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Optimization of Extraction Parameters of Total Phenolic Compounds from Henna *(Lawsonia inermis)* Leaves

(Pengoptimuman Parameter Pengekstrakan Jumlah Sebatian Fenolik daripada Daun Inai (*Lawsonia inermis*)

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ABSTRACT

Response surface methodology (RSM) in conjunction with central composite rotatable design (CCRD) was performed in the present study to optimize the extraction parameters for assessing maximum yield of total phenolic content (TPC) from henna (Lawsonia inermis) *leaves. The range of the independent variables, namely acetone concentration (20-90%, v/v), extraction time (10-90 minutes) and extraction temperature (25-45°C) were identified by a first set of single factor experiments. The actual values of the independent variables coded at five levels were selected based on the results of single factor experiments. The optimum conditions for extraction of TPC were found to be at acetone concentration 48.07%, extraction time 73.78 minutes and extraction temperature 39.57°C. Under these optimized conditions, the experimental maximum yield of TPC was 7203.74 mg GAE/100g DW, which was in close agreement with predicted values, thus indicating the suitability of the models developed and the success of RSM in optimizing the extraction conditions.*

Keywords: Central composite rotatable design; Henna (Lawsonia inermis) *leaves; response surface methodology; total phenolic content*

ABSTRAK

Kaedah Permukaan Respons (RSM) bersama-sama reka bentuk komposit putaran tengah (CCRD) telah dijalankan dalam kajian ini untuk mengoptimumkan parameter pengekstrakan bagi penentuan jumlah sebatian fenolik (TPC) daripada daun henna (Lawsonia inermis). *Julat parameter tak bersandar iaitu kepekatan pelarut aseton, (20-90% v/v), masa pengekstrakan (10-90 minit) dan suhu pengekstrakan (25-45°C) dikenal pasti daripada kajian awalan uji kaji faktor tunggal. Nilai sebenar parameter tak bersandar yang berkod pada lima aras telah dipilih berdasarkan ujikaji faktor tunggal tersebut. Keputusan menunjukkan parameter optimum untuk mengekstrak TPC adalah pada kepekatan aseton, 48.07%; masa pengekstrakan, 73.78 minit dan suhu pengekstrakan, 39.57°C. Dalam keadaan optimum pengekstrakan ini, nilai eksperimen bagi jumlah sebatian fenolik (TPC) maksimum ialah 7203.74 mg GAE/100g berat kering yang sepadan dengan nilai yang diramal menunjukkan model yang dibangunkan menggunakan kaedah RSM adalah sesuai.*

Kata kunci: Daun inai (Lawsonia inermis)*; jumlah sebatian fenolik; kaedah permukaan respons; reka bentuk komposit putaran tengah*

Introduction

Medicinal plants have been used in almost all cultures as a source of medicine (Hoareau & DaSilva 1999). The use of traditional medicine and medicinal plants in most developing countries as a normative basis for the maintenance of good health has been widely observed. Moreover, in nowadays societies, herbal remedies have become more popular in the treatment of minor ailments, on account of the increasing costs of personal health maintenance (Hoareau & DaSilva 1999). About 1400 herbal preparations are used widely, according to a recent survey in Member States of the European Union. Herbal plants such as henna (*Lawsonia inermis*) contains high amount of flavanol and phenolic acid (Surveswaran et al. 2007) also known as antioxidants to help reduce free radicals by many ways from medication to food additives.

Surveswaran et al. (2007) noticed that traditional knowledge of medicinal plants has always guided the search for new cures. Eventhough there have been modern drug discovery and screening techniques, traditional knowledge systems have given clues to the discovery of valuable drugs. Traditional medicinal plants are often cheaper, locally available, easily consumable (raw) and has simple medicinal preparations (Surveswaran et al. 2007). Nowadays, traditional medicinal practices are a form of alternative medicine. Although their efficacy and mechanisms of action have not been tested scientifically

in most cases, these simple medicinal preparations often mediate beneficial responses due to their active chemical constituents (Alasalvar et al. 2006).

Plant phenolics comprise a great diversity of compounds such as flavonoids and several classes of non-flavonoids. Plant derived products contain a wide range of phytochemicals and phenolic compounds such as phenolic acids, flavonoids, tannins, lignin and others. It possesses antioxidant and antiradical activities (Shahidi & Naczk 2004), anticarcinogenic and antimutagenic effects as well as antiproliferative potential (Yang et al. 2005; Alasalvar et al. 2006). Chirinos et al. (2007) reported that plant based products contain health related effects such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities. Most of the beneficial characteristics of phenolic compounds have been attributed to their antioxidant activity which tells why Henna (*Lawsonia Inermis*) an indian herbal plant rich in phenolic compound was chosen for research purposes. Henna is easily found, cheap and contains high medicinal values (Dasgupta et al. 2003).

The phytochemical investigations have shown that henna is rich in phenolic antioxidants such as lawsone, flavonoids, tannins and coumarins (Khare 2007). In addition, response surface methodology (RSM) has been used successfully to model and optimize biochemical process including extraction processes, such as extraction of phenolic compounds from *Inga edulis* (Silva et al. 2007) and phenolic compounds from wheat (Liyana-Pathirana & Shahidi 2005). However, to the best of our knowledge, optimization of extraction of phenolic antioxidants from henna (*Lawsonia inermis)* leaves using RSM has not been reported yet. Therefore, the objective of this study was to determine the best extraction conditions for henna stems, in order to maximize simultaneously the yield of total phenolic content (TPC) by using response surface methodology.

Materials and Methods

SAMPLES

Local henna (*Lawsonia inermis*) leaf sample of 3 kg was freshly plucked from Cheras, Kuala 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.

CHEMICALS AND REAGENTS

All the solvents and chemicals used were of analytical grade. Deionised water which was produced by Milipore system (Millipore Corporation, USA) was used in the entire experiment.

SAMPLE PREPARATION

All the leaves were removed from the stems immediately upon picking. The leaves were then washed under running tap water thoroughly, 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 hours 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 0.5 mm 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.

SOLVENT EXTRACTION

Approximately 1 g of sample was weighed using analytical balance (Mettler Toledo AB204-S). The sample was then transferred into a 50 ml conical flask wrapped with aluminium foil to avoid direct sunlight. For each conical flask, 10 ml of extracting solvent was added to the sample with solvent ratio of 1:10. The upper part of the conical flask was then covered with parafilm and later covered with aluminium foil at the top. The conical flask was then placed on a shaker (VS-202D Green Sseriker, Germany) at 130 rpm or in a water bath shaker (Memmert, Germany) if temperature manipulation is required for extraction. A shaker is used for efficient mixing of solvent with sample. The extracts were than filtered using Whatmann No.1 filter paper and the filtrates were then transferred into an amber bottle and diluted 80 times before determination of Total Phenolic Content (TPC). All the extractions were carried out in replicates.

EXPERIMENTAL DESIGN

Basically, experimental design used in this present study comprised of single-factor experimental design, which was used for in screening, and central composite rotatable design (CCRD) that was employed for the purpose of response surface methodology (RSM).

SINGLE FACTOR EXPERIMENTS

Before using response surface methodology (RSM), preliminary experiments was carried out to choose the relevant variables in phenolic antioxidants recovery as well as the experimental range for the independent factors. Based on the mean values of total phenolic content (TPC),

the three levels (upper, middle and lower level) of each design variables (acetone concentration, extraction time and temperature) were determined for RSM.

SOLVENT TYPE

Extraction of antioxidant using different solvent mixture of acetone-water 60% (v/v), methanol-water 60% (v/v) and ethanol-water 60% (v/v) respectively. Each was done at fixed condition of room temperature (≈25°C), shaking at 130 rpm, and a fixed extraction time of 180 min. The best solvent type was chosen based on the highest value of total phenolic content (TPC) express as mg Gallic Acid Equivalents/100 g dry weight.

SOLVENT CONCENTRATION

The solvent concentration was investigated using the best solvent with concentration ranging from 20% to 100% (v/v). The best solvent concentration was selected based on the highest value of TPC (mg GAE/ 100 g DW).

EXTRACTION TIME

The impact of extraction time on the TPC was varied from 30, 90, 180, 270, 360 and 450 minutes. Extraction was accomplished by applying the best solvent composition, under room temperature. The extraction procedures were repeated as described. The best extraction time was chosen according to the highest value of TPC (mg GAE/ 100 g DW).

EXTRACTION TEMPERATURE

Lastly, the extraction was executed by using the best solvent composition and extraction time, under various temperatures, which were 25, 35, 45 and 55°C. The extraction procedures were repeated. The best extraction temperature was chosen due to the highest value of TPC (mg GAE/ 100 g dry weight).

MULTIPLE FACTOR EXPERIMENT

A five level, three factor rotatable central composite rotatable design (CCRD) (Design-Expert 6.0.10) was utilized to examine the optimum combination of extraction variables based on the TPC of henna leaves samples. The CCRD design comprised of 20 experimental runs with eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68 from the design center) and six replicates at the centre point. The low and high factor values were entered in term of alpha as extreme points (axial), thus all other design points will be located within those extremes. The design variables were the solvent concentration, X_1, the extraction temperature, X_2, and the extraction time, X_3 while the response was the total phenolic content (TPC) (mg GAE/ 100 g DW. The variables X_i were coded as x_i based on Equation (1):

$$x_i = \left(X_i - \bar{X}_i\right) / \Delta X_i \quad (1)$$

where x_i is the coded value (-α, -1, 0, +1, + α) of an independent variable, $\bar{X}_i$ is the real value of an independent variable at the center point, and ΔX_i is the step change value. The variables and their levels, with both coded values and natural values investigated in this work are represented in Table 1.

Table 2 shows the actual experimental parameters equivalent to the designed levels, which were performed for generating the second order polynomial model. Each experiment treatment was carried out in triplicate and the average TPC was taken as response, *Y*. The whole experiment was repeated for second time under the same conditions. Randomizing the order of experiments reduced the effects of unexplained inconsistency in the observed response due to the irrelevant factors.

VERIFICATION OF MODEL

Optimal extraction conditions on TPC of henna leave samples crude extract were obtained using the predictive equations generated by RSM. TPCwas tested using Folin-Ciocalteu method after solvent extraction under specific optimal conditions. Each set of experiment was conducted in two replicates, and the experimental and predicted values were compared in order to examine the validity of the model.

TOTAL PHENOLIC CONTENT (TPC) ASSAY

The TPC of henna stem extracts was determined spectrophotometrically using Folin-Ciocalteu's reagent according to the method described by Lim et al. (2007)

TABLE. 1 Independent variables and the coded and natural levels employed in a central composite rotatable design for optimisation of henna leaves extracts

Independent variable	Units	Coded levels				
		-α (-1.68)	-1	0	+1	+α (+1.68)
		Natural levels				
Acetone concentration, X_1	% (v/v)	20	34.19	55	75.81	90
Extraction time, X_2	min	10	26.22	50	73.78	90
Extraction temperature, X_3	°C	25	29.05	35	40.95	45

TABLE 2. Experimental design of five-level, three-variable central composite rotatable design, and the predicted and experimental results [a]

Test set	X_1, Acetone concentration	X_2, Extraction temperature	X_3, Extraction time	TPC (mg GAE/ 100 g DW)	
				Experimental	Predicted
1	34.19 (-1)	29.05 (-1)	26.22 (-1)	5778.99	5702.73
2	75.81 (+1)	29.05 (-1)	26.22 (-1)	6575.17	6487.52
3	34.19 (-1)	29.05 (-1)	73.78 (+1)	5886.01	5911.54
4	75.81 (+1)	29.05 (-1)	73.78 (+1)	7224.20	7070.97
5	34.19 (-1)	40.95 (+1)	26.22 (-1)	6602.44	6722.27
6	75.81 (+1)	40.95 (+1)	26.22 (-1)	5957.65	5898.71
7	34.19 (-1)	40.95 (+1)	73.78 (+1)	6768.76	6822.99
8	75.81 (+1)	40.95 (+1)	73.78 (+1)	6331.22	6374.06
9	55 (0)	35 (0)	50 (0)	7006.72	7058.53
10	55 (0)	35 (0)	50 (0)	7125.29	7058.53
11	55 (0)	35 (0)	50 (0)	7109.06	7058.53
12	55 (0)	35 (0)	50 (0)	6859.37	7058.53
13	20 (-1.68)	35 (0)	50 (0)	5167.02	5077.58
14	90 (+1.68)	35 (0)	50 (0)	5223.31	5360.01
15	55 (0)	35 (0)	10 (-1.68)	6894.98	6940.13
16	55 (0)	35 (0)	90 (+1.68)	7513.34	7515.44
17	55 (0)	25 (-1.68)	50 (0)	6411.60	6568.88
18	55 (0)	45 (+1.68)	50 (0)	6950.21	6840.18
19	55 (0)	35 (0)	50 (0)	7142.33	7029.22
20	55 (0)	35 (0)	50 (0)	7057.89	7029.22

[a] Experimental TPC values were average of triplicate

with slight modifications. Approximately 0.3 mL sample (15x dilutions) was added into test tubes followed by 1.5 mL of Folin-Ciocalteau reagent (10% v/v) and 1.2 mL of sodium carbonate (7.5% w/v). The test tubes were covered with parafilm and aluminium foil, mixed for 10 seconds using vortex and allowed to stand at room temperature for 30 minutes in dark environment. Absorption was measured at 765 nm using Uvi light spectrophotometer (Model XTD 5, SECOMAM). Blank sample was prepared by adding 0.3 mL solvent without the extract. Gallic acid was used as standard and TPC were expressed in gallic acid equivalents, mg GAE/ 100 g DW. Analysis was done in triplicate. The calibration equation for gallic acid is shown in equation (2):

$$y = 10.422x + 0.0042 \ (R^2 = 0.9977). \quad (2)$$

Statistical analysis All measurements of TPC assay were conducted in triplicate. The experimental results were mentioned as means ± standard deviation (SD) of three measurements. Relative standard deviation percentage (%RSD) was calculated using equation (3):

$$\%RSD = SD/ \text{ mean} \times 100\%. \quad (3)$$

Statistical analysis for data of single factor experiments was performed using one-way analysis of variance (ANOVA) (Minitab statistical software, version 15.1.1.0.). Data was analyzed using Tukey's test at $P< 0.05$.

Multiple linear regression analysis was performed by the software Design-Expert (Version 6.0.10, Stat-Ease Inc., Minneapolis) statistical software. Experimental data were fitted into a second-order polynomial model as shown in equation (4):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (4)$$

where X_i and X_j are the independent variables influencing the responses Y; β_0,β_i,β_{ii},β_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, correspondingly; k is the number of variables ($k = 3$). The quality of fit of the polynomial model was expressed by the coefficient of determination R^2, and the statistical

significances were evaluated by determining the F-value at a probability (p) at 0.001, 0.01, or 0.05.

Results and Discussion

Single factor experiments

Effect of solvent type on extraction of total phenolic compounds. Aqueous methanol, ethanol and acetone separately or mixed with water are commonly used to extract phenolic compounds from sample (Liu & Yao 2007). The ability of different solvents in extracting phenolic compounds was compared by performing Folic-Ciocalteu assay method. The results were expressed as gallic acid equivalents (mg GAE/ 100 g dry weight of henna leaves). Figure 1(a) shows that all solvents were capable of extracting phenolics but aqueous acetone (60%) was a more effective solvent than methanol (60%) and ethanol (60%) for extracting henna leaf samples. Acetone (60%) solvent gave the highest TPC (mg GAE/100 g DW) which is 6298.0 followed by ethanol (60%) 4684.7 and methanol (60%) 4028.4. The different types of solvent has a significant effect (p<0.05) on total phenolic content (TPC).

Acetone is a more effective solvent for extraction of condensed tannins as tannins have a relatively high molecular weight compounds (Alasalvar et al. 2006). It is strongly believed that the higher the molecular weight of the solvent, the lower the polarity which allows other substances of about the same molecular weight to be easily extracted. This can be correlated to "like dissolve like" or "polarity versus polarity" principle as both acetone and tannins are of high molecular weight. Acetone has the lowest polarity but contains the highest TPC value. Solubility of phenolics is affected by the polarity of solvents used which is why it is very difficult to develop an extraction procedure suitable for extraction of all plant phenolics (Naczk & Shahidi 2004). Acetone extract provides the most complete extraction of phenolic compounds from lentil seeds especially for flavonols and condensed tannins and other high-tannin plant material (Alasalvar et al. 2006).

Effect of acetone concentration on extraction of total phenolic compounds. As can be seen from Figure 1(b), the total phenolic content (TPC) as a function of acetone concentration follows a parabolic shape. TPC of henna leaf extracts increased with increasing of acetone concentration from 20% (4725 mg GAE/100 g dry weight, DW) to 60% (6298.0 mg GAE/100 g dry weight, DW) after which, it began to decrease until it reached a minimum of 708.5 mg GAE/100 g DW at 100% which was lower than acetone 20% of 4680.9 mg GAE/100g DW.

Alcoholic solvents have been commonly employed to extract phenolics from natural sources where they gave quite a high yield of total extract even though they are not highly selective for phenols. Mixtures of alcohols and water have revealed to be more efficient in extracting phenolic constituents than compared to mono-component solvent system (Spigno et al. 2007). Addition of small quantity of water to organic solvent usually creates a more polar medium which facilitate the extraction of polyphenols as suggested by Spigno et al. (2007). By increasing the proportion of water to acetone, the polarity of the solvent also increases. When this is achieved, the solvent system is able to extract phenolic substances from both ends of the polarity (highest polarity substances and low polarity substances), as well as those of moderate polarity (Zhang et al. 2007). Thus, acetone concentration (60%) was chosen for the determination of the effects of extraction time and extraction temperature on TPC.

Effect of extraction time on extraction of total phenolic compounds. Figure 1(c) presents the TPC extracted from henna leaf samples using various range of extraction time. As observed in Figure 1(c), the highest value of TPC for

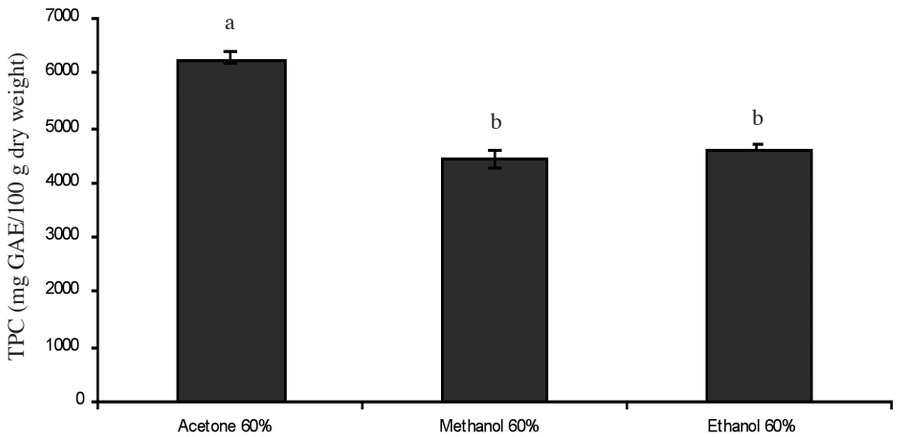

FIGURE 1a. Effect of the solvent type on the extraction of total phenolics from henna (*Lawsonia inermis*) leaves. The vertical bars represent the standard deviation (n = 2). Values marked by the same letter are not significantly different (p>0.05)

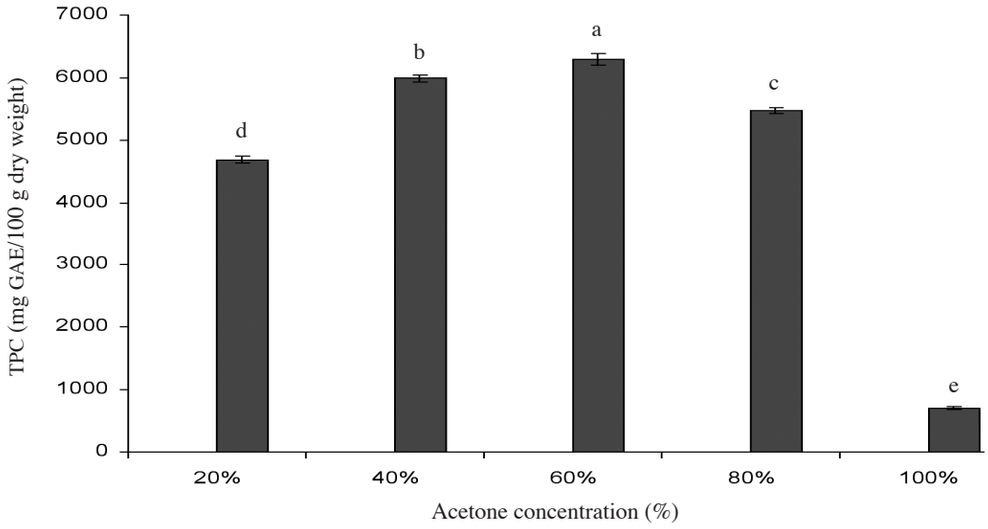

FIGURE 1b. Effect of the acetone concentration on the extraction of total phenolics from henna (*Lawsonia inermis*) leaves using acetone. The vertical bars represent the standard deviation ($n = 2$). Values marked by the same letter are not significantly different ($p>0.05$)

extraction time in extraction of phenolic compound was at 30 minutes accompanied by a drastic decrease at 90 minutes. The rest of the extraction time followed closely behind 90 minutes. The total phenolic content (TPC) at 30 minutes and 90 minutes was 6649.8 and 5910.3 mg GAE/100 g dry weights, DW, respectively. The lowest total phenolic content (TPC) reading was at 360 minutes extraction time 5791.3 mg GAE/100 g dry weights, DW as seen in Figure 1(c). Extraction time has significant effect on the extraction yield of phenolic compounds ($p<0.05$). However no significant difference ($p>0.05$) was observed at extraction time of 90 to 450 minutes.

The range of time was determined based on the practical and economical aspects. It was probably because longer time will increase cost. However, even at longer time, there was not much difference in extraction of phenolic compounds when compared to shorter time. Time does have a significant effect on extraction of phenolic compound as shown in Figure 1(c). It was obvious that a shorter time will extract the same amount of phenolic extracts as longer time while saving cost and is more practical. Excessive extraction time is not useful to extract more phenolic antioxidants (Silva et al. 2007). Polymers and wall-bound phenolics retained in cells that was extracted out as well as the polymerization reaction that occurs and new components produced probably a reason to the increase of total phenolic contents at a longer extraction time as reported by Spigno & De Faveri (2007). According to Mane et al. (2007), short extraction time was aimed to decrease tannin degradation and long ones to maximize extraction, but the concentration of tannins in the extracts tend to fall rather than rise after few hours. Taking into account of these facts, the lower, middle and upper levels of the extraction time were set as 10 minutes, 30 minutes and 90 minutes respectively for response surface methodology (RSM) optimisation.

Effect of extraction temperature on extraction of total phenolic compounds. The selection of the extraction temperature was the final step in a series of experiment. As shown in Figure 1(d), temperature has a significant ($p<0.05$) effect on extraction of phenolic compounds from henna leaves. Total phenolic yield at extraction temperature 25˚C is significant ($p<0.05$), while the other extraction time shows no significant difference between each other ($p>0.05$). The extraction of phenolic compounds as shown in Figure 1(d) was at its peak at 25°C with the value of total phenolic content (TPC) showing 5840.0 mg GAE/100 g DW. The value then decreases slightly to 45°C. After 45°C, the value of TPC decreased drastically as shown in Figure 1(d). Wang and Zheng (2001) reported that temperature strongly alters antioxidant properties in strawberries. When there is an increase in temperature, it favours extraction thus enhancing both solubility of solute and diffusion coefficient but beyond a certain temperature, phenolic compounds can be denatured (Spigno et al. 2007). This can be associated with extraction temperature reading at 55°C which shows the lowest TPC reading of 3446.3 (mg GAE/100 g DW). It was observed that high temperature may encourage solvent loss since boiling point of acetone is very close to 55°C through vapourisation and hence increase the cost for extraction process from the industry point of view. Vapourisation will create a more concentrated solvent extraction system where high concentration will increase high organic solvent content which reduces polarity and so eventually disturbs the phenolic extraction process as high concentration yield a lower extraction of phenolic compound. Therefore, moderate extraction temperatures of 25, 35 and 45°C were chosen as the lower, middle and upper levels, respectively, for the optimisation.

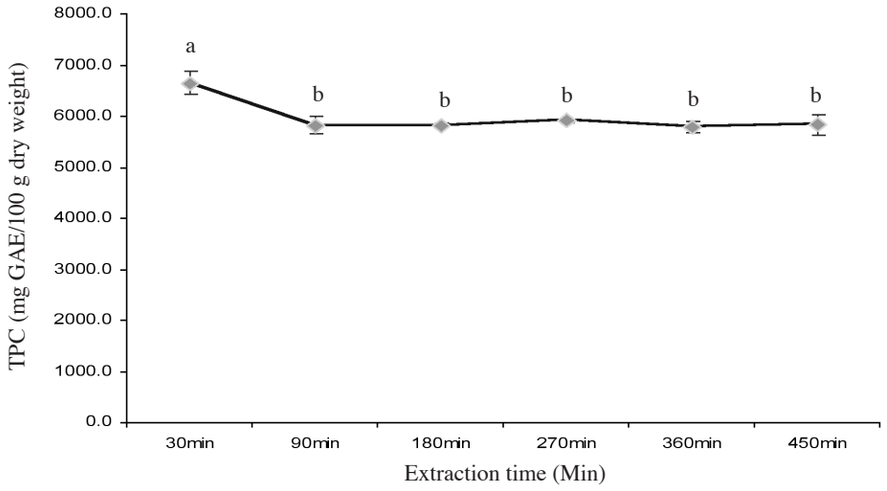

FIGURE 1c. Effect of the extraction time on the extraction of total phenolics from henna (*Lawsonia inermis*) leaves using 60% acetone. The vertical bars represent the standard deviation ($n = 2$). Values marked by the same letter are not significantly different ($p>0.05$)

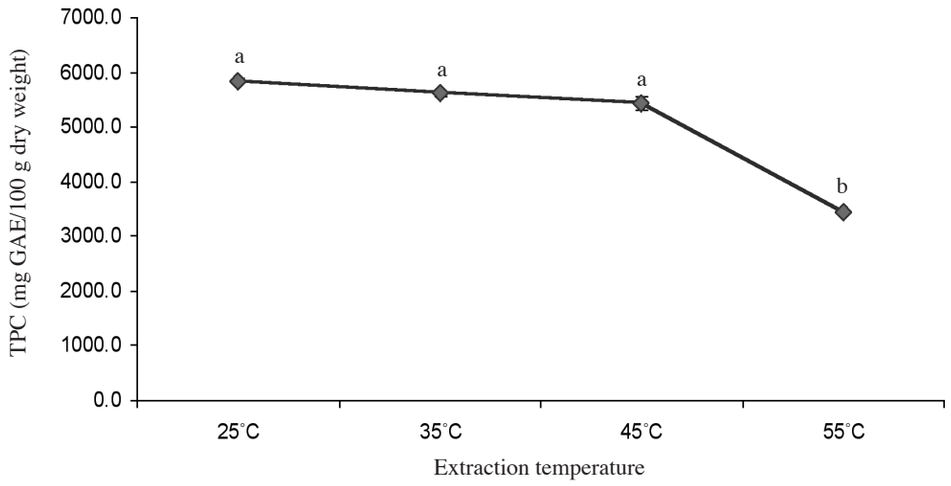

FIGURE 1d. Effect of the extraction temperature on the extraction of total phenolics from henna (*Lawsonia inermis*) leaves using 60% acetone for 30 min. The vertical bars represent the standard deviation ($n = 2$). Values marked by the same letter are not significantly different ($p>0.05$)

MULTIPLE FACTOR EXPERIMENT

Optimisation of extraction condition by response surface methodology (RSM). Based on the results of acetone concentration, extraction time and extraction temperature on the TPC of henna leaf extracts, these parameters were taken into account in the experimental design of RSM approach. A set of experiments was generated to optimize the extraction condition by employing the following factor ranges: acetone concentration, 20-90% (v/v); extraction time, 10-90 minutes; and extraction temperature, 25 - 45°C. The central points of CCRD were 55% (v/v), 50 minutes, and 35°C (Table 1). CCRD were conducted in 2 blocks. The first block consisted of 8 factorial points with 2 center points, while the second block contained 6 axial points with additional 4 center points.

The experimental design of five-level, three-variable CCRD and the predicted and experimental results of extraction are shown in Table 2. High TPC of the residue extract of henna leaf extract demonstrated its high commercial potential as an antioxidant resource. The maximum content of total phenolics (7513.34 mg GAE/ 100 g DW) was recorded under test set No.16 with experimental parameters of acetone concentration of 55% (v/v), extraction temperature of 35°C, and extraction time of 90 minutes. The lowest TPC (5167.02 mg GAE/ 100 g DW) was found at 20% (v/v) acetone concentration, 50 minutes extraction time, and 35°C extraction temperature.

TPC values obtained in single factor experiment were much higher than the TPC values acquired in multiple factor experiment. This was due to the interactive effect between

the three variables on the TPC of henna leaf extracts. Besides, the leaves were collected at different times and have differences in maturity, colour and climate which may lead to inconsistency in total content of phenolic antioxidants. According to Balasundram et al. (2005), TPC of plant extracts rely on numerous intrinsic factors such as species and genus, and also extrinsic factors for instances environment and storage.

REGRESSION COEFFICIENT ANALYSIS

The regression coefficients and the response surface were used to study the impacts of variables on the extraction of total phenolics and their total antioxidant capacity. Regression coefficients of the predicted second-order polynomial models for TPC are shown in Table 3.

Linear, quadratic and interaction effect of acetone concentration, extraction temperature and time was highly significant ($p < 0.01$), which showed the existence of the optimal value within the experimental area. This suggested that the change in either factor will influence TPC distinctly as shown in Table 3.

There were three regression terms which were highly significant ($p < 0.01$) with satisfactory coefficient of determination (R^2), which was 0.9774 for TPC. High coefficient of determination (R^2) illustrated that the model was well adapted to the response (Ven et al. 2002). Besides, the CV for TPC (Table 3) was within the tolerable range. The small values of CV (< 5%) exhibited superior reproducibility as it was a measure which representing relative standard deviation (%RSD). According to Pathirana and Shahidi (2005), a high CV demonstrated that variation in the mean value was large and did not sufficiently generate an acceptable response model. Therefore, CV < 10% was suggested suitable in predicting the responses surface model. The lack of fit of the model indicated whether the estimated response surface represents the actual shape of the surface. The lack of fit was not significant (p>0.05) in the models, meaning that the model was well fit.

TABLE 3. Regression coefficients of the predicted second-order polynomial models for TPC of star fruit residues

Model parameter	Regression coefficient
	Total phenolic content (TPC)
Linear	
X_1 - Concentration	83.97*
X_2 - Temperature	80.66*
X_3 - Time	171.04*
Quadratic	
X_1^2	-640.08*
X_2^2	-114.80*
X_3^2	70.20*
Interaction	
$X_1 X_2$	-402.09*
$X_1 X_3$	93.66*
$X_2 X_3$	-27.02*
Other statistics	
R^2 [a]	0.9774
Mean	6579.28
SD [b]	144.83
CV [c]	2.20
Lack of fit	0.2180

[a] Coefficient of multiple determination.
[b] Standard deviation.
[c] Coefficient of variance (SD/ mean × 100%).
* Significant at $p < 0.01$

ANALYSIS OF RESPONSE SURFACES

The effect of acetone concentration and extraction temperature on TPC was illustrated in the response surface and contour plots (Figure 2) with constant extraction time of 50 minutes. Figure 2 reveals the effects of the selected parameters on TPC which gave a significant result during optimisation. As reflected in Figure 2, the predicted response surface showing the effect of acetone concentration and extraction temperature on TPC at constant time (50 minutes) is saddle shaped. As seen in Figure 2, a higher amount of phenolic content approximately yielded in the region of acetone concentration between 55.00 and 65.41% where its peak was at 60 – 63% and extraction temperature between 29.05 and 32.05°C.

The experimental results of interaction effect between X_1X_2 showed that total phenolic content (TPC) of henna leaves samples varied at 5167-7513 mg GAE/100 g dry weight, DW. The highest TPC value (7513 mg GAE/100 g DW) was associated with experiment number 16 (Table 2), in which the acetone concentration was set at 55%, the extraction temperature at 35°C and the extraction time at 90 minutes. In contrast, the lowest TPC (5167 mg GAE/100 g DW) corresponded to experiment 13 (Table 2), with acetone concentration of 20%, extraction temperature of 35°C and the extraction time of 50 minutes.

It is believed that a high (> 60%, v/v) solvent concentration would give a low total phenolic content. According to Zhang et al. (2007) utilization of high solvent concentration in extraction of phenolic compounds should be avoided. In addition, Chirinos et al. (2007) mentioned in his study that solvents with high polarity do not give good extraction results. The use of water in combination with other organic solvents contributes to creation of moderately polar medium that ensures the extraction of polyphenols (Chirinos et al. 2007). Excessive water addition to acetone together with heating tends to extract high content of impurities (example organic acids, sugars and soluble proteins), which could interfere in phenolic identification and quantification (Chirinos et al. 2007). Acetone has distinct specificities in extraction of polyphenolic substances (Tabart et al. 2007). Acetone is the least polar solvents (The Merck Index 2001) compared to methanol, water and ethanol, which means that they

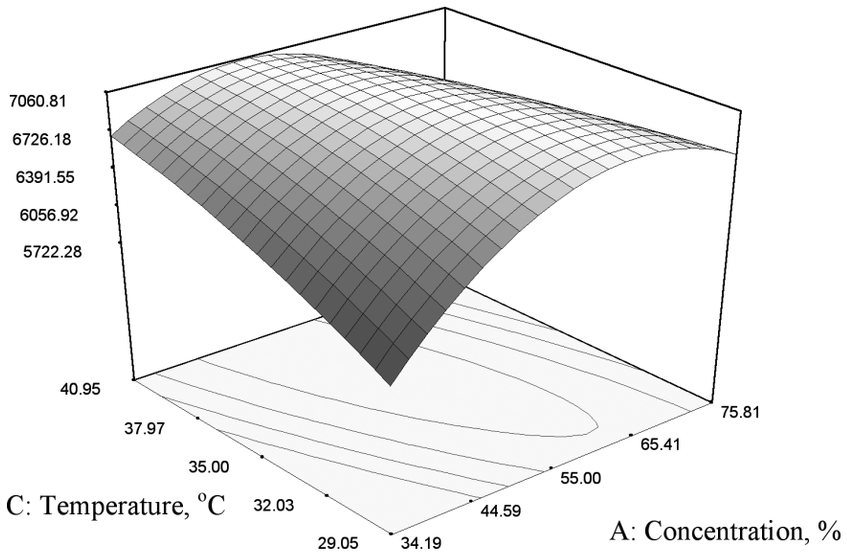

FIGURE 2. Response surface showing the effect of acetone concentration (%) and extraction temperature (°C) on Total Phenolic Compound (mg GAE/100 g) of henna (*Lawsonia inermis*) leaves. Extraction time was constant at 60 minutes

are more efficient in cell wall degradation which have nonpolar character which allows anthocyanins and other polyphenols to be released (Lapornik et al. 2005).

According to Table 3, all the linear and quadratic terms of acetone concentration, X_1, extraction temperature, X_2, and extraction time, X_3, showed significant effect (p<0.05) on TPC. This suggested that the change in either factors will influence TPC distinctly as shown in Table 2.

VERIFICATION OF OPTIMAL EXTRACTION CONDITIONS

In order to verify the predictive capability of the model, optimum conditions were established by RSM and comparisons between the predicted results and the practical values were done by experimental rechecking using those presumed optimal conditions. Table 4 presented the optimum conditions for TPC, and its predicted and experimental value.

The optimal extraction conditions of TPC from henna leaf extracts acquired using the model was as follows: acetone concentration, 48.07% (v/v); extraction temperature, 39.57°C; and extraction time, 73.78 minutes. Under these optimal conditions, the model predicted a maximum response of 7231.34 mg GAE/ 100 g DW of henna leave extracts. A mean value of 7203.74 ± 197.8 mg GAE/ 100 g DW of henna leaf extracts was acquired from real experiments.

From Table 4, it can be observed that the difference between the predicted result and the experimental value under the optimal extraction conditions for TPC was small (< 10%). This demonstrated that the response model was adequate to reflect the expected optimisation.

CONCLUSION

The RSM was successfully employed to optimize the extraction conditions on TPC of henna (*Lawsonia inermis*) leaf extracts. The regression coefficient and p-value indicated that acetone concentration (p<0.01) and time (p<0.01) was the most significant factor affecting extraction of TPC, followed by extraction temperature. The optimum operating conditions that maximize the extraction of TPC were acetone concentration, 48.07% (v/v); extraction temperature, 39.57°C; and extraction time, 73.78 min. TPC extracted under these conditions was 7203.74 ± 197.8 mg GAE/ 100 g DW, which in accordance to the predicted value (7231.34 mg GAE/ 100 g DW). Further study should be conducted to distinguish, isolate and purify the extracted phenolic compounds for the benefit of consumers as food supplements. A study should

TABLE 4. Optimal extraction conditions and the predicted and experimental value for TPC

Optimum conditions		TPC (mg GAE/ 100 g DW)		
		Experimental [a]	Predicted	Difference (%)
Acetone concentration	48.07%	7203.74 ± 197.8	7231.34	0.38%
Extraction temperature	39.57°C			
Extraction time	73.78mins			

[a] Means ± standard deviation of triplicate determinations (n = 2).

be carried out to determine what are the major contributors to the high phenolic content in henna leaf extract.

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