Journal of Applied Pharmaceutical Science 2025. Article in Press
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2025.266427
ISSN 2231-3354

Supercritical fluid extraction, LC-MS profiling, and QbD-guided green HPLC method for standardization of *Careya arborea* Roxb. nanoemulsion

Abhijit S. Salokhe[1], Archana S. Patil[1*], Yadishma Gaude[1], Pooja Rayanade[1], Rahul Koli[2], Namdeo S. Jadhav[3]

[1]Department of Pharmaceutics, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi, India.
[2]Department of Quality Assurance, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi, India.
[3]Department of Pharmaceutics, Krishna College of Pharmacy, Karad, India.

ARTICLE HISTORY

Received on: 24/06/2025
Accepted on: 26/07/2025
Available Online: XX

Key words:
Supercritical fluid extraction, green RP-HPLC method, quality by design, LC-MS characterization, *Careya arborea* Roxb. nanoemulsion.

ABSTRACT

Careya arborea Roxb. was investigated for its phytotherapeutic potential, focusing on two key bioactive constituents, (−)-epigallocatechin-3-gallate (EGCG) and azelaic acid (AzA). These compounds were efficiently extracted using supercritical fluid extraction (SFE), a green and selective technique. The chemical composition of the extract was confirmed through liquid chromatography–mass spectrometry (LC-MS), verifying the presence of both EGCG and AzA. To improve bioavailability and dermal absorption, the extract was formulated into a nanosuspension using suitable stabilizers and homogenization techniques. A robust and reproducible reverse-phase high-performance liquid chromatography method was developed for the quantitative estimation of EGCG and AzA in both the crude extract and nanosuspension. Method development was guided by a quality by design approach using central composite design, optimizing key chromatographic parameters with minimal experimentation. EGCG was separated using a Chemsil ODS C18 column with methanol and 0.1% orthophosphoric acid (60:40 v/v) at 276 nm, while AzA was analyzed using a Phenomenex Luna C18 column with acetonitrile and 0.1% formic acid (80:20 v/v) at 227 nm. The method was validated as per ICH Q2(R1) guidelines, showing high accuracy, precision, and robustness. Green chemistry metrics such as Analytical Eco-Scale and Analytical GREEnness confirmed the method's environmental sustainability.

INTRODUCTION

The increasing global preference for plant-based medicines, especially in developed countries, is driven by their broad therapeutic efficacy and comparatively low side effect profile compared to synthetic pharmaceuticals [1]. Yet, the lack of standardized manufacturing and quality control protocols limits their acceptance in mainstream healthcare. Ensuring safety, efficacy, and batch consistency requires rigorous authentication of botanical materials and reproducible extraction and analysis procedures [2].

Careya arborea Roxb. (Kumbi), a deciduous Lecythidaceae tree native to tropical Asia, has been traditionally used to address wounds, inflammation, dyspepsia, dysentery, toothache, snakebite, coughs, colds, and parasitic infections [3,4]. Despite its broad ethnomedical profile, *C. arborea* Roxb. has rarely been standardized or formulated in modern dosage forms. Recent interest in nanoemulsion-based topical systems offers a promising platform to realize its therapeutic potential more effectively.

Nanoemulsions provide exceptional advantages in wound healing due to their thermodynamic stability, ultrafine droplet size, enhanced bioavailability, and controlled release of bioactive compounds [5]. Several comparative evaluations show that nanoemulsions outperform conventional formulations

*Corresponding Author
Archana S. Patil, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi, India. E-mail: archanapatil @ klepharm.edu

in achieving faster tissue repair, deeper skin penetration, and reduced inflammation. For instance, lecithinbased herbal nanoemulsions demonstrated superior physicochemical stability and hydration benefits compared to regular emulsions [6,7].

Conventional extraction techniques such as maceration, Soxhlet, or ultrasound-assisted extraction remain cost-effective but suffer limitations such as high solvent consumption, long durations, poor selectivity, and degradation of heat-sensitive molecules [7]. In contrast, supercritical fluid extraction (SFE) using CO_2, often with ethanol modifiers, offers a cleaner, more selective, and rapid alternative. SFE preserves thermolabile compounds, reduces environmental risks, and achieves higher purity and yield [7,13] through parameter control, as demonstrated in advanced two-step SFE protocols that sequentially isolate volatiles and phenolics in under 80 minutes across diverse plant species [15].

Analytical standardization is a cornerstone of reliable herbal drug development. High-performance liquid chromatography (HPLC) remains critical for quantitative profiling and fingerprinting, while liquid chromatography–mass spectrometry (LC-MS) enables structural elucidation of complex phytochemicals [8,9]. More recently, supercritical fluid extraction (SFE) has emerged as a green and powerful alternative to HPLC, offering rapid separation, reduced solvent use, and chiral analysis capabilities—meeting stringent regulatory demands with renewed efficiency [8,14].

Development of robust analytical methods benefits significantly from quality by design (QbD) workflows combined with design of experiment (DoE) software. These strategies allow predictive modeling of chromatographic conditions with fewer experimental trials, reducing development time and solvent use while improving method robustness [10]. In alignment with Green Analytical Chemistry (GAC) principles, the Analytical GREEnness (AGREE), Complementary Green Analytical Procedure Index (ComplexGAPI), and Eco-Scale Analytical (ESA) frameworks provide quantitative assessments of method sustainability—encouraging adoption of energy-efficient instruments, safe solvents, and minimal waste practices [11,12,17].

Taken together, the current literature reflects improved therapeutic outcomes from nanoemulsion-based herbal formulations, eco-friendly extraction strategies such as SFE, green chromatographic innovations, and data-driven method design. Yet, no published study has integrated all these elements-SFE extraction, nanoemulsion formulation, LC-MS, or SFE profiling, QbD-optimized HPLC quantification, and greenness evaluation into a single, standardized workflow for *C. arborea* Roxb.

Thus, this study seeks to fill a crucial gap by developing a comprehensive and eco-conscious quality control platform for *C. arborea* Roxb. nanoemulsion designed for wound healing. It merges supercritical CO_2 extraction for extracting bioactives, followed by LC-MS characterization, nanoemulsion development, QbD-based optimization of HPLC quantification method, and greenness assessment via contemporary GAC tools.

By uniting traditional botanical knowledge with modern analytical rigor and sustainability principles, this work offers a scalable, reproducible methodology that supports the therapeutic credibility, regulatory readiness, and environmental stewardship of plant-based wound healing agents.

MATERIALS AND METHODS

Chemicals and reagents

Otto Chemie Pvt. Ltd. in Mumbai supplied the (−)-epigallocatechin-3-gallate (EGCG). Azelaic acid (AzA) was obtained through Yuka Enterprises, Mumbai. HPLC-grade methanol, acetonitrile, and formic acid were supplied by HiMedia Laboratories Pvt. Ltd., Mumbai. Milli-Q water used in the study was provided by KLE College of Pharmacy, Belagavi.

Collection, authentication, and taxonomic validation of *C. arborea* Roxb. plant material

The bark of *C. arborea* Roxb. was collected during the post-monsoon season (October) from Jamboti, southwest of Belagavi, Karnataka, and was authenticated (CRF/Auth/119/2023) at Shri B.M.K. Ayurveda Mahavidyalaya, Central Research Facility, Shahpur, Belgaum. A total of 1,200 g of *C. arborea* Roxb. bark was collected, and after shade drying and grinding, approximately 800 g of coarse powder was obtained for further extraction.

Green isolation of phytoconstituents via SFE

The recently gathered *C. arborea* Roxb. bark was cleaned and kept to dry under the shade for 15 days. The dried bark was powdered using a grinder mixer. The extraction relies on a supercritical fluid, commonly CO_2, which reaches a unique phase, intermediate between gas and liquid, under controlled temperature and pressure. The high-pressure vessel was loaded with a 150-g sample, and the oven temperature was maintained within 45°C to 60°C. A high-pressure pump (Thar Technologies, Southern California, USA) then introduced pressurized CO_2 into the vessel at 2 l per minute, maintaining a pressure of 100 to 350 bar throughout the extraction. Once the supercritical CO_2 containing the extract went through a temperature-controlled micrometer and was exposed to air, the extract was gathered in a glass vial after each extraction. The extract was stored in a refrigerator (Remi, India) maintained at 4°C ± 1°C [12,13].

LC-MS-based metabolite fingerprinting and profiling of *C. arborea* Roxb. extract

Ultra-HPLC on a Shimadzu Nexera XR LC 40 system connected to a Shimadzu LCMS 8060 mass spectrometer (Shimadzu Italy, Milan, Italy) was used to ascertain the chemical profile of the SFE from *C. arborea* Roxb. While the heating gas flow rate was set at 10 l/min, the nebulization gas flow rate was found to be 2.9 l/min. The drying gas flow rate was 10 l/min. The dissolution line, heating block, and interface were all kept at 250°C, 400°C, and 300°C, respectively [13]. Using a Phenomenex C18 column (3 × 100 mm, 2.6 µm, Torrance, CA) and a mobile phase made up of acetonitrile (A) and water with 0.01% formic acid (B), compound separation was accomplished. To prepare the extract for injection, it was first mixed with a 1:1 ratio of acetonitrile and water, then diluted 50-fold with acetonitrile. Ion detection was performed

using negative electrospray ionization and multiple reaction monitoring [14].

Nanoemulsion development and characterization of *C. arborea* Roxb. extract

Nanoemulsions were prepared with a precise amount of *C. arborea* Roxb. bark extract weighed and dissolved in a measured volume of clove oil, along with a predetermined amount of Transcutol P (Mixture A). Continuous stirring was maintained using a magnetic stirrer to ensure uniform mixing. Tween 80 (surfactant) and water were mixed separately to form Mixture B, which was thoroughly stirred at 700 rpm for proper blending. Mixture B was then added dropwise into Mixture A (containing the dissolved drug, oil, and co-surfactant) while continuously stirring. The slow addition helped achieve uniform dispersion and prevented the formation of large droplets. The resulting mixture was subjected to high-speed homogenization using a homogenizer operating at 15,000 rpm for approximately 15 minutes. This step reduced droplet size, enhanced emulsion stability, and yielded a clear nanoemulsion. After homogenization, the nanoemulsion was visually inspected for clarity. The *C. arborea* Roxb. bark extract nanoemulsion was also characterized for its zeta potential, polydispersity index (PDI), droplet size, and entrapment efficiency [15]. To quantify and standardize the prepared nanoemulsion, a green HPLC method was established using a central composite design strategy.

Stability assessment of prepared nanoemulsion

To assess the shelf-life and robustness of the *C. arborea Roxb*. extract-loaded nanoemulsion, a stability study was performed under ICH Q1(A) guidelines. The formulation was stored in tightly sealed glass containers under two different temperature conditions: refrigerated (4°C ± 2°C) and room temperature (25°C ± 2°C), for 60 days. Samples were withdrawn at predetermined intervals (0, 15, 30, and 60 days) and evaluated for various physicochemical parameters. These included visual inspection for any changes in physical appearance, color, or phase separation, as well as measurement of particle size, PDI, and zeta potential using dynamic light scattering. The pH of the formulation was measured using a calibrated digital pH meter. Additionally, encapsulation efficiency (%EE) for EGCG and AzA was determined at each time point by assessing the free and total drug content using appropriate analytical techniques [42].

Strategic development of a robust RP-HPLC method

Instrumental specifications and analytical configuration

The analytical process involved chromatographic analysis using a Waters Alliance e2695 system that has a Chemsil ODS C18 column, which was kept at ambient temperature for optimal performance. A steady flow rate of 1 ml/min was used to provide the mobile phase, which was a 60:40 combination of methanol and 0.1% orthophosphoric acid [16]. To prevent dissolved gases from interfering, the mobile phase underwent degassing by sonication through a 0.45 µm polyvinylidene difluoride membrane filter before its use. To precisely measure and identify EGCG, samples were injected at a constant 10 µl volume and detected at 276 nm [17].

The analysis of AzA, an Agilent 1220 Infinity II liquid chromatography system (LC-20AD, Japan) was employed. This system comprised a quaternary pump with an integrated degasser (G7111A), an autosampler (G7129A), and a photodiode array (PDA) detector (G7115A). OpenLab CDS software facilitated data acquisition and interpretation. A Phenomenex Luna C18 analytical column (C-18(2) 100) with dimensions of 250 × 4.6 mm and a particle size of 5 µm was used to achieve chromatographic separation. The optimal mobile phase, acetonitrile with 0.1% formic acid in an 80:20 ratio, was supplied at a steady flow rate of 1 ml/min. Before use, the mobile phase was sonicated and passed through a 0.45 µm polyvinylidene difluoride membrane filter to eliminate interference from dissolved gases. A constant volume of injection of 10 µl was used for all samples, and detection was performed at 227 nm for accurate quantification and identification of AzA [18].

Preparation of stock and working standard solutions

To prepare stock solutions, 10 mg of EGCG and 10 mg of AzA were precisely weighed into separate 10 ml volumetric flasks. Every bioactive component was dissolved in 10 ml of methanol, yielding a stock concentration of 1,000 µg/ml for each sample. Each stock solution was then diluted with its corresponding mobile phase to develop working standard solutions, each with a 100 µg/ml concentration. Serial dilutions of these working standards resulted in 5 to 30 µg/ml concentration ranges for EGCG and 2 to 12 µg/ml for AzA. A 0.22 µm syringe filter was used to filter all samples before chromatographic analysis to guarantee the lack of particulate matter and sufficient clarity [19].

Preliminary method development studies

Due to the limited aqueous solubility of EGCG, methanol was used as an organic solvent for dissolution. Therefore, methanol was selected as a key component of the mobile phase for chromatographic analysis. Early attempts using water as a diluent were unsuccessful, as no detectable peaks appeared in the chromatogram even after a 30-minute analysis. This problem was addressed by substituting water with a 60:40 mixture containing 0.1% orthophosphoric acid. This modification led to peak detection; however, slight tailing was observed, and retention times exceeded 15 minutes. Further optimization was conducted to improve separation and peak characteristics [20].

AzA showed good solubility in methanol, and hence, it was selected as a key component of the mobile phase for chromatographic analysis. Early attempts using water as a diluent were unsuccessful, as no detectable peaks appeared in the chromatogram even after a 30-minute analysis. To address this, water was replaced with 0.1% formic acid, and the mobile phase composed of Methanol: 0.1% formic acid in an 80:20 ratio was selected. This modification facilitated peak detection; however, slight tailing was observed, and retention times exceeded 15 minutes. Further optimization was undertaken to improve separation and peak characteristics [21].

To achieve optimal separation and peak characteristics, a QbD approach was employed. This systematic strategy facilitated the optimization of chromatographic conditions, ensuring robust and reliable analytical performance.

QbD-driven method optimization

Analysis of risk assessment

To identify variables potentially impacting method performance, preliminary risk evaluation studies are essential as a key precursor to chromatographic optimization. Ishikawa fishbone diagram was constructed using Minitab® 18 software (Minitab, LLC, USA) to illustrate the connections between Critical Method Parameters and Critical Analytical Attributes within the Analytical Target Profile. In this risk assessment, method parameters were evaluated based on their severity, probability of occurrence, and detectability to estimate their potential impact. To calculate the Risk Priority Number for each parameter, the allocated scores were mathematically multiplied. The parameters were then divided into three risk groups based on these risk priority number (RPN) values: high, medium, and low. Parameters exhibiting an RPN above 60 were selected for further optimization of factors to improve chromatographic conditions [22].

Central composite design

To optimize both independent and dependent variables, this study employs response surface methodology coupled with HPLC. The optimization procedure utilized a central composite design (CCD) through version 13.0 of DoE software. This investigation examined two independent variables: the percentage of organic phase (X_1) and the flow rate (X_2). The resulting dependent variables were retention time (Y_1), tailing factor (Y_2), and theoretical plate number (Y_3). Specifically, for EGCG optimization, the organic phase percentage (X_1) was varied at three levels: 50%, 60%, and 70%, while the flow rate (X_2) was held constant at 0.8, 1.0, and 1.2 ml/min as detailed in Table 1. In the optimization of AzA, the organic phase percentage (X_1) was set to 70%, 80%, and 90%, and the flow rate (X_2) was likewise adjusted to 0.8, 1.0, and 1.2 ml/min as detailed in Table 2. The gathered data were transformed into a polynomial equation, and a comprehensive ANOVA was performed to determine the best values of X_1 and X_2 for the desired results. The chosen model was assessed based on the response variables: RT (Y_1), tailing factor (Y_2), and theoretical plate number (Y_3) [23,24].

Comprehensive validation of the analytical method

ICH guidelines were followed in the validation of the developed HPLC technique to ensure the correctness and dependability of the analytical results. Validation included assessing linearity, accuracy (% recovery), system suitability, limits of detection (LOD), limit of quantification (LOQ), precision, and robustness [25–27].

Linearity

To determine the linearity of the developed method, EGCG and AzA standard solutions were prepared at various concentrations. For EGCG, the concentration range studied was 5 to 30 µg/ml with a mobile phase made up of methanol and 0.1% orthophosphoric acid (60:40). In contrast, AzA concentrations were examined from 2 to 12 µg/ml using a mobile phase of acetonitrile and 0.1% formic acid (80:20). Each concentration was analyzed in six replicates ($n = 6$). The generated data was statistically evaluated using the least squares method after the peak regions were displayed against the relevant concentrations. To prove linearity, the y-intercept and regression coefficient (R^2) were calculated from the response versus concentration linear regression plot [33,34].

LOD and LOQ

The standard deviation method was employed to determine the LOQ and LOD and the HPLC technique [35]. The subsequent equations were used to determine these limits:

$$LOD = 3.3 \times \frac{sd}{s}$$

$$LOQ = 10 \times \frac{sd}{s}$$

System suitability

System suitability parameters were assessed as part of the HPLC method validation to make sure the chromatographic system was functioning properly and producing accurate analytical results. To calculate the theoretical plates, peak area, and tailing factor for EGCG and AzA, the procedure involved six iterations of sample analysis [36].

Precision

Precision in HPLC method validation assesses the consistency and reproducibility of analytical results. For intra-day precision assessment, EGCG standard solutions (10, 15, and 20 µg/ml) and AzA standard solutions (4, 8, and 12 µg/ml) were made in three separate batches and examined three times in a single day. Three different solutions were prepared to evaluate the inter-day precision of each concentration of EGCG (10, 15, 20 µg/ml) and AzA (4, 8, and 12 µg/ml), and then analyzed these solutions once per day for three consecutive days. The samples were stored in tightly sealed containers in the dark between analyses [37,38].

Accuracy

The accuracy was evaluated by examining placebo samples that had been tampered with EGCG and AzA at three different concentration levels (50%, 100%, and 150% of the target) within the method's validated linear range. The accuracy of the procedure for standard EGCG, standard AzA, and *C. arborea* Roxb. nanoemulsion samples was expressed as a percentage recovery [39].

Robustness

To assess the robustness of the modified chromatographic process, important analytical parameters were purposefully changed. These included alterations in the mobile phase composition for EGCG (58:42 and 62:38) and AzA

Table 1. Execution of the central composite experimental design and the corresponding outcomes obtained for the proposed HPLC method for EGCG.

		Factor 1	Factor 2	Response 1	Response 2	Response 3
Std	Run	A: Organic phase (%)	B: Flow rate (ml/min)	RT	TF	TP
11	1	60	1	2.835	1.868	3,016.21
8	2	60	1.2	2.732	1.921	2,909.87
6	3	70	1	2.456	1.974	2,709.01
5	4	50	1	3.532	1.731	2,387.57
10	5	60	1	2.847	1.865	3,015.14
2	6	70	0.8	2.564	1.957	2,798.34
9	7	60	1	2.864	1.862	3,014.11
3	8	50	1.2	3.467	1.747	2,403.65
1	9	50	0.8	3.905	1.687	2,343.39
7	10	60	0.8	3.131	1.784	2,998.43
4	11	70	1.2	2.378	1.991	2,691.22

Table 2. Execution of the central composite experimental design and the corresponding outcomes obtained for the proposed HPLC method for azelaic Acid.

		Factor 1	Factor 2	Response 1	Response 2	Response 3
Std	Run	A: Organic phase (%)	B: Flow rate (ml/min)	RT	TF	TP
8	1	80	1.2	4.654	1.576	4,583.54
2	2	90	0.8	3.943	1.848	4,299.23
1	3	70	0.8	5.764	1.245	4,194.54
5	4	70	1	5.643	1.296	4,201.27
4	5	90	1.2	3.645	1.924	4,101.39
9	6	80	1	4.833	1.421	4,602.67
6	7	90	1	3.765	1.901	4,178.31
11	8	80	1	4.831	1.432	4,621.19
7	9	80	0.8	5.043	1.394	4,596.41
3	10	70	1.2	5.124	1.374	4,287.59
10	11	80	1	4.742	1.415	4,667.54

(78:22 and 82:18), adjustments to the flow rate (0.8 and 1.2 ml/min), and small changes in the detection wavelengths for EGCG (274 and 276 nm) and AzA (226 and 228 nm). The method was demonstrated to be dependable and to yield consistent results under the many experimental settings examined based on the findings of these evaluations [40,41].

Greenness evaluation using advanced eco-metrics and sustainability indices

AGREE, blue applicability grade index (BAGI), ComplexGAPI, and Analytical Eco-Scale were among the comprehensive assessment tools used to analyze the proposed method for environmental impact.

Complementary green analytical procedure index software

ComplexGAPI is an updated version of the well-known GAPI indicator, designed to more comprehensively assess the environmental impact of analytical methods. This tool aims to evaluate how environmentally friendly and sustainable analytical procedures are by examining key factors such as the waste produced, energy consumed, sample handling methods, reagents used, and the overall environmental burden. ComplexGAPI to compare different methods and identify the most ecologically friendly option. It assigns scores based on specific criteria, and the assessment is presented through a color-coded system: green indicates low environmental risk, yellow signifies medium risk, and red represents high risk [28].

AGREE metric approach

The AGREE metric serves as a well-established and validated means to gauge the environmental effects of analytical processes. AGREE's comprehensive assessment is fundamentally based on the 12 guiding principles of GAC. It is available via a mathematical application that may be downloaded at https://mostwiedzy.pl/AGREE. These guidelines concentrate on cutting waste, improving safety, and using less energy and chemicals. Greater adherence to the green chemistry principles is indicated by higher scores on the AGREE scoring

system, which runs from 0 to 1. The resulting aggregated scores are presented as a radar chart, which visually illustrates the method's overall environmental impact. This comprehensive assessment not only assesses environmental effects but also identifies areas for increased sustainability, facilitating ongoing development in laboratory procedures [29].

Utilizing the ESA

The Environmental Sustainability Assessment is a key tool for evaluating how analytical methods affect the environment. It is founded on the 12 principles of GAC. The ESA software assesses an analytical method's environmental friendliness by looking at factors like how many resources it uses, how much waste it creates, and any safety concerns, then gives it a score. Each principle of GAC, such as promoting, reducing energy consumption, safer solvents, and limiting hazardous reagents, is quantitatively evaluated with a range of 0 to 100. The Eco-Scale Score provides a straightforward measure of how environmentally efficient a method. Additionally, by pointing out areas for improvement, it promotes the ongoing development of analytical techniques, which results in the adoption of more environmentally friendly approaches. The Eco-Scale Score is determined by subtracting total penalty points from 100, with the various environmental effects of the procedure being taken into consideration when assigning these penalty points [30].

Blue applicability grade index

One important indicator in analytical chemistry, particularly when discussing white chemistry, is the BAGI. Based on performance characteristics, this index evaluates the appropriateness and efficacy of analytical techniques. High dependability and appropriateness for the desired analysis are indicated by a high BAGI score. Employing ten criteria, the BAGI framework evaluates methods and generates a pictogram and numerical score reflecting overall practicality. BAGI offers a comprehensive evaluation of analytical methods' environmental effects and utility by bringing together various assessment instruments. This helps to cultivate an all-encompassing perspective on white chemistry principles [31].

Standardization and quantitative estimation of EGCG and AzA in nanoformulation

For HPLC analysis, a 20 ml sample of the nanoemulsion underwent dissolution in 200 ml of methanol with 30 minutes of sonication to ensure complete solubility. The EGCG and AzA content of the *C. arborea* Roxb. nanoemulsion was quantified using the validated HPLC technique after a 20 ml aliquot of the generated solution was diluted with the mobile phase to reach a final concentration of 10 µg/ml [32].

RESULTS AND DISCUSSION

Phytochemical yield and extraction efficiency via SFE

Using solvents, the yield of crude extracts from the bark of *C. arborea* Roxb. was calculated. From an initial 150 g of bark powder, 11.34 g of extract were obtained on a w/w basis, indicating the yield of the air-dried bark material.

Phytochemical analysis by LC-MS/MS

The *C. arborea* Roxb. extract showed a rich phytochemical profile by LC-MS. Qualitative screening confirmed the presence of various phytoconstituents, including phytosterols, amino acids, carbohydrates, flavonoids, phenolic compounds, glycosides, proteins, and volatile oils. Analogous to parsley phytoconstituent identification, LC-MS of the SFE found 13 molecules depicted in Table 3 and the LC-MS Chromatogram shown in Figure 1.

Nanoemulsion development and characterization

The *C. arborea* Roxb. extract-loaded nanoemulsion was successfully formulated using the homogenization

Table 3. LCMS proposed compound.

Sl No	Proposed compounds	RT (min)	Precursor m/z	Adduct	Formula
1	3-Amino-Beta-Pinene	2.719	188.128	$[M+H]^+$	$C_{10}H_{18}CIN$
2	3-Hydroxy-3-methylglutarate	3.174	163.089	$[M+H]^+$	$C_6H_{10}O_5$
3	Valylvaline	3.224	217.156	$[M+H]^+$	$C_{10}H_{20}N_2O_3$
4	Azelaic acid	3.881	206.134	$[M+NH4]^+$	$C_9H_{16}O_4$
5	(4R)-3-methylidene-4-[(E)-3-methyl-4-(4-methyl-5-oxooxolan-2-yl)but-2-enyl] oxolan-2-one	4.234	287.124	$[M+Na]^+$	$C_{15}H_{20}O_4$
6	Daphnetin	4.386	179.1592	$[M+H-H2O]^+$	$C_9H_6O_4$
7	2-[[(2R,3S)-2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-3,4-dihydro-2H-chromen-7-yl] oxy] oxane-3,4,5-triol	4.588	271.130	$[M+Na]^+$	$C_{15}H_{20}O_3$
8	(-)-Epigallocatechin-3-gallate	4.638	458.372	$[M+H]^+$	$C_{22}H_{18}O_{11}$
9	(9E)-11a-hydroxy-3,6,10-trimethyl-6,7,8,11-tetrahydro-4H-cyclodeca[b]furan-2,5-dione	5.295	287.117	$[M+H]^+$	$C_{15}H_{20}O_4$
10	(2R)-1-(5-Hydroxy-3-methylpentyl)-2,5,5,8a-tetramethyldecahydro-2-naphthalenol	6.153	293.281	$[M-H2O+H]^+$	$C_{20}H_{38}O_2$
11	2-Penten-1-ol, 5-[(1S,4aS,8aS)-decahydro-5,5,8a-trimethyl-2-methylene-1-naphthalenyl]-3-methyl-, (2E)-	7.012	291.259	$[M+H]^+$	$C_{20}H_{34}O$
12	Mestarolone	7.315	289.251	$[M-H2O+H]^+$	$C_{20}H_{34}O_2$
13	4-Methylumbelliferyl acetate	8.678	219.230	$[M+H]^+$	$C_{12}H_{10}O_4$

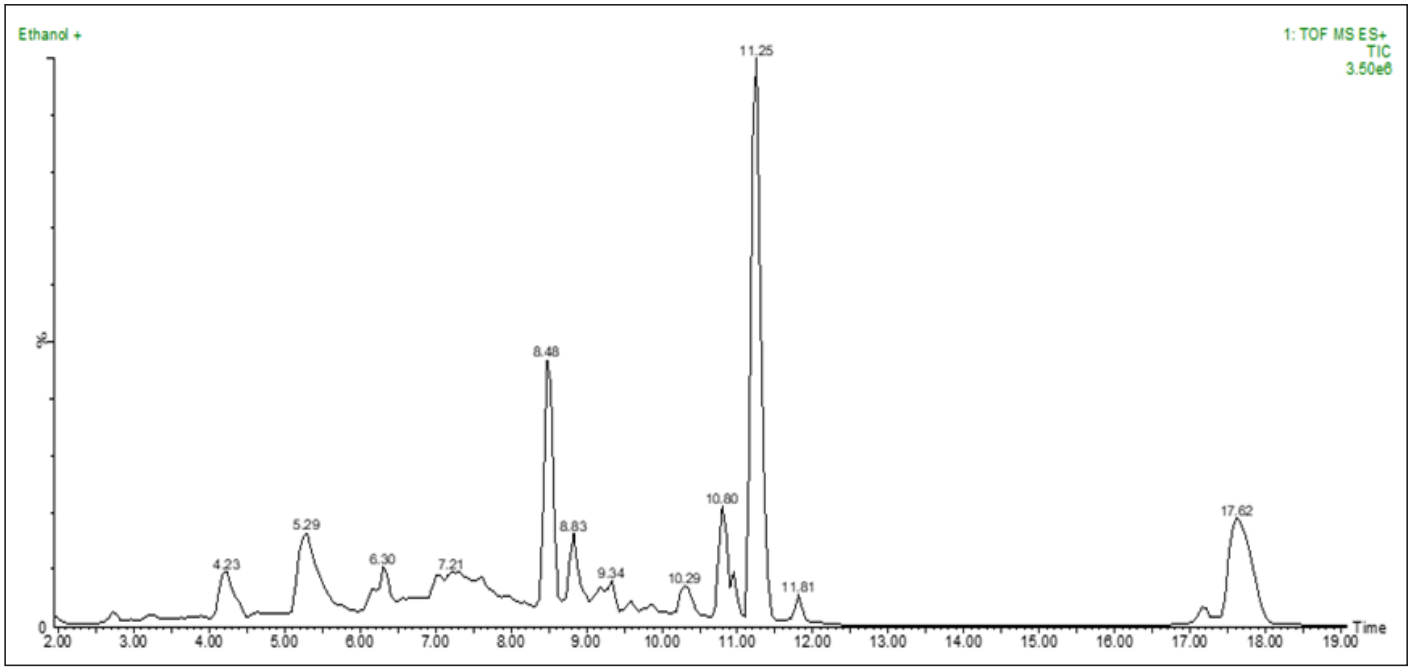

Figure 1. LCMS chromatograph.

technique. The formulation exhibited an average particle size of 153.52 ± 0.27 nm with a narrow particle size distribution (PDI 0.167 ± 0.23), confirming uniformity. The zeta potential was recorded at −38.9 mV, indicating good colloidal stability. The nanoemulsion demonstrated high encapsulation efficiency (%EE) of 84.2% ± 0.24% for EGCG and 79.43% ± 0.39% for AzA, attributed to the optimized surfactant concentration. Additionally, the pH of the nanoemulsion was found to be 6.12 ± 0.08, which falls within the acceptable range for topical applications and suggests good skin compatibility. These physicochemical characteristics collectively support the nanoemulsion potential as a stable and effective.

Stability assessment of prepared nanoemulsion

To assess the shelf-life and robustness of the formulation, a stability study was conducted under different storage conditions (4°C and 25°C) over 60 days. The nanoemulsion was evaluated at regular intervals for changes in physical appearance, phase separation, particle size, PDI, zeta potential, pH, and encapsulation efficiency. Throughout the study period, no signs of creaming, cracking, or phase separation were observed, indicating physical stability. The particle size was 165.52 ± 0.31 nm and PDI 0.167 ± 0.29 within acceptable limits, with only minimal variations, while the zeta potential −32.6 mV remained above −30 mV, suggesting maintained colloidal stability. The pH remained relatively stable (6.08–6.18), and encapsulation efficiency showed negligible loss (<5%) for both EGCG and AzA. These findings confirm that the *C. arborea* Roxb. extract-loaded nanoemulsion possesses satisfactory physicochemical stability under varied storage conditions, supporting its suitability for long-term topical application.

Systematic optimization of RP-HPLC conditions for dual marker separation

Risk assessment studies

Based on early testing and existing understanding of the RP-HPLC method, risk assessment studies were carried out to determine possible method risk characteristics. Using an Ishikawa diagram, we identified over 30 method risk characteristics and categorized them into six groups: material, measurements, techniques, personnel, environment, and machines (Fig. 2). RPN ranking and filtering were used to evaluate the risk of possible technique risk characteristics. We used RPN ranking and filtering to assess the risk of potential technique-related issues. The percentage flow rate, organic phase, and column temperature were identified as high-risk method parameters, each having an RPN score above 60. For the desired method to be developed, these parameters had to be under control. Other technique risk factors were modified for suitable chromatographic settings and were deemed medium- and low-risk. When high-risk method parameters in the measurements category changed, the critical analytical attributes (CAAs) most affected were the tailing factor, retention time, and theoretical plates. Consequently, these three CAAs (tailing factor, retention time, and theoretical plates) for both AzA and EGCG were selected for the factor optimization tests.

CCD-assisted RP-HPLC method development for EGCG and AzA

Chromatographic conditions were optimized via CCD, using organic phase volume and flow rate as independent variables. The tailing factor, theoretical plates, and retention time for both EGCG and AzA were the dependent variables

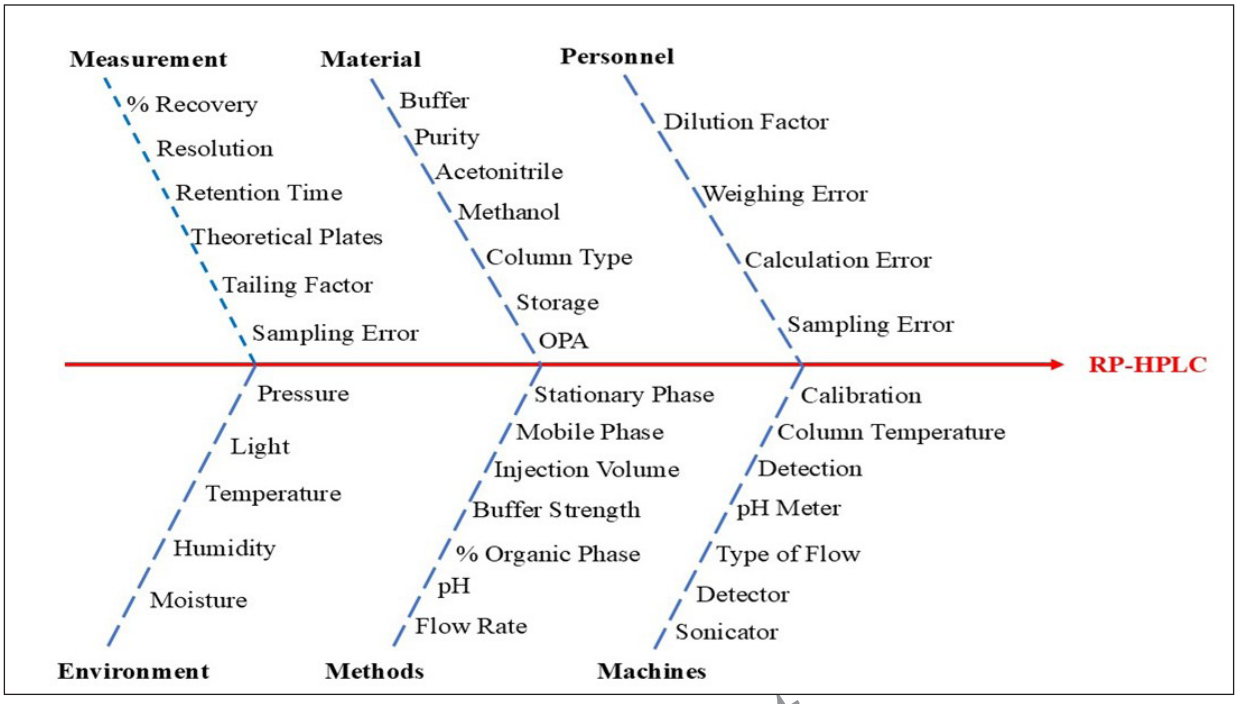

Figure 2. Ishikawa diagram for the identification of potential method risk parameters for the development of the chromatography method.

in the optimization procedure. The Design-Expert program, version 13.0, was used to evaluate the data produced by the CCD studies. The software generated a total of 11 experimental runs, including three center points, as detailed in Tables 1 and 2. The significance of the covariates and how they interacted with the response variables was evaluated using ANOVA. Tables S1–S6 provide a summary of the statistical analysis results for EGCG, while Tables S7–S12 show the results for AzA. The relationships between the independent (factor) and dependent (response) variables were modeled using polynomial equations. Positive coefficients indicated synergistic effects, while negative coefficients suggested antagonistic effects within the model. Strong correlations among all equations confirmed the achievement of optimal values for the selected responses, as outlined in equations 1 to 6.

1. $RT\ EGCG = +2.85 - 0.5843 * A - 0.1705 * B + 0.0630 * AB + 0.1462 * A2 + 0.0837 * B2$
2. $TF\ EGCG = +1.86 + 0.1262 * A + 0.0385 * B - 0.0065 * AB - 0.0096 * A2 - 0.0096 * B2$
3. $TP\ EGCG = +3000 + 177.33 * A - 22.57 * B - 41.85 * AB - 429.04 * A2 - 23.18 * B2$
4. $RT\ AzA = +4.83 - 0.8630 * A - 0.2212 * B + 0.0855 * AB - 0.1672 * A2 - 0.0227 * B2$
5. $TF\ AzA = +1.44 + 0.2930 * A + 0.0645 * B - 0.0133 * AB + 0.1426 * A2 + 0.0291 * B2$
6. $TP\ AzA = +4615.00 - 17.33 * A - 19.67 * B - 72.75 * AB - 403.00 * 22 - 3.00 * B2$

The response surface plots for EGCG illustrate how changes in chromatographic parameters affect its retention time (Y_1), tailing factor (Y_2), and theoretical plates (Y_3). These figures show that the retention time of EGCG reduces with increasing organic phase content and flow rate, indicating an enhanced elution procedure. Plotting for Y2 (tailing factor) correlates: a larger tailing factor was achieved by utilizing a quicker flow rate and increasing the organic phase concentration (Fig. 3a and b). About Y_3 (theoretical plates), increasing the organic phase concentration to 60% v/v and the flow rate to 1 ml/min increased the number of theoretical plates, indicating higher separation efficiency. However, exceeding these optimums leads to a drop in theoretical plates, suggesting a loss of chromatographic resolution (Fig. 3c).

The response surface plots for AzA illustrate how chromatographic parameters affect its retention time (Y_1), tailing factor (Y_2), and theoretical plates (Y_3). According to these plots, a faster elution of AzA is suggested by the decrease in retention time that occurs when the flow rate and organic phase concentration are increased. The plots show a clear correlation concerning Y_2 (tailing factor): higher flow rate and organic phase content resulted in a higher tailing factor (Fig. 4a and b). For AzA Y_3 (theoretical plates), Figure 4c demonstrates that separation was enhanced by raising the organic phase 60% v/v and the flow rate to 1 ml/min. Once these thresholds were surpassed, theoretical plates and resolution decreased.

Using Stat-Ease, Inc.'s Design-Expert software (version 13.0), we identified the ideal chromatographic conditions as an 80% v/v organic phase and a 1 ml/min flow rate. These predicted values closely matched the experimental outcomes, with the prediction error being limited to

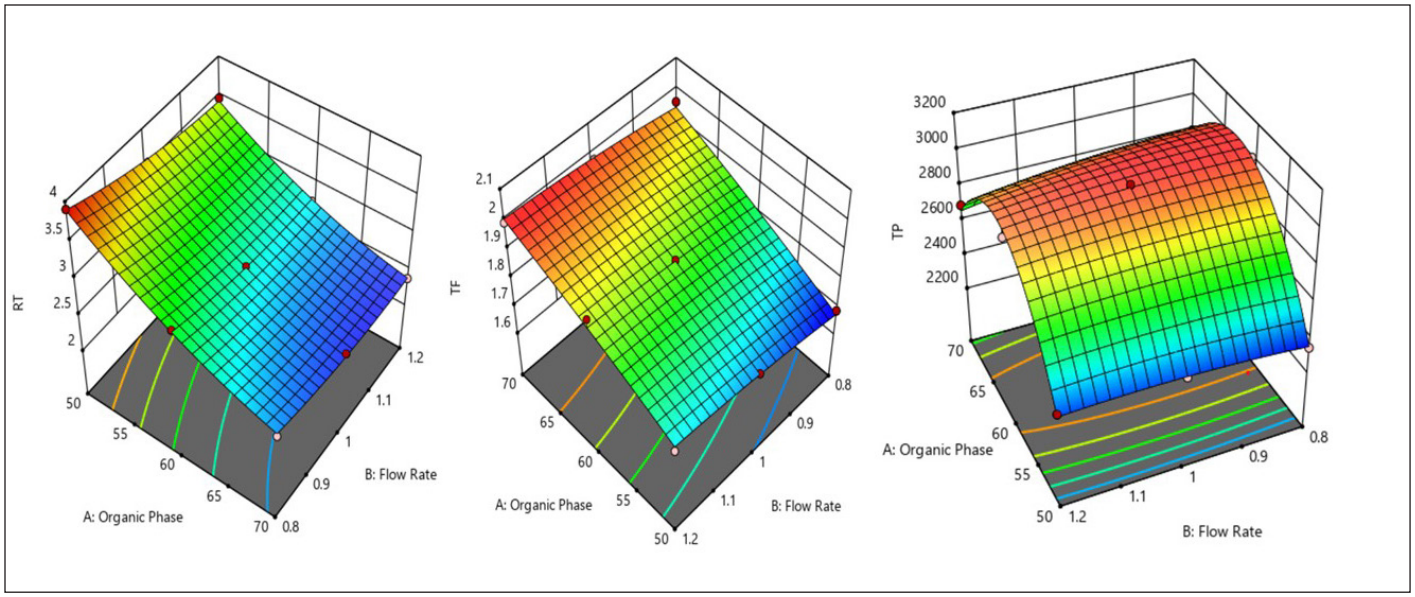

Figure 3. Response surface plot showing the effect of % organic phase (X_1), and flow rate (X_2), on (A) RT, (B) TF, and (C) TP of EGCG.

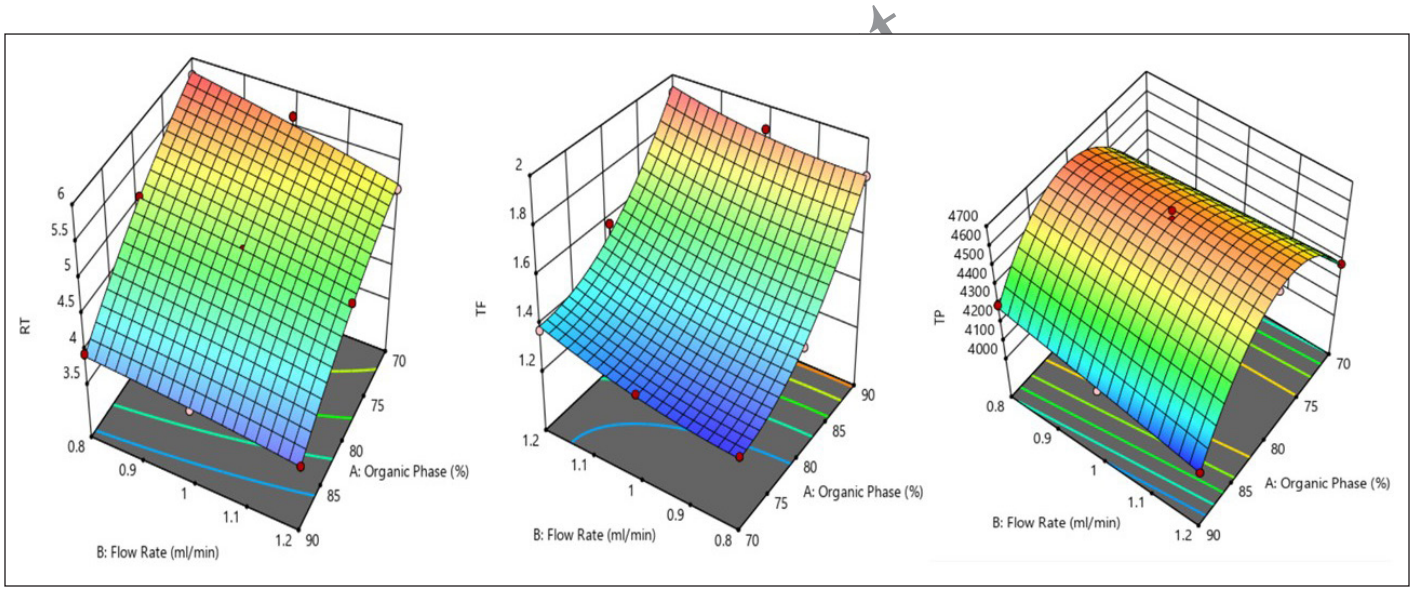

Figure 4. Response surface plot showing the effect of % organic phase, flow rate (X_2), on (A) RT, (B) TF, and (C) TP of AzA.

5%, particularly in Run 9. The use of these optimized chromatographic conditions led to excellent separation of the drug, characterized by high resolution and efficient method performance (Figs. 5 and 6).

Method validation

Plotting the average peak area of EGCG and AzA against their respective concentrations, which varied from 2 to 12 µg/ml for AzA and 5 to 30 µg/ml for EGCG, developed a calibration curve that was used to confirm linearity. The linear regression equation was used to get the LOD and LOQ. The suitability of the method was confirmed by calculating the % RSD for the four key analytical parameters: theoretical plates, tailing factor, peak area, and RT. The method's precision and acceptability were confirmed by all % RSD values being less than 2%. Using minor changes to the HPLC parameters (wavelength, column oven temperature, and mobile phase composition), robustness was further evaluated. The findings demonstrated that % RSD values for both peak area and RT remained well below the acceptable threshold of ≤2%. Additionally, intra-day and inter-day precision assessments confirmed the method's high reproducibility. Accuracy, evaluated via percentage recovery experiments at concentrations of 50%, 100%, and 150%, further supported the method's reliability. These findings, detailed in Table 4, conclude that the developed method exhibits robustness and precision.

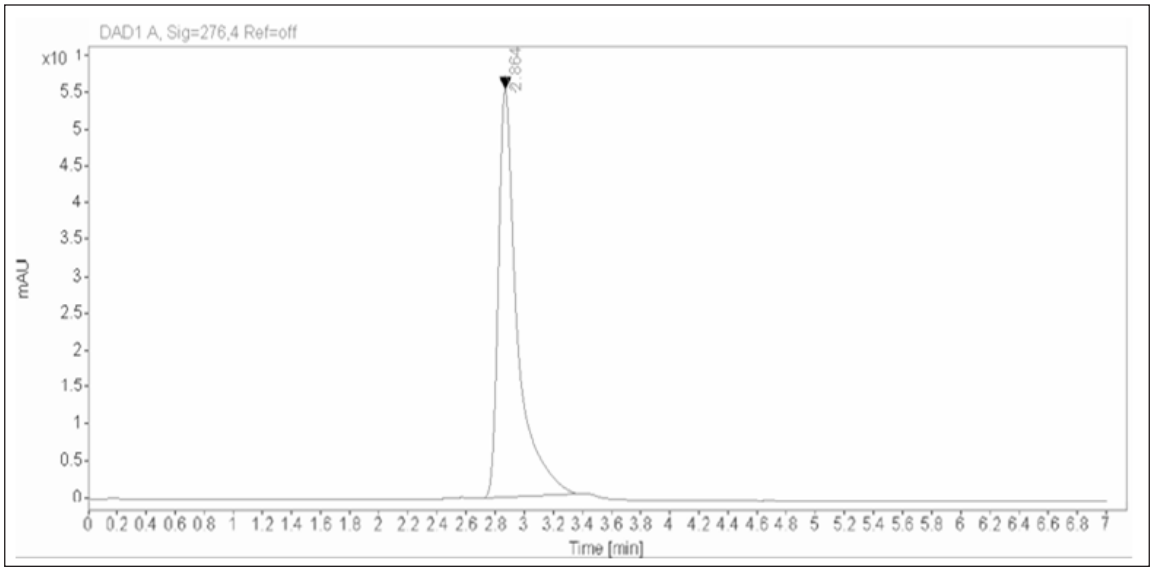

Figure 5. HPLC chromatogram of EGCG under the optimized analytical conditions.

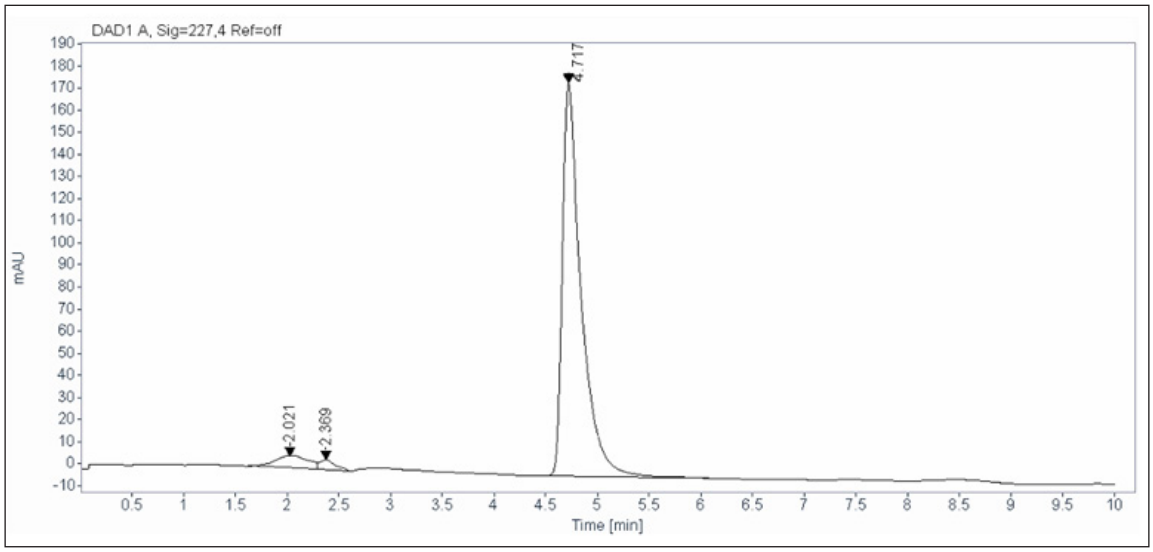

Figure 6. HPLC chromatogram of under the optimized analytical conditions of azelaic acid.

Greenness and whiteness evaluation of the proposed method

Using ComplexGAPI, we assessed the proposed method's environmental impact, which was determined. The results are visually represented by a color-coded pictogram. This pictogram includes five pentagrams, each denoting a specific aspect, such as sample preparation, reagent/solvent use, apparatus, and the type of procedure, along with a hexagon for the pre-analysis phase. Red, yellow, and green indicate high, moderate, and minimal environmental impact, respectively. Ten green, one red, and four yellow indications were produced by the HPLC method designed for EGCG, together with an E factor of 2 (Fig. 7a). Similarly, the AzA technique displayed five yellow, one red, and nine green signs, with an E factor of 2 (Fig. 8a).

A clock-style pictogram was created using the AGREE tool, which includes 12 evaluation criteria, to further assess the method's environmental sustainability. In addition to displaying a central score, this graphic depicts the environmental impact using a color gradient that goes from deep green, which denotes good sustainability, to deep red, which denotes low sustainability. The AGREE tool evaluation of the developed HPLC method resulted in scores of 0.75 for EGCG and 0.71 for AzA, predominantly showing a green profile (Fig. 7b and 8b). The procedure is a great green strategy, according to the Analytical Eco-Scale, a semi-quantitative tool that rates EGCG and AzA at 78 and 74, respectively, based on waste creation, energy consumption, and the usage of dangerous compounds. The BAGI tool also confirmed the feasibility and applicability of the developed method, yielding scores of 75 for EGCG and 72.5 for AzA (Fig. 7c and 8c), indicating its potential for regular implementation.

Quantitative standardization of EGCG and AzA in *C. arborea* Roxb. nanoformulation

The quantification of EGCG and AzA in the formulated *C. arborea* Roxb. nanoemulsion was successfully performed using the developed method in six replicate analyses. Table 5 recovery rates, which varied from 95.5% to 97.8%, demonstrate satisfactory performance. The quantitative analysis's results demonstrated clear, distinct peaks for both AzA and EGCG, highlighting the new method's excellent accuracy, sensitivity, and precision. The reliability of these quantitative findings is confirmed and presented in Figures 9 and 10.

Table 4. Summary of validation parameters.

Sr. no	Validation parameters	EGCG	AzA
1	Linearity		
	Linearity range (µg/ml)	5-30	2–12
	Correlation-coefficient	<0.998	<0.999
2	LOD (µg/ml)	1.35	0.57
	LOQ (µg/ml)	4.05	1.71
3	System suitability		
	Peak area (%RSD)	1.74	0.78
	Retention time (%RSD)	0.67	0.96
	Tailing factor (%RSD)	0.59	1.23
	Theoretical plates (%RSD)	0.56	0.95
4	Precision		
	Intra-day (%RSD)	1.07	0.93
	Inter-day (%RSD)	1.29	1.19
5	Robustness		
	Mobile phase ratio (%RSD)	0.61	0.78
	Flow rate (%RSD)	0.29	0.83
	Detection wavelength (%RSD)	0.41	0.96
6	Accuracy		
	50% recovery	95.12 ± 0.23	96.51 ± 0.27
	100% recovery	97.01 ± 0.54	99.45 ± 0.65
	150% recovery	99.09 ± 0.36	98.92 ± 0.32

This investigation presents a robust analytical and formulation framework aimed at the standardization and effective delivery of phytoconstituents derived from *C. arborea* Roxb., a plant traditionally revered for its medicinal properties. A yield of 11.34 g was obtained from 150 g of bark powder using SFE, showcasing the advantages of this eco-friendly technique. Compared to conventional methods, which often involve lengthy procedures and high solvent consumption, SFE provides a more sustainable and efficient approach to isolating bioactive compounds [13,14].

Comprehensive LC-MS/MS analysis of the extract revealed a wide spectrum of bioactives, including phenolics, flavonoids, sterols, and glycosides. These findings support previous literature on the medicinal value of *C. arborea* Roxb. [3,4,9]. However, our application of LC-MS/MS post-SFE allowed for better preservation of sensitive phytochemicals, addressing the shortcomings observed in earlier studies that relied on alcohol or aqueous-based extraction systems [1,2,13].

The formulation of a nanoemulsion encapsulating *C. arborea* Roxb. extract represents a significant leap in botanical delivery systems. The nanoformulation demonstrated excellent physical characteristics—average particle size around 153.52 nm, zeta potential of 38.9 mV, and encapsulation efficiency exceeding 84%. These results align well with previously reported nanoemulsion systems based on medicinal plant extracts such as *Curcuma longa* and *Plantago major*, both recognized for their role in wound healing [5,7]. The improved solubility and bioavailability typically associated with nanoscale formulations further emphasize the therapeutic potential of this delivery strategy.

One of the study's major innovations lies in the QbD-based development of a robust RP-HPLC method for the simultaneous quantification of EGCG and AzA. Employing CCD, key variables such as flow rate and organic phase composition were systematically optimized to influence method parameters such as retention time, peak shape, and theoretical plates. This approach, which replaces traditional trial-and-error strategies, reflects the modern trend of QbD integration into analytical method development in pharmaceutical sciences [11].

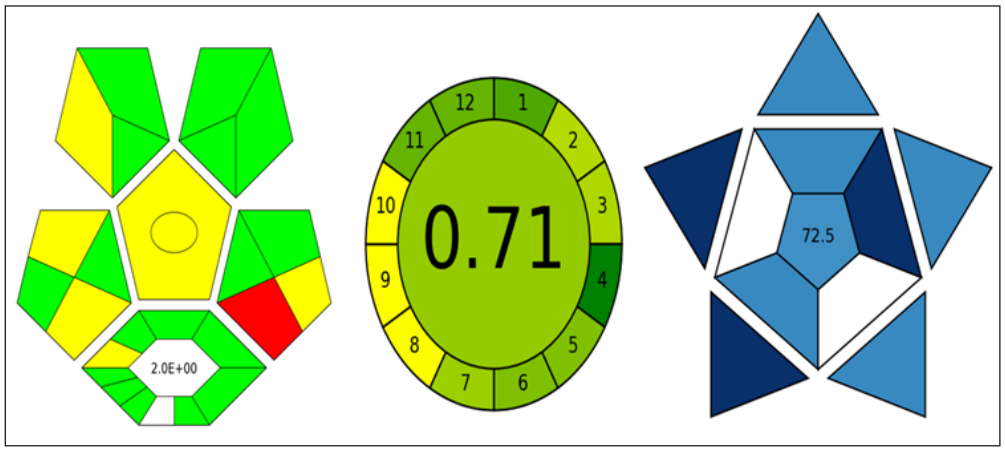

Figure 7. Evaluating the "greenness and whiteness" of the suggested technique, using (a) ComplexGAPI, (b) AGREE, and (c) BAGI for AzA.

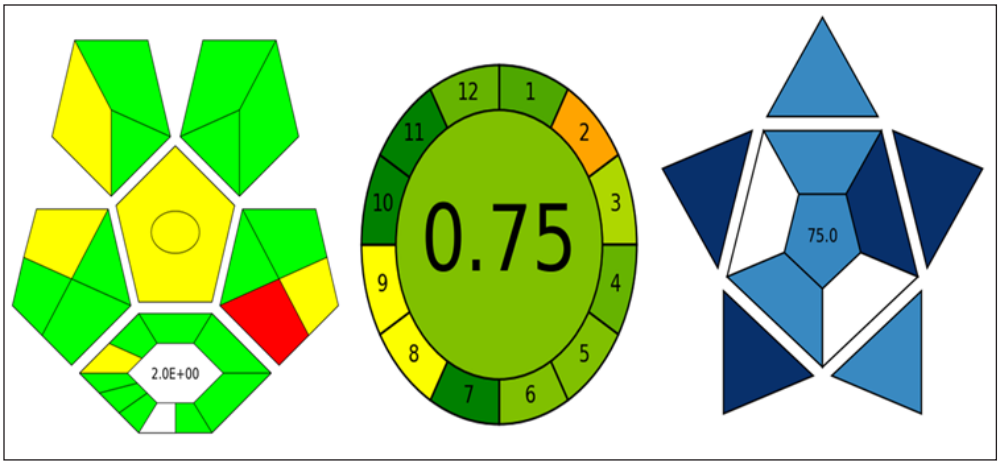

Figure 8. Evaluating the "greenness and whiteness" of the suggested technique, using (a) ComplexGAPI, (b) AGREE, and (c) BAGI for EGCG.

Validation data confirmed that the developed method was precise, reproducible, and reliable, with all RSD values below the 2% threshold. These outcomes are in line with recent validations of chromatographic methods tailored for polyphenols and AzA [10,11,15], reaffirming the method's robustness and suitability for routine analysis.

Environmental assessment of the developed method was performed using tools such as Complex GAPI, AGREE, and the Analytical Eco-Scale. The method scored well across all platforms, with AGREE values of 0.75 (EGCG) and 0.71 (AzA), and Eco-Scale ratings above 70. Such scores highlight its compliance with sustainable analytical practices, as advocated in recent green chemistry frameworks [12,13,16].

Finally, the validated method was applied for the quantitative determination of EGCG and AzA in the developed nanoemulsion. Recovery values ranging between 95.5% and 97.8% confirmed its applicability for formulation analysis. The ability to accurately measure key phytoconstituents post-formulation is crucial for maintaining quality and efficacy in herbal therapeutics, a need emphasized in modern phytopharmaceutical research [2,17].

Table 5. Recovery assay of the proposed method from the prepared nanoemulsion.

Drug	Excess drug added to analytes (%)	Spiked (µg/ml)	Found (µg/ml)	Recovery (%)
EGCG	50	15	14.54	96.2
	100	20	18.98	95.6
	150	25	24..03	96.0
AzA	50	6	5.76	96.1
	100	8	7.56	97.8
	150	10	8.97	95.5

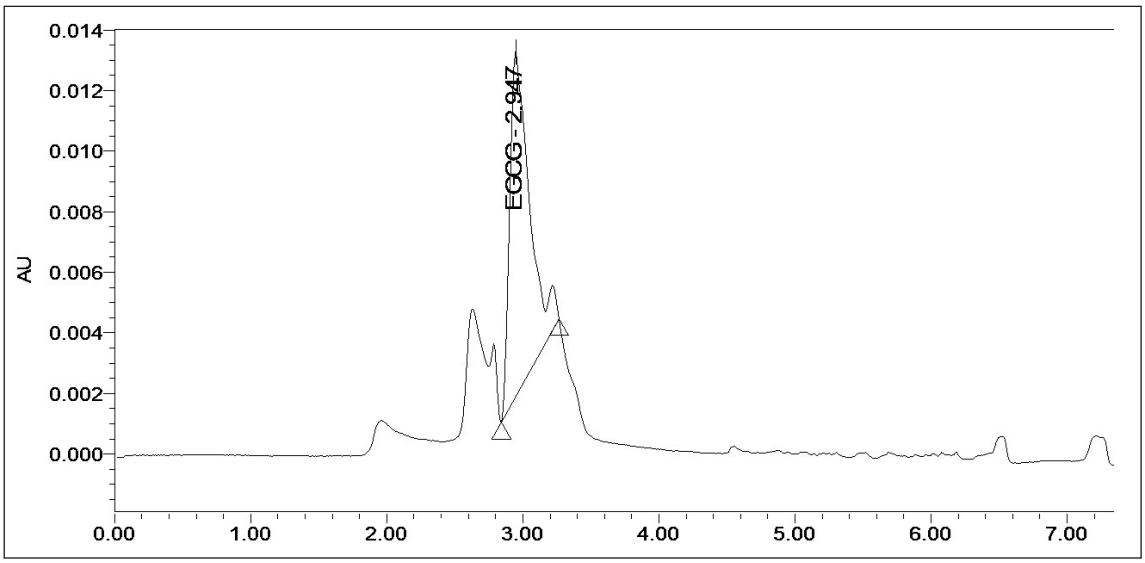

Figure 9. HPLC chromatogram of nanoemulsion under the optimized analytical conditions.

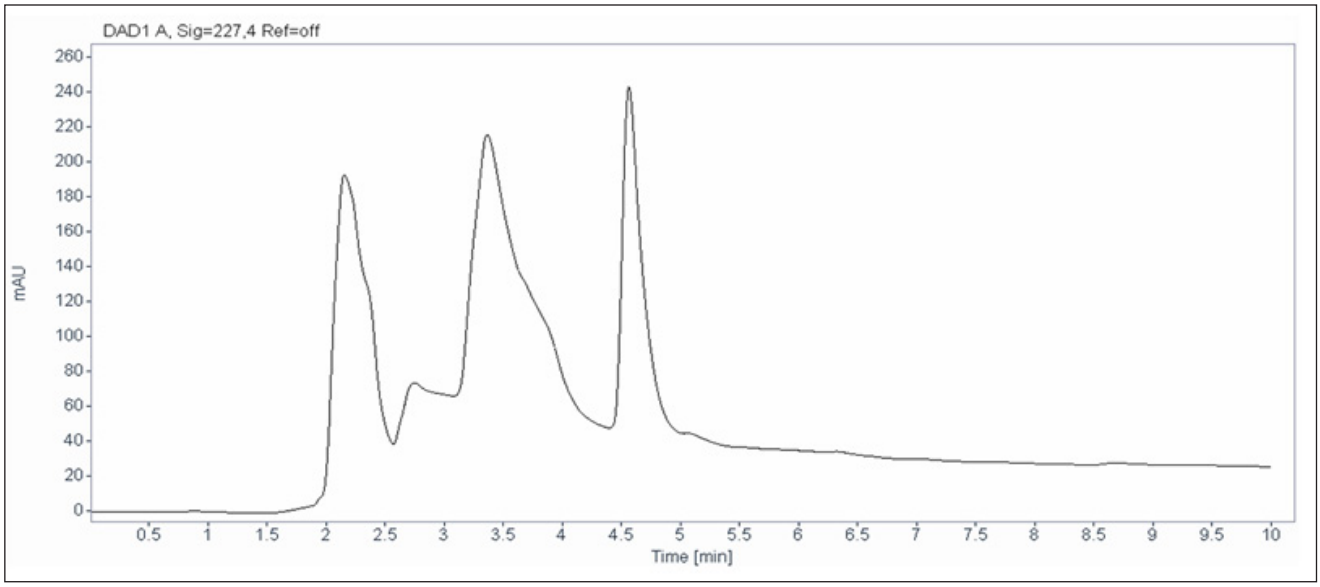

Figure 10. HPLC chromatogram of nanoemulsion under the optimized analytical conditions of azelaic acid.

CONCLUSION

This research presents a sustainable and scientifically sound framework for the analytical standardization of *C. arborea* Roxb. extract formulated into a nanoemulsion. SFE proved effective in achieving a high yield of bioactive compounds, supporting a cleaner and more efficient alternative to conventional extraction methods. The resulting nanoemulsion displayed favorable properties, including uniform particle size, strong surface charge, and high encapsulation efficiency, indicating its potential for improved phytochemical delivery. A significant highlight of the study is the use of CCD under the QbD approach to optimize RP-HPLC conditions. The developed method demonstrated excellent sensitivity, accuracy, and precision for the simultaneous estimation of EGCG and AzA. Environmental sustainability of the method was also confirmed through recognized greenness evaluation tools. The study contributes meaningfully to the field of green pharmaceutical analysis, offering a practical path forward for reliable herbal formulation development and quality control.

LIST OF ABBREVIATIONS

AGREE, Analytical GREEnness; AzA, azelaic acid; BAGI, blue applicability grade index; CAAs, critical analytical attributes; CCD, central composite design; CMPs, critical method parameters; ComplexGAPI, Complementary Green Analytical Procedure Index; DoE, design of experiment; EGCG, (-)-epigallocatechin-3-gallate; ESA, Eco-Scale Analytical; GAC, green analytical chemistry; HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; TF, tailing factor; tR, retention time.

ACKNOWLEDGMENTS

The authors are very thankful to Principal Dr. S.S. Jalalpure and the Vice Principal Dr. M. B. Patil, KLE College of Pharmacy, Belagavi, for their support and guidance. The authors are also thankful to the Department of Pharmaceutics and KLE College of Pharmacy, Belagavi.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to the conception and design, data acquisition, or data analysis and interpretation; participated in drafting or critically revising the article for important intellectual content; agreed to submit it to the current journal; gave final approval of the version to be published; and accepted responsibility for all aspects of the work. All authors meet the criteria established by the International Committee of Medical Journal Editors (ICMJE) guidelines.

FINANCIAL SUPPORT

There is no funding to report.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

PUBLISHER'S NOTE

All claims expressed in this article are solely those of the authors and do not necessarily represent those of the publisher, the editors, and the reviewers. This journal remains neutral concerning jurisdictional claims in published institutional affiliations.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI) tools for writing and editing the manuscript, and no images were manipulated using AI.

REFERENCES

1. Chaachouay N, Zidane L. Plantderived natural products: a source for drug discovery and development. Drugs Drug Candidates. 2024;3(1):184–207. doi: https://doi.org/10.3390/ddc3010011
2. Paul T, Kumar KJ. Standardization of herbal medicines for lifestyle diseases. In: Dhara AK, Mandal SC, editors. Role of herbal medicines: management of lifestyle diseases. Singapore: Springer; 2024. pp: 545–557. doi: https://doi.org/10.1007/9789819977031_27
3. Khaliq HA. Pharmacognostic, physicochemical, phytochemical and pharmacological studies on *Careya arborea* Roxb.; a review. J Phytopharmacol. 2016;5(1):27–34.
4. Gupta P, Patil D, Patil A. Qualitative HPTLC phytochemical profiling of *Careya arborea* Roxb. bark, leaves and seeds. 3 Biotech. 2019;9(8):311. doi: https://doi.org/10.1007/s132050191846x
5. Kumari M, Nanda DK. Potential of Curcumin nanoemulsion as antimicrobial and wound healing agent in burn wound infection. Burns 2023;49(5):1003–16. doi: https://doi.org/10.1016/j.burns.2022.10.008
6. Liakopoulou A, Letsiou S, Avgoustakis K, Hatziantoniou S. Comparative study between nanoemulsions and conventional emulsions as carriers of plant oils: physicochemical and invivo performance. Cosmetics 2025;12(3):102. doi: https://doi.org/10.3390/cosmetics12030102
7. Jahanimoghadam F, Javidan A, Ranjbar M, Torabi M, Kakooei S, Sharififar F. The healing effect of nanoemulsified *Plantago major* L extract on oral wounds in a Wistar rat model. BMC Complement Med Ther. 2024;24:327. doi: https://doi.org/10.1186/s12906024040001
8. Myint YL, Kyu KM, Sint TT. Phytochemical, physicochemical and antimicrobial activities on flowers of *Careya arborea* Roxb. J Myanmar Acad Arts Sci. 2021;19(4B):77.
9. De França Ferreira ÉL, de Carvalho Oliveira JP, de Araújo MR, Rai M, Chaves MH. Phytochemical profile and ethnopharmacological applications of Lecythidaceae: an overview. J Ethnopharmacol. 2021;274:114049. doi: https://doi.org/10.1016/j.jep.2021.114049
10. AlSulaimi S, Kushwah R, Abdullah Alsibani M, El Jery A, Aldrdery M, Ashraf GA. Emerging developments in separation techniques and analysis of chiral pharmaceuticals. Molecules 2023;28(17):6175. doi: https://doi.org/10.3390/molecules28176175
11. Tome T, Žigart N, Časar Z, Obreza A. Development and optimization of liquid chromatography analytical methods by using AQbD principles: Overview and recent advances. Org Process Res Dev. 2019;23(9):1784–802. doi: https://doi.org/10.1021/acs.oprd.9b00238
12. Meher AK, Zarouri A. Green analytical chemistry—recent innovations. Analytica 2025;6(1):10. doi: https://doi.org/10.3390/analytica6010010
13. Uwineza PA, Waśkiewicz A. Recent advances in supercritical fluid extraction of natural bioactive compounds from plant materials. Molecules 2020;25(17):3847. doi: https://doi.org/10.3390/molecules25173847.
14. Toribio L, Martín MT, Bernal J. Supercritical fluid chromatography in bioanalysis: a review. J Sep Sci. 2024;47(21):e70003. doi: https://doi.org/10.1002/jssc.70003.
15. Chouhan B. Supercritical fluid chromatography: a review on a key green analytical technique in pharmaceutical spectral analysis. J Sep Sci. 2025;1(1):1–10. doi: https://doi.org/10.69557/n70bvv08
16. Pilařová V, Plachká K, Herbsová D, Kosturko Š, Svec F, Nováková L. Comprehensive twostep supercritical fluid extraction for green isolation of volatiles and phenolic compounds from plant material. Green Chem. 2024;26(11):6480–9. doi: https://doi.org/10.1039/d4gc00371c
17. Nitthikan N, Preedalikit W, Supadej K, Chaichit S, Leelapornpisid P, Kiattisin K. Exploring the wound healing potential of a *Cuscuta chinensis* extract-loaded nanoemulsion-based gel. Pharmaceutics. 2024;16(5):573. doi: https://doi.org/10.3390/pharmaceutics16050573
18. Rodrigues VH, Portugal I, Silva CM. Economic analysis of the supercritical fluid extraction of lupane-triterpenoids from *Acacia dealbata* Link bark. Ind Crops Prod. 2023;200:116838. doi: https://doi.org/10.1016/j.indcrop.2023.116838
19. Herzyk F, Piłakowska-Pietras D, Korzeniowska M. Supercritical extraction techniques for obtaining biologically active substances from a variety of plant byproducts. Foods 2024;13(11):1713. doi: https://doi.org/10.3390/foods13111713
20. Patel N, Dave K, Patel R, Vegad K, Kanaki N. HPLC-based metabolomic fingerprint profiling as a comprehensive quality assessment tool, why not for standardization of ayurvedic herbs and formulations. Pharmacognosy Res. 2024;16(4):677–87. doi: http://dx.doi.org/10.5530/pres.16.4.79
21. Jouaneh TM, Motta N, Wu C, Coffey C, Via CW, Kirk RD, et al. Analysis of botanicals and botanical supplements by LC-MS/MS-based molecular networking: approaches for annotating plant metabolites and authentication. Fitoterapia 2022;159:105200.
22. Aayush K, Sharma K, Singh GP, Chiu I, Chavan P, Shandilya M, et al. Development and characterization of edible and active coating based on xanthan gum nanoemulsion incorporating betel leaf extract for fresh produce preservation. Int J Biol Macromol. 2024;270:132220. doi: https://doi.org/10.1016/j.ijbiomac.2024.132220
23. Semysim FA, Hussain BK, Hussien MA, Azooz EA, Snigur D. Assessing the greenness and environmental friendliness of analytical methods: modern approaches and recent computational programs. Crit Rev Anal Chem. 2024;12:1–4. doi: https://doi.org/10.1080/10408347.2024.2304552
24. Koli R, Mannur VS. Green RP-HPLC method for simultaneous quantification of epigallocatechin-3-gallate and rosmarinic acid in lipid-based nanocarriers and biological fluids: quality by design-driven optimization and lean six sigma approach. Green Anal Chem. 2024;11:100153. doi: https://doi.org/10.1016/j.greeac.2024.100153
25. Prajapati SK, Jain A, Bajpai M. Development and validation of the RP-HPLC method for quantification of tavaborole. Anal Methods. 2024;16(30):5280–7. doi: https://doi.org/10.1039/D4AY00943F
26. Gulawani S, Mahajan S, Waghole RJ, Srivastava P. Comprehensive metabolic profiling of Mandarin Peel different extracts obtained by conventional and green methods by Sciex LC-MS/MS-Qtrap. J Food Compos Anal. 2025;21:107254. doi: https://doi.org/10.1016/j.jfca.2025.107254
27. Maan P, Chauhan S, Gupta N, Rani D. Dual-drug quantification: HPLC method validation for hesperidin and piperine in ethosomal delivery systems. Int J Appl Pharm. 2025;17(2):240–9. doi: https://dx.doi.org/10.22159/ijap.2025v17i2.53262
28. Lee SG, Ko H, Choi EJ, Oh DR, Bae D, Choi C. Isolation and analytical method validation for phytocomponents of aqueous leaf extracts from *Vaccinium bracteatum* Thunb. in Korea. Processes 2021;9(11):1868. doi: https://doi.org/10.3390/pr9111868
29. Sezgin B, Soyseven M, Arli G. Greenness assessment and comparison of the developed and validated green HPLC-PDA, HPLC-FLD, and HPLC-ELSD methods for the determination of melatonin in various products using analytical eco-scale, NEMI, GAPI, and AGREE greenness metric tools. Microchem J. 2024;205:111211. doi: https://doi.org/10.1016/j.microc.2024.111211
30. Magdy MA, Abdelfatah RM, Abdelrahman MM, Elsayed MA. Feasible HPLC method for simultaneous determination of triple therapy prescribed for cancer patients with application to human plasma: method's greenness, whiteness, and blueness appraisal. Microchem J. 2025;209:112731. doi: https://doi.org/10.1016/j.microc.2025.112731

31. Shrigod NM, Swami Hulle NR, Prasad RV. Supercritical fluid extraction of essential oil from mint leaves (*Mentha spicata*): process optimization and its quality evaluation. J Food Process Eng. 2017;40(3):e12488. doi: https://doi.org/10.1111/jfpe.12488
32. Slighoua M, Mahdi I, Moussaid FZ, Kamaly OA, Amrati FE, Conte R, et al. LC-MS/MS and GC/MS profiling of *Petroselinum sativum* Hoffm. and its topical application on burn wound healing and related analgesic potential in rats. Metabolites 2023;13(2):260. doi: https://doi.org/10.3390/metabo13020260
33. Chandrakasan G, García-Trejo JF, Feregrino-Pérez AA, Aguirre-Becerra H, García ER, Nieto-Ramírez MI. Preliminary screening on antibacterial crude secondary metabolites extracted from bacterial symbionts and identification of functional bioactive compounds by FTIR, HPLC and gas chromatography–mass spectrometry. Molecules 2024;29(12):2914. doi: https://doi.org/10.3390/molecules29122914
34. Gandu S, Gandla K. Development of a quality by design-based ultra-performance liquid chromatography method for the simultaneous estimation of casirivimab and imdevimab with greenness metrics. Green Anal Chem. 2025;13:100248. doi: https://doi.org/10.1016/j.greeac.2025.100248
35. Spaggiari C, Annunziato G, Spadini C, Montanaro SL, Iannarelli M, Cabassi CS, et al. Extraction and quantification of azelaic acid from different wheat samples (*Triticum durum* Desf.) and evaluation of their antimicrobial and antioxidant activities. Molecules 2023;28(5):2134. doi: https://doi.org/10.3390/molecules28052134
36. Ádám AL, Kátay G, Künstler A, Király L. Detection of lipid peroxidation-derived free azelaic acid, a biotic stress marker and other dicarboxylic acids in tobacco by reversed-phase HPLC-MS under non-derivatized conditions. Methods Mol Bio. 2022;2526:191–200. doi: https://doi.org/10.1007/978-1-0716-2469-2_14
37. Kunchanur M, Mannur VS, Koli R. High-performance liquid chromatography–based standardization of stigmasterol in *Moringa oleifera*: method development and validation through design of experiment approach. SSC Plus. 2024;7(9):e202400105. doi: https://doi.org/10.1002/sscp.202400105
38. Wadie M, Abdel-Moety EM, Rezk MR, Tantawy MA. Eco-friendly chiral HPLC method for determination of alfuzosin enantiomers and solifenacin in their newly pharmaceutical combination: method optimization via central composite design. Microchem J. 2021;165:106095. doi: https://doi.org/10.1016/j.microc.2021.106095
39. Singh BK, Verma H, Singh N, Singh P, Chaudhary A, Rajpoot AK. Optimization of HPLC method by using central composite design for simultaneous estimation of montelukast and ebastine dosage form. RJPT. 2024;17(4):1844–50. doi: http://dx.doi.org/10.52711/0974-360X.2024.00293
40. Mady MS, Sobhy Y, Orabi A, Sharaky M, Mina SA, Abo-Zeid Y. Preparation and characterization of nano-emulsion formulations of Asparagus densiflorus root and aerial parts extracts: evaluation of *in-vitro* antibacterial and anticancer activities of nano-emulsion versus pure plant extract. Drug Dev Ind Pharm. 2024;50(7):658–70. doi: https://doi.org/10.1080/03639045.2024.2386001
41. Siddique I. High-performance liquid chromatography: comprehensive techniques and cutting-edge innovations. Eur J Adv Eng Technol. 2023;10(9):66–70. doi: https://dx.doi.org/10.2139/ssrn.4885931
42. Mohannaik K, Basha SS, Bhaskar B, Malarvannan M. An overview of developments in stability-indicating chromatographic methods: an essential part of regulatory considerations. J Anal Chem. 2023;78:S1–3. doi: https://doi.org/10.1134/S1061934824030092

How to cite this article:
Salokhe AS, Patil AS, Gaude Y, Rayanade P, Koli R, Jadhav NS. Supercritical fluid extraction, LC-MS profiling, and QbD-guided green HPLC method for standardization of *Careya arborea* Roxb. nanoemulsion. J Appl Pharm Sci. 2025. Article in Press.
http://doi.org/10.7324/JAPS.2025.266427

SUPPLEMENTARY MATERIAL

The supplementary material can be accessed at the link here: [https://japsonline.com/admin/php/uploadss/4658_pdf.pdf]