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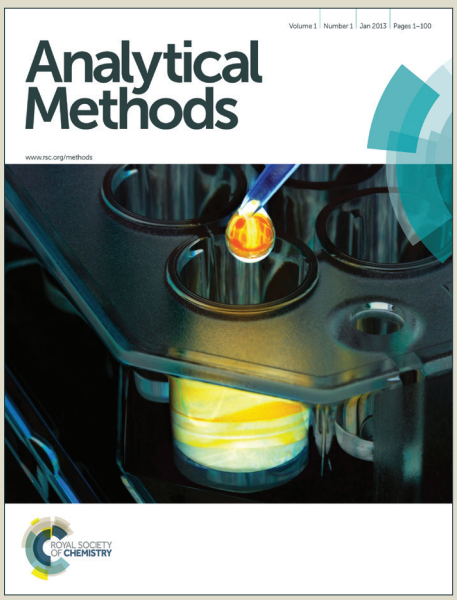

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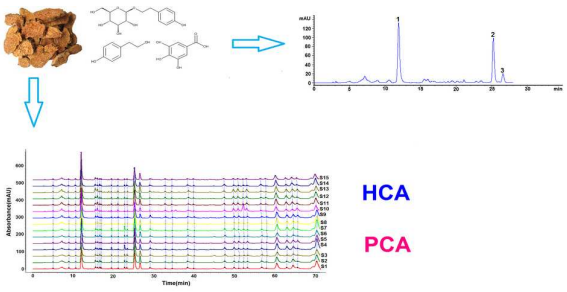

Combinative method using multi-components quantitation by single reference standard and HPLC fingerprint for comprehensive evaluation of *Rhodiola crenulata* H.Ohba

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Abstract

A combinative method using HPLC-DAD quantitative analysis and fingerprint were developed to evaluate the quality of *Rhodiola crenulata* H.Ohba (*R. crenulata*) from different origins. In this study, three phenolic compounds were first simultaneously determined by single standard to determine multi-components (SSDMC) method. The chromatographic separation was performed on a Merck Purospher STAR RP-18 column (4.6×250mm, 5μm) with a gradient elution program within 28 min at 275nm wavelength. The method has achieved desired specificity, linearity ($r^2 \geqslant 0.9999$), precision, accuracy (95-105%), stability and robustness. Compared with the normal external standard method (ESM), this alternative method can be used for simultaneous determination of multiple indexes effectively and accurately, and can resolve the problem of lacking of chemical standards. Additionally, the herbal chromatographic fingerprint was calculated using similarity evaluation system and SPSS 19.0 software as a result of analysing all the *R. crenulata* samples. The obtained data showed good repeatability and stability of the

chromatographic fingerprint. The 15 *R. crenulata* samples from different origins were classified by hierarchical clustering analysis (HCA) and principal components analysis (PCA) according to the characteristic of common peaks and the similarity values were all above 0.90. This study demonstrated that a combination of the chromatographic quantitative analysis and fingerprint offers an efficient way to quality consistency evaluation of *R. crenulata*.

Keywords: Quality consistency evaluation, *Rhodiola crenulata* H.Ohba, Single standard to determine multi-components (SSDMC), Quantitative determination, HPLC fingerprint.

1. Introduction

The plants of the genus *Rhodiola* are widely distributed in the high cold region of Asia, Europe and North America. There are approximately 90 species recorded in the world, and more than 70 species are found in China, mainly the Hengduan Mountains region including eastern Tibet, northern Yunnan and western Sichuan. In China, *Rhodiola* species have been used for maintaining body health and treating various diseases in traditional Tibetan medicines (TTM) for thousands of years.[1] Among the variety of *Rhodiola* species,[2-4] *R. crenulata* is known for best quality and has been accepted by the Pharmacopoeia of China since 2005.[5]

R. crenulata consists of the dried roots and rhizome of *Rhodiola crenulata* (Hook. f. et Thorns.) H. Ohba (Fam. Crassulaceae). Phytochemical studies on *R. crenulata* revealed that it contained a variety of chemical constituents, mainly including phenolic compounds, organic acids, flavonoids and tannins.[6-8] Phenolic compounds are the major active constituents for the standardization of *R. crenulata* which exhibit many biological activities such as adaptogenic, anti-oxidation, anti-hypoxia, anti-fatigue, anti-arrhythmic, anti-cancer and enhancement in learning and memory.[9-17]

Separation-based techniques, such as capillary zone electrophoresis and high speed counter current chromatography have been used for the determination of *R. crenulata*.[3, 18] However, on the basis of satisfactory accuracy and effectiveness, a simple method is preferred to be developed for quality evaluation of *R. crenulata*. High performance liquid chromatography coupled with diode array detector (HPLC-DAD) has proved to be one of

the most powerful tools for the quality control of traditional Chinese medicine (TCM) because of the simplicity, speed and stability of the method.[19-23] Only few studies using HPLC on the evaluation of *R. crenulata* have been reported.[24] But it is insufficient to determine merely one or two markers for completely evaluating the inner quality of *R. crenulata*. Therefore a comprehensive and systematic quality standard for quality assessment of *R. crenulata* is imperative.

In this study, SSDMC method, which has already applied in some TCM for resolving the problem of the expensive price and the limited availability of reference substances, [25, 26] was first used to determine phenolic compounds in *R. crenulata*. The three phenolic compounds salidroside, tyrosol and gallic acid which have specific pharmacological activity and abundant in *R. crenulata* were simultaneously determined with salidroside as reference standard. The SSDMC method was validated, and its results were compared with that obtained by traditional ESM. Additionally, the same chromatographic condition was applied to fingerprint analysis for the further assessment of *R. crenulata*. The similarity evaluation system, HCA and PCA were performed to classify the samples from different origins according to the characteristic of common peaks in fingerprint chromatogram. The aim of the present study was to develop a direct, rapid and reliable HPLC-DAD method for quantitative analysis and establish the chromatographic fingerprint analysis for distinguishing *R. crenulata* from various locations. This combinative method can evaluate the quality of *R. crenulata* more comprehensively.

2. Experimental

2.1 Materials and reagents

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fisher Scientific, USA). Phosphoric acid for HPLC was obtained from Kemiou Chemical Reagent Co. Ltd. (Tianjin, China). Redistilled water was used for the preparation of two-phase mobile solvent system. All solvents and samples were filtered through 0.45μm membrane filters before analysis.

Salidroside and tyrosol were purchased from the Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and gallic acid was obtained

from Acros Organics (New Jersey, USA). The purity of salidroside, tyrosol and gallic acid were all determined by peak area normalization method on HPLC. Through calculation, the purity of the standards was all above 98.0%.

Fifteen batches of *R. crenulata* from different provinces in China were collected, including Tibet Linzhi (No.5), Tibet Lasa (No.1, 2, 3, 4, 13, 14), Yunnan Kunming (No.7, 8), Yunnan Lijiang (No.6), Sichuan Leshan (No.9), Sichuan A-bazhou (No.10), Qinghai Xining(No.11, 12), Gansu (No.15). All of the samples were identified by Professor Ying Jia (Department of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang, China)

2.2 Apparatus

Agilent 1100 HPLC system (Agilent Technologies, USA) consisted of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and a diode array detector (DAD). System control and data analysis were processed with Agilent ChemStation software. Additional different HPLC instrument was used. Agilent 1260 HPLC system comprised a quaternary solvent delivery system, an on-line degasser, an auto-sampler, and photodiode array detector coupled with an analytical workstation. A KH5200b sonicated bath (HeChuang, KunShan Co. Ltd) was used for sample preparation.

2.3 Preparation of solutions

2.3.1 Preparation of standard solution

Stock solutions of the reference standards (salidroside, tyrosol and gallic acid) were prepared by dissolving accurately weighed standards in methanol to yield the concentrations of 3.016, 1.618 and 2.020 mg/mL respectively, and stored in a 10 mL volumetric flask. These solutions were stored at 4^{o}C before use.

2.3.2 Sample solutions

Transfer about 500mg powder of *R. crenulata*, accurately weighed to a 100mL glass-stoppered conical flask. 15 mL methanol-water (6:4) was added and sonicated for 30 min, and then adjust to the initial weight by adding methanol-water (6:4) as needed. The extracts were filtered with a 0.45μm membrane filter prior to HPLC analysis.

2.4 Chromatographic conditions

The chromatographic separation was carried out on a Merck Purospher STAR RP-18 (4.6×250mm, 5μm) with a guard column (4.6×10mm, 5μm; Merck). Agilent Zorbax SB C_{18} (4.6×250mm, 5μm). The mobile phase consisted of water containing 0.02% phosphoric acid (A) and methanol: acetonitrile=9:1(B). The segmented gradient profile was 5-5% (B) in 0-10 min, 5-17% (B) in 10-15 min, 17-17% (B) in 15-28 min. 17-23% (B) in 28-45min, 23-25.5% (B) in 45-72min. The assay determination was stop at 28 min. The mobile phase flow rate was 1.0 mL per minute. The DAD detector was operated at 275 nm with 8 nm bandwidth and no reference wave. The column temperature was at 25°C. All injection volumes of sample and standard solutions were 10μL.

2.5 Data analysis

To calculate the RRFs, three different concentration calibration curves was prepared. The RRFs of tyrosol and gallic acid at each concentration level of calibration curve were calculated as Eq. (1). The content of reference standard salidroside was calculated by external standard method. The final RRFs of each analyte were obtained as the mean values calculated from the triplet of six gradient concentrations level.

$$\text{RRF}= \frac{A_s / C_s}{A_{sx} / C_{sx}} \quad (1)$$

In which A_s and C_s were the peak area and the concentration of salidroside obtained from standard solution; A_{sx} and C_{sx} were the the peak area and the concentration of analyte obtained from standard solution.

The relative retention time of the analyte (RRT_x) was calculated as the ratio of retention time of the analyte (t_x) and the salidroside (t_{SA}).

$$\text{RRT}_x= \frac{t_x}{t_{SA}} \quad (2)$$

A reference chromatographic fingerprint was calculated by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (version 2009A). The software was to employ the correlative coefficient in evaluating the similarities of different chromatograms. The HCA and PCA were done by SPSS 19.0 software. P value was the probability and it was used to compare

the differences between two groups of data by paired *t*-test. If $P<0.05$ meant there were significant difference between two groups of data.

3. Results and discussion

3.1 Optimization of extraction conditions

Sample pretreatment conditions were optimized by investigating extraction solvents, extraction methods, solvent volumes, extraction time and extraction frequency on the extraction efficiencies for chemical markers which used for HPLC quantification and fingerprinting. In the preliminary studies for the selection of different proportions of extraction solvents, methanol-water (6:4) was found to be the most effective solvent for extracting phenolic compounds based on the HPLC results. Extraction methods including ultrasonication, heat-reflux and maceration were then investigated for extraction efficiencies for marker compounds. In consideration of the convenience and shorter time-length of sonication, ultrasonication was chosen for further experiment. The appropriate volume of solvent (5mL, 15mL, 30mL), extraction time (15minutes, 30 minutes, 45 minutes) and extraction frequency (once, twice) were also investigated, and the results showed that 15mL solvent volume for extraction once in 30 minutes was the appropriate condition for extraction in consideration of convenience operation and the relatively higher value achieved. The optimal extraction conditions for *R. crenulata* used in this study are presented in detail in Section 2.3.2.

3.2 Optimization of HPLC chromatographic conditions

HPLC conditions including detection wavelength and mobile phase were investigated for optimization of chromatographic separations for marker compounds. The wavelength for the detection of the target compounds in the preparation was scanned in the entire UV range (200-400nm) to determine the λmax. The chemical structures of these reference compounds were shown in Fig. 1(A). Most chemical constituents had the best responses at 275nm, thus the detection wavelength was set at 275nm.

In the selection of mobile phase, mobile phase system, pH of mobile phase, program of mobile phase, flow rate, column temperature and injection volume were investigated to achieve better separation. It was found that good resolution and symmetric peak shape

were obtained when methanol: acetonitrile=9:1–water containing 0.02% phosphoric acid was selected as a mobile phase with a step linear gradient. Meanwhile, the results showed that column temperature maintained at 25°C, flow rate was set at 1 mL/min and the injection volume was 10μL which could obtain good resolution and acceptable peak parameters. Optimal HPLC condition used in this study are shown in Section 2.4.

3.3 Selection of reference standard

In order to generate the'standard'RRF for each analyte to the reference analyte, it is essential to choose suitable reference analyte. Selected reference analyte should be abundant in sample, stable, easily accessible and has a clear pharmacological effect or is related to clinical efficacy. Meanwhile, it should achieve a good separation under the chromatographic conditions. Therefore, salidroside was selected as reference analyte because of its low cost, high stability, high content and significant pharmacological activities in *R. crenulata*.

3.4 Calculation of relative reference standards and relative retention time

Using salidroside as reference standard, the results of RRFs and RRT gained by six concentration standard solutions are shown in Table 1. Additionally, the influence of concentrations of standard solution on the RRFs was studied. It was prepared through diluting the mixed standard solution in order. Triplicate experiments were performed, and average RRFs were calculated based on the RRFs obtained in each experiment. The results showed that the RRF of gallic acid was 0.091, and the RRF of tyrosol was 0.43. The average retention time of gallic acid, salidroside and tyrosol were 12.0min, 25.1min and 26.5min respectively. And the RRT of gallic acid was 0.48, the RRT of tyrosol was 1.05. It also proved that the RRFs and RRT were stability at different of standard solution.

3.5 Method validation

3.5.1 Specificity

The specificity was estimated by comparing the consistency of the retention time of each analyte between a sample and the corresponding reference standard. The integration peak in the chromatogram of the sample solution was corresponding in time to the peak in the chromatogram of standard solution (Fig. 1(B)).

3.5.2 Linearity, limits of quantification and detection

Methanol stock solutions containing three phenolics were prepared and diluted into six appropriate concentrations for the construction of calibration curves. The calibration curve was constructed by plotting the peak area of each compound against the concentration of each compound. The results exhibited good linearity ($r^2 \geqslant 0.9999$) over the concentration range. The limits of detection (LOD) and quantification (LOQ) for each analyte under the chromatographic conditions were determined at the signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD and LOQ of the 3 analytes were 0.075-0.60 and 0.25-2.00 μg/mL, respectively (Table 1).

3.5.3 Repeatability, precision and accuracy

The repeatability test was analyzed by three replicates at three different concentrations. The RSD values of the content of the three markers were less than 1.1%. The intermediate precision including different analysts and days were also analyzed to ensure the validity of the method. The RSD values of the content of the three phenolics with different analysts and days were found in the range of 0.6-1.0% and 1.4-1.9%, respectively. Accuracy was calculated as the percentage of recovery by the assay of the known added amount of gallic acid, salidroside and tyrosol in the sample. Recovery was between 95.6% and 103.6% with RSD values of less than 3.7% for all the three marker compounds. The results obtained by the ESM and SSDMC method showed no remarkable differences using paired *t*-test ($p=0.435>0.05$).

3.5.4 Stability

The stability of sample solution was investigated. It was carried out by comparing the peak areas of gallic acid, salidroide and tyrosol in the chromatograph of the same sample solution after storing at room temperature for different time (0, 2, 4, 6, 8, 12, 24 hours). Stability was evaluated by calculating the RSD of area obtained. The analytes were found to be stable within 24 hours in sample solution (RSD<1%).

3.5.5 Robustness

In order to introduce the SSDMC method into different laboratories, the robustness test was performed to investigate the significant affecting factors for this method. Through six concentration standard solutions, the robustness of RRT and RRFs was

evaluated with different equipment and columns. The results showed that the RRT and RRFs were fluctuated in a relative narrow range. The robustness of the established method was also evaluated by examining its stability with small variations of procedural parameters, including pH of mobile phase, ratio of components in mobile phase, programs of mobile phase, wavelength, flow rate, injection volume, and column temperature. It was proved that a slight variation of ratio of mobile phase and flow rate had great influence on the RRT of gallic acid; a little variation of wavelength had a significant effect on RRFs of gallic acid(RSD>4%). Other Factors had slight influence on the RRFs and RRT and the fluctuation was proportionable (RSD<2%). The initial ratio of organic phase had significant influence on the retention time of gallic acid which was the first peak in the chromatogram, and it was also sensitive to flow rate mainly because the chromatography behavior of gallic acid, tyrosol and salidroside were very different at different flow rate. The variation of RRFs of gallic acid was great influenced may due to the UV spectrums of gallic acid, tyrosol and salidroside were different. The results showed that RRFs and RRT of gallic acid were greatly sensitive to the variation of conditions. Therefore it was recommended that the ratio of organic phase and flow rate should not be changed too much, and the wavelength should be controlled strictly. The pH of mobile phase, the time program of mobile phase, the injection volume and the column temperature can be slightly adjusted to meet the system suitability.

3.6 Quantitative determination of three phenolics in *R. crenulata* samples

The ESM and SSDMC method were employed for quantitative analysis of three phenolics in *R. crenulata* samples from different sources. All samples were analyzed by the optimized extraction procedure under the optimized HPLC conditions. Three main phenolics, salidroside, tyrosol and gallic acid were selected as analytes to evaluate the quality. The contents of three phenolics were calculated using these two methods are shown in Table 2.

It was proved that there was no significant differences between these two methods using the paired *t*-test($p=0.164>0.05$). The values of RRFs determination is complimentary and can be effectively used as an alternative method to conventional HPLC method especially in the absence of suitable reference materials for the active

pharmaceutical ingredients.

3.7 Establishment of chromatographic fingerprint of *R. crenulata* and similarity evaluation

Fifteen batches of materials from various locations, including almost all of the growing places of *R. crenulata* in China, were obtained to ensure that the reference fingerprint thus developed was geographically representative and authentic. Peaks existing in all chromatograms of the samples were assigned as the 'common peak'. Using the software of Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2009A), the chromatograms of *R. crenulata* containing 31 common peaks within 72 min were shown in Fig. 2. It was found that these samples had similar HPLC profiles. The representive standard fingerprint was generated by the median method were shown in Fig. 3. Similarity analysis was conducted based on the standard fingerprints, and the results are shown in Table 3. Five peaks were identified comparing with standard compounds, including gallic acid (4), salidroside (13), tyrosol (14), epicatechin (20), *p*-counaric acid (26). Peak 13 (salidroside) was chosen to calculate the RRT and RPA. The similarity values of all the 15 samples were more than 0.90 indicating that the chemical constituents of the 15 batches *R. crenulata* samples were similar. Therefore, if 0.90 is set as an appropriate threshold, it is easy to identify *R. crenulata* based on the chromatographic fingerprint.

3.8 HCA of the samples

In order to evaluate the variation of *R. crenulata* from various origins, HCA was used to find natural cluster of samples according to RPA was performed from HPLC profiles. The clustering analysis was operated in SPSS software, and the results were shown in Fig. 4. It was found that 15 tested samples of *R. crenulata* were divided into two main clusters (I and II) containing 7 and 8 samples, respectively. The results indicated that samples 3, 14, 1, 2, 5, 4 and 13 which all collected from Tibet province were classified in the same cluster (cluster I). Sample 3 and 14 were classified in the same group may due to their similarity values were relative low in the cluster I. And samples 6 and 7 (from Yunnan province, China) were classified in another group. The other samples were classified in one cluster (cluster II) might be due to the constituent of *R. crenulata* from different

cultivation regions were similar.

3.9 PCA of the samples

PCA was implemented as a data reduction technique to generate a visual scatterplot for qualitative evaluation of resemblances and differences between the studied samples. The first two PCs explained more than 97.2% of total variability, and the other principal components which had a minor effect on the model were discarded. Hence it met a sufficient condition for studying the relation between *R. crenulata* samples in a bi-dimensional plate (Fig. 5). From the scatter points, the PC1 values of samples were all comparatively concentrated. However, the samples can be classified into two groups according to the difference of the PC 2 values and marked as group I and II, respectively. In group I, sample 3, 14, 2, 4, 5, 1 and 13 were all collected from Tibet. And the content of salidroside from group I was higher than group II. The result was closely related to the unique climatic ecological environment in the plateau of Tibet and the categorized data were corresponded with the results of the HCA.

The presented method suggested that the quality of different samples for sale was not consistent, and it may be helpful for discriminate *R. crenulata* according to their cultivation regions. The *R. crenulata* from Tibet occupies an important place in the *R. crenulata* resource of the world, thus it would be significant to classify and identify them.

4. Conclusions

The proposed HPLC quantitative analyse combined with fingerprint method is an efficient and comprehensive tool for quality control of *R. crenulata*. In this research, SSDMC method was first developed to quantify the chromatographically diverse constituents in *R. crenulata*. Comparison with accurate values obtained from external standard method proved that it was a fast, convenient, and accurate method to determine the contents of phenolics in *R. crenulata*. The fingerprinting analysis using similarity, HCA, and PCA approaches has produced the desirable results with high accuracy. This study revealed that quantitation of pharmacologically active constituents by SSDMC method combined with chromatographic fingerprints offers an efficient way for monitoring of the *R. crenulata* quality consistency.

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Table1 The results of linearity, LOD, LOQ, RRFs and RRT. (n=2)

Analytes	Linearity equation	R^2	Range (μg/mL)	LOD	LOQ	RRFs		RRT	
				(μg/mL)	(μg/mL)	RRFs	RSD%	RRT	RSD%
Salidroside	Y=2.881x-6.406	0.9999	55.10-1102	0.60	2.00	1.0	0	1.0	0
Tyrosol	Y=6.711x-8.06	0.9999	19.42-388.4	0.29	0.97	0.43	1.0	1.05	0.1
Gallic acid	Y=31.81x-31.18	0.9999	16.16-323.2	0.075	0.25	0.091	0.5	0.47	0.4

Table 2 Content of 15 batches of *Rhodiola crenulata* H.Ohba determined with ESM and SSDMC method (n=2)

No.	origin	ESM				SSDMC Method			
		Total phenolics(%)	Gallic acid(%)	Tyrosol(%)	Salidroside(%)	Total phenolics(%)	Gallic acid(%)	Tyrosol(%)	Salidroside(%)
1	Tibet Lasa	2.02	0.22	0.28	1.52	2.02	0.22	0.28	1.52
2	Tibet Lasa	2.29	0.20	0.27	1.82	2.29	0.20	0.27	1.82
3	Tibet Lasa	2.54	0.11	0.23	2.20	2.54	0.11	0.23	2.20
4	Tibet Lasa	2.42	0.28	0.22	1.92	2.42	0.27	0.22	1.92
5	Tibet Linzhi	2.17	0.25	0.15	1.77	2.17	0.25	0.15	1.77
6	Yunnan	1.86	0.46	0.71	0.69	1.86	0.46	0.71	0.69
7	Yunnan	1.56	0.30	0.40	0.87	1.56	0.29	0.40	0.87
8	Yunnan	2.35	0.32	0.33	1.70	2.35	0.32	0.33	1.70
9	Sichuan	1.76	0.31	0.13	1.33	1.76	0.31	0.13	1.33
10	Sichuan	1.51	0.25	0.16	1.10	1.51	0.25	0.16	1.10
11	Qinghai	2.69	0.59	0.33	1.77	2.68	0.58	0.33	1.77
12	Qinghai	2.50	0.41	0.27	1.82	2.50	0.40	0.28	1.82
13	Tibet Lasa	1.77	0.26	0.31	1.20	1.76	0.25	0.31	1.20
14	Tibet Lasa	2.51	0.18	0.16	2.17	2.51	0.17	0.16	2.17
15	Gansu	1.93	0.28	0.28	1.37	1.93	0.28	0.28	1.37
Average		2.13	0.29	0.28	1.55	2.12	0.29	0.28	1.55

Total phenolics were the sum of the content of salidroside, tyrosol and gallic acid.

Table 3 The similarities of chromatograms of 15 samples

No.	Similarities
1	0.976
2	0.961
3	0.901
4	0.972
5	0.981
6	0.918
7	0.944
8	0.974
9	0.951
10	0.948
11	0.971
12	0.996
13	0.977
14	0.921
15	0.998

Figure caption

Fig.1 (A) The chemical structures of the investigated compounds.

Fig.1 (B) (I) mixed standards: (1) gallic acid (2) salidroside (3) tyrosol, (II) sample of *Rhodiola crenulata* H.Ohba (Tibet Linzhi).

Fig.2 Chromatographic fingerprints for all the *Rhodiola crenulata* H.Ohba samples.

Fig.3 The representative standard fingerprint was generated by the median method. Gallic acid (4), salidroside(13), tyrosol(14), epicatechin(20), *p*-counaricacid(26).

Fig.4 Dendrograms of hierarchical cluster analysis for the 15 tested samples of *Rhodiola crenulata* H.Ohba samples.

Fig.5 PCA scatterplot for all the *Rhodiola crenulata* H.Ohba samples.