

Article

Development of Aqueous Two-Phase System for Eco-Friendly and Decolorized Triterpenoids Extraction of *Centella asiatica* Optimized by Response Surface Methodology

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Abstract

Centella asiatica (L.) Urban extracts are widely used in food, cosmetic, and pharmaceutical products. Conventional solvent extraction requires organic solvents and often produces dark green extracts due to chlorophyll co-extraction, limiting their industrial applications. This study developed an environmentally friendly aqueous two-phase system (ATPS) for the simultaneous extraction of triterpenoids of *Centella asiatica*. Response surface methodology with a central composite design was employed to optimize ethanol concentration, ammonium sulfate concentration, and temperature. A rapid and validated HPLC method for quantification of four triterpenoids—madecassoside, asiaticoside, madecassic acid, and asiatic acid—was developed and validated on a Poroshell 120 SB-C18 column (3.0 × 150 mm, 2.7 microns) at 40 °C. The developed method for all four triterpenoids was rapid (14 min run time) with acceptable system suitability, specificity, linearity ($R > 0.9995$), accuracy (98.42–101.52%), precision (0.16–1.36%), LOD (0.04–0.64 µg/mL), and LOQ (0.13–1.93 µg/mL). The ANOVA data from the central composite design of response surface methodology for total triterpenoid content were fitted with quadratic models, yielding acceptable R^2 (>0.97), adjusted R^2 (>0.95), and predicted R^2 (>0.80). The optimum extraction conditions were 18.29% *w/w* ethanol, 33.93% *w/w* ammonium sulfate, and 60.04 °C, yielding a total triterpenoid content of 30.53 mg g^{−1} dry weight. The optimized ATPS produced higher yields of madecassoside (13.15 mg g^{−1}), asiaticoside (9.02 mg g^{−1}), madecassic acid (5.26 mg g^{−1}), and asiatic acid (3.10 mg g^{−1}) than conventional ethanol extraction while simultaneously reducing chlorophyll-derived coloration. This ATPS extraction strategy provides a sustainable approach for producing suitable *Centella asiatica* extracts for cosmeceutical and pharmaceutical applications.



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Keywords: aqueous two-phase system; *Centella asiatica*; triterpenoids; eco-friendly extraction; sustainability

1. Introduction

Centella asiatica (L.) Urban, known as gotu kola, is a medicinal herb that has been extensively used in traditional medicine throughout Asia and has gained increasing recognition in modern Western healthcare [1]. Its therapeutic applications are officially documented in several pharmacopoeias, including those of China, India (Ayurveda), and France [2]. Several studies have demonstrated that *Centella asiatica* exhibits a broad spectrum of biological

activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, neuroprotective, anti-ulcer, memory-enhancing, and anti-asthmatic effects [3–7]. The pharmacological activities of *Centella asiatica* are mainly attributed to its pentacyclic triterpenoids, particularly madecassoside (M), asiaticoside (A), madecassic acid (MA), and asiatic acid (AA) (Figure 1), which are widely accepted as quality markers for herbal preparations [8]. Asiaticoside and madecassoside also exhibit wound-healing activity, possibly through the production of glycosaminoglycan, hydroxyproline, and collagen in wound tissue [9,10]. Madecassic acid and asiatic acid are utilized in dermatology, traditional medicine, and pharmacology for their anti-inflammatory, antioxidant, and neuroprotective properties [5,6].

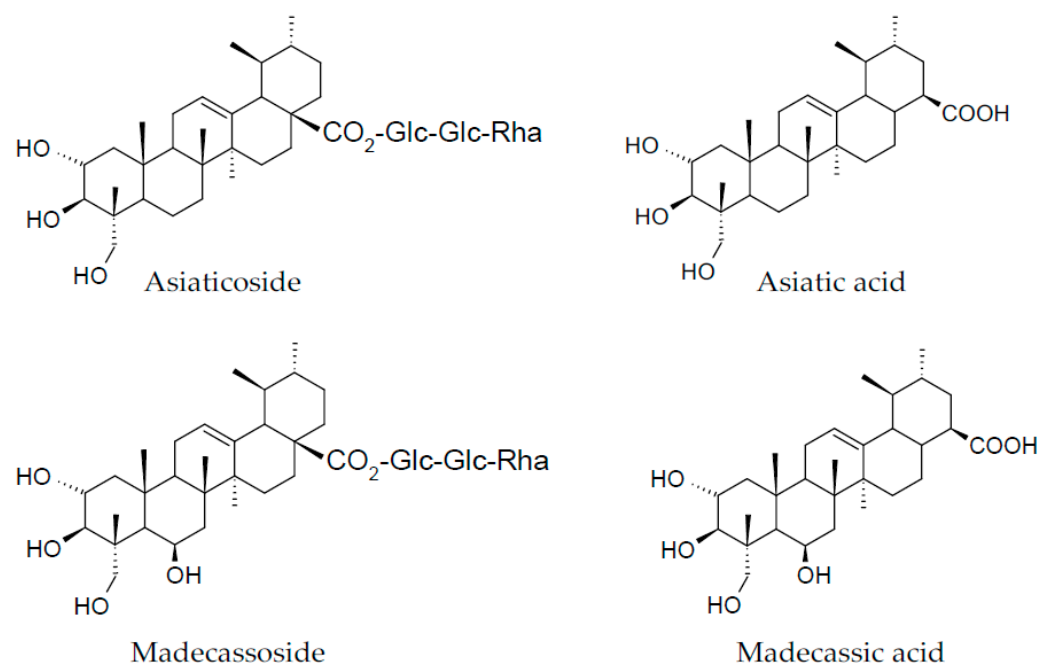


Figure 1. The chemical structures of asiaticoside (A), madecassoside (M), asiatic acid (AA), and madecassic acid (MA) [11].

Several extraction techniques for *Centella asiatica* have been developed to improve the recovery of valuable triterpenoid compounds from *Centella asiatica*, including supercritical fluid extraction [12], microwave-assisted extraction [13,14], ultrasound-assisted extraction [15,16], enzyme-assisted extraction [2], and pressurized liquid extraction [17]. These methods rely largely on conventional solvent extraction techniques requiring large volumes of organic solvents, resulting in increased production costs, safety concerns, and environmental burdens. Moreover, the solvent extraction process often co-extracts substantial amounts of chlorophylls and other plant pigments, producing dark green extracts that are undesirable for food, cosmetic, and pharmaceutical applications [13]. The presence of these pigments not only affects the visual appearance and consumer acceptance of the final products but may also interfere with downstream purification, analytical quantification, and formulation processes.

Industrial production strategies typically begin with mechanical pulverization of dried *Centella asiatica* biomass to maximize surface area contact, followed by solid–liquid extraction using hydroethanolic solvents via automated heat reflux configurations. However, conventional methods often suffer from extended processing times and substantial solvent consumption [2]. Consequently, contemporary industrial processing is shifting toward green and sustainable approaches. Process-intensified technologies are increasingly focused on eco-friendly extraction approaches with lower energy requirements and reduced thermal degradation of secondary metabolites [18]. Downstream operations necessitate

rigorous filtration, vacuum concentration for solvent recovery, and specialized purification via macroporous adsorption resin columns to selectively isolate target triterpenoids from interfering plant matrices, pigments, and sugars. Final polishing using activated carbon decolorization coupled with industrial spray- or freeze-drying transforms the liquid fraction into a highly stable, uniform powder. These procedures are multi-step extraction and purification, causing downstream separation inefficiencies.

In recent years, the aqueous two-phase system (ATPS) has emerged as a promising liquid–liquid fractionation technique with considerable potential for the extraction, separation, purification, and enrichment of various biomolecules, including proteins, enzymes, and nucleic acids [19]. Typically, an ATPS is formed by combining two water-soluble components, such as polymer/polymer (e.g., PEG–dextran) or polymer/salt (e.g., PEG–phosphate, PEG–sulfate), which separate into two immiscible aqueous phases above a critical concentration [20]. ATPS contains two types of insoluble aqueous phase systems spontaneously formed by mixing aqueous solutions of two hydrophilic compounds in a certain mass fraction. Similar to the traditional solvent extraction principle, the partitioning behavior of target compounds between these phases is governed by their physicochemical properties such as intermolecular hydrogen bonds, van der Waals forces, hydrophobicity, interface properties, and other factors. In the two immiscible aqueous phases of ATPS, the difference in polarity and hydrophobicity between the phases causes compounds to partition selectively according to their different physicochemical properties. Recent studies have demonstrated that this technique has been effectively applied to the detection of veterinary drug residues in food products, the recovery of valuable metals, wastewater treatment, and several other industrial and environmental applications [21]. Chlorophylls are highly hydrophobic molecules with long phytol chains, whereas the relatively more polar triterpenoid glycosides and triterpene acids exhibit different partitioning behavior [22]. This selective distribution reduces the co-extraction of green pigments, producing a lighter-colored extract without requiring an additional bleaching or purification step. To the best of our knowledge, this is the first report on the application of ATPS for the simultaneous recovery of triterpenoid compounds and reduced co-pigmentation of *Centella asiatica* extracts. This study aimed to develop and optimize an environmentally friendly ethanol–ammonium sulfate ATPS for the simultaneous extraction, depigmentation, and enrichment of the major triterpenoids from *Centella asiatica*. A central composite design combined with response surface methodology was employed to investigate the effects of ethanol concentration, ammonium sulfate concentration, and temperature on asiaticoside and total triterpenoid recovery. In addition, a rapid HPLC–UV method was developed and validated for the simultaneous quantification of madecassoside, asiaticoside, madecassic acid, and asiatic acid. The performance of the optimized ATPS was subsequently compared with conventional hydroethanolic extraction in terms of triterpenoid recovery and chlorophyll co-extraction.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemical reagents were of analytical grade, including ammonium sulfate (99.5%), acetone (99.5%), and ethyl alcohol (99.9%) from Sigma-Aldrich, St. Louis, MO, USA. Reference standards of madecassoside (98.06%), asiaticoside (99.67%), and madecassic acid (92.32%) were obtained from MedChemExpress (MCE, Monmouth Junction, NJ, USA), and asiatic acid (97.00%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was procured from Merck (Boston, MA, USA).

2.2. Plant Sample and Preparation

Centella asiatica leaves were collected from a local source in Nakhon Pathom province, Thailand. Identification of *Centella asiatica* leaves was performed according to the Thai Herbal Pharmacopoeia 2021 [23]. The leaves of *Centella asiatica* were thoroughly washed with deionized water and dried in a hot-air oven at 50 °C for 24 h. The dried leaves were subsequently ground into a fine powder and passed through a No. 60 sieve to obtain a uniform particle size. The appearance of *Centella asiatica* powder is shown in Figure 2. The prepared powder was stored in a desiccator under dark and oxygen-free conditions until further use.



Figure 2. *Centella asiatica* (L.) Urban dried leaf powder obtained after oven drying and grinding.

2.3. Extraction

Stock solutions of ethanol and ammonium sulfate were prepared using deionized water. The aqueous two-phase system was prepared using 18.29–26.70% *w/w* ethanol, 30.80–39.20% *w/w* salt, and a temperature of 38.18–71.82 °C. The system was temperature-controlled to ensure that the required conditions were established before the experiment. The leaf powder 1 g was then added to the mixture. The solution was stirred at 150 rpm for 90 min. The extract solutions resulted in the separation into two clear phases: a top alcohol-rich phase and a bottom salt-rich phase. Each phase was carefully collected. The alcohol-rich phase was diluted into methanol (1:1) and filtered through a 0.22-micron nylon membrane prior to HPLC analysis.

2.4. Method Validation and Quantification of Triterpenoid Contents Using High-Performance Liquid Chromatography (HPLC-UV)

The HPLC-UV method for the quantification of madecassoside (M), asiaticoside (A), madecassic acid (MA), and asiatic acid (AA) has been developed through modifications of the method established by Rafi et al. [11]. The HPLC system (Agilent, Santa Clara, CA, USA) comprised the 1290 Infinity II binary pump, autosampler, column thermostat, and photodiode array detector. The separation process occurred in a Poroshell 120 SB-C18 column (Agilent, Santa Clara, CA, USA) (3×150 mm, 2.7 microns). The mobile phase, consisting of water (A) and acetonitrile (B) with a gradient elution of 0–3 min, 15%B; 3–3.5 min, 15–30%B; 3.5–7 min, 30%B; 7–7.5 min, 30–60%B; and 7.5–14 min, 60%B, maintained a steady flow rate of 0.6 mL/min. Prior to use, the mobile phase underwent degassing and was freshly prepared for each analysis. After each run, the column was washed with 90% acetonitrile for 3 min and re-equilibrated with 15% acetonitrile for 3 min. The detection wavelength was set at 206 nm, with an injection volume of 5 μ L. The needle was washed with acetonitrile for 1 min. The analysis was performed at an operating temperature of 40 °C. Under these specified chromatographic conditions, the average retention times of M, A, MA, and AA were 6.04, 7.08, 10.99 and 12.64 min, respectively.

The calibration curves of four standard solutions in methanol were in the range of 11–113 μ g/mL of M, 17–170 μ g/mL of A, 3.5–35 μ g/mL of MA, and 9.1–91 μ g/mL of AA, respectively [24]. System suitability testing was performed prior to the analysis to ensure adequate chromatographic performance for the simultaneous determination of M, A, MA, and AA in *Centella asiatica* extracts. A mixed reference standard solution and a sample solution containing M, A, MA, and AA at the working concentrations were injected six consecutive times under optimized chromatographic conditions. System suitability parameters, including retention time repeatability, peak area repeatability, theoretical plate number (N), tailing factor (T), and resolution (R) between adjacent peaks, were evaluated. The analytical method was validated for specificity, linearity, precision, accuracy, LODs and LOQs according to AOAC guidelines [25]. The contents of M, A, MA, and AA were expressed as mg/g dry powder weight. Total triterpenoid content was calculated as the sum of these four compounds. All experiments were conducted in triplicate, and results were reported as the mean.

2.5. Optimization Using Central Composite Design (CCD) and Response Surface Methodology (RSM)

Factors for CCD included ethanol concentration (X1, 18.29–26.70% w/w), ammonium sulfate concentration (X2, 30.80–39.20% w/w), and temperature (X3, 38.18–71.82 °C) (Table 1). CCD was employed as a statistical tool to investigate the effects of input parameters on process outcomes and to identify the most effective operating conditions [26]. This design approach offers the ability to analyze both main and interaction effects among variables, reduce the number of required experimental runs, save time and resources, and efficiently determine the optimal conditions for process improvement. CCD also provides a robust framework for supporting the development and scale-up of industrial processes [27]. Experimental CCD parameters of *Centella asiatica* ATPS extraction were investigated at five coded levels ($-\alpha$, -1 , 0 , $+1$, $+\alpha$) (Table 1), allowing the construction of a quadratic polynomial model to describe the relationship between the responses (including asiaticoside and total triterpenoid contents) and the experimental factors. A total of 20 experimental runs were performed, consisting of eight factorial points, six axial (star) points, and six center points to estimate pure error and ensure model reproducibility (Table 2). The experiments were randomized to minimize the influence of uncontrolled variability. The experimental data were fitted to a second-order polynomial model according to Equation (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y represents the response variable, β_0 is the intercept, β_1 – β_3 are linear coefficients, β_{11} – β_{33} are quadratic coefficients, and β_{12} – β_{23} are interaction coefficients.

Table 1. Independent factors and studied ranges in the experimental design of *Centella asiatica* extraction using aqueous two-phase system (ATPS).

Factor	Symbol	Unit	− α	−1	0	+1	+ α
Ethanol	A	%w/w	18.29	20	22.5	25	26.70
Ammonium sulfate	B	%w/w	30.80	32.5	35	37.5	39.20
Temperature	C	°C	38.18	45	55	65	71.82

Table 2. Experimental CCD parameters of *Centella asiatica* extraction using ATPS.

Run	Ethanol (%w/w)	Ammonium Sulfate (%w/w)	Temp. (°C)
1	20	32.5	45
2	25	32.5	45
3	20	37.5	45
4	25	37.5	45
5	20	32.5	65
6	25	32.5	65
7	20	37.5	65
8	25	37.5	65
9	18.29	35	55
10	26.70	35	55
11	22.5	30.80	55
12	22.5	39.20	55
13	22.5	35	38.18
14	22.5	35	71.82
15	22.5	35	55
16	22.5	35	55
17	22.5	35	55
18	22.5	35	55
19	22.5	35	55
20	22.5	35	55

Statistical analysis and model development were performed using Design-Expert software (version 12, Stat-Ease Inc., Minneapolis, MN, USA). Analysis of variance (ANOVA) was applied to evaluate the significance and adequacy of the model, while model fitness was assessed using the coefficient of determination (R^2). The predicted optimal conditions were validated through confirmatory experiments.

2.6. Determination of Chlorophyll Intensity

The method for chlorophyll determination followed a previous study with some modifications [13]. The intensity of chlorophyll was evaluated by absorbance. The samples were determined using a microplate reader (CLARIOstar®, BMG LABTECH, Ortenberg, Germany). One hundred microliters of sample solution were mixed with one hundred microliters of ethanol in a 96-well plate. The absorbance of chlorophyll was measured at 660 nm, which is the maximum absorbance wavelength. The solvent of each extract was used as a blank.

2.7. Antioxidant Activity Analysis

DPPH and ABTS scavenging activities of the extracts were determined using a previously described method [12]. For the DPPH assay, the extract sample solution was 2-fold diluted in methanol and then filtered through 0.22 microns Nylon membrane filter. Fifty microliters of sample were mixed with fifty microliters of 0.6 mM ethanolic DPPH solution in the wells of a 96-well plate. The reaction mixture was incubated at room temperature for 30 min in the dark. The absorbance was measured at 515 nm against a blank (methanol) using a microplate reader. Gallic acid at 100 µg/mL was used as the positive control. All experiments were performed in triplicate. The percentages of the DPPH radical scavenging activity were calculated by Equation (2):

$$\text{DPPH scavenging activity (\%)} = (A_{\text{control}} - A_{\text{sample}}) \times 100 / A_{\text{control}} \quad (2)$$

where A sample is the absorbance of the DPPH solution with the extract solution, and A control is the absorbance of the DPPH solution.

For the ABTS assay, 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate solution (1:1) and kept in the dark at room temperature for 16 h. The working solution was then diluted in ethanol to obtain a spectrophotometric absorbance of 0.74 ± 0.02 at 734 nm. The extract solution was 25-fold diluted with methanol and then filtered through 0.22 microns Nylon membrane filter. Fifty microliters of sample were mixed with fifty microliters of the ABTS solution in the wells of a 96-well plate. The reaction mixture was incubated at room temperature for 30 min in the dark, and the absorbance was recorded at 734 nm using a microplate reader. Gallic acid at 100 µg/mL was used as the positive control. All experiments were performed in triplicate. The percentages of the ABTS radical scavenging activity were calculated by Equation (3):

$$\text{ABTS scavenging activity (\%)} = (A_{\text{control}} - A_{\text{sample}}) \times 100 / A_{\text{control}} \quad (3)$$

where A sample is the absorbance of the ABTS solution with the extract solution, and A control is the absorbance of the ABTS solution.

3. Results

3.1. Method Validation and Quantification of Triterpenoid Contents Using HPLC-UV

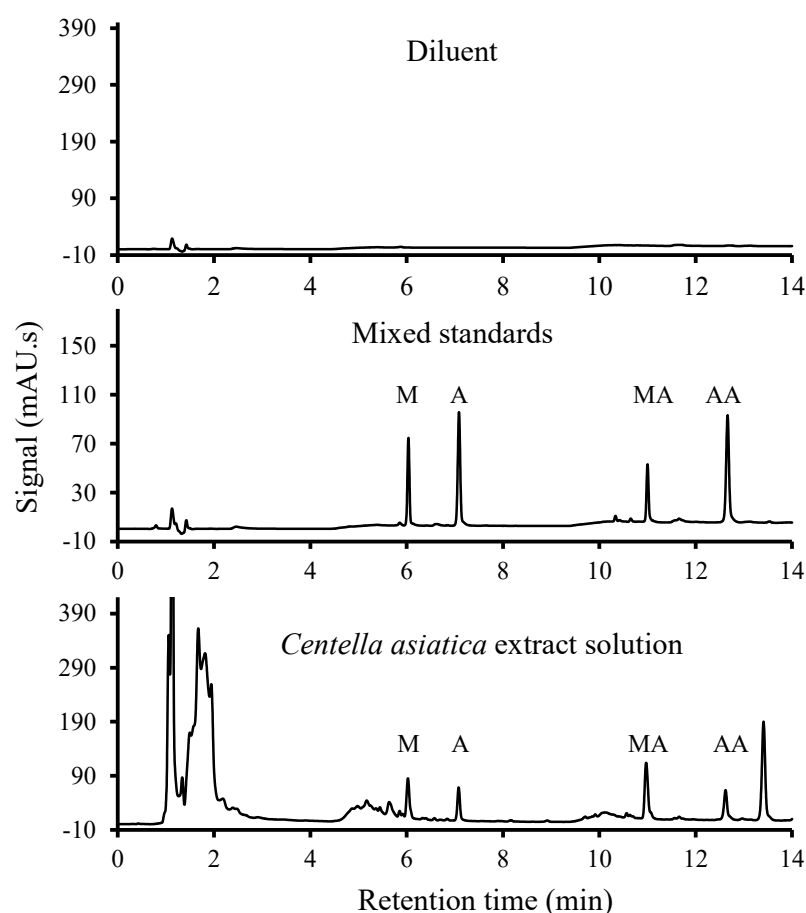
System suitability testing demonstrated that the HPLC system was adequate and reliable for the analysis of M, A, MA, and AA in *Centella asiatica* extracts. Across six replicate injections, the %RSD of retention time and peak area were low ($\leq 1\%$), confirming excellent repeatability and detector precision. All analytes showed appropriate retention behavior, high theoretical plate numbers indicating good column efficiency, and acceptable peak symmetry with tailing factors below 1.5. Baseline separation was achieved for all peaks, with resolution values exceeding the minimum requirement ($R > 2.0$); these results confirm that the chromatographic system was suitable for subsequent method validation and routine quantitative analysis (Table 3). Then, validation of the analytical method was conducted in adherence to the guidelines established by the Association of Official Analytical Chemists (AOAC) [25].

Table 3. System suitability parameters using mixed standard solution and *Centella asiatica* extract solution (n = 6).

Analyte	%RSD of Retention Time	%RSD of Peak Area	Capacity Factor	Plate Number	Tailing Factor	Resolution	Peak Purity
Mixed standard solution							
Madecassoside (M)	0.03	0.58	4.35	98,583	1.01	30.52	99.99
Asiaticoside (A)	0.03	0.76	5.27	87,648	1.02	12.06	100.00
Madecassic acid (MA)	0.00	0.89	8.74	301,367	1.14	44.56	99.99
Asiatic acid (AA)	0.01	0.49	10.20	196,267	1.26	16.99	99.33
<i>Centella asiatica</i> extract solution							
Madecassoside (M)	0.05	0.69	4.35	49,805	1.16	2.02	99.17
Asiaticoside (A)	0.06	0.80	5.28	83,550	1.06	10.24	99.94
Madecassic acid (MA)	0.02	0.39	8.73	122,304	1.48	37.77	99.10
Asiatic acid (AA)	0.02	0.27	10.19	192,591	1.23	13.70	99.11

3.1.1. Specificity

The chromatographic profile was obtained from the *Centella asiatica* sample solution. The chromatogram demonstrates the separation and detection of M, A, MA, and AA within the complex matrix of the herbal extraction. The retention times of M, A, MA, and AA were observed to be 6.04, 7.08, 10.99, and 12.64 min, respectively. The chromatograms of the standards and the sample exhibited identical retention times. The peak purity of these peaks in the mixed standard solution and the sample solution was not less than 99.0%, and the resolutions of these peaks with the adjacent peaks were not less than 2.0 (Figure 3).

**Figure 3.** HPLC chromatograms of diluent, reference standards, and *Centella asiatica* extract solution.

3.1.2. Linearity and Range

The analysis of the mixed standard solutions in methanol was conducted in the range of 11–113 µg/mL of M, 17–170 µg/mL of A, 3.5–35 µg/mL of MA, and 9.1–91 µg/mL of AA, respectively, to provide average peak areas and corresponding concentrations to construct calibration curves. The results indicate that calibration curves 1, 2, and 3 from three different validation days exhibited correlation coefficient values of at least 0.9995 (Table 4).

Table 4. Linearity, LOD, and LOQ for madecassoside (M), asiaticoside (A), madecassic acid (MA), and asiatic acid (AA).

Analyte	Linear Equations	R	LOD (µg/mL)	LOQ (µg/mL)
Madecassoside (M)	$y = 1.8156x + 0.4358$	0.9999	0.44	1.32
	$y = 1.8081x + 0.3937$	0.9999		
	$y = 1.8168x + 0.8288$	0.9999		
Asiaticoside (A)	$y = 2.015x - 2.9101$	0.9995	0.62	1.87
	$y = 2.2032x - 2.8669$	0.9995		
	$y = 2.027x - 2.2138$	0.9995		
Madecassic acid (MA)	$y = 4.2774x + 0.3591$	0.9997	0.04	0.13
	$y = 4.4586x + 0.4521$	0.9997		
	$y = 4.3500x + 0.4550$	0.9999		
Asiatic acid (AA)	$y = 4.1202x + 7.6863$	0.9997	0.64	1.93
	$y = 4.1392x + 8.1820$	0.9997		
	$y = 4.1523x + 9.2503$	0.9997		

3.1.3. LOD and LOQ

Utilizing the linear equation data, the limit of detection (LOD) and limit of quantification (LOQ) were calculated employing the following formulas: $LOD = 3.3 \times (\text{standard deviation of } y\text{-intercept}) / (\text{mean of slope})$ and $LOQ = 10 \times (\text{standard deviation of } y\text{-intercept}) / (\text{mean of slope})$. The calculated LOD values for M, A, MA, AA were determined to be 0.44, 0.62, 0.04 and 0.64 µg/mL, and the calculated LOQ values for M, A, MA, AA were found to be 1.32, 1.87, 0.13, and 1.93 µg/mL, respectively (Table 4).

3.1.4. Accuracy and Precision

The analysis of triterpenoids in *Centella asiatica* extracts employed a UV detector; accuracy and precision tests were conducted using M, A, MA, and AA standard solutions at three concentration levels to be spiked into the sample extract according to the standard addition method to represent the closeness to the real samples, with triplicate preparations for each concentration. As presented in Table 5, M, A, MA, and AA exhibited average recovery rates ranging from 98.42% to 101.52%. The relative standard deviation (%RSD) for precision ranged from 0.16% to 1.36%. The comprehensive validation of the analytical method for triterpenoid quantification in the *Centella asiatica* extracts has confirmed its reliability, accuracy, and suitability for its intended purpose. This analytical approach provides a foundation for the quantification of triterpenoids in the ATPS.

Table 5. Accuracy and precision for madecassoside (M), asiaticoside (A), madecassic acid (MA) and asiatic acid (AA) (n = 3).

Analyte	Conc. (µg/mL)	Average Recovery (µg/mL)	%Recovery	SD	%RSD
Madecassoside (M)	22.65	22.16	98.42	0.16	0.16
	33.98	34.18	100.60	0.69	0.69
	45.30	46.28	101.37	0.60	0.60
Asiaticoside (A)	33.89	34.08	100.56	1.23	1.23
	50.83	51.18	100.68	1.26	1.25
	67.78	68.95	101.52	0.78	0.77
Madecassic acid (MA)	6.92	6.96	100.62	1.07	1.07
	10.39	10.51	101.24	0.91	0.90
	13.85	13.87	100.18	0.98	0.98
Asiatic acid (AA)	18.14	17.89	98.62	0.32	0.32
	27.21	27.33	100.45	1.36	1.36
	36.28	36.71	101.18	0.37	0.36

3.1.5. Quantification of Triterpenoid Contents and Chlorophyll Intensity

The contents of the four major triterpenoids varied considerably across the experimental conditions and are presented in Table 6. A ranged from 5.92 to 10.06 mg/g, M from 9.32 to 12.71 mg/g, MA from 2.72 to 5.51 mg/g, and AA from 2.06 to 2.94 mg/g. The total triterpenoid content (TTC), calculated as the sum of these compounds, ranged between 20.87 and 30.86 mg/g. Among the 20 CCD experiments, Run 9 (ethanol = −1.68, ammonium sulfate = 0, temperature = 0) produced the highest total triterpenoid content (30.86 mg/g) and also yielded the highest asiatic acid content (2.94 mg/g). In contrast, Run 10 (ethanol = +1.68, ammonium sulfate = 0, temperature = 0) gave one of the lowest total triterpenoid values (21.84 mg/g), suggesting that excessive ethanol concentration adversely affected the extraction efficiency. The center point experiments (Runs 15–20) exhibited good reproducibility, with total triterpenoid values ranging from 27.74 to 28.26 mg/g.

Table 6. The central composite design matrix and experimental results.

Run	Ethanol (%w/w)	Ammonium Sulfate (%w/w)	Temp (°C)	Experimental Results (mg/g)					Absorbance at 660 nm *
				A	M	MA	AA	Total Triterpenoid Content	
1	−1	−1	−1	7.74	11.11	4.51	2.71	26.07	0.124
2	1	−1	−1	6.05	10.33	3.30	2.23	21.91	0.098
3	−1	1	−1	6.41	10.24	3.93	2.53	23.11	0.446
4	1	1	−1	6.68	9.408	2.72	2.06	20.87	0.235
5	−1	−1	1	7.54	12.23	5.51	2.86	28.14	0.151
6	1	−1	1	6.17	10.76	4.30	2.38	23.61	0.085
7	−1	1	1	6.90	11.28	4.93	2.68	25.79	0.384
8	1	1	1	6.22	10.52	3.72	2.21	22.67	0.332
9	−1.68	0	0	10.06	12.63	5.23	2.94	30.86	0.252
10	1.68	0	0	5.92	10.58	3.20	2.14	21.84	0.198
11	0	−1.68	0	6.70	10.39	4.70	2.68	24.47	0.090
12	0	1.68	0	6.57	9.32	3.73	2.39	22.01	0.458
13	0	0	−1.68	6.10	9.97	3.09	2.18	21.34	0.138

Table 6. Cont.

Run	Ethanol (%w/w)	Ammonium Sulfate (%w/w)	Temp (°C)	Experimental Results (mg/g)					Absorbance at 660 nm *
				A	M	MA	AA	Total Triterpenoid Content	
14	0	0	1.68	6.71	12.71	4.77	2.44	26.63	0.186
15	0	0	0	9.97	11.54	4.21	2.54	28.26	0.246
16	0	0	0	9.82	11.49	4.25	2.58	28.14	0.208
17	0	0	0	9.90	11.12	4.25	2.47	27.74	0.210
18	0	0	0	9.86	11.32	4.20	2.44	27.82	0.208
19	0	0	0	9.93	11.20	4.17	2.56	27.86	0.207
20	0	0	0	9.88	11.36	4.19	2.49	27.92	0.208
70% (v/v) Ethanol				4.36	10.90	2.25	1.91	19.42	0.615

* The absorbances of chlorophyll in the extracts at 660 nm were subtracted from the absorbance of their solvent as a blank.

The chlorophyll content, represented by the absorbance at 660 nm after blank correction, ranged from 0.085 to 0.458 among the CCD runs. The lowest absorbance was observed in Run 6 (0.085), whereas the highest was recorded for Run 12 (0.458). Notably, extraction using the conventional 70% (v/v) ethanol solvent resulted in a substantially higher chlorophyll absorbance (0.615) while producing a lower TTC (19.42 mg/g). These findings suggest that the ATPS not only improved triterpenoid extraction but also effectively reduced chlorophyll co-extraction, thereby enhancing extract selectivity. The CCD results demonstrated that the extraction variables influenced both the recovery of triterpenoids and the level of chlorophyll contamination.

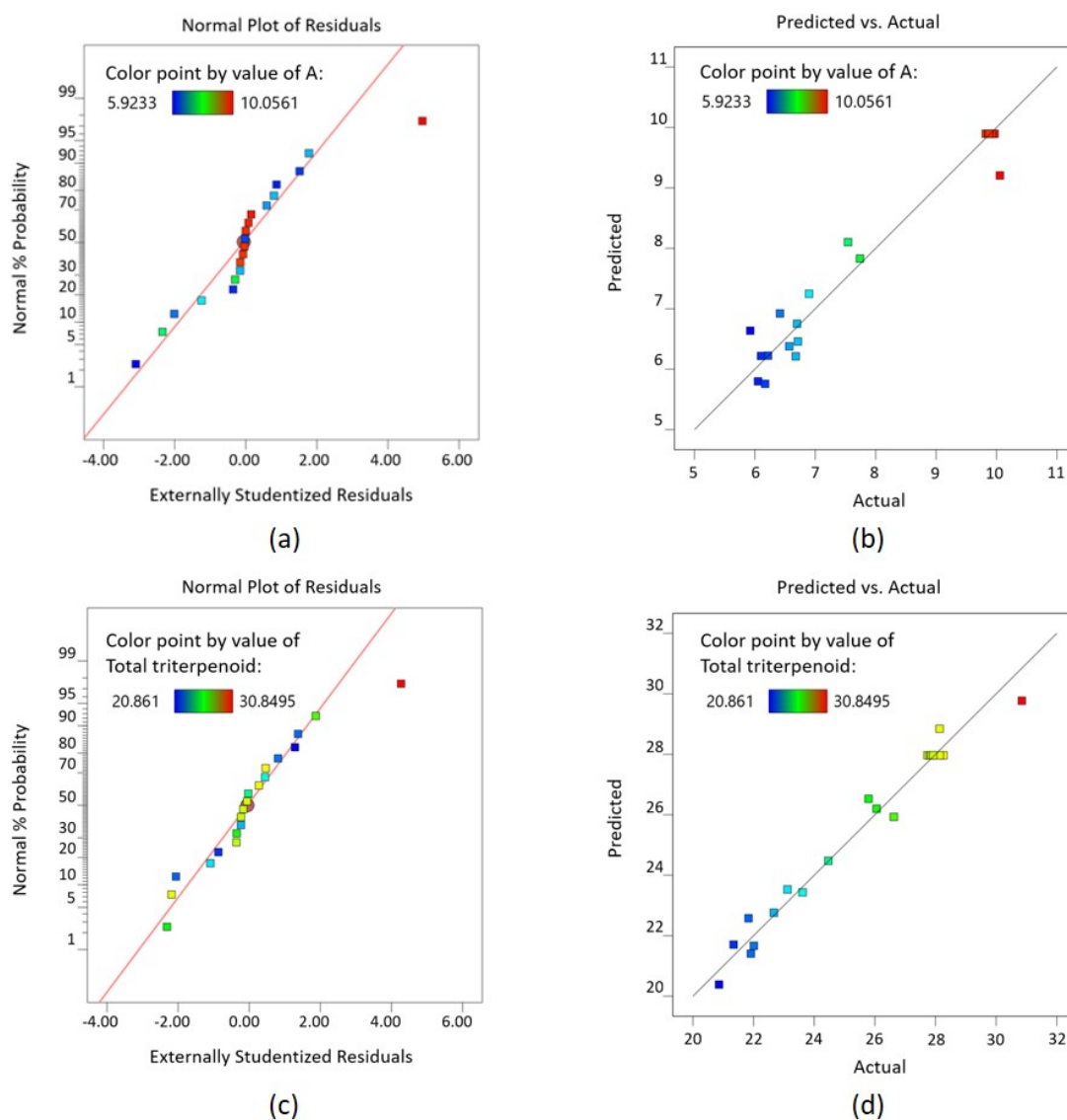
3.2. Central Composite Design Experiment

The ANOVA results demonstrated that the quadratic models for both asiaticoside and total triterpenoid contents were statistically significant ($p < 0.0001$) (Table 7). For asiaticoside content, ethanol concentration (A) showed a significant linear effect ($p = 0.0002$), whereas the linear effects of ammonium sulfate concentration (B) and temperature (C) were not significant ($p = 0.4343$ and 0.6124 , respectively). In contrast, all quadratic terms (A^2 , B^2 , and C^2) significantly affected asiaticoside content ($p \leq 0.0004$), indicating pronounced curvature in the response within the investigated experimental range. For total triterpenoid content, all three linear factors significantly influenced the response. Ethanol concentration exhibited the strongest linear effect ($p < 0.0001$), followed by temperature ($p < 0.0001$) and ammonium sulfate concentration ($p = 0.0009$). The quadratic terms A^2 , B^2 , and C^2 were also significant ($p \leq 0.0047$), further demonstrating nonlinear relationships between the extraction conditions and total triterpenoid recovery. The models showed good overall fit, with R^2 values of 0.9533 for asiaticoside and 0.9739 for total triterpenoid content. The corresponding adjusted R^2 values were 0.9113 and 0.9504, respectively. The predicted R^2 was 0.6414 for asiaticoside and 0.8088 for total triterpenoid content. Model diagnostic plots for asiaticoside and total triterpenoid content demonstrated the normal probability plots. The predicted versus actual plots showed satisfactory agreement between experimental and model-predicted values, with most data points located close to the diagonal line (Figure 4). These results confirm that the quadratic models adequately describe the experimental responses within the studied design space.

Table 7. ANOVA summary and fit statistics of the quadratic models for asiaticoside and total triterpenoid content.

Source	Asiaticoside Content		Total Triterpenoid Content	
	F-Value	p-Value	F-Value	p-Value
Model	22.70	<0.0001	41.44	<0.0001
A (Ethanol)	31.65	0.0002	141.71	<0.0001
B (Salt)	0.6635	0.4343	21.63	0.0009
C (Temp)	0.2735	0.6124	48.86	<0.0001
A ²	27.93	0.0004	13.10	0.0047
B ²	79.42	<0.0001	97.81	<0.0001
C ²	90.61	<0.0001	70.28	<0.0001
R ²	0.9533		0.9739	
Adjusted R ²	0.9113		0.9504	
Predicted R ²	0.6414		0.8088	

Bold values indicate significant model terms ($p < 0.05$)

**Figure 4.** Model diagnostic plots: (a) normal plot of residuals for asiaticoside, (b) predicted and actual values for asiaticoside; (c) normal plot of residuals for total triterpenoid content, (d) predicted and actual values for total triterpenoid content.

3.3. Effect of Ethanol, Ammonium Sulfate, and Temperature on ATPS Efficiency

The experimental asiaticoside content ranged from 5.92 to 10.06 mg/g, with a mean value of 7.76 mg/g (Table 6). Asiaticoside content increased as ethanol (%w/w) increased from 20% to approximately 22–23%. Further increases in ethanol resulted in a decline in asiaticoside content. This behavior can be explained by the co-solvent effect of ethanol in ATPS. Moderate ethanol concentrations enhance solubility and mass transfer of glycosidic triterpenoids into the alcohol-rich phase. The lowest asiaticoside contents (approximately 5.9–6.7 mg/g) were observed at higher ethanol (%w/w) combined with non-optimal salt or temperature levels, supporting the predicted curvature effect. Ammonium sulfate (%w/w) mainly influenced asiaticoside content through a significant quadratic term. The contour plots (Figures 5 and S1) show that asiaticoside content increased as salt concentration reached 34–35%, followed by a slight decline at higher salt (%w/w) factors. Temperature exhibited a moderate but significant quadratic effect (Table 7). When temperature increased to approximately 55 °C, asiaticoside content increased. When temperature was more than 55 °C, a slight decrease was observed. Optimal temperature enhances diffusion and mass transfer, facilitating extraction, whereas higher temperatures may reduce partition selectivity or promote partial degradation of thermolabile glycosides. The optimized model was revealed to be 22–23% w/w ethanol, 34–35% w/w ammonium sulfate, and 55 °C, with an asiaticoside content of 9.8–10.0 mg/g.

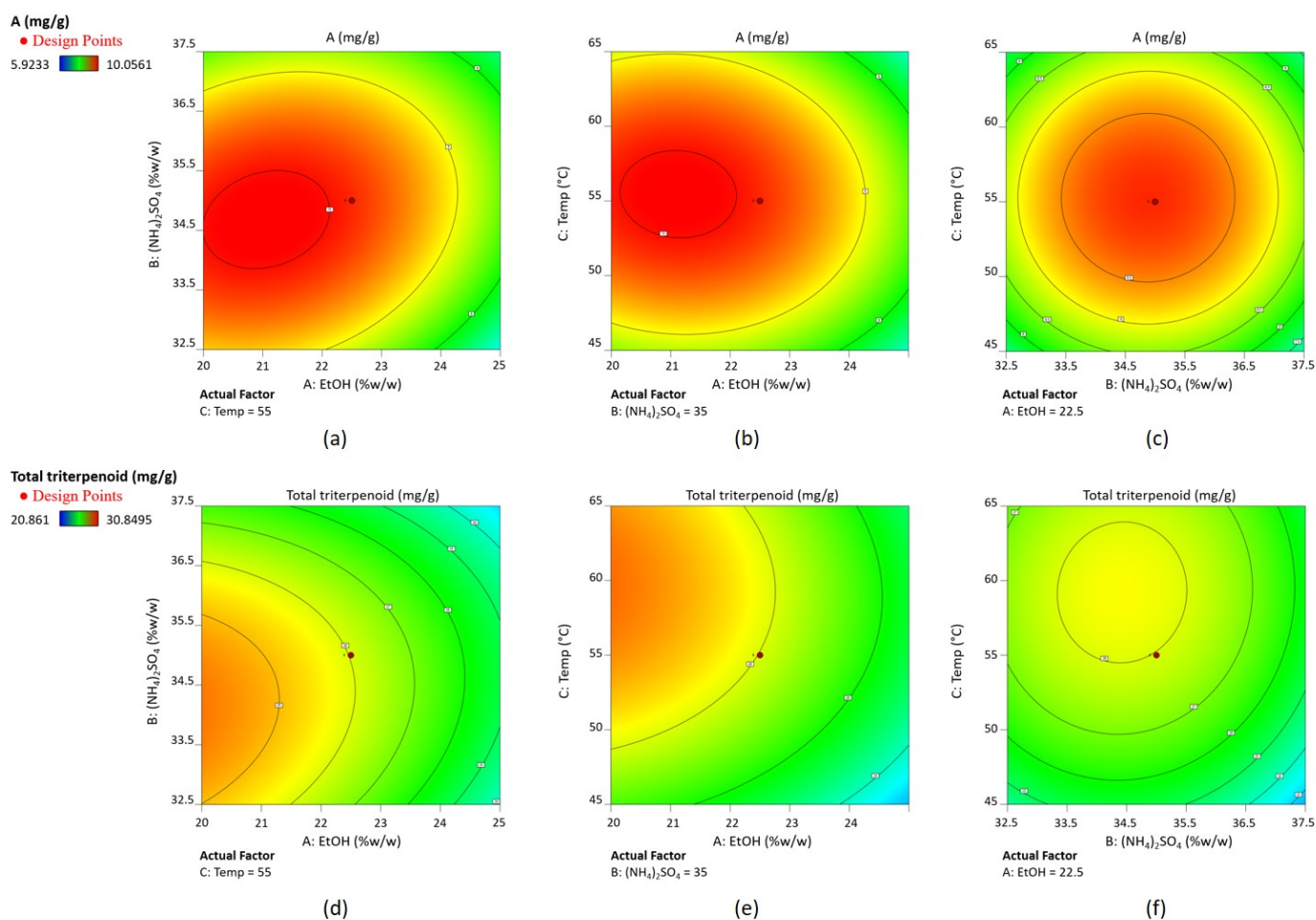


Figure 5. Contour plots for asiaticoside: (a) ethanol and ammonium sulfate; (b) ethanol and temperature; (c) ammonium sulfate and temperature. Contour plots for total triterpenoid content: (d) ethanol and ammonium sulfate; (e) ethanol and temperature; (f) ammonium sulfate and temperature.

Total triterpenoid content ranged from 20.87 to 30.86 mg/g (Table 6). The quadratic RSM model revealed that ethanol (%w/w), salt (%w/w), and temperature significantly influenced total triterpenoid content. Ethanol (%w/w) exhibited a predominantly negative influence on total triterpenoid content within the investigated range; increasing ethanol from approximately 20 to 25% led to a progressive decrease in total triterpenoid yield. The highest values (~29–31 mg/g) were achieved at lower ethanol levels combined with moderate salt concentrations. At elevated ethanol, the alcohol-rich phase becomes less selective toward triterpenoids, reducing overall recovery. Total triterpenoid content increased when salt (%w/w) was increased to ~34–35% before plateauing or slightly declining at higher salt levels. In alcohol/salt ATPS, salt can change solvent polarity and phase composition, which enhances partitioning of hydrophobic bioactive compounds into the alcohol-rich phase up to an optimum by increasing ionic strength and reducing solubility in the salt-rich phase [28,29]. Higher salt concentrations beyond the optimum can increase phase volume asymmetry, viscosity, or aggregation/precipitation, reducing effective recovery. This qualitative behavior agrees with mechanistic observations that salt significantly affects partition coefficients through salting-out mechanisms in aqueous biphasic systems [21], and has been demonstrated experimentally in ethanol/ammonium sulfate systems where solute partitioning strongly depends on ionic composition [30]. Temperature showed a positive linear effect, with the maximum values of 55–60 °C (Figures 5 and S2). Increasing temperature enhances mass transfer and solute diffusivity, facilitating extraction. Higher temperature than the optimum could reduce recovery, due to reduced partition selectivity or thermal sensitivity of triterpenoid components [31,32]. Interaction terms among the three factors were relatively small, indicating that total triterpenoid extraction was primarily governed by individual factor effects rather than strong synergistic interactions. The elliptical contour patterns confirm that quadratic effects dominate within the studied range (Figure 5). Based on the fitted model and experimental data, the optimal region for maximizing total triterpenoid content was located at approximately low-to-moderate ethanol (20–22% w/w), moderate salt (34–35% w/w), and intermediate-to-elevated temperature (~55 °C), where total triterpenoid content approached 30.86 mg/g.

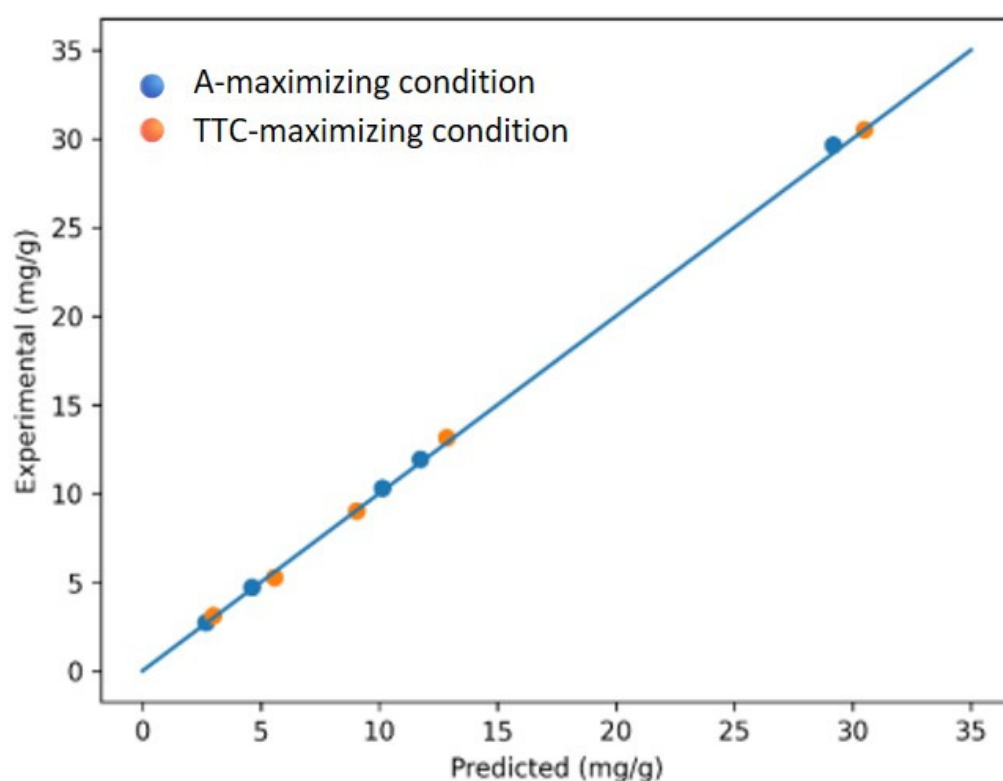
3.4. Optimization and Model Validation

The asiaticoside-maximizing condition (21.05% w/w ethanol, 34.68% w/w ammonium sulfate, and 55.46 °C) (Figure S3) and the total triterpenoids-maximizing condition (18.29% w/w ethanol, 33.93% w/w ammonium sulfate, and 60.04 °C) (Figure S4) were tested. Predicted and experimental values of individual triterpenoids at both optimized ATPS conditions are shown in Table 8. At both optimized conditions, asiaticoside (A) and madecassoside (M) were present at substantially higher concentrations than their corresponding aglycones, asiatic acid (AA) and madecassic acid (MA). At the optimized condition for asiaticoside content, the model predicted A and M contents of 10.13 and 11.74 mg/g, respectively, while the confirmatory experiment yielded 10.29 and 11.94 mg/g. At the optimized condition for total triterpenoid content, the model predicted a total triterpenoid content of 30.48 mg/g, comprising 9.05 mg/g A, 12.85 mg/g M, 5.58 mg/g MA, and 3.00 mg/g AA. The corresponding experimental value was 30.53 mg/g, with the individual contents of 9.02, 13.15, 5.26, and 3.10 mg/g for A, M, MA, and AA, respectively. Overall, the predicted values showed good agreement with the experimental results (Figure 6).

Table 8. Predicted and experimental values of individual triterpenoids at optimized ATPS conditions with percent error.

Optimization Condition	A (mg/g)	M (mg/g)	MA (mg/g)	AA (mg/g)	Total (mg/g)
Asiaticoside-maximizing condition ¹					
Predicted values	10.13	11.74	4.63	2.67	29.16
Experimental values	10.30	11.94	4.70	2.72	29.66
% Error	1.67	1.68	1.66	2.03	1.70
Total triterpenoids-maximizing condition ²					
Predicted values	9.05	12.85	5.58	3.00	30.48
Experimental values	9.02	13.15	5.26	3.10	30.53
% Error	−0.40	2.33	−5.79	3.52	0.15

¹ 21.05% *w/w* ethanol, 34.68% *w/w* ammonium sulfate, and 55.46 °C; ² 18.29% *w/w* ethanol, 33.93% *w/w* ammonium sulfate, and 60.04 °C.

**Figure 6.** Predicted vs observed scatter.

3.5. Antioxidant Activity and Chlorophyll Intensity

The antioxidant activities of the *Centella asiatica* extracts from ATPS are shown in Table 9. The total triterpenoid-maximizing extract exhibited higher DPPH radical-scavenging activity ($66.02 \pm 1.38\%$) than the asiaticoside-maximizing extract ($59.12 \pm 0.33\%$). A similar trend was observed in the ABTS assay, with scavenging activities of $53.02 \pm 5.23\%$ and $48.82 \pm 6.20\%$, respectively. These results suggest that optimization toward total triterpenoid content may provide an extract with greater overall antioxidant capacity than optimization based solely on asiaticoside content. The absorbance values measured at 660 nm were low and comparable for the asiaticoside-maximizing (0.121 ± 0.002) and total triterpenoid-maximizing (0.114 ± 0.006) extracts, with the latter showing a slightly lower value.

Table 9. Antioxidant activities and absorbance at 660 nm of *Centella asiatica* extracts from ATPS methods.

Biological Activity	DPPH Scavenging Activity (%)	ABTS Scavenging Activity (%)	Absorbance at 660 nm *
Asiaticoside-maximizing extract	59.12 ± 0.33	48.82 ± 6.20	0.121 ± 0.002
Total triterpenoids-maximizing extract	66.02 ± 1.38	53.02 ± 5.23	0.114 ± 0.006
Gallic acid solution (100 µg/mL)	87.78 ± 0.63	97.39 ± 0.93	-

* The absorbances of chlorophyll in the extracts at 660 nm were subtracted from the absorbance of their solvent as a blank.

4. Discussion

The present study demonstrates the feasibility of using an ethanol–ammonium sulfate aqueous two-phase system (ATPS) as an integrated extraction and fractionation process for recovering the four principal triterpenoids from *Centella asiatica*. Unlike conventional hydroethanolic extraction, which commonly co-extracts chlorophyll and requires subsequent concentration, adsorption, or decolorization operations, the proposed ATPS simultaneously enhanced triterpenoid recovery and reduced pigment contamination. The combination of ATPS with CCD-RSM also enabled the extraction conditions to be adjusted according to the desired product profile, either asiaticoside or total triterpenoid-enriched extract.

The reliability of the extraction results was supported by the validated HPLC–UV method for simultaneous determination of madecassoside, asiaticoside, madecassic acid, and asiatic acid within 14 min. The use of a 2.7 µm superficially porous C18 column provided efficient separation despite the structural similarities between the two glycosides and their corresponding aglycones. The suitability of the chromatographic system was acceptable. In addition, correlation coefficients of at least 0.9995, recoveries of 98.42–101.52%, and precision values of 0.16–1.36% demonstrated that the method was sufficiently accurate and reproducible for monitoring changes caused by the extraction variables. The analytical performance of this work was comparable for linearity, accuracy, and precision but faster than the previous study that required 40 min of analysis time [11].

The extraction behavior observed in the present study can be explained by the combined effects of solvent polarity, salting-out, phase composition, mass transfer, and the physicochemical properties of the triterpenoids. Short-chain alcohol and salt in ATPSs are formed because strongly hydrated salt ions compete with alcohol molecules for water, resulting in the separation of an alcohol-rich upper phase and a salt-rich lower phase [20,21]. Ammonium sulfate has a strong salting-out capacity and can promote the transfer of organic solutes from the salt-rich phase into the ethanol-rich phase. However, the extent of this transfer depends on the polarity and hydrogen-bonding capacity of each solute, as well as the ethanol concentration, salt concentration, temperature, and relative volumes of the two phases.

Ethanol concentration was the most influential linear factor for both asiaticoside and total triterpenoid recovery. Moderate ethanol concentrations can improve wetting of the powdered plant material, facilitate penetration of the solvent into the cellular matrix, and increase the solubility of amphiphilic triterpenoids. Nevertheless, increasing the ethanol concentration beyond the optimal region reduced the total triterpenoid content. For example, the CCD run performed at the highest ethanol level produced only 21.84 mg/g total triterpenoids, whereas the lowest ethanol axial condition produced 30.86 mg/g. This result indicates that increasing solvent strength alone does not necessarily improve recovery in an ATPS. Excessive ethanol may alter phase polarity, phase–volume ratio, water activity, and the distribution coefficients of the analytes, thereby moving the system away from the most selective phase composition. It may also increase the extraction of competing plant constituents, which can affect triterpenoid partitioning. Similar composition-dependent

behavior has been described for short-chain alcohol–salt systems used to extract plant bioactive compounds [28,29].

Ammonium sulfate produced a nonlinear effect on the responses. At an appropriate concentration, the salt promoted phase formation and enhanced the transfer of triterpenoids into the ethanol-rich phase through salting-out. In contrast, excessive salt concentration did not further improve recovery and was associated with increased chlorophyll absorbance in some experimental runs. Very high ionic strength may decrease water availability, modify the relative volumes and compositions of the phases, and increase solution viscosity, thereby restricting solid–liquid mass transfer. These competing effects explain the significant negative quadratic term for ammonium sulfate and the occurrence of an optimum within the investigated range rather than at the highest salt concentration.

Temperature similarly exhibited a quadratic relationship with extraction efficiency. Increasing temperature generally decreases solvent viscosity and improves diffusion, solute solubility, and penetration of the extraction medium into plant particles. These effects likely contributed to higher total triterpenoid content observed at intermediate-to-high temperatures. However, further increases in temperature may alter phase equilibrium and reduce favorable partitioning between the salt-rich and alcohol-rich phases. Elevated temperature may also increase the extraction of interfering matrix components or affect the stability of susceptible constituents [31,32]. Therefore, the optimal temperatures of approximately 55–60 °C represent a balance between enhanced mass transfer and favorable phase partitioning.

The predominance of madecassoside and asiaticoside in the collected phase is consistent with their structural characteristics. Madecassoside and asiaticoside contain trisaccharide groups that increase their polarity and hydrogen-bonding capacity compared with the corresponding aglycones, madecassic acid and asiatic acid [33,34]. These glycosides therefore exhibit favorable interactions with the polar ethanol–water phase. Conversely, the more hydrophobic aglycones may be more sensitive to relatively small changes in ethanol concentration, ionic strength, temperature, and phase ratio. This difference may also explain why madecassic acid showed the largest prediction error at the total-triterpenoid optimum, whereas the predicted and experimental concentrations of the glycosides were in closer agreement.

ATPS composition can be used to control extract selectivity. The asiaticoside-maximizing condition, consisting of approximately 21.05% *w/w* ethanol, 34.68% *w/w* ammonium sulfate, and 55.46 °C, produced 10.30 mg/g asiaticoside. In contrast, 18.29% *w/w* ethanol, 33.93% *w/w* ammonium sulfate, and increasing the temperature to 60.04 °C produced 30.53 mg/g total triterpenoids. The increase in total triterpenoid content was primarily associated with improved madecassoside and madecassic acid recovery rather than an increase in asiaticoside alone. Thus, relatively small adjustments in phase composition and temperature changed the relative recovery of individual triterpenoids. This process flexibility could be valuable industrially because different standardized *Centella asiatica* extracts may be required for different pharmaceutical, cosmeceutical, or nutraceutical applications.

The optimized total-triterpenoid ATPS substantially outperformed conventional extraction with 70% ethanol. Based on the experimental concentrations, the ATPS increased the total triterpenoid content from 19.42 to 30.53 mg/g, corresponding to an improvement of approximately 57%. The concentrations of asiaticoside, madecassoside, madecassic acid, and asiatic acid were approximately 107%, 21%, 134%, and 62% higher, respectively, than those obtained using 70% ethanol. These results indicate that the ATPS did not merely increase the recovery of one marker compound but enhanced the overall recovery of both glycosylated and aglycone triterpenoids. The antioxidant activities of the extracts under the optimized conditions showed that the total triterpenoid-maximizing extract exhibited

higher DPPH and ABTS radical-scavenging activities than the asiaticoside-maximizing extract, suggesting that overall antioxidant activity may be related to total triterpenoids and influenced by the combined contribution of multiple triterpenoids.

The quadratic models showed high coefficients of determination for asiaticoside and total triterpenoid content, indicating that most of the observed response variability was explained by the selected factors. The total-triterpenoid model showed particularly good agreement among its R^2 , adjusted R^2 , and predicted R^2 values. The asiaticoside model had a lower predicted R^2 , indicating more moderate predictive performance across the entire design space. Nevertheless, confirmatory experiments demonstrated close agreement between predicted and observed values at both optimized conditions. Mean absolute prediction errors were approximately 1.8% for the asiaticoside-focused condition and 2.4% for the total-triterpenoid condition.

An additional advantage of the ATPS was its lower chlorophyll co-extraction. The absorbance at 660 nm of the conventional 70% ethanol extract was 0.615, whereas values for the ATPS experiments ranged from 0.085 to 0.458. Thus, all investigated ATPS conditions produced lower chlorophyll-related absorbance than the conventional solvent, with reductions ranging from approximately 26% to 86%. The reduced coloration is likely attributable to the lower ethanol concentration and selective distribution or exclusion of chlorophyll and pigment-associated matrix components during phase formation. Chlorophyll possesses a highly hydrophobic phytol chain, and its distribution may differ markedly from that of the more polar triterpenoid glycosides. The phase-separation step therefore functions as partial in-line purification and may decrease the need for activated carbon treatment or adsorption-resin purification, both of which can cause product loss and increase processing costs. The observed reduction in pigment co-extraction is consistent with the development of colorless *Centella asiatica* extracts using selective solvent systems such as natural deep eutectic solvent–microwave-assisted extraction (NADES-MAE) [13].

Compared with previous extraction studies, the optimized ATPS in this work delivered asiaticoside of 10.30 mg/g and total triterpenoid content of 30.53 mg/g at the experimental maximizing conditions (Table 10). The yields fall within the reported range for assisted extraction techniques such as (NADES-MAE) [13] and ultrasound-assisted extraction (UAE) under optimized conditions [16]. The ATPS-RSM approach combines respectable yields with pronounced process advantages: single-step phase separation that acts as partial in-line purification, markedly reduced volatile solvent usage compared with other methods, and straightforward scale-up using standard phase-separation equipment. By contrast, NADES can achieve very high target concentrations but requires custom natural deep eutectic solvent formulation and additional consideration for solvent removal; solvent-free microwave extraction (SFME) can reduce volatile organic compound usage but needs specialized reactors/pressure control. Thus, although absolute yields may be slightly higher in some NADES or MAE reports, ATPS-RSM provides a balanced combination of yield, green metrics and industrial applicability.

Table 10. Comparison of selected extraction methods for *Centella asiatica*.

Method	Solvent	Condition	Yield	Advantages
ATPS (This work)	Ethanol 21.05% w/w; ammonium sulfate 34.68% w/w	90 min stirring; Temp 55.46 °C	A $\approx$ 10.30 mg/g	Single-step partitioning; low ethanol fraction; tunable selectivity; scalable; reduced pigment contamination
	Ethanol 18.29% w/w; ammonium sulfate 33.93% w/w	90 min stirring; Temp 60.04 °C	TTC $\approx$ 30.53 mg/g	

Table 10. Cont.

Method	Solvent	Condition	Yield	Advantages
NADES-MAE [13]	Acetylcholine chloride: malic acid: water (1:2:2)	MAE with RSM (ratio, microwave power, irradiation time)	M $\approx$ 21.7 mg/g A $\approx$ 12.7 mg/g	High yields; colorless extracts; requires microwave and NADES design
UAE [15]	35% aqueous ethanol	Solid:solvent ratio 1:15; sonication 18 min; optimized using RSM and artificial neural network (ANN)	A $\approx$ 0.198% w/w	Fast extraction; solvent-efficient; ANN improved predictive modeling
UAE [16]	Methanol	Ultrasonic bath, 40 kHz, 250 W, 25 °C, 15 min; solid-to-solvent ratio 2 g:50 mL	M $\approx$ 8.21 mg/g A $\approx$ 7.82 mg/g MA $\approx$ 4.44 mg/g AA $\approx$ 3.38 mg/g	High triterpenoid yields; rapid; requires solvent cleanup
SFME [35]	No solvent	Microwave and vacuum	A $\approx$ 158.8 μ g/mL	Solvent-free (green); concentrated extract; specialized equipment required
Maceration/Soxhlet [36,37]	Ethanol, methanol, water, or aqueous alcohol mixtures	Maceration: typically several hours to days at room or moderately elevated temperature; Soxhlet: 6–8 h under solvent reflux	Extraction efficiency depends strongly on solvent composition, temperature, and duration; generally longer processing times than UAE/MAE	Simple and widely accessible equipment; however, conventional methods generally require longer extraction times and relatively large solvent volumes

The present study was conducted using one plant material source and at laboratory scale. Natural variation in cultivar, geographical origin, harvesting season, and drying conditions may influence the triterpenoid profile and extraction behavior [38]. Future studies should therefore evaluate multiple batches and establish process robustness against raw-material variability. Determination of phase volumes, partition coefficients, mass balances, overall extraction recoveries, residual ammonium sulfate, and solvent-recycling efficiency would provide further mechanistic and process-development information. Confirmatory measurement of chlorophyll or instrumental color parameters at both optimized conditions would also strengthen the evidence for simultaneous extraction and reduced pigment contamination. Finally, pilot-scale studies using continuous or semicontinuous mixing and phase separation are required to establish scalability and the economic feasibility of producing standardized triterpenoid-rich *Centella asiatica* extracts.

5. Conclusions

This work demonstrates that an ethanol/ammonium sulfate aqueous two-phase system (ATPS) is an effective platform for the eco-friendly extraction, selective enrichment of triterpenoids and reduced co-pigmentation from *Centella asiatica*. Validated HPLC–UV analysis enabled rapid and reliable quantification of madecassoside, asiaticoside, madecassic acid, and asiatic acid. Partition behavior of ATPS was primarily governed by ethanol polarity and salt-induced salting-out effects, enabling controllable selectivity through adjustment of phase composition and temperature. The ATPS–RSM approach offers a balanced combination of competitive yield, reduced co-pigmentation, mild operating conditions, and potential for scale-up in the production of low-pigment triterpenoid-rich *Centella asiatica* extracts for pharmaceutical and cosmeceutical applications.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/pr14182873/s1>, Figure S1: 3D response surface plots for asiaticoside; Figure S2: 3D response surface plots for total triterpenoid content; Figure S3: Optimization and validation of asiaticoside; Figure S4: Optimization and validation of total triterpenoid content.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Definition
A	Asiaticoside
AA	Asiatic acid
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATPS	Aqueous two-phase system
CCD	Central composite design
CV	Coefficient of variation
DPPH	2,2-Diphenyl-1-picrylhydrazyl
HPLC–UV	High-performance liquid chromatography with ultraviolet detection
LOD	Limit of detection
LOQ	Limit of quantification
M	Madecassoside
MA	Madecassic acid
MAE	Microwave-assisted extraction
NADES	Natural deep eutectic solvent
PEG	Polyethylene glycol
R ²	Coefficient of determination
RSD	Relative standard deviation
RSM	Response surface methodology
SD	Standard deviation
SFME	Solvent-free microwave extraction
TTC	Total triterpenoid content
UAE	Ultrasound-assisted extraction

References

1. Gohil, K.J.; Patel, J.A.; Gajjar, A.K. Pharmacological review on *Centella asiatica*: A potential herbal cure-all. *Indian J. Pharm. Sci.* **2010**, *72*, 546–556.
2. Razzaq, W.; Mazzitelli, J.B.; Fabiano-Tixier, A.S.; Vian, M.A. *Centella asiatica* as a Model Biomass for Sustainable Production of Biochemicals via Green Extraction and Purification Technologies: A Comprehensive Field-to-Market Review. *Molecules* **2026**, *31*, 526. [[CrossRef](#)]

3. Kandasamy, A.; Aruchamy, K.; Rangasamy, P.; Varadhaiyan, D.; Gowri, C.; Oh, T.H.; Ramasundaram, S.; Athinarayanan, B. Phytochemical analysis and antioxidant activity of *Centella asiatica* extracts: An experimental and theoretical investigation of flavonoids. *Plants* **2023**, *12*, 3547. [\[CrossRef\]](#)
4. Bylka, W.; Znajdek-Awizeń, P.; Studzińska-Sroka, E.; Dańczak-Pazdrowska, A.; Brzezińska, M. *Centella asiatica* in Dermatology: An Overview. *Phytother. Res.* **2014**, *28*, 1117–1124. [\[CrossRef\]](#)
5. Orhan, I.E. *Centella asiatica* (L.) Urban from traditional medicine to modern medicine with neuroprotective potential. *Evid. Based Complement. Altern. Med.* **2012**, *2012*, 946259. [\[CrossRef\]](#)
6. Gray, N.E.; Alcazar Magana, A.A.; Lak, P.; Wright, K.M.; Quinn, J.; Stevens, J.F.; Maier, C.S.; Soumyanath, A. *Centella asiatica*: Phytochemistry and mechanisms of neuroprotection and cognitive enhancement. *Phytochem. Rev.* **2018**, *17*, 161–194. [\[CrossRef\]](#)
7. Brinkhaus, B.; Lindner, M.; Schuppan, D.; Hahn, E.G. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. *Phytomedicine* **2000**, *7*, 427–448. [\[CrossRef\]](#)
8. Dubery, J.T.J.; Urban, I.A. Pentacyclic Triterpenoids from the Medicinal Herb, *Centella asiatica* (L.) Urban. *Molecules* **2009**, *14*, 3922–3941. [\[CrossRef\]](#)
9. Razif, R.; Mohd Fadzi, N.I.; Ahmad, H.; Looi Qi Hao, D.; Maarof, M.; Busra Fauzi, M. Asiaticoside-Loaded Multifunctional Bioscaffolds for Enhanced Hyperglycemic Wound Healing. *Biomedicines* **2025**, *13*, 277. [\[CrossRef\]](#)
10. Yulianti, L.; Meiyanto, E.; Bramono, K.; Freisleben, H.J. Asiaticoside induces cell proliferation and collagen synthesis in human dermal fibroblasts. *Univ. Med.* **2015**, *34*, 112–120. [\[CrossRef\]](#)
11. Rafi, M.; Handayani, F.; Darusman, L.K.; Rohaeti, E.; Wahyu, Y.; Sulistiyan; Honda, K.; Putri, S.P. A combination of simultaneous quantification of four triterpenes and fingerprint analysis using HPLC for rapid identification of *Centella asiatica* from its related plants and classification based on cultivation ages. *Ind. Crops Prod.* **2018**, *122*, 93–97. [\[CrossRef\]](#)
12. Ruksiriwanich, W.; Khantham, C.; Sringarm, K.; Sommano, S.; Jantrawut, P. Depigmented *Centella asiatica* extraction by pretreated with supercritical carbon dioxide fluid for wound healing application. *Processes* **2020**, *8*, 277. [\[CrossRef\]](#)
13. Phaisan, S.; Makkliang, F.; Putalun, W.; Sakamoto, S.; Yusakul, G. Development of a colorless *Centella asiatica* (L.) Urb. extract using a natural deep eutectic solvent (NADES) and microwave-assisted extraction (MAE) optimized by response surface methodology. *RSC Adv.* **2021**, *11*, 8741–8750. [\[CrossRef\]](#)
14. Yingngam, B.; Brantner, A.H. Modeling and optimization of microwave-assisted extraction of pentacyclic triterpenes from *Centella asiatica* leaves using response surface methodology. *Ind. Crops Prod.* **2020**, *147*, 112261. [\[CrossRef\]](#)
15. Kumari, P.; Kumar, P.; Kumar, V.; Pandey, B.; Nazir, R.; Katoch, K.; Dwivedi, P.; Dey, A.; Pandey, D.K. Response surface methodology and artificial neural network modeling for optimization of ultrasound-assisted extraction and rapid HPTLC analysis of asiaticoside from *Centella asiatica*. *Ind. Crops Prod.* **2022**, *176*, 114304. [\[CrossRef\]](#)
16. Khursheed, T.; Khan, A.A.; Akhtar, M.N.; Khalid, A.; Tariq, M.R.; Alsulami, T.; Mugabi, R.; Nayik, G.A. Ultrasound-assisted solvent extraction of phenolics, flavonoids, and major triterpenoids from *Centella asiatica* leaves: A comparative study. *Ultrason. Sonochem.* **2024**, *111*, 106981. [\[CrossRef\]](#)
17. Hein, Z.M.; Gopalakrishna, P.K.; Kanuri, A.K.; Thomas, W.; Hussan, F.; Naik, V.R.; Shantakumari, N.; Che Ramli, M.D.; Mohd Moklas, M.A.; Che Mohd Nassir, C.M.N.; et al. *Centella asiatica*: Advances in extraction technologies, phytochemistry, and therapeutic applications. *Life* **2025**, *15*, 1081. [\[CrossRef\]](#)
18. Plyduang, T.; Monton, C.; Suksaeree, J. Sustainable green extraction approaches for herbal phytochemicals supporting environmentally friendly and climate-responsive product development. *Sustain. Chem. Clim. Action* **2026**, *8*, 100184. [\[CrossRef\]](#)
19. Rosa, P.A.J.; Azevedo, A.M.; Sommerfeld, S.; Bäcker, W.; Aires-Barros, M.R. Aqueous two-phase extraction as a platform in the biomanufacturing industry: Economical and environmental sustainability. *Biotechnol. Adv.* **2011**, *29*, 559–567. [\[CrossRef\]](#)
20. Zhang, Y.; Li, Y.; Zhao, J.; Zheng, W.; Zhan, J.; Zheng, H.; Luo, F. Emerging delivery systems based on aqueous two-phase systems: A review. *Acta Pharm. Sin. B* **2024**, *14*, 110–132. [\[CrossRef\]](#)
21. Iqbal, M.; Tao, Y.; Xie, S.; Zhu, Y.; Chen, D.; Wang, X.; Huang, L.; Peng, D.; Sattar, A.; Shabbir, M.A.B.; et al. Aqueous two-phase system (ATPS): An overview and advances in its applications. *Biol. Proced. Online* **2016**, *18*, 18. [\[CrossRef\]](#)
22. Kim, S.B.; Bisson, J.; Friesen, J.B.; Pauli, G.F.; Simmler, C. Selective chlorophyll removal method to “degreen” botanical extracts. *J. Nat. Prod.* **2020**, *83*, 1846–1858. [\[CrossRef\]](#)
23. Department of Medical Sciences, Ministry of Public Health. *Thai Herbal Pharmacopoeia 2021*; Department of Medical Sciences, Ministry of Public Health: Nonthaburi, Thailand, 2021.
24. Rafamantanana, M.H.; Rozet, R.; Raelison, G.E.; Cheuk, K.; Ratsimamanga, S.U.; Hubert, P.; Quetin-Leclercq, J. An improved HPLC-UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of *Centella asiatica* (L.) Urb (APIACEAE). *J. Chromatogr. B.* **2009**, *877*, 2396–2402. [\[CrossRef\]](#)
25. AOAC INTERNATIONAL. Appendix K: Guidelines for dietary supplements and botanicals. In *Official Methods of Analysis of AOAC INTERNATIONAL*, 22nd ed.; Latimer, G.W., Jr., Ed.; AOAC INTERNATIONAL: Rockville, MD, USA, 2023.
26. Somadasan, S.; Shaji, G.; Athisayaraj, M.S.; Sukumaran, S.K. Central Composite Design: An Optimization Tool for Developing Pharmaceutical Formulations. *J. Young Pharm.* **2024**, *16*, 400–409. [\[CrossRef\]](#)

27. Chen, X.; Peng, Y.; Wang, X.; Zhou, W.; Jiang, L. Response Surface Methodology—Central Composite Design Optimization Sugarcane Bagasse Activated Carbon under Varying Microwave-Assisted Pyrolysis Conditions. *Processes* **2024**, *12*, 497. [\[CrossRef\]](#)
28. Wang, Y.; Wang, S.; Liu, L. Recovery of natural active molecules using aqueous two-phase systems comprising of ionic liquids/deep eutectic solvents. *Green Chem. Eng.* **2022**, *3*, 5–14. [\[CrossRef\]](#)
29. Xi, J.; Zhang, X.; Wang, Y.; Wei, S. Short-chain alcohol–salt-based aqueous two-phase system as a novel solvent for extraction of plant active ingredients: A review. *Trends Food Sci. Technol.* **2023**, *138*, 74–84. [\[CrossRef\]](#)
30. Arbianti, R.; Lesmana, L.P.; Fadli, F.; Larasati, A.; Utami, T.S.; Muharam, Y.; Slamet. The effects of aqueous two-phase system on the enrichment of quercetin from *Strobilanthes crispus* L. In *Proceedings of the 5th International Tropical Renewable Energy Conference, Online, 28–29 October 2020*; AIP Publishing: Melville, NY, USA, 2021; Volume 2344, p. 020015.
31. Spigno, G.; Tramelli, L.; De Faveri, D.M. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *J. Food Eng.* **2007**, *81*, 200–208. [\[CrossRef\]](#)
32. Olugbami, B.D.; Kavaz, D. Effect of extraction temperature and solvent type on the bioactive potential of *Ocimum gratissimum* L. extracts. *Sci. Rep.* **2020**, *10*, 18540. [\[CrossRef\]](#)
33. Azerad, R. Chemical structures, production and enzymatic transformations of saponin and saponins from *Centella asiatica* (L.) Urban. *Fitoterapia* **2016**, *114*, 168–187. [\[CrossRef\]](#)
34. Pillai, A.R.S.; Bhuyan, Y.K.; Roy, S. Extraction of Bioactive Compounds From *Centella asiatica* and Enlightenment of Its Utilization Into Food Packaging: A Review. *Int. J. Food Sci.* **2024**, *2024*, 8863214. [\[CrossRef\]](#)
35. Idris, F.N.; Mohd Nauman, M.; Abd Shukor, S.R. Optimization of solvent-free microwave extraction of essential oil from *Centella asiatica* using response surface methodology. *J. Appl. Res. Med. Aromat. Plants* **2021**, *22*, 100293.
36. Idris, F.N.; Nadzir, M.M. Comparative studies on different extraction methods of *Centella asiatica* and extracts bioactive compounds effects on antimicrobial activities. *Antibiotics* **2021**, *10*, 457. [\[CrossRef\]](#)
37. Sabaragamuwa, R.; Perera, C.O. Total triterpenes, polyphenols, flavonoids, and antioxidant activity of bioactive phytochemicals of *Centella asiatica* by different extraction techniques. *Foods* **2023**, *12*, 3972. [\[CrossRef\]](#)
38. Panichayupakaranant, P.; Gritsanapan, W. Factors affecting the content of pentacyclic triterpene acids in *Centella asiatica* raw materials. *Pharm. Biol.* **2012**, *50*, 1508–1512.

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