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OPTIMIZATION OF SOXHLET EXTRACTION PARAMETER OF *Annona muricata* LEAVES USING BOX-BEHNKEN DESIGN (BBD) EXPERT AND ANTIOXIDANT ANALYSIS

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Graphical abstract

Abstract

Response surface methodology (RSM) was employed to optimize the extraction of herbal plant of *Annona muricata* L. using Box-Behnken Design (BBD). The Box-Behnken Design (BBD) with three factors and two responses (yield and total solid content) was implemented. The processing parameters of *A. muricata* leaves by soxhlet extraction were solvent to raw material ratio, ethanol concentration, and duration of extraction using soxhlet extractor. The presence of acetogenins compounds was screened by High Performance Liquid Chromatography. Optimum condition with the solvent to raw material ratio (1:5.92), ethanol concentration (1.67 %), and duration of extraction (6.84 hours) was obtained and further analyzed with antioxidant tests. Analyses showed the *A. muricata* leaves contained high total phenolic and flavonoid contents which corresponding to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test. The DPPH result showed that the extract has the highest percentage of scavenging activity with the inhibitory concentration (IC_{50}) value of leaves is 0.243 mg/mL $\pm$ 0.001 nearly comparable to butylated hydroxyanisole (BHA) and ascorbic acid (Vit. C), which indicated that the leaves also have higher free radical scavenging activity compared to these commercial standards. Besides, the presence of primary; polysacharide (21.85 % $\pm$ 0.001), protein (19.59 % $\pm$ 0.000), glycosaponin (18.87 % $\pm$ 0.001) and secondary; phenolic (47.26 mg GAE/1g $\pm$ 0.001), flavonoid (22.12 mg CE/1g $\pm$ 0.001) metabolites in *A. muricata* leaves suggested that the leaves contained strong antioxidant properties that are believed can act as anticancer agent for the body.

Keywords: *Annona muricata,* Soxhlet extraction, HPLC, Box-Behnken design

Abstrak

Response Surface Methodology (RSM) digunakan untuk mengoptimumkan pengekstrakan tumbuhan herba *Annona muricata L.* menggunakan *Box-Behnken Design (BBD). Box-Behnken Design (BBD) expert* dengan tiga faktor dan dua tindak balas (hasil dan jumlah kandungan pepejal) telah dilakukan. Pemprosesan pengekstrakan daun *A. muricata* melibatkan nisbah pelarut dengan bahan mentah, kepekatan etanol, dan tempoh pengekstrakan menggunakan pengekstrak *Soxhlet.* Kehadiran sebatian asetogenin telah dikesan oleh Kromatografi Cecair Tekanan Tinggi (HPLC). Keadaan optimum dengan nisbah pelarut kepada bahan mentah (1: 5.92), kepekatan etanol (1.67%), dan tempoh pengekstrakan (6.84 jam) telah diperolehi dan seterusnya dianalisis dengan ujian antioksidan. Analisis menunjukkan daun *A. muricata* mengandungi jumlah fenolik dan

flavonoid yang tinggi bertepatan dengan ujian aktiviti radikal 1,1-difenil-2-pikrilhidrazil (DPPH). Hasil DPPH menunjukkan bahawa ekstrak yang mempunyai peratusan aktiviti tertinggi memerangkap dengan nilai kepekatan perencatan (IC_{50}) daun 0.243 mg/mL ± 0.001 hampir setanding dengan hidroksianisol terbutil (BHA) dan asid askorbik (Vit. C), yang menunjukkan bahawa daun juga mempunyai aktiviti radikal bebas yang lebih tinggi berbanding dengan piawaian komersial. Selain itu, kehadiran metabolit primer; polisakarida (21.85% ± 0.001), protein (19,59% ± 0.000), glikosaponin (18.87% ± 0.001) dan metabolit sekunder; fenolik (47,26 mg GAE / 1 g ± 0.001), flavonoid (22.12 mg CE / 1 g ± 0.001) dalam daun *A. muricata* menunjukkan daun mempunyai sifat antioksidan yang tinggi yang dipercayai boleh bertindak sebagai agen anti kanser untuk badan.

Kata kunci: Annona muricata, pengekstrakan Soxhlet, HPLC, Box-Behnken design

1.0 INTRODUCTION

Herbs and spices have been valuable sources of natural products for maintaining human health for many years; more than 35,000 plants around the world are consumed by human for medical purposes [1]. Since the ancient time, the leaves of *Annona muricata* L (Annonaceae) have been used as herbal remedies in treating diabetes, hypertension, fever, vomiting and against worm [2]. Other than that, it also has been used in treating headaches, cough, asthma and as a sedative [3-5].

Cancer is characterized by the alteration in the expression of multiple genes, leading to dysregulation of the normal cellular program for cell proliferation and cell differentiation. It is notable that imbalance of cell proliferation and cell death will lead cancer cell to invade a population of cells and ultimately metastasize to distant sites, causing significant morbidity and, if untreated, death of the host [6].

In term of cytotoxic study, the previous study indicated that the ethanol extract of *A. muricata* leaves have cytotoxic effects on breast and cervical cancer with IC_{50} 17.1 and 97 µg/mL, respectively [7,8]. Meanwhile, the aqueous extract of *A. muricata* leaves also showed cytotoxic effects on pancreatic cancer by inhibit pancreatic oxidative damage produced by streptozotocin [9]. There are two effective methods mostly applied in extracting *A. muricata* leaves including traditional and conventional method which are boiling, maceration and soaking, soxhlet extraction and supercritical fluid extraction (SFE).

Annonaceous acetogenins is a series of polyketide derived fatty acid compound which composed of 12,15-Cis-squamostatin-A, squamostatin-A, bullatacin, squamostatin-D, squamocin, isodesacetyluvaricin, asiminecin, and desacetyluvaricin [10]. Figure 1 shows bullatacin, one of the acetogenins compound that is believed to have anticancer properties. The increasing importance of these compounds as potent ingredient in pharmaceutical anticancer supplements besides their traditional role in treating inflammation, but also as antioxidant donor has opened up wide opportunities for global marketing. With the endless impressive medicinal properties of *acetogenins*, they are truly the spice of life that yet needs to be discovered and investigated further in the research field.

Antioxidants properties in *acetogenins* also have numerous benefits to the human body. As it combats the free radical it actually protects the cells from oxidative damage that leads to aging and other diseases. Besides, it can improve blood vessel function by preventing injury to the blood vessel membranes thus, optimizing the blood flow to the heart and brain [11]. Moreover, antioxidant may help the cells defend against cancer-causing DNA damage that usually will lead to uncontrolled cell growth by decrease the proliferation rates of the cell [12].

The soxhlet extractor has been used for years in extracting herbal plants. The extraction process using soxhlet extraction technique is one of the methods to extract the compound that has poor solubility in a solvent which the impurity is insoluble in that solvent. The choosing of the soxhlet extractor as standard extraction method in this study was based on its advantages which include; environmental friendly, lower operating cost and all parameters that were involved in the extraction process were easy to control and manipulate.

The extraction parameters were optimized using response surface methodology (RSM) in order to determine the best operation conditions to extract the nonpolar compounds from *A. muricata* leaves. Traditionally, folk people consumed *Annona muricata* leaves as herbal tea in treating pancreatic cancer. However, not much study has been performed on this herb in Malaysia especially in the optimization of the extraction. According to the usage for medical fields, *A. muricata* leaves is extremely needed due to the finding of *annonaceous acetogenins* compound which is proven beneficial in treating health problems.

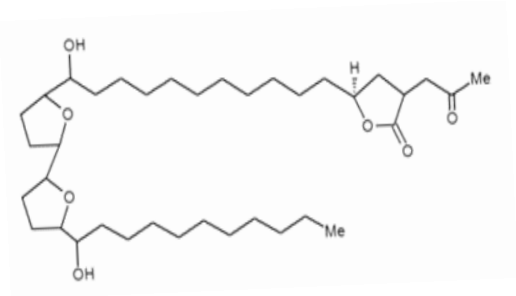

Figure 1 Chemical structure of Bullatacin

2.0 EXPERIMENTAL

2.1 Plant Materials

The ground-dried leaves of *A. muricata* were purchased from Toko Tenaga Keluarga Sdn. Bhd, Kota Tinggi, Johor, Malaysia and identified by botanist, Dr Shamsul Khamis from Institute of Bioscience, Universiti Putra Malaysia (UPM) and the voucher specimen numbers of plant samples were deposited in the Herbarium, Biodiversity Unit, Institute of Bioscience, UPM, Serdang, Selangor, Malaysia. The distilled water and Ethanol approx. 95% from QREC was used as a solvent extractor.

2.2 Extraction

The dried leaves were extracted in a soxhlet by using different ratio of solvent to raw material (1:1, 1:4, 1:6), with different ethanol concentration (0, 5, 10 %), and extraction time (1, 4, 8 hr). The extracts were filtered using Whatman No. 1 filter paper. The filtrate was then evaporated to dryness using rotary evaporator (Buchi Evaporator, Heidolph, Germany) at 50°C. The dried crude extract was obtained from oven drying method at 40°C (Mermet). The samples were stored in a freezer of (-20) ° C for further analysis. The yield of *Annona muricata* leaves extract and total solid content obtained was calculated based on equation below:

$$\text{Yield of extract (\% w/w)} = \frac{\text{g of solid content}}{\text{g of raw material}} \times 100\ \% \quad (1)$$

$$\text{Total solid content (mg/g)} = \frac{\text{g of dried sample + petri dish}}{\text{g of sample as received}} \quad (2)$$

2.3 Experimental Design and Statistical Analysis

In the optimization process, three operating parameters of soxhlet extraction have been optimized by Response Surface Methodology (RSM) version 6.0.8. It is a software tool for experiment design and analysis (DOE). An experimental design with 17 experimental runs was performed using three factorial variables (Table 1)to optimize the extraction of *Annona muricata* leaves.

The Box-Behnken design was employed to obtain high extract yield and total solid content. The soxhlet extraction is chosen prior to the form of active compound, *acetogenin* found in the leaves. Design Expert software version 6.0 was utilized to carry out the regression analysis and to analyze the points of the data which was obtained from the preliminary experiment. The BBD technique was applied to select the optimum conditions of the three extraction factors in different experiments. The low, middle, and high levels for all the independent variables are shown in Table 1. Each variable to be optimized was coded using coding scheme to denote the level of the factor at three levels. The -1 was indicated as lower level, 0 as medium level, meanwhile +1 show as the higher level. Five replicates at the center of the design were executed to allow the estimation of the pure error.

Table 1 Processing Parameters of the *A. muricata* Leaves Extraction Process

Factor	Factor name	Factor levels		
		(-1)	(0)	(+1)
A	Solvent to raw material ratio (g/g)	1:1	1:3.5	1:6
B	Concentration of ethanol solvent (%)	0	5	10
C	Duration of extraction (hour)	1	4	8

A full quadratic polynomial regression model was expected to express for the presumed responses. All experiments were carried out in a randomized order to minimize the effect of unexpected variability in the observed response due to extraneous matter [13]. The model proposed for each response of Y was:

$$Y = A + B + C + A^2 + B^2 + C^2 + AB + AC + BC \quad (3)$$

where, Y is the presumed response variables; yield of extract or total solid content. The proposed model equation predicts the response as a function of the different levels of independent variables; A, B, C. The significance of each coefficient of the resulted model was determined by using the F-test and p-value.

The Analysis of Variance (ANOVA), the coefficient of multiples determination, and R^2 was applied to analyze the adequacy of the quadratic polynomial model for the determination of the significance of the model. Besides, the relationship between response and experimental levels of each factor can be visualized and the optimum condition of the

process can be deduced through the quadratic equation. The fitted quadratic polynomial equation can be presented as the surface plot or contour plot [14].

2.4 HPLC-PDA Screening

Acetogenin qualitative analysis was carried out using HPLC with operating conditions that matched the previous study with slightly modification [10]. A water HPLC system (Milford, MA, USA) consisting of double pump and system controller (Model 2695), an auto-sampler and photo-diode array detector (Model 966) was used. The column configuration consisted of a reversed phase column (4.6 × 150 mm, 4 µm; Phenomemex, Torrance, CA, USA). Detection wavelength was set at 220 nm. The mobile phase consisted of A (acetonitirile) and B (deionized water), using a linear gradient: 0-40 min (85%), and 40-60 min (85-95% A). The flow rate was 1.0 ml/min. The column temperature was maintained at 30°C.

2.5 Total Phenolic and Flavonoids Content

Folin-Ciocalteau method was used for investigation of total phenolic content with a slight modification from [15]. The extracts were mixed together with Folin-Ciocalteau reagent (FCR). Sodium carbonate solution was added to the mixture after 3 minutes. Then, the mixture was allowed to stand in a dark for 1 hour at room temperature and the absorbance of the samples was taken at 725 nm. Gallic acid was used as a standard to obtain the standard calibration curve. Total phenol content was obtainedfrom the regression equation and expressed as grams of Gallic acid equivalent (g GAE) per 1g of extract (g extract).

The colorimetric assay method was used in total flavonoid content test with a slight modification [16]. Sodium nitride was added to the extracts. After 5 minutes, Aluminium chloride was added, followed by sodium hydroxide after 1 minute of reaction. In this test, the absorbance of the samples was measured at 510 nm and catechin was used as the standard to measure the flavonoid activity present in the sample. Total flavonoid content was obtained from the regression equation. Catechin was used as the basis of standard curve and the results was showed as gram of catechin equivalent per 1 g (g CE/1g) of extract.

2.6 DPPH Assay

The radical scavenging activity of samples was determined using method by the 2, 2, diphenyl-2-picryl-hydrazyl (DPPH) methods with slightly modification [17]. Each sample was prepared in a series of dilution 1 to 0.0078 mg/mL with 70 % ethanol. 0.5 mL of each sample was added into 1 mL of 0.1 mM DPPH reagents and mixed thoroughly. After 30 minutes of incubation at room temperature in the dark, the absorbance was measured against blank of 70 % ethanol at 517 nm using visible spectrophotometer. Standard free radical scavenger, ascorbic acid (vitamin C) was used as positive controls under the same assay conditions. Meanwhile, the negative control was prepared with 2 mL of 0.1 mM DPPH reagent and 1 mL of 70 % ethanol without extracts or standard. At the end of experiment, the result was expressed in mean average and the free radical scavenging activity was calculated according to the following equation by using Excel software:

$$\% \text{ Inhibition} = \frac{\text{A control - A sample}}{\text{A control}} \times 100\ \% \quad (4)$$

2.7 Total Polysaccharide Measurement

Total polysaccharide content in *Annona muricata* leaves was determined by a phenol-sulfuric acid colorimetric assay [18]. 1 mL of extract was combined with 4 mL of 99.5 % ethanol in a plastic tube. The mixture was incubated in an ice bath for one hour. After one hour, the mixture was centrifuged at 13000 rpm for 10 minutes. The supernatant was removed without disturbing the pellet. The pellet was washed twice with 4 mL of 99.5 %. The pellet was air-dried and dissolved in a final volume of 3 mL distilled water. 1 mL of sample was transferred into 1 mL of 4 % (w/v) phenol. 5 mL of concentrated sulphuric acid on ice was added into the mixture. The reaction mixture was kept constant temperature at 80°C in water bath. After cooling to room temperature, the absorbance of the reaction mixture was determined at 492 nm of absorbance using UV-Vis Spectrophotometer. Glucose solution was used as a standard to determine the total polysaccharide content.

2.8 Total Protein Measurement

To determine the total protein in *Annona muricata* leaves extract, Lowry's method was applied for this analysis [19], [20]. The lowry solution was prepared fresh at the day of measurement. Lowry's solution was prepared by mixing solution A (alkaline solution; 0.8598 g NaOH and 4.29252g Na_2CO_3 were dissolved in water to produce 150 mL solution), B (1.4232 g $CuSO_4$. 5(H_2O) was dissolved in water to produce 100 mL solution) and C (2.85299 g Na_2Tartrate. 2(H_2O) was dissolved in water to produce 100 mL solution) with a ratio (v/v) of 100:1:1. 1 mL of extract was mixed with 1.4 mL of Lowry's solution into the glass tube and was capped and vortexed. The sample was incubated for 20 minutes at room temperature in the dark. The diluted Folin reagent was prepared and 0.2 mL of diluted Folin reagent was added into the sample. The sample was incubated once more for 30 minutes or longer at room temperature in the dark and was measured using UV mini-1240 of UV-Vis Spectrophotometer at 750 nm of absorbance. Bovine Serum Albumine (BSA) was used as a standard to determine the total protein content.

2.9 Total Glycosaponins Measurement

Total glycosaponins content in *Annona muricata* leaves was determined using the method which was described in previous studies [18,21]. 1 mL from sample was mixed with 1 mL of 8% w/v vanillin and 10 mL of 72 % sulfuric acid. The mixture was incubated at 60°C for 10 minutes and cooled in an ice bath for 15 minutes. The absorbance of sample was read at 560 nm using UV-Vis spectrophotometer.

2.10 Statistical Analysis

SPSS16.0 software (SPSS 16.0 for Windows Evaluation Version software, SPSS Inc., USA) and Microsoft Excel 2010 were used to analyze the data. The normality of data was determined using One Way ANOVA, Post Hoc and Tukey HSD test. The data was considered to be significant if the probability $p < 0.05$.

3.0 RESULTS AND DISCUSSION

3.1 Model Fitting

In this study, Table 2 shows the results of independent process variables; A, B, C for both dependent responses; yield of extract and total solid content. The multiple regressions was used to analyze the experimental values of yield of extract and total solidcontent in order to fit the quadratic polynomial equations; Eq.1 and 2 shown in Table 3 and 4, respectively. The magnitude and sign of the regression coefficient were incorporated in the equation to check the quality of fit to the equations. The coefficient of determination (R^2) for yield of *A. muricata* leaves extract and total solid content was reported as 0.8900 and 0.8433, respectively. The positive sign of the coefficient implied that the independent variable was directly proportional to the response variable and *vice versa* [22]. The coefficient for yield of extract and total solid content in this study showed an acceptable agreement between predicted and actual responses. Other than that, statistical analysis from p values also indicated that equation for yield of extract and total solid content can satisfactorily predict the experimental results, since the fit was significant p<0.050. Furthermore, Figure 1 and 2 show the yield of extract and its total solid content obtained for the experiments against the predicted values displayed an acceptable level of agreement. Both graphs illustrates that the model of yield of extract and total solid content have a good equation fit. These results were implied as a satisfactory mathematical description of the extraction process through the fitted models from Eq. 1 and 2:

Table 2 Box-Benhken Design Arrangement and Responses Value for Extraction Process

Experiments		**Independent variables**			**Responses**	
Std. order	Run order	X_1 A	X_2 B	X_3 C	Y_1 YOE (%)	Y_2 TSC (g/g)
1	8	1	1	0	2.52	30.2
2	4	0	1	1	3.33	32
3	10	0	0	0	1.94	28
4	1	1	-1	0	14.7	39.3
5	15	0	0	0	6.39	36.3
6	7	-1	0	1	4.8	34
7	6	1	0	-1	1.26	28
8	11	-1	-1	0	1.12	26.6
9	9	0	-1	-1	0.05	24
10	13	-1	1	0	2.24	30
11	17	1	0	1	14.6	38.8
12	2	0	0	0	1.98	29.3
13	3	0	1	-1	0.05	24.2
14	5	0	0	0	5.81	35.6
15	12	-1	0	-1	0.78	25.8
16	16	0	0	0	3.92	31.4
17	14	0	-1	1	6.39	36.4

Table 3 Analysis of variance for the yield of *Annona muricata* leaves extract

Effect	**SS**	**df**	**MS**	**F-value**	**p-value**	**R^2**
Regression	278.34	9	30.93	6.29	0.0121	0.8900
Residual	34.41	7	4.92			
Total	312.75	16				

Table 4 Analysis of variance for the total solid content

Effect	**SS**	**df**	**MS**	**F-value**	**p-value**	**R^2**
Regression	322.13	9	35.79	4.19	0.0361	0.8433
Residual	59.84	7	8.55			
Total	381.98	16				

Yield of extract = + 4.01 + 3.02 *A - 1.76 * B + 3.37 * C + 2.02 * A^2 - 0.88 * B^2 - 0.67 * C^2 - 3.33 * A * B + 2.33 * A *C-0.77*B*C (5)

Total solid content = + 32.12 + 2.49 * A - 1.24 * B + 4.90 * C + 0.95 * A^2 - 1.55 * B^2 - 1.42 * C^2 - 3.12 * A * B + 0.65 *A*C-1.15*B*C (6)

The polynomial response model and response for the actual value of experiments for all 17 experimental runs are shown in Figure 2 and 3. In response to the predicted and actual values of the experiments shown in Figure 2 and 3, the differential values or known as residual values illustrated in Figure 4 and 5 show the values were significant to both responses. The outlier T detection showed that all the experiments data point were located within the ranges +3.50 and -3.50 of standard deviation for yield of extract and total solid content. Therefore, no outlier needs to be omitted in this model since both normal plots also were normal and acceptable.

3.2 Effect of Processing Parameters

The 3D-plotted graphs were response surface graphs which were drawn to demonstrate the interactive effects of the independent variables on the dependent variables; yield of extract and total solid content. Figure 6 shows the interaction between the effect of solvent to raw material ratio and concentration of ethanol solvent on the yield of extract. Ethanol was used in this study prior to the fact that many semi-polar compounds show a maximum solubility in it [23] and since the acetogenins is a polyketide compounds, it is more preferable rather than used any other solvent extractor. A quadratic effect for both variables was observed through the graph, though solvent had greater influence on the yield of *Annona muricata* leaves extract. Yield of *Annona muricata* leaves was increased by increment of solvent to raw material ratio along with the decrement of concentration of ethanol solvent. A higher value of yield could be noticed with the increment of solvent to raw material ratio . This findings is agreed with another reported study which clearly stated that different ratio of solvent to raw material will significantly affect extract yield [24]. If the ratio is too small, the compounds in it cannot be completely extracted up but if the ratio is too big, this will cause high process cost. Therefore, it is necessary to select the suitable ratio of solvent to raw material for extraction of compounds. The yield is continued to increase evidently with the increasing ratio of solvent to raw material due to the increase diffusivity of the solvent into cells and enhance desorption of the compounds from the cells. Figure 7 shows the effect of solvent to raw material ratio and duration of extraction on the yield of *A. muricata* leaves extract.

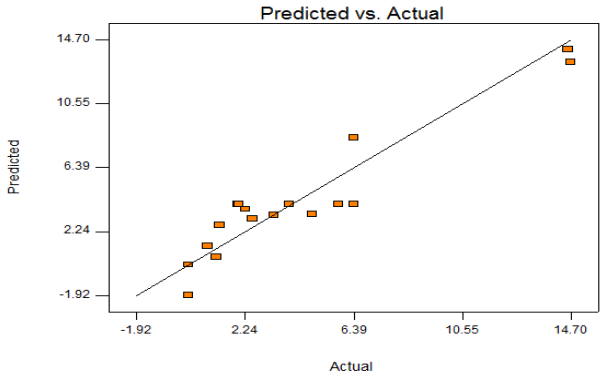

Figure 2 Relationship between the predicted and actual value of the yield of extract

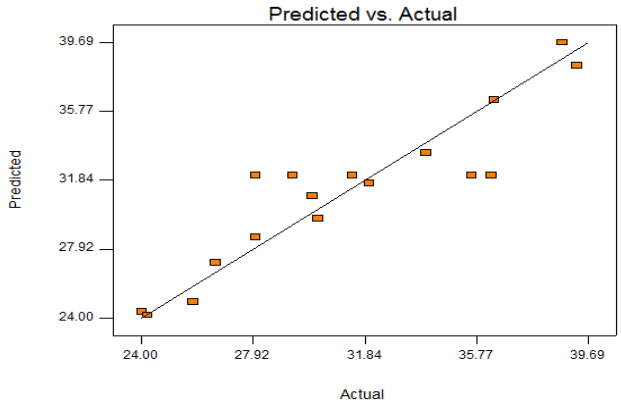

Figure 3 Relationship between the predicted and actual value of the total solid content

The yield of extract was increased by duration of extraction. The reason was that the extraction has reached its equilibrium state [25]. At that time, additional duration of extraction will no longer affect the rate of extraction due to the completed extraction. The higher yield extract is obtained by increasing the duration of extraction since the extraction time enhanced the extraction of most components [26].

Table 5 The predicted solution of the optimization of processing parameter of *A. muricata* leaves

Ratio (g/g)	Conc. solvent (%)	Duration (h)	Yoe (%)	Tsc (g/g)	Db
1:5.92	1.67	6.84	15.56	41.15	1.00

*Yoe:Yield of extract, Tsc:Total solid content, Db : Desirability

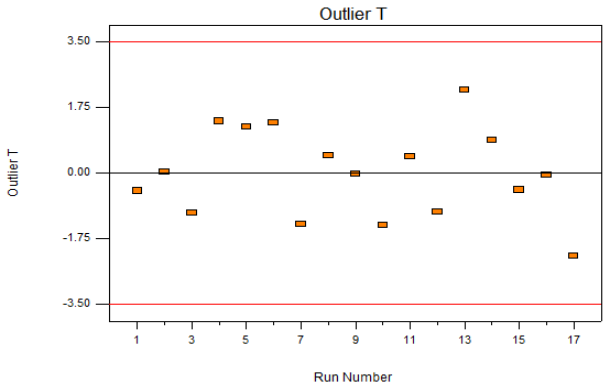

Figure 4 Outlier T plot for 17 Experiments Data Points of the yield of extract

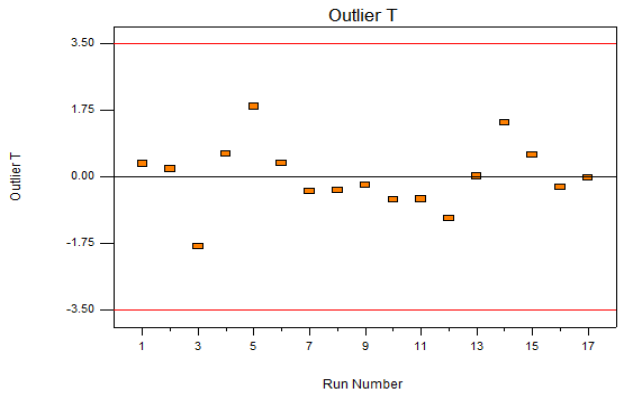

Figure 5 Outlier T plot for 17 Experiments Data Points of the total solid content

In this study, the optimum processing parameter to produce the highest amount yield of extract (15.56 % w/w) was obtained from sample with solvent to raw material ratio (1:5.92) and 1.67 % concentration of ethanol . The response surface or 3D-plotted graph on the total solid content is also shown in Figure 8 and 9. Figure 8 shows the effect of solvent to raw material ratio and concentration of ethanol solvent on total solid content. Quadratic effect for both variables through solvent to raw material ratio with great influence on the total solid content was observed. Total solid content increased in increment of solvent to raw material ratio along with the decrement in concentration of ethanol solvent. This isbecause the characteristic of acetogenins can range from very polar, such as those extracted by water and ethanol, to nonpolar such as hexane. However, environmental side would suggest the use of more polar solvents [27-28]

The higher value of total solid content could also be noted with the low concentration of ethanol solvent. Figure 9 shows the effect of solvent to raw material ratio and duration of extraction on the total solid content; quadratic effect of solvent to raw material ratio and a linear effect of duration of extraction on the total solid content can be seen. The total solid content was increased with solvent to raw material ratio and duration of extraction. The optimum processing parameter to produce the highest amount total solid content (41.15 w/w) was obtained from sample with solvent to raw material ratio (1:5.92) and 6.84 h duration of extraction. These results showed that the response surface had a maximum point within the experimental range of the independent variables.

3.3 Optimization of The Process

Ten solutions were obtained after the optimization process using design expert software version 6.0.8. The parameter was chosen based on the value of desirability is equal or nearly to 1. In this study, the first solution or parameter was selected as an optimal processing parameter since the desirability is nearly equal to 1. Therefore, the prediction solution from the first parameter chosen was reported in Table 5.

The optimum yield of *A. muricata* leaves extract and total solid content was obtained from crude extract and was further analyzed with phytochemical screening like antioxidant tests. The agreement of optimal data among solvent to raw material ratio, concentration of ethanol solvent and duration of extraction gave the best optimum condition for the highest amount of yield and total solid content.

The highest value for yield of extract was obtained with the increment of solvent to raw material ratio and the decrement of concentration of ethanol solvent. Meanwhile, the highest value for total solid content was obtained with the same pattern of solvent to raw material ratio and concentration of ethanol solvent but along with the increment of duration of extraction which is 6.84 hours not more than 8 hours.

3.4 HPLC analysis

Representative chromatograms of the *acetogenins* analytes for the sample with optimum condition was illustrated in Figure 6. Figure 6 displayed that the six *acetogenins* compound analytes were well separated and detected in HPLC screening process. The resolution between any two compounds in the sample was greater than 1.5. Other compounds in the sample did not interfere with analysis of the six *acetogenins* compounds analytes, as shown in Figure 10.

The chromatographic peaks were identified by comparing their retention time with that of each reference compounds reported from the previous journal [21]. Retention time for the analytes of interest will not change significantly from day to day or from laboratory to laboratory if the method is considered robust [29]. The determination of the robustness of chromatographic methodology involved in the alteration of the experimental condition and the chromatographic characteristics were evaluated. The retention times of the compound in the extract were remains same and the coefficient of variation

for retention time was less than 1 % make a confirmation of the presence of *acetogenins* in the extract. The content of these components varied in different *A. muricata* leaves which might be due to the differences in soils and climates in each region. Thus, it is necessary to control the main bioactive ACGs in different *Annona muricata* plant leaves by good agricultural practice (GAP). Then, the quality of *A. muricata* leaves could be assured.

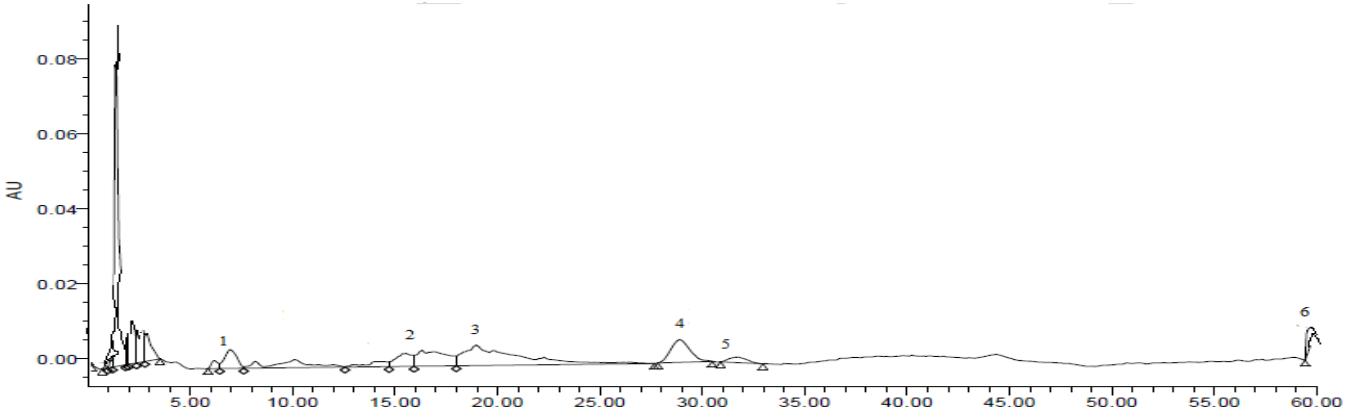

Figure 6 Reprehensive HPLC chromatograms of acetogenins compound analytes.in Annona muricata leaves. Peak identification: (1) 12,15-cis-squamostatin-A; (2) squamostatin-A; (3) bullatacin; (4) Squamocin; (5) Isodesacetyluvaricin; (6) Desacetyluvaricin

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3.5 TPC, TFC and DPPH Assay Analysis

Results of TPC assay were expressed as gallic acid equivalent. TPC of each sample was calculated from calibration curve of gallic acid where the calibration equation was determined to be y = 30.963x + 0.0657 (R^2 = 0.9986), whereby y = absorbance at 725 nm and x = concentration of total phenolic compounds in mg per 1 mL of the extract. The reaction mixture for the sample of *A. muricata* leaves extract was dark blue in color which indicated high phenolic content in leaves (47.26±0.001). On the other hand, reaction mixture for the sample of low concentration was light blue in color indicating low phenolic content.

Results of TFC assay were expressed as catechin equivalent. TFC of each sample was calculated from calibration curve of catechin where the calibration equation was determined to be y = 1.7315x + 0.0337 (R^2 = 0.9959), whereby y = absorbance at 510 nm and x = concentration of total flavonoid compounds in mg per 1 mL of the extract. The reaction mixture for the samples was yellow in color which indicated high flavonoid content, as same trend as TPC test (22.12±0.001). On the contrast, reaction mixture for the sample of low concentration usually was faded in color indicating low flavonoid content.

In DPPH reaction mixture, ascorbic acid was used as positive control gave the fastest color change from purple to yellow. As for the samples, high concentration of an optimum sample showed bleaching of purple color during eye observation. However, low concentration of *A. muricata* leaves extract showed no obvious purple bleaching effect, even though there were decreases in the absorbance readings.

Likewise, Figure 11 shows that for positive control, butylated hydroxyanisole (BHA) contained higher radical scavenging activity compared to ascorbic acid (Vit. C). Meanwhile extract sample also showed the highest percentage of scavenging activity with the IC_{50} value (0.243± 0.001 mg/mL). However, the IC_{50} value for samples was still nearly the range of both positive controls, where IC_{50} value for BHA and Vit. C were (0.155 ± 0.001 and 0.232 ± 0.001 mg/mLl) respectively.

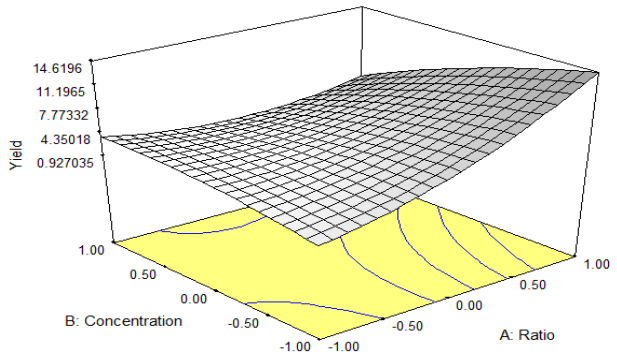


Figure 7 Response surface plot showing the combine effect of concentration of ethanol solvent and solvent to raw material ratio on the yield of extract

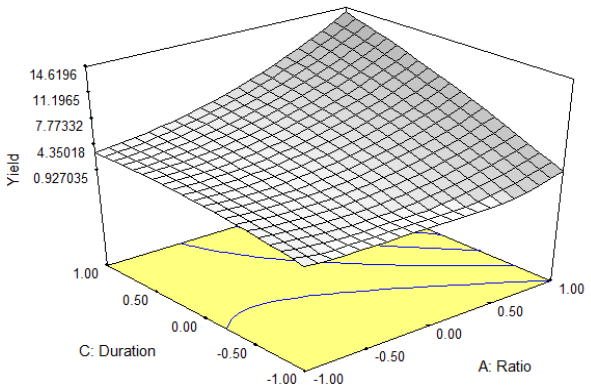
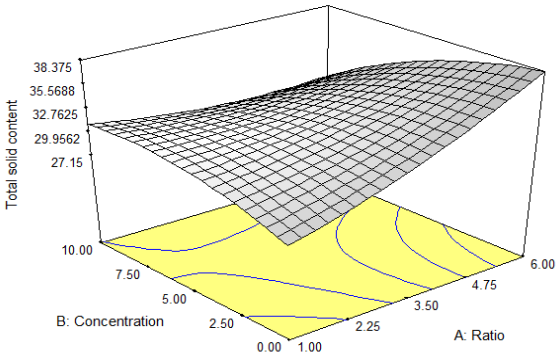


Figure 8 Response surface plot showing the combine effect of duration of extraction and solvent to raw material ratio on the yield of extract



Fig

Figure 9 Response surface plot showing the combine effect of solvent to raw material ratio and concentration of ethanol solvent on the total solid content

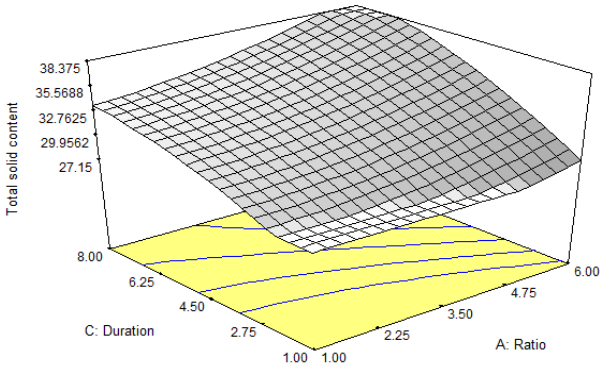


Figure 10 Response surface plot showing the combine effect of solvent to raw material ratio and duration of extraction on the total solid content

3.6 Polysaccharide, Protein, and Glycosaponins Analysis

The extracts were further investigated for some primary metabolites content. Total protein contents were estimated using linear regression equation (y = 0.0015x + 0.1152, R^2 = 0.9536), which was obtained from the calibration curve of bovine serum albumin. Total polysaccharides were estimated by linear regression equation (y = 0.0015x + 0.1152, R^2 = 0.9536), which was obtained from the standard curve of glucose. Total glycosaponins were estimated by gravimetric assay. The results of total primary metabolites along with contents are shown in Table 6, which indicates the percentage contents of total primary metabolites and their distribution in various parts of the plant as well as the effect of different chemical tests on the extraction on these metabolites. The distribution of total metabolic contents was in the order: phenolic > flavonoid > polysaccharide > protein > glycosaponins with 47.26 ± 0.001, 22.12 ± 0.001, 21.85 ± 0.001, 19.59 ± 0.000, and 18.87 ± 0.001, respectively.

Table 6 Percentage total contents of protein, polysaccharides and glycosaponins of *A. muricata* leaves

Name of Compound	Primary and Secondary Metabolites
Polysaccharide	21.85±0.001*
Protein	19.59±0.000*
Glycosaponin	18.87±0.001*
Phenolic	47.26±0.001*
Flavonoid	22.12±0.001*

*Refer to Standard Deviation (± SD)

3.7 Correlation of Radical Scavenging Activity and Antioxidant Contents

There were some correlations between free radical scavenging activity with the antioxidant contents including primary and secondary metabolites. The total phenolic content in sample followed by flavonoid, polysaccharide, protein and glycosaponins, the correlations were stronger for its free radical scavenging activity compared to ascorbic acid and butylated anisole. In this study, the results showed the differences in the percentage of metabolites distribution suggested that these antioxidant contents might be one of the contributors to the free radical scavenging activity with only to a certain limits.

A validated analytical method for qualification of *annonaceous acetogenins* from A. *muricata* leaves has been developed. Six *acetogenins* were detected from the sample including peak identification: 12,15-*cis*-squamostatin-A (1); Squamostatin A (2); Bullatacin (3); Squamocin (4); Isodes acetyluvaricin (5) and Desacetyluvaricin (6). The antioxidants result showed that the leaves contained a significant amount of polyphenols compounds including primary and secondary

metabolites such as polysaccharides, protein, glycosaponins, phenolic and flavonoid.

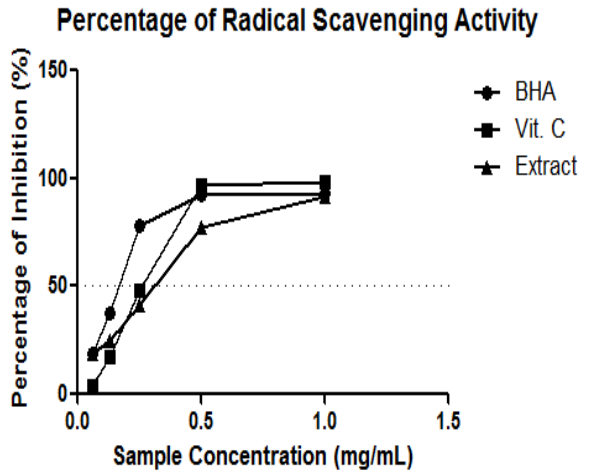


Figure 11 Comparison of radical scavenging activity between the positive control; ascorbic acid (Vit. C), butylated hydroxyanisole (BHA) and sample. IC_{50} value (in mg/mL) for each sample was derived from the graph at 50 % radical scavenging activity.

The identification of antioxidant activity in the leaves was evidently proved by DPPH radical scavenging activity assay performed on it. The finding showed that IC_{50} value of leaves was nearly comparable with ascorbic acid, which means that the leaves have higher free radical scavenging activity compared to the commercial standard. Besides, statistical significance was assessed and showed that $p < 0.001$ using One-Way ANOVA (SPSS 11.5 software package) implied that all value have a significance difference in this study. Thus, this showed that the optimum condition was seen to be the best extract out of both types of standards.

4.0 CONCLUSION

Response surface methodology was successfully applied for optimization of *Annona muricata* leaves extraction by using Soxhlet extractor. The high regression coefficient of quadratic polynomial of the response showed that model fitted with the data well. After the optimization process using Box-Behnken design technique of design expert software version 6.0.8, the optimal processing parameter that fulfilled the requirement for yield of *Annona muricata* leaves extract and total solid content were found to be 1:5.92 solvent to raw material ratio, 1.67 % concentration of ethanol solvent, and 6.84 hour duration of extraction. While, the optimal yield of extract and total solid content was 15.56 % w/w 41.15 g w/w.

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