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Optimizing Polyphenol Recovery and Antioxidant Capacity of *Orthosiphon aristatus* Leaves via Split-Plot Central Composite Design

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Abstract: *Orthosiphon aristatus* (Blume) Miq., a traditional medicinal plant rich in polyphenolic and flavonoid compounds, holds significant potential as a natural antioxidant source. This study aimed to optimize the extraction conditions to maximize the bioactive compound yield and antioxidant capacity of O. aristatus leaves using maceration extraction combined with a Split-plot Central Composite Design (CCD) approach, specifically to address operational constraints associated with hard-to-change factors. The optimization was conducted in two stages. In the first stage, temperature and extraction time were evaluated to maximize phenolic recovery and antioxidant capacity (FRAP, DPPH). The second stage assessed pH, solvent-to-solid ratio, and methanol concentration to enhance flavonoid yield and antioxidant capacity (CUPRAC, ABTS). Optimal conditions in the first stage at 67.4°C and 219.1 min resulted in high total phenolic content (21.07 mg GAE/g DW) and enhanced FRAP and DPPH activities (92.30 and 2.35 µmol TE/g DW, respectively). The second stage yielded optimal values at pH 2, 15 mL/g, and 86.05%, respectively. These conditions maximized total flavonoid content (7.21 mg QE/g DW) and antioxidant activities as measured by CUPRAC (366.71 µmol TE/g DW) and ABTS (102.48 µmol TE/g DW). Model validation confirmed high accuracy with desirability values >0.90 and RSE <5%. This study highlights the effectiveness of optimized maceration-based extraction in enhancing antioxidant compound recovery from *O. aristatus*, supporting its development for functional and therapeutic applications.

Keywords: flavonoids; maceration; pH; phenolic; temperature.

1. Introduction

Orthosiphon aristatus (Blume) Miq. (*O. aristatus*), commonly referred to as "kidney tea" or "misai kucing" in Southeast Asia, is a medicinal plant rich in secondary metabolites. [1,2]. Over 200 chemical constituents have been identified from *O. aristatus*, with polyphenolic compounds—including phenolics, flavonoids, diterpenoids, triterpenoids, and their derivatives—being the predominant bioactive component. Nearly 50 phenolic compounds, such as rosmarinic acid and more than 20 flavonoid compounds, primarily flavones with polymethoxy substitutions, have been isolated from this species [3]. Contemporary pharmacological research has demonstrated that *O. aristatus* possesses a broad spectrum of

bioactivities, including antioxidant, anti-inflammatory, nephroprotective, antibacterial, antitumor, immunomodulatory, and notably, antidiabetic effects [4]. To fully harness these therapeutic benefits, the extraction of bioactive compounds is a critical step that requires optimization.

Maceration, a diffusion-based method employing organic solvents based on polarity, is among the most widely used due to its operational simplicity, scalability, and cost-effectiveness. Selecting an appropriate solvent system is essential for maximizing compound recovery. While various solvents exist, da Silva [5] highlighted that binary solvent systems outperform single solvents in extracting phytochemicals. Consequently, a maceration technique combined with a methanol-water binary solvent system was adopted for this study due to its high efficiency in recovering the broad polarity range of *O. aristatus* polyphenols.

The efficiency of extraction is influenced by several parameters, including temperature, duration, pH, solvent-to-solid ratio, and solvent concentration [6]. To systematically optimize these factors, this study employed a Split-plot Central Composite Design (Split-plot CCD), a variant of the Response Surface Methodology (RSM). Unlike standard designs, this approach accommodates constraints in randomization arising from Hard-to-Change (HTC) variables, specifically temperature and pH, which require time to stabilize [7]. To the best of our knowledge, this is the first study to apply Split-plot CCD to optimize *O. aristatus* extraction. The objective of this study was to determine the optimal extraction conditions—including temperature, time, pH, solvent-to-solid ratio, and solvent concentration—to maximize polyphenol content and antioxidant capacity. It is hypothesized that this targeted optimization will yield superior extraction efficiency and provide a robust model for industrial scaling.

2. Materials and Methods

2.1. Chemicals and reagents.

All chemicals utilized in this study were of analytical grade and used without further purification. Merck-Millipore (Darmstadt, Germany) supplied pro-analysis methanol, Folin–Ciocalteu reagent, gallic acid, ammonium acetate buffer, $CuCl_2$, $K_2S_2O_8$, neocuproine, $AlCl_3$, $FeCl_3$, HCl, NaOH, and quercetin. Reagents such as Trolox, ABTS, DPPH, sodium carbonate, and glacial acetic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA). Furthermore, 2,4,6-tripyridyl-s-triazine (TPTZ) and acetic acid were sourced from Sisco Research Laboratories Pvt. Ltd. (Maharashtra, India).

2.2. Sample preparation.

Leaves of *O. aristatus* were obtained from the Tropical Biopharmaca Research Center, IPB University (Bogor, Indonesia). The leaves were oven-dried at 50°C for 48 hours and subsequently ground into a fine powder, passed through an 80-mesh sieve for uniformity.

2.3. Sample extraction.

Extraction was performed using a multifactorial maceration method with methanol as the solvent, aided by a water bath shaker (Memmert, Schwabach, Germany, 3 bar). The optimization process was conducted in two phases. In the first phase, temperature (°C) served as the Hard-to-Change (HTC) factor and extraction time (minutes) as the Easy-to-Change (ETC) factor. Optimal conditions identified were then used in the second phase, where pH was

treated as the HTC factor, and solvent-to-solid ratio (mL/g) and solvent concentration (%) as ETC factors. Extraction results were filtered through Whatman No. 42 filter paper, vacuumed, and the final filtrate adjusted to the original extraction volume.

2.4. Experimental design – first optimization phase.

Split-plot Central Composite Design (CCD) was applied using Design Expert 13.0 software to optimize extraction parameters. Temperature and extraction time were varied across five levels (±α, ±1, 0), as shown in Table 1. Twenty experimental runs were conducted, and responses included total phenolic content (TPC) and antioxidant capacities (DPPH and FRAP). The CCD matrix is shown in Table 2.

Table 1. Factor levels in the first optimization phase.

Independent variable	Parameter level				
	-α	**-1**	**0**	**+1**	**+α**
Temperature (°C)	55	60	70	80	85
Extraction time (min)	165	180	210	240	255

Table 2. CCD matrix for first-stage optimization.

Run	Group	Temperature (°C)	Extraction time (minutes)
1	1	55	210
2		55	210
3	2	70	255
4		70	165
5	3	60	180
6		60	240
7	4	60	180
8		60	240
9	5	70	210
10		70	210
11	6	80	180
12		80	240
13	7	70	210
14		70	210
15	8	70	210
16		70	210
17	9	85	210
18		85	210
19	10	80	180
20		80	240

2.5. Determination of total phenolic content.

TPC was quantified using the Folin–Ciocalteu method with gallic acid as the standard, following Khumaida [8]. A standard curve was prepared (25–200 ppm). Each well of a 96-well microplate received 20 µL sample/standard, 120 µL of 10% Folin–Ciocalteu reagent, incubated in the dark for 5 minutes, followed by 80 µL of 10% Na_2CO_3, and a 30-minute room temperature incubation. Absorbance was measured at 750 nm using a SPECTROstarNano spectrophotometer (BMG LABTECH, Germany), and results were expressed as mol gallic acid equivalents (mol GAE/g DW).

2.6. DPPH radical scavenging capacity.

DPPH assay followed Calvindi [9], using Trolox equivalents (TE). Trolox standards (10–50 µM) were mixed with 100 µL of 125 µM DPPH solution. After 30 minutes of dark incubation, absorbance was read at 517 nm. Results were expressed as µmol TE/g DW.

2.7. FRAP antioxidant capacity.

FRAP analysis was based on Calvindi [9]. Trolox standards (100–700 µM) and 300 µL of FRAP reagent were combined with 10 µL of the sample. After 30 minutes, absorbance was measured at 593 nm and expressed as µmol TE/g DW.

2.8. Experimental design – second optimization phase.

Design Expert 13.0 software was again used for the second optimization phase. The independent variables were pH, solvent-to-solid ratio, and methanol concentration. Each factor was tested at five levels (±α, ±1, 0). Responses included total flavonoid content (TFC) and antioxidant activities via ABTS and CUPRAC assays. Table 3 presents the factor levels, and Table 4 details the experimental matrix.

Table 3. Factor levels in the second optimization phase.

Variable	Parameter Level				
	-α	**-1**	**0**	**+1**	**+α**
pH	1.5	2	3	4	1.5
Solvent: solids ratio (mL/g)	2.5	5	10	15	17.5
Methanol concentration (%)	40	50	70	90	100

Table 4. CCD matrix for second-stage optimization.

Run	Group	pH	Solvent: solids ratio (mL/g)	[Methanol] (%)
1	1	2	15	90
2		2	5	90
3		2	15	50
4		2	5	50
5	2	1.5	10	70
6		1.5	10	70
7	3	3	10	70
8		3	10	70
9		3	10	70
10	4	4.5	10	70
11		4.5	10	70
12	5	4	5	90
13		4	5	50
14		4	15	90
15		4	15	50
16	6	3	10	70
17		3	10	70
18		3	10	70
19	7	3	10	100
20		3	10	40
21		3	2.5	70
22		3	17.5	70
23	8	3	10	70
24		3	10	70
25		3	10	70

2.9. Determination of total flavonoid content.

TFC was determined using the aluminum chloride colorimetric method as per Khumaida [8]. A quercetin standard curve (100–900 ppm) was prepared. A 96-well plate received 10 µL of sample/standard, 60 µL methanol, 10 µL 10% $AlCl_3$, 10 µL 1 M glacial acetic acid, and 120 µL distilled water. After 30 minutes of incubation, absorbance was read at 415 nm. Results were expressed as mg quercetin equivalents (mg QE/g DW).

2.10. ABTS radical scavenging capacity.

ABTS assay followed Calvindi [9]. Trolox standards (50–500 µM) were mixed with 180 µL of ABTS radical solution and 20 µL of the sample. After 6 minutes of incubation in darkness, absorbance was read at 734 nm and reported in µmol TE/g DW.

2.11. CUPRAC antioxidant capacity.

CUPRAC assay was conducted following Nurcholis [10]. A reaction mixture of 50 µL each of $CuCl_2$, neocuproine, and ammonium acetate buffer (pH 7) was combined with 50 µL of sample/standard (Trolox 50–600 µM). After 30 minutes, absorbance was read at 450 nm and expressed in µmol TE/g DW.

2.12. Data analysis and optimization verification.

Data were analyzed using the REML (Restricted Maximum Likelihood) method with Kenward–Roger approximation via Design Expert 13.0. Optimal conditions were selected based on the highest desirability values, with values close to 1 indicating excellent model fit. Experimental verification was performed, and model accuracy confirmed by calculating the Residual Standard Error (%RSE) using:

$$\text{RSE}\ (\%) = \frac{(\text{verification value} - \text{prediction value})}{\text{prediction value}} \times 100 \qquad (1)$$

An RSE within ±5% was considered non-significant.

3. Results and Discussion

3.1. First optimization phase – temperature and time.

Experimental data (Table 5) indicated that extraction at 70°C generally yielded the highest bioactive recovery across all parameters. To determine the precise optima, statistical analysis was conducted using REML with Kenward-Roger correction. This rigorous approach confirmed that temperature and time significantly influenced TPC, FRAP, and DPPH values (Table S1). The developed regression models demonstrated exceptional quality, characterized by high determination coefficients ($R^2 > 0.98$) and low coefficients of variation (CV < 2.5%), confirming both the high reproducibility and statistical adequacy of the models.

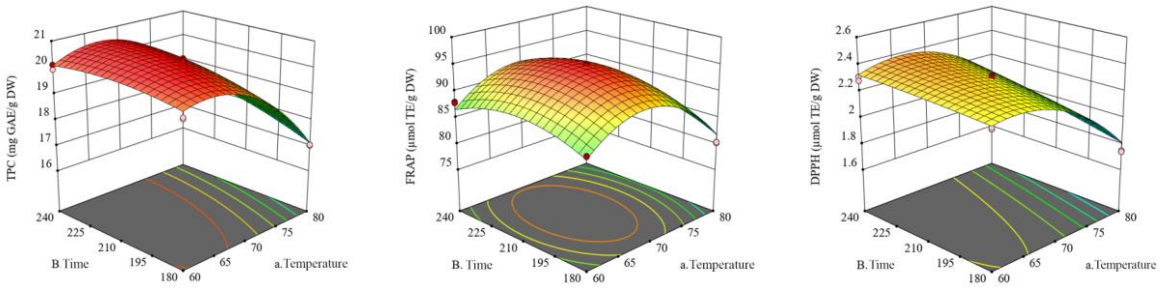
Table 5. Independent variables and first optimization response using Split-plot CCD.

Run	Group	Factor		Response		
		Temperature (°C)	Extraction time (min)	TPC (mg GAE/g DW)	FRAP (µmol TE/g DW)	DPPH (µmol TE/g DW)
1	1	55	210	19.53	80.22	2.18
2		55	210	19.56	80.15	2.21
3	2	70	255	20.24	87.85	2.49
4		70	165	19.56	88.3	2.24
5	3	60	180	19.76	86.89	2.27
6		60	240	20.12	87.85	2.28
7	4	60	180	19.71	87.04	2.26
8		60	240	19.94	88.07	2.31
9	5	70	210	20.21	94.74	2.32
10		70	210	20.24	94.67	2.31
11	6	80	180	17.04	80.37	1.74
12		80	240	18.49	83.41	1.96
13	7	70	210	20.27	94.81	2.29

Run	Group	Factor		Response		
		Temperature (°C)	Extraction time (min)	TPC (mg GAE/g DW)	FRAP (µmol TE/g DW)	DPPH (µmol TE/g DW)
14		70	210	20.33	95.04	2.3
15	8	70	210	20.36	94.89	2.3
16		70	210	20.3	94.59	2.31
17	9	85	210	16	77.56	1.71
18		85	210	16.06	77.7	1.7
19	10	80	180	17.01	80.22	1.75
20		80	240	18.67	83.11	1.98

Regarding TPC and FRAP, both responses exhibited a marked positive linear relationship with temperature as illustrated in (Figure 1A and 1B). The regression coefficients in (Table S2) show positive values for both the linear temperature term (a) and the interaction term (aB). For TPC specifically, the significant positive interaction ($p < 0.001$) indicates a synergistic advantage where simultaneous increases in temperature and time initially enhance yield. These results indicate that moderate thermal input enhances solubility and mass transfer of phenolic compounds by breaking cell wall structures and decreasing solvent viscosity [11].

The parallel trends observed for TPC and FRAP are attributable to their shared mechanistic basis: both assays operate primarily via the Single Electron Transfer (SET) mechanism, in which antioxidants reduce oxidants through redox reactions involving electron donation. However, a limitation was observed at elevated temperatures, where extraction efficiency declined beyond 70°C. Further heating may induce degradation of thermosensitive phenolics. This behavior is captured by the highly significant negative quadratic terms (a^2) in both models, which statistically outweigh the positive interaction, confirming that thermal degradation becomes the limiting factor at 80°C.



(a) **(b)** **(c)**

Figure 1. 3D response surface plots for the first optimization phase showing the interaction between Temperature vs. Extraction Time. **(a)** Total Phenolic Content (TPC); **(b)** FRAP antioxidant capacity; **(c)** DPPH radical scavenging capacity. Units: Temperature (°C), time (min).

Similarly, the DPPH radical scavenging capacity (Figure 1C) was positively driven by temperature (+0.19a) and its interaction with time (+0.00016aB). Unlike FRAP, the DPPH assay relies heavily on the Hydrogen Atom Transfer (HAT) mechanism, assessing the ability of antioxidants to neutralize free radicals via hydrogen donation. While this interaction suggests that prolonged heating can aid extraction to some extent, the negative quadratic term (-0.0017a2) again played a decisive role, reducing radical scavenging potential at the upper limits of the design space (>70°C). This trend highlights a critical weakness of uncontrolled heating: specific antioxidants responsible for DPPH inhibition are susceptible to oxidation under prolonged heat exposure.

Consequently, the global optimal conditions were established at 67.4°C and 219.1 min to balance the synergy between extraction and antioxidant preservation effectively. Model validation under these predicted optimal conditions demonstrated high accuracy, with a

desirability of 0.937 and RSE below 2.5% (Table 6). Notably, the optimized TPC (21.07 mg GAE/g DW) obtained here is superior to yields reported in previous studies utilizing uncontrolled heating [12, 13]. Furthermore, this yield is competitive with results reported for other medicinal plants extracted using advanced techniques, such as combined Ultrasound and Microwave-Assisted Extraction (UMAE) [14], suggesting that optimized maceration remains a highly efficient and cost-effective method.

Table 6. Comparison of predicted and experimental values of the first optimization.

Parameters	a	B	TPC	FRAP	DPPH	Desirability
Prediction	67.367	219.105	20.556	94.620	2.352	0.937
Experimental	67.367	219.105	21.068	92.296	2.348	
%RSE			2.49%	-2.46%	-0.17%	

3.2. Second optimization phase – ph, solvent: solids ratio, and methanol concentration.

Following the first phase, the second optimization evaluated pH (HTC factor), solvent-to-solid ratio, and methanol concentration, with detailed experimental results listed in Table 7. Statistical analysis utilizing the same REML approach (Table S3) confirmed that the proposed models fitted the data well, exhibiting high determination coefficients ($R^2 > 0.95$) and low coefficients of variation (CV < 2.5%), indicating a strong agreement between observed and predicted values, thus ensuring the reliability of the optimization process. The mathematical models are provided as Equations 4–6 in Table S4.

Table 7. Independent variables and the second optimization response using Split-plot CCD.

Run	Group	Factor			Response		
		pH	Solvent: solids ratio (mL/g)	Solvent concentration (%)	TFC (mg QE/g DW)	CUPRAC (µmol TE/g DW)	ABTS (µmol TE/g DW)
1	1	2	15	90	7.76	386.98	111.84
2		2	5	90	1.91	73.32	21.55
3		2	15	50	2.81	308.57	94.34
4		2	5	50	0.54	109.07	28.22
5	2	1.5	10	70	5.13	323.74	70.71
6		1.5	10	70	5.16	324.05	70.92
7	3	3	10	70	4.54	257.53	77.79
8		3	10	70	4.61	257.23	76.75
9		3	10	70	4.6	259.35	77.27
10	4	4.5	10	70	5.85	217.83	80.08
11		4.5	10	70	5.87	217.98	79.88
12	5	4	5	90	3.14	53.54	20.51
13		4	5	50	1.94	158.23	54
14		4	15	90	7.13	160.84	65.75
15		4	15	50	5.16	284.25	114.97
16	6	3	10	70	4.62	257.83	78.63
17		3	10	70	4.61	257.98	78
18		3	10	70	4.63	256.92	77.27
19	7	3	10	100	5.01	97.92	32.06
20		3	10	40	3.07	276.62	93.63
21		3	2.5	70	1.14	57.94	24.55
22		3	17.5	70	5.24	286.81	110.8
23	8	3	10	70	4.69	257.08	77.69
24		3	10	70	4.69	260.86	77.48
25		3	10	70	4.7	260.41	77.58

TFC was influenced by complex interactions among all variables, as visualized in Figure 2A–C. Specifically, (Figure 2C) illustrates the strong interaction between solvent-to-solid ratio and methanol concentration (BC), where increasing both factors simultaneously maximizes yield. The pH factor also played a crucial role; as seen in (Figure 2A and 2B), TFC recovery tends to curve upwards at acidic extremes (pH 2). This supports the quadratic effect

identified in Equation 4. Acidic conditions likely promote the hydrolysis of ester bonds in phenolic glycosides and stabilize the polyphenol structure. This is corroborated by findings that show enhanced recovery of flavonoids at low pH values [15, 16].

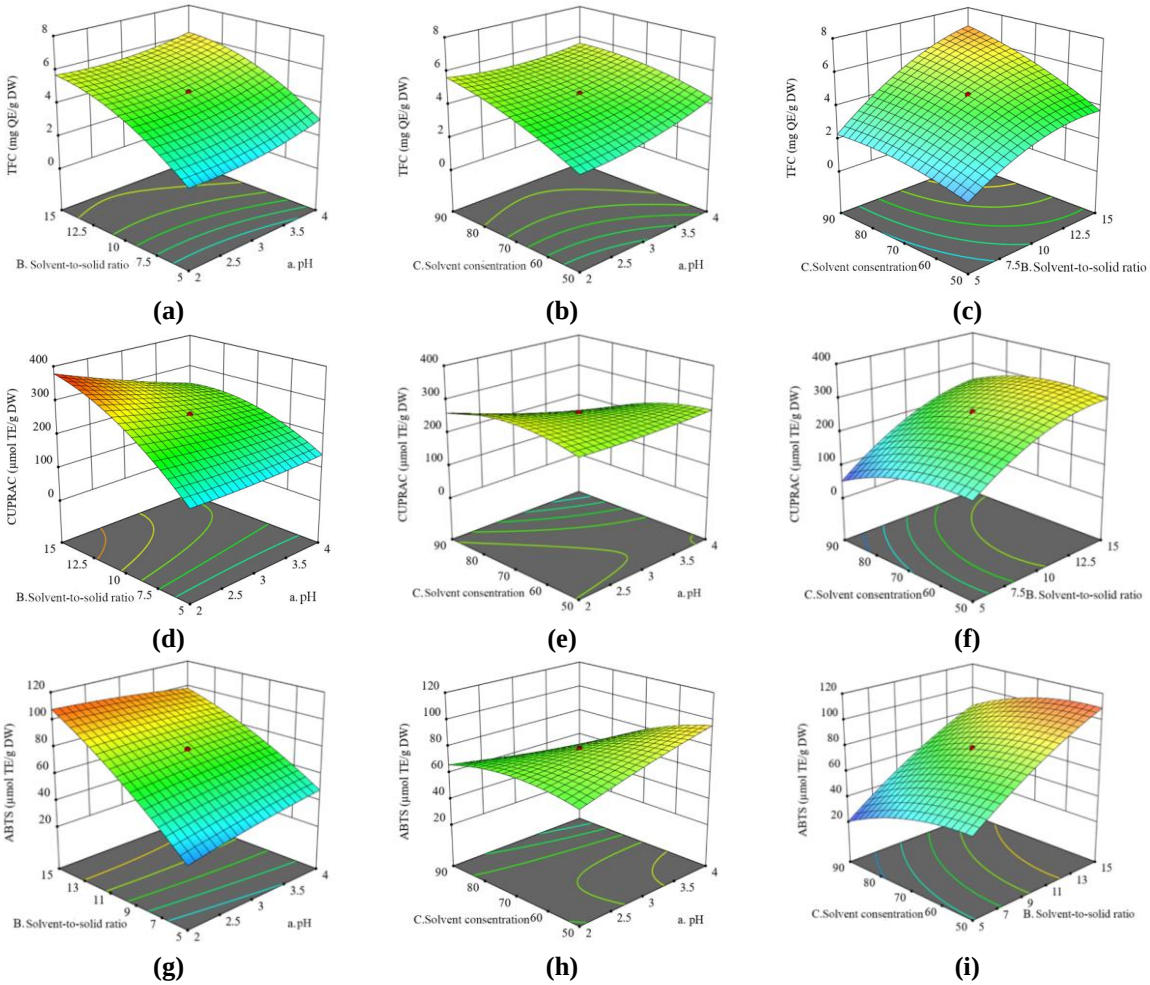


Figure 2. Matrix of 3D response surface plots for the second optimization phase showing interactions between pH, Solvent-to-solid ratio, and Methanol concentration. **(a–c)** Total Flavonoid Content (TFC); **(d–f)** CUPRAC antioxidant capacity; **(g–i)** ABTS radical scavenging. Units: Solvent-to-solid ratio (mL/g), Methanol concentration (%).

Distinct dependencies were also observed for antioxidant activities, highlighting the impact of solvent choice on the types of antioxidant compounds extracted and their specific modes of action. For CUPRAC capacity (Figure 2D-F), pH was the dominant factor. (Figure 2D) demonstrates a steep increase in activity at lower pH values combined with a high solvent ratio (aB interaction). This aligns with the SET mechanism governing CUPRAC, where acidic conditions facilitate the protonation of phenolic hydroxyl groups, thereby enhancing their electron-donating capacity. Conversely, the ABTS scavenging capacity (Figure 2G-I) was primarily driven by solvent factors rather than pH. As shown in (Figure 2I), both solvent-to-solid ratio and methanol concentration exhibited strong positive linear effects. Unlike TFC, the surfaces in (Figure 2G and 2H) are relatively flatter concerning the pH axis. This indicates that ABTS scavenging, which relies on the HAT mechanism, is less sensitive to acidity changes and more dependent on the solvent's ability to solubilize hydrogen-donating compounds.

The global optimization predicted that the optimal conditions for maximizing all three responses were pH 2, a solvent-to-solid ratio of 15 mL/g, and a methanol concentration of 86.05%. These conditions successfully balance the need for acidic stabilization of phenolics

with the optimal polarity for flavonoid solubility. Experimental verification confirmed the model's reliability, yielding a composite desirability of 0.901 with RSE below 2.3% for all parameters (Table 8). Notably, the optimized flavonoid yield (7.21 mg QE/g DW) significantly exceeds those reported in previous studies using unoptimized maceration and ethyl acetate solvents [12, 17]. Moreover, the antioxidant capacity obtained demonstrates that precise parameter control can elevate the efficiency of conventional maceration to levels comparable to energy-intensive modern instrumentation used for other bioactive-rich species [14], confirming its industrial viability.

Table 8. Comparison of predicted and experimental values of the second optimization.

Parameters	a	B	C	TFC	CUPRAC	ABTS	Desirability
Prediction	2	15	86.047	7.031	365.578	102.738	0.901
Experiment	2	15	86.047	7.21	366.71	102.48	
%RSE				2.25%	0.31%	-0.25%	

4. Conclusions

This study successfully optimized the extraction parameters to maximize the yield of phenolic and flavonoid compounds from *O. aristatus* leaves using the maceration method supported by Split-plot Central Composite Design (CCD), demonstrating a novel approach to address operational constraints of hard-to-change factors. In the first optimization phase, temperature and extraction time significantly influenced the total phenolic content (TPC), FRAP, and DPPH antioxidant capacities, with the optimal extraction condition identified at 67.4°C and 219.1 minutes. These parameters yielded phenolic-rich extracts with higher antioxidant activities than previously reported under uncontrolled conditions.

The second optimization phase revealed that pH, solvent: solids ratio, and methanol concentration significantly impacted total flavonoid content (TFC), CUPRAC, and ABTS antioxidant capacities. The optimal conditions were pH 2, solvent: solids ratio of 15 mL/g, and methanol concentration of 86.05%, which produced markedly higher yields of flavonoids and antioxidant capacity. Experimental validations confirmed the high reliability and predictive strength of the developed models with desirability values above 0.90 and minimal relative standard errors.

Overall, the findings demonstrate that careful control and multivariate optimization of extraction parameters—particularly temperature, pH, solvent ratio, and methanol concentration—are critical for enhancing the recovery and functional quality of bioactive compounds from *O. aristatus*, providing a scalable and cost-effective protocol. These optimized conditions can support the development of functional ingredients, nutraceuticals, or phytopharmaceuticals with potent antioxidant properties, although further in vivo studies and stability testing are recommended to validate their clinical efficacy.

Author Contributions

Conceptualization, I.D. and W.N.; methodology, D.F. and W.N.; software, D.F. and W.N.; validation, D.F. and W.N.; formal analysis, D.F. and W.N.; investigation, D.F.; resources, W.N.; data curation, D.F. and W.N.; writing—original draft preparation, D.F. and W.N.; writing—review and editing, D.F. and W.N.; visualization, D.F.; supervision, I.D. and W.N.; project administration, W.N.; funding acquisition, W.N. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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