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Ultrasonic-assisted extraction, antimicrobial and antioxidant activities of *Cyclocarya paliurus* (Batal.) Iljinskaja polysaccharides

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ABSTRACT

Recently, renewed interest has grown in the use of ultrasonic-assisted extraction as an alternative approach to the traditional extraction methods. In this study, this novel extraction method was used to isolate polysaccharides from *Cyclocarya paliurus* (Batal.) Iljinskaja, and response surface methodology (RSM) was employed to optimize the extraction conditions. The optimal conditions for the extraction of polysaccharides were determined to be the ratio of liquid to solid of 8, extraction time of 59 min and extraction temperature of 58 °C. Under these optimal conditions, the yield of polysaccharides obtained was 4.91 ± 0.11%, which was well matched with the value predicted by the model. *In vitro* antioxidant assays showed that the polysaccharides exhibited strong DPPH radicals (92.09% at 0.25 mg/ml) and self-oxidation of 1,2,3-phentriol (37.22% at 1 mg/ml) scavenging activities, moderate hydroxyl radicals (43.18% at 1 mg/ml) scavenging activity and lipid peroxidation inhibitory effect (31.66% at 1 mg/ml). In addition, the polysaccharides showed moderate antimicrobial activity.

1. Introduction

Cyclocarya paliurus (Batal.) Iljinskaja (*C. paliurus*), commonly known as "sweet tea tree", is a well-known edible and medicinal plant, grown on foggy highlands in southern China. In recent years, *C. paliurus* has gained increasing attention due to its unique biological activities (Xie & Xie, 2008), such as antihypertensive activity (Kurihara, Fukami, et al., 2003), hypolipidaemic (Kurihara, Asami, Shibata, Fukami, & Tanaka, 2003), hypoglycemic activity (Kurihara, Fukami, et al., 2003; Xie, Li, Nie, Wang, & Lee, 2006), enhancement of mental efficiency (Li et al., 2000) and antioxidant activity (Dong et al., 2007). The constituents isolated from its leaves consist of protein, polysaccharides, triterpenoids, flavonoids, steroids, saponins, phenolic compounds, etc. (Jiang, Zhang, Zhou, Qiu, & Chen, 2006; Kennelly et al., 1995; Shu, Xu, Li, & Yu, 1995; Xie et al., 2006; Xie, Wang, Yi, & Wang, 2004; Xie & Xie, 2008; Xie, Xie, Nie, et al., 2010; Xie, Xie, Shen, et al., 2010; Zhang et al., 2010). Among these compounds, the unique characteristics of its polysaccharides have drawn much attention (Xie et al., 2006). Crude polysaccharide from the leaves of *C. paliurus* has been reported to exhibit hypoglycemic activity in diabetic mice (Xie et al., 2006). In addition, CPP-1, a water-soluble polysaccharide from the leaves of *C. paliurus*, was shown to possess strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging activity (Xie, Xie, Nie, et al., 2010). Until now, there has been little information on the antimicrobial potentials of polysaccharides from *C. paliurus*.

Conventional techniques to obtain polysaccharides, such as heating, boiling, or refluxing, usually require long extraction time and high extraction temperature, but the extraction efficiency was low. Recently, the use of ultrasonic-assisted extraction of constituents from different materials has been shown to have tremendous research potential (Tsochatzidis, Guiraud, Wilhelm, & Delmas, 2001). Compared with traditional methods, this extraction approach has many advantages, such as shorter extraction time, use of less solvent and higher extraction rate (Chen et al., 2010; Chen & Zhang, 2007). So far, it has been widely employed to extract polysaccharides from different materials with great extraction efficiency (Hromadkova, Ebringerova, & Valachovic, 1999; Wang, Cheng, Mao, Fan, & Wu, 2009; Wu, Cui, Tang, & Gu, 2007; Yan et al., 2011; Zhong & Wang, 2010). This great extraction efficiency by ultrasonic treatment is mainly attributed to its mechanical effects, which greatly facilitate mass transfer between immiscible phases through a super agitation (Zhong & Wang, 2010).

To the best of our knowledge, there were no reports available in the literature regarding the optimization of ultrasonic-assisted extraction of polysaccharides from the leaves of *C. paliurus* by RSM. In this study, the ultrasonic-assisted extraction parameters (ratio

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Table 1
Program and test results of RSM.

Experiment number	Coded variables			Actual variables			Observed yield (%)
	Ratio of liquid to solid (ml/g) x_1	Temperature (°C) x_2	Time (min) x_3	Ratio of liquid to solid (ml/g) X_1	Temperature (°C) X_2	Time (min) X_3	
1	−1	−1	0	8	50	60	4.69
2	−1	1	0	8	50	60	4.12
3	1	−1	0	16	70	60	3.59
4	1	1	0	16	70	60	3.94
5	0	−1	−1	12	50	50	3.89
6	0	−1	1	12	50	70	3.99
7	0	1	−1	12	70	50	4.23
8	0	1	1	12	70	70	3.89
9	−1	0	−1	8	60	50	4.63
10	1	0	−1	16	60	50	3.92
11	−1	0	1	8	60	70	4.68
12	1	0	1	16	60	70	4.36
13	0	0	0	12	60	60	4.83
14	0	0	0	12	60	60	4.80
15	0	0	0	12	60	60	4.76

of liquid to solid, extraction temperature and time) of polysaccharides from the leaves of *C. paliurus* was firstly investigated and optimized using a three-level, three-variable Box–Behnken design (BBD). Antioxidant properties of the polysaccharides were investigated by various *in vitro* assays. Additionally, the polysaccharides were tested for antimicrobial activity against bacteria, yeast and fungi.

2. Materials and methods

2.1. Materials and chemicals

The leaves of *C. paliurus*, grown in Xiushui County, Jiangxi Province, China, were provided by Jiangxi Xiushui Miraculous Tea Industry Co. (Jiangxi, China). The leaves were air dried and ground into fine powder before extraction.

DPPH, ascorbic acid and D-glucose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the study were purchased from Shanghai Chemicals and Reagents Co. (Shanghai, China) and were of analytical grade. Aqueous solutions were prepared with ultra pure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Extraction procedure

Extraction of polysaccharides was conducted by the method of Xie, Shen, Nie, Li and Xie (2011) with some modifications. The powder of *C. paliurus* leaves (5 kg) was firstly extracted with 10 l of 80% ethanol for 24 h to remove interference components such as monosaccharide, disaccharide, oligosaccharide and polyphenols in the samples. The pretreated samples were separated from the organic solvent through the nylon cloth (pore diameter: 38 μm). The extraction of polysaccharides from *C. paliurus* leaves by ultrasonic treatment was performed with a power of 100 W ultrasonic bath (KQ2200E, Kunshan Ultrasonic Instrument Co., Jiangsu, China). Each pretreated power (2 g) was put into a 100 ml beaker and extracted by water in a designed ratio of liquid to solid, extraction temperature and time. After extraction, the water extraction solutions were separated from insoluble residue by centrifugation (8400 × *g* for 10 min, at 20 °C), followed by precipitation with the addition of alcohol to a final concentration of 80% (v/v). The precipitates collected by centrifugation at 8000 × *g* for 5 min in a high speed centrifuge (3K3D, Sigma, Germany) were washed by precipitation using 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifuging, the precipitates were collected and vacuum-dried. Finally the precipitates were lyophilized in a vacuum freeze dryer (ALPHA 2-4, Christ, Germany) to obtain CPP.

A comparative study was conducted between the conventional heat reflux extraction and the developed ultrasonic-assisted extraction after the optimization of the latter. The conventional heat reflux extraction used in this study was similar to that described by Xie, Xie, Shen, et al. (2010). The procedure was performed in a water-bath (HH-4, Guohua Electric Co., Jiangsu, China) using the following extraction conditions: ratio of water to material of 20 ml/g, extraction in 100 °C water bath for 180 min. After extraction, the post-treatment of the water extraction solutions was the same as that mentioned in the ultrasonic-assisted extraction. In this study, all the experiments were conducted in triplicate.

2.3. Experimental design

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for improving and optimizing processes, and it is used to examine the relationship between one or more response variables and a set of quantitative experimental variables or factors (Xie, Xie, Shen, et al., 2010). In this study, RSM was used to determine the optimal extraction condition of polysaccharides. A three-level three-factor BBD was chosen to evaluate the combined effect of three independent variables: ratio of liquid to solid, extraction temperature and time, coded as X_1, X_2 and X_3, respectively. For each factor, the experimental range was determined based on the results of single factor experiment. Each independent variable had coded levels of −1, 0 and 1. The experimental designs of the coded (x) and actual (X) levels of variables were shown in Table 1. The three independent variables were coded according to the following equation (Shan, Xie, Zhu, & Peng, 2012):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3 \tag{1}$$

where x_i is the coded value of an independent variable, X_i is the actual value of the independent variable, X_0 is the actual value of the independent variable at the center point, and ΔX is the step change value.

CPP yield was considered as the dependent variable or response. For a Box–Behnken design with three independent variables at three levels, 15 experimental runs were required. The design of experiments was given in Table 1.

A second-order polynomial model corresponding to the BBD was fitted to correlate the relationship between the independent

variables and the response to predict the optimized conditions. The general form of quadratic polynomial equation was expressed according to the following equation:

$$Y = B_0 + \sum_{i=1}^{n} B_i X_i + \sum_{i=j=1}^{n} B_{ij} X_i X_j \quad (2)$$

In this study, Eq. (2) could be converted into the following equation according to the value of three variables:

$$Y = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_{12}x_1x_2 + B_{13}x_1x_3 + B_{23}x_2x_3 + B_{11}x_1^2 + B_{22}x_2^2 + B_{33}x_3^2 \quad (3)$$

where Y is the predicted response, x_1, x_2 and x_3 are the coded values of ratio of liquid to solid, extraction temperature (°C) and extraction time (min), respectively.

The significance in the model was evaluated by analysis of variance (ANOVA) for each response. The adequacy of model was checked accounting for the coefficient of determination (R^2) and adjusted coefficient of determination (R^2_{Adj}). The three-dimensional (3D) response surface plots were used for interpretation of the interaction effects of two independent variables on the responses or dependent variables when a third factor was kept at a constant level.

2.4. Determination of CPP content

CPP was precipitated by 95% (v/v) ethanol, and then separated by centrifugation (8400 × *g* for 10 min, at 20 °C). The precipitate was dissolved in water and CPP content determined by phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with D-glucose as a standard at 490 nm.

The yield of polysaccharides in leaves of *C. paliurus* was calculated by the following equation:

$$\text{Polysaccharides yield } \%(\text{w/w}) = \frac{\text{polysaccharides weight}}{\text{leaves weight}} \times 100 \quad (4)$$

2.5. Scavenging activity of hydroxyl radicals

The hydroxyl radical-scavenging abilities of polysaccharide samples were measured by the deoxyribose assay (Halliwell, Gutteridge, & Aruoma, 1987). Samples were dissolved in distilled water to form final concentrations of 0.125, 0.25, 0.375, 0.5, 0.75 and 1 mg/ml, respectively. 1 ml of CPP solution was mixed with 2 ml 0.05 M phosphate buffer (PBS) (pH 7.4), 1.5 ml 5 mM 1,10-phenanthroline, 1 ml 7.5 mM $FeSO_4$ and 1 ml H_2O_2 (0.1%) were added into a tube. After incubation for 60 min at 37 °C, the absorbance of the mixture was measured at 536 nm, with ascorbic acid as a positive control. The scavenging activity of hydroxyl radicals was calculated using the following formula:

$$\text{Scavenging activity}\,(\%) = \frac{A_2 - A_1}{A_0 - A_1} \times 100$$

where A_1 is the absorbance of control, A_2 is the absorbance of polysaccharide sample and A_0 is the absorbance of the solution without polysaccharide sample and H_2O_2.

2.6. Scavenging activity of DPPH radical

Here DPPH radical scavenging activity is based on the determination by DPPH at a steady state in ethanol solution after adding the mixture of sample solutions or control. The DPPH radical scavenging activities of polysaccharide samples were determined according to the method of our previous study (Xie, Xie, Nie, et al., 2010). Briefly, 0.1 mM DPPH in 95% ethanol was freshly prepared before measurements, 2 ml of various concentrations (0.016, 0.031, 0.063, 0.125 and 0.25 mg/ml) of the samples was thoroughly mixed with 2 ml of 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 by ultraviolet–visible spectrophotometer (TU-1900, Pgenenal, Beijing, China). Lower absorbance values of the reaction mixture indicated higher free radical scavenging activity. 95% ethanol was used as the blank control, and ascorbic acid was used as a positive control. The ability to scavenge the free radical DPPH in percentage of sample was calculated according to the following equation:

$$\text{Scavenging activity}\,(\%) = \frac{1 - (A_2 - A_1)}{A0} \times 100$$

where A_0 is the absorbance of the DPPH solution without addition of the sample or positive control, A_1 is the absorbance of the sample without DPPH solution, and A_2 is the absorbance of the incubation mixture containing both the sample and DPPH solution.

2.7. Inhibitory effect on self-oxidation of 1,2,3-phentriol

The scavenging capacities for self-oxidation of 1,2,3-phentriol of all polysaccharide samples were assessed according to the method of Marklund and Marklund (1974) with a minor modification. In this experiment, 4.5 ml 0.05 M Tris–HCl buffer (pH 8.2) was mixed with 3.9 ml ultra pure water and 1 ml of CPP solution at different concentrations (0.063, 0.125, 0.25, 0.5 and 1 mg/ml). The solution was incubated in a water bath at 25 °C, then 0.3 ml 0.045 M 1,2,3-phentriol at the same temperature was added to the solution and the mixture was shaken rapidly. The change speed of absorbance (A/min) of the reactive solution was measured at 325 nm per 10 s, against a blank (water and 50 mM PBS instead of sample). The scavenging activity for self-oxidation of 1,2,3-phentriol was determined as follows:

$$\text{Scavenging activity}\,(\%) = \frac{1 - A_1}{A_0} \times 100$$

where A_0 is the change speed of absorbance of the control group in the superoxide radical generation system and A_1 is the change speed of absorbance of the sample.

2.8. Inhibitory effect on lipid peroxidation

Lipid peroxidation was estimated by the method of Yin, Nie, Zhou, Wan and Xie (2010) with a few modifications. In brief, an equal volume yolk was added into PBS (pH 7.45, 0.1 M). The mixture was stirred by magnetic force for 10 min and diluted with 24 times volume of the PBS. The yolk homogenates (1 ml), sample (0.5 ml), PBS (1 ml) and $FeSO_4$ (1 ml, 25 mM) were mixed in a tube and shaken at 37 °C for 15 min. The reaction was stopped by trichloroacetic acid and the mixture was centrifuged. After 1 ml thiobarbituric acid (8.0 g/l) was added into 3 ml of the supernatant, the mixture was heated at 100 °C for 10 min. The absorbance of mixture was recorded at 532 nm. The inhibition ratio was calculated according to the formula below:

$$\text{Inhibiting ratio}\,(\%) = \frac{1 - A}{A_0} \times 100$$

where A_0 is the absorbance value of the control and A is the absorbance in the presence of polysaccharide sample.

2.9. Antimicrobial activity

Bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Aerobacter aerogenu*, *Proteus vulgari*), yeast (*Saccharomyces cerevisiae*, *Candida* sp.) and fungi (*Aspergillus niger*, *Mucor*, *Penicillium*

Table 2
Regression coefficient, standard error, and Student's *t*-test results of response surface for the yields of CPP extracted with ultrasonic-assisted extraction.

Parameter	DF	Estimate	Standard error	*t*-Value	Pr > |*t*|
x_1	1	−0.285	0.053	−5.412	0.003
x_2	1	0.003	0.053	0.047	0.964
x_3	1	0.035	0.053	0.665	0.536
$x_1 * x_1$	1	−0.153	0.078	−1.978	0.105
$x_2 * x_1$	1	0.230	0.074	3.088	0.027
$x_2 * x_2$	1	0.105	0.074	1.410	0.217
$x_3 * x_1$	1	−0.558	0.078	−7.203	0.001
$x_3 * x_2$	1	−0.110	0.074	−1.477	0.200
$x_3 * x_3$	1	−0.238	0.078	−3.075	0.028

DF: degrees of freedom.

sp.) were selected as test organisms in this study. The microorganisms used for the assay were obtained from Microbiology Laboratory, College of Life Science and Food Engineering, Nanchang University. Bacteria were cultured in nutrient broth (Difco, Sparks, MD, USA) at 37 °C for 18 h. Yeast and fungi were cultured in Sabouraud medium at 28 °C for 24 h. Antibacterial activity was evaluated by the filter disc diffusion plate method (Li, Zhou, & Han, 2006) with a few modifications. The polysaccharides were dissolved in a minimum value of water and added to the nutrient medium. Nutrient agar was prepared by autoclaving before the addition of the polysaccharides. A suspension of an overnight culture of each test organism containing 10^6 cells/ml was added to the medium. A paper disk, 6 mm in diameter and 1.5 mm thick, containing 0.4 ml of sample (0.5 mg/ml and 1.0 mg/ml) was placed in the center of the plate after the top agar had solidified. Bacterial plates were incubated at 37 °C for 24 h while yeast and fungi plates were incubated at 28 °C for 48 h. The antimicrobial activity was assessed by the diameter (mm) of the inhibition zones, measured using calipers. A blank plate containing only nutrient agar served as a control. Each test was replicated three times.

2.10. Statistical analysis

All experimental results were expressed as mean ± SD of three parallel measurements and the data of RSM were analyzed using SAS (SAS Institute Inc., Cary, NC, USA). Fischer's test was used to determine the type of model equation, while the Student's *t*-test was performed for the determination of statistical significance of regression coefficients. *P* values less than 0.01 and 0.005 were regarded as significant and highly significant, respectively.

3. Results and discussion

3.1. Model fitting and statistical analysis

The effects of three ultrasonic-assisted extraction conditions, including ratio of liquid to solid, extraction temperature and time on the responses (yields of CPP) were examined using the BBD of RSM (Table 1).

By applying multiple regression analysis and the data shown in Table 1 the predicted response *Y* for the yield of CPP could be obtained by the following second-order polynomial equation:

$$Y = 4.797 - 0.2888X_1 + 0.0025X_2 + 0.0313X_3 - 0.1571X_1^2 + 0.23X_1X_2 + 0.0975X_1X_3 - 0.5546X_2^2 - 0.116X_2X_3 - 0.2421X_3^2 \quad (5)$$

The coefficients of the above Eq. (5) were calculated using RSM, and statistical analysis of the experimental data was used to establish the best-fitted models for the independent variables. To choose the best model matched with the data, the analysis of variances (the linearity and quadratic effect of the treatments variables, their interactions and coefficients on the response variables) was employed (Tables 2 and 3). It can be observed that the first order main effect of ratio of liquid to solid (x_1) was highly significant ($P < 0.005$, Table 3), suggesting that the ratio of liquid to solid was directly related to the CPP yield.

ANOVA showed that the selected quadratic models adequately represented the data obtained for CPP. The corresponding variables would be more significant if the absolute *F*-value becomes greater and the *P*-value becomes smaller. It can be seen that the variables with the largest effect were the linear terms of ratio of liquid to solid. *F*-test suggested that the model had a very high *F* value ($F = 11.4765$) and a very low *P*-value ($P < 0.01$), indicating that this model was highly significant (Table 3). In addition, the value of the determination coefficient R^2 was 0.9578, implying that the sample variation of 95.78% for the yield of CPP was attributable to the independent variables, and the adjusted correlation coefficient of determination (R^2_{Adj}) of the equation was 0.8712, suggesting an excellent correlation between the independent variables.

3.2. Analysis of response surfaces

The 3D response surface plots provided a method to visualize the relationship between responses and experimental levels of each variable, the type of interactions between two test variables, and to determine the optimum concentration of each factor for maximum CPP yield. In each plot, the interaction of two variables was investigated simultaneously, while the third one was in its middle level value. The 3D response surfaces plotted by SAS software of the independent variables on response (*Y*) were shown in Fig. 1.

Fig. 1A was the 3D surface plot showing the effects of ratio of liquid to solid (X_1) and extraction temperature (X_2) on the yield of CPP, while the extraction time (X_3) was fixed at its central levels. Both ratio of liquid to solid and extraction temperature exerted a quadratic effect on CPP production (Fig. 1A). Moreover, the ratio of liquid to solid had a positive linear effect on the CPP yield, and the CPP yield increased as the ratio of liquid to solid was raised from 8 to 16 during the extraction process. And a greater increase in the yield of polysaccharides occurred when the temperature was increased from 50 to 60 °C (Fig. 1A). For very high extraction temperatures (60–70 °C), however, the negative quadratic effect also became significant, higher extraction temperatures beyond 60 °C did not show any significant improvement in the extraction yield. This could be attributed to the thermal degradation of CPP at high temperature conditions (Li, Ding, & Ding, 2007). These results were in accordance with the extraction of polysaccharides from *Zizyphus jujuba* cv. *Jinsixiaozao* (Li et al., 2007) and non-polar compounds from *Anastatica hierochuntica* (Norulaini et al., 2009). Fig. 1B represents the effects of ratio of liquid to solid (X_1) and extraction time (X_3), and their reciprocal interactions on CPP yield at a constant temperature of 60 °C. The extraction yield initially increased when there was an increase in the ratio of liquid to solid and extraction time. The ratio of liquid to solid gave a slight increase in CPP yield whereas

Table 3
ANOVA for the regression model of ultrasonic-assisted extraction conditions.

Source	DF	SS	Mean square	*F*-value	Pr > *F*
x_1	1	0.6498	0.6498	29.2922	0.0029
x_2	1	0.0001	2.6340	0.0023	0.9640
x_3	1	0.0098	0.0098	0.4418	0.5357
$x_1 * x_1$	1	0.0868	0.0868	3.9133	0.1048
$x_2 * x_1$	1	0.2116	0.2116	9.5387	0.0273
$x_2 * x_2$	1	1.1510	1.1510	51.887	0.0008
$x_3 * x_1$	1	0.0441	0.0441	1.9880	0.2176
$x_3 * x_2$	1	0.0484	0.0484	2.1818	0.1997
$x_3 * x_3$	1	0.2097	0.2097	9.4545	0.0276
Model	9	2.2913	0.2546	11.4765	0.0076
Error	5	0.1109	0.0222		
Total model	14	2.4022			
$R^2 = 0.9578$Adj. $R^2 = 0.8712$					

DF: degrees of freedom; SS: sum of squares.

extraction time gave an exponential increase to CPP yield within the first 60 min and gradually became almost constant at the following time from 60 to 70 min. The CPP yield of the extract ranged from 4.22% to 4.91% at 60 °C (Fig. 1B). However, results showed that interactions between the variables were insignificant ($P > 0.05$, Table 2). Fig. 1C shows the effects of extraction temperature (X_2) and time (X_3) on the yield of CPP when the ratio of liquid to solid (X_1) was fixed at 1:12. The extraction temperature and time also had a similar effect on the CPP yield when the ratio of liquid to solid was fixed (Fig. 1C). Nevertheless, longer or shorter time led to a decrease in the CPP yield (Fig. 1C). At this fixed ratio of liquid to solid, the maximal yield (4.81%) was obtained when a time of 60 min and a temperature of 60 °C were used.

3.3. Optimization of extraction parameters and validation of the model

Through these 3D plots and their respective contour plots (Fig. 1), the optimal values of the tested variables of ultrasonic-assisted extraction process for obtaining the highest CPP yield can be predicted as follows: ratio of liquid to solid, 7.46; extraction temperature, 58.01 °C; and extraction time, 59.05 min. Under these

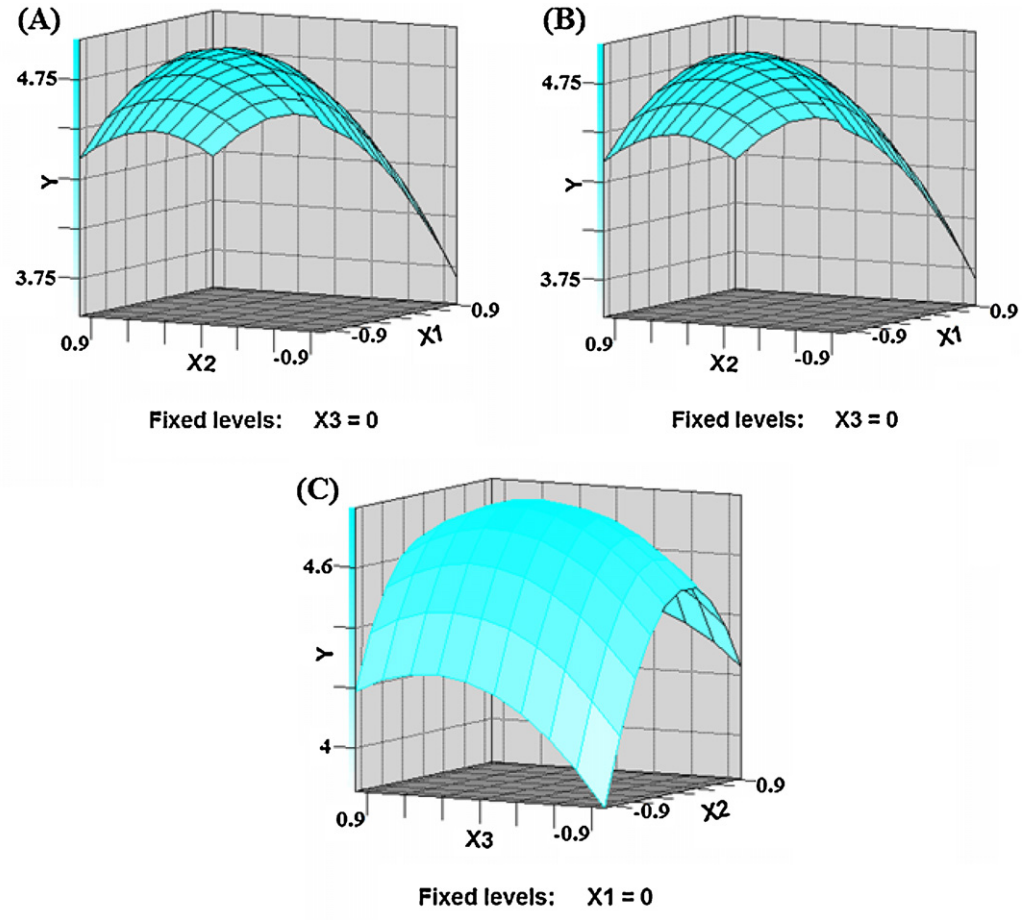

Fig. 1. Response surface plots showing the interaction between variables in the extraction of polysaccharides from *C. paliurus* using ultrasonic-assisted extraction. (A) Interaction between ratio of liquid to solid and extraction temperature; (B) interaction between ratio of liquid to solid and extraction time; (C) interaction between extraction temperature and extraction time, while keeping other variables at their respective '0' levels.

optimal conditions, the model predicted a maximum response of 4.96%. However, considering the operability in actual production, the optimal conditions can be modified as follows: ratio of liquid to solid of 8, extraction temperature of 58 °C and extraction time of 59 min.

To validate the adequacy of the model equations, a verification experiment was performed under the optimal conditions mentioned above. A mean value of 4.91 ± 0.11%, obtained from the experiments, validated the RSM model. There was not statistically difference between the experimental and predicted values ($P > 0.05$). The good correlation between these results confirmed that the response model was adequate for reflecting the expected optimization.

Furthermore, CPP was extracted with a conventional heat reflux extraction method (ratio of water to material of 20 ml/g, extraction in 100 °C water bath for 180 min), and a yield of 3.89% was obtained, which is significantly less than that obtained with the ultrasonic extraction method. Ultrasonic-assisted extraction can greatly reduce the extraction time for the same level of extraction, the quantity of solvent is less and the processing time is shorter. This opinion was in agreement with Li, Ding and Ding who examined the ultrasonically assisted extraction of polysaccharides from *Z. jujuba* cv. *jinsixiaozao* (Li et al., 2007). They reported that the yield and purity of polysaccharides extracted by the ultrasound-assisted procedures exceeded those of the classical procedures. Similar result was also observed in the study of extraction and processing of *Flammulina velutipes* polysaccharides (Yang, Fang, Liang, & Hu, 2011). The high efficiency of ultrasonic assistant extraction found in this work is suggested, because the cells of *C. paliurus* leaves were broken by the ultrasonic treatment, so that polysaccharide dissolved more easily in the solvent (Huang & Ning, 2010). The results suggested that ultrasonic assistant extraction of polysaccharides from *C. paliurus* was a time and energy saving and high yielding method.

3.4. Scavenging activity of hydroxyl radicals

Hydroxyl radicals, which are well known as the most reactive free radicals, can react with almost all the biomacromolecules functioning in living cells and induce severe damage to the adjacent biomolecules (Rollet-Labelle et al., 1998). Therefore, the removal of hydroxyl radical is important for antioxidant defense in cell or food systems. CPP was found to have the ability to scavenge hydroxyl radicals at concentrations between 0.125 mg/ml and 1 mg/ml (Fig. 2A). Interestingly, the scavenging ability of CPP on hydroxyl radicals was in a concentration-dependent manner. The scavenging ability of CPP on hydroxyl radicals was lower than ascorbic acid. Previous studies have suggested that the antioxidant mechanism may be due to the supply of hydrogen by polysaccharide, which combines with radicals and forms a stable radical to terminate the radical chain reaction (Chen et al., 2011). Another hypothesis for the antioxidant mechanism is due to the supply of hydrogen by polysaccharide, which combines with radical ions such as Fe^{2+} and Cu^{2+} (Qi et al., 2005). These results indicated that CPP might act as electron or hydrogen donor to scavenge hydroxyl radicals.

3.5. Scavenging activity of DPPH radicals

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants (Chen, Xie, Nie, Li, & Wang, 2008). It is an organic nitrogen radical with a ultraviolet–visible absorption at 517 nm, and its color fades upon reduction. It was found that CPP exhibited notable DPPH radical-scavenging activity, and the DPPH radical scavenging effects were increased with increasing

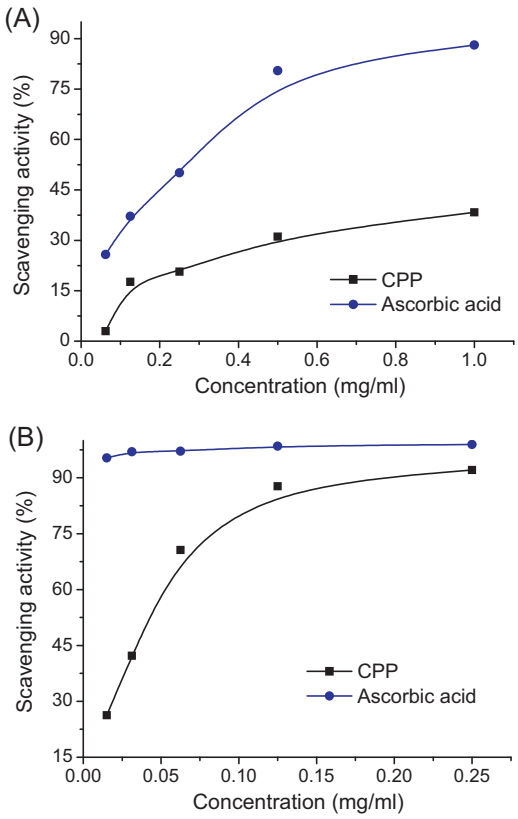

Fig. 2. (A) Scavenging effect of CPP on hydroxyl radicals and (B) scavenging effect of CPP on DPPH radicals compared with that of ascorbic acid (standard control). Results were means ± SD of three parallel measurements.

concentrations (Fig. 2B). At concentrations of 0.02–0.25 mg/ml, the scavenging abilities of CPP on DPPH radicals were in the range of 26.26–92.09%. At the concentration of 0.125 mg/ml, CPP possessed strong free radical scavenging effects of DPPH radicals (scavenging activity 87.72%). The scavenging activity of CPP on DPPH radicals of CPP reached 92.02% at 0.25 mg/ml, which was slightly higher than that of CPP-1 isolated from the leaves of *C. paliurus* (90.21%) (Xie, Xie, Nie, et al., 2010). The results indicated that CPP had a noticeable effect on scavenging DPPH free radicals, especially at high concentrations. However, the radical-scavenging activity of CPP was lower than that of ascorbic acid used in this study. The possible mechanism by which CPP acts as an antioxidant may be attributed to their electron donation power to the free radicals, thereby terminating the radical chain reaction (Lai, Wen, Li, Wu, & Li, 2010).

3.6. Scavenging activity of self-oxidation of 1,2,3-phentriol

Superoxide radical is considered as an initial free radical, formed from mitochondrial electron transport systems, to create other cell-damaging free radicals, such as H_2O_2, hydroxyl radical, or singlet oxygen in living systems (Bloknina, Virolainen, & Fagerstedt, 2003). It is generated by auto-oxidation of pyrogallol and can produce a colored compound. Due to the color change from purple to yellow, the absorbance at 320 nm increased when the superoxide anion was scavenged by antioxidant, which can represent the content of superoxide radicals and indicate the antioxidant activity of the sample (Chen et al., 2008).

As shown in Fig. 3A, the scavenging effect of self-oxidation of 1,2,3-phentriol of CPP was in a concentration-dependent manner. At lower concentrations (0.1–0.3 mg/ml), CPP exhibited higher superoxide radical scavenging activity, which was close to that of ascorbic acid. However, at higher concentrations (0.4–1.0 mg/ml),

Table 4
Antimicrobial activities (diameters of inhibition zone; mm) of polysaccharide from the leaves of *C. paliurus*[a].

Microorganisms	Diameters of inhibition zone (mm)	
	Polysaccharide (1 mg/ml)	Polysaccharide (0.5 mg/ml)
Escherichia coli	6.54 ± 0.23	–
Staphylococcus aureus	6.57 ± 0.11	–
Bacillus subtilis	6.93 ± 0.45	–
Aerobacter aerogenu	–	–
Proteus vulgari	–	–
Saccharomyces cerevisiae	9.72 ± 0.16	7.9 ± 0.42
Candida sp.	10.21 ± 0.37	8.3 ± 0.25
Aspergillus niger	–	–
Mucor	–	–
Penicillium sp.	–	–

–: no inhibition.
[a] The data are presented as the means ± standard deviation (SD) of triplicate determinations.

the radical scavenging activity of CPP was lower than that of ascorbic acid. At the concentration of l mg/ml, the scavenging activity of CPP was 37.22%,which was higher than those values of polysaccharides from *Plantago asiatica* L. and *Ganoderma atrum* against superoxide radicals at the same concentration (Chen et al., 2008; Yin et al., 2010). These results suggest that CPP has a stronger scavenging activity for superoxide radicals.

3.7. Inhibitory effect on lipid peroxidation

As shown in Fig. 3B, CPP exhibited a strong inhibitory effect on lipid peroxidation and the inhibitory effect was concentration dependent (Fig. 3B). The inhibition ratios of CPP ranged from 3.19% to 31.66% when the concentrations varied from 0.125 mg/ml to 1 mg/ml, which was lower than that of ascorbic acid. The inhibition percentage of CPP reached 31.66% at 1 mg/l, which was higher than that of polysaccharides derived from *P. asiatica* L (16.76%) (Yin et al., 2010). Our data suggest that CPP has a significant effect on inhibiting lipid peroxidation. According to previous studies, most polysaccharides derived from plants are relatively nontoxic and do not cause significant side effects. Therefore, CPP may be developed as an effective natural antioxidant with little side effects by the food industry.

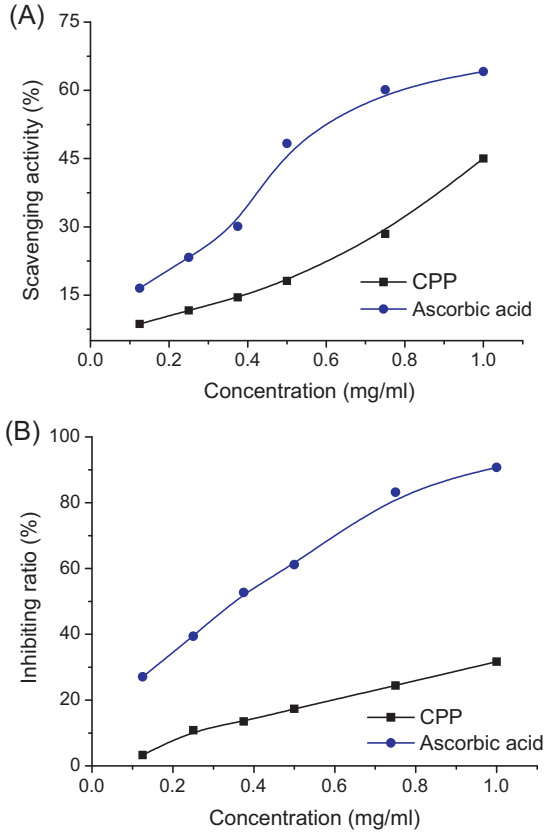

Fig. 3. (A) Inhibition effect of CPP on self-oxidation of 1,2,3-phentriol and (B) inhibition effect of CPP on lipid peroxidation compared with that of ascorbic acid (standard control). Results were means ± SD of three parallel measurements.

3.8. Antimicrobial activity

CPP, at a concentration of 1 mg/ml, showed good antimicrobial activities against *S. cerevisiae* and *Candida* sp. with inhibition zones of 9.72 ± 0.16 mm and 10.21 ± 0.37 mm, respectively (Table 4). Previous studies have also shown that polysaccharide from *Lygodium japonicum* was the most effective against microzymes tested especially toward *Hansenula anomala*, *Candida albicans* and *S. cerevisiae* (Li et al., 2006). CPP (1 mg/ml) also showed moderate antimicrobial activity to *E. coli*, *S. aureus* and *B. subtilis*, with diameters of the inhibition zones of 6.54 ± 0.23, 6.57 ± 0.11 and 6.93 ± 0.45 mm, respectively. However, the polysaccharides had no inhibitory effects on three fungi; *A. niger*, *Mucor* and *Penicillium* sp. at a concentration of 1 mg/ml. Previous reports showed that polysaccharides from *L. japonicum* possess significant broad-spectrum anti-microorganism activity at a concentration of 10 mg/ml (Li et al., 2006). Significant antibacterial and antifungal activities were also shown for polysaccharide isolated from the broth of *Streptomyces virginia* H03 (He, Yang, Yang, &Yu, 2010). The mechanisms involved in these antimicrobial effects of CPP are worthy of further investigation.

4. Conclusions

In this study, ultrasonic-assisted extraction technique was employed to extract polysaccharides from the leaves of *C. paliurus* and RSM was used to determine the main and interaction effects of the variables that are important in the extraction process. The ratio of liquid to solid had highly significant effect on the response value and the three variables investigated in the present study could be ranked as follows in terms of influence on the extraction performance: ratio of liquid to solid > extraction time > extraction temperature. The optimal predicted CPP yield (4.91 ± 0.11%) by ultrasonic-assisted extraction was obtained when the procedure was carried out at ratio of liquid to solid of 8, extraction temperature of 58 °C, and extraction time of 59 min. Under these optimized conditions, the experimental yield of CPP agreed closely with the predicted yield. The polysaccharide exerted strong antioxidant activity and free radical-scavenging activity, as well as moderate antimicrobial activities on yeast (*S. cerevisiae* and *Candida* sp.). The

results indicated that ultrasonic-assisted extraction may be used as an recommended alternative for the extraction of polysaccharides from *C. paliurus* and RSM was an useful tool for the optimization of the experimental variables. Our results also suggested that the polysaccharide extracted from *C. paliurus* should be explored as a novel natural antioxidant for use in functional foods or medicine.

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References

Bloknina, O., Virolainen, E., & Fagerstedt, K. V. (2003). Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Annals Botany*, *91*(2), 179–194.

Chen, R. Z., Liu, Z. Q., Zhao, J. J., Chen, R. P., Meng, F. L., Zhang, M., et al. (2011). Antioxidant and immunobiological activity of water-soluble polysaccharide fractions purified from *Acanthopanax senticosu*. *Food Chemistry*, *127*(2), 434–440.

Chen, X. P., Wang, W. X., Li, S. B., Xue, J. L., Fan, L. J., Sheng, Z. J., et al. (2010). Optimization of ultrasound-assisted extraction of Lingzhi polysaccharides using response surface methodology and its inhibitory effect on cervical cancer cells. *Carbohydrate Polymers*, *80*(3), 944–948.

Chen, Y., Xie, M. Y., Nie, S. P., Li, C., & Wang, Y. X. (2008). Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. *Food Chemistry*, *107*(1), 231–241.

Chen, X. Q., & Zhang, Y. (2007). Ultrasonic-associated extraction of water soluble polysaccharides from defatted Korean pine kernel. *Journal of Forestry Research*, *18*(2), 133–135.

Dong, C. J., Xie, M. Y., Nie, S. P., Xie, J. H., Jia, S. H., & Huang, P. (2007). Study on antioxidant activity of *Cyclocarya paliurus* (Bata1.) Iljinskaya extracts in vitro. *Food Science*, *28*(10), 31–34.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Halliwell, B., Gutteridge, J. M. C., & Aruoma, O. I. (1987). The deoxyribose method: Simple 'test tube' assay for determination of rate constants for reaction of hydroxyl radicals. *Analytical Biochemistry*, *165*(1), 215–219.

He, F., Yang, Y., Yang, G., & Yu, L. J. (2010). Studies on antibacterial activity and antibacterial mechanism of a novel polysaccharide from *Streptomyces virginia* H03. *Food Control*, *21*(9), 1257–1262.

Hromadkova, Z., Ebringerova, A., & Valachovic, P. (1999). Comparison of classical and ultrasound-assisted extraction of polysaccharides from *Salvia officinalis* L. *Ultrasonics Sonochemistry*, *5*(4), 163–168.

Huang, S. Q., & Ning, Z. X. (2010). Extraction of polysaccharide from *Ganoderma lucidum* and its immune enhancement activity. *International Journal of Biological Macromolecules*, *47*(3), 336–341.

Jiang, Z. Y., Zhang, X. M., Zhou, J., Qiu, S. X., & Chen, J. J. (2006). Two new triterpenoid glycosides from *Cyclocarya paliurus*. *Journal of Asian Natural Products Research*, *8*(1–2), 93–98.

Kurihara, H., Asami, S., Shibata, H., Fukami, H., & Tanaka, T. (2003). Hypolipemic effect of *Cyclocarya paliurus* (Batal) Iljinskaja in lipid-loaded mice. *Biological & Pharmaceutical Bulletin*, *26*(3), 383–385.

Kurihara, H., Fukami, H., Kusumoto, A., Toyoda, Y., Shibata, H., Matsui, Y., et al. (2003). Hypoglycemic action of *Cyclocarya paliurus* (Batal.) Iljinskaja in normal and diabetic mice. *Bioscience, Biotechnology, and Biochemistry*, *67*(4), 877–880.

Kennelly, E. J., Cai, L. N., Long, L. N., Shamon, L., Zaw, K., Zhou, B. N., et al. (1995). Novel highly sweet secodammarane glycosides from *Pterocarya puliurus*. *Journal of Agricultural and Food Chemistry*, *43*(10), 2602–2607.

Lai, F. R., Wen, Q. B., Li, L., Wu, H., & Li, X. F. (2010). Antioxidant activities of water-soluble polysaccharide extracted from mung bean (*Vigna radiata* L.) hull with ultrasonic assisted treatment. *Carbohydrate Polymers*, *81*(2), 323–329.

Li, J. W., Ding, S. D., & Ding, X. L. (2007). Optimization of the ultrasonically assisted extraction of polysaccharides from *Zizyphus jujuba* cv. *Jinsixiaozao*. *Journal of Food Engineering*, *80*(1), 176–183.

Li, L., Xie, M. Y., Sun, Z. H., Wu, X. H., Sun, D. H., & Wang, X. R. (2000). The study on the element transference characteristics and element speciation in the extract of *Cyclocarya paliurus* (Batal.) Iljinskaja leaves. *Chemical Journal of Chinese Universities-Chinese*, *21*(5), 707–709.

Li, X. L., Zhou, A. G., & Han, Y. (2006). Anti-oxidation and anti-microorganism activities of purification polysaccharide from *Lygodium japonicum* in vitro. *Carbohydrate Polymers*, *66*(1), 34–42.

Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, *47*(3), 469–474.

Norulaini, N. A. N., Anuar, O., Abbas, F. M. A., Fatehah, M. O., Omar, A. K. M., Sahena, F., et al. (2009). Optimization of supercritical CO_2 extraction of *Anastatica hierochuntica*. *Food and Bioproducts Processing*, *87*(2), 152–158.

Qi, H. M., Zhang, Q. B., Zhao, T. T., Chen, R., Zhang, H., Niu, X. Z., et al. (2005). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) in vitro. *International Journal of Biological Macromolecules*, *37*(4), 195–199.

Rollet-Labelle, E., Grange, M. J., Elbim, C., Marquetty, C., Gougerot-Pocidalo, M. A., & Pasquier, C. (1998). Hydroxyl radical as a potential intracellular mediator of polymorphonuclear neutrophil apoptosis. *Free Radical Biology and Medicine*, *24*(4), 563–572.

Shan, B., Xie, J. H., Zhu, J. H., & Peng, Y. (2012). Ethanol modified supercritical carbon dioxide extraction of flavonoids from *Momordica charantia* L. and its antioxidant activity. *Food and Bioproducts Processing*, doi:10.1016/j.fbp.2011. 09. 004

Shu, R. G., Xu, C. R., Li, L. N., & Yu, Z. L. (1995). Cyclocariosides II and III: Two secodammarane triterpenoid saponins from *Cyclocarya paliurus*. *Planta Medica*, *61*(6), 551–553.

Tsochatzidis, N. A., Guiraud, P., Wilhelm, A. M., & Delmas, H. (2001). Determination of velocity, size and concentration of ultrasonic cavitation bubbles by the phase-Doppler technique. *Chemical Engineering Science*, *56*(5), 1831–1840.

Wang, Y. J., Cheng, Z., Mao, J. W., Fan, M. G., & Wu, X. Q. (2009). Optimization of ultrasonic-assisted extraction process of *Poria cocos* polysaccharides by response surface methodology. *Carbohydrate Polymers*, *77*(4), 713–717.

Wu, Y., Cui, S. W., Tang, J., & Gu, X. H. (2007). Optimization of extraction process of crude polysaccharides from boat-fruited sterculia seeds by response surface methodology. *Food Chemistry*, *105*(4), 1599–1605.

Xie, M. Y., Li, L., Nie, S. P., Wang, X. R., & Lee, F. S. C. (2006). Determination of speciation of elements related to blood sugar in bioactive extracts from *Cyclocarya paliurus* leaves by FIA-ICP-MS. *European Food Research and Technology*, *223*(2), 202–209.

Xie, J. H., Shen, M. Y., Nie, S. P., Li, C., & Xie, M. Y. (2011). Decolorization of polysaccharides solution from *Cyclocarya paliurus* (Batal.) Iljinskaja using ultrasound/H_2O_2 process. *Carbohydrate Polymers*, *84*(1), 255–261.

Xie, M. Y., Wang, Y. X., Yi, X., & Wang, X. R. (2004). A study on the structure and contents of flavonoids in the leaves of *Cyclocarya paliurus* (Batal.) Iljinsk. *Chinese Journal of Analytical Chemistry*, *32*(8), 1053–1056.

Xie, M. Y., & Xie, J. H. (2008). Review about the research on *Cyclocarya paliurus* (Batal.) Iljinskaja. *Journal of Food Science and Biotechnology*, *27*(1), 113–121.

Xie, J. H., Xie, M. Y., Nie, S. P., Shen, M. Y., Wang, Y. X., & Li, C. (2010). Isolation, chemical composition and antioxidant activities of a water-soluble polysaccharide from *Cyclocarya paliurus* (Batal.) Iljinskaja. *Food Chemistry*, *119*(4), 1626–1632.

Xie, J. H., Xie, M. Y., Shen, M. Y., Nie, S. P., Wang, Y. X., & Li, C. (2010). Optimisation of microwave-assisted extraction of polysaccharides from *Cyclocarya paliurus* (Batal.) Iljinskaja using response surface methodology. *Journal of the Science of Food and Agriculture*, *90*(8), 1353–1360.

Yan, Y. L., Yu, C. H., Chen, J., Li, X. X., Wang, W., & Li, S. Q. (2011). Ultrasonic-assisted extraction optimized by response surface methodology, chemical composition and antioxidant activity of polysaccharides from *Tremella mesenterica*. *Carbohydrate Polymers*, *83*(1), 217–224.

Yang, W. J., Fang, Y., Liang, J., & Hu, Q. H. (2011). Optimization of ultrasonic extraction of *Flammulina velutipes* polysaccharides and evaluation of its acetylcholinesterase inhibitory activity. *Food Research International*, *44*(5), 1269–1275.

Yin, J. Y., Nie, S. P., Zhou, C., Wan, Y., & Xie, M. Y. (2010). Chemical characteristics and antioxidant activities of polysaccharide purified from the seeds of *Plantago asiatica* L. *Journal of the Science of Food and Agriculture*, *90*(2), 210–217.

Zhang, J., Shen, Q., Lu, J. C., Li, J. Y., Liu, W. Y., Yang, J. J., et al. (2010). Phenolic compounds from the leaves of *Cyclocarya paliurus* (Batal.) Ijinskaja and their inhibitory activity against PTP1B. *Food Chemistry*, *119*(4), 1491–1496.

Zhong, K., & Wang, Q. (2010). Optimization of ultrasonic extraction of polysaccharides from dried longan pulp using response surface methodology. *Carbohydrate Polymers*, *80*(1), 19–25.