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Optimization of Ultrasound-Assisted Extraction of Total Flavonoids Content from the White Flowering Variety of *Melastoma Malabathricum*

(Mengoptimumkan Jumlah Kandungan Flavonoid dari *Melastoma Malabathricum* (Jenis Bunga Putih) Menggunakan Kaedah Ultrasonik Ekstrak)

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ABSTRACT

Melastoma malabathricum or Senduduk Putih is one of the herbs listed under National Key Economic Area (NKEA) of Malaysia. It has the potential to be used as remedies that is rich in natural flavonoids. One of the innovations for extraction in modern technology is Ultrasound-Assisted Extraction (UAE) that showed several advantages. Therefore, this study aimed to explore using UAE to extract the total flavonoids in M. malabathricum. After that, UAE is optimized coupled with a stirrer for total flavonoid extraction. Five operating parameters: ethanol concentration, plant material to solvent ratio, extraction temperature, extraction time and ultrasound power were investigated by one-factor-at-a-time (OFAT) method to choose the most important parameters. After that, the response surface methodology coupled with face-centered central composite design (FCCD) was employed to study the interaction of the three keys parameters. The optimized conditions to extract total flavonoids content by UAE with constant mixing speed (300 ± 6 rpm) were at a ratio of 1g of plant material: 30 mL of 100% (v/v) ethanol, extraction temperature at 70°C, 19 min of extraction time and 280 W of ultrasound power. The experimental yields of total flavonoids content were 64.94 mg/g, which is well matched with the predicted value of 63.75 mg/g.

Keywords: Melastoma malabathricum; Flavonoids; Optimization; Ultrasound-assisted extraction.

ABSTRAK

Melastoma malabathricum atau Senduduk Putih merupakan salah satu herba yang disenaraikan di bawah National Economic Area (NKEA) Malaysia, berpotensi untuk digunakan sebagai ubatan remedy yang kaya dengan flavonoid. Ultrasonik Ekstrek (UAE) merupakan keadeah yang digunakan dalam teknologi moden untuk mengekstrek herba mempunyai beberapa kelebihan. Kajian ini bertujuan untuk menyelidik M. malabathricum dengan menggunakan kaedah UAE untuk mengekstrak flavonoid. UAE dioptimumkan bersama dengan pengaduk untuk mengekstrak flavonoid. Terdapat lima operasi parameter: kepekatan etanol, bahan tumbuhan kepada nisbah pelarut, suhu pengekstrakan, masa pengekstrakan dan kuasa ultrasonik dikaji oleh kaedah one-factor-at-a-time (OFAT) untuk memilih parameter yang penting dan kemudian response surface methodology bersama dengan face-centered central composite design (FCCD) digunakan untuk mengkaji interaksi tiga parameter. Keadaan optimum untuk proses pengekstrakan Senduduk Putih menggunakan kaedah UAE telah dicapai pada kelajuan pencampuran (300 ± 6 rpm), nisbah 1g bahan tumbuhan kepada 30 mL 100% (v / v) etanol, suhu pengekstrakan pada 70°C, 19 min masa pengekstrakan dan 280 W kuasa ultrasonik. Jumlah flavonoid ekstrek adalah 64.94 mg/g berbanding dengan nilai jangkakan (63.75 mg/g).

Kata kunci: Melastoma malabathricum; Flavonoids; Mengoptimumkan; Ultrasonik ekstrak

INTRODUCTION

Increasing acute chronic diseases, patients are trying to seek supplementary and alternative remedies such as plant-based products to prevent major illness have been the main focus (Mamat et al. 2013). Most people understand that antioxidant properties can recover from many chronic health problems such as cardiovascular, cancer and aging by capturing the free radicals. There is a group of antioxidants receiving a lot of attention but less pronounceable: flavonoids. Flavonoids are extremely common and widespread in the plant kingdom (Bruneton 2012). They are abundant in plants, especially

in fruits and vegetables. These flavonoids are commonly consumed in the human diet. Therefore, biochemical and nutritional researchers are paying more and more attention to flavonoids because they have multiple biological benefits for humans (Sheng et al. 2013). Among the many benefits attributing to flavonoids are reduced the cancer risk, heart disease risk, asthma and stroke (Babu & Liu 2009; Bruneton 2012). *Melastoma malabathricum* which belongs to the *Melastomataceae* family, with different place names such as India-rhododendron (India), Straits Rhododendron (Singapore) and Malabar melastome (Australia) (Alnajar et al. 2012). Most important facts about *M. malabathricum* in Malaysia is a well-known plant with the local name, Senduduk Putih and has been extensively used in traditional Malay medicine. It has also been listed in the National Key Economic Area (NKEA) since 2014 (FRIM 2015). Susanti et al. (2007) described, this plant consists three varieties with different flowers colours which are dark-purple, light pink and white. From these varieties, the attractive flower of *M. malabathricum* with light pink magenta petals is the most commonly found variety in Malaysia whereas the white magenta colour flower is considered rare (Norshazila et al. 2010; Susanti et al. 2007). Previous studies showed that various parts of *M. malabathricum* have been extracted but the variety was not specified (Rajenderan 2010). Scientifically, *M. malabathricum* was reported to contain several phytochemical metabolites namely flavonoids and phenolic compounds that have different types of medicinal or therapeutic activities such as anticancer, antibacterial, antioxidant, anti-nociceptive, anti-parasitic, anti-venom, anticoagulant, antiviral, antiulcer, anti-inflammatory, antipyretic and wound healing (Zakaria et al. 2011; Mamat et al. 2013). A review of the literature shows that limited phytochemical and pharmacological studies have been carried on *M. malabathricum* (Susanti et al. 2007).

Various researches have been conducted by the pharmaceutical and food industries aimed to identify and extract new plant-based components that has the potential to be developed into drugs, functional food ingredients or nutraceuticals (Gil-Chavez et al. 2013). However, the supplies for plants are limited to serve the global demand. The conventional extraction methods namely Soxhlet extraction are not acceptable for industrial applications due to consumption of hazardous and expensive solvents, heavily use of raw materials, time consuming, labour intensive to perform the extraction and the risk of decomposition of some bioactive secondary metabolites (oxidation, hydrolysis etc.) (De Castro & Garcıa-Ayuso 1998; Wang & Weller 2006). New innovative technology and method have emerged to increase the production of plant extracts and at the same time preserve natural bioactive compounds. Some of the innovative technology such as Microwave-Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE) are not cost effective and also the needs of setting up sophisticated instruments (Wang & Weller 2006; Gil-Chavez et al. 2013). The other emerging innovation for extraction in modern technology is Ultrasound-Assisted Extraction (UAE) that has several advantages such as costly effective, time saving, lower volume of extraction solvent, increasing extraction yield, higher extract quality and environmental friendly (Wang & Weller 2006). With the development of the "green" chemistry concept during the last decades, many attentions were cast on UAE technique by many researchers. The bioactive ingredients from different natural sources were extracted under the ultrasonic treatment that is promising to offer the remarkable advantages. Moreover, during ultrasound extraction, creating an extra vibration at the sample molecules that facilitates the recovery of bioactive compounds from the solid material to the liquid solvent phase could bring many advantages such as facilitating mass transfer of solutes from the plant cells to solvent (Chemat et al. 2017).

Previous researchers have reported that the effects of ultrasound extraction variables (including ultrasound power, temperature, solvent to solid ratio and time) on the recoveries of the bioactive compound from different plant source resulted in better yield of targeted compounds obtained by UAE optimized process (Samaram et al. 2015). According to study has been reported UAE technique used to be to extract Phenolic compounds from *Citrus unshiu*, the total flavonoids content from *Flos populi*, the oil from papaya seed, and the antioxidant contents from *M. sanguineum* fruit (Ma et al. 2008; Sheng et al. 2013; Samaram et al. 2015; Zhou et al. 2017). The above studies have clearly shown the advantage of using the UAE technique under optimal conditions that have resulted in a higher yield of targeted compounds. This is due to ultrasound waves that generates an acoustic effects in the solvent resulting faster movement of molecules and higher penetration of solvent into the targeted material (Chemat et al. 2017). The successful operation of the UAE mentioned above was the motivation of this study, which aims to use this technique and determine the optimal conditions to extract the total flavonoid content from the white flower variety of *M. malabathricum.*

From the literature, it was hypothesized that UAE could be a reliable extraction method for the recovery of the total flavonoids content from white flower variety of *M. malabathricum*. Additionally, there has not been much studies performed on this species in Malaysia especially on the use of the UAE process. In this regard, the effect of UAE extraction variables on the total flavonoids content of *M. malabathricum* investigation was carried out. The extraction process parameters are to be investigated before optimizing. Therefore, one-factor-at-a-time (OFAT) method was employed to examine its performance over five selected process parameters (ethanol concentration, plant material to solvent ratio, extraction temperature, extraction time and ultrasound power). The determined operating conditions were then used for Response Surface Methodology (RSM) for optimization. Optimization studies were carried out by using the RSM coupled with face-centred composite design (FCCD) since it accounts for interaction effects between variables. This powerful tool is able to provide the optimized conditions for the process (Baş & Boyacı 2007; Amir et al. 2017; Irmayani 2017).

METHODOLOGY

PLANT SAMPLE COLLECTION AND PREPARATION

The aerial parts (leaves, stems, flowers and fruits) of white flower variety of *M. malabathricum* were collected from Kampung Ulu Choh, Johor, Malaysia. The species was further validated by Forest Research Institute Malaysia (FRIM) with the voucher specimen number (SBID: 029/18) of plant sample. The aerial parts of *M. malabathricum* plant were thoroughly cleaned and washed with potable water and then with distilled water. After that, the plant material was dried at 40°C until a constant weight obtained in a drying oven (UM 100, Memmert). After drying, it was ground and sieved to obtain the uniformly sized particles (between 30 to 40 mesh) (Sukhdev et al. 2008). Then, it was stored in a chiller at 4°C until future use. The obtained powdered sample from aerial parts of *M. malabathricum* is referred as plant material throughout this study.

SOXHLET EXTRACTION

Soxhlet extraction served as a benchmark for the amount of flavonoids content extracted from the aerial parts of *M. malabathricum* (Webster 2006). The same method was adapted from Dauda et al. (2015) with minor modification. The plant material was weighed with plant material to solvent ratio (g/mL) at 1:10 in 100 (v/v %) concentration of ethanol and soaked for 6 hours. Extraction at boiling point of solvent (78.37°C) was carried out. After extraction, the extract from *M. malabathricum* was filtered using Whatman No. 1 filter paper with Buchner filter under vacuum. The recovered filtrate was concentrated in a rotary evaporator (Model Heidolph, Germany) at 50°C under a reduced pressure of 200 mbar to prevent and minimize the degradation of the phytochemicals in the extract which then was dried at 40°C in an oven until a constant weight is obtained. The extracted sample was then kept in air tight glass jar and purged with nitrogen at the head space to maintain the stability of the sample. The sample was then stored in a freezer (-20°C) until future use.

ULTRASOUND-ASSISTED EXTRACTION

The UAE was conducted in a digital ultrasonic bath (XUB 25, Grant) comprising 400 W of ultrasound power with a capacity of 25 L (the dimensions: 365 x 385 x 546 mm). In this study, UAE was coupled with a stirrer (RZR 2041, Heidolph) to facilitate the mixing evenly (Figure 1). The mixing speed was fixed at 300 ± 6 rpm throughout the study. The sample with different plant material to solvent ratio (g/mL) was measured and filled into a beaker (2 L), made up to the volume with the extracting solvent and sonicated for different power ranges and time at the required temperature according to the OFAT and RSM experiments parameters. Before the extraction, water temperature in the ultrasonic bath was counter checked with a mercury thermometer (ZEAL, London) to ensure the correct water temperature. After UAE, the sample extract was filtered using Whatman No. 1 filter paper with Buchner filter apparatus under vacuum and the solution was collected and concentrated under reduced pressure in a rotary evaporator (200 mbar) at 50°C. Finally, the extract was dried at 40°C in the oven until a constant weight was obtained. The extracted sample was then kept in air tight glass jar and purged with nitrogen at the head space to stabilize the sample. The sample was then stored in a freezer (-20°C) until future use.

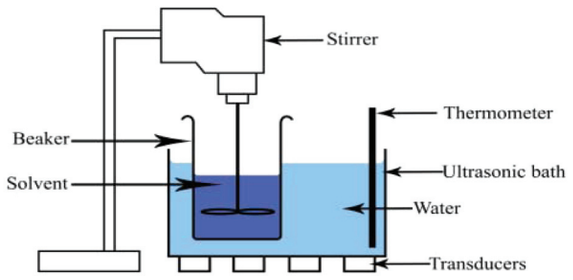

FIGURE 1. Schematic diagram of UAE coupled with mechanical stirrer

SINGLE FACTOR EXPERIMENT

Initial tests were conducted to screen the appropriate five parameters namely ethanol concentration, plant material to solvent ratio, temperature, extraction time, and ultrasound power by OFAT method in order to determine the experimental domain for an appropriate RSM design (Sheng et al. 2013; Heleno et al. 2016). The ranges of each parameter were tabulated in Table 1.

TABLE 1. Ranges of different parameters for OFAT method

Parameters	Ranges
Ethanol concentration (%)	20, 40, 60, 80, 100
Plant material to solvent ratio (g/mL)	1:10, 1:20, 1:30, 1:40, 1:50
Temperature (°C)	30, 40, 50, 60, 70
Extraction time (min)	10, 20, 30, 40, 50
Ultrasound power (W)	240, 280, 320, 360, 400

DESIGN OF EXPERIMENTAL (DOE) AND PROCESS OPTIMIZATION USING RSM

Based on the results in the OFAT, RSM was used to estimate the process conditions for maximum yield of total flavonoids content extraction. As shown in Table 2, three factors were selected in this study, namely temperature (X_1), extraction time (X_2) and ultrasound power (X_3), while the total flavonoids content (Y_1) was selected as the response for this design. The face-centred central composite design (FCCD) was used in this study, therefore there were seventeen experimental runs corresponding to eight factorial points, six axial points and three replicates at centre points in the design. The experiment was randomised to prevent the errors caused by uncontrolled variables (Bezerra et al. 2008). The experiment data was fitted to a second-order-polynomial model (Eq. (1)) to correlate

the relationship between the independent variables and the response (total flavonoids content).

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

Where Y is the predicted response; β_0 is a constant coefficient; β_i, β_{ii}, and β_{ij} are the linear, quadratic and interaction coefficients, respectively; the terms X_i and X_j are the coded value of independent variables; the terms X_iX_j and X_i^2 are the interaction and quadratic terms, respectively.

TABLE 2. Independent variables ranges and levels for optimization of total flavonoid content

Independent variable	Coded symbols	Coded level		
		-1	0	1
Temperature (°C)	X_1	50	60	70
Extraction time (min)	X_2	10	20	30
Ultrasound power (W)	X_3	240	280	320

ANALYSIS OF TOTAL FLAVONOIDS CONTENT

The aluminum chloride colorimetric method was adapted to determine the total flavonoid content (Chandra et al. 2014) with some modifications. Stock quercetin (standard) and sample extract solution were prepared separately by dissolving 10 mg of standard/extract in 10 mL of methanol. Then, an amount of 600 µL of diluted standard quercetin solution and sample extract was separately mixed with 600 µL of 2% aluminium chloride. The mixtures were incubated at room temperature for 60 min. In this test, the absorbance of the mixture was measured against the blank sample (same mixture without the sample) and recorded spectrophotometrically at 415 nm with a UV-Vis spectrophotometer (UVmini-1240, Shimadzu Corp). All analyses were performed in triplicate. Quercetin was used as the standard to measure the flavonoids content in the samples expressed as mg quercetin equivalent (QE)/1g of extract from the standard curve () with a standard deviation of $R^2 = 0.9981$.

STATISTICAL ANALYSIS

All the experimental runs were performed in triplicates unless mentioned. Statistical analysis of the experimental data from the OFAT method was carried out using one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post hoc test. The SPSS statistic version 23.0 software (IBM SPSS Inc., Chicago, IL, USA) was used for the data processing and analysis. The mean values at $p < 0.05$ were used to verify the statistical significance of the result. The experimental design and statistical analysis in RSM experiment were performed using Minitab software (version 16). The adequacy of the model was determined by evaluating the lack of fit, the coefficient of determination (R^2) and the F-value.

RESULTS AND DISCUSSION

SINGLE FACTOR EXPERIMENTAL ANALYSES

OFAT technique was employed to evaluate the extraction parameters on flavonoids content of the aerial parts of *M. malabathricum*. The UAE parameters were first examined with this method to determine the most important variable (X) that affected the response (Y). The results are shown in Figure 2 and its details are described in the section below:

EFFECT OF ETHANOL CONCENTRATION ON TOTAL FLAVONOIDS CONTENT

Ethanol is one of the most commonly used solvent to extract bioactive components from plant materials. Ethanol was selected as a solvent in this study with the consideration that it was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant materials and an adequate solvent for extraction of flavonoids (Ijaiya et al. 2014). In addition, ethanol for plant extraction has been considered easy to evaporate at low temperature, low toxicity, safe for human beings and the environment and green compared to toxic solvents dangerous in industrial processes (Pandey & Tripathi 2014; Zhou et al. 2017). Hence, ethanol was selected strengthened as the solvent to be used in the extraction process for this study. In order to study the effect of ethanol on the extraction performance, different concentrations of ethanol (20, 40, 60, 80 and 100%) were used and other experimental parameters were set as follows: plant material to solvent ratio (1:30 g/mL), extraction temperature (50°C), extraction time (30 min) and ultrasound power (240 W). The results obtained from the analysis are presented in Figure 2a. It was clearly observed that when the ethanol concentration increased from 20% (v/v) to 100% (v/v), the flavonoids content significantly increased ($p < 0.05$). The flavonoids content was at the highest 49.52 ± 0.65 mg QE/g when 100% (v/v) ethanol was used. According to the principle "like-dissolves-like", it has been found that 100% (v/v) ethanol was benefic to the extraction of flavonoids from *M. malabathricum* aerial parts (Bayliak et al. 2016; Zhou et al. 2017). Meanwhile, solvent properties such as vapour pressure, viscosity and surface tension could affected the cavitation effect involved in the UAE process. Theoretically, solvent which contained low viscosity and surface tension will produce good cavitation effect in UAE process (Hemwimol et al. 2006). The results shown in Figure 2a, can be attributed to the combination of the polarity of ethanol and the cavitation effect. The results also showed in statistical analysis (Table 3) that 100% (v/v) ethanol has the significant difference among 20, 40, 60 and 80 ($p < 0.05$). It showed that the mean difference generated was the highest with 100% (v/v) ethanol. From the economic perspective, it is affordable to use 100% (v/v) ethanol concentration as a solvent since it can be recovered easily by reduced pressure distillation and do not require high energy to remove the solvent as compared to lower concentration of

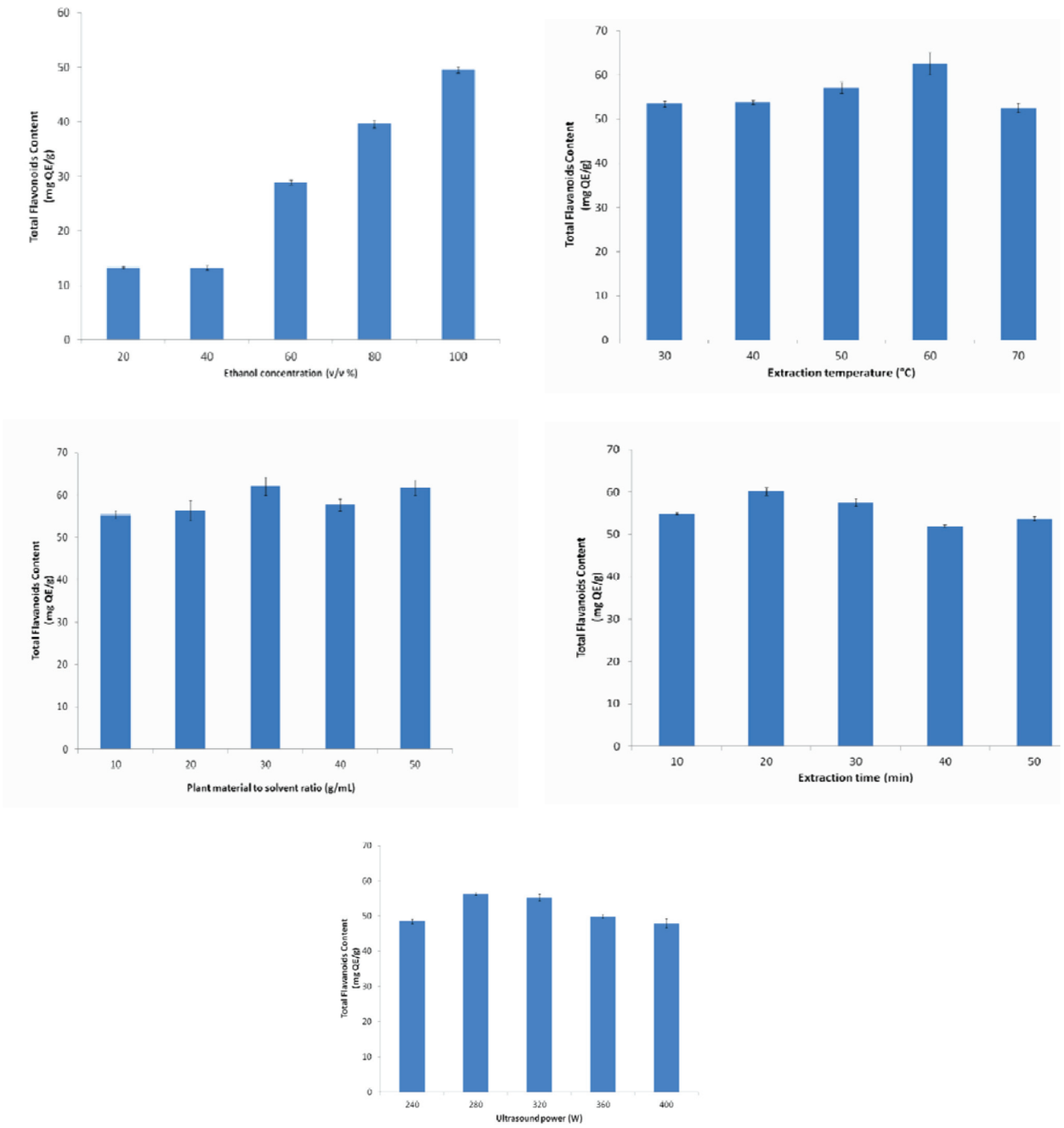

FIGURE 2. The effects of different extraction parameters on total flavonoids content of M. malabathricum aerial parts using OFAT. (a) Ethanol concentration; (b) Extraction temperature; (c) Plant material to solvent ratio; (d) Extraction time; (e) Ultrasound power. Data values were expressed as mean ± standard deviation (n = 3); means were compared by LSD test ($p < 0.05$)

TABLE 3. One-Way ANOVA, LSD Post Hoc test with comparison of different extraction parameters

Parameters	(I) VAR	(J) VAR	Mean Difference (I-J)	Std.Error	Sig	95% Confidence Interval	
						Lower Bound	Upper Bound
Ethanol concentration (%)	100	20	36.24*	0.413	0.00	35.32	37.16
		40	36.29*	0.413	0.00	35.37	37.21
		60	20.57*	0.413	0.00	19.65	21.49
		80	9.84*	0.413	0.00	8.92	10.76
Extraction temperature (°C)	60	30	9.08*	1.13	0.00	6.56	11.60
		40	8.69*	1.13	0.00	6.18	11.21
		50	5.44*	1.13	0.00	2.93	7.96
		70	10.03*	1.13	0.00	7.52	12.55
Extraction time (min)	20	10	5.32*	1.09	0.00	3.01	7.64
		30	2.70*	1.09	0.03	0.39	5.01
		40	8.22*	1.09	0.00	5.91	10.53
		50	6.52*	1.09	0.00	4.21	8.83
Plant material to solvent ratio (g/mL)	30	10	7.11*	2.61	0.02	1.29	12.92
		20	6.37*	2.61	0.04	0.55	12.18
		40	4.93	2.61	0.09	-0.88	10.75
		50	1.51	2.61	0.58	-0.43	7.32
Ultrasound power (W)	280	240	7.79*	0.66	0.00	6.32	9.26
		320	0.97	0.66	0.17	-0.50	2.44
		360	6.35*	0.66	0.00	4.88	7.82
		400	8.33*	0.66	0.00	6.86	9.80

*The mean difference is significant at the 0.05 level

ethanol (Boonkird et al. 2008). With the above reasons and results, 100% (v/v) ethanol concentration was not evaluated further in the RSM study as it was not a continuous variable. Thus, 100% (v/v) ethanol concentration was used in all the followed study.

EFFECT OF TEMPERATURE ON TOTAL FLAVONOIDS CONTENT

In order to study the effect of temperature on the yield of the total flavonoids extraction, different temperatures (30°C to 70°C) were tried while keeping the other parameters set at 100% (v/v) ethanol, 30 min of extraction time, plant material to solvent ratio (1:30 g/mL) and 240 W of ultrasonic power. As shown in Figure 2b, the extracted flavonoids content increased gradually with the increasing of temperature from 30°C to 60°C ($p < 0.05$). The highest yield (62.55 ± 2.51 mg QE/g) was achieved at 60°C and then dropped at 70°C (52.52 ± 1.05 mg QE/g). Based on the statistical analysis in Table 3, the mean yield difference was highest at 60°C and it showed significant differences among 30, 40, 50 and 70°C. This could be due to sufficiently high temperature (60°C) thus promoting the diffusion of the solvent through the cell walls and increases the acoustic cavitations effects (Zhou et al. 2017). Sheng et al. (2013) also obtained similar results and explained that solvent viscidity declined and the movement of molecular accelerated with the increase of temperature. In such view, with the increasing of extraction temperature, it was beneficial for bioactive compounds to release from plant cells. However, when the temperature rose higher than 60°C, degradation of some thermo-sensitive compounds could have taken place. In short, the yield of flavonoids extraction increased with the temperature until 60°C. However, it decreased after 60°C drastically and could be due to some heat sensitive compounds are denatured. Thus, the extraction temperature at 60°C was used to evaluate further in the RSM study.

EFFECT OF PLANT MATERIAL TO SOLVENT RATIO ON TOTAL FLAVONOIDS CONTENT

Plant material to solvent ratio is an important parameter that will significantly affect the total flavonoids extraction (Sheng et al. 2013; Zhou et al. 2017). If the ratio of plant material to solvent is too small, it would cause the incomplete extraction whereas if the ratio is too big, this would cause a higher processing cost (Sheng et al. 2013). Therefore, suitable ratio between plant material and solvent should be investigated for the flavonoids extraction. The effect of different contents of the plant material to solvent ratio (1:10, 1:20, 1:30, 1:40 and 1:50 g/mL) was studied while the other parameters were set at the investigated value earlier: 100% (v/v) ethanol, extraction temperature (60°C), extraction time (30 min) and ultrasound power (240 W). The results are shown in Figure 2c. The flavonoids content gradually increased with the ratio from 1:10 g/mL to 1:30 g/mL ($p < 0.05$). This could be due to the increase diffusivity of the solvent into the cells and enhance the dissolution of solute (Zhou et al. 2017). It has been found

that the highest flavonoids content (62.04 ± 2.18 mg QE/g) was obtained with the ratio 1:30 g/mL. However, when the later increased from 1:30 g/mL to 1:50 g/mL, no improvement in total flavonoids extraction was observed. This finding was in good agreement with the results obtained by Zhou et al. (2017). This phenomenon could be explained by the fact that the dissolution process reached its equilibrium at this ratio (1:30 g/mL), consequently, an increase in this plant material to solvent ratio does not lead to any improvement in the extraction. As shown in Table 3, the mean difference (1:30 g/mL) showed significant differences among 1:10 g/mL and 1:20 g/mL ($p < 0.05$) but there was no significant difference for 1:40 g/mL and 1:50 g/mL ($p < 0.05$). Insignificant differences of the extraction yield exist from 1:30 g/mL to 1:50 g/mL, which indicated that the flavonoids content has reached its equilibrium. In view of the effective extraction aspects, the discontinuity of the parameters and also cost effectiveness, the ratio 1:30 g/mL was adapted in all the following work.

EFFECT OF EXTRACTION TIME ON TOTAL FLAVONOIDS CONTENT

A longer extraction time could improve the extraction yield by prolonging contact between the solvent and the plant material. However, a longer extraction time could, on the other hand, reduce the efficiency of the process (Sheng et al. 2013; Zhou et al. 2017). Extraction time is therefore a considerable parameter to be evaluated. The effect of extraction time was analyzed at different durations (10, 20, 30, 40 and 50 min) maintaining the three previously studied parameters set to: 100% (v/v) ethanol, 60°C of extraction temperature, plant material to solvent ratio (1:30 g/mL), and using 240 W of ultrasound power. Figure 2d showed that the flavonoids content increased with the extraction time from 10 min to 20 min. The statistical analysis (Table 3) showed significant differences for the first 20 min with peak yield value at 60.19 ± 0.88 mg QE/g ($p < 0.05$) and indicated that UAE could extract the total flavonoids from the plant material in a short time (Zhou et al. 2017). However, when the extraction time was extended to 50 minutes, the flavonoid content decreased slightly ($p < 0.05$). This could be interpreted by a possible degradation of some flavonoids during the extended ultrasound time (Sheng et al. 2013; Zhou et al. 2017). Thus, the 20 min of extraction time was used in the RSM experiment.

EFFECT OF ULTRASOUND POWER ON TOTAL FLAVONOIDS CONTENTS

With the above investigated parameters: 100% (v/v) ethanol, 60°C of extraction temperature, plant material to solvent ratio (1:30 g/mL) and 20 min of extraction time, the effect of ultrasound power (240 W, 280 W, 320 W, 360 W and 400 W) on the extraction yield of total flavonoids was studied. The results given in Figure 2e showed that the extraction yield of flavonoids increases with the increase of ultrasound power from 240 to 280 W. The statistical analysis (Table 3) showed significant differences between 240 W and 280 W of ultrasound power with a peak value at 56.23 ± 0.35 mg QE/g ($p < 0.05$). As the ultrasound power rose from 280 W to 400 W, the yield of flavonoids decreased ($p < 0.05$). This result indicated that the extraction efficiency was improved with the increasing of the ultrasound power (from 240 W to 280 W). But, when the ultrasound power exceeded 280 W, some flavonoids components are possibly degraded (Zhou et al. 2017). Similar results were obtained by Boonkird et al. (2008); Chemat et al. (2017); Zhou et al. (2017) who concluded that the ultrasound power could enhance the effect of cavitation. This conclusion was also supported by the fact that increasing the ultrasound power, it accelerates swelling and caused an enlargement in the pores of the cell walls (Boonkird et al. 2008). It resulted in a better mass transfer of constituents from the plant material to the solvent from 240 W to 280 W of ultrasound power. Hence, the 280 W of ultrasound power was evaluated further in the RSM experiment.

UAE OPTIMIZATION BY RSM

RSM is a robust statistical tool used to predict the relationship between measurable response variables and a set of experimental factors presumed to affect the responses (Bezerra et al. 2008). In this study, ethanol concentration and plant material to solvent ratio were not evaluated in the RSM experiment as it showed the discontinuity of the parameter. Thus, the RSM experiment was designed based on the OFAT results obtained: extraction temperature (60°C), extraction time (20 min) and ultrasound power (280 W) on the flavonoids content of *M. malabathricum* aerial parts.

MODEL FITTING AND STATISTICAL ANALYSIS

FCCD was adapted in RSM as it required three levels of setting for each parameter. Another consideration of using FCCD was due to it is a simpler design to perform and could reduce the prediction error (Zhang & Xiaofeng 2009). As shown in Table 4, the response ranges (flavonoid content) between 47.60 mg QE/g and 63.73 mg QE/g.

$$Y_1 = 22.750 - 8.010X_1 + 3.228X_2 + 1.665X_3 - 0.010X_1X_2 - 1.844E-04X_1X_3 - 4.344E-04X_2X_3 + 0.071X_1^2 - 0.062X_2^2 - 0.003X_3^2 \quad \text{Eq. (2)}$$

Where X_1, X_2, and X_3 are the coded values of temperature, extraction time and ultrsound power, resectively.

The reliability of the fitted quadratic model (Eq. (2)) was analyzed using ANOVA, while the statistical significance of the regression equation was checked by F-test (Table 5). The p-value assocaited with each linear, quadratic and interaction term in the model indicated the significance of the effect represented by the term. The smaller the p-value, the greater the significance of the corresponding coefficient (Sheng et al. 2013). Based on Table 5, the F-value (40.50) and the p-value ($p < 0.0001$) of the model indicate the validity of the model. Moreover, the coefficient of determination ($R^2 = 0.9812$) and the adjusted coefficient of determination (Adj. $R^2 = 0.9569$) are close to unity, demonstrating the closeness of

TABLE 4. Experimental design and the experimental data for total flavonoids contents

Run	X_1 (temperature)	X_2 (extraction time)	X_3 (ultrasound power)	Y_1(Total flavonoids contents)
				Actual value (mg QE/g)
1	60	10	280	48.15
2	70	20	280	63.73
3	60	30	280	49.63
4	60	20	240	50.21
5	60	20	280	54.40
6	60	20	320	50.65
7	50	30	240	48.96
8	50	30	320	49.87
9	60	20	280	54.63
10	70	10	320	54.78
11	60	20	280	53.33
12	50	10	240	47.60
13	50	10	320	48.06
14	50	20	280	60.61
15	70	30	320	51.30
16	70	30	240	51.83
17	70	10	240	53.47

the experimental data to the values predicted by the software. Meanwhile, the lack of fit test with F-value (2.01) and p-value (0.3649) was insignificant relative to the pure error. Insignificant lack of fit test indicated an adequate fit of the models to the experimental data for all response variables. Apart from that, Table 5 also showed the significance of each coefficient as in Eq. (2). From the p-value of each model term, it was observed that the linear term (X_1), quadratic terms (X_1^2, X_1^2, X_1^2) and interaction terms (X_1X_2, X_3X_2) significantly affected the total flavonoids content ($p < 0.05$).

TABLE 5. ANOVA analysis for flavonoids extraction

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value	Significant
Model	301.85	9	33.54	40.50	< 0.0001	Significant
X_1	40.04	1	40.04	48.35	0.0002	
X_2	0.022	1	0.022	0.027	0.8749	
X_3	0.67	1	0.67	0.81	0.3980	
X_1^2	133.46	1	133.46	161.6	< 0.0001	
X_2^2	103.73	1	103.73	125.26	< 0.0001	
X_3^2	58.74	1	58.74	70.93	< 0.0001	
X_1X_2	8.59	1	8.59	10.37	0.0146	
X_1X_3	0.044	1	0.044	0.053	0.0146	
X_3X_2	0.24	1	0.24	0.29	0.6059	
Residual	5.80	7	0.83			
Lack of fit	4.83	5	0.97	2.01	0.3649	Not significant
Pure error	0.96	2	0.48			
Cor total	307.65	16				
R^2	0.9812					
Adj. R^2	0.9569					
Pred. R^2	0.8640					
Adequate precision	23.501					

RESPONSE SURFACE ANALYSIS

Three-dimensional response surface plots that visualized the relationships of the independent variables on the response are presented in Figure 3. Figure 3a shows the total flavonoids content increased with the extraction time from 10 min to 20 min, and subsequently decreased with the extraction time above 20 min. This trend is similar to the OFAT experiment result (Section 3.1.4) which was also observed by Sheng et al. (2013). Figure 3b shows as the ultrasound power increased from 240 W to 280 W, flavonoids content increased. Nevertheless, the trend reversed upon further increase of the ultrasound power. Such observation is also consistent with the OFAT experiment findings (Section 3.1.5). Extraction

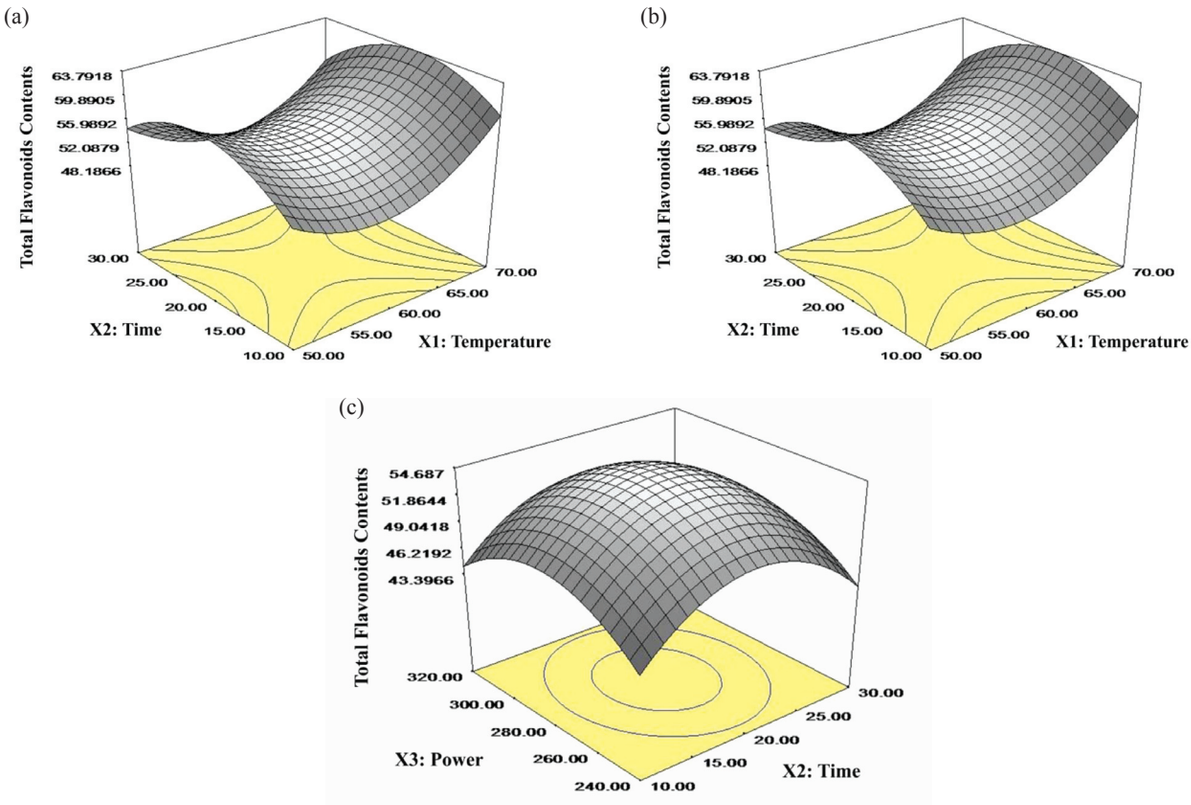

FIGURE 3. Response surface plots of total flavonoids contents. (a) Effect of temperature and extraction time; (b) Effect of temperature and ultrasound power; (c) Effect of extraction time and ultrasound power

temperature exerted similar effect on the total flavonoid content in both Figures 3a and 3b. The response decreased with the temperature from 50°C to 60°C, then increased again at 70°C. Besides, Figure 3c illustrated the surface plot for the effect between the ultrasound power and extraction temperature. An increased in response value was observed with the increase of ultrasound power and extraction time at in the beginning but the trend was reversed when the ultrasound power and extraction time reached its optimum point (20 min and 280 W, respectively).

VALIDATION OF PREDICTED VALUE

The maximum response (Y_1) was determined using the Response Optimizer function in the Minitab software. A maximum of 63.75 mg QE/g content was predicted under the following conditions: temperature (70°C), extraction time (19 min) and ultrasound power (280 W) as shown in Table 6. The observed value under such conditions was 64.94 mg QE/g, with 1.83% error (Table 6). As the error was small (within 5%), therefore the optimized condition for the response model was verified.

TABLE 6. Model validation from predicted values at optimum conditions

Optimum condition			Total flavonoids contents (mg QE/g)				
			Experimental				Predicted
Extraction temperature (°C)	Extraction time (min)	Ultrasound power (W)	Test 1 Test 1	Test 2 Test 2	Test 3 Test 3	Average	
70	19	280	65.05	65.65	64.13	64.94 ± 0.77	63.75

COMPARISON BETWEEN UAE AND SOXHLET TECHNIQUES

In order to evaluate the process of UAE in this study, the optimized UAE was compared with the Soxhlet method for the extraction of the total flavonoids from *M. malabathricum* (Table 7). Comparing the results from the two different techniques, it was found that the total flavonoids content extracted by UAE (64.94 ± 0.77 mg QE/g) was significantly higher than that obtained by the use of the Soxhlet extraction technique (40.97±1.05 mg QE/g). It should also be noted that the optimum result by UAE technique was obtained in short extraction time (19 min) while the respective result with the Soxhlet extraction method obtained after 6 hours. Therefore, the UAE technique was found to be more efficient than the conventional Soxhlet extraction method, thus allowing to recover a significantly higher content of flavonoids from the aerial parts of the plant *M. malabathricum.* The results were consistent with other reports (Xu et al. 2015; Xu et al. 2016; Chanioti & Tzia 2017; Zhou et al. 2017). This could be due to the mechanical effect and cavitation induced by ultrasound which certainly disrupte the cell walls of the plant and resulting an increased on the mass transfer compared to the Soxhlet extraction method (Ma et al. 2008). The thermosensitivity of the target bioactive compounds (flavonoids), could facilitate their possible degradation or denaturation (oxidation, hydrolysis etc.) during extraction with Soxhlet due to the high boiling point of ethanol (78.37°C) and the relatively long extraction time (6 hours). This could caused the lower yield in Soxhlet method (Chanioti & Tzia 2017). Overall, the results indicated that UAE used lower extraction temperature, shorter extraction time and lower ultrasund power (70°C, 19 min and 280 W, respectively) to produce higher total flavonoids content. UAE technique can be considered for an industrial scale in the future.

TABLE 7. Quantification of phytochemical composition from UAE and Soxhlet techniques

Extraction method	Total flavonoids content (mg QE/g)
Soxhlet extraction	40.97 ± 1.05
UAE	64.94 ± 0.77

CONCLUSION

This study investigated the effects of different UAE's extraction parameters namely, extraction temperature, extraction time and ultrasound power on the yield of total flavonoids content of *M. malabathricum* aerial parts. Optimization process revealed that the optimum extraction parameters were set as follows: extraction temperature, 70°C; extraction time, 19 min; and ultrasound power, 280 W. This optimized extraction process provided the desirable yield (64.94 ± 0.77 mg QE/g). Our findings indicated the definite effectiveness of UAE for the extraction of total flavonoids from *M. malabathricum* aerial parts compared to the Soxhlet extraction method. The experimental validation verified the reliability of the optimum UAE conditions.

Compared to Soxhlet extraction method, the UAE technique has proven its potency for extracting enough more flavonoids (24 mg QE/g more) from the aerial parts of *M. malabathricum*. In addition, this study is the first trial of the use of UAE for the extraction of flavonoids from the aerial parts of this medicinal plant. The positive and encouraging results from this study demonstrated that the UAE process could also be considered as an alternative for the industrial to develop into full production scale in the future.

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