Application of Response Surface Methodology for the Optimization of the Extraction of Triterpenoid Saponins from *Azadirachta excelsa* Leaves

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Azadirachta excelsa originates from the Meliaceae family and is often subjected to bioactivity studies due to the presence of various types of compounds such as phenolics, flavonoids and terpenes. Triterpenoid saponins are diverse groups under terpenoids and are secondary metabolites produced by plants for carrying out antagonistic and mutualistic interactions. These compounds are extensively investigated for bioactivities such as anticancer, antimicrobial and pesticidal properties due to their wide range of action mechanism. This study aimed to optimize *A. excelsa* leaves extraction process using response surface methodology (RSM) for obtaining extract with high extract yield and triterpenoid saponins content. The independent variables selected for the extraction process included temperature, ethanol-to-chloroform ratio, time and sample-to-solvent ratio. The individual and interactional effects of extraction parameters on the yield of extract and triterpenoid saponins content were evaluated using Central composite design (CCD) of RSM. The responses were predicted using second order polynomial model. One-way analysis of variance (ANOVA) revealed correlation coefficients (R^2) of developed model for yield of extract and triterpenoid saponins contents were 0.9792 and 0.9509. High F-statistics value and lower p-value (<0.0001) revealed sample-to-solvent ratio had major effect on extract yield and triterpenoid saponins content. Larger surface area exposure of plant material to solvent elevates interaction between solid and liquid matter resulting in higher extract yield and triterpenoid saponins content. The optimum extraction conditions were 45 °C of temperature, ethanol-to-chloroform ratio of 90:10, 60 min of extraction duration, 1:50 g/mL of sample-to-solvent ratio. The actual yield of extract and the triterpenoid saponins contents at optimum extraction conditions were 10.63% and 0.45% respectively. These values differed by 2.9 % for yield of extract and 11.3 %, for triterpenoid saponins content from the values predicted by the model. Some plants triterpenoid saponins have demonstrated pesticidal properties. This study was conducted as an initial step in the potential utilization of *A. excelsa* leaves extract as a natural pesticide. Results obtained in this study can be utilized as a guidance for extraction of *A. excelsa* leaves and to compare the extraction efficiency of established method with possible methods developed in future.

1. Introduction

Azadirachta excelsa is a lowland monsoon forest species of Azadirachta genus from the mahogany family, Meliaceae and is native to the Southeast Asian-Pacific region. Pesticidal activity were exhibited by extracts from the seeds and wood of *A. excelsa* against various species via multiple action mechanism. Seeds of *A. excelsa* were often investigated due to highest concentration of bioactive compound called Azadirachtin or its derivatives which is a triterpenoid mainly found in Azadirachta genus. Other than seeds, *A. excelsa* leaves has been studied for antioxidant, antidiabetic properties due to the synergism between flavonoids and phenolics compounds (Nur et al., 2016). Seasonal fruiting of *A. excelsa* from June to August limits accessibility to seeds compared to continuous availability *A. excelsa* leaves which led to further investigation on the leaves specifically in terms of

extraction as this were the primary step in separating chemical compounds from raw material. Triterpenoid saponins are secondary plant metabolites found in abundance among plants and have diverse chemical structures due to enzymatic reactions, gene evolution through selection and mutation, and modification reaction altering terpene skeletons (Cárdenas et al., 2019). Their structural diversity has led to various studies which reported broad range of bioactivity. Extraction of triterpenoid saponins from plant material varies based on their chemical complexity from nonpolar, small hydrocarbons to large compounds with higher physical complexity caused by chemical alterations.

Nur et al. (2016) performed extraction of *A. excelsa* leaves by macerating powdered leaves at room temperature for two days using 70% ethanol which resulted in extract with total flavonoid and phenolic contents of 198 ± 0.67 mg rutin/g extract and 202 ± 0.42 mg gallic acid/g extract. However, the extraction process was not optimized in the mentioned study and the parameters influencing the yield of extract as well as content of compounds in *A. excelsa* leaf extract was not resolved in any formerly conducted studies. Optimization studies were never performed on *A. excelsa* leaves and this study was the first pursuing the subject in matter for maximizing extract yield and triterpenoid saponins content in extract using Response surface methodology (RSM). RSM is a statistical model which develops the best factor and level settings for optimizing process parameters using randomized and minimal experimental runs. Central composite design (CCD) of the RSM provides data for multivariable system modelling and was utilized in this research work for interpreting the individual and interactional effect of extraction parameters on extract yield and triterpenoid saponins content (Fadjare et al., 2021).

This research was performed to optimize *A. excelsa* leaves extraction process using RSM for obtaining extract with high extract yield and triterpenoid saponins content.

2. Materials and methodology

2.1 Chemicals and reagents

Reagents used in the extraction of *A. excelsa* leaves included 95 % ethanol (v/v) (Systerm, Malaysia) and chloroform (R & M Chemicals, India). For colorimetric testing of total terpenoid content, ursolic acid (Sigma Aldrich, USA), vanillin (Ajax Chemicals, Australia), acetic acid (R & M Chemicals, India), perchloric acid (Systerm, Malaysia) and ethyl acetate (EMSURE, Germany) were utilized. All chemicals were used as received unless otherwise stated.

2.2 Sample preparation

Fresh *A. excelsa* leaves obtained from Forest Research Institute Malaysia (FRIM) were dried in the oven at 50 °C (Genlab Large Capacity Multi-Purpose Incubators, UK) for five days. After the drying process was completed, the leaves were ground and powdered using a blender (Panasonic MX-GM1011, Japan). Powdered samples were sieved using a laboratory sieve (Endecotts 200SIW.500) to ensure uniform particle size of 500 μm and the sample were then stored at a dry and cool place.

2.3 Experimental design

The optimization of *A. excelsa* leaves extraction process was performed based on RSM via Design Expert® software (Version 13, StatEase, Minneapolis, USA). Four independent variables and respective values were selected for the optimization process which were ethanol-to-chloroform ratio, A (90:10, 55:45, 20:80), sample-to-solvent ratio, B (1:10, 1:30, 1:50 g/mL), temperature, C (25, 35, 45 °C) and time, D (30, 45, 60 min) (Hismath et al., 2011). Experimental design consisted of 30 randomized runs which included sixteen factorial points, eight axial points, one central edge point and five central points including the replicates. The responses of the study were the yield of extract and triterpenoid saponins content.

2.4 Extract preparation

The extraction of *A. excelsa* leaves was performed using the maceration method due to its simplicity and safer recovery of triterpenoid saponins compounds. The extractions were performed based on the method reported by Jovanović et al. (2017) with modifications. The particle size of *A. excelsa* leaves was kept constant at 500 μm. Extraction temperature and duration, weight of powdered *A. excelsa* leaves as well as volume and composition of extraction solvent were based on the central composite response surface experimental design. Plant material and solvent were added into a 250 mL conical flask and covered using aluminium foil and further sealed using parafilm to minimize solvent loss. A shaker incubator (LABEC, ILM-570D, Australia) was utilized to maintain the extraction conditions while mixing the contents of the conical flasks. The shaking motion was maintained at 100 rpm. After the extraction period was over, the contents of conical flask were filtered using 320 mm filter paper. Recovered filtrate was dried using rotary evaporator (Eyela, Japan) to remove excess solvent

at 40 °C resulting in crude extract. The extracts were stored in a dry and cool place. The yield of extract was expressed in % and calculated using the Eq 1 below:

$$\text{Extract yield (\%)} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of plant material (g)}} \times 100 \text{ \%} \tag{1}$$

2.5 Quantification of triterpenoid saponins content using colorimetric method

The content of triterpenoid saponins in *A. excelsa* leaves was determined according to method mentioned by Chen et al. (2007) with slight modification and expressed as % of dry plant material. Stock solution of ursolic acid with a concentration of (1 mg/mL) was prepared and 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL was pipetted into a 10 mL test tube specific for colorimetry analysis. Next, those test tubes were transferred into a water bath and heated until reaching evaporation followed by, addition of 1.2 mL of perchloric acid and 0.2 mL of newly mixed 5 % (w/v) vanillin-acetic acid. Content of test tubes were mixed well and settled for incubation at 70 °C for 15 min. After incubation period was over, the test tubes were exposed in running water for 2 min to reduce temperature of sample solution and then, total volume of test tube content was increased to 5mL by gradual addition of ethyl acetate. UV/VIS Spectrophotometer (Agilent BioTek Epoch Microplate Reader, USA) was utilized to obtain the absorbance of test solution at 550nm with a blank solution for reference. A standard curve was then plotted.

The content of triterpenoid saponins in *A. excelsa* leaves were measured by pipetting 0.2 mL of 1 mg/mL of *A. excelsa* extract obtained via experimental runs into the test tube. The absorbance was obtained via colorimetric method described previously in standard curve preparation. Standard curve was utilized to attain concentration of triterpenoid saponins and Eq 2 shown below was used to calculate the content of triterpenoid saponins in dry material:

$$\text{Triterpenoid saponins content (\% in dry material)} = \frac{\text{Triterpenoid saponins in extract (mg)}}{\textit{A. excelsa} \text{ leaves (mg)}} \times 100 \text{ \%} \tag{2}$$

2.6 Statistical analysis

Experimental design data were analyzed and the predicted responses were calculated using Design Expert® software (Version 13, StatEase, Minneapolis, USA). Analysis of variance (ANOVA) was accomplished to evaluate significance of obtained values by computing the F-value at the level of 95 % confidence ($p < 0.05$). By utilizing the obtained data, a second order polynomial equation was established for prediction of optimal conditions fitted to comprehend the effect of extraction parameters on extract yield and triterpenoid saponins content. The common second order polynomial model utilized is as shown in Eq(3):

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 + \varepsilon \tag{3}$$

Y displays the predicted response, b_0 stands for constant, *bi*, *bii* and *bij* are regression coefficients for the developed model, *Xi* and *Xj* are the extraction parameters and ε shows error. Response surface and contour plots were generated by the software to provide visualization of the interaction between extraction parameters on the response values.

2.7 Optimization and validation of model

Optimized condition for the developed second-order polynomial model was determined using RSM via Expert design software. Three validation runs were performed based on optimal conditions given by the software to determine suitability of developed model in predicting response values and to calculate percentage of difference. Actual and predicted response values were compared to validate the developed model in terms of extract yield and triterpenoid saponins content under optimized condition (Dhawane et al., 2015).

3. Results and discussion

3.1 Analysis of response surface models

Full factorial design of CCD was applied to evaluate the effect of between the process parameters on response values along with identifying optimized extraction conditions. Second order polynomial quadratic model in terms of actual factors was developed to estimate the response value by carrying out multiple regression analysis (Eq 4) and (Eq 5) where actual values of extraction parameters are represented as A, B, C and D.

Yield of extract = -17.77245 -0.096790A + 0.789075B + 0.449999 C + 0.221302D + 0.000969AB + 0.001057AC - 0.000152AD - 0.003347BC - 0.000194BD + 0.000673 CD + 0.000115A^2 - 0.008440B^2 - 0.004053C^2- 0.002757D^2 $\hspace{1cm}$ (4)

Triterpenoid saponins content = - 0.537904 - 0.005798A + 0.031416B + 0.011880C + 0.000588D + 0.000028AB + 0.000041AC + 0.000018AD - 0.000092BC + 0.000064BD +5.60741E-06CD + 0.000026A^2 - 0.000384B^2 - 0.000105C^2 - 0.000029D^2 $\hspace{1cm}$ (5)

The best fitted quadratic model for the four extraction parameters was obtained via ANOVA analysis which was performed according to 95 % confidence intervals (Table 1). The coefficient of determination, R^2 values of yield of extract and triterpenoid saponins contents were 0.9792 and 0.9509 which were not very close to unity but is still viable due to high significance of developed model proven by low probability value ($p < 0.0001$). R^2 value larger than 80% indicated adequate agreement among actual and predicted value which proves the developed model is statistically sound and can be used to study the effects of extraction parameters on response values (Che Yunus et al., 2011). The CV values of the yield of extract and triterpenoid saponins content were 4.62% and 7.76% proving the models were reproducible due to both values are less than 10. For Lack of fit analysis, replicated design points residual error was compared to pure error, indicating the differences among responses around the fitted model. Based on the results obtained, MS of pure error was zero due to no variations among replicates which implied the F-value and p-value of lack of fit test to be considered as negligible in the developed model. ANOVA analysis revealed that sample-to-solvent ratio had the most significant influence on yield of extract and triterpenoid saponins content. Further interpretation of RSM surface plots were carried out to understand the interaction effects of sample-to-solvent ratio with other parameters on response.

Table 1: ANOVA for the response surface quadratic model for optimization of extraction parameters of A. excelsa leaves

Source	Yield of extract (%) (R^2 = 0.9792)					Triterpenoid saponins content (% in dry plant) (R^2= 0.9509)				
	SS	df	MS	F-value	p-value	SS	df	MS	F-value	p-value
Model	115.27	14	8.23	50.56	<0.0001	0.1162	14	0.0083	20.75	<0.0001
A-Ethanol to chloroform ratio	1.15	1	1.15	7.07	0.0179	0.0034	1	0.0034	8.63	0.0102
B-Extraction Temperature	17.18	1	17.18	105.50	<0.0001	0.0282	1	0.0282	70.57	<0.0001
C-Extraction Time	9.03	1	9.03	55.44	<0.0001	0.0109	1	0.0109	27.19	0.0001
D-Sample to solvent ratio	33.63	1	33.63	206.50	<0.0001	0.0354	1	0.0354	88.46	<0.0001
AB	1.84	1	1.84	11.31	0.0043	0.0015	1	0.0015	3.79	0.0704
AC	4.93	1	4.93	30.25	<0.0001	0.0075	1	0.0075	18.72	0.0006
AD	0.1811	1	0.1811	1.11	0.3084	0.0025	1	0.0025	6.20	0.0249
BC	4.19	1	4.19	25.71	0.0001	0.0031	1	0.0031	7.86	0.0134
BD	0.0250	1	0.0250	0.1534	0.7008	0.0027	1	0.0027	6.86	0.0194
CD	0.6769	1	0.6769	4.16	0.0595	0.0000	1	0.0000	0.1176	0.7364
A²	0.0610	1	0.0610	0.3748	0.5496	0.0031	1	0.0031	7.67	0.0143
B²	9.64	1	9.64	59.22	<0.0001	0.0200	1	0.0200	49.98	<0.0001
C²	11.32	1	11.32	69.49	<0.0001	0.0077	1	0.0077	19.18	0.0005
D²	3.46	1	3.46	21.27	0.0003	0.0004	1	0.0004	0.9846	0.3368
Residual	2.44	15	0.1629			0.0060	15	0.0004		
Lack of Fit	2.44	11	0.2221			0.0060	11	0.0005		
Pure Error	0.0000	4	0.0000			0.0000	4	0.0000		
Cor Total	117.72	29				0.1221	29			

3.2 Effects of independent variables on the yield of extract and triterpenoid saponins content

RSM plots shown in Figure 1(a) and 1(c) displayed the interaction effect of sample-and-solvent ratio with extraction temperature and ethanol-to-chloroform ratio on yield of extract. Figure 1(a) revealed that as the sample-to-solvent ratio increases along with temperature the yield of extract continues to escalate. The extract prepared using 1:50 (w:v) sample-to-solvent ratio had the largest yield of 10.63 % compared to the extract prepared using 1:10 (w:v) where the extract yield was 7.87 %. Figure 2(a) and 2(b) showed the effects of sample-to-solvent ratio with varying ethanol-to-chloroform ratio and extraction time on triterpenoid saponins content. Extracts prepared using 1:50 (w:v) had the highest triterpenoid saponins content compared to 1:10 (w:v) with the values of 0.45 and 0.24 %. Main reason behind the dramatic escalation is the higher driving force for the mass transfer of compounds from *A. excelsa* leaves to extraction solvent and acceleration of dissolution of chemical constituents into extraction solvent. High sample-to-solvent ratio escalated rate of leaching-out of compounds into the solvent as higher concentration gradient exposed more surface areas of the raw material

to be in contact with the solvent (Shi et al., 2002). The increase of contact between solid and liquid increased the yield of extract and the extraction of triterpenoid saponins from *A. excelsa* leaves.
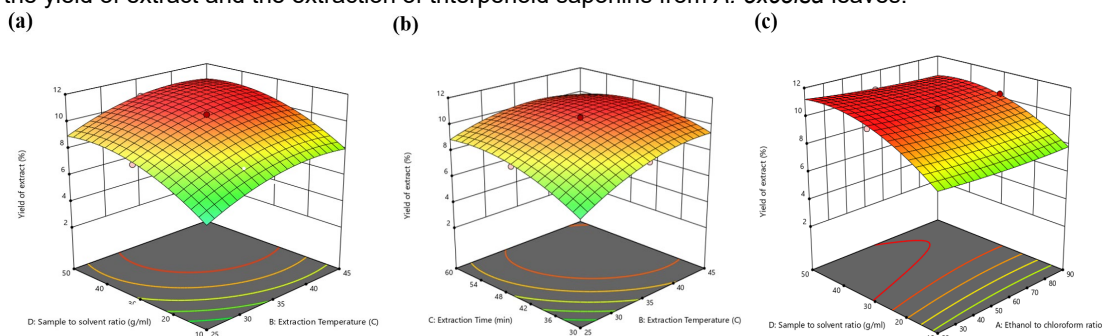
(a) (b) (c)

Figure 1: Response surface plots on yield of extract (a) sample-to-solvent ratio vs extraction temperature, (b) extraction time vs extraction temperature, and (c) sample-to-solvent ratio vs ethanol-to-chloroform ratio

The significant influence of extraction temperature on yield of extract is displayed by the RSM plots in Figure 1(a) and 1(b), and can be further proved by the high F-value and SS as shown in Table 1. When the yield of extract is compared between two extraction temperatures such as 25 and 45 °C while other parameters are kept constant, the response values are 6.2 and 7.87 %. Moreover, RSM plots in figure 2(c) revealed the positive impact of extraction temperature on triterpenoid saponins content. These results are due to higher extraction temperature softened plant tissues which compromised cell integrity allowing release of compounds out of cell membrane. Kinetic energy of molecules elevated due to higher temperature caused better dissociation of released compounds into extraction solvent which increased yield of extract and triterpenoid saponins content. RSM plots showing the influence of extraction time on extract yield is shown in Figure1(b) and for triterpenoid saponins content is displayed in Figure 2(b) and 2(c). Extraction performed for 30 and 45 min while fixing other parameters resulted in extract yield of 8.75 and 10.54 % and triterpenoid saponins content of 0.2276 and 0.2845%. These results indicated elevation of extraction time provided longer duration of diffusion allowing compounds to be released into extraction solvent which improved extract yield and triterpenoid saponins content. Figure 1(c) and 2(a) revealed the influence of ethanol-to-chloroform ratio on yield of extract and triterpenoid saponins content. The curvature of plots revealed that this parameter did not cause apparent changes on both responses and ANOVA results (Table 1) further proves this finding. By maintaining other parameters and changing the values of ethanol-to-chloroform ratio to 90:10 - 20:80, the triterpenoid saponins content obtained were 0.45 - 0.28%. These results might be due to higher concentration of medium to non-polar triterpenoid compounds in *A. excelsa* leaves. This observation is supported by Wei et al. (2015) stating extraction of polar triterpenoid favors lower concentration of ethanol while medium to non-polar triterpenoid can be effectively extracted using higher concentration of ethanol. Based on the polarity index of ethanol-to-chloroform ratio, different type of compounds in varying quantities are being extracted from *A. excelsa* leaves and interaction with other parameters is necessary for accentuating the capabilities of extraction solvent to improve response outcomes.
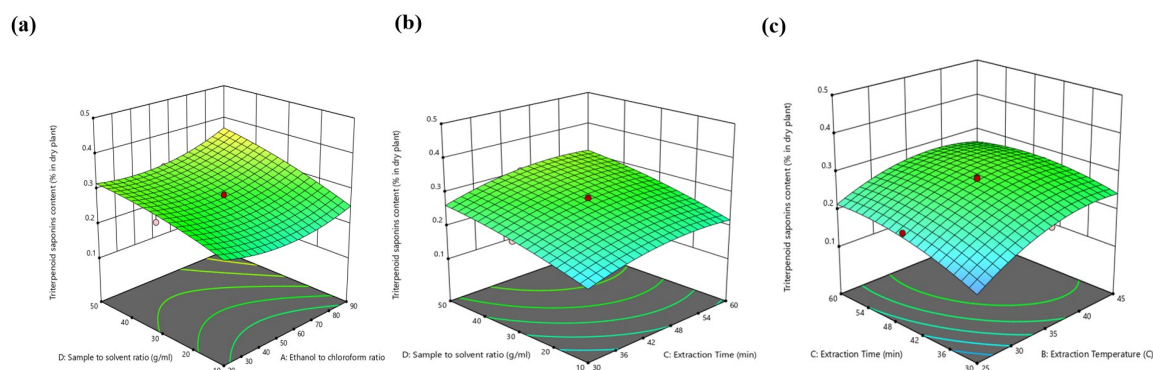
(a) (b) (c)

Figure 2: Response surface plots on triterpenoid saponins content (% in dry plant) (a) sample-to-solvent ratio vs ethanol-to-chloroform ratio, (b) sample-to-solvent ratio vs extraction time, (c) extraction time vs extraction temperature

3.3 Extraction conditions optimization and model validation

Optimal conditions to achieve high yield of extract and triterpenoid saponins content in extract were 45 °C of temperature, ethanol-to-chloroform ratio of 90:10, 60 min of extraction duration, 1:50 g/mL of sample-to-solvent ratio. Optimized conditions resulted in 10.63 % of extract yield and triterpenoid saponins content of 0.45 %, while the predicted values were 10.94 % of extract yield and triterpenoid content of 0.40 %. Minimal deviation between experimental and predicted values of extract yield was proven by low percentage of difference of 2.9 %. The developed model was still acceptable for studying triterpenoid saponins content although the percentage of difference was 11.3 % because there was minimal variation between experimental (0.45 %) and predicted values (0.40 %) as well as the ANOVA results (Table 1) proved the significance ($p < 0.0001$) of the model.

4. Conclusions

This study revealed that optimization carried out using RSM resulted in the most fitted extraction condition to produce *A. excelsa* leaf extract with high extract yield and triterpenoid saponins content with minimal usage of solvent within shorter duration rather than utilizing classical method. RSM revealed that sample-to-solvent ratio was the parameter with the most significant effect on extract yield and triterpenoid saponins due to elevated mass transfer between solid and liquid matter. The optimized condition established by RSM were as follows: 45 °C of temperature, ethanol-to-chloroform ratio of 90:10, 60 min of extraction duration, 1:50 g/mL of sample-to-solvent ratio which led to extract yield of 10.63 % and 0.45 % of triterpenoid saponins content. Optimization outcomes in this study could not be compared to any previous works but can be utilized by upcoming researches to compare effectiveness of other extraction methods with the current method under the described optimization condition. Possible future studies can be conducted on isolating triterpenoid from extract produced under established optimized condition to identify the compound possibly accountable for the pesticidal activity of *A. excelsa* leaves.

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