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OPTIMIZED HEAT-ASSISTED EXTRACTION OF BIOACTIVE PHENOLICS FROM *ANDROGRAPHIS PANICULATA*: A RESPONSE SURFACE MODELLING AND HPLC PROFILING STUDY

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ABSTRACT

This study investigated the heat-assisted extraction (HAE) of phenolic compounds from Andrographis paniculata leaves and optimized the process using D-optimal response surface methodology (RSM). The effects of extraction temperature (30–50 °C), solid-to-liquid ratio (1:20–1:60 g/mL), and extraction time (45–180 min) on total phenolic content (TPC), extraction yield (EY), and antioxidant activity (AA) were systematically evaluated. The developed quadratic models showed strong predictive accuracy with high coefficients of determination ($R^2 > 0.97$) and non-significant lack-of-fit ($p > 0.05$). Among the parameters investigated, the solid-to-liquid ratio exhibited the most significant influence on all responses, followed by extraction time and temperature. Multi-response numerical optimization predicted optimal conditions at 44.87 °C, 45 min, and 1:60 g/mL, yielding 125.33 mg GAE/g dw TPC, 25.88 % EY, and 41.83 µM AAE/g dw AA. Experimental validation confirmed the model's reliability with relative standard deviations below 10%. HPLC analysis of the optimized extract identified key phenolic constituents, including botulinic, gallic, chlorogenic, caffeic, ellagic, and ferulic acids, along

with rutin, catechin, and epicatechin, confirming a rich phenolic composition. The findings establish HAE as a robust and efficient green extraction technique for recovering bioactive phenolics from A. paniculata, providing a scientific foundation for its application in functional food and pharmaceutical product development.

Keywords: *Andrographis paniculata, Heat-assisted extraction, Phenolic compounds, Response surface methodology, Antioxidant activity.*

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1. INTRODUCTION

Phenolic compounds are vital secondary metabolites that play key roles in plant physiology, including pigmentation, reproduction, and defense against oxidative and microbial stress. Structurally, they consist of aromatic rings bearing hydroxyl groups and occur mainly as phenolic acids, flavonoids, tannins, and stilbenes. These compounds are recognized for their strong antioxidant capacity, acting through radical scavenging, hydrogen donation, and metal chelation. In biological systems, phenolics protect against oxidative stress by neutralizing free radicals and enhancing endogenous antioxidant defense mechanisms. Numerous studies have linked dietary phenolics to the prevention of cardiovascular diseases, cancer, diabetes, and neurodegenerative disorders. Consequently, phenolic-rich natural products have gained increasing attention as potential substitutes for synthetic antioxidants and preservatives, which are often limited by toxicity and instability. The growing demand for safe,

sustainable, and natural bioactives underscores the need to develop efficient extraction and characterization strategies for phenolic compounds (Shahidi and Ambigaipalan, 2015; Ruskovska et al., 2020).

Among medicinal plants, *Andrographis paniculata* (Burm. f.) Nees—commonly known as "king of bitters"—has received significant scientific and industrial interest for its therapeutic potential. Traditionally used in Asian and African medicine to manage fever, diabetes, and inflammatory conditions, *A. paniculata* possesses a diverse phytochemical profile rich in diterpenoid lactones, flavonoids, and phenolic acids (Akbar, 2011). These compounds collectively contribute to the plant's antioxidant, antimicrobial, anti-inflammatory, and hepatoprotective activities. Notably, the phenolic fraction, including gallic, chlorogenic, caffeic, and ferulic acids, has been associated with strong radical-scavenging capacity and modulation of oxidative pathways. However, the yield and bioactivity of these phenolics are largely influenced by the extraction method and operational parameters such as solvent composition, temperature, time, and solid-to-liquid ratio. Efficient optimization of these parameters is critical to maximize recovery, preserve compound integrity, and improve extract quality for industrial applications (Wang et al., 2018).

Conventional extraction methods, such as maceration and Soxhlet extraction, remain widely used but are limited by long extraction times, high solvent consumption, and potential thermal degradation of heat-sensitive bioactives. Modern green extraction techniques have emerged to overcome these drawbacks, emphasizing energy efficiency, process selectivity, and environmental compatibility. Among them, heat-assisted

extraction (HAE) has gained attention due to its simplicity, scalability, and ability to enhance mass transfer by increasing solute diffusion and solvent permeability. Moderate heating reduces solvent viscosity and improves extraction kinetics, thereby facilitating efficient solubilization of phenolic compounds. However, excessive temperature or prolonged extraction may promote degradation or polymerization, resulting in reduced antioxidant potential. Therefore, process conditions must be carefully optimized to achieve the desired balance between yield and compound stability (Mustafa and Turner, 2011; Castro-López et al., 2017).

Response surface methodology (RSM) has been recognized as a powerful statistical tool for optimizing multivariable processes in a minimal number of experimental trials. It enables the evaluation of individual and interactive effects of process factors and allows for model-based prediction of responses such as total phenolic content (TPC), extraction yield (EY), and antioxidant activity (AA). The D-optimal design, in particular, efficiently handles experimental constraints while maintaining model accuracy. Applying RSM to bioactive compound extraction has been shown to enhance process efficiency, improve product reproducibility, and reduce energy and solvent costs. Through multi-response optimization, it is possible to achieve balanced conditions that simultaneously maximize yield and bioactivity. Additionally, confirmatory experiments and statistical diagnostics such as ANOVA and R^2 are essential to validate model reliability and ensure predictive accuracy for real-world applications (Myers et al., 2016).

Beyond process optimization, the identification and quantification of individual phenolic compounds are crucial for

understanding extract functionality and quality assurance. High-performance liquid chromatography (HPLC) is widely employed for profiling bioactive constituents, allowing for precise detection of phenolic acids and flavonoids in plant extracts. Establishing correlations between extraction parameters and compositional profiles enhances understanding of process–structure–function relationships and supports the standardization of *A. paniculata* extracts for commercial applications. In view of these considerations, the major objectives of this study were to (i) examine how extraction temperature, time, and solid-to-liquid ratio affect TPC, EY, and antioxidant activity of *A. paniculata* leaf extracts under HAE, (ii) build and validate predictive quadratic models, (iii) perform multi-response optimization to maximize phenolic recovery and antioxidant efficacy, and (iv) analyze the phenolic composition of the optimized extract via HPLC.

2. REVIEW OF LITERATURE

Previous studies on *Andrographis paniculata* have largely focused on evaluating its pharmacological and antioxidant potential, with particular emphasis on diterpenoid lactones and, to a lesser extent, phenolic constituents. Several authors have reported the presence of phenolic acids and flavonoids such as gallic acid, chlorogenic acid, caffeic acid, ferulic acid, rutin, and catechins in leaf and aerial parts of the plant, attributing its antioxidant and therapeutic effects partly to these compounds (Akbar, 2011; Kumar and Pandey, 2013). Extraction of these bioactives has commonly been carried out using conventional solvent-based techniques, including maceration (Coelho et al., 209), reflux extraction (Ma et al., 2022), and Soxhlet extraction (Alara, 2019), employing aqueous or hydroalcoholic solvents.

While these approaches have confirmed the phenolic richness of *A. paniculata*, they are often characterized by long extraction times, high solvent consumption, and limited control over compound degradation, which may compromise extract quality and reproducibility (Oke et al., 2023).

More recent investigations have explored assisted extraction techniques, such as ultrasound- and microwave-assisted extraction (Garcia-Vaquero et al., 2020), to improve recovery of phenolic compounds from medicinal plants, including *A. paniculata*. These studies generally report enhanced extraction efficiency and antioxidant activity compared to conventional methods, primarily due to improved mass transfer and cell wall disruption (Mustafa and Turner, 2011; Castro-López et al., 2017). However, in many cases, extraction conditions are selected empirically or optimized using one-factor-at-a-time approaches, which fail to capture interaction effects among critical variables such as temperature, extraction time, and solid-to-liquid ratio (Adeyi et al., 2026). As a result, reported optimal conditions vary widely across studies, limiting comparability and hindering scale-up and industrial application of *A. paniculata* phenolic extracts.

Multivariate optimization tools, particularly response surface methodology (RSM), have been successfully applied to phenolic extraction from various plant matrices, demonstrating improved process efficiency and predictive capability (Ferreira et al., 2007; Myers et al., 2016). Nevertheless, studies applying RSM to *A. paniculata* remain scarce, and those available predominantly employ conventional experimental designs (Chao and Lin 2020; Kumar et al., 2014) without leveraging the flexibility of D-optimal designs under constrained experimental

domains. Moreover, many optimization studies focus solely on global indices such as total phenolic content or antioxidant activity, without adequate validation or integration of compositional analysis. The absence of robust model validation and predictive assessment further limits the reliability of such studies for process design and scale-up (Adeyi et al., 2023).

In addition, detailed characterization of individual phenolic compounds in optimized *A. paniculata* extracts has received limited attention. While HPLC has been used sporadically to identify selected compounds, few studies systematically link optimized extraction conditions with comprehensive phenolic profiling and antioxidant performance. Consequently, the relationship between process parameters, phenolic composition, and functional quality of *A. paniculata* extracts remains insufficiently understood. This reveals a clear knowledge gap in the integrated optimization of heat-assisted extraction using advanced experimental design tools, coupled with rigorous model validation and HPLC-based phenolic profiling. Addressing this gap is essential for developing reproducible, efficient, and industrially relevant extraction strategies for phenolic-rich *A. paniculata* extracts.

3. RESEARCH METHODOLOGY

3.1 Materials and Chemical Reagents

Fresh *Andrographis paniculata* leaves were collected from a medicinal plantation and washed thoroughly under running water to remove surface impurities. The cleaned leaves were air-dried at room temperature for about two weeks, ground, and sieved to a uniform particle size of 0.1 mm. The powdered

samples were stored in black polyethylene bags until analysis. Analytical-grade reagents, including absolute ethanol (99%), sodium carbonate, ascorbic acid, Folin–Ciocalteu reagent, glacial acetic acid, 2,4,6-tripyridyl-s-triazine, sodium acetate trihydrate, hydrochloric acid, and ferric chloride hexahydrate, were obtained from Sigma-Aldrich (Poole, UK). HPLC standards—betulinic acid, gallic acid, caffeic acid, ellagic acid, chlorogenic acid, ferulic acid, rutin, and quercetin—were used, and distilled water served for all laboratory procedures.

3.2 Heat-assisted Extraction of Bioactive Compounds

Heat-assisted extraction (HAE) of bioactive compounds from *A. paniculata* leaves was performed using a water bath, following the approach outlined by Adeyi et al. (2023). A measured amount of leaf powder was mixed with distilled water solvent in a beaker and heated at a controlled temperature with continuous stirring. To minimize solvent evaporation, the beaker was covered with aluminum foil during extraction. Preliminary trials were carried out at 40 °C, 45 min, and a solid-to-liquid ratio of 1:20 g/mL to identify the most suitable solvent mixture for antioxidant recovery. Subsequent optimization experiments employed the selected solvent and were conducted according to the Box–Behnken design conditions. After extraction, the mixtures were centrifuged at 25 °C and 500 rpm for 10 min to separate the solid residue. The resulting extracts were collected and stored in sealed plastic bottles for further analyses

3.3 Experimental Design and Statistical Analysis

The heat-assisted extraction (HAE) process for recovering bioactive compounds from *A. paniculata* leaves was designed and optimized using the Box–Behnken response surface methodology (BBD–RSM). Three process variables—operating temperature (OT, X_1), solid-to-liquid ratio (S:L, X_2), and extraction time (ET, X_3)—were examined at three levels (−1, 0, +1). The coded values corresponded to 35, 45, and 55 °C for OT; 20, 35, and 50 mL for S: L; and 100, 150, and 200 min for ET. The measured responses were total phenolic content (TPC, mg GAE/g dw), antioxidant activity (AA, µM AAE/g dw), and extraction yield (EY, %). The relationships between independent and response variables were expressed by a second-order polynomial model, as shown in Eq. (1)

$$Y = b_o + \sum_{i=1}^{3} b_i X_i + \sum \sum_{i<j=1}^{3} b_{ij} X_i X_j + \sum_{i=j}^{3} b_{ii} X_i^2 \qquad (1)$$

where b_o, b_i, b_{ij} and b_{ii} denote the intercept, linear, interaction, and quadratic coefficients, respectively. Model adequacy and significance were evaluated at a 95 % confidence level using Design-Expert software, based on statistical parameters including R^2, R^2_{adj} and Pred R^2.

3.4 Process Optimization and Model Validation

The optimal conditions for heat-assisted extraction (HAE) of antioxidant compounds from *A. paniculata* leaves were established using the numerical optimization tool in Design-Expert software. The desirability function approach, as described by Adeyi et al. (2022), was applied to simultaneously optimize process variables and responses. Optimization criteria,

including goals, weights, and importance levels, were defined for each variable prior to analysis. The overall desirability index (D) for multiple responses was calculated using Eq. (2):

$$D(x) = (d_1 \times d_2 \times d_3 \dots \dots \dots \dots \dots \dots \dots \dots \dots . d_n)^{1/n} \quad (2)$$

where each individual desirability ranges between 0 and 1, with 1 representing the most desirable condition and 0 indicating unacceptable results. The objective was to concurrently maximize total phenolic content (TPC), antioxidant activity (AA), and extraction yield (EY). The optimized conditions predicted by the model were experimentally validated, and the accuracy of prediction was assessed using the relative standard deviation (RSD) given by Eq. (3)

$$\% \text{ RSD} = \frac{SD_{1-2}}{M_v} * 100 \quad (3)$$

where (SD_{1-2})) is the standard deviation between predicted and experimental values, and (M_v) is their mean.

3.5 Determination of Extraction Yield

The extraction yield (EY) was determined following the method described by Alara et al. (2021). The separated extracts obtained from *A. paniculata* leaves were collected, concentrated, and oven-dried at a controlled temperature until a constant weight was reached. The yield was then calculated using Eq. (4)

$$\% \text{ EY} = \frac{W_1}{W_2} * 100 \quad (4)$$

where W_1 is the weight of the dried extract (g) and $W_2$is the weight of the leaf sample (g) used in the extraction.

3.6 Determination of Total Phenolic Content

The TPC of the aqueous extracts was determined using the Folin–Ciocalteu method as described by Gan and Latiff (2019). Briefly, 1 mL of *A. paniculata* extract diluted (1:10, v/v) with distilled water was mixed with 1.8 mL of Folin–Ciocalteu reagent and allowed to react for 5 minutes. Subsequently, 1.2 mL of 7.5% (w/v) sodium carbonate solution was added, and the mixture was stirred and incubated at 20 °C for 1 hour. The absorbance was measured at 765 nm, and TPC (mg GAE/g dw) was calculated from the gallic acid calibration curve ($y = 0.037x + 0.0025$, $R^2 = 0.997$) using Eq. (5)

$$\mathrm{TPC} = \frac{\mathrm{C} * \mathrm{V}}{\mathrm{m}} \quad (5)$$

where C is the gallic acid concentration (mg/mL) from the standard curve, V is the extract volume (mL), and mmm is the dry weight (g) of the *A. paniculata* sample used.

3.7 Determination of Antioxidant Activity (FRAP Assay)

The antioxidant activity of the extracts was determined using the ferric reducing antioxidant power (FRAP) assay following the procedure of Uddin et al. (2016). This method evaluates the ability of antioxidants to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. The FRAP reagent was freshly prepared by combining 0.1 M acetate buffer, 0.01 M TPTZ solution, and 0.02 M $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio. A 50 µL portion of the diluted extract (1:10, v/v) was mixed with 1.5 mL of the FRAP reagent, and the reaction mixture was incubated at 25 °C for 30 minutes. The absorbance was then recorded at 593 nm using a UV–Vis spectrophotometer. Antioxidant activity (AA), expressed as µM

AAE/g dw, was calculated from an ascorbic acid calibration curve ($y = 0.0051x + 0.0015$, $R^2 = 0.995$).

3.8 HPLC Characterization of Phenolic Compounds

The characterization of *A. paniculata* leaf extracts was carried out using high-performance liquid chromatography (HPLC) following the procedure described by Krishna and Manohar (2014). Analysis was performed on the bioactive extract obtained under optimized conditions. Before injection, the extract was filtered through a 0.45 µm membrane to eliminate residual particles. A 10 µL aliquot of the filtrate was introduced into a Shimadzu HPLC system (Kyoto, Japan) equipped with a UV diode array detector (UV-DAD) operating within a wavelength range of 190–800 nm. Separation of phenolic constituents was achieved using a VP-ODS column (150 × 4.6 mm, 5 µm particle size) with detection at 220 nm. The mobile phase consisted of 0.2% (v/v) formic acid and acetonitrile under gradient elution. Chromatograms were recorded and processed using LC Solution software, which enabled compound identification and quantification by comparing retention times and peak areas with those of reference standards. A chromatographic fingerprint was subsequently developed to represent the phenolic profile of the *A. paniculata* extract.

4. DISCUSSION

4.1 Effect of Extraction Process Parameters on Total Phenolic Content, Antioxidant Activity, and Extract Yield of *A. paniculata* Leaves

Table 1 presents the D-optimal experimental design matrix and corresponding responses of extraction yield (EY), total phenolic content (TPC), and antioxidant activity (AA) for heat-assisted extraction (HAE) of *Andrographis paniculata* leaves. The three independent factors—operating temperature (OT), solid-to-liquid ratio (S/L), and extraction time (ET)—showed distinct influences on the responses. The solid-to-liquid ratio was one of the most influential variables, with higher solvent availability (1:60 g/mL) consistently producing higher TPC and AA compared to lower ratios (1:20 g/mL). For example, at 30 °C and 45 min (Run 2), TPC and AA values reached 110.46 mg GAE/g dw and 39.63 µM AAE/g dw, respectively, whereas at similar conditions but with a ratio of 1:20 (Run 9), the corresponding values dropped sharply to 43.41 mg GAE/g dw and 6.94 µM AAE/g dw. This finding is consistent with previous reports, which demonstrated that increasing solvent volume improves solute–solvent interactions, enhances concentration gradients, and facilitates mass transfer of phenolic compounds into the extraction medium (Alara *et al.*, 2018; Cacace and Mazza, 2003).

Temperature also exerted a notable effect. Moderate heating (40–50 °C) generally supported higher phenolic recovery and antioxidant activity compared to either low or excessive thermal exposure. For instance, Run 11 (40 °C, 1:60, 112.5 min) gave the highest TPC (133.26 mg GAE/g dw) and AA (39.60 µM

AAE/g dw), while Run 14 (50 °C, 1:60, 45 min) similarly achieved high values (123.23 mg GAE/g dw, 40.96 µM AAE/g dw). By contrast, prolonged extraction at elevated temperature (e.g., Run 7: 50 °C, 1:20, 180 min) yielded much lower phenolic content (33.87 mg GAE/g dw) and antioxidant activity (8.07 µM AAE/g dw). These results align with literature noting that while moderate temperatures enhance solvent penetration and compound solubilization, excessive heating promotes degradation and oxidative polymerization of thermolabile phenolics, ultimately reducing extract quality (Mustafa and Turner, 2011; Wang *et al*., 2018).

Table 1. Experimental design and measured responses

Run	HAE conditions			Experimental values		
	Temperature (°C)	Solid to liquid ratio (g/mL)	Time (min)	EY (w/w %)	TPC (mg GAE/g dw)	AA (µM AAE/g dw)
1	50.00	1:20	45.00	22.30	44.29	15.00
2	30.00	1:60	45.00	23.03	110.46	39.63
3	50.00	1:60	45.00	24.98	120.40	41.76
4	40.00	1:20	112.50	17.13	56.25	10.81
5	30.00	1:20	180.00	15.78	44.94	11.28
6	50.00	1:60	180.00	19.56	125.87	32.30
7	50.00	1:20	180.00	17.75	33.87	8.07
8	50.00	1:20	45.00	21.79	45.76	14.92
9	30.00	1:20	45.00	17.59	43.41	6.94
10	30.00	1:20	45.00	16.65	47.33	7.21
11	40.00	1:60	112.50	22.85	133.26	39.60
12	30.00	1:60	180.00	22.20	128.72	43.88
13	40.00	1:40	180.00	22.57	89.58	28.94
14	50.00	1:60	45.00	26.60	123.23	40.96
15	30.00	1:60	45.00	22.65	109.74	39.00
16	40.00	1:40	45.00	26.49	90.09	28.61
17	50.00	1:40	112.50	23.26	85.08	27.36
18	30.00	1:40	112.50	20.74	86.58	28.75

Note: OT is operating temperature; S:L refers to solid to liquid ratio; ET represents extraction time; EY is extracting yield; TPC refers to total phenolic content; AA represents antioxidant activity.

Extraction time influenced responses in a saturation-like manner. TPC and AA increased substantially during shorter times (45–112.5 min), but extending extraction to 180 min resulted in marginal gains or even decreases. For instance, Run 16 (40 °C, 1:40, 45 min) achieved 90.09 mg GAE/g dw and 28.61 µM AAE/g dw, which were comparable to Run 13 (40 °C, 1:40, 180 min: 89.58 mg GAE/g dw, 28.94 µM AAE/g dw). This indicates that equilibrium between the plant matrix and solvent was achieved before 180 min, beyond which prolonged exposure likely caused degradation of phenolic compounds. Similar equilibrium-limited extraction dynamics have been reported in phenolic recovery from other plant matrices (Singleton and Rossi, 1965; Brand-Williams *et al.*, 1995). Interestingly, extraction yield (EY) did not always parallel TPC and AA trends. While the highest EY was observed at 50 °C, 1:60, 45 min (Run 14, 26.60%), not all conditions yielding high EY corresponded to equally high phenolic recovery. For instance, Run 5 (30 °C, 1:20, 180 min) gave a modest EY (15.78%) despite relatively low TPC (44.94 mg GAE/g dw). This suggests that EY reflects the combined extraction of both phenolic and non-phenolic constituents, whereas TPC and AA specifically represent bioactive phenolics and antioxidant potential. Such divergence between yield and phenolic content has been highlighted in earlier studies, emphasizing the importance of measuring quality-specific indices in addition to bulk yield (Dahmoune *et al.*, 2015).

4.2 D-Optimal-RSM modelling, evaluation of model adequacies and statistical analysis

Using the D-Optimal experimental matrix, empirical second-order polynomial equations were derived to describe how

extraction temperature (OT), solid-to-liquid ratio (S/L) and extraction time (ET) affect total phenolic content (TPC), extraction yield (EY) and antioxidant activity (AA). The fitted models are given in coded form as Equations (6)–(8), where A, B and C represent OT, S/L and ET, respectively. In these expressions, positive coefficient values identify terms (main, interaction or quadratic) that raise the predicted response, while negative coefficients indicate terms that reduce it. Examination of the coefficient signs and magnitudes thus reveals that both the individual factors and their pairwise and quadratic interactions contribute to the observed behavior of TPC, EY and AA, with the relative importance and direction of influence varying for each response

$$\mathrm{TPC} = +94.27 - 0.39 * \mathrm{A} + 39.48 * \mathrm{B} + 1.13 * \mathrm{C} - 8.44 * \mathrm{A}^2 + 0.48 * \mathrm{B}^2 - 4.44 * \mathrm{C}^2 + 2.69 * \mathrm{A} * \mathrm{B} - 3.18 * \mathrm{A} * \mathrm{C} + 4.22 * \mathrm{B} * \mathrm{C} \quad (6)$$

$$\mathrm{EY} = +23.08 + 0.96 * \mathrm{A} + 2.31 * \mathrm{B} - 1.63 * \mathrm{C} - 1.08 * \mathrm{A}^2 - 3.09 * \mathrm{B}^2 + 1.45 * \mathrm{C}^2 - 0.71 * \mathrm{A} * \mathrm{B} - 1.05 * \mathrm{A} * \mathrm{C} - 0.12 * \mathrm{B} * \mathrm{C} \quad (7)$$

$$\mathrm{AA} = +28.60 - 0.62 * \mathrm{A} - 14.43 * \mathrm{B} - 0.73 * \mathrm{C} - 0.54 * \mathrm{A}^2 - 3.39 * \mathrm{B}^2 + 0.18 * \mathrm{C}^2 - 1.67 * \mathrm{A} * \mathrm{B} - 3.09 * \mathrm{A} * \mathrm{C} - 0.23 * \mathrm{B} * \mathrm{C} \quad (8)$$

Table 2 presents the analysis of variance (ANOVA) for the quadratic models developed to describe the effects of operating temperature (OT), solid-to-liquid ratio (S/L), and extraction time (ET) on total phenolic content (TPC), extraction yield (EY), and antioxidant activity (AA) of *A. paniculata* leaves. The high model F-values (TPC = 837.9, EY = 36.80, AA = 783.35; all $p < 0.0001$) indicate that the quadratic models are statistically significant, capturing the majority of variability in the experimental data. The very low residual sum of squares across all responses suggests that the models fit the data well

without overfitting. Among the linear terms, the S/L ratio (factor B) emerged as the most influential parameter across all three responses. It contributed significantly to TPC (F = 6991.3, p < 0.0001), EY (F = 126.1, p < 0.0001), and AA (F = 5955.7, p < 0.0001). This strong dependence on solvent volume is consistent with earlier studies, which reported that higher solvent-to-solid ratios enhance mass transfer and solute solubilization, leading to improved phenolic extraction (Alara *et al.*, 2018; Cacace and Mazza, 2003). In contrast, temperature (A) showed limited direct influence, being significant only for EY (p = 0.0017) and AA (p = 0.0105), while its effect on TPC was not statistically significant (p = 0.4363). This suggests that temperature primarily enhances extraction efficiency through its interaction with other variables rather than as an independent factor, as has been noted in heat-assisted extraction studies (Mustafa and Turner, 2011).

Table 2. Analysis of variance (ANOVA) for TPC, EY and AA quadratic models

Source	TPC (mg GAE/g d.w)				EY (%)				AA (µM AAE/g d.w)			
	Sum of squares	df	F-value	P-value P>F	Sum of squares	df	F-value	P-value P>F	Sum of squares	df	F-value	P-value P>F
Model	21297.1	9	837.9	< 0.0001	178.16	9	36.80	< 0.0001	3121.2	9	783.35	<0.0001
A-OT	1.90	1	0.67	0.4363	11.61	1	21.58	0.0017	4.89	1	11.05	0.0105
B-S/L	19742.9	1	6991.3	< 0.0001	67.84	1	126.1	< 0.0001	2636.65	1	5955.7	<0.0001
C-ET	16.09	1	5.70	0.0441	33.48	1	62.24	< 0.0001	6.75	1	15.24	0.0045
A^2	179.02	1	63.40	0.0001	2.92	1	5.42	< 0.0001	0.74	1	1.68	0.2307
B^2	0.59	1	0.21	0.6604	23.95	1	44.52	0.0002	28.95	1	65.39	<0.0001
C^2	49.45	1	17.51	0.0031	5.30	1	9.85	0.0138	0.077	1	0.17	0.6869
AB	87.16	1	30.86	0.0005	6.11	1	11.35	0.0098	33.50	1	75.67	< 0.0001
AC	110.06	1	38.89	0.0002	11.95	1	22.22	0.0015	103.97	1	234.86	< 0.0001
BC	193.49	1	68.52	< 0.0001	0.16	1	0.29	0.6037	0.57	1	1.28	0.2910
Residual	22.59	8			4.30	8			3.54	8		
Lack of fit	9.56	4	0.73	0.6141	2.35	4	1.20	0.4320	2.98	4	6.45	0.0667
Pure error	13.03	4			1.96	4			0.56	4		

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Cor. Total	21319.7	17		182.47	17		3124.69	17
CV%	1.68			3.44			2.58	
PRESS	118.43			28.15			27.46	
Adeq Precision	80.794			18.717			74.459	
R^2	0.9989			0.9764			0.9989	
Adj R^2	0.9977			0.9499			0.9976	
Pred R^2	0.9944			0.8457			0.9912	

Note: OT stands for operating temperature; S:L refers to the solid-to-liquid ratio; ET represents extraction time; EY is the extract yield; TPC indicates total phenolic content; AA denotes antioxidant activity; df stands for degree of freedom; CV refers to the coefficient of variation; Adeq Precision measures the adequacy of precision; Adj R^2 is the adjusted R-squared value; and Pred R^2 is the predicted R-squared value.

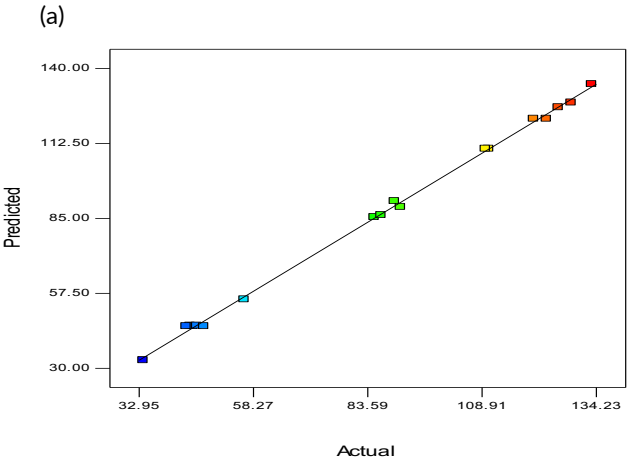
Extraction time (C) exhibited a moderate but significant effect on all responses (TPC: p = 0.0441; EY: p < 0.0001; AA: p = 0.0045), consistent with the saturation-type extraction kinetics commonly reported for phenolic compounds (Singleton & Rossi, 1965). However, quadratic effects revealed additional nuances. For example, quadratic terms for OT (A^2, p = 0.0001) and ET (C^2, p = 0.0031) were significant for TPC, indicating that phenolic recovery follows a curvilinear trend, increasing up to a threshold before declining due to thermal or oxidative degradation (Wang et al., 2018). Similarly, quadratic effects of S/L (B^2) and ET (C^2) were significant for EY, confirming nonlinear response patterns, while AA was influenced by quadratic S/L (B^2, p < 0.0001), reflecting diminishing returns at excessively high solvent ratios. Interaction terms also played critical roles. The interactions of OT with ET (AC) and OT with S/L (AB) were significant for all responses, highlighting the synergistic effects of moderate heating, solvent ratio, and time in enhancing solubilization while minimizing degradation. For example, the AC term strongly influenced AA (F = 234.86, p < 0.0001), demonstrating that antioxidant recovery depends on an optimal balance between

temperature and time. By contrast, the BC interaction (S/L × ET) was only significant for TPC (p < 0.0001), suggesting that solvent loading and contact time predominantly affect phenolic solubilization.

Model adequacy diagnostics further confirmed robustness. The coefficients of determination (R^2) were exceptionally high for TPC (0.9989) and AA (0.9989), and satisfactory for EY (0.9764). Adjusted R^2 values (TPC = 0.9977, EY = 0.9499, AA = 0.9976) and predicted R^2 values (TPC = 0.9944, EY = 0.8457, AA = 0.9912) showed close agreement, indicating excellent model generalizability. Adeq Precision values were well above the threshold of 4 (TPC = 80.794, EY = 18.717, AA = 74.459), confirming adequate signal-to-noise ratios for navigating the design space. Low coefficients of variation (CV < 3.5%) reflected good experimental reproducibility and minimal random error (Ferreira *et al.*, 2007; Myers *et al.*, 2016). Importantly, lack-of-fit tests were non-significant (p > 0.05), supporting the adequacy of the quadratic models in capturing the true response behavior. Taken together, the ANOVA results demonstrate that the developed quadratic models are statistically sound, highly predictive, and capable of explaining the effects of extraction parameters on phenolic recovery and antioxidant activity of *A. paniculata*. The dominant role of S/L ratio, the nonlinear effects of temperature and time, and the significant interactions highlight the complex dynamics of HAE and the necessity of predictive modeling for process understanding and future optimization

Figures 2a–c show observed-versus-predicted scatterplots for TPC, EY and AA. In each panel, the measured values cluster closely around the identity line (y = x), indicating only small

discrepancies between experimental results and model estimates and no evident systematic bias across the response range. This tight clustering and lack of pronounced deviation from the 45° line demonstrate that the fitted quadratic models reproduce the experimental behaviour well and yield reliable predictions within the investigated domain (Adeyi *et al.*, 2022; Olalere *et al.*, 2022).



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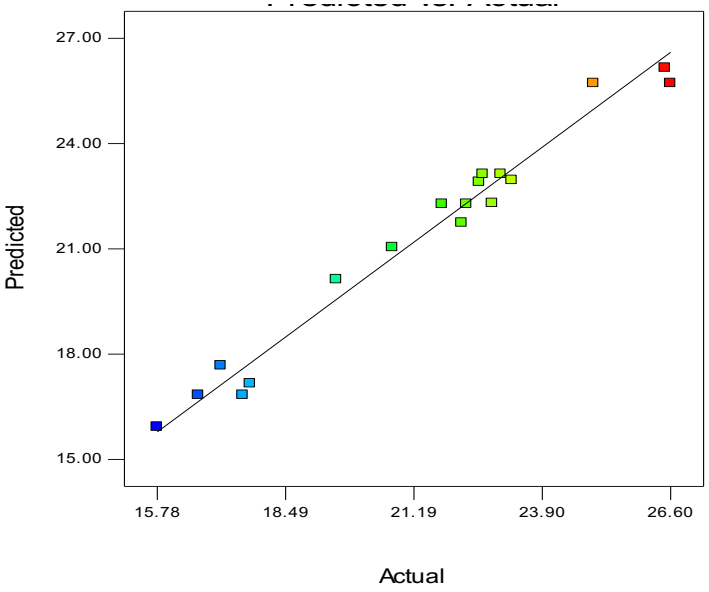
(b)



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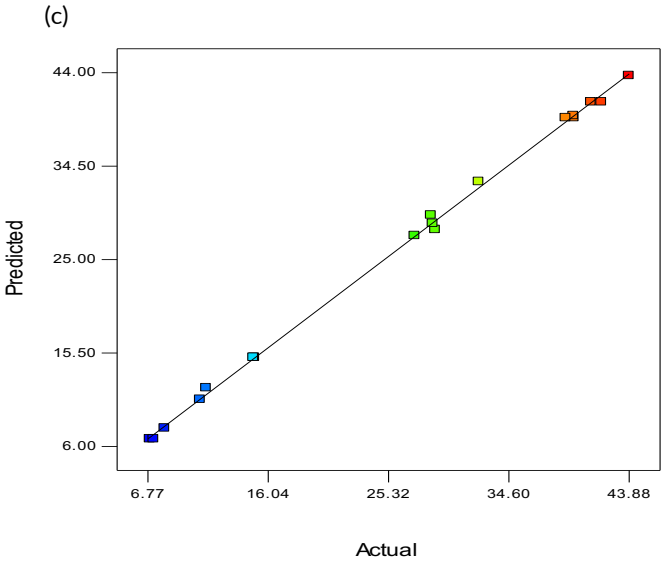


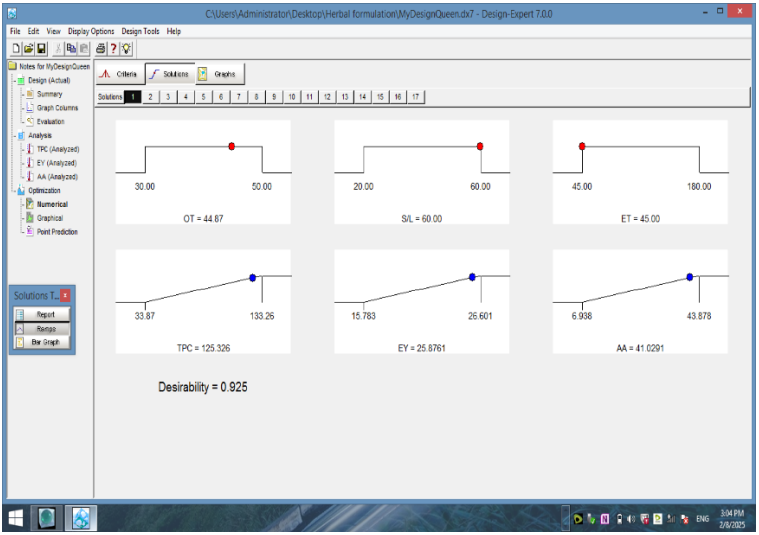
Figure 2. Parity graphs of experimental and predicted data for (a) TPC (b) EY and (c) AA

4.3 Extraction Process Optimization and Validation

A multi-objective optimization strategy, adapted from the framework of Alanzi et al. (2024), was employed to simultaneously enhance the total phenolic content (TPC), extraction yield (EY), and antioxidant activity (AA) of crude bioactive extracts from *A. paniculata* leaves. The optimization procedure, executed using Design-Expert software, identified the best solution based on the highest composite desirability index. As illustrated in Figure 3a, the predicted optimal responses corresponded to TPC of 125.326 mg GAE/g dw, EY of 25.8761%, and AA of 41.8291 µM AAE/g dw, achieved at an extraction temperature (OT) of 44.87 °C, extraction time (ET) of

45.0 min, and a solid-to-liquid ratio (S/L) of 1:60 g/mL. The desirability profile (Figure 3b) revealed that each process variable (OT, ET, and S/L) attained a desirability score of 1.0, reflecting their strong alignment with the optimization criteria. By contrast, the response factors—TPC, EY, and AA—attained desirability scores of 0.9201, 0.9329, and 0.9229, respectively, with an overall composite desirability value of 0.9376

(a)



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(b)

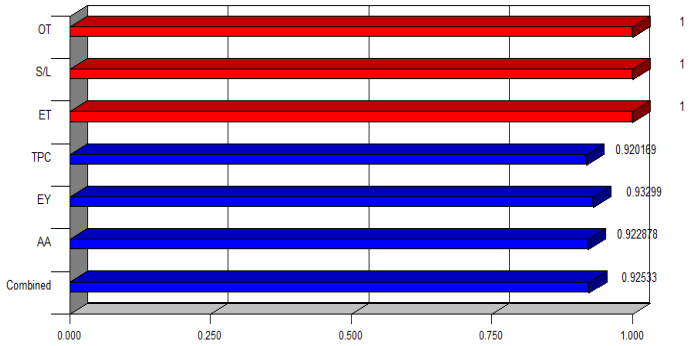

Figure 3. (a) Numerical optimization ramp and (b) desirability values for process and response variables

To verify the model predictions, confirmatory experiments were performed under the optimized conditions. The experimental results yielded values of 128.41 mg GAE/g dw for TPC, 28.96% for EY, and 42.45 µM AAE/g dw for AA. Relative standard deviation (RSD) analysis showed differences of 1.72% (TPC), 7.95% (EY), and 2.69% (AA) between experimental and predicted values. Since all RSDs were below 10%, the experimental outcomes were considered statistically consistent with the model forecasts (Adeyi *et al.*, 2022). These findings support the reliability and robustness of the constructed models in predicting extraction performance for heat-assisted recovery of bioactive compounds. Minor variations between predicted and experimental values are likely attributable to external and uncontrolled influences, such as fluctuations in ambient laboratory conditions, instrument-related limitations or human

errors during measurement. Despite these potential sources of discrepancy, the close agreement between the predicted and validated responses underscores the precision of the optimization models and their suitability for practical application.

4.4 Elucidation of Bioactive Contents of *A. paniculata* Leaves

Figure 4 presents the composition of the bioactive constituents identified in the leaf extract of *A. paniculata*. The HPLC chromatogram illustrates the separation and identification of major phenolic compounds at 280 nm, a wavelength characteristic of aromatic polyphenols (Tahiri *et al.*, 2025). Peaks correspond to betulinic acid (2.372 min), gallic acid (3.175 min), chlorogenic acid (3.629 min), and caffeic acid (4.362 min). Betulinic acid, the most prominent peak, is widely reported for its anticancer, anti-inflammatory, and antiviral properties, including activity against HIV (Wang *et al.*, 2018). Gallic acid, a strong antioxidant, contributes to cardiovascular protection and has been associated with antidiabetic effects through modulation of oxidative stress pathways (Suganthy *et al.*, 2018). Chlorogenic acid is recognized for regulating glucose metabolism and improving lipid profiles, making it relevant in diabetes and obesity management (Upadhyay and Mohan Rao, 2013). Similarly, caffeic acid exhibits antioxidant and anti-inflammatory activity and has been linked to reduced risk of chronic diseases such as atherosclerosis and certain cancers (Heleno *et al.*, 2015).

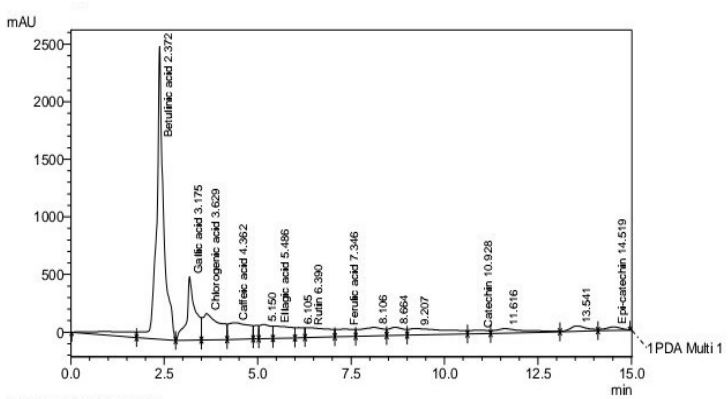

Figure 4. HPLC chromatogram of A. paniculata leaves

Additional peaks, including ellagic acid (5.150 min), rutin (5.486 min), and ferulic acid (7.346 min), further highlight the extract's diversity. Ellagic acid possesses antimutagenic and chemopreventive activity, while rutin is valued for vascular strengthening and antihypertensive effects (Kumar and Pandey, 2013). Ferulic acid is known to scavenge free radicals and enhance skin photoprotection (Zduńska *et al*., 2018). Late-eluting flavan-3-ols, catechin (10.928 min) and epicatechin (14.519 min), are potent antioxidants associated with improved endothelial function and neuroprotection (Pervin *et al*., 2019). The chromatographic resolution confirms a broad spectrum of phenolics with multiple bioactivities, supporting the potential health-promoting value of the *A. paniculata* leaf extract and underscoring its importance in nutraceutical and functional food research.

5. CONCLUSION

This study established heat-assisted extraction (HAE) as an efficient method for recovering phenolic compounds and antioxidant constituents from *Andrographis paniculata* leaves. The extraction temperature, solid-to-liquid ratio, and extraction time significantly influenced total phenolic content (TPC), extract yield (EY), and antioxidant activity (AA), with the solid-to-liquid ratio emerging as the most critical parameter. The developed D-optimal quadratic models showed excellent statistical validity ($R^2 > 0.97$) and non-significant lack-of-fit, confirming their robustness and predictive accuracy. Multi-response optimization identified the optimum extraction conditions at 44.87 °C, 45 min, and a 1:60 g/mL solid-to-liquid ratio, yielding experimental values of 128.41 mg GAE/g dw for TPC, 28.96% for EY, and 42.45 µM AAE/g dw for AA, with relative deviations below 10%. These validated results confirm the models' reliability for predicting and optimizing process parameters. HPLC profiling revealed diverse phenolic constituents, including betulinic, gallic, chlorogenic, caffeic, ellagic, and ferulic acids, along with rutin, catechin, and epicatechin, highlighting the extract's rich bioactive potential. Overall, the study provides a scientific basis for optimizing phenolic extraction from *A. paniculata*, supporting its development as a natural source of antioxidants for potential applications in food, nutraceutical, and pharmaceutical industries.

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