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Full Length Article

Identification and Determination of Total Saponins from Radix (*Pulsatilla chinensis*)

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Abstract

In this research, the thin layer chromatography (TLC) was performed to identify saponins from root of *Pulsatilla chinensis* (Bunge) Regel (*Pulsatilla chinensis*) extracts qualitatively and the total saponins content was determined by ultraviolet spectrophotometry, and orthogonal experiment was used to optimize the determination condition. The results showed that the Radix *Pulsatilla* extracts contained *Pulsatilla* saponin D, A and F, and Oleanolic acid 3-O-α-D-Pyranoglucosyl-(1→4)-α-D-Pyranoglucosyl-(1→3)-α-L-Pyridine rhamnosyl-(1→2)-α-L-Pyranosine arabinoside. Moreover, the optimized conditions of content determination were as follows: adding 5% vanillic-acetic acid (0.2 mL) and perchloric acid (0.8 mL) for water bath at 90℃ for 40 min. The content of total saponins from Radix *Pulsatilla* extracts of five batches was greater than 70%. In conclusion, the method reported in this paper can be used to identify saponins from Radix *Pulsatilla* qualitatively. The determination method of total saponins was simple and had good repeatability, which can provide the basis for the further study of quality standard of Radix *Pulsatilla*.

Keywords: Radix *Pulsatilla* extracts; TLC identification; Ultraviolet spectrophotometry; Orthogonal experiment; Content determination

Introduction

Radix *Pulsatilla*, which had an effect of "heat-clearing and cooling blood for the diarrhea", was first published in Shennong's Herbal Classic (Anonymous, 1998; National Pharmacopoeia Commission, 2015). It belongs to the roots of the Ranunculaceae plant, and often inhabits at the sunny side of the hillside and exists in the North Temperate Zone. The surface of Radix *pulsatilla* root is yellowish brown with irregular lines (Li, 2018) and can be distinguished by its characteristics of tissue structure and bast fiber under the microscope (Qian *et al.*, 2017).

Recent studies indicated that Radix *Pulsatilla* extracts had anti-tumor, anti-inflammatory and immune-enhancing activities (Liu *et al.*, 2008; Zhang and Jiang, 2009; Hu *et al.*, 2018; Zhong *et al.*, 2019). However, studies of *Pulsatilla* extracts in quality control were less reported. In order to provide a basis for further study of the quality standards of *Pulsatilla* extract and lay a foundation for the collection of pharmacies of *Pulsatilla* saponins, this paper intended to identify several saponins with clear drug effects in Radix *Pulsatilla* extracts by thin layer chromatography (Sun *et al*., 2010; Shu *et al*., 2011; Luo *et al*., 2018), and determine the content of total saponins by ultraviolet spectrophotometry.

Materials and Methods

Instruments and Reagents

TU-1950 UV spectrophotometer (Beijing Pu Analysis General Instrument Co., Ltd.), Digital thermostat water bath (Gongyi City Yuhua Instrument Co., Ltd.), Ultrasonic cleaner (KQ5200DE, Kunshan Ultrasonic Instrument Co., Ltd.), Electronic balance (LBA-520, Kunshan Yuheng Electronic Weighing Co., Ltd, Thin layer chromatography expansion cylinder (Shanghai Xinyi Instrument Co., Ltd.), Silica gel G board (Qinghai Marine Chemical Plant).

Standard *Pulsatilla* saponin D, *Pulsatilla* saponin A, *Pulsatilla* saponin F, Oleanolic acid 3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-α-L--rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl (National Engineering Research Center for Traditional Chinese Medicine Solid Preparation Manufacturing Technology, with the batch numbers of 2013062, 20130407, 20140521

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and 20140521, Purity≥98%), Methanol (AR), Perchloric acid, N-butanol, Chloroform, Ethyl acetate (Xiqiao Chemical Co., Ltd.), Glacial acetic acid (Tianjin Fuchen Chemical Reagent Factory), Vanillin (National Pharmaceutical Group Chemical Reagent Co., Ltd.), etc.

Dried Radix of *P. chinensis* was purchased from a Chinese herbal store in Suzhou City, which was grown in the wild and collected in April, 2007 in Heilongjiang Province, China. This plant was identified by Professor Xiaoran Li at College of Pharmaceutical Sciences, Soochow University. A voucher specimen (No. 08-02-15-18) was deposited at Soochow University. The dried root was extracted with 70% alcohol under reflux for three times. The solvent was under reduced pressure conditions. The residue was separated on a D101 resin column, which was eluted with different concentrations of ethanol, the fraction eluted with 60% alcohol was combined and then lyophilized, and the resulting powder (*Pulsatilla* extracts) was obtained and subjected to the following studies.

Preparation of Test Solution

Different batches of *Pulsatilla* extracts were accurately weighed, dissolved with methanol by Ultrasonic heating, and placed to a constant volume to get the test solution with the concentration of 5.0 mg·mL^{-1}.

Preparation of Reference Solution

Appropriate amount of standard *Pulsatilla* saponin D, *Pulsatilla* saponin A, *Pulsatilla* saponin F and Oleanolic acid 3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl--(1→3)- α-L- rhamnopyranosyl-(1→2)- α-L-arabinopyranosyl were weighed and dissolved with methanol to make the reference solutions to the concentration of 0.5 mg·mL^{-1}, respectively. Then the four kinds of reference substances were taken together, and dissolved in methanol to make the mixture reference solution with the concentration of 0.4 mg·mL^{-1}.

Thin-layer Identification

In the light of experiment of thin layer chromatography (General rule 0520), 5 μL of the above solutions were pipetted and performed on silica gel G thin layer plate, respectively. After being developed with chloroform-methanol-acetic acid (7:3:1), the thin layer plate was dried and immersed in the ethanol solution of 10% sulfuric acid, and then took out and heated at 105°C until the spots were clear.

Determination of Total Saponins Content

The orthogonal test was designed to optimize conditions of the experiment. The content of total saponins in *P. chinensis* extract was determined by ultraviolet spectrophotometry.

Preparation of Reference Solution

Proper amount of standard *Pulsatilla* saponin D was accurately weighed and added in methanol to make the reference solution with the concentration of 0.0575 mg·mL^{-1}.

Preparation of Test Solution

Pulsatilla extracts was weighed precisely, placed in a 10 mL volumetric flask, added with methanol, and dissolved by Ultrasound, then shaken evenly to get the solution with the concentration of 1.5 mg·mL^{-1}. The solution was placed for a few min and diluted with methanol to the scale to make the mother solution. Then 1mL mother solution was precisely pipetted to a 25 mL volumetric flask, diluted with methanol to the scale, and shaken evenly to get the test solution.

Selection of Detection Wavelength

A 0.5 mL each of the reference solution and test solutions were precisely and respectively pipetted into a 10 mL test tube with glass stopper and the two solutions were evaporated at 80°C in water bath. A 0.2 mL of 5% Vanillin-glacial acetic acid and 0.8 mL of perchloric acid were added in the two test tubes with glass stopper, shaken evenly, and placed in water bath at 90℃ for 40 min. Then the tubes were transferred to ice bath immediately for 5 min, added with 5 mL of glacial acetic acid, and shaken evenly. Blank control was a kind of solution processed by the same method but without reference or test substance. The reference solution and test solution were scanned in the range of 400~800 nm using the UV spectrophotometer.

Optimization of Chromogenic Condition

Proper amount of test solution was pipetted to make the orthogonal test, in which the absorbance was the indicator, and the reaction temperature, reaction time, volume of 5% Vanillin-Glacial acetic acid and perchloric acid were the influencing factors (Table 1). $L_9(3)^4$ orthogonal test table was designed to conduct the experiment (Luo *et al*, 2018), and the results are shown in Table 2.

Methodological Investigation

Drawing of standard curve: Reference solution to concentrations of 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mL were pipetted precisely in the respective test tube with glass stopper, evaporated at 80°C, added precisely with new 0.2 mL of 5% Vanillin-glacial acetic acid and 0.8 mL perchloric acid, and shaken evenly. The test tubes were heated in water bath with a constant temperature at 90°C for 40 min, and placed immediately in ice bath for 5 min. Then the solutions were added with 5.0 mL of glacial acetic acid, respectively, and shaken evenly. The first tube was regarded as a blank control and the absorbance was measured at the

Table 1: Levels of experiment factors for orthogonal tests

Level	Reaction temperature (°C)	Reaction time /min	Volume of 5% vanillin-glacial acetic acid /mL	Volume of perchloric acid /mL
	A	B	C	D
1	60	20	0.2	0.4
2	80	30	0.4	0.8
3	90	40	0.6	1.6

Table 2: Results of orthogonal tests for optimized chromogenic condition

No.	A	B	C	D	Absorbance
1	1	1	1	1	0.114
2	1	2	2	2	0.153
3	1	3	3	3	0.197
4	2	1	2	3	0.273
5	2	2	3	1	0.225
6	2	3	1	2	0.564
7	3	1	3	2	0.321
8	3	2	1	3	0.340
9	3	3	2	1	0.536
K1	0.155	0.236	0.339	0.292	
K2	0.354	0.239	0.320	0.346	
K3	0.399	0.432	0.247	0.270	
R	0.244	0.196	0.092	0.076	

wavelength of 531 nm. The standard curve was drawn by regarding the absorbance as the ordinate (Y) and the quality of *Pulsatilla* saponin D as the abscissa (X). The regression equation was obtained as: Y=10.05X+0.001 (r=0.9997).

Precision test: The same reference solution (0.4 mL) was accurately pipetted in a 10 mL test tube with glass stopper for six times and the value of absorbance was measured by the method mentioned in drawing of standard curve.

Repeatability test: The same batches of *Pulsatilla* extracts were accurately weighed for six times, while a blank control was made by the method mentioned in preparation of test solution and color-processed by the method of drawing of standard curve. The absorbance was measured at 531 nm.

Stability test: The same test solution was accurately pipetted. The blank solution was regarded as comparison and measured by the method mentioned in drawing of standard curve. Its absorbance was respectively scanned after 10, 20, 30, 40, 60, 90 min.

Average recovery rate of sample addition test: The same batches of *Pulsatilla* extracts with known content of total saponins were accurately weighed with the concentration of high, medium and low. Standard *Pulsatilla* saponin D was added respectively and accurately for three times, which was equal to 80%, 100%, 120% of the content of total saponins in the sample. The sample solution was made following the method mentioned in preparation of test solution, color-processed and measured by the method which was used in drawing of standard curve.

Determination of Sample Content

Five different batches of *Pulsatilla* extracts were accurately weighed. The sample solutions were made by the method of preparation of test solution and measured by the method of drawing of standard curve (Three tests were prepared in parallel for each batch and the results were averaged). The content of total saponins in *Pulsatilla* extracts was calculated by comparing with the standard curve.

Results

Thin-layer Identification of Saponins in *Pulsatilla* Extract

The result of thin-layer identification shows that in the chromatogram of the sample, the same color spots appeared at the corresponding position with the reference substance (Fig. 1).

Determination of Total Saponins Content

We can find that both of reference solution and test solution had a maximum absorption peak at 531 nm (Fig. 2). Finally, 531 nm was selected as the measured wavelength. From the result of orthogonal test, it can be inferred from the value R that the sequence of the four influencing factors were A, B, C and D, the optimal condition of reaction was $A_3B_3C_1D_2$: the chromogenic agents were 0.2 mL 5% Vanillin-glacial acetic acid and 0.8 mL perchloric acid, the reaction temperature was 90℃, and the reaction time was 40 min. Thus, it can be concluded from the data of methodological investigation that the quality of *Pulsatilla* saponin D in the range of 0.0115~0.0805mg had a good linear relationship with absorbance (Fig. 3). After calculating and analyzing, the results show that the relative standard deviation (RSD) of precision test was 0.36%, the RSD of repeatability test was 2.38%, the RSD of repeatability test was 1.70%, which illustrated that the test solution was stable within 90 min under this chromogenic method. Furthermore, the average recovery rate of sample addition was 101.1% and RSD was 1.75%, which indicated that the method of determination of total saponins content conformed the requirements. The result of determination of sample content indicated that the content of total saponins was more than 70% of the 5 batches of *Pulsatilla* extract, but there were big differences among the different batches (Table 3).

Discussion

According to the properties of saponin components and the relevant literature (Wang *et al*., 2011; Li *et al*., 2014; Zhou, 2014), the system of corresponding expansion was selected in this study. N-butanol-acetic acid-water (5:1:2,7:1:2,8:1:3), chloroform-methanol (7:3,7:4), chloroform-methanol-acetic acid (7:4:0.5, 7:4:1, 7:3:1) and ethyl acetate-acetic acid-water (8:2:1; 4:1:1, 4:1:2, 8:2:3) were investigated, respectively. The results indicated that the deployment effect was the best and the developed time was the shortest when chloroform-methanol-acetic acid was

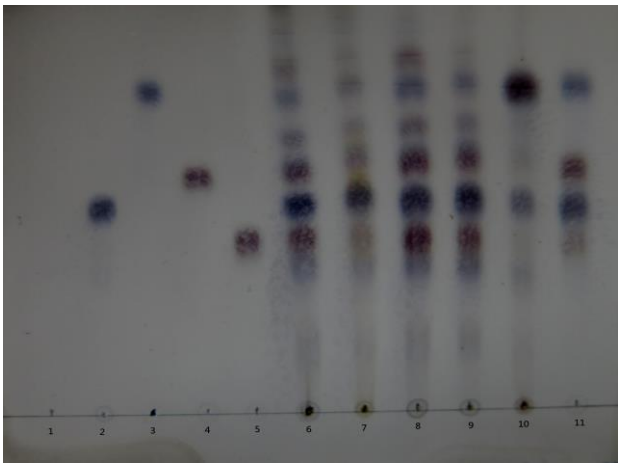

Fig. 1: Developed image after TLC run and identification of saponins in Radix *Pulsatilla* extracts
1: Blank solution; 2:Standard *Pulsatilla* saponin D; 3: Standard *Pulsatilla* saponin A; 4: Standard *Pulsatilla* saponin F; 5: Standard Oleanolic acid 3-O-α-D-Pyranoglucosyl-(1→4)-α-D-Pyranoglucosyl-(1→3)-α-L-Pyridine rhamnosyl-(1→2)- α-L-Pyranosine arabinoside; 6~10: Different batches of *Pulsatilla* extract; 11: Mixed reference

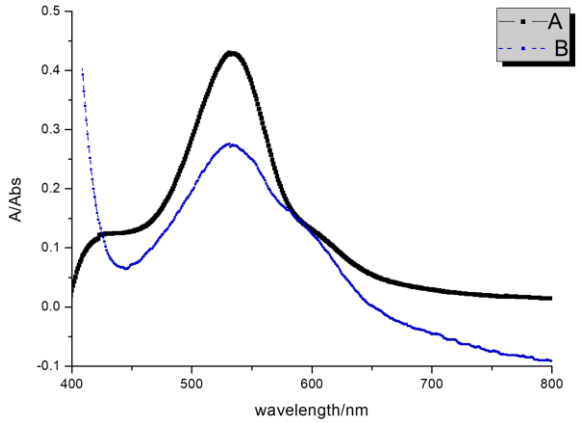

Fig. 2: The spectrophotometer scanning for maxim absorbance of saponins identification
A: *Pulsatilla* saponin D; B: *Pulsatilla* extract

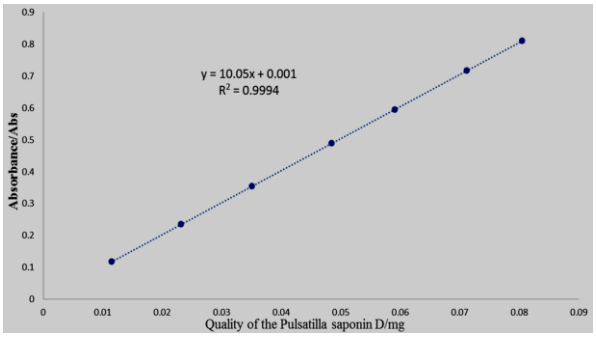

Fig. 3: The standard curve of content determination of total saponins

used as developed system. Therefore, chloroform-methanol-acetic acid (7:3:1) was selected as the optimal condition of development.

Table 3: Result of determination of the 5 batches of *Pulsatilla* extracts (n=3)

Batch number	Absorbance	Sampling quality /mg	Measured mass /mg	Content /%
20110804	0.386	0.0502	0.0383	76.31
20120330	0.357	0.0501	0.0354	70.70
20120723	0.403	0.0478	0.0400	83.68
20121022	0.425	0.0500	0.0422	84.38
20130617	0.368	0.0510	0.0365	71.60

The chromogenic agent of determination of total saponins usually included vanillin-glacial acetic acid-concentrated sulfuric acid and vanillin-glacial acetic acid-perchloric acid (Zhang *et al*., 2013). The two kinds of chromogenic agents were investigated in the early stage of the experiment. The result of the experiment illustrated that the latter was better. In addition, vanillin-ethanol-perchloric acid was also investigated, but the coloration of this reagent was too fast and it was difficult to control. Therefore, vanillin-glacial acetic acid-perchloric acid was finally selected as the chromogenic agent of the experiment.

In this paper, determination of total saponins was more susceptible to the conditions of reaction because of chromogenic agent. It was preliminarily determined by the literature that the determination will be influenced by reaction temperature, reaction time and the amount of the chromogenic agent (Zhu, 2011; Fan *et al*., 2015; Peng *et al*., 2015; Wei, 2015). Therefore, in order to clarify the trend of corresponding change, the single factor parallel test was firstly used in the pre-experiment，and the $L_9(3)^4$ orthogonal test was used to further select the optimal reaction condition in this experiment.

In this experiment, the optimal color development condition for the identity test was first determined, and the color development condition can also be applicable to the determination of saponin components from other plants. The determination wavelength was then determined to provide a basis for the determination of saponin components. The determination method and color development condition in this paper are innovative and simple compared to that in other references (Chen *et al*., 2016).

Conclusion

Thin layer chromatography method reported here can be used to identify saponins from Radix *Pulsatilla* qualitatively. The results showed that *Pulsatilla* extracts contain *Pulsatilla* saponin D, *Pulsatilla* saponin A, *Pulsatilla* saponin F and Oleanolic acid 3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl.
Meanwhile, the ultraviolet spectrophotometry method, which was used for quantitative analysis of total saponins,

was simple and had good repeatability. The study can provide the basis for the further study of quality standard of Radix *Pulsatilla*, and it may also be applied in other experiments for identification and determination of saponins in plants and their extracts.

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