Application of microscopy technique and high performance liquid chromatography for quality assessment of *Polygonum multiflorum* Thunb. (Heshouwu)

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ABSTRACT

Background: The technique of microscopy has been applied for identification of Chinese materia medica (CMM) since decades. However, very few scientific publications report the combination of conventional microscopy and high performance liquid chromatography (HPLC) techniques for further application to quality assessment of CMM. **Objective:** The objective of this study is to analyze the quality of the dried root tuber of *Polygonum multiflorum* Thunb. (Heshouwu) and to establish the relationships between 2,3,5,4′-tetrahydroxystilbene-2-O-β-glucoside, combined anthraquinone (CAQ) and quantity of clusters of calcium oxalate. **Materials and Methods:** In this study, microscopy and HPLC techniques were applied to assess the quality of *P. multiflorum* Thunb., and SPSS software was used to establish the relationship between microscopic characteristics and chemical components. **Results:** The results showed close and direct correlations between the quantity of clusters of calcium oxalate in *P. multiflorum* Thunb. and the contents of 2,3,5,4′-tetrahydroxystilbene-2-O-β-glucoside and CAQ. From these results, it can be deduced that Polygoni Multiflori Radix with a higher quantity of clusters of calcium oxalate should be of better quality. **Conclusion:** The established method can be helpful for evaluating the quality of CMM based upon the identification and quantitation of chemical and ergastic substance of cells.

Key words: 2,3,5,4′-tetrahydroxystilbene-2-O-β-glucoside, clusters of calcium oxalates, combined anthraquinone, *Polygonum multiflorum* Thunb, quantitative analysis

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INTRODUCTION

Authentication of Chinese materia medica (CMM) has been enhanced by using a combination of four methods, namely origin identification, macroscopic identification, microscopic identification, and physicochemical identification.[1] Microscopic identification plays an important role in CMM identification because of its advantages of simplicity, reliability, low cost and low sample amount requirement, and fast speed. When macroscopic features do not sufficiently distinguish plant material, microscopic identification can further identify taxa based on the size and shape of cells, the color and nature of ergastic substances, such as crystalline inclusions.[2] Crystals and stone cells are often considered as characteristic features for microscopic identification of CMM powder due to their stable nature and the differences in their size and shape.[3]

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Recently, researchers have focused upon application of microscopy techniques along with high performance liquid chromatography (HPLC) for identification and quantitative analysis. In microscopic identification of powdered CMMs, the cellular components with the higher stability could be selected for quantitative analysis.[4-9] In such quantitative determinations, microscopic characteristic constant (MCC), representing the quantity of a particular characteristic is calculated. The number of times one certain characteristic appears on a slide or in a view, is counted, and then extrapolated to determine the frequency of occurrence in every milligram of a powdered CMM. The frequency of occurrence in every milligram is usually called the MCC, representing the quantity of that particular characteristic. It is calculated as follows: MCC = (X × V)/(V' × W), where X is the number of times that characteristic appears on one slide; V is the total volume of the CMM suspension from which the drop on the slide was drawn; V' is the volume of suspension on each slide; and W is the weight of powdered material that is put into suspension (mg, measured by dry product).[3]

In addition to quantitative analysis, MCC can be used for qualitative identification and authentication of different plant species in the same genus. The technique has also been applied for determining the percentage of a specific CMM in Chinese patent medicines (CPM) and mixtures of herbal medicines.[3,10]

The objective of the current study was to explore the relationship between quantity of microscopic characteristics and various phytoconstituents present in the dried root tuber of *Polygonum multiflorum* (Polygoni Multiflori Radix, Heshouwu).[11] This plant is widely distributed in Sichuan, Guizhou, Guangxi, Hubei, and Henan province. *P. multiflorum* Thunb. is known to remove toxins, disperse abscesses, interrupt malaria, and moisten the intestines to relax the bowels. It is used to treat sores and abscesses, scrofula, to relieve itching caused by rubella, to restore constitutions weakened by long-term malaria, and to relieve constipation caused by intestinal dryness.[11]

In microscopic examination of the powdered *P. multiflorum* Thunb., all microscopic characteristics are shown in Figure 1, clusters of calcium oxalate 10-80 (160) μm in diameter are found, and these clusters are occasionally joined with prisms. The cluster is a reliable feature of this powder, and occurs in significant numbers; hence, it is easy to observe and count the number of clusters of calcium oxalate under the microscope.[1,12] In terms of the chemical components of *P. multiflorum* Thunb., previous studies have indicated that it contains anthraquinones and diphenyl ethylene glycosides as major components.[13] Combined anthraquinone (CAQ) and 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside and are reported as active components of this CMM and are also used as chemical markers for this plant material in the Pharmacopoeia of the People's Republic of China.[11]

The purpose of this study was to establish an analytical method for quantitation of a microscopic component and provide scientific data for quality evaluation of *P. multiflorum* Thunb. In this study, clusters of calcium oxalate were quantified by microscopic examination, and chemical components were determined by HPLC analysis. Based upon the results obtained, the relationships between quantity of calcium oxalate clusters and chemical components were established.

MATERIALS AND METHODS

Apparatus

Microscopy was performed using an Olympus-BX51 system biologic microscope equipped with an Olympus-DP72 camera and an Olympus U-POT polarizer. (Olympus Corporation) HPLC analysis was carried out using an Agilent Series 1100 liquid chromatograph, coupled with HP ChemStation. A Waters Nova-Pak C_{18} column (3.9 mm × 150 mm) at a column temperature of 30°C was used for HPLC analysis. For 2,3,5,4'-tetra hydroxystilbene-2-O-β-glucoside, we used a mixture of methanol and 0.1% solution of phosphoric acid (30:70) as the mobile phase with a flow rate of 1 mL/min and detection wavelength set at 320 nm. For physcion and emodin, we used a mixture of methanol and 0.1% solution of phosphoric acid (80:20) as the mobile phase with a flow rate of 1 mL/min and detection wavelength set at 254 nm. CAQ was calculated as the total amount of physcion and emodin.

Reagents

Microscopic reagents used were glycerin and chloral hydrate. Chloral hydrate solution and dilute glycerin were prepared according to the procedures described in Appendix XV B of the Pharmacopoeia of the People's Republic of China.[11] HPLC-grade methanol was used

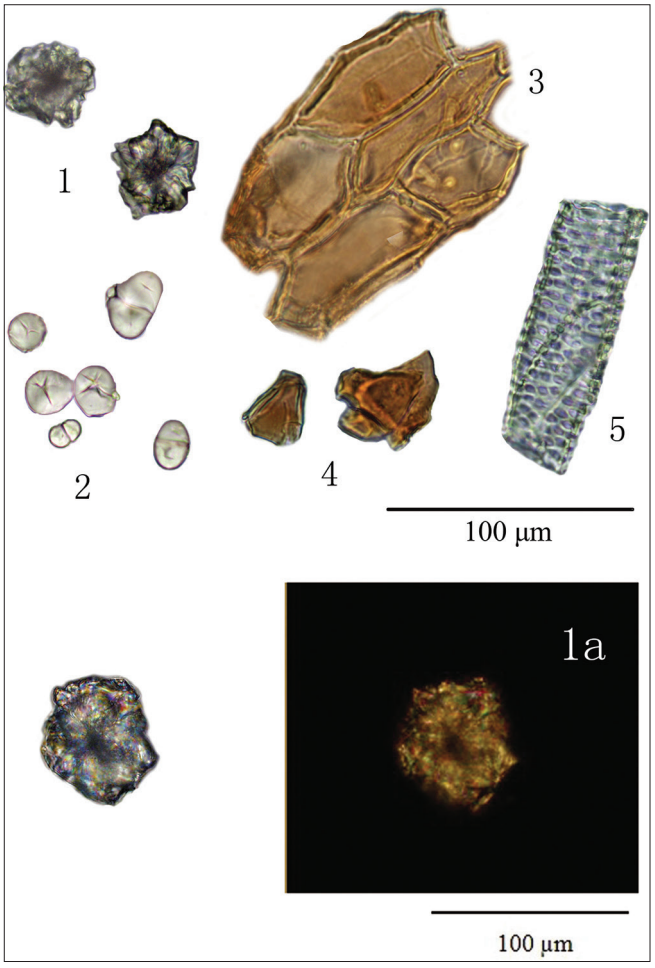

Figure 1: Microscopic characteristics of root tubers of Polygoni Multiflori Radix (Heshouwu) (1) clusters of calcium oxalate; (2) starch granules; (3) cork cells; (4) brown contents; (5) vessel; (1a) clusters of calcium oxalate under the polarized light microscope at column width

for preparation of the mobile phase (Merck, Germany). Analytical-grade methanol was used for preparation of standards and sample extraction.

Materials

Reference compounds of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, physcion, and emodin were purchased from the National Institute for Food and Drug Control of China (Batch NOs. 110844-200908, 110758-201013, 110756-200110, respectively).

Six different herbal samples (HSW-01–HSW-06) of *P. multiflorum* Thunb. were cultivated ones with the age of 18 months and collected from the same growing area in Guizhou Province of China in 2013. Nine batches of Polygoni Multiflori Radix (HSW-07–HSW-15) were wild ones with uncertain age and collected from different areas of China in 2011. All samples were authenticated by Professor Tingguo Kang and deposited in the School of Pharmacy of Liaoning University of Traditional Chinese Medicine in China. The samples used to determine the amount of calcium oxalate clusters were dried herbs; they necessarily contained some water, which could skew the results. Thus, it was necessary to determine moisture content of the test samples. Determination of moisture content was performed according to the procedures described in Appendix IX H Drying in Oven Method of the Pharmacopoeia of the People's Republic of China. Sample details were shown in Table 1.

Preparation of slides

Slides preparation was performed under optimized conditions. In brief, samples were ground into powders of 0.15-0.18 mm in particle size. For each sample, 400 mg powder was weighed accurately, ground with 2 mL chloral hydrate 5 times, 8 mL dilute glycerin was then added, transferred to a 25 mL volumetric flask and was adjusted to the volume with chloral hydrate, mixed well. Each mixture was used to prepare 50 slides with 20 μL on each slide. This procedure of slide preparation for each sample was repeated for 3 times. In total, 150 slides were prepared and examined for each batch of sample.[5-8]

Preparation of standard and sample solutions for high performance liquid chromatography analysis

The three reference compounds, 2,3,5,4'-tetrahydroxy stilbene-2-O-β-glucoside, physcion and emodin, were accurately weighed and then dissolved in 70% methanol to produce standard solutions. Each powdered sample of 0.5 g was refluxed using 50 mL 70% methanol in a water bath for 60 min. The supernatant was used as test solution A for 2,3,5,4'-tetrahydroxystilbene-2-O-β-glu coside and free anthraquinone (FAQ). Test solution A of 10 mL together with 1.5 mL hydrochloric acid was refluxed in a water bath for 60 min and then transferred to a 25 mL volumetric flask, diluted with 70% solution of methanol to volume, and mixed well. The supernatant of this prepared solution was used as test solution B to determine total anthraquinone (TAQ) content. Content of CAQ = Content of TAQ − Content of FAQ. CAQ was calculated as the total amount of physcion and emodin. Standard solutions, Test solutions A and B were filtered through 0.45 μm membranes separately, and 10 μL of each standard and sample solutions were analyzed by HPLC 3 times.[11,14]

Table 1: Details of samples, quantity of clusters of calcium oxalate, contents of chemical components

Sample number	Collection place (wild)	Collection time DD/MM	Water (%)	Average value of clusters of calcium oxalate of each suspension				Quantity of clusters of calcium oxalate (MCC)	2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside (%)	CAQ (%)
				Test-01	Test-02	Test-03	Mean±SD			
HSW-01	Kaili City, Guizhou Province	23/02	6.39	3.64	3.52	3.44	3.53±0.10	11.68	3.52	2.33
HSW-02	Kaili City, Guizhou Province	23/02	7.42	1.98	1.92	2.12	2.01±0.10	6.75	3.41	1.99
HSW-03	Kaili City, Guizhou Province	23/02	6.85	3.56	3.74	3.42	3.57±0.16	11.91	3.78	0.67
HSW-04	Kaili City, Guizhou Province	23/02	7.24	4.66	4.78	4.70	4.71±0.06	15.83	4.55	2.40
HSW-05	Kaili City, Guizhou Province	23/02	6.69	2.88	3.18	2.94	3.00±0.16	10.05	4.22	2.36
HSW-06	Kaili City, Guizhou Province	23/02	7.02	6.26	6.18	6.32	6.25±0.07	21.01	4.66	2.81
HSW-07	Zigong City, Sichuan Province	07/04	5.84	27.64	28.24	27.70	27.86±0.27	92.64	3.70	1.76
HSW-08	Chengdu City, Sichuan Province	01/05	6.76	3.58	3.52	3.78	3.63±0.11	12.12	3.84	0.30
HSW-09	Zigong City, Sichuan Province	24/04	8.68	11.56	10.72	10.82	11.03±0.37	37.78	4.69	0.22
HSW-10	Haile City, Yunnan Province	14/05	6.62	28.66	26.88	28.26	27.93±0.76	93.38	4.55	1.33
HSW-11	Shiyan City, Hubei Province	20/05	5.77	32.58	33.00	33.18	32.92±0.25	109.58	5.30	4.09
HSW-12	Zunyi City, Guizhou Province	15/05	5.43	3.64	3.62	3.74	3.66±0.06	12.60	4.55	0.88
HSW-13	Shennongjia City, Hubei Province	01/05	8.97	4.94	5.04	4.76	4.91±0.14	16.91	3.73	1.25
HSW-14	Guangyuan City, Sichuan Province	10/05	9.90	22.52	21.20	21.34	21.69±0.59	72.87	5.06	1.03
HSW-15	Lushan City, Henan Province	23/05	6.83	50.74	49.60	50.66	50.33±0.52	174.67	6.04	2.95

MCC: Microscopic characteristic constant; SD: Standard deviation; CAQ: Combined anthraquinone

RESULTS

Optimization of preparation procedure for Chinese materia medica suspensions

In the process of preparing CMM suspensions, the parameters such as weight of sample (150 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg), particle size (0.13-0.15 mm, 0.15-0.18 mm), amount of dilute glycerin (3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL) were studied by uniform design experiment [Table 2].[15]

Six suspensions were prepared according to Table 2, ground with 2 mL chloral hydrate 5 times, and transferred to a 25 mL volumetric flask. The suspensions were diluted with chloral hydrate to 25 mL, and mixed well. Each mixture was used to prepare 50 slides with 20 μL on each slide. The slides were observed under the microscope as showed in Figure 2. The number of clusters of calcium oxalate found on each slide was recorded, and then divided randomly into five groups. The relative standard deviation (RSD) of the average value of each group was calculated as 7.20%, 5.89%, 4.76%, 4.03%, 3.43%, and 2.82%, respectively.

To determine the better method for preparing CMM suspensions, the weight of sample (parameter A), particle size (parameter B), amount of dilute glycerin (parameter C) and the obtained RSD value were analyzed with SPSS 17.0 statistics software (International Business Machines Corporation). The results showed: RSD = 9.404-0.017A, $R^2 = 0.970$, $F = 128.861$, $P = 0.000$, $RSD_{min} = 2.604(\%)$. This calculation revealed that only the weight of sample contributed to the RSD, whereas the particle size of samples and the amount of dilute glycerin were not significantly influential factors. Thus, the particle size of samples and the amount of dilute glycerin could be chosen according to what was convenient and appropriate for each sample. Based on these optimized conditions, CMMs were ground into powders of 0.15–0.18 mm in particle size. Four hundred milligram powder was taken and mixed with 8 mL dilute glycerin.

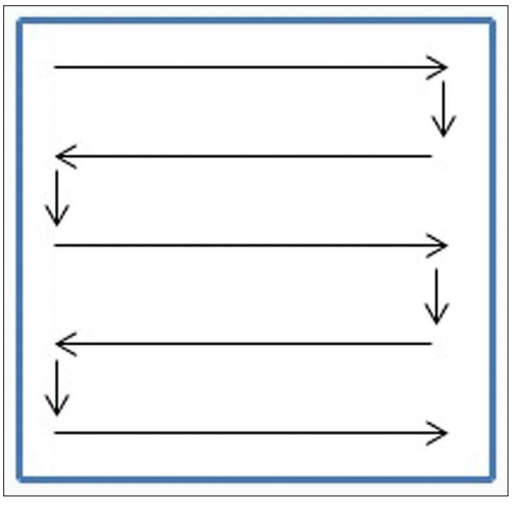

Figure 2: Diagrammatic representation of the method used for observation of slides at column width

Microscopy method validation

Linearity of microscopy method was examined with a series of HSW-07 sample solutions. The calibration curves were constructed by plotting the mean value of the clusters of calcium oxalate found on each slide versus the amount of sample (mg). The liner regression equation and correlation coefficient (r^2) was $y = 0.0695x + 0.5398$ ($r^2 = 0.9903$, $n = 6$).

Method precision was investigated by repeatedly analyzing the same HSW-07 sample solution. The RSD was 1.63% for the quantity of the clusters of calcium oxalate.

Method repeatability was evaluated by six replicated analyses of HSW-07 sample. The RSD was 2.18% for the quantity of the clusters of calcium oxalate.[5]

High performance liquid chromatography method validation

For 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, emodin and physcion, linearity of each compound was examined with a series of standard solution, respectively. Satisfactory linearity of each compound was obtained [Table 3].

Method precision of each compound was determined on the standard solutions at a certain concentration

Table 2: Uniform design for optimization of preparation of CMM suspensions

Number	Weight of sample (mg)	Particle size (mm)	Amount of dilute glycerin (mL)
1	150.0	0.15-0.18	5.0
2	200.0	0.13-0.15	8.0
3	250.0	0.13-0.15	4.0
4	300.0	0.15-0.18	7.0
5	350.0	0.15-0.18	3.0
6	400.0	0.13-0.15	6.0

CMM: Chinese materia medica

Table 3: Results of linearity studies

Compound	Linear range (μg/mL)	Regression equation	Correlation coefficient (r^2)
2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside	107.2-643.2	y=22.018 x−90.18	0.9999
Emodin	5.0-200.0	y=19.953 x+1.72	0.9998
Physcion	46.0-920.0	y=0.249 x+1.45	0.9998

respectively. The values of RSDs were 0.46%, 0.89% and 0.88% (n = 5) for 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, emodin and physcion respectively.

Stability testing was performed on a sample solution after standing for 0, 2, 4, 6, 8 and 24 h. The results showed that the RSD of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, emodin and physcion was 0.85%, 1.40% and 1.40% (n = 6) respectively.

Method repeatability of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside was evaluated by five replicated analyses of herbal samples and the RSD was 1.36%. Method repeatability of emodin and physcion were evaluated by five replicated analyses of herbal samples and the RSDs were 1.27% and 1.19%, respectively.

Recovery study was conducted on a sample spiked with about 100% of known amounts of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, emodin and physcion in the samples with five replicated analyses. The spiked samples were extracted and the amounts of these analytes were quantified. All recoveries were in the range of 99.8–105.8%. The results showed that the average recoveries were estimated to be 95.79% ±0.88% (mean ± SD, n = 5) for 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, 96.30% ±1.75% (mean ± SD, n = 5) for emodin and 96.14% ±1.43% (mean ± SD, n = 5) for physcion.

The above results were considered to be satisfactory for quantitative analysis of herbal samples.[14]

Quantitation of clusters of calcium oxalate

The slides were observed under microscope as shown in Figure 2. The number of clusters of calcium oxalate on one slide was recorded, and its average value was calculated. The test was repeated three times, and the results were shown in Table 1. The MCC, which represents the total quantity of clusters of calcium oxalate, was then calculated. The MCC from different samples varied from 6.75 to 174.67. The detail results were shown in Table 1.

Chemical contents of 2,3,5,4′-tetrahydroxystilbene-2-O-β-glucoside (%) and combined anthraquinone (%)

Under the above analytical method, 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, emodin and physcion were well separated in the HPLC chromatograms [Figures 3 and 4]. Nine batches of *P. multiflorum* Thunb. were assayed in triplicate using the HPLC method. The results indicated that the contents of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside and CAQ varied in different samples. For example, the highest content of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside was 6.04% in the sample of HSW-15 collected from Henan province while the highest content of CAQ was 4.09% in the sample of HSW-11 collected from Hubei province. More information was shown in Table 1.

Linear regression

The relationship between the quantity of clusters of calcium oxalates and the content of 2,3,5,4'-tetrahyd

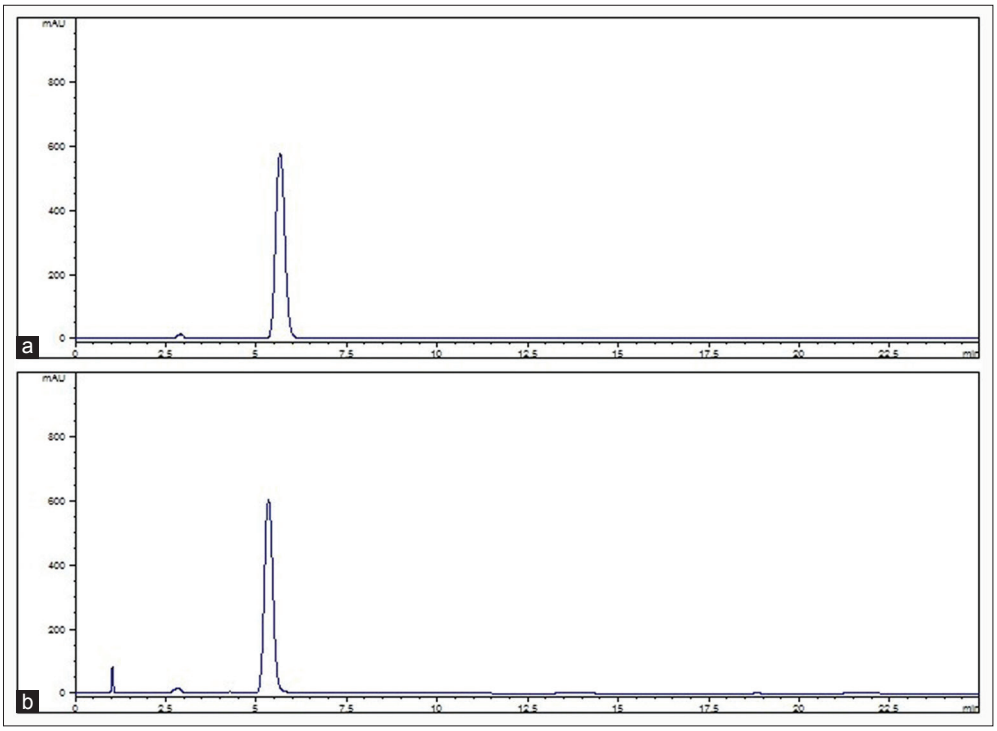

Figure 3: The high performance liquid chromatography chromatographs of standards and samples of Polygoni Multiflori Radix (Heshouwu). (a) Standard solution of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside; (b) sample solution at column width

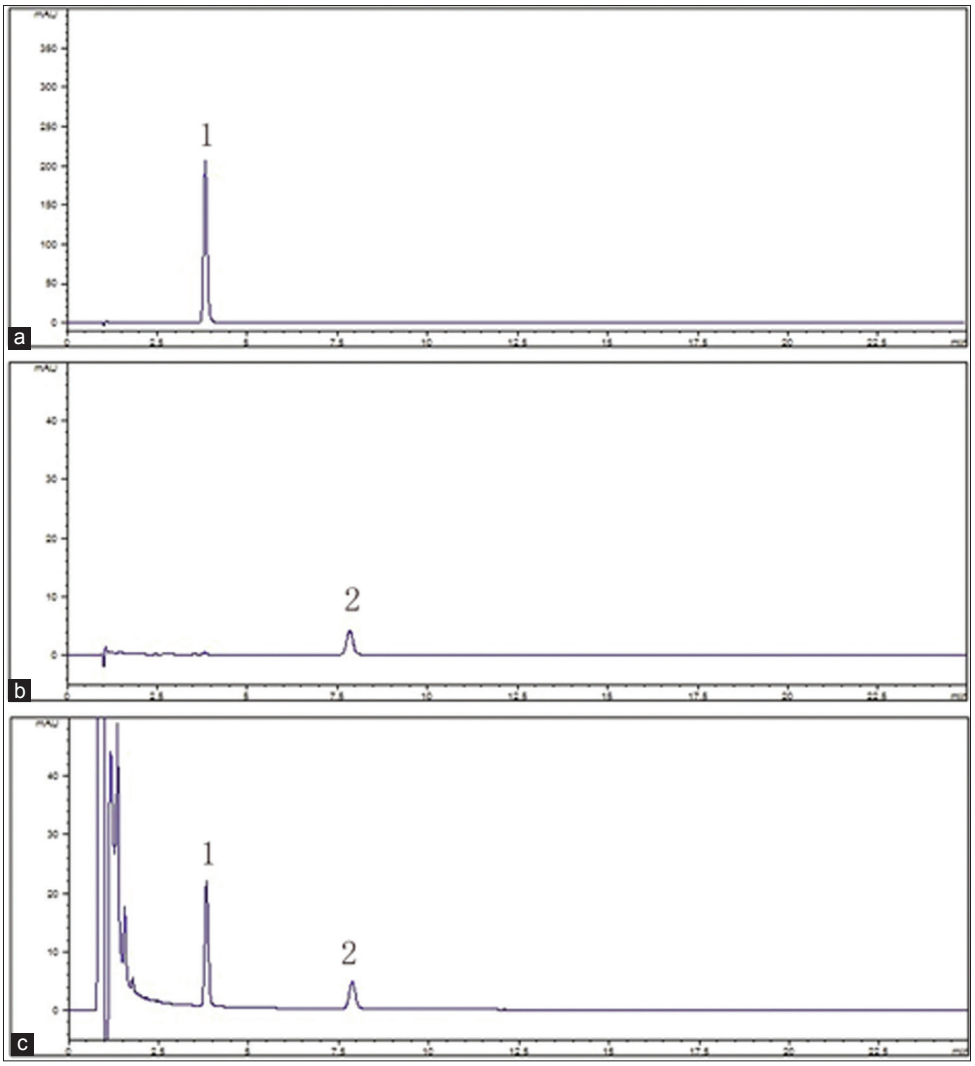

Figure 4: The high performance liquid chromatography chromatographs of standards and samples of Polygoni Multiflori Radix (Heshouwu). (a) Standard solution of emodin; (b) standard solution of physcion; (c) sample solution. Peak 1: Emodin; peak 2: Physcion at column width

roxystilbene-2-O-β-glucoside was analyzed by linear regression using SPSS 17.0 (International Business Machines Corporation) statistics software. As the growing environments between cultivated and wild samples were totally different, which could influence the chemical contents, to exclude the additional factor, the linear regression analysis was carried out on cultivated and wild samples, respectively. For the six batch cultivated samples (HSW-01–HSW-06), the result showed that a significantly positive correlation between the quantity of clusters of calcium oxalate and the content of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside ($Y = 0.088x + 2.894$, $r = 0.819$, $F = 8.127$, $P = 0.046$, $P < 0.05$); for the nine batch wild samples (HSW-07–HSW-15), the result also showed that a significantly positive correlation between the quantity of clusters of calcium oxalate and the content of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside ($Y = 0.01x + 3.893$, $r = 0.721$, $F = 7.565$, $P = 0.028$, $P < 0.05$).[16] It indicated that larger numbers of clusters of calcium oxalate of this medicinal material would contain higher amounts of 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside.

The relationship between the quantity of clusters of calcium oxalate and the content of CAQ was also analyzed by linear regression using SPSS 17.0 statistics software. For the six batch cultivated samples (HSW-01–HSW-06), the result showed a significantly positive correlation between the quantity of clusters of calcium oxalates and the content of CAQ ($Y = 0.047x + 1.820$, $r = 0.814$, $F = 7.837$, $P = 0.049$, $P < 0.05$); for the nine batch wild samples (HSW-07–HSW-15), the result showed a significantly positive correlation between the quantity of clusters of calcium oxalates and the content of CAQ ($Y = 0.17x + 0.344$, $r = 0.751$, $F = 9.080$, $P = 0.020$, $P < 0.05$).[16] It indicated that *P. multiflorum* Thunb. with more clusters of calcium oxalate would contain more CAQ.

DISCUSSION

Preparing the sample suspension and microscopic analysis slides are significant factors in quantitative analysis of clusters of calcium oxalate. Three main factors which would influence the amount of calcium oxalate clusters in the suspension are the weight of original sample, the particle size of original sample and the amount of dilute glycerin added into the suspension. Considering that variation in each factor would have different impacts on the final count, optimization was performed using uniform design experiment. When making slides, in order to reduce errors, it is essential to well-disperse the original suspension. The suspension on the glass slide should be completely covered but it should not spillover. We found that 20 μL was the appropriate amount of suspension for each slide.

To avoid miscounting, observations were performed in specific pattern, as shown in Figure 2. Although some of the clusters of calcium oxalate were broken after blended into powder and passed through sieve, we only counted whole ones of clusters of calcium oxalate.[6,7]

In this study, a new method using microscopy coupled with HPLC was established as a convenient and effective approach to assess the quality of *P. multiflorum* Thunb. The relationships between quantity of one microscopic character and chemical components of *P. multiflorum* Thunb. were established. Clusters of calcium oxalate were taken as the microscopic characteristic; 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside and CAQ were taken as major chemical components. In statistics, the value of regression coefficient (r) ≥0.7 reveals high correlation between two factors; $0.7 > r \geq 0.3$ reveals a middle degree correlation between two factors; $r < 0.3$ reveals a low correlation between two factors. The correlation only can be established in the situation sig(P) <0.05. Results revealed a direct correlation between 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside and CAQ and the quantity of clusters of calcium oxalate. In other words, results indicated that herbal samples with more clusters of calcium oxalate would contain greater amounts of the pharmacologically active components in *P. multiflorum* Thunb., thus be of better quality in clinical application. Previous studies have shown that Polygoni Multiflori Radix with a broader cortex should be of better quality.[17] As clusters of calcium oxalate mainly exist in cortex, the results of the current study are in agreement with previously reported studies. In general, the microscopic feature-quantity of calcium oxalate crystals can be used not only for identification, but also for quality assessment of *P. multiflorum* Thunb.

The established method provides a useful application of evaluation of chemical and ergastic substance of cells for quality control of medicinal materials. This approach can be further applied to other medicinal herbs, extending the use of microscopic techniques to the field of quality control of medicinal materials.

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