TRENDS IN SCIENCES 2026; 23(3): 11303
https://doi.org/10.48048/tis.2026.11303 RESEARCH ARTICLE

Homogenization-Assisted Extraction of Antioxidants from *Centella asiatica* Using a Natural Deep Eutectic Solvent (NADES): Optimization and Evaluation

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Received: 18 July 2025, Revised: 26 August 2025, Accepted: 1 October 2025, Published: 1 December 2025

Abstract

Centella asiatica (L.) Urban is a valued medicinal herb rich in antioxidant compounds, notably phenolics and flavonoids. This study aimed to develop a green and effective method for extracting these bioactives using natural deep eutectic solvents (NADES) coupled with homogenization-assisted extraction (HAE). Twelve NADES systems were screened, and choline chloride:glycerol (1:2) demonstrated the highest efficiency due to its moderate viscosity and strong solvation capacity. A Box-Behnken Design combined with response surface methodology was employed to optimize 4 key factors: Liquid-to-solid ratio (30 - 70 mL/g), solvent concentration (10% - 30%), homogenization speed (5,000 - 15,000 rpm), and extraction time (10 - 30 min). Optimal conditions (50 mL/g, 20% solvent, 10,000 rpm, 20 min) achieved a 31.79% extraction yield, 66.91 mg GAE/g of total phenolic content, 45.13 mg QE/g of total flavonoid content, and 80.72% DPPH radical scavenging activity. Statistical models exhibited strong predictive power ($R^2 > 0.91$), validating the process efficiency. The integration of NADES and HAE provides a scalable, solvent-free alternative to conventional extraction, supporting sustainable production of antioxidant-rich extracts. This method holds promise for applications in functional foods, pharmaceuticals, and cosmetics where clean-label, eco-friendly ingredients are in demand.

Keywords: *Centella asiatica*, Natural deep eutectic solvents (NADES), Homogenization-assisted extraction (HAE), Box-Behnken design, Green extraction, Antioxidant capacity

Introduction

Centella asiatica (L.) Urban, commonly known as Gotu kola, is a medicinal plant broadly distributed in Southeast Asia and traditionally utilized for wound healing, cognitive enhancement, and anti-inflammatory purposes [1,2]. Phytochemical investigations have revealed that the pharmacological efficacy of *C. asiatica* arises from a complex array of bioactive constituents, notably triterpenoid saponins (asiaticoside, madecassoside), phenolic acids, and flavonoids [3,4]. Relative to commonly studied herbs such as *Rosmarinus officinalis* [1], *Thymus serpyllum* L [2] and *Origanum vulgare* [3] as investigated in prior researchs, while these common herbs exhibit high total phenolic contents or radical scavenging capacity, *C. asiatica* remains distinctive due to its broad-spectrum bioactivity and the presence of unique triterpenoid compounds. Reported total phenolic contents in *C. asiatica* extracts (20 - 35 mg GAE/g DW) are comparable to or higher than many culinary herbs, while its triterpenoid concentration (up to 8% DW) is substantially greater, reinforcing its selection as a superior source of multifunctional phytochemicals. These compounds have drawn increasing interest from the food, pharmaceutical, and cosmeceutical industries due to their proven antioxidant, antimicrobial, neuroprotective, and dermo-regenerative activities [5-7]. Furthermore, they are particularly notable for their anti-inflammatory, wound healing and neuroprotective properties in attenuating spinal cord injury-activities not commonly found in typical phenolic-rich herbs. However, the recovery of these compounds from plant matrices presents a significant challenge. Conventional extraction methods typically employ organic solvents (ethanol, methanol, acetone) under heat or ultrasound conditions, which, despite their effectiveness, are associated with environmental concerns, solvent toxicity, energy consumption, and limited selectivity for target compounds [8-10]. Moreover, high temperatures or prolonged extraction durations may lead to the degradation of thermolabile phytochemicals, particularly phenolic and flavonoid contents [11].

In response to these limitations, natural deep eutectic solvents (NADES) have emerged as promising green extraction media [12,13]. NADES are designer solvents formed by mixing hydrogen bond donors and acceptors, such as choline chloride with glycerol, lactic acid, or sugars, which upon mixing form a eutectic system stabilized by extensive hydrogen bonding. These solvents possess green characteristics such as biodegradable, non-volatile, non-flammable, and ability to be tailored to achieve a wide range of polarities [14-16]. More importantly, NADES have demonstrated remarkable efficacy in extracting phenolic compounds and stabilizing them against oxidation and degradation [17,18]. Recent studies have highlighted the advantages of NADES over conventional solvents in recovering phytochemicals from *Rosmarinus officinalis* [19], *Camellia sinensis* [20], and *Glycyrrhiza glabra* [21].

To further enhance extraction efficiency, homogenization-assisted extraction (HAE) has gained attention as a physical intensification method. HAE applies strong mechanical shear forces that rupture plant cell walls, promote solvent penetration, and facilitate the release of intracellular contents [22]. Compared to traditional solid-liquid extraction or Soxhlet extraction, HAE enables shorter extraction times, lower temperatures, and higher yields without extensive degradation of target compounds [23]. When combined with NADES, HAE offers a sustainable, solvent-efficient, and thermally mild platform for phytochemical extraction, although such integration remains relatively underexplored in the literature. Few studies to date have focused on the NADES-HAE approach for *Centella asiatica*. While prior works have demonstrated the potential of NADES for static maceration or ultrasound-assisted extraction from *C. asiatica* [24-26], the synergistic effect of homogenization parameters (e.g., homogenization speed, which indirectly determines shear rate, and extraction time) with NADES composition has not been fully investigated. In this study, shear rate was not directly measured but considered indirectly via homogenization speed (rpm), which controls the rotor tip velocity and thus the effective shear forces in the extraction medium. Importantly, multivariate optimization techniques, such as response surface methodology (RSM) using Box-Behnken design, are essential to understand the interactive effects between operational variables, such as liquid-to-solid ratio, NADES concentration, homogenization speed, and extraction time, on extraction performance [25]. These 4 variables were selected based on preliminary single-factor screening and supported by recent studies. The liquid-to-solid ratio directly influences solvent penetration and mass transfer gradients [27], while the level of NADES dilution (water content) modulates viscosity and solvation capacity - both critical for efficient recovery of phenolics and flavonoids [28]. Homogenization speed determines the intensity of shear force applied for cell disruption, thereby affecting the release of intracellular compounds, whereas extraction time governs diffusion efficiency and simultaneously limits possible degradation of thermo- or shear-sensitive constituents [29].

The Box-Behnken design (BBD) is particularly suitable because it minimizes the number of experimental runs while efficiently estimating linear, quadratic, and interaction effects without requiring extreme factor levels [27]. In natural product research, design of experiments (DoE) approaches such as BBD have increasingly been applied to optimize solvent systems and process variables, offering reproducible and statistically robust conditions for maximizing the recovery of phenolic and flavonoid compounds [30]. Therefore, applying BBD in this study enables not only the determination of significant variables but also the visualization of their interactions, leading to a more precise and efficient optimization strategy for NADES-based extraction of *Centella asiatica*. These variables jointly determine the efficacy of compound recovery, solvent penetration, and energy efficiency, particularly when working with viscous, non-volatile solvents like NADES [31].

Therefore, the present study aims to optimize a homogenization-assisted extraction protocol using natural deep eutectic solvents for the green and efficient recovery of bioactive compounds from *Centella asiatica*. In particular, the study was to (i) screen various NADES formulations for extraction efficiency; (ii) evaluate the influence of key variables (liquid-to-solid ratio, solvent concentration, homogenization speed, and extraction time), and (iii) apply Box-Behnken Design and RSM to maximize extraction yield, total phenolic content, total flavonoid content, and antioxidant activity. This study provides new insights into process intensification for plant-based extractions and establishes a scalable, eco-friendly method for producing high-value phytochemical-rich extracts suitable for functional applications.

Materials and methods

Materials

Fresh *Centella asiatica* leaves were collected from Phuc An Ecolodge located at coordinates 10°34′51.7″N 106°20′36.9″E, in My An, Thu Thua district, Long An province, Vietnam. The leaves were thoroughly washed under running tap water to remove surface impurities, followed by rinsing with deionized water. They were then air-dried in a ventilated oven (Memmert UF110, Memmert GmbH, Germany) at 40 ± 2 °C until a constant weight was achieved. The dried leaves were milled using a laboratory-scale grinder (Retsch ZM 200, Retsch GmbH, Germany) equipped with stainless steel blades, and the ground material was passed through a 60-mesh stainless steel sieve using a mechanical sieve shaker (Retsch AS 200 basic, Retsch GmbH, Germany) with 60-mesh sieve (250 µm) to ensure uniform particle size. The sieved powder was stored in airtight, light-protected containers at ambient temperature until use.

Choline chloride (≥ 98%), glycerol (≥ 99%), lactic acid (≥ 85%), citric acid (≥ 99.5%), glucose (≥ 99%), Folin-Ciocalteu reagent, gallic acid (≥ 98%), aluminum chloride (≥ 98%), quercetin (≥ 98%), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (≥ 95%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents used in this study were of analytical grade and used without further purification.

Screening of natural deep eutectic solvents (NADES)

A total of twelve different natural deep eutectic solvents (NADES) were prepared by combining choline chloride (ChCl) as the hydrogen bond acceptor (HBA) with 4 selected hydrogen bond donors (HBDs): Glycerol, lactic acid, citric acid, and glucose, at varying molar ratios. The HBA:HBD ratios tested included 1:1, 1:2 and 2:1 for glycerol, lactic acid, and citric acid systems, and 1:1, 2:1 and 3:1 for the glucose-based system.

For each NADES system, the respective components were accurately weighed and mixed in clean glass beakers. The mixtures were then heated at 80 °C under continuous magnetic stirring for approximately 30 - 60 min until a clear and homogeneous liquid (or semi-solid phase, in the case of some sugar-based NADES) was formed. All prepared NADES were stored in sealed amber glass containers at room temperature (25 ± 2 °C), shielded from light until further use. The physicochemical properties of each NADES, including pH and viscosity, were measured at 25 ± 1 °C using a calibrated digital pH meter (Mettler Toledo SevenCompact™) and a Brookfield DV2T viscometer (Spindle no. 61). Each NADES formulation was coded and summarized as shown in **Table 1**.

Table 1 Composition and properties of NADES systems used in the preliminary screening.

Code	HBD	HBA:HBD Molar ratio	pH	Viscosity (mPa·s)	Appearance at 25 °C	TPC (mg GAE/g)	DPPH Inhibition (%)
NADES-1	Glycerol	1:1	5.82	187.2	Clear, moderately viscous liquid	61.8	82.2
NADES-2	Glycerol	1:2	5.42	123.5	Clear, low-viscosity liquid	68.25	87.45
NADES-3	Glycerol	2:1	6.08	234.6	Slightly turbid, sticky	53.25	78.9

Code	HBD	HBA:HBD Molar ratio	pH	Viscosity (mPa·s)	Appearance at 25 °C	TPC (mg GAE/g)	DPPH Inhibition (%)
NADES-4	Lactic acid	1:1	2.67	89.4	Clear, fluid	58.4	81.2
NADES-5	Lactic acid	1:2	2.21	74.6	Clear, low-viscosity liquid	63.1	83.7
NADES-6	Lactic acid	2:1	2.95	138.3	Clear, slightly viscous	49.85	76.9
NADES-7	Citric acid	1:1	2.12	245.8	Opaque, gel-like	45.6	74.3
NADES-8	Citric acid	1:2	1.96	198.1	Semi-gel, cloudy	50.1	75.65
NADES-9	Citric acid	2:1	2.45	310.2	Turbid, semi-solid	40.75	71.1
NADES-10	Glucose	1:1	4.18	367.2	Sticky, highly viscous	32.8	55.4
NADES-11	Glucose	2:1	4.39	425.7	Semi-solid, poorly flowing	29.45	52.1
NADES-12	Glucose	3:1	4.75	472.5	Solid-like at room temperature	25.35	48.8

Homogenization-assisted extraction

Extraction of bioactive compounds from *Centella asiatica* was carried out using homogenization-assisted extraction (HAE) with the prepared NADES. For each extraction, 1.0 g of dried *C. asiatica* powder was accurately weighed into a 50 mL centrifuge tube and mixed with the NADES at a predetermined liquid-to-solid (L/S) ratio (30 - 70 mL/g). The NADES solution used was diluted with distilled water corresponding to 10% - 30% of the NADES mass (w/w) to reduce viscosity and improve diffusivity. The mixture was homogenized using a high-speed homogenizer (Model D-500, Dlab Scientific, USA) at speeds ranging from 5,000 to 15,000 rpm for a fixed extraction time (10 - 30 min), depending on experimental conditions, equipped with a standard rotor–stator generator (diameter 10 mm) with the tip immersed approximately 1.5 cm below the liquid surface, and extractions were performed in 50 mL cylindrical glass beakers (internal diameter 40 mm) to ensure consistent shear conditions. During homogenization, samples were kept in an ice-water bath, and the extraction temperature was monitored with a digital thermometer to ensure it remained below 30 °C (typically 25 ± 2 °C), thereby avoiding excessive heating and degradation of thermolabile compounds.After homogenization, the extract was centrifuged at 6,000 rpm for 15 min at 4 °C, and the supernatant was collected and filtered through Whatman No. 1 filter paper. The clarified extract was then subjected to further analysis for determination of yield of extraction, total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity. All extractions were performed in triplicate, and results were reported as mean ± standard deviation.

Analytical methods for extract characterization

Yield of extraction

The extraction yield was determined using an ethanol-induced precipitation method to overcome the non-volatility of NADES [32,33]. Briefly, 5.0 mL of the NADES extract was mixed with 20.0 mL of absolute ethanol (1:4 v/v) to precipitate the solubilized compounds. The mixture was vortexed and allowed to stand at 4 °C for 12 h to ensure complete precipitation. The resulting suspension was centrifuged at 6,000 rpm for 15 min, and the solid precipitate was collected, dried in an oven at 50 °C to constant weight, and weighed. The extraction yield was calculated using the following equation:

$$Yield(\%) = \frac{Mass\ of\ dried\ precipitate}{Mass\ of\ dry\ plant\ material} \times 100 \qquad (1)$$

Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu colorimetric method with slight modifications. Briefly, 0.2 mL of diluted extract previously diluted with methanol was mixed with 1.0 mL of 10 %(v/v) Folin-Ciocalteu reagent, followed by the addition of 0.8 mL of 7.5 %(w/v) sodium carbonate. The mixture was vortexed and incubated in the dark at room temperature for 30 min. Absorbance was measured at 765 nm using a UV-vis spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, USA). Gallic acid was used as the standard, and results were expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW) [31].

Total Flavonoid Content (TFC)

TFC was measured using the aluminum chloride colorimetric method. In brief, 0.5 mL of extract diluted in methanol when necessary was mixed with 0.5 mL of 2 %(w/v) $AlCl_3$ in methanol and allowed to react for 30 min at room temperature in the dark. The absorbance was read at 420 nm. Quercetin was used as a reference standard, and TFC was expressed as mg quercetin equivalents per gram of dry weight (mg QE/g DW) [31].

DPPH radical scavenging activity

The antioxidant capacity of the extracts diluted in methanol when necessary was assessed using the DPPH free radical scavenging assay. A 0.1 mL aliquot of extract was added to 3.9 mL of 0.1 mM DPPH methanolic solution. The mixture was vortexed and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm, and radical scavenging activity was calculated as a percentage using the following equation [31]:

$$DPPH\ scavenging\ activity\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad (2)$$

where: $A_{control}$: The absorbance of the control solution; A_{sample}: The absorbance of the sample solution.

Experimental design

The Box-Behnken Design model (BBD) was used to optimize the extraction conditions. The 4 independent factors and their 3 levels in the Box-Behnken design are represented in **Table 2**. The 4 independent factors at 3 levels (−1, 0 and +1) for 29 experiments were chosen to quantify the response data. The conditional range of 4 independent factors (Liquid/Solid ratio, Homogenization speed and Extraction time) was selected from the results of 1-factor experiments. The condition amounts produced the best yield and DPPH radical scavenging activity, representing the proper condition (corresponding to 0 level). The boundary conditions were the lower and upper points of proper conditions, corresponding to the level of −1 and 1, respectively. A second-order polynomial model was employed to determine the correlation between response data and independent factors utilizing equation:

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X_i^2 + \sum_{i=1}^{k}\sum_{j=1}^{k} B_{ij} X_i X_j \qquad (3)$$

In this equation, B_0, B_i, B_{ii}, and B_{ij} were the regression coefficient for intercept, linear, quadratic, and interaction terms, respectively. X_i and X_j represented independent factor values, while k expressed the number of independent factors (k = 4). The 4 independent factors and their 3 levels were A, Liquid/solid ratio: 30, 50 and 70 (mL/g); B, NADES dilution level: 10, 20 and 30 %(v/v); C, Homogenization speed: 5,000, 10,000 and 15,000 (rpm); D, Extraction time: 10, 20 and 30 (min). Dependent responses (Y) were yield (%), total phenolic content (mg GAE/g DW), total flavonoid content (mg QE/g DW) and DPPH radical scavenging activity (%).

Numerical optimization was performed using the desirability function in Design-Expert 25.0.3, targeting maximization of yield, TPC, TFC, and DPPH. The regression models generated by BBD-RSM were further used for multi-response optimization. A numerical optimization procedure was performed in Design-Expert software (version 25.0.3, Stat-Ease Inc., Minneapolis, MN, USA) using the desirability function, where all responses (yield, TPC, TFC, and DPPH inhibition) were set to be maximized simultaneously. The predicted optimal conditions were then validated experimentally in triplicate, and the obtained values were compared with model predictions to evaluate accuracy and reliability.

Table 2 Experimental design factors and their levels.

Variables	Unit	The value of independent factors		
		Low (−1)	**Middle (0)**	**High (+1)**
A, Liquid/solid ratio (L/S)	mL/g	30	50	70
B, NADES dilution level	%, v/v	10	20	30
C, Homogenization speed	rpm	5,000	10,000	15,000
D, Extraction time	minute	10	20	30

Dependent variable	Constraints
Y_1 = Yield (%)	Maximize
Y_2 = Total phenolic content (mg GAE/g)	Maximize
Y_3 = Total flavonoid content (mg QE/g)	Maximize
Y_4 = DPPH radical scavenging activity	Maximize

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) in SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Tukey's post-hoc test was applied to determine significant differences between means, with a probability level of $p < 0.05$ considered statistically significant. All results are expressed as the mean ± standard deviation (SD) of 3 independent experimental replicates.

Prior to ANOVA, assumptions of normality and homogeneity of variance were verified using the Shapiro-Wilk and Levene's tests, respectively. For the response surface methodology (RSM), model adequacy was assessed through analysis of variance (ANOVA), including lack-of-fit testing and determination coefficients (R^2), and residual analysis was performed to confirm normal distribution and homoscedasticity.

Results and discussion

Screening of NADES

To identify the most suitable natural deep eutectic solvent (NADES) for extracting phenolic and flavonoid compounds from *Centella asiatica*, twelve ChCl-based NADES systems (NADES-1 to NADES-12) were systematically prepared by combining choline chloride with 4 hydrogen bond donors (HBDs): Glycerol, lactic acid, citric acid, and glucose at various molar ratios (**Table 1**). All systems were diluted with 20% (w/w) distilled water and tested under uniform homogenization-assisted extraction (HAE) conditions. The extraction performance of each NADES was evaluated based on total phenolic content (TPC, mg GAE/g) and DPPH radical scavenging activity (%). The results revealed pronounced differences across NADES systems, strongly influenced by both the chemical nature of the HBD and the HBA:HBD molar ratio.

Glycerol-based systems (NADES-1 to NADES-3) showed superior extraction performance compared to other HBD types. Among them, NADES-2 (ChCl:Glycerol, 1:2) delivered the best overall results, yielding 68.25 mg GAE/g TPC, and 87.45% DPPH inhibition. The high efficacy of this system is attributed to its moderate polarity, optimal hydrogen bonding network, and relatively low viscosity (123.5 mPa·s), which collectively promote solubilization and facilitate mass transfer during homogenization [27]. Increasing the glycerol content (1:2) has also been reported in previous studies to enhance phenolic recovery due to a favorable balance between fluidity and hydrogen-bond donor density [34,35]Lactic acid-based NADES (NADES-4 to NADES-6) also performed well, particularly NADES-5 (1:2), which had a low pH (2.21) and low viscosity (74.6 mPa.s), facilitating the release of phenolic acids. However, their highly acidic environment may destabilize certain flavonoid structures, as flavonoids generally contain hydroxyl groups with pKa values ranging from 6 to 10, making them prone to protonation and reduced resonance stabilization under strongly acidic conditions [36]. In addition, such protonation can in turn promote structural rearrangements or hydrolysis of glycosidic bonds, which have been reported to compromise flavonoid stability and assay response in low-pH NADES [37]. Citric acid-based systems (NADES-7 to NADES-9) showed moderate performance in antioxidant activity but generally underperformed in TPC and TFC extraction. Their poor efficiency can be attributed to high viscosities (198.1 - 310.2 mPa·s) and gel-like textures, impeded solvent penetration and effective disruption of plant cell walls during HAE [38].Moreover, their strong acidity (pH < 2.5) may have negatively affected flavonoid stability or caused aggregation of certain extractable compounds [39]. Glucose-based NADES (NADES-10 to NADES-12) consistently exhibited the lowest extraction efficiency across all metrics. These sugar-rich systems had the highest viscosities (> 360 mPa·s) and semi-solid to solid-like consistencies at room temperature, significantly limiting their practicality in shear-dependent processes like HAE [40]. Even with 20% water dilution, the high internal hydrogen bonding within glucose-based solvents likely restricted solvation capacity and mobility of target compounds. Taken together, these results indicate that viscosity alone can not fully account for the divergent performance of NADES. Instead, extraction outcomes reflect a combined influence of solvent polarity, acidity (pH/pKa), and solvation capacity, in addition to rheological properties. Notably, previous studies have shown that NADES with intermediate polarity and balanced hydrogen-bonding networks are particularly effective for polyphenol recovery [34,35] Likewise, the observation that ~20% (w/w) water dilution produced optimal performance agrees with reports that moderate water addition simultaneously reduces viscosity and fine-tunes polarity, whereas excessive water disrupts the eutectic network and decreases solvating power [41].

Interestingly, the HBA:HBD molar ratio had a non-linear impact across all HBD types. A 1:2 ratio often yielded the best performance (NADES-2 and NADES-5), likely due to an optimal balance between hydrogen bond donor density, fluidity, and polarity. In contrast, ratios of 2:1 or 3:1 (NADES-3, NADES-6, NADES-9, NADES-11 and NADES-12) tended to produce more rigid hydrogen-bonding networks with higher viscosities and less favorable polarity matching, which collectively reduced extraction efficacy. Therefore, the results demonstrate that both the choice of HBD and the molar ratio critically influence extraction efficiency. Among all tested formulations, NADES-2 (ChCl:Glycerol, 1:2) was selected as the optimal solvent for subsequent process optimization based on its superior extraction of phenolic antioxidants and favorable physicochemical properties. Notably, the extraction efficiency of NADES-2 (68.25 mg GAE/g) compares favorably with conventional organic solvents, as ethanol- and methanol-based extractions of *C. asiatica* have generally been reported in the range of 40 - 60 mg GAE/g under similar conditions [42,43]. This outcome indicates that NADES-2 not only outperformed the other NADES formulations in our screen but also rivaled, and in some cases exceeded, the performance of traditional solvents while offering the added benefit of green, non-volatile characteristics.

Factors affecting the extraction conditions

Effect of liquid-to-solid ratio

The liquid-to-solid (L/S) ratio plays a pivotal role in determining extraction efficiency by influencing mass transfer dynamics and solvent saturation. As illustrated in **Figure 1**, increasing the L/S ratio from 30 to 50 mL/g led to significant improvements in all measured parameters ($p < 0.05$), including extraction yield, total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity, as determined by ANOVA with Tukey's test. This enhancement can be attributed to improved solvent penetration and solute diffusion at moderate solvent volumes, which increase the concentration gradient between the plant matrix and the solvent and facilitate shear-mediated cell disruption under homogenization [27]. The optimal performance was observed at 50 mL/g, where the highest values were recorded: 14.79% yield, 70.73 mg GAE/g TPC, 43.32 mg QE/g TFC, and 90.41% DPPH inhibition. However, further increases to 60 and 70 mL/g resulted in slight declines across all responses, likely due to a dilution effect that reduced the concentration gradient between plant matrix and solvent, thereby diminishing the driving force for mass transfer [44,45]. Furthermore, turbulence-related inefficiencies may arise under high solvent volumes, disturbing shear distribution and lowering mixing efficiency during homogenization [46].

Additionally, an excessive volume of NADES may increase handling costs and reduce sustainability of the extraction process. Therefore, 50 mL/g was selected as the optimal liquid-to-solid ratio for subsequent extractions.

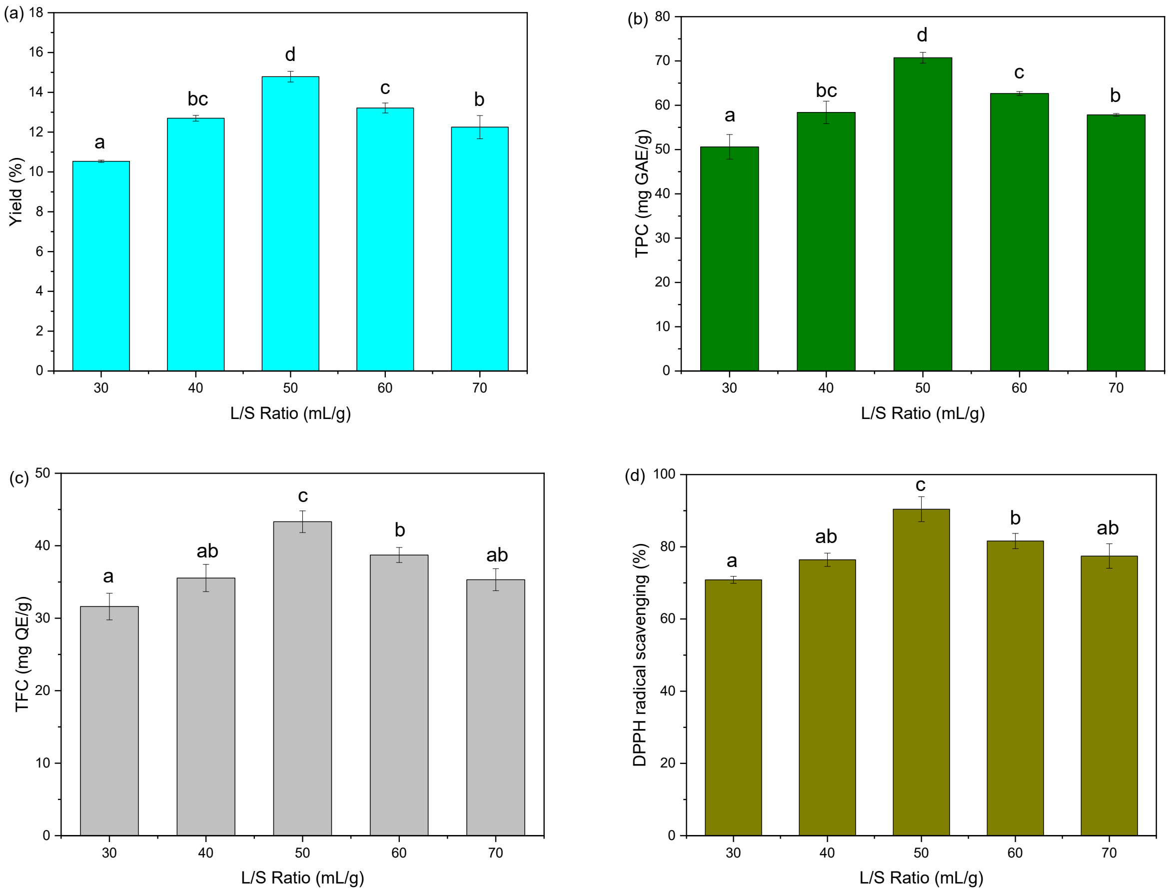


Figure 1 Effect of liquid-to-solid ratio on (a) extraction yield, (b) TPC, (c) TFC, and (d) DPPH radical scavenging activity.Values are expressed as mean ± SD (n = 3). Error bars represent the standard deviation (n = 3). Different letters indicate significant differences among samples according to Tukey's test ($p < 0.05$).

Effect of solvent concentration

Solvent concentration, referring to the percentage of distilled water added to the NADES system, had a notable impact on the extraction efficiency (**Figure 2**). Among the tested levels (10 - 30 %w/w), 20% dilution yielded the highest extraction yield (15.08%), total phenolic content (70.27 mg GAE/g), total flavonoid content (44.38 mg QE/g), and DPPH radical scavenging activity (89.54%). This suggests that moderate dilution effectively reduces the intrinsic viscosity of the NADES, improving solvent penetration into plant tissues and enhancing mass transfer of solutes. At lower dilution levels (10% - 15%), the high viscosity of the NADES restricted the effective formation of shear forces during homogenization, thereby reducing cell disruption and extractability. On the other hand, excessive dilution (25% - 30%) appeared to decrease the solvating power of the NADES system, likely due to disruption of the hydrogen bonding network and decreased polarity match with phenolic compounds, leading to reduced extraction performance [47,48]. The observed trend confirms that a critical balance between viscosity reduction and solvating capacity is necessary to maximize extraction efficiency. These findings are consistent with earlier studies that reported optimal water content between 20% - 30% in NADES-based extraction of phenolics [49]. As a result, 20% water dilution was selected as the optimal solvent concentration for further optimization studies.

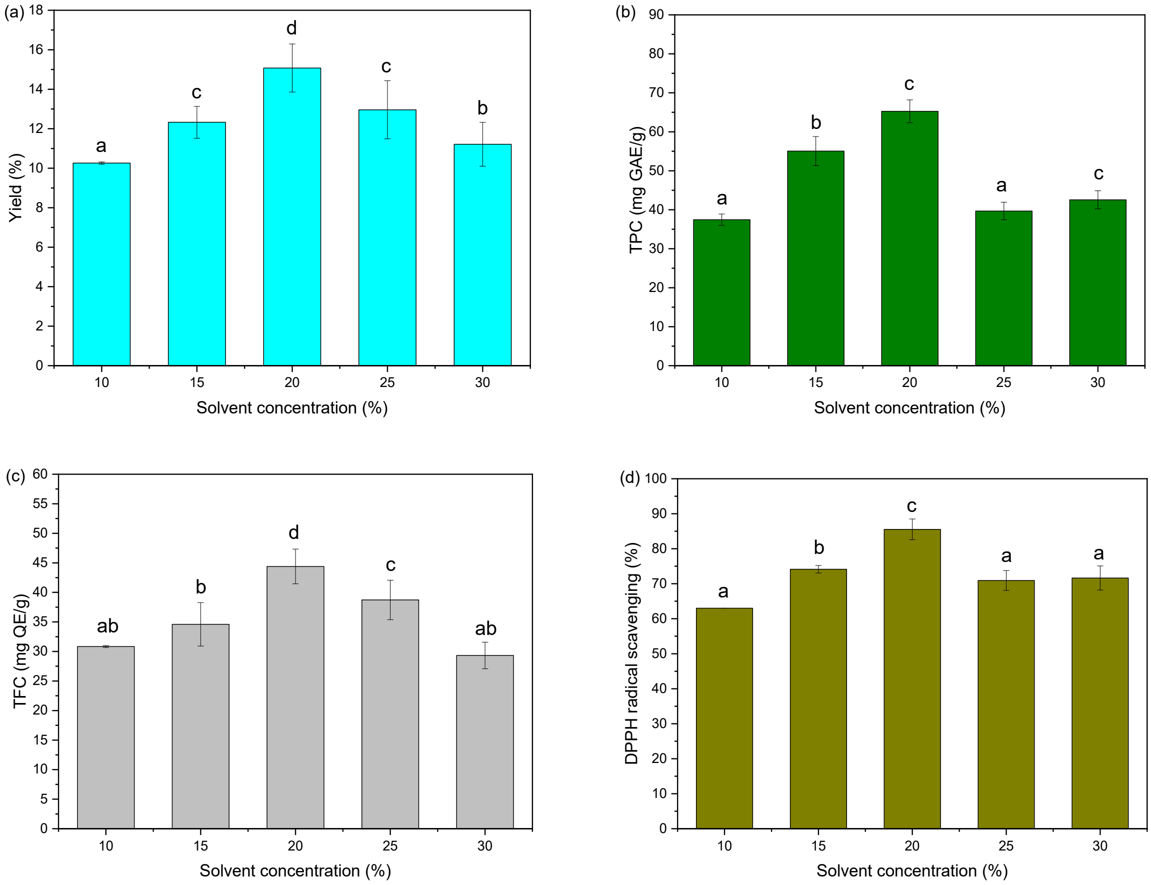


Figure 2 Effect of solvent concentration on (a) extraction yield, (b) TPC, (c) TFC, and (d) DPPH radical scavenging activity.Values are expressed as mean ± SD (n = 3). Error bars represent the standard deviation (n = 3). Different letters indicate significant differences among samples according to Tukey's test ($p < 0.05$).

Effect of homogenization speed

Homogenization speed critically affects the degree of cellular disruption and solute diffusion during extraction. As shown in **Figure 3**, increasing the speed from 5,000 to 10,000 rpm led to a substantial improvement in all response variables ($p < 0.05$). At 10,000 rpm, the extraction yield peaked at 15.21%, with TPC reaching 71.67 mg GAE/g, TFC at 45.25 mg QE/g, and DPPH radical scavenging activity at 91.70%. The improvement at moderate to high speeds can be attributed to enhanced shear forces, which promote more efficient breakdown of plant cell walls and release of intracellular compounds. However, further increasing the speed beyond 10,000 rpm did not improve results and even showed slight declines. This may be due to turbulent flow regimes or localized heating, which could damage sensitive bioactives or hinder uniform mixing [50,51]. Therefore, 10,000 rpm was identified as the optimal homogenization speed, striking a balance between effective physical disruption and preserving compound integrity. These results are in agreement with previous studies using homogenization-assisted extraction in NADES, where excessive speeds were shown to cause degradation or emulsification problems [52].

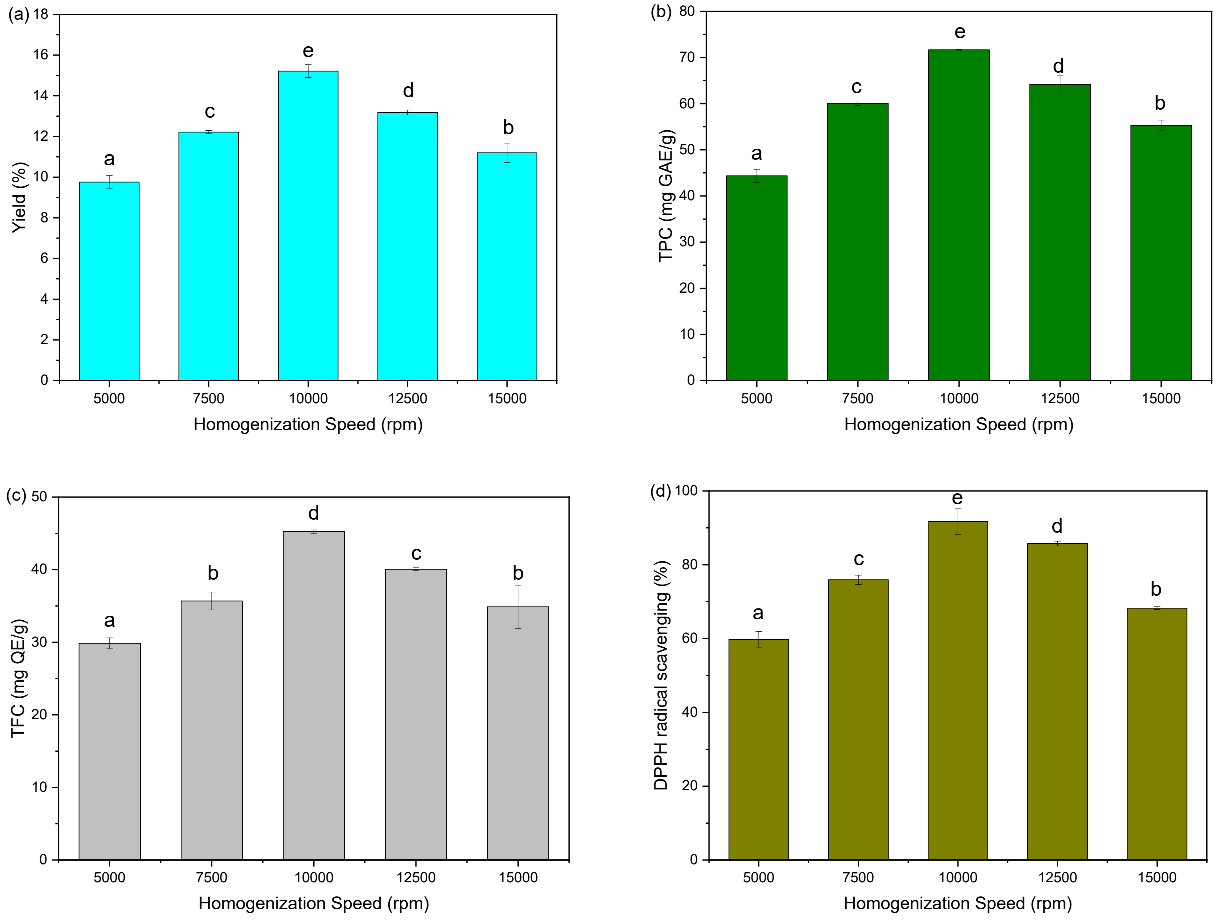


Figure 3 Effect of homogenization speed on (a) extraction yield, (b) TPC, (c) TFC, and (d) DPPH radical scavenging activityValues are expressed as mean ± SD (n = 3). Error bars represent the standard deviation (n = 3). Different letters indicate significant differences among samples according to Tukey's test ($p < 0.05$).

Effect of extraction time

Extraction time significantly influences the efficiency of solute transfer from plant matrices into solvents. As illustrated in **Figure 4**, extending the homogenization time from 10 to 20 minutes led to clear increases in extraction yield (15.02%), total phenolic content (70.95 mg GAE/g), total flavonoid content (45.89 mg QE/g), and DPPH inhibition (88.50%). This suggests that prolonged homogenization up to an optimal point enhances cellular rupture and facilitates diffusion of target compounds. However, further prolonging the extraction beyond 20 min resulted in a gradual decline in all response parameters ($p < 0.05$). This may be due to oxidative degradation or structural breakdown of heat- or shear-sensitive phytochemicals, particularly phenolics and flavonoids, when exposed to mechanical stress and oxygen over extended durations [53,54]. Therefore, 20 min was selected as the optimal extraction time.

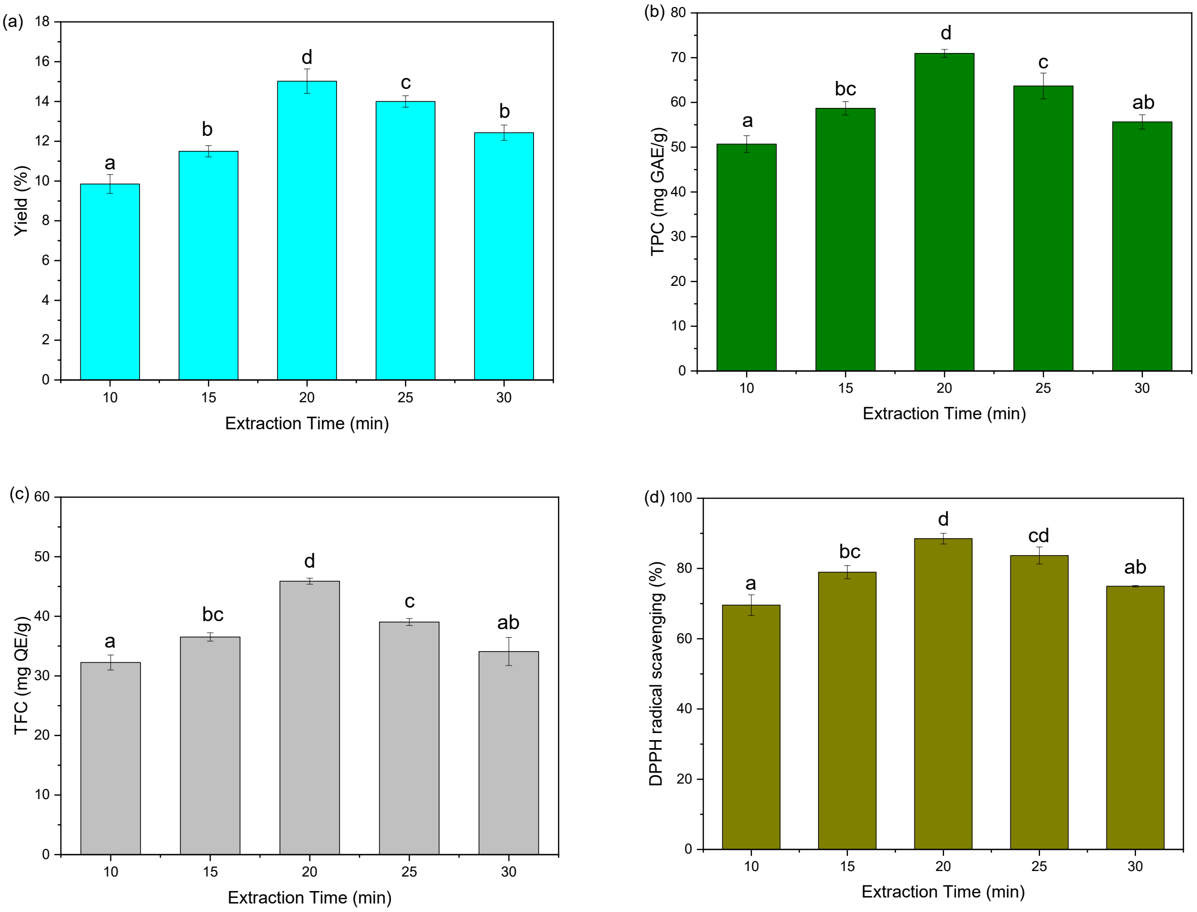


Figure 4 Effect of extraction time on (a) extraction yield, (b) TPC, (c) TFC, and (d) DPPH radical scavenging activity. Values are expressed as mean ± SD (n = 3). Error bars represent the standard deviation (n = 3). Different letters indicate significant differences among samples according to Tukey's test ($p < 0.05$).

Optimization of the extraction conditions using RSM

The factors influencing the extraction conditions were optimized using a Box-Behnken Design (BBD) combined with response surface methodology (RSM). Unlike the OFAT screens, which identified around 15% as the highest yield under fixed single-factor conditions, the multivate RSM revealed a higher global optinum of approximately 31% when variable interactions were considered. The range of independent variables was determined based on experimental findings presented in *Sections 3.2.1 (Effect of liquid-to-solid ratio)*, 3.2.2 *(Effect of solvent concentration)*, 3.2.3 *(Effect of homogenization speed)* and 3.2.4 *(Effect of extraction time)*. Each factor was assessed at 3 levels: Optimal (coded as 0), upper-optimal (+1), and lower-optimal (−1). The experimental results from the BBD model are summarized in **Table 3**. Regression analysis (**Table 4**) identified statistically significant coefficients that were used to develop predictive models for yield, total phenolic content, total flavonoid content, and DPPH radical scavenging activity. Quadratic polynomial equations (Eqs. (4) - (7)) were formulated to describe the interactions among independent variables and their effects on the responses. The high coefficients of determination (R^2), ranging from 0.9164 to 0.9500, indicate a strong correlation between experimental and predicted values, supporting the robustness of most models (**Table 5**). However, the yield (Y_1) model displayed a comparatively low predicted R^2 (0.6719), raising concerns about its predictive utility and the possibility of overfitting. Similarly, the TPC (Y_2) and TFC (Y_3) models showed notable discrepancies between adjusted and predicted R^2 values (e.g., 0.9284 vs. 0.7821 for Y2; 0.9164 vs. 0.7652 for Y3), which suggests that extrapolations outside the studied parameter space should be made with caution. Even so, the relatively small differences (< 0.2) between adjusted and predicted

R^2 values suggest internal consistency, although the modest predictive accuracy of Y_2 and Y_3 (predicted $R^2 < 0.55$) should be acknowledged when applying the models beyond the tested domain. The significant F-values highlight the crucial role of the independent variables in determining extraction efficiency.

In the quadratic model for yield (Y_1), the adjusted R^2 value of 0.8861 suggests that the independent variables accounted for a significant portion of the variability in yield. The most influential factors affecting yield were the liquid-to-solid ratio (p = 0.0069), homogenization speed (p = 0.0012), and extraction time (p = 0.0005), with the quadratic terms A^2, B^2, C^2 and D^2 also showing statistical significance ($p < 0.05$). An increase in liquid-to-solid ratio positively influenced yield by enhancing solvent-solute contact and diffusion, although overly high ratios may have diluted the extracts, lowering efficiency [55]. Notably, homogenization speed exerted the greatest influence, as reflected by its highest coefficient in Eq. (4). This emphasizes the essential role of homogenization speed in optimizing extraction, likely due to its effect on the efficient breakdown of the solid matrix and the release of bioactive compounds. As higher shear improved matrix breakdown and solvent penetration, but excessive shear could cause degradation of sensitive compounds [56]. Furthermore, the interaction terms between factors (AB, AC, AD, BC, BD and CD) were not statistically significant ($p > 0.05$), reinforcing the predominance of individual variables in determining yield. This lack of significant interactions suggests an additive system, where each factor influences yield independently, thereby simplifying optimization protocols. This trend is consistent with previous NADES-based extractions, where solvent composition and mechanical forces independently dominate process performance [17]. While the model fit was satisfactory (p = 0.1062), the lower predicted R^2 (0.6719) compared to the adjusted R^2 suggests potential overfitting, necessitating further validation for improved predictive accuracy. Such divergence underscores the need for confirmatory experiments to validate reproducibility and ensure the practical relevance of the optimization results.

The quadratic model for total phenolic content (Y_2) exhibited an adjusted R^2 of 0.8344, indicating that the independent variables contributed significantly to explaining the variability in the phenolic content. The most influential factors for phenolic content were the liquid-to-solid ratio (p = 0.0082) and extraction time (p = 0.0021), followed by the homogenization speed (p = 0.0203). Increasing liquid-to-solid ratio improved phenolic recovery by facilitating solvent accessibility and diffusion into plant tissues [57]. Extraction time positively contributed up to an optimal point, beyond which extended exposure likely induced oxidation or polymerization of phenolics, decreasing recovery [58] Homogenization speed exerted a moderate effect by enhancing solvent penetration, though excessive mechanical stress may destabilize certain phenolic structures. Particularly, the quadratic terms, A^2 and D^2, were statistically significant ($p < 0.05$), emphasizing the importance of these factors in determining the phenolic yield. Homogenization speed was found to have a moderate effect, likely due to its role in improving solvent penetration into the solid matrix. However, the interaction terms between the factors (AB, AC and AD) were not statistically significant ($p > 0.05$), which suggests that optimizing each factor individually was sufficient to maximize phenolic content. This result indicates that phenolic extraction is mainly influenced by the physical properties of the extraction process, with little dependence on interactions between the variables, thereby suggesting an additive behavior that simplifies optimization. Such independence has been observed in other DoE-guided phenolic extraction studies, where solvent polarity and process duration exert direct but non-synergistic contributions [56]. The relatively high R^2 value (0.9172) and the satisfactory model fit (p = 0.0696) further support the reliability of the predictive model for total phenolic content within the tested range. Although the adjusted R^2 was high (0.8344), the relatively low predicted R^2 (0.55) indicates limited predictive power outside the studied parameter space. Therefore, maximizing phenolic recovery primarily depends on optimizing solvent ratio, extraction time, and homogenization individually, with little benefit gained from adjusting their interactions

For total flavonoid content (Y_3), the quadratic model demonstrated an adjusted R^2 of 0.8328, indicating that the independent variables had a considerable influence on the flavonoid yield. The liquid-to-solid ratio (p = 0.0045) and homogenization speed (p = 0.0167) were the primary factors affecting flavonoid content. The quadratic term for B^2 was significant ($p < 0.05$), suggesting a non-linear influence of solvent concentration on flavonoid release, though it was not among the primary determinants of the model. Interestingly, the extraction time and solvent concentration did not show a significant effect on flavonoid extraction ($p > 0.05$), indicating that other variables, such as solvent characteristics and temperature, may play a more important role in determining flavonoid yield. Within the explored domain, increasing the liquid-to-solid ratio exerted a positive effect on TFC by enhancing solvent-solute contact and diffusion; however, excessively high ratios may dilute solute concentration and lower process efficiency [27]. Homogenization speed showed a positive effect up to an optimum, where improved shear promoted matrix disruption and flavonoid release; beyond that, over-shear may foster oxidation or structural rearrangement that offsets gains. Given that extraction time and solvent concentration were not significant ($p > 0.05$), they can be fixed at economical mid-levels without compromising TFC, while optimization should prioritize liquid-to-solid ratio and homogenization conditions. Interaction terms between factors (AB, AC, AD, BC, BD and CD) were not significant, indicating that flavonoid recovery depends primarily on the additive contributions of individual factors. This independence simplifies optimization protocols, as each factor can be tuned separately without complex multi-factor adjustments. The model for total flavonoid content provided a strong correlation between experimental and predicted values, with a high R^2 value (0.9164), and the model fit was satisfactory ($p = 0.0924$). Although the adjusted R^2 was high (0.8328), the relatively low predicted R^2 (0.55) indicates that predictive power outside the studied parameter space is limited. Nonetheless, within the tested conditions, the model reliably shows that flavonoid recovery can be optimized by focusing on solvent loading and homogenization speed, without requiring complex multi-factor interactions.

Together, the DPPH radical scavenging activity (Y_4) model also demonstrated a strong adjusted R^2 of 0.8999, indicating a high degree of correlation between the experimental and predicted values. The liquid-to-solid ratio ($p < 0.0001$), homogenization speed ($p = 0.0004$) and extraction time ($p < 0.0001$) were the most significant factors influencing antioxidant activity. Similar to the phenolic and flavonoid models, the quadratic terms for A^2 and B^2 were statistically significant ($p < 0.05$). The extraction time ($p < 0.0001$) also showed a significant effect on DPPH scavenging activity, though its impact was less pronounced than the liquid-to-solid ratio and homogenization speed. In the tested range, increasing the liquid-to-solid ratio enhanced the release of antioxidant compounds by improving solvent-solute mass transfer, though excessive dilution may reduce the effective concentration of bioactives in the extract. Homogenization speed exerted a strong positive influence by facilitating cell rupture and diffusion of polyphenols, but very high shear could promote oxidation or degradation of sensitive antioxidants. Extraction time showed a dual effect, with antioxidant activity increasing initially due to improved release but decreasing at prolonged times, likely because of oxidative breakdown of flavonoids and phenolics. Interestingly, solvent concentration did not significantly affect antioxidant activity ($p > 0.05$), suggesting that the extraction process itself (particularly homogenization speed and liquid-to-solid ratio) has a dominant role in determining the antioxidant potential. The high R^2 value (0.9500) and the satisfactory model fit ($p = 0.0555$) support the robustness of the model, and the minimal difference between adjusted R^2 and predicted R^2 values further confirms the predictive accuracy. These results indicate that antioxidant recovery can be maximized by balancing solvent loading, homogenization intensity, and extraction duration. By contrast, solvent concentration showed no significant contribution ($p = 0.9155$) and thus does not require adjustment within the tested range. Its lack of significance in the flavonoid (Y3) and antioxidant (Y4) models contrasts with the clear influence observed during OFAT screening. This discrepancy likely reflects the narrower parameter space examined in RSM and the predominance of mass-transfer-driven variables (liquid-to-solid ratio, homogenization speed), a pattern also reported in DOE-guided extractions.

Table 3 Box-Behnken Design (BBD) model with independent variables and the corresponding experimental responses.

Run	Independent variables				Responses			
	A	**B**	**C**	**D**	**Y_1**	**Y_2**	**Y_3**	**Y_4**
1	50	20	15,000	30	27.1382	61.4832	38.2157	74.9247
2	50	20	10,000	20	29.5125	65.4035	42.5124	77.6236
3	70	10	10,000	20	21.4197	57.2486	34.9265	68.2315
4	50	10	10,000	30	23.6784	53.9871	36.1324	70.5128
5	30	10	10,000	20	19.8243	52.6214	31.4152	64.2785
6	30	20	10,000	30	20.4738	56.8395	32.7458	66.8772
7	50	20	10,000	20	29.2732	65.8375	43.4112	79.5129
8	50	10	5,000	20	19.5124	48.313	30.2178	60.1047
9	50	10	15,000	20	25.3158	60.4027	36.6459	72.3769
10	50	20	5,000	10	17.5842	50.1536	31.0974	59.9934
11	50	30	15,000	20	26.1457	54.1043	39.6782	62.4217
12	70	20	10,000	10	20.6213	56.3812	33.7524	67.6799
13	50	20	10,000	20	29.5921	65.2173	43.1589	79.1357
14	30	20	5,000	20	19.3287	50.7641	32.4781	57.1294
15	50	20	10,000	20	31.7897	66.9123	45.1297	79.512
16	50	20	15,000	10	23.6123	52.5493	38.0297	69.2423
17	50	20	5,000	30	22.8374	58.1485	34.4183	67.8824
18	50	30	5,000	20	19.3287	55.7893	32.4781	65.3157
19	70	20	10,000	30	22.5934	59.6048	35.5392	71.9758
20	70	30	10,000	20	21.2574	57.4789	34.1286	69.1297
21	50	10	10,000	10	17.6412	55.3248	30.0376	58.7389
22	30	30	10,000	20	15.9783	54.4192	28.6395	55.8751
23	30	20	15,000	20	19.4837	56.5147	32.0148	62.4978
24	50	30	10,000	10	21.3127	57.8923	33.3492	65.8174
25	50	20	10,000	20	30.0821	63.9824	44.6573	80.7249
26	70	20	15,000	20	23.0294	60.2483	36.3257	72.1834
27	30	20	10,000	10	16.1349	50.7368	29.0872	57.0327
28	50	30	10,000	30	27.4721	63.7295	40.1829	74.8205
29	70	20	5,000	20	21.5142	56.9183	33.3147	66.0329

Note: A (Liquid/Solid ratio - mL/g), B (Solvent concentration - %), C (Homogenization speed - rpm), D (Extraction time - min) and Y_1 (Yield - %), Y_2 (Total phenolic content - mg GAE/g), Y_3 (Total flavonoid content - mg QE/g), Y_4 (DPPH radical scavenging activity - %).

Table 4 Analysis of variance (ANOVA) for the fitted quadratic polynomial "Quadratic model" for optimization of extraction based on a Box-Behnken design matrix for response surface analysis.

Source	SS	df	MS	F-value	*p*-value	
			Response Y_1			
Model	501.22	14	35.80	11.63	< 0.0001	significant
A-L/S ration (mL/g)	30.76	1	30.76	10.00	0.0069	
B-Solvent concentration (%)	1.40	1	1.40	0.4559	0.5105	
C-Homogenization speed (rpm)	50.51	1	50.51	16.41	0.0012	
D-Extraction time (min)	62.05	1	62.05	20.16	0.0005	
AB	3.39	1	3.39	1.10	0.3115	
AC	0.4625	1	0.4625	0.1503	0.7041	
AD	1.40	1	1.40	0.4551	0.5109	
BC	0.2568	1	0.2568	0.0835	0.7769	
BD	0.0037	1	0.0037	0.0012	0.9727	
CD	0.7459	1	0.7459	0.2424	0.6301	
A^2	249.54	1	249.54	81.09	< 0.0001	
B^2	106.34	1	106.34	34.56	< 0.0001	
C^2	70.90	1	70.90	23.04	0.0003	
D^2	92.31	1	92.31	30.00	< 0.0001	
Residual	43.08	14	3.08			
Lack of Fit	38.95	10	3.90	3.77	0.1062	not significant
Pure Error	4.13	4	1.03			
Cor Total	544.30	28				
			Response Y_2			
Model	660.02	14	47.14	11.08	< 0.0001	significant
A-L/S ration (mL/g)	56.27	1	56.27	13.22	0.0027	
B-Solvent concentration (%)	20.06	1	20.06	4.71	0.0476	
C-Homogenization speed (rpm)	52.99	1	52.99	12.45	0.0033	
D-Extraction time (min)	78.82	1	78.82	18.52	0.0007	
AB	0.6143	1	0.6143	0.1443	0.7097	
AC	1.46	1	1.46	0.3442	0.5667	
AD	2.07	1	2.07	0.4870	0.4967	
BC	47.44	1	47.44	11.15	0.0049	
BD	12.87	1	12.87	3.02	0.1040	
CD	0.2204	1	0.2204	0.0518	0.8233	

Source	**SS**	**df**	**MS**	**F-value**	***p*-value**	
A^2	156.73	1	156.73	36.83	< 0.0001	
B^2	144.73	1	144.73	34.01	< 0.0001	
C^2	193.65	1	193.65	45.50	< 0.0001	
D^2	105.54	1	105.54	24.80	0.0002	
Residual	59.58	14	4.26			
Lack of Fit	55.08	10	5.51	4.90	0.0696	not significant
Pure Error	4.50	4	1.12			
Cor Total	719.60	28				
Response Y_3						
Model	584.46	14	41.75	10.96	< 0.0001	significant
A-L/S ration (mL/g)	38.90	1	38.90	10.22	0.0065	
B-Solvent concentration (%)	6.87	1	6.87	1.80	0.2005	
C-Homogenization speed (rpm)	60.33	1	60.33	15.84	0.0014	
D-Extraction time (min)	39.90	1	39.90	10.48	0.0060	
AB	0.9779	1	0.9779	0.2568	0.6202	
AC	3.02	1	3.02	0.7924	0.3884	
AD	0.8759	1	0.8759	0.2300	0.6389	
BC	0.1490	1	0.1490	0.0391	0.8460	
BD	0.1365	1	0.1365	0.0358	0.8526	
CD	2.46	1	2.46	0.6451	0.4353	
A^2	277.70	1	277.70	72.92	< 0.0001	
B^2	153.21	1	153.21	40.23	< 0.0001	
C^2	102.48	1	102.48	26.91	0.0001	
D^2	118.02	1	118.02	30.99	< 0.0001	
Residual	53.32	14	3.81			
Lack of Fit	48.60	10	4.86	4.12	0.0924	not significant
Pure Error	4.72	4	1.18			
Cor Total	637.78	28				
Response Y_4						
Model	1,414.30	14	101.02	18.99	< 0.0001	significant
A-L/S ration (mL/g)	221.39	1	221.39	41.61	< 0.0001	
B-Solvent concentration (%)	0.0621	1	0.0621	0.0117	0.9155	
C-Homogenization speed (rpm)	115.25	1	115.25	21.66	0.0004	
D-Extraction time (min)	195.93	1	195.93	36.82	< 0.0001	

Source	SS	df	MS	F-value	*p*-value	
AB	21.63	1	21.63	4.07	0.0634	
AC	0.1529	1	0.1529	0.0287	0.8678	
AD	7.70	1	7.70	1.45	0.2490	
BC	57.50	1	57.50	10.81	0.0054	
BD	1.92	1	1.92	0.3607	0.5577	
CD	1.22	1	1.22	0.2288	0.6398	
A^2	432.28	1	432.28	81.25	< 0.0001	
B^2	324.79	1	324.79	61.04	< 0.0001	
C^2	296.93	1	296.93	55.81	< 0.0001	
D^2	152.06	1	152.06	28.58	0.0001	
Residual	74.49	14	5.32			
Lack of Fit	69.53	10	6.95	5.61	0.0555	not significant
Pure Error	4.96	4	1.24			
Cor Total	1,488.79	28				

Note: A (Liquid/Solid ratio - mL/g), B (Solvent concentration - %), C (Homogenization speed - rpm), D (Extraction time - min) and Y_1 (Yield - %), Y_2 (Total Phenolic Content - mg GAE/g), Y_3 (Total Flavonoid Content - mg QE/g), Y_4 (DPPH radical scavenging activity - %); df: Degree of freedom; SS: Sum of Squares; MS: Mean Square; and Significant ($p < 0.05$).

Table 5 Analyses of regression fit for different responses.

Responses	SD	R^2	Adjusted R^2	Predicted R^2	Suggested model
Y_1	1.45	0.9430	0.8861	0.6719	Quadratic
Y_2	2.96	0.9172	0.8344	0.5493	Quadratic
Y_3	1.95	0.9164	0.8328	0.5495	Quadratic
Y_4	2.31	0.9500	0.8999	0.7258	Quadratic

$$Y_1 = -63.80811 + 1.56375A + 1.36679B + 0.002957C + 1.96453D + 0.004605AB + 3.4005\times10^{-6}AC - 0.002959AD + 5.068\times10^{-6}BC + 0.000305BD - 8.6365\times10^{-6}CD - 0.015506A^2 - 0.04049B^2 - 1.32242\times10^{-7}C^2 - 0.037724D^2 \quad (4)$$

$$Y_2 = -53.70897 + 1.50884A + 2.44671B + 0.006377C + 1.64402D - 0.001959AB - 6.0515\times10^{-6}AC - 0.003599AD - 0.000069BC + 0.017937BD + 4.695\times10^{-6}CD - 0.012289A^2 - 0.047236B^2 - 2.18554\times10^{-7}C^2 - 0.040337D^2 \quad (5)$$

$$Y_3 = -60.81689 + 1.6363A + 1.82055B + 0.00343C + 2.12537D + 0.002472AB + 8.68575\times10^{-6}AC - 0.00234AD - 3.86\times10^{-6}BC + 0.001847BD - 0.000016CD - 0.016358A^2 - 0.048601B^2 - 1.58991\times10^{-7}C^2 - 0.042656D^2 \quad (6)$$

$$Y_4 = -85.80405 + 2.14227A + 3.13876B + 0.007672C + 2.93645D + 0.011627AB + 1.95525\times10^{-6}AC - 0.006936AD - 0.000076BC - 0.006927BD - 0.000011CD - 0.020409A^2 - 0.070761B^2 - 2.70635\times10^{-7}C^2 - 0.048418D^2 \quad (7)$$

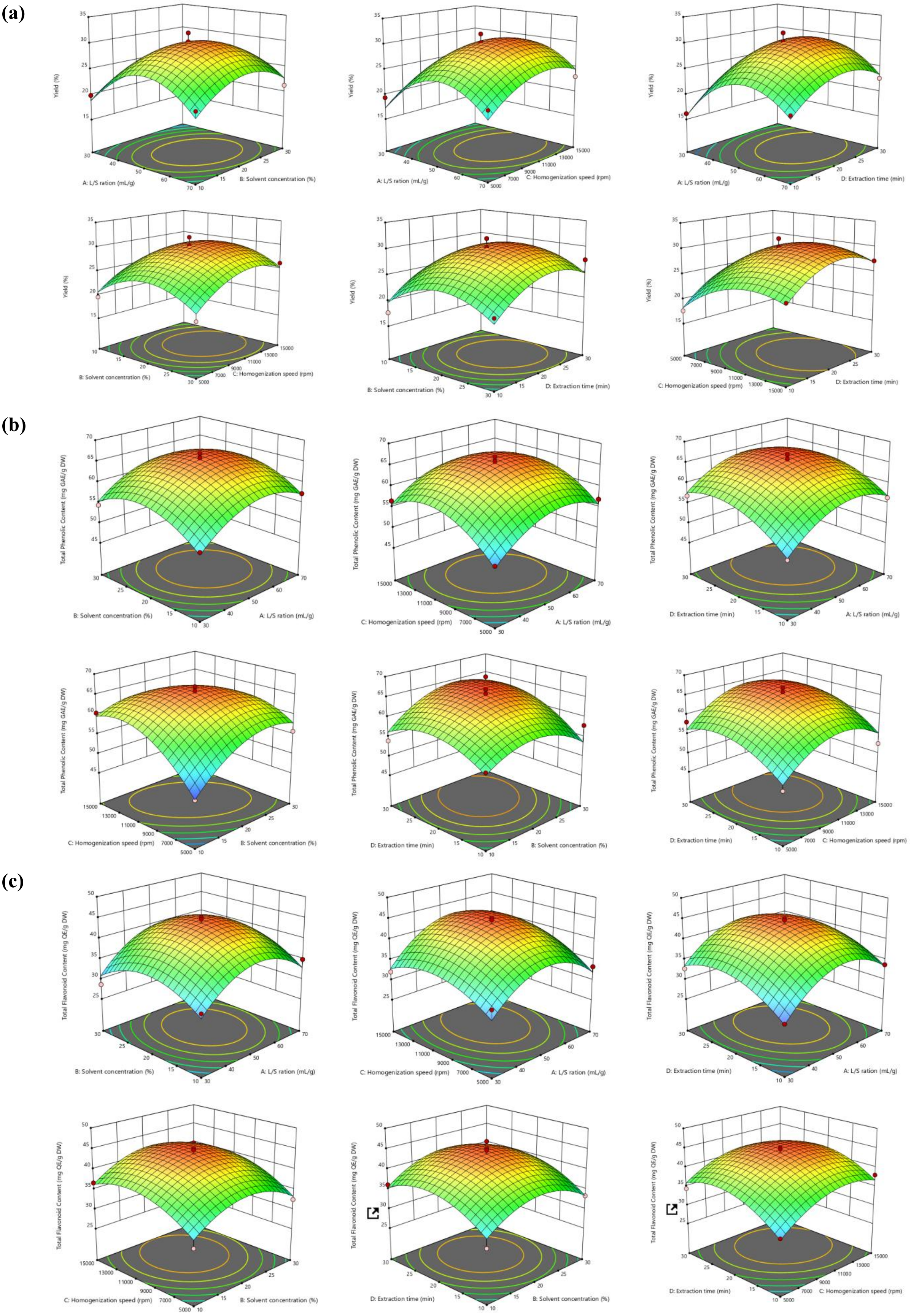
The optimization of homogenization-assisted extraction using natural deep eutectic solvents (NADES) for *Centella asiatica* was successfully conducted through a Box-Behnken Design (BBD) in conjunction with Response Surface Methodology (RSM) to evaluate the effects of key formulation variables on the extraction responses: yield, total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity, as illustrated in **Figure 5**. Each response was examined through 3D surface plots and contour plots, offering a detailed insight into the interactions between variables and their collective influence on extraction efficiency. The plots illustrate paired independent variables along the X- and Y-axes, with the corresponding response on the Z-axis. Most surfaces displayed quadratic curvature, and significance contours ($p < 0.05$) were marked to highlight regions of optimal response.

The response surface plots and contour maps for yield (**Figure 5(a)**) indicated a significant dependency on the liquid-to-solid ratio, solvent concentration, homogenization speed, and extraction time. Under the optimized conditions of 50 mL/g liquid-to-solid ratio, 20% solvent concentration, 10,000 rpm homogenization speed, and 20 min of extraction time, the highest yield (31.7897%) was obtained. These results underscore the importance of maintaining an optimal balance between solvent volume and the surface area of solid material to maximize mass transfer efficiency. Notably, when either the liquid-to-solid ratio or solvent concentration was reduced, a significant decline in yield was observed, highlighting the essential role of solvent availability in the extraction process. This trend aligns with established observations that sufficient solvent volume facilitates effective extraction, enabling more complete solubilization of bioactive compounds.

As shown in **Figure 5(b)**, the total phenolic content (TPC) was significantly influenced by both solvent concentration and homogenization speed. The highest TPC (66.9123 mg GAE/g) was recorded under the same optimized conditions that yielded the highest extraction efficiency. These observations support the view that solvent concentration and homogenization speed are important parameters for enhancing phenolic compound release. This outcome aligns with previous research, which suggests that higher solvent concentrations and increased homogenization speeds facilitate better solubilization and recovery of phenolic compounds from plant materials. The enhanced extraction of phenolics under these conditions is attributed to the increased solvent availability and effective disruption of cell walls, promoting the release of bioactive molecules.

Similarly, the total flavonoid content (TFC) exhibited a strong dependence on solvent concentration and homogenization speed, as depicted in **Figure 5(c)**. The highest TFC (45.1297 mg QE/g) was achieved under the same optimized conditions. Adequate solvent concentration allowed for efficient solubilization of flavonoids, while the appropriate homogenization speed ensured effective disruption of the plant cell walls, thereby releasing the flavonoids into the solvent. However, excessive homogenization speeds may lead to the degradation of sensitive bioactive compounds, resulting in a decrease in TFC. These results underscore the importance of homogenization speed in influencing extraction efficiency, as higher shear promotes matrix breakdown and enhances the release of bioactive compounds.

Furthermore, the DPPH radical scavenging activity, a well-established indicator of antioxidant potential, was closely linked to the recovery of phenolic and flavonoid compounds. As illustrated in **Figure 5(d)**, the highest DPPH radical scavenging activity (80.7249%) was achieved under the optimized conditions that maximized both TPC and TFC. Although solvent concentration did not exert a statistically significant effect ($p = 0.9155$), moderate levels may still facilitate solubilization of antioxidant compounds. In contrast, homogenization speed and liquid-to-solid ratio were the dominant factors driving antioxidant recovery. The positive correlation between phenolic and flavonoid contents and DPPH scavenging activity highlights the strong antioxidant potential of the extract. These optimized conditions enabled the extraction of bioactive compounds with robust antioxidant properties, making them valuable for potential applications in food, pharmaceutical, and cosmetic industries.


(a)
Yield (%)
A: L/S ration (mL/g)
B: Solvent concentration (%)
C: Homogenization speed (rpm)
D: Extraction time (min)
(b)
Total Phenolic Content (mg GAE/g DW)
A: L/S ration (mL/g)
B: Solvent concentration (%)
C: Homogenization speed (rpm)
D: Extraction time (min)
(c)
Total Flavonoid Content (mg QE/g DW)
A: L/S ration (mL/g)
B: Solvent concentration (%)
C: Homogenization speed (rpm)
D: Extraction time (min)

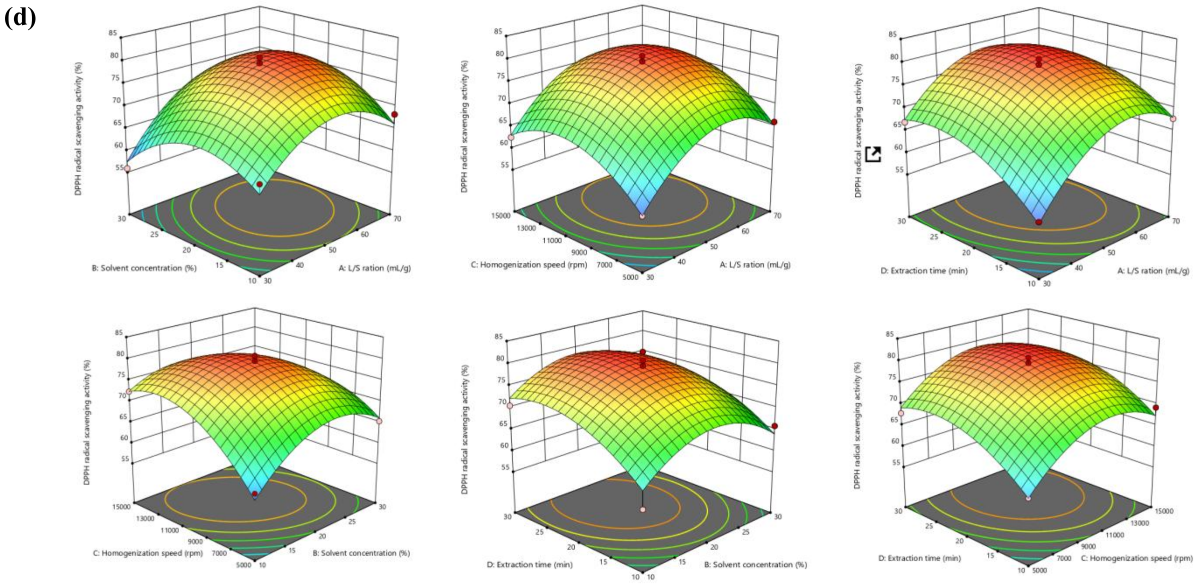


Figure 5 3D response surface methodology (RSM) plots for formulation optimization where the effects of variables on (a) yield, (b) total phenolic content, (c) total flavonoid content and (d) DPPH radical scavenging activity. The assumptions of normality and homogeneity of variance were confirmed prior to ANOVA.

Table 6 The experimental and predicted values of four responses at optimal conditions.

Variables				Dependent responses			
LSR (mL/g)	**NADES concentration (%)**	**Homogenization speed (rpm)**	**Time (min)**		**Predicted Value**	**Actual Value**	**Prediction error (%)**
50	20	10,000	20	Yield (%)	30.05	31.20 ± 1.0	3.82
				TPC (mg GAE/g)	65.47	65.81 ± 1.2	0.52
				TFC (mg QE/g)	43.44	44.58 ± 0.9	2.62
				DPPH (%)	79.03	80.54 ± 1.2	1.91

Model verification

The reliability of the regression models was assessed by conducting experiments under the predicted optimal conditions, and the corresponding responses are presented in **Table 6**. Numerical optimization, informed by the 3D response surface plots, indicated that the most favorable conditions consisted of a liquid-to-solid ratio of 50 mL/g, a NADES concentration of 20 %(w/w), a homogenization speed of 10,000 rpm, and an extraction time of 20 min. Under these parameters, the models predicted an extraction yield of 30.05%, a total phenolic content of 65.47 mg GAE/g DW, a total flavonoid content of 43.44 mg QE/g DW, and a DPPH inhibition of 79.03%. Validation experiments performed in triplicate produced results in close agreement with these predictions, with relative errors below 5%. This consistency demonstrates that the response surface methodology coupled with the Box-Behnken design provides accurate predictive power and can be applied effectively for optimizing NADES-based homogenization-assisted extraction.

Conclusions

In this work, we have demonstrated the effect of combining natural deep eutectic solvents with homogenization-assisted extraction as a sustainable strategy for recovering antioxidants from *Centella asiatica*. By coupling solvent innovation with process intensification, the study provides a green alternative to conventional organic solvents, capable of preserving the stability of thermolabile phytochemicals while enhancing extraction efficiency. The approach underscores the role of NADES design, particularly the choline chloride-glycerol system, in achieving high solubilization capacity under mild conditions, reinforcing the value of tailoring solvent properties to target plant matrices. From a methodological perspective, the use of response surface methodology offered a statistically robust framework to optimize interacting parameters and establish reliable predictive models. This not only supports reproducibility but also provides practical insights into scaling laboratory findings toward industrial practice. Importantly, the results highlight the broader relevance of NADES-HAE for green chemistry and natural product valorization, positioning it as a versatile platform applicable to diverse botanical sources and bioactive compounds. Despite these promising outcomes, several limitations must be acknowledged. The recyclability and reusability of NADES remain a critical challenge, and the energy requirements of homogenization may limit cost-effectiveness in large-scale operations. Moreover, the current study focused primarily on antioxidant contents, leaving the quantitative profiling of specific bioactives such as asiaticoside and madecassoside for future investigation. Addressing these issues through solvent recovery protocols, pilot-scale validation, advanced analytical methods (e.g., HPLC-MS/MS), and in vitro and in vivo bioactivity assessments will be essential to confirm efficacy, safety, and economic feasibility. Such efforts will accelerate the translation of this green extraction technology into functional foods, nutraceuticals, and cosmeceuticals, thereby bridging laboratory innovation with industrial application.

Acknowledgements

We acknowledge Ho Chi Minh City University of Technology (HCMUT), VNU-HCM, Vietnam for supporting this study.

Declaration of generative AI in scientific writing

During the preparation of this work the author(s) used ChatGPT in order to improve language and readability, with caution. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Long Phuoc Lieu: Writing - review & editing, Writing - original draft, Visualization, Methodology, Investigation, Project administration, Formal analysis, Data curation, Conceptualization. **Thi Thanh Huyen Nguyen**: Writing - review & editing, Methodology, Investigation, Visualization, Formal analysis, Conceptualization. **Duy Thanh Phu Quan Dao**: Writing - original draft, Investigation, Formal analysis, Validation, Visualization. **Vo Yen Nhi Pham**: Methodology, Software, Data curation, Conceptualization, Investigation, Formal analysis. **Dinh Quan Nguyen**: Writing - original draft, Writing - review & editing, Project administration, Visualization, Supervision.

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