MICROSCOPIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF FLOWER BUDS OF TUSSILAGO FARFARA

*Authors: Researcher **Abdiravidov Temurbek Abdumannonovich**, 3rd year student of Clinical Pharmacy, Tashkent Pharmaceutical Institute*
*Scientific researcher, Tashkent Pharmaceutical Institute, 3rd year student of clinical pharmacology department, **Hikmatova Shukrona Ismatovna***
*Researcher **Murtazakulov Ilyos Ismailovich**, 3rd year student of Clinical Pharmacy, Tashkent Pharmaceutical Institute*

Key words: Chemical components, pollen grains, quantitative analysis, Tussilago farfara L, tussilagone

Abstract

Background: Quality control is one of the bottleneck problems limiting the application and development of traditional Chinese medicine (TCM). In recent years, microscopy and high-performance liquid chromatography (HPLC) techniques have been frequently applied in the quality control of TCM. However, studies combining conventional microscopy and HPLC techniques for the quality control of the flower bud of Tussilago farfara L. (Kuandonghua) have not been reported. Objective: This study was undertaken to evaluate the quality of the flower bud of T. farfara L. and to establish the relationships between the quantity of pollen grains and four main bioactive constituents: tussilagone, chlorogenic acid, rutin and isoquercitrin. Materials and Methods: In this study, microscopic examination was used to quantify microscopic characteristics of the flower bud of T. farfara L., and the chemical components were determined by HPLC. The data were analyzed by Statistical Package for the Social Sciences statistics software. Results: The results of the analysis showed that tussilagone, chlorogenic acid, rutin and isoquercitrin were significantly correlated with the quantity of pollen grains in the flower bud of T. farfara L. There is a positive correlation between them. From these results, it can be deduced that the flower bud of T. farfara L. with a greater quantity of pollen grains should be of better quality. Conclusion: The study showed that the established method can be helpful for evaluating the quality of the flower bud of T. farfara L. based on microscopic characteristic constants and chemical quantitation.

INTRODUCTION

Over its long history, traditional Chinese medicine (TCM) has contributed to the healthcare of mankind and has become popular worldwide. Chinese medicinal materials (CMM) are fundamental components used in TCM therapy, so the quality of

CMM has a major impact on clinical safety and efficacy. However, CMM uses complex mixtures containing many chemical components, which makes quality control extremely difficult. Currently, there are many methods used in the assessment of CMM, such as high-performance liquid chromatography (HPLC), but it is hard to establish a comprehensive quality assurance with any single approach.[1,2] Therefore, the combination of two or more methods offers a more comprehensive approach for the quality evaluation of CMM. Recently researchers have focused upon the application of microscopy techniques along with HPLC for identification and quantitative analysis.[3-9] Microscopic identification has been widely accepted as a crucial method for authentication of CMM because it offers many advantages over other methods, such as simplicity of operation, low requirements in terms of sample amounts, and less solvent consumption. As a routine technique, microscopic identification has been applied for authenticating mixtures of herbal medicines, and can also reveal detailed microscopic features, such as pollen grains, in cases when macroscopic features do not sufficiently distinguish plant material.[3,4]. Microscopic characteristic constants (MCC) of CMM are usually expressed as the quantity of a particular characteristic of a CMM per milligram. The MCC is calculated using following formula MCC = (X × V)/(V′ × W) where X is the number of times that the 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).[2,3] The cellular components with the highest stability are selected as the particular characteristic of a given CMM for quantitative analysis. In this paper, we studied the quality of the flower bud of Tussilago farfara L. by applying a new method that combines microscopy techniques and the HPLC method. The flower bud of T. farfara, which is known as Kuandonghua, has been used to relieve cough and resolve phlegm in China for a long time. The flower bud of T. farfara is a plant in the family Asteraceae that is used in traditional medicine. This plant is widely used for the treatment of coughs, bronchitis and asthmatic disorders in TCM. It is distributed in Hebei, Gansu, Shanxi, and Henan provinces.[10] Pollen grains are often considered as characteristic features in the microscopic identification of CMM powder due to their stable nature and specificity. It is easy to observe and count the number of pollen grains under the microscope.[4,5] In microscopic examination of powdered the flower bud of T. farfara, pollen grains 28–40 μm in diameter are found.[10,11] All microscopic characteristics are shown in Figure 1. The flower bud of T. farfara has been extensively investigated in phytochemistry, and the results indicate that tussilagone, chlorogenic acid, rutin, and isoquercitrin are the main active components in the flower bud of T. farfara. [12,13] Tussilagone is reported as an active component of this CMM and is also used as a chemical marker for this plant material in the Pharmacopoeia of the

People's Republic of China.[10] In this study, microscopic examination was used to quantify pollen grains of the flower bud of T. farfara, and chemical components were determined by HPLC. The data were analyzed by Statistical Package for the Social Sciences(SPSS) statistics software (IBM SPSS Statistics 19.0). On the basis of the results obtained, the relationships between the quantity of pollen grains and chemical components were established. We expect that the method will be helpful and will provide scientific data for the quality control of the flower bud of T. farfara in the future.

MATERIALS AND METHODS

Standards and reagents

Tussilagone, chlorogenic acid, rutin and isoquercitrin (Batch Nos. 111884–201102, 110753–200413, 100080–200707, 111809–201001, respectively) were purchased from the National Institute for Food and Drug Control (Beijing, China). Chloral hydrate and dilute glycerin (prepared according to the procedures described in Appendix XV B of the Pharmacopoeia of the People's Republic of China) were used for preparation of slides.[10] Analytical-grade methanol and ethanol (Tianjin Company Inc., Tianjin, China) were used for preparation of sample extraction. Methanol, acetonitrile and formic acid (Merck, Germany) of HPLC-grade were used for preparation of the mobile phase and water was distilled water.

Plant materials

Eighteen batches of the flower bud of T. farfara samples were collected in all, including eight batches (KDH-01 to KDH-08) of the flower bud of T. farfara samples provided by cultivation bases in Hebei Province of China, and 10 wild batches (KDH-09 to KDH-18) collected from different regions of China. The samples were identified by Professor Tingguo Kang (Liaoning University of TCM, China). It was necessary to determine the moisture content of all collected samples prior to determining the amount of pollen grains and performing chemical analysis. 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.[11]

Preparation of slides

Slide preparation was performed under optimized conditions. In brief, samples were ground into powder with a particle size of 0.15–0.18 mm. For each sample, 400 mg of powder was weighed accurately, ground with 2 mL chloral hydrate 5 times, 4 mL dilute glycerin was added; the solution was transferred to a 25 mL volumetric flask, adjusted to the volume with chloral hydrate, and 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 three times. In total, 150 slides were prepared and examined for each batch of sample.[3,4]

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

Four reference compounds were accurately weighed and dissolved in 85% methanol for tussilagone and methanol for chlorogenic acid, rutin, and isoquercitrin. These were then diluted to an appropriate concentration for the establishment of calibration curves. All stock and working standard solutions were stored at 4°C until used for analysis.[10,13] Preparation of sample solutions One pulverized sample (1.0 g) was accurately weighed and extracted with 20 mL ethanol under an ultrasonic water bath for 60 min. The supernatant was used as test solution A for tussilagone. Another pulverized sample (0.3 g) was accurately weighed and extracted with 35 mL methanol under an ultrasonic water bath for 45 min. The supernatant was used as test solution B for chlorogenic acid, rutin, and isoquercitrin. The injection volume was 10 μL for solution A and 20 μL for solution B.[10,13]

Instrument and chromatographic conditions

The microscopy equipment used was an Olympus-BX51 system biologic microscope equipped with an Olympus-DP72 camera. The HPLC equipment used was an Agilent Series 1100 liquid chromatograph coupled with HP chemstation. A waters symmetry C18 column (250 mm × 4.6 mm, 5 μm) was used with column temperature set at 30°C. For tussilagone, the mobile phase consisted of methanol (A) and water (B) (85:15) at a flow rate of 1.0 mL/min and the detection wavelength was set at 220 nm. For chlorogenic acid, rutin and isoquercitrin, we used the mobile phase with a flow rate of 1.2 mL/min and the detection wavelength was set at 255 nm. The mobile phase was a mixture of methanol (A), acetonitrile (B) and 0.1% formic acid-water (C), with an optimized linear gradient elution as follows: 0–14 min, 22% A, 78% C; 14–20 min, 22–13% A, 0–15% B, 78–72% C; 20–50 min, 13% A, 15% B, 72% C.[10,13]

Results

To optimize the preparation procedure for CMM suspensions, variables involved in the procedure such as the weight of the sample, particle size and the amount of dilute glycerin were optimized by a uniform design experiment. Six suspensions were prepared according to the method of uniform design [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 pollen grains were observed and counted on each slide with olympus, and then divided randomly into five groups.[3,4] The relative standard deviation (RSD) of the average value of each group was calculated as 8.60%, 7.59%, 6.94%, 4.71%, 4.49% and 2.85%, respectively. We want to obtain the better method for preparing CMM suspensions. Count with(IBM SPSS Statistics 19.0)statistics software relevant relations in the two analyze, must produce the equation of coming back then, important intensity of analyzing every factor. On the

basis of the results obtained above, CMMs were ground into powders of 0.15–0.18 mm in particle size. 400 mg powder was taken and mixed with 4 mL dilute glycerin. Microscopy method validation The series of KDH-01 sample was examined for the linearity of microscopy. The calibration curve was generated to confirm the linear relationship between the pollen grains found on each slide versus the amount of sample (mg) in the test samples. The liner regression equation and correlation coefficient (R2) was y = 0.1054x– 7.7584 (R2 = 0.9938, n = 6). The precision was evaluated with the solution of sample KDH-01 under the selected optimal conditions, and the RSD was 1.95% for the quantity of the pollen grains. The repeatability was evaluated with the solution of sample KDH-01 under the optimal conditions six times, and the RSD was 1.99% for the quantity of the pollen grains.[3,4]

High-performance liquid chromatography method validation

The analyte solutions at six different concentrations were injected in triplicate, and the calibration curves were established by plotting the peak area (y) versus the concentration (x) of each component. The detailed information regarding the calibration curves and linear ranges of the tussilagone, chlorogenic acid, rutin and isoquercitrin are listed in Table 3. The calibration curves showed good linear regression, with a correlation coefficient over 0.9994 within test ranges. The precision was evaluated with solutions of sample KDH-01 under different concentrations. The values of RSDs were 0.82%, 0.89%, 0.77% and 0.98% (n = 5) for tussilagone, chlorogenic acid, rutin and isoquercitrin, respectively. The repeatability was examined by the injection of six different working solutions A and B of sample KDH-01, which were prepared with the same sample preparation procedure. The RSDs were 1.33% 1.68%, 1.74% and 1.75% for tussilagone, chlorogenic acid, rutin, and isoquercitrin, respectively. The solution of sample KDH-01 was injected into the apparatus to evaluate the stability of the solution. The RSDs were 0.93%, 1.13%, 1.25% and 1.03% (n=6) for tussilagone, chlorogenic acid, rutin and isoquercitrin, respectively. The recovery test was performed by adding known quantities of the four standards to the samples. Then, the extraction and analysis were performed five times according to the above sample preparation procedure. The mean recovery of the four marks was 97.19–103.33%. The results showed that the average recoveries were estimated to be 99.47% ±1.28% (mean ± standard deviation [SD], n = 5) for tussilagone, 99.64 ± 1.68% (mean ± SD, n = 5) for chlorogenic acid, 100.8 ± 1.71% (mean ± SD, n = 5) for rutin, and 100.18 ± 1.34% (mean ± SD, n = 5) for isoquercitrin.[13]

Linear regression

The relationship between the quantity of pollen grains and the content of tussilagone was analyzed by linear regression using SPSS 19.0 statistics software. Since no difference was observed between cultivated samples (KDH-01 to KDH-08) and wild samples (KDH-10 to KDH-18), the linear regression analysis was carried out

on cultivated and wild samples together. The results showed significant positive correlation between the quantity of pollen grains and the content of tussilagone, as well as a positive correlation between the quantity of pollen grains and chlorogenic acid, rutin and isoquercitrin. Thus, medicinal material with greater numbers of pollen grains likely contains higher amounts of tussilagone, chlorogenic acid, rutin, and isoquercitrin.[14,15].

DISCUSSION

Capacity analysis was used to quantify pollen grains of the flower bud of T. farfara. When preparing the microscopic analysis slides and sample suspension, in order to reduce possible systematic errors and improve measurement accuracy, three main factors(the weight of original sample, the particle size of original sample and the amount of dilute glycerin added into the suspension) should be considered. Therefore, we used a Uniform Design experiment in order to reduce the influence on the number of pollen grains from the variation in each factor and we only counted whole pollen grains. The results showed: RSD = 12.192–0.023A, R2 = 0.969, F = 123.971, P = 0.000, RSDmin = 2.99(%), where parameter A is the weight of the sample, parameter B is the particle size, and parameter C is the amount of dilute glycerin. This calculation indicated that only parameter A contributed to the RSD, 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.[3,4] In the present study, the combination of microscopy and HPLC offers a comprehensive and effective method for the quality assessment of the flower bud of T. farfara. This study established a relationship between the quantity of one microscopic characteristic and the components of the flower bud of T. farfara. Pollen grains were taken as the microscopic characteristic; tussilagone, chlorogenic acid, rutin, and isoquercitrin were taken as major chemical components. Based on the measuring data of MCC and chemical components, the correlation between MCC and chemical components was analyzed in this paper. The value of regression coefficient $r \geq 0.7$ represents data that has a high correlation between the two factors; $0.7 > r \geq 0.3$ represents a middle degree correlation; $r \geq 0.7$ represents a low correlation. The correlation only can be established in the situation sig $P < 0.05$. The results of the analysis showed that the tussilagone, chlorogenic acid, rutin, and isoquercitrin were significantly and positively correlated with the quantity of pollen grains in the flower bud of T. farfara. In other words, the results indicated that the herbal samples with more pollen grains contain greater amounts of the pharmacologically active components in the flower bud of T. farfara, and would thus be of better quality in clinical application.[16] Many papers regarding the analysis of chemical components have shown that there is a close relationship between the chemical components and harvest time. Dynamic analysis and evaluations illustrate that the traditional harvest time in November to December is a suitable time for

harvesting the flower bud of T. farfara. During this time, the bioactive components dynamically accumulate in plants.[10,17] Meanwhile, during the growth process and with maturity of the flower bud of T. farfara, pollen grains also accumulate.[16] Thus, the increase in the number of pollen grains accompanies an increase in bioactive components. We deduced that this is the reason why herbal samples with more pollen grains contain greater amounts of the pharmacologically active components in the flower bud of T. farfara. In general, the microscopic feature of the quantity of pollen grains can be used for identification, as well as for quality assessment of the flower bud of T. farfara. Due to the complex nature of Chinese herbal preparations, any one component may not be representative of the overall quality. However, if a given CMM has a positive correlation between MCC and chemical components, we could use the established method to evaluate its quality based on the MCC and chemical quantitation.[18] This is the first report for the simultaneous determination of the MCC and chemical components of the flower bud of T. farfara. The established method provides a useful application of the evaluation of chemical and pollen grains for quality control of the flower bud of T. farfara. It is valuable for improving the quality control of Chinese herbal preparations that contain the flower bud of T. farfara and can be applied in the quality control of other medicinal materials.

Conclusion: The study showed that the established method can be helpful for evaluating the quality of the flower bud of T. farfara L. based on microscopic characteristic constants and chemical quantitation.

REFERENCES

1. Kang TG. Authentication of Chinese Medicinal Material. Beijing: China Press of Traditional Chinese Medicine; 2003. p. 28-56.

2. Kang TG. Authentication of Chinese Medicinal Material Monographs. Beijing: China Press of Traditional Chinese Medicine; 2009. p. 140-8.

3. Liang L, Zhao Z, Kang T. Application of microscopy technique and high performance liquid chromatography for quality assessment of Polygonum multiflorum Thunb. (Heshouwu). Pharmacogn Mag 2014;10:415-21.

4. Li Da, Zhang J, Zhang JK, Xu L, Kang TG. Research of correlation between microscopic characteristic constants and chemical components of Carthami Flos. Chin J Exp Tradit Med Formulae 2014;19:57-60.

5. Chen CH, Kang TG. Research of correlation between microscopic characteristic constants and chemical components of honeysuckle. Zhong Yao Cai 2011;34:1373-6.

6. Liang L, Zhao ZZ, Li N, Kang TG. Research on the correlation between microscopic characteristic constant and chemical component of sophorae flos. Zhong Yao Cai 2013;36:572-4.

7. Li N, Li B, Liu XY, Kang TG. Research of correlation of microscopic characteristic constants and chemical components of cortex mouton. Chin Arch Tradit Chin Med 2009;27:1094-5.

8. Yuan DM, Liu Y, Luan XJ, Kang TG. The study on Cortex phellodendri Chinese's by the microscopic quantitative method. Chin Arch Tradit Chin Med 2007;25:964-6.

9. Yuan DM, Luan XJ, Kang TG. Astudy on quantitative microscopic method applied to the identification of Chinese material medica. Liaoning J Tradit Chin Med 2006;33:444-5.

10. State Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China. Beijing: China Medical Science and Technology Press; 2010.

11. Xu GJ. Microscopic Identification of Chinese Medicinal Materials. Beijing: The People's Health Publishing House; 1986.

12. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26:343-56.