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Chemical characteristics for different parts of *Panax notoginseng* using pressurized liquid extraction and HPLC-ELSD

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Abstract

The chemical characteristics for different parts of *Panax notoginseng*, including root, fibre root, rhizome, stem, leaf, flower and seed, were determined using high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) and pressurized liquid extraction (PLE). Eight major saponins, namely notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rc, Rb2, Rb3 and Rd were also quantitatively compared among the different parts of *P. notoginseng*. The chromatograms showed that there was significant difference between underground (root, fibre root, rhizome) and aerial (leaf and flower) parts from *P. notoginseng*, though the similarities of entire chromatographic patterns among tested samples from underground (0.965 ± 0.029, $n = 12$) and aerial parts (0.987 ± 0.014, $n = 5$) were similar, respectively. Especially, no saponin was detected in the seed of *P. notoginseng*. Hierarchical clustering analysis based on eight investigated saponins or the ratios of contents for ginsenoside Rg1/Rb1 and ginsenoside Rb3/Rb1 showed that the samples from different parts of *P. notoginseng* were divided into three main clusters. One cluster was underground parts, which contained rich protopanaxatriol and protopanaxadiol types saponins. The leaf and flower were in the same cluster, which contained protopanaxadiol type saponins only. Especially, ginsenoside Rc, Rb2 and Rb3, rare in the underground parts, were rich in aerial parts of *P. notoginseng*. The stem of *P. notoginseng* was another cluster. Based on the cluster analysis, the chemical characteristics for different parts of *P. notoginseng* were revealed. They are composite cluster (underground parts), protopanaxadiol cluster (aerial parts) and interim (stem) cluster, which was the one between the two typical clusters, respectively. The result shows that chemical characteristics of underground parts and aerial parts from *P. notoginseng* are obviously different, which is helpful for pharmacological evaluation and quality control of *P. notoginseng*.

Keywords: *Panax notoginseng*; Ginsenoside; Notoginsenoside; High performance liquid chromatography-evaporative light scattering detection; Pressurized liquid extraction

1. Introduction

Root of *Panax notoginseng* (Burk.) F.H. Chen, known as Sanqi in Chinese, is a well-known and commonly used Chinese medicine. It has been cultivated for about 400 years and more than 85% of *P. notoginseng* was from Wenshan, Yunnan Province, China [1]. Modern pharmacological studies have demonstrated that *P. notoginseng* possesses anticarcinogenic [2,3] and hepatoprotective [4] properties, as well as protective effects on cardiovascular and cerebrovascular systems [5–7]. Saponins with aglycon of 20(S)-protopanaxatriol or 20(S)-protopanaxadiol are considered as the major active components in *P. notoginseng* [8–11]. Actually, the biological activities of saponins were related with their structures [12,13]. It was reported that the effect of ginsenoside Rg1 with protopanaxatriol moiety was contrary to that of ginsenoside Rb1 with protopanaxadiol as aglycon [12]. Indeed, the saponins from leaf of *P. notoginseng* have been developed for treatment of insomnia [14]. Therefore, chemical characteristics are very important for ensuring efficacy and quality of *P. notoginseng*.

In this paper, chemical characteristics of different parts, including root, fibre root, rhizome, stem, leaf, flower and seed from *P. notoginseng*, were determined using pressurized liquid extraction (PLE) and high performance liquid chromatography coupled with evaporative light scattering detection (HPLC-ELSD). Eight major saponins, namely notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rc, Rb2, Rb3 and Rd were also quantitatively compared. The data were analyzed and evaluated

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Table 1
Summary for the tested samples of *Panax notoginseng*

No.	Code	Sources	Sampling parts
1	RT-1	Wenshan, Yunnan, China	Root
2	RT-2	Wenshan, Yunnan, China	Root
3	RT-3	Wenshan, Yunnan, China	Root
4	RT-4	Xichou, Yunnan, China	Root
5	RT-5	Mengzi, Yunnan, China	Root
6	RT-6	Maguang, Yunnan, China	Root
7	RT-7	Yanshan, Yunnan, China	Root
8	RT-8	Guangnan, Yunnan, China	Root
9	RT-9	Qiubei, Yunnan, China	Root
10	RT-10	Guanxi Province, China	Root
11	RE	Wenshan, Yunnan, China	Rhizome
12	FT	Wenshan, Yunnan, China	Fibre root
13	SM	Wenshan, Yunnan, China	Stem
14	LF	Wenshan, Yunnan, China	Leaf
15	FR-1	Purchased from Tongrentang drugstore, Guangzhou, China (habitat: Yunnan)	Flower
16	FR-2	Wenshan, Yunnan, China	Flower
17	FR-3	Purchased from Jianming drugstore, Guangzhou, China (habitat: Yunnan)	Flower
18	FR-4	Purchased from Jianming drugstore, Zhuhai, China (habitat: Yunnan)	Flower
19	SD	Wenshan, Yunnan, China	Seed

using hierarchical clustering analysis and "similarity evaluation system for chromatographic fingerprint of TCM".

2. Materials and methods

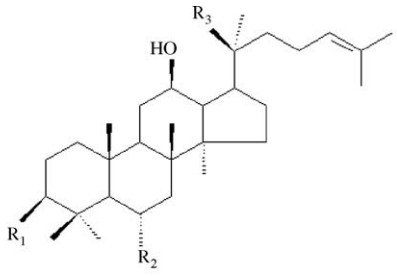
2.1. Materials and chemicals

Ten batches root of *P. notoginseng* were from Yunnan and Guangxi Province of China. The samples of fibre root, rhizome, stem, leaf and seed of *P. notoginseng* were obtained from Wenshan, Yunnan Province, China. Flowers were obtained from Wenshan, Yunnan (one batch) or purchased from the local drugstores (three batches) (Table 1). The botanical origin of materials was identified by Dr. Cui Xiuming, Wenshan Prefecture Sanqi Research Institute, Yunnan Province. The voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

Notoginsenoside R1 was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China); ginsenosides Rg1, Re, Rf, Rb1, Rc, Rb2, Rb3 and Rd were purchased from Chromadex Company (Santa Anna, CA, USA); ginsenoside Rg2 and Rg3 (Fig. 1) were obtained from International Laboratory (CA, USA). HPLC-grade methanol and acetonitrile were products of Merck (Darmstadt, Germany). Deionized water was purified by Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Pressurized liquid extraction

PLE were performed on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA) under optimized conditions. In brief, dried powder of *P. notoginseng* (0.5 g) was placed into an 11 ml stainless steel extraction cell. The optimized conditions [15] were: particle size, 0.3–0.45 mm; solvent, methanol; temperature, 150 °C; static time, 15 min; pressure, 6.895×10^3 kPa; static cycle, 1; number of extraction, 1. PLE extract was transferred into a 25 ml volumetric flask which was brought up to its volume with the same solvent and filtered through a 0.45 μm Econofilter (Agilent Technologies) prior to injection into the HPLC system.



No.	Compounds	R_1	R_2	R_3
1	NG-R1	-OH	-Oglc(2-1)xyl	-Oglc
2	G-Rg1	-OH	-Oglc	-Oglc
3	G-Re	-OH	-Oglc(2-1)rha	-Oglc
4	G-Rf	-OH	-Oglc(2-1)glc	-OH
5	G-Rg2	-OH	-Oglc(2-1)rha	-OH
6	G-Rb1	-Oglc(2-1)glc	-H	-Oglc(6-1)glc
7	G-Rc	-Oglc(2-1)glc	-H	-Oglc(6-1)araf
8	G-Rb2	-Oglc(2-1)glc	-H	-Oglc(6-1)arap
9	G-Rb3	-Oglc(2-1)glc	-H	-Oglc(6-1)xyl
10	G-Rd	-Oglc(2-1)glc	-H	-Oglc
11	G-Rg3	-Oglc(2-1)glc	-H	-OH

Fig. 1. Chemical structures of identified compounds in the different parts of *P. notoginseng*. NG, notoginsenoside; G, ginsenoside; Glc, β-D-glucose; Rha, α-L-rhamnose; Arap, α-L-arabinose (pyranose); Araf, α-L-arabinose (furanose); Xyl, β-D-xylose.

2.3. HPLC-ELSD analysis

Agilent 1100 series HPLC apparatus (Palo Alto, CA, USA), equipped with vacuum degasser, quaternary gradient pump, autosampler was used. The signal from Alltech ELSD 2000 (Alltech, Deerfield, IL, USA) was transmitted to the Chemstation for processing through an Agilent 35900E (Agilent Technologies, USA). A Zorbax ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and a Zorbax ODS C_{18} guard column (12.5 mm × 4.6 mm i.d., 5 μm) were used at 40 °C. A binary gradient elution system consisted of water (A) and acetonitrile (B) and separation was achieved using the following gradient program: 0–30 min, 18–19% B; 30–40 min, 19–31% B; 40–60 min, 31–56% B. The flow-rate was at 1.5 ml/min and sample injection volume was 10 μl. The Alltech ELSD impactor was set at ON mode, and the drift tube temperature was 60 °C and nebulizer nitrogen gas flow-rate was at 1.4 l/min.

2.4. Data analysis

Hierarchical clustering analysis was performed by SPSS 11.5 for windows (SPSS Inc., Chicago, IL, USA), which comprise a number of "procedures" – graphical, statistical, reporting, processing and tabulating procedures – that enable simple and rapid data evaluation. A method named as the between groups linkage was applied, and Pearson correlation, which is a pattern similarity measure, was selected as measurement for hierarchical clustering analysis.

The correlation coefficients and the similarities of entire chromatographic patterns among tested samples, and the simulative mean chromatogram were calculated and generated using a professional software named "Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine" (Version 2004 A), which was recommended by State Pharmacopoeia Committee of the People's Republic of China.

3. Results and discussions

3.1. HPLC separation of saponins in P. notoginseng

Under the HPLC-ELSD conditions mentioned above, the investigated saponins were well separated within 60 min. The typical chromatograms of PLE extracts from different parts of *P. notoginseng* are shown in Fig. 2. The peaks of analytes were identified by two means: (i) by comparing the retention times of the peaks with those of the reference compounds eluted under the same conditions and (ii) by spiking the sample with stock standard solutions of saponins. Ginsenoside Rg3 was not detected in all samples of different parts from *P. notoginseng*.

3.2. Validation of HPLC-ELSD method

To develop chemical characteristics, it is necessary to validate the analytical procedure. Aqueous methanol stock solution containing eight main saponins (**1**–**3**, **5**, **7**–**10**) in *P. notoginseng* was prepared and diluted to appropriate concentration for establishment of calibration curves. The result showed that the correlation coefficient values ($r > 0.991$) indicated good correlations between investigated compounds concentrations and their peak areas within the test ranges. Intra- and inter-day repeatability was determined for five times within 1 day and 5 separate days, respectively. The data are shown in Table 2.

Recovery was used to evaluate the accuracy of the method. Known amounts of eight saponins were added into the root sample (RT-1) and extracted at specified conditions mentioned above ($n = 2$). The mean recovery of notoginsenoside R1, ginsenoside Rg1, Re, Rb1, Rc, Rb2, Rb3 and Rd were 98.5, 97.7, 99.4, 101.1, 104.2, 97.7, 100.0 and 101.1%, respectively (Table 3).

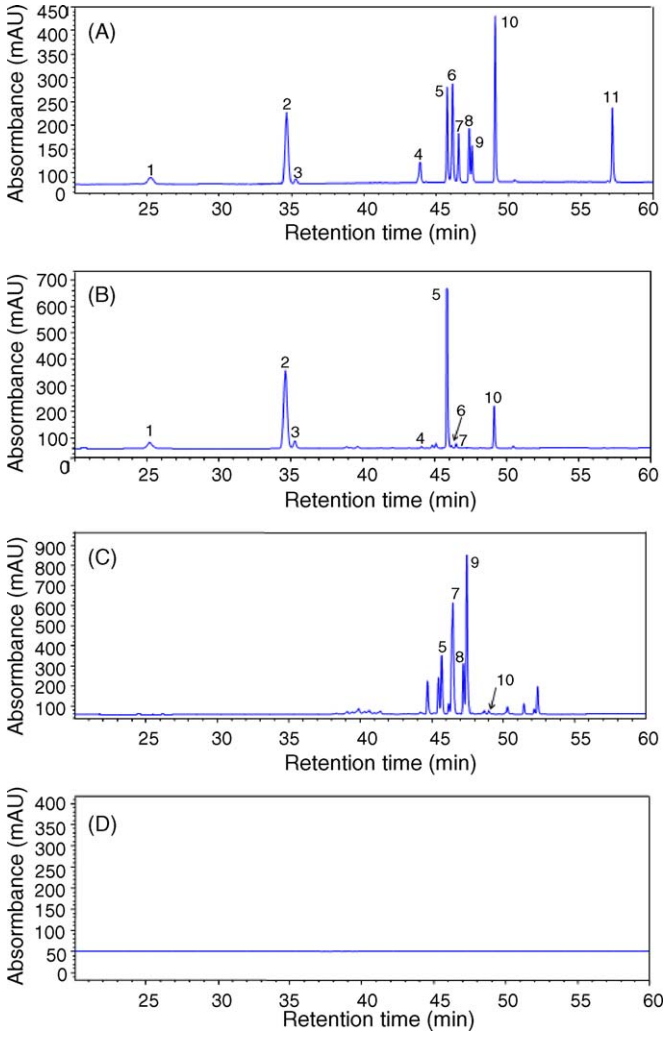


Fig. 2. HPLC-ELSD chromatograms of (A) mixed standards and typical PLE extracts of (B) underground parts (root), (C) aerial parts (flower) and (D) seed from *P. notoginseng*. (**1**) Notoginsenoside R1; (**2**–**11**) ginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rb2, Rb3, Rd and Rg3, respectively. HPLC was performed using a Zorbax ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and a Zorbax ODS C_{18} guard column (12.5 mm × 4.6 mm i.d., 5 μm) at 40 °C. The mobile phase consisted of water (A) and acetonitrile (B) using the following gradient program: 0–30 min, 18–19% B; 30–40 min, 19–31% B; 40–60 min, 31–56% B. The flow-rate was at 1.5 ml/min and the sample injection volume was 10 μl. Alltech ELSD impactor was set at ON mode, and the drift tube temperature was at 60 °C and nebulizer nitrogen gas flow-rate was at 1.4 l/min.

Table 2
Linear regression data and precision for eight saponins

Analytes	Linear regression data				Precision, R.S.D. (%)	
	Linear range (mg/ml)	Slope	Intercept	r (n = 6)	Intra-day (n = 5)	Inter-day (n = 5)
NG-R1	0.050–0.250	3593.4	−170.2	0.994	1.4	2.0
G-Rg1	0.142–1.420	6288.7	−910.9	0.994	1.3	2.3
G-Re	0.036–0.180	4037.8	−130.9	0.991	1.0	1.7
G-Rb1	0.118–1.180	8122.9	−719.2	0.997	1.8	1.7
G-Rc	0.115–1.035	7754.5	−572.4	0.998	2.2	1.7
G-Rb2	0.143–0.430	8150.9	−633.8	0.997	1.6	1.9
G-Rb3	0.163–1.470	6472.0	−758.7	0.997	1.3	2.0
G-Rd	0.016–0.195	6788.9	−177.1	0.998	1.6	2.3

NG: notoginsenoside; G: ginsenoside.

3.3. Chemical characteristics of different parts from *P. notoginseng*

The chemical characteristics of 12 samples for underground parts (root, rhizome and fibre root) of *P. notoginseng* were very similar, which contained both 20(S)-protopanaxatriol and 20(S)-protopanaxadiol type saponins. Fig. 2B shows the typical HPLC profile of 12 tested underground parts samples. The correlation coefficient of each chromatogram to their simulative mean chromatogram was 0.965 ± 0.029 (mean ± S.D., $n = 12$). Similarly, the chemical characteristics of aerial parts (except stem and seed), leaf and flower, from *P. notoginseng* were also similar (Fig. 2C) and the correlation coefficient of each chromatogram to their simulative mean chromatogram was 0.987 ± 0.014 (mean ± S.D., $n = 5$). However, chemical characteristics of underground and aerial parts from *P. notoginseng* were significantly different. Especially, ginsenoside Rc, Rb2 and Rb3, rare in the underground parts, were rich in aerial parts of *P. notoginseng*. It was also interesting that no saponins were detected in seed of *P. notoginseng* (Fig. 2D). Furthermore, the relative retention time (RT_R) and relative peak area (RPA) of investigated peaks to reference peak (ginsenoside Rb1), which was a high peak in all tested samples, were calculated. The data indicated that the ratios of Rg1/Rb1 and Rb3/Rb1 were obviously different among underground parts, stem and aerial parts of *P. notoginseng* (Table 4).

Using the ratios of Rg1/Rb1 and Rb3/Rb1 characteristics, hierarchical cluster analysis of the tested 18 samples (except seed) was performed as mentioned above. The result was very similar to the one derived from eight investigated

Table 3
Recoveries for the assay of eight saponins in *P. notoginseng*

Analytes	Original (mg)	Spiked (mg)	Found (mg)	R.S.D.[a] (%)	Recovery[b] (%)
NG-R1	0.358	0.376	0.713	5.0	94.4
		0.188	0.551	1.2	102.7
G-Rg1	2.092	0.964	2.989	1.4	93.1
		0.482	2.586	2.9	102.5
G-Re	0.284	0.252	0.541	4.5	102.1
		0.084	0.365	3.0	96.7
G-Rb1	2.930	0.925	3.902	1.4	105.1
		0.370	3.290	0.9	97.2
G-Rc	0.075	0.150	0.221	2.7	97.6
		0.050	0.130	1.7	110.8
G-Rb2	Tr[c]	0.064	0.059	1.4	92.2
		0.032	0.033	0.4	103.1
G-Rb3	Tr	0.046	0.050	2.3	108.7
		0.023	0.021	0.1	91.3
G-Rd	0.316	0.234	0.562	2.3	105.0
		0.078	0.392	4.6	97.2

NG: notoginsenoside; G: ginsenoside.
[a] Recovery (%) = 100 × (amount found − original amount)/amount spiked.
[b] R.S.D. (%) = 100 × S.D./mean.
[c] Tr, below the limit of quantification.

Table 4
The content (CNT, mg/g) of investigated saponins and their relative peak area (RPA, ginsenoside Rb1 as reference compound) in different parts of *Panax notoginseng*

Samples		NG-R1	G-Rg1	G-Re	G-Rb1	G-Rc	G-Rb2	G-Rb3	G-Rd	PTS[a]	PDS[b]	PTS/PDS
RT-1[c]	CNT	6.8[d]	39.1	5.1	30.4	1.5	–[e]	–	7.0	51.0	38.8	1.3
	RPA	7.2	93.9	6.7	100	1.4	0	0	18.1			
RT-2	CNT	6.3	33.1	4.2	29.1	1.2	–	–	6.7	43.6	37.0	1.2
	RPA	7.1	82.1	5.3	100	1.1	0	0	18.6			
RT-3	CNT	5.3	31.7	4.9	27.0	1.2	–	–	5.7	41.9	34.1	1.2
	RPA	5.5	84.0	6.9	100	1.0	0	0	15.8			
RT-4	CNT	6.0	32.9	4.0	29.7	1.2	–	–	5.9	42.9	36.8	1.2
	RPA	6.5	77.7	4.8	100	1.1	0	0	15.2			
RT-5	CNT	7.2	31.1	4.2	30.5	1.2	–	–	7.5	42.5	39.2	1.1
	RPA	8.2	71.1	5.1	100	0.9	0	0	19.5			
RT-6	CNT	6.3	30.1	3.8	27.7	1.1	–	–	6.6	40.2	35.5	1.1
	RPA	7.6	78.0	4.7	100	1.0	0	0	19.2			
RT-7	CNT	5.5	31.2	4.7	28.1	1.1	–	–	5.9	41.3	35.1	1.2
	RPA	6.0	76.6	6.7	100	0.9	0	0	16.2			
RT-8	CNT	6.5	33.5	4.9	32.4	1.3	–	–	8.4	44.9	42.2	1.1
	RPA	6.99	73.31	6.28	100	1.05	0	0	22.25			
RT-9	CNT	6.6	29.6	4.1	26.7	1.1	–	–	5.9	40.2	33.7	1.2
	RPA	8.4	77.1	5.5	100	1.0	0	0	17.1			
RT-10	CNT	5.9	29.6	4.8	30.6	1.1	–	–	7.3	40.4	38.9	1.0
	RPA	6.0	64.6	6.1	100	0.8	0	0	18.5			
FT	CNT	4.7	21.8	3.4	20.8	1.8	–	–	3.8	29.9	26.3	1.1
	RPA	6.3	64.1	4.8	100	2.5	0	0	12.2			
RE	CNT	12.6	52.8	6.4	48.8	4.4	+[f]	+	12.7	71.7	65.8	1.1
	RPA	9.7	78.7	5.4	100	1.4	0.2	0.7	20.9			
SM	CNT	–	–	–	10.79	+	+	+	+	–	10.8	0
	RPA	0	0	0	100	4.55	3.31	6.13	5.72			
LF	CNT	–	–	–	7.58	32.89	9.47	56.79	2.50	–	109.2	0
	RPA	0	0	0	100	894.2	183.1	1311.8	35.2			
FR-1	CNT	–	–	–	22.0	54.4	17.7	79.1	2.6	–	178.8	0
	RPA	0	0	0	100	275.3	79.2	329.9	5.9			
FR-2	CNT	–	–	–	22.2	56.0	16.5	79.2	2.7	–	176.7	0
	RPA	0	0	0	100	281.2	72.0	332.0	6.9			
FR-3	CNT	–	–	–	17.7	44.1	14.3	57.8	2.7	–	136.5	0
	RPA	0	0	0	100	285.5	77.1	308.5	8.2			
FR-4	CNT	–	–	–	19.8	52.9	17.0	72.7	2.6	–	165.0	0
	RPA	0	0	0	100	309.5	86.1	347.7	<0.1			
SD	CNT	–	–	–	–	–	–	–	–	–	–	–
	RPA	0	0	0	0	0	0	0	0			

NG: notoginsenoside; G: ginsenoside.
[a] Total amount of 20(S)-protopanaxatriol saponins including ginsenoside Rg1, Re and notoginsenoside R1.
[b] Total amount of 20(S)-protopanaxadiol saponins including ginsenoside Rc, Rb1, Rb2, Rb3 and Rd.
[c] The same as that described in Table 1.
[d] The data present as average of duplicates.
[e] Undetected.
[f] Under the limit of quantification.

peaks characteristics (Fig. 3), which suggested there were two clusters, composite cluster (underground parts) contained both 20(S)-protopanaxatriol and 20(S)-protopanaxadiol type saponins and protopanaxadiol cluster (aerial parts) contained 20(S)-protopanaxadiol type saponins only, among different parts of *P. notoginseng*. Chemical characteristic for stem of *P. notoginseng* belonged to interim cluster, which was the one between the two clusters. Therefore, the characteristics of peaks, especially ginsenoside R1, Rb1 and Rb3, from HPLC profiles could be used as markers for quality control of *P. notoginseng*. The

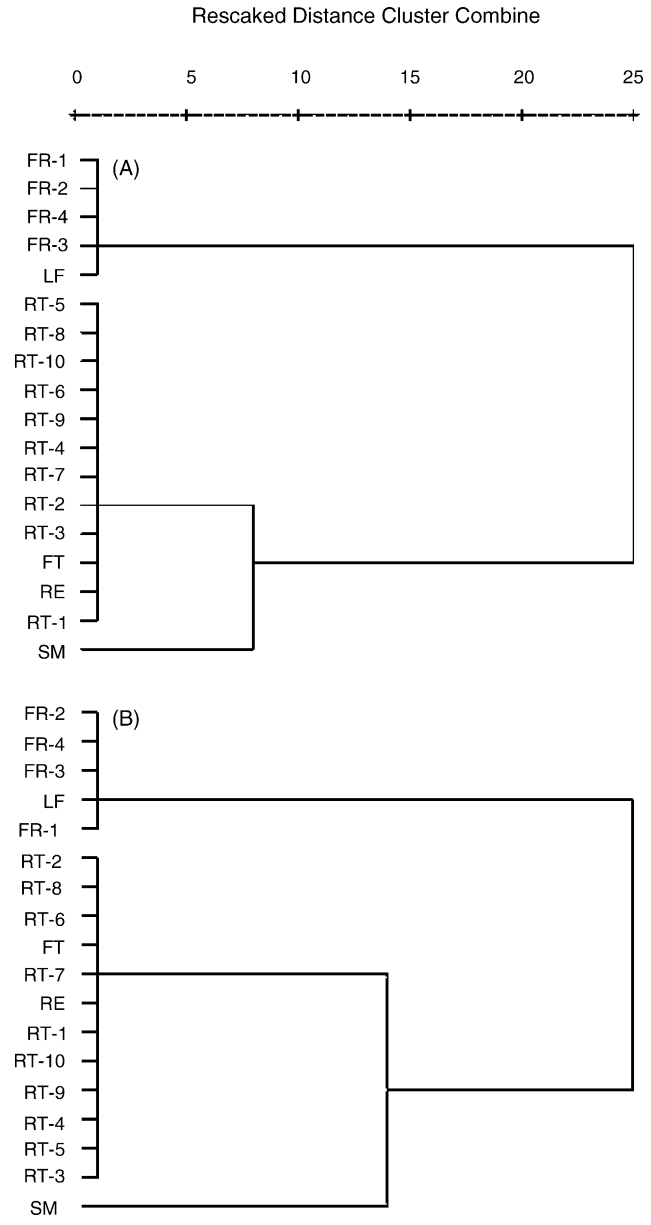


Fig. 3. Dendrograms resulting from single linkage between groups hierarchical cluster analysis. The hierarchical clustering was done by SPSS 11.5 for windows. A method named as the between groups linkage was applied, and Pearson correlation was selected as measurement. (A) Dendrogram resulting from the content of eight investigated saponins in 18 tested samples (except the seed) of *P. notoginseng*. (B) Dendrogram resulting from the ratios of ginsenoside Rg1/Rb1 and Rb3/Rb1 in the tested samples.

contents of eight investigated saponins in different parts of *P. notoginseng* are shown in Table 4.

4. Conclusion

The chemical characteristics, composite cluster and protopanaxadiol cluster, of different parts from *P. notoginseng* were revealed using HPLC-ELSD. The result is helpful for pharmacological evaluation and quality control of *P. notoginseng*.

Acknowledgements

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