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Full Length Research Paper

HPLC-DAD separation and determination of major active constituents in an important Tibetan medicine *Meconopsis quintuplinervia* from different regions of Qinghai-Tibet Plateau

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This study describes a simple and sensitive method for the simultaneous determination of major flavonoids active constituents in a traditional Tibetan medicine *Meconopsis quintuplinervia* collecting from 29 different localities of Qinghai-Tibet Plateau by high-performance liquid chromatography coupled with diode array detection (HPLC-DAD). The optimal conditions of separation and detection were achieved on a Zorbax Eclipse XDB-C_{18} column (4.6 × 250 mm, 5 µm) with a gradient of methanol and 0.05% aqueous acetic acid (*v*/*v*), at a flow rate of 1.0 ml min^{-1}, detected at 370 nm. The complete separation was obtained within 40 min for the four analytes. The recovery of the method was >100%, and all the flavonoids showed good linearity (r≥0.9990) in a relatively wide concentration range. The limits of detection (LOD) are (injection volume 10 µl, at a signal-to-noise ratio of 3, S/N = 3:1) between 0.09 and 0.36 µg ml^{-1}. Furthermore, the main characteristics of flavonoids in *M. quintuplinervia* were also analyzed by principle component analysis (PCA).

Key words: HPLC-DAD, flavonoids, *Meconopsis quintuplinervia*, Tibetan medicine.

INTRODUCTION

Meconopsis quintuplinervia Regel Gartenfl, belonging to the Papaveraceae family and widely distributing in the Qinghai-Tibet Plateau of China, is a traditional Tibetan folk medicine called as "Wu ba la" in Tibetan. The common medicinal part is the whole herb and the processing of harvesting time is generally between August and September during the growth periods. According to the record in the classical books of Jing Zhu Ben Cao and Tibetan Medicines, *M. quintuplinervia* had a long drug application history by Tibetan people. For example, in Tibetan Medicines, it is often used as an important constituent in many compound prescriptions, such as Ershiwuwei Lv Rong Hao pill, and could be used to treat various diseases, such as anti-inflammation, relieving pain, improving hepatitis and tuberculosis (Yang et al., 1991). Presently, with the increasing conception and attraction of traditional medicinal value of *M. quintuplinervia*, relative pharmaceutical studies had revealed that the chemical constituents in this medicinal plant mainly included alkaloids (Shang et al., 2003a, b; Wu et al., 2009), flavonoids (Shang et al., 2002, 2006) and volatile oils (Wu et al., 2006). Flavonoids are considered as the major bioactive constituents of many medicinal plants with health-related properties, which are especially based on their special antioxidant activity. It has been demonstrated that flavonoids are one of the most important bioactive constituents in *M. quintuplinervia* and possess a number of significant biological activities for human health, such as antibacterial (Rice-Evans et al., 1996), reducing risk of cardiovascular diseases (Wang et al., 2003; Yochum et al., 1999) and certain types of cancer (Alcocer et al., 2002; Wattenberg, 1990). Therefore, flavonoids could be

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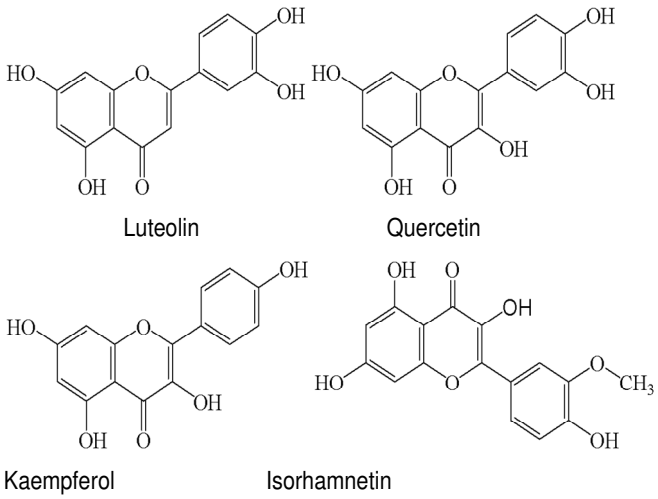

Figure 1. The chemical structures of the four analytes.

considered as one of the important "marker compounds" for the chemical evaluation and quality control of *M. quintuplinervia*. For this reason, the quality control of *M. quintuplinervia* was focused mainly on the determination of these major flavonoids bioactive constituents. To our knowledge, the specific studies on the determination of profile bioactive constituents of *M. quintuplinervia* have not been reported yet. Thus, a reliable method for the qualitative and quantitative analysis of major flavonoids constituents in *M. quintuplinervia* is highly desirable.

Currently, high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) is widely used in the determination and separation of traditional herbal medicines and has been proven to be repro--ducible, practicable and also resulted in comparable results (Drasar and Moravcova, 2004). In this paper, four major flavonoids constituents (luteolin, quercetin, kaempferol and isorhamnetin) in 29 *M. quintuplinervia* samples were simultaneously determined by HPLC-DAD and a simple, rapid and accurate analysis method was presented. Their contents in *M. quintuplinervia* could be used to evaluate the crude drug's quality. Characteristics of flavonoids in *M. quintuplinervia* were also discussed by principle component analysis (PCA).

MATERIALS AND METHODS

Instrumentation

Experiments of the liquid chromatography were performed using an Agilent 1200 series consisting of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a diode array detector (DAD) (model G1321A). The HPLC analysis was separated on a Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm, 5 µm i.d., Agilent Co.) by a gradient elution. The mobile phase was filtered through a 0.2 mm nylon membrane filter (Alltech, Deerfiled, IL). The HPLC system was controlled by HP Chemstation software.

Chemicals and materials

The four standards (luteolin, quercetin, kaempferol and isorhamnetin) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and their chemical structures were listed in Figure 1. HPLC grade methanol was purchased from Merck Co. (Germany). Acetic acid (CH_3COOH) and hydrochloric acid (HCl) were of analytical grade from Shanghai Chemical Reagent Co. Water was purified on a Milli-Q System (Millipore, Bedford, MA, USA).

The whole *M. quintuplinervia* plant samples were collected from 29 different localities of Qinghai-Tibet Plateau in August, 2006. At an individual sampling locality, distance more than 1 m apart was set and 20 - 30 individual plant materials were collected randomly. When brought back to the laboratory, the whole plant sample was cut, mixed and washed with deionized water, and then dried under the room temperature until constant weight was reached. Each sampling material was pulverized in a stainless steel. When not in use, samples were preserved in a refrigerator.

Preparation of standard solutions

An amount of the four standards equivalent to 1.36 mg (luteolin), 0.94 mg (quercetin), 1.44 mg (kaempferol) and 1.80 mg (isorhamnetin) was dissolved with methanol in a 10 ml flask, respectively. The corresponding individual standard was obtained at the concentration of 0.136 mg ml^{-1} for luteolin, 0.094 mg ml^{-1} for quercetin, 0.144 mg ml^{-1} for kaempferol and 0.180 mg ml^{-1} for isorhamnetin. A standard mixture solution were prepared by take luteolin (2 ml), quercetin (4 ml), kaempferol (2 ml) and isorhamnetin (1 ml) solution respectively and mix into a 10 ml flask with methanol. All the solutions were filtered through 0.45 µm membrane filters (Millipore), and directly injected. When not in use, all the solutions were kept at -4℃ in a refrigerator.

Preparation of sample solutions

Two extraction methods of flavonoids were listed as follows:

Ultrasound-assisted extraction

Powdered sample (2.0 g) was dissolved in a 50 ml mixture solution of methanol / 25% HCl (4:2, *v*/*v*) and extracted in an ultrasound bath under 80℃ for 2 h. The extraction solutions were leached for three times and combined into a 50 ml flask with methanol. All the sample solutions were filtered by 0.45 µm membrane filter before the chromatographic analysis.

Extraction by hot reflux

Plant samples (2.0 g) were extracted three times, each time for 30 min at 80℃, with a mixture (50 ml) of methanol and 25% HCl (4:2, *v*/*v*), by use of a hot reflux equipment, and the extracts were combined in a 50 ml flask with methanol.

HPLC conditions

HPLC separation of the four active constituents was carried out on a Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm, 5µm i.d.) on conjunction with a gradient elution. Eluent A was methanol (100%); eluent B was 0.05% CH_3COOH aqueous solution. Gradient

conditions: initial = 35% A and 65% B; 30 min = 50% A and 50% B; 40 min = 90% A and 10% B. Before injection of the next sample, the column was equilibrated with the initial elution condition for 10 min. The flow rate was constant at 1.0 ml min^{-1} and the column temperature was at 25℃. The injection volume was 10 µl.

Data analysis

All the statistical data analysis was performed using the SPSS 11.0 software package. The values of the concentrations of four active compounds were presented by mean ± SD (standard deviation). The relative standard deviation (RSD) was taken as a measure of detection limits, precision, repeatability and recovery.

RESULTS AND DISCUSSION

Optimal extraction

In order to achieve the highest extraction yields, two methods for extraction of flavonoids from *M. quintuplinervia* were evaluated by comparison of the detector responses obtained from analysis of flavonoids. One method was the ultrasound extraction, while another was extraction by hot reflux. The results indicated flavonoids were extracted most efficiently from *M. quintuplinervia* samples by use of ultrasonication. Lower efficiency was usually observed if extraction was performed by hot reflux. In tests with different solvents the efficiency of extraction was highest when a mixture of methanol and HCl (4:2, *v*/*v*) was used, possibly because the flavonoids are more soluble in a mixture of methanol and HCl than in ethanol in this study. In general, the solubility of flavonoids in the solvents tested seemed to decrease in the order methanol > ethanol. All subsequent experiments in this study were therefore performed using ultrasonic extraction with a mixture of methanol and HCl as solvent.

HPLC separation optimization

Optimization of parameters in HPLC was done through investigating the influence of the mobile phase and detection wavelength, because these two parameters played a key role on resolution and sensitivity. The selection of the HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within a short time especially when large amount of samples were analyzed. In this study, a HPLC method was successfully developed to analyze multiple components in the medicinal plant *M. quintuplinervia*. Different ratios of water and methanol were compared but no satisfied separation was reached. Thus, CH_3COOH was added to the mobile phase and optimizes the separation. Eventually, it was found that CH_3COOH in the mobile phase could better improve the separation and baseline separation of the four standards within 40 min was achieved. The solvent system of A-B (A, methanol; B, 0.05% CH_3COOH aqueous solution) was tested by changing the volume ratio of the solvent to obtain the optimal composition. When the ratio of A-B (35:65, *v*/*v*) of the solvent system at a flow rate of 1.0 ml min^{-1} was utilized so as to ensure that each run of analysis was completed within 40 min with better resolution of adjacent peaks and low solvent consumption.

Generally, not all the components were detectable to the same extent at a given wavelength due to the different chromophoric characteristics of the analytes. However, with a DAD, detection at more than one wavelength at the same time is possible. Finally, 370 nm was chosen as the detection wavelength, as it is close to the maximum absorbency of these flavonoids compounds. HPLC chromatograms of four standards were presented in Figure 2.

Linearity and detection limits

Under the optimal chromatographic conditions, a mixture solution of the four standards was diluted according to 1, 2, 4, 8, 16 and 32 times and then injected, respectively. All these four calibration curves exhibited good linear regressions as shown in Table 1, and the correlation coefficient of the regression equation (r^2) was ≥ 0.9990. The LOD were evaluated on the basis of a signal-to-noise ratio of 3 (S/N = 3) and in the range of 0.09 - 0.36 µg ml^{-1} for these four analytes.

Method validation

A standard mixture solution was analyzed for six times to determine the method precision (corresponding injected volume 10 ìL). The RSD of the peak areas were from 0.54 - 1.24% (shown in Table 1). The repeatability of the method was confirmed by analyzing the samples (each for 2.0 g) and the RSD of the peak areas was from 0.98 - 1.83% (n = 6). The results demonstrated that the developed analytical method was repeatable with good accuracy and sensitivity for all analytes examined.

Analysis of samples and recovery

The chromatogram for the analysis of four analytes extracted from medicinal plant *M. quintuplinervia* with DAD detection was shown in Figure 2. Chromatographic peaks were identified by contrasting chromatograms of real samples with that of four standards by retention time, and simultaneously confirmed by DAD spectrometry identification. All the analytes were quantified by linear regression equations. Concentrations of the four active constituents from 29 different localities were showed in Table 2, indicating that quercetin had higher concentration level than the other flavonoids constituents.

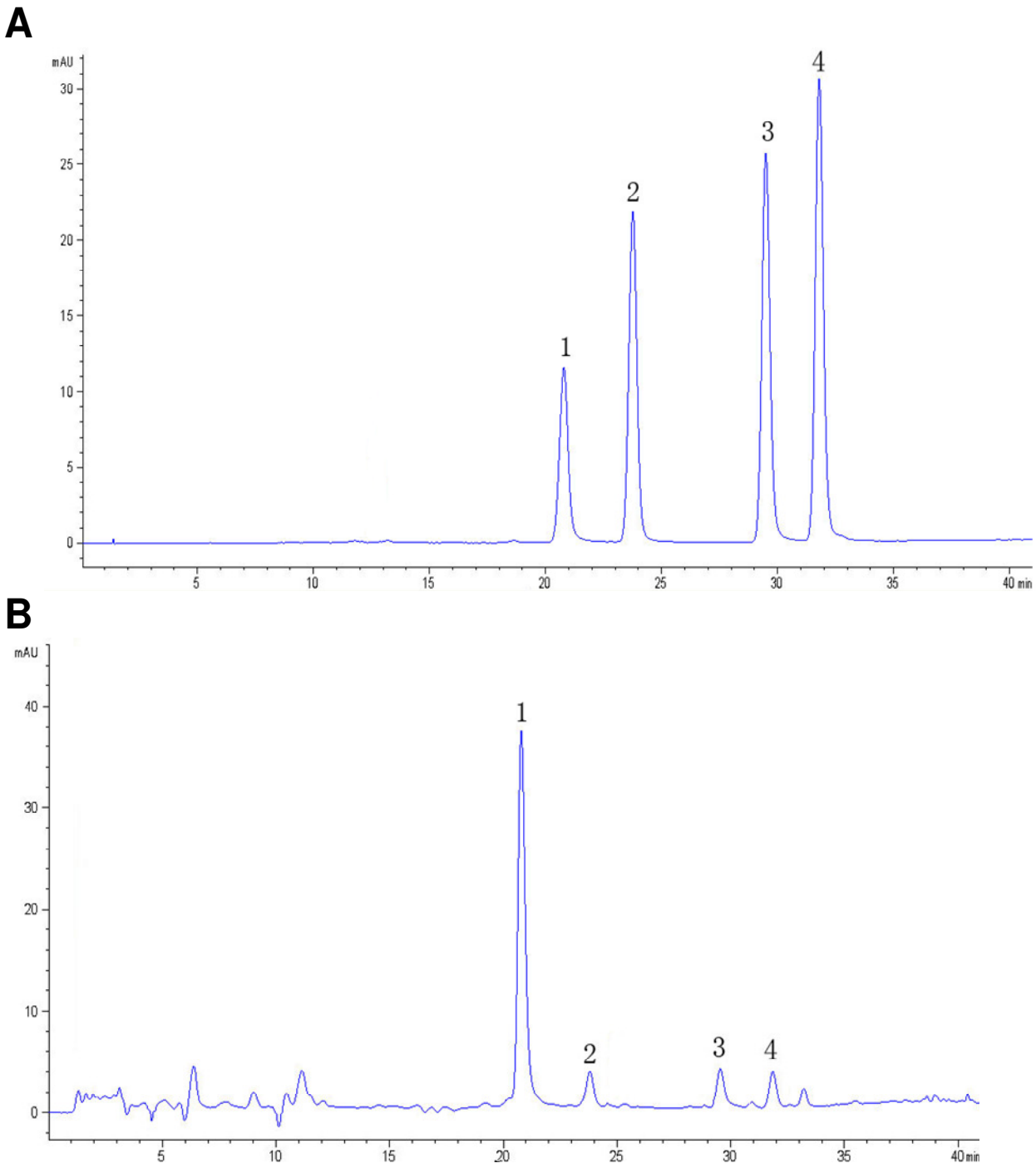

Figure 2. HPLC chromatograms of the four standards (A) and real samples (B) HPLC conditions: Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm, i.d.5 µm), detection wavelength was set at 370 nm, the flow rate was constant at 1.0 ml min^{-1}. Peak 1 for quercetin, 2 for luteolin, 3 for kaempferol and 4 for isorhamnetin.

The recoveries of four analytes were investigated by the addition of known amounts of samples into the standard solution, in which the contents of four analytes were in accordance with the calculation from linear regression equations. The experimental recoveries were in the range of 100.15 - 100.63% (Table 1).

Characteristic of flavonoids in *M. quintuplinervia*

In order to further understand the chemical characteristics of major flavonoids in *M. quintuplinervia*, a PCA method was used and discussed. PCA is a multivariate statistical technique that reduces the data

Table 1. Method validation results of the four analytes in *M. quintuplinervia.*

Analyte	Linearity equation	Correlation coefficient r^2	Linear range (μg ml^{-1})	LOD (μg ml^{-1})	Repeatability (n=6) RSD (%)	Precision (n=6) RSD (%)	Recovery (n = 6)	
							Mean	RSD (%)
Luteolin	*Y*=38566*X*+5.2315	0.9999	0.85-27.20	0.09	1.83	0.54	100.63	1.85
Quercetin	*Y*= 32886*X*-12.025	0.9999	1.18-37.60	0.17	1.57	0.65	100.15	1.62
Kaempferol	*Y*= 47575*X*-44.667	0.9990	0.90-28.80	0.36	1.21	1.24	100.28	0.78
Isorhamnetin	*Y*= 43041*X*-25.679	0.9998	0.56-18.00	0.22	0.98	0.94	100.61	1.22

Y=peak area, *X*=injected amount (μg ml^{-1}).

Table 2. Contents of four analytes in 29 different localities of *M. quintuplinervia* (mg g^{-1}) (n=3).

Sample no.	Luteolin	Quercetin	Kaempferol	Isorhamnetin
1	0.060±0.016	0.104±0.036	0.048±0.011	0.031±0.009
2	0.089±0.021	0.122±0.032	0.036±0.009	0.028±0.010
3	0.082±0.014	0.128±0.026	0.049±0.005	0.030±0.009
4	0.095±0.023	0.491±0.050	0.082±0.011	0.053±0.010
5	0.081±0.026	0.069±0.015	0.030±0.008	0.031±0.005
6	0.066±0.017	0.094±0.011	0.035±0.013	0.028±0.010
7	0.069±0.011	0.046±0.018	0.034±0.009	0.023±0.011
8	0.050±0.018	0.600±0.056	0.060±0.018	0.065±0.022
9	0.033±0.010	0.222±0.035	0.039±0.011	0.023±0.009
10	0.124±0.020	0.123±0.018	0.027±0.010	0.030±0.014
11	0.081±0.019	0.059±0.015	0.040±0.015	0.025±0.012
12	0.144±0.049	0.123±0.011	0.037±0.010	0.027±0.015
13	0.095±0.006	0.098±0.021	0.054±0.006	0.026±0.009
14	0.133±0.013	0.127±0.009	0.039±0.008	0.029±0.010
15	0.222±0.010	0.109±0.021	0.032±0.009	0.028±0.008
16	0.033±0.007	0.222±0.010	0.039±0.006	0.023±0.009
17	0.134±0.032	0.040±0.008	0.031±0.007	0.024±0.003
18	0.116±0.019	0.164±0.021	0.049±0.009	0.025±0.008
19	0.124±0.017	0.123±0.022	0.027±0.007	0.030±0.006
20	0.081±0.010	0.059±0.009	0.040±0.011	0.025±0.005
21	0.144±0.015	0.123±0.006	0.037±0.008	0.027±0.006
22	0.118±0.031	0.036±0.009	0.033±0.007	0.022±0.002
23	0.134±0.024	0.025±0.007	0.048±0.010	0.022±0.004
24	0.174±0.021	0.056±0.005	0.037±0.007	0.021±0.003

Table 2. Cont'd.

25	0.116±0.011	0.120±0.011	0.036±0.010	0.030±0.005
26	0.095±0.022	0.098±0.017	0.054±0.006	0.026±0.007
27	0.133±0.031	0.127±0.020	0.039±0.003	0.029±0.004
28	0.222±0.016	0.109±0.027	0.032±0.002	0.028±0.006
29	0.183±0.030	0.052±0.009	0.045±0.009	0.031±0.009

Table 3. Variance percentage for the score vectors of PC1 obtained for each flavonoid in *M. quintuplinervia.*

Item	Eigen values
Principle component 1	
Variance	1.624E-02
Percentage	88.544
Cumulative %	88.544
Score vectors	
Luteolin	-0.203
Quercetin	1.000
Kaempferol	0.681
Isorhamnetin	0.915

dimensionality by the linear transformation of the original data and then set them in a new and smaller set of uncorrelated variables, which has been widely applied in the chemometrics studies of bioactive compounds (Beebe et al., 1998).

The results of PCA obtained from the four flavonoid components in *M. quintuplinervia* were presented in Table 3. It was found that the first component (PC1) was responsible for 88.544% of the original data and could express the most flavonoids information from *M. quintuplinervia*. Therefore, PC1 was drawn out so as to deep analyze its biological significance. Among the four variables, quercetin, kaempferol and isorhamnetin held much higher score vectors than luteolin. In fact, quercetin, kaempferol and isorhamnetin were all flavonols, while luteolin was flavanones. Therefore, flavanones constituents could be considered as the main characteristics of flavonoids in medicinal plant *M. quintuplinervia*.

Conclusion

This was the first report on the simultaneous separation and determination of four major flavonoids bioactive constituents in Tibetan medicine *M. quintuplinervia* from 29 different samples collecting from Qinghai-Tibet Plateau. A simple, accurate and rapid assay method was successfully established. The results suggested that this HPLC-DAD method could be considered as a valid and applicable method for the quality control of Tibetan medicine *M. quintuplinervia*, and accurate results were obtained as a consequence. In addition, chemical characteristics of flavonoids were also discussed by PCA and flavanones could be considered as the main characteristics of flavonoids in Tibetan medicinal plant *M. quintuplinervia*.

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REFERENCES

Alcocer F, Whitley D, Salazar-Gonzalez JF, Jordan WD, Sellers MT, Eckhoff DE, Suzuki K, Macrae C, Bland KI (2002). Quercetin inhibits human vascular smooth muscle cell proliferation and migration. Surg. 131: 198-204.

Beebe KR, Pell RJ, Seasholtz MB (1998). Chemometrics: A Practical Guide, Wiley-Interscience, New York p. 5.

Drasar P, Moravcova J (2004). Rencent advances in analysis of Chinese medicinal plants and traditional medicines. J. Chromatogr. 812: 3-21.

Rice-Evans CA, Miller NJ, Paganga G (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical. Biol. Med. 20: 933-956.

Shang XY, Jiao HS, Yang YC, Shi JG (2003a). A morphinane alkaloid from *Meconopsis quintuplinervia*. Chin. Chem. Lett. 14: 597-598.

Shang XY, Shi JG, Yang YC, Liu X, Li C, Zhang CZ (2003b). Alkaloids from a Tibetan medicine *Meconopsis quintuplinervia* Regel. Acta. Pharmaceut. Sin. 38: 276-278.

Shang XY, Wang YH, Li C, Zhang CZ, Yang YC, Shi JG (2006). Acetylated flavonol diglucosides from *Meconopsis quintuplinervia*. Phytochem. 67: 511-515.

Shang XY, Zhang CZ, Li C, Yang YC, Shi JG (2002). Studies on chemical constituents of *Meconopsis Quintuplinervia* Regel. Chin. Med. Mater. 25: 250-252.

Wang YF, Wang XH, Zhu YT (2003). Advancement of researches in quercetin. Nat. Prod. Res. Dev. 15: 171-173.

Wattenberg LW (1990). Inhibition of carcinogenesis by minor anutrient constituents of the diet. Proc. Nutr. Soc. 49: 173-183.

Wu HF, Ding LS, Shen JW, Zhu HJ, Zhang XF (2009). A new proaporphine alkaloid from *Meconopsis horridula*. Fitoterapia 80: 252-254.

Wu HF, Pan L, Zou DS, Yang SJ, Zhang XF (2006). Analysis on volatile oils from three species of *Meconopsis* by GC-MS. Chin. Pharm. J. 41: 1298-1300.

Yang YC, Ho TN, Lu SL, Huang RF, Wang ZX (1991). Tibetan Medicines. Qinghai People's Press, Xining, China p. 465.

Yochum L, Kushi LH, Meyer K, Folson AR (1999). Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. Am. J. Epidemiol. 149: 43-949.