Microwave-assisted Extraction of Flavonoids from *Phyllostachys heterocycla* Leaves: Optimization, Mechanism, and Antioxidant Activity *in vitro*

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Flavonoids were extracted from *Phyllostachys heterocycla* leaves by adopting microwave-assisted extraction technology. Based on the single factor experiment and Plackett-Burman design results, the extraction process of flavonoids was further optimized using the response surface methodology. The optimum conditions were as follows: an ethanol concentration of 78.1%, an extraction time of 24.9 min, and a microwave power of 559 W. Under these conditions, the extraction yield of flavonoids was 4.67%, which was in close proximity to the predicted value (4.70%) and higher than the extraction yield from traditional Soxhlet extraction (3.35%). Moreover, the possible extraction mechanisms of these two extraction methods were further derived to explain why the microwave-assisted extraction of flavonoids was more efficient compared with traditional Soxhlet extraction. Ultimately, the antioxidant activities *in vitro* of flavonoids from *Phyllostachys heterocycla* leaves were evaluated *via* DPPH and ABTS radical scavenging assay. The flavonoids from *Phyllostachys heterocycla* leaves exhibited excellent antioxidant activities *in vitro* and *Phyllostachys heterocycla* leaves could be a new natural source for developing antioxidants. Overall, the findings of this research could provide a theoretical reference for the further comprehensive development and utilization of bamboo resources.

Keywords: Phyllostachys heterocycla leaves; Microwave-assisted extraction; Flavonoids; Antioxidant activity; Extraction mechanism

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INTRODUCTION

Bamboo is widely distributed throughout the world and is a perennial evergreen herb that belongs to the Gramineae family and the Bambuseae subfamily (He *et al*. 2014). In recent years, research on promoting the utilization of bamboo resources has been a very active field of study throughout the world. Bamboo resources have been considered as an important ingredient in traditional Asian medicine, especially in China and India (Ayurveda) (Yuan *et al.* 2020). Bamboo leaves are a type of forestry resource with potential utilization value worldwide. It has long history of consumption in China since it contains many secondary plant metabolites with medicinal and nutritive properties, which are related to human health (Gong *et al.* 2015). Bamboo leaf extract can be constituted as a light brown powder extracted from bamboo leaves and contains a large amount of

biologically active components, which primarily include flavonoids, phenolic acids, and coumaric lactones (Gao *et al*. 2019).

Phyllostachys heterocycla is native to China. It grows as a thick rod, and the surface of the stalk is covered with a layer of white powder. It is one of several vital scattered bamboo species, primarily distributed in southern China (Tao *et al.* 2019; Yuan *et al.* 2021). Flavonoids are secondary plant metabolites and present in nearly all parts of bamboo, such as leaves, roots, and xylem. Flavonoids play an important role in plants growth and development. Besides, they have been related to the defense mechanism in the aerial parts (leaves and stems) of plants (Orsat and Routray 2017). Flavonoids have attracted much attention due to their beneficial health effects, including antioxidant, antifungal, antitumor, and antidiabetic effects (Fang *et al.* 2020; Ma *et al.* 2020; Li *et al.* 2021). Presently, flavonoids have great application potential in chemical, pharmaceutical and other industries, and the market demand for flavonoids is gradually increasing. In addition, the research on the extraction of flavonoids from vegetal materials mostly focuses on the traditional heat reflux extraction (Liu *et al.* 2021; Shamsuddin *et al.* 2021; Xia *et al.* 2021). This extraction process is time-consuming, laborious, and inefficient, and the quantity and quality of the flavonoids obtained from this method are not satisfactory (Proestos and Komaitis 2008; Zhang *et al.* 2011). Therefore, it is urgent to exploit a high-efficiency extraction method with optimum parameters for achieving the maximum yield of flavonoids.

Microwave-assisted extraction is one of the advanced methods of extraction, which has been increasingly applied for the extraction of bioactive compounds. When compared with the traditional heat reflux extraction method, microwave-assisted extraction is more efficient in many cases and has many advantages, including a strong penetrating force, high selectivity, shorter extraction time, reduced solvent consumption, and a higher yield with a high quality of the targeted compound (Routray and Orsat 2012; Routray and Orsat 2014; Odabas and Koca 2021; Petrotos *et al.* 2021). Many reports have been published on the application of microwave-assisted extraction of secondary metabolites from plants (Zhong *et al.* 2016; Alara *et al.* 2019; Alirezalu *et al.* 2020; Carbone *et al.* 2020; Chaves *et al.* 2020; Yadav *et al.* 2020; Kaanin-Boudraa *et al.* 2021). Nevertheless, there is little information about the extraction of flavonoids from *Phyllostachys heterocycla* leaves using microwave-assisted extraction. Therefore, the primary purpose of this study was to optimize the microwave-assisted extraction of flavonoids using the response surface methodology and to elucidate the mechanism underlying microwave-assisted extraction of flavonoids from *Phyllostachys heterocycla* leaves and compare it with traditional Soxhlet extraction. In addition, the antioxidant activities of flavonoids were also investigated to expand its applications in the healthcare domain.

EXPERIMENTAL

Materials and Chemicals

Phyllostachys heterocycla leaves were collected from the Yibin region (Sichuan, China). These fresh leaves were rinsed with distilled water three times and dried in a vacuum oven at a temperature of 50 °C for 24 h. Then, the leaves were ground in a domestic mixer grinder and sieved with a 60-mesh screen. Finally, the prepared samples were stored in a desiccator for subsequent experiments. The rutin, pyrogallic, salicylic acid, vitamin C and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Aladdin Co., while the

aluminum nitrate, sodium nitrite, sodium hydroxide, and 2,2-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS) were procured from MacLeans Co. All chemicals were of analytical grade.

Methods

Microwave-assisted extraction

A microwave synthesis reactor (MCR-3KT, Zhengzhou Ketai Experimental Instrument Co. Ltd., Zhengzhou, China) with a maximal microwave power of 800 W at a frequency of 2450 MHz was employed to extract the flavonoids. A schematic diagram of the MCR-3KT device is presented in Fig. 1. The pre-prepared *Phyllostachys heterocycla* leaf powder (4 g) was transferred into a 250 mL round-bottom flask containing 120 mL of 70% (v/v) ethanol. Then, the flask was moved into the chamber of this device connected with condensing tubes. Next, the chamber door was closed, and programs of different microwave powers and extraction times were set. After the microwave-assisted extraction, the treated mixture was cooled to room temperature. Then, the supernatant was collected *via* centrifugation (5000×g, 15 min) and concentrated at a temperature of 50 °C using a rotary evaporator. Finally, the concentrate was made up to a volume of 100 mL by adding 30% ethanol for the subsequent determination of the total flavonoid content. All experiments were investigated in triplicate.

Traditional Soxhlet extraction

A Soxhlet apparatus was used to extract the flavonoids from *Phyllostachys heterocycla* leaves using the following optimal conditions: the sample (4 g) was extracted with 120 mL of ethanol (60%) for 7 h at an extraction temperature of 100 °C based on the preliminary orthogonal experiment (4^4). After the Soxhlet extraction, the extracts were evaporated at a temperature of 50 °C using a rotary evaporator. Then, the extracts were transferred to brown glass bottles and stored at a temperature of 4 °C until later use. All Soxhlet extractions were performed in triplicate.

Experimental Design

Single factor experiment and Plackett-Burman design

The microwave-assisted ethanol extraction method was implemented to extract the flavonoids from *Phyllostachys heterocycla* leaves. The reason why this approach was chosen was that ethanol as an extraction solvent can achieve efficient recovery of flavonoids and the extraction process conforms to the principle of "green" chemistry. The extraction yield is affected by several factors, including ethanol concentration, solid to liquid ratio, temperature, and time (Su *et al.* 2019). Considering the multiple effects of various factors on the extraction of target compounds, it is of great importance to explore the optimal extraction conditions in terms of designing a complete extraction process. For this study, the ethanol concentration, solid to liquid ratio, extraction time, and microwave power were chosen as single factors. Then, based on the results of the single factor experiment, the Plackett-Burman design with four independent variables, *i.e.*, ethanol concentration (X_1), solid to liquid ratio (X_2), extraction time (X_3), and microwave power (X_4), at two levels (±1) was applied to design 12 experimental points to explore effects of the above independent variables on the extraction yield of flavonoids from bamboo leaves. The significance of each variable was determined *via* a Student's t-test.

Experimental design of the response surface methodology (RSM)

The response surface methodology (RSM) is widely applied to explore the estimated functional relationship between a response variable and design variables as well as search for the optimal conditions of factors for desirable responses. According to the preliminary results of the single factor and Plackett-Burman design experiments, the independent variables and their ranges were chosen for the further optimization. In order to obtain the optimal extracting conditions and investigate the effects of the independent variables on the flavonoid yield (Y), a Box-Behnken design with three variables, *i.e.*, concentration of ethanol (A), extraction time (B), and microwave power (C), at three levels (Table 4) was performed using Design Expert 8.0 software (Stat-Ease, Minneapolis, MN). In addition, Y was used as the dependent variable, and its values in each trial were the average of the triplicates.

To predict the optimal extracting conditions, a second-order polynomial model concerning the relationship between the independent and response variables was established, as shown in Eq. 1,

$$Y_k = a_{k0} + \sum_{i=1}^{3} a_{ki} x_i + \sum_{i=1}^{3} a_{kii} x_i^2 + \sum_{i<j}^{3} a_{kij} x_i x_j \quad (1)$$

where Y_k is the response, a_{k0}, a_{ki}, a_{kii}, and a_{kij} are coefficients for the intercept, linear, quadratic, and interactive terms, respectively, and x_i, x_i^2, and $x_i x_j$ are the linear, quadratic, and interactive terms of the coded independent variables, respectively.

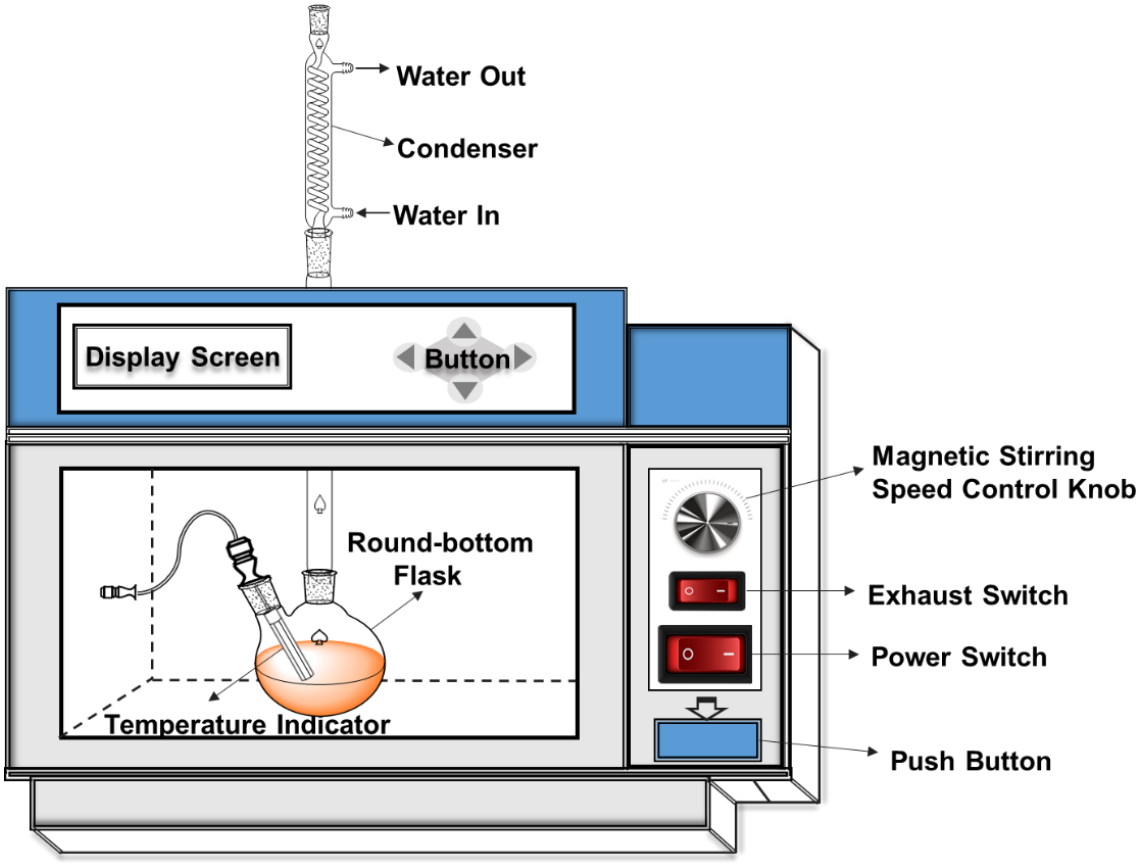

Fig. 1. Schematic diagram of the experimental setup for microwave-assisted extraction

Analytical Method

Determination of total flavonoids content

The calibration curves were constructed using rutin solutions with different concentrations, *i.e.*, 0.008, 0.016, 0.024, 0.032, 0.04, 0.048, 0.056, and 0.064 mg/mL. According to the analysis of the calibration curves, the regression model of the linear relationship between the concentration of the rutin standard solution (*C*) and the absorbency (*A*) was obtained using Eq. 2,

$$A = 9.8938C + 0.0051 \quad (2)$$

where R^2 equals 0.9965.

The total flavonoid content was determined by the colorimetric method with some modifications (Zhu *et al.* 2010; Lu *et al.* 2013). First, 1 mL of the sample solution and 1 mL of a $NaNO_2$ (5%) solution were accurately added to a volumetric flask (25 mL); the mixture was mixed thoroughly and kept at room temperature (25 °C) for 5 min. Second, 1 mL of an $Al(NO_3)_3$ (10%) solution was removed to the above flask and reacted for 6 min. Finally, 10 mL of a NaOH (4%) solution was added to the above volumetric flask, which was followed by adding alcohol (30%) to the scale. After 20 min, the absorbance of the resulting solution was measured at 510 nm using an ultraviolet-visible detector. The sample solution without coloration was used as a reference solution and the final data was presented as the mean of the three replicate measurements. The formula for calculating the extraction yield of flavonoids is shown in Eq. 3,

$$Y = \frac{C \times V \times N}{1000m} \times 100\% \quad (3)$$

where *Y* (%) is the extraction yield of flavonoids, *C* (mg/mL) is the flavonoid content of the test solution calculated by the linear regression model, *V* (mL) is the volume of the test solution, *N* is the total dilution value, and *m* (g) is the mass of test sample.

Fourier-transform infrared (FTIR) analysis

Fourier-transform infrared (FTIR) spectra of the extracts were obtained using a Nicolet 6700 (Thermo Fisher Scientific, Waltham, MA) in the wavenumber range of 4000 to 400 cm^{-1} using a KBr disc containing approximately 1% finely ground samples.

Scanning electron micrographs

The surface morphology of the *Phyllostachys heterocycla* leaves after either the microwave-assisted extraction or traditional Soxhlet extraction were examined using a field-emission scanning electron microscope (SEM) (ZEISS Gemini 500, Oberkochen Germany) with InLens mode. The sample was fixed on the sample holder with conductive adhesive and a thin layer of gold was sputtered on the sample to render the surface conductive prior to examination.

Evaluation for Antioxidant Activity

DPPH radical scavenging activity assay

The scavenging effect of the extracted flavonoids on the DPPH radicals was measured according to the previous report by Ge *et al.* (2020), with some modifications. Next, 600 μL of different concentrations of the samples (0.018 to 0.18 mg/mL) were added to separate glass test tubes. Then, 4 mL of 0.1 mM DPPH (0.1 mM DPPH in ethanol was freshly prepared) was poured into each test tube. These two solutions were mixed

thoroughly and stored at room temperature (25°C) in the dark for 30 min. Finally, the absorbance of the resulting solution was measured at 517 nm using a UV/VIS spectrophotometer (TU-1900, Beijing Purkinje General Instrument Co., Ltd, Beijing, China). Vitamin C was used as a positive control. The DPPH radical-scavenging activity (%) was calculated by Eq. 4,

$$DPPH\ scavenging\ effect\ (\%) = \left(1 - \frac{A_x - A_{x0}}{A_0}\right) \times 100\% \quad (4)$$

where A_0 is the absorbance of the DPPH-ethanol solution mixed with ethanol, A_x is the absorbance of the DPPH-ethanol solution mixed with the sample solution, and A_{x0} is the absorbance of ethanol mixed with the sample solution.

ABTS radical scavenging activity assay

The scavenging effect of the samples on the ABTS radicals was determined as described previously by Hu *et al.* (2016), with some modifications. First, ABTS stock solution with the concentration of 7.4 mM was prepared, and then 5 mL of ABTS stock solution was mixed with 5 mL of 2.6 mM potassium persulfate to prepare the ABTS reagent solution. This solution was stored at room temperature in the dark for 12 h. In addition, the first thing to do when starting the ABTS assay is to dilute the ABTS reagent solution with a phosphate-buffered saline (pH 7.4) to maintain an absorbance of 0.70 ± 0.02 at 734 nm. Then, 400 μL of the sample of different concentrations was added to 4 mL of the diluted ABTS solution to react in the dark at a temperature of 30 ℃ for 6 min. Finally, the absorbance of the mixture was measured at a wavelength of 734 nm. Vitamin C was used as a positive control. ABTS radical-scavenging activity (%) was calculated with Eq. 5,

$$ABTS\ scavenging\ effect\ (\%) = \left(1 - \frac{A_x}{A_0}\right) \times 100\% \quad (5)$$

where A_0 is the absorbance of the diluted ABTS solution mixed with ethanol, and A_x is the absorbance of the diluted ABTS solution mixed with the sample solution.

RESULTS AND DISCUSSION

Analysis of the Singal Factor and Plackett-Burman Experimental Results

The effects of different factors on the total flavonoid extraction yield are shown in Figs. 2a through 2d. For instance, in Fig. 2a, the total flavonoid extraction yield increased as the ethanol concentration increased, before achieving the highest extraction yield (4.26%) at an ethanol concentration of 70%, and then the yield rapidly decreased. A possible explanation for this phenomenon might be that the increase in the ethanol proportion in the extraction solvent will gradually reduce the solvent polarity (Butsat and Siriamornpun 2016). Furthermore, a high concentration of ethanol may hold back the dissolution of polyphenols, thereby affecting the extraction yield (Bouras *et al*. 2015; Ghaffar *et al.* 2020; Petrotos *et al.* 2021). In consideration to the limitation of the ethanol proportion, a 60% to 80% (v/v) ethanol concentration was used as the optimal range for the Plackett-Burman experiment. Regarding the influence of the solid to liquid ratio (as shown in Fig. 2b), the flavonoid extraction yield showed a downward trend when the solid to liquid ratio exceeded 1 to 30, which may be explained by the fact that the solid to liquid ratio of 1 to 30 was more suitable to completely extract the soluble flavonoids.

Regarding the influence of the extraction time (as shown in Fig. 2c), the extraction yield showed a prominent increase during the range of 5 to 20 min. However, the yield began to decrease when the extraction time exceeded 20 min, which indicated that too long an extraction time may lead to the decomposition of flavonoids (Su *et al.* 2019). In terms of microwave power (Fig. 2d), as the microwave power increased from 160 to 640 W, the flavonoid yield gradually increased until it reached its maximum value at 480 W. However, the extraction yields dropped as the microwave power was further increased. The preliminary improvement to the yield at an elevated microwave power could be attributed to the increased destruction of the cellular structure, which can be explained by the rapid pressure built up in the cellular matrix and the corresponding increase in temperature due to dielectric heating (Wang *et al.* 2012; Routray and Orsat 2014; He *et al.* 2016; Zhong *et al.* 2016). However, a high microwave power might increase the temperature of the flavonoids too much and decrease the extraction yield due to the breakdown of the flavonoid structure (Ma *et al.* 2009).

Based on the above results, the optimal conditions amongst the four parameters tested were evaluated as follows: an ethanol concentration of 70%, solid to liquid ratio of 1 to 30, extraction time of 20 min, and the microwave power of 480 W, which resulted in a flavonoid extraction yield of 4.26%. Similar studies have been investigated by Lu *et al.* (2013), who found that the highest flavonoid yield (2.70%) extracted from *Cryptotaenia japonica* Hassk was achieved at a solid to liquid ratio of 1 to 20 using 70% (v/v) ethanol for 15 min.

To further select the significant variables for the extraction of flavonoids, the ethanol concentration, solid to liquid ratio, extraction time, and microwave power were tested and identified *via* the Plackett-Burman design experiment. The experimental design with the name, symbol code, and actual level of the variables are presented in Table 1. The corresponding responses are shown in Table 2. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened *via* Student's t-test for ANOVA (Table 3).

In addition, factors were selected for further optimization studies with *p*-values of less than 0.05 suggesting significant effects on the response. The ethanol concentration was determined to be the most significant factor with a probability value of 0.000, followed by microwave power (0.004), and extraction time (0.007). A lower probability values indicates a more significant factor on the flavonoid extraction yield. The insignificant variable, *i.e.*, the solid to liquid ratio, was neglected, and the optimum levels of the three variables, *i.e.*, the ethanol concentration, extraction time and microwave power, were further determined *via* an RSM design.

The Pareto chart indicating the significant factors for the flavonoid extraction process is shown in Fig. 3. It showed the standardized effect of each parameter or the combination of parameters, as calculated by Eq. 6,

$$Y = 3.3592 + 0.01842X_1 - 0.00492X_2 + 0.01817X_3 - 0.09916X_4 \quad (6)$$

where Y is the response (the flavonoid extraction yield), and the coefficient of each parameter reveals the effect of the parameters on the extraction process. Among these parameters, the ethanol concentration and extraction time exerted positive effects, whereas the microwave power and solid to liquid ratio exerted negative effects.

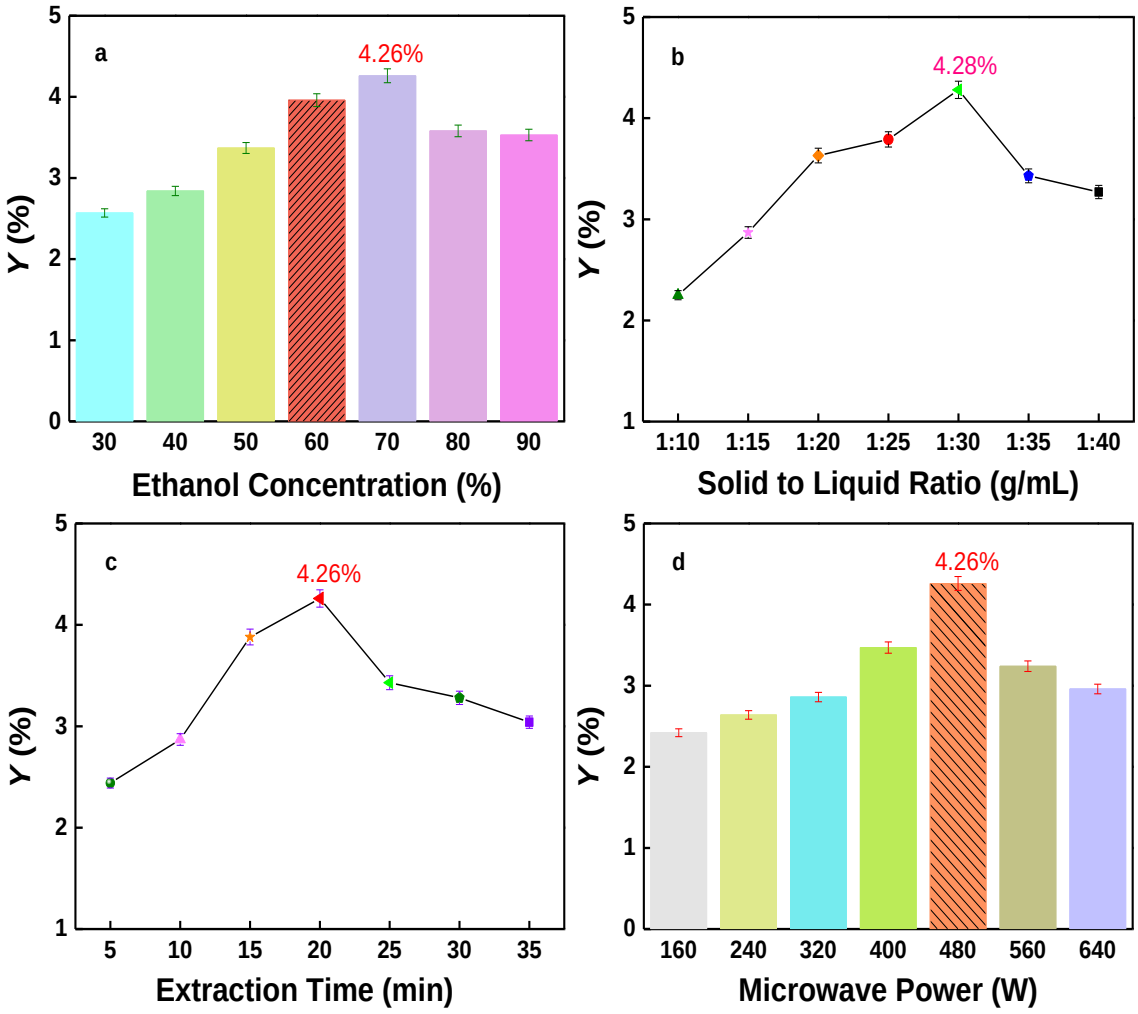

Fig. 2. Effect of the ethanol concentration (a); solid to liquid ratio (b); extraction time (c); and microwave power (d) on the total flavonoid extraction yield

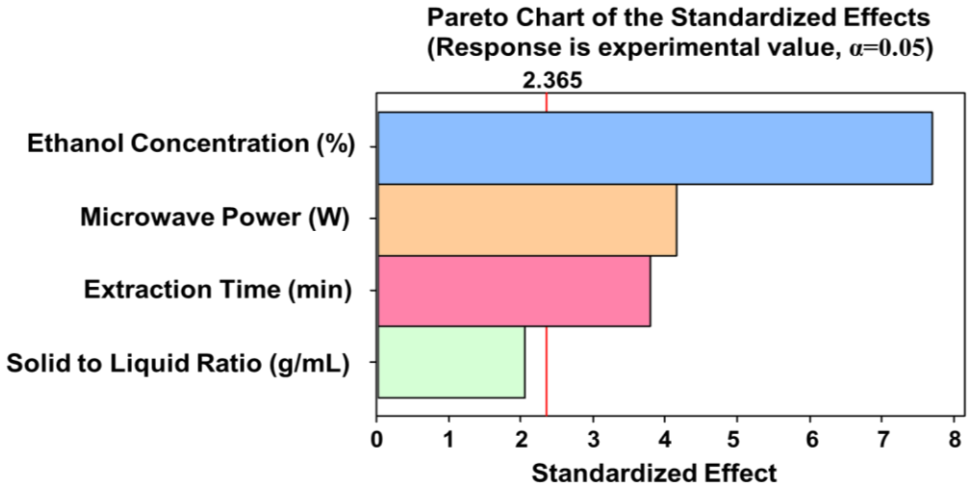

Fig. 3. Pareto chart indicating the significant parameters

Table 1. Experimental Variables at Different Levels Used for the Microwave-Assisted Extraction of Flavonoids Using the Plackett-Burman Design

Variables	Symbol Code	Experimental Values	
		Low (-1)	High (+1)
Ethanol concentration (%)	X_1	60	80
Solid to liquid ratio (g/mL)	X_2	1:20	1:40
Extraction time (min)	X_3	15	25
Microwave power (W)	X_4	400	560

Table 2. The Twelve-Trial Plackett-Burman Design Matrix of the Four Variables with Actual Values

Number	X_1 (%)	X_2 (g/mL)	X_3 (min)	X_4 (W)	*Y* (%)
1	60	20	15	400	4.23
2	60	40	25	400	4.27
3	80	40	15	560	4.24
4	60	20	25	560	4.21
5	80	40	15	560	4.24
6	80	40	25	400	4.63
7	60	20	15	560	3.86
8	60	40	25	560	3.96
9	80	20	25	400	4.58
10	80	20	25	560	4.51
11	60	40	15	400	3.98
12	80	20	15	400	4.52

Table 3. Estimated Effect, Regression Coefficient, and Corresponding t-value and *p*-value for the Flavonoid Extraction Yield of the Four Variables Plackett-Burman Design Experiment

Variables	Effect	Coefficient	Standard Error	t-value	*p*-value
Ethanol Concentration (%)	0.368	0.184	0.024	7.70	0.000[b]
Solid to Liquid Ratio (g/mL)	-0.098	-0.049	0.024	-2.06	0.079[a]
Extraction Time (min)	0.182	0.091	0.024	3.80	0.007[b]
Microwave Power (W)	-0.198	-0.099	0.024	-4.15	0.004[c]
Note: a is non-significant at a *p*-value greater than 0.05; b is a significant positive effect; and c is a significant negative effect					

Statistical Analysis and Model Fitting of the Flavonoid Extraction Process

The three independent variables, *i.e.*, concentration of ethanol (*A*), extraction time (*B*), microwave power (*C*), and their levels in the response surface design are shown in the Table 4. The response surface analysis of the flavonoid yield varying with *A*, *B*, and *C* is presented in Table 5. The second-order polynomial model for the relationship between the independent variables and the flavonoid extraction yield (*Y*) was established as shown in Eq. 7,

$$Y = 4.10 + 0.1150A + 0.0988B + 0.0662C + 0.2025AB - 0.1725AC - 0.0400BC - 0.0250A^2 + 0.1225B^2 + 0.2625C^2 \quad (7)$$

It can be seen from the data in Table 6 that the proposed regression model for the flavonoid extraction yield had a satisfactory determination coefficient (R^2) value and no significant value of the lack of fit (0.1644). The R^2 value was 99.07%, which indicated a reasonable correlation between the experimental results and the theoretical values. In addition, the *p*-values were applied to check the significance of each variable. As shown in Table 6, the linear terms of ethanol concentration (*A*), extraction time (*B*), and microwave power (*C*), as well as the quadratic terms of extraction time (B^2) and microwave power (C^2) showed significant (*p*-value was less than 0.05) effects on the flavonoid extraction process. Among the interaction terms, *AB* and *AC* were significant (*p*-value was less than 0.05) for this extraction process.

Table 4. Independent Variables and Their Levels in the Response Surface Design

Independent Variables		Coded Factor Level		
		-1	0	1
A	Ethanol concentration (%)	60	70	80
B	Extraction time (min)	15	20	25
C	Microwave power (W)	400	480	560

Table 5. Results of the Response Surface Analysis of the Variation in Flavonoid Yield

Number	*A* (%)	*B* (min)	*C* (W)	*Y* (%)
1	70	20	480	4.12
2	70	15	400	4.25
3	70	25	560	4.64
4	60	20	400	3.99
5	70	25	400	4.58
6	60	20	560	4.46
7	70	20	480	4.06
8	70	15	560	4.47
9	80	20	400	4.56
10	80	15	480	4.04
11	60	15	480	4.21
12	80	25	480	4.59
13	80	20	560	4.34
14	70	20	480	4.11
15	70	20	480	4.12
16	70	20	480	4.09
17	60	25	480	3.95

The scatter diagram of the predicted results *versus* the actual values for the flavonoid yield is shown in Fig. 4. The predicted values obtained by the regression equation matched the experimental values reasonably well with an R^2 value of 99.07%. Moreover, adequate precision (AP) was applied to compare the range of the predicted values at the design points to the average prediction error (Fig. 4). According to Table 6, the AP values (24.08) higher than four confirmed that the predicted model can be used to navigate the

design space. The coefficient of variance (CV) as the ratio of the estimated standard error to the average of the observed response defines the reproducibility of the model. The CV values (0.81%) in Table 6 was less than 10%, which demonstrated that the model can be considered reproducible (Ghafari *et al.* 2009). Similarly, the normal probability distribution diagram of the residuals is shown in Fig. 5. It was found that the externally studentized residuals were also distributed on the straight line with a 45° slope, which satisfied the normal hypothesis. From this analysis, it was found that the established regression model could approximately predict the flavonoid extraction yield.

Table 6. Variance Analysis of Regression Model

Source	Degree of Freedom	Sum of Square	Mean Square	*F* Value	$P_r > F$
Model	9	0.8770	0.0974	82.43	< 0.0001
A	1	0.1058	0.1058	89.50	< 0.0001
B	1	0.0780	0.0780	65.99	< 0.0001
C	1	0.0351	0.0351	29.70	0.0010
AB	1	0.1640	0.1640	138.75	< 0.0001
AC	1	0.1190	0.1190	100.69	< 0.0001
BC	1	0.0064	0.0064	5.41	0.0529
A^2	1	0.0026	0.0026	2.23	0.1793
B^2	1	0.0632	0.0632	53.45	0.0002
C^2	1	0.2901	0.2901	245.43	< 0.0001
Residual	7	0.0083	0.0012		
Lack of fit	3	0.0057	0.0019	2.91	0.1644
Note: R^2 equals 99.07%; AP equals 24.08; CV equals 0.81%; and P_r was less than 0.0001					

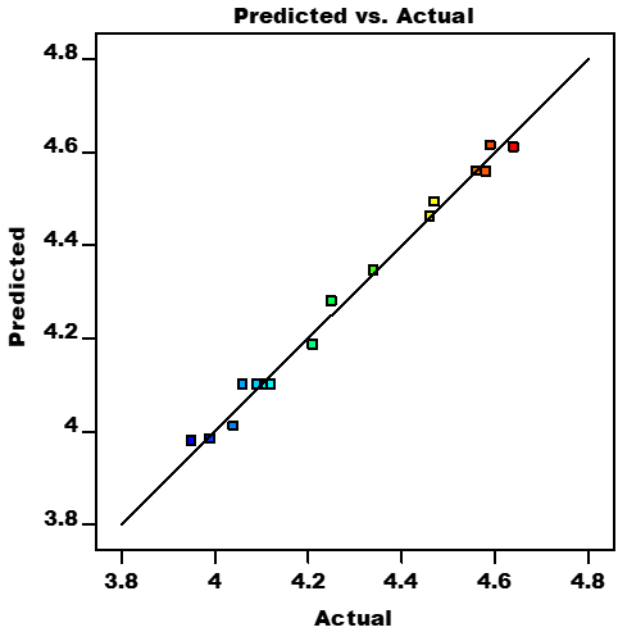

Fig. 4. Scatter diagram of the predicted results *versus* the actual values for the flavonoid yield

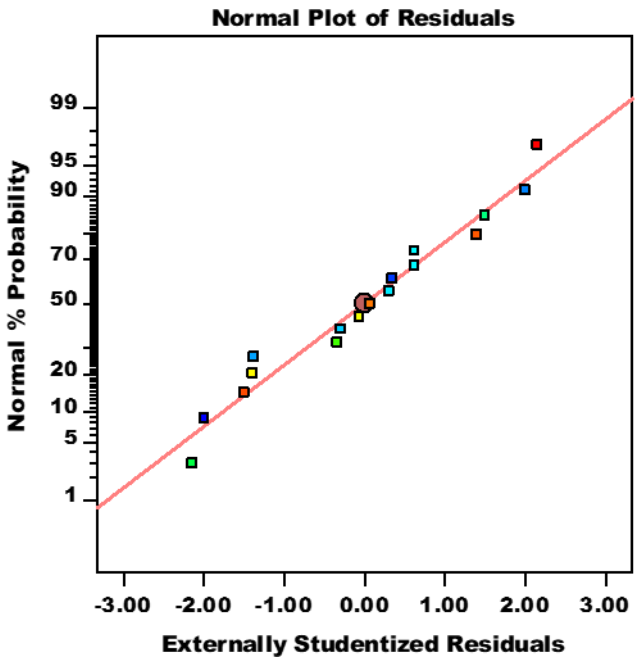

Fig. 5. The normal probability distribution of the residuals

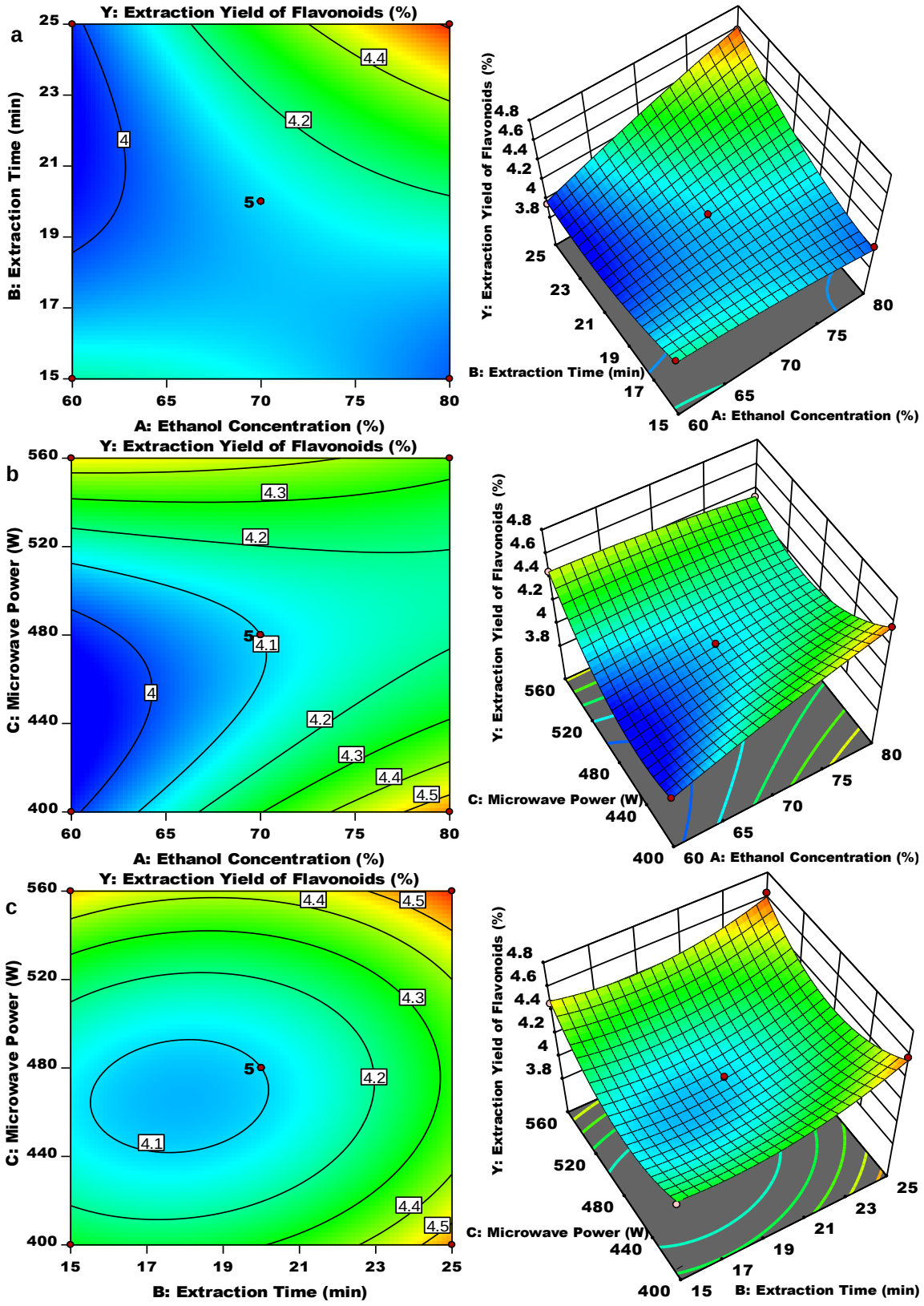

Fig. 6. The contour plots and response surface demonstrating the effect of a (ethanol concentration and extraction time), b (ethanol concentration and microwave power), and c (extraction time and microwave power) on the yield of flavonoids.

Optimization of the Microwave-Assisted Extraction Conditions and Validation of the Models

The contour plots and 3-D response surface plots obtained using the Design Expert 8.0 software are shown in Figs. 6a through 6c. Through these graphs, the visual interaction between two extraction variables on the flavonoid yield can be determined (Cardoso-Ugarte *et al. 2*014). Figure 6a demonstrated the effect of the ethanol concentration and extraction time on the flavonoid yield. The positive coefficient of the term *AB* (refer to Eq. 7) confirmed the prominent interaction effect on the flavonoid yield. It was evident that with an increase in ethanol concentration or extraction time, the flavonoid yield gradually increased. In addition, the *p*-value of *AB*, which was less than 0.05, indicated significant interaction of the ethanol concentration and extraction time. The plot of the effect of the ethanol concentration and microwave power to raw material on the flavonoid yield is shown in Fig. 6b. The flavonoid yield significantly increased as the ethanol concentration and microwave power increased. Figure 6c predicted the mutual effect of the extraction time and microwave power on the flavonoid yield. It can be observed from the plot that there was a marginal increase in the flavonoid yield when the extraction time and the microwave power increased from 400 W and 15 min to 480 W and 20 min, respectively.

Based on the established quadratic regression model, the optimal extraction conditions were as follows: an ethanol concentration of 78.1%, an extraction time of 24.9 min, and a microwave power of 559 W. Under these optimal conditions, the predicted flavonoid yield was 4.70%. The validation was performed under these conditions, modified for convenience, as follows: the ethanol concentration was 78%, the extraction time was 25 min, and the microwave power was 560 W. The obtained actual flavonoid yield was 4.67%, which not only indicated that there were no remarkable differences between the experimental and predicted values, but also verified that this quadratic regression model was feasible in terms of simulation and able to predict experimental results in practice.

Comparison of the Microwave-Assisted Extraction and the Traditional Soxhlet Extraction

The application of microwave-assisted extraction had a positive effect on the flavonoid yield compared with traditional Soxhlet extraction, and the results are summarized in Table 7. Under the optimal conditions of microwave-assisted extraction, the flavonoid yield increased from 3.35% to 4.67% with a notably shorter extraction time (25 min), which demonstrated that this method could not only increase the extraction yield, but also greatly shorten the extraction time (Ho *et al.* 2019). These findings were consistent with those reported in the literature, which have shown that applying microwave-assisted extraction to any number of materials can considerably reduce the extraction time compared to conventional extraction methods (Zhang *et al.* 2011; Tanongkankit *et al.* 2013; Geetha *et al.* 2019).

Table 7. Comparison of the Microwave-Assisted Extraction and Traditional Soxhlet Extraction in Terms of the Flavonoid Yield from *Phyllostachys heterocycla* Leaves

Methods	Extraction Time (min)	Y (%)
Microwave-assisted extraction	25	4.67
Traditional Soxhlet extraction	420	3.35

In order to explore the essential differences between the microwave-assisted extraction and traditional Soxhlet extraction, a possible extraction mechanism was proposed (Fig. 7). When dry bamboo leaf particles were extracted with ethanol, the extraction process was divided into three steps: First, the ethanol surrounded the bamboo leaf particles to create a solvent film and swelling occurs (Vinatoru *et al.* 2017). The shape of the bamboo leaf particles, which were initially irregular, became rounded and smooth. During the swelling process, the solvent entered the bamboo leaf particle matrix and the flavonoids were released into the solvent (type II diffusion, as shown in Fig. 7). At the end of the swelling period, the bamboo leaf particles were surrounded by a solvent layer to form a "stagnant layer", which will hinder the extraction. Second, the flavonoids diffused from the solvated bamboo leaf particles to the stagnant layer (type I diffusion, as shown in Fig. 7). Third, the flavonoids proliferated from the stagnant layer into the bulk solvent (type III diffusion, as shown in Fig. 7). However, the major difference between these two extraction methods is that the bamboo leaf particles can quickly reach a higher temperature under microwave irradiation than traditional Soxhlet extraction and steam is created within the cellular structures, which increases the inside cell pressure, leading to the rupture of the cell membranes; hence the extraction efficiency is improved (Gao *et al.* 2004; Li *et al.* 2013; Lee *et al.* 2016). In addition, this effect also enhances the rinsing out of the contents of the broken cells and facilitates the release of more active compounds.

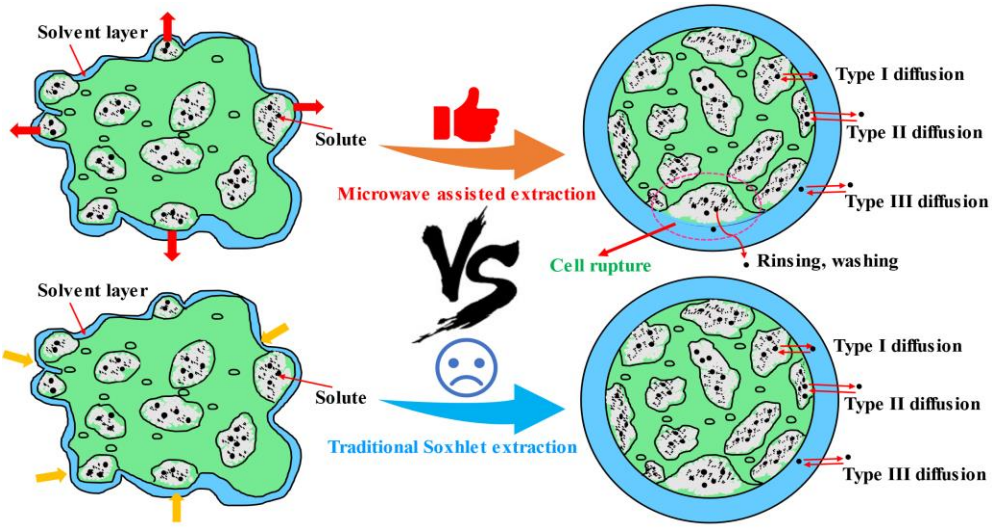

Fig. 7. Possible extraction mechanism (microwave-assisted extraction *versus* traditional Soxhlet extraction)

The scanning electron micrographs of the raw material (*Phyllostachys heterocycla* leaves) and the materials treated *via* microwave-assisted extraction and traditional Soxhlet extraction are shown in Fig. 8. The surface of the *Phyllostachys heterocycla* leaves before extraction (Fig. 8a) are planar and smooth. After the traditional Soxhlet extraction, no appreciable changes could be found in the surfaces of the samples (Fig. 8c). However, the scanning electron micrograph of the *Phyllostachys heterocycla* leaves after the microwave-assisted extraction revealed the presence of several long cracks distributed across the entire particle. This phenomenon was attributed to the sudden thermal stresses and the high localized pressures, thereby causing ruptures and creases in the cell structure (Kaderides *et al.* 2019). Similarly, studies carried out by Alara *et al.* (2019) have revealed that microwave

radiation plays a vital role in the rupture of vegetal cell walls and this structural change in the cell walls allows the solvent to easily enter the cellular channels with high extraction efficiency. All of these analyses confirmed that microwave-assisted extraction should be more suitable for extracting flavonoids from *Phyllostachys heterocycla* leaves than the traditional Soxhlet extraction.

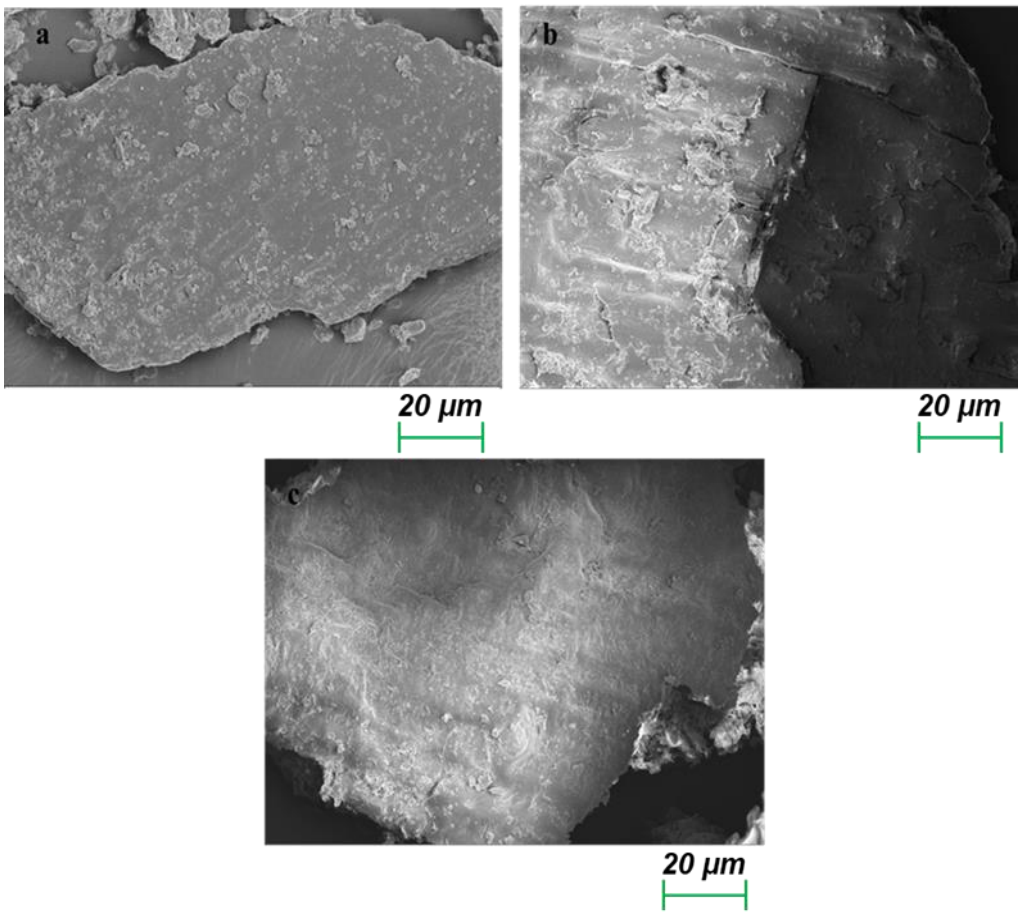

Fig. 8. Scanning electron micrographs (magnification 500 X) of the *Phyllostachys heterocycla* leaves: (a) untreated; (b) after microwave-assisted extraction; and (c) after traditional Soxhlet extraction

Fourier-transform Infrared (FTIR) Analysis

Fourier-transform infrared spectroscopy is a powerful non-destructive analytical technique for the chemical characterization of different compounds, which provides basic information on the molecular structure of organic compounds in plant extracts (Patle *et al.* 2020). In order to demonstrate the effect of microwave-assisted extraction and traditional Soxhlet extraction on the flavonoid functional groups, the infrared spectrum of the flavonoids extracted from *Phyllostachys heterocycla* leaves *via* two methods, within a frequency range of 4000 to 400 cm^{-1}, were analyzed (Fig. 9). The FTIR bands in the range of 4000 to 1500 cm^{-1} and 1500 to 500 cm^{-1} represented the functional group and the fingerprint region, respectively. There was a broad absorption peak at 3419 cm^{-1}, which was assigned to the stretching vibration of the O-H group. The low-intensity absorption band at 2930 cm^{-1} was attributed to the -CH stretching (Karpagasundari and Kulothungan 2014). A prominent peak at 1628 cm^{-1} was due to the stretching of the carbonyl (C=O)

group in the compound and the minor changes in bands at 1500 to 1400 cm^{-1} can be attributed to the stretching of C-O. The absorption peak nearby 1118 to 1100 cm^{-1} was assigned to the stretching vibration of the C-O-C group and the band at 800 to 600 cm^{-1} was allocated to the substitutions of the Ar-H group (Meenakshi *et al.* 2009; Hamad *et al.* 2012; Patle *et al.* 2020). From this analysis (Fig. 9), the functional groups of the flavonoids extracted *via* microwave-assisted extraction and traditional Soxhlet extraction were fundamentally identical, which confirmed that microwave-assisted extraction had no effect on the flavonoid functional groups and should be an efficient and suitable technique.

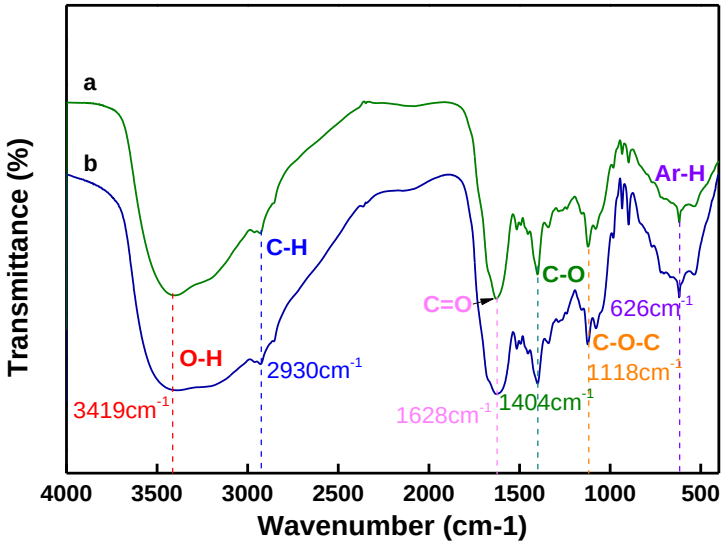

Fig. 9. The FTIR spectra of the flavonoids from *Phyllostachys heterocycla* leaves extracted with different methods (Note: 'a' means microwave-assisted extraction; and 'b' means Soxhlet extraction)

Evaluation of the Antioxidant Activities of the Flavonoids Obtained Under Optimal Conditions

The antioxidant potential of plant extracts is often determined by *in vitro* antioxidant activity assays, *e.g.*, the DPPH radical scavenging assay (Mišan *et al.* 2020; Chen *et al.* 2021). In the present work, the antioxidant activities of the flavonoids obtained under optimal microwave-assisted extraction conditions were performed by two antioxidant assays, *i.e.*, DPPH and ABTS. As shown in Fig. 10, the free radical scavenging activities of the flavonoids and vitamin C in the DPPH and ABTS experiments follow the same trend as the concentration increased. The DPPH radical scavenging activity of the flavonoids at each concentration was lower than radical scavenging activity of vitamin C. According to the different concentrations of the clearance curve, the half-maximal inhibitory concentration (IC50) was used to compare the radical scavenging activity of the flavonoids, and the lower IC50 value, the better it can scavenge radicals. As observed from Fig. 10, the flavonoids had a considerably higher IC50 value (0.0734 mg/mL) compared to vitamin C (0.0277 mg/mL), which demonstrated that the antioxidant activities of the flavonoids extracted from *Phyllostachys heterocycla* leaves were lower than the antioxidant activities of vitamin C. However, the result of the flavonoids in the DPPH assay was 84.1% based on a 0.18 mg/mL sample, which revealed that the flavonoids extracted from the *Phyllostachys heterocycla* leaves had excellent antioxidant activity. In addition, the radical scavenging activity of the flavonoids in the ABTS assay was higher than the

radical scavenging activity of vitamin C, and at the highest concentration (0.18 mg/mL), 98.4% of scavenging potential was observed. The IC50 value for the flavonoids (0.019 mg/mL) in the ABTS assay was found to be lower when compared to vitamin C, which indicated that the ABTS radical scavenging activity of the total flavonoids extracted from *Phyllostachys heterocycla* leaves was higher than vitamin C. Overall, the results suggested that *Phyllostachys heterocycla* leaves could be a new natural source for developing antioxidants in the clinical industry.

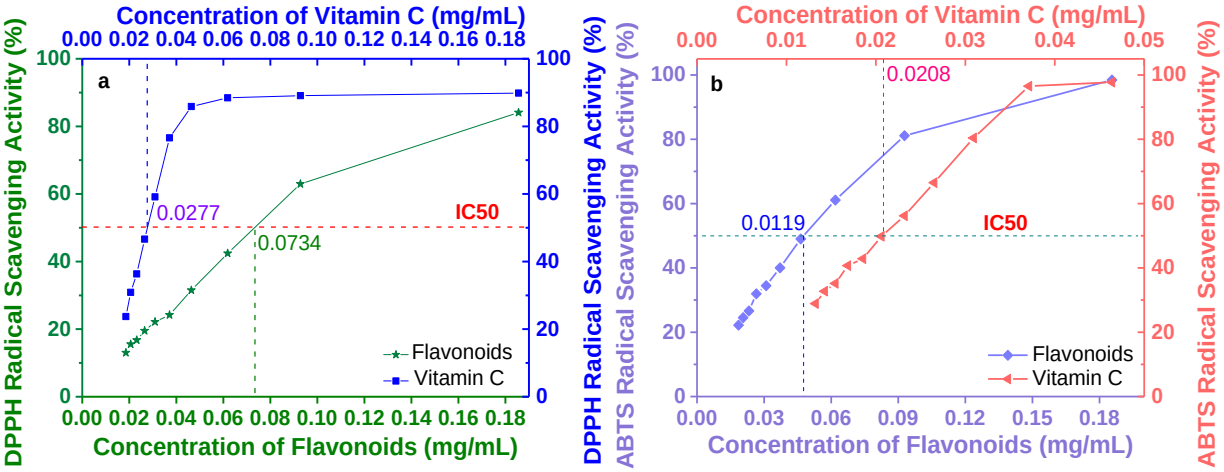

Fig. 10. Antioxidant activities of the flavonoids and vitamin C: (a) DPPH assay; and (b) ABTS assay

CONCLUSIONS

1. Based on the results of the single-factor experiments, the three significant variables, *i.e.*, ethanol concentration, extraction time, and microwave power, for the extraction of flavonoids were selected through the Plackett-Burman experiment. In addition, the optimal conditions for extracting flavonoids were further determined by an RSM design. The optimal microwave-assisted extraction conditions for flavonoids were obtained, as follows: an ethanol concentration of 78.1%, an extraction time of 24.9 min, and a microwave power of 559 W. Compared with traditional Soxhlet extraction, the microwave-assisted extraction technique not only increased the extraction yield, but also greatly shorten the extraction time.
2. The possible extraction mechanisms of the microwave-assisted extraction and the traditional Soxhlet extraction were derived and confirmed *via* scanning electron micrographs. Microwave radiation plays a vital role in the rupture of the vegetal cell walls, which led to the improvement in the extraction efficiency. Furthermore, the results of the FTIR analysis showed that the functional groups of the flavonoids extracted *via* microwave-assisted extraction and traditional Soxhlet extraction were fundamentally identical.
3. The antioxidant activities *in vitro* of flavonoids from *Phyllostachys heterocycla* leaves were evaluated *via* DPPH and ABTS radical scavenging assays. The results indicated that the flavonoids from *Phyllostachys heterocycla* leaves had higher antioxidant

activities *in vitro*. In addition, this confirmed that *Phyllostachys heterocycla* leaves could be a new natural source for developing antioxidants in the clinical industry.

4. The application of microwave-assisted extraction is a potential strategy for improving the yield of flavonoids from *Phyllostachys heterocycla* leaves. This research could provide a theoretical reference for the development of novel microwave set-ups for possible commercial production of various flavonoids and promote the exploitation of new extraction method based on microwave, such as dynamic microwave extraction.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (No. 51704292 and 51974311), the National Key Research and Development Program of China (No. 2019YFC1904302), and the Open Sharing Fund for the Large-scale Instruments and Equipment of China University of Mining and Technology.

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Article submitted: September 3, 2021; Peer review completed: October 9, 2021; Revised version received and accepted: October 11, 2021; Published: October 17, 2021.
DOI: 10.15376/biores.16.4.8060-8081