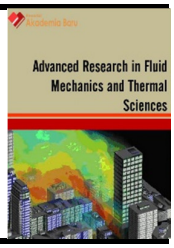
Penerbit Akademia Baru

Journal of Advanced Research in Fluid Mechanics and Thermal Sciences

Journal homepage: www.akademiabaru.com/arfmts.html
ISSN: 2289-7879



Response Surface Optimization of High Antioxidative Extraction from *Curcuma Zedoaria* Leaves

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ARTICLE INFO

Article history:
Received 2 December 2017
Received in revised form 30 January 2018
Accepted 10 February 2018
Available online 10 March 2018

Keywords:
Curcuma zedoaria leaves, antioxidant activity, RSM, DPPH, FRAP, BCB

ABSTRACT

Response surface methodology (RSM) has been fully utilized in this study to model and optimize the extraction conditions for maximum antioxidant compounds activity from *Curcuma zedoaria* leaves. This work was carried out based on three factor/5 level of central composite rotatable design (CCRD) and three independent variables, namely extraction temperature (60-80^{o}C), processing time (80-120min) and solvent concentration (70-90 v/v %). The experimental data was subjected to total antioxidant activity measured by the 1, 1-diphenyl-2-picrylhdrazyl (DPPH)-radical scavenging, β-carotene bleaching (BCB) inhibition as well as ferric ion reducing antioxidant power (FRAP). By using the desirability functions, the optimal conditions were found to be at temperature 75 ^{o}C, 92 min and (90:10 v/v%) ethanol concentration for the highest total antioxidants values for DPPH, BCB and FRAP were 85.76%, 81.35 % and 5.08 mM Fe^{2+} g/DW respectively, which closely agreed with the predicted values of 87.59%, 82.29%, 5.25 mM Fe^{2+} g/DW respectively. Thus, indicating the suitability of the model employed in RSM to optimizing the extraction conditions.

1. Introduction

Antioxidants are widely recognized as an agent in promoting health and lowering the risk of getting serious diseases [1]. The highly reactive compounds help to delay or inhibit the initiation and propagation of oxidizing chain reactions involving uncontrollable metabolic processes from reactive oxygen species (ROS) and free radical species [2]. Practically, radical damage can be treated by both synthetic and natural antioxidants. However, the use of artificial antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are suspected to increase the risk of cancer and liver damage in humans [3]. Therefore, the research for new bio-efficient

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antioxidants from natural plant sources has gained greater attention because natural antioxidants tend to be safer and possess anti-viral, anti-inflammatory, and anti-cancer properties [2,4].

Fortunately, Malaysia is one of world's richest tropical rainforest that can provide useful primary medicinal plants and herbs. In recent times, Zingiberaceae from the largest family of Zingiberales is getting recognition from scientists all over the world for their potential benefit to living organisms [5]. One of the genus of Zingiberaceae families that has higher potential is *Curcuma zedoaria*, which is popularly known as white turmeric, zedoaria or gajutsu [6]. Curcuma zedoaria is widely cultivated in East-Asian countries including China, Malaysia, Indonesia, India, Vietnam and Bangladesh [7]. Recently, it has been investigated that numerous isolated functional active compounds were found from zedoaria plant exhibits curcumenol, dihydrocurdione, curcumin, dihydrocurcumin, tetrahydromethoxycurcumin, tetrahydrobismethoxycurcumin which can act as analgesic properties, curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcumenol as cytotoxic activities against various cancer cell lines, furanodiene, germacrone, curdione, neocurdione, curcumenol, isocurcumenol, aerugidiol, zedoarondiol, curcumenone and curcumin as anti-hepatotoxicity along with curzenone and dehydrocurdione as anti-inflammatory agents [8]. Therefore, it is indisputable that this plant has been used since ancient times as traditional medicines to treat various health problems such as treatment for dropsy and leprosy, stomachic, vomiting, menstrual disorders, dyspepsia, cancer, flatulence, cough, cold and fever [6,9].

In each plant, the bio-actives compounds are responsible for its anti-inflammatory, antioxidative, analgesic, antimicrobial activities and etc. Therefore, the discovery of effective extraction process in plant extracts is a very crucial stage in determining extract efficacy since each plant has its unique properties in terms of the value of functional active components. According to Aybastier *et al*., [10] many factors contribute to the efficiency of extractions such as the type of solvent, the concentration of solvent, temperature, time, pH and solid-liquid ratios [10]. Presently, scientists are exploring a more comprehensive statistical mathematical tools to develop intensive and user friendly methods to analyze experimental results. Response surface methodology (RSM) is one of the powerful mathematical techniques widely used in many industries for technological operations which introduce excellent optimization in experimental conditions. RSM is utilized to maximize or minimize various independent variables by evaluating the effects of multiple factors and their interactions on one or more response variables simultaneously. Besides, RSM not only serves as a visual aid to have a clearer picture about the effects of various factors on extraction but also helps to locate the region where the extraction is optimized. Furthermore, according to Vuong *et al*., [11] this technique is time and cost effective because it simultaneously evaluates the key experimental parameters and studied response.

Despite numerous studies on Curcuma zedoaria such as rhizomes and roots which cater enormous functional components, however very limited studies have been conducted on zedoaria leaves for the therapeutic value of antioxidants compounds. Chanda and Nagani [2] reported that generally leaves are selected for their potential in phytochemical studies especially on evaluation of total antioxidants activity. Therefore this research attempted an RSM technique using central composite rotatable design (CCRD) to obtain the optimum extraction conditions such as solvent concentration, temperature and time on the highest total anti-oxidants of free radical scavenging activity (DPPH), β-carotene bleaching (BCB) and ferric reducing antioxidant power assay (FRAP) from the Curcuma zedoaria leaves.

2. Materials and Methods

2.1 Materials

Curcuma zedoaria leaves were obtained from a supplier from Kedah, Malaysia. 1-diphenyl-2-picrylhydrazyl (DPPH) radical and *β*-carotene were purchased from Sigma-Aldrich Co., Ltd. (Sigma Chemical Co., St. Louis, MO, USA). Ethanol, linoleic acid, chloroform, sodium acetate trihydrate, acetic acid, TPTZ [2,4,6-tri (2-pyridyl)-s-triazine], ferric chloride. All other chemical reagents used in this study were of analytical grade and double distilled water was used throughout the experiment.

2.2 Plant Extraction

The air dried leaves of *Curcuma zedoaria* plant were cut into pieces and ground into powder form using mechanical blender. About 0.5g of powdered leaves were exactly weighed into a 150mL round bottomed conical flask and mixed with ethanol. The extraction process was performed using a reflux system equipped with a temperature controller and digital timer. The extract was then filtered through normal filtration using Whatman filter paper to collect the supernatant and vacuum-dried in rotary evaporator, at 40^{o}C until the excess solvent was completely removed.

2.3 Determination of Antioxidant Activity (DPPH)

The DPPH free radical scavenging activity method was carried out according to the method described by Blois [12] with some modifications. Briefly, 10 mg of the dried extracts were weighed and dissolved in 10 mL ethanol in a volumetric flask (1000mg/L). 10mg of 1-diphenyl-2-picrylhydrazyl (DPPH) radical was dissolved in ethanol and made up to 100 mL in a volumetric flask (0.1 mM). 50uL of the extract sample was mixed with 150uL ethanolic solution of DPPH in 96-well plate microliter plate. The mixture was incubated in the dark for 30 minutes and the absorbance values of the samples were measured at 515 nm using a UV-VIS microplate reader. A sample control was prepared by omitting sample extract from DPPH working solutions. All the analyses were performed in triplicate The DPPH scavenging activities of the extracts were calculated using the following equation

$$\text{DPPH scavenging activity (\%)} = \frac{(A0-A1)}{A0} X\ 100 \quad (1)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.4 Determination of β-carotene bleaching test (BCB)

The *β*-carotene bleaching method was evaluated based on the reduction of the yellow-colour of *β*-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion [13]. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants. The method used for determination of total antioxidant potential through the *β*-carotene/linoleic acid system was adopted from Lai and Lim [14] with some modifications to fit the present study. For the reactive mixture, 0.2 mL of linoleic acid, 2 mL of tween 20 and 2mg of *β*-carotene were placed into a flask containing 100uL of chloroform. Then, 50 mL of distilled water was added slowly to the mixture with vigorous agitation to form an emulsion. In each tube, 200uL of emulsion mixture was added to 20uL of the sample and the tubes were placed in a microwave oven at 50^{o}C for 180 mins to catalyze the oxidation reaction and discoloration of the *β*-carotene. For the control tube, the

sample was replaced with the extraction solvent. The absorbance reading was measured at 20 min intervals for 180 min at 450 nm. All the analyses were performed in triplicate. The antioxidant activity was calculated in terms of the successful bleaching of *β*-carotene using the formula

$$\text{Degradation rate (DR)} = \frac{\ln(\frac{a}{b})}{t} \quad (2)$$

where In is natural log, *a* is the initial absorbance (470 nm) at time 0, *b* is the absorbance at 180 mins and t is the time at 180 mins.

$$\text{Antioxidant activity, AA (\%)} = \frac{DR\ control - DR\ sample}{Dr\ control} X\ 100 \quad (3)$$

2.5 Determination Ferric Ion Reducing Antioxidant Power (FRAP)

The FRAP was assayed according to the method developed by Benzie and Strain [15] with minor modifications. The FRAP solution includes acetate buffer (300mM, pH 3.6), a 10mM TPTZ solution in 40mM of HCl and 20mM $FeCl_3.6H_2O$ solution. A working FRAP solution was freshly prepared by mixing 100 mL acetate buffer, 10 mL TPTZ solution and 10 mL $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 at the time of use. A sample blank was prepared by excluding sample extract from the FRAP solution and measured by reading the absorbance at 593 nm (0min) using a spectrophotometer UV-VIS microplate reader. Subsequently, each extracts of 60uL was mixed with 1.8 mL FRAP solution and second reading at 593 nm was performed after 4min. The FRAP value of the samples were determined by subtracting the final reading of the FRAP solution plus sample with the initial blank. The standard curve was prepared using ferrous sulphate solution (1mM) at different concentrations ranging from 0.1mM to 1.0mM. The activity was then expressed as mM Fe^{2+}/g DW. Analysis was done in triplicate for standard and each extract.

2.6 Experimental Design

The optimization of the extraction conditions from the *Curcuma zedoaria* leaves were established by using response surface methodology (RSM) where a Design Expert software Version 7.0.0, (Stat ease Inc., Minneapolis, USA) was used in this study. The experimental plan was based on three factor/5 level design referred to as rotatable central composite design (CCRD). In this study, the selection of CCRD as the experimental design because it is more precise for estimating factor effects [16]. Hence, the interaction effect between factors can be evaluated and optimized in the full factor space.

The design consisted of 20 experimental runs, 8 factorial points, 6 axial points and 6 replicates of center points at 5 levels of each variable. The center points were utilized to define the experimental error and the reproducibility of the data. The independent variables in this study were extraction temperature (*A*: 60-80^{o}C), time (*B*: 80-120 mins) and ethanol concentrations (*C*: 70-90 %v/v ethanol/water). The five levels of values for the independent variables were explicit of their coded and uncoded forms in Table 1. The value of independent variables were expressed in their coded as -1, 0, +1 interval shows the low, center, and high level of each variable, respectively. The axial points α (±1.682) were placed at (±α, 0, 0), (0, ±α, 0), and (0, 0 ±α) where α is the axial point distance from center and makes the design rotatable.

Table 1
Independent test variables and their coded and uncoded value used for CCRD matrix

Variables	Units	Coded and uncoded level of variables				
		-α	-1	0	+1	+ α
Temperature	°C	53	60	70	80	87
Time	Min	66	80	100	120	133
Solvent ratio Ethanol : Water	v/v%	63	70	80	90	97

The experimental data were analyzed by response surface regression analysis procedure and the results were statistically analyzed by the corresponding analyses of variances [16]. An appropriate polynomial model was chosen based on the statistical significance of the model ($p<0.05$) and the lack of fit value of the model provided by Design Expert software was not significant [16].

2.7 Optimization and Statistical Analysis

The optimization of the interest study was carried out to determine the optimum condition of the independent variables when predicting the variation of preparation conditions in the Design Expert software. The desired goal for each variable was chosen. The optimal extraction conditions of ethanolic *Curcuma zedoaria* leaves were chosen based on the condition of attaining highest antioxidant capacity for each assay. The responses were then analyzed jointly by conferring to them either the same importance or weight for simultaneous optimization of the multiple responses [16]. The Design Expert software overlaying all the responses to obtained the optimal condition. Moreover, the optimal condition that depended on the independent variables was also obtained using the predicted equations determined by RSM. By employing the polynomial regression equation, the response surface behavior was examined for the response function (Y). The generalized response surface model is shown below (Eq. 4)

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j>i}^{k} \beta_{ij} X_i X_j + e \quad (4)$$

Where Y represents the response variables to be modeled; β_o is a constant, β_i, β_{ii} and β_{ij} are the linear, quadratic and cross-product coefficients, respectively. X_i and X_j are the levels of the independent variables. k is the number of variables and e is the random error of the model.

The statistical significance of the term in the regression equations and best fitted model of response can be achieved by highlighting these statistical parameters, including the adjusted multiple correlation coefficients (*adjusted* R^2), multiple correlation coefficient (R^2), coefficient variation (C.V %), lack of fit, regression *F-value* and regression *P-value* by using analysis of variance (ANOVA). This statistical approach was used to summarize the results obtained under all experimental conditions with confidence interval of 95%, set to test the significant effect of the factors and their interaction. The fitted polynomial regression equation was expressed in the form of three dimensional (3-D) surface plots in order to illustrate the relationship between responses and the experimental variables used.

3. Results and Discussion

3.1 Fitting the Response Surface Models

RSM uses mathematical and statistical method to describe the behaviour of the data by obtaining highest percentage of responses and to achieve optimal conditions. Based on the CCRD matrix, the experimental design and corresponding three response variables are presented in Table 2. In the present study, according to the sequential model sum of squares, the highest order polynomials were utilized to select the models where the additional coefficients estimates were significant and the models were not aliased. Hence, for all three independent variables and responses, a quadratic polynomial model was selected and fitted well as suggested by the software.

The final empirical regression model of the relationship between responses and the three tested variables for antioxidant DPPH radical scavenging, β-carotene assay (BCB), and FRAP reducing assay were obtained by the following equations (Eq. (5)-(7)):

$$DPPH\ radical\ scavenging = +84.06+2.94A-0.82B+0.70C-9.60AB+0.73AC+0.70BC-1.42A^2 -2.72B^2-0.39C^2 \quad (5)$$

$$Beta\ carotene\ assay = +75.80+2.41A+1.28B+8.93C+0.065AB-4.25AC-8.27BC-2.53A^2- 3.34B^2-2.87C^2 \quad (6)$$

$$FRAP\ reducing\ assay = +5.30-0.047A-0.066B+0.17C+0.22AB-0.11AC+0.21BC-0.052A^2+0.022B^2-0.018C^2 \quad (7)$$

where *A* is the temperature, *B* is the time and *C* is the solvent. A negative sign in each equation represents an antagonistic effect of the variables and a positive sign represents a synergistic effect of the variables

The RSM model coefficients were validated by analysis of variance (ANOVA) of the response variables for the quadratic polynomial model summarized in Table 3. The ANOVA results were calculated based on 95% confidence intervals and this analysis was crucial to determine the best fitted quadratic model for three independent variables. Regression model was evaluated by using *F* statistics and lack of fit test. The results, showed that the model is highly significant when the computed *F*-value is greater than the tabulated *F*-value and a probability value is low *(P<0.0001)* indicating that the individual terms in each response model was significant on the interaction effect.

The performance of the models was also checked by calculating the determination coefficients R^2, adjusted R^2, regression (*P-value*), regression (*F-value*), lack of fit (*P-value*), coefficient variation (C.V%) and probability values related to the effect of the three independent variables. Based on the results, higher coefficients of determination for DPPH, BCB and FRAP were (R^2 =0.9989), (R^2 =0.9988) and (R^2 =0.9927) respectively, which confirmed that the quadratic polynomial models are highly significant and acceptable to interpret actual relationship between the responses and variables tested. Furthermore, the calculated adjusted R^2 values for studied responses variables were higher than 0.80, hence there is a close agreement between the experimental values and the theoretical values predicted by the proposed models. The coefficients of variations (CV) for DPPH, β-carotene assay and FRAP reducing assay were 0.41, 0.72 and 0.60 respectively, which indicates that a relatively lower value of CV showed a better reliability of the response model and high degree of precision. It was observed that the lack of fit gave no indication of significance ($p<0.05$) for all the models tested, thus proving the satisfactory fitness of the response surface model were

within the chosen range and significant ($p<0.05$) to the factors effect.

Based on ANOVA factors, any terms from quadratic polynomial coefficients model, large *F-values* and a small *P-values* indicated a more significant effect on the respective response variables. The 3-D contour plots of the fitted polynomial regression equations were generated by the software to better visualize the interaction effect of independent variables on responses.

Table 2
The experimental data obtained for the three responses based on CCRD matrix

Run no	Type	Temp (*A*)	Time (*B*)	Solvent ratio (*C*)	DPPH %	BCB %	FRAP assay mM Fe^{2+} g/DW
1	Fact	80.0	80.0	90.0	93.70	80.97	4.90
2	Fact	60.0	120.0	70.0	84.82	61.03	4.53
3	Center	70.0	100.0	80.0	84.40	74.90	5.34
4	Fact	80.0	120.0	70.0	70.40	74.10	5.08
5	Axial	53.18	100.0	80.0	75.10	64.08	5.25
6	Center	70.0	100.0	80.0	84.20	76.25	5.33
7	Axial	70.0	66.36	80.0	77.93	64.30	5.49
8	Axial	86.82	100.0	80.0	84.79	73.26	5.05
9	Axial	70.0	100.0	96.82	84.20	82.40	5.52
10	Fact	80.0	80.0	70.0	92.10	54.70	5.20
11	Axial	70.0	100.0	63.18	81.50	53.02	4.97
12	Fact	80.0	120.0	90.0	74.20	67.13	5.66
13	Fact	60.0	80.0	70.0	68.70	42.04	5.48
14	Fact	60.0	80.0	90.0	66.79	85.15	5.65
15	Center	70.0	100.0	80.0	83.70	75.89	5.29
16	Center	70.0	100.0	80.0	84.29	76.15	5.25
17	Fact	60.0	120.0	90.0	86.30	71.20	5.49
18	Axial	70.0	133.64	80.0	74.59	68.43	5.23
19	Center	70.0	100.0	80.0	83.95	75.80	5.27
20	Center	70.0	100.0	80.0	83.90	75.79	5.30

3.2 Response Surface Analysis

The analysis of the response surfaces obtained for the dependent variables allowed selecting the best extraction conditions for obtaining extracts with high antioxidant activity. This methodology has demonstrated to be a powerful tool for optimizing the experimental conditions [17]. Temperature, time and ethanol concentration are the main factors that affect the extraction condition of the highest percentage of the total antioxidants activity (DPPH), *β*-carotene assay and FRAP reducing assay. This section discusses how these conditions work on natural antioxidants extraction. Three dimensional model graphs were plotted as shown in the respective figures. The response surface plots of the model were done by varying two variables, within experimental range under investigation and holding the other variables at its central level (0 levels).

Table 3
Analysis of variance (ANOVA) for the model

	DPPH			BCB			FRAP			
Source	Mean squares	F value	P-value	Mean squares	F value	P-value	Mean squares	F value	P-value	
model	111.68	1016.83	<0.0001	243.89	955.97	<0.0001	0.15	151.02		*significant
A-Temperature	117.66	1071.35	<0.0001	85.79	311.02	<0.0001	0.031	30.46	0.0003	
B-Time	9.16	83.44	<0.0001	19.30	88.36	<0.0001	0.060	60.00	<0.0001	
C-Solvent ratio	6.62	60.31	<0.0001	1089.70	4271.32	<0.0001	0.40	397.45	<0.0001	
AB	737.86	6718.26	<0.0001	0.034	0.13	0.7234	0.38	381.11	<0.0001	
AC	4.25	36.86	<0.0001	144.32	565.74	<0.0001	0.090	89.91	<0.0001	
BC	3.91	35.56	0.0001	547.47	2145.95	<0.0001	0.35	347.06	<0.0001	
A^2	28.89	263.03	<0.0001	92.16	361.23	<0.0001	0.039	38.64	<0.0001	
B^2	106.52	969.83	<0.0001	161.13	631.58	<0.0001	0.007	7.17	0.0232	
C^2	2.18	19.83	0.0012	181.56	464.71	<0.0001	0.004	4.81	0.0531	
Residual	0.11			0.26			0.001			
Lack of fit	0.15	2.10	0.2175	0.28	1.23	0.4123	0.0008	0.69	0.6514	*not significant
Pure error	0.071			0.23			0.0001			
	R^2=0.9989 , Adj. R^2=0.9979 , CV%= 0.41			R^2=0.9988 , Adj. R^2=0.9978, CV%= 0.72			R^2=0.9927, Adj. R^2=0.9861, CV%= 0.60			

3.2.1 Antioxidant capacity- 3-D contour plots

The DPPH free radical scavenging, *β*–carotene and FRAP reducing assay of *Curcuma zedoaria* leaves extracts showed excellent antioxidant activity ranging from 66.79% to 93.7%, 42.04% to 85.15 % and 4.53 to 5.66 mM Fe^{2+}/g DW respectively. The mean percentage of DPPH, *β*–carotene and FRAP of all prepared extracts were 80.98 %, 69.83% and 5.26 mM Fe^{2+}/g DW respectively. The leaves extracts obtained with experimental run no. 1 showed the best antioxidants activity with the highest percentage for DPPH radical scavenging, run no.14 for *β*-carotene and run no 12 for FRAP assay. The ANOVA showed the model *F* value of 1016.83, 955.97 and 151.02 for DPPH, *β*-carotene and FRAP with probability ($p<0.0001$) which implies that the model is significant and there is only 0.01% chances that this large *F* value could occur due to noise. DPPH radical scavenging activity was significantly influenced at ($p< 0.05$) by all three linear (*A, B, C*), interaction parameters (*AB*, *AC, BC*) and quadratic parameters (A^2, B^2, C^2). *β*- Carotene assay was significantly influenced at ($p< 0.05$) by three linear (*A, B, C*), two interaction parameters (*AC*, *BC*) and three quadratic parameters (A^2, B^2, C^2). FRAP reducing activity assay was significantly influenced at ($p< 0.05$) by all three linear (*A, B, C*), interaction parameters (*AB*, *AC, BC*) and quadratic parameters (A^2, B^2).

The 3D surface plots showed that the DPPH scavenging activity and FRAP assay of *Curcuma zedoaria* leaves extracts were simultaneously influenced by the factor of temperature (A) and time (B) at a fixed ethanol to water (80:20) v/v% ratio in figure 1(A) and 3(A). However, for *β*–carotene assay the AB interactions were not significant ($P>0.05$). This result can be supported with ANOVA factors where large (*P*-value) (0.7234) demonstrates that the interactions between AB terms are not important in producing higher percentage of antioxidants in *Curcuma zedoaria* leaves.

From the response analysis, it was observed that increasing the extraction temperature and time resulted in a higher percentage of DPPH scavenging activity and FRAP. The optimum amount of DPPH scavenging activity can be obtained at almost 80^{o}C and 80mins extraction time as shown on figure 1(A). Meanwhile it was found that the FRAP value increased with increasing extraction temperature at constant extraction time as shown in figure 3(A). The 3D reveals that the optimum FRAP value can be obtained at about 70^{o}C and 100mins of extraction temperature and time, respectively and there was no significant effect when increased to longer extraction time of 120mins as the FRAP value declined beyond the optimal conditions. This occurrence might be due to decomposition of antioxidant compounds during the prolonged extraction time [18]. To date, our results were in line with those reported by Archana *et al.*, [19]. They also found a similar trend as increase in the FRAP value when increasing extraction temperature at a fixed extraction time in capcicum annuum. From the observations, the high temperatures used in this study might have increased the diffusion and solubility rate of many compounds resulting in antioxidants compounds being extracted at a higher rate [20]. However, DPPH scavenging activity and FRAP assay exhibited a decreasing trend above the optimum level of extraction time. This was indicated with the working high temperature employed in this study, which requires short periods of time to avoid the degradation of the antioxidants compounds. At short periods of time, the temperature enhances the extraction process but for relatively long periods, the effect is inverted and the antioxidants compounds are threatened by oxidation or degradation [21]. Roseiro *et al.*, [22] also found a similar phenomenon in extracting antioxidants from carob pods. It seems that the degradation of antioxidant compound happens after a longer time of extraction.

Figure 1, 2 and 3 (B) demonstrates the effect of varying of ethanol concentration (C) and temperature (A) at constant 100mins of extraction time. The results showed that increasing the ethanol concentration and temperature, resulted in a higher percentage of DPPH, *β*-carotene and FRAP assay. The results obtained for DPPH are generally in agreement with previous reports which

reveal that the DPPH scavenging ability increases with increasing ethanol concentrations and temperature [23]. The response surface plots in figure 1(B) shows that the optimum ethanol concentration and extraction temperature for higher antioxidants content in *Curcuma zedoaria* leaves was in the range of (80-90 v/v %) and 75-80^{o}C, respectively. Meanwhile, in figure 2(B) shows the optimum antioxidants for *β*-carotene can be achieved at the highest ethanol concentrations 85-90% under constant temperature of 70-75^{o}C. However, this current results were not in agreement with literature reported by Morelli and Prado [24] which revealed that highest ethanol concentrations above 65% and temperature under 50^{o}C showed the weakest extraction of antioxidants for *β*-carotene assay. The differences can be explained by the type of material and by considering some plant may synthesize and accumulate different amounts of secondary metabolites (*β*- carotene) and also the optimization extractions range used in the study. In figure 3(B) shows the highest FRAP value was observed when conducted at about highest ethanol solvent ratio 85-90% at extraction temperature of 65-70^{o}C. This result showed resemblance with the study done by Sai-Ut *et al*., [20] which reported that an increase in the amount of solvent leads to a significant increase in FRAP values from mango materials. The higher scavenging activity of DPPH, *β*-carotene and FRAP could be explained by the natural polarity of the solvents used [25]. Ethanol and water were used in this study because they are safer to handle as compared to other organic solvents and more importantly, they are acceptable for human consumption. Moreover, according to Samuagam *et al*., [26] a suitable solvent ratio is able to improve the efficiency of extraction. Therefore, it can be justified that solvent polarity played an important role in the extraction of antioxidants, resulting in highest antioxidant activity.

Figure 1, 2 and 3 (C) illustrates the effect of time (B) and ethanol concentration (C). The response surface plots in figure 1 (C), 2 (C) and 3 (C) shows that the antioxidant for DPPH, *β*-carotene and FRAP increased as increasing extraction time and ethanol concentrations. In figure 1 (C) it can be seen that maximum DPPH radical scavenging activity can be obtained at about 100mins extraction time and 75-80 v/v% ethanol concentrations, and thereafter, deliberately dropped. This phenomenon can be explained by the prolonged extraction time which would increase the chance for occurrence of degradation of antioxidant compounds [20]. Meanwhile in figure 2 (C), the maximum antioxidants for *β*-carotene assay can be yielded at range 85-90 v/v% and 90-100mins of ethanol concentrations and extraction time, respectively. The 3D also reveals that the time curve started to level off at about 100mins and subsequently, the percentage of inhibition activity in *β*-carotene from *Curcuma zedoaria* leaves decreased. This behavior can also be explained by the instability of the compounds in unfavorable conditions which forces the compounds to stop the reaction that produces more antioxidants constituents [27]. The 3D response surface in figure 3 (C) showed that the that higher FRAP values in *Curcuma zedoaria* leaves can be achieved when conducted at higher ethanol concentration of 90:10 v/v% at any level of elevated extraction time until the maximum equilibrium achieved.

3.3 RSM Optimization

By using Design Expert software, the desirability function was probed to achieve an optimized extraction conditions where they were evaluated by considering the simultaneous response surface and contour plot from the interaction between the independent variables and responses of interest (radical scavenging activity- DPPH, β-carotene assay and FRAP assay). The final results for the simultaneous optimization using the desirability function approach suggested that the optimal ethanolic extraction conditions for *Curcuma zedoaria* leaves extract were at 75^{o}C, 92mins and 90:10 v/v % of ethanol concentrations to achieve the best solution for highest antioxidants capacity.

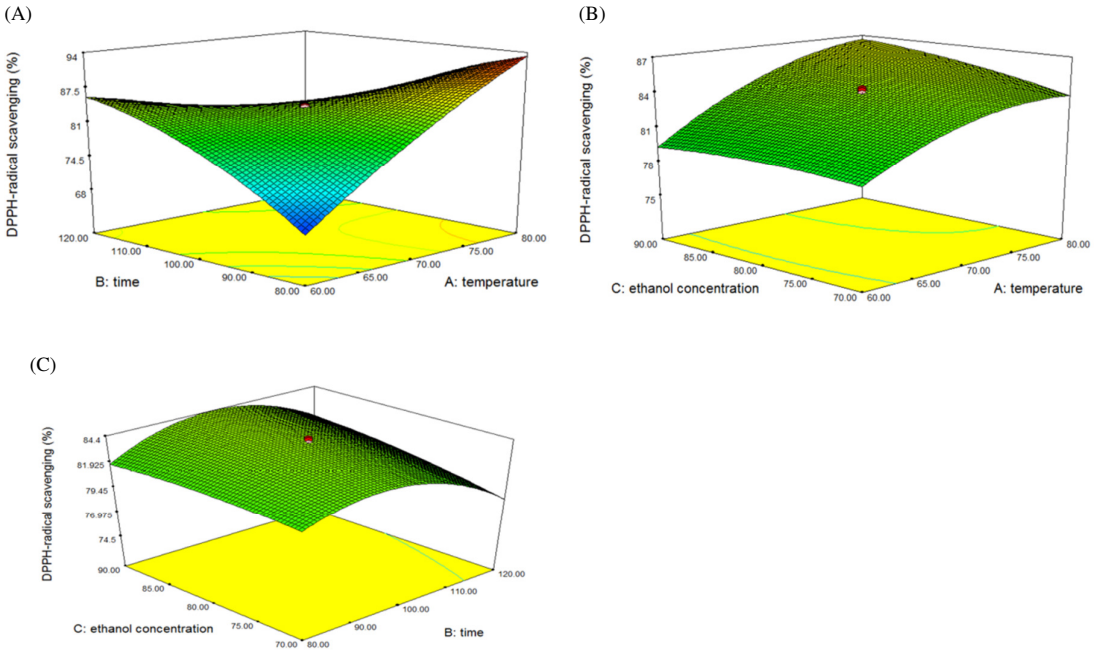

Fig. 1. Response surface plots for the effects of temperature, time and ethanol concentration on DPPH scavenging activity of *Curcuma zedoaria* leaves extracts. *(A)* temperature versus time; *(B)* ethanol concentration versus temperature; *(C)* time versus ethanol concentration

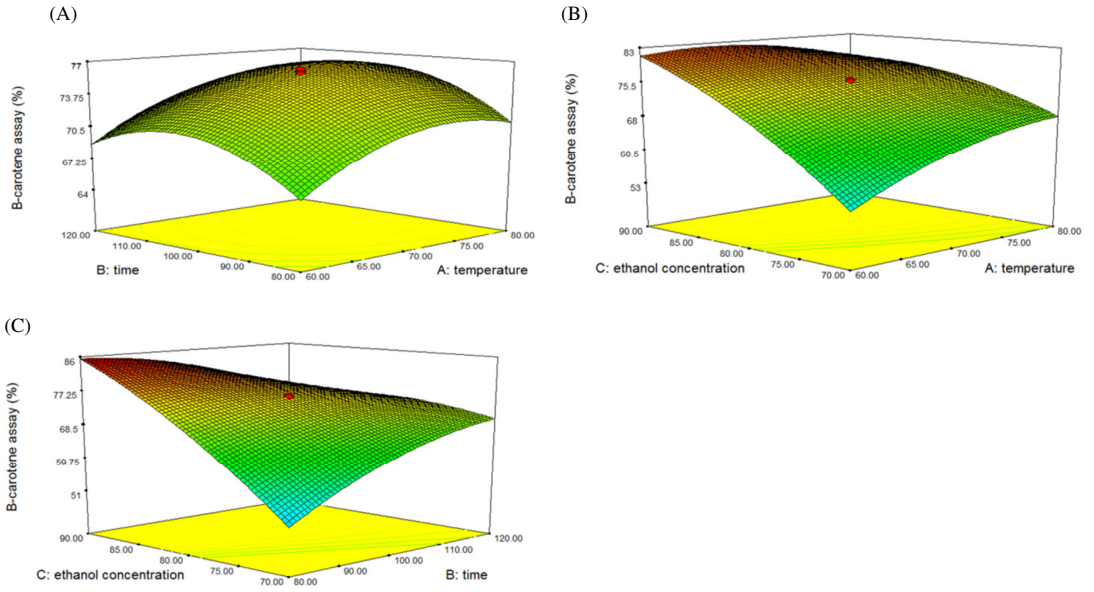

Fig. 2. Response surface plots for the effects of temperature, time and ethanol concentration on β-carotene (%) activity of *Curcuma zedoaria* leaves extracts. *(A)* temperature versus time; *(B)* ethanol concentration versus temperature; *(C)* time versus ethanol concentration

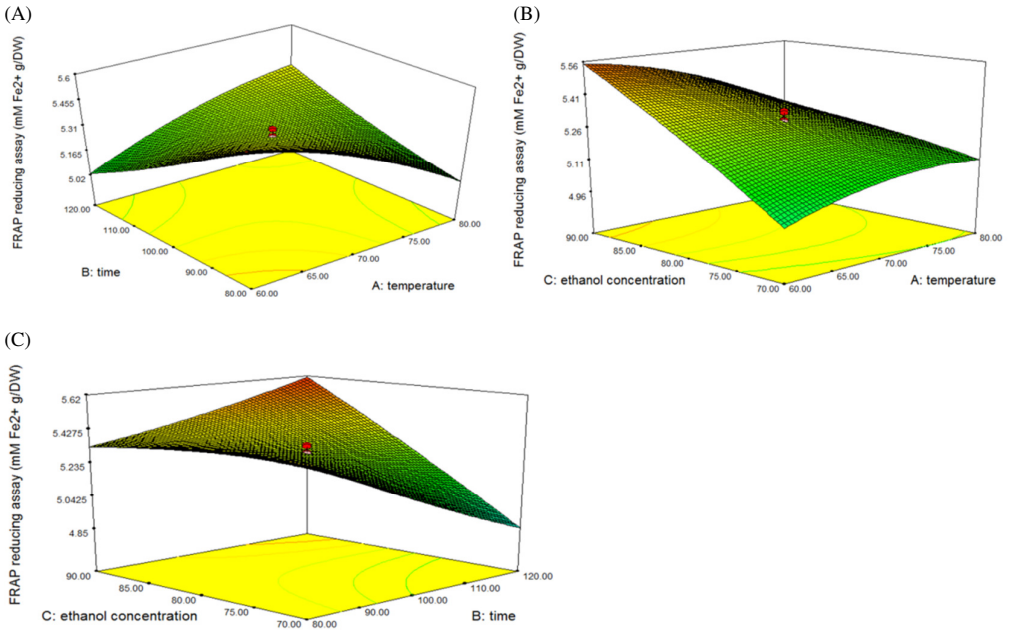

Fig. 3. Response surface plots for the effects of temperature, time and ethanol concentration on percentage FRAP assay of *Curcuma zedoaria* leaves extracts. *(A)* temperature versus time; *(B)* ethanol concentration versus temperature; *(C)* time versus ethanol concentration

In order to verify the optimum conditions, the *Curcuma zedoaria* leaves were subjected to optimal conditions and the results were statistically compared to the predicted values of the mathematical model. Based on the results, the predicted values of responses were found within the range and to be not statistically different at 95% confidence level. Based on the optimum conditions, the predicted values of DPPH, β-carotene and FRAP assay were 87.59% 82.29% and 5.25 mM Fe^{2+} g/DW respectively. The analysis showed that the experimental extraction condition had the DPPH, β-carotene and FRAP mM Fe^{2+} g/DW value of 85.76%, 81.35% and 5.08 mM Fe^{2+} g/DW as shown in table 4.

Table 4
Comparison between the predicted and experimental values for antioxidants from extracts of *Curcuma zedoaria* leaves

Condition	Response Values		
	DPPH scavenging %	β-carotene assay %	FRAP reducing assay mM Fe^{2+} g/DW
Predicted	87.59	82.29	5.25
Experimental	85.76	81.35	5.08

3. Conclusions

In this study, response surface methodology proved to be effective in estimating the effect of three independent variables, namely extraction temperature, time processing and ethanol

concentration to optimize the antioxidant capacities of *Curcuma zedoaria* leaves. A design called central composite rotatable design (CCRD) was successfully developed to determine the optimum process parameters and the second order polynomial models for predicting responses were obtained. The recommended optimal conditions were as follows: temperature 75^{o}C, extraction time 92mins and 90:10 v/v% of ethanol concentration. Under the optimal conditions, the maximum antioxidants activities in *Curcuma zedoaria* leaves was attained with respect to the highest DPPH, β-carotene assay and also FRAP assay. In addition, this present study also showed that the *Curcuma zedoaria* leaves can be considered a good source of antioxidant compounds. There could be clear potential for the utilization of *Curcuma zedoaria* leaves for formulation of cosmeceuticals, food additive, or as medicinal source for industrial needs. Appendixes, if needed, appear before the acknowledgment.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgement

The authors gratefully acknowledge the financial support from Graduate Research Fellowship (GRF) under Universiti Putra Malaysia for the scholarship.

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