Optimisation of Ultrasonic-Microwave-Assisted Extraction Conditions for Polysaccharides from Mulberry (Morus atropurpurea Roxb) Leaves and Evaluation of Antioxidant Activities in vitro

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Abstract

Background: In this study, ultrasonic-microwave-assisted extraction (UMAE) was first applied to extract polysaccharides from the leaves of mulberry (PLM). The optimal conditions for polysaccharides extraction were determined by response surface methodology (RSM) along with central composite design (CCD) based on the single-factor experiments. Extraction temperature (°C), extraction time (s), pH value, and ratio of water to raw material were investigated. Also, the antioxidant activities of PLM with UMAE were evaluated.

Results: According to statistical analysis, extraction temperature and pH value significantly affected extraction yield. The central composite design (CCD) showed that polynomial regression models were in good agreement with the experimental results with the coefficients of determination of 0.9263 for PLM. The optimal conditions were extraction temperature 76°C, extraction time 807 s, pH value 6.16 and the ratio of water to raw material 40:1, and the maximal yield of PLM was 10.29 ± 0.41(%). Meanwhile, DPPH radical-scavenging at EC50 values of PLM was found to be 128.3 μg/ml.

Conclusion: Under the optimal conditions, the experimental values agreed with value predicted by the model. The results suggested that UMAE could be used for the extraction of PLM, and the polysaccharides from mulberry leaves have significant antioxidant activities, which can be used as a source of potential antioxidant.

Keywords: M. atropurpurea Roxb.; Polysaccharides; Optimization; Response surface methodology; Antioxidant activities

Introduction

During the past decades, the polysaccharides from medicinal plants have attracted great attentions, due to their diverse and potentially significant pharmacological activities. The leaves of mulberry, as a folk medicine, has been traditionally used in Asian countries for purposes to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate the discharge of urine, reduce blood glucose and lower blood pressure [1]. Mulberry leaves extracts have shown multi-direction biological activities, such as the antioxidant, antimicrobial, anti-inflammatory, anti-allergic, anti-atherosclerotic, and significant hypoglycemic effect [2-7]. A large amount of polysaccharides was contained in mulberry leaves, and their useful pharmacological effects, such as hypoglycemic and lipid-lowering activities, have been verified [8]. Polysaccharides from the leaves of mulberry (PLM) have displayed a broad application prospect in medicine and functional food.

Extraction of polysaccharides is an important processing for its application or further research and development, which has prompted many research papers on the extraction technology of polysaccharides from plentiful of plants or fungus in recent years. Response surface methodology (RSM) was successfully used for optimizing the hot water extraction of polysaccharides from the roots of Codonopsis pilosula [9] and Prediction of optimum reaction conditions for the thermotolerant acetylxylan esterase from Neocallimastix patriciarum [10]. The advantage of RSM was that it could reduce the number of experimental trials and evaluate the interactions between multiple parameters. It is more effective and precise than many approaches. Ultrasonic technology and microwave technology were respectively applied to extract polysaccharides from Poria cocos [11] and cultured Cordyceps militaris [12], and the optimal extracted conditions were both obtained by RSM.

However, there were few reports about optimization of ultrasonic-microwave-assisted extraction (UMAE) of polysaccharides, especially PLM. Therefore, the first objective of this study was to optimize conditions of UMAE by RSM. Moreover, there was little information about antioxidant activities of PLM. Consequently, the other purpose was to evaluate the potential antioxidant activities of PLM with UMAE and to broaden its application in medicine field.

Materials and Methods

Materials and equipments

Mulberry (Morus alba L.) leaves of Kangqing 10, widely grown in southern China, were harvested from an experimental field in Guangzhou, which was managed by the Sericultural & Agri-Food Research Institute of the Guangdong Academy of Agricultural Sciences, in September 2013. Ethanol was purchased from Sinopharm Chemical Reagent Co. Ltd. (China). All the chemicals used in the experiment were of analytical grade. The extraction procedure was carried out in the ultrasonic-microwave-assisted-extractor (Xin Tuo Microwave Decomposition and Testing Technology Co. Ltd. Shanghai, China).

Extraction of crude PLM

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Mulberry leaves powder was immersed overnight in ethanol and refluxed extraction with ethanol at 80°C for 2 h, and 80% (v/v) ethanol at 50°C for 4 h to remove some pigments, oligosaccharides and some small molecule materials. Then the resulting residue was extracted in water by ultrasonic-microwave.

The extraction was performed at different temperature, time, pH value, and ratio of water to raw material. The extract was filtered through a Whatman No.1 filter paper and the filtrate was then concentrated to 25 ml with vacuum evaporator at 70°C. The crude polysaccharide fraction was obtained through precipitation by adding ethanol until the concentration of the ethanol reached 80% (24 h, 4°C). The precipitant was collected by centrifugation (5000 rpm, 15 min), and lyophilized (Free Zone 7948030, Labconco, USA) and finally weighted.

The polysaccharide yield (%) was calculated using Equation (1):

\[ \text{Yield} (%) = \frac{\text{Weight of polysaccharides}}{\text{Weight of raw material}} \times 100 \]  

(1)

where M, the weight of crude polysaccharides; W, the mass of mulberry leaves powder.

**Experimental design**

The extraction parameters were optimized using RSM. The central composite design (CCD) was applied in this study. The range and center point values of four independent variables, presented in Table 1, were based on the results of preliminary experiments. CCD in the experimental design consists of sixteen factorial points, eight axial points and seven replicate of the central points (Table 2). All trials were performed in triplicate. Extraction temperature (X1), extraction time (X2), pH value (X3) and ratio of water to raw material (X4) were chosen for independent variables. Yield of polysaccharides was selected as the response for the combination of the independent variables given in Table 2. An SAS Software Version 9 (SAS Institute Inc., NC, USA) was used to generate the experimental designs, statistical analysis and regression model. Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. The variables were coded according to Equation (2):

\[ x_i = \frac{(X_i - X_0)}{\Delta X} \]  

(2)

where Xi, the real value of variable; X0, the real value of the Xi at the center point; ΔX, the step change in Xi; xi, the coded value of the variable; i = 1, 2, 3, 4.

The response Y (PLM yield) was analyzed by using a second-order polynomial equation in four independent variables and the data were fitted into the equation by multiple regression procedure. The model equation for analysis was given below Equation (3):

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

(3)

where \( \beta_0 \), \( \beta_i \), \( \beta_{ii} \), and \( \beta_{ij} \) represent the constant process effect in total, the linear, quadratic effect of Xi and the interaction effect between Xi and Xj on PLM production, respectively. The fitness of the second-order model was expressed by the regression coefficient R² and its statistical significance was determined by F test. T-test was used for evaluating regression significance. Subsequently, three extra confirmation experiments were conducted to verify the validity of the statistical experimental strategies.

**Evaluation of antioxidant activities of PLM**

The free radical-scavenging activity of the crude polysaccharides was measured by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test, according to the method of Shimada, Fukushima, Yahara, and Nakamura (1992) [13], with some modifications. The 0.1 mmol/L solution of DPPH• in 95% ethanol was prepared daily before UV measurements. Two ml of various concentrations (15.6, 31.3, 62.5, 125, 250, and 400 μg/ml) of the polysaccharides in ultra-pure water were thoroughly mixed with 2 ml of freshly prepared DPPH•. The mixture was shaken vigorously and allowed to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank with an ultraviolet–visible spectrophotometer (UV-1800, Shimadzu, Japan). Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. Ascorbic acid and butylated-hydroxytoluene (BHT) were used as positive controls. All tests and analyses were carried out in triplicate and averaged. The ability to scavenge the free radical, DPPH• in percent (I %) was calculated using the following equation:

\[ I \% = \frac{[A_0 - (A_2 - A_1)]}{A_0} \times 100 \]  

(4)

where A0 is the absorbance of the incubated DPPH• solution without addition of the sample or positive controls, A1 is the absorbance of the sample plus 95 % ethanol without DPPH• and A2

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<th>X2(Time, s)</th>
<th>X3(pH)</th>
<th>X4(ratio, v/w)</th>
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**Table 2:** Central composite arrangement for independent variables and their response
is the absorbance of the incubation mixture containing both the test sample and DPPH• solution.

EC50 values were expressed in terms of μg/ml of PLM to show the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50%. The data were presented as mean values (n=3).

Result and Discussion

Effect of temperature on extraction yield of PLM

The temperature varied from 50°C to 100°C, other extraction variables were set as follows: ratio of water to raw material 30:1, pH value 7.00 and extraction time 540 s. Polysaccharides yield was increased with temperature until 80°C and began to decrease, and the maximum extraction yield was 8.49 ± 0.04(%) at 80°C, while the extraction yield reached 8.40 ± 0.07(%) at 70°C, and the difference between them was not significant. Therefore, 70°C was selected as the optimal in the present experiment with considering saving cost. This result suggested that temperature improved the polysaccharides extraction from mulberry leaves into the water to a certain level followed by their possible loss, due to decomposition at a higher temperature.

Effect of time on extraction yield of PLM

When the extraction time increased from 180 s to 900 s, other extraction conditions were as follows: extraction temperature 80°C, the ratio of water to raw material 30:1, and pH value 7.00. The results showed that the extraction yield increased slightly from 7.03 ± 0.06(%) to 9.51 ± 0.14(%) with time increasing, which indicated a longer extraction time totally represented a positive effect on the yield of polysaccharides.

Effect of pH value on extraction yield of PLM

When the pH value varied from 5.00 to 9.00, the extraction temperature, time and ratio of water to raw material were fixed at 80°C, 540 s and 7.00, respectively. The extraction yield increased slightly with the ratio of water to raw material until 40:1, and then decreased. Therefore, the ratio of water to raw material 40:1 was considered as the optimal.

According to the single-parameter study, we adopted extraction temperature 50-90°C, extraction time 360-1080 s, pH value 5.00-9.00, and ratio of water to raw material 20:1-60:1 for RSM experiments.

Predicted model and statistical analysis

The process variables and the experiment results of extraction yield according to the factorial design were presented in Table 2. The extraction yield ranged from 4.24% to 9.92%. The maximum yield of polysaccharides (9.92%) was recorded under the experimental conditions of extraction temperature 70°C, extraction time 720 s, pH value 7.00, and ratio of water to raw material 40:1. By employing multiple regression analysis on the experimental data, the response variable and the test variables were related by the following second-order polynomial equation:

\[ Y_1 = 9.832857 + 0.949167 \times X_1 + 0.0125 \times X_2 - 0.357589 \times X_3 + 0.17 \times X_4 - 0.847589 \times X_1 \times X_1 + 0.11875 \times X_1 \times X_2 + 0.09375 \times X_1 \times X_3 - 0.15375 \times X_1 \times X_4 - 0.353839 \times X_2 \times X_2 - 0.295 \times X_2 \times X_3 - 0.145 \times X_2 \times X_4 - 0.400089 \times X_3 \times X_3 + 0.11 \times X_3 \times X_4 - 0.357589 \times X_4 \times X_4 \]

As shown in Table 3, the fitness of the model was examined by determination coefficient (R2= 0.9263), which pointed out that the sample variation of more than 92% was attributed to the variable. In addition, a low value 6.52 of the coefficient of the variation (CV)
clearly indicated a high degree of precision and a good deal of reliability of the experimental values. The model was found to be adequate for prediction within the range of experimental variables. Statistical testing of the model by analysis of variance was shown in Table 3, which was required to test the significance and adequacy of the model. Here the ANOVA of the regression model demonstrated that the model was highly significant, as was evident from the calculated F value (14.37258) and very low probability value (0.0001). It can be seen from this table that two linear coefficients (X1, X3), interactive terms (X2X3) and all the quadratic terms were significant, with very small P-values (P < 0.05). The other term coefficients were not significant (P > 0.05).

Response surface plot and contour plot

The graphical representations of the regression function, called the response surfaces and the contour plots, were obtained by SAS version 9.0, and the results of extraction yield of PLM affected by extraction temperature, extraction time, pH value, and ratio of water to raw material were presented in Figure 1 and Figure 2. The interactions between two
tested variables and the relationship between responses and experiment levels of each variable were determined by plotting the response surface curves. Different shapes of the contour plots indicated different interactions between the variables. Circular contour plot suggested that the interactions were negligible between the corresponding variables, while elliptical or saddle nature of the contour plot indicated otherwise [14]. As shown in Figure 1a and 2a, when pH value (X3) and ratio of water to raw material (X4) were fixed at 0 levels, extraction temperature and extraction time displayed a quadratic effect on the response yield. From Figure 1b and 2b, it showed that when extraction time (X2) and ratio of water to raw material (X4) were fixed at 0 levels, the extraction yield of PLM decreased with the increase of pH value rapidly increased at first and then dropped with the increasing extraction temperature. The 3-D response surface plot and the contour plot in Figure 1c and 2c, which gave the extraction yield of polysaccharides with varying extraction temperature and ratio of water to raw material at fixed extraction time (X2) (0 level) and pH value (X3) (0 level), indicated that extraction temperature demonstrated a quadratic effect on the response yield, and the variations of yield were negligible with the extraction time increase. In Figure 1d and 2d, it showed that the mutual interactions between extraction time and pH value were significant at fixed extraction temperature (X1) (0 level) and ratio of water to raw material (X4) (0 level). The Figure 1e and 2e presented the extraction yield of polysaccharides affected by different extraction time and ratio of water to raw material when extraction temperature (X1) and pH value (X3) were fixed at 0 levels. It can be seen that extraction time and ratio of water to raw material displayed quadratic effects on the extraction yield. The Figure 1f and 2f showed the 3-D response surface plot and the contour plot at varying pH value and ratio of water to raw material when extraction temperature (X1) and extraction time (X2) were fixed at 0 levels. It presented that pH value had a negative impact on the extraction yield of PLM. The extraction yield increased with the increasing ratio of water to raw material and reached the maximum value at a certain level, but beyond this level, it decreased.

Verificaiton of the predictive model

By analyzing the plots, the model predicted a maximum value of 10.33(%) of the tested variables for polysaccharides, which lied in the following condition: extraction temperature 76°C, extraction time 807 s, pH value 6.16 and ratio of water to raw material 40:1. In the optimal conditions, the experiment yield of PLM was 10.29 ± 0.41(%) (n=3), which indicated a good correlation with the predicted value. Therefore, the results indicated suitability of the model employed and the success of RSM in optimizing the extraction conditions.

The experiment yield (10.29%) was greatly better than 4.67 % by the hot water extraction process [15]. This great extraction efficiency by UMAE might be attributed to the mechanical effects of ultrasonic wave, which greatly facilitate mass transfer between immiscible phases through a super agitation [16], and the non-ionising radiation energy of microwave, which causes motion of molecule and rotation of dipoles to heat solvents to promote targeted compounds to move from the sample matrix into the solvent [17].

Antioxidant activities analysis

The model of scavenging the stable DPPH radical is a widely used method for evaluating the free radical-scavenging ability of various antioxidants [18,19]. The method is based on the reduction of ethanolic DPPH radical solution at 517 nm in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. It can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations. Herein the above-mentioned model was used to determine inhibitory activities of the water-soluble polysaccharide on DPPH radicals. Figure 3 depicted the DPPH•-scavenging power of PLM. Obviously, the polysaccharides scavenging effect was increased with increasing concentrations. At concentrations of 30-125 μg/ml, the scavenging abilities of PLM from filtrate on DPPH radicals were in the range of 14.88 % - 53.27 % (Figure 3). At the concentration of 400 μg/ ml, PLM was observed to possess strong free radical-scavenging effects against DPPH radicals, with a value of around 77.68 %, and scavenging effects of ascorbic acid and BHT on the DPPH radical were 95.54 % and 49.40 %, respectively. Finally, results were expressed using the term EC50. DPPH radical-scavenging at EC50 values of PLM can be indirectly seen in Figure 3. The EC50 value of PLM was found to be 128.3 μg/ml. However, the DPPH free radical-scavenging of PLM was less than that of vitamin C, at the same concentration, and more than that of BHT, a synthetic antioxidant, when the concentration of PLM was more than 120 μg/ml. These results indicated that PLM had a noticeable effect in scavenging free radicals, especially at a high concentration. However, the antioxidant mechanism of PLM is still not fully understood. Therefore, it is suggested that further work could be performed on the possible antioxidant mechanism of PLM.

Conclusion

In this study, the single-factor experiments and CCD along with RSM were applied for optimizing the ultrasonic-microwave-assisted extraction technology of polysaccharides from mulberry leaves. The optimal conditions of UMAE were given as follows: extraction temperature 76°C, extraction time 807 s, pH value 6.16 and ratio of water to raw material 40:1, the experiment yield was 10.29 ± 0.41(%), which was agreed with the predicted value, greatly better than 4.67 % by the hot water extraction process [15]. Therefore, UMAE of polysaccharides had great potential and efficiency. The experimental conditions allow a fast and cost-saving process in extraction of PLM. What’s more, the result of the free radical-scavenging activity of PLM, measured by the DPPH test, indicated that PLM possessed significant antioxidative activity and could be possibly developed as a potential natural antioxidant functional ingredient in the food industry. Further studies on the chemical structures and other biological functions of PLM are in process.

Acknowledgments

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Figure 3: Free radical-scavenging activity of PLM at different concentrations by DPPH· method. Each sample was assayed in triplicate for each concentration. Experimental results were means ± SD of three parallel measurements.
References


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