Note

Response Surface Optimization of Extraction and Antioxidant Activity of Total Flavonoids from Seed Shell of *Juglans mandshurica*

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The seed shell of *Juglans mandshurica* is a waste product from seed kernel processing in the food industry. For the first time, the optimal conditions of ultrasonic-assisted extraction for total flavonoids from seed shell of *Juglans mandshurica* (TFSSJM) were investigated and the antioxidant activity of TFSSJM was analyzed. Box-Behnken Design (BBD) combined with response surface methodology (RSM) was used. The optimal conditions were obtained as flows: ultrasonic power 250 W, ultrasonic time 31.2 min and solid-liquid ratio 1:31.4 (g/mL). The flavonoids yield was 6.62 mg/g under these optimal conditions. And TFSSJM had better capability of scavenging ABTS, DPPH and hydroxyl radical than BHT, there was dose effect relationship. IC\(_{50}\) of ABTS and hydroxyl radical-scavenging was 33 µg/mL and 0.762 mg/mL respectively. The results indicated the ultrasonic-assisted method could be used as an effective and feasible method to extract total flavonoids from seed shell of *Juglans mandshurica*, and TFSSJM had excellent antioxidant activity, which can be used as a potential source of natural antioxidant.

Keywords: response surface, flavonoids, *Juglans mandshurica*, seed shell, antioxidant activity

Introduction

Nowadays, nature and health have been the main lifestyle we seek. Thus, a great deal of attention has been focused on natural bioactive agents and their function (Jacquemin et al., 2010; Pezzuto, 1997). And plant-derived flavonoids were one of these natural bioactive agents (Chidambara Murthy et al., 2012; Wang et al., 2011). Flavonoids are widely present in many plants, such as fruits, vegetables, grains, flowers, tea and some medicinal plants, herb (Peterson and Dwyer, 1998). They have a wide spectrum of biological activities including antioxidant, antiviral, antiplatelet, anti-mutagenic, anti-inflammatory and antitumor activities, and have been attracted much attention in both food and medicine areas (Hodek et al., 2002; Tripoli et al., 2007). Some reports also showed that biological activity of flavonoids is mainly attributed to their antioxidant properties (Duffy et al., 2008; Hughes, 2008; Pham-Huy et al., 2008).

*Juglans mandshurica*, belonging to Juglandaceae, is a fast-growing walnut tree native to many Asian countries, especially in China. This plant has been used as folk and traditional medicines for the treatment of cancer in some countries (Graham et al., 2000; Machida et al., 2009). Some research also showed that *Juglans mandshurica* had marked effects in many aspects. Flavonoids such as taxifolin and quercetin were found in roots, leaves, stem-barks and fresh unripe fruits of this plant (Li et al., 2003; Min et al., 2002; Park et al., 2012). But the study on flavonoids in the seed shell of *Juglans mandshurica* is not reported. *Juglans mandshurica* seed is the byproducts of forestry. And its kernel has recently received a great deal of attention because of rich nutrition. During the processing kernel, a lot of seed shells were produced as garbage. Currently besides some seed shells were made into...
handicraft products or used as fuel woods, many of them were discarded leading to waste. For the better utilization of seed shell of *Juglans mandshurica*, more research of composition and bioactivity are required about it. Moreover, agricultural and industrial residues have become focused as materials to obtain antioxidants (Bagchi et al., 2000; Graham et al., 2000; Guangyan Pan, 2012; Lafka et al., 2007).

In order to prepare the bioactive compounds, currently, various new extraction techniques have been utilized including microwave-assisted extraction (Ballard et al., 2010), ultrasonic-assisted extraction (Lin et al., 2013), supercritical fluid extraction (Sovová, 2012) and accelerated solvent extraction (Hossain et al., 2011). Compared with conventional extraction techniques as heating, boiling, or refluxing and above other extraction techniques, ultrasonic-assisted extraction is a much easier, inexpensive and efficient alternative procedure with lower temperature (Huang et al., 2009; Tian et al., 2013), which can prevent possible degradation and loss of target compounds. The effects of mechanical vibration and cavitation in the solvent can break cell walls mechanically and improve material transfer during the ultrasonic-assisted extraction. Therefore, ultrasonic-assisted extraction has been used in preparing many natural products including polyphenol, flavonoids and alkaloid (Wang and Weller, 2006).

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Baş and Boyacı, 2007). The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interaction (Khuri and Mukhopadhyay, 2010; Yin et al., 2011). Therefore, it is less laborious and time-consuming. RSM has been widely applicable for different purposes in chemical, biochemical, engineering processes and industrial research.

Hence, the aim of the present study is to select and validate the optimum conditions of ultrasonic-assisted extraction for total flavonoids from seed shell of *Juglans mandshurica* using Box-Behnken design (BBD) with RSM. Moreover the antioxidant activities of the extract were evaluated in vitro for the first time, including ABTS, DPPH and hydroxyl radical scavenging effects. We hope this study will be helpful to further provide bioactivity information about flavonoids from seed shell of *Juglans mandshurica* for development and application of the resource.

**Materials and Methods**

*Materials and instruments* *Juglans mandshurica* seeds were provided by Forest Enterprise of Mudanjiang in Heilongjiang Province (China). Rutin was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). ABTS (2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH(2, 2-Di(4-tert-octylphenl)-1-pierylhydrazyl) and BHT (2,6-Di-tert-butyl-4-methylphenol) were purchased from Sigma Chemicals Co. (Shanghai, China). VC was purchased from Tianjin Kermel Chemical Reagent Co. (Tianjin, China). All other chemical reagents used in experiments were of analytical grade.

Ultrasonic cleaner (KQ-500DE, Kunshan Ultrasonic Instrument Co. Jiangsu, China) was used for ultrasonic-assisted extraction of total flavonoids, TU-1810 spectrophotometer (Persee Universal Instrument Co., Beijing, China) for analyzing total flavonoids content and antioxidant activity, RE-52A rotavapour (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) for concentration of sample. The kernels of seeds were taken out, and its shells were pulverized in a knife mill and passed through a 60-mesh sieve. The powder (2.5 g) of seed shell was weighed accurately and loaded into a 200-mL extraction vessel with ethanol solvent for 1h at room temperature. Total flavonoids were extracted by Ultrasonic-assisted extraction with different ethanol concentration (30%, 40%, 50%, 60%, 70%, 80% 90%) with a solid-liquid ratio (g/mL) (ranging from 1:10 to 1:70) for a given time (ranging from 10 to 70 min), while extraction power ranged from 200 to 500 W and the temperature of the water bath was kept 55°C. The extract were filtrated, the solution was collected and added to a defined volume in a volumetric flask (100 mL) for further analysis. Total flavonoids yield and reducing power were chosen as the indexes of the single-factor experiment.

**Box-Behnken Design (BBD)** On the basis of the single-factor experimental results, major influencing factors were confirmed. And BBD was applied to evaluate the main and interaction effects of the factors in the experimental region: ultrasonic power (**X**), ultrasonic time (**X**), solid-liquid ratio (**X**), on total flavonoids yield (**Y**) obtained. The independent variables were coded at three levels (−1, 0, 1), and the complete design consisted of 15 experimental points including three replications of the center points (all variables were coded as zero). The actual and coded levels of the independent variables used in the experimental design are shown in Table 1. In the research, a second order polynomial Eq. (1) was used to describe the effects of variables on the response:

\[
Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ij} X_i X_j \quad \text{----- Eq. 1}
\]

Where **Y** is the predicted response, \(\beta_0\), \(\beta_i\), \(\beta_{ij}\) and \(\beta_{ij}\) are the regression coefficients for intercept, linearity, square and interaction, respectively, while \(X_i\) and \(X_j\) are coded the independent variables. All the experiments were repeated three times, and the flavonoids yields were given as average values.

**Determination of total flavonoids content** The total flavonoids content was determined using a Sodium nitrite and Aluminum chloride colorimetric method described previously (Gong et al., 2012) and slightly modified. Briefly, 2 mL of sample was placed in a 10 mL volumetric flask, 1 mL ethanol/water (60:40 v/v) and 0.4 mL of sodium nitrite (5 g/100 mL) were added and mixed. 0.4 mL of aluminium chloride solution (10 g/100 mL) was added...
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after 6 min. 6 min later, 4 mL of sodium hydroxide solution (1 mol/L) was added, and the total volume was made up to 10 mL with ethanol/water (60:40 v/v) and mixed. After 15 min, the absorbance was measured against a blank at 510 nm. The total flavonoids content was calculated from a calibration curve of rutin and expressed as rutin equivalent (mg/g dry basis). The calibration curve \( Y = 13.536X - 0.0005 \), where \( Y \) is absorbance value of sample, \( X \) sample concentration) ranged 0 – 300 µg/mL (\( R^2 = 0.9994 \)).

The flavonoids yield of ultrasonic-assisted extraction was calculated using the following formula (1):

\[
\text{Flavonoids yield (mg/g)} = \frac{CNV}{W}
\]

Where \( C \) is mass concentration of the sample fluid calculated through calibration curve of rutin, mg/mL, \( N \) is dilution, \( V \) is extract volume, mL, \( W \) is quantity of powder, g.

**Determination of reducing power** The determination of reducing power was performed as described (Zhang *et al.*, 2011) and slightly modified. 2 mL extract sample (varying concentration) was mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). After the mixture was incubated at 50°C for 20 min, and then the mixture was quickly chilled in ice bath, trichloroacetic acid (2.5 mL, 10%) was added, and the mixture was centrifuged at 4000 rpm for 10 min (TGL-16G Centrifuge, Shanghai Anting scientific Instrument Factory, Shanghai, China). The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%) for 10 min, and then the absorbance was measured at 700 nm against a blank. Increasing absorbance of the reaction mixture indicated increasing reducing power.

**Determination of total flavonoids antioxidant activity** The total flavonoids were extracted using the optimal conditions by RSM and named TFSSJM. Its antioxidant activity was investigated by ABTS, DPPH and Hydroxyl radical scavenging activity.

a) ABTS radical-scavenging activity

The activity of TFSSJM to scavenge ABTS\(^+\) was carried out according to the method (Gülsen Tel, 2012) with slight modifications. ABTS\(^+\) was prepared by mixing an ABTS\(^+\) stock solution (7.4 mmol/L in water) with 2.6 mmol/L potassium persulfate. This mixture was stored at room temperature in the dark for 16 h. Before usage, ABTS\(^+\) stock solution was diluted to get an absorbance of 0.700 ± 0.020 at 734 nm with methanol (MeOH). And then 0.15 mL of the appropriately diluted sample was added to 2.85 mL of ABTS\(^+\) working solution and mixed thoroughly. The reaction mixture was kept at room temperature in the dark for 10 min, and then the absorbance was recorded at 734 nm. The absorbance of reagent control was measured by the same way in each time.

The radical scavenging activity was calculated using the following formula (2):

\[
\text{Radical scavenging activity \%} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (2)
\]

Where \( A_{\text{sample}} \) is the absorbance of the reaction mixture, \( A_{\text{control}} \) is the absorbance of the reaction mixture without sample solution. VC and BHA were used as antioxidant standards for comparison of the activity.

b) DPPH radical scavenging activity

The activity of TFSSJM to scavenge DPPH radical was carried out according to the method (Gülsen Tel, 2012) with slight modifications.

DPPH stock solution (0.15 mmol/L) was prepared with ethanol and stored in the dark. Before usage, it was diluted to get an absorbance of 0.700 ± 0.020 at 734 nm with ethanol. And then 2 mL of the appropriately diluted sample was added to 2 mL of DPPH working

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**Table 1. Factors and levels in Box–Behnken Design arrangement and experimental results**

<table>
<thead>
<tr>
<th>Run</th>
<th>( X_1 ) Ultrasonic power (W)</th>
<th>( X_2 ) Ultrasonic time (min)</th>
<th>( X_3 ) Solid-liquid ratio (g/mL)</th>
<th>Flavonoids yield (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1(200)</td>
<td>1(20)</td>
<td>0(1:30)</td>
<td>5.687</td>
</tr>
<tr>
<td>2</td>
<td>1(300)</td>
<td>1(20)</td>
<td>0(1:30)</td>
<td>5.952</td>
</tr>
<tr>
<td>3</td>
<td>1(200)</td>
<td>1(40)</td>
<td>0(1:30)</td>
<td>5.888</td>
</tr>
<tr>
<td>4</td>
<td>1(300)</td>
<td>1(40)</td>
<td>0(1:30)</td>
<td>6.028</td>
</tr>
<tr>
<td>5</td>
<td>1(200)</td>
<td>0(30)</td>
<td>1(1:20)</td>
<td>5.782</td>
</tr>
<tr>
<td>6</td>
<td>1(300)</td>
<td>0(30)</td>
<td>1(1:20)</td>
<td>6.008</td>
</tr>
<tr>
<td>7</td>
<td>1(200)</td>
<td>0(30)</td>
<td>1(1:40)</td>
<td>6.108</td>
</tr>
<tr>
<td>8</td>
<td>1(300)</td>
<td>0(30)</td>
<td>1(1:40)</td>
<td>6.393</td>
</tr>
<tr>
<td>9</td>
<td>0(250)</td>
<td>1(20)</td>
<td>1(1:20)</td>
<td>5.447</td>
</tr>
<tr>
<td>10</td>
<td>0(250)</td>
<td>1(40)</td>
<td>1(1:20)</td>
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<td>1(40)</td>
<td>1(1:40)</td>
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<td>1(40)</td>
<td>1(1:40)</td>
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</tr>
<tr>
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<td>0(30)</td>
<td>0(1:30)</td>
<td>6.593</td>
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<tr>
<td>14</td>
<td>0(250)</td>
<td>0(30)</td>
<td>0(1:30)</td>
<td>6.888</td>
</tr>
<tr>
<td>15</td>
<td>0(250)</td>
<td>0(30)</td>
<td>0(1:30)</td>
<td>6.717</td>
</tr>
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</table>
solution and mixed thoroughly. The reaction mixture was kept at room temperature in the dark for 30 min, and then the absorbance was recorded at 517 nm. The absorbance of reagent control and sample solution was measured by the same way in each time.

The radical scavenging activity was calculated using the following formula (3):

\[
\text{DPPH Radical-scavenging activity } \% = 1 - \left( \frac{A_1 - A_2}{A_{\text{control}}} \right) \times 100
\]  

Where \(A_1\) is the absorbance of the reaction mixture, \(A_2\) is the absorbance of the reaction mixture without DPPH solution, \(A_{\text{control}}\) is the absorbance of the reaction mixture without sample solution. VC and BHA were used as antioxidant standards for comparison of the activity.

\(c)\) Hydroxyl radical scavenging activity

The hydroxyl radicals scavenging activity was evaluated using the hydroxyl radical system generated by the Fenton reaction, according to the method (Zhao et al., 2012) with slight modification. 1 mL of the appropriately diluted sample was added to the mixture of \(\text{FeSO}_4\cdot\text{7H}_2\text{O}(2\text{ mL}, 6\text{ mmol/L})\) and \(\text{H}_2\text{O}_2(2\text{ mL}, 6\text{ mmol/L})\), mixed and then placed for 10 min. Salicylic acid (2 mL, 6 mmol/L) was added, the mixture was at 37°C water bath for 30 min, and then was centrifuged at 4000 rpm for 10 min (TGL-16G Centrifuge, Shanghai Anting scientific Instrument Factory, Shanghai, China). The absorbance of the supernatant was measured at 510 nm. The radical scavenging activity was calculated as indicated in the ABTS method.

Statistical analysis All analyses were performed in triplicate. The experimental results obtained were expressed as means ± SD. Statistical analysis was performed using the software Minitab 15.0. Data were analyzed by analysis of variance (ANOVA) and \(P < 0.05\) was regarded as significant.

Results and Discussion

The effects of the main parameters on flavonoids yield and reducing power Before the optimised experiment, the effects of ultrasonic-assisted extraction parameters involving ultrasonic time, ultrasonic power, ethanol concentration and solid-liquid ratio on flavonoids yield and reducing power were investigated and the results were showed in Fig. 1.

It can be seen from Fig. 1A that flavonoids yield and reducing power started to increase with increasing ultrasonic time, and reached a maximum at 30 min, followed by a decrease with further increase in the ultrasonic time, and reducing power decreased more quickly. These may be made by too much ultrasonic time results in changes of composition and loss of activities. Therefore, 30 min was determined as the best ultrasonic time.

It can be seen from Fig. 1B that flavonoids yield and reducing power started to increase with increasing ultrasonic power, and reached a maximum at 250 W, followed by a decrease with further increase in the ultrasonic power and reducing power decreased more quickly. These may be made by too stronger ultrasonic effect results in changes of composition and loss of activities. Therefore, 250 W was determined as the best ultrasonic power.

In Fig. 1C, it was noticed that flavonoids yield and reducing power started to increase with increasing ethanol concentration, and flavonoids yield were similar at ethanol concentration of 40%, 50% and 60%, but reducing power reached a maximum at 60%, followed by both flavonoids yield and reducing power quickly decreased with further increase in the ethanol solution concentration. These may be made because different ethanol concentrations had different polarities. Lower ethanol concentration yielded stronger polarity of the solution, which favored the extraction of polar compounds. On the contrary, higher ethanol concentration was suitable for the slightly polar compounds. And during experiment, the extracts using lower ethanol concentration were filtrated difficultly. Hence, ethanol concentration of 60% was determined as the best solvent.

In Fig. 1D, there was a significant increase in both flavonoids yield and reducing power with increasing solid-liquid ratios from 1:10 to 1:30, afterwards, the further increase of solid-liquid ratio led to a slight increase in both flavonoids yield and reducing power. It is because that the increase of solvent volume would increase interfacial area between tiny bubbles and samples, the cavitation effect of bubbles collapse would be more intense. While considering the cost of experiment and further works, 1:30 was determined as the best solid-liquid ratio.

In this section, ultrasonic power, ultrasonic time, ethanol concentration and solid-liquid ratio were analyzed by single-factor experiment. It is indicated that different factors had different effects on flavonoids yield and their reducing power. Ultrasonic power, ultrasonic time and solid-liquid ratio had stronger effects on flavonoids yield and reducing power than other factors. Hence, the three factors were chosen for further optimization in BBD test.

Analysis of BBD results

a) Model fitting

According to the values obtained in the single-factor experiment, three significant factors were subjected to BBD with RSM. Experiments were randomized and the results were shown in Table 1.

The experimental data were analyzed using Minitab 15.0 software for statistical analysis of variance (ANOVA), regression coefficients and regression equation. And the following second-order polynomial stepwise equation was yielded as Eq. (2):

\[
\begin{align*}
Y = & 6.73293 + 0.11456X_1 + 0.11709X_2 + 0.13766X_3 - 0.38320X_1^2 - 0.46086X_2^2 - 0.27702X_3^2 - 0.03112X_1X_2 \\
& + 0.01510X_1X_3 - 0.28526X_2X_3 \quad \cdots \cdots \text{Eq. 2}
\end{align*}
\]

The significance of each coefficient was determined using the T-test and p-value and shown in Table 2.

As show in Table 2, the coefficient of determination (\(R^2\)) was the proportion of variability in the data explained or accounted for by the model. The \(R^2\) of 96.49% was therefore desirable (Baş and Boyacı, 2007).

The linear variables \(X_1, X_2 \) and \(X_3\) had significant influences (\(P < 0.05\)) on the flavonoids yield, the quadratic variables \(X_1^2, X_2^2 \) and \(X_3^2\) were statistically very significant (\(P < 0.01\), two-variable interaction
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Fig. 1. Flavonoids Yield and reducing power during the evaluation of the following ultrasonic-assisted extraction parameters: (A) Ultrasonic time (min), (B) Ultrasonic power (W), (C) Ethanol concentration (%), (D) Solid-liquid ratio (g/mL). (The results were expressed as the means ± SD, n = 3).
$X_2X_4$ had significant influences ($P<0.05$) on the flavanoids yield, whereas the two-variable interaction $X_2X_5$ and $X_3X_4$ had no significant influence ($P > 0.05$) on the flavanoids yield. By observing linear and quadratic coefficients, we concluded that the order of factors influencing the response value of the flavonoids yield was as follows: solid-liquid ratio > ultrasonic time > ultrasonic power.

ANOVA was used to analyze the model for significance and suitability, and the results were given in Table 3.

As shown in Table 3, the model F-value of 15.25 with a low probability P-value (p < 0.01), indicated high significance of the model. And lack of fit P-value was 0.722, $p > 0.05$, indicated no significance of lack of fit. Lack of fit was bad, so the model was significant (Khuri and Mukhopadhyay, 2010). These results showed that the build model could describe the relationship between the independent coded variables and response value, and it was desirable.

b) Response surface analysis

To investigate the interactive effects of operational parameters on flavonoids yield, three-dimensional (3D) response surfaces plots and two-dimensional (2D) isograms were presented in Fig. 2 and Fig. 3 for independent variables (ultrasonic power, ultrasonic time, solid-liquid ratio), respectively, when the other parameter was kept constant, which indicated the changes in flavonoids yield under different conditions. From these 3D and 2D plots, it is easy to see the interactions between any two factors.

The combined effect of ultrasonic power and ultrasonic time on the flavonoids yield was shown in Fig. 2A and Fig. 3A, it revealed that at low and high levels of the ultrasonic power and ultrasonic time the flavonoids yield was minimal. Increase of ultrasonic power from 200 W to 255 W with the increase of ultrasonic time from 20 min to 31 min, the flavonoids yield increased. The flavonoids yield reached the maximum when ultrasonic power and ultrasonic time were up to a certain value, respectively. However, their further increase appeared to be disadvantaged. The same trend can be seen from Fig. 2B, 3B, 2C and 3C. As the ultrasonic power, ultrasonic time and solid-liquid ratio increased, the flavonoids increased to a maximal value and then decreased. And it can be seen that none of the interactive effects of ultrasonic time and ultrasonic power, ultrasonic power and solid-liquid ratio excepting for ultrasonic time and solid-liquid ratio. These results are in accordance with the results obtained in Table 2.

c) Optimization and verification

By computation, the optimal conditions to obtain the highest flavonoid yield of seed shell of Juglans mandshurica were determined as follows: ultrasonic power 255.7 W, ultrasonic time 31.2 min and solid-liquid ratio:1:31.4 (g/mL), the flavonoids yield of the model prediction was 6.70 mg/g. For considering the practical operation, adjusted extraction conditions were as follows: ultrasonic power 250 W, ultrasonic time 31.2 min and solid-liquid ratio:1:31.4 (g/mL). To further test the reliability of the experimental method, verification experiments were carried out for three times under these optimal conditions. The resulting mean flavonoids yield was 6.62 mg/g with relative standard deviation (RSD) 1.47%. The result showed that the experimental values were consistent with the predictive values.
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Therefore, the extraction conditions obtained by RSM were effective, reliable and feasible.

**Antioxidant activity analysis of TFSSJM**  Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, and binding of transition metal ion catalysts (Zhao *et al*., 2012). Some reports showed that the flavonoids exhibited extensive free radical scavenging activities through their reactivity as hydrogen or electron-donating agents, and metal ion chelating properties (Erlund, 2004; Fraga *et al*., 1987; Heim *et al*., 2002; Pham-Huy *et al*., 2008). The antioxidant activity of TFSSJM was analyzed by ABTS, DPPH and Hydroxyl radical scavenging test.

a) **ABTS radical-scavenging activity**

ABTS$^-$ often is used to evaluate the total antioxidant activity of natural product (Kim and Son, 2011). The scavenging activity of TFSSJM on ABTS$^-$ were investigated and the results were shown in Fig. 4.

As show in Fig. 4, the scavenging activity increased with TFSSJM concentration increasing. Its IC$_{50}$ was 33 µg/mL. But TFSSJM exhibited lower activity of ABTS radical-scavenging compared with VC. The scavenging percentage of VC has been reached 98.22% at 40 µg/mL concentration. However, TFSSJM exhibited much higher activity of ABTS radical-scavenging compared with BHT. At concentration of 100 µg/mL, the scavenging percentage of TFSSJM on ABTS$^-$ was 96.26%, but latter was only 40.97%. The result showed that TFSSJM had a significant activity of ABTS radical-scavenging.

b) **DPPH radical-scavenging activity**

DPPH radicals-scavenging is considered a good in vitro model widely used to assess antioxidant efficacy (Rao *et al*., 2010). The scavenging activity of TFSSJM on DPPH radical was investigated and the results were shown in Fig. 5.

As show in Fig. 5, the scavenging capability increased with TFSSJM concentration increasing. But TFSSJM exhibited lower capability of DPPH radical-scavenging compared with VC. The scavenging percentage of VC has been reached 80% at 1.5 mg/mL concentration. However, TFSSJM exhibited higher capability of DPPH radical-scavenging compared with BHT. At concentration of 2.5 mg/mL, the scavenging percentage of TFSSJM on DPPH radical was 48.37%, but latter was only 14.11%. These results indicated that TFSSJM had a better activity of DPPH radical-scavenging.

c) **Hydroxyl radical-scavenging activity**

Hydroxyl radicals are considered to cause the ageing of human body and some diseases (Siddhuraju and Becker, 2007), interact with the purine and pyrimidine bases of DNA as well as, leading to the formation of sulphur radicals able to combine with oxygen to generate oxysulphur radicals, a number of which damage biological molecules (Huang *et al*., 2009). The scavenging activity of TFSSJM on Hydroxyl radical was investigated and the results were shown in Fig. 6.

As show in Fig. 6, the scavenging capability increased with TFSSJM concentration increasing. Its IC$_{50}$ was 0.762 mg/mL. But TFSSJM exhibited lower capability of hydroxyl radical-scavenging compared with VC. The scavenging percentage of VC has been reached 99.87% at 2.5 mg/mL concentration. However, TFSSJM exhibited higher capability of hydroxyl radical-scavenging compared with BHT. At concentration of 4.5 mg/mL, the scavenging percentage of TFSSJM on hydroxyl radical was 66.29%, but latter was only 46.97%. These results indicated that TFSSJM had a significant activity of hydroxyl radical-scavenging.

Above data demonstrated that TFSSJM could scavenge ABTS, DPPH and hydroxyl radical. Although the antioxidant activity of TFSSJM falled behind VC, it was better than BHT.

The results studied will possibly cause interest in utilizing
flavonoids from seed shell of *Juglans mandshurica* as an inexpensive source of health-promoting additives, e.g. natural antioxidants. To identify the component of flavonoids from seed shell of Juglans mandshurica and evaluate other bioactivities, further study is in progress.

Conclusions

The extraction condition and antioxidant activity of total flavonoids from seed shell of *Juglans mandshurica* were investigated for the first time. The ultrasonic-assisted extraction could be used as an effective, reliable and feasible method to extract total flavonoids from
seed shell of \textit{Juglans mandshurica}. The optimal conditions were obtained by RSM as flows: ultrasonic power 250 W, ultrasonic time 31.2 min and solid-liquid ratio 1:31.4 (g/mL). In addition, another main finding of this work was the fact that TFSSJM could scavenge ABTS, DPPH and hydroxyl radical, showed excellent antioxidant activity. Thus, flavonoids from seed shell of \textit{Juglans mandshurica} may be beneficial to the antioxidant protection system in food industry even in human body against oxidative damage.

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\textbf{Conflict of Interest} The authors declare no conflict of interest.

\textbf{References}


