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Optimisation of Microwave-Assisted Extraction of Flavonoids and Phenolics from Celery (*Apium graveolens* L.) Leaves by Response Surface Methodology

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Abstract

He Q., Li Y., Zhang P., Zhang A., Wu H. (2016): **Optimisation of microwave-assisted extraction of flavonoids and phenolics from celery** (*Apium graveolens* L.) **leaves by response surface methodology**. Czech J. Food Sci., 34: 341–349.

The conditions for microwave-assisted extraction (MAE) of total flavonoids (TF) and total phenols (TP), and antioxidant activity from celery (*Apium graveolens* L.) leaves by response surface methodology (RSM) were optimised. The 3-level, 3-factorial Box-Behnken design (BBD) was employed to study three main extraction conditions: microwave power (300–500 W), solid-solvent ratio (15–30 ml/g), and ethanol concentration (50–80%). It was found that microwave power of 500 W at 30 ml/g solid-solvent ratio with 75.6% (v/v) ethanol concentration was the most optimum conditions for the extraction of TF and TP from celery leaves with the consequent high antioxidant activity measured by the DPPH inhibition rate. Using the optimum extraction conditions, the extraction yields of TF and TP were 0.62 g RUE/100 g DW, 3.01 g GAE/100 g DW, respectively, and the DPPH inhibition rate was 88%. The results indicated that the nutritional quality of celery (*Apium graveolens* L.) leaves could be improved significantly by optimising the extraction process of MAE using response surface methodology.

Keywords: Box-Behnken experimental design; total flavonoids; total phenolics; antioxidant activity

Nowadays, many scientists and food manufacturers are gradually concerned in the use of modern techniques of extraction of bioactivity compounds from vegetables and traditional Chinese medicinal plants. The new extraction techniques include Soxhlet extraction, heat reflux extraction, supercritical fluid extraction, ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE). Compared with conventional extraction methods, MAE has been widely applied as an alternative for the extraction of bioactive ingredients from various plants due to its many advantages, such as higher drying rate, higher energy efficiency, rapid extraction, and less solvent and extraction time (Camel 2000). The dipole interaction of water molecules and the solvent in the microwave field causes the temperature and pressure of the solvent to rise, which leads to a diffusion from material to the solvent with the high extraction rate of MAE (Spigno & De Faveri 2009). During the MAE process, it is very necessary to determine the most advantageous combination of process parameters because of the diversity of biological materials existing in different plant materials (Wettasinghe & Shahidi 1999). The main parameters affecting the microwave processing are time, power, and solvent volume, and so on. Response surface methodology (RSM) is a useful statistical technique based on the Box-Behnken design (BBD) for optimising conditions for desirable responses (Szydłowska-Czerniak *et al*. 2011). Recently, RSM has been shown to be a very important design tool applied to investigate the best combination of experimental conditions. The MAE of total flavonoids (TF) and total phenol (TP) components from a large amount of fruits and vegetables, including *Apium graveolens* L. plant (Zhu & Row 2011), has been successfully studied using RSM.

Celery (*Apium graveolens* L.) is a popular daily vegetable in China due to its attractive taste and nu-

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tritional composition. In traditional medicine, celery has been used to treat asthma, bronchitis, spleen, and liver diseases (Singh & Handa 1995). Recently, there has been a growing interest in the plant for its health-beneficial activities, such as antioxidant activities (Ninfali & Bacchiocca 2003), anti-inflammatory activity (Ovodova *et al*. 2009), anticancer activities (Sultana *et al*. 2005), anti-hyperlipidaemic effect (Tsi & Tan 2000; Iyer & Patil 2011), and so on. Numerous studies have confirmed that flavonoids consist of a common basic structure comprising two aromatic rings linked by three carbons that form an oxygenated heterocyclic compound, and phenol compounds are the main antioxidant substance which can promote the human health through reducing oxidative damage (Wang & Stoner 2008; Mousavinejad *et al*. 2009). Thus, several reports have been published on investigating the composition of flavonoids and phenolic compounds and antioxidant capacities of *Apium graveolens* L. (Yildiz *et al*. 2008; Yao *et al*. 2010). Especially, Zhang and coworkers optimised the appropriate MAE conditions for TF by RSM and found that the yield of TF from celery was enhanced to 2.443 mg/g at optimum extraction conditions of 520 W microwave power, 32 ml/g solid-liquid ratio and 80% ethanol concentration (Zhang *et al*. 2010).

However, to the best of our knowledge, few previous studies have been performed using MAE technology for flavonoid and phenolic extraction from celery leaves until now. Therefore, the purpose of this study was to optimise the best combinations of three MAE process parameters for the extraction of TF and TP from celery leaves on the application of the RSM.

MATERIAL AND METHODS

Material. Fresh Jinnan celery was bought from the Hong Qi market (Tianjin, China). The leaves were collected, washed, and shade-dried at room temperature. A relatively homogenous powder was obtained by grinding with a blade mill and sieved through the 60-mesh sifter. The powder was kept at 4°C until use.

Reagents. Rutin and 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from the Sigma Chemical Co. (Shanghai, China). Gallic acid (GA), and Folin-Ciocalteu phenol reagent were purchased from the Sinopharm Chemical Reagent Co. (Shanghai, China). The other reagents used were of analytical grade.

MAE extraction procedure. The process of MAE of celery leaves was performed in a domestic microwave extraction apparatus (CW-2000; Shanghai XTrust Instruments Company, Shanghai, China) equipped with a 250 ml quartz vessel and a cool water circulation system. Precisely 2.0 g of dried sample was placed in a round bottom flask with different volumes of extraction solvent according to the BBD. After the extraction procedure, the leaf extracts were filtered through Whatman filter paper. The filtrate was transferred into a volumetric flask and diluted to 100 ml for a quantitative analysis.

RSM optimisation procedure. Response surface methodology was employed to optimise extraction parameters of the MAE process (Cheng *et al*. 2014). The optimisation was performed according to the BBD with 3 variables at 3 levels on the yields of TF, TP, and antioxidant activity (AC) by the DESIGN EXPERT software (Trial Version 8.0.6). Based on our previous single-factor experiments, microwave power (MP), solid-solvent ratio (SSR), and ethanol concentration (EC) were chosen as independent variables with ranges of 300–500 W, 10–30 ml/g, and 50–80%, respectively. Data from the BBD were analysed by multiple regressions to fit the quadratic model. The second-order model equation for each response was as follows:

$$Y = \beta_0 \pm \sum_{i=1}^{3}\beta_i X_i \pm \sum_{i=1}^{3}\beta_i X_i^2 \pm \sum_{\substack{i=1 \\ i<j}}^{2}\sum_{i=2}^{3}\beta_j X_i X_j \quad (1)$$

where: Y – predicted response; β_0 – intercept; β_i, β_{ii}, β_{ij} – regression coefficients of linear, quadratic, and interactive effects, respectively; X_i, X_j – independent coded variables that affect the responses

Table 1 shows the coded levels of the independent variables and the parallel parameter values. Analyses of variance (ANOVA) were used to determine the statistical significance of the model ($P < 0.05$). The adequacy of the models was predicted by evaluating the coefficient of determination (R^2) and the lack of fit. In addition, three-dimensional (3D) response surface generated by keeping one response variable

Table 1. Coded values and corresponding actual values of the independent variables used in the Box-Behnken design

Code	Microwave power (W)	Solid-solvent ratio (ml/g)	Ethanol concentration (%)
–1	300	10	50
0	400	20	65
1	500	30	80

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at its optimal level and plotting this against two independent variables was used in order to decide the relationship between the response and independent variables.

Determination of TF. The determination of TF in the plant extracts was carried out according to Shao *et al*. (2012). Briefly, 6.0 ml of the extract solution was added separately 1 ml 5 wt% $NaNO_2$, 1 ml 10 wt% $Al(NO_3)_3$, 10 ml 5 wt% KOH. After mixing, the absorbance of the sample was measured at 500 nm with an ultraviolet spectrophotometer (UV-5200; Shanghai Metash Instruments Co., China). Rutin standards were employed to prepare the calibration curve:

$$y = 9.7868x + 0.0263, \quad R^2 = 0.9908 \qquad (2)$$

where: y – absorbance at 500 nm; x – concentration of flavonoids (mg/ml)

TF yield of the samples was calculated using the calibration standard and expressed as rutin equivalents per 100 grams of dry weight (g RUE/100 g DW).

Determination of TP. TP contents of the extracts were determined by the Folin-Ciocalteu reagent (McDonald *et al*. 2001). Briefly, 2 ml of the sample were mixed with 4.0 ml of 2% Na_2CO_3. After incubation at room temperature for 2 min, 5 ml of 10% Folin-Ciocalteu phenol reagent (diluted with distilled water) were added. The reaction mixture was mixed thoroughly and allowed to stand for 15 min in the dark at room temperature. Absorbances of the samples were measured at 765 nm using the spectrophotometer (7200; Unico, Shanghai, China). Measurements were based on the calibration curve with gallic acid:

$$y = 0.00199x + 0.00154, \quad R^2 = 0.9908 \qquad (3)$$

where: y – absorbance at 765 nm; x – concentration of total phenols (μg/ml)

TP yields were calculated and expressed as gallic acid equivalents per 100 g of dry weight (g GAE/100 g DW).

Determination of antioxidant activity. The AC of the extracts was measured by a DPPH inhibition assay according to the method described by de Ancos *et al*. (2002). Briefly, 10 ml of the sample was mixed with 3.99 ml of 70% ethanol of DPPH (25 mM). The reaction mixture was vortexed for 1 min and incubated at room

Table 2. Three-factor and three-level Box-Behnken design (BBD) used for response surface methodology (RSM), observed actual data and predicted data

Run	Std	Microwave power (W)	Solid-solvent ratio (ml/g)	Ethanol concentration (%)	TF (g RUE/100 g DW)		TP (g GAE/100 g DW)		DPPH scavenging activity (%)	
					actual	predicted	actual	predicted	actual	predicted
1	8	500	20	80	0.56	0.56	2.38	2.36	82	80.63
2	16	400	20	65	0.45	0.43	2.51	2.48	68	68.00
3	12	300	30	80	0.49	0.49	2.19	2.33	76	75.38
4	5	400	20	50	0.17	0.17	0.87	0.89	50	51.38
5	15	300	20	65	0.43	0.43	2.49	2.48	65	68.00
6	3	300	30	65	0.39	0.37	1.50	1.44	49	50.25
7	7	500	20	80	0.35	0.37	1.52	1.44	52	51.38
8	4	400	30	65	0.62	0.62	3.05	2.93	79	81.00
9	10	400	30	50	0.32	0.35	1.33	1.37	61	58.38
10	11	400	30	80	0.37	0.34	1.28	1.24	50	52.63
11	14	500	10	65	0.42	0.43	2.58	2.48	70	68.00
12	6	400	20	50	0.46	0.44	1.46	1.54	62	62.63
13	17	400	20	65	0.39	0.45	2.39	2.48	68	68.00
14	2	500	10	65	0.41	0.43	1.33	1.39	57	55.75
15	13	400	20	65	0.47	0.43	2.42	2.48	71	68.00
16	9	400	10	50	0.16	0.16	0.96	0.82	51	51.63
17	1	300	10	65	0.40	0.43	1.21	1.33	48	46.00

TF – total flavonoids; TF – total phenols; RUE – rutin equivalents; GAE – gallic acid equivalents; DPPH – 1-diphenyl-2-picrylhydrazyl

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temperature for 30 min in the dark. The light absorption of the reaction mixture was measured spectrophotometrically at 515 nm. Ethanol instead of the sample solution was used as a control. The DPPH scavenging rate was calculated using the following equation:

$$\text{DPPH scavenging rate (\%)} = 100 \times (Ac - As)/Ac \quad (4)$$

where: Ac, As – absorbances at 515 nm of the control and sample, respectively

RESULTS AND DISCUSSION

Model fitting. BBD based on 3-level incomplete factorial designs is a class of rotatable second-order designs for multivariate optimisation (Ferreira *et al.* 2007). In this study, three effectual variables (MP, EC, and SSR) that affect the MAE of *Apium graveolens* L. leaves on yields of TF and TP were optimised using BBD. All data were received from 17 experimental runs. As shown in Table 2, the highest yields of TF and TP were obtained using treatments 8, with the respective values of 0.62 g RUE/100 g DW and 3.05 g GAE/100 g DW, and the lowest yields of TF and TP were obtained using treatments 4, with the respective values of 0.17 g RUE/100 g DW and 0.87 g GAE/100 g DW. In addition, when treatments 17 and 1 were employed, the AC measured by the DPPH inhibition rate ranged from 48% to 82%, respectively. After the regression analysis of the data shown in Table 2, the second-order polynomial equation developed for TF and TP in terms of coded units was as follows:

$$Y_{TF} = 0.43 + 0.12A + 0.084B + 0.083C + 0.0075AB - 0.01BC - 0.02AC + 0.012A^2 - 0.038B^2 - 0.061C^2 \quad (5)$$

$$Y_{TP} = 2.48 + 0.39A + 0.41B + 0.34C + 0.36AB + 0.068AC + 0.13BC - 0.29A^2 - 0.41B^2 - 0.63C^2 \quad (6)$$

$$Y_{AC} = 68 + 10.13A + 7.38B + 4.5C + 5.25AB + 4.50AC + 4BC - 3.88A^2 - 5.88B^2 - 2.63C^2 \quad (7)$$

ANOVA of models. ANOVA was used to determine the significance of regression coefficients of the quadratic polynomial model. The coefficient of determination (R^2) indicates that the model accurately represents the relationship between the variables

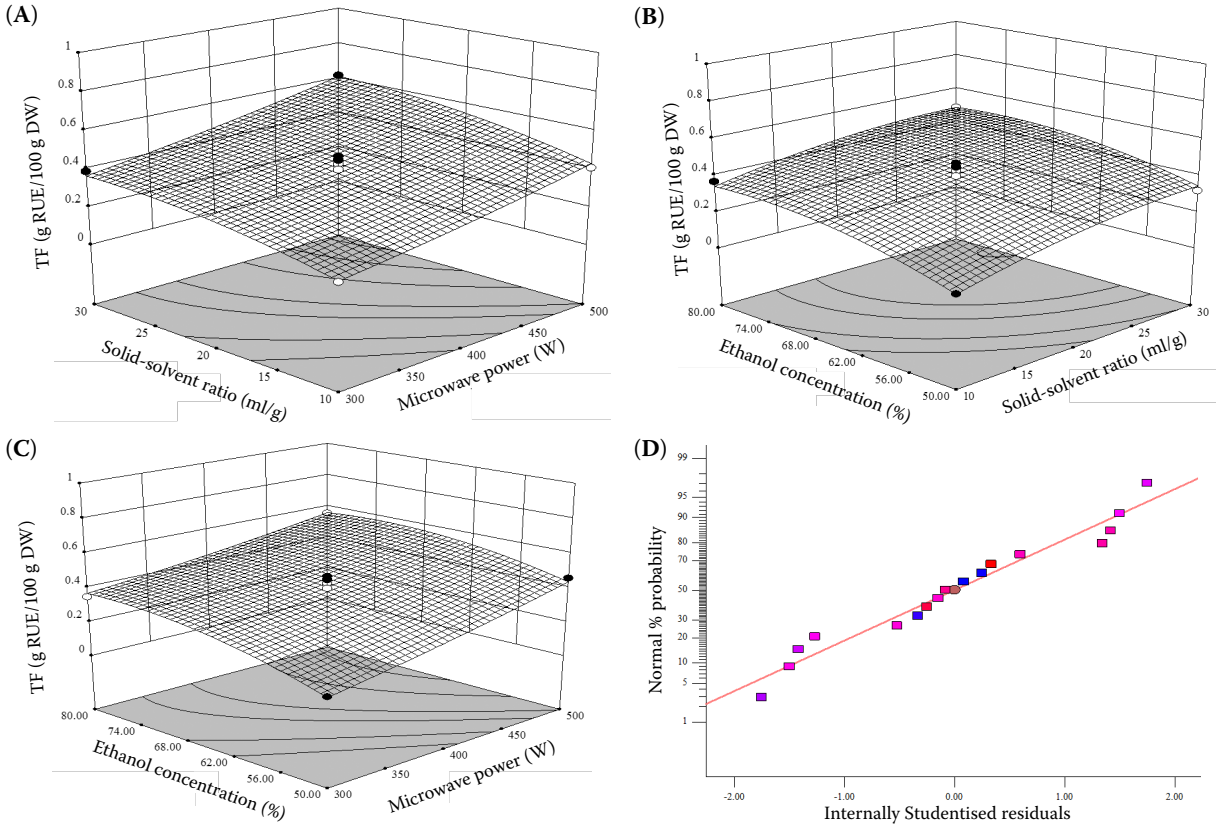

Figure 1. Response surface plots for extracting total flavonoids (TF) by microwave-assisted extraction (MAE): (**A**) solid-solvent ratio and microwave power, (**B**) ethanol concentration and solid-solvent ratio, (**C**) ethanol concentration and microwave power, and (**D**) actual versus predicted values obtained from an estimated model

RUE – rutin equivalents

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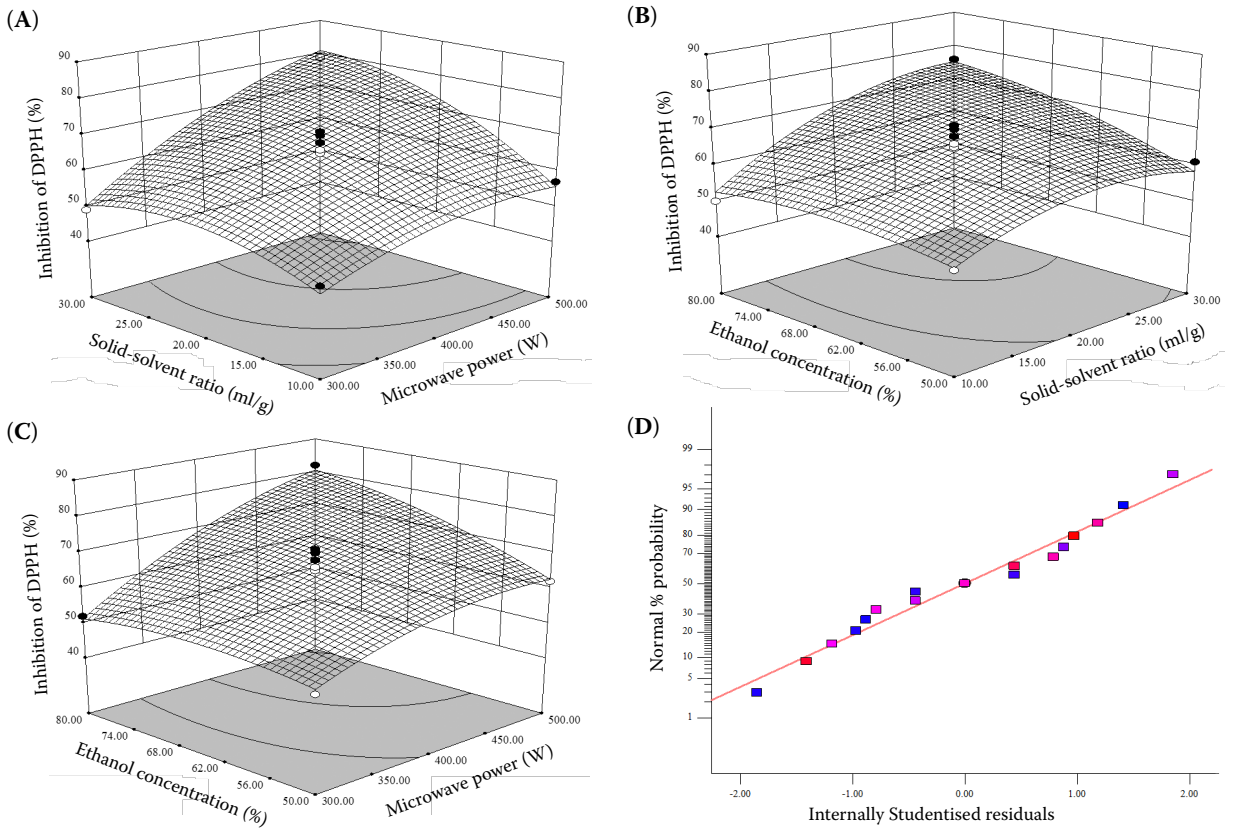

Figure 2. Response surface plots for 1-diphenyl-2-picrylhydrazyl (DPPH) inhibition by microwave-assisted extraction (MAE): (**A**) solid-solvent ratio and microwave power, (**B**) ethanol concentration and solid-solvent ratio, (**C**) ethanol concentration and microwave power, and (**D**) actual versus predicted values obtained from an estimated model

chosen. The model with $R^2 > 0.75$ was considered acceptable (Yang *et al.* 2010). Moreover, the lack of fit need not be significant for a valid model development by RSM (Noordin *et al.* 2004). As shown in Table 3, the models of the TF extraction were highly significant (F-value 20.13, $P < 0.0001$) with the determination coefficient $R^2 = 0.9748$ ($R^2_{adj} = 0.9542$), while the models of the TP component extraction were also highly significant (F-value 48.48, $P < 0.0001$) with the determination coefficient $R^2 = 0.9842$ ($R^2_{adj} = 0.9639$), and the predicted models for AC implied that the model was highly significant (F-value 26.74, $P = 0.0001$) with a good determination coefficient ($R^2 = 0.9717$; $R^2_{adj} = 0.9354$). Furthermore, no significance ($P < 0.05$) of the lack of fit indicated that a few unknown variables can influence the responses. Meanwhile, the coefficients of variation (CV) of TF, TP, and AC were 7.63, 6.9, and 4.6% respectively. The small value of CV implied that variation in the mean value is low and can satisfactorily develop an adequate response model (Liyana-Pathirana & Shahidi 2005). Figures 1D–3D depicts the plot of actual values versus predicted values for the three estimated models. In all three figures, the actual point cluster around the diagonal line reveals the experimental values fitted well with the predicted values. With all written above in mind, the obtained predicted model could optimise the conditions for maximum response.

Response surface analysis. The relationship between variables is illustrated in a 3D response surface, which graphically shows the sensitivity of the response value towards the change in the independent variable, namely microwave power, ethanol concentration, and solid-solvent ratio (Figures 1–3). Figure 1A shows the effect of the interaction of MP and SSR on the TF yield of the extract at a fixed EC of 65%; the minimum TF yield was obtained at the lowest MP and the maximum TF value was obtained at 500 W at the fixed SSR of 30 ml/g. Figure 1B depicts that an increase of microwave power from 300 W to 500 W and an increase of SSR from 10 ml/g to 30 ml/g enhanced the TF yield. Figure 1C indicates that the TP increased rapidly with an increase of SSR at a fixed EC, while with an increase in EC beyond 74%, the increase of TP was slight. The result of this study showed that MP is the critical factor that

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Table 3. ANOVA for the response surface quadratic models

Source	Sum of squares	*df*	Mean of square	*F*-Value	*P*-value	
Total flavonoids						
Model	0.24	9	0.027	30.13	< 0.0001	significant
A	0.11	1	0.11	120.22	< 0.0001	
B	0.056	1	0.056	62.40	< 0.0001	
C	0.054	1	0.054	60.55	0.0001	
AB	2.250E-004	1	2.250E-004	0.25	0.6323	
AC	1.600E-003	1	1.600E-003	1.78	0.2240	
BC	4.000E-004	1	4.000E-004	0.44	0.5262	
A^2	5.813E-004	1	5.813E-004	0.65	0.4478	
B^2	6.160E-003	1	6.160E-003	6.85	0.0345	
C^2	0.016	1	0.016	17.28	0.0043	
Residual	6.295E-003	7	8.993E-004			
Lack of fit	3.375E-003	3	1.125E-003	1.54	0.3343	not significant
Pure error	2.920E-003	4	7.300E-004			
Total phenol						
Model	7.13	9	0.79	48.48	< 0.0001	significant
A	1.22	1	1.22	74.48	< 0.0001	
B	1.35	1	1.35	82.82	< 0.0001	
C	0.95	1	0.95	57.87	0.0001	
AB	0.51	1	0.51	31.29	0.0008	
AC	0.018	1	0.018	1.12	0.3260	
BC	0.073	1	0.073	4.46	0.0725	
A^2	0.36	1	0.36	22.28	0.0022	
B^2	0.71	1	0.71	43.64	0.0003	
C^2	1.65	1	1.65	101.16	< 0.0001	
Residual	0.11	7	0.016			
Lack of fit	0.092	3	0.031	5.39	0.0687	not significant
Pure error	0.023	4	5.670E-00			
DPPH inhibition rate						
Model	1934.22	9	214.91	26.74	0.0001	significant
A	820.13	1	820.13	102.06	< 0.0001	
B	435.13	1	435.13	54.15	0.0002	
C	162.00	1	162.00	20.16	0.0028	
AB	110.25	1	110.25	13.72	0.0076	
AC	81.00	1	81.00	10.08	0.0156	
BC	64.00	1	64.00	7.96	0.0257	
A^2	63.22	1	63.22	7.87	0.0263	
B^2	145.33	1	145.33	18.09	0.0038	
C^2	29.01	1	29.01	3.61	0.0992	
Residual	56.25	7	8.04			
Lack of fit	30.25	3	10.08	1.55	0.3322	not significant
Pure error	26.00	4	6.50			

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Table 4. Coefficinet of determination (R^2), R^2_{adj}, and coeficient of variation (CV) of the predicted models

	R^2	R^2_{adj}	CV%
Total flavonoids	0.9748	0.9542	7.63
Total phenols	0.9842	0.9639	6.90
DPPH	0.9717	0.9354	4.56

influenced the response. The extraction of flavonoids can be improved as a result of enhancement of component solubility in the extraction solvent, diffusion rate, solvent viscosity, and the decreased surface tension (Ju & Howard 2003). However, we cannot say that this trend will continue even for high MP, because a further increment of MP may result in an increase in the temperature of the extraction solvent, the resulting excessive heat can influence the stability of flavonoids and phenolic components due to destabilisation by a reaction with other chemical components or enzymes, chemical, and enzymatic degradation or thermal decomposition, thus reducing the extraction efficiency (Durling *et al*. 2007).

Figure 2A reveals the effect of interaction between MP and SSR on TP. With the increase of MP and SSR, the TP yield was enhanced to a maximum value at 500 W and 30 ml/g SSR. The increase of MP from 300 W to 500 W with the increase of EC from 50% to 70% resulted in an increase of the TP yield, however, at an increase of EC over 70% there was a gradual decline in the response (Figure 2B). The same trend was observed for the interactive effect of EC and SSR (Figure 2C). Considering the safe manipulation, environmental pollution, and economic savings (Pompeu *et al*. 2009; Ballard *et al*. 2010), ethanol-water mixtures were used as the solvent in this work. Although the increment of EC can increase the TP yield, a further increase in the ethanol concentration appeared to be disadvantageous for the TP yield. The phenomenon was similar to that of the study conducted by Liu *et al*. (2015). Increasing the ethanol concentration may increase the amount of impurities and change the extraction solvent polarity.

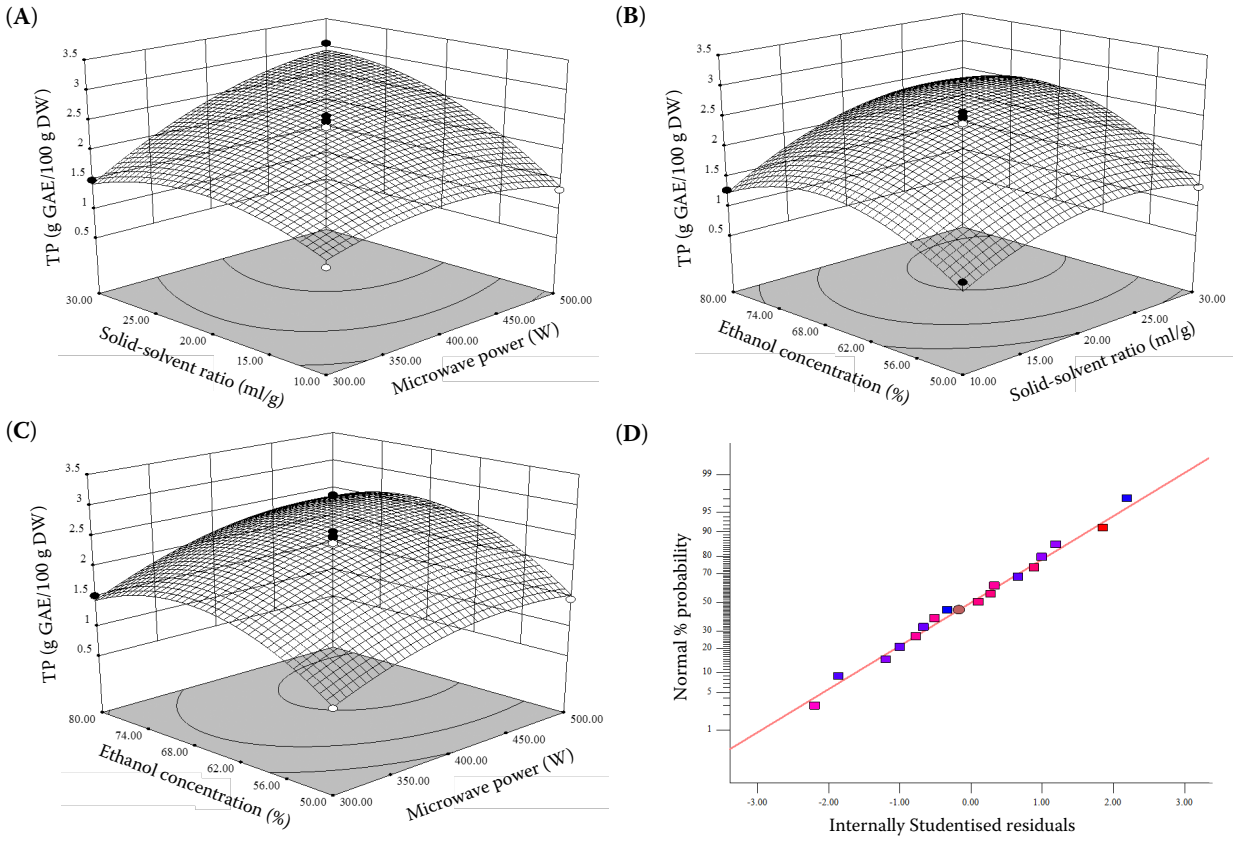

Figure 3. Response surface plots for extracting total phenols (TP) by microwave-assisted extraction (MAE): (**A**) solid-solvent ratio and microwave power; (**B**) ethanol concentration and solid-solvent ratio, (**C**) ethanol concentration and microwave power, and (**D**) actual versus predicted values obtained from an estimated model

GAE – gallic acid equivalents

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Table 5. Optimum conditions for the microwave-assisted extraction (MAE) of total flavonoids (g RUE/100 g DW), total phenols (mg GAE/100 g), and antioxidant activity (DPPH) (%) from celery leaves

	Actual	Predicted	95% CI
Total flavonoids	0.59 ± 0.07	0.62	(0.55, 0.69)
Total phenols	3.05 ± 0.18	3.01	(2.70, 3.31)
DPPH (%)	89.53 ± 0.82	88.84	(82.16, 95.52)

Figure 3 shows the effect of interaction of independent variables on the DPPH inhibition rate. The interactive effects of two variables are similar, namely, when one factor was fixed at the central level, the increase of the other two factors results in the increase of the response to a peak value at maximum levels of the variables. The DPPH inhibition activity was significantly affected by MP, SSR, and EC ($P < 0.01$) with three linear effects (A, B, and C), two quadratic (A^2 and B^2), and three interactive effects (AB, BC, and AC). It is generally acceptable that the DPPH assay has been widely accepted as a method to evaluate the free radical-scavenging activity of natural compounds (Nagai *et al*. 2003). In the present study, both TF and TP had a significant ($P < 0.01$) correlation with AC ($r = 0.76$, $r = 86$, respectively). This result was further evidence that flavonoids and phenolic constituents from celery had a noticeable effect of scavenging on DPPH radical.

Verification of the models. In the present study, for all responses only one optimal condition was obtained: 500 W MP, 30 ml/g SSR, and 75.54% EC. Samples were repeatedly extracted 3 times under the optimum conditions, the observed and predicted values for TF, TP, and DPPH inhibition rate were: TF (g RUE/100 g DW) (observed 0.59 ± 0.07; predicted 0.62), TP (g GAE/100 g DW) (observed 3.05 ± 0.18; predicted 3.01), and DPPH inhibition (%) (observed 89.53 ± 0.82, predicted 90.37). The experimental data were within the 95% confidence interval of predicted values. Furthermore, predicted values are reasonably close to the experimental values, which verified the validity and the adequacy of the predicted models.

CONCLUSION

In this study, MAE extractions of TF, TP, and antioxidant capacity of celery leaves were successfully optimised using RSM. The results indicated that MP, SSR, and EC all influenced significantly the investigated response variables. The second-order polynomial model can be applied to optimise the parameters of celery leaf extraction to obtain an extract with high yield of TF and TP, and high antioxidant capacity. It was found that microwave power of 500 W, 30 ml/g solid-solvent ratio, and 75.54% ethanol concentration were the appropriate conditions for maximum values of TF and TP from celery leaves with the DPPH inhibition rate. It could be concluded that flavonoids and phenol compounds from the curry leaves contributed to the antioxidant activity of the extract.

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