

Seasonal Variation, Microscopic and Chromatographic Analysis of Leaves in *Malus hupehensis*: A Protocol for Its Quality Control

SHEN Tao¹, XIANG Lan¹, REN Dong-mei¹, WANG Shu-qi¹, YANG Ming-ren², LOU Hong-xiang^{1*}

1. School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China

2. Linyi Shansong Pharmaceutical Company, Linyi 276036, China

Abstract: **Objective** To establish a quality control protocol based on microscopic, TLC, and HPLC methods, and to verify the optimal harvesting time for the leaves of *Malus hupehensis* (LMH). **Methods** The LMH were pulverized into powder for microscopic identification or TLC and HPLC analysis after ultrasonic extraction with methanol. Seasonal variations of the phlorizin content and average leaf weight were determined by HPLC analysis and weighing up the leaves collected from May to October. **Results** Microscopic and macromorphologic characteristics have been described for the leaf identification. A qualitative TLC assay and a quantitative HPLC method have been established for the quality control of LMH. Phlorizin was selected as a reference marker, which resolved at R_f 0.53 in TLC assay and at 14.0 min in HPLC assay. The content of phlorizin decreased gradually from 17.0% in leaves collected in May to 7.5% in October. The average leaf weight reached the level of 0.6 g in August and maintained until its falling. **Conclusion** These methods are simple, selective, accurate, and reliable for the quality control of LMH. The period from late August to early September is suggested as the optimal harvesting time of the LMH.

Key words: HPLC; *Malus hupehensis*; optimal harvesting time; phlorizin; quality control; TLC

DOI: 10.3969/j.issn.1674-6348.2013.02.012

Introduction

Malus hupehensis (Pamp.) Rehd., belonging to the Rosaceae family, mainly distributes in the south region of Yellow River and areas beneath alt. 2900 m in China (Chinese Academy of Sciences, 2004). Anti-oxidative, antidiabetic, antimicrobial, antifatigue, antihypoxia, and antiproliferative activities of the constituents in the leaves of *M. hupehensis* (LMH) have been investigated *in vivo* and *in vitro* (Zhang *et al*, 2008a, 2008b; Wang, Wang, and Zhang, 1999; Qu *et al*, 2000; Xue *et al*, 2011). Flavonoids are responsible for the diverse biological activities of LMH. The dihydrochalcone phlorizin (Fig. 1) is the main constituent in LMH (State Administration of Traditional Chinese Medicine, 1999), and accounts for the hypoglycemic property of LMH. The mechanism of phlorizin targeting hyperglycemia has been investigated *in vivo* (Dong *et al*, 2006; Dudash, Zhang, and Zeck, 2004), and the inhibition of sodium-glucose cotransporters (SGLTs) and promotion of

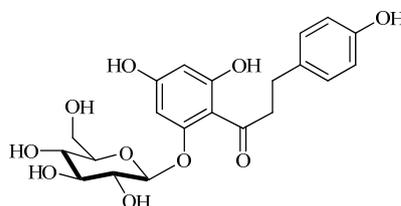


Fig. 1 Chemical structure of phlorizin

glucose excretion and lower postprandial blood glucose are found to be potential functions.

The LMH have been used as tea for the treatment of hyperglycemia in Chinese folk culture. Owing to this traditional use, its potential as an antidiabetic substance has attracted lots of interests, and some pharmaceuticals and health products of this plant have been sold in the market. The extract from LMH has been developed to be a hospital internal-used pharmaceuticals for the treatment of type 2 diabetes in Shandong province. A literature reported a quality control study of LMH collected in Hubei province (Zhu *et al*, 2010). However,

* Corresponding author: Lou HX Address: School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan 250012, China Tel: +86-531-8838 2012 Fax: +86-531-8838 2019 E-mail: loughongxiang@sdu.edu.cn

Received: May 8, 2012; Revised: October 17, 2012; Accepted: December 28, 2012

Fund: Science and Technology Development Program of Shandong Province (2011GSF11909)

Online time: April 20, 2013 Online website: <http://www.cnki.net/kcms/detail/12.11410.R.20130420.1111.002.html>

it was found that the quality of LMH was largely affected by the place of origin. For instance, the phlorizin content of LMH grown in Shandong province is about 10%, compared to that of less than 5% grown in Hubei province. In addition, seasonal variation of the content of phlorizin and the optimal harvesting time of LMH have not been investigated. To ensure the batch-to-batch quality consistency and clinical efficacy of this pharmaceuticals, the quality control and optimal harvesting time on this plant material should be established in the first place.

The present paper aims to verify the macro-morphologic characteristic and microscopic features of LMH, establish TLC and HPLC methods for the qualitative and quantitative analyses, and optimize the harvesting time of LMH.

Materials and methods

Plant material

The leaves were collected from *M. hupehensis* plantation of Shansong Pharmaceutical Company (Linyi, China) from May to October in 2009. The leaves were authenticated by Prof. XIANG Lan from Department of Pharmacognosy, Shandong University (Jinan, China). Voucher specimens (MH090511, MH090630, MH090729, MH090831, MH090918, and MH091030) were deposited at Department of Pharmacognosy.

Reagents and chemicals

Phlorizin was isolated from the LMH and its structure was established based on its MS and NMR data (Lu and Foo, 1998). The purity was greater than 98% by HPLC analysis. Acetonitrile (HPLC-grade) was purchased from Tianjin Siyou Company (China). Other chemical reagents used in the experiment were of analytical grade.

Microscopy

Microscopic identification was carried out according to the protocols in *Chinese Pharmacopoeia* 2010 (Pharmacopoeia Committee of P. R. China, 2010). For preparation of transverse section slides, the dried LMH were softened and then cut by a sliding microtome to a thickness of 10–20 μm . The samples were examined under a microscope (Nikon E200) after treated with chloral hydrate test solution. For the preparation of powder slides, a small quantity of LMH powder was spread on a slide and treated with chloral

hydrate test solution. For the preparation of surface slides, the upper and lower epidermis were torn out from the moistened and softened LMH material, the chloral hydrate test solution was added, and the microscopic examination was carried out.

Reference solution

The stock solution was prepared by dissolving phlorizin in methanol to obtain a concentration of 1.0 mg/mL. The working reference solution was prepared by diluting the stock solution with methanol.

Extraction procedure

The LMH were pulverized into powder and sifted through a 24 mesh sieve. The powdered sample (0.2 g) was ultrasonically extracted with methanol (50 mL) at room temperature for 30 min. The extraction solution was filtered and the filtrate was adopted for TLC analysis. For HPLC quantitative analysis, the working sample solution was prepared by dissolving 1 mL of above solution in a 10 mL volumetric flask with methanol, and then filtered through a 0.22 μm membrane before injection.

Chromatographic conditions

TLC chromatography was performed on pre-coated silica gel GF254 plates (Qingdao Ocean Company, China). The sample solution (1.0 μL) and the reference solution (1.0 mg/mL) were loaded for TLC analysis. HPLC analysis was performed using an Agilent 1200 system equipped with degasser, auto sampler, and diode array detector (Agilent Technologies, USA). An Agilent Zorbax C₁₈ column (250 mm \times 4.6 mm, 5 μm) was used for the separation. The mobile phase consisted of acetonitrile (A) and water with 0.5% formic acid (B) (22:78) at a flow rate of 1.0 mL/min. The column temperature was maintained at 25 $^{\circ}\text{C}$, and the injection volume was 10 μL . Detection wavelength was set at 280 nm.

Results

Macromorphology

The LMH were flat or shrink, ovate to elliptic with a petiole of 1–3 cm; The leaf size was 5–10 cm in length and 2.5–4.0 cm in width; The apex is acuminate and margin is serrulate, with a broad cuneate base; The reticulate venation was pinnate with 5–6 pairs of lateral veins and obvious main longitudinal veins in lower surface; There was pubescence in main

vein and petiole; It was dark green in upper surface and little thinner in lower one (Fig. 2).



Fig. 2 Leaf of *M. hupehensis*

Anatomical feature

Mesophyll is differentiated into upper 2—3 layer palisade and lower loose spongy tissue (Fig. 3a). The epidermis was composed of a layer of rectangular cells. Upper epidermis is covered with cuticle (Fig. 3b). There are nonglandular trichomes on the surface of lower epidermis. Stomata type is anomocytic (Fig. 3c). The central midrib contains the semilune-type main vascular bundle, the large xylem vessels are visible, and the phloem tissue is observed below xylem (Fig. 3d). The prismatic and clustered crystals of calcium oxalate existed in parenchyma tissue.

Powder analysis

The powder of LMH was green in color and coarse to moderate coarse. In microscopic study, the upper epidermal cells of LMH are more or less polygonal with straight anticlinal walls, the cells of lower epidermis have more sinuous anticlinal walls. Stomata type is

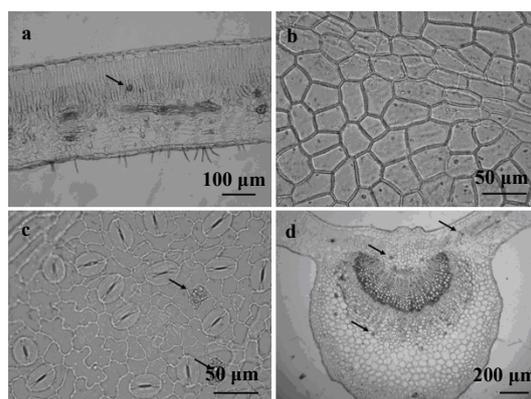


Fig. 3 Anatomical features of *M. hupehensis*

a: leaflet lamina b: upper epidermal cells c: lower epidermal cells and anomocytic stoma d: calcium oxalate crystals

anomocytic with 4—5 subsidiary cells. Besides, prismatic crystal, clustered crystal, and crystal fiber (Fig. 4a—4c), together with spiral and reticulate vessels (Fig. 4d and 4e), and nonglandular trichome (Fig. 4f) have been found to be the characteristic features of the powder.

TLC analysis

The developing solvent for TLC analysis has been optimized in order to obtain high resolution, round-shape spot and optimum R_f value. Chloroform-methanol-formic acid (16:4:1) was selected as the most suitable developing solvent. In the view of the hydroxy groups in the molecules of phlorizin and flavonoids, 2% FeCl₃-2% K₃[Fe(CN)₆] solution was adopted as color-developing agent before detection. As shown in Fig. 5, phlorizin was detected as a major constituent in

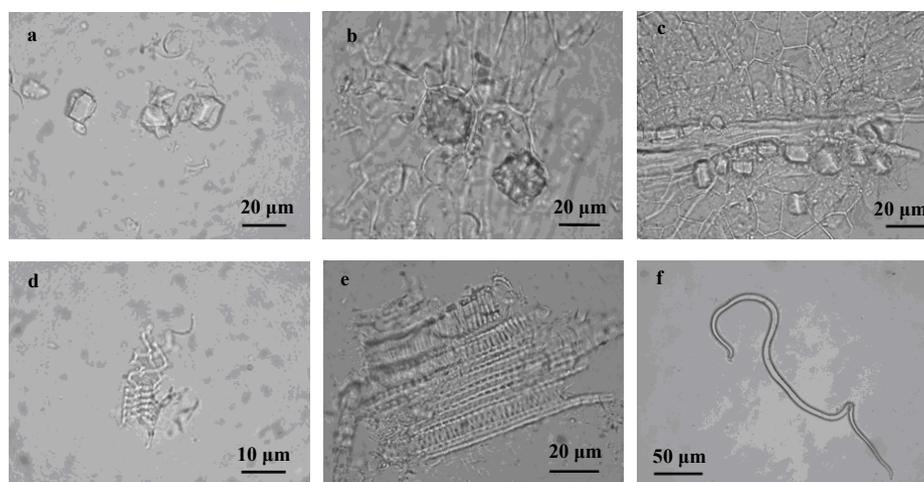


Fig. 4 Powdered elements of *M. hupehensis*

a: prismatic crystal b: clustered crystal c: crystal fiber d: spiral vessel e: reticulate vessel f: nonglandular trichome

methanol extract from LMH with Rf value of 0.53 on TLC plate. In addition, two minor constituents resolving at Rf values of 0.34 and 0.94 were also observed.

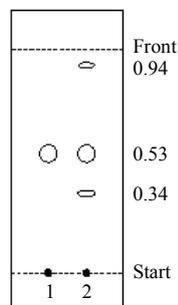


Fig. 5 TLC chromatogram of reference phlorizin (1.0 µg/spot) (1) and methanol extract from LMH (2)

Method validation of HPLC quantitative analysis

The HPLC method was validated in terms of linearity, limit of detection, limit of quantification (LOQ), precision, stability, repeatability, and recovery tests.

The calibration curve for phlorizin was constructed

using five points corresponding to 100, 300, 600, 1200, and 1800 ng of phlorizin injected on-column, and then the peak areas (Y) versus the amounts of phlorizin (X) were plotted. The results showed that phlorizin had a good linearity with a calibration equation of $Y = 1806.1X - 38.307$ ($R^2 = 0.9996$) in the range from 100 to 1800 ng.

The limit of detection (LOD) for phlorizin, defined as a signal-to-noise ratio of 3:1, was 0.4 ng. The LOQ was defined as a signal-to-noise ratio of 10:1, and the LOQ value for phlorizin was 2.0 ng.

Precision of the method was assessed by replicate analysis of phlorizin (20 µg/mL). The stability was estimated by analyzing the sample solution (MH090918) at 0, 4, 8, 12, and 24 h at room temperature. Five independently prepared samples of LMH (MH090918) were analyzed to evaluate the repeatability of this method. The results of precision, stability, and repeatability (Table 1) were expressed as relative standard deviation (RSD), which are 0.075%, 2.26%, and 1.79%, respectively.

Table 1 Precision, stability, and repeatability of HPLC analysis of phlorizin

Batch No.	Precision		Stability		Repeatability	
	Peak area	t / h	Peak area	Amount / g	Peak area	Content / %
1	377.1	0	878.7	0.201 02	878.7	11.7
2	377.5	4	879.5	0.200 96	877.2	11.7
3	377.5	8	880.7	0.201 14	902.8	12.1
4	377.9	12	870.5	0.201 27	913.9	12.2
5	377.5	24	834.3	0.198 89	872.8	11.7
Mean	377.5		868.7		11.9	
RSD / %	0.075		2.26		1.79	

Recovery test was performed in six replicates through adding 25 mg phlorizin to 0.2 g of the known MH090918 leaf sample (with phlorizin content of 11.6%), and then the extraction and analysis were carried out as described above. Recovery was calculated according to the formula as follows: recovery = (amount detected – original amount) / amount added. The recovery of phlorizin was 97.6%, with a RSD of 1.36%.

These results indicate that the established HPLC method is reliable and applicable for the determination of the content of phlorizin in *M. hupehensis*.

HPLC analysis of LMH samples and validation of optimal collecting time

The established HPLC method was applied to

quantitative analysis of LMH. The typical chromatograms of phlorizin reference solution and LMH sample are shown in Fig. 6. The retention time of phlorizin is 14.0 min, and the analytical process could be completed within 25 min. Addition of 0.5% formic acid avoided the asymmetry of chromatographic peak. Under the operating condition, the running time, resolution, and symmetry were satisfied with the quantitative requirements.

The contents of phlorizin in six LMH samples collected from May to October were determined. The results were shown in Table 2. It displayed that the content of phlorizin gradually decreased from 17.0% in May to 7.5% in October. When the leaves turned yellow and fell in October, the content of phlorizin

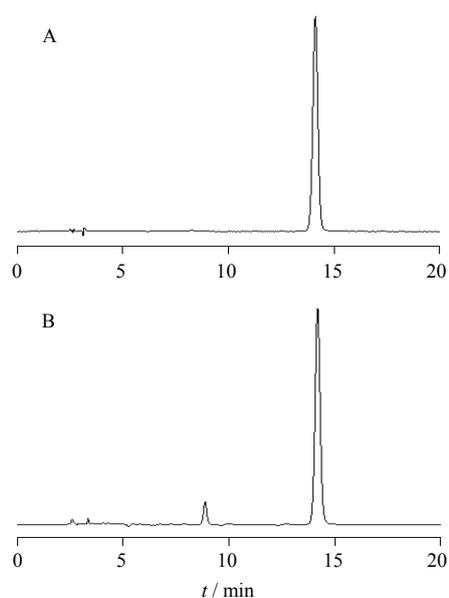


Fig. 6 HPLC chromatograms of phlorizin reference solution at 20 µg/mL (A) and *M. hupehensis* sample solution for MH090918 (B)

dropped rapidly during this period.

Besides the content of phlorizin, the dry weight of LMH, which decides the absolute quantity of phlorizin, should be concerned in validating the optimal collecting time for *M. hupehensis*. As shown in Table 2, the average dry weight of LMH increased with the leaf growth, from 0.101 to 0.637 g, which is contrary to the variation trend of phlorizin content. Moreover, the weight of LMH increased slightly since August, and kept above 0.6 g. Taking account of absolute quantity of phlorizin in LMH, the late August and the early September are suggested to be the appropriate harvesting time.

Discussion

Macromorphologic and microscopic features and TLC identification would be beneficial for the qualification of LMH. It was observed that calcium oxalate

Table 2 Color, contents of phlorizin, and average weights of LMH collected in different time ($\bar{x} \pm s$, $n = 3$)

Samples	Collecting time	Color	Contents of phlorizin / %	Average weight / g
MH090511	2009.5.11	green	17.0 ± 0.529	0.101 ± 0.0068
MH090630	2009.6.30	green	16.8 ± 0.265	0.230 ± 0.0087
MH090729	2009.7.29	green	14.9 ± 0.556	0.294 ± 0.0105
MH090831	2009.8.31	green	13.1 ± 0.458	0.611 ± 0.0075
MH090918	2009.9.18	green	11.6 ± 0.300	0.624 ± 0.0061
MH091030	2009.10.30	yellowish-brown	7.5 ± 0.215	0.637 ± 0.0089

crystal, particular prismatic crystal, clustered crystal and crystal fiber, spiral and reticulate vessels, together with nonglandular trichome and anomocytic-type stomata in lower epidermis (Figs. 3 and 4) were of significance in the recognition of fragmented leaflets. These results were similar with the reported data (Zhu *et al.*, 2010). In the TLC assay, the coloration of phlorizin using 10% H₂SO₄-EtOH solution is unclear and needs heating. We improved previous methods and adopted 2% FeCl₃-2% K₃[Fe(CN)₆] solution as color-developing agent to improve the property of being selective, and avoid the interference from miscellaneous constituents of LMH. A clear phlorizin dot was detected in the TLC plate with R_f value of 0.53 (Fig. 5). The application of combined microscopy and TLC assay was useful in distinguishing *M. hupehensis* from the other plant species in Rosaceae family.

Phlorizin, the main constituent in LMH, could

lower the blood glucose level *in vivo* (Dong *et al.*, 2006; Dudash, Zhang, and Zeck, 2004), and be responsible for the traditional use of LMH as an antidiabetic medicine in China. Hence, it was the first choice as the reference marker in HPLC assay. The addition of formic acid to the mobile phase provided an acid environment (pH < 7) to keep phlorizin in molecular form, and a satisfying chromatographic peak shape fulfilling with the quantitative requirement was received. Phlorizin revealed a retention time of 14.0 min and the whole analytical process was completed within 25 min. The LOD and LOQ were only 0.4 ng and 2.0 ng for phlorizin. The RSD values in precision and the results of stability and repeatability tests were less than 3.0% as shown in Table 1. The established method was simple, sensitive, and accurate, which was reliable for the quantitative analysis.

The phlorizin contents of LMH collected in Linyi

city, Shandong province from May to October were determined using the HPLC method. The contents decreased gradually from 17% in May to 7.5% in October, and were about two-folds higher than that (about 4%) in the leaves collected in Hubei province (Zhu *et al.*, 2010). This result indicated that the planting region influenced the quality of LMH largely.

The dry weight of LMH is an important factor to verify the harvesting time. It reaches the level of 0.6 g from August and has little increase until leaf falling in October. By consideration of the dry weight of LMH and the phlorizin contents, the late August and the early September are found to be the optimal harvesting time.

References

- Chinese Academy of Sciences, 2004. *Flora of China*. Vol. 36. Science Press: Beijing.
- Dong HQ, Ning ZX, Yu LJ, Li L, Lin LC, 2006. Anti-hyperglycemia and effects of flavonoid phloridzin from *Lithocarpus polystachyus* Rehd on diabetic model mice. *Food Sci* 27(12): 714-718.
- Dudash J, Zhang X, Zeck R, 2004. Glycosylated dihydrochalcones as potent and selective sodium glucose cotransporter 2 (SGLT2) inhibitors. *Bioorg Med Chem Lett* 14(20): 5121-5125.
- Lu Y, Foo LY, 1998. Constitution of some chemical components of apple seed. *Food Chem* 61(1/2): 29-33.
- Pharmacopoeia Committee of P. R. China, 2010. *Pharmacopoeia of People's Republic of China*. China Medical Science and Technology Press: Beijing.
- Qu KY, Hu HH, Du YY, Guo LJ, 2000. Experiment study of pharmacodynamics of *Malus hupehensis* decoction. *Lishizhen Med Mater Med Res* 11(2): 107.
- State Administration of Traditional Chinese Medicine, 1999. *Chinese Materia Medica*. Vol. 10. Shanghai Science and Technology Press: Shanghai.
- Wang CL, Wang P, Zhang ZW, 1999. Effect of *Malus hupehensis* on blood glucose in rat. *Prim J Chin Mater Med* 13(2): 19-20.
- Xue BJ, Cao D, Zhou JG, Zou KN, Wang JZ, 2011. Effects of total flavonoids of the *Malus hupehensis* on proliferation and differentiation of osteoblasts and activity of osteoclasts. *Chin J Tradit Med Traumatol Orthop* 19(5): 12-14.
- Zhang HQ, Wang JZ, Zou K, Dan FJ, Wang XQ, Li L, Guo ZY, Yang J, 2008a. Study on *in vitro* antioxidant effects of extractive from *Malus hupehensis* (Pamp.) Rehd. *Food Sci Technol* 33(11): 183-186.
- Zhang X, Fei Y, Wei W, Yan J, 2008b. Study on antioxidant activity of flavonoids from Hubei wingnut (*Malus hupehensis*) leaf. *Acad Period Farm Prod Proc* 2008(4): 38-40.
- Zhu J, Liu C, Zhang H, Wang X, Wang J, Guo Z, Zou K, 2010. Study on the quality standard of leaves of *Malus hupehensis*. *J Chin Med Mater* 33(5): 707-710.