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Optimization of Polysaccharide Extraction from *Polygonatum odoratum* by Response Surface Methodology and Evaluation of its Antitumor Activity

Zhan Xiao-ri[1,2], Xu Li-li[1], Xiong Feng[3], Li Xiao-lin[2], Yu Chun-na[1], Xie Tian[2] and Li Xia[3]*

[1]College of Life and Environment Science, Hangzhou Normal University, Hangzhou 310036, People's Republic of China
[2]Resource Center for Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, People's Republic of China
[3]Guangxi Key Laboratory of Electrochemical and Magneto-chemical Functional Materials, College of Chemistry and Bioengineering, Guilin University of Technology, Guilin, Guangxi 541004, People's Republic of China

Abstract

This work established a microwave-assisted procedure for polysaccharide extraction from *Polygonatum odoratum*. Response surface methodology was used to optimize microwave-assisted extraction parameters (extraction temperature, water-to-raw material ratio, microwave power, and extraction time) by implementing a three-level, four-variable Box–Behnken experimental design in a single-factor investigation. Three-dimensional response surfaces were plotted by Design-Expert, and result indicated the absence of interaction effects of extraction temperature and microwave power. A polysaccharide yield of approximately 17.49% was obtained under the following optimized conditions: temperature of 57°C, microwave power of 300 W, extraction time of 10 min, and water-to-raw material ratio of 23:1. The results of preliminary *in vitro* antitumor activity tests showed that polysaccharides derived from *P. odoratum* could inhibit growth of A549 cells in a dose-dependent manner. Moreover, 4-400 μg/mL of these polysaccharides exerted no significant cytotoxicity to Panc-1 cells.

Keywords: Microwave-assisted extraction; Polysaccharides; *Polygonatum odoratum*; Response surface methodology

Introduction

Polysaccharides are essentially polymeric carbohydrates consisting of monosaccharides linked by glycosidic bonds. Depending on their structure, polysaccharides display functional properties distinct from those of their constituent monosaccharides; as a result, they play a wide array of important roles in nature [1]. Plant-derived polysaccharides demonstrate a variety of biological properties, including anti-oxidative [2-4], anti-viral [5], and anti-complementary activities [6,7]. Over the years, polysaccharides have become important raw materials for health foods, drugs, and cosmetics.

Rhizoma Polygonati Odorati (Fragrant Solomonseal Rhizome, Yu Zhu) is the dried rhizome of *Polygonatum odoratum* (Mill.), which has been used for centuries in traditional herbal medicine as an important source of medicine and as base for a valuable nourishing tonic. The Ministry of Health of the People's Republic of China has defined *P. odoratum* as an affinal drug and a diet material. Traditionally, *P. odoratum* is described to possess the capability to nourish the yin and to promote fluid production to quench thirst. This medicinal plant is applied to treat diabetes, palpitations, lung illnesses, and upset stomachs. Currently, *P. odoratum* is usually used as a functional food given that long-term consumption of this plant does not damage the stomach [8-10]. *P. odoratum* contains various chemical components, such as polysaccharides, flavonoids, steroidal saponins, and alkaloids. Studies have shown that *P. odoratum* polysaccharides (POP) demonstrate immunological, anti-tumor, and anti-aging activities [11]. However, studies on POP extraction remain low.

Microwave assists in solvent extraction of bioactive compounds from herbs [12-15]. Microwave extraction has many advantages, including reduced consumption of organic solvent and shorter extraction time. Thus, microwave extraction is more effective than traditional extraction methods.

When many factors and interactions affect desired response, response surface methodology (RSM) is an effective tool for optimizing the process, which was originally described by Box and Wilson. RSM is a collection of statistical and mathematical techniques used to determine the effects of several variables and to optimize various processes [16]. RSM has been successfully applied to optimize conditions in food and pharmaceutical research [16,17]. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple variables and their interactions. Therefore, it is less laborious and time consuming than other approaches required optimizing a process. Usually, it applies an experimental design such as Box–Behnken (BBD), central composite (CCD) and Doehlert designs (DDD) to fit a second-order polynomial by a least squares technique. An equation is used to describe how the test variables affect the response and determines the interrelationship among the variables.

This study investigated significant variables (ratio of water to raw material, extraction temperature, extraction time, microwave power) and further optimize the extraction process of polysaccharides from *P. odoratum* using RSM, while employing a three-level, four-variable BBD. Inhibitory effect of POP extraction on A549 and Panc-1 cell lines was investigated.

***Corresponding author:** Xia Li, Guangxi Key Laboratory of Electrochemical and Magneto-chemical Functional Materials, College of Chemistry and Bioengineering, Guilin University of Technology, Guilin, Guangxi 541004, People's Republic of China, Tel: +8618107735083; Fax: +865896839; E-mail: biology754@163.com

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Materials and Methods

Materials and reagents

A549 lung adenocarcinoma cells and Panc-1 human pancreatic carcinoma cells were obtained from the Department of Research Center for Biomedicine and Health, Hangzhou Normal University (Hangzhou).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). RPMI 1640 and FBS were obtained from Gibco. MAS-I Microwave Synthesis System was obtained from Shanghai Sineo Microwave Chemistry Technology Co., Ltd (Shanghai).

Preparation of POP

P. odoratum was dried at 40°C for 48 h and then pulverized in a blender to obtain a fine powder (particle diameter is <500 μm). The pulverized *P. odoratum* was soaked in 95% ethanol twice for defatting and removal of colored materials, oligosaccharides, and small molecules.

Each pretreated dried sample (5.0 g) was extracted with water under specified extraction temperature, water-to-raw material ratio, microwave power, and extraction time. The water extraction solutions were centrifuged at 3000 rpm for 15 min, and then the supernatant was separated from insoluble residue with four-layer filter cloth, which were precipitated by the addition of ethanol to a final concentration of 80% (v/v) and incubated overnight. The precipitates collected through centrifugation (3000 rpm for 15 min) were washed three times with dehydrated alcohol and ethyl ether and then dried under reduced pressure to obtain crude polysaccharides.

Determination of polysaccharides yield

The polysaccharide content was determined through the phenol–sulfuric acid method by using D-glucose as standard [18]. The crude polysaccharides were accurately weighed and dissolved in distilled water in a 100 mL volumetric flask. Afterwards, the sample solution was carefully transferred into a 10 mL cuvette. Phenol (5%, 1 mL) was added into the cuvette and then shaken. Sulfuric acid (7.0 mL) was subsequently added into the mixture, shaken, incubated at 40°C in a water bath for 30 min, and then allowed to stand in an ice bath for 5 min. Absorbance values were recorded by a UV spectrophotometer at 490 nm. The wash solution served as blank control and was measured in a similar manner. The regression equation between microgram value of glucose and OD value were obtained as: A=6.0667C+0.1078 (A: 490 nm OD, C: μg/mL), r=0.9972; linearity range: 10-40 μg/mL.

Experimental design

After the range of extraction variables was preliminarily determined through a single-factor test, a three-level BBD was performed using four independent variables (X1, extraction temperature; X2, water-to-raw material ratio; X3, microwave power; and X4, extraction time). For statistical calculation, the variables were coded according to

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where χ_i is a coded value of a variable; X_i is the actual value of a variable; X_0 is the actual value of X_i at the center point; and ΔX_i is the step change value. Table 1 shows the range of independent variables and their levels. The independent variables and their ranges were determined in our preliminary experiments. Three experiments for each condition were performed and the mean values were considered as observed responses. The complete design consisted of 27 experimental points, and the experiments were performed in a random order (Table 2).

Data from the BBD were analyzed by multiple regressions to fit the following quadratic polynomial model.

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

Y represents the response function. β_0 is an intercept. β_i, β_{ii}, and β_{ij} are the coefficients of the linear, quadratic, and interactive terms, respectively. χ_i and χ_j represent the coded independent variables, respectively. The fitted polynomial equation was expressed as surface and contour plots to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions [19]. The regression coefficients of individual linear, quadratic, and interaction terms were determined through analysis of variance. These regression coefficients were used in statistical calculation to generate dimensional and contour maps from the regression models. Design-expert version 8.0.4 (Trial Version, State-Ease Inc., Minneapolis, MN, USA) was used to analyze the experimental data. P-values of less than 0.05 indicated statistical significance.

Cytotoxicity assay *in vitro*

POP was dissolved in distilled water at a concentration of 10 g/L and then filtered through a 0.22 μm filter and stored at 4°C. The solution was further diluted to different concentrations (800 μg/mL, 80 μg/mL, 8 μg/mL) with RPMI 1640 medium in tissue plates separately.

Cell proliferation was analyzed through colorimetric MTT assay as described previously [20]. Briefly, cells were seeded in a 96-well microplate (1×10^4 cells/well, in a volume of 100 μL), treated with or without 5-Fu (1 μg/mL), DDP (10 μg/mL) and incubated in RPMI 1640 medium containing 10% FBS with 100 μL sample polysaccharides at a concentration of 400 μg/mL, 40 μg/mL, 4 μg/mL. The experiments were performed in triplicate. The cells were subsequently incubated at 37°C under a humidified 5% CO_2 atmosphere.

MTT (5 mg/mL, 20 μL) was added after 72 h. After the plates were incubated at 37°C for 4 h, the supernatant was aspirated and 150 μL of DMSO was added into each well. Absorbance was measured at 570 nm by a 96-well microplate reader (Bio-Rad, USA). The inhibition rate of cell growth was calculated as follows:

(mean value for control group−mean value for treated group)/ control group × 100%.

Results and Discussion

Effect of extraction temperature on extraction yield of polysaccharides

Different extraction temperature was set at 30, 40, 50, 60 and 70°C, respectively, to investigate the influence of extraction temperature on the yield of POP. The other reaction conditions were as follows: water-to-raw material ratio of 20:1, microwave power of 200 W, and extraction time of 10 min. Figure 1a indicates that the maximum yield was obtained when the extraction temperature was increased from 40°C to 50°C. However, when the extraction temperature was further increased, the yield declined linearly. Therefore, the optimal extraction temperature adopted in this work is 50°C.

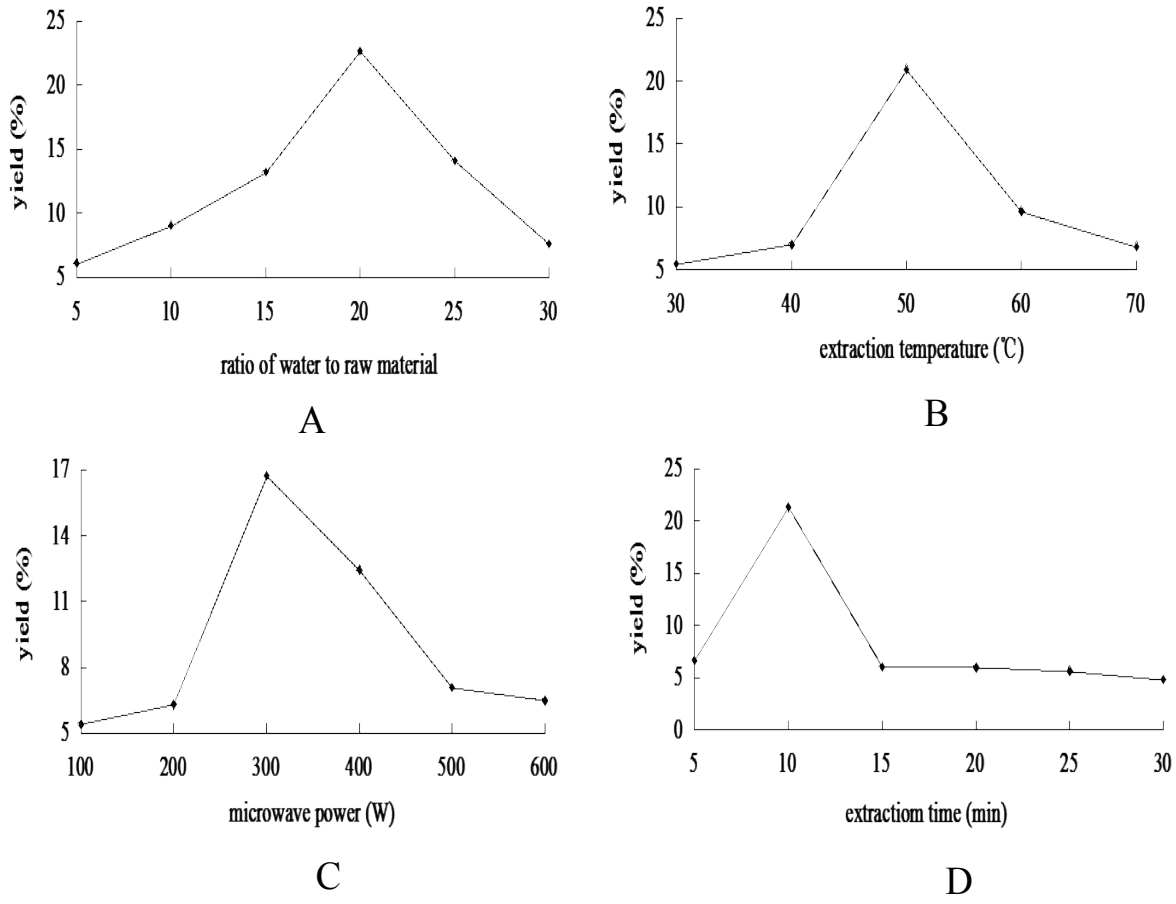

Figure 1: Effect of different extraction parameters (a: ratio of water to raw material; b: extraction temperature, °C; c: microwave power, W; d: extraction time, min) on yield of polysaccharides.

Effect of water to raw material ratio on extraction yield of polysaccharides

Figure 1b shows the effect of different water-to-raw material ratios (5:1, 10:1, 15:1, 20:1, 25:1, and 30:1) on POP yield. When the extraction temperature (50°C), microwave power (200 W), and extraction time (10 min) were set at level 0, the yield suddenly increased to the critical value of 22.68% under 20:1 ratio. This result indicated that when the ratio is higher than 20:1, the polysaccharide yield decreases possibly because polysaccharides can be excessively dissolved in low-concentration solvent, resulting in considerable yield loss during product collection. Therefore, the water-to-raw material ratio of 20:1 was adopted in the following work.

Effect of microwave power on the extraction yield of polysaccharides

Figure 1c shows the effect of microwave power on POP yields. When the extraction temperature, water-to-raw material ratio, and extraction time were set at 50°C, 20:1, and 10 min, respectively, the yield obviously increased under microwave power of 100-600 W. The polysaccharide yield was 16.74% when the samples were extracted at 300 W. However, the yield decreased significantly when the microwave power further increased, indicating that 200 W is sufficient for polysaccharide extraction in the present work.

Effect of extraction time on extraction yield of polysaccharides

Extraction time influences the extraction efficiency and selectivity of a fluid. A longer extraction time increases polysaccharide yields [21,22]. This study investigated the effect of extraction time (5, 10, 15, 20, 25, and 30 min) on polysaccharide yield when the extraction temperature is 50°C, water-to-raw material ratio is 20:1, and microwave power is 200 W. Figure 1d shows that the maximum yield was obtained when the extraction time was 10 min; beyond this period, the yield decreased significantly; more over a descending dynamic equilibrium was observed with extended extraction time. Therefore, the extraction time used in this work is 5-15 min.

Optimization of the extraction parameters of polysaccharides

Statistical analysis and the model fitting: RSM optimization is more advantageous than the traditional single-parameter optimization because the former saves time, space, and raw materials [23]. A total of 29 runs were performed to optimize the four individual parameters in BBD. Table 2 shows the experimental conditions and the polysaccharide yields according to the factorial design. By applying multiple regression analysis on the experimental data, the response variables and the test variables were related by the following second-order polynomial equation:

Independent variables	Factor level		
	-1	0	1
X_1: Extraction temperature (ºC)	40	50	60
X_2: Ratio of water to raw material	15	20	25
X_3: Microwave power (W)	200	300	400
X_4: Extraction time (min)	5	10	15

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

Run	X_1	X_2	X_3	X_4	Yield (%)
1	1	0	0	-1	13.98
2	0	-1	1	0	9.26
3	-1	0	-1	0	6.99
4	-1	-1	0	0	11.07
5	0	0	1	-1	9.24
6	0	0	0	0	16.74
7	1	0	1	0	10.58
8	0	-1	-1	0	2.37
9	-1	0	1	0	7.07
10	-1	0	0	1	10.27
11	1	-1	0	0	1.12
12	0	0	-1	1	6.02
13	1	0	-1	0	9.68
14	0	0	0	0	16.74
15	0	-1	0	-1	3.37
16	0	0	1	1	10.83
17	0	0	-1	-1	10.22
18	1	0	0	1	8.80
19	1	1	0	0	17.23
20	0	0	0	0	16.74
21	0	1	1	0	8.84
22	0	0	0	0	16.74
23	0	1	0	1	13.75
24	0	1	0	-1	6.56
25	0	1	-1	0	9.54
26	-1	1	0	0	1.16
27	0	0	0	0	16.74
28	0	-1	0	1	4.06
29	-1	0	0	-1	5.03

Table 2: Box–Behnken experimental design and results for extraction yield.

$$Y=16.78+1.62\times X_1+1.61\times X_2+0.92\times X_3-0.097\times X_4+6.59\times X_1\times X_2+0.20\times X_1\times X_3-2.52\times X_1\times X_4-1.90\times X_2\times X_3+3.25\times X_2\times X_4+1.45\times X_3\times X_4-4.03\times X_1\times X_1-5.01\times X_2\times X_2-4.28\times X_3\times X_3-3.28\times X_4\times X_4 \quad (3)$$

Table 3 shows the ANOVA results for the experimental BBD data. The determination coefficient (R^2=0.9910) indicated that less than 1.0% of the total variations were not explained by the model. Moreover, the adjusted determination coefficient (AdjR^2=0.9437) confirmed that the model was highly significant. Low values of coefficient of variance (6.81) clearly indicated that the model was reproducible and reliable [24]. A Pred R-Squared of 0.9457 was reasonably consistent with an Adj R-Squared of 0.9820. Adequate Precision was used to measure the signal-to-noise ratio. A ratio greater than 4 is desirable, and a ratio of 34.650 indicated adequate signal. This model can be used to navigate the design space.

The P value was used as a tool to determine the significance of each coefficient; moreover, it indicated the strength of interaction between independent variables. The smaller the P value, the more significant the corresponding coefficient would be [21]. Table 3 shows that the linear coefficients (X_1, X_2, and X_3), quadratic term coefficients (X_1^2, X_2^2, X_3^2, and X_4^2), and cross product coefficients (X_1X_2, X_1X_4, X_2X_3, X_2X_4, and X_3X_4) were significant, and their P-values were very low (P<0.05). The other term coefficients were not significant (P>0.05). The full model filled Eq. (3) was made into three-dimensional and contour plots to predict the relationships between the independent and dependent variables.

Optimization of polysaccharide extraction conditions: Response surfaces were plotted using Design-Expert (version 8.0) software to study the effect of parameters on polysaccharide yield, as well as the interactions of these parameters. 3-D response surface plots and 2-D contour plots (Figures 2 and 3) were very useful to assess the interaction effects of the factors on the responses. These types of plots show the effects of two factors on the response at a time. In all of the plots, the two other factors were kept at level 0. In the two plots, the maximum predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when a perfect interaction occurs between independent variables.

Figures 2a and 3a show the effect of water-to-raw material ratio, extraction temperature, and their reciprocal interaction on extraction yield when extraction time and microwave power were fixed at level 0. A strong interaction between these factors were observed. The polysaccharide yield decreased at the designed extraction temperature range (40°C-60°C), and a quadratic effect on the response yield was observed when the water-to-raw material ratio increased from 15 to 25. The maximum extraction yield could be achieved at a water-to-raw material ratio and extraction temperature of approximately 20 and 50°C, respectively.

Figures 2b and 3b show the 3-D response surface plot and contour plot under varying extraction temperatures and microwave powers at a fixed water-to-raw material ratio (level 0) and extraction time (level 0). The plots indicate that the polysaccharide yield increased at 40°C-52°C

Source	SS	DF	MS	F value	P value Prob.>F
Model	671.27	14	47.95	110.32	< 0.0001
X_1	32.42	1	32.42	74.60	< 0.0001
X_2	29.68	1	29.68	68.29	< 0.0001
X_3	10.08	1	10.08	23.20	0.0003
X_4	0.11	1	0.11	0.25	0.6276^{ns}
X_1X_2	190.08	1	190.08	437.34	< 0.0001
X_1X_3	0.17	1	0.17	0.39	0.5440^{ns}
X_1X_4	27.67	1	27.67	63.67	< 0.0001
X_2X_3	14.40	1	14.40	33.14	< 0.0001
X_2X_4	36.67	1	36.67	84.38	< 0.0001
X_3X_4	8.38	1	8.38	19.28	0.0006
X_1^2	103.48	1	103.48	238.08	< 0.0001
X^2_2	162.60	1	162.60	374.12	< 0.0001
X_3^2	116.86	1	116.86	268.86	< 0.0001
X_4^2	69.88	1	69.88	160.79	< 0.0001
Residual	6.08	14	0.43		
Lack of Fit	6.08	10	0.61		
Pure Error	0.000	4	0.000		
Cor Total	677.35	28			
SD	0.66		**R^2**		0.9910
Mean	9.68		**Adj R^2**		0.9820
CV (%)	6.81		**Pred R^2**		0.9457
PRESS	36.79		**Adequate Precision**		34.650

Table 3: Analysis variance of experimental of the BBD.

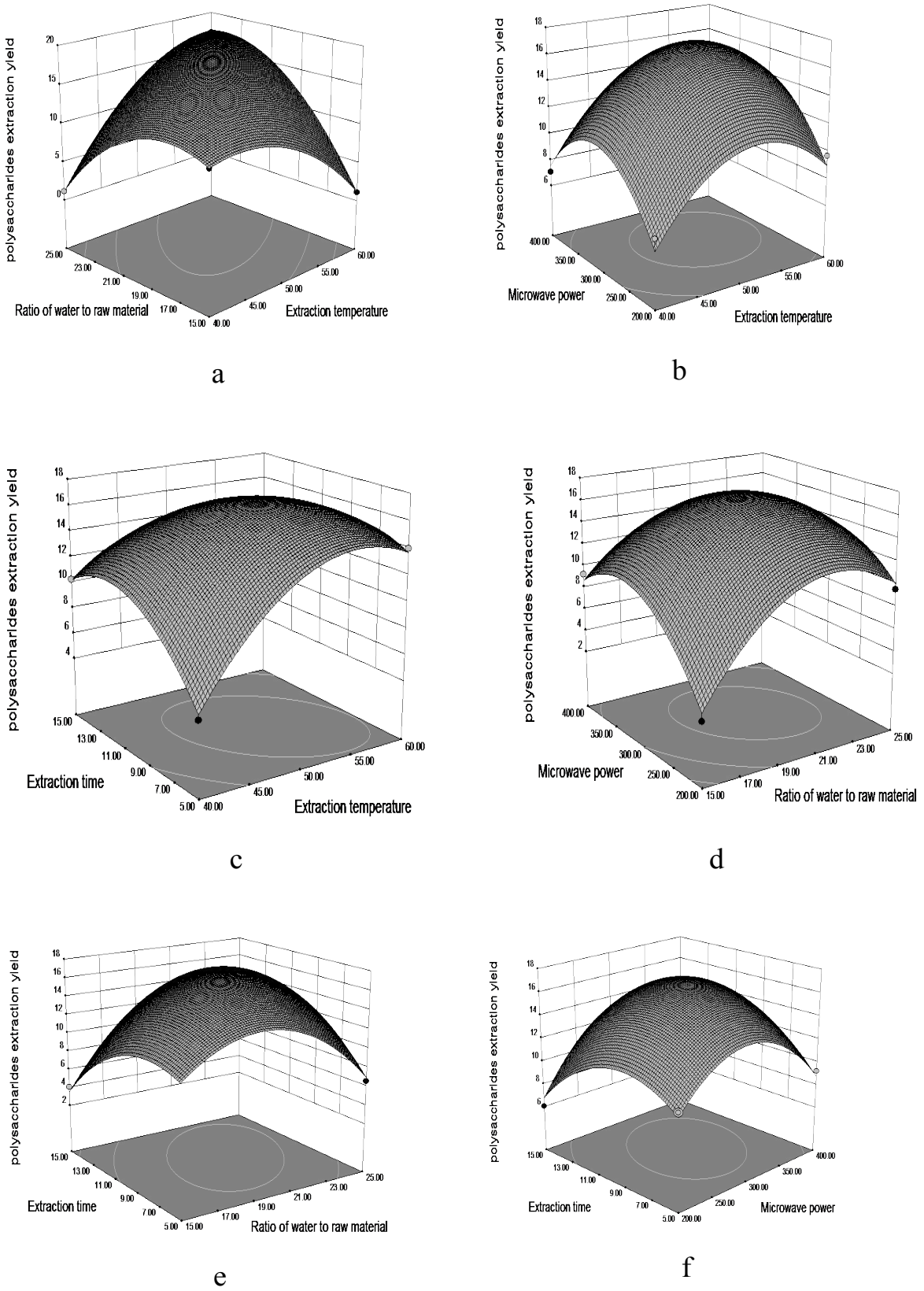

Figure 2: Response surface plots (3-D) showing the effects of variables (X1: extraction temperature, ºC; X2: ratio of water to raw material; X3: microwave power, W; and X4: extraction time, min) on the response Y.

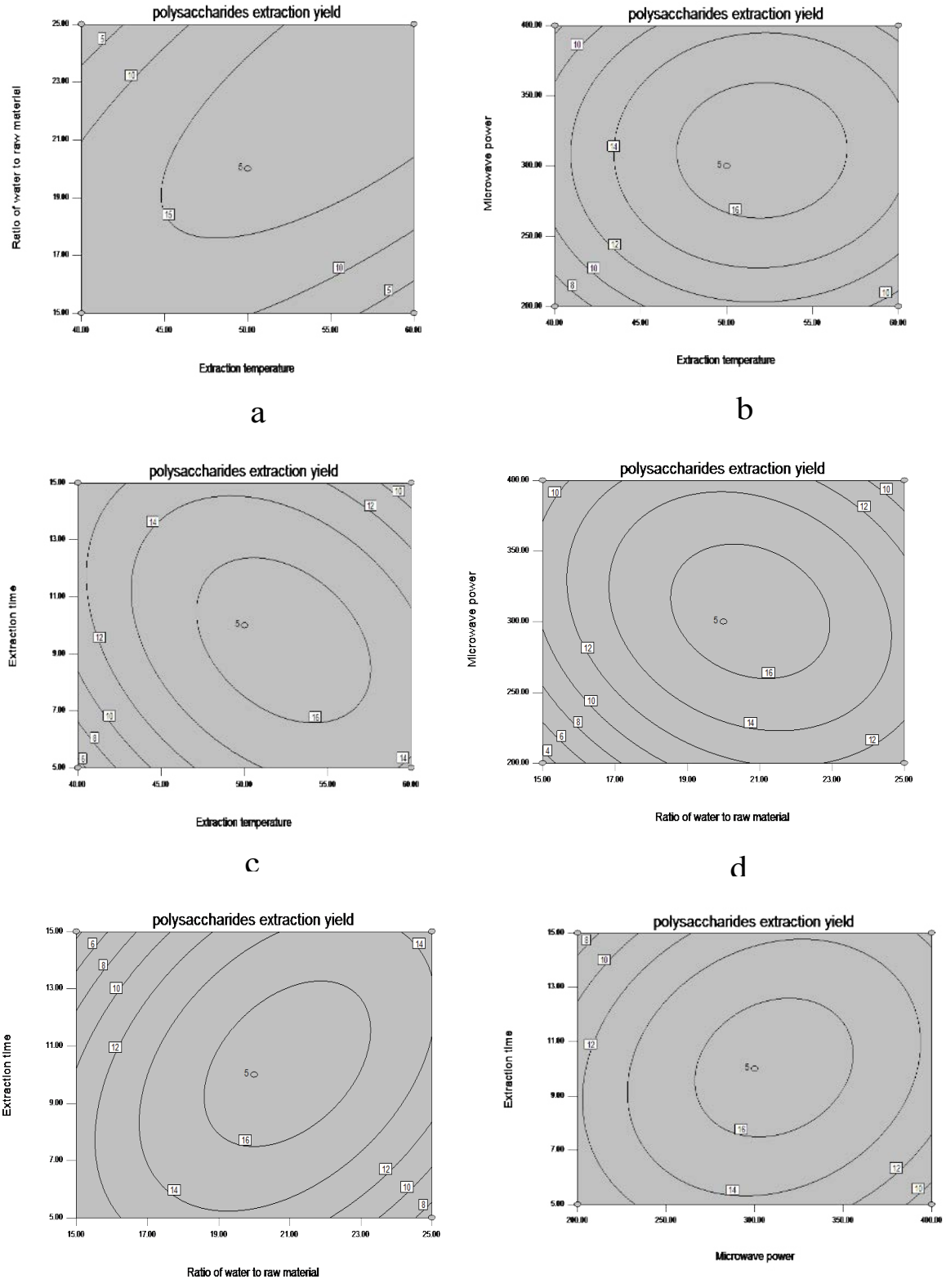

Figure 3: Contour plots (2D) showing the effects of variables (X1: extraction temperature, ºC; X2: ratio of water to raw material; X3: microwave power, W; and X4: extraction time, min) on the response Y.

and then decreased slightly at 52°C-60°C. The polysaccharide extraction yield increased evidently with increased microwave power from 200 W to 311 W; the polysaccharide yield decreased beyond 311 W.

The POP yield was affected by different extraction temperatures and extraction times (Figures 2c and 3c) when the water-to-raw material ratio and microwave power were fixed at level 0. The maximum polysaccharide yield was obtained when the extraction temperature and extraction time.

Figures 2d and 3d show the 3-D response surface plot and the contour plot under varying water-to-raw material ratios and microwave power values at a fixed extraction temperature (level 0) and extraction time (level 0). During polysaccharide extraction, the water-to-raw material ratio and microwave power demonstrated quadratic effects on the extraction yields. The optimal range of microwave power was 250-350 W and that of water-to-raw material ratio was 18-22.5.

Figures 2e and 3e show the plots based on the independent variables water-to-raw material ratio and extraction time when the extraction temperature and microwave power were kept at level 0. The maximum extraction yield could be achieved when the extraction time and water-to-raw material ratio were 10 min and 20, respectively.

Figures 2f and 3f show the plots under varying microwave power and extraction time values under a fixed extraction temperature (level 0) and water-to-raw material ratio (level 0). The extraction yield increased with increased microwave power from 200 W to 325 W; beyond 325 W, the yield decreased with increasing extraction time. The influence of extraction time was similar to that of the microwave power, and 10 min was the critical extraction time. Extraction time and microwave power both exerted a positive impact on POP yields.

Based on Figures 2 and 3 and on the results of the above single-parameter study, the optimal extraction conditions for POP extraction are as follows: a temperature of 57.21°C, a water-to-raw material ratio of 23.23, a microwave power of 298.51 W, and an extraction time of 10.12 min. Among the four extraction parameters studied, extraction temperature was the most significant factor affecting the POP extraction yield successively followed by water-to-raw material ratio, microwave power, and extraction time based on the significance of regression coefficients of the quadratic polynomial model (Table 3) and on gradient of slope in the 3-D response surface plot (Figure 2).

Verification of the predictive model: The suitability of the models in predicting optimum response values was tested under the following conditions: extraction temperature of 57°C, water-to-raw material ratio of 23:1, microwave power of 300 W, and extraction time of 10 min. This set of conditions was determined to be optimum through RSM optimization; moreover, this set of condition was used to experimentally validate and predict response values by using the model. A mean value of 17.49% ± 0.64% (n=3) was obtained from real experiments. The results indicated that the RSM approach effectively optimized the conditions for POP extraction, suggesting that the regression model was accurate and adequate for polysaccharide extraction (Table 4).

Anti-tumor activity *in vitro*: The inhibition effects of different POP concentrations (4, 40, 400 μg/mL) were tested in two human tumor cell lines for 72 h and measured using the MTT method *in vitro*. In this experiment, POP showed different degrees of antitumor effects. Panc-1 human pancreatic carcinoma cells were incubated with different concentrations of the polysaccharides, and the results showed that POP exerted no significant toxic effect on Panc-1 cells (Figure 4). Although 40 and 400 μg/mL of the polysaccharides exerted a certain degree of inhibitory effect on Panc-1, their effects were not statistically significant [25,26].

Growth of A549 cells was inhibited by POP in a dose-dependent manner compared with the control, suggesting that POP (400 μg/mL) are significantly cytotoxic to A549 cells *in vitro* (P<0.01) (Figure 5).

Conclusion

The extraction conditions significantly influenced the POP yields. The contour and surface plots in RSM effectively estimated the effect of four independent variables (extraction temperature, water-to-raw material ratio, microwave power, and extraction time). The optimum set of the independent variables was achieved graphically in order to obtain the desired levels of crude polysaccharide. The optimal

	Extraction temperature (ºC)	Ratio of water to raw material	microwave power (W)	Extraction time (min)	Yield (%)
Optimum condition	57.21	23.23	298.51	10.12	17.87 (Predicted)
Modified conditions	57	23	300	10	17.49 (actual)

Table 4: Predicted and experimental values of the responses at optimum conditions.

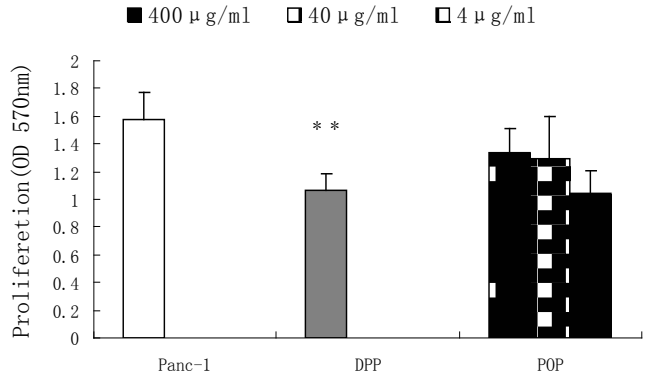

Figure 4: Effect of POP on the Panc-1 cells *in vitro*. Panc-1 was stimulated with POP at the concentrations of 400, 40, 4 μg/mL for 72 h. After the incubation, cell proliferation was measure by the MTT assay. DDP, positive, 10 μg/mL. Values were expressed as mean ± SD. *P<0.05 vs control, **P<0.01 vs control.

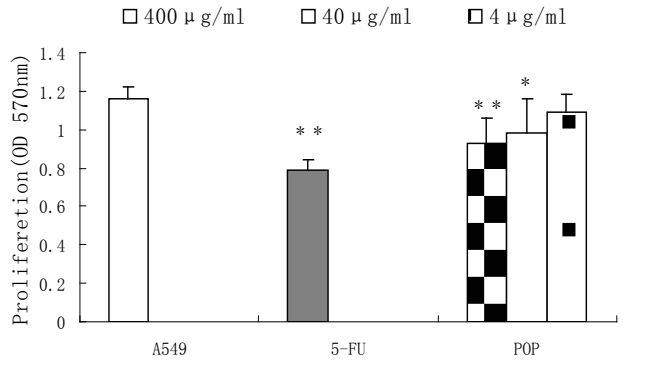

Figure 5: Effect of POP on the A549 cells in vitro. A549 was stimulated with POP at the concentrations of 400, 40, 4 μg/mL for 72 h. After incubation, cell proliferation was measured by the MTT assay. 5-Fu, positive, 1 μg/mL. Values were expressed as mean ± SD. *P<0.05 vs control, **P<0.01 vs control.

experimental yield of 17.49% was obtained when the conditions for POP extraction were as follows: extraction temperature of 57°C, water-to-raw material ratio of 23, microwave power of 300 W, and extraction time of 10 min. Under these optimized conditions, the experimental extraction yield is close to the predicted yield.

Acknowledgements

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