Optimization of extraction conditions for phenolic compounds from neem (*Azadirachta indica*) leaves

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Abstract: The objective of this study was to optimise the extraction conditions for phenolic compounds from neem (*Azadirachta indica*) leaves using response surface methodology (RSM). A central composite rotatable design (CCRD) was applied to determine the effects of acetone concentration (%), extraction time (mins), and extraction temperature (°C) on total phenolic content (TPC) from neem (*Azadirachta indica*) leaves. The independent variables were coded at five levels and their actual values were selected based on the results of single factor experiments. Results showed that acetone concentration and extraction time were the most significant ($p < 0.001$) factor affecting the TPC. The optimum extraction conditions were found to be acetone concentration of 48.49%, extraction time of 59.25 mins, and extraction temperature of 40.88°C. Under the optimised conditions, the experimental value for TPC was 4661.17 mg GAE/100 g DW, which reasonably close to the predicted value (4649.16 mg GAE/100 g DW).

Keywords: Neem (*Azadirachta indica*) leaves, phenolic compounds, antioxidants, total phenolic content (TPC), response surface methodology (RSM)

Introduction

The use of plant substances for medication is believed to be less toxic compared to that of synthetic chemical compounds (Pramono, 2002; Muhammad and Muhammad, 2005). The medicinal uses of the plants are attributed by the plant's secondary metabolites and are unique resources for pharmaceuticals, food additives, and fine chemicals (Zhao *et al*., 2005). Numerous investigations have proved that these secondary metabolites contain diverse classes of bioactive phenolic compounds such as polyphenols, tocopherols and alkaloids. Among them flavonoids and phenolic acids are particularly attractive as they are known to exhibit various pharmacological properties such as vasoprotection, anticarcinogenic, antimicrobial, anti-inflammatory as well as antiallergic and antiproliferative activity on tumour cells (Tsao and Deng, 2004; Cai *et al*., 2006).

Many researches have been done in order to find out the presence and the content of antioxidant phenolic compounds from various plants and fruits (Perez-Jimenez and Saura-Calixto, 2006; Dzingirai *et al*., 2007; Rufian-Henares and Morales, 2007). However, there is no universal standardized set of optimum condition for the extraction of phenolic compounds from different plants (Chirinos *et al*., 2006; Chen *et al*., 2007). The nature of bioactive phenolic compounds and the presence of interfering substances recovery were reported by Chirinos *et al*. (2006) to be affected by several extraction factors such as extraction methods, type of solvent, pH, temperature, sample-solvent ratio and extraction time. *Azadirachta indica*, commonly known as Neem is a fast growing evergreen tree belonging to mahogany family (Meliaceae) which can grow up to 200 years and is found in most tropical countries. Especially neem leaf and its constituents, which is also the focus of this study, have demonstrated antioxidant, immunomodulatory, anti-inflammatory, antiulcer, antimutagenic and anticarcinogenic properties (Biswas *et al*., 2002). Most of these beneficial characteristics were reported attributed by the presence of phenolic compounds.

However, to the best of our knowledge, optimization of extraction of phenolic antioxidants from neem (*Azadirachta indica*) leaves using response surface methodology (RSM) has not been reported yet. Therefore, the objective of this study was to determine the best extraction conditions for neem leaves, in order to maximize simultaneously the yield of total phenolic content (TPC) by using RSM.

Materials and Methods

Plant material

Local neem (*Azadirachta indica*) leaves sample of 3 kg was freshly plucked from Cheras, Kuala

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Lumpur, Malaysia. The leaves were a mix of both young and matured leaves. The leaves were chosen based on its bright green colour, have little or no pigmentation, no blemishes or diseased leaves. The leaves are also of the correct species with uniformity in shape, size and length.

Chemical reagents

All the solvents and chemicals used were of analytical grade. Deionized water used for the preparation of all the solutions was purified by Milli-Q purification system (Millipore) (Massachusetts, USA).

Sample preparation

Upon arrival at the laboratory, samples were thoroughly washed with tap water, manually peeled and cut into smaller size approximately about 1 cm wide using kitchen knife. The smallest strips of leaves were then spread evenly onto a tray lined ≈ 50 cm by 50 cm with aluminium foil (Diamond USA). The tray was then placed into the conventional oven (Memmert, Germany) for 24 hour drying at 45°C. Once after the sample has been dried, the sample was then passed through a miller (MF 10 basic IKA, Germany) at 4000 rpm speed where the sample size exited at 0.5mm sieve. The milled sample was then mixed, weighed into amounts of 10 grams each in a nylon-linear low density polyethylene (LDPE) bag (Flexoprint, Malaysia) and was vacuum packed using a vacuum package machine (Model DZQ400/500) (Zhejiang, China). The sample was then wrapped in a newspaper and stored in a sealed container (dark, dry and room temperature environment) for further experiments. The dried sample was stored at room temperature for a maximum period of one month.

Preparation of extracts

Approximately 2 g of dried sample was weighed and extracted with 20 mL of the extracting solvent in a conical flask. Conical flask was covered with parafilm (Pechiney plastic packaging) and aluminium foil to prevent light exposure. The mixture was shaken at constant rate using a water bath shaker (Memmert, Germany) for different times at required temperature. After the extraction, the neem leaves extract was then filtered through a Whatman No. 1 filter paper, and the clear solution was collected in an amber reagent bottle. The filtrate was subsequently used for the determination of TPC. All the extractions were replicated once.

Experimental design

The experimental design for this study was divided into two major parts. Firstly, single factor experiments were performed to determine the appropriate range of conditions for neem leaves phenolics extraction, namely, solvent type, solvent concentration, extraction time, and extraction temperature by varying one independent variable at a time while keeping the others constant. Secondly, the optimisation of phenolic compounds extraction was carried out using RSM and a second order polynomial model was developed.

Single factor experiments

Selection of solvent type

By fixing extraction time (180 mins) and extraction temperature (25°C), samples were extracted with 60% (v/v) acetone, 60% (v/v) ethanol, 60% (v/v) methanol, distilled water, and boiling water respectively. The extraction procedures were described in solvent extraction section. The best solvent type was selected according to the value of TPC (mg GAE/100 g DW).

Effect of solvent concentration on extraction of phenolic compounds

Using the best solvent type selected in single factor experiments section (a), samples were extracted with solvent ranging from 20% (v/v) to 100% (v/v) by fixing the extraction time and extraction temperature at 180 mins and 25°C, respectively. The best solvent concentration was selected according to the value of TPC (mg GAE/100 g DW).

Effect of extraction time on extraction of phenolic compounds

Samples were extracted using the best solvent type and the best solvent concentration selected in single factor experiments sections (a) and (b), respectively. The extraction procedures were repeated as described in section of single factor experiments by varying the extraction time from 30 to 450 mins while fixing the extraction temperature constant at 25°C. The best extraction time was selected according to the value of TPC (mg GAE/100 g DW).

Effect of extraction temperature on extraction of phenolic compounds

Using the best solvent type and the best solvent concentration selected in single factor experiments sections (a) and (b), samples were extracted at various extraction temperature ranged from 25 to 55°C at the optimum time determined in single factor experiments section (c). The extraction procedures were repeated as described in solvent extraction section. The best

extraction temperature was selected according to the value of TPC (mg GAE/100 g DW). Based on the results of single factor experiment, the ranges of three factors (solvent concentration, extraction time and extraction temperature) were determined for RSM.

Experiment of RSM

A three-factor (X_1, X_2 and X_3) and five level (-α, -1, 0, 1, and +α) central composite rotatable design (CCRD) was applied to optimise the phenolics extraction from neem leaves. The complete CCRD design comprised of twenty experiments with eight factorial points, six axial points and six center points (Table 1). Six replicate runs at the centre of the design were performed to allow a good estimation of pure error (Sin *et al.*, 2006). The independent variables studied were acetone concentration (X_1, %), extraction time (X_3, mins) and extraction temperature (X_2, ºC) and while the dependent variable (response variable) measured was TPC (Y, mg GAE/100 g dry weight, DW). Each experiment was performed in replicate and the average values were taken as the response, Y.

Determination of total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu reagent according to the method described by Lim *et al.* (2007) with slight modifications. Crude extracts obtained from extraction were diluted 40 times before use. Approximately 0.3 mL of diluted samples was added into aluminium foil-wrapped test tubes followed by 1.5 mL of Folin-Ciocalteu's reagent (10 folds dilution) and 1.2 mL of 7.5% (w/v) sodium carbonate. The blank sample was prepared by replacing 0.3 mL of sample with 0.3 mL of deionised water. The test tubes were covered with parafilm, vortexed for 10 s and allowed to stand in the dark environment at room temperature for 30 mins. Absorbance was measured against the blank sample at 765 nm using UV light spectrophotometer (Model XTD 5; Secomam) (Ales Cedex, France). Each extract was analyzed in triplicate. A calibration curve of gallic acid was plotted by plotting absorbance vs concentrations of gallic acid (mg/L).

Statistical analysis

The experimental results in single factor experiments were analyzed using Minitab software (Minitab Version 15.1.1.0.). All data were expressed as means ± standard deviations of triplicate measurements. One-way analysis of variance (ANOVA) with Tukey's test was used to determine the significant differences ($p<0.05$) between the means.

The Design Expert (Version 6.0.10, Stat-Ease Inc., Minneapolis) statistical software was employed to design the CCRD and to analyze the experimental data in RSM. Experimental data were fitted to the following second order polynomial model and regression coefficients were obtained. The generalized second-order polynomial model proposed for the response surface analysis was given as follows:

Equation (1)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, β_{ij} are regression coefficients for intercept, linear, quadratic and interactions terms, respectively. X_i and X_j are coded value of the independent variables while k equals to the number of the tested factors (k=3). The ANOVA tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were analyzed statistically by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05.

Verification of model

Optimal conditions for the extraction of phenolic compounds from neem leaves were obtained using the second-order polynomial model of RSM. The suitability of the model equation for predicting the response values was verified by conducting the extractions under the recommended optimal conditions. In this study, a numerical optimisation method was adopted to find a point that maximizes the response. A series of solutions was generated and the solution to be employed for the verification would be selected based on its desirability and suitability. The experimental and predicted values of TPC were compared in order to determine the validity of the model. To confirm the results, runs were carried out in replicate under the selected optimised conditions.

Results and Discussion

A calibration curve of gallic acid was constructed to measure the amount of phenolic compounds in the limau purut peels. The calibration equation for gallic acid was y = 10.278x + 0.0085 (R^2 = 0.997). All the results in this study were computed from the above calibration curve and expressed as gallic acid equivalent (GAE) in mg per 100 g dry weight (DW).

Single factor experiments

Effect of solvent type on extraction of phenolic compounds

The selection of extraction solvents is critical

Table 1. Three factors and five levels CCRD together with the experimental and predicted values under different extraction conditions

| Run | Independent variables | | | Dependent variable | |
| | X_1, Ethanol concentration (%) | X_2, Time (mins) | X_3, Temperature (°C) | Total phenolic content (mg GAE/100g DW) | |
				Experimental	Predicted
1	34.19	26.22	29.05	4221.07	4240.50
2	75.81	26.22	29.05	3680.33	3670.70
3	34.19	73.78	29.05	3660.72	3684.25
4	75.81	73.78	29.05	3071.25	3032.57
5	34.19	26.22	40.95	4363.08	4393.15
6	75.81	26.22	40.95	3172.59	3140.44
7	34.19	73.78	40.95	4303.83	4305.85
8	75.81	73.78	40.95	2999.30	2971.26
9	55.00	50.00	35.00	4608.71	4637.47
10	55.00	50.00	35.00	4615.11	4637.47
11	55.00	50.00	35.00	4643.25	4637.47
12	55.00	50.00	35.00	4648.37	4637.47
13	20.00	50.00	35.00	3685.03	3636.84
14	90.00	50.00	35.00	1975.07	2035.44
15	55.00	10.00	35.00	4215.95	4207.21
16	55.00	90.00	35.00	3576.28	3597.20
17	55.00	50.00	25.00	4350.28	4349.31
18	55.00	50.00	45.00	4412.97	4426.12
19	55.00	50.00	35.00	4612.55	4611.55
20	55.00	50.00	35.00	4647.09	4611.55

for the complex food samples as it will determine the amount and type of phenolic compounds being extracted. Aqueous alcohols particularly acetone, ethanol and methanol are most commonly employed in phenolics extraction from botanical materials (Naczk and Shahidi, 2004; Hayouni *et al.*, 2007). Figure 1(a) showed that aqueous acetone significantly ($p<0.05$) higher than other type of solvent in extracting phenolics from neem leaves under the same extraction conditions (60%, 25°C, and 180 mins). This indicating that phenolic compounds extracted from neem leaves might cover from moderate polarity to low polarity. Acetone was chosen as the extraction solvent for the next experiments.

Effect of acetone concentration on extraction of phenolic compounds

The effects of acetone concentration on extraction of phenolic compounds from neem leaves were shown in Figure 1(b). TPC increased with the increment of the ethanol concentration up to 60% (3502.8 mg GAE/100 g dry weight, DW) followed by a reduction until reaching a minimum of 547.8 mg GAE/100 g DW at 100%. Similarly, Yap *et al.*, (2009) revealed that maximum total phenolics in star fruit residues extracts was obtained at about 60% acetone followed by a decrease with further increase in concentration.

Uma *et al.* (2010) also found that increased the acetone concentration beyond 60% will dramatically reduced the amount of phenolics extracted from henna (*Lawsonia inermis*) leaves. A remarkable drop in TPC at 100% ethanol revealed that absolute solvent do not ensure a good recovery of phenolic compounds as compared to aqueous acetone. Thus, moderate acetone concentration of 20%, 55% and 90% were selected as the lower, middle and upper levels, respectively, to be employed in RSM optimisation.

Effect of extraction time on extraction of phenolic compounds

Extraction time was another main parameter in the extraction procedure. The extraction time can either be as short as few minutes or very long up to 24 hours (Laponik *et al.*, 2005; Lee *et al.*, 2005). In this study, the range of extraction time was designed based on the practical and economical aspects. Figure 1(c) showed that an increase in extraction time increased from 30 to 90 mins was accompanied by a small increment in TPC from 3489.8 to 3886.2 mg GAE/100 g dw. After 180 mins, further increase in process duration did not significantly ($p>0.05$) improve the recovery of phenolics. This observation was well explained by Fick's second law of diffusion, which stated that final equilibrium will be achieved between the solute

concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) after a certain time, hence, an excessive extraction time was not useful to extract more phenolic antioxidants (Silva *et al*., 2007). Furthermore, prolonged extraction process might lead to phenolics oxidation due to light or oxygen exposure. Taking into account of these facts, an extraction time of 10–90 mins was selected for RSM optimisation.
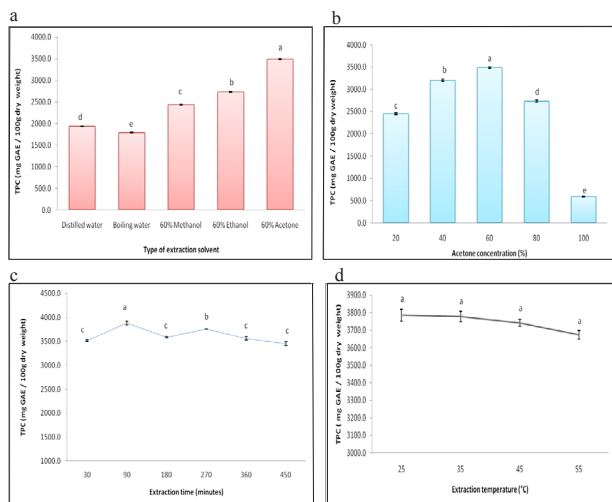

Figure 1. Effect of (a) solvent type; (b) acetone concentration; (c) extraction time; and (d) extraction temperature on total phenolic content from neem leaves. Values marked by different letters are significantly different (*p*<0.05)

Effect of extraction temperature on extraction of phenolic compounds

The selection of an appropriate extraction temperature was the final step in a series of single factor experiments. The extraction of phenolic compounds was decreased slightly when extraction temperature increased from 25 to 55ºC as reflected in Figure 1(d). This result was in accordance with the study of Chan *et al*., (2009), which reported that temperature did not showed significant (*p*<0.05) effect on TPC of neem leaves in acetone aqueous system. In general, increasing the temperature beyond certain values may encourage possible concurrent decomposition of phenolic compounds which were already mobilized at lower temperature or even the break down of phenolics that are still remained in the plant matrix. Additionally, high temperature may encourage solvent loss through vaporization and increase the cost for extraction process from the industrialization point of view. Therefore, moderate extraction temperature of 25, 35 and 45ºC were chosen as the lower, middle and upper levels, respectively, to be applied in RSM optimisation.

Response surface methodology (rsm) experiments

Fitting the model

Based on the observations from single factor experiments, the ranges of each independent variable (acetone concentration, extraction time and extraction temperature) that influence TPC were selected. In this study, the lower and upper values for the factors were set at +alpha (+α=1.682) and –alpha (-1.682) and thus all the factor levels was chosen within the limits that were desirable and practical. In RSM, natural variables are transformed into coded variables that have been defined as dimensionless with a mean zero and the same standard deviation (Liyana-Pathirana & Shahidi 2005). The experimental and predicted values for response (TPC) under different combination of extraction conditions were given in Table 1. The results showed that TPC of neem leaves ranged from1975.07 to 4648.37 mg GAE/100 g DW. By applying multiple regression analysis, relationship between the tested independent variables and the response was explained in Equation 2:

$$Y = 4624.51 - 476.10X_1 - 181.36X_2 + 22.84X_3 - 627.70X_1^2 - 250.79\ X_2^2 - 79.14\ X_3^2 - 20.47\ X_1 X_2 - 170.73\ X_1 X_3 + 117.24\ X_2 X_3$$

Equation (2)

To fit the response function and experimental data, the linearity and quadratic effect of the independent variables, their interactions and regression coefficients on the response variables were evaluated by analysis of variance (ANOVA) (Table 2). The ANOVA of the regression model showed that the model was highly significant due to a very low probability value (*p*<0.0001). The fitness and adequacy of the model was judged by the coefficient of determination (R^2) and the significance of lack-of-fit. R^2 which was defined as the ratio of the explained variation to the total variation, used as a measure of the degree of fit (Wang *et al*., 2008). The closer the R^2 value to unity, the better the empirical model fits the actual data (Fan *et al*., 2007). By referring to Table 2, R^2 value for the regression model of TPC was 0.9986, which was closed to 1. This suggested that the predicted second order polynomial models defined well the real behaviour of the system. In addition, the value of adjusted R^2 (0.9971) was also very high to advocate for a high significance of the model. The adjusted R^2 was a corrected value for R^2 after the elimination of the unnecessary model terms. If there were many non-significant terms have been included in the model, the adjusted R^2 would be remarkably smaller than the R^2 (Myers & Montgometry 2002). In this study, the adjusted R^2 was very close to the R^2

value. Besides, the absence of any lack of fit ($p>0.05$) also strengthened the reliability of the models. A small coefficient of variation (1.02) revealed that the experimental results were precise and reliable.

Table 2. The estimated regression coefficients of the second order polynomial model for total phenolic content of Neem leaf extract

Model parameters	Regression coefficients
Intercept	
X_0	4624.51
Linear	
X_1, Acetone concentration	-476.10***
X_2, Extraction time	-181.36***
X_3, Extraction temperature	22.84
Quadratic	
X_1^2	-627.70***
X_2^2	-250.79***
X_3^2	-79.14***
Interaction	
$X_1 X_2$	-20.47
$X_1 X_3$	-170.73***
$X_2 X_3$	117.24***
Mean	3973.19
Standard deviation	40.34
R^2	0.9986
Adjusted R^2	0.9971
Coefficient of variation	1.02
F value	688.79
p value	<0.0001

*   Significant at 0.05 level
**   Significant at 0.01 level
***   Significant at 0.001 level

The multiple regression results and the significance of regression coefficients for the TPC model were tabulated in Table 2. The *P*-values were used as a tool for checking the significance of each coefficient, which in turn might indicated the interaction patterns between the variables (Hou & Chen 2008). The smaller the *P*-value, the more significant was the corresponding coefficient. It could be observed from Table 2 that both the linear and quadratic term of all parameters (acetone concentration, X_1; extraction time, X_2; and extraction temperature, X_3) had significant (at least at $p<0.05$) effect on TPC. In addition, TPC was also significantly influenced by the interactions between acetone concentration and extraction temperature, X_{13} ($p<0.001$) and extraction time and extraction temperature, X_{23} ($p<0.001$). Among all the three extraction parameters studied, acetone concentration had the most critical role in the extraction of phenolic compounds from neem leaves followed by extraction time and extraction temperature.

Analysis of response surface plot

Figure 2 illustrated three-dimensional response surfaces plots by presenting the response in function of two factors and keeping the other constant at its middle level. Each figure revealed the effects of the selected parameters on TPC.
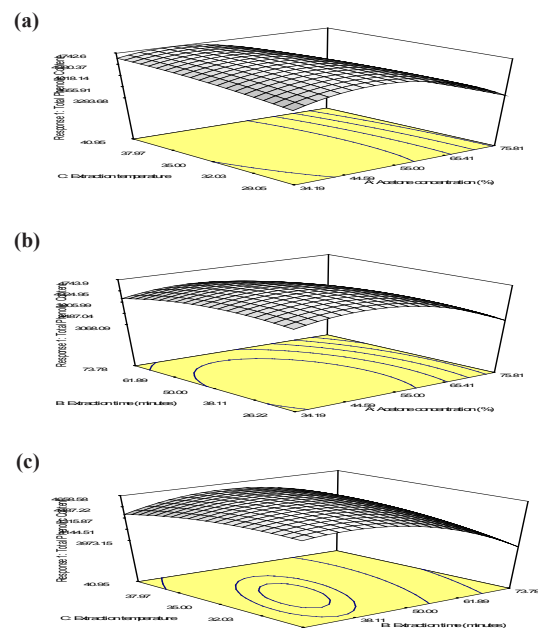

Figure 2. Response surface plot corresponding to total phenolic content (TPC) of neem leaves as a function of (a) acetone concentration and extraction temperature; (b) acetone concentration and extraction time; and (c) extraction temperature and extraction time. The value of the missing independent variable in each plot was kept at the middle level

The predicted response surface showing the effect of acetone concentration and extraction temperature on TPC at constant time (45 mins) appeared as a saddled shape (Figure 2a). Figure 2(a) depicted a higher amount of phenolic content yielded in the region at acetone concentration between 45 and 55% and extraction temperature between 35 and 41°C. Both acetone concentration and extraction temperature showed significant negative quadratic effects on TPC at $p<0.001$ and $p<0.001$, respectively (Table 2). Therefore, TPC gradually mounted up with the increase of acetone concentration and extraction temperature, and achieved optimum value at about 50% and 38°C, before it began to decrease. However, the contour gradient in extraction temperature coordinate direction was less than that in acetone concentration coordinate direction, namely acetone concentration is more important than extraction temperature as reflected by its higher negative quadratic coefficient (β_{11} = -627.70) compared to latter (β_{22} = -250.79). In general, the polarity of acetone-water mixture would increase continuously with the addition of water to acetone. More polar phenolic compounds such as may be extracted according to "like dissolves

like" principle. Thus, it could be seen that phenolics extracted using 60% acetone was higher than that of 90% acetone (Figure 2a).

Figure 2(b) denoted the effects of acetone concentration and extraction time on total phenolic content (TPC) at fixed extraction temperature of 35°C. Acetone concentration demonstrated a pronounced influence on TPC in linear and quadratic manner ($p<0.001$) (Table 2). Its linear and quadratic effects on TPC were both negative, which explained the nature of the curve as shown in Figure 2(b). At lower and upper levels of time, TPC went up corresponsive with the increase of acetone concentration up to 50% and further increase in acetone concentration leads to deceleration of phenolics extraction. However, extraction of phenolic compounds was observed to be negatively influenced by the synergism between acetone concentration and extraction temperature ($p<0.05$). This implicated that the extraction was largely favoured in two cases: low extraction temperature in the presence of high acetone concentration or high extraction temperature in the presence of low acetone concentration. From the industrialization point of view, high acetone concentration with low extraction temperature would be more adequate as high extraction temperature rendered the extraction procedure uneconomical.

The relationship of extraction temperature and extraction time with TPC was shown in Figure 2(c). Both of the factors displayed significant quadratic effect (at $p<0.001$) on TPC but in term of linear effect, extraction temperature showed no significant ($p>0.05$) (Table 2). With regard to extraction temperature, TPC of neem leaves extracts increased readily with increasing temperature up to 38°C and followed by a slight decrease afterwards. This suggested that incubation in warm water did improve phenolics extraction, yet was gentle enough to avoid heat degradation of the target phenolic antioxidants. Mild heating might soften the plant tissue, weaken the cell wall integrity, hydrolyze the bonds of bound phenolic compounds (phenol-protein or phenol-polysaccharide) as well as enhance phenolics solubility, thus more phenolics would distribute to the solvent (Juntachote *et al.*, 2006; Li *et al.*, 2006; Spigno *et al.*, 2007). At optimum extraction temperature (about 38°C), higher amounts of phenolic contents were obtained with short extraction time. In other words, long extraction time may compensate the beneficial effects of moderate temperature by inducing oxidation or degradation of phenolic compounds, yielding low TPC.

By combining all the results presented in Figure 2, the following conclusions can be drawn. It was clear that acetone concentration had the most critical role in the the extraction of phenolic compounds from neem leaves followed by extraction time and extraction temperature. Solubility of phenolic compounds could be enchanced using an aqueous acetone over a limited compositional range. In general, it was found that acetone that ranged from 40-60% had greater efficiency in the extraction of polyphenol compounds compared to pure acetone (Yap *et al.*, 2009). This seems to be agreed with 45-55% acetone reported in the present study. On the other hand, time and temperature of extraction were important variable to be optimised in order to minimize the energy cost of the process. The results revealed that extraction carried out at moderate temperature (32-40°C) for shorter time (30-60 min) was enough to saturate the solutions with phenolic compounds. In the meantime, this condition was able to minimize the possible impact on plant phenolics which might heat and light sensible.

Verification of predictive model

The experimental result for phenolic content (4661.17 ± 19.76 mg GAE/100 g, DW) were very close to the predicted one (4649.16 mg GAE/100 g DW). This implied that there was a high fit degree between the values observed in experiment and the value predicted from the regression model. Hence, the response surface modeling could be applied effectively to predict extraction of phenolic compounds from neem leaves.

Conclusion

The present study confirmed the advantages of RSM over classical method in optimising the extraction conditions for phenolic antioxidants from neem leaves. The results from RSM showed that TPC of neem leaves were most affected by acetone concentration followed by extraction time and extraction temperature. Using the numerical optimisation method, the optimum conditions for maximum TPC were as follows: acetone concentration, 51.52%; extraction time, 59.25 mins; and extraction temperature, 40.88°C. Under the mentioned conditions, 4661.17 mg GAE/100 g DW of phenolics were extracted from the neem leaves, which well agreed with the predicted value (4649.16 mg GAE/100 g DW). The second-order polynomial models developed were satisfactory in describing and predicting the phenolics extraction from neem leaves. With the application of RSM, the interaction effects among the extraction factors can be accessed as well the solvent usage and extraction time and temperature can be reduced as compared to single

factor experiment. Further works may carry out under the optimum conditions to elucidate the identity of phenolic compounds responsible for the antioxidant properties of neem leaves.

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