Carbohydrate Polymers 80 (2010) 944–948

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimization of ultrasound-assisted extraction of Lingzhi polysaccharides using response surface methodology and its inhibitory effect on cervical cancer cells

XiaoPing Chen [a,*], WanXiang Wang [b], ShuiBing Li [c], JinLing Xue [a], LiJun Fan [a], ZhongJi Sheng [d], YouGuo Chen [d]

[a] The Department of Obstetrics and Gynecology, YanCheng 1th People Hospital, YanCheng City 224000 Jiangsu, China
[b] Department of Science Education, YanCheng 1th People Hospital, YanCheng City 224000 Jiangsu, China
[c] Department of Facility, YanCheng 1th People Hospital, YanCheng City 224000, China
[d] Department of Obstetrics and Gynecology, First Affiliated Hospital of Soochow University, Suzhou 215006, China

ARTICLE INFO

Article history:
Received 27 November 2009
Received in revised form 6 January 2010
Accepted 7 January 2010
Available online 11 January 2010

Keywords:
Lingzhi polysaccharides
Ultrasonic-assisted extraction
Optimization
Response surface methodology (RSM)
CaSki
HeLa

ABSTRACT

In this work, an ultrasound-assisted procedure for the extraction of Lingzhi polysaccharides was established. Response surface methodology (RSM) was used to optimize the ultrasound-assisted extraction parameters (ultrasonic frequency (*X*1), extraction temperature (*X*2), extraction time (*X*3), and ratio of water to raw material (*X*4)) for enhancing the forward extraction efficiency of Lingzhi polysaccharides by implementing a three-level, four-variable Box–Behnken experimental design. The independent variable with the largest effect on response was *X*1 (ultrasonic frequency), followed by *X*2 (extraction temperature) and *X*3 (extraction time). The optimum extraction conditions were found to be ultrasonic frequency 8 kHz, extraction temperature 95 °C, extraction time 3 h and ratio of water to raw material 12. Under these conditions, the forward extraction efficiency of Lingzhi polysaccharides can increase from 42% to 75%. Pharmacological experiment showed that Lingzhi polysaccharides could decrease CyclinB1 mRNA expression in CaSki cells and inhibit CaSki and HeLa cells proliferation. Results indicated that Lingzhi polysaccharides possessed strong antitumor activity.

1. Introduction

The traditional approach of "one variable at a time (OVAT)" is well accepted, but this technique is not only time and work demanding, but completely lacks in representing the effect of interaction between different factors. Therefore an alternate strategy involving statistical approach, e.g. factorial experimental design and response surface methodology (RSM) should be adopted to solve this complexity involved in effluent treatment (Korbhati, Aktas, & Tanyolac, 2007). RSM is a collection of statistical and mathematical techniques useful for developing, improving and optimizing process. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions (Chen, Chen, & Lin, 2005; Gunawan, Basri, Rahman, Salleh, & Rahman, 2005; Jafari, Nateghi & Rabbani, 2010). RSM has been largely used for optimizing the media for enzyme production or citric acid production (Lofty, Ghanem, & El-Helow, 2007). This methodology can be used in developing suitable treatment technology considering the effects of operational conditions on the removal process or to determine a region that satisfies the operating specifications (Ravikumar, Pakshirajan, Swaminathan, & Balu, 2005; Zhao, Wang, & Lu, 2009).

Ganoderma lucidum (Fr.) Karst. (Polyporaceae) is a medicinal mushroom known to the Chinese as 'Lingzhi'. Its fruiting bodies have been used for their medicinal properties in traditional Chinese medicine for over 2000 years. This mushroom is described in detail in the Chinese Materia Medica classics, Shen Nung Ben Cao Jin (dated 206 BC–8 AD). Lingzhi allegedly has multiple health benefits for a broad range of conditions including hepatitis, hypertension, arthritis, bronchitis, and tumorigenic diseases (Li & Wang, 2006; Wasser & Weis, 1999; Yuen & Gohel, 2005), and has therefore attained an unparalleled reputation in the East as the ultimate herbal substance. Its growing popularity in patients with cancer may be supported by its suppressive effects of tumor growth in vitro (Sliva, 2006; Yuen & Gohel, 2005). Lingzhi has also been reported to have antioxidant, anti-inflammatory and analgesic effects (Lakshmi, Ajith, Sheena, Gunapalan, & Janardhanan, 2003; You & Lin, 2003). In vitro studies have shown that it can inhibit histamine release from mast cells (Tasaka, Akagi, Miyoshi, Mio, & Makino, 1988), and modulate peripheral mononuclear cell production of several cytokines, such as tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), IL-2, and IL-6 (Ho et al., 2007).

Ultrasonics has been proven to assist the solvent extraction of bioactive compounds from herbs (Zhong and Wang, in press). The application of ultrasound-assisted extraction offers many advantages including the reduction of solvents, temperature and the time for extraction, which is very useful for the extraction of

* Corresponding author. Tel./fax: +86 54 67984623.
E-mail address: chenxpdr@gmail.com (X. Chen).

0144-8617/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.01.010

thermolabile and in unstable compounds. As well-known, ultrasound extraction is the new technology that attracts much more attention in the department of separation and extraction in recent years. In the current study, the ultrasonics extraction condition of polysaccharides from Lingzhi mushroom is firstly investigated and optimized using RSM. Inhibitory effect of Lingzhi polysaccharides on cervical cancer cells was investigated.

2. Materials and method

2.1. Materials

The samples of Lingzhi mushroom were collected in Jiangsu Province (2008), in China. All the collected mushroom were immediately dried at 90 °C and stored in a dry and dark place.

2.2. Experimental design

A three-level-four-factor, Box–Behnken factorial design (BBD) was employed in this optimization study. Ultrasonic frequency ($X1$), extraction temperature ($X2$), extraction time ($X3$), and ratio of water to raw material ($X4$) were the independent variables selected to be optimized for the extraction of Lingzhi polysaccharides. Extraction yield (Y) was taken as the response of the design experiments. The coded and uncoded (actual) levels of the independent variables are given in Table 1. Twenty-seven experiments were augmented with three replications were carried out at the center points to evaluate the pure error.

Once the experiments are performed, the response variable (extraction yield) was fitted a second-order model in order to correlate the response variable to the independent variable. The general form of the second-order polynomial equation is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i 2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y is the response (dependent variables), β_0 the constant coefficient, β_i, β_{ii} and β_{ij} the coefficient for the linear, quadratic and interaction effect, x_i and x_j the factors (independent variables) and ε is the error.

2.3. MTT assay

The cell viability was checked using MTT assay. The MTT test is a colorimetric assay that measures the cell survival as percentage of cell survival compared to untreated controls (Williams, Liu, Knipp, & Sinko, 2002). One hundred microliters of 0.45 g/l MTT solution was added to wells. Cells were incubated at 37 °C for 45–60 min to allow colour development and thereafter, 100 µl of 20% SDS in DMF:H_2O 1:1 solution was added to the wells. Plates were incubated overnight at 37 °C to solubilize the formazan products. Absorbances were measured at the wavelength of 570 nm. The levels of blue colour development in the control wells were designated as 100% viability, and all further comparisons were based on that reference level. Blank values, indicating the absorbance of MTT and 20% SDS in DMF:H_2O 1:1 solutions only, were subtracted from all samples.

Table 1
Factors and levels.

Factor	Low	Center	High
Ultrasonic frequency (kHz, $X1$)	−1 (4)	0 (6)	1 (8)
Extraction temperature (°C, $X2$)	−1 (75)	0 (85)	1 (95)
Extraction time (h, $X3$)	−1 (2.5)	0 (3)	1 (3.5)
Ratio of water to raw material (m/v, $X4$)	−1 (8)	0 (12)	1 (16)

2.4. RT-PCR

To determine the amounts of Cyclin B 1 mRNA, standard curves were constructed with serially diluted PCR products. PCR products were obtained by amplification cDNA from normal mammary gland using specific primers as follows: sense: 5′CCATTATTGA-TCGGTTCATGCAGA3′; antisense: 5′CTAGTGCAGAATTCAGCTGTG-GTA3′ for cycling B 1; sense: 5′CGAAGTCAACGGATTTGGTCGTAT3′, antisense: 5′AGCCTTCTCGGTGGTGAAGAC3′ for GAPDH. PCR was carried out in a final volume of 50 µl using 25 pmol of each of the primers, 40 µM of each of dNTPs, 1.5 U Taq polymerase (Finnzymes, Finland), 5 µl 10-fold PCR buffer and 5 µl cDNA. PCR was carried out under the following conditions: 5 min at 95 °C, 1 min denaturation at 95 °C, 1 min annealing at 60 °C, 1 min extension at 72 °C for 40 cycles, with an additional 10 min extension for the last cycle. Amplified products were separated on a 2% (w/v) agarose gel, extracted and purified from agarose slices using DNA Gel Extraction Kit (Millipore, USA), quantified by the use of One Dscan/Zero Dscan software (Scanalytics Inc., USA) and then serially diluted in sterile water.

All RT-PCR reactions were performed using ABI Prism 7000 Sequence Detection System (Perkin–Elmer Applied Biosystems, USA). For each PCR run, a master mix was prepared with 10 ml 2× Taq Man Universal PCR Master Mix (Applied Biosystems), 1 µl 20× Assays-on Demand Gene Expression Assay Mix (Applied Biosystems), 5 µl cDNA or diluted standard and sterile water to final volume of 20 µl.

3. Result and discussion

3.1. Optimization of extraction conditions for Lingzhi polysaccharides

The extraction conditions certainly affected the extraction yield of Lingzhi polysaccharides. The experiments were designed to evaluate the impact of four factors, ultrasonic frequency ($X1$), extraction temperature ($X2$), extraction time ($X3$), and ratio of water to raw material ($X4$) on polysaccharides extraction from

Table 2
Experiment of ultrasonic extraction of polysaccharides from Lingzhi mushroom.

Run	$X1$	$X2$	$X3$	$X4$	Y
1	−1	−1	0	0	0.42
2	−1	1	0	0	0.49
3	1	−1	0	0	0.63
4	1	1	0	0	0.77
5	0	0	−1	−1	0.52
6	0	0	−1	1	0.56
7	0	0	1	−1	0.58
8	0	0	1	1	0.65
9	−1	0	0	−1	0.55
10	−1	0	0	1	0.54
11	1	0	0	−1	0.71
12	1	0	0	1	0.75
13	0	−1	−1	0	0.48
14	0	−1	1	0	0.56
15	0	1	−1	0	0.54
16	0	1	1	0	0.63
17	−1	0	−1	0	0.53
18	−1	0	1	0	0.51
19	1	0	−1	0	0.64
20	1	0	1	0	0.69
21	0	−1	0	−1	0.58
22	0	−1	0	1	0.62
23	0	1	0	−1	0.65
24	0	1	0	1	0.70
25	0	0	0	0	0.64
26	0	0	0	0	0.64
27	0	0	0	0	0.66

Table 3
ANOVA for *Y*.

Source	Master model				
	DF	SS	MS	*F*	Pr > *F*
*X*1	1	0.110208	0.110208	84.95717	0.0001
*X*2	1	0.020008	0.020008	15.42398	0.002008
*X*3	1	0.010208	0.010208	7.869379	0.015889
*X*4	1	0.004408	0.004408	3.398287	0.090088
*X*1 ∗ *X*1	1	0.001268	0.001268	0.977159	0.342415
*X*1 ∗ *X*2	1	0.001225	0.001225	0.944325	0.350349
*X*1 ∗ *X*3	1	0.001225	0.001225	0.944325	0.350349
*X*1 ∗ *X*4	1	0.000625	0.000625	0.481799	0.500826
*X*2 ∗ *X*2	1	0.00669	0.00669	5.157031	0.042368
*X*2 ∗ *X*3	1	0.000025	0.000025	0.019272	0.891892
*X*2 ∗ *X*4	1	0.000025	0.000025	0.019272	0.891892
*X*3 ∗ *X*3	1	0.01789	0.01789	13.79086	0.002961
*X*3 ∗ *X*4	1	0.000225	0.000225	0.173448	0.684418
*X*4 ∗ *X*4	1	0.000268	0.000268	0.206281	0.657803
Model	14	0.173574	0.012398	9.557459	0.000185
Error	12	0.015567	0.001297		
Total	26	0.189141			

Table 4
Fit statistics for *Y*.

	Master model	Predictive model
Mean	0.601481	0.601481
R-square	91.77%	91.77%
Adj. *R*-square	82.17%	82.17%
RMSE	0.036017	0.036017
CV	5.988043	5.988043

Lingzhi mushroom using ultrasonic extraction method. Results (Table 2) suggest that these variables significantly affect extraction yield. The significance of each coefficient was determined using the *F*-test and *p*-value in Table 3. The corresponding variables would be more significant if the absolute *F*-value becomes greater and the *p*-value becomes smaller (Atkinson & Donev, 1992). It can be seen that the variables with the largest effect were the linear terms of ultrasonic frequency (*X*1), extraction temperature (*X*2), and extraction time (*X*3) and the quadratic term of extraction temperature (*X*2*X*2), and extraction time (*X*3*X*3).

Analysis of variance (ANOVA) for the model was given in Table 4. The coefficient of determination (R^2) of the predicted model was 0.92, suggesting a good fit, the predicted model seemed to reasonably represent the observed values. Thus, the response was sufficiently explained by the model.

$$\begin{aligned} Y = {} & 0.646667 + 0.095833 * X1 + 0.040833 * X2 \\ & + 0.029167 * X3 + 0.019167 * X4 - 0.015417 * X1 * X1 \\ & + 0.0175 * X1 * X2 + 0.0175 * X1 * X3 + 0.0125 * X1 * X4 \\ & - 0.035417 * X2 * X2 + 0.0025 * X2 * X3 + 0.0025 * X2 * X4 \\ & - 0.057917 * X3 * X3 + 0.0075 * X3 * X4 + 0.007083 * X4 * X4 \end{aligned} \quad (2)$$

The 3D response surface plots were obtained by plotting the response (percentage conversion) on the *Z*-axis against any two variables while keeping other variable at its '0' level. Figs. 1 and 2 shows the isoresponse contour and surface plots for the optimization conditions of ultrasonic extraction of polysaccharides from Lingzhi mushroom. The effects of ultrasonic frequency (*X*1), extraction temperature (*X*2) on extraction yield are shown in Figs. 1a and 2a. The extraction ratio was increased with increases in ultrasonic frequency and extraction temperature (*X*2). Figs. 1b and 2b shows the effect of ultrasonic frequency (*X*1) and extraction time (*X*3) on extraction yield. The extraction ratio was increased with increases in ultrasonic frequency. A further increase in extraction time resulted in reversal of this trend. Figs. 1c and 2c shows the effect of ultrasonic frequency (*X*1) and ratio of water to raw material

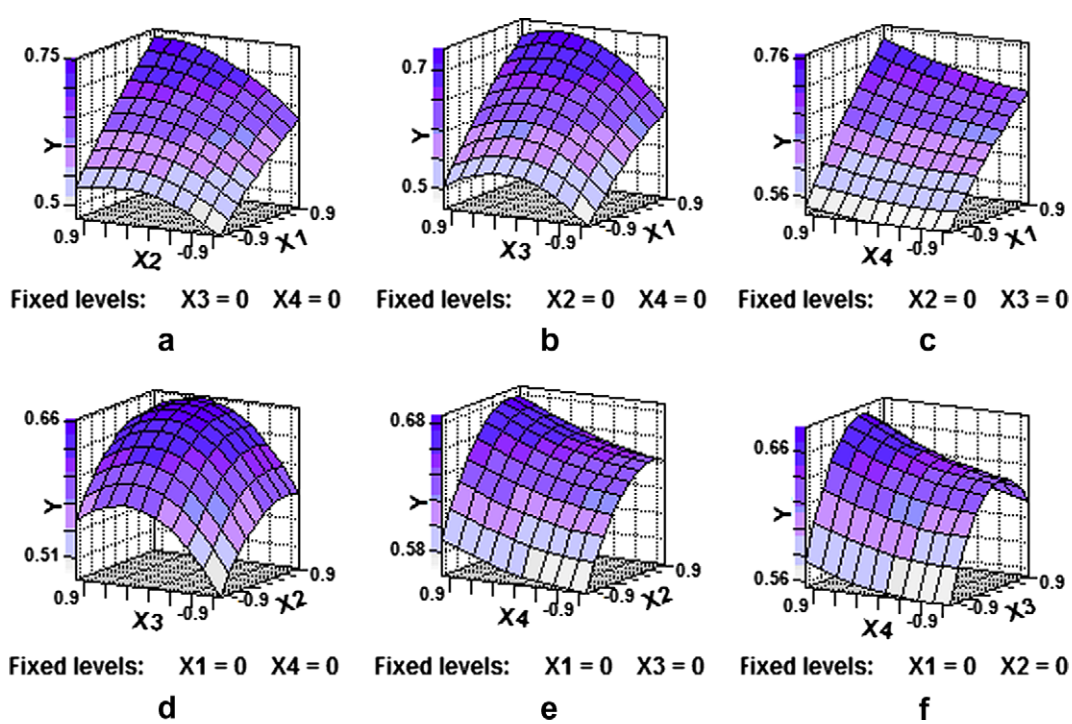

Fig. 1. (a) Response surface plots showing the effect of ultrasonic frequency (*X*1) and extraction temperature (*X*2) on extraction yield (*Y*) of Lingzhi polysaccharides. (b) Response surface plots showing the effect of ultrasonic frequency (*X*1) and extraction time (*X*3) on extraction yield (*Y*) of Lingzhi polysaccharides. (c) Response surface plots showing the effect of ultrasonic frequency (*X*1) and ratio of water to raw material (*X*4) on extraction yield (*Y*) of Lingzhi polysaccharides. (d) Response surface plots showing the effect of extraction temperature (*X*2) and extraction time (*X*3) on extraction yield (*Y*) of Lingzhi polysaccharides. (e) Response surface plots showing the effect of extraction temperature (*X*2) and ratio of water to raw material (*X*4) on extraction yield (*Y*) of Lingzhi polysaccharides. (f) Response surface plots showing the effect of extraction time (*X*3) and ratio of water to raw material (*X*4) on extraction yield (*Y*) of Lingzhi polysaccharides.

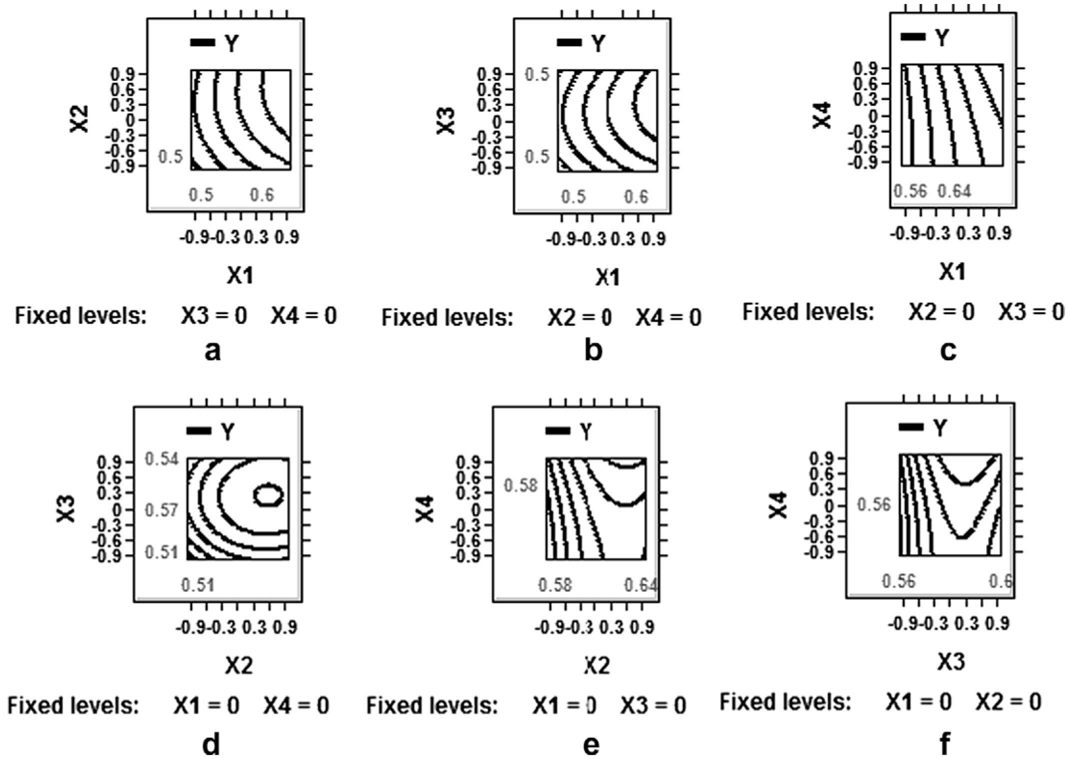

Fig. 2. (a) Contour plots for the effects of ultrasonic frequency (*X*1) and extraction temperature (*X*2) on extraction yield of Lingzhi polysaccharides. (b) Contour plots for the effects of ultrasonic frequency (*X*1) and extraction time (*X*3) on extraction yield of Lingzhi polysaccharides. (c) Contour plots for the effects of ultrasonic frequency (*X*1) and ratio of water to raw material (*X*4) on extraction yield of Lingzhi polysaccharides. (d) Contour plots for the effects of extraction temperature (*X*2) and extraction time (*X*3) on extraction yield of Lingzhi polysaccharides. (e) Contour plots for the effects of extraction temperature (*X*2) and ratio of water to raw material (*X*4) on extraction yield of Lingzhi polysaccharides. (f) Contour plots for the effects of extraction time (*X*3) and ratio of water to raw material (*X*4) on extraction yield of Lingzhi polysaccharides.

(*X*4) on extraction yield. The extraction ratio was increased with increases in ultrasonic frequency and ratio of water to raw material. Figs. 1d and 2d shows the effect of extraction temperature (*X*2) and extraction time (*X*3) on extraction yield. The extraction ratio was increased with increases in extraction temperature. A further increase in extraction time resulted in reversal of this trend. Figs. 1e and 2e shows the effect of extraction temperature (*X*2) and ratio of water to raw material (*X*4) on extraction yield. The extraction ratio was increased with increases in extraction temperature and ratio of water to raw material. Figs. 1f and 2f shows the effect of extraction time (*X*3) and ratio of water to raw material (*X*4) on extraction yield. The extraction ratio was increased with increases in ratio of water to raw material. A further increase in extraction time resulted in reversal of this trend.

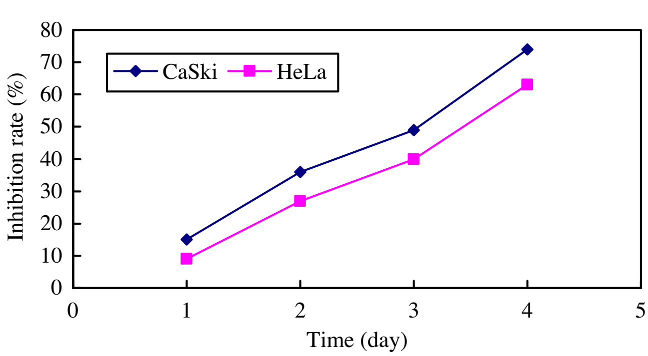

Fig. 3. Effect of Lingzhi polysaccharides on the growth of human cervical cancer cells.

3.2. Effect of Lingzhi polysaccharides on the growth of human cervical cancer cells

We investigated effect of Lingzhi polysaccharides on the growth of human cervical cancer cells. As shown in Fig. 3, these results implicate a role of Lingzhi polysaccharides in Lingzhi polysaccharides-resulted inhibition of CaSki and HeLa cells proliferation. Lingzhi polysaccharides treatment resulted in a much higher inhibition of CaSki cells proliferation compared to that of HeLa (Fig. 3).

3.3. Effect of Lingzhi polysaccharides with various concentration on cyclinBl mRNA expression in CaSki cells

To determine the effect of Lingzhi polysaccharides on Cyclin B 1 mRNA expression, CaSki cells were incubated with different amounts of Lingzhi polysaccharides for 24 h. The Cyclin B 1 mRNA levels were decreased by 14%, 30%, 43% and 49% in response to 50, 100, 200 and 300 mg/L polysaccharides as determined with real-time RT-PCR (Table 5).

Table 5
Effect of Lingzhi polysaccharides with various concentration on cyclinBl mRNA expression in CaSki cells.

Content	Relative level of Cyclin B 1 mRNA expression Cyclin B 1:GAPDH	Inhibitory effect (%)
0	1.26 ± 0.11	–
50	1.08 ± 0.09	14
100	0.88 ± 0.07	30
200	0.71 ± 0.08	43
300	0.64 ± 0.07	49

4. Conclusion

RSM was sufficient to describe and predict the extraction process of bioactive compounds from gardenia fruits. Furthermore, the optimal extraction condition regarding of three responses could be well induced by graphical optimization method which combined RSM with desirability function. The optimum extraction parameters were generated and the predicted values for yields of the bioactive compounds were well consistent to the experimental ones. Lingzhi polysaccharides could decrease cyclinBl mRNA expression in CaSki cells and inhibit CaSki and HeLa cells proliferation.

References

Atkinson, A. C., & Donev, A. N. (1992). *Optimum experimental designs*. Oxford: Oxford University Press (pp. 132–189).

Chen, M. J., Chen, K. N., & Lin, C. W. (2005). Optimization on response surface models for the optimal manufacturing conditions of dairy tofu. *Journal of Food Engineering., 68*, 471–480.

Gunawan, E. R., Basri, M., Rahman, B. A. M., Salleh, A. B., & Rahman, R. N. Z. A. (2005). Study on response surface methodology (RSM) of lipase-catalyzed synthesis of palm-based wax ester. *Enzyme and Microbial Technology, 37*, 739–744.

Ho, Y. W., Yeung, J. S., Chiu, P. K., Tang, W. M., Lin, Z. B., Man, R. Y., et al. (2007). *Ganoderma lucidum* polysaccharide peptide reduced the production of proinflammatory cytokines in activated rheumatoid synovial fibroblast. *Molecular and Cellular Biochemistry, 301*, 173–179.

Jafari, M., Nateghi, M., & Rabbani, A. (2010). Interaction of sulfur mustard with rat liver salt fractionated chromatin. *International Journal of Biological Macromolecules, 46*, 104–108.

Korbhati, B. K., Aktas, N., & Tanyolac, A. (2007). Optimization of electrochemical treatment of industrial paint wastewater with response surface methodology. *Journal of Hazardous Materials, 148*, 83–90.

Lakshmi, B., Ajith, T. A., Sheena, N., Gunapalan, N., & Janardhanan, K. K. (2003). Antiperoxidative, anti-inflammatory, and antimutagenic activities of ethanol extract of the mycelium of *Ganoderma lucidum* occurring in South India. *Teratogenesis, Carcinogenesis, and Mutagenesis Supplement, 185*, 197.

Li, Y. Q., & Wang, S. F. (2006). Anti-hepatitis B activities of ganoderic acid from *Ganoderma lucidum*. *Biotechnology Letters, 28*, 837–841.

Lofty, W. A., Ghanem, K. M., & El-Helow, E. R. (2007). Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. *Bioresource Technology, 98*, 3470–3477.

Ravikumar, K., Pakshirajan, K., Swaminathan, T., & Balu, K. (2005). Optimization of batch process parameters using response surface methodology for dye removal by a novel adsorbent. *Chemical Engineering Journal, 105*, 131–138.

Sliva, D. (2006). *Ganoderma lucidum* in cancer research. *Leukemia Research, 30*, 767–768.

Tasaka, K., Akagi, M., Miyoshi, K., Mio, M., & Makino, T. (1988). Anti-allergic constituents in the culture medium of *Ganoderma lucidum*. (I). Inhibitory effect of oleic acid on histamine release. *Agents and Actions, 23*, 153–156.

Wasser, S. P., & Weis, A. L. (1999). Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: A modern perspective. *Critical Reviews in Immunology, 19*, 65–96.

Williams, G. C., Liu, A., Knipp, G., & Sinko, P. J. (2002). Direct evidence that saquinavir is transported by multidrug resistance-associated protein (MRP1) and canalicular multispecific organic anion transporter (MRP2). *Antimicrobial Agents and Chemotherapy, 46*, 3456–3462.

You, Y. H., & Lin, Z. B. (2003). Antioxidant effect of Ganoderma polysaccharide peptide. *Acta Pharmaceutica Sinica, 38*, 85–88.

Yuen, J. W., & Gohel, M. D. (2005). Anticancer effects of *Ganoderma lucidum*: A review of scientific evidence. *Nutrition and Cancer, 53*, 11–17.

Zhao, H. Z., Wang, J., & Lu, Z. X. (2009). Optimization of process parameters of the *Pholiota squarrosa* extracellular polysaccharide by Box–Behnken statistical design. *Carbohydrate Polymers, 77*, 677–680.

Zhong, K., & Wang, Q. (in press). Optimization of ultrasonic extraction of polysaccharides from dried longan pulp using response surface methodology. *Carbohydrate Polymers*. doi:10.1016/j.carbpol.2009.10.066.