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ORIGINAL ARTICLE

Rapid Quantitative Analysis of Naringenin in the Fruit Bodies of *Inonotus vaninii* by Two-phase Acid Hydrolysis Followed by Reversed Phase-high Performance Liquid Chromatography-ultra Violet

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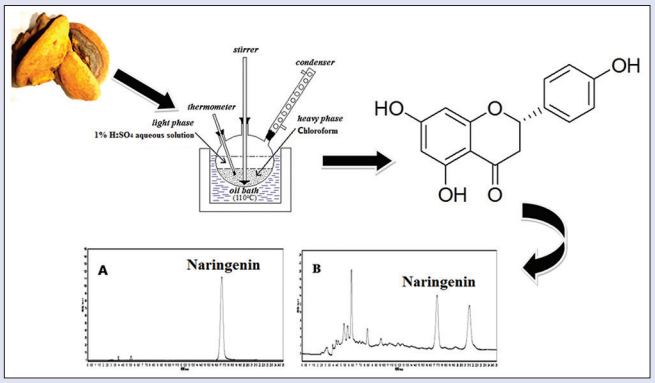
ABSTRACT

Introduction: Sanghuang is one of mystical traditional Chinese medicines recorded earliest 2000 years ago, that included various fungi of *Inonotus* genus and was well-known for antitumor effect in modern medicine. *Inonotus vaninii* is grown in natural forest of Northeastern China merely and used as Sanghuang commercially, but it has no quality control specification until now. This study was to establish a rapid method of two-phase acid hydrolysis followed by reversed phase-high performance liquid chromatography-ultra violet (RP-HPLC-UV) to quantify naringenin in the fruit body of *I. vaninii*. **Materials and Methods:** Sample solution was prepared by pretreatment of raw material in two-phase acid hydrolysis and the hydrolysis technology was optimized. After reconstitution, analysis was performed using RP-HPLC-UV. The method validation was investigated and the naringenin content of sample and comparison were determined. **Results:** The naringenin was obtained by two-phase acid hydrolysis method, namely, 10.0 g of raw material was hydrolyzed in 200 mL of 1% sulfuric acid aqueous solution (v/v) and 400 mL of chloroform in oil bath at 110°C for 2 h. Good linearity (r = 0.9992) was achieved between concentration of analyte and peak area. The relative standard deviation (RSD) of precision was 2.47% and the RSD of naringenin contents for repeatability was 3.13%. The accuracy was supported with recoveries at 96.37%, 97.30%, and 99.31%. The sample solution prepared using the proposed method contained higher content of naringenin than conventional method and was stable for 8 h. **Conclusion:** Due to the high efficiency of sample preparation and high reliability of the HPLC method, it is feasible to use this method for routine analysis of naringenin in the fungus.

Key words: *Inonotus vaninii*, naringenin, reversed phase-high performance liquid chromatography-ultra violet, Sanghuang, two-phase acid hydrolysis

SUMMARY

- A convenient two-phase acid hydrolysis was employed to produce naringenin from raw material, and then an efficient and reliable reversed phase-high performance liquid chromatography-ultra violet method was established to monitor naringenin in the fruit bodies of *Inonotus vaninii*. The newly established method could be used to control the quality of the herb.



Abbreviations used: RP-HPLC-UV: Reversed Phase-High Performance Liquid Chromatography-Ultra Violet, RSD: Relative Standard Deviation, EtOAc: Ethyl acetate, ACN: Acetonitrile, MeOH: Methanol, RH: Relative Humility.

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INTRODUCTION

Inonotus vaninii, used as one of ancient traditional Chinese medicines named Sanghuang recorded early in the Newly Revised Materia Medica (the world's earliest pharmacopoeia) issued in the 7th century,[1] is grown in natural forest of Northeastern China merely.[2] Sanghuang are various fungi of *Inonotus* genus used to treat dysentery, neight sweat, and metrorrhagia throughout China, Japan, and Korea, and are well-known for anticancer effect in modern medicine.[3-6] In addition, for scarce resources they went by the name of "Forest Gold."

The fruit body of *I. vaninii* contains the high content of naringenin [Figure 1] that shown various pharmacological actions such as antitumor, anti-inflammatory, antibacterial, spasmolysis, and cholagogue.[7-12] Naturally, this compound exists mainly as naringin in the fruit body, where the aglycone is linked with glucose and rhamnose by a 7-C-O glucosidal bond. Therefore, it is necessary to perform hydrolysis before this marker can be determined and interpreted for quality control purpose.

At present, there is a conventional method to determine naringenin content in *I. vaninii*.[13] The naringin is extracted from raw material and

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then hydrolyzed in strong acid. Ethyl acetate (EtOAc) is then added to extract naringenin for determination with reversed phase-high performance liquid chromatography (RP-HPLC) method. Since the naringenin and naringin expose simultaneously to strong acid during the hydrolysis prolonged period using this method, it is not easy to achieve a high yield of naringenin. Besides the drawback of a low yield, the sample preparations are tedious and time-consuming. As such, there is imperative need to develop a more efficient method for quantitative analysis of naringenin.

In the present study, a two-phase acid hydrolysis is introduced to straight extract aglycones.[14-16] The released naringin was dissolved and hydrolyzed in top phase composed of sulfuric acid and water, and the naringenin produced was promptly transferred into the bottom organic phase made up of chloroform. The two-phase system has not only protected the chemical group of naringenin effectively, but also combined the extraction and hydrolysis of naringin with the isolation of naringenin into one step. Consequently, this method has simplified the sample preparation for quantitative analysis of naringenin from *I. vaninii* using RP-HPLC-ultra violet (UV) analysis.

MATERIALS AND METHODS

Chemicals and plant material

Naringenin standard (purity >98.0% by HPLC, B/N: ZL78156YPS) was purchased from Nanjing Zelang Medical Technology Co., Ltd., China. Acetonitrile (ACN) and methanol (MeOH) of HPLC grade was obtained from OmniChem Co., Ltd., (USA). All the other reagents were analytical grade from Sinopharm Chemical Reagent Co., Ltd., (China).

The fruit bodies of *I. vaninii*, a parasite on poplar, were collected from Mount Paektu in Yanji city, Jilin Province (China). The voucher specimen (No.: SP20160405) had been authenticated by Prof. Yanru Ge (School of Pharmacy, Jiangsu University, Zhenjiang, Jiangsu, China) and was deposited at the Pharmacognosy Research Facility in Jiangsu University. The fruit bodies were then dried in an oven at 60°6 to remove moisture before lyophilization for another 24 h. The raw material was then grounded into powder and passed through 40-mesh sieve. Fine powder was collected and stored in a dry cabinet (relative humility <40%) at room temperature.

Apparatus and chromatographic conditions

Shimadzu Prominence HPLC instrument (Japan) was equipped with DGU-20A$_5$ degasser, LC-20AT pump, CTO-10AS column oven, FCV-10AL mixer SUS and SPD-20A UV-Vis detector. The data were acquired and processed using N2000 software for chromatographic analysis (Zhejiang University, China). Ultrapure water was produced by Milli-Q Biocel Purification Systems (Millipore, USA).

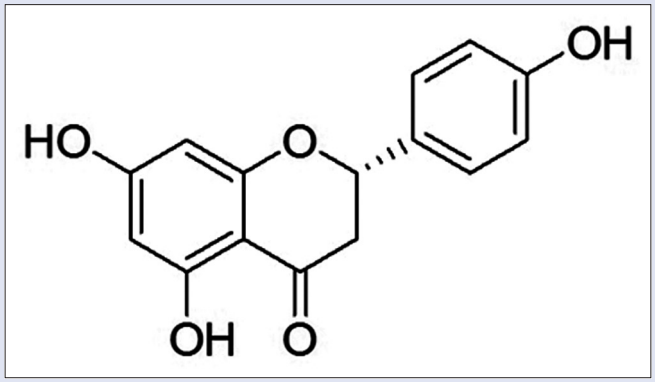

Figure 1: Chemical structures of naringenin

An Omni Bond Hubble C18 (4.6 mm × 250 mm, 5 μm; USA) was used for the HPLC analysis of naringenin. The mobile phase, composed of ACN-water (30:70, v/v), was set to elute at 1.0 mL/min for 25 min at 35°C. The injection volume was 20 μL and UV wavelength for monitoring the analysis was 288 nm.

Preparation of standard solutions

Naringenin, 25.4 mg was dissolved in MeOH and made up to 25 mL. A series of dilution was carried out to give six standard solutions ranging from 25.4 to 280.4 μg/mL. Before injection, the solutions were filtered through 0.45 μm polytetrafluoroethylene membrane syringe filters (Thermo Fisher Scientific, USA).

Preparation of sample solutions

Two-phase acid hydrolysis method

Sample powder, 10.0 g, was hydrolyzed in sulfuric acid aqueous solution and chloroform in oil bath synchronous. The chloroform phase containing naringenin was collected and the top phase was washed by fresh chloroform twice. All chloroform containing naringenin was pooled together for further analysis. Based on single factor experiment, the hydrolysis technology was optimized by orthogonal design with production ratio of naringenin as index [Table 1].

Conventional method

Sample powder, 10.0 g, was extracted by an ultrasonic method with 350 mL of 70% ethanol (EtOH) (v/v) for 30 min twice. The suspension was then subjected to suction filtration, and the extracts were collected and pooled together. The solvent was removed under reduced pressure using rotor evaporator (Büchi R-200, Germany) at below 60°C. The residue was refluxed with 100 mL of 2.0% sulfuric acid (*v*/v) for 30 min to hydrolyze naringin, and then 40% NaOH (*g*/v) was used to raise pH to 7.0. 100 mL of EtOAc was poured into the mixture for sequential extraction of naringenin and the organic phase containing naringenin was collected for further analysis.[13]

Solution preparation

The extract solutions from the above methods were concentrated using rotor-vap under reduced pressure until dryness. The residue was reconstituted in MeOH, made up to 10 mL, and filtered through 0.45 μm membrane syringe filters before being injected into HPLC system for analysis of naringenin.

Method validation

Linearity

Linearity between the analyte concentration and peak area was established by analyzing six different concentrations of naringenin. The solutions were injected consecutively and linear regression line of naringenin concentration (C) to peak area (A), was plotted.

Table 1: Orthogonal experimental design for technology of two-phase acid hydrolysis

Level	Temperature (A, °C)	Extraction duration (B, h)	Concentration of sulfuric acid (C, %)	Ratio of material to chloroform (D, g/mL)
1	90	2	1	20:1
2	110	4	2	30:1
3	130	6	4	40:1

Precision

The intra-day precision of developed method was evaluated by analyzing the same sample solutions on the same day for six times.

Repeatability

Six sample solutions were concurrently prepared and injected into the HPLC system to evaluate the repeatability of the method.

Stability

A sample solution freshly prepared from the fungus was analyzed at room temperature at 0 h, 2 h, 4 h, 6 h, and 8 h.

Recovery

Recovery test was conducted to evaluate the accuracy of the developed method. Naringenin standard was spiked into known amount of the fungus, and analyzed in triplicate at different concentrations. The recoveries in percentage were then calculated by (Detected amount − Original amount)/Spiked amount × 100%.

Determination of naringenin in samples and comparison

The content of naringenin in the fruit body of *I. vaninii* was determined for three times using developed method and conventional method.

The contents of naringenin in *I. vaninii* of the biennial, 3 years and 4 years growths were determined for 3 times using developed method.

The contents of naringenin in the fruit bodies of *I. vaninii* (on *Populus*), *Inonotus sanghuang* (on *Morus*) and *Inonotus baumii* (on *Betula*) were determined for three times using developed method. The morphology of three fruit bodies is illustrated in Figure 2.

RESULTS

Optimization of hydrolysis conditions by orthogonal experimental design

According to the results obtained from orthogonal experiments [Tables 2 and 3], the significance of the factors decreased in the order, C > D > B > A, and the best hydrolysis condition of the method was $A_2B_1C_1D_3$ by verification test.

Method validation

A high correlation coefficient of linear, $r = 0.9992$, was obtained from the regression line, A = 3378.4C−4440.9. This demonstrates a good linear relationship between the analyte peak area and analyte concentration in the range. Typical HPLC chromatograms of naringenin standard and naringenin product are illustrated in Figure 3.

Intra-day precision was evaluated by analyzing the same sample solutions, and relative standard deviation (RSD) of analyte peak area was 2.47%.

RSD of naringenin content for the repeatability was 3.13%.

RSD of peak area was 2.04%, indicating the stability of the prepared sample solution under normal storage condition.

Figure 2: Sanghuang from *Inonotus sanghuang* (a), *Inonotus vaninii* (b) and *Inonotus baumii* (c)

The results of recovery are tabulated in Table 4 and the recoveries of naringenin were within the range of 96.37%–99.31% (RSD <4%), indicating the high accuracy of the developed method.

Determination of naringenin in sample and comparisons

The average content of naringenin in the fruit body of *I. vaninii* was 129 μg/g (129 μg per 1 g of raw material) using developed method and 116 μg/g using the conventional method, indicating the high yield of naringenin using developed method.

The average contents of naringenin in *I. vaninii* of biennial, 3 years and 4 years growths were 109, 125, and 129 μg/g, respectively.

The average contents of naringenin in the fruit bodies of *I. vaninii*, *I. sanghuang* and *I. baumii* were 129, 154, and 67 μg/g, respectively.

DISCUSSION

The Sanghuang mushrooms, including various fungi of *Inonotus* genus in the *Hymenochaetales* (*Basidiomycota*), were legendary and huge development-potential medicine throughout the world. *I. vaninii* was the primary commodities used as Sanghuang for high effectiveness and harvest, while *I. baumii* was considered as an adulterate. In this study, the naringenin contents were determined in the fruit bodies of *I. sanghuang* (on *Morus*), *I. baumii* (on *Betula*) and *I. vaninii* (on *Populus)* of different growth years, that shown the wide difference and that could be one of the appraisal indexes of Sanghuang from separate sources.

According to the results, the naringenin content determined by developed method was higher compared to the conventional method. Since the naringenin produced was promptly transferred into the organic phase, the hydrolysis reaction could proceed completely and the target compounds were protected. In addition, the analysis procedures were simplified using developed method.

To optimize the hydrolysis, seven organic solvents including EtOAc, petroleum ether (boiling range 60°C–90°C), methylbenzene, dimethylbenzene, dichloromethane, chloroform and n-butanol were

Table 2: Results of orthogonal experiments

n	A	B	C	D	Production rate (μg/g)
1	1	1	1	1	99.79
2	1	2	2	2	75.40
3	1	3	3	3	52.16
4	2	1	2	3	108.88
5	2	2	3	1	39.88
6	2	3	1	2	90.13
7	3	1	3	2	43.82
8	3	2	1	3	116.31
9	3	3	2	1	40.73
K_1	75.783	84.163	102.077	60.133	
K_2	79.630	77.197	75.003	69.783	
K_3	66.953	61.007	45.287	92.450	
R	12.677	23.156	56.790	32.317	

Table 3: Analysis of variance of orthogonal experiments

Factors	Sum of squared deviations	Freedom	*F*	The critical value of *F*	Significance
A	253.464	2	1.000	19.000	
B	846.882	2	3.341	19.000	
C	4841.150	2	19.100	19.000	*
D	1651.267	2	6.515	19.000	
Error	7592.76	2			

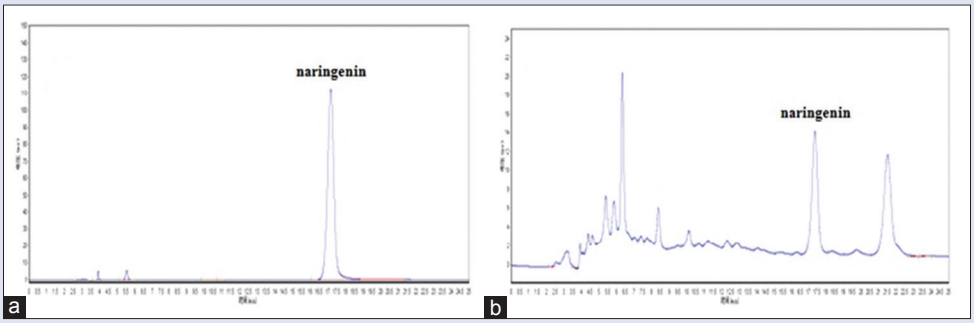

Figure 3: Typical high performance liquid chromatography chromatograms of naringenin standard (a) and product (b)

Table 4: Results of naringenin recovery

Level	Original (mg)	Spiked (mg)	Detected (mg)	Recovery (%)	Mean (%)	RSD (%)
Low	1.316	1.016	2.149	92.15	96.37	3.88
	1.316	1.016	2.278	97.68		
	1.316	1.016	2.315	99.27		
Middle	1.316	1.321	2.549	96.66	97.30	1.03
	1.316	1.321	2.596	98.45		
	1.316	1.321	2.552	96.78		
High	1.316	1.626	2.904	98.71	99.31	3.85
	1.316	1.626	3.043	103.4		
	1.316	1.626	2.819	95.82		

RSD: Relative standard deviation

compared. EtOAc had the highest solubility to naringenin among all solvents; nevertheless, it was hydrolyzed into acetic acid and EtOH in sulfuric acid aqueous solution when heated. Petroleum ether, methylbenzene, and dimethylbenzene were not chosen in this method for low extraction rate. Dichloromethane was bottom phase contained sample powder in addition to the low boiling point that hydrolysis was difficult to complete. N-butanol had high solubility to naringenin and high boiling point, but its extract was too complex to be analyzed by HPLC, therefore chloroform was chosen as organic phase in this method.

CONCLUSION

In this study, a rapid and high-reliability method of two-phase acid hydrolysis followed by RP-HPLC-UV was established for quantify naringenin in the fruit body of *I. vaninii*. The method was more accurate compared with conventional approaches for the high efficiency of sample preparation. Using this method, the contents of naringenin in Sanghuang harvested at various times and of different species were determined respectively, that shown the wide difference and that could be one of the appraisal indexes of Sanghuang.

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Conflicts of interest

There are no conflicts of interest.

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