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ORIGINAL PAPER

Ethanolic extraction of bioactive compounds from *Vernonia amygdalina* leaf using response surface methodology as an optimization tool

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Abstract
In this study, ethanolic microwave-assisted extraction of total phenolic content (TFC), total flavonoid content (TFC) and antioxidant from *Vernonia amygdalina* leaf was investigated using response surface methodology. Four extraction variables, including irradiation time (1–5 min), microwave power level (500–700 W), feed-to-solvent ratio (1:8–1:12 g/ml), and ethanol concentration (60–80%) were optimized to obtain optimal yields. The analysis of variance results showed that ethanol concentration, microwave power level and irradiation time mostly affected the TFC, TPC and antioxidant activities significantly ($p < 0.05$), whereas the feed-to-solvent ratio was insignificant. The validated optimal yields of TPC (113.76 mg GAE/g d.w.), TFC (94.08 mg QE/g d.w.), DPPH (97.98%), and ABTS (99.34%) were obtained at extraction conditions, viz, 4 min of irradiation time, 558 W of microwave power, feed-to-solvent ratio of 1:10 g/ml, and ethanol concentration of 76%. In addition, the phytochemical profiling of the extract at optimal conditions confirmed the presence of phenolic and flavonoid compounds.

Keywords Response surface methodology · Phenols · Flavonoids · *V. amygdalina* leaf · Antioxidant activities

Introduction

Bioactive compounds like polyphenols and flavonoids derived from plants have been reported to possess antioxidant potentials associated with the prevention of several human diseases like cancer, diabetes, inflammation, cardiovascular diseases, and aging [1, 2]. Antioxidants are chemical compounds that possess the ability to scavenge free radicals and/or reduce their production rate and lipid peroxidation in human bodies [3]. Thus, these bioactive compounds are being extracted from plant matrix for food and pharmaceutical industries utilization. In addition, World Health Organization had reported that traditional medicine will continue to play an important role in health care system since 80% population of the third world countries relies on the use of traditional medication [4]. Although, medicinal plants are prone to extinction, hence, the bioactive phytochemicals needs to be identified and isolated for use in therapeutic drugs.

Bitter leaf, scientifically known to be *V. amygdalina* is an important medicinal plant mostly found in Africa and Asia. The leaves of this plant are popularly consumed as food and medicine. It has been used to treat different ailments like inflammation, diabetes, malaria, oxidative stress diseases, cancer, infertility and hypoglycaemia without any record of toxicity [5–8]. These pharmacological activities had been associated with the presence of polyphenols, flavonoids, alkaloids, saponins, steroidal glycosides, sesquiterpenes lactones, tannins, and terpenoids in the leaf extracts [8, 9]. More so, the greenish powders are being produced by Medical Traditional Healer Association in Rukararwe, Uganda for consumption as a tea for patient suffering from malaria [10].

In general, the conventional methods including Soxhlet, maceration and decoction used in the extraction of phenolic compounds responsible for the antioxidant property from plant matrix are very time-consuming and require large quantities of solvents [11, 12]. Therefore, the use of microwave-assisted extraction technique has been found to

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be more effective and environmental friendly in obtaining higher and quality yields of phenolic compounds from plant matrix using a lesser solvent in a shorter time of extraction [12, 13]. The mechanism at which microwave-assisted extraction (MAE) works is different from other types of extraction methods because the extraction occurs as a result of changes in the cell structure caused by electromagnetic waves [11]. Thus, involves a synergistic combination of mass and heat transfers working in the same direction. In contrary, the mass transfer in conventional methods occurs from inside to outside of the substrates and heat transfer occurs from the outside to inside of substrate [13]. The efficiency of MAE is usually influenced by variables like irradiation time, microwave power level, feed-to-solvent ratio, and solvent concentration, therefore optimization of the process variables are inevitable. Response surface methodology (RSM) is an optimization tool that involves the compilation of mathematical and statistical techniques for the evaluation of several experimental parameters interactions. The main advantage of this approach over single factor experiment is that it involves the evaluation of multiple variables and their interactions on the independent variables with a minimal number of experimental runs [14, 15].

Thus, this study evaluate the optimization of the MAE extraction conditions (irradiation time, microwave power level, feed-to-solvent ratio, and ethanol concentration) in obtaining the optimal yields of phenolics, flavonoids and antioxidant activities from *V. amygdalina* leaf ethanolic extract using RSM, and to further identify the polyphenols and flavonoids which are responsible for its antioxidant activities using liquid chromatography mass spectrometry quadrupole time of flight (LCMS QTOF).

Materials and methods

Plant sample and extract preparation

Vernonia amygdalina fresh leaves were procured from a garden located in Gambang, Malaysia. The leaves were manually removed from the stalks, washed and shade-dried until constant weight was observed. The recorded moisture content before grinding was 0.012 ± 0.15 g water/g dry. The blended sample was sieved to an average particle size of 105 mm, sealed in a dark container and stored in 4 °C refrigerator before extraction. For the extraction process, 10 g of the sample was extracted using a desired concentration of ethanol, irradiation time, microwave power level, and feed-to-solvent ratio based on the experimental design matrix (Table 1). An enclosed ethos reflux microwave extractor (1000 W, Frequency 2450 MHz, Milestone, Italy) using 3-levels of heating: 2 min of preheating to desire temperature, irradiation based on experimental design, and 2 min of cooling to 30 °C were used. Thereafter, the extract was filtered through Whatman qualitative filter paper No. 1, concentrated to dryness using a rotary evaporator (Buchi Rotavapor R-200 coupled with Buchi Vac V-500 pump, Switzerland) and stored in a 4 °C refrigerator until further analysis. The experimental processes were carried out thrice and the average values were computed.

Chemicals and reagents

Folin–Ciocalteu phenol reagent, aluminium chloride salt, quercetin, 2,2-diphenyl-picrylhydrazyl (DPPH), and 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+\cdot}$), methanol, ethanol, and sodium carbonate anhydrous were purchased from Sigma-Aldrich (M) Sdn Bhd, Selangor. The distilled water was collected from Faculty of Chemical Engineering and Natural Resources laboratory. All chemicals and reagents used in this study were of analytical grade.

Total phenolic content determination

The total phenolic content in *V. amygdalina* leaf extract was evaluated using Folin–Ciocalteu reagent following the procedure described by Alara et al. [16]. Concisely, *V. amygdalina* leaf extract (100 µl) was thoroughly mixed with 0.2 ml of FC reagent. Thereafter, the mixture was allowed to stand in dark for 5 min at room temperature, 0.6 ml of 0.2 mM Na_2CO_3 solution was then added and the mixture was left to incubate for the next 2 h. The absorbance of the mixture was recorded at 765 nm using a UV–Vis Spectrophotometer (Hitachi U-1800, Japan). Afterward, the sample concentration was calculated using the gallic acid (50–500 mg/ml) standard curve equation ($y = 0.0006x + 0.0169$, $R^2 = 0.9903$) and the result was expressed as mg gallic acid equivalents per gram of dried weight sample (mg GAE/g d.w.). The total phenolic contents of the extracts were calculated using Eq. (1). Ethanol was used as the blank and the analyses were performed thrice.

$$TPC = \frac{c \times V}{m} \quad (1)$$

where c is the sample concentration from the calibration curve (mg/ml), V is the volume (ml) of the solvent used in the extraction, and m represents the weight (g) of the dried sample used.

Total flavonoid content determination

Total flavonoid content in the extract was evaluated using the procedure outlined by Alara et al. [16]. Concisely, 1 ml of 2% $AlCl_3$ dissolved in ethanol was thoroughly mixed with 1 ml of *V. amygdalina* leaf extract (or standard). The absorbance of the mixture was measured at 420 nm using a UV–Vis Spectrophotometer (Hitachi U-1800, Japan)

Table 1 Coded and actual values for the independent variables

Independent variables	Unit	Coded levels		
		−1	0	+1
Irradiation time (X_1)	min	1	2	5
Microwave power (X_2)	W	500	600	700
Feed:solvent (X_3)	g/ml	1:8	1:10	1:12
Ethanol concentration (X_4)	%	60	70	80

Std	Irradiation time	Microwave power	F:S	Ethanol concentration	TPC (mg GAE/g d.w.)	TFC (mg QE/g d.w.)	Antioxidant activity	
							DPPH (%)	ABTS (%)
1	−1	−1	−1	−1	90.56	73.63	77.34	81.22
2	+1	−1	−1	−1	91.87	73.36	77.18	80.91
3	−1	+1	−1	−1	91.21	73.01	76.83	80.78
4	+1	+1	−1	−1	92.51	74.10	77.75	81.76
5	−1	−1	+1	−1	92.29	73.99	77.59	81.59
6	+1	−1	+1	−1	91.12	72.71	76.62	80.58
7	−1	+1	+1	−1	94.72	76.32	80.06	83.68
8	+1	+1	+1	−1	89.99	71.43	75.32	79.49
9	−1	−1	−1	+1	107.02	88.82	92.58	94.72
10	+1	−1	−1	+1	106.74	88.29	92.33	94.54
11	−1	+1	−1	+1	101.65	83.11	86.99	89.78
12	+1	+1	−1	+1	95.50	77.26	81.11	84.68
13	−1	−1	+1	+1	109.44	91.00	94.84	95.76
14	+1	−1	+1	+1	105.08	86.62	90.49	92.83
15	−1	+1	+1	+1	97.72	79.49	83.24	86.35
16	+1	+1	+1	+1	90.44	72.08	75.94	80.02
17	−1	0	0	0	108.40	89.85	93.65	95.72
18	+1	0	0	0	107.71	89.22	93.00	95.16
19	0	−1	0	0	111.82	93.51	97.28	98.90
20	+1	0	0	0	107.49	89.02	92.87	94.91
21	0	0	−1	0	107.69	89.27	93.09	95.19
22	0	0	+1	0	104.52	86.10	89.87	92.35
23	0	0	0	−1	98.20	79.92	83.68	86.86
24	0	0	0	+1	109.45	90.98	94.77	96.78
25	0	0	0	0	108.90	90.57	94.35	96.32
26	0	0	0	0	110.78	90.36	96.14	97.86
27	0	0	0	0	110.95	92.61	96.39	98.02

Table 1 (continued)

Std	Irradiation time	Microwave power	F:S	Ethanol concentration	TPC (mg GAE/g d.w.)	TFC (mg QE/g d.w.)	Antioxidant activity	
							DPPH (%)	ABTS (%)
28	0	0	0	0	110.72	92.43	96.19	97.89
29	0	0	0	0	110.82	92.33	96.21	97.92
30	0	0	0	0	110.97	92.15	96.37	98.02

after it has been left in the dark for 60 min at room temperature. The sample concentration was determined using the quercetin (50–500 mg/ml) standard curve equation ($y = 0.112x + 0.178$, $R^2 = 0.9945$) and the result was expressed as mg quercetin equivalents per gram of dried weight sample (mg QE/g d.w.). The total flavonoid content in the extract was calculated using Eq. (2). Ethanol was used as the blank and the analyses were repeated thrice.

$$TFC = \frac{c \times V}{m} \tag{2}$$

where c is the sample concentration from the calibration curve (mg/ml), V is the volume (ml) of the solvent used in the extraction, and m represents the weight (g) of the dried sample used.

Antioxidant activities determination

The DPPH and ABTS free radical assays were carried out based on the procedure outlined by Alara et al. [3]. Briefly, 0.2 ml of the extract was mixed with 2 ml of 0.1 mM DPPH solution. After 30 min of incubation, the absorbance of the mixture was determined at 517 nm using a UV–Vis Spectrophotometer (Hitachi U-1800, Japan). Methanol was used as blank and the percentage inhibition was determined using Eq. (3).

For ABTS free radical assays, 2 ml of ABTS stock solution (a mixture of 2.45 mM potassium persulfate and 7 mM $ABTS^{+\cdot}$ solutions at a ratio of 1:1) was diluted with 120 ml methanol. Thereafter, 2.85 ml of the freshly prepared solution was added to 0.15 ml of *V. amygdalina* leaf extract. The mixture was incubated at room temperature for 120 min and absorbance was measured at 734 nm using a UV–Vis Spectrophotometer (Hitachi U-1800, Japan). Methanol was used as blank and the percentage inhibition was determined using Eq. (3).

$$\%\, DPPH/ABTS^{+\cdot}\ inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \tag{3}$$

where $A_{control}$ is absorbance of the mixture of methanol and DPPH/$ABTS^{+\cdot}$ solution; and A_{sample} is absorbance of the mixture of sample extract and DPPH/$ABTS^{+\cdot}$ solution.

Experimental design

Response surface methodology (RSM) with face central centered composite design (FCCCD) consisting of 30 experimental runs with six centre points was used to optimize the extraction factors, including, irradiation time (X_1), microwave power level (X_2), feed-to-solvent ratio (X_3), and ethanol concentration (X_4) which were selected based on the results obtained from two-level factorial

screening [16]. The coded levels and range of values of all the four process variables are shown in Table 1. The obtained experimental results were fitted to a second-order polynomial equation as shown in Eq. (4). Four dependent responses presented in this study are Y_{TPC} = total phenolic content (mg GAE/g d.w.), Y_{TFC} = total flavonoid content (mg QE/g d.w.), Y_{DPPH} = percentage inhibition of DPPH (%), and Y_{ABTS} = percentage inhibition of DPPH (%).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_1 x_1 + \sum_{\substack{i=1 \\ i<j}}^{k=1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \sum_{i=2}^{k} \beta_{ii} x_i^2 \quad (4)$$

where various x_i values are independent variables affecting the dependent responses Y; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; and k is the number of variables.

Model validation

The MAE conditions were numerically optimized for the optimum recovery of TPC, TFC and antioxidant activities based on analysis of variance and 3D response surface plots of the independent variables. The obtained models were verified by comparing the predicted data with experimental data.

LCMS-QTOF analysis of the extract

The phenolic and flavonoid compounds present in the extract at optimized condition were identified by LC-ESI-MS/MS analysis using Q-TOF mass spectrometer equipped with PDA detector (Waters Vion IMS, USA) and a symmetry C_{18} column of 100 mm × 2.1 mm, 1.8 µm particle size (Waters Acquity UPLC HSS T3, USA). The composition of mobile phase were: A, water with 0.1% formic acid and 100% acetonitrile as solvent B. The gradient elution was 30% A and 70% B (0.69 min), 50% A and 50% B (1.16 min), 50% A and 50% B (1.85 min), 30% A and 70% B (3.47 min), 99% A and 1% B (4.63 min) with the flow rate of 0.6 ml/min and injection volume of 20 µl. Identification of the polyphenols were carried out using SYNAPT mass spectrometer (Waters) coupled with an electrospray ionization operating in negative and positive ion mode, mass range of 100–1000 m/z, spray voltage 4 keV, column temperature of 40 °C, sample temperature of 10 °C, gas flow of 50 l/h, desolvation temperature of 555 °C, and desolvation gas flow rate of 800 l/h. Polyphenols in the *V. amygdalina* leaf ethanolic extract was characterized with MS/MS fragmentation pattern reference standards.

Fourier transform infrared transmission (FTIR) analysis

Fourier transform infrared transmission is a powerful analyser for identifying different functional groups in plant extracts. *V. amygdalina* leaf extract at optimum condition was analysed using FTIR (Nicolet iS5 iD7 ATR; Thermo Scientific, Germany) equipped with OMNIC software. The extract was analysed using KBr standard procedure to obtain IR spectra in the scanning wave number ranging from 600 to 4000 cm^{-1} with a resolution of 4 cm^{-1} [17]. The spectra obtained for the extract was interpreted with a chart for characteristics infrared absorption frequencies of functional groups.

Statistical analysis

The experimental design, analysis of the results and response predictions were done using Design Expert 7.0 software® (Version 7.1.6, Stat-Ease Inc., Minneapolis, USA). The experimental runs and the analyses were performed thrice and the results were expressed as mean ± SD. Paired *t* test was used to compare the replicate results.

Results and discussion

Fitting of models

Four variables (irradiation time, microwave power, feed-to-solvent ratio, and ethanol concentration) were optimized using RSM for optimal recoveries of phenolic, flavonoid and antioxidant activities from *V. amygdalina* leaf. The analysis of variance (Table 2) shows that the quadratic models can best represent the experimental data in obtaining the optimal recoveries. All the models were most significant ($p < 0.0001$). Generally, closer relationship between the predicted and experiment data reflects satisfactory of the generated models. The coefficients in terms of coded variables for the predicted models are shown in Eqs. (5, 6, 7, 8) for TPC, TFC, %DPPH and %ABTS, respectively.

$$\begin{aligned} Y_{TPC} = {} & 110.43 - 1.23x_1 - 2.48x_2 - 0.52x_3 + 5.03x_4 \\ & - 0.77x_1x_2 - 0.86x_1x_3 - 0.92x_1x_4 - 0.61x_2x_3 \\ & - 2.85x_2x_4 - 0.64x_3x_4 - 2.28x_1^2 - 0.68x_2^2 \\ & - 4.23x_3^2 - 6.51x_4^2 \end{aligned} \quad (5)$$

$$\begin{aligned} Y_{TFC} = {} & 91.78 - 1.34x_1 - 2.56x_2 - 0.62x_3 + 4.95x_4 \\ & - 0.66x_1x_2 - 0.77x_1x_3 - 0.80x_1x_4 \\ & - 0.52x_2x_3 - 2.75x_2x_4 - 0.54x_3x_4 \\ & - 2.28x_1^2 - 0.55x_2^2 - 4.13x_3^2 - 6.36x_4^2 \end{aligned} \quad (6)$$

Table 2 Analysis of variance for the response surface quadratic models

Source	Sum of squares	df	Mean square	F value	p value Prob > F
TPC (mg GAE/g d.w.)					
Model	1883.65	14	134.55	102.80	< 0.0001
x_1-Irradiation time	27.01	1	27.01	20.64	0.0004
x_2-Microwave power	111.05	1	111.05	84.85	< 0.0001
x_3-Feed:Solvent	4.94	1	4.94	3.77	0.0711
x_4-Ethanol concentration	445.72	1	445.72	348.18	< 0.0001
x_1x_2	9.55	1	9.55	7.29	0.0164
x_1x_3	11.76	1	11.76	8.99	0.0090
x_1x_4	13.65	1	13.65	10.43	0.00503
x_2x_3	5.93	1	5.93	4.53	0.1063
x_2x_4	129.73	1	129.73	99.12	< 0.0001
x_3x_4	6.50	1	6.50	4.97	0.0415
x_1^2	13.47	1	13.47	10.29	0.0059
x_2^2	1.20	1	1.20	0.92	0.3539
x_3^2	46.36	1	46.36	35.42	< 0.0001
x_4^2	109.80	1	109.80	83.89	< 0.0001
Residual	19.63	15	1.31		
Lack of fit	16.42	10	1.64	2.56	0.1557
Pure error	3.21	5	0.64		
Cor total	1903.29	29			
C.V. %	1.12				
PRESS	121.51				
Adeq precision	28.369				
R^2	0.9897				
Adj R^2	0.9801				
Pred R^2	0.9362				
TFC (mg QE/g d.w.)					
Model	1804.96	14	128.93	83.11	< 0.0001
x_1-Irradiation time	32.40	1	32.40	20.89	0.0004
x_2-Microwave power	118.12	1	118.12	76.14	< 0.0001
x_3-Feed:Solvent	6.86	1	6.86	4.42	0.0604
x_4-Ethanol concentration	441.84	1	441.84	284.82	< 0.0001
x_1x_2	7.02	1	7.02	4.53	0.0504
x_1x_3	9.61	1	9.61	6.19	0.0250
x_1x_4	10.27	1	10.27	6.62	0.0212
x_2x_3	4.39	1	4.39	2.83	0.1133
x_2x_4	120.78	1	120.78	77.86	< 0.0001
x_3x_4	4.67	1	4.67	3.01	0.1034
x_1^2	13.46	1	13.46	8.68	0.0100
x_2^2	0.78	1	0.78	0.50	0.4887
x_3^2	44.18	1	44.18	28.48	< 0.0001
x_4^2	104.94	1	104.94	67.65	< 0.0001
Residual	23.27	15	1.55		
Lack of fit	18.25	10	1.82	1.82	0.2646
Pure error	5.02	5	1.00		
Cor total	1828.23	29			
C.V. %	1.25				
PRESS	139.34				
Adeq precision	25.754				
R^2	0.9873				

Table 2 (continued)

Source	Sum of squares	df	Mean square	F value	p value Prob > F
Adj R^2	0.9754				
Pred R^2	0.9238				
DPPH inhibition (%)					
Model	1848.88	14	132.06	91.96	< 0.0001
x_1-Irradiation time	30.37	1	30.37	21.15	0.0003
x_2-Microwave power	118.27	1	118.27	82.36	< 0.0001
x_3-Feed:Solvent	7.01	1	7.01	4.88	0.0432
x_4-Ethanol concentration	449.20	1	449.20	312.81	< 0.0001
x_1x_2	7.94	1	7.94	5.53	0.0328
x_1x_3	8.99	1	8.99	6.26	0.0244
x_1x_4	10.29	1	10.29	7.16	0.0172
x_2x_3	4.23	1	4.23	2.95	0.1066
x_2x_4	122.05	1	122.05	84.99	< 0.0001
x_3x_4	5.05	1	5.05	3.52	0.0803
x_1^2	14.20	1	14.20	9.89	0.0067
x_2^2	0.90	1	0.90	0.63	0.4399
x_3^2	45.39	1	45.39	31.61	< 0.0001
x_4^2	107.48	1	107.48	74.84	< 0.0001
Residual	21.54	15	1.44		
Lack of fit	18.45	10	1.84	2.98	0.1196
Pure error	3.09	5	0.62		
Cor total	1870.42	29			
C.V. %	1.37				
PRESS	137.93				
Adeq precision	26.972				
R^2	0.9885				
Adj R^2	0.9777				
Pred R^2	0.9263				
ABTS inhibition (%)					
Model	1434.61	14	102.47	97.41	< 0.0001
x_1-Irradiation time	21.41	1	21.41	20.35	0.0004
x_2-Microwave power	87.12	1	87.12	82.81	< 0.0001
x_3-Feed:Solvent	6.64	1	6.64	6.31	0.0239
x_4-Ethanol concentration	343.13	1	343.13	326.17	< 0.0001
x_1x_2	6.52	1	6.52	6.19	0.0251
x_1x_3	6.06	1	6.06	5.76	0.0298
x_1x_4	6.26	1	6.26	5.95	0.0276
x_2x_3	2.92	1	2.92	2.77	0.1167
x_2x_4	92.30	1	92.30	87.74	< 0.0001
x_3x_4	5.56	1	5.56	5.28	0.0363
x_1^2	10.96	1	10.96	10.42	0.0056
x_2^2	0.91	1	0.91	0.86	0.3678
x_3^2	35.98	1	35.98	34.21	< 0.0001
x_4^2	84.49	1	84.49	79.37	< 0.0001
Residual	15.78	15	1.05		
Lack of fit	13.57	10	1.36	3.06	0.1142
Pure error	2.21	5	0.44		
Cor total	1450.39	29			
C.V. %	1.14				
PRESS	102.03				

Table 2 (continued)

Source	Sum of squares	df	Mean square	F value	p value Prob > F
Adeq precision	27.560				
R^2	0.9891				
Adj R^2	0.9790				
Pred R^2	0.9297				

$$Y_{DPPH} = 95.80 - 1.30x_1 - 2.56x_2 - 0.62x_3 + 5.00x_4 - 0.70x_1x_2 - 0.75x_1x_3 - 0.80x_1x_4 - 0.51x_2x_3 - 2.76x_2x_4 - 0.56x_3x_4 - 2.34x_1^2 - 0.59x_2^2 - 4.19x_3^2 - 6.44x_4^2 \quad (7)$$

$$Y_{ABTS} = 97.58 - 1.09x_1 - 2.20x_2 - 0.61x_3 + 4.37x_4 - 0.64x_1x_2 - 0.62x_1x_3 - 0.63x_1x_4 - 0.43x_2x_3 - 2.40x_2x_4 - 0.59x_3x_4 - 2.06x_1^2 - 0.59x_2^2 - 3.73x_3^2 - 5.68x_4^2 \quad (8)$$

where x_1, x_2, x_3, and x_4 are the coded variables for irradiation time, microwave power, extraction temperature, and feed-to-solvent ratio, respectively.

The lack-of-fit test ($p > 0.05$) was used to assess the 'fitness' of the models. It can be clearly seen from Table 2 that the lack-of-fit for TPC (0.1557), TFC (0.2646), %DPPH (0.1196) and %ABTS (0.1142) were insignificant, showing the good fit of the models. More so, the obtained p-value illustrates the suitability of the models to accurately predict the variations. The qualities of the fitted models were evaluated based on the coefficient of determination (R^2) which were 0.9897, 0.9873, 0.9885 and 0.9891 for TPC, TFC, %DPPH and %ABTS, respectively. Although, a larger value of R^2 value does not always show a sound regression model [14]. Thus, the R^2 value should be comparable to adj R^2 for a good statistical model. The obtained R^2 values were not differed greatly from adj R^2 (Table 2). In addition, the significant adequacy of the models was verified at 0.0001% level of probability with the R^2, adjusted R^2 and predicted R^2 of greater than 90%. The coefficient of variation (CV) explains the extent to which data were dispersed. Thus, the CV for TPC, TFC and antioxidant activities from *V. amygdalina* leaf were lower which show a better reproducibility [18].

Effect of extraction variables on the total phenolic and flavonoid contents

Extraction variables including irradiation time, microwave power, feed-to-solvent ratio, and ethanol concentration significantly influence the recovery yields of total phenolic content and total flavonoid content. Amongst the variables, ethanol concentration and microwave power greatly affected the polyphenols significantly ($p < 0.0001$) followed by irradiation time ($p < 0.05$). Similar resulted were obtained for optimal recoveries of polyphenols from *Prunus domestica, Punica granatum* leaf and *Haematococcus pluvialis* using microwave-assisted extraction techniques [18–20]. In contrary, the feed-to-solvent ratio was insignificant ($p > 0.05$) in obtaining optimal yields of polyphenols from *V. amygdalina* leaf. Parallel observations were made when extracting total phenolic compounds from *Eucalyptus robusta* leaf whereby sample-to-solvent ratio pose the greatest impact on the recovery [21].

Solvent polarity plays an important role because of its ability to extract polyphenols through solubilization [22]. Generally, phenolic compounds have a wide spectrum of solubility in ethanol–water solution as compared to mono-component solvent. The addition of a little amount of water to organic solvent increases its polar medium which can facilitate the extraction of polyphenols [23]. Thus, the permeability of this solvent into plant matrix may require using a higher concentration. In this study, variation in the ethanol concentration from 60 to 80% showed an increased in the TPC and TFC yields where optimal values of 112.32 mg GAE/g d.w. and 93.68 mg QE/g d.w., respectively were obtained at 76% v/v of ethanol concentration. In the same vein, microwave power had a pivotal effect on the TPC and TFC recoveries from *V. amygdalina* leaf. The linear term was statistically significant on the yields. At microwave power level between 500 and 600 W, optimal yields of polyphenols were obtained. The irradiation time is another variable that influences the optimal recoveries of TPC and TFC from *V. amygdalina* leaf. The linear term was significant in obtaining optimal yields. The quadratic terms of irradiation time, feed-to-solvent ratio and ethanol concentration were significant ($p < 0.05$) but microwave power was insignificant (Table 2). All the independent extraction variables interactions contributed significantly to the optimal recoveries of both TPC and TFC except the interaction between microwave power level and feed-to-solvent ratio that was insignificant ($p > 0.05$, Table 2).

Effect of extraction variables on antioxidant activities

The effect of microwave-assisted extraction on the antioxidant activity of *V. amygdalina* leaf was evaluated using DPPH and ABTS antioxidant assays, as the antioxidant ability of extracts from plant sample tends to fluctuate depending on the assays used [21]. The models for both assays were significant ($p < 0.0001$) and all linear variables significantly contributed in the order: ethanol concentration > microwave power > irradiation time > feed-to-solvent ratio (depending on the p-value, Table 2) to the antioxidant activities. All the quadratic terms were significant ($p < 0.05$) except microwave power in attaining optimal antioxidant inhibition from *V. amygdalina* leaf. Nevertheless, the interaction between microwave power × feed-to-solvent ratio and feed-to-solvent ratio × ethanol concentration were insignificant ($p > 0.05$) in obtaining optimum DPPH antioxidant inhibition but only the interaction between microwave power × feed-to-solvent ratio was insignificant for ABTS antioxidant inhibition. As the ethanol concentration increases from 60 to 80%, the antioxidant inhibition tends to increase until ethanol concentration of 76%, where optimal DPPH of 97.71% and ABTS of 99.23% were obtained.

The antioxidant capacity of a plant extract may spring up from the combined or synergetic action of each polyphenol that can as well be evaluated by their chemical structure [22]. Thus, the antioxidant activity can be affected by any change in polyphenol content in the extracts of the plant. This can simply be explained through a positive correlation between total polyphenol content and antioxidant activity

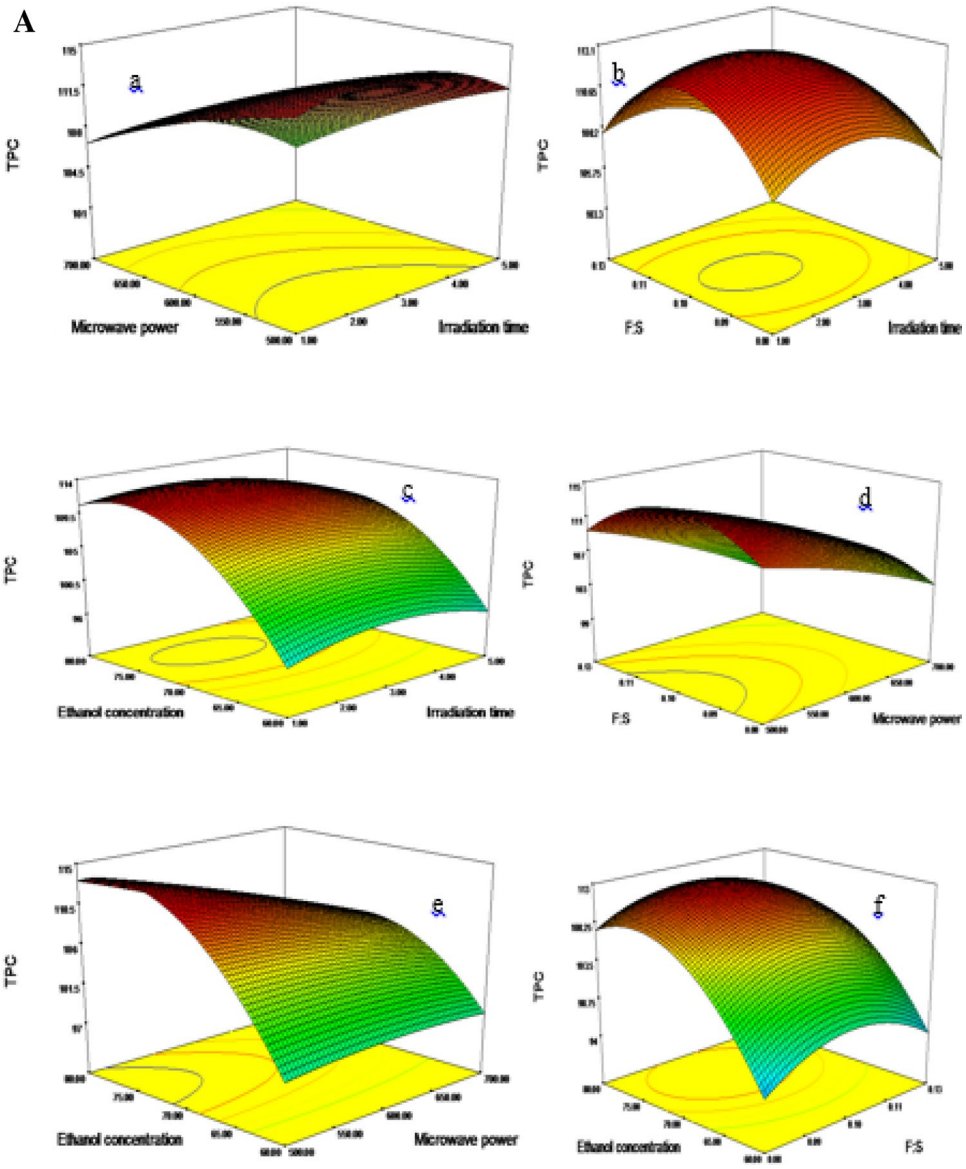
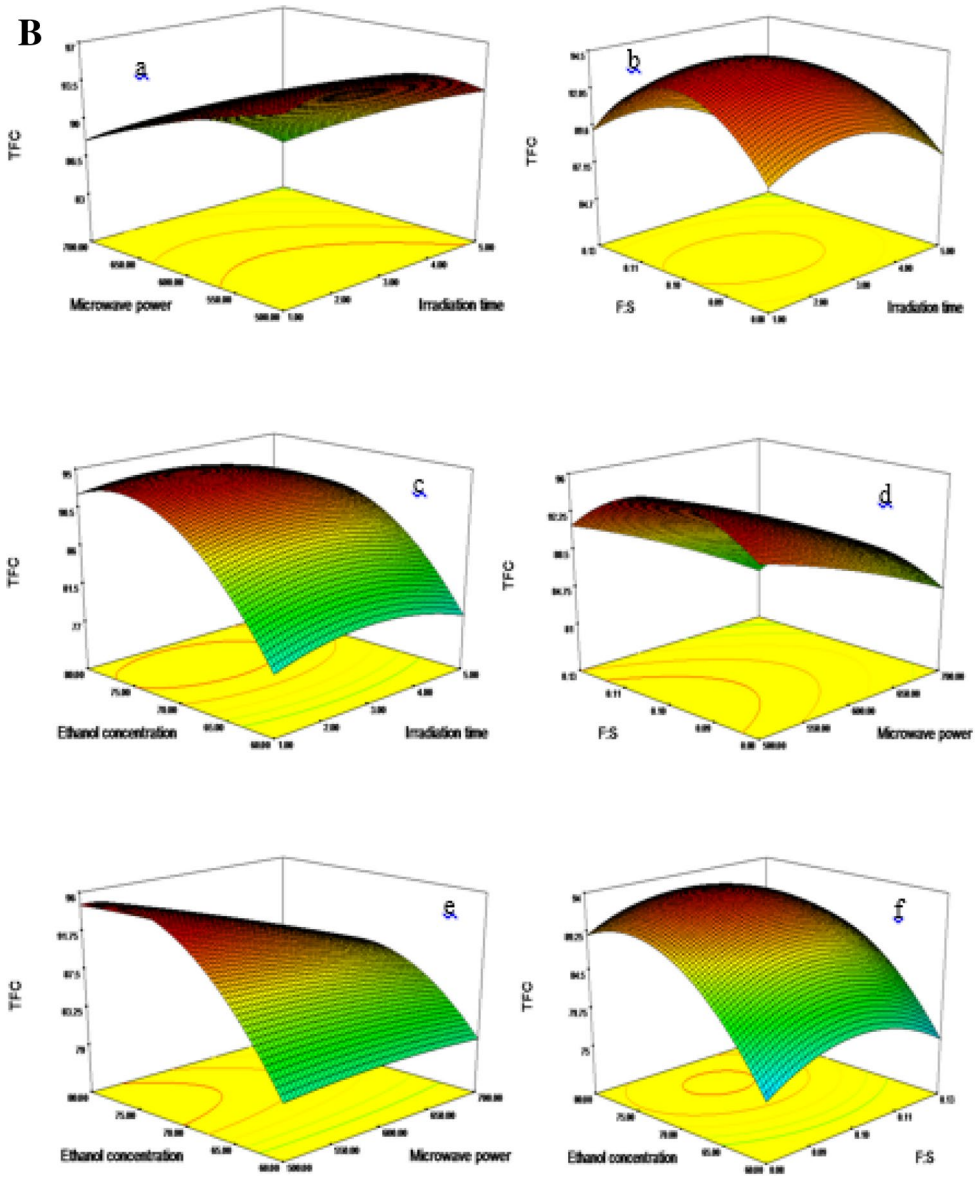


Fig. 1 Effects of irradiation time, microwave power level, feed-to-solvent ratio, and ethanol concentration on the total phenolic content (**a**); total flavonoid content (**b**); DPPH inhibition (**c**); and ABTS inhibition (**d**) from *Vernonia amygdalina* leaf

Fig. 1 (continued)

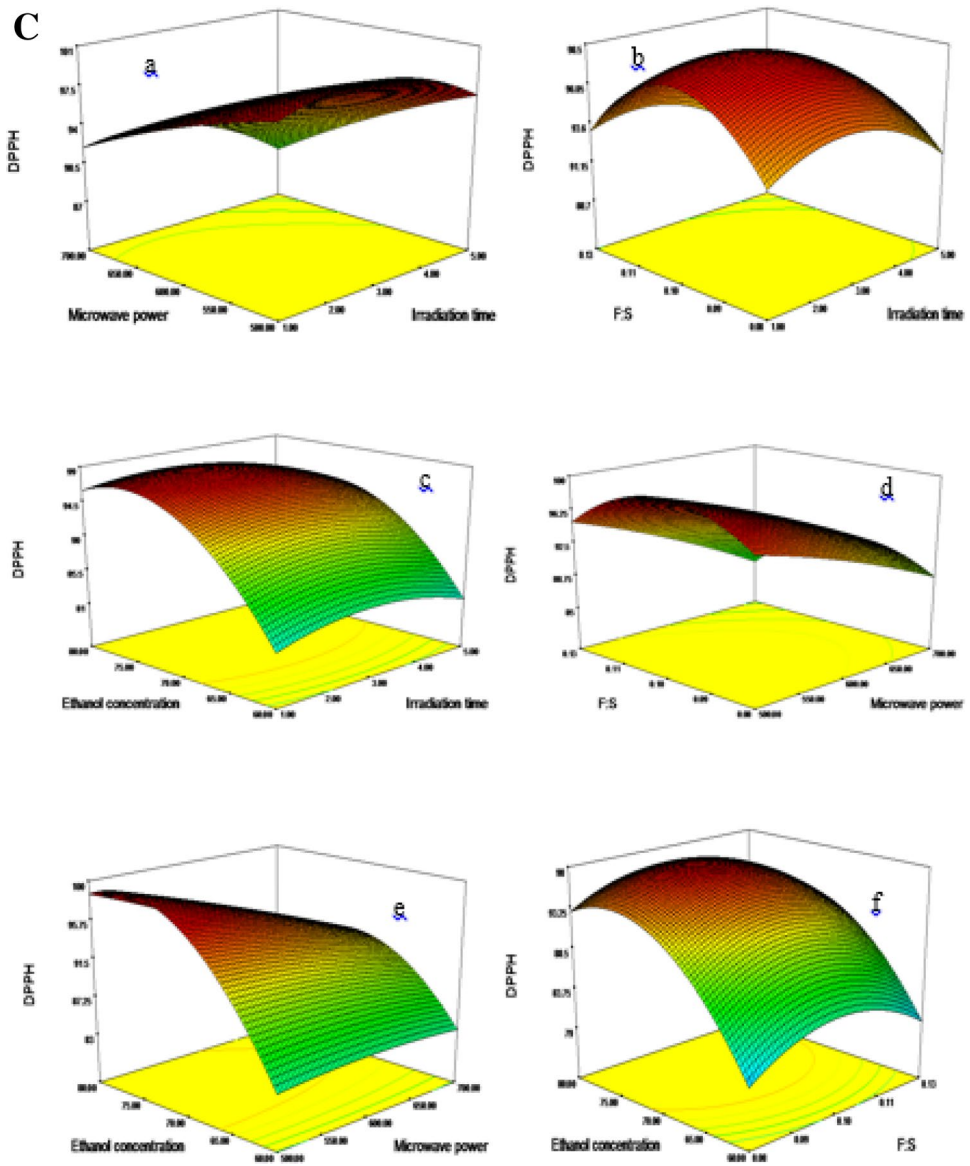


of the extract. The obtained results from this study results are in good agreement with the earlier reports whereby the ferric reducing antioxidant power (FRAP), DPPH and total antioxidant capacity of photochemiluminescence (TAC-PCL) scavenging activities were correlated with total polyphenols [23, 24]. In general, the phenolic contents and activities of bioactive compounds from the plant are primarily influenced by the genotypes (species and cultivars of the plant).

Optimization responses and validation of the predictive models

The influences of extraction conditions on the recovery yields of TPC, TFC and antioxidant activities from *V. amygdalina* leaf were evaluated through 3D response surface plots (Fig. 1). The x-, y- and z- axes of the 3D plot represent two factors and response, for instance, microwave power, irradiation time, and TPC (Fig. 1Aa). The response surface slope is associated with evaluation index implying that the larger the slope, the more the evaluation index increases. More so, the interaction between two variables is reflected in the contour of the plot whereby a rounded contour line shows a significant interaction between the two variables and a distorted contour reflects a weak (or insignificant) interaction (Table 2; Fig. 1). The predicted optimal conditions were: irradiation time of 3.49 min, microwave power level of 558.09 W, feed-to-solvent ratio of 1:10, and ethanol concentration of 75.56%.

Fig. 1 (continued)

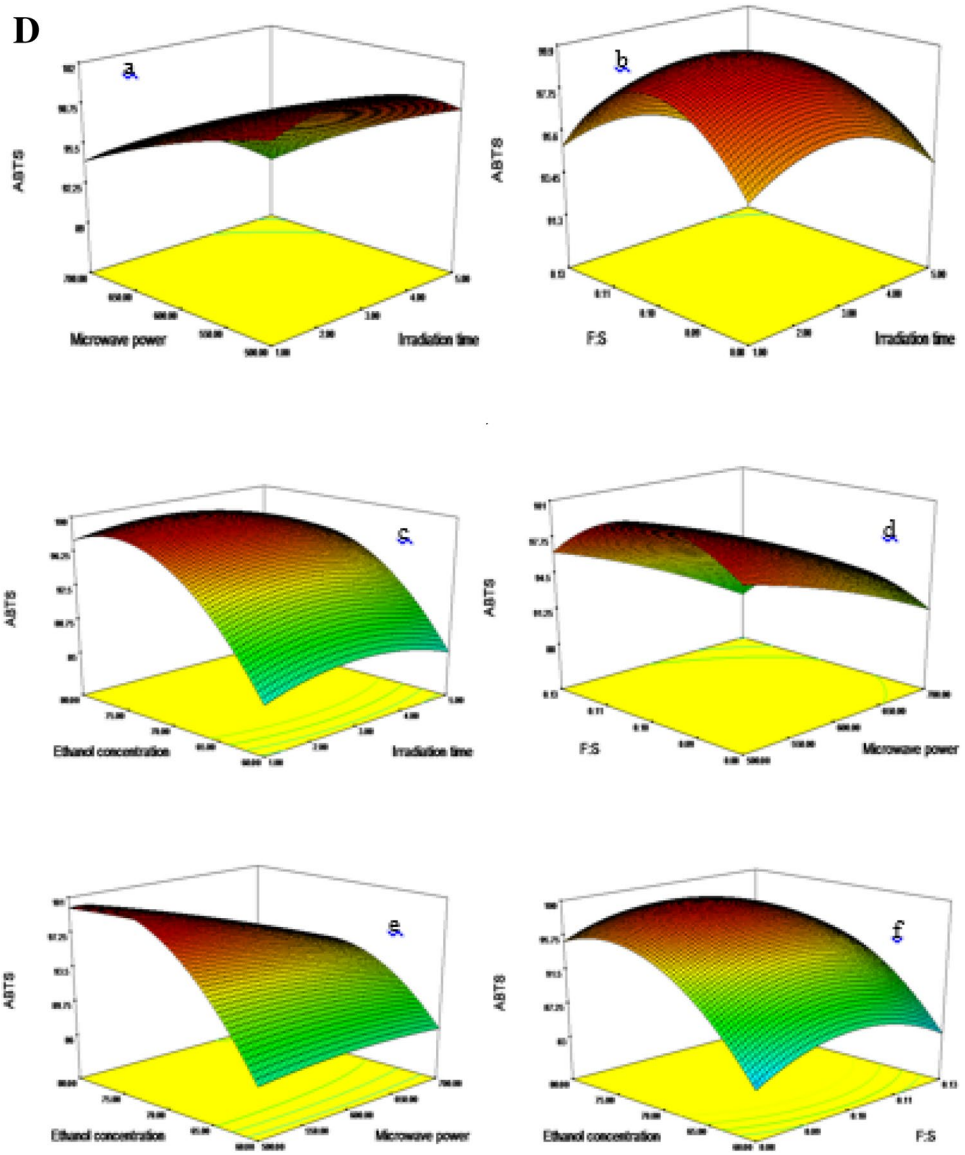


The models were verified by extracting the *V. amygdalina* leaf under the predicted optimal conditions. For the experimental verification runs (n = 3), the MAE conditions were set at irradiation time of 4 min, microwave power level of 558 W, feed-to-solvent ratio of 1:10, and 76% of ethanol concentration. Thereafter, the TPC, TFC and antioxidant activities of the optimum extract were determined to validate the reliability of the predicted optimal conditions. The validation showed that the optimal extraction conditions gave the desirable TPC (113.76 ± 2.34 mg GAE/g d.w.), TFC (94.08 ± 1.89 mg QE/g d.w.), %DPPH (97.98 ± 0.73%), and %ABTS (99.34 ± 0.56%) which were found to be not significantly different from predicted values at $p > 0.05$ using a paired t-test [25]. The close agreement between predicted and experimental data confirmed the validity of the generated models.

Identification of phenolic compounds in the extract from *V. amygdalina* leaf using LCMS-QTOF

Phenolic compounds are inevitable active compounds in medicinal plants since they disrupt the bacterium cell wall, altering its membrane potential and prevent the ATP pool which results in the death of bacterium. The polyphenols profiling of the extract was carried out using LCMS QTOF analysis. The parent compounds being confirmed by their respective daughter ions are shown in Fig. 2. Results from this analysis revealed that the ethanolic extract of *V. amygdalina* leaf contains appreciable amounts of polyphenol and flavonoid compounds, including, 3-4-*O*-dicaffeoylquinic acid at m/z 515.1201, tran-ferulaldehyde at m/z 223.0617, 3,7-dihydroxy-2-4-dimethoxyphenanthrene-3-*O*-glucoside at m/z 431.1351, 1,3,5-*O*-tricaffeoyl-quinic acid at

Fig. 1 (continued)



m/z 677.1511, (±)-isoduartin at m/z 377.1242, coniferol at m/z 179.0719, hematine at m/z 290.0568, caesalpins P at m/z 290.0568, 6-gingerol at m/z 293.1766, ginkgol at m/z 347.2593, 1,3-*O*-dicaffeoylquinic acid at m/z 555.0914, trigonelline at m/z 138.0551, kaempferol-3-*O*-β-D-glucopyranoside at m/z 449.1091, kaempferol at m/z 287.0552, kaempferol-3-*O*-β-D-glucuronide at m/z 463.0875, neoline at m/z 438.2855, ephedradine A at m/z 515.2640, ephedradine C at m/z 555.20901, polycancanthine at m/z 262.1419, hirsutine at m/z 391.1992, corypalline at m/z 194.1182, dauricinoline at m/z 611.3116, and songorine at m/z 358.2371. The combined action of these compounds may be responsible for the antioxidant and other biological activities of the extract. In addition, studies have shown that phenolic compounds in plants exert several biological activities like modulation of cellular enzymes, inhibition of cancerous cell proliferation, cell metabolisms, caspase-dependent pathways and cell survival, that may be independent of their conventional antioxidant mechanisms. For instance, ginkgol has been reported to significantly inhibit the migration, proliferation, and invasion of human tumor cells in a dose-dependent manner [26]. 1,3,5-*O*-tricaffeoyl-quinic acid has been found to be a potent RNase H inhibitor and antiviral agent when human peripheral blood mononuclear cells infected with HIV-1 was tested through cellular assay [27]. Folwarczna et al. had reported that trigonelline possess antidiabetic properties whereas kaempferol compounds could scavenge free radical cells [28].

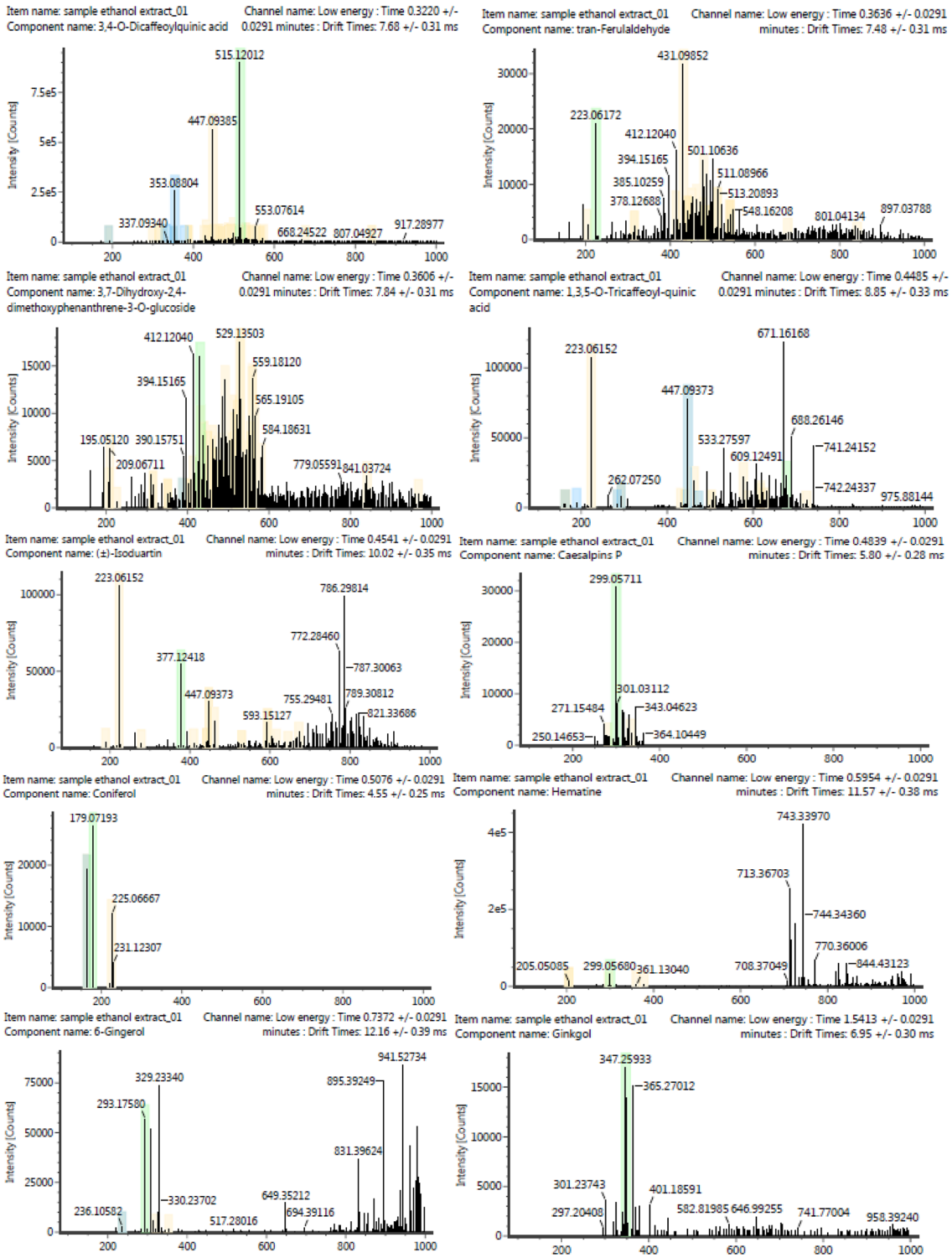


Fig. 2 LCMS QTOF analysis of an optimized ethanolic extract of *Vernonia amygdalina* leaf for phenolic and flavonoid compounds identification

FTIR spectra interpretation

The FTIR spectra of *V. amygdalina* leaf extract is shown in Fig. 3. Since the sample was extracted using aqueous ethanol, there could be an ethanol band in the spectra. Thus, characteristic ethanol band would be 3345.51 cm^{-1} which shows the stretching vibration of O–H group or O-H wagging of phenolic compounds. This strongly suggested

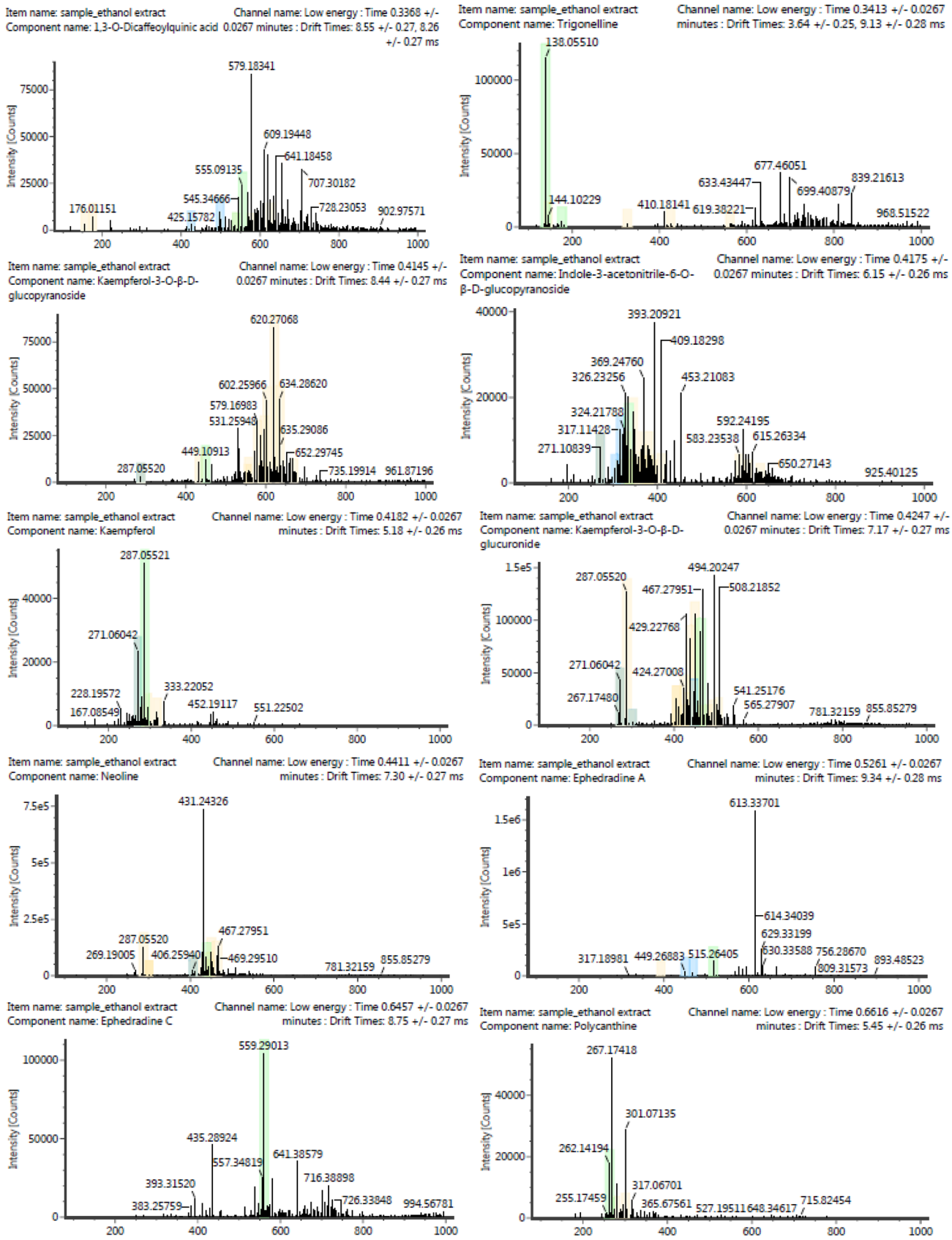


Fig. 2 (continued)

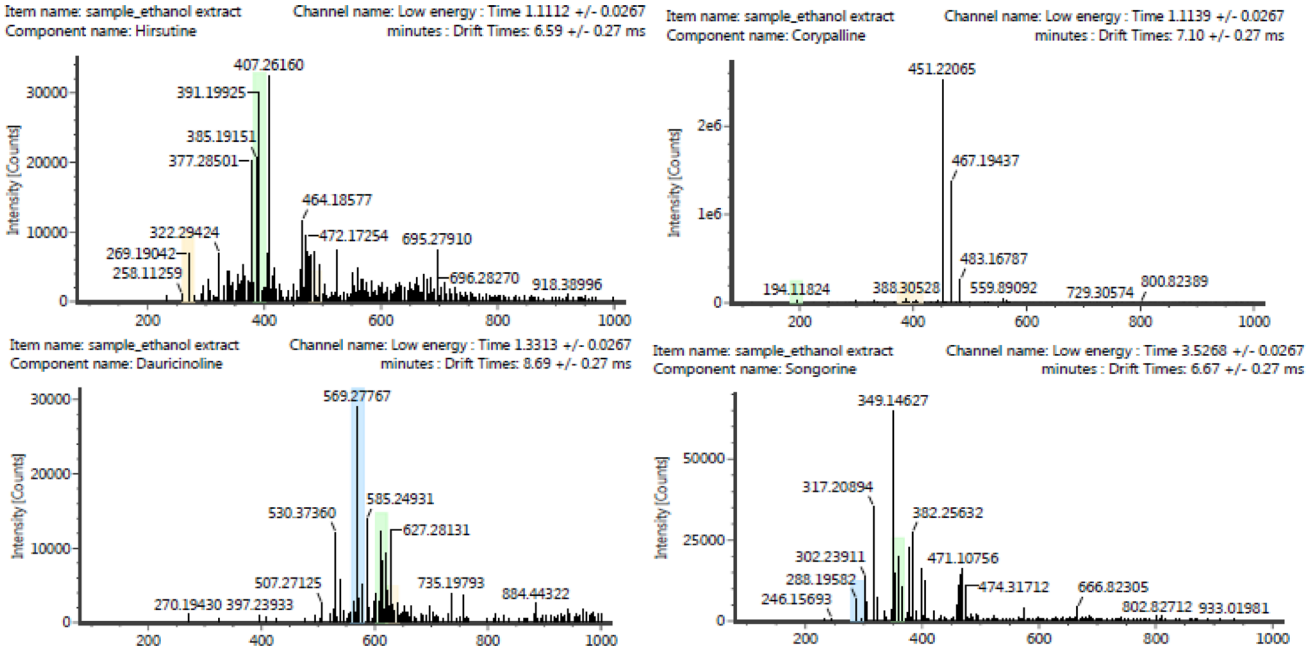


Fig. 2 (continued)

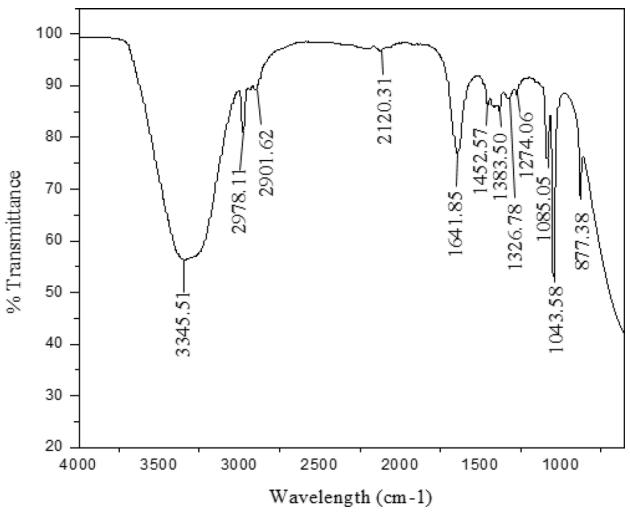


Fig. 3 FTIR spectra of ethanolic extract of *Vernonia amygdalina* leaf

the presence of phenolic compounds in the extract. The observed bands at 2978.11 and 2901.62 cm^{-1} would be due to stretching vibrations of CH_2 and CH_3 groups. A band at 1641.85 cm^{-1} could be due to stretching vibration of C=C groups, aromatic ring deformations, and the presence of flavonoids and amino acids. More so, the band at 1452.57 cm^{-1} could be attributed to CH_2, CH_3, flavonoids, and aromatic rings. Bands at 1383.50 and 1326.78 cm^{-1} may be due to (CH_2) and (CH_3) bending of methyl which can be associated with the presence of proteins [29]. The band at 1274.06 cm^{-1} could be attributed to the presence of C–O groups of polyols like hydroxyflavonoids [30]. The identified bands at 1085.05 and 1043.58 cm^{-1} could be due to the presence of C–O- stretching ester group and/or secondary alcohols. In addition, a band at 877.38 cm^{-1} could be due to aromatic ring vibration. Therefore, the observed functional groups in the ethanolic extract of *V. amygdalina* leaf show the presence of amino acids, lipids, phenolic compounds, flavonoids, carbohydrates, saponins, and tannins.

Conclusion

The effect of microwave-assisted extraction conditions on the recovery yields of total phenolic content, total flavonoid content and antioxidant activities of ethanolic extract of *V. amygdalina* leaf has been investigated using response surface methodology. The results showed that TPC and TFC were significantly affected by ethanol concentration, microwave power and irradiation time. In contrary, antioxidant activities (DPPH and ABTS) were affected by the four considered extraction variables. The optimal yields of TPC (113.76 ± 2.34 mg GAE/g d.w.), TFC (94.08 ± 1.89 mg QE/g d.w.), DPPH (97.98 ± 0.73%), and ABTS (99.34 ± 0.56%) were obtained at the optimal condition, viz, irradiation time of 4 min, microwave power level of 558 W, feed-to-solvent ratio of 1:10, and 76% of ethanol concentration. In addition, the identified compounds from LCMS QTOF and FTIR analysis confirmed the presence of phenolic and flavonoid compounds in the extract. The combined actions of the present compounds

may be responsible for its antioxidant activity. Thus, the obtained models can be applied in the scale-up production of polyphenols for usage in food and pharmaceutical industries.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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