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TWO-LEVEL FACTORIAL SCREENING OF MICROWAVE-ASSISTED EXTRACTION PARAMETERS FOR THE RECOVERY OF PHENOLIC COMPOUNDS FROM *Vernonia cinerea* LEAF

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ABSTRACT

Vernonia cinerea is one of the medicinal plants with several potentials for treating different ailments. In the present study, Microwave-assisted extraction (MAE) was employed in extracting phenolic compounds from this plant. However, different factors that affect this extraction method in the recovery of phenolic compounds abound, these factors need to be screened to determine actual contributing factor in order to minimize cost. Irradiation time (1-5 min), ethanol concentration (20-60% v/v), microwave power (40-80 W), extraction temperature (40-80 ºC), and feed/solvent (1:10-1:18 g/mL) have been screened using the two-level factorial design for the recoveries of phenolic compounds from *V. cinerea* leaves. The results obtained in this study indicated that only microwave power, ethanol concentration, irritation time, and feed/solvent contributed to recoveries of total phenolic content (TPC) and total flavonoid content (TFC) from *V. cinerea* leaves. Thus, these factors at these ranges can further be optimized to obtain optimal yields of phenolic compounds from *V. cinerea* leaves.

Keywords: Two-level factorial design; Total flavonoid content; Total phenolic content; *Vernonia cinerea*; Screening

1.0 INTRODUCTION

Nowadays, the consumptions of vegetables and fruits have grown higher due to the biochemical and epidemiological effects associated with them. Several studies have reported that regular intake of fruits and vegetables can minimize cancers, cardiovascular and degenerative diseases (Alara and Abdurahman, 2019; Tuberoso and Orrù, 2008). These activities might be due to the presence of phenolic compounds in the plants (Garcia-Salas*et al*., 2010). Phenolic compounds that are extensively distributed in plants, contribute to their nutritive qualities. Thus, evaluation of the phenolic compounds in vegetables, fruits and other food sources are being studied in recent times. *Vernonia cinerea* mostly found in tropical areas is one of the phenolic-rich plants with different pharmacological activities such as antioxidant, anti-cancer, anti-malaria, antibacterial, anti-inflammation, anti-diabetic, and among others (Alara and Abdurahman, 2019; Prasopthum*et al*., 2015; Youn *et al*., 2014). This plant can be found abundantly in Malaysia as one of the major weeds (Alara *et al.*, 2018).

However, extraction process has been appraised as the first and paramount step in distinguishing bioactive compounds in the plant samples (Liu*et al*., 2017). Microwave-assisted extraction (MAE) is an efficient and effective method of extraction due to the relatively lower cost of implementation and ability to quickly heat-up the sample-solvent mixture in shorter extraction time compare to other techniques (Dahmoune *et al.*, 2014). This is the reason why MAE was chosen as the extraction technique in this study. Moreover, several factors hinder the operation of MAE which includes irradiation time, ethanol concentration, microwave power, extraction temperature, and feed/solvents. It is therefore important to evaluate the significance levels of individual factors for excellent recoveries of phenolic compounds. In the screening process, a two-level factorial design is being used for minimizing the number of experimental runs without the need to replicate experiments to draw statistically valid conclusions.
Thus, this study investigates the screening of MAE parameters such as irradiation time, ethanol concentration, microwave power, extraction temperature, and feed/solvent in recovering total phenolic and total flavonoid contents from *V. cinerea* leaves using a two-level factorial design.

2.0 MATERIALS AND METHODS

2.1 Procurement of sample and chemicals used

The fresh leaves of *V. cinerea* were collected from Universiti Malaysia Pahang premises. Ethanol, gallic acid, Folin-Ciocalteu phenol reagent, $AlCl_3$, Na_2CO_3, and quercetin were supplied by Sigma Aldrich, Selangor, Malaysia.

2.2 Extraction procedure

The *V. cinerea* leaves were thoroughly washed with water and dried at the room temperature for two weeks. The dried plant samples were pulverized with a grinder, sieved to an average particle size of 0.105 mm and kept in a dark container before extraction. Aqueous ethanol was used as the extracting solvent. MAE method was employed in the extraction of phenolic compounds from the plant sample. The extraction process was performed according to the design matrix from two-level factorial (Table 1). Then, the extract solution was filtered and dried in a rotary evaporator set at 60 ºC. Thereafter, the TPC and TFC in the extract were evaluated.

2.3 Methods of analysis

In order to estimate the contents of phenolics and flavonoids in the extract of *V. cinerea* leaves, Folin-Ciocalteu phenol and aluminium chloride colourimetric assays, respectively. The methods used in the previous study was adopted for these analyses (Alara*et al*., 2019). For TPC determination, the dried extract of *V. cinerea* leaves and gallic acid were dissolved in ethanol solution separately to make 5 g/L. Then, gallic acid solutions at the different concentration between 50-500 mg/L were used to prepare a standard curve. A 1000 µL of extract and 200 µL of Folin-Ciocalteu reagent were thoroughly mixed and allow to stay at room temperature for 5 min before 0.2 mM Na_2CO_3 solution (600 µL) was added to the mixture. This mixture was then allowed to react for the next 2 h and the absorbance was recorded against a blank (ethanol) at 765 nm using a UV-vis spectrophotometer. The concentration of the phenolic was evaluated based on the standard curve of gallic acid and yield of TPC expressed in mg GAE/g d.w. was calculated using Equation (1).

Table 1: Two-level factorial experimental design matrix for the extraction of *V. cinerea* leaves

Run	A	B	C	D	E
1	1	400	40	1:18	60
2	5	400	40	1:18	20
3	1	600	40	1:18	20
4	5	600	40	1:18	60
5	1	400	80	1:18	20
6	5	400	80	1:18	60
7	1	600	80	1:18	60
8	5	600	80	1:18	20
9	1	400	40	1:10	20
10	5	400	40	1:10	60
11	1	600	40	1:10	60
12	5	600	40	1:10	20
13	1	400	80	1:10	60
14	5	400	80	1:10	20
15	1	600	80	1:10	20
16	5	600	80	1:10	60

A - Irradiation time (min); B - Microwave power (W); C - Extraction temperature (ºC); D - Feed-to-solvent ratio (g/mL); E - Ethanol concentration (% v/v)

$$Y_{ph} = \frac{c_{ph} * V}{m} \quad (1)$$

where Y_{ph} indicates the yield of TPC, c_{ph} is the concentration of TPC estimated from the gallic acid standard curve and m denotes the weight of plant sample used in the extraction.

In the case of TFC determination, the dried plant sample was re-dissolved in ethanol and 5 mg of quercetin stock in methanol to make 1 g/L concentration individually. Quercetin solutions at the different concentration between 50-500 mg/L were used to prepare a standard curve. A 1000 µL of extract and 1000 µL of 0.02 g/mL Al_2Cl_3 solution were thoroughly mixed and allow to stay at room temperature for 60 min before the absorbance was recorded against a blank (ethanol) at 420 nm using a UV-vis spectrophotometer. The concentration of the flavonoid was evaluated based on the standard curve of quercetin and yield of TFC expressed in mg QE/g d.w. was calculated using Equation (2).

$$Y_{fl} = \frac{c_{fl} * V}{m} \quad (2)$$

where Y_{fl} indicates the yield of TFC, c_{fl} is the concentration of the flavonoids estimated from standard curve of quercetin and m denotes the weight of plant sample used in the extraction.

2.4 Analysis of influential factors

Two-level factorial screening experimental design of Design Expert 7.0 software® (Version 7.1.6 Stat-Ease Inc., Minneapolis, USA) was used to examine the statistical contributions of MAE factors, namely: irradiation time, microwave power, ethanol concentration, feed/solvent, and temperature in the recoveries of TPC and TFC from

leaves of *V. cinerea*. Tables 1 and 2 illustrate the experimental matrix and their ranges, respectively. These ranges were obtained between their low (-1) and high (+) levels as earlier reported in the previous study (Alara *et al.*, 2018). The experimental processes were repeated thrice and mean ± standard deviation were computed.

Table 2: Ranges for the MAE independent factors for two-level factorial design

MAE independent factors	Unit	Coded levels		
		-1	0	+1
Irradiation time (A)	min	1	2	5
Microwave power (B)	W	400	500	600
Temperature (C)	ºC	40	60	80
Feed/solvent (D)	g/mL	1:10	1:14	1:18
Ethanol concentration (E)	% v/v	20	40	60

3.0RESULTS AND DISCUSSION

The main importance of two-level factorial design is to economically study the cause-and-effect relationship of significance in the considered experimental process (Sibanda and Pretorius, 2011). A previous study had investigated that the use of less contributing factor in an extraction process may alter the results (Jalbani *et al.*, 2006). Then, five factors of MAE namely: microwave power, temperature, irradiation time, ethanol concentration, and feed/solvent were screened for their contributions in the recovery of phenolic and flavonoid from the leaves of *V. cinerea*. A total of 16 experimental trials was randomly performed as shown in Table 1 using 2^{5-1} factorial design. Moreover, analysis of variance (ANOVA) describing the percentage of contribution of individual extraction factor and significant levels are presented in Table 3 and Figure 1. Moreover, to ascertain the significant factors to the recovery of TPC and TFC from *V. cinerea* leaf, the generated second-order model equations illustrating the relationship between the dependent and independent factors are provided as follows:

$$Y(TPC) = 77.10 - 1.81A - 2.21B - 5.00\times10^{-3}C - 1.16D + 2.43E - 2.44AB - 1.52AE - 0.52BC - 1.02CE + 0.65DE \quad (1)$$

$$Y(TFC) = 44.78 - 1.19A - 1.11B - 0.33C + 1.08D + 2.38E - 1.37AB - 1.76AE - 0.63BC - 1.29CE \quad (2)$$

where A, B, C, D, and E represent irradiation time, microwave power, temperature, feed/solvent ratio, and ethanol concentration, respectively; Y (TPC) and Y (TFC) are the dependent factors for TPC and TFC, respectively.

Table 3: ANOVA analysis and contribution of individual MAE factors and the p-values for the responses

Extraction factors	TFC (Y_1)				TFC (Y_2)				Percentage of contribution		p-values for responses	
	Sum of Squares	df	Mean Square	F-value	Sum of Squares	df	Mean Square	F-value	Y_1	Y_2	Y_1	Y_2
Model	406.59	10	40.66	81.01	266.51	9	29.61	64.11	-	-	<0.0001	<0.0001
A	52.20	1	52.20	104.01	22.78	1	22.78	49.31	12.76	8.46	0.0002	0.0004
B	78.32	1	78.32	156.06	19.74	1	19.74	42.31	19.15	7.33	<0.0001	0.0006
C	4.00×10^{-4}	1	4.00×10^{-4}	7.97×10^{-4}	1.75	1	1.75	3.79	9.77×10^{-5}	0.65	0.9786	0.0996
D	21.67	1	21.67	43.18	18.68	1	18.68	40.45	5.30	6.94	0.0012	0.0007
E	94.58	1	94.58	188.44	90.77	1	90.77	196.52	23.12	33.71	<0.0001	<0.0001
AB	95.06	1	95.06	189.41	29.89	1	29.89	64.72	23.24	11.10	<0.0001	0.0002
AC	-	-	-	-	-	-	-	-	0.05	0.10	-	-
AD	-	-	-	-	-	-	-	-	0.08	0.22	-	-
AE	37.03	1	37.03	73.78	49.81	1	49.81	107.83	9.05	18.50	0.0004	<0.0001
BC	4.35	1	4.35	8.66	-	-	-	-	1.06	0.29	0.0321	-
BD	-	-	-	-	-	-	-	-	0.18	0.07	-	-
BE	-	-	-	-	6.39	1	6.39	13.83	8.80×10^{-4}	2.37	-	0.0099
CD	-	-	-	-	-	-	-	-	0.31	0.18	-	-
CE	16.65	1	16.65	6.73	26.70	1	26.70	57.81	4.07	9.92	0.0022	0.0003
DE	6.73	1	6.73	0.50	-	-	-	-	1.65	0.16	0.0145	-
Residual	2.51	5	2.51	-	2.77	6	0.46	-	-	-	-	-
Cor total	409.09	15	-	-	269.28	15	-	-	-	-	-	-
CV%	0.92	-	-	-	1.52	-	-	-	-	-	-	-

PRESS	25.70	-	-	-	19.71	-	-	-	-	-	-	-
R^2	0.9939	-	-	-	0.9897	-	-	-	-	-	-	-
Adj. R^2	0.9816	-	-	-	0.9743	-	-	-	-	-	-	-
Pred. R^2	0.9372	-	-	-	0.9268	-	-	-	-	-	-	-
Adeq. Precision	28.41	-	-	-	22.14	-	-	-	-	-	-	-

A – Irradiation time (min); B – Microwave power (W); C – Temperature (ºC); D – Feed-to-solvent ratio (g/mL); 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

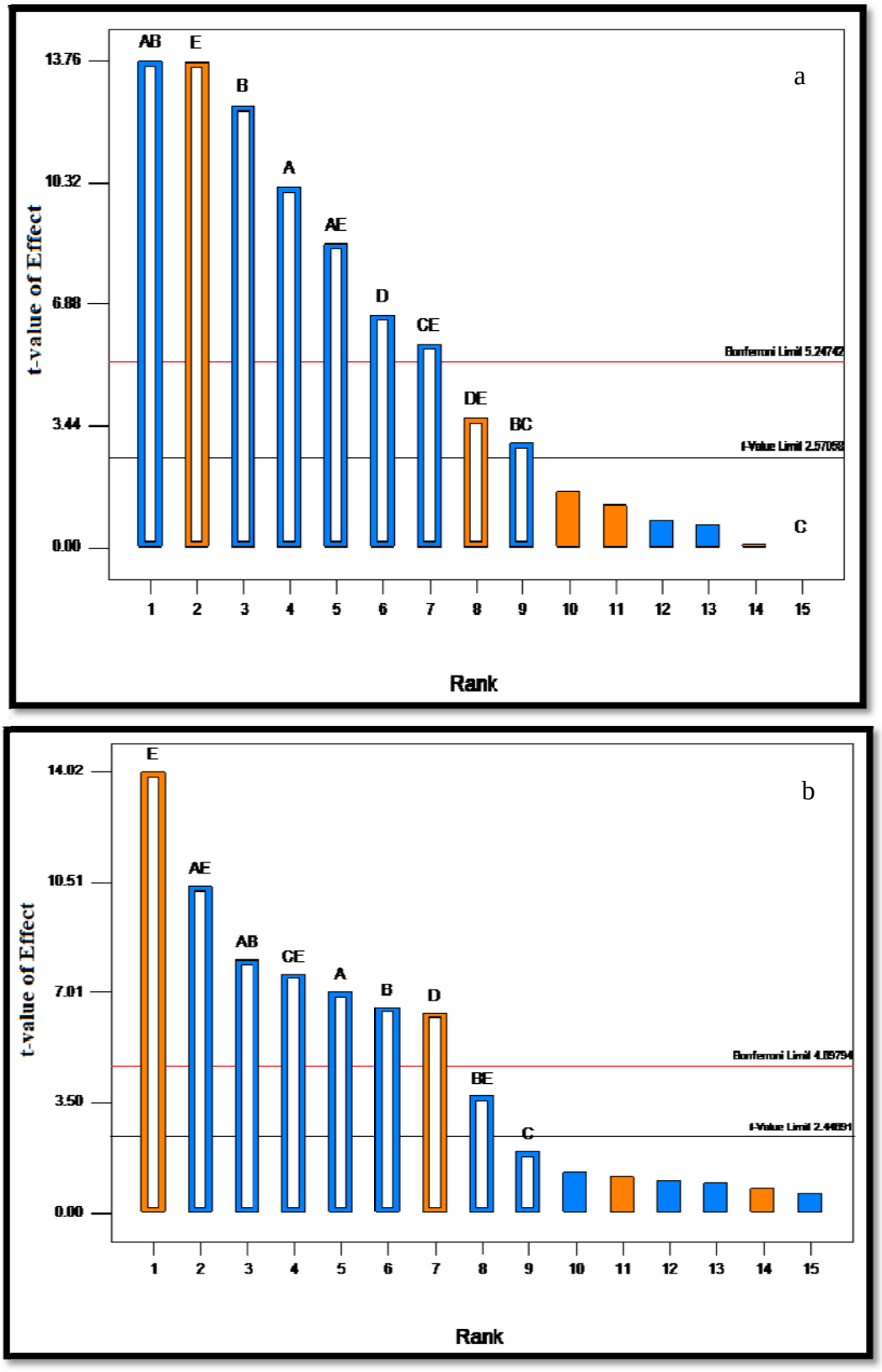

Figure 1: Pareto chart showing the contribution level of each MAE factor on recovery of TPC from *V. cinerea* leaves (a) and TFC from *V. cinerea* leaves (b)

The results illustrated in Table 3 reflects that the yields of TPC and TFC were significantly influenced by ethanol concentration, microwave power, irradiation time, and feed/solvent. However, extraction temperature insignificantly influenced the recoveries of phenolic compounds from *V. cinerea* leaves since the p-values were estimated as 0.9786 and 0.0996 for TPC and TFC, respectively ($p > 0.05$). Moreover, the percentage of contributions of the significant factors are as follows: ethanol concentration of 23.12 and 33.71%; microwave power of 19.15 and 7.33%; irradiation time of 12.76 and 8.46%; and the feed-to-solvent ratio of 5.30 and 6.94%, respectively. It can be clearly seen that extraction temperature showed the least contribution in recovering of the yields. This might be due to rupturing of plant matrix in leaching out of phenolic compounds that alter the membrane anatomy of plant sample, resulting in reduced activity of the phytochemicals (Chew *et al.*, 2011).

In the same manner, Figure 1 presents the Pareto plot which evaluates the sampling error of each extraction factor with their interactions using standard deviation (Alara *et al.*, 2019). This plot ranks the contribution of an individual factor against t-value of effect. However, any of the extraction factors that drops below t-value limit is insignificant. It can be seen in Figure 1 that ethanol concentration had the highest contributing percentage compared to other considered extraction factors. Meanwhile, temperature showed the least contribution. The highest significance level of ethanol concentration might be due to the fact that its concentration can make extraction solution to have a varied polarity that can enhance the leaching of phenolic compounds from *V. cinerea* leaves (Spigno and De Faveri, 2009). In addition, feed/solvent enhances the mass concentration difference between outside and inside of plant sample which might result in the improvement of mass transport driving force and rate of internal diffusion (Liu *et al.*, 2017).

Moreover, Figures 2 and 3 outline the relationships between the TPC, TFC and most significant MAE variables. It can be observed that the yields of TPC declined with increasing irradiation time, microwave power and feed/solvent ratio. However, it increased with increasing ethanol concentration for both TPC and TFC yields. This can be justified by the fact that the overexposure of plant extract to excessive microwave power at prolonging extraction time tends to degrade the phenolic compounds (Alara *et al.*, 2019). On the other hand, aqueous ethanol can improve the recoveries of phenolic compounds from plant extract because it can make extraction solution to have a varied polarity that can enhance the leaching of phenolic compounds from plant samples (Spigno and De Faveri, 2009). This shows the importance of estimating the actual time, power, feed/solvent ratio, and ethanol concentration needed to obtain the highest yields. Furthermore, the interactions between the MAE variables relative to the responses (TPC and TFC) are presented in Figures 4 and 5, respectively. There were clear interactions between the following factors to achieve higher yields of TPC and TFC from *V. cinerea* leaf, these are microwave power × irradiation time and temperature × microwave power. Whereas, no interactions existed between the following: Ethanol concentration × irradiation time; ethanol concentration × temperature; ethanol concentration × F:S; and ethanol concentration × microwave power. Thus, the irradiation time, ethanol concentration, feed/solvent, and microwave power are the contributing factors for MAE of *V. cinerea* leaf in order to recover higher and quality phenolic compounds.

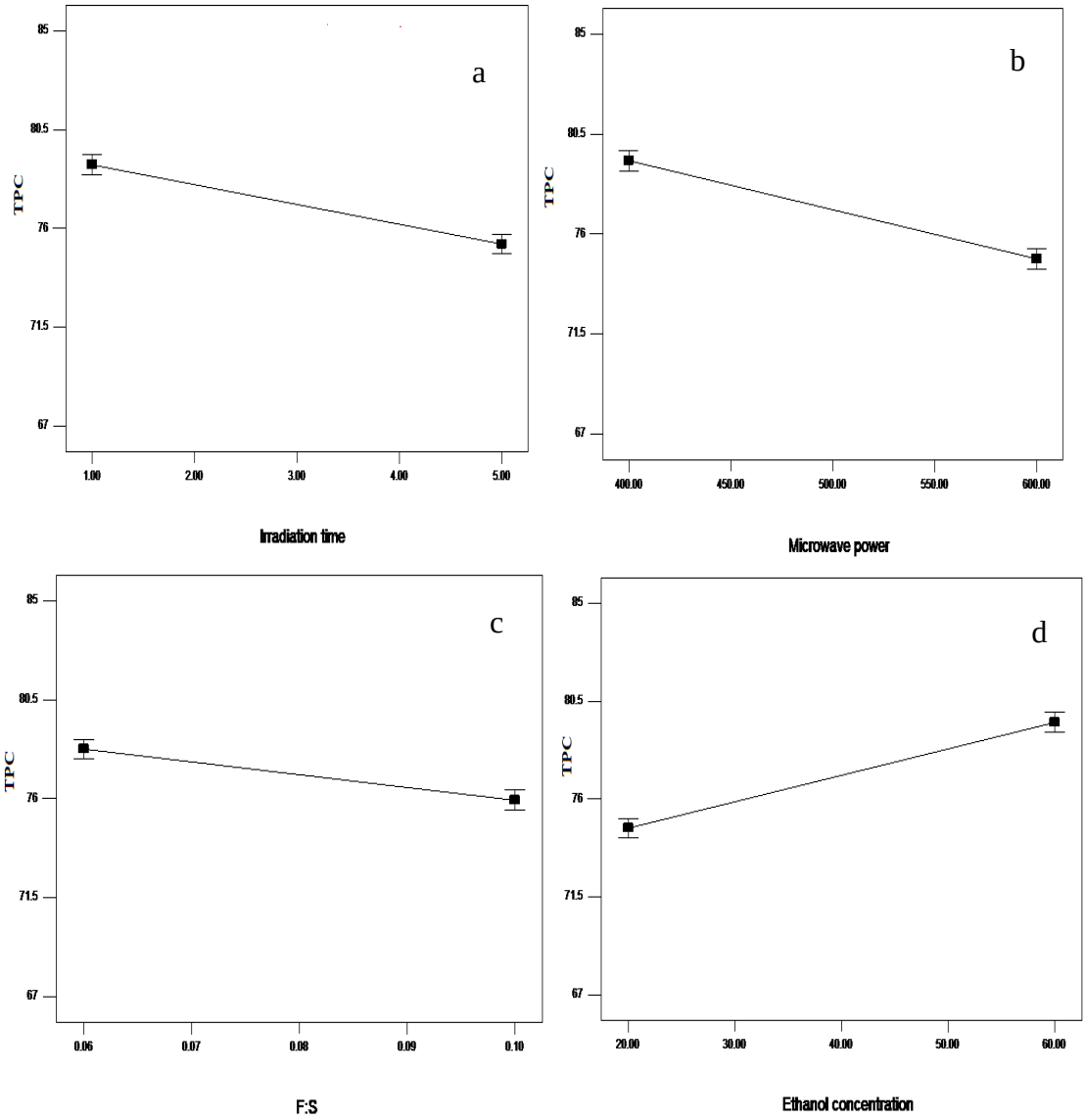

Figure 2: Impact of irradiation time (a); microwave power (b); feed/solvent ration; and ethanol concentration (d) of the total phenolic content recovery from *V. cinerea* leaf

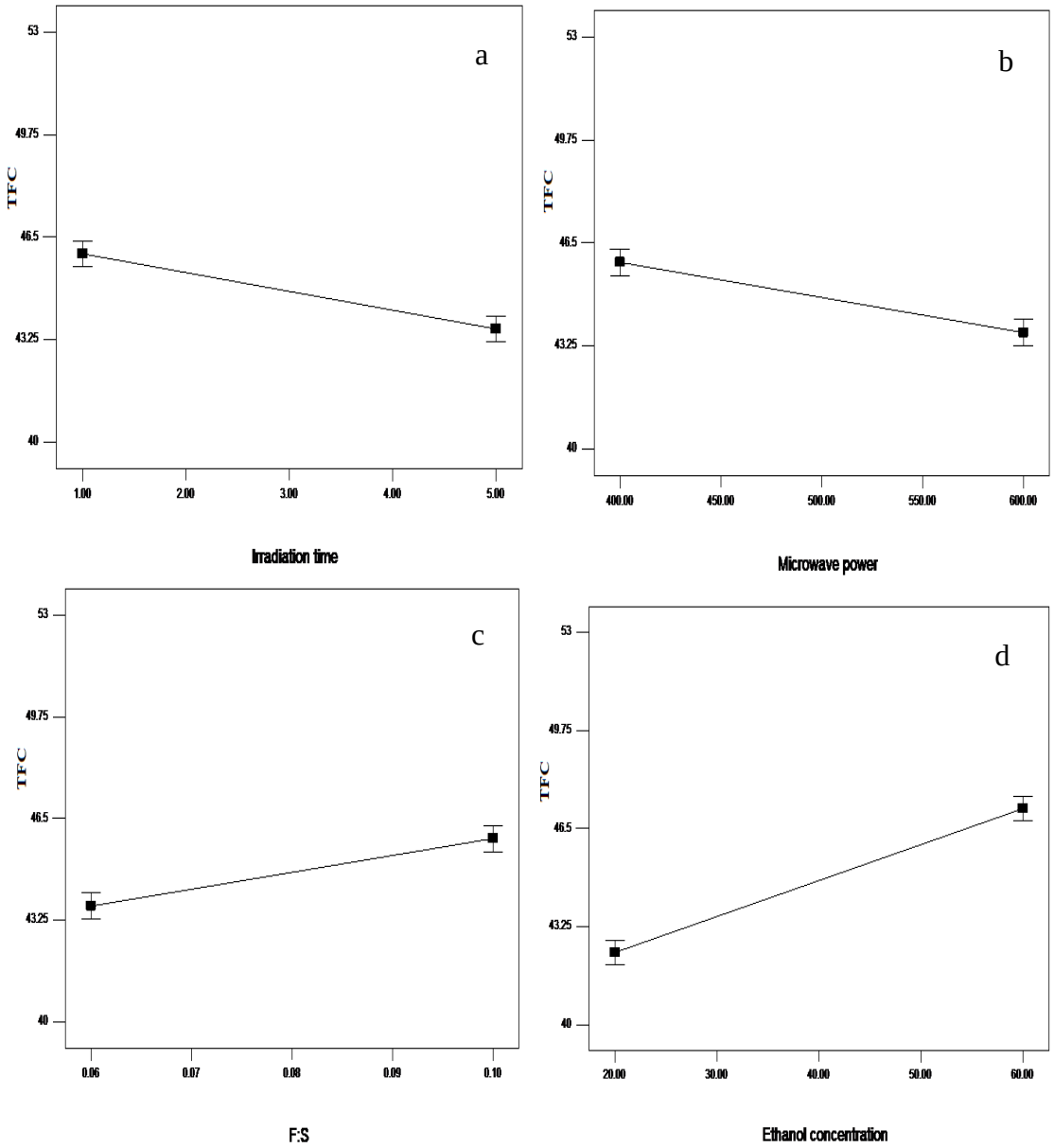

Figure 3: Impact of irradiation time (a); microwave power (b); feed/solvent ration; and ethanol concentration (d) of the total flavonoid content recovery from *V. cinerea* leaf

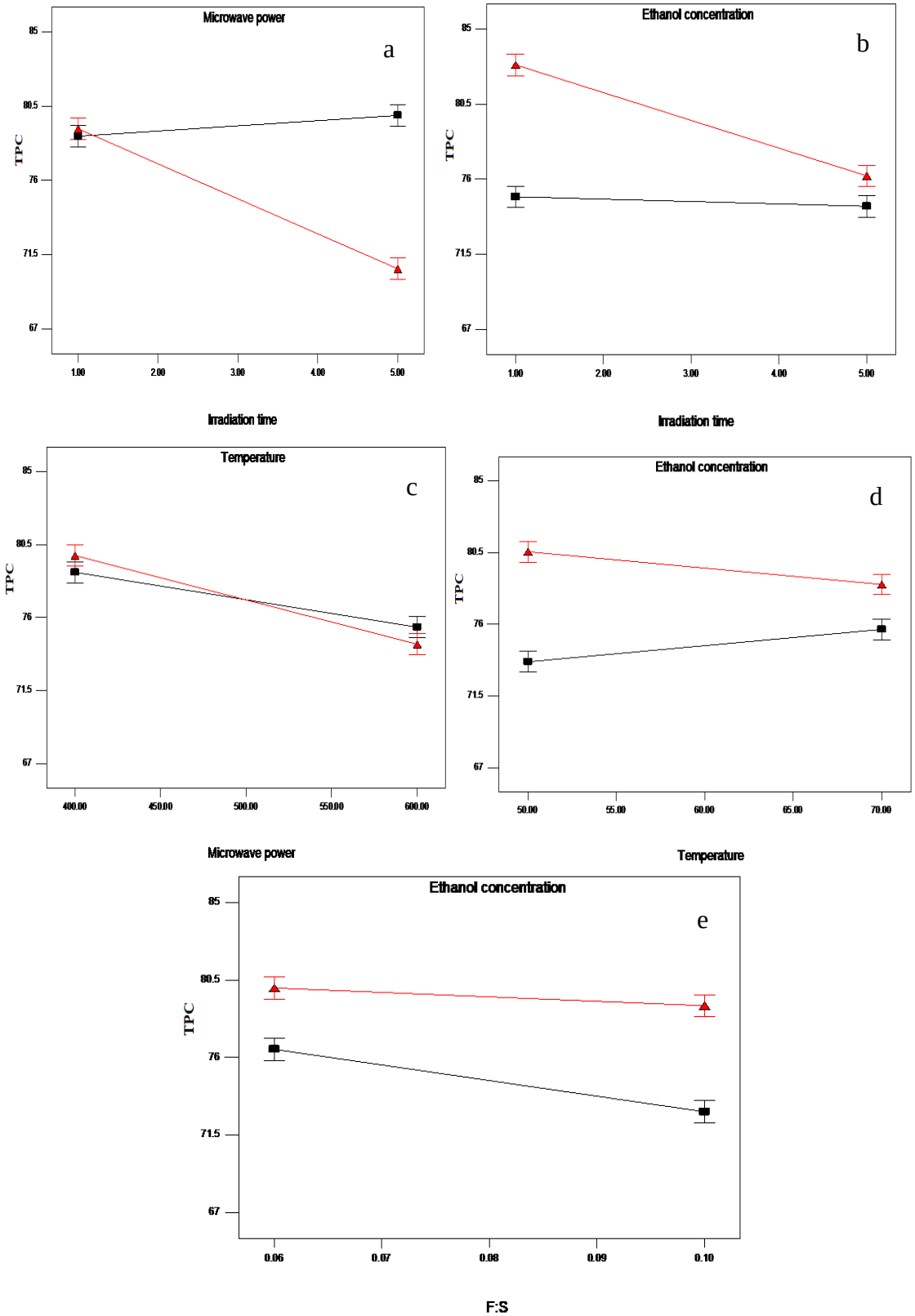

Figure 4: Interactions between the microwave power and irradiation time (a); ethanol concentration and irradiation time (b); temperature and microwave power (c); ethanol concentration and temperature (d); and ethanol concentration and feed/solvent ratio on the TPC recovery

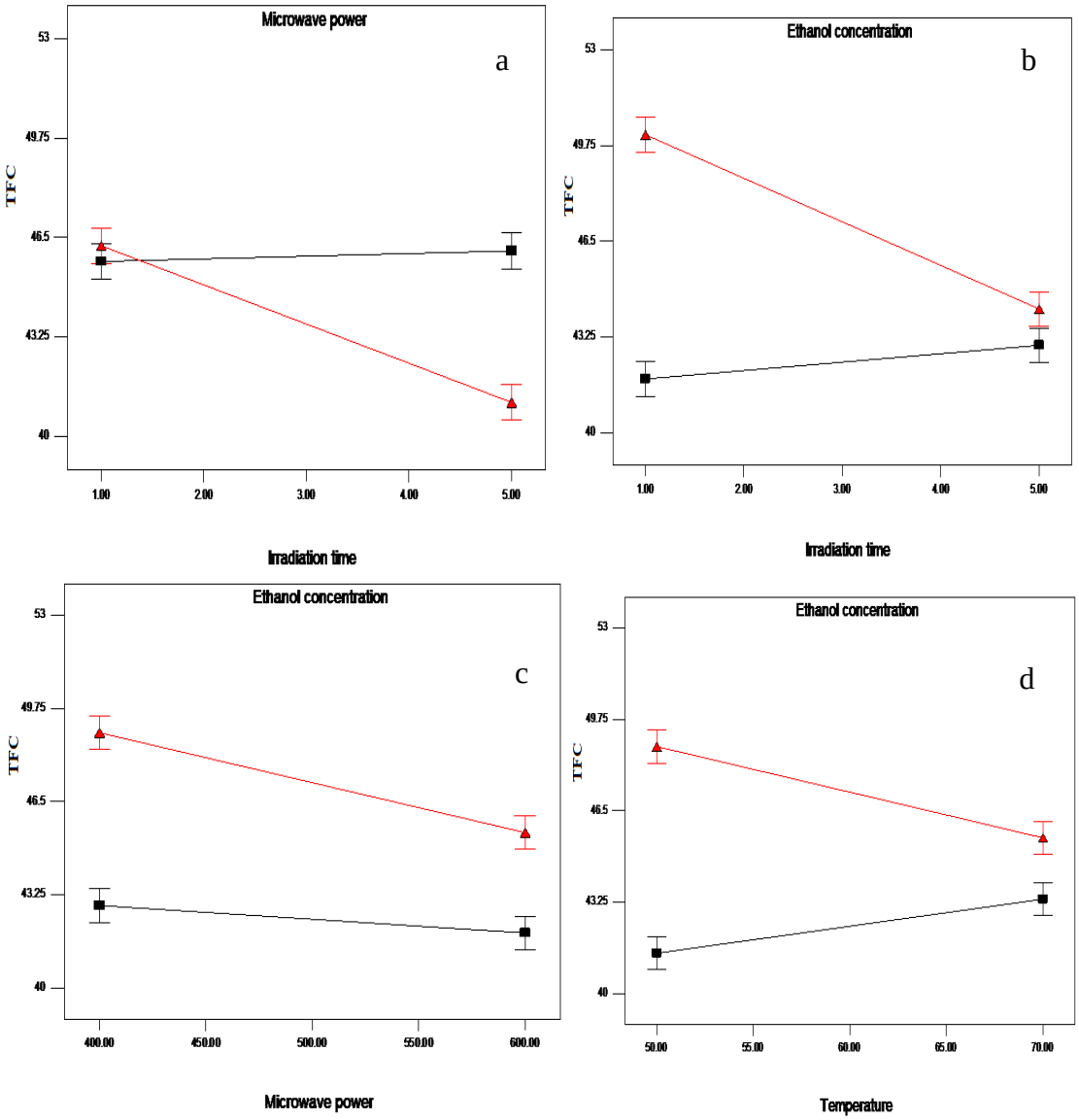

Figure 5: Interactions between the microwave power and irradiation time (a); ethanol concentration and irradiation time (b); ethanol concentration and microwave power (c); and ethanol concentration and temperature on the TFC recovery

4.0 CONCLUSIONS

This study has reflected that phenolic-rich extracts can be achieved from *V. cinerea* leaves using MAE. However, the screening of MAE factors, namely irradiation time, ethanol concentration, feed/solvent, and microwave power showed that only extraction temperature was insignificant ($p > 0.05$). These factors (irradiation time, ethanol concentration, microwave power, and feed/solvent ratio) can further be optimized to achieve optimal yields of TPC and TFC from *V. cinerea* leaves. This will show the optimal MAE conditions to achieved higher yields of TPC and TFC from *V. cinerea* leaf. Additionally, these results have shown that *V. cinerea* leaf is rich in phenolic compounds.

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