Quantitative Study of Multiple Components in *Tetracera asiatica* Based on High-Performance Liquid Chromatography

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Abstract [**Objectives**] To establish an HPLC method for the quantitative determination of multiple phenolic acid components in *Tetracera asiatica* medicinal material, providing a basis for establishing its quality standards. [**Methods**] An Inertsil ODS-C_{18} column (250 mm×4.6 mm, 5 μm) was used. The mobile phase consisted of acetonitrile-0.2% phosphoric acid solution (10∶90). The flow rate was 1.0 mL/min. The detection wavelength was 274 nm. The column temperature was 25 ℃. The injection volume was 10 μL. The content of three components, gallic acid, protocatechuic acid, and protocatechualdehyde, was determined in 13 batches of *T. asiatica*. [**Results**] Gallic acid showed good linearity within the range of 0.020 – 6.400 μg/mL, protocatechuic acid within 0.201 – 6.432 μg/mL, and protocatechualdehyde within 0.202 – 6.464 μg/mL (r > 0.999 0). The average recovery rates ranged from 98.61% to 101.17%, with *RSD*s between 1.21% and 2.69%. [**Conclusions**] The quantitative determination method established in this study is simple and feasible, and can provide a basis for the quality evaluation of *T. asiatica*.

Key words *Tetracera asiatica*, High-performance liquid chromatography (HPLC), Components, Quantitative determination

0 Introduction

Tetracera asiatica originates from the roots, stems, and leaves of the Dilleniaceae plant *Tetracera asiatica* (Lour.) Hoogland. It has the effects of astringency, arresting prolapse, reducing swelling, and relieving pain. It is used to treat chronic diarrhea and dysentery, rectal prolapse, traumatic swelling, and pain[1]. It is mainly distributed in tropical and subtropical regions such as Guangxi, Guangdong, and Yunnan, growing in sparse forests and shrublands at low-altitude mountainous areas[2]. Modern research indicates that it contains components such as flavonoids, phenolic acids, and alkaloids[3-5], and exhibits effects including antioxidant[6], antimalarial[7], antibacterial[8], hepatoprotective[9], and anti-HIV activity[10]. *T. asiatica* is widely used among ethnic minorities in regions such as Guangxi, Guangdong, and Hainan. Zhuang medicine often uses its roots and vine stems as medicine. Currently, *T. asiatica* is included in *Guangxi Zhuang Autonomous Region Zhuang Medicine Quality Standards* (Volume II)[11]. However, the standard content is simple and lacks provisions for content determination, which is not conducive to the quality control of *T. asiatica*.

In previous work, this research group screened the bioactivity of *T. asiatica* through antibacterial, antitumor, and hepatoprotective experiments. They discovered that the main active fraction in the stems of *T. asiatica* was the ethyl acetate extract obtained after alcohol extraction. Component analysis of this extract revealed that it contained phenolic acid components such as gallic acid, protocatechuic acid, and protocatechualdehyde. Literature reports also mention that these components possess antibacterial, antitumor, and hepatoprotective effects[12-14]. Multi-component quantitative analysis is a primary method for effectively studying the quality of medicinal materials. Given that there are currently no literature reports on the use of HPLC for determining gallic acid, protocatechuic acid, and protocatechualdehyde in *T. asiatica*, this experiment employed HPLC to quantify these three components. This aims to provide an experimental basis for the quality control of *T. asiatica* and also lay a foundation for the research and development of *T. asiatica* medicinal materials and related products.

1 Materials

1.1 Instruments HX-200A High-speed Herbal Grinder (Yongkang Xian Hardware Medical Apparatus Factory, Zhejiang Province, China); KQ-500DA Numerical Control Ultrasonic Cleaner (Kunshan Ultrasonic Instruments Co., Ltd., China); Sartorius BP210S Electronic Analytical Balance[Sartorius Scientific Instruments (Beijing) Co., Ltd., China]; Ultrapure Water System (Nanning Bomei Biotechnology Co., Ltd., Guangxi, China); Agilent 1260 High-performance Liquid Chromatograph (Agilent Technologies, USA); Inertsil ODS-C_{18} Chromatographic Column (250 mm×4.60 mm, 5 μm).

1.2 Materials and reagents Thirteen batches of samples were collected from Guangxi (Lingshan, Wuming, Xingning, Qinbei, Heng County, Ningming, Bobai, Rong County), Guangdong (Meilin Reservoir, Wutong Mountain, Huizhou, Xianhu), and Hong Kong. The vine stems were identified as belonging to the Dilleniaceae plant *T. asiatica* (Lour.) Hoogland by Associate Professor Dai Zhonghua from the College of Pharmacy, Guangxi University of Chinese Medicine. Gallic acid (Batch No.:110831-

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201605, purity ≥ 98%), protocatechuic acid (Batch No.: 110809-201205, purity ≥ 98%), protocatechualdehyde (Batch No.:110110-201608, purity≥98%) were all purchased from the National Institutes for Food and Drug Control (NIFDC), China; acetonitrile [chromatographic grade, Thermo Fisher Scientific (China) Co., Ltd.], methanol, hydrochloric acid, phosphoric acid (analytical grade, Tianjin Komiou Chemical Reagent Co., Ltd., China); ultrapure water.

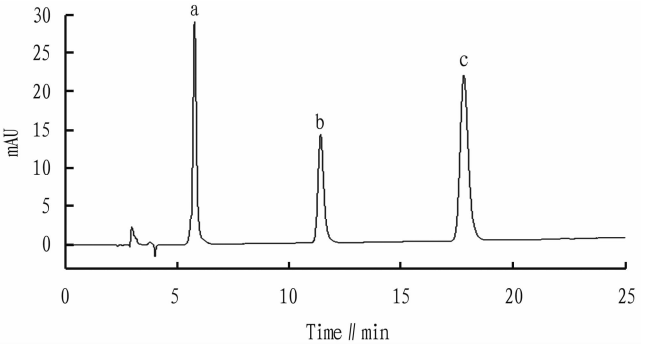
2 Methods and results

2.1 Preparation of reference standard solutions Accurately weighed amounts of gallic acid (2.000 0 mg), protocatechuic acid (2.010 0 mg), and protocatechualdehyde (2.020 0 mg) reference standards were dissolved in methanol in separate volumetric flasks. The solutions were shaken well to dissolve. These were then used to prepare a mixed reference standard solution containing 0.016 0, 0.016 1, and 0.016 2 mg/mL of gallic acid, protocatechuic acid, and protocatechualdehyde, respectively.

2.2 Preparation of test sample solutions 2 g of *T. asiatica* stem powder was accurately weighed into a 50 mL conical flask. 25 mL of methanol-hydrochloric acid mixed solvent (23.5∶1.5) was precisely added into the flask. The flask was accurately weighed, allowed to stand for 30 min, then subjected to ultrasonic extraction for 60 min. The solution was filtered through a 0.45 μm microporous membrane to obtain the final extract.

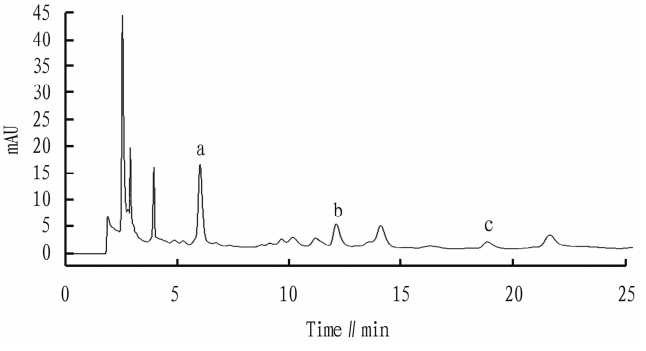
2.3 Chromatographic conditions Stationary phase: Octadecylsilane chemically bonded silica gel filler. Mobile phase: Acetonitrile-0.2% phosphoric acid (10∶90). Detection wavelength: 274 nm. Injection volume: 10 μL. Column temperature: 25 ℃. Flow rate: 1.0 mL/min. The chromatograms for the mixed reference standard solution and the *T. asiatica* stem test sample solution are shown in Fig. 1-2, respectively.

2.4 Linearity investigation Three mixed standard solutions with volumes of 0.125, 0.25, 0.5, 1, 2, and 4 mL were precisely measured and transferred into 10 mL brown volumetric flasks. The solutions were then diluted to volume with methanol. The analyses were conducted according to the chromatographic conditions specified in Section **2.3**. The peak area was obtained. A standard curve was plotted with the injection volume as the abscissa (X) and the corresponding peak area as the ordinate (Y). The results showed that the reference standard solutions exhibited good linear relationships within their respective concentration ranges (Table 1).



NOTE a. Gallic acid, b. Protocatechuic acid, c. Protocatechualdehyde.

Fig. 1 HPLC chromatogram of the mixed reference standard solution



NOTE a. Gallic acid, b. Protocatechuic acid, c. Protocatechualdehyde.

Fig. 2 HPLC chromatogram of the *Tetracera asiatica* stem test sample solution

Table 1 Linearity data for three components in *Tetracera asiatica* medicinal material

Component	Regression equation	r	Linear range // μg/mL
Gallic acid	$y = 59\,214.265\,1x - 0.394\,743\,80$	0.999 81	0.020 – 6.400
Protocatechuic acid	$y = 39\,687.209\,5x + 3.993\,675\,10$	0.999 73	0.201 – 6.432
Protocatechualdehyde	$y = 85\,129.561\,9x + 9.108\,996\,21$	0.999 74	0.202 – 6.464

2.5 Methodological study

2.5.1 Precision test. 10 μL of the same mixed standard solution was precisely injected six times consecutively under the chromatographic conditions described in Section **2.3**. The peak area was measured. The calculated *RSD* values for the peak area of gallic acid, protocatechuic acid, and protocatechualdehyde were 0.93%, 1.51%, and 0.82%, respectively. This indicates that the method provides good injection precision.

2.5.2 Stability test. 10 μL of the same *T. asiatica* test sample solution was precisely injected at 0, 2, 4, 6, 8, and 10 h. The peak area was measured. The calculated *RSD* values for the peak area of gallic acid, protocatechuic acid, and protocatechualdehyde were 0.51%, 2.28%, and 2.21%, respectively. This indicates that the test sample solution remained stable for at least 10 h.

2.5.3 Repeatability test. Six portions of *T. asiatica* stem medicinal powder (2 g each, all sourced from Xingning District, Nanning City) were accurately weighed. The test solutions were prepared according to the method described in Section **2.2**. The analysis was performed under the chromatographic conditions specified in Section **2.3**, and the peak area was measured. The calculated *RSD* values for the peak area of gallic acid, protocatechuic acid, and protocatechualdehyde were 0.55%, 2.91%, and 2.20%, respectively. This indicates that the method has good repeatability.

2.5.4 Spike recovery test. Six portions of *T. asiatica* stem pow-

der (each 2 g, all sourced from Xingning District, Nanning City) were accurately weighed. 1 mL of a mixed standard solution (containing 0.062 4 mg/mL gallic acid, 0.060 6 mg/mL protocatechuic acid, and 0.061 2 mg/mL protocatechualdehyde) was precisely added to each portion. A methanol : hydrochloric acid (23.5 : 1.5) mixed solution was then precisely added to each, and the volume was made up to 25 mL in volumetric flasks. Ultrasonic extraction was performed for 60 min, followed by shaking to homogenize, and filtration through a microporous membrane (0.45 μm) to obtain the final solutions. The solutions were analyzed under the chromatographic conditions specified in Section **2.3**. The spike recovery rate for each component was calculated, and the results are shown in Table 2. The average spike recovery rates for gallic acid, protocatechuic acid, and protocatechualdehyde were all within 98% – 102%, with *RSD* values all less than 3.0%, indicating that this method provides good accuracy.

Table 2 Results of spike recovery test for three components in *Tetracera asiatica* ($n=6$)

No.	Sample weight // g	Sample amount // mg	Spiked amount // mg	Measured amount // mg	Recovery rate // %	Average // %	*RSD* // %
Gallic acid	2.000 2	0.035 0	0.014 6	0.049 7	100.56	101.17	1.21
	2.000 1	0.035 5	0.014 6	0.049 5	95.86		
	2.000 2	0.035 0	0.014 6	0.050 0	102.79		
	2.000 5	0.035 2	0.014 6	0.051 2	109.24		
	2.000 6	0.035 2	0.014 6	0.049 8	99.76		
	2.000 3	0.035 4	0.014 6	0.049 8	98.80		
Protocatechuic acid	2.000 2	0.011 9	0.014 6	0.025 7	94.51	100.48	2.50
	2.000 1	0.011 3	0.014 6	0.026 8	105.92		
	2.000 2	0.012 5	0.014 6	0.027 4	101.81		
	2.000 5	0.012 0	0.014 6	0.027 1	103.51		
	2.000 6	0.011 9	0.014 6	0.025 8	94.90		
	2.000 3	0.011 7	0.014 6	0.026 6	102.26		
Protocatechualdehyde	2.000 2	0.003 4	0.008 0	0.011 9	106.28	98.61	2.69
	2.000 1	0.003 4	0.008 0	0.010 9	93.42		
	2.000 2	0.003 6	0.008 0	0.011 4	98.19		
	2.000 5	0.003 3	0.008 0	0.011 5	102.39		
	2.000 6	0.003 6	0.008 0	0.011 2	94.68		
	2.000 3	0.003 4	0.008 0	0.011 2	96.72		

2.6 Sample content determination Approximately 2 g of *T. asiatica* stem powder from each of 13 batches was accurately weighed into 50 mL conical flasks. The exact weight was recorded. Test sample solutions were prepared according to the conditions specified in Section **2.2**. These solutions were injected under the chromatographic conditions described in Section **2.3**. Parallel determinations were performed in triplicate ($n=3$), and the peak area of these three phenolic acid components was measured. The content of gallic acid, protocatechuic acid, and protocatechualdehyde in different batches from various origins was presented in Table 3. The content ranged from 0.019 6 to 0.064 1 mg/g for gallic acid, 0.006 7 to 0.034 8 mg/g for protocatechuic acid, and 0.003 5 to 0.017 3 mg/g for protocatechualdehyde.

Table 3 Content determination results of three components in *Tetracera asiatica* (mg/g, $n=3$)

No.	Origin	Gallic acid	Protocatechuic acid	Protocatechualdehyde
1	Wuming District, Nanning City, Guangxi	0.032 4	0.034 8	0.007 2
2	Xingning District, Nanning City, Guangxi	0.034 9	0.014 7	0.006 5
3	Heng County, Nanning City, Guangxi	0.037 1	0.011 6	0.004 6
4	Lingshan County, Qinzhou City, Guangxi	0.040 1	0.010 4	0.004 5
5	Qinbei District, Qinzhou City, Guangxi	0.050 2	0.012 1	0.004 1
6	Ningming County, Chongzuo City, Guangxi	0.050 4	0.010 6	0.008 0
7	Rong County, Yulin City, Guangxi	0.019 7	0.006 7	0.017 3
8	Bobai County, Yulin City, Guangxi	0.027 3	0.009 3	0.005 1
9	Wutong Mountain, Shenzhen City, Guangdong	0.034 7	0.016 2	0.005 7
10	Luohu District, Shenzhen City, Guangdong	0.042 8	0.025 7	0.006 6
11	Futian District, Shenzhen City, Guangdong	0.053 5	0.030 2	0.011 6
12	Huiyang District, Huizhou City, Guangdong	0.064 1	0.014 3	0.009 1
13	Wong Tai Sin District, Hong Kong	0.019 6	0.007 0	0.003 5

3 Conclusions and discussions

3.1 Selection of extraction method Prior to formal experiments, we also investigated extraction conditions for *T. asiatica* stem test solutions. Various methods involving different extraction solvents, extraction techniques, extraction durations, and solvent volumes were employed. An optimal extraction method was ultimately selected: using a methanol : hydrochloric acid (23.5 : 1.5) mixture as the extraction solvent, followed by a 30-min standing period and 60-min ultrasonic extraction, which yielded satisfactory content determination results. Compared to conventional methanol extraction, the addition of an appropriate amount of hydrochloric acid facilitated better dissolution of the phenolic acid components. The results indicated that the content of gallic acid, protocatechuic acid, and protocatechualdehyde in *T. asiatica* stems from various origins was relatively low. This may be attributed to the harvesting time occurring in late summer to early autumn, whereas the period when rhizome components typically reach their peak storage levels is during autumn and winter. Therefore, this implies that the harvesting time should be postponed.

3.2 Selection of chromatographic conditions This study explored the conditions affecting content determination. For wavelength selection, content determinations were investigated at 210, 214, 274, 280, and 320 nm. Three-dimensional full-wavelength spectra were obtained using a DAD detector. At 274 nm, all chromatographic peaks exhibited good UV absorption, provided rich information, and had a relatively flat baseline. Therefore, 274 nm was selected as the detection wavelength. For mobile phase selection, the following systems were investigated: methanol-water, acetonitrile-water, acetonitrile-0.1% phosphoric acid, and acetonitrile-0.2% phosphoric acid. The results showed that methanol-water and acetonitrile-water systems provided less chromatographic peak information, the acetonitrile-0.1% phosphoric acid system yielded poor resolution between peaks, while the acetonitrile-0.2% phosphoric acid system demonstrated the best separation efficiency and good peak shape. Therefore, acetonitrile-0.2% phosphoric acid was selected. For temperature selection, conditions of 25, 30, 35 ℃, and no temperature control were investigated. At 25 ℃, the separation of all peaks was optimal, and the required analysis time was moderate. For flow rate selection, chromatogram at different flow rates (0.8, 1.0, and 1.2 mL/min) was investigated. At 0.8 mL/min, not all chromatographic peaks eluted within the set time, while at 1.2 mL/min, the peaks eluted too rapidly. The flow rate of 1.0 mL/min was found to be optimal.

The results indicated that using a mobile phase of acetonitrile-0.2% phosphoric acid (10 : 90), at a wavelength of 274 nm and a flow rate of 1.0 mL/min, all peaks were well separated with a relatively stable baseline.

3.3 Factors influencing content variation The content of components in *T. asiatica* stem materials from different origins showed significant variation. Among these 13 batches, the maximum content of gallic acid (one of the three phenolic acids) was 0.064 1 mg/g (Huiyang District, Huizhou City, Guangdong), and the minimum was 0.019 6 mg/g (Wong Tai Sin District, Hong Kong). The maximum content of protocatechuic acid was 0.034 8 mg/g (Wuming District, Nanning City, Guangxi), and the minimum was 0.006 7 mg/g (Rong County, Yulin City, Guangxi). The maximum content of protocatechualdehyde was 0.017 3 mg/g (Rong County, Yulin City, Guangxi), and the minimum was 0.003 5 mg/g (Wong Tai Sin District, Hong Kong). The significant variation in phenolic acid content observed among the 13 batches of *T. asiatica* stem collected from different origins indicates that the origin has a substantial impact on the quality of the medicinal material. Emphasizing the authenticity of medicinal materials is a fundamental principle in the development of Zhuang medicine. Furthermore, the quality of medicinal materials may be related to various ecological factors such as local climate, soil, sunlight, vegetation type, and geographical location. In a sense, the 13 samples from different sources may not fully represent the diversity and uniformity of *T. asiatica* stem materials. A larger number of samples from more origins, different harvesting periods, and varying storage durations should be collected. Substantial research data needs to be accumulated to ensure the objectivity and impartiality of the research findings.

In summary, the HPLC method established in this study for the simultaneous determination of the three phenolic acid components in *T. asiatica* is simple, accurate, and reproducible. It can serve as a reference for the quality control of *T. asiatica*.

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tion: Integration of molecular breeding with traditional breeding techniques to develop new dzo varieties possessing both hybrid vigor and fertility. Through multidisciplinary collaboration and the application of new technologies, it is anticipated that the bottleneck of male sterility in dzo can be overcome, thereby promoting the sustainable development of yak crossbreeding improvement and its associated industry chain.

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