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Optimization of the Extraction Parameters of Gardenia (*Gardenia jasminoides* Ellis) Fruits for the Maximum Antioxidant Capacity

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Abstract Response surface methodology (RSM) was used for the optimization of antioxidant capacity in gardenia extracts. The antioxidant capacities of gardenia fruit extracts were investigated by ferric reducing ability (FRA) and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging activity (RSA) assays. The optimum extraction parameters for the strongest antioxidant capacity were the ethanol concentration (EtOH) of 48.9%, extraction temperature of 72.9°C, and extraction time of 29.9 min. Analysis of variance (ANOVA) showed that the quadratics of EtOH and extraction temperature had highly significant effect on the antioxidant capacity ($p<0.001$). The antioxidant capacity was correlated with contents of bioactive components [crocin, geniposide, and total phenolic (TP) compounds] in gardenia extracts and mainly attributed to the content of the TP compounds.

Keywords: extraction optimization, antioxidant capacity, response surface methodology (RSM), gardenia fruit extract

Introduction

Gardenia (*Gardenia jasminoides* Ellis), an evergreen shrub cultivated in many temperate regions, belongs to the Rubiaceae family. Its oval shaped fruits attain a reddish yellow color when ripe in late autumn and are used in traditional Chinese herbal medicine for curing a number of ailments (1). The most important components in gardenia extract are carotenoids (2-4) which are esterified with one or two glucoses and gentibiose sugar moieties and are known for their coloring properties owing to their peculiar water soluble behavior especially different from most families of carotenoids (2). Carotenoids have been studied for many years and there has been a growing interest in the antioxidant properties of carotenoids in the past few years (5-7). Carotenoids can be used as antioxidants by quenching singlet oxygen (8) or free radicals (9). Crocin, the digentiobiosyl ester of *trans*-crocetin which is the principal coloring power of gardenia fruit extracts, is non-toxic and chemically stable compared with many other natural pigments (10). It might be found numerous applications as a food colorant or antioxidant (11-13) and the information on the reaction of crocin with linoleic acid and oxygen has been reported (14). Gardenia extract is also rich in phenolic compounds with high antioxidant capacity (15,16). However, the antioxidant activity of gardenia extracts which were obtained under different extraction parameters has not yet been studied.

As a powerful statistical and mathematical tool, response surface methodology (RSM) which has a major advantage over the single-factor approach can be used for evaluating interaction among factors, exploring the relationships between the response and the independent variables and optimizing the processes or products where multiple variables may influence the outputs (17-19).

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The objective of the present study was to investigate the effect of extraction parameters [ethanol concentration (EtOH), extraction temperature, and extraction time] on the antioxidant capacity of the gardenia extract and to estimate the relationship between antioxidant capacities and contents of the main bioactive components in gardenia extracts.

Materials and Methods

Materials The dried gardenia fruits were provided by Fuzhou Tianshun Industry Development Co., Ltd. (Jiangxi, China) and stored in the dark at room temperature until used. Whole gardenia fruits were dehulled (water content was 6.15%) and ground to pass through 0.9, 0.6, and 0.3-mm mesh sieves. The distribution of the particle sizes was as follows: <0.3 mm (16.7%), 0.3-0.6 mm (29.0%), 0.6-0.9 mm (46.6%) and >0.9 mm (7.7%).

Chemicals and reagents Standards of crocin, self-prepared [>99.5% high performance liquid chromatography (HPLC) grade], was used in the assay for crocin quantification. Geniposide standard (99.8% HPLC grade) and chlorogenic acid was purchased from Shanghai U-sea Biotech Co., Ltd. (Shanghai, China) and Xi'an Jiangxing Biology Technology Co., Ltd. (Xi'an, China), respectively. Folin-Ciocalteau reagent, 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris-2-pyridy-s-triazine (TPTZ), and 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals (analytical grade) were from Beijing Chemical Co. (Beijing, China), unless otherwise stated.

HPLC assay of crocin and geniposide Crocin and geniposide were determined according to the procedure reported before (2,20) with slight modifications. HPLC analysis was performed on an Agilent 1100 liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) with an SB-C_{18} column (250×4.6 mm, i.d.) at 25°C. The mobile phase for crocin delivered at 0.8 mL/min was 80:20 (v/v) mixture of 0.25% aqueous formic

acid and acetonitrile which was changed linearly to 20:80 (v/v) over 15 min. Geniposide was eluted at the same flow rate for 10 min by a isocratic mobile phase composed of methanol and 0.25% aqueous formic acid at 35:65 (v/v). The diode-array detector was performed at 440 and 238 nm for crocin and geniposide, and their concentrations were calculated from the calibration curves, respectively. All assays were performed in triplicate.

Determination of the total phenolic (TP) compounds content The concentration of TP compounds was measured with the method described by Matthäus (21) and expressed as chlorogenic acid equivalents (CAE) using a calibration curve with chlorogenic acid as the standard (mg/mL) (22).

Assay of ferric reducing ability (FRA) The FRA of each extract sample was measured with TPTZ method described by Benzie and Strain (23) with minor modifications. Reagents included 0.3 M acetate buffer (pH 3.6); 0.01 M TPTZ in 0.04 M HCl; 0.02 M $FeCl_3 \cdot 6H_2O$. Working reagent (23) was prepared as required by mixing acetate buffer, TPTZ solution and $FeCl_3 \cdot 6H_2O$ solution (10:1:1). A 0.5 mL aqueous solution of the extract was added to 3.5 mL distilled water and 1 mL of working reagent. The mixture was shaken vigorously for 20 sec and allowed to stand for 10 min in the dark at 37°C. The absorbance of the mixture was measured at 593 nm. The FRA of each gardenia extract was expressed as Trolox equivalent antioxidant capacity (TEAC) using a calibration curve with Trolox antioxidant activities as the standard (10^{-3} M).

Assay of DPPH free radical scavenging activity (RSA) The stable DPPH RSA of gardenia extracts was determined by the method of Von-Gadow *et al.* (24) with minor modifications. Two mL of ethanolic DPPH (10^{-4} M) solution was added to 2 mL of aqueous solution of extract, and 2 mL of distilled water was used as the blank. The mixture was shaken vigorously for 20 sec and allowed to stand for 30 min in the dark at ambient temperature. The decrease in absorbance of the mixture was measured at 517 nm against the blank using a UV-Vis spectrophotometer. The RSA of the sample was expressed as percentage inhibition of DPPH,

$$\%\text{Inhibition} = \left(1 - \frac{A_i - A_j}{A_c}\right) \times 100 \qquad (1)$$

where A_c is the absorbance of the blank; A_i is the absorbance of the sample and A_j is the background absorbance of the sample. The RSA of each gardenia extract was expressed TEAC with a calibration curve with Trolox antioxidant activities as the standard (10^{-3} M).

Extraction process of gardenia fruits About 2.0 g of gardenia powder was immersed into 12 mL solvent of aqueous ethanol. The EtOH, extraction temperature, and extraction time were set according to the preliminary experimental results. In brief, 100 μL of the extract was filled up with distilled water to 10 mL and filtrated with a 0.22-μm filter membrane. Each sample was analyzed by the methods described above.

Experimental design RSM was applied to identify optimum levels of EtOH (%), extraction temperature (°C), and extraction time (min) on the antioxidant capacity (10^{-3} M TEAC) of gardenia extracts. The coded and uncoded independent variables used in the RSM design were listed in Table 1. Ranges of EtOH (X_1), temperature (X_2), and time (X_3) and the central point were selected based on preliminary experimental results. The experiments were designed according to the central composite design using a 2^3 factorial and star design with 6 central points as shown in Table 2. The order of the experiments has been fully randomized. Analytical determinations were performed in triplicate. Data were analyzed by multiple regressions through the least-square method. A second-order polynomial equation was used to express the responses as a function of the independent variables as follows:

Table 1. Uncoded and coded levels of independent variables used in the RSM design

Symbol	Independent variable	Coded level				
		-1.5267	-1	0	1	1.5267
X_1	EtOH (%)	19.5	30	50	70	80.5
X_2	Temperature (°C)	27.1	35	50	65	72.9
X_3	Time (min)	7.1	15	30	45	52.9

$$Y_k = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_{ii} + \sum_{i \neq j=1}^{3} a_{ij} X_i X_j \qquad (2)$$

where Y_k represents the measured response variables, a_0 is a constant, a_i, a_{ii}, and a_{ij} are the linear, quadratic, and interactive coefficients of the model, respectively. X_i and X_j are the levels of the independent variables. Three-dimensional surface response plots were generated by varying 2 variables within the experimental range and holding the other constant at the central point. The mathematical analyses of the data were conducted using Statgraphics Centurion XV (25). The test of statistical significance was based on the total error criteria with a confidence level of 95.0%.

Results and Discussion

Antioxidant capacities of gardenia extracts The FRA and RSA of gardenia extracts were expressed as Trolox equivalent antioxidant capacity in the study. The standard curves of FRA (Y_1) and DPPH RSA (Y_2) of Trolox were established with the determination coefficients of 0.9997 and 0.9991, respectively. The antioxidant capacities of gardenia extracts obtained from all the experiments were calculated according to the standard curves and listed in Table 2. Integrated surveying, average antioxidant capacity (Y) of the extracts was calculated by the average of FRA and DPPH RSA.

Fitting the model The experimental data in Table 2 were used to calculate the coefficients of the second-order polynomial equation and the results of analysis of variance (ANOVA) for the antioxidant capacity were listed in Table 3. ANOVA was used to evaluate the significance of the coefficients of the models. For any of the terms in the model, a larger regression coefficient and a smaller p value

Table 2. Contents of bioactive constituents (crocin, geniposide, and TP compounds) and antioxidant capacities of gardenia extracts (10^{-3} M TEAC)

No	EtOH	Temperature	Time	Antixoidant capacity[1)]			Content (mg/mL)		
	X_1 (%)	X_2 (°C)	X_3 (min)	FRA (Y_1)	RSA (Y_2)	Y	Crocin	Geniposide	TP[2)] compounds
1	50.0	27.1	30.0	4.629	4.813	4.721	1.151	14.808	2.724
2	50.0	50.0	30.0	6.939	6.608	6.773	1.274	17.002	3.679
3	50.0	50.0	52.9	6.620	6.308	6.464	1.270	16.616	3.521
4	70.0	65.0	45.0	6.954	6.640	6.797	1.354	17.573	3.701
5	30.0	35.0	15.0	5.078	5.346	5.212	1.107	14.600	2.671
6	30.0	35.0	45.0	5.374	5.565	5.469	1.233	16.427	3.039
7	70.0	65.0	15.0	6.605	6.171	6.388	1.207	15.484	3.370
8	19.5	50.0	30.0	5.556	5.694	5.625	1.025	13.834	2.738
9	50.0	50.0	30.0	6.939	6.575	6.757	1.275	16.859	3.669
10	30.0	65.0	45.0	6.658	6.365	6.512	1.222	15.895	3.588
11	70.0	35.0	45.0	5.807	5.799	5.803	1.159	14.634	2.679
12	30.0	65.0	15.0	6.856	6.551	6.703	1.316	16.584	3.564
13	50.0	72.9	30.0	7.380	7.028	7.204	1.389	18.048	4.008
14	50.0	50.0	30.0	6.970	6.583	6.776	1.286	17.205	3.674
15	80.5	50.0	30.0	4.979	4.829	4.904	1.111	14.622	2.703
16	50.0	50.0	30.0	6.939	6.616	6.777	1.279	17.102	3.677
17	70.0	35.0	15.0	4.454	4.619	4.537	0.964	12.106	2.307
18	50.0	50.0	30.0	6.947	6.600	6.773	1.271	16.896	3.685
19	50.0	50.0	30.0	6.985	6.583	6.784	1.273	16.913	3.679
20	50.0	50.0	7.1	5.351	5.565	5.458	1.188	14.761	2.859

[1)]FRA, ferric reducing ability; RSA, radical scavenging ability; Y, average value of Y_1 and Y_2.
[2)]Total phenolic, mg CAE/mL.

(less than 0.05) would indicate a more significant effect on the respective response variables. The high coefficient of determination (R^2=0.9356) and insignificant result of lack of fit analysis (p>0.05) indicated the model fitted the experimental data very well and could explain 93.56% of the antioxidant capacity variability.

Response surface analysis of the data in Table 2 demonstrated that the relationship between average antioxidant capacities (Y, average of Y_1 and Y_2) of the extracts and EtOH, extraction temperature and time is quadratic with good regression coefficient (R^2 = 0.9356). Eq. 3 shows the relationship between the average antioxidant capacity and extraction parameters.

$$Y=-2.10222+0.158012X_2+0.0768233X_3-0.00128186X_1^2 -0.00094432X_2^2-0.000947X_3^2 \quad (3)$$

Analysis of response surface Three-dimensional graphs, clearly visualizing the influence of each independent variable on the dependent-average antioxidant capacity, were presented in Fig. 1. These graphs revealed that the significant influence of temperature and EtOH. Contour plots, each showing the response of antioxidant capacity to 2 independent variables, located the point of maximum activity.

Figure 1A is a response surface plot showing the effect of the EtOH and extraction temperature on the average antioxidant capacity at fixed extraction time of 30 min. The concentration of ethanol had a significant negative quadratic effect on the antioxidant capacity (p<0.001, Table 3). The average antioxidant capacity first increased and then decreased when the concentration of ethanol increased. EtOH had a positive effect on average antioxidant capacity at low levels of EtOH. This is most likely because of the improvement of the phenolic compound solubility resulted from the increased ethanol molecule in the aqueous solution and the breakage degree of the raw material cell membrane was also enhanced by higher EtOH (26,27). However, at high levels of EtOH, the negative quadratic effect on the average antioxidant capacity became important. This is probably because EtOH at higher levels could lead to the coagulation of protein in the raw material and in turn resulted in an increasing diffusion resistance of antioxidant compounds in cells of raw material which is essential to the extraction process according to the previous studies (26-28).

Figure 1B-C show the effect of extraction temperature and time on the average antioxidant capacity of gardenia extracts. Extraction temperature showed a positive linear effect on the average of antioxidant capacity (p<0.001) within the experimental range at fixed EtOH of 50%. This is mostly attributed to the increase in diffusion activities of bioactive compounds with the rise of temperature. Similarly, extraction time also played a positive role in the extraction process (p<0.01). With the extension of extraction time, more antioxidant compounds released from the materials to the extract, therefore the average antioxidant capacity of the extract became stronger.

Optimization of extraction condition The optimal extraction parameters predicted by response surface analysis were the EtOH of 48.9%, extraction temperature

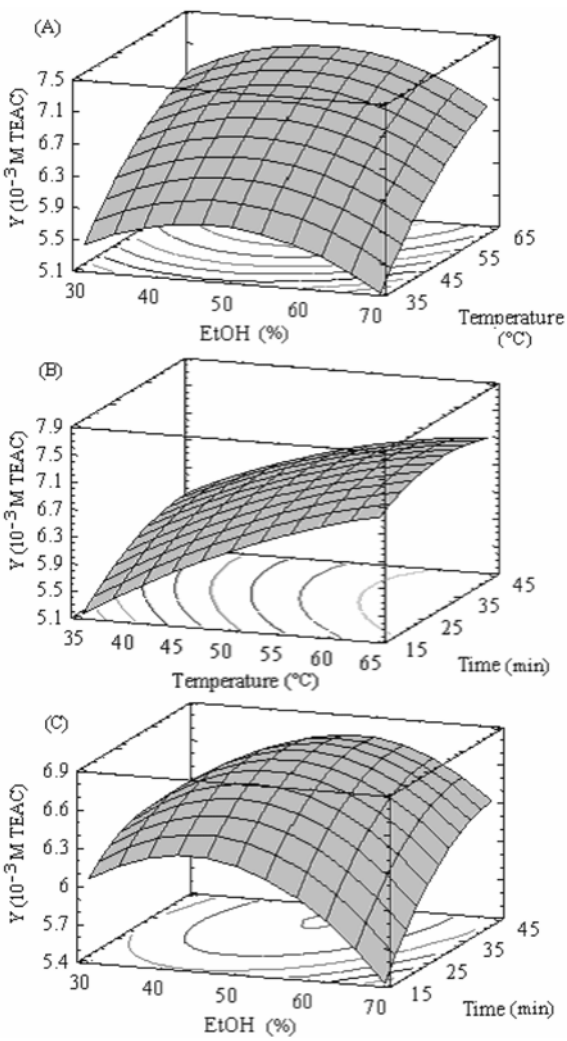

Fig. 1. Three-dimensional and contour plots for the effect of extraction parameters on the antioxidant capacity. (A) Effect of extraction temperature and EtOH (extraction time of 30 min); (B) effect of extraction time and temperature (EtOH of 50%); and (C) effect of extraction time and EtOH (extraction temperature of 50°C).

of 72.9°C, and extraction time of 29.9 min, and the maximum average antioxidant capacity calculated by the model was 7.33 M TEAC while the experimental value is 7.62±0.18 M TEAC.

Multiple linear regressions Regression analysis of the relationship between the antioxidant capacity and contents of crocin, geniposide, and total phenol in gardenia extract was performed by SAS software according to stepwise procedure, selecting variables step by step, and the results were shown in Table 4. FRA (Y_1) and DPPH RSA (Y_2) were linearly correlated to the content of TP compounds in the extract of gardenia. The average antioxidant capacity of the gardenia extract was also linearly related to the content of TP compounds with R^2 of 0.920. The results indicated that geniposide, the major iridoid glycoside in the extract of gardenia, did not contribute to the antioxidant capacity of the extract, which is consistent with the previous literature reported by Chen *et al.* (29). This could be explained by its similarity to the chemical structure of iridoid glycosides. However, the crocin, which possesses antioxidant activity on the basis of some published literature (8,14), showed little contribution to the overall antioxidant capacity of the gadenia extract. The possible explanation might be that the crocin content was much less than that of TP compounds in the crude gardenia extract, therefore TP compounds were dominating over the antioxidant capacity of the extract.

The current study showed that the second-order polynomial model was sufficient to describe and predict the response of the average antioxidant capacity of gardenia extracts in the extraction processes. The average antioxidant capacity of the extract was more significantly affected by the independent parameters of extraction temperature and the quadratics of EtOH ($p<0.001$). The extraction time also had significant effect on the average antioxidant capacity ($p<0.05$). The graphical optimization method was adopted to find the best extraction condition and the predicted average antioxidant capacity/mL of the gardenia extract was consistent with the experimental value. The antioxidant capacity of the gardenia extract was mainly correlated to TP compounds.

Table 3. Analysis of variance for the response of antioxidant capacity in the gardenia extracts

Source	F-value	*p*-value
EtOH	2.07	0.1811
Temperature	80.20	0.0000***
Time	10.25	0.0095**
EtOH^2	34.31	0.0002***
EtOH×Temperature	0.15	0.7096
EtOH×Time	3.91	0.0761
Temperature^2	5.89	0.0356*
Temperature×Time	2.58	0.1395
Time^2	5.93	0.0352*
R^2	0.9356	

*$p<0.05$, **$p<0.01$, ***$p<0.001$.

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Table 4. Regression statistic between antioxidant activities and contents of bioactive component

	F-value	*p*-value	CV[1)]	R^2	Adj R^2	Regression model
Y_1	225.92	<0.0001	4.20497	0.9262	0.9221	Y_1=0.30470+1.79946TP
Y_2	160.87	<0.0001	3.86154	0.8994	0.8938	Y_2=1.59028+1.35881TP
Y	207.50	<0.0001	3.90054	0.9202	0.9157	Y=0.94716+1.57927TP

[1)]Coefficient of variation.

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