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Application of Box-Behnken Design for Ultrasound-Assisted Extraction and Recycling Preparative HPLC for Isolation of Anthraquinones from *Cassia singueana*

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ABSTRACT:

Introduction – *Cassia singueana* Del. (Fabaceae) is a rare medicinal plant used in the traditional medicine preparations to treat various ailments. The root of *C. singueana* is a rich source of anthraquinones that possess anticancer, antibacterial and antifungal properties.

Objective – The objective of this study was to develop ultrasound-assisted extraction (UAE) method for achieving a high extraction yield of anthraquinones using the response surface methodology (RSM), Box-Behnken Design (BBD), and a recycling preparative HPLC protocol for isolation of anthraquinones from *C. singueana*.

Methodology – Optimisation of UAE was performed using the Box-Behnken experimental design. Recycling preparative HPLC was employed to isolate anthraquinones from the root extract of *C. singueana*.

Results - The BBD was well-described by a quadratic polynomial model (R^2 = 0.9751). The predicted optimal UAE conditions for a high extraction yield were obtained at: extraction time 25.00 min, temperature 50°C and solvent-sample ratio of 10 mL/g. Under the predicted conditions, the experimental value (1.65 ± 0.07%) closely agreed to the predicted yield (1.64%). The obtained crude extract of *C. singueana* root was subsequently purified to afford eight anthraquinones.

Conclusion - The extraction protocol described here is suitable for large-scale extraction of anthraquinones from plant extracts.

Keywords: Optimization; Box-Behnken Design; HPLC; *Cassia singueana*; Fabaceae; anthraquinone; ultrasound-assisted extraction.

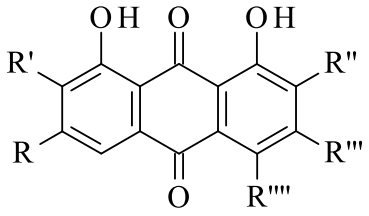
Introduction

Anthraquinonoids, simply anthraquinones, like physcion (**5**) and chrysophanol (**7**), are major bioactive constituents of many medicinal plants, traditionally used in the oriental medicinal formulations, e.g., the traditional Chinese medicine (TCM). Additionally, anthraquinones, e.g., chrysophanol and rhein, are widely valued for their diverse therapeutic and pharmacological properties, e.g., antioxidant, antimicrobial, antitumour, insecticides and pesticides, and their uses as cathartic, detoxifying agent and laxative (Hemen and Lalita, 2012; Lim *et al.*, 2017; Zhang *et al.*, 2017). The use of anthraquinone containing plants, e.g., species from the genera *Cassia* and *Aloe*, as constituents in many traditional oriental herbal formulations has gained commercial success, owing to various scientific studies revealing various pharmacological activities of anthraquinones and their glycosides (Shivjeet and Ashutosh, 2013). The *Cassia* species, well-known for producing anthraquinones and their glycosides, are popular in Southeast Asia and India because of their medicinal applications to treat eczema, skin diseases and ulcers, and as anthelmintic, expectorant, and liver- and cardio-tonic (Shivjeet and Ashutosh, 2013). *Cassia singueana* Del. of the family Fabaceae is a medicinal plant native to Africa and has been traditionally used for treating various diseases such as cancer, stomach disorders and as a post-partum tonic (Ifeanyi and Ode, 2012). Previous studies showed that the root of *C. singueana* is a good source of anthraquinones (Endo and Naoki, 1980; Mutasa *et al.*, 1990).

The biological activity of plant-based extracts may depend on the physical and chemical aspects of the crude suspension obtained from the extraction process, which often dictates the quality and quantity of certain group of secondary metabolites in the extract (Majeed *et al*., 2016). Thus, the selection of an appropriate extraction method and its optimization are essential for ensuring the quality of the extract (Sarker and Nahar, 2012). The most common method used in the extraction of *C*. *singueana* is the maceration process (Ifeanyi and Ode, 2012; Ode and Asuzu, 2014). Only a few anthraquinones were reported from this plant using this extraction process (Endo and Naoki, 1980). In this perspective, the use of ultrasound-assisted extraction (UAE) is expected to give better extraction yield of anthraquinones, since it involves mechanisms such as cell disruption and improved solvent penetration, thereby enhancing swelling and proper hydration process (Yang and Huang, 2013). This method is simple and economical, and can be easily scaled-up to industrial level (Yang and Huang, 2013). The extraction conditions using UAE are influenced by several parameters such as extraction time, extraction temperature, solvent to solid ratio and ultrasonic power (Zhao *et al*., 2011). For optimizing UAE conditions, the use of Response surface methodology (RSM) would be advantageous. It is an efficient mathematical and statistical tool for optimization of a process (Liu and He, 2016). The RSM technique has advantages over the conventional techniques where only one variable is studied at a time. While being economical and time saving, RSM can study the interaction between independent variables and provides vast information (Ahmad and Raish, 2015). For this study, the Box-Behnken design (BBD) was selected as it does not include an embedded factorial or fractional design (Box and Behnken, 1960).

Preparative HPLC has been in use for separation and purification of natural products for high purity, resolution and selectivity (Sticher, 2008; Sarker and Nahar, 2012). Unfortunately, this high quality purification technique requires the use of a long column and

large amounts of solvents. In contrast, recycling preparative HPLC uses a short column and require considerably lesser quantities of solvent for higher recovery yield of the target compounds, purity and resolution, as well as able to separate small quantitative bioactive constituents with close similar structures (Sidana and Joshi, 2013). In this study, for the very first time, a BBD was used to optimize the extraction conditions (extraction time, extraction temperature and solvent to sample ratio) for extracting anthraquinones from the roots of *C. singueana* and recycling preparative HPLC technique was employed to isolate the anthraquinones. The anthraquinones were subsequently identified as islandicin (**1**) (Fiaz *et al.*, 2013), 1,8-dihydroxy-2,6-dimethyl-3,7-dimethoxyanthraquinone (**2**), xanthorin (**3**) (Zhao *et al.*, 2011), 7-methylphyscion (**4**) (Kitanaka *et al.*, 1985), physcion (**5**) (Renuka *et al.*, 2010), erythroglaucin (**6**) (Jiang *et al.*, 1990), chrysophanol (**7**) (Renuka *et al.*, 2010) and 1-*O*-methylparietin (**8**) (Manojlovic *et al.*, 2005) (Figure 1). To the best of our knowledge, this is the first ever report describing the isolation of compounds (**1)**, (**2)**, (**3)**, (**6)** and (**8)** from the roots of *C. singueana*.



Anthraquinones	R	R’	R’’	R’’’	R’’’’
Islandicin (**1**)	H	H	H	CH_3	OH
1,8-Dihydroxy-2,6-dimethyl-3,7-dimethoxyanthraquinone (**2**)	CH_3	OCH_3	CH_3	OCH_3	OH
Xanthorin (**3**)	OCH_3	H	H	CH_3	OH
7-Methylphyscion (**4**)	OCH_3	CH_3	H	CH_3	H
Physcion (**5**)	OCH_3	H	H	CH_3	H
Erythroglaucin (**6**)	OCH_3	H	H	CH_3	OH
Chrysophanol (**7**)	H	H	H	CH_3	H
1-*O*-Methylparietin (**8**)	CH_3	H	H	OCH_3	H

Figure 1: Chemical structure of anthraquinones (**1**-**8**) isolated from the root of *C. singueana*

Experimental

Plant materials and chemicals

The roots of *Cassia singueana* Del. were collected in August 2014, from Bauchi State, Nigeria. The plant was identified by Mr. Baha’uddeen Said Adam and a voucher specimen (BUKHAN 0316) has been deposited at the Herbarium of the Department of Plant Biology, Bayero University Kano, Nigeria. The plant sample was air-dried, ground and sieved through a mesh to obtain 60 mesh particle sizes. Chloroform (HPLC grade) and methanol were purchased from Sigma Aldrich (St. Louis, USA). Ultrasonic cleaning bath (Elmasonic, Elma Schmidbauer GmbH Germany) with a frequency of 60 kHz and a power of 750 W, equipped with time and temperature controller, a recycle preparative HPLC, Japan Analytical Industry (JAI LC-9110 II

NEXT) instrument equipped with JAIGEL polystagel column (20 mm id × 600 mm length) 1H-40 (exclusion limit of 1 × 103) and 2.5H-40 (exclusion limit of 2 × 104) and UV flash as a detector were used.

Experimental design

RSM was employed using a three-variable BBD (software Design Expert V 7.1.6) to evaluate the behaviour in the optimum region of extraction yield, Y (%), for an UAE of *C. singueana* root. The relevant independent variables considered for optimizing the extraction protocol was as follows: *(A)*: extraction time (min), *(B)*: extraction temperature, and *(C)*: solvent to sample ratio, the effects of unexplained variability in the response (extraction yield) due to extraneous factors were minimized by randomizing the order of the experiments. Twelve experiments were enhanced with five replications to assess the pure error to afford a BBD that consisted of 17 runs, each one evaluated at three different levels (−1, 0, +1). Table 1 depicts the rank of the independent variables, levels and experimental design in coded and decoded terms.

Table 1. BBD matrix showing the independent variables rank, levels and experimental data for a three-factor-3-level response surface analysis, represented in both coded and decoded terms.

Independent Variables	Symbols	Levels		
		−1	0	+1
Time	*A*	10	25	40
Temperature	*B*	40	45	50
Solvent-sample ratio	*C*	10	20	30

The runs were performed in random order.

Data analysis

As describing the experimental data for the curvature of the system was pivotal in this study, a second-order polynomial equation was employed for the fitting process. The variables were expressed individually as a function of the independent variables. The generalised second-order polynomial model for predicting the optimal point in the response surface analysis is illustrated below (Equation **1**);

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad Equation\ \mathbf{1}$$

where Y is the predicted response, β_0 , β_i , β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j are the independent variables. Contour and response surface plots were generated by varying two variables within the experimental range and one variable was held constant at the central point.

The terms in the regression equations were examined for statistical significance using ANOVA for each response and only variables that are significant ($p \leq 0.05$) were fitted. The

optimisation was obtained by the desirability function. The optimized conditions as per the Derringer's desirability prediction tool of response surface methodology for the independent variables were used to validate the model. The quality of the fit of the polynomial model used to evaluate the major and interactive effects between the dependent and independent variables was expressed by the value of coefficient of determination, R^2. The main indicators to represent the significance and adequacy of the generated model in this study cover the model F-value and lack of fit, probability value (*Prob* > P), Adjusted coefficient of determination (*Adj,* R^2*)* and Adequate precision.

Verification of model

For verifying the prediction power and adequacy of the model, the optimum conditions predicted were validated using the results obtained under specific experimental conditions. Experiments were performed in triplicates using the optimised parameters proposed by the BBD optimization software. The theoretical predicted data were then compared with the experimental data as shown in Table 2.

Table 2. BBD matrix along with the experimental and predicted values for the extraction yield of *C. singueana*

Run *	*A,* time (min)	*B,* temperature (°C)	*C*, sample to solvent ratio (mL/g)	*Yield,* (%)	
				Actual	Predicted
1	25 *(0)*	40 *(−1)*	30 *(+1)*	0.76	0.80
2	10 *(−1)*	40 *(−1)*	20 *(0)*	1.69	1.63
3	25 *(0)*	45 *(0)*	20 *(0)*	1.13	1.18
4	25 *(0)*	45 *(0)*	20 *(0)*	1.61	1.56
5	25 *(0)*	45 *(0)*	20 *(0)*	0.90	0.85
6	10 *(−1)*	50 *(+1)*	20 *(0)*	1.76	1.81
7	10 *(−1)*	45 *(0)*	10 *(−1)*	1.23	1.18
8	25 *(0)*	40 *(−1)*	10 *(−1)*	1.38	1.43
9	25 *(0)*	45 *(0)*	20 *(0)*	1.19	1.20
10	40 *(+1)*	45 *(0)*	30 *(+1)*	1.65	1.64
11	40 *(+1)*	45 *(0)*	10 *(−1)*	1.45	1.46
12	40 *(+1)*	50 *(+1)*	20 *(0)*	1.34	1.33
13	40 *(+1)*	40 *(−1)*	20 *(0)*	1.50	1.52
14	25 *(0)*	50 *(+1)*	30 *(+1)*	1.47	1.52
15	25 *(0)*	50 *(+1)*	10 *(−1)*	1.54	1.52
16	10 *(−1)*	45 *(0)*	30 *(+1)*	1.60	1.52
17	25 *(0)*	45 *(0)*	20 *(0)*	1.49	1.52

* The runs were carried out in random order.

Extraction and Isolation

Air-dried and powdered root of *C. singueana* (20 g) was extracted with methanol (300 mL) using UAE under the optimized conditions of 50 min extraction time, 50°C extraction temperature and 10 mL/g solvent to sample ratio. The solvent was removed using a rotary

evaporator to yield a dark brown residue (0.55 g). The residue was fractionated over silica gel column chromatography using *n*-hexane, *n*-hexane/ethyl acetate and ethyl acetate gradient as eluting solvents. Similar fractions were combined based on their TLC profile to obtain four fractions (F1–F4). Fraction three (F3) (320 mg) was further subjected to silica gel chromatography (70 – 230 mesh) to obtain 8 sub-fractions (f1-8). Based on the TLC profile the sub-fractions, f2-5 was further purified by recycling preparative HPLC. Fraction, f2-5 (250 mg) was dissolved in chloroform (HPLC grade, 3 mL) and filtered using microfiltration membrane (0.45 µm). Sample fraction f2-5 (2.5 mL) was injected into the recycling preparative HPLC (JAIGEL column) and eluted under isocratic condition using degassed chloroform (HPLC grade, 100%), at a flow rate of 3.5 min/mL. The eluates were subjected to four cycles and components at various retention times in the eluate were detected by UV detector at 238, 254, 280 and 300 nm to afford compounds, **1** (t_R = 104 min, 12 mg), **2** (t_R = 108 min, 2 mg), **3** (t_R = 187 min, 5 mg), **4** (t_R = 226 min, 16 mg), **5** (t_R = 229 min, 60 mg), **6** (t_R = 234 min, 2 mg), **7** (t_R = 297 min, 90 mg) and **8** (t_R = 301 min, 12 mg). These compounds were identified by spectroscopic means and were compared with respective published data.

Results and discussion

Model fitting of parameters based on the extraction yield of *C. singueana*

To optimize extraction of the *C. singueana* roots, the ranges for extraction time (10 – 40 min), extraction temperature (40 – 50°C) and solvent-sample ratio (12:1 – 25:1, v/w) were selected based on the logic range obtained from the one variable at-a-time (OVAT) experiments (results not shown) and these variables were optimized by the Box-Behnken design (BBD). The analysis of variance (ANOVA) for the extraction yield of the *C. singueana* roots from BBD is shown in Table 3. The *P* value < 0.05 indicates the model term is significant and *P*-value < 0.0001 is suggestive of a highly significant term.

Table 3. ANOVA for the second-order polynomial model of the BBD

Source of variation	Sum of squares	Mean squares	Degree of freedom	F value	P value
Model	1.18	0.13	9	30.47	< 0.0001***
Residual	0.030	4.305E-003	7		
Lack of fit	0.020	6.677E-003	3	2.64	0.1855
Pure error	1.21		16		

*** indicates that the effect is very significant, no asterisk indicates that the effect is not significant

The fitting of the model was assessed by a numerical method, which included determinant coefficient (R^2) and adjusted determinant coefficient (*Adj*. R^2). The very high value of the determinant coefficient, (R^2 = 0.9751) of the model showed that 97.51% of the variation in the extraction yield of the *C. singueana* roots could be explained by the generated model. Likewise, the adjusted determinant coefficient (*Adj*. R^2 = 0.9431) was found to be relatively close to the value of R^2, indicative of an excellent fit as well as a good statistical model. Furthermore, the lack of fit test, which determines the adequacy of the selected model to describe variations in the experimental data around the fitted model, revealed a

very small *F*-value = 2.64, and an insignificant P value = 0.1855. These values indicated the lack of fit was not significant relative to the pure error, inferring the model was appropriate. A model with ill-fitting data might exhibit a significant lack of fit (P value > 0.05), therefore proceeding with the optimization of the fitted response could yield poor or misleading results. The suitability of the model in this study was also reflected in the high value for adequate precision (*Ad_{eq} precision*) which measures the signal to noise ratio. A value of *Ad_{eq} precision* > 4 is deemed desirable, hence and *Ad_{eq} precision* of 20.033 for the model indicated an adequate signal. All the above data strongly signified reliability and accuracy of the model to represent the relationship between the response (% *yield*) and the variables.

Analysis of the regression coefficients and the response surface

The linear and quadratic effect of the independent variables and their regression coefficients on the response variables were analysed and illustrated in Table 4. The linear effects were confirmed to be statistically significant to affect the extraction yield, as indicated by the P value with *A* (P value < 0.0001) being the most significant (Table 4). The data confirmed that the increase in time and temperature of the sonication process would improve the yield of anthraquinones extracted from the *C. singueana* roots. Consequently, a higher extraction temperature tends to increase the solubility and diffusion coefficient of anthraquinones (Roque *et al.*, 1974) that favour a higher extraction rate. Similarly, a longer extraction time improves the phenomenon of cavitation that will be further discussed below. The exception, however, was *C* (P value = 0.6067). Interaction terms *AB, AC and AB,* as well as the quadratic term, A^2 were also significant. A negative quadratic effect of A^2 was attained for extraction yield implying that there is a maximum for the extraction yield of *C. singueana* at a certain extraction time; the yield starts to decline when the duration is exceeded. The phenomenon occurring beyond this point might be explained by Fick's second law of diffusion, which predicts a final equilibrium has been reached between the concentrations of solute in the solution and in the solid matrix. Therefore, extending the sonication time beyond this point is futile for extracting more anthraquinones. In contrary, the linear and quadratic terms for *C* were not significant in improving the extraction yield of *C. singueana* roots, due to large P-values (> 0.05) (Table 4). Consequently, the surface and contour plots were generated by the model to facilitate the visualisation of the significant factors derived from the statistical analysis (Figures 2-4).

The interactive effect of extraction time (*A*) and temperature (*B*) on the extraction yield (*Y*) of *C. singueana* roots at a fixed ratio of solvent to sample (20 mL/g) is depicted as surface and contour plots in Figures 2a and 2b, respectively. The interactions of the two variables were significant due to a small P-value (0.0116) (Table 4**)**. The effect of extraction time (F value = 169.35) was more significant when compared to the extraction temperature (F value = 11.71), in yielding higher extraction yields of *C. singueana*.

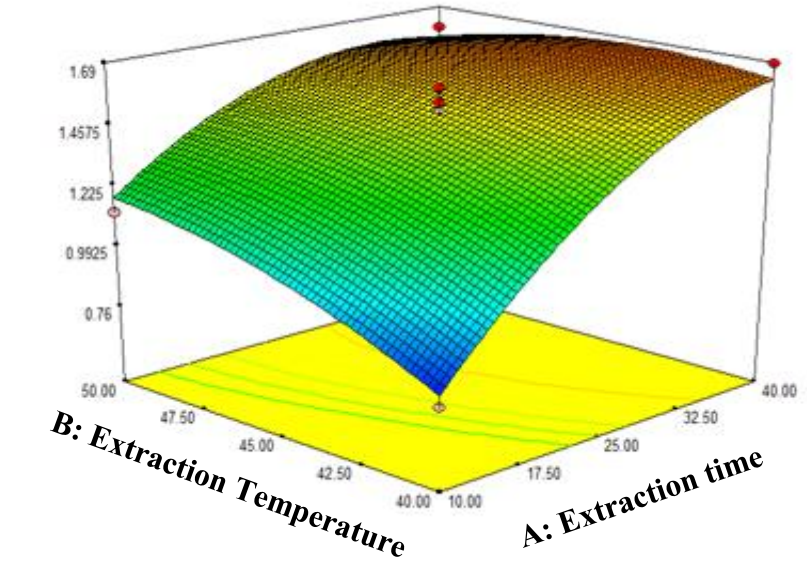
An extraction yield as high as 1.65% was attainable when the extraction time (*A*) and temperature (*B*) were set anywhere between 32 – 40 min and 42 – 47°C, respectively (Figure 2). Further increasing the sonication conditions beyond the maximum points of each variable only led to the decline in the response. Interestingly, a positive interaction term (+ 0.11 *AB*) between extraction time and temperature (Table 4), indicated a synergistic effect between the two variables. The data inferred the extraction yield of the *C. singueana* root extract was

influenced by the extent of time and temperature of sonication used in the extraction process. This means the extraction yield might generally increase with longer extraction time and higher temperature. A similar phenomenon has been reported in the literature (García-Pérez *et al.*, 2007). The higher extraction yields observed between 32 – 40 min could be explained by the increase in cavitation effect when longer sonication time is applied. This permits sufficient contact time for the mechanical effect of ultrasound (cavitation) to disrupt the cell wall of the *C. singueana* hence, concomitantly facilitating internal diffusion and enhancing mass transfer of anthraquinones into the bulk liquid. Aside from enabling greater penetration of methanol into the matrix of *C. singueana* roots, the cell wall pores are enlarged from the swelling and hydration, and finally disrupted to release more of the anthraquinones. Higher sonication temperatures also promote cavitation as diffusion of anthraquinone contents out from the plant sample into the bulk liquid are accelerated (Soria and Villamiel, 2010).

Table 4. ANOVA for the second-order polynomial models and coefficient values for extraction yield obtained from the root of *C. singueana*

Variables	*Regression coefficient*	*Sum of squares*	*Mean square*	*F-values*	*p-value*
Interaction	1.52				
Linear					
A	+ 0.30	0.73	0.73	169.35	< 0.0001**
B	+ 0.079	0.05	0.05	11.71	0.0111*
C	- 0.0126	1.250E-003	1.250E-003	0.29	0.6067
Interaction					
AB	+ 0.11	0.05	0.05	11.50	0.0116*
AC	- 0.18	0.13	0.13	29.27	0.0010*
BC	- 0.14	0.081	0.081	18.87	0.0034*
Quadratic					
A^2	- 0.16	0.11	0.11	24.39	0.0017*
B^2	- 0.068	0.019	0.019	4.51	0.0713
C^2	- 0.44	8.217E-003	8.217E-003	1.91	0.2096

**indicates that the effect is highly significant, * indicate that the effect is significant; no asterisk indicates that the effect is not significant



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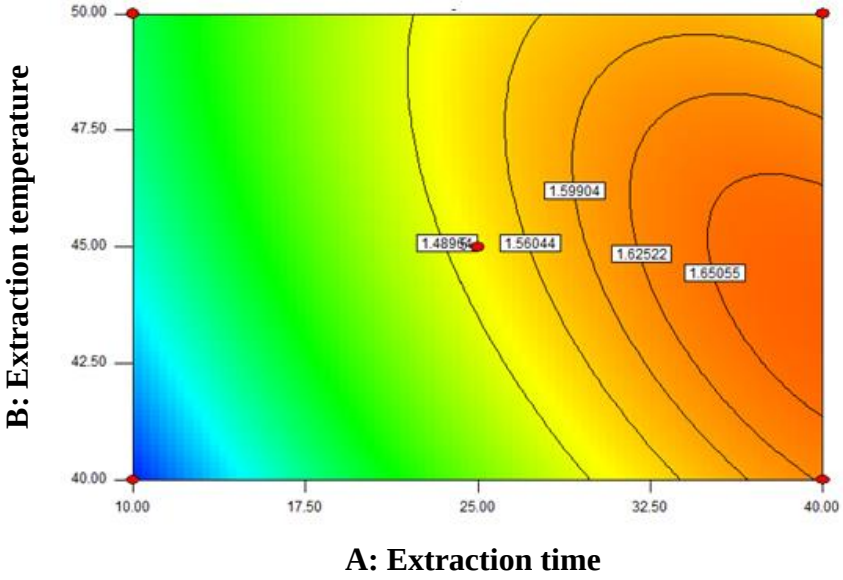
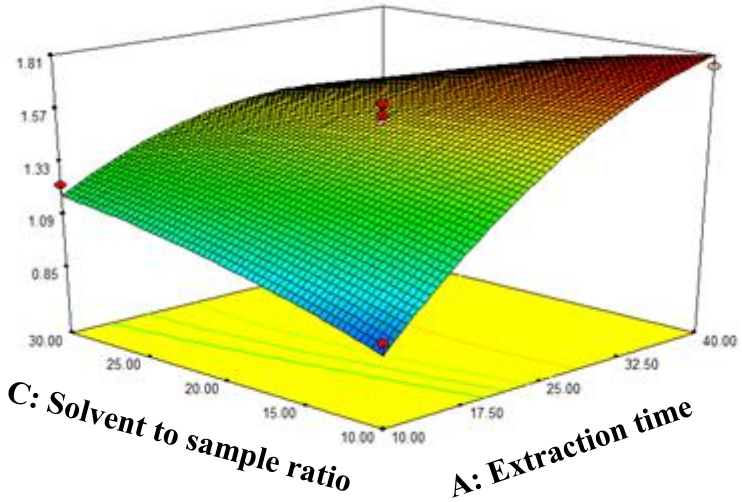


(b)

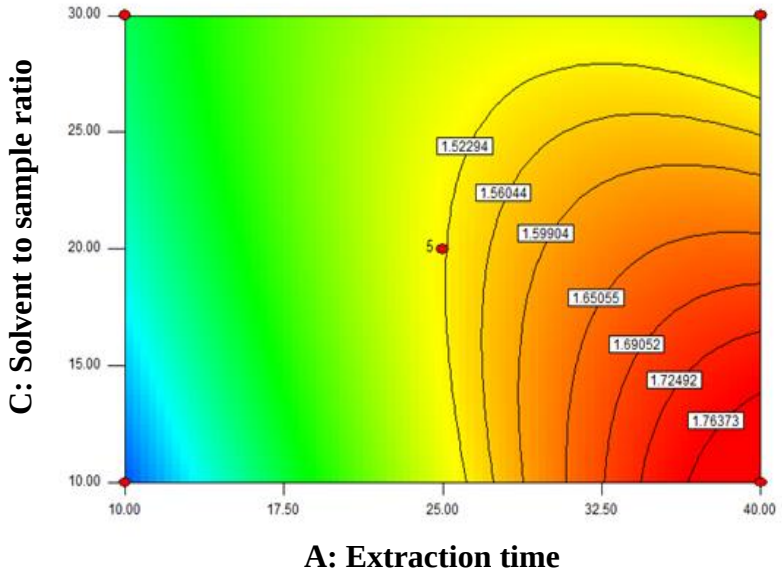
Figure 2. Graph illustrating the (**a**) response surface and (**b**) contour plot showing the effect of extraction time and extraction temperature ratio, and their mutual interaction for the UAE of *C. singueana* root at a constant solvent to sample ratio (20 mL/g)

Figures 3a and 3b illustrates the surface and contour plots for the interactive effect between extraction time (*A*) and solvent to sample ratio (*C*) on extraction yield. The mutual interaction between extraction time and solvent to sample ratio was highly significant (P value = 0.001) with extraction time (F value = 169.35) being more significant than the effect of solvent to sample ratio (F value = 0.29) (Table *4*). A maximum extraction yield of approximately 1.76% could be achieved from the plant roots when the sonication time was extended between 35−40 min using lower solvent sample to sample ratios that ranged between 10−14 mL/g. The negative interactive term, *AC* (- 0.18) was suggestive of an inverse

proportionality or antagonistic effect (Table **4**). The outcome observed here agrees with Fick's second law of diffusion, owing to the attainment of equilibrium as previously mentioned (Silva *et al.*, 2007). Pertinently, the higher solvent to sample ratio was found to be counterproductive to the sonication process and gave lower extraction yields even when the longest extraction time was used.



(a)



(b)

Figure 3. Graph illustrating the (**a**) response surface and (**b**) contour plot showing the effect of extraction time and solvent to sample ratio, and their mutual interaction for the UAE of *C. singueana* root at constant extraction temperature (45°C)

The surface and contour plots for the interactive effect of extraction temperature (*B*) versus solvent to sample ratio (*C*) on the extraction yield of *C. singueana* roots is illustrated in Figures 4a and 4b. It was clear the linear effect of extraction temperature (F value = 11.71)

was more significant than the variable, solvent to sample ratio (F value = 0.29) and a small P-value (0.0034) indicates their interaction was significant. Notably, the negative model term (–0.14 *BC*) (Table 4) reveals antagonistic behaviour between the variables, indicating that increasing both variables to their maximum values will not improve the extraction yield of the *C. singueana* roots.

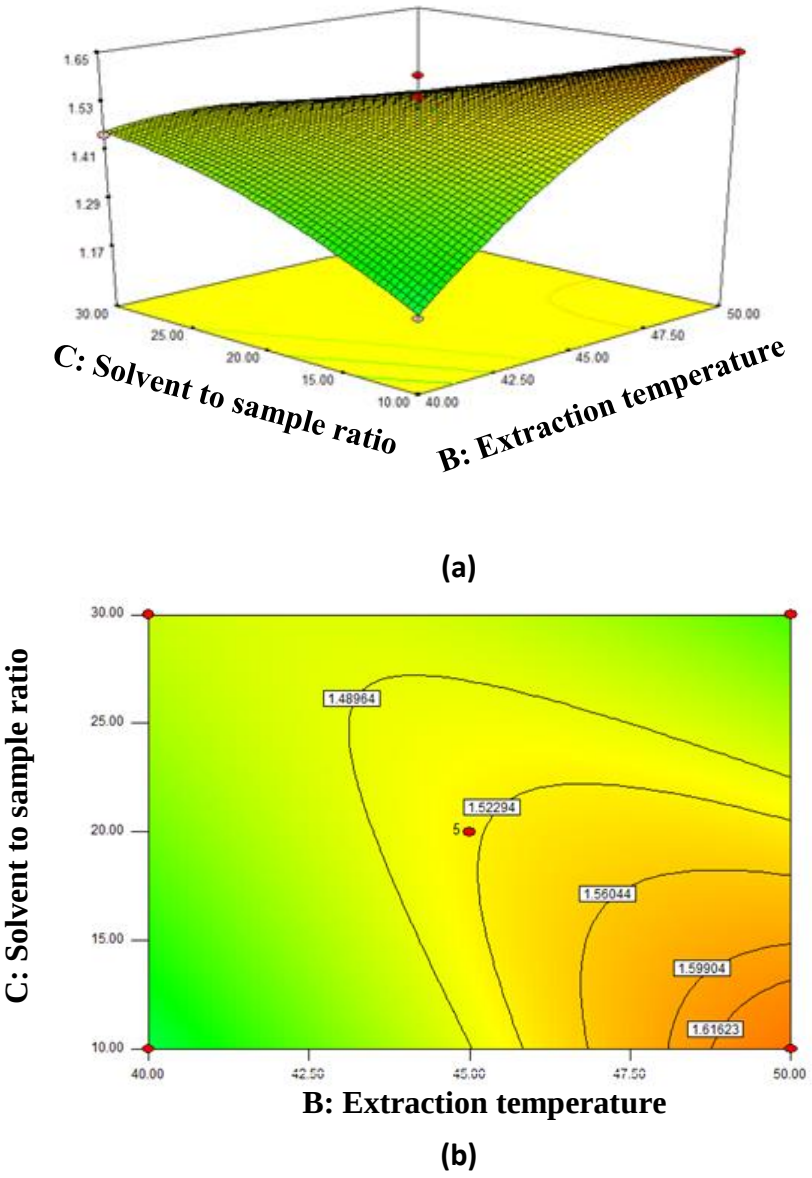


Figure 4. Graph illustrating the (**a**) response surface and (**b**) contour plots showing the effect of extraction temperature and solvent to sample ratio, and their mutual interaction for the UAE of *C. singueana* root at constant extraction time (25 min)

An extraction yield as high as 1.61% was apparent when the solvent to sample ratios and the extraction temperatures are set below 15 mL/g and 48−50°C (Figure 3b), respectively, which corroborates the negative regression coefficient. It was evident that the higher extraction temperature of the system was beneficial in yielding higher extractions yields. The higher kinetic energy of the system would have promoted high velocity collisions, and

accelerated the penetration of methanol into the cell wall of *C. singueana*. Hence, the diffusion of anthraquinones out from the sample into the solvent are facilitated. Also, viscosity and density the solvent (methanol) are reduced at higher extraction temperatures (Vilkhu *et al.*, 2008). On the other hand, lower extraction yields were obtained when solvent to sample ratio were increased > 15 mL/g and using lower extraction temperatures < 48˚C were presumably due to reduced velocity of inter-particle collisions. Hence, the diffusion of anthraquinones out of the sample into the bulk liquid became less efficient.

Verification of the model

The adequacy of the predictive extraction model was realized under the optimum response values i.e., highest extraction yield of *C. singueana* roots. The highest yield was verified using an optimum extraction condition proposed by the model with the highest desirability value (93.9%). The optimum condition generated by the BBD are as follows: extraction time (*A*) of 25.00 min, extraction temperature (*B*) of 50˚C, solvent to sample ratio (*C*) of 10 mL/g, whereby the predicted yield (*Y*) was 1.64%. The actual extraction yield (1.65 ± 0.07%) in the verification experiment agreed closely with that of the predicted, suggesting the obtained optimized condition was reliable and the RSM model accurate.

Isolation and identification of anthraquinones from the roots extract of *C. singueana*

The isolation of anthraquinones (**1**-**8**) from the roots of *C. singueana* was carried out by recycling preparative HPLC coupled with a UV detector using a JAIGEL polystagel column and isocratic mobile phase of chloroform (100%) at a flow rate of 3.5 mL/min and wavelengths of 238, 254, 280 and 300 nm. Four cycles were accomplished (Figure 5) to obtain, islandicin (**1**) as a reddish powder (t_R = 104 min), 1,8-dihydroxy-2,6-dimethyl-3,7-dimethoxyanthraquinone (**2**) as an orange-yellow powder (t_R = 108 min), xanthorin (**3**) as yellow powder (t_R = 187 min), 7-methylphyscion (**4**) as yellow solid (t_R = 226 min), physcion (**5**) as an orange yellow solid (t_R = 229 min), erythroglaucin (**6**) as yellow solid (t_R = 234 min), chrysophanol (**7**) as yellow powder (t_R = 297 min), and 1-*O*- methylparintent (**8**) as yellow solid (t_R = 301 min). Table 5 and 6 show the ^{1}H and ^{13}C NMR data for compounds (**1**-**8**) respectively.

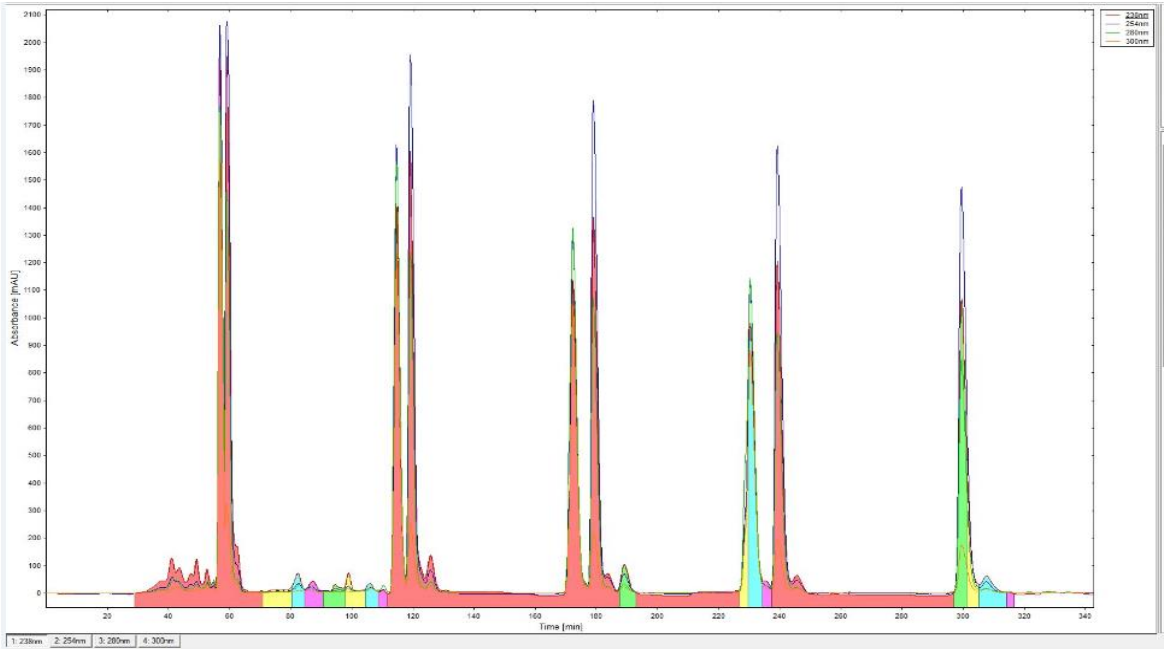


Figure 5: Chromatogram for recycling prep-HPLC for f2-5 of *C. singueana* roots extract (**1** = islandicin, **2** = 1, 8-dihydroxy-2, 6-dimethyl-3, 7-dimethoxyanthraquinone, **3** = xanthorin, **4** = 7-methylphyscion, **5** = physcion, **6 =** erythroglaucin, **7 =** chrysophanol, **8** = 1-*O*-methylparietin)

The response surface methodology using the BBD with the ultrasound-assisted extraction technique, together with recycling preparative HPLC, was successfully used for the extraction of *C. singueana* roots and isolation of anthraquinones. Pertinently, the coupling of RSM optimization of UAE of the *C. singueana* root with recycling preparative HPLC was advantageous in yielding high resolution separation of similar anthraquinones, whilst requiring a considerably shorter extraction time.

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