HPLC Determination of Eight Polyphenols in the Leaves of *Crataegus pinnatifida* Bge. var. major

Xixiang Ying[1,2], Rongxiang Wang[1], Jing Xu[1], Wenjie Zhang[1], Haibo Li[3], Chaoshen Zhang[1], and **Famei Li[2,*]**

[1]School of Pharmacy, Liaoning University of TCM, 79 Chongshan East Road, Shenyang, Liaoning Province, P.R. China, 110032; [2]School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, Liaoning Province, P.R. China, 110016; and [3]School of Basic Medical Sciences, Liaoning University of TCM, 79 Chongshan East Road, Shenyang, Liaoning Province, P.R. China, 110032

Abstract

A simple high-performance liquid chromatographic (HPLC) assay using the internal standard method is developed for the simultaneous determination of eight polyphenols. The analyzed compounds isolated from the leaves of *Crataegus pinnatifida* Bge. var. major include chlorogenic acid, vitexin-4″-O-glucoside, vitexin-2"-O-rhamnoside, vitexin, rutin, hyperoside, isoquercitrin, and quercetin. HPLC analysis is performed on a Diamonsil C18 analytical column (150 × 4.6 mm, i.d., 5-µm) using solvent (A) acetonitrile–tetrahydrofuran (95:5, v/v) and (B) 1% aqueous phosphoric acid as the mobile phase with UV absorption at 270 nm. The calibration curves of the eight polyphenols are linear (r^2 > 0.9992) over the concentration range of 0.0894–120.0 µg/mL. The mean recoveries are 95.4% to 98.1%. The results indicate that the HPLC method developed can easily be applied to the determination of eight polyphenols in the leaves of *Crataegus pinnatifida* Bge. var. major.

Introduction

The leaves of *Crataegus pinnatifida* Bge. var. major recorded in the Chinese Pharmacopoeia are a well-known traditional Chinese medicine (TCM) for the treatment of qi-stagnancy and blood stasis, chest distress, palpitation, loss of memory, dizziness, and tinnitus (1). Vitexin-4"-O-glucoside and vitexin-2"-O-rhamnoside, being as flavonoids glycoside, are main components of the leaves of *Crataegus pinnatifida* Bge. var. major (2). Recently, pharmacological studies also showed that vitexin-2"-O-rhamnoside strongly inhibits DNA synthesis in MCF-7 human breast cancer cells (3). There are some published reports of quantitation of seven flavonoid constituents including hyperin, quercetin, etc. in *Potentilla multifida* by high-performance liquid chromatographic (HPLC) method (4) and four flavonoid constituents including vitexin-2"-O-rhamnoside, hyperoside, rutin, and vitexin in *Crataegus pinnatifida* using capillary zone electrophoresis (CZE) method (5). HPLC (6,7) and ultra-performance liquid chromatography electrospray ionization tandem mass spectrometric (UPLC–ESI-MS–MS) methods (8) have been employed to investigate the pharmacokinetics of vitexin-2"-O-rhamnoside and vitexin-4"-O-glucoside. However, there are no published reports of measuring the eight polyphenols simultaneously in the leaves of *Crataegus pinnatifida* Bge. var. major by HPLC using internal standard method.

The aim of this study is to develop an assay to fully evaluate the contents of ployphenols in the leaves of *Crataegus pinnatifida* Bge. var. major from different locations and growth stages in order to exploit and utilize them reasonably.

Experimental

Plant material

Dried leaves of *Crataegus pinnatifida* Bge. var. major were collected weekly in Liaozhong, Liaoyang, and Shenyang (Liaoning, China) from May 20 to October 22, 2005. Voucher specimens were maintained at Liaoning University of Traditional Chinese Medicine, China.

Reagents

The eight standard substances of polyphenol were isolated from the leaves of *Crataegus pinnatifida* Bge. var. major including chlorogenic acid (CHA), vitexin-4"-O-glucoside (VOG), vitexin-2"-O-rhamnoside (VOR), vitexin (VIT), rutin (RUT), hyperoside (HYP), isoquercitrin (ISOQ), and quercetin (QUE). Baicalin, the internal standard (IS), was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), and the water used in all experiments was purified by a Milli-Q Ultrapure Water System (Millipore, MA). All other chemicals were of analytical reagent grade purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

* Author to whom correspondence should be addressed.

Chromatographic system and conditions

HPLC analysis was carried out on an Agilent 1100 series HPLC (Palo Alto, CA) incorporating a UV detector. The analytes were determined at room temperature on an analytical column (Diamonsil C18, 150 × 4.6 mm, i.d., 5-µm particle size) (Dikma Technologies, Beijing, China). The mobile phase consisted of the solvent (A) acetonitrile–tetrahydrofuran (95:5, v/v) and (B) 1% aqueous phosphoric acid (v/v) using a gradient elution of 13–18% (A) at 0–11 min, 18–19% (A) at 11–25 min, 19–20% (A) at 25–30 min, 20–22% (A) at 30–35 min, 22–25% (A) at 35–40 min, 25–28% (A) at 40–45 min, 28–30% (A) at 45–50 min, 30–32% (A) at 50–55 min, 100% (A) at 55–65 min, and then returned to initial condition for a 5 min re-equilibration, with total run time 70 min. The mobile phase was passed under vacuum through a 0.45-µm membrane filter before use. The analysis was carried out at a flow rate of 1 mL/min with the detection wavelength set at 270 nm.

Sample preparation

To a conical flask with lid, 0.2 g of the dried and powdered leaves of *Crataegus pinnatifida* Bge. var. major and 0.3 mL of IS were added, then extracted with 20 mL of a mixture of methanol–water (50:50, v/v) in an ultrasonic bath for 30 min. The supernatants were filtered with 0.45-µm membrane filter to obtain the filtered solution, and an aliquot (20 µL) of filtrate was injected into the HPLC system. Analysis was carried out through the internal standard method.

Extract and separation

A sample of the leaves (3.0 kg) of *Crataegus pinnatifida* Bge. var. major, collected from Liaozhong (Liaoning, China) on October 8, 2005, was cut in small pieces and refluxed with 70% ethanol (3 × 24 L, 2 h for each). After removal of the solvent under reduced pressure, the crude extract was concentrated, then adsorbed on a porous-polymer resin (AB-8, Tianjin, China) column (Φ20 × 150 cm), initially, eluted with 5 L water to eliminate the impurity, then eluted with 15 L 70% ethanol for total polyphenols. The fraction of 70% ethanol eluant was evaporated under reduced pressure then partitioned with chloroform and *n*-butanol successively. The fraction of *n*-butanol dried to afford the crude extract (150 g), which was chromatographed on a silica gel (280–300 mesh) column (Φ12 × 200 cm), and eluted with chloroform–methanol (50:1, 25:1, 10:1, 4:1, 2:1) in gradient. Fractions of similar composition were pooled on the basis of TLC analysis (UV monitoring at 254 nm and 365 nm) to afford F_1–F_{10}, and each fraction was repeatedly subjected to silica gel column (Φ2 × 100 cm) chromatography and eluted with ethyl acetate–butanone–formic acid–water (4:3:1:1) to obtain eight compounds (> 99.0% purity checked by HPLC) including 1 (QUE), 2 (ISOQ), 3 (HYP), 4 (RUT), 5(VIT), 6 (VOR), 7 (VOG), and 8 (CHA). The structures of eight polyphenols were fully characterized by ^{1}H-NMR, ^{13}C-NMR, and ESI-MS compared with the data in the literature (2).

Method validation

Stock standard solutions of the eight polyphenols and IS were prepared with methanol. The concentrations of CHA, VOG, VOR, VIT, RUT, HYP, ISOQ, QUE, and IS were 0.0772, 2.00, 1.856, 0.332, 0.388, 0.342, 0.398, 0.0894, and 0.24 mg/mL, respectively. Six calibrators of the eight polyphenols with IS were prepared by dilution of stock solutions. The calibration curves for the eight polyphenols were generated by plotting the peak area ratio of the eight polyphenols to IS versus the nominal concentrations. The regression equation was obtained by weighted ($1/c^2$) least-square linear regression. The limit of detection (LOD) was determined in signal to noise ratio (S/N) of 3:1, and the lower limit of quantitation (LOQ) was determined in S/N of > 10. The acceptable accuracy (RE) within ± 20% and a precision (RSD) not exceeding 20% should be obtained.

The recovery was determined by adding known amounts of the standard substance of polyphenols, approximately 1 times the levels detected in unspiked samples prior to extraction, and these spiked samples were prepared as described in the "Sample preparation" section. The found amount of polyphenol subtracted

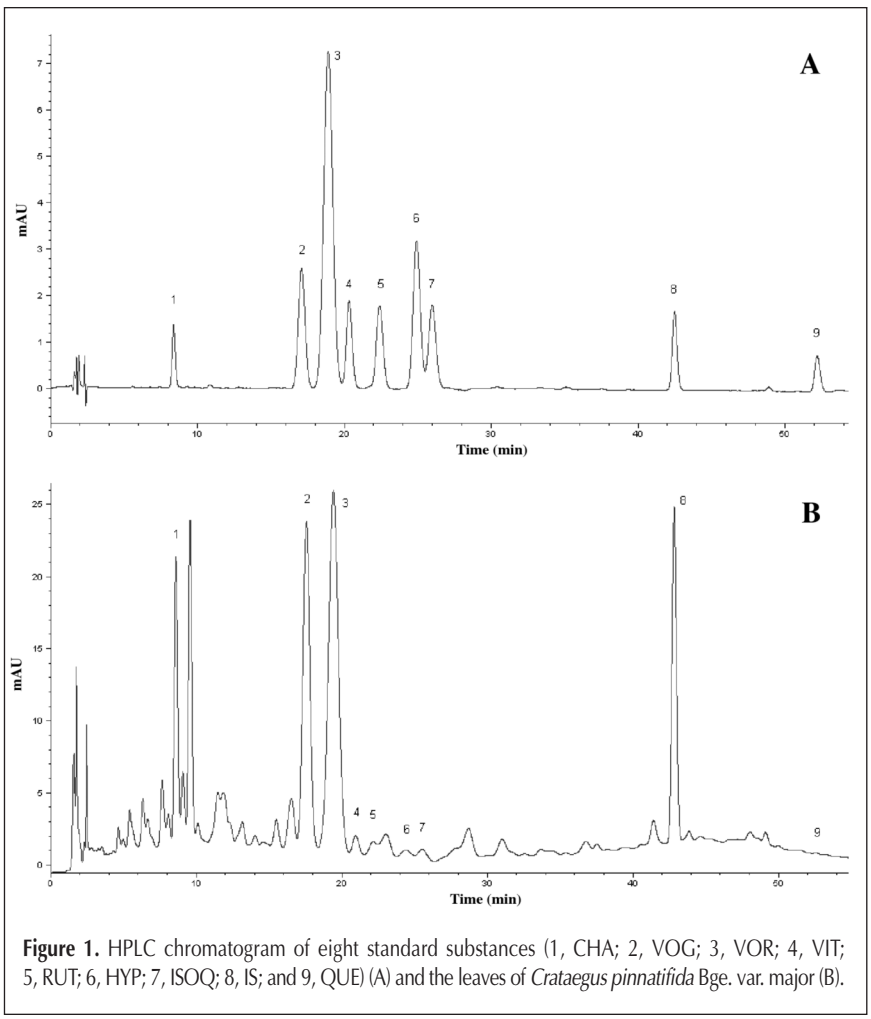

Figure 1. HPLC chromatogram of eight standard substances (1, CHA; 2, VOG; 3, VOR; 4, VIT; 5, RUT; 6, HYP; 7, ISOQ; 8, IS; and 9, QUE) (A) and the leaves of *Crataegus pinnatifida* Bge. var. major (B).

from that of unspiked sample was divided by the added known amount of the standard substance of polyphenol, and the extraction recovery was calculated as a percentage.

The stabilities of the eight polyphenols in the mixture of methanol–water (50:50, v/v) were investigated by comparing the peak area ratio of the eight polyphenols to IS at 0, 6, 12, 18, and 24 h.

Results and Discussion

HPLC analysis

Our attempts to use the method with isocratic elution for the determination of eight polyphenols were unsuccessful. The gradient elution method was, therefore, used for the separation of the polyphenols. During method development, the mobile phase composition varied using different combinations of methanol, tetrahydrofuran, and phosphoric acid. It demonstrated a longer retention time with a decrease in the organic composition. Here, the mobile phase consisting of (A) 95% methanol and 5% tetrahydrofuran, and (B) 1% phosphoric acid was chosen to achieve good peak shape, satisfactory resolution, and relatively short analysis time. Figure 1 shows typical chromatograms of the standard substances with IS (A), and the leaves of *Crataegus pinnatifida* Bge. var. major with IS (B). The retention times of CHA, VOG, VOR, VIT, RUT, HYP, ISOQ, IS, and QUE were approximately 8.4, 17.0, 19.9, 20.3, 22.4, 24.8, 25.6, 42.5, and 52.2 min, respectively, and the total chromatographic run time was 70 min.

Simultaneous determination of the eight polyphenols was carried out with the IS method to prevent possible errors brought on by changes of injection volume. Baicalin, as it is commercially available and has the similar property and structure to polyphenols, was chosen as the IS, the separation of which offered baseline resolution with adjacent peaks. IS was found with the maximum absorption at 280 nm from the spectrophotometry. If the detector was set at 280 nm, the absorption of Baicalin was slightly weak, and CHA was nearly not detected; in addition, HYP and ISOQ were not well separated from adjacent interference peaks. Finally, a detection wavelength of 270 nm was chosen in the assay and was suitable for the simultaneous determination of the eight polyphenols.

Initially, the absolute methanol was used as the extraction solvent, but CHA was not found when observing the chromatogram. After decreasing the organic composition, the extraction recovery of QUE was not acceptable. Thus, a mixture of methanol–water (50:50, v/v) was chosen as the extraction solvent. A simple ultrasonic method was used for sample preparation, which was timesaving and may not lead to losses of polyphenols. The sample clean-up steps to a minimum was a primary concern; thus, the filtrate, after ultrasonication, was directly analyzed to decrease assay variability, apart from RUT, which had slight interference from the adjacent peaks, all other polyphenols showed good resolution and high recovery.

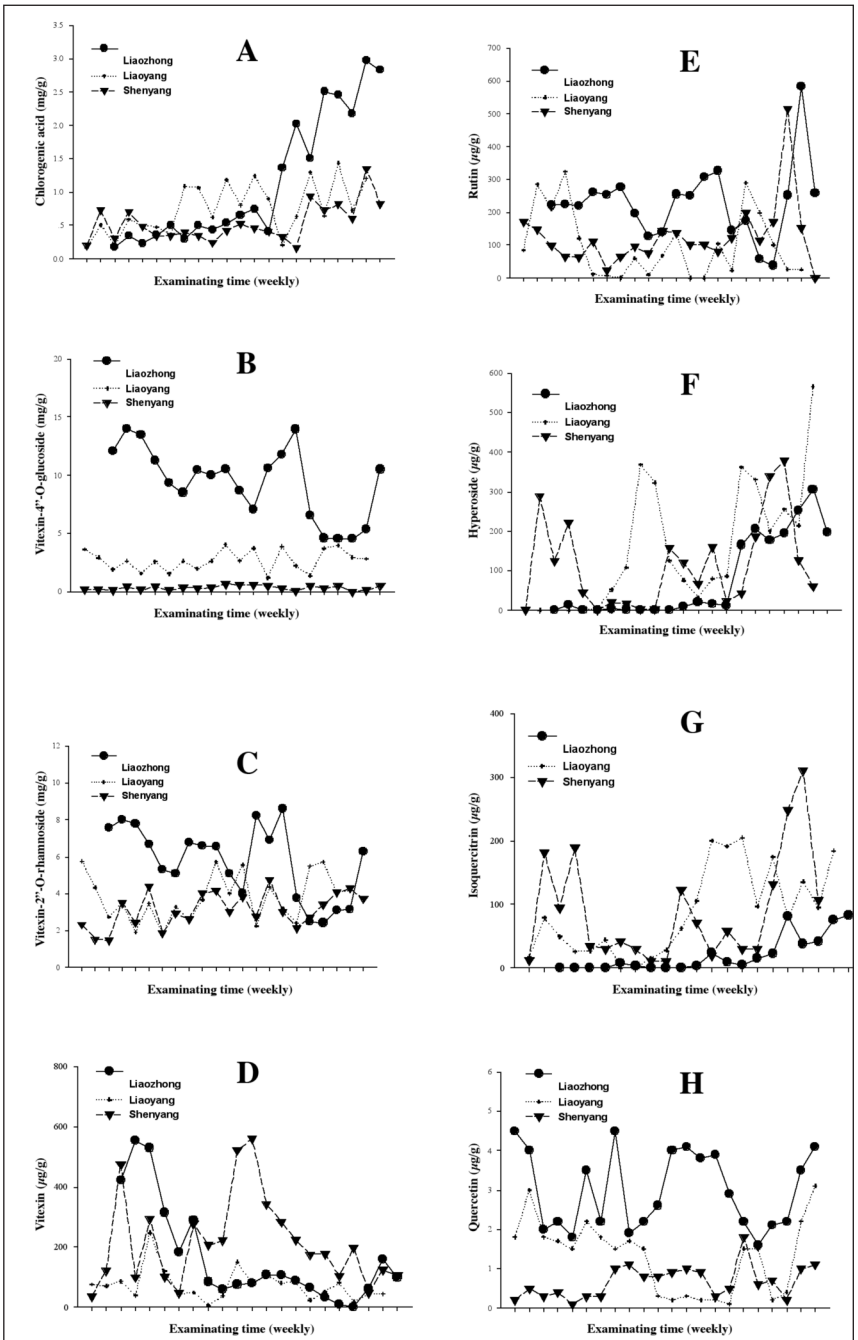

Figure 2. Content trendgraphs of chlorogenic acid, vitexin-4''-O-glucoside, vitexin-2''-O-rhamnoside, vitexin, rutin, hyperoside, isoquercitrin, and quercetin in the leaves of *Crataegus pinnatifida* Bge. var. major from different locations (Liaozhong, Liaoyang, and Shenyang) and growth stages from May 20 to October 22.

Method validation

Linearity, LLOQ, and LOD

The eight calibration curves in the concentration ranges of CHA, VOG, VOR, VIT, RUT, HYP, ISOQ, and QUE were 0.0965–19.3, 1.2–120.0, 3.712–371.2, 0.1992–19.92, 0.97–97.00, 0.3078–30.78, 0.597–59.70, and 0.0894–4.469 µg/mL, respectively. The calibration curve was constructed using six different concentrations by plotting the peak area versus the polyphenolic concentration. The regression equations and coefficients were: $y = 0.0728x + 0.0081$, $r = 0.9993$; $y = 0.02x + 0.0222$, $r = 0.9997$; $y = 0.0543x + 0.0727$, $r = 0.9995$; $y = 0.0622x - 0.0004$, $r = 0.9992$; $y = 0.0304x + 0.0123$, $r = 0.9996$; $y = 0.0574x + 0.0145$, $r = 0.9993$; $y = 0.0484x + 0.0147$, $r = 0.9997$; $y = 1.8444x + 0.0621$, $r = 0.9995$, respectively, where y is the peak area ratio of polyphenol to IS, and x is the concentration of polyphenol.

The LOD of the eight polyphenols were 0.0233, 0.320, 0.374, 0.0498, 0.188, 0.0864, 0.102, and 0.0162 µg/mL, and the lower limit of quantification (LLOQ) of the eight polyphenols, defined as the lowest concentration on the calibration curve, were 0.0965, 1.2, 3.712, 0.1992, 0.97, 0.3078, 0.597, and 0.0894 µg/mL, with precision and accuracy within 20% verified by repeated analysis.

Recovery

For a validation of the extraction recoveries of the eight polyphenols, the analyisis for each polyphenol was carried out in six replicates. The results showed that the mean extraction recoveries were acceptable, suggesting that there was negligible loss during the leaves extraction (Table I).

Stability

The stock solutions of the eight polyphenols and IS were found to be stable at room temperature based on a comparison of the peak area ratio of the eight polyphenols to IS over the time range of 0–24 h. The stabilities of the eight polyphenols in the solution of a mixture methanol–water (50:50, v/v) were investigated during the storing period of 0–24 h. The eight polyphenols were considered to be stable after the samples were treated as in the "Sample preparation" section under the experimental conditions of the regular analytical procedure (Table II).

Simultaneously determine eight polyphenols

HPLC incorporating UV detector using the IS method was employed to simultaneously determine the eight polyphenols in the leaves of *Crataegus pinnatifida* Bge. var. *major*. Considering the output of the leaves, May 20 to October 22 (2005) was taken as the collecting time. The dried and powdered leaves were treated as in the "Sample preparation" section. The results indicated the variation of the eight polyphenols in the leaves from different locations and growth stages (Figure 2). Figure 2H showed that the highest content 4.5 µg/g of QUE existed in the leaves of Liaozhong at July 11, which was obviously low among the eight polyphenols. The contents of VIT, RUT, HYP, and ISOQ were over the concentration range from 562–309 µg/g in Figure 2D–2G, and the optimum collecting time was at October 4–16, apart from that of VIT, which ranged from August 4–11. Figures 2A–2C indicated that CHA, VOG, and VOR were the main components of the leaves, in which the highest contents of them were 2.97 mg/g (October 16), 14.3 mg/g (June 7, September 13, October 22), and 13.5 mg/g of VOR (August 4,19, October 16), respectively, and the data demonstrated that the optimum collecting time of the leave in Liaozhong ranged from October 16–22. VOG and VOR varied insignificantly, being the main components of the leaves of *Crataegus pinnatifida* Bge. var.

Table I. Results of Recovery Experiments (%)

NO.	CHA	VOG	VOR	VIT	RUT	HYP	ISOQ	QUE
1	97.8	95.3	98.1	98.1	96.3	98.5	95.1	95.0
2	98.2	97.8	97.6	97.9	96.5	98.1	95.3	95.1
3	98.1	98.0	97.6	97.3	95.7	97.4	95.4	95.1
4	98.1	99.0	97.3	97.1	96.1	97.3	95.4	95.8
5	97.5	95.3	98.0	98.0	95.9	97.9	95.1	95.1
6	98.8	98.8	99.6	99.9	98.5	99.1	97.3	96.1
X ± SD	98.1 ± 0.436	97.4 ± 1.67	98.0 ± 0.821	98.1 ± 0.991	96.5 ± 1.02	98.1 ± 0.680	95.6 ± 0.844	95.4 ± 0.463
RSD (%)	0.44	1.7	0.84	1.0	1.1	0.69	0.88	0.49

Table II. Results of Stability Experiments

Time (h)	A_{CHA}/A_{IS}	A_{VOG}/A_{IS}	A_{VOR}/A_{IS}	A_{VIT}/A_{IS}	A_{RUT}/A_{IS}	A_{HYP}/A_{IS}	A_{ISOQ}/A_{IS}	A_{QUE}/A_{IS}
0	0.0783	2.81	9.39	0.166	0.297	0.571	0.280	0.0378
6	0.0801	2.82	9.43	0.169	0.298	0.576	0.277	0.0390
12	0.0807	2.83	9.43	0.169	0.297	0.562	0.277	0.0390
18	0.0800	2.83	9.43	0.160	0.297	0.562	0.281	0.0377
24	0.0812	2.83	9.44	0.168	0.291	0.568	0.272	0.0391
x ± SD	0.0801 ± 0.0011	2.82 ± 0.089	9.42 ± 0.019	0.166 ± 0.0034	0.296 ± 0.0028	0.568 ± 0.0060	0.277 ± 0.0035	0.0385 ± 0.00072
RSD (%)	1.4	0.32	0.21	2.1	0.96	1.1	1.2	1.9

major, and can be taken as the indices to control the quality of the leaves. However, VOG varied significantly from different locations, suggesting that VOG cannot be used as the index in the quality control of the preparation. Therefore, the contents of the eight polyphenols in the leaves from different locations and growth stages must be considered when we exploit and utilize the leaves.

Conclusion

A simple HPLC method using the IS was developed for the simultaneous determination the eight polyphenols in the leaves of *Crataegus pinnatifida* Bge. var. major. The linearity, recovery and stability of the developed method were validated, respectively. From the results of this study, we know that the contents of polyphenols of the leaves of *Crataegus pinnatifida* Bge. var. major varied from different locations and growth stages. In a word, the results can be taken as the evidence for the exploitation and utilization of the leaves reasonably.

Acknowledgments

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References

1. The Pharmacopoeia Commission of PRC. The Pharmacopoeia of the People's Republic of China. Part I. Chemical Industry Publishing House, Beijing, China, 2005, p. 22.
2. R.H. Liu and B.Y. Yu. Study on the chemical constituents of the leaves from *Crataegus pinnatifida* Bge. var. major. *N.E. Br. J. Chinese Med. Mater.* **29:** 1169–73 (2006).
3. P. Ninfali, M. Bacchiocca, A. Antonelli, E. Biagiotti, A.M. Di Gioacchino, G. Piccoli, V. Stocchi, and G. Brandi. Characterization and biological activity of the main flavonoids from Swiss chard (*Beta vulgaris* subspecies cycla). *Phytomedicine*. **14:** 216–22 (2007).
4. P.F. Xue, Y.Y. Zhao, B. Wang, and H. Liang. Simultaneous determination of seven flavonoids in *Potentilla multifida* by HPLC. *J. Chromatogr. Sci.* **45:** 216–19 (2007).
5. W.M. Liu, G.H. Chen, and T. Cui. Determination of flavones in *Crataegus pinnatifida* by capillary zone electrophoresis. *J. Chromatogr. Sci.* **41:** 87–91 (2003).
6. G. Ma, X.H. Jiang, Z. Chen, J. Ren, C.R. Li, and T.M. Liu. Simultaneous determination of vitexin-4-O-glucoside and vitexin-2"-O-rhamnoside from hawthorn leaves flavonoids in rat plasma by HPLC method and its application to pharmacokinetic studies. *J. Pharm. Biomed.* **44:** 243–49 (2007).
7. X.X. Ying, S. Gao, W.L. Zhu, Y.J. Bi, F. Qin, X.Q. Li, and F.M. Li. High-performance liquid chromatography determination and pharmacokinetics study of vitexin-2″-O-rhamnoside in rat plasma after intravenous administration. *J. Pharmaceut. Biomed.* **44:** 802–806 (2007).
8. X.X. Ying, X.M. Lu, X.H. Sun, X.Q. Li, and F.M. Li. Determination of vitexin-2″-O-rhamnoside in rat plasma by ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry and its application to pharmacokinetic study. *Talanta*. **72:** 1500–1506 (2007).

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