Optimization Of Microwave-Assisted Extraction Of Bioactive Compounds From Anogeissus Leiocarpus Guill. & Perr. Stem Bark Using Response Surface Methodology

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ABSTRACT: The optimal conditions of Microwave-Assisted Extraction (MAE) of antioxidants from Anogeissus leiocarpus Guill. & Perr stem bark were determined. A second-order regression for central composite design (CCD) was used to investigate the effects of four independent variables, namely extraction time (s), irradiation power (W), solvent-to-solids ratio (ml/g) and methanol concentration (%) on the responses. The second-order regression for CCD consisted of 24 experimental points and 4 replications at the central point. Data were analyzed using Statgraphics software. The optimal conditions based on combination responses were: extraction time of 83 s, irradiation power of 538 W, solvent-to-solids ratio of 16.3 ml/g and methanol concentration of 51.84% according to the analysis of response surface. These optimum conditions yielded total phenolic contents (TPC) and total flavonoid content (TFC) of 498 mg Gallic Acid Equivalent (GAE)/ gDM and 3068 µg Quercetin Equivalent (QE)/gDM, respectively, with %DPPHsc of 53.21 % and total antioxidant activity (TAA) of 96206 µg Vitamin C Equivalent (VCE)/gDM. Close agreement between experimental and predicted values was found.

Keywords: Anogeissus leiocarpus, antioxidants, Microwave-Assisted Extraction, Optimal conditions

1. INTRODUCTION

The medicinal uses of the plants are attributed by the plant's secondary metabolites and are unique resources for pharmaceuticals, food additives, and fine chemicals [1]. Numerous investigations have proved that these secondary metabolites contain diverse classes of bioactive phenolic compounds such as polyphenols, tocopherols and alkaloids. Among them favonoids and phenolic acids are particularly attractive as they are known to exhibit various pharmacological properties such as anticarcinogenic, antimicrobial, antioxidant as well as antiallergic and antiproliferative activity on tumour cells [2,3]. Anogeissus leiocarpus Guill. & Perr. (synonym: Anogeissus schimperi Hochst. ex Hutch & Dalziel) (Combretaceae)) is a woody species commonly found in forest savannahs of West Africa. It belongs to the phylum, Tracheophyta; Order; Myrtales and Family: Combretaceae (combretoideae). It is commonly called Axle-wood tree, and in Nigeria, it is referred to as Marke (Hausa), Kojoli (Fulani), Annum (Kanuri), Ayin or Orin-odan Ainy (Yoruba), Atara (Igbo) and Kukunchi (Nupe). It is a very graceful tropical tree which grows up to 28m and occurs in the most of the savannah areas from the driest regions to the borders of the forest zone. In Africa, its occurrence extends from Senegal in West Africa to Sudan and Ethiopia in East Africa. Those growing in the driest area tend to have smaller leaves and more hairy flowers than those growing under wetter conditions, but both differences are not sufficiently marked to create distinct varieties [4]. Extraction is the initial and the most important step in the recovery and purification of bioactive compounds from plant materials. Such traditional methods as soxhlet extraction, which have been used for many decades, are very time-consuming and require relatively large quantities of solvents [5]. There is an increasing demand for new extraction techniques to shorten the extraction time, reduce organic solvent consumption, and to prevent environmental pollution. Novel extraction methods including Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Supercritical Fluid Extraction (SFE) and Accelerated Solvent Extraction (ASE) are fast and efficient for extracting chemicals from solid plant matrixes [5, 6, 7]. The use of SFE or ASE, however, requires greater financial investment, and the presence of water in samples can cause blockage in both techniques [8]. However, many factors such as solvent composition, extraction temperature, solvent-to-solid ratio, solvent pH, and pressure may significantly influence the extraction efficiency, antioxidant activity and phenolic content [9, 10, 11, 12]. Hence, it is necessary to optimize the extraction conditions to obtain highest phenolic recovery and antioxidant activity. The aim of the present study was to describe the response surface optimisation of solvent extraction of bioactive compounds from Anogeissus leiocarpus stem bark for the enhanced recovery of total phenolic content (TPC) and total flavonoids content (TFC)

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with two in vitro methodologies of measuring antioxidant activities: Total antioxidant activity (TAA) and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (%DPPHsc).

2. MATERIALS AND METHODS

2.1. Materials

One batch of 5 kg of A. leiocarpus stem bark was collected in Yagoua locality, Cameroon. These stem bark were air dried for 24h and milled. The powder obtained was stored in a sealed container for later use**.** All chemicals (analytical grade) used in the experiment were purchased from VWR.

2.2. Preparation of A. leiocarpus bark extracts

The process of MAE was performed with the use of a household microwave (DAE WOO, KOG-360, Combi Grill, AHYEON-DONG MAPO-GU SEOUL, KOREA) with CAVITY DIMENSIONS (WXHXD) of 290X290X220mm. Extraction of polyphenolic compounds from A. leiocarpus bark powders was carried out using aqueous methanol solvent. Desired weight of a dried bark powder and solvent were extracted at different periods (X_1), irradiation power (X_2) after mixed at different solvent-to-solids ratio (X_3) in each extraction with the corresponding concentration of solvent (X_4) (Table 1). The mixtures were then filtrated and the filtrates were collected as the extracts. Four replicates were performed in each extraction.

Table 1: *Experimental domain of central composite design (CCD).*

Xj	Factor levels				
	-1.607	- 1	0	1	1.607
Time (s)	63.9	70	80	90	96.1
Irradiation Power (W)	295.37	350	440	530	584.63
Solvent to solid ratio (ml/g)	16.96	10	15	20	23.03
Solvent concentration (%)	30.89	40	55	70	79.10

2.3. Determination of total phenolic content (TPC)

Total phenolic content of A. leiocarpus extracts obtained was determined according to the method of Singleton and Rossi [13] and then expressed as milligram gallic acid equivalent (GAE)/gram of dry weight. In brief, a 4-µL aliquot of the sample was added to 0.5 mL Folin–Ciocalteu reagent (pre-diluted at a ratio of 1:16) and allowed to stand at room temperature for 5 min, and then 2 ml of sodium carbonate (20%, w/v) were added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 760 nm against blank using a spectrophotometer (Spectroquant® Pharo 100M). The blank consisted of all reagents and solvents without the sample. The total phenolic content was determined using the standard gallic acid calibration curve.

2.4. Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) of A. leiocarpus extracts obtained by the above method was determined according to the method of Dowd as described by Nana et al. [14] and then expressed as microgram quercetin equivalent (QE)/gram of dry weight. In brief, a 40 µL aliquot of the sample was added to 1ml of methanolic solution of aluminium chloride (2%). After standing for 20 min at room temperature, absorbance was measured at 415 nm against blank (40 µL of methanol and 1ml of methanolic solution of $AlCl_3$) using a spectrophotometer (Spectroquant® Pharo 100M). The TFC was determined using the standard quercetin calibration curve.

2.5. Total Antioxidant activity by phosphomolybdenum method (TAA)

Antioxidant capacities were determined by the method of Prieto et al. [15]. An aliquot (20 µL) of the sample fractions was mixed with 2 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was covered and incubated at 95 °C for 90 min. After the mixture was cooled, the absorbance was measured at 695 nm against blank. A typical blank solution contained 2 mL of reagent solution and the appropriate volume of the same solvent used for extraction, and it was incubated under the same conditions. The antioxidant activity was expressed as microgram vitamin C equivalent (VCE)/gram dry weight.

2.6. DPPH radical scavenging test (%DPPHsc)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity of the extracts (pre-diluted at a ratio of 1:100) was determined as described by Liu et al., [16]. Aliquots of each extracts (30 µl) were added to 1 ml of methanolic DPPH solutions (0.1 mM). Discolourations were measured at 517 nm after incubation for 30 min at 25±1°C in the dark. The %DPPH which was scavenged (%DPPHsc) was calculated using the formula: %DPPHsc = (Acont – Asample)× 100/Acont Where Acont was defined as absorbance of the control whereas Asample was defined as absorbance of the sample (the extracts).

2.7. Experimental design

The extraction parameters were optimized using response surface methodology (RSM). A central composite design (CCD) was employed in this regard. Irradiation time (X_1), irradiation power (X_2), solvent-to-solids ratio (X_3) and methanol concentration (X_4) were chosen for independent variables. The range and centre point values of four independent variables presented in Table 1 were based on the results of preliminary experiments, a modified design from Pompeu et al. [17]. The experimental design consists of sixteen factorial points, eight axial points at a distance of ±1.607 from the centre and four replicates of the central point (Table 2). TPC, TFC, TAA and %DPPHsc were selected as the responses for the combination of the independent variables given in Table 2. Four experiments were carried out at each experimental design point and the mean values were stated as observed responses. Experimental runs were randomised, to minimise the effects of unexpected variability in the observed responses. The variables were coded according to the equation:

$$x = (Xi - Xo)/\Delta X \quad (1)$$

Where x is the coded value, Xi is the corresponding actual value, Xo is the actual value in the centre of the domain and ΔX is the increment of Xi corresponding to a variation of 1

unit of x. The mathematical model corresponding to the composite design is:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

Where Yi is the dependent variables (TPC, TFC, TAA and %DPPHsc), βo is the model constant, βi, βii and βij are the model coefficients, and ε is the error. They represent the linear, quadratic and interaction effects of the variables. Analysis of the experimental design data and calculation of predicted responses were carried out using Statgraphics centurion software (Version XVI.I). Additional confirmation experiments were subsequently conducted to verify the validity of the statistical experimental design.

2.8. Statistical analysis

Comparison of means was performed by one-way analysis of variance (ANOVA) followed by Duncan's test. Statistical analyses ($p < 0.05$) were performed using Statgraphics centurion software (Version XVI.I). The optimal extraction conditions were estimated through regression analysis and three-dimensional (3D) response surface plots (obtained using Sigmaplot 12.0 software) of the independent variables and each dependent variable.

3. RESULTS AND DISCUSSION

The effects of the four process variables, i.e., time (X_1: 70–90 s), irradiation power (X_2: 350–530 w), solvent-to-solids ratio (X_3: 10–20 ml/g), and methanol concentration (X_4: 40-70%), were investigated during the study. The four responses of interest were TPC, TFC, TAA and %DPPHsc. The results of 28 runs using CCD design are shown in Table 2, which include the design, observed responses and the predicted values. A close agreement between experimental and predicted values was found. In addition, it was observed that the yield of TPC and TFC ranged from 264 - 498 mg GAE/gDM and 434 - 3394 µg QE/gDM, respectively. The highest TPC (498 mg EAG/gMS) was obtained under the experimental conditions of X_1=70s, X_2=530 W, X_3= 20/1 ml/g and X_4= 40%; whereas the highest TFC was obtained under conditions of X_1=90s, X_2=530 W, X_3= 20/1 ml/g and X_4= 70%. A wide range of antioxidant activities were also found (TAA : 63043 - 109791 µgVCE/gDM, %DPPHsc : 11 - 76%) and the maximum points were found under the conditions of X_1= 90s, X_2 = 530 W, X_3= 10/1 ml/g and X_4= 40 % for %DPPHsc (76 %) and X_1=80s, X_2=440 W, X_3= 23/1 ml/g et X_4= 55% for TAA (109791 µg VCE/gDM). Therefore, an optimisation process was investigated, in order to obtain desirable antioxidant contents and activities.

3.1. Model fitting

Table 3 shows the results of fitting quadratic models to the data. The results of analysis of variance (ANOVA) indicate that the contribution of the quadratic model was significant. The fitted quadratic models for TPC, TFC, TAA and %DPPHsc in coded variables are given in Eqs. (3) – (6), respectively. In order to obtain simpler quadratic regression model insignificant factors and their interactions have been omitted from the equations. The significance of each coefficient was determined using the F-test and p-value in Table 3. This table shows only those which are significant. The corresponding variables would be more significant if the absolute F-value becomes greater and the p-value becomes smaller [18]. Lack of fit was also given in Table 3 in order to check the quality of the fitted models.

$$TPC = 539.526 + 17.675X_1 + 19.195X_2 + 49.404X_3 - 33.181X_4 - 29.902X_1^2 + 12.267X_1X_3 - 37.805\ X_2^2 - 52.052\ X_3^2 - 20.806X_3X_4 - 62.642\ X_4^2 \quad (3)$$

$$TFC = 2692.56 + 125.527X_1 + 281.587X_2 - 74.1092X_3 - 88.8645X_4 - 148.863X_1^2 + 99.6941X_1X_4 + 163.596X_2^2 + 101.701X_2X_3 \quad (4)$$

$$TAA = 101325.0 + 4293.08X_1 + 4685.63X_2 + 5852.76X_3 - 2966.92X_4 - 6054.5X_1^2 + 3441.6X_1X_4 - 5910.07X_2^2 - 8869.08X_4^2 \quad (5)$$

$$\%DPPHsc = 61.13 + 3.90X_1 + 6.40X_2 - 17.31X_3 - 3.57X_1^2 - 3.63X_3^2 - 6.35X_4^2 \quad (6)$$

3.1.1 TPC

The effect of extraction time (X_1), irradiation power (X_2), liquid to solid ratio (X_3) and solvent concentration (X_4), was significant ($p < 0.05$) both in first-order linear effect (X_1, X_2, X_3 and X_4) and second-order quadratic effect (X_1^2, X_2^2, X_3^2 and X_4^2) as shown the table 3. These results obtained suggest that the change of different independent variables had significant effects ($p < 0.05$) on the yield of TPC. Interaction terms X_1X_3 and X_3X_4 were equally significant ($p > 0.05$). The coefficient of determination (r^2) of the predicted models in this response was 96.40%. However, p-value for lack of fit was 0.1208, which suggesting a relative good fit to the mathematical model Eq. (3).

3.1.2 TFC

For TFC (Y_2), effect of extraction time, incubation power, liquid to solid ratio and solvent concentration, was significant ($p < 0.05$) in first-order linear effect (X_1, X_2, X_3 and X_4), second-order quadratic effect (X_1^2 and X_2^2) and interactive effect (X_1X_4 and X_2X_3). The coefficient of determination (r^2) of the predicted models in this response was 94.61%. However, p-value for lack of fit was 0.0020, which suggesting not a good fit to the mathematical model Eq. (4).

Table 2: *CCD with the observed responses and predicted values for TPC, TFC, TAA and %DPPHsc*

N°	CODED VARIABLES LEVELS				Observed responses				Predicted values			
	X_1	X_2	X_3	X_4	Y_{TPC} (mgGAE/gDM)	Y_{TFC} (µgQE/gDM)	Y_{TAA} (µgVCE/gDM)	$Y_{\%DPPHsc}$ (%)	Y_{TPC} (mgGAE/gDM)	Y_{TFC} (µgQE/gDM)	Y_{TAA} (µgVCE/gDM)	$Y_{\%DPPHsc}$ (%)
1	70	350	10/1	40	296.710	2702.27	70605.9	61.674	308.164	2778.57	70031.1	60.516
2	90	350	10/1	40	294.238	2868.62	73555.9	65.456	311.850	2856.31	76331.8	64.294
3	70	530	10/1	40	330.780	3095.02	75511.5	75.894	340.962	2972.00	78601.2	73.606
4	90	530	10/1	40	314.570	3274.81	81976	76.346	319.399	3203.94	83303.5	73.233
5	70	350	20/1	40	391.858	2415.20	80949.5	22.601	404.211	2494.60	82534.4	17.707
6	90	350	20/1	40	465.825	434.47	80649.1	29.566	456.969	328.19	81237.9	29.790
7	70	530	20/1	40	478.904	3027.10	95531.3	36.662	476.685	3044.84	95047.3	37.364
8	90	530	20/1	40	484.394	3020.44	90148.4	45.860	504.192	3032.63	92152.5	45.296
9	70	350	10/1	70	264.289	2249.35	55840.1	60.709	265.280	2207.79	56555.7	56.179
10	90	350	10/1	70	296.928	2715.47	74210.1	64.389	308.475	2684.31	76622.9	63.859
11	70	530	10/1	70	276.653	2499.06	63043.7	66.360	294.837	2591.90	64383.7	66.308
12	90	530	10/1	70	304.346	3331.41	81717.5	70.039	312.783	3222.62	82852.5	69.838
13	70	350	20/1	70	273.603	2025.22	70516.5	11.563	278.102	2082.66	71117.8	14.849
14	90	350	20/1	70	359.763	2221.38	83957.6	33.639	370.370	2315.03	83587.7	30.833
15	70	530	20/1	70	344.159	2840.65	82944.7	35.479	347.335	2823.59	82888.6	31.546
16	90	530	20/1	70	416.477	3299.88	91256.6	42.049	414.352	3210.15	93760.2	43.379
17	63.928	440	15/1	55	456.193	2230.02	80469	39.947	433.879	2121.50	78786.4	45.639
18	96.0717	440	15/1	55	515.020	2319.80	98102	56.254	490.695	2494.61	92585.8	58.185
19	80	295.37	15/1	55	434.327	2769.74	81155.7	48.764	411.025	2677.76	78528.5	53.649
20	80	584.63	15/1	55	496.062	3394.24	98161.4	71.511	472.725	3552.51	93589.7	74.249
21	80	440	16.96	55	363.304	2708.88	94617.4	73.771	325.673	2824.75	89198.3	79.566
22	80	440	23.03	55	493.483	2636.12	109791	22.076	484.475	2586.54	108011	23.903
23	80	440	15/1	30.892	457.430	2864.63	87415.3	41.931	431.049	2923.43	83184.4	47.241
24	80	440	15/1	79.107	344.653	2599.94	76615.6	39.902	324.394	2607.42	73647.7	42.215
25	80	440	15/1	55	528.036	2719.80	99122	61.722	539.526	2692.56	101325	61.135
26	80	440	15/1	55	518.340	2709.69	98897.4	66.826	539.526	2692.56	101325	61.135
27	80	440	15/1	55	507.516	2699.81	97144.2	61.882	539.526	2692.56	101325	61.135
28	80	440	15/1	55	538.122	2734.90	99934.6	64.912	539.526	2692.56	101325	61.135

3.1.3 Total Antioxidant activity (TAA)

In term of total antioxidant activities, it can be observed that all linear, quadratic terms except X_3^2 and interactive effect (X_2X_3) gave a significant effect whereas other terms were not significant ($p > 0.05$). The coefficient of determination (r^2) of the predicted models in TAA was 95.45%, whereas p-values for lack of fits were 0.0269. The predicted models can reasonably represent the observed values. Thus, the responses were sufficiently explained by the models.

3.1.4. DPPH radical scavenging acticity (%DPPHsc)

For %DPPHsc, the extraction time, incubation power, liquid-to-solid ratio and solvent concentration, were significant ($p < 0.05$) in three linear effects (X_1, X_2 and X_3), three quadratic effects (X_1^2, X_2^2 and X_4^2). The coefficient of determination (r^2) of the predicted models in this response was 96.81% and p-value for lack of fit was 0.1267. These values would give a relative good fit to the mathematic model in Eq. (6).

3.2. Influence of extraction parameters on phenolic compounds

The influence of four independent variables towards total phenolic content was reported through the significant ($p < 0.05$) coefficient of the second-order polynomial regression equation. 3D response surfaces curves in Fig. 1 demonstrated the effects of the independent variables and their mutual interactions on the TPC values. They were obtained by keeping two of the variables constant. The constant was equal to the natural value of zero level. As evidence, the fig. 1e shows that at lower irradiation power, increasing power led to a gradual increase in the TPC value over time. This phenomenon is considered to be caused by the low rate of mass transfer at low temperatures, which would require more time for the phenolic compounds to dissolve from the raw materials into the solution.

Table 3: *ANOVA for response surface quadratic model: estimated regression model of relationship between response variables and independent variables (X_1, X_2, X_3, X_4).*

Source	Sum of square	DF	Mean square	*F-value*	*P-value*
TPC (µg GAE/ g DM)[a]					
X_1	6.61×10^{9}	1	6.61×10^{9}	38.48	0.0084
X_2	7.80×10^{9}	1	7.80×10^{9}	45.38	0.0067
X_3	5.17×10^{10}	1	5.17×10^{10}	300.63	0.0004
X_4	2.33×10^{10}	1	2.33×10^{10}	135.61	0.0014
X_1^2	1.19×10^{10}	1	1.19×10^{10}	69.43	0.0036
X_2^2	1.90×10^{10}	1	1.90×10^{10}	110.98	0.0018
X_3^2	3.61×10^{10}	1	3.61×10^{10}	210.39	0.0007
X_4^2	5.23×10^{10}	1	5.23×10^{10}	304.70	0.0004
X_1X_3	2.41×10^{9}	1	2.41×10^{9}	14.01	0.0333
X_3X_4	6.93×10^{9}	1	6.93×10^{9}	40.31	0.0079
Pure error	5.15×10^{8}	3	1.71×10^{8}		
Lack of fit	7.75×10^{8}	10	7.75×10^{8}	4.51	0.1208
Total	2.35×10^{11}	27			
TFC (µg QE/ gDM)[b]					
X_1	2.85×10^{5}	1	2.85×10^{5}	19.95	0.0006
X_2	1.56×10^{6}	1	1.56×10^{6}	109.63	0.0000
X_3	1.16×10^{5}	1	1.16×10^{5}	8.13	0.0136
X_4	2.04×10^{5}	1	2.04×10^{5}	14.31	0.0023
X_1^2	2.95×10^{5}	1	2.95×10^{5}	20.68	0.0005
X_2^2	3.57×10^{5}	1	3.57×10^{5}	24.98	0.0002
X_1X_4	1.59×10^{5}	1	1.59×10^{5}	11.12	0.0054
X_2X_3	1.27×10^{5}	1	1.27×10^{5}	8.90	0.0106
Pure error	673.46	3	224.48		
Lack of fit	1.85×10^{5}	10	18520.9	82.50	0.0020
Total	3.45×10^{6}	27			
TAA					
X_1	3.90×10^{8}	1	3.90×10^{8}	26.26	0.0002
X_2	4.64×10^{8}	1	4.64×10^{8}	31.28	0.0001
X_3	7.25×10^{8}	1	7.25×10^{8}	48.81	0.0000
X_4	1.86×10^{8}	1	1.86×10^{8}	12.54	0.0036
X_1^2	4.89×10^{8}	1	4.89×10^{8}	32.93	0.0001
X_2^2	4.66×10^{8}	1	4.66×10^{8}	31.38	0.0001
X_4^2	1.04×10^{9}	1	1.04×10^{9}	70.66	0.0000
X_1X_4	1.89×10^{8}	1	1.89×10^{8}	12.76	0.0034
Pure error	4.13×10^{6}	3	1.37×10^{6}		
Lack of fit	1.88×10^{8}	10	1.88×10^{7}	13.69	0.0269
Total	4.24×10^{9}	27			
%DPPHsc (%)[c]					
X_1	322.43	1	322.431	14.72	0.0021
X_2	869.37	1	869.371	39.70	0.0000
X_3	6347.12	1	6347.12	289.84	0.0000
X_1^2	170.12	1	170.129	7.77	0.0154
X_3^2	176.73	1	176.736	8.07	0.0139
X_4^2	538.38	1	538.38	24.59	0.0003
Pure error	18.384	3	6.12818		
Lack of fit	266.29	10	26.6297	4.35	0.1267
Total	8931.9	27			

[a] The coefficient of determination (r2) of the model was 96.40%

These results are similar to the research findings by Karabegovic´et al. [19]. Moreover the microwave irradiation accelerates cell rupture by sudden temperature rise and internal pressure increase inside the cells of plant sample, which promotes the destruction of sample surface and in turns the exudation of the chemical substance within the cells into the surrounding solvents takes place [20, 21]. At higher irradiation power, however, dissolution of the phenolic compounds can reach the equilibrium in a shorter time then decreased by changes in the extraction time. This suggests that a higher irradiation power and a short extraction time are more effective in extracting antioxidative phenolic compounds from A. leiocarpus bark using MAE. The decreasing observed at higher values of irradiation power and long period of extraction may be due to thermal degradation of the phenolics [22]. The total phenolic content increased with increasing liquid-to-solid ratio. When the liquid-to-solid ratio increased from 8:1 to 16:1, the total phenolic content also increased, which was probably due to the fact that more solvent could enter cells while more phenolic compounds could permeate into the solvent under the higher solid-to-liquid ratio conditions [23, 24]. With further increase in liquid-to-solid ratio, a decline in TPC content was observed (Fig. 1a, Fig. 1b and Fig. 1c). Pompeu et al. [17] have reported that extraction of phenolics compounds was highly dependent on liquid/solid ratio. They have reported liquid/solid ratio of 40:1 (mL/g) was sufficient to extract high quantities of phenolics from fruits of E. oleraeceae. Gan and Latiff [11] reported liquid/solid ratio (20 mL/g) played a significant role in the yield of phenolics, while extraction temperature did not make any significant contribution towards TPC When methanol concentration increased from 31 to 50 %, increase in the phenolic content from 443 to 544 mg GAE/g DM, was observed (Fig. 1d). This is probably due to the increased solubility of phenolic compounds in the mixture of methanol and water. The methanol concentration was also an important variable contributing to the extraction of phenolic compounds from other natural sources, such as mashua tubers [25], brewer's spent grains [26]. Mussatto et al [12] showed that both of solvent-to-solids ratio and concentration of methanol had significant effects on extraction of phenolic compounds from spent coffee grounds.

3.3. Influence of extraction parameters on Antioxidant activities (%DPPHsc and TAA)

The 3D response surfaces curves in Fig. 2 demonstrated the effects of the independent variables and their mutual interactions for %DPPHsc. Overall, results show that lower irradiation power of extraction (~350 W) would a give lower %DPPHsc value (~50%), respective of incubation time (Fig. 2a). As the irradiation power of extraction increased, the %DPPHsc increased to 70%. For extracts with lower solvent-to-solids ratio (~10 ml/g), the DPPH scavenging activity was higher (~70%), compared to higher solvent-to-solids ratio (Fig. 2d, 2e and 2f). %DPPHsc started to increase with increased proportion of methanol concentration in the extraction medium (Fig. 2b, 2c and 2d). Thus, the proportion of methanol concentration in the extraction medium had a significant influence on the antioxidant properties of A. leiocarpus extracts.

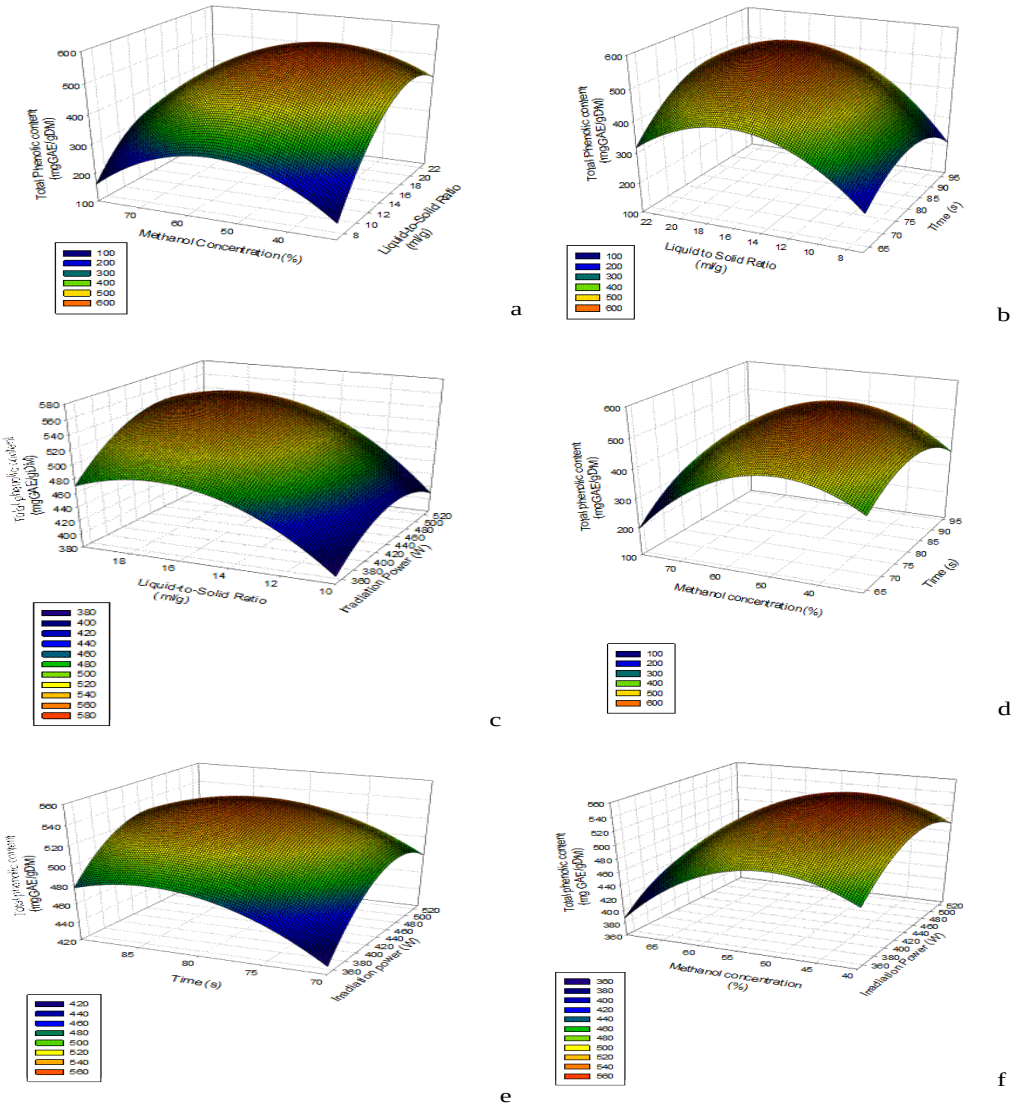

Fig. 1. 3D *Response surface plots showing the effects of variables on TPC*

Fig. 3 shows the 3D response surfaces for total antioxidant activity. It shows that at lower irradiation power, increasing power led to a gradual increase in the TAA value over time (Fig. 3f). For the time of 63.88 to 85.55s, there is an increase of 78755 to 101736 µg VCE/gDM of TAA. This corresponds to positive influence of the time (X_1) to this response. This phenomenon is considered to be caused by the low rate of mass transfer at low irradiation power, which would require more time for the antioxidants to dissolve from the raw materials into the solution. At higher irradiation power, however, dissolution of the phenolic compounds can reach the equilibrium in a shorter time then decreased by changes in the extraction time, the Fig. 3f shows that the TAA decreased from 100230 to 93569 µg VCE/gDM when the irradiation power rise from 460 to 520 W. This suggests that a higher irradiation power and a short extraction time are more effective in extracting antioxidative compounds from A. leiocarpus bark using MAE. Total antioxidant activity increase linearly with solvent liquid-to-solid ratio (Fig. 3c, 3d and 3e). For this case, an increase in the volume of solvent would result in a diffusion of phenolic compounds, their stability (cooling system). Gan and Latiff [11] showed that the solvent liquid-to-solid ratio of 20 ml/g was required for high antioxidant activity. This activity increases equally with methanol concentration in extraction medium. There is an increase in the TAA of 83176.7 to 101,569 µg VCE / gDM when the methanol concentration increased from 30.95 to 52.87 % (Fig. 3a). The extraction of active substances is favored in aqueous-alcoholic medium, pure methanol is not effective enough for the extraction of phenolic compounds. Prasad et al. [27] show that the ethanol 68% is optimum for optimal total antioxidant activity. However there is a reduction in activity when the concentration of methanol is greater than 52%. This could be explained by the fact that the solubility of the bioactive substance concentration decreases with methanol.

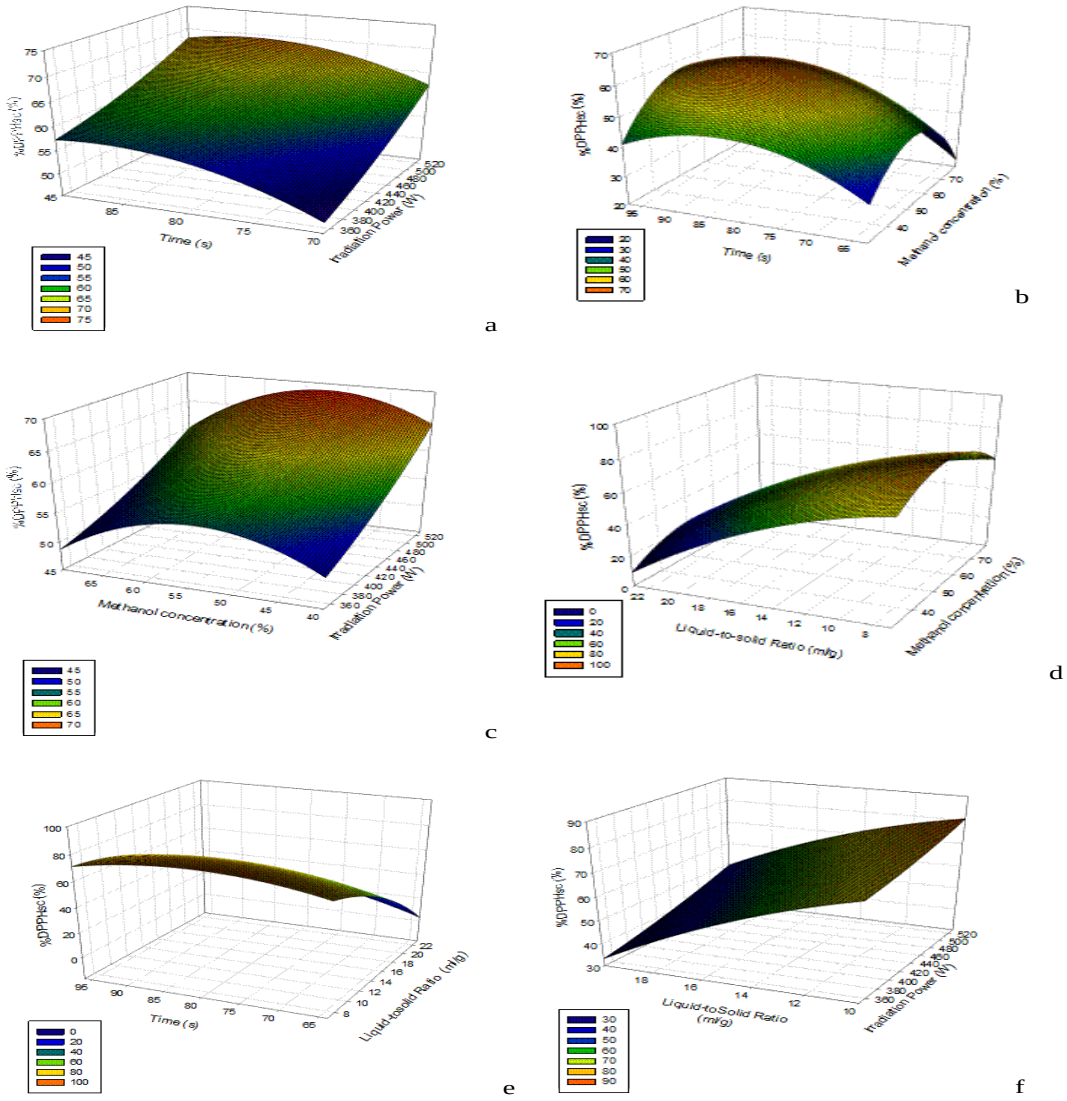

Fig. 2. 3D *Response surface plots showing the effects of variables on %DPPHsc*

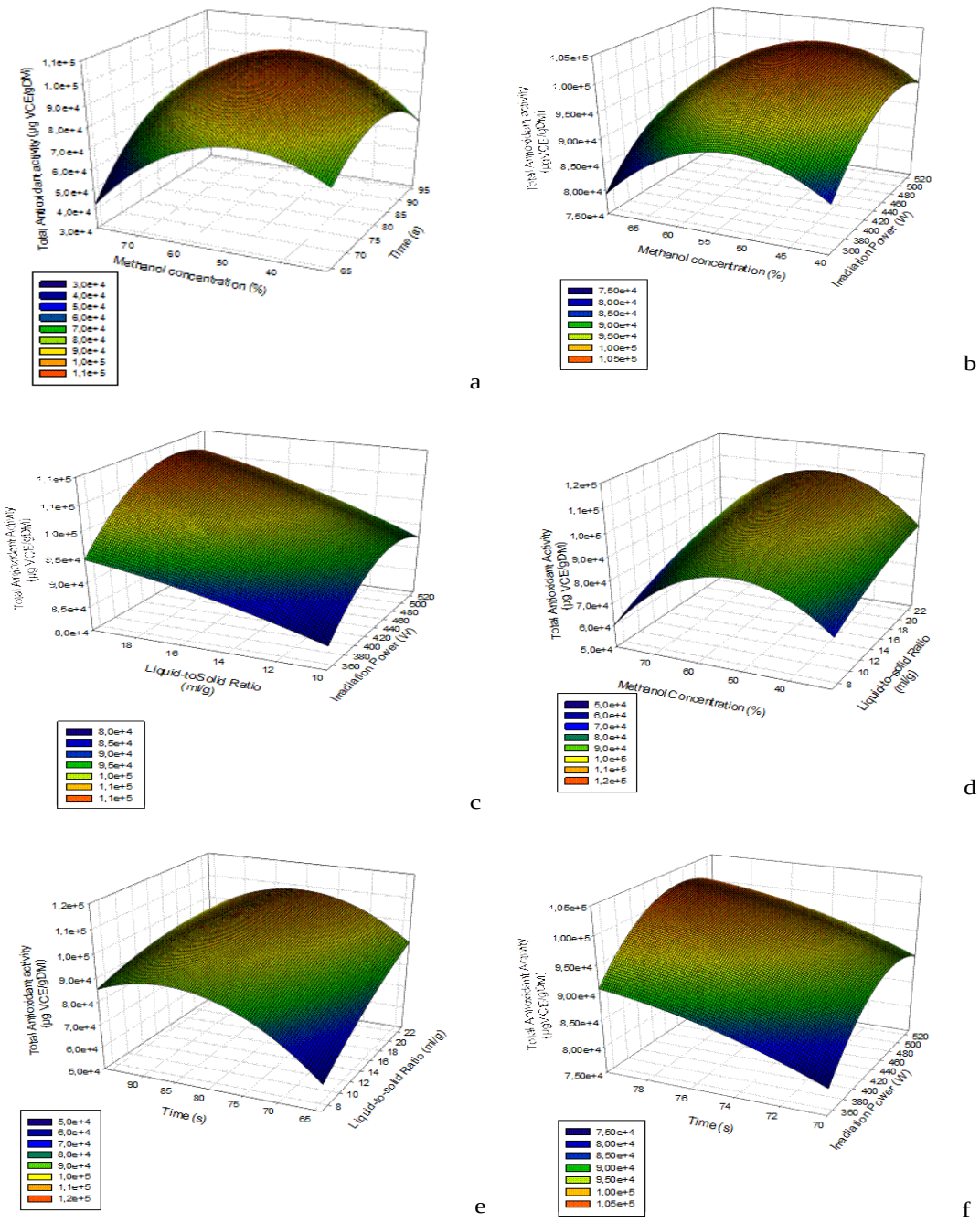

Fig. 3.: *3D Response surface plots showing the effects of variables on TAA*

3.5. Verification of predictive models

Based on the above findings, an optimisation study was performed to evaluate the optimal operating conditions for individual response as well as combination of all responses. The target was to obtain high phenolic compounds yields with high antioxidant activities within the extraction parameters, where consideration of the efficiency, the energy conservation and the feasibility of the experiment were taken into account. Table 4 shows the optimal conditions for each individual response with the predicted and experimental values. Optimal conditions for TPC were extraction time of 80 s, incubation power of 468 W, solvent-to-solids ratio of 18 ml/g and methanol concentration of 50%. On the other hand, optimal conditions for TFC were extraction time of 92 s, incubation power of 584 W, solvent-to-solids ratio of 14.4 ml/g and methanol concentration of 79%, whereas optimal conditions for TAA were extraction time of 80 s, incubation power of 487 W, solvent-to-solids ratio of 23 ml/g and methanol concentration of 54.4%. Optimal conditions for %DPPHsc were extraction time of 78 s, incubation power of 584 W, solvent-to-solids ratio of 7 ml/g and methanol concentration of 51%.

Table 4: *Predicted and experimental values under optimum conditions based on both individual and combination of all responses*

	Experimental Variables				Responses		
	Time (s)	Power (W)	liquid -solid Ratio (ml/g)	Methanol Conc. (%)	Calculated	Expt$_1$	Expt$_2$
TPC (mgGAE/gMS)	83	468	18	50	566	445	511
TFC (µgQE/gMS)	92	584	14.4	79	3762	3805	3660
TAA (mgVCE/gMS)	80	487	23	54.4	109	93.6	89.9
(%DPPHsc)	78	584	7	51	89	71	82
Combined Optimum	83	560	16.5	51.8			

These conditions gave TPC, TFC, TAA and %DPPHsc values of 445 - 511 mg GAE/ gDM, 3660 - 3805 µg QE/ gDM, 89.9 – 93.6 µg VCE/gDM and 71 – 82%, respectively. Table 4 shows the four optimum conditions based on combination of all responses. These optimal conditions extraction are time of 83 s, irradiation power of 560 W, solvent-to-solids ratio of 16.5 ml/g and methanol concentration of 51.8%, yielded TPC and TFC of 498 mg GAE/ gDM and 3068 µg QE/gDM, respectively, with %DPPHsc of 63.21 % and TAA of 99206 µg VCE/gDM. It could be observed that only small deviations were found between the experimental values and predicted values in Table 4.

CONCLUSION

RSM was used to determine the optimum process parameters that yield high antioxidant contents and activities. ANOVA (The F-test and P-value) indicated that the effects of extraction time, irradiation power, solvent-to-solids ratio and methanol concentration were significant in TPC yield, TFC and TAA. Whereas the effect of methanol concentration was not significant in %DPPHsc. Quadratic models were used in predicting all the responses. The optimal conditions based on both individual and combination of all responses were determined. Based on the combination of all responses, these optimal conditions of extraction time of 83s, irradiation power of 560 W, solvent-to-solids ratio of 16.5 ml/g and methanol concentration of 51.8% yielded TPC and TFC of 498 mg GAE/ gDM and 3068 µg QE/gDM, respectively, with %DPPHsc of 53.21 % and TAA of 96206 µg VCE/gDM. Results showed that predicted and experimental values were not significantly different. Therefore, it is suggested the models obtained can be used to optimize the process of bioactive compounds extraction from A. leiocarpus stem bark.

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