Carbohydrate Polymers 112 (2014) 396–403

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Optimum extraction of polysaccharides from motherwort leaf and its antioxidant and antimicrobial activities

Saeed Tahmouzi*, Mitra Ghodsi

Department of Food Science and Technology, Faculty of Animal Science and Food Technology, Ramin Agriculture and Natural Resources University, Mollasani, Ahvaz, Iran

ARTICLE INFO

Article history:
Received 12 March 2014
Received in revised form 23 May 2014
Accepted 4 June 2014
Available online 19 June 2014

Keywords:
Leonurus cardiaca
Polysaccharide
RSM
Antioxidant
Antimicrobial

ABSTRACT

Box–Behnken design was employed to optimize the extraction conditions for polysaccharides from the leaves of motherwort (*Leonurus cardiaca* L.). Three independent variables including extraction temperature (60–100 °C), extraction time (60–120 min), and the ratio of water to raw material (20–60) were investigated. The results revealed that the quadratic and linear terms of three factors had strong effects on the extraction yield of polysaccharides from motherwort leaf. The best extraction conditions for the yield of polysaccharide (LCLP) was extraction temperature of 81.4 °C, time of 106.6 min and the ratio of water to raw material of 45.2. Under the optimal conditions, the extraction yield of LCLP was 9.17 ± 0.39%, which was well matched with value predicted by the model 9.26%. The results indicated that the purified LCLP exerted obvious scavenging effects on free radicals in vitro. Furthermore, motherwort polysaccharides could be used as a novel antimicrobial additive.

1. Introduction

Leonurus cardiaca L. commonly called motherwort is an interesting member of the mint family, Lamiaceae that grows in temperate areas of the world such as Iran, China and Japan. The leaf shape varies somewhat by location, but are generally opposite, lobed and toothed (Chen, Zhao, Bai, Hu, & Si, 2009). Motherwort is a medicinal plant employed in Asia as traditional anti-cancer and antiinflammatory, and is now found in producing valuable products which have potential application as an antibacterial, antifungal and antioxidant. It has also been reported to interfere with blood clotting (Nagasawa, Inatomi, Suzuki, & Mori, 1992; Park, Vepachedu, Sharma, & Vivanco, 2004). Motherwort leaves and flowers are used in the treatment of stomach gas, cramping, menopausal problems, insomnia, and hypertension (Matkowski & Piotrowska, 2006). It may potentially benefit people at risk of coronary heart disease (CHD), bronchial asthma, climacteric symptoms, flatulence amenorrhea, and hyperthyroidism (Agnihotri, Elsohly, & Smillie, 2008).

Oxygen-derived free radicals (•OH) are the most common type of radicals produced in the body naturally as a byproduct of metabolism (Li & Zhou, 2007). Excessive free radicals can cause many disorders, such as oxidative stress, cataract, aging, inflammatory lung diseases, tubulointerstitial nephritis, chronic renal failure, proteinuria, uremia and rheumatoid arthritis. Free radicals can obviously damage the cell membranes and other structures including deoxyribonucleic acid, lipids, lipoproteins, and proteins (Galle, 2001; Sun et al., 2008; Ye, Wang, Zhou, Liu, & Zeng, 2008).

In recent years, some bioactive polysaccharides extracted from natural sources have attracted great attention in the field of biochemistry and pharmacology due to their beneficial activities. As an example, plant polysaccharides, a kind of natural biomacromolecule or their glycoconjugates were shown to exhibit multiple biological activities including anti-tumor, anticarcinogenic, anticoagulant, immunostimulating, antioxidant, etc (Mizuno et al., 2000; Ooi & Liu, 2000; Wang, Chen, Jia, Tang, & Ma, 2012; Xu et al., 2009).

As an important subject in the statistical design of experiments, the response surface methodology (RSM) is an effective statistical tool involving mathematical approach, which applies in the optimization of conditions in food and pharmaceutical research frequently. It can be applied for the approximation of both experimental and numerical responses (Kshirsagar & Singhal, 2007; Masmoudi et al., 2008; Montgomery, 2005; Qiao et al., 2009). The main application of RSM to design optimization is aimed at reducing the number of experimental trials. RSM was extensively applied in optimizing the polysaccharide and bioactive compounds extraction process variables (Gan, Abdul Manaf, & Latiff, 2010; Guo, Zou, & Sun, 2010; Zhu, Heo, & Row, 2010).

* Corresponding author. Tel.: +98 6123224341; fax: +98 6123222425/+98 6123224341.
E-mail address: stahmouzi@gmail.com (S. Tahmouzi).

http://dx.doi.org/10.1016/j.carbpol.2014.06.024
0144-8617/

Response surface methodology has been used to optimize the hot water extraction process of polysaccharides from plants such as *Radix astragali* leaf, fruiting bodies of *Camellia oleifera* and *Ganoderma lucidum* (Huang & Ning, 2010; Liu, Miao, Wen, & Sun, 2009). However, up to now, no detailed investigation has been conducted on optimization of polysaccharide extraction from the leaves of motherwort. Therefore, the objectives of present work were (i) to optimize the extraction process using RSM, applying a three-level, three-variable Box–Behnken design to investigate the effects of extraction temperature, extraction time, and water/solid ratio on the purity of LCLP from the leaves of *L. cardiaca*, (ii) to find the antioxidant abilities (scavenging activities of hydroxyl radical and DPPH radical) and antimicrobial activities (against bacteria, yeast and mold) of extracted polysaccharides in vitro assays.

2. Materials and methods

2.1. Samples and chemicals

The leaves of motherwort were collected from Tabriz province, Iran, washed, freeze-dried, and powdered for this work. All other reagents and solvents used were of the highest quality and purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultra-pure water was used throughout the experiments.

2.2. Extraction of LCLP and determination of the yield

The extraction of crude polysaccharides from motherwort leaf was conducted following the method reported by Li, Ai, Yang, Liu, and Shan (2013) with minor modifications. The leaves of *L. cardiaca* was powdered in a blender and then extracted with 85% ethanol at the temperature of 70 °C for 4 h to defat and remove interference components, including polyphenols, oligosaccharides, and amino acids under reflux in the Soxhlet's apparatus. The pretreated samples were separated from the organic solvent by centrifugation ($7000 \times g$ for 15 min), and then extracted by water in a designed temperature, time and water/solid ratio. The water extraction solutions were separated from insoluble residue by centrifugation ($4000 \times g$ for 10 min, at 20 °C), and then precipitated by the addition of ethanol to a final concentration of 70% (v/v) to obtain crude polysaccharides (composed of 73.4% neutral sugar and 19.8% acid sugar). The sugar content and composition of LCLP were analyzed according to the published method (Jiang, Wang, Liu, Gan, & Zeng, 2011). Protein was removed as described by Matthaei, Jones, Martin, & Nirenberg (1962). The precipitate was separated by centrifugation (10,000 × *g* for 15 min) and air-dried for 18 h. The purity (%) of LCLP is calculated as follows:

$$\text{LCLP extraction yield \% (w/w)} = \frac{N_0}{N} \times 100\% \quad (1)$$

N_0 (g) is the dried LCLP weight; N (g) is the dried powder of *L. cardiaca* leaf weight.

2.3. Experimental design of RSM

Based on single-factor experimentation, preliminary proper ranges of the extraction temperature (X_1), extraction time (X_2) and the ratio of water to raw material (X_3) were obtained. A BBD with three independent variables at three levels was performed to determine the best combination of extraction factors for the optimization of LCLP. On the basis of single-factor experiments, the key parameters were determined to be extraction temperature, extraction time and the ratio of water to raw material. Tables 1 and 2 show the Box–Behnken design and response values carried out for developing the model. The whole design consisted of 17 trials carried out in random order. The experimental design included five central points to determine the repeatability of the method (Montgomery, 2001). Regression analysis was performed for the experimental data and was matched into an empirical second-order polynomial using below equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 \beta_{23} X_2 X_3 \quad (2)$$

here Y represented the dependent variable of (purity of LCLP %). β_0 was a constant coefficient of the models. (β_1, β_2 and β_3), (β_{11}, β_{22} and β_{33}) and (β_{12}, β_{13} and β_{23}) were the coefficients of the linear, quadratic and interactive terms, respectively.

Table 1
Independent variables and their levels used for Box–Behnken design.

Levels	Variables		
	Temperature (X_1) (°C)	Extraction time (X_2) (min)	Ratio of water to raw material (X_3)
−1	60	60	20
0	80	90	40
+1	100	120	60

2.4. Determination of antioxidant activities of LCLP in vitro

2.4.1. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging capacity of polysaccharides extract was measured with slight modification by Fenton-type reaction (Wang, Gao, Zhou, Cai, & Yao, 2008). Reaction mixture consisted 1.5 mL of 6 mM $FeSO_4$, 1.0 mL of sample solutions in water at various concentrations (3, 6, 9, 12 and 15 mg/mL) and 0.5 mL sodium salicylate (10 mM). The reaction mixture was incubated at ambient temperature for 1.5 h. The absorbance of the mixture was measured at 562 nm, and the scavenging capacity of LCLP was calculated using the following equation:

$$\text{Scavenging rate (\%)} = \frac{k_0 - k_1}{k_0} \times 100(\%) \quad (3)$$

K_0 and K_1 are the absorbance of the control (without extract) and LCLP, respectively.

2.4.2. DPPH free radical scavenging assay

The free radical scavenging capacity of the crude polysaccharide from *L. cardia* was determined by the previously reported method (Yang et al., 2009) with some modifications. Briefly, 2.0 mL

Table 2
Box–Behnken experimental with the independent variables.

Trial	X_1 (°C)	X_2 (min)	X_3	Extraction yield of LCLP (%)	
				Experimental	Predicted
1	80	120	20	8.53	8.62
2	80	90	40	9.22	9.12
3	80	90	40	9.31	9.33
4	60	90	20	7.89	7.93
5	80	90	40	8.95	8.88
6	100	90	60	8.01	8.10
7	60	120	40	8.25	8.18
8	100	120	40	8.96	9.02
9	100	90	20	8.22	8.31
10	80	90	40	9.21	9.27
11	100	60	40	8.32	8.29
12	60	90	60	8.60	8.53
13	80	90	40	9.16	9.21
14	80	60	20	7.92	7.96
15	80	120	60	9.13	9.22
16	60	60	40	7.96	7.83
17	80	60	60	8.99	9.07

of polysaccharides in water (3, 6, 9, 12 and 15 mg/mL) was added to 2 mL DPPH ethanol solution (0.5 mM). The mixture was shaken and then incubated for 30 min at room temperature in the dark. The absorbance was recorded at 517 nm with a UV–vis spectrophotometer. Synthetic antioxidant, butylated hydroxytoluene (BHT), was used as a positive control. The DPPH radical scavenging ability was calculated with the following equation:

$$\text{Scavenging rate } (\%) = \frac{D_0 - D_1}{D_0} \times 100(\%) \quad (4)$$

where D_0 is the absorbance of control (without extract) and D_1 is the absorbance of motherwort leaf polysaccharide (LCLP) solution.

2.5. Antimicrobial activity

The antimicrobial effect of *L. cardia* was evaluated by the filter disk diffusion plate method. Bacteria (*Bacillus polymixa* and *Escherichia coli*), yeast (*Debaryomyces hansenii*) and fungi (*Aspergillus flavus*) were selected as test organisms in this work. After the inoculum solution (100 μL) of each test organism containing 10^6 cells/mL was inoculated to the medium (nutrient medium for bacteria and Sabouraud medium for yeast and fungi), filters (8 mm in diameter and 2 mm thick) containing samples (3, 6, 9, 12 and 15 mg/mL) were placed in the center of the plate after the top agar had solidified. Bacteria were incubated at 37 °C for 24 h, while yeast and fungi plates were stored at 28 °C for 48 h. Sterilized water was applied as a control. The antimicrobial activity was analyzed with calipers and expressed by the diameter (mm) of the inhibition zones (Xie et al., 2012).

2.6. Statistical analysis

Analysis of the experimental design and data was performed using Design Expert software of version 7.0 (Stat-Ease Inc., Minneapolis, USA). All experimental results were shown as the means ± standard deviations of three measurements (triplicate) and the data of RSM were analyzed by SAS (SAS Institute Inc., Cary, NC, USA). Results were analyzed by ANOVA, *p*-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Single-factor experimental analysis

3.1.1. Effect of different temperature on extraction yield of LCLP

Temperature is an efficient factor that would influence the extraction yield of polysaccharides (Ye & Jiang, 2011). Effect of different temperatures (20, 40, 60, 80, 100 and 120 °C) on the extraction efficiency of LCLP was investigated, and other experimental variables were set as follows: extraction time 90 min and the ratio of water to material 40. As shown in Fig. 1(a), the yield significantly increased along with the increase of temperature from 20 to 60 °C, and then the yields were almost unchanged from 60 to 100 °C. The extraction rate increased up to maximum level of 9.42% at 100 °C, and then decreased with the further increase of temperature. So, the temperature range of 60–100 °C was adopted as optimal in the Box–Behnken design experiment. The good effect of extraction temperature could be explained by the enhanced solubility of polysaccharides in the extracting solvent, the higher diffusion coefficient of the extracted molecules and the improved mass transfer at higher temperature. This tendency is in accordance with reports of other authors in extracting polysaccharides (Vinogradov, Brade, Brade, & Holst, 2003; Ye & Jiang, 2011).

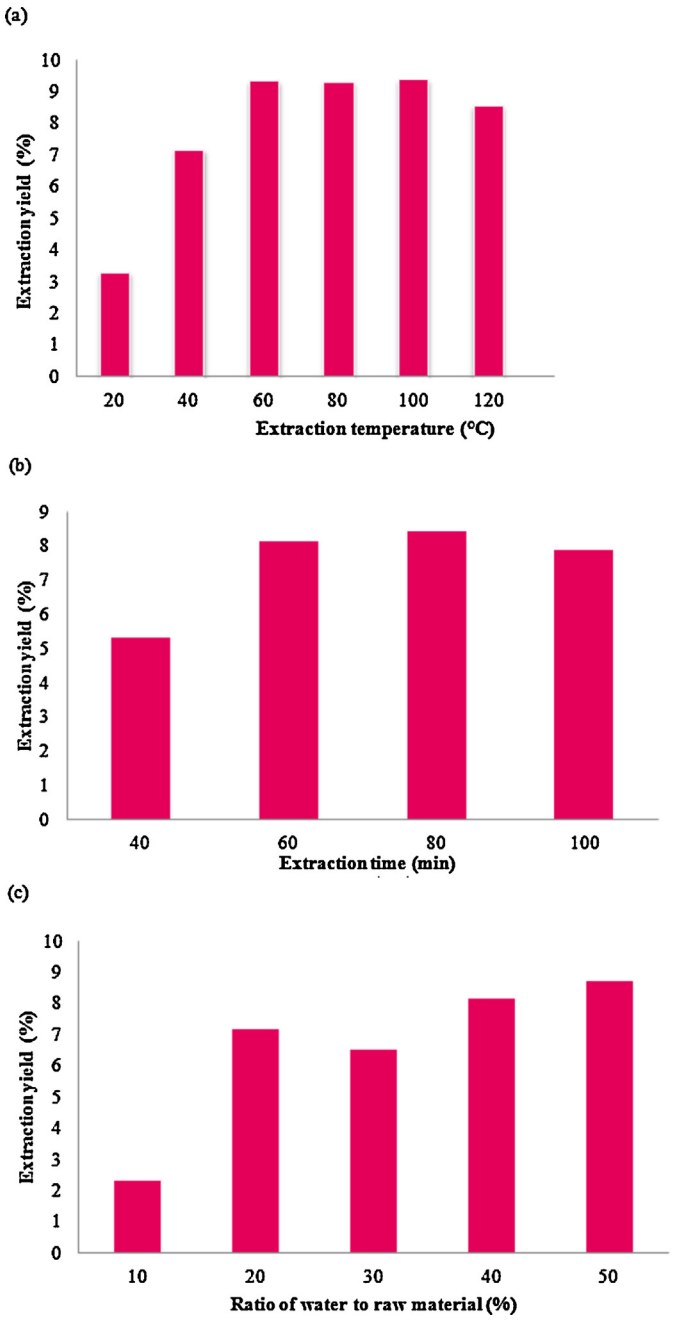

Fig. 1. Effects of extraction temperature (a), extraction time (b), and the ratio of water to raw material (c) on the extraction yield of LCLP (%).

3.1.2. Effect of different times on extraction yield of LCLP

Extraction time is one of the important variables which affect the efficiency of polysaccharides (Yin, You, & Jiang, 2011). In present work, extraction time was set at 40, 60, 80, 100, and 120 min to investigate its influence on the extraction yield of LCLP when other factors were set as follows: extraction temperature 80 °C and the ratio of water to material 40. As shown in Fig. 1b, the extraction yield increased significantly when the time ranged from 40 to 60 min. After 60 min, there was a minor increase and then maintained an equilibrium with increasing the extraction time (Eskilsson, Davidsson, & Mathiasson, 2002). The maximum yield (8.44%) was achieved at the time of 80 min. When extraction time was higher than 80 min, the yield decreased with the further increase of time. These results suggest that using a higher extraction time may lead to degradation, aggregation and hydrolysis of the

Table 3
Analysis of variance for the fitted models.

Source	CE[a]	SS[b]	DF[c]	SE[d]	MS[e]	*F* value	*p*-Value
Model	9.17	3.84	9	0.12	0.43	16.42	0.0114
X_1	0.1	0.082	1	0.091	0.082	11.23	0.0030
X_2	0.21	0.35	1	0.091	0.35	15.30	<0.0001
X_3	0.27	0.59	1	0.091	0.59	8.85	0.0059
X_1X_2	0.088	0.031	1	0.13	0.031	0.46	0.3843 ns
X_1X_3	−0.23	0.21	1	0.13	0.21	3.18	0.0391
X_2X_3	−0.12	0.055	1	0.13	0.055	0.83	0.1191 ns
X_1^2	−0.63	1.67	1	0.13	1.67	25.04	0.0016
X_2^2	−0.17	0.12	1	0.13	0.12	1.75	0.0022
X_3^2	−0.36	0.54	1		0.54	8.16	0.0024
Residual		0.47	7		0.067		
Lack of fit		4.11	3		0.13	7.60	0.093 ns
Pure error		0.069	4		0.017		
Cor total		4.31	16				
SD		0.26		R^2		0.953	
Mean		8.62		Adj-R^2		0.891	
C.V. (%)		2.99		Pred-R^2		o.790	
Press		6.45		Adequate precision		8.039	

ns: not significant.
[a] Coefficient estimate.
[b] Sum of squares.
[c] Degree of freedom.
[d] Standard error.
[e] Mean square.

LCLP (Sun, Liu, & Kennedy, 2010). Therefore, 80 min was selected as the optimum extraction time.

3.1.3. *Effect of different ratio of water to the raw material on extraction yield of LCLP*

The ratio of water to the raw material is also one of the important variables which influence the extraction of LCLP from *L. cardiaca*. To find the optimum range of water ratio (%) for the efficient extraction of polysaccharide, extraction process was carried out at different water ratios 10, 20, 30, 40, 50, 60 and 70. For this process, extraction temperature was set at 80 °C and extraction time 90 min. The yield of LCLP was increased continuously with increasing ratios from 30 to 50 (Fig. 1c) (Zhu & Liu, 2013). The crude protein extracted from motherwort leaf varied from 0.82% ± 0.15% to 1.19% ± 0.26%, with little variation with ratio of water to raw material. It is indicated that increasing the water to raw material ratio will decrease the concentration of LCLP (Zhong & Wang, 2010). The maximum yield (8.72%) was obtained at the ratio of water to raw material 50 mL/100 g. The ratio of water to the raw material range of 20–60 was chosen to be optimal in this study.

Therefore, in this research, it was employed extraction temperature 60–100 °C, extraction time 60–120 min and the ratio of water to the raw material 20–60 for RSM experiments.

3.2. *Model fitting and statistical analysis*

RSM is more efficient than the traditional single parameter optimization in that it reduces the number of experiments and saves time. There were a total of 17 runs for optimizing the three individual variables (X_1: extraction temperature, X_2: extraction time and X_3: the ratio of water to raw material) in the current Box–Behnken design, as shown in Table 2.

Second order polynomial equation was used to correlate the independent parameters with the yield of LCLP. The matched model for yield (Y) to predict the relationships between the independent variables and the dependent variable can be expressed by:

$$Y = 9.17 + 0.10X_1 + 0.21X_2 + 0.27X_3 - 0.23X_1X_3 - 0.63X_1^2 - 0.17X_2^2 - 0.36X_3^2 \quad (5)$$

The process variables and experimental data for yield of the LCLP under various conditions are depicted in Table 2. The analysis of variance for the experimental results of the Box–Behnken design is presented in Table 3. The data revealed that the proposed regression model for yield of polysaccharide was adequate with satisfactory R^2 value (measure of the degree of fit). The R^2 and R^2_{adj} values for yield were 0.953 and 0.891, respectively, which confirmed a better degree of correlation between the experimental and the theoretical values predicted by the polynomial model (Sin, Yusof, Sheikh Abdul Hamid, & Rahman, 2006). The model was reliable and reproducible for prediction within the range of test parameters (*C.V.* = 2.99) (Prakash Maran, Mekala, & Manikandan, 2013).

The significance of regression coefficient was evaluated for fitness and adequacy using the *p*-value in Table 3. The quadratic term coefficients (X_1^2, X_2^2, X_3^2) and linear coefficients (X_1, X_2, X_3) were significant ($p < 0.05$). Among the interaction terms, X_1X_2 and X_2X_3 were insignificant ($p < 0.05$) to the yield of LCLP. Therefore, X_1^2, X_2^2, X_3^2, X_1, X_2, X_3 and X_1X_3 were important variables in the extraction process of the LCLP. The Model *F*-value of 16.42 implied that the model was significant. Results of the ANOVA showed that the lack of fit was insignificant (0.093). It implied that the model equation is adequate for predicting the yield of LCLP under any combination of extraction temperature, extraction time and the ratio of water to raw material. This is in agreement with reports of Tahmouzi (2014) in extracting polysaccharides from zagros oak leaf.

3.3. *Optimization for LCLP extraction*

Response surfaces were obtained using Design-Expert (version 7.0) software to evaluate the effects of variables and their interactions on the yield of motherwort leaf polysaccharides. 3-D response surface plots and 2-D contour plots, as shown in Figs. 2a–c, were very helpful to judge interaction effects of the parameters on the responses. These plots represent effects of two variables on the response at a time. In all figures, the other one factor was adjusted at central point.

The plots in Fig. 2a, which gives the ratio of water to raw material (40), shows that extraction yield of LCLP increased with increasing of extraction temperature and extraction time from 60 to 81.4 °C and 60 to 104.8 min, respectively. It was obvious that the yield of LCLP was declined with the increasing extraction time from 105 to

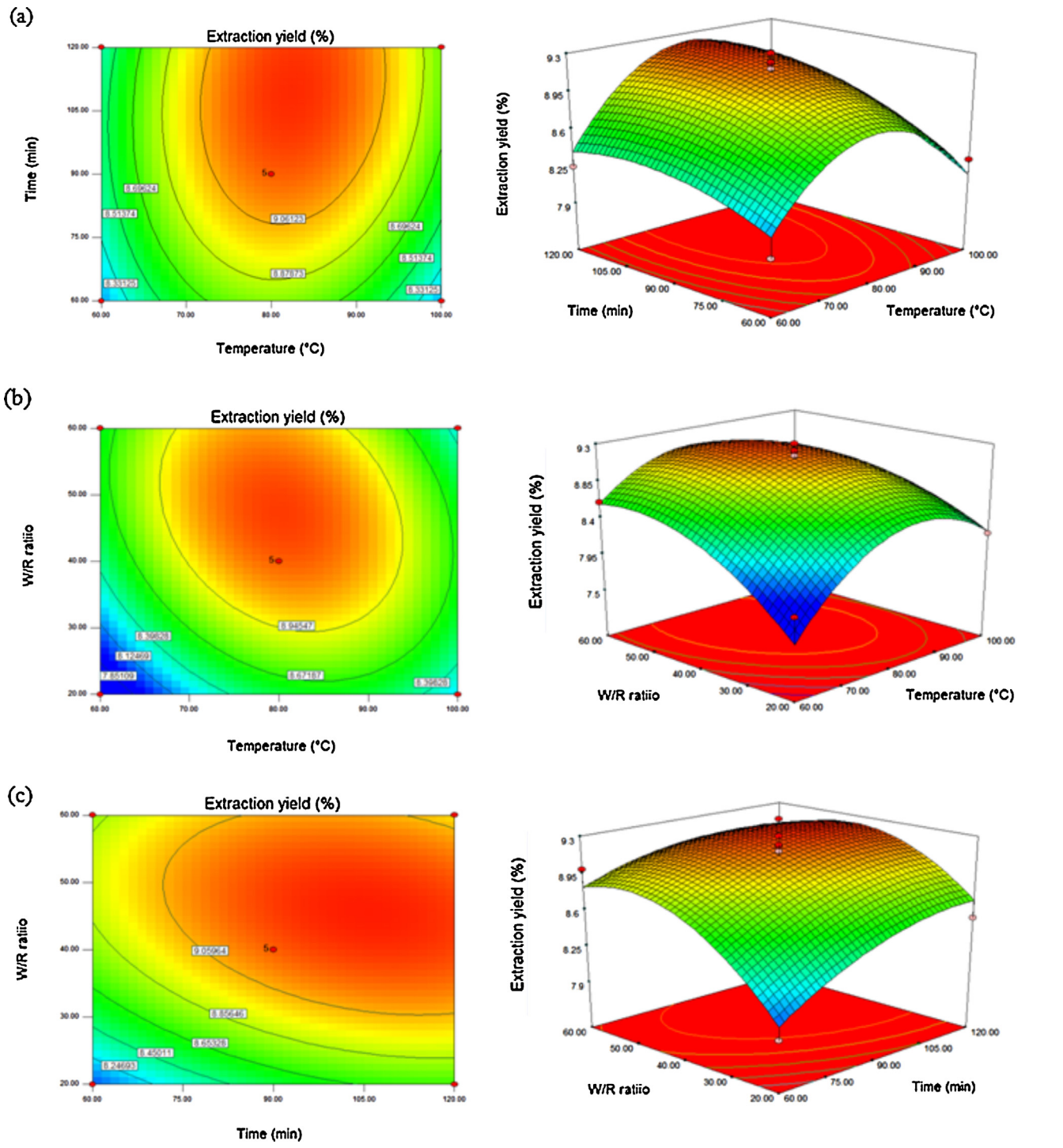

Fig. 2. Response surface (3-D) and contour plots showing the effects of (a) extraction time and extraction temperature, (b) the ratio of water to raw material and extraction temperature and (c) extraction time and the ratio of water to raw material on the LCLP extraction yield.

120 min, meaning that further increases of time would not improve the yield of polysaccharides any longer. It is suggested that increasing the time could deform pores on plant cell membranes (Sun et al., 2010).

Fig. 2b represents the 3-D plot and the contour plot at different extraction temperature and ratio of water to raw material at fixed extraction time (90 min). It can be observed that the polysaccharide efficiency was sensitive to even slight changes in extraction temperature or the water to raw material ratio. However, the interactive influence of these two variables was not significant (Chen, Xie, & Gong, 2007). As shown in Fig. 2b, maximum extraction yield of LCLP can be obtained when extraction temperature and ratio of water to raw material were 82.7 °C and 38.9, respectively. The extraction yield of polysaccharides increased evidently with increasing of extraction temperature from 60 to 82.7 °C, but beyond 82.7 °C, extraction yield of LCLP declined as extraction temperature increased. The extraction yield of polysaccharides ascended evidently with increasing of the ratio of water to raw material from 20 to 38.9, but beyond 38.9, the extraction yield of LCLP decreased with increasing water ratio.

The plots based on independent variables ratio of water to raw material and extraction time were shown in Fig. 2c, while the extraction temperature was fixed at central point of design (80 °C). An increase in the yield efficiency of polysaccharides could be strongly obtained with the increases of the ratio of water to raw material. It was clear that the extraction yield of LCLP was increased with the ascending time from 60 to 106.5 min, meaning that further increases of extraction temperature would decrease the extraction yield of crude polysaccharides. It is speculate that high temperature may affect the activity and chemical structure of LCLP, i.e., and the temperature obviously decomposed polysaccharides to monosaccharide, which can influence the extraction yield (Chen et al., 2007).

It was concluded that optimal extraction condition of polysaccharides from *L. cardiaca* were extraction temperature 81.4 °C, extraction time 106.6 min and the ratio of water to raw material 45.2 and the model predicted a maximum response of 9.26% with a desirability value of 0.976.

3.4. Verification of predictive model

According to Table 4, and the above single factor study, it could be concluded that the optimized extraction variables of the LCLP from motherwort leaf are as follows: extraction temperature 81.4 °C, extraction time 106.6 min and the ratio of water to raw material 45.2. Under these conditions, the model predicted a maximum response of 9.26%. For their verification of the adequacy of the model equation, five confirmatory experiments were conducted under the optimal conditions, and the extraction yield of LCLP was obtained as 9.17 ± 0.39% (which indicated that the regression model was adequate and accurate for the extraction of motherwort leaf polysaccharides) (Table 4).

3.5. Antioxidant activity

3.5.1. Scavenging activity of hydroxyl radicals

Hydroxyl radical is the neutral form of the hydroxide ion, can damage easily all types of adjacent biological molecules including proteins, carbohydrates, lipids and nucleic acids (Sakanaka, Tachibana, & Okada, 2005). There are two types of antioxidation mechanism for hydroxyl radical; one suppresses the formation of the hydroxyl radical, and the other scavenges the hydroxyl radicals produced (Qi et al., 2006). Some studies have explained that polysaccharides isolated from the leaves have considerable bioactivities, being the most important modulation of the immune system. It is suggested that motherwort contains bufadienolic glycosides, which are supposed to possess cardiotonic property. *L. cardia* extract has also been reported to prevent lipid oxidation (Park et al., 2004). It has been employed in the treatment of hyperchlosterolemia (Matkowski & Piotrowska, 2006). Complicated carbohydrates from plants play important roles in the prevention of several disorders, such as inflammation, infections and cancer. The mechanism of biological activity of motherwort leaf may involve several pathways (Cai, 2005). The antioxidant property not only depended on the plant origin but also on the solution conformation, molecular weight, primary structure and polyanionic charge (Li et al., 2012).

In the previously studies, there is only little information about radical scavenging effect of motherwort leaf extract. However, the methods used for those researches were different, so the results are not clearly comparable. The results of scavenging abilities of *L. cardia* suggest that motherwort leaf polysaccharide has a good hydroxyl radical scavenging capacity (94.8%) at a concentration of 13.5 mg/mL in the reaction mixture (Fig. 3a). The hydroxyl radical scavenging capacity rose as the increase of LCLP concentration, while it had not very significant changes from 3 mg/mL to 5 mg/mL.

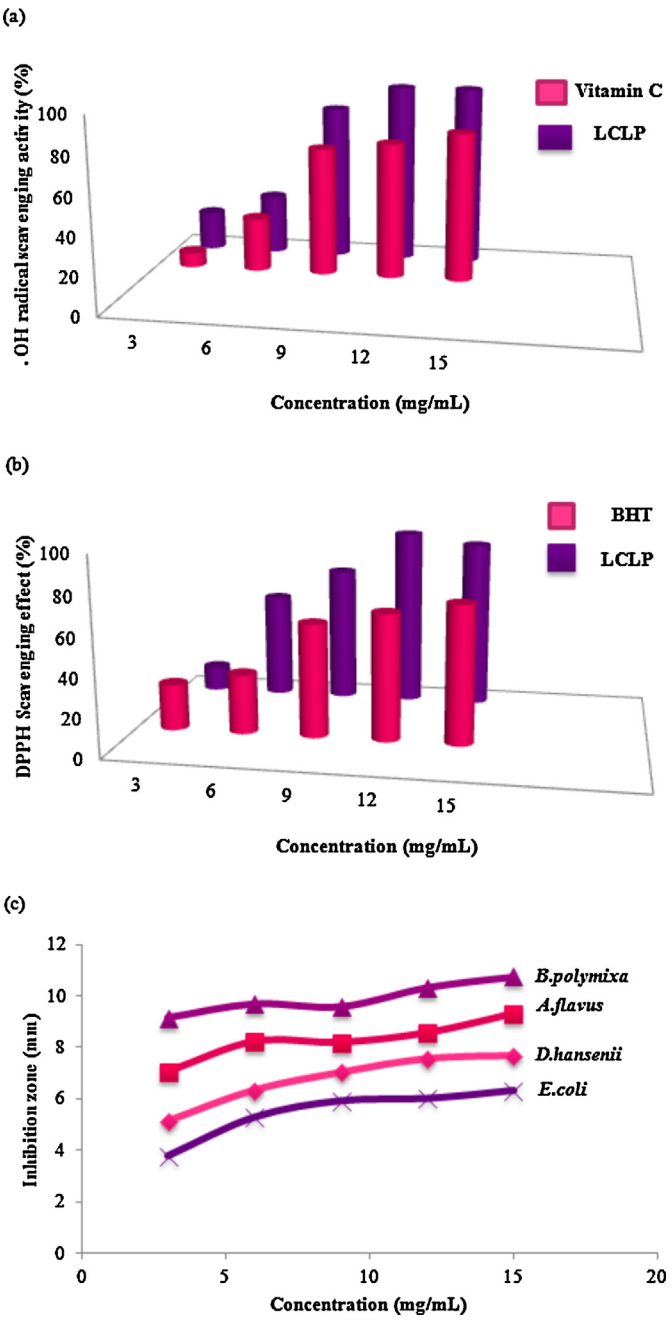

Fig. 3. Scavenging effect of LCLP on (a) hydroxyl radicals and (b) DPPH radicals compared with that of vitamin C and butylated hydroxytoluene (BHT). (c) Antimicrobial activity of LCLP on *B. polymixa*, *E. coli*, *D. hansenii* and *A. flavus*.

The scavenging activity of crude polysaccharides of *L. cardiaca* on hydroxyl groups was higher than ascorbic acid as positive control.

The half maximal inhibitory concentration (IC_{50}) represents the concentration needed to prevent a biological or biochemical function by half (e.g. inhibition of oxidation) (Wood, Gulati, & Forgiarini, 1985). According to the FDA, the inhibitory potency of a compound shows the concentration of a sample that is required for 50% inhibition in vitro. The IC_{50} values for hydroxyl radical with crude polysaccharides of motherwort leaf and vitamin C were found to be 6.98 ± 0.87 mg/mL and 7.59 ± 0.94 mg/mL, respectively.

3.5.2. DPPH free radical scavenging activity

The DPPH is a well-known radical with a maximum absorption at about 520 nm that can readily undergo scavenging by

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

Optimum conditions			Yield of polysaccharides (%)		
Temperature (°C)	Extraction time (min)	Ratio of water to raw material	Experimental	Predicted	Desirability
81.4	106.6	45.2	9.17 ± 0.39	9.26	0.976

antioxidants (Ionita, 2005). The reaction of DPPH with numerous plant polysaccharides has been reported and the stoichiometry characterized (Yuan, Zhang, Fan, & Yang, 2008). Motherwort alcoholic extract has been reported to have DPPH radical scavenging effect, may be related to the antioxidant ability of their constituents (Nagasawa et al., 1992). European motherwort has been published to have efficient antioxidant activities and known to have significant amounts of polysaccharides. This is probably due to the fact that the polysaccharides contained other components such as amino acids, carotenoids, polyphenols, proteins, peptides and vitamins (Li & Zhou, 2007). The anti-oxidative abilities of the aqueous extract of *Leonurus heterophyllus* on ischemic rat hearts were investigated in vitro and in vivo (Sun et al., 2005).

Fig. 3b represents DPPH scavenging capacities of different levels of LCLP. The IC_{50} values of motherwort leaf polysaccharides and BHT (as positive control) were 5.54 ± 1.04 mg/mL and 7.98 ± 0.95 mg/mL, respectively. When the concentration of LCLP was 12.14 mg/mL, the scavenging influences of LCLP on DPPH increased at 92.8%. Fig. 3b shows that the DPPH scavenging ability rose as the increase of polysaccharide concentration. It suggested that the effects of scavenging DPPH radicals were concentration related. Compared with Butylated Hydroxytoluene, LCLP showed significant radical-scavenging capacity under the same conditions. The mechanism of motherwort leaf polysaccharides might be explained to the supply of hydrogen (Lai, Wen, Li, Wu, & Li, 2010).

3.6. Determination of antimicrobial activities of LCLP

Crude polysaccharides from species of *Quercus*, *Echium* and *Chromolaena* have been reported to possess antimicrobial ability (Dall'Agnol, Ferraz, & Bernardi, 2003). Figs. 3c shows the results of antimicrobial activity of the motherwort leaf polysaccharides against *B. polymixa*, *E. coli*, *D. hansenii* and *A. flavus*. Tahmouzi (2014) reported the results of inhibition zones for *Salmonella typhi*, *Staphylococcus aureus*, *Candida albicans* and *Planococcus citri*. In general, the LCLP showed significant antimicrobial activity against these microorganisms. This highest ability of crude polysaccharides may be due to the disruption of cell wall and membrane of microbes (He, Yang, Yang, & Yu, 2010). From the results it was observed that, *E. coli* (Gram-negative) was found to be very resistant and *B. polymixa* (Gram-positive) was found to be more sensitive among the four organisms applied in the experiment. As the concentration increasing, the antimicrobial abilities of LCLP improved significantly (He et al., 2010). The antimicrobial activity of LCLP in maximum concentration (15 mg/mL) was in the following order: *B. polymixa* (Inhibition zone = 10.76 mm) > *A. flavus* (Inhibition zone = 9.30 mm) > *D. hansenii* (Inhibition zone = 7.67 mm) > *E. coli* (Inhibition zone = 6.32 mm). The *MIC* (minimum inhibitory concentration) values of polysaccharides were determined using broth dilution assay. The *MIC* values for LCLP were in the range of 8.9 mg/mL to 12.5 mg/mL. Among the microorganisms tested, *B. polymixa* was found to be more sensible (*MIC* = 8.9 mg/mL) and *E. coli* was found to be more resistant (*MIC* = 12.5 mg/mL). *A. flavus* and *D. hansenii* showed moderate *MIC* values against the polysaccharides.

4. Conclusion

Based on the single-factor experiments, a three-level, three-variable Box–Behnken design was used to determine the optimal variables for extraction of LCLP from Tabriz motherwort (*L. cardiaca* L.) leaf. A second-order polynomial model was employed to optimize polysaccharides extraction from motherwort leaf. Through optimization, a maximum predicted extraction efficiency of 9.26% was obtained with the following optimized conditions: extraction temperature of 81.4 °C, extraction time of 106.6 min and the ratio of water to raw material of 45.2. Under the optimal conditions of extraction temperature 82 °C, extraction time 106 min and the ratio of water to raw material 45, an actual experimental yield of 9.17 ± 0.39% was obtained. There was no significant difference ($p < 0.05$) between the experimental and predicted values. The results presented that the regression model was adequate and accurate for the extraction of LCLP. Motherwort leaf polysaccharides showed a dose-dependent antioxidant activity within the concentration range of 3–15 mg/mL, and LCLP possessed appreciable scavenging abilities on hydroxyl radical and DPPH radical in vitro. The inhibitory concentration 50 values (IC_{50}) of motherwort leaf polysaccharides for hydroxyl radical and DPPH radical were found to be 6.98 ± 0.87 mg/mL and 5.54 ± 1.04 mg/mL, respectively. The polysaccharides of *L. cardiaca* indicated inhibitory activity at different extent especially toward *B. polymixa* (*MIC* = 8.9 mg/mL) and *A. flavus* (*MIC* = 9.2 mg/mL). The antimicrobial activity of LCLP against some microorganisms was in the following order: *B. polymixa* > *A. flavus* > *D. hansenii* > *E. coli*. Therefore, motherwort leaf polysaccharides are natural antioxidant and preservative, and have a potential for clinical use.

Acknowledgement

This work was financially supported by the Ramin Agricultural and Natural Resources University, Ahvaz, Iran (Project no: 922/105).

References

Agnihotri, V. K., Elsohly, H. N., & Smillie, T. J. (2008). New labdane diterpenes from *Leonurus cardiaca*. *Planta Medica*, *74*(10), 1288–1290.
Cai, X. H. (2005). Studies on the chemical constituents of Leonurus japonicus Houtt. In *Master thesis*. Shenyang: Shenyang Pharmaceutical University (in Chinese).
Chen, Y., Xie, M. Y., & Gong, X. F. (2007). Microwave-assisted extraction used for the isolation of total triterpenoid saponins from *Ganoderma atrum*. *Journal of Food Engineering*, *81*, 162–170.
Chen, L., Zhao, L., Bai, Y., Hu, R., & Si, J. (2009). Genetic relationship analysis of different provenances of *Leonurus japonicus* by ISSR marker. *Zhongguo Zhong Yao Za Zhi*, *34*(11), 1343–1345.
Dall'Agnol, R., Ferraz, A., & Bernardi, A. P. (2003). Antimicrobial activity of some *Hypericum species*. *Phytomedicine*, *10*, 511–516.
Eskilsson, C. S., Davidsson, R., & Mathiasson, L. (2002). Harmful azo colorants in leather: Determination based on their cleavage and extraction of corresponding carcinogenic aromatic amines using modern extraction techniques. *Journal of Chromatography A*, *955*, 215–227.
Galle, J. (2001). Oxidative stress in chronic renal failure. *Nephrology Dialysis Transplantation*, *16*, 2135–2142.
Gan, C.-Y., Abdul Manaf, N. H., & Latiff, A. A. (2010). Optimization of alcohol insoluble polysaccharides (AIPS) extraction from the *Parkia speciosa* pod using response surface methodology (RSM). *Carbohydrate Polymers*, *79*, 825–831.

Guo, X., Zou, X., & Sun, M. (2010). Optimization of extraction process by response surface methodology and preliminary characterization of polysaccharides from *Phellinus igniarius*. *Carbohydrate Polymers*, *80*, 344–349.
He, F., Yang, Y., Yang, G., & Yu, L. J. (2010). Studies on antibacterial activity and antibacterial mechanism of a novel polysaccharide from *Streptomyces virginia* H03. *Food Control*, *21*(9), 1257–1262.
Huang, S., & Ning, Z. (2010). Extraction of polysaccharide from *Ganoderma lucidum* and its immune enhancement activity. *International Journal of Biological Macromolecules*, *47*, 336–341.
Ionita, P. (2005). *Is DPPH stable free radical a good scavenger for oxygen active species? Chemical papers* (59) , 1.
Jiang, C., Wang, M., Liu, J., Gan, D., & Zeng, X. (2011). Extraction, preliminary characterization, antioxidant and anticancer activities in vitro of polysaccharides from *Cyclina sinensis*. *Carbohydrate Polymers*, *84*, 851–857.
Kshirsagar, A. C., & Singhal, R. S. (2007). Optimization of starch oleate derivatives from native corn and hydrolyzed corn starch by response surface methodology. *Carbohydrate Polymers*, *69*, 455–461.
Lai, F., Wen, Q., Li, L., Wu, H., & Li, X. (2010). Antioxidant activities of water-soluble polysaccharide extracted from mung bean (*Vigna radiata* L.) hull with ultrasonic assisted treatment. *Carbohydrate Polymers*, *81*(2), 323–329.
Li, J., Ai, L., Yang, Q., Liu, Y., & Shan, L. (2013). Composition and antioxidant activity of polysaccharides from jujuba by classical and ultrasound extraction. *International Journal of Biological Macromolecules*, *55*, 83–87.
Li, N., Li, L., Fang, J. C., Wong, J. H., Ng, T. B., Jiang, Y., Wang, C. R., Zhang, N. Y., Wen, T. Y., & Qu, L. Y. (2012). Isolation and identification of a novel polysaccharide-peptide complex with antioxidant, anti-proliferative and hypoglycaemic activities from the abalone mushroom. *Bioscience Reports*, *32*, 221–228.
Li, X. L., & Zhou, A. G. (2007). Evaluation of the antioxidant effects of polysaccharides extracted from *Lycium barbarum*. *Medicinal Chemistry Research*, *15*(9), 471–482.
Liu, J., Miao, S., Wen, X., & Sun, Y. (2009). Optimization of polysaccharides (ABP) extraction from the fruiting bodies of *Agaricus blazei Murill* using response surface methodology (RSM). *Carbohydrate Polymers*, *78*, 704–709.
Masmoudi, M., Besbes, S., Chaabouni, M., Robert, C., Paquot, M., & Blecker, C. (2008). Optimization of pectin extraction from lemon by-product with acidified date juice using response surface methodology. *Carbohydrate Polymers*, *74*, 185–192.
Matkowski, A., & Piotrowska, M. (2006). Antioxidant and free radical scavenging activities of some medicinal plants from the Lamiaceae. *Fitoterapia*, *77*(5), 346–353.
Matthaei, J. H., Jones, O. W., Martin, R. G., & Nirenberg, M. W. (1962). Characteristics and composition of RNA coding units. *Proceedings of the National Academy of Sciences of the United States of America*, *48*(4), 666–667.
Mizuno, M., Shiomi, Y., Minato, K., Kawakami, S., Ashida, H., & Tsuchida, H. (2000). Fucogalactan isolated from *Sarcodon aspratus* elicits release of tumor necrosis factor-α and nitric oxide from murine macrophages. *Immunopharmacology*, *46*, 113–121.
Montgomery, D. C. (2001). *Design and analysis of experiments* (5th New ed., pp. 455–492). York: Wiley.
Montgomery, D. C. (2005). *Design and analysis of experiments: Response surface method and designs*. Hoboken, NJ: John Wiley and Sons, Inc.
Nagasawa, H., Inatomi, H., Suzuki, M., & Mori, T. (1992). Further study on the effects of motherwort (*Leonurus sibiricus* L.) on preneoplastic and neoplastic mammary gland growth in multiparous GR/A mice. *Anticancer Research*, *12*, 141–143.
Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, *7*, 715–729.
Park, S. W., Vepachedu, R., Sharma, N., & Vivanco, J. M. (2004). Ribosome inactivating proteins in plant biology. *Planta*, *219*, 1093–1096.
Prakash Maran, J., Mekala, V., & Manikandan, S. (2013). Modeling and optimization of ultrasound-assisted extraction of polysaccharide from *Cucurbita moschata*. *Carbohydrate Polymers*, *92*, 2018–2026.
Qi, H., Zhang, Q., Zhao, T., Hu, R., Zhang, K., & Li, Z. (2006). In vitro antioxidant activity of acetylated and benzoylated derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). *Bioorganic and Medicinal Chemistry Letters*, *16*, 2441–2445.
Qiao, D., Hu, B., Gan, D., Sun, Y., Ye, H., & Zeng, X. (2009). Extraction optimized by using response surface methodology, purification and preliminary characterization of polysaccharides from *Hyriopsis cumingii*. *Carbohydrate Polymers*, *76*, 422–429.
Sakanaka, S., Tachibana, Y., & Okada, Y. (2005). Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). *Food Chemistry*, *89*, 569–575.
Sin, H. H., Yusof, S., Sheikh Abdul Hamid, N., & Rahman, R. A. (2006). Optimization of hot water extraction for sapodilla juice using response surface methodology. *Journal of Food Engineering*, *74*, 352–358.
Sun, J., Huang, S. H., Zhu, Y. C., Whiteman, M., Wang, M. J., Tan, B. K. H., & Zhu, Y. Z. (2005). Anti-oxidative stress effects of *Herba leonuri* on ischemic rat hear. *Life Science*, *76*, 3043–3056.
Sun, Y., Wang, S., Li, T., Li, X., Jiao, L., & Zhang, L. (2008). Purification, structure and immunobiological activity of a new water-soluble polysaccharide from the mycelium of *Polyporus albicans* (Imaz.) Teng. *Bioresource Technology*, *99*(4), 900–904.
Sun, Y., Liu, J. C., & Kennedy, J. F. (2010). Extraction optimization of antioxidant polysaccharides from the fruiting bodies of *Chroogomphis rutilus* (Schaeff.: Fr.) O.K. Miller by Box–Behnken statistical design. *Carbohydrate Polymers*, *82*, 209–214.
Tahmouzi, S. (2014). Optimization of polysaccharides from Zagros oak leaf using RSM: Antioxidant and antimicrobial activities. *Carbohydrate Polymers*, *106*, 238–246.
Vinogradov, E. V., Brade, L., Brade, H., & Holst, O. (2003). Structural and serological characterisation of the *O*-antigenic polysaccharide of the lipopolysaccharide from *Acinetobacter baumannii* strain 24. *Carbohydrate Research*, *338*, 2751–2756.
Wang, H., Gao, X. D., Zhou, G. C., Cai, L., & Yao, W. B. (2008). In vitro and in vivo antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit. *Food Chemistry*, *106*, 888–895.
Wang, R. F., Chen, P., Jia, F., Tang, J., & Ma, F. (2012). Optimization of polysaccharides from *Panax japonicus* C.A. Meyer by RSM and its anti-oxidant activity. *International Journal of Biological Macromolecules*, *50*, 331–336.
Wood, J. M., Gulati, N., & Forgiarini, P. (1985). Effect of a specific and long-acting rennin inhibitor in the marmoset. *Hypertention*, *7*, 797–803.
Xie, J. H., Shen, M. Y., Xie, M. Y., Nie, S. P., Chen, Y., & Li, C. (2012). Ultrasonic-assisted extraction, antimicrobial and antioxidant activities of *Cyclocarya paliurus* (Batal.) Iljinskaja polysaccharides. *Carbohydrate Polymers*, *89*(1), 177–184.
Xu, W. T., Zhang, F. F., Luo, Y. B., Ma, L. Y., Kou, X. H., & Huang, K. L. (2009). Antioxidant activity of a water-soluble polysaccharide purified from *Pteridium aquilinum*. *Carbohydrate Research*, *344*, 217–222.
Yang, X. M., Yu, W., Ou, Z. P., Ma, H. L., Liu, W. M., & Ji, X. L. (2009). Antioxidant and immunity activity of water extract and crude polysaccharide from *Ficus carica* L. fruit. *Plant Foods for Human Nutrition*, *64*(2), 167–173.
Ye, C. L., & Jiang, C. J. (2011). Optimization of extraction process of crude polysaccharides from *Plantago asiatica* L. by response surface methodology. *Carbohydrate Polymers*, *84*, 495–502.
Ye, H., Wang, K., Zhou, C., Liu, J., & Zeng, X. (2008). Purification, antitumor and antioxidant activities in vitro of polysaccharides from the brown seaweed *Sargassum pallidum*. *Food Chemistry*, *111*(2), 428–432.
Yin, X., You, Q., & Jiang, Z. (2011). Optimization of enzyme assisted extraction of polysaccharides from *Tricholoma Matsutake* by response surface methodology. *Carbohydrate Polymers*, *86*(3), 1358–1364.
Yuan, J. F., Zhang, Z. Q., Fan, Z. C., & Yang, J. X. (2008). Antioxidant effects and cytotoxicity of three purified polysaccharides from *Ligusticum chuanxiong* Hort. *Carbohydrate Polymers*, *74*, 822–827.
Zhong, K., & Wang, Q. (2010). Optimization of ultrasonic extraction of polysaccharides from dried longan pulp using response surface methodology. *Carbohydrate Polymers*, *80*, 19–25.
Zhu, C., & Liu, Z. (2013). Optimization of extraction process of crude polysaccharides from Pomegranate peel by response surface methodology. *Carbohydrate Polymers*, *92*, 1197–1202.
Zhu, T., Heo, H. J., & Row, K. H. (2010). Optimization of crude polysaccharides extraction from *Hizikia fusiformis* using response surface methodology. *Carbohydrate Polymers*, *82*, 106–110.