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Optimization of an extraction method for the simultaneous quantification of six active compounds in the aril part of *Orostachys japonicus* using HPLC–UV

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Abstract: In this study, we describe the development of a new high-performance liquid chromatography (HPLC) method for the simultaneous analysis of six bioactive compounds (including gallic acid, epicatechin 3-gallate, quercitrin, afzelin, quercetin, and kaempferol) from *Orostachys japonicus*. The extraction method was investigated and optimization of the extraction time (min), solvent composition (%), and solvent to material ratio were conducted. As a result, 30 min extraction with 50% methanol and 40:1 mL/g of solvent: material ratio achieved the highest extraction efficiency with a yield of 3.32 mg/g. Furthermore, the developed HPLC method was validated and the correlation coefficient (R) values were within the satisfactory range of 0.9995-0.9999 over the linearity range of 1.53-417 μg/mL. The limit of detection and limit of quantification for the six active components were between 0.03-0.08 μg/mL and 0.08-0.26 μg/mL, respectively. With these newly optimized and developed methods, four batches of *O. japonicus* were analyzed to confirm the high extraction efficiency of the method and the feasibility of an application.

Key words: HPLC, *O. Japonicus*, quality control, ultrasonic-assisted extraction, method validation

1. Introduction

Orostachys japonicus A Berger (Crassulaceae) is a perennial herb that contains a vast number of compounds that are both beneficial and essential within folk medicine.[1,2] In South Korea, *O. Japonicus* is known as Wa-song, and water extracts are used in the treatment of various diseases, including metritis, fever, intoxication, gingivitis, and cancers.[3-5] The pharmacological effects of the herb decoction have been analyzed including the calpain repressive effect, improvement of hepatic alcohol dehydrogenase, protective effectiveness of neuronal cells, and oxidative stress.[6-8] The water decoction is drunk by patients from a dried aerial part to treat the disease.[9] The homemade juice of fresh *O. Japonicus* has a slightly sweet and sour taste, consumed often as a healthy beverage by un-diseased, healthy people.[2,8,10] However,

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many studies focus on the evaluation of new biological activities of *O. Japonicus* through the isolation of new active compounds. However, high-quality analysis of these new compounds has been neglected. Therefore, the development of an effective method to evaluate the quality of commercial *O. Japonicus* is needed.

In our previous studies, we reported that flavonoids are the main components in *O. Japonicus*, and characterized five major flavonoids: epicatechin, kaempferol, epicatechin 3-gallate, quercitrin, afzelin, and quercetin.[11] The anticancer, antioxidant, inhibit obesity, and anti-inflammatory activities are believed to be due to these flavonoid components.[10,12-14] Therefore, these compounds could be used as marker compounds for the evaluation of the quality of *O. Japonicus*.

So far extraction of flavonoids was achieved tediously from natural products with wide range of extraction methods (refluxing, ultrasonic, maceration, microwave, soxhlet pressurized liquid and pulsed-electric field). Among them, ultrasonic-assisted extraction is normally regarded as an environment-friendly, energy-saving and time-saving technology.[15,16] Ultrasonic wave could rapidly induce acoustic cavitation of the herb cell wall to improve the extraction of beneficial constituents from plant samples.[17] Moreover, the extraction parameters of ultrasonic-assisted extraction including solvent type, the concentration of applied solvent, extraction time and temperature, and the material to solvent ratio can affect the extraction efficiency of bio-active compounds.[18-20] Thus, investigation of the effects of independent variables of ultrasonic-assisted extraction on the maker compounds contents of *O. Japonicus* is needed.

In this study, we aimed to establish a simple and efficient high-performance liquid chromatographic ultraviolet (HPLC-UV) method for the quality control and identification of *O. Japonicus* compounds. Moreover, we optimized the extraction method to maximize the yield of the marker compounds in *O. Japonicus*.

2. Experimental

2.1. Materials and reagents

O. Japonicus was purchased online from the herbal markets (https://shopping.naver.com/ and http://www.khwasong.kr/) in December 2020. The species was identified by Prof. J. S. Kang and Y. H. Kim. A voucher specimen (CNU-201201) was deposited in the herbarium, College of Pharmacy, Chungnam National University (CNU).

The Laboratory of Natural Products donated the

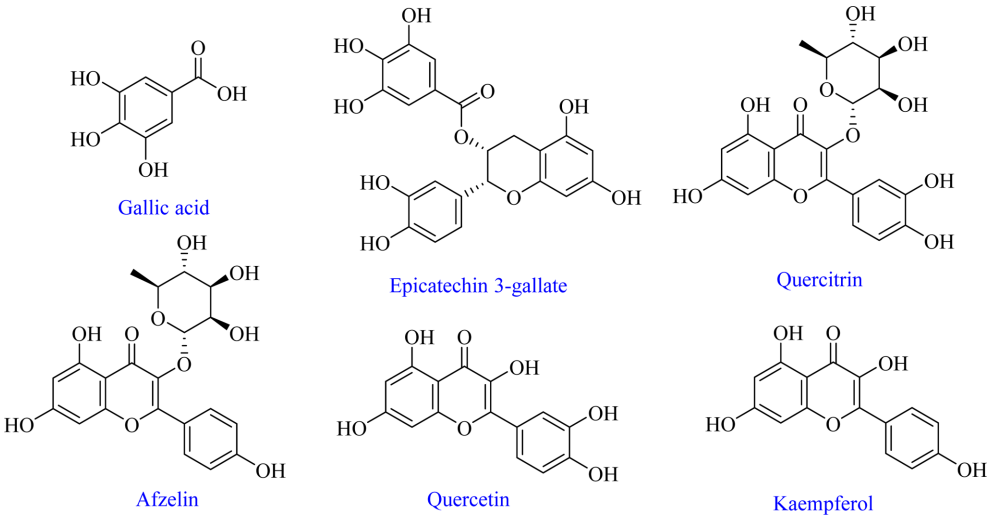

Fig. 1. The chemical names and structures of marker compounds.

reference compounds epicatechin 3-gallate, quercitrin, afzelin, quercetin, and kaempferol (*Fig.* 1). The purity of these compounds was determined to be >98 % by HPLC–UV. Gallic acid was purchased from Sigma-Aldrich Chemical Co. (St Louis, Mo, USA). Special grade acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, MI, USA); a buffer containing formic acid and acetic acid (HPLC-MS grade) was purchased from Sigma Aldrich (St. Louis, Mo, USA). A Milli-Q water purification machine was used to obtain deionized water (Sinhan, Seoul, Korea).

2.2. Sample and standard preparation

To 10 mL of 50 % ethanol, 1 g of *O. Japonicus* powder was added into a conical flask. This solution was sonicated (40 kHz, 280 W) at 50 ºC for 30 min using a Mujigae ultrasonic machine (Seoul, Korea). The extract solution was cooled to ambient temperature, and an additional 50 % ethanol was added to compensate for the loss in volume after the ultrasonic extraction process. Standards were dissolved in methanol and prepared as 1 mg/mL stock. All solutions used in this study were filtered using a 0.22 μm Polyvinylide fluoride syringe filter and stored at 4 ºC.

2.3. HPLC analysis condition

Both qualitative and quantitative analyses of the *O. Japonicus* samples were performed using a Shimadzu LC-20 A series system (Shimadzu Corporation, Kyoto, Japan) equipped with SPD-20A ultraviolet-visible detector. The chromatographic separation was performed on a Hector M C18 column (4.6 mm × 250 mm, 5 μm (RStech, Daejeon, Korea)). The separation system was a mixture of solvent A (water: formic acid 1000:1, v/v) and solvent B (acetonitrile: methanol 2:1, v/v) with a gradient elution of 5 %-60 % at 0-60 min under a column oven temperature of 30 ºC and a flow rate of 1.0 mL/min.

2.4. Optimization of extraction method

Various sample extraction methods and parameters were evaluated and optimized. To begin, the ultrasonic-assisted and reflux extraction methods were compared to determine the optimal extraction approach. To analyze the extraction medium for *O. Japonicus*, a 0.5 g of sample powder was extracted using 10 mL of varying types of solvents (water, ethanol, methanol, and acetonitrile) conducted in triplicate. To study the optimal concentration of ethanol, 0.5 g of sample was added to 10 mL of different concentrations of ethanol (10 %, 30 %, 50 %, 70 %, and 100 %). For optimizing the extraction time and extraction method, 0.5 g of sample was added to 10 mL of optimal extracting solvent (ethanol) and subjected to ultrasonication. This was subsequently tested at 0, 15, 30, 45, 60, and 90 min time points. Optimization of solvent to sample ratio (mL of 50 % ethanol: sample powder) was conducted in different solvent to sample ratios including 10:1, 20:1, 40:1, 80:1, and 100:1.

2.5. Method validation

The established HPLC method was validated based on accuracy, precision linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and specificity, by following the guideline of the International Council for Harmonization.[21]

2.6. Statistical analysis

All statistical analysis was performed using GraphPad Prism 8.02 (GraphPad Software Inc., La Jolla, CA, USA). * P-value < 0.05 was considered statistically significant difference.

3. Results and Discussion

3.1. Optimized HPLC condition

To obtain simultaneous separation of flavonoids from *O. Japonicus*, HPLC conditions including column, mobile phase, buffer type, and the column temperature was optimized. After comparison of the Waters ODS C18 column (4.6 mm × 250 mm, 5 μm), Optimapak C18 column (4.6 mm × 250 mm, 5 μm), and the Hector M C18 column (4.6 mm × 250 mm, 5 μm), we selected the latter to achieve the best separation efficiency. Various mobile phase compo- sitions (0.1 % formic acid-acetonitrile, 0.1 % formic acid-methanol, 0.1 % formic acid-water, 0.1 % formic acid-acetonitrile: methanol (2:1 v/v), 0.1 % acetic acid–acetonitrile)

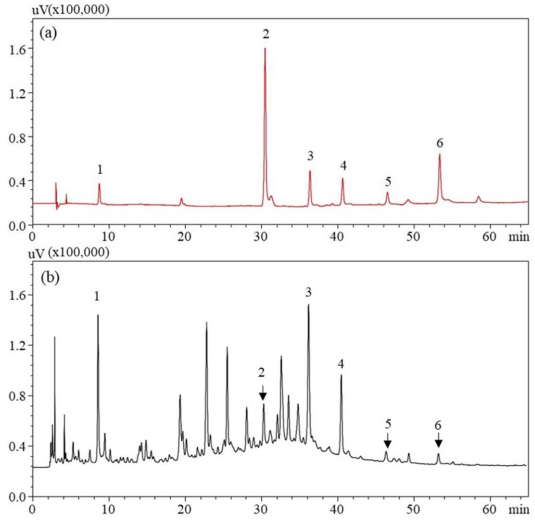

Fig. 2. The typical chromatograms of (a) mixed standard solutions and (b) sample solutions (1: gallic aicd; 2: epicatechin 3-gallate; 3: quercitrin; 4: afzelin; 5: quercetin; 6: kaempferol).

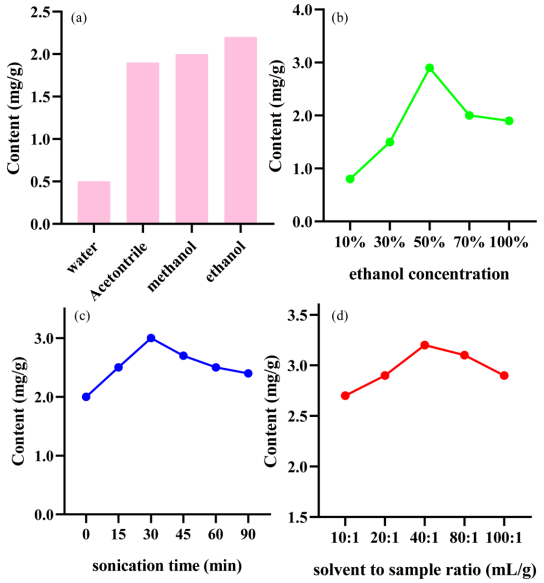

Fig. 3. Effect of (a) solvent type, (b) ethanol concentration, (c) sonication time, (d) solvent to material ratio on the yields of marker compounds in single-factor experiments.

and column temperature (30, 35, and 40 °C) were evaluated. Finally, 0.1 % formic acid-acetonitrile: methanol (2:1, v/v) and 0.1 % formic acid-water were used as the mobile phase, and the column temperature was 30 °C which had a sharp peak shape, acceptable resolution, and a stable baseline. The detection wavelength was 261 nm as flavonoids had a strong UV absorption at this wavelength.[22-25] The HPLC chromatograms of the standard mixture and *O. Japonicus* are shown in *Fig.* 2.

3.2. Optimization of extraction method

To begin, the extraction method of both the ultrasonic-assisted and reflux were compared, and the results demonstrate that the most efficient method was the ultrasonic extraction ($P < 0.05$). Subsequent experiments were designed to appraise the effects of each experimental parameter (solvent type, solvent concentration, ultrasonic time, and solvent to material ratio) on the yields of the marker compounds. As a result, ethanol had the best extraction efficiency for both safety and efficiency (*Fig.* 3(a)). Thus, ethanol was selected for subsequent experiments as the extraction solvent. The influence of ethanol concentration on the yield of the marker compounds was evaluated, and the extraction efficiency increased with ethanol concentration from 10 %-50 % (v/v) and decreases with an ethanol concentration of >70 % (*Fig.* 3(b)). Therefore, 50 % ethanol had the best extraction efficiency. The effect of ultrasonic time on the yield of the marker compounds was investigated from 0-90 min along with other fixed extraction parameters (*Fig.* 3(c)). The yield of the marker compounds increased with an increase in ultrasonication from 0-30 min, with the yield of marker compounds decreasing after 30 min. This may be the result of structural degradation induced by extended ultrasonication time. Therefore, 30 min was selected as the extraction time. The effect of the solvent to sample ratio on the yield of the marker compounds is shown in *Fig.* 3(d). As the solvent to material ratio increases, the extraction yield of flavonoids also increases, with a maximum extraction yield of these marker compounds at a 40:1 mL/g ratio. It was observed that a slight decrease in the marker compounds occurred after the solvent to sample ratio went above 40:1 mL/g. This may be explained by a higher ratio of solvent to solids can cause a greater concentration difference, accelerating

Table 1. Validation data of marker compounds

Parameters	Gallic acid	Epicatechin 3-gallate	Quercitrin	Afzelin	Quercetin	Kaempferol
Linearity range (μg/mL)	1.55-18.55	34.75-417.00	2.70-32.35	1.94-23.32	1.53-18.33	3.44-41.26
R^2	0.9996	0.9999	0.9996	0.9997	0.9995	0.9997
Equation	y = 228x-4	y = 104x + 13	y = 275x-2	y = 302x-3	y = 222x-6	y = 43x-9
LOD (μg/mL)	0.03	0.08	0.03	0.03	0.05	0.03
LOQ (μg/mL)	0.09	0.26	0.09	0.09	0.15	0.08
Precision						
Intra-day	0.84-1.24	0.66-1.44	0.14-1.94	0.44-1.97	0.72-1.66	1.10-1.57
Inter-day	1.00-2.99	0.97-2.07	1.32-3.28	1.44-2.06	1.85-3.66	1.49-3.17
Accuracy						
Intra-day	99.5-103.7	97.6-102.7	100.4-103.7	99.5-103.9	98.9-103.8	100.6-104.7
Inter-day	98.5-106.5	99.4-107.2	101.0-107.5	96.5-102.6	98.3-104.2	98.7-102.9
Repeatability						
Retention time (%RSD)	0.21	1.30	0.56	1.23	0.72	0.65
Content (%RSD)	0.16	1.24	0.43	0.96	0.35	1.25

the mass transfer, and facilitates the diffusion of all marker compounds into the extraction medium.[15,26,27] However, once the mass transfer reached its maximum, a further increase in the ratio of solvent to material extended the distance of diffusion from solvent to interior matrix, and could therefore not improve the yield of flavonoid.[28,29]

3.3. Validation of the HPLC method

The developed HPLC method was validated in terms of linearity range, LOD, LOQ, precision (intra-day and inter-day), and repeatability. The results demonstrated that this developed method was reliable, stable, and conducive for the quantification of the marker compounds in *O. Japonicus* (*Table* 1).

3.3.1. Linearity, LOD and LOQ

With this newly optimized and developed method, the linearity of the peak area versus the concentration of the marker compounds was observed by increasing the calibration curves with five different concentrations. The linearity range and regression equation of the flavonoids are shown in *Table* 1. Moreover, the correlation coefficient of the marker compounds was >0.9996. The LOD and LOQ were determined by setting the response signal of the detector to a noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ of these marker compounds ranged from 0.03-0.08 μg/mL and 0.08-0.26 μg/mL, respectively, which demonstrates that this optimized method had satisfactory sensitivity.

3.3.2. Precision and accuracy

Precision and accuracy were evaluated by determining three concentrations (low, middle, and high) of the standard of marker compounds, repeated five times. These data are articulated as the relative standard deviation (RSD). The results demonstrate that the precision of the intra-day and inter-day for the marker compounds were 0.14 %-1.97 % and 0.97 %-3.66 %, respectively, and the method accuracy ranged from 96.5 %-107.5 % (*Table* 1).

3.3.3. Repeatability

The repeatability of the assay is utilized to evaluate the stability of the HPLC instrument after consecutive sample injection. In this study, the repeatability was expressed as the RSD of the retention times and contents of the marker compounds in *O. Japonicus* samples. The results demonstrated that the RSD of the retention time and concentrations of the marker compounds were 0.21 %-1.30 % and 0.16 %-1.25 %, respectively. This suggests that this established method was both effective and accurate.

3.4. Application to the analysis of *O. Japonicus* sample

To assess the reliability of this developed HPLC method, four batches of *O. Japonicus* samples were analyzed. The contents of the analyte were calculated using the standard calibration curves and the results demonstrated that this new HPLC method developed in this study can be used to monitor *O. Japonicus* quality within the industrial routine analysis.

4. Conclusions

This is the first report on the development of an extraction method of flavonoids in *O. Japonicus* and validation of a reliable and fast analytical method for quantifying these bioactive components in *O. Japonicus*. It was observed that the optimum extraction condition of flavonoids was 30 min of sonication, an ethanol concentration of 50 %, and the solvent to material ratio of 40:1 mL/g. This reliable HPLC–UV method had acceptable linearity, accuracy, precision, and repeatability, which could be further used to evaluate the quality of *O. Japonicus* samples.

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