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Original Article

Comparative investigation for raw and processed Aconiti Lateralis Radix using chemical UPLC-MS profiling and multivariate classification techniques



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ABSTRACT

A strategy combining chemical UPLC-MS profiling and multivariate classification techniques has been used for the comparison of raw and processed Aconiti Lateralis Radix. UPLC-MS was used to identify 18 characteristic compounds, which were selected for discrimination of the raw and two processed products (Heishunpian and Baifupian). Chemometric analyses, including the combination of a heat map and hierarchical cluster analysis (HCA) and principal component analysis (PCA), were used to visualize the discrimination of raw and two processed products. HCA and PCA provided a clear discrimination of raw Aconiti Lateralis Radix, Heishunpian and Baifupian. Finally, the counter-propagation artificial neural network (CP-ANN) was applied to confirm the results of HCA, PCA and to explore the effect of 18 compounds on samples differentiation and the rationality of processing. The results showed that this strategy could be successfully used for comparison of raw and two processed products of Aconiti Lateralis Radix, which could be used as a general procedure to compare herbal medicines and related processed products to elaborate the rationality of processing from the perspective of chemical composition.

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1. Introduction

Chinese herbal medicines are gaining increasing interest because of their long history of clinical application, reliable therapeutic efficacies, wide pharmacological activities [1,2]. Herbal processing is a characteristic pharmaceutical skill in Chinese herbal medicines, which promotes their therapeutic effects, reduces toxic constituents and their side effects, and moderates their drastic effects [2,3]. The traditional processing approaches include steaming with water, wine, or black bean juice; braising with black bean juice or herbal liquids; and frying with oil or sand [4]. For many herbs, it is necessary to reduce the toxicities and side effects, but retain the pharmacological effects of toxic Chinese herbal medicines before their clinical applications.

Aconiti Lateralis Radix (“Fuzi” in Chinese), recorded in Chinese Pharmacopoeia 2015, is the daughter root obtained from *Aconitum carmichaelii* Debx., and is widely used in Chinese herbal medicines and prescriptions with “hot” and “toxic” properties [5,6]. The main bioactive constituents of Aconiti Lateralis Radix are alkaloids, including diester-diterpenoid alkaloids (DDAs), monoester-diterpenoid

alkaloids (MDAs), aminoalcohol-diterpenoid alkaloids (ADAs), and lipo-alkaloids, which are both responsible for many pharmacological activities as well as being highly toxic [5,7–11]. Additionally, there are also some flavonoids, glucides, fatty acids, saponins, glycosides, and ceramides found in this herbal medicine [7]. The majority of phytochemicals contribute to various pharmacological activities of Fuzi. It affects the cardiovascular, immune, and metabolic systems; it has cardiogenic activities that protect cardiomyocytes with an anti-arrhythmia effect; it has anti-inflammation activity, anti-tumor activity, and anti-aging activity; it has hypoglycemic and hypolipidemic effects, which protect against lipopolysaccharide-induced acute lung injury; and it has an analgesic effect [7,12–16]. Due to its high toxicity, raw Aconiti Lateralis Radix cannot be directly used, the processing is a necessary step to reduce the toxicities and side effects. Only the processed products of Aconiti Lateralis Radix are allowed to use to oral administration in clinic according to State Food and Drug Administration of China. The commercial varieties after processing include Heishunpian, Baifupian, and Yanfuzi, with the former two being the most popular varieties [17]. Heishunpian (black slices products) and Baifupian (white slices) show many similarities in the ways of processing form,

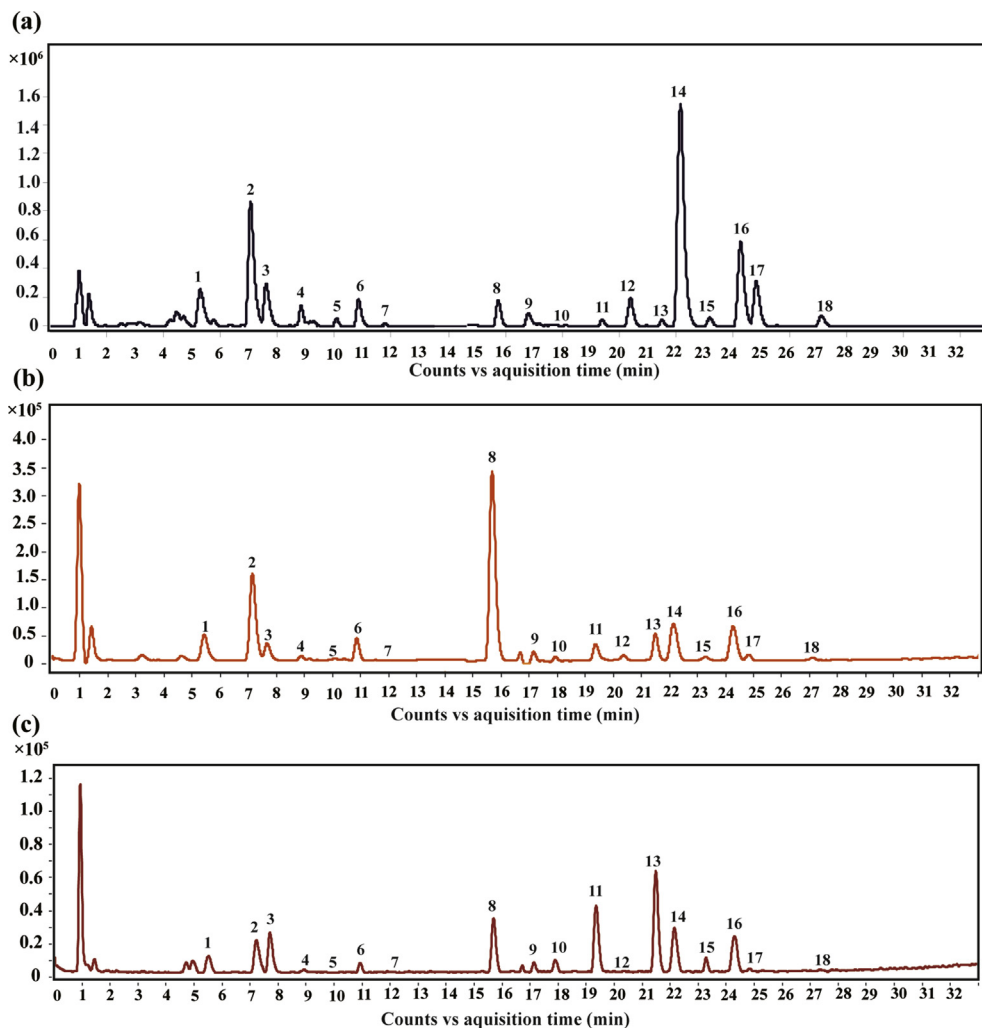


Fig. 1 – Base peak chromatograms of raw Fuzi (a), Heishunpian (b), and Baifupian (c).

Table 1 – Compounds identified in Aconiti Lateralis Radix.

No.	$t_{R/min}$	$[M+H]^+$ (m/z)		Formula	Typical fragment ions (MS^2) m/z	Identification	Type
		Measured	actual				
1	5.924	358.2	358.2	$C_{22}H_{31}NO_3$	$340.2 [M+H-H_2O]^+$, $312.2 [M+H-H_2O-C_2H_4]^+$	Songorine	Veatchine
2	7.535	454.2	454.3	$C_{24}H_{39}NO_7$	$436.1 [M+H-H_2O]^+$, $418.2 [M+H-2H_2O]^+$	Fuziline	ADA
3	7.983	438.2	438.3	$C_{24}H_{39}NO_6$	$420.0 [M+H-H_2O]^+$, $370.3 [M+H-2H_2O-CH_3OH]^+$	Neoline	ADA
4	9.079	422.2	422.3	$C_{24}H_{39}NO_5$	$390.1 [M+H-CH_3OH]^+$, $358.4 [M+H-2CH_3OH]^+$	Talatisamine	ADA
5	10.199	452.2	452.3	$C_{24}H_{37}NO_7$	$388.2 [M+H-2CH_3OH]^+$, $370.2 [M+H-2CH_3OH-H_2O]^+$	Chasmanine	ADA
6	11.027	480.2	480.3	$C_{26}H_{41}NO_7$	$462.2 [M+H-H_2O]^+$, $430.3 [M+H-H_2O-CH_3OH]^+$	14-Acetylincoline	ADA
7	11.855	464.2	464.3	$C_{26}H_{41}NO_6$	$432.2 [M+H-CH_3OH]^+$, $372.2 [M+H-CH_3OH-AcOH]^+$	14-O-acetylaltatisamine	ADA
8	15.742	590.2	590.3	$C_{31}H_{43}NO_{10}$	$572.2 [M+H-H_2O]^+$, $540.1 [M+H-H_2O-CH_3OH]^+$	Benzoylmesaconine	MDA
9	17.172	604.2	604.3	$C_{33}H_{45}NO_{10}$	$586.3 [M+H-H_2O]^+$, $540.2 [M+H-2CH_3OH]^+$	Benzoylaconitine	MDA
10	18.047	574.2	574.3	$C_{31}H_{43}NO_9$	$510.3 [M+H-3CH_3OH]^+$, $478.1 [M+H-3CH_3OH]^+$	Benzoylhypaconitine	MDA
11	19.323	701.4	701.9	$C_{37}H_{52}N_2O_{11}$	$683.2 [M+H-H_2O]^+$, $640.1 [M+H-AcOH]^+$	Demethyldelevaine A/B	ADA
12	20.308	648.2	648.3	$C_{33}H_{45}NO_{12}$	$598.3 [M+H-2CH_3OH]^+$, $538.4 [M+H-AcOH-CH_3OH-H_2O]^+$	Beiwutine	DDA
13	21.420	814.1	814.0	$C_{46}H_{72}NO_{11}$	$618.0 [M+H-3CH_3OH]^+$, $572.1 [M+H-pdcl]^+$	8-pdc-Benzoylmesaconine ^a	LDA
14	21.987	632.2	632.3	$C_{33}H_{45}NO_{11}$	$572.2 [M+H-AcOH]^+$, $538.2 [M+H-AcOH-CH_3OH]^+$	Mesaconitine	DDA
15	23.121	662.2	662.3	$C_{34}H_{47}NO_{12}$	$602.2 [M+H-AcOH]^+$, $570.3 [M+H-AcOH-CH_3OH]^+$	10-OH-aconitine	DDA
16	24.217	616.2	616.3	$C_{33}H_{45}NO_{10}$	$556.1 [M+H-AcOH]^+$, $524.1 [M+H-AcOH-CH_3OH]^+$	Hypaconitine	DDA
17	24.799	646.3	646.3	$C_{34}H_{47}NO_{11}$	$586.0 [M+H-AcOH]^+$, $526.0 [M+H-AcOH-CH_3OH-CO]^+$	Aconitine	DDA
18	27.082	630.3	630.3	$C_{34}H_{47}NO_{10}$	$570.3 [M+H-AcOH]^+$, $538.2 [M+H-AcOH-CH_3OH]^+$	Deoxyaconitine	DDA

^a pdc-pentadecanoic acid.

however, the values of thickness were about 0.5 cm, and 0.3 cm, respectively, and the former was dyed, the latter was peeled the skin during the process. The median lethal for ethanol extracts of raw Fuzi, Heishunpian and Baifupian on SPF-level are 22.169, 49.853, and 42.550 g/kg, respectively; processing, decoction and compatibility make substantial contributions to detoxification of Aconiti Lateralis Radix [18]. Both the two approaches decompose the poisonous DDAs into less or non-toxic derivatives to decrease toxicity of raw Fuzi. The two processed products have different efficacy, Heishunpian presents stronger pharmacological activities and widely used as analgesic and anti-inflammatory agent in clinics [7].

In the present investigation, many studies of Aconiti Lateralis Radix and related processed products have been focused on the phytochemistry and pharmacological activities [7,11]. After processing, the chemical composition would be changed and the toxicity reduced [7,11]. The combination of chromatographic fingerprints coupled with mass spectrometry and chemometrics can provide a comprehensive and multivariate description of chemical composition of herbal medicines [19]. However, few studies have been reported multivariate chemometric methods to comprehensively analyze and compare the chemical composition and obtain significant compounds for discrimination of raw and processed products. In our study, chemical UPLC-MS profiling and multivariate classification techniques were combined to compare the raw and processed Aconiti Lateralis Radix. First, UPLC-MS of Aconiti Lateralis Radix was used to identify the main compounds and obtain related information of the common components of raw Fuzi, Heishunpian, and Baifupian. A combination of heat map, hierarchical cluster analysis (HCA), and principal component analysis (PCA) were used to characterize the compounds obtained after processing, and to develop an appropriate classification scheme. Counter-propagation artificial neural network (CP-ANN), a supervised recognition pattern, was then used to explore the effect of main compounds on samples differentiation and the rationality of processing. Together, this methodology provided comprehensive analyses for comparison of raw and processed Aconiti Lateralis Radix, which would be valuable, especially for toxic Chinese herbal medicines.

2. Materials and methods

2.1. Materials and reagents

14 batches of crude Fuzi (F1–F14), 14 batches of Baifupian (B1–B14), and 19 batches of Heishunpian (H1–H19) from Jiangyou City, Sichuan Province, China were collected from different suppliers of Chinese herbal medicine at Bozhou (Anhui Province), Anguo (Hebei Province), and the Bozhou Jingwan Chinese Herbal Medicine Factory (Bozhou, Anhui). All samples were authenticated in our laboratory according to the Pharmacopeia of the People's Republic of China (2015 edition), and each sample conformed to standard specifications.

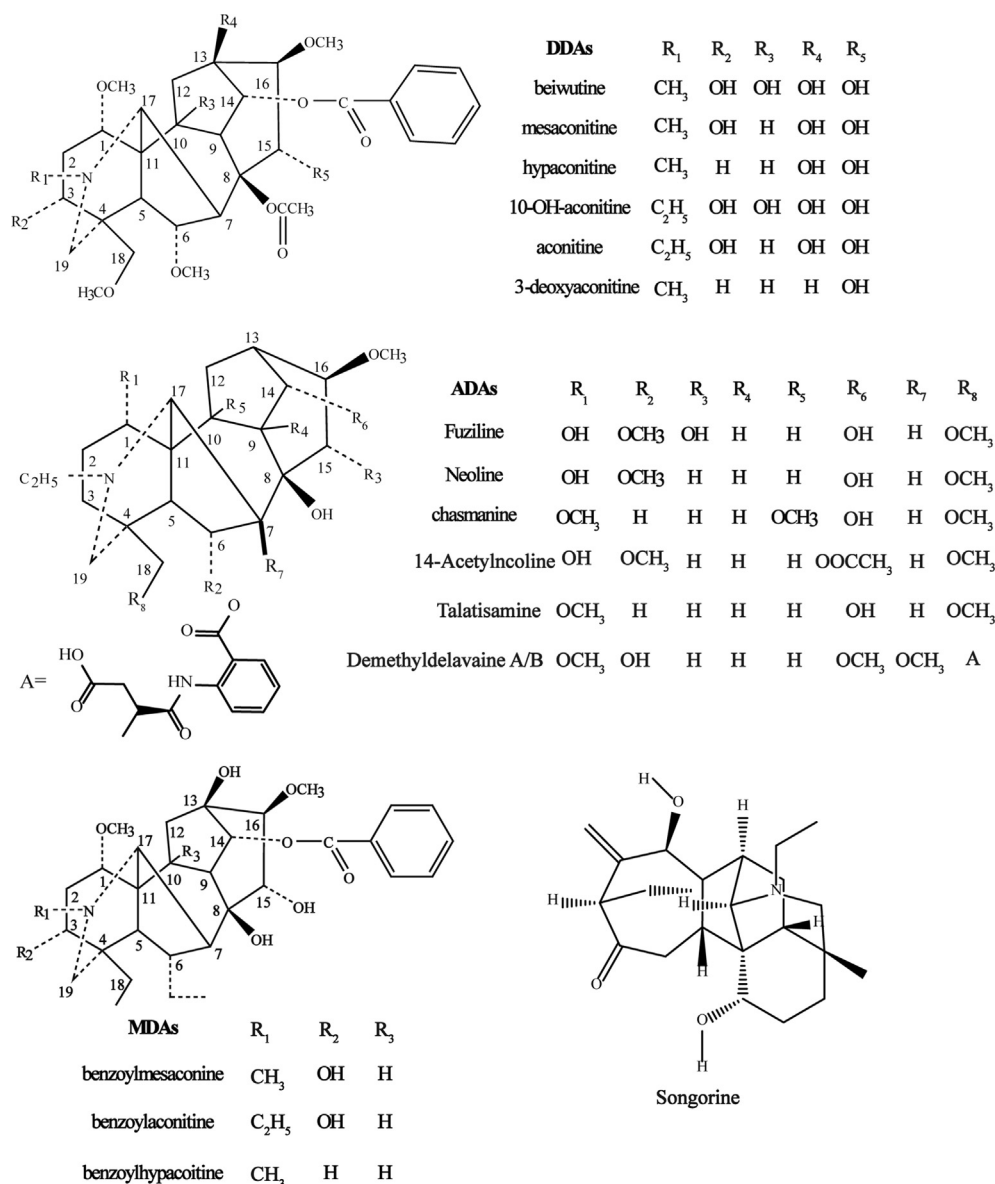


Fig. 2 – Most chemical structures of Fuzi.

All the standards, including mesaconitine, hypoconitine, aconitine, benzoylmesaconine, benzoylaconitine, and benzoylhypaconitine, were obtained from Jiangsu Yongjian Pharmaceutical Co., Ltd.. MS-grade acetonitrile and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The Milli-Q system (Millipore, Bedford, MA, USA) was used to obtain purified water for the UPLC-MS analyses.

2.2. Sample preparation

Each sample of *Aconiti Lateralis Radix*, including raw Fuzi, Baifupian, and Heishunpian, was powdered by a pulverizer (Huangcheng HC-150, Zhejiang province, China), and passed through a 50-mesh sieve, then 200 mg powder of each sample was dissolved in 0.05 M hydrochloric acid solution (5 mL), followed by shaking and sonication for 30 min. The supernatant was obtained, and was diluted five times with 0.05 M

hydrochloric acid solution, each solution was filtered through a 0.22 μm nylon membrane.

2.3. UPLC-MS analysis

UPLC-MS analysis was performed using an Agilent Technology 1290 Infinity UPLC equipped with an Agilent Technology 6460 Triple Quad liquid chromatography/mass spectrometer instrument (Agilent, Santa Clara, CA, USA). Chromatographic separations were performed using an Acquity UPLC BEH shield RP18 column (2.1 mm × 100 mm, 1.7 mm particle size; Waters, Milford, MA, USA) maintained at 25 °C. The mobile phase consisted of 0.3% formic acid (solvent A) and acetonitrile (solvent B) with a gradient as follows: 2% B for 0–2 min, 2%–5% B for 2–5 min, 5%–15% B for 5–10 min, and 15%–35% B for 10–32 min. The flow rate was kept at 0.3 mL/min, and the injection volume was 2 μL. The UV detection wavelength was set at 235 nm.

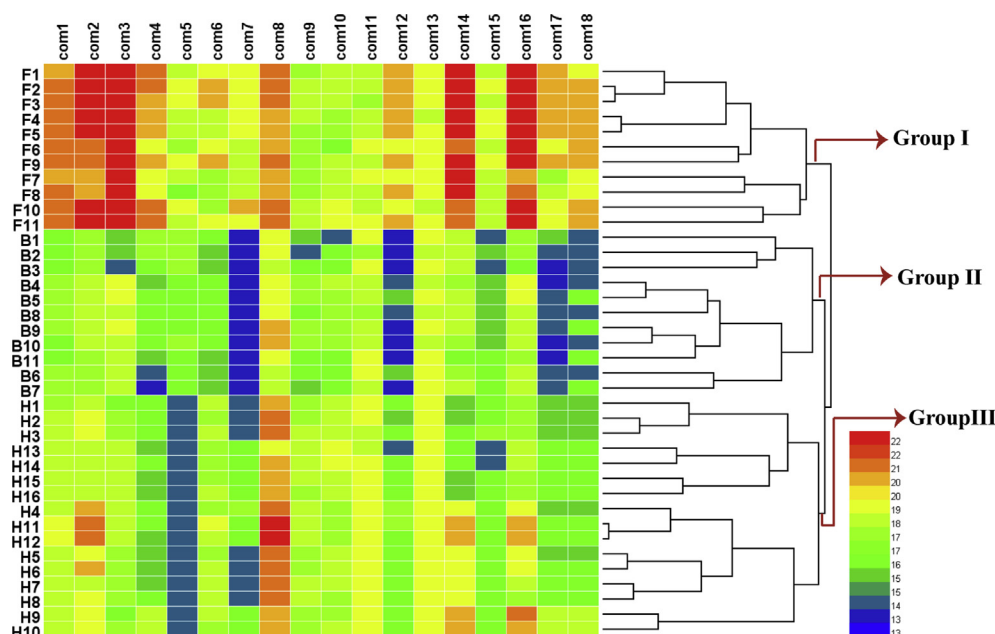


Fig. 3 – The heat map and dendrogram obtained from hierarchical cluster analyses.

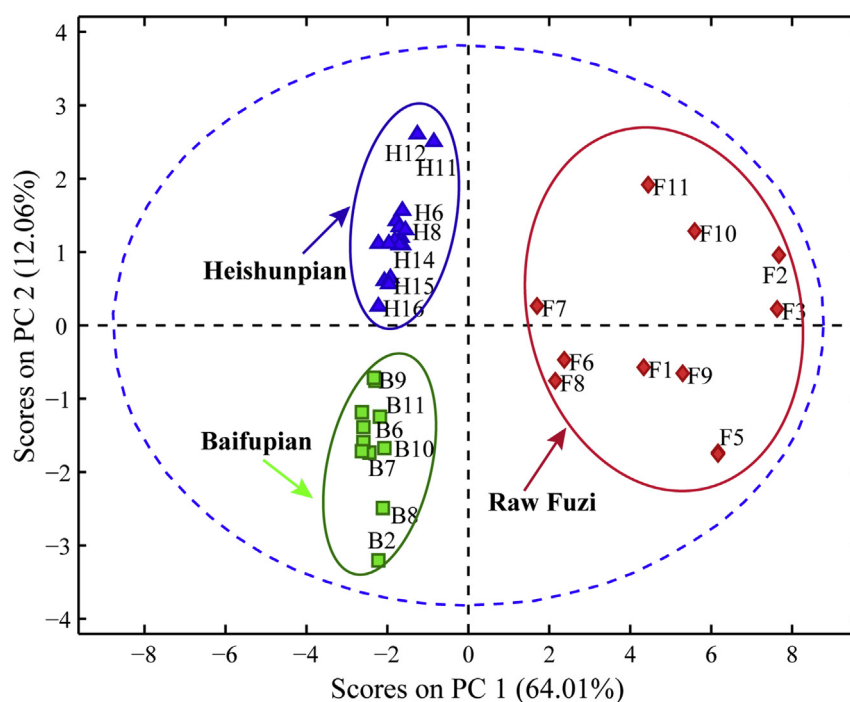


Fig. 4 – A principal component analysis two-dimensional score plot of Aconiti Lateralis Radix samples.

The MS analyses were performed using an electrospray ionization ion source under a positive ion mode with the full scan mass from 100 to 1000 m/z . The voltage of the capillary was set at 7 kV. The gas temperature was 350 °C, and the gas flow was 13 L/min. The nebulizer was maintained at 60 psi.

2.4. Data analysis

UPLC-MS data were imported into the Agilent Mass Hunter to identify the compounds and obtain peak information from the

Aconiti Lateralis Radix samples, including the crude Fuzi, Baifupian, and Heishunpian. Common peaks in the base peak chromatogram obtained from UPLC-MS were selected to perform chemometric analyses. Heatmap Illustrator 1.0.3 (<http://hemi.biocuckoo.org/down.php>) was used to perform hierarchical cluster analyses, and PLS-Toolbox, version 7.9 (Eigenvector Research, Wenatchee, WA, USA) was used for principal component analysis [18]. The counter propagation artificial neural network (CP-ANN) was used based on Matlab R2014a (MathWorks, Natick, MA, USA) and Kohonen and

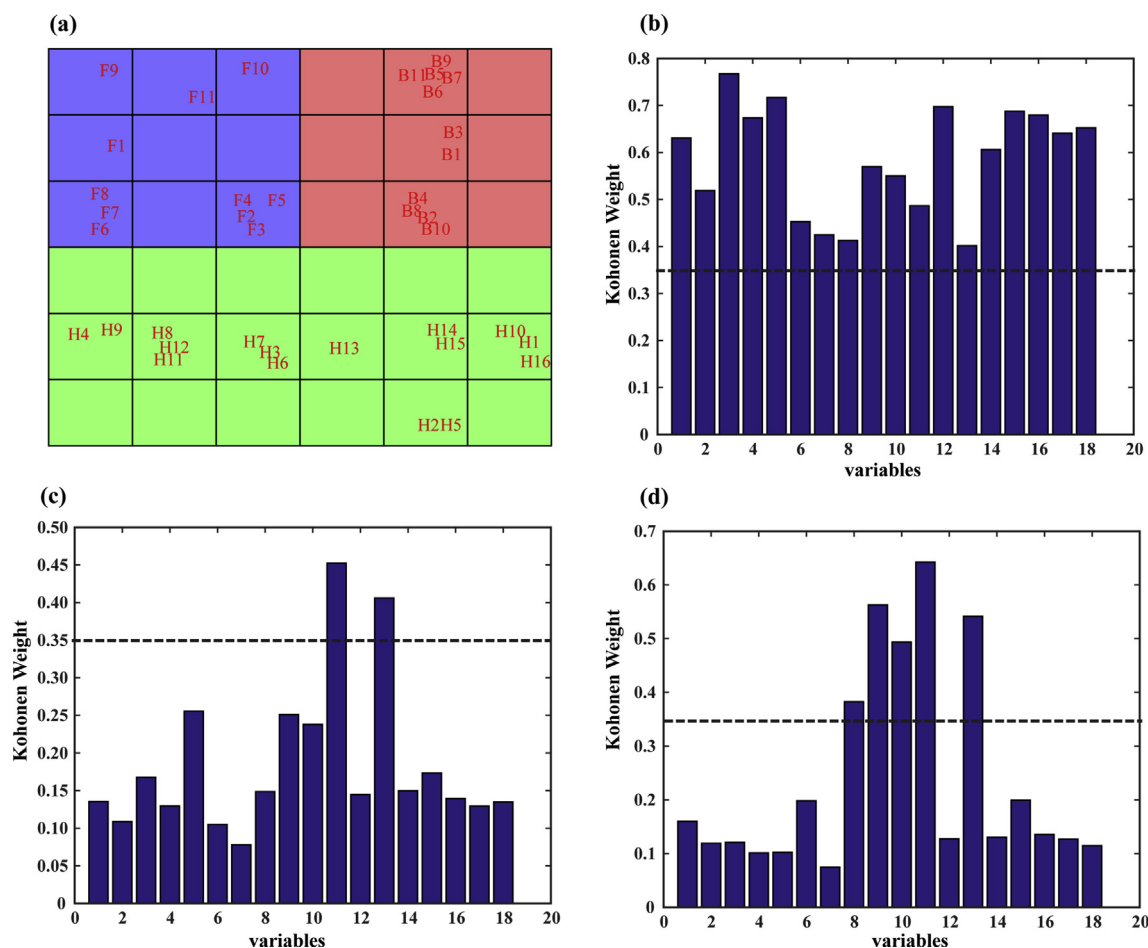


Fig. 5 – Results of counterpropagation artificial neural network analyses. (a) The distribution of each sample on the Kohonen map; Kohonen weights of the 18 variables in three groups: (b) raw group, (c) Baifupian group and (d) Heishunpian group.

CP-ANN toolbox, version 3.6 (Milano Chemometrics and QSAR Research Group, Milano, Italy) [20–22]. The 38 samples (F1–F11, B1–B11, H1–H16) $\times$ 18 variables data matrix was used to establish HCA, PCA and CP-ANN model. The rest samples $\times$ 18 variables data composed the prediction set for CP-ANN model.

3. Results and discussion

3.1. UPLC-MS identification

The base peak chromatograms of raw Fuzi, Baifupian, and Heishunpian samples are shown in Fig. 1, while Table 1 shows the identification results for 18 peaks, including retention times, MS information, and formulas according to references and standards. A total 18 compounds were identified, including one veatchine alkaloid, seven ADAs, six DDAs, three MDAs, and one lipo-diterpenoid alkaloid [7–9,23]. Chemical structures are shown in Fig. 2. The raw Fuzi contained high amounts of DDAs, including aconitine, hypaconitine, mesaconitine, deoxyaconitine, etc, which possessed curariform toxicity and aconitine-type toxicity. As can be seen, the amounts of DDAs dramatically decreased in the two products,

and the lower toxic MDAs play a role in the chemical composition.

3.2. Chemometric analysis

3.2.1. Heat map and HCA

The data matrix (38 samples $\times$ 18 variables) was used to perform intuitive heat mapping and HCA to determine the intuitive changes of compounds after processing, and to calculate the interpoint distances among raw Fuzi, Baifupian, and Heishunpian samples. Logarithmic base 2 was used for normalization, average linkage with the Pearson distance was selected as cluster method. Fig. 3 shows the obtained heat map and dendrograms of 38 samples. Heat map indicated that the contents of most compounds reduced in processed samples compared with the raw samples. Samples could be classified into two independent clusters, in which all the ingredients from the raw Fuzi sample were included in the same cluster, and all ingredients from the Baifupian and Heishunpian were included in another cluster. Additionally, samples of Baifupian and Heishunpian could also be divided into two clusters. Therefore, all the samples could be considered as three clusters, where the raw samples (group I), Baifupian (group II) and Heishunpian (group III), composed three separate clusters. Together, the

results showed that there were significant differences between raw and two processed *Aconiti Lateralis Radix*.

3.2.2. PCA

PCA was used to perform further exploratory data analyses by importing the data matrix into PLS-Toolbox software, version 7.9. Autoscaling was used as the preprocessing method, and five principal component (PCs) sharing 92.63% variance were selected to develop a PCA model. The root mean square error of calibration (RMSEC) and root mean square error of cross validation (RMSECV) were 0.072 and 0.6167, respectively, which showed good fit and recognition abilities. The two-dimensional (PC1 = 64.01% versus PC2 = 12.06%) score plots are shown in Fig. 4, where objects could be classified into three separate groups. As can be seen, all ingredients of raw Fuzi were found to be closely located to each other and were included in an independent group in a greater distance from the positions of ingredients of Heishunpian and Baifupian found in the other two separate clusters. The classification results were in good agreement with those obtained from HCA. All samples were classified into three groups, which were the raw Fuzi group (group I), Baifupian group (group II), and Heishunpian group (group III) according to the results from HCA and PCA. To confirm the results of HCA and PCA, and to investigate the effects of compounds in sample differentiation, a supervised recognition pattern model was developed.

3.2.3. CP-ANN analysis

CP-ANN based on Kohonen maps, but combining both supervised and unsupervised results, was performed for a supervised recognition pattern and investigation the effect of compounds in the differentiation of raw Fuzi and two processed products [20,21]. Firstly, all the samples were separated into three different classes according to the PCA plots. The matrix (38 samples $\times$ 18 variables) was input to the CP-ANN network. The values of 1, 2, 3 were defined the assigned class for each sample. A genetic algorithm (GA) was used to optimize the architecture of the network [24,25]. The optimal architecture of 8 $\times$ 8 (64 neurons) and the epochs of 50 were obtained and utilized to develop a CP-ANN model, and venetian blinds with ten data splits were used for cross-validation. The developed CP-ANN model performed best during classification and recognition with sensitivity, specificity, and precision values of 1.00. The results of CP-ANN analyses are shown in Fig. 5a–d. Fig. 5a showed the distribution of each sample on the Kohonen map. All samples occupied different neurons on the Kohonen map obtained from CP-ANN analyses, samples from same class were located to each other. Prediction set (9 samples $\times$ 18 variables) was input to the established CP-ANN model to confirm the classification results. Results showed that the model performed well for discrimination samples of raw Fuzi, Heishunpian and Baifupian with 100% correct prediction.

Fig. 5b–d show the Kohonen weights of 18 variables in the three groups obtained using CP-ANN analyses. According to the figure, all the variables present high effect in raw samples, variables of 11 (demethyldelavaine A/B) and 13 (8-pdc-benzoylmesaconine) were the most discrimination variables for Baifupian, variables of 8 (benzoylmesaconine), 9

(benzoylconitine), 10 (benzoylhypaconitine), 11 (demethyldelavaine A/B) and 13 (8-pdc-benzoylmesaconine) for Heishunpian. The main reason for the similarity between two processed samples was the similar effect of variables of 11 and 13. The most discrimination variables of Baifupian, and Heishunpian were MDA, ADAs or LDA that possessed lower toxicity than DDAs. Based on the results, it could be concluded that the chemical composition was changed, most of the compounds were reduced and high toxic compounds decomposed to the lower toxic derivatives after processing which had led to the lowered toxicity of the processed products.

4. Conclusions

Processing before clinical use is an important step for toxic traditional Chinese medicines. In the present study, a strategy comprising UPLC-MS and chemometric methods was developed for the comprehensive analysis and comparison among raw and two processed *Aconiti Lateralis Radix*. UPLC-MS was used to identify the chemical composition of *Aconiti Lateralis Radix* samples, including raw samples, and two kinds of processed samples (Heishunpian and Baifupian). HCA and PCA described the relationships among raw and processed samples, and were used to develop the appropriate classification methodology. According to the results from the HCA and PCA, the samples were classified into three groups (the raw samples, Heishunpian and Baifupian). The CP-ANN model was developed to facilitate a supervised recognition pattern and for investigation the effect of compounds in the differentiation of raw and two processed products of *Aconiti Lateralis Radix* before and after processing. Together, the proposed strategy discriminated compounds between the raw and processed *Aconiti Lateralis Radix* samples, and provided a generalizable approach for comprehensive comparison of chemical composition for Chinese herbal medicines before and after processing.

Conflicts of interest

The authors declare no conflicts of interests.

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