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Short communication

Chemical fingerprinting of *Isatis indigotica* root by RP-HPLC and hierarchical clustering analysis

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Abstract

The aim was to establish a method for extraction and chemical fingerprinting of extracts of *Isatis indigotica* roots ("Ban–Lan–Gen") and to apply the method developed to 18 Ban–Lan–Gen samples. RP-HPLC with gradient elution was performed on authentic reference standards of powdered *I. indigotica* roots, indigotin and indirubin purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) of China. Eighteen "Ban–Lan–Gen" samples (including the reference powdered herb) were bought from Singapore and different regions in China. Comparisons of the chromatograms showed that the samples can be divided into three groups. The chromatograms of the extracts of five samples were found to be similar to that of the extract of the authentic sample. Eight other samples had similar peaks as the authentic sample but the intensities of the peaks were generally lower, except for the peaks between retention times of 10–40 min. Peaks in these regions were more intense than those found in the extract of the authentic sample. Forty-five characteristic peaks could be found in the extracts of all the above samples. Peaks at retention times 52 and 53 min were determined to be indigotin and indirubin, respectively. The remaining four samples had similar chemical fingerprints to each other but were different from that of the authentic sample. Hierarchical clustering analysis gave similar results as the visual comparison. The RP-HPLC method developed allows simple identification and comparisons of *I. indigotica* roots. This is the first report of hierarchical clustering analysis of *I. indigotica* root.

Keywords: *Isatis indigotica*; HPLC; Chemical fingerprinting; Hierarchical clustering analysis

1. Introduction

Isatis indigotica Fort. (Chinese woad) is a biennial herbaceous plant species distributed widely in China and used as a traditional Chinese medicine. It is different from *Isatis tinctoria* (European woad) which was used for production of blue dye indigo [1]. The root of *I. indigotica* (Radix Isatidis), named "Ban–Lan–Gen" in China, is used for the treatment of influenza, epidemic hepatitis and epidemic encephalitis B for hundreds of years in China [2]. As one of the most popular herbal medicines, the root of *I. indigotica* has been recorded in Chinese Pharmacopoeia since 1985 and its purified extracts have been formulated for clinical use [3a].

Many chemical compounds have been isolated from *I. indigotica* roots, including indigotin, indirubin, isatin, isatan A, isatan B, trytanthrin, purin, isaindigotidione, organic acids and many amino acids [4,5]. Indigotin and indirubin have been reported to be biologically active. Their structures are shown in Fig. 1. Indirubin has been shown to have an anti-cancer activity in the treatment of chronic granulocytic leukemia [6]. Recently, it was found that indigotin and indirubin were potent aryl hydrocarbon receptor (AhR) agonists [7] and also that the organic acids in *I. indigotica* roots had in vitro anti-endotoxic action and antiviral action [8]. The alkaloid isaindigotone from *I. indigotica* was reported [9] to be a scavenger of superoxide generated

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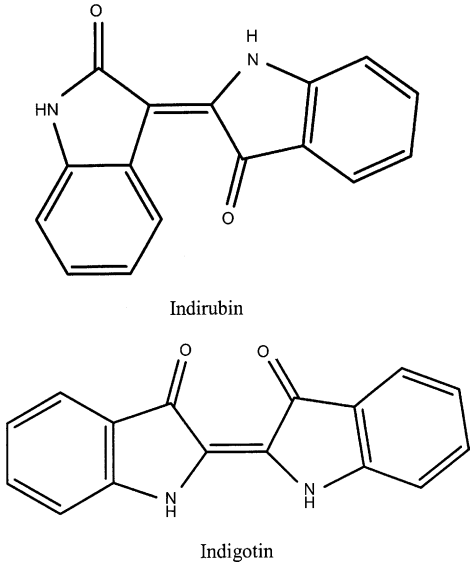

Fig. 1. Chemical structures of indirubin and indigotin.

either by the hypoxanthine/xanthine oxidase system or stimulated human neutrophils. Isaindigotone also inhibited 5-lipoxygenase activity and leukotriene B_4 production in human neutrophils. It is likely that none of the above compounds can fully account for all the activities of Ban–Lan–Gen. Therefore, a holistic approach for quality control is necessary.

It is well known that herbs collected at different times and planted in different regions may differ in the types and quantities of chemical components, thereby affecting the efficacy. Due to its wide applications, *I. indigotica* roots are in great demand and sometimes in shortage. In the official Chinese Pharmacopoeia, *Baphicacanthus cusia* is listed as "Nan–Ban–Lan–Gen" [3b] and often sold as a "Ban–Lan–Gen" substitute. Scientific methods are needed to control the quality of *I. indigotica* roots. One common method used by the industry is to quantify indigotin and indirubin [10]. Although it has been the adopted method for quality control, the presence of the chemical markers does not always guarantee identity and efficacy, especially if the chemical markers have been spiked into the product. It is difficult to evaluate a sample without any information of the other components other than one or two marker compounds. Chromatographic fingerprinting is a useful method in the identification and quality control of botanical medicines [11–16] and HPLC fingerprinting analysis of the roots and leaves of *Isatis* species has been reported [17,18]. However, the reported extraction and analysis methods provided incomplete fingerprints of *I. indigotica* roots. Extraction with water or ethyl acetate gave incomplete components. None of the peaks for *I. indigotica* were identified in the reported chemical fingerprints.

The objectives of this work were to establish a new and simple HPLC method for the identification and quality evaluation of *I. indigotica* roots. The chromatograms of extracts of samples from different regions are compared visually and analyzed using hierarchical clustering analysis.

2. Experimental

2.1. Plant materials

Authentic reference standard of powdered *I. indigotica* roots (Sample 1) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) of China. Seventeen other samples were purchased from different medical halls in Singapore and different regions of China as "Ban–Lan–Gen" (Table 1).

Table 1
Sources of samples

Group no.	Sample no.	Sources	Physical appearance of samples
1	1	NICPBP	Brownish-yellow powder
	2	TS medical hall, Singapore	Cut roots of about 0.5 cm in diameter, 10–20 cm long, brownish-yellow and thin root, with rootlet scars, yellowish-white in bark, yellow in wood, slight odour. Agree with the descriptions of *I. indigotica* root in Chinese Pharmacopoeia
	3	Hebei, China	
	4	Sinch medical hall, Singapore	
	5	CBS medical hall, Singapore	
	6	Zoucheng, Shandong, China	
2	7	TRT I, Beijing, China	Cut roots of about 0.5 cm in diameter, 10–20 cm long, brownish-yellow and thin root, with rootlet scars, yellowish-white in bark, yellow in wood, slight odour. Agree with the descriptions of *I. indigotica* root in Chinese Pharmacopoeia
	8	TRT II, Beijing, China	
	9	WYN I medical hall, Singapore	
	10	BJTRT, Singapore	
	11	Quxian, Anhui, China	
	12	Congming, Shanghai, China	
	13	Station medical hall, Beijing	
	14	Gansu, China	
3	15	EYS medical hall, Singapore	Cut roots of 0.1–1.0 cm in diameter, 10–30 cm long, greyish-brown, texture hard and fragile, greyish-blue in wood.
	16	WYN II medical hall, Singapore	
	17	DRXZ medical hall, Singapore	
	18	DX medical hall, Singapore	

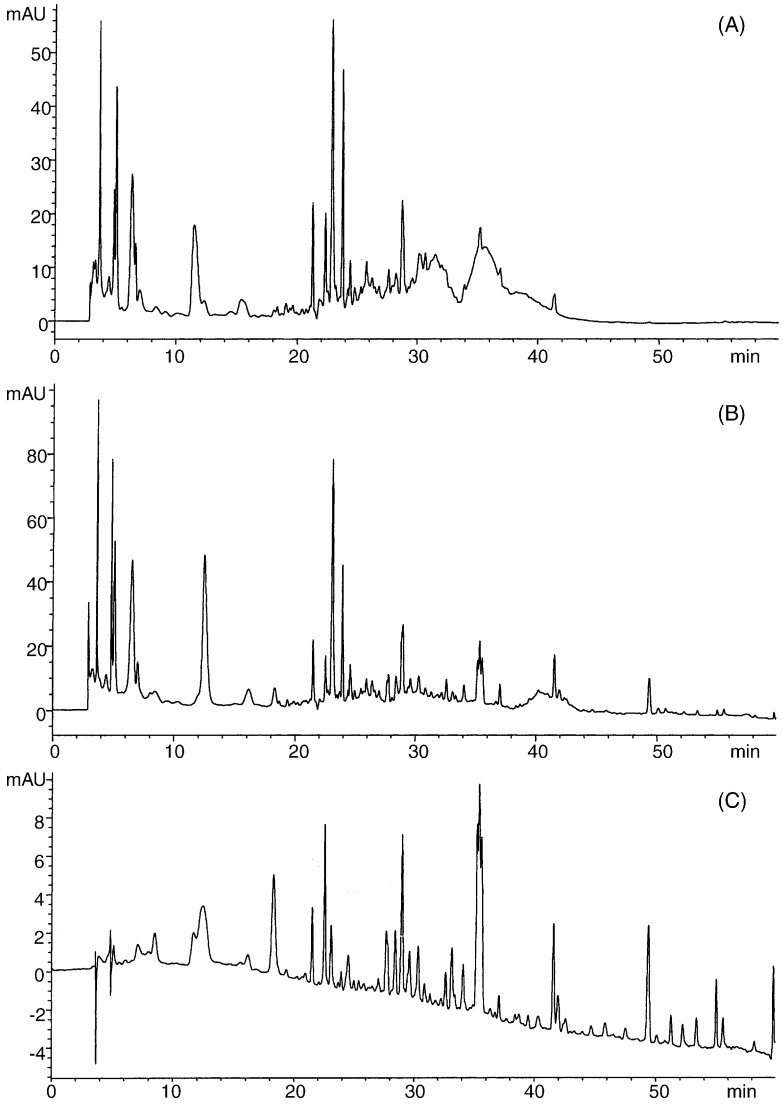

Fig. 2. HPLC chromatograms of extracts of Sample 14 (2 g) obtained by Soxhlet extraction with (A) hot water, (B) methanol and (C) chloroform.

2.2. Standards and solvents

Authentic reference standards indigotin and indirubin were purchased from NICPBP. HPLC grade acetonitrile and AR grade methanol were bought from Fisher Scientific (USA). MilliQ Water was obtained using a Synergy Purification System (France).

2.3. Instrumentation and chromatographic conditions

All HPLC analyses were performed using an Agilent 1100 series HPLC chromatograph with diode-array detector. An ODS-3 Inertsil 5 μm column (25 cm × 4.6 mm i.d.) was used for chromatographic separations. The mobile phase was water A–acetonitrile B system. The gradient elution profile was: 0–10 min water A:acetonitrile B (95:5, v/v), 10–45 min water A:acetonitrile B (95:5, v/v) to water A:acetonitrile B (50:50, v/v), 45–60 min water A:acetonitrile B (50:50, v/v) to 100% B. The flow rate was 1 ml/min, the column temperature was 35 °C and the injection volume was 10 μm. The UV detector was set at 210, 240, 260, 280 and 310 nm.

2.4. Sample preparation

Approximately 2 g of ground plant material was accurately weighed and extracted with water, methanol or chloroform in a Soxhlet apparatus for 6 h. The extract was filtered and evaporated under vacuum and reconstituted with 10 ml methanol. 2.0 ml was filtered through a 0.45 μm Nylon filter (Whatman, UK) for analysis. Subsequent extractions were carried out using 4 g of ground plant material, accurately weighed and extracted with methanol as above.

2.5. Hierarchical clustering analysis

The hierarchical clustering analysis (HCA) of Samples 1–14 was performed using SPSS software (SPSS for

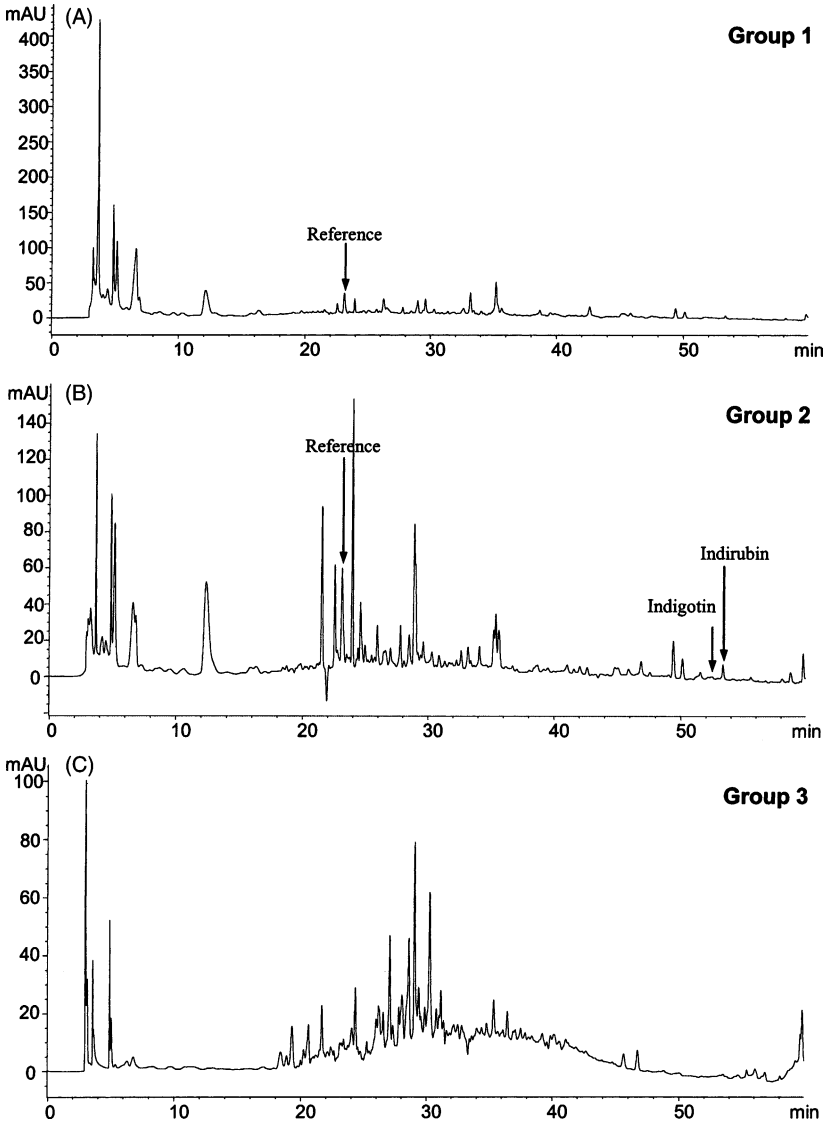

Fig. 3. Representative HPLC chromatograms of extracts from (A) Group 1 (Sample 1, the reference herb from NICPBP), (B) Group 2 (Sample 13) and (C) Group 3 (Sample 15).

Windows 11.5, SPSS Inc., USA). A method called average linkage between groups was applied and Pearson correlation was selected as a measurement [19]. Forty-five Characteristic peaks in the chromatograms were selected and the peak at retention time 23.1 min was used as a reference.

2.6. Precision

To assess the repeatability of the method, Samples 2 and 3 were each extracted three times and each of these extracts was injected three times for HPLC analyses. The R.S.D. of the retention time and peak area of the reference peak in the three extracts of both Samples 2 and 3 were determined. One of the extracts of Sample 2 and one of the extracts of Sample 3 were analyzed by HPLC again one week and one month after extraction. The inter day variations of the retention time and peak area of the reference peak in one of the extracts of Samples 2 and 3 were also determined.

3. Results and discussion

All 18 samples were primarily identified according to the descriptions in Chinese Pharmacopoeia. The physical appearances of Samples 1–14 agreed with the descriptions of *I. indigotica* root in the Chinese Pharmacopoeia while those of Samples 15–18 agreed with the descriptions of *B. cusia* (Table 1).

Traditional Chinese herbal medicines are commonly decocted by boiling with water. However, the concentrations of most of the components extracted from *I. indigotica* roots were found to be lower in the aqueous extract than in the methanol extract (Fig. 2). Some low polarity components

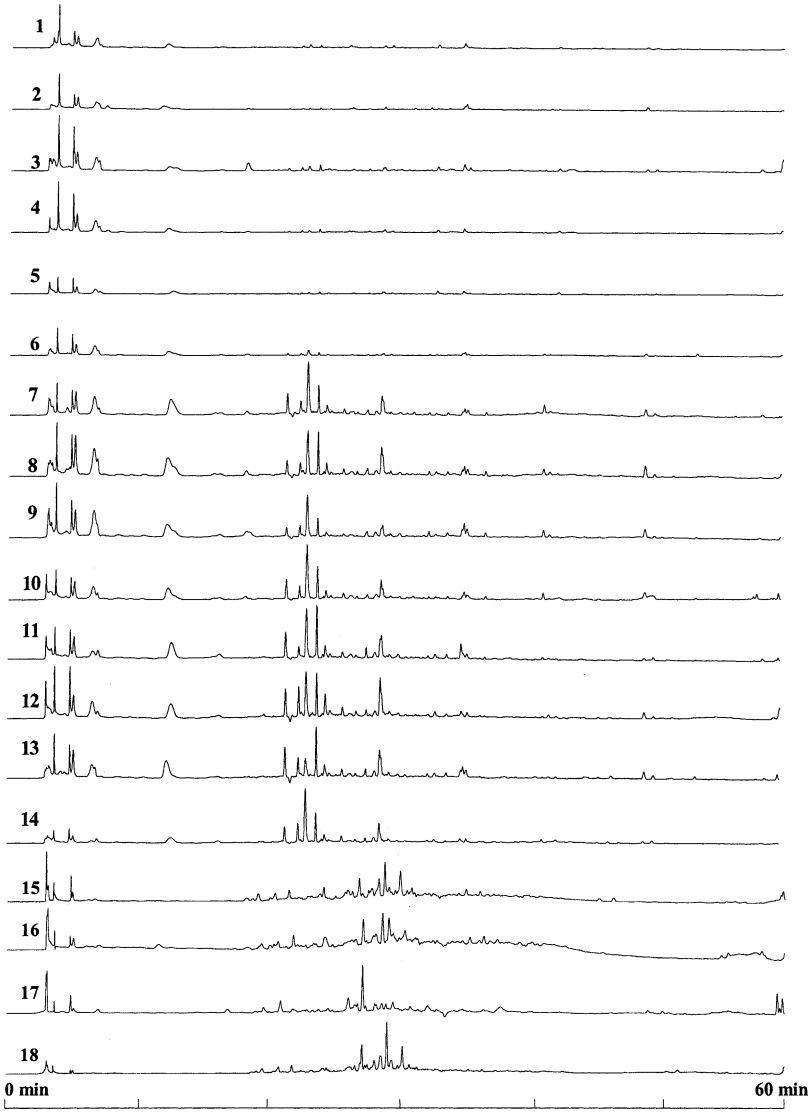

Fig. 4. Overlaid HPLC chromatograms of extracts of Samples 1–18.

were not found in the chromatogram of the aqueous extract. The concentrations of most peaks in the chromatogram of the chloroform extract were lower than those in the methanol extract and some high polarity components typically eluting between 0 and 10 min were not detected in the chloroform extract. Hence, chloroform is undesirable as an extraction solvent because high polarity components such as organic acids were reported to have anti-endotoxic activity and were of great interest in chemical fingerprinting. To extract and detect as many components as possible, methanol was hence selected as the extraction solvent. DAD full scan (200–400 nm) was used. However, for the purpose of HCA, five wavelengths (210, 240, 260, 280 and 310 nm) were also specified, as 3-D data are not suitable for HCA. It was found that there were more constituents of measurable levels at wavelengths 210 and 280 nm. Indigotin and indirubin had maximum absorbances at 280 nm. Hence, the results at 280 nm were used for HCA.

Peaks of the chemical markers indigotin and indirubin were assigned in the HPLC chromatograms by comparing individual peak retention times and the UV spectra with those of the authentic reference standards. Peaks at retention times 52.2 and 53.3 min were determined to be indigotin and indirubin respectively (Fig. 3). The peak corresponding to indirubin was found in the HPLC chromatograms of all the 14 samples but the peak corresponding to indigotin was only found in 9 samples.

With the same chromatographic conditions, comparisons of the chromatograms showed that all the 18 samples could be divided into three groups (Fig. 4). The chromatograms of Samples 1–14 were similar in peak retention times but differed in peak areas. Chromatograms of the extracts of Group 1 (Samples 2–6) were similar to that of the extract of the authentic sample (Sample 1). Members of Group 2 (Sample 7–14) had similar peaks as the authentic sample but the intensities of the peaks were generally lower, except for the

peaks between retention times of 10–40 min. Peaks in these regions were more intense than those found in the extract of the authentic sample (Fig. 3). Chromatograms of extracts of Samples 15–18 were found to be different from those of the other 14 samples (Fig. 4), which suggested that they served as substitutes of *I. indigotica*. These were classified as Group 3. From the physical appearance of the cut roots, these samples resembled *B. cusia*.

The quality of herbal medicine is closely related to the concentrations of their chemical constituents. Although it is possible to visually differentiate the different chromatograms, however, the process is subjective and not quantitative. In addition, minor differences between very similar chromatograms might be missed. Hence, HCA was used to compare the chromatograms of Samples 1–14. The peak at retention time 23.1 min was selected as a reference peak because it was a strong single peak in the middle of the chromatograms of all the 14 samples (Fig. 3). Forty-five characteristic peaks were selected and the relative retention times of these constituents were calculated with respect to the reference peak at retention time 23.1 min. The relative areas of the 45 characteristic peaks were calculated by using the area of the reference peak as a reference standard. Relative areas of the 45 constituents of Samples 1–14 formed a matrix of 14×45. Similarities among the 14 *I. indigotica* roots were calculated using the SPSS software. The results of HCA were shown in Fig. 5. It was clear that the samples could be divided into two clusters: Samples 1–6 in Cluster one and Samples 7–14 in Cluster two. This result agreed very well with visual comparisons of their chromatograms (Fig. 4). HCA provided a quantitative comparison of the samples. HCA was not performed on the Group 3 samples as their chemical fingerprints were entirely different from those of Groups 1 and 2, and they did not contain all of the 45 characteristic peaks, including the reference peak at retention time 23.1 min.

The difference in contents of the components may be due to many factors such as different geographical origins, different genetic sources, processing methods and age of roots when harvested. In this study, the results showed that some of the herbal samples bought in Singapore were similar to these from China. One reason could be that those samples originated from China. However, the provinces of origin were not made known to us. In this study, concentrations of indirubin and indigotin were found to be very low in all samples. This suggests that assessing the quality of *I. indigotica* roots using these two marker compounds alone is not recommended, a view shared by other workers [20,21].

The repeatability of the extraction technique, HPLC method and HCA was determined by replicate analyses of Samples 2 and 3. The two samples were selected because of the similarity in their chromatograms. Each sample was extracted three times and each extract was injected three times. The 45 peaks were detected in all the 18 chromatograms. The HCA results agreed well with the previous results showed in Fig. 5. The different extracts of the same sample (e.g. Sample 2) were similarly clustered with samples in the sample

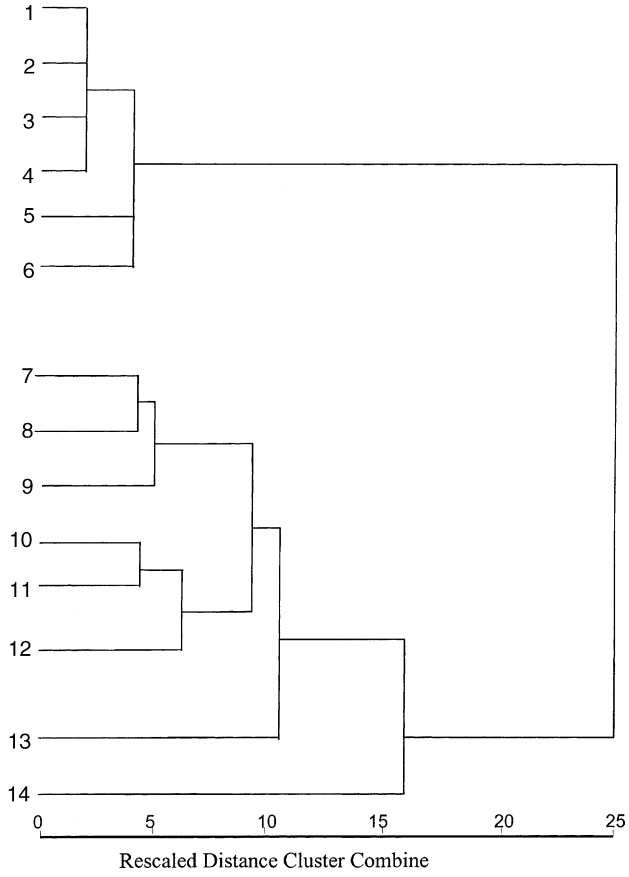

Fig. 5. Results of hierarchical clustering analysis of 14 *I. indigotica* roots (dendrogram using average linkage between groups).

group (Samples 1–6). The R.S.D. of the retention time and peak area of the reference peak in the three different extracts of Sample 2 were 0.05 and 9.3%, respectively. The R.S.D. of the retention time and peak area of the reference peak in the three different extracts of Sample 3 were 0.07 and 10.5%, respectively. The intermediate precision was assessed by replicate injections (three times) of the second extract of Samples 2 and 3 one week and one month after extraction. The inter day variations of the peak retention time and peak area of the reference peak were typically acceptable (R.S.D.. < 0.3 and 13.4%, respectively). One week after extraction, all the 45 peaks were detected in the chromatograms of the second extracts of Samples 2 and 3. These extracts of Samples 2 and 3 could still be differentiated in the HCA results. This suggests that the extraction method, HPLC analysis and HCA method are able to differentiate the *I. indigotica* roots.

A new batch of *I. indigotica* root was also purchased from a local medical hall (about 6 months later) and analyzed. The results showed that 42 characteristic peaks out of the 45 were detected. The other three minor components (accounting for 0.62 ± 0.15, 0.64 ± 0.21 and 0.74 ± 0.35% of the total area) could not be detected. The HCA method was also applicable when only 12 peaks were used. To apply the

method to a sample which did not possess all the 45 characteristic peaks, 12 peaks out of these 45 peaks were selected as markers in the fingerprint. Each of these 12 peaks contributed >1.0% to the total peak area. In Samples 1–14, the total area of these 12 peaks represented about 31.4–43.7% of the total area of all peaks in each chromatogram. Similar clustering results were obtained when the 12 peaks were evaluated by the hierarchical clustering analysis. The relative retention times of the 12 peaks were 0.16, 0.21, 0.22, 0.29, 0.53, 0.98, 1.00, 1.28, 1.44, 1.52, 1.84 and 2.14, respectively. They can serve as characteristic peaks for identification of "unknown" samples.

4. Conclusion

In conclusion, the HPLC method developed allows the identification and comparison of the *I. indigotica* roots and can be utilized to assess the quality of *I. indigotica* roots. This method has been validated for precision. The use of SPSS software allows quantitative comparison of *I. indigotica* roots. This method will also help to identify possible spiking with synthetic compounds and prevent counterfeits. Chemical fingerprinting with HCA is one step towards ensuring the quality of Chinese traditional medicines.

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