Screening of Microwave-Assisted-Batch Extraction Parameters for Recovering Total Phenolic and Flavonoid Contents from *Chromolaena odorata* Leaves through Two-Level Factorial Design

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Abstract: *Microwave-assisted-extraction (MAE) of phenolic compounds from* Chromolaena odorata *leaves was investigated using one-factor-at-a-time (OFAT) and two-level factorial design. The MAE parameters studied were irradiation time (A: 1–5 min); microwave power level (B: 400-800 W); extraction temperature (C: 60–80 °C); solvent/feed ratio (D: 8:1–14:1 mL/g); and ethanol concentration (E: 20–60% v/v). The optimum yields of TPC and TFC were 56.13 mg GAE/g d.w. and 44.78 mg QE/g d.w., respectively were achieved from MAE of* C. odorata *leaf at irradiation time of 2 min, microwave power of 600 W, temperature of 60 °C, solvent:feed ratio of 10:1 mL/g, and ethanol concentration of 40% v/v through one-factor-at-time (OFAT) experimental trials. The results obtained from a two-level factorial design experiments demonstrated that only ethanol concentration (20–60% v/v), irradiation time (1–5 min) and microwave power level (400–800 W) had significant effects on the yields of total phenolic content (TPC) and total flavonoid content (TFC) from* C. odorata *leaves ($p < 0.05$). However, temperature and solvent/feed ratio was not significant. In addition, the interactions AB (irradiation time and microwave power) and AE (irradiation time and ethanol concentration) contributed greatly to the recovery yields.*

Keywords: Chromolaena odorata; *phenolics; flavonoids; microwave-assisted extraction*

INTRODUCTION

Over the few decades, studies on functional foods have increased, leading to the discovery of new bioactive components that can boost food processing and lead the reduction in oxidative-stress diseases. Plants are the source of natural phytochemicals such as secondary metabolites which are important as part of the food supplement and pharmaceutical active ingredients. Phenolics and flavonoids are important parts of secondary metabolites that can inhibit free radicals cells and minimize the possibility of cancer [1]. A lot of pharmacological activities which include antioxidant, anti-inflammatory, anti-diabetes, anti-microbial, and among others could be associated with the occurrence of phenolic compounds [2-4].

Chromolaena odorata, known to be bush bitter, is an invasive shrub widely found in sub-tropical regions of the world. This plant had been studied to be full of potentials as a medicinal shrub. In fact, it has been reported to improve soil properties, act as good phytoremediating and bioremediating agent against crude oil polluted soils [5]. The leaves are rich in carbohydrate, proteins, fiber, ash, and energy. Traditionally, this plant has been exploited as a source of medicine in the treatment of several ailments such as the human small cell, lungs, and breast cancer, inflammation, and burn wounds intestine diseases [5-6].

Different phenolic compounds had been isolated from *C. odorata* leaves, including *p*-coumaric, vanillic acids, protocatechuic, quercetin, kaempferol, luteolin, and others, which can reduce the oxidative degradation of lipids [7]. Phenolic compounds are known to possess antioxidant activity through various mechanisms, including free radical inhibition, hydrogen donors, metal chelation, gene expression, and signaling pathways [8]. Different extraction methods had been employed in previous studies to recover phenolic compounds from this plant, which include maceration, decoction and Soxhlet [7,9-10]. Nevertheless, lower recovery yields of the phenolic compounds had been reported through these conventional extraction methods. Therefore, microwave-assisted extraction method has been employed in this study for higher recoveries of phenolic compounds from *C. odorata* leaves.

Microwave-assisted-extraction (MAE) is a modern method with the advantage of recovering higher yield of phenolic compounds from plant matrix within a shorter irradiation time and a small amount of solvent [11]. Comparing to other unconventional extraction methods, such as ultrasonic-assisted-extraction, MAE can recover higher yield faster than other methods [12]. Nevertheless, different factors including extraction temperature, solvent/feed ratio, irradiation time, the concentration of solvent, and microwave power can alter the performance of MAE [13]. The efficiency of MAE had been previously compared with ultrasound-assisted-and conventional solvent extraction of TPC from *Pistacia lentiscus* leaves. The results reflected that MAE gave TPC in higher yield of comparing to the other two extraction methods [12]. In addition, MAE had been previously employed to recover TPC and TFC from *Vernonia cinerea* leaves. The results indicated that higher yields of phenolic compounds can be achieved through MAE [14]. To the best of our knowledge, there is no study on the application of MAE in the recovery of TPC and TFC from *C. odorata* leaves had been found.

MAE is influenced by several parameters which include microwave power, temperature, feed/solvent ratio, solvent concentration, and irradiation time. In order to determine the ranges of value for these parameters, one-factor-at-a-time experimental trials are usually used by varying one parameter when keeping the others fixed [13]. However, to affirm the significant contribution of individual extraction parameter, the two-level factorial design is mostly employed. Therefore, this study focuses on the use of a two-level factorial design in filtering the MAE variables to determine the contribution of individual variables in the recoveries of total phenolic and flavonoid contents from *C. odorata* leaves, the ranges for individual MAE variables were determined through single factor experiment.

■ EXPERIMENTAL SECTION

Materials and Chemicals

The leaves of *C. odorata* were harvested from Universiti Malaysia Pahang, Gambang premises. The sample was rinsed using running tap water to eliminate earth debris and air-dried to achieve a stable weight. Then, the dried leaves were blended using an electric grinder and kept in a sealed dark container at 4 °C prior to the extraction process. The average moisture content of the sample before extraction was estimated to be 0.017±0.03 g water/ dry sample.

Quercetin, anhydrous aluminum chloride, Folin-Ciocalteu phenol reagent, sodium carbonate salt, and ethanol (99 wt.% purity), and gallic acid were purhased from Sigma-Aldrich (M) Sdn Bhd, Selangor.

Procedure

Microwave-assisted-extraction

The ranges of MAE variables, including microwave power level (200–800 W); extraction temperature (40–100 °C); irradiation time (1-20 min); ethanol concentration (20-80% v/v); and solvent/feed ratio (6:1–16:1 mL/g) were randomly examined using a one-factor-at-a-time experiment (Fig. 1) [13]. The crushed sample of *C. odorata* leaves (5 g) was mixed with the binary mixture of ethanol and water based on the single factor and two-level experimental design matrix (Fig. 1, Table 1). The suspension was placed in an enclosed Milestone microwave reflux extractor (ATC-FO-300 model, N. America). The single factor experiment was carried out by changing the values of a factor and fixing others

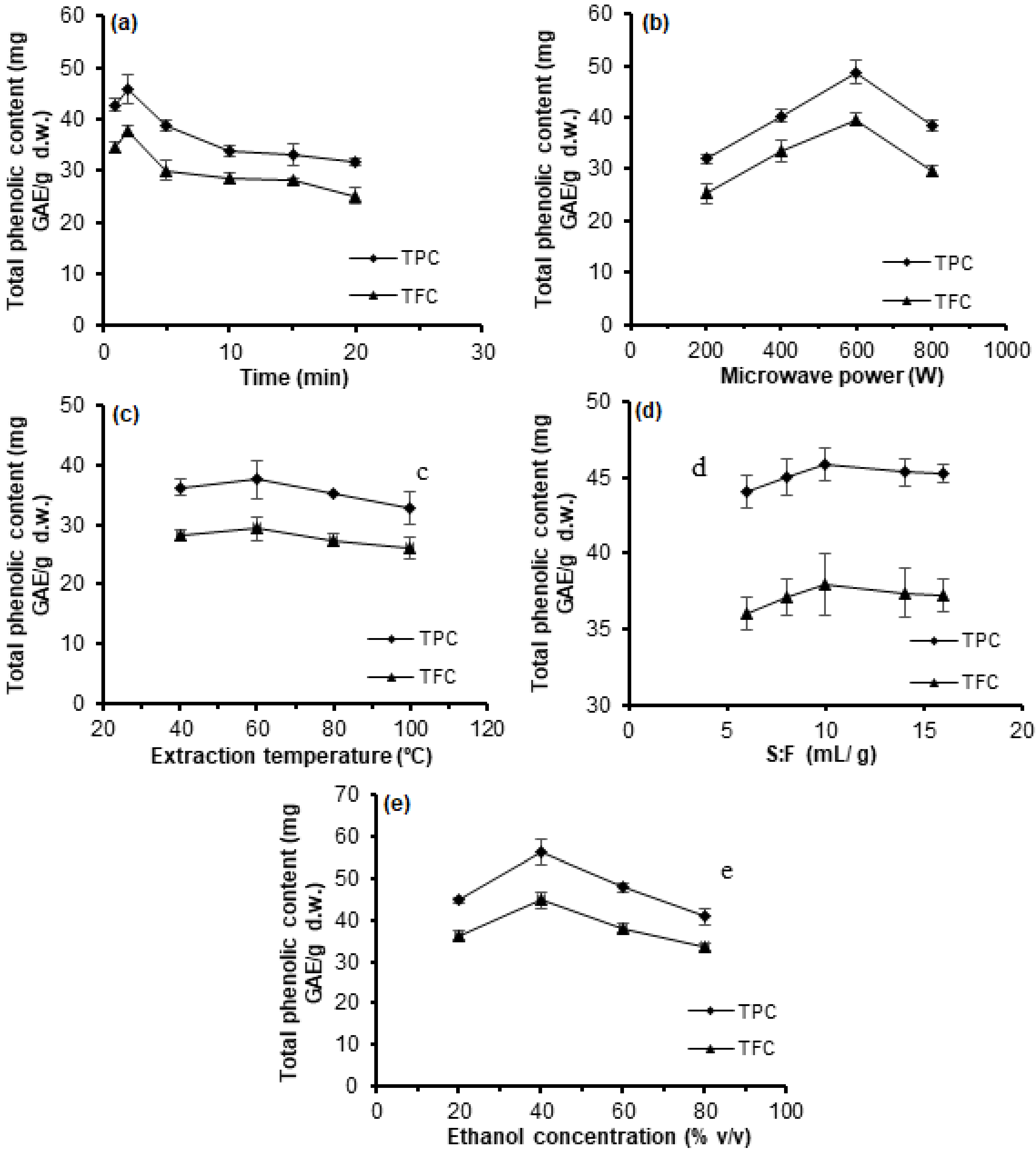

Fig 1. Influence of irradiation time (a), microwave power (b), extraction temperature (c), solvent/feed ratio (d), and ethanol concentration (e) on the TPC and TFC yields from *C. odorata* leaves

constantly. In the case of irradiation time, the variation was randomly ranged from 1 to 20 min, while keeping solvent/feed ratio, microwave power, extraction temperature, and ethanol concentration at 6:1 mL/g, 200 W, 40 °C, and 20% v/v, respectively. The suspension was filtered and dried using a rotary evaporator. Thereafter, the extracts were analyzed to determine TPC and TFC recovery yields.

Total phenolic content determination

TPC in the *C. odorata* leaf extract was determined according to the procedure employed in a previous study [15]. The TPC was calculated using Eq. (1). For the purpose of error correction, the analysis was done in triplicate and the obtained results were expressed as mean±standard deviation.

$$\text{TPC (mg GAE / g d.w.)} = \frac{\text{Conc. (mg / mL)} \times \text{vol. of solvent used (mL)}}{\text{Weight of dried sample used (g)}} \quad (1)$$

Evaluation of total flavonoid content

Total flavonoid content in the *C. odorata* leaf extract was determined using the procedure used in a previous study [15]. Then, TFC was calculated using Eq. (2). The analysis was repeated thrice, and the obtained results were reported as mean±standard deviation.

$$\text{TFC (mg QE / g d.w.)} = \frac{\text{Conc. (mg / mL)} \times \text{vol. of solvent used (mL)}}{\text{Weight of dried sample used (g)}} \quad (2)$$

Statistical analysis

In order to examine the effect of five MAE variables, a two-level half factorial experimental design with 16 runs was employed. Each run was carried out thrice to determine the experimental error. The resulting data and statistical analysis were evaluated using Design Expert 7.0 software® (Minneapolis, USA) and Microsoft Excel 2013®.

■ RESULTS AND DISCUSSION

One-factor-at-a-time Experiment

The one-factor-at-a-time experiments were carried out to evaluate the effect of each MAE variable on the TPC and TFC yields from *C. odorata* leaf and to determine the range limit at which the experimental processes need to be performed. Five MAE factors, including microwave power level, extraction temperature, irradiation time, ethanol concentration, and solvent/feed ratio were considered. Basically, this experimental process was performed by changing the values of a parameter while others were fixed.

For the irradiation time variation, microwave power (200 W), extraction temperature (40 °C), solvent/feed ratio (6:1 mL/g), and ethanol concentration (20% v/v) were fixed at various irradiation time between 1 and 20 min (Fig. 1(a)). The irradiation time is one of the main factors that affect MAE. It can be seen that an improvement in the irradiation time from 1 to 2 min was followed by the increase in TPC and TFC. Beyond 2 min of irradiation, the yields gradually decrease which implies that final equilibrium had been achieved between the solute concentration in the plant materials and bulk solution as explained by Fick's second law of diffusion [16]. Thus, excessive irradiation time was unnecessary to extract phenolic compounds from *C. odorata* leaves. In addition, prolonged extraction might degrade the phenolic compounds due to oxygen exposure. Considering this fact, irradiation time at the range of 1 to 5 min was selected for the two-level factorial design experimental processes.

The selection of microwave power levels is another important variable in MAE. Microwave power level was varied between 200 to 800 W at fixed irradiation time of 2 min, extraction temperature of 40 °C, the solvent/feed ratio of 8:1 mL/g, and ethanol concentration of 20% v/v. Fig. 1(b) illustrates the influence of microwave power on TPC and TFC. There was a noticeable increase in the TPC and TFC recoveries as microwave power level improved from 200 to 600 W, prolong increase in power decline the yield recoveries. This might be due to the thermal degradation of phenolic compounds at elevated microwave power [17]. Therefore, a range of 400–800 W microwave power level was chosen for further experimentation.

The influences of extraction temperature on MAE of TPC and TFC from *C. odorata* leaf are illustrated in Fig. 1(c). TPC and TFC slightly increased as the extraction temperature increased from 40 to 60 °C. There was an insignificant increment in the yields as the extraction temperature increases. This implies that extraction temperature might have little or no effects on the recovery of phenolic compounds from *C. odorata* leaves using MAE. More so, it should be noted that increasing extraction temperature beyond a certain limit may cause concurrent degradation of phenolic compounds that would have been mobilized at a lower temperature. Likewise, a higher extraction temperature leads to solvent loss through vaporization, this can increase the cost of the extraction process [10,12]. In order to affirm the significant contribution of extraction temperature to this process, the extraction temperature in a range of 40 to 80 °C was chosen for further experimentation.

Similarly, the effects of solvent/feed ratio on MAE of phenolic compounds from *C. odorata* leaves at a constant microwave power level of 600 W, the irradiation

Table 1. Experimental design matrix using two-level factorial

Run	Variables					Responses	
	A	B	C	D	E	Y_1	Y_2
1	1	400	40	8:1	60	68.02	43.55
2	5	400	40	8:1	20	65.02	40.12
3	1	800	40	8:1	60	50.00	26.34
4	5	800	40	8:1	60	53.49	28.09
5	1	400	80	8:1	20	54.18	34.65
6	5	400	80	8:1	60	66.43	44.43
7	1	800	80	8:1	60	66.97	45.11
8	5	800	80	8:1	20	53.15	31.96
9	1	400	40	14:1	20	51.00	32.01
10	5	400	40	14:1	60	66.33	45.34
11	1	800	40	14:1	60	77.85	56.99
12	5	800	40	14:1	20	53.24	29.87
13	1	400	80	14:1	60	67.03	43.02
14	5	400	80	14:1	20	65.88	42.45
15	1	800	80	14:1	20	54.86	30.25
16	5	800	80	14:1	60	43.15	21.09

A - Irradiation time (min); B - Microwave power (W); C - Temperature (°C); D – Solvent/feed ratio (mL/g); E - Ethanol concentration (% v/v); Y_1 – Total phenolic content (mg GAE/g d.w.); Y_2 – Total flavonoid content (mg QE/g d.w.); d.w. – dry weight

time of 2 min, extraction temperature of 60 °C, and 20% v/v of ethanol concentration are shown in Fig. 1(d). It can be observed that gradual increase in TPC and TFC only occurred when solvent/feed ratio was increased from 6:1 to 10:1 mL/g, there was an insignificant increase in yield as the volume of the extracting solvent increased beyond 10:1 mL/g. In general, the solvent/feed ratio showed little or no influence on the recovery of phenolic compounds from *C. odorata* leaves. The two-level factorial design was further employed to confirm the contributing level of solvent/feed ratio to the recoveries of TPC and TFC from *C. odorata* leaf. Thus, the recovery of TPC and TFC was maximized at a solvent/feed ratio of 10:1 mL/g. The ratio of 8:1-14:1 mL/g was selected for the two-level full factorial experiments.

The influences of ethanol concentration on the TPC and TFC yields from MAE of *C. odorata* leaves are presented in Fig. 1(e). Ethanol concentration was varied between 20 and 80% v/v at fixed irradiation time of 2 min, 600 W microwave power, extraction temperature of 60 °C, and solvent/feed ratio of 10:1 mL/g. Increase in phenolic compounds was observed as the ethanol concentration inclined from 20 to 40% v/v followed by a reduction in yields (ethanol concentration beyond 40% v/v). The optimum yields of TPC and TFC were 56.13 mg GAE/g d.w. and 44.78 mg QE/g d.w., respectively were achieved from MAE of *C. odorata* leaf at irradiation time of 2 min, microwave power of 600 W, the temperature of 60 °C, S:F ratio of 10:1 mL/g, and ethanol concentration of 40% v/v. A similar effect has been reported for the extraction of phenolic compounds from *Pistacia lentiscus* leaves, increasing ethanol concentration beyond 50% v/v resulted in drastic reduction of phenolic compounds [12]. This study revealed that 100% v/v absolute ethanol can not recover higher phenolic compounds as compared with aqueous ethanol. This phenomenon can simply be explained based on the fact that molecular movement and solvent polarity tends to decrease as the ethanol concentration increases, which result in the reduced dissolution of phenolic compounds due to the declined solubility and diffusion coefficient [12]. The range between 20 and 60% v/v was selected for further experimentation.

Table 2. ANOVA and contributing level of each MAE factor and p-values for the responses

MAE variables	Percentage contribution (%)		p-values for the responses	
	Y_1	Y_2	Y_1	Y_2
Model			0.0006	0.0004
A	2.63	3.90	0.0139	0.0044
B	12.77	14.93	0.0008	0.0003
C	0.86	0.42	0.0748	0.1300
D	0.021	0.22	0.7271	0.2406
E	18.71	17.21	0.0004	0.0003
AB	23.95	21.34		
AC	0.16	0.027		
AD	2.16	1.63		
AE	29.46	24.03		
BC	1.87	1.29		
BD	0.39	0.21		
BE	0.011	0.16		
CD	2.30	9.88		
CE	4.66	4.68		
DE	0.049	0.067		

A - Irradiation time (min); B - Microwave power (W); C - Temperature (°C); D- Solvent/feed ratio (mL/g); E - Ethanol concentration (% v/v); Y_1 – Total phenolic content (mg GAE/g d.w.); Y_2 – Total flavonoid content (mg QE/g d.w.); d.w. – dry weight

Influence of MAE Parameters on the TPC and TFC Yields from *C. odorata* Leaf Extract Using a Two-Level Full Factorial Design

The influences of five MAE variables such as irradiation time; extraction temperature; solvent/feed ratio; ethanol concentration, and microwave power on the yields of TPC and TFC from *C. odorata* leaves were examined using the two-level half factorial design. The evaluation of five MAE variables required a total number of 16 experimental trials (Table 1). The effects of individual variable and their interactions on the recovery yields were determined based on the percentage of their contributions and significant levels at $p < 0.05$. Table 2 illustrates the ANOVA and percentage contribution of each variable alongside their interactions. It can be observed that the models for both responses are significant with the p-value less than 0.05. Ethanol concentration (E) was the most contributing factors with 18.71 and 17.21% for TPC and TFC, respectively while the solvent/feed ratio was the least contributing factor. This indicates that ethanol concentration is more important in the recovery if TPC and TFC from *C. odorata* leaves as it had the most significant influence on yields with the highest percentage of contribution. This phenomenon can simply be explained based on the fact that molecular movement and solvent polarity tends to decrease as the ethanol concentration increases, which result in the reduced dissolution of phenolic compounds due to declined solubility and diffusion coefficient [12].

Likewise, the ethanol concentration, irradiation time and microwave power had noticeable influences on the responses ($p < 0.05$), however, solvent/feed ratio and extraction temperature were insignificant. The higher contributing levels of irradiation time, ethanol concentration and microwave power had been previously reported for MAE of Reb-A, stevioside and total extract from *Stevia rebaudiana* leaves [19]. The contribution level of individual variables is in order of ethanol concentration > microwave power level > irradiation time > temperature > solvent/feed ratio. However, the interaction between variables as well accorded to the recoveries of TPC and TFC from *C. odorata* leaf. From the obtained results, the interactions between irradiation time and microwave power,

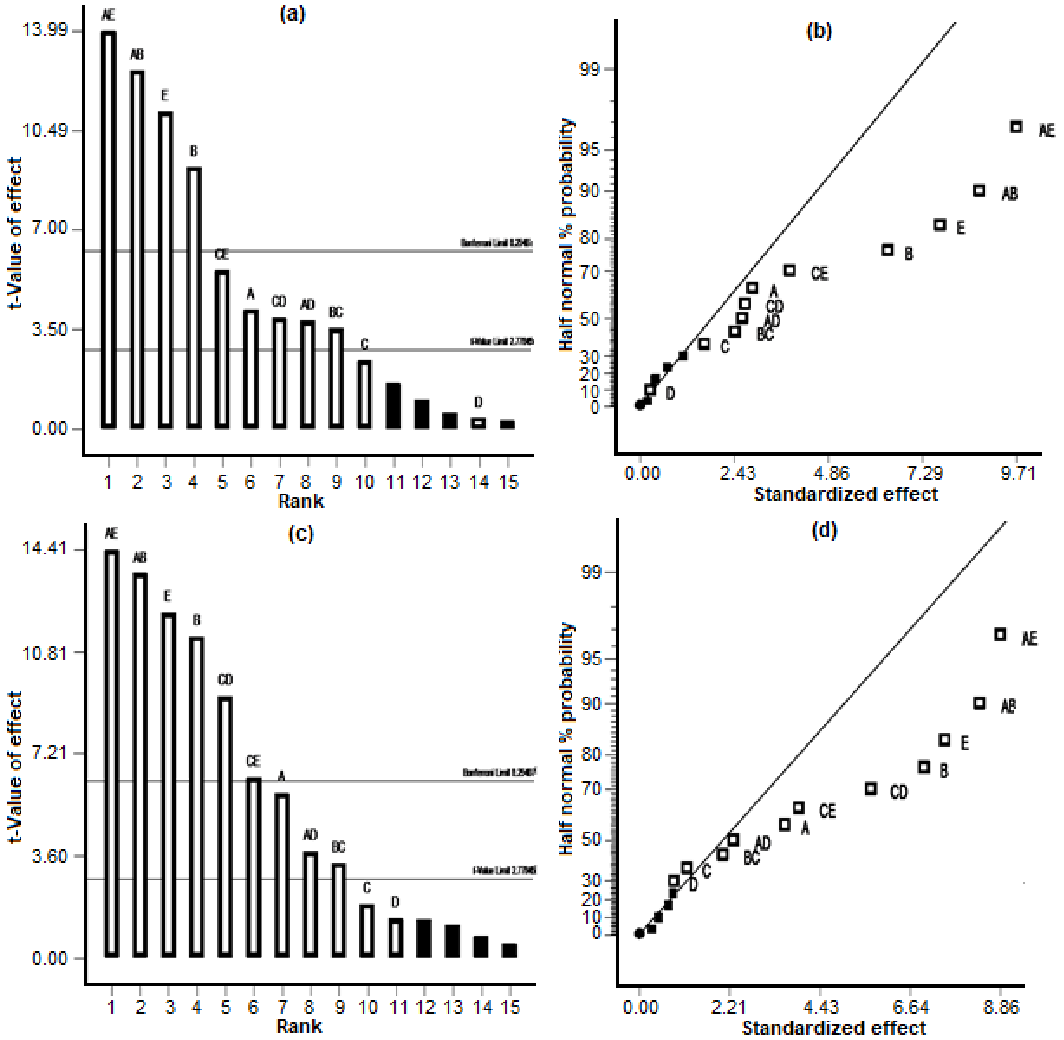

Fig 2. Pareto and half normal probability plots for the recovery of TPC (a and b) and TFC (c and d)

irradiation time and ethanol concentration greatly contributed to the yields of TPC and TFC. It has been reported that solvent concentration, irradiation time and microwave power level are the main MAE variable that determines the quantity and quality of recovery yields from plant materials [9,14-15]. Thus, the interactions between these three MAE factors play important role in recovering TPC and TFC from *C. odorata* leaves. A related result had been obtained in the extraction of phenolic compounds from *Vernonia amygdalina* leaves [15]. Likewise, the obtained results from two-level factorial design were in line with the OFAT experiments (Fig. 1).

Moreover, Pareto and half normal probability plots shown in Fig. 2 are employed to compare the statistical significance of the MAE independent variables and their interaction terms on the TPC and TFC yields from *C. odorata* leaf. The sampling errors through the standard deviation of MAE variables are being measured using Pareto plot. From the plots, the effect of interactions between irradiation time x ethanol concentration and irradiation time x microwave power were the main contributing factors to the recoveries. Ethanol concentration was the most contributing parameter with a positive effect (Fig. 2). However, solvent/feed ratio and temperature were the least contributing factors. This indicates that temperature might have little or no effects on the recovery of phenolic compounds from *C. odorata* leaves using MAE. Moreover, it should be noted that increasing extraction temperature beyond a certain limit

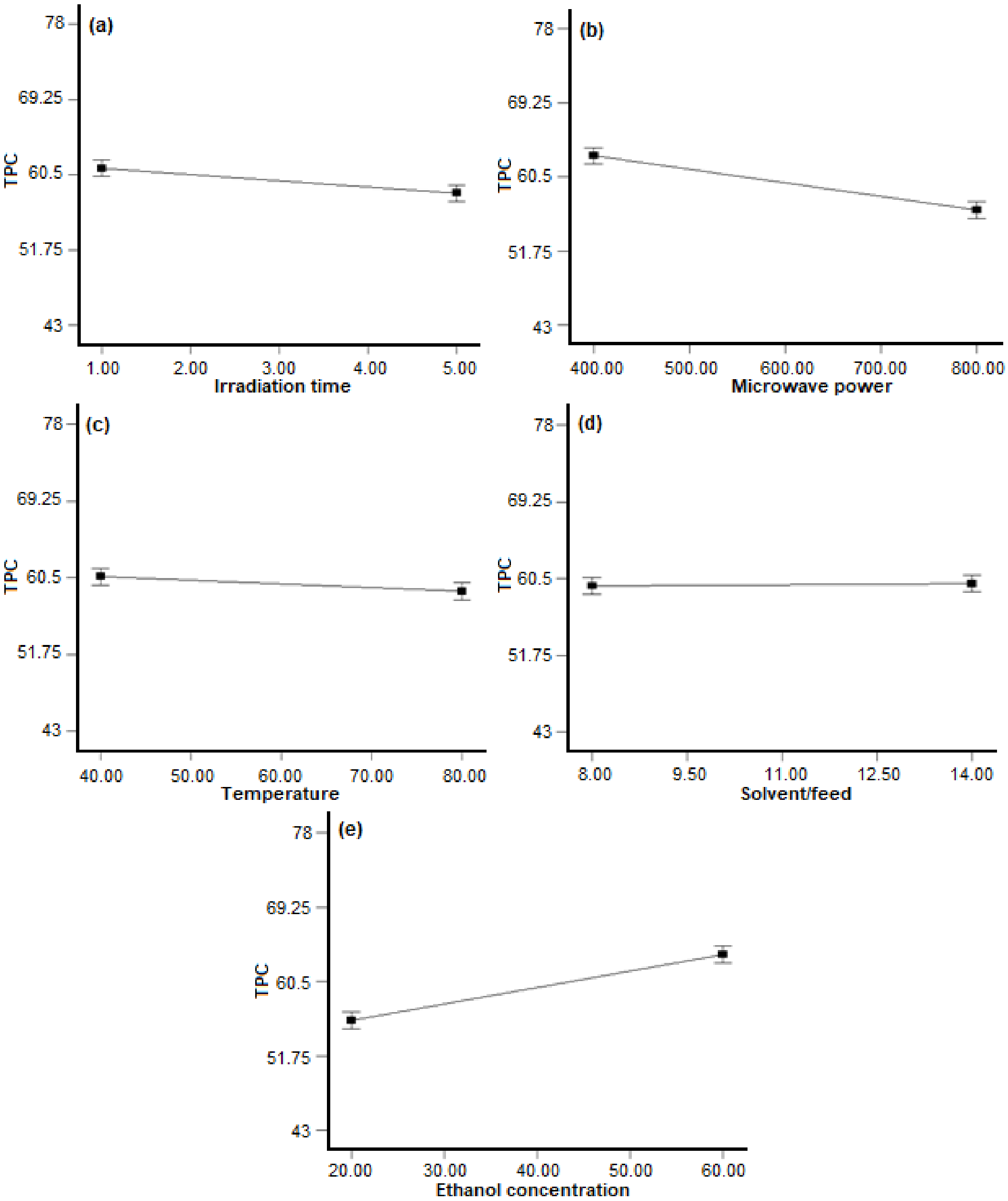

Fig 3. Effect of irradiation time (a), microwave power (b) temperature (c), solvent/feed ratio (d), and ethanol concentration (e) on the TPC yields from *C. odorata* leaf

may cause concurrent degradation of phenolic compounds that would have been mobilized at a lower temperature. A higher extraction temperature can results in solvent loss through vaporization, this can increase the cost of the extraction process [10,12]. Previous reports by Ameer et al. showed that the interaction between irradiation time and ethanol concentration, and irradiation time and microwave power enhanced the Reb-A from powdered stevia leaves [19].

In addition, half normal probability plots confirmed the outcome from Pareto charts. Insignificant variables with zero means and variances were distributed along the straight line of the plot while the significant ones were located at the right side of the plots according to their level of contribution. Thus, the contributing effect of main- and interaction terms to the recoveries of TPC and TFC were in order: AE > AB > E > B > CE > A > CD >AD > BC. Therefore, the three variables: irradiation time (1–5 min), ethanol concentration (20–60% v/v) and microwave power (400–800 W) at fixed temperature of 60 °C and solvent/feed ratio of 10:1 mL/g are suggested for the optimization process using response surface methodology so as to obtain optimal recoveries of TPC and TFC from *C. odorata* leaves.

On the other hand, it can be observed that the TPC and TFC increase with increasing ethanol concentration to obtain the highest values of 63.66 mg GAE/g d.w. and 40.95 mg QE/g d.w., respectively at 3 min of irradiation time, microwave power of 600 W, the temperature of 60 °C, and solvent/feed ratio of 11:1 mL/g (Fig. 3 and 4).

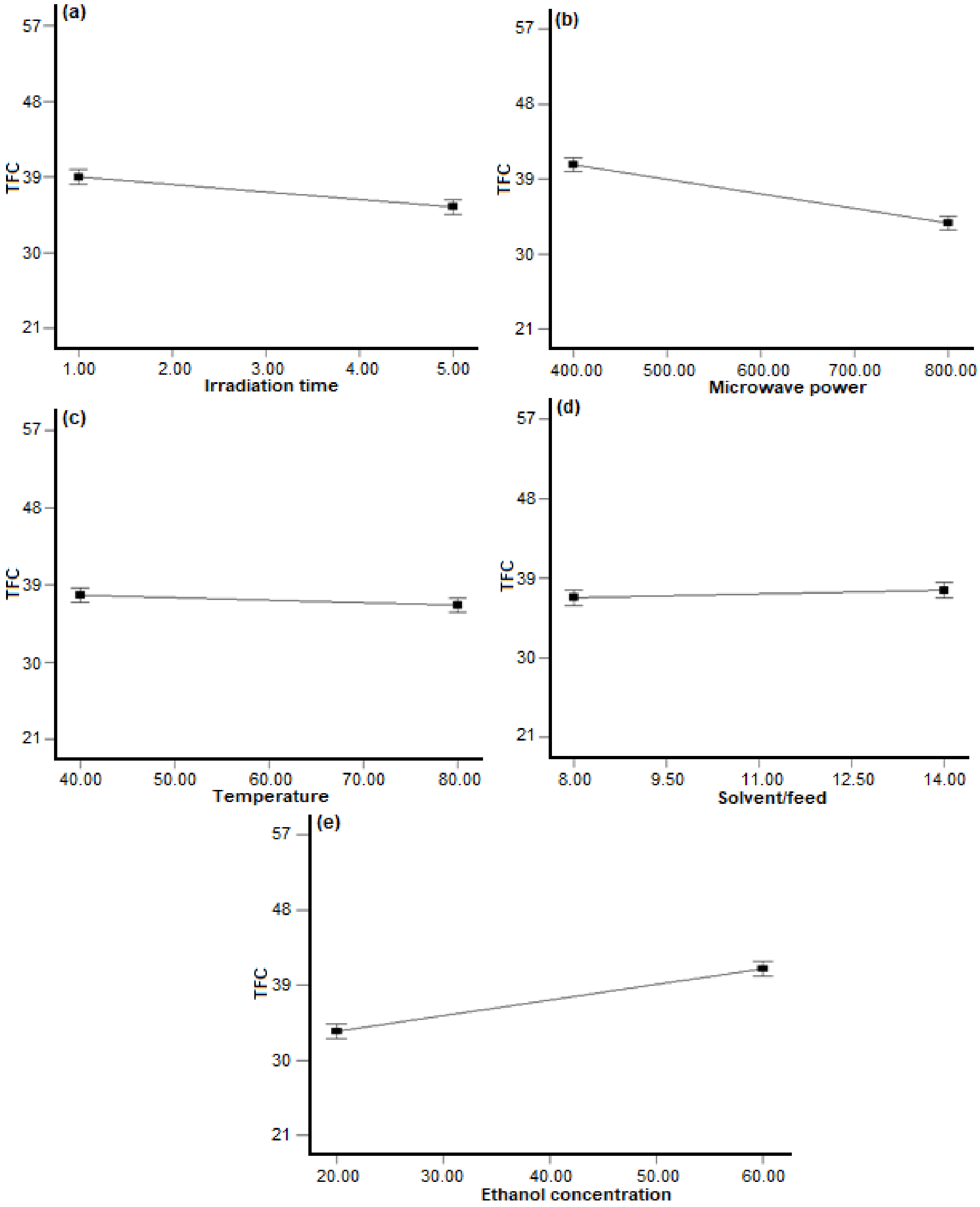

Fig 4. Effect of irradiation time (a), microwave power (b) temperature (c), solvent/feed ratio (d), and ethanol concentration (e) on the TFC yields from *C. odorata* leaf

This showed that increasing ethanol concentration from 20 to 60% v/v improves the yields of TPC and TFC from *C. odorata* leaves. In contrast, the TPC and TFC yields declined with the increasing irradiation time and microwave power (Fig. 3 and 4). This can be associated with the influence of microwave power in degrading phenolic compounds at prolonging irradiation time [17]. Thus, increasing irradiation time from 1 through 5 min declined TPC from 61.24 to 58.34 mg GAE/g d.w. and TFC from 38.99 to 35.42 mg QE/g d.w., respectively. It can be clearly observed that varying temperature between 40 and 80 °C, and solvent/feed ratio between 8:1 and 14:1 mL/g only showed little variations in the yields of TPC and TFC which were not significant based on the ANOVA results with $p > 0.05$ (Table 2).

■ CONCLUSION

The screening of MAE variables for the recoveries of TPC and TFC from *C. odorata* leaves was successfully achieved through a two-level factorial design. Effects of individual variables, including: irradiation time (1–5 min); temperature (60–80 °C); microwave power (400–800 W); ethanol concentration (20–60% v/v); and solvent/feed ratio (8:1–14:1 mL/g) showed that solvent/feed ratio and extraction temperature had unnoticeable effects on the recovery yields ($p > 0.05$). The optimum yields of TPC and TFC were 56.13 mg GAE/g d.w. and 44.78 mg QE/g d.w., respectively were achieved from MAE of *C. odorata* leaf at irradiation time of 2 min, microwave power of 600 W, temperature of 60 °C, solvent:feed ratio of 10:1 mL/g, and ethanol concentration of 40% v/v through one-factor-at-time (OFAT) experimental trials. Moreover, the interactions AB (irradiation time and microwave power) and AE (irradiation time and ethanol concentration) greatly contributed to TPC and TFC yields from *C. odorata* leaves. Thus, the significant variables which include microwave power (400–800 W), irradiation time (1–5 min) and ethanol concentration (20–60%) at fixed temperature (70 °C) and solvent/feed ratio (10:1 mL/g) can be further optimized to achieve maximum yields of TPC and TFC from *C. odorata* leaves.

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