MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Selahuddeen *et al.* | Malaysian Journal of Fundamental and Applied Sciences, Vol. 20 (2024) 497-513

RESEARCH ARTICLE

Investigation on Optimized Flavonoid Extraction from *Leucas zeylanica* and Its Anthelmintic Activity

Muhammad Luqman Selahuddeen[a], Marouane Bouguerra[e], Siti Ernieyanti Hashim[a], Roswanira Abdul Wahab[a,d], Abdul Fatah A. Samad[b], Faizuan Abdullah[a,c]*

[a]Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [b]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [c]Centre for Sustainable Nanomaterials (CSNano), Ibnu Sina Institute for Scientific and Industrial Research (ISI-SIR), Level 4, Block T02, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [d]Advanced Membrane Technology Research Centre (AMTEC), Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [e]Department of Agri-Food, Environmental and Animal Sciences, University of Udine, Via Sondrio 2A, 33100 Udine, Italy

Abstract Extraction has been the primary method of concentrating and obtaining a crude essence of plants, and to an extend fruits and nuts. In modern times, there are many methods of extraction developed from simple maceration to using Soxhlet extractor to microwave assisted extraction and ultrasound assisted extraction. However, the more time saving and energy efficient method for the extraction of plants needs to be investigated. This research explored optimization of ultrasound assisted extraction of flavonoids from *Leucas zeylanica* and the optimization was done on parameters of sonication time (minutes), water bath temperature (°C), volume of solvent to solid ratio (ml/g) and solvent concentration (%) using response surface methodology (RSM). By measuring the total flavonoid content using aluminium colorimetric method and UV visible spectrophotometry, highest flavonoid yield is achieved with 30.38% increase compared to unoptimized method. This occurred with sonication time of 42 minutes, temperature of 55°C, solvent to solid ratio of 40 ml/g and solvent concentration of 100%. The resulting extract was then tested for its anthelmintic (anti-worm) ability, obtaining 40% efficacy of that of Albendazole.

Keywords: Optimization, Extraction, Flavonoid, Leucas zeylanica, Anthelmintic.

***For correspondence:**
faizuan@utm.my

Received: 21 Feb. 2024
Accepted: 25 March 2024

Introduction

Leucas zeylanica (L.) W.T.Aiton as per the taxonomy on World Flora Online ("World Flora Online," 2022), or Ceylon slitwort is a tropical plant common to parts of Asia including India, Myanmar, and Malaysia, with a maximum height of 30 cm and abundantly found on sandy soil [1-4]. The curative and medicinal properties of this plant are not extensively studied, even though it has been the central ingredient in many local and traditional medicinal concoction in parts of India Bangladesh and Malaysia [5]. Specifically, in Malaysia, in parts of the state of Kelantan, this plant is known to be used in their cooking and to cure worms in children [1].

The traditional recipe is to grind the plant, that grows wild on the sandy soil and beaches, with some limestone paste and applied topically on the stomach of children with worms [1]. This recipe has been passed down through generations among the Kelantanese people, but its mechanism has not been studied or its properties analysed scientifically, hence its absence in modern medicine. Studies on the

Leucas genus, specifically its chemical constituents show to be more than 10 groups of flavonoids and phenolic compounds present, including apigenin and tricin [3, 4, 6-8]. It can be proposed that these secondary metabolites, whether individually or in synergy, may be responsible for the medicinal properties of this plant, including anthelmintic [2, 3, 6, 9, 10].

Once inside the body, these worms take up residence in the gastrointestinal system of the infected individuals, where they feed on nutrients derived from digested food and even directly from blood vessels. This parasitic infestation can result in malnutrition, poor weight gain, and hinder both physical and intellectual growth in affected children. The life cycle of helminths is complex, involving egg, larvae, and adult stages [11] and current prescribed drug such as albendazole works by disrupting growth requirements of the helminth [12]. As a result of the drug's action, it eliminates mature or hatched worms but does not affect the eggs, which makes it an ineffective one-time solution. Patients often require multiple doses to completely rid themselves of the parasites.

While research into natural anthelminthic agents has been conducted, it has primarily focused on addressing helminth infections in livestock rather than humans [13-15]. Developing more effective and accessible treatments for human helminth infections remains an important area of study and public health concern, especially in regions with limited access to healthcare and sanitation facilities [13-16]. Not only natural anthelminthic is more sustainable, but the cost of production could also be less than conventional drugs. Also, the suggested use of *Leucas zeylanica* (LZ) extract is topical and not ingested like current drugs, making it easier to apply to children of all ages.

Current extraction methods are not optimized for the purpose of extracting flavonoid compound from *Leucas zeylanica* for maximum flavonoid yield [3, 6, 17]. In order to identify and quantify the bioactive compound of *Leucas zeylanica*, an optimized extraction method is crucial especially by utilizing response surface methodology (RSM) [18].

Given that LZ is primarily used in traditional medicine, there is currently not many published records detailing its chemical compounds and active ingredients [3, 4, 8]. The application of chromatographic techniques, such as liquid chromatography with tandem mass spectrometry (LCMS-MS), could provide valuable insights into the chemical constituents of LZ. This analysis has the potential to uncover not only the plant's active compounds but also other potential medicinal uses beyond anthelminthic properties.

Conducting an assay would also allow for the determination of LZ's anthelmintic potential, as well as establishing dosage information or the lethal concentration (LC50). This research could contribute to a better understanding of the plant's therapeutic properties and broaden its potential applications in modern medicine.

Materials and Methods

The LZ plant are obtained from a local area in Kelantan, Malaysia (6°09'42.7"N 102°19'51.2"E) during the month of April 2019. Identification of the plant was authenticated at herbarium of Universiti Kebangsaan Malaysia (UKM) with voucher number of UKMB40376. For the extraction method, methanol, CH_3OH (70%) solvent of industrial grade by QRec company will be used. A few standard solutions that will be used are tannic acid, gallic acid, apigenin, and quercetin purchased from Sigma-Aldrich.

Besides that, 7.5% Sodium Carbonate, sodium nitrite, aluminium (III) chloride, and sodium hydroxide will be used for antioxidant assays preparation. Folin- Ciocalteu and Folin – Denis reagents purchased from Sigma-Aldrich.

All the glassware such as conical flasks, beakers, vials, measuring cylinders, volumetric flasks, are washed with distilled water before use while used glassware will be soaked in an acid bath followed by rinsing with distilled water before washing with detergent and dried in an oven at a temperature of 95˚C to 100˚C.

The plants are washed with distilled water upon arrival and then rinsed to remove excess water before leaving to dry in the oven (Memmert UF55). After drying, the leaves would be separated and used in further testing while the branch discarded. The dried leaves are then grinded to powder using Wellmac Grinder.

Sample Extraction and Optimization using RSM

For optimization using Response Surface Methodology (RSM), factors like extraction temperatures (℃), extraction periods (minutes), solvent to solid ratio (ml/g), and ethanol concentrations (%) will be varied and the output value was that of the TFC value of each experiment. The minimum and maximum varied values were determined from preliminary single factor experiment (SFE). Using an analytical balance, 3 g of powdered material was weighed and then put in a 250 mL round bottomed flask, which was then filled with ethanol to the desired concentration. The round-bottomed flask was linked to a Liebig condenser, which was then put within the ultrasonic generator for use. The ultrasonic assistance enabled the mixture to reflux for a short period of time. Following the completion of the reflux procedure, the mixture was filtered through filter paper into a conical flask and allowed to dry on a hot plate to remove any remaining moisture. Total flavonoid content (TFC) will be the desired output. Table 1 below shows the parameters for the SFE.

Table 1. The value of each independent variable for SFE

Extraction temperatures			
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)
30	20	40	40
40	20	40	40
50	20	40	40
60	20	40	40
70	20	40	40
Extraction Time			
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)
30	10	40	40
30	20	40	40
30	30	40	40
30	40	40	40
30	50	40	40
Solid to solvent ratio			
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)
30	20	10	40
30	20	20	40
30	20	30	40
30	20	40	40
30	20	50	40
Concentration of ethanol			
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)
30	20	40	20
30	20	40	40
30	20	40	60
30	20	40	80
30	20	40	100

The highest TFC value achieved in each varying category would be the highest value of that parameter to be considered in the RSM.

RSM, or Response Surface Methodology, can be characterized as a method involving intricate calculations to optimize processes. This approach entails creating an appropriate experimental design that encompasses all independent variables and utilizes the experimental data to derive a set of equations for estimating theoretical output values. These output predictions are derived through a meticulously planned regression analysis based on controlled independent variable values. Subsequently, the dependent variable can be forecasted using the updated values of independent variables. Employing the Box-Behken method, the following table was generated within the Design Expert software (Design Expert 13, version 13.0.5.0 64 bit) to optimize extraction, focusing on various factors with the desired outcome being the yield percentage.

In the Design Expert, a model equation is used in order to approximate the optimum value and determine the interaction between the variables, and is usually a quadratic equation model which is

stated as below in equation 1:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X^2{}_j + \sum_{i=1}^{j=1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \varepsilon$$

(Equation 1)

In this equation, Y represents the response, i is the linear and j is the quadratic coefficients, Xi is the uncoded independent variables and Xj is the regression coefficient, k is the number of studied and optimized variables, β0 is a constant coefficient, while βj, βjj, and βij are the interaction coefficients of linear, quadratic and second-order items, and ε is the error.

Table 2 Experimental design for extraction of LZ

Time of extraction (minutes)	Temperature of water bath (°C)	Solvent concentration (%)	Solvent to solid ratio (ml/g)	Run
40	60	80	40	exp 10
40	60	80	40	exp 13
40	60	80	40	exp 16
50	60	80	40	exp 11
40	60	80	40	exp 19
40	60	60	40	exp 29
40	60	80	40	exp 8
40	60	80	40	exp 2
40	50	80	40	exp 7
40	60	80	50	exp 30
50	50	100	30	exp 17
50	50	100	50	exp 23
30	50	100	50	exp 15
40	60	100	40	exp 3
40	60	80	30	exp 28
50	70	100	50	exp 4
30	50	100	30	exp 27
50	70	100	30	exp 5
30	70	60	50	exp 25
40	70	80	40	exp 20
50	50	60	30	exp 1
50	50	60	50	exp 18
30	70	100	50	exp 24
30	70	60	30	exp 26
50	70	60	30	exp 9
50	70	60	50	exp 21
30	70	100	30	exp 6
30	60	80	40	exp 14
30	50	60	50	exp 22
30	50	60	30	exp 12

All the 30 runs which have been proposed by Design Expert are represented in Table 2 and were done using the same apparatus and machine. The average (mean) value of the TFC was calculated from the 3 replicates and inserted to the Design-Expert software, additionally the experiments were done randomly to reduce the effect of systematic errors. This model suggested by Design Expert was a quadratic model and the mathematical relationship of the variables and the response is demonstrated by the second-order polynomial.

Occasionally, the model derived from fitting a function to the data may struggle to accurately represent the entire experimental domain. To evaluate the model's quality, the Analysis of Variance (ANOVA) is commonly employed. ANOVA helps distinguish differences attributed to adjustments in the mixing of variable levels from variations arising due to random errors inherent to the response. In this particular study, ANOVA was conducted to validate the significance of the experimental design recommended by the Box-Behnken Design (BBD) Response Surface Methodology (RSM) and to confirm that the model aligns well with the experimental data.

In order to verify the model sufficiency, several methods have been done through ANOVA, which includes residual analysis, testing lack of fit, F-value, p-value, prediction error sum of squares (PRESS), pure error, coefficient of determination (R2), adjusted R2 and predicted R2. Coefficient of determination (R2) is commonly used to describe the model desirability for the experimental domain and is calculated from PRESS, however the method does not necessarily have a good regression if R2 has a large value, because addition of variables can increase the value of R2, so a model with high R2 value still can have low response. Usually a model with R2 of above 80% is significant [19].

The F-test is a statistical measure used to assess the overall significance of a method, with reference to the F-distribution table. In contrast, the p-value is employed to indicate the individual significance of each variable within the method. Typically, a variable is considered highly significant when it has a p-value of less than 0.05 and an F-test value greater than 0.1. In this context, smaller p-values are associated with greater significance.

The Lack of Fit (LOF) test is used to analyse the variation of the response compared to the fitted model. In this case, it is desirable for the LOF test to be non-significant, indicating that the model adequately fits the obtained results. In other words, a non-significant LOF test suggests that any unexplained variation in the response is random and can be attributed to noise rather than inadequacies in the model's fit.

The presentation of results from RSM is typically done using 3-dimensional graphical displays, which illustrate the relationships between variables while keeping other factors constant. Alternatively, these results can be visualized to showcase the influence of a single factor on the response. Another approach involves using a cube representation to depict the interaction of three factors on the response, although this is less common.

However, the most frequently utilized graphical models are either contour plots or 3-D views. These formats offer a clear and insightful visualization of the data, allowing for a better understanding of the relationships between variables and their impact on the response.

Total Flavonoid Content (TFC)

Total flavonoid content was measured by the aluminium chloride colorimetric assay. The standard solutions of quercetin (20, 40, 60, 80 and 100 ppm) (25 µL) were added in each microplate wells and a reagent blank was prepared using distilled water. The 0.066 M of $NaNO_2$ (110 µL) was added to the solutions and gently swirled. The mixtures were incubated in the dark for 5 minutes. Then, 0.75 M $AlCl_3$ (15 µL) was added into the mixtures and swirled again, then incubated for 6 minutes at room temperature. After incubation, 1 M of NaOH (100 µL) was added. The steps were repeated for an aliquot (25 µL) of crude methanol extracts (100, 200 and 1000 ppm) in the microplate wells. The absorbance against the reagent blank was measured at 510 nm. The quantification of TFC in the sample was compared to standard curve of quercetin. The total flavonoid content was expressed as mg quercetin equivalents per gram of dried extract (mg QE/ g DE).

Anthelmintic Assay

Anthelmintic assay is done to measure or qualify the anthelmintic activity of a compound or subject in response to a model organism, usually *C. elegans* or other suitable nematodes. The compound of interest or subject is introduced to the worm and the worm motility is observed as the criteria of whether the subject kills the worms.

Cultivation and Maintenance of *C. elegans*

Strain was grown on Nematode Growth Medium (NGM) in Petri dishes containing a lawn of the bacterium *Escherichia coli* OP50, grown at 20°C as per the studies using *C. elegans* [20]. Briefly, worm culture plates with eggs and egg laying adults were washed with M9 media and incubated with freshly prepared bleaching solution (4 ml of commercial bleach, in 9 ml of 1 M NaOH in water) for 3.5 min, followed by three washings with M9 [21]. Eggs in M9 buffer were kept on a rotator at 20 C overnight to hatch into L1s. L1s were put onto NGM plates the next morning and grown at 20 C. L3s were isolated from those plates after 24 h, L4s after 36 h and young adults after 48 h. These worms were washed with M9 before being used for assay.

Analysis of the Anthelmintic Activity

For determination and study of the anthelminthic effect of LZ, worm motility assay (WMA) was adapted and modified from a previous study [22, 23]. Adult earthworms were gathered from soil near the laboratory. Each earthworm was separately released into 10ml of desired formulation of control, reference drug and extracts in clean petri dishes. Group 1 earthworms were released in 10ml distilled water with 0.5 ml DMSO in a clean petri dish respectively which acts as a negative control. Group 2 earthworms were released in 10ml normal saline containing standard drug albendazole (25mg/ml) which is the reference drug in this study. Group 3,4 and 5 earthworms were released in each 10ml methanolic extracts solution while Group 6,7 and 8 earthworms in 10ml ethanolic extracts solution, both at concentrations of 25mg/ml, 50mg/ml and 100mg/ml respectively. Similarly Group 9,10 and 11 earthworms were released in 10ml normal saline containing 25mg/ml, 50mg/ml, and 100mg/ml of aqueous extracts, respectively. Before the initiation of experiment, the earthworms were washed with normal saline to remove all the faecal matter and waste surrounding their body. Earthworms were observed; the time taken for paralysis and the time taken for death was monitored and documented in minutes. Paralysis was said to occur based on the behaviour of earthworm with no revival body state in normal saline medium. Death was concluded after ascertaining that the earthworms neither moved when shaken vigorously nor when dipped in warm water (50°C) with faded body colour.

A 96 – well plate was prepared for 5 concentrations of extract with a positive and negative control in 3 replicates. From the dried extract, 80 mg was diluted with 2970 µL of M9 buffer and 30 µL DMSO [9]. From that stock solution, 10, 15, 20, 25, and 30 mg/mL plant extract solution were prepared. Corresponding volumes of 93.75, 140.6, 187.5, 234.3 and 281.2 µL were transferred to the 96 - well plate and made up to 300 µL with M9 buffer. 10 mg/mL of Albendazole suspension diluted with M9 buffer was used as positive control and DMSO diluted in M9 buffer was negative control. 10 young adult, healthy motile worms are transferred into each well. After 24 hours incubation in 20°C, number of non - motile worms in each plate were counted.

Worm mortality (%) = 100 x [(number of non - motile worms per well)/ (total number of worms per well)).

Results and Discussion

Optimization of Flavonoid Extraction using RSM

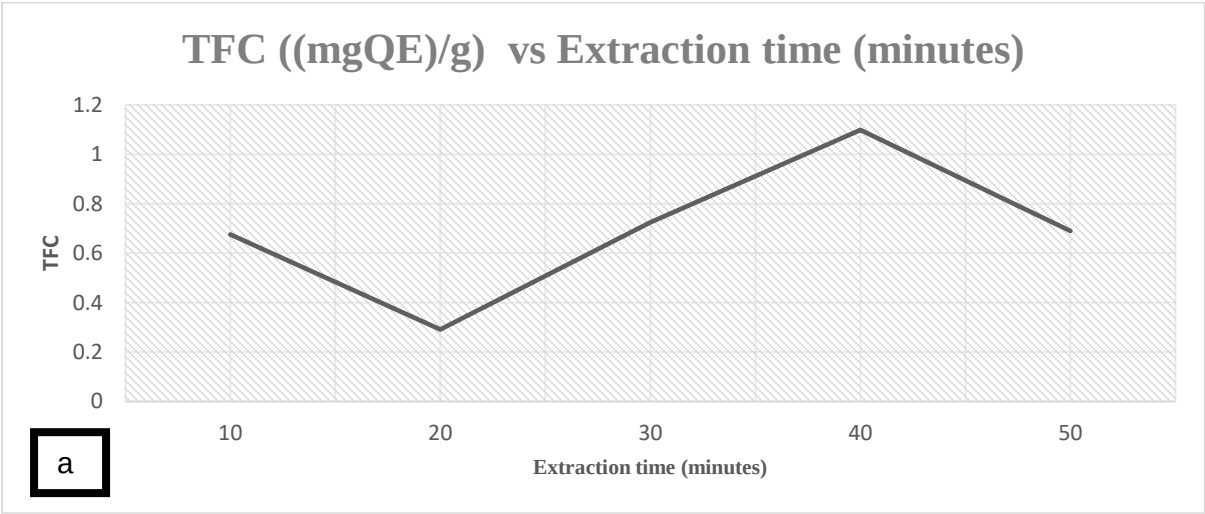
Data from the SFE is used as benchmarks for parameters in the RSM. Table 3 below is the TFC yield for each variable of the SFE.

Table 3 The TFC value of each variable of SFE

Extraction temperatures				
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)	Total flavonoid content ((mgQE)/g)
30	20	40	40	0.051982
40	20	40	40	0.008561
50	20	40	40	0.057064
60	20	40	40	0.832318
70	20	40	40	0.273112

Extraction Time				
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)	Total flavonoid content ((mgQE)/g)
30	10	40	40	0.675492
30	20	40	40	0.291219
30	30	40	40	0.724336
30	40	40	40	1.098496
30	50	40	40	0.689982
Solvent to solid ratio				
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)	Total flavonoid content ((mgQE)/g)
30	20	10	40	1.297169
30	20	20	40	1.335270
30	20	30	40	1.387601
30	20	40	40	1.808176
30	20	50	40	1.759818
Concentration of ethanol				
Extraction Temperature (℃)	Extraction time (minutes)	Solvent to solid ratio (ml/g)	Ethanol concentration (%)	Total flavonoid content ((mgQE)/g)
30	20	40	20	0.910681
30	20	40	40	0.748360
30	20	40	60	0.679804
30	20	40	80	4.196456
30	20	40	100	1.180756

Based on these results, the Figure 1 below was drawn to further illustrate the minimum and maximum values of each variable. It shows the range for extraction temperature 60°C to 70°C, 30 to 50 minutes of extraction time, 60% to 100% of solvent concentration, and 30 ml/g to 50 ml/g for solvent to solid ratio.



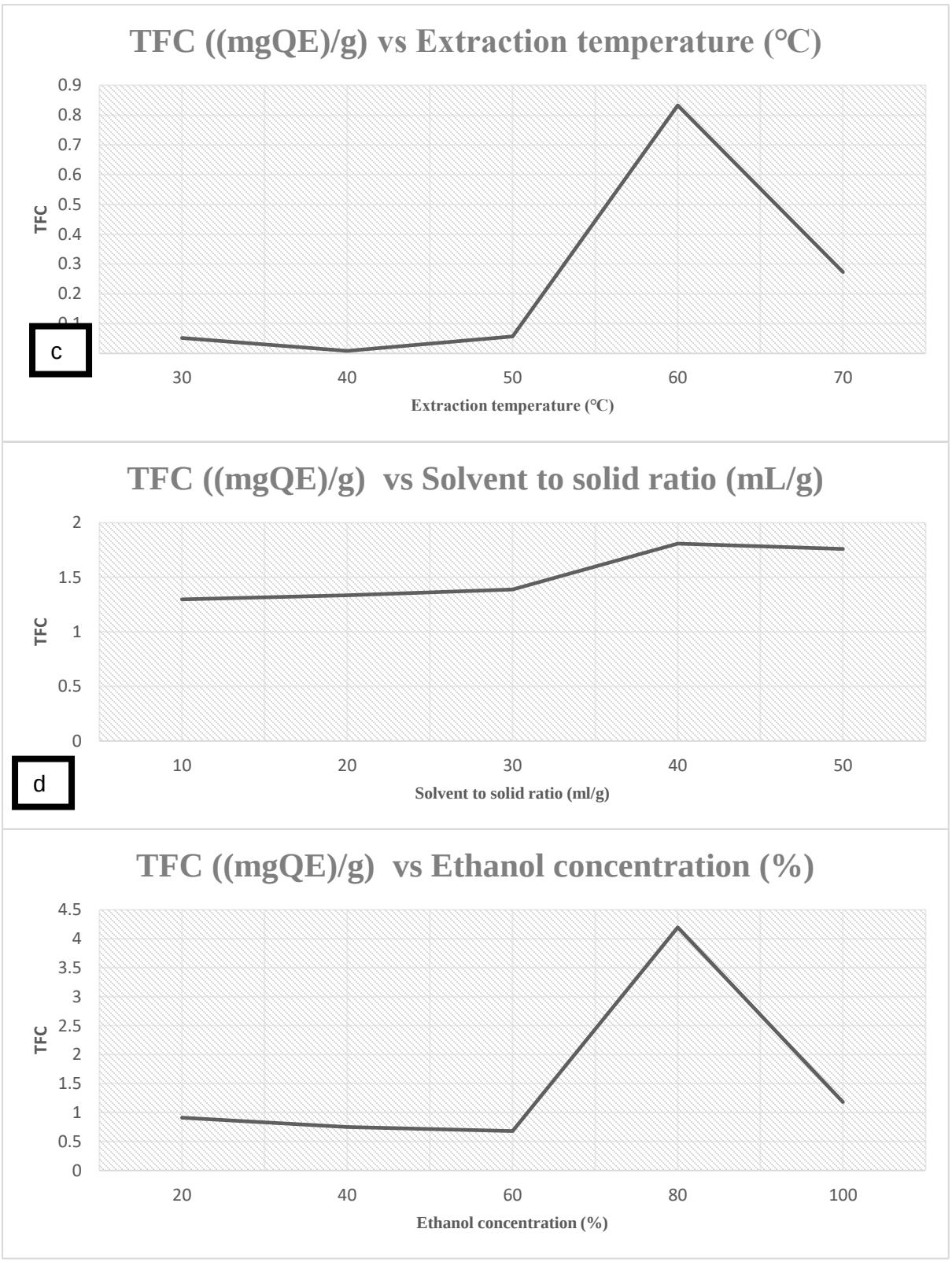


Figure 1 Graphs of each variable of SFE, (a) TFC against extraction time, (b) TFC against extraction temperature, (c) TFC against solvent to solid ratio, (d) TFC against ethanol concentration

From Table 3 and Figure 4, values for the parameters to be optimized were used as follows; extraction time is from 30 minutes to 50 minutes, extraction temperature is from 50°C to 70°C, solvent concentration is 60% to 100% and solvent to solid ratio is between 30 mg/ml to50 mg/ml. For comparison to RSM optimized method, values of 40 minutes extraction time, 60°C of extraction temperature, 40 mg/ml of solvent to solid ratio and 80% solvent concentration was used as the unoptimized method.

Optimized Extraction Method

According to BBD-RSM and Design-Expert software, 30 experiments were proposed for optimizing four variables, which have been done with three replicates. The actual values of the variables along with the response for each experiment are listed in Table 4, and the obtained results were analysed statistically and demonstrated graphically using 3-dimensional contour plots of the response model.

Table 4 RSM experiment

Run	Factor 1 A: Time (minutes)	Factor 2 B: Temperature (℃)	Factor 3 C: Solvent Concentration (%)	Factor 4 D: Solvent to solid ratio (ml/g)	Output)	
					TFC value	Percent compared to unoptimized method
1	40	60	80	40	0.785	37.41659
2	40	60	80	40	0.685	32.65014
3	40	60	80	40	0.635	30.26692
4	50	60	80	40	0.635	30.26692
5	40	60	80	40	0.585	27.8837
6	40	60	60	40	0.535	25.50048
7	40	60	80	40	0.535	25.50048
8	40	60	80	40	0.485	23.11725
9	40	50	80	40	0.585	27.8837
10	40	60	80	50	0.435	20.73403
11	50	50	100	30	0.335	15.96759
12	50	50	100	50	0.385	18.35081
13	30	50	100	50	0.385	18.35081
14	40	60	100	40	0.335	15.96759
15	40	60	80	30	0.335	15.96759
16	50	70	100	50	0.285	13.58437
17	30	50	100	30	0.335	15.96759
18	50	70	100	30	0.285	13.58437
19	30	70	60	50	0.285	13.58437
20	40	70	80	40	0.235	11.20114
21	50	50	60	30	0.235	11.20114
22	50	50	60	50	0.235	11.20114
23	30	70	100	50	0.235	11.20114
24	30	70	60	30	0.185	8.817922
25	50	70	60	30	0.185	8.817922
26	50	70	60	50	0.185	8.817922
27	30	70	100	30	0.185	8.817922
28	30	60	80	40	0.135	6.4347
29	30	50	60	50	0.085	4.051478
30	30	50	60	30	0.035	1.668255

Analysis of Variance (ANOVA) for Flavonoid Extraction of LZ

The extraction has been analysed by analysis of variance (ANOVA) with a 5% level of significance. Table 5 demonstrates the ANOVA for percentage of TFC value compared to normal Soxhlet extraction, where the model was significant with low p-value (0.0001) which is less than 0.05 thus support the quadratic model to describe the correlation of response and significant factors, and F-value of 11.58, that is greater than the tabulated F0.05 (14,15) which is 2.40, therefore the null hypothesis is negated, with zero as regression coefficients. Generally, F-value is obtained from the ratio between the mean of square regression or model and mean of square residual, while these two values are obtained from the ratio of sum of squares and sum of residuals with degree of freedom (DF), respectively [24].

Some individual parameter affect the response significantly, such as A (Time),C (solvent concentration) and some combined parameters like BC (temperature – solvent concentration), and D2 (solvent volume squared) significantly influence the response with p-values of less than 0.05, meanwhile the "Prob>F" values of greater than 0.1 is indicated as insignificant terms, so the lack of fit of the model with a p-value of 0.5446 was not significant relative to the pure error, which means there is a 20.35% chance that a "Lack of Fit F-value" this large could occur due to noise, thus non-significant lack of fit is good since we want the model to be fit.

Table 5 Analysis of variance (ANOVA) for flavonoid extraction of LZ

Source	Sum of Squares	Df	Mean square	F value	p-value	
Model	360.85	14	25.77	3.33	0.0001	Significant
A-Time	9.04	1	9.04	1.17	0.2968	
B-Temperature	12.19	1	12.19	1.58	0.2286	
C-Solvent concentration	14.47	1	14.47	1.87	0.1917	
D-Solvent Volume	2.56	1	2.56	0.33	0.5733	
AB	12.30	1	12.30	1.59	0.2266	
AC	4.33	1	4.33	0.56	0.4661	
AD	35.13	1	35.13	4.54	0.0501	
BC	27.05	1	27.05	3.50	0.0812	
BD	53.50	1	53.50	6.91	0.0190	
CD	17.31	1	17.31	2.24	0.1555	
A^2	15.44	1	15.44	2.00	0.1782	
B^2	24.02	1	24.02	3.10	0.0985	
C^2	12.80	1	12.80	1.65	0.2178	
D^2	84.18	1	84.18	10.88	0.0049	
Residual	116.08	15	7.74			
Lack of Fit	85.49	11	7.77	1.02	0.2035	not significant
Pure Error	30.59	4	7.65			
Cor Total	476.93	29				

Figure 2 shows the graph of experimental results against the predicted values for the flavonoid extraction of LZ with an R^2 value of 0.9018, which indicates that the data points are approximately linear and most of the values are predicted by the model, sustaining that the model and actual values from experimentation are close enough to make the model significant [24-26]

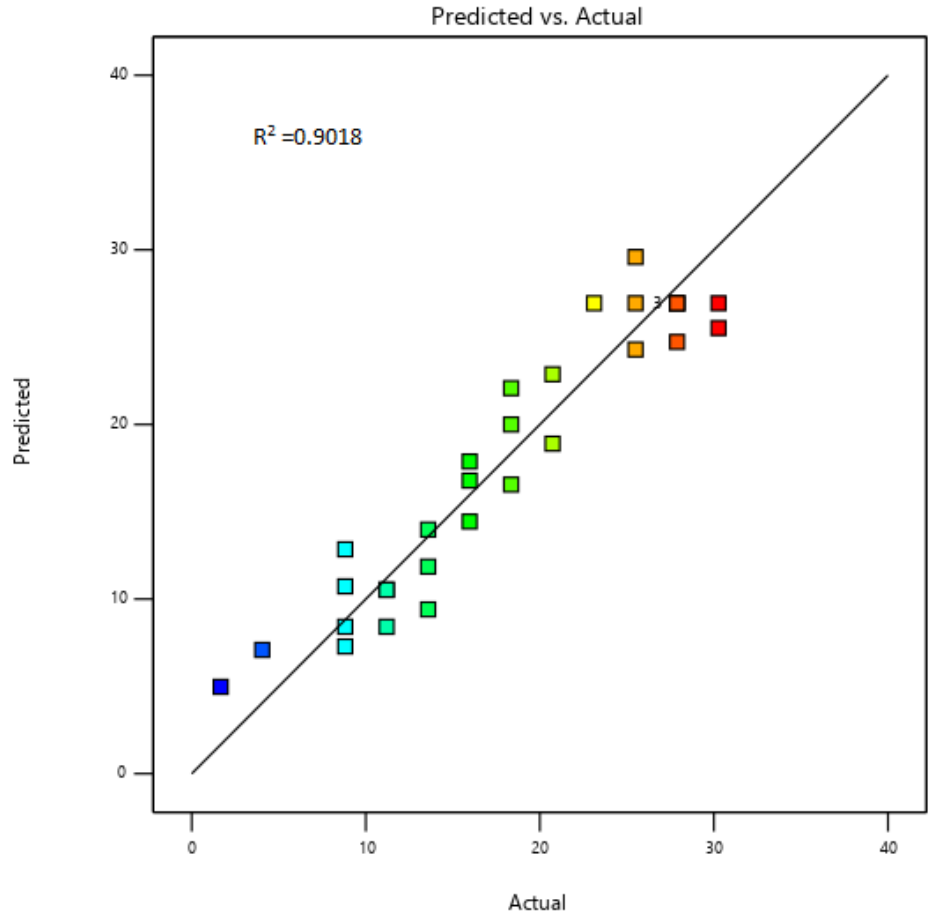
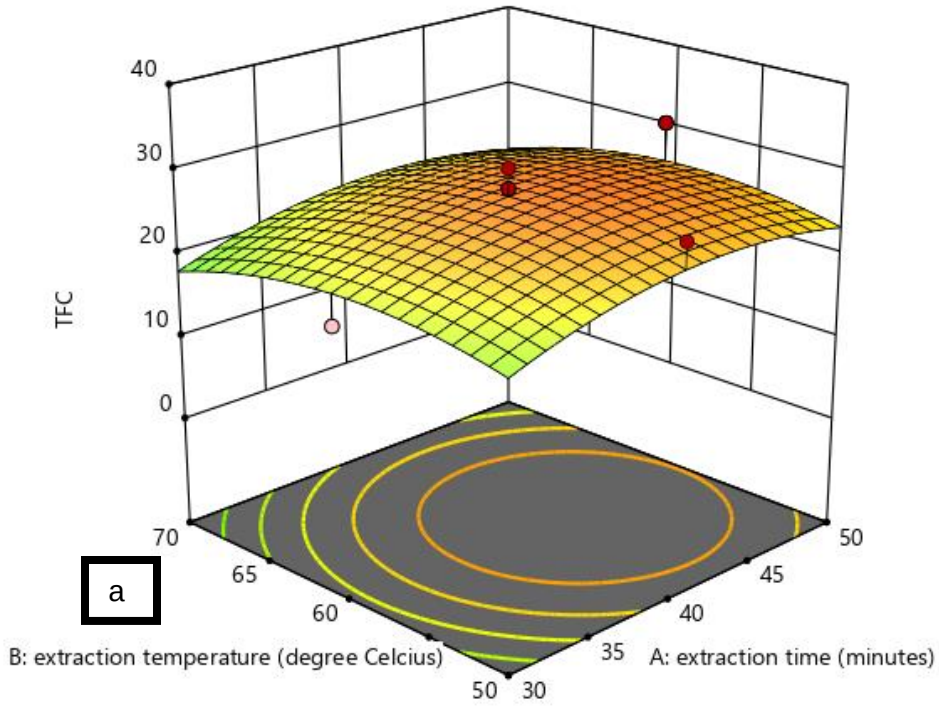


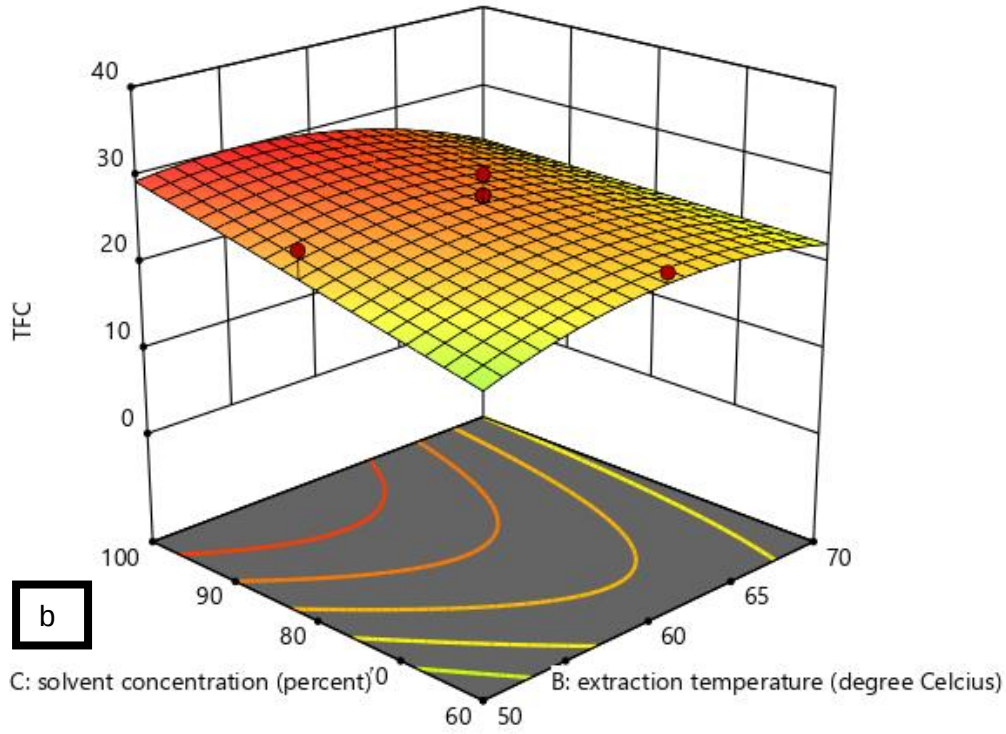
Figure 2 Comparison of actual versus predicted value of extraction output

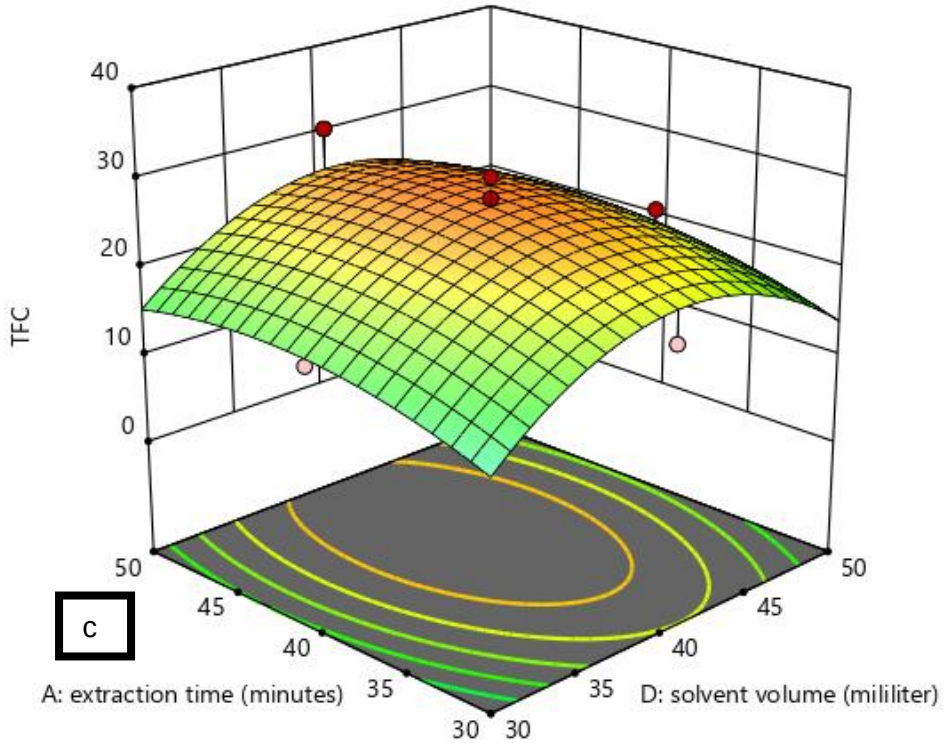
Interpretation of 3-Dimensionl Contour Plots

The regression model is graphically displayed by 3-dimensional contour plots to illustrate the interactive effect of factors on the TFC yield of extraction process. The 3-dimensional contour plots of the combined two variables' effect are displayed in Figure 3. Time of extraction combined with solvent concentration have a significant interactive effect on TFC yield of (±95%), at concentration of 80 % and 40 minutes. The increasing of time of extraction from 50 minutes does not influence the yield of TFC as the increase of concentration.

The time of extraction combined with the temperature of water bath is another significant interaction with a percentage removal of (±95%), where increase of both variables have not any influence on TFC yield and is at its maximum range at temperature 70℃ and extraction time of 50 minutes. Furthermore, the combination of solvent volume and time of extraction also has a remarkable role on TFC yield with a percentage of (±94%) at 40 mL and 40 minutes of extraction time. Meanwhile the increasing of time of extraction has no significant role in TFC yield compared to solvent volume.


TFC
40
30
20
10
0
70
65
60
50
50
45
40
35
30
B: extraction temperature (degree Celcius)
A: extraction time (minutes)
a


TFC
40
30
20
10
0
100
90
80
70
60
50
70
65
60
50
C: solvent concentration (percent)
B: extraction temperature (degree Celcius)
b



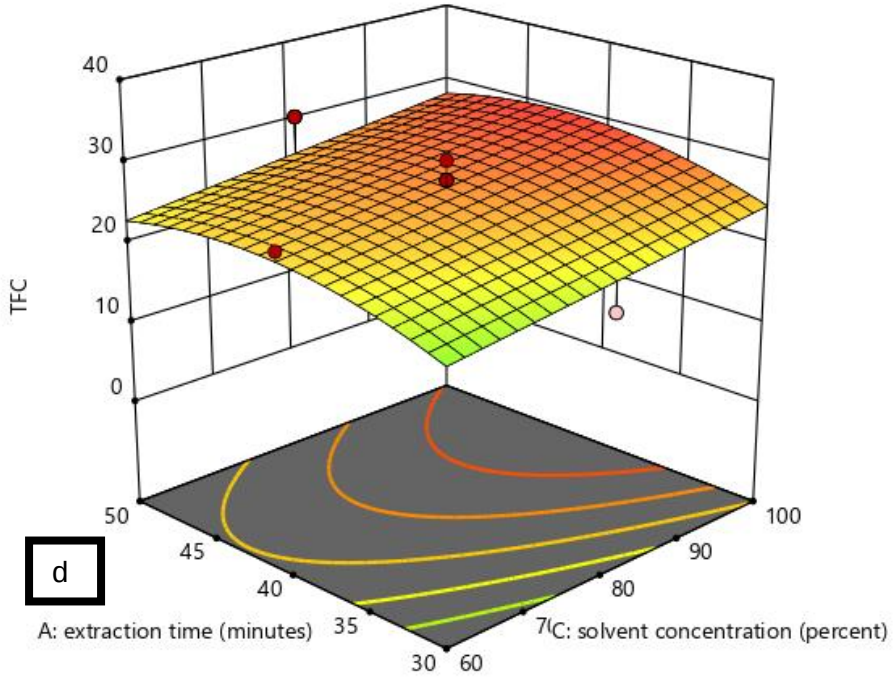


Figure 3 3D contour plots of interactives variables (a) extraction temperature against extraction time, (b) solvent concentration against extraction temperature, (c) extraction time against solvent volume, (d) extraction time against solvent concentration

Optimization of Flavonoid Extraction

To optimize the flavonoid extraction of LZ using UAE method, optimization experiments were done to obtain the maximum output that is TFC yield. The optimization was conducted according to the numerical values of variables suggested by the Design-Expert software based on BBD RSM for all combined responses. The TFC yield was set in its maximum range while the variables involved in this study were set in range with the suggested values from the model, as listed in Table 6.

Table 6 Optimized parameters and ranges proposed by BBD RSM

Constraints	Goal	Lower Limit	Upper Limit	Importance
Time of extraction (minutes)	In range	30	50	-
Temperature of water bath (℃)	In range	50	70	-
Solvent concentration (%)	In range	60	100	-
Solvent Volume (mL)	In range	30	50	-
TFC yield (%)	Maximize	1.668	30.267	3

The Design-Expert software, based on BBD RSM, has suggested 100 solutions of constraints (variables and goals), however for the optimization experiment the solution with the highest desirability (1.000) was conducted with seven replicates and considering the proposed values for the parameters by the software. The variables along with their proposed values by the software and suggested removal and actual results after conducting the optimization experiment have been listed in Table 7 and can be concluded from that the model is valid for replications of experiments in the proposed range and the TFC yield from optimization experiment is within the range of optimized goals suggested by the software.

Table 7 Optimization experiment with the suggested solution parameters and actual results

Constraints	Solutions suggested	Results
Time of extraction (minutes)	42.4	-
Temperature of water bath (℃)	55.7	-
Solvent concentration (%)	100	-
Solvent Volume (mL)	40.3	-
TFC yield (%)	30.5	31.3

Anthelmintic Assay

LZ is a well-known medicinal plant and is widely used in folks medicine and ayurvedic system of medicine. In the present study solvents namely methanol, ethanol and water were used sequentially for crude extraction of LZ aerial part of plants. This study attempts to efficiently evaluate the anthelmintic activity of this plant to justify the ethnomedicinal claims of LZ.

Time taken for paralysis and death of earthworms for leave extracts and standard drug are given in Table 8. Earthworm belonging to control group showed paralysis and death only after a week which occurred under natural circumstances. Meanwhile standard drug in this study Albendazole at 25mg/ml showed paralysis and death time at 17.33 and 23 minutes, respectively which was used to compare the significance of the extracts. Generally, all extracts showed better anthelmintic activity from highest concentration of 100 mg/ml to lowest concentration of 25 mg/ml. Of them all, methanolic extract of L. zeylanica is the most effective extract compared to all extracts at all concentrations 25, 50 and 100 mg/ml.

Table 8 Earthworm anthelmintic assay

Test Samples	Concentration (mg/ml)	Time taken for paralysis (min)	Time taken for death (min)
Control (Distilled Water + 0.5ml DMSO)	-	More than 2 days	More than 2 days
Albendazole	25	17.33 ± 0.88	23.00 ± 1.53
Methanolic extract	25	45.67 ± 3.48	54.00 ± 2.08
	50	31.00 ± 1.15	43.00 ± 1.53
	100	15.67 ± 1.76	23.00 ± 1.53
Ethanolic extract	25	49.67 ± 0.88	64.00 ± 2.08
	50	41.33 ± 0.88	61.33 ± 0.88
	100	23.33 ± 1.76	42.00 ± 1.15
Aqueous extract	25	54.67 ± 2.40	85.00 ± 2.89
	50	48.00 ± 1.53	63.67 ± 2.03
	100	33.33 ± 2.40	45.00 ± 2.89

Methanolic extract was the most effective at the highest concentration 100 mg/ml where paralysis time was 15.67 minutes and death time was 23 minutes which was comparable to the reference drug (Albendazole) at 25 mg/ml. At concentration of 25 mg/ml and 50 mg/ml methanolic extracts the paralysis time were 45.67 and 31 minutes while death was recorded at 57 minutes for 25mg/ml and for 50 mg/ml it was at 43 minutes. Second most effective extract is ethanol extract which is also the most effective at the highest concentration of 100 mg/ml all the way to the lowest and least effective concentration of 25 mg/ml.

Ethanol extract at the concentration of 25 mg/ml, 50mg/ml and 100 mg/ml have paralysis time of 49, 41.33 and 23.33 minutes respectively while death time was 64, 61 and 42 minutes. As being said earlier significant results is recorded at ethanolic extract concentration of 100 mg/ml. On the other hand, aqueous extracts at concentration 25 mg/ml and 50 mg/ml were considered least effective as the paralysis and death time were 54.67 minutes and 85 minutes for 25 mg/ml and 48 minutes and 63.67 minutes for 50 mg/ml. However, aqueous extract at 100 mg/ml shows a comparable time taken for paralysis and death at 33.33 minutes and 45 minutes, respectively. This is comparable to the time recorded for methanolic and ethanolic extract at higher concentration of 100 mg/ml.

Therefore, this investigation revealed that the efficiency of extract from the most to least effective is methanolic, ethanolic and aqueous extracts while in terms of concentration, the most effective concentration is at 100 mg/ml whereas the least effective concentration is 25 mg/ml for all extracts. This investigation revealed that methanolic extract of LZ showed significant anthelmintic activity against earthworm when compared to ethanolic and aqueous extracts. This is due to different polarity of solvents extracting different types of compounds from the plant and these compounds may work in synergy [27]. Further study is needed to determine the compounds that are extracted with methanol, ethanol, and aqueous solvents.

However, methanolic extract did not prove to be as efficient as the standard drug at 25 mg/ml. It can be reported that this study showed that LZ extracts have anthelmintic property as claimed in traditional practice, which is in dose dependent manner, but still not better than the reference drug. Further research should be done to establish the precise mechanism of action and dosage. Besides the extracts can be tested on other helminths at different life cycle to ascertain the anthelmintic activity on a broader scale.

Mortality percentage of the optimized flavonoid extracts against *C. elegans* is presented in Table 9. From the table, it can be observed that concentration of 10 mg/ml of extract kills 6.7 % of the worms while same concentration of Albendazole as positive control kills 100 % of the worms. Concentrations 25 and 30 mg/ml extract is as effective as 10 mg/ml Albendazole extract, giving a 100% mortality rate of *C. elegans*. The need for higher concentration of extract compared to Albendazole gives a 40 % efficiency of the extract against Albendazole. The predicted LC50 of the extract is 17.6 mg/ml.

Table 9 Anthelmintic activity of *Leucas zeylanica* extract against *C. elegans*

Extract concentration	**10 mg/mL**			**15 mg/mL**			**20 mg/mL**			**25 mg/mL**			**30 mg/mL**			**Positive control**			**Negative control**		
Number of non – motile worms	1	0	1	2	2	3	8	9	8	10	10	10	10	10	10	10	10	10	0	0	0
Mortality percentage % Mean ± SD	6.7 ± 5.78			23 ± 5.78			83 ± 5.78			100 ± 0			100 ± 0			100 ± 0			0 ± 0		

Conclusions

An extraction method using ultrasound assistance was optimized for LZ in yielding highest possible flavonoid content. With Time of 40 minutes, temperature of 55°C, solvent concentration of 100% and solvent to solid ratio of 40 ml/g, the TFC yield was 30.38% higher (0.61 mg of quercetin equivalent to 1 mg of extract) than that of unoptimized extraction method. Anthelmintic assay shows the extract to be as 40% effective as conventional Albendazole medication in the same dosage of 10 mg/ml. Further studies should be done on the chemical constituents of LZ to determine the chemicals responsible for its anthelmintic capacity and the use of this plant extract as a substitute or complement to current medication, its dosage, cytotoxicity, and practicality.

Conflicts of Interest

The authors declares that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

The authors would like to express their gratitude to the Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, for their facilities. The work was funded by Tier 2 UTM Research University Grant Q.J130000.2654.16J57.

References

[1] Abdullah, F., *et al*. (2019). *Potential of Leucas zeylanica extract to eliminate E. coli and S. aureus in Corbicula fluminea ("Etak") tissue. Malaysian Journal of Fundamental and Applied Sciences*, *15*(4), 597-599.

[2] Babu, A., *et al*. (2016). In-vitro antifungal activity of leaf extracts of Leucas aspera and Leucas zeylanica*. International Journal of Pharmaceutical Sciences and Research*, *7*(2), 752.

[3] Napagoda, M., *et al.* (2018). Lipophilic extracts of Leucas zeylanica, a multi-purpose medicinal plant in the tropics, inhibit key enzymes involved in inflammation and gout. *Journal of ethnopharmacology, 224*, 474-481.

[4] Kumar, S., *et al.* (2023). The genus Leucas: A review on phytochemistry and pharmacological activities. *Fitoterapia*, 105492.

[5] Radhika, B. and C. Bindu. (2018). Anti-helminthic activity of Leucas zeylanica linn leaves. *International Journal of Pharmaceutical & Biological Archive, 9*(2), 70-73.

[6] Choudhary, A. K., P. Sunojkumar, and G. Mishra. (2017). Fatty acid profiling and multivariate analysis in the genus Leucas reveals its nutritional, pharmaceutical and chemotaxonomic significance. *Phytochemistry*, *143*, 72-80.

[7] Gunathilake, K. P. P. and K. Ranaweera. (2016). Antioxidative properties of 34 green leafy vegetables. *Journal of Functional Foods*, *26*, 176-186.

[8] Nidhal, N., *et a*l. (2020). Chemical constituents of Leucas zeylanica and their chemotaxonomic significance*. Biochemical Systematics and Ecology*, *89*, 104006.

[9] Katiki, L. M., *et al.* (2013). Anthelmintic effect of plant extracts containing condensed and hydrolyzable tannins on Caenorhabditis elegans, and their antioxidant capacity*. Veterinary Parasitology, 192(*1), 218-227.

[10] Islam, A., *et al*. (2017). Investigation of in vitro thrombolytic and anti-helminthic activity and in vivo anxiolytic and antidepressant potentiality with phytochemical nature of methanolic extract of

Leucas lavandulifolia. *Sustainable Chemistry and Pharmacy*, *6*, 61-66.
[11] Anisuzzaman and N. Tsuji. (2020). Schistosomiasis and hookworm infection in humans: Disease burden, pathobiology and anthelmintic vaccines. *Parasitology International, 75*, 102051.
[12] Hong, S.-T. (2018). Albendazole and praziquantel: review and safety monitoring in Korea. *Infection & Chemotherapy*, *50*(1), 1-10.
[13] Imani-Baran, A., *et al*. (2020). Anthelmintic activity of crude powder and crude aqueous extract of Trachyspermum ammi on gastrointestinal nematodes in donkey (Equus asinus): An in vivo study. *Journal of Ethnopharmacology*, *248*, 112249.
[14] Mansur, F., *et al.* (2014). The anthelmintic efficacy of natural plant cysteine proteinases against two rodent cestodes Hymenolepis diminuta and Hymenolepis microstoma in vitro. *Veterinary Parasitology*, *201*(1-2), 48-58.
[15] Romero-Benavides, J. C., *et al.* (2017). Medicinal plants used as anthelmintics: Ethnomedical, pharmacological, and phytochemical studies. *European Journal of Medicinal Chemistry*, *129*, 209-217.
[16] Nath, T. C., *et al.* (2022). An update of Intestinal helminth infections among urban slums communities in Bangladesh. IJID Regions.
[17] Cheng, Y., *et al.* (2021). Subcritical water extraction of natural products. *Molecules*, *26*(13).
[18] Romes, N. B., *et al*. (2022). *D*-optimal design-assisted Elaeis guineensis leaves extract in olive oil-sunflower seed nanoemulsions: development, characterization, and physical stability. *Journal of Dispersion Science and Technology, 43*(2), 289-301.
[19] Furmuly, M., *et al*. (2020). Enhancement of Mercury removal by utilizing catalytic chelation technique.
[20] Ismail, M. A. H. *Caenorhabditis elegans: a Model Organism for Research. Language*, *3*, 91456321.
[21] Peña-Espinoza, M., *et al.* (2020). Anthelmintic and metabolomic analyses of chicory (Cichorium intybus) identify an industrial by-product with potent in vitro antinematodal activity. *Veterinary Parasitology*, *280*, 109088.
[22] Murugamani, V., *et al*. (2012). The new method developed for evaluation of anthelmintic activity by housefly worms and compared with conventional earthworm method. *International Scholarly Research Notices.*
[23] Agarwal, S., *et al.* (2011). Evaluation of in vitro anthelminthic activity of Leucas aspera extracts. *Pharmacognosy Journal, 3*(24), 77-80.
[24] Wan Omar, W. N. N. and N. A. Saidina Amin. (2011). Optimization of heterogeneous biodiesel production from waste cooking palm oil via response surface methodology. *Biomass and Bioenergy, 35*(3), 1329-1338.
[25] Hagquist, C. and M. Stenbeck. (1998). Goodness of fit in regression analysis–R 2 and G 2 reconsidered. *Quality and Quantity*, *32*(3), 229-245.
[26] Said, K. A .M. and M. A. M. Amin. (2015). Overview on the response surface methodology (RSM) in extraction processes. *Journal of Applied Science & Process Engineering*, *2*(1), 8-17.
[27] Ri, H.-i., *et al*. 2019. Effect of different polarity solvents on total phenols and flavonoids content, and In-vitro antioxidant properties of flowers extract from Aurea Helianthus. arXiv preprint arXiv:1906.12006.