

# Quality Assessment of *Moringa* Seed Shells Based on Fingerprinting Using HPLC-DAD

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A fingerprint analysis method was established for the quality control of *Moringa* seed shells by high-performance liquid chromatography with diode array detection (HPLC–DAD). The HPLC–DAD separation was performed on a Thermo Hypersil Gold C18 (4.6 mm × 250 mm, 5 μm) column by gradient elution with acetonitrile–water as mobile phase. The fingerprint of *Moringa* seed shells was established with good precision, reproducibility, and stability obtaining within 60 min, and 13 common peaks in the fingerprint were designed. Similarity analysis, principal component analysis (PCA), and hierarchical clustering analysis (HCA) were carried out to analyze the obtained fingerprints. The similarity among 11 batches of samples in addition to No. 5 and 6 was no less than 0.92. Eleven samples could be classified into 2 clusters. The HPLC fingerprint technology and application of chemical pattern recognition can provide a more comprehensive reference for the quality control of medicinal plants.

**Keywords:** *Moringa* seed shells, fingerprint, similarity evaluation, PCA, HCA, HPLC–DAD

## 1 Introduction

*Moringa oleifera* belongs to the genus *Moringaceae*, which consists of 14 known species. Among these species, *M. oleifera* is the most widely known and utilized. The plant is a native of the sub-Himalayan regions of North-West India and is now indigenous to many countries in Africa, Arabia, South East Asia, the Pacific, the Caribbean islands, and South America [1, 2]. The tree is prized mainly for its edible fruits, leaves, flowers, roots, and seed oil [3]. The root extract possesses antimicrobial activity [4]. The extracts from the flowers are found to have hepatoprotective effect [5]. Different parts of a *M. oleifera* tree contain 17 amino acids, protein, vitamin C, glucosinolates, carotenoids, tocopherols, polyunsaturated fatty acids, and other nutritive compositions [6]. *Moringa* seed shells contain bioactive ingredients, such as polyphenols, flavonoids, and etc., and they are used in the prevention and treatment of diabetes, high blood pressure, skin disease, anemia, scurvy, obesity, arthritis, and other diseases [7–13]. The nutritive value and medicinal value of *Moringa* are getting more and more attention [14].

Medicinal plants have diversified types of components that vary in assay; therefore, quality control of these components remains a challenge. In recent years, chromatography methods, such as high-performance liquid chromatography (HPLC) [15–17], gas chromatography–mass spectrometry (GC–MS) [18], and HPLC–MS [19–21], have been used for quantitative analysis and quality control of *Moringa*. In 2013, Coppin and co-workers performed the detailed HPLC with ultraviolet and mass spectroscopy (HPLC–UV–MS) research that focused on establishing and comparing the flavonoid profiles in leaf samples from different African countries [19]. In addition, in 2016, Nouman et al. performed the assessment of polyphenolics in hydro-methanolic extracts obtained from the germplasm leaves of 7 cultivars of *M. oleifera* using HPLC

with photodiode array detection and electrospray ionization–MS (HPLC–PDA–ESI–MS) analysis [20].

Fingerprinting techniques can provide an overall profile of almost every component of medicinal plants and are considered to be an important method for evaluating the quality of medicinal plants [22]. Many health organizations, including the U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the State Food and Drug Administration of China (SFDA), have recommended this strategy to assess the quality and consistency of botanical products. HPLC fingerprinting can be used to detect the complex constituents in plant extracts both qualitatively and quantitatively, and it is highly suitable for quality control of various plants. According to the literature published in the recent years, to the best of our knowledge, no literature study has been documented where a high-performance liquid chromatography fingerprint was used to evaluate the quality of *Moringa* seed shells.

This study aims to offer an efficient and affordable HPLC with diode array detection (HPLC–DAD) method for fingerprinting and reveal the correlation and consistency of quality control in *Moringa* seed shells preparations. The chemical fingerprints of *Moringa* seed shells from various regions were established and investigated by similarity analysis (SA), principal component analysis (PCA) and hierarchical clustering analysis (HCA). The combination of chromatographic fingerprint analysis afford a more comprehensive strategy for the quality evaluation of *Moringa* seed shells.

## 2 Experimental

**2.1 Chemicals and Reagents.** 4(α-L-rhamnosyloxy)-benzyl isothiocyanate was purchased from Shanghai Ann Spectrum Experiment Technology Co., Ltd. (Shanghai, China). The purity of the component was determined to be higher than 98.0% by HPLC. The HPLC grade methanol, acetonitrile, phosphoric acid

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**Table 1.** Representative samples of *Moringa* seed shells investigated in this study

Sample No.	Herbal species	Locations	Collection time
S1	<i>Moringa oleifera</i> Lam.	Hot-Dry Valley, Sichuan Province	2015-9-11
S2	<i>Moringa oleifera</i> Lam.	Lum palmer bottom, Yunnan Province	2015-9-22
S3	<i>Moringa oleifera</i> Lam.	Baoshan Conggang, Yunnan Province	2015-10-11
S4	<i>Moringa oleifera</i> Lam.	Dehong palmer bottom, Yunnan Province	2015-10-11
S5	<i>Moringa oleifera</i> Lam.	Northwestward, India	2015-9-16
S6	<i>Moringa oleifera</i> Lam.	Kokang, Myanmar	2015-9-20
S7	<i>Moringa oleifera</i> Lam.	Dehong Mangshi, Yunnan Province	2015-10-12
S8	<i>Moringa oleifera</i> Lam.	Lucheng Jiang Ba, Yunnan Province	2015-10-11
S9	<i>Moringa oleifera</i> Lam.	Baoshan, Yunnan Province	2015-10-12
S10	<i>Moringa oleifera</i> Lam.	Jinghong County, Yunnan Province	2015-10-16
S11	<i>Moringa oleifera</i> Lam.	Pu'er, Yunnan Province	2015-10-16

and acetic acid were purchased from Merck (Merck Co., Darmstadt, Germany). Deionized water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). Other chemicals and solvents used in sample preparation were of analytical grade.

**2.2 Plant Materials.** *Moringa* seed samples were collected respectively from different areas (Table 1). Then, the samples were processed, decorticated, and pulverized for later use.

**2.3 Instrumentation and Chromatographic Conditions.** HPLC fingerprint analysis was performed on a DIONEX U3000 system (DIONEX Corp., USA), equipped with a dual ternary solvent manager, an auto-sampler, and a diode array detector, and a class-up station was used for liquid chromatographic analysis. Chromatographic data were accessed, processed, and handled, employing Chromeleon 7 (DIONEX Corp., USA) software. The influence of some chromatographic conditions for the HPLC analysis, such as column length, column temperature, wave length and mobile phase was investigated. Different types of columns were tested. Thermo Hypersil Gold C18 column is better than that of Agilent Eclipse XDB-C18 column. A SB3200 ultrasonic cleaning instrument (Branson Ultrasonic Instrument Co., Shanghai, China) was used for extraction. Comparing the chromatograms at 3 different temperatures, namely, 25 °C, 30 °C, and room temperature, we found no obvious separation difference, but after overall consideration of the analysis time and separating effect, the column temperature was set at room temperature.

Selection of an appropriate detection wavelength was of great importance to ensure precise detection of some essential constituents and to achieve more peaks. DIONEX U3000 with DAD was used in the analysis, and full scan runs were made first to choose the optimum wavelength that provided the best results in chromatographic fingerprinting analysis. Chromatogram at 203 nm showed the most abundant component information and the steadiest baseline, compared with the other wavelengths. Ultimately, we selected 203 nm as the monitoring wavelength.

The effect of the composition of the mobile phase in the chromatographic separation of the samples was researched in this study. Different mobile phases were tried, such as acetonitrile–phosphoric acid/water (1:100, v/v), methanol–acetic acid/water (1:100, v/v), methanol–water, acetonitrile–water, etc. Finally, acetonitrile–water was selected as an appropriate mobile phase with gradient elution. The gradient program was set as follows: 0–20 min, 10–30% (A); 20–40 min, 30–50% (A); 40–45 min, 50–90% (A); 45–50 min, 90% (A); 50–52 min, 90–10% (A); 52–60 min, 10% (A). The injection volume of each sample, as well as standard solution, was 5 µL, and the flow rate was 1.0 mL/min.

**2.4 Sample Preparation.** All samples were ground to fine powder (40 mesh) using a pulverizer. The 11 pulverized samples of *Moringa* seed shells from different locations were accurately weighed (approximately 5.0 g), soaked for 24 h in 30 mL of 50% methanol, and extracted by an ultrasonic cleaner at room temperature with 30 min. The extracted

solutions were filtered through a 0.45-µm filter membrane prior to injection into the HPLC–DAD system.

**2.5 Similarity Analysis.** Characteristic peaks were present in all chromatograms of detected samples. Identification of the characteristic peaks and similarities of the fingerprint data were performed on the basis of the relative retention time (RRT) and relative peak area (RPA) using the professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (2004A). The software was used to employ the correlative coefficient in evaluating the similarities of different chromatogram. Matching among the fingerprints of samples was performed by a multipoint calibration mode based on the retention time and UV spectra. In this test, all 11 samples were examined to generate a reference chromatogram as the representative standard fingerprint, and the similarity of each chromatogram against this standard chromatogram was then calculated. Furthermore, the RRT and RPA of each characteristic peak related to a reference peak were also constructed for quantitative measurement of the chemical composition of *Moringa* seed shells samples.

**2.6 Hierarchical Cluster Analysis.** Hierarchical clustering analysis is a multivariate analytical technique used to classify samples into different groups. In this study, the *Moringa* seed shells samples were grouped by using Statistics software with the v.18.0 SPSS system (SPSS Inc., USA) to discover the differences in samples caused by space factors.

### 3 Results and Discussion

**3.1 Optimization of Extracting Conditions.** To achieve satisfactory extraction efficiency, different extraction conditions were studied by comparing reflux extraction, Soxhlet extraction, ultrasonic extraction, and soak extraction methods. According to the temperature effect and the withdrawal rates of 4(α-L-rhamnosyloxy) benzyl isothiocyanate, the soak extraction of 24 h and the ultrasonic extraction for 30 min were chosen as the extracting method. Meanwhile, various extraction solutions such as petroleum ether (boiling range: 30–60 °C), *n*-hexane, acetone, and 50% methanol were studied. As a result, the optimal extraction conditions are as follows: soak extraction of 24 h and then ultrasonic extraction for 30 min in 50% methanol.

**3.2 Methodology Validation.** All tests below were carried out on the sample extract solutions prepared as described in the section Sample Preparation. The method precision was evaluated by carrying out 6 independent HPLC runs of the same sample solution and by analyzing fingerprints of 5 parallel extraction solutions of the same sample. Precision of sample stability was determined with measurements from a single sample solution stored at room temperature for 0, 2, 4, 6, 8, and 12 h. The reproducibility of the experiment was evaluated by analyzing 6 separate extract solutions of one sample. RRT and RPA of each characteristic peak were calculated for the estimation of precision, stability, and

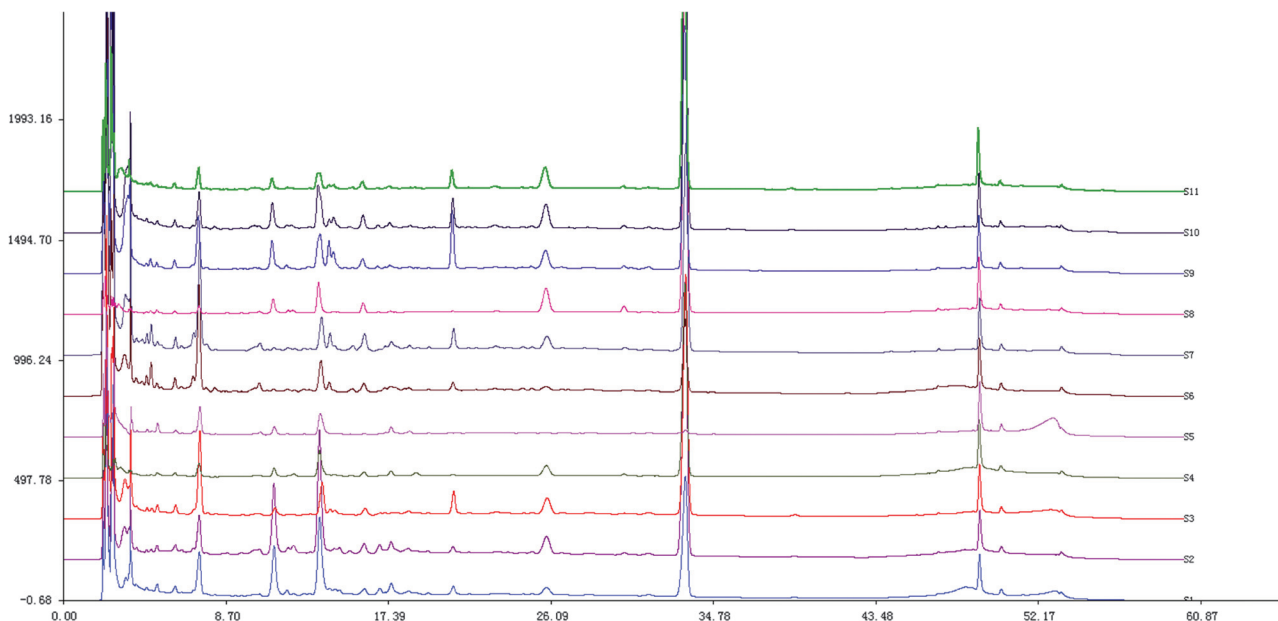


Figure 1. Chromatogram of the fingerprints of the 11 samples

reproducibility, and all the results of the relative standard deviation (RSD) of RRT and RPA were found not to exceed 3.0%. Therefore, all results demonstrated that the quality of the studied samples and the HPLC–DAD measurements were stable and under control.

**3.3 Analysis of High-Performance Liquid Chromatography Fingerprint.** The 11 sample solutions of *Moringa* seed shells and standard substances were prepared and analyzed, and chromatograms of the samples were recorded in 60 min, which is shown in Figure 1. According to the results of HPLC fingerprint, we found 13 common characteristic peaks. Among them, peak 11 at retention time of 33.28 min, which was designated as the reference peak. Because it was present with a stable time and a large peak area in the chromatogram, the RSD value is below 3.0%. Furthermore, its comparative standing time of common peaks approximately fits the requirements of “Technological Requirements of Fingerprint Chromatography”. Consequently, the established fingerprint method for *Moringa* seed shells is convenient and feasible as a tool for species authentication and quality assessment of the raw material.

**3.4 Relative Peak Area and Similarity of the Sample.** The 11 sample solutions of *Moringa* seed shells were prepared and analyzed, and chromatograms of the samples were recorded at 203 nm. The chromatographic peaks in different samples with the same relative retention time were defined as the characteristic peaks. Thirteen peaks were identified as the characteristic peaks. Among the characteristic

peaks, peak 11 at retention time of 33.28 min was designated as the reference peak for the calculation of RPA. On the basis of this reference, RRT and RPA of these 13 peaks in 11 samples were shown in Table 2. The data of the chromatographic fingerprints were imported into the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese medicine (TCM) (Version 2004 A) software. In this study, a similarity analysis based on matching against the median of the fusion vectors of all the samples was performed. The reference chromatogram was generated by the median method. As a result, the similarity indexes of the 11 samples are listed in Table 3.

**3.5 Identification of Chromatographic Peaks.** Standard substances coupled with HPLC-DAD had been generally used for qualitative or quantitative analysis of components in analytes separated. In this study, 4( $\alpha$ -L-rhamnosyloxy) benzyl isothiocyanate (peak 11) was explicitly identified in the samples of *Moringa* seed shells. The other compounds of common peaks were needed to be identified in a further study. The HPLC chromatogram of sample No.1 obtained at 203 nm was shown in Figure 2.

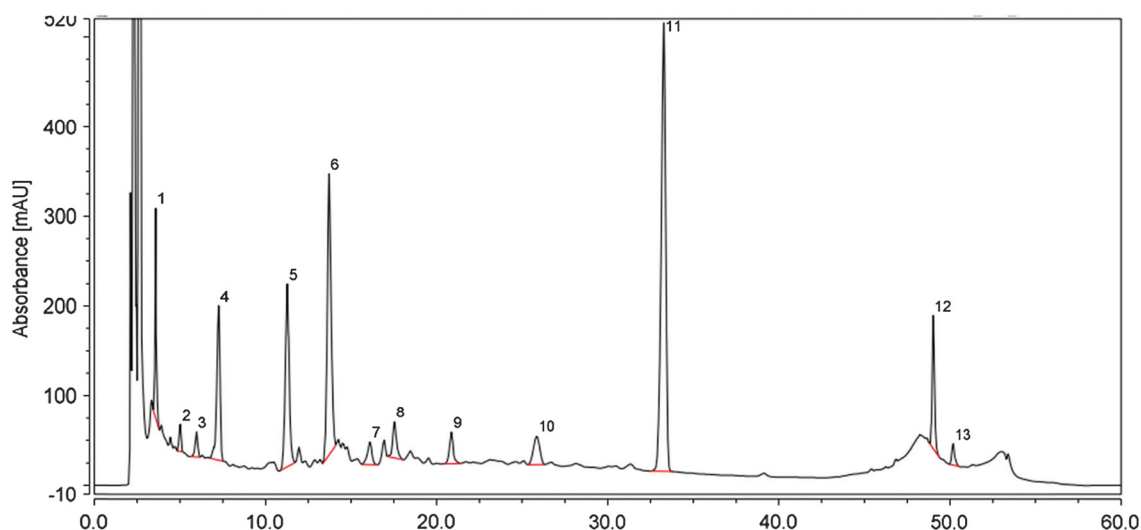
Compared with the reference chromatogram generated by the median method, the similarity indexes of the 11 samples in addition to No. 5 and 6 were all above 0.92 (Table 3). It showed that 9 batch *Moringa* seed shells samples had a high similarity matching, which means that the qualities of 9 batch *Moringa* seed shells samples collected from different locations

Table 2. The calculation result of the relative peak area of the communion peak

RRT	RSD (%)	RPA											RSD (%)
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	
0.108	0.66	0.119	0.047	0.047	0.003	2.466	0.324	0.124	0.001	0.046	0.031	0.013	71.60
0.150	2.22	0.028	0.010	0.014	0.012	1.593	0.029	0.009	0.004	0.014	0.005	0.002	44.86
0.180	0.36	0.032	0.012	0.020	0.009	0.595	0.087	0.028	0.003	0.019	0.010	0.010	44.57
0.218	0.30	0.227	0.082	0.211	0.047	6.059	0.931	0.319	0.011	0.159	0.070	0.044	68.47
0.338	0.45	0.342	0.203	0.027	0.035	2.448	0.018	0.007	0.030	0.086	0.065	0.028	112.58
0.412	0.53	0.548	0.355	0.108	0.122	7.926	0.381	0.142	0.065	0.157	0.138	0.061	71.00
0.483	0.29	0.052	0.035	0.025	0.022	0.288	0.126	0.069	0.022	0.042	0.037	0.023	44.22
0.527	0.66	0.064	0.027	0.001	0.020	1.418	0.022	0.018	0.002	0.007	0.008	0.007	83.87
0.627	0.38	0.056	0.019	0.068	0.007	0.173	0.093	0.074	0.001	0.175	0.061	0.040	99.94
0.776	0.22	0.090	0.086	0.092	0.077	0.333	0.085	0.094	0.092	0.115	0.105	0.097	53.69
1.000	0.07	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	52.17
1.473	0.05	0.152	0.076	0.103	0.134	9.178	0.383	0.135	0.072	0.119	0.084	0.093	8.19
1.508	0.05	0.030	0.013	0.016	0.017	1.634	0.062	0.020	0.009	0.017	0.010	0.010	12.62

**Table 3.** Similarity comparison of chromatographic patterns of the 11 samples

No.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Reference
S1	1.000	0.973	0.896	0.888	0.538	0.825	0.900	0.857	0.917	0.904	0.864	0.929
S2	0.973	1.000	0.951	0.962	0.369	0.769	0.935	0.947	0.963	0.971	0.948	0.978
S3	0.896	0.951	1.000	0.984	0.294	0.830	0.991	0.977	0.991	0.989	0.985	0.994
S4	0.888	0.962	0.984	1.000	0.252	0.744	0.958	0.996	0.978	0.996	0.996	0.991
S5	0.538	0.369	0.294	0.252	1.000	0.659	0.372	0.165	0.316	0.247	0.192	0.324
S6	0.825	0.769	0.830	0.744	0.659	1.000	0.892	0.701	0.811	0.758	0.728	0.810
S7	0.900	0.935	0.991	0.958	0.372	0.892	1.000	0.944	0.979	0.966	0.957	0.981
S8	0.857	0.947	0.977	0.996	0.165	0.701	0.944	1.000	0.969	0.993	0.998	0.981
S9	0.917	0.963	0.991	0.978	0.316	0.811	0.979	0.969	1.000	0.990	0.979	0.993
S10	0.904	0.971	0.989	0.996	0.247	0.758	0.966	0.993	0.990	1.000	0.996	0.996
S11	0.864	0.948	0.985	0.996	0.192	0.728	0.957	0.998	0.979	0.996	1.000	0.986
Reference	0.929	0.978	0.994	0.991	0.324	0.810	0.981	0.981	0.993	0.996	0.986	1.000

**Figure 2.** The HPLC chromatogram of sample No. 1 acquired at 203 nm

were stable and consistent. The 13 characteristic peaks in chromatograms were the main components of *Moringa* seed shells. In practice of quality control, the herbs which have the smallest similarity indexes or are below a certain value can be regarded as not qualified. Consequently, for quality control of *Moringa* seed shells, 0.90 was set as a suitable value, and it is easy to find that the samples of *Moringa* seed shells in addition to No. 5 and 6 with similarity indexes above 0.92 have credible qualities.

**Table 4.** Total variance explained

Components	Initial eigenvalues		
	Total	% of variance	Cumulative %
p 1	4.699	36.149	36.149
p 2	3.266	25.127	61.276
p 3	3.208	24.678	85.953
p 4	0.957	7.364	93.317
p 5	0.388	2.985	96.302
p 6	0.213	1.641	97.943
p 7	0.144	1.110	99.054
p 8	0.082	0.630	99.684
p 9	0.034	0.262	99.946
p 10	0.007	0.054	100.000
p 11	8.245E-17	6.342E-16	100.000
p 12	5.173E-17	3.979E-16	100.000
p 13	-2.877E-17	-2.213E-16	100.000
Components	Extraction sums of squared loadings		
	Total	% of variance	Cumulative %
1	4.699	36.149	36.149
2	3.266	25.127	61.276
3	3.208	24.678	85.953

Extraction method: principal component analysis.

**3.6 PCA.** PCA is an effective data technique, which has long been used as an exploratory method to study the classification of samples [23–28]. PCA was employed to analyze the relationship of the 11 *Moringa* seed shells samples from different sources, implemented by performing singular value decomposition on the data array of areas of the 13 common peaks (Table 2 and 3), each row representing a sample and each column containing the values of 13 peak areas. As is shown in Tables 4 and 5, 3 components were extracted. It is apparent that the 3 extracted components explain 100% of the variance of the data matrix and represent the dominant information. With these figures, differences between the 3 groups were discovered according to principal

**Table 5.** Component matrix

	Components		
	1	2	3
Zscore(p1)	0.691	0.647	0.199
Zscore(p2)	0.721	-0.301	-0.257
Zscore(p3)	0.522	0.780	0.228
Zscore(p4)	0.504	0.843	-0.025
Zscore(p5)	0.461	-0.490	0.720
Zscore(p6)	0.557	-0.358	0.725
Zscore(p7)	-0.048	0.556	0.763
Zscore(p8)	.659	-0.526	0.385
Zscore(p9)	-0.088	0.563	0.306
Zscore(p10)	-0.660	0.041	0.701
Zscore(p11)	-0.642	-0.043	0.709
Zscore(p12)	-0.793	0.390	-0.277
Zscore(p13)	0.855	0.110	-0.333

Extraction method: principal component analysis. Three components extracted.

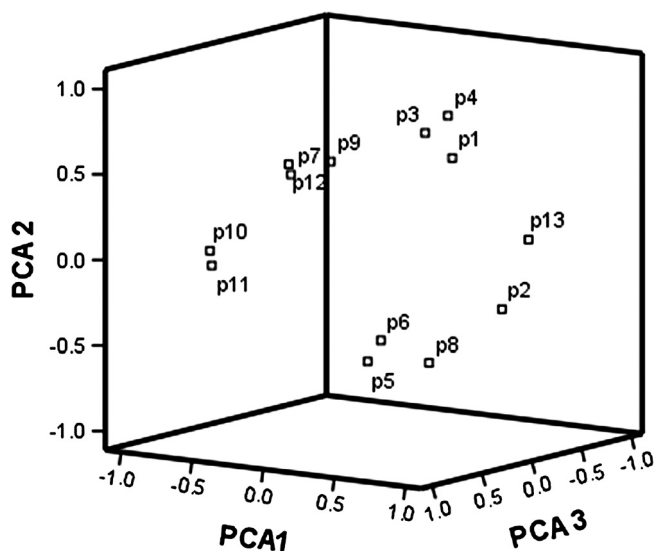


Figure 3. PCA score figure

component analysis (Figure 3). It indicates that PCA combined with similarity analysis can be used for discrimination and classification of *Moringa* seed shell samples.

**3.7 HCA.** The areas of 13 characteristic peaks were selected for HCA. As we know, hierarchical clustering can efficiently organize biological measurements and produce a qualitative and quantitative representation of original experimental results through statistical organization and graphic display [29–33]. In the analysis, the peak areas of the 13 characteristic peaks of samples 1–11 formed a matrix of  $11 \times 13$ . Similarities among samples 1–11 were calculated using the Statistics software. It was clear that the samples could be divided into 2 clusters at a rescaled distance of 25: samples 5 and 6 in cluster 1, and the others could be grouped into cluster 2. HCA provided a quantitative comparison of the samples. The dendrogram constructed according to hierarchical cluster analysis, based on the squared Euclidean distance, shows the relationships among the *Moringa* seed shells samples collected from 11 locations (Figure 4).

The findings of HCA show that there was a larger difference of chemical components between India and Myanmar because of the size of *Moringa* seeds. Similar to the appearance

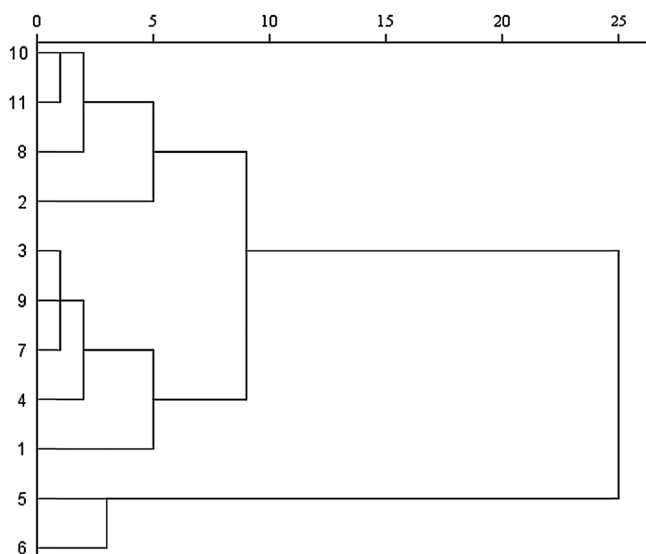


Figure 4. Results of hierarchical cluster analysis of 11 samples

analysis, the hierarchical cluster analysis in this study also demonstrates the high similarity in the different areas. Hence, the researching results of hierarchical cluster analysis and similarity analysis are consistent. Meanwhile, hierarchical cluster analysis proved that the 11 samples could be separated into 2 distinct groups based on their compositional fingerprints. This finding further suggests that HCA represents an effective method for the categorization of the *Moringa* seed shells.

#### 4 Conclusions

In conclusion, the chemical fingerprints of 11 batches of *Moringa* seed shells samples from various sources were established using HPLC–DAD. Eleven samples were performed by this method, and the chromatographic data were processed by similarity analysis and hierarchical cluster analysis. The result of the similarity analysis shows that *Moringa* seed shells samples have relatively constant quality, and this HPLC–DAD fingerprinting method could be applied in the quality control as a powerful tool. The result of hierarchical cluster analysis indicates that the components in *Moringa* seed shells from the 11 districts have very similar contents. The HPLC–DAD fingerprinting method of *Moringa* seed shells established in this paper is precise, efficient, practical, and reliable. As a result, the HPLC fingerprint technology could be applied further to evaluate and control effectively the quality of *Moringa* seed shells. Similarly, the strategy of qualitative and quantitative reliability analysis is the most promising for the assessment of the investigated plant material.

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