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Classification of Fangchi Radix Samples by Multivariate Analysis

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ABSTRACT

A total of 37 commercial samples of Fangchi Radix, including *Sinomenium acutum*, *Stephania tetrandra*, *Aristolochia fangchi*, and *Cocculus orbiculatus*, were collected from herb markets in Taiwan and China. The contents of 18 constituents in these samples were analyzed by high-performance liquid chromatography and were used to assess the potential relationships with their plant origins. A multivariate analysis, including a principal component analysis (PCA), cluster analysis (CA), and linear discriminant analysis (LDA) were used as the classification strategies. The successful natural groupings of the samples into 4 sets, namely, 5 *S. acutum*, 11 *S. tetrandra*, 15 *A. fangchi*, and 6 *C. orbiculatus*, were achieved by PCA and CA. The application of LDA gave the correct assignation percentages of 100.0% for all 4 groups.

Key words: Fangchi Radix, high-performance liquid chromatography, species classification, multivariate analysis

INTRODUCTION

Fangchi Radix is frequently used for the treatment of edema, arthritic swelling, gonorrhea, urinary difficulty, rheumatic pain, and beriberi and is derived from *Sinomenium acutum*, *Stephania tetrandra*, *Aristolochia fangchi*, and *Cocculus orbiculatus*[1,2]. Those herbs contain a series of alkaloids as their biologically active components, which have been well separated by high-performance liquid chromatography where each peak has been identified by a liquid chromatography-mass procedure[3]. In this study, 37 batches of Fangchi Radix samples collected from different herb markets in Taiwan and China were identified and categorized based on the pharmacognostic histological anatomy[4]. It was found, as shown in Figure 1, that *S. acutum* contains acutumidine (**1**)[5], magnoflorine (**2**)[6], stepharine (**3**)[7], sinomenine (**4**)[8], and acutumine (**5**)[9], *S. tetrandra* has sinomenine (**4**), cyclanoline (**6**)[10], fangchinoline (**7**)[7], berbamine (**8**)[11], tetrandrine (**9**), and isotetrandrine (**10**)[11], *A. fangchi* contained magnoflorine (**2**), aristolochic acid II (**12**)[12], aristolochic acid I (**13**)[13], aristololactam (**14**)[14], and an unknown component **X**, and *C. orbiculatus* has magnoflorine (**2**), sinomenine (**4**), trilobine (**11**)[15], isotrilobine (**15**)[15], and unknown components **A** and **B** as their most abundant components. A total of those 18 components were identified and the results were applied to the differentiation of plant origins.

A number of classification methods were effectively employed to distinguish the origins of various different herbal drug materials[16-22]. Although the radar chart (Figure 2) was convenient for the differentiation of the origins of Fangchi Radix in this study, the rapid advances in informatics technology, that had heralded the development of several types of statistics software for multivariate analysis of empirical data and led to many significant conclusions[23-29], might be worthy applying. In this report, the principal component analysis (PCA), cluster analysis (CA), and linear discriminant analysis (LDA) were used to process the quantitative data from 37 samples of commercial Fangchi Radix. Here, PCA and CA do not require prior knowledge about the groups to which individual cases belong. To the contrary, grouping is performed during the analysis process by sorting cases based on the similarity / homogeneity among the variables. In other words, variables sharing higher similarity / homogeneity are sorted into one group. On the other hand, LDA does require prior knowledge about the groups to which individual cases belong. The variable values of the corresponding groups are thus analyzed separately to obtain the distinction function, which can be used as a standard to sort variables of different cases into groups. Overall, PCA and CA are more convenient for sample prediction. Besides, our previous results showed that those two techniques could also provide effective grouping and sample prediction capabilities well in the differentiation and classification of Aurantii Fructus[30].

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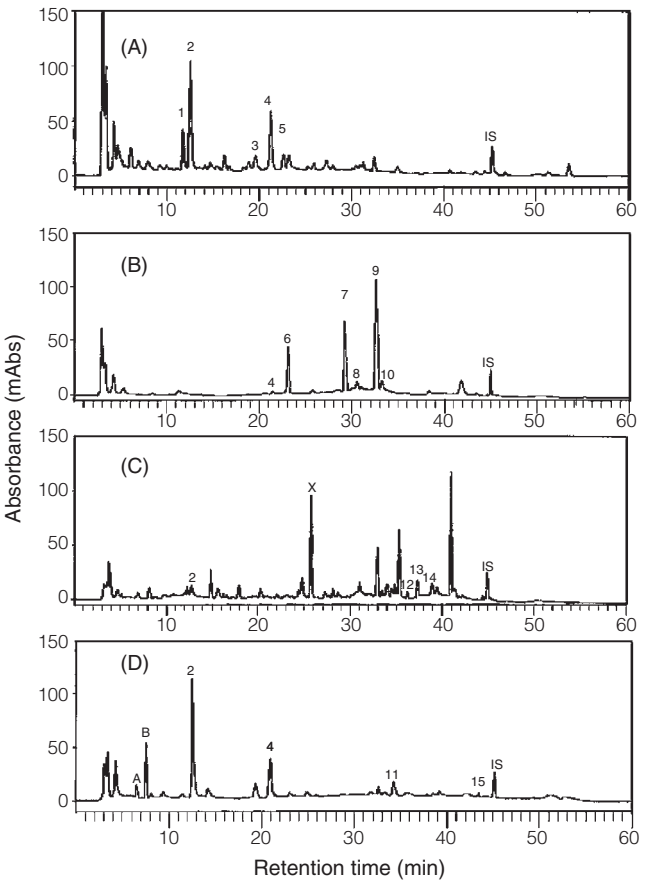

Figure 1. HPLC chromatograms: (A) *Sinomenium acutum*, (B) *Stephania tetrandra*, (C) *Aristolochia fangchi*, (D) *Cocculus orbiculatus*. acutumidine (**1**), magnoflorine (**2**), stepharine (**3**), sinomenine (**4**), acutumine (**5**), cyclanoline (**6**), fangchinoline (**7**), berbamine (**8**), tetrandrine (**9**), isotetrandrine (**10**), trilobine (**11**), aristolochic acid II (**12**), aristolochic acid I (**13**), aristololactam (**14**), isotrilobine (**15**), and unknown components **X** , **A**, and **B**.

MATERIALS AND METHODS

I. *Reagents and Materials*

Sinomenine**,** tetrandrine**,** aristolochic acid I, and aristolochic acid Π were purchased from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan), propyl 4-hydroxybenzoate from Aldrich (Milwaukee, WIS, USA), acetic acid (CH_3COOH), acetonitrile (CH_3CN), and methanol (CH_3OH) from Merck (Darmstadt, Germany), and ammonium acetate (CH_3COONH_4) from Nacalai Tesque (Kyoto, Japan). Other marker substances were isolated from Fangchi Radix[5-15]. Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all the buffers and sample solutions. Thirty-seven herb samples were collected from different herbal shops throughout Taiwan, Japan, and China and identified based on their external appearance and pharmacognostic histological anatomy[4]. They were found to be derived from four species—*S. acutumn* (S1–S5), *S. tetrandra* (T1–T11), *A. fangchi* (A1–A15), and *C. orbiculatus* (C1–C6).

Table 1. Peak-area ratios (constituent peak-area / internal standard peak-area) of each component in *Sinomenium acutum*

Sample No.	A/IS	B/IS	1/IS	2/IS	3/IS	4/IS	5/IS	6/IS	X/IS	7/IS	8/IS	9/IS	10/IS	11/IS	12/IS	13/IS	14/IS	15/IS
S1	–	–	1.408	4.637	0.730	1.526	0.407	–	–	–	–	0.519	–	–	–	–	–	–
S2	–	–	1.035	4.418	0.649	1.395	0.435	–	–	–	–	0.661	–	–	–	–	–	–
S3	–	–	0.809	4.009	0.739	1.439	0.831	–	–	–	–	0.488	–	–	–	–	–	–
S4	–	–	1.263	4.913	0.766	1.720	0.486	–	–	–	–	0.574	–	–	–	–	–	–
S5	–	–	1.102	4.511	1.019	1.800	0.865	–	–	–	–	0.857	–	–	–	–	–	–
Ave. ± SD			1.123 ± 0.228	4.498 ± 0.331	0.781 ± 0.140	1.576 ± 0.177	0.605 ± 0.224					0.619 ± 0.148						
max			1.408	4.913	1.019	1.800	0.865					0.661						
min			0.809	4.009	0.649	1.395	0.407					0.488						

– : The symbol stands for undetected.

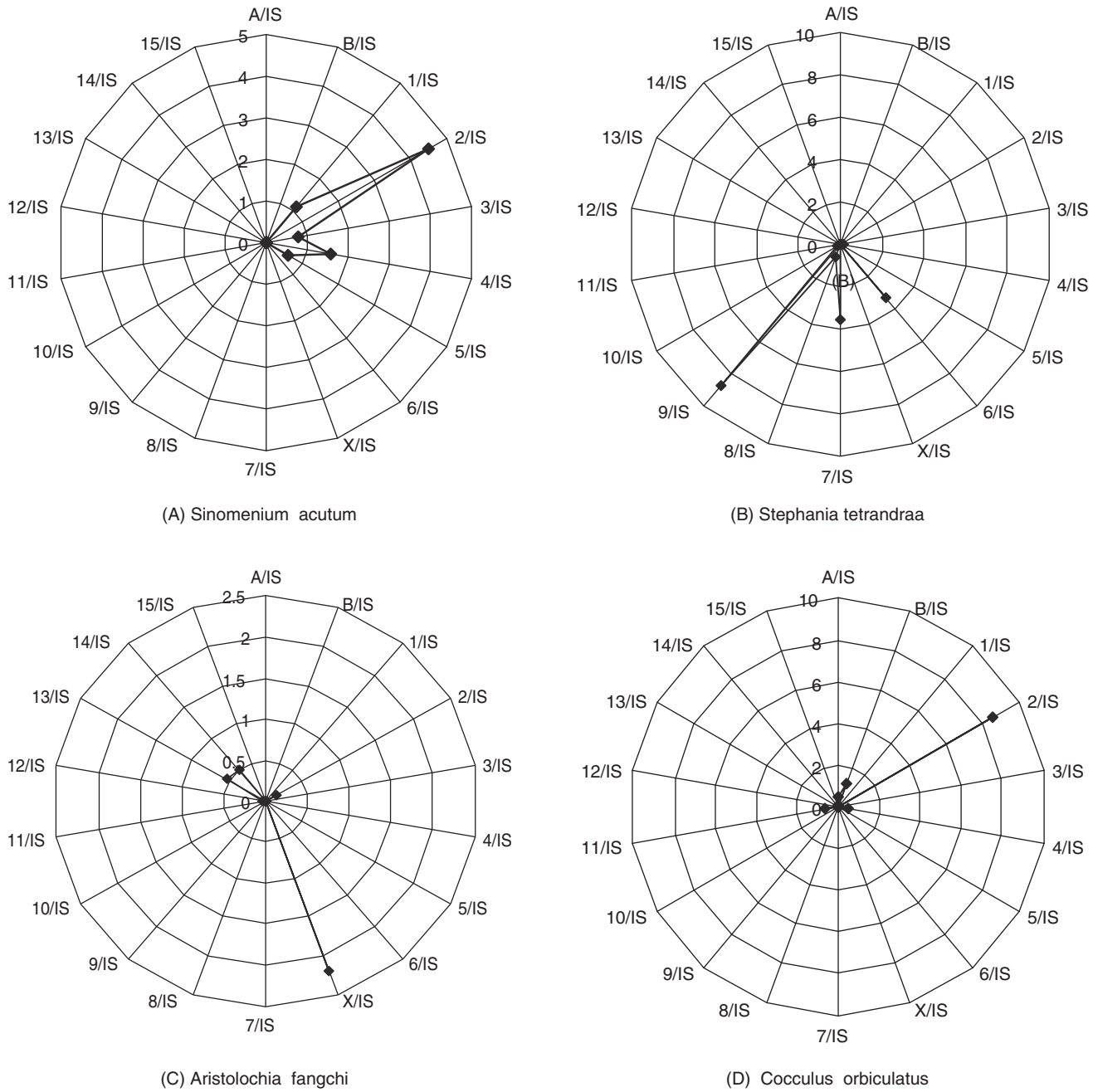

Figure 2. Radar chart of the ratios of the areas of the components in (A) *Sinomenium acutum*, (B) *Stephania tetrandra*, (C) *Aristolochia fangchi*, (D) *Cocculus orbiculatus*.

Π. *Preparation of Fangchi Radix Extracts*

1.25 g pulverized Fangchi Radix was extracted by refluxing in 70% methanol (15 mL) for 15 min, followed by centrifugation of the suspension at 1500 g (Universal, Hettich Zentrifugen) for 5 min. The extraction was repeated three times and the extracts were combined and filtered through a 0.45 μm filter. After the addition of 1 mL of internal standard (51.9 mg of propyl-4-hydroxybenzoate in 100 mL of 70% methanol), the extract was diluted to 50 mL with 70% methanol. This solution was passed through a 0.45 μm filter and injected directly into the HPLC system.

Ш. *Apparatus and Conditions*

HPLC was performed on a Jasco 890 instrument equipped with a Jasco 890 system controller and a Jasco 975 detector (254 nm). The substances were separated on a reverse-phase column (Cosmosil $5C_{18}$-MS, 5 μm, 25 cm × 4.6 mm I.D.; Nacalai Tesque, Kyoto, Japan) and eluted at a flow rate of 1.0 mL/min in linear solvent gradients of A-B [A = 20 mM CH_3COONH_4-CH_3COOH (500:0.25 v/v, pH 4.81); B = H_2O-CH_3CN (20:80 v/v)] using the following protocol: 0 min, 10%B; 10 min, 20%B; 15 min, 25%B; 20 min, 30%B; 25 min, 37%B; 30 min, 40%B; 35

min, 55%B; 40 min, 60%B; 45 min, 80%B; and 60 min, 10%B.

IV. *Data Analysis*

The contents of the 18 constituents in the 37 Fangchi Radix samples were analyzed by multivariate statistical analysis that included unsupervised methods,—PCA and CA,—and a supervised method, LDA. The calculations were performed on the **SPSS** software (**SPSS** for Windows, Ver. 12.0, SPSS Inc., Chicago, IL, USA). All of the data matrices were mean-centered and autoscaled before analyses.

RESULTS AND DISCUSSION

I. *Basic Statistics*

Thirty-seven batches of the Fangchi Radix samples were collected, 5 batches (S1–S5) of the *S. acutum* samples, 8 batches (T1–T8) of the *S. tetrandra* samples, 13 batches (A1–A13) of the *A. fangchi* samples, 6 batches (C1–C6) of the *C. orbiculatus* samples, and 5 batches of samples were used as blind test samples (A14, A15, T9, T10, T11). The HPLC chromatograms are illustrated in Figure 1. The data from the quantitative analysis are summarized in Tables 1-4. It was found that peak-area ratios (constituent peak-area / internal standard peak-area) of the 18 constituents could be easily used to differentiate the origin of Fangchi Radix. The results are plotted in the radar figures as shown in Figure 2. In order to confirm the accuracy of the origins of Fangchi Radix and the blind test (Table 5), statistical methods including PCA, CA, and LDA were utilized for the classification.

(I) *PCA and CA*

PCA is used to achieve a reduction in dimensions and to obtain a preliminary evaluation of between-class similarities. PCA also aids in identifying how one sample differs from another and which variables contribute most to the difference. CA is an unsupervised classification procedure that involves the measurement of similarities between objects to be clustered. A similarity measurement is based, among other factors, on the squared Euclidean distance.

For this study, PCA was performed on data from 32 Fangchi Radix samples (5 *S. acutum*, 8 *S. tetrandra*, 13 *A. fangchi*, and 6 *C. orbiculatus*). PC1, PC2, and PC3 exhibited 95.324% of the total variability. The contents of **2**, **13**, **14**, and **X** peaks were the dominating features in the first principal component (PC1, 36.020% of the total variability), and **6**, **7**, **9**, and **10** were the features with the highest weights in the second principal component (PC2, 34.820% of the total variability). The **1**, **3**, and **5** contents showed the highest weights in the third principal compo-

Table 2. Peak-area ratios (constituent peak-area / internal standard peak-area) of each component in Stephania tetrandra

Sample No.	A/IS	B/IS	1/IS	2/IS	3/IS	4/IS	5/IS	6/IS	X/IS	7/IS	8/IS	9/IS	10/IS	11/IS	12/IS	13/IS	14/IS	15/IS
T1	–	–	–	–	–	0.113	–	2.158	–	2.292	0.491	7.684	0.116	–	–	–	–	–
T2	–	–	–	–	–	0.121	–	4.765	–	4.102	0.906	12.239	0.127	–	–	–	–	–
T3	–	–	–	–	–	0.149	–	4.652	–	4.059	0.473	7.216	0.241	–	–	–	–	–
T4	–	–	–	–	–	0.198	–	4.125	–	2.963	0.799	8.812	0.139	–	–	–	–	–
T5	–	–	–	–	–	0.111	–	2.202	–	3.296	0.685	9.628	0.194	–	–	–	–	–
T6	–	–	–	–	–	0.138	–	2.836	–	3.835	0.372	10.348	0.139	–	–	–	–	–
T7	–	–	–	–	–	0.136	–	2.287	–	3.763	0.567	7.797	0.152	–	–	–	–	–
T8	–	–	–	–	–	0.186	–	3.505	–	3.986	1.073	6.205	0.199	–	–	–	–	–
Ave. ± SD						0.144 ± 0.033		3.316 ± 1.098		3.537 ± 0.641	0.671 ± 0.241	8.741 ± 1.941	0.163 ± 0.043					
max						0.198		4.765		4.102	1.073	12.239	0.199					
min						0.111		2.202		2.292	0.372	6.205	0.116					

–: The symbol stands for undetected.

Table 3. Peak-area ratios (constituent peak-area / internal standard peak-area) of each component in *Aristolochia fangchi*

Sample No.	A/IS	B/IS	1/IS	2/IS	3/IS	4/IS	5/IS	6/IS	X/IS	7/IS	8/IS	9/IS	10/IS	11/IS	12/IS	13/IS	14/IS	15/IS
A1	–	–	–	0.168	–	–	–	–	1.609	–	–	1.228	–	–	0.038	0.465	0.340	–
A2	–	–	–	0.115	–	–	–	–	2.457	–	–	1.775	–	–	0.057	0.596	0.568	–
A3	–	–	–	0.136	–	–	–	–	2.443	–	–	1.937	–	–	0.028	0.498	0.451	–
A4	–	–	–	0.155	–	–	–	–	2.453	–	–	1.854	–	–	0.032	0.646	0.453	–
A5	–	–	–	0.143	–	–	–	–	2.731	–	–	2.102	–	–	0.021	0.478	0.455	–
A6	–	–	–	0.141	–	–	–	–	2.738	–	–	1.651	–	–	0.032	0.627	0.595	–
A7	–	–	–	0.164	–	–	–	–	2.711	–	–	1.623	–	–	0.040	0.615	0.570	–
A8	–	–	–	0.104	–	–	–	–	1.905	–	–	1.899	–	–	0.022	0.411	0.276	–
A9	–	–	–	0.168	–	–	–	–	2.835	–	–	2.199	–	–	0.035	0.513	0.580	–
A10	–	–	–	0.105	–	–	–	–	2.931	–	–	1.187	–	–	0.044	0.583	0.551	–
A11	–	–	–	0.140	–	–	–	–	1.672	–	–	1.320	–	–	0.053	0.275	0.281	–
A12	–	–	–	0.160	–	–	–	–	2.894	–	–	2.229	–	–	0.049	0.676	0.499	–
A13	–	–	–	0.116	–	–	–	–	1.342	–	–	1.299	–	–	0.052	0.475	0.333	–
Ave. ± SD				0.140 ± 0.023					2.363 ± 0.544			1.716 ± 0.367			0.039 ± 0.012	0.527 ± 0.111	0.457 ± 0.116	
max				0.168					2.931			2.229			0.053	0.676	0.595	
min				0.104					1.342			1.187			0.021	0.275	0.333	

–: The symbol stands for undetected.

Table 4. Peak-area ratios (constituent peak-area / internal standard peak-area) of each component in *Cocculus orbiculatus*

Sample No.	A/IS	B/IS	1/IS	2/IS	3/IS	4/IS	5/IS	6/IS	X/IS	7/IS	8/IS	9/IS	10/IS	11/IS	12/IS	13/IS	14/IS	15/IS
C1	0.422	1.264	–	9.448	-	0.415	–	–	–	–	–	0.128	–	0.711	–	–	–	0.063
C2	0.405	1.255	–	9.240	-	0.385	–	–	–	–	–	0.121	–	0.661	–	–	–	0.061
C3	0.399	1.242	–	9.001	-	0.432	–	–	–	–	–	0.119	–	0.694	–	–	–	0.060
C4	0.412	1.205	–	8.842	-	0.443	–	–	–	–	–	0.164	–	0.626	–	–	–	0.069
C5	0.613	1.193	–	7.003	-	0.519	–	–	–	–	–	0.177	–	0.539	–	–	–	0.098
C6	0.555	1.001	–	7.232	-	0.534	–	–	–	–	–	0.194	–	0.540	–	–	–	0.088
Ave. ± SD	0.468 ± 0.092	1.193 ± 0.098		8.462 ± 1.064		0.455 ± 0.059						0.151 ± 0.032		0.628 ± 0.075				0.073 ± 0.016
max	0.613	1.264		9.448		0.534						0.194		0.711				0.098
min	0.399	1.001		7.003		0.385						0.119		0.539				0.060

– The symbol stands for undetected.

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nent (PC3, 24.564% of the total variability). Examining a three-dimensional score plot in the space defined by the PC1, PC2, and PC3, 32 samples of *S. acutum* (1), *S. tetrandra* (2), *A. fangchi* (3), and *C. orbiculatus* (4) could be distinguished. Consequently, five blind samples of the Fangchi Radix (A14, A15, T9, T10, T11) were included in the training set, and analyzed by PCA. The localization of a total of 37 samples, according to their score values, on planes defined by the new variable factors 1, 2, and 3, are shown in Figure 3. It is shown that *S. acutum* is clustered at the middle right-hand side of the PCA plot, *S. tetrandra*, on the upper left-hand side, *A. fangchi*, on the lower left-hand side; and *C. orbiculatus*, at the middle. By comparing the three dimensional presentation graphs before and after adding the 5 blind samples, it was found that the localization of the training sets changed slightly, and all the blind samples fell into the right groups. Meanwhile, the samples A14 and A15 were grouped under *C. orbiculatus* and samples T9, T10, and T11 under *S. tetrandra*, as shown in Figure 3.

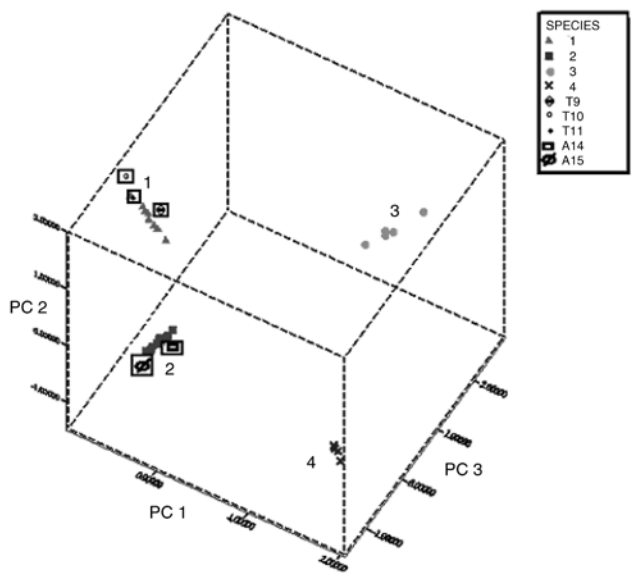

Figure 3. PCA analysis plot from 37 HPLC data (including 5 prediction set data) for *Sinomenium acutum* (1), *Stephania tetrandra* (2), *Aristolochia fangchi* (3), and *Cocculus orbiculatus* (4).

Similar results were obtained after the application of cluster analysis at a distance of 3.5 (full scale: 25). As Figure. 4 shows, the first cluster comprised 13 samples from *A. fangchi* and A14, A15. The two blind samples, A14 and A15, were grouped into this cluster. The second cluster comprised of 5 samples from *S. acutum*. The third

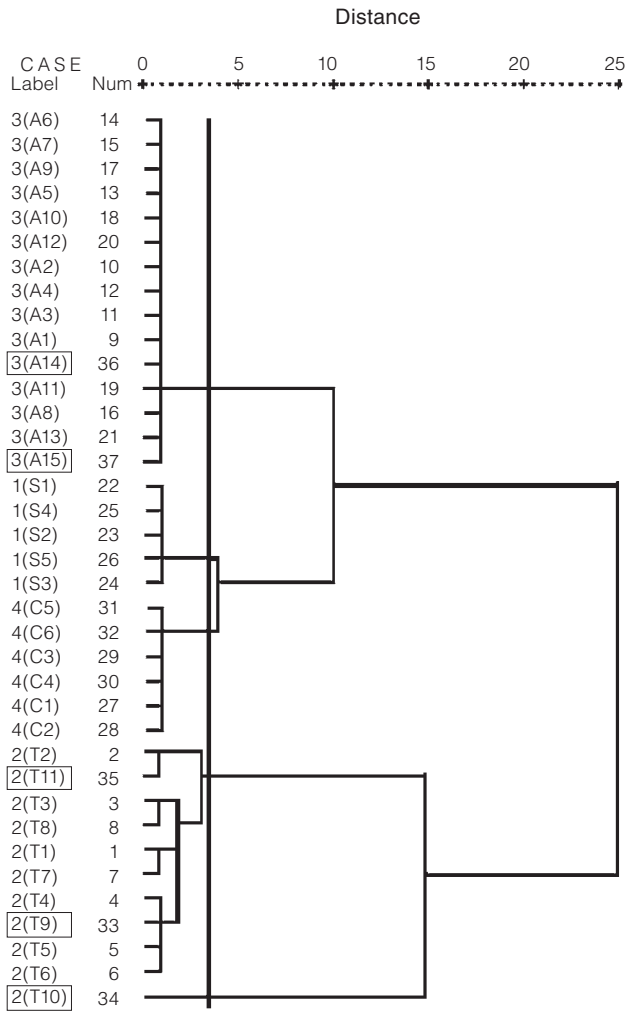

Figure 4. Dendrogram of cluster analysis: *Sinomenium acutum* (1), *Stephania tetrandra* (2), *Aristolochia fangchi* (3), *Cocculus orbiculatus* (4), and the samples acting as unknown articles: A14, A15, T9, T10, T11.

Table 5. Peak-area ratios (constituent peak-area / internal standard peak-area) of each component in Fangchi Radix as the unknown samples

Sample No.	A/IS	B/IS	1/IS	2/IS	3/IS	4/IS	5/IS	6/IS	X/IS	7/IS	8/IS	9/IS	10/IS	11/IS	12/IS	13/IS	14/IS	15/IS
T9	–	–	0.461	4.176	3.327	0.732	9.382	–	–	–	–	0.091	–	–	–	–	–	–
T10	–	–	0.315	4.108	10.818	0.556	3.803	–	–	–	–	0.433	–	–	–	–	–	–
T11	–	–	0.112	4.194	5.701	0.481	10.978	–	–	–	–	0.239	–	–	–	–	–	–
A14	–	–	–	0.140	–	–	–	–	1.616	–	–	1.505	–	–	0.038	0.405	0.502	–
A15	–	–	–	0.182	–	–	–	–	0.508	–	–	1.591	–	–	0.074	0.646	0.962	–

–: The symbol stands for undetected.

cluster comprised 6 samples from *C. orbiculatus*. Finally, the fourth cluster comprised 8 samples from *S. tetrandra* and T9–T11, the latter were the other three prediction samples.

(II) *LDA*

The classification procedure for LDA maximizes the variance between categories, but minimizes the variance within categories. LDA renders the number of orthogonal linear discriminant functions, which is one less than the number of categories.

The recognition of the four classes (*S. acutum*, *S. tetrandra*, *A. fangchi*, and *C. orbiculatus*) by LDA procedure was highly satisfactory (Table 6, Figure 5). A cross-validation process in which each case was classified based on the functions derived from all the cases other than a particular case was performed. In this procedure, the data point obtained from a particular case was eliminated in each iteration of the calculation and the discriminant function of the remaining (n−1) data points was computed. The resulting discriminant function was then used to categorize the previously eliminated data point. The results showed that all Fangchi Radix samples were correctly classified by the LDA method, either in the original grouped cases or in the cross-validated grouped cases. The discrimination function is also shown in Table 6, with **15**, **2**, and **11** showing variables with the highest weights in discriminant function 1 (+5.833, +3.328, and +2.096, respectively). Discriminant function 2 was constituted mainly by **A** (+3.019), **2** (+2.966), and **15** (–2.962), and discriminant function 3 mainly by **10** (+2.296), **9** (+1.379), and **7** (–1.242), respectively. In addition, these variables were found to be the dominating features in PC1, PC2, and PC3.

CONCLUSIONS

Thirty-seven commercial samples of Fangchi Radix

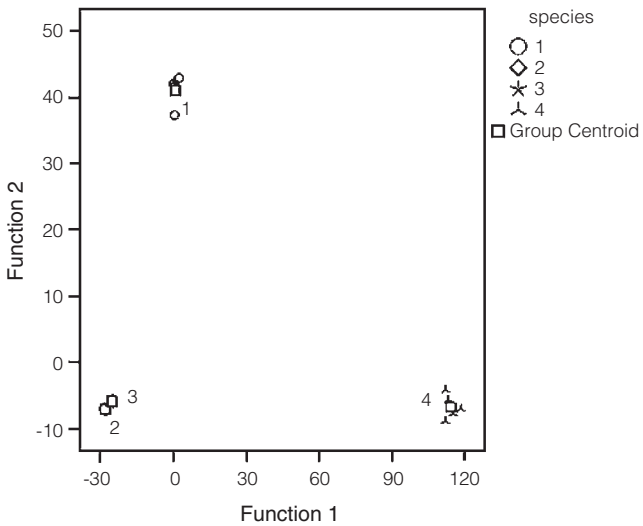

Figure 5. LDA analysis plot from 37 HPLC data for *Sinomenium acutum* (1), *Stephania tetrandra* (2), *Aristolochia fangchi* (3), and *Cocculus orbiculatus* (4).

Table 6. Classification with LDA of Fangchi Radix

		Predicted Group Membership			
Actual group	Group size	*Simomenium acutum*	*Stephania tetrandra*	*Aristolochia fangchi*	*Cocculus orbiculatus*
original					
Simomenium acutum	5	**5 (100%)**	0 (0%)	0 (0%)	0 (0%)
Stephania tetrandra	11	0 (0%)	**11 (100%)**	0 (0%)	0 (0%)
Aristolochia fangchi	15	0 (0%)	0 (0%)	**15 (100%)**	0 (0%)
Cocculus orbiculatus	6	0 (0%)	0 (0%)	0 (0%)	**6 (100%)**
Cross-validated					
Simomenium acutum	5	**5 (100%)**	0 (0%)	0 (0%)	0 (0%)
Stephania tetrandra	11	0 (0%)	**11 (100%)**	0 (0%)	0 (0%)
Aristolochia fangchi	15	0 (0%)	0 (0%)	**15 (100%)**	0 (0%)
Cocculus orbiculatus	6	0 (0%)	0 (0%)	0 (0%)	**6 (100%)**

Discriminant function 1 = –1.088**A/IS** – 0.75**B/IS** – 0.051**1/IS** + 3.328**2/IS** – 0.567**3/IS** – 0.307**4/IS** + 0.938**5/IS** + 0.086**6/IS** + 0.003**X/IS** + 0.304**7/IS** – 0.035**8/IS** – 0.219**9/IS** – 0.437**10/IS** + 2.096**11/IS** – 0.013**12/IS** – 0.028**13/IS** – 0.051**14/IS** + 5.833**15/IS**

Discriminant function 2 = 3.019**A/IS** – 0.128**B/IS** + 1.486**1/IS** + 2.966**2/IS** –1.748**3/IS** + 0.056**4/IS** + 2.792**5/IS** - 0.007**6/IS** - 0.045**X/IS** + 0.014**7/IS** – 0.073**8/IS** – 0.122**9/IS** – 0.137**10/IS**- 2.789**11/IS** – 0.056**12/IS** – 0.042**13/IS** – 0.040**14/IS** – 2.962**15/IS**

Discriminant function 3 = –0.220**A/IS** + 0.146**B/IS** – 0.038**1/IS** – 0.325**2/IS** – 0.695**3/IS** + 0.953**4/IS** + 0.256**5/IS** – 0.308**6/IS** – 0.408**X/IS** – 1.242**7/IS** + 0.450**8/IS** + 1.379**9/IS** + 2.296**10/IS** + 0.204**11/IS** – 0.431**12/IS** – 0.166**13/IS** + 0.057**14/IS** – 0.023**15/IS**

were classified into four species, *S. acutum*, *S. tetrandra*, *A. fangchi*, and *C. orbiculatus*, by PCA and CA techniques. They were also proven correct by the LDA procedure. This result clearly demonstrates that differentiation and classification of Fangchi Radix samples from different species are possible using the profiles of constituent-contents (peak-area rations of the HPLC chromatograms) in the samples and by the application of multi-dimensional chemometric techniques.

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