Optimization of ultrasonic extraction of polysaccharides from *Hovenia dulcis* peduncles and their antioxidant potential

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**A B S T R A C T**

An ultrasonic-assisted extraction of polysaccharides from the ripe peduncles of *Hovenia dulcis* (HDPs) was investigated. Response surface methodology along with a Box–Behnken design based on single-factor experiments was employed to optimize and model the extraction conditions of HDPs, namely extraction temperature (40–60 °C), ultrasonic power (320–480W) and extraction time (35–65 min). The optimized conditions were extraction temperature 60 °C, ultrasonic power 362W and extraction time 65 min. Under these conditions, the maximal yield of crude HDPs was 25.12 ± 0.145 mg/g DW, which is consistent with the predictive yield of 25.33 mg/g DW. The polysaccharides were graded by an ethanol precipitation method and three fractions (HDPs1, HDPs2 and HDPs3) were harvested. These had final ethanol concentrations of 40%, 60% and 80%, respectively and were acidic polysaccharides. The preliminary characterization was mainly composed of Ara, Rha, Glu and Gal, and exhibited an almost similar characteristic absorption peak by gas chromatograph and infrared spectra analysis. The antioxidant activity assays *in vitro* revealed that HDPs can be used as natural antioxidants in functional foods and pharmaceutical industries.

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1. Introduction

HDPs have been reported to have biological functions such as antioxidant, hepatoprotective effect and immunomodulatory activities [1,2]. Currently, there is a great deal of need to supply the market with high-quality polysaccharides. However, little information about the extraction and purification of HDPs is available. Heating or boiling was normally used to extract water-soluble polysaccharides, but many bioactive compounds are easily lost due to ionization, hydrolysis and oxidation during the extraction [3]. Compared with conventional extraction methods of polysaccharides, ultrasonic-assisted extraction is one of the most inexpensive, simple and efficient techniques, which can increase the yield of extracted components, reduce extraction time, make higher processing throughput and reduce energy consumption [4–8]. Ultrasonic-assisted extraction technology has been widely used in the extraction of natural products from different materials. Response surface methodology (RSM) is a statistical method, which can find the optimal parameters by analyzing the regression equation or by simultaneously solving multivariate problems [9]. The main advantage of RSM is the reduced number of experiments needed to evaluate optimum values of the variables and their interactions. *Hovenia dulcis* (*Rhamnaceae*), is a medicinal and edible fruit. It is widely distributed in China, India, Korea and Japan. *H. dulcis* tastes sweet, containing many nutrients [10] and no toxins [11]. It peduncles have a long history as traditional herbal medicine with the effects of quenching thirst, detoxifying alcoholic intoxication, helping digestion, anti-lipid peroxidation and so on [2]. Peduncles and seeds of some species of *Hovenia* have been used for treating alcoholism and liver disease in China [12]. Previous studies have demonstrated that the extracts of *H. dulcis* have anticancer activity [13] and protective effects against liver damage [1]. HDPs have an important protective effect against acute alcohol-induced liver injury probably via its antioxidant capacity to protect biological systems and prevent oxidative stress [1].

The objectives of this study were to optimize and model the extraction conditions of HDPs by ultrasonic-assisted extraction using RSM. The Box–Behnken design (BBD), one of the RSMs, based on a 3 level and 3 variable central composite, was employed to obtain the best possible combination of extraction temperature, ultrasonic power and extraction time to maximize the yield of polysaccharides from *H. dulcis*.

Grading of crude polysaccharides by different concentrations of ethanol and a preliminary characterization of HDPs was performed.
Furthermore, the *in vitro* antioxidant activities of the HDPs were evaluated (by studying the scavenging activities against DPPH, hydroxyl and ABTS radicals) to assess their applications as natural antioxidants in functional foods or medicine.

2. Materials and methods

2.1. Materials and chemicals

The ripe peduncles of *H. dulcis* were harvested in October of 2013 on the campus of Hefei University of Technology (Hefei City, Anhui Province, China). The peduncles of *H. dulcis* were dried and ground into powder to go through a 60-mesh sieve and kept at −20 °C until use. DPPH, ABTS, glucose and galacturonic acid (GaA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were analytical grade or chromatographic grade.

2.2. Extraction of polysaccharides

The powder of the *H. dulcis* peduncles was defatted and decolorized with acetone for 12 h and subsequently with ether for another 12 h in a Soxhlet extractor system. Then the sample was dried and the extraction of HDPs with distilled water was carried out in an ultrasonic cell disintegrator according to the experimental design. The supernatant was collected after centrifugation at 3000 × g for 15 min. The supernatant was concentrated with a rotary evaporator and ethanol (95%) was added to obtain a final concentration of 80% (v/v). This was then kept at 4 °C for 12 h. The precipitate was collected from centrifugation (10,000 × g for 10 min) and washed with deionized water. The proteins were removed by Sevag methods [14], and then dialyzed (Mw cutoff 3500) and lyophilized to gain the polysaccharides. The polysaccharide content was determined by the phenol–sulfuric acid method, using glucose as a standard [15]. The HDPs yield (mg/g DW) was measured by the following equation:

\[
\text{Yield of HDPs (mg/g DW) = } \frac{\text{weight of dried crude HDPs (mg)}}{\text{weight of sample (g)}}
\]

2.3. Experimental design

2.3.1. Single-factor experimental design

A single-factor test was employed to determine the preliminary range of the extraction variables including extraction temperature (30, 40, 50, 60 and 70 °C), ultrasonic power (160, 240, 320, 400 and 480 W) and extraction time (5, 20, 35, 50 and 65 min). One factor was changed when the other conditions were kept constant in each experiment. All trials were performed in triplicate.

2.3.2. Box–Behnken design

Based on the single factor experiments, a three-factor-three-level BBD was used to determine the optimal conditions for ultrasonic-assisted extraction of HDPs, requiring 17 experiments shown in Table 1. The extraction yield was taken as the response. A multiple quadratic regression model was used to analyze the response, using the following equation:

\[
Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j
\]

where \(Y\) is the estimated response, \(\beta_0, \beta_i, \beta_{ii}\) and \(\beta_{ij}\) are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and \(x_i\) and \(x_j\) are the independent variables.

2.4. Statistical analysis

Design-Expert software (Version 8.0.6) was adopted to analyze the experimental data. The three-dimensional (3D) surface and contour plots of RSM expressed the interaction between different variables. The best fit of the multiple quadratic models was determined by the determination coefficients \(R^2\) and \(R^2_{adj}\). The significance of the statistic and regression coefficients were tested by F-test (if \(p < 0.05\) is significance).

2.5. Grading of crude polysaccharides

After the supernatant was concentrated by a rotary evaporator, the concentrated solution was graded by the ethanol precipitation method at different final concentrations. HDPs was found at a final ethanol concentration of 80% (v/v). A final ethanol concentration of 40% (v/v) was centrifuged (10,000 × g for 10 min), and the precipitate was HDPs1. Ethanol was added to the supernatant (E60) to a final ethanol concentration of 60% (v/v), after centrifugation, the precipitate was HDPs2. Then, HDPs3 was found from the supernatant (E80) at a final ethanol concentration of 80% (v/v).

2.6. Preliminary characterization of polysaccharides

2.6.1. Determination of the contents of protein and uronic acid

Protein content of polysaccharides was determined by the Bradford method [16] with bovine serum albumin as a standard. The content of uronic acid was measured by a m-hydroxydiphenyl colormetric method and with GaA as a standard [17].

2.6.2. Fourier-transform infrared spectra (FT-IR)

The FT-IR spectra of the polysaccharides were recorded by the KBr disk method using a Nicolet 67 spectrometer (Thermo Nicolet, USA). All spectra were scanned between 400 and 4000 cm⁻¹.

2.6.3. Analysis of monosaccharides

The HDPs and three graded fractions of polysaccharides (5 mg) were respectively hydrolyzed by 4 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 4 h in a sealed tube. Methanol was added to the hydrolysate to remove the residual TFA, and mixture was concentrated by drying with a rotary vacuum evaporator. Subsequently, the treated samples and monosaccharides including glucose (Glu), mannose (Man), xylose (Xyl), arabinose (Ara), rhamnose (Rha) and galactose (Gal) were respectively reduced by adding of NaBH₄ (30 mg) at room temperature for 3 h, and were acetylated by adding of ACOH (3 mL) and pyridine (3 mL) for 1 h at 100 °C. The acetylated alditol acetates and monosaccharides were analyzed by gas chromatography (GC) (Agilent 7980A, USA) with a fused silica capillary column (Agilent Co. Model: HP-5, 30 m × 0.32 mm × 0.25 μm, USA) and a flame ionization detector. The operation conditions were according to the reported method [18].

2.7. Analysis of antioxidant activity in vitro

2.7.1. DPPH radical scavenging assay

The scavenging activity of HDPs against DPPH radicals was determined according to the reported method [19]. The different concentrations of sample solutions (2.0 mL) were mixed with 2.5 mL of DPPH (100 μM in methanol). The absorbance of mixtures was measured at 517 nm after it had been kept at room temperature for 30 min and ascorbic acid (VC) was chosen as a positive control. The scavenging activity was calculated according to the equation:

\[
\text{DPPH scavenging activity (%) = } \left( \frac{A_0 - A_1 + A_2}{A_0} \right) \times 100
\]

where \(A_0\) is the absorbance of DPPH solution (water instead of the sample), \(A_1\) is the absorbance of the sample and \(A_2\) is the absorbance of the sample without DPPH solution.
Table 1
Box–Behnken design and results for extraction yield of HDPs.

<table>
<thead>
<tr>
<th>No.</th>
<th>X1 (°C)</th>
<th>X2 (W)</th>
<th>X3 (min)</th>
<th>Yield (mg/g)</th>
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</thead>
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<td></td>
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<td>1(480)</td>
<td>0(65)</td>
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<td>0(50)</td>
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<td>0</td>
<td>1</td>
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<td>1</td>
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<td>1</td>
<td>-1</td>
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2.7.2. Hydroxyl radicals (OH•) scavenging assay

The hydroxyl radical scavenging activity was determined according to Gao et al. [20]. 1 mL of sample solutions of different concentrations were mixed with 1 mL of FeSO4 (9 mM), 1 mL of salicylic acid–ethanol (9 mM) and 1 mL of H2O2 (9 mM). After incubation at 37 °C for 30 min, the absorbance was determined at 510 nm. Vitamin C (Vc) served as positive control and distilled water was used as control. The hydroxyl radical scavenging activity was calculated as follows:

\[ \text{OH• scavenging activity (%) = } \left( \frac{A_0 - A_1 + A_2}{A_0} \right) \times 100 \]  

where \( A_0 \) is the absorbance of control (water instead of sample), \( A_1 \) is the absorbance of the sample and \( A_2 \) is the final absorbance of the sample without OH•.

2.7.3. ABTS radicals scavenging assay

The ABTS radical scavenging activity was determined according to the method of Cheng et al. [21]. 0.2 mL samples of different concentrations were mixed with ABTS (4 mL, 7 M). After incubation at room temperature for 6 min, the absorbance was measured at 734 nm and ascorbic acid was used as a positive control. The ABTS radical scavenging activity was calculated with the formula below:

\[ \text{ABTS scavenging activity (%) = } \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \]  

where \( A_0 \) is the absorbance of the control (without sample) and \( A_1 \) is the absorbance of the sample.

3. Results and discussion

3.1. Single factor experiment

3.1.1. Effect of temperature on extraction yield of HDPs

The effect of temperature (30–70 °C) on the extraction yield of HDPs was investigated with an ultrasonic power of 160 W and an extraction time of 20 min. As shown in Fig. 1A, the yield of HDPs increased sharply when the extraction temperature was increased from 30 to 50 °C. The results were similar with the conclusion of Wang et al. [22]. At a high extraction temperature, the liquid viscosity and density decreased, resulting in an increase in mass transfer. Moreover, the number of cavitation bubbles and surface contact area increased with the temperature [23]. The maximum extraction yield was 17.266 ± 0.31 (mg/g DW) at 50 °C. Therefore, extraction temperature range of 40–60 °C was considered to be optimal in the present study.

3.1.2. Effect of ultrasonic power on extraction yield of HDPs

Ultrasonic power was another factor that would influence the extraction yield. Fig. 1B exhibits the effect of ultrasonic power on extraction yield of HDPs, while extraction temperature and time were 50 °C and 20 min, respectively. The yield of HDPs increased with increasing ultrasonic power up to 400 W and then decreased as the extraction proceeded. The maximum extraction yield was 19.998 ± 0.17 (mg/g DW) at an ultrasonic power of 400 W. This result indicated that ultrasonic power of 320–480 W was favorable for producing higher yields of HDPs. Ultrasonic power enhanced the HDPs in water to a certain level followed by their possible loss at a higher power due to decomposition. The mechanical effects of ultrasound result in a greater penetration of liquid into cellular materials and improved mass transfer as well. The main factors responsible for the enhancement of extraction yield were reported as due to efficient cell disruption by ultrasonic power that results in effective mass transfer [24].

3.1.3. Effect of extraction time on the yield of HDPs

Extraction time influences the extraction efficiency [5,8]. When the extraction time was increased from 5 min to 65 min, and the other experimental parameters were kept constant (extraction temperature at 50 °C and ultrasonic power of 400 W). The results are shown in Fig. 1C. A longer extraction time represented a positive effect on the extraction yield of HDPs, which increased in the initial 50 min. After 50 min, the yield decreased because of the degradation of polysaccharides [25]. The maximum extraction yield was 19.435 ± 0.22 (mg/g DW) at 50 min. It indicated that the extraction time of 35–65 min was sufficient to obtain an optimum yield of polysaccharides.

3.2. Optimization of procedure by RSM

3.2.1. Statistical analysis and the model fitting

A 17-run BBD was applied to optimize the three independent variables (X1: extraction temperature, X2: ultrasonic power, and X3: extraction time) and performed to determine the best combination of the three factors on the yield of HDPs. Table 1 shows the layout of the BBD and the corresponding results obtained with each run. Based on the analysis of multiple regressions, the results exhibited that the response variable and the three variables followed the second-order polynomial equation:

\[ Y = 20.01 + 3.67X_1 - 1.40X_2 + 1.67X_3 + 1.20X_1X_2 + 1.31X_1X_3 - 0.91X_2X_3 - 2.03X_1^2 - 1.18X_2^2 + 0.43X_3^2 \]  

(6)
where $Y, X_1, X_2, X_3$ represent the yield of HDPs, extraction temperature (°C), ultrasonic power (W), and extraction time (min), respectively.

Analysis of variance (ANOVA) was used to determine the fitting degree of the model. In Table 2, the F-value of 16.94 and p-value of 0.0006 indicated the quadratic polynomial regression model was significant. The lack-of-fit p-value of 0.2778 indicated that the lack-of-fit was not significant relative to pure error. Meanwhile, the determination coefficient ($R^2 = 0.9561$) also confirmed that the fitted model could explain 95.61% of the total variability within the scope of values studied.

Additionally, a signal-noise ratio of 17.146 implied an adequate signal, and a ratio greater than 4 is desirable. Thus, the model was considered to be reliable for designing this experiment.

By analysis of the significance for regression coefficient, it was found that $X_1, X_2, X_3, X_1X_3$ and $X_2^2$ had significant effect on the extraction yield ($p < 0.05$). The other term coefficients were insignificant ($p > 0.05$). The coefficient of $X_1$ was highly significant ($p < 0.001$), which means extraction temperature was the most important of the independent variables. And the interaction effect of extraction temperature and time was significantly correlated with the yield of HDPs.

3.2.2. Optimization of extraction conditions and verification of predictive model

The 3D response surfaces and contour plots were created by using the Design-Expert software (Version 8.0.6) based on the regression Eq. (2), as shown in Fig. 2. The effect of extraction temperature ($X_1$) and ultrasonic power ($X_2$) on the yield of HDPs were shown in Fig. 2A and B when extraction time ($X_3$) was fixed at 0 level.

Fig. 1. Effect of different factors on extraction yield of polysaccharides. (A) Extraction temperature, (B) ultrasonic power, and (C) extraction time.

Obviously the extraction yield increased with increasing extraction temperature. However, the yield of HDPs was lower with high ultrasonic power. Fig. 2C and D shows the combined effect of extraction temperature and time on the yield of HDPs at constant ultrasonic power. The maximum yield of HDPs was 25.29 mg/g DW when extraction temperature was 60 °C and the extraction time was 65 min. In Fig. 2E and F, when the extraction temperature was fixed at 50 °C, a low ultrasonic power level and higher level of extraction time were good for the extraction of HDPs.

The optimum extraction yield of HDPs was obtained when the three variables were 60 °C, 362 W and 65 min for extraction temperature, ultrasonic power, and extraction time, respectively. Under these conditions, the extraction yield of HDPs was $25.12 \pm 0.145$ mg/g DW ($n = 3$), which was similar with the predicted value of 25.33 mg/g DW, and meant that the model was valid for the extraction process.

3.3. Preliminary characterization of polysaccharides

The contents of uronic acid in HDPs, HDPs1, HDPs2 and HDPs3 were $2.9 \pm 0.05\%, 2.35 \pm 0.03\%, 11.1 \pm 0.05\%$ and $2.15 \pm 0.08\%$, respectively. These values suggested that they were all acid polysaccharides. Their protein contents were $8.35 \pm 0.35\%, 2.37 \pm 0.05\%, 5.98 \pm 0.16\%$ and $11.77 \pm 0.35\%$, respectively. As shown in Table 3, the monosaccharides of HDPs, HDPs1, HDPs2 and HDPs3 were mainly composed of Ara, Rha, Glu and Gal in comparison to the standard monosaccharides by GC. However, the contents were different. The contents of Glu in HDPs and HDPs1 were much higher than the others, while Xyl and Man were found in trace amounts. However, HDPs2 and HDPs3 were mainly
composed of Gal, Ara and Glu. Polysaccharides rich in galactose and arabinose have been reported to display relatively higher antioxidant activity [26].

The FT-IR spectra of the polysaccharides scanning from 400 cm\(^{-1}\) to 4000 cm\(^{-1}\) were shown in Fig. 3. It could be found that all samples exhibited an almost similar characteristic absorption peak. The broad peak at 3412 cm\(^{-1}\) is attributed to the hydroxyl groups stretching vibration. A weak band at around 2930 cm\(^{-1}\) was the characteristic absorption of polysaccharides which was due to C–H stretching and bending vibration. The bands at 1611 cm\(^{-1}\) and
The presence of uronic acid in polysaccharides. This may be due to the absorption with the range. The percentages of scavenging activities of HDPs2, HDPs3, HDPs1, HDPs3, and HDPs1 at the concentration of 1.0 mg/mL were 93.33 ± 0.38%, 90.92 ± 0.58%, 89.84 ± 0.4% and 82.79 ± 0.61%, respectively. The percentage of scavenging activity of Vc was in the range of 95% to 96% at the tested concentrations. Obviously, the HDPs had strong DPPH radical scavenging activities, which were higher than those of other active polysaccharides [8,28,29].

3.4.2. ABTS radicals scavenging assay

Fig. 4B shows the percentage of scavenging activity of the polysaccharides against ABTS radicals. The percentages of scavenging activities of HDPs2, HDPs3, HDPs1, and HDPs3 at the concentration of 5.0 mg/mL were 99.81 ± 0.08%, 99.76 ± 0.08%, 95.88 ± 0.37%, and 61.48 ± 0.59%, respectively. The ABTS radicals scavenging ability of HDPs3 was very close to vitamin C, and higher than other fractions. Meanwhile, ABTS radicals scavenging ability of HDPs3 exhibited concentration dependence at concentrations ranging from 1.0 to 5.0 mg/mL. The results demonstrated that polysaccharides from *H. dulcis* had high scavenging activities against ABTS radicals.

3.4.3. Hydroxyl radical scavenging assay

Hydroxyl radicals are the most active free radicals formed in biological systems. They could easily cross cell membranes and lead to the breaking of DNA strands, and have the capacity of carcinogenesis, mutagenesis and cytotoxicity [30]. The percentages of scavenging activities of HDPs1, HDPs2 and HDPs3 exhibited concentration dependence at lower concentration (1–4 mg/mL), which were 13.23 ± 0.47%, 14.47 ± 0.69%, 26.76 ± 0.71% and 23.69 ± 1.02% respectively at the concentration of 5.0 mg/mL, and lower than that of Vc within the test dosage range (Fig. 4C). The results showed that the polysaccharides revealed some hydroxyl radicals scavenging ability. HDPs2 and HDPs3 were
mainly composed of Gal, Ara and Glu by GC. The monosaccharide composition Gal and Ara in polysaccharides was reported to possess relatively higher antioxidant activity [26]. According to the antioxidant activity analysis, the antioxidant activities of HDPs2 and HDPs3 graded by the ethanol precipitation method were higher than crude HDPs, and this observation is in accordance with their composition of Gal and Ara as determined by GC.

4. Conclusions

In this study, an ultrasonic-assisted technology was first used for extraction of HDPs using RSM to optimize and model the extraction conditions. The results showed that the independent variables (extraction temperature, ultrasonic power and extraction time), and the interaction effects between extraction temperature and extraction time, and the quadratic terms of extraction temperature had significant effects on the yield of polysaccharides. BBD based on the single-factor experiments was employed to optimize polysaccharides extraction from *H. dulcis* by ultrasonic technology. The optimum conditions were extraction temperature at 60 °C, ultrasonic power of 362 W and extraction time for 65 min. Under the optimal conditions, the extraction yield of HDPs (25.12 ± 0.145 mg/g DW) agreed with the predictive value of 25.33 mg/g DW. The HDPs exhibited strong scavenging activities against DPPH and ABTS radicals, and moderate scavenging activity against hydroxyl radicals in vitro. The preliminary characterizations were acidic polysaccharides; the FT-IR spectra of the four fractions exhibited an almost similar characteristic absorption peak. The monosaccharides of HDPs, HDPs1, HDPs2 and HDPs3 were mainly composed of Ara, Rha, Glu and Gal by GC. However, the contents were different. Taken together, the results suggest that polysaccharides from *H. dulcis* have great potential to be developed as antioxidants for nutraceutical and pharmaceutical industries.

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References


Fig. 4. Antioxidant activity tests of HDPs. (A) DPPH radical scavenging activity, (B) ABTS radical scavenging activity, and (C) hydroxyl radical scavenging activity.