OPEM
www.opem.org

Oriental Pharmacy and Experimental Medicine 2006 **6(4)**, 293-299

KHUP

Regions in China identification and quality control of radix Codonopsis by chemical fingerprint: Evaluation of lobetyolin from different cultivated

Gui X Chou, Qiu T Gao, Jun LI, Ran Duan, Anna WH Cheung, Glanice KY Chu, Zhi Y Jiang, Tina TX Dong and Karl WK Tsim

Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong SAR, China

SUMMARY

By using high-performance liquid chromatography-photodiode array detection, a simple and accurate chromatographic fingerprint method was developed for the identification of Radix Codonopsis (roots of *Codonopsis*) from different sources. Eighteen herbs of *Codonopsis* at different habitats in China, including roots from *Codonopsis pilosula*, *Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen* were analyzed by the fingerprint. The amount of lobetyolin was calibrated, which was found to be more consistent in roots of *Codonopsis pilosula* as compared to that of *Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen*. Having the fingerprint results, hierarchical clustering analyses were performed to classify the eighteen herbs into three groups: *Codonopsis pilosula*, *Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen*. This clustering analysis agrees very well with the pharmacognostic identification result, and which could be used as a tool in the quality control of Radix Codonopsis.

Key words: Radix Codonopsis; *Codonopsis pilosula*; *Codonopsis pilosula* var *modesta*; *Codonopsis tangshen*; Fingerprint; Chinese medicine; Quality assurance

INTRODUCTION

Radix Codonopsis (roots of *Codonopsis*; Dangshen) is one of the commonly used traditional Chinese medicines (TCMs) that has been used over thousands of years for replenishing the energy and invigorates the spleen (Zheng, 2005). Recently, Radix Codonopsis was found to have effects on anti-aging (Jin *et al.*, 1996), enhance memory acquisition and retention (Singh *et al.*, 2004), helping remembering dysfunction (Pan, 1992) and inhibiting erythrocyte hemolysis (Ng *et al.*, 2004). Three species including *Codonopsis pilosula* (Franch.) Nannf., *Codonopsis pilosula* Nannf. var. *modesta* (Nannf.) L.T. Shen and *Codonopsis tangshen* Oliv. are being recorded in Chinese Pharmacopoeia (Zheng, 2005) as the source plant. Traditionally, Shanxi, Henan, Gansu, Heilongjiang and Sichuan are considered as the geo-authentic habitats of *Codonopsis pilosula*, *Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen*. In Hong Kong herbal market, the root of *Codonopsis pilosula* var. *modesta* is found to be the main source of Radix Codonopsis. The active constituents of Radix Codonopsis have been previously investigated and shown to be polyacetylens (e.g. lobetyolin), alkaloids, sterides, triterpenoids and polysaccharides, which have been shown to have the effects on gastric ulcer (Wang *et al.*, 1997), exciting the gastric smooth muscle

*Correspondence: Karl WK Tsim, Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong SAR, China. Tel: +852-2358-7332; Fax: +852-2358-1559; E-mail: botsim@ust.hk

(Zheng *et al.*, 1998) and potentiating neurite outgrowth (Liu *et al.*, 2003). However, the amount of these active constituents could vary according to *Codonopsis* species. Besides, the amounts of active constituents within *Codonopsis* roots could vary significantly according to their geographical sources. These problems in quality control, therefore, compromise the values of TCMs or even jeopardize the safety of the consumers.

As to ensure the clinical efficacy, an evaluation of the constituents of Radix Codonopsis is the first priority. Chromatographic fingerprint is a comprehensive identification method to show chemical information of herbal medicines by using chromatogram, spectrograms and other graphs derived from analytic evaluations; the fingerprint could show not only naturally occurring active constituents but also the chemically characteristic ratios of them (Gu *et al.*, 2003; Lu *et al.*, 2005; Xia *et al.*, 2005; Yan *et al.*, 2005; Zou *et al.*, 2005). Both Food and Drug Administration (FDA) of the USA (US Food and Drug Administration, 2000) and European Medicines Agency (European Medicines Agency, 2001) clearly denoted that the appropriate fingerprint chromatogram should be applied to assess the consistency of the botanical drugs. State Food and Drug Administration (SFDA) of China has also required that all the injections made from herbal medicines or their raw materials should be standardized by chromatographic fingerprint (State Food and Drug Administration of China, 2000). Here, we developed HPLC methods in optimizing the chemical fingerprint of Radix Codonopsis from different sources; the developed methods were being used for the determination of lobetyolin, as well as for the hierarchical clustering analysis. The results suggested that roots of *Codonopsis pilosula*, *Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen* are three chemically distinct groups of Radix Codonopsis.

MATERIALS AND METHODS

Plant materials and sample preparation

Amongst the 18 raw herbs of Radix Codonopsis, 14 were collected from different habitats in China, and 4 were purchased from Hong Kong local market. The herbs were identified by one of the authors Dr. Tina T.X. Dong according to morphological characteristics. The voucher specimens were deposited in Hong Kong University of Science and Technology. The dried samples were kept in silica gel. For chemical analysis, the roots of different *Codonopsis* species were collected from various regions of China; their notations were listed in Table 1. The chosen area for the herbal collection was based on the popularity of Radix Codonopsis that was cultivated. The plant materials were collected in Fall of 2003; they were ~2 - 3 years of growth. About 10 batches of individual species having similar but not identical geographical properties at the same region were tested. Individual samples were prepared from ~500 g of powder (0.10 - 0.15 mm) that was grounded from ~20 plants of the same population. The powder was passed through a No. 3 sieve and stored with silica gel, which stabilized the chemical constituents. Before HPLC analysis, the sieved sample 1.5 g was put into a 250 ml conical flask and 30 ml of 0.1 N HCl-methanol (1 : 1, v/v) was added. The mixture was sonicated for 60 min. The extract was filtered and then centrifuged at 3,000 × *g* for 10 min. The result solution was filtered with 0.45 mm PTFE filter membrane, and the filtrate was stocked at 4°C.

HPLC conditions and determination of lobetyolin

The reference standard lobetyolin (Fig. 1) having purity of over 99% was purchased from Shanghai R & D Center for Standardization of TCM, Shanghai, China. Methanol and acetonitrile (Merck, Germany) were of chromatographic grade. HPLC grade reagents were from Fischer and Labscan (Dublin, Ireland). The HPLC system having a LC-10ATvp pump and a SPD-M10Avp photo diode array detector were from Shimadzu (Tokyo, Japan). Chromatographic separation was achieved on C-18 columns of different packing materials (250 × 4.6 mm, 5 μm Symmetry; 150 × 3.9 mm,

5 μm Delta PAK and 250 × 4.6 mm, 5 μm Alltima) at room temperature. Symmetry provided a much better resolution than others, and thus it was used for further analysis. The on-line UV spectra were recorded with photodiode detector from 200 to 400 nm. The flow rate was set at 1.0 ml/min. The mobile phase was composed of 0.2% acetate acid methanol (A) and acetonitrile (B) with a gradient program of: 10 - 20% (B) in 0 - 20 min and 20 - 100% (A) in 20 - 60 min. Twenty ìl aliquot was injected into HPLC system for analysis. Data acquisition was performed with Shimadzu Class-vp 6.1 software. In the calibration of lobetyolin, the standards were weighed and dissolved in 1 ml of methanol to give serial concentrations, and three injections were performed for each dilution. The standard curve was calibrated by using the linear least-squares regression equation derived from the peak area; the concentration was calculated according to the regression parameters derived from the standard curves.

Data analysis

The hierarchical clustering analyses of samples from different habitats were performed using SPSS software (SPSS for Windows 13.0). A method called average linkage between groups was applied and Pearson correlation was selected as a similarity measure (Zhao *et al.*, 2003).

RESULTS AND DISCUSSION

Optimization of HPLC system

Amongst different types of polyacetylens, lobetyolin is the major form in Radix Codonopsis. Because of this abundance, lobetyolin was chosen for further analysis in this report. Preliminary study showed that the use of methanol as an extracting solvent could provide better yield of lobetyolin from Radix Codonopsis (data not shown). In addition, different conditions of extraction were calibrated in the yield of lobetyolin. By determining the contents of lobetyolin, the optimized extraction condition was revealed for Radix Codonopsis. Four extraction methods were tested: reflux, Soxhlet, sonication and cold extraction. Fig. 1 shows sonciation could be a better choice of extraction, at least in view of the yield of lobetyolin, and therefore which was used in the subsequent experiments.

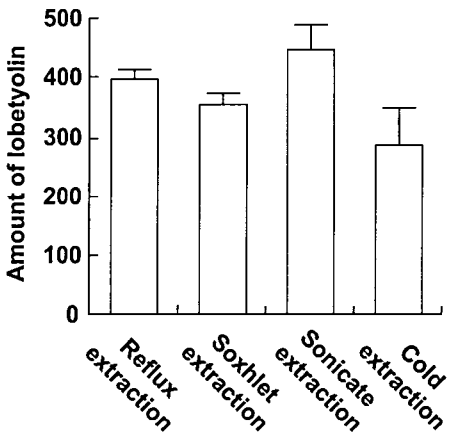

Fig. 1. The yields of lobetyolin under different extraction conditions. Power of Radix Codonopsis at 1.5 g was put into a 250 ml conical flask and 30 ml of methanol was added for extraction. By using a C18 column in HPLC analyses, the amounts of lobetyolin in mg/kg of fried root were determined in extracts from reflux, Soxhlet, sonication and cold extraction. The result solution was filtered with 0.45 mm filter and then subjected to HPLC analysis for lobetyolin.

In order to achieve the best resolution in HPLC fingerprints as well as the calibration of lobetyolin, the extracting condition of Radix Codonopsis was determined. Methanol extract of Radix Codonopsis showed a poor resolution of peaks from 20 to 30 min of retention time (Fig. 2A); this problem could not be resolved even the extract was further purified by HLB cartridge (Fig. 2B). In contrast, the addition of acid environment greatly enhanced the resolution of fingerprints (Fig. 2C). Thus, 0.1 N HCl-methanol (1 : 1) extract was used for HPLC, which could also give good reproducibility of peaks and better chromatographic patterns for identifying Radix Codonopsis.

An ideal fingerprint chromatogram should contain sufficient information of constituents and

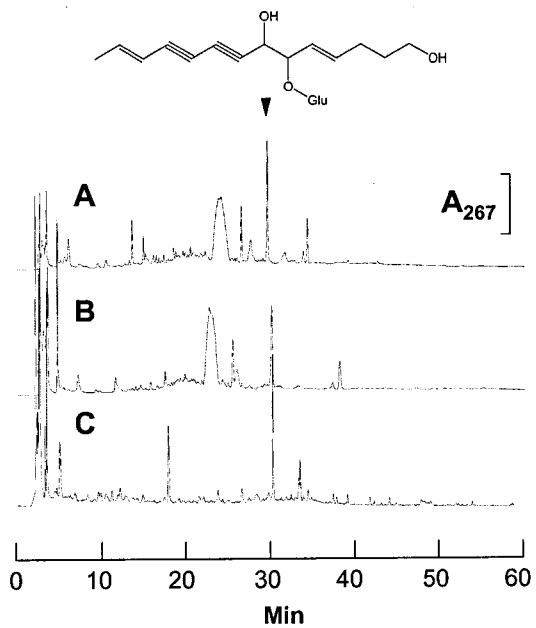

Fig. 2. HPLC chromatograms of extracts from Radix Codonopsis. Under different conditions of extraction. The HPLC system having a LC-10ATvp pump and SPD-M10Avp photodiode array detector were from Shimadzu. Chromatographic separation was achieved on C-18 column (250 × 4.6 mm, 5 μm Symmetry) at room temperature. The flow rate was set at 1.0 ml/min. The mobile phase was described in Materials and Methods. Twenty μl aliquot was injected into HPLC system for analysis. Typical chromatograms at absorbance of 267 nm under different conditions are shown: (A) methanol extract; (B) methanol extract was further purified by HLB cartridge; (C) 0.1 N HCl-methanol (1 : 1) extract. The peak of lobetyolin is indicated by an arrowhead. The scale is 0.01.

represent characteristic of the plants e.g. separation of characteristic peaks, appropriate retention time and height of peaks and steady baseline with larger signal-to-noise ratio. In order to achieve an optimized chromatogram, a full-scan on the chromatogram from 200 nm to 400 nm was performed. Based on the aforementioned criteria of an ideal chromatogram, the wavelength at 267 nm was chosen as to achieve more peaks as well as a smooth baseline. Besides, this wavelength provided the best resolution of lobetyolin (retention time at 29.9) (Fig. 2C)., which was identified by two means: (i) by comparing the retention times of the unknown peaks with those of the standards eluted under the same conditions; and (ii) by spiking the sample with stock standard solutions (data not shown). In the choice of mobile phase, the chromatograms with the mobile phase adding 0.1% acetic acid showed better pattern than the chromatograms using the mobile phases only with different ratio of water: acetonitrile.

Lobetyolin content and chemical fingerprint

Having lobetyolin as a reference standard (retention time at 29.9 min) in the extracts of Radix Codonopsis, the HPLC calibration curve of lobetyolin exhibited good linearity in a range from 0.01 mg/ml to 0.2 mg/ml. The coefficients of correlation were 0.9992. Precision of relative retention times and relative peak areas were found in the range of 0.1 - 1.3% and 0.6 - 2.9% of RSD ($n = 5$), respectively. The repeatability of the constituent was excellent of having the RSD of 1.0%. The recovery experiment was carried out to evaluate the accuracy of the method. Known amounts of lobetyolin was added to the sample and extracted accordingly; the extracted material was subjected for analysis, and the content of the marker was calibrated. The average recoveries of the tested lobetyolin was from 99.1% to 102.0% ($n = 5$). The stability of the sample solution was further evaluated by determining their peak areas after 48 h of storage. By comparing the chromatographic peaks, RSD of relative retention time and relative peak area were less than 2% and 5%, respectively. This indicated that the extracts in 0.1 N HCl-methanol were stable at least for 48 h.

The content of lobetyolin was determined from roots of *Codonopsis. pilosula, Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen* derived from different regions of China. Eighteen different populations of *Codonopsis* roots from various geographical regions were collected; each chosen population contained over 10 different batches of samples. Table 1 shows there are a great variation regarding the content of lobetyolin from different regions of China, even if they are from the same

region of cultivation. The variation of lobetyolin within the roots from *Codonopsis pilosula* was rather small. The highest amount of lobetyolin at 815.4 mg/kg was found in roots from Nanzhen of Shannxi, while Hanzhong of Shannxi showed the lowest amount at 391.19 mg/kg. In contrast, the amount of lobetyolin could be varied greatly in roots of *Codonopsis pilosula* var. *modesta* e.g. from 148.11 mg/kg (Yuxi of Yunnan) to 1,417.44 mg/kg (Pingwu of Sichuan); this problem of chemical variation was revealed also for *Codonopsis tangshen* roots. In view of this aspect, *Codonopsis pilosula* could be a better source of Radix Codonopsis. In addition, the herbal products from Hong Kong local marker also showed a significant variation in containing lobetyolin.

The quality of crude drugs is closely related to their chemical constituents and could be assessed by chemical pattern recognition method. Fig. 3 shows the calibrated HPLC fingerprints from the collected 18 *Codonopsis* roots. It could be observed that the peak at retention time of 45.0 min was found only in *Codonopsis pilosula* var. *modesta*. In contrast, the peak at retention time of 19.0 min was significantly higher in *Codonopsis pilosula* and *Codonopsis tangshen* than in *Codonopsis pilosula* var. *modesta* roots. Radix Codonopsis purchased from local market in Hong Kong contained the small peaks at 19.0 min and 45 min, which indicated that these samples were, indeed, from roots of *Codonopsis pilosula* var. *modesta*. (Fig. 3). Therefore, these peaks could serve as markers to identify *Codonopsis pilosula*, *Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen*.

Hierarchical clustering analysis is one of the statistical methods that could evaluate the resemblance, as well as the classification, of different herbs by measuring the peak migration and height from their corresponding HPLC fingerprints. By using the results from HPLC analyses, different samples of *Codonopsis* roots were subject to hierarchical clustering analysis. The HPLC peaks at absorbance of 267 nm that had good resolution and peak

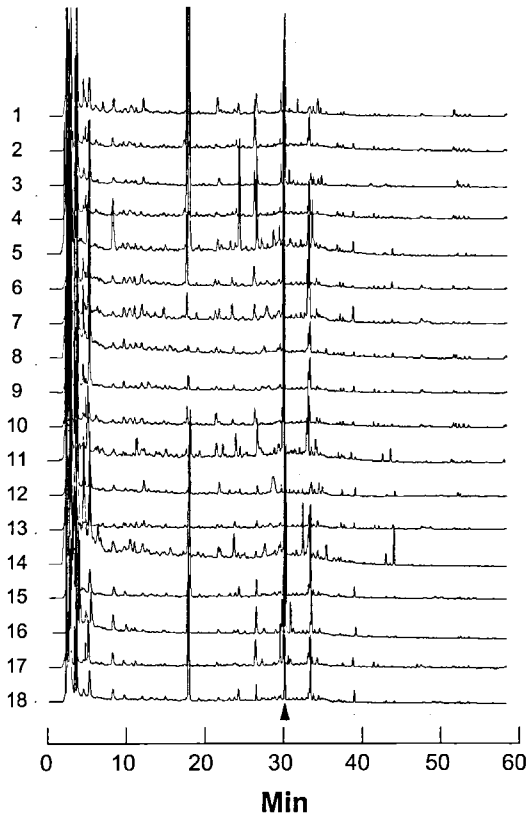

Fig. 3. Chemical fingerprintings of Radix Codonopsis. HPLC were performed as in Fig. 2C with absorbance at 267 nm. Superimpose of HPLC fingerprints from the tested 18 samples including the 3 different species of Radix Codonopsis including *Codonopsis pilosula*, *Codonopsis pilosula* var. *modest* and *Codonopsis tangshen* were done. These samples were chosen randomly from the 18 populations, one single sample from each population, as listed in Table 1. The peak of lobetyolin is indicated by an arrowhead.

shape, after the normalization, were selected, and therefore 15 constituents including lobetyolin were eluted and calibrated. These 15 constituents were quantified based on their peak areas by using the peak area of lobetyolin as a reference standard. A matrix of 18 × 15 was obtained, which gave the content differences of 15 constituents among the tested 18 populations, as listed in Table 1. In the hierarchical clustering analysis, a method called average linkage between groups was applied, and Pearson correlation was selected as measurement,

Table 1. The contents of lobetyolin from different populations of Radix Codonopsis

Sample[a]	Voucher#	Species	Habitats[b]	Lobetyolin (mg/kg)[c]
1	LDS001	*Codonopsis pilosula*	Xinzhou, Shanxi	510.89
2	LDS002	*Codonopsis pilosula*	Fanshi, Shanxi	532.90
3	LDS005	*Codonopsis pilosula*	Lushi, Henan	484.60
4	LDS006	*Codonopsis pilosula*	Hanzhong, Shannxi	391.19
5	LDS008	*Codonopsis pilosula*	Nanzheng, Shannxi	815.40
6	LDC001	*Codonopsis pilosula* var. *modesta*	HK Market	703.40
7	LDC002	*Codonopsis pilosula* var. *modesta*	HK Market	804.97
8	LDC003	*Codonopsis pilosula* var. *modesta*	HK Market	105.87
9	LDC004	*Codonopsis pilosula* var. *modesta*	HK Market	130.46
10	LDS007	*Codonopsis pilosula* var. *modesta*	Luonan,Shannxi	413.116
11	LDS010	*Codonopsis pilosula* var. *modesta*	Pingwu, Sichuan	1417.44
12	LDS011	*Codonopsis pilosula* var. *modesta*	Yuxi, Yunnan	148.11
13	LDS013	*Codonopsis pilosula* var. *modesta*	Anshun, Guizhou	355.96
14	LDS014	*Codonopsis pilosula* var. *modesta*	Bijie, Guizhou	821.71
15	LDS004	*Codonopsis tangshen*	Enshi, Hubei	291.00
16	LDS015	*Codonopsis tangshen*	Ganzi, Sichuan	1239.51
17	LDS016	*Codonopsis tangshen*	Wushan, Sichuan	601.29
18	LDS017	*Codonopsis tangshen*	Yichang, Hubei	490.46

[a]Eighteen populations of *Codonopsis* roots were collected as described in the experimental section. [b]From 10 to 15 individual samples from each population were analyzed. All roots were collected in Fall of 2003; they were ~2 - 3 years of growth. [c]Values are (mean ± S.D.) (n = 10 - 15).

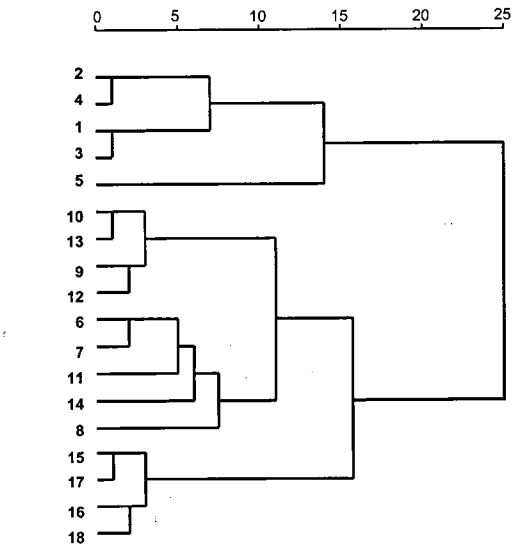

Fig. 4. The hierarchical clustering analysis of different Radix Codonopsis. The clustering was done by SPSS software from 15 HPLC peaks analyzed from the tested 18 samples of Radix Codonopsis; these samples were chosen randomly from the 18 populations, one single sample from each population, as listed in Table 1.

and the result was shown in Fig. 4. The tested 18 populations of *Codonopsis* roots were divided into two main clusters: samples 1 - 5 from *Codonopsis pilosula* as cluster one; samples 6 - 14 from *Codonopsis pilosula* var. *modesta* as cluster two and samples 15 - 18 from *Codonopsis tangshen* as cluster three. This result was in good agreement with the pharmacognostic identification.

CONCLUSION

Precise identification of crude drugs is a prerequisite for chemical and pharmacological investigations of TCM and for their clinical applications. This requirement is also important in achieving good agricultural practice (GAP) farming of herbal medicines in China. In the case of Radix Codonopsis, a simple and accurate chromatographic fingerprint method was developed and validated using high-performance liquid chromatography-photodiode

array detection. The method was used to determine the amount of lobetyolin within Radix Codonopsis from different sources. With the chemical fingerprint results, hierarchical clustering analysis was used to group different populations of *Codonopsis* roots, which subsequently could classify the tested Radix Codonopsis into 3 groups: *Codonopsis pilosula*, *Codonopsis pilosula* var. *modesta* and *Codonopsis tangshen*. The chemical fingerprint analysis therefore could be used for the identification and quality control of Radix Codonopsis.

ACKNOWLEDGEMENTS

The research was supported by grants from the Area of Excellence Scheme established under the University Grants Committee of Hong Kong (AoE/B-10/01) to KWKT. This work is part of a project on the study of some common Chinese Material Medica in Hong Kong being undertaken by Department of Health, Hong Kong SAR Government, China.

REFERENCES

European Medicines Agency. (2001) Note for Guidance on Quality of Herbal Medicinal Products, pp. 6, London.

Gu M, Ouyang F, Su ZG. (2003) Comparison of high-speed counter-current chromatography and high-performance liquid chromatography on fingerprinting of Chinese traditional medicine. *J. Chromatogr.* A **1022**, 139-144.

Jin ZL, Wen J, Li SF. (1996) Anti-aging effect of shenqi mixture tonic. *Chin. Trad. Herb Drugs* **27**, 90-92.

Liu JH, Bao YM, Song JJ, An LJ. (2003) *Codonopsis pilosula* (Franch) Nannf total alkaloids potentiate neurite outgrowth induced by nerve growth factor in PC12 cells. *Acta Pharmacol. Sin.* **24**, 913-917.

Lu GH, Chan K, Liang YZ, Leung K, Chan CL, Jiang ZH, Zhao ZZ. (2005) Development of high-performance liquid chromatographic fingerprints for distinguishing Chinese *Angelica* from related umbelliferae herbs *J. Chromatogra.* A **1073**, 383-392.

Ng TB, Liu F, Wang HX. (2004) The antioxidant effects of aqueous and organic extracts of Panax quinquefolium, Panax notoginseng, Codonopsis pilosula, Pseudostellaria heterophylla and Glehnia littoralis. *J. Ethnopharmacol.* **93**, 285-288.

Pan SY. (1992) Circadian effects of scopolamine on memory, exploratory behavior, and muscarinic receptors in mouse brain. *Acta Pharmacol. Sin.* **13**, 323-326.

Singh B, Song H, Liu XD, Hardy M, Liu GZ, Vinjamury SP, Martirosian CD. (2004) Dangshen (*Codonopsis pilosula*) and Bai guo (*Gingko biloba*) enhance learning and memory. *Altern. Ther. Health Med.* **10**, 52-56.

State Food and Drug Administration of China. (2000) Note for Studying Fingerprint of Traditional Chinese Medicine Injections, pp. 3, Shanghai.

US Food and Drug Administration. (2000) FDA Guidance for Industry - Botanical Drug Products, pp. 4, Rockville.

Wang ZT, Du Q, Xu GJ, Wang RJ, Fu DZ, Ng TB. (1997) Investigations on the protective action of *Condonopsis pilosula* (Dangshen) extract on experimentally-induced gastric ulcer in rats. *Gen. Pharmacol.* **28**, 469-473.

Xia Q, Zhao KJ, Huang ZG, Zhang P, Dong, TTX, Li SP, Tsim KWK. (2005) Molecular genetic and chemical assessment of Rhizoma Curcumae in China. *J. Agric. Food Chem.* **53**, 6019-6026.

Yan SK, Xin WF, Luo GA, Wang YM, Cheng YY. (2005) An approach to develop two-dimensional fingerprint for the quality control of Qingkailing injection by high-performance liquid chromatography with diode array detection. *J. Chromatogra.* A **1090**, 90-97.

Zhao KJ, Dong TTX, Tsim KWK. (2003) Molecular genetic and chemical assessment of Radix Angelica (Danggui) in China. *J. Agric. Food Chem.* **51**, 2576-2583.

Zheng TZ, Li W, Qu SY, Ma YM, Ding YH, Wei YL. (1998) Effects of Dangshen on isolated gastric muscle strips in rats. *World J. Gastroenterol.* **4**, 354-356.

Zheng XY. (2005) Pharmacopoeia of the People's Republic of China, pp. 199, Chemistry Industry Press: Beijing, China.

Zou P, Hong Y, Koh HL. (2005) Chemical fingerprinting of *Isatis indigotica* root by RP-HPLC and hierarchical clustering analysis, *J. Pharm. Biomed. Anal.* **38**, 514-520.