Development of *Phaleria macrocarpa* (Scheff.) Boerl Fruits Using Response Surface Methodology On Phenolic, Flavonoid and Antioxidants Properties

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Abstract:

In this study, the optimal condition for the extraction of antioxidants from the fruit Buah Mahkota Dewa (*Phaleria macrocarpa)* was determined by using Response Surface Methodology (RSM). The optimization was applied using central composite design (CCD) to investigate the three independent variables, namely extraction temperature (°C), extraction time (minutes) and extraction solvent to-feed ratio (%v/v) on the responses of free radical scavenging activity (DPPH), ferric ion reducing power assay (FRAP), total phenolic content (TPC) and total flavonoid content (TFC).The optimal conditions for the antioxidants extraction were found to be extraction temperature (64°C), extraction time (66 minutes) and solvent to-feed ratio (75 %v/v) with the highest percentage yield of DPPH, FRAP, TPC and TFC were 86.85%, 7.47%, 292.86 mg/g and 3.22 mg/g respectively. Moreover, the data were subjected to response surface methodology (RSM) and the results showed that the polynomial equations for all models were significant, did not show lack of fit, and presented adjusted determination coefficients (R^2) above 99%, proving the yield of phenolic, flavonoid and antioxidants activities obtained experimentally were close to the predicted values and the suitability of the model employed in RSM to optimize the extraction conditions. Hence, in this study, the fruit from *P.macrocarpa* could be considered to have the strong antioxidant ability and can be used in various cosmeceutical or medicinal applications.

Keywords: *Phaleria macrocarpa*; extraction; free radical scavenging activity (DPPH); ferric ion reducing power assay (FRAP); total phenolic content (TPC); total flavonoid content (TFC); response surface methodology (RSM)

1. Introduction

Nowadays, natural and healthy lifestyle has become the attention of people worldwide since 2000, and there is a thriving attention in the natural bioactive compound in the human diet or applied as natural medicines (Gong et al. 2012). The medicinal properties have been investigated in the recent scientific evolution all over the world due to abundance of antioxidant properties, no side effects and economic viability (Kumar et al. 2007). Besides, traditional uses of plants are the main preferences for research because medicine from plants have good advantages such as low toxicity, low cost, fewer side effect and easy to obtain if it is used in right dose. One of the plants is *Phaleria macrocarpa* (Scheff.) Boerl, belongs to Thymelaeceae family and widely known as 'Crown of God', 'Mahkota Dewa' and 'Pau'. It is a native plant from the tropical areas of Papua Island, Indonesia and grows up to 5-18 m tall. It can be found up to 1200 m above the sea level (Winarto, 2003). It is a complete tree with stem, leaves, flowers and fruits. The leaves are green and acuminate with length and diameter range from 7-10 cm and 3-5 cm approximately. The flowers can make compounds of 2-4 with colors from green to maroon. The pit is round, white and poisonous (Sufi A, 2007). The fruits are green when they are not ripen and become red when fully ripen (Hendra A, 2011) while the seeds have 1to 2 seeds per fruit with brown, egg-shaped and anatropous.

Traditionally, the locals have used *P.macrocarpa* as herbal drink either by singly or mixed with other medicinal plants to treat illness such as cancer, hypertension and diabetes mellitus (Kurnia et al. 2008). The biological and pharmacological activities for the parts of stem, leaves, fruit and seed had been examined by several researchers (Ali et al. 2012). The fruits can cure high blood pressure, gout, skin disease, liver, cancer and diabetes (Winarto, 2007). The stems are used to treat bone cancer; the egg shells of the seeds are used to antidote the breast cancer, cervix cancer, lung disease and heart disease, meanwhile leaves consist of constituents that treat impotence, allergies, blood disease and tumors (Hending W et al. 2009). Regarding on beneficial of this plant, it had been reported *P.macrocarpa* has medicinal activities such as anti-tumor, anti-hyperglycemia, anti-inflammation, anti-diarrhoel, anti-oxidant, anti-viral, anti-bacterial, anti-fungal and vasodilator effect (Altaf et al. 2013). Moreover, the major bioactive components that can be found are alkaloid, flavonoid, lignin, saponin, terpenoid and polyphenol (Azmir et al. 2014). The alkaloid constituents were suggested to possess anticancer activity (Sugiwati, Kardono & Bintang, 2006) such as dodecanoic acid, palmitic acid, ethyl stearate, sucrose, vasorelaxant icariside C3 and

mangiferin. The presence of flavonoid content as kaempferol, naringin, mycricetin, rutin and quercetin in fruit extract of *P.macrocarpa* provide to antibacterial activity with mechanism of action against pathogenic microorganism (Hendra A, 2011). Other than that, each part of plant in *P.macrocarpa* has their presences of compounds. In fruits, the isolated constituents are icariside C3, magniferin, gallic acid (Boerl & Saufi, 2007) and two triterpenoids (24-methylenecycloartan-3-one and 24-mehyl-9,19-cyclolanost-25-en-3-ol) (Singh & Ghanapriya, 2014). The leaves consist of benzophenone glycoside by (Hartati et al. 2005). In seeds, consists of phorboesters, des-acetyl flavicordin-A and 29-norcucurbitacin derivatives, while mesocarp and seed have naringin and quercitin (Hendra A, 2011).

Response surface methodology was introduced by Box and Wilson in 1951 within the context of Chemical Engineering in an attempt to construct empirical models that able to find effective statistical relationship between all the variables making up an industrial system (Jiménez-Contreras et al. 2009). The advantages of using RSM are empower evaluation of the effects of certain process variables and their interaction of response variables, less number of required experiments, more rapid, less expensive, less laborious and less time consuming (Bae et al. 2015). Moreover, the two major common and popular designs in RSM are Central Composite Design (CCD) and Box-Behnken Design (BBD). There is a research regarding on optimization of antioxidants, phenolic and flavonoid content by using RSM such as in extraction of rambutan peel extract (Mizrahi, 1997), defatted Dabai parts (Khoo, Azlan, Ismail, & Abas, 2013), *Annona crassiflora* Mart. (Araticum) (Arruda, Pereira, & Pastore, 2016) and others based on research band gap.

In this research, CCD was applied to determine the optimum condition for extraction due to the advantages as more useful for uniform precision with lower run required, chronological investigation and reasonable information of lack of it (Islam Shishir et al. 2016). Hence, the extraction was carried out by using reflux set apparatus by focusing on three independent variables of the extraction temperature (˚C),extraction time (minutes) and solvent to-feed ratio (%v/v) on the responses of free radical scavenging activity (DPPH), ferric ion reducing power assay (FRAP), phenolic and flavonoid content. These three factors were choose based on optimization of oil yield of *P.macrocarpa* seed using RSM and its fatty acid constituents where it influences the presence of major fatty acid found in this plant (Azmir et al., 2014). However, there is no wide research yet on determining antioxidant properties by using response surface methodology (RSM). Hence, the main objective is to study RSM to optimize the extraction temperature, extraction time and extraction solvent ratios that maximize the highest percentage yield on the antioxidant properties of fruit extract *P.macrocarpa.*

2. Material and Methods

2.1 Plant Material and Chemical

Phaleria macrocarpa was obtained from Skudai in Johor Bahru, Malaysia with latitude of 1°32'16.47"N and a longitude of 103°39'44.02"E respectively on November 2015. The fresh fruit of this plant which is in the red color was cut into small pieces and let it dried at room temperature for two weeks. The dried sample was grounded to powder form by using fine grinding machine with 0.5 mm mesh size. Solvent ethanol was purchased from Dinamik Sains Sdn.Bhd, Shah Alam, Selangor and other chemical reagents used in this study were analytical grade and purchased from Malaysia Sigma-Aldrich.

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2.2 Extraction Procedure

For the extraction process, about 10 g of dried *P.macrocarpa* fruit was added to 200 mL of extraction solvent ethanol and heated according to RSM. The mixture was extracted by using reflux set apparatus at extraction time from 66.30 to 133.64 minutes, temperature from 53.18 to 80 ˚C and solvent to-feed ratio at range 63.18 to 96.82 mL/g according to the experimental design with parameter combination provided by Central Composite Design, CCD. The solvent was then filtered out through a Whatman No. 1 filter paper to obtain the clear extract. The crude of each extracts were collected by using rotary evaporator (EYELA, N-N series, Tokyo, Japan) at 45°C and weighed.

2.3 Experimental Design

P.macrocarpa fruit extraction using ethanol was optimized by employed central composite design (CCD) with2^2 factorial designs consisting of seven factorial points, seven axial points and six central points with 20 experiments were conducted. The independent variables in this study were extraction temperature (*X1*: 53.18-88.82˚C), time (*X2*: 66.36-133.64 minutes) and ethanol concentrations (*X3*: 63.18-96.82 %v/v ethanol/water) which coded into five levels (-1.682, -1.000, 0.000, +1.000, +1.682). The five levels of values for the independent variables were explicit of their coded and uncoded forms in Table 1. The value of independent variables was expressed in their coded values as -1, 0, +1 interval shows the low, center and high level of each variable, respectively. The multiple regression analysis was performed on the data of response variables such as DPPH, FRAP, TPC and TFC content as affected by the extraction conditions and was fitted by the response surface regression procedure using the following second-order polynomial equation:

$$Y = \sum A_0 + \sum_{i=1}^{k} A_{ij} X_i + \sum_{i=1}^{k} A_{ij} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} A_{ij} X_i X_j \qquad (1)$$

where Y is the response variables, A_o, A_{ij}, A_{ii} and A_{ij} are the linear, quadratic and interaction coefficients, respectively and X_i and X_j represent the coded value of the i^{th} and j^{th} independent variables. The variables X_iX_j represents the first order interaction between X_j and X_i for ($i<j$).

Table 1: Independent test variables and their coded and uncoded values for CCD matrix

Variables	Coded and Uncoded Level of Variables				
	-1.682	-1	0	-1	+1.682
Temperature (˚C), X_1	53.18	60	70	80	86.82
Time (min), X_2	66.36	90	100	120	133.64
Solvent Ratio Ethanol:Water v/v (%), X_3	63.18	70	80	90	96.82

2.4 Antioxidant Test

2.4.1 DPPH Free Radical Scavenging

Anti-oxidant activity of the crude ethanol extract of *P.macrocarpa* fruit was carried out by determination of free-radical scavenging activity, measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. This method was proposed by (Lay et al. 2014) with minor modification. 50 µL of ethanolic solution of crude extract (10 mg/mL) was placed in micro plate and then, mixed with 195 µL of DPPH (50 mg/mL). The mixture was incubated for 30 minutes in the dark condition at room temperature. After the reaction, the absorbance was recorded at a wavelength 517 nm on a Microplate Reader (Spectra Max Plus 384, Molecular Devices Co.,Ltd.,America). It was done by triplicate for each of the crude extract. The positive control used in this method was ascorbic acid, while ethanol was used as a

negative control (blank). The radical scavenging activity of the tested sample was expressed as inhibition percentage of the DPPH radical scavenging activity, calculated using the equation (2) below:

$$\% \text{ Inhibition} = [(A_O - A_{1)})/A_0] \text{ X } 100\% \quad (2)$$

where A_o is absorbance control and A_1 is absorbance sample

2.4.2 Ferric reducing-antioxidant power (FRAP)

This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the ferrous form at low pH. The FRAP of fruit extract *P.macrocarpa* was determined by using the method of (Singh, Sharma, & Sarkat, 2012). First, acetate buffer was prepared by 3.10 g sodium acetate trihydrate was added with 16 mL acetic acid and made up the volume until 1L using distilled water. Secondly, 0.031 g TPTZ was prepared in 10 mL of 40 mM HCl. Thirdly, 0.054 g of ferric chloride solution, $FeCl_3.H_2O$ was prepared in 10 mL of distilled water. Lastly, FRAP reagent was prepared by mixing 100 mL acetate buffer, 10 mL TPTZ and 10 mL ferric chloride. The samples were run by mixing 3.0 mL of working FRAP reagent to 100 µL of each crude extract (10 mg/mL). This step was carried out in triplicate and measured immediately the absorbance at 593 nm using spectrometer. The procedure was repeated with ascorbic acid as a positive control.

2.4.3 Determination of Total Phenolic Content (TPC)

The total phenolic content of ethanol extracts was determined by using Folin–Ciocalteau's reagent adapted to a 96-wells plate with minor modifications (Hendra et al. 2011). The reaction mixture was prepared by mixing 100 µL of ethanolic crude extract (10mg/mL), 7.9 mL of distilled water and 2.5 mL of 10% Folling-Ciocalteu's Reagent for 8 seconds in a tube. Then, 2.5 mL of 7.5% sodium carbonate was prepared by dissolving in distilled water. The amount of 1,500 µL of sodium carbonate will mixed in previous tube solution. The mixture was shaken for 5 minutes by vortex and incubated for 2 hours at 20°C in dark condition at room temperature. While, blank test was prepared by containing 100 µL of ethanol, 2.5 mL 10% Follin-Ciocalteu's Reagent dissolved in water and 2.5 mL of $NaHCO_3$. The absorption was measured at 765 nm using a spectrophotometer. The samples prepared in triplicate for each of the crude extract. The same procedures were repeated for the standard solution of gallic acid and a calibration graph of various concentrations was constructed. The phenolic contents (TPC) of the crude extracts were expressed as (mg gallic acid equivalent/g of dry weight).

2.4.4 Determination of Total Flavonoid Content (TFC)

The total flavonoid content in each ethanol extract was measured using (Lay et al. 2014) with minor modification. The sample contained 100 µL of ethanol solution of the extract in the concentration of 10 mg/mL and 100 µL of 2% $AlCl_3$ solution dissolved in ethanol. The samples were incubated in dark for 30 minutes at room temperature and the absorbance was measured at 406 nm. The same procedure was repeated for the standard solution of quercetin and the calibration line was constructed. Based on the measured absorbance, the concentration of flavonoids (mg/mL) was read from the calibration plot and the content of flavonoid in extract was expressed in terms of quercetin equivalent (mg of quercetin/g of extract).

2.5 Gas Chromatography Mass Spectrometry (GCMS)

The determination of the compound was carried out using a BPX-5 column (30 m x 0.25 mm i.d., 0.25 µm film thickness; SGE Analytical Science, Australia) on a Shimadzu GC-MS system (Shimadzu Corporation, Kyoto, Japan) consisting of GC-2010 with a QP2010 mass spectrometer. The temperature programming was set at 50 °C for 3 min with the ramp rate of 10 °C/min to 340 °C and held for 10 min. The injector and ion source temperature were set at 250 °C and 200 °C respectively. The scanning mass range was 50-750 *m/z* with a flow 35 rate of 1.0 mL/min. The sample injection volume was 0.5 µL

2.6 Fourier Transform Infrared Spectroscopy (FTIR)

The analysis was conducted at Department of Chemistry, Faculty of Science, Univerisiti Putra Malaysia, UPM. 1.0 mg of the extract sample of *P.macrocarpa* fruit was thoroughly mixed with 100.0 mg of the potassium bromide (KBr) in ratio of 1:100. The mixture was milled to homogenize the mixture. An interferogram for the background correction was determined before the sample analysis. Then, the mixture was compressed to produce thin layer pellets in the form of disc shape. Next, the disc was placed in a beam of infrared radiation and the sample absorbed the radiation at frequencies corresponding to molecular vibration frequencies. The instrument then measured the spectrum of the scattered radiation relative to the spectrum of the non-absorbing standard (KBr). Infrared spectra were recorded on a FTIR (Agilent Technologies-630 Spectrophotometer System). All the spectra were run over the range from 280 cm-1 to 4000 cm-1 at room temperature.

3. Results and Discussion

3.1 Model Fitting

Twenty experiments were carried out using different combination of the independent variables by using response surface methodology of central composite design (CCD). It was used to identify the relationship between the response functions and process variables. The experimental and predicted values for the responses of DPPH, FRAP, TPC and TFC under different concentration are presented in Table 2. The predicted values were obtained with a model fitting technique by using software design expert 7.0.0 (Trial version, Stat-Ease Inc., Minneapolis, MN, USA). In the present study, according to the sequential model sum of squares, the highest order polynomials were utilized to select the models where the additional coefficients estimates were significant and the models are not aliased. Hence, for all three independent variables and responses, a quadratic polynomial model was selected and fitted well as suggested by the software

The independent variables were focusing mainly on three factors because it gives impact to the result obtained. Solvent extraction was the main commonly used extraction method to recover a wide range of antioxidants and phenolic compounds (Abad-Garcia et al. 2007, Chirinos et al. 2007). The effectiveness of extraction is also affected by factor such as storage time, extraction method, solvent type, the pH, extraction temperature, solvent-to-solid ratio, particle size and solvent concentration (Pinelo et al. 2005, Silva et al. 2007). Furthermore, solvent polarity plays an important role in increasing the solubility of phenolic compounds (Arruda, Pereira, & Pastore, 2016). The factor of extraction temperature was considered because increasing the extraction temperature improves extraction by increasing the solubility, hence producing better antioxidant activity (Liyanapathirana and Shahidi, 2005), while set to higher extraction temperature can cause bioactive compound decomposed particularly in flavonoids, concluded in decreasing of antioxidant activity (Silva et al. 2007). Moreover, from an industrial point, the longer extraction time means the efficiency of equipment utilization become low. It needs to be in a range of suitable extraction time (Shi et al., 2003).

Table 2: The experimental and predicted values for the responses of DPPH, FRAP, TPC and TFC under different concentration

Run Order[a]	DPPH[b]		FRAP[c]		TPC[d]		TFC[e]	
	Exp.[f]	Pred.[g]	Exp.[f]	Pred.[g]	Exp.[f]	Pred.[g]	Exp.[f]	Pred.[g]
1	86.70	86.70	7.47	7.47	226.44	226.44	3.22	3.22
2	85.88	85.88	7.29	7.29	212.21	212.20	2.60	2.60
3	84.66	84.68	7.20	7.20	221.64	221.64	2.47	2.47
4	83.70	83.71	7.12	7.12	190.01	190.02	2.32	2.32
5	86.06	86.06	7.21	7.20	242.45	242.47	2.35	2.36
6	86.35	86.35	7.14	7.13	292.33	292.35	1.92	1.92
7	86.25	86.26	7.25	7.28	201.04	201.04	2.11	2.11
8	86.42	86.42	7.30	7.32	233.55	233.55	2.13	2.14
9	86.18	86.18	7.29	7.29	197.95	197.95	2.40	2.43
10	85.60	85.60	7.19	7.17	213.32	213.32	1.93	1.94
11	86.85	86.84	7.31	7.31	292.86	292.88	2.75	2.74
12	84.20	85.20	7.23	7.24	239.38	239.39	2.30	2.30
13	85.41	84.50	7.26	7.26	186.22	186.22	2.90	2.91
14	86.25	86.25	7.19	7.20	236.31	236.30	2.03	2.04
15	85.79	85.79	7.13	7.13	231.20	231.20	2.39	2.39
16	85.79	85.79	7.13	7.13	231.20	231.20	2.39	2.39
17	85.79	85.79	7.13	7.13	231.20	231.20	2.39	2.39
18	85.79	85.79	7.13	7.13	231.20	231.20	2.39	2.39
19	85.79	85.79	7.13	7.13	231.20	231.20	2.39	2.39
20	85.79	85.79	7.13	7.13	231.20	231.20	2.39	2.39

[a] Run order – randomized

[b] Free radical scavenging activity in mg/mL

[c] Ferric ion reducing power assay in mg/mL

[d] Total phenolic content in (mg/g GAE)

[e] Total flavonoid content in (mg QE/ g of extract)

[f] Experimental value

[g] Predicted value

Based on Table 2, the result showed that the antioxidant activities of DPPH, FRAP, TPC and TFC on crude extracts of *P.macrocarpa* ranged from 83.70% to 86.85% , 7.12-7.47%, 186.22-292.86 mg of gallic acid/g of extract and 1.92-3.22 mg of quercetin/g of extract respectively for the samples treated under different extraction condition. The maximum value for DPPH and TPC obtained for the extraction time, temperature and solvent ratio were 66.36 min, 70˚C, 80% (v/v). While, maximum value for FRAP and TFC obtained were 80 min, 60˚C, 70 % (v/v) respectively. The minimum condition for antioxidants of DPPH and FRAP found was 120 min, 80˚C and 70% (v/v). Lastly, the minimum condition for phenolic and flavonoid were 100 min, 70 ˚C and 63.18% (v/v) and 80 min, 80 ˚C and 90% (v/v).

In order to fit the response function and experimental data to the second-order polynomial, the linearity and quadratic effect of the independent variables, their interactions and regression coefficients on the response variables were evaluated from variance analysis ANOVA as given in Table 3. The ANOVA results were calculated based on 94% confidence intervals and this analysis was crucial to determine the best fitted quadratic model for three independent variables. A regression model was evaluated by using *F* statistics and lack of fit test. Based on the results, it showed that the model is highly significant when the computed *F*-value is greater than the tabulated *F*-value and the probability value is low *(P<0.0001)* indicating that the individual terms in each response model were significant on the interaction effect.

The performance of the models was determined by calculating the determination coefficients R^2, adjusted R^2, predicted R^2, regression (*P-value*), regression (*F-value*), lack of fit (*P-value*), coefficient variation (CV%), lowest PRESS and probability values related to the effect of the three independent variables. Coefficient of determination, R^2 and the significance of lack of fit are the two important aspects that judges the fitness and adequacy of the model obtained. R^2 can be defined as the ratio of the explained variation to the total variation was a measure of the degree of fit (Chan et al., 2009). In practice, high values (>70%) of determination coefficients R^2 are reasonable indicators of suitability of regression models to describe the influences of the independent variables on the dependent variables (Bas and Boyaci 2007). It indicate the modeling of experimental data allowed for the generation of useful mathematical equations for general use, within the experimental range tested in this study, to investigated the behavior of the system under different factor combinations (Bassani, Nunes, & Granato, 2014). Hence, the coefficient of determination R^2 values for regression model predicted for DPPH, FRAP, TPC, TFC were 0.9999, 0.9918, 1.000 and 0.9998 respectively, suggesting a good fit. The closer R^2 value to unity, the better and significant empirical model fits the actual data. While, the absence of lack of fit (p>0.05) for all the responses also strengthened the accuracy of the models.

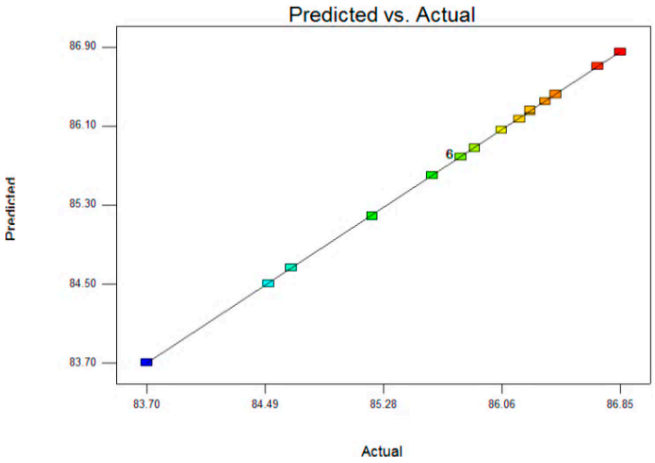
Moreover, the calculated adjusted R^2 values for studied responses variables were higher than 0.80, hence there is a close agreement between the experimental results and the theoretical values predicted by the proposed models. Furthermore, the coefficient variation (CV) is a measure of deviation from the mean value, indicating the reliability of the experiment. Generally, when CV<10% it shows the better reproducibility (Myers, 2002). The CV values obtained for DPPH, FRAP, TPC and TFC were 9.65, 0.15, 2.32 and 0.25 respectively. It is indicating the model is highly reliable and precise.

Table 3: ANOVA for the response surface quadratic model for optimization of DPPH, FRAP, TPC and TFC on the extraction parameters from fruits *P.macrocarpa*

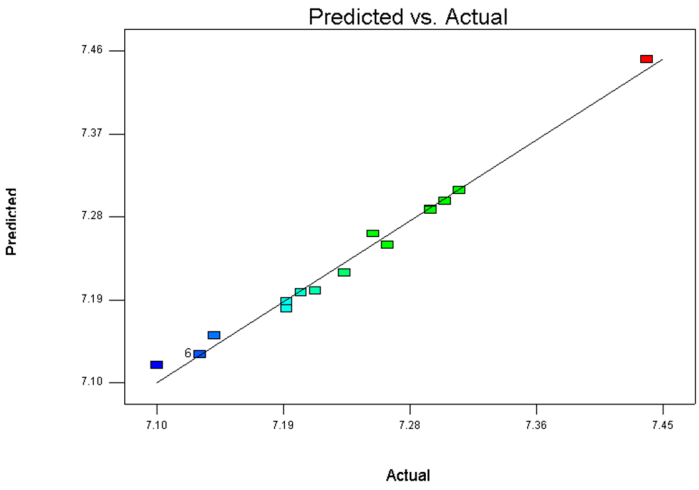
			DPPH			FRAP			TPC			TFC		
Variance Sources	dF	*p*-value	Sum of squares	Mean square	*F*-value	Sum of squares	Mean square	*F*-value	Sum of squares	Mean square	*F*-value	Sum of squares	Mean square	*F*-value
Model	9	<0.0001	10.98	1.22	17783.61	0.14	0.016	134.76	14152.19	1573.58	5.585E+007	1.84	0.20	5593.88
A	1	<0.0001	0.54	0.54	7811.46	0.013	0.013	108.74	48.05	48.05	1.705E+006	0.46	0.46	12619.20
B	1	<0.0001	4.50	4.50	65592.56	0.055	0.055	465.96	38891.80	3889.18	1.380E+008	0.30	0.30	8167.97
C	1	<0.0001	1.80	1.80	26305.77	0.031	0.031	262.11	5221.77	5221.77	1.853E+008	0.80	0.80	21729.86
Interaction														
AB	1	<0.0001	8.450E-003	8.45	123.23	3.612E-003	3.612E-003	30.55	151.12	151.12	5.363+006	0.10	0.10	2763.26
AC	1	<0.0001	0.63	0.63	9146.45	6.163E-003	6.613E-003	55.93	2056.01	2056.01	7.297E+007	0.014	0.014	394.36
BC	1	<0.0001	2.51	2.51	36585.80	0.050	0.050	419.62	669.60	669.60	2.376E+007	0.13	0.13	3549.26
Square														
A^2	1	<0.0001	0.016	0.016	229.34	0.019	0.032	271.98	1176.92	1176.92	4.177E+007	0.10	0.10	2769.65
B^2	1	<0.0001	0.094	0.09	1370.74	0.032	0.014	119.63	2196.13	2196.13	7.794E+007	0.037	0.037	1000.07
C^2	1	<0.0001	0.31	0.31	4557.82	0.014	1.182E-004		715.94	715.94	2.541E+007	0.011	0.011	296.27
Residual	10		6.857E-004	6.857E-005		1.182E-003	2.365E-004		2.818E-004	2.818E-005		3.664E-004	3.664E-005	
Lack of Fit	5		6.857E-004	1.371E-004		1.182E-003	0.000		2.818E-004	5.635E-005		3.664E-004	7.328E-004	
Pure Error	5		0.00	0.000		0.000			0.00	0.000		0.00	0.000	
Total	19		10.98			0.14			14162.19			1.85		
R^2			0.9999			0.9918			1.000			0.9998		
Adj-R^2			0.9999			0.9845			1.000			0.9996		
Pre-R^2			0.9999			0.9313			1.000			0.9985		
Adeq. Pre.			536.669			43.252			28409.729			302.131		
CV%			9.65			0.15			2.32			0.25		
PRESS			5.269E-003			9.926e-003			2.316E-003			2.781E-003		

Other than that, the significance of each coefficient was determined using the *F*-test and *p*-value. The corresponding variables are more significant if the absolute F value become greater and the p-value become smaller (Wang et al., 2007). The *F*-value for all responses model are greater than the tabulated F-value which indicating the adequacy of the models to predict different responses at different extraction conditions. Adequate precision, AP is a comparative measure between the predicted values and mean prediction error. It also measured the signal to noise ratio. A ratio greater than 4 is more desirable which ensure the predicted models are consistent with independent variables (P Manivannan& M Rajasimman, 2011). Lastly, the PRESS values were considered to be minimums to obtain a well-accorded model.

It can be concluded from the analysis of ANOVA, any terms from quadratic polynomial coefficients model, large *F-values* and a small *P-values* indicated a more significant effect on the respective response variables. The suitability model was investigated based on the results of the fitted-line plot of predicted versus experimental as shown in Figure 1. The diagnostic line plot proved the intimate closeness between predicted and experimental values for all these responses.



(DPPH, I)



(FRAP, II)

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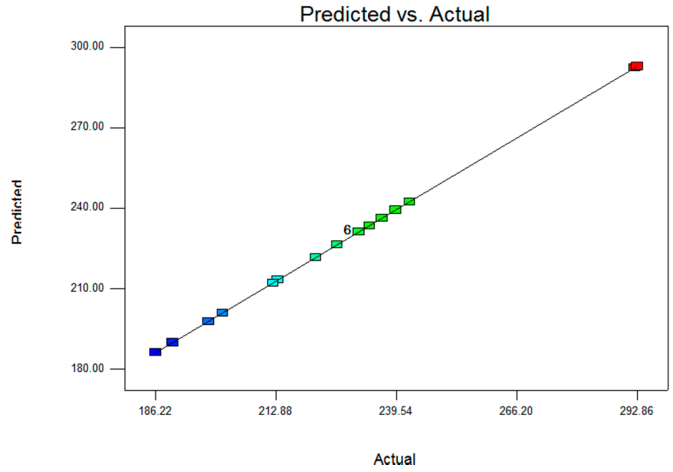
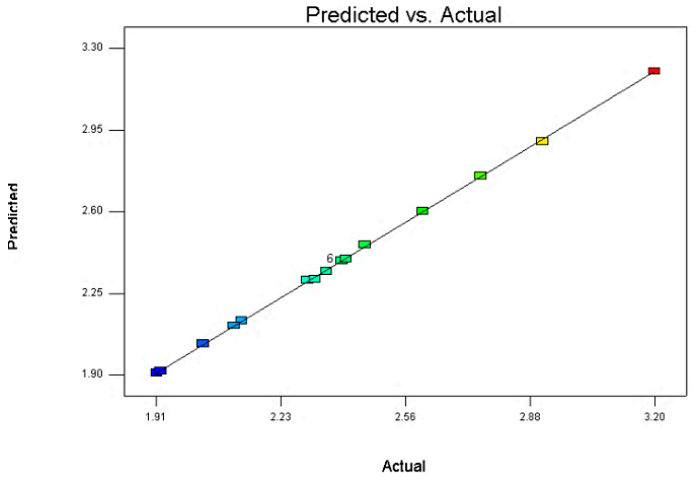


(TPC, III)



(TFC, IV)

Figure 1: The fitted line plot signifying the closeness between predicted values and experimental values for (DPPH, I), (FRAP, II), (TPC, III) and (TFC, IV)

3.2 Response surface analysis

Temperature, time and ethanol concentration are the main factors that affect the extraction condition of the maximum total antioxidant of DPPH, FRAP, total phenolic and flavonoids content. This section discusses how these conditions work on natural antioxidants extraction. Three dimensional (3D) model graphs were plotted as shown in the respective figures. The response surface plots of the model were done by varying two variables, within experimental range under investigation and holding the other variables at its central level (0 levels).

3.2.1 Free Radical Scavenging Activity, DPPH

The amount of extracted antioxidant of free radical scavenging activity (DPPH) content from *Phaleria macrocarpa* fruit extract ranged from 83.70 to 86.70 sample extract The values of mean recorded was 85.76 of total fruit extracts. The highest DPPH content was reported at experimental no.1 while the lowest DPPH content was observed at experiment no.4. The ANOVA showed the model *F*- value of 17783.61 with probability ($p<0.0001$) which implies that the model is significant and there is only 0.01% chances that this large *F* value could occur due to noise. Free radical scavenging activity was significantly influenced at ($p< 0.05$) by all three linear

(*A, B, C*), interaction parameters (*AB, AC, BC*) and quadratic parameters (A^2, B^2, C^2) as shown in Table 3. The effect of their variables and their interaction on the responses can be seen in Figure 2(A), (B) and (C). The interactions also give a significant effect on DPPH for each of the parameters. Ethanol concentration showed the most critical effect on the DPPH of *P.macrocarpa* fruit as it displayed the largest negative regression coefficient for linear effect and interaction effect with extraction temperature. The predicted model of the DPPH scavenging activity was obtained from the following second-order polynomial equation:

$$Y_{DPPH} = 85.80 - 0.45\ A - 1.00\ B + 0.63\ C - 0.080\ AB + 0.69\ AC + 1.01\ BC + 0.11\ A^2 + 0.15\ B^2 - 0.26\ C^2 \quad (3)$$

Effects of the two independent variables on DPPH, when the third variable is fixed in the middle level is shown by three dimensional (3D) response plots in Figure 2. Based on Figure 2(A), it is revealed the predicted response surface of the effect of temperature and time on DPPH at a constant ethanol concentration of 90% (v/v). There was a rapid increase in antioxidant activity of the compound extracted as the temperature increased from 60-80 °C and time from 80-120 minutes respectively. However, at the constant ethanol concentration at 80% (v/v), the antioxidant activity of DPPH declined at the time of 100 minutes with the temperature at 53.18°C and 70°C.According to Pinelo et al. (2005) state that high DPPH scavenging activity could be obtained by increasing the extraction time because it will improve the solubility of solute and increased the extraction coefficients.

DPPH also decreases with increasing ethanol concentration and temperature from 63.18-80% (v/v) and 53.18-70°C respectively at a constant time of 100 min as shown in Figure 2(B). The increases of DPPH antioxidant activity can only be selective when the extraction concentration increases to 80% (v/v) at lower temperature 60°C which indicate solvent polarity plays a vital role in extraction of antioxidant compounds. It was safer to use ethanol, less toxicity compared to other solvents such as methanol, acetone and others. Therefore, addition of appropriate amount of water to organic solvents increases its polarity which consequently increases the efficiency of antioxidant compounds extraction.

Furthermore, Figure 2(C) showed DPPH decrease when the temperature was kept constant at 70°C in the range of time of 100-133.64 min and ethanol concentration of 63.18-80% (v/v). In order to obtain high antioxidant of DPPH, the extraction temperature plays a more critical role in extend to extraction time (Yim et al. 2013). In this experiment, it revealed that as the temperature increase until 80°C the value of DPPH scavenging activity did not significantly increase as the time increased due to the decomposition of the anti-oxidative compound which is heat-sensitive.

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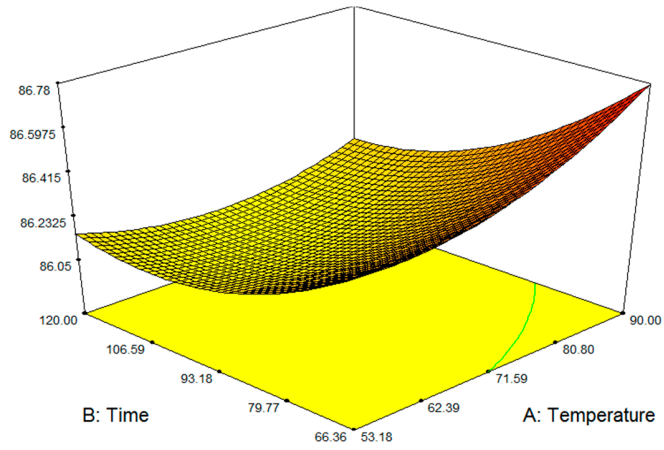
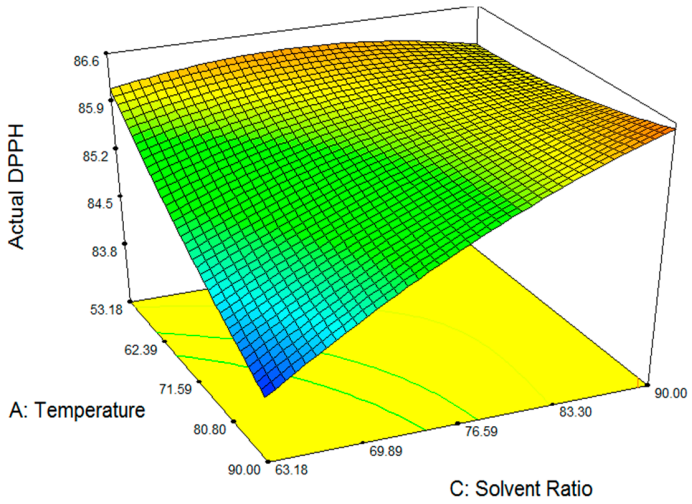
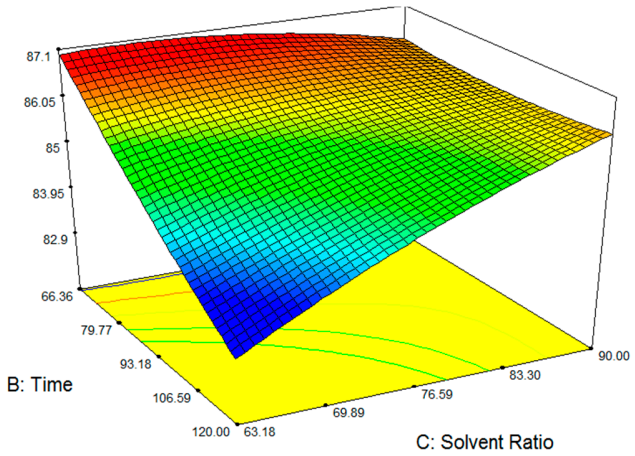


(A) DPPH Scavenging Activity (%)



(B) DPPH Scavenging Activity (%)



(C) DPPH Scavenging Activity (%)

Figure 2: Response surface analysis (3D) on the effect of extraction temperature, ethanol concentration and extraction time on DPPH of *P.macrocarpa* fruit extract

3.2.2 Ferric Ion Reducing Power Assay, FRAP

The amount of extracted antioxidant ferric ion reducing power assay, FRAP content from *Phaleria macrocarpa* fruit extract ranged from 7.12 to 7.47 samples extract. The value of mean recorded was 7.21 of total fruit extracts. The highest FRAP content was reported at experimental no.1 while the lowest FRAP content was observed at experiment no.4. The ANOVA showed the model *F*- value of 134.76 with probability ($p<0.0001$) which implies that the model is significant. There is only 0.01% chances that this large *F* value could occur due to noise. Ferric ion reducing power assay (FRAP) was significantly influenced at ($p< 0.05$) by all three linear (*A, B, C*), interaction parameters (*AB, AC, BC*) and quadratic parameters (A^2, B^2, C^2) as shown in Table 3. The effect of their variables and their interaction on the responses can be seen in Figure 3(A), (B) and (C). The interactions also give a significant effect on FRAP for each of the parameters. Temperature show the most critical effect on FRAP of *P.macrocarpa* fruit as it displayed the largest negative regression coefficient for linear effect and interaction effect with extraction temperature. The predicted model of the FRAP was obtained from the following second-order polynomial equation:

$$Y_{FRAP} = 7.16 - 0.069\ A - 0.11\ B - 0.083\ C + 0.052\ AB + 0.071\ AC + 0.14\ BC + 0.12\ A^2 + 0.085\ B^2 - 0.056\ C \quad (4)$$

Effects of the two independent variables on FRAP, when the third variable is fixed in the middle level is shown by three dimensional (3D) response plots. Based on Figure 3(A), it is revealed that the predicted response surface of the effect of temperature and time on FRAP at a constant ethanol concentration of 90% (v/v). There was a rapid increase in antioxidant activity of the compound extracted as the temperature increased up to 60-80 °C and time at 80-120 minutes respectively. However, at constant ethanol concentration of 70% (v/v), the antioxidant decreases as extraction time and extraction temperature increases. This revealed that antioxidant activity undergo heat degradation of the thermo-sensitive bioactive compounds, leading to reduce the antioxidant activity (Silva et al.2007). FRAP also showed decreases with increasing of ethanol concentration and temperature from 70-90% (v/v) and 60-80°C respectively at a constant time of 80 min as shown in Figure 3(B). The decreases of FRAP antioxidant activity indicates that solvent polarity plays a vital role in the extraction of antioxidant compounds. Methanol extract of sea buckthorn seed had higher reducing power than the extract using low polarity chloroform as reported by Negi et al. (2005).

Furthermore, in Figure 3(C) showed that FRAP showed fluctuated condition when the temperature was kept constant at 70°C in the range of extraction time of 66.36-133.64 min and extraction ethanol concentration of 63.18-80% (v/v). In order to obtain higher FRAP antioxidant activities, Turken et al. (2007) reported that polar aqueous solvents dissolve more polar plant polyphenols with higher reducing power at all different extraction time. The reducing power of antioxidants components associated with their total phenolic content. The plant extracts higher levels of total phenolic also exhibit greater reducing power (Cheng et al. 2006). It also had been reported that reducing properties are generally associated with the presence of reductones that been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Shadidi 2000). Moreover, reductones are efficient reducing agents and their efficiency can attribute to their hydrogen-donating ability (Duh 1998). The result of the reducing power reaction is to terminate the radical chain reactions that may otherwise be damaging (Yen and Chan 1995).

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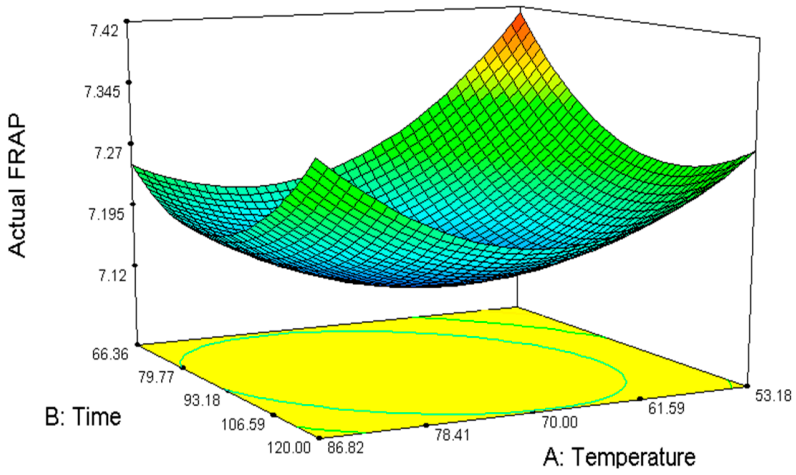
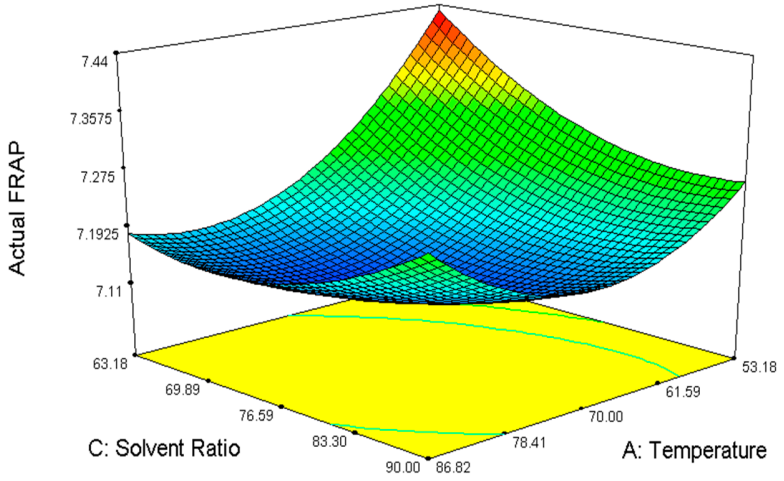
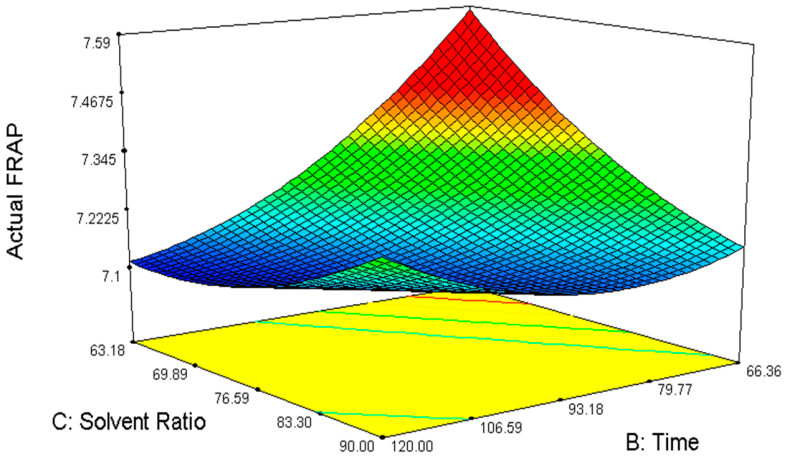


(A) Ferric Ion Reducing Power Assay, FRAP (%)



(B) Ferric Ion Reducing Power Assay, FRAP (%)



(C) Ferric Ion Reducing Power Assay, FRAP (%)

Figure 3: Response surface analysis (3D) on the effect of extraction temperature, ethanol concentration and extraction time on FRAP of *P.macrocarpa* fruit extract

3.2.3 Total Phenolic Content, TPC

The amount of extracted total phenolic content, TPC from *P.macrocarpa* fruit extract ranged from 186.22 to 292.86 samples extract, measured as gallic acid equivalent (GAE). The value of mean recorded was 228.65 mg/g GAE of total leaves extracts. The highest TPC content was reported at experimental no.11 while the lowest TPC content was observed at experiment no.13. The ANOVA showed the model *F*- value of 5.585E + 007 with probability ($p<0.0001$) which implies that the model is significant. There is only 0.01% chances that this large *F* value could occur due to noise. Total phenolic content, TPC was significantly influenced at ($p< 0.05$) by all three linear (*A, B, C*), interaction parameters (*AB, AC, BC*) and quadratic parameters (A^2, B^2, C^2) as shown in Table 3. The effect of their variables and their interaction on the responses can be seen in Figure 4(A), (B) and (C). The interactions also give a significant effect on TPC for each of the parameters. From the Table 3, both the linear and quadratic terms of all parameters were significant with at least $p<0.05$ on total phenolic content. The interactions also give a significant effect on TPC for each of the parameters. Ethanol concentrations show the most critical effect on the TPC of *P.macrocarpa* fruit as it displayed the largest positive regression coefficient for linear and interaction effect with extraction time. The predicted model of the TPC was obtained from the following second-order polynomial equation:

$$Y_{TPC} = 230.96 - 4.22\ A - 29.35\ B + 34.01\ C - 10.73\ AB + 39.58\ AC - 16.45\ BC - 30.62\ A^2 + 22.19\ B^2 - 12.67\ C^2 \quad (5)$$

Based on Figure 4(A), it is revealed the predicted response surface of the effect of temperature and time on TPC at a constant ethanol concentration of 90% (v/v). The phenolic compound stated the higher value at the range of time 80-120 minutes and temperature of 60-80˚C. As revealed by Cacace and Mazza (2003) higher solubility and diffusion coefficient of polyphenol when temperature increased which allowing more extraction rate. Moreover, increased in extraction temperature risings both solubility of solute and diffusion coefficient, but beyond a certain extend phenolic compounds could be decomposed. Compound stability could be affected due to chemical and enzymatic degradation or losses by thermal decomposition (Yim et al. 2013)

Nevertheless, TPC also showed increase with increasing of ethanol concentration and temperature from 70-90% (v/v) with 60°C and 80°C respectively at a constant time of 120 min as shown in Figure 4(B). Solvent polarity plays an important role in the extraction of antioxidant compounds. Ethanol was chosen because it is safer and less toxicity compared to other solvents such as methanol, acetone and others. It was reported that binary-solvent system demonstrated higher yield of phenolic compounds and flavonoid as compared to mono-solvent system which comprises of pure solvent or pure water only (Chirinos et al. 2007). In Figure 4(C), the highest TPC was attained when the temperature was kept constant at 60°C was in the range of time of 80-120 min and ethanol concentration of 70-90% (v/v). The phenolic content began to decrease as the extraction duration approached 120 min because it was believed that long extraction time increases the exposure to oxygen that leads to oxidation of antioxidant compounds (Naczk et al. 2004).

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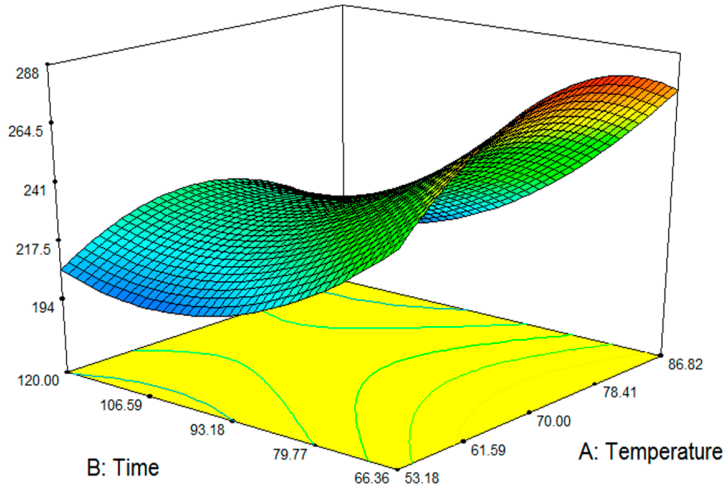
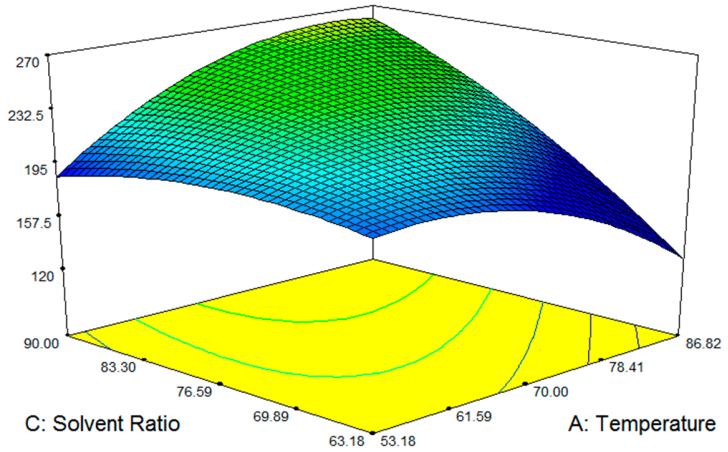
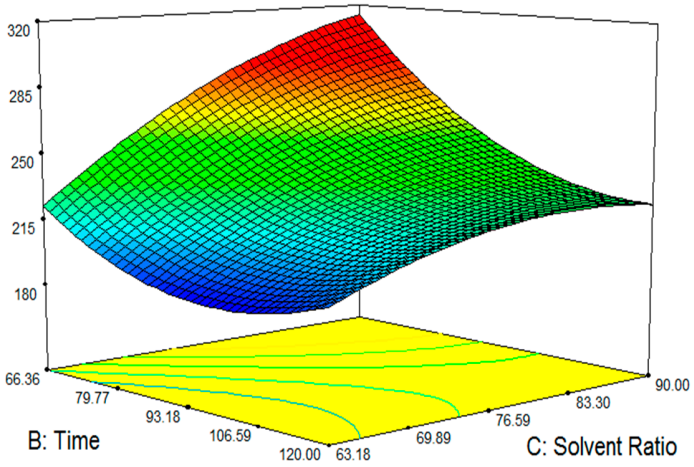


(A) Total Phenolic Content (mg/g)



(B) Total Phenolic Content (mg/g)



(C) Total Phenolic Content (mg/g)

Figure 4: Response surface (3D) on the effect of extraction temperature, ethanol concentration and extraction time on TPC of *P.macrocarpa* fruit extract

3.2.4 Total Flavonoid Content, TFC

The amount of extracted total flavonoid content, TFC from *P.macrocarpa* fruit extract ranged from 1.92 to 3.22 sample extract, measured as gallic acid equivalent (GAE). The value of mean recorded was 2.39 mg/g GAE of total leaves extracts. The highest TFC content was reported at experimental no.1 while the lowest TFC content was observed at experiment no.6. The ANOVA showed the model *F*- value of 5593.88 with probability ($p<0.0001$) which implies that the model is significant. There is only 0.01% chances that this large *F* value could occur due to noise. Total flavonoid content, TPC was significantly influenced at ($p< 0.05$) by all three linear (*A, B, C*), interaction parameters (*AB, AC, BC*) and quadratic parameters (A^2, B^2, C^2) as shown in Table 3. The effect of their variables and their interaction on the responses can be seen in Figure 5(A), (B) and (C). The interactions also give a significant effect on TFC for each of the parameters.

$$Y_{TFC} = 2.51 - 0.41A - 0.26B - 0.42C + 0.28\ AB + 0.10\ AC + 0.23\ BC - 0.28\ A^2 + 0.091\ B^2 + 0.049\ C^2 \quad (6)$$

Based on Figure 5(A), when the ethanol concentration was fixed at 80% (v/v), the flavonoid content was decreased as the temperature increased from 53.18˚C to 70˚C at the range of time 66.36 min to 133.64 min. At higher temperature it will decrease the fluid density which leads to reduce the extraction efficiency (He et al. 2005). However, at the constant ethanol concentration at 70% (v/v), with the suitable temperature of 60˚C and time of 80 minutes the flavonoid content increased. It may be the greater speed of the molecule movements in higher temperature can cause flavonoid diffusion more quickly from cell to extracting agent (Kumar et al. 2008). However, an upper limit of temperature must be controlled to prevent the decomposition of thermo-sensitive compounds in particular flavonoid (Silva et al. 2007).

Figure 5(B) of 3D response surface plot showed at varying temperature and ethanol concentration while the extraction time kept constant at 80 min. It can be seen that the maximum flavonoid of fruit extracts *P.macrocarpa* was attained around the range of temperature and ethanol concentration of 60-80°C and 70-90% (v/v) respectively. According to Kumar et al. (2008) also, the optimal extraction yield can be fulfilled when the polarity of the fluid and its flavonoid are coincident. Moreover, TFC increased with the concentration of ethanol 55-75% or above 75%. Flavonoid content was decreased in leaves of *Tabernaemontana heyneana Wall.* Ethanol interacts with the flavonoid possibly through non-covalent interactions and promotes a rapid diffusion into the solution (Luque de Castro and Tena 1996). Various concentration of ethanol used exhibited different effect in changing the fluid polarity. Hence, it effect on the solubility enhancement of the flavonoid (He et al. 2005).

While, in Figure 5(c) showed the relationship between ethanol concentration and extraction time at a constant temperature of 70°C. The flavonoid content was observed to be positively influenced by the synergism between the ethanol concentration and extraction time. Fruit extracts of *P.macrocarpa* displayed maximum flavonoid content of 3.22 mg of quercetin/g of extract at ethanol concentration of 140% ethanol and 60% water while the extraction time was 80 minutes.

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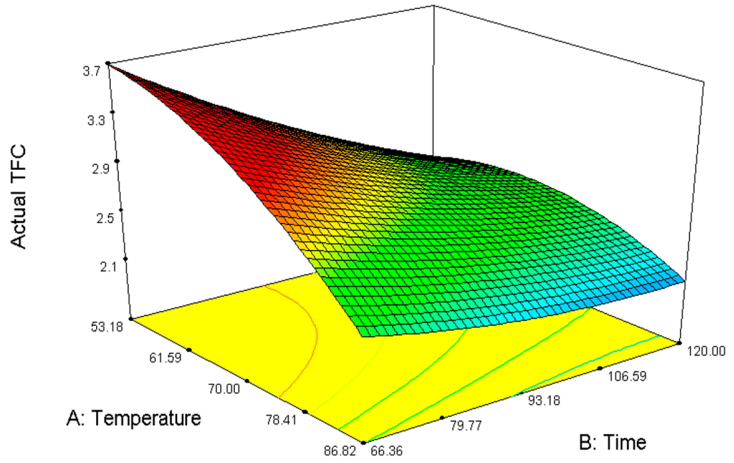
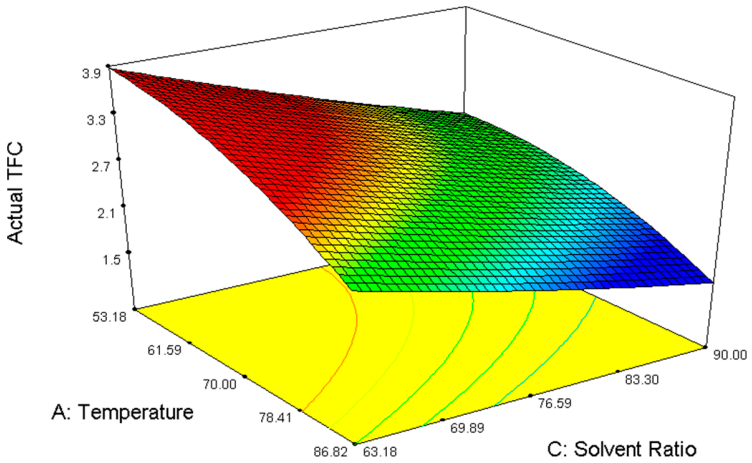
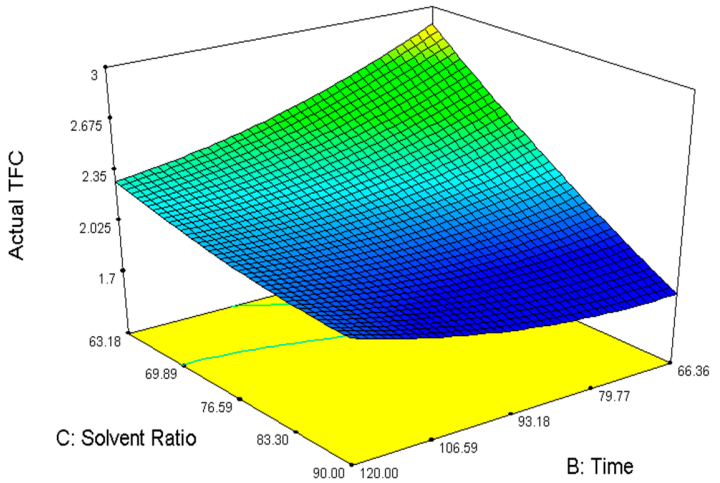


(A) Total Flavonoid Content (mg/g)



(B) Total Flavonoid Content (mg/g)



(C) Total Flavonoid Content (mg/g)

Figure 5: Response surface (3D) on the effect of extraction temperature, ethanol concentration and extraction time on TFC of *P.macrocarpa* fruit

3.3 GCMS Analysis

The compounds composition of *P.macrocarpa* fruit extract is very important for further analysis. Table 4 and Figure 6 shows the compounds constituents in *P.macrocarpa* extracted using ethanol. From the analysis obtained, seventeen were identified. The mass spectrum of the compounds was compared with the NIST08 mass spectral database for identification of compounds. The library generally will offer three possible matches of compounds through comparison with the ion spectrum obtained from the GC-MS. Only compounds with similarity index (SI) of ≥80 were chosen as a possible match with the unknown compounds of the fractions (Information, Advanced, & Handling, n.d.). The presence of saturated fatty acid such as palmitic acid (Lay et al., 2014) which one of the alkaloids compounds are strongly recommended to have anticancer activity (Tsuda et al., 2004) that can be found in the fruit and leaves extract of *P.macrocarpa.* There is also reported presence of benzophenone from the isolation of ethanol extract (Singh & Ghanapriya, 2014).

Table 4: Composition compound of ethanol extract *P.macrocarpa* fruit as determined by gas chromatography–mass spectroscopy

Retention Time (RT)	Area (%)	Height (%)	Compounds
5.228	0.23	0.37	1,3-diethoxy-1,1,3,3-tetramethyldisiloxane
5.407	0.01	0.03	m-Ethyltoluene
5.454	0.77	0.74	Benzaldehyde
5.586	1.08	0.72	Ethyl orthosilicate
5.722	1.10	1.09	β-myrcene
5.835	0.10	0.11	Pseudocumene
6.118	0.08	0.10	p-cymene
7.033	0.01	0.02	Isodurene
7.286	0.06	0.08	Camphor
7.413	0.03	0.03	Benzoic acid
7.687	0.71	0.63	Napthalene
8.190	0.11	0.12	Nonanoic acid
9.756	0.32	0.35	2,4-di-tert-butylphenol
10.547	2.01	1.79	Spathulenol
10.658	1.53	0.97	Neocurdione
12.089	2.24	1.07	Palmitic acid
13.798	0.31	0.34	Bisoflex DOA

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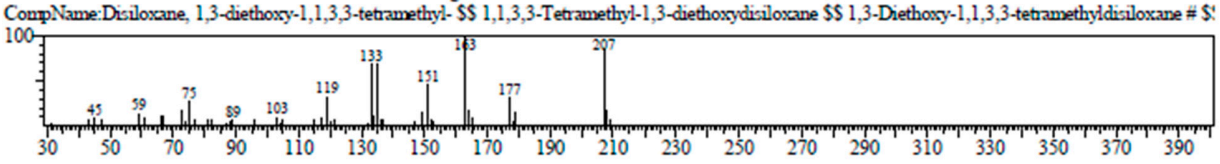
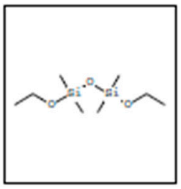
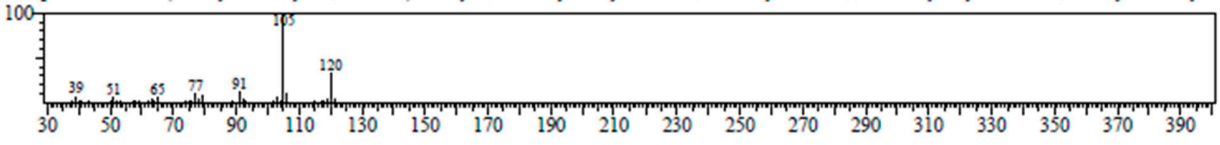
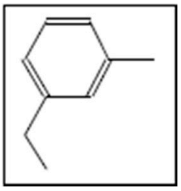
SI:90 Formula:C8H22O3Si2 CAS:18420-09-2 MolWeight:222 RetIndex:864
CompName:Disiloxane, 1,3-diethoxy-1,1,3,3-tetramethyl- $$ 1,1,3,3-Tetramethyl-1,3-diethoxydisiloxane $$ 1,3-Diethoxy-1,1,3,3-tetramethyldisiloxane # $:



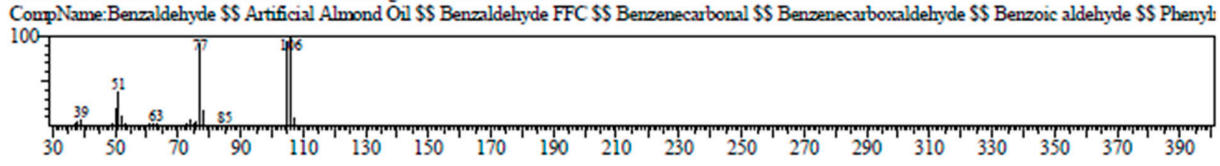


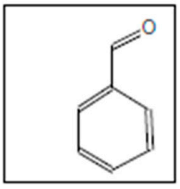
SI:85 Formula:C9H12 CAS:620-14-4 MolWeight:120 RetIndex:1006
CompName:Benzene, 1-ethyl-3-methyl- $$ Toluene, m-ethyl- $$ m-Ethylmethylbenzene $$ m-Ethyltoluene $$ m-Methylethylbenzene $$ 1-Ethyl-3-methylb



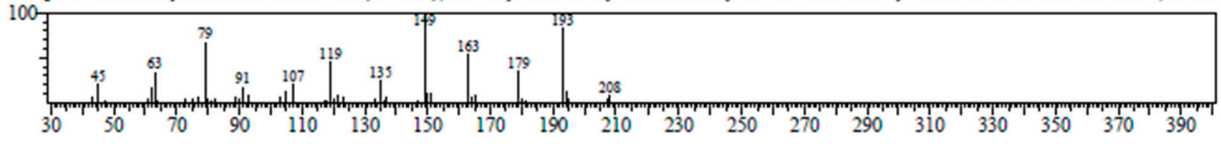


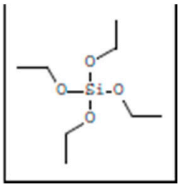
SI:98 Formula:C7H6O CAS:100-52-7 MolWeight:106 RetIndex:982
CompName:Benzaldehyde $$ Artificial Almond Oil $$ Benzaldehyde FFC $$ Benzenecarbonal $$ Benzenecarboxaldehyde $$ Benzoic aldehyde $$ Phenyl



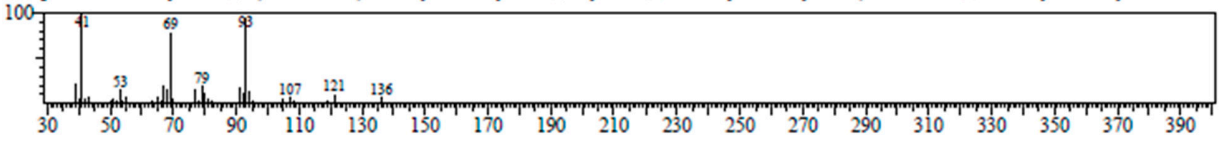


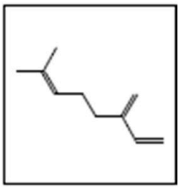
SI:93 Formula:C8H20O4Si CAS:78-10-4 MolWeight:208 RetIndex:1030
CompName:Tetraethyl silicate $$ Silicic acid (H4SiO4), tetraethyl ester $$ Dynasil A $$ Ethyl orthosilicate $$ Ethyl silicat $$ ES 28 Ester $$ Silane, tetraet



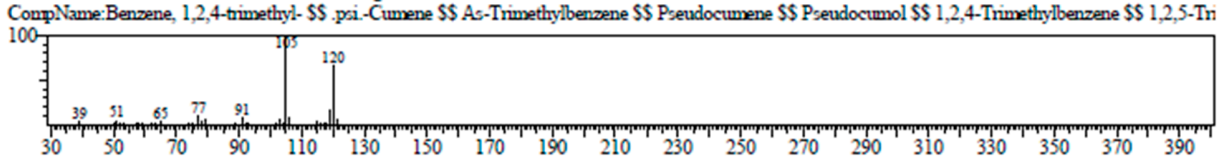


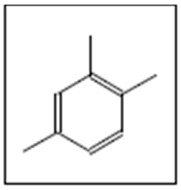
SI:94 Formula:C10H16 CAS:123-35-3 MolWeight:136 RetIndex:958
CompName:.beta.-Myrcene $$ 1,6-Octadiene, 7-methyl-3-methylene- $$ Myrcene $$ 7-Methyl-3-methylene-1,6-octadiene $$ 7-Methyl-3-methyleneoctadie



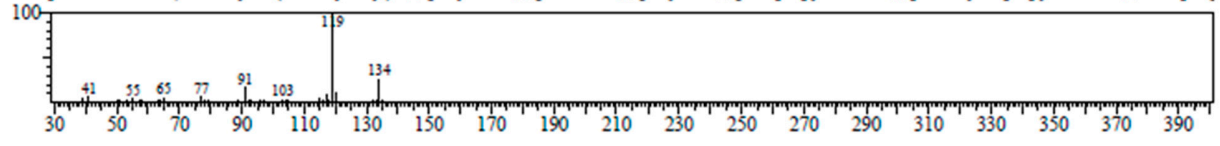


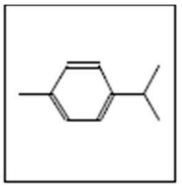
SI:87 Formula:C9H12 CAS:95-63-6 MolWeight:120 RetIndex:1020
CompName:Benzene, 1,2,4-trimethyl- $$ .psi.-Cumene $$ As-Trimethylbenzene $$ Pseudocumene $$ Pseudocumol $$ 1,2,4-Trimethylbenzene $$ 1,2,5-Tri



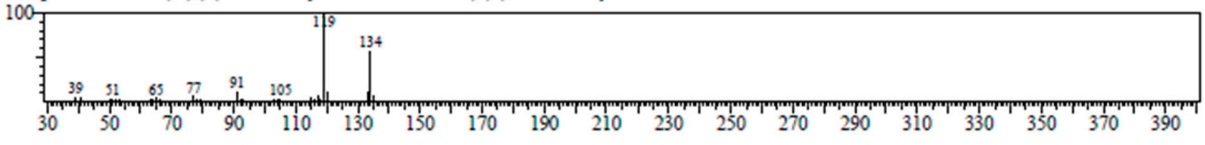


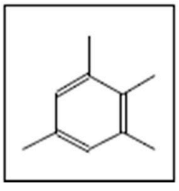
SI:80 Formula:C10H14 CAS:99-87-6 MolWeight:134 RetIndex:1042
CompName:Benzene, 1-methyl-4-(1-methylethyl)- $$ p-Cymene $$ p-Cimene $$ p-Cymol $$ p-Isopropyltoluene $$ p-Methylisopropylbenzene $$ Campho



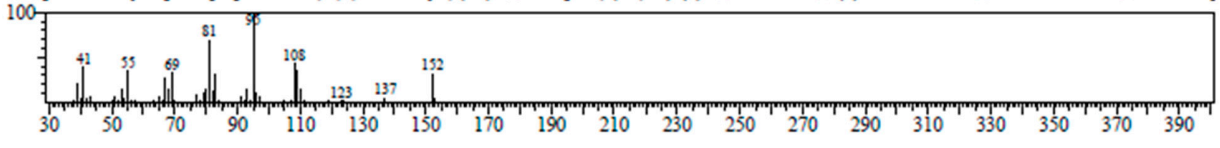


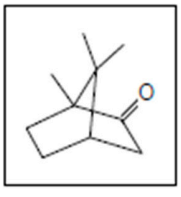
SI:80 Formula:C10H14 CAS:527-53-7 MolWeight:134 RetIndex:1133
CompName:Benzene, 1,2,3,5-tetramethyl- $$ Isodurene $$ 1,2,3,5-Tetramethylbenzene $$





SI:81 Formula:C10H16O CAS:464-49-3 MolWeight:152 RetIndex:1121
CompName:Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)- $$ Camphor, (1R,4R)-(+)- $$ Alcanfor $$ (+)-2-Bornanone $$ d-2-Bornanone $$ d-2-Camp





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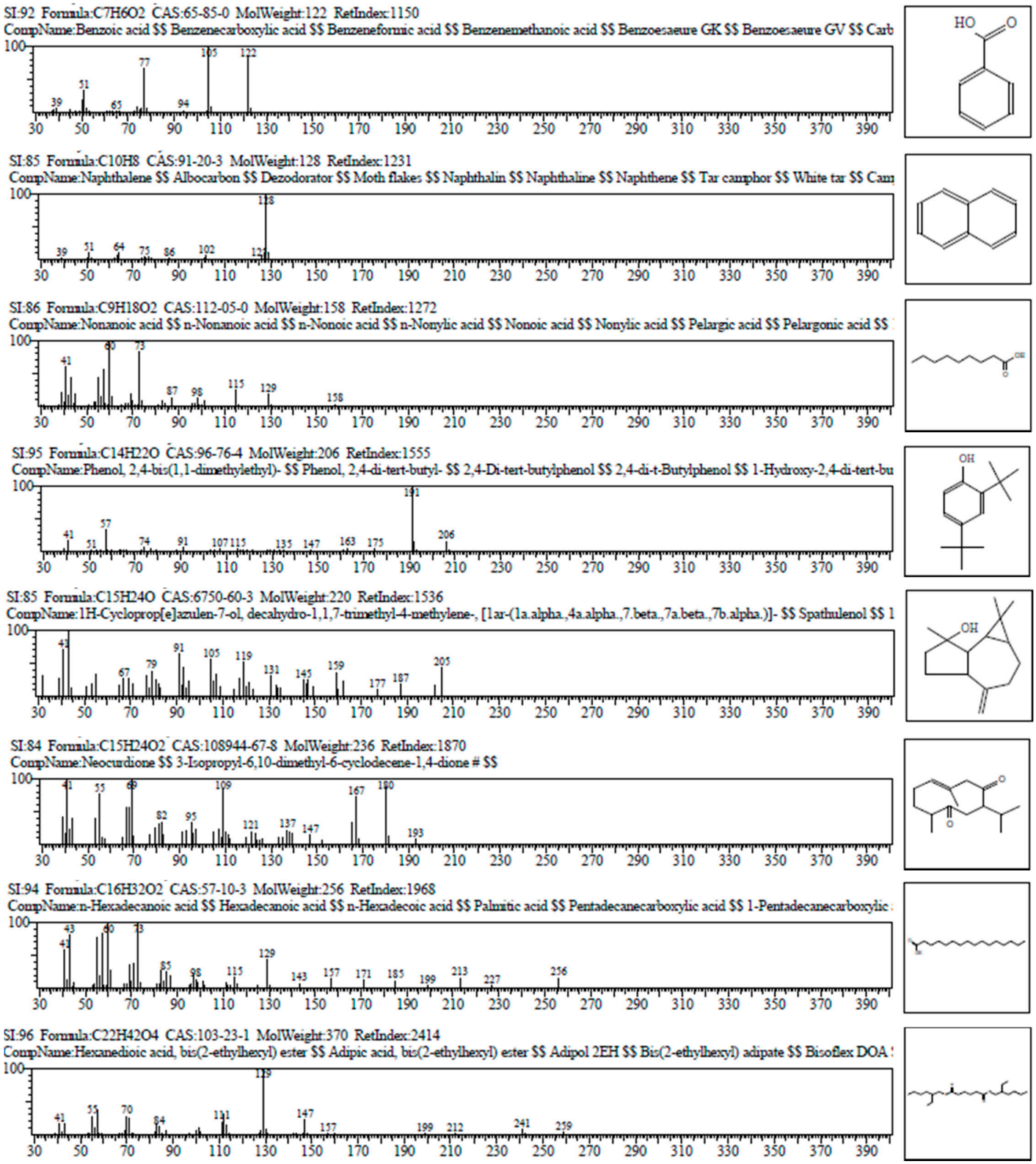

Figure 6: Mass spectra of compound in *P.macrocarpa* fruit of ethanol extract

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3.4 FTIR Analysis

The spectral features of *P.macrocarpa* fruit extract was displayed in Figure 7. The absorption peak at 3290.21 cm^{-1} is due to presence of hydroxyl group of H bonded O-H stretch. The absorption peak at 2922.46 cm^{-1} is due to saturated aliphatic group of methylene C-H asymmetric (>CH_2). Moreover, the aromatic ring stretch was presence at the absorption peak 1604.12 cm^{-1} (C=C=C^a). The 1272.34 cm^{-1} and 1046.30 cm^{-1} were indicated the presence of aromatic amino group of primary amine, C-N stretch and hetero-oxy for silicon-oxy compounds, respectively (Coates, 2000).

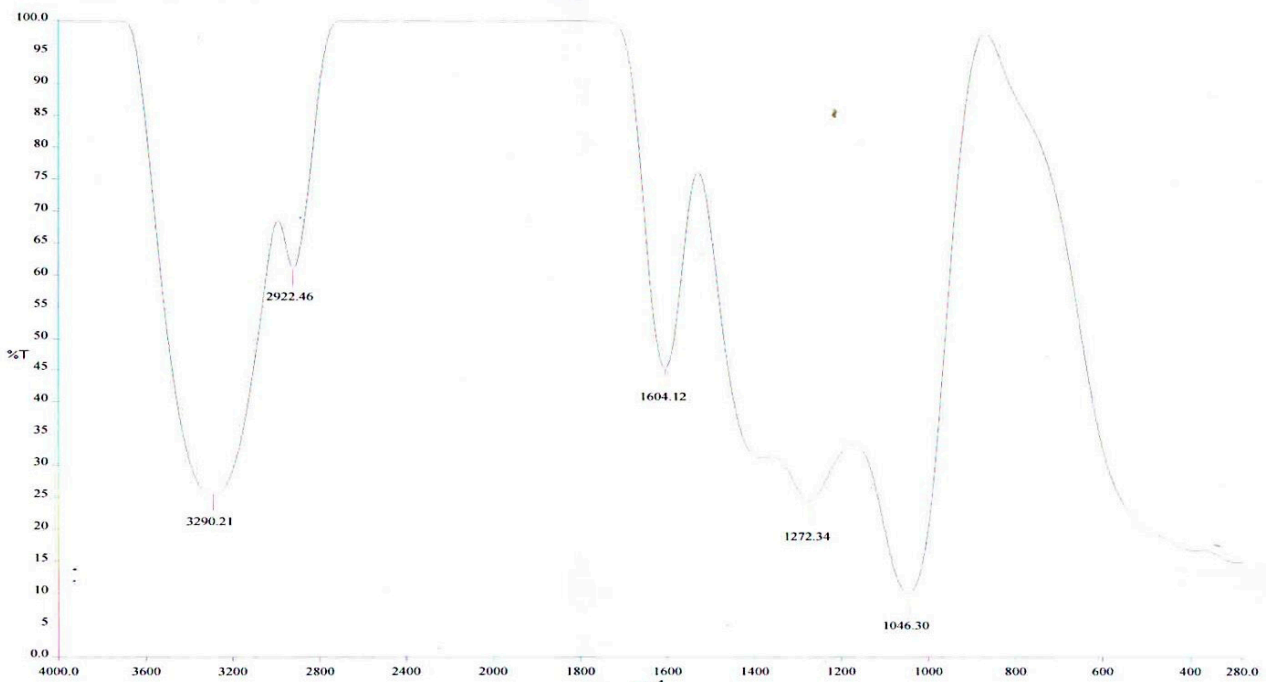

Figure 7: FT-IR of ethanol fruit extracted *P.macrocarpa*

3.5 Optimization of extraction conditions

The main objective of response surface methodology in this research to investigate the levels of experimental factors which give the highest antioxidant activity on the crude extracts. Four individual verification experiments for free radical scavenging activity (DPPH), ferric ion reducing power assay (FRAP), total phenolic content (TPC) and total flavonoid content (TFC) were carried out under respective optimal extraction time, extraction temperature and extraction solvent to-feed ratio within the experimental range. The final result for the simultaneous optimization using the desirability function approach suggested that the optimal ethanolic extraction conditions for *P.macrocarpa* fruit extract were 66.36 minutes, 64.10°C temperature and 74.59% v/v solvent ratio with desirability of 94%. These optimum extraction conditions were evaluated by considering the simultaneous response surface and contour plot from the interaction between the independent variables and responses of interest as shown in Figure 7. The value of the missing independent variable in plot was kept at the center point. In order to verify the optimum condition, the *P. macrocarpa* fruit was subjected using the optimal condition above and the results were statistically compared to the predicted values given by the design expert 7.0.0 software of the response surface methodological (RSM) model.

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Based on the results, the predicted values of responses were found to be quite comparable with experimental values at 94% confidence level in Table 4. It showed the suitability of the model equation for the prediction of maximum responses was verified using respective responses optimal condition. When constraint in the range were selected, the optimum conditions obtained were. However, practically it is difficult to maintain the conditions during processing and some deviations were expected. Therefore, optimum conditions were targeted as 66 minutes, 64°C temperature and 75% v/v solvent ratio.

Table 5: Optimized level (in the range), optimum level (targeted), predicted optimum value and experimental value of DPPH, FRAP, TPC and TFC

		Optimum value (In range)	Optimum value (Targeted)
Variables	Time	66.36 minutes	66 minutes
	Temperature	64.10 ˚C	64˚C
	Solvent to-feed ratio	74.59 % (v/v)	75 % (v/v)
		Predicted value	Experimental value
Responses	DPPH	86.84 %	86.85%
	FRAP	7.47 %	7.47%
	TPC	292.88 mg/g	292.86 mg/g
	TFC	3.22 mg/g	3.22 mg/g

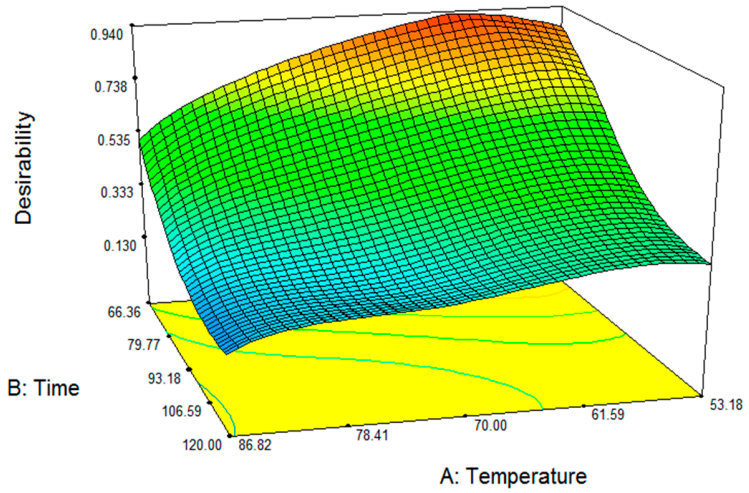

Figure 7: Response surface for desirability, in function of temperature and time

4. Conclusion

In this research, response surface methodology (RSM) employing central composite design (CCD) was successfully used to determine the optimal conditions for the extractions of antioxidants crude extract from *P.macrocarpa* fruits. The results from RSM proved the extraction of phenolic, flavonoid and antioxidant activities are significantly affected by the factors chosen from the preliminary study. The optimal conditions for the nutraceutical extraction of *P.macrocarpa* fruits were found to be extraction temperature 64°C, extraction time 66 minutes and extraction solvent ratio 75% v/v. Under the optimum conditions, the experimental values of DPPH, FRAP, TPC and TFC were in agreement with those predicted. Therefore indicating suitability of the model employed.

This study suggested the models obtained can be utilized to optimize the extraction time, temperature and solvent ratio for the maximum yield of antioxidants activities. In future research work, the good optimized result obtained from RSM can be further used for the extracted fruit of *P.macrocarpa* in cosmeceuitcal properties such as investigating the physical characterization of the total carotenoid, total anthocyanin, total ascorbic acid, the *in-vitro* sun protection factor (SPF), anti-tyrosinase, anti-collagenase and anti-elastase.

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6. Competing Interest

The authors declare that they have no competing interest regarding the publication of this paper.

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