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OPTIMISATION OF THE MICROWAVE-ASSISTED EXTRACTION OF NATURAL ANTIOXIDANTS FROM DEFATTED BLACK RICE BRAN OF *ORYZA SATIVA* L. CV. HOMNIN

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ABSTRACT

This research aimed to improve antioxidant extraction of Hom Nin rice bran using microwave-assisted extraction by Response Surface Methodology. The results showed that the predicted values i.e. total phenolic content, total flavonoids, anthocyanin and antioxidant activities *(*DPPH*)*, were close to the predicted results which were 89.89 mg GAE/g DM, 76.98 mg CE/g DM, 18.08 mg C3G/g DM and 107.72 µmol TE/g DM, respectively. Comparing with the results obtained from the optimum extraction condition which the three factors were used as followings*:* 648 watt of power energy, 0.076 mol/dm^3 of citric acid concentration and 83 second of extraction time, the experimental values were 90.59 mg GAE/g DM, 77.53 mg CE/g DM, 18.82 mg C3G/g DM*)*, 109.35 µmol TE/g DM, respectively.

Keywords: Microwave assisted extraction ; Antioxidant ; Hom Nin rice bran

INTRODUCTION

Rice (*Oryza sativa* L.) is the principle cereal food in Asia and staple food of nearly half of the world's population. Rice is the most common crop, and very important in the world. In Asia, rice is so central to the culture that the word is almost synonymous with food **(Danielski *et al.*, 2005; Xia *et al.*, 2006; Lu *et al.*, 2008)**. It is commonly been believed that rice provides more health benefits than other carbohydrate sources since it contains antioxidant compounds and several nutrients **(Vijittra *et al.*, 2011)**. Furthermore, the consumption of colored rice increased dramatically **(Kong and Lee, 2010)**. It has been reported that colored rice comprised higher levels of antioxidative content in its seed coat and pericarp than white rice **(Yodmanee *et al.*, 2011)**. Rice bran contains the strongest antioxidants and significant amount of natural phytochemicals (**Godber & Juliano, 2004**). The major of antioxidative compounds in the colored rice seed coat are phenolic compounds, mainly anthocyanins, a subgroup of flavonoids **(Abdel *et al.*, 2006)**. Phenolic compounds provide health benefits forasmuch their antioxidant activities, which have inhibited effects on carcinogenesis and mutagenesis (**Vattem *et al.*, 2005**). In addition, black rice or purple rice has been known to relieve the risk of chronic diseases; such as, cardiovascular, cancers problems, diabetes and its complications **(Xia *et al.*, 2006; Walter and Marchesan, 2011)**. It also has been reported that black rice has more therapeutic values and nutritional compared to white rice **(Choi *et al.*, 2007; Chen *et al.*, 2006)**.

Oryza sativa L. CV. Homnin rice is one type of black rice and it has higher nutritional value than other rice varieties such as protein, minerals and vitamins **(Suzuki *et al.*, 2004)**. Furthermore, the amount of vitamin B, vitamin E, calcium, niacin, iron, zinc and magnesium were higher when compared to non-colored rice and and it could be important fountainhead of anthocyanin. Anthocyanins in natural's source offer health benefits in anti-cancer, decrease of dyslipidemia, reduction of coronary heart disease, prevention of heart disease, liver protection and improvement of visual acuity **(Mazza and Miniati, 1993; Chen *et al.*, 2006; Lee, 2010)**. Rice bran is an abundant and underutilized by-product of rice milling, and constitutes around 10% of the total weight of rough rice, which is generally used for animal feed. In addition, it offers nutritional value and antioxidants, as well as unpolished rice, such as tocopherols, phytic acid, tocotrienols and tricin and also pigments in which major of them are stored in the pericarp or bran of the rice grain, and the distinct pigmented are related to different colors; such as, red, black and purple **(Hu *et al.*, 2003; Vijittra *et al.*, 2011; Anek *et al.*, 2018)**.

Extraction of antioxidative compounds has become an alternative method for improving by-product from colored rice to be a more valuable product. Howsoever, the antioxidant stability of the extract is an affair of large concern due to the effects of the extraction procedure and also the charge of extraction. Therefore, the extraction temperature needs to be considered due to some instability of the antioxidative compounds with heat **(Benchahem *et. al.*, 2015).** For those antioxidantive compounds that react to alkaline compounds and acidic aqueous solvents, neutral solvents have been used for extraction, so to break the cell wall and cell membranes and at the meanwhile dissolve the water-soluble antioxidants. Normally, acetic acid, citric acid and hydrochloric acid are selected for acidulating the extraction solvent but using citric acid is also safe for consumer **(Li *et al.*, 2012; Mateus and Freitas, 2009; Anek *et al.*, 2018)**. Usually, conventional solvent extraction of antioxidative compounds are long time and solvent consuming while the efficiency is low. In addition, thermal extraction with a long time could cause the degradation of anthocyanins and then decrease the antioxidant capacity of the extracts **(Lapornik *et al.* 2005)**. Microwave-assisted extraction (MAE) utilizes the energy of microwaves to cause molecular rotation and movement of aqueous solution with a permanent dipole leading to a very fast heating of the sample and the solvent, providing advantages such as reduced extraction time, improved efficiency, high level of automation and low solvent consumption compared to conventional solvent extraction methods **(Sun *et al.*, 2007)**.

The objective of this research was to investigate the optimization conditions of microwave-assisted extraction on total phenolic and total flavonoid content as well as to evaluate the antioxidant capacity from the defatted Homnin rice bran extract. Therefore, natural antioxidative compound and also the natural color from the extracts of by-product materials could then be probably used to supersede synthetic colors and antioxidants in foods for safety concern.

MATERIALS AND METHODS

Rice bran sample

Homnin rice bran (must be within 24 hours after milling) was collected from the Nongpingkai Rice Mill Community enterprise, Kamphaeng Phet province, Thailand. The Homnin rice bran was sieved to separate the bran from the rice grains and then vacuum packed in laminate bag and stored at -20°C.

Chemicals and reagents

(+)-Catechin hydrate, Gallic acid, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl- 1-picrylhydrazyl (DPPH), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin-Ciocalteu's reagent,

methanol, sodium carbonate and ethanol were purchased from Merck (Darmstadt, Germany). Aluminum chloride hydrate was purchased from Ajax Finechem Pty Ltd. (Auckland, New Zealand). All chemicals and other reagents used in this study were analytical grade.

Sample preparation

Homnin rice bran ground to passed through 100 mesh sieves and heated by using oven at 100±3℃ for 15 minutes (Memmert UNE 500, Germany) to inactivate the endogenous lipase **(Juliano, 1985; Anek *et al*., 2018)**. Briefly, Homnin rice bran was defatted twice using hexane in bran to solvent ratio of 1:10 at a setting of 100 rpm in a rotary shaker for 2 hours and filter through Whatman No. 1 filter paper at room temperature (RT). The defatted Homnin rice bran (DHRB) was air-dried overnight (12 hours). The DHRB was packed in laminate bags and stored at -20∘C. **(Kim *et al*., 2013; Anek *et al*., 2018)**.

Extraction of defatted Homnin rice bran (DHRB)

Two grams of DHRB was placed into Erlenmeyer flasks and extracted with different proportion of power energy from 298-800 watt, citric acid concentration in water from 0.033-0.117 mol/dm^3 (1:20 w/v), and extraction time from 13-147 seconds (Table 1). The mixtures were carried out in microwave reactor. Then, the extracts were centrifuged at 10℃ and 5,000 rpm for 10 min and the supernatants were collected and stored at -20℃ until use.

Table 1 Variables and their levels employed in a central composite design for optimization of DHRB extracts

Variable	Range and levels (coded)				
	-α(-1.68)	**-1**	**0**	**+1**	**+α(+1.68)**
Power energy (Watt)	298	400	550	700	800
Citric acid concentration (mol/dm^3)	0.033	0.05	0.75	0.1	0.117
Extraction time (second)	13	40	80	120	147

Determination of total phenolic content (TP)

TP of each DHRB sample was determined using the colorimetric method described by **Singleton *et al* (1999)** with modifications.
Briefly, 100 µl of DHRB extract solution were mixed with distilled H_2O (3 ml) and 10% v/v of Folin- Ciocalteu's reagent for 6 min. Sodium carbonate solution (2 mL, 7.5%) was added to the mixture. The absorbance of the resulting solution was measured after 2 hours at 750 nm. (Genersis10, Thermo Scientific, USA). Gallic acid was used as a standard and expressed as mg gallic acid per gram of DHRB dry matter (mg/g DM).

Determination of total flavonoid content (TF)

TF was determined according to the method described by **Yang *et al.* (2009) and Anek *et al.* (2018)** with some modifications. 100 µl of the extract was mixed with 1.25 ml of distilled H_2O, and then mixed with sodium nitrite solution (75 µl, 5%w/v). After 6 min, aluminum chloride solution (150 µl of 10%w/v) was added, the mixture was allowed to stand for another 5 min, and sodium hydroxide solution (0.5 ml, 1.0 M) was then added. The reaction solution was well mixed and kept for 15 min, and the absorbance was determined at 510 nm using a UV-Vis spectrophotometer (Genersis10, Thermo Scientific, USA) compared to the catechin standard. The TF of the extracts was expressed as mg of catechin equivalents per gram of the DHRB dry matter (mg CE/g DM).

Determination of total anthocyanin content (TA)

Total anthocyanin content in the samples were measured using a spectophotometricpH differential method of **Finocchiaro *et al.* (2010) and Anek *et al.* (2018)** with some modifications. Aliquots of appropriately diluted solutions were mixed thoroughly with potassium chloride buffer (25 mM, pH 1.0). The absorbance of the mixture was then measured at 520 and 700 nm against distilled water blank. The aliquots of appropriately diluted solutions were then combined similarly with sodium acetate buffer (400 mM, pH 4.5), and the absorbance of these solutions was measured at the same wavelengths.
The TA was calculated as equivalent to cyanindin-3-glucoside according to the following equation:

Total anthocyanin content = (ΔA × MW × DF × 1000) / e)

Where

ΔA= (Absλ520-Absλ700) pH 1.0 - (Absλ520-Absλ700) pH 4.5
MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside
DF = dilution factor
e = is the molar extinction coefficient, equaling 26,900 L/mol cm for cyanidin-3-glucoside.
1000 = conversion factor from g to mg.

The total anthocyanin was expressed as mg of cyanidin-3-glucoside equivalents per 1 gram of DHRB.

DPPH radical scavenging assay

The DPPH assay was carried out with slight modification **(Brand-Williams *et al.* 1995; Zhang *et al,* 2007; Anek *et al*., 2018)**. 100 µl of the DHRB extract was added to 3 ml of 0.1 mM DPPH solution (prepared in methanol). After 30 min of incubation at room temperaturein the dark, the absorbance at 517 nm was measured. The DPPH free radical scavenging activities of the DHRB extracts were expressed as µM of the Trolox equivalents (TE) per gram of rice bran dry matter using a standard curve of Trolox (µmol TE/g DM).

Experimental design and data analysis

The optimum of extraction parameters from DHRB by using RSM was employed in the optimum study. A central composite design (CCD) was used to investigate the effect of independent variables as power energy (X_1), citric acid concentration (X_2), extraction time (X_3) to be optimized for the extraction (Table 2). The complete design consisted of 20 experimental points including eight factorial points, six axial points and six center points. The data was fitted with a second order polynomial equation, which expressed total phenolic content (Y_1), total flavonoid (Y_2), anthocyanin (Y_3) and DPPH (Y_4). The equation was as follows:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ij} X_i^2 + \sum_{i=1}^{3}\sum_{j=1}^{3} a_{ij} X_i X_j \qquad (1)$$

Where Y indicates the response variables, b_0 is a constant, b_i , b_{ii} *and* b_{ij} are the linear, quadratic and interactive coefficients, respectively. X_i and X_j are the levels of the independent variables. Three-dimensional surface response plots were generated by varying two variables within the experimental range and holding the other constant at the central point. The analytical steps as analysis of variance (ANOVA), regression analysis and coefficients of the response surface equation were estimated by using Design expert v 6.10. Fisher's F-test determined the second-order polynomial model equation at a probability (P) of 0.001, 0.01 or 0.05. The test of statistical significance was based on the total error criteria with a confidence level of 95% ($p < 0.05$).

Verification of model

The predictive performance of the models depending on the combined effect of power energy (X_1), citric acid concentration (X_2), extraction time (X_3) was validated with an optimum extraction condition as predictive models of RSM. The criterion of fitting efficiency of data to the model was the average mean deviation Eq. (2).

$$E\% = \frac{1}{\eta_e}\sum_{i=1}^{n}\left\|\frac{V_E - V_P}{V_E}\right\| \times 100 \qquad (2)$$

Where η_e is the number of experiment data, V_P is the predicted value and V_E is the experimental value (Hossain et al., 2012).

RESULTS AND DISCUSSION

Optimization of DHRB extraction using MAE such as power energy, citric acid concentration and extraction time variables on total phenolic content (TP), total flavonoids (TF), anthocyanin (AT) and antioxidant activitiy (DPPH) of DHRB extracts.

The experimental data from central composite design demonstrates that the power energy (X_1), citric acid concentration (X_2) and extraction time (X_3) had revealed quadratic effect on TP (Y_1), TF (Y_2), AT (Y_3), DPPH (Y_4) (Table 1). The observed values of TP, TF and AT of DHRB extracts varied from 21.97 to 86.78 mg GAE/g DM, 15.00 to 78.33 mg CE/g DM and 4.16 to 16.98 mg C3G/g DM, respectively. The antioxidant activities of the DHRB extracts determined by the DPPH method was between 17.22 -40.27 µmol TE/g DM.
The experimental data allowed the development of mathematic equations resulting the predicted results. The second order models and regression coefficients of the intercept, linear, quadratic and interaction terms of model had significant effects of P<0.0001, P<0.001, P<0.01 or P<0.05 (Table 3). For all responses, the quadratic polynomial models were significant with P-values for P<0.0001.
The analysis of variance (ANOVA) to assess the goodness of fit of all parameters of the models and to estimate the statistic significant of the factor and interactions between terms is shown in Table 3. The R-squared (R^2) values were in the range between 0.962 to 0.989 and were in agreement with the Adjust R-squared (Adj R^2) values in the range between 0.927 to 0.968. Both R^2 and Adj R^2 values

indicated that the general availability and accuracy of the polynomial model were sufficient **(Han, *et al.*, 2016)**.

Table 2 Experimental design of central composite design (CCD) of TP, TF, AT and DPPH of DHRB extracts

Run no.	Factors			Responses			
	(X_1) [a] Power energy (Watt)	(X_2)[b] Citric acid concentration (mol/dm^3)	(X_3)[c] Extraction time (second)	TP (mg GAE/ g DM)	TF (mg CE/ g DM)	TA (mg C3G/ g DM)	DPPH (μ mol TE/g DM)
1	-1	1	-1	26.97	25.00	6.40	59.54
2	0	-1.682(α)	0	50.48	41.67	10.94	85.54
3	0	0	-1.682(α)	45.34	30.82	9.58	80.21
4	1	-1	1	62.34	52.37	13.99	85.58
5	-1	-1	-1	42.90	25.00	7.71	68.58
6	1.682(α)	0	0	80.76	57.67	16.19	95.03
7	0	0	1.682(α)	43.40	23.57	8.36	66.36
8	-1	-1	1	32.64	15.00	4.16	44.89
9	1	1	1	63.59	61.73	14.35	95.56
10	-1	1	1	21.97	20.19	5.17	45.11
11	1	1	-1	50.06	48.33	10.74	90.25
12	0	1.682(α)	0	38.25	34.07	9.82	82.38
13	1	-1	-1	55.26	47.47	11.65	86.97
14	-1.682(α)	0	0	36.30	26.57	5.57	40.27
15	0	0	0	84.79	67.30	16.25	99.36
16	0	0	0	85.40	70.83	17.02	102.69
17	0	0	0	83.32	71.11	17.22	99.83
18	0	0	0	86.59	78.33	16.42	101.97
19	0	0	0	85.17	75.00	16.98	103.25
20	0	0	0	86.78	74.67	16.89	104.26

a Level of power energy: -α(298), -1(400), 0(550), 1(700), +α(800)
b Level of citric acid concentration: -α(0.033), -1(0.050), 0(0.075), 1(0.100), +α(0.117)
c Level of extraction time: -α(13), -1(40), 0(80), 1(120), +α(147)

Table 3 Second order polynomial equations and regression coefficients and analysis of the models for four response variables

Coefficient	Responses			
	TP	**TF**	**AT**	**DPPH**
b_0	85.42^d	72.76^d	16.81^d	101.93^d
X_1	13.29^d	12.96^d	3.31^d	17.01^d
X_2	-3.74^d	0.19^{ns}	-0.20^{ns}	-0.06^{ns}
X_3	0.15^{ns}	-0.64^{ns}	-0.06^{ns}	-4.21^d
X_1^2	-9.99^d	-10.15^d	-2.18^d	-12.37^d
X_2^2	-15.00^d	-11.66^d	-2.35^d	-6.61^d
X_3^2	-15.00^d	-15.43^d	-2.85^d	-10.38^d
X_{12}	2.83^b	0.63^{ns}	-0.03^{ns}	2.76^b
X_{13}	4.48^d	4.14^{ns}	1.34^d	5.26^d
X_{23}	1.46^a	1.71^{ns}	0.45^a	2.00^a
Regression model				
F-value	326.17	27.97	165.10	244.24
P -value	<0.0001	<0.0001	<0.0001	<0.0001
Lack of fit				
F -value	3.03^{ns}	3.32^{ns}	2.67^{ns}	1.00^{ns}
P -value	0.125	0.107	0.153	0.500
R^2	0.987	0.962	0.963	0.976
R^2-*adj*	0.964	0.927	0.957	0.951

[ns] Not significant
[a] Significant at $P < 0.05$
[b] Significant at $P < 0.01$
[c] Significant at $P < 0.001$
[d] Significant at $P < 0.0001$

The lack of fit indicates the variation of the data around the fitted model. If the model were significant, it did not fit the data well **(Ghafari *et al.*, 2009; Han *et al.*, 2016)**. The P-value for lack of fit presents in Table 3, showing the non-significant, implying model had a significant correlation between the responses and variables. Therefore, the four responses suggested quadratic polynomial models could be adequately used to predict models. These results were corresponding with the finding of **González-Centeno *et al*, (2014)** who reported the significant of regression and non-significant lack of fit of model would be fitted to the experimental data which were suitable to predict within the system.

The effects of power energy, citric acid concentration and extraction time on TP, TF, AT and DPPH values of DHRB extract are depicted with the three dimension response surface plots. Fig.1-4 presents three dimension response surface showing interaction of two variables, while another variable was fixed constant at their respective zero level. The results indicated that power energy, citric acid concentration and extraction time had significantly affected (P<0.01) by the quadratic terms of TP, TF, AT and DPPH.

Response surface of TP, TF, AT and DPPH

The response surface of TP, TF, AT and DPPH as presented in Table 1 demonstrates that power energy (X_1), citric acid concentration (X_2) and extraction time (X_3) had a quadratic effect. The second-order polynomial equations of TP, TF, AT and DPPH were attained as follows:

$$Y_1 = 85.42 + 13.29X_1 - 3.74X_2 + 0.15X_3 - 9.99\ X_1^2 - 15.00\ X_2^2 - 15.00\ X_3^2 + 2.83X_1X_2 + 4.48X_1X_3 + 1.46X_2X_3 \quad (3)$$

$$Y_2 = 72.76 + 12.96X_1 + 0.19X_2 - 0.64X_3 - 10.15\ X_1^2 - 11.66\ X_2^2 - 15.43\ X_3^2 + 0.63X_1X_2 + 4.14X_1X_3 + 1.71X_2X_3 \quad (4)$$

$$Y_3 = 16.81 + 3.31X_1 + 0.20X_2 - 0.06X_3 - 2.18\ X_1^2 - 2.35\ X_2^2 - 2.85\ X_3^2 - 0.03X_1X_2 + 1.34X_1X_3 + 0.45X_2X_3 \quad (5)$$

$$Y_4 = 101.93 + 17.01X_1 - 0.06X_2 - 4.21X_3 - 12.37\ X_1^2 - 6.61\ X_2^2 - 10.38\ X_3^2 + 2.76X_1X_2 + 5.26X_1X_3 + 2.00X_2X_3 \quad (6)$$

As depicted in Table 3, each parameter had more significant influence on TP, TF and AT contents. All of equations were extremely significant (P<0.0001) and the

lack of fit was non-significant (P>0.05). Hence the results indicated that the equations were reasonable. ANOVA of model regression coefficients were significance. The effects of power energy (X_1) citric acid concentration (X_2) from linear, quadratic (X_1^2, X_2^2 and X_3^2) and interaction (X_1X_2, X_1X_3 and X_2X_3) of TP were significant (P<0.05), while the effects of citric acid concentration (X_2), extraction time (X_3) of TF and AT model from linear and interaction were non-significant but all quadratic parameters were significant (P<0.05). The R^2 for all responses TP, TF and AT were 0.987, 0.962 and 0.963, respectively, indicated that the model could explain 98.7, 96.2 and 96.3% variability of the response variables. These results were in accordance with **González-Centeno *et al.* (2014)** and **Sai-Ut *et al.* (2015)** who found that the R^2 values had to be higher than 0.80 which indicated a good correlation between responses and independent variable of model. The Adj R^2 of TP, TF and AT (0.964, 0.927 and 0.957) were agreed with the R^2. The P-value for lack of fit shows non-significant, implying the effects of independent variables on the TP, TF and AT were described by the obtained models.

The effect of independent variables i.e., power energy (X_1), citric acid concentration (X_2) and extraction time (X_3) on antioxidant activity obtained from DHRB with MAE was investigated. Antioxidant activity of DHRB determined by DPPH methods ranged between 40.27-104.26 µmol TE/g DM. The ANOVA indicated that three quadratic polynomial equations were highly significant (P<0.0001) while lack of fits from three equations were not significant (P>0.05), indicating the models were good fitted to the experimental data. These results were confirmed by coefficient of determination (R^2) of DPPH models, (0.976), which explained good regression values of all variances of the data.

The response surface three dimension plots are the representation of regression equation indicating the relationship between dependent and independent variables. The response surface plots are different shapes indicating whether the variables or mutual interactions are significant or not **(Liu *et al.*, 2013)**. The response surface plots based on equations 3-6, showing the effects of two variables within the experiment and another one variable was kept constant at their optimum values are presented in Fig. 1-4. The effects of power energy and citric acid concentration on TP, TF and AT contents are shown in Fig. 1a, 2a and 3a, respectively. Fig. 1a, 2a and 3a demonstrate the power energy (X_1), citric acid concentration (X_2) and their reciprocal interaction on TP, TF and AT contents, where the extraction time (X_3) was fixed at 80 seconds. The results indicated that TP, TF and AT increased slowly with increasing citric acid concentration while the increase of power energy resulted the rapidly increasing of TP, TF and AT content. However, beyond 0.075 mol/dm^3 and 625 watts, TP content decreases slightly.

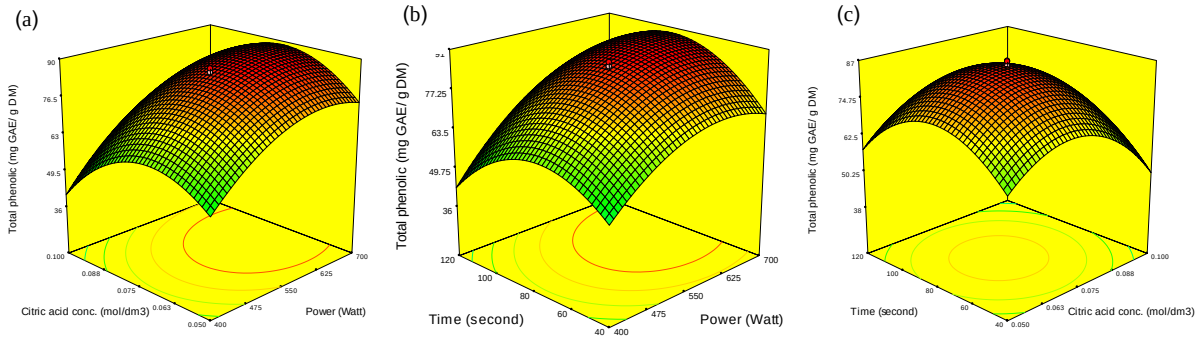

Figure 1 Response surface plots of DHRB showing the effects of power energy,citric acid concentration and extraction time on total phenolic content

The response surface three dimension plots in Fig. 1b, 2b and 3b, which kept the citric acid concentration at zero level (0.075 mol/dm^3), showed that TP, TF and AT contents increased with increasing extraction time at the initial stage and then slightly decreased of trend, while the increase of power energy resulted the rapidly increasing of TP, TF and AT content. However, beyond 80 second and 625 watts, TP, TF and AT content was decreased slightly. As shown in Fig. 1c, 2c and 3c, TP, TF and AT contents were increased when citric acid concentration increased from 0.050 to 0.075 mol/dm^3 and the extraction time from 40 to 90 second, respectively and then they began to decrease afterwards.

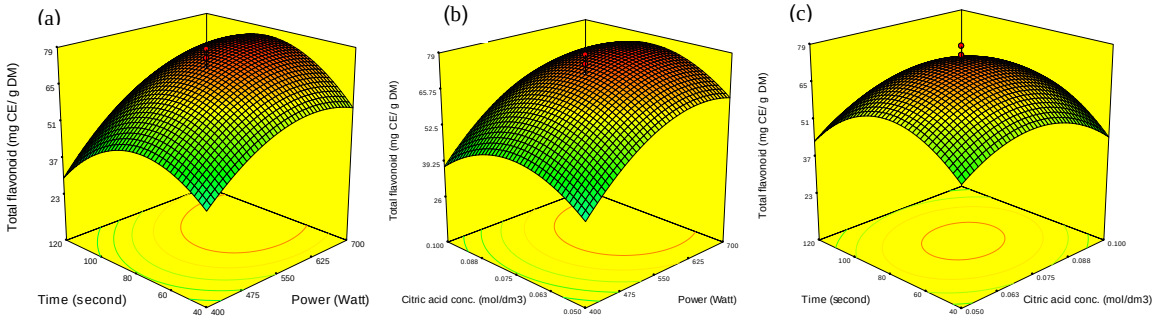

Figure 2 Response surface plots of DHRB showing the effects of power energy, citric acid concentration and extraction time on total flavonoid content

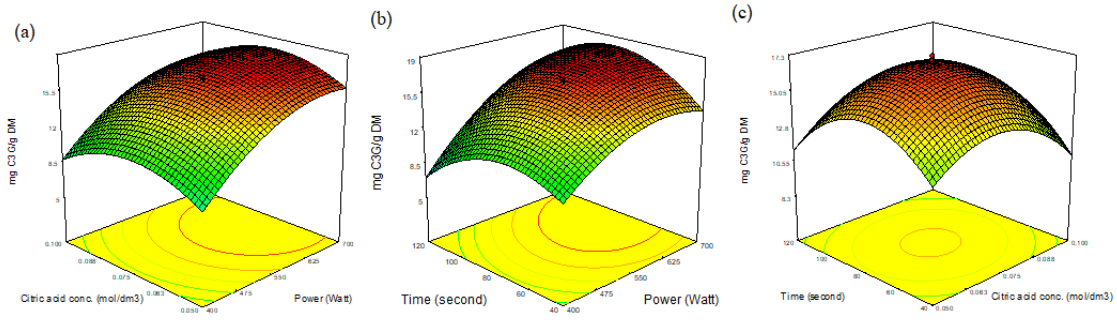

Figure 3 Response surface plots of DHRB showing the effects of power energy, citric acid concentration and extraction time on anthocyanin content

The relationship between the extraction factors and antioxidant activity investigated by three dimensional response surface plots, are presented in Fig. 4. The three-dimension response surface plots of DPPH assay were similar to the trend of TP, TF and AT. The response surface plots showed that DPPH increased slowly with increasing citric acid concentration as well as extraction time at the initial stage and then the antioxidant activity slightly decreased, while the increase of power energy resulted the rapidly increase of DPPH. However, beyond 625 watts and 0.075 mol/dm^3, the DPPH decreased slightly. Our results were in concordance with **Bachir Bey *et al*, (2014)** who reported the polarity played an important role in antioxidant activity. This might be assumed to the fact that citric acid is a strong organic acid, and there are three hydrogen ions (H^+) which can be ionized, so the pH value is much lower in the solution with higher citric acid concentration. In addition, microwave can make those polar ions move in the solution, and then heat the solution and there is much more H^+ in low pH solution, consequently, the low pH solution has higher temperature than the high pH solution **(Li *et al*., 2012)**. It was noticed that both citric acid concentration and power energy had significant interaction effect on DPPH.

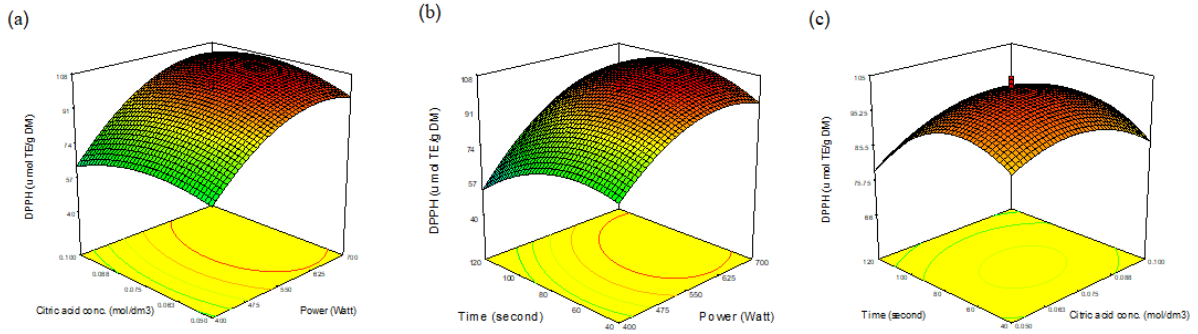

Figure 4 Response surface plots of DPPH of DHRB as affected by power energy, citric acid concentration and extraction time

The similar results were also reported in the extraction of anthocyanin content from grape peel by **Li *et al*, (2012)**, found that increasing power energy and citric acid concentration resulted in higher anthocyanin. In addition, the factors that mostly affected the total anthocyanin content were the concentration of citric acid, extraction time, power of the microwave, and ratio between the solvent and the sample, respectively. Citric acid concentration affecting anthocyanin extraction contents which may be attributed to the change of polarity of solvent as citric acid concentration was changed. More polar phenolic compounds may be extracted according to "like dissolves like" principle and solvent would extract compounds which have similar polarity with solvents **(Chew *et al*., 2011; Gong *et al*., 2012)**. Basically, phenolics could easily be dissolved from material cells when solvent polarity are similar to phenolics **(Sai-Ut *et al*., 2015)**. Furthermore, using acids to assist the phytochemical extraction would help disrupt the cell walls and cell membrane of the plant samples, consequently, anthocyanins could be released effectively in higher amounts.

When increasing citric acid concentration, the pH of solvent was lower and the TP, TF and AT were decreased. The result were similar to **Bahar *et al*. (2009)** studied the effect of solvent and citric acid concentration on the extraction of phenolic compounds from olives and found that when using a higher concentration of citric acid, the phenolic content was decreased. This was due to phenolic generally found in most plants include 3 different groups such as a free form, bound form and conjugated form. The bound form is chiefly found in the layer of lignin extracted by using alkaline or acidic hydrolysis, whilst the free form is the group that the structure is gently stable so that when using acidic extraction of phenolic compounds, it would be partially damaged. Howsoever, when using high concentration of acid, the plant cell walls and cell membrane were ruptured, so that the more active ingredients were released.

From the investigation of influence of extraction time on TP, TF and AT content found that TP, TF and AT were increased in the early stage until they reacted the maximum release and then they were decreased since longer extraction causes more degradation of the extract due to phenolics could more react with surrounding environment such as heat, oxygen and light **(Chew *et al*., 2011)**.

Optimum conditions from DHRB and model validation

The RSM procedure is used to determine the experimental factors and levels which would allow to obtain an extract with high phytochemicals and antioxidant activities. The predictive ability of the models was examined by extraction at optimum conditions. Table 4 shows the optimum extracting condition which the three factors obtained from the model were followings: 648 watts of power energy, 0.076 mol/dm^3 of citric acid concentration and extraction time of 83 seconds and the experimental values of 90.59 mg GAE/g DM (Y_1), 77.53 mg CE/g DM (Y_2), 18.82 mg C3G/g DM (Y_3) and 109.35 µmol TE/g DM (Y_4), were obtained. The experimental results were close to the predicted values of 89.89 mg GAE/g DM (Y_1), 76.98 mg CE/g DM (Y_2), 18.08 mg/g DM (Y_3), 107.72 µmol TE/g DM (Y_4), by the regression models. Validation step was done to ensure that the predicted results were not biased towards the practical value with each response to obtain maximum values **(Yim *et al*., 2012)** .The predicted values match well with the experimental values obtained by using optimum extracting conditions which the validation between the predicted and experimental values were within an acceptable error range as depicted by average mean deviation with the E% range between -1.67 to 3.95% (Table 4). Comparing with the conventional extraction method, MAE provided higher extraction efficiency as Halee *et al.* (2018) studied the extraction of defatted Homnin rice bran and found that TP, TF, AT and DPPH of the methanolic extract with 0.1 mol/dm^3 of citric acid were 86.63 mg of GAE/g DM, 14.67 mg CE/g DM, 6.18 mg C3G/g DM. and 29.02 µmol TE/g DM, respectively.

Table 4 Validation of predicted and experimental values of the TP, TF, AT and DPPH values from DHRB extract with different extraction conditions

Responses	Extracting conditions			
	Worse[a]	High[b]	Medium[c]	Optimum[d]
Predicted value				
TP	44.51	63.92	85.42	89.89
TF	29.49	54.53	72.77	76.98
AT	8.15	14.24	16.81	18.08
DPPH	69.84	95.32	101.94	107.72
Experimental value				
TP	46.02±0.49	65.08±0.43	86.59±0.85	90.59±0.46
TF	30.08±0.56	56.03±0.34	72.61±0.46	77.53±0.55
AT	8.02±0.43	14.62±0.66	17.06±0.52	18.82±0.49
DPPH	70.68±1.06	97.16±1.79	100.26±2.52	109.35±2.62
E%				
TP	3.27	1.78	1.35	0.77
TF	1.96	2.69	0.21	0.71
AT	-1.57	2.63	1.45	3.95
DPPH	1.18	1.89	-1.67	1.49

[a] Worse(run 5): $X_1 : X_2 : X_3$ as 400 : 0.05 : 40
[b] High (run 15) : $X_1 : X_2 : X_3$ as 700 : 0.10 : 120
[c] Medium (run 9) : $X_1 : X_2 : X_3$ as 550 : 0.075 : 80
[d] Optimum : $X_1 : X_2 : X_3$ as 648 : 0.076 : 83

The obtained optimum condition of power energy was in the same range as **Chen *et al.* (2016)** who reported the optimum power energy of 704 watts for extraction of antioxidant from tangerine peels. In addition **Oussaid *et al.* (2018)** reported the values of 600 watts of power energy and 69 second for extraction of phenolic compound from *Scirpus holoschoenus* L. The increase of power energy level beyond 500 watt could have speed-up the mass transfer of intracellular bioactive compounds. However, excessive microwave power could lead to the degradation of the total flavonoid content chargeable for the antioxidant capacity **(Dahmoune *et al.*, 2014; Spigno and De Faveri, 2009)**.

CONCLUSION

This study reveals that the optimum microwave assisted extraction condition was following: 648 watt of power energy, 0.076 mol/dm^3 of citric acid concentration and 83 second of extraction time and the experimental values of TP, TF, AT and antioxidant activity (DPPH) were 90.59 mg GAE/g DM, 77.53 mg CE/g DM, 18.82 mg C3G/g DM, 109.35 µmol TE/g DM, respectively. The predicted values matched well with the experimental values which the validation between the experimental values and predicted values were within an acceptable error range as depicted by average mean deviation with the E% ranged from -1.85 to 3.27.

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