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Optimization and characterization of *Mytilus coruscus* polysaccharide and investigation on antitumor activity

Mingyan Ma*, Yubo Zhou, Jiajia Zhang, Aijuan Yan, Wen Cao

Pharmacy Faculty, Zhejiang Pharmaceutical University, Ningbo, China

***Corresponding Author:** Mingyan Ma, Pharmacy Faculty, Zhejiang Pharmaceutical University, Ningbo 315010, China. Email: 894025537@qq.com

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ORIGINAL ARTICLE

Abstract

This study aimed to optimize the enzymatic extraction of polysaccharides from *Mytilus coruscus* using the response surface method, characterize its composition, and investigate its antitumor activity in vivo. Employing a three-level and four-variable Box–Behnken design and setting the *Mytilus coruscus* polysaccharides (MCP) extraction yield (%) as response value, the optimum enzymatic extraction conditions were as follows: extraction time was 173.34 min, extraction temperature was 56.82°C, extraction pH was 5.64, and the ratio of liquid to fresh flesh was 19.82. Under the optimized conditions, the predicted extraction yield indicated by the response surface methodology (RSM) model was 19.68%, which showed compliance to the experimental yield (19.53%). GC-MS was used to detect the monosaccharide composition and methylation analysis, it was showed that MCP was composed of Rhamnose, Arabia sugar, fructose, mannose, glucose, and galactose with a molar ratio of 1.92:1.29:8.18:27.90:27.63:29.50 and the main linkage type was 1→4 linked glucose, the molecular weight of the polysaccharide was about 57 kDa determined by high-performance gel permeation chromatography (HPGPC). The antitumor activity was investigated in mice. The tumor growth inhibitory rate, spleen index, and thymus index were calculated. The tumor inhibition rates of medium and high-dose MCP were 21.8% and 32.9%, respectively, which were significantly different with the model group. Spleen index and thymus index significantly increased in medium and high-dose MCP group. It was obvious that MCP could improve immunity and inhibit the growth of tumors.

Keywords: antitumor in vivo; extraction; *Mytilus coruscus*; polysacchrides; processing optimization; response surface method

Introduction

Polysaccharide is an important biological macromolecule which is widely existing in animals, plants, and microbials. With the progress in molecular biology research, people have gradually found that different sources of polysaccharides have extensive and complex biological activities (Jia *et al*., 2020; Mirzadeh *et al*., 2021; Sun *et al*., 2018; Wen *et al*., 2009, 2022; Yang *et al*., 2022; Ye *et al*., 2021; Zhou *et al*., 2021). Ocean is an important source of biopolysaccharides. *Mytilus coruscus* is one of the important and rich shellfish resources in China. It was shown that *M. coruscus* extract has functions such as anti-tumor, anti-aging, anti-viral and anti-bacterial, enhancing immunity, and lowering blood lipid content (Chu *et al*., 2008; Luan *et al*., 2010; Xu *et al*., 2007; Zhou *et al*., 2009). Polysaccharide is one of the most important active ingredients in it.

The response surface methodology (RSM) is an effective statistical tool for optimization of a process when independent variables have a combined effect on the desired response. It has been successfully used by many researchers in extracting processes. The main advantage of RSM is the usage of less number of experimental trials to evaluate multiple parameters and their

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interactions (Galai *et al*., 2012; Zhao *et al*., 2023; Zhong *et al*., 2010).

In this study, the extraction time, the extraction temperature, the extraction pH, and the liquid to fresh flesh ratio were optimized by RSM for getting the maximum *M. coruscus* polysaccharides (MCP) extraction yield.

It has been reported that *M. coruscus* polysaccharides possess a broad spectrum of biological, pharmacological, and therapeutic activities, including antioxidant activity, antiviral activity, antibacterial activity, anti-aging activity, and antitumor activity. Various extraction methods for polysaccharides are hot water extraction (Yu *et al.*, 2010; Zhang *et al.*, 2022), alkali extraction (Chen *et al.*, 2021; Zeng and Li, 2009), and enzyme extraction (Li *et al.*, 2018; Wen *et al.*, 2010). Our team compared the effect of different extraction methods on the extraction efficiency of polysaccharides from *M. coruscus* (Ma *et al.*, 2015). It was found that not only the enzyme extraction proved to be highly efficient and stable method for extraction of polysaccharides from *M. coruscus*, but also the chemical characters of MCP remained the best. Therefore, we first optimize the enzyme extraction method of MCP with RSM to get the optimal conditions. In addition, the antitumor activity in vitro of MRP was studied much, and there was a small report about whether the MCP had the antitumor activity in vivo. To know more about the antitumor activity of MRP, the mice weight, tumor growth inhibitory rate, spleen index, and thymus index of mice further investigation needed.

Materials and Methods

Materials and reagents

Mytilus coruscus was purchased from a local market in Ningbo, China. After the shells and algae were removed, the flesh of *M. coruscus* was homogenized and stored in a refrigerator at –20°C for standby. S180 cell was purchased from Kunming Pharmaceutical Research Institution. Cyclophosphamide injection was purchased from Jiangsu Hengrui Co. All reagents were purchased from Ningbo Aubo Chemical and Reagents Co. (Ningbo, China) and were of analytical grade.

Instrument

HH-3 electric-heated thermostatic water bath was supplied by Changzhou Guohua Co., LXJ-IIB centrifuge was supplied by Shanghai Feige Co., 722s Ultraviolet spectrophotometer was supplied by Shanghai Qinghua Co., BS124S balance (Satoris Corporation, Germany), MCO-18AC CO_2 incubator (Panasonic, Japan), and BCD-221TMBA refrigerator (Haier Corporation, China) were employed in the experiment.

Extraction procedure

The frozen flesh of *M. coruscus* was taken out of the refrigerator and kept at room temperature to be thawed. The flesh of *M. coruscus* was pre-extracted in a Soxhlet apparatus with petroleum ether, and afterwards with 95% ethanol twice at 60°C in a water bath for 2 h, to remove coloring matter, monosaccharides, disaccharides, oligosaccharides, and other small molecular weight materials. The obtained residue was extracted by the enzyme at different conditions including temperature, time, enzyme, pH, and ratio of liquid–solid. Then the extraction solution was centrifuged at 6000 rpm for 30 min. The supernatant was collected, followed by adding 95% ethanol successfully. After the mixture was stored in a refrigerator at 4°C for 12 h, it was centrifuged at 6000 rpm for 30 min. The precipitate was washed three times with Sevag reagent to remove the proteins and then freeze-dried to obtain crude MCPs. The polysaccharides extraction yield (%) was calculated as follows:

$$\text{Polysaccharides extraction yield (\%)} = \frac{\text{Dried polysaccharides weight (g)}}{\text{flesh weight (g)}} \times 100\% \quad (1)$$

Single-factor extraction design

At the beginning, cellulase, papain, and trypsin were optimized; it was found that the MCP yield (%) with papain was the best, so the papain was used in the following experiments. First, the extraction time was set at 30, 60, 90, 120, 150, 240, and 360 min when extraction pH, extraction temperature, and liquid-flesh ratio were fixed at 6.0, 55°C, and 10:1, respectively. Second, the extraction pH was set at 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 when extraction time, extraction temperature, and liquid-flesh ratio were fixed at 150 min, 55°C, and 10:1, respectively. Third, the extraction temperature was set at 40, 50, 60, 70, 80, 90, and 100°C when extraction time, extraction pH, and liquid-flesh ratio were fixed at 150 min, 6.0, and 10:1, respectively. Fourth, the liquid-flesh ratio was set at 5:1, 10:1, 20:1, 30:1, and 40:1 when extraction pH, extraction temperature, and extraction time were fixed at 6.0, 55°C, and 150 min, respectively. The effect of extraction time, extraction pH, extraction temperature, and liquid-flesh ratio on MCP yield (%) were investigated.

RSM extraction design

On the basis of single-factor experiments, a three-level, four-factor Box–Behnken design of RSM was performed.

As shown in Table 1, extraction temperature (X1), extraction time (X2), extraction pH (X3), and liquid-to-fresh flesh ratio (X4) were the independent variables selected to be optimized for the extraction of MCPs, and the three different levels were coded as +1, 0, and –1. The polysaccharides extraction yield was taken as the response for the combination of the independent variables given in Table 2. The extraction yield of MCPs was fitted using a second-order polynomial equation and multiple regression of data was carried out to obtain an empirical model related to the investigated factors. The forms of the response model were as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where, Y is the response variable, X_i and X_j are independent variables, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient. Design-Expert 8.0.6 (STAT-EASE Inc., Minneapolis, USA) was used for the statistical analysis of the experimental data. The analysis of variance (ANOVA) was used to estimate the statistical parameters.

Table 1. Independent variables and their levels used in the response surface design.

		Factor levels		
Independent variables	Symbol	-1	0	1
Extraction temperature (°C)	X1	40	55	70
Extraction time (min)	X2	60	150	240
Extraction pH	X3	4	6	7
Liquid–solid ratio (v/w)	X4	10	20	30

Table 2. Box–Behnken design and the extraction yield of MCP.

					Extraction yield (%)	
No.	X1	X2	X3	X4	Actual	Predicted
1	55	60	6.0	20	10.16	10.04
2	40	150	4.0	10	12.03	12.24
3	40	150	7.0	10	13.63	13.75
4	70	150	6.0	20	17.37	16.91
5	40	150	6.0	30	14.53	14.72
6	55	60	6.0	30	15.32	15.29
7	55	240	7.0	10	13.87	14.27
8	40	60	6.0	10	11.89	13.32
9	55	60	7.0	10	12.29	11.50
10	55	150	7.0	20	17.39	17.22
11	55	150	7.0	30	12.20	12.35
12	55	240	6.0	30	14.86	15.73
13	70	60	6.0	10	10.39	9.88
14	40	150	6.0	20	14.83	15.19
15	70	150	6.0	30	11.74	13.23
16	55	150	6.0	20	15.93	16.72
17	70	240	6.0	10	12.77	12.90
18	55	240	6.0	20	13.78	14.06
19	55	150	4.0	30	14.67	12.91
20	40	240	6.0	10	17.42	16.29
21	70	150	4.0	10	12.33	11.09
22	55	60	4.0	10	13.72	13.66
23	70	150	7.0	10	11.76	12.57
24	55	240	4.0	10	13.54	11.67
25	55	150	4.0	20	19.53	19.68

Characterization of MCP

General analysis

The content of MCP was determined using the phenol-sulfuric acid colorimetric method, choosing glucose as the standard. Protein content was determined according to Bradford's method using bovine serum albumin (BSA) as standard. Total uronic acids were assayed as anhydro galacturonic acid employing *m*-phenol color reagent.

Monosaccharide composition of polysaccharide

Monosaccharide composition of polysaccharides was determined by gas chromatography (GC) (Agilent 6890, USA). 10 mg polysaccharide was mixed with 10 mg hydroxylamine hydrochloride and 0.5 mL pyridine at 90°C for 30 min, after cooled, 0.5 mL acetic anhydride was added at 90°C for 30 min. Standards (mannose, glucose, galactose, rhamnose, fructose, xylose, and Arabia sugar) were carried out using the same method as above described for the sample. Lastly, the prepared aldononitrile acetate derivative solution was decompressed and was loaded on a DP-5 column (Agilent, USA) for further analysis under the following conditions: He_2, 1.0 mL/min; injection temperature, 250°C; MS detector; and column temperature programmed, 150°C for 2 min and then increased to 300°C at 15°C/min and maintained for 5 min at 300°C.

Determination of purity and molecular weight

The purity and molecular weight of the polysaccharide were determined by high-performance gel permeation chromatography (HPGPC) (Agilent 1200, USA) with a carbohydrate analysis column (TSK-G3000SWXL, TSK-G3000SWXL), using a mobile phase (0.1 mol/L sodium dihydrogen phosphate at a rate of 0.8 mL/min) and a refractive index detector (Agilent 1200, USA). The calibration curve was made based on a series of different

molecular sizes of dextrans (120, 100, 70, 40, and 10 kDa) as standard.

Methylation analysis

Methylation analysis was performed following the method of Needs and Selvendran (Galai *et al.*, 2012). 5 mg of MCP was dissolved in dimethyl sulfoxide and methylated using sodium cyanide and iodomethane, the completion of methylation was indicated by the disappearance of the hydroxyl absorption peak in the FTIR spectrum (Bruker) at 3400 cm^{-1}. After hydrolysis with 90% formic acid at 105°C for 6 h, then further hydrolyzed with 2 mol/L trifluoroacetic acid at 105°C for 6 h, the methylated sugar residues were converted to partially methylated alditol acetates by reduction with sodium borohydride, followed by acetylation with acetic anhydride. The derivatized sugar residues were extracted into toluene and evaporated to dryness, and dissolved again in chloroform. The products were quantitatively analyzed by GC-MS method.

In vivo antitumor activity

ICR mice (18–22 g) were purchased from the animal center of Zhejiang province and maintained in barrier facilities on a 12-h light/dark cycle and received food and water ad libitum. All animals were cared for in accordance with the National Institutes of Health guide for the care and use of experimental animals (NIH Publications No. 8023, revised 1978). S180 cells were cultured in RPMI 1640 medium supplemented with 10% FBS in a 5% CO_2 incubator at 37°C (Chen *et al.*, 2014; Qin *et al.*, 2022). Except for the control group, 0.2 mL (1×10^7 cells/mL) of seven-day-old S180 cells was transplanted into the right axilla of the mouse. The whole operation was finished in 30 min. These treatments were started 24 h after tumor inoculation and the MCPs were given once a day for 14 days. Fifty mice were randomly divided into six groups of 10 animals: the control group was not disturbed and received with normal saline orally, the model group was injected S180 cells without any treatment and received with normal saline orally, the low dose, medium dose and high dose group were injected S180 cells and received MCPs of 2 g/kg, 8 g/kg, and 16 g/kg, respectively, and the CTX group was injected S180 cells and was given CTX 20 mg/kg daily.

Animals were observed daily for activity, thinness, appearance of skin and hair, appetite, and irritability. Tumor size was measured daily using a ruler, and when the tumor size was plotted against time to measure tumor growth velocity. Twenty-four hours after the last treatment administration on day 14, the mice were weighed and sacrificed by cervical dislocation. The thymus, spleen, and solid tumors were removed and weighed. The anticancer activity in vivo was expressed as an inhibitory rate calculated by the formula: $[(A-B)/A] \times 100\%$, where A and B were the mean tumor weights of the model control and experimental groups, respectively. The spleen, liver, and thymus were evaluated by the organ index formula: spleen or thymus weight (g)/body weight (g).

Result and Discussion

Effect of extraction time on the yield of MCP

Extraction time is an important factor to associate with final polysaccharides extraction efficiency and energy cost (Xie *et al*., 2022; Ye *et al*., 2011). In this study, the effect of extraction time on the extraction yield of MCP was investigated and the result was shown in Figure 1. When extraction pH, extraction temperature, and liquid-flesh ratio were fixed at 6.0, 55°C, and 10:1, respectively. It could be found that the extraction yield increased rapidly from 30 to 200 min, and then increased slowly after 240 min (Figure 1A). This may be due to that MCP permeates into the raw material with the increase of extraction time, and then the enzyme activity decreases accordingly. So it is indicated that 240 min of extraction time was enough for the polysaccharides in the present work.

Effect of extraction temperature on the yield of MCP

Normally, with an increase in temperature, the enzyme activity may decrease. Polysaccharides can also be hydrolyzed and dissolved. So as shown in Figure 1B, the extraction yield increases with the increase of temperature between 40°C and 70°C. When the temperature exceeds 70°C, the extraction yield starts to decline when extraction time, extraction pH, and liquid-flesh ratio are fixed at 150 min, 6.0, and 10:1, respectively.

Effect of extraction pH on the yield of MCP

As we all know, there are two polysaccharides, acidic and alkaline polysaccharides. The amount of acidic polysaccharides is far more than that of alkaline polysaccharides. So the pH selection is important. The results showed that the extraction yield increased when the extraction pH was between 4.0 and 7.0, and then declined when the pH exceeded 7.0 (Figure 1C). This may be due to the structural destruction of MCP under alkaline conditions.

Effect of liquid-to-fresh flesh ratio on the yield of MCP

Liquid-to-fresh flesh ratio can significantly affect extraction yield (Manafu *et al.*, 2022). If the ratio of

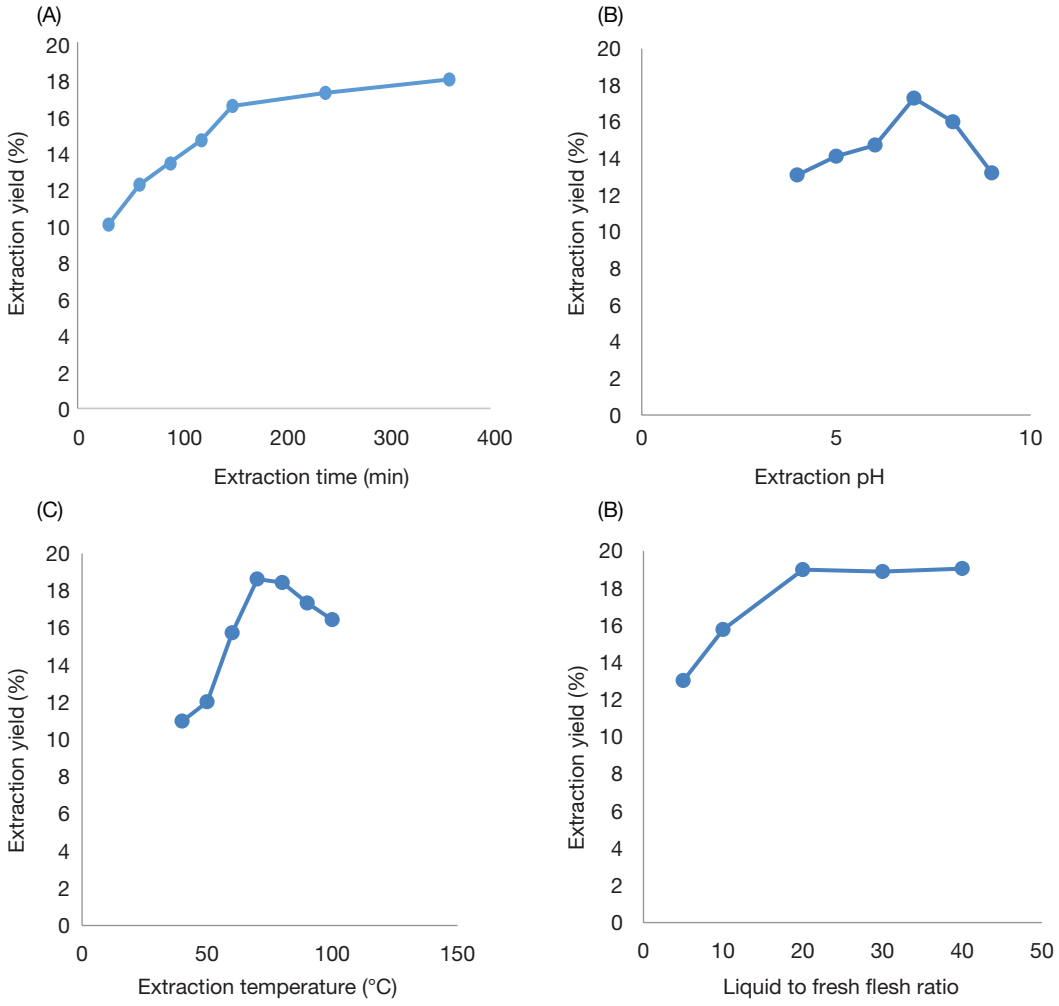

Figure 1. Effect of extraction parameters on the yield of MCP. (A) extraction time, (B) extraction temperature, (C) extraction pH, (D) liquid-to-fresh flesh ratio.

liquid-to-fresh flesh is too small, polysaccharides cannot be completely extracted. If the ratio of liquid-to-fresh flesh is too large, polysaccharides will be dissolved in the liquid which may lead to lower extraction yield (Du *et al.*, 2022; Zhu *et al.*, 2010). Therefore, when extraction pH, extraction temperature, and extraction time were fixed at 6.0, 55°C, and 150 min, respectively, the extraction yield of MCPs increases gradually with liquid-to-fresh flesh ratio. But when the ratio of liquid-to fresh-flesh was above 20, the extraction yield of MCPs does not increase (Figure 1D).

Statistical analysis and the model fitting

On the basis of preliminary study, BBD response surface methodology was applied to optimize the extraction conditions [extraction temperature (X_1), extraction time (X_2), extraction pH (X_3), and liquid-fresh flesh ratio (X_4)] of polysaccharides from *M. coruscus* by enzyme extraction. Twenty-five different combination of the investigated variables were carried out to optimize the four individual parameters. The experimental design and result is shown in Table 2. The following response surface model was obtained by multiple quadratic regression analysis of the experimental data, which was used to predict polysaccharide extraction yield (Y) based on the different values of extraction parameters (X1, X2, X3, and X4):

$$\begin{aligned} Y = {} & 1.49 + 0.075X_1 - 0.035X_2 + 0.02X_3 - 0.41X_4 \\ & -0.077X_1X_2 - 0.51X_1X_3 - 0.82X_1X_4 + 0.10X_2X_3 \\ & -0.11X_2X_4 + 0.083X_3X_4 - 0.93X_1^2 - 0.30X_2^2 \\ & -0.21X_3^2 - 0.38X_4^2 \end{aligned} \quad (3)$$

The statistical significance of the model equation was evaluated by ANOVA as shown in Table 3. The model P-value was less than 0.01, which implied that the model was extremely significant and the tests were reliable. The P-value of lack of fit was 0.034 which is less than 0.05

Table 3. The fitted quadratic polynomial model of ANOVA.

Origin	SS	DF	MS	F-value	P-value	Significance
Model	13.45	14	3.15	21.42	<0.0001	*
X_1	4.79	1	4.79	14.72	0.0039	***
X_2	2.55	1	2.55	15.19	0.0078	***
X_3	0.16	1	0.16	0.88	0.6872	
X_4	0.85	1	0.85	16.31	0.0233	**
X_1X_2	0.013	1	0.013	0.73	0.4399	
X_1X_3	0.65	1	0.65	2.57	0.3072	
X_1X_4	0.024	1	0.024	8.64	0.2783	
X_2X_3	0.0036	1	0.0036	3.49	0.8078	
X_2X_4	4.53	1	4.53	15.66	0.0656	
X_3X_4	6.02	1	6.02	9.37	0.2356	
X_1^2	0.59	1	0.59	2.88	0.0349	**
X_2^2	0.0067	1	0.0067	6.73	0.0061	***
X_3^2	2.26	1	2.26	19..85	0.0076	***
X_4^2	0.055	1	0.055	7.98	0.0866	
Residual	1.67	14	0.01			
Lack of fit	0.51	10	0.27		0.034	**
Pure error	0.26	4	0.02			
Cor total	12.01	28				
R^2	0.957					
R^2_{adj}	0.923					

*No significant difference (P > 0.05), **Significant difference (P < 0.05), ***Extremely significant difference (P < 0.01).

indicating that the model fits well. For the model fitted, the coefficient of determination (R^2) was 0.957 which indicated that the fitting result of 95.7% is consistent with the actual situation, and the error is small, which can fully reflect the relationship between the extraction temperature, extraction time, extraction pH, the ratio of liquid-to-fresh flesh and the extraction yield of MCP. And the adjusted coefficient of determination (R^2_{adj}) was 0.923 which indicated a high consistency between the actual and predicted extraction yield. As the P-values implied the degree of significance of each extraction condition, the P-value was smaller and the degree of significance was higher. It can be seen that X_1, X_2, X_2^2, and X_3^2 showed extremely significant differences ($P < 0.01$), X_4 and X_1^2 showed significant effects ($P < 0.05$).

Optimization of extraction conditions of MCP

The 3D response surface plots and 2D contour plots can be made according to the regression model equation as shown in Figures 2 and 3. With the help of the plots, we can find the effect of the interaction of two extraction parameters on the extraction yield easily. When the shape of the contour is close to the ellipse, it means that the interaction between the two extraction conditions is significant. When the shape of the contour is closer to the circle, the interaction between the two variables is small. When the response surface graph is steeper, it indicates that extraction conditions have a more significant impact on the extraction yield of polysaccharides.

Figures 2A and 3A illustrate that while the extraction pH and liquid-to-fresh flesh ratio were fixed, the extraction yield of MCP increased evidently with increasing of extraction time from 60 to 150 min, but beyond 200 min, the extraction yield of MCP declined and the extraction yield of MCP increased slowly with increasing of extraction temperature.

Figures 2B and 3B show that while the extraction time and liquid-to-fresh flesh ratio were fixed, the extraction yield of MCP increased evidently with increasing of extraction pH from 4.0 to 5.6, but beyond 6.0, the extraction yield of MCP declined.

The effect of various extraction temperatures and ratios of liquid-to-fresh flesh on extraction yield of MCP at fixed extraction time and pH are shown in Figures 2C and 3C. When liquid-to-fresh flesh ratio ranged from 16 to 25 and the extraction temperature from 40°C to 60°C, the extraction yield of MCP was increased significantly with the increasing of liquid-to-fresh flesh ratio and extraction temperature.

Figures 2D and 3D showed obviously that the increase of extraction yield of MCP could be achieved significantly with increasing of extraction time from 60 min to 180 min and the extraction yield of MCP was increased rapidly with the increasing extraction pH from 4.0 to 5.6, but beyond 6.0, the extraction yield of MCP declined, while the extraction temperature and liquid-to-fresh flesh ratio were fixed.

In Figures 2E and 3E, the extraction yield of MCP was affected by different liquid-to-fresh flesh ratio and extraction time. The extraction yield of MCP increased

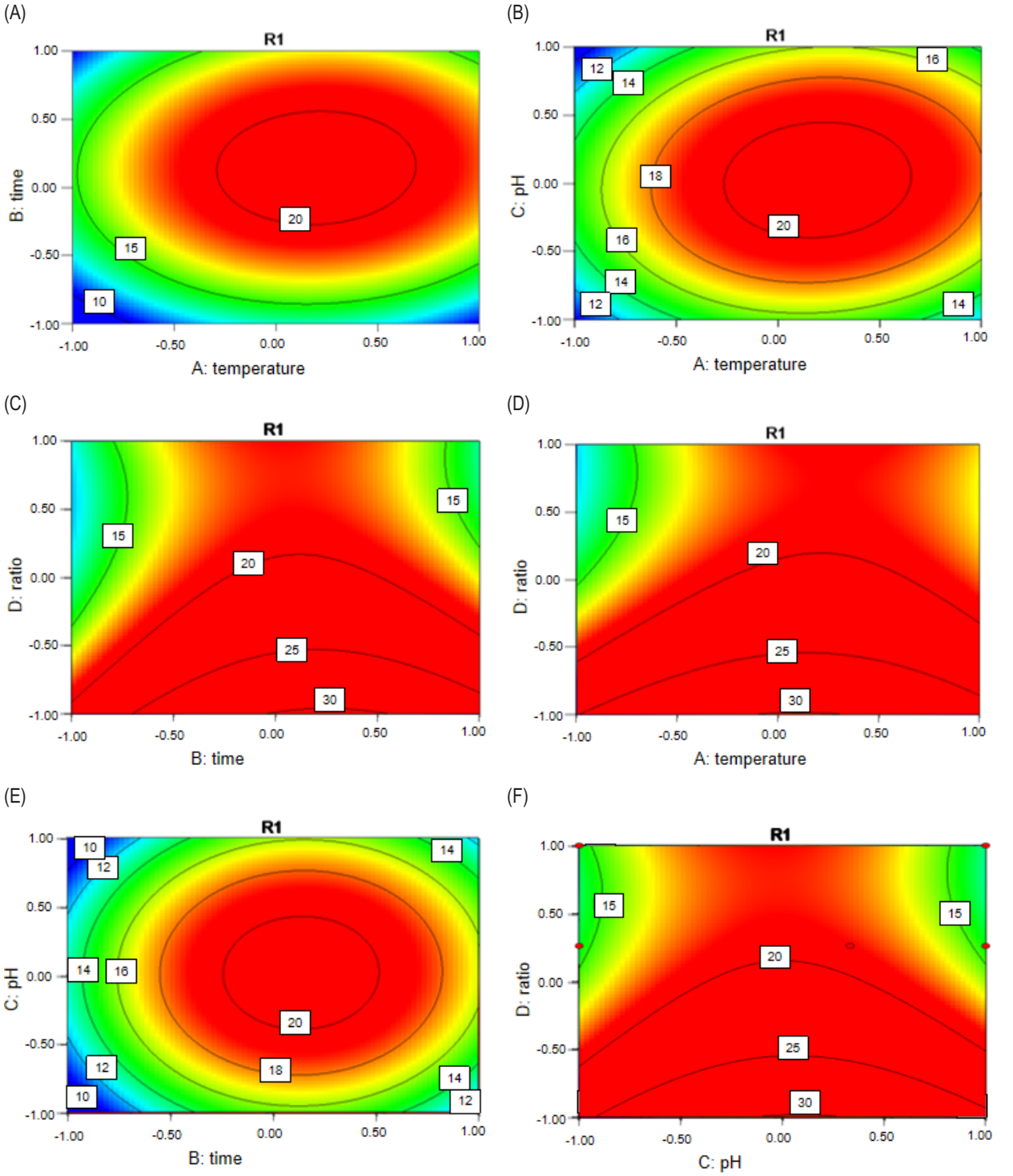

Figure 2. Contour plots showing the interaction between two test variables (extraction temperature, extraction time, extraction pH, and liquid-to-fresh flesh ratio) while the other factors were fixed at zero.

with the increase of liquid-to-fresh flesh ratio from 18 to 25 and extraction time from 100 to 190 min.

According to 3D and contour plots shown in Figures 2F and 3F, while the extraction time and temperature were fixed, the extraction yield of MCP increased with the increase of liquid-to-fresh flesh ratio from 18 to 25.5 and the extraction pH from 4.1 to 5.6.

According to the above study, it can be concluded that the optimal extraction condition of MCP is as follows: extraction time was 173.34 min, extraction temperature was 56.82°C, extraction pH was 5.64, and ratio of liquid-to-fresh flesh was 19.82. Among the four variables, the extraction time and temperature were most significant to affect the extraction yield of MCP, and liquid-to-fresh flesh ratio and extraction pH affect the extraction yield of MCP less according to the regression coefficients' significance of quadratic polynomial model.

Verification of the model

In order to validate the reliability of the model equations, experiments were carried out under the following conditions: extraction time of 173.34 min, extraction temperature of 56.82°C, extraction pH of 5.64, and ratio of liquid-to-fresh flesh of 19.82. A mean value of 19.53 ± 0.21%, obtained from three experiments, is basically consistent with the predicted value of the model (19.68%). Thus, it was confirmed that the response surface model was suitable for the optimization of MCP extracting conditions.

Characterization of MCP

MCP was white powder which was soluble in hot water. Monosaccharide composition analysis by GC-MS showed that MCP consisted of Rhamnose, Arabia sugar, fructose, mannose, glucose, and galactose with a molar ratio of 1.9

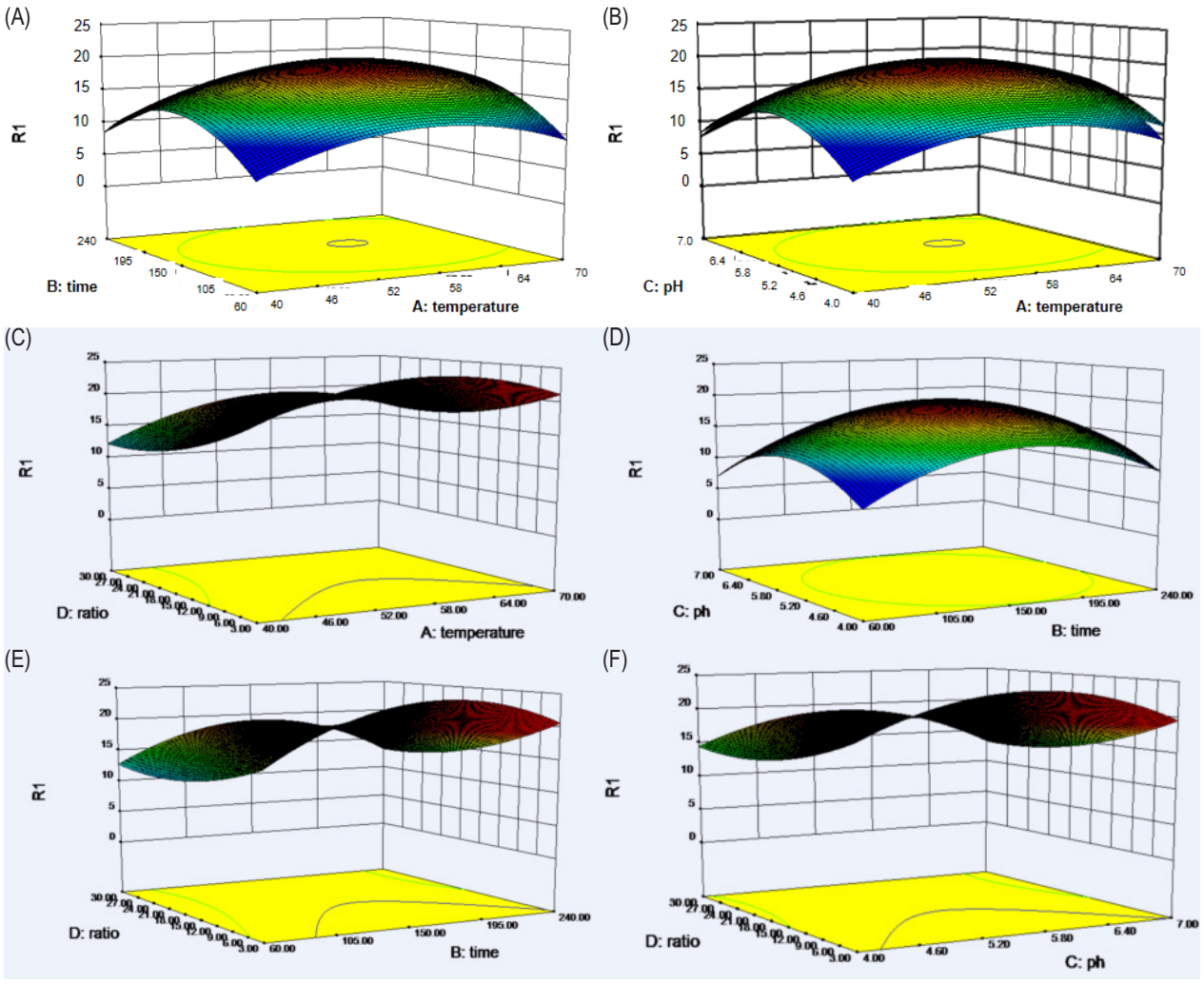

Figure 3. Response surface plots for the mutual effects of four variables on the extraction rate of MCP.

Table 4. Methylation analysis of MCP.

Methylated product	Molar ratio	Linkage pattern
1,5-di-O-acetyl-2,3,4-tri-O-methyl-galactitol	2.1	Gal*p*(1→
1,5-di-O-acetyl-2,3,4,6-quadra-O-methyl-galactitol	0.8	Gal*p*(1→
1,3,5-tri-O-acetyl-2,4-di-O-methyl-mannitol	1.0	→3)Man*p*(1→
1,3,5-tri-O-acetyl-2,4-di-O-methyl-mannitol	1.0	→3)Man*p*(1→
1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol	3.5	→4)Glc*p*(1→
1,4,5,6-quadra-O-acetyl-2,3-di-O-methyl-glucitol	2.8	→4,6)Glc*p*(1→
1,4,5-tri-O-acetyl-2-acetyl amine-3,6-di-O- methyl-glucitol	2.2	→4)Glc*p*(1→

Table 5. Effect of treatment with CTX or MCP in mice bearing S180 tumor on body weight, spleen index, and thymus index.

Group	Dosage (g/kg)	Body weight (g)	Spleen index (mg/g)	Thymus index (mg/g)	Tumor inhibition (%)
Control		31.72±1.25	5.22±0.39	2.19±0.26	
Model		29.89±2.03	5.13±0.55	2.01±0.57	
Low dose	2	27.67±1.02	5.27±0.76	2.23±0.34	5.2
Medium dose	8	29.03±1.27	6.01±0.74	2.45±0.29	21.8
High dose	16	28.78±1.43	6.68±0.98	2.69±0.37	32.9
CTX	0.02	22.04±0.67	3.54±0.45	1.66±0.24	30.9

2:1.29:8.18:27.90:27.63:29.50. The m-hydroxydiphenyl method revealed that it contained 10.58% uronic acid. In correlation with the calibration curve of dextran standards, the molecular weight of MCP was about 57 kDa. MCP contained 93.6% total carbohydrate and minor amounts of protein (0.33%).

To obtain linkage information for monosaccharides, MCP was subjected to methylation analysis (Table 4). 1,5-di-O-acetyl-2,3,4-tri-O-methyl-galactitol, 1,5-di-O-acetyl-2,3,4,6-quadra-O-methyl-galactitol, 1,3,5-tri-O-acetyl-2,4-di-O-methyl-mannitol, 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-mannitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol, 1,4,5,6-quadra-O-acetyl-2,3-di-O-methyl-glucitol,1,4,5-tri-O-acetyl-2-acetyl amine-3, 6-di-O-methyl-glucitol were detected, indicating that there were four types of monosaccharide linkage structure in which the main linkage was 1→4 linked glucose, 1→3 linked mannose, 1→4,6 linked glucose, and 1→4 linked galactose.

Antitumor activity in vivo

As shown in Table 5, the tumor inhibition rates of low, medium, and high dose MCP were 5.2%, 21.8%, and 32.9%, respectively, which were significantly different with the model group ($P < 0.01$). The result showed that different dose of MCP had different degree of inhibition to tumor. It was worth to pay attention to the body weight of the mice, when the tumors were inhibited by CTX, the body weight of the mice decreased. When the tumors were inhibited by MCP, there was a slight change in the body weight of the mice. The result showed that when MCP works on the tumor, the other functions may not be affected.

The spleen index and thymus index decreased in the CTX group, which indicates that CTX had obvious effect of immunity suppression. There was no difference in the spleen index and thymus index between low dose group and the model group, but the spleen index and thymus index were significantly higher in medium and high dose MCP group than in the model group ($P < 0.05$). It illustrated that the medium and high dose MCP activated the immune system of mice to compete with tumors.

Conclusions

The present study is undertaken to optimize the enzyme extraction conditions of polysaccharides from *M. coruscus* and investigate the antitumor activity in vivo. The response surface method proved to be useful for the optimization of experimental variables based on single-factor experimental design. It was revealed that an optimum yield of MCP could be obtained by using extraction time of 173.34 min, extraction temperature of 56.82°C, extraction pH of 5.64, and ratio of liquid-to-fresh flesh of 19.82. Under these conditions, the actual yield of MCP was 19.53 ± 0.21%, which was in accordance with the

predicted value (19.68%). It was shown that MCP was composed of Rhamnose, Arabia sugar, fructose, mannose, glucose, and galactose with a molar ratio of 1.92:1 .29:8.18:27.90:27.63:29.50 and the main linkage type was 1→4 linked glucose by detecting the monosaccharide composition and methylation analysis using GC-MS. Tumor growth inhibitory rate, spleen index, and thymus index in mice were used to investigate the antitumor activity of MCP. Because the immune function of tumor patients receiving chemotherapy is very weak, the recurrence and prognosis of tumor are closely related to the low immune function of the body, so improving the immune function of the body is essential to prevent cancer. This study suggested that MCPs have the activity of antitumor and can enhance the immune system, which maybe provide support for the future application in enhancing the body function and treating cancer. As for the mechanism of MCP, improving the immune function and whether it could be used with chemotherapy drugs to repair the damage of chemotherapy drugs to the body is to be further studied.

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