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.

**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, is grown in natural forest of Northeastern China merely. Sanghuang are various fungi of *Inonotus* genus used to treat dysentery, night sweat, and metrorrhagia throughout China, Japan, and Korea, and are well-known for anticancer effect in modern medicine. 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. 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*. The naringin is extracted from raw material and 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.

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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°C to remove moisture before lyophilization for another 24 h. The raw material was then ground into powder and passed through 40-mesh sieve. Fine powder was collected and stored in a dry cabinet (relative humidity <40%) at room temperature.

**Apparatus and chromatographic conditions**

Shimadzu Prominence HPLC instrument (Japan) was equipped with DGU-20A, 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).

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 rotov evaporator (Buchi 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 A2B3C2D1 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.

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 compared using orthogonal experiments.

**Table 2: Results of orthogonal experiments**

| n  | A  | B  | C  | D  | Production rate (µg/g) |
|----|----|----|----|----|------------------------|
| 1  | 1  | 1  | 1  | 1  | 99.79                  |
| 2  | 2  | 2  | 2  | 2  | 75.40                  |
| 3  | 3  | 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                  |

**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       |       |                          |             |

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

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**Figure 3:** Typical HPLC chromatograms of naringenin standard (a) and naringenin product (b)
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 quantifying 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.

REFERENCES

1. Wu SH, Dai YC, Hatton T, Yu T, Wang D, Parmasto E, et al. Species clarification for the medicinally valuable ‘sanghuang’ mushroom. Bot Stud 2012;53:135-49.
2. Dai YC. A new medicinal fungus-Phellinus vaninii. Edible Fungi China 2003;22:7-8.
3. New Hospital of Jiangsu. Great Dictionary of Chinese Medicine [M]. Shanghai of China: Shanghai Scientific and Technical Publishing House; 1995, 1976.
4. Huang HY, Chieh SY, Tso TK, Chien TY, Lin HF, Tsai YC. Orally administered mycelial culture of Inonotus linteus exhibits antitumor effects in hepatoma cell-bearing mice. J Ethnopharmacol 2011;133:460-6.
5. Kim BC, Choi JW, Hong HY, Lee SA, Hong S, Park EH, et al. Heme oxygenase-1 mediates the anti-inflammatory effect of mushroom Phellinus linteus in LPS-stimulated RAW264.7 macrophages. J Ethnopharmacol 2006;108:364-71.
6. Lee YS, Kim YH, Shin EK, Kim DH, Lim SS, Lee JY, et al. Anti-angiogenic activity of methanol extract of Phellinus linteus and its fractions. J Ethnopharmacol 2010;131:56-62.
7. Shiomwar SS, Chidrawar VR. Combined effects of p-coumaric acid and naringenin against doxorubicin-induced cardiotoxicity in rats. Pharmacogn Res 2011;3:214-9.
8. Yang HL, Tian H, Li PB. Bioactivity research of naringin and naringenin. J Chin Med Mater 2005;28:792-4.
9. Muthaiah VP, Venkitasamy L, Michael FM, Chandrasekar K, Venkatachalam S. Neuroprotective role of naringenin on carbaryl induced neurotoxicity in mouse neuroblastoma cells. J Pharmacol Pharmacother 2013;4:192-7.
10. Sak K. Cytotoxicity of dietary flavonoids on different human cancer types. Pharmacogn Rev 2014;8:122-46.
11. Dwevedi A, Sharma K, Sharma YK. Cadamba: A miraculous tree having enormous pharmacological implications. Pharmacogn Rev 2015;9:107-13.
12. Naran N, Jinaugkoorsru W. Anticancer activity of key lime, Citrus aurantifolia. Pharmacogn Res 2016;8:118-22.
13. Xia GH, Ge YR, Qi XY. Determination of naringenin content in Phellinus Vaninii by RP-HPLC. Acta Edulis Fungi 2012;19:84-6.
14. Yang H, Yin-H, Shen Y, Xia GH, Zhang B, Wu XY, et al. A more ecological and efficient approach for producing diosgenin from Dioscorea zingiberensis tubers via pressurized biphasic acid hydrolysis. J Clean Prod 2016;131:10-9.
15. Yang H, Yin HW, Wang XW, Li ZH, Shen YP, Ja XE. In situ pressurized biphasic acid hydrolysis, a promising approach to produce bioactive diosgenin from the tubers of Dioscorea zingiberensis. Pharmacogn Mag 2015;11:636-42.
16. Yang H, Chen B, Wang XB, Chuw PW, Shen YP, Xia GH, et al. Rapid quantitative analysis of diosgenin in the tubers of Dioscorea zingiberensis C.H. Wright by coupling cellulose enzymolysis and two-phase acid hydrolysis in tandem with HPLC-UV. Nat Prod Res 2013;27:1933-5.