

Response surface-based optimization of antioxidant-rich extracts from *Orthosiphon aristatus* (Blume) Miq. via microwave-assisted extraction

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Abstract: This study aimed to optimize the extraction of total phenolic content (TPC) and antioxidant capacity from *Orthosiphon aristatus* (Blume) Miq. leaves using microwave-assisted extraction (MAE) and Response Surface Methodology (RSM) with a Box-Behnken Design. The optimization considered three independent variables: extraction time, ethanol concentration, and solid-liquid ratio. Antioxidant activity was evaluated using four different assays: DPPH, ABTS, FRAP, and CUPRAC. The optimal extraction conditions—3.465 minutes extraction time, 60% ethanol concentration, and a 1:20 solid-liquid ratio—produced high levels of TPC and antioxidant activities. The optimization model exhibited strong predictive accuracy, yielding a desirability score of 0.81 and minimal deviation between predicted and experimental values. These findings confirm that MAE, in combination with RSM, provides a robust and efficient approach for extracting bioactive compounds from *O. aristatus*, establishing a solid foundation for scaling up applications in the pharmaceutical, nutraceutical, and functional food industries.

INTRODUCTION

Orthosiphon aristatus (Blume) Miq., widely known as kumis kucing in Indonesia, is a medicinal herb prevalent in Southeast Asia, including Indonesia. Its therapeutic applications are primarily attributed to its rich content of phenolic and flavonoid compounds, which exhibit potent antioxidant properties. In Indonesia, the plant occurs in three phenotypes: purple-flowered, intermediate purple-white, and white-flowered varieties (Febjislami *et al.*, 2019). Notably, the purple-flowered phenotype contains higher levels of bioactive compounds, exhibiting superior pharmacological properties compared to the other types, thereby positioning *O. aristatus* as a promising botanical source for antioxidant-based therapeutic formulations (Suhaimi *et al.*, 2019). The importance of optimizing the extraction of bioactive compounds from *O. aristatus* cannot be overstated. A study by Mahendra (2023) demonstrated that microwave-assisted extraction (MAE) of the purple-flowered leaves yielded higher total phenolic content (TPC) and stronger antioxidant activities—including DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric reducing antioxidant power), and CUPRAC (cupric reducing antioxidant capacity)—compared

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to the white-flowered variant. Specifically, the purple-flowered phenotype exhibited a TPC of 5.89 mg GAE/g DW and antioxidant capacities of 4.34, 40.55, 87.02, and 154.96 $\mu\text{mol TE/g DW}$ for DPPH, ABTS, FRAP, and CUPRAC, respectively, whereas the white-flowered phenotype yielded significantly lower values. These findings align with Batubara *et al.* (2020), reinforcing the importance of optimizing extraction conditions to maximize the recovery of bioactive compounds.

Extraction efficiency of phytochemicals is highly influenced by various operational parameters, including solvent concentration, extraction duration, and the solid-to-liquid ratio. Qomaliyah *et al.* (2019) emphasized the importance of solvent selection, Soós *et al.* (2019) highlighted the influence of extraction time, and Sajid *et al.* (2019) stressed the impact of the solid–liquid ratio. Traditional extraction methods often suffer from extended processing times and excessive solvent consumption (Jiang *et al.*, 2020), thereby necessitating the development of more sustainable and efficient extraction techniques, particularly for industrial-scale applications.

Among modern optimization techniques, Response Surface Methodology (RSM) offers a statistically rigorous and cost-effective framework for evaluating multivariable experimental systems. RSM enables simultaneous assessment of multiple factors and their interactions, using a reduced number of experimental runs—thus improving efficiency compared to traditional full factorial designs (Chelladurai *et al.*, 2020). Its strengths include enhanced interpretability, reduction in experimental cost, and identification of significant variable interactions (Jitolis *et al.*, 2021). RSM has been widely and successfully applied in optimizing extraction processes of phenolic compounds and other secondary metabolites in various plant studies (Nurcholis *et al.*, 2023, 2024; Yatma *et al.*, 2023).

In this context, the present study aims to optimize the extraction of phenolic antioxidants from *O. aristatus* leaves by employing MAE in combination with RSM based on a Box–Behnken Design (BBD). BBD is particularly advantageous because it excludes extreme experimental conditions—often impractical or costly—while enabling accurate modeling and prediction of optimal outcomes (Beg & Akhter, 2021). The study focuses on optimizing three independent variables—extraction time, ethanol concentration, and solid–liquid ratio—to maximize the yield of total phenolic content (TPC) and antioxidant activities, evaluated through DPPH, FRAP, ABTS, and CUPRAC assays. Although prior research has demonstrated the variation in bioactive content among different *O. aristatus* phenotypes and highlighted the influence of extraction conditions, comprehensive optimization studies integrating MAE with RSM-BBD remain limited. This research addresses that gap by systematically evaluating and modeling the effects of process parameters to develop a more efficient, reproducible, and scalable extraction method. The novelty of this study lies in its integrative approach, combining an advanced extraction technique with a statistical design, specifically applied to the purple-flowered phenotype, which exhibits the highest antioxidant potential. The outcomes are expected to contribute not only to academic understanding but also to support the development of industrial applications of *O. aristatus*-based products in the pharmaceutical, nutraceutical, and functional food sectors.

METHOD

Plant Material and Sample Preparation

Purple-flowered *O. aristatus* plants were harvested from Tropical Biopharmaca Research Center, IPB University, Bogor, Indonesia. Leaves were selected from mature plants (≥ 35 days). The harvested leaves were washed with distilled water, oven-dried at 50 °C for 48 hours, ground into powder, and sieved using an 80-mesh stainless steel filter.

Experimental Design Using Response Surface Methodology

Optimization of the extraction process was conducted using Response Surface Methodology (RSM) based on a Box–Behnken Design (BBD). Three independent variables were tested: extraction time (1, 3, and 5 minutes), ethanol concentration (40%, 60%, and 80%), and solid–

liquid ratio (1:10, 1:15, and 1:20 g/mL). Design-Expert® version 23.0 software (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate the experimental matrix, analyze data, and calculate the desirability function to identify optimal extraction conditions. The responses evaluated were total phenolic content (TPC) and antioxidant activities (DPPH, FRAP, ABTS, and CUPRAC).

Extraction Procedure

The powdered *O. aristatus* leaves were subjected to MAE using a Sharp R-21D0(S)-IN microwave oven (Sharp Corporation, Osaka, Japan) at 135 W, modified from Nurcholis *et al.* (2022). For each run, 4.0 g of sample was mixed with ethanol (Merck, Germany) at the specified concentration according to the experimental design (Table 1), then irradiated for the designated time. After extraction, the mixture was filtered using Whatman No. 4 filter paper (GE Healthcare, UK), and the filtrate was collected. To standardize extract concentration, the filtrate was adjusted back to its original volume using additional ethanol.

Total Phenolic Content

Total Phenolic Content (TPC) was measured using the Folin–Ciocalteu method, following Nurcholis *et al.* (2022) with slight modification. A 20 µL aliquot of extract was mixed with 120 µL of 10% Folin–Ciocalteu reagent (Merck, Germany) and incubated in the dark at room temperature for 5 minutes. Then, 80 µL of 10% sodium carbonate solution (Sigma-Aldrich, USA) was added. After 30 minutes of incubation, absorbance was recorded at 750 nm using a BMG LABTECH NanoSpectrophotometer (Germany). Gallic acid (Sigma-Aldrich, USA) was used for the standard curve, and TPC was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Antioxidant Assays

Antioxidant activity was assessed using four assays: DPPH, FRAP, ABTS, and CUPRAC. DPPH: The method was modified from Arista *et al.* (2023). A 100 µL aliquot of extract was mixed with 100 µL of 125 µM DPPH solution (Sigma-Aldrich, USA) and incubated in the dark for 30 minutes at room temperature. Absorbance was measured at 517 nm. FRAP: Based on Arista *et al.* (2023), 10 µL of extract was mixed with 300 µL of freshly prepared FRAP reagent consisting of acetate buffer, TPTZ (Sigma-Aldrich, USA), and FeCl₃•6H₂O (Merck, Germany) in a 10:1:1 ratio. After 30 minutes, absorbance was measured at 593 nm. ABTS: According to Nurcholis *et al.* (2022), 20 µL of extract was reacted with 180 µL of ABTS solution (ABTS 7.7 mM and K₂S₂O₈ 2.4 mM, both from Sigma-Aldrich, USA). Absorbance was read at 734 nm after 6 minutes. CUPRAC: Following Nurcholis *et al.* (2022), 50 µL of extract was combined with 50 µL each of 0.01 M CuCl₂, 0.0075 M neocuproine, and pH 7 ammonium acetate buffer (all from Sigma-Aldrich, USA), then incubated in the dark for 30 minutes. Absorbance was measured at 450 nm. Trolox (Sigma-Aldrich, USA) was used as the calibration standard, and all results were expressed in µmol Trolox equivalents per gram dry weight (µmol TE/g DW).

Statistical Analysis and Model Verification

All response variables were analyzed using Analysis of Variance (ANOVA) in Design-Expert® version 23.0 software (Stat-Ease Inc., Minneapolis, MN, USA). Regression models were constructed to identify significant individual and interaction effects among variables. Model adequacy was assessed based on F-values, p-values, coefficient of determination (R²), and adjusted R². To validate the optimized conditions, the experiment was repeated using the predicted optimal parameters. Model performance was confirmed by comparing predicted versus actual values using residual standard error (RSE) and p-values. A p-value > 0.05 and %RSE within ±5% were considered acceptable thresholds for model accuracy and reliability.

RESULTS

Evaluation of Experimental Responses in Box-Behnken Design

The optimization process was conducted to maximize the extraction of antioxidant-rich compounds from *O. aristatus* through a statistically efficient experimental design. The experimental responses, including total phenolic content (TPC) and antioxidant capacities, were influenced by variations in extraction time, ethanol concentration, and solid–liquid ratio (Table 1). The maximum TPC (17.84 mg GAE/g DW) was achieved using 1 minute extraction time, 60% ethanol, and a 1:15 solid-to-solvent ratio. The highest antioxidant capacities were recorded as follows: DPPH (0.87 $\mu\text{mol TE/g DW}$ at 5 min, 80%, 1:20), FRAP (421.13 $\mu\text{mol TE/g DW}$ at 3 min, 60%, 1:20), CUPRAC (355.57 $\mu\text{mol TE/g DW}$ at 3 min, 60%, 1:20), and ABTS (2057.74 $\mu\text{mol TE/g DW}$ at 1 min, 80%, 1:20).

Table 1. Experimental results from Box-Behnken design showing TPC and antioxidant responses (DPPH, FRAP, ABTS, CUPRAC) under different extraction conditions.

Run	Factors			Response				
	Time (min)	[Ethan ol] (%)	Solid-to-solvent (mL/g)	TPC (mg GAE/g DW)	DPPH ($\mu\text{mol TE/g DW}$)	FRAP ($\mu\text{mol TE/g DW}$)	ABTS ($\mu\text{mol TE/g DW}$)	CUPRAC ($\mu\text{mol TE/g DW}$)
1	3	80	1:15	6.01	0.57	341.42	1196.29	243.88
2	3	80	1:15	11.17	0.66	278.54	853.00	249.35
3	3	80	1:15	11.64	0.37	271.23	1156.64	214.55
4	1	80	1:10	15.58	0.26	225.56	699.21	164.90
5	3	100	1:10	3.31	0.10	139.28	567.02	121.25
6	5	80	1:10	13.36	0.30	239.41	648.81	146.85
7	1	60	1:15	17.84	0.33	378.34	1449.11	174.68
8	3	60	1:20	12.51	0.77	421.13	1593.41	355.57
9	3	80	1:15	11.65	0.38	331.04	1022.79	237.61
10	1	80	1:20	5.39	0.32	361.64	2057.74	226.51
11	5	80	1:20	3.31	0.87	295.23	1320.76	234.15
12	3	80	1:15	12.35	0.51	264.88	936.04	212.68
13	3	100	1:20	2.64	0.55	286.51	1257.96	218.01
14	1	100	1:15	6.12	0.28	161.04	705.53	190.01
15	5	100	1:15	6.31	0.30	158.92	758.82	245.35
16	3	60	1:10	17.47	0.36	261.33	770.26	163.39

Note: TPC = total phenolic content; GAE = gallic acid equivalent; DW = dry weight; DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = ferric reducing antioxidant power; TE = Trolox equivalent; ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CUPRAC = cupric reducing antioxidant capacity.

Regression Modeling and ANOVA Analysis

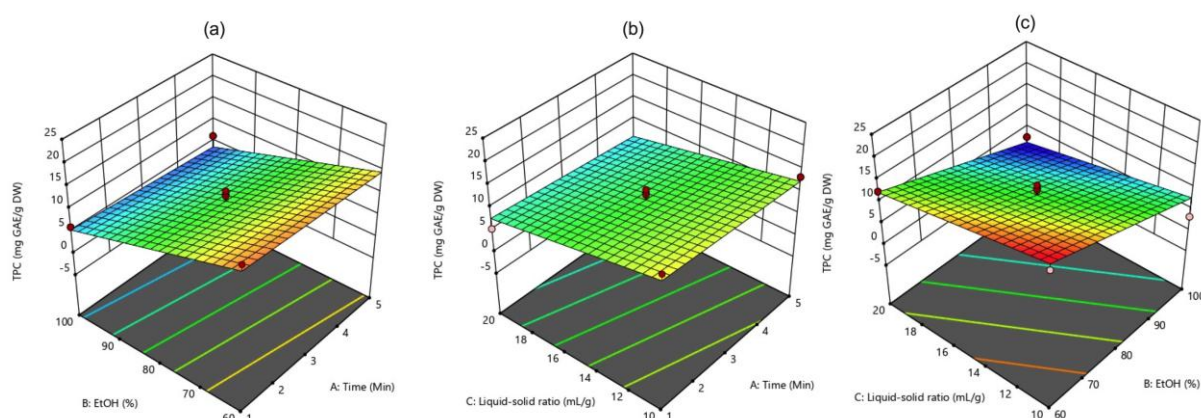
The response surface modeling revealed that TPC, FRAP, ABTS, and CUPRAC were best fitted by linear models, whereas the DPPH response followed a two-factor interaction (2FI) model (Table 2). Negative coefficients in the TPC model suggested a decline in phenolic yield with increasing factor levels, while the antioxidant responses exhibited improvements under specific extraction conditions. Analysis of variance (ANOVA) confirmed that all models were statistically significant ($p < 0.05$). The high F-values and strong agreement between R^2 and adjusted R^2 values indicated that the models provided a good fit to the data.

Table 2. Regression equations and ANOVA statistics for each response variable in the optimization model.

Variables	TPC	DPPH	FRAP	ABTS	CUPRAC
	Linear Model	2FI Model	Linear Model	Linear Model	Linear Model
F	16.36	10.16	22.80	19.4	6.80
p	< 0.0002	0.0014	< 0.0001	< 0.0001	0.0062
R ²	0.8035	0.8714	0.8507	0.8291	0.6300
Adjusted R ²	0.7544	0.7857	0.8134	0.7864	0.5374

Total Phenolic Content (TPC) Surface Response

The 3D response surface plots (Figure 1) demonstrated that shorter extraction times and moderate ethanol concentrations (particularly 60%) produced higher TPC values. Similarly, the solid–liquid ratio significantly affected phenolic yield, where lower ratios (e.g., 1:10 and 1:15) favored higher TPC. The optimal combination for TPC maximization was observed at short extraction duration, 60% ethanol concentration, and a reduced solid–liquid ratio.

**Figure 1.** 3D response surface plots for TPC showing the effect of (a) extraction time and ethanol concentration, (b) extraction time and solid-liquid ratio, and (c) ethanol concentration and solid-liquid ratio.

Antioxidant Capacity Surface Responses

The response surface plots (Figures 2 and 3) showed that DPPH radical scavenging activity peaked under 5 min extraction time, 60% ethanol, and 1:20 solid–liquid ratio. The FRAP and ABTS assays exhibited maximum values at 1 min, 60% ethanol, and 1:20 ratio, while CUPRAC peaked at 5 min, 60% ethanol, and 1:20. Among all assays, DPPH showed the lowest antioxidant capacity, whereas ABTS provided the highest readings. Prolonged extraction and increased solvent volume improved DPPH and CUPRAC outcomes, whereas shorter extraction times were more favorable for FRAP and ABTS activities.

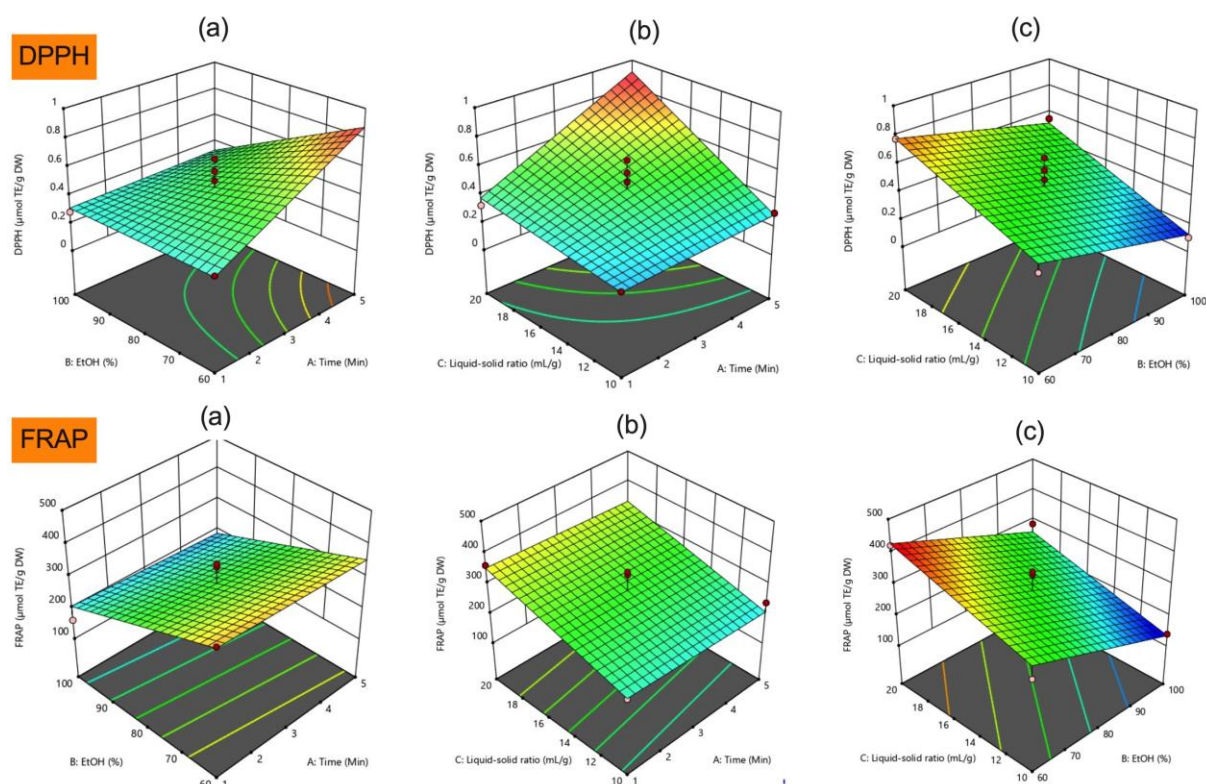


Figure 2. 3D response surface plots for DPPH and FRAP showing the effect of (a) extraction time and ethanol concentration, (b) extraction time and solid-liquid ratio, and (c) ethanol concentration and solid-liquid ratio.

Optimization Validation and Model Accuracy

The optimal extraction condition was identified at 3.465 minutes, 60% ethanol concentration, and a 1:20 solid–liquid ratio, yielding a desirability score of 0.81 (Table 3). The predicted values closely matched the actual experimental results, confirming model validity. The residual standard error (%RSE) for all response variables ranged between 0.001% and 0.043%, and *p*-values exceeded 0.05, indicating that there were no statistically significant differences between predicted and actual values. These findings validated the reliability and predictive accuracy of the optimization model.

Table 3. Comparison of predicted and actual values under optimal extraction conditions.

Factors	A (Time, min)	B (EtOH, %)	C (Solid-solvent, g/mL)	TPC	DPPH	FRAP	ABTS	CUPRAC	Desirability
Predicted	3.465	60	1:20	12.150	0.870	424.355	1688.660	294.226	0.81
Actual	3.465	60	1:20	12.673	0.874	424.718	1690.899	294.459	
%RSE				0.043	0.005	0.001	0.001	0.001	
<i>p</i> -value				0.096	0.074	0.35	0.301	0.583	

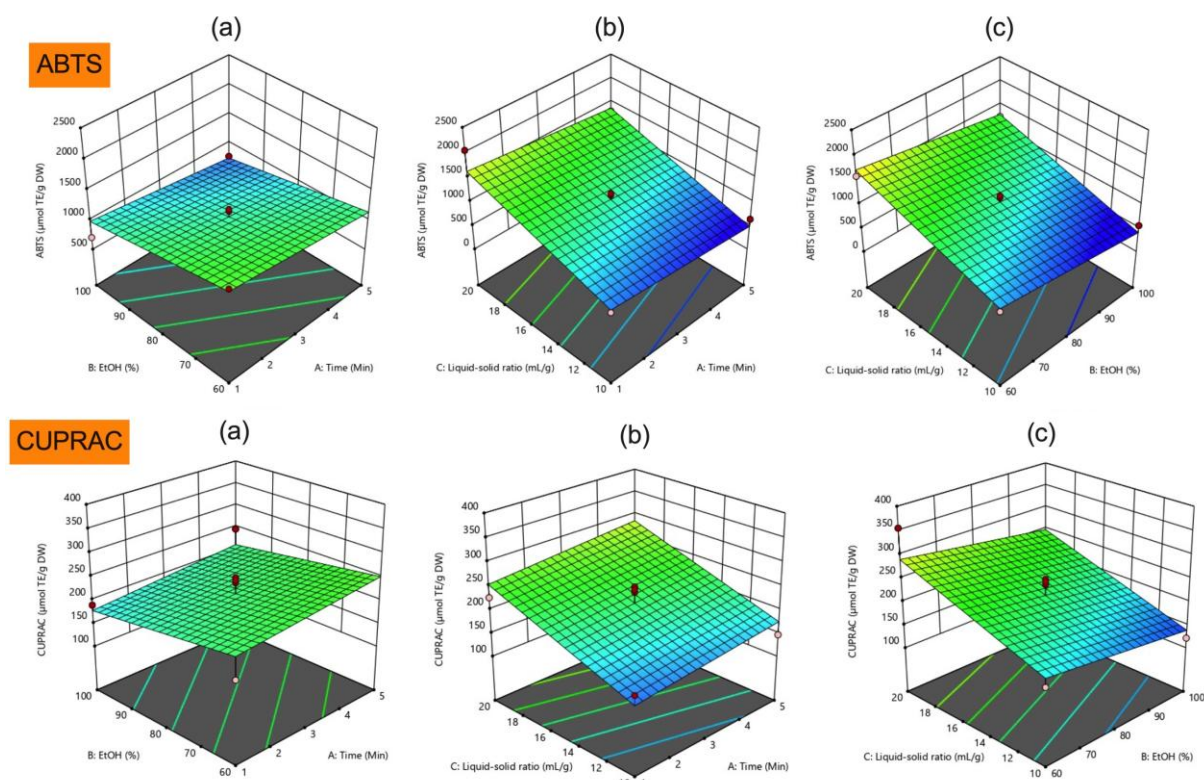


Figure 3. 3D response surface plots for ABTS and CUPRAC showing the effect of (a) extraction time and ethanol concentration, (b) extraction time and solid-liquid ratio, and (c) ethanol concentration and solid-liquid ratio.

DISCUSSION and CONCLUSION

The findings from this study reinforce the critical role of extraction parameters in maximizing the recovery of bioactive compounds from *O. aristatus* leaves. The observed variability in TPC and antioxidant capacities across different treatment conditions highlights the sensitivity of phytochemical yields to extraction dynamics, particularly under MAE. MAE is well-documented for its efficiency in cell wall disruption and solute mobilization, attributed to rapid internal heating and solvent penetration (Osorio-Tobón, 2020), which supports the enhanced yields obtained under the optimized settings in this study.

The desirability function-based optimization identified 3.465-minute extraction time, 60% ethanol, and a 1:20 solid–liquid ratio as the most effective parameters. These conditions promote efficient solvent penetration and diffusion, facilitating compound release without excessive thermal degradation (Frempong *et al.*, 2021). Furthermore, the findings support prior evidence that semi-polar solvents, such as ethanol–water mixtures, are optimal for extracting phenolic antioxidants due to their intermediate polarity, which aligns well with the polarity of target phytochemicals (Kaczorová *et al.*, 2021; Salehi *et al.*, 2019).

The differential responses observed across antioxidant assays reflect the distinct mechanisms and chemical reactivities inherent to each method. ABTS and DPPH primarily assess radical scavenging ability, while FRAP and CUPRA evaluate the reducing capacity of antioxidants (Atere *et al.*, 2018; Munteanu & Apetrei, 2021). The notably high ABTS values indicate a strong hydrogen atom transfer (HAT) capability of the extract constituents, whereas moderate FRAP and CUPRAC activities point to the presence of compounds with electron transfer (ET) capacity. These mechanistic differences emphasize the importance of using multiple complementary assays for a comprehensive evaluation of antioxidant potential (Bibi Sadeer *et al.*, 2020). The statistical model was validated by the close agreement between predicted and experimental values, characterized by high R^2 values and low residual standard errors, confirming its reliability. This robustness is consistent with findings from previous studies

employing RSM in optimizing phytochemical extractions (Dos Santos *et al.*, 2020), underscoring the applicability of this approach in predictive modeling.

This study successfully employed RSM combined with a Box–Behnken Design (BBD) to optimize MAE conditions for extracting phenolic compounds and antioxidants from *O. aristatus*. The optimal parameters—3.465 min extraction time, 60% ethanol, and a 1:20 solid–liquid ratio—yielded high TPC and antioxidant activities, achieving a desirability score of 0.81. The strong concordance between predicted and observed values further validated the precision and robustness of the optimization model.

Nonetheless, this study presents certain limitations. It focused exclusively on the purple-flowered phenotype, which, although known for its high antioxidant potential, may not represent the full metabolic diversity of the species. Additionally, the study did not conduct comprehensive metabolite profiling. Future research should investigate multiple phenotypes and incorporate advanced analytical tools such as LC-MS/MS or NMR spectroscopy to identify specific active compounds and further assess their biological relevance through *in vitro* and *in vivo* validation.

These findings offer practical applications for industries utilizing botanical extracts. The validated model supports the development of efficient, scalable, and cost-effective extraction protocols suitable for pharmaceutical, nutraceutical, and functional food production. Furthermore, the optimization framework demonstrated in this study may serve as a reference model for scaling up extraction processes in other medicinal plants with similar phytochemical profiles.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

R.P.B.: Contributed to conceptualization, investigation, data curation, software, formal analysis, visualization, and writing—original draft. **L.A.:** Contributed to methodology, supervision, validation, and writing—review and editing. Waras Nurcholis contributed to supervision, validation, methodology, and writing—review and editing. All authors have read and approved the final manuscript.

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