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Simultaneous Determination of Three Quassinoids in *Brucea javanica* by High Performance Liquid Chromatography

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Abstract

Objective: To establish a method for quantitative determination of three quassinoids (bruceoside B, bruceoside A and brusatol) in *Brucea javanica* by HPLC.

Method: The determination was carried out on a Cosmosil (4.6×250 mm, 5 μm) C_{18} column eluted by a gradient elution program of H_2O (A)-CH_3OH (B) at a flow rate of 1.0 mL min^{-1}, and the detection wavelength was 221 nm.

Result: The calibration curve of bruceoside B, bruceoside A and brusatol were linear in the range of 0.722-2.166, 2.074-6.222 and 0.503-1.509 μg, respectively with all the correlations of 0.9999. The average recovery of bruceoside B, bruceoside A and brusatol were 96.1%, 106.3% and 96.7%, with the RSD of 4.4%, 5.9% and 4.8% (n=5), respectively.

Conclusion: The results showed that the content of bruceoside B was in the range of 0.05%~0.12%, bruceoside A was in the range of 0.19%~0.38% and brusatol was in the range of 0.07%~0.18%, indicating that the contents of bruceoside B, bruceoside A and brusatol varied among different sources. Furthermore, the fingerprinting chromatograms of eight samples of *Brucea javanica* were established and the characteristic peaks were identified by mass spectrometry. The method was accurate, simple and convenient, which could be used for the quality control of *Brucea javanica*.

Keywords: *Brucea javanica*; HPLC; Quassinoids; Quantitative; Fingerprint

Introduction

Brucea javanica (L.) Merr. is an evergreen shrub from Simaroubaceae family distributing from southeast Asia to northern Australia. The dried ripe fruits or seeds of this plants ("Ya-Dan-Zi" in Chinese) were used as a Traditional Chinese Medicine (TCM) since Ming Dynasty (1364-1644 AD) [1]. The fruit of this herb was currently recorded in the Pharmacopoeia of the People's Republic of China (2010 edition) for removing fever and toxicity [2].

Modern pharmacological studies showed that this herb has antitumor, anti-inflammation, and anti-malaria activities and could also be used to treat amoebic dysentery [3-5]. Phytochemical studies revealed that the major ingredients include quassinoids, e.g. bruceosides A-G, bruceines A-H and brusatol), triterpenoids, e.g. bruceajavanin A bruceajavanin B, dihydrobruceajavanin A), and fatty or other organic acids, e.g. crotonic acid, cis-oleic acid, azelaic acid and vanillic acid. In addition, some minor alkaloids were also reported, e.g. 5-methoxycanthin-6-one, 11-hydroxycanthin-6-one and canthin-6-one) [6-10].

Among the above three kind of ingredients (quassinoids, fatty acids and alkaloids), quassinoids were reported to show significant antitumor, anti-malarial and anti-amoebic activities, which were consistent with the function of this herb. Thus quassinoids were considered as the characteristic and active components of *Brucea javanica*. For example, early reports showed that bruceoside B, bruceoside A and brusatol had anti-leukemia activities [11,12]. Furthermore, brusatol was reported to show antimalarial activities, and could be used to sensitize the activity of chemotherapy [13]. Though content determinations of some quassinoids were reported [14], simultaneous determinations on bruceoside B, bruceoside A and brusatol were not reported. Furthermore, bruceoside B, bruceoside A and brusatol were the major active ingredients in *Brucea javanica*. Accordingly, we reported herein the simultaneous determination of these major quassinoids in *Brucea javanica*, and provided a quality control method for this herb, which was similar to the method reported for Radix Stemonae [15].

Materials and Methods

Instrumentation and materials

Agilent 1200 HPLC system (calibrated by the Analytical and Testing Center of Jinan University) coupled with a C18 column (Cosmosil 4.6×250 mm, 5 μm). Reference compounds bruceoside B, bruceoside A and brusatol were prepared by our lab, and identified through NMR and LC-MS analyses. Their purities were over 98% by HPLC analysis. The solvent methanol and water were chromatographic grade, and other reagents were analytical grade.

Method development and validation

Chromatographic conditions: C_{18} column (Cosmosil 4.6×250 mm, 5 μm); mobile phase H_2O (A)-CH_3OH (B), gradient system: 0~10 min, 15%→35% B, 10~30 min, 35%→45% B, 30~36 min and 45%→48% B, flow rate 1.0 mL/min, detection wavelength 221 nm, column temperature 29°C. According to the above chromatographic conditions, 10 μL of the mixed reference substance (Bruceoside B, bruceoside A and brusatol) and the test solution were separately injected. All three

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reference peaks could be identified in the chromatogram of the test solution by comparison of the retention time and the online UV spectra Figure 1.

Preparation of testing solution: Weigh accurately 1.0000 g powder of *B. javanica*, and extract three times with methanol under ultrasonic conditions. The solutions were combined and concentrated under reduced pressure. The obtained extract was dissolved in a 10 mL volumetric flask with methanol. Finally, the methanol solution was filtered with a 0.45 μm membrane

Preparation of reference solution: The three reference compounds brusatol 5.0300 mg, bruceoside A 20.7400 mg and bruceoside B 7.2200 mg were precisely weighed, respectively. All three compounds were mixed and dissolved with methanol in a 50 mL volumetric flask.

Selection of UV wavelength: DAD analysis of the three components showed that the maximum absorption occurred at 221 nm. So the detection wavelength was set at 221 nm.

Limits of quantification and detection: When the signal-to-noise ratio is 3, the detection limits of bruceoside B, bruceoside A, and brusatol are 4.3, 3.6, and 2.9 ng/mL, respectively. When the signal-to-noise ratio is 10, the limits of quantitation of the three components are 12.2, 10.4 and 9.1 ng/mL, respectively.

Linearity and range: The concentrations of brusatol, bruceoside A and bruceoside B in the mixed reference solution were 0.1000, 0.4150 and 0.1444 g/L respectively. A certain volume was (5, 7.5, 10, 12.5 and 15 μL) accurately injected into HPLC, and the peak area of these compounds in each chromatogram was calculated. The injection amount (μg) was plotted on the abscissa (X) and the peak area of the chromatogram was plotted on the ordinate (Y). The results are shown in Table 1.

Reference Solution	Linearity range /μg	Equation of linear regression	R^2	Limit of detection (ng/mL)	Limit of quantitation (ng/mL)
Bruceoside B	0.722-2.166	Y=1194X-1.56	0.9999	4.3	12.2
Bruceoside A	2.074-6.222	Y=1296.2X + 9.76	0.9999	3.6	10.4
Brusatol	0.503-1.509	Y=1717.3X + 0.08	0.9999	2.9	9.1

Table 1: Standard regression equations and linear ranges of Bruceoside B, Bruceoside A and Brusatol.

Precision: Bruceoside A was continuously injected 5 times (10 μL), and the contents and RSD were calculated. The results show that the RSD of bruceoside A content was 0.44%, and RSD of retention time was 0.12%, indicating that the good precision of the instrument Table 2.

Stability: The test solution of sample S3 (10 μL) was injected into HPLC at 0, 6, 12, 18 and 24 h, respectively. The peak area of bruceoside A was determined, and the content was calculated. The results were shown in Table 3. The RSD of the bruceoside A content was 0.52%, and the retention time RSD was 0.08%. The experimental results indicated

Injection Sequence	Content (Bruceoside A/μg)	Retention Time (Bruceoside A)
1	3.6109	28.658
2	3.5892	28.575
3	3.6251	28.652
4	3.6156	28.631
5	3.5904	28.643
RSD	0.44%	0.12%

Table 2: Precision tests.

Injection Sequence	Content (Bruceoside A/μg)	Retention Time (Bruceoside A/min)
1	3.6288	28.857
2	3.6071	28.813
3	3.5803	28.829
4	3.6183	28.871
5	3.6193	28.847
RSD	0.52%	0.08%

Table 3: Stability tests

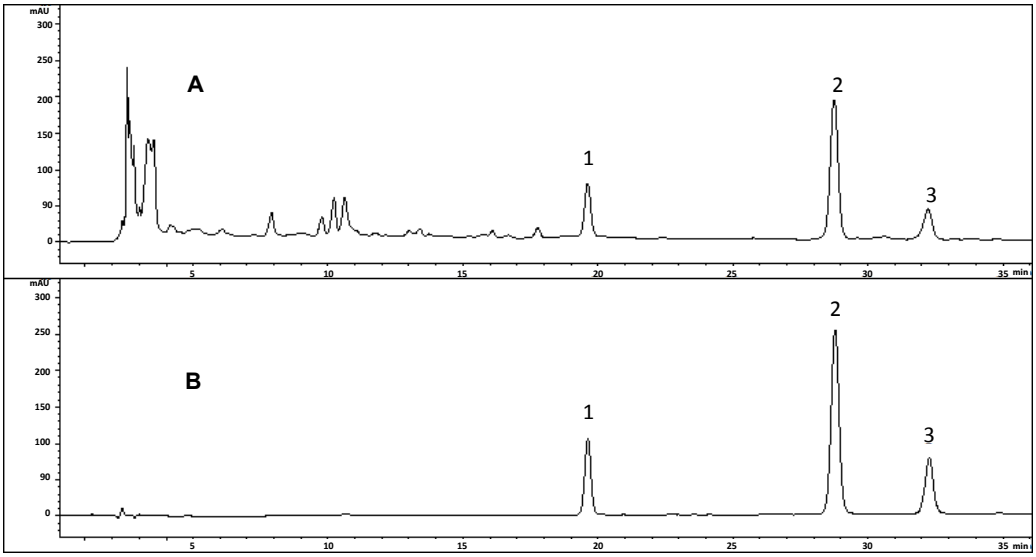

Figure 1: HPLC chromatograms (0-36 min) of the test solution of *Brucea javanica* (A) and Reference standards (B). Reference standards: 1) Bruceoside B; 2) Bruceoside A; 3) Brusatol.

that samples were stable in 24 h.

Reproducibility: Five samples from the same batch S3 were carefully weighted (each 1.0000 g). The test solution was prepared according to the method under "2.2" and 10 μL was injected. The contents of bruceoside A in these sample was determined and found to be 3.6188, 3.4618, 3.5295, 3.5267 and 3.6464 μg. RSD for the contents was 2.11% and RSD for the retention time was 0.04. The results were shown in Table 4. The experiment results indicated that the good reproducibility of this method.

Recovery: Six samples of *Brucea javanica* powder (S3), each about 0.5 g, were accurately weighted and extracted using the same method as section 2.2. Appropriate amount of reference solution was added. 10 μL of each sample was injected into the HPLC system. The peak areas of the three reference components were recorded, and the contents of these compounds were calculated. The average recovery rates of bruceoside B, brusatol and bruceoside A were 96.1%, 106.3% and 96.7%, and RSD were 4.4%, 5.9% and 4.8%, respectively. The results were shown in Tables 5-7.

Fingerprinting analysis: The testing solution was prepared according to section 2.2. The HPLC analysis conditions were as follow: Cosmosil (4.6x250 mm, 5 μm) C_{18} column, mobile phase H_2O (A)-CH_3OH (B), gradient system (0-15 min, 15%→30% B; 15~30 min, 30%→45%B; 30~50 min, 45%→60%), flow rate1.0 mL min^{-1}, detection wavelength 254 nm, injection volume 10 μL. Altogether eight samples of *Brucea javanica* were tested, and their fingerprinting chromatograms were shown in Figure 2. Some characteristic peaks were prepared and

Injection Sequence	Content (Bruceoside A/μg)	Retention time (Bruceoside A/min)
1	3.6188	28.464
2	3.4618	28.441
3	3.5295	28.450
4	3.5267	28.441
5	3.6464	28.467
RSD	2.11%	0.04%

Table 4: Method repeatability tests.

Sampling quantity/g	Sample content/mg	Addition/mg	Determination/mg	Recovery/%	Average/%	RSD/%
0.5072	0.4061	0.4052	0.7796	92.1	96.1	4.4
0.5041	0.4032	0.4053	0.7969	97.1		
0.5062	0.4052	0.4053	0.8234	103.1		
0.5093	0.4071	0.4052	0.7932	95.2		
0.5072	0.4053	0.4051	0.7812	92.7		

Table 5: Recovery rate of bruceoside B.

Sampling quantity/g	Sample content/mg	Addition/mg	Determination/mg	Recovery/%	Average/%	RSD/%
0.5072	0.6092	0.6051	1.2082	98.9	106.3	5.9
0.5041	0.6051	0.6052	1.2805	111.5		
0.5062	0.6072	0.6051	1.2438	105.2		
0.5093	0.6113	0.6052	1.2322	113.2		
0.5072	0.6082	0.6053	1.2305	102.8		

Table 6: Recovery rate of brusatol.

Sampling quantity/g	Sample content/mg	Addition/mg	Determination/mg	Recovery/%	Average/%	RSD/%
0.5073	1.8252	1.8241	3.5873	96.6	96.7	4.8
0.5041	1.8153	1.8262	3.6816	102.1		
0.5062	1.8201	1.8252	3.6525	100.3		
0.5092	1.8332	1.8274	3.4776	89.9		
0.5073	1.8242	1.8263	3.5483	94.4		

Table 7: Recovery rate of bruceoside A.

Peaks	Retention time	$[M+Na]^+$	$[2M+Na]^+$	$[M-H]^-$	$[2M-H]^-$	Identification
2	11.52	433.3	843.2	-	-	bruceine D
4	23.18	-	-	285.3	570.9	Luteolin
7	26.22	705.4	1387.3	-	-	bruceoside B
9	33.65	705.5	1387.3	-	-	bruceoside A
10	36.90	543.2	1063.0	-	-	brusatol
11	40.13	707.4	1391.3	-	-	yadanzioside A
12	41.85	791.4	1559.4	-	-	yadanzioside G
Note: "-" means not found.						

Table 8: Identification of some of the peaks in the chromatograms of *Brucea javanica*.

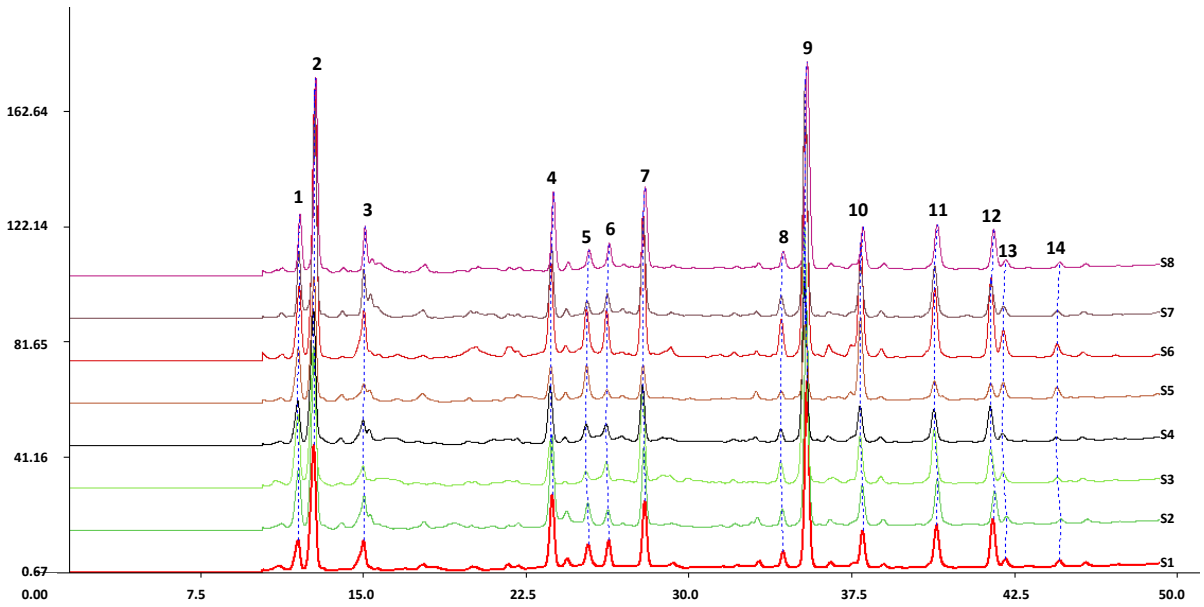

Figure 2: Fingerprinting analysis of *Brucea javanica*.

Batches	Bruceoside B	Brusatol	Bruceoside A
S1	0.08 ± 0.006	0.12 ± 0.006	0.36 ± 0.014
S2	0.12 ± 0.010	0.14 ± 0.008	0.34 ± 0.018
S3	0.06 ± 0.003	0.07 ± 0.004	0.31 ± 0.013
S4	0.09 ± 0.007	0.11 ± 0.005	0.28 ± 0.012
S5	0.10 ± 0.006	0.18 ± 0.009	0.19 ± 0.011
S6	0.10 ± 0.004	0.12 ± 0.007	0.38 ± 0.016
S7	0.05 ± 0.002	0.08 ± 0.005	0.29 ± 0.017
S8	0.07 ± 0.005	0.13 ± 0.008	0.30 ± 0.015
S9	0.09 ± 0.006	0.12 ± 0.007	0.29 ± 0.017
S10	0.08 ± 0.004	0.10 ± 0.005	0.33 ± 0.014
S11	0.10 ± 0.006	0.11 ± 0.004	0.35 ± 0.018
S12	0.11 ± 0.005	0.14 ± 0.007	0.36 ± 0.015

Table 9: Results of content determination of twelve samples (n=3).

analyzed by mass spectrometry using either positive mode or negative mode. The identification of these peaks was shown in Table 8.

Results

Twelve batches of medicinal materials were purchased or collected from different regions of the country and treated separately according to the method of preparation of test solutions (section 2.2). The test solution (10 μL) for each sample was injected into the HPLC. The measurement was carried out according to the above chromatographic method (section 2.1), and the operation was performed in parallel three times. The contents were calculated using the external standard method. The results are shown in Table 9.

From the fingerprinting chromatograms Figure 2, it could be seen that all eight samples of *B. javanica* showed similar patterns with 14 common peaks. Peaks 2, 4, 7, 9, 10, 11 and 12 were further identified by mass spectrometry (positive mode: $[M+Na]^+$and $[2M+Na]^+$ or negative mode: $[M-H]^-$ and $[2M-H]^-$) as bruceine D, Luteolin, bruceoside B, bruceoside A, brusatol, yadanzioside A, yadanzioside G, respectively.

Discussion

DAD analysis was carried out on three reference substances (bruceoside B, bruceoside A and brusatol), which showed that the maximum absorption occurred at 221 nm. So the detection wavelength was set at 221 nm. Using the gradient elution program of method-water, three quassinoids can be completely separated.

In this paper, the method of ultrasonic extraction with methanol was used to prepare the test solution of *B. javanica*. Then we established a method for quantitative determination of three quassinoids (bruceoside B, bruceoside A and brusatol) in *B. javanica* by HPLC. The analytical method was simple, quick, and showed good reproducibility, and the chromatographic peaks of the measured components are well separated from other chromatographic peaks. Therefore, this method can be used as one of the quality control methods of *B. javanica*.

Conclusion

The contents of bruceoside B, bruceoside A and brusatol in 12 bath of *B. javanica* were simultaneously determined. The results showed that the content of bruceoside B was in the range of 0.05~0.12%, bruceoside A was in the range of 0.19%~0.38% and brusatol was in the range of 0.07%~0.18%, indicating that the contents of bruceoside B, bruceoside A and brusatol varied among different sources. Bruceoside B bruceoside A and brusatol were the main active components, and their pharmacological activities were consistent with those of *B. javanica*. Furthermore, the fingerprinting chromatograms of eight samples of *Brucea javanica* were established which showed similar patterns with 14 common peaks. Peaks 2,4,7,9,10,11 and 12 were identified by

mass spectrometry. Accordingly, the simultaneous determination of these three components in *B. javanica* and the fingerprinting analysis provided a useful method for the quality control of *B. javanica.*

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