Effects of Postharvest Processing and Geographical Source on Phytochemical Variation of *Corydalis Rhizoma*

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Abstract: **Objective** To assess the relative contributions of postharvest processing and geographical source to phytochemical variation of *Corydalis Rhizoma*, and rhizome of *Corydalis yanhusuo*, and to examine what phytochemical components are the most sensitive to the differences of each factor and how they change. **Methods** HPLC fingerprinting and LC-MS coupled with chemometric approaches were applied. **Results** The results of principal component analysis (PCA) and hierarchical cluster analysis (HCA) explicitly demonstrated the postharvest processing could produce a greater impact on the phytochemical profiles of *Corydalis Rhizoma* than geographical source. The contents of most compounds increased after water boiling while decreased after sulphur-fumigation. Protopine, coptisine, and palmatine were the most variable components in processing. Geographical sources also led to a remarkable phytochemical differentiation, in which the environmental variation of the three regions might play a role. Dehydrocorybulbine, coptisine, dehydrocorydaline, and protopine varied most among the three production regions and decreased sequentially in Zhejiang, Shaanxi, and Jiangsu provinces, China. **Conclusion** Both postharvest processing and geographical source should be enhanced with the priority for the former in the quality control of *Corydalis Rhizoma*. The application of boiling is supported but the consistency should be improved in practice. Sulphur-fumigation is strongly suggested to be abandoned.

Key words: chemometrics; *Corydalis yanhusuo*; geographical source; HPLC fingerprinting; phytochemical variation; postharvest processing

DOI: 10.3969/j.issn.1674-6348.2013.02.013

Introduction

Corydalis Rhizoma, the dried rhizome of *Corydalis yanhusuo* W. T. Wang ex Z. Y. Su et C.Y. Wu (Fumariaceae), is one of the most well-known Chinese herbal medicines (CHM), and is reputed to be beneficial for blood activation and pain relief in traditional remedies of emmeniopathy and injuries from falls (Pharmacopoeia Committee of P. R. China, 2010). Quality control (QC) plays a critical role in guaranteeing the efficacy of CHM. Phytochemicals underlying pharmaceutical quality of CHM could be affected by an array of intrinsic and extrinsic factors, of which postharvest processing, i.e., primary processing after harvest, and geographical source may be the most highlighted in the stage of local production (Dong, Zhang, and Lian, 2009). There are a number of geographical regions in China producing *Corydalis Rhizoma*, of which Zhejiang gained a reputation of geographical authenticity. In practice, the harvested rhizomes of *C. yanhusuo* are subject to the insolation directly or following water boiling to produce raw and boiled *Corydalis Rhizoma*, respectively. Sulphur fumigation is occasionally applied for raw materials to prevent mildew diseases during drying and storage despite its prohibited application.

Among the rich and characteristic alkaloids, tetrahydropalmatine (THP), of which the bioactivities have been intensively investigated (He, Gao, and Zhao,

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Received: June 12, 2012; Revised: October 21, 2012; Accepted: January 25, 2013
Fund: Key Breeding Project of Zhejiang Province (2012C12912) and Zhejiang Provincial Natural Science Foundation of China (Y3080087)
Online time: April 20, 2013 Online website: http://www.cnki.net/kcms/detail/12.11410.R.20130420.1112.004.html

2007), was stipulated as the marker compound for QC of *Corydalis Rhizoma* in *Chinese Pharmacopeia*. Previous quantitative analyses indicated that THP content varies greatly among either postharvest processing methods (Ren, Xu, and Yu, 2009; Wang *et al*, 2010a) or different geographical sources of *Corydalis Rhizoma* (Chen, Lu, and Zhang, 2003; Ma *et al*, 2006; Zhou *et al*, 2006; Ding *et al*, 2007; Dou *et al*, 2007). The changing tendencies, however, were inconsistent. Chromatographic fingerprinting gained a rising application in QC of CHM in recent years due to more comprehensive reflection on the phytochemical background (Drasar and Moravcova, 2004; Liang, Xie, and Chan, 2010; Xie *et al*, 2006). Much more alkaloids were qualified and spontaneously quantified in *Corydalis Rhizoma* for QC based on HPLC fingerprinting (Ding *et al*, 2007; Cheng *et al*, 2010; Li *et al*, 2011; Shen *et al*, 2011). The results of its combination with principal component analysis (PCA) supported the differentiation of *Corydalis Rhizoma* among the different geographical sources (Zhou *et al*, 2006).

The impacts of postharvest processing and geographical source have hitherto been assessed separately so that their unclear relative importance could weakly guide the priority of QC for *Corydalis Rhizoma*. Furthermore, it was rarely examined what compounds were the most sensitive to the differences of each factor and how they responded. Therefore, based on a reliable sampling strategy, the authors specifically aimed to explore the relative contributions of postharvest processing and geographical source to the phytochemical variation of *Corydalis Rhizoma*, and to unravel the most responsible alkaloids for the phytochemical variation resulted from the above factors and their changing patterns. Our results would better serve QC and good agricultural practice (GAP) of *Corydalis Rhizoma*.

Materials and methods

Plant materials and sample preparation

We collected the analyzed samples of *Corydalis Rhizoma* with a similar size from different local producers in Zhejiang, Shaanxi, and Jiangsu provinces (Table 1) which were all major producing regions in China. To better assess the impact of the primary processing methods, we collected differentially the processed samples from the same or neighboring village(s). Multiple accessions for each of the three processing types, i.e., raw, boiling, and sulphur-fumigated, were collected from various towns and villages in the three regions for a satisfactory representation. The species was authenticated as *Corydalis yanhusuo* W. T. Wang ex Z. Y. Su et C.Y. Wu by Prof. FU Cheng-xin (Zhejiang University). Voucher specimens were deposited in the Herbarium of Zhejiang University, China. All the samples were dried to a constant weight at 60 ℃ with preceding pulverization and 40-mesh sieving. An accurately weighed sample powder (1.0 g) was ultrasonically extracted with 30 mL 70% ethanol for 30 min, followed by centrifugation for 1.5 min at 1000 r/min. The supernatant was filtered through a 0.45 μm membrane filter prior to HPLC analysis.

HPLC analysis

Chromatograms were generated on an Agilent 1100 series HPLC system, consisting of a quaternary pump, a diode array detector, a vacuum degasser, an ALS auto-injector, a column oven, and a data system (Agilent ChemStation, USA). The chromatographic separation was performed on a Zorbax Extend C_{18} column (250 mm × 4.6 mm, 5 μm) using a gradient of acetonitrile (A) and water (0.06% triethylamine and 0.6% acetic acid, B) programmed as follows: 0—5 min, 5% A; 5—15 min, 5%—10% A; 15—45 min, 10%—30% A; 45—65 min, 30% A; 65—75 min, 30%—85% A. The solvent flow rate was 0.8 mL/min,

Table 1 Sampling information of *Corydalis Rhizoma* surveyed for phytochemical variation

Codes	Geographical sources	Processing methods	Batch No.	Vouchers
ZJR	Pan'an County, Zhejiang Province	raw	7	J. Fan 401—403
ZJB	Pan'an County, Zhejiang Province	boiled	13	J. Fan 404—409
ZJF	Pan'an County, Zhejiang Province	sulphur fumigated	7	J. Fan 410—412
SXR	Chenggu County, Shaanxi Province	raw	7	J. Fan 425—427
JSR	Nantong City, Jiangsu Province	raw	7	J. Fan 511

and the column temperature was maintained at 40 ℃. After screening, the monitor wavelength of 280 nm was selected to visualize a sufficiently large number of detectable peaks in the chromatograms.

LC-MS analysis

The same chromatographic conditions were applied. The mass spectra were acquired in the positive ion mode (ESI+) in Thermo Finnigan LCQ^{DECA} XP system equipped with an electrospray ionization source (Thermo LC/MS Division, San Jose, CA, USA). The mass spectrometry detector (MSD) parameters were set as follows: nebulizer sheath gas, nitrogen (80 units); nebulizer auxiliary gas, nitrogen (20 units); capillary temperature, 350 ℃; spray voltage, 5 kV; capillary voltage, −30 V; atmospheric pressure ionization housing and drying gas temperature, 50 and 250 ℃; collision gas, argon; and collision energy, 20 eV.

Method validation

The developed HPLC quantification method of the four alkaloids, protopine, coptisine, tetrahydropalmatine, and palmatine, was validated in terms of calibration curve, limit of detection (LOD), limit of quantification (LOQ), precision, stability, and accuracy. Calibration curve was plotted with peak area against concentration by duplicate injections of the six series of methanol-diluted working solutions of the reference compounds. The determination of LOD and LOQ for each analyte was based on the signal-to-noise ratio (S/N) of 3 and 10, respectively. Precision was evaluated by six consecutive analyses (n = 6). Stability test was carried out in 72 h (n = 6). Variations were assessed using relative standard deviation (RSD), which was calculated by the following formula: RSD (%) = (SD/Mean) × 100%. The same volume of a mixture of the four analytes was triply spiked into the sample for recovery calculation. All the reference compounds were purchased from National Institute for Food and Drug Control.

Data analysis

Areas of characteristic peaks were extracted from chromatogram data with Agilent Chemstation software for the following analyses. PCA was employed to produce both score and loading plots, which were expected to reveal the clustering pattern of the analyzed samples and the most associated variables (compounds) with the first two components, respectively. Parallel analysis of clustering was performed using hierarchical cluster analysis (HCA) based on Ward's method measured by Euclidean distance with min-max normalization (0 to 1 range). PCA and HCA are complementary in their ability to present results although they both aim to reduce the multivariate dimensionality (Massart, 1998). With the reference to the mean data set generated by the seven raw materials from Zhejiang (ZJR), similarity in terms of angle cosine was calculated using between-groups linkage method in HCA. Angle cosine values allow a statistical comparison among different categories. All quantitative data were subject to the statistical test of one-way ANOVA. Variance homogeneity of the submitted data was tested prior to multiple comparisons. Given an insignificant difference, LSD method was applied, which assumed equal variances, otherwise, Tamhane's T2 method. All above analyses were implemented using program SPSS16.0 for Windows.

Results

HPLC fingerprint characterization and method validation

A total of 13 chromatographic peaks detected by HPLC were characterized by LC-MS^n, of which nine were identified by comparing with published data (Ding *et al*, 2007; Cheng *et al*, 2008; Wang *et al*, 2010b; Zhang *et al*, 2009) (Table 2 and Fig. 1). All the nine identified compounds were alkaloids, four tertiary and five quaternary. All samples shared the 13 peaks which varied in peak intensity, presenting similar chromatographic profiles. However, the greatest and smallest peak intensities appeared in the boiled and sulphur-fumigated samples, respectively, and the raw samples intermediated between them (Fig. 1 and Table 1). Method validation demonstrated satisfactory precision (RSD < 3%), stability (RSD < 3%), linearity (R^2 > 0.99), accuracy (recovery > 96%, RSD < 5%) and sensitivity (LOD ≤ 0.09 μg/mL; LOQ ≤ 0.02 μg/mL).

PCA

The PCA score plot (Fig. 1) of all samples based on the 13 compounds matrix (Table 1) illustrated a clear clustering pattern. The five categories of *Corydalis Rhizoma* were well clustered. Apparently, ZJB were most scattered while each of the three categories of raw materials (ZJR, JSR, and SXR) was most closely

Table 2 Retention time (t_R) and MS data for major alkaloids presented in a boiled samples of *Corydalis Rhizoma* from Zhejiang

Peak No.	t_R / min	Identification	λ_{max} / nm	$[M + H]^+$ *m/z*	$[M]^+$ *m/z*	ESI-MS/MS *m/z*
1	20.21	–	280	332		–
2	30.94	THP	279	342		178, 165, 151
3	32.93	protopine	286	354		206, 188, 149
4	35.62	coptisine	234	320		318, 292, 290, 262
5	36.36	fumaricine	254	370		354, 206, 165
6	37.03	THP	279	356		192, 165, 151
7	38.55	–	272		352	337
8	39.03	palmatine	272		352	337, 336, 322, 308
9	39.81	–	269	352		334
10	40.98	dehydrocorybulbine	270		352	336, 322, 320
11	41.76	corydaline	280		370	192, 165, 151
12	43.62	dehydrocorydaline	263		366	351, 350, 334, 322
13	63.31	–	271	332		–

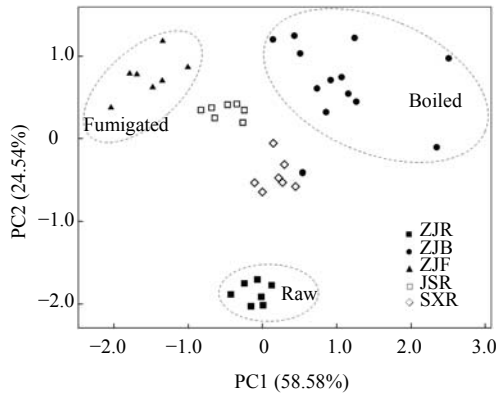

Fig. 1 Score plot of PCA for all analyzed samples of *Corydalis Rhizoma* based on chromatographic peak areas (13 variables)

Each symbol indicates a single sample. Sample abbreviations correspond to those in Table 1

gathered, implying the greater variation of overall phytochemical profile among the boiled *Corydalis Rhizoma*. The three categories of materials from Zhejiang with different postharvest processing methods (ZJR, ZJB, and ZJF) were evidently separated from each other, surrounding the remaining two clusters consisting of raw materials from Jiangsu (JSR) and Shaanxi (SXR), respectively. The loading plot suggested the top four compounds contributing to the differentiation, protopine, coptisine, palmatine, and the unidentified peak 9 (Fig. 2 and Table 3). Since two factors, harvest processing method and geographical source, were involved, further PCAs regarding each factor were implemented. The corresponding accessions were expectedly well clustered and differentiated in both score plots (Fig. 3). Both PCAs remarkably differed in loading plots. In the processing group, all the 13 compounds exhibited positive correlations with the first principal component with the largest variance (PC1) (Fig. 2A), indicating their same changing tendencies along the sequence of ZJR, ZJB, and ZJF. Alternatively, in the geographical source group, eight and five compounds were positively and negatively correlated with PC1, respectively (Fig. 2B), suggesting their opposite changing trends with the order of ZJR, JSR, and SXR. The top four contributive compounds to PC1 included protopine (0.936), coptisine (0.923), peak 7 (0.936), and palmatine (0.881) for the processing group and protopine (0.893), coptisine (0.906), dehydrocorybulbine (0.980), and dehydrocorydaline (0.905) for the geographical source group (Table 3). Both protopine and coptisine were most responsible for the phytochemical differentiation caused by either postharvest processing or geographical source. The marker compound for the quality control of *Corydalis Rhizoma*, THP, was little responsible (0.114) for the differentiation in the geographical source group although it presented a high correlation with PC1 (0.875) in the other group.

HCA

The HCA dendrogram showed three major clades corresponding to the three postharvest processing methods, raw, boiling, and fumigation (Fig. 3). The raw and boiling clades were jointed preceding fumigated clade, suggesting the lowest similarity between the fumigated samples and the remaining ones. The raw clade was composed of three subclades representing the raw materials with different geographical sources (ZJR, JSR, and SXR). The latter two (JSR and SXR) were

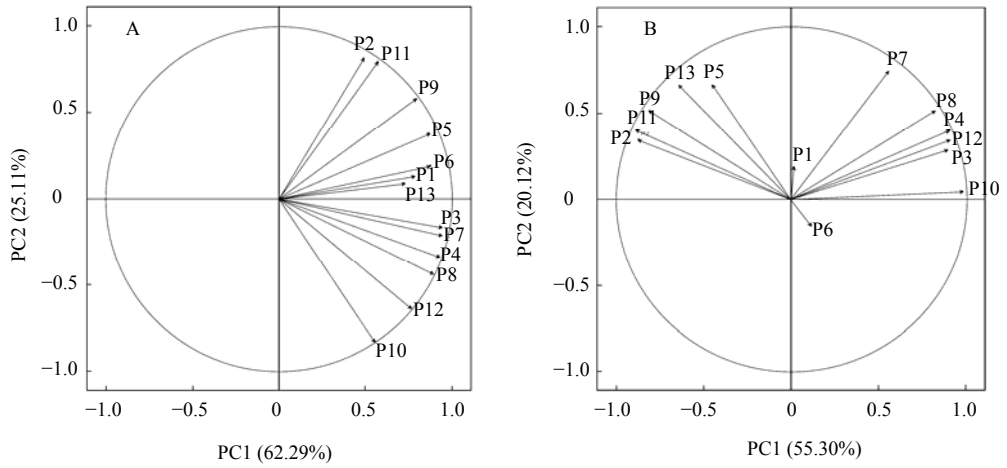

Fig. 2 Loading plot of PCA for analyzed samples with different harvest processing methods (A) or geographical sources (B)

P1—P13 represent the compounds corresponding to those in Table 2

Table 3 Comparison on compound contents (mg/g) and fingerprint similarity coefficient of *Corydalis Rhizoma* samples with different harvest processing methods and geographical sources

Groups	Codes	Protopine	Coptisine	Tetrahydropalmatine	Palmatine	Angle cosine
Processing	ZJR	0.32 ± 0.04 b	0.90 ± 0.06 a	0.74 ± 0.12 b	0.79 ± 0.12 a	0.997 ± 0.001 a
	ZJB	0.40 ± 0.07 a	0.99 ± 0.18 a	0.98 ± 0.16 a	0.80 ± 0.16 a	0.900 ± 0.049 b
	ZJF	0.10 ± 0.03 c	0.11 ± 0.06 b	0.59 ± 0.12 b	0.12 ± 0.12 b	0.684 ± 0.075 c
Geographical source	ZJR	0.32 ± 0.04 a	0.90 ± 0.06 a	0.74 ± 0.12 a	0.79 ± 0.12 a	0.997 ± 0.001 a
	SXR	0.27 ± 0.03 b	0.79 ± 0.08 b	0.70 ± 0.12 a	0.76 ± 0.12 a	0.972 ± 0.007 b
	JSR	0.19 ± 0.03 c	0.42 ± 0.08 c	0.71 ± 0.03 a	0.39 ± 0.03 b	0.933 ± 0.015 c

Lowercases represent significant differences at 0.05 level revealed by multiple comparison based on either LSD or Tamhane's *t* method

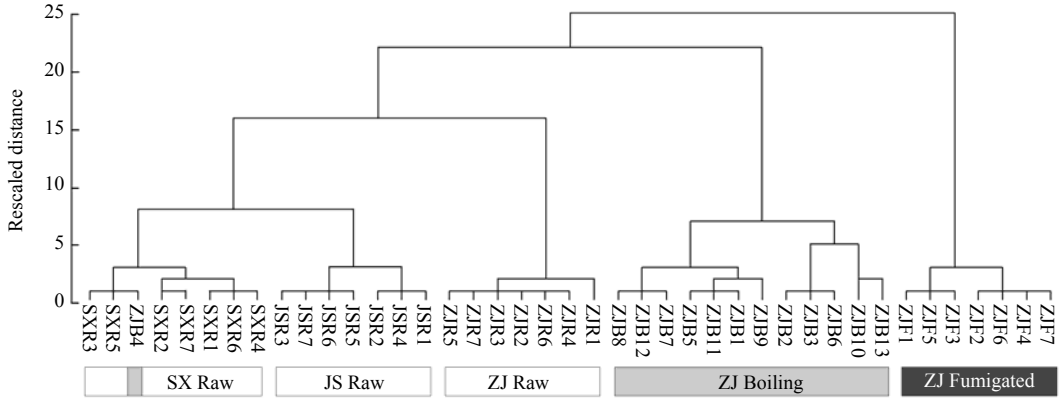

Fig. 3 Dendrogram resulted from HCA of all analyzed samples of *Corydalis Rhizoma* using 13 chromatographic peak areas as variables

closer to each other. Similar to that in PCA, an outlier of a boiled sample (ZJB4) fell into the SXR clade. The parameter of angle cosine calculated via HCA produced a compatible result (Table 3). The average of cosine decreased sequentially in ZJR, SXR, JSR, ZJB, and ZJF with significance. Fumigated samples demonstrated lowest similarity to raw samples.

Content comparisons

Table 3 presented the contents of three most contributive compounds, protopine, coptisine, and palmatine as well as the marker compound, THP. In the processing group, boiled samples produced the highest mean contents of the four alkaloids in contrast to the fumigated samples with the lowest values. Boiling significantly improved the contents of THP and palmatine while fumigation led to the significant reductions of all four alkaloids except THP. Regarding the geographical source group, three alkaloids significantly differed in contents whereas THP presented an insignificant difference. JSR demonstrated

the lower contents of these three alkaloids relative to ZJR and SXR significantly.

The changing patterns of relative contents of all 13 compounds represented by peak areas among different categories of *Corydalis Rhizoma*. In the processing group, ZJB exhibited the highest contents of 11 compounds, of which nine were significantly higher than ZJR, while the other two were reduced after boiling. Fumigation led to decrease in seven compounds, increase in three compounds, and stabilization in the remaining three ones. In the geographical source group, significant content differences were suggested for 11 analyzed compounds (except unidentified P1 and THP). The levels of the four compounds most contributive to differentiation, protopine, coptisine, dehydrocorybulbine, and dehydrocorydaline, identically decreased along the order of ZJR, SXR, and JSR. The changing tendencies of the compound contents in either groups coincided with those revealed by PCA (Fig. 2).

Discussion

Impressively, the contents of nine of all 13 compounds were increased by boiling. The growth of THP was also consistent with that in Ren *et al* (2009). However, abrupt decreases of compounds after boiling were observed in a number of plant materials, like *Scutellaria baicalensis* (Song *et al*, 2006) and garlic (Gorinstein *et al*, 2005). Xu and Chang (2009) argued that thermal effects on phytochemical profiles were considerably complex based on the analyses of four legumes, depending on legume type and processing method as well as compound type. This argument was also supported by the contrasting changing patterns of different alkaloids after boiling in the present study. The content increase could be resulted from the release of bonded forms enhanced by thermal processing (Xu and Chang, 2009). In addition, since the phytochemical similarity of ZJR was quite high but varied greatly in ZJB, the boiling procedure should be responsible. The processing method of *Corydalis Rhizoma* stipulated in *Chinese Pharmacopeia* is boiling to the point when the white color of the central part of the rhizome disappears (Pharmacopoeia Committee of P. R. China, 2010). In practice, the producers processed the raw materials empirically and cared quantitative operation insufficiently. Therefore, boiling procedure should be standardized to produce more consistent medicinal materials. Further examination of phytochemical dynamics during boiling processing and responsible mechanism(s) would be critical to achieve such a goal. Regarding sulfur-fumigation, the ZJF was even more different from the ZJR with significantly lower contents of most detected alkaloids. In addition to the toxic residue, sulphur fumigation was strongly proposed to be abandoned for processing in practice although the official prohibition has been issued.

Corydalis Rhizoma with various geographical sources also presented noticeable, although relatively less than processing and phytochemical differentiation which were frequently observed in many other plant materials and well accepted. Genetic and environmental variation could generally account for this difference (Huang, Chen, and Xiao, 2004). Our genetic analysis of *C. yanhusuo* showed highly similar genetic diversity of Zhejiang and Jiangsu populations and obvious differentiation between Shaanxi population and Zhejiang and Jiangsu (Qiu *et al*, 2009). However, among the three populations, the genetically closer ones, Zhejiang and Jiangsu, were phytochemically farther (Figs. 1 and 3). This incongruence should be at least partly resulted from the environmental contribution, which has been demonstrated in a variety of plants, such as *Juniperus communis* Linn. (Filipowicz *et al*, 2006), *Tribulus terrestris* L. (Dinchev *et al*, 2008), and *Leontodon autumalis* L. (Grass *et al*, 2006). Our ongoing research on relative contribution of genetic and environmental variation would provide further stronger evidences.

In conclusion, our present results explicitly demonstrated that the postharvest processing could produce a greater impact on phytochemical profile of *Corydalis Rhizoma* than geographical source. For the purpose of QC of *Corydalis Rhizoma*, processing deserves priority of concerns with comparison to geographical source. The application of boiling was supported by the increased contents of most compounds but should also be improved in consistency. Sulphur-fumigation was strongly suggested to be abandoned. Protopine, coptisine, and palmatine were the most variable components in processing. Geographical sources should also be concerned. Environmental variation of the three regions may play a role in the

phytochemical differentiation of *Corydalis Rhizoma*. Dehydrocorybulbine, coptisine, dehydrocorydaline, and protopine varied most among the three production regions and sequentially declined in Zhejiang, Shaanxi, and Jiangsu provinces.

Acknowledgements

The authors are indebted to CHEN Bin-long and PAN Lan-lan as well as the local producers for facilitating sampling. We also thank JIANG Xin-hang for his technical assistance in LC-MS analysis. Dr. Boufford DE made a linguistic editing.

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