing regions. J Sep Sci 2014;37:1067-74.
11. Kim N, Kim K, Lee D, Shin YS, Bang KH, Cha SW, *et al.* Nontargeted metabolomics approach for age differentiation and structure interpretation of age-dependent key constituents in hairy roots of *Panax ginseng*. J Nat Prod 2012;75:1777-84.
12. Lim CB, Ky N, Ng HM, Hamza MS, Zhao Y. *Curcuma wenyujin* extract induces apoptosis and inhibits proliferation of human cervical cancer cells *in vitro* and *in vivo*. Integr Cancer Ther 2010;9:36-49.
13. Xiao Y, Yang FQ, Li SP, Hu G, Lee SM, Wang YT, *et al.* Essential oil of *Curcuma wenyujin* induces apoptosis in human hepatoma cells. World J Gastroenterol 2008;14:4309-18.
14. Xie CY, Yang W, Ying J, Ni QC, Pan XD, Dong JH, *et al.* B-cell lymphoma-2 over-expression protects δ-elemene-induced apoptosis in human lung carcinoma mucoepidermoid cells via a nuclear factor kappa B-related pathway. Biol Pharm Bull 2011;34:1279-86.
15. Zhang Q, Yang F, Zhu J, Yang L, Wang Z, Zhang Q, *et al.* Essential oil composition of three herbal medicines from *Curcuma wenyujin* by GC-MS. Zhongguo Zhong Yao Za Zhi 2010;35:2590-3.
16. Yang FQ, Li SP, Zhao J, Lao SC, Wang YT. Optimization of GC-MS conditions based on resolution and stability of analytes for simultaneous determination of nine sesquiterpenoids in three species of *Curcuma rhizomes*. J Pharm Biomed Anal 2007;43:73-82.
17. Dang YY, Li XC, Zhang QW, Li SP, Wang YT. Preparative isolation and purification of six volatile compounds from essential oil of *Curcuma wenyujin* using high-performance centrifugal partition chromatography. J Sep Sci 2010;33:1658-64.
18. Kim MK, Choi GJ, Lee HS. Fungicidal property of *curcuma longa* L. Rhizome-derived curcumin against phytopathogenic fungi in a greenhouse. J Agric Food Chem 2003;51:1578-81.
19. Baldovini N, Tomi F, Casanova J. Identification and quantitative determination of furanodiene, a heat-sensitive compound, in essential oil by 13C-NMR. Phytochem Anal 2001;12:58-63.
20. Ishii H, Tozyo T, Nakamura M, Takeda K. Components of the root of *Lindera strychnifolia* VIII-XIII: Structure of isogemafurene and lineroxide. Tetrahedron 1968;24:625-31.
21. Liu F, Bai X, Yang FQ, Zhang XJ, Hu Y, Li P, *et al.* Discriminating from species of *Curcumae radix* (Yujin) by a UHPLC/Q-TOFMS-based metabolomics approach. Chin Med 2016;11:21.
22. Jiang H, Timmermann BN, Gang DR. Use of liquid chromatography-electrospray ionization tandem mass spectrometry to identify diarylheptanoids in turmeric (*Curcuma longa* L.) rhizome. J Chromatogr A 2006;1111:21-31.
23. Shen Y, Han C, Chen X, Hou X, Long Z. Simultaneous determination of three curcuminoids in *Curcuma wenyujin* Y.H.Chen et C.Ling. By liquid chromatography-tandem mass spectrometry combined with pressurized liquid extraction. J Pharm Biomed Anal 2013;81-82:146-50.
24. Ji, ZZ, Zeng SJ. Curdione quantificationally converted to curcumol. J Shenyang Pharm Univ 1985;2:56-60.
25. Wan JB, Bai X, Cai XJ, Rao Y, Wang YS, Wang YT, *et al.* Chemical differentiation of Da-Cheng-Qi-Tang, a Chinese medicine formula, prepared by traditional and modern decoction methods using UPLC/Q-TOFMS-based metabolomics approach. J Pharm Biomed Anal 2013;83:34-42.