Original Article

Orthogonal Test Design for Optimization of the Extraction of Flavonid from the Fructus Gardeniae*

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Abstract

Objective It is imperative to provide some consistent experimental results for the extraction of flavonid from Fructus Gardeniae.

Methods The key extraction parameters that influenced the yield of flavonid from Fructus Gardeniae were optimized by employing an orthogonal experiment [$L_9(3)^4$], including the ratio of buffer solution ($Na_2B_4O_7 \cdot 10H_2O$) to raw material, concentration of Fructus Gardeniae in extracting solution, extraction time and pH of buffer solution. An UV/Vis detector was used to perform the qualitative and quantitative analyses of the extracted flavonid with the using of the standard sample.

Results The maximum extraction yield of the crude extract was 5.0533 (mg/g) after 20 min when the mass ratio of $Na_2B_4O_7 \cdot 10H_2O$ to raw material was 0.4%, the concentration of Fructus Gardeniae in the extraction solution was 1/12 (g/mL), and pH of buffer solution was 4.5. The positive reactions to the Molish and HCl-Mg tests suggested that the extracted compound was flavonoid, and FTIR measurements also identified the presence of flavonoid in the extracts.

Conclusion This work is expected to provide a basis for further research, development, and utilization of Fructus gardenia in flavonid extraction.

Key words: Fructus Gardeniae; Flavonid; Extraction; Orthogonal experiment

Biomed Environ Sci, 2011; 24(6):688-693 doi:10.3967/0895-3988.2011.06.015 ISSN:0895-3988

www.besjournal.com(full text) CN:11-2816/Q

INTRODUCTION

Viruses are acellular, ultra-microscopic and metabolically inert nucleoprotein particles. They are not cells, but consist of single or double stranded RNA or DNA surrounded by a protein shell called a capsid[1-5]. Viruses are obligate intracellular parasites, so they do not have all the characteristics of living organisms and must utilize the host cell to propagate new viruses. Each strain of viruses has its own unique surface molecule configuration, which can enable the surface molecules of viruses to precisely fit with the target cell's molecules. Therefore, viruses can enter into host cells by this invasion strategy. As a consequence, viruses have adapted to all forms of life and occupied numerous ecological niches resulting in widespread diseases in humans, livestock and plants[6-10].

The discovery of novel anti-viral drugs deserves great efforts. In recent years, the development of novel anti-viral drugs has achieved significant progress. These newly developed anti-viral drugs belong to three categories: nucleoside analogues,

*This work was supported by National Natural Science Foundation of China (No. 21067004), Research Fund for the Doctoral Program of Jiangxi University of Science and Technology (22020).

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Received: January 5, 2011; Accepted: April 25, 2011

thymidine kinase-dependent nucleotide analogues and specific viral enzyme inhibitors. Since viral enzymes are crucial for virus replication and disease progression, inhibitors against viral enzymes are the most desirable strategy. The application of antibiotics is a major route for the annihilation of viruses, which are commonly used in human and animal. However, recent studies have shown that the prevalence of antibiotic has brought about increasing resistance to these drugs. Owing to the fear of side effects of common drugs over the counter medicines, there has been huge upsurge in people preferring to use more and more natural plant product for prevention and treatment of serious ailments.

Based on the above analysis, it can be concluded that the development of novel efficient and safe anti-viral drugs is necessary. At present, several reports have shown that traditional medicines, like Ayurvedic, traditional Chinese (TCM), Chakma medicines, are good and potential sources for promising anti-viral drugs[11-16]. These medicinal herbs have many potential clinical and therapeutic applications in the modern medical setting, and numerous studies have revealed that they contain bioactive components. These medicinal herbs are traditionally said to provide safe and effective treatments against many diseases. The medicinal herbs also can be used to extract anti-microbial agents, and several plant extracts and plant-derived compounds (phytochemicals) have been reported to have anti-viral activity[17-18].

Fructus Gardeniae is the ripe fruit of Gardeniajasminoides Ellis (family Rubiaceae), which was stir-baked to a brown or charcoal color before it is medicinally used. In Chinese clinics, this preparation is widely used to treat leukecythemia, thyroid carcinoma, uterine cervix cancer, primary hepatic carcinoma, icterohepatitis, coronary artery disease, and so on[19]. Flavonoids are naturally occurring in biological compounds that are often found in plants. Flavonoids possess antioxidant and free radical scavenging activity, and epidemiological studies have indicated that consumption of these compounds is associated with a reduced risk of cancer and cardiovascular disease[20].

It will be of great significance to research the feasibility of extraction of flavonoid from Fructus Gardeniae since this plant is widely distributed in China. However, so far few reports whatsoever have been made for the potential extraction of flavonid from Fructus Gardeniae. With no scientific background, no quality control or consistent results for the extraction of flavonid from Fructus Gardeniae can be provided. Thus, it is imperative to search for the possibility of extraction of flavonoid from the Fructus Gardeniae. The objective of this work was to optimize the operating conditions of extraction of flavonoid from Fructus Gardeniae and the investigation will be expected to result in a better understanding of the Fructus Gardeniae's therapeutic and clinical merits.

MATERIALS AND METHODS

Chemicals

The ethanol, $NaNO_2$, $Al(NO_3)_3$, NaOH, $AlCl_3$, $FeCl_3$, $PbAc_2$, H_2SO_4, HCl, benzene, borax, lime milk, magnesium powder were of analytical grade and supplied by Shanghai Fine Chemical Reagent Corporation. The standard sample of flavonid (chromatograph pure) was supplied from Sigma-Aldrich (Steinheim, Germany).

Preparation of Fructus Gardeniae Extracts

The Fructus Gardeniae was picked from the wild mountain of Ganzhou. The pre-treatment of the Fructus Gardeniae was carried out as follows: first, the sample was washed with hot de-ionized water (>80 °C) to remove any impurities; second, the cleaned sample was filtrated and dried at 120 °C under vacuum for 4 h; third, the dried sample was grinded to obtain the Fructus Gardeniae powder. The extraction of flavonid from the Fructus Gardeniae was carried out as follows: first, the Fructus Gardeniae powder (30 g) was infused with 200 mL of $Na_2B_4O_7 \cdot 10H_2O$ solution in a 250 mL flask; second, the mixed solution was heated to 200 °C under reflux and agitation for 10 h, with the addition of lime milk to maintain the pH at 8~9; third, the mixed solution was placed into a separate funnel, and the supernate layer of the mixed solution was taken away by the filtration, with the repetition of the above operations twice; fourth, the pH of the filtrate was adjusted with 2 mol/L HCl, then lay aside over night to obtain the flavonid crystal; fifth, the centrifugal separation was used to purify the crystal, and the sample was washed with hot de-ionized water (>80 °C) to remove any impurities, with the repetition of the washing for 2~3 times; and finally, the flavonid crystal was placed on a labeled glass slide, dried at 50 °C under vacuum for 5 h to give desire products.

UV/Vis Analysis of Flavonid

A UV/Vis detector was used to perform the qualitative and quantitative analyses of the flavonid that was extracted from the fructus gardeniae with authentic standard samples. The analysis was carried out as follows: first, 100 mg of the sample was put into a 1 000 mL volumetric flask that contains 500 mL of ethanol; second, 5 mL solution was taken and put in a 100 mL volumetric flask that contains 10 mL of ethanol to obtain the 0.1 mg/mL flavonid standard solution; third, other concentrations of the flavonid standard solutions also were obtained as well. The absorption of the UV/Vis detector was set in the range of 200-700 nm. After 2 h, the absorbance of blue colouration was measured at 360 nm against a blank sample. The results were expressed as mg/mL of flavonid. All measurements were performed in triplicate.

Optimization of Fructus Gardeniae Extraction

An orthogonal experiment [$L_9(3)^4$] test design in the extraction mode was used for optimizing the extraction conditions. In this study, extraction was accomplished with $Na_2B_4O_7 \cdot 10H_2O$ buffer. The key parameters that influenced the yield of extracts were analyzed, including the ratio of buffer solution ($Na_2B_4O_7 \cdot 10H_2O$) to raw material (A), concentration of Fructus Gardeniae in extraction solution (B), extraction time (C), and pH of buffer solution (D). And every factor has three levels to be optimized. Nine extractions were carried out when the ratio of $Na_2B_4O_7 \cdot 10H_2O$ to raw material (%) were 0.3, 0.4, and 0.5, concentration of Fructus Gardeniae in the extraction solution（g/mL）were 1/8, 1/10, and 1/12, extraction time were 10, 15, and 20 min, and pH of the buffer solution were 4.5, 5.0, and 5.5. Table 1 shows the experimental conditions for the extraction of flavonid from Fructus Gardeniae.

Table 1. Orthogonal Experiment Design (four factors and three levels)

Level	Factor			
	A, Ratio of buffer solution ($Na_2B_4O_7 \cdot 10H_2O$) to raw material (%)	*B*, Concentration of fructus gardeniae in extraction solution（g/mL）	*C*, Extraction time (min)	*D*, pH of buffer solution
1	0.3	1/8	10	4.5
2	0.4	1/10	15	5.0
3	0.5	1/12	20	5.5

RESULTS AND DISCUSSION

UV/Vis Absorption Spectra

The positive reactions to the Molish and HCl-Mg tests suggested that the extracted compound was flavonoid. Figure 1 shows the UV/Vis spectra of the extracted flavonid after the dryness treatment. The absorption was carried out in the range 200-700 nm, and a maximum at 360 nm was selected for each spectrum. This is consistent with the results obtained in prior studies[21]. This phenomenon can be explained as follows: under the UV irradiation, the turbidity of the solution increases as the radiation causes changes in the structure of the collagen molecule (helix-coil transition). After 1 and 2 h irradiation, the maximum of absorption/ scattering is almost the same. It can be seen from Figure 1 that two broad ultraviolet absorption bands have appeared: one is the linking of cinamyl (300-400 nm), and the other is the linking of benzoyl (240-285 nm).

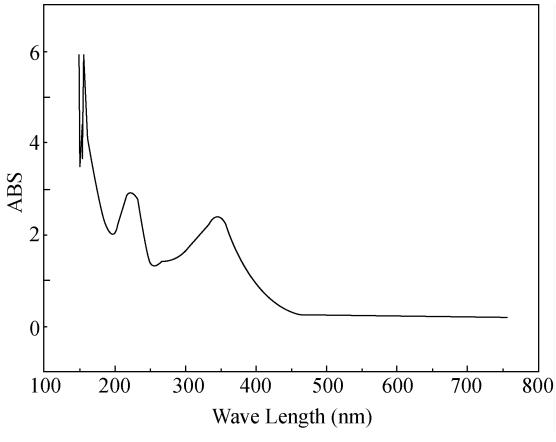

Figure 1. UV/Vis spectra of extract from Fructus Gardeniae.

Figure 2 shows the specification curve of the standard samples of flavonid, and the absorption was carried out at 360 nm.

The least squares method was used to obtain the regression equation, which was carried out as Equation (1) to (4); and then, the regression equation can be obtained and expressed as equation (5); finally, the related coefficient (r) was calculated as equation (6).

$$\bar{x} = \frac{\sum_{i=1}^{7} x_i}{7} = \frac{0.00 + 0.01 + 0.02 + 0.03 + 0.04 + 0.05 + 0.06}{7} = 0.03 \quad (1)$$

$$\bar{y} = \frac{\sum_{i=1}^{7} y_i}{7} = \frac{0.000 + 0.196 + 0.481 + 0.739 + 1.063 + 1.315 + 1.507}{7} = 0.7573 \quad (2)$$

$$a = \frac{\sum_{i=1}^{7}\left(x_i - \bar{x}\right)\left(y_i - \bar{y}\right)}{\sum\left(x_i - \bar{x}\right)^2} = \frac{\sum_{i=1}^{7}(x_i - 0.03)(y_i - 0.7573)}{\sum_{i=1}^{7}(x_i - 0.03)^2} = 26.2143 \quad (3)$$

$$b = \bar{y} - a\bar{x} = 0.7573 - 26.2143 \times 0.03 = -0.0291 \quad (4)$$

$$y = 26.2143x - 0.0291 \quad (5)$$

$$r = \frac{\sum_{i=1}^{7}\left(x_i - \bar{x}\right)\left(y_i - \bar{y}\right)}{\sqrt{\sum_{i=1}^{7}\left(x_i - \bar{x}\right)^2 \sum_{i=1}^{7}\left(y_i - \bar{y}\right)^2}} = \frac{\sum_{i=1}^{7}(x_i - 0.03)(y_i - 0.7573)}{\sqrt{\sum_{i=1}^{7}(x_i - 0.03)^2 \sum_{i=1}^{7}(y_i - 0.7573)^2}} = 0.9982 \quad (6)$$

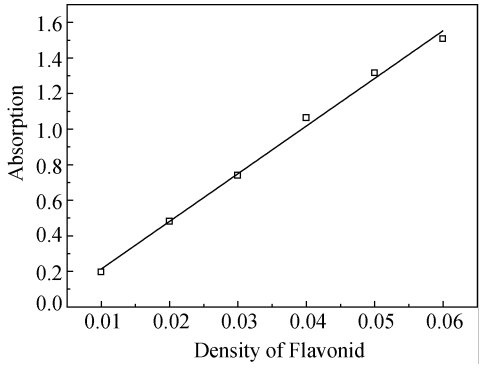

Figure 2. Specification curve of the standard flavonid sample.

FT-IR Spectra

FTIR spectroscopy was used to investigate the functional groups on the extracted flavonid after the dryness treatment. This is an effective tool for a semi-quantitative estimation of structural information on functional groups in complex solids. The FTIR spectrum of the extracted flavonid was shown in Figure 3.

As shown in this spectrum: the strong band appearing at 604.9 cm^{-1} can be connected with the two isolation hydrogen expansion vibration of the benzene ring; the band at 805.6 cm^{-1} is the linking of ortho-position hydrogen; two bands at 1 264.4 cm^{-1} and 1 026.1 cm^{-1} are the antisymmetry and the symmetrical expansion vibration linking of the C-O-C ether; two wide and strong absorption peaks appearing at 1 539.5 and 1 622.8 cm^{-1} are mainly produced by the C=O stretching vibration.

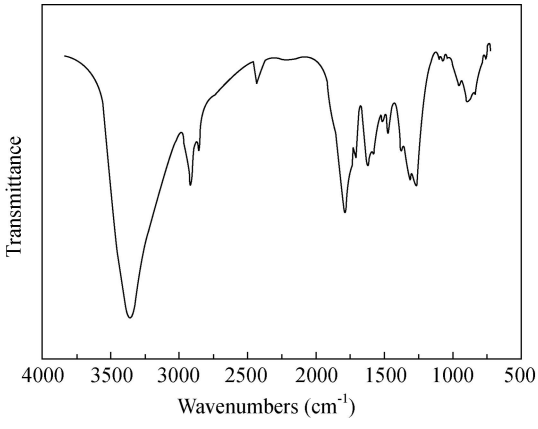

Figure 3. FTIR spectra of extract from Fructus Gardeniae.

Optimization of the Extraction Parameters of Flavonid from Fructus Gardeniae

Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of a solvent extraction method. Based on the above considerations, the extraction parameters were optimized to obtain an efficient extraction of the flavonid from fructus gardeniae. In

fact, the ratio of buffer solution ($Na_2B_4O_7 \cdot 10H_2O$) to raw material (%), extraction time, pH of the extraction solution, and ratio of fructus gardeniae to extracting solution are generally considered to be the most important factors. Optimization of the suitable extraction conditions in the fructus gardeniae extraction can be carried out by using an experimental design. In the present study, all selected factors were examined by using an orthogonal [$L_9(3)^4$] test design. The results of the orthogonal test and the extreme difference analysis are presented in Table 2.

Table 2. Orthogonal Array of the Experiments on Extraction of Flavonid from Fructus Gardeniae

Experiment No.	Factor				Ratio of Flavonid to Fructus Gardeniae（mg/g）
	Ratio of buffer solution ($Na_2B_4O_7 \cdot 10H_2O$) to raw material (%)	concentration of fructus gardeniae in extraction solution（g/mL）	extraction time (min)	pH of buffer solution	
1	1	1	1	1	3.9833
2	1	2	2	2	4.3633
3	1	3	3	3	3.9133
4	2	1	2	3	4.0967
5	2	2	3	1	5.1200
6	2	3	1	2	5.0533
7	3	1	3	2	4.0733
8	3	2	1	3	4.0300
9	3	3	2	1	4.4667
K1	12.2599	12.1533	13.0666	13.5700	
K2	14.2700	13.5133	12.9267	13.4899	
K3	12.5700	13.4333	13.1066	12.0400	
R	0.6701	0.4533	0.06	0.51	

Note. Ki is obtained by adding any number of columns corresponding to i factor. R is the difference between the maximum value and the minimum value of Ki of any columns.

The extract obtained from each test in the fructus gardeniae extraction was weighted and quantitatively analyzed and then the extraction yields of the crude extract were calculated. The results of the experiments presented in Table 2 indicated that the maximum extraction yield of the crude extract was 5.0533 (mg/g). However, we cannot select the best extraction conditions only based on these results in Table 2, and a further orthogonal analysis was warranted. Thus, the K, and R values were calculated and listed in Table 2. As seen from Table 2, we can find that the influence on the mean extraction yields of the compounds decreases in the order: A>C>D>B according to the R values. The ratio of buffer solution ($Na_2B_4O_7 \cdot 10H_2O$) to raw material (%) was found to be the most important determinant of the yield. In other words, the maximum yield of the flavonid was obtained when the ratio of $Na_2B_4O_7 \cdot 10H_2O$ to raw material was 0.4%, the extraction time was 20 min, the pH of buffer solution was 4.5, and the concentration of Fructus Gardeniae in the extraction solution was 1/12 (g/mL), respectively.

CONCLUSION

Fructus Gardeniae is widely distributed in China, but reports on the extraction of flavonid from Fructus Gardeniae are few. This work was made to optimize the operating conditions of the extraction of flavonoid from Fructus Gardeniae. An orthogonal experiment [$L_9(3)^4$] was applied to get the best extraction conditions. Results showed that the best extraction conditions were: the ratio of $Na_2B_4O_7 \cdot 10H_2O$ to raw material was 0.4%, the extraction time was 20 min, the pH of buffer solution was 4.5, and the concentration of Fructus Gardeniae in the extraction solution was 1/12 (g/mL). This investigation will be expected to result in a better understanding of the fructus gardeniae's therapeutic and clinical merits.

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