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Ultrasonic-assisted extraction of pigments from *Hylocereus undatus* flowers: Optimization, antioxidant activity, and HPLC analysis

Yong Liu*, Huifang Zhang and Shoulian Wei

School of Chemistry and Chemical Engineering, Zhaoqing University, Zhaoqing, P.R. China

Abstract: The ultrasonic-assisted extraction of pigments from the *Hylocereus undatus* flowers was modeled using response surface methodology. A three-level, three-factor Box-Behnken design was employed to optimize three extraction variables, including liquid-solid ratio (X_1), extraction time (X_2), and ethanol concentration (X_3), for the achievement of high absorbance. The statistical analysis shows that the model is significant to optimize the extraction variables ($p < 0.01$), and the independent variable (X_3) and the quadratic term (X_3^2) have significant effect on the absorbance ($p < 0.01$). The optimized conditions are X_1 of 42:1 mL/g, X_2 of 33.2 min, and X_3 of 77.5%. Under these conditions, the experimental absorbance is 2.240±0.095% (n=3), which is in good agreement with the predicted value of 2.243. The evaluation of antioxidant activity by DPPH and hydroxyl free radicals, reducing power, and metal chelating ability indicates that the pigments from the *H. undatus* flowers possess significant antioxidant activity. HPLC analysis reveals that gallic acid, rutin, quercetin, kaempferol and isorhamnetin are the major composition in the pigments.

Keywords: *Hylocereus undatus*; pigments; response surface methodology; antioxidant activity; ultrasonic extraction.

1. Introduction

In recent years, various pigments are extensively used as additives or supplements in food industry.[1] Pigments play an important role in foodstuffs for improving food color, giving food attractive color, and identifying visual clues and taste thresholds, which can give consumers good sensing enjoyment and strong purchasing desire.[2,3] Edible pigments are divided into synthetic and natural pigments according to their resources. Synthetic pigments are widely

*Corresponding author at: School of Chemistry and Chemical Engineering, Zhaoqing University, Zhaoqing, 526061, P.R. China. E-mail: lygdut@163.com (Yong Liu).

used in food industry due to their bright color, low cost and strong stability. However, applications of synthetic pigments in food products are under strict regulation due to the toxicity, carcinogenicity and mutagenicity in the human body.[4-6] In contrast, natural pigments mainly obtained from plants, microorganisms and animal tissues by physical extraction have bright color and a history of safe use.[7] Furthermore, the natural pigments reveal certain functional properties, such as nutritional, pharmaceutical and antioxidant agents,[5,8,9] which makes them of particular interest to processors and consumers. Consequently, development and utilization of the natural pigments as alternatives to the synthetic pigments for both health benefits and commercial needs have attracted global interest among researchers.

The *Hylocereus undatus* flowers, known as "Bawanghua" or "Jianhua" in China, belong to the Cactaceae family from the subfamily Cactoidea of the tribe Cactea[10-12] and are the common vegetable in southern China and are used to prepare various healthy and tasty soups.[13,14] Glycosides and flavonoids are found in the *H. undatus* flowers,[15,16] and the flowers are also used traditionally for many medicinal purposes, including clearing "heat-fire", moisturizing the lung, eliminating phlegm and relieving cough.[17] The *H. undatus* flower is rich in yellow pigments. However, to the best of our knowledge, there are no reports about the extraction, antioxidant activity and HPLC analysis of the pigments from the *H. undatus* flowers.

Ultrasonic-assisted extraction (UAE) is one of the most inexpensive, rapid, simple and efficient techniques compared with conventional extraction,[18] and has been applied to extract bioactive compounds from different materials owing to its high reproducibility at shorter time, simplified manipulation, significant reduction in solvent consumption and temperature, and lower energy input.[19,20] Therefore, the ultrasound technology has been used in some industries, such as food industry, chemical industry, and material industry.[21]

Response surface methodology (RSM), an effective statistical technique for modeling and optimization of complex processes, has been used increasingly to optimize processing parameters owing to more efficient and easier arrangement and interpretation of experiments compared with others.[22-24] The advantages of RSM are the reduced number of experimental trials and the convenience to evaluate multiple parameters and their interactions. Therefore, it is widely used to optimize the extraction parameters, such as pigments,[25] polysaccharides,[26]

phenolic compounds,[27] and protein[28] from different materials.

In this work, ultrasonic-assisted extraction technique and response surface methodology were used to investigate the extraction variables (liquid-solid ratio, extraction time, and ethanol concentration), and optimize these variables for the pigments extraction from *H. undatus* flowers. Additionally, the antioxidant activity and the major composition of the pigments were evaluated in detail.

2. Experimental

2.1 Materials and chemicals

H. undatus was obtained from a farm in Zhaoqing City (China) and was identified by Prof. Gang Chen, a botanist in Zhaoqing University. Sodium salicylate, H_2O_2, polassium ferricyanide, trichloroacetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, ferrozine, vitamin C, gallic acid, rutin, quercetin, kaempferol, and isorhamnetin were from Aladdin (Shanghai, China). Methanol was HPLC grade, other reagents were analytical grade and used as received.

2.2 Extraction and spectral characteristics of pigments

The dry *H. undatus* flowers were powdered by a pulverizer (XS-10B, Longxin, China) and passed through an 80 mesh sieve. The extraction of pigments from the *H. undatus* flowers by ultrasonic-assisted treatment was performed in an ultrasonic generator (500 W, 53kHz, SK8200H, Kedao, China). 0.2000 g of the powders was used for each case in a sealed beaker. The beaker was held in the ultrasonic generator and exposed to extract pigments for different extraction time at various ethanol concentrations in different liquid-solid ratios. After ultrasonic treatment, the extracted slurry was centrifuged at 12000 rpm for 15 min to collect the supernatant. To determine the maximum absorption wavelength of the pigments, the supernatant was diluted to 100 mL in a volumetric flask. Then the diluted solution was scanned using a UV-vis spectrophotometer (UVmini-1240, Shimadzu, Japan) in the wavenumber range of 200 to 600 nm to obtain the spectral characteristics of the pigments. To investigate the extraction effect of the pigments, the supernatant was diluted for each case to 50 mL in a volumetric flask. Then the absorbance of diluted solution was determined at 262 nm to indicate the concentration of pigments. The pigment powders were obtained by freeze-drying the supernatant using a freeze dryer (LL3000, Heto, Germany).

2.3 Antioxidant activity assays

2.3.1 DPPH radical scavenging activity

The DPPH radical scavenging activity of the pigments was measured according to Li[29] with slight modifications. Briefly, 0.1 mmol/L solution of DPPH in ethanol was prepared and 1 mL of this solution was added to 3 mL of sample solution at different concentrations. The mixture was vortexed thoroughly and incubated in the dark at room temperature for 30 min, and then the absorbance of the mixture was measured at 517 nm. Vitamin C was used as positive control. The DPPH radical scavenging effect is calculated using the following equation:

$$\text{Scavenging effect}\ (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

where A_{control} is the absorbance of control solution without sample, and A_{sample} is the absorbance of sample solution.

2.3.2 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the pigments was measured according to Ge[30] with slight modifications. 1.0 mL of sample solution was mixed with 1.0 mL of $FeSO_4$ solution (1.5 mM) and 0.3 mL of sodium salicylate solution (20 mM), and then reacted with 0.7 mL of 6 mM H_2O_2. The mixture was incubated at 37 °C for 60 min, and then the absorbance of the mixture was measured at 562 nm. Vitamin C was used as positive control. The hydroxyl radical scavenging effect is calculated using the following equation:

$$\text{Scavenging effect}\ (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (2)$$

where A_{control} is the absorbance of control solution without sample, and A_{sample} is the absorbance of sample solution.

2.3.3 Reducing power

The reducing power of the pigments was measured according to Ge[30] with slight modifications. 1.0 mL of sample solution was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide solution (1%, w/v). The mixture was incubated at 50 °C for 20 min, and then 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1%, w/v) in a

test tube. After 10 min reaction, the absorbance of the mixture was measured at 700 nm. Vitamin C was used as positive control.

2.3.4 Ferrous ions chelating ability

The ferrous ions chelating ability of the pigments was measured according to Feng[31] with slight modifications. 0.5 mL of sample solution was mixed with 3.7 mL of distilled water, 0.1 mL of $FeSO_4$ solution (2.0 mM) and 0.2 mL of ferrozine solution (5.0 mM). After 10 min reaction, the absorbance of the mixture was measured at 562 nm. EDTA-2Na was used as positive control. The ferrous ions chelating ability is calculated using the following equation:

$$\text{Chelating ability}\ (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (3)$$

where A_{control} is the absorbance of control solution without sample, and A_{sample} is the absorbance of sample solution.

2.4 HPLC analysis

The pigments were analyzed using a HPLC (Agilent 1200 series, Agilent Technologies, USA) equipped with a UV detector (G1314B, Agilent) and an Eclipse XDB-C18 column (5μm, 250 mm × 4.6 mm, Agilent). 0.1% H_3PO_4 was used as solvent A and 100% methanol as solvent B (A:B=3:7). The solutions of the standards and the pigments were filtered through a 0.45μm syringe filter. The operating conditions were: column temperature, 25 °C; injection volume, 20μL; detection wavelength, 262 nm; flow rate, 0.8 mL/min. The identification and peak assignment of the pigments was based on comparison of retention times and spectral data with those of the standards. The identified components were quantified according to respective standard calibration curves.

2.5 Experimental design

A three-level, three-factor Box-Behnken design (BBD) was employed to determine the best combination of extraction variables for the pigments based on the results of preliminary single-factor-test. Liquid-solid ratio (X_1), extraction time (X_2), and ethanol concentration (X_3) were the independent variables, and their coded and uncoded levels were presented in Table 1. Absorbance (Y) taken as the response for the design experiment was given in Table 2. Experimental data were fitted to a quadratic polynomial model and the model was explained by the following quadratic equation:[32]

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j \quad (4)$$

where Y is the dependent variable; A_0, A_i, A_{ii}, and A_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively; X_i and X_j are the independent variables.

2.6 Statistical analysis

All the data were determined in triplicate and the results were averaged. Design Expert software version 8.0.6 (Stat-Ease, Minneapolis) was employed for the regression analysis and the optimization.

3. Results and discussion

3.1 Spectral characteristics of pigments

The pigment solution was scanned in the wavelength range of 200 to 600 nm to measure the spectral characteristics of the pigments and depicted in Fig. 1. As shown in Fig. 1, the pigments have significant absorption peak at 262 nm and 349nm, which indicates that the pigments may contain mainly polyphenols.[33,34] Because the maximum absorption peak is at 262 nm, measuring the absorbance value of the pigment solution at 262 nm is expressed as the extraction efficiency of the pigments and taken as the response for the experimental design.

3.2 Single factor test

3.2.1 Effect of liquid-solid ratio on absorbance

Extraction was carried out using liquid-solid ratio from 10:1 to 50:1 mL/g, while other parameters were as follows: extraction time 20 min and ethanol concentration 70% (v/v). The effect of liquid-solid ratio on absorbance is shown in Fig. 2A. As liquid-solid ratio increases, the absorbance slowly increases; but after the ratio exceeds 30:1 mL/g, the increase is not significantly different ($p > 0.05$). This phenomenon may be attributed to the mass transfer principle. The driving force is the concentration gradient between the bulk of the liquid and the solid.[35] Higher liquid-solid ratio means more solvent can enter cells to permeate more pigments, which is good for the pigments diffusion into the solvent under higher liquid-solid ratio.[36] Therefore, higher liquid-solid ratio can result in higher absorbance. When the ratio is over 30:1 mL/g, the increase of the absorbance is not significantly different because most of

the pigments are extracted. The tendency of this parameter was similar to the polyphenols extraction from grape seeds[37] and the polysaccharides extraction from *Lilium davidii* var. *unicolor* Salisb.[38] Therefore, the liquid-solid ratio of 30:1 mL/g is sufficient for extracting the pigments.

3.2.2 Effect of extraction time on absorbance

Extraction was carried out using extraction time from 10 to 50 min, while other parameters were as follows: liquid-solid ratio 20:1 mL/g and ethanol concentration 70%. The effect of extraction time on absorbance is shown in Fig. 2B. When extraction time increases, the absorbance increases initially with a maximum achieved at 30 min and then starts slightly decreasing. Ultrasound has the mechanical agitation, cavitation and thermal effects, which can increase the movement and penetration speed of solvent molecules,[21,39] and consequently make the solvent molecules rapidly enter cells to permeate more pigments. But as the extraction proceeds, the absorbance decreases due to the structural destruction and the decomposition of the pigments.[39] Therefore, 30 min is favorable for extracting the pigments.

3.2.3 Effect of ethanol concentration on absorbance

Extraction was carried out at different ethanol concentration in the range of 60% to 100%, while liquid-solid ratio and extraction time were fixed at 20:1 mL/g and 20 min, respectively. The effect of ethanol concentration on absorbance is shown in Fig. 2C. The variance of the absorbance increases initially and then decreases with the increase of ethanol concentration, and peaks at 90%. It is reported that water is acted as the plant swelling agent, while ethanol is believed to disrupt the bonding between the solutes and plant matrices.[40] In addition, water has a high dielectric constant, which leads to different ethanol concentrations with different polarities.[41] Therefore, this phenomenon may be related to the solvent polarity and the solubility of the pigments, and the ethanol concentration of 90% is good for extracting the pigments.

3.3 Optimization of extraction parameters for pigments

Table 2 shows the process variables and experimental data of 15 runs containing 3 replicates at center point. By applying Design Expert software to multiple regression analysis on the experimental data, the model for the response variable could be expressed by the following quadratic polynomial equation in the form of coded values:

$$Y=2.22 + 0.011X_1 + 0.019X_2 - 0.12X_3 - 0.041X_1^2 - 0.018X_2^2 - 0.22X_3^2 + 0.009X_1X_2 - 0.013X_1X_3 + 0.038X_2X_3 \quad (5)$$

Analysis of variance (ANOVA) for the model is shown in Table 3. The determination coefficient (R^2=0.9653) indicates that only 3.47 % of the total variations are not explained by the model. For a good statistical model, the adjusted determination coefficient (R^2_{adj}) should be close to R^2. As shown in Table 3, R^2_{adj} (0.9027) is close to R^2, indicating that the model is highly significant. The lack of fit test determines whether the selected model is adequate to explain the experimental data, or whether another model should be reselected. The value of lack of fit test (0.0645) is higher than 0.05, which is not significant relative to the pure error and indicates that the fitting model is adequate to describe the experimental data. At the same time, a relatively low value of coefficient of variation (CV) (2.28) indicates a better precision and reliability of the experimental values. Therefore, the model is adequate for prediction in the range of experimental variables.

The significance of each coefficient measured using *p*-value and *F*-value is listed in Table 4. Smaller *p*-value and greater *F*-value mean the corresponding variables would be more significant. The *p*-value of the model is less than 0.01, which indicates that the model is significant and can be used to optimize the extraction variables. The independent variable (X_3) and quadratic term (X_3^2) significantly affect the absorbance within a 99 % confidence interval, and the interactions between liquid-solid ratio (X_1), extraction time (X_2) and ethanol concentration (X_3) are not significant ($p > 0.05$). Meanwhile, ethanol concentration (X_3) is the most significant factor affecting the absorbance.

3D response surface and 2D contour plots are the graphical representations of regression equation and are very useful to judge the relationship between independent and dependent variables. Different shapes of the contour plots indicate whether the mutual interactions between the variables are significant or not. Circular contour plot means the interactions between the corresponding variables are negligible, while elliptical contour suggests the interactions between the corresponding variables are significant.[42] The three-dimensional representation of the response surfaces and two-dimensional contours generated by the model are shown in Fig. 3-5. In these three variables, when two variables are depicted in three-dimensional surface plots, the third variable is fixed at zero level. It is found in Figs. 3-5

that all the three response surfaces are convex in shape, which indicates that the ranges of variables were chosen properly.

As shown in Fig. 3, the absorbance increases slowly when liquid-solid ratio (X_1) and extraction time (X_2) increase in the range of 30:1 to 42.14:1 mL/g and 20 to 33.21 min, respectively; but beyond 42.14:1 mL/g and 33.21 min, the absorbance decreases slightly. This demonstrates that the effect of liquid-solid ratio (X_1) and extraction time (X_2) on the absorbance is not significant and is in good agreement with the results in Table 4. Moreover, the contour shapes in Fig. 3 mean that there is not a significant interaction between the two variables, which also agrees with the results in Table 4.

From Fig. 4, the absorbance increases slowly with increasing of liquid-solid ratio (X_1) from 30:1 to 42.14:1 mL/g and decreases slowly after 42.14:1 mL/g, which indicates that the effect of liquid-solid ratio (X_1) on absorbance is not significant. However, the absorbance increases rapidly with increasing of ethanol concentration (X_3) from 70 to 77.45% and decreases rapidly after 77.45%, which indicates that the effect of ethanol concentration (X_3) on the absorbance is significant. Also, the contour shapes in Fig. 4 mean that there is not a significant interaction between the two variables. These results also agree with the results in Table 4.

It is obvious in Fig. 5 that the absorbance increases slowly with increasing of extraction time (X_2) from 20 to 33.21 min and decreases slowly after 33.21 min; while the absorbance increases at first and then decreased quickly with increasing of ethanol concentration (X_3), and a maximum absorbance is achieved at 77.45%. These phenomena indicate that the effect of extraction time (X_2) on the absorbance is not significant but ethanol concentration (X_3) is significant. Additionally, the contour shapes in Fig. 5 mean that there is not a significant interaction between the two variables. These results also agree with the results in Table 4.

3.4 Verification of the model

The suitability of the model equation for predicting the optimum response values are tested using the selected optimum conditions. The optimum conditions are liquid-solid ratio (X_1) of 42.14:1 mL/g, extraction time (X_2) of 33.21 min, and ethanol concentration (X_3) of 77.45%, under which the predicted absorbance is 2.243. The model is experimentally verified at liquid-solid ratio (X_1) of 42:1 mL/g, extraction time (X_2) of 33.2 min, and ethanol concentration (X_3) of 77.5%, under which the experimental value is 2.240±0.095% (n=3),

agreeing closely with the predicted value and consequently indicating the RSM model is satisfactory and accurate.

3.5 Antioxidant activity of pigments

3.5.1 Radical scavenging activity

DPPH assay was used to evaluate the radical scavenging activity of the pigments because DPPH radicals are the stable free radicals and the model of scavenging them is a most widely used method to evaluate radical scavenging activity in a relatively short time compared with other methods.[43] The effect of samples on scavenging DPPH radicals is related to the ability of their hydrogen donation. The decrease in absorbance of the DPPH radicals by samples is determined at 517 nm. Fig. 6A shows the scavenging effect of the pigments on the DPPH radicals compared with vitamin C as a positive control. The effect of scavenging DPPH radicals increases significantly ($p < 0.05$) with the increases in the concentrations of vitamin C and pigments. The scavenging effect of vitamin C is 41.53% at 0.1 mg/mL, and increases rapidly to 89.86% at 0.6 mg/mL; the scavenging effect of the pigments is 33.62% at 0.1 mg/mL, and increases linearly to 86.78% at 1.0 mg/mL. The results show that the scavenging effect of pigments at the concentration of 1.0 mg/mL is close to that of vitamin C at the concentration of 0.6 mg/mL, and the pigments have a noticeable effect on scavenging DPPH free radicals.

Hydroxyl radicals, well known as the most reactive free radicals, can react with almost all the biomacromolecules functioning in living cells and severely induce tissue damage or cell death.[30] Moreover, there is no specific enzyme to defend against the hydroxyl radicals in humans.[44] Therefore, it is important to discover chemicals with good scavenging effect for removing the hydroxyl radicals to prevent cell damage.[45] The scavenging effect of the pigments on the hydroxyl radicals is shown in Fig. 6B. The effect of scavenging hydroxyl radicals increases significantly ($p < 0.05$) with the increases in the concentrations of pigments and vitamin C. The scavenging effect of vitamin C is higher than that of pigments, and is 36.58% at 0.1 mg/mL and increase rapidly to 87.11% at 0.6 mg/mL but increase slowly after 0.6 mg/mL; the scavenging effect of the pigments is 31.59% at 0.1 mg/mL, and increases linearly to 84.67% at 1.0 mg/mL. These results show that the pigments possess a significant effect on scavenging hydroxyl radicals.

3.5.2 Reducing power and ferrous ions chelating ability

It has been reported that the reducing power of a sample may serve as a significant indicator of potential antioxidant activity and is positively related to the antioxidant activity.[46] The reducing power of the pigments determined at 700 nm is shown in Fig. 7A. It can be seen in Fig. 7A that the reducing power of the pigments and vitamin C is strong and in a concentration-dependent manner and a linear relationship. At the concentration from 0.025 to 0.2 mg/mL, the reducing power of the pigments and vitamin C increases from 0.209 to 1.579 and 0.290 to 1.779, respectively. A higher slope of the line means a higher reducing power. The slopes of the pigments and vitamin C obtained by linear regression are 7.71 and 8.18, respectively, which indicates that the reducing power of pigments is lower than that of vitamin C. These results demonstrate that the pigments have strong reducing power and can be used as reducing agent.

Fe^{2+}, known as the most powerful prooxidant due to its high reactivity, can trigger process of free radical reaction to magnify the cellular damage through stimulating lipid peroxidation.[47] Therefore, chelating Fe^{2+} is very important to reduce this damage. Chelating ability measures the effect of samples to compete with ferrozine for ferrous ions.[48] The ferrous ions chelating ability of the pigments and EDTA at different concentrations is shown in Fig. 7B. As shown in Fig. 7B, both the pigments and EDTA have strong chelating ability in a concentration-dependent manner. Compared with EDTA at the concentration from 0.025 to 0.2 mg/mL, the pigments exhibit a relatively weak chelating ability. At the concentration of 0.1 mg/mL, the chelating ability of EDTA is 97.17%, whereas that of pigments is 70.73% but reaches 94.56% at the concentration of 0.2 mg/mL. These results indicate that the pigments have high chelating ability and can be used to effectively chelate Fe^{2+}.

3.6 HPLC analysis of pigments

Fig. 8 shows the chromatograms of the standard mixture and the pigments. The HPLC chromatograms reveal that gallic acid is the major phenolic compound in the *H. undatus* flowers, while rutin, quercetin, kaempferol and isorhamnetin are the major flavonoid compounds. The structures of gallic acid, rutin, quercetin, kaempferol and isorhamnetin are shown in Fig. 9. The content of gallic acid, rutin, quercetin, kaempferol and isorhamnetin in the pigments is calculated from respective standard calibration curves, and the values for

gallic acid, rutin, quercetin, kaempferol and isorhamnetin are 48.55%, 20.12%, 2.77%, 5.39% and 5.41%, respectively. These results indicate that gallic acid (48.55%), rutin (20.12%) and unknown (17.76%) may be mainly responsible for the antioxidant activity.

4. Conclusions

The ultrasonic-assisted extraction technology was performed for the extraction of the pigments from the *H. undatus* flowers and optimized by response surface methodology. Based on the single-factor-test, Box-Behnken design was used to evaluate and optimize the extraction variables for the absorbance. The regression analyses show that the variable (ethanol concentration) is significant and a high correlation of quadratic model obtained is satisfactory and accurate to predict the absorbance. The optimized conditions are as follows: liquid-solid ratio 42:1 mL/g, extraction time 33.2 min, and ethanol concentration 77.5%. Under these conditions, the absorbance is 2.240±0.095% (n=3), agreeing closely with the predicted value. The HPLC analysis and the antioxidant activity assays indicate that the pigments are composed of gallic acid, rutin, quercetin, kaempferol and isorhamnetin, and have significant antioxidant activity. The results indicate that the pigments can be used as a source of potential antioxidant or functional coloring agent in food system.

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Table 1 Independent variables and their levels for Box-Behnken design

Independent variables	Levels		
	−1	0	1
Liquid-solid ratio (X_1) (mL/g)	30:1	40:1	50:1
Extraction time (X_2) (min)	20	30	40
Ethanol concentration (X_3) (%)	70	80	90

Table 2 Box-Behnken design for independent variables and their absorbance

Run	X_1 (liquid-solid ratio, mL/g)	X_2 (extraction time, min)	X_3 (ethanol concentration, %)	Absorbance
1	0	0	0	2.239
2	1	−1	0	2.172
3	0	0	0	2.221
4	0	1	1	1.971
5	0	−1	1	1.815
6	−1	−1	0	2.159
7	0	0	0	2.208
8	−1	1	0	2.138
9	−1	0	1	1.816
10	1	0	1	1.805
11	0	−1	−1	2.075
12	1	0	−1	2.133
13	1	1	0	2.187
14	−1	0	−1	2.094
15	0	1	−1	2.079

Table 3 Analysis of variance for fitted quadratic model of extraction of pigments

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value (Prob. > *F*)
Model	0.31	9	0.034	15.43	0.0038
Residual	0.011	5	2.231×10^{-3}		
Lack of fit	0.011	3	3.557×10^{-3}	14.68	0.0645
Pure error	4.847×10^{-4}	2	2.423×10^{-4}		
Cor. total	0.32	14			

R^2=0.9653; R^2_{adj}=0.9027; C.V.%=2.28.

Table 4 Regression coefficients estimate and their significance test for quadratic model

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value (Prob. > *F*)
X_1	1.012×10^{-3}	1	1.012×10^{-3}	0.45	0.5304
X_2	2.964×10^{-3}	1	2.964×10^{-3}	1.33	0.3011
X_3	0.12	1	0.12	53.16	0.0008
X_1^2	6.156×10^{-3}	1	6.156×10^{-3}	2.76	0.1576
X_2^2	1.174×10^{-3}	1	1.174×10^{-3}	0.53	0.5007
X_3^2	0.18	1	0.18	79.99	0.0003
X_1X_2	3.24×10^{-4}	1	3.24×10^{-4}	0.15	0.7188
X_1X_3	6.25×10^{-4}	1	6.25×10^{-4}	0.28	0.6192
X_2X_3	5.776×10^{-3}	1	5.776×10^{-3}	2.59	0.1685

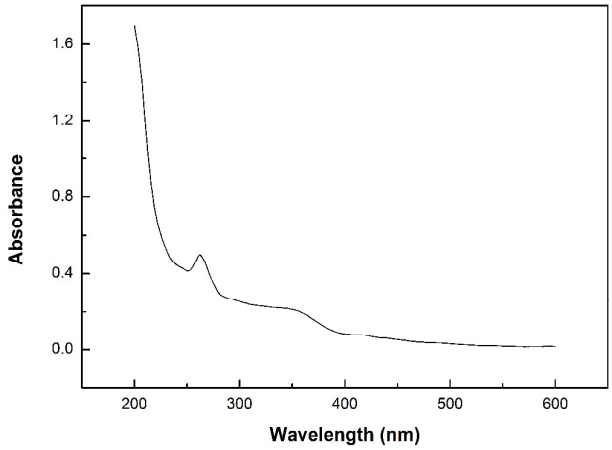

Fig. 1 UV-vis spectral curve of pigments in the wavelength range of 200 to 600 nm.

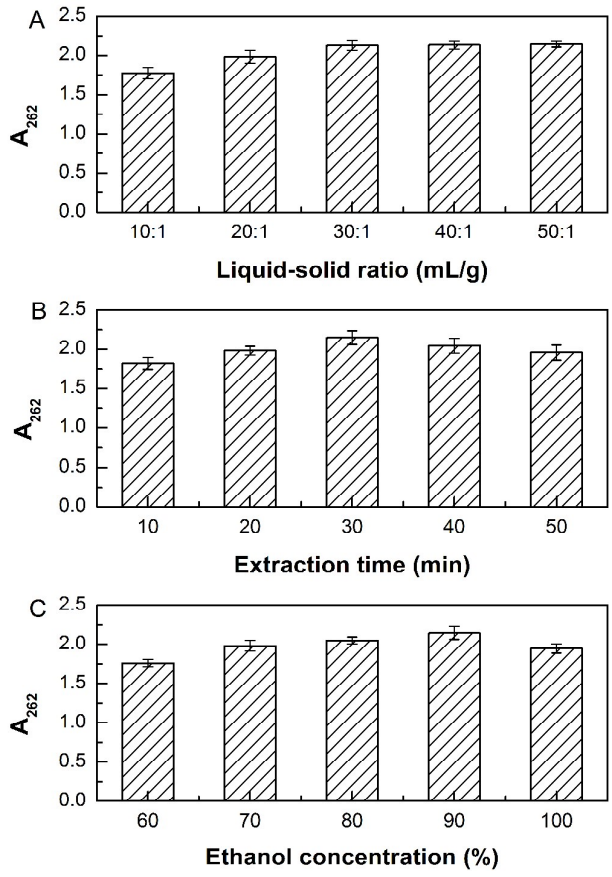

Fig. 2 Effect of different extraction variables on absorbance.

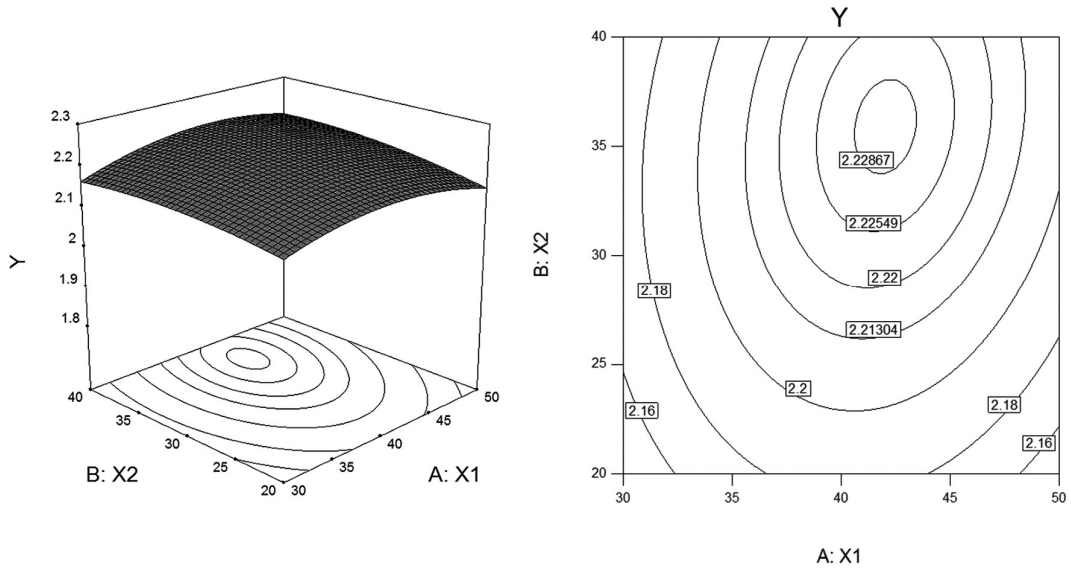

Fig. 3 Response surface and contour plots showing effect of liquid-solid ratio (X_1) and extraction time (X_2).

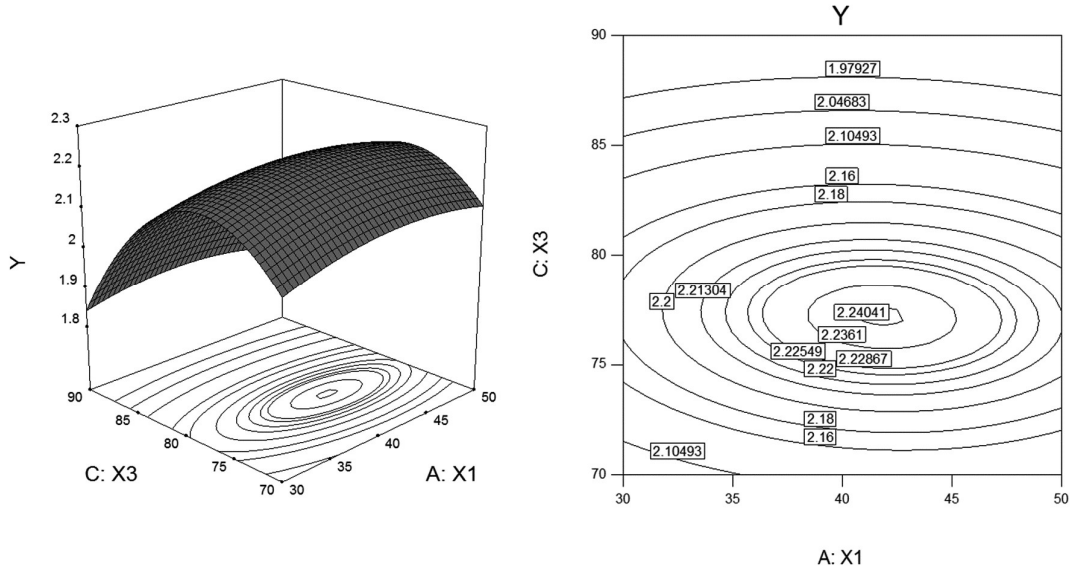

Fig. 4 Response surface and contour plots showing effect of liquid-solid ratio (X_1) and ethanol concentration (X_3).

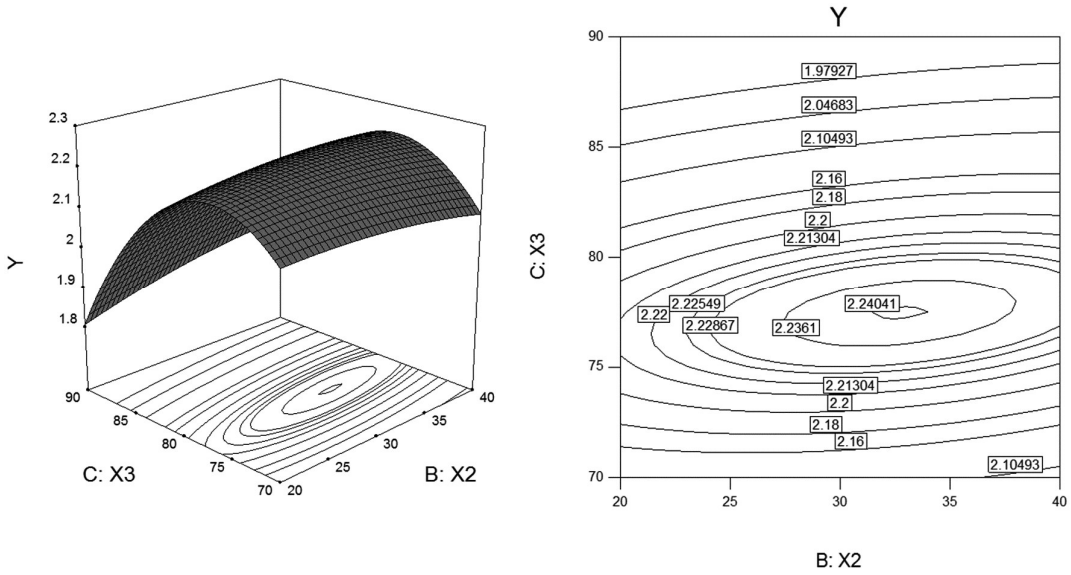

Fig. 5 Response surface and contour plots showing effect of extraction time (X_2) and ethanol concentration (X_3).

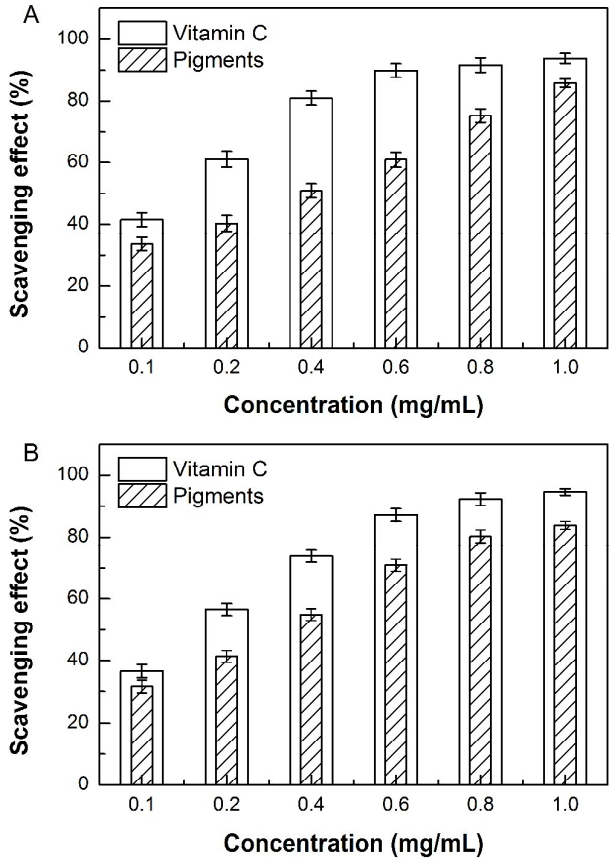

Fig. 6 DPPH radicals (a) and hydroxyl radicals (b) scavenging effect of pigments as function of concentration.

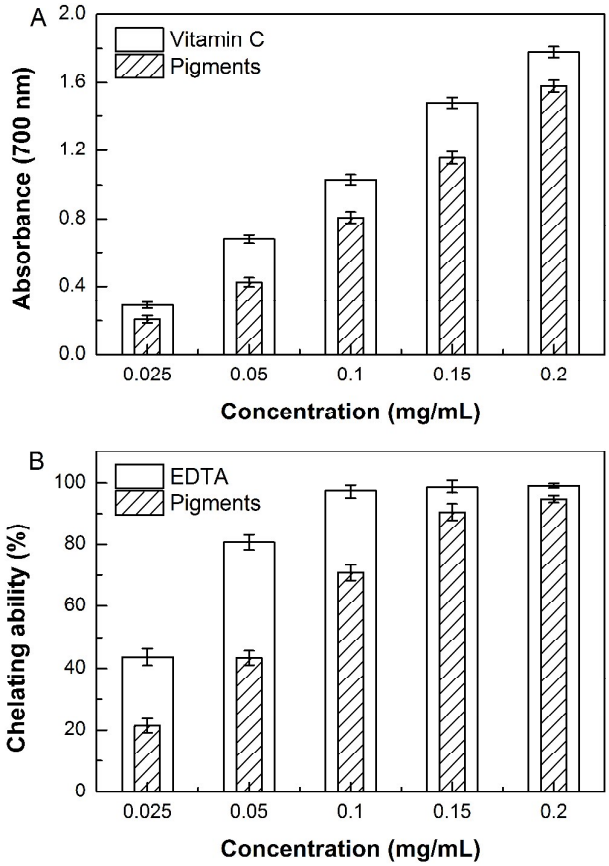

Fig. 7 Reducing power (a) and metal chelating ability (b) of pigments as function of concentration.

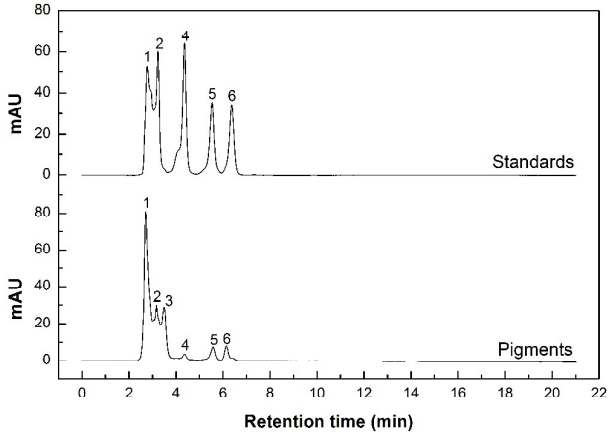

Fig. 8 HPLC chromatograms of standards and pigments: (1) gallic acid, (2) rutin, (3) unknown, (4) quercetin, (5) kaempferol, and (6) isorhamnetin.

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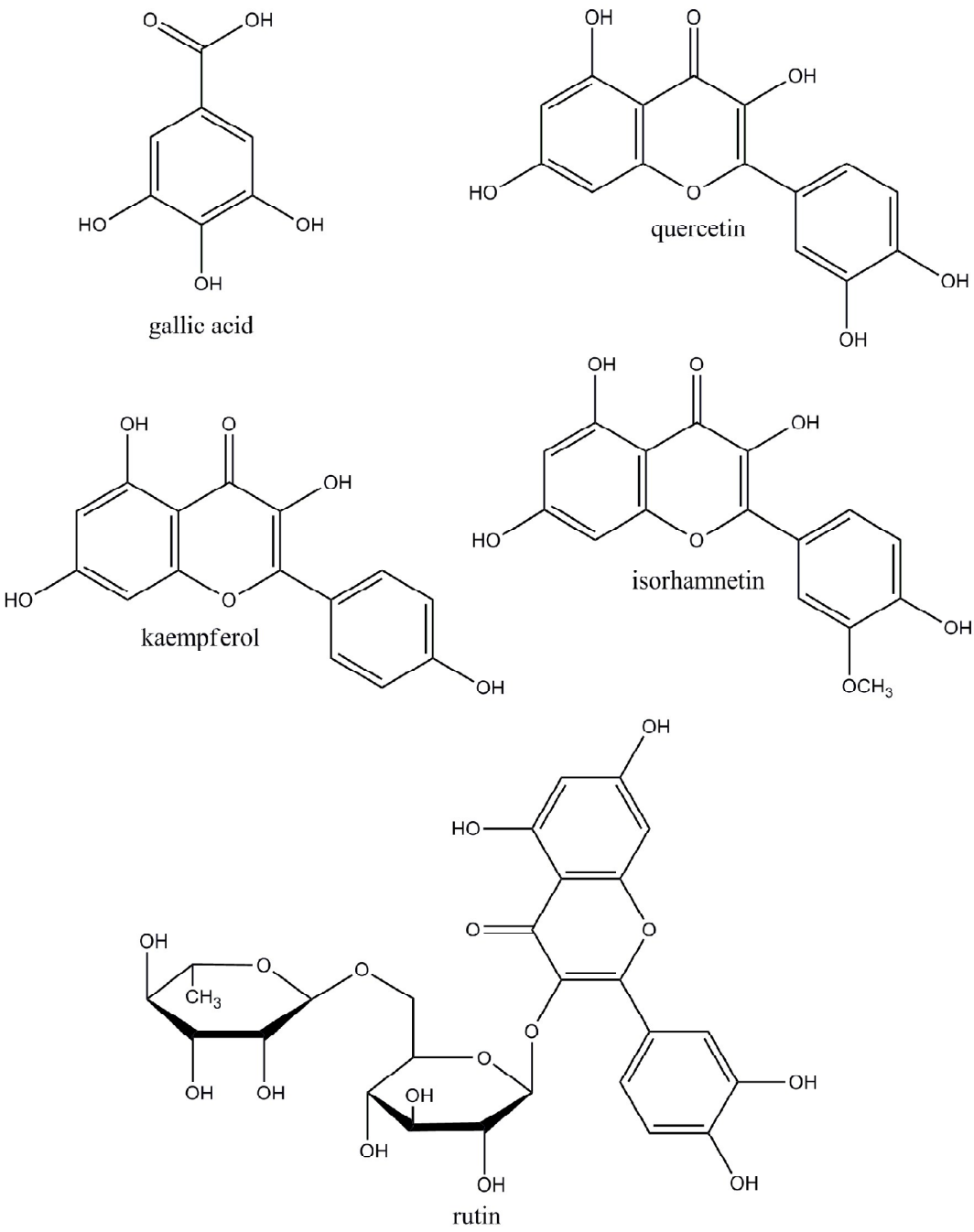

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3 Fig. 9 Structures of gallic acid, rutin, quercetin, kaempferol and isorhamnetin.

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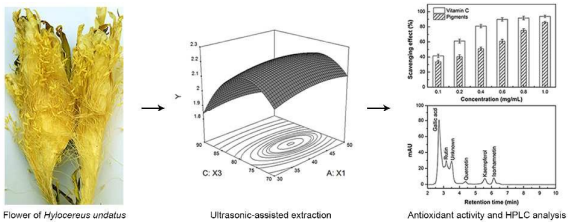

Optimization extraction, antioxidant activity, and HPLC analysis of pigments from *Hylocereus undatus* flowers