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Original Research Article

Chromatographic determination of siphonodin content: A rapid and simple strategy for discriminating between *Hemsleya omeiensis* and other sources of Xuedan

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Abstract

Purpose: *To develop a rapid and simple siphonodin content-based high performance liquid chromatography (HPLC) method to distinguish Hemsleya omeiensis from other sources of xuedan.*
Methods: *Siphonodin was isolated from Hemsleya omeiensis and identified by x-ray crystallographic analysis. An optimized HPLC method was applied for the determination of siphonodin contents of H. omeiensis, H. dolichocarpa and H. gigantha.*
Results: *Siphonodin was successfully separated by the optimized HPLC method in < 10 min, and the results of validation showed that the HPLC method was stable and very accurate for the quantification of siphonodin. The mean content of siphonodin in 10 batches of H. omeiensis was 3.78 mg/g, but the compound was not detectable in H. dolichocarpa and H. gigantha using the developed HPLC method.*
Conclusion: *These results indicate that the developed HPLC method is suitable for distinguishing H. omeiensis from other sources of xuedan.*

Keywords: *Xuedan, Hemsleya omeiensis, Hemsleya giganth, Hemsleya dolichocarpa, Siphonodin, Discrimination*

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INTRODUCTION

It is known that herbal medicines are beneficial for protecting humans from diseases, and they are important sources of new drugs for the cure and prevention of diseases [1,2]. In Chinese folk medicine, x*uedan* is a traditional herbal medicine used for *clearing heat* and removing toxins. It has for long been widely used in clinics as a folk and conventional herb for the treatment of ulcers, bacillary dysentery and all kinds of inflammations [3,4]. *Xuedan* is derived mainly from three varieties of *Hemsleya*: *Hemsleya omeiensis*, *Hemsleya gigantha* and *Hemsleya dolichocarpa*. Compared with other varieties, *Hemsleya omeiensis* is not bitter, and it is associated with better clinical compliance and high price. Several studies have reported appreciable differences in compositions among the three sources of *xuedan*, especially in their bitter taste [5-8].

However, it is usually difficult to distinguish them in morphology when these medicinal herbs are dried. Previous studies revealed that *xuedan* contains chikusetsu saponin IVa, hemslosides Ma_1, hemslosides G_1, hemslosides H_1, dihydrocucurbitacin F-25-O-acetate, dihydrocucurbitacin F and oleanolic acid-3-O-a-L-arabinopyranosyl glucurropyranoside E [7]. Siphonodin [4-hydroxymethyl-2(5H)-furanone] is a natural compound found in a variety of *Euonymus* leaves and small nest moths, and it has also been isolated from *Rambutan* seeds and *H. Ellipsoidea* [9-11]. It is one of the 2(5)-furanone compounds structurally similar to the bacterial quorum sensing system signaling molecule N-acyl homoserine lactone (AHL), and it is widely studied as bacterial quorum sensing inhibitor [12-14]. Previous studies reported that siphonodin exerted bacterial biofilm inhibitory activity against *Pseudomonas aeruginosa*, and that its structure can be modified [15]. Consequently, the aim of this study was to develop a rapid and simple siphonodin content-based HPLC method for distinguishing *H. omeiensis* from other sources of *xuedan*.

EXPERIMENTAL

Plant materials, processing and reagents

Fresh tubers of *H. omeiensis* and *H. dolichocarpa* were collected at *Emei* Mount and *Peng* County, respectively, in Sichuan Province of China, in May 2017. Fresh tubers of *H. gigantha* were collected at *Shimian* County, Sichuan Province of China in August 2017. The samples were identified by Prof Yue-cheng Li, a taxonomist at the Sichuan Institute for Food and Drug Control, Chengdu, China. Voucher specimens were deposited in the herbarium of the College of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, China. Fresh, whole tubers were cut into slices and oven-dried at 55 ^{o}C. The dried tubers were ground with a universal high-speed milling machine (Bingdu Electric Co. Ltd, Shanghai, China), and then sieved through a 65 mesh prior to use in subsequent experiments. The water used was purified with a UPH-1-10T model water purifier system (Chengdu ultrapure Technology Co., Ltd, China). All other chemicals were of HPLC or reagent grade. Silica gel (200 – 300 mesh, and 60 - 80 mesh) were supplied by Qingdao Marine Chemical Corporation, China.

Sample purification

For thin layer chromatography (TLC) analysis, powdered tuber (2 g) was dissolved with 20 mL of methanol in a tightly sealed conical flask. It was then subjected to ultrasonic processing for 40 min, and filtered. Thereafter, 10 mL of the filtrate was concentrated to 4 mL. For HPLC analysis, powdered tuber (0.2 g) was weighed precisely and placed in a weighed 50 mL conical flask to which HPLC-grade methanol (25 mL) was added, and the flask and contents were subjected to ultrasonic treatment for 30 min. On cooling and weighing, some methanol (MeOH) was added, with shaking, and the solution was through 0.45 μm polytetrafluoroethylene filter before injection.

TLC analysis

The TLC separations were performed on G plates (Qingdao Marine Chemical Corporation, China). The sample solutions (5 μL) were applied at the origin on the TLC plate, and developed with petroleum ether/ethyl acetate/methanol at a volume ratio of 5:1:0.5. The developed plates were air-dried and the spots were made visible by spraying with 2 % vanillic aldehyde in 10 % ethanol sulfate solution, followed by heating at 105 °C for 2 min. The spots were visualized at 365 nm in a UV trans-illuminator (Baoshan Gucun Electro Optic Instrument Factor, Shanghai, China).

Extraction and isolation of siphonodin

The powdered tubers of *H. omeiensis* L. T. Shen. et. W.J. Chang (1.04 kg) were extracted 7 times with methanol (MeOH) under reflux (MeOH 4 L x 7, 6 h each), and filtered. After evaporation of the solvent under reduced pressure, the MeOH extract (217 g) was dissolved in warm water and then partitioned in ethyl acetate (EtOAc). The EtOAc fraction (34 g) was subjected to silica gel column chromatography with a gradient elution system of petroleum ether: EtOAc (10:1, 8:1, 5:1, 3:1, and 1:1 v/v) to give eight fractions (Fr.A - Fr.H). The combined Fr.F (17 g) was re-chromatographed over silica gel using petroleum ether: EtOAc (3:1, 1:1 v/v) to give three fractions (Fr.I -FrIII). The Fr.III (14 g) was recrystallized from petroleum ether to give siphonodin (12 g).

HPLC analysis

Instrument and chromatographic conditions

The HPLC separations were carried out on a Shimadzu LC-2030C3 D model instrument (Shimadzu Corp., Kyoto Japan) using a reverse-phase Shim-pack GIST C_{18} (250 × 4.6 mm, 5 μm) column (Shimadzu Corp., Kyoto Japan) at a column temperature of 30 °C. The samples were eluted that using a gradient program of the mixture of acetonitrile and 0.1 % phosphoric acid

(Table 1). The flow rate was 0.8 mL/min, and the sample injection volume was 5 μL. The UV detection wavelength was set at 210 nm.

Table 1: Gradient elution program

Time (min)	PAA	Acetonitrile (%)
0.01	95	5
10	90	10

Preparation of standard solution

The standard solution was prepared with siphonodin dissolving in methanol to a final concentration of 0.2224 mg/mL.

Method validation

The method validation contains for linearity and range, limit of detection (LOD) and limit of quantification (LOQ), accuracy, precision, and recovery test [16].

Linearity assessment

Linearity of the method was obtained by the determination of the peak area of the same reference solution with sample sizes of 1, 2, 3, 5, 7, 9 and 10 µL. Calibration curves were constructed by plotting the peak areas against the corresponding concentrations of the compound. The regression equation was calculated by using the reference quantity of the control sample as the horizontal axis (*x*), and the peak area of the chromatogram as the ordinate (y).

Precision assessment

The precision was obtained by injecting the same reference solution of 5µl sample size 6 times into the HPLC column. From the areas obtained, the % relative standard deviation (RSD) value for siphonodin was calculated.

Repeatability

The repeatability was obtained from six copies of determinations of a 5-µL sample solution (SI) from *H. Omeiensis.* The RSD was calculated based on the mass fraction of siphonodin.

Solution stability

Stability was tested with one sample solution stored at room temperature at several time points (0, 3, 6, 9, 12, 15, 18 and 24 h) after preparation, and the RSD values were calculated. The sample size used was 5 µL.

Recovery test

Six samples (S6, 0.1 g) of *H. omeiensis*, determined already, were weighed precisely. Then, 1 mL reference solution in which the concentration of the siphonodin was 2.251 mg/mL was added to each sample. Using the above method of sample preparation to make one concentration level (100 %), the peak areas of mixed reference solutions were determined, with sample size of 5 µL, and the recovery was obtained. The average recovery was estimated as percentage of analyte.

Limits of detection and of quantification

The LOD and LOQ values were the corresponding concentrations when the signal-to-noise ratio was 3:1 and 10:1.

RESULTS

TLC differentiated *H. omeiensis* from other *Xuedan*

For TLC analysis, three sources of *Xuedan* were extracted in methanol, and the reference compound was dissolved in methanol and applied on the bottom of a silica gel-coated TLC plates as spots. Under the experimental condition, a light green fluorescence spot appeared only in the samples of *H. omeiensis* under 365 nm with retention factor (Rf) of 0.53 (Figure 1). For the samples of *H. dolichocarpa* and *H. gigantha,* the signal at Rf 0.53 was absent. It was observed that 25-O-acetyl-23,24-dihydrocucurbitacin F and 23, 24-dihydrocucurbitacin F appeared as yellow spots under the fluorescent lamp and 365 nm, with Rf values of 0.68 and 0.39, respectively. The yellow spots occurred at Rf of 0.68 and Rf of 0.39 for *H. dolichocarpa* and *H. gigantha,* respectively when their bitter tubers were extracted in methanol, whereas the yellow spots with Rf values of 0.0.68 and 0.39 were absent in the samples of *H. omeiensis*.

Identification of compounds

Siphobodin: $C_5H_6O_3$, light yellow crystal (petroleum ether). The absolute configurations of this compound were identified by X-ray crystallographic analysis (Figure 2). Comparing the published physical and NMR data, the compound was siphonodin [4-hydroxymethyl-2(5H)-furanone] [17]. This structurally simple butenolide was first isolated from *H. omeiensis* while it was reported that siphonodin in the

Hemsleya genus was first isolated from *H. ellipsoidea*.

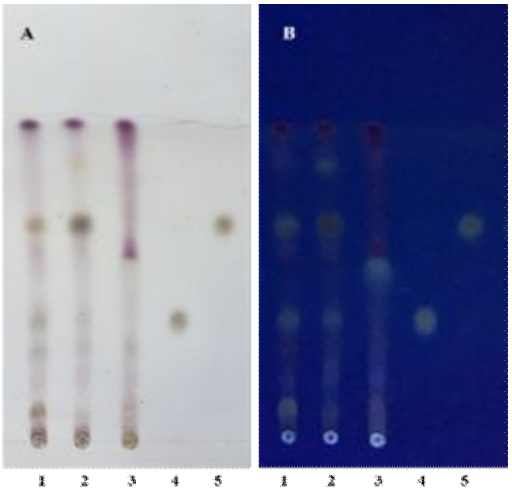

Figure 1: TLC identification of three sources of *Xuedan.* The plate was visualized under fluorescent lamp (A), and at 365 nm (B). TLC samples were prepared by methanol extraction. *H. dolichocarpa* (lane 1); *H. gigantha*, (lane 2); *H. Omeiensis* (lane 3); 23, 4-dihydrocucurbitacin F (lane 4) and 25-O-acetyl-23,24-dihydrocucurbitacin F 9lane 5)

Figure 2: ORTEP representation of siphonodin

Method validation results

Linearity

Linear calibration curves of siphonodin were obtained over the calibration range of 0.2224 - 2.224 µg. The linear regression equation was:
y = 7,056527.51 x + 83684.35 ……. (1) (r^2 = 0.9998 for siphonodin).

Precision

In the precision test, the RSD value of the peak area of the siphonodin was 0.33 %, which indicated that the instrument had high precision.

Repeatability

In the repeatability test, the RSD values of each component was less than 0.99 %, indicating that the method had good repeatability.

Solution stability

In the stability test, the RSD value of siphonodin was 0.64 %. The solution stability results showed that peak area of siphonodin remained approximately unchanged up to 24 h: no significant degradation was observed within the indicated period.

Recovery

In the recovery test, average recovery was 96.50 % and RSD value was 1.34 %, as shown in Table 2.

Limits of detection and of quantification

The LOD and LOQ for siphonodin were estimated at signal-to-noise ratios of 3:1 and 10:1 as 0.0741 ng and 0.2964 ng, respectively.

Content of siphonodin in three sources of *Xuedan*

The results of analysis of 30 batches of samples in the three sources of *xuedan* revealed that siphonodin was detected only in *H. omeiensis*, while there was no chromatographic evidence of siphonodin in *H. gigantha* and *H. dolichocarpa* (Figure 3). Thus, the new HPLC–UV method for determination of siphonodin would be useful for discriminating between *H. omeiensis* and other sources of *xuedan*.

Under the above chromatographic conditions, chromatographic peaks of the sample solution and reference solution had the same retention time. The degree of separation of siphonodin in all samples was greater than 1.5, the theoretical plates were greater than 60000, and the method gave good specificity.

Table 2: Recovery of the HPLC method for determination of siphonodin

Original found	Amount spiked (mg)	Amount found	Recovery (%)	Mean recovery (%)	RSD (%)
2.1418	2.251	4.078	97.42	96.50	1.34
2.1418	2.251	4.038	95.32		
2.1418	2.251	4.103	98.40		
2.1418	2.251	4.038	95.30		
2.1418	2.251	4.047	95.59		
2.1418	2.251	4.076	96.96		

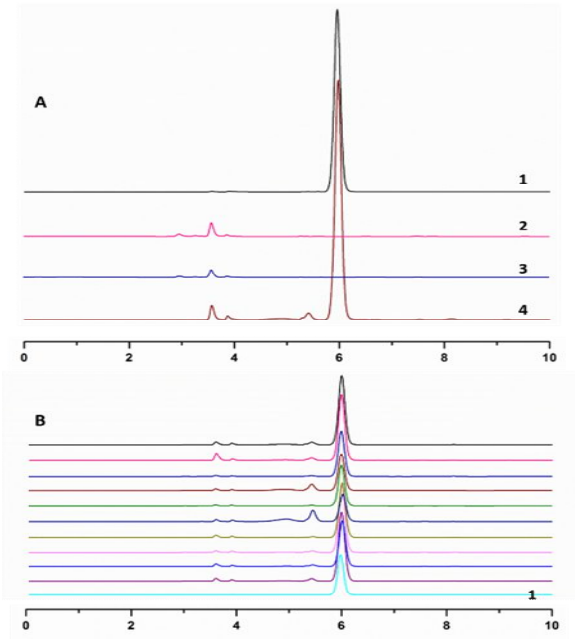

Figure 3: HPLC chromatograms: comparison of three sources *Xuedan* (A) and 10 batches of test samples from *H. omeiensis* (B); 1 - 4 in Figure A represent siphonodin, *H. dolichocarpa* ,*H. gigantha* and *H. omeiensis* respectively

The quantitative results on siphonodin from the 30-batch samples in the HPLC-PDA assays are shown in Table 3. In *H. omeiensis*, the average content of siphonodin was 3.78 mg/g. However, the contents of siphonodin in *H. dolichocarpa* and *H. gigantha* were 2.02 x 10^{-3} and 1.56 x 10^{-3} mg/g, respectively, and were approximately below the limit of quantitation. Therefore, siphonodin content could be a marker for identification of *H. omeiensis*.

Table 3: Content of siphonodin from the three sources of *xuedan*

No.	*H. omeiensis* (mg/g)	*H. dolichocarpa* (mg/g)	*H. gigantha* (mg/g)
S1	4.14	0.90x10^{-3}	1.69x10^{-3}
S2	4.73	2.34x10^{-3}	2.73x10^{-3}
S3	3.66	1.82x10^{-3}	1.00x10^{-3}
S4	3.04	2.11x10^{-3}	2.07x10^{-3}
S5	3.23	1.99x10^{-3}	0.81x10^{-3}
S6	2.14	1.77x10^{-3}	0.81x10^{-3}
S7	3.97	4.49x10^{-3}	1.31x10^{-3}
S8	4.30	1.92x10^{-3}	1.18x10^{-3}
S9	3.49	1.29x10^{-3}	2.67x10^{-3}
S10	5.11	1.52x10^{-3}	1.34x10^{-3}
Mean	3.78	2.02x10^{-3}	1.56x10^{-3}

DISCUSSION

In a previous study, 25-O-acetyl-23,24-dihydrocucurbitacin F and 23, 24-dihydro-cucurbitacin F were identified as responsible for the bitter taste in *xuedan* [18]. These cucurbitacins are usually used as markers of quality control, and for distinguishing between various *xuedans* [19]. However, these compounds are not suitable markers for *H. omeiensis*. From the results obtained in this study, the light green spot with Rf 0.53 was set as a specific marker for the identification of *H. omeiensis*. This was the basis of subsequent guided isolation using TLC analysis.

Analysis of siphonodin in 30-batch samples from three sources of *xuedan* revealed that siphonodin was detected only in *H. omeiensis*. Chromatographic characteristics of siphonodin were absent in *H. gigantha* and *H. dolichocarpa*. Thus, this new HPLC–UV method for determination siphonodin would be useful for distinguishing *H. omeiensis* from other xuedan sources.

CONCLUSION

A simple, selective, accurate and durable HPLC method for the determination of siphonodin contents of *xuedan* has been successfully developed. The developed method is the first reported HPLC method for the analysis of siphonodin contents of three sources of *xuedan*. The results of the analysis of the *xuedan* samples suggest that this method can be applied for the successful identification of crude drugs from *H. omeiensis*.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

Authors' contribution

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. QWH is corresponding author on the study. YQ was first author and responsible for collecting materials,

doing experiment, writing the paper. JLS and JW edited in the article pictures. HLZ and QWH analyzed the article and made recommendations. RCY provided samples. All authors read and approved the final manuscript.

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