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Optimization for the Extraction of Polysaccharide from Walnut (*Juglans regia L.*) Leaves: Antioxidant Activities in Vitro

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Abstract: Cellulase-assisted extraction of Walnut Leaf Polysaccharide (WLP) was studied. Single factor test and response surface design were employed to optimize the technological conditions. The consequences indicated that the highest WLP yield of 5.21% was achieved with raw material ratio of 50 mL/g, cellulase dose of 41488.52 U/g, enzymolysis pH of 5.88, enzymolysis time of 29.74 min and enzymolysis temperature of 52.07°C. Moreover, in vitro antioxidant assays revealed that WLP has significant eliminating capability against 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydroxyl free radicals in a dose-dependent manner and exhibited a strong reducing power. These results suggest that WLP could be developed as a promising natural antioxidant agent in the pharmaceutical and functional food industries.

Keywords: Polysaccharide, Walnut Leaf, Antioxidant

Introduction

With the advancement of separation and identification technologies, the extraction of active substances from plants becomes the principal pathway to further utilize agricultural wastes (Jeddou *et al*., 2016). Walnut is a kind of economic crop planted widely in the world and its fruit has abundant functional activities (Carvalho *et al*., 2010). Nevertheless, there are few studies on walnut leaves at present, which results in a huge waste of resources. For a long time, walnut leaves have been used not only in the production of tea but also in traditional medicine for the treatment of various diseases (Almeida *et al*., 2008; Forino *et al*., 2016; Pereira *et al*., 2007). In addition, reports indicate that walnut leaves have anti-diabetes and anti-inflammatory effects (Forino, *et al*., 2016; Mollica *et al*., Bellagamba, *et al*., 2017). Therefore, the development of walnut leaves is conducive to maximize resource utilization.

It is universally knowledge that free radicals can cause degenerative diseases, such as cancer, atherosclerosis, neurodegenerative diseases, aging, diabetes and immune system decline (Gao *et al*., 2015; Lai *et al*., 2010). Therefore, antioxidant substances play an important role in cleaning up excessive free radicals and maintaining human health (Kurd and Samavati, 2015). Plant polysaccharides are good sources of antioxidants. According to the report, three purified polysaccharides extracted from *chuanxiong rhizome*, LCX0, LCX1 and LCX2, exhibited strong antioxidant activities (Hu *et al*., 2016). Potatoe peel polysaccharide and *arthrocnemum indicum* leaf polysaccharide exhibited fairly strong cleaning capacities on DPPH and ABTS radicals *in vitro* (Jeddou *et al*., 2016; Mzoughi *et al*., 2018). What's more, *cordyceps sinensis* polysaccharide restrained PDGF-BB-induced ROS generation through ERK/Akt pathway (Yu *et al*., 2018). Judging from these, the antioxidant function of polysaccharides has received extensive attention. However, the antioxidant capability of polysaccharides from walnut leaves has not been investigated.

Compared with traditional hot water extraction, enzyme-assisted extraction which has become an alternative method to extract natural products is more environmental-friendly, efficient and easier to operate (Zhu *et al*., 2014). Cellulase is a complex enzyme system consisting of a variety of hydrolytic enzymes which generally exist in organisms. It can effectively destroy the cell wall structure and facilitate the release of intracellular biological active components (Yang *et al*., 2017). Therefore, cellulase has significant application value in the process of polysaccharide extraction. Response Surface Methodology (RSM) is a statistical

method to optimize complex processes by evaluating the interaction between various factors to find the optimal process parameters (Kurd and Samavati, 2015; Mzoughi *et al*., 2018). Because of its high efficiency, RSM has been widely used by researchers to optimize the extraction process of bioactive substances (Xu *et al*., 2016; Yin *et al*., 2018). So far, there has been no report about enzyme-assisted extraction of polysaccharide from walnut leaves using RSM.

Base on the above, this study reported the optimum extraction process of WLP assisted by cellulase and investigated its antioxidant activities through DPPH, ABTS, hydroxyl radical scavenging capacities and Ferric Reducing/Antioxidant Power (FRAP) assay. Our work will help to further understand the functional activities of walnut leaves and provide a basis for the development and utilization of walnut leaves in the future.

Materials and Methods

Materials

Fresh leaves of walnut were gathered from Zibo city, North of Shangdong, in July 2018. The leaves were shade dried and ground into fine powder. Then, the resulting pulverescent samples were stored in a desiccator for future use.

Cellulase was purchased from Beijing Solarbio Science and Technology Co., Ltd (Beijing, China). Ascorbic acid was purchased from Tianjin Fuchen Chemical Reagent Co., Ltd (Tianjin, China). DPPH and ABTS were purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). All other reagents were analytical grade. Ultrapure water was prepared for the experiment.

DZK thermostatic electric oscillating water bath (Shanghai Yiheng Scientific Instrument Co., Ltd.); FW100 high speed pulverizer (Tianjin Tester Instrument Co., Ltd.); RE-52AA rotary evaporators (Shanghai Yarong Biochemical Instrument Factory); 5810R supercentrifuge (German Eppendorf Vo., LTD); UV-2600 ultraviolet spectrophotometer (Shimadu Co., Ltd.).

Methods

Single-Factor Experimental Design of Polysaccharide Extraction

The single-factor design was employed to analyze the effects of extraction variables on polysaccharide extraction. Prepared samples (5 g) were churned in the cellulase-added ultrapure water according to a certain proportion (the cellulase dosage ranging from 1×10^4 to 5×10^4 U/g; liquid-material ratio ranging from 20 to 60 mL/g) and then the prescriptive enzymolysis pH (4-7) value was adjusted. Throughout the course of extraction, the beaker was placed in a vibrating water bath at a given enzymolysis temperature (30-70°C) for a certain period of time (10-60 min). Each experiment was performed in triplicate.

Following extraction, the solution was collected by centrifugation (4000 rpm, 10 min) and evaporated using rotary evaporator at 60°C under vacuum. Sevage method was used to remove the free proteins in concentrated solutions. Ethanol was added to a final concentration of 95% (v/v) and kept at 4°C for 12 h. The precipitate was obtained by centrifugation (4000 r/min, 10 min) and then lyophilized to obtain WLP (Yun *et al*., 2019). The polysaccharide content was measured using the phenol-sulfuric method. Extraction yield of the polysaccharide weight (Equation 1):

$$Y(\%, w/w) = W_0 / W \times 100$$

W_0 (g) is the weight of WLP; W (g) is the weight of pretreated powder of walnut leaves.

Box-Behnken Design (BBD) for Extraction Optimization

On the basis of the single-factor experiment, a three-level four-factor BBD was used to determine optimal levels of extraction variables including the cellulase dosage (U/g, X_1), enzymolysis pH (X_2), enzymolysis time (min, X_3), enzymolysis temperature (°C, X_4) for the extraction yield of WLP (Wu *et al*., 2014). The coded and uncoded levels of independent variables were shown in Table 1 and 29 experimental runs in random order were required. The general form of the quadratic regression model was expressed as the following Equation 2 (Yin *et al*., 2018):

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_i^2 + \sum_{i<j=2}^{4}\beta_{ij} X_i X_j$$

Where:

Y = The predicted WLP extraction ratio
β_0 = The constant term
β_i, β_{ii} and β_{ij} = The regression coefficients for linear, quadratic and interactive terms, respectively
X_i and X_j = The coded independent variables

DPPH Free Radical Scavenging Assay

DPPH method was proposed in 1958 and widely used for quantitative determination of antioxidant capacity of biological samples and food. The scavenging power on DPPH free radical of WLP was investigated according to previous method with slight modifications (Gao *et al*., 2015). In short, polysaccharide solutions of different concentrations were prepared. Two milliliter of sample solution with variable concentrations (0.05-0.8 mg/mL) was mixed with 2 mL of DPPH in 95% ethanol (0.1 mM).

Table 1: Factors and coding value of response surface experimental designment

Levels	Cellulase dosage (X_1)/ U/g	Enzymolysis pH (X_2)	Enzymolysis time (X_3)/min	Enzymolysis temperature (X_4)/°C
-1	3×10^4	5	20	40
0	4×10^4	6	30	50
1	5×10^4	7	40	6

The reaction mixture was incubated at room temperature for 30 min in the dark. The absorbance was measured against a blank at 517 nm. Ascorbic acid was used as the positive control. The DPPH radical scavenging ratio of WLP was calculated according to the following equation (Equation 3):

$$DPPF\ radical\ scavenging\ ability\ (\%) = \left(1 - \frac{A_1 - A_2}{A_3}\right) \times 100$$

where, A_1 is the absorbance of the various test samples, A_2 refers to the absorbance of the sample without the DPPH solution, A_3 is the absorbance of the control (ethanol instead of sample). Tests were executed in triplicate.

ABTS Free Radical Scavenging Assay

The ABTS free radical scavenging capacity of WLP was performed based on a modified edition of method reported by Wang *et al*. (2015). Briefly, the ABTS radical solution was obtained through 10 mL of 7 mM ABTS solution was mixed with 10 mL of 2.45 mM potassium persulfate. After blending, the mixture was preserved in the dark at room temperature for 12 h. Then the solution was diluted with distilled water to an absorbance of 0.7±0.02 at 734 nm before used.

To estimate the depolarization activity, 2 mL different concentrations of polysaccharide samples (0.05-0.8 mg/mL) was mixed with 2 mL of the diluted ABTS reagent. The blend was allowed to react at room temperature for 1 h and the absorbance was measured at 734 nm. Ascorbic acid was used as the positive control. The scavenging activity of ABTS radicals was calculated using the following formula (Equation 4):

$$ABTS\ radical\ scavenging\ ability\ (\%) = \left(1 - \frac{A_1}{A_2}\right) \times 100$$

Where:

A_1 = The absorbance of the diluted *ABTS* solution plus samples at different concentrations and

A_2 = The absorbance of distilled water mixed with *ABTS*

FRAP Assay

The FRAP of WLP was assayed according to the literature reported by Wu *et al*. with some modifications (Wu *et al*., 2014). In brief, the working solution contained 2.5 mL polysaccharide samples of various concentrations (0.4-6.4 mg/mL), 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%, w/v). After hatched in a water bath at 50°C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v) was appended to the working solution to terminate the reaction. The mixture was then centrifuged (3500 r/min) for 10 min. Thereafter, 2.5 mL of the supernatant was mixed with 0.5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%, w/v). After allowing the reaction to continue for 15 min at room temperature, the absorbance of the samples and ascorbic acid were measured at 700 nm. A higher absorbance manifested a greater reducing capability. Values presented are the mean of triplicate analyses.

Hydroxyl Radical Scavenging Assay

Method reported by Yin *et al*. was used to investigate hydroxyl radical scavenging activity of WLP with some changes (Yin *et al*., 2018). Samples of various concentrations (2-10 mg/mL) were mixed with 1 mL of $FeSO_4$ (10 mM), 1 mL of salicylic acid-ethanol (10 mM) and 0.5 mL H_2O_2 (10 mM). The final mixture was kept for 30 min at 37°C and the absorbance at 510 nm was recorded. The following formula was used (Equation 5):

$$Hydroxyl\ radicalsscavenging\ rate\ (\%) = \left(1 - \frac{A_1 - A_2}{A_3}\right) \times 100$$

Where:

A_1 = The absorbance of the mixture with sample solution

A_2 = The absorbance of the H_2O_2 replaced by distilled water

A_3 = The absorbance of the blank reaction (distilled water instead of the sample)

Statistical Analysis

The experimental design and statistically analysis of the results of RSM were executed by Design-Expert Version software (version 8.0.6.1, State-Ease, Inc., Minneapolis, MN, USA). All data were indicated as the means ± Standard Deviation (SD) and evaluated using Analysis Of Variance (ANOVA). For all comparisons, a significant difference was judged to be statistically significant if $P<0.05$.

Results and Discussion

Effect of Cellulase Dosage on Extraction Yield

Cellulase is used to hydrolyze or degrade the cell walls of plants to improve the extraction rate. The cellulase amounts can markedly affect the yield of the effective constituent in the enzyme-assisted

extraction. As a consequence, different cellulase dosages (1, 2, 3, 4 and 5×10^4 U/g) were investigated with enzymolysis pH of 6, enzymolysis time of 30 min, liquid-material ratio of 30 mL/g and enzymolysis temperature of 50°C. As shown in Fig. 1, the extraction rate of WLP increased from 1.52% to 4.57% and then fall slowly as the cellulase dosage increased from 1×10^4 to 5×10^4 U/g, which was consistent with the results in previous studies (Yin *et al*., 2016). Possible reason for this phenomenon is that WLP glucosidic bonds is partially hydrolyzed on account of the saturated substrate.

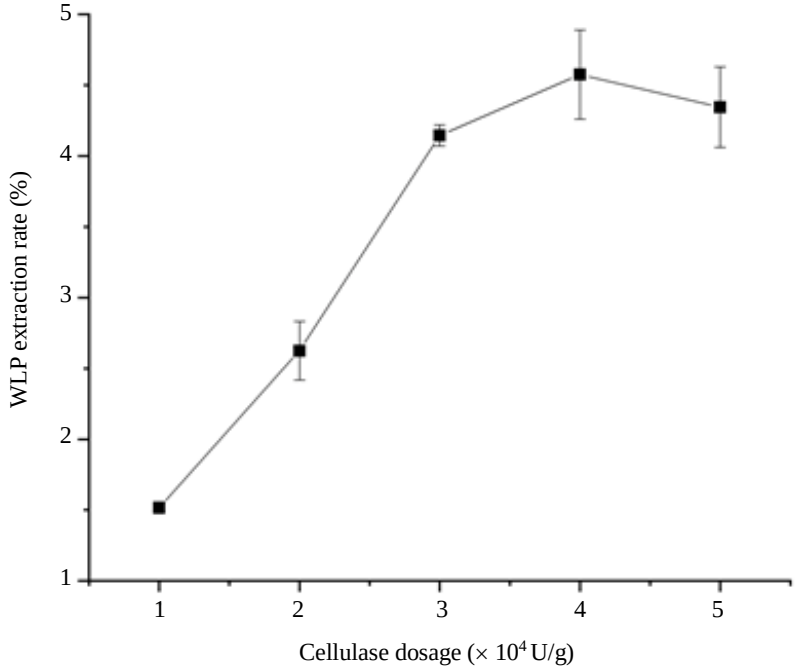


Fig. 1: Effects of cellulase dosage on extraction yield of WLP. Each value represents the mean ± SD of three determinations

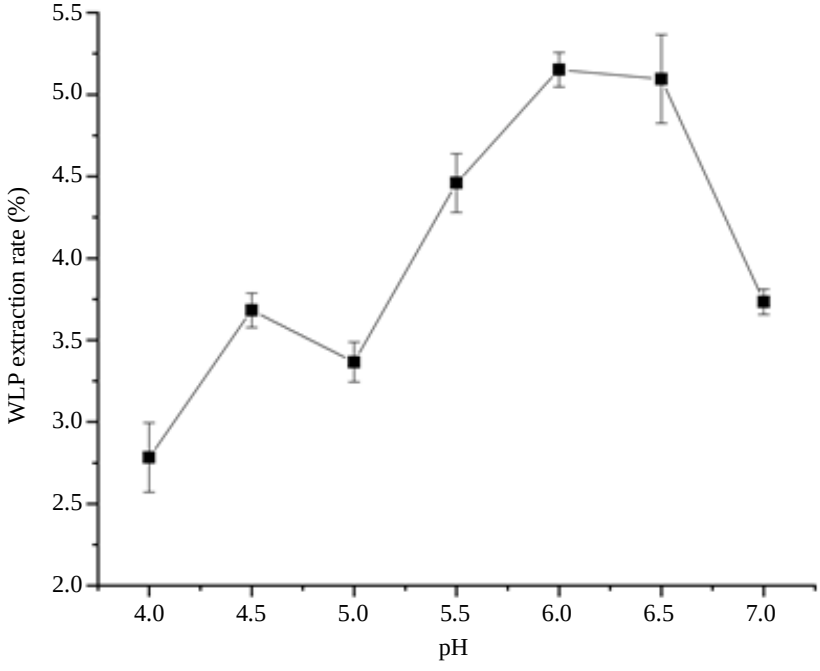


Fig. 2: Effects of enzymolysis pH on extraction yield of WLP. Each value represents the mean ± SD of three determinations

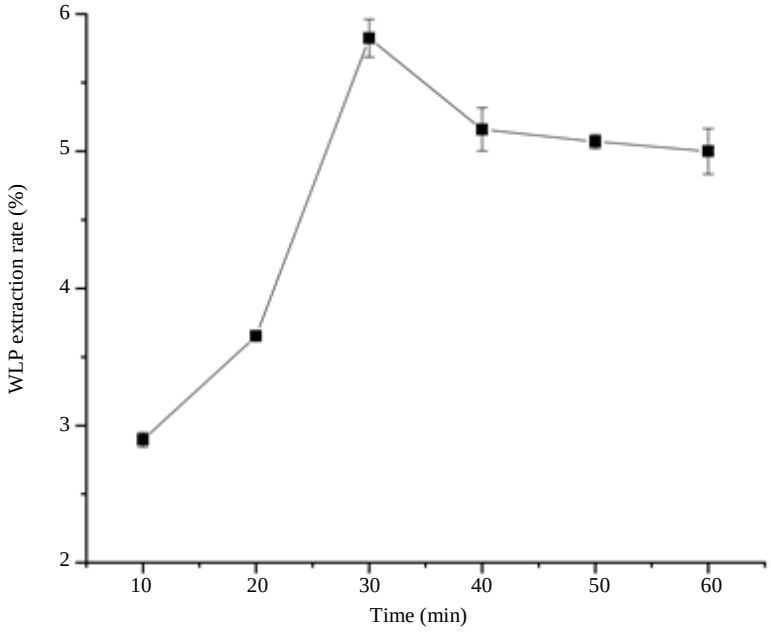


Fig. 3: Effects of enzymolysis time on extraction yield of WLP. Each value represents the mean ± SD of three determinations

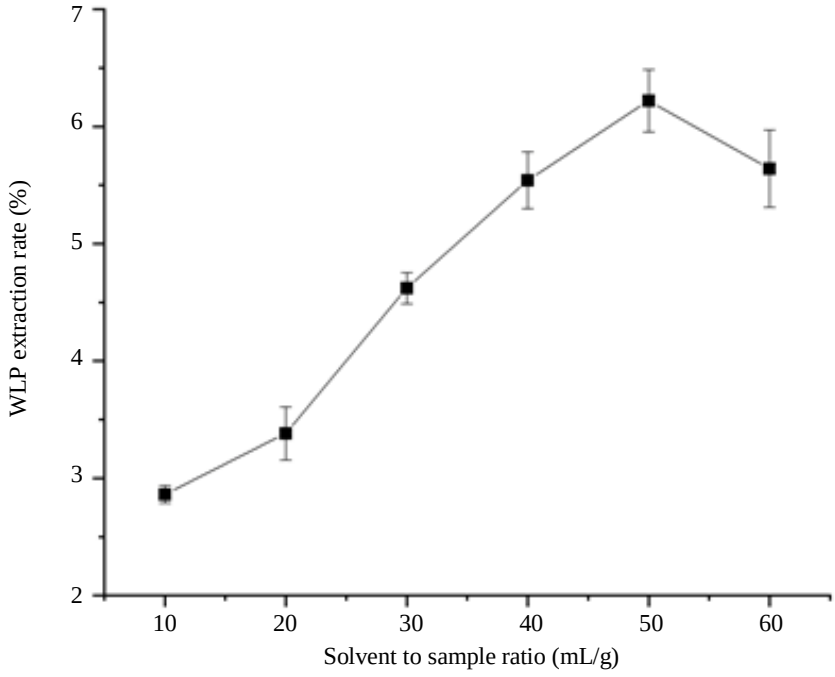


Fig. 4: Effects of solvent to sample ratio on extraction yield of WLP. Each value represents the mean ± SD of three determinations

Effect of Enzymolysis pH on Extraction Yield

In the process of enzymatic reaction, the extracting amount of target constituents can be maximized by different enzymes at the optimum pH value. Extraction technology was performed using various pH range from 4 to 7 and other variables such as cellulase dosage, liquid-material ratio, enzymolysis time and enzymolysis temperature were fixed at 4×10^4 U/g, 30 mL/g, 30 min and 50°C, respectively. As can be seen in Fig. 2, the extraction rate of WLP gradually increased and then declined with the increase of pH value of the sample solution and the yield reached its maximum (5.15%) at pH 6. The interpretation for the

result above is that the steric configuration of the cellulase may change under different acid and base conditions and then alters the enzyme conformation and vitality (Yin *et al*., 2018).

Effect of Enzymolysis Time on Extraction Yield

The effect of enzymolysis time on the extraction yield of WLP at a settled condition including the enzymolysis temperature of 50°C, enzymolysis pH of 6, liquid-material ratio of 30 mL/g and cellulase dosage of 4×10^4 U/g was illustrated in Fig. 3, According to the results, the output of WLP was the highest (5.82%) at the time of 30 min, after more than 30 min, the WLP yield showed a slight decline trend. This tendency of results was also in keeping with Yin *et al*.' s inquiry (Yin *et al*., 2018). The appearance of this phenomenon may be due to the long enzymolysis time leading to degradation of polysaccharides (Chen *et al*., 2017). We will discuss this issue through further experiments.

Effect of Liquid-Material Ratio on Extraction Yield

Extraction yield of bioactive compounds was significantly affected by the ratio of solvent to raw material. The polysaccharides in the raw materials will not be fully extracted with the small ratio of liquid to solid. However, too large ratio of liquid to material will lead to increased processing expenses (Kurd and Samavati, 2015). As shown in Fig. 4, a favorable impact was presented when the liquid-material ratio ranged from 10 to 50 mL/g. After the maximal output (6.21%) was obtained, the extraction yield of WLP declined by degrees with the ulteriorly increase of liquid-material ratio. These results above might be attribute to the increased driving force of mass transfer with the increase of liquid-material ratio, which was beneficial to the desorption of WLP from the cells (Yang *et al*., 2017). Nevertheless, potentially on account of the oversaturation of WLP in water, the productive rate inclined to decline as the liquid- material ratio continued to rised, which is consistent with the previous report by (Yun *et al*., 2019).

Effect of Enzymolysis Temperature on Extraction Yield

The temperature of enzymatic hydrolysis exerts a significant effect on enzyme activity. Moreover, proper heat treatment plays an important role in extraction efficiency and quality loss (Yang *et al*., 2017). In this study, several temperatures including 30, 40, 50, 60 and 70°C were used and the cellulase dosage, enzymolysis pH, enzymolysis time and liquid-material ratio were fixed at 4×10^4 U/g, 6, 30 min and 50 mL/g, respectively. As shown in Fig. 5, with the rising temperature from 30 to 50°C, the extraction yield of WLP increased gradually to a maximum of 4.21% and then decreased with the further increase of enzymolysis temperature. These results were consistent with what has been described in previous studies and might be attributed to the optimum hydrolytic activity of enzymes at the preferred temperature and lower hydrolytic activity of enzymes at other temperatures (Kurd and Samavati, 2015; Mazarei *et al*., 2017). on the other side, higher temperature can accelerate the motion of molecules which improve the electrical conductivity of the solvent to the raw material. The reaction mentioned above enhances the dissolvability and diffusion rate of the materials, thus further increase the extraction rate (Kurd and Samavati, 2015).

Optimization of the Extraction Process by RSM

Models fitting and Statistical Analysis

According to the results of single-factor experiments, we found that cellulase dosage, enzymolysis pH, enzymolysis time and enzymolysis temperature played an important role in the extraction field of WLP. Thus, the extraction technology was further optimized by means of a three level and four-factor RSM employing BBD method. The design matrix and the corresponding results of the WLP yield were listed in Table 2. A variety of multiple regression analysis was used to process the experiment data. The relationship between the response for extraction yield of WLP and the test variables were indicated in terms of following second-order polynomial equations (Equation 6):

$$Y = 5.13 + 0.24X_1 - 0.43X_2 + 0.003703X_3 + 0.43X_4 + 0.27X_1X_2 + 0.038X_1X_3 + 0.10X_1X_4 + 0.42X_2X_3 + 0.66X_2X_4 - 0.18X_3X_4 - 0.78X_1^2 - 2.04X_2^2 - 0.6X_3^3 - 0.89X_4^2$$

where, Y is the yield of *WLP*, X_1, X_2, X_3 and X_4 are the coded variables for the cellulase dosage, enzymolysis pH, enzymolysis time, enzymolysis temperature, respectively.

The quadratic model based on multivariate regression analysis can fully describe the obtained data by evaluating the experimental data. The significance of parameters and the interaction of each coefficient were checked by F-test and P-value. Meanwhile, the precision and validity of the model were evaluated by the values of determination coefficient (R^2) and adjusted determination coefficient (R^2_{adj}) (Zhao *et al*., 2016). Table 3 listed the variance analysis (ANOVA) for the response surface quadratic model of WLP extraction. According to analysis of the model, the high-level *F*-value (15.66) and low-grade *P*-value (< 0.0001) implied that the model was sky-high statistically significance (Chen *et al*., 2017). The lack of fit (F-values of 2.83 and p-value of 0.1641) of the model was insignificant ($p > 0.05$) relative to the pure error. Furthermore, the high degree of correlation between the observed values was

confirmed by the close proximity of the value of the determination coefficient (R^2=0.94) and the adjusted coefficient of determination (R^2_{adj}=0.8799), which also recommended the reasonable and availability of the model (Kurd and Samavati, 2015). Base on the above, the regression model was reasonable.

Table 2: Box-behnken experimental design and results for extraction yield of WLP

Runs	X_1（U/g）	X_2	X_3 (min)	X_4 (°C)	Y (%)
1	-1	0	-1	0	3.77
2	0	0	0	0	4.93
3	0	1	1	0	3.41
4	0	1	0	1	3.49
5	1	0	1	0	3.75
6	0	-1	1	0	3.69
7	0	0	0	0	5.09
8	0	0	0	0	5.37
9	-1	1	0	0	2.29
10	-1	0	1	0	3.33
11	0	0	-1	-1	2.68
12	0	0	0	0	4.96
13	0	0	-1	1	4.26
14	1	-1	0	0	3.74
15	0	-1	0	1	3.62
16	1	0	0	1	4.14
17	0	0	1	-1	3.21
18	-1	-1	0	0	3.59
19	-1	0	0	1	3.49
20	0	1	-1	0	3.12
21	-1	0	0	-1	3.01
22	0	1	0	-1	1.51
23	1	0	-1	0	4.04
24	1	1	0	0	3.52
25	0	0	0	0	5.29
26	0	-1	-1	0	3.56
27	0	-1	0	-1	4.29
28	0	0	1	1	4.08
29	1	0	0	-1	3.23

Table 3: Analysis of the variance (ANOVA) for the second-order polynomial model

Source	Sum of squares	df	Mean square	F value	P value
Model	19.65	14	1.40	15.66	<0.0001[a]
X_1	0.71	1	0.71	7.97	0.0136[b]
X_2	2.21	1	2.21	24.60	0.0002[a]
X_3	1.646×10^{-4}	1	1.646×10^{-4}	1.836×10^{-3}	0.9664[c]
X_4	2.20	1	2.20	24.59	0.0002[a]
X_1X_2	0.30	1	0.30	3.30	0.0909[c]
X_1X_3	5.765×10^{-3}	1	5.765×10^{-3}	0.064	0.8035[c]
X_1X_4	0.044	1	0.044	0.49	0.4960[c]
X_2X_3	6.944×10^{-3}	1	6.944×10^{-3}	0.077	0.7848[c]
X_2X_4	1.76	1	1.76	19.59	0.0006[a]
X_3X_4	0.13	1	0.13	1.41	0.2548[c]
X_1^2	3.92	1	3.92	43.68	<0.0001[a]
X_2^2	6.97	1	6.97	77.80	<0.0001[a]
X_3^2	2.75	1	2.75	30.73	<0.0001[a]
X_4^2	5.13	1	5.13	57.24	<0.0001[a]
Residual	1.25	14	0.09		
Lack of fit	1.10	10	0.11	2.83	0.1641[b]
Pure error	0.16	4	0.039		
Cor total	20.90	28			

[a]$P < 0.01$.
[b]$p < 0.05$.
[c]Not significant.

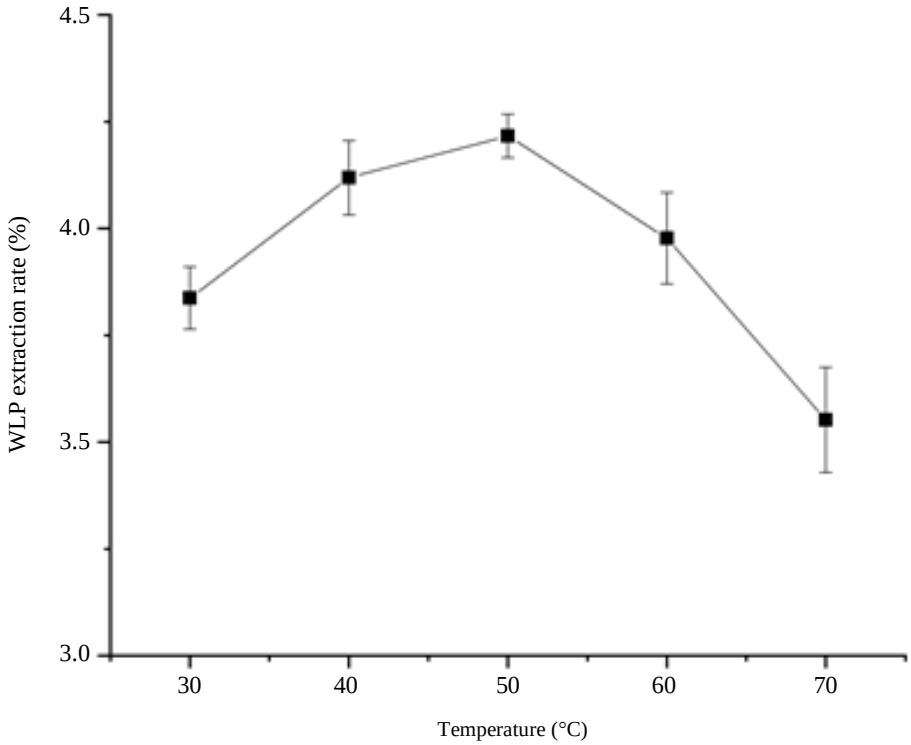
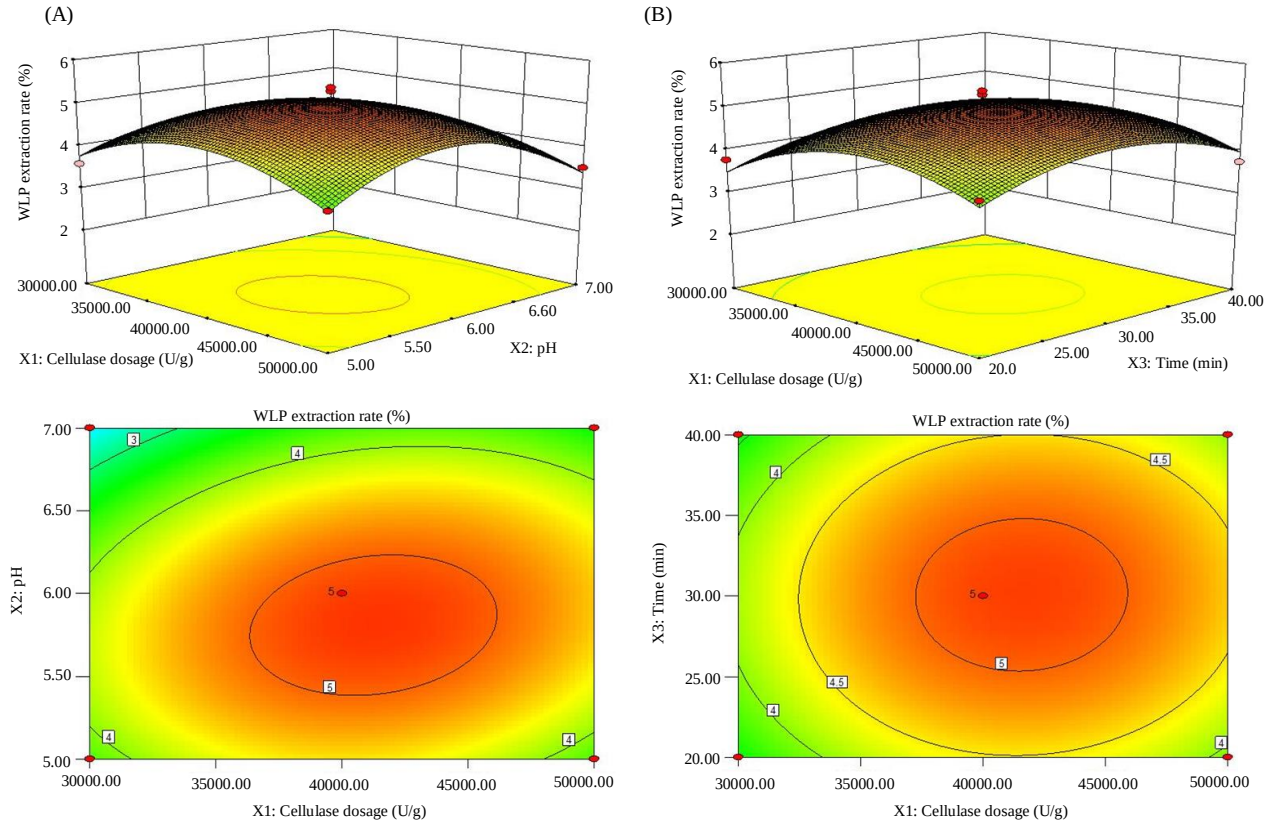


Fig. 5: Effects of enzymolysis temperature on extraction yield of WLP. Each value represents the mean ± SD of three determinations



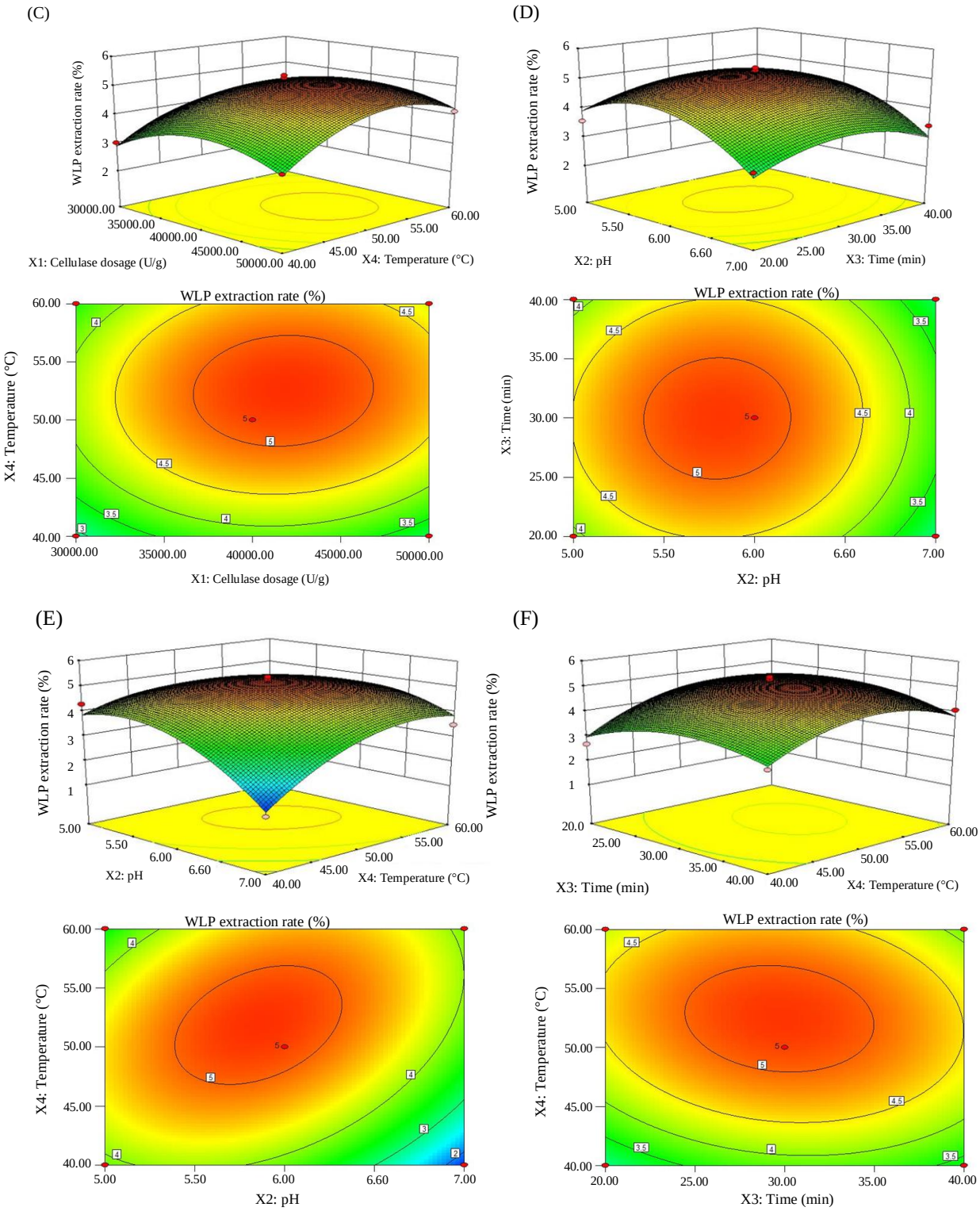
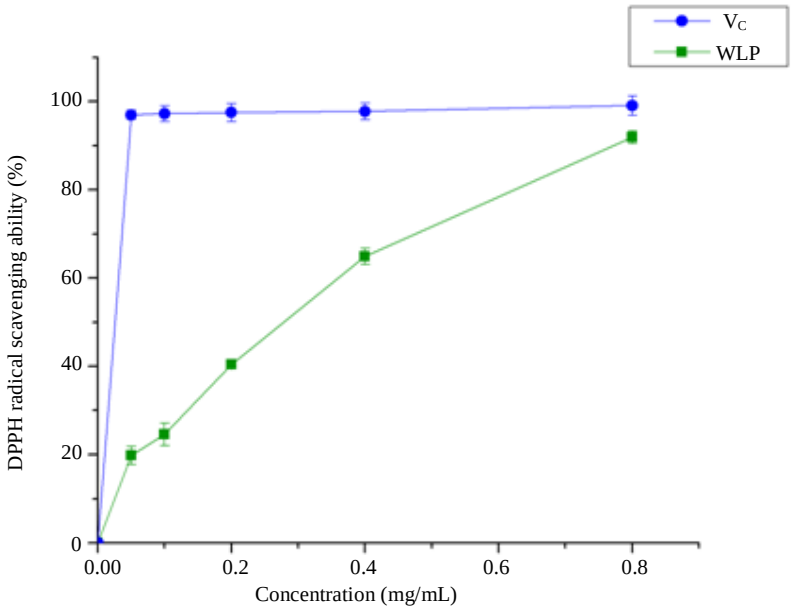
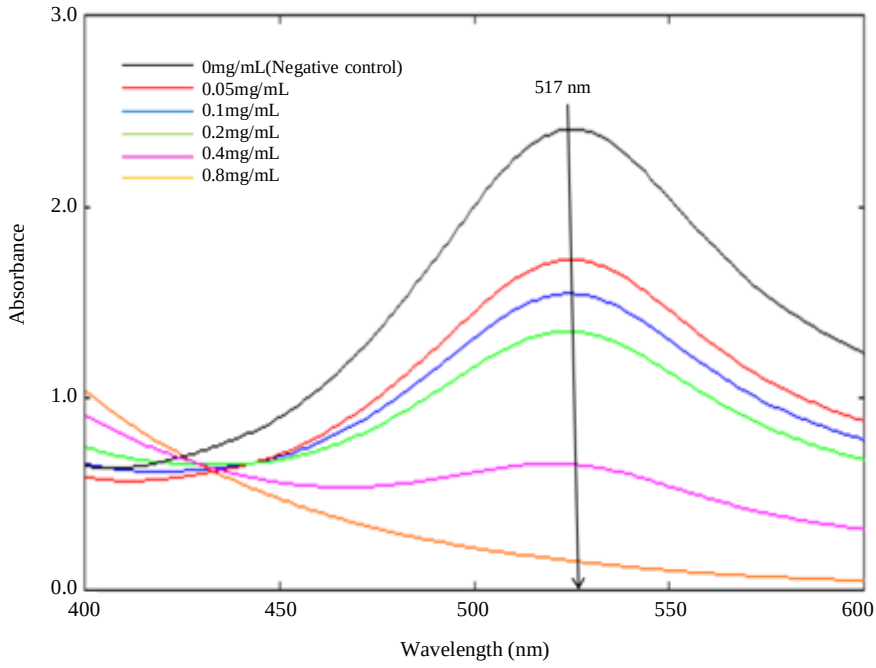


Fig. 6: Response surface plots (2D) and contour plots (3D) (A-F) showing the effect of different variables on the extraction yield of WLP. (A) The effect of cellulase dosage and enzymolysis pH on the WLP extraction rate; (B) The effect of cellulase dosage and enzymolysis time on the WLP extraction rate; (C) The effect of cellulase dosage and enzymolysis temperature on the WLP extraction rate; (D) The effect of enzymolysis pH and enzymolysis time on the WLP extraction rate; (E) The effect of enzymolysis pH and enzymolysis temperature on the WLP extraction rate; (F) The effect of enzymolysis time and enzymolysis temperature on the WLP extraction rate



(a)



(b)

Fig. 7: Inhibition effects of WLP on DPPH free radicals. (a) Concentration-dependent inhibitions of WLP and V_C (positive control) on DPPH free radicals. (b) Changes of UV absorbance spectra after the addition of different concentrations of WLP

The F-values and P-values of each model item are presented in Table 3 as a tool to test the significance of each coefficient. The F-test had a larger F-value and smaller p-value indicated that the more notable of relevant coefficient. Hence, the linear coefficients (X_1, X_2, X_4), cross coefficients (X_2X_4) and quadratic coefficients (X_1^2, X_2^2, X_3^2, X_4^2) all revealed a significant influence ($p<0.05$) on the extraction yield of WLP. The enzymolysis pH (X_2) and enzymolysis temperature (X_4) were the major parameter which influenced the WLP extraction yield followed by cellulase dosage (X_1) and enzymolysis time (X_4). However, coefficient of other terms was not significant ($p>0.05$).

Analysis of Response Surface Plot and Contour Plot

In order to determine the interaction effect of various parameters on the response, triaxial section of the model was need to be generated, which were implemented by altering two parameters in experimental scopes and maintaining the other two variables at zero. As shown in Fig. 6, the interaction of the factors on the WLP extraction yield were inquired employing a three-dimensional response surface and two-dimensional outline plots.

As can be seen from Fig. 6A, the WLP extraction yield was increased in response to the increase of cellulase dosage (X_1) and enzymolysis pH (X_2) when enzymolysis time and temperature were fixed at 0 levels. After acquiring the topmost WLP extraction yield relevanted to the pH-cellulase dosage reveal, undue pH-cellulase dosage exposure would lead to a decline in the extraction rate.

Figure 6B showed the interactions of cellulase dosage (X_1) and enzymolysis time (X_3) on the extraction ratio of WLP. Firstly, the output of WLP increased along with the increase of cellulase dose and enzymolysis time. After the WLP yield reached the maximum, the extraction yield of WLP did not increase even further.

Effect of enzymolysis temperature (X_4) on extraction rate was more obvious than that of the cellulase dosage (X_1), illustrating that the enzymolysis temperature generated a greater effect on WLP yield than cellulase dose (Fig. 6C). Fig. 6C showed that the WLP extraction yield increased firstly and then gradually declined with the increase of cellulase dosage and enzymolysis temperature, which was consistent with our results in Fig. 1A and 1E. Moreover, the maximum extraction yield of WLP was obtained when cellulase dosage and enzymolysis temperature were 41488.52 U/g and 52.07°C, respectively.

Fig. 6D displayed the effects of pH (X_2) and enzymolysis time (X_3) on the extraction yield of WLP. The extraction yield of WLP increased as pH ranged from 5 to 5.88 and enzymolysis time ranged from 20 to 29.74 min, respectively and then decreased slightly.

The contour plots and 3-D response surface at different enzymolysis pH (X_2) and enzymolysis temperature (X_4) as cellulase dose (X_1) and enzymolysis time (X_3) were fixed at 0 levels are shown in Fig. 6E. The highest WLP extraction yield was obtained when the pH and the enzymolysis temperature was 5.88 and 52.07°C, respectively. With further increase of pH and temperature, the declined trend of the extraction yield of WLP was displayed.

In Fig. 6F, the production gained along with the increase of enzymolysis time (X_3) and temperature (X_4). Nevertheless, extended time and higher temperatures slightly reduced the extraction yield of WLP within the test zone.

Verification of Predictive Model

Based on the single factor experiment and 3D response surface, optimum extraction conditions for WLP were as follows: cellulase dosage of 41488.52 U/g, enzymolysis pH of 5.88, enzymolysis time of 29.74 min and enzymolysis temperature of 52.07°C. Under these optimum extraction conditions, the maximum predicted value of extraction yield of WLP was 5.21%.

To verify the feasibility and reliability of the model, triplicate confirmatory experiments were executed under conditions including cellulase dosage of 41000 U/g, enzymolysis pH of 5.8, enzymolysis time of 30 min and enzymolysis temperature of 52°C and the extraction yield of WLP was 5.24%, which was closely approached the predicted value. Base on the above, the model used in this study was adequate for assessing the extraction field of WLP.

DPPH free Radical Scavenging Activity

DPPH free radical is a stable lipophilic free radical with a maximum absorption at 517 nm. Thereby, it has been widely used to investigate the antioxidant property of natural compounds in vitro (Huang *et al*., 2016; Mzoughi *et al*., 2018). The DPPH radical scavenging capacities of WLP at different concentrations (0.05-0.8 mg/mL) were studied with ascorbic acid as a reference. As can be seen in Fig. 7A and B, both WLP and ascorbic acid exhibited obvious scavenging effect on DPPH radical in a dose-dependent manner at a comparatively lower concentration. At the concentration of 0.8 mg/mL, the scavenging activities of WLP and ascorbic acid were 81.93% and 98.06%, respectively. Moreover, the variational tendency in absorption bands at 517 nm from the ultraviolet-visible (UV−vis) spectral scanning confirmed the DPPH radical scavenging ability.

Studies showed that polysaccharide from olive leaves also had good scavenging ability to DPPH free radicals (Khemakhem *et al*., 2018).

ABTS Free Radical Scavenging Activity

ABTS is extensively usedto evaluate the scavenging power of natural products extracted from various plants (Meng *et al*., 2017). Therefore, ABTS free radical was also applied to assess the antioxidant property of WLP in our examination. The antioxidant ability of all samples on ABTS free radicals were displayed in Fig. 8A and B. Obviously, the outcomes declared the WLP behaved strongly ABTS scavenging capacities at all tested concentrations and the ABTS scavenging ability of WLP strengthened drastically with the increase of WLP dosage. Additionally, WLP exhibited the highest ABTS scavenging ability of 94.8% at a concentration of 0.8 mg/mL, which was close to the highest ABTS scavenging ability of ascorbic acid (99.42%). Previous studies have reported that the ABTS scavenging activity of *gracilaria rubra* crude polysaccharides was 55.34% at the concentration of 2.5 mg/mL and a polysaccharide named DJP-2 from diaphragma juglandis fructus demonstrated ABTS radical scavenging function of 78.31% at a concentration of 4 mg/mL (Di *et al*., 2017; Meng *et al*., 2017).

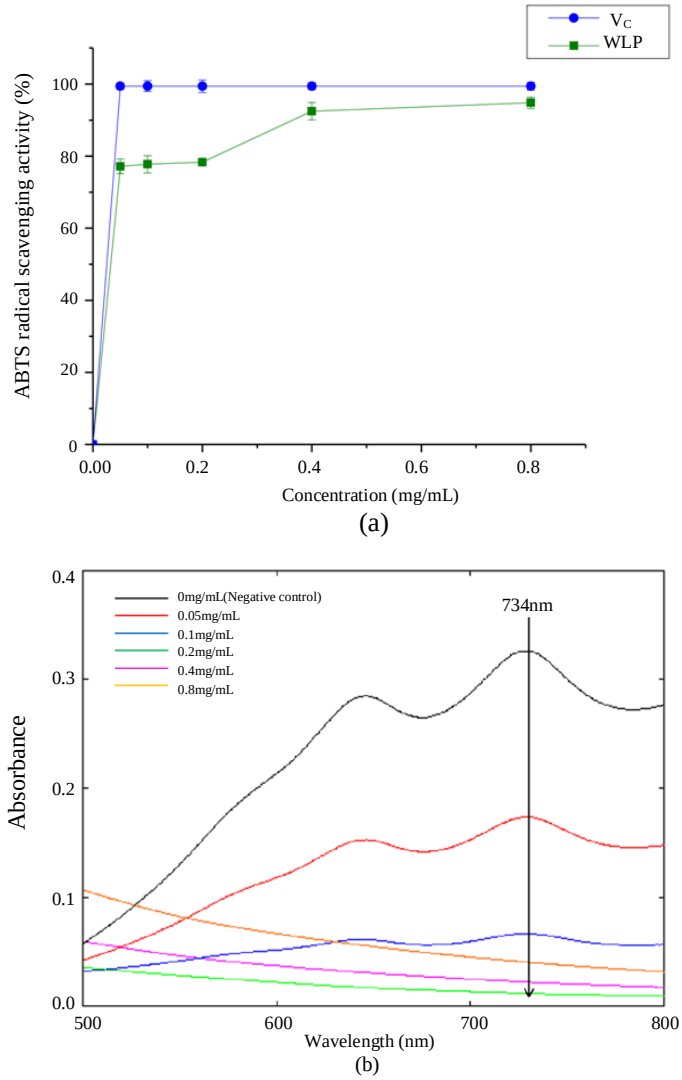
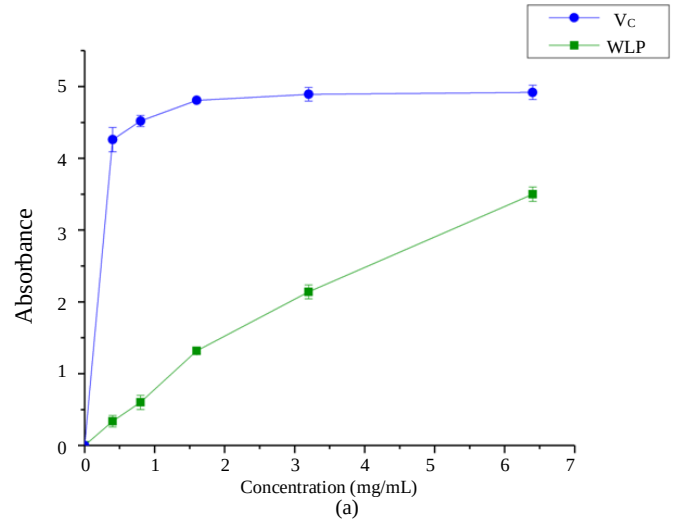


Fig. 8: Inhibition effects of WLP on ABTS free radicals. (a) Concentration-dependent inhibitions of WLP and V_C (positive control) on ABTS free radicals. (b) Changes of UV absorbance spectra after the addition of different concentrations of WLP



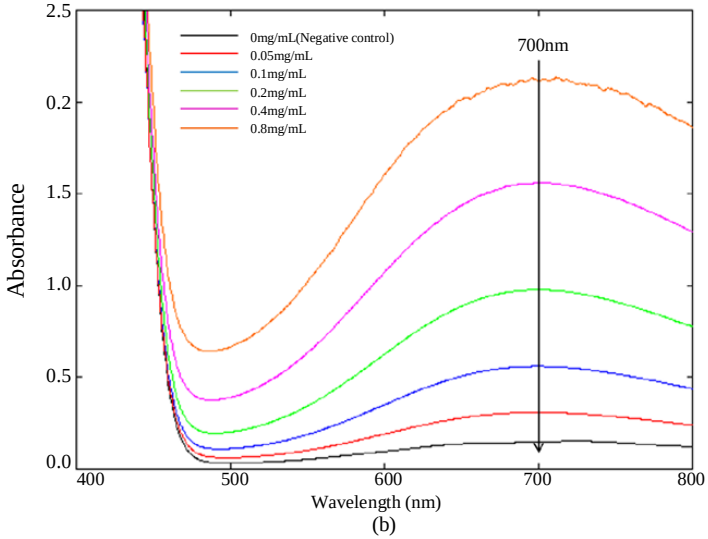


Fig. 9: Reducing power of WLP. (a) Concentration-dependent reducing power of WLP and V_C (positive control). (b) Changes of UV absorbance spectra after the addition of different concentrations of WLP

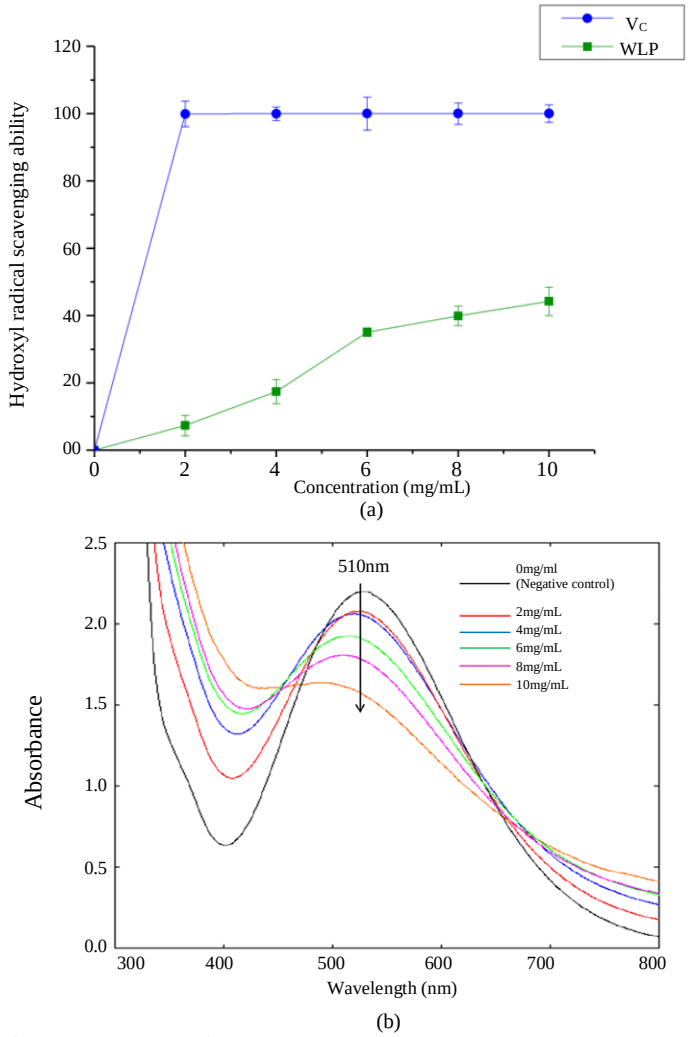


Fig. 10: Inhibition effects of WLP on hydroxyl free radicals. (a) Concentration-dependent inhibitions of WLP and V_C (positive control) on hydroxyl free radicals. (b) Changes of UV absorbance spectra after the addition of different concentrations of WLP

FRAP Assay

It has been reported that the antioxidant activity of active substance is directly related to its reducing power (Khemakhem *et al*., 2018). Ferrous ion is considered to be an effective food pro-oxidant because of its role in promoting lipid peroxidation (Huang *et al*., 2016). Therefore, the FRAP test using the principle of ferrous ion and TPTZ (tripyridyl-triazine) to generate blue-purple complex is widely used in analyzing antioxidant capacity of natural active components and is determined by measuring the absorbance of reaction blend at 700 nm. The higher absorbance value manifested stronger reducing power of natural antioxidants (Yin *et al*., 2018).

The FRAP of WLP was determined in this study and the results were shown in Fig. 9A and B. WLP displayed significant FRAP activities in a concentration-dependent pattern within the test concentration range (0.4-6.4 mg/mL). Nevertheless, the FRAP of WLP was weaker than that of ascorbic acid. According to the previous reports, polysaccharides extracted from *Lentinus edodes* and *Euryale ferox* salisb. exhibited obvious FRAP activities (Wu *et al*., 2014; Yin *et al*., 2018). The consequences disclosed that WLP could act as an electron donor in radical chain reactions, react with free radicals to transform them into further stabilized end-product.

Hydroxyl Radical Scavenging Activity

Hydroxyl radical is the most oxidizing radical and can non-specifically oxygenate different types of biomolecules in the tissue cells which result in cytotoxicity, carcinogenesis and other diseases (Li and Shah, 2014; Xu *et al*., 2012). Hence, the hydroxyl radical eliminating power is also one of the crucial indications of antioxidant abilities. As illustrated in Fig. 10A and B, WLP exhibited a reversely limited scavenging activities on hydroxyl radicals compared with ascorbic acid within a concentration range of 2 to 10 mg/mL. At the concentration of 10 mg/mL, the scavenging effects of WLP and ascorbic acid on hydroxyl free radical were 44.23% and 100%, respectively, which was similar to the interpretations of (He *et al*., 2011; Xu *et al*., 2012).

Conclusion

In this study, the crude polysaccharide was successfully extracted from walnut leaves using optimization of cellulase assisted water extraction process. The antioxidant activities of WLP *in vitro* were determined by four indexes including DPPH, ABTS and hydroxyl radical scavenging abilities as well as reducing power. The results indicated that WLP exhibited a higher free-radical scavenging ability in a dose-dependent manner. Based on the above, polysaccharide acquired from walnut leaves is a potential natural antioxidant agent, which is expected to be used in functional food or medicine industry. What's more important, future work will focus on exploring the mechanism of its functional activity.

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Author's Contributions

Shuqing Yang: Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

Haifang Xiao: Contributed to the study design, the interpretation of the results and manuscript preparation.

Shuyan Yu: Participated in part of the experimental design.

Zhike Xie: Participated in part of the experimental design.

Shaoxuan Yu: Ameliorated the manuscript.

Wanting Sun: Contributed to the experiment of polysaccharide extraction.

Yuanda Song: Contributed to the guidance of experimental design and ameliorated the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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