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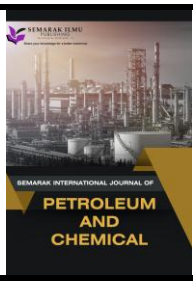

Optimization of Extraction Temperature and Time for Antioxidant Lentinus Edodes Using Response Surface Methodology

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ABSTRACT

The extraction conditions influencing the yield of crude polysaccharide and antioxidant activity in *Lentinus edodes* were studied and optimized using response surface methodology (RSM). Central composite design (CCD) was applied to optimize the extraction time and temperature. The results showed that the data were well-fitted to second-order polynomial models. The independent variables, temperature (A) and time (B), had significant linear, quadratic, and interaction effects on total carbohydrate content (TCC), total flavonoid content (TFC), and DPPH radical-scavenging activity. ANOVA analysis revealed that the models were highly significant ($p < 0.001$) for TCC, TFC, and DPPH radical-scavenging activity. The optimal extraction conditions were 225 minutes at 65°C, producing 55 mg/mL of carbohydrate content (TCC); 420 minutes at 100°C, yielding 53 mg/mL of flavonoid content (TFC); and 225 minutes at 65°C, resulting in 75.5% DPPH radical-scavenging activity. The models were validated, and the experimental values exceeded the predicted optimal values, reaching 66 mg/mL, 67 mg/mL, and 84.6% for TCC, TFC, and DPPH radical-scavenging activity, respectively.

1. Introduction

1.1 Research Background

The use of natural products has gained much attention in providing alternative treatment for various diseases in the las few decades. Based on health issues and dietary supplement, the fungal food sources consumption has been increasing globally [24]. One of the well- known mushrooms, Lentinus edodes which is known as Xianggu in Chinese and Shiitake in Japanese have become world's second largest edible and cultivated medicinal mushroom [12]. Lentinus edodes consumed world-wide as a food choice in commercial market due to its abundant nutritional value and peculiar flavour.

One of the active constituents mainly obtain from Lentinus edodes are crude polysaccharides. It was reported that bioactive compound extracted from Lentinus edodes fruiting bodies exhibited

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antioxidant, anticancer, anticarcinogenic and immunoregulating properties [10] (Lancy et al., 2013 & Bisen *et al.,* 2010). Besides polysaccharides, phenolic compounds which possess antioxidant properties are also contain Lentinus edodes fruiting bodies [3] and [4].

An antioxidant is a biomolecule which can prevent or slowing down the oxidation process of other molecules. The antioxidants activity of Lentinus edodes fruiting bodies extract, mainly polyphenolic compound has been reported widely [14].

1.2 Literature Review

Conventional extraction method normally was carried out through traditional heating method using water with one-factor-at-a-time approach. In conventional extraction method, the variables mainly set up and manipulated manually and as consequences the extraction process will be time consuming, high cost, low level of accuracy as there will be no interaction between variables were considered. Response Surface Methodology (RSM) is an assortment of mathematical and statistical device for developing and optimizing the experimental process by Nicolai and Dekker [11] was applied in this study to overcome the inconvenience situation of extraction process. Extraction process which sometimes referred as sample preparing procedure normally will be the initial crucial step and carried significant effect for high yield of product. Thus, an optimize extraction condition are crucial for the quantitative determination of phenolic compound extracted from the mushroom.

1.2.2 Extraction process

Extraction process is an initial downstream process which are crucial on determining the recovery of wide range of bioactive compounds such as antioxidants and phenolic compounds [17,22]. During extraction processes, various physical and chemical parameters are involved, and the effectiveness of the process are depending on these parameters such as storage time, extraction method, solvent time, pH, extraction temperature, solvent-to-solid ratio, particle size and solvent extraction [18,21]. Solvent extraction process always been chosen due to its simple and easy step of conducting method. However, this extraction method has arisen several drawbacks such as time consuming, laborious, and exhibited low extraction yield after large volume of solvents have been used by Herrero *et al.,* [16].

1.2.3 Response surface methodology (RSM)

A statistical technique known as Response surface methodology (RSM) are widely used nowadays to optimize the process variables where it can demonstrate the optimal conditions of a certain process. The optimal conditions suggested by RSM normally might improve the product yields of a process if it used adequately [20,21]. By using RSM not only the direct effect of independent variables, the combination effect between independent variables also will be considered and could be estimated by Bas and Boyaci [19]. Thus, RSM is a useful which clearly provide a better manipulation and interaction of various independent variables in optimizing processes. This statistical technique also had overcome the disadvantage of the one-factor-at-a-time method which is relatively time-consuming process. In the present study, the extraction process of Lentinus edodes mushroom fruiting bodies powder were extracted using RSM to obtain the optimize extraction conditions for its polysaccharide concentration and antioxidant activities.

2. Methodology

2.1 Materials and Reagents

Fruiting bodies of Lentinus edodes were purchased from local supermarket, Negeri Sembilan, Malaysia. The fruiting bodies were dried in an oven (50°C) until constant weight was obtained. The dried mushrooms were ground into powder form using waring blender and store it in a dry place for further usage.

2.2 Experimental Design

The preliminary data for extraction time and temperature was referred to previous study reported by Yim *et al.,* [2]. Design Expert Version 6.0 was used to create the experimental design by chosen the Central Composite Design (CCD) as a quadratic model. CCD is a non-linear model and used to create various operating conditions where the regression model equations from the suitable limited experiments will be calculated to acceptance of the second order model by Tanyildiz [13].

In this study, two factors namely extraction time and temperature has been manipulated for optimization of total carbohydrate content (TCC), total flavonoid content (TFC) and DPPH radical scavenging activity as antioxidant properties. Extraction temperature (A) time (B) were implied into coded level table as shown in Table 1.

Table 1
Experimental range and coded level of independent variables

Independent variables	Range and levels				
	-α	-1	0	+1	+ α
Temperature, (A) (°C)	30	45	65	80	100
Time, (B) (min)	30	50	180	420	500

The empirical model was developed based on the second-order quadratic model of TCC, DPPH-activity and TFC to analyze the effect of independent variables and its interaction as shown in Eq. (1);

$$y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=2}^{2} X_i^2 + \sum_{i \neq j=1}^{2} \beta_i X_i X_j \quad (1)$$

where y is the predicted response β_0 is the linear coefficient, β_i, β_{ii}, β_{ij} is the interaction coefficient, β_{ij} is the quadratic coefficient, and $X_i X_j$ are the coded levels.

2.3 Extraction process

Approximately 6 g of dried mushroom powder were dissolved in 100 mL of distilled water at a required time and temperature in water bath. After that, the solution was cool down and filtered using filter paper (Whatman No 1.). The filtered solution was then used for carbohydrate and antioxidant analysis (DPPH & TFC analysis).

2.3.1 DPPH-free radical scavenging

The DPPH-free radical scavenging assay was carried out on all 13 mushrooms test samples to determine the radical scavenging activity. According to Burits and Bucar [9], 4 mL of 0.004% of

methanol DPPH solution was added in the test tube containing test samples (1 mL) and keep in the dark area for 30 minutes. The absorbance for each test samples were read at 492 nm. Inhibition of free radical by DPPH in percent (%) was calculated as follows:

Scavenging effect (%) = (A_{sample}/A_{blank}) x 100

where, A_{blank} is represent as the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test samples. IC_{50} (value of 50% inhibition) of every test sample was calculated from the graph plotted inhibition percentage against extract concentrations. Test were carried out in triplicates where ascorbic acid was used as a standard.

2.3.2 Total flavonoid content (TFC)

Total flavonoid content in the filtered solution were determined according to the method by Park *et al.,* [7]. Briefly, 1 mL of mushroom test samples was mixed with 4.3 mL of 80% aqueous ethanol, added with 0.1 mL of 10% aluminum nitrate followed by addition of 0.1 mL of aqueous potassium acetate (1M). The mixtures were kept for 40 minutes (room temperature) prior of absorbance reading at 415 nm. Quercetin was used as a standard compound and the total flavonoid content were calculated and was depicted as mg of Quercetin equivalents per gram of extract.

3. Result

3.1 Fitting the models

In this research, water was selected as the extracting solvent due to its properties which act as universal solvent where possess easy availability and convenient to be used in the extraction process. Water extraction method also classified as most effective solvent for antioxidant extraction as it is non-toxic and suitable for edible sources like mushroom as compared to other solvents [15]. Previous research also had reported that water display powerful activity as extracting solvent as compared to the other solvents such as hexane, ethanol, acetone, and methanol [2].

The actual and predicted values of total carbohydrate content (TCC), DPPH radical-scavenging activity (DPPH) and total flavonoid content (TFC) in Lentinus edodes extract are shown in Table 2. The second-order polynomial equations and the regression coefficients of the models were fitted by the experimental results and analysis of variance (ANOVA) were calculated and exhibited in Table 3, 4 and 5. The lack of fit test is represented the acceptability of the experimental model. Lack of fit for three responses was not significant ($p<0.05$) showing that the model was effectively accepted to the experimental data. Significant p-value with greater coefficient regression model shows a greater outcome on the response [23].

Table 2
The experimental design of RSM and CCD for three responses, total carbohydrate content (TCC), DPPH radical-scavenging activity (DPPH) and total flavonoid content (TFC)

Run	Independent variables		Responses					
	Temperature (^{0}C)	Time (minutes)	TCC (mg/mL)		DPPH (%)		TFC (mg/mL)	
			Actual Time	Predicted value	Actual Time	Predicted value	Actual Time	Predicted value
1	65	50	0.21	0.21	55.6	62.1	0.28	0.27
2	30	420	0.68	0.70	63.9	71.2	0.60	0.60
3	100	225	0.69	0.69	84.6	84.6	0.67	0.62
4	65	225	0.62	0.60	87.2	88.8	0.66	0.63
5	65	225	0.33	0.38	67.3	69.0	0.32	0.41
6	30	30	0.67	0.69	85.4	81.9	0.61	0.66
7	65	225	0.43	0.42	72.2	62.9	0.44	0.41
8	100	30	0.61	0.66	85.1	87.9	0.63	0.66
9	65	500	0.52	0.54	75.0	75.5	0.57	0.53
10	30	225	0.54	0.54	78.3	75.5	0.52	0.53
11	65	225	0.55	0.54	72.3	75.5	0.56	0.53
12	80	225	0.56	0.54	74.5	75.5	0.56	0.53
13	100	420	0.58	0.54	79.6	75.5	0.52	0.53

3.1.1 *Response surface analysis of TCC*

The ANOVA analysis of response surface model for total carbohydrate content (TCC) is shown in Table 3. It was clearly shown that model was highly significant with p<0.0001. The R^2 = 0.9535 obtained indicating that 95% of the variability could be explained by the model and only 5% of the variations could not be explained. The adjusted determination coefficient (Adj. R^2 = 0.9379) was within reasonable agreement with the predicted R^2 of 0.8705 and it also implied to the significance of the model. Extraction temperature and time had both significant linear effect with p<0.0001. Results also demonstrated that the interaction between temperature and time variables had strongest effect on the TCC content (p<0.0001). The model in terms of actual variables of carbohydrate content was regressed by mainly in Eq. (2) as follows:

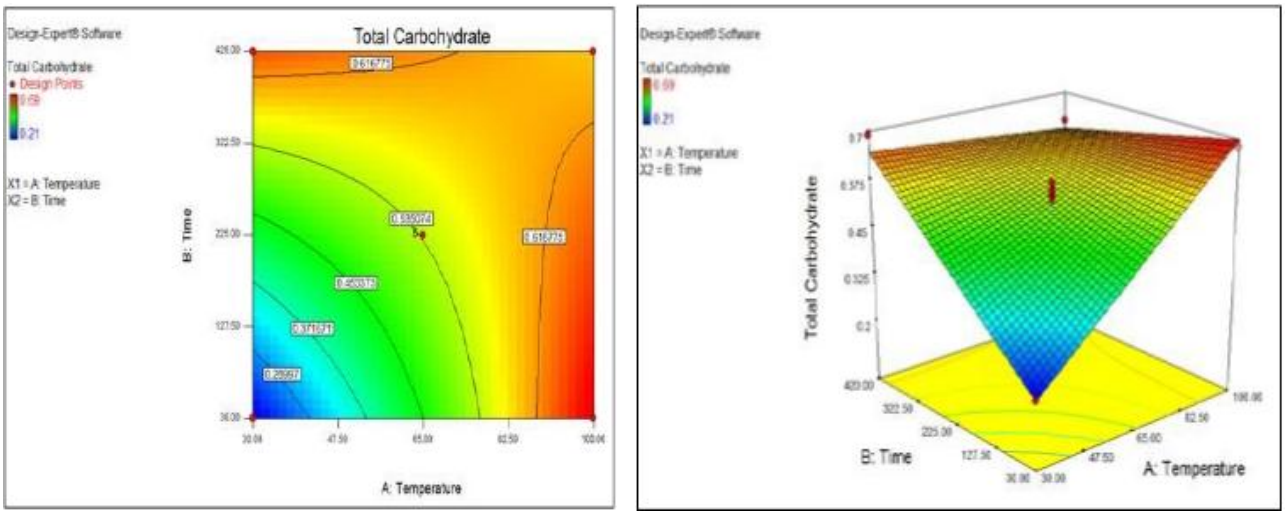
$$Total\ carbohydrate = -0.053364 + 7.59638E-003 \times Temperature + 1.7181E-003 \times Time - 1.97802E-005 \times Temperature \times Time \quad (2)$$

It is known that extraction plays a big role as the first step of sample processing procedure in mushroom polysaccharide extraction. For water soluble polysaccharides, hot water extraction was normally applied. Although various types of extraction method can be applied depending on the structure and water solubility of polysaccharides, however the suitable temperature range determined the effect to rupture the hard cell wall from the outer layer to the inner layer with weak-to-strong-extraction conditions. In this study, it was clearly showed that the increases of the temperature had significantly increase the TCC content and the optimize temperature obtained was 65°C with 225 minutes of extraction time with 54 mg/mL of TCC as shown in Figure 1.

Table 3
Analysis of cariance (ANOVA) for the experimental results of the CCD quadratic model for TCC

Source	Sum of squares	DF	Mean of square	F value	Prob>F	
Model	0.23	5	0.076	61.46	<0.0001	*Significant*
A: Temperature	0.097	1	0.097	78.86	<0.0001	*Significant*
B: Time	0.57	1	0.057	46.25	<0.0001	*Significant*
AB	0.073	1	0.073	59.27	<0.0001	*Significant*
Residual	0.011	9				
Lack of fit	9.069E-003	5	3.63	3.63	0.1179	*Not Significant*
Pure Error	2.00E-003	4				
Cor Total	0.24	12				

Std. Deviation = 0.035 R^2 = 0.9535
Mean = 0.54 Adj R^2 = 0.9379
Predicted R^2 = 0.8705 Adeq. Precision =25.199



(a) (b)

Fig. 1. Contour plot (a) and response surface plot (b) of total carbohydrate content

3.1.2 Response surface analysis of DPPH scavenging ability

Antioxidants is a compound responsible for protecting cells in the body from damaging caused by free radicals. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable organic molecule at room temperature, is reduced in the presence of an antioxidant molecule, decolorizes color of DPPH-methanol solution [5]. The antioxidant properties of a crude extract can be measured by the percentage of scavenging activity through the DPPH assay. The highest percentage of the DPPH scavenging activity indicates the highest ability of the crude extract from the mushrooms to scavenge the DPPH radical molecules. The DPPH assay was performed on 13 mushrooms extracts (Table 4) to determine the radical scavenging ability in each extraction condition. The ability of the radical scavenging activity of the extracts were determined by the capability to decrease the intensity of purple colour of DPPH-methanol solution [8].

The analysis of variance (ANOVA) for DPPH scavenging ability responses were shown in Table 4. The quadratic model for DPPH scavenging activity showed that the model was very significant p<0.001 with R2 = 0.7477. This indicates that 74.77% of the variability in the response could be explained by the model and around 25% were not explained by the model. The adjusted

determination coefficient value (Adj. R2 = 0.6972) was within the rationale agreement. The extraction time (B) variables have shown significant effect ($p<0.001$) meanwhile temperature (A) had showed not significant effect towards the DPPH scavenging activity. The model in terms of actual variables and relationship between the DPPH scavenging activity and the extraction conditions tested are presented in Eq. (3) as below:

$$DPPH\ activity = 75.46 + 4.56 \times Temperature + 8.82 \times Time \quad (3)$$

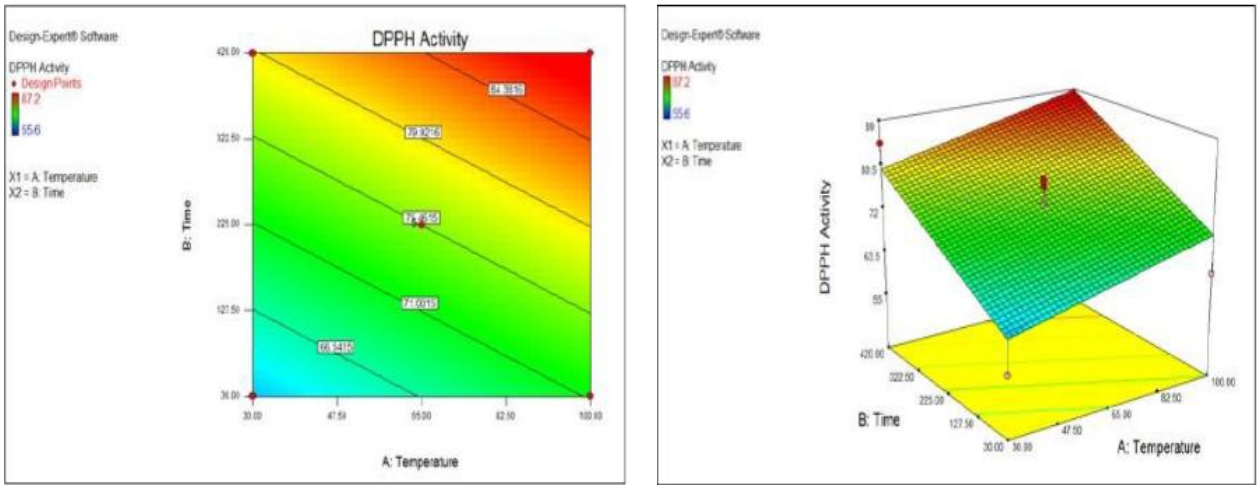
In general, high DPPH scavenging activity is correlate with the increase of extraction temperature [18]. Conversely, this study exhibited that the increased of extraction temperature shows a negative linear effect of the DPPH scavenging ability. Increase of extraction temperature will enhance the solubility of the solute, however extraction temperature above 50°C will affect the stability of the phenolic compounds based on Yim *et al.,* [2] consequently decrease the DPPH radical scavenging activity. The decrease of the scavenging ability with the increase of extraction temperature in the present study might possible due to the decomposition of the antioxidative compounds that are heat sensitive.

The present results showed a similarity with the previous research which showed a negative linear and quadratic effect of extraction temperature in the optimization of extraction process of *Schizophyllum commune* [23]. These results suggested that the maximum DPPH scavenging activity only will be achieved at a certain temperature and the DPPH scavenging activity is decreased as the temperature increased. The maximal DPPH scavenging activity obtained was 75.46% with optimum extraction time of 225 minutes and temperature of 65°C as shown in Figure 2.

Table 4
The analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for DPPH radical scavenging activity

Source	Sum of squares	DF	Mean of square	F value	Prob>F	
Model	788.55	2	394.28	14.81	0.0010	Significant
A: Temperature	166.5	1	166.51	6.26	0.0314	Not Significant
B: Time	622.05	1	622.05	23.37	0.0007	Significant
Residual	266.14	10	26.61			
Lack of fit	230.97	6	38.49	4.38	0.0872	Not Significant
Pure Error	35.17	4	8.79			
Cor Total	1054.69	12				
Model	788.55	2	394.28	14.81	0.0010	Significant

Std. Deviation = 5.16 R2 = 0.7477
Mean = 75.46 Adj R2 = 0.6972
Predicted R2 = 0.4786 Adeq. Precision =10.798



(a) (b)

Fig. 2. Contour plot (a) and response surface plot (b) of DPPH activity

3.1.3 Response surface analysis of TFC

The total flavonoid content analyzed by ANOVA demonstrated that the model was also significant ($p<0.0001$) with regression coefficient of R2 = 0.8903 indicating that 89.03 of changeability in the response able to analyze by the model and less than 20% could not be analyzed. The predicted value of R2 was 0.6976 and the adjusted determination coefficient value Adj R2 = 0.8538 implied the significance of the model. It was found that both variables tested (Temperature, A and Time, B) were significant for linear effect with $p<0.001$ meanwhile the interaction between these two variables were only significant at $p<0.01$. Results indicated that as the temperature and time of extraction increased, the TFC was increased. Comparatively, the TFC obtained in this study was in similar range with previous study which applied 100°C and 380 minutes of extraction conditions where the TFC were varied considerable from 0.24 to 0.59 mg/g by Ahmad *et al.,* [6]. This results also similar with previous research which suggested that the extraction time play a major effect on the entire flavonoid content value by Babatunde and Oseni [1]. The model in terms of actual variables and relationship between the total flavonoid in Eq. (4);

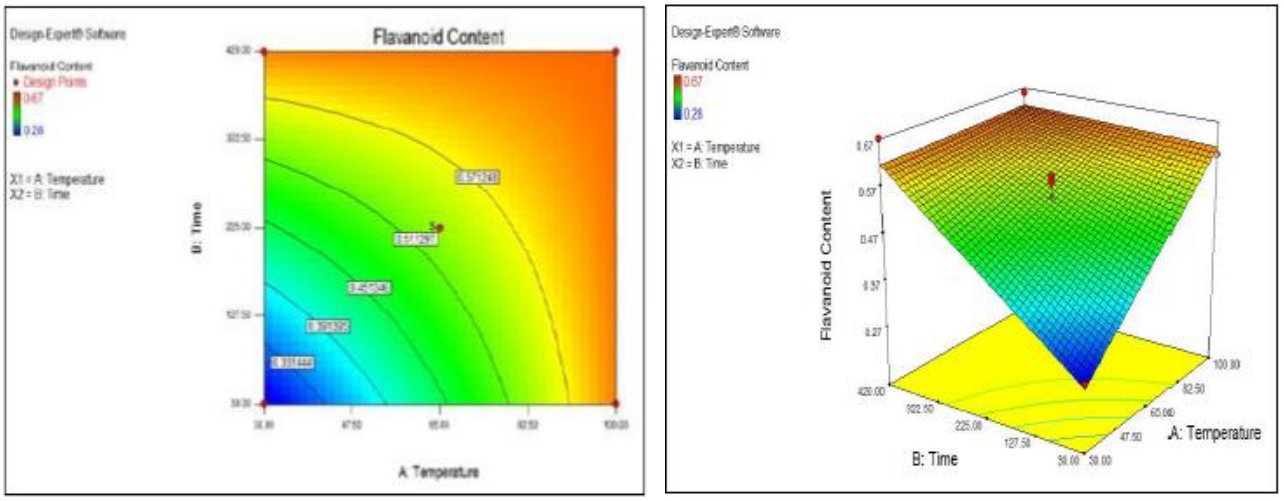
$$Total\ Flavonoid\ Content = 0.53 + 0.09 \times Temperature + 0.09 \times Time - 0.082 \times Temperature \times Time \quad (4)$$

The maximum TFC obtained from the crude extract was 0.67 mg/mL at extraction conditions of 100°C with 420 minutes of extraction time. Figures 3(a) and (b) shows the contour and 3D plot of the linear and quadratic effect of temperature (X1) and time (X2) on the TFC. While, Table 5 shows the analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for TFC.

Table 5
The analysis of variance (ANOVA) for the experimental results of the CCD quadratic model for TFC

Source	Sum of squares	DF	Mean of square	F value	Prob>F	
Model	0.16	3	0.052	24.35	0.0001	Significant
A: Temperature	0.065	1	0.065	30.24	0.0004	Significant
B: Time	0.065	1	0.065	30.12	0.0004	Significant
AB	0.027	1	0.027	12.70	0.0061	Significant
Residual	0.019	9	2.144-003			
Lack of fit	0.017	5	3.395E-003	5.85	0.0558	Not Significant
Pure Error	2.320E-003	4	5.800E-			
Cor Total	0.18	12				

Std. Deviation = 0.046 R^2 = 0.8903
Mean = 0.53 Adj R^2 = 0.8538
Predicted R^2 = 0.6976 Adeq. Precision =15.171



(a) (b)

Fig. 3. Contour plot (a) and response surface plot (b) of total Flavonoid content

3.2 Verification of Predictive Model

The statistical model was verified on TCC, DPPH and TFC by applying the optimized conditions suggested by RSM within the experimental range. Table 6 shows that the verification of the model equation for the predicted and experimental value. The experimental values of TCC, DPPH and TFC were 0.55 g/L, 74.46% and 0.56 g/L respectively near to the predicted values. The excellent correlation between predicted and measured values justifies the validity of the response model Eq. (2), Eq. (3) and Eq. (4).

Table 6
Verification of predicted extraction parameters

Responses	Temperature (°C)	Time (min)	Predicted value	Experimental value	% Differences
Total carbohydrate content (TCC)	65	225	0.54	0.55	0.01
DPPH (%)	65	225	74.46	76	2.5
Total flavonoid Content (TFC)	100	420	0.53	0.56	0.2

4. Conclusions

In conclusion, temperature and time variables revealed the significant effects on the three responses (TCC, DPPH and TFC). The optimized extraction conditions for TCC and DPPH were 65°C in 225 minutes and 100°C with 420 minutes of extraction time for TFC. These optimum conditions produced 0.55 g/L of TCC, 74.46% of DPPH radical scavenging activity and 0.56 g/L of TFC.

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