Optimization of ultrasound-assisted extraction of indigo and indirubin from *Isatis indigotica* Fort. and their antioxidant capacities

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Abstract An effective method for the ultrasound-assisted extraction of indigo and indirubin from *Isatis indigotica* Fort. was established and their antioxidant activities were investigated. Response surface methodology based on a three-level, four-factor Box–Behnken design was used to optimize the extraction conditions. Analysis of variance showed that the quadratic model was significant for the extraction of indigo and indirubin ($112.72\% \pm 1.65\%$ and $116.42\% \pm 1.27\%$, respectively) under the optimal conditions (methanol concentration, 80%; extraction time, 25 min; ratio of solid to liquid, 1:34 g/mL; and extraction temperature, 41 °C) and was in good agreement with the predicted value. Moreover, evaluation of the antioxidant activities suggested that indigo and indirubin presented better scavenging effects on 1,1-diphenyl-2-picrylhydrazyl free radical and superoxide radical than the extract and the extract revealed certain antioxidant activities in hydroxyl radical scavenging and reducing power, and indigo and indirubin could be used as natural antioxidants in the food or medicine industry.

Keywords *Isatis indigotica* Fort. · Indigo · Indirubin · Ultrasound-assisted extraction · Antioxidant activity

Introduction

Isatis indigotica Fort. (Cruciferae), a biennial herbaceous plant, is widely used as an indigo-producing and medicinal plant [1]. The dried leaves, named “Da-Qing-Ye,” are popular as traditional Chinese medicine, showing anti-bacterial [2], anti-virus [3], anti-endotoxin [4], immuno-regulatory [5], and anti-tumor [6] effects. It was illustrated that “Da-Qing-Ye” mainly contain various bioactive components, such as indigo, indirubin, organic acids, nucleosides, glucosinolates, lignans, ceramides, steroids, and polysaccharides [7]. Here indigo and indirubin are structural isomers. Indigo is one of the oldest natural blue dyes for blue jeans, denims, and other fabrics [8]. Moreover, it was proved that both indigo and indirubin showed biological activities on liver microsomes in mice [9]. They could also induce ethoxyresorufin-O-dealkylase and methoxyresorufin-O-dealkylase activities mediated by the aryl hydrocarbon receptor [10]. Indirubin was used as an anti-leukemia active ingredient in mice and anti-cancer drug in humans, showing inhibiting cell growth and inducing differentiation and apoptosis of leukemic cells [11]. Moreover, it possesses an anti-inflammatory effect by inhibiting the production of interferon gamma [12]. Recently, indirubin was found to inhibit the regulation on the activation in normal T cell expressed and secreted [5]. Therefore, both indigo and indirubin are important active ingredients received much attention in *I. indigotica*.

In recent years, ultrasound-assisted extraction (UAE), hot reflux extraction (HRE), microwave-assisted extraction (MAE), and solvent extraction (SE) have been widely applied to extract bioactive components from medicinal plants [13]. In particular, UAE is confirmed as a more economic and effective extraction method with advantages of shorter extraction period, higher yield, lower

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temperature, and less solvent consumption [14]. To date, the extraction efficiency of indigo and indirubin by HRE, UAE, MAE, and SE had been discussed. Dr. Jiang pointed out that UAE is more effective than SE [15] and HRE [16] by single-factor and orthogonal experiments. Response surface method (RSM), a mathematical and statistical method, has been broadly used to optimize the experimental process and examine the relation between the variables and the response, which has successfully been applied in many areas such as food, chemical, or biological processing. To the best of our knowledge, optimization of the extraction condition of indigo and indirubin using RSM, which could describe the effects of the main parameters of UAE, namely, extraction time, solvent concentration, ratio of solid to liquid, and extraction temperature, on the extraction efficiency, showing high-efficiency, low-cost, and continuous analysis characteristics, has not been discussed in the literature [17].

In addition, Chen et al. [18] showed that the extract from *I. indigotica* exhibited hydroxyl radical ($\cdot\text{OH}$) scavenging ability and good inhibitory effect on oils peroxidation. However, the kind of components that contributed to the antioxidant activity was not clear. Therefore, the antioxidant properties of indigo and indirubin extracted under optimal UAE conditions were evaluated via 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion radical, $\cdot\text{OH}$, and reducing power test in this study. The results of this experiment will be beneficial to further exploit and use *I. indigotica* resources.

Materials and methods

Plant materials

The plant materials were obtained from Shaanxi Geo-Authentic Medicinal Plant Co. Ltd. (Xi'an, China) and authenticated as the leaves of *I. indigotica* by the National Engineering Laboratory for Resource Developing of Endangered Chinese Crude Drugs in Northwest China. The leaves were dried to a constant mass at 60 °C (Beijing Kewei Instrument Co. Ltd., China) and then ground into powder (Tianjin Taisite Instrument Co. Ltd., China).

Chemical reagents

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH $\cdot$) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), indigo and indirubin from Sima-lab (Tianjin, China), and ascorbic acid (V_C) from Tianli Chemical Reagent Co. (Beijing, China). Other chemicals and solvents were analytical or chromatographic grade. Ultra-pure water was used throughout the experiment.

Determination of indigo and indirubin content

Identification and determination of indigo and indirubin were performed using HPLC (SHIMADZU, Japan) and a diode array detector. Solvents and samples were filtered through a 0.45- μm filter. Chromatographic analysis was performed using Agilent C-18 (5 μm $\times$ 4.6 mm $\times$ 250 mm i.d.). Simultaneous monitoring of indigo and indirubin was performed at wavelength of 289 nm and a flow rate of 1.0 mL/min. The running temperature was at 30 °C, and the injection volume was 10 μL . The mobile phase, Phase A, was 100% HPLC grade methanol, whereas Phase B was a mixture of 0.02% (v/v) phosphoric acid in distilled water.

Hot reflux extraction

HRE was performed in a water bath (Guohua Electrical Appliance Co. Ltd., China). First, 0.50 g dried leaf powder was placed in a 150-mL round bottom flask with 15 mL 80% (v/v) methanol solution and then hot bathed for three cycles at 80 °C, 2 h per cycle. The filtrate was collected and analyzed using HPLC (SHIMADZU, Japan). Each extraction was performed in triplicate. In this research, the extraction efficiency of indigo and indirubin by HRE was set at 100% recovery.

Ultrasound-assisted extraction

The leaf powder (0.50 g) with a predetermined volume of 80% (v/v) methanol solution was placed in a 50-mL centrifuge tube. UAE was performed at different temperatures and time periods in an ultrasonic cleaning bath (Ningbo Xinzhi Biological Technology Co. Ltd., China). Recycled water was provided to maintain a constant extraction temperature. The filtrate was collected and analyzed using HPLC (SHIMADZU, Japan). Each extraction was performed in triplicate. The extraction efficiency of indigo and indirubin by UAE was compared with that of HRE.

Experimental design by the response surface method

The effects of extraction parameters including methanol concentration (55, 65, 75, 85, and 95% v/v), extraction time (5, 15, 25, 35, and 45 min), ratio of solid to liquid (1:10, 1:20, 1:30, 1:40, and 1:50 g/mL), and extraction temperature (20, 30, 40, 50, and 60 °C) on the extraction efficiency of indigo and indirubin were initially investigated using single-factor tests. The results showed that the best conditions of the extraction parameters, including methanol concentration, extraction time, ratio of solid to liquid and extraction temperature, were 75%, 25 min, 1:30, and 40 °C, respectively. Next, the proper ranges of the

parameters were determined as follows: methanol concentration (65, 75, and 85%, v/v), extraction time (15, 25, and 35 min), ratio of solid to liquid (1:20, 1:30, and 1:40 g/mL), and extraction temperature (30, 40, and 50 °C). A three-level, four-variable Box–Behnken design (BBD) was employed to optimize the extraction process, including 24 factorial and 5 axial points. For the multi-parameters test design, the different weight coefficients represent the importance of different parameters, and the comprehensive score could be conveniently used as the response [19]. In this study, the weight coefficient of indigo and indirubin were set to 0.5, and the comprehensive extraction efficiency of indigo and indirubin was described as a composite indicator (Y) calculated as Eq. (1)

$$\text{Composite indicator } (Y) = \frac{0.5 \times 100 \times A_1}{A} + \frac{0.5 \times 100 \times B_1}{B}, \quad (1)$$

where A_1 is the extraction efficiency of indigo, A is the maximum extraction efficiency of indigo, B_1 is the extraction efficiency of indirubin, and B is the maximum extraction efficiency of indirubin. The composite indicator (Y) was considered as the response of this experiment. The actual and coded levels of the independent variables are given in Table 1. The second-order polynomial (Eq. 2) including all interaction terms was used to calculate the predicted response:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{i \neq j} \beta_{ij} X_i X_j, \quad (2)$$

where Y , comprehensive extraction efficiency of indigo and indirubin, is the predicted response; β_0 , β_i , β_{ii} , and β_{ij} are the regression coefficients for the intercept, linear, quadratic, and interaction terms, respectively; X_i and X_j are the independent variables ($i \neq j$).

Assay of 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity

The scavenging activities on DPPH $\cdot$ of indigo, indirubin, and the extract were measured as described previously [20] with slight modifications. Briefly, 2.0-mL sample solutions,

including V_C solution (0.002, 0.006, 0.010, 0.014, 0.018, 0.022, and 0.026 mg/mL), extract solution (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/mL), and indigo and indirubin solutions (both as follows: 0.02, 0.06, 0.10, 0.14, 0.18, 0.22, and 0.26 mg/mL), were mixed with 4.0-mL DPPH $\cdot$ solution (1×10^{-4} mol/L). Then, the mixture was incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm. V_C was used as the positive control. Lower absorbance indicates higher free radical scavenging activity. The DPPH radical scavenging activity was calculated by the following equation (Eq. 3):

$$\left(\text{Scavenging activity } (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100 \right), \quad (3)$$

where A_0 is the absorbance of the blank control (without the sample or positive control), A_1 is the absorbance of the sample without DPPH $\cdot$ solution, and A_2 is the absorbance of the mixture including the sample and DPPH $\cdot$ solution.

Assay of superoxide anion radical scavenging activity

Superoxide anion radical was generated in the pyrogallol auto oxidation system under alkaline conditions. The scavenging effects of indigo, indirubin, and the extract on the superoxide anion radical were measured as described previously [21] with minor modifications. Sample solutions of 0.4 mL, including V_C solution (0.04, 0.20, 0.36, 0.52, 0.68, 0.74, and 0.90 mg/mL), extract solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 mg/mL), and indigo and indirubin solutions (both as follows: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mg/mL), and 4.5-mL Tris–HCl buffer (50 mM, pH 8.2) were incubated at 25 °C for 15 min and 0.1-mL pyrogallol acid (3.0 mM) was then added for another 3 min. The absorbance change speed (A/min) of the mixture was measured at 320 nm. V_C was used as the positive control. The scavenging activity of the superoxide anion radical was calculated by the following formula (Eq. 4):

$$\text{Scavenging activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100, \quad (4)$$

Table 1 Independent variables and their levels for the experimental design

Levels	Variables			
	Methanol concentration (% , X_1)	Extraction time (min, X_2)	Ratio of solid to liquid (g/mL, X_3)	Extraction temperature (°C, X_4)
–1	65	15	1:20	30
0	75	25	1:30	40
1	85	35	1:40	50

where A_0 is the absorbance change speed of the control, and A_I is the absorbance change speed of the sample.

Assay of hydroxyl radical scavenging activity

$\cdot\text{OH}$ scavenging activity was measured according to Fenton's reaction [22]. Positive control and the samples were dissolved to different final concentrations (V_C solution of 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, and 2.6 mg/mL, indigo and indirubin solutions of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 mg/mL, and the extract solution of 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0 mg/mL) and then incubated at 37 °C for 30 min with the reaction solution, including 2.0-mL FeSO_4 (6.0 mM), 2.0-mL H_2O_2 (6.0 mM), and 2.0-mL $\text{C}_7\text{H}_6\text{O}_3$ ethanol solution (6.0 mM). The total volume of the mixture in each tube was made up to 7.0 mL with distilled water as needed. The absorbance of the mixture was recorded at 510 nm against the blank. The scavenging activity on $\cdot\text{OH}$ was calculated by the following equation (Eq. 5):

$$\text{Scavenging activity (\%)} = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100, \quad (5)$$

where A_0 is the absorbance of the blank control (distilled water instead of sample solution), A_1 is the absorbance of the test sample mixed with the reaction solution, and A_2 is the absorbance when H_2O_2 and the sample were replaced with distilled water.

Assay of the total reducing power

The total reducing power of indigo, indirubin, and the extract was measured according to the method of Oyaizu [23]. Positive control and the samples were dissolved to different final concentrations (V_C solution of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, and 0.07 mg/mL, indigo and indirubin solutions of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mg/mL, and the extract solution of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mg/mL). The reaction system, including 1.0-mL sample solution and 2.5-mL $\text{K}_3[\text{Fe}(\text{CN})_6]$ (1%, w/v), was incubated at 50 °C for 20 min. Then, 1.0-mL $\text{C}_2\text{Cl}_3\text{O}_2$ 10% (w/v) was added to the mixture and centrifuged at 3000 r/min for 10 min, followed by the addition of 0.5-mL FeCl_3 (0.1%, w/v) and 2.0-mL distilled water. The absorbance was measured at 700 nm. The greater the absorbance, the stronger will be the reducing power.

Statistical analysis

All the experiments were performed in triplicate, and the data were expressed as mean $\pm$ SD. Response surface

design and data analysis were performed using Design-Expert.V8.0.6, and the other data were analyzed using the Origin Pro (Version 9.1) software.

Results and discussion

Fitting the model

The BBD matrix and the results are presented in Table 2, showing the independent variables and the response. The data was fitted by the Design-Expert 8.0 software to the following second-order polynomial formula (Eq. 6):

$$Y = 95.14 + 11.12X_1 + 2.44X_2 + 5.48X_3 + 3.19X_4 - 2.73X_1X_2 - 0.41X_1X_3 - 1.71X_1X_4 - 4.18X_2X_3 + 3.40X_2X_4 - 0.96X_3X_4 - 10.9X_1^2 - 7.6X_2^2 - 6.03X_3^2 - 7.5X_4^2, \quad (6)$$

where Y is the comprehensive extraction efficiency of indigo and indirubin and X_1 , X_2 , X_3 , and X_4 are the coded variables for methanol concentration, extraction time, ratio of solid to liquid, and extraction temperature, respectively.

Regression coefficient and analysis of variance (ANOVA) of the reduced polynomial cubic models are presented in Table 3. ANOVA analysis showed that the model was significant ($p < 0.0001$) and the lack of fit was not significant ($p > 0.05$) at 95% confidence level, which demonstrated that the fitted model could well describe the true relations between the variables and the response [24]. The coefficient of determination (R^2), which represents the consistency between the observed and predicted values, was used to test the fitting of equation [25]. In our study, R^2 was 0.95, which illustrated that 95% of the actual levels could be matched with the model-predicted levels. Moreover, the adjusted determination coefficient (R_{Adj}^2) was 0.90, which further suggested that the model could fully represent a better correlation between the observed and predicted values. Furthermore, the value of coefficient of the variance (c.v. %) was 4.39, indicating that the model was reproducible [26]. The predicted residual sum of squares for the model was 784.25, which indicated that this model could fit the 29 points in the design. As can be seen from Table 3, the extraction efficiency of indigo and indirubin was affected most significantly by X_1 ($p < 0.0001$), followed by X_3 ($p < 0.001$), X_4 ($p < 0.01$), and X_2 ($p < 0.05$). It was obvious that all the quadratic terms (X_1^2 , X_2^2 , X_3^2 , X_4^2) and one interaction terms (X_2X_3) were significant ($p < 0.05$ or $p < 0.01$), whereas the other interaction quadratic terms were not ($p > 0.05$).

Table 2 Box-Behnken design of the process variables along with the observed and predicted values for the composite indicator

Run	X_1^1	X_2^1	X_3^1	X_4^1	Indigo content ($\mu\text{g/g}$)	Indirubin content ($\mu\text{g/g}$)	Indigo extraction efficiency (%)	Indirubin extraction efficiency (%)	Composite indicator, Y	
									Actual value	Predicted value
1	−1	−1	0	0	94.88	199.01	60.52 $\pm$ 1.21	81.80 $\pm$ 0.45	63.71	60.33
2	1	−1	0	0	165.26	230.60	105.41 $\pm$ 0.98	94.78 $\pm$ 0.71	89.15	88.04
3	−1	1	0	0	117.02	217.96	74.65 $\pm$ 1.01	89.59 $\pm$ 1.31	72.79	70.68
4	1	1	0	0	137.58	265.35	87.76 $\pm$ 0.45	109.06 $\pm$ 1.47	87.29	87.44
5	0	0	−1	−1	112.28	224.28	71.62 $\pm$ 0.59	92.18 $\pm$ 0.92	72.67	71.96
6	0	0	1	−1	132.05	268.51	84.23 $\pm$ 0.12	110.36 $\pm$ 1.01	85.96	84.85
7	0	0	−1	1	138.37	236.92	88.26 $\pm$ 0.34	97.38 $\pm$ 0.46	82.39	80.28
8	0	0	1	1	162.88	249.55	103.90 $\pm$ 0.35	102.57 $\pm$ 0.79	91.82	89.32
9	−1	0	0	−1	94.88	189.53	60.52 $\pm$ 1.45	77.90 $\pm$ 0.86	61.16	60.71
10	1	0	0	−1	162.09	236.92	103.40 $\pm$ 0.89	97.38 $\pm$ 0.43	89.77	86.36
11	−1	0	0	1	113.07	205.33	72.12 $\pm$ 1.11	84.39 $\pm$ 0.12	69.54	70.51
12	1	0	0	1	168.42	236.92	107.43 $\pm$ 0.74	97.38 $\pm$ 0.42	91.32	89.34
13	0	−1	−1	0	105.16	224.28	67.08 $\pm$ 0.67	92.18 $\pm$ 1.61	70.28	69.39
14	0	1	−1	0	129.67	265.35	82.72 $\pm$ 0.56	109.06 $\pm$ 0.86	84.93	82.63
15	0	−1	1	0	143.12	265.35	91.29 $\pm$ 0.47	109.06 $\pm$ 1.66	88.86	88.72
16	0	1	1	0	139.95	259.03	89.27 $\pm$ 1.34	106.47 $\pm$ 1.59	86.77	85.23
17	−1	0	−1	0	81.44	195.85	51.95 $\pm$ 1.13	80.50 $\pm$ 0.99	58.52	61.18
18	1	0	−1	0	132.84	233.76	84.73 $\pm$ 0.96	96.08 $\pm$ 0.54	80.88	84.24
19	−1	0	1	0	103.58	227.44	66.07 $\pm$ 0.73	93.48 $\pm$ 0.52	70.66	72.97
20	1	0	1	0	154.19	262.19	98.35 $\pm$ 1.85	107.76 $\pm$ 1.03	91.38	94.38
21	0	−1	0	−1	114.65	233.76	73.13 $\pm$ 1.09	96.08 $\pm$ 1.89	75.03	77.80
22	0	1	0	−1	109.91	230.60	70.11 $\pm$ 0.65	94.78 $\pm$ 1.54	72.95	75.87
23	0	−1	0	1	123.35	217.96	78.68 $\pm$ 0.95	89.59 $\pm$ 0.79	74.65	77.39
24	0	1	0	1	134.42	265.35	85.74 $\pm$ 0.55	109.06 $\pm$ 0.83	86.19	89.07
25	0	0	0	0	163.67	281.14	104.40 $\pm$ 0.28	115.55 $\pm$ 1.26	97.65	95.13
26	0	0	0	0	171.58	274.82	109.45 $\pm$ 1.57	112.96 $\pm$ 0.28	98.61	95.13
27	0	0	0	0	161.30	277.98	102.89 $\pm$ 1.12	114.26 $\pm$ 1.25	96.27	95.13
28	0	0	0	0	159.72	236.92	101.88 $\pm$ 1.24	97.38 $\pm$ 1.19	88.70	95.13
29	0	0	0	0	157.35	271.67	100.37 $\pm$ 0.88	111.66 $\pm$ 0.68	94.46	95.13

¹ X_1 : methanol concentration (%), X_2 : extraction time (min), X_3 : ratio of solid to liquid (g/mL), and X_4 : extraction temperature ($^{\circ}\text{C}$)

Response surface analysis

Three-dimensional response surface graphs (Fig. 1) are the graphical representations of regression equation, which can clarify the interactions between the independent variables and response [27], and propose the optimal extraction condition for indigo and indirubin. Each graph shows the effects of two independent variables on the response while the others are held at zero level [28].

Based on the similarity–intermiscibility theory, bioactive compound extraction largely depends on the polarity of the solvents. In this study, the extraction efficiency of indigo and indirubin increased significantly when the concentration of methanol solution increased from 65 to

80% (Fig. 1A–C) and reached the maximum when the concentration of methanol solution was 80%. However, as the concentration of methanol solution increase to 85%, the extraction efficiency clearly decreased. Higher concentration of methanol solution made more liposoluble components out, which caused to the adverse effects on indigo and indirubin extraction.

Extraction time also affects the extraction efficiency of indigo and indirubin. From Fig. 1(A, C, and D), it was obvious that 25 min was an optimum extraction time for indigo and indirubin, and a peak value of the extraction efficiency was observed at that point, after which, the extraction efficiency decreased. The ultrasonic acoustic cavitation actions could break the plant cells, dissolve the

Table 3 Analysis of variance for the second-order response surface model

SD ¹	SS ¹	DF ¹	MS ¹	F value	Prob > F	S ²
Model	3364.53	14	240.32	18.58	<0.0001	***
X ₁ -methanol concentration	1483.60	1	1483.60	114.74	<0.0001	***
X ₂ -extraction time	71.22	1	71.22	5.50	0.0342	*
X ₃ -ratio of solid to liquid	360.74	1	360.74	27.89	0.0001	**
X ₄ -extraction temperature	122.62	1	122.62	9.48	0.0082	**
X ₁ X ₂	29.90	1	29.90	2.31	0.1505	
X ₁ X ₃	0.68	1	0.68	0.05	0.8219	
X ₁ X ₄	11.65	1	11.65	0.90	0.3586	
X ₂ X ₃	70.02	1	70.02	5.41	0.0355	*
X ₂ X ₄	46.31	1	46.31	3.58	0.0793	
X ₃ X ₄	3.71	1	3.71	0.28	0.6004	
X ₁ ²	771.68	1	771.68	59.68	<0.0001	***
X ₂ ²	374.97	1	374.97	29.00	<0.0001	***
X ₃ ²	236.44	1	236.44	18.28	0.0008	***
X ₄ ²	364.61	1	364.61	28.19	0.0001	***
Residual	181.02	14	12.93			
Lack of fit	119.45	10	11.94	0.776099	0.6621	
Pure error	61.56	4	15.39			
Cor total	3545.55	28				
R ²	0.9489			c.v. %	4.39	
R _{Adj} ²	0.8979			PRESS ³	784.25	

¹ SD: source of deviation, DF: degree of freedom, SS: sum of squares, and MS: mean square

² S: significant

³ PRESS: predicted residual sum of squares

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

effective components, and improve the extraction efficiency. At the same time, various non-target components, maybe insoluble substances and cytosol, could also release and flow into the solvents, which reduced the mass transfer efficiency and permeability of the solvents [29]. Furthermore, exposure to ultrasound for longer time could result in the structural destruction and decomposition of indigo and indirubin and reduce the extraction efficiency at the later stage of extraction.

The ratio of solid to liquid contributed to the extraction efficiency of indigo and indirubin. When the ratio of solid to liquid increased from 1:20 to 1:35 g/mL, the extraction efficiency increased significantly, which is depicted in Fig. 1(B, C, and E). Increment of the proportion of liquid to solid could enhance the contact area between materials and solvents, improve indigo and indirubin dissolution, and increase the viscosity of solvents. However, the intensity of cavitation decreased with increase in the viscosity of the solvents [30]. In this experiment, when the ratio of solid to liquid continued to increase, the extraction efficiency of indigo and indirubin no longer increased.

Besides, the extraction temperature is considered as an important factor for bioactive compound extraction. In this

study, the extraction temperature played both positive and negative roles on indigo and indirubin extraction (Fig. 1D–F), and the phenomena was similar to the results of Yang and Zhai's [31]. There was a linear increase of extraction efficiency from 30 to 40 °C. A possible explanation is that higher temperature could accelerate the solubility and diffusivity of materials in solvents. Over 50 °C, the extraction efficiency represented a decreasing tendency; this may be because the higher temperature decreased the surface tension and viscosity of the solvent, which could alter not only the peculiarity of ultrasonic cavitation but also the intensity of mass transfer.

Validation of the model

The process conditions were optimized through the numerical optimization technique. The optimum conditions for the maximum extraction efficiency of indigo and indirubin were determined through the developed regression models. The asserted optimal conditions were validated by triplicating the experiments at the optimum conditions and comparing the average values of the triplicated experiment and the predicted values at optimum conditions. In this

Fig. 1 Response surface plots showing the effects of variables (X_1 : methanol concentration, %; X_2 : extraction time, min; X_3 : ratio of solid to liquid, g/mL; and X_4 : extraction temperature, °C) on the composite indicator

study, the optimal condition predicted by the model was as follows: methanol concentration of 79.95%, extraction time of 24.79 min, ratio of solid to liquid of 1:34.35 (g/mL), and extraction temperature of 41.24 °C. Moreover, the optimal conditions were modified according to the extraction operability during the actual production process. The modified optimal conditions were methanol concentration of 80%, extraction time of 25 min, ratio of solid to liquid of 1:34 (g/mL), and extraction temperature of 41 °C (Table 4).

Selected optimal conditions were tested experimentally to examine the suitability of optimum conditions for predicting the optimum response. The mean value of the triplicate experiments conducted under the optimized conditions was 101.87, which was well consistent with the actual value (99.25). These results confirmed that the response model was adequate for reflecting the expected optimization process.

Table 4 Optimum conditions and the predicted and actual values of the response under the optimum conditions

Optimum extraction conditions				Extraction efficiency of indigo (%)	Extraction efficiency of indirubin (%)	Composite indicator	
X_1 (%) ¹	X_2 (min) ¹	X_3 (g/mL) ¹	X_4 (°C) ¹			Actual value	Predicted value
80	25	34	41	112.72 ± 1.65	116.42 ± 1.27	101.87	99.25

¹ X_1 : methanol concentration (%), X_2 : extraction time (min), X_3 : ratio of solid to liquid (g/mL), X_4 : extraction temperature (°C)

Fig. 2 The scavenging activity of DPPH free radical (A), superoxide anion radical (B), hydroxyl radical (C), and the reducing power (D)

DPPH free radical scavenging activity

DPPH $\cdot$ is a relatively stable free radical that has been extensively used to evaluate the antioxidant capacity of natural compounds, showing maximum absorption at 517 nm. When DPPH $\cdot$ meets a proton-donating substance, such as an antioxidant, its absorbance will reduce with an obvious color change from purple to yellow [32]. Here the DPPH $\cdot$ scavenging effects of indigo, indirubin, and the extract were measured (Fig. 2A). The scavenge rate of indigo (28.31% $\pm$ 1.21%) and indirubin (22.35% $\pm$

0.99%) is higher than that of the extract (20.56% $\pm$ 1.56%) at the concentration of 0.10 mg/mL ($p < 0.01$). But after the concentration of 0.20 mg/mL, the extract showed better scavenging rate (40.25% $\pm$ 1.89%) than indigo (39.11% $\pm$ 1.02%) and indirubin (28.41% $\pm$ 1.59%). Moreover, the EC₅₀ of the extract was 0.29 mg/mL, showing a noticeable effect on scavenging DPPH $\cdot$ compared with that of the ultrasonic-assisted extracted polysaccharides from *Trametes orientalis*, which was 0.87 mg/mL [33]; however, this value was lower than that of V_C (EC₅₀ = 0.007 mg/mL). Unfortunately, the EC₅₀ of indigo and indirubin could not

been calculated because their scavenging activities did not reach 50%. To date, there are no studies on the DPPH· scavenging effects of indigo and indirubin. The obtained results showed that indigo, indirubin, and the extract clearly exhibited scavenging activities on DPPH· in a concentration-dependent manner. Moreover, indigo and indirubin had better scavenging activities on DPPH· at a lower concentration but the activities decreased with increasing concentration. The extract showed higher scavenging activities at higher concentration than indigo and indirubin; this may be attributed to the other unknown components that played the role of antioxidants in the extract, such as monosaccharides. Some researchers proved that the hydroxyl group of the monosaccharide unit could donate the proton to combine with DPPH· to form the stable DPPH-H, showing higher scavenging activities [7].

Superoxide anion scavenging activity

Superoxide anion is known as the precursors of some reactive oxygen species, such as hydroxyl radicals or singlet oxygen, which could indirectly trigger lipid peroxidation and pathological incidents [34]. Superoxide anion scavenging activities of indigo, indirubin, and the extract are shown in Fig. 2(B). For all the samples, the scavenging activities of superoxide anion increased with increasing concentration, and indigo and indirubin showed higher scavenging activities than the extract. The plateau was reached for indigo (55.46%) and indirubin (58.45%) at the concentration of 1.4 and 1.2 mg/mL, respectively, and the plateau of the extract (62.01%) was reached at 3.5 mg/mL. The results illustrated that indigo and indirubin were more effective for superoxide anion scavenging owing to the presence of some electrophilic groups, such as keto or aldehyde, facilitating hydrogen liberation from the O-H bond and stabilizing superoxide anion. According to the EC₅₀ value, the order of the superoxide anion scavenging activities of the samples were as follows: V_C (0.20 mg/mL) > indigo (0.61 mg/mL) > indirubin (0.74 mg/mL) > extract (1.75 mg/mL). The mechanism of scavenging superoxide anion might be connected with the presence of some types of electrophilic groups, which promoted the release of hydrogen from hydroxyl and formed the non-radical superoxide anion [35].

Hydroxyl radical scavenging activity

·OH is one of the most harmful reactive radicals for some biomolecules including carbohydrates, lipids, proteins, and DNA, which could cause tissue damage and cell death [36]. Therefore, removing ·OH is very important for biological system protection. The ·OH scavenging activities of indigo, indirubin, and the extract were determined in this study. According to the preliminary test results, there were no

apparent ·OH scavenging activities for indigo and indirubin (data not shown). The extract manifested concentration-dependent scavenging activity on ·OH, as shown in Fig. 2(C). It was observed that the scavenging rate increased with increasing concentration and reached a plateau at the concentration of 12.0 mg/mL. The EC₅₀ of the extract was 10.34 mg/mL, which is much higher than V_C. It was reported that the scavenging rate of the extract was 15.4% at the concentration of 16.95 mg/L [16], which was different from our results; this may be attributed to the different materials, extraction process, and purity of the extract. Moreover, the absence of hydroxyl in the chemical structure of indigo and indirubin could cause low scavenging activities on the ·OH. Some studies reported that the effect of the antioxidants on ·OH scavenging owing to the hydroxyl group in samples could donate the hydrogen, which would combine with the ·OH [20, 37, 38].

Reducing power

The reducing power of a natural compound is an important index of its potential antioxidant capacity. Many studies revealed that there was a direct correlation between the antioxidant activity and reducing power [39]. The reducing power of indigo, indirubin, and the extract were determined in this study. There were no apparent reducing powers for indigo and indirubin according to the preliminary experimental results (data not shown). Nevertheless, the extract exhibited effective reducing power in a concentration-dependent manner, as shown in Fig. 2(D). The reducing power of the extract represented its potential to break and terminate the free radical chain by donating electrons [39]. Higher absorbance value means stronger reducing ability. In the range of 0.2–1.4 mg/mL, the reducing power of the extract increased from 0.161 to 0.663. Indigo and indirubin did not exhibit the obvious reducing power.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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