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Statistical modeling of process parameters for the recovery of polysaccharide from *Morus alba* leaf

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ABSTRACT

A central composite rotatable design (CCRD) was used to evaluate the effects of extraction time, extraction temperature, particle size of mulberry leaf, number of extraction and water to the mulberry leaf ratio on extraction yield of mulberry leaf crude polysaccharides (MLCP). The response surface methodology (RSM) showed that the significant quadratic regression equation with high R^2 (=0.9782) was successfully fitted for extraction yield of MLCP as function of independent variables. The overall optimum region was found to be at the combined level of extraction time 5 h, extraction temperature 85 °C, particle size of mulberry leaf (mesh) 40, number of extraction 4 and water to mulberry leaf ratio 18. At this optimum point, extraction yield of MLCP was 12.0017 ± 0.42%. No significant ($p > 0.05$) difference was found between the actual and predicted (11.6286 ± 0.19) values. The results demonstrated that MLCP had strong scavenging activities on DPPH and hydroxyl radicals. Overall, MLCP may have potential applications in the medical and food industries.

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1. Introduction

Morus alba a deciduous tree belonging to the family of Moraceae, is widely cultivated in tropical, subtropical and temperate areas (Agarwal & Kanwar, 2007). Its leaves have been used in East Asia as herbal medicine for healing of various diseases (i.e., treat fever, protect the liver, improve eye-sight and lower blood pressure) for thousands of years (Zhishen et al., 1999). Leaves and extracts of *Morus alba* are commonly used as functional foods in Asian regions, including China, Korea, Japan, Thailand and Iran (Kimura et al., 2007; Sattayasai, Tiamkao, & Puapairoj, 2008). Traditionally, mulberry leaves have been consumed regionally as herbal tea. The consumption of mulberry-leaf tea has increased over the past decades because of its hypoglycemic, antidepressant, antioxidant and hepatoprotective effects (Nookabkaew, Rangkadilok, & Satayavivad, 2006; Yang, Wang, Wang, & Zhang, 2012; Zhong, Furne, & Levitt, 2006). Recently, various other food-grade mulberry products (i.e., drink powders, snacks and tablets) have been developed and marketed in these countries (Yoshihashi, Do, Tungtrakul, Boonbumrung, & Yamaki, 2010).

Hydrocolloids are high molecular weight macromolecules that can be easily dissolved and dispersed in water under appropriate conditions. They can modulate rheological properties of foods, and are generally used as food thickeners, texture modifier, stabilizers and emulsifiers for various applications (Lai & Lin, 2004; Salazar-Montoya, Ramos-Ramırez, & Delgado-Reyes, 2002). Plant hydrocolloid preparation generally begins with extraction from the source using water or an alkaline solution (Lin & Lai, 2009). The nutritional and physiological aspects of mulberry leaves and mulberry leaf hydrocolloid have been reported extensively (Gai et al., 2005; Jia, Tang, & Wu, 1999; Wu, 1994). However, studies on the rheological aspects of mulberry leaf hydrocolloid or mulberry leaf extracts are quite limited.

Plant-derived hydrocolloids are generally extracted from different parts of plants with water or alkaline solution (Lai & Yang, 2007; Lin & Lai, 2009; Yamazakia, Kuritaa, & Matsumurab, 2008). They can modulate rheological properties of foods, and are broadly used as food texture modifier, stabilizers, thickeners and emulsifiers for various applications (Lai & Lin, 2004; Phillips & Williams, 2000). An acidic hydrocolloid has also been found in mulberry leaves (Hosseinzadeh & Sadeghi, 1999; Lin & Lai, 2009; Ouyang, Chen, & Li, 2005). The chemical composition analysis revealed that mulberry leaf hydrocolloids were mainly polysaccharide with high levels of uronic acid and diverse sugar compositions, including rhamnose, arabinose, galactose and glucose (Lin & Lai, 2009). In addition, mulberry leaf polysaccharide is reported to be one of the key components in mulberry leaf showing antihyperglycemic effects (Chen et al., 1996).

Hot water technology is the main and most conventional extraction method for polysaccharides mentioned in recent studies (Shao, Deng, Shen, Fang, & Zhao, 2011; Ye, Hu, & Dai, 2011). Therefore, efficient extraction conditions for FCPs are needed to be optimum. In

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general, efficiency of the extraction of crude polysaccharide from plant source is influenced by multiple parameters such as extraction temperature, time and ratio of water to raw material, among others, and their effects may be either independent or interactive (Chen, Zhang, Li, Chen, & Jia, 2012; Han, Jiang, & Zhang, 2011; Wu, Cui, Tang, & Gu, 2007). The traditional one-factor-at-a-time approach to process optimization is time consuming. Moreover, the interactions among various factors may be ignored (Liyana-Pathirana & Shahidi, 2005).

Response surface methodology (RSM) is a collection of mathematical and empirical techniques useful for establishing models, and for optimizing processes even in the presence of complex interactions. It not only determines the interaction between parameters, but also reduces the number of experimental trials, development time and overall cost (Gharibzahedi, Mousavi, Khodaiyan, & Hamedi, 2012). Employing RSM could lead to simplifying the complexity of experimental trials needed to evaluate multiple variables and their interactions. Therefore, it is labor and time-saving than other methods required to optimize a process (Qiao et al., 2009; Zhu, Gao, Li, Zhao, & Deng, 2010; Zhu, Heo, & Row, 2010).

RSM has been successfully applied for optimizing conditions in food and pharmaceutical research (Batistuti, Barros, & Areas, 1991; Ibanoglu & Ainsworth, 2004; Samavati, 2013; Shieh, Koehler, & Akoh, 1996; Varnalis, Brennan, MacDougall, & Gilmour, 2004; Vega, Balaban, Sims, O'Keefe, & Cornell, 1996).

Usually, it applies an experimental design such as Central Composite Rotatable Design (CCRD) 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 determine the interrelationship among the variables (Wu, Li, Tu, Yang, & Zhan, 2013).

The objective of this present study was to optimize the process for extraction of the polysaccharides from the leaf of Iranian mulberry (*Morus alba*), using response surface methodology (RSM), employing a five-level, five-variable central composite rotatable design (CCRD).

2. Materials and methods

2.1. Materials

Mulberry (*Morus alba*) leaves were collected at mollasani, khouzestan province in Iran in July 2009. All other chemicals and solvents used were of analytical grade and obtained from Merck Co., Germany.

2.2. Methods

2.2.1. Extraction of mulberry leaf crude polysaccharides (MLCP)

The extraction of crude polysaccharides from mulberry leaf was performed using a method modified from that by Komae and Misaki (1989). The mulberry leaves were washed, dried and ground to obtain fine powders with different particle size (10–60 mesh), then were extracted in a Soxhlet apparatus with aether (30 °C), and pretreated with 80% aether twice to remove some colored materials, oligosaccharides and some small molecule materials. The organic solvent was volatilized and pretreated dry powder was obtained, as described by Yang, Qu, and Cheng (2004).

The pretreated dry powder of mulberry leaf (10–60 mesh) (20 g) were stirred in deionized water (water to the raw material ratio ranging from 3 to 30) at pH 5.6 (adjusting the suspension pH by sodium acetate–acetic acid buffer), while the temperature of the water bath was kept steady for a given temperature (within ±1.0 °C, extraction temperature ranging from 50 to 100 °C). The slurry in a 2.0 L stainless steel in the hot water bath was stirred with an electric mixing paddle for a given time (extraction time ranging from 1 to 9 h) during the entire extraction process. The extracted slurry was centrifuged at 5000 rpm/min for 20 min to collect the supernatant, and the insoluble residue was treated again (extraction times ranging from 1 to 7) as mentioned above. An equal volume of ethanol was added to the resultant viscous solution to precipitate the crude polysaccharide extract. The precipitate was washed successively with 50% ethanol until a clear polysaccharide was obtained. It was then dewatered by washing successively with 80% ethanol and acetone. The extract was air-dried at 50 °C until its weight was constant, and then was weighted with a balance (BS2202S, Sartorius, Germany). The percentage polysaccharides extraction yield (%) is calculated as follows:

$$\text{MLCP extraction yield}\,\%(\text{w/w}) = \frac{W_o}{W} \quad (1)$$

W_o (g) is the dried MLCP weight; W (g) is the dried powder of mulberry leaf weight.

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

Independent variables	Factor level				
	$-2\ (-\alpha)$	-1	0	1	$2\ (+\alpha)$
Extraction time (h)	0.5	2	3.5	5	6.5
Extraction temperature (°C)	55	65	75	85	95
Particle size (mesh)	10	20	30	40	50
Number of extraction	1	2	3	4	5
Water to mulberry leaf ratio	3	8	13	18	23

2.2.2. Experimental design of RSM

In this study, for obtaining a relationship between the responses, i.e., the extraction yield of MLCP (%), and five process parameters (extraction time X_1, extraction temperature X_2, the raw material particle size X_3, the number of extraction X_4 and water to the mulberry leaf ratio X_5), the Central Composite Rotatable Design (CCRD) was employed (Table 1). Central Composite Rotatable Design based on RSM provides a design of experiments through which not only optimum conditions are achieved, but also the number of experiments is reduced (Gharibzahedi et al., 2012). RSM constitutes an experimental methods category for the determination of a relationship between independent and dependant variables (Can, Kaya, & Algur, 2006; Pouralinazara, Yunusa, & Zahedi, 2012). The RSM was applied to the experimental data using a commercial statistical package, Design-Expert version 8.0.7.1 (Statease Inc., Minneapolis, USA). In RSM development, at the beginning, a first order model was developed to find a relationship between input and output variables. It was found that the model is not accurate and in this case a second-order model was developed in the next attempt (Bayraktar, 2001).

All the experiments were done in triplicate and the average Y obtained was taken as the response (Table 2). A non-linear regression method was used to fit the second order polynomial (Eq. (1)) to the experimental data and to identify the relevant model terms. Considering all the linear terms, square terms and interaction items, the quadratic response model can be described as:

$$Y = \sum A_o + \sum_{i=1}^{5} A_{ii}X_i + \sum_{i=1}^{5} A_{ii}X_i^2 + \sum_{i=1}^{4}\sum_{j=i+1}^{5} X_iX_j \quad (2)$$

where Y is the response variable, A_o, A_i, A_{ii}, A_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively, and X_i, X_j are the independent variables (i–j). The coefficients of the second polynomial model and the responses obtained from each set of experimental design were subjected to multiple nonlinear regressions using software Design-Expert 8.

Table 2
Response surface central composite design (uncoded) and results for extraction yield of MLCP.

Run	X_1 (h)	X_2 (°C)	X_3 (mesh)	X_4	X_5	Extraction yield (%)		
						Experimental	Predicted	$(Y_{Exp} - Y_{Pred})$
1	5	65	20	4	8	3.20	2.95	0.246
2	5	85	40	4	8	4.00	4.23	−0.226
3	3.5	75	30	3	13	3.90	3.73	0.174
4	2	85	20	2	8	4.90	5.19	−0.286
5	2	85	20	4	18	3.50	3.28	0.216
6	2	85	20	4	8	5.20	5.37	−0.169
7	5	85	40	4	18	4.20	4.62	−0.419
8	2	65	40	4	8	7.20	6.89	0.309
9	5	65	40	4	8	3.70	4.00	−0.299
10	3.5	75	30	3	13	7.50	7.13	0.366
11	2	65	20	4	18	4.60	4.33	0.266
12	2	65	40	2	18	7.40	7.66	−0.256
13	5	85	40	2	18	4.10	4.12	−0.016
14	2	85	20	2	18	7.80	8.06	−0.264
15	5	65	40	4	18	4.80	5.01	−0.214
16	5	65	40	2	18	9.40	9.15	0.251
17	3.5	75	30	3	13	3.90	3.88	0.016
18	3.5	75	30	5	13	5.10	5.12	−0.019
19	2	85	40	2	8	4.90	4.77	0.131
20	6.5	75	30	3	13	5.90	6.19	−0.291
21	3.5	75	30	3	3	5.00	5.40	−0.401
22	3.5	75	30	1	13	7.40	7.45	−0.049
23	2	85	40	4	18	6.60	6.85	−0.249
24	3.5	75	50	3	13	9.30	9.08	0.216
25	3.5	55	30	3	13	4.80	5.22	−0.416
26	3.5	75	30	3	13	8.40	8.31	0.086
27	3.5	75	30	3	13	5.40	5.66	−0.264
28	2	65	20	2	18	9.20	8.95	0.251
29	5	85	20	4	18	6.90	6.52	0.379
30	3.5	75	30	3	13	10.20	10.43	−0.231
31	5	85	20	2	18	7.80	7.53	0.269
32	3.5	75	10	3	13	10.90	11.63	−0.729
33	5	85	20	2	8	4.20	4.11	0.087
34	5	65	20	2	8	9.80	9.48	0.317
35	5	65	20	4	18	5.70	5.51	0.187
36	2	85	40	2	18	7.70	7.48	0.217
37	2	65	20	2	8	4.40	4.34	0.057
38	2	65	20	4	8	7.70	7.35	0.347
39	2	65	40	4	18	5.00	4.80	0.197
40	2	65	40	2	8	8.60	8.39	0.207
41	3.5	95	30	3	13	5.00	5.04	−0.043
42	5	85	20	4	8	8.90	8.45	0.447
43	3.5	75	30	3	13	8.00	7.65	0.350
44	5	65	20	2	18	7.40	7.65	−0.251
45	5	65	40	2	8	7.40	7.65	−0.251
46	0.5	75	30	3	13	7.30	7.65	−0.351
47	3.5	75	30	3	23	8.10	7.65	0.450
48	2	85	40	4	8	7.30	7.65	−0.351
49	5	85	40	2	8	8.10	7.65	0.450
50	3.5	75	30	3	13	7.20	7.65	−0.451

The uncoded independent variables (X_1,X_2,...,X_k) are coded in accordance with the transfer equation given below (Adinarayana & Ellaiah, 2002):

$$x_i = \frac{X_i - X_o}{\Delta X_i} \quad i = 1, 2, 3, \ldots, \mathrm{k} \tag{3}$$

where x_i is the dimensionless value of an independent variable; X_i is the real value of an independent variable; X_o is the real value of an independent variable at the center point; and ΔX_i is the step change of the real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i.

The statistical significance of the terms in the regression equations was examined. The significant terms in the model were found by analysis of variance (ANOVA) for each response. The adequacy of the model was checked accounting for R^2, adjusted-R^2 and PRESS in Eqs. (2)–(4), respectively (Gharibzahedi et al., 2012):

$$R^2 = 1 - \frac{\mathrm{SS_{Residual}}}{\mathrm{SS_{Residual}} + \mathrm{SS_{Model}}} \tag{4}$$

$$R^2_{\mathrm{Adj}} = 1 - \frac{\mathrm{SS_{Residual}}/\mathrm{DF_{Residual}}}{(\mathrm{SS_{Residual}} + \mathrm{SS_{Model}})/\mathrm{DF_{Residual}} + \mathrm{DF_{Model}}} \tag{5}$$

$$\mathrm{PREES} = \sqrt{\sum_{i=1}^{N} (y_{\mathrm{Pred},i} - y_{\mathrm{Exp},i})^2} \tag{6}$$

Adequate precision compares the range of the predicted values at the design points to the average prediction error. The definition of adequate precision is in Eqs. (5) and (6):

$$\text{adequate precision} = \frac{\mathrm{Max}(\tilde{y}) - \overline{(y)}}{\sqrt{\overline{\nabla(y)}}} \tag{7}$$

$$\tilde{V}(\tilde{y}) = \frac{1}{n}\sum_{i=1}^{N} V(\tilde{y}) = \frac{P\sigma^2}{n} \tag{8}$$

In Eqs. (4)–(8), SS is the sum of squares, DF is the degrees of freedom, $y_{i,\,exp}$ is the experimental responses, $y_{i,\,per}$ is the predicted responses, $\bar{y}$ is the predicted value, p is the number of model parameters, σ^2 is the residual mean square from ANOVA table, and n is the number of experiments.

Numerical and graphical optimization technique of the Design Expert software was used for simultaneous optimization of the multiple responses. The desired goal for variables and response was chosen. All the independent variables were kept within range while the response was maximized.

2.3. Antioxidant activity of MLCP

2.3.1. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH•) scavenging assay

The DPPH radical scavenging activity of MLCP was measured by the method of Zhong, Lin, Wang, and Zhou (2012) with a minor modification. The MLCP was dissolved in distilled water with different concentrations (40–300 mg/L). 1.5 mL of solution was added to equivalent aliquot DPPH (0.1 mM) in 95% ethanol. The reaction solution was shaken vigorously and incubated at room temperature (25 ± 1 °C) for 30 min, and then the absorbance of the resulting solution at 517 nm was measured. Blank solution contains 95% ethanol instead of DPPH solution for the baseline correction. A lower absorbance represented a higher DPPH scavenging activity. The same procedure was repeated with butylated hydroxytoluene (BHT), as a positive control. The DPPH radical scavenging percentage (A) was calculated as follows:

$$A(\%) = \left[1 - \frac{A_s}{A_c}\right] \times 100 \quad (9)$$

where A_s is absorbance of sample and A_c is absorbance of control. The control solution contained equivalent distilled water instead of samples solution. All tests were performed in triplicate.

2.3.2. Hydroxyl radicals scavenging assay

Assessment of the scavenging ability of MLCP on Hydroxyl radicals was performed by the method previously described by Halliwell, Gutteridge, and Aruoma (1987) and Wu et al. (2013) with a little modification. Reaction mixtures in a final volume of 1.0 mL contained deoxyribose (60 mM), phosphate buffer (pH 7.4, 20 mM), ferric trichloride (100 mM), EDTA (100 mM), H_2O_2 (1 mM), ascorbic acid (100 mM) and different concentrations of MLCP (40–240 mg/L). The reaction solution was incubated for 1 h at 38 °C, and then 1 mL of 1% thiobarbituric acid (TBA) and 1 mL of 20% (v/v) HCl were added to the mixture. The mixture was boiled for 15 min and cooled on ice. Distilled water and butylated hydroxytoluene (BHT) served as blank and positive control, respectively. The absorbance of the mixture was measured at 532 nm. The scavenging activity of Hydroxyl radicals (%) was calculated according to the following equation:

$$\text{Hydroxyl radicals scavenging activity}\,(\%) = \left[\frac{A_c - A_s}{A_c}\right] \times 100 \quad (10)$$

where A_c is the absorbance of the control (water instead of the sample) and A_s is the absorbance of the sample.

3. Results and discussion

To evaluate the effect of process variables on the extraction yield of MLCP (%), experiments were performed on the basis of the design matrix of central composite retortable design (CCRD) with five replicated points. Design points, experimental and predicted results of MLCP extraction yield (%) are shown in Table 2. To minimize the effects of uncontrolled factors, experiments were performed in random sequence.

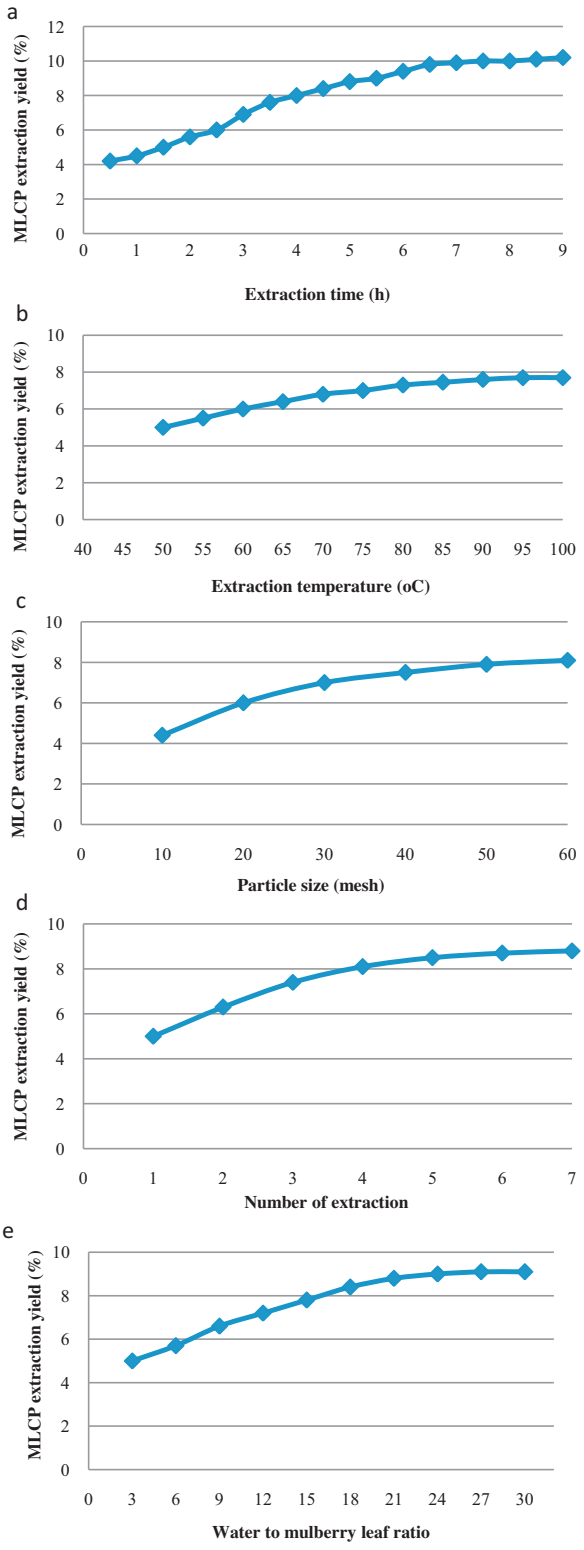


Fig. 1. Effects of different (a) extraction times, (b), temperatures extraction, (c) particle size of mulberry leaf (mesh), (d) numbers of extraction cycle, and (e) water to the water to mulberry leaf ratios, on extraction yield of MLCP (%).

3.1. Effect of extraction time on extraction yield of MLCP

The effect of extraction time on yield of MLCP was shown in Fig. 1a. The extraction time was set at 0.5–9 h while other extraction parameters were given as the followings: extraction temperature

75 °C, particle size of mulberry leaf 30 (mesh), number of extraction 3 and the water to mulberry leaf ratio 13. The extraction yields of the MLCP significantly increased from 4.2 ± 0.11% to 9.8 ± 0.17% as time of extraction increased from 0.5 to 6.5, and then the extraction yield of MLCP no longer obviously changed, when the extraction time increased. It was reported that a long extraction time favors the production of polysaccharides (Cho & Hwang, 2000; Hou & Chen, 2008; Liu, Wei, Guo, & Kennedy, 2006; Ros et al., 2004). This might be due to the time requirement of the exposure of the polysaccharides to the release medium where the liquid penetrated into the dried raw material, dissolved the polysaccharides and subsequently diffused out from the raw material. In addition, increasing extraction temperature would increase the solubility of the extracted polysaccharides, giving a higher rate of extraction. Also, the diffusion coefficient will increase and thus improves the rate of diffusion (Masmoudi et al., 2008). This indicated that extraction time of 6.5 h was sufficient to obtain the MLCP extraction.

3.2. Effect of extraction temperature on extraction yield of MLCP

The extraction yield of MLCP influenced by different extraction temperature from 50 to 100 °C is shown in Fig. 1b. The extraction was carried out under the following conditions: extraction time 3.5 h, particle size of mulberry leaf 10 (mesh), number of extraction 3 and water to the mulberry leaf ratio of 13. The extraction yield of polysaccharides had been increasing when he extraction temperature increased from 50 to 95 °C. And then there was no increase when extraction temperature continued to rise. As shown in Fig. 1b, the maximum yield of MLCP was observed when the extraction temperature was around 90 °C. The extraction yield increased with increasing the extraction temperature due to the increase of the polysaccharides solubility (Braga, Moreschi, & Meireles, 2006). Although the extraction yield of polysaccharides was also high at 100 °C, increasing temperature will bring about the increase in cost for extraction process from the industrialization point of view (XuJie & Wei, 2008). Therefore, the extraction temperature range of 90–100 °C was considered to be optimal in the present experiment.

3.3. Effect of different particle sizes on extraction yield of MLCP

Particle sizes were set at 10–60 meshes to examine the effect of different particle sizes on extraction yield of polysaccharides while the other independent variables, extraction time, extraction temperature, number of extraction and water to the mulberry leaf ratio were kept at 3.5 h, 75 °C, 3 and 13, respectively. As seen from Fig. 1c, it can be found that the extraction yield of the MLCP significantly increased as particle sizes of the mulberry leaf increased from 10 to 50 meshes. The maximum yield (8.0 ± 0.21%) was also was obtained when particle size was 50 mesh but beyond this particle size, extraction yield of MLCP reached the plateau region where the yield was maximized and did not further increase the yield. The small size of the mulberry leaf would lead to high yield of the MLCP. The reason for this was that a smaller amount of the polysaccharides could be transported from the interior parts of smaller mulberry leaf particles to the bulk of liquid extract than from the larger ones, due to a smaller resistance and short path to the polysaccharides diffusion (Biais, LeBail, Robert, Pontoire, & Buleon, 2006; Sun & Tomkinso, 2002; XuJie & Wei, 2008).

3.4. Effect of number of extraction on extraction yield of MLCP

The extraction yield of MLCP affected by different extraction number (1–7 times) is seen in Fig. 1d, when other four factors were fixed as follows: extraction time 3.5 h, extraction temperature 75 °C, particle size of mulberry leaf 30 (mesh) and water to raw material ratio 13. The results showed that the extraction yield of MLCP had obviously increased accompanying the increase of extracting times, but there was no significant increase after 4 times. In order to save the production cost and time for industrialization, 4 times were sufficient for the extraction of MLCP. Therefore 4 times were chosen as the extracting number through all the extraction optimization experiments. This result is in agreement with reports of other authors in extracting polysaccharides (Liang, 2008; Sun, Li, Yan, & Liu, 2010).

3.5. Effect of different ratio of water to mulberry leaf on extraction yield of MLCP

The different ratio of water to raw material will significantly affect extract yield. The extraction yield (%) of MLCP extracted by different ratio of water to mulberry leaf from 3 to 30 was shown in Fig. 1e. The extraction time, extraction temperature, particle size of mulberry leaf and number of extraction were fixed at 3.5 h, 75 °C, 30 (mesh) and 3, respectively. If ratio of water to raw material is too small, crude polysaccharide in raw material cannot be completely extracted up. If ratio of water to raw material is too big, this will cause high process cost (Zhu, Gao, et al., 2010; Zhu, Heo, et al., 2010; Zhu & Liu, 2013). Therefore, it is necessary to select a suitable ratio of water to raw material for extraction of targeted MLCP. The extraction yields of the polysaccharides significantly increased from 5.0 ± 0.14 to 9.1 ± 0.25 as the ratio of water to mulberry leaf increased from 3 to 23 shown in Fig. 1e, due to the increase of the driving force for the mass transfer of the polysaccharides (Bendahou, Dufresne, Kaddami, & Habibi, 2007). However, when the ratio continued to increase, the extraction yields no longer changed.

According to the single-parameter study, we adopted extraction time 0.5–6.5 h, extraction temperature 55–95 °C, particle size of mulberry leaf 10-50 (mesh), number of extraction 4 and water to the mulberry leaf ratio 3–23 for RSM experiments.

3.6. Fitting the response surface models to significant independent variables

Because RSM saves time, space and raw material, it is superior to the traditional single factor optimization. The experimental data for extraction of the crude polysaccharides of mulberry leaf under different treatment conditions were presented in Table 2. The extraction yield of mulberry leaf ranged from 3.2 to 10.9%. By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the MLCP extraction regardless of the significance of the coefficients:

$$\begin{aligned} Y = {} & -22.564 + 0.002X_1 + 0.453X_2 + 0.131X_3 + 2.182X_4 \\ & + 0.146X_5 + 0.003X_1X_2 + 0.013X_1X_3 + 0.310X_1X_4 \\ & - 0.001X_1X_5 + 0.001X_2X_3 - 0.011X_2X_4 + 0.006X_2X_5 \\ & - 0.005X_3X_4 + 0.006X_3X_5 + 0.014X_4X_5 - 0.095X_1^2 \\ & - 0.003X_2^2 - 0.005X_3^2 - 0.263X_4^2 - 0.009X_5^2 \end{aligned} \quad (11)$$

where Y is the predicted response (yield extraction of MLCP (%)); X_1, X_2, X_3, X_4, and X_5 are experimental values of extraction time, extraction temperature, particle size of mulberry leaf and number of extraction and water to the mulberry leaf ratio, respectively.

As shown in Table 3, the predicted model was fitted significantly ($p < 0.0001$). The value of lack of fit was 0.5772 (Table 3), which implied that the lack of fit was not significantly relative to the pure error (Myers & Montgomery, 2002). As revealed in Table 3, the significant response surface model with high R^2 and adj-R^2 values were 0.9782 and 0.9632, respectively. These

Table 3
Analysis of variance for the fitted models.

	Source	DF	Coefficient	Sum of square	Mean square	*F*-Value	*p*-Value
Extraction yield (%)	Model	20		192.6359	9.6318	65.0515	<0.0001
	Residual	29		4.2939	0.1481		
	Lack of fit	22		3.2139	0.1461	0.9468	0.5772 ns
	Pure error	7		1.0800	0.1543		
	Total	49		196.9298			
	R^2		0.9782				
	Adj-R^2		0.9632				
	CV		5.9217				
	PRESS		13.0400				
	Standard deviation		0.3848				
	Adequate precision		34.7872				

Table 4
The significance of each response variable effect showed by using *F* ratio and *p* value in the nonlinear second order model.

	Variables	DF[a]	SS[b]	MS[c]	*F*-Value ($F_{cal.}$)	*p*-Value[d] (probability)
Linear effects	X_1	1	72.0923	72.0923	486.8985	<0.0001
	X_2	1	9.7023	9.7023	65.5273	<0.0001
	X_3	1	22.6503	22.6503	152.9758	<0.0001
	X_4	1	32.2203	32.2203	217.6100	<0.0001
	X_5	1	29.0703	29.0703	196.3354	<0.0001
Quadratic effects	X_1^2	1	1.4535	1.4535	9.81677	0.0039
	X_2^2	1	2.6565	2.6565	17.94162	0.0002
	X_3^2	1	6.4980	6.4980	43.88645	<0.0001
	X_4^2	1	2.2155	2.2155	14.96319	0.0006
	X_5^2	1	1.6290	1.6290	11.00207	0.0025
Interaction effects	X_1X_2	1	0.0703	0.0703	0.4749	0.4962 ns
	X_1X_3	1	1.3203	1.3203	8.9172	0.0057
	X_1X_4	1	6.9378	6.9378	46.8568	<0.0001
	X_1X_5	1	0.0028	0.0028	0.0190	0.8913 ns
	X_2X_3	1	0.6328	0.6328	4.2739	0.0477
	X_2X_4	1	0.3828	0.3828	2.5854	0.1187 ns
	X_2X_5	1	0.0253	0.0253	0.1710	0.6823 ns
	X_3X_4	1	0.0903	0.0903	0.6100	0.4411 ns
	X_3X_5	1	2.8203	2.8203	19.0479	0.0001
	X_4X_5	1	0.1653	0.1653	1.1165	0.2994 ns

[a] Degrees of freedom.
[b] Sum of squares.
[c] Mean sum of squares.
[d] *p*-Values < 0.05 were considered to be significant.
[e] ns: not significant.

values showed a good agreement between the experimental and the predicted values. The low PRESS (13.040) value suggest for the adequacy of the fitted quadratic model for predictive application (Table 2). The value of the coefficient of variation (CV) was 5.9217 (Table 3). Adequate precision measures the signal-to noise ratio. A ratio greater than 4 is desirable (Gharibzahedi, Mousavi, Hamedi, & Khodaiyan, 2011; Myers & Montgomery, 2002). In the proposed model, this value was 34.7278 (Table 3), a very good signal-to-noise ratio. All these statistical parameters show the reliability of the models.

The *p*-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the *p*-values, the bigger the significance of the corresponding coefficient (Liu, Weng, Zhang, Xu, & Ji, 2003). The parameter estimates and the corresponding *p*-values (Table 4) suggest that, among the independent variables, extraction time X_1, temperature extraction X_2, particle size of mulberry leaf X_3, number of extraction X_4 and water to the mulberry leaf ratio X_5, have a significant effect on extraction yield. The positive coefficients for the all variables (X_1, X_2, X_3, X_4 and X_5) indicate a linear effect to increase extraction yield. The quadratic terms of these five variables and interaction terms of X_1X_3, X_1X_4 X_2X_3 and X_3X_5 also had a significant effect. However, the interaction terms (X_1X_2, X_1X_5, X_2X_4, X_2X_5, X_3X_4 and X_4X_5) were found insignificant ($p > 0.05$).

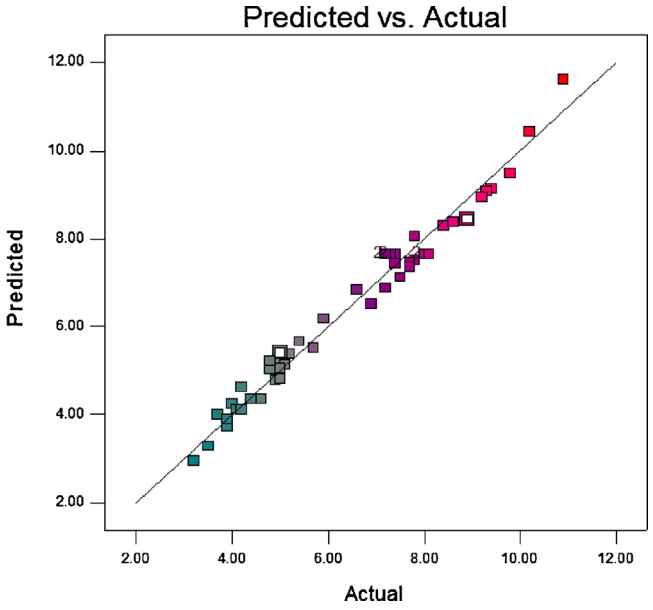


Fig. 2. Actual extraction yield vs. the predicted extraction yield under optimum extraction conditions.

3.7. Comparison of observed and predicted extraction yield

A regression model can be used to predict future observations on the response Y (extraction yield of MLCP) corresponding to particular values of the regressor variables. In predicting new observations and in estimating the mean response at a given point, one must be careful about extrapolating beyond the region containing the original observations (Li, Cui, Liu, Xud, & Zhao, 2007). It is very possible that a model that fits well in the region of the original data will no longer fit well outside the region. Fig. 2 shows observed extraction yield of MLCP (the response) versus those from the empirical model Eq. (11). The figure proves the predicted data of the response from the empirical model is in agreement with the observed ones in the range of the operating variables. No significant ($p > 0.05$) difference was observed between the experimental and predicted values of extraction yield of MLCP.

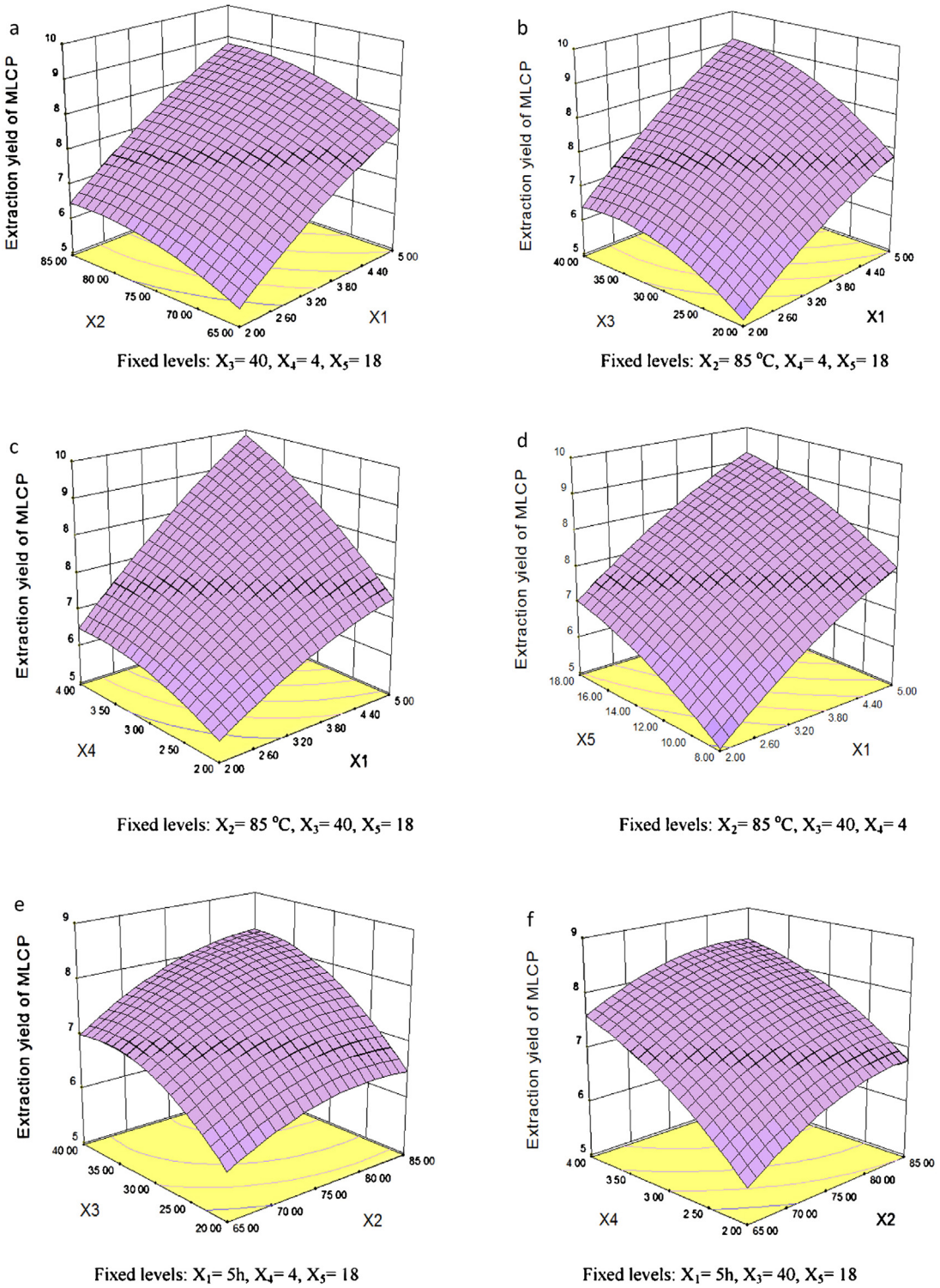
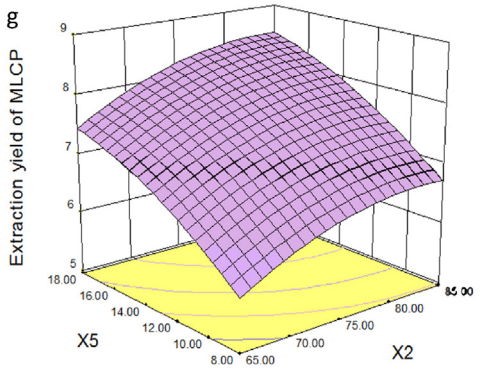
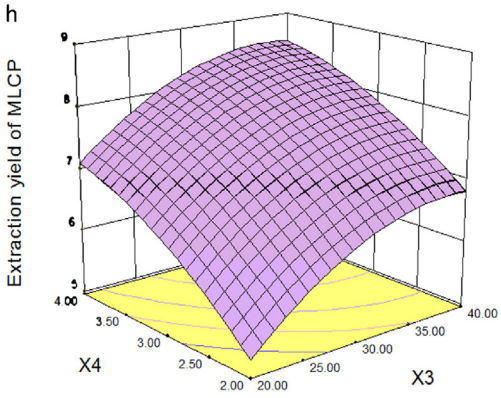


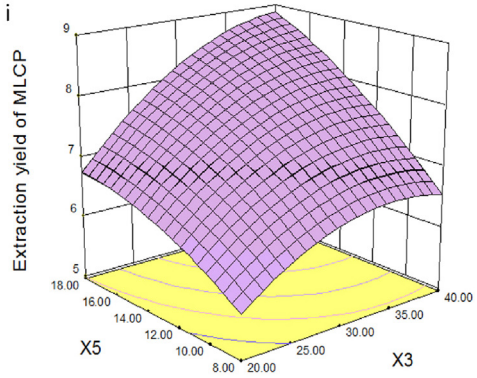
Fig. 3. Response surface (3D) showing the effect of the extraction time (X_1), extraction temperature (X_2), Particle size of mulberry leaf (mesh) (X_3), number of extraction (X_4), and water to mulberry leaf ratio (X_5) on the extraction yield of MLCP (%).



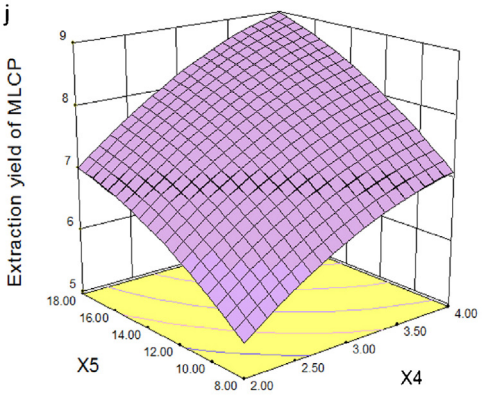
Fixed levels: X_1=5h, X_3= 40, X_4= 4



Fixed levels: X_1= 5h, X_2= 85 °C, X_5= 18



Fixed levels: X_1= 5h, X_2= 85 °C, X_4= 4



Fixed levels: X_1= 5h, X_2= 85 °C, X_3= 40

Fig. 3. (*Continued*).

3.8. Optimization of extraction conditions of MLCP

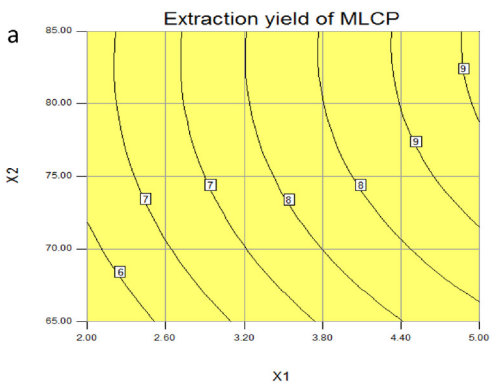
Response surfaces were plotted by using Design expert software (version 8.0) to study the effects of parameters and their interactions on MLCP extraction yield. RSM played a key role in identifying the optimum values of the independent variables efficiently, under which dependent variable could reach the maximum response. The independent variables and maximum predicted values from the figures corresponded with the optimum values of the dependent variables obtained by the equations. The results of extraction yield of MLCP affected by extraction time, extraction temperature, particle size of mulberry leaf, number of extraction and water to the mulberry leaf ratio are presented in Figs. 3 and 4. In the response surface plot and contour plot, the data were generated through keeping three variables at their respective zero level (central value of the testing ranges) and varying the other two within the experimental range. In the two figures, the maximum predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar, Chirumamil, Marchant, & Nigam, 2001). The independent variables and maximum predicted values from the figures corresponded with the optimum values of the dependent variables obtained by the equations.

Figs. 3a and 4a illustrate the 3D response surface plot and the contour plot at varying the extraction time, extraction temperature; at the fixed particle size of mulberry leaf 30 (mesh), number of extraction 3, and water to mulberry leaf ratio 13. A longer extraction time also presents a positive effect on the yield of polysaccharides. The results indicate that there is a positively significant effect of extracting time on the yield of MLCP when less than 5 h, and the effect is not significant when extracting time is higher than 5 h. Therefore, 5 h was selected as the center point of extracting time in the RSM experiments as higher time will bring about the energy waste and the cost increase for the extraction process. The extraction coefficient increased with increasing the extraction temperature due to the increase of the polysaccharides solubility (Braga et al., 2006). This phenomenon may be due to partial of the polysaccharide was hydrolyzed under the same temperature and long extraction time (Liu, Miao, Wen, & Sun, 2009). It can be seen that the maximum extraction yield of MLCP can be achieved when extraction temperature and extraction time were 5 h and 85 °C, respectively.

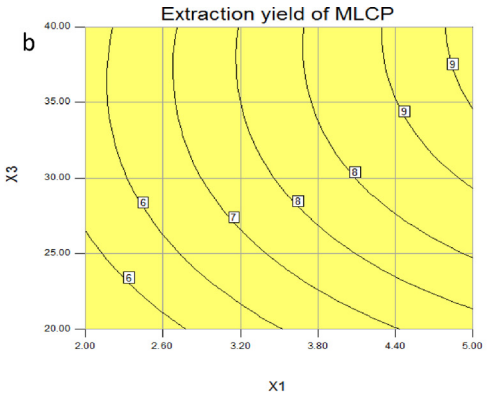
Figs. 3b and 4b show the interaction effect of extraction time and particle size of mulberry leaf while the other three factors were fixed as follow: extraction temperature 75 °C, number of extraction 3, water to mulberry leaf ratio 13. The extraction yield of MLCP reached a maximum percentage of 8.9 when the extraction time was 5 h. After this point, the extraction yield of MLCP reached the plateau region where the yield was maximized and did not further increase the yield. The yield extraction of MLCP increased with the

increasing the particle size of mulberry leaf (mesh) from 20 to 40 and reached the maximum value at extraction time of 5 h.

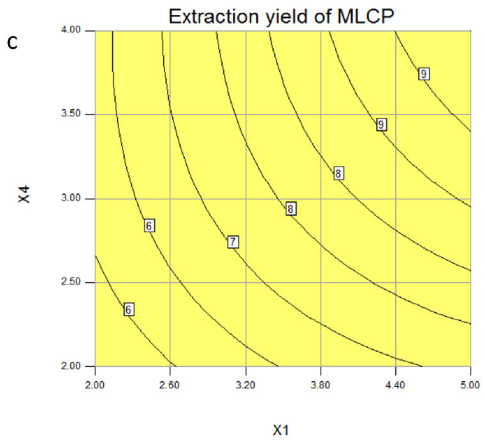
The 3D response surface plot in Figs. 2c and 3c, which gives the extraction yield of MLCP as a function of extraction time and number of extraction at fixed extraction temperature (75 °C), particle size of mulberry leaf (30 (mesh)) and water to mulberry leaf ratio (13), shows that extraction yield of polysaccharides increase with increase in extraction time, and extraction yield of polysaccharides is found to increase rapidly with increase of number of extraction from 1 to 4, but beyond 4, extraction yield of MLCP reached the



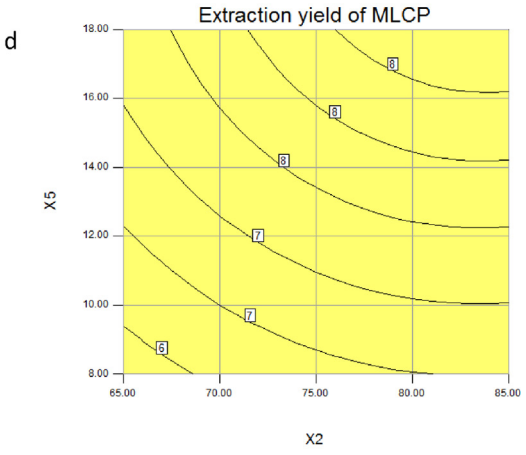
Fixed levels: X_3= 40, X_4= 4, X_5= 18



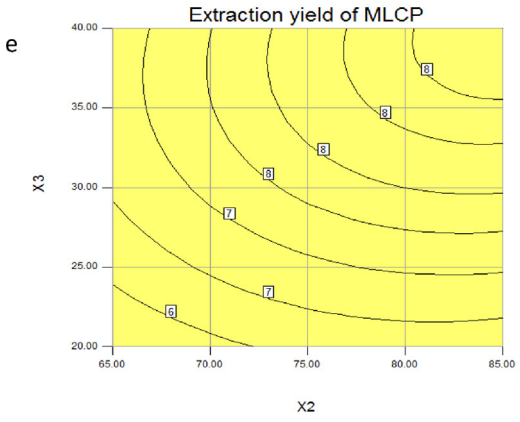
Fixed levels: X_2= 85 °C, X_4= 4, X_5=18



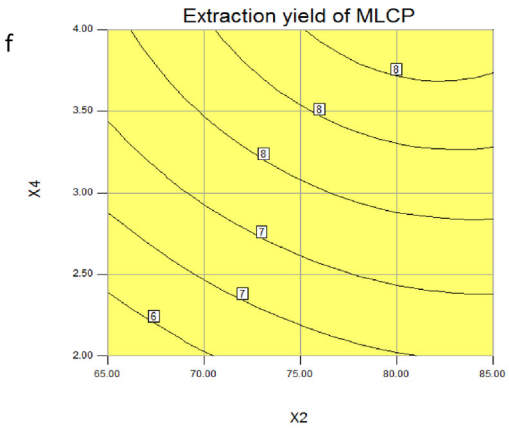
Fixed levels: X_2= 85 °C, X_3= 40, X_5= 18



Fixed levels: X_2= 85 °C, X_3= 40, X_4= 4



Fixed levels: X_1= 5h, X_4= 4, X_5= 18



Fixed levels: X_1= 5h, X_3= 40, X_5= 18

Fig. 4. Contour plots the effect of the extraction time (X_1), extraction temperature (X_2), Particle size of mulberry leaf (mesh) (X_3), number of extraction (X_4), and water to mulberry leaf ratio (X_5) on the extraction yield of MLCP (%).

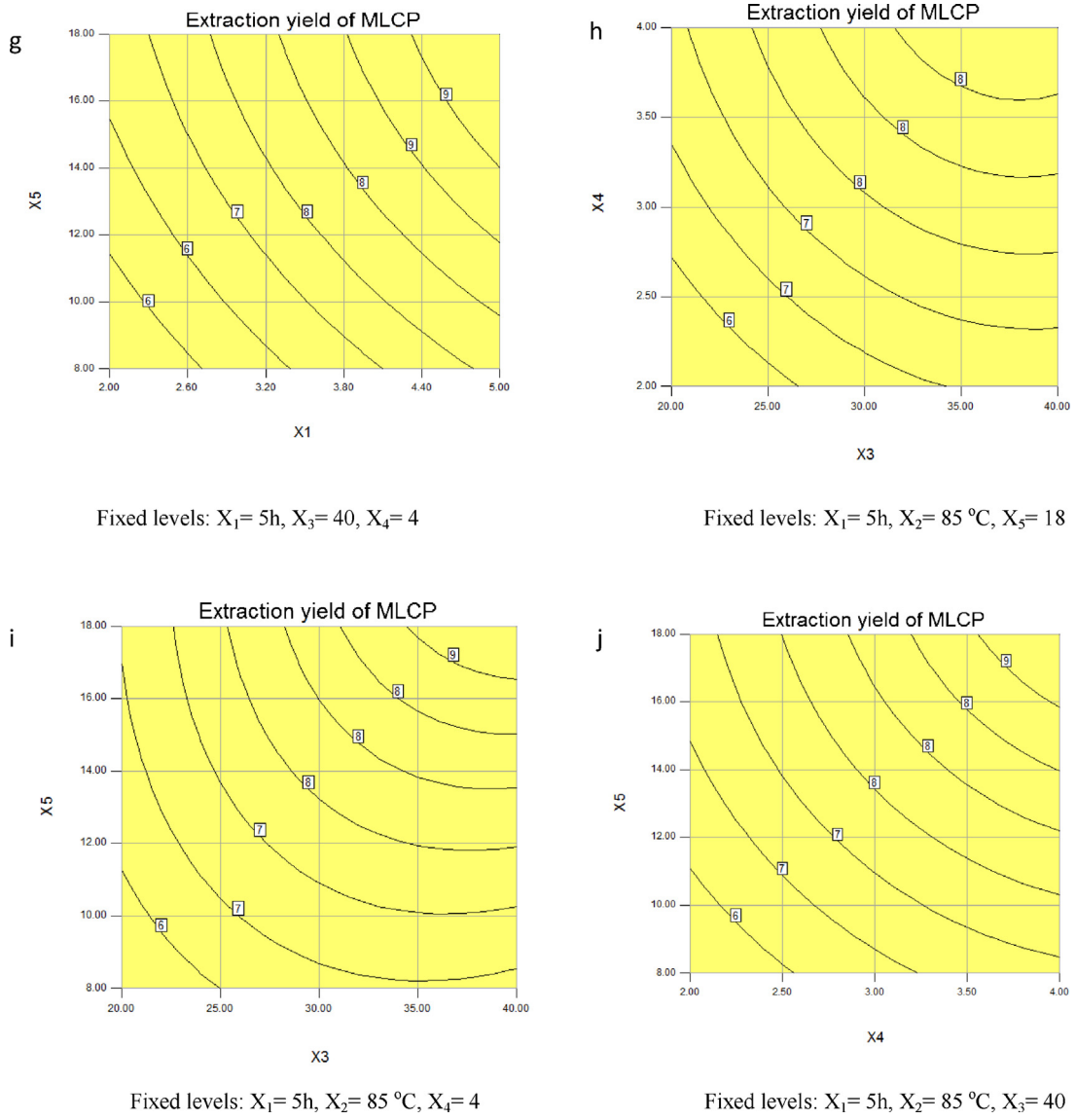


Fig. 4. (*Continued*).

plateau region where the yield was maximized and did not further increase the yield.

Figs. 3d and 4d illustrate the 3D response surface plot and the contour plot at varying extraction time and water to the raw material ratio at fixed extraction temperature 75 °C, particle size of mulberry leaf 30 (mesh) and number of extraction 3. It indicated that the maximum extraction yield of MLCP can be achieved when extraction time and the ratio of water to raw material at the threshold level of 5 h and 18, respectively.

The extraction yield of MLCP affected by different extraction temperature and particle size of mulberry leaf is seen in Figs. 3e and 4e, when the extraction time, number of extraction and water to the mulberry leaf ratio were fixed at 3.5 h, 3 and 13, respectively. It can be seen that the maximum extraction yield of MLCP can be achieved when extraction temperature and particle size of mulberry leaf were 85◦C and 40 (mesh), respectively.

The extraction yield of MLCP affected by different extraction temperatures and numbers of extraction was seen in Figs. 3f and 4f, when other three factors (extraction time, particle size of mulberry leaf and water to mulberry leaf ratio) were fixed at 3.5 h, 30 (mesh) and 13, respectively. It can be seen that the extraction yield of MLCP increased with increase of number of extraction from 1 to 4, then did not further increase with increasing number of extraction, and reached the maximum value when the extraction temperature was at 85 °C, and beyond this level, extraction yield of MLCP did not further increase.

In Figs. 3g and 4g, when the 3D response surface plot and the contour plot were developed for the extraction yield of MLCP with varying extraction temperature and water to the mulberry leaf ratio at the fixed extraction time (3.5 h), particle size of mulberry leaf (30 (mesh)) and number of extraction (3). The yield increased with temperature of extraction. At higher temperatures, the viscosity of mucilage, decreases and makes the slurry less sticky. As a result, the mucilage can be easily released and the extraction yield increases (Koocheki et al., 2008). The extraction yield of MLCP increased with the increasing ratio of water to mulberry leaf from 8 to 18, and reached the maximum value at extraction temperature around 85 °C.

The extraction yield of MLCP affected by different particle size of mulberry leaf and number of extraction is seen in Figs. 3h and 4h, when the extraction time, extraction temperature and water to the mulberry leaf ratio were fixed at 3.5 h, 75 °C and 13. It can be seen that the maximum extraction yield of MLCP can be achieved when

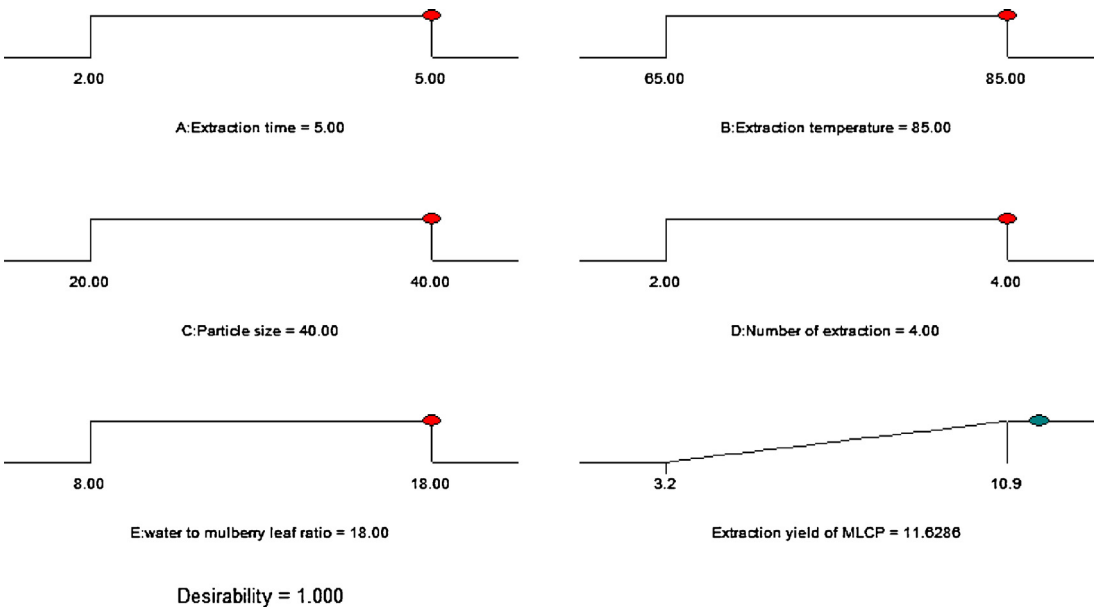


Fig. 5. Schematic representation of the optimum values of factors, response, and the corresponding levels.

the particle size of mulberry leaf and number of extraction were 40 (mesh) and 4, respectively.

The 3D response surface plot and the contour plot based on the independent variable particle size of mulberry leaf and water to the mulberry leaf ratio were shown in Figs. 3i and 4i, while the other three independent variables, extraction time, extraction temperature and number of extraction were kept at 3.5 h, 75 °C and 3, respectively. An increase in the extraction yield of MLCP could be significantly achieved with the increases of water to the mulberry leaf ratio. It is observed that the extraction yield of MLCP was increased with the increasing particle size of mulberry (mesh) from 20 to 40, meaning that a further increase of particle size of mulberry leaf mesh would not increase the extraction yield of MLCP any longer.

In Figs. 3j and 4j, when the 3-D response surface plot and the contour plot were developed for the extraction yield of MLCP with varying number of extraction and water to the mulberry leaf ratio at the fixed extraction time 3.5 h, extraction temperature 75 °C and particle size of mulberry leaf (30 (mesh)). The extraction yield of MLCP increased with the increasing the number of extraction and water to the mulberry leaf ratio, and reached the peak value at of extraction and water to the mulberry leaf ratio, 4 and 18, respectively.

A desirability ramp was developed from optimal points via numerical optimization technique (Fig. 5). According to Fig. 5, and the above single parameter study, it can be concluded that optimal extraction condition of MLCP from the mulberry leaf were extraction time 5 h, extraction temperature 85 °C, particle size of mulberry leaf 40 (mesh), number of extraction 4 and the ratio of water to raw material 18. According to these results, the corresponding predicted response value under the optimum conditions for the yield extraction of MLCP was 11.628%.

3.9. Model adequacy checking

Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface likely give poor or misleading results (Li et al., 2007). The residuals from the least squares fit play an important role in judging model adequacy (Myers & Montgomery, 2002). By constructing a normal probability plot of the residuals, a check was made for the normality assumption, as given in Fig. 6. The normality assumption was satisfied as the residual plot approximated along a straight line. Fig. 7 presents a plot of residuals versus the predicted response. The general impression is that the residuals scatter randomly on the display, suggesting that he variance of the original observation is constant for all values of *Y*. Both of the plots (Figs. 6 and 7) are satisfactory, so we conclude that the empirical model is adequate to describe the MLCP extraction yield by response surface.

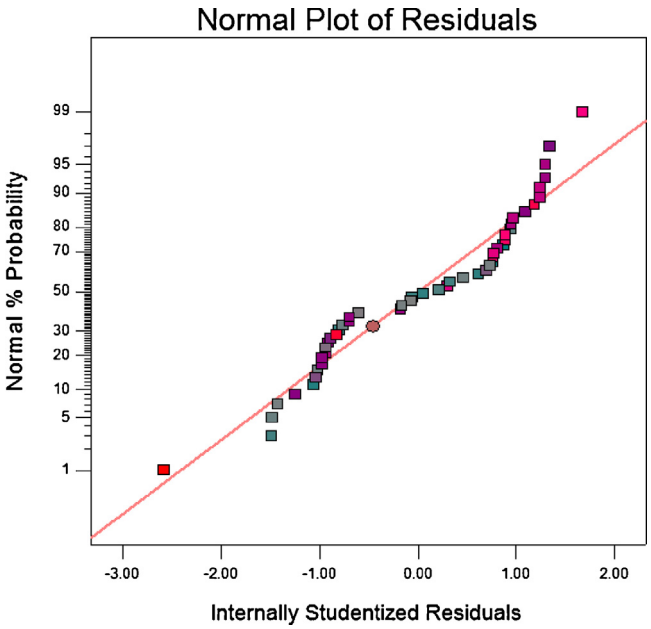


Fig. 6. Normal probability of internally studentized residuals.

3.10. Validation of the model

In order to validate the adequacy of the model equation, four verification experiments were carried out to test the suitability of the optimal extracting variables under the following conditions:

Table 5
Predicted and experimental values of the responses at optimum conditions.

Optimum condition					Extraction yield of MLCP (%)	
Extraction time (h)	Extraction temperature (°C)	Particle size of mulberry leaf (mesh)	Number of extraction	Water to mulberry leaf ratio	Experimental	Predicted
5	85	40	4	18	12.0017 ± 0.42[a]	11.6286 ± 0.19

[a] Mean ± standard deviation ($N = 3$).

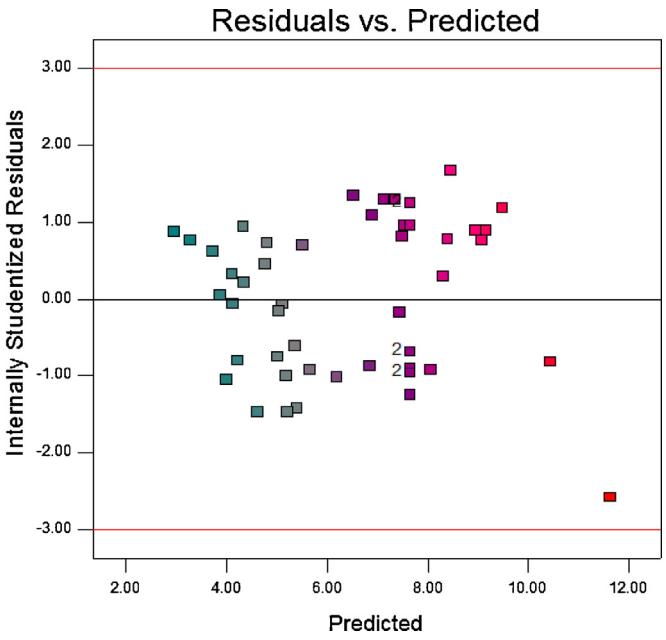


Fig. 7. Plot of internally studentized residuals vs. predicted response.

extraction time 5 h, extraction temperature of 85 °C, particle size of mulberry leaf 40 (mesh), number of extraction 4 and water to the mulberry leaf ratio 18 with a desirability value of 1.000. Table 5 presents the experimental results and theoretical values predicted by the regression equation. A mean value of 12.0017 ± 0.42 ($N = 3$) from real experiments indicated the validation of the RSM model. The results indicated that the experimental values were in coincide with the predicted ones, and also suggested that the regression model was accurate and adequate for the extraction of MLCP.

3.11. Antioxidant activity of MLCP

3.11.1. Scavenging activity of MLCP on hydroxyl radicals

The formation of some diseases, such as cancer, can be directly induced by free radicals, while the radical scavenging activity is one of the important functional properties for bioactive compounds (Zhong et al., 2012). The result of hydroxyl free radical-scavenging ability of the MLCP is shown in Fig. 8 and compared with that of BHT. Fig. 8 shows MLCP exhibited scavenging activity toward hydroxyl radicals in a concentration-dependent manner and the scavenging effect increased based on the concentration of MLCP. As shown in Fig. 8, MLCP scavenging effect on hydroxyl radicals increased by increasing the concentration of MLCP up to 240 mg/L. MLCP was found to have a higher scavenging effect (86%) on hydroxyl radicals than ascorbate acid (73%), suggesting that MLCP has stronger scavenging activity on hydroxyl radicals.

3.11.2. DPPH scavenging activity of MLCP

The DPPH free radical is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant. So it has been widely accepted as a tool for evaluating

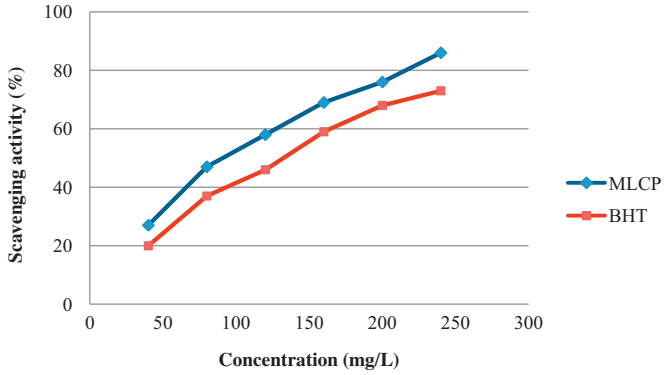


Fig. 8. Inhibition effects of the MLCP and BTH on DPPH radical. Data are mean ± SD for three measurements.

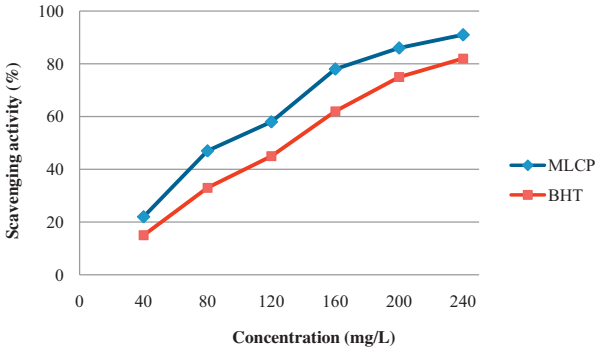


Fig. 9. Hydroxyl radical-scavenging activity of MLCP and BTH at different concentrations. Data are mean ± SD for three measurements.

the free radical scavenging activities of natural compounds (Zhong et al., 2012). On interacting with DPPH, antioxidants transfer either an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character (Naik et al., 2003). As shown in Fig. 9, MLCP was found to have a strong scavenging effect on DPPH at concentrations from 40 to 240 mg/L The scavenging effect of MLCP increased in a dose-dependent manner. Compared with BHT, MSLCP showed a higher degree of free radical-scavenging activity under the same conditions. At 240 mg/L concentration, MLCP was observed to possess significantly ($p < 0.01$) higher (91%) free radical-scavenging activity when compared to BHT (82%), suggesting that MSLCP has stronger DPPH radical-scavenging activity.

4. Conclusion

The extraction conditions have significant effects on the yield, purity and relative viscosity of crude polysaccharides. Using the contour plots in the response surface methodology, the optimum set of the independent variables was obtained graphically in order to obtain the desired level of crude polysaccharides extraction. Optimum conditions for the extraction procedure of crude polysaccharides from mulberry leaf were identified as follows: extraction time 5 h, extraction temperature 85◦C, particle size of mulberry

leaf 40 (mesh), number of extraction 4 and the ratio of water to raw material 18. The results indicated that the experimental values were in coincide with the predicted ones, and also suggested that the regression model was accurate and adequate for the extraction of MLCP.

Acknowledgement

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