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Optimization of polysaccharides extraction from *Clematis huchouensis* Tamura and its antioxidant activity

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ABSTRACT

In this study, response surface methodology (RSM), based on Box–Behnken design, was employed to optimize the extraction conditions of polysaccharide from *Clematis huchouensis* Tamura (CP). And then the antioxidant activities of the samples were investigated including scavenging effects of superoxide and hydroxyl radicals and their reducing power. The results of chemical composition and FT-IR spectrum analysis showed the polysaccharide was acidic proteoglycan. And moreover, CP showed excellent antioxidant activity in these three assays. The purification and structure of CP need to be further studied.

1. Introduction

Clematis, a genus of approximately 350 species, is one of the largest genera in Ranunculaceae. This cosmopolitan genus is native in the temperate zone of both hemispheres but with some species distributed in tropical areas (Tamura, 1995; Wang & Li, 2005). The genus is extremely diverse in temperate and subtropical regions of the Northern Hemisphere, especially in eastern Asia, with more than 147 species reported in China, 93 of which are endemic to the country (Wang & Bartholomew, 2001).

Many Clematis species are of horticultural interest (e.g. *Clematis montana*, *Clematis patens*, and *Clematis viorna*), and some others are regarded as pharmaceutically important (e.g. *Clematis chinensis*, *Clematis henryi*, and *Clematis armandii*) (Xie & Li, 2012). It was reported that the entire genus contains essential oils and compounds which are extremely irritating to the skin and mucous membranes (Kizu, Shimana, & Tomimori, 1995). Native Americans used very small amounts of clematis as an effective treatment for migraine headaches and nervous disorders. It was also used as an effective treatment of skin infections. Leaf extracts from two Ethiopian species (*Clematis longicauda* and *Clematis burgensis*) are used locally to treat ear disorders and eczema. Phytochemical screening of the extracts from both of these species showed antibacterial and antifungal activity (Hawaze, Deti, & Suleman, 2012). The extracts of these plants also possess wound healing and anti-inflammatory activities which could also be attributed to the phytoconstituents (Hawaze, Deti, & Suleman, 2013).

Clematis huchouensis Tamura belongs to the family Ranunculaceae which is distributed in Zhejiang, Hunan, Jiangsu and Jiangxi provinces of China (Nanjing University of Chinese Medicine, 2006). The Traditional Chinese Herbal Medicine prepared from this herb has been used for rheumatoid arthritis. In folklore traditions, herbal preparations from this plant are considered to have anti-tumor/anti-cancer, anti-inflammatory, anti-allergy, antithrombotic, anti-mutagenic and other bioactivities in conjunction with other herbs (Gu, Wang, & Xu, 2004). However, to our knowledge, little information is available on the polysaccharides from this plant. The objective of this present study was to optimize the process for production of the crude polysaccharides from *C. huchouensis* using response surface methodology (RSM) and evaluate its antioxidant activity in vitro.

2. Materials and methods

2.1. Chemicals

C. huchouensis Tamura was obtained from Huzhou, Zhejiang province in October 2012. The leaf and stem were soon washed; sun dried and kept in plastic bags at room temperature for use.

Ferrozine, nicotinamide adenine dinucleotide (NADH), reduced hydrogen peroxide (H_2O_2), ethylene diamine tetra-acetic acid (EDTA), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), ferric chloride and potassium ferricyanide were of analytical grade.

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Table 1
Box–Behnken experimental design with the independent variables.

Run	X_1/temperature (°C)	X_2/time (h)	X_3/ratio of water to raw (mL/g)	Yield of CP (%)	
				Actual value	Predicted value
1	−1 (80)	−1 (3)	0 (40)	5.19	5.18
2	1 (100)	−1	0	6.16	6.14
3	−1	1 (5)	0	5.33	5.35
4	1	1	0	6.35	6.36
5	−1	0 (4)	−1(30)	4.97	5.04
6	1	0	−1	6.05	6.12
7	−1	0	1 (50)	5.46	5.39
8	1	0	1	6.34	6.28
9	0 (90)	−1	−1	5.87	5.82
10	0	1	−1	6.04	5.96
11	0	−1	1	5.93	6.01
12	0	1	1	6.21	6.26
13	0	0	0	6.93	6.89
14	0	0	0	6.90	6.89
15	0	0	0	6.81	6.89
16	0	0	0	6.88	6.89
17	0	0	0	6.95	6.89

Total sugar content was determined by phenol-sulfuric acid method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956) using glucose as standard. Uronic acid was estimated in a modified carbazole method using D-glucuronic acid as standard (Bitter & Muir, 1962). Sulfate content was determined by barium chloride method (Kawai, Seno, & Anno, 1966). The protein was determined by Bradford assay as previously described (Kweon, Sung, & Yang, 1994).

The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000–400 cm^{-1} region.

2.2. Preparation of crude polysaccharides

C. huchouensis (500 g) was added into 95% EtOH with 85 °C water bath for 2 h. After incubation, the residue was dried in an oven at 50 °C, and each pretreated sample (10 g) was extracted by water in a designed extraction times, extraction temperatures and the ratio of water to raw material. The solution was dialyzed against tap water for 48 h and against distilled water for 24 h, and then the solution was concentrated under reduced pressure. The extraction solution was centrifugated and then precipitated by the addition of ethanol to a final concentration of 75% (v/v). The precipitate was air-dried to give *C. huchouensis* polysaccharide (named after CP) as a white powder.

2.3. Experimental design of RSM

After determining the preliminary range of the extraction parameters by a single-factor experiment for the polysaccharides production, a Box–Behnken design with three variables was used for the optimization of CP. Based on the results of preliminary experiments, three independent variables considered were extraction temperature (X_1, A), extraction time (X_2, B) and ratio of water to raw material (X_3, C) while the response variable was the yield of crude polysaccharides. In Table 1, these three factors were prescribed into three levels coded +1, 0 and −1 for high, medium and low. In order to predict the optimized conditions, experimental data were analyzed using software Design-Expert and fitted to an empirical second-order polynomial regression model:

$$Y = \beta_0 + \sum_{i-1}^{k} \beta_i X_1 + \sum_{i-1}^{k} \beta_{ii} X_i^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j$$

where Y is the estimated response (extraction yield of CP); β_0 is the constant, β_i, β_{ii}, and β_{ij} are the regression coefficients for linearity, square, and interaction, respectively; and X_i and X_j are the independent variables. And k equals to the number of the tested factors ($k = 3$).

2.4. Antioxidant activity

2.4.1. Superoxide radical assay

The superoxide radical scavenging abilities of all samples were assessed by the modified method of Nishimiki, Rao, and Yagi (1972). In this experiment, the sample in 4.5 mL of Tris–HCl buffer (16 mM, pH 8.0) was added into 0.5 mL of NBT (300 μM) solution, 0.5 mL of NADH (468 μM) and 0.5 mL of PMS (60 μM). The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer against blank samples. The capability of scavenging the superoxide anion radicals was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \left(1 - \frac{A_{\text{sample 560}}}{A_{\text{control 560}}}\right) \times 100\%$$

2.4.2. Hydroxyl radical assay

The reaction mixture, containing all different derivatives, was incubated with EDTA-Fe^{2+} (2.0 mM), saffron (360 μg/mL), and H_2O_2 (3%) in potassium phosphate buffer (150 mM, pH 7.4), and was incubated for 30 min at 37 °C (Wang et al., 1994). The absorbance was read at 520 nm against a blank. Hydroxyl radical bleached the saffron, so decreased absorbance of the reaction mixture indicated a decrease in hydroxyl radical scavenging ability. The capability of scavenging hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100\%$$

where A_0 is the absorbance of the control (without samples) and A_1 is the absorbance of the mixture containing samples.

2.4.3. Reducing power assay

The reducing power was determined as described previously by Yen and Chen (1995). Briefly, 1.0 mL of different concentration of samples in phosphate buffer (0.2 M, pH 6.6) was mixed with 1.0 mL of potassium ferricyanide (1%, w/v), and was incubated at 50 °C for 20 min. Afterwards, 2.0 mL of trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. Then the solution was mixed with 1.2 mL ferric chloride (0.1%, w/v) and

Table 2
Analysis of variance of the Box–Behnken design experimental results.

Variables	Sum of squares	DF	Mean square	*F* value	*P* value
Model	6.42	9	0.71	97.02	<0.0001
X_1	1.95	1	1.95	265.37	<0.0001
X_2	0.076	1	0.076	10.35	0.0147
X_3	0.13	1	0.13	17.35	0.0042
X_1X_2	0.000	1	0.000	0.085	0.7790
X_1X_3	0.010	1	0.010	1.36	0.2816
X_2X_3	0.003	1	0.003	0.41	0.5416
X_1^2	2.19	1	2.19	298.65	<0.0001
X_2^2	0.72	1	0.72	98.43	<0.0001
X_3^2	0.92	1	0.92	124.95	<0.0001
Residue	0.051	7	0.007		
Lack of fit	0.040	3	0.013	4.52	0.0896
Pure error	0.012	4	0.003		
Total	6.47	16			

the absorbance was measured at 700 nm. Increased absorbance of reaction mixture indicated increased reducing power.

3. Results and discussion

3.1. Optimization of the extraction conditions

3.1.1. ANOVA for response surface quadratic model

The designed matrix as well as the measured and predicted values for response *Y* (yield of CP) is given in Table 1. By employing multiple regression analysis on the experimental data, the predicted response model *Y* was obtained by the following second-order polynomial equation as follows:

$$Y = 6.89 + 0.49X_1 + 0.098X_2 + 0.13X_3 + 0.012X_1X_2 - 0.05X_1X_3 + 0.028X_2X_3 - 0.72X_1^2 - 0.41X_2^2 - 0.47X_3^2$$

Table 2 showed the analysis result of variance (ANOVA) by the Box–Behnken design. In ANOVA, *F*-test is used to check the statistical significance of regression equation. The *P* values are used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables (Hou & Chen, 2008). In other words, the smaller the *P*-values are, the bigger the significance of the corresponding coefficient (Murthy, Swaminathan, Rakshit, & Kosugi, 2000), which is implied the model is suitable for use. As shown in Table 3, the high *F*-test (97.02) and the low *P*-value ($P < 0.0001$) indicated that the model was highly significant. And furthermore, The *F*-value (4.52) and *P*-value (0.0896) of "lack-of-fit" which indicated that this was insignificant relative to the pure error. It indicates that the model equation is adequate for predicting the yield of polysaccharides under any combination of values of the variables. The calculated coefficient for the parameter optimization suggests that the linear model terms (X_1, X_2, and X_3) and the quadratic model terms (X_1^2, X_2^2, X_3^2) significantly affected the extraction yield ($P < 0.05$). Therefore, it can be seen from this table that the temperature (A), the time (B) and the ratio of water to raw material (C) were important factors in the extraction process of the polysaccharides.

Table 3 showed the analysis results of variance for the fitted quadratic polynomial model. The fitness of the model was further confirmed using the determination coefficient (R^2). Both R^2 and Adj R^2 values indicated that the accuracy and general availability of the polynomial model were adequate. In Table 3, the "Pred $R^{2\prime\prime}$ of 0.8989 is in reasonable agreement with the "Adj $R^{2\prime\prime}$ of 0.9818. And in addition, the very low value of coefficient of the variation (C.V.%, 1.40) clearly suggested that a very high degree of precision and a good deal of reliability of the experimental values. Finally, the "Adeq. Precision" (28.274), used to measure the signal to noise ratio, indicated that this model could be used to navigate the design space.

3.1.2. Diagnostics case statistics of the extraction conditions

The three-dimensional (3D) response surface and two-dimensional contour plots simulated by Design-Expert software are the graphical representations of regression equation. The response surface curves show the interactions of the variables and their optimal levels for a maximal response, and the contour plot indicates whether the interaction between variables is significant (Ma et al., 2012). Fig. 1(a) showed the effect of temperature (A), time (B) and their reciprocal interaction on yield of CP, when ratio of water to raw material (C) was fixed at 30 mL/g. The results revealed that the yield of polysaccharides increased with the temperature and time. When the yield reached to then peak, it changed slightly. The same phenomenon was observed in Fig. 1(b) and (c), indicating that the extraction time and temperature had significant effect on the yield of CP.

Through prediction by this built model, the optimal conditions to obtain the highest yield of lentinan were determined as follows: temperature 93.38 °C, time 4.13 h, ratio of water to material 31.21 mL/g.

3.1.3. Verification of the predictive model

A verification experiment was carried out under the aforementioned modified conditions. Under these conditions, the predicted values were 6.99% from fitted equations, and the experimental yield was 6.94% ($n = 3$). The result of the analysis confirmed that the response model adequately reflects the expected optimization, and the model equation was satisfactory and accurate.

3.2. Chemical analysis

The chemical compositions of CP indicate that CP consisted of 81.27% total sugar and 9.73% uronic acid, which suggested this sample was a acidic polysaccharide. And what is more, 5.06% of protein

Table 3
Analysis of variance for the fitted quadratic polynomial model.

Item	Std. dev	Mean	C.V.%	Press	R^2	Adj. R^2	Pred. R^2	Adeq precision
Value	0.086	6.14	1.40	0.65	0.9920	0.9818	0.8989	28.274

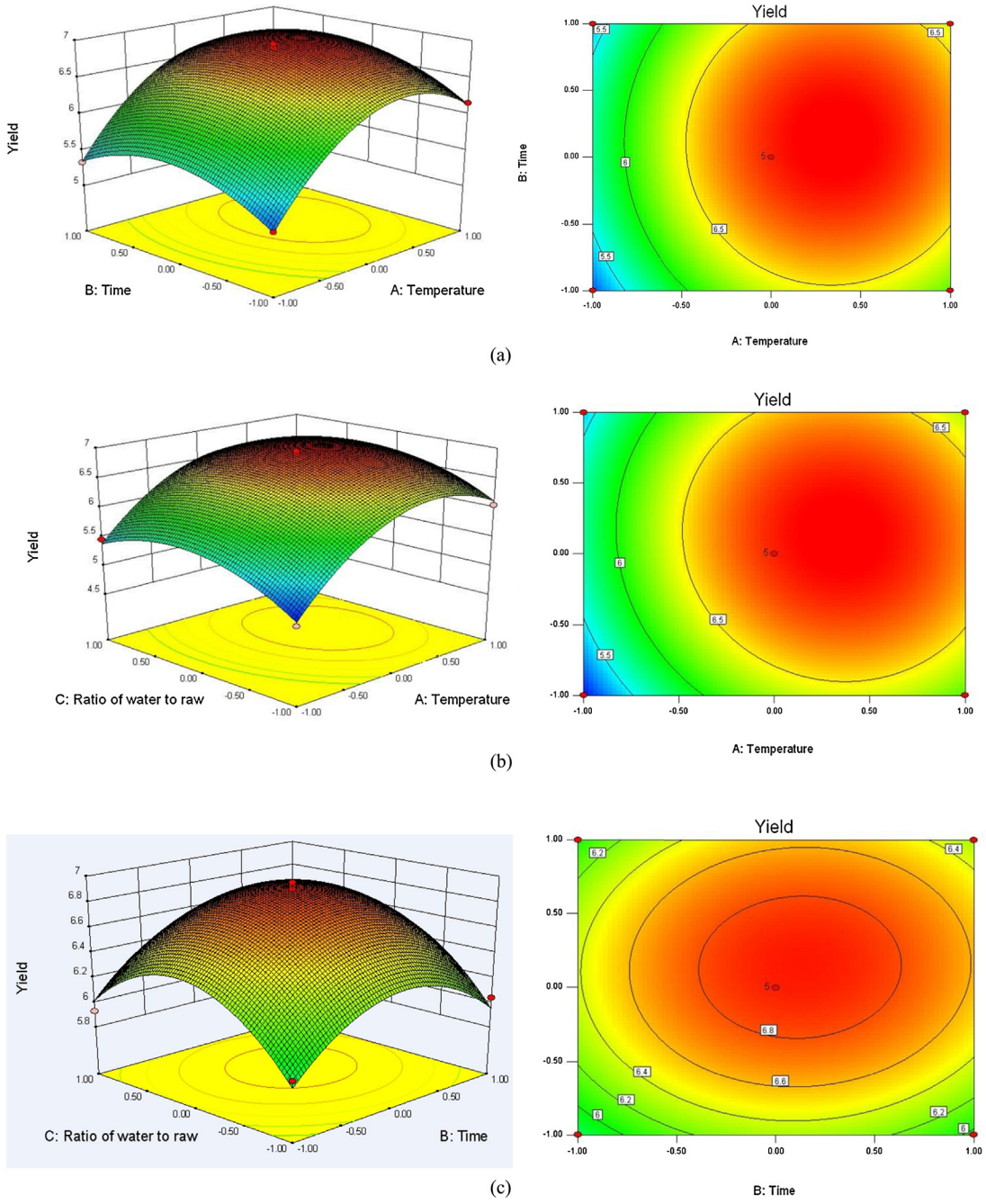

Fig. 1. Response surface plots and contours plots showing the effect of temperature (X_1), time (X_2) and ratio of water to raw material (X_3) on the yield of CP.

in sample showed this was proteoglycan. Sulfate (3.31%) was also found in CP indicated it was sulfated polysaccharide.

Fig. 2 showed the FT-IR spectrums of the CP. Typical signals of polysaccharide at 3365, 2931, 1612, 1398 and 1073 cm^{-1} were clear for CP. They correspond to the O—H stretching vibrations, the C—H stretching vibrations, the carbonyl C=O vibrations in uronic acid, the carbonyl C—O stretching vibrations, and the C—O—H in glucosidal bond or C—O—C stretching vibrations in ring. The signal at 1247 and 841 cm^{-1} were the S=O asymmetric stretching vibration and C—O—S in sulfate group.

3.3. Superoxide radical assay

In a PMS/NADH system, the superoxide radical ($^{\bullet}O_2^-$) was generated for being assayed in the reduction of NBT and was a highly toxic species (Banerjee, Dasgupta, & De, 2005). Fig. 3 depicted the inhibitory effects on the superoxide radical of CP and Vc. For these two samples, the scavenging effects significantly increased with increasing concentration. The IC_{50} values of Vc and CP were 8.4 and 27.1 μg/mL, respectively.

It was reported that addition of electron-withdrawing groups to the pyrrole enhanced antioxidant activity (Yanagimoto, Lee, Ochi, & Shibamoto, 2002). In CP, sulfate was strong electron-withdrawing group, which could significantly increase the activity of scavenging radicals. Although superoxide was a relatively weak oxidant, it decomposed to form stronger, reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids. Furthermore, superoxides were also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Dahl & Richardson, 1978). The

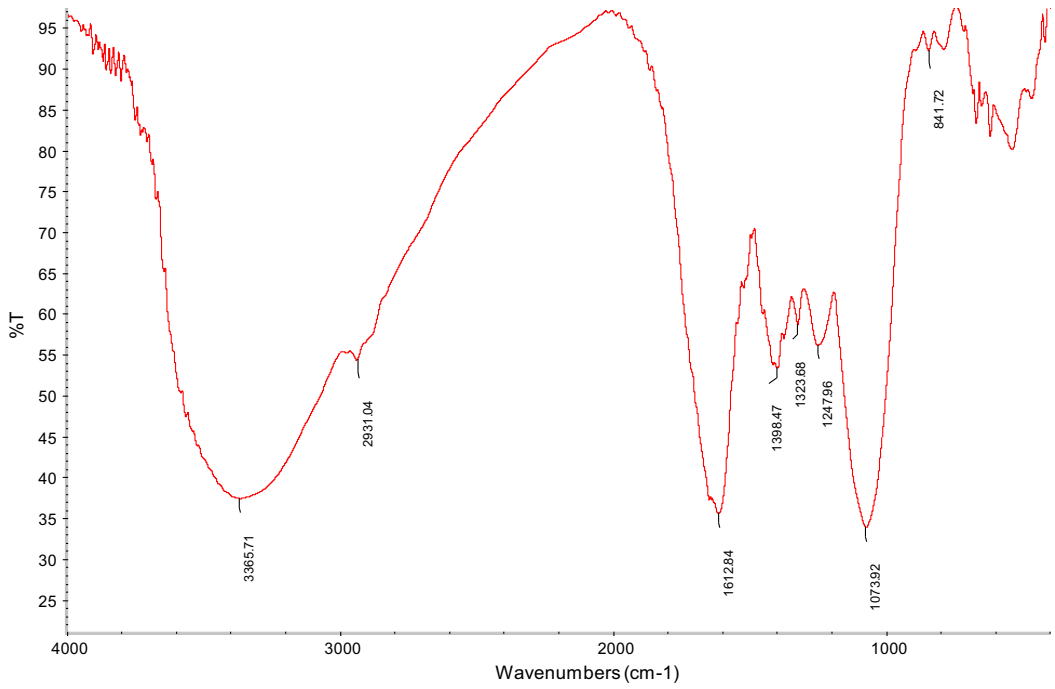

Fig. 2. The FT-IR spectrums of the CP.

results above indicated that the antioxidant activity of CP was related to their abilities to scavenge superoxide.

3.4. Hydroxyl radical assay

The scavenging effect on hydroxyl radical of two samples was shown in Fig. 4. The hydroxyl radical, known to be generated through the Fenton reaction in this system, was scavenged by samples. For CP and Vc, the effects of scavenging hydroxyl radicals were in a concentration-dependent manner. The IC_{50} values of CP and Vc were 0.64 and 1.47 mg/mL, respectively. CP showed excellent scavenging effects (78.1%) at the concentration of 2.52 mg/mL.

For hydroxyl radical, there were two types of antioxidation mechanism; one suppresses the generation of the hydroxyl radical, and the other scavenges the hydroxyl radicals generated. In the former, the antioxidant activity may ligate to the metal ions which react with H_2O_2 to give the metal complexes. The metal complexes thus formed cannot further react with H_2O_2 to give hydroxyl radicals (Ueda, Saito, Shimazu, & Ozawa, 1996). Iron, a transition metal, is capable of generating free radicals from peroxides by the Fenton reaction and is implicated in many diseases. Fe^{2+} has also been shown to produce oxyradicals and lipid peroxidation, and reduction of Fe^{2+} concentrations in the Fenton reaction would protect against oxidative damage (Singh & Rajini, 2004). Romera, González, Ballester, Blázquez, and Muñoz (2006) reported the biosorption with kinds of algae, and found the adsorption capacity of brown algae was directly to the presence of carboxylic groups on alginate polymer. In the present study, –COO– groups was generally the most abundant acidic functional group, and could chelate with metal ion, so showed the higher hydroxyl radical scavenging activities. The relation of uronic acid and –OH scavenging activities was also previously reported (Wang, Jin, Peng, & Wei, 2009; Xue et al., 2000).

3.5. Reducing power assay

Fig. 5 showed the reducing power of CP and Vc. As shown in the figure, the reducing power of the samples correlated well with increasing concentrations. The reducing powers of CP and Vc were 0.499 and 1.162 at the concentration of 10 mg/mL, respectively.

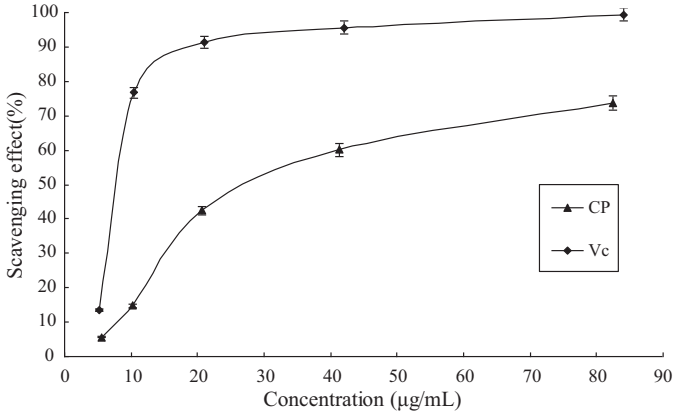

Fig. 3. The inhibitory effects on the superoxide radical of CP and Vc.

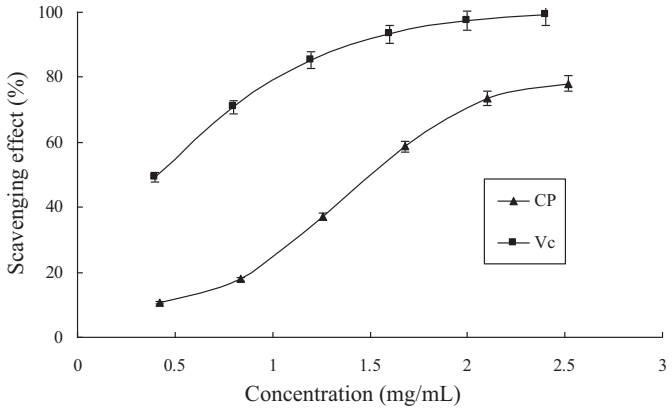

Fig. 4. The scavenging effect on hydroxyl radical of CP and Vc.

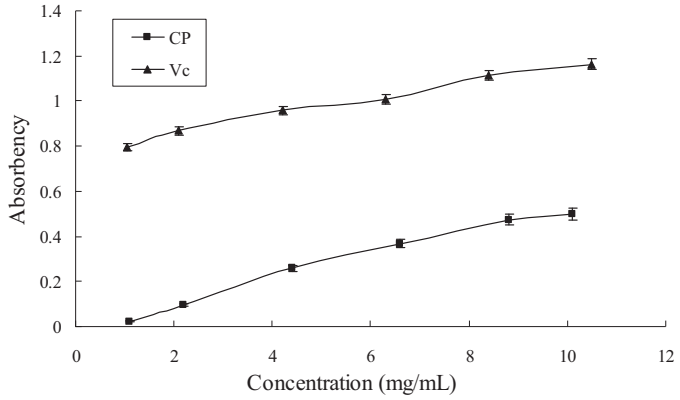

Fig. 5. The reducing power of CP and Vc.

It has been previously reported that there was a direct correlation between antioxidant activities and reducing power of certain plant extracts (Duh, Du, & Yen, 1999). The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, CP showed reducing power probably because of the –OH, which contributed to reduce the Fe^{3+}/ferricyanide complex and then close the reaction.

4. Conclusion

The results of the present work indicated that the polysaccharide from *C. huchouensis* Tamura was acidic proteoglycan. And moreover, CP showed excellent antioxidant activity in these three assays. The purification and structure need to be study in further. CP may have a use as a possible supplement in the food and pharmaceutical industries. However, factors effecting and attributing to radical scavenging effect need to be further studied.

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