Optimization of Antioxidant Activity and Phenolic Extraction from *Ainsliaea acerifolia* Stem Using Ultrasound-Assisted Extraction Technology

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Ultrasound-assisted extraction for bioactive compound retrieval is a viable alternative to traditional extraction methods. Employing ultrasound-assisted extraction, this study aimed to enhance the scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), total polyphenol content (TPC), and flavonoid content in *Ainsliaea acerifolia* (*A. acerifolia*) through response surface methodology (RSM). Initially, the impact of extraction temperature, time, and ethanol concentration on DPPH scavenging capacity, ABTS, TPC, and flavonoid content was assessed. Optimal conditions for maximizing antioxidant activity and TPC were determined as 78% (v/v) ethanol, 60 °C extraction temperature, and 91 min of extraction time. High-performance liquid chromatography (HPLC) analysis of the optimized extract revealed dicaffeoylquinic acid as the primary polyphenol in *A. acerifolia* extracts, comprising the majority of phenolic compounds (102.06 mg/g DW). This model enabled the optimization of conditions for phenolic compound extraction with antioxidant properties from *A. acerifolia*, highlighting its potential as a source of antioxidant compounds for industrial, pharmaceutical, and food applications.

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INTRODUCTION

Ainsliaea acerifolia, a perennial herb of the Compositae family, is indigenous to the mountainous regions of South Korea. Traditionally, this bitter mountain vegetable has been employed for treating rheumatic arthritis and enteritis (Choi *et al*. 2006). The predominant secondary metabolites extracted from its aerial parts, referred to as quinic acid derivatives, are renowned for their diverse biological activities, encompassing antioxidative, anti-diabetic, anti-viral, anti-thrombotic, hepatoprotective, and neuro-protective properties (Park 2010). These beneficial effects are often attributed to the presence of polysaccharides and phenolic compounds, which demonstrate significant scavenging abilities against free radicals and reactive oxygen species (Fogarasi *et al*. 2021). The efficacy of extracting such bioactive compounds hinges on the efficiency and effectiveness of the chosen extraction methods (Azmir *et al*. 2013). Consequently, employing an extraction technique capable of maximizing the yield of these compounds from *A. acerifolia*, while also adhering to principles of environmental sustainability, rapidity, and cost-effectiveness, holds paramount importance.

Traditional techniques such as maceration and Soxhlet extraction are typically straightforward and uncomplicated, relying on solvent properties and external variables such as temperature, time, and agitation to enhance compound solubility (Jha and Sit 2022). However, these conventional approaches often entail prolonged extraction times, necessitate large solvent volumes, and they yield relatively low extraction efficiencies (Chávez-González *et al*. 2020). Moreover, conventional methods such as solvent extraction, distillation, and pressing exhibit limitations such as reduced efficiency, diminished yields, and substantial solvent or resource consumption. Additionally, some methods may require elevated temperatures, potentially compromising the integrity of certain bioactive constituents. Consequently, researchers are actively exploring environmentally sustainable and efficient extraction technologies.

Ultrasound-assisted extraction (UAE) has emerged as a viable alternative to traditional extraction methods (Shen *et al*. 2023). UAE offers several advantages including reduced extraction time, energy consumption, and solvent usage (Aslam *et al*. 2022). Recognized for its environmentally friendly nature and high efficiency, UAE is a versatile and user-friendly extraction technology (Carpentieri *et al*. 2021). Notably, UAE requires lower investment compared to other advanced extraction techniques such as supercritical fluid extraction (SFE), pressurized solvent extraction, or accelerated solvent extraction (ASE) (Tiwari 2015). Moreover, UAE demonstrates superiority in preserving the structure of volatile bioactive compounds during extraction from material by-products (Kumar *et al*. 2021). However, the extraction of antioxidants and polyphenols is influenced by solvent concentration, extraction time, and temperature, *etc*. Optimizing extraction conditions tailored to each specific raw material is essential for maximizing the recovery of target compounds.

There is a dearth of literature exploring the extraction of phenolic compounds from *A. acerifolia*. The objective of this study was to optimize extraction conditions employing response surface methodology (RSM), focusing on variables such as extraction temperature, time, and ethanol concentration, while utilizing UAE. The aim was to enhance the recovery of antioxidant phenolic compounds from *A. acerifolia*. Additionally, the study to confirm of main component content in the extract obtained under optimized conditions using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

A. acerifolia plants were purchased from Yeoju Natural Farm (99-31, Majang-ro, King Sejong-myeon, Yeoju-si, Gyeonggi-do) and used in the experiment, in March 2023. After identification at the Research Forest of the Gyeongsang National University, the fresh stems were cut into small pieces, which were 0.5 cm in diameter and 1 cm in length. They were dried in a drying oven at 40 °C, and then ground to pass through a 40 mesh (425 μm) sieve. The samples were stored at 4 °C in airtight containers until use.

UAE Methodology

The UAE technique was used to extract bioactive compounds from *A. acerifolia*, utilizing ultrasound waves to disrupt cell walls. Ethanol served as the solvent due to its common use in phenolic compound extraction, partly owing to its cost-effectiveness. Initially, 5 g of dried powder was combined with 100 mL of ethanol at varying

concentrations: 50%, 75%, and 100% (v/v). Extraction procedures were conducted using the Kodo Technical Research Co., LTD ultrasonic processor device (Model: JAC 2010, GyeongGi, South Korea), operating at 200 Watts and 40 kHz, for times of 60, 90, and 120 min (contact time). To prevent thermodegradation of phenolic compounds, samples were maintained within an appropriate temperature range by placing them in an ice bath during sonication. Following extraction, samples underwent centrifugation (4500× g for 10 min at 4 °C), and the resulting supernatants were filtered using Whatman no. 2 filter paper, then stored at −20 °C until analysis. All experiments were conducted in triplicate.

Experimental Design

Experiments were conducted with varied extract temperatures, times, and ethanol concentrations (Table 1). These factors were amalgamated based on a central composite design. After sonication, each extract underwent centrifugation and filtration as previously outlined, with the resulting supernatants stored until commencement of analyses. The impact of three independent numeric variables—extract temperature (°C, X_1), extraction time (min, X_2), and ethanol concentration (% v/v, X_3)—on the efficacy of phenolic compound extraction, DPPH scavenging capacity, and ABTS in *A. acerifolia* was assessed utilizing a central composite design. These variables were coded at levels -1, 0, and 1 (Table 2), with seventeen runs established under specific conditions. Total phenolic content, DPPH, ABTS, TPC, and flavonoid content were quantified in the resulting extracts. Response surface analysis was conducted using Design Expert 13.0 (Stat-Ease, Minneapolis, MN), while the significance of primary variable effects, variable interactions, and model validity were evaluated *via* Analysis of Variance (ANOVA).

Table 1. Experimental Design of the Central Composite Design

Run	Temp. (°C)	Time (min)	Ethanol Concentration (%)	DPPH (%)	ABTS (%)	TPC (mg/g)	Flavonoid Content (mg/g)
1	80	120	75	R1	R1	R1	R1
2	40	60	75	R2	R2	R2	R2
3	40	120	75	R3	R3	R3	R3
4	60	120	50	R4	R4	R4	R4
5	60	60	50	R5	R5	R5	R5
6	80	90	50	R6	R6	R6	R6
7	40	90	100	R7	R7	R7	R7
8	60	90	75	R8	R8	R8	R8
9	80	60	75	R9	R9	R9	R9
10	60	90	75	R10	R10	R10	R10
11	60	90	75	R11	R11	R11	R11
12	60	90	75	R12	R12	R12	R12
13	60	120	100	R13	R13	R13	R13
14	60	60	100	R14	R14	R14	R14
15	60	90	75	R15	R15	R15	R15
16	40	90	50	R16	R16	R16	R16
17	80	90	100	R17	R17	R17	R17

Temp: Temperature; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); TPC: Total polyphenol content

Table 2. Independent Variables and their Corresponding Levels for *A. acerifolia* Extracts

Independent variables	-1	0	1
Temperature (°C)	40	60	80
Time (min)	60	90	120
Ethanol concentration (%)	50	75	100

DPPH radical scavenging assay

The reaction commenced with the addition of 0.4 mM DPPH, dissolved in 150 µL of ethanol, to the 50 µL test sample. Subsequently, the reaction proceeded for 30 min at room temperature under dark conditions. The absorbance of the resultant solution was then measured at 517 nm.

ABTS radical scavenging assay

To prepare the ABTS-mixture solution, equal volumes of 7.4 mM ABTS and 2.6 mM potassium peroxydisulfate solutions were mixed and rotated for 15 h in the dark at room temperature. Subsequently, the ABTS-working solution was prepared by diluting 150 µL of the ABTS-mixture solution in 2.9 mL of methanol. The reaction commenced by adding 190 µL of the ABTS-working solution to varying concentrations of the test sample (10 µL). This reaction, constituting a final volume of 200 µL, proceeded at room temperature for 2 h in the dark. The absorbance of the resulting solution was measured at 734 nm.

Determination of TPC

Total polyphenol content was determined following a standardized protocol. In brief, a mixture comprising 25 µL of test samples and 125 µL of Folin-Ciocalteu phenol reagent solution was incubated with 125 µL of 10% sodium carbonate solution for 30 min at room temperature. The resulting assay mixture underwent colorimetric measurement at 750 nm using a grating microplate reader (SpectraMax 190, Molecular Devices LLC, CA, USA). A calibration curve was prepared using gallic acid as the control standard. Results were expressed as milligrams of gallic acid per gram of dry weight of the raw material (mg GA/g DW).

Determination of flavonoid content

The test samples (25 µL) were combined with 125 µL of water and 7.5 µL of 5% sodium nitrite solution, and the mixture was allowed to react for 6 min. Subsequently, 15 µL of 10% aluminum chloride solution was added, and the mixture was further incubated for 5 min at room temperature. Following this, 50 µL of 1 mM NaOH and 27.5 µL of water were introduced to the mixture, which was then incubated for 10 min with continuous shaking. The resulting assay mixture underwent colorimetric measurement at 510 nm using a microplate reader (SpectraMax 190, Molecular Devices LLC, CA, USA). A calibration curve was prepared using (+)-Catechin as the control standard.

HPLC Analysis

The HPLC analysis parameters are detailed in Table 3. Dicaffeoylquinic acid standard (Merck KGaA, Darmstadt, Germany; Product Number: SMB00131; CAS Number: 2450-53-5; Molecular weight: 516.45) was procured for analysis using HPLC. The standard solution was prepared by dilution and injected into the HPLC instrument. Upon conducting HPLC analysis of the dicaffeoylquinic acid standard solution, a retention

time of 6.01 min was observed.

Table 3. HPLC Analysis Conditions

Contents	Conditions
Specifications	YL9120NUV/VIS detector, Y9110 Plus quaternary pump, YL9101 vacuum degasser
Column	Eclipse plus C18 column (5 μm, 4.6 x 250 mm)
Mobile phase	A: Water; B: Methanol
Gradient	0 min: 85% A : 15% B 0-15 min: 10% A : 90% B 15-20 min: 85% A : 15% B
Flow rate	1.00 mL/min
Injection volume	10 μL
Wavelength	330 nm

Statistical Analysis

Statistical analysis of the experimental design aimed at maximizing extraction efficiency was performed using Design Expert 13.0 software (Stat-Ease, Minneapolis, MN).

RESULTS AND DISCUSSION

Optimization of the Extraction Conditions

Various extraction conditions were explored to optimize the retrieval of antioxidant phenolic compounds from *A. acerifolia*. Several factors were found to influence the antioxidant activity of extracts and the kinetics of phenolic compound release from the solid matrix. These crucial variables are directly linked to extract yield and encompass factors such as extraction methodology, solvent type and concentration, extraction time, solvent-to-solid ratio, and extraction temperature, among others.

The optimization of extraction conditions to achieve maximum antioxidant activity, total polyphenol content (TPC), or flavonoid content involved testing the variables of contact temperature, extraction time in the UAE method, and ethanol concentration. Table 4 outlines the conditions for each experimental assay along with the corresponding measured and predicted values for DPPH, ABTS, TPC, and flavonoid content. These values underwent multiple regression analysis to fit a second-order polynomial equation, resulting in quadratic models describing the fluctuations of the responses concerning the significant process variables (extract temperatures (°C, X_1), extraction time (min, X_2), and ethanol concentrations (% v/v, X_3)) as delineated in Table 5.

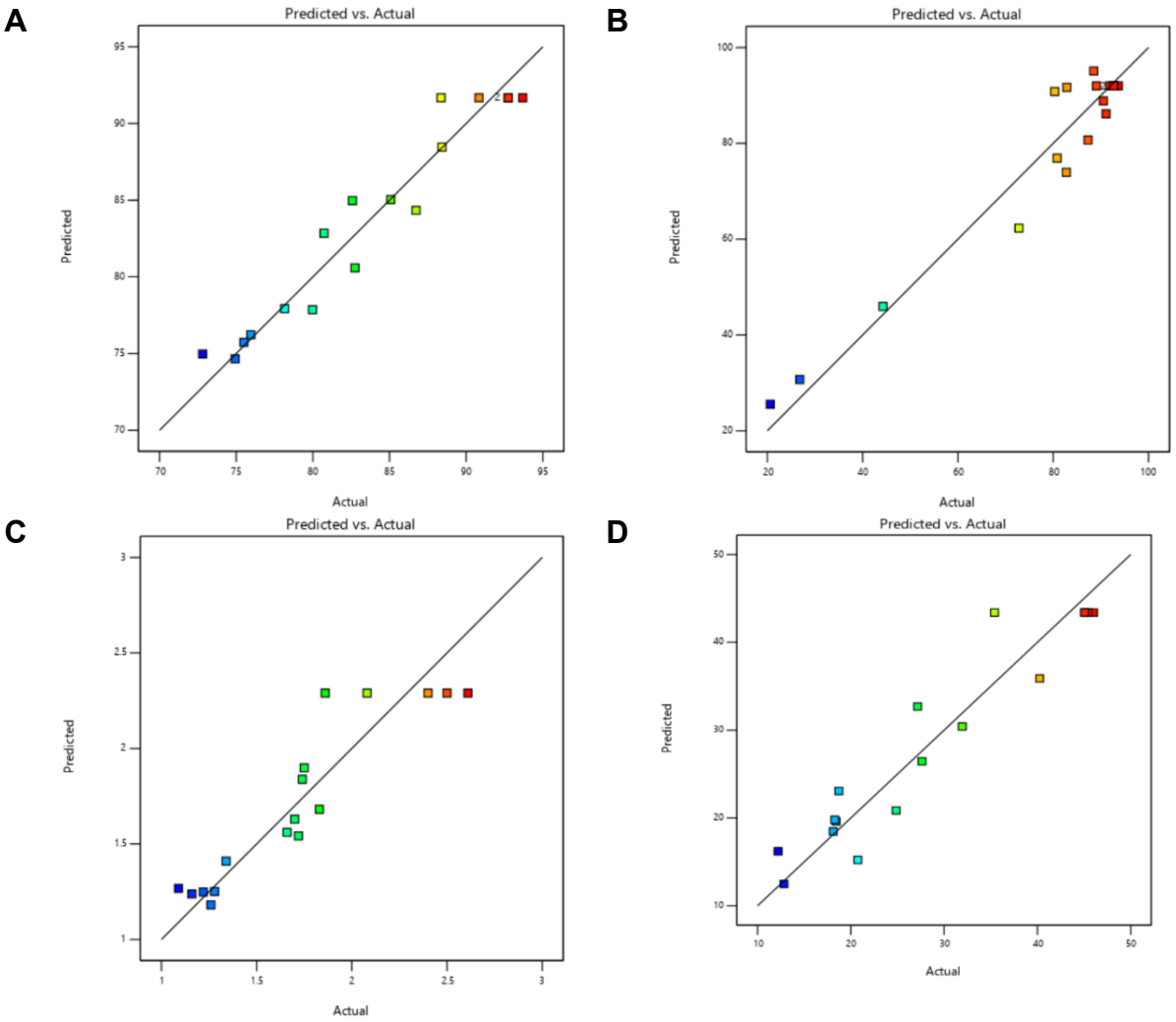


Fig. 1. Predicted versus actual values for the RSM design for (A) DPPH, (B) ABTS, (C) TPC, and (D) flavonoid content. DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPC, total polyphenol content.

Table 4. Effect of Processing Variables on DPPH, ABTS, TPC, and Flavonoid Content of *A. acerifolia* Extract by RSM

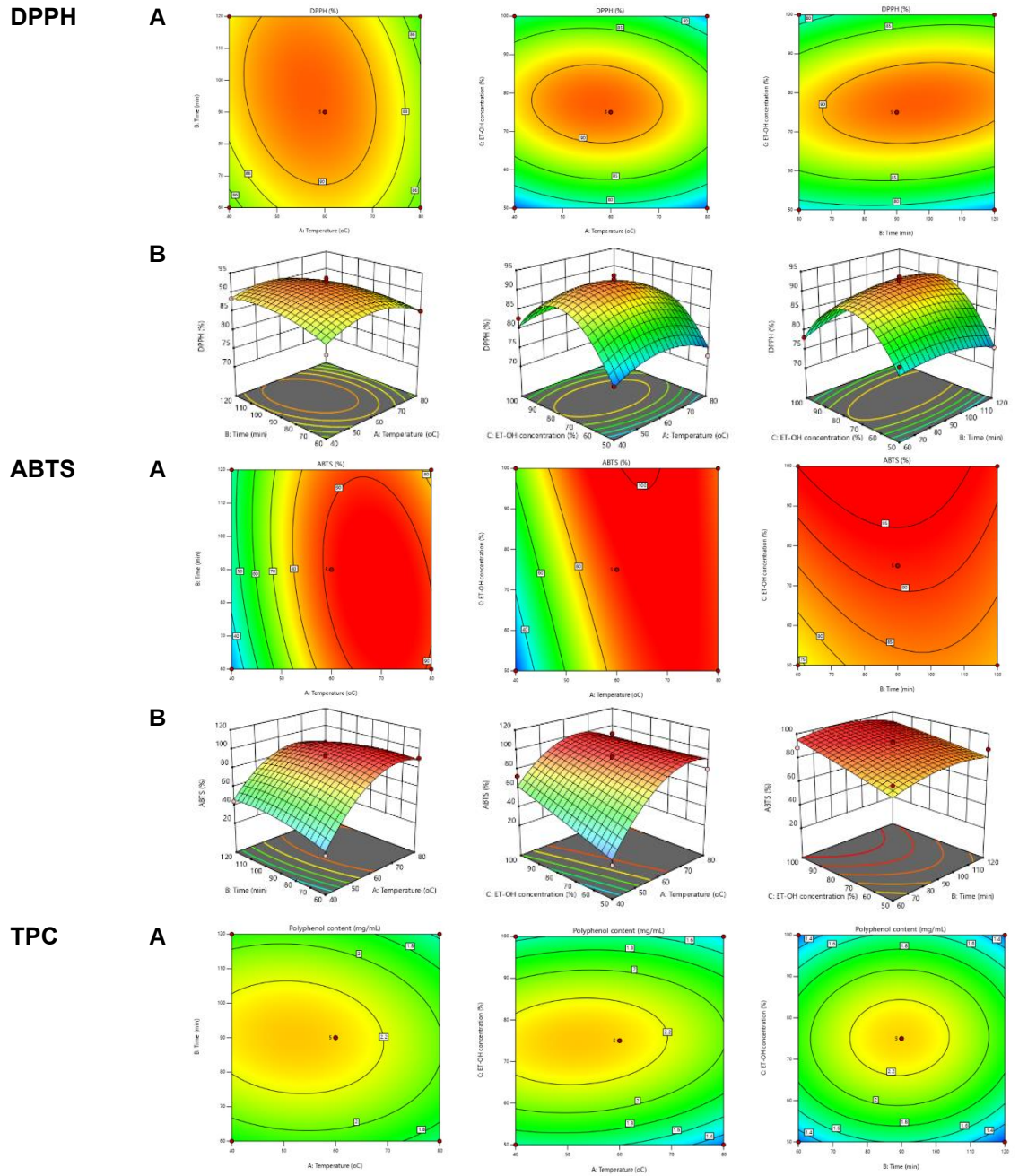
Run	Coded Level			DPPH (%)		ABTS (%)		TPC (mg/g)		Flavonoid Content (mg/g)	
	Temp. (°C)	Time (min)	Ethanol concentration (%)	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	1	1	0	86.73	85.21	80.81	80.99	1.66	1.69	12.2	12.37
2	-1	-1	0	82.58	83.19	26.81	26.86	1.74	1.76	24.86	24.93
3	-1	1	0	88.42	88.51	44.28	44.32	1.75	1.81	27.63	27.69
4	0	1	-1	75.5	76.33	87.31	87.39	1.26	1.28	18.28	18.32
5	0	-1	-1	79.98	79.81	82.76	82.82	1.28	1.31	18.72	18.88
6	1	0	-1	72.81	72.88	80.32	80.53	1.09	1.12	20.75	20.69
7	-1	0	1	82.75	83.69	72.8	72.81	1.72	1.83	27.16	27.02
8	0	0	0	88.36	88.55	89.07	89.21	1.86	1.88	35.4	35.31
9	1	-1	0	85.08	85.12	90.54	90.61	1.83	1.86	18.43	18.39
10	0	0	0	92.75	92.69	91.86	91.92	2.08	2.11	46.02	46.23
11	0	0	0	90.84	91.12	93.66	93.71	2.5	2.63	45.07	45.06
12	0	0	0	93.69	94.16	92.7	92.69	2.61	2.67	45.06	45.18
13	0	1	1	80.73	80.69	82.86	82.96	1.22	1.28	40.24	40.35
14	0	-1	1	78.16	78.18	88.5	88.61	1.16	1.19	31.94	31.33
15	0	0	0	92.75	92.76	92.7	92.83	2.4	2.48	45.4	45.59
16	-1	0	-1	74.93	74.99	20.62	20.66	1.7	1.87	12.83	12.84
17	1	0	1	75.95	75.91	91.1	91.254	1.34	1.49	18.12	18.18
Temp: Temperature; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); TPC: Total polyphenol content;											

Table 5. F-values and *p*-values for Each Coefficient and Polynomial Equations Calculated by the Central Composite Design for the Extraction Conditions of *A. acerifolia*

Source	DPPH		ABTS		TPC		Flavonoid content	
	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value
Model	11.33	0.0021	11.23	0.0022	4.34	0.0330	7.51	0.0073
X_1	1.20	0.3101	49.70	0.0002	1.59	0.2473	2.11	0.1898
X_2	0.5669	0.4761	0.0692	0.8001	0.0234	0.8827	0.0773	0.7890
X_3	3.76	0.0937	6.46	0.0386	0.0197	0.8924	8.78	0.0210
X_1X_2	0.6392	0.4503	2.31	0.1720	0.1053	0.7550	0.6469	0.4477
X_1X_3	0.7975	0.4015	5.36	0.0537	0.1720	0.6908	2.30	0.1734
X_2X_3	1.81	0.2205	0.3248	0.5865	0.0208	0.8894	0.6101	0.4603
X_1^2	9.71	0.0169	33.64	0.0007	1.34	0.2855	30.65	0.0009
X_2^2	2.44	0.1619	1.97	0.2029	8.28	0.0238	7.60	0.0282
X_3^2	75.41	<0.0001	0.0143	0.9083	24.67	0.0016	9.90	0.0162
Lack of fit	2.22	0.2288	59.11	0.2009	0.5181	0.6920	2.30	0.2191

The *p*-value was used to assess the quality of fit, which was 0.0021 for DPPH, 0.0022 for ABTS, 0.0330 for TPC, and 0.0073 for flavonoid content. These findings suggest a highly significant agreement between the results experimentally obtained and those predicted by the equations for DPPH, ABTS, TPC, and flavonoid content, which can adequately predict the experimental results (Fig. 1). The model F-values of 11.33 (DPPH), 11.23 (ABTS), 4.34 (TPC), and 7.51 (flavonoid content) mean that the generated model was meaningful.

Based on the constructed regression model, 2D contour lines (Fig. 2A) and 3D response graphs (Fig. 2B) were generated for each of the analyzed responses. These figures illustrate the resemblance in the optimized responses for DPPH, ABTS, TPC, and flavonoid content.



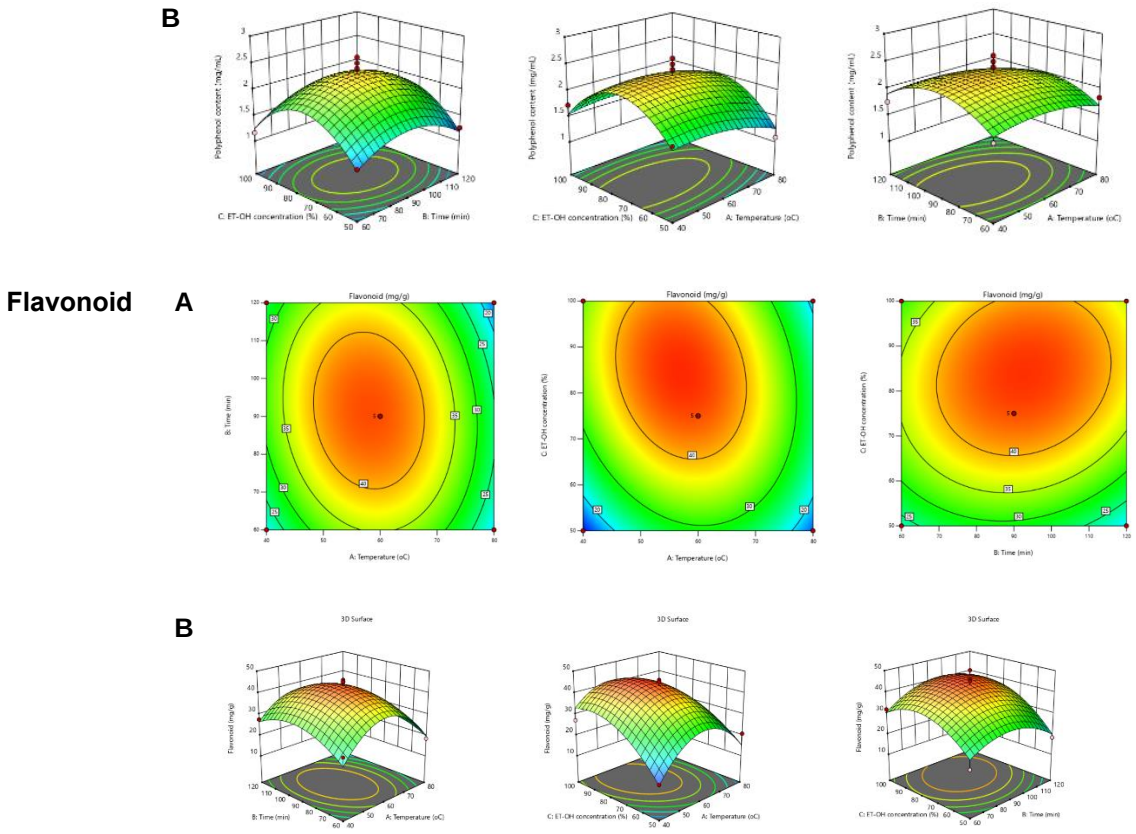


Fig. 2. Three-dimensional response surface plot on DPPH, ABTS, TPC, and flavonoid of *A. acerifolia*

The data presented in Table 4 reveal that the peak DPPH activity was attained at a temperature of 60 °C, with a sonication time of 90 min in 75% ethanol. Similarly, the optimal conditions for ABTS, TPC, and flavonoid assays coincided with these parameters. It is evident that both the phenolic compound content and antioxidant activity, as determined by DPPH and ABTS assays, varied with the sonication conditions.

Consistent with these findings, previous studies on ethanolic extracts from various sources such as fruit (Krishnan *et al*. 2020), herb leaves (Rout *et al*. 2021), plant roots (Osae *et al*. 2019), and wood (Vivek *et al*. 2017) have reported changes in antioxidant activity or TPC in response to sonication conditions. The ultrasonic waves in UAE induce the formation and collapse of bubbles, which creates localized pressure to disrupt cell walls, thereby facilitating the release of intracellular substances into the solvent. This result enhances antioxidant extraction and efficiency while reducing extraction time. Consequently, diffusion rates increase, resulting in enhanced extraction of solids by the solvent.

Notably, the extraction time in the present study had minimal impact on the evaluated responses, suggesting that a sonication period of 90 min may suffice for extracting the compounds of interest. This observation aligns with findings by Ballesteros *et al*. (2014), who investigated the ethanolic extraction of antioxidant phenolic compounds and found that extraction time did not significantly influence TPC or antioxidant activity. From an economic standpoint, shorter extraction times are advantageous, as they reduce energy consumption.

The resulting ramp function graph (Fig. 3) depicted a region where all specified conditions were met. Within this region, an optimal point was identified, aligning with a contact time of 90 min, temperature of 60 °C, and ethanol concentration of 78%.

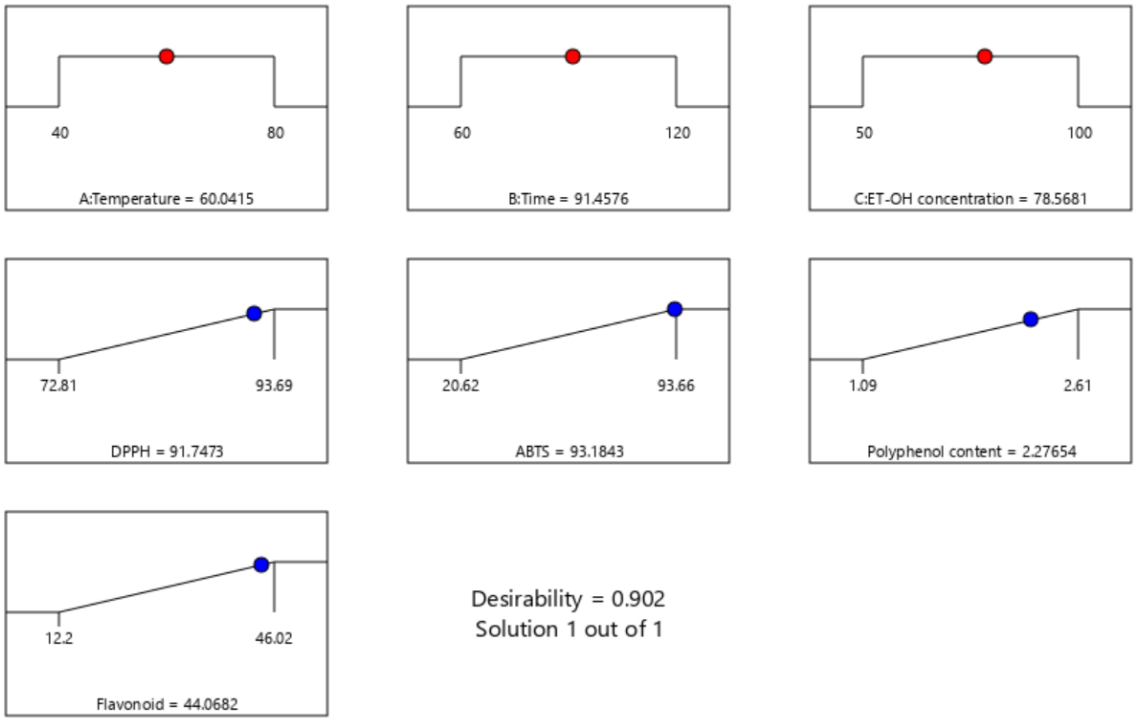


Fig. 3. Ramp function graph of optimal extraction conditions for DPPH, ABTS, TPC, and flavonoid content

Table 6. Validation of the Optimal Extraction Conditions for DPPH, ABTS, TPC, and Flavonoid Content from *A. acerifolia* Extract

Assays	Responses	
	Actual	Predicted
DPPH, %	90.54	91.7473
ABTS, %	91.11	93.1843
TPC, mg/g	2.19	2.27654
Flavonoid content, mg/g	42.39	44.0682

After determining the optimal conditions for the three independent variables, extractions were conducted in triplicate under these conditions to validate the model. The obtained data for DPPH (90.54%), ABTS (91.11%), TPC (2.19 mg GA/g DW), and flavonoid content (42.39 mg Catechin/g DW) closely corresponded with the predicted results (DPPH (91.7473%), ABTS (93.1843%), TPC (2.27654 mg GA/g DW), and flavonoid content (44.0682 mg Catechin/g DW)) from the statistical analysis (Table 6).

The relationship between phenolic compounds and antioxidant activity in extracts from various raw materials has been explored in prior research (Terpinc *et al*. 2012). The present findings align with previous investigations demonstrating a consistent trend in extracts, where higher levels of phenolics correspond to increased antioxidant activity (Burri *et al*. 2017), indicating a direct association between phenolic compounds and antioxidant potential. However, some studies have reported a weak correlation between TPC and DPPH values, potentially attributable to the presence of other compounds, aside from phenols, that could also exhibit DPPH radical scavenging activity (Wojdyło *et al*. 2007). Similarly, the correlation observed between DPPH and ABTS implies that both assays share a similar mechanism of action, such as electron transfer from antioxidant to oxidant (Apak *et al*. 2016).

Table 7. Dicaffeoylquinic Acid Identified and Quantified from *A. acerifolia* Extracts at the Optimized Condition

No.	Temp. (°C)	Time (min)	Ethanol concentration (%)	Dicaffeoylquinic acid (mg/g)
1	80	120	75	77.71
2	40	60	75	67.75
3	40	120	75	79.74
4	60	120	50	57.97
5	60	60	50	62.16
6	80	90	50	54.03
7	40	90	100	67.18
8	80	60	75	71.88
9	60	90	75	95.05
10	60	120	100	64.20
11	60	60	100	60.69
12	40	90	50	55.58
13	80	90	100	57.32
Optimization condition	60	91	78	102.06

HPLC Analysis of Dicaffeoylquinic acid

The analysis of phenolic compound content, specifically dicaffeoylquinic acid, in extracts obtained under various extraction conditions was conducted using HPLC, with results presented in Table 7. Previous studies have highlighted the efficacy of compounds belonging to dicaffeoylquinic acid in anti-acetylcholinesterase and peroxynitrite scavenging activities (Nugroho *et al*. 2019). Additionally, Lee *et al*. (2020) reported the potential of *A. acerifolia* water extract, containing dicaffeoylquinic acid, in mitigating LPS/D-GalN-induced acute liver injury in human HepG2 cells. A specific antioxidant compound, 3,4-dicaffeoylquinic acid, found in various plant materials such as coffee beans, fruits, and vegetables, has demonstrated the ability to reduce oxidative stress both *in vitro* and in vivo, thus exhibiting cytoprotective properties (Liang and Kitts 2015; Budryn *et al*. 2017). Based on our findings, it is plausible that the observed antioxidant and reducing power in the extract of *A. acerifolia* under optimized conditions is primarily attributable to the presence of dicaffeoylquinic acid. This inference is supported by the consistency between the content of dicaffeoylquinic acid in *A. acerifolia* and the observed trend in antioxidant activity or TPC.

These findings hold significant relevance due to the multifaceted functional properties of these compounds and their potential applications in cosmeceutical, pharmaceutical, and food industries. Despite the favorable *in vitro* potential of the *A. acerifolia* extract, it is imperative to ascertain the pharmacokinetics and toxicity of the extracted compounds through in vivo toxicological studies.

CONCLUSIONS

This study focused on optimizing the UAE extraction process of phenolic compounds from *A. acerifolia* using RSM design.

1. The utilization of 78% (v/v) ethanol proved to be efficient for extracting phenolics with antioxidant capacity at a temperature of 60°C and a sonication time of 91 min.
2. *A. acerifolia* represents an underexplored bioresource, and for the first time, optimal conditions to maximize the extraction of antioxidant phenolic compounds were successfully determined.

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Data Availability

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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