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STATISTICAL OPTIMIZATION OF ANTIOXIDANT AND EXTRACTION YIELD FROM CENTELLA ASIATICA LEAVES

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Abstract:

This study focuses on the statistical optimization of antioxidant and extraction yield from Centella asiatica leaves using Central Composite Designs (CCD) and response surface methodology. The primary objective was to identify influential variables affecting the extraction process, such as extraction time and temperature, using ultrasonic-assisted extraction. The optimized extraction conditions yielded an antioxidant activity of 52.4420%, Total Phenolic Content (TPC) of 0.5000 mg/g, and Total Flavonoid Content (TFC) of 15.3125 mg/g. Statistical analysis revealed significant model terms with an R^2 value of 0.930, indicating that 93% of the variability in antioxidant yield could be explained by the model. The 'Adeq Precision' ratio of 15.479 confirmed an adequate signal, and the Model F-value of 15.16 ($p = 0.0043$) highlighted the significance of the model. The findings demonstrate a viable and efficient technique for maximizing the extraction yield of bioactive components from C. asiatica, providing a robust framework for enhancing the efficiency of extracting valuable antioxidants from medicinal plants, which is crucial for natural product research and herbal medicine.

Keywords:

Yield Of Antioxidant Activity; Plant Extraction; Centella Asiatica; Free Radical Scavenging

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Introduction

Centella asiatica, commonly known as Gotu Kola or pegaga in Malay, is a versatile and medicinal herb that has been treasured for centuries in traditional herbal medicine systems such as Ayurveda and Traditional Chinese Medicine (Gamage et al., 2021). This remarkable plant belongs to the Apiaceae family and is native to various regions of Asia, including India, Southeast Asia, and China. The plant is characterized by its small, kidney-shaped leaves and creeping stems that spread along the ground. It thrives in moist, marshy areas and is often found near water bodies.

The herb has a long history of use for its therapeutic properties and has been utilized to address a wide range of health concerns. One of the primary constituents responsible for C. asiatica's potential health benefits is triterpenoid compounds, including asiaticoside and asiatic acid (Wu et al., 2012). These compounds are believed to contribute to its anti-inflammatory, antioxidant, and wound-healing properties. Additionally, C. asiatica has been explored for its potential cognitive-enhancing effects and its role in promoting healthy skin (Gray et al., 2018), which can be seen many cosmetics products now use C. asiatica extract as its ingredient. For the research the chemical that is focused on is antioxidant inside the C. asiatica. This is because antioxidant is good in maintaining health and reducing the risk of chronic diseases, including heart disease, cancer, and neurodegenerative conditions, which has been extensively studied (Zhang et al., 2015).

To collect the extract, the herbs need to undergo extraction process which is a technique used in organic chemistry to isolate a target compound (Zhang & Pawliszyn, 1993). There are lot types of extraction available for example maceration process, ultrasonic assisted, Soxhlet and Supercritical Fluid Extraction. For this research, ultrasonic assisted extraction was used to extract C. asiatica as it helps to decrease extraction and processing time, the amount of energy and solvents used. Antioxidant composition in the extract is checked by running the antioxidant test, which is 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay on each sample which this test can measure antiradical activities of various antioxidants from the extract (Brand-Williams, Cuvelier, & Berset, 1995).

The aims of this research are to optimize the antioxidant and extraction yield from C. asiatica leaves statistically and to identify the variables that can influence the extraction such as extraction time and extraction temperature. Optimization of extraction processes is essential for maximizing the yield of bioactive compounds. Central Composite Design (CCD) and Response Surface Methodology (RSM) are powerful statistical tools used for this purpose. CCD is a common experimental design method for fitting a second-order (quadratic) model, which is particularly useful when the number of factors is less than five. RSM helps in evaluating the interactions between variables and identifying optimal conditions. Ultrasonic assisted extraction process was used to extract the herbs with temperature and time of extraction as the main factor of the experiment. Temperature and time of extraction are great factor in extraction process which both can give impact to the extraction yield. According to the research from (Ghoreishi, Shahrestani, & Ghaziaskar, 2009), as the extraction time rises from 10 to 90 minutes, the yield increases and with further increase up to 180 minutes, it did

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not cause any changes. (Iylia Arina & Harisun, 2019) conclude that to efficiently distil the active components from plant extracts, extraction temperatures are crucial which high temperatures can either increase or decrease the number of active compounds that can be extracted. From the statement, it shows that to gain the highest yield extract, we need to extract the target material with optimum temperature. For this research, the experiment that is done is optimized by Central Composite Designs (CCD) from Design Expert Software which can help to assist in carrying out experimental designs, such as identifying the best preparation's recipe (Sopyan et al., 2022). Despite the extensive documentation of the benefits of C. asiatica, there remains a gap in optimizing the extraction parameters specifically for maximizing the antioxidant yield using CCD and RSM. While previous studies have focused on different aspects of C. asiatica's pharmacological properties, few have applied a systematic approach to optimize the extraction conditions for antioxidant yield. This research aims to fill this gap by employing CCD and RSM to identify the optimal extraction temperature and time, thereby enhancing the yield of antioxidants, total phenolic contents (TPC), and total flavonoid contents (TFC) from C. asiatica leaves. By refining these specific variables, this study presents a viable and efficient technique for extracting numerous beneficial components, offering new insights and practical applications in the field of natural product extraction and optimization.

Materials and Methodology

Materials

Centella asiatica leaves (pegaga leaves) were purchased from the local market at Seberang Perai, Penang, Malaysia. Chemicals that were used for the experiment were Ethanol with 96% purity, Gallic Acid 97.5-102.5%, Aluminum Chloride reagent grade, 98%, and 2,2-Diphenyl-1-picrylhydrazyl was purchased from Sigma Aldrich. Sodium Carbonate 7% from R&M Chemicals, Folin-Ciocalteu 10% was supplied by Merck KGaA, Sodium Hydroxide by 2N Universal Lab & Chemical Supplies, Quercetin hydrate 95% by ACROS Organics, and Sodium Nitrite by EMSURE®.

Preparation and Extraction of Centella asiatica

C. Asiatica leaves (pegaga leaves) was left to dried for 24 hours in the oven at 50℃. Dried C. asiatica leaves were grounded to powder form by using grinder and stored in a container which the leaves were used in powder form in the extraction process. Ultrasonic-assisted extraction (UAE) was selected for this experiment due to its efficiency and ability to enhance the extraction yield of bioactive compounds. UAE utilizes ultrasonic waves to create cavitation bubbles in the solvent, which collapse and disrupt plant cell walls, thereby improving solvent penetration and the release of intracellular compounds. This method offers several advantages over traditional extraction techniques, including reduced extraction time, lower solvent consumption, and higher extraction efficiency. UAE significantly reduces extraction time compared to conventional methods like maceration and Soxhlet extraction. Additionally, it is more energy-efficient and requires less solvent, making it a more sustainable and cost-effective option. The extraction was performed using an ultrasonic bath with a frequency of 40 kHz and power of 300 W. 1 gram of dried c. asiatica powder was extracted by 30 ml of 96% ethanol throughout the entire experiment. The extract was then subjected to a centrifuge at 60000 rpm for 20 minutes to separate the liquid from the solid.

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Optimization using Central Composite Design (CCD) and Response Surface Methodology

Central Composite Design (CCD) and response surface methodology were applied in this experiment with the use from Design Expert Software version 7 which helps to optimize the experiment with efficient experiment. According to the (Ogbonna et al., 2017), the development of pharmaceutical dosage forms takes less time thanks to optimization using factorial designs, which also improves research and development work by lowering the number of experimental trials required to evaluate multiple parameters and their interactions, making the process less time-consuming. The circumscribed central composite experimental design was used in which extraction temperature and time of extraction were the independent factors in the experiment. The values for extraction temperature were 36℃, 40℃, 50℃, 60℃, and 65℃. For time of extraction the values were 24 minutes, 30 minutes, 45 minutes, 60 minutes, and 67 minutes. Values for extraction temperature and extraction time for each run were according to the table below:

Table 1: List of Parameters with the Range of Value

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded
A	Extraction temperature	℃	Numeric	40.00	60.00	-1.000	-1.000
B	Extraction time	min	Numeric	30.00	60.00	-1.000	-1.000

Table 2: Yield of Antioxidant with Various Value of Parameters (Extraction Temperature and Extraction Time)

Standard	Run	Extraction Temperature, ℃	Extraction Time, min
1	5	40	30
2	2	60	30
3	7	40	60
4	10	60	60
5	11	36	45
6	13	65	45
7	3	50	24
8	6	50	66
9	9	50	45
10	12	50	45
11	1	50	45
12	4	50	45
13	8	50	45

Antioxidants Activities using DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

The DPPH free radical scavenging assay was used to measure the extracts' antioxidant activity. An antioxidant substance reduces DPPH by giving it an electron. When DPPH is reduced, its color changes from deep violet to yellow. The significant absorbance at 517 nm that DPPH solutions exhibit appears deep violet in color.

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Preparation of DPPH Solution

A volumetric flask containing 0.005 mg of DPPH was filled with methanol to a final volume of 125 ml to create a DPPH solution (40µg/ml).

Evaluation of Antioxidant Content

1 ml of optimized extract with extraction time 115 minutes and solid liquid ratio 1:15, was added to 3 ml of DPPH reagent. The solution was shaken well and incubated in room temperature for 30 minutes. The absorbance was measured at wavelength 517 nm against blank sample (DPPH reagent) using UV-vis spectrophotometer. The value of absorbance was used to calculate the antioxidant content. The percentage of inhibition was calculated using the formula below:

$$I\% = \frac{AC - AO}{AC} \times 100\%$$

Where I% = Percentage of inhibition, AC = Absorbance of the control, and AO = Absorbance of the sample solution.

Determination of Total Phenolic Compound

With more than 8000 phenolic structures now known, phenolic compounds, also known as polyphenols, are one of the most abundant and widely distributed classes of secondary metabolites found in plants (Soobrattee et al., 2005). Phenolic chemicals are important parts of plants, and their redox properties enhance their antioxidant action.

Preparation of Standard Gallic Acid for Calibration Curve

To prepare the standard gallic acid solution, 50 mg of gallic acid was dissolved in 50 mL of ethanol, resulting in a concentration of 1 mg/mL. From this standard solution, two diluted concentrations of 0.050 mg/mL and 0.10 mg/mL were prepared. For each of these concentrations, 5 mL of 10% Folin-Ciocalteu reagent (FCR) and 4 mL of 7% sodium carbonate (Na_2CO_3) were added, and the volume was adjusted to a final total of 10 mL. The mixture was then covered and allowed to react for 90 minutes at room temperature. After the incubation period, the absorbance of each solution was measured at 765 nm using a UV-visible spectrophotometer, with ethanol serving as the blank. The absorbance values obtained for the 0.050 mg/mL and 0.10 mg/mL solutions were then used to plot the calibration curve.

Preparation of Samples for Total Phenolic Content

For each of the 13 samples, the same steps as those for standard gallic acid were used, and the solution was diluted using deionized water with dilution factor 1:11, 1 ml of solution and 10 ml of deionized water. The absorbance of the extract samples was recorded. Results were expressed as mg per g of sample dry weight. The total phenolic content was calculated using the formula below:

$$C = c\,\frac{V}{M}$$

Where C = Total phenolic content (mg/g), c = Concentration of sample obtained from the calibration curve in mg/ml, V = Volume of extract in ml, and m = Mass of extract in gram.

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Preparation of Optimized Sample for Total Phenolic Content

Optimized extraction time (115 minutes) and solid-liquid ratio (1:15) was used to extract new sample. The same step in preparation of samples for total phenolic content was used to obtain the total phenolic content.

Determination of Total Flavonoid Content

The extracts' total flavonoid content was determined using the aluminium chloride colorimetric assay. The quantity and location of free OH groups determine the potency of flavonoids, which are secondary metabolites with antioxidant action (Panche et al., 2016).

Preparation of Standard Quercetin for Calibration Curve

To determine the total flavonoid content, a standard quercetin solution was prepared. First, 10 mg of quercetin was dissolved in 96% ethanol, and then diluted to concentrations of 50, 100, and 200 µg/mL. For each sample solution and each standard solution, 1 mL was mixed with 0.3 mL of 5% sodium nitrate ($NaNO_3$), 0.3 mL of 10% aluminum chloride ($AlCl_3$), 2 mL of sodium hydroxide (NaOH), and 6.4 mL of distilled water. The mixtures were incubated at room temperature for 10 minutes. After incubation, the absorbance of each mixture was measured at 510 nm using a T-9200 UV-vis spectrophotometer, with a blank sample as the reference. The absorbance values obtained for the 50, 100, and 200 µg/mL quercetin solutions were then used to plot the calibration curve.

Preparation of Optimized Sample for Total Flavonoid Content

1 ml of optimized extract with extraction time 115 minutes and solid liquid ratio 1:15, was added to 10 ml of volumetric flask. 6.4 ml of H_2O, 0.3 ml of 5% NaNO3, 0.3 ml of 10% AlCl3 and 2 ml of NaOH were added to the same volumetric flask, making final volume of 10 ml. The solution was shaken well and incubated in room temperature for 10 minutes. The absorbance was measured at wavelength 510 nm against blank sample (ethanol) using UV-vis spectrophotometer. The value of absorbance was used to calculate the total flavonoid content.

Results and Discussion

Yield of inhibition for antioxidants activities was determined using DPPH free radical scavenging assay. The result for each run and parameters were according to the table below:

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Table 3: Yield of Antioxidant with Various Value of Parameters (Extraction Temperature and Extraction Time)

Run	Run	Extraction Temperature, ℃	Extraction Time, min	Yield of Antioxidant (%)
1	5	40	30	50.53
2	2	60	30	48.02
3	7	40	60	45.38
4	10	60	60	43.93
5	11	36	45	56.00
6	13	65	45	42.20
7	3	50	24	50.40
8	6	50	66	36.26
9	9	50	45	48.28
10	12	50	45	52.24
11	1	50	45	49.74
12	4	50	45	49.60
13	8	50	45	47.76

The antioxidant yield varied across the 13 experimental runs, ranging from 36.26% to 56.00%. These variations can be attributed to the diverse parameter values from Table 2, which were determined using the experimental Central Composite Design (CCD) and the conditions suggested by Design Expert Software version 7.1 (Table 1). To minimize systematic errors and biases, all experiments were conducted in a randomized manner following the standard order specified by the design matrix.

The influence of extraction parameters on antioxidant activity recovery was further explored using analysis of variance (ANOVA). As detailed in Table 4, both extraction temperature and time were identified as significant factors affecting antioxidant yield. Higher temperatures and longer extraction times generally led to increased antioxidant yields, aligning with previous studies on extraction kinetics and heat sensitivity of antioxidants.

Additional measures of model adequacy, including R^2, Adjusted R^2, and Predicted R^2, were also examined. The R^2 value of 0.930, exceeding 90%, underscores the robustness and statistical significance of the model in explaining the variability in antioxidant yield. Moreover, the 'Adeq Precision' ratio of 15.479, well above the desired threshold of 4, confirms a high signal-to-noise ratio in the data, suggesting reliable predictions from the model.

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The Model F-value of 15.16 further supports the significance of the overall model. The probability (Prob > F) of 0.43% indicates a minimal likelihood that such a high F-value could occur by chance alone, reinforcing the model's validity. Specifically, parameter 'B' emerged as a statistically significant contributor to the model, as indicated by Prob > F values below 0.0500. This finding highlights the importance of extraction time in maximizing antioxidant yield, corroborating literature that emphasizes the role of extraction kinetics in optimizing bioactive compound recovery.

Conversely, the Lack of Fit F-value of 0.37 suggests that the lack of fit relative to pure error is not significant, with a 57.47% probability that this value could be due to noise. This non-significant lack of fit enhances the reliability of the model predictions, indicating that the selected experimental conditions adequately capture the relationship between extraction parameters and antioxidant yield.

Table 4: ANOVA Table of The Selectivity for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F	
Model	280.52	7	40.07	15.16	0.0043	significant
A-Extraction temperature	95.22	1	95.22	36.03	0.0018	
B-Extraction time	99.97	1	99.97	37.83	0.0017	
AB	0.28	1	0.28	0.11	0.7576	
A^2	4.176E-003	1	4.176E-003	1.58E-003	0.9698	
B^2	58.89	1	58.89	22.28	0.0052	
A^2B	14.46	1	14.46	5.47	0.0664	
AB^2	30.25	1	30.25	11.45	0.0196	
Residual	13.21	5	2.64			
Lack of fit	1.13	1	1.13	0.37	0.5747	Not significant
Pure Error	12.09	4	3.02			
Cor Total	293.73	12				
Std. Dev.	1.63					
Mean	47.72					
C.V.%	3.41					
PRESS	90.89					
R-Squared	0.9550					
Adj R-Squared	0.8920					
Pred R-Squared	0.6906					
Adeq Precision	15.479					

Figure 3 illustrates the relationship between the actual and predicted values of antioxidant activity yield. The plot reveals that the residuals, representing the differences between observed and predicted values, are consistently small across the range of measurements. Importantly, these residuals closely follow the diagonal line, indicating a high degree of agreement between the model predictions and actual experimental outcomes. This alignment signifies the adequacy of the developed model in accurately predicting antioxidant yield under varying extraction

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conditions. The close fit of data points to the diagonal suggests that the model effectively captures the complex interactions between extraction parameters and antioxidant activity. Such a validation is crucial for ensuring the reliability and applicability of the model in optimizing extraction processes for maximum antioxidant yield.

Moreover, the consistency of residuals around the diagonal line supports the model's robustness against potential outliers or deviations from expected values. This robustness enhances confidence in the model's ability to generalize findings beyond the specific experimental conditions tested, providing valuable insights into the factors influencing antioxidant yield in similar extraction processes.

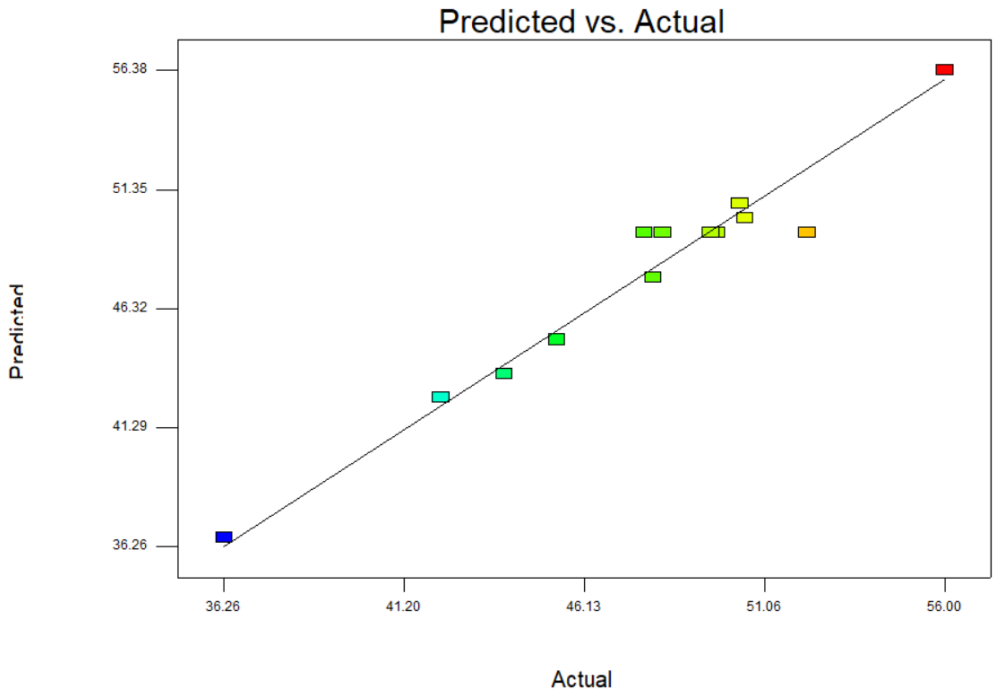

Figure 1: Scatter Diagram of Predicted Versus Actual Yield of Antioxidant Activities

Figures 2 and 3 depict 3D surface and 2D contour plots, respectively, analyzing the influence of extraction temperature and solid extraction time on antioxidant activity yield. These visual representations highlight the interactive effects of these two factors on the response variable. The plots clearly demonstrate that both extraction temperature and extraction time significantly impact the yield of antioxidant activities. Higher temperatures and longer extraction times generally lead to increased yields, as indicated by the contour lines and surface contours showing higher levels of antioxidant activity yield.

According to the plots, the optimal conditions for maximizing antioxidant activity yield are observed at an extraction temperature of 40°C and a solid extraction time of approximately 42.16 minutes. These conditions represent the points on the plots where the contours or surface peaks, indicating the highest predicted yields, are most pronounced. This finding is supported by the observed trends in the plots, where deviations from these optimal conditions generally lead to decreased antioxidant activity yields. Such insights are crucial for optimizing extraction

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processes to achieve maximum yield of bioactive compounds, ensuring efficient and effective utilization of resources in industrial applications.

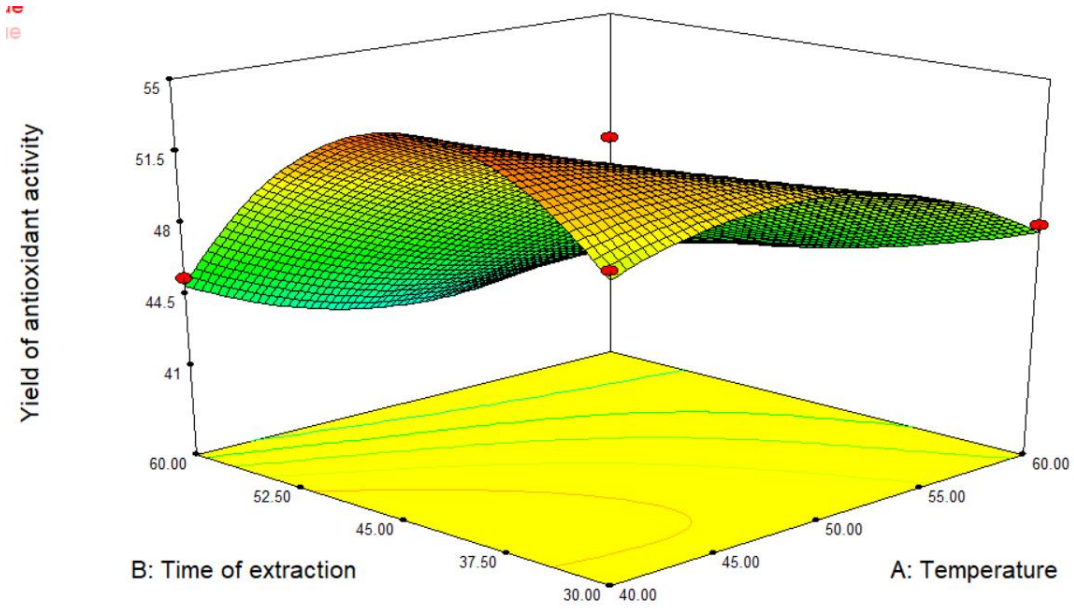

Figure 3: 3D Surface Plot of Yield of Antioxidant Activity Versus Extraction Time and Extraction Temperature

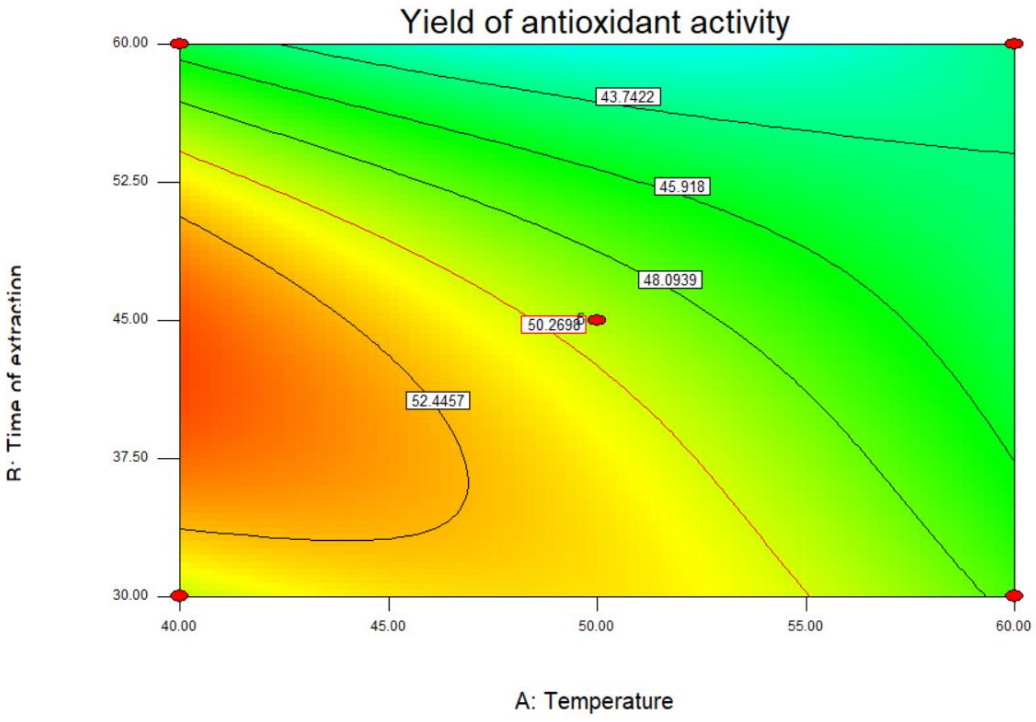

Figure 4: Contour Plot of Yield of Antioxidant Activity Versus Extraction Time and Extraction Temperature

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The optimal process parameters identified were an extraction temperature of 40°C and a solid extraction time of 42.16 minutes. These conditions were determined through analysis of the experimental data and validated using predictive modeling techniques. Under these optimal extraction conditions, the actual yield of antioxidant activities was measured at 52.4420%. This result closely aligns with the predicted value of 54.6224%, as indicated by the small deviation between the observed and expected outcomes. This consistency verifies the accuracy and reliability of the developed model in predicting antioxidant yield under varying extraction parameters. The close agreement between actual and predicted values underscores the robustness of the model in capturing the complex interactions between extraction variables and antioxidant activity. Such validation is essential for demonstrating the model's applicability and effectiveness in optimizing extraction processes to achieve desired bioactive compound yields.

Moreover, the high level of agreement between actual and predicted values enhances confidence in the model's predictive capabilities beyond the tested conditions, providing valuable insights for scaling up extraction processes in industrial applications. This validation strengthens the basis for utilizing the identified optimal parameters to enhance efficiency and productivity in antioxidant extraction processes.

Table 5: Optimized Extraction Temperature and Extraction Time

Number	Extraction Temperature, ℃	Extraction Time, min	Yield of Antioxidant Activity	Desirability	
1	40.00	42.16	54.6224	0.930	Selected
2	40.00	41.85	54.6195	0.930	
3	40.00	42.58	54.6169	0.930	

Table 6: Result Validation and Percentage Error of the Actual and Predicted Yield of Antioxidant Activities

Run	A: Extraction temperature (℃)	B: Extraction time (min)	Yield of antioxidant activity (%)		
			Predicted	Actual	Error, %
Optimized	40	42.16	54.6224	52.4420	3.99

The optimized extract was evaluated for flavonoid and total phenolic content, and the results are summarized in Table 7. The data demonstrate that Centella Asiatica leaves extracted using ultrasound-assisted extraction (UAE) exhibit significant antioxidant activity compared to various other plants, as detailed in Table 7. The analysis reveals that the extract from Centella Asiatica leaves achieved a notably high yield of antioxidant activities, surpassing those reported for other plant extracts listed in Table 7. This finding underscores the efficacy of UAE in extracting bioactive compounds, such as flavonoids and total phenolics, from Centella Asiatica leaves.

These results contribute to the growing body of evidence supporting UAE as a promising method for obtaining antioxidant-rich extracts from botanical sources. The comparative data presented in Table 7 highlight Centella Asiatica as a valuable candidate for further investigation and potential applications in pharmaceutical and nutraceutical industries.

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Table 7: Comparison of Yield of Antioxidant Activities, Total Phenolic Contents, Total Flavonoid Contents

Type of plant	Yield of antioxidant activities	Total Phenolic contents (mg/g)	Total Flavonoid contents (mg/g)	References
Centella Asiatica leaves (*Pegaga*)	52.4420%	0.5000	15.3125	This work
Manihot Esculenta stems (Cassava)	0.518 %	29.82	N/A	(Yi et al., 2011)
Artemisia arborescens L (Tree wormwood)	8.47 (mmol/100gDW)	10.9100	16.4100	(Piluzza & Bullitta, 2011)
Dipsacus fullonum L (Wild teasel)	4.01(mmol/100gDW)	19.5200	16.2500	(Piluzza & Bullitta, 2011)
Opuntia ficusindica L. (Prickly pear cactus)	2.35(mmol/100gDW)	12.7900	2.6200	(Piluzza & Bullitta, 2011)
Lemon peel Petasites hybridus (butterbur)	N/A	16.71	14.26	(Durmus & Kilic-Akyilmaz, 2023)
Lemon peel Petasites hybridus (butterbur)	N/A	4.37	N/A	(Gündüz, Çiçek, & Topuz, 2023)
Artocarpus heterophyllus Lam. (Jackfruit pulp)	80.92	2.4	N/A	(Cheng et al., 2023)

Conclusion

In conclusion, this study successfully optimized the extraction process of Centella Asiatica leaves using ultrasound-assisted extraction (UAE), achieving a high yield of antioxidant activities. The experiment demonstrated that extraction temperature and time significantly influence antioxidant yield, with optimal conditions identified at 40°C and 42.16 minutes. The accuracy of the predictive model was validated, showing close agreement between predicted and actual antioxidant yields. Moreover, the Centella Asiatica extract exhibited superior antioxidant activity compared to extracts from various other plants, as evidenced by its high flavonoid and total phenolic content. These findings underscore the effectiveness of UAE in extracting bioactive compounds from medicinal plants like Centella Asiatica. For future research, exploring additional bioactive compounds beyond flavonoids and phenolics in Centella Asiatica could provide deeper insights into its medicinal properties. Additionally, applying the optimization technique to different plant materials could extend the applicability of UAE in enhancing bioactive compound extraction across diverse botanical sources. Overall, this study not only contributes to the understanding of extraction optimization and antioxidant activity in Centella Asiatica but also opens avenues for broader applications in pharmaceutical and nutraceutical industries.

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