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Optimization of Ultrasound-Assisted Extraction of Flavonoids from *Cryptotaenia japonica* Hassk. and Evaluation of Antioxidant Activity

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Abstract

The present study optimized the ultrasound assisted extraction (UAE) conditions using Box-Behnken design (BBD) with response surface methodology (RSM) and evaluated the antioxidant activity of total flavonoids from *Cryptotaenia japonica* Hassk. The results showed that the optimum conditions were 55% ethanol concentration, 1:30 g/mL solid to solvent ratio, 400 W ultrasound power, 70 min extraction time and 3 cycles. Under these optimum conditions, the extraction yield of total flavonoids was 33.110 ± 0.742 mg/g DW (dry weight), which was well consisted with value predicted by the model. The RSM optimized extract was subsequently checked for antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, hydroxyl scavenging assay and reducing power assay. Under the flavonoid concentration of 3.0 mg/mL, antioxidant scavenging activity were of 79.87±2.7% and 71.60±2.6% for DPPH and hydroxyl assay while for reducing power and absorbance of 1.200±0.053 was obtained. Furthermore, the composition in antioxidants were investigated, and the luteolin and apigenin were determined. The results indicated that ultrasound assisted extraction is a promising technique for extraction of flavonoids from *C. japonica Hassk* and the flavonoids could be explored as a potential antioxidant agent for use in medicine or functional foods.

Keywords: flavonoids, *Cryptotaenia japonica* Hassk., ultrasound-assisted extraction, optimization, response surface methodology, antioxidant activity

1. Introduction

Studies of active components from natural sources such as fruits and vegetables, especially flavonoids compounds, have greatly increased in recent years. Flavonoids include a large number of compounds which have been proven to possess a broad spectrum of biochemical and pharmacological activities such as antioxidant (Floreset, Wu, Negrin, & Kennelly, 2015; Zielinski et al., 2014), antibacterial (Cushnie & Lamb, 2011), antiviral (S. Y. Kang, J. Y. Kang, & Oh, 2012), etc. Therefore, highly efficient methods of extracting and isolating total flavonoids for supplements or medicinal use attract world-wide interest and studies (Cherng, Chiag, & Chiang, 2008; Pourcel, Routaboul, Cheynier, Lepiniec, & Debeaujon, 2007; Joana Gil-Chávez et al., 2013). *Cryptotaenia japonica* Hassk. (Yaojiaoban, in Chinese) is a popular forestry vegetable due to its flavor and Chinese folk medicine use its medical effect for skin itch and traumatic injury (Yang, Xia, Li, & Cheng, 2010). Some studies have reported on the compositions and antioxidant activity of *C. japonica* (Cheng, Lin, Yen, & Yu, 2010; Cheng Lin, Yu, & Peng, 2008; Okude & Hayashi, 1970; Yao, Sang, & Zhou, 2010; Yao & Ren, 2011), but few studies refer to the extraction of flavonoids (Li, Zhu, & Deng, 2006). Ultrasound-assisted extraction is the new technology that has become widely applied in the extraction of a variety of bioactive ingredient (Kiani, Sun, Zhang, Al-Rubeai, & Naciri, 2013; Şahin & Şamlı, 2013; Wang et al., 2013). It has the notable advantages for decreasing the consumption of solvent and extraction time, increasing yield and ease of use (Bimakr et al., 2011; Grigonis, Venskutonis, Sivik, Sandahl, & Eskilsson, 2005) comparing with other extraction techniques such as heating, boiling, refluxing and soxhlet extraction. However, there is no report about using ultrasonic technique to extract total flavonoids from *C. japonica* and evaluate the antioxidant potential. In this study, we use the ultrasound assisted extraction to separate total flavonoids from *C. japonica* by RSM and to evaluate the

antioxidant activity by hydroxyl radical, DPPH radicals and reducing power assays. Finally, the main flavonoid compositions in extract were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC).

2. Materials and Methods

2.1 Materials and Reagents

Cryptotaenia japonica was harvested in May from west region of Hunan Province, China. The plant was authenticated by Prof. Zhonghai Li and voucher specimen (YY.201105) deposited at the College of Food Science and Engineering, Central South University of Forestry and Technology, Changsha, Hunan, China. All plant materials were washed and separated into root and aerial parts. The latter were dried in an oven for 24 h at 60 ºC and powdered to a 60 mesh by a disintegrator, and stored in sealed polyethylene bag in a dry and dark place until use. Rutin was purchased from National Institutes for Food and Drug Control (analytical grade), DPPH were purchased from Sigma Chemicals Co., Ltd, sodium nitrite, sodium carbonate, aluminum nitrite, sodium hydroxide and ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. All other chemicals and solvents were of analytical grade.

2.2 Sonication Treatment

Ultrasound-assisted extraction experiments were carried out with an ultrasonic cell disruption machine which was equipped with a cylindrical titanium alloy probe (12.70 mm diameter). (JY92-Ⅲ, Ningbo Shizhi Biotechnology Co., Ltd, China). A powdered sample of 3.0 g was placed into a 200 mL of glass flask, soaked with different ratio of ethanol solvent (varing ethanol concentration from 50% to 70%; varing liquid to solid ratio from 1:30 to 1:50), then sonicated for 40-60 min at a constant working frequency of 60 kHz and pulse 7 s on and 5 s off with different times at the designed ultrasound power. The extract solution was filtered by a Busher funnel and the filtrate was centrifuged for 10 min at 6000 rpm. The supernatant was collected to detect the concentration of total flavonoids. The combined extracts solution under the optimized condition were evaporated under vacuum and were lyophilized with a freeze dryer system (Ningbo Shizhi Biotechnology Co., Ltd, China) at -55 ºC. The crude flavonoid was later redissolved in ethanol to obtain the designed concentration for the evaluating antioxidant activity.

2.3 Determination of Total Flavonoids Content

Total flavonoids were measured spectrophotometrically using a modified colorimetric method (Zhu, Wang, Liu, Xia, & Tang, 2010) with slight modification. Briefly, 1 mL of the sample solution was accurately removed and placed in a volumetric flask (10 mL) where 0.6 mL of NaNO2 (5%) solution was added. The solution was shaken and stood for 6 minutes at room temperature. Additionally 0.5 ml of 10% (W/V) $Al(NO_3)_3$ was added and allowed to stand for an additional 6 minutes prior to the addition of 3.0 mL of 4.3% NaOH, followed by addition of 70% ethanol (v/v) to the scale, shaken, and left to stand for 15 min before detection. The absorbance was measured at 507 nm using an ultraviolet-visible spectrophotometer (Shanghai precision instrument Co., Ltd, Shanghai, China), using the sample solution without coloration as blank solution. Rutin was used as a reference for the standard curve, the regression equation was A = 1.0286C-0.0161 (R2 = 0.9993). All data were reported as mean ± SD for three replicate measurements. When calculating extraction yield of flavonoids, the following formula was used:

$$X = (C \times V \times D) / M \quad (1)$$

Where, X (mg/g) = extraction yield of flavonoids; C (mg/mL) = flavonoids content of test solution calculated by standard curve; V (mL) = volume of test solution; D = total dilution value; M (g) = mass of test sample.

2.4 Evaluation of Antioxidant Activity

The free radical scavenging activity was measured by DPPH test according to the method described by Zhang, He, and Hu (2011) with some modifications. The 0.4 mmol/L solution of DPPH in 95% ethanol was prepared daily before UV measurements. Two milliliter of the tested sample was thoroughly mixed with 2.0 mL of freshly prepared DPPH solution. The mixture was shaken vigorously and left to stand in the dark for 30 min, and the absorbance was then measured at 517 nm against a blank. The antioxidant capacity of samples was also estimated in another assay according to the procedure described by Guo et al. (2011). The reagents were added into a reaction tube in the following order: 0.3 mL of 20 mM sodium salicylate, 1.0 mL of 1.5 mM FeSO4, 1.0 mL of various concentrations of sample solution, 0.7 mL of 6 mM H_2O_2. The solution was mixed immediately, and placed in a 37 ºC water bath for 1 h. The absorbance measurement of the mixture was recorded at 510 nm against a blank.The reducing power was measured according to the method described by Guo et al. (2011) with slight modifications. An aliquot of each sample (1.0 mL), of different concentrations, was mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) followed by 2.5 mL of 1% potassium ferricyanide $[K_3Fe(CN)_6]$.The mixture

was incubated for 20 min in a water bath at 50 ºC. After the incubation, 1.0 mL of 10% trichloroacetic acid (TCA) was added, followed by centrifugation at 3000 r/min for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3$); then the absorbance was measured at 700 nm against a blank in the spectrophotometer.

2.5 HPLC Analysis

The analysis of total flavonoids was carried out by a Shimadzu LC-20A HPLC system (Shimadzu, Japan) via a reversed-phase C18 column (150 mm 4.6 mm, I.D., 3 μm) (CNW), maintained at 25 ºC.The mobile phase was methanol water (58:42, v/v) with a pH of 3 adjusted with phosphoric acid (pH = 4.2). All standards and sample were dissolved in methanol and filtrated through a 0.25 μm membrane filter prior to use. The flow rate was set at 0.7 mL/min throughout the runs. The injection volume was 10 μL for each run and the PDA detector was set to a wavelength of 270 nm. The calibration curve, based on six kinds of flavonoid standard solutions, showed excellent linearity over the range of 0.221-122 μg/mL. The retention times of luteolin and apigenin were 7.79 min and 11.56 min, respectively. The chromatographic peaks of the analytes were identified by comparing their retention times and UV spectra with those of the reference standards. Quantity calculations were made according to the linear calibration curves of standards.

2.6 Box-Behnken Design

A three-variable, three-level Box-Behnken design (BBD) with 15 experimental runs was used in this optimization study based on the results of preliminary experiments. The ethanol concentration (X1), ultrasonic time (X2), extraction times (X3) were independent variables selected to be optimized for the extraction of *Cryptotaenia japonica* Hassk flavonoids. Each variable set at the three levels. Extraction yield (Y) was taken as the response of the designed experiments. The coded and uncoded (actual) levels of the independent variables are given in Table 1. A second-order polynomial regression model was used to express the yield as a function of the independent variables as follows:

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

Where, Y represents the response variables, α_0 is a constant, α_i, a_{ii} and a_{ij} are the linear, quadratic and interactive coefficients, respectively. X_i and X_j are the levels of the independent variables.

Table 1. Box-Behnken design of the levels of factors

Independent variables	Coded symbols	-1	0	1
Ethanol concentration (%)	X1	50	60	70
Ultrasonic time(min)	X2	50	60	70
Extraction times	X3	1	2	3

2.7. Statistical Analysis

The Design expert (Version 8.0.5b, Stat-Ease Inc., USA) were used to design BBD experiment as well as for regression and graphical analysis of the experimental results. All analyses were performed in triplicate and all experimental results were expressed as mean ± SD. Analysis of variance was performed by an ANOVA procedure. *P* values < 0.05 were regarded as significant and *P* values < 0.01 as very significant.

3. Results and Discussion

3.1 BBD and Response Surface Analysis

Results of Box-Behnken design (BBD) analysis were shown in Table 2. The observed responses of the fifteen experiments were presented in Table 2. Multiple regression analysis was used to analyze the data and the second-order polynomial equation was derived from regression analysis as follows:

$$Y = -97.87717 + 2.99776X1 + 0.57261X2 + 12.95783X3 - 2.83E - 3X1X2 - 1.0307X1X3 + 0.064275X2\ X3 - 0.022583X1^2 - 2.94833E - 3X2^2 - 1.74408X3^2 \quad (3)$$

Table 3 showed the ANOVA results of response surface for extraction yield. As shown in Table 3, the model F-value of "Prob > F" <0.0001 demonstrated that the model is highly significant at the 1% level, and the F-value for lack of fit (*P* = 0.138) also indicated that the model was a good fit. The value of determinations coefficient (R^2) was 0.9927, indicating a good agreement between experimental and predicted values which can explain

99.27% variability of the responses. The value of adjusted determinant coefficient (adj-R^2 = 0.9797) suggested that the total variation of 97.97% for total flavonoids is attributed to the independent variables and only about 2.03% of the total variation cannot be explained by the model. The lower value of CV (1.9142%) indicated that the experimental was highly reliable. The model was very adequate for the prediction with the range of variable employed.

Table 2. Experimental design and results of Box-Behnken design

Run	Ethanol concentration (%) X1	Ultrasonic time (min) X2	Extraction times X3	Extraction yield (mg/g)
1	-1	0	-1	21.020±0.139
2	1	0	-1	19.507±0.276
3	1	0	1	26.517±1.232
4	-1	1	0	28.533±1.180
5	1	1	0	25.611±1.518
6	-1	-1	0	24.770±0.867
7	1	-1	0	22.343±1.274
8	0	1	1	33.312±0.500
9	0	0	0	28.413±0.363
10	0	-1	1	26.612±1.764
11	0	-1	-1	21.261±0.903
12	0	1	-1	23.977±0.553
13	0	0	0	27.620±1.207
14	-1	0	1	30.110±2.243
15	0	0	0	27.707±1.445

Table 3 also showed that the quadratic coefficients of ethanol concentration (X1) (P = 0.0042), ultrasonic time (X2) (P = 0.0002), extraction times (X3) (P < 0.0001), and the interaction between ultrasonic time and extraction times (X1X3) (P = 0.0093) for flavonoids extract yield are significant at the level of P < 0.05 or P < 0.01, while the cross product coefficients of X1X2 and X2X3 were non-significant at the 0.05 level. These results indicated that the changes in ultrasonication time and extraction times had a significant effect on the flavonoid extraction yield.

Table 3. ANOVA of response for UAE experiments

Source	Sum of Squares	DF	Mean Square	F Value	P-value (Prob > F)
Model	172.4425	9	19.1603	75.9727	< 0.0001
X1	6.2216	1	6.2216	24.6695	0.0042
X2	25.2228	1	25.2228	100.0111	0.0002
X3	106.7845	1	106.7845	423.4128	< 0.0001
X1X2	0.3204	1	0.3204	1.2702	0.3109
X1X3	4.2498	1	4.2498	16.8509	0.0093
X2X3	1.6525	1	1.6525	6.5524	0.0507
$X1^2$	18.831	1	18.831	74.6672	0.0003
$X2^2$	0.321	1	0.321	1.2726	0.3105
$X3^2$	11.2314	1	11.2314	44.5336	0.0011
Residual	1.261	5	0.2522		
Lack of Fit	1.1421	3	0.3807	6.4057	0.138
Pure Error	0.1189	2	0.0594		
Cor Total	173.7035	14			

Note. R^2 = 0.9927, Adj-R^2 = 0.9797, CV = 1.9142%.

The three-dimensional response surface shown in Figure 1 was based on Equation 3 and displayed the influence of the variable on extraction yield. One variable was kept constant at an intermediate level while the other two were changed within their respective experimental ranges. The other less significant parameters, ultrasonic power and solvent to solid ratio were set at 400 W and 30:1 mL/g, respectively. It was concluded from Figure 1A that the extraction yield has a positive linear relationship with ultrasonic time. The extraction yield increased slowly with increased ultrasonic time and an increases in ethanol concentration from 50% to 55%, and the resulting response surfaces showed a relatively steep curve with sonication time while the changed the ethanol concentration yield a slightly decrease. Prolonged sonication time and a balanced ethanol/water solvent may have enhanced the extraction yield by water's swelling effect on the plant material and an increase of contact surface area between the plant matrix and solvent. resulting in increased of the extraction yield (Huang et al., 2009).The interactive effects of the extraction times and ethanol concentration on extraction yield were shown in Figure 1B, the extraction yield mainly depended upon extraction times. When the ethanol concentration was kept at the lower level, the extraction yield increased with the increase of extraction times. Furthermore an increase of the ethanol concentration more than 55% caused a slight decrease in extraction yield. Therefore, the interactive effect of ethanol concentration and extraction times on extraction yield was significant, and could be derived from the Table 3, as the "Prob > F" < 0.001 was significant. It was observed in Figure 1C that when other parameters were kept constant, varying the extraction times from 1 to 3, and the ultrasonic time from 50 to 70 min, the extraction yield increased with increasing ultrasonic time and extraction times. Maximum flavonoids extraction yield were observed at a higher ultrasonic time and ultrasonic times.

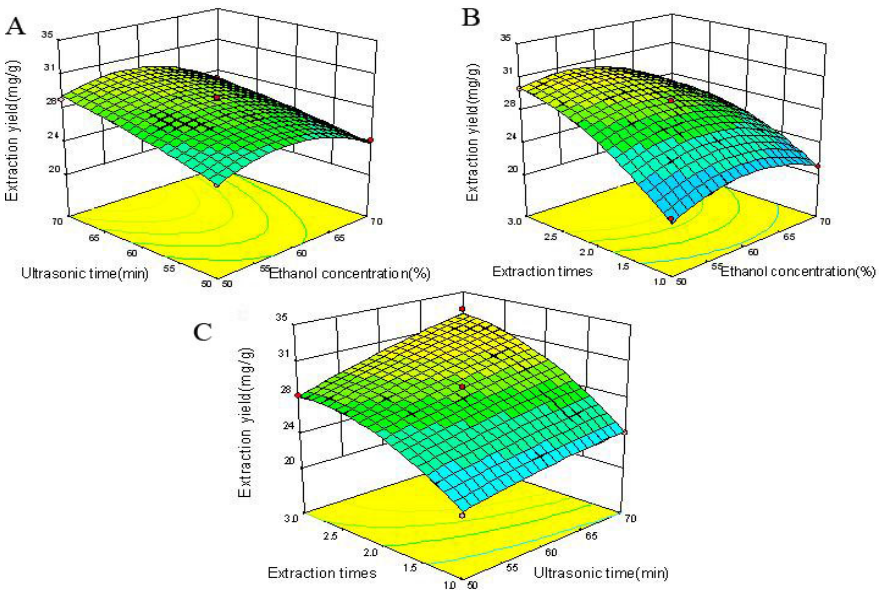

Figure 1. Response surface plots of *Cryptotaenia japonica* Hassk (A) showing the effect of ultrasonic time and ethanol concentration, (B) showing the effect of extraction times and ethanol concentration, (C) showing the effect of extraction times and ultrasonic time on total flavonoids extraction yield

3.2 Validation of the Models

An optimization study was performed to validate the adequacy of the models equation. The optimal extraction conditions determined by the models were as follows: ethanol concentration of 55.13%, an extraction time of 70 min and three cycles, and the maximum results were 33.093 mg/g. To ensure the operation convenience, experiment was performed using the modified optimal conditions: 55% ethanol concentration, ultrasonic time of 70 min, three cycles, respectively. The Experimental value was 33.112 ± 0.742 mg/g which was in agreement with the predicted value (33.093 mg/g) and validated the model.

3.3 Antioxidant Activity and Antioxidant Compositions Analysis

The extracts under the optimum condition were combined together to reduce ethanol under the vacuum and were further lyophilized with lyophilizer to obtain the crude flavonoids (0.101 ± 0.031g/g dry weight powder). The antioxidant capacities of crude flavonoids were shown in Figure 2. When the concentration of crude flavonoids

increased from 0.1 to 3.0 mg/mL, the DPPH radical scavenging activity increased from 23.18 ± 0.1 % to 79.87 ± 2.7%, the values of hydroxyl scavenging effects ranged from 0 to 71.60 ± 2.6%, and the reducing power from 0.035 ± 0.013 to 1.200 ± 0.053. With regards to EC50, the EC50 values for DPPH radical, hydroxyl radical scavenging activity and reducing power were found to be 0.861 ± 0.068 mg/mL, 1.259 ± 0.106 and 1.818 ± 0.560 mg/mL, respectively. The results indicated that the crude flavonoids from *C. japonica* have relative high antioxidant activities and the effects increased with increasing the concentration of flavonoids. At a relatively high concentration (3.0 mg/mL) of flavonoids, much higher antioxidant activity was not generated which could have resulted from a lower purity of crude flavonoid. The major flavonoid compositions were identified and quantified by HPLC and was showed in Figure 3B. Only several constituent peaks were found in the extract, where the main components of the flavonoids were identified as luteolin and apigenin which were consist with Li and Niu (2010). Upon quantification of luteolin and apigenin, the results indicated 15.18 mg/g and 0.985 mg/g, respectively. A strong correlation between luteolin and apigenin with above antioxidant activities indicated that luteolin and apigenin were major contributors of antioxidant activity of *C. japonica*. As these two antioxidants have been demonstrated to have high antioxidant capacity by various research investigations (Gao et al., 2012; Zhang, Gan, Shelar, Ng, & Chew, 2013; Žugić et al., 2014).

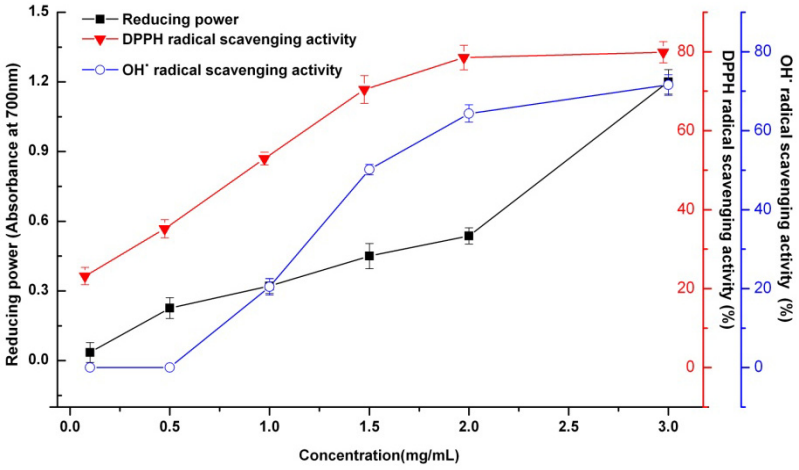

Figure 2. Antioxidant activity of different flavonoids concentration (A). (■) reducing power, (▼) DPPH radical scavenging activity and (○) OH• radical scavenging activity (mean±SD, n = 3)

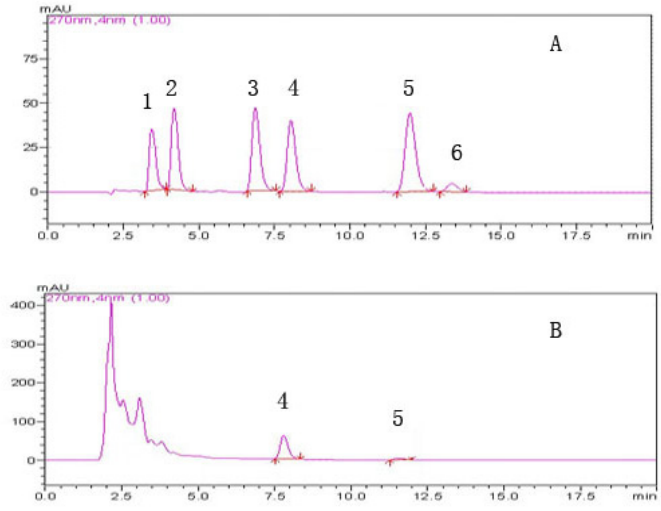

Figure 3. HPLC chromatogram of (A) standard reference and (B) crude flavonoids extracted by UAE (1, hyperin; 2, quercetin-3-rhamnoside; 3, quercetin; 4, luteolin; 5, apigenin; 6, isorhamnetin)

4. Conclusions

In this study, the optimum conditions of ultrasound-assisted extraction (UAE) and antioxidant activity of flavonoids from *Cryptotaenia japonica* Hassk. were investigated. The optimal conditions of UAE were achieved based on response surface methodology with Box-Behnken design. Under these optimal extraction conditions (ultrasound power: 400 W, ethanol concentration: 55%, extraction time: 70 min, solvent to solid ratio: 30 mL/g, and 3 cycles extraction). The highest extraction yield of flavonoids was 33.112 ± 0.742 mg/g, which was well consistent with the predicted value (33.093 mg/g). It revealed that the ultrasonic-assistance extraction is an effective method for *C. japonica* flavonoids extraction. The antioxidant activities evaluation and HPLC analysis showed that the extracts had moderate antioxidant capacities and luteolin and apigenin were the major flavonoid component in the extracts. Our results provide basic of scientific evidence to further the exploitation and application of the resource. The present study also indicated that *C. japonica* can be considered as good sources of antioxidant flavonoid compounds.

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