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Optimization of Phenolic Extraction and Antioxidant Activity from Curcuma aeruginosa Rhizomes using Box-Behnken Experimental Design

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ABSTRACT

Curcuma aeruginosa or black turmeric is a medicinal plant whose rhizomes contain bioactive compounds, including phenolic compounds that act as antioxidants. The extraction efficiency of these compounds is significantly influenced by several process variables, making optimization crucial for achieving maximum yield and activity. This study aimed to optimize extraction conditions for antioxidant activity from Curcuma aeruginosa rhizomes using the Response Surface Method (RSM) combined with a Box–Behnken experimental design from Design-Expert 12.0.3.0. The independent variables used were the varying ethanol concentration, the liquid to solid ratio, and the extraction time during the maceration process. Antioxidant activity was the main response parameter. The results showed that these three variables significantly influenced the results of the antioxidant test as observed in the response. The resulting second-order polynomial model showed good uniformity in predicting optimal extraction conditions. The optimum conditions were obtained at 73% ethanol concentration, a liquid to solid ratio of 1:20 (300 mL:15 g), and an extraction time of 1 day, which produced the highest total phenolics and antioxidant activity

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Model statistics.

1. Introduction

Curcuma aeruginosa Roxb., commonly known as Temu Ireng is a medicinal plant widespread in Southeast Asia and traditionally used for various medicinal purposes. The rhizome of this plant have been reported to be rich in bioactive compounds, including phenolics, flavonoids, and essential oils.[1]. *C. aeruginosa* Roxb., is believed to be able to treat rheumatism, asthma, enteritis, stomach ache, as well as help increase appetite and overcome obesity [2]. Along with the development of scientific research, various studies have revealed that *C. aeruginosa* rhizomes not only have traditional benefits, but also show important biological activities, one of which is as a source of natural antioxidants [3]. This antioxidant potential is related to the content of phenolic compounds and other bioactive components that can ward off free radicals, making this plant increasingly interesting for further study, especially in the development of natural-based products.

Degenerative diseases in humans are closely associated with the presence of free radicals, which can induce oxidative stress and lead to cellular damage over time. In this context, antioxidants play a crucial role by stabilizing these reactive species, effectively transforming them into more stable and non-reactive forms, thereby reducing their harmful effects on biological systems. A number of studies have highlighted the diverse biological activities of

Curcuma aeruginosa, demonstrating its potential not only as an antimicrobial agent [4], and anticancer compound [5], but also as a natural source of antioxidants [6].

The Response Surface Method (RSM) has been used in previous studies to optimize the extraction process and evaluate the biological activity of natural compounds through the Brine Shrimp Lethality Test (BSLT) to assess the potential toxicity of C. Aeruginosa extracts [7]. However, the use of the Box–Behnken design specifically for Curcuma aeruginosa, especially in the context of optimizing antioxidant activity, still requires further research. Therefore, this study was directed to determine the most effective combination of extraction parameters, including ethanol concentration, solid-liquid ratio, and extraction duration, to achieve optimal antioxidant yield.

2. Method

2.1. Extraction Process and Determination of DPPH Radical Scavenging Bioactivity

The experimental design of C. aeruginosa rhizome samples was made using response surface methodology with Box-Behnken Design using *Design-Expert version 11 (State Ease, Inc.)* based on [8]. The extraction optimization stage was carried out to obtain the best extract rich in phenolics and flavonoids. The independent variables used were the solvent ratio, ethanol concentration, and extraction time. The research began by extracting 500g of *C. Aeruginosa* rhizome. The samples were then mixed evenly and extracted through a maceration process using a shaker operated at a speed of 235 rpm, the aim being to ensure optimal contact between the solvent and the material during the process. Upon completion of the extraction stage, the obtained mixture was subjected to filtration using Whatman No. 5 filter paper to separate the liquid extract from residual solid particles. The clear filtrate was subsequently concentrated by evaporating the solvent at 50°C with the aid of a rotary evaporator (BUCHI, R-250, Switzerland) until a crude extract was obtained, after which the extraction yield was calculated and expressed as a percentage on a weight basis (%, w/w). The antioxidant potential of the extract was then assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method, employing a microplate reader (Epoch BioTek, USA) for measurement. In the assay procedure, 100 µL of the sample solution was combined with an equal volume (100 µL) of DPPH solution (125 µM in ethanol) in each well of a microplate. The reaction mixture was allowed to stand for 30 minutes in dark conditions to ensure optimal interaction between the extract and DPPH radicals. Following incubation, the absorbance was recorded at 517 nm using the microplate reader. The percentage of inhibition of antioxidant activity against DPPH radicals is expressed as the IC50. Each experiment was done in triplicate (n = 3) to ensure data reliability and reproducibility.

2.2. Experimental Design

Experimental design is defined as a study to determine the basic framework for collecting information on objects that have variations, based on statistical principles [9]. This study used RSM and Box-Behnken design to obtain optimal extraction results and the highest DPPH radical scavenging bioactivity. The first variable variation is the solvent ratio. The solvent ratios used are 1:5, 1:10, and 1:20. The second variable is the ethanol concentration. The ethanol concentrations used are 50%, 70%, and 100%. The last variable variation was the duration of extraction left in a closed space. The time was varied between 1 day, 2 days, and 3 days. The range values and midpoints are presented in Table 1. The experimental design used in this study included 12 experimental combinations arranged based on a factorial approach, and equipped with three replications at the central point, the overall results and details of which are presented systematically in Table 2.

Table 1. Code of three variables

Variable	Variable Code		
	-1	***0***	***+1***
Solvent ratios (gr/mL) / A	1 : 5	1 : 10	1 : 20
Ethanol concentrations (%) / B	50	70	96
Extraction times (day) / C	1	2	3

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Table 2. Box-Behnken Experimental and results

Test Number	Variable Code		
	A	***B***	***C***
1	+1	0	+1
2	-1	-1	0
3	0	-1	+1
4	+1	0	-1
5	0	0	0
6	0	-1	-1
7	0	+1	+1
8	+1	+1	0
9	-1	0	+1
10	+1	-1	0
11	0	0	0
12	-1	0	-1
13	0	0	0
14	0	+1	-1
15	-1	+1	0

3. Results and Discussion

3.1. Modeling

This study used the Box-Bhenken model with RSM to obtain the best combination of solvent percentage, solid-solvent ratio, and extraction time. The mathematical model of the RSM model includes linear, interaction, and quadraticterms effect. The process system involves three process variables, x1, x2 dan x3, so general form of the model fitting equation is:

$$y = b_o + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + \varepsilon$$

The regression model was developed using coded variables, namely factor values that have been normalized into the range −1 to +1, to facilitate the analysis of the influence and interaction between variables in the RSM design. From the experimental results, the recommended model for testing Total Phenolics and DPPH was the quadratic model Table 3. In the total phenolic and DPPH tests, R2 values of 0.9212 and 0.6849 were obtained. The R2 value is used to indicate the suitability of the quadratic model equation [10] so that it can describe the level of suitability with the model used. [11] explain that an R2 value close to 1 indicates the suitability between the prediction results and the experimental results.

Table 3. Polynomial equations for the six responses tested

Test Parameters	Model	Equation
Total Phenolic	Kuadrat	$Y = -70.00476 + 0.415210A + 3.45535B - 20.26731C - 0.001299AB + 0.008642AC + 0.131643BC - 0.000850A^2 - 0.022851B^2 + 2.68981C^2$
DPPH	Kuadrat	$Y = 90.75412 - 0.050898A + 0.526771B - 3.04330C - 0.000760AB - 0.000805AC - 0.062041BC - 2.63806E\text{-}06A^2 - 0.003434B^2 + 1.90948C^2$

Table 4. ANOVA results from each test performed

ANOVA	Test Parameters	
	Total Phenolic	DPPH
Model F-value	6.49	1.21
Model p-value	0.0266	0.4396
LOF F-value	1.36	4.29
LOF p-value	0.4501	0.1949
R^2	0.9212	0.6849
Adj R^2	0.7792	0.1178
Pred R^2	0.0970	-3.4580
Adeq Pres.	8.5387	3.7617
Std. Dev.	4.52	2.35
C.V.	6.23	2.26
PRESS	33.64	3.71

From the ANOVA table in Table 4, the Adj R2 for total phenolic was 0.7792 and for DPPH was 0.1178 at a 95% confidence level. The F value of 6.49 for total phenolics indicates that the model is significant, meaning that the extraction parameters have an effect on the total phenolics obtained. However, the F value for DPPH is not significant at 1.21. The adequacy precision for total phenolics is 8.5387. This value can be considered good because it is greater than 4.0, so the model is accepted for navigating design optimization [12]. Meanwhile, the adequacy precision for DPPH is 3.7617, which is close to 4.0. The predicted values vs. actual values for total phenolic content and DPPH are presented in Fig 1.

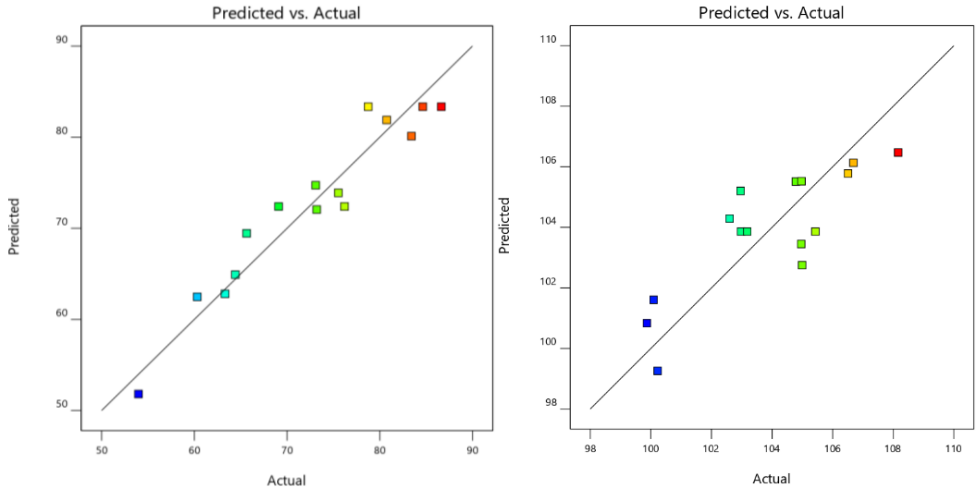

Fig 1. Predicted value vs. actual value for (a) total phenolic and (b) antioxidant activity

3.2. Total Phenolic Response Results

The total phenolic content was determined by adding Folin-Ciocalteau reagent. Folin-Ciocalteau reagent is an inorganic reagent that is expected to react with phenolic compounds to produce a colored complex solution. The resulting color is then measured for its absorbance. According to [13], the Folin–Ciocalteau method is based on a colorimetric redox reaction used to determine the total phenolic compound content in a sample. The Folin–Ciocalteau reagent is a polymer ion complex solution composed of phosphomolybdic acid and heteropolyphosphotungstic acid. In this reaction, the reagent oxidizes the phenolic hydroxyl group under alkaline conditions, causing the reduction of heteropoly acid into a colored molybdenum–tungsten complex.

The determination of total phenolic content in this study was carried out using gallic acid as the calibration standard due to its well-established role as a representative phenolic compound. According to (Viranda 2009), gallic acid belongs to the hydroxybenzoic acid group and is categorized as a simple phenolic acid, making it suitable for expressing phenolic content in equivalent units. In the analytical procedure, the sample extract was reacted with the Folin–Ciocalteu reagent, which serves as an oxidizing agent and initiates a color change to a yellowish hue, indicating the initial presence of phenolic constituents. To further promote the reaction, a sodium carbonate (Na_2CO_3) solution was subsequently introduced to provide an alkaline environment, which is essential for the reduction process to occur more effectively. Under these conditions, phenolic compounds in the sample are able to reduce the Folin–Ciocalteu reagent, leading to the formation of a colored complex whose intensity is proportional to the total phenolic concentration. This color development can then be quantitatively measured using spectrophotometric analysis, allowing the phenolic content of the sample to be expressed in terms of gallic acid equivalents.

In the next stage, the hydroxyl groups present in the phenolic compounds react with the Folin–Ciocalteu reagent to produce a blue complex composed of molybdenum and tungsten, although its exact structure is not yet fully understood. The intensity of the blue color formed is directly proportional to the amount of phenolic ions produced. In other words, the higher the phenolic compound content in a sample, the greater its reducing ability towards heteropoly

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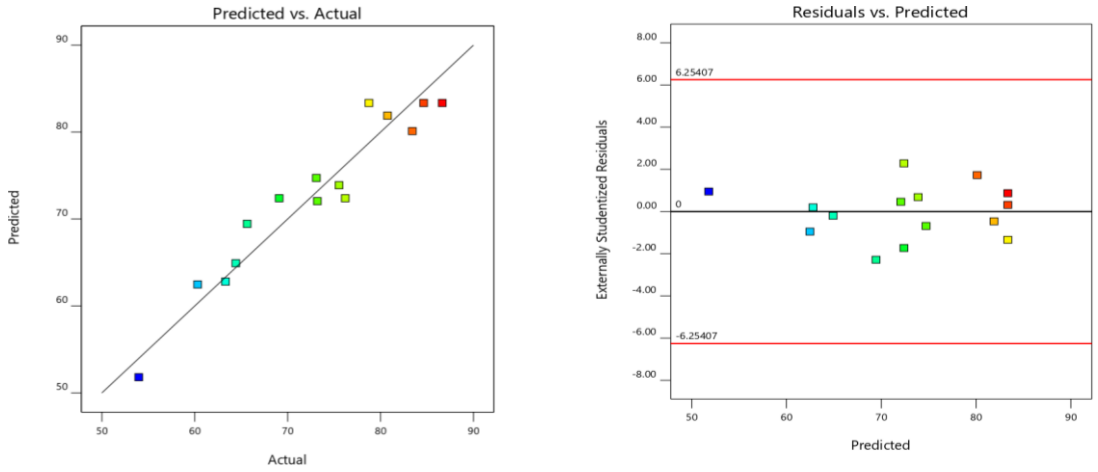
acids (phosphomolybdic–phosphotungstic), resulting in a more intense blue color that can be analyzed quantitatively using a spectrophotometer.

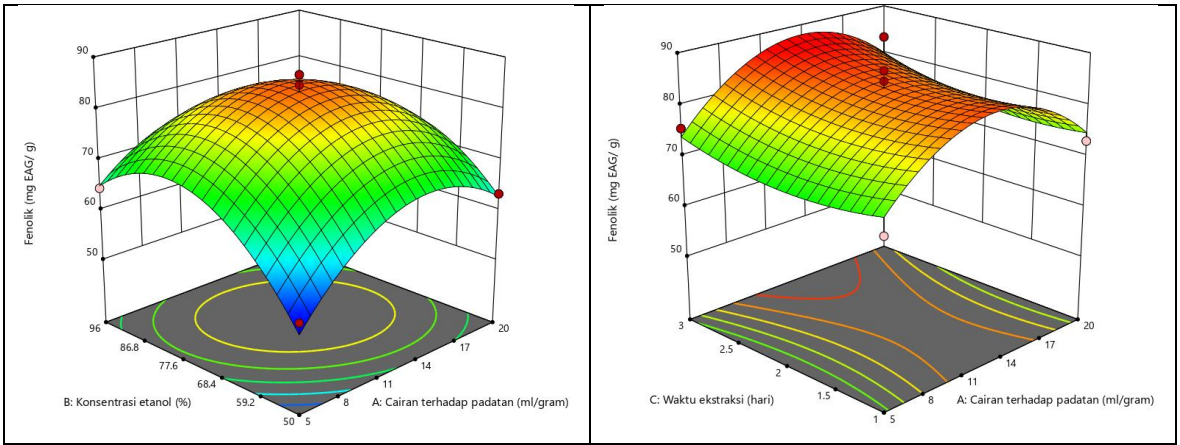
Based on ANOVA analysis, the three variables used show an effect on obtaining optimal yield, which is reflected in the proximity between the predicted and actual values in Fig. 2. The total of six points are very close to the diagonal line and there are no points that deviate far from the line. This indicates that the regression model equation is able to accurately represent the experimental data.



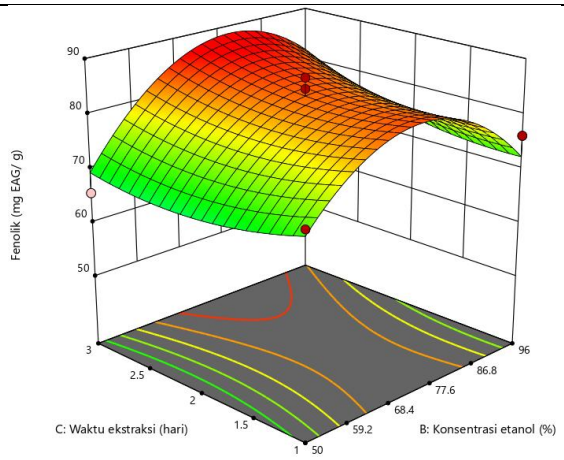
(a) (b)

Fig 2. Grafik permukaan respon plot 2D total fenolik; (a) perbandingan nilai aktual dan prediksi (b) Plot normal antara respons yang diperkirakan vs residual

The effect of solvent-to-solids ratio, ethanol concentration, and extraction time on the total phenolic response is visualized in a three-dimensional (3D) response surface graph. The 3D graph provides a more comprehensive picture of the interactions between variables in determining the resulting response value. The graph is shown in Fig 3. The color differences in the graph represent variations in total phenolic content, where red areas indicate higher total phenolic values, while light blue areas indicate lower values. Fig. 3 (a) and 3 (c) show that the phenolic compound content increases with increasing ethanol concentration until it reaches the optimum condition, then decreases at higher ethanol concentrations.



(a) (b)



(c)

Fig 3. Total phenolic yield from the combination effect of (a) ethanol vs. liquid-to-solid ratio, (b) time vs. liquid-to-solid ratio, and (c) time vs ethanol

The ethanol concentration significantly affects the extraction process. The use of low-concentration ethanol tends to extract impurities [14], while high ethanol concentrations are more dominant in extracting lipid components [15]. Based on the results of this study, the optimum ethanol concentration for obtaining the highest total phenolic content from C. aeruginosa rhizomes is 73%. This finding is in line with a study [16] which reported that the highest total phenolic content was obtained in samples dissolved with ethanol solvent with a concentration of 75% in the crude extract of *Eucheuma denticulatum* seaweed.

3.3. DPPH Radical Scavenging Activity

The antioxidant activity test method used the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. DPPH was chosen because it can evaluate a compound's ability to capture or reduce free radicals. This method is expected to provide a clear picture of the compound's potential antioxidant activity. DPPH is a stable purple-colored free radical. This method is used to assess single electron transfer ability, including H• transfer activity, as well as the ability to inhibit free radicals. According to [17], the DPPH method is an easy, fast, and sensitive antioxidant test method. In addition, this method does not require a large amount of sample for antioxidant activity evaluation, making it more cost-effective. The percentage of inhibition of antioxidant activity against DPPH radicals is expressed as the IC50 value [18]. [19] states that the IC50 value can be interpreted as the concentration of the sample solution required to inhibit or reduce up to 50% of the number of DPPH free radicals, so this parameter is often used as an indicator to show the level of antioxidant activity of a compound or extract being tested. The reaction process between antioxidant compounds and DPPH free radicals is indicated by a color change from purple to yellow, which signifies stoichiometric electron capture [20]. This color change is an indication of radical scavenging activity in accordance with the reaction in Fig. 4.

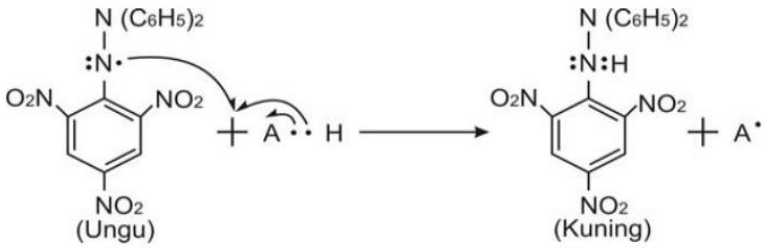


Fig 4. DPPH Reaction and Antioxidant [21]

The results of the combined effect of variations in the liquid to solid ratio, the concentration of ethanol used as a solvent, and the length of extraction time on the DPPH free radical scavenging activity are visualized in three dimensions (3D) surface response graph. The graph

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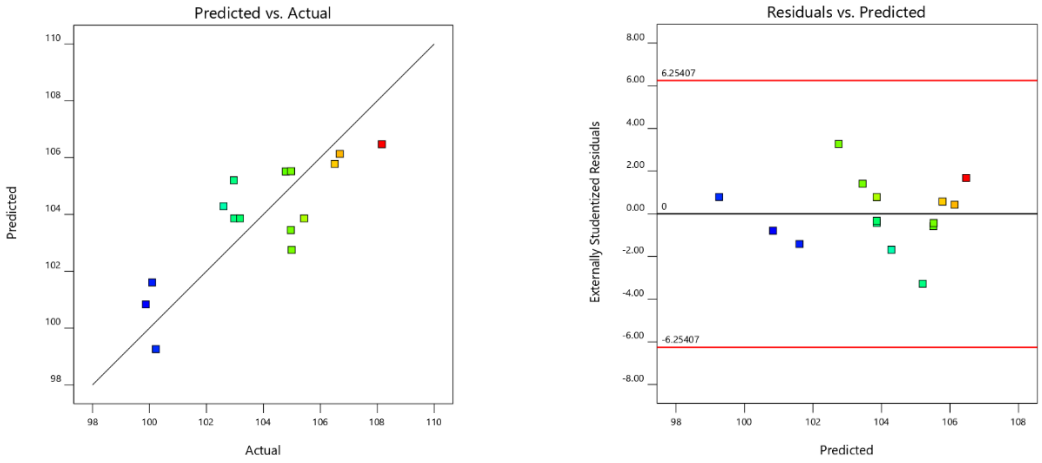
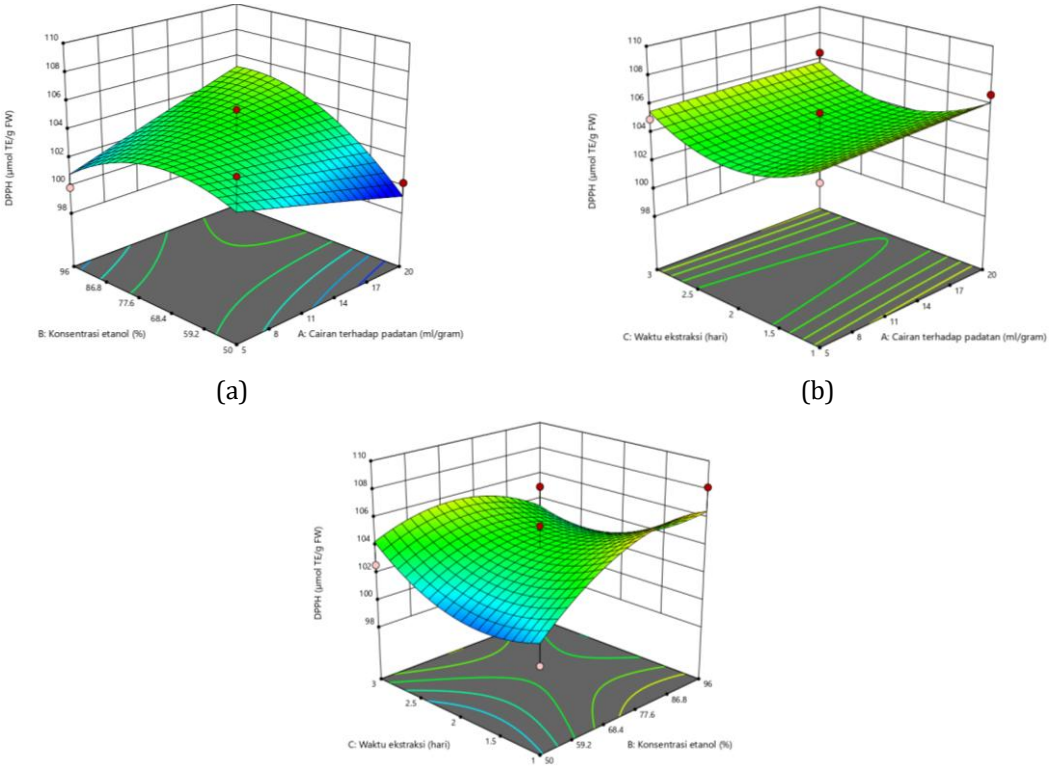
is depicted in Figure 6, which provides a clearer picture of the interaction pattern between variables in influencing the resulting antioxidant activity value. Fig. 6 (a) shows that antioxidant activity increases with increasing solvent ratio up to an ethanol concentration of 96%.



(a) (b)

Fig 5. 2D plot response surface graph of DPPH antioxidant activity; (a) comparison of actual and predicted values (b) Normal plot between predicted vs residual response

Meanwhile, in Fig. 6 (b), the increase in antioxidant activity is not as rapid. However, a solvent ratio of 1:20 has been proven to provide maximum antioxidant activity. This is in line with the opinion [22] that the greater the amount of ethanol solvent used, the more target compounds are dissolved in ethanol. The effect of extraction time is illustrated in Fig. 6 (b) and (c), showing that the extraction time that maximizes antioxidant activity is 1 day. The longer the extraction time, the more target compounds can be extracted with ethanol.



(a) (b)

(c)

Fig 6. The bioactivity test of DPPH radical scavenging from the combined effects of (a) ethanol vs. liquid-to-solid ratio, (b) time vs. liquid-to-solid ratio, and (c) time vs ethanol

However, on the other hand, the statistical model for the DPPH response in this study was declared invalid because it was not statistically significant ($p = 0.4396 > 0.05$), indicating that the tested variables did not have a significant influence on the response. In addition, the negative predicted R^2 value (−3.458) indicates a low predictive ability of the model. This condition indicates that the model is not suitable for use for optimization purposes. This invalidity is likely caused by several factors, such as the selection of an inappropriate variable distance that does not capture a significant effect, high or inconsistent variation in experimental data, a limited number of replications, model inconsistencies, or the presence of other important factors that are not included in the model so that the relationship between variables and responses cannot be explained properly.

4. Conclusion

This study demonstrates the successful use of the Response Surface Method (RSM) through a Box-Behnken Design experimental plan to find the optimal point for three extraction parameters for antioxidant activity from Curcuma aeruginosa Roxb rhizomes. The results showed that the highest antioxidant activity was obtained from samples macerated at 96% ethanol concentration with a 1:20 sample-to-solvent ratio (300 mL:15 g) and an extraction time of 1 day.

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