TLC Identification of Yao Medicine *Pileostegia tomentellal* and Extraction Technology and Content Determination of Umbelliferone

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Abstract [**Objectives**] To establish a TLC and content determination method of *Pileostegia tomentellal*, with umbelliferone as the indicator component. [**Methods**] TLC identification was performed by silica gel G thin layer plate with n-hexane-ethyl acetate (4∶3) as the developing agent, and the plate was examined by UV lamp (365 nm). The umbelliferone content was determined by HPLC: Inertsil ODS-3 C_{18} column (4.60 mm×250 mm, 5 μm); mobile phase acetonitrile-0.2% phosphoric acid gradient elution; detection wavelength 320 nm, flow rate 1.0 mL/min, column temperature 30 ℃, injection volume 10 μL. [**Results**] The chromatogram of *P. tomentellal* showed the same color spot in the same position as that of reference medicinal material, and the spot was clear with good specificity. Umbelliferone showed a good linear relationship when the injection volume was 2.63 - 131.27 μg/mL (R^2 = 0.999 7). The average recovery of umbelliferone in the low, middle and high adding groups of *P. tomentellal* was 99.57% and the *RSD* was 2.15%. [**Conclusions**] The method can effectively identify Yao medicine *P. tomentellal* and accurately determine the content of umbelliferone in medicinal materials, which will provide a scientific basis for the development and utilization of medicinal resources of Yao medicine *P. tomentellal*.

Key words *Pileostegia tomentellal*, TLC identification, Extraction technology, Umbelliferone, Content determination

1 Introduction

Pileostegia tomentellal Hand. Mazz, the vine or stem of *Pileostegia* Hook. f. et Thoms. plants of the Saxifragaceae family, is mainly distributed in Guangxi, Guangdong, Hunan and other places of China, and the whole plant plays a role of promoting blood circulation and dispersing blood stasis[1-2]. It has been collected in many Chinese medicinal plant works, with the effects of dispelling wind and removing dampness, dispersing blood stasis and relieving pain, and strengthening bones, and is commonly used in clinical practice for waist and leg soreness, rheumatism and numbness, and for external treatment of injuries, fractures, and traumatic bleeding[1-2]. Years of clinical practice of folk Yao medicine in Guangxi has proved that Yao medicine *P. tomentellal* has anti-tumor effect and can be used to treat a variety of malignant tumors[3]. It has been collected in *Chinese Materia Medica*[4], *List of Medicinal Plants of Guangxi*[5], *Flora of Guangxi*[6] and other monographs. Modern pharmacological studies have shown that Yao medicine *P. tomentellal* has significant potential anti-tumor activity[3] and is the main drug used in the treatment of various tumors in the Oncology Department of Yao Medicine Hospital, Jinxiu Yao Autonomous County, Guangxi. At present, it has been reported in relevant literature that *P. tomentellal* is mainly composed of effective components such as flavonoids[7], coumarins[8], iridoid[8], *etc.*, and the major active components are total coumarins. Studies have found[9] that coumarins and their derivatives have strong anti-tumor effects *in vivo* and *in vitro*, such as umbelliferone. *P. tomentellal* is often used for anti-tumor in Guangxi, but it has not been widely exploited so far.

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2 Materials

2.1 Instruments 2695 High performance liquid chromatograph (Waters Corporation, USA); Simplicity ultra-pure water system (Millipore China Co., Ltd.); Practum224-1CN analytical balance [Sartorius Scientific Instruments (Beijing) Co., Ltd.].

2.2 Reagents Umbelliferone (Chengdu Herb Substance Pure Biotechnology Co., Ltd., batch No.: 230309); methanol and acetonitrile (chromatographically pure, Fisher, 4 L); ethanol, phosphoric acid and other reagents (analytically pure); ultra-pure water.

Ten batches of *P. tomentellal* were collected from different areas of Guangxi in 2022, and were identified as the vine or stem of *Pileostegia* Hook. f. et Thoms. plants of the Saxifragaceae family by Zhong Wen, a director pharmacist of Chinese medicine at Guangxi International Zhuang Medical Hospital affiliated to Guangxi University of Chinese Medicine. Specific sources are shown in Table 1.

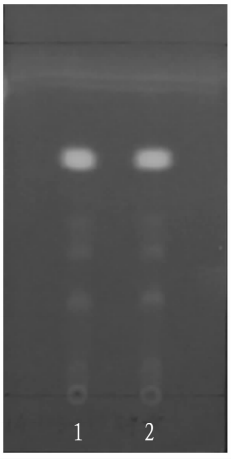
3 Methods and results

3.1 TLC identification Appropriately 2 g of crude powder of *P. tomentellal* was added with 30 mL of ether, and treated by ul-

Table 1 Sample information of *Pileostegia tomentellal*

No. of medicinal materials	Source of medicinal materials	Collection month
ZLT-1	Wuzhou City	May
ZLT-2	Cenxi City	May
ZLT-3	Quanzhou County	May
ZLT-4	Longsheng County	March
ZLT-5	Gongcheng County	March
ZLT-6	Jinxiu County	March
ZLT-7	Zhongshan County	August
ZLT-8	Fuchuan County	August
ZLT-9	Hechi City	September
ZLT-10	Baise City	September

trasound for 10 min. After filtered, the filtrate was evaporated to dryness, and the residue was dissolved in l mL of methanol as the test solution. In addition, 2 g of reference medicinal material was prepared into the reference solution by the same method. According to the general rules of the *Chinese Pharmacopoeia* 2020 edition volume IV (0502), 5 μL of each solution were dotted on the same silica gel G thin layer plate, and spread with n-hexane-ethyl acetate (4∶3) as the developing agent. Subsequently, the plate was taken out, dried, and examined under ultraviolet lamp (365 nm). In the chromatogram of the test product, the fluorescence spots of the same color appeared at the corresponding position of the reference medicinal material (Fig. 1).



NOTE **1**. Reference medicinal material; **2**. *P. tomentellal* sample.

Fig. 1 TLC identification of medicinal materials

3.2 Content determination

3.2.1 Sample preparation. *P. tomentellal* samples collected were dried naturally, crushed and passed through No. 2 sieve for subsequent content determination.

3.2.2 Chromatographic conditions. Chromatographic column: Inertsil ODS-3 C_{18} (4.60 mm × 250 mm, 5 μm); mobile phase: acetonitrile-0.2% phosphoric acid; detection wavelength: 320 nm[10]; flow rate: 1.0 mL/min; column temperature: 30 ℃; injection volume: 10 μL. The gradient elution schedule is shown in Table 2. According to the above conditions, all components were well separated.

Table 2 Gradient elution schedule

Time//min	Mobile phase	
	Acetonitrile//%	0.2% phosphoric acid//%
0	8	92
8	22	78
16	35	65
26	46	54
40	58	42

3.2.3 Preparation of reference solution. Umbelliferone reference product was accurately weighed, dissolved with methanol and diluted to a constant volume, and the umbelliferone reference solution with a concentration of 262.54 μg/mL was obtained.

3.2.4 Preparation of test solution. Accurately 1.0 g of crude powder of *P. tomentellal* was weighed, extracted by reflux with 80% methanol for 1 h, and centrifuged at a radius of 0.65 cm and 13 000 r/min for 10 min. The supernatant was filtered by 0.45 μm microporous filter membrane to obtain the test solution.

3.2.5 Preparation of standard curve. Precisely 0.1, 0.5, 1.0, 2.0, 3.0, 5.0 mL of umbelliferone reference solution (concentration: 262.54 μg/mL) were absorbed and set to a constant volume of 10 mL with methanol. The solutions were determined according to the chromatographic conditions described in Section **3.2.2**. With the concentration of reference solution as the abscissa and the peak area as the ordinate, the regression equation and correlation coefficient were calculated: $Y = 42\ 108X - 6\ 912.5$ (R^2 = 0.999 7). Umbelliferone showed a good linear relationship when the injection volume was 2.63 – 131.27 μg/mL.

3.2.6 Precision test. Proper amount of reference solution (262.54 μg/mL) was precisely measured, and injected for 6 consecutive times according to the conditions described in Section **3.2.2**. The *RSD* of umbelliferone peak area was 0.54%, less than 3.0%, indicating good precision of the instrument.

3.2.7 Stability test. Precisely 1.0 g of *P. tomentellal* crude powder of the same batch was weighed, and prepared into test solution according to the method described in Section **3.2.4**. The test solution prepared was determined at 0, 2, 4, 8, 12 and 24 h according to the conditions described in Section **3.2.2**. The *RSD* of umbelliferone peak area from *P. tomentellal* was 1.85%, less than 3.0%, indicating that the test solution had good stability within 24 h.

3.2.8 Repeatability test. Precisely 1.0 g of *P. tomentellal* crude powder of the same batch was weighed for 6 copies, and prepared into test solution according to the method described in Section **3.2.4**. The test solution prepared was determined according to the conditions described in Section **3.2.2**. The average content of *P. tomentellal* was 0.42 mg/g, and its *RSD* was 2.58%, less than 3.0%, indicating that the method had good repeatability.

3.2.9 Recovery test. Precisely 0.5 g of *P. tomentellal* crude powder of the same batch (content 0.42 mg/g) was weighed with a total of 9 copies, and divided into 3 groups, namely low, medium and high adding groups (adding amount 0.168, 0.210, 0.252 mg). The average recovery of umbelliferone in the low, middle and high adding groups of *P. tomentellal* was 99.57% and its *RSD* was 2.15% (n = 9), indicating that the method was accurate (Table 3).

Table 3 Recovery test of umbelliferone from *Pileostegia tomentellal* ($n=9$)

No.	Weight // g	Content // mg	Adding amount // mg	Measured amount // mg	Recovery // %	Average recovery // %	*RSD* // %
1	0.500 6	0.210 3	0.168	0.380 2	101.16	99.57	2.15
2	0.500 2	0.210 1	0.168	0.376 5	99.06		
3	0.501 0	0.210 4	0.168	0.382 1	102.19		
4	0.500 8	0.210 3	0.210	0.413 2	96.60		
5	0.501 5	0.210 6	0.210	0.425 3	102.22		
6	0.501 2	0.210 5	0.210	0.417 6	98.62		
7	0.500 5	0.210 2	0.252	0.461 5	99.72		
8	0.500 7	0.210 3	0.252	0.453 2	96.39		
9	0.500 8	0.210 3	0.252	0.460 6	99.31		

4 Extraction technology of umbelliferone from *P. tomentellal*

4.1 Single factor investigation

4.1.1 Determination of maximum absorption wavelength. The umbelliferone reference solution was dissolved and diluted with methanol, and was scanned in the wavelength range of 200 – 400 nm by UV-VIS spectrophotometer, with methanol as the reference solution. According to the absorption curve of umbelliferone, the best absorption wavelength was determined to be 320 nm.

4.1.2 Investigation of extraction method. Using umbelliferone as the performance indicator, we extracted umbelliferone from *P. tomentellal* through reflux extraction method, ultrasonic extraction method and solvent impregnation method, with extraction time of 1 h and 25 mL 80% methanol as extraction conditions. The results showed that reflux extraction method had high extraction rate of umbelliferone, so reflux extraction method was selected in this experiment.

4.1.3 Investigation of solvent concentration. The extraction time of 1 h and 25 mL solvent were selected as fixed conditions for the extraction of umbelliferone. We examined the effect of different concentrations of ethanol and methanol on the yield of umbelliferone from *P. tomentellal* by reflux extraction method, and the concentrations of methanol and ethanol were designed as 0%, 20%, 40%, 60%, 80% and 95%. The results showed that 80% methanol achieved higher reflux extraction rate.

4.1.4 Investigation of extraction volume. The extraction time of 1 h and 80% methanol were selected as fixed conditions for the extraction of umbelliferone. We examined the effect of different solvent extraction volumes on the yield of umbelliferone from *P. tomentellal* by reflux extraction method, and the solvent extraction volumes were 10, 20, 30, 40 and 50 mL. The results showed that the extraction rate was the highest when the volume was 30 mL.

4.1.5 Investigation of extraction time. By setting a fixed condition of 30 mL 80% methanol, we examined the effect of different extraction time on the yield of umbelliferone from *P. tomentellal* by reflux extraction method, and the extraction time were 0.5, 1.0, 1.5, 2.0, 2.5 h. The results showed that the yield of umbelliferone was higher when the extraction time was 1.0 h.

4.2 Orthogonal experimental design[11] According to relevant literature and the 2020 edition of *Chinese Pharmacopoeia*, the extraction method with different methanol concentrations was finally adopted in this experiment, and three conditions including solvent concentration (60%, 80% and 95% methanol), solvent volume (20, 30 and 40 mL) and extraction time (1, 1.5 and 2 h) were investigated. According to the orthogonal design assistant software, $L_9(3^4)$ orthogonal experiment design was selected, as shown in Table 4.

Table 4 Factors and levels of orthogonal test

Level	Factor		
	A (methanol concentration) // %	B (solvent volume) // mL	C (extraction time) // h
1	60	20	1
2	80	30	1.5
3	95	40	2

Precisely 1.0 g of *P. tomentellal* powder were weighed, extracted according to the extraction method of umbelliferone from *P. tomentellal*, and operated according to the preparation method of test solution. The experiment was carried out according to the $L_9(3^4)$ orthogonal design schedule, and the content of umbelliferone in *P. tomentellal* was determined by HPLC (Table 5).

Table 5 Design and results of orthogonal test for extraction of umbelliferone from *Pileostegia tomentellal*

No.	Factor				Umbelliferone
	A	B	C	D (error)	mg/g
1	1	1	1	1	0.354 2
2	1	2	2	2	0.428 3
3	1	3	3	3	0.443 1
4	2	1	2	3	0.431 3
5	2	2	3	1	0.461 4
6	2	3	1	2	0.457 5
7	3	1	3	2	0.296 2
8	3	2	1	3	0.371 2
9	3	3	2	1	0.367 6
K_1	0.408 5	0.360 6	0.394 3	0.394 4	
K_2	0.450 1	0.420 3	0.409 1	0.394 0	
K_3	0.345 0	0.422 7	0.400 2	0.415 2	
R	0.105 1	0.062 2	0.014 8	0.021 2	

The results of variance analysis showed (Table 6) that methanol concentration had a significant effect on the extraction rate of umbelliferone, followed by solvent volume and extraction time. There were significant differences in extraction rate of umbelliferone among methanol concentrations ($P<0.05$), while there was no significant difference among solvent volumes and extraction time ($P>0.05$), and the strength of each influencing factor was A > B > C.

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of the peak area of 5-HMF, and finally determined as 70% ethanol, ultrasonic for 30 min. The chromatographic conditions were determined according to the peak shape and resolution. In this experiment, the maximum absorption wavelength was determined between 200 – 210 nm by DAD full-wavelength scanning, and finally 200 nm was determined as the optimal wavelength.

From the experimental results, it can be seen that there are great differences in the chemical composition of the nine batches of samples with different processing degrees. If we need to explore which specific chemical components are different, we need to further analyze them with the help of mass spectrometry. Because the quality of Polygonati Rhizoma from different varieties and different places may be different, the best steaming times of Polygonati Rhizoma with large quality difference may be different. In the future, more batches of Polygonati Rhizoma from different varieties and different places can be selected for further study.

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Therefore, methanol concentration was the factor that had statistical significance on the extraction effect of umbelliferone from *P. tomentellal*. In consequence, the extraction schedule of this experiment was $A_2B_3C_2$, whereas the optimal extraction schedule of this experiment was $A_2B_2C_2$ combined with the experimental cost and energy saving, that is, the extraction effect was the best when the methanol concentration, extraction solvent volume and reflux extraction time were 80%, 30 mL, and 1.5 h, respectively.

Table 6 Analysis of variance for extraction of umbelliferone from *Pileostegia tomentellal*

Source of error	*SS*	*f*	*S*	*F*	*P*
A	0.016 8	2	0.008 4	19.043 0	<0.05
B	0.007 4	2	0.003 7	8.431 6	>0.05
C	0.000 3	2	0.000 2	0.375 5	>0.05
D (error)	0.000 9	2	0.000 4		

5 Validation test

Three batches of validation tests were conducted according to the optimal technology determined by orthogonal test. The results showed that the content of umbelliferone in *P. tomentellal* was 0.425 0 mg/g, and the *RSD* was 2.36%, indicating that the process had good reproducibility and could be used as the extraction technology of umbelliferone from *P. tomentellal*.

6 Discussion

The content of umbelliferone in *P. tomentellal* was determined by HPLC, and the effects of different pH, composition and flow rates of mobile phase were studied. The results showed that the method had good precision, reproducibility and stability, and could be used for the determination of umbelliferone content in *P. tomentellal*, which provides a basis for establishing and improving the quality standard of Yao medicine *P. tomentellal*.

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