ORIGINAL ARTICLE

Enhancement of Phenolics and Antioxidant Activity via Heat Assisted Extraction From *Moringa oleifera* Using Response Surface Methodology and Its Potential Bioactive Constituents

Ammar Akram Kamarudin[1], Norazalina Saad[2], Nor Hafiza Sayuti[1], Nor Asma Ab. Razak[1], Norhaizan Mohd. Esa[1,3]

[1] Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia;
[2] Laboratory of Cancer Research UPM-MAKNA (CANRES), Institute of Bioscience, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia
[3] Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia

ABSTRACT

Introduction: *Moringa oleifera* Lam. is a miracle tree that has been widely utilised in folklore medicine due to its immense amount of phenolic constituents that could treat various ailments. Different techniques have been implemented to extract the phenolic but the parameters may not be optimised to further enhance the amount of phenolic extracted. Thus, the work aimed to enhance phenolic content and antioxidant activity of *M. oleifera* through RSM methodology, which is rapid and convenience. **Methods:** At first, antioxidant activity of different parts of *M. oleifera* (leaves, stem, pod and seed) were investigated. The plant part with the highest antioxidant activity was selected for the optimisation of extraction condition using RSM. In RSM, temperature (X_A), extraction time (X_B) and solid-liquid ratio (X^C) were employed to study the effects on yield, total phenolics, flavonoids and antioxidant activity. Then, the optimum extraction condition obtained via RSM was utilised in LC-MS and HPLC analysis to determine the potential bioactive constituents. **Results:** The leaves of *M. oleifera* displayed the highest antioxidant activity as compared to other plant parts. The optimum extraction condition obtained for the leaves extract was: temperature (X_A): 82°C, extraction time (X_B): 48 min and solid-liquid ratio (X_C): 1:30 g/mL (w/v). Meanwhile, LC-MS revealed the presence of gallic acid, chlorogenic acid, quercetin, kaempferol and 3-O-glucoside kaempferol. HPLC analysis detected six compounds; gallic acid, epicatechin gallate, chlorogenic acid, myricetin, quercetin and kaempferol. **Conclusion:** The optimisation are promising to improve yield and antioxidant activity in *M. oleifera* as compared to non-conventional extractions.

Keywords: Preliminary, Optimization, CCD, Phenolics, Chromatography analysis

Corresponding Author:
Norhaizan Mohd Esa, PhD
Email: nhaizan@upm.edu.my
Tel: +603-97692427

INTRODUCTION

Moringa oleifera (*M. oleifera*) belongs to the family of Moringaceae and widely known as "the miracle tree". It is widely cultivated throughout the tropical region such as Asia and Africa (1). *M. oleifera* is locally known as the horseradish or drumstick tree due to its root's taste and pod's shape respectively (2). Besides high in protein, vitamin and mineral, the increasing interest for *M. oleifera* plant are also due to their unique bioactive constituents, namely phenolic acids and flavonoids that contribute to antioxidant and anti-inflammatory properties which promote harmful free radical scavenging and suppress enzyme oxidation (3).

In order to maximise the therapeutic effects of the bioactive compounds from *M. oleifera*, the extraction process plays a crucial role. The efficiency of extraction can be affected by various factors such as extraction time, temperature, volumes, type of solvent used and solid-liquid ratios (4). These parameters need to be optimised to avoid missing the potential antioxidant properties from the sample. Although numerous studies have analysed the antioxidant activity of *M. oleifera*, the sample usually extracted by the conventional procedure that consumes time as process variables need to be optimised sequentially with only limited yield produced.

Response surface methodology (RSM) has been extensively used to optimise the experimental conditions. It is an effective mathematical tool used to calculate the region of interest. It enables users to maximise or minimise the process variables as it evaluates multiple

responses simultaneously (5). Apart from that, RSM can also reduce time consumption as it evaluates multiple responses without avoiding the effects of the interaction between variables. Conventional optimisation such as one-variable-at-a-time approach is completely ineffective to determine the optimum condition when interaction effects between process variables are present throughout the model responses studied (6).

In the earlier part of this study, the antioxidant activity of leaves, stem, pod and seed of *M. oleifera* was analysed and the part that showed the highest antioxidant were forwarded to the optimisation of phenolics extraction via RSM. The optimum extraction conditions were employed in chromatographic analysis to determine the potential bioactive constituents in *M. oleifera* extract. To the best of our knowledge, there was no study conducted to improve phenolic extraction from different parts of *M oleifera* by using RSM.

MATERIALS AND METHODS

Sample collection and preparation

The leaves, stem, pod and seed of *M. oleifera* were collected from Port Dickson, Negeri Sembilan, Malaysia. The samples were dry heated in a warm air oven at 40°C for 24 h. Then, dried samples were ground to a fine powder and sieved before stored at 4°C for further analysis.

Chemicals and reagents

Gallic acid, sodium carbonate, Folin Ciocalteau reagent, rutin, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and potassium persulfate were purchased from Sigma-Aldrich, (Missouri, United States). Aluminum chloride was procured from R&M Marketing (Essex, United Kingdom), 6-hydroxy-2, 5, 7, 8-tetra-methylchroman 2- carboxylic acid (Trolox) was purchased from Calbiochem (San Diego, United States). 2,2'-azinobis (3-ethylbenzo-thiazoline-6-sulphonic acid) disodium salt (ABTS), methanol, formic acid and acetonitrile were obtained from Merck KGaA (Germany). All chemicals were analytical and HPLC grade.

Extraction of aqueous extract from M. oleifera

For the preliminary study of antioxidant activity, all parts of the plant (1 g each) were extracted in 30 mL deionised water at 60°C. Whereas during optimisation, the part with the best antioxidant activity was mixed with different ratios of deionised water (20-40 mL). The mixtures were exposed to various temperatures ranging from 50 to 90°C. All parameters in the experimental design were generated by Design Expert version 6.0 (State Ease, Inc.). The aqueous extract was filtered with Whatman No.1 filter paper, dried and stored at 4°C for further analysis.

Experimental design

Central composite design has been chosen to optimize three independent variables (XA = temperature, XB = extraction time, XC = solid-liquid ratio) at five coded levels. The process parameters were coded at -1.682, -1, 0, 1 and 1.682 and 20 runs of experimental designs were generated, as shown in Table I. The observed values were then fitted into second order polynomial and the regression coefficient (β) was generated as shown in Table IIa. Hence, the predicted optimum condition in the quadratic model was displayed as:

$$Y_k = b_{k0} \sum_{i=1}^{3} b_{ki} x_i + \sum_{i=1}^{3} b_{kii} x_i^2 + \sum_{j=1}^{3} b_{kij} x_i x_j \quad (1)$$

where b_{k0}, b_{ki}, b_{kii} and b_{kij} were the constant regression estimate (β) whereas xi and xj were the process variables.

Phytochemical analysis

Total phenolic content (TPC)

Total phenolic content was determined according to Folin Ciocalteau method described by Yusri et al. (7) with slight modifications. Briefly, an aliquot of the extract (100 µL) was mixed with 500 µL of 10% Folin Ciocalteau reagent. The mixture was reacted with 7.5% sodium carbonate (400 µL). The mixture was next incubated for 1 h in the dark environment at 40°C and read at 765 nm. Gallic acid served as a standard and expressed in mg gallic acid equivalent per mg sample (mg GAE/mg sample).

Total flavonoid content (TFC)

Total flavonoid content was conducted as described by Yusri et al. (7) with slight modifications. Aqueous extract (150 µL) was mixed with 150 µL aluminium chloride (20 mg/mL in methanol). The reaction mixture was incubated for 10 min and later read at 435 nm. Rutin acted as standard and expressed in mg rutin equivalent per mg sample (mg RE/mg sample).

Antioxidant activities

DPPH free radical scavenging assay

The procedure was conducted as performed by Pandey et al. (8) with several adjustments. The aqueous extract (50 µL) was mixed with 195 µL of DPPH solution (0.2 mM). The mixture was incubated for 1 h in the dark environment and read at 540 nm. Trolox (1 mg/mL) was used as standard and expressed in mg Trolox equivalent per mg sample (mg TE/mg sample).

ABTS scavenging assay

ABTS scavenging assay was conducted according to the procedure described by Re et al. (9) with minor modifications. Potassium persulfate (13 mg) and ABTS (76.8 mg) were mixed separately with 2 mL of deionised water. The total volume for both solutions were brought to 20 mL and later mixed together. The mixture was incubated for 16 h in a dark environment. The absorbance of the mixture was adjusted to 0.7 ± 0.2 at 735 nm with deionised water. The aliquot (24 µL) of the extract was mixed with 216 µL of ABTS solution. The mixture was incubated for 1 h and read at 734

nm. Trolox served as standard and expressed similar to DPPH scavenging assay.

Validation and statistical analysis

Analysis of variance (ANOVA) was conducted to identify significant models that fitted the second-order polynomial. Percentage of coefficient variation (% CV) and Student's t-test were performed to determine the significant effects between observed and predicted values for optimum extraction condition. The optimum extraction condition was later utilised in chromatographic separations.

Chromatographic separations

Screening of bioactive compounds via LC-MS analysis

The optimum extraction condition was utilised in LC-MS analysis using MicroTOF-Q model (Bruker Daltonics Inc., USA) supplemented with reverse phase C18 column (150 x 2.0 mm) according to the method described by Skendi et al. (10). The system was coupled to electrospray ionization (ESI) interface operating in positive ionisation mode and the gradient elution was scanned from m/z 50 to 1000. Two types of mobile phases used; Solvent A: 0.1% formic acid, Solvent B: acetonitrile. The elution was as followed:
0-5 min (5% acetonitrile); 10-25 min (95% acetonitrile); 26-30 min (5% acetonitrile). The flow rate was operated at 0.3 mL/min and the injection volume was 20 µL. The chemical composition was determined using MassBank (https://massbank.eu/) and METLIN Metabolite Database (https://metlin.scripps.edu/).

Quantification of bioactive compounds via HPLC analysis

The optimum extraction condition was also utilised in HPLC analysis using Shimadzu model (Shimadzu, Japan) supplemented with reverse-phase Zorbax Eclipse Plus C18 column (4.6 x 150 mm) according to the method described by Rodrнguez-Pйrez et al. (11) with slight modifications. The dried extract was dissolved with deionised water and passed through a 0.45 µm nylon syringe filter. Two types of mobile phases used; Solvent A: 0.5% formic acid, Solvent B: acetonitrile. The elution as followed: 0-9 min (5% B); 10-14 min (45% B); 15-17 min (65% B) and 19-21 min (5% B). The flow rate was 0.5 mL/min and the injection volume was 10 µL. The separation was detected by UV detector at 280 nm. The chemical composition was determined by comparing the retention times with the corresponding standards.

RESULTS

Antioxidant activities of M. oleifera plant parts

The leaves of *M. oleifera* displays the highest antioxidant activity through DPPH and ABTS scavenging assay with 48.30 TE mg/sample and 36.33 TE mg/sample respectively in Fig. 1a (i) and (ii). In contrast, the seed of *M. oleifera* recorded the lowest antioxidant activity in both assays with 12.62 TE mg/sample and 5.38 TE mg/sample respectively. Stem and pod of *M. oleifera* recorded 35.13 TE mg/sample and 15.6481 TE mg/sample in DPPH assay while 24.58 TE mg/sample and 11.05 TE mg/sample in ABTS assay respectively. Hence, the antioxidant activity of *M. oleifera* could be arranged as followed: leaves > stem > pod > seed as displayed in both DPPH and ABTS scavenging assays. Fig. 1b (i) showed the highest extraction of phenolic content recorded was from leaves with 69.98 GAE mg/sample while the lowest total phenolic content recorded was from the pod with 24.58 GAE mg/sample respectively. Stem also displayed a high amount of phenolic content with 55.66 GAE mg/sample while the seed of *M. oleifera* recorded only 26.28 GAE mg/sample.

Fig. 1b (ii) illustrated the stem of *M. oleifera* displayed the highest amount of flavonoid with 25.66 RE mg/sample while seed recorded the lowest flavonoid content with 6.93 RE mg/sample respectively. The leaves

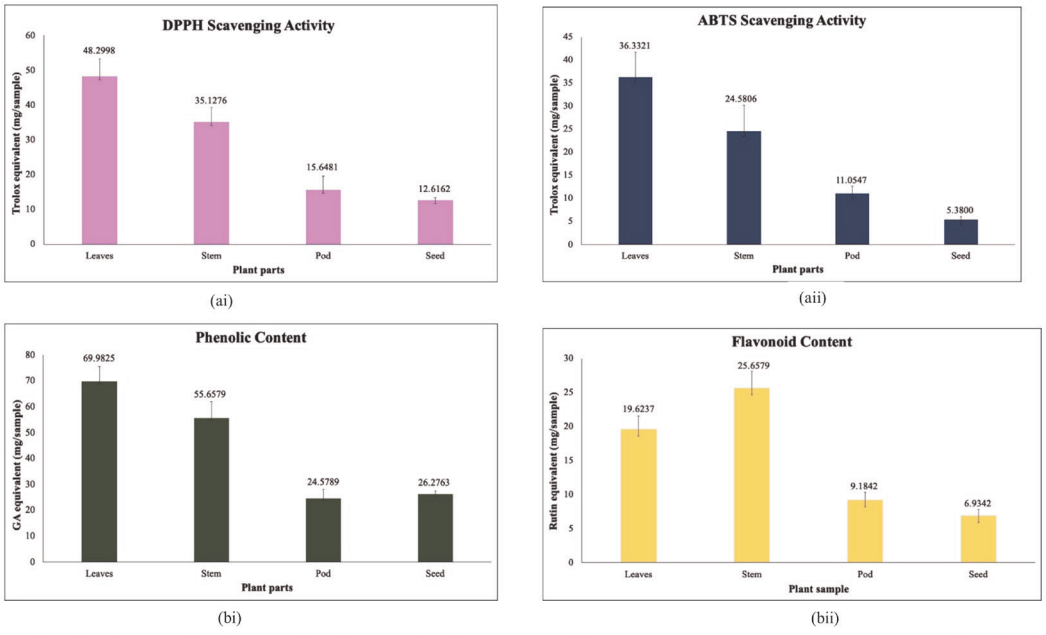

Figure 1: (a) Phytochemical analysis of M. oleifera parts; (ai) DPPH, (aii) ABTS, (bi) Total phenolic content, (bii) Total flavonoid content

of *M. oleifera* recorded 19.62 RE/mg sample whereas pod recorded 9.18 RE/mg sample. Thus, the pattern of flavonoid content for *M. oleifera* could be arranged as followed: stem > leaves > pod > seed.

Fitting the model

The CCD comprises of three process parameters, five coded levels and six centre points. The observed values of 20 runs were presented as shown in Table I. In the meantime, the results of ANOVA were tabulated in Table IIa. The level of significance where $p < 0.05$, 0.01 and 0.001 indicated that the model terms were significant, highly significant and remarkably significant respectively. The values that were greater than 0.05 indicated that the model was insignificant.

Overall, ANOVA table revealed that the model responses particularly yield and DPPH were significant ($p < 0.05$), TPC and TFC were highly significant ($p < 0.01$) while ABTS was remarkably significant ($p < 0.001$). The lack of fit was non-significant for all model responses ($p > 0.05$). The non-significant lack of fit indicated that the model term adequately explains the relationship between process parameters and model responses. The coefficient of determination (R^2) was nearly fitted to 1 for all model responses which displayed a strong correlation between observed and predicted values. Three dimensional (3D) response surface graphs displayed interaction effects between process parameters towards the model responses, if any (Fig. 2a-b).

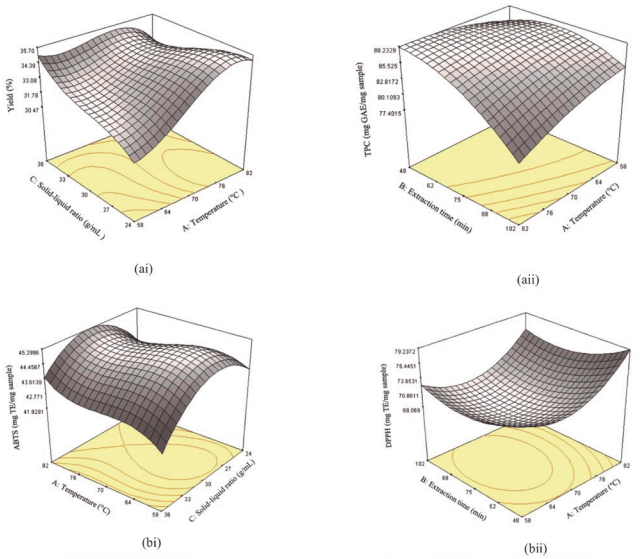

Figure 2: The significant interaction effects of process parameters on (ai) Yield, (aii) TPC, (bi) ABTS scavenging assay and (bii) DPPH scavenging assay

The effect of process parameters on total recovery yield The analysis of variance (ANOVA) revealed that temperature and extraction time displayed significant ($p < 0.05$) linear models on total recovery yield while a significant ($p < 0.05$) quadratic model only in temperature. In cross effect, there was a significant effect between temperature and solid-liquid ratio ($p < 0.05$) while in reduced cubic model, only temperature displayed a highly significant model ($p < 0.01$). By

Table I: The experimental data observed in all responses through CCD matrix

Run	X_A (°C)	X_B (min)	X_C (w/v)	Yield (%)	TPC (mg GAE/mg sample)	TFC (mg RE/mg sample)	ABTS (mg TE/mg sample)	DPPH (mg TE/mg sample)
1	58 (-1)	102 (1)	36 (1)	37.42 ± 0.01	83.67 ± 3.49	20.79 ± 1.42	40.14 ± 4.31	77.73 ± 0.11
2	58 (-1)	48 (-1)	24 (-1)	32.24 ± 0.02	86.19 ± 4.35	18.60 ± 0.63	41.15 ± 0.57	72.82 ± 1.90
3	70 (0)	75 (0)	30 (0)	34.26 ± 0.05	87.09 ± 2.99	21.26 ± 0.55	44.07 ± 2.62	71.71 ± 1.01
4	82 (1)	48 (-1)	36 (1)	32.80 ± 0.03	93.31 ± 12.27	22.45 ± 1.28	40.97 ± 3.81	77.10 ± 0.56
5	70 (0)	75 (0)	30 (0)	32.69 ± 0.03	88.80 ± 2.51	21.31 ± 0.65	45.40 ± 2.77	67.30 ± 2.95
6	82 (1)	102 (1)	24 (-1)	36.22 ± 0.05	74.84 ± 1.35	20.01 ± 1.02	41.69 ± 3.81	76.38 ± 0.45
7	82 (1)	48 (-1)	24 (-1)	36.13 ± 0.04	82.05 ± 1.02	21.57 ± 1.49	41.08 ± 3.68	84.23 ± 1.90
8	82 (1)	102 (1)	36 (1)	36.10 ± 0.01	76.01 ± 5.54	19.95 ± 0.31	42.41 ± 1.34	81.06 ± 1.90
9	58 (-1)	102 (1)	24 (-1)	30.67 ± 0.001	80.42 ± 5.41	19.43 ± 0.77	42.13 ± 0.75	79.87 ± 0.90
10	58 (-1)	48 (-1)	36 (1)	34.52 ± 0.02	83.58 ± 4.48	21.26 ± 0.48	39.64 ± 4.10	85.57 ± 5.15
11	70 (0)	75 (0)	30 (0)	34.64 ± 0.02	89.52 ± 3.26	21.57 ± 0.59	44.68 ± 1.32	68.98 ± 0.60
12	70 (0)	75 (0)	30 (0)	34.97 ± 0.01	85.20 ± 1.80	20.42 ± 0.54	43.64 ± 2.02	70.76 ± 2.35
13	90 (1.682)	75 (0)	30 (0)	26.29 ± 0.10	83.40 ± 1.36	26.46 ± 6.33	49.69 ± 6.44	84.80 ± 0.58
14	70 (0)	120 (1.682)	30 (0)	33.47 ± 0.01	77.90 ± 0.87	18.03 ± 0.39	40.83 ± 1.27	67.46 ± 4.09
15	70 (0)	75 (0)	30 (0)	34.78 ± 0.05	89.61 ± 8.72	22.92 ± 0.41	46.70 ± 1.85	66.86 ± 6.04
16	70 (0)	75 (0)	40 (1.682)	38.16 ± 0.01	90.33 ± 1.56	19.95 ± 0.16	40.47 ± 3.03	71.63 ± 6.80
17	70 (0)	75 (0)	30 (0)	33.61 ± 0.03	82.50 ± 5.31	21.31 ± 0.74	45.59 ± 0.58	65.34 ± 3.51
18	70 (0)	75 (0)	20 (-1.682)	28.87 ± 0.02	81.14 ± 0.87	17.09 ± 0.39	41.33 ± 1.84	64.97 ± 4.44
19	50 (-1.682)	75 (0)	30 (0)	34.13 ± 0.01	87.27 ± 4.61	18.86 ± 0.31	41.84 ± 1.50	68.41 ± 5.65
20	70 (0)	30 (-1.682)	30 (0)	33.03 ±0.03	84.48 ± 1.33	20.99 ± 1.45	42.31 ± 4.74	74.80 ± 1.35

X_A = Temperature (°C), X_B = Extraction time (min), X_C = Solid-liquid ratio (w/v), Yield = Total recovery yield (%), TPC = Total phenolic content (mg GAE/mg sample), TFC = Total flavonoid content (mg RE/mg sample), ABTS and DPPH (mg TE/mg sample).

All measurements were conducted in triplicate and expressed as mean ± standard deviation.

removing the non-significant process variables from the model, the second order polynomial equation was presented as:

$$Y_Y = 34.14 + 2.51X_A + 0.84X_B - 0.89X_A^2 - 1.56X_{AC} - 1.71X_A^3 \quad (2)$$

In terms of an interaction effect, temperature and solid-liquid ratio (X_{AC}) illustrated a significant negative outcome ($p < 0.05$) (Table IIa). Fig. 2a (i) displayed the amount of yield decreases with increasing extraction temperature and solid-liquid ratio.

Table IIa: The regression coefficient (β), coefficient of determination (R^2) and the probability value (p-value) to fit the second order polynomial models for antioxidant study

	Yield	TPC	TFC	ABTS	DPPH
Intercept	34.14	87.15	21.45	44.96	68.46
	2.51*	-1.04	-0.48	-0.68	2.18
Linear	0.84*	-3.02**	-0.23*	0.92*	-1.25
	-0.43	2.09*	0.47*	-0.42	1.42
	-0.89*	-0.93	0.50	-0.046	4.42**
Quadratic	0.19	-2.40*	-0.62*	-1.53***	2.54*
	0.28	-0.79	-0.97**	-1.76***	1.54
	0.26	-2.35*	-0.55	0.072	-0.39
Cross product	-1.56*	1.48	-0.40	0.51*	-1.63
	0.96	-0.53	-0.28	0.045	-0.39
Reduced Cubic	-1.71**	-0.11	0.97**	1.06***	2.42
	-0.25	0.99	-0.23	-0.48*	-0.87
	1.13*	0.60	0.13	0.056	0.52
R^2	0.93	0.80	0.94	0.97	0.92
F-value (model)	7.47*	5.53**	11.02**	31.53***	4.81*
Lack of fit	3.70	0.60	0.97	0.16	4.49

X_A = Temperature (°C), X_B = Extraction time (min), X_C = Solid-liquid ratio (w/v)
TPC = Total phenolic content (mg GAE/mg sample), TFC = Total flavonoid content (mg RE/mg sample), ABTS and DPPH (mg TE/mg sample)
Level of significance = $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

Table IIb: Predicted versus actual values at optimal extraction condition.

Responses	Predicted values	Actual values	% Difference (CV)
Yield	33.56	22.48 ± 0.01	27.95
TPC	88.21	88.71 ± 1.09	0.40
TFC	23.03	22.87 ± 0.39	0.25
ABTS	43.33	43.51 ± 0.43	0.29
DPPH	79.24	78.28 ± 0.90	0.86

TPC = Total phenolic content (mg GAE/mg sample), TFC = Total flavonoid content (mg RE/mg sample), ABTS and DPPH (mg TE/mg sample)

The effect of process parameters on total phenolic content (TPC)

ANOVA displayed a statistically significant difference ($p < 0.01$ and $p < 0.05$ respectively) for extraction time (X_B^2) and solid-liquid ratio (X_C^3) on TPC in linear model. Concurrently, the extraction time (X_B^2) displayed significant quadratic model ($p < 0.05$) on TPC. The interaction effect was seen between temperature and extraction time (X_{AB}).

Thereby, by removing the non-significant variables, the final equation that fitted with the second-order polynomial was presented as follows:

$$Y_{TPC} = 87.15 - 3.02X_B + 2.09X_C - 2.40X_B^2 - 2.35X_{AB} \quad (3)$$

The effect of process parameters on total flavonoid content (TFC)

ANOVA table exhibited statistically significant difference for extraction time (X_B, X_B^2) and solid-liquid ratio (X_C, X_C^2) in linear ($p < 0.05$) and quadratic models ($p < 0.05$ and $p < 0.01$ respectively). Nevertheless, there was no significant difference observed in other process variables and the interaction effect. By removing the non-significant terms and applying the second order polynomial equation, the final equation was presented as followed:

$$Y_{TFC} = 21.45 - 0.23X_B + 0.47X_C - 0.62X_B^2 - 0.97X_C^2 + 0.97X_A^3 \quad (4)$$

Table I depicted that the highest amount of total flavonoid content was observed in Run 13 (26.46 mg RE/mg sample) whereas the lowest TFC recorded was from Run 18 (17.09 mg RE/mg sample).

The effect of process parameters on antioxidant activity (AA)

Extraction time (X_B) exhibited a significant effect ($p < 0.05$) in linear model of ABTS assay whereas in quadratic model, temperature (X_A^2) displayed highly significant effect ($p < 0.01$) on DPPH assay. Meanwhile, extraction time (X_B^2) recorded significant effects for both ABTS and DPPH assays ($p < 0.001$ and $p < 0.05$ respectively). Solid-liquid ratio (X_C^2) was remarkably significant ($p < 0.001$) with the experimental values for ABTS assay. In terms of interaction effect, temperature and solid-liquid ratio (X_{AC}) displayed significant effect on ABTS assay ($p < 0.05$) while temperature (X_A^3) and extraction time (X_B^3) displayed significant effects in reduced cubic model ($p < 0.001$ and $p < 0.05$ respectively). By removing the non-significant terms and applying the highest order polynomial, it could be stated that the final equation for both models were:

$$Y_{ABTS} = 44.96 + 0.92X_B - 1.53X_B^2 - 1.76X_C^2 + 0.51X_{AC} + 1.06X_A^3 - 0.48X_B^3 \quad (5)$$

$$Y_{DPPH} = 68.46 + 4.42X_A^2 + 2.54X_B^2 \quad (6)$$

Optimisation of design parameters and model validation

The determination of optimum condition for phenolic extraction was carried out using Design Expert Version 6.0 (Stat-Ease, Inc.). Hence, the optimum extraction condition to determine the highest yield, TPC, TFC, ABTS and DPPH was a temperature of 82°C; extraction time of 48 min and the solid-liquid ratio of 1 g in 30 mL (w/v). This optimisation was validated as the actual values were in agreement with the predicted values in which the differences of the coefficient of variation ranging from 0.28 to 27.95%, illustrating that the overall model was useful and reliable for optimum extraction of phenolic compounds (Table IIb).

Chromatographic analysis

Screening of bioactive compounds through LC-MS analysis

Fig. 3 revealed five phenolic compounds such as gallic acid (Peak 1), chlorogenic acid (Peak 2), quercetin (Peak 3), kaempferol (Peak 4) and 3-O-glucoside kaempferol (Peak 5) that could potentially contribute to the antioxidant activity of the leaves extract. In the meantime, the peak assignments were tabulated as in Table IIIa.

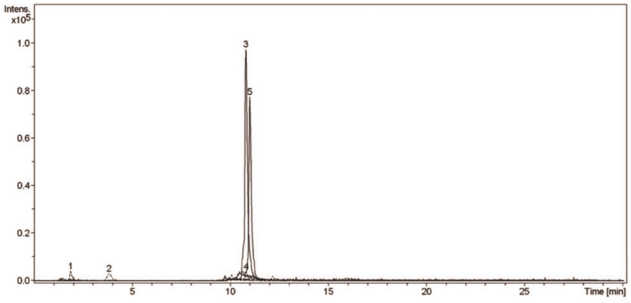

Figure 3: LC-MS chromatogram of polyphenol in M. oleifera aqueous leaves extract. Peak 1: Gallic acid, Peak 2: Chlorogenic acid, Peak 3: Quercetin, Peak 4: Kaempferol and Peak 5: 3-O-glucoside kaempferol

HPLC analysis

The bioactive compounds under optimised extraction conditions were quantified through HPLC analysis. The analysis was also conducted to confirm the presence of phenolics detected in LC-MS analysis. Briefly, six phenolics were detected at 280 nm (Fig. 4). The amount of each phenolic was presented in Table IIIb which was based on peak area calculated. Thus, the amount was as follows: gallic acid > chlorogenic acid > epicatechin gallate > kaempferol > myricetin > quercetin.

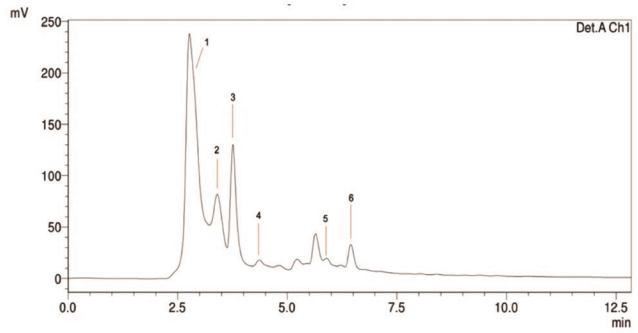

Figure 4: HPLC profiles of phenolic compounds detected at 280 nm. The chromatograms shown were Gallic acid (1); Epicatechin gallate (2); Chlorogenic acid (3); Myricetin (4); Quercetin (5) and Kaempferol (6)

DISCUSSION

M. oleifera leaves are rich in functional phytochemicals such as phenolic acids that contribute to potent antioxidant properties. Published literature displayed that the leaves of *M. oleifera* possess gallic acid, chlorogenic acid, ellagic acid, caffeic acid and syringic acid which explained the antioxidant activity in DPPH and ABTS scavenging assay (12).

Table IIIa: Peak assignment for LC-MS analysis of M. oleifera aqueous leaves extract through databases

No.	R_t (min)	Formula	$[M+H]^+$ (m/z)	Mass (g/mol)	Compounds
1	1.87	$C_7H_6O_5$	171.029	170.120	Gallic acid
2	3.83	$C_{16}H_{18}O_9$	355.102	354.311	Chlorogenic acid
3	10.79	$C_{15}H_{10}O_7$	303.050	302.236	Quercetin
4	10.81	$C_{15}H_{10}O_6$	287.055	286.239	Kaempferol
5	10.99	$C_{21}H_{19}O_{11}$	449.108	447.372	3-O-glucoside kaempferol

R_t = Retention time (min)

Table IIIb: The amount of phenolic compounds detected under optimal extraction condition

Compounds	Retention time (min)	Content (mg/g sample)	Regression equation
Gallic acid	2.761	221.17	Y= 3098x + 29147, R^2 = 0.9986
Epicatechin gallate	3.398	151.99	Y= 1603.7x - 32866, R^2 = 0.9947
Chlorogenic acid	3.755	171.34	Y = 1315.8x + 771.91, R^2 = 0.9941
Myricetin	4.355	26.23	Y = 1977.5x + 15896, R^2 = 0.9947
Quercetin	5.889	5.04	Y = 7630.5x + 18786, R^2 = 0.9923
Kaempferol	6.444	41.56	Y = 1419.4x + 13629, R^2 = 0.9985

Besides phenolic acids, the plant is also rich in flavonoid constituents such as kaempferol, quercetin, rutin, myricetin and epicatechin. These compounds are very good in scavenging free radicals that could cause oxidative stress where potential antioxidant activity could be clearly seen in DPPH and ABTS assays respectively.

Temperature is a crucial parameter for production of yield as heat activation initiates the diffusion of the phenolic compound from the plant matrices, with the aid of solvent extraction. However, at a certain threshold of temperature, the yield may be affected solely due to thermal lability (13). The amount of yield obtained was comparable with the study conducted by Mahdi et al. (14) (37.84%). This shows that this optimisation was useful though different extraction techniques were applied.

Temperature significantly affects TPC in the interaction model as heat activation initiates the diffusion of phenolic compounds from the plant matrices. However, prolonged extraction time gave a negative effect since the amount of phenolic contents would be reduced due to degradation of the bioactive components (15). It was noticed that the highest production of phenolic compound was from Run 4 (X_A= 82°C, X_B = 48 min, X_C = 1:36 w/v) while the lowest production of total phenolic

content was from Run 6 (X_A= 82°C, X_B = 102 min, X_C = 1:24 w/v). Thus, the pattern of phenolic contents could be observed such that temperature enhances the extraction of phenolic compounds while reducing extraction time and increase solid-liquid ratio will maximize the amount of TPC obtained.

The amount of phenolics obtained were immensely improved compared to similar studies by Rodríguez-Pérez et al. (11, 16) (24.0 mg GAE/g dry leaf and 59.0 mg GAE/g dry leaf respectively). In contrast to Naeem et al. (17), the amount of TPC was relatively low (12.28 mg GAE/g DW) as compared to this study which enhances the production of TPC. This indicated that RSM methodology was useful in terms of yield and cost effectiveness compared to previous studies.

Based on the process parameters, the highest production of flavonoid was found at 90°C with time at 75 min and the solid-liquid ratio at 1:30 (w/v). Prolonged extraction time with high extraction temperature would not be suggested as flavonoids may degrade as shown by low flavonoid content in Run 18 (X_A= 70°C, X_B = 120 min, X_C = 1:30 w/v). In the meantime, solid-liquid ratio (X_C^2) displayed an inverse relationship with TFC in which lower solid-liquid ratio allows higher production of the amount of flavonoid produced and vice versa (Run 18). Surprisingly, this result was not anticipated because of the lowest flavonoid content which could be due to insufficient extraction temperature that is required to extract enough flavonoid compounds. According to Chen et al. (18), an increase in extraction temperature could immensely intensify the total solubility of flavonoid thereby, increase the penetration to plant matrices. With this, the amount of flavonoid produced would be greatly increased.

Table I highlighted that the highest antioxidant activity detected in Run 13 for ABTS assay while Run 10 in DPPH assays. The lowest antioxidant activity was identified in Run 10 and Run 18 for ABTS and DPPH assays respectively. It could be deduced that the extract exhibited a notable antioxidant activity in both assays. It could also be stated that ABTS and DPPH radical scavenging assay were solely affected by temperature and extraction time while solid-liquid ratio seems to affect only ABTS assay (Table IIa). Third, it could be stated that the high antioxidant activity displayed in *M. oleifera* aqueous leaves extract was associated with the total phenolic and flavonoid content obtained (19).

Total antioxidant activities of *M. oleifera* leaves in this study was comparable to previous studies conducted by Fombang and Saa (20) (80.94%). Besides, the amount of antioxidant activity in this study was also higher as compared to Rodríguez-Pérez et al. (16) (12.3 mmol TE/100 g dry leaf) study which used non-conventional extraction techniques.

CONCLUSION

The current work explained the potential application of RSM to optimise the extraction of polyphenolic compounds from *M. oleifera* leaves. The mathematical models calculated using RSM software effectively showed that the observed values with the process variables significantly ($p < 0.05$) affected the studied responses. In addition, LC-MS analysis revealed five biologically active compounds of interest, which were gallic acid, chlorogenic acid, quercetin, kaempferol and 3-O-glucoside kaempferol. While HPLC profiles successfully detected six phenolic compounds such as gallic acid, epicatechin gallate, chlorogenic acid, myricetin, quercetin and kaempferol. The optimisation was promising to improve yield and antioxidant activity in *M. oleifera* as compared to non-conventional extractions.

ACKNOWLEDGEMENTS

Special thanks to Universiti Putra Malaysia (UPM/700-2/1/GPB/2017/9549900) for the financial support to complete the works. Besides, authors would also present a token of appreciation to Dr. Haniza Hassan from the Department of Human Anatomy, Faculty of Medicine and Health Sciences, UPM for her precious help in utilising the RSM software. Finally, special gratitude to Laboratory of Molecular Biomedicine, Institute of Bioscience for providing the facilities during the work.

REFERENCES

1. Booth FE, Wickens GE. Non-timber uses of selected arid zone trees and shrubs in Africa. Food & Agriculture Org.; 1988.
2. Shih MC, Chang CM, Kang SM, Tsai ML. Effect of different parts (leaf, stem and stalk) and seasons (summer and winter) on the chemical compositions and antioxidant activity of *Moringa oleifera*. International journal of molecular sciences. 2011 Sep;12(9):6077-88.
3. Moundipa PF, Beboyl NS, Zelefack F, Ngouela S, Tsamo E, Schill WB, et al. Effects of *Basella alba* and *Hibiscus macranthus* extracts on testosterone production of adult rat and bull Ley dig cells. Asian Journal of Andrology. 2005 Dec;7(4):411-7.
4. Aybastıer Lİ, Işık E, Şahin S, Demir C. Optimization of ultrasonic-assisted extraction of antioxidant compounds from blackberry leaves using response surface methodology. Industrial crops and products. 2013 Jan 1;44:558-65.
5. Azahar NF, Gani SS, Mokhtar NF. Optimization of phenolics and flavonoids extraction conditions of *Curcuma zedoaria* leaves using response surface methodology. Chemistry Central Journal. 2017 Dec;11(1):96.
6. Ibrahim HM, Elkhidir EE. Response surface method

as an efficient tool for medium optimisation. Trends in Applied Sciences Research. 2011 Feb 1;6(2):121-9.
7. Yusri N, Chan K, Iqbal S, Ismail M. Phenolic content and antioxidant activity of *Hibiscus cannabinus* L. seed extracts after sequential solvent extraction. Molecules. 2012;17(11):12612-21.
8. Pandey A, Belwal T, Sekar KC, Bhatt ID, Rawal RS. Optimization of ultrasonic-assisted extraction (UAE) of phenolics and antioxidant compounds from rhizomes of Rheum moorcroftianum using response surface methodology (RSM). Industrial Crops and Products. 2018 Sep 1;119:218-25.
9. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biology and medicine. 1999 May 1;26(9-10):1231-7.
10. Skendi A, Irakli M, Chatzopoulou P. Analysis of phenolic compounds in Greek plants of Lamiaceae family by HPLC. Journal of applied research on medicinal and aromatic plants. 2017 Sep 1;6:62-9.
11. Rodríguez-Pérez C, Quirantes-Piné R, Fernández-Gutiérrez A, Segura-Carretero A. Optimization of extraction method to obtain a phenolic compounds-rich extract from *Moringa oleifera* Lam leaves. Industrial Crops and Products. 2015 Apr 1;66:246-54.
12. Leone A, Spada A, Battezzati A, Schiraldi A, Aristil J, Bertoli S. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: an overview. International journal of molecular sciences. 2015 Jun 5;16(6):12791-835.
13. Roselly-Soto E, Martн-Quijal FJ, Cilla A, Munekata PE, Lorenzo JM, Remize F, et al. Influence of Temperature, Solvent and pH on the Selective Extraction of Phenolic Compounds from Tiger Nuts by-Products: Triple-TOF-LC-MS-MS Characterization. Molecules. 2019 Jan;24(4):797.
14. Mahdi HJ, Yousif EM, Khan NAK, Mahmud R, Murugaiah V and Asmawi MZ. Optimizing extraction conditions of *Moringa oleifera* LAM leaf for percent yield, total phenolics content, total flavonoids content and total radical scavenging activity. International Journal of Advanced Research. 2016 Oct 25;4(11):682-695.
15. Naczk M, Shahidi F. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. Journal of pharmaceutical and biomedical analysis. 2006 Aug 28;41(5):1523-42.
16. Rodríguez-Pérez C, Gilbert-Lypez B, Mendiola JA, Quirantes-Piné R, Segura-Carretero A, Ibácez E. Optimization of microwave-assisted extraction and pressurized liquid extraction of phenolic compounds from *Moringa oleifera* leaves by multiresponse surface methodology. Electrophoresis. 2016 Jul;37(13):1938-46.
17. Naeem S, Ali M, Mahmood A. Optimization of extraction conditions for the extraction of phenolic compounds from *Moringa oleifera* leaves. Pakistan journal of pharmaceutical sciences. 2012 Jul 1;25(3):535-41.
18. Chen Y, Xie MY, Gong XF. Microwave-assisted extraction used for the isolation of total triterpenoid saponins from *Ganoderma atrum*. Journal of Food Engineering. 2007 Jul 1;81(1):162-70.
19. Irakli M, Tsifodimou K, Sarrou E, Chatzopoulou P. Optimization infusions conditions for improving phenolic content and antioxidant activity in *Sideritis scardica* tea using response surface methodology. Journal of applied research on medicinal and aromatic plants. 2018 Mar 1;8:67-74.
20. Fombang E.N. and Saa W.R. Production of a functional tea from *Moringa oleifera* LAM leaf powder: Optimization of phenolic extraction using response surface methodology. Journal of Nutrition and Food Sciences. 2016 Sep 22,6(6) DOI: 10.4172/2155-9600.100056