Full Length Article

Optimization of microwave-assisted extraction of mulberry twigs (*Morus alba* Linn.) on antityrosinase and antioxidant potential using response surface methodology

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Received: 21 July 2021 *Revised: 21 August 2021* *Accepted: 23 August 2021*

Abstract

Response surface methodology (RSM) has been used to optimize the extraction conditions of bioactive components with relatively high antityrosinase and antioxidant activity from mulberry twigs by using microwave-assisted extraction (MAE). The results showed that the highest antityrosinase (2.51 mg VE/g dw) and antioxidant activity (79.03 % scavenging and 250.03 mg VEAC/100 g dw for DPPH assay, and 1342.75 mg Fe(II)/100g dw for FRAP assay) were obtained with an extraction time of 5 min, 45% ethanol, and 70 ml/g liquid to solid ratio. In this study, MAE can be used as an alternative to conventional immersion extraction with respect to the recovery of bioactive compounds from mulberry twigs, with the advantages of shorter extraction time and reduced solvent consumption.

Keywords: mulberry twigs, microwave-assisted extraction, antityrosinase activity, antioxidant activity

Introduction

Mulberry (*Morus alba* L.) is a fast-growing deciduous plant that grows under different climatic conditions. Mulberry has been used in traditional Chinese medicine as an anti-diabetic, anti-hypertensive, anti-headache, and diuretic agent (Choi et al., 2013). In particular, mulberry twigs have been widely used for the healing of aching and numbness of joints in oriental medicine (Zhu, 1998). Mulberry contains bioactive compounds including phenolic compounds especially stilbene groups which can inhibit enzyme tyrosinase activity and as an antioxidant, so its potential in cosmetic (Batubara et al., 2010). Oxyresveratrol was an aglycone of mulberroside A and showed strong inhibit enzyme tyrosinase activity (Kim et al., 2010).

Bioactive substances especially phenolic groups could be extracted from mulberry twigs by several conventional methods, such as maceration, percolation, soxhlet extraction and reflux extraction. However, these traditional methods are often inefficient, as well as solvent-

and time-consuming. Therefore, some new extraction methods came into being to overcome these problems, including Ultrasonic-assisted extraction (UAE), pressurized liquid extraction, enzyme-assisted extraction and microwave-assisted extraction (MAE). MAE is a novel method used to improve production yield. Microwave radiation has a destructive effect on cell structure, which makes the active substance dissolve into the solvent quickly, thus obtaining higher extraction efficiency in a shorter time (Vinatoru et al., 2017). At the same time, the microwave-assisted extraction also has strong advantages in stability and reproducibility (Chan et al., 2011).

To our knowledge, no report could be found in the literature on the extraction of antioxidants from mulberry twigs by microwave-assisted extraction. The objective of this study was to investigate the effect of microwave on the extraction efficiency of mulberry twigs on antityrosinase and antioxidant capacities, as well as to optimize the parameters of this process by response surface methodology.

Materials and Methods

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), and FeCl3 were purchased from Sigma-Aldrich (USA); Folin-Ciocalteus's phenol reagent and TPTZ (2,4,6-tripyridyl-S-triazine) were purchased from Fluka (Switzerland); acetonitrile and milli-Q water were of HPLC grade and other chemicals were of analytical grade.

Microwave-Assisted Extraction (MAE)

Mulberry twigs (*Morus alba* L.) of the cultivars, Nakhonratchasima 60 was obtained from Silk Innovation Center, Mahasarakham University, Thailand. The MAE process was performed by microwave equipment with controlled microwave power (2000 W). Dried mulberry twigs sample (1.000 g) was placed in a centrifuge tube and then mixed with ethanol aqueous solutions of different concentrations and volumes. After extraction, the mixture was cooled with running water, centrifuged at 4200xg for 10 min and the supernatant was kept at 4^{o}C for subsequent experiments.

Experimental design and statistical analysis

Microwave -assisted extraction optimized the experimental design using RSM. A Central Composite Design (CCD) consisting of twenty experimental runs was employed including six-star points (α = 1.682) points, eight factorial points and six central points. The independent variables were the ethanol concentration (X1, 30-60%), liquid to solid ratio (X2, 50-90 ml/g), and extraction time (X3, 1-10 min) (Table 1) while dependent variables (response) were antityrosinase (Y1) and antioxidant activity (Y2). The range values of the three independent variables were determined by preliminary study. Experiments were performed in replicate and the average values were used as the response, Y.

Table 1. Independent variables and code levels of central composite design (CCD).

Independent Variable	Units	Level				
		-∞	-1	0	+1	+∞
Ethanol concentration (X1)	% v/v	15	30	45	60	75
Liquid-to-Solid ratio (X2)	ml/g	30	50	70	90	110
Extraction time (X3)	min	1	3	5	7	9

The CCD matrix contains 20 experiments with 6 replicates of center points. The response values of the model were analyzed by Design-Expert 7.0 and the data were subjected to multiple regression analysis to fit the quadratic equation:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \varepsilon, \quad (1)$$

where Y refers to the predicted response values of antityrosinase and antioxidant activity; β_0 is the constant coefficient; β_i, β_{ij} and β_{ii} represent the coefficients of linear, quadratic and interaction terms, respectively; X_i and X_j are independent variables; ε is the residual error.

Analysis of variance (ANOVA) was used to analyze the statistical significance of the fitting model and each term of the fitted model. The interaction effect of each variable on the response value was shown on the 3D surface plot.

Determination of antioxidant activity

DPPH scavenging activity

The radical scavenging activity of medicinal plant extracts were evaluated according to the method of Brand-Williams et al. (1995) with some modification. One hundred microliters of plant extracts were mixed with 100 µl of the 0.2 mM DPPH solution. The mixture was thoroughly mixed and left to stand for 1 h at room temperature in the dark. The absorbance was read at 520 nm using a microplate reader spectrophotometer (Synergy HT, BiotTek instruments, USA). The antioxidant activity of medicinal plant extracts was expressed as EC_{50} (mg/ml), the extract dose required to scavenge 50% of DPPH free radicals. A smaller EC_{50} value corresponds to a higher antioxidant activity.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the method of Benzie and Strain (1996) with some modifications. Briefly, 270 µl of freshly FRAP reagent containing 300 mM acetate buffer (pH 3.6), 20 mM $FeCL_3.6H_2O$, 10 mM TPTZ in 40 mM HCl in the proportion of 10:1:1 (v/v), respectively, was mixed with 30 µl medicinal plant extracts. After incubated in the dark at room temperature for 30 min, the absorbance at 595 nm was measured using a microplate reader spectrophotometer (Synergy HT, BiotTek instruments, USA). A standard curve of ferrous sulfate solution ($FeSO_4.7H_2O$) was used for calculation of FRAP and expressed as mg Fe (II)/100g dw.

Tyrosinase inhibition

Tyrosinase inhibition activity was determined using the modified dopachrome method with L-DOPA as the substrate (Masuda et al., 2005). A 96-well microtiter plate was used to measure

absorbance at 490 nm. Each well contained 40 μL of sample with 80 μL of phosphate buffer (0.1M, pH 6.8), 40 μL of tyrosinase (100 units/mL). After incubated at room temperature for 10 min, 40 μL of L-DOPA (2.5 mM) was added. The mixture was incubated for 10 min at room temperature and absorbance was measured at 490 nm using a microplate reader spectrophotometer (Synergy HT, BiotTek instruments, USA). Each sample was accompanied by a blank containing all components except L-DOPA. L-ascorbic acid was used as positive controls. The results were compared with a control consisting of phosphate buffer in place of the sample. The percentage of tyrosinase inhibition was calculated as follows: $[(A_{control} - A_{sample})/A_{control}] \times 100\%$

Statistical Analysis

All experiments were performed in triplicate and the results were displayed as mean. The data were analyzed by Design Expert 7.0 software and SPSS 16.0 statistics software (IBM Corp., Armonk, NY, USA). The statistical significance was investigated by one-way ANOVA and the significance level was $p < 0.05$.

Results and Discussion

Fitting the Models

An optimization of extraction conditions for the extraction of mulberry twigs with relatively high antityrosinase and antioxidant capacities was conducted using RSM. The extraction efficiency of bioactive compounds was affected by extraction conditions including extraction solvent concentration, extraction temperature and extraction time (Tan et al., 2011). RSM is accepted as a powerful tool in optimizing experimental conditions to maximize various responses (Zhao et al., 2011). For RSM, the levels of independent variables for the extraction of mulberry twigs with relatively high antityrosinase and antioxidant activities was selected based on the results obtained from our preliminary experiments. The experimental design and corresponding response data are shown in Table 2. Twenty experiments were designated and six were zero-point tests performed to estimate the errors. The ranges of ethanol concentration (15%–75%), liquid to solid ratio (30–110 ml/g), and extraction time (1–9 min) were used. Antityrosinase and antioxidant activities (DPPH, FRAP) were used as responses in the RSM experimental design. Predicted response Y for extraction of mulberry twigs could be obtained by applying multiple regression analysis on the experimental data. The predicted quadratic polynomial models are shown in Table 3. The models were checked using a numerical method including the coefficient of determination (R^2). R^2 provided a measure of how well future outcomes are likely to be predicted by the model. In the models, X_2 and X_2X_3 were associated with synergistic effects on the antityrosinase activity whereas X1 and X_3 were associated with antagonistic effects. In addition, X_1, X_2 and X_2X_3 were associated with synergistic effects on the antioxidant activity whereas X_3 and X_1X_3, were associated with antagonistic effects. The R^2 of the models for antityrosinase and antioxidant activity was 0.9545 and 0.8647-0.9787, respectively, which showed suitable fitting of the model in the designed experiments (Table 4).

Moreover, the coefficient of variation (CV) was 12.14 and 4.40-7.93, respectively, which indicates that a relatively lower value of CV showed a better reliability of the response model.

Response surface optimization of MAE

To visualize the relationship between the response and experimental levels of the independent variables for the antityrosinase and antioxidant capacities, three-dimensional (3D) surface and contour plots were constructed according to the quadratic polynomial model equations of Table 3.

The influence of the variables and their interaction on the responses can be seen Figures 1-4. As shown in Figure 1A, shows the effect of the interaction of ethanol concentration and liquid to solid ratio on antityrosinase activity. The inhibition activity of tyrosinase was lower when the extraction conditions had higher ethanol concentrations and the inhibition of tyrosinase enzyme was higher when the ratio between ethanol to mulberry twigs as higher. The optimum extraction ethanol concentration was in the range of 30-45% and the ratio of ethanol per mulberry twigs was in the range of 70-90 ml/gram which has an inhibitory effect on tyrosinase greater than 2.74 mg VE/g dw. Figure 1B shows the effect of the interaction of ethanol concentration and extraction time on antityrosinase activity. Tyrosinase inhibitory activity was higher with lower ethanol concentration and shorter extraction time. At the highest point of the graph, the concentration of ethanol used for extraction was suitable in the range of 30-45% and the extraction time was in the range of 3-5 minutes, which would inhibit the tyrosinase enzyme activity greater than 2.43 mg VE/g dw. Figure 1C shows the effect of the interaction of liquid to solid ratio and extraction time on antityrosinase activity. The inhibition of tyrosinase was higher with higher liquid to solid ratio and shorter extraction time. At the highest point of the curve, the optimum liquid to solid ratio was in the range of 70-90 ml/gram and the extraction time was 3-5 minutes, which would have an inhibitory effect on the tyrosinase enzyme greater than 2.70 mg VE/g dw.

Figure 2A, 3A and 4A shows the effect of the interaction of ethanol concentration and liquid to solid ratio on the antioxidant activity (DPPH and FRAP). The antioxidant activity of DPPH and FRAP was higher when the ethanol concentration decreased and the liquid to solid ratio increased. The optimum point was in the region where the concentration of ethanol extracted was in the range of 30-45% and the liquid to solid ratio was in the range of 70-90 ml/g. The antioxidant DPPH was greater than 271.23 mg VEAC/100 g dw and the reducing ability was greater than 1332.05 mg Fe(II)/100g dw.

Figure 2B and 3B shows the effect of the interaction of ethanol concentration and extraction time on the antioxidant activity (DPPH). From the graph, it was found that when the ethanol concentration and extraction time were increased, the DPPH scavenging activity was increased, indicating that the optimum point was in the concentration region. The ethanol concentration was in the range of 45-60% and the extraction time was 3-5 min. The DPPH scavenging activity was higher than 79.34% and 244.08 mg VEAC/100 g dw.

Figure 4B shows the effect of the interaction of ethanol concentration and extraction time on the antioxidant activity (FRAP). The results in the same way as DPPH, from the curves of FRAP, it was found that the reducing ability was increased when the ethanol concentration and extraction time increased. The optimum point is in the region where the ethanol concentration and extraction time were in the range of 30-45% and 3-5 min, respectively, resulting in the reducing ability greater than 1349.06 mg Fe(II)/100g dw.

Figure 2C and 3C shows the effect of the interaction of extraction time and liquid to solid ratio on the antioxidant activity (DPPH). The DPPH scavenging activity was lower when the liquid to solid ratio increased whereas the extraction time increased resulting in the DPPH scavenging activity was higher. This indicates that the optimum point is in the region where the liquid to solid ratio and extraction time were in the range of 50-70 ml/g and 3-5 min,

Table 2. The design and the corresponding actual values of CCD.

Run	X1	X2	X3	Antityrosinase activity (mg VE/g dw)		DPPH assay				FRAP (mg Fe(II)/100g dw)	
						%Scavenging		mM VEAC/100 g dw			
	Ethanol concentration (%)	Liquid-to-Solid ratio (ml/g)	Extraction time (min)	Observed	predicted	Observed	predicted	Observed	predicted	Observed	predicted
1	30 (-1)	50 (-1)	3 (-1)	1.74	1.88	80.48	76.62	211.97	186.24	1134.9	1029.27
2	60 (1)	50 (-1)	3 (-1)	1.24	1.46	85.58	81.98	187.83	181.51	1300.16	1245.89
3	30 (-1)	90 (1)	3 (-1)	3.39	3.17	68.31	63.27	374.63	365.11	1344.84	1329.55
4	60 (1)	90 (1)	3 (-1)	2.72	2.93	83.71	83.03	383.31	364.07	1509.74	1456.53
5	30 (-1)	50 (-1)	7 (1)	1.11	1.30	80.34	77.71	123.12	138.74	693.97	740.51
6	60 (1)	50 (-1)	7 (1)	0.63	0.71	78.98	80.72	65.48	71.38	654.23	662.85
7	30 (-1)	90(1)	7 (1)	2.97	3.15	68.02	68.32	300.67	303.37	1407.21	1454.81
8	60 (1)	90 (1)	7 (1)	1.94	2.20	85.16	85.72	217.58	239.69	1188.52	1287.50
9	15 (-2)	70 (0)	5(0)	2.77	2.56	60.44	64.40	235.74	242.40	1069.64	1079.65
10	75 (2)	70 (0)	5 (0)	1.37	1.19	87.83	87.17	177.03	174.00	1132.43	1128.97
11	45 (0)	30 (-2)	5 (0)	0.63	0.51	79.87	82.39	63.35	66.81	604.80	653.81
12	45 (0)	110 (2)	5 (0)	4.11	3.83	73.27	74.09	412.83	413.99	1621.20	1578.73
13	45 (0)	70 (0)	1 (-2)	3.01	2.77	68.82	73.76	283.77	312.37	1158.24	1269.05
14	45 (0)	70 (0)	9 (2)	1.62	1.46	79.17	77.54	165.47	140.49	915.52	811.26
15	45 (0)	70 (0)	5(0)	2.29	2.51	79.03	79.03	247.12	250.03	1304.09	1342.72
16	45 (0)	70 (0)	5(0)	2.68	2.51	78.70	79.03	253.11	250.03	1339.3	1342.72
17	45 (0)	70 (0)	5(0)	2.60	2.51	78.27	79.03	248.32	250.03	1343.25	1342.72
18	45 (0)	70 (0)	5(0)	2.62	2.51	78.33	79.03	251.14	250.03	1367.51	1342.72
19	45 (0)	70 (0)	5(0)	2.64	2.51	78.30	79.03	249.52	250.03	1332.26	1342.72
20	45 (0)	70 (0)	5(0)	2.62	2.51	78.23	79.03	247.37	250.03	1327.46	1342.72

Table 3. Coded and processed variables levels used in experimental design for RSM.

Responses	Model equations	R^2	CV (%)
Antityrosinase activity	$Y = -2.85168 + 0.069023X_1 + 0.083864X_2 + 0.13733X_3 - (3x10^{-4})X_1X_2 - (1.41667x10^{-3})X_1X_3 + (1.25x10^{-4})X_2X_3 - (7.08586x10^{-4})X_1^2 - (2.1108x10^{-4})X_2^2 - 0.024545X_3^2$	0.9545	12.14
DPPH (%Scavenging)	$Y = +93.86884 - 0.038205X_1 - 0.69727X_2 + 1.74216X_3 + 0.012000X_1X_2 - 0.019667X_1X_3 + 0.024688X_2X_3 - (3.60051x10^{-3})X_1^2 - (5.03409x10^{-4})X_2^2 - 0.21128X_3^2$	0.8647	4.40
DPPH (VEAC)	$Y = -194.48276 + 5.43866X_1 + 5.48976X_2 + 22.98852X_3 + (3.07083x10^{-3})X_1X_2 - 0.52196X_1X_3 - 0.089031X_2X_3 - 0.046487X_1^2 - (6.02102x10^{-3})X_2^2 - 1.47523X_3^2$	0.9787	7.93
FRAP	$Y = -801.57622 + 42.15406X_1 + 21.79947X_2 + 61.10364X_3 - 0.074704X_1X_2 - 2.45238X_1X_3 + 2.58759X_2X_3 - 0.26490X_1^2 - 0.14153X_2^2 - 18.91011X_3^2$	0.9596	6.51

CV, coefficient of variation; X1, ethanol concentration; X2, liquid to solid ratio; X3, extraction time.

Table 4. Regression coefficients for different antioxidant potential as responses.

Term	Antityrosinase activity (mg VE/g dw)	DPPH assay		FRAP (mg Fe(II)/100g dw)
		%Scavenging	mM VEAC/100 g dw	
X_1	-0.34***	5.69***	-17.10***	12.33
X_2	0.83***	-2.09*	86.80***	231.24***
X_3	-0.33***	0.94	-42.97***	-114.45***
X_1X_2	-0.090	3.60**	0.92	-22.41
X_1X_3	-0.042	-0.59	-15.66*	-73.57*
X_2X_3	0.005	0.99	-3.56	103.50***
X_1^2	-0.16**	-0.81	-10.46**	-59.60***
X_2^2	-0.084	-0.20	-2.41	-56.61***
X_3^2	-0.098	-0.85	-5.90	-75.64***
C.V. %	12.14	4.40	7.93	6.51
R^2 of model	0.9545	0.8647	0.9787	0.9596

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$.

respectively, resulting in the DPPH scavenging activity was higher than 7859% and 245.28 mg VEAC/100 g dw.

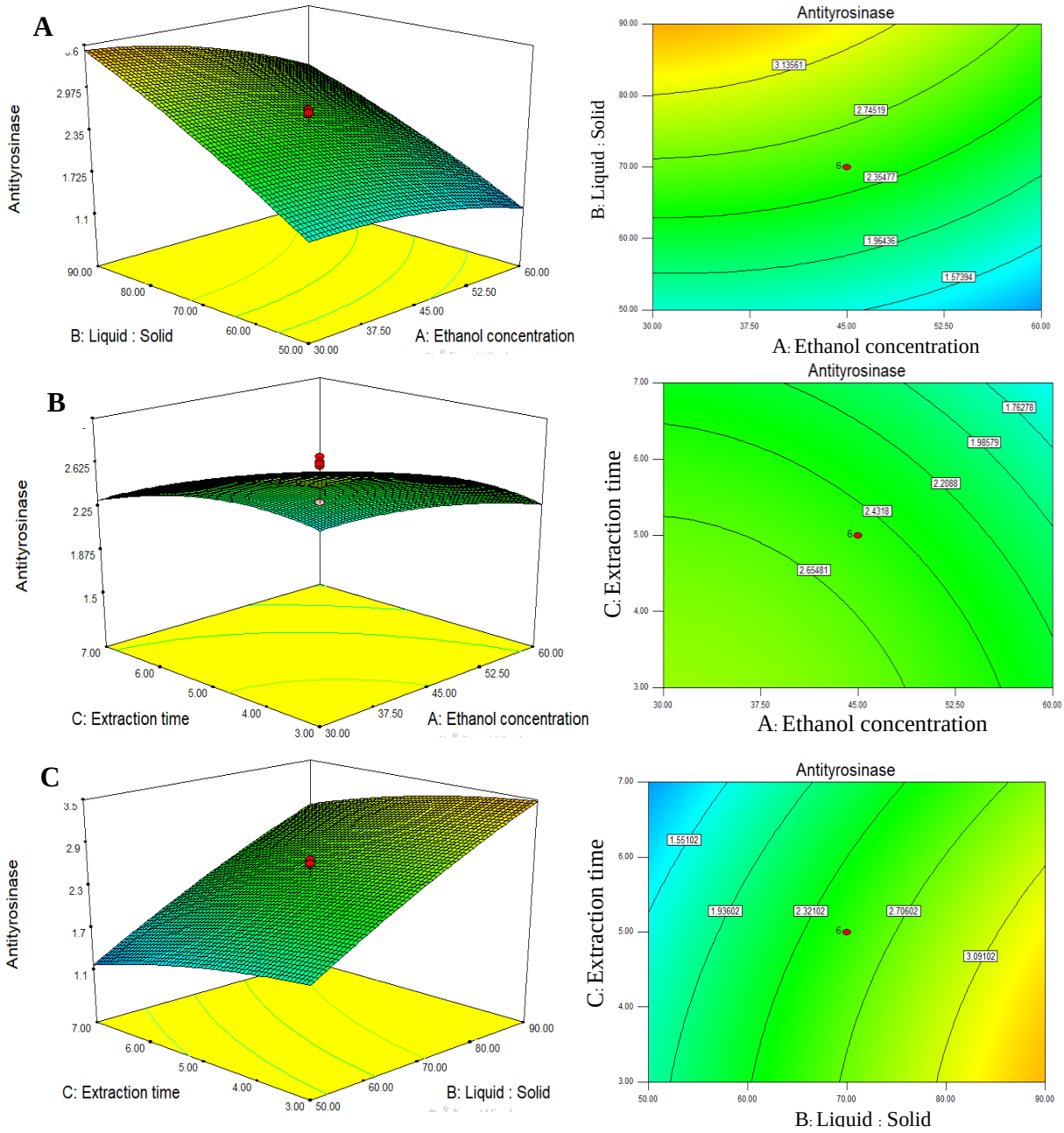

Figure 1. Response surface plot and contour plot of the interactions between different factors. Interaction effect of ethanal concentration and liquid to solid (A), ethanal concentration and extraction time (B), extraction time and liquid to solid (C) on antityrosinase activity.

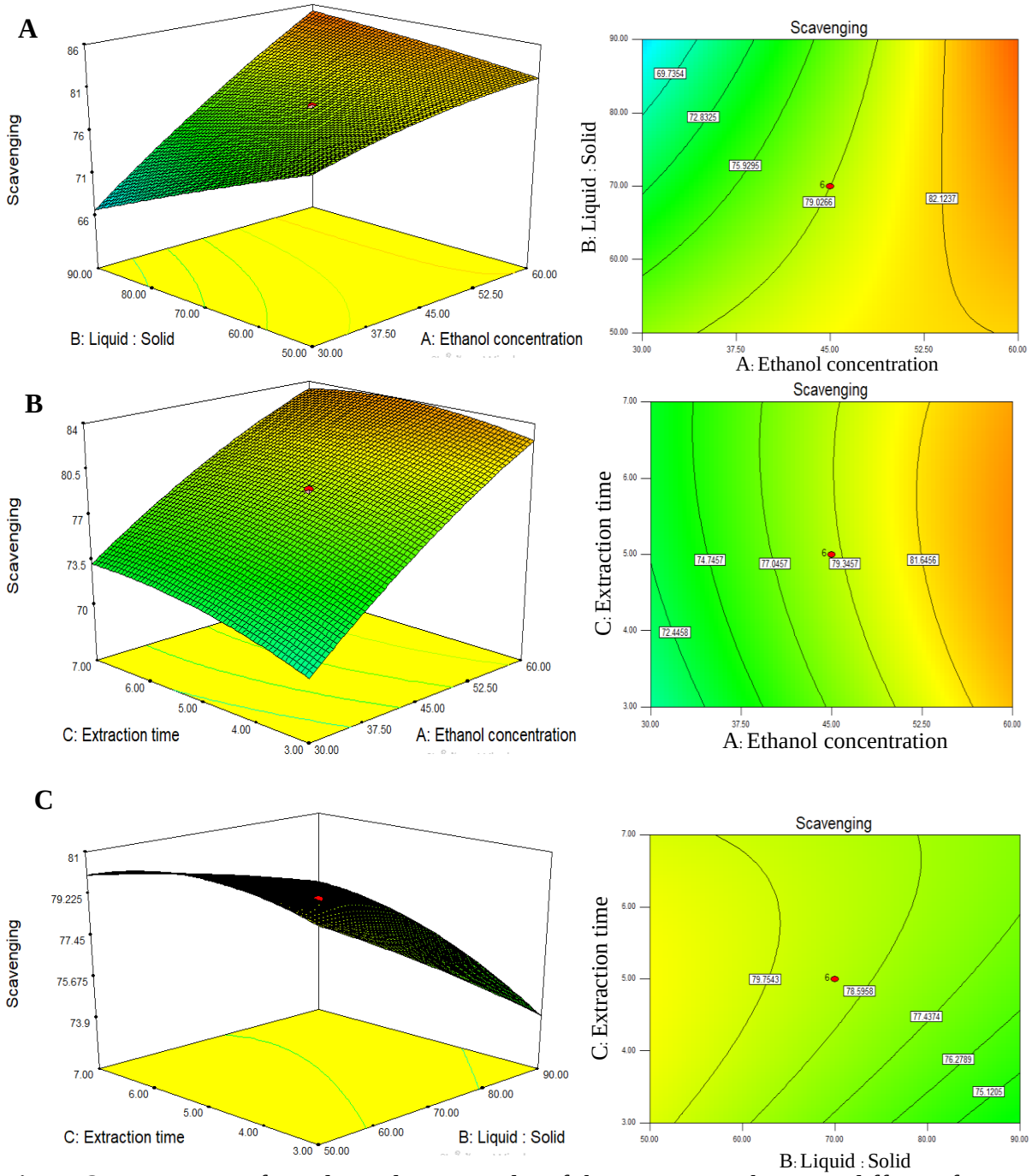

Figure 2. Response surface plot and contour plot of the interactions between different factors. Interaction effect of ethanal concentration and liquid to solid (A), ethanal concentration and extraction time (B), extraction time and liquid to solid (C) on antioxidant activity (%scavenging).

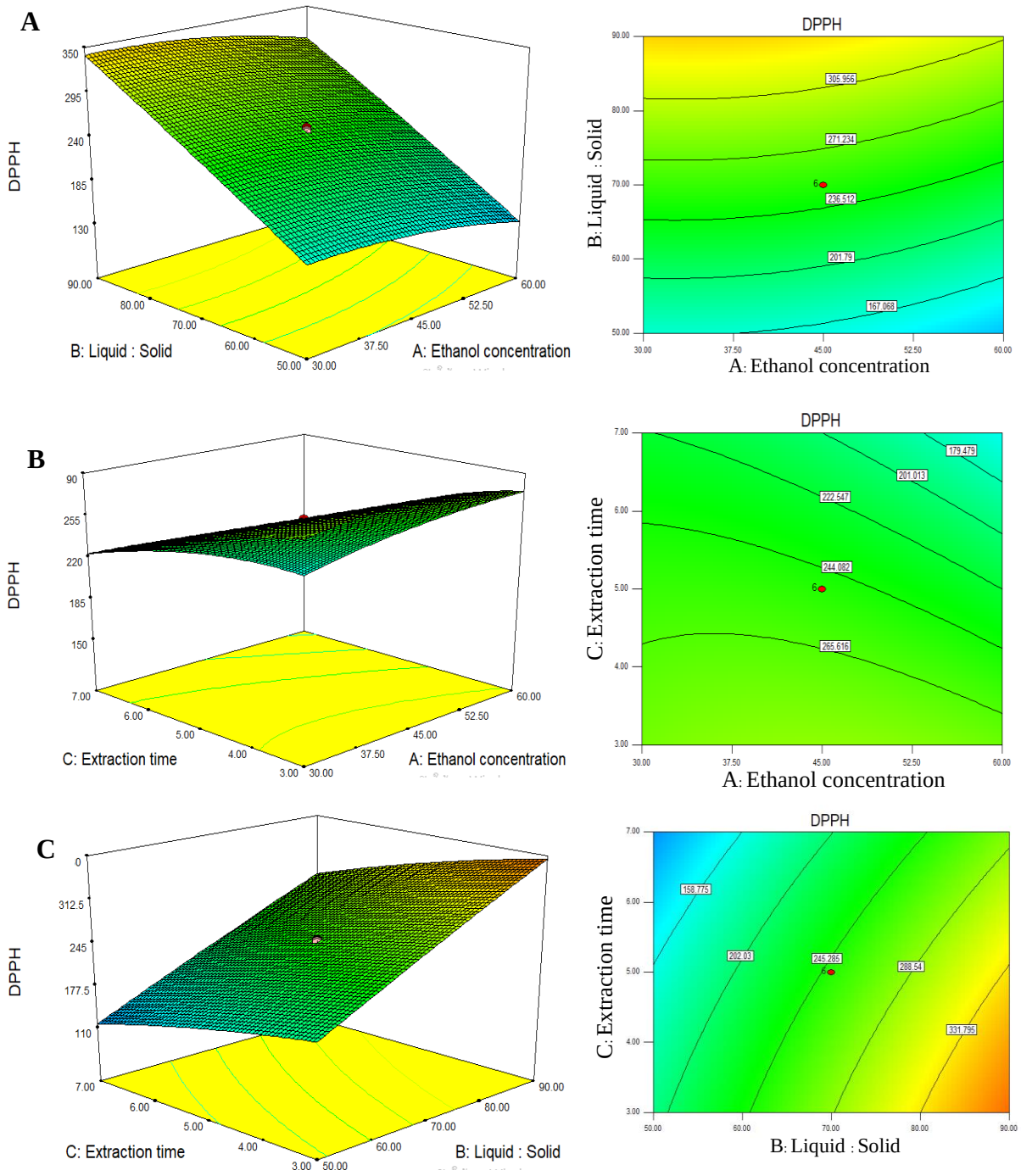

Figure 3. Response surface plot and contour plot of the interactions between different factors. Interaction effect of ethanal concentration and liquid to solid (A), ethanal concentration and extraction time (B), extraction time and liquid to solid (C) on antioxidant activity (mM VEAC/100 g dw).

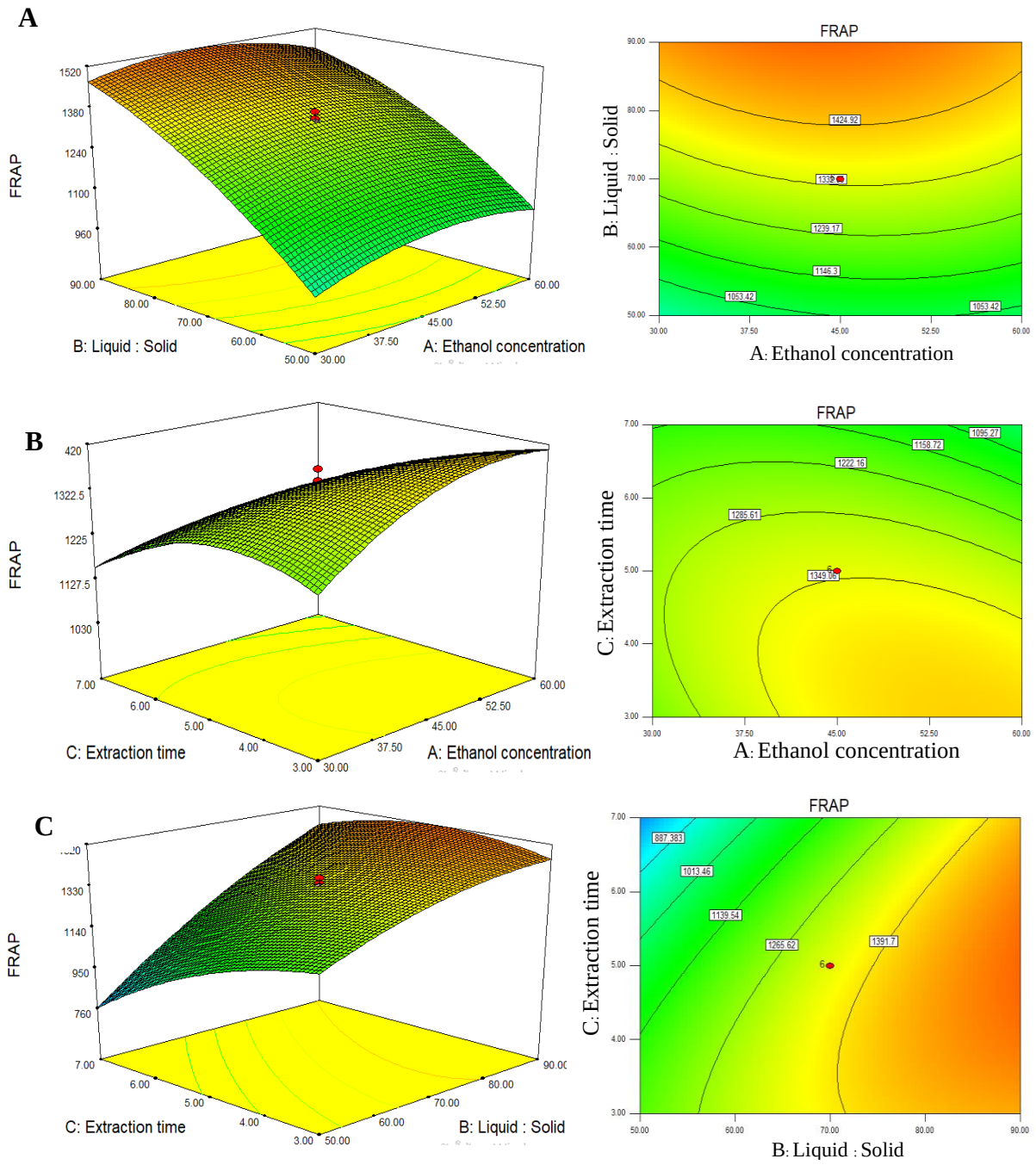

Figure 4. Response surface plot and contour plot of the interactions between different factors. Interaction effect of ethanal concentration and liquid to solid (A), ethanal concentration and extraction time (B), extraction time and liquid to solid (C) on antioxidant activity (FRAP, mg Fe(II)/100g dw).

Figure 4C shows the effect of the interaction of extraction time and liquid to solid ratio on the antioxidant activity (FRAP). It was found that the reducing ability was increased when the liquid to solid ratio increased whereas the extraction time increased resulting in the reducing ability decreased. The optimum point is in the region where the liquid to solid ratio

and extraction time were in the range of 50-75 ml/g and 5-7 min, respectively, resulting in the reducing ability greater than 1265.62 mg Fe(II)/100g dw.

Conclusion

In the present study, response surface methodology was used to optimize the microwave-assisted extraction (MAE) of bioactive compounds with relatively high antityrosinase and antioxidant activities from mulberry twigs. A central composite design was used to determine the optimum process parameters and the second order polynomial models for predicting responses were obtained. Ethanol concentration, liquid to solid ratio and extraction time were the most significant factor affecting antityrosinase and antioxidant capacities and the optimal extraction conditions were 45% ethanol, 70 ml/g and 5 min. Under optimized conditions the experimental values were very close to the predicted values. As such, it may be said that MAE is an effective and practical method for obtaining bioactive compounds with relatively high antityrosinase and antioxidant activities.

Acknowledgements

The authors would like to thank Department of Biotechnology, Mahasarakham University for providing laboratory facilities to complete this study.

Conflicts of Interest

The authors declare no conflict of interest.

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