Original Research Paper

Response Surface Optimized Ultrasonic Assisted Extraction of Total Flavonoids from Walnut Leaves and *In Vitro* Antibacterial Activities

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Abstract: In this paper, the optimum extraction conditions of total flavonoids extracted from walnut leaves subjected to Ultrasonic Assisted Extraction (UAE) were optimized by Response Surface Methodology (RSM). The mathematical model showed the high coefficient of measurement (R^2 = 0.9938) which indicated that this model could be used to guide the response surface methodology. The optimum extraction parameters for extracting flavonoids from walnut leaves determined in this study were extraction temperature 47.73°C, extraction time 30.79 min, ethanol concentration 72.89% (v/v). Under the optimal extraction conditions, the flavonoids yield was about 3.5315%. Statistical analysis of the results showed that extraction temperature, extraction time and ethanol concentration significantly affected the extraction yield of total flavonoids. In addition, the antibacterial activity assays of the flavonoids were carried out and it was demonstrated that the total flavonoids extracted at the optimum conditions had pronounced antibacterial effects against the four bacterial species. Therefore, this study suggested that walnut leaves are promising resources with antibacterial properties for the development of phytomedicines.

Keywords: Ultrasound-Assisted Extraction, Response Surface Methodology, Walnut Leaf, Flavonoids

Introduction

Walnut (*Juglans regia L.*), which belongs to the Juglandaceae family, is a local deciduous tree in northwestern Chain (2012; Moser, 2012). Walnut leaves are known to possess many biological properties and are easily available in abundant amounts (Derebecka *et al.*, 2012). They have been used as a traditional medicine in China and have shown various health benefits for the treatment of skin inflammations, venous insufficiency and ulcers (Cheniany *et al.*, 2013). Moreover, researches in pharmacology and therapeutics have shown that walnut leaves have hypoglycemic, antioxidative, antimicrobial and antihypertensive effects (Gîrzu *et al.*, 1988). In recent years, there has been an in-depth study on substances having considerable antimicrobial properties. It is well known that certain chemicals produced by plants are naturally toxic to bacteria and fungi. Various medicinal plant extracts containing flavonoids are reported to have antimicrobial activity (Basile *et al.*, 1999).

Walnut leaves are good sources of flavonoids (Zhao *et al.*, 2014). As natural products, flavonoids exert an extensive biochemical and pharmacological properties. They are described as dietary supplements that promote health, prevention of disease and active cancer preventive agents (Duarte *et al.*, 1993; Hodek *et al.*, 2002). Flavonoids are present in photosynthetic cells and are therefore widespread in the plant kingdom (Manthey *et al.*, 2001). They are common ingredient in the human diet and are found in vegetables and fruits (Xie *et al.*, 2007; Harborne and Baxter, 1999). Flavonoids have been shown to possess a series of important biological activities, including antifungal and antibacterial activities (Galeotti *et al.*, 2008; Kabir *et al.*, 2015; Alarcón *et al.*, 2008). Flavonoids compounds can form complexes with soluble proteins and extracellular matrix and bacterial cell walls, which probably lead to their antibacterial

activities (Cushnie and Lamb, 2005). Presence of flavonoids in plants might have some or significant contribution to the antimicrobial activity of plants.

Up-to now, several traditional extraction methods have been applied to the extraction of flavonoids from walnut leaves such as Maceration Extraction (ME) (Djozan and Assadi, 1995), Heat Reflux Extraction (HRE) (Zhang and Liu, 2004), soxhlet extraction (Shang and Yuan, 2003) and Microwave-Assisted Extraction (MAE) (Xia *et al*., 2006). This extraction process usually takes several hours or even days and requires a large amount of solvents, which may result in the damage of flavonoids due to hydrolysis and oxidation (Camel, 2000). Ultrasonic Assisted Extraction (UAE) method can extract bioactive molecules at lower temperature, shorter time and also can relatively reduces the structural damage of compounds in plants than using other traditional extraction (Yuan *et al*., 2015).

Response Surface Methodology (RSM) is a collection of improvement method of optimization mathematical and statistical process (Talebpour *et al*., 2009). This is a useful tool for studying the mutual effect between various factors on their measurement and quantification of the influence of reaction parameters (Al-Matani *et al*., 2015; Teng and Choi, 2014). Box–Behnken Design (BBD) is a commonly used process of RSM which make it easier to arrange the experiments result (Borges *et al*., 2009). Therefore, BBD technology was employed to analyze the influence of various process variables, including extraction temperature, extraction time and ethanol concentration on the yield of the flavonoids extracted from walnut leaves.

In the current study, UAE was used to extract flavonoids from walnut leaves using one factor and RSM experimental design to optimize extraction conditions. Furthermore, the antibacterial effects of the total flavonoids extracted at the optimum conditions were determined by using the diffusion methods of agar well. The purpose of this study was to determine the best extraction process parameters for flavonoids extraction from walnut leaves by ultrasonic assisted method and to explore its potential antibacterial properties, so as to establish a scientific basis for the development and utilization of flavonoids.

Materials and Methods

Plant Material

The samples of walnut leaves were harvested between June and July at Zibo city, Shandong province of China and the experimental materials were dried in oven and ground to a powder and then filtered using a 10 mesh sieve.

Chemicals and Reagents

Rutin (purity>98%), sodium hydroxide, aluminum chloride and aluminum nitrate were obtained from Sigma-Aldrich Chemicals Co, Germany and all are analytical grade.

The Total Flavonoids Extraction from Walnut Leaves

The powders of walnut leaves (1g) were placed in 50 mL^{-1} centrifuge tubes and mixed with ethanol. After ultrasonic extraction, the samples were centrifuged at 5000 rpm and the supernatant was collected 15 min later. The residue continued to be extracted twice according to the above mentioned conditions, then all supernatants were mixed up and concentrated by a rotary evaporator, then, flavonoids were separated and purified used large hole resin, finally, the collected fractions were freeze dried to powder.

Determination of the Content of Total Flavonoids

The total flavonoids content in the extracted solution was measured by an aluminum-chloride-colorimetric method (Qadir *et al*., 2015). In brief, the Rutin standard and the extracted solution with different concentrations were appropriately diluted by 30% ethanol to 5 mL and added 0.3 mL of 5% sodium nitrite solution, placed 6 min then added 0.3 ml of 10% alchlor solution, placed 6 min, then added 4 mL of 5% sodium hydroxide solution. Finally, adjusted the volume of the mixture to 10 mL by 30% ethanol and placed for 15 min. The absorbance of the mixture was measured at 510 nm and distilled water was used as a blank control. The reference standard was Rutin, while the contents of total flavonoids in extracts were presented as Rutin equivalents. All determinations were performed in triplicate.

In this work, the total flavonoids of the total extract obtained from the walnut leaves were calculated from the equation of the standard plots (Fig. 1) as follows:

Absorbance = 8.66×total flavonoids
+ 0.0004 (R^2 = 0.9997)

Single Factor Experiments

Total flavonoids extraction yield of walnut leaves was influenced by many factors. Therefore, choosing appropriate extraction solvent and extraction method is an important consideration. Based on the preliminary experiments results ethanol and UAE were selected as reasonable options.

The experiment used ethanol as the extraction solvent and UAE as extraction method, respectively. The maximum total flavonoids content were determined by single factor experiments. Before RSM analysis, an initial experiment was performed to screen for important factors affecting the experimental responses.
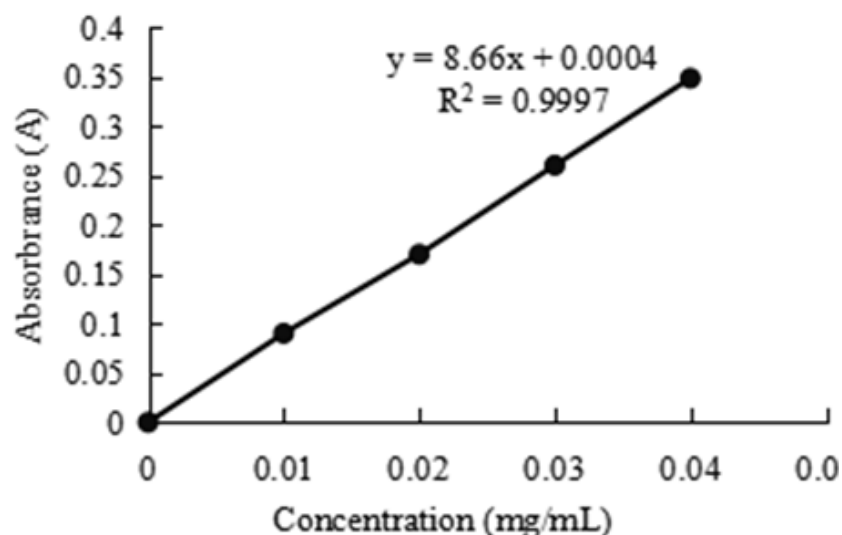
Fig. 1. Calibration curve of standard Rutin for determination of total flavonoids content in total extract of walnut leaves

Ethanol concentration of 30, 40, 50, 60, 70 and 80%, extraction time of 10, 20, 30, 40, 50 and 60 min, extraction temperature of 30, 40, 50, 60, 70 and 80°C were investigated using single factor tests to select the impact areas for further investigations.

Box-Behnken Design (BBD) Optimized UAE Conditions

Box-Behnken Design (BBD) is a frequently used method of RSM that is composed of several intermediate points and a central point (Saniah and Samsiah, 2012). BBD was employed to design the experiments, optimize the extraction conditions and analyze the interactions between the above-mentioned parameters. In the present study, three main factors to RSM were used to describe the relationship between responses and variables to obtain the best extraction conditions. Therefore, the influences of three variables X_1 (ethanol concentration, 60 to 80%), X_2 (extraction temperature, 30°C to 50°C) and X_3 (extraction time, 20 to 40 min) were considered (Table 1). The BBD method was consisted of three factors and levels of 17 experimental operations. In the observed response, the experiment was randomized to maximize the effect of unexplained variability. A quadratic equation was used for this model as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{i2} + \sum_{i \neq j=1}^{3} \beta_{ij} X_i X_j$$

Where:
Y = The response variable measurement
β_0 = A constant index
β^i = The *ith* liner regression coefficient
β_{ii} = *ith* quadratic coefficient,
β_{ij} = *ijth* interaction coefficient

X_i, X_j = The leves of independent variable and the term X_i, X_j and X_i^2 represent the interaction and quadratic terms, respectively

Bacterial Strains and Cultures

Pure bacterial strains used in this study, including *Staphylococcus aureus, Escherichia coli, Salmonella typhi and Bacillus subtilis,* were obtained from the Department of Microbiology, Agriculture Culture Collection of China. Separate sterile nutrient agar slants were prepared and the bacterial strains were individually inoculated under aseptic conditions and incubated at 37°C for 24 h. Colonies were harvested separately under aseptic condition from the slants and individually inoculated into sterile nutrient broths in separate test tubes and kept in refrigerated condition (Channabasava *et al.*, 2014).

Active cultures were achieved by dispensing a tube of cells into 100 mL of nutrient broth and incubating at 37°C for 10 h. The turbidity of the cell suspension was adjusted to the initial concentration 10^8 CFU /mL according to the McFarland standard (Lv *et al.*, 2011).

Antimicrobial Assay

The effective antibacterial capacity of total flavonoids against bacterial strains was determined by diffusion method of agar well and further confirmed by analyzing the Minimal Inhibitory Concentration (MIC) (Vutuc and Holzer, 2014). The walnut leaves extract was diluted with sterile water to 100 mg/mL. Then pour 100 μL bacteria suspension (108 CFU mL) on the solid medium, evenly distributed. Oxford cup of 5 mm diameter were sterilized, then the cups were set on the medium and different concentration of the extraction (100 μL) were filled respectively.

Table 1. Independent variables and their levels for Box Behnken rotatable design

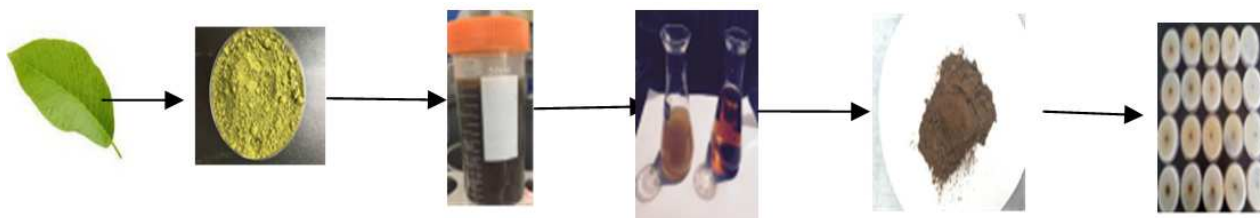| Factor	symbol	Levels		
		-1	0	1
Extraction temperature (°C)	X_1	30	40	50
Extraction time (min)	X_2	20	30	40
Ethanol concentration (%)	X_3	60	70	80

Fig. 2. Schematic representation of total flavonoids extraction from walnut leaves and its antibacterial activity assay

After 48 h of incubation, the diameter of the inhibitory area was measured with micro scale. The procedure for the extraction of flavonoids and determination of its antibacterial activity was shown in Fig. 2.

Minimum Inhibitory Concentration (MIC) Determination

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of antibacterial agents that inhibits the proliferation of the bacteria (Doss *et al.*, 2011). For the determination of MIC, 100µL bacteria suspension (10^8 CFU /mL) was added on the medium evenly distributed. Sterilized oxford cup of 5 mm diameter were prepared, then added with 100 µL different concentrations of the extract, which were 100, 50, 25, 12.5, 6.25, 3.125 mg/mL, respectively and kept at 37°C for 24 h. For each microorganism, at least three replicated experiments were carried out for date analysis.

Analysis of Statistical

Experiments were repeated three times; the mean and standard deviation (X ± SD) of the data were calculated. The statistical analyses were carried out using Design-Expert 8.0, spass 20.0 version and Microsoft Excel program (2007).

Results and Discussion

Single Factor Experiment

To evaluate the effect of various factors on the extraction of total flavonoids from walnut leaves and to analyze the influences of different variables we designed the single factor experiments (Wang *et al.*, 2012).

Extraction of Total Flavonoids Influenced by Ethanol Concentration

A fundamental aspect of solvent selection is 'similar dissolution', which indicated that there is a high degree of solubility in the selected solvents (Mustafa and Turner, 2011). A mixture of ethanol and water is usually used to extract flavonoids from different herbs (Garcia-Castello *et al.*, 2015; Luthria *et al.*, 2007). The main reason is that a large amount of phenolic and flavonoids compounds could be dissolved in water and ethanol mixture (Alothman *et al.*, 2009). In addition, the important aspects of solvent selection are economy, security and sustainability. Due to the volatility of ethanol, it is a better fit polar modifier in the choice of extraction solvent. In addition, ethanol has been considered as one of the most safe and environmentally friendly solvents (Otero-Pareja *et al.*, 2015).

To study the influence of ethanol concentration on the total flavonoids extraction from walnut leaves, ethanol concentrations of 30, 40, 50, 60, 70 and 80% were used. Figure 3 indicated the influence of the ethanol concentration on the extraction yield of flavonoids, the extraction yield of flavonoids was not significantly affected by 30-60% ethanol; however, peak extraction of total flavonoids was achieved when the alcohol concentration reached 70%, then the extraction yield decreased with ethanol concentration higher than 70%.

Different products are extracted under different conditions. Because of the different chemical structure and polarity of the extracts, solvent has different extraction capacities. Existing studies have shown that the binary solvent system is better than the single-solvent system in extracting flavonoids. It was observed in this study that the optimum yield of flavonoids was obtained at 70% ethanol, which suggested that the flavonoids in walnut leaves were highly soluble in ethanol-water mixture

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and the yield difference of walnut leaves could be due to different polar and chemical constituents of flavonoids.

Extraction of Total Flavonoids Influenced by Extraction Temperature

Extraction temperature affects the movement of molecule and heat could promote the dissolution of a large number of compounds (Pompeu *et al*., 2009). In the present study, the temperatures of 30, 40, 50, 60, 70 and 80°C were selected to study temperature influence on total flavonoids extraction from walnut leaves. Figure 4 presented the influence of the extraction temperature on the extraction yield of flavonoids. When the temperature was increased from 30 to 45°C, the extraction yield increased and then the extraction yield decreased when the extraction temperature was over 45°C. The increase in molecular motion is caused by the increase in temperature, so it accelerating the dissolution of flavonoids from plant cells (Lai *et al*., 2014). As in this study, the appropriate temperature increase in plant cell decomposition and solubility helping to release flavonoids from the substances. But the temperature was too high; it may also cause the damage of the flavonoids. Similar results have also been reported for total flavonoids extraction from alfalfa (Jing *et al*., 2015). Therefore, 45°C is selected as the optimal extraction temperature.
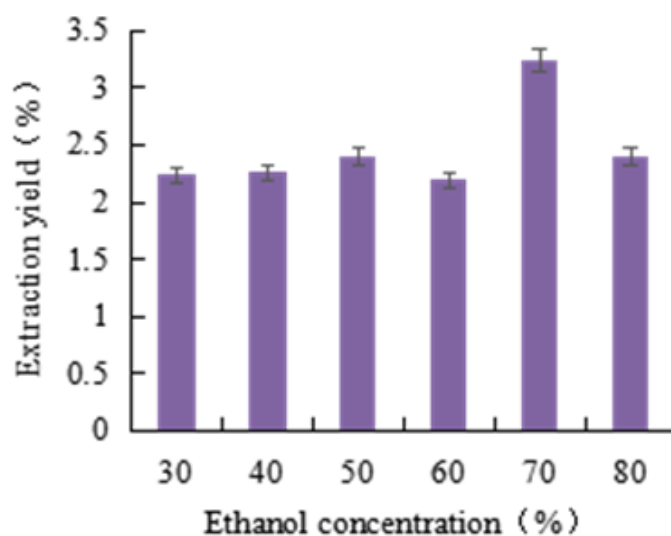

Fig. 3. Effect of the concentration of ethanol on the extraction yield of the walnut leaves
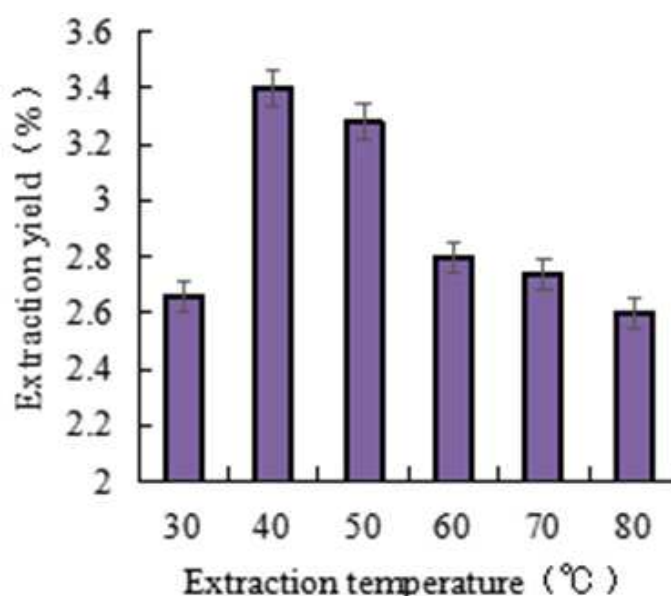

Fig. 4. Effect of the extraction temperature on the extraction yield of the walnut leaves

Extraction of Total Flavonoids Influenced by Extraction Time

The time range required of ultrasonic extraction was the third factor investigated, when the other two factors were fixed, e.g., extraction temperature was set at 45°C and ethanol concentration was set at 70%, respectively. As indicated in Fig. 5, the extraction time has a significant effect on total flavonoids and the yield increased with the increase of time and then decreased at long extraction time. The maximum yield was achieved at 30 min. The presence of different degrees of flavonoids polymerization and their interaction, may have caused this phenomenon, as the equilibrium between the bulk solution and the solution in the material being reached at different times) (Lissi *et al.*, 1999). Therefore, the optimum extraction time is 30 min.

From the above analysis, we can find that the ethanol concentration, extraction time, extraction temperature are the main factors of the preparation technology and the best extraction conditions were ethanol concentration 70%, extraction time 30 min and extraction temperature 45°C.

Data Analysis and Evaluation of RSM Model

The experiments for RSM model were conducted based on the design matrix under the defined conditions and the responses from the experimental runs were obtained by using 'design expert' (Table 2). A total of 17 runs of experiments were carried out and three individual parameters that affect the flavonoids extraction yield were optimized. Analysis of variance (ANOVE) and the resulting model regression coefficients were presented in Table 3 which demonstrated the contribution of the variable to the quadratic model. A multivariate regression equation was established and the response variable coding level of the independent variable was analyzed. The quadratic polynomial model of walnut leaves flavonoids was predicted by the least square method and the multiple regression coefficients were determined. The responses of flavonoids extraction ratio of walnut leaves were considered in studying the influence of process variable. The extraction yield of total flavonoids and independent variables of walnut leaves were studied and an empirical model was proposed (Equation 1):

$$Y\% = 3.7 - 0.069X_1 + 0.059X_2 + 0.11X_3 + 0.025X_1X_2 + 0.11X_1X_3 - 0.10X_2X_3 - 0.25X_1^2 - 0.31X_2^2 - 0.32X_3^2 \qquad (1)$$

The variance analysis of the extraction yields of the total flavonoids from the walnut leaves using Box-Behnken design was shown in Table 3. The determination coefficient (R^2) was 0.9938, which is greater than 0.8, indicating a very high correlation (Mirhosseini *et al.*, 2009). The F value and P value were 124.75 and 0.9679, respectively, which indicated the suitability of model that can accurately predict the change of variations. Based on this, the model was used to predict the response. The regression equation coefficients and p-values coefficients for total flavonoids extraction were shown in Table 3. The second-order terms of extraction time, extraction temperature and ethanol concentrations (X_1^2, X_2^2, X_3^2), one interaction parameters (X_1X_3, X_2X_3) and the first-order term of extraction time, extraction temperature and ethanol concentrations （X_1, X_2, X_3） were extremely significant with a small P value ($p < 0.01$), whereas parameters (X_1X_2) model term were significant ($p < 0.05$).
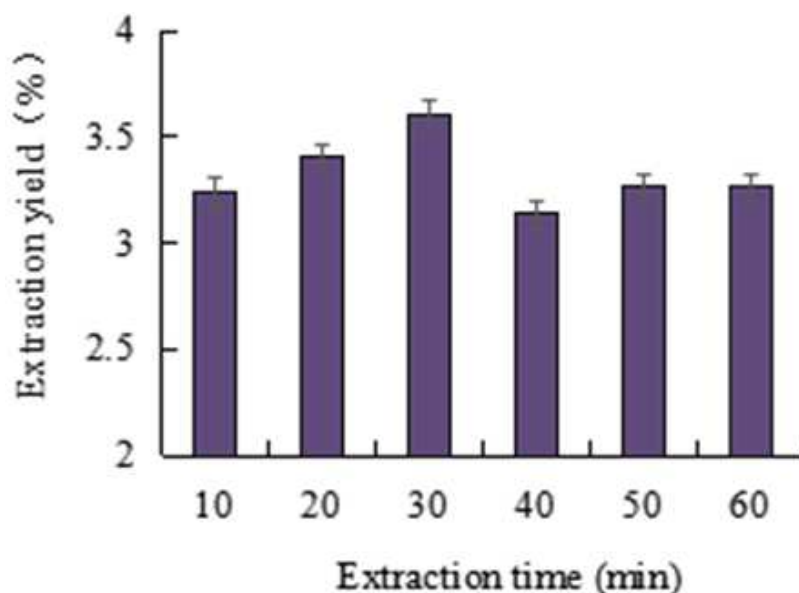

Fig. 5. Effect of the extraction time on the extraction yield of the walnut leaves

Table 2. Experimental design matrix and result of wanlut leaves extraction yield

Run	X_1 Extraction temperature (°C)	X_2 Extraction time (min)	X_3 Ethanol concentrations (%)	Extract yield (%)
1	40	40	60	3.15
2	40	20	80	3.20
3	40	20	60	2.80
4	40	30	70	3.70
5	40	30	70	3.75
6	30	40	70	3.24
7	40	30	70	3.67
8	40	40	80	3.14
9	30	20	70	3.20
10	40	30	70	3.65
11	30	30	60	3.20
12	50	40	70	3.14
13	50	30	80	3.30
14	50	20	70	3.00
15	30	30	80	3.20
16	50	30	60	2.85
17	40	30	70	3.73

Table 3. ANOVA result for the experiment response at different factor level

Source	Sum of Squares	df	F	Mean Square	P-value Value	Prob > F
Model	1.46	9	0.16	124.75	<0.0001	significant
X_1-Reaction temperature	0.038	1	0.038	29.17	0.0010	
X_2-Reaction time	0.028	1	0.028	21.30	0.0024	
X_3-Ethanol concentrations	0.088	1	0.088	68.03	<0.0001	
X_1X_2	2.500E-003	1	2.500E-003	1.93	0.2075	
X_1X_3	0.051	1	0.051	39.05	0.0004	
X_2X_3	0.042	1	0.042	32.42	0.0007	
X_1^2	0.25	1	0.25	194.95	<0.0001	
X_2^2	0.4	1	0.4	312.11	<0.0001	
X_3^2	0.42	1	0.42	327.40	<0.0001	
Residual	9.075E-003	7	1.296E-003			
Lack of fit	2.275E-003	3	7.583E-003	0.45	0.7334	not significant
Pure Error	6.800E-003	4	1.700E-003			
Cor Total	1.46	16				
R^2	0.9938					
Adj.R^2	0.9858					

Response Surface Analysis

The three dimensional response surface plots can make predictive model equations more intuitive. Therefore, the surface response plot of the model is established and the influence of independent variables on the dependent variables is visualized through the adjustment of one factor at the same time (Samimi *et al.*, 2015). In the present experiment, Fig. 6 and 7 showed the three-dimensional (3d) curved surface and two-dimensional (2d) contour plot of the experiment.

The influence of extraction temperature and extraction time on the total flavonoids extract yield was analyzed (Fig. 6a and 7a). At a definite extraction time, increasing extraction temperature resulted an increase in extraction yield. However, when extraction temperature was higher than 45°C the extraction yield was slightly decreased. It was similar for the extraction time. Therefore we can predict that the optimum extraction

time is about 30 min and optimum extraction temperature is about 45°C. In addition, the effects of the extraction time and temperature on flavonoids extraction from walnut leaves were very significant.

Figure 6b and 7b showed the 3D plot for the extraction yield of flavonoids from walnut leaves with respect to ethanol concentration and reaction temperature. With an increasing extraction temperature the extract yield slightly increased but as the temperature was exceeded 45°C the extract yield decreased extremely. When the extraction temperature was constant, the extraction yield increased with the ethanol concentration, then decreased slightly when the ethanol concentration is too high. The optimum ethanol concentration is about 70%.

The influences of extraction time and ethanol concentration on the extraction yield of flavonoids were shown in the Fig. 6c and 7c. The extraction yield increased with the increase in ethanol concentration,

however when ethanol concentration was higher than 70%, the extraction yield of flavonoids tend to be stable and slightly decreased. When the concentration of ethanol was constant, the extraction yield of total flavonoids increase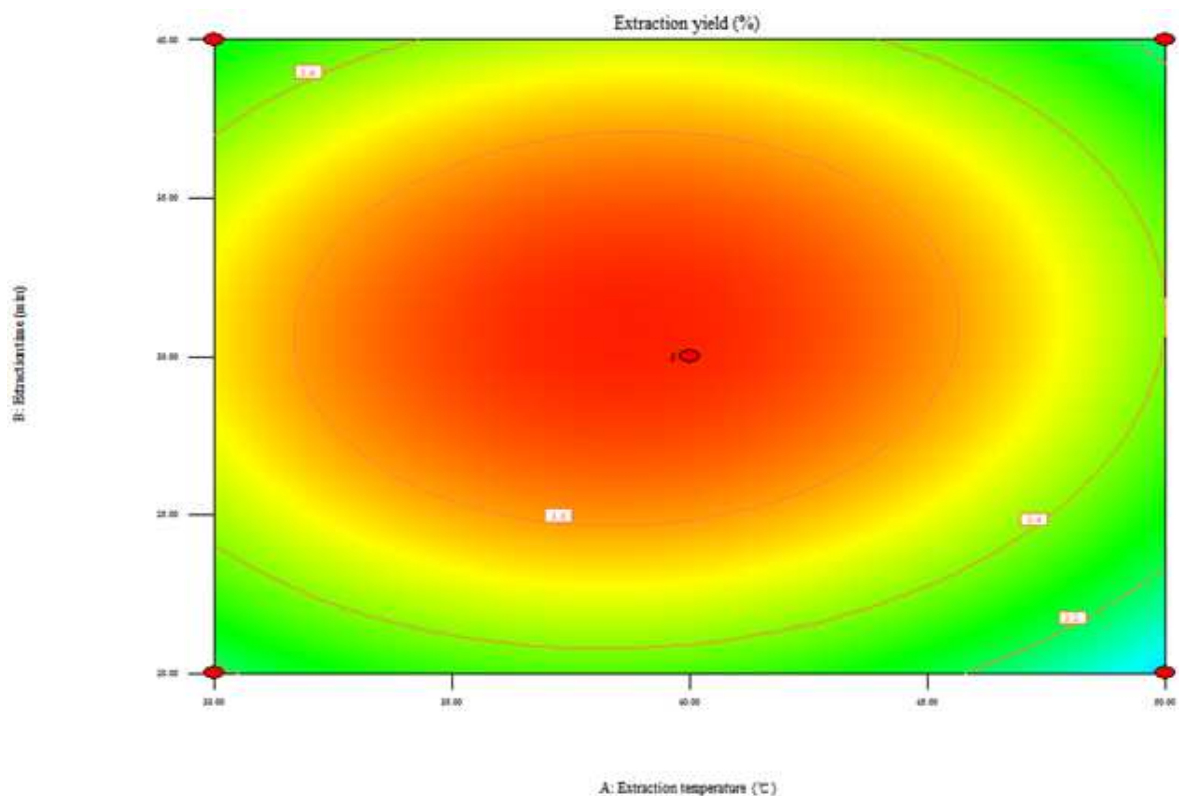d with extraction time, as extraction time reached 30 min the best extraction yield was attainted. While extract yield slightly decreased with the extraction time exceeded 30 min. We can conclude that the optimum extraction time is about 30 min and the optimum ethanol concentration is about 70%.
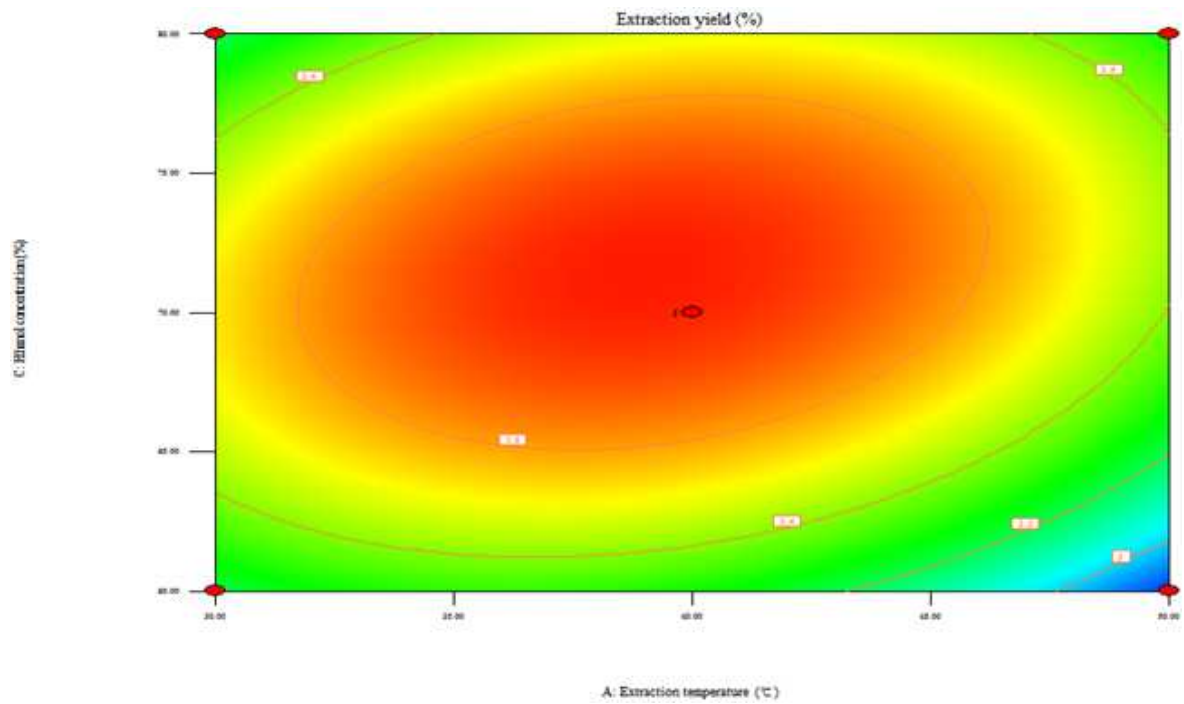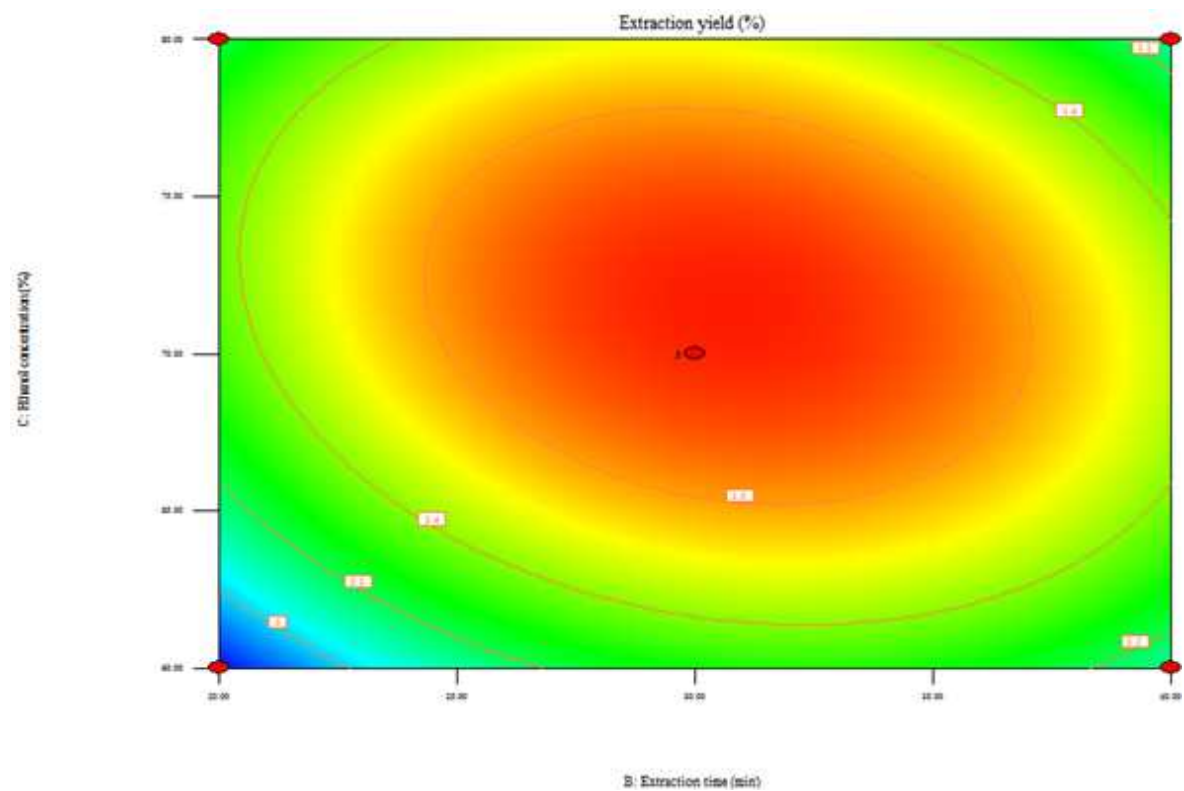

(a)

(b)

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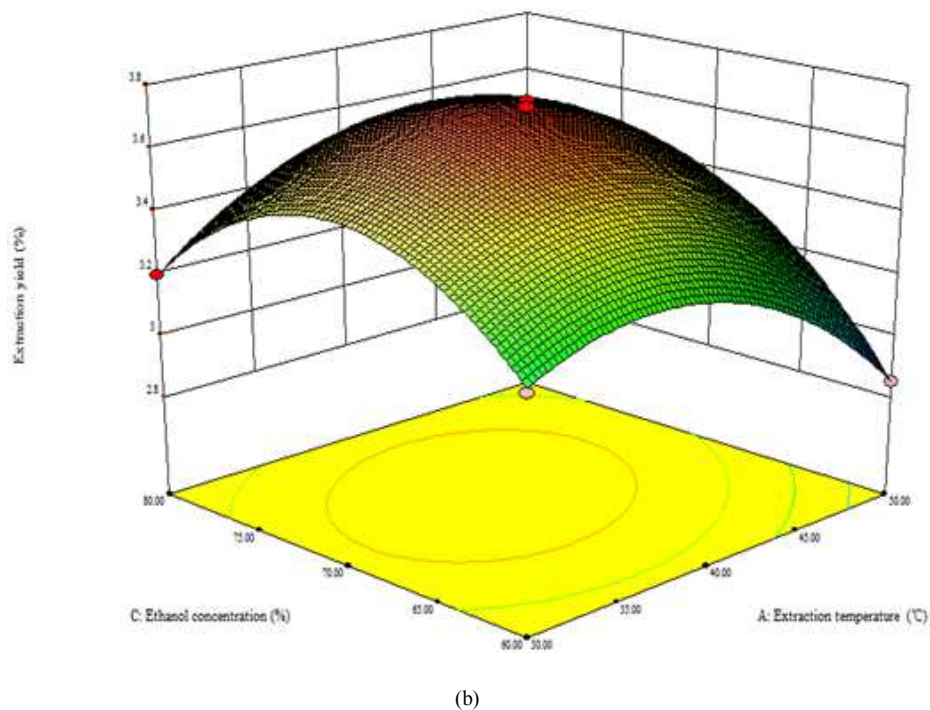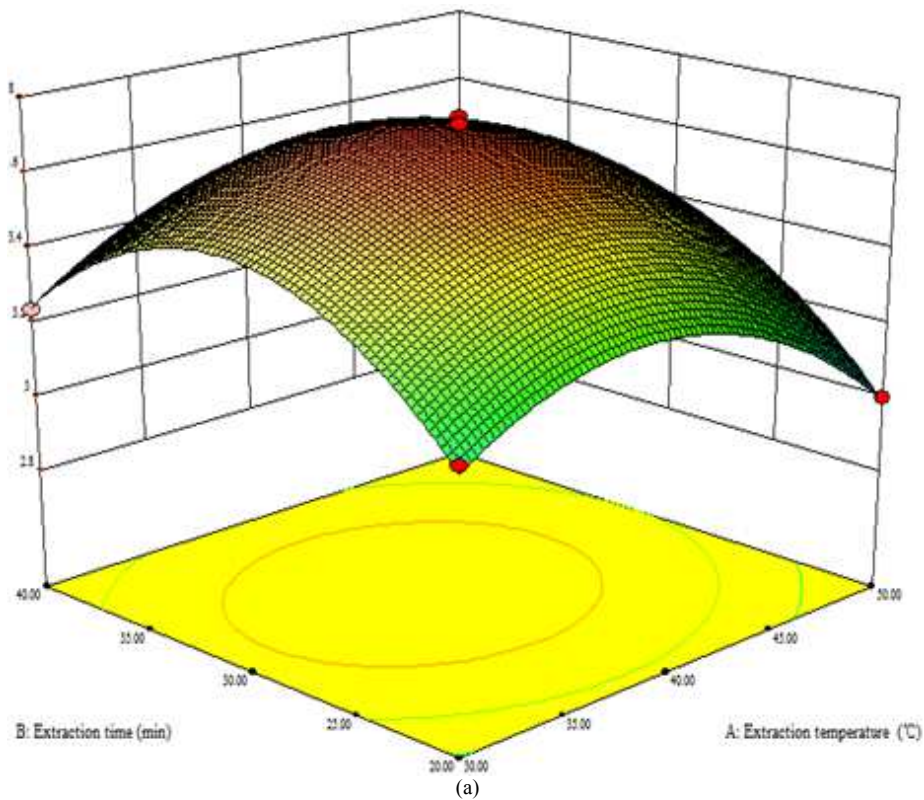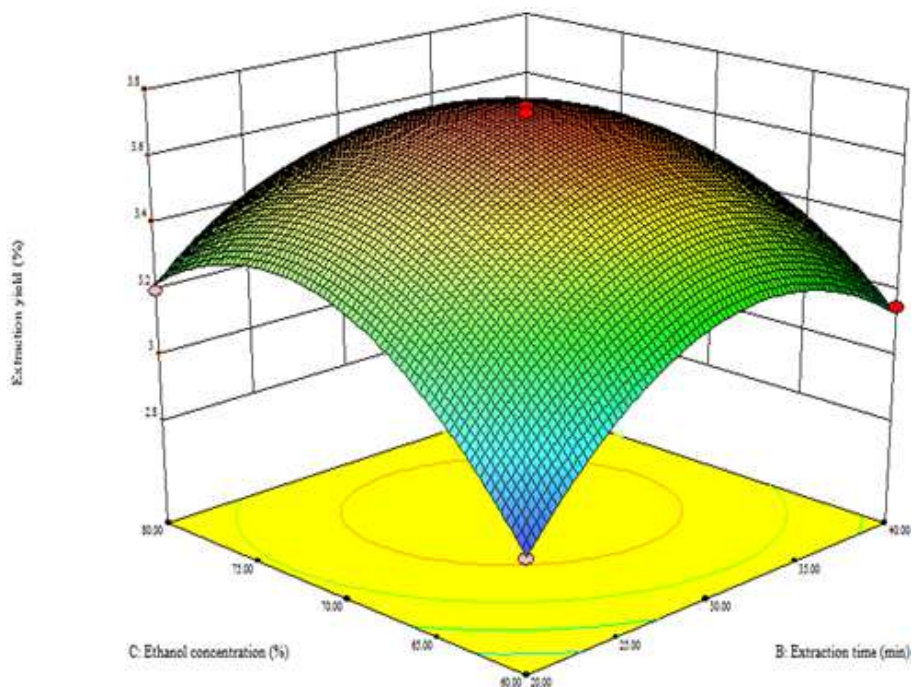
(c)

Fig. 6. 3D surface plots of the average particle size of materials at different experimental factors (a) Extraction temperature versus Extraction time (b) Extraction temperature versus ethanol concentration (c) Extraction time versus ethanol concentration

(a)

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(b)

(c)

Fig. 7. 2D contour plots of the average particle size of materials at different experimental factors (a) Extraction temperature versus Extraction time (b) Extraction temperature versus ethanol concentration (c) Extraction time versus ethanol concentration

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Table 4. Antibacterial activities of the flavonoids extracts from walnut Leaves

Zone of inhibition (mm)

Extraction concentration (mg/ml)	*Salmonella typhi*	*Escherichia col*	*Bacillus subtilis*	*Staphylococcus aureus*
0	—	—	—	—
100	11.3±0.2	20.69±0.1	14.73±0.2	22.32±0.3

Table 5. Minimum Inhibitory Concentration of the flavonoids extracts from walnut leaves

Zone of inhibition (mm)

Extraction concentration (mg/ml)	*Salmonella typhi*	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*
0	—	—	—	—
3.125	—	—	—	—
6.25	—	—	—	—
12.5	—	10.35±0.1	—	11.32±0.2
25	—	13.23±0.3	9.76±0.35	15.3±0.2
50	8.75±0.4	15.57±0.2	11.57±0.3	17.98±0.1
100	11.3±0.2	20.69±0.3	14.73±0.2	22.32±0.35

The Antibacterial Activity Analysis

Antimicrobial activities of the flavonoids extracts from walnut leaves were tested against selected microorganisms (Table 4). The results indicated that the flavonoids obtained from walnut leaves have antibacterial activity at 100mg/mL^{-1} and the bacterial inhibition ring of flavonoids was measured. It indicated that the flavonoids have best antimicrobial activity against *Staphylococcus aureusm*, followed by *Escherichia coli* and then *Salmonella typhi*.

MIC of the extracts recorded was in the range of 12.5-50 mg/mL (Table 5). In this investigation the MIC value of 12.5 mg/mL was recorded for *Escherichia coli* and *Staphylococcus aureus*. Whereas, MIC for *Bacillus subtilis* was 25 mg/mL and for *Salmonella typhi* was 50mg/mL, indicating that the walnut leave extracts have significant antimicrobial potential. Because the MIC of the extracts were very small, indicating they were highly efficient. An overview of the biological activity data obtained from the current survey can emphasize that the tested extracts have great potential for inhibiting bacteria. *Staphylococcus aureus* is of considerable importance because it is considered to be one of the main pathogens of many hospitals and community infections.

Conclusion

Through this study, RSM was applied in optimizing the total flavonoids compounds extraction from walnut leaves and it was also showed that the UAE is a valid method for obtaining flavonoids from walnut leaves. The maximum extraction yield of 3.53% was achieved at temperature of 47.73°C, ethanol concentration of 72.89% and extraction time of 30.79 min. Then, the extracts of flavonoids were used to determine their antibacterial activity. The results showed that the flavonoids extracted from walnut leaves have comparatively significant antibacterial activities with good MIC values. Our results showed that walnut leaves can be a potential source of important bioactive compounds and play an important role in controlling the growth of disease-causing bacteria but further phytochemical analysis is needed to identify the extract types of compounds that presented in walnut leaves.

Acknowledgment

Author's Contribution

Jingjing Fu: Performed the bench work and drafted the manuscript.

Yuanda Song: Supervised the work, reviewed and revised the manuscript.

Haifang Xiao: Participated in data analysis.

Shaoxuan Yu: Reviewed the manuscript.

Han Wu: Involved in antibacterial assay.

Conflicts of Interest

The authors declare no conflict of interest.

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