Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(5):166-172

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

HPLC fingerprint spectrum analysis of *Rehmannia gtutinosa*

Li Jianjun[1], Wang Jun[1], Wang Ying[1], Chen Xuejiao[1], Xu Yuge[1], Zhang Zhongyi[2], Jiang Daohui[3] and Zhao Zhengwei[4]

[1]*Henan Normal University College of Life Sciences, Engineering Technology Research Center of Nursing and Utilization of Genuine Chinese Crude Drugs, University of Henan Province, Henan Xinxiang, China*
[2]*Institute of Chinese Medicinal Materials, Henan Agriculture University, Zhengzhou, China*
[3]*The Wanxi Production Base of Pharmaceutical Huaiyao, Jiaozuo Wenxiang, China*
[4]*The Agriculture Science Institute of Wenxian, Jiaozuo Wenxiang, China*

ABSTRACT

The aim of this study was to compare the HPLC fingerprints of different Rehmannia Species to build a standard for strengthening the quality control and species identification. The method was HPLC and the Chinese Medicine Chromatography Similarity Evaluation System and similarity and clustering analyses to the HPLC fingerprint spectrum of different kinds of Rehmannia was performed. An optimized HPLC technique was obtained, and the Rehmannia HPLC comparison fingerprint spectrum containing 18 featured fingerprint peaks was established. The 9 agriculture species were first divided into two broad categories and then further divided into four detailed categories. It was concluded that the established spectrum could be used as the standard spectrum for Rehamnin quality control since the chemical fingerprint spectrums of different Rehmannia species were different, and could provide theoretical and technical support for quality control, species identification and classification of Rehmannia.

Keywords: *Rehmannia gtutinosa*, Planting Strains, HPLC, Fingerprint Spectrum

---

INTRODUCTION

Rehmannia gtutinosa Libosch is a perennial herb belonging to the Scrophulariaceae family, and is a kind of common Chinese medical material[1-2]. There are many agricultural strains of Rehmannia distributed across China. Among them, “Guhuaiqingfu” from Henan is the best with the longest history, and is one of the “Four huai medicines”. After generations of natural selection and artificial screening, the germplasms of Rehmannia have shown significant genetic varieties, forming many agricultural species and strains. The productivities and qualities of different agricultural species vary significantly. The proportions of major effective components catalpol and acetoside are not always the same. Based on the producing methods, Rehmannia could be divided into fresh, raw and matured ones. Since Rehmannia’s chemical components are complicated, catalpol and acetoside defined in the 2010 “Chinese Pharmacopoeia” could not thoroughly represent the quality and efficiency of Rehmannia. Current quality control of Chinese medicine adopts mainly the Chemical fingerprint spectrum monitoring system. However, the reported HPLC fingerprint spectrums are mostly about honeysuckle rather than Rehmannia. Therefore, building a standard chemical fingerprint spectrum is important for providing scientific reference for the quality evaluation, species identification and classification of Rehmannia [5-14]. This research utilized seven samples from “Beijing No3” and nine traditional agricultural species in Guhuanqingfu of Daodi producing area. Through HPLC fingerprint spectrum analysis, 18 feature peaks of Rehmannia were collected. Using similarity calculation and clustering analysis, we identified and classified the agricultural species and evaluated the qualities, and built the standard chemical fingerprint spectrum.

EXPERIMENTAL SECTION

1.1 Samples

Nine agricultural planting species from "Guhuanqingfu" of Daodi producing area were collected as table 1; Among them, the No.6 agricultural specie "Beijing No.3" was the most broadly planted. The samples were collected from seven different plots of the Huai region as table 2. In each normal-growth region, 10 random samples were selected, collected in November, 2011 and dried. All samples were identified as raw Rahmannia by Prof. Chongyi Zhang from Henan Agricultural University. We smashed all samples and filtered them with No.4 filter. The details of the samples are shown in Table 1 and Table 2.

Table 1 Nine agricultural species of *Rehmannin gtutinos*a

NO.	1	2	3	4	5	6	7	8	9
Name	shengjin	hongshu King	new taisi	Qinhuai	Beijing No.1	Beijing No.3	85-5	Detoxified 85-5 (G2)	Detoxified 85-5(G1)
Producingarea	Wen county	Wen county	Wuzhi	Wuzhi xiyuhui	Wen county	Wen county	Wen county	Mengzhou Huagong viliage	Wen county Zhaobao

Table 2 Beijing NO.3 from seven different plots

NO.	1	2	3	4	5	6	7
Producing area	Wuzhi Xiyuhui	Wen county	Wen county	Mengzhou Huagong viliage	Mengzhou Nankaiyi	Wen county	Wen county
Soil	loam	Yellow River Sand	Sandy loam	Clay loam	Sandy loam	Mixed soil	Mixed soil
Drying methods	Coal fire	Coal fire	Steam	Coal fire	Coal fire	Coal fire	Coal fire

1.2 Equipment

The main instruments employed in the experiment included AGILENT 1200 HPLC (including 1200 pump, automatic sample injector, column oven, and diode-array detector (DAD)), Solvent Filter A, Ultra-Water Purifier, Ultrasound cleaner, and Electronic balance (Shanghai precision scientific instrument limited company).

1.3 Reagents

The main reagents used in the experiments were Acetonitrile(chromatographic pure, DeEn Chemical Reagent limited Company, Tianjin), glacial acetic acid(chromatographic pure, Fuyu precision chemical industry limited company, Tianjin), Ultra-pure water(Fresh prepared), Catalpol reference(Chinese Food Medicine identification Institute # 110808-201009), and Acteoside reference (Chinese Food Medicine identification Institute # 111530-201007).

2 Methodology

2.1 Chromatography condition

The filler was octadecyl silane chemically bonded silica. The fluid phase and detection wavelength were chosen after referring to many articles and comparison. The final fluid phase was 0.4% acetic acid and acetonitrile. The detection wavelength was 260 nm, the column temperature was 30 ℃, the fluid speed was 1 ml/min, and the sample sice was 10 μl. The gradient wash conditions are shown in Table 3.

Table 3 The conditions of gradient elution

Time(min)	0.3%acetic acid (%)	acetonitrile(%)	wavelength（nm）
0	97	3	260
10	90	10	
25	67	33	
35	50	50	
40	20	80	
55	20	80	

2.2 Preparation of sample solutions

3.92 mg of catalpol and 4.02 mg of acteoside were weighed and put into 5 ml and 10 ml volumetric flasks, respectively, dissolved by 50% methanol to the scale and shaken to mix well. We precisely measured 2 ml of catalpol solution and 1 ml of acteoside solution, moved to a 10 ml volumetric flask, and adjusted to scale volume as the reference solution.

2.3 Preparation of Rehmannia solution

The collected samples were smashed and filtered. We weighed 1 g of each power, added into a 50 ml flask with plug, then added 50 ml of 50% methanol, weighed and recorded. After 90 min of sonication, we weighed after cooling down the solution, and added weight to scale. The solution was then shaken to mix well. We collected the filtered solution and made the marker.

2.4 Methodology Investigation

2.4.1 Precision test

One gram of the powdered Rehmannia (Sample No.1) was weighed precisely and used for preparing solution according to the method in 2.3. Using the condition in 2.1, the sample was eluted by 5 consecutive fillings of 10.0 μl. The result indicated that there was no obvious change in the peak areas and peak maintaining time for five chromatographic peaks whose areas were larger than 2%. The measured RSD% was smaller than 3%, showing the good precision of the equipment.

2.4.2 Stability test

Sample solution was extracted as in section 2.3 and filled at time points of 0, 2, 4, 8, and 10 hour. After the elution according to the chromatographic condition in 2.1, we got the spectrum figure. The result indicated that there was no obvious change on the peak areas and peak maintaining time for five chromatographic peaks whose areas were larger than 2%. The measured RSD% was smaller than 3%, showing the stability of the sample within 10 hours.

2.4.3 Reproducibility test

The same sample was made 5 copies as the method in section 2.3. The relative maintaining duration and peak area of the chromatographic peaks were measured. The RSD% was smaller than 3%, showing good reproducibility.

RESULT AND DISCUSSION

3.1 Establishment of HPLC Fingerprint Spectrum

The sample solution was analyzed based on the chromatographic condition above and the reference sample position is shown in Figure 1. The Beijing No.3 samples from seven different plots were made into test solution and analyzed using the same chromatographic condition. The HPLC spectrum was analyzed using the *Chinese Medicine Chromatography Fingerprint Spectrum Similarity Evaluation System Version A*. In the standard fingerprint spectrum we got from Beijing No.3, 17 peaks could be used as the feature peaks for all Rehmannia fingerprint spectrum, as shown in Figures 2-3 and Table 4.

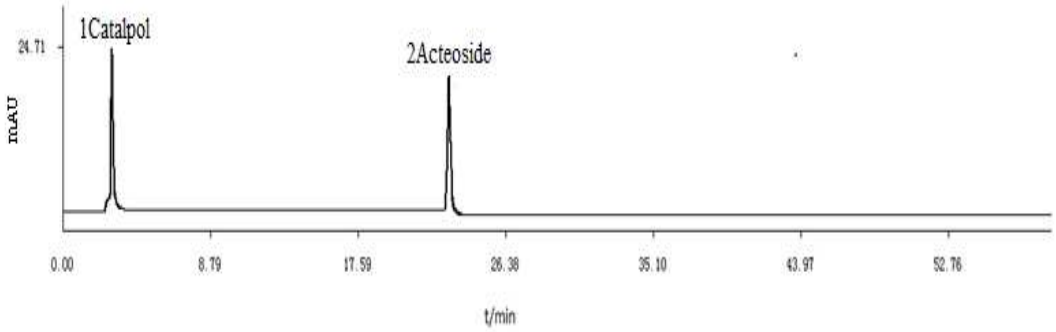


Fig. 1 HPLC of Mixed reference solution

Table 4 Similarity evaluation of Beijing NO. 3 from 7 different plots

No.	S1	S2	S3	S4	S5	S6	S7	R
S1	1	0.996	0.995	0.993	0.991	0.991	0.992	0.995
S2	0.996	1	0.996	0.995	0.997	0.995	0.992	0.995
S3	0.995	0.996	1	0.995	0.993	0.996	0.995	0.997
S4	0.993	0.995	0.995	1	0.996	0.997	0.991	0.993
S5	0.991	0.997	0.993	0.996	1	0.995	0.998	0.997
S6	0.991	0.995	0.996	0.997	0.997	1	0.994	0.996
S7	0.992	0.992	0.995	0.991	0.998	0.994	1	0.996
R	0.995	0.995	0.997	0.993	0.997	0.996	0.996	1

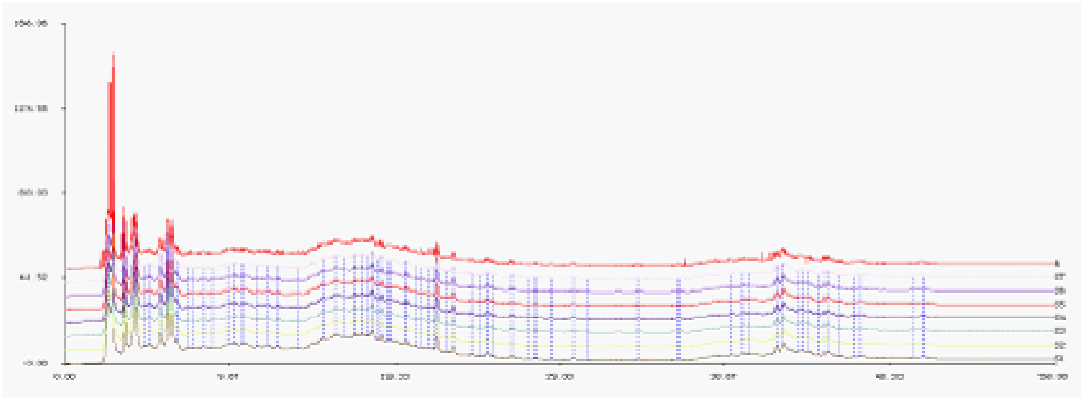


Fig. 2 Spectrum peak match of Beijing NO.3 from 7 different plots

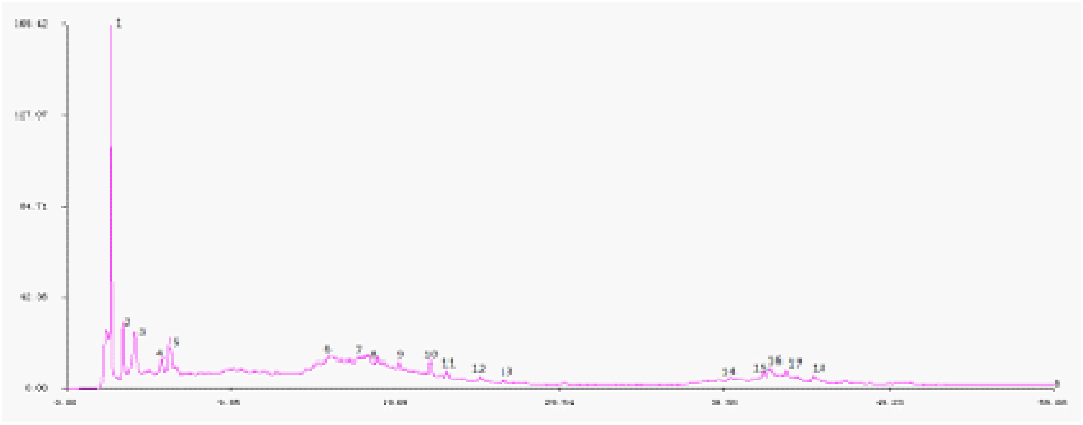
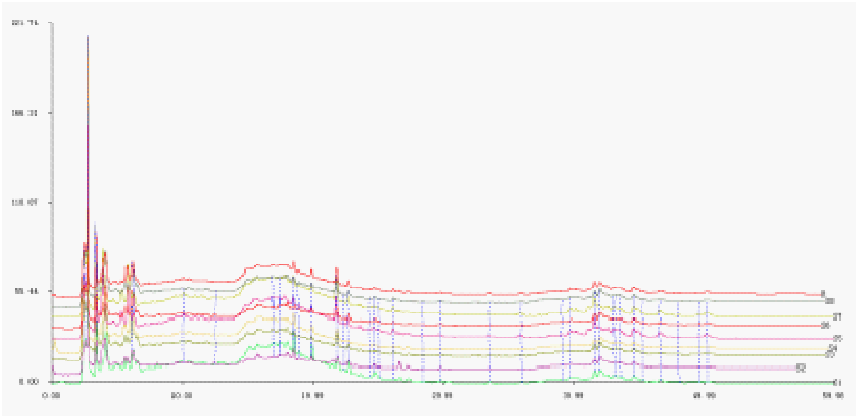


Fig. 3 Beijing NO.3 reference fingerprint spectrum

3.2 Comparison of HPLC chemical fingerprint spectrums of 9 different Rehmannia agricultural species

Rehmannia samples of 9 different agricultural species were made into test solutions. We compared the HPLC fingerprint spectrums with that of Sample No.6 using the *Chinese Medicine Chromatography Fingerprint Spectrum Similarity Evaluation System Version A*; the results are shown in Figure 4 and Figure 5.



R.Beijing NO.3；S1. Samples1；S2. Samples 2；S3. Samples 3；S4. Samples 4；S5. Samples 5；S6. Samples 6；S7. Samples 7；S8. Samples 8；S9. Samples 9

Fig. 4 PLC fingerprint spectrums of 9 agricultural rehmannia glutinosa species

Table 5 Similarity evaluation of 9 agricultural species

No.	S1	S2	S3	S4	S5	S6	S7	S8	S9	R
S1	1	0.986	0.969	0.974	0.968	0.969	0.961	0.978	0.981	0.990
S2	0.986	1	0.974	0.970	0.967	0.979	0.974	0.977	0.976	0.992
S3	0.969	0.974	1	0.961	0.969	0.970	0.971	0.982	0.970	0.993
S4	0.974	0.970	0.961	1	0.967	0.969	0.973	0.967	0.974	0.995
S5	0.968	0.967	0.969	0.967	1	0.968	0.970	0.967	0.968	0.994
S6	0.969	0.979	0.970	0.969	0.968	1	0.961	0.964	0.976	0.994
S7	0.961	0.974	0.961	0.963	0.960	0.961	1	0.963	0.974	0.993
S8	0.978	0.977	0.982	0.967	0.967	0.964	0.963	1	0.974	0.990
S9	0.981	0.976	0.970	0.974	0.968	0.976	0.974	0.974	1	0.992
R	0.990	0.992	0.993	0.995	0.994	0.994	0.993	0.990	0.992	1

The result indicated that the similarity of the HPLC fingerprint spectrums of samples No.6 from 7 different plots were all greater than 0.99, which met the standard of the *Technical requirements of Chinese medicine injection solution fingerprint spectrum research (temporary)* version 2000. Therefore, the reference fingerprint spectrum of Sample No.6 was eligible. Comparing Figure 1 and Figure 3, we could know that the peak 1 in Figure 3 was catalpol, and peak 10 was acteoside. Tables 4 and 5 show that the similarity degrees of the HPLC fingerprint spectrums obtained from all 9 species and those grown in all 7 plots were greater than 0.96. Therefore, the spectrum could be used as the standard chemical fingerprint spectrum of Rehmannia

Using inter-group averaging method as the identification method, we took relative area values from 20 shared feature peaks of 6 agricultural species in Table 5 and performed cluster analysis as shown in Figure 5.

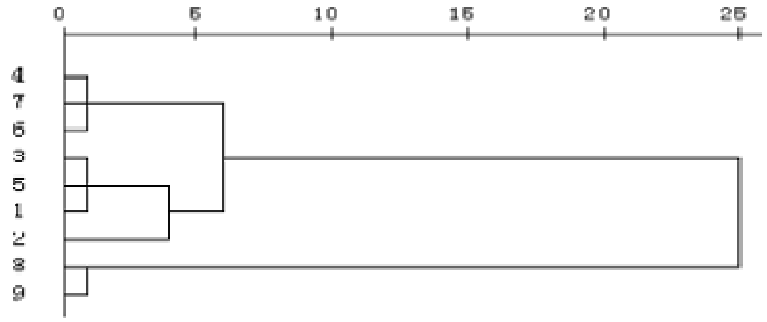


Fig. 5 Clustering analysis of 9 agricultural species of Rehmannia Glutinosa

From Figure 5 we could know that the nine samples could be clustered into 4 categories: No8 Mengzhou Detoxified 85-5 and No9 Wen county Detoxified 85-5 belonged to one category, No2 Hongshu King belonged to one category, No3 Xintaisi, No 5 Beijing No.1 and No1 Shengjin belonged to one category, and No4 Qinhuai, No7 85-5 and No6 Beijing No.3 belonged to one category.

Based on the 20 feature peaks' areas and heights, we could divide the HPLC fingerprint spectrums of the 9 agricultural species into 2 general categories. 20 peaks of Detoxified No8 and No9 were not obvious with relative small peak heights and areas, while 20 peaks of Sample No1~No7 were obvious with relative large peak heights and areas. The clustering analysis showed the same trend. Feature peak 1 catalpol and peak 12 acteoside were dramatically different. If we sort them by peak areas, it would be No6>No7>No4>No1>No3>No5>No2>No8>No9, which showed the same trend with clustering analysis. No.6 peak's peak areas and heights of Sample No8 and Sample No 9 were the smallest, while the relative peak areas of other samples were all larger than 182. Peak areas and heights of other peaks were also different.

RESULTS AND DISCUSSION

4.1 Optimization of Chromatographic conditions

Based on the chromatographic result, we compared the chromatographic difference between fluid phases of 0.3% acetic acid, 1% acetic acid-methanol and acetonitrile. The result showed that 0.4% acetic acid and acetonitrile were the best fluid phases for separation with plenty of stable peaks. The spectrum difference under 260 nm and 300 nm

wavelengths was compared and the result showed that the peak absorption under 260 nm was large and with a stable baseline. Therefore, this research adopted 0.4% acetic acid as the fluid phase and 260 nm as the detection wavelength.

4.2 Optimization of extracting conditions

We explored the spectrums based on types, condition, duration and quantity of extracting solutions. The results showed that sonication of 1 h in 50% methanol gave the most peaks and the best peak separation.

4.3 Establishment of the standard fingerprint spectrum of Rehmannia

So far, only Yumei Jia et.al use fresh Rehmannia to get juice, and identified 12 peaks in common and built fingerprint spectrum of fresh Rehmannia, using acetonitrile-0.1 phosphate buffer solution as the fluid phase and 215 and 230 nm as the detect wavelengths. Beijing No.3 Rehmannia from 7 different plots, which occupies the largest planting area and yields the highest productivity in Daodi producing area, was chosen as the study sample. The sample was eluted with the condition in table 3. A reference fingerprint spectrum of Rehmannia from Daodi, Henan was built and 17 relative large peaks were identified as the feature peaks for Rehmannia. The result provided a quality control reference for Rehmannia in Daodi, Henan.

4.4 Common features of different agricultural Rehmannia species

After comparison, it was found that the group feature of major peaks in fingerprint spectrums for all 8 samples were basically identical: 17 shared peaks had correlated relative maintaining durations while the peaks-not-in-common represented a relatively smaller proportion in the spectrum. Similarity degrees of 9 Rehmannia agricultural species were all greater than 0.9, which represented the relative stability of the internal quality of Rehmannia in Daodi, Henan. Therefore, the HPLC fingerprint spectrum made in this research could be used as the common spectrum for different agricultural Rehmannia species. Meanwhile, we proved the scientific value of the “One test, multiple evaluation” method provided by Zhimin Wang et al. [16], who provided scientific reference for identification of the two representing components defined in the 2010 pharmacopoeia: catalpol and acteoside.

4.5 Differences in various agricultural Rehmannis species

There are certain differences between peak areas and peak heights of different agricultural species. Based on the peak areas and peak heights of 17 feature peaks, we divided the agricultural species into two general categories using clustering analysis-- Detoxified and Other, and then further classified them into 4 small categories. Detoxified Species had 17 relatively small feature peaks, which matched the low content for two parameters catalpol and acteoside of Detoxified 85-5 mentioned in the research of Jianjun Li et al. [4]. The mechanism of that needs further investigation. After combining fingerprint spectrum, similarity and clustering analysis to evaluate the quality of agricultural Rehmannia species, the species identification would be more scientific, comprehensive and fitting the real situation. Our research provids theoretical and technical supports for the quality control and germplasm identification of Rehmannia.

Acknowledgments

This work was supported by Traditional Chinese medicine public health special (wealth club [2011] no. 76），special scientific research of traditional Chinese medicine industry (201207002)， Eleventh Five National science and technology supporting plan (2006BRI06R12-06), Henan education department natural science research plan (2009B180012), Henan Key science and technology funding (122102310283), Henan education depart science and technology key research funding (12B180010), and henan Social sciences planning project (2013 bjj084).

REFERENCES

[1] National pharmacopoeia committee, Pharmacopoeia of People’s Republic of China(2005), Chemical Industrial Press, Beijing, **2005**：152.

[2] Jianjun Li, Ying Wang, Xiaojie Chen et,al, *Henan Agriculture University Journal* **2012**,46（2）：127-130.

[3] Jianjun Li, Ying Wang, Xiaojie Chen et,al, *Henan Agriculture University Journal*, **2011**（4）：13-16.

[4]Jianjun Li, Ying Wang, Yanqing Zhou et,al, *Zhengzhou University Journal*, **2012**，2 (44) 102~107.

[5] Xuesen Wen, Shilin Yang, Jianhe Wei et,al, *Chinese herb medicine*, **2002**，33（10）:946-949.

[6] Yanqing Zhou, Qingxiang Yang, Gaina Zhang, Genetic markers and application, Chemical Industrial Press, Beijing, **2008**:132-174.

[7] Li X B,Tu P F. *Chin Tradit Herb Drugs*，**2003**，34（5）：385-387.

[8] Xiao R,Zhang L T. *J Hebei Med Uvin*，**2003**，24(5):301-303.

[9] Lin Shao, Qingmei Guo, Rong Ran et,al, *Pharmacology of traditional Chinese medical formulae*, **2011**,17（19）：117-120.

[10] Zengxu Xiang, Shanlin Gao, *Chinese Medicine Journal of China*, **2008**,33（9）：996.
[11] Yan Xion， Huimin Gao, Zhimin Wang, et,al, *Chinese Medicine Journal of China*, **2008**,33（9）：996.
[12] Junying Shi, Huimin Zhang, Ying Wang et,al, *Shandong Chinese Medicine University Journal*, **2008**,32（1）：69.
[13] Jun Cheng, Guisheng Li, Zhaoyan Ren et,al *Chinese Doctor and Chinese medicine* **2006**,17（2）：238.
[14] Qian Wang, Jianxin Wang, Zhiguo Yu et,al, *Chinese Patent Drug*, **2005**,27（7）：751.
[15] Yumen Jia, Hongfu Duan, Ying Cun et,al, *Chinese Patent Drug*, **2005**,27（7）：751.
[16] Zhimin Wang, Zhongzhi Qian, Qiwei Zhang et,al, *Chinese Medicine Journal of China*, **2011** ，36（6）,657.